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Multiple APOBEC3 Restriction Factors for HIV-1 and One Vif to Rule Them All

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Abstract

Several members of the APOBEC3 family of cellular restriction factors provide intrinsic immunity to the host against viral infection. Specifically, APOBEC3DE, APOBEC3F, APOBEC3G, and APOBEC3H haplotypes II, V, and VII provide protection against HIV-1 Δ vif through hypermutation of the viral genome, inhibition of reverse transcription, and inhibition of viral DNA integration into the host genome. HIV-1 counteracts APOBEC3 proteins by encoding the viral protein Vif, which contains distinct domains that specifically interact with these APOBEC3 proteins to ensure their proteasomal degradation, allowing virus replication to proceed. Here, we review our current understanding of APOBEC3 structure, editing and non-editing mechanisms of APOBEC3-mediated restriction, Vif-APOBEC3 interactions that trigger APOBEC3 degradation, and the contribution of APOBEC3 proteins to restriction and control of HIV-1 replication in infected patients.

Keywords

APOBEC3G; APOBEC3F; APOBEC3H; cytidine deamination; Vif; restriction factor

Introduction

Over millions of years, eukaryotes have been exposed to bacterial and viral infections. In response to these pressures, mammalian hosts have not only developed powerful immune systems to detect and neutralize foreign invaders, but also acquired host restriction factors, i.e. intrinsic immunity, to defend against these infections. Restriction factors provide a first line of defense for the host to suppress replication of the pathogens. One important family of cellular cytidine deaminases is the apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide (APOBEC) family of proteins, which act on single-stranded DNA (ssDNA) or RNA substrates.¹ In the human genome, this family of proteins consists of 11 members: activation-induced cytidine deaminase (AID), APOBEC1 (A1), APOBEC2 (A2), APOBEC3 (A3:A3A, A3B, A3C, A3DE, A3F, A3G, A3H) and APOBEC4 (A4), all of which contain a conserved zinc-coordinating cytidine deamination domain (Figure 1).

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The main function of the APOBEC family of proteins is to alter the nucleotide sequence through cytidine deamination, converting cytidines to uridines (C-to-U) or deoxycytidines to deoxyuridines (dC-to-dU). It is known that A1 is involved in editing the apolipoprotein B pre-mRNA to introduce a stop codon to create two distinct mRNAs important in lipid metabolism.^{2; 3} AID functions in somatic hypermutation and antibody diversification by class-switch recombination of immunoglobulin genes.⁴ A2, a muscle-specific APOBEC family member, has recently been shown to have a role in normal muscle differentiation and maintenance of muscle fiber-type ratios.^{5; 6; 7; 8} In addition, A2 is involved in regulating TGF- β signaling to specify the left-right axis in vertebrates during embryogenesis, although gene loss is not lethal.^{5; 9} The function of A4, primarily expressed in testis, is still not known.¹⁰ In 2002, A3G was discovered to inhibit HIV-1 infection in the absence of the virally encoded protein, virion infectivity factor (Vif), marking the A3 proteins as important restriction factors against viral infection.¹¹ A3G was further shown to induce C-to-U deamination in the minus-strand DNA of the replicating virus, resulting in G-to-A hypermutation in the HIV-1 double-stranded DNA (dsDNA) genome, which introduces substitution mutations and stop codons that ultimately disrupt expression of functional viral proteins (Figure 2).^{12; 13} HIV-1 has evolved to counteract the effects of A3G by coding for the accessory protein Vif. Vif binds A3G in the cytoplasm, directing it for polyubiquitination and proteasomal degradation, allowing HIV-1 to exclude A3G from the nascent virions, and thereby overcoming A3G-mediated inhibition of viral replication in infected cells.^{14; 15; 16; 17; 18; 19; 20}

The A3 proteins are true restriction factors because they block HIV-1 infectivity, they are induced by the innate immune response, and they exhibit positive selection due to their direct interactions with the viral “counter-restriction factor” Vif, reflecting an ongoing host-pathogen arms race resulting in co-evolution.²¹ Since this discovery, the A3 proteins have been intensely studied. The human A3 locus, found on chromosome 22, encodes 7 A3 proteins with A3A, A3C, and A3H containing a single cytidine deaminase domain and A3B, A3DE, A3F and A3G containing two deaminase domains (CD1 and CD2), which likely formed as a result of gene duplication (Figure 1).^{1; 22} The A3 proteins are widely expressed in different tissues and cell types, especially targets of HIV-1 such as CD4⁺ T cells, macrophages and dendritic cells.^{23; 24} The fact that A3 proteins act on ssDNA intermediates implies that their antiviral activity is not limited to HIV but extends to other retroviruses including gammaretroviruses (murine leukemia virus, mouse mammary tumor virus),^{25; 26} simian immunodeficiency virus,²⁷ equine infectious anemia virus,²⁸ human T-cell leukemia virus type-1,^{29; 30; 31} Rous sarcoma virus,³² and foamy viruses.^{33; 34; 35} In addition, A3 proteins also inhibit both LTR- and non-LTR retrotransposons,³⁶ and other viruses such as hepatitis B virus³⁷ and human papillomavirus.³⁸

In this review, we summarize our current knowledge of A3 proteins and their mechanisms of HIV-1 inhibition, with special emphasis on A3G, which seems to be the most important, and most-studied, inhibitor of HIV-1. We survey which A3 proteins are the main players in blocking HIV-1 infection, either through hypermutation or non-editing mechanisms (e.g. inhibition of reverse transcription and integration). We analyze the current structural and biochemical data examining how A3 proteins function and interact with Vif, and how they are regulated not only in cells, but also in HIV-1 infected patients.

Domain organization and tertiary structures of A3 proteins

The presence of either one (A3A, A3C, A3H) or two (A3B, A3DE, A3F, A3G) catalytic domains (CD1 and CD2) sandwiched between an N-terminal short α -helical domain and a C-terminal short linker peptide is a hallmark of the structural arrangement of A3 proteins (Figure 1).²² A3B, A3DE, A3F, and A3G have a duplication of the entire unit consisting of

6 α -helices and 5 β -strands.²² Each CD is organized as a HXEX₂₃₋₂₈-PCX₂₋₄C motif containing four invariable residues where X represents any amino acid and the histidine and the two cysteines are the essential residues that coordinate Zn²⁺, while the glutamic acid is important for the deaminase activity of the proteins by shuttling protons in the catalytically active domains.²² Although structures of full-length A3G or A3F are not yet available, high resolution crystal and nuclear magnetic resonance (NMR) structures of human A3G-CTD, A3F-CTD, A3A, A3C, and A2 (the closest paralog of A3) have been determined.^{39; 40; 41; 42; 43; 44; 45; 46; 47} Moreover, the NMR structure of murine A2 has been determined through the RIKEN Structural Genomics/Proteomics Initiative (see PDB entry 2RPZ; <http://www.rcsb.org/pdb/explore/explore.do?structureId=2RPZ>).

A3G structure

Several groups have successfully resolved high resolution crystal^{39; 40; 44} or NMR^{46; 48} structures of the C-terminal fragment of A3G (A3G-CTD), using either a wild-type or a soluble mutant of A3G-CTD (A3-2K3A) containing L234K, F310K, C234A, C321A, and C356A substitutions. The structures of the A3G-CTD exhibit a canonical polynucleotide cytidine deaminase fold that is composed of a five β -strand core surrounded by six α -helices with the catalytic site composed of two α -helices (α 1 and α 2) and a β 3-strand that is accessible for large RNA or ssDNA binding (Figure 3A, B), common to all APOBEC proteins whose structures are known; i.e., A2, A3A, A3C, and A3F-CTD proteins (Figure 3C)^{39; 40; 41; 42; 43; 44; 45} Despite this progress, many important aspects of the structures of A3 proteins have not been elucidated. Currently, it is unknown how the two domains of the A3 proteins are positioned relative to each other. In addition, the assignments of residues that are involved in ssDNA binding in different models have little correlation with each other and are speculative largely because none of the structures incorporated a substrate.

A3C structure

Recently, a high resolution crystal structure of full-length A3C has also been determined (Figure 3C).⁴⁹ Interestingly, the crystal structure of A3C provided an important glimpse on the essential Vif-interacting residues on A3C protein, which forms a shallow cavity where Vif binds.⁴⁹ Using structure-guided mutagenesis, Kitamura *et al.* identified 10 hydrophobic or negatively charged residues between the α 2 and α 3 helices, which are conserved in the homologous domain of A3F and A3DE proteins but not in A3G (Figure 4).⁴⁹ Notably, the shallow cavity in the interface is composed of the hydrophobic (L72, I79 and L80), aromatic (F75, Y86, F107 and H111) and hydrophilic (C76, S81 and E106) residues of A3C. Furthermore, mutational analysis of the equivalent ten residues in A3F (L255, F258, C259, I262, L263, S264, Y269, E289, F290 and H294) and A3DE (L268, F271, C272, I275, L276, S277, Y282, E302, F303 and H307) revealed clustering of these residues that form a Vif-binding cavity homologous to that observed in the A3C structure.⁴⁹

A3F structure

Similar to A3G, A3F is notoriously insoluble and readily precipitates in solution, making it unsuitable for structural studies. Therefore a minimal A3F-CTD construct was engineered in which 11 amino acids were substituted (A3F₁₈₅₋₃₇₃-11X) to increase the solubility of the protein, while maintaining its catalytic activity and susceptibility to HIV-1 Vif-mediated degradation (Figure 3C).⁴⁵ The A3F-CTD crystallizes as an elongated rod-shaped asymmetric unit composed of four molecules assembled through two different monomer-monomer interfaces that can be viewed as two homodimers joined head-to-head.⁴⁵ Such structural configurations, distinct from the canonical square-shaped tetramer structure of free nucleotide cytidine deaminase where the loops from two neighboring monomers cover the active site so that only small free nucleotides can bind to the buried sites,⁵⁰ could provide

an explanation for the ability of APOBEC family members to bind and deaminate long polynucleotide substrates. The head-to-head and the two head-to-tail interfaces of the crystal structure of A3F-CTD are held together by extensive salt bridges, hydrogen bonding and hydrophobic packing. The largest interface, a symmetric/isologous head-to-head interface, is principally formed as a result of hydrophobic β -sheet interactions of identical regions of homodimers.⁴⁵ The second interface, an asymmetric/heterologous interface, is a head-to-tail interaction between monomers within the homodimer unit cells, which involves the catalytic sites.⁴⁵

Unlike the A3G-CTD region, the CTD of A3F is involved both in the cytidine deaminase activity and Vif interaction (Figure 1). Therefore, the A3F-CTD crystal structure further elucidated an HIV-1 Vif binding surface. Interestingly, the majority of the A3F-CTD structure surface is negatively charged including the previously identified motif composed of ²⁸⁹EFLARH²⁹⁴⁵¹ and E324⁵² that determine the interaction between A3F and HIV-1 Vif (Figure 4). Consistent with this, a shallow groove was identified in the crystal structure of A3C as a binding pocket of HIV-1 Vif,⁴⁹ albeit significant differences were observed between the two structures (Figure 4). Another important observation was that the intermolecular contact surfaces involving ³⁰⁵RLYYFWD³¹¹ and ²²³EVVKHHSPVS²³² regions of the asymmetric tetramer crystal structure of A3F were suggested as a potential oligomerization surfaces.⁴⁵ Interestingly, these motifs showed certain sequence similarities with ¹⁰⁸HLYYFD¹¹³ and ²³SLVKHHMYVS³² regions of HIV-1 Vif sequence. This suggests that perhaps HIV-1 Vif evolved to mimic this oligomerization interface so that it could bind to A3F and induce its degradation. It is necessary to point out however, that the interfaces between A3F-CTDs in the crystal structure may not represent the protein-protein interactions that occur with the intact A3F protein and could form as a result of removal of A3F-NTD. In this regard, the A2 crystal structure,⁴² obtained from a truncated A2, indicated that it is a dimer, but the NMR structure of the intact A2 protein indicates that it is a monomer.⁴⁷ Further mutational analyses of these regions of Vif and A3F should verify these intriguing predictions and provide novel insights into Vif-A3F interactions.

A3A structure

The recently reported NMR structure of A3A⁴³ closely resembles the crystal structure of A3G-CTD quintuple mutant (A3G-2K3A) (Figure 3C).⁴⁴ Detailed analysis of the A3A NMR structure with particular emphasis around the active site, where the sequences significantly differ from the A3G-CTD,³⁹ reveals the presence of additional hydrophobic residues that distort the conformation of loop 7 (Figure 3C, magenta color) situated next to the active site.⁴³ A3A and A3G purportedly possess a different substrate specificity, which is believed to be due to certain structural differences at the substrate binding surface.^{39; 43; 48; 53} Although A3A can bind to both 5'-TC- and 5'-CC-containing oligonucleotides using the same interaction surface, 5'-TC is the preferred dinucleotide substrate. Byeon *et al.* used NMR to map ssDNA substrate binding to A3A to a large surface covering loop 3, loop 5, loop 7 (Figure 3C, blue, orange, and magenta colors, respectively), and helix α 4 clustered around the active site.⁴³ Interestingly, the clustering of the A3A substrate-interacting residues and the conformational changes of the ssDNA permit the reactive cytosine and only the immediate neighboring (-1, -2 and +1) nucleotides access the active site. Earlier studies also demonstrate DNA contraction during A3G scanning of ssDNA substrate⁵⁴ and RNA bending in a co-crystal structure of tRNA adenosine deaminase Tada/RNA complex,⁵⁵ suggesting that ssDNA/RNA substrate bending or extensive conformational changes are required because the active site of polynucleotide cytidine deaminases can only accommodate few nucleotides.

Unlike A3A, A3G preferentially binds to the 5'-CC dinucleotide in the ssDNA and the interaction surface is localized in loops spanning residues 207 to 217 and 313 to 320.^{39; 40; 56; 57} Although the proposed ssDNA binding surfaces of A3G are not clustered, a recent observation demonstrates that protonation of H216, which is spatially close to the active site and is located in loop 1 composed of residues 207 to 217, was found to be important for determining substrate binding and HIV restriction.⁵⁸ Moreover, using mutagenesis and domain swapping, several groups have identified several amino acids, which include loop 7 residues that govern the intrinsic dinucleotide specificity (5'-CC vs 5'-TC context) of several cytidine deaminases.^{55; 57; 59; 60; 61} Notably, a recent study showed that replacing the aspartic acid for tyrosine at position 317 (the corresponding residue in A3A is Y132) in loop 7 in A3G was sufficient to alter A3G's substrate specificity from 5'-CC to 5'-TC.⁵⁹

In summary, the structural similarities of A3 proteins parallel the similarities in their primary sequences.²² The core structures, composed of six helices (α 1- α 6) intermixed with five β -strands (β 1- β 5), containing the zinc ion coordinated in the active site are highly conserved structural hallmarks of the APOBEC family of proteins.⁶² The catalytic loop of α 1 helix of all A3 proteins exhibits the same conformation, whereas it is oriented perpendicularly in the A2 crystal structure (Figure 3C). In both the A2 and A3F-CTD asymmetric tetrameric crystal structures, the β 1- β 2 loop exists in two distinct conformations depending on the interface involved in the asymmetric tetramer unit and the long continuous β 2 strand was found to mediate homodimerization.^{42; 45} However, this structural organization is not a common feature of all A3 proteins structures (Figure 3C). For instance, the NMR structures of A3A,⁴³ and wild-type A3G-CTD,⁴⁸ and the crystal and NMR structures of A3G-CTD (A3G-2K3A)^{39; 44; 46} exhibit a discontinuous β 2 strand, whereas the crystal and NMR structures of A2,^{42; 47} as well as crystal structures of A3C,⁴⁹ A3G-CTD,⁴⁰ and A3F-CTD⁴⁵ revealed a long continuous and extended β 2 strand (Figure 3C). While these structural differences may reflect the structures of the proteins in solution, they may also be the result of differences in the methods used to determine the structure or to the engineered protein constructs used for the structural studies. Nevertheless, a combination of these structural and sequence differences are believed to dictate the differential substrate specificity of the APOBEC family of cytidine deaminases. The remarkable advances that were made on the structural biology of the APOBEC family of proteins in the past few years have led to (i) an understanding of the architecture of their catalytic core, (ii) the identification of a determinant of their substrate specificity, and (iii) elucidation of the structures of A3 protein determinants involved in interaction with HIV-1 Vif. These advances will hopefully facilitate structure-based drug design and perhaps identification of small molecule inhibitors that specifically target A3-Vif interactions and allow the A3 proteins to potentially block HIV replication.

Structural determinants of Vif-A3 interactions, virion incorporation, and substrate preference

Vif-A3 interaction determinants

As described earlier, due to the poor solubility of these proteins, detailed views of the A3G/A3F-Vif and/or A3G/A3F-ssDNA complexes are lacking.^{39; 40; 44; 45; 46; 48} To achieve a more comprehensive picture of the enzymatic activities of A3 proteins, their interaction with HIV-1 Vif, and their regulation in cells, co-crystal structures that encompass full-length protein and their ssDNA substrate as well as Vif are essential. Nevertheless, the available structural data afford important glimpses into the antiviral mechanisms of the A3 proteins, their interaction with HIV-1 Vif, and their substrate specificity.^{39; 43; 44; 45; 46; 49} Furthermore, using site-directed mutagenesis along with functional and biochemical

analyses, the critical residues involved in Vif-A3 protein interactions have been revealed.^{14; 49; 51; 63; 64; 65; 66; 67}

A3 residues critical for A3-Vif interactions

Mapping critical residues in A3 proteins has demonstrated the clustering of critical residues in well-defined patches on the surface of structural models^{45; 46; 48; 49; 51; 66; 67; 68} (Figure 4). Initially, we and others identified D128 as a critical residue in human A3G that was involved in interaction with HIV-1 Vif.^{69; 70; 71; 72} Subsequently, we and others demonstrated that the ¹²⁸DPD¹³⁰ motif is critical for the interaction between A3G and Vif, and Vif-mediated degradation of A3G (Figure 4).^{65; 66} More precisely, HIV-1 Vif fails to neutralize A3G from African green monkey (agm) or rhesus macaque that contains a lysine (K) instead of aspartic acid (D) at position 128; however, the agm and rhesus macaque simian immunodeficiency virus (SIV_{agm} and SIV_{mac}, respectively) can induce the degradation of human A3G. Moreover, substitution of D for K in human A3G confers resistance to HIV-1 Vif.^{69; 70; 71; 72} Interestingly, recent studies demonstrated that substitution of the aspartic acid at position 121 to lysine in A3H haplotype II, the equivalent residue of D128 A3G, render A3H resistant to HIV-1 Vif-mediated degradation.^{73; 74; 75}

Even though D128 in A3G and the equivalent residue in A3H are critical for interaction with HIV-1 Vif, they are not critical in all human A3 proteins. Our analysis using functional A3G and A3F chimeras identified amino acids 283-300 in the C-terminus of A3F as critically important residues for A3F-Vif interaction.⁶⁶ Further analysis of this region of A3F led to the identification of the ²⁸⁹EFLARH²⁹⁴ region in A3F, with E289 being the most critical residue for interaction with HIV-1 Vif, and ²⁹²ARH²⁹⁴ having a significant effect on Vif binding when all three residues are simultaneously mutated to alanines (Figure 4).⁵¹ The E324 residue of A3F was also shown to be critical for Vif interaction (Figure 4).⁵² Interestingly, these residues are conserved in A3C, A3DE and A3F but not in A3G.^{49; 51}

HIV-1 Vif residues important for A3-Vif interactions

Since Vif is also an extremely difficult protein to purify and maintain in solution for structural analysis, structural features of Vif that enable interactions with its partners have not been elucidated. Moreover, recombinant Vif is highly dynamic and likely to populate an ensemble of heterogeneous conformational states in solution, which is not amenable for structural studies.⁷⁶ Nevertheless, using site-directed mutagenesis several domains in Vif have been identified as being important for interactions with proteasomal components to induce degradation of A3 proteins. For example, several investigators have identified an ¹⁴⁴SLQYLA¹⁴⁹ domain that is important for interaction with elongin C (EloC) and an ¹⁰⁸HX₅-CX₁₇₋₁₈-CX₃₋₅-H¹³⁹ domain critical for docking with cullin 5 (CUL5) of the E3 ubiquitin ligase complex.^{77; 78; 79; 80; 81} In addition, through mutational analysis of the N-terminal regions of Vif, we and others have identified two distinct regions in HIV-1 Vif that specifically contribute to interactions with A3G or A3F proteins.^{67; 82} The first region, ¹⁴DRMR¹⁷, and to a lesser extent W11 and Q12, are important for degradation of A3F. Interestingly, its substitution with ¹⁴SERQ¹⁷ or ¹⁴SEMQ¹⁷ (equivalent residues in SIV_{agm} Vif) endows the mutant HIV-1 Vif to degrade agmA3G as well as D128K-A3G.^{67; 69; 70; 71; 72; 83} This species specificity was attributed to the two polar amino acids (E15 and Q17) in Vif and their interaction with the negatively charged D128 in human A3G. However, Russell *et al.* demonstrated that a second region, the ⁴⁰YRHHY⁴⁴ domain, is actually the region that is essential for the binding of Vif to A3G in co-immunoprecipitation experiments.⁶⁷ Others also independently identified this same region to be essential for its interaction with A3G.⁸² These two distinct domains of Vif show a differential effect on interaction of Vif with A3G or A3F, which are the two most potent antiviral A3 proteins. Interestingly, the amino acid variation at position 48 was found to dictate the differential

sensitivity of A3H haplotype II to the two commonly used laboratory strains, NL4-3 and LAI, which contain N48 and H48 residues, respectively. While Vif containing N48 cannot neutralize the antiviral activity of A3H haplotype II, substitution to H48 as in the case of LAI, induces proteasomal degradation of A3H.⁸⁴

Alanine scanning mutagenesis in other regions of Vif identified Y69 and L72 amino acids in the ⁶⁹YXXL⁷² motif to be critical for neutralizing the antiviral activity of both A3G and A3F.^{82; 85; 86} In these studies, Vif proteins with mutations in this region were shown to retain binding to CUL5, confirming that the mutations did not result in expression of misfolded proteins that had lost all functions associated with Vif. Because of the absence of crystal or NMR structures of Vif and the complexity of its interaction in the context of E3 ubiquitin ligase, the identification of different regions of Vif involved in polyubiquitination and degradation of A3 proteins provided important insights in understanding the details of the Vif-A3 complex.^{67; 82; 85; 86; 87; 88; 89; 90; 91; 92} However, much still remains to be determined including elucidation of the order of interaction of Vif, A3G, CBF- β - a cellular cofactor of Vif that facilitates A3G degradation, and E3 ubiquitin ligase complex. It is also important to note that in the absence of a structure, it cannot be determined whether the amino acids critical for interaction in A3 proteins or Vif are directly involved in the interactions, or they result in conformational changes that indirectly facilitate the interactions. Nonetheless, identification of these determinants that are critical for Vif-A3 protein interactions will hopefully assist rational design of inhibitors that inhibit Vif's capacity to induce degradation of the A3 proteins, thereby preserving their anti-HIV-1 function.

Structural determinants of A3G virion incorporation

Mutational analysis of A3 proteins has demonstrated the clustering of critical residues in well-defined patches on the surface of structural models.^{45; 46; 48; 49; 51; 66; 67; 68} A3G from the producer cells, not the target cells, blocks HIV-1 replication in the subsequent round of infection; thus, it is the A3G carried by the virion that exerts the antiviral effects.^{18; 28} To understand this essential aspect of the antiviral actions of A3G, we determined the stoichiometry of A3G in Vif-deficient virions produced from activated CD4⁺ T cells.⁹³ We found that about seven molecules of A3G were incorporated per virion; yet the infectivity of the virions is severely impaired in this physiologically relevant system, suggesting that the catalytic activity of A3G may play an important role in its antiviral activity. In the absence of Vif, A3G was shown to be incorporated into nascent virions through interactions with viral and non-viral RNAs and none of the viral proteins appeared to be required for virion incorporation.⁹⁴ Some studies have suggested that A3G interacts with the nucleocapsid (NC) domain of Gag in an RNA-dependent manner, which contributes to A3G virion incorporation.^{95; 96; 97; 98; 99; 100} A3G encapsidation was also analyzed with HIV-1 Gag constructs in which the NC domain was replaced with a heterologous leucine zipper dimerization motif.⁹⁷ These Gag constructs produce viral particles that contain little or no RNA, and it was observed that these particles contain very little if any A3G, suggesting that the primary mechanism by which A3G is incorporated into virions is through its interaction with viral or non-viral RNA. Hydrophobic residues ¹²⁴YYFW¹²⁷ in the linker region between CD1 and CD2, and adjacent to the D128 residue that is critical for interaction with Vif, have been shown to be important for A3G virion encapsidation and were identified to interact with RNA and nucleocapsid in an RNA-dependent manner.^{65; 101} Moreover, the W94/W127 residues in A3G were recently reported to be critical for its interaction with RNA, HMM complex assembly, and virion incorporation.¹⁰²

It is known that A3G is an RNA-binding protein^{103; 104} and is associated with an array of cellular ribonucleoprotein (RNP) complexes in the cytosol.^{56; 105; 106} Although the identity of the RNA(s) that promote the virion encapsidation of A3G remains unclear, HIV-1

genomic RNA and 7SL RNA have been implicated to play essential roles in the process.^{96; 98; 107; 108} Interactions of A3G with cellular 7SL RNA was suggested to be necessary for its virion incorporation.¹⁰⁸ However, other studies have suggested that binding to 7SL RNA is dispensable for A3G virion incorporation.¹⁰⁹ We recently examined the role of 7SL RNA in A3G virion incorporation by sequestering the available 7SL RNA in cells by overexpression of SRP19, a protein that binds to 7SL RNA, and found that 7SL RNA availability did not have any effect on the efficiency of A3G virion incorporation.¹¹⁰ Although binding to viral and/or non-viral RNAs by A3 proteins is essential for their virion incorporation, it is thought that given the vast amount of RNA present in cells, simple RNA binding cannot explain virion incorporation of A3G, and that some unidentified mechanisms may contribute to the specificity of the interactions of A3G with viral RNA, leading to their efficient virion incorporation.

Host proteins that regulate A3G functions and its Vif-mediated degradation

As recounted above, the anti-HIV action of A3G was discovered through efforts to understand the function of Vif that preserves viral infectivity. Subsequent studies revealed that Vif induces polyubiquitination and proteasomal degradation of A3G by hijacking the E3 ubiquitin ligase complex composed of CUL5, EloB, EloC and a RING-box subunit 2 (RBX2) and CBF- β , the allosteric regulator of Vif (Figure 5).^{14; 16; 18; 19; 20; 111; 112} A few studies have also reported that Vif binds to A3G mRNA and downregulates its expression and stability, and directly inhibits the deaminase activity of A3G.^{19; 113; 114; 115} However, it is generally accepted that the primary mechanism by which Vif overcomes the antiviral activity of A3 proteins is by inducing their proteasomal degradation. In addition to these proteasomal components, several cellular factors including NEDD8, IFNs, HSP70, protein kinase A and others have been reported to modulate the expression of A3G and/or its antiviral activities (Table 1).

CBF- β

Core binding factor- β (CBF- β) normally forms functional heterodimers with one of the three DNA-binding RUNX transcription factors and is involved in the regulation of hematopoiesis as well as development and differentiation of T lymphocytes.¹¹⁶ Recently, two groups simultaneously reported that CBF- β can interact with Vif and this interaction is required for Vif-induced A3G polyubiquitination and degradation.^{111; 112} Several lines of evidence suggest that Vif hijacks CBF- β , presumably before it recruits A3G to the Vif-CUL5-EloB/C-E3 ubiquitin ligase complex, to promote polyubiquitination and proteasomal degradation of A3G (Figure 5).^{111; 112} Moreover, stable knockdown of CBF- β in 293T cells reduces the stability of Vif and abolishes the assembly of Vif-CUL5-EloB/C-E3 ubiquitin ligase complex resulting in reduced HIV-1 infectivity primarily by preventing A3G degradation and preserving its antiviral activity.^{111; 112}

Vif binds to both isoforms of CBF- β ^{117; 118} and the N-terminus of CBF- β containing amino acids 15-126 was identified as the Vif binding region,¹¹⁷ which appears to be distinct from its domain that interacts with the RUNX transcription factors.^{112; 117; 118} However, when the N-terminal domain of CBF- β was extended to include residues 15-130, both Vif and RUNX were found to interact with CBF- β , suggesting that both proteins interact with CBF- β in a mutually exclusive manner.^{117; 119} In fact, Hultquist *et al.* identified a single amino acid substitution, F68D, in CBF- β , which was sufficient to abrogate the interaction between CBF- β and Vif, while preserving its ability to form CBF- β -RUNX heterodimers.¹¹⁸ Moreover, it has been shown that CBF- β prevents Vif oligomerization and promotes an ordered binding of Vif to the CUL5-E3 ubiquitin ligase complex.^{119; 120} Although the molecular basis of Vif-CBF- β interaction is still unclear, a dual hijacking mechanism was recently proposed.¹¹⁹ First, Vif recruits CBF- β to efficiently evade the innate immunity by

promoting A3G degradation. Second, Vif sequesters CBF- β into the CUL5-Vif complex and perturbs the transcription of genes governed by RUNX-associated transcription factors. Disrupting the interaction between Vif and CBF- β may be an attractive pharmacological intervention for the development of anti-HIV therapies.¹¹² Furthermore, co-expression of Vif and CBF- β profoundly improves full-length Vif purification and solubility which will facilitate future structural and biochemical studies.¹²¹

Nedd8

HIV-1 Vif recruits host factors to form an E3 ubiquitin ligase complex including CUL5, RBX, EloB, EloC, and CBF- β to degrade A3G (Figure 5).^{20; 111; 112} The cullin-RING ubiquitin ligase must be activated by the covalent modification of a conserved lysine in the C-terminal domain of all cullins with the ubiquitin-like protein NEDD8,¹²² which requires the action of a three-enzyme E1-E2-E3 cascade much like that of ubiquitin.¹²³ Given that CUL5 NEDD8ylation is required for the activity of Vif, pharmacological inhibition of the NEDD8 E1 by a small molecule MLN4924¹²⁴ or knockdown of NEDD8-conjugating enzyme E2 UBE2F abrogates the action of Vif-induced degradation of A3G and restores the restriction of HIV.¹²⁵ Thus NEDD8 is also required to regulate the turnover of A3G.

Interferons

Understanding the regulation of the innate immune response, which includes cellular restriction factors such as A3 proteins, upon exposure to HIV-1 is essential. In fact, exposure to any foreign invaders including viral infections induces the innate immune response. One of the essential arms of the immune response is the type I interferon (IFN- α and - β) pathway that senses foreign dsDNA via its intracellular receptor, activated Toll-like receptor-3 (TLR-3), and generates an overwhelming antiviral state and curbs propagation of infection in immune cells.¹²⁶ *In vitro* studies showed that IFN- α is a potent inhibitor of HIV-1, particularly in the early stages of infection.^{127; 128; 129} Interestingly, the IFN- α response seems to modulate the expression levels of restriction factors in response to exposure to HIV. For example, in macrophages, IFN- α and - β upregulate the expression of A3G, TRIM5 α , tetherin, and anti-HIV miRNAs, and block viral replication.¹³⁰ Furthermore, it has been shown recently that even low levels of IFN- α expression can induce expression of A3 proteins that is enough to restrict HIV-1 replication in monocyte-derived dendritic cells.¹³¹ The induction of type I IFN-inducible antiviral factors, including A3G and tetherin, is believed to be through the JAK-STAT signaling pathway that fine-tunes the transcription of several essential cellular proteins.¹³² In addition, it has been reported that A3G expression is critically dependent on the partnering of NFATc1/NFATc2 and IRF-4 that regulate the A3G promoter activity in T cells. When either NFATc1 or NFATc2 and IRF-4 were co-expressed in CEM-SS cells that normally do not express A3G and challenged with HIV-1 Δ vif, these cells avert infection primarily by inducing the expression of A3G.¹³³ Conversely, other host factors such as nerve growth factor (NGF) that can be released from HIV-1 infected macrophages were found to blunt IFN- γ -induced expression of A3G, favoring the propagation of HIV-1 infection in macrophages.¹³⁴

Hsp70

Prostaglandin A1 (PGA1) inhibits the replication of a wide variety of DNA and RNA viruses, including HIV-1.¹³⁵ The proposed underlying mechanism is modulation of the expression of chaperone heat-shock proteins (HSP), especially HSP70 by regulating the action of nuclear factor- κ B (NF- κ B) and proliferator-activated receptor γ (PPAR γ) in infected cells.^{136; 137} Interestingly, HSP70 over-expression abrogates Vif-mediated degradation of A3G,¹³⁸ which suggests a potential role for PGA1 in innate immune

response by upregulating the expression of HSP70 that prevents Vif-mediated A3G degradation.¹³⁹

Serine/threonine kinases and A3G phosphorylation

The archetypal mammalian cytidine deaminases, AID and A3G, are believed to be regulated by post-translational modifications, more specifically through phosphorylation by protein kinase A with functional consequences. For example, phosphorylation of certain serine/threonine residues of AID and A3G was claimed to be important in fine-tuning their role in adaptive and innate immune responses, respectively, and proposed to prevent potential deleterious mutagenic effects on the host genome.^{140; 141; 142} Additionally, Shirakawa *et al.* reported that phosphorylation of A3G at position T27 renders it less susceptible to Vif-mediated polyubiquitination and degradation.¹⁴³ However, others showed that the serine/threonine phosphorylation of Vif and A3G is dispensable for Vif-mediated degradation of A3G.¹⁴⁴ Thus, the role of phosphorylation in regulating the antiviral activity of A3 proteins remains an open question that requires further study.

Other host factors that modulate A3G expression and function

The regulation of A3G gene expression and Vif-mediated degradation are affected by many other host factors. For example, recently, FLIP, also known as Fas-associated death domain (FADD) interleukin-1 β -converting enzyme (FLICE)-like inhibitory protein, an inhibitor of caspase, was reported to inhibit HIV-1 replication in T cells including primary CD4⁺ T-cells and peripheral blood mononuclear cells (PBMCs).¹⁴⁵ Cellular FLIP (cFLIP) was found to regulate the expression of multiple HIV restriction factors including A3G, TRIM5, and Bst2/tetherin.¹⁴⁵ Interestingly, Oguariri *et al.* observed an anti-HIV-1 activity of interleukin-2 (IL-2) in HTLV-1 transformed laboratory T-cell lines such as MT-2, MT-4, SLB-1 and ATL-2 but not in other cell lines.¹⁴⁶ Although the loss of infectivity of the virus seems to be due to upregulation of A3G and possible incorporation into virions produced in these cell lines treated with IL-2, the relevance of this observation to the physiologically relevant target cells remains unknown.

Other cellular factors that are also believed to influence the function and/or expression of A3G include the human replication protein A (RPA), TLR2/3, PKC, SP3, PIN1, C/EBP- β , IFN type III, TNF- α , and others (Table 1). Although a considerable amount of knowledge is emerging in understanding the global landscape of host-pathogen interactions, much still remains to be determined, particularly in unraveling the delicate balance between the mechanism of evasion of host restriction factors and the hijacking of essential cellular cofactors by the pathogens to promote their own replication.

Mechanisms by which A3 proteins inhibit viral replication: hypermutation, inhibition of reverse transcription, and integration

The current weight of evidence indicates that A3G, and to a lesser extent A3DE, A3F and haplotypes II, V, and VII of A3H inhibit HIV-1 in the absence of Vif.^{147; 148; 149; 150; 151; 152} A3A, A3B, and A3C have also been reported to inhibit retroviral replication in a limited number of studies.^{60; 148; 153; 154; 155; 156}

The phenomenon of extensive G-to-A mutations called hypermutation, initially described in a simple retrovirus,¹⁵⁷ is the result of C-to-U deamination of the viral minus-strand DNA by A3 proteins during reverse transcription.^{158; 159} This results in G-to-A substitution in the complementary plus-strand often at a frequency that can exceed 10% of all G residues in the viral genome. The global hypermutation in the viral transcripts results in lethal mutational loads that preclude the subsequent generation of infectious particles and propagation of

infection.^{12; 28; 158; 160; 161; 162} Although hypermutation clearly results in lethal mutagenesis and inactivation of the viral genomes,^{18; 28; 160} there is accumulating evidence that A3G and A3F also inhibit viral replication by reducing viral DNA synthesis^{102; 163; 164; 165; 166; 167; 168; 169; 170; 171; 172; 173; 174} and integration of the viral DNA into the host genome.^{172; 173; 175}

Biochemical mechanisms of cytidine deamination and viral inhibition

Several lines of evidence, including recent data from primary target cells infected with HIV-1,¹⁴⁷ indicate that editing and non-editing mechanisms are separate and distinct modes of action of A3 proteins in blocking HIV-1 infection.^{28; 167; 168; 172; 176; 177}

Editing mechanism: hypermutation

As minus-strand DNA synthesis progresses during reverse transcription, the RNase H activity of reverse transcriptase (RT) degrades the RNA template, allowing A3G access to the newly synthesized ssDNA for cytidine deamination. A3G prefers to deaminate C residues in 5'-CCC or 5'-CC motifs on ssDNA, deaminating the 3' C (5'-CCU or 5'-CU); A3G lacks cytidine deaminase activity on dsDNA or RNA templates.^{56; 148; 162; 178} Site preferences for hypermutation are not fully understood, but the sequence context of the cDNA as well as the secondary structure (stem loops) of the substrate are known to influence cytidine deamination patterns.¹⁷⁹ For example, numerous studies have shown that A3G favors the consensus sequence 5'-(T/C)CC(G/A/C)-3'.^{148; 179; 180} Although A3G prefers to deaminate the second C in 5'-CC, resulting in 5'-GG-to-AG mutations on the viral cDNA,^{148; 161} the remaining A3 proteins prefer to deaminate the C in the 5'-TC dinucleotide context, resulting in 5'-GA-to-AA mutations on the viral cDNA.^{60; 148; 152; 154; 155; 156; 181; 182; 183} As discussed in later sections, patient-derived HIV-1 proviral sequences contain G-to-A hypermutations in either the GG-to-AG^{184; 185; 186} or GA-to-AA dinucleotide context,^{181; 187; 188} suggesting that A3G and at least one of the other A3 proteins contribute to HIV-1 restriction *in vivo*. The overrepresentation of GA-to-AA dinucleotide hypermutation reported from one set of patients was based in part on the sequence data using a short fragment of HIV-1 proviral region coding for protease and on the observation that A3F is partially resistant to HIV-1 Vif.^{181; 188} A3F is the most likely A3 protein that is responsible for the GA-to-AA hypermutation,¹⁸¹ although possible contributions from A3H haplotypes II, V, and VII and A3DE cannot be excluded. A3H haplotype II likely restricts HIV-1 in a deaminase-independent manner,⁷³ although A3H haplotype II (RDD mutant) and even the macaque A3H have shown GA-to-AA hypermutation profiles.^{155; 189} It is unclear at this time whether hypermutations are more frequent in the GA or GG dinucleotide context in patients, and a systematic analysis of large proviral sequence databases derived from patient cohorts which include infections with different subtypes is needed to answer this question.

Insights into the biochemical mechanisms by which A3 proteins induce cytidine deamination can be gained by analyzing the patterns of G-to-A hypermutations in proviral genomes. First, hypermutation is often observed throughout the viral genome, suggesting that the A3 proteins must be able to access the viral minus-strand DNA to access multiple target sites. Second, some regions of the genome are hypermutated more extensively than others. Hypermutated proviral DNAs exhibit an overall "twin gradient", one gradient with increasing frequency of mutation from the primer binding site to the central polypurine tract (cPPT), and a second gradient with increasing frequency of mutation from the cPPT to the polypurine tract (PPT)¹⁹⁰. These twin gradients are thought to be largely the direct result of increased access of the minus-strand ssDNA by the A3 proteins during reverse transcription. As minus-strand DNA synthesis is extended in the 5' to 3' direction by RT, RNase H

removes the genomic RNA from the RNA-DNA hybrids, increasing access to the minus-strand DNA by the A3 proteins.

A deamination bias within very short stretches of substrate DNA has been observed *in vitro*, whereby the deamination occurs more frequently at the 5' end of the ssDNA, while the 3' end of the ssDNA is never deaminated ("dead zone").^{56; 191} A3G bound in the active orientation with the CD1 domain oriented at the 3' end of the template would physically block deamination in this region. Recent *in vitro* data using FRET analysis shows that the observed 3' to 5' polarity is due to the binding of A3G to the ssDNA in an asymmetric catalytically active orientation with its CD2 active domain facing the 5' end of the ssDNA.⁵⁴ A3G bound in the active orientation deaminates the 3' C in the 5'-CCC-3' motif more efficiently than the A3G bound in the inactive orientation.^{162; 191} It has been suggested that this intrinsic directionality to the processive movement of A3G could partly explain the twin gradient observed over the entire viral genome.

Processivity refers to the ability of A3 proteins to catalyze multiple cytidine deamination reactions before dissociating from the template. It has been proposed that A3G induces hypermutation by binding its target through facilitated diffusion scanning mechanisms involving sliding and jumping motions,^{56; 191; 192; 193} resulting in directional deamination. Recent results suggest that A3G can slide 69 nucleotides per second on ssDNA.¹⁹⁴ During reverse transcription, RNase H leaves fragments of RNA still attached to the newly synthesized DNA, which are likely to impede the sliding motions of A3 proteins. Perhaps to overcome this barrier, the A3 proteins have evolved to jump from one region of the template to another. When A3 physically leaves the template and reassociates elsewhere on the template (i.e. jumping); these jumps can be micro- or macroscopic depending upon the size of the jump. When A3G binds two ssDNA templates simultaneously, and switches to the alternate template, this is termed intersegmental transfer.¹⁹⁵ Whether A3G can undergo sliding motions, jumping, and/or intersegmental transfers is currently being investigated.^{196; 197}

The catalytically inactive CD1 of A3G, which can bind RNA and DNA,^{103; 178} mediates the processivity of A3G,^{56; 196} while the catalytically active CD2 mediates cytidine deamination and hypermutation.^{103; 178} As described earlier, the A3G mutant W127A is deficient in binding to RNA and remains monomeric. The monomeric A3G double mutant F126A/W127A, which is unable to jump efficiently, and mutant H186R, which is unable to slide efficiently, both deaminate ssDNA less efficiently than wild-type A3G, supporting the notion that a dimer or oligomer is the functional form of A3G, and the sliding and jumping movements are essential for efficient cytidine deamination.¹⁹³

Non-editing mechanisms: blocking reverse transcription and integration

In addition to inducing lethal mutagenesis, the A3 proteins employ non-editing mechanisms to inhibit retroviral replication. It was observed early that catalytic site mutants of A3G could inhibit viral replication,¹⁷¹ and A3F catalytic site mutants could inhibit accumulation of reverse transcripts.¹⁷⁰ Other studies showed that A3G can inhibit reverse transcription by physically blocking RT template binding, reducing tRNA binding, inhibiting efficient tRNA processing, impairing elongation of cDNA synthesis, and blocking minus- and plus-strand transfer events.^{165; 166; 167; 168; 169; 172; 198} Furthermore, the presence of dU in the minus-strand DNA was shown to decrease the efficiency of plus-strand DNA synthesis during reverse transcription.¹⁹⁹ In these *in vitro* studies and transient transfection assays, it was not clear whether the high levels of A3 proteins resulted in the non-editing modes of viral inhibition that were absent under physiological levels of the A3 proteins.

Two distinct mechanisms were thought to contribute to the reduction in accumulation of viral DNA products; first, A3G could bind to viral RNA or cDNA and inhibit the initiation or elongation of viral DNA synthesis; second, removal of the uracil base by uracil DNA glycosylase (UDG), followed by cleavage of the abasic site by apurinic-apyrimidinic endonucleases could result in degradation of reverse transcripts. To gain insights into the mechanisms by which A3G inhibits HIV-1 replication, we performed transient transfection assays in which the transfected cells expressed A3G at levels similar to those observed in primary activated CD4⁺ T cells, and quantified the synthesis and integration of viral DNA using quantitative real-time PCR.¹⁷² In agreement with other studies,^{200; 201} our results showed that the inhibition of host UDG had no impact on the amount of viral DNA synthesized, suggesting that UDG was not inducing degradation of viral DNA subjected to cytidine deamination by A3G. We also observed that a 35-fold decrease in viral infectivity was accompanied by a 5 to 7-fold reduction in viral DNA synthesis, and in agreement with another study,¹⁷³ a 5-fold reduction in integration of the viral DNA. Moreover, the inhibition of reverse transcription and integration required the catalytic activity of A3G, and was the result of aberrant primer tRNA processing that subsequently reduced the efficiency of plus-strand DNA transfer and integration. Subsequently, we found that A3F also inhibits viral DNA integration, but it does so by inhibiting the 3' processing reaction catalyzed by integrase.¹⁷⁵ Overall, the results indicated most of the 35-fold decrease in viral infectivity could be explained by the 5 to 7-fold reduction in viral DNA synthesis and a 5-fold reduction in integration.

Recently, a detailed analysis of the editing and non-editing mechanisms of viral inhibition in primary CD4⁺ T cells was performed.¹⁴⁷ The results indicated that both hypermutation and reduction of viral DNA synthesis occurred when physiological levels of A3 proteins were present, and that A3G was predominantly responsible for the observed cytidine deamination.

A3G Oligomerization

Several lines of evidence indicate that A3G oligomerization and binding to RNA or ssDNA are inextricably linked.^{105; 191; 202; 203; 204; 205; 206; 207} Chelico *et al.* concluded that A3G oligomerization is primarily dependent on its interaction with ssDNA. Co-immunoprecipitation and cross-linking studies indicated that the Y124 and W127 residues were important for A3G's ability to bind to RNA and to form oligomers in an RNA-dependent manner. Consistent with the models of dimer interaction,^{42; 45; 204} it has been shown that A3G, A3F and A3DE form homodimers and heterodimers in cells.^{152; 182} We used a bimolecular fluorescence complementation system to investigate A3G oligomerization and RNA binding *in situ*, and found that wild-type A3G molecules oligomerized and reconstituted fluorescence, while RNA-binding defective mutants of A3G were monomeric and failed to reconstitute fluorescence.²⁰⁷ In these and other studies, it is unclear whether the RNA-dependent oligomers of A3G form protein-protein interactions after binding to RNA, or whether they represent single A3G molecules that are bound to the same RNA. Interestingly, we found that a wild-type A3G can weakly reconstitute fluorescence with an RNA-binding defective mutant of A3G, suggesting a model in which conformational changes in the wild-type A3G that occur after RNA binding can expose protein determinants that can interact with another A3G molecule.

McDougall *et al.* have analyzed the composition of oligomers formed *in vitro* in the presence of ssDNA and correlated the oligomeric forms with their deaminase activity; their results suggest that the homodimeric forms had low deaminase activity whereas homotetramers and higher order multimers were more efficient at carrying out cytidine deamination²⁰⁸. Recently, high-speed atomic force microscopy (AFM) has been used to study the association of A3G with ssDNA;^{194; 203} it was observed that the stoichiometry of

A3G binding to ssDNA was independent of its 5' to 3' polarity and bound to gapped ssDNA substrates as well as tail-DNA, indicating that a DNA end is not required for binding. The authors observed that the assembled multimeric complexes could dissociate from the ssDNA as a whole complex or as monomers in a stepwise fashion. These results confirm the *in situ* results²⁰⁷ and provide direct biochemical evidence to support the view that A3G is primarily monomeric in cells in the absence of RNA binding, and forms higher order multimers upon binding to single-stranded nucleic acid. Because of the abundance of RNA in cells, A3G largely exists as a high molecular mass complex (HMM) in association with RNA.¹⁰⁵

Anti-HIV activities of other APOBEC3 proteins: A3DE, A3F and A3H

A3G has been identified as a potent restriction factor of HIV-1 Δ vif in the natural target cells of infection - PBMCs, monocyte-derived macrophages, and dendritic cells; however, the role of A3DE, A3F, and A3H in HIV-1 restriction is more controversial. A3DE, A3F, and A3G are expressed in T cells and B cells, whereas A3H is expressed at higher levels in T cells than in B cells.²³ The expression levels of these proteins vary in different tissues (e.g., spleen, lymph node, lung, etc), and are largely dependent on the size of the lymphocyte population in each tissue.²³ Depletion of A3G from the nonpermissive T cell line CEM2n by gene targeting resulted in a significant decrease in the 5'-GG-to-AG hypermutation and a 10-fold increase in virus replication. However, restoration of the replication capacity of the virus remained 2-fold less compared to that in the permissive T cell line CEM-SS, indicating the presence of additional restriction factors that induce 5'-GA-to-AA hypermutations.

Although A3F was initially found to restrict HIV when overexpressed in virus producer cells,¹⁸² in some studies it was observed to have little or no effect on HIV-1 replication under physiological conditions. Mulder *et al.*²⁰⁹ reported no difference in HIV replication in PBMCs using wild-type virus (which can degrade both A3F and A3G) and the W11R Vif mutant (which can degrade only A3G), whereas replication of the Δ SLQ Vif mutant (which does not degrade A3G) was significantly restricted. Moreover, A3F had no significant effect on virus infectivity when it was stably expressed in HeLa cells, even though a significant amount of A3F was packaged into progeny virions.²¹⁰ Interestingly, a selection experiment using HIV-1 Δ vif virus in stably transfected T cell lines that express A3F resulted in the selection of a mutant that restored Vif function, suggesting that A3F can impose selection pressure to maintain Vif function *in vivo*.²¹¹ Moreover, HIV-1 containing a Vif mutant (DRMR>4A) that could degrade A3G, but not A3F, was restricted in PBMCs, albeit to a lesser extent than virus that contains Vif (YRHHY>5A) that is capable of degrading A3F, but not A3G.¹⁵⁰ Overall, these studies indicate that both A3G and A3F can restrict HIV-1 replication in their natural target cells of infection, but A3G is a more potent inhibitor of HIV-1 than A3F.

Subsequent studies have revealed that A3DE and A3H haplotypes II, V, and VII, have restriction activity when stably expressed, further expanding the antiviral A3 repertoire in the producer cells.^{151; 212} Viruses produced from a CEM cell line in which A3F was specifically knocked down showed increased infectivity compared to viruses produced from the parental cell line with normal A3F expression.²¹² Interestingly, the 5'-GA-to-AA mutational pattern was unchanged, consistent with the idea that other A3 proteins are responsible for the 5'-GA-to-AA mutations.

Virus produced from A3F-null CEM cells in which A3DE was also depleted by shRNA had increased infectivity and decreased 5'-GA-to-AA mutations relative to virus produced from A3F-null CEM cells, indicating that A3DE is likely a true restriction factor in this cell line, potentially expanding the antiviral A3 proteins repertoire in T cells. Virus produced from

A3F-null CEM cells in which A3H was also depleted by shRNA had increased infectivity, but no difference in mutation rate or pattern relative to virus produced from A3F-null CEM cells. Interestingly, the A3H mRNA level was 20-fold lower in the A3F-null CEM cell line compared to primary CD4⁺ T cells, indicating that A3H can restrict virus even at subphysiological levels.²¹² A3H haplotype II-, but not haplotype I-, overexpression in producer cells has been shown to restrict the replication of some wild-type laboratory adapted HIV-1 strains.^{153; 155} Moreover, only A3H haplotypes II, V, and VII that express stable proteins could incorporate into virions and restrict virus replication in 293T cells.¹⁵³ Interestingly, A3H Haplotype II is not completely neutralized by Vif primarily due to a single amino acid polymorphism at position 121 which is prevalent in people of African descent.^{74; 75; 153; 155} Overall, while A3G is the most potent restriction factor, these members of A3 proteins can restrict HIV-1 replication. Their contribution to the inhibition of HIV-1 replication appears to be quantitatively less than that of A3G, but it is possible that they play important roles in certain physiological conditions or in specific HIV-1 infected populations.

Inhibition of HIV-1 replication by A3 proteins in humanized mice

Animal models for HIV-1 infection are essential tools for studying the *in vivo* mechanism of host-virus interactions such as transmission, replication, and pathogenesis. Nonhuman primates such as rhesus macaques infected with SIVmac or SIV/HIV chimeric viruses (SHIVs) represent well-characterized animal models of immunodeficiency virus infection. However, there are key limitations to these primate studies, including the inherent genetic variability of the host animals, and the fact that SIVmac, while closely related to HIV-2, is genetically distinct from HIV-1. Although chimpanzee immunodeficiency virus (SIVcpz) is believed to be a closely related ancestor of HIV-1, chimpanzees are not a suitable animal model for HIV-1 infection because of ethical concerns, availability of chimpanzees, and high cost. Therefore, humanized mouse models have been developed that support HIV-1 infection and recapitulate many aspects of the human infection. Three different strains of mice have been transplanted with human hematopoietic stem cells and have been confirmed to be permissive to HIV-1 infection: NOD/SCID, Rag2^{null}γC^{null} (double knockout; DKO), and NOD/SCID/γC^{null} (NOG of NSG).^{213; 214; 215; 216; 217; 218; 219}

Results from HIV-1 infected humanized mice have been shown to positively correlate with infection in humans. Ince *et al.* reported that the divergence rate of the HIV-1 *env* gene in the virus population in DKO-humanized mice was similar to that observed in patients during a similar period of infection.²²⁰ The observed mutations in the *env* region were associated with increased CD4 binding efficiency, with one mouse even exhibiting a CCR5 to CXCR4 coreceptor switch. Moreover, A3G-associated hypermutation in proviral DNA was reported in wild-type HIV-1 infected NOG humanized mice,²²¹ indicating that A3G mediated G-to-A hypermutation abrogated HIV-1 replication *in vivo*.

Interestingly, Krisko *et al.* recently demonstrated that A3G and A3F proteins efficiently restrict R5-tropic HIV-1Δ*vif* in bone marrow liver thymus (BLT) mice.²²² In stark contrast, the X4-tropic HIV-1Δ*vif* virus escaped the actions of A3G and A3F in this murine model. BLT mice were developed by transplanting human hematopoietic stem cells along with human fetal thymus and liver tissue into NOD/SCID mice so that the T lymphocyte progenitors migrate and undergo the maturation process in the thymus. To date, BLT mice represent the only murine system designed to encompass the physiological T cell maturation process in the implanted human thymus.^{223; 224} Interestingly, the expression levels of A3F and A3G in the thymus are much lower compared to PBMCs or other organs. Moreover, whereas 30-40% of thymocytes express CXCR4 coreceptor, <5% of the population was found to be CCR5 positive,²²² indicating that R5-tropic viruses have far fewer target cells

in the thymus compared to the X4-tropic virus. Together, the reduced expression of A3G and A3F proteins may explain the differential resistance of the X4-tropic virus in thymocytes, with or without Vif expression. This was further corroborated by the sensitivity of the X4-tropic Vif-deficient virus to A3 restriction as extensive G-to-A hypermutation was observed in PBMCs and other organs. These data strongly indicate that the BLT mouse model may provide an excellent tool to study the impact of the antiviral actions of A3 proteins on HIV-1 infection *in vivo* and further aid in understanding the role of A3 proteins in the pathogenesis of HIV infection. Importantly, Vif-independent HIV-1 replication in the thymus highlights the complexity of the mechanisms by which HIV-1 can escape inhibition by A3 proteins.

A3 restriction in HIV-1 infected patients

The diversification of the HIV-1 population in an infected individual and its continuous adaptation to its host is the result of a combination of several factors: a large viral population, high replication and mutation rates, recombination, and various host selective pressures.²²⁵ The high mutation rate is mainly attributed to error prone reverse transcription.²²⁵ Although sublethal G-to-A hypermutations induced by the A3 proteins have also been proposed to contribute to viral variation,^{226; 227; 228} its contribution to viral diversification and potential accumulation of resistance mutations in patients on therapy is controversial;^{159; 229; 230; 231; 232; 233} and other studies have suggested that even a single incorporated A3G is likely to cause extensive and lethal hypermutation and may be an “all or nothing” phenomenon.²³³ It is important to determine whether A3G has the capacity to contribute to viral variation, because potential therapeutic measures targeting the interaction between Vif and A3G may contribute to viral variation and selection of drug resistant variants.

A3 protein expression levels and/or natural variation of A3 proteins can influence their antiviral activity in patients. In agreement with this, several investigators reported that A3G expression levels correlate inversely with HIV-1 viral load and disease progression and directly with the mutational loads in the viral genome (Table 2).^{234; 235; 236; 237; 238; 239} Interestingly, A3G mRNA levels are higher in long-term nonprogressors than in uninfected controls, while the lowest A3G mRNA levels are found in HIV progressors.²³⁴ In a South African cohort, significantly higher A3G expression in PBMCs was shown for HIV-1-exposed seronegative individuals compared to HIV-1-seropositive patients or healthy controls,²⁴⁰ but another recent study could not demonstrate any difference in the expression levels of A3G in HIV-1-exposed seronegative patients.²⁴¹ Contrary to the aforementioned cohorts, Gandhi *et al.* showed that the frequency of hypermutated viral genomes in elite controllers was not significantly different from that observed in patients on highly active antiretroviral therapy, and no correlation was found between A3G expression and the rate of hypermutation.²⁴² Moreover, recent studies found no correlation between the expression levels of A3G and A3F and disease progression in HIV-1 infected children.^{243; 244; 245}

Sporadic G-to-A hypermutation is readily detected in significant proportions of the integrated proviral DNA from patient samples.^{89; 246; 247} Interestingly, such mutational loads could not be found in viral RNA from plasma samples of the same HIV-1 infected patients, indicating that the hypermutated proviruses do not generate progeny virions. These *in vivo* results are in agreement with our analysis in cell culture using HIV-1 Δ vif virus, which showed a gradient hypermutation from proviral DNA>cellular RNA>viral RNA.²⁴⁸ This hypermutation gradient results from purifying selection acting at the level of transcription and expression of functional viral proteins, leading to the preferential packaging of mostly unmutated viral genomes into virions.

Genetic variation in A3G and vif

Single nucleotide polymorphisms (SNPs) in A3G can alter its antiviral activity and hence the rate of HIV-1 disease progression. An SNP in A3G (H186R) in exon 4, which is abundant in African Americans (37%) but rare in Caucasians, is strongly associated with a more rapid CD4⁺ T cell loss and accelerated disease progression (Table 2).^{249; 250} This mutation was also found at a similar frequency in ART-naïve African HIV-infected adults and was significantly associated with increased viral load and a rapid decline in CD4⁺ T-cell count.²⁵¹ Singh *et al.* also identified another mutation in A3G (F119F), a synonymous genetic variant, which is associated with reduced rate of disease progression in HIV-1 infected children.²⁴⁹ Although it is possible that this SNP may be in linkage disequilibrium with other as yet unidentified SNPs that alter the expression and/or function of A3G, the underlying mechanism is not completely understood. Other studies indicate that A3G- and A3F-induced hypermutation is associated with increased CD4⁺ T-cell count²⁴⁶ and a reduction in viremia.²⁵² However, some studies have not found a correlation between A3G and A3F mRNA levels or polymorphisms and viral load and/or CD4⁺ T-cell count (Table 2).^{242; 251; 253; 254; 255}

Not surprisingly, the *vif* gene has high genetic variability^{89; 256; 257; 258; 259} and subtype dependent amino acid substitutions^{188; 260; 261} in patient samples with varying degrees of activity against A3 proteins. Interestingly, defective Vif alleles such as K22H that cannot efficiently neutralize A3G and A3F are readily detected in HIV-1 infected patients.^{89; 259} Recently, a Vif mutant (I107T) was identified in one long-term nonprogressor African patient that failed to interact with A3G and induce A3G degradation.²⁵⁷ Given that I107 is in the ⁹⁶T(Q/D/E)X₅ADX₂(I/L)¹⁰⁷ motif of HIV-1 Vif that has been shown to be critical for Vif activity to induce A3G and A3F degradation as well as its interaction with CUL5,²⁶² the I107T mutant likely attenuates viral infection and profoundly impacts disease progression. Overall, both host and viral genetic diversity play important roles in viral fitness and host innate immunity.

Summary

The main function of A3 proteins is to provide intrinsic immunity to the host by preventing the spread of foreign nucleic acids either through editing (G-to-A hypermutation in the retroviral DNA) and non-editing mechanisms (inhibition of reverse transcription and integration). The host innate immune response is a first line of defense against foreign invaders. A3G, A3F, A3H haplotypes II, V, and VII, (and possibly A3DE) are the main human A3 proteins that block HIV-1 infection. Understanding the interactions between A3 proteins and Vif could provide a unique opportunity to develop novel therapeutic strategies for HIV-1 infected patients.

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Highlights

- APOBEC3DE, APOBEC3F, APOBEC3G, and APOBEC3H haplotypes II, V, and VII inhibit HIV-1 replication.
- HIV-1 Vif protein antagonizes the antiviral activity of APOBEC3 proteins by targeting them for proteasomal degradation.
- Vif-APOBEC3 protein interactions provide a novel target for antiviral drug development.

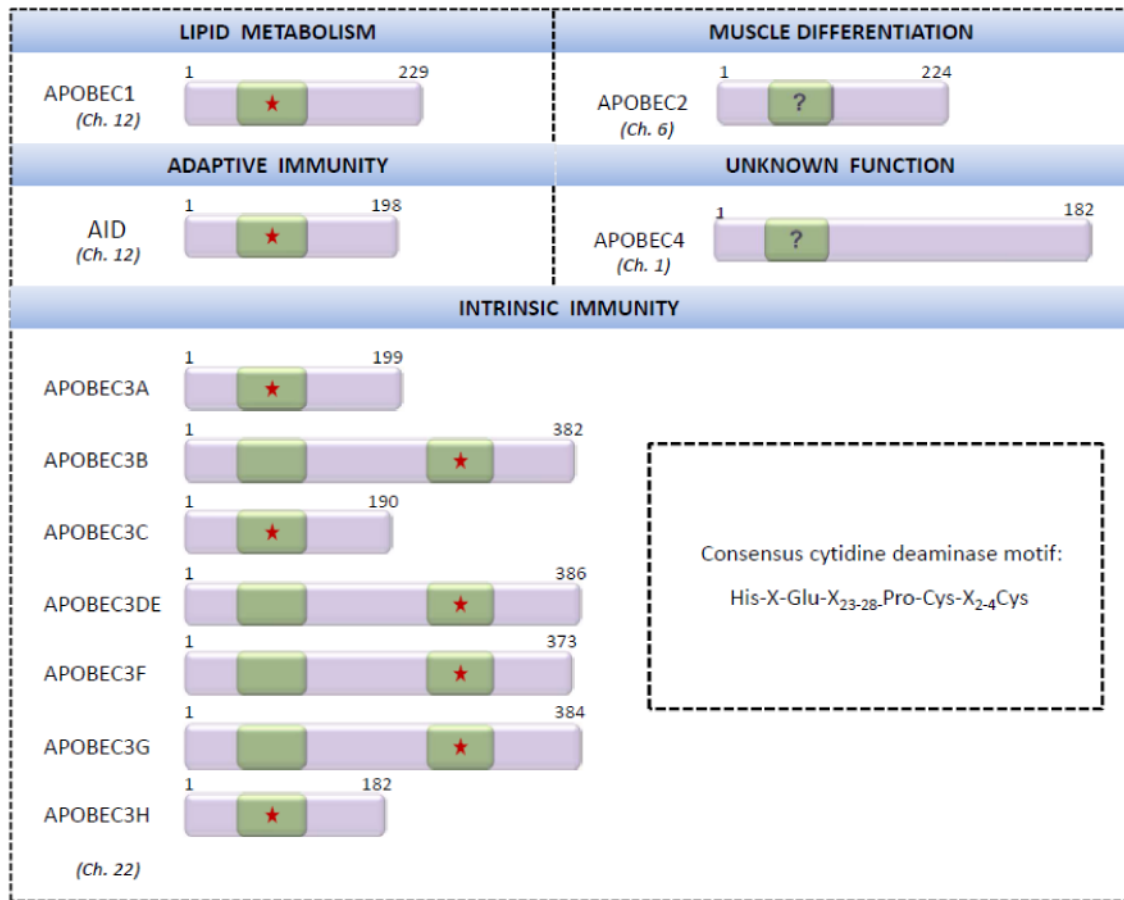


Figure 1. Functional roles of the APOBEC family of proteins

Each APOBEC protein is shown, containing one or two conserved Zn²⁺ coordinating catalytic domains (green); active catalytic domains (red star). Numbers correspond to length of amino acid sequence. Both the catalytically active and non-active domains contain a conserved sequence His-X-Glu-X₂₃₋₂₈-Pro-Cys-X₂₋₄Cys, in which X represents any amino acid. Ch. 1, 6, 12, and 22 refer to human chromosome numbers.

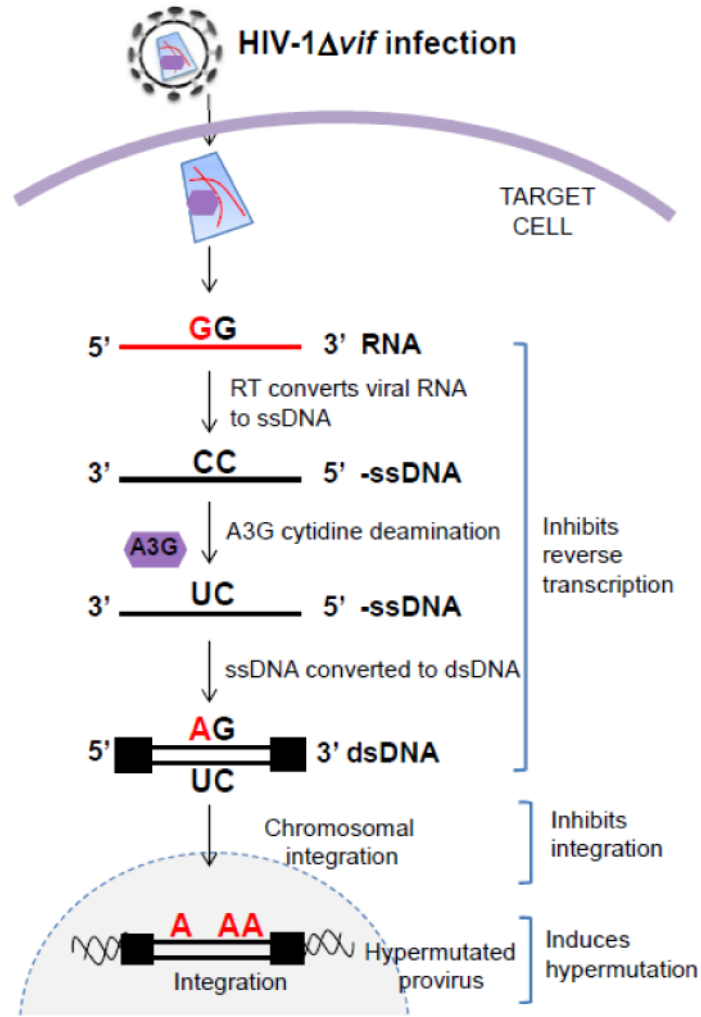


Figure 2. Mechanism of action of A3G during infection with HIV-1 Δ vif virions

In virus producer cells, in the absence of a functional Vif protein, A3G (purple hexagon) is packaged into the viral particles. In the target cell, A3G exerts its antiviral activity by inhibiting reverse transcription, blocking integration, and inducing G-to-A hypermutation. In the hypermutation process, A3G mainly deaminates deoxycytidines in minus-strand DNA to deoxyuridines, which ultimately results in G-to-A hypermutation in the plus-strand DNA. Although hypermutated viral DNA may integrate into the host chromosomal DNA to form proviruses, they are largely defective.

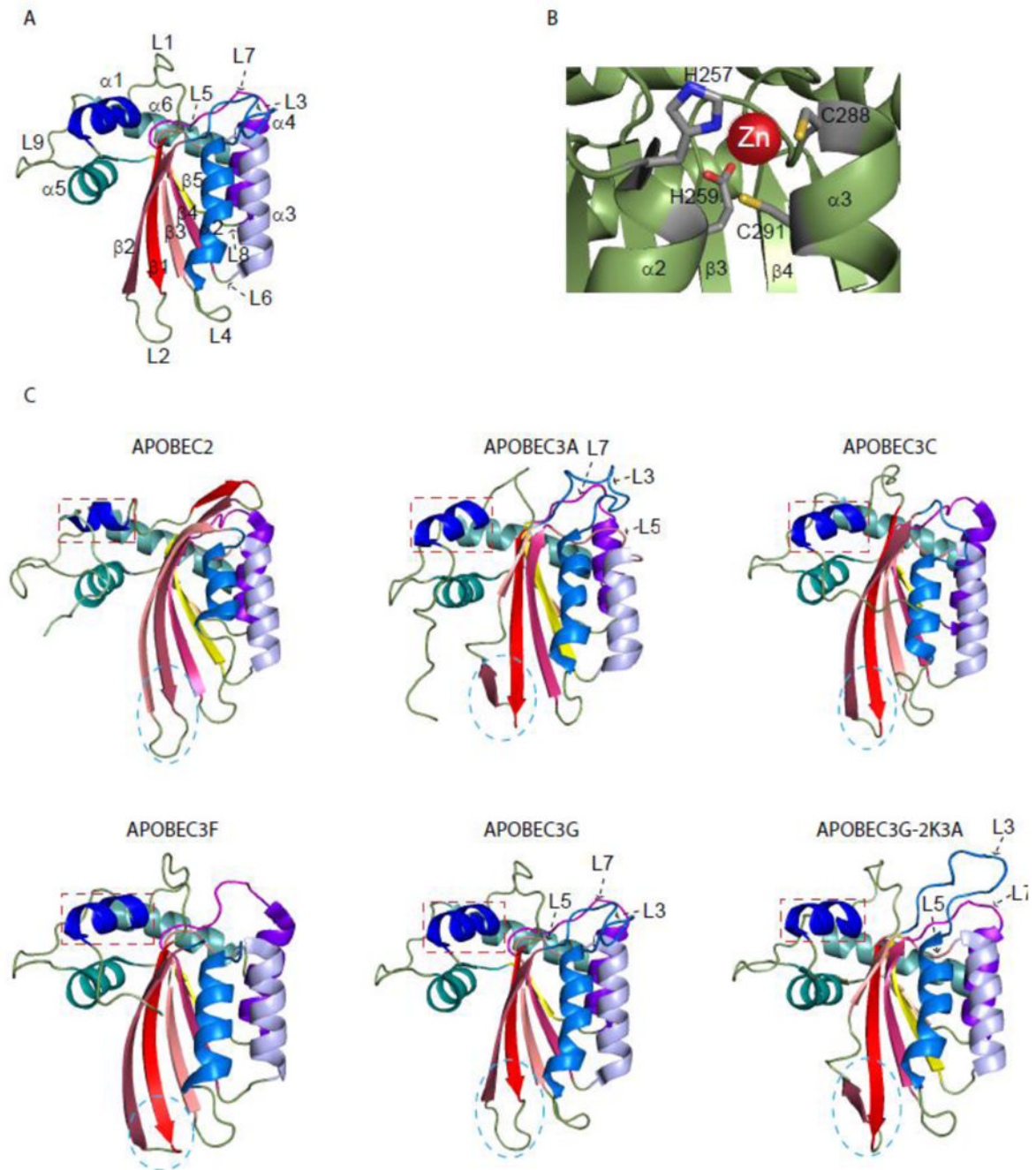


Figure 3. Structural comparison of APOBEC family members' crystal structures

(A) The APOBEC proteins have a canonical deaminase core composed of five β strands and six α helices. As a prototype, the wild-type A3G-CTD monomer structure (PDB entry 3IQS) with the indicated α -helices, β -strands and loops is shown in a ribbon representation. Loop 3, 5, and 7 (L3, L5, and L7, respectively) are shown in blue, orange, and magenta colors, respectively. (B) The active site of A3G-CTD. A zinc atom is coordinated by the three residues H257, C288 and C291, and indirectly via a water molecule (view occluded by zinc atom) with E259. (C) Comparison of ribbon representations of A2 (PDB entry 2NYT), A3A (PDB entry 2M65), A3C (PDB entry 3VOW), A3F-CTD (PDB entry 4IOU), A3G-CTD (PDB entry 3ISQ) and A3G-CTD-2K3A (PDB entry 3IR2) crystal structures. The β 1/ β 2

region is highlighted (blue oval) to emphasize similarities and the conformational plasticity in the β 1- β 2 loop region across all A3 proteins. The α 1 helix (red box) exhibits a similar orientation across all A3 proteins but differs in A2 crystal structure. The corresponding loops (L3, L5, and L7) in Fig. 3A are also labeled in A3A and A3G structures.

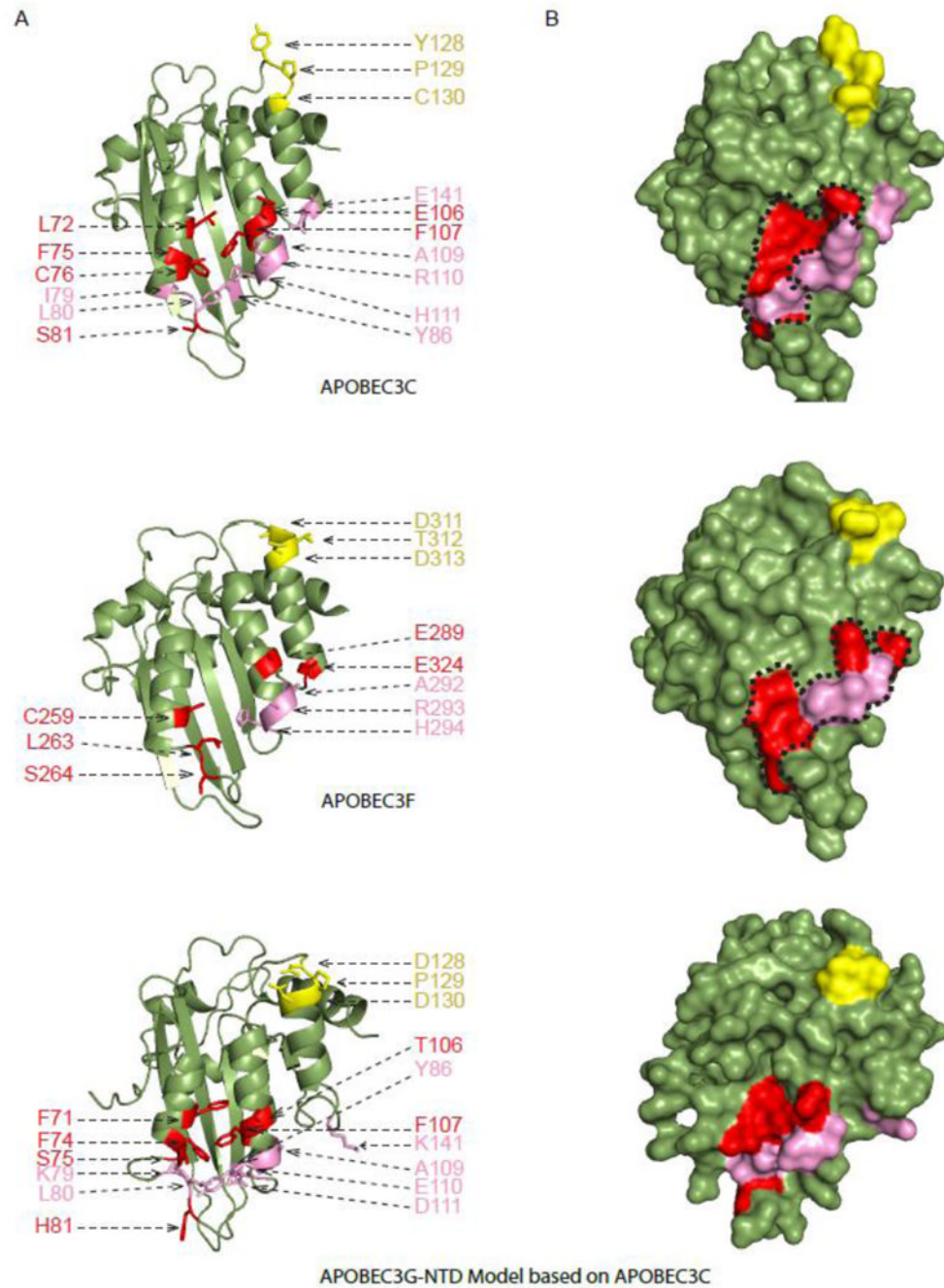


Figure 4. Ribbon diagrams and surface representations of the A3C, A3F-CTD and A3G-NTD model structures

(A) Ribbon diagrams of the structure of A3C (PDB entry 3VOW), A3F-CTD (PDB entry 4IUU) and A3G-NTD, a possible model generated through homology modeling based on the A3C structure (PDB entry 3VOW) by using SwissModel Workspace (<http://swissmodel.expasy.org/>),^{276; 277} showing the known Vif-binding residues labeled in red (critical) and in pink (less critical). The equivalent critical residues in A3G are shown in yellow in A3C and A3F-CTD. The designations of critical and less critical residues are based on mutational analyses.^{49; 51; 52; 65; 66} (B) Surface representation of the Vif-binding

groove delineated by the dashed line in A3C (PDB entry 3VOW), A3F-CTD (PDB entry 4IOU), and A3G-NTD depicted with the same colors used as in (A).

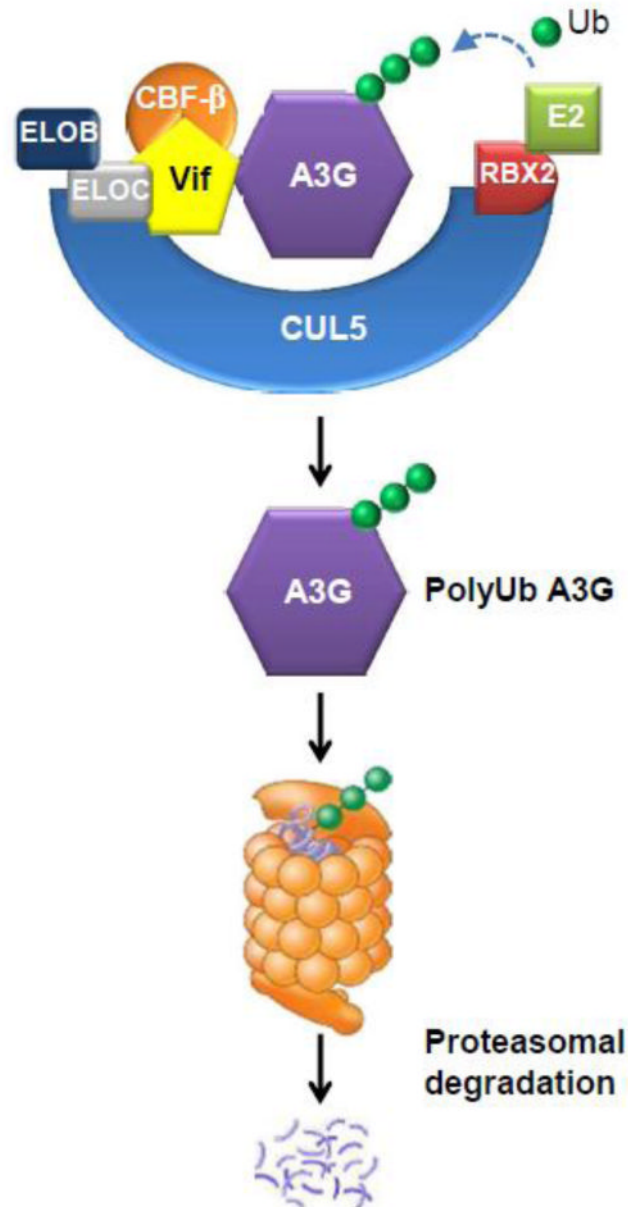


Figure 5. The role of CBF- β in Vif-mediated polyubiquitination and degradation of A3G
 Schematic depiction of different components of the A3G polyubiquitination and degradation pathway. CUL5 (scaffold) directly interacts with Elob/C (a component of the Elob/C adaptors) and RING finger protein 2 (RBX2; regulator of the stepwise cascade of substrate polyubiquitination), which also associates with E2 (ubiquitin conjugating enzyme). Vif (a substrate receptor for A3G) associates with the CUL5-Elob/C-RBX2-E2 complex by binding directly to CUL5 via a conserved Zinc ion coordinating HCCH motif and to Eloc via its SOCS box motif. A3G is recruited by Vif to this complex. Subsequently, A3G is polyubiquitinated and is targeted for 26S proteasomal degradation. CBF- β , a recently discovered cofactor of Vif, has been proposed to be required to facilitate the Vif-CUL5 interaction and to suppress the antiviral activity of A3G in HIV-1 infection.^{111; 112}

Table 1
Host factors that influence the A3G antiviral activity

Host Proteins	Effects on A3G Expression, Function, or Degradation	Possible Mechanism	Ref
CBF- β	Promotes the polyubiquitination and degradation of A3G	Unclear	111; 112
IFN- α , β , γ	Upregulate the expression of A3G	Activation of JAK-STAK signaling pathway; activation of NFATc1/NFATc2 and IRF-4	129; 130; 131; 132; 133; 263
HSP70	Inhibits Vif-mediated A3G degradation	Unclear	138; 139
NEDD8	Promotes the polyubiquitination and degradation of A3G	Activates the cullin-RING ubiquitin ligases by Neddylation	125
FLIP	Upregulates A3G expression	Unclear	145
IL-2, -15, -7	Enhance the expression of A3G	Unclear	146; 264
PKA	Reduces the binding of A3G with Vif	Binds and phosphorylates A3G	143
RPA	Protects ssDNA from A3G binding and blocks the processivity of A3G	Non-specific steric hindrance	265
TLR3	Induction of A3G expression	Unclear	266
PKC α / β /MEK/ERK	Control A3G's mRNA and protein levels	Activates transcription	267
SP1, SP3	Control A3G's mRNA levels	Binds to GC BOX of A3G's promoter	268
IL-1 α , TNF- α	Upregulate the expression of A3G	Unclear	269; 270; 271
PIN1	Reduces the expression of A3G	Interacts with A3G	272
C/EBP- β	Inhibits the activity of A3G	Interacts with A3G	273
TLR2	Downregulates the expression of APOBEC3G	Unclear	274

CBF- β : core binding factor β ; HSP70: heat shock protein 70; FLIP: Fas-associated death domain (FADD) interleukin-1 β -converting enzyme (FLICE)-like inhibitory protein; PKA: protein kinase A; RPA: replication protein A; TLR: Toll-like receptor; MEK: mitogen-activated extracellular signal-related kinase; ERK: extracellular signal-regulated kinases; SP1, SP3: specificity Protein 1/3; PIN1: peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; NF-IL6 (C/EBP- β): CCAAT/enhancer-binding protein β ; IRF-4: interferon regulatory factor 4; NFATc1/2: nuclear factor of activated T-cells, cytoplasmic 1 or 2.

Table 2
The effect of A3G natural variation on HIV-1 pathogenesis

A3G Expression or Polymorphisms	Findings	Ref
Increased expression is associated with	Increased proviral hypermutation; higher CD4 ⁺ T-cell count	237; 238; 246
	Lower viral set point	235
	Resistance to HIV-1 infection after exposure	240
	Lower viral load in long term nonprogressors	234
	Higher CD4 ⁺ cell count in highly exposed seronegative individuals compared to healthy controls and HIV ⁺ patients	236
Unaffected expression	No correlation with control of viremia in elite controllers	242
	In HIV-1 exposed seronegative subjects	241
Decreased expression	Observed upon HIV-1 infection	251
	No correlation with CD4 ⁺ T cell count or viral load	
H186R polymorphism	Association with significantly increased viral load and decreased CD4 ⁺ T cell count with rapid progression to AIDS	244; 250; 251
C40693T polymorphism	An intronic sequence polymorphism associated with increased risk of infection	275
F119F	Associated with protection against disease progression in pediatric patients	249