HIV-1 Vif Requires Core Binding Factor Beta to Degrade the APOBEC3 Restriction Factors and Facilitate Viral Replication

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This work is dedicated to

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for his unwavering support,

eternal patience, soaring spirit, and boundless love.

ABSTRACT

While there are a number of highly active antiretroviral drugs for the treatment of Human Immunodeficiency Virus (HIV), they are all expensive, invasive, susceptible to resistance, and none of them provide a cure. Therefore, one's HIV status is not so much a condition as it is a facet of one's identity. Over 34 million people today are living with HIV and all of the associated physical, psychological, economic, and social implications. My thesis research has focused on identifying novel drug targets for therapeutics that may be used alone or in combination with existing programs to improve treatments and, ultimately, provide an effective cure.

One intriguing drug target is the interaction between the human antiviral APOBEC3 proteins and the HIV counterdefense protein, Vif. HIV Vif binds to and neutralizes the DNA-mutating APOBEC3 proteins by recruitment of an E3 ubiquitin ligase complex that targets them for degradation. Design of small molecule therapeutics to disrupt this interaction and free the antiviral APOBEC3 proteins has been hampered by an incomplete understanding of the Vif E3 ubiquitin ligase complex and conflicting reports as to which of the seven different APOBEC3 proteins may contribute to HIV restriction *in vivo*.

To definitively determine which APOBEC3 proteins contribute to HIV restriction and, thus, may be leveraged to combat HIV infection, we performed a comprehensive analysis of both seven-member human and rhesus macaque APOBEC3 families in T cells. Based on six criteria (expression, virion incorporation, HIV restriction, viral genome mutation, neutralization by Vif, and conservation in the rhesus macaque), we found that four APOBEC3 proteins have the potential to be involved in HIV restriction *in*

vivo. Thus, small molecules that prevent Vif-mediated degradation of all four restrictive APOBEC3 proteins will likely be more effective than those that rescue any one alone.

To better understand the Vif E3 ligase complex responsible for degrading these four, restrictive APOBEC3 proteins, we performed extensive purification experiments with HIV Vif and discovered that Vif interacts with the cellular transcription factor CBFβ. We discovered that CBFβ not only allows for reconstitution of the Vif E3 ligase complex *in vitro*, but also stabilizes Vif *in vivo*, subsequently facilitating ligase assembly and allowing for the efficient degradation of APOBEC3G. Furthermore, this functional interaction is highly conserved, being required to enhance the steady-state levels of Vif proteins from all tested varieties of HIV and required for the degradation of all restrictive human and rhesus APOBEC3 proteins by their respective lentiviral Vif proteins. Surface mutagenesis screening indicated that CBFβ interacts with Vif and its normal RUNX transcription partners on genetically separable interfaces, indicating this essential virus-host interaction may serve as a viable drug target with minimal off-target effects.

Overall, my research shows that HIV is targeted by a swarm of APOBEC3 proteins, all of which are subverted by the viral Vif protein. For Vif to neutralize the APOBEC proteins, it must fist hijack the cellular protein, CBFβ. Disruption of this newly identified, unique, and highly conserved CBFβ-Vif interaction would release the entire multitude of restrictive APOBEC3 proteins and significantly inhibit HIV infection, making this interaction a promising new target for small molecule therapeutics.

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List of Abbreviations

HUMAN GENES

APOBEC3 = Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-like 3
A3A = APOBEC3A
A3B = APOBEC3B
A3C = APOBEC3C
A3D = APOBEC3D
A3F = APOBEC3F
A3G = APOBEC3G
A3H = APOBEC3H
BC-box = Vif SOCS-box motif (ELONGINC binding helix)
CBFβ = Core Binding Factor β
CRL5-Vif-CBFβ = CUL5/RBX2/ ELOB/ELOC/Vif/CBFβ complex
CUL5 = CULLIN5
ELOB = ELONGINB
ELOC = ELONGINC
MDM2 = Murine Double Minute 2
TBP = TATA-binding Protein

VIRUSES, VIRAL GENES, & DISEASES

AIDS = Acquired Immunodeficiency Syndrome
CMV = Cytomegalovirus
EIAV = Equine Infectious Anemia Virus
FFV = Feline Foamy Virus
HAART = Highly Active AntiRetroviral Therapy
HERV = Human Endogenous Retrovirus
HIV = Human Immunodeficiency Virus
HIV-1(2) = Human Immunodeficiency Virus type 1 (type 2)
HTLV-1 = Human T cell Leukemia Virus type 1
LTR = Long Terminal Repeat
MLV = Murine Leukemia Virus
MMTV = Mouse Mammary Tumor Virus
Nef = Negative Regulatory Factor
PERV = Porcine Endogenous Retrovirus
PFV = Primate Foamy Virus
SIV = Simian Immunodeficiency Virus
SIV_{agm} = Simian Immunodeficiency Virus of the African Green Monkey
SIV_{mac} = Simian Immunodeficiency Virus of the Rhesus Macaque
Vif = Viral infectivity factor
Vpr/u/x = Viral protein r/u/x
VSVG = Vesicular Stomatitis Virus G glycoprotein

REAGENTS & METHODS

3D-PCR = Differential DNA Denaturation PCR
AP-MS = Affinity Tag Purification-Mass Spectrometry
DMEM = Dulbecco's Modified Eagle Medium
FBS = Fetal Bovine Serum
EST = Expressed Sequence Tag
GFP = Green Fluorescent Protein
HA tag = Influenza Hemagglutinin tag

HEK293(T) = Human Embryonic Kidney 293 (plus T cell antigen)
HRP = Horseradish Peroxidase
IP = Immunoprecipitate
MiST = Mass Spectrometry Interaction Statistics
MOI = Multiplicity of Infection
PBS = Phosphate Buffered Saline
PCR = Polymerase Chain Reaction
P/S = Penicillin/Streptomycin
qPCR = quantitative PCR
RACE = Rapid Amplification of cDNA Ends
RPMI = Roswell Park Memorial Institute medium
SDS-PAGE = Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
VLP = Virus Like Particle

GENERAL BIOLOGY

ATP = Adenosine Triphosphate
cDNA = complementary DNA
C-terminus(al) = Carboxy-terminus(al)
C-to-U = Cytosine-to-Uracil
DNA = Deoxyribonucleic Acid
dNTP = Deoxyribonucleoside Triphosphate
E. coli =*Escherichia coli* bacteria
gRNA = genomic RNA
GST tag = Glutatione S-Transferase tag
G-to-A = Guanine-to-Adenosine
HMM = High Molecular Mass
mRNA = messenger RNA
N-terminus(al) = Amino-terminus(al)
ORF = Open Reading Frame
PBMC = Peripheral Blood Mononuclear Cell
RNA = Ribonucleic Acid
RNP = Ribonucleoprotein
shRNA = short hairpin RNA
ssDNA = single-strand DNA
Ub = Ubiquitin
UTR = Untranslated Region
Z-domain = Zinc-binding domain

MEASUREMENTS

Da = Daltons
kDa = kiloDaltons
mg = milligram
ml = milliliter
ng = nanogram
nmol = nanomole
pmol = picomole
μg = microgram
μl = microliter
μmol = micromole

CHAPTER 1

Introduction - Leveraging APOBEC3 Proteins to Alter the HIV Mutation Rate and Combat AIDS

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Authors' contributions

J.F. Hultquist and R.S. Harris both contributed to the writing, editing, and figure preparation for the manuscript.

FOREWORD

As the HIV epidemic enters its fourth decade, we can look forward with some hope. The tireless efforts of basic medical researchers and public health activists have advanced our understanding of this deadly virus to the point that we are beginning to turn the tide and are seeing, for the first time, a decline both in AIDS-related deaths and in new HIV infections. However, this fight is still far from over and success will ultimately depend on massive efforts from the global community in developing best practice public health measures, robust treatment options, new preventative measures, and, eventually, effective cures. Until such time as a viable preventative measure or effective cure becomes widely available, many efforts are focused on the improvement and development of new antiviral therapies. Recent FDA approval of a new class of HIV antiretroviral drugs, the entry inhibitors, serves as a proof-of-principle that one practical, and largely untapped, means to inhibit viral replication is by inhibiting essential interactions between host and viral proteins. One such essential interaction for HIV pathogenesis is that between the antiviral APOBEC3 family of human innate immune defense proteins and the HIV counterdefense protein, Vif. In the absence of Vif modulation, the APOBEC3 proteins lethally restrict HIV replication. My research has focused on the APOBEC3 proteins and their counteraction by HIV Vif with the ultimate goal of manipulating this interaction for therapeutic benefit. Here, I briefly delineate the current state of the HIV epidemic and the basic principles underlying antiviral therapy before providing a more detailed review of the APOBEC3 restriction factors, HIV Vif, and how these proteins may be leveraged by novel, small molecule therapeutics to advance human health.

The Global HIV Epidemic

Human Immunodeficiency Virus (HIV) currently infects approximately 34 million people worldwide in a global pandemic particularly severe in sub-Saharan Africa[1]. HIV is a lentivirus, a genus of retrovirus distinguished by its long incubation period. Left untreated, HIV infects and destroys $CD4^+$ T lymphocytes and macrophages, eventually causing Acquired Immunodeficiency Syndrome (AIDS)[2]. Most AIDS patients succumb to opportunistic infection or disease shortly after diagnosis. HIV type 1 (HIV-1) is thought to have originated via cross-species transmission from chimpanzees early in the twentieth century and comprises the bulk of the current day epidemic[3-5]. HIV type 2 (HIV-2) is thought to have originated from the African sooty mangabey, has a longer incubation time, lower morbidity, and is less prevalent[3,6,7]. While there are several treatments for HIV infection, over twenty-five years of focused research efforts have failed to yield a cure.

Like all retroviruses, HIV is a positive-strand RNA virus that reverse transcribes its genome in the host cell for insertion into the host genome as a DNA provirus[2] (**Figure 1-1**). The HIV genome contains three genes required for the mechanics of HIV replication: *gag*, which encodes the structural subunits of the viral core including capsid and nucleocapsid, *pol*, which encodes the reverse transcriptase, integrase, and protease enzymes essential for genome replication, proviral integration, and gag polyprotein processing respectively, and *env*, which encodes the outer envelope protein of the virus essential for infection of susceptible host cells. Besides these three genes common to all retroviruses, HIV also encodes two auxiliary proteins, Tat and Rev, which regulate transcription and splicing respectively (reviewed in[8]).

Additionally, HIV encodes a number of accessory proteins required for replication *in vivo* and in some, but not all, immortalized T cell lines. These proteins generally function as adaptors to E3 ubiquitin ligase complexes that target various host proteins, such as restriction factors and immune signaling components, for polyubiquitination and subsequent degradation (reviewed in[9-13]). HIV negative regulatory factor (Nef), for example, downregulates major histocompatibility complex class I (MHC-I) cell surface expression to prevent viral antigen presentation and subsequent clearance of infected cells by cytotoxic T lymphocytes[14,15]. Viral infectivity factor (Vif) prevents hypermutation and clearance of the viral complementary DNA (cDNA) by degradation of the APOBEC3 family of retrovirus restriction factors[16-23]. HIV-1 specific viral protein U (Vpu) enhances viral particle budding by degradation and sequestration of the BST-2/tetherin restriction factor while HIV-2 specific viral protein X (Vpx) facilitates reverse transcription by degradation of the myeloid lineage restriction factor, SAMHD1[24-27]. Viral protein R (Vpr) forms an E3 ubiquitin ligase complex and induces G2 cell cycle arrest, but the cellular targets are unknown[28-31].

Reigning in the burgeoning HIV epidemic will likely require a combination of public health measures, robust treatment options, preventative measures (pre-exposure prophylaxis or protective vaccination), and, potentially, the development of an effective cure (*i.e.* latency purging drug regimens combined with antiretroviral therapy). All of these require a detailed knowledge of basic HIV biology and pathogenesis, many aspects of which are still being discovered. Until such time as a viable preventative measure or effective cure becomes available, many current efforts are focused on the improvement of existing and on the development of novel antiviral therapies.

Basic Concepts in Antiviral Therapy

The most straightforward and traditional approach to blocking viral replication is through the use of compounds that are engineered to be highly specific inhibitors of viral proteins. Such inhibitors are pervasive among therapeutics today and are often used as standard treatments for chronic viral infections. The treatment of HIV-1, for example, generally includes one or more compounds that inhibit essential HIV-1 proteins such as reverse transcriptase, protease, and integrase. In general, these compounds have few off-target effects and avoid most problems with cytotoxicity. Despite the numerous successes of this approach, a major problem with virus-specific compounds is the evolution of drug resistance. Rapidly evolving viruses such as HIV-1 invariably mutate to alter their amino acid composition and resist the drug. The invariant correlate that all effective drugs eventually select drug resistant viruses is called Coffin's razor. This phenomenon has demanded the application of multi-drug cocktails that potently suppress viral replication and which require multiple, simultaneous alterations in the virus to be overcome. While these Highly Active AntiRetroviral Therapies (HAART) have been employed with great success to help treat and control the HIV-1 epidemic, such drug cocktails are expensive, require strict patient adherence, can elicit invasive side-effects, and fail to provide an effective cure. Improved treatments and treatment strategies are, therefore, in high demand, especially those that exploit new viral targets as these will most readily be able to be incorporated into existing multi-drug programs.

A second general strategy takes advantage of the fact that viruses are dependent on host proteins for replication and pathogenesis. If inessential to the host, such proteins are good targets for antiviral compounds. Cellular proteins are many magnitudes more

stable (less mutable) than viral proteins and therefore much less likely to contribute to the evolution of drug resistance. While this may increase the long-term efficacy of the drug, a potential drawback to this approach lies in the risk that disrupting a cellular process may have unintended consequences or off-target effects. Thus, a thorough knowledge of the human interactome within the realm of the targeted protein and extensive preclinical studies are required for any therapeutic compound utilizing this strategy. As with viral protein inhibitors, administration requires careful calculation and consideration of the cost-to-benefit ratio. This strategy has recently been employed in the development of a new class of HIV-1 therapeutics, the entry inhibitors. This class of drugs works by blocking the interaction between the viral envelope protein and the host co-receptor CCR5, effectively preventing HIV entry into the host cell. HIV is dependent on hundreds of host proteins for replication and pathogenesis; elucidating which of these dependency factors are absolutely essential to viral replication and yet non-essential for host cell survival and function is an important goal towards advancing this therapeutic strategy.

A third and relatively new antiviral strategy has been realized with the discovery of cellular proteins that function to inhibit viral replication. These virus 'restriction factors' are: (i) dominant-acting cellular proteins that potently block a specific stage of the retroviral life cycle, (ii) susceptible to neutralization (or evasion) by viral counter-measures, (iii) under positive selective pressure and so generally more diverse than most other cellular proteins, and (iv) often induced by interferon or by viral infection itself (*i.e.*, as part of the innate immune system) (**Figure 1-2**)[9,11-13]. Examples include APOBEC3G[17,32-35], TRIM5α[36-40], BST-2/tetherin[24,27], and SAMHD1[25,26]. The discovery of these antiviral factors has opened the door to novel therapeutic approaches intended to

facilitate, supplement, or improve endogenous restriction strategies already in place *i.e.* by disruption of the viral counter-restriction mechanism. Indeed, one such lead compound that disrupts the ability of HIV-1 to neutralize the restriction factor APOBEC3G has already been reported, though much work remains to be done defining the optimal targets for disruption, determining the efficacy and safety of such a strategy, and in optimizing the effective compounds for *in vivo* delivery[41].

Attacking the Viral Genome – the APOBEC3 Protein Family

APOBEC3G is the archetype of the APOBEC3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3) subfamily of single-strand DNA (ssDNA) cytidine deaminases. This seven-member group of DNA mutators – APOBEC3A, B, C, D (formerly DE), F, G, & H (hereafter A3A, A3B, A3C, A3D, A3F, A3G, & A3H) - plays a central role in innate immunity, defending the genome against mutation induced by: (i) the invasion of exogenous pathogens such as retroviruses[35,42-53] and (ii) the movement of endogenous retroelements such as Human Endogenous Retrovirus (HERV), LINE and Alu[54-63] (**Table 1-1**). A3G was originally identified as a potent ssDNA mutator[32] with significant homology to the RNA-editing enzyme and family namesake APOBEC1[64]. Concurrently, A3G was identified as a dominant inhibitor of Vif-deficient HIV-1 replication, whose effects could be completely overcome by the neutralizing effects of the Vif accessory protein[17]. The potent DNA deaminase activity of A3G and its ability to restrict HIV-1 replication were quickly found to be linked[32-35]. The mutation and subsequent restriction of Vif-deficient HIV-1 by A3G has been extensively studied and has help set the paradigm for continuing mechanistic studies on the role of DNA

deamination in retroviral restriction and innate immunity.

In the absence of Vif, A3G is encapsidated into the core of budding virions in a manner dependent on an interaction with both RNA and nucleocapsid protein[65-71]. Once the virion fuses to a target cell and deposits its core, the availability of deoxyribonucleoside triphosphates (dNTPs) enables the start of reverse transcription. Within the deposited core, A3G is hypothesized to bind the viral genomic RNA (gRNA) and this action alone is thought to be sufficient to partially inhibit accumulation of reverse transcripts though a deaminase independent mechanism[72-79]. During first (or minus) strand synthesis, RNaseH degrades the gRNA to allow for second strand synthesis, not only liberating A3G, but also exposing its ssDNA substrate[80]. A3G binds and deaminates cytosines-to-uracils (C-to-U) on the exposed minus strand[33-35,42,44,46,81]. This heavily edited cDNA is highly susceptible to degradation, though the identity of the responsible factors remains unclear[74,82-85]. Edited viral cDNA copies that escape degradation template second-strand synthesis. The uridines code for plus-strand adenosines, thus ultimately manifesting as guanine-to-adenine (G-to-A) mutations[33-35,42]. The generation of frequent nonsense and missense mutations usually renders the resulting provirus incapable of further replication and infection (**Figure 1-3**).

The APOBEC family is characterized by a conserved zinc(Z)-binding motif, H-x-E-x_{25-31}-C-x_{2-4}-C, required for deaminase activity[32,42,64,86-88]. These domains are organized in a modular fashion at the APOBEC3 locus to give rise to a series of single or double Z-domain proteins. The human APOBEC3 locus encodes four double Z-domain proteins (A3B, A3D, A3F, and A3G) and three single Z-domain proteins (A3A, A3C, and A3H) from seven genes located in tandem on chromosome 22[64,86] (**Table 1-1**). These loci vary

dramatically across species with, for example, the sheep and cattle loci encoding three single Z-domain proteins and one double Z-domain protein from only three genes[88] or the cat locus encoding four single Z-domain proteins and one double Z-domain protein from four genes[89]. The Z-domains fall into three phylogenetic clusters based on conserved amino acid variations within the zinc-binding motif and are designated type Z1, Z2, or Z3[87,88]. While no functional difference has yet been ascribed to the different domain types, the Z-domain nomenclature has been useful for making cross species comparisons and modeling the evolutionary history of the family[87,88].

The human, double-domain APOBEC3s have several properties that distinguish them from their single-domain family members that may offer clues to their specific physiological functions. First, the double-domain APOBEC3s tend to display a separation of function between domains; one determines ssDNA cytidine deaminase activity and sequence specificity, while the other the other is responsible for subcellular localization, RNA-binding and encapsidation. For example, the amino(N)-terminal catalytic domain of A3G is responsible for viral RNA-binding and is required for encapsidation into the viral core, but it lacks DNA deaminase activity[90]. The carboxy(C)-terminal catalytic domain, on the other hand, confers both DNA deaminase activity and sequence specificity (A3G prefers to deaminate cytidines in a 5'-CC context), but is unable to encapsidate on its own. A3F has a similar functional distribution, though it prefers cytidines in a 5'-TC context[44,86,90,91]. A3B mostly follows this rule with the C-terminal domain being catalytically dominant and determining preference for cytidines in a 5'-TC context[92], but the N-terminal domain still elicits activity capable of mutating the HIV-1 genome[71]. The N-terminal domain of these three proteins also determines subcellular localization,

nuclear for A3B and cytoplasmic for A3F and A3G[57,62,63,93-96]. Notably, the active determinants of subcellular localization overlap with those that determine RNA-binding capacity suggesting the two may go hand-in-hand, though protein-protein interactions have also been implicated in APOBEC3G trafficking and localization[95-97].

Second, the double-domain APOBEC3s are distinguishable from their single-domain family members in their capacity to homo- and hetero-oligomerize to form higher order multimers in cells. A3G has long been known to be capable of homo-oligomerization, an interaction greatly facilitated by RNA[64,70,97-99]. It is also capable of assembling into much larger, heterogeneous high molecular mass (HMM) ribonucleoprotein (RNP) complexes[100]. These HMM RNPs lack definitive identification as either specific RNA granules, such as Staufen-containing RNA granules, or nonspecific stress granules and processing bodies[60,93,101,102]. Either way, A3G in HMM complexes has been shown to be catalytically inactive and incapable of HIV-1 restriction[100]. Like A3G, A3F is also known to self-oligomerize and assemble into HMM complexes[91,102,103]. The relevance of these higher order complexes is not yet known, though several reports provide clues that they may be functionally important to the restriction of retroelements such as L1 and Alu[60,104]. Furthermore, A3G is not restricted to homo-oligomers, but has been shown to hetero-oligomerize with A3B, A3D, and A3F[52,91,105]. It is unknown whether or not these hetero-oligomers are incorporated into HMM complexes and what their physiological relevance may be.

Finally, and perhaps most notably, all of the double-domain APOBEC3s appear to be capable of restricting Vif-deficient HIV-1 while the single-domain APOBEC3s only do so weakly, if at all[106,107] (**Table 1-1**). Double-domain A3G and A3F have consistently

demonstrated an impressive ability to restrict Vif-deficient HIV-1 in both single cycle assays and spreading infections experiments performed by numerous labs[17,33-35,42,44,46,86,91,108]. Likewise, A3B and A3D show clear inhibition by single cycle assays, though there are a number of conflicting reports[44,49,52,57,92,105]. On the other hand, single-domain A3C, while highly efficient at restricting SIV, has only been reported to weakly inhibit HIV-1[44,52,57,58,91,92,105,109]. Of the four A3H variants, only haplotype II appears capable of HIV-1 restriction, though to a lesser extent than either A3F or A3G[110-112]. A3A is also unable to restrict Vif-deficient HIV-1[44,49,52,57-59,74,91,92].

While it is still not known which APOBEC3s are relevant to HIV restriction *in vivo*, several APOBEC3s have been implicated. Analysis of proviral sequences obtained from clinical samples of HIV-1 infected patients shows a mutational spectrum consisting of not only GG-to-AG mutations indicative of A3G, but also of GA-to-AA and GC-to-AC mutations indicative of A3F/A3B and A3D, respectively[52,86,113,114]. While perhaps not an absolute correlation, the ability of the double-domain APOBEC3s to restrict HIV is striking and may be linked with one of the unique physical attributes delineated above.

Besides HIV-1, the APOBEC3 subfamily has been shown to act on ssDNA replication intermediates of other retroviruses such as Simian Immunodeficiency Virus (SIV), Murine Leukemia Virus (MLV), Human T cell Leukemia Virus (HTLV), Porcine Endogenous Retrovirus (PERV), Primate Foamy Virus (PFV), *etc*[35,42-45,47-53,92,115] and retroelements such as HERV, LINE and Alu elements, *etc*[54-63,85,116-118]. While working overall to promote the genomic integrity of the cell, it is unclear what specifically drove the maintenance and rapid expansion of the APOBEC3 locus from one to several unique genes. The single APOBEC3 homolog present in mice was found to be inessential,

though the knockout mice were found to be more susceptible to Mouse Mammary Tumor Virus (MMTV) and MLV infection[119-121]. Did selective pressure from exogenous retrovirus transmission sculpt the locus and/or was the pressure derived from some endogenous retroelement run amuck in the genome? Regardless of factors responsible for shaping the APOBEC3 locus, the current APOBEC3 repertoire provides a potent, intrinsic means by which to defend the host genome from both endogenous and exogenous parasitic elements.

Defending the Viral Genome – Vif and Other Inhibitors

Perhaps nothing speaks to the significance of the APOBEC3 family in viral restriction more clearly than the fact that nearly every relevant retroviral pathogen has evolved a way to neutralize, bypass, or otherwise overcome the APOBEC3 replication block. As any effective antiviral drug obeys Coffin's razor and selects for resistance over time, so too do the APOBEC3s. The APOBEC3s have pressured viruses to evolve a variety of resistance mechanisms that include avoidance, sequestration, and degradation. The emerging theme is that all of these mechanisms appear to work by preventing the encapsidation of APOBEC3s, thereby protecting the viral nucleic acid. The main paradigm for APOBEC3 antagonism has been set by the HIV-1 accessory protein Vif. All lentiviruses but Equine Infectious Anemia Virus (EIAV) encode a Vif accessory protein that, while divergent in sequence, has a conserved function and a largely conserved mechanism of action.

HIV-1 Vif is a highly basic, 23kD accessory protein that, while not strictly required for viral replication, is essential for efficient pathogenesis in certain cell types

including primary $CD4^+$ T cells and macrophages[122-124]. For over two decades, the field has known that a Vif-deficient virus could sustain a spreading infection on certain 'permissive' cell lines, but could not on other 'nonpermissive' cell lines[122-124]. While Vif-deficient virions produced on permissive cells could infect nonpermissive cells, Vif-deficient virions produced on nonpermissive cells lacked the ability to efficiently infect any target cell[122,124]. This indicated that either Vif was required to overcome some viral restriction factor expressed by nonpermissive cells or that the absence of Vif required supplementation by a positive factor in permissive cells. Hybrids formed by the fusion of permissive and nonpermissive cells were unable to support Vif-deficient virion replication arguing for the presence of a dominant negative factor in nonpermissive cells[125,126]. Subtractive hybridization between a parental nonpermissive line and a nearly isogenic, but permissive, daughter line identified A3G as this dominant restriction factor[17]. The ability of A3G to restrict, however, required the absence of Vif. A Vif-proficient virus can overcome APOBEC3-mediated restriction and replicate almost equally well on either type of cell line.

Vif counters A3G-mediated restriction primarily by decreasing the steady-state level of A3G protein in an infected cell. Vif acts as an adaptor protein, linking A3G to an E3 ubiquitin ligase complex additionally composed of ELONGINB (ELOB), ELONGINC (ELOC), CULLIN5 (CUL5), and RBX1[18,20,23]. Bound A3G is then polyubiquitinated and degraded by the 26S proteasome[16,19-23]. In the presence of Vif, the half-life of A3G has been reported to fall from more than 8 hours to anywhere between 4 hours and 5 minutes[16,19,20,22]. While degradation is clearly a main contributor towards the exclusion of A3G from viral particles, several auxiliary mechanisms have been proposed

based on reports showing that the ability of Vif to degrade A3G doesn't necessarily correlate with its ability to restore infectivity or inhibit deaminase activity[127-129]. While the degradation-independent mechanism of A3G inhibition remains unclear, several hypotheses have been posited including direct inhibition of enzymatic activity, indirect inhibition by promoting the incorporation of A3G into HMM complexes, steric hindrance of the interactions required for encapsidation, and inhibition of messenger RNA (mRNA) translation[22,43,128-131] (**Figure 1-4**).

Structural data on the HIV-1 Vif protein remain largely elusive due to the difficulty of expressing high levels of soluble, recombinant protein. Nevertheless, comparative studies between SIV, HIV-1, and HIV-2 have identified several conserved interaction domains that subsequent mutagenesis studies proved essential for coordinating A3G degradation (reviewed by[132]). Two of the most highly conserved motifs serve to recruit the E3 ligase complex: a HCCH zinc-coordinating motif and a SOCS-box motif. The HCCH motif consists of broadly conserved histidine(H)/cysteine(C) pairs and several flanked hydrophobic residues of highly conserved spacing. Zinc-binding by the HCCH residues is thought to maintain a structural conformation that aligns the hydrophobic residues forming the CUL5 binding surface[133-136]. The SOCS-box (or BC-box) motif includes a highly conserved $_{144}$SLQ(Y/F)LA$_{149}$ sequence responsible for the binding of ELOC[18,137-139]. Substitution of the SLQ residues with alanines results in a dramatic loss of HIV-1 infectivity on nonpermissive cell lines due to the inability of this Vif variant to recruit the E3 ligase complex and degrade A3G[23,140]. *In silico* modeling of this conserved amino acid sequence, based on the structures of analogous SOCS-box motifs, predict an alpha helical

form with the conserved hydrophobic residues clustered, allowing for binding in the hydrophobic pocket of ELOC[18]. This prediction was confirmed by recent crystallographic studies showing the HIV-1 Vif BC-box bound to CUL5[141].

The N-terminal domain of HIV-1 Vif is largely responsible for binding the APOBEC3 proteins[19,142-147]. The Vif residues involved are arranged in a nonlinear fashion indicating the involvement of multiple surfaces. For instance, A3G binding is dependent on a hydrophilic patch that includes the conserved $_{23}$SLVK$_{26}$ and $_{40}$YRHHY$_{44}$ motifs as well as on a hydrophobic patch that includes four tryptophans at the very N-terminus and a $_{69}$YWxL$_{72}$ cluster[142,143,145,146,148]. Thus, A3G binding by Vif requires the proper arrangement of hydrophilic and hydrophobic residues on multiple surfaces. This complex binding scheme likely exists to ensure partial A3G binding and neutralization even if Vif has incurred one or more mutations in a binding motif. Vif also can bind and neutralize several other APOBEC3 family members including A3C, A3D, A3F, and A3H[52,91,92,108,109,149] (**Table 1-1**). The N-terminal domain of Vif is similarly responsible for the binding of these APOBEC3s, though through other residues. A3F binding, for example, is mediated by residues $_{14}$DRMR$_{17}$ and an exclusive set of two tryptophans at the N-terminus[146,147,150]. A3C, A3D, and A3F are thought to bind Vif through similar residues, and the recent crystallographic structure of A3C implicates a conserved cleft between alpha helices 2 and 3 for Vif binding, though a co-crystal structure will be required to tease apart the complete interface[151].

Several other Vif domains are also crucial for HIV infectivity in nonpermissive cells. The central, hydrophilic $_{88}$EWRKKR$_{93}$ motif is essential for protein stability, mutation of which causes a dramatic drop in Vif steady-state levels[152]. The $_{161}$PPLP$_{164}$

proline-rich domain is required for Vif homo-multimerization. Disruption of multimerization by either mutation of the domain or by expression of a peptide antagonist ablates the ability of Vif to prevent A3G encapsidation[153-155]. Finally, an RNA-binding domain exists at the N-terminus that mediates Vif interaction with viral genomic RNA[156-158]. Mutations of key residues within this domain also render HIV-1 incapable of replication on nonpermissive cells[158]. This RNA interaction is also required for efficient Vif incorporation into virions, though the role of Vif within the virions is unclear[157,159-161]. It may be that Vif acts in the particle to inhibit A3G in a degradation-independent manner (as discussed earlier) or that Vif is required to fulfill some other role in the particle required for replication. For example, Vif is thought to have several roles similar to an RNA chaperone and it may function during genome folding and processing[162,163].

In contrast to APOBEC3G, which can effectively inhibit a broad array of retroviruses from other species including SIV, EIAV, HTLV, PERV and MLV[35,42-45,92,115], the potency of Vif and Vif-like molecules appears somewhat species-specific. For example, HIV-1 Vif can neutralize human A3G, but not African green monkey (Agm) A3G. Similarly, SIV_{agm} Vif can neutralize African green monkey A3G, but not human A3G[43,164]. This specificity has been traced to a single amino acid in human A3G at position 128, which as an aspartate allows for the binding of HIV-1 Vif and as a lysine allows for the binding of SIV_{agm} Vif[165-168]. This tendency towards species specificity likely reflects the strong positive selection Vif is under to neutralize the particular APOBEC3 repertoire of its host species[43]. This is contrasted by the selective pressure exerted on the APOBEC3s to restrict a diverse set of targets including not only human lentiviruses, but other viruses and retroelements both human and non-human. This mutual

selection for broad antiviral activity by the APOBEC3 repertoire and for species specificity by the viral APOBEC3 antagonist may help explain why retroviral zoonotic transmissions are relatively rare[88,106,115].

While Vif-directed proteasomal degradation has provided a useful paradigm, several other mechanisms exist by which APOBEC3-mediated restriction is successfully avoided (**Figure 1-4**). For example, the foamy viruses are a family of complex viruses that infect a variety of mammals and whose replication can be inhibited by APOBEC3 proteins[48,50,53]. The Primate Foamy Virus (PFV) accessory protein Bet functions similarly to Vif and can rescue infectivity of Vif-deficient HIV-1. Similarly, PFV Bet appears to function in a somewhat species-specific manner as it can bind to both human A3G and African green monkey A3G, but not to mouse APOBEC3[50]. However, neither PFV Bet nor the related Feline Foamy Virus (FFV) Bet has been shown to decrease steady-state levels of their target APOBEC3s. It is hypothesized that Bet instead functions to sequester the APOBEC3s in the cell, thereby preventing encapsidation and restriction[48,50] (**Figure 1-4**).

The ability of a virus to neutralize the APOBEC3s is not even always dependent on the presence of a specialized accessory protein such as Vif or Bet. For example, MLV is a simpler virus that lacks accessory genes. It has been found to be resistant to its host APOBEC3 (mouse APOBEC3), though not to human A3G[47]. This is due to a failure of mouse APOBEC3 to encapsidate, likely due to its failure to efficiently bind MLV capsid protein. It has been hypothesized that MLV Gag has evolved to avoid mouse APOBEC3 binding, and that this may be aided by an inhibitory effect the viral RNA has on this interaction[47,51]. This strategy appears similar to that used by HTLV-1 to evade human

A3G. HTLV-1 similarly lacks a Vif or Bet-like accessory protein, but rather uses a novel motif at the C-terminus of the nucleocapsid to prevent A3G encapsidation[169]. A3G packaging is dependent on RNA and on a direct or indirect association with viral nucleocapsid[65,68,70,97,170]. It is hypothesized that this C-terminal motif precludes or disrupts the crucial A3G/nucleocapsid association[169] (**Figure 1-4**).

The prevailing trend is that every APOBEC3 susceptible retroelement has evolved a means to escape restriction. In addition to the mechanisms reviewed here - degradation, neutralization, and avoidance - it is likely that viruses as a whole possess many other novel strategies for evading the APOBEC3 proteins. For example, recent data from our lab demonstrate that HIV-1 is capable of overcoming APOBEC3-mediated restriction by yet another means, tolerance[171]. Passaged continuously on CEM-SS cells stably expressing A3G, a Vif-deficient virus can evolve A3G resistance by accumulating both a pyrimidine at position 200 and a null mutation in the accessory gene Vpr. While the role of the Vpr mutation is unknown, the A200C/T mutation was shown to dramatically increase viral titer by increasing translational efficiency[172]. This increase in viral titer serves to effectively titrate out available A3G, dropping the mutational load back to tolerable levels[171,172]. As our knowledge of host-virus interactions continues to expand, other APOBEC3 evasion mechanisms are certain to emerge.

Modulating the APOBEC3s to Facilitate Virus Eradication

The remarkable success of HIV as a pathogen can be partially attributed to an optimal mutation rate that allows for stable transmission to successive generations while seeding sufficient diversity to allow for quick evolution in response to selective pressure

(**Figure 1-5A**). Therapeutically, then, alteration of the viral mutation rate provides two conceivable means by which to restrict the virus *in vivo*. Either the mutation rate must be increased to a level that prohibits stable transmission (hypermutation), or the mutation rate must be decreased to inhibit the evolution of resistance and so foster susceptibility to existent selective pressure (hypomutation). The A3G/Vif interaction provides a putative means by which to control the mutation rate and investigate both of these novel drug design strategies.

Hypermutation: A3G functions to increase the viral mutation rate and preclude stable transmission, while Vif functions to rein in A3G and restore the mutation rate to sublethal levels (middle panel, **Figure 1-5B**). Thus, if the A3G/Vif interaction could be disrupted or if Vif function could be disabled, the viral mutation rate would be predicted to soar to lethal levels (top panel, **Figure 1-5B**). Hypermutated viral cDNA would be subject to degradation or would encode frequent nonsense and missense mutations rendering the virus incapable of replication as seen *ex vivo* and as detailed above. In other words, A3G-mediated hypermutagenesis would push the genome beyond the threshold for genomic stability, causing catastrophic error and replication failure. Recently, a screen to identify small molecule inhibitors of the A3G/Vif interaction discovered a number of promising compounds[41]. One inhibitor, RN-18, functions to alter targeting of the A3G/Vif/E3 ligase complex to polyubiquitinate Vif instead of A3G. This results in Vif degradation, increased encapsidation of A3G, and inhibition of viral replication.

While an attractive strategy, the use of hypermutagenesis to destroy the virus is inherently risky. First of all, very little is known about the mechanisms controlling the ability of A3G to discriminate between self from non-self DNA. A3G can act as a

genomic DNA mutator in heterologous systems and poses an intrinsic hazard if not properly regulated[32,56]. While A3G is cytoplasmic in human cells and sequestered from the genome, several redundant mechanisms most likely exist to ensure proper discrimination and must exist for those APOBEC3s residing in the nucleus. Despite these systems, there is evidence that the nuclear APOBEC3s come at a cost to the host with maintenance dependent upon the cost-to-benefit ratio. For example, some human populations have lost nuclear-localizing A3B[173]. While there are no clear clinical manifestations of this event, it is possible that maintenance of the deletion is being driven by unintended, off-target deamination events whose costs outweigh the benefits of normal A3B function. Were any therapeutic to disrupt the enigmatic processes regulating APOBEC3 discrimination of self from non-self, the results could be catastrophic with the resultant genomic G-to-A hypermutation leading to massive cell death and/or to cancer.

Hypermutagenesis is also a risky strategy because it depends on reaching a lethal level of mutation in the viral genome sufficient to induce collapse of the population (**Figure 1-5A**). Failure to reach that threshold would promote further genetic diversification and could enhance the virus' ability to become resistant to immune responses and/or therapeutics. If A3G wasn't completely neutralized by the virus, for example, it could be used to alter the mutation rate in the face of selective pressure and could contribute to the creation of a new resistant population. In this respect, Vif may act as a regulator of viral mutation rate and serve to modulate APOBEC3 levels rather than eliminate them[174] (middle panel, **Figure 1-5B**). In other words, the optimal mutation rate of the virus may be dependent on the exploitation of the host's APOBEC3 proteins to seed genetic diversity and so the APOBEC3 proteins may be beneficial to the virus in

instances of sublethal mutation. Thus, any therapeutic designed to enhance or restore APOBEC3 function would have to do so fully and without altering its normal regulatory mechanisms.

Finally, the A3G/Vif interaction may be a non-ideal target for disruption based on the genetic flexibility of the Vif gene itself. While other therapeutics inhibit the action of essential viral proteins with limited mutagenic potential, such as integrase, protease, or reverse transcriptase, Vif is an accessory protein whose primary function is to overcome APOBEC3-mediated restriction. Thus, it is unclear how readily HIV-1 Vif can and will mutate to overcome small molecule inhibition of the interaction. Moreover, as discussed above, Vif-deficient HIV-1 has been shown to evolve resistance to A3G in a Vif-independent manner[171]. Based on the wide range of mechanisms employed by various viruses to overcome the APOBEC3s (**Figure 1-4**), much more research is required on the ability of HIV-1 to evolve resistance before therapeutics are developed and tested clinically.

Hypomutation: As opposed to hypermutagenesis, which comes with a fair number of caveats, it is tempting to consider what would happen if one could render HIV less mutable (**Figure 1-5A**). HIV-1 depends on an optimal mutation rate to ensure genetic variability in the face of an unending series of selective pressures. When selective pressure is exerted on the population in the form of an adaptive immune response or drug, there typically exist a number of resistant clones that survive and serve to repopulate the host. Hence, Coffin's razor is dependent on a basal level of genetic variability in the population. By dropping the mutation rate of the virus, it has been hypothesized that the genetic variation of the virus could be sufficiently diminished such that it is no longer

able to evade the host immune system and antiviral compounds[174] (bottom panel, **Figure 1-5B**).

One contributing factor to the genetic variation of an HIV-1 population is likely the APOBEC3 family itself[175-178]. The main function of Vif is to rein in APOBEC3-mediated hypermutation and restore the mutation rate to sublethal levels. However, it is known that Vif doesn't always neutralize A3G completely. For example, early in infection, A3G levels have not yet been depleted and Vif levels are still on the rise resulting in a window where neutralization is incomplete[171]. Also, defective Vif alleles, which would lead to incomplete neutralization of A3G, often arise *in vivo* and are detected in HIV-1 isolates from infected patients[179]. Furthermore, in clinical samples from patients infected with HIV-1, some proviral sequences show evidence of G-to-A hypermutation demonstrating a failure by Vif to completely neutralize the APOBEC3 proteins[113]. While it is unclear to what extent the viruses depend on the APOBEC3s as sources of 'beneficial' mutations, there is some evidence that these mutations can lead to drug resistance. Considering all mutations associated with HIV-1 drug resistance, G-to-A mutations are the most frequent[175]. While reverse transcriptase itself is responsible for a number of G-to-A mutations even a minor dependency on the APOBEC3s for variation can be exploited by designing specific APOBEC3 inhibitors as therapeutics[174]. Even if APOBEC3 inhibitors do not diminish the genetic variability of the virus enough to allow the adaptive immune system to clear infection by itself, they may still be useful as adjuvants that can enhance the long-term efficacy of other currently available therapies.

This approach is also not without its risks. Again, the normal physiological function of the APOBEC3s in the absence of viral infection is unknown and it is unclear

what effects complete inhibition may have on the cell. Current data are consistent with an exclusive role in retroelement restriction, but more studies are needed to bolster this important point. For instance, while mouse APOBEC3 is inessential[119-121] and human A3B null populations lack a definitive clinical symptom[173], unintended effects cannot be ruled out at this time. Furthermore, secondary viral infections in the absence of APOBEC3-mediated restriction may be significantly exacerbated. Such a therapy must also be careful to preserve the function of Activation Induced Deaminase (AID), an APOBEC3-related deaminase responsible for antibody gene diversification (reviewed in[180]). All therapeutics and antiviral drugs come with their own benefits and risks and so all require extensive pre-clinical research and rigorous clinical testing. However, the use of small compounds to alter the mutation rate of HIV-1 by leveraging APOBEC3 function is likely to provide, either alone or in conjunction with other antiviral drugs, a viable therapeutic option in the future.

ADDITIONAL CONTRIBUTIONS

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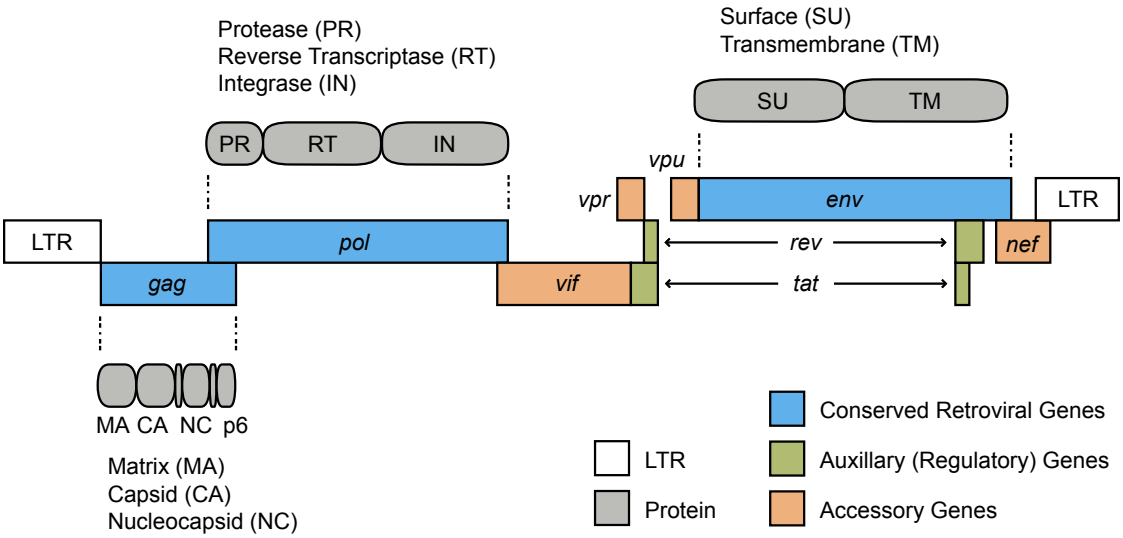

Figure 1-1. Schematic of the HIV-1 Genome and Encoded Polyproteins.

The HIV-1 genome contains three genes (blue) that are common to all retroviruses and that encode polyproteins, which are subsequently processed within the cell and/or virus particles to their mature forms (represented in gray). These include *gag*, which encodes structural components of the viral core, *pol*, which encodes essential viral enzymes, and *env*, which encodes the outer viral envelope. HIV-1 also contains two auxillary genes (green), *tat* and *rev*, whose gene products regulate transcription and splicing respectively. Finally, it contains four accessory genes (orange) with roles in immune evasion/modulation: *vif, vpr*, *vpu*, and *nef*. These nine genes are flanked by non-coding long terminal repeats (LTRs, white) that allow for successful reverse transcription, proviral integration, and HIV-1 gene expression.

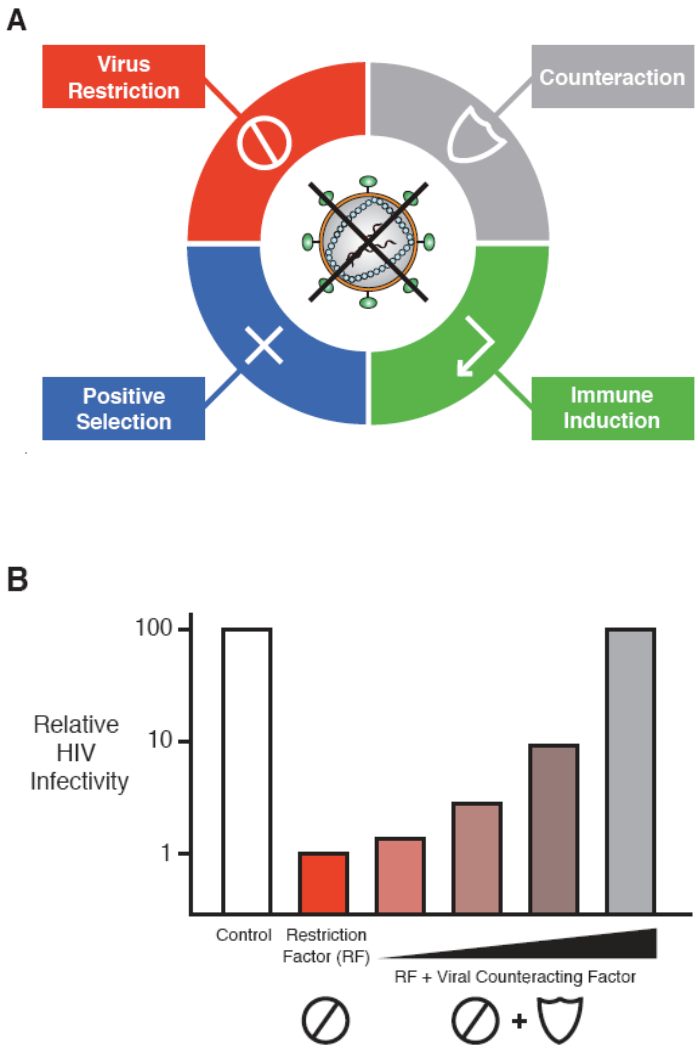

Figure 1-2. The Hallmarks of Restriction Factors.

(A) Four defining hallmarks of an HIV restriction factor include dominant restriction of virus replication (null sign), a virus encoded counteraction mechanism (shield sign), interferon-responsiveness (promoter sign), and positive selection signatures (plus sign; clockwise from top left).

(B) Histogram depicting virus infectivity in the presence of a restriction factor and a dose-response of the viral counteraction mechanism. Reprinted with permission from[11].

	A3A	A3B	A3C	A3D	A3F	A3G	A3H
Domains	Z1	Z2 Z1	Z2	Z2 Z2	Z2 Z2	Z2 Z1	Z3
Subcellular Localization	Cell Wide	Nuclear	Cell Wide	Cytoplasmic	Cytoplasmic	Cytoplasmic	Cell Wide
ΔVif HIV Restriction	No	Yes	Weak	Yes	Strong	Strong	Some Variants
HIV Vif Interaction	No	No	Yes	Yes	Yes	Yes	Yes
Expression	PBLs, spleen, bone marrow, lung	PBLs, bone marrow, lung, stem cells	PBLs, thymus, spleen, lymph node, testis, ovary, small intestine, colon, liver, pancreas, heart, lung, adipose	PBLs, thymus, spleen, lymph node, ovary, lung, liver, adipose	PBLs, thymus, spleen, lymph node, testis, ovary, uterus, brain, lung, colon, liver, kidney, and pancreas	PBLs, thymus, spleen, lymph node, testis, ovary, uterus, brain, lung, small intestine, colon, liver, kidney, and pancreas	PBLs, thymus, testis, ovary, brain, small intestine, colon, skin

Table 1-1. The Human APOBEC3 Repertoire.

See text for references. Expression data compiled from[181,182].

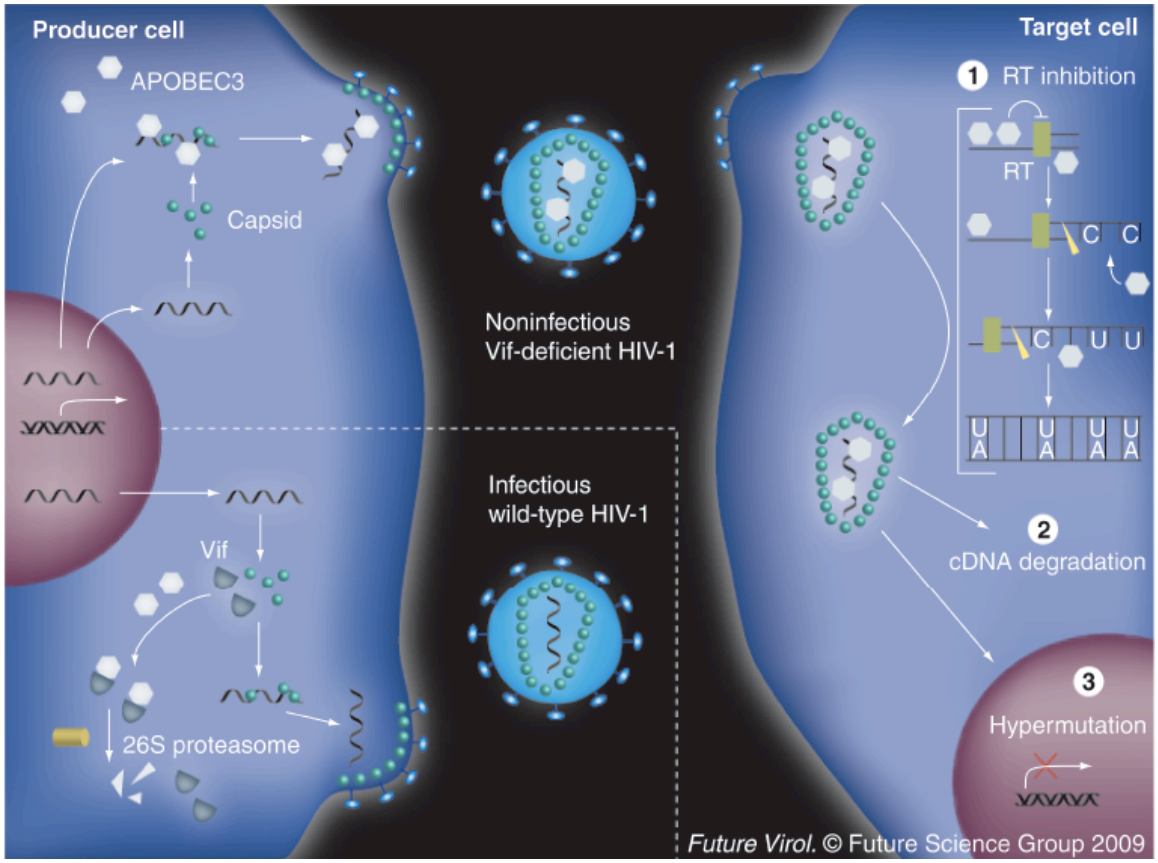

Figure 1-3. Mechanism of APOBEC3G-mediated HIV-1 Restriction.

The integrated provirus in the producer cell is transcribed and either packaged as genomic RNA (gRNA) or processed and translated to produce essential viral proteins such as capsid, reverse transcriptase, and integrase. In the absence of Vif (upper left), APOBEC3G can be encapsidated within the nucleic acid containing core of the budding virus dependent on an interaction with both gRNA and nucleocapsid protein. This virion will be rendered replication deficient by three APOBEC3G-dependent mechanisms that occur after the viral core is deposited in the target cell. 1) APOBEC3G binds the gRNA inhibiting reverse transcription in a deaminase-independent manner. 2) Closely following reverse transcription, the gRNA is degraded by RNaseH both freeing APOBEC3G from

its RNA-bound state and exposing the single-stranded DNA of the minus strand. APOBEC3G deaminates available cytidines to uridines resulting in hypermutation. These mutations are fixed as G-to-A transitions upon second strand synthesis. This heavily edited viral cDNA is highly susceptible to degradation by yet unidentified factors. 3) If the cDNA escapes degradation and is integrated, the frequent nonsense and missense mutations within the provirus prevent further replication. In the presence of Vif (lower left), APOBEC3G is polyubiquitinated and degraded by the 26S proteasome. Thus, the budding virions are fully infectious.

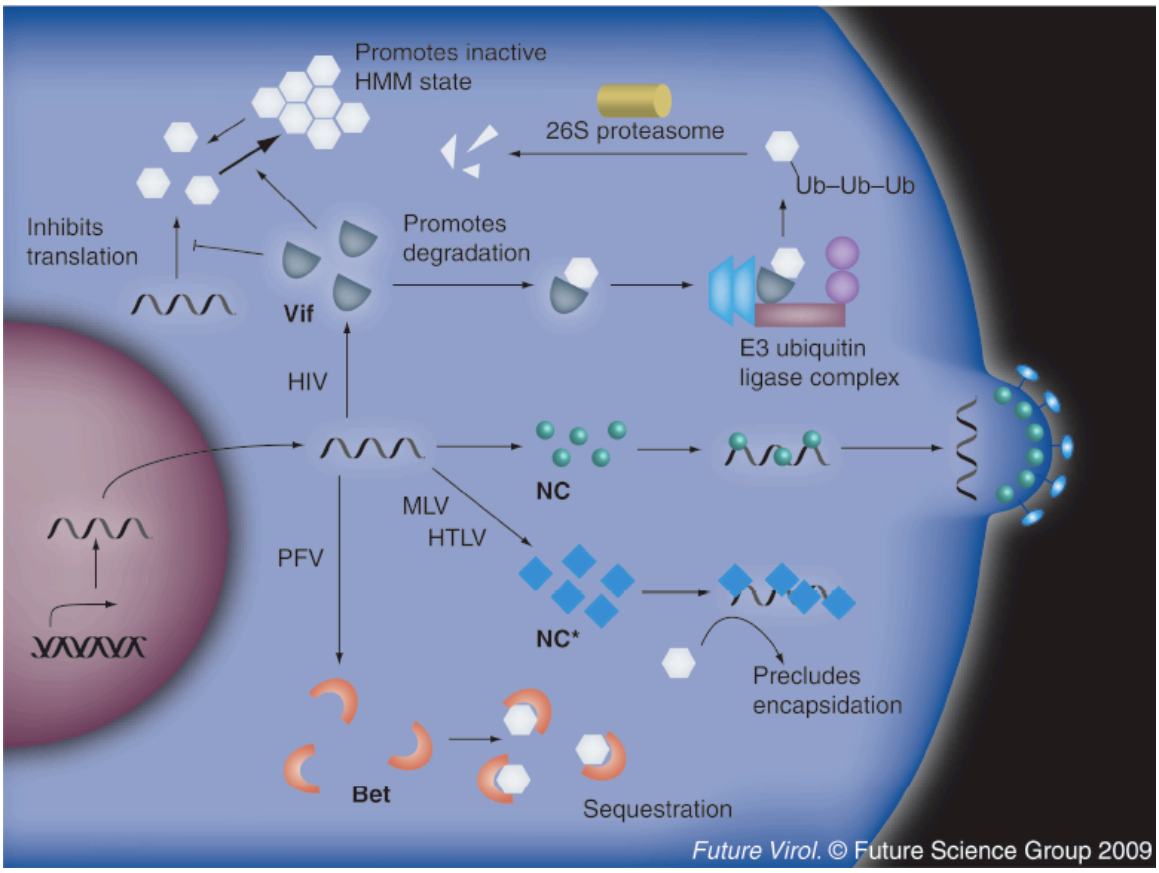

Figure 1-4. Mechanisms of Natural APOBEC3 Resistance.

Every relevant retroviral pathogen has evolved a mechanism to overcome APOBEC3-mediated restriction. For example, the HIV-1 accessory protein Vif protein acts as an adaptor between several human APOBEC3s and an ELOB/C-CUL5-RBX1 E3 ubiquitin ligase complex which polyubiquitinylates APOBEC3, targeting it for degradation by the 26S proteasome. This effectively decreases the steady-state levels of APOBEC3 in the cell, preventing sufficient encapsidation for viral inhibition. Vif is also hypothesized to have secondary, degradation-independent mechanisms to overcome the APOBEC3 replication block. These include translational inhibition and promotion of APOBEC3 accumulation in inactive HMM complexes. All of these mechanisms serve to inhibit the

encapsidation of APOBEC3 and allow for the production of infectious viruses. The foamy viruses such as PFV similarly encode an accessory protein, Bet, that binds APOBEC3G and prevents it from encapsidating. Bet doesn't promote APOBEC3 degradation, however, but rather is hypothesized to sequester APOBEC3 away from the sites of virion budding. MLV and HTLV don't require a specific accessory protein to overcome APOBEC3-mediated restriction. They both encode specialized nucleocapsid proteins that preclude APOBEC3 binding and encapsidation.

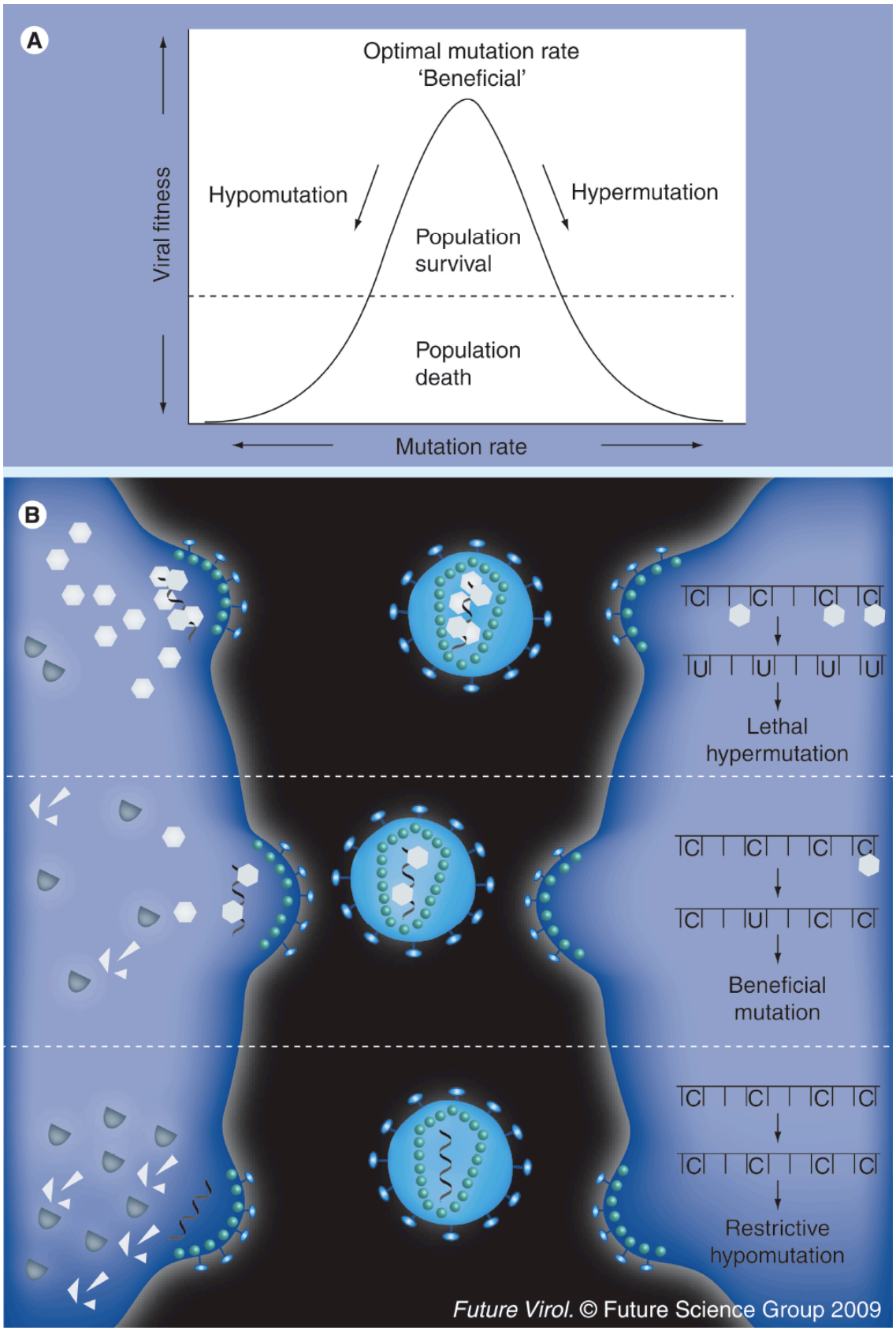
A
Optimal mutation rate
'Beneficial'
Hypomutation
Hypermutation
Viral fitness
Population
survival
Population
death
Mutation rate
B
Lethal
hypermutation
Beneficial
mutation
Restrictive
hypomutation
Future Virol. © Future Science Group 2009

Figure 1-5. Leveraging the Activity of the APOBEC3s to Alter the HIV-1 Mutation Rate.

(A) HIV-1 depends on an optimal mutation rate that allows for stable transmission to successive generations while seeding sufficient diversity to allow for quick evolution in response to selective pressure. Large increases in the mutation rate (hypermutagenesis) will prevent stable transmission of the genetic material to successive generations, decreasing viral fitness to lethal levels. On the other hand, decreases in the mutation rate (hypomutagenesis) will impede the viruses' ability to evolve resistance to other sources of selective pressure and so can be equally detrimental to viral fitness. Figure adapted from[174].

(B) The optimal mutation rate of HIV-1 is modulated at least partially by the APOBEC3 mutators and their viral inhibitor, Vif, making them attractive targets for therapeutic compounds (middle panel). If Vif function could be inhibited or APOBEC3 activity augmented, high levels of APOBEC3 would be packaged into budding virions (top panel). This would increase the viral mutation rate and the resulting hypermutated viral cDNAs would either be degraded or code nonfunctional proteins. On the other hand, if APOBEC3 activity could be abolished or Vif function enhanced, virtually no APOBEC3 would be encapsidated (bottom panel). This would decrease the viral mutation rate and inhibit accumulation of genetic diversity, impairing the virus' ability to evolve resistance to the adaptive immune system and other antiviral compounds.

CHAPTER 2

Human and Rhesus APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H Demonstrate a Conserved Capacity to Restrict Vif-deficient HIV-1

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Authors' contributions

J.F. Hultquist performed all experiments, wrote/revised the manuscript, and designed all figures except as follows. J. Lengyel created the stable rhesus APOBEC3 expressing SupT11 cell lines and performed the spreading infection experiment on those lines. E.W. Refsland performed qPCR on the infected, primary $CD4^+$ T lymphocytes and helped with primary cell isolation. R.S. LaRue cloned rhesus APOBEC3A. L. Lackey captured the microscopy images. W.L. Brown aided in the creation of the stable human APOBEC3 expressing SupT11 cell lines. R.S. Harris aided in the design of the experiments and in manuscript preparation/revision.

FOREWORD

While there is considerable evidence that the APOBEC3-Vif axis may be leveraged by novel therapeutics for the treatment of HIV, one of the major hurdles lies in deciding which of the many functional interactions to target. This becomes particularly difficult if multiple APOBEC3 family members are involved, *i.e.* if the family demonstrates functional redundancy. For example, treatment by hypomutation is not likely to be successful unless all APOBEC3 proteins contributing to HIV mutation are significantly inhibited. On the other hand, treatment by hypermutation carries the inherent risk of seeding its own resistance mutation and so success will depend on presenting a near insurmountable restriction barrier that, ideally, requires multiple adaptations to bypass. Indeed, recent evidence suggests that, even in the absence of Vif, HIV can adapt to bypass restriction mediated by physiologic levels of APOBEC3G. This implies that efforts to disrupt the ability of Vif to counteract the APOBEC3 proteins will be more successful if they are able to liberate multiple, restrictive APOBEC3 proteins rather than any one alone. As either therapeutic strategy relies on a complete definition of the restrictive APOBEC3 repertoire, and as this remains an active point of debate in the field, we decided to perform a comprehensive analysis of both seven-member human and rhesus APOBEC3 families to determine which ones may contribute to lentiviral restriction and mutation *in vivo*. We found that four APOBEC3 proteins, A3D, A3F, A3G, and A3H, demonstrate a conserved capacity to restrict HIV-1 and that all four are counteracted by their respective lentiviral Vif protein. Thus, either therapeutic route will require consideration of all four of these proteins for ultimate success.

SUMMARY

Successful intracellular pathogens must evade or neutralize the innate immune defenses of their host cells and render the cellular environment permissive for replication. For example, to replicate efficiently in $CD4^+$ T lymphocytes, HIV-1 encodes a protein called Vif that promotes pathogenesis by triggering degradation of the retrovirus restriction factor, APOBEC3G. Other APOBEC3 proteins have been implicated in HIV-1 restriction, but the relevant repertoire remains ambiguous. Here, we present the first comprehensive analysis of the complete, seven-member human and rhesus APOBEC3 families in HIV-1 restriction. In addition to APOBEC3G, we find that three other human APOBEC3 proteins, APOBEC3D, APOBEC3F, and APOBEC3H, are all potent HIV-1 restriction factors. These four proteins are expressed in $CD4^+$ T lymphocytes, package into and restrict Vif-deficient HIV-1 when stably expressed in T cells, mutate proviral DNA, and are counteracted by HIV-1 Vif. Furthermore, APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H of the rhesus macaque also package into and restrict Vif-deficient HIV-1 when stably expressed in T cells, and they are all neutralized by the SIV_{mac} lentiviral Vif protein. On the other hand, neither human nor rhesus APOBEC3A, APOBEC3B, or APOBEC3C had a significant impact on HIV-1 replication. These data strongly implicate a combination four APOBEC3 proteins – APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H – in HIV-1 restriction.

INTRODUCTION

Restriction factors are dominant-acting cellular proteins that provide an innate defense against invasive pathogens. APOBEC3G (A3G) is a prototypical example, which functions to block the replication of a broad number of endogenous mobile elements and exogenous viral pathogens, such as Human Immunodeficiency Virus type 1 (hereafter HIV). For a pathogen to replicate efficiently and be successful, it must evade or neutralize the relevant restriction factors of its host. HIV and related lentiviruses, for example, encode a viral infectivity factor (Vif) protein that promotes pathogenesis by triggering A3G degradation (reviewed by[9,12,13,183]). A3G is a DNA cytosine deaminase, which restricts retroviruses by incorporating into budding virions, inhibiting reverse transcription, and subsequently mutating the viral complementary DNA (cDNA) by deamination of cytosines-to-uracils. To overcome this replication block, HIV Vif targets A3G for polyubiquitination and subsequent degradation by the proteasome. Efforts are ongoing to develop therapeutics that disrupt the A3G/Vif interaction and thus render HIV susceptible to A3G-mediated restriction (*e.g.*[41]).

APOBEC3-mediated deamination of cytosine-to-uracil (C-to-U) in viral replication intermediates templates the insertion of plus-strand adenines and accounts for the well-documented occurrence of guanine-to-adenine (G-to-A) hypermutation in patient-derived viral DNA sequences[113,114,184,185]. A3G is unique as it strongly prefers to deaminate the second cytosine of 5'-CC dinucleotide motifs, resulting in 5'-GG-to-AG mutations, whereas the six other APOBEC3 proteins prefer to deaminate cytosines in 5'-TC dinucleotide motifs, resulting in 5'-GA-to-AA mutations (*e.g.*[42,44,52,86,91,105,109,111,186]).

Patient-derived HIV DNA sequences show both G-to-A hypermutation signatures, strongly implicating A3G and at least one other APOBEC3 protein in HIV restriction.

Determining the restrictive APOBEC3 repertoire in $CD4^+$ T lymphocytes is critical for identifying normal innate defenses that may be leveraged by therapeutics to combat HIV. With the exception of A3G, there is little consensus as to which of the other six APOBEC3 proteins contribute to HIV restriction *in vivo* (see aforementioned reviews). APOBEC3F (A3F) has been implicated in Vif-deficient HIV restriction when expressed transiently in HEK293T cells and stably in T cell lines, but two recent studies question its importance[187,188]. The restrictive capacity of the other five APOBEC3 proteins, APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3C (A3C), APOBEC3D (A3D; formerly A3DE), and APOBEC3H (A3H), has primarily been examined through transient expression in Human Embryonic Kidney (HEK) 293 cells with widely varying results and no overall consensus.

Based on the mechanistic paradigm provided by A3G, we predict that all APOBEC3 proteins that contribute to HIV restriction should all share at least five, and possibly six, commonalities. First, they should be expressed in physiologically relevant $CD4^+$ T lymphocytes. Second, they should package into Vif-deficient HIV virions when stably expressed in T cells. Third, they should restrict Vif-deficient HIV when packaged into virions. Fourth, they should be neutralized by HIV Vif, as Vif-proficient viruses can replicate without significant restriction in primary $CD4^+$ T lymphocytes. Fifth, they should result in the significant accumulation of G-to-A hypermutations in integrated proviruses during infection. Finally, they may be functionally conserved in closely related species such as the rhesus macaque, though this may not be true in all cases as the

ongoing evolutionary struggle between host and pathogen leads to rapid diversification[189].

The APOBEC3 locus has undergone major expansion during the evolutionary radiation of primates[88]. Humans, chimpanzees, and at least one Old World monkey, the rhesus macaque, appear to share similar locus architectures with a seven-protein coding capacity of analogous domain organization ([88,112] and this study). These proteins undergo high rates of amino acid substitution, or positive selection, which, together with the locus expansion, is consistent with numerous historical and ongoing host-pathogen conflicts[190]. While the distinct and overlapping functions of each of the seven APOBEC3 proteins are still under investigation, we hypothesize that homologous proteins among the hominids and Old World monkeys may still have similar functional properties.

Here, we present a comprehensive, functional analysis of both the human and rhesus macaque APOBEC3 repertoires to determine which human APOBEC3 proteins contribute to HIV restriction based on the six criteria above. Each human and rhesus APOBEC3 protein was expressed either stably in isogenic T cell lines or transiently in HEK293T cells and challenged with Vif-proficient or Vif-deficient HIV while APOBEC3 expression, packaging, viral infectivity, and proviral mutation were monitored. These results indicate that A3D, A3F, A3G, and A3H can all contribute to HIV restriction and may all be valid targets for mono- or combinatorial therapeutic development. Furthermore, the homologous APOBEC3 proteins of the rhesus macaque are similarly able to restrict Vif-deficient HIV and are targeted for degradation by SIV_{mac239} Vif, suggesting that a similarly complex story may underlie innate restriction of

other lentiviruses and possibly other exogenous pathogens in humans, related primates, and other mammals.

RESULTS

T Cell Stimulation and HIV Infection Alter *APOBEC3* Expression Profiles

$CD4^+$ T lymphocytes express six of the seven human *APOBEC3* mRNAs, *A3B*, *A3C*, *A3D*, *A3F*, *A3G*, and *A3H*, the five latter of which are induced upon stimulation with IL-2/PHA[182]. To determine if any of these are similarly induced by HIV infection, naïve $CD4^+$ T lymphocytes were isolated from peripheral blood mononuclear cells (PBMCs) of three independent donors by negative selection. All selections achieved greater than 90% purity of $CD4^+$ cells (*i.e.* **Figure 2-1A** and data not shown). Purified $CD4^+$ cells were stimulated with either IL-2/PHA or CD2/CD3/CD28 beads as verified by CD25 staining (**Figure 2-1B** and data not shown). 72 hours after stimulation, these cells were mock-infected or infected with HIV in several parallel cultures. Every few days, mRNA was isolated from one culture per condition and *APOBEC3* expression monitored by qPCR. Periodic infection of a reporter line, CEM-GFP, with culture supernatant indicated that viral titers peaked at 7 days post-infection in the cultures from each donor. Representative data from the T lymphocytes of one donor stimulated with CD2/CD3/CD28 beads are shown in **Figure 2-1C**.

Consistent with prior studies, *A3C*, *A3D*, *A3F*, *A3G*, and *A3H* were all expressed in naïve $CD4^+$ T lymphocytes and all induced upon stimulation (**Figure 2-1C,** naïve vs day 0, $p<0.01$)[182]. *A3B* was barely above the threshold for detection in naïve and stimulated cells while *A3A* was undetectable. Up to nine days post-infection, *APOBEC3*

levels in the mock-infected cultures remained at or slightly below the levels observed soon after stimulation (**Figure 2-1C**, day 0 vs mock-infected). In the HIV-infected cultures, on the other hand, expression levels of particularly *A3C, A3G,* and *A3H* were significantly higher than in the mock-infected cultures throughout the experiment (**Figure 2-1C,** mock vs HIV-infected at each timepoint, $p<0.01$). Similar results were also observed in T lymphocytes from an independent donor stimulated with CD2/CD3/CD28 and in T lymphocytes from a third donor stimulated with IL-2/PHA (data not shown). Thus, while six of the seven *APOBEC3*s are detectable in $CD4^+$ T lymphocytes, five of them are induced upon stimulation and at least three of them*, A3C, A3G* and *A3H*, are further induced upon HIV infection. While these levels may be further modulated by immune signaling *in vivo*, at least six of the seven APOBEC3 proteins are expressed in $CD4^+$ T lymphocytes and so are appropriately placed to contribute to HIV restriction.

Human A3D, A3F, A3G, and A3H Package Efficiently into Viral Particles and Restrict Vif-deficient HIV in T Cells

Most of our knowledge concerning the restrictive potential of the APOBEC3 proteins is based on the effects of transient over-expression on the infectivity of a replication-deficient reporter virus produced on HEK293T cells during a single infectious cycle. In order to assay the ability of each APOBEC3 protein to restrict replication-proficient HIV over numerous infectious cycles, we generated a clonal set of T cell lines stably expressing each human APOBEC3 protein with a carboxy(C)-terminal triple hemagglutinin (HA) tag. These panels of clones exhibited similar and overlapping ranges of expression by immunoblotting (representative sets in **Figures 2-2A** and **2-S1**) and fall

in a near physiologic range (data not shown). The parental T cell line, SupT11, is nearly devoid of all APOBEC3 expression and, accordingly, is permissive to infection by Vif-deficient HIV ([191] and **Figure 2-S1**). With the exception of A3H, all APOBEC3 proteins match the consensus human sequences, including A3B, whose toxicity to *Escherichia coli* (*E. coli*) was overcome with an intron-disrupted cDNA (**Materials & Methods**). The documented instability of some A3H alleles was mitigated by using the cDNA for stable haplotype II[192].

For an APOBEC3 protein to access viral cDNA during reverse transcription and restrict the virus, it must be packaged into the budding virion from the producer cell. To test which APOBEC3 proteins are capable of packaging into Vif-deficient HIV virions, each stable T cell line was infected with Vesicular Stomatitis Virus G glycoprotein (VSVG) pseudotyped, Vif-deficient HIV to achieve 25% initial infection. After allowing 12 hours for viral entry, the cells were washed and placed in fresh media. After another 36 hours to allow for new virus production, the supernatants were collected and virus-like particles (VLPs) were isolated by centrifugation. Immunoblots confirmed the accumulation of similar amounts of viral p24 in each culture, indicating that each line is equally capable of producing mature virions (**Figure 2-2A**). Furthermore, immunoblotting for the HA-tagged APOBEC3 proteins in the viral lysates indicated that only four, A3D, A3F, A3G, and A3H, are efficiently packaged into HIV viral particles (**Figure 2-2A**), while A3A, A3B, and A3C are undetectable.

To assay the effects of stable expression of each APOBEC3 protein on the replication of HIV over time, concurrent spreading infections with Vif-proficient and Vif-deficient HIV were initiated on panels of independently-derived SupT11 clones each

stably expressing an individual APOBEC3 protein. Every two to three days for three weeks, supernatant was removed from each culture and used to infect a reporter cell line, CEM-GFP, which expresses GFP upon HIV infection[193]. The percent of GFP-positive reporter cells directly correlates with live viral titer, which, when plotted over time, indicates the kinetics of the viral infection. For each APOBEC3, several independent lines were assayed to control for clonal variation (**Figure 2-S1**). Spreading infection data from one representative line from each set of APOBEC3-expressing clones is shown in **Figure 2-2B**. In this system, APOBEC3-mediated restriction of viral infection manifests as either a delay in or ablation of Vif-deficient viral replication in comparison to the Vif-proficient virus.

At 1% multiplicity of infection (MOI), the Vif-proficient virus peaked just prior to the Vif-deficient virus in both our parental SupT11 line and our lines with stably integrated empty vector (**Figures 2-2B** and **2-S1**). This may be due to the loss of some secondary function of Vif that aids in viral replication[163,194]. Vif-deficient virus replicated with similar kinetics on lines stably expressing various levels of A3A, A3B, or A3C. On A3D expressing lines, however, Vif-deficient viruses displayed an extended delay to peak in comparison to the Vif-proficient virus (**Figures 2-2B** and **2-S1**). In contrast, A3F, A3G, and A3H all caused a complete suppression of detectable Vif-deficient viral replication over the course of the experiment (**Figures 2-2B** and **2-S1**). Surprisingly, most A3H stable cell lines also caused a complete suppression of detectable Vif-proficient viral replication, indicating a possible resistance to the antagonistic effects of HIV Vif as previously reported[192,195,196]. Reconstruction experiments with untagged A3H supported this likelihood as these lines appear more permissive for Vif-proficient, but not

Vif-deficient, HIV replication (**Figure 2-2C**). While these data suggest a C-terminal tag may provide A3H some protection from Vif-mediated degradation, higher levels of untagged A3H were also sufficient to overcome the antagonistic effects of HIV Vif (**Figure 2-2C**), likely indicating some level of innate resistance. Overall, these data show that human A3D, A3F, A3G, and A3H all are capable of restricting Vif-deficient HIV in T cell lines and that this activity correlates directly with packaging into viral particles.

Human A3B, A3D, A3F, A3G, and A3H Package Efficiently into Viral Particles and Restrict Vif-deficient HIV in HEK293T Cells

To compare the above SupT11 T cell data with the numerous accounts of transient APOBEC3 expression in HEK293T cells, we used the same APOBEC3 expression vectors as used to generate our stable T cell clones to perform a complementary set of single cycle experiments. HEK293T cells were transiently transfected with either Vif-proficient or Vif-deficient HIV proviral construct alongside a gradient of each APOBEC3. After 48 hours to allow for virus production, viral supernatants were purified and used to infect CEM-GFP cells to assay infectivity. Cell and VLP lysates were collected concurrently to assay APOBEC3 expression and packaging efficiency.

As in SupT11 T cells, transiently expressed A3D, A3F, A3G, and A3H all restricted Vif-deficient HIV (**Figure 2-3A**). A3G achieved greater than 50% restriction at only a 1:40 APOBEC3:virus cotransfection ratio, while a similar level of restriction required four times as much A3F and A3H or eight times as much A3D. Surprisingly, unlike in T cells, A3B also was able to restrict and with just as much potency as A3G.

A3A and A3C were unable to achieve 50% restriction even at the highest expression levels. Restriction correlated strongly with packaging efficiency. A3B, A3D, A3F, A3G, and A3H were all able to package even at the lowest level of detection in the cell lysates (**Figure 2-3C**). A3A and A3C could be detected in virions, but only at the highest transfection ratios. In the absence of virus, no APOBEC3 was detected in purified supernatants (**Figure 2-3C**, far right).

HIV Vif, however, was able to counteract A3D, A3F, and A3G-mediated restriction, all of which only reached 50% restriction of Vif-proficient HIV at the highest APOBEC3:virus cotransfection ratio (**Figure 2-3B**). A3H restriction was only slightly counteracted in the presence of HIV Vif and only at low transfection ratios, in agreement with the spreading infection data (**Figure 2-2B**). HIV Vif did not effect A3B restriction while A3A and A3C remained unrestrictive. These observations correlate well with expression and packaging efficiency. Compared to expression achieved in the presence of Vif-deficient HIV virions, cellular steady-state levels of A3C, A3D, A3F, A3G, and, to a lesser extent, A3H were all decreased in the presence of HIV Vif (**Figure 2-3D**). Packaging of each was similarly decreased or undetectable. The expression level and packaging efficiency of A3A and A3B were unaffected by the presence of HIV Vif.

Overall, A3D, A3F, A3G, and A3H are all able to package into and restrict Vif-deficient HIV in both T cells and HEK293T cells. Similarly, all are Vif-sensitive, though A3H to a lesser extent. One major difference between model systems is that, while A3B can package into and restrict Vif-deficient HIV in HEK293T cells, it can do neither when stably expressed in the SupT11 T cell line. To determine if this was a SupT11-specific phenomenon, a panel of clones stably expressing A3B was constructed in the permissive

T cell line, CEM-SS. Vif-deficient and Vif-proficient HIV replication on these lines was also unaffected by A3B as compared to vector controls (data not shown). Moreover, although the same plasmid DNA stock was used in the aforementioned transient and stable expression experiments, the A3B cDNA was recovered from representative clones and confirmed correct by DNA sequencing. Therefore, this discrepancy likely reflects an intrinsic and potentially critical difference between the two cell-based systems.

Rhesus A3D, A3F, A3G, and A3H Package Efficiently into Viral Particles and Restrict Vif-deficient HIV in T Cells

The rhesus macaque is currently the best animal model for testing the efficacy and safety of many HIV therapeutics *in vivo*[197]. It has been previously reported that rhesus A3F, A3G, and A3H can restrict Vif-deficient HIV in single cycle assays in HEK293T cells while rhesus A3B, A3C, and A3D cannot[43,164,198]. No rhesus A3A ortholog has been reported. We hypothesize that, if human A3D, A3F, A3G, and A3H are antiretroviral factors, their homologs may show some functional conservation in the rhesus macaque. To test this hypothesis, we performed a comprehensive analysis of the entire rhesus macaque APOBEC3 repertoire in both T cells and HEK293T cells using HIV again as our model retrovirus.

To begin these analyses, we first aligned the publicly available rhesus macaque genomic sequence with trace files from the National Center for Biotechnology Information (NCBI) Whole Genome Shotgun (WGS) sequencing project for rhesus macaque, as well as with available rhesus high-throughput genomic sequencing (HTGS) and expressed sequence tag (EST) sequences to generate a locus assembly[199]. This

assembly displayed remarkable similarity to the human locus and contained evidence for a few exons of the previously unidentified rhesus A3A. Using 3' Rapid Amplification of cDNA Ends (3'RACE) on cDNA from rhesus PBMCs, we were able to clone the complete rhesus A3A transcript encoding a 202 amino acid protein of 82% identity and 87% similarity to human A3A. With the identification of rhesus A3A, the copy number and domain organization of the human and rhesus APOBEC3 repertoires appear perfectly syntenous with each homologous pair displaying at least 77% identity (**Figure 2-4A**). We detected no functional difference between the two rhesus A3D isoforms, A3D-I and A3D-II[164], and so only data for A3D-I are shown here.

The entire rhesus APOBEC3 repertoire was cloned with either a C-terminal Green Fluorescent Protein (GFP) or triple HA tag to facilitate localization and retroviral restriction assays, respectively. The human and rhesus GFP-tagged repertoires were transfected into HeLa cells and imaged by fluorescent microscopy (**Figure 2-4A**). Rhesus A3D, A3F, A3G, and A3H all localized solely to the cytoplasm while rhesus A3B was mainly nuclear. Rhesus A3A and A3C distributed cell-wide. This is in concurrence with the human repertoire; human A3D, A3F, A3G, and A3H localized to the cytoplasm, human A3B was mainly nuclear, and human A3A and A3C distributed cell-wide (**Figure 2-4A** and *e.g.*[62,63]).

To assay the ability of each rhesus APOBEC3 protein to package into and restrict replication-proficient HIV over numerous infectious cycles, we generated a clonal set of SupT11 T cell lines expressing each rhesus APOBEC3 protein with a C-terminal triple HA-tag. To assay viral production and APOBEC3 packaging, each stable T cell line was first infected with VSVG-pseudotyped, Vif-deficient HIV to achieve 25% initial

infection. Immunoblots of VLP lysates collected 48 hours after infection confirmed the accumulation of similar amounts of viral p24 in each culture, indicating each line is equally capable of producing mature virions (**Figure 2-4B**). Furthermore, immunoblots of the viral lysates indicated that only rhesus A3D, A3F, A3G, and A3H are efficiently packaged into HIV viral particles when produced in T cell lines (**Figure 2-4B**).

To assay the effects of stable expression of each rhesus APOBEC3 protein on the replication of HIV over time, concurrent spreading infections with Vif-proficient and Vif-deficient HIV were initiated on panels of independently derived cell lines stably expressing each rhesus APOBEC3 protein (**Figures 2-4C** and **2-S2**). As with the human repertoire, the Vif-proficient virus peaked just prior to the Vif-deficient virus in our vector control lines as well as in our lines stably expressing rhesus A3A, A3B, or A3C. On the other hand, all cell lines expressing rhesus A3D, A3F, A3G, or A3H caused complete suppression of Vif-deficient virus replication. Vif-proficient virus was also unable to replicate on cell lines expressing rhesus A3F and A3H, but replicated comparably to vector controls in rhesus A3D lines and with delayed kinetics on rhesus A3G lines. This suggests that HIV Vif is able to fully neutralize rhesus A3D and partially neutralize rhesus A3G, while it is ineffective against rhesus A3F and A3H (**Figures 2-4C**, **2-S2**, and [164]). Thus, apart from differential Vif sensitivities, these data are in perfect concordance with the human repertoire; rhesus A3D, A3F, A3G, and A3H all are capable of restricting Vif-deficient HIV in T cell lines while rhesus A3A, A3B, and A3C are not.

Rhesus A3D, A3F, A3G, and A3H Package Efficiently into Viral Particles and Restrict Vif-deficient HIV in HEK293T Cells

To test the sensitivity of each rhesus APOBEC3 protein to HIV Vif and their ability to restrict in the HEK293T model system, we cotransfected either Vif-proficient or Vif-deficient HIV proviral construct into HEK293T cells alongside a gradient of each rhesus APOBEC3. As in T cells and in correspondence with the human repertoire, rhesus A3D, A3F, A3G, and A3H were all able to restrict Vif-deficient HIV in HEK293T cells (**Figure 2-5A**). All four restrictive rhesus APOBEC3 proteins achieved greater than 50% restriction at near a 1:40 APOBEC3:virus cotransfection ratio, though rhesus A3H appeared particularly restrictive. Unlike its human homolog, rhesus A3B was unable to significantly restrict Vif-deficient HIV. Similar to rhesus A3B, rhesus A3A and A3C were unable to achieve 50% restriction even at the highest expression level. Again, restriction correlated strongly with packaging efficiency. Rhesus A3D, A3F, A3G, and A3H were all able to package even at the lowest level of detection in the cell lysates (**Figure 2-5C**). Rhesus A3A and A3C could be detected in virions, but only at the highest expression levels. Rhesus A3B, while packaging better than rhesus A3A and A3C, was much less efficient than the restrictive APOBEC3s. Again, without virus, APOBEC3 proteins were undetectable in purified supernatants (**Figure 2-5C**, no virus controls).

As predicted from the spreading infection, HIV Vif did not appear to affect the restrictive capacity, expression, or packaging efficiency of rhesus A3F and rhesus A3H (**Figures 2-5B** and **2-5D**). Similarly, no significant effect on the restrictive capacity, expression, or packaging of rhesus A3G was observed despite the ability of Vif-proficient HIV to replicate, albeit poorly, on stable rhesus A3G-expressing T cell lines (**Figures 2-**

4C, 2-5B, and **2-5D**). On the other hand, HIV Vif was able to counteract rhesus A3D, leading to a decrease in its restriction capacity (**Figure 2-5B**), a decrease in its steady-state level of expression, and a near ablation of its capacity to package (**Figure 2-5D**). Unrestrictive rhesus A3A, A3B, and A3C appeared unaffected by the presence of HIV Vif.

Simian Immunodeficiency Virus of the rhesus macaque strain 239 (SIV_{mac239}) requires Vif to replicate *in vivo* and overcome restriction by the APOBEC3 proteins of the rhesus macaque[200]. Thus, we hypothesized that, while HIV Vif does not, SIV_{mac239} Vif should neutralize all the restrictive APOBEC3s of the rhesus macaque. To determine the sensitivity of the rhesus APOBEC3 proteins to SIV Vif, single cycle assays in HEK293T cells were performed with a constant amount of provirus cotransfected alongside a constant amount of rhesus APOBEC3 and either an empty vector, SIV Vif, or an SIV $Vif_{SLQ->AAA}$ mutant unable to degrade the APOBEC3 proteins[18,23,133,138]. Viral supernatants were monitored for infectivity and VLPs collected for immunoblots. We observed that SIV Vif was able to partially rescue viral infectivity in the presence of rhesus A3D, A3F, A3G, and A3H. Accordingly, SIV Vif inhibited the packaging efficiency of rhesus A3D, A3F, A3G, and A3H as well as rhesus A3C and to a lesser extent rhesus A3B (**Figure 2-6**). Rhesus A3F was less sensitive to SIV Vif than the other restrictive APOBEC3 proteins, in concordance with prior literature[164,198]. Thus, in agreement with the human repertoire, all of the restrictive, rhesus APOBEC3 proteins are targeted by their host lentiviral Vif.

Overall, rhesus A3D, A3F, A3G, and A3H are all able to restrict Vif-deficient HIV in both T cells and HEK293T cells. Of these, only rhesus A3D appears sensitive to

HIV Vif, but rhesus A3D, A3F, A3G, and A3H are all sensitive to degradation by SIV Vif. Thus, four lines of evidence support a role for human A3D, A3F, A3G, and A3H in retroviral restriction: 1) their ability to restrict Vif-deficient HIV in T cell and HEK293T model systems, 2) their degradation and neutralization by HIV Vif, 3) the conserved capacity of rhesus A3D, A3F, A3G, and A3H to restrict Vif-deficient HIV in T cells and HEK293Ts, and 4) the conserved capacity of SIV Vif to neutralize rhesus A3D, A3F, A3G, and A3H.

Human A3D, A3F, A3G, and A3H Cause Proviral G-to-A Hypermutation

A3G restricts Vif-deficient HIV largely through its ability to deaminate the viral cDNA prior to second-strand synthesis[77,79,85,201]. To varying degrees, all human APOBEC3s have been shown to result in the accumulation of G-to-A mutations in HIV proviral sequences (see **Introduction**). While A3G uniquely prefers cytosines in a 5'-CC context resulting in GG-to-AG mutations, the other six APOBEC3s prefer to deaminate cytosines in a 5'-TC context resulting in GA-to-AA mutations. Consequently, we expect that if A3D, A3F, A3G, and A3H are truly restrictive, significant levels of G-to-A mutations should accumulate in the integrated proviruses of our restrictive T cell lines, but not in those of our nonrestrictive A3A, A3B, and A3C lines.

To gauge overall mutational loads, we analyzed integrated proviruses in our SupT11 stable lines two weeks after infection with Vif-deficient HIV using both DNA sequencing and differential DNA denaturation (3D) PCR[202]. For sequencing, the *vif-vpr* region of integrated proviruses was amplified from total genomic DNA obtained from the infected culture, cloned, and sequenced. At least five kilobases from a minimum of 10

sequences was analyzed per condition. For 3D-PCR, a region of *pol* was amplified from the same genomic DNA samples. This amplicon was quantified by real-time PCR and a constant amount used to seed a second PCR reaction carried out over a range of denaturation temperatures. An accumulation of G-to-A mutations in an amplicon will effectively lower its denaturation temperature by decreasing its GC content. Thus, more mutation results in amplification at lower denaturation temperatures.

3D-PCR on proviral amplicons from permissive, APOBEC3-low lines SupT11 and CEM-SS allowed for amplification down to the same denaturation temperature as the HIV proviral plasmid (around 85˚C), indicating little mutational accumulation occurred in those sequences over the two weeks of prior spreading infection (**Figure 2-7A**). The same held true for proviruses amplified from the nonrestrictive, stable SupT11 lines expressing A3A, A3B, A3C, or the E259Q catalytic mutant of A3G. On the contrary, the APOBEC3-high nonpermissive cell lines CEM and H9 resulted in a significant accumulation of mutation and allowed proviral amplification at temperatures up to 6 degrees lower than the permissive controls (**Figure 2-7A**). Similarly, stable SupT11 lines expressing either A3D, A3F, A3G, or A3H allowed for amplification at significantly lower denaturation temperatures, indicating an accumulation of proviral mutation. This accumulation is specific to proviral sequences as 3D-PCR over a region of the *MDM2* genomic locus yielded no difference in minimum denaturation temperature regardless of the *APOBEC3* expression profile (**Figure 2-7B**).

Accumulation of G-to-A mutations in these proviruses was confirmed by unbiased sequencing of the *vif-vpr* region (*i.e.*, of amplicons from the highest denaturation temperature). In agreement with the 3D-PCR data, proviruses integrated into

the permissive cell lines CEM-SS and SupT11 had few or no mutations while those integrated into the nonpermissive lines CEM and H9 had high levels of mutation (data not shown). These mutations were primarily G-to-A in either a 5'-GG or 5'-GA dinucleotide context, indicative of the APOBEC3 proteins. Of the SupT11 stable lines, A3A, A3B, and A3C all showed little to no mutation like the parental SupT11 line. On the contrary, A3D, A3F, A3G, and A3H expressing lines all showed significant G-to-A hypermutation (**Figure 2-7C**). A3D, A3F, and A3H all displayed a primarily 5'-GA dinculeotide bias while A3G displayed a primarily 5'-GG dinucleotide bias as previously reported (**Figure 2-7C** and **Introduction**). All sequences displayed a consistent, low-level frequency of other base substitutions. These data show a strong correlation between restriction capacity and the ability to induce significant levels of G-to-A proviral hypermutation: A3D, A3F, A3G, and A3H are restrictive in T cells and result in proviral hypermutation while A3A, A3B, and A3C are nonrestrictive and do not result in the accumulation of proviral hypermutation.

DISCUSSION

Here, we present the first comprehensive analysis of the human and rhesus macaque APOBEC3 repertoires and demonstrate a conserved capacity for A3D, A3F, A3G, and A3H to restrict Vif-deficient HIV (**Figure 2-8**). Our data indicate that these four proteins may contribute to the nonpermissive phenotype of $CD4^+$ T lymphocytes for Vif-deficient HIV based on six criteria: 1) expression in $CD4^+$ T lymphocytes, 2) incorporation into Vif-deficient HIV virions in T cells, 3) restriction of Vif-deficient HIV in T cells, 4) neutralization by HIV Vif, 5) mutation of viral cDNA in a physiologically

relevant spreading infection system, and 6) functional conservation with homologous proteins of rhesus macaque.

Despite approximately 25-35 million years of independent evolution, the human and rhesus APOBEC3 repertoires appear remarkably similar in organization and function. The genomic loci are syntenic, each composed of seven genes arranged in tandem to encode seven proteins, three with one zinc-coordinating domain and four with two zinc-coordinating domains. Although the rhesus macaque genomic sequence assembly has a few intergenic gaps, we have no reason to suspect that the *APOBEC3* gene order will differ with that of humans. As we show here, the homologous proteins of humans and rhesus macaques have comparable steady-state localization and HIV restriction capabilities. Such similarities may be attributable to evolutionary conservation, *i.e.* activities retained from common ancestors, convergent evolution, or may be pure coincidence. While the striking similarity between all seven human and rhesus homologs suggests the former, more work will be required to determine the restrictive APOBEC3 repertoires of more primates against their lentiviral pathogens. Nevertheless, these data suggest that future rhesus macaque studies with candidate molecules designed to leverage the APOBEC3/Vif axis may be an informative step prior to testing in humans, though more work will be required to verify that the exact mechanisms used by SIV and HIV Vif to neutralize the restrictive APOBEC3 repertoires of their hosts are analogous.

Three APOBEC3 proteins, A3A, A3B, and A3C, appear irrelevant to HIV restriction when expressed individually. Human A3A is not expressed in $CD4^+$ T lymphocytes, does not package into HIV virions in T cells or HEK293T cells, does not restrict HIV in T cells or HEK293T cells, is not targeted for degradation by HIV Vif, and

does not result in proviral hypermutation. Rhesus A3A similarly does not package into or restrict HIV and is not targeted for degradation by SIV Vif. Prior reports of A3A restriction can be attributed either to fusions with protein domains that direct encapsidation[186,203], to direct restriction of the transfected proviral DNA[204], and/or to overexpression artifacts (*i.e.*, highest transfected DNA levels in this study).

Similarly, neither human nor rhesus A3C is able to package into or restrict Vif-deficient HIV in HEK293T or T cells. Nevertheless, both human and rhesus A3C are targeted for degradation by their host-specific lentiviral Vif. One plausible explanation for this *non sequitor* is that Vif may inadvertently target A3C due to its high sequence homology to A3D and A3F. The deaminase domains of the APOBEC3 proteins cluster phylogenetically into three groups: Z1, Z2, and Z3[88]. A3C, A3D, and A3F are all composed of related Z2-type deaminase domains and Vif-mediated degradation of all three proteins is influenced by the same conserved residues[205,206]. Also note that while human A3C is expressed in $CD4^+$ T lymphocytes, it is also highly expressed in a wide variety of other tissues and cell lines, both permissive and nonpermissive to Vif-deficient HIV infection[182]. Therefore, while we conclude A3C is not directly relevant to HIV infection, its near ubiquitous expression may indicate a more general biological or immune function.

While HEK293T cells have proved a valuable model system and yield data largely congruent with our studies in T cells, human A3B behaves differently between the two systems. In agreement with prior studies that used A3B hypomorph alleles ([63,92,105] and others), we found that the human consensus A3B protein packages into and restricts HIV in HEK293T cells. On the other hand, when stably expressed in the SupT11 T cell

line, human A3B fails to both package into and restrict HIV. Rhesus A3B, similarly, while having some packaging and restriction capacity in HEK293T cells, does not package or restrict in SupT11 T cells. In both cases, the same preparations of the same A3B plasmid and proviral DNA construct were used for transfection of both the HEK293T and SupT11 T cells. This discrepancy between HEK293T and T cells is also observed with at least one A3B hypomorph and is evident in other T cell lines such as CEM-SS ([171] and data not shown). The reason for these disparate observations is unknown, but they emphasize a limitation of the HEK293T system for APOBEC3 studies. These data, alongside the nearly undetectable expression of *A3B* in $CD4^+$ T lymphocytes and the fact that it is not targeted for degradation by HIV Vif, lead us to conclude that A3B does not have a front-line role in HIV restriction.

Of the seven-member family, our studies demonstrate that four APOBEC3 proteins are directly relevant for HIV restriction: A3D, A3F, A3G, and A3H. While A3G has already been implicated due to its unique 5'-GG-to-AG mutational signature discovered in patient derived HIV sequences, A3D, A3F, and A3H all share a common 5'-GA-to-AA mutational signature and their contribution has been the subject of controversy. A3F has been the most widely considered source for the 5'-GA-to-AA mutations in patient derived sequences, but two recent papers have questioned its relative importance[187,188]. Both human and rhesus A3F package into and restrict Vif-deficient HIV when transiently expressed in HEK293T cells and when stably expressed in SupT11 T cells. Human A3F is furthermore counteracted by HIV Vif, expressed in $CD4^+$ T lymphocytes, and can cause viral hypermutation during a spreading infection, behaving

analogously to its restrictive family member, A3G. We therefore conclude that A3F can contribute to HIV restriction.

Unlike A3G and A3F, there is much less agreement on the restrictive capacity of A3D. Previous reports of restriction in HEK293T cells relied on a nearly 16:1 APOBEC3:virus transfection ratio[52] while the only report of stable A3D expression found no restriction activity as a result of very low expression levels[171]. Nevertheless, human A3D is found to be under the highest level of positive selection among any human APOBEC3 protein[190], implicating a likely role in innate immune defense. Here, we show that human and rhesus A3D package into and restrict Vif-deficient HIV in both HEK293T and T cells. Human A3D is not detectable by immunoblot as readily as the rest of its family members and its restrictive activity is likewise weaker than A3F or A3G, though the reason for this is unclear. Rhesus A3D appears stable and restricts equally well as rhesus A3F and rhesus A3G. Furthermore, both human and rhesus A3D are sensitive to their host-specific lentiviral Vif proteins. Rhesus A3D is even sensitive to HIV Vif, though none of the other rhesus APOBEC3 proteins appear to be so. HIV Vif is thought to neutralize A3C, A3D, and A3F by way of a common binding surface[205,206], which would therefore be predicted to remain intact in rhesus A3D, but not rhesus A3C or rhesus A3F. Comparative analyses between these proteins may aid in further elucidation of this binding surface. All together, these data strongly support a role for A3D in HIV restriction.

There is likewise little agreement concerning the restrictive potential of human A3H, though this has been made somewhat clearer with the recent appreciation of various haplotypes and splice variants[111,192,196,207-209]. For all of our studies here, we used stable

haplotype II, which has previously been shown to restrict Vif-deficient HIV in HEK293T cells[192]. We advanced these studies further by demonstrating that not only does A3H package and restrict HIV in T cells, but it is also induced in $CD4^+$ T lymphocytes upon stimulation and infection, just like A3G. Restriction and packaging ability are conserved in rhesus A3H and both are similarly neutralized by their host-specific lentiviral Vif (this study and [195]). The C-terminal HA tag used in our studies seems to offer some protection to human A3H from HIV Vif, implicating the C-terminal end of A3H in Vif-binding. Therefore, we predict that A3H will also play a role in HIV restriction *in vivo*, though this is likely to be influenced significantly by an individual's haplotype[192].

An important implication from our systematic and comprehensive comparison of the human and rhesus APOBEC3 repertoires is that four proteins – A3D, A3F, A3G, and A3H – likely contribute to HIV restriction in T cells, not one or two as previously inferred (**Figure 2-8**). While the relative importance of each will vary based on expression level, haplotype, and viral strain, all four proteins can contribute and may be leveraged by novel therapeutics to combat HIV infection. It is likely that small molecules that prevent Vif-mediated degradation of all four restrictive APOBEC3 proteins will be much more effective than those that rescue any single one (analogous to current combinatorial therapies). The strong parallels between the human and rhesus macaque APOBEC3 repertoires further suggest that the rhesus macaque may be an excellent model system for testing such candidate pan-APOBEC3/Vif influencing compounds prior to human trials.

The breadth of the APOBEC3 response to retroviral pathogens suggests that the family acts endogenously as an innate immune network wherein overlapping subsets of

proteins are specialized to restrict various exogenous pathogens and endogenous mobile elements. Rigorous testing of the entire repertoire against each affected pathogen in a physiologically relevant system will be required to clearly define the APOBEC3 proteins relevant to each. Such overlap in function may indicate a need for a cumulative effect (*i.e.* all are needed to reach the threshold for effective restriction) or may even indicate an emergent property (*i.e.* four slightly different proteins place more restrictions on the flexibility of a pathogen to evolve resistance, and so form a better zoonotic barrier, than would one protein alone). Regardless, each APOBEC3 protein is under positive selection, though it is unclear if this is due to multiple, independent pressures exerted on the divergent functions of individual proteins or due to shared pressures exerted on overlapping functions of subsets of proteins. A better understanding of the repertoires in other primates and mammals would aid in reconstructing the selective pressures that have sculpted the present day locus and help to elucidate the genetic principles that underlie redundancy and innate restriction.

MATERIALS & METHODS

Virus Constructs. Vif-proficient and Vif-deficient ($X_{26}X_{27}$) HIV-1_{IIIB} A200C proviral expression constructs have been reported previously[171]. SIV_{mac239} Vif and the SIV_{mac239} $Vif_{SLQ->AAA}$ expression constructs with C-terminal Myc tags in pVR1012 (Vical Co.) have also been previously reported[195].

APOBEC3 Expression Constructs. Isogenic constructs expressing the coding sequence of human *A3A* (NM_145699), *A3B* (NM_004900), *A3C* (NM_014508), *A3D*

(NM_152426), *A3F* (NM_145298), *A3G* (NM021822), and *A3H* (haplotype II: FJ376615) with C-terminal triple HA tags in pcDNA3.1(+) (Invitrogen) or C-terminal GFP tags in pEGFP-N3 (Clontech) were created and used for transient expression in HEK293T cells, transient expression in HeLa cells, or stable transfection into SupT11 T cells. A3A, A3D, A3F, A3G, and A3H-GFP constructs have been previously reported[63,195,204]. The *A3C* and *A3H* coding regions were amplified from preexisting plasmids by PCR using primers 5'-NNN GAG CTC GGT ACC ACC ATG AAT CCA CAG ATC AGA AAC CCG-3' / 5'-NNN GTC GAC TCC CTG GAG ACT CTC CCG TAG C-3' and 5'-NNN GAG CTC AGG TAC CAC CAT GGC TCT GTT AAC AGC CGA-3' / 5'-NNN GTC GAC GGA CTG CTT TAT CCT G-3' respectively. PCR products were digested with SacI/SalI, PCR purified (GeneJET™ PCR Purification Kit from Fermentas) and ligated into similarly digested pEGFP-N3. The resulting vectors were subsequently digested with KpnI/SalI and the coding region was subcloned into a KpnI/XhoI digested, modified pcDNA3.1(+) vector with a C-terminal 3xHA tag.

To prevent expression of the highly mutagenic, consensus human A3B protein (NP_004891) in *E. coli*, an intron was inserted into the *A3B* coding region between exons five and six. The 5' end of *A3B* was amplified by PCR from image clone 5539942 using primers 5'-NNN AAG CTT ACC GCC ATG AAT CCA CAG ATC-3' and 5'-CAT CTC CTG GAC TCA CCT CGT TGC ATA GAA AGC C-3'. The beta-globin intron was amplified from a preexisting plasmid using the primers 5'-GTG AGT CCA GGA GAT GTT TCA GCA CTG TTG CC-3' and 5'-CTG TTG AGA TGA AAG GAG ACA ATA AAG ATG AC-3'. The 3' end of *A3B* was amplified from image clone 4707934 using primers 5'-CCT TTC ATC TCA ACA GGC TAA GAA TCT TCT C-3' and 5'-GTC

GAC GTT TCC CTG ATT CTG GAG AAT GGC CC-3'. The fragments were gel purified (GeneJET^{TM} Gel Extraction Kit from Fermentas) and connected by overlapping PCR. The full-length 2 kilobase segment was amplified by PCR, gel purified, and digested with HindIII/SalI. This insert was ligated into a HindIII/SalI digested pEGFP-N3 vector and a HindIII/XhoI digested pcDNA3.1(+) vector with a C-terminal triple HA tag.

As with the human repertoire, isogenic constructs expressing the coding sequence of rhesus *A3A* (JF714484), *A3B* (JF714485), *A3C* (JF714486), *A3D-I* (JF714487), *A3D-II* (JF714488), *A3F* (NM_001042373), *A3G* (AY331716), and *A3H* (NM_001042372) with C-terminal triple HA tags in pcDNA3.1(+) (Invitrogen) or C-terminal GFP tags in pEGFP-N3 (Clontech) were similarly created. The rhesus macaque *A3B*, *A3C*, *A3D-I*, *A3D-II*, *A3F*, *A3G*, and *A3H* cDNAs were generously provided by T. Hatziioannou[164]. The coding regions were amplified by PCR using primers 5'-NNN NGA GCT CGG TAC CAC CAT GAA TCC ACA GAT CAG-3' and 5'-NNN GTC GAC TCC CTG GAG AATCTC CCG-3' for rhesus *A3C*, 5'-NNN NGA GCT CGG TAC CAC CAT GAA TCC ACA GAT CAG-3' and 5'-NNN GTC GAC TCC CTG GAG AATCTC CCG-3' for rhesus *A3D-I*, 5'-NNN NGA GCT CGG TAC CAC CAT GAA TCC ACA GAT CAG-3' and 5'-NNN GTC GAC TCC CTG GAG AAT CTC CTG-3' for rhesus *A3D-II*, 5'-NNN NGA GCT CGG TAC CAC CAT GAA GCC TCA CTT CAG-3' and 5'-NNN GTC GAC TCC CTC GAG AAT CTC CTG CAG C-3' for rhesus *A3F*, 5'-NNN NGA GCT CGG TAC CAC CAT GGT GGA GCC AAT GG-3' and 5'-NNN GTC GAC TCC GTT TTC CTG ATT CTG GAG AAT G-3' for rhesus *A3G*, and 5'-NNN NGA GCT CGG TAC CAC CAT GGC TCT GCT AAC AGC-3' and 5'-NNN GTC GAC TCC TCT TGA GTT GCG TAT TGA CGA TG-3' for rhesus *A3H*. PCR products were digested with

SacI/SalI and ligated into similarly digested pEGFP-N3. The resulting vectors were subsequently digested with KpnI/SalI and the coding region was subcloned into a KpnI/XhoI digested, modified pcDNA3.1(+) vector with a C-terminal triple HA tag.

The rhesus macaque genome assembly that contains the *APOBEC3* locus (AANU00000000.1) has a large gap in the region syntenous with the human *A3A* locus. To look for evidence of a rhesus *A3A* homolog, the human A3A protein sequence was blasted against trace files from the WSG project for rhesus macaque[199]. The main genome assembly contained evidence for *A3A*-like exon one, while two other assemblies (AANU01133640.1 and AANU01300720.1) contained evidence for *A3A*-like exons two, three and four. Outer and inner nested primers were designed in the predicted macaque *A3A* exon three in each direction, one set for 3' RACE (5'-CGT GGA GCT GCG CTT CCT GTG TGA GGT T-3' outer and 5'-AGG AGG GGC TGT GCC GGG CAA GTG-3' inner) and one set paired with a forward primer (5'-CGG CAG CCC AGC ATC CAG GCC CA-3') just downstream from the start site in the predicted *A3A* exon 1 (5'-CAC TTG CCC GGC ACA GCC CCT CCT-3' outer and 5'- AAC CTC ACA CAG GAA GCG CAG CTC CAC G-3' inner). RNA was extracted from rhesus PBMCs (gift from Ed Stevens at the University of Kansas) using the QIAamp RNA Blood mini kit (Qiagen). cDNA production and subsequent 3' RACE was performed using reagents from the FirstChoice RLM-RACE kit (Ambion). Products were amplified using the Phusion high-fidelity polymerase (NEB), gel purified, TOPO-cloned (Invitrogen), and sequenced (BioMedical Genomics Center at the University of Minnesota). An alignment (not shown) of the sequenced products confirmed the predicted rhesus *A3A* exons one

through four and also identified exon five. Full-length rhesus *A3A* cDNA was subsequently amplified, cloned, and sequenced.

To prevent expression of the potentially mutagenic, consensus rhesus A3A and rhesus A3B proteins in *E.coli*, an intron was inserted into the coding regions between exons two and three. The 5' end of rhesus *A3A* was amplified by PCR from rhesus PBMC cDNA using primers 5'-NNN NGA GCT CAG GTA CCA CCA TGG ACG GCA GCC CAG CA-3' and 5'-CAT CTC CTG GAC TCA CCT TGT TGC ATA GAA A-3' and the 3' end of rhesus *A3A* was amplified using primers 5'-CTT TCA TCT CAA CAG GCT AAG AAT GTT CCC-3' and 5'-NNN NGT CGA CGT TTC CCT GAT TCT GGA G-3'. The human beta-globin intron was amplified as described above. These three fragments were gel purified and connected by overlapping PCR. The full-length segment was amplified by PCR, gel purified, and digested with KpnI/SalI. This insert was ligated into into a KpnI/SalI digested pEGFP-N3 vector and a KpnI/XhoI digested pcDNA3.1(+) vector with a C-terminal triple HA tag. The 5' end of rhesus *A3B* was amplified by PCR from cDNA provided by T. Hatziioannou using primers 5'-NNN NAA GCT TGG TAC CAC CAT GAA TCC ACA GAT CAG-3' and 5'-CAT CTC CTG GAC TCA CCT GGC CTC GAA AGA C-3' and the 3' end was amplified using primers 5'-CTT TCA TCT CAA CAG ATG TAT TCC AAG CCT G-3' and 5'-NNN GTC GAC TCC GTT TCC CTG ATT CTG G-3'. The fragments were gel purified and connected to the human beta-globin intron again by overlapping PCR. The full-length segment was amplified by PCR, gel purified, and digested with HindIII/SalI. This insert was ligated into a HindIII/SalI digested pEGFP-N3 vector and a HindIII/XhoI digested pcDNA3.1(+) vector with a C-terminal triple HA tag.

Cell lines. Human Embryonic Kidney (HEK) 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin (P/S). CEM-GFP cells (obtained from the AIDS Research and Reference Reagent Program or ARRRP) and SupT11 cells (APOBEC3-devoid subclone of SupT1[191]) were maintained in Roswell Park Memorial Institute (RPMI) medium with 10% FBS and 0.5% P/S. APOBEC3-expressing SupT11 clones were generated by electroporation of $2*10^7$ SupT11 cells with 20 μg of PvuI-linearized plasmid, using the isogenic human and rhesus APOBEC3 constructs with C-terminal triple HA tags in pcDNA3.1(+) as described above. Cells were serial plated and outgrown for three weeks in selective growth media containing 1 mg/ml G418 (Mediatech). Individual clones were expanded, maintained in selective media, and screened by immunoblotting.

Primary Cells. PBMCs were isolated from blood (Memorial Blood Center, St. Paul, MN) by Ficoll gradient centrifugation and $CD4^+$ T lymphocytes isolated by negative selection using the Miltenyi Biotec $CD4^+$ T Cell Isolation Kit II as previously described[182]. Stimulation was performed either by treatment with IL-2/PHA[182] or CD2/CD3/CD28 beads (Miltenyi Biotec T Cell Activation/Expansion Kit) according to the manufacturer's protocol. CD4-PE (Miltenyi Biotec) and CD25-PE (Miltenyi Biotec) antibodies were used to verify purification and stimulation respectively by cell staining according to the manufacturer's protocol. Primary cells were maintained in RPMI with 10% FBS, 0.5% P/S, and 20U/ml IL-2.

Immunoblotting. Cell lysates were prepared by resuspension of washed cell pellets directly in 2.5x Laemmli Sample Buffer (25mM Tris pH 6.8, 8% glycerol, 0.8% SDS, 2%

2-mercaptoethanol, 0.02% bromophenol blue), and homogenization at 95°C for 5-10 minutes. Virus-like particles were isolated from culture supernatants by purification through 0.45μm PVDF filters (Millipore) followed by centrifugation (13,000 rpm for 2 hours) through a 20% sucrose, 1xPBS cushion and lysis directly in 2.5x Laemmli Sample Buffer. Samples were run on 12.5% Tris-HCl SDS-PAGE resolving gels with a 4% stacker each at a 37.5 acrylamide : 1 bis-acrylamide ratio (BioRad Criterion system) at 150V for 90 minutes. Proteins were transferred to PVDF membranes by methanol-based electrotransfer (BioRad Criterion Blotter) at 100V for 2 hours. Membranes were blocked in 4% Milk in PBS, 0.1% Tween-20 overnight prior to blotting. APOBEC3 expression was detected using an HA-specific, mouse monoclonal antibody (Covance). Vif expression was detected using a c-myc-specific, rabbit monoclonal antibody (Sigma-Aldrich). Protein loading for cell lysates was monitored by detection of tubulin with a tubulin-specific, mouse monoclonal antibody (BioRad). Protein loading for viral particles was monitored by detection of p24 with a p24-specific, mouse monoclonal antibody (ARRRP). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (BioRad) were detected using Hyglo HRP detection reagents (Denville Scientific). Blots were incubated in a 1x PBS, 0.2M glycine, 1.0% SDS, 1.0% Tween-20, pH 2.2 stripping buffer before reprobing.

qPCR. mRNA isolation, reverse transcription and qPCR were carried out as previously described[182]. Significance was determined using paired, two-tailed Student t-tests.

Flow Cytometry. HIV-infected CEM-GFP cells were prepared for flow cytometry by fixation in 4% paraformaldehyde, 1x PBS. GFP fluorescence was measured on a

Beckman Coulter Cell Lab Quanta™ SC-MPL. All data was analyzed using FlowJo Flow Cytometry Analysis Software (Version 8.8.6). Quantification was done by first gating the live cell population, followed by gating on the GFP^+ cells.

HIV Single Cycle with Replication Proficient Virus. At 50% confluency, HEK293T cells were transfected (TransIt, Mirus) with 1 μg Vif-proficient or Vif-deficient HIV proviral expression construct alongside either 0, 25, 50, 100, 200, or 400 ng of HA-tagged APOBEC3 expression construct supplemented with appropriate empty vector. CEM-GFP cells were infected after 48 hours to monitor infectivity and cell and viral particle lysates were prepared for immunoblotting. Data were normalized to the 0 ng APOBEC3 control as 0% restriction.

HIV Single Cycle Infectivity Assays with SIV_{mac239} Vif. At 50% confluency, HEK293T cells were transfected (TransIt, Mirus) with 1 μg Vif-deficient ($X_{26}X_{27}$) HIV-1_{IIIB} A200C proviral construct, 200 ng of rhesus A3 expression construct or empty vector, and 50 ng of codon-optimized Vif or empty vector. SIV_{mac239} Vif and the SIV_{mac239} $Vif_{SLQ->AAA}$ constructs with C-terminal Myc tags in pVR1012 (Vical Co.) have been reported previously[195]. All experiments were performed concurrently in duplicate. After 48 hours, virus-containing supernatants were harvested and any remaining producer cells removed by centrifugation. Infectivity was monitored by infection of CEM-GFP with viral supernatant. Supernatants from one replicate were processed for immunoblotting to monitor packaging.

HIV Spreading Infection. Spreading infection was carried out as previously described[191]. HIV was generated by transfection of HEK293T cells with 4 μg of Vif-

proficient or Vif-deficient HIV proviral expression construct. Viral stocks were titered and used to initiate infections on $5x10^4$ T cells at 1% initial infection. Infection was monitored by infection of CEM-GFP every 2-3 days. As needed, infected cultures were split and fed. Infections were allowed to proceed until the viral growth curve peaked and returned to baseline.

HIV Packaging in T cells. At 50% confluency, HEK293T cells were transfected (TransIt, Mirus) with 3.2 μg Vif-deficient ($X_{26}X_{27}$) HIV-1_{IIIB} A200C proviral construct alongside 0.8 μg of a VSVG expression construct[171]. After 48 hours to allow for VSVG-pseudotyped virus production, supernatant was harvested, filtered to eliminate cellular contaminants, and frozen in aliquots at -80°C. Virus stocks were titered by infection with 150 ul of stock on 25,000 CEM-GFP cells per well in a 96-well round bottom plate. Cells were fixed in 4% paraformaldehyde at approximately 48 hours post infection, and analyzed via flow cytometry to determine the stock titer. $2.5x10^5$ cells of each stable APOBEC3-expressing SupT11 cell line were plated in 2 ml RPMI in six-well plates and infected to achieve 25% initial infection. Twelve hours after infection, cells were pelleted, washed once in PBS to remove residual VSVG-pseudotyped virus, and resuspended in 2 ml fresh RPMI. 36 hours after washing, the culture was aliquoted into microcentrifuge tubes and the cells were pelleted. Approximately 1 ml of viral supernatant was removed and filtered through a 0.45 μm PVDF filter. This 1 ml of filtered supernatant was subsequently processed for immunoblotting.

Fluorescence Microscopy. Microscopy experiments were performed as described in [96]. Briefly, HeLa cells in LabTek chambered cover glasses (Nunc) were transfected with

isogenic pEGFP-N3 APOBEC3 expression plasmids (described above) and incubated overnight. A Deltavision deconvolution microscope (Applied Precision) at 40x magnification was used to collect the images and deconvolution was performed using SoftWoRx, the Deltavision software (Applied Precision). Images were cropped and adjusted for contrast in Photoshop 3.0 and assembled in Adobe Illustrator CS3.

Sequencing of Integrated Provirus. Fourteen days after infection with Vif-deficient HIV, genomic DNA was prepared from the infected cultures using the Qiagen DNeasy Kit. Two 1.2 kilobase amplicons over the *vif-vpr* region of integrated proviruses were PCR amplified using primers 5'-CAA GGC CAA TGG ACA TAT CA-3' with 5'-TTT GCT GGT CCT TTC CAA AC-3' and 5'-AGC AGG AAG ATG GCC AGT AA-3' with 5'-CAA ACT TGG CAA TGA AAG CA-3'. These amplicons were PCR purified and cloned into pJET1.2 using the CloneJET^{TM} PCR Cloning Kit (Fermentas). These amplicons were cloned and sequenced at the BioMedical Genomics Center at the University of Minnesota. Sequences were analyzed using Sequencher 4.6 (Gene Codes Corp.). Only 500 base pairs from the 5' end of either amplicon was used for analysis. Duplicate sequences arising from PCR bias were discarded.

Semi-quantitative 3D-PCR. Fourteen days after infection with Vif-deficient HIV, genomic DNA was isolated from the infected cultures using the Qiagen DNeasy Kit. An 875 base pair amplicon from the *pol* gene of integrated proviruses was amplified by Taq polymerase (Roche) for 20 cycles using degenerate primers 5'-TCC ART ATT TRC CAT AAA RAA AAA-3' and 5'-TTY AGA TTT TTA AAT GGY TYT TGA-3'. The relative amount of this outer amplicon was quantified by Real-Time PCR (Roche,

LightCycler 480) using degenerate primers 5'-AAT ATT CCA RTR TAR CAT RAC AAA AAT-3' and 5'-AAT GGY TYT TGA TAA ATT TGA TAT GT-3' along with a fluorescent hydrolysis probe for visualization (Universal ProbeLibrary #58, Roche). Quantification was used to normalize the amount of integrated provirus for a subsequent second PCR over a range of denaturation temperatures using the same primers as used for Real-Time. Nested PCR reactions used Phusion DNA polymerase (NEB), with other reagents according to the manufacturer's protocol. Reaction conditions were: a gradient of denaturation temperatures (T_d) for 30 seconds, then 25 cycles of the T_d gradient for 15 seconds, 52˚C for 30 seconds, and 72˚C for 2 minutes, and a final extension at 72˚C for 7 minutes. T_d gradients are indicated in the figures. PCR products were run on agarose gels and detected by ethidium-bromide staining. 3D-PCR over the *MDM2* genomic locus was performed as described[204].

ADDITIONAL CONTRIBUTIONS

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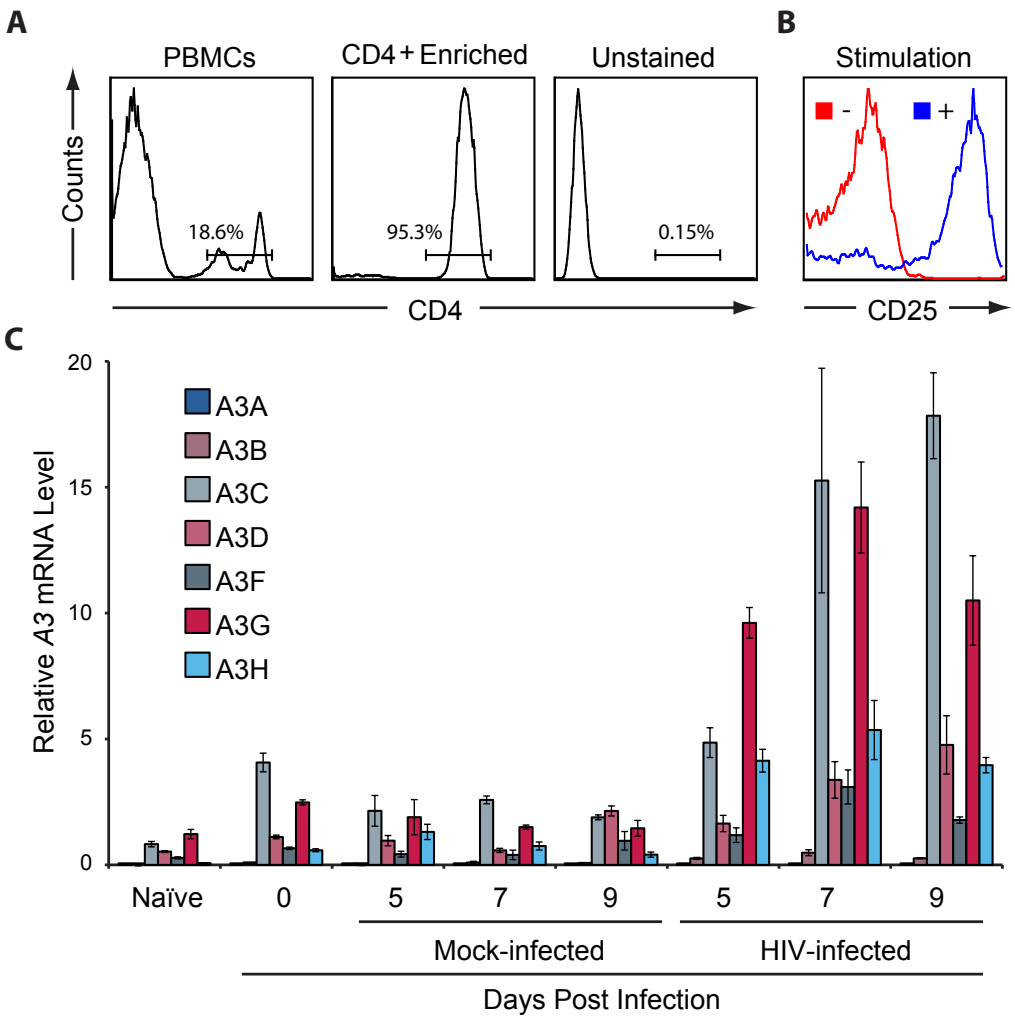

Figure 2-1. Human *A3C*, *A3G*, and *A3H* are Induced in HIV-infected CD4$^+$ T Lymphocytes.

(A) Histograms depicting CD4-PE antibody staining of PBMCs and naïve CD4$^+$ T lymphocytes after purification by negative selection alongside the unstained control. Naïve CD4$^+$ T lymphocytes were enriched to 95% purity.

(B) Histogram depicting CD25-PE antibody staining of naïve $CD4^+$ T lymphocytes before (red) and after (blue) stimulation with CD2/CD3/CD28 beads. CD25 is a marker of T cell activation.

(C) Quantitative PCR profiles for each *APOBEC3* mRNA in $CD4^+$ T lymphocytes shown before and after stimulation with CD2/CD3/CD28 beads as well as over the course of mock-infection or infection with HIV. Mean values and standard deviations of three independent qPCR reactions are shown for each condition. Expression is normalized to the reference gene *Tata Binding Protein* (*TBP*) and the level of *A3G* in naïve cells is set to 1 to facilitate comparison.

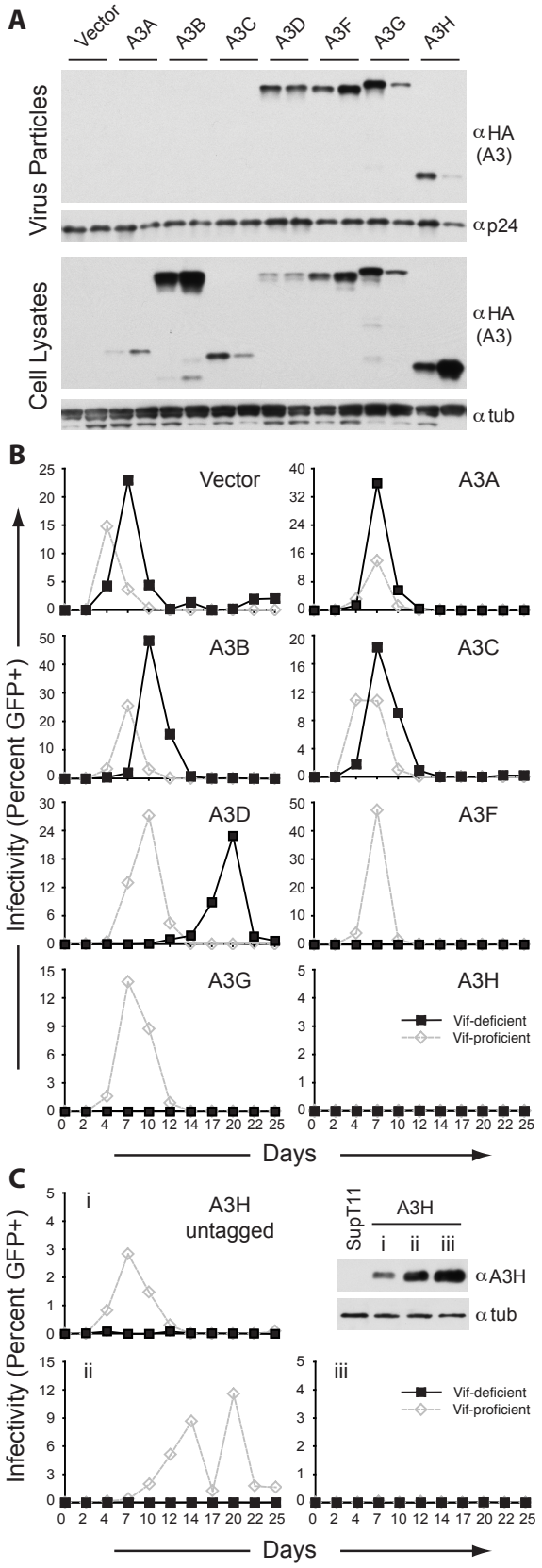
A
Vector
A3A
A3B
A3C
A3D
A3F
A3G
A3H
Virus Particles
αHA (A3)
αp24
Cell Lysates
αHA (A3)
αtub
B
Vector
A3A
A3B
A3C
A3D
A3F
A3G
A3H
Infectivity (Percent GFP+)
Vif-deficient
Vif-proficient
Days
C
i
A3H untagged
SupT11
A3H
i ii iii
αA3H
αtub
ii
iii
Infectivity (Percent GFP+)
Vif-deficient
Vif-proficient
Days

Figure 2-2. Human A3D, A3F, A3G, and A3H Package into and Restrict Vif-deficient HIV in T Cells.

(A) Paired immunoblots of representative SupT11 clones show stable expression of each HA-tagged, human APOBEC3 protein in cells (lower panels) and incorporation into Vif-deficient HIV virus particles produced by those cells (upper panels). Tubulin (tub) and p24 served as cell and viral lysate loading controls.

(B) Replication kinetics of Vif-proficient (gray diamonds) and Vif-deficient (black squares) HIV in representative SupT11 stable clones expressing the indicated APOBEC3 protein. Infectivity was monitored over 25 days by periodic infection of the CEM-GFP reporter line, which expresses GFP upon infection, and subsequent flow cytometry to quantify GFP^+ cells.

(C) Replication kinetics of Vif-proficient (gray diamonds) and Vif-deficient (black squares) HIV in representative SupT11 stable clones expressing various levels of untagged A3H as indicated by immunoblotting.

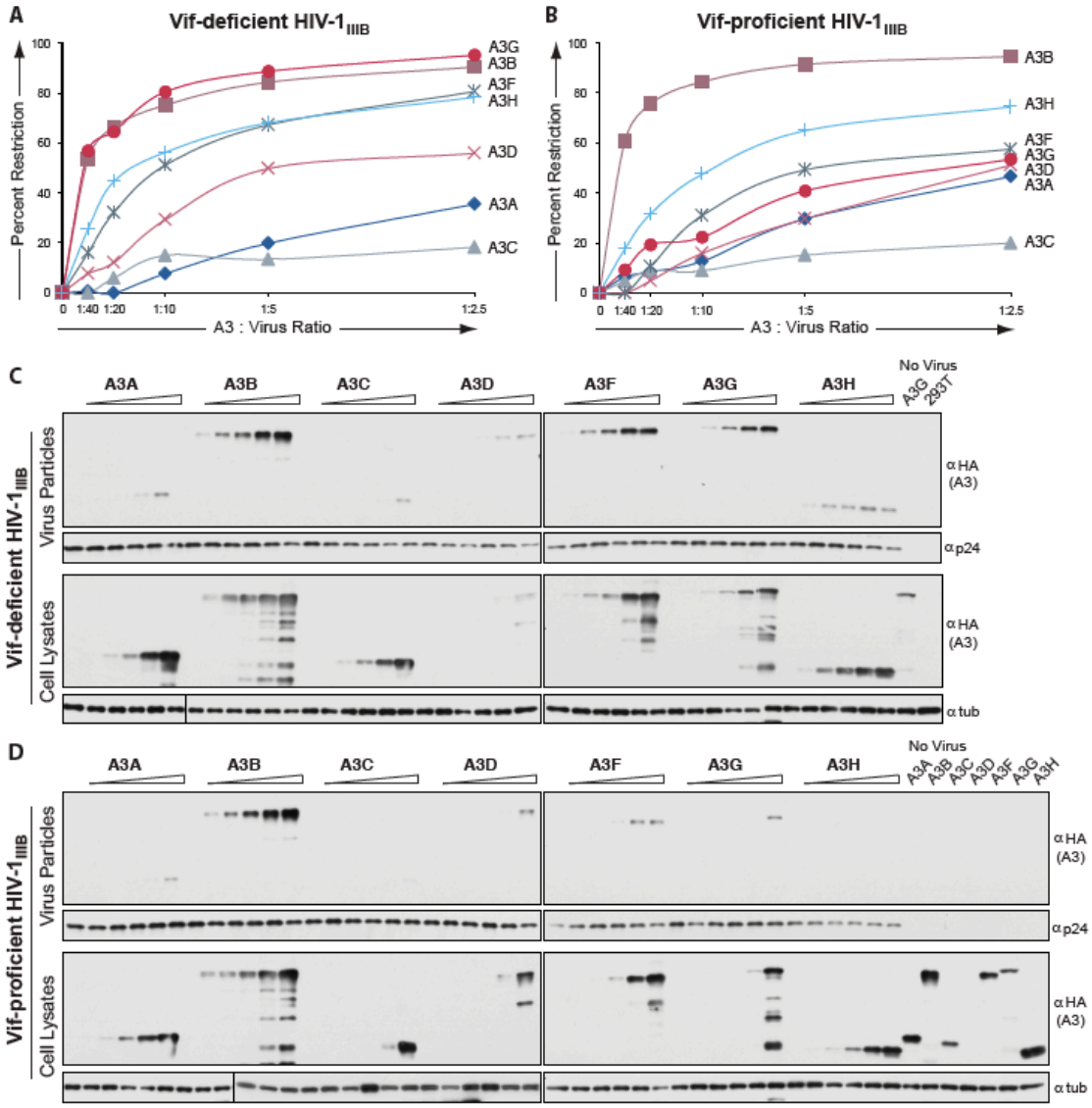

Figure 2-3. Human A3B, A3D, A3F, A3G, and A3H Package into and Restrict Vif-deficient HIV in HEK293T Cells.

(A) Percent restriction achieved by each APOBEC3 protein in HEK293T cells at the indicated cotransfection ratios with a constant amount of Vif-deficient HIV proviral construct (1 μg) and increasing amounts of APOBEC3 expression construct (0, 25, 50,

100, 200, 400 ng). Infectivity of the resultant viruses was monitored by infection of CEM-GFP. Percent restriction was calculated as the inverse of infectivity and was normalized to the corresponding no APOBEC3 control.

(B) Percent restriction achieved by each APOBEC3 protein in HEK293T cells at the indicated cotransfection ratios with a constant amount of Vif-proficient HIV proviral construct and increasing amounts of APOBEC3 expression construct as in (A).

(C) Immunoblots of HEK293Ts transfected in (A) of each HA-tagged, human APOBEC3 protein in cells (lower panels) and in Vif-deficient HIV virus particles produced by those cells (upper panels). Tubulin (tub) and p24 served as cell and viral lysate loading controls. No virus control cells (far right) were transfected with the maximum amount of APOBEC3 (400ng) and no proviral DNA.

(D) Immunoblots of HEK293Ts transfected in (B) of each HA-tagged, human APOBEC3 protein in cells (lower panels) and in Vif-proficient HIV virus particles produced by those cells (upper panels).

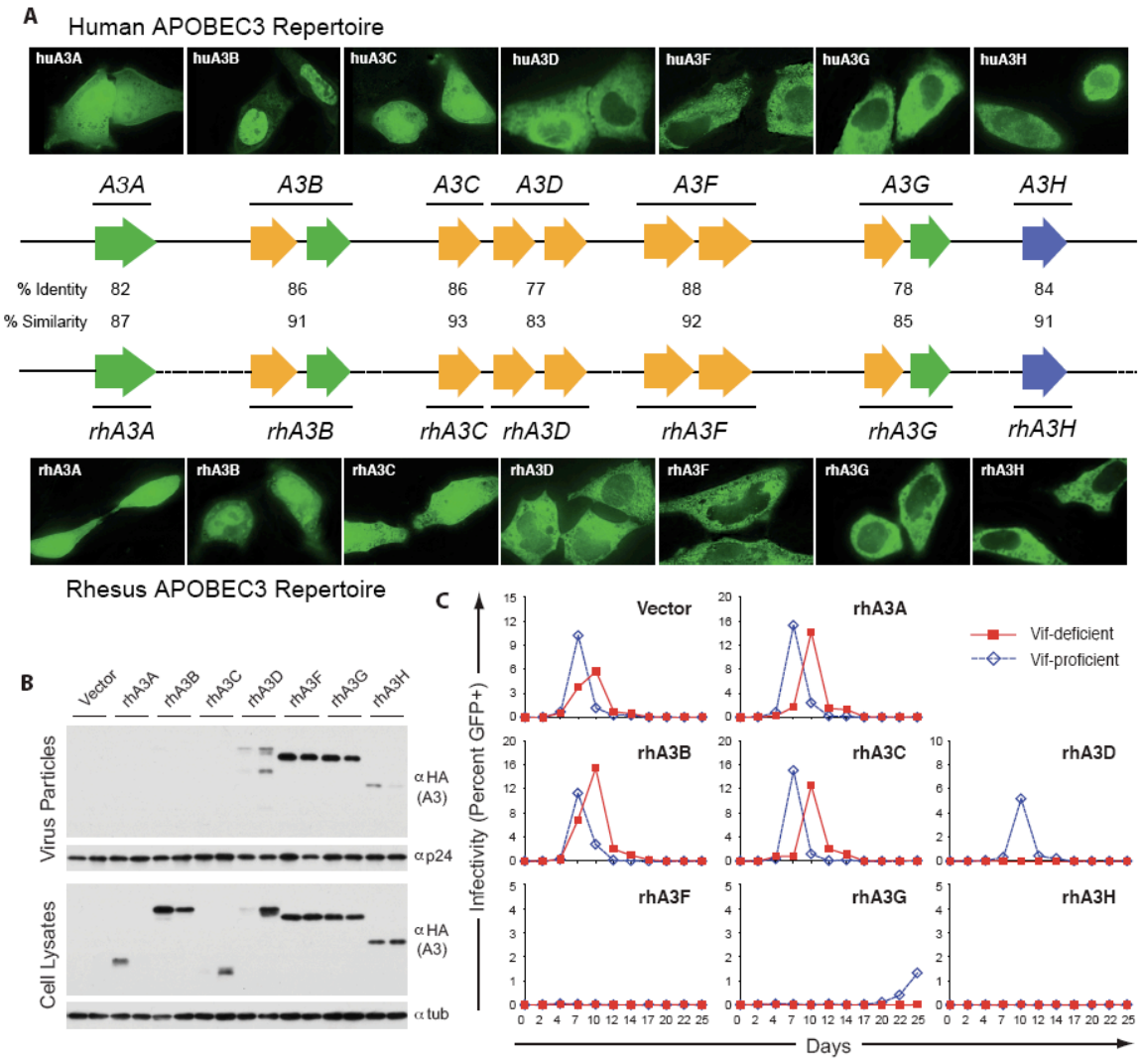

Figure 2-4. The Human and Rhesus APOBEC3 Repertoires are Analogous in Organization and Restriction Capacity.

(A) Schematics of the human and rhesus *APOBEC3* loci. Percent identity and similarity were calculated by pairwise protein alignment via the Basic Local Alignment Search Tool (BLAST). Each arrow represents one deaminase domain, colored to reflect phylogenetic groups (green – Z1, orange – Z2, blue – Z3). Representative images of

HeLa cells expressing the indicated GFP-tagged APOBEC3 constructs are shown above and below.

(B) Paired immunoblots of representative SupT11 clones show stable expression of each HA-tagged, rhesus APOBEC3 protein in cells (lower panels) and incorporation into Vif-deficient HIV virus particles produced by those cells (upper panels). Tubulin (tub) and p24 served as cell and viral lysate loading controls.

(C) Replication kinetics of Vif-proficient (blue diamonds) and Vif-deficient (red squares) HIV in representative SupT11 stable clones expressing the indicated rhesus APOBEC3 proteins. Infectivity was monitored over 25 days by periodic infection of the CEM-GFP reporter line and flow cytometry to quantify GFP^+ cells.

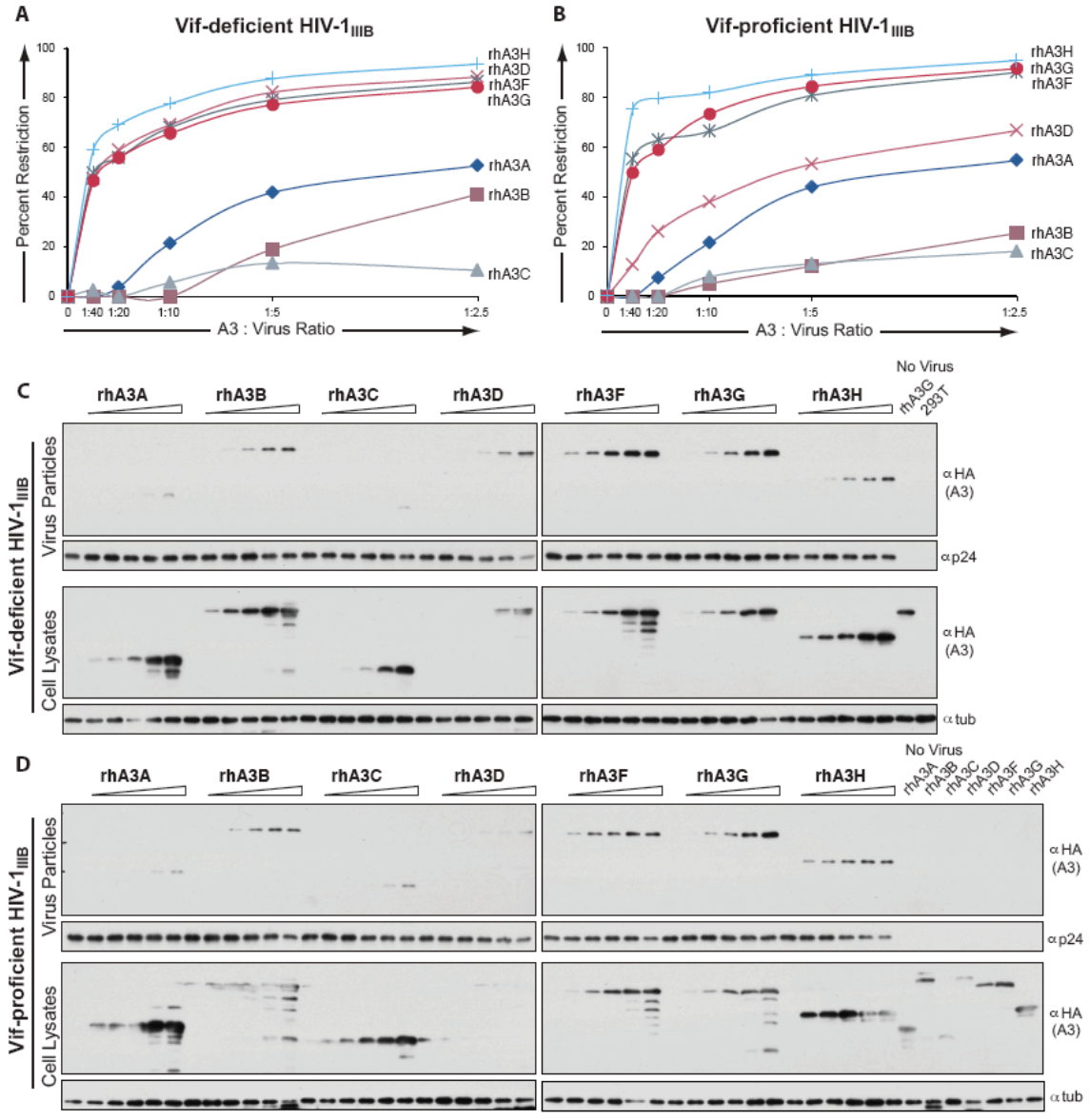

Figure 2-5. Rhesus A3D, A3F, A3G, and A3H Package into and Restrict Vif-deficient HIV in HEK293T Cells.

(A) Percent restriction achieved by each rhesus APOBEC3 protein in HEK293T cells at the indicated cotransfection ratios with a constant amount of Vif-deficient HIV proviral construct (1 μg) and increasing amounts of rhesus APOBEC3 expression construct (0, 25,

50, 100, 200, 400ng). Infectivity of the resultant viruses was monitored by infection of CEM-GFP. Percent restriction was calculated as the inverse of infectivity and normalized to the corresponding no APOBEC3 control.

(B) Percent restriction achieved by each rhesus APOBEC3 protein in HEK293T cells at the indicated cotransfection ratios with a constant amount of Vif-proficient HIV proviral construct and increasing amounts of rhesus APOBEC3 expression construct as in (A).

(C) Immunoblots of HEK293Ts transfected in (A) of each HA-tagged, rhesus APOBEC3 protein in cells (lower panels) and in Vif-deficient HIV virus particles produced by those cells (upper panels). Tubulin (tub) and p24 served as cell and viral lysate loading controls. No virus control cells (far right) were transfected with the maximum amount of rhesus APOBEC3 (400ng) and no proviral DNA.

(D) Immunoblots of HEK293Ts transfected in (B) of each HA-tagged, rhesus APOBEC3 protein in cells (lower panels) and in Vif-proficient HIV virus particles produced by those cells (upper panels).

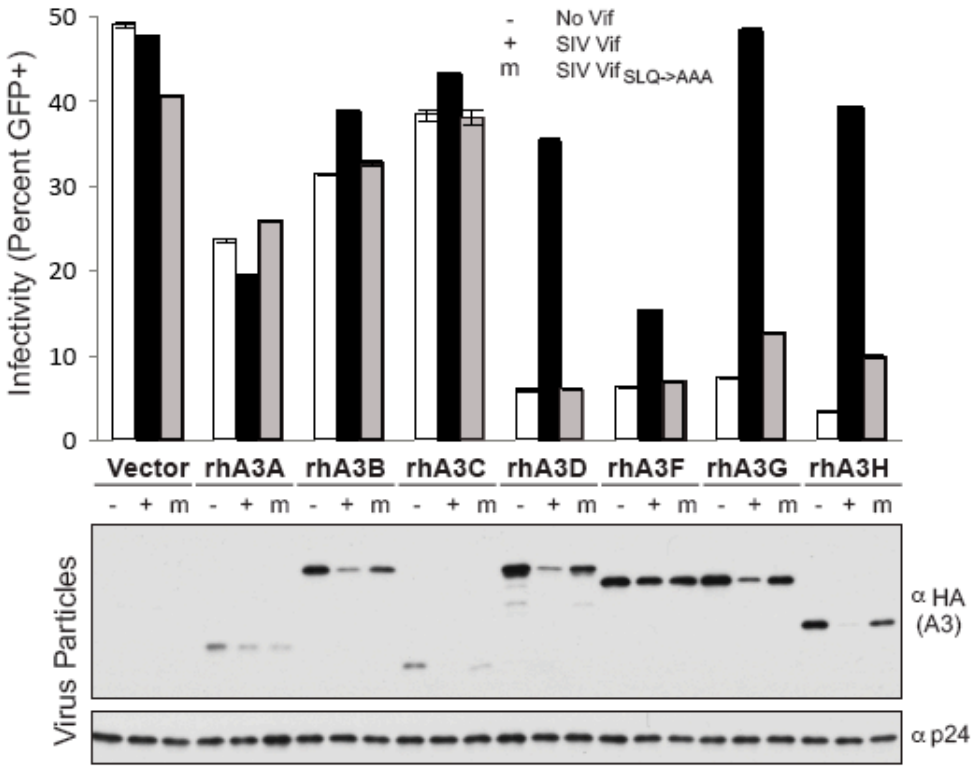

Figure 2-6. Neutralization of the Rhesus APOBEC3 repertoire by SIV_{mac239} Vif.

Rhesus A3D, A3F, A3G, and A3H are all neutralized by SIV_{mac239} Vif. A constant amount of Vif-deficient HIV proviral expression construct (1 μg) was transfected into HEK293T cells alongside a constant amount of rhesus APOBEC3 (200 ng) and either an empty vector (-), SIV_{mac239} Vif (+), or SIV_{mac239} $Vif_{SLQ->AAA}$ (m), a Vif mutant that is unable to degrade the APOBEC3 proteins (50 ng). Infectivity was determined by infection of CEM-GFP with viral supernatant. Error bars indicate the standard deviation between two biological replicates (miniscule in several instances). Viral particle lysates were concurrently collected to monitor APOBEC3 packaging.

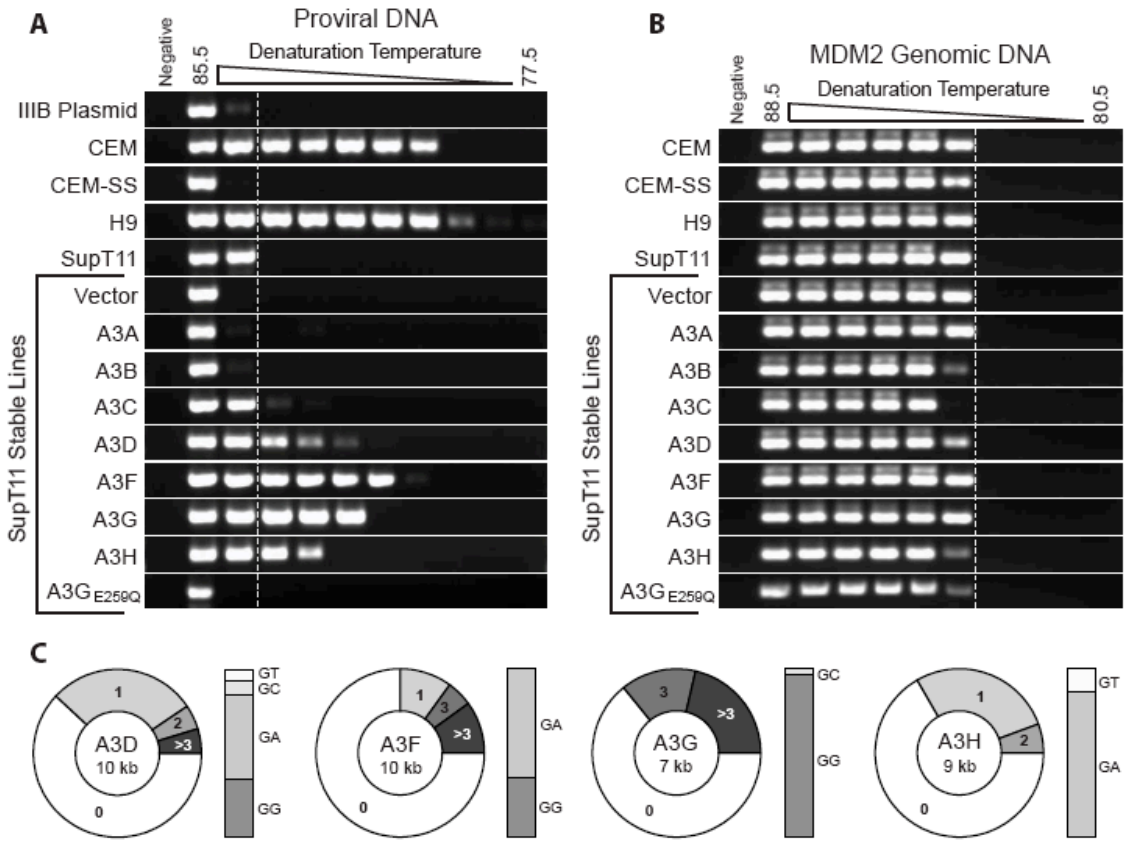

Figure 2-7. Human A3D, A3F, A3G, and A3H Cause Proviral G-to-A Hypermutation as Evidenced by Semi-quantitative 3D-PCR and Proviral DNA Sequencing.

(A) Integrated provirus from the indicated cell lines was amplified fourteen days after infection with Vif-deficient HIV. Amplicons were quantified and constant amounts used to seed a secondary PCR over a 77.5 - 85.5˚C range of denaturation temperatures. Products were run on agarose gels and visualized by ethidium bromide staining. The dotted line indicates the lowest denaturation temperature at which this product amplifies from the permissive, parental SupT11 cell line.

(B) In order to verify specific accumulation of mutations in proviral sequences, 3D-PCR was also performed on a small genomic amplicon of the *MDM2* gene. Regardless of the APOBEC3 repertoire of the cell line, amplification occurs to nearly the same minimum denaturation temperature.

(C) Sequence analysis of the *vif-vpr* region of integrated proviruses amplified from SupT11 cell lines stably expressing the indicated APOBEC3 fourteen days after infection with Vif-deficient HIV. At least five kilobases from ten clones was analyzed for each condition. The pie charts reflect the percent of sequences analyzed with the indicated number of G-to-A mutations. The accompanying bar graph indicates the dinucleotide context of these mutations.

A

		Human Repertoire					Rhesus Repertoire				
		Restriction		Packaging		Vif	Restriction		Packaging		Vif
		T cells	HEK293	T cells	HEK293	HIV	T cells	HEK293	T cells	HEK293	SIV
APOBEC3	A3A	-	-	-	-	-	-	-	-	-	-
	A3B	-	++	-	++	-	-	-	-	+	+
	A3C	-	-	-	-	++	-	-	-	-	++
	A3D	+	+	++	+	++	++	++	++	++	++
	A3F	++	++	++	++	++	++	++	++	++	+
	A3G	++	++	++	++	++	++	++	++	++	++
	A3H	++	++	++	++	+	++	++	++	++	++

B

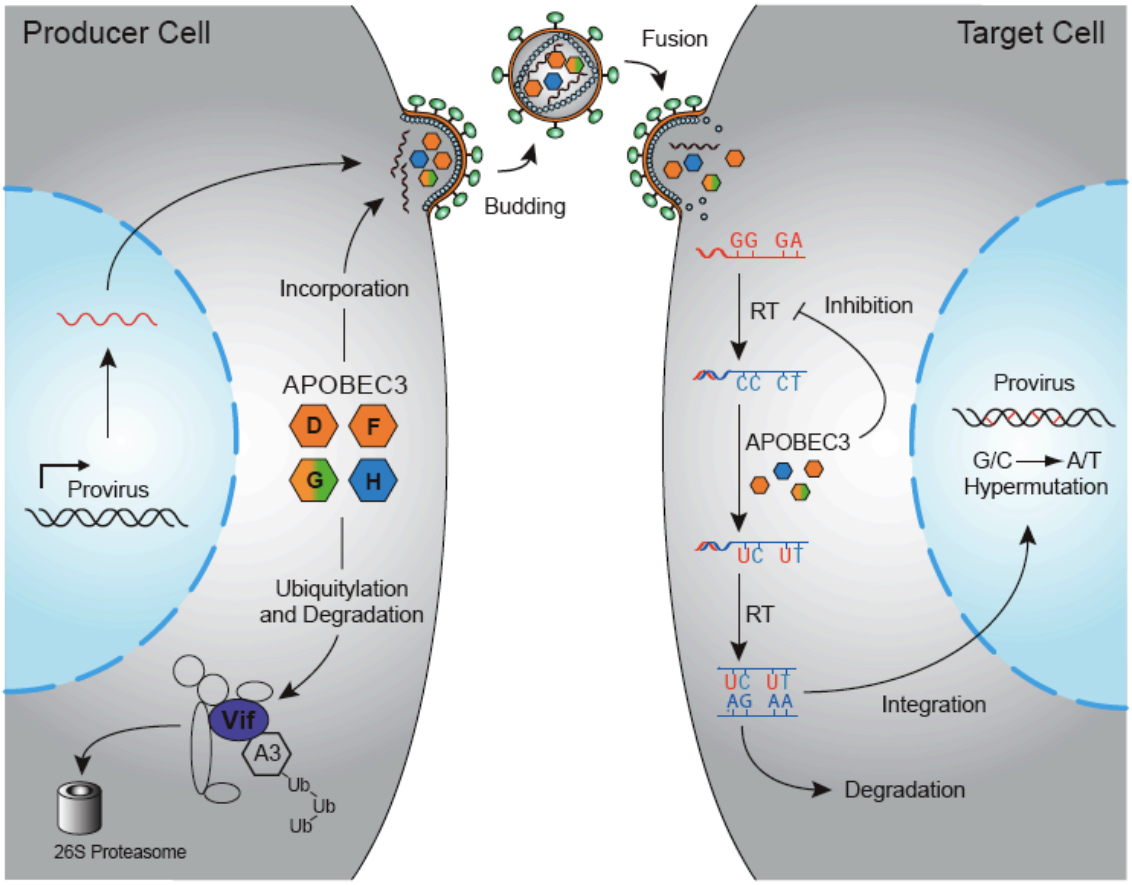

Figure 2-8. Human and Rhesus A3D, A3F, A3G, and A3H Demonstrate a Conserved Capacity to Restrict Vif-deficient HIV and Are Neutralized by their Species-specific Lentiviral Vif.

(A) Summary of the restriction, packaging, and Vif sensitivities of each human and rhesus APOBEC3 protein in T cells and HEK293T cells. '+' indicates a capacity to restrict Vif-deficient HIV, package into Vif-deficient HIV, or be degraded by the indicated Vif in the indicated cell line.

(B) Model for APOBEC3-mediated restriction of HIV and related lentiviruses. In the absence of HIV Vif, four different APOBEC3 proteins, A3D, A3F, A3G, and A3H, may be incorporated into budding virions along with the viral RNA. After viral fusion with a target cell and the initiation of reverse transcription, the APOBEC3 proteins may prevent successful replication by three mechanisms. First, they can bind to the viral RNA and directly inhibit reverse transcription in a deaminase-independent manner. Second, they can deaminate cytosines to uracils on the minus-strand of the viral cDNA resulting in G-to-A hypermutations and the creation of nonfunctional proviruses. Third, they can hypermutate the viral cDNA leading to its degradation prior to integration. HIV Vif protects budding viruses in the producer cell by effectively lowering the steady-state levels of A3D, A3F, A3G, and A3H. Vif acts as an adaptor molecule between these APOBEC3 proteins and an E3 ubiquitin ligase complex, which polyubiquitinates and targets the APOBEC3 proteins for degradation by the 26S proteasome.

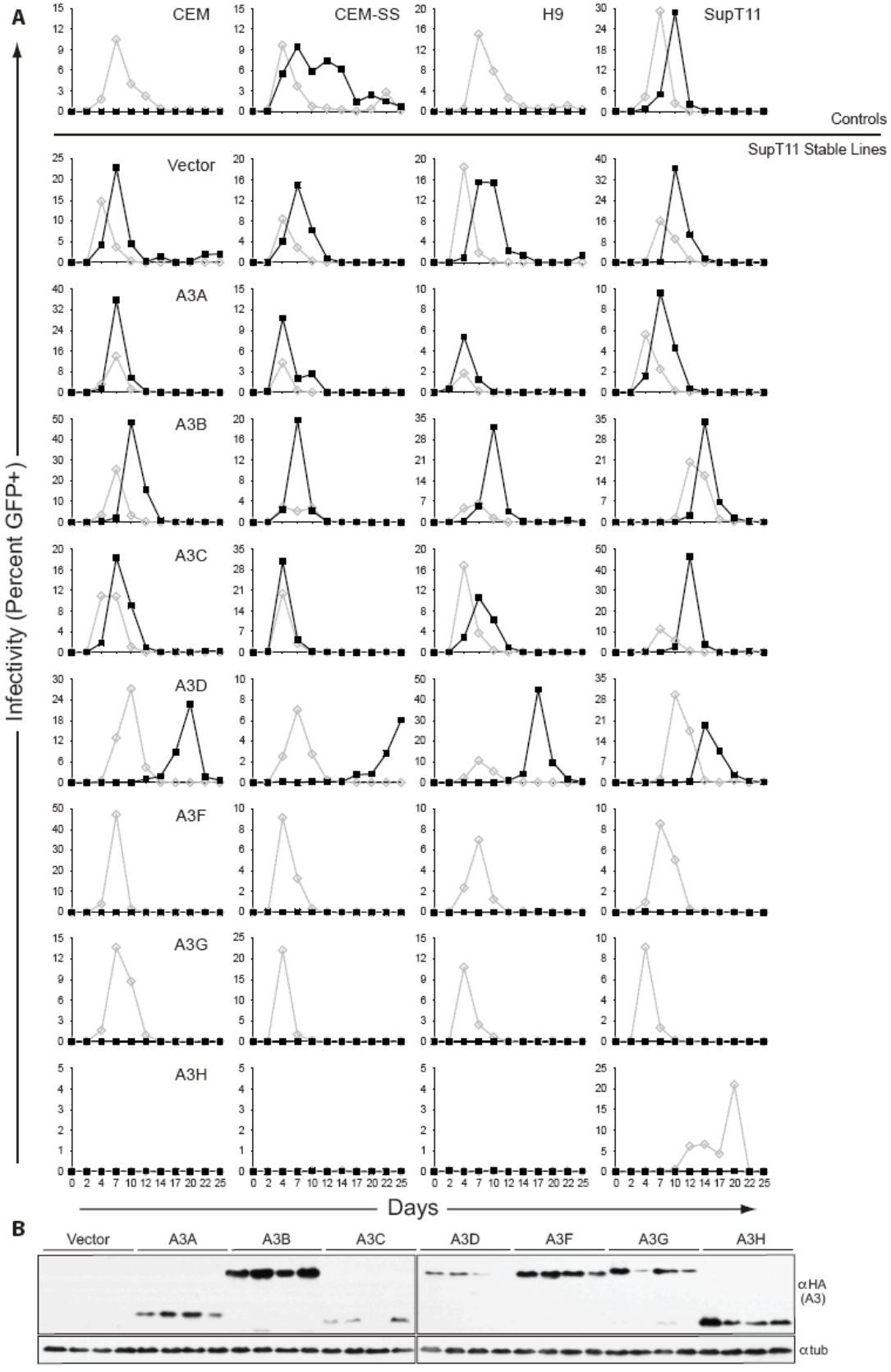
A
CEM
CEM-SS
H9
SupT11
Controls
SupT11 Stable Lines
Vector
A3A
A3B
A3C
A3D
A3F
A3G
A3H
Infectivity (Percent GFP+)
Days
B
Vector
A3A
A3B
A3C
A3D
A3F
A3G
A3H
αHA (A3)
αtub

Figure 2-S1. Replication Kinetics of Vif-proficient and Vif-deficient HIV-1_{IIIB} on Panels of Human APOBEC3-Expressing SupT11 T Cell Lines.

(A) The replication kinetics of Vif-proficient (gray diamonds) and Vif-deficient (black squares) HIV-1_{IIIB} were monitored over three weeks of a spreading infection on panels of SupT11 clones stably expressing each APOBEC3 protein by periodic infection of the reporter line CEM-GFP. The results for four clones from each panel are shown here. Vif-proficient HIV-1_{IIIB} replicates with similar kinetics on permissive (APOBEC3-low) cell lines, such as CEM-SS and SupT11, and nonpermissive (APOBEC3-high) cell lines, such as CEM and H9. Vif-deficient HIV-1_{IIIB} only replicates on permissive cell lines. Stable expression of A3A, A3B, or A3C in the permissive SupT11 line results in no inhibition of Vif-deficient HIV-1_{IIIB} replication relative to Vif-proficient HIV-1_{IIIB}. On the other hand, Vif-deficient HIV-1_{IIIB} replication is significantly delayed or undetectable on cell lines expressing A3D, A3F, A3G, or A3H. In cell lines expressing moderate to high levels of A3H, Vif-proficient HIV-1_{IIIB} replication is also undetectable or delayed, reflecting the inability of HIV-1_{IIIB} Vif to completely neutralize C-terminally tagged A3H as previously reported[195,196] and as in Figure 2-2C.

(B) Immunoblots of the SupT11 clones, whose infectivity plots are shown above, demonstrate stable expression of each HA-tagged APOBEC3. The order of the infectivity plots corresponds to the loading order of the samples.

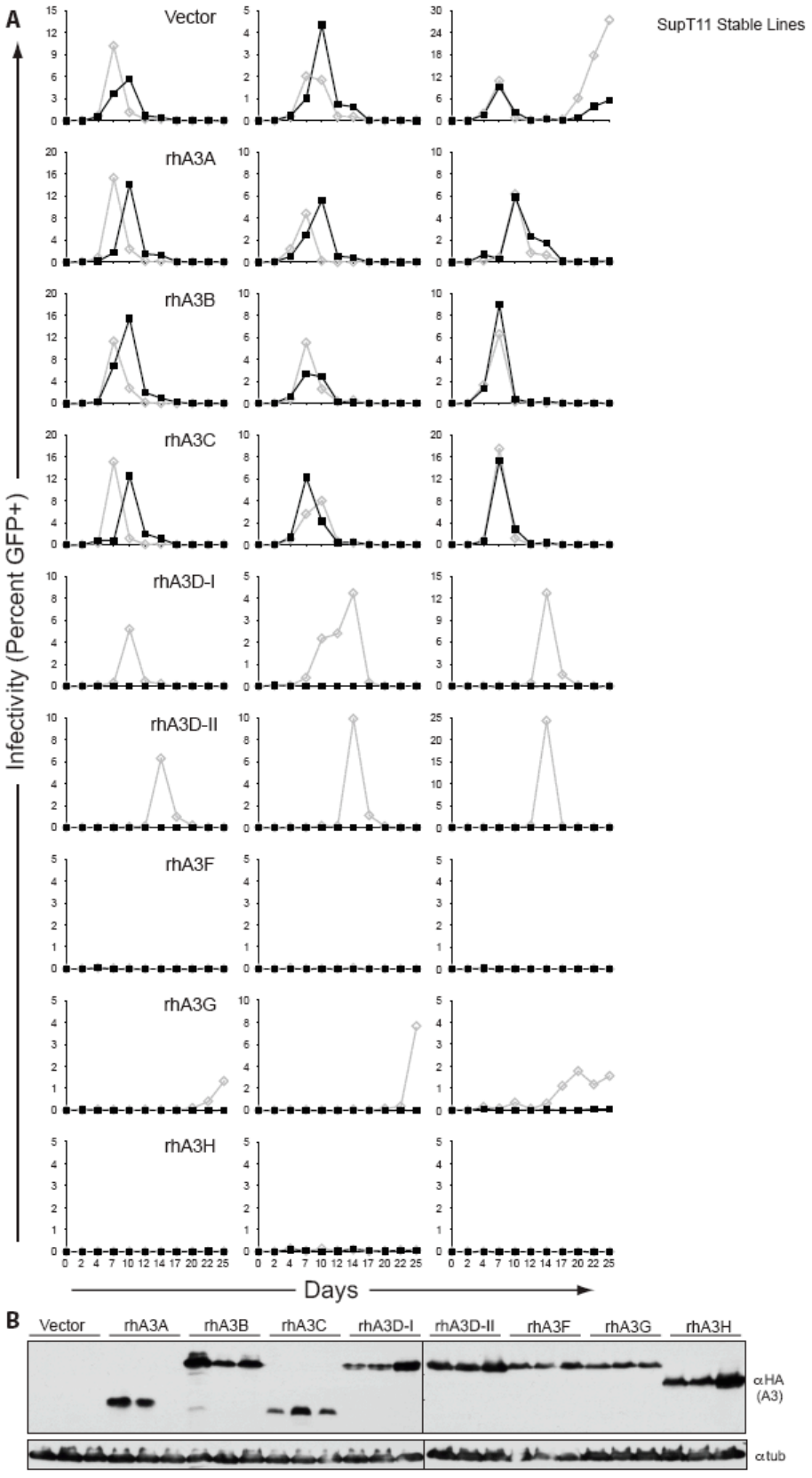
A
SupT11 Stable Lines
Vector
rhA3A
rhA3B
rhA3C
rhA3D-I
rhA3D-II
rhA3F
rhA3G
rhA3H
Infectivity (Percent GFP+)
Days
B
Vector
rhA3A
rhA3B
rhA3C
rhA3D-I
rhA3D-II
rhA3F
rhA3G
rhA3H
αHA
(A3)
αtub

Figure 2-S2. Replication Kinetics of Vif-proficient and Vif-deficient HIV-1_{IIIB} on Panels of Rhesus APOBEC3-Expressing SupT11 T Cell Lines.

(A) The replication kinetics of Vif-proficient (gray diamonds) and Vif-deficient (black squares) HIV-1_{IIIB} were monitored over three weeks of a spreading infection on panels of SupT11 clones stably expressing each rhesus APOBEC3 protein by periodic infection of the reporter line CEM-GFP. The results for three clones from each panel are shown here. Stable expression of rhesus A3A, A3B, or A3C in the permissive SupT11 line results in no inhibition of Vif-deficient HIV-1_{IIIB} replication relative to Vif-proficient HIV-1_{IIIB} similar to the vector control lines. On the other hand, Vif-deficient HIV-1_{IIIB} replication is undetectable on cell lines expressing rhesus A3D isoform I, A3D isoform II, A3F, A3G, or A3H. Vif-proficient HIV-1_{IIIB} replication is similarly suppressed on rhesus A3F and A3H lines and is delayed on rhesus A3G lines, reflecting the inability of HIV-1_{IIIB} Vif to completely neutralize these proteins.

(B) Immunoblots of the SupT11 clones, whose infectivity plots are shown above, demonstrate stable expression of each HA-tagged rhesus APOBEC3. The order of the infectivity plots corresponds to the loading order of the samples.

CHAPTER 3

Vif Hijacks CBFβ to Degrade APOBEC3G and Promote HIV-1 Infection

Reprinted with permission from: Jäger[*], S., D.Y. Kim[*], J.F. Hultquist[*], K. Shindo[^], R.S. LaRue[^], E. Kwon[^], M. Li, B.D. Anderson, L. Yen, D. Stanley, C. Mahon, J. Kane, K. Franks-Skiba, P. Cimermancic, A. Burlingame, A. Sali, C. Craik, R.S. Harris[#], J.D. Gross[#], and N.J. Krogan[#]. (2012) Nature, 481, 371-375.

[*] Equal primary contributions.

[^] Equal secondary contributions.

[#] Corresponding author.

Authors' contributions

This work represents a massive collaborative effort between the lab of R.S. Harris at the University of Minnesota and the labs of J.D. Gross and N.J. Krogan at the University of California – San Francisco. Generally, the lab of N.J. Krogan performed the proteomics and associated work highlighted in Figure 1, the lab of J.D. Gross performed the in vitro purification and characterization of Vif complexes highlighted in Figure 2, and the lab of R.S. Harris performed the functional and virological work highlighted in Figure 3. All primary and corresponding authors had significant roles in the design and execution of the experiments as well as in the preparation and revision of the manuscript/figures. For further clarity, authors' contributions have been appended to each figure legend.

FOREWORD

APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H demonstrate a conserved capacity to restrict Vif-deficient HIV-1. This presents a particular difficulty for HIV treatment by hypomutation as successful implementation of this strategy would depend on the enzymatic inhibition of all four deaminases. On the other hand, it implies that treatment by hypermutation may be successful, especially if such a treatment could liberate the entire repertoire of restrictive APOBEC3s. Unfortunately, efforts to develop such inhibitory compounds have been stifled by an incomplete knowledge of the Vif E3 ubiquitin ligase complex responsible for APOBEC3 degradation. HIV-1 Vif itself has not been amenable to *in vitro* or structural studies. In collaboration with a pair of labs at UCSF, we undertook a series of affinity purification-mass spectrometry experiments with HIV-1 Vif in order to better understand its cellular interactome. Through a series of immunoprecipitation, genetic knockdown, and complementation studies, we discovered a hereto unreported member of the Vif E3 ligase complex, the cellular transcription factor CBFβ. In the absence of CBFβ, Vif is rendered unstable, is unable to assemble the E3 ligase complex, and is unable to alleviate APOBEC3G-mediated inhibition of viral replication. Our working model is that Vif requires CBFβ for stability and/or folding and for ultimate assembly of the E3 ligase complex. We further found that coexpression of Vif and CBFβ allowed for purification of a monodisperse complex and reconstitution of the functional Vif E3 ligase complex *in vitro*. This not only gives us a new platform for the screening of potential small molecule inhibitors of the Vif E3 ligase complex, but also identifies a new potential drug target, the interaction between HIV-1 Vif and CBFβ.

SUMMARY

Restriction factors, such as the retroviral complementary DNA (cDNA) deaminase APOBEC3G (A3G), are cellular proteins that dominantly block virus replication[9,12,13]. The Acquired Iummunodeficiency Syndrome (AIDS) virus, Human Immunodeficiency Virus type 1 (HIV-1), produces the accessory protein Viral infecitivty factor (Vif), which counteracts the host's antiviral defense by hijacking a ubiquitin ligase complex, containing CULLIN5 (CUL5), ELONGINC (ELOC), ELOGINB (ELOB) and a RING-Box protein, and targeting A3G for degradation[16-23,138]. Here we reveal, using an affinity tag purification/mass spectrometry approach, that Vif additionally recruits the transcription cofactor Core Binding Factor β (CBFβ) to this ubiquitin ligase complex. CBFβ, which normally functions in concert with RUNX DNA-binding proteins, allows the reconstitution of a recombinant six-protein assembly that elicits specific polyubiquitination activity with A3G, but not the related deaminase APOBEC3A. Using RNA knockdown and genetic complementation studies, we also demonstrate that CBFβ is required for Vif-mediated degradation of A3G and therefore for preserving HIV-1 infectivity. Finally, Simian Immunodeficiency Virus (SIV) Vif also binds to and requires CBFβ to degrade rhesus macaque A3G, indicating functional conservation. Methods of disrupting the CBFβ-Vif interaction might enable HIV-1 restriction and provide a supplement to current antiviral therapies that primarily target viral proteins.

INTRODUCTION

Mammals have evolved cellular proteins termed restriction factors that function to prevent the spread of mobile genetic elements including retroviruses[9,13,183]. As a counter-defense, most retroviruses, including the human pathogen HIV-1, have developed mechanisms to prevent restriction, often through subversion of the host's ubiquitin-proteasome system. In eukaryotic cells, 8.6 kDa ubiquitin moieties are added to a target protein by sequential action of one of two ubiquitin activating enzymes (E1), which transfer ubiquitin to a pool of dozens of ubiquitin conjugating enzymes (E2) that, in turn, collaborate with hundreds of ubiquitin ligases (E3) to catalyze transfer to specific substrates[210]. If more than four ubiquitins are joined together through K48 linkages, the target protein is usually degraded by the 26S proteasome[211]. At least three HIV-1 proteins, Vif, Viral protein U (Vpu), and Viral protein R (Vpr), hijack cullin(CUL)-RING E3 ligases consisting of CUL5, CUL1 and CUL4A to promote ubiquitination and degradation of APOBEC3 family members (for example, A3G), BST-2/tetherin and an unknown, putative restriction factor, respectively[9,13]. Understanding the composition of CUL-RING E3 ligase complexes and the underlying cellular signaling components may provide therapeutic routes for treating a variety of human diseases, including infection by HIV-1.

HIV-1 Vif is recruited to CUL5 by virtue of its SOCS box, which contains an ELONGINC binding helix (the BC-box), a conserved HCCH Zn-binding motif and a short Cullin Box[20,23,138]. Though a structure of the BC-box peptide in complex with the heterodimer of ELOB and ELOC has been reported[141], the architecture of the full-length Vif in complex with host factors has remained elusive, in part because Vif complexes

have poor solubility and activity. We therefore reasoned that Vif may bind an additional host factor and that such a factor may render it more tractable *in vitro*.

RESULTS

We took an unbiased proteomic approach to identify host factors that bind all 18 HIV processed and polyproteins using an affinity tag purification/mass spectrometry (AP-MS) approach[212]. To this end, 2xStrep and 3xFlag was fused to the carboxy (C) terminus of these factors, including Vif. The tagged Vif construct was both transiently transfected into HEK293 cells and used to make a stable, tetracycline-inducible Vif-Strep-Flag Jurkat T cell line (**Figure 3-1A**). Epitope-tagged Vif was purified from both cell types using antibodies specific to either Strep or Flag and aliquots of the co-purifying proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure 3-1B**). Materials from each step were analyzed by mass spectrometry[212].

Using a new scoring system for data derived from AP-MS studies, termed Mass Spectrometry Interaction Statistics (MiST)[212], we identified 24 Vif-human protein-protein interactions with seven of them found in both cell types (**Figure 3-1C**). Seventeen of these were verified independently by co-immunoprecipitation (**Figure 3-S1)**. Among these were the components of the E3 ubiquitin ligase complex, CUL5, ELOB, and ELOC, known to interact with Vif and trigger A3G degradation[20,23,138,141]. Although the RING-box protein RBX1 was originally reported as part of this complex[23], only RBX2 was found above the MiST score threshold used, consistent with recent work showing that it binds CUL5[213,214]. We did not find endogenous A3G, probably because of its poor expression in the HEK293 and Jurkat cell lines exacerbated by further depletion through

Vif-mediated degradation. We did find Vif associating with two proteins that function in autophagy, AMRA1 and SQSTM, as well as with the transcriptional corepressor complex NCOR1/HDAC3/GPS2/TBL1R (the latter only in T cells) (**Figure 3-1C**). Also, in both cell types, Vif was found to interact with the transcription cofactor CBFβ, which is known to heterodimerize with the RUNX family of transcription factors[215].

To determine if any of the newly defined Vif interactors belong to the Vif-CUL5 complex, we performed double affinity purifications using cells co-transfected with Vif-2xStrep and either A3G- or CUL5-3xFlag (**Figure 3-1D** and **3-1E**). Following purification first with Strep-Tactin and second with anti-Flag beads, mass spectrometry analysis of the final elution revealed the presence of CUL5, ELOB, ELOC, RBX2, and invariably CBFβ, strongly suggesting that this latter protein may be a new component of the Vif E3 ubiquitin ligase complex. To confirm this interaction and the composition of the complex, we performed an additional double affinity purification experiment using Vif-2xStrep and CBFβ-3xFlag. This strategy also yielded CUL5, ELOB, ELOC, and RBX2, in addition to the epitope-tagged bait proteins (**Figures 3-1D** and **3-1E**).

To determine if the association of CBFβ with the CUL5 ligase complex was dependent on Vif, we immunoprecipitated CUL5-haemagglutinin (HA) or ELOB-HA in the presence or absence of Vif in HEK293T cells and blotted for endogenous CBFβ. Only in the presence of Vif did CBFβ co-immunoprecipitate with tagged CUL5 or ELOB, indicating that recruitment of CBFβ to the CUL5 ligase is dependent on Vif (**Figure 3-1F**). SIV Vif also associated with CBFβ by immunoprecipitation, suggesting the interaction is conserved (**Figure 3-1G**).

We next asked if the Vif-CUL5 ligase could be reconstituted with CBFβ using recombinant proteins purified from *Escherichia coli* (*E. coli*). Initial purification attempts without CBFβ yielded aggregated and inactive complexes, assayed by size-exclusion chromatography and autoubiquitination activity, suggesting that CBFβ may be required for complex formation (data not shown). Therefore, full-length Vif, ELOB, ELOC, and CBFβ were co-expressed, purified to homogeneity and found to form a stable, monodisperse complex with recombinant CUL5/RBX2, as shown by size-exclusion chromatography and SDS-PAGE analysis (**Figures 3-2A** and **3-2B**). Pull-down experiments performed with purified, His-tagged APOBEC3 enzymes immobilized on cobolt-chelating resin showed that the four protein complex containing Vif, CBFβ, ELOB, and ELOC binds A3G, but not the related Vif-resistant deaminase, A3A (**Figure 3-2C**). These observations suggested that Vif, CBFβ, ELOB, and ELOC form a substrate adaptor for CUL5/RBX2 that enables specific interaction with susceptible APOBEC3 proteins.

To test the activity of the reconstituted six protein complex, CUL5/RBX2/ ELOB/ELOC/Vif/CBFβ (CRL5-Vif-CBFβ), we assayed substrate and Vif ubiquitination activities using two distinct and well characterized ubiquitin conjugating enzymes, UBE2R1 (hCDC34a) and UBCH5b, which are capable of forming specific K48 and heterogenous ubiquitin chain linkages, respectively[216,217]. With UBE2R1, CRL5-Vif-CBFβ catalyzed formation of high-molecular weight K48 chains on A3G, but not A3A (**Figures 3-2D** and **3-2E**), mirroring the chain linkage and substrate specificity observed in cells[20,23,204,218,219]. As with most ubiquitin ligase assemblies, the CRL5-Vif-CBFβ complex also possessed autoubiquitination activity that was only marginally affected by

substrate APOBEC3s (**Figure 3-S2**). These experiments were done with NEDD8-modified CUL5, since NEDD8ylation is required for CUL5 to degrade A3G *in vivo*[23] (**Figure 3-S3**). Similarly, with UBCH5b, CRL5-Vif-CBFβ was able to promote the specific polyubiquitination of A3G and elicit Vif autoubiquitination activity (**Figure 3-S4**). We conclude that the reconstituted Vif E3 ligase is specific for A3G, supports K48 chain formation, and can function with at least two ubiquitin conjugating enzymes *in vitro*. It is conceivable that these two ubiquitin conjugating enzymes work together in cells to promote multi-monoubiquitination of A3G followed by specific chain elongation, as described for other RING E3s[220,221], but additional work will be necessary to rule out other E2s *in vivo*.

To determine if CBFβ is required for Vif folding and/or stability in living cells, we transfected a constant amount of Vif into HEK293T cells expressing either a scrambled short hairpin (sh)RNA or a CBFβ-specific shRNA. The levels of steady-state Vif were 3-fold lower in CBFβ-depleted cells than in the scrambled control cells (**Figure 3-3A**). Proteasome inhibitor MG132 reversed this effect, suggesting that Vif degradation is accelerated without CBFβ. Analogous data were obtained when Vif was expressed from a proviral plasmid in CBFβ-depleted cells and complemented with a CBFβ expression plasmid (**Figure 3-S5**).

Based on these observations, we predicted that CBFβ knockdown should result in less functional Vif and less infectious HIV-1 particles when produced in the presence of A3G. To test this prediction, shRNA was used to deplete CBFβ stably in HEK293T cells, and a knockdown clone was used to produce replication competent Vif-proficient HIV-1 in the presence or absence of A3G and CBFβ expressed from plasmids (**Figure 3-3B**). In

CBFβ-depleted cells, steady-state Vif levels were very low despite equivalent levels of virus production as indicated by capsid. Moreover, Vif levels increased when CBFβ was replenished by complementation, and this correlated with decreases in cellular and viral A3G levels and corresponding increases in viral infectivity. In the absence of A3G, no difference in infectivity was observed regardless of cellular CBFβ or Vif levels. Titration experiments showed that CBFβ complementation is dose-responsive (**Figure 3-S6**). Analogous results were obtained with a multi-vector HIV-Green Fluorescent Protein (GFP) system (**Figures 3-3C** and **3-S7**). The Vif/CBFβ interaction was confirmed in virus producing cells by coimmunoprecipitation experiments (**Figure 3-S8**). Furthermore, SIV_{mac239} Vif requires CBFβ to degrade rhesus macaque A3G and promote viral infectivity (**Figure 3- 3D**). Interestingly, in contrast to HIV Vif, lower steady-state levels of SIV Vif were observed in the presence of CBFβ, which may be functionally significant or may be a consequence of the heterologous assay system (that is, expressing SIV/rhesus proteins in human cells). Nevertheless, these results demonstrate the essential and conserved nature of CBFβ for Vif function in promoting A3G degradation and efficient virus replication.

DISCUSSION

Our proteomic, biochemical, and genetic studies combine to suggest a model in which HIV-1 Vif hijacks the cellular transcription factor CBFβ to facilitate Vif folding and/or stability as well as nucleation of the rest of the E3 ubiquitin ligase complex (**Figure 3-4**). CBFβ is required for A3G substrate binding and, ultimately, for polyubiquitination and degradation, thereby enabling the production of infectious viral

particles. Because genetic studies have shown that Vif is also capable of degrading APOBEC3F and several other human APOBEC3 proteins[9,13,183], most of which are expressed in primary $CD4^+$ T lymphocytes[181,182], it is quite likely that CBFβ is required for counteracting multiple endogenous APOBEC3s and thus for rendering T lymphocytes permissive for HIV-1 replication. We anticipate that the development of antiviral therapies that antagonize the CBFβ-Vif interaction will be more powerful than those that specifically target the A3G-Vif interaction, because they have the potential to unleash the simultaneous restriction potential of multiple APOBEC3s analogous to current combinatorial therapies.

MATERIALS & METHODS

Cell culture and affinity purification. HEK293(T) cells (ATCC# CRL-1573) were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose supplemented with 10% Fetal Bovine Serum (FBS) and Penicillin/Streptomycin (P/S). For affinity purification, $2.5x10^6$ cells were seeded in 15 cm plates and the next day transfected with 3-10 µg plasmid using calcium phosphate. 42 hours after transfection cells were detached and washed with Phosphate Buffered Saline (PBS). Jurkat TRex cells (Invitrogen) were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, P/S, and 10 µg/ml Blasticidin. Stable Jurkat cell clones were generated by transfection with the linearized vector, selection with 300 µg/ml Zeocin followed by limiting dilution. For affinity purification, $2.5x10^8$ cells were induced with 1 µg/ml doxycyclin for 16 hours. In case of the Vif-2xStrep-3xFlag and Vpr-2xStrep-3xFlag clones, 0.5 µM MG132 (Calbiochem) was added 12 hours before harvest. Cells were

lysed in 1 ml cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, complete protease inhibitor (Roche) and phosphostop (Roche)). Cells were dounced 20x on ice and spun at 2800xg for 20 minutes. The supernatant was incubated with 60 µl preclearing beads (mouse IgG agarose, Sigma or Sepharose 4FF) for 2 hours. The precleared lysate was incubated with 30 µl immunoprecipitation beads over night. Flag affinity purifications were performed with anti-Flag M2 Affinity Gel (Sigma) and Strep affinity purifications with Strep-Tactin Sepharose (IBA). The beads were washed 5x with lysis buffer containing 0.05% Nonidet P40 followed by one wash with lysis buffer without detergent. Proteins were eluted with 40 µl 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA containing either 100 µg/ml 3xFlag peptide (ELIM) and 0.05% RapiGest (Waters), or 2.5 mM Desthiobiotin (IBA). 4 µl of the eluate was analyzed by 4-20% SDS PAGE (Biorad) and silver staining.

Sample preparation for mass spectrometry. For gel-free mass spectometry analysis, 10 µl of the immunoprecipitated (IP) eluate were reduced with 2.5 mM DTT at 60ºC for 30 minutes followed by alkylation with 2.5 mM iodoacetamide for 40 minutes at room temperature. 100 ng sequencing grade modified trypsin (Promega) was then added to the sample and incubated overnight at 37ºC. The resulting peptides were concentrated on ZipTip C18 pipette tips (Millipore) and eluted in a final 20 µl solution of 0.1% formic acid. For gel-based analysis, 20 µl IP eluate was separated by 4-20% SDS-PAGE and stained with GelCode Blue (Thermo Scientific). Each lane was cut into fifteen pieces. Each gel piece was diced into small (1 mm^2) pieces and washed three times with 25 mM NH_4HCO_3/50% ACN. Gel pieces were dehydrated and incubated with 10 mM DTT in 25 mM NH_4HCO_3 and incubated for 1 hour at 56ºC. The supernatant was removed and

the gel pieces were incubated with 55 mM iodoacetamide and incubated for 40 minutes. Gel pieces were washed with 25 mM NH_4HCO_3, then 25 mM NH_4HCO_3/50% ACN and were then dehydrated. 10 ng/µl trypsin in 25 mM NH_4HCO_3 was then added to the gel pieces and incubated overnight at 37ºC. Finally, peptides were extracted from the gel pieces with 50% ACN/5% formic acid and the solvent evaporated. The final peptide sample was resuspended in 20 µl 0.1% formic acid.

Mass spectrometry. All samples were analyzed on a Thermo Scientific LTQ Orbitrap XL mass spectrometer equipped with a nanoACQUITY UPLC (Waters) chromatography system and a nanoelectrospray source. 5 µl of each sample was injected onto a nanoACQUITY Symmetry C18 trap (5 µm particle size, 180 µm x 20 mm) in buffer A (0.1% formic acid in water) at a flow rate of 4 µl/minute and then separated over a nanoACQUITY BEH C18 analytical column (1.7 µm particle size, 100 µm x 100 mm) over one hour with a gradient from 2% to 25% buffer B (99.9% ACN/0.1% formic acid) at a flow rate of 0.4 µl/minute. The mass spectrometer continuously collected data in a data-dependent manner, collecting a survey scan in the Orbitrap mass analyzer at 40,000 resolution with an automatic gain control (AGC) target of 1×10^6 followed by collision-induced dissociation (CID) MS/MS scans of the 10 most abundant ions in the survey scan in the ion trap with an AGC target of 5,000, a signal threshold of 1,000, a 2.0 Da isolation width, and 30 millisecond activation time at 35% normalized collision energy. Charge state screening was employed to reject unassigned or 1+ charge states. Dynamic exclusion was enabled to ignore masses for 30 seconds that had been previously selected for fragmentation. Raw mass spectrometric data were converted into peaklists using Bioworks 3.3.1 SP1. The spectra were searched using Prospector v.5.3

(http://prospector.ucsf.edu)[222] against a human-restricted UniProt database (downloaded October 2009) supplemented with HIV protein sequences from 40 strains. Trypsin was specified as the enzyme; one missed cleavage and zero non-specific cleavages at the peptide termini were permitted. Mass accuracy was set to 25 parts per million for precursor ions and 0.8 Da for fragment ions. Carbamidomethylation of Cys residues was set as fixed modification, and acetylation of protein N-termini and Met oxidation as variable modifications. Protein Prospector results were filtered by applying a minimum Protein Score of 22.0, a minimum Peptide Score of 15.0, a maximum Protein E-Value of 0.01 and a maximum Peptide E-Value of 0.05. Details of the MiST scoring system are described in the accompanying manuscript[212].

Cloning of genes for co-immunoprecipitation confirmation. Coding regions of human cDNA sequences were Polymerase Chain Reaction (PCR) amplified from HEK293 cDNA and cloned into pcDNA4/TO (Invitrogen) carrying amino(N)- or carboxy(C)-terminal 3xFlag tag sequences. Identities of Open Reading Frames (ORFs) were confirmed by sequencing.

Co-immunoprecipitation of putative Vif interactors. HEK293T cells were co-transfected with plasmids encoding a human 3xFlag-tagged protein, and EGFP, Nef or Vif fused to a C-terminal 2xStrep-tagII. Approximately 42 hours post-transfection, cells were lysed in 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, protease inhibitor (Roche). The lysate was clarified by centrifugation (5200xg, 5 minutes, 4ºC) and incubated with Strep-Tactin sepharose (IBA) for 2 hours at 4ºC. After 5 washes in buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P40), beads

were eluted by boiling in SDS sample buffer. Cell lysates and eluates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk/TBST and incubated with mouse anti-Flag antibody (SIGMA), and Strep-TagII antibody HRP conjugate (Novagen). Immunoreactive bands were detected by chemiluminescence (ECL kit; Amersham).

Reconstitution of Vif E3 ligase complex. The Vif substrate adaptor or CUL5/RBX2 heterodimer were coexpressed in *E. coli* using Duet vectors harboring HXB2 Vif, HIS_6-tagged CBFβ and ELOB/ELOC or His-GB1-CUL5/RBX2. Affinity tags were removed by TEV protease after immobilized metal affinity chromatography and subcomplexes were fractionated by size-exclusion chromatography (SEC). The monodisperse fractions of the Vif substrate adaptor and CUL5/RBX2 were mixed and subjected to a final round of SEC to obtain copurified hexamer. Recombinant A3A- and A3G-myc-His_6 were purified from HEK293T cells as described[204,223]. Ubiquitination assays were performed at room temperature with the ubiquitin activating system containing: 2 mM ATP, human ubiquitin activating enzyme (UBE1) (200 nM), wild-type, methyl-ubiquitin or mutant K48R ubiquitin (75 mM), 4 mM E2 (UBE2R1 or UBCH5b) in addition to 0.625 mM Vif E3 and 200 nM APOBEC3 proteins in buffer containing 30 mM Tris-Cl (pH 7.3), 100 mM NaCl, 5 mM $MgCl_2$, in a total reaction volume of 10 µl. For reactions with UBE2R1, the Vif E3 was NEDD8ylated in conditions that included: 50 mM NaCl, 50 mM Tris-Cl pH 7.6, 2.5 mM $MgCl_2$, 2 mg/ml BSA, 2 mM ATP, 100 nM NEDD8 activating enzyme (NAE), 2 mM UBE2F, 30 mM NEDD8 and 4 mM Vif E3 ligase. After 1 hour, the NEDD8 reaction mixture was diluted ~6 fold upon the addition of the ubiquitin activating system and substrate, with final concentration and buffer conditions identical to the

assays done with UBCH5b. The ubiquitination reactions were quenched after 1 hour by the addition of 2x SDS loading dye. UBE1, NEDD8, ubiquitin, Me-ubiquitin, K48R-ubiquitin, and K48-only ubiquitin were purchased from Boston Biochem. GST-NAE (Courtesy of Dr. Brenda Schulman), HIS_6-UBCH5b, HIS_6-UBE2F and HIS_6-UBE2R1 were expressed in *E. coli*, purified by IMAC or GST affinity chromatography. HIS_6 tags were removed by TEV protease. All proteins were subjected to size exclusion chromatography for the final purification. Ubiquitinated Vif or A3 proteins were detected using a polyclonal anti-Vif antibody or monoclonal anti-c-Myc antibody respectively.

***In vitro* pulldowns.** A3A- and A3G-myc-His_6 were purified from HEK293T cells as described[204,223]. 5 µl Talon Metal Affinity Resin (Clontech) was washed with 20 mM HEPES, pH7.4, 0.5 M NaCl, 10% Glycerol and 0.1% Triton X-100, and then blocked with 1 ml of 20 mM HEPES, pH7.4, 0.5 M NaCl, 10% Glycerol, 0.1% Triton X-100 and 10% BSA for 2 hours. The blocked resin was then incubated with 20 pmol of A3G-MycHis or A3A-MycHis protein in 1 ml of 20 mM HEPES, pH7.4, 0.8 M NaCl, 10% Glycerol, 0.1% Triton X-100 and 5% BSA for 2 hours with no APOBEC protein as control. These resins were incubated with 10 pmol Vif substrate adaptor complex in 20 mM HEPES, pH7.4, 0.5 M NaCl, 10% Glycerol, 0.1% Triton X-100, 10% BSA and 20 mM imidazole for 2 hours. The bound proteins were washed with 1 ml of 20mM HEPES, pH7.4, 0.5 M NaCl, 10% Glycerol, 0.5% Triton X-100, 10% BSA and 20mM imidazole, boiled in 30 µl 1×SDS-PAGE loading buffer, and subjected to SDS-PAGE and immunoblotting with appropriate primary (anti-Vif, anti-CBFβ, anti-myc (A3A or A3G)) antibody.

Single cycle HIV-GFP infectivity studies. A non-epitope-tagged CBFβ expression plasmid was constructed by amplifying CBFβ (NM_001755.2) from HEK293T cell cDNA using primers 5'-NNN NGA ATT CAC CAT GCC GCG CGT CGT GCC CGA CCA-3' and 5'-NNN NTC TAG ACT AGG GTC TTG TTG TCT TCT-3', cleaving with EcoRI/XbaI enzymes, and inserting into pcDNA3.1 (Invitrogen) at the compatible sites. The CBFβ shRNA expression construct and non-silencing control construct (Open Biosystems, catalog number RHS4430-99161432 and RHS4346) were obtained through the Biomedical Genomics Center, University of Minnesota. Knockdown of CBFβ was performed by lentiviral transduction, followed by limiting dilution onto 96-well plates and selection of puromycin at a concentration of 0.03 μg/ml. Non-epitope-tagged A3G and HIV-1_{IIIB} Vif expression plasmids were described previously[171]. GFP encoding HIV-1 particles were produced by transiently transfecting HEK293T cells at 50% confluency using 0.7 μg HIV-GFP cocktail [0.3 μg of pCS-CG (LTR flanked GFP), 0.2 μg of pRK5/Pack1(Gag-Pol), 0.1 μg pRK5/Rev, 0.1 μg of pMDG (VSV-G Env)], 140 ng of A3G or empty vector, and 35 ng of codon-optimized Vif or empty vector. 200 ng of CBFβ or empty vector was used for genetic complementation. All reactions were performed in triplicate. After 48 hours, virus-containing supernatants were harvested through PVDF filter with 0.22 μm pores (Millipore) to remove any remaining producer cells. Infectivity of each supernatant was measured by challenging fresh HEK293T cells, incubating 48 hours, and quantifying GFP-positive cells by flow cytometry (FACSCalibur, BD). Sample preparation and immunoblotting were done as described[171,218].

Replication-competent HIV-1 infectivity studies. A stable HEK293T-shCBFβ clone (described above) was used for all the replication-competent HIV-1 infectivity studies. For Figure 3-3B, cells were transfected in triplicate with 1 μg HIV-1_{IIIB} molecular clone in the presence or absence of 100 ng A3G expression plasmid and 100 ng of CBFβ expression plasmid (both untagged). For Figure 3-3D, cells were transfected in triplicate with 1 μg Vif-deficient HIV-1 molecular clone in the presence or absence of 100 ng human or rhesus A3G-HA, 25 ng HIV-1_{IIIB} or SIV Vif-Myc, and 25 ng of CBFβ expression plasmid. After 48 hours, virus-containing supernatants were harvested through PVDF filters with 0.45 μm pores (Millipore) to remove any remaining producer cells. Infectivity of each supernatant was measured by challenging CEM-GFP reporter cells, incubating 48 hours, and quantifying GFP-positive cells by flow cytometry (FACSCalibur, BD)[171,193]. The remaining supernatant was used to collect viral particles. Sample preparation and immunoblotting were done as described[171,218].

Antibodies. Immunoblots used antibodies to A3G (NIH ARRRP #10201 courtesy of Dr. J. Lingappa), CBFβ (Santa Cruz Biotechnology), HA (HA.11; Covance), TUB (tubulin; Covance), c-Myc (Sigma), Vif (NIH ARRRP #2221 courtesy of Dr. D. Gabuzda), and p24/capsid (NIH ARRRP #3537 courtesy of Drs. B. Chesebro and K. Wehrly). Secondary antibodies used were goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (Bio-Rad).

ADDITIONAL CONTRIBUTIONS

We thank members of the Krogan, Gross and Harris labs for helpful comments, B.

Leonard for sharing unpublished data, M. Shales for help with figures, and B. Chesebro, D. Gabuzda, J. Lingappa, M. Malim, K. Wehrly, X.F. Yu, A. Bullock, B. Schulman and the AIDS Research and Reference Reagent Program (ARRRP) for reagents. This research was funded by grants from the National Institutes of Health (NIH P50 GM082250 and P01 AI090935 to NJK; NIGMS U54 RR022220 to AS, R01 AI064046 and P01 GM091743 to RSH; P50 GM082250 to JDG; P41RR001614 to AB). NJK is a Searle Scholar and a Keck Young Investigator.

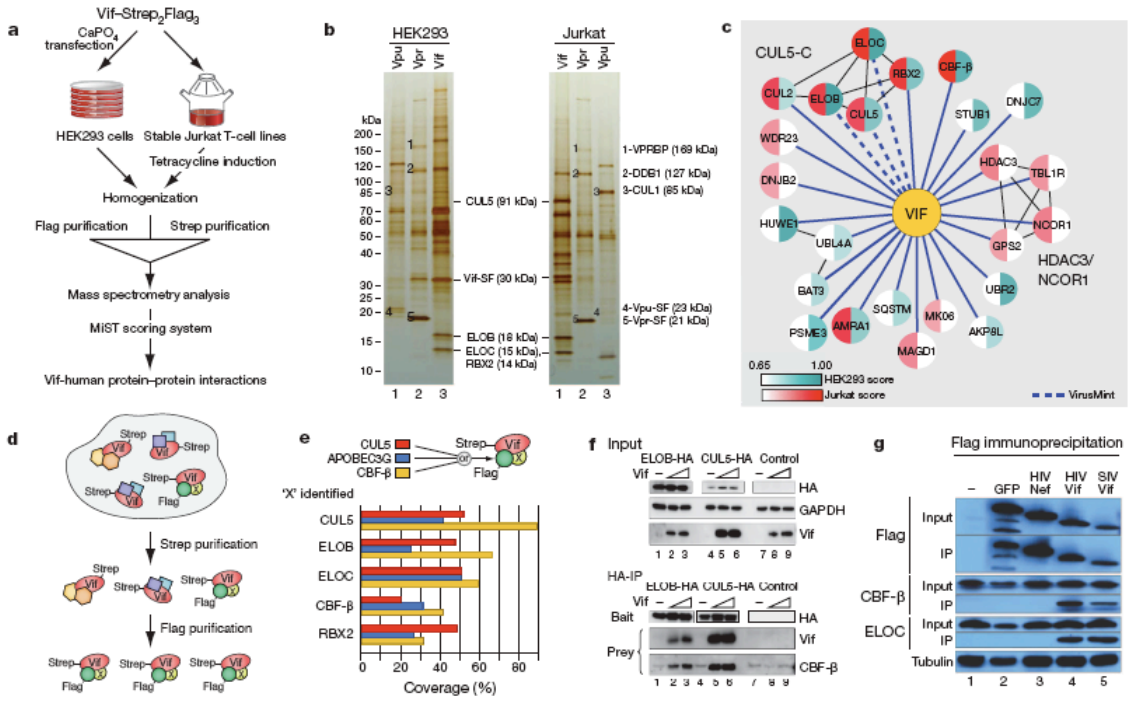

Figure 3-1. AP-MS Experiments Identify CBFβ as a Vif-dependent Component of the Vif-CUL5 Ubiquitin Ligase Complex.

(A) Flow-chart of the proteomic analysis carried out during the course of the study.

(B) Affinity tagged versions of Vif, Vpu and Vpr were purified using 3x-Flag from HEK293 and Jurkat cells, subjected to SDS-PAGE and stained with silver. Visible bands corresponding to interactions that are known for each accessory factor are labeled. Note Vif and CBFβ run at a similar place on the gel. Tagged versions of Vpr and Vpu were used as specificity controls.

(C) A network representation of Vif-host protein-protein interactions from both HEK293 (blue) and Jurkat T cells (red) after subjecting the data derived from the AP-MS analysis to the MiST scoring system[212]. The intensity of the node colors corresponds to the

quantitative MiST score. Blue edges represent interactions derived during this work; black edges are previously described interactions between host factors; dashed edges correspond to previously described Vif-host interactions present in the database VirusMint.

(D) Cartoon schematic of the double purification approach, which allows for the identification of stable, stoichiometric protein complexes.

(E) Double purifications were carried out in triplicate using 3x-Flag tagged CUL5, A3G or CBFβ with 2x-Strep tagged Vif in HEK293 cells. Proteins that were identified in all three double purifications, following trypsin digestion and analysis by mass spectrometry, are represented. The percent coverage corresponds to the percent of protein identified via tryptic peptides.

(F) Immunoblots showing that Vif recruits CBFβ to the CUL5/ELOB/ELOC/RBX2 ubiquitin ligase complex. HA-tagged ELOB or CUL5 were immunoprecipitated in the presence or absence of increasing amounts of Vif, and endogenous CBFβ was monitored via immunoblot.

(G) HIV and SIV Vif co-immunoprecipitate CBFβ and ELOC. GFP and HIV Nef were analyzed in parallel as specificity controls.

S. Jäger and K. Franks-Skiba generated the Vif protein-protein interaction map (A-C); P. Cimermancic developed the MiST scoring system (C); E. Kwon and S. Jäger performed double purification analyses (D-E); C. Mahon and J. Kane performed immunoprecipitation analyses (F-G).

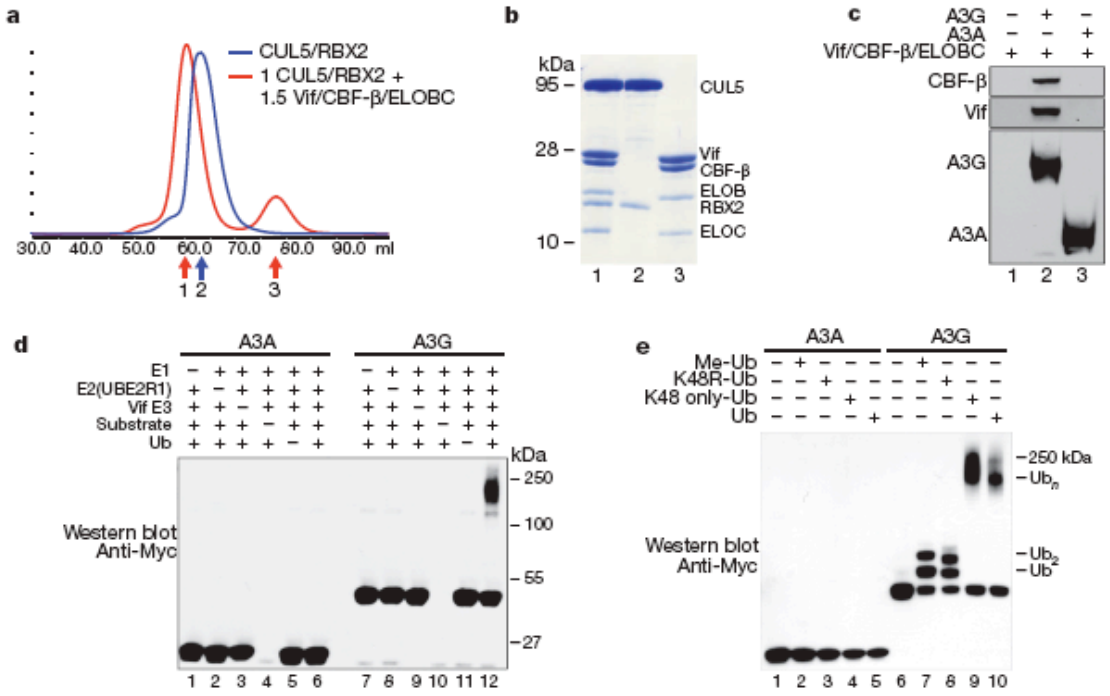

Figure 3-2. CBFβ is a Stoichiometric Component of the Vif E3 Ubiquitin Ligase.

(A) Size exclusion chromatography of recombinant purified CUL5/RBX2 (blue) overlayed with CUL5/RBX2 mixed with 1.5 equivalents of purified Vif substrate adaptor containing Vif, ELOB, ELOC, and CBFβ (red).

(B) Coomassie stained SDS-PAGE of fractions labeled 1-3 in (A) indicating the Vif substrate adaptor and a six protein assembly (CRL5-Vif-CBFβ) copurify as stable monodisperse species.

(C) A3G, but not A3A, directly binds the tetrameric Vif substrate adaptor in pull down experiments *in vitro*.

(D) CRL5-Vif-CBFβ is an E3 ligase that promotes polyubiquitination of A3G, but not A3A (detected using an anti-c-myc antibody to the C-terminal tag on the deaminases).

(E) CRL5-Vif-CBFβ and UBE2R1 catalyze formation of K48 linked chains on A3G. Immunoblot of substrate in ubiquitination reactions containing UBE2R1 as E2, no ubiquitin, chain-terminating methyl-ubiquitin (Me-Ub), K48R mutant ubiquitin (K48R-Ub), ubiquitin containing only K48, with other lysines mutated to arginine (K48 only) and wild-type ubiquitin (Ub). Reactions with Me-Ub indicate at least two distinct sites are modified on A3G; K48R recapitulates pattern observed with Me-Ub, whereas both wildtype and K48R-only Ub result in extensive polyubiquitin chains[220].

D.Y. Kim, L. Yen, and D. Stanley reconstituted the Vif E3 ligase from recombinant components and performed ubiquitination assays (A-B & D-E). M. Li expressed and purified A3 proteins and did in vitro pulldowns (C).

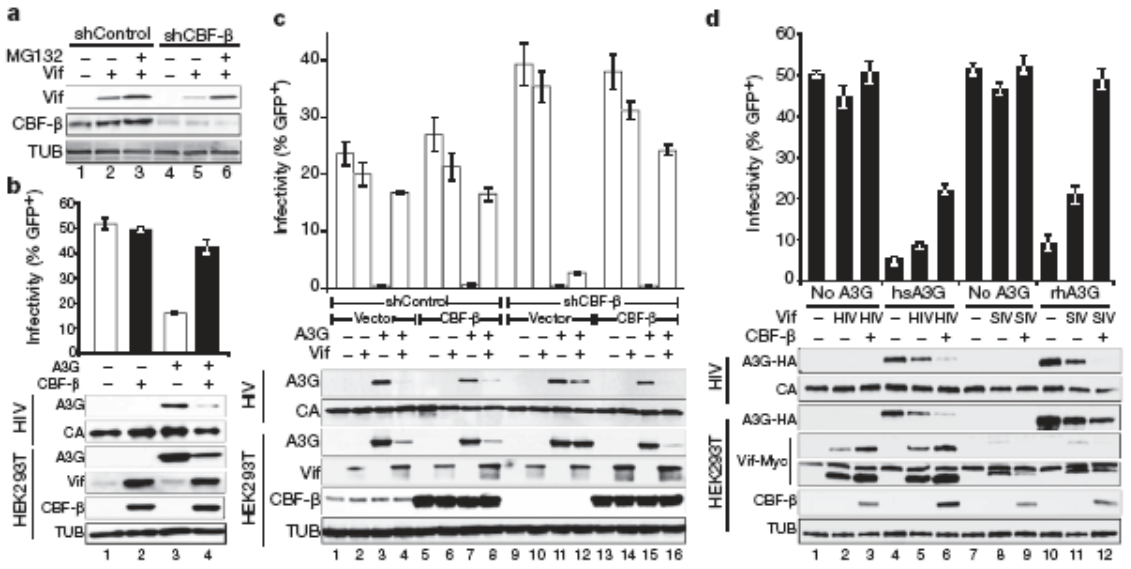

Figure 3-3. CBFβ and Vif Collaborate to Degrade APOBEC3G and Enable HIV-1 Infectivity.

(A) CBFβ-depleted HEK293T cells have lower steady-state Vif levels than control cells. Vif levels recover upon treatment with 2.5 μM proteasome inhibitor MG132.

(B) Infectivity of replication-competent Vif-proficient HIV-1 in the presence and absence of CBFβ and A3G (n=3; mean +/- standard deviation). Immunoblots are shown for the indicated proteins in virus-producing cells and viral particles.

(C) Infectivity of HIV-GFP produced using HEK293T-shCBFβ or HEK293T-shControl clones transfected with the single cycle virus cocktail, A3G, Vif, and CBFβ as indicated (n=3; mean +/- standard deviation). The corresponding immunoblots are shown below.

(D) Infectivity of a Vif-deficient HIV-1 molecular clone produced in the presence or absence of human or rhesus A3G-HA, HIV or SIV Vif-myc, and CBFβ as indicated (n=3; mean +/- standard deviation). Immunoblots are shown for the indicated proteins in virus-

producing cells and viral particles with two exposures of the anti-myc (Vif) blot shown to clarify the SIV Vif signal (the longer exposure also shows endogenous c-myc).

J.F. Hultquist, K. Shindo, and R.S. LaRue performed CBFβ knockdown, complementation, and virus infectivity experiments (A-D).

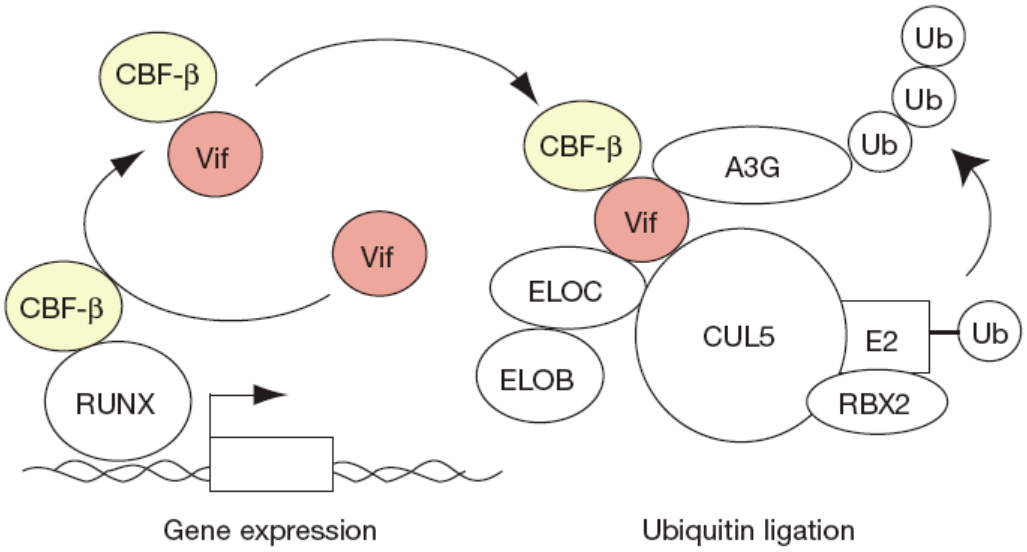

Figure 3-4. Model for Vif-CBFβ E3 Ligase Formation and APOBEC3G Polyubiquitination and Degradation.

Vif is depicted hijacking cellular CBFβ to effect the nucleation of the six-protein E3 ubiquitin ligase complex required for A3G polyubiquitination and degradation. Vif may recruit newly translated CBFβ (not shown) and/or hijack existing CBFβ from RUNX transcription complexes.

All authors contributed to the data and discussion supporting this model. Model design by R.S. LaRue.

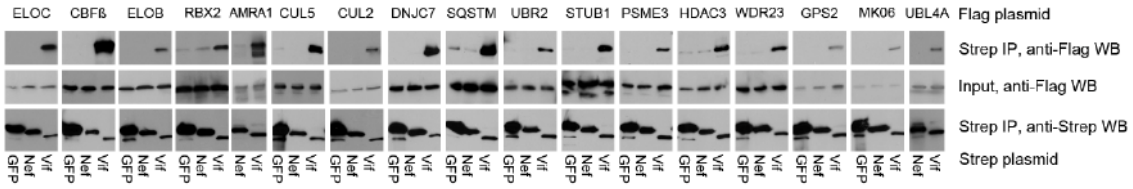

Figure 3-S1. Co-immunoprecipitation Confirmation of Vif Interactors.

Seventeen Flag-tagged human proteins were co-expressed with Strep-TagII-tagged HIV Nef, Vif or GFP in HEK293T cells, and a Strep-Tactin pulldown was performed. Eluates were analyzed by SDS-PAGE and anti-Flag (upper blots), as well as anti-Strep-TagII (lower blots) western blotting. Cell lysates were probed against Flag (middle blots).

C. Mahon and J. Kane performed immunoprecipitation analyses.

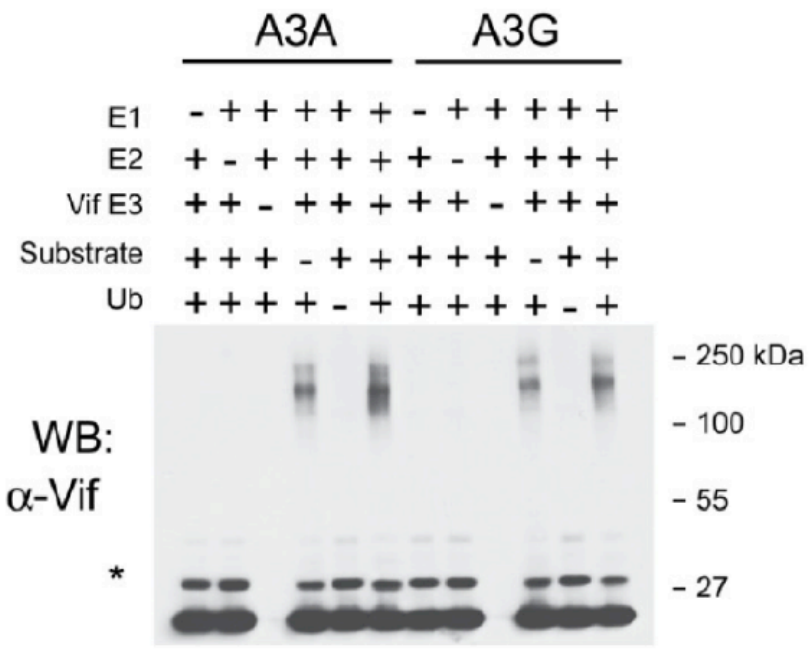

Figure 3-S2. The Presence of APOBEC3 Proteins Does Not Significantly Affect Autoubiquitination Activity of CRL5-Vif-CBFβ.

Reactions products of experiment in Figure 3-2D were probed with a polyclonal anti-Vif antibody. In addition to unmodified Vif and poly-Ub Vif, a small fraction of Nedd8ylated Vif was detected (indicated by the asterisk) under our assay conditions (documented further below in Figure 3-S3).

D.Y. Kim, L. Yen, and D. Stanley reconstituted the Vif E3 ligase from recombinant components and performed autoubiquitination assays.

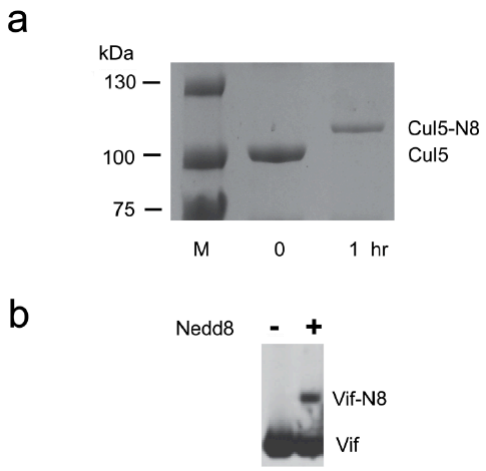

Figure 3-S3. Nedd8ylation of the Ubiquitin Ligase Complex by the NEDD8 Conjugating Enzyme UBE2F.

(A) Coomassie stained SDS-PAGE showing NEDD8 "pulse" reaction 0 and 1 hours after addition of ATP, NEDD8, NEDD8 E1, and UBE2F showing complete modification of CUL5. See methods for reaction conditions. M designates molecular weight standards.

(B) Immunoblot of "pulse" reaction with and without NEDD8 sampled after 1 hour of initiation, indicating a small (<5%) fraction of Nedd8ylated Vif, quantified by a fluorescent secondary antibody. UBE2F was was previously shown to promote Nedd8ylation of CUL5/RBX2 but not complexes between RBX1 and CUL1-4 *in vivo*[213]. Consistent with this observation and our proteomic data, knockdown of UBE2F blocks HIV infectivity by reducing CUL5 NEDD8ylation in HIV producing cells, abrogating Vif mediated ubiquitination and degradation of A3G (Stanley *et al*., in preparation).

D.Y. Kim, L. Yen, and D. Stanley reconstituted the Vif E3 ligase from recombinant components and performed NEDD8ylation assays (A-B).

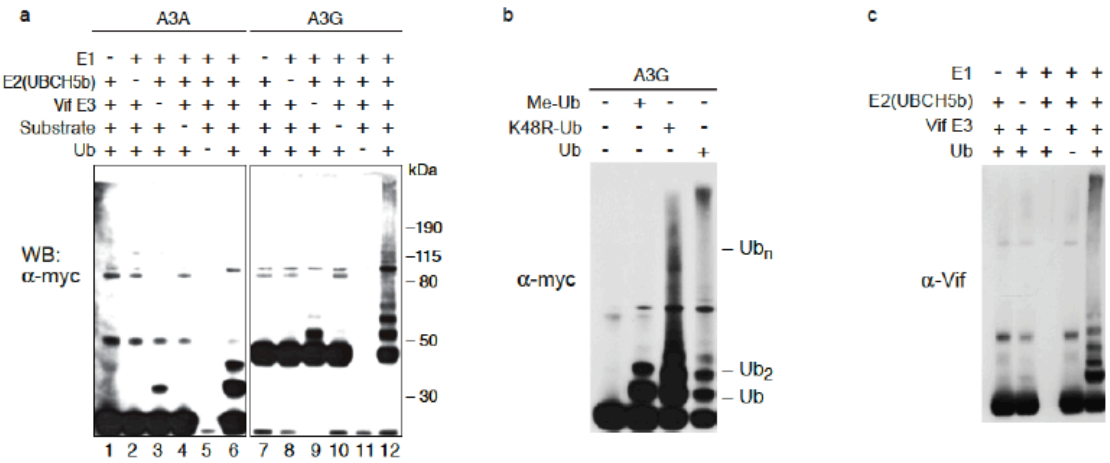

Figure 3-S4. CRL5-Vif-CBFβ Catalyzes Polyubiquitin Chain Formation on APOBEC3G in the Presence of Ubiquitin E2 UBCH5b.

(A) Immunoblots of substrate ubiquitination in the presence of ATP, E1 (UBE1), UBCH5b and CRL5-Vif-CBFβ.

(B) Immunoblots of A3G in reactions lacking Ub, containing Me-Ub, K48R Ub or wildtype Ub. As with UBE2R1 (**Figure 3-2E**), two sites are modified on A3G; however, the chain is not K48 linked consistent with previous reports that UBCH5b generates heterogeneous chain linkages[217]. Me-Ub experiments on A3A reveal a single site modified (data not shown); the di-ubiquitin chain observed in panel (A) is unlikely to trigger proteasomal degradation because tetra-ubiquitin chains are the minimal chain length required for efficient recognition by the proteasome[211].

(C) Immunoblot showing CRL5-Vif-CBFβ autoubiquitination activity with UBCH5b.

D.Y. Kim, L. Yen, and D. Stanley reconstituted the Vif E3 ligase from recombinant components and performed ubiquitination assays.

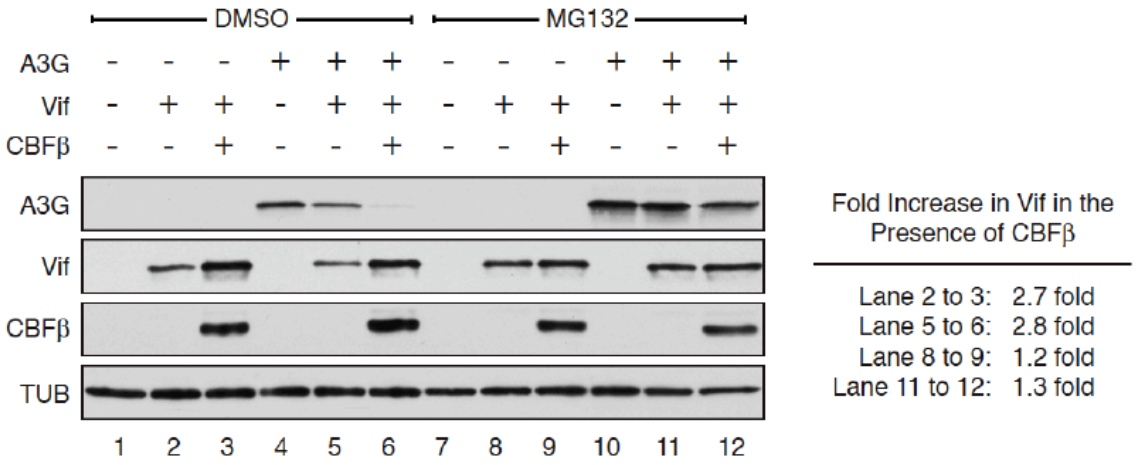

Figure 3-S5. CBFβ is Essential for Vif Expression and/or Stability.

A HEK293T-shCBFβ clone was transfected with plasmids expressing A3G, CBFβ, and either a Vif-proficient or Vif-deficient HIV-1_{IIIB} molecular clone, as indicated. 24 hours post-transfection, cells were treated with either 2.5 μM proteasome inhibitor MG132 or with a volumetric equivalent of DMSO. 16 hours later, cells were processed for immunoblotting as described in the methods. The percent increase in Vif steady-state levels was determined as the amount of Vif (relative to tubulin) in the $CBF\beta^+$ lane over the amount of Vif (relative to tubulin) in the $CBF\beta^-$ lane. Quantification was done using Image J.

J.F. Hultquist and K. Shindo performed CBFβ knockdown and complementation assays in the presence of proteasome inhibitor.

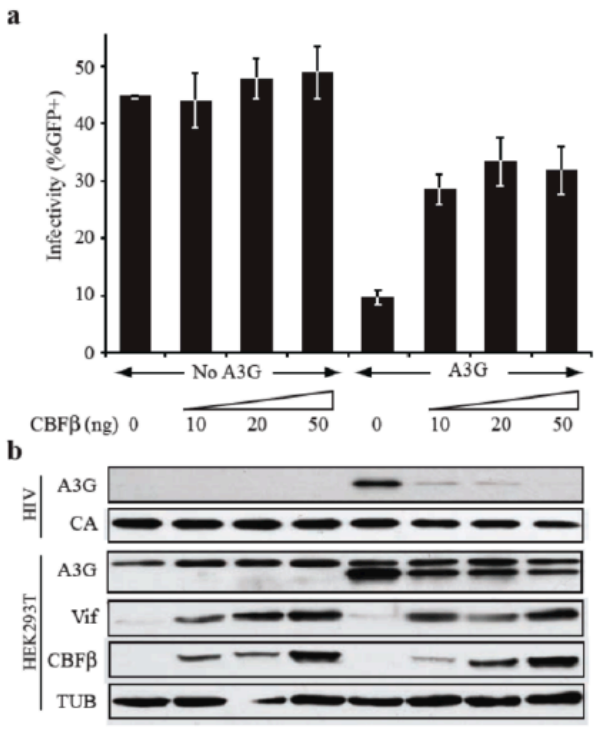

Figure 3-S6. CBFβ Titration Experiment.

The infectivity of replication-competent Vif-proficient HIV-1_{IIIB} produced using a HEK293T-shCBFβ clone in the presence of 100 ng control plasmid or 100 ng untagged A3G expression plasmid and the indicated amount of untagged CBFβ expression plasmid. Immunoblots were performed as described in the methods.

J.F. Hultquist and R.S. LaRue performed CBFβ knockdown, complementation, and virus infectivity experiments with replication-competent HIV-1.

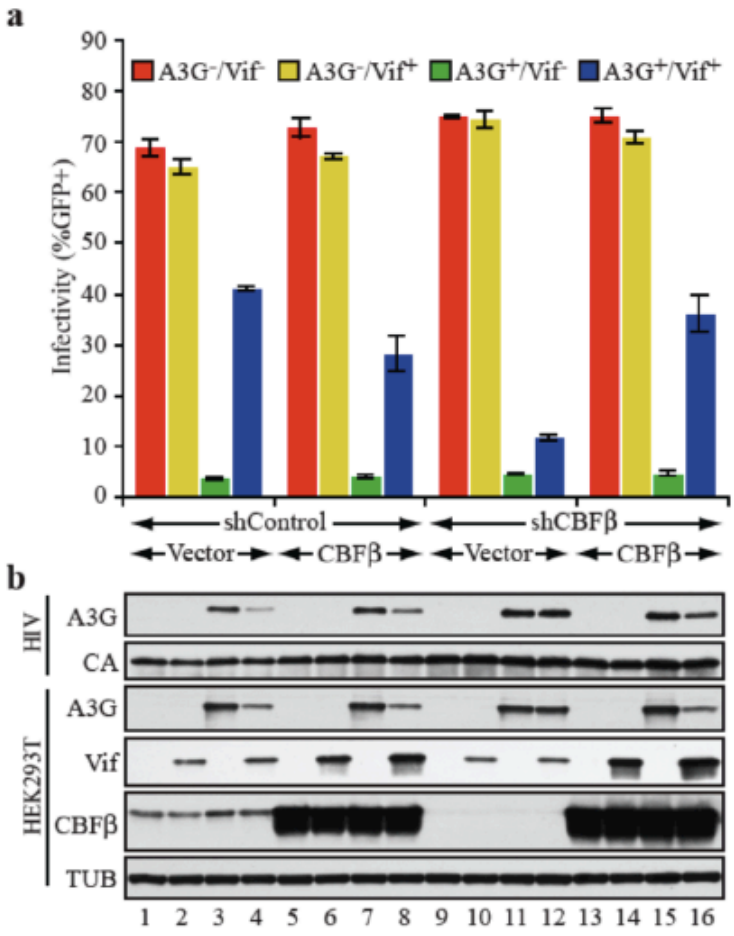

Figure 3-S7. CBFβ and Vif Collaborate to Degrade APOBEC3G and Enable HIV-1 Infectivity.

(A) Infectivity of Vif-deficient HIV-GFP produced in clonal HEK293T stably transduced with control shRNA or CBFβ-specific shRNA constructs and transiently expressing A3G, Vif, and/or CBFβ as shown. This experiment is an independent replicate of the one shown in Figure 3-4C, except here the virus producing cells are puromycin selected shControl or shCBFβ transduced populations.

(B) Immunoblots of A3G and CA in viral particles (top 2 panels) and A3G, Vif, CBFβ, and tubulin in the producer cell lysates (bottom 4 panels).

K. Shindo performed CBFβ knockdown, complementation, and virus infectivity experiments with replication-incompetent HIV-1 cocktails.

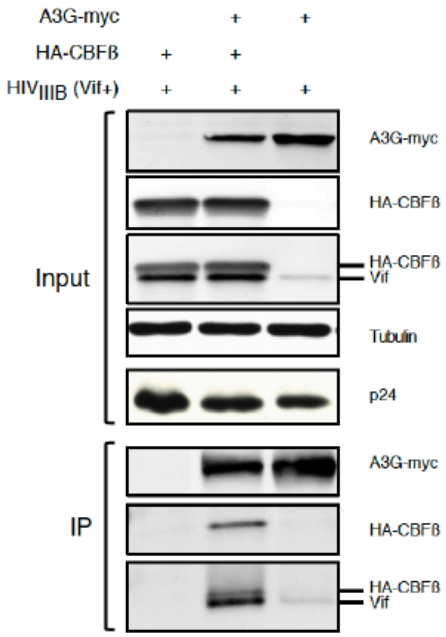

Figure 3-S8. Co-immunoprecipitation of CBFβ and Vif in HIV-1 Producing Cells.

An HA-tagged CBFβ expression construct was co-transfected with infectious Vif-proficient HIV-1_{IIIB} and A3G-myc expression plasmids into HEK293T-shCBFβ knockdown cells. 24 hours post-transfection, 2.5 μM MG132 was added to inhibit the proteosome and 16 hours later lysates were prepared [25mM HEPES pH7.4, 150mM NaCl, 1mM EDTA, 1mM $MgCl_2$, 1mM $ZnCl_2$, 0.1% Triton-X100, 10% glycerol, and complete EDTA free protease inhibitor (Roche)], treated with DNase I and RNase A, and used for anti-myc (A3G) immunoprecipitation [anti-c-myc 9E11 (Thermo Scientific)]. Input and co-IP samples were fractionated by SDS-PAGE, transferred to Immobilon-FL PVDF (Millipore) and immunoblotted. Similar results were obtained in analogous experiments with untagged CBFβ in place of HA-tagged CBFβ (data not shown).

J.F. Hultquist and B.D. Anderson performed CBFβ-Vif co-IP experiments.

CHAPTER 4

Vif Proteins of Human and Simian Immunodeficiency Viruses Require Cellular CBFβ to Degrade APOBEC3 Restriction Factors

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Authors' contributions

This work was done in collaboration with the lab of V. Simon at Mount Sinai School of Medicine in New York. J.F. Hultquist performed all experiments, wrote/revised the manuscript, and created all of the figures except as follows. M. Binka performed the work with Vif proteins from other HIV subtypes shown in Figure 2. R.S. Harris and V. Simon aided in the design of the experiments and in manuscript revision.

FOREWORD

HIV-1 Vif requires CBFβ for stability and/or folding and for the ultimate assembly of the APOBEC3-degrading E3 ligase complex. Not only does the identification of CBFβ allow for the first *in vitro* studies of the Vif E3 ligase complex, but it also raises the possibility that the Vif-CBFβ interaction may be a druggable target. This is an attractive target for two reasons. First, if CBFβ is required for E3 ligase assembly, disruption of this interaction has the potential to release the entire restrictive repertoire of APOBEC3 proteins. Second, CBFβ does not associate with the E3 ligase complex in the absence of Vif. Therefore, unlike targeting one of the other E3 ligase components, targeting the Vif-CBFβ interaction for therapeutic disruption does not risk inhibiting normal, and possibly essential, E3 ligase functions. It is unknown, however, if the requirement of Vif for CBFβ is broad and fairly well conserved or if it is a unique property of a few select HIV subtypes. To gauge the general conservation of the Vif/CBFβ/APOBEC3 functional interplay, we set out to determine which isoforms of CBFβ contribute to Vif stabilization, whether CBFβ is required to stabilize Vif proteins of multiple different HIV subtypes, and, finally, if CBFβ is required by Vif to neutralize the entire repertoire of Vif-sensitive APOBEC3 proteins. We found that the functional requirement for CBFβ is highly conserved, required to enhance the steady-state levels of Vif proteins from all tested subtypes of HIV and required for the degradation of all restrictive human and rhesus APOBEC3 proteins by their respective lentiviral Vif proteins. This further implies that the Vif-CBFβ interaction would be a good candidate for therapeutic disruption as such small molecule inhibitors would be broadly applicable.

SUMMARY

Human Immunodeficiency Virus type 1 (HIV-1) requires the cellular transcription factor Core Binding Factor β (CBFβ) to stabilize its accessory protein Viral infectivity factor (Vif) and promote APOBEC3G (A3G) degradation. Here, we demonstrate that both isoforms of CBFβ allow for increased steady-state levels of Vif, enhanced A3G degradation, and increased viral infectivity. This conserved functional interaction enhances the steady-state levels of Vif proteins from multiple HIV-1 subtypes and is required for the degradation of all human and rhesus Vif-sensitive APOBEC3 proteins by their respective lentiviral Vif proteins.

INTRODUCTION

HIV-1 and related lentiviruses require the viral accessory protein Vif to neutralize members of the APOBEC3 family of retroviral restriction factors and render host cells permissive for productive viral replication. HIV-1 Vif neutralizes the APOBEC3 proteins by recruitment of an E3 ubiquitin ligase complex that polyubiquitinates APOBEC3 proteins and targets them for proteasomal degradation ([23], reviewed by [9,12,183]). Recently, the cellular transcription factor CBFβ was found to be associated with this complex and allows for its reconstitution *in vitro*[224]. Furthermore, CBFβ was found to be required for the stability of HIV-1_{IIIB} Vif *in vivo*, allowing for efficient degradation of A3G and increased viral infectivity[224]. The current model is that HIV-1 Vif hijacks cellular CBFβ to facilitate Vif folding and/or stability as well as nucleation of the E3 ubiquitin ligase complex. While it has been shown that Simian Immunodeficiency Virus of the rhesus macaque strain 239 (SIV_{mac239}) Vif also requires CBFβ to degrade rhesus A3G[224], the generality of the CBFβ/Vif/APOBEC3 functional interplay remains to be determined. The goal of the current study was to determine which isoforms of CBFβ contribute to Vif stabilization, whether CBFβ is required to stabilize Vif proteins of multiple different HIV subtypes, and, finally, if CBFβ is required by Vif to neutralize the entire repertoire of Vif-sensitive APOBEC3 proteins.

RESULTS & DISCUSSION

Alternative splicing generates at least two isoforms of CBFβ in human cells (Genbank accessions NM_022845.2 and NM_001755.2). Though they differ in size and

amino acid sequence at their C-terminal end, these splice variants share 165 N-terminal residues, including the RUNX heterodimerization domain, and a clear functional difference has yet to be delineated. To determine if HIV-1 Vif distinguishes between these CBFβ isoforms, a stable CBFβ knockdown clone of HEK293T was created using a stably integrated short hairpin (sh)RNA that targets both isoforms[224]. This line was transiently transfected with a Vif-proficient or Vif-deficient A200C HIV-1_{IIIB} molecular clone[172] in the presence or absence of human A3G and complemented with either the 187 amino acid CBFβ isoform 1 (cloned from CEM cDNA by PCR and standard molecular biology techniques) or the shorter 182 amino acid CBFβ isoform 2 (as used previously; [224]). 48 hours after transient transfection, cell lysates and viral particles were collected for immunoblotting and viral infectivity was monitored by infection of the reporter cell line CEM-GFP[218]. Both isoforms resulted in a comparable increase in HIV-1_{IIIB} Vif steady-state levels, enhanced degradation of A3G, and a rescue of viral infectivity (**Figure 4-1**). In the absence of A3G, neither CBFβ isoform impacted viral infectivity.

Most laboratory strains of HIV-1, including HIV-1_{IIIB}, HIV-1_{NL4-3}, and HIV-1_{LAI}, are subtype B, but over ten different HIV-1 subtypes are found worldwide with subtype C being the most prevalent[225]. To determine if CBFβ can stabilize Vif proteins from multiple subtypes, representative Vif alleles from HIV-1 subtypes A, B, C, D, AE, F, and G (as described[226]) were co-transfected into the HEK293T CBFβ knockdown cell line with A3G in the presence or absence of CBFβ isoform 2 and glutatione S-transferase (GST) as a transfection control. In every case, CBFβ increased the steady-state level of the Vif variant and resulted in increased degradation of A3G (**Figure 4-2**). While basal Vif expression levels varied, CBFβ increased the steady-state level of each Vif variant by

an average of approximately 4-fold. Furthermore, while each variant also differs in its ability to neutralize A3G[226], steady-state levels of A3G were decreased upon CBFβ complementation in every case by an average of 2-fold. A3G levels were not affected by CBFβ in the absence of Vif or in the presence of HIV-1_{IIIB} Vif C133S, which fails to recruit the E3 ubiquitin ligase complex[133,227]. CBFβ did not effect the expression of the GST control. Thus, the dependency of Vif for CBFβ is broadly conserved across multiple HIV-1 subtypes.

Human $CD4^+$ T cells express six APOBEC3 proteins, of which HIV-1 Vif degrades five: A3C, A3D, A3F, A3G, and A3H[182,218]. To determine if HIV-1 Vif requires CBFβ to neutralize not only A3G, but the other Vif-sensitive APOBEC3 proteins as well, HIV-1_{LAI} molecular clone was transfected into HEK293T CBFβ knockdown cells with increasing amounts of each human APOBEC3 protein in the presence or absence of CBFβ isoform 2. CBFβ increased Vif steady-state levels and resulted in decreased cellular levels of all Vif-sensitive APOBEC3 proteins (A3C, A3D, A3F, A3G, and A3H haplotype II, **Figure 4-3**). In the presence of CBFβ, packaging of A3D, A3F, A3G, and A3H was also decreased and viral infectivity increased accordingly. Neither A3A nor A3B are sensitive to HIV-1_{LAI} Vif and consequently their expression, packaging, and impact on viral infectivity were not affected by CBFβ. Thus, HIV-1 Vif requires CBFβ to neutralize not only A3G, but the entire repertoire of Vif-sensitive human APOBEC3 proteins.

Rhesus macaques also encode seven distinct APOBEC3 proteins, of which rhesus A3D, A3F, A3G, and A3H can restrict Vif-deficient HIV-1 and SIV[164,218]. SIV_{mac239} Vif neutralizes all four restrictive rhesus APOBEC3 proteins and also degrades rhesus A3B

and rhesus A3C[218]. To determine if SIV_{mac239} Vif requires CBFβ to neutralize the rhesus APOBEC3 proteins, Vif-deficient $HIV\text{-}1_{IIIB}$ molecular clone was transfected into HEK293T CBFβ knockdown cells alongside SIV_{mac239} Vif and increasing amounts of each rhesus APOBEC3 protein in the presence or absence of CBFβ isoform 2. Human CBFβ isoforms 1 and 2 are identical at the amino acid level to rhesus CBFβ isoforms 1 and 2, respectively. While there is no antibody for SIVmac239 Vif, the addition of CBFβ resulted in decreased cellular levels of all Vif-sensitive rhesus APOBEC3 proteins (rhesus A3B, A3C, A3D, A3F, A3G, and A3H, **Figure 4-4**). In the presence of CBFβ, packaging of rhesus A3D, A3F, A3G, and A3H was also decreased and viral infectivity consequently increased. Rhesus A3A is not sensitive to SIV_{mac239} Vif and so its expression, packaging, and effect on viral infectivity were unaltered by CBFβ. Thus, SIV Vif demonstrates a conserved requirement for CBFβ to neutralize the rhesus repertoire of APOBEC3 proteins.

$HIV\text{-}1_{IIIB}$ Vif was previously shown to require CBFβ isoform 2 for stable expression and neutralization of A3G[224]. Here, we demonstrate that both CBFβ isoform 1 and isoform 2 may be hijacked to stabilize $HIV\text{-}1_{IIIB}$ Vif and degrade A3G. This functional interaction was conserved across all tested HIV-1 subtypes and was required for the neutralization of not only A3G, but all Vif-sensitive human APOBEC3 proteins. SIV_{mac239} Vif also required CBFβ to neutralize all restrictive rhesus APOBEC3 proteins. Taken together, the CBFβ-Vif interaction appears to be broadly conserved and essential for Vif function, implicating this interface as a candidate for disruption by small molecule therapeutics that would alleviate repression of multiple restrictive APOBEC3 proteins.

MATERIALS & METHODS

All materials and methods are described in the text or figure legends and published previously in detail[218,224].

ADDITIONAL CONTRIBUTIONS

We thank N. Krogan and J. Gross for discussion and data sharing prior to publication, and the National Institute of Health (NIH) AIDS Research and Reference Reagent Program (ARRRP) for materials. This research was funded by NIH R01 AI064046 and P01 GM091743 to RSH and NIH R01 AI064001 and NIH R01 AI089246 to VS. JFH was supported by a National Science Foundation (NSF) Predoctoral Fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

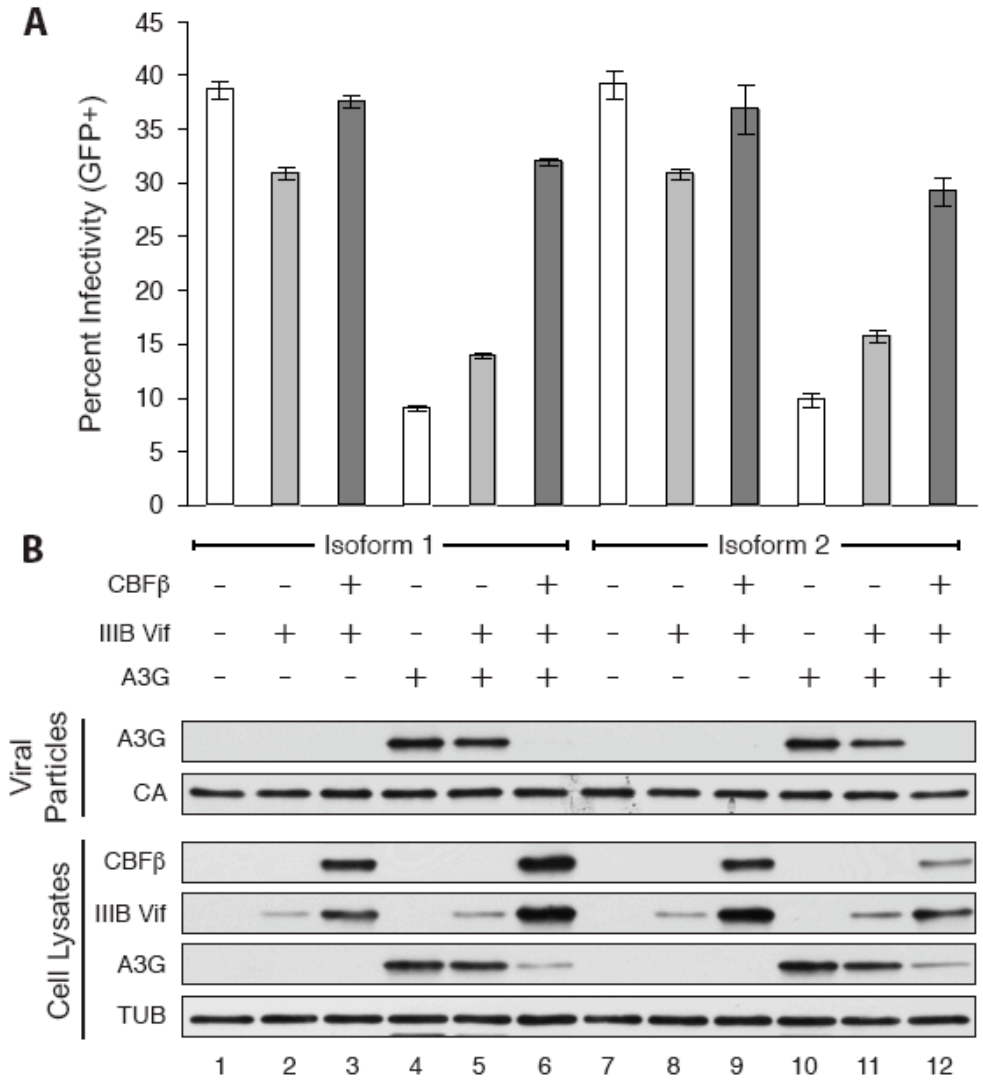

Figure 4-1. CBFβ Isoform 1 and Isoform 2 Stabilize HIV-1 Vif to Degrade APOBEC3G and Increase Viral Infectivity.

(A) Percent infectivity of HIV-1_{IIIB} measured by duplicate infection of CEM-GFP and flow cytometry, reported as the mean +/- the standard deviation of the technical replicate. A constant amount of Vif-deficient or Vif-proficient A200C HIV-1_{IIIB} molecular clone (1

μg) was co-transfected with A3G or empty plasmid (50 ng) in the presence or absence of CBFβ complementation vector (25 ng) as indicated.

(B) Immunoblots of CBFβ, Vif, and HA-tagged human A3G in cell lysates (lower panels) and of A3G in HIV-1 particles produced by those cells (upper panels). Tubulin (TUB) and p24 (CA) served as cell and viral lysate loading controls.

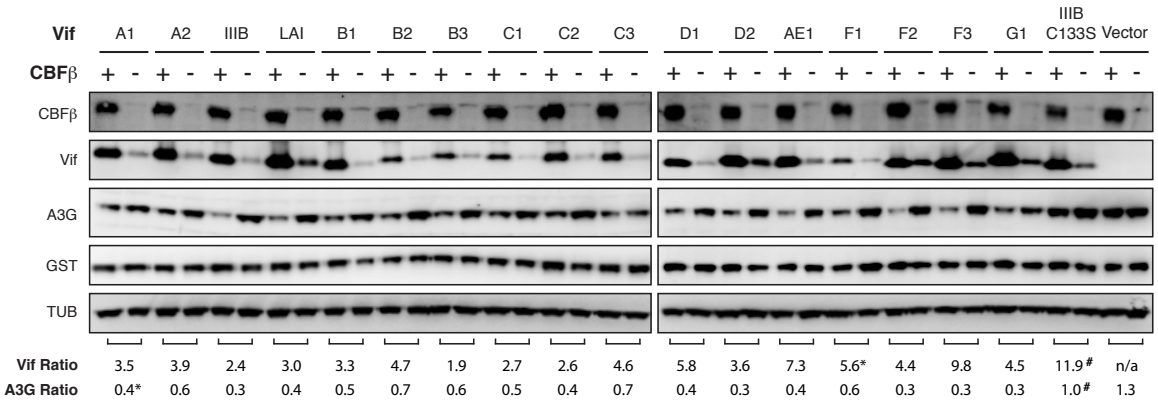

Figure 4-2. CBFβ Stabilizes Vif Proteins from Multiple HIV-1 Subtypes.

Immunoblots of HA-tagged CBFβ, HIV-1 Vif, and FLAG-tagged human A3G in cell lysates. Tubulin (TUB) and V5-tagged GST served as cell lysate loading and transfection controls respectively. A constant amount of the indicated Vif variants (pCRV1 expression vector, 50 ng) were co-transfected with A3G (300 ng), GST (200 ng), and either CBFβ isoform 2 (100 ng) or empty vector. The untagged Vif variants were detected with a polyclonal rabbit anti-Vif antibody (NIH Cat#2221). One representative experiment of three independent transfections is shown. The Vif ratio represents the average ratio of Vif in the presence versus the absence of CBFβ (relative to GST) over three experiments unless otherwise noted (*n=2, #n=6). The A3G ratio was calculated analogously. Quantification was performed using Image Gauge v4.0.

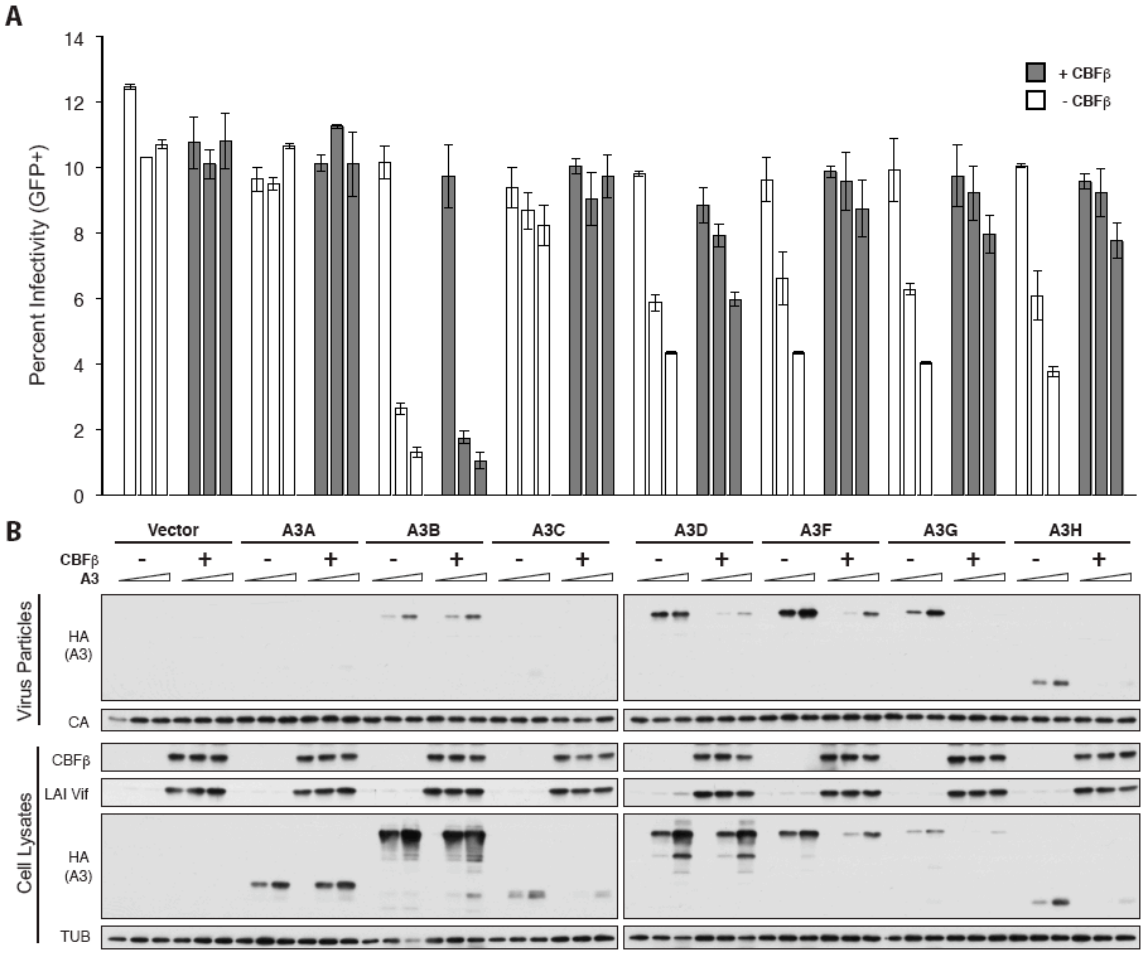

Figure 4-3. CBFβ Is Required for HIV-1 Vif to Degrade all Vif-sensitive Human APOBEC3 Proteins.

(A) Percent infectivity of HIV-1_{LAI} measured by duplicate infection of CEM-GFP and flow cytometry, reported as the mean +/- the standard deviation of the technical replicate. A constant amount of Vif-proficient HIV-1_{LAI} proviral construct (1 μg) was co-transfected with an increasing concentration of each human HA-tagged APOBEC3 protein (0, 50, or 100 ng) in the presence or absence of CBFβ isoform 2 complementation vector (25 ng) as indicated.

(B) Immunoblots of CBFβ, Vif, and the HA-tagged human APOBEC3 proteins in cell lysates (lower panels) and of the APOBEC3 proteins in HIV virus particles produced by those cells (upper panels). Tubulin (TUB) and p24 (CA) served as cell and viral lysate loading controls.

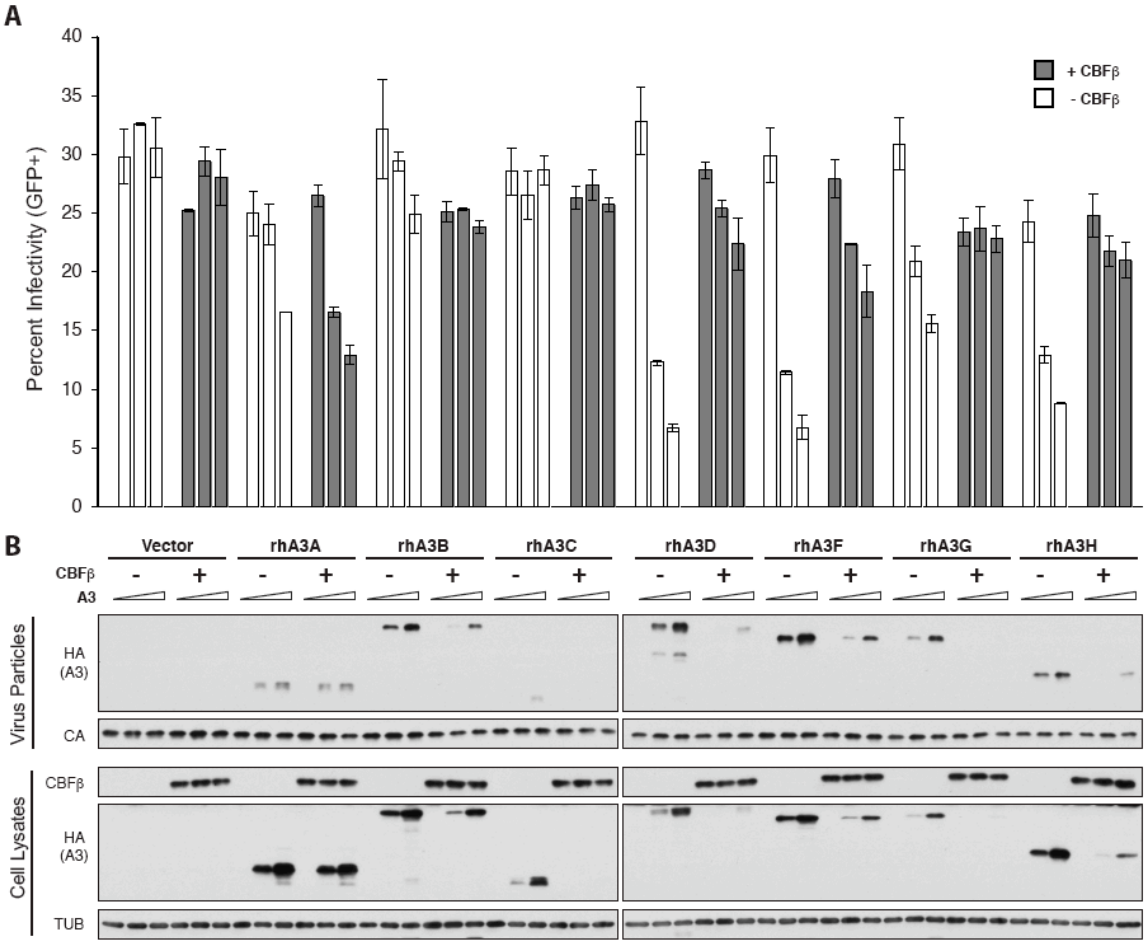

Figure 4-4. CBFβ Is Required for SIV Vif to Degrade all Vif-sensitive Rhesus APOBEC3 Proteins.

(A) Percent infectivity of Vif-deficient HIV-1_{IIIB} supplemented with SIV_{mac239} Vif measured by duplicate infection of CEM-GFP and flow cytometry, reported as the mean +/- the standard deviation of the technical replicate. A constant amount of Vif-deficient A200C HIV-1_{IIIB} proviral construct (1 μg) was co-transfected with untagged SIV_{mac239} Vif (pVR1012 expression vector, 50 ng) and an increasing concentration of each rhesus,

HA-tagged APOBEC3 protein (0, 50, or 100 ng) in the presence or absence of CBFβ isoform 2 complementation vector (25 ng) as indicated.

(B) Immunoblots of CBFβ, Vif, and the HA-tagged rhesus APOBEC3 proteins in cell lysates (lower panels) and of the rhesus APOBEC3 proteins in HIV virus particles produced by those cells (upper panels). Tubulin (TUB) and p24 (CA) served as cell and viral lysate loading controls.

CHAPTER 5

Human Immunodeficiency Virus type 1 Viral Infectivity Factor and the RUNX Transcription Factors Interact with CBFβ on Genetically Distinct Surfaces

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Authors' contributions

J.F. Hultquist performed all experiments, wrote/revised the manuscript, and designed all figures except as follows. R.M. McDougle made most CBFβ variant constructs and helped screen for their Vif stabilization activity as part of Table 1. R.S. Harris and B.D. Anderson aided in the design of the experiments and in manuscript revision.

FOREWORD

Disruption of the Vif-CBFβ interaction as a means of HIV treatment by hypermutation is enticing because: 1) this interaction is broadly conserved among HIV subtypes indicating broad applicability, 2) disruption of this interaction would liberate the entire restrictive APOBEC3 repertoire eliciting a strong inhibitory barrier, and 3) this interaction represents a unique drug target unlikely to alter the activity of other essential E3 ligase complexes. However, this does not rule out the possibility of off-target effects that may impact ordinary CBFβ function. CBFβ acts normally in conjunction with the RUNX family of transcription factors to alter promoter activity at genes essential for hematopoiesis and osteogenesis. Off-target effects of a putative Vif-CBFβ inhibitor are of particular concern if Vif is acting as a RUNX mimic and binds CBFβ on a similar molecular interface. To address this possibility, we carried out a mutagenesis screen of CBFβ surface residues and discovered a number of separation-of-function mutants that cleanly distinguish between RUNX binding and Vif binding activities. As Vif and RUNX interact with CBFβ on genetically separable interfaces, there is the potential to develop specific Vif-CBFβ inhibitors with minimal off-target effects. This further indicates that Vif and the RUNX proteins need not be in direct competition for CBFβ, though they may be if CBFβ is limiting in the cell. This leaves open the possibility the Vif may be altering transcription at CBFβ-RUNX dependent genes by hijacking CBFβ from its normal cellular function. This implies that Vif may be multifunctional, required for multiple activities in the course of HIV pathogenesis.

SUMMARY

Human Immunodeficiency Virus type 1 (HIV-1) requires the cellular transcription factor Core Binding Factor β (CBFβ) to stabilize its viral infectivity factor (Vif) protein and neutralize the APOBEC3 restriction factors. CBFβ normally heterodimerizes with the RUNX family of transcription factors, enhancing their stability and DNA-binding affinity. To test the hypothesis that Vif may act as a RUNX mimic to bind CBFβ, we generated a series of CBFβ mutants at the RUNX/CBFβ interface and tested their ability to stabilize Vif and impact transcription at a RUNX-dependent promoter. While several CBFβ amino acid substitutions disrupted promoter activity, none of these impacted the ability of CBFβ to stabilize Vif or enhance degradation of APOBEC3G (A3G). A mutagenesis screen of CBFβ surface residues identified a single amino acid change, F68D, that disrupted Vif binding and its ability to degrade A3G. This mutant still bound RUNX and stimulated RUNX-dependent transcription. These separation-of-function mutants demonstrate that HIV-1 Vif and the RUNX transcription factors interact with cellular CBFβ on genetically distinct surfaces.

INTRODUCTION

HIV-1 and most related lentiviruses encode a Vif protein required to neutralize the APOBEC3 restriction factors of their hosts. The human APOBEC3 family consists of seven distinct single-stranded DNA deaminases, of which APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H combine to restrict the replication of Vif-deficient HIV-1 by incorporating into budding virions, inhibiting reverse transcription, and subsequently mutating the viral complementary DNA (cDNA) by deamination of cytosines-to-uracils ([218,228] and many others; reviewed in[12,183]). HIV-1 Vif neutralizes the APOBEC3 proteins by recruitment of an E3 ubiquitin ligase complex that polyubiquitinates the APOBEC3s and targets them for proteasomal degradation ([23] and others; reviewed in[9,183]).

Recently, the cellular transcription cofactor CBFβ was found to be associated with the HIV-1 Vif E3 ubiquitin ligase complex[224]. *In vitro*, CBFβ allowed for the reconstitution of an active Vif E3 ubiquitin ligase complex, additionally composed of CULLIN5 (CUL5), ELONGINB (ELOB), ELONGINC (ELOC), and RBX2[224]. *In vivo*, knockdown of endogenous CBFβ resulted in lower steady-state levels of HIV-1 Vif, attenuated degradation of APOBEC3G (A3G), and decreased viral infectivity[224]. The current working model is that HIV-1 Vif hijacks cellular CBFβ to facilitate Vif folding and/or stability as well as nucleation of the APOBEC3-degrading E3 ubiquitin ligase complex[224]. The HIV-1 Vif/CBFβ/APOBEC3 functional interplay is highly conserved as Vif proteins from multiple HIV-1 subtypes require cellular CBFβ for stability and for degradation of all Vif-sensitive, human APOBEC3 proteins[229]. Furthermore, Simian Immunodeficiency Virus of the rhesus macaque strain 239 (SIV_{mac239}) Vif requires CBFβ to degrade the Vif-sensitive APOBEC3 proteins of the rhesus macaque[229].

CBFβ is the non-DNA-binding subunit of the core binding factor family of transcription factors. CBFβ heterodimerizes with RUNX1, RUNX2, or RUNX3 (generally referred to as RUNX proteins) to activate or repress transcription at several loci important for hematopoiesis and osteogenesis (reviewed in[230-232]). For example, CBFβ heterodimerizes with RUNX1 and RUNX3 to regulate activity of the *FOXP3* promoter, an essential factor in regulatory T cell development[233,234]. Heterodimerization induces a conformational change in the RUNX proteins that renders them more stable and increases their DNA-binding affinity[235-239]. This is thought to occur by way of a conformational change that removes autoinhibition of the RUNX DNA-binding domain[240-242]. The CBFβ heterodimerization domain that contacts the RUNX proteins lies within the first 141 amino acids of the protein and forms a stable beta-barrel like structure[237,243,244]. The structure of the CBFβ heterodimerization domain bound to RUNX1 and in complex with DNA has been solved and the interaction surfaces have been mapped[245-248].

While the CBFβ/RUNX1 structure has been solved, the macromolecular structure of the HIV-1 Vif E3 ubiquitin ligase complex is unknown. Vif is thought to interact directly with the first cullin repeat of CUL5 dependent on an HCCH zinc-coordinating motif, directly with a hydrophobic pocket of ELOC dependent on a highly conserved SLQ(Y/F)LA motif, and directly with CBFβ, though specific interaction surfaces have yet to be thoroughly defined[18,23,133,134,136,138,224,249,250]. Towards mapping the Vif interaction surface on CBFβ, it has recently been shown that both human isoforms of CBFβ can function to stabilize HIV-1 Vif[229,251]. These splice variants share 165 N-terminal residues, including the RUNX heterodimerization domain, but differ in C-

terminal amino acid sequence and overall size (187 and 182 amino acids for isoforms 1 and 2, respectively)[229,236,237]. Therefore, the binding surface for Vif on CBFβ likely resides within the first 165 N-terminal residues of the protein and possibly within the RUNX heterodimerization domain.

As molecular mimicry of host proteins is a common viral strategy for hijacking cellular factors[252], here we test the hypothesis that HIV-1 Vif may act as a mimic of RUNX and utilize an overlapping set of interacting residues on CBFβ. To test this hypothesis, we created several CBFβ variants that no longer interact with RUNX1. While these variants have diminished capacities to activate transcription from RUNX1-dependent promoters, they retain their full ability to interact with and stabilize HIV-1 Vif. Subsequent mutagenesis screening of CBFβ surface residues revealed a single amino acid substitution that completely disrupts its ability to bind and stabilize HIV-1 Vif, but does not impact its ability to heterodimerize with RUNX1. These separation-of-function mutants demonstrate that cellular CBFβ uses genetically distinct surfaces to bind RUNX1 and Vif and that HIV-1 Vif is not a molecular mimic of the RUNX transcription factors.

RESULTS

Amino Acid Substitutions that Disrupt the CBFβ/RUNX Heterodimer Do Not Disrupt the CBFβ/Vif Interaction

Based on the available structural and biochemical data, we created several amino acid substitutions in CBFβ isoform 2 predicted to disrupt the interaction with RUNX1[245-248]. To confirm that these substitutions significantly disrupt the CBFβ/RUNX1 heterodimer, we performed a series of dual luciferase assays using the CBFβ/RUNX1-

dependent *FOXP3* promoter in a CBFβ-depleted HEK293T cell line[233]. This cell line stably expresses a short hairpin (sh)RNA targeting the 3' untranslated region (3'UTR) of both endogenous CBFβ isoforms, allowing for complementation with wildtype or variant coding sequences[224]. These cells were transiently transfected with Firefly luciferase under control of the *FOXP3* promoter and Renilla luciferase under control of the Cytomegalovirus (CMV) immediate early constitutive promoter in the presence of RUNX1 and each hemagglutinin (HA) tagged CBFβ variant. 48 hours after transfection, cell lysates were collected, luciferase activity was quantified, and promoter activity determined by normalizing the Firefly luciferase signal to the Renilla luciferase transfection control. Significance was determined by pairwise t-tests at a 0.05 significance threshold.

In the absence of CBFβ and RUNX1, this fragment of the *FOXP3* promoter has a low basal activity (normalized to one; **Figure 5-1A**). Expression of either CBFβ or RUNX1 alone results in no significant increase in promoter activity (**Figure 5-1A**). However, expression of both CBFβ and RUNX1 together allows for reconstitution of the heterodimeric transcription factor and results in a significant increase in activity of the *FOXP3* promoter reporter (**Figure 5-1A**). This promoter is sensitive to a dose-dependent increase in CBFβ/RUNX1, saturating at near 8-fold over baseline (data not shown). In all subsequent experiments, CBFβ/RUNX1 levels were chosen to achieve a 2.0 to 2.5-fold increase in promoter activity in order to stay within the linear range of the assay. Seven of the nine amino acid substitutions resulted in either a significant reduction (Q8R, G61A, N63K, I102E, N104A, E135R) or complete ablation (N104K) of promoter activity relative to complementation with wildtype CBFβ (**Figure 5-1B**).

To determine if these substitutions also disrupt the interaction with HIV-1 Vif, we performed a series of single cycle HIV-1 replication assays in the same CBFβ-depleted HEK293T cell line (**Figure 5-1C**). These cells were transiently transfected with a Vif-proficient A200C HIV-1_{IIIB} molecular clone in the presence of human A3G and an increasing amount of each HA-tagged CBFβ variant. 48 hours after transfection, cell lysates and viral particles were collected for immunoblotting and viral infectivity was monitored by infection of the reporter cell line CEM-GFP. In the absence of CBFβ complementation, HIV-1 Vif steady-state levels are low, A3G levels are high, A3G is able to package efficiently into the viral particles, and infectivity is restricted (**Figure 5-1C**). Upon complementation with wildtype CBFβ, HIV-1 Vif steady-state levels increase, A3G is degraded, less A3G incorporates into the viral particles, and viral infectivity is rescued (**Figure 5-1C**). In every case, complementation with the CBFβ variants phenocopied the wildtype protein, resulting in increased Vif stability, increased degradation of A3G, and a rescue of viral infectivity. As the seven amino acid substitutions that diminished CBFβ/RUNX1-dependent transcription have no effect on Vif binding, it is unlikely that Vif is acting as a RUNX mimic.

A Mutagenesis Screen of CBFβ Surface Residues Reveals F68 as a Key HIV-1 Vif Interaction Determinant

As none of the amino acid substitutions that disrupted the RUNX interaction had an impact on Vif function, we carried out a mutagenesis screen of CBFβ surface residues based on the crystal structure of the unbound RUNX heterodimerization domain (CBFβ residues 1-141)[243]. This screen was focused on changing charged or hydrophobic residues

to oppositely charged or hydrophilic residues, respectively. Each CBFβ variant was assayed for its capacity to activate the RUNX1-dependent luciferase reporter and to stabilize Vif/rescue viral infectivity in the presence of A3G as above (summarized in **Table 5-1**). Of the thirty-three variants tested, thriteen resulted in a significant reduction or complete ablation of RUNX1-dependent promoter activity relative to wildtype CBFβ (t-test, p-value <0.05). As above, none of these amino acid substitutions had any impact on the ability of CBFβ to stabilize Vif, enhance A3G degradation, or rescue viral infectivity.

In our screen, two amino acids, a pair of phenylalanines at positions 68 and 69, did impact the ability of CBFβ to stabilize HIV-1 Vif (**Table 5-1** and **Figure 5-2**). The F68D F69D CBFβ variant did not visibly stabilize HIV-1 Vif, did not enhance the degradation of A3G, and did not rescue viral infectivity (**Figure 5-2A**). Making the substitutions singly, F68D behaves indistinguishably from the double substitution variant and has no appreciable ability to stabilize Vif or rescue viral infectivity. The F69D substitution alone displays a slight defect in its ability to stabilize HIV-1 Vif, but is still able to rescue viral infectivity comparable to complementation with wildtype CBFβ (**Figure 5-2A**). This slight defect may simply be due to proximity to F68. All three variants, CBFβ F68D F69D, F68D alone, and F69D alone, are able to activate transcription at the RUNX1-dependent promoter, indicating that these variants are structurally intact and still able to form functional heterodimers with RUNX1 (**Figure 5-2C**).

To determine if the F68D substitution disrupts the physical interaction between CBFβ and Vif or if this substitution is acting by a different mechanism, FLAG affinity-

tagged versions of CBFβ, CBFβ F68D, and CBFβ N104K were coexpressed with RUNX1 and HIV-1_{IIIB} Vif from a full molecular clone in CBFβ knockdown HEK293T cells and immunoprecipitated. These cells were treated with MG132 to stabilize Vif in the absence of wildtype CBFβ and lysed by sonication to break open the nuclei. Wildtype CBFβ is able to pull down both RUNX1 and Vif (**Figure 5-2B**). CBFβ F68D, while still able to pull down RUNX1 with similar efficiency to wildtype CBFβ, is greatly diminished in its ability to pull down HIV-1 Vif. CBFβ N104K, on the other hand, is able to precipitate Vif, but not RUNX1 (**Figure 5-2B**). CBFβ F68D and CBFβ N104K are therefore true separation-of-function variants, specifically disrupting the ability of CBFβ to bind and functionally interact with HIV-1 Vif and RUNX1 respectively.

An Alanine Scan of CBFβ Regions Previously Implicated in HIV-1 Vif Binding Reveals No Additional Interacting Residues

A previous study implicated two regions of CBFβ, loop three from amino acids 69 to 91 and helix four from amino acids 129 to 140, in HIV-1 Vif binding based on immunoprecipitation experiments with deletion constructs[249]. These regions lie in close proximity to F68D and may hold additional Vif interacting residues. As deletion mutants within the RUNX heterodimerization domain of CBFβ often render it nonfunctional or unstable[237,238,253], we generated a series of alanine scan substitutions that covered the entirety of both of these regions three to four amino acids at a time. Again, complementation with wildtype CBFβ, but not the F68D variant, resulted in increased HIV-1 Vif steady-state levels, increased A3G degradation, and a rescue in viral infectivity (**Figure 5-3A**). The first set of alanine substitutions 69-FPAS-73 included the

F69 residue previously assayed. This mutant again displayed an intermediate phenotype, resulting in reduced, but appreciable Vif stabilization and a partial rescue in viral infectivity (**Figure 5-3A**). Relative to their respective wildtype controls, this defect appears more severe than the F69D mutation alone, potentially indicating a role for the additionally altered amino acids in the CBFβ/Vif interaction or reflecting a larger structural alteration in the F68 region. All remaining alanine scan CBFβ variants stabilized Vif and rescued viral infectivity comparable to the wildtype protein (**Figure 5-3A**). Furthermore, none of these variants had a significant defect in activating the RUNX1-dependent promoter relative to wildtype CBFβ (**Figure 5-3B**).

DISCUSSION

Molecular mimicry is a commonly observed viral strategy to hijack host proteins through already evolutionarily optimized binding surfaces[252]. HIV-1 Vif is known to hijack the host protein CBFβ to enhance its stability and degrade the antiviral family of APOBEC3 restriction factors[224,229,249]. CBFβ is a transcription cofactor that normally heterodimerizes with one of three RUNX proteins to activate the transcription of genes involved in hematopoeisis and osteogenesis[230-232]. We tested the hypothesis that HIV-1 Vif may act as a RUNX mimic to bind CBFβ by creating a series of amino acid substitutions that disrupted the ability of CBFβ to heterodimerize with RUNX1. While none of these substitutions altered the ability of CBFβ to interact with HIV-1 Vif, a mutagenesis screen of surface residues identified F68 as a crucial determinant of this interaction. The F68D substitution does not impact the ability of CBFβ to heterodimerize with RUNX1. The ability to create distinct separation-of-function substitutions in CBFβ

that either specifically disrupt Vif or RUNX binding indicates that the two proteins interact with CBFβ on genetically distinct surfaces and that HIV-1 Vif is not acting as a RUNX1 mimic.

In total, thirteen distinct CBFβ variants disrupted the ability of CBFβ to heterodimerize with RUNX1 and activate transcription of our reporter construct (shaded blue, **Figure 5-3C**). These substitutions map to the extensive CBFβ/RUNX1 interface in the co-crystal structure[245,246]. Surprisingly, of all tested CBFβ variants, only F68D strongly disrupted the interaction with HIV-1 Vif and failed to enhance A3G degradation (shaded orange, **Figure 5-3C**). While substitutions at F69 and adjacent residues resulted in minor defects in Vif stability, it is unclear if this is due to direct binding disruption or due to indirect disruption at the adjacent F68 position. To identify the remainder of the interaction surface, a majority of the residues on the same surface of CBFβ as F68 were altered, but no clear candidates were identified.

One possibility is that the alanine substitutions near F68 were not dramatic enough to disrupt Vif binding. For example, a CBFβ E135R substitution resulted in partial disruption of RUNX1 heterodimerization, but no disruption was observed for E135A in an alanine scan variant over the same region (**Table 5-1** and **Figure 5-3B**). Alternatively, the remainder of the Vif interaction surface may reside on the carboxy(C)-terminal end for which there is no available structure and which, therefore, was not mutagenized in our surface screen. This is unlikely, however, as both CBFβ isoforms are able to interact with HIV-1 Vif despite divergent C-termini and as recent evidence has shown that the first 140 amino acids of CBFβ are sufficient to bind HIV-1 Vif[229,251]. Additionally, the structures of the apo and RUNX1/DNA bound forms of CBFβ revealed

significant conformational differences between the free and complexed protein[243-246]. It is probable that binding to HIV-1 Vif also induces a conformational change in CBFβ and this may reveal buried residues otherwise inaccessible for interaction. Structural studies will therefore likely be necessary for elucidation of the full CBFβ/Vif interaction surface.

The ability to cleanly separate function definitively shows that the impact of CBFβ on HIV-1 Vif, including enhanced stability and an enhanced capacity to neutralize the APOBEC3 proteins, is a result of the direct interaction between the two proteins and not an indirect effect dependent on RUNX transcription. We hypothesize that CBFβ may increase Vif steady-state levels by decreasing its rate of proteasomal turnover dependent on a direct protein-protein interaction[224]. CBFβ similarly protects the RUNX proteins from ubiquitin-mediated degradation, though that mechanism is also unclear[235]. It is possible that the interaction of CBFβ with the RUNX proteins and with Vif renders these proteins inaccessible for E3 ubiquitin ligase turnover either through conformational influence or steric hinderance[224,235].

While the two binding surfaces are genetically separable, it does not rule out the possibility that the RUNX/CBFβ and the HIV-1 Vif/CBFβ interaction surfaces are partially overlapping and therefore mutually exclusive. If CBFβ were limiting, it is possible the RUNX proteins and HIV-1 Vif may compete for CBFβ in an infected cell and may therefore impact the functionality of one another. These separation-of-function mutants will prove essential tools in answering these questions going forward. Furthermore, we envision that a better definition of the CBFβ/Vif interface will help inform the search for small molecules therapeutics designed to work by disruption of Vif function.

MATERIALS & METHODS

Expression Constructs. A3G and Vif-proficient HIV-1_{IIIB} A200C proviral expression constructs have been reported[171]. To generate the HA-tagged CBFβ expression construct, the coding sequence of CBFβ isoform 2 (NM_001755.2) was excised from a previously reported pcDNA3.1-CBFβ construct with EcoRI and XbaI and ligated into the same sites of a pcDNA3.1-HA animo(N)-terminal tag expression vector using standard molecular biology techniques[224]. The pcDNA4/TO-3xFLAG-CBFβ and pcDNA4/TO-3xFLAG expression constructs were provided by Dr. N. Krogan (UCSF), and the pcDNA3-RUNX1 expression construct by Dr. J. Westendorf (Mayo Clinic). The CBFβ variants were generated by site-directed mutagenesis of the FLAG-CBFβ or HA-CBFβ constructs (sequences available upon request).

To generate the *FOXP3* promoter luciferase reporter construct, a 594 base pair fragment of the *FOXP3* promoter previously shown to respond to RUNX1/CBFβ was cloned from CEM genomic DNA using primers 5'-NNN NGG TAC CCG GGT TGG CCC TGT GAT TTA T-3' and 5'-NNN NCT CGA GAC CTT ACC TGG CTG GAA TCA CG-3' [233]. This product was gel purified (Fermentas GeneJet Gel Extraction Kit), digested with KpnI and XhoI, and ligated into a similarly digested pGL3-Basic Firefly luciferase vector (E1751; Promega). The CMV-Renilla luciferase vector transfection control was obtained from Promega (E2261; phRL-CMV).

Cell Lines. The CBFβ knockdown Human Embryonic Kidney 293T (HEK293T) cell line stably expressing a CBFβ-specific shRNA has been reported previously[7] and was

maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin (P/S). CEM-GFP cells (obtained from the National Institutes of Health AIDS Research and Reference Reagent Program or NIH ARRRP) were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS and 0.5% P/S.

HIV Single Cycle Assay with Replication Proficient Virus. At 50% confluency, CBFβ knockdown HEK293T cells were transfected (TransIt, Mirus) with 1 μg Vif-proficient HIV-1_{IIIB} A200C proviral expression construct alongside 50 ng of APOBEC3G expression construct and either 25 or 50 ng of the appropriate HA-CBFβ expression construct. CEM-GFP cells were infected after 48 hours to monitor infectivity, and cell and viral particle lysates were prepared for immunoblotting.

Immunoblotting. Cell lysates were prepared by resuspension of washed cell pellets directly in 2.5x Laemmli Sample Buffer (25mM Tris pH 6.8, 8% glycerol, 0.8% SDS, 2% 2-mercaptoethanol, 0.02% bromophenol blue), and homogenization at 95°C for 30 minutes. Virus-like particles were isolated from culture supernatants by purification through 0.45μm PVDF filters (Millipore) followed by centrifugation (13,000 rpm for 2 hours) through a 20% sucrose, 1x PBS cushion and lysis directly in 2.5x Laemmli Sample Buffer. Samples were run on 12.5% Tris-HCl SDS-PAGE resolving gels with 4% stacking gels each at a 37.5:1 acrylamide:bis-acrylamide ratio (BioRad Criterion) at 150V for 90 minutes. Proteins were transferred to PVDF membranes by methanol-based electrotransfer (BioRad Criterion Blotter) at 90V for 2 hours. Membranes were blocked in 4% Milk in PBS, 0.1% Tween-20 overnight prior to overnight incubation with primary

antibody against A3G (NIH ARRRP 10201 courtesy of J. Lingappa), HA to detect HA-tagged CBFβ (HA.11; Covance), FLAG to detect FLAG-tagged CBFβ (F7425; Sigma), TUB (tubulin; Covance), Vif (NIH ARRRP 2221 courtesy of D. Gabuzda), p24/capsid (NIH ARRRP 3537 courtesy of B. Chesebro and K. Wehrly), or RUNX1 (sc-28679; Santa Cruz). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (BioRad) were detected using Hyglo HRP detection reagents (Denville Scientific). Blots were incubated in a 1xPBS, 0.2M glycine, 1.0% SDS, 1.0% Tween-20, pH 2.2 stripping buffer before reprobing.

Flow Cytometry. HIV-infected CEM-GFP cells were prepared for flow cytometry by fixation in 4% paraformaldehyde, 1x PBS. GFP fluorescence was measured on a Becton Dickinson FACS Canto II flow cytometer. All data was analyzed using FlowJo Flow Cytometry Analysis Software (Version 8.8.6). Quantification was done by first gating the live cell population, followed by gating on the GFP^+ cells.

Dual Luciferase Reporter Assay. At 50% confluency, CBFβ knockdown HEK293T cells were transfected (TransIt, Mirus) with 250 ng Firefly luciferase *FOXP3* promoter reporter construct, 5 ng Renilla luciferase CMV promoter transfection control, 150 ng RUNX1 expression construct, and 75 ng of each HA-CBFβ variant expression construct in triplicate. After 48 hours, cells were lysed and Firefly and Renilla luciferase activity was quantified using the Promega Dual-Luciferase Reporter Assay System by the manufacturer's protocol. Luminescence was read on a SynergyMx plate reader (courtesy of Dr. S. McIvor).

Coimmunoprecipitation. At 50% confluency, CBFβ knockdown HEK293T cells were transfected (TransIt, Mirus) with 1 μg Vif-proficient HIV-1_{IIIB} A200C proviral expression construct alongside 1 μg RUNX1 expression construct and either 1 μg of pcDNA4/TO-3xFLAG empty vector or 1 μg pcDNA4/TO-3xFLAG-CBFβ variant. 32 hours after transfection, the media was replaced with fresh DMEM supplemented with 2.5 mM MG132 to stabilize HIV-1 Vif. After 16 hours, the cells were washed with 1x PBS and lysed in 0.5% NP40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 50 mM MG132, 0.5% NP40, Roche protease inhibitor cocktail) for 1 hour. Samples were sonicated briefly to lyse nuclei, treated for 1 hour with DNase (Roche), and cleared by centrifugation. Input samples were suspended directly in 2.5x Laemmli Sample Buffer. The remainder of each sample was cleared with Mouse IgG Agarose beads (A0919; Sigma) prior to FLAG immunoprecipitation using anti-FLAG M2 affinity agarose gel (A2220; Sigma). Beads were washed with 0.1% NP40 lysis buffer four times and resuspended directly in 2.5x Laemmli Sample Buffer.

ADDITIONAL CONTRIBUTIONS

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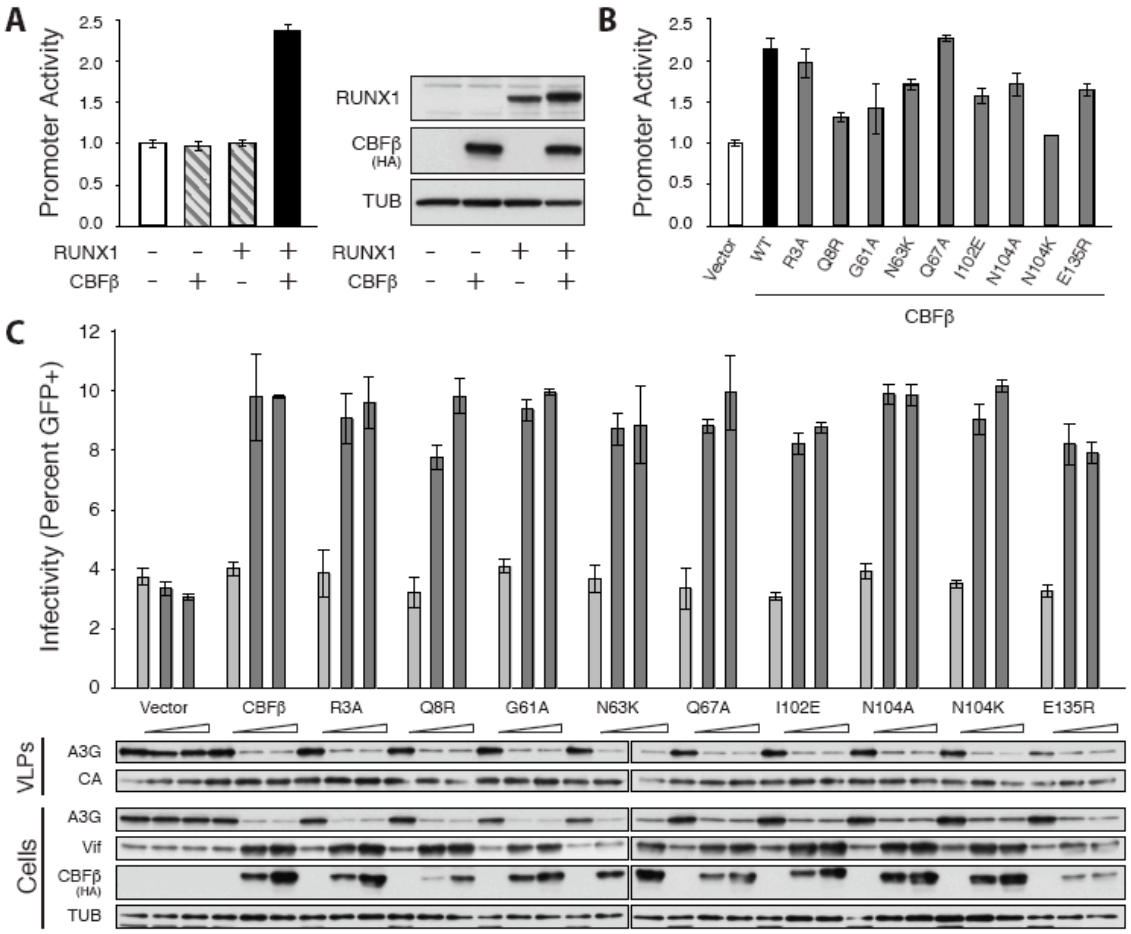

Figure 5-1. Amino Acid Substitutions that Disrupt the CBFβ/RUNX1 Heterodimer Do Not Disrupt the CBFβ/Vif Interaction.

(A) Activity of the *FOXP3* promoter reporter gauged by the activity of Firefly luciferase relative to the Renilla luciferase transfection control and reported as the mean +/- standard deviation of three independent biological replicates, normalized to the no RUNX, no CBFβ control (white bar). A constant amount of each luciferase construct was cotransfected with empty vector (white bar), HA-tagged CBFβ or RUNX1 alone (striped bars), or both HA-tagged CBFβ and RUNX1 (black bar) into a stable CBFβ knockdown

HEK293T cell line. Immunoblots of RUNX1 and HA-CBFβ in cell lysates are shown with tubulin (TUB) as a loading control.

(B) Activity of the *FOXP3* promoter reporter gauged by the activity of Firefly luciferase relative to the Renilla luciferase transfection control and reported as the mean +/- standard deviation of three independent biological replicates and normalized to the no CBFβ control (white bar). A constant amount of each luciferase construct was cotransfected with RUNX1 and either empty vector (white bar), HA-tagged CBFβ (black bar) or the indicated HA-CBFβ variant (gray bars) into a stable CBFβ knockdown HEK293T cell line. Significance was determined by t-test, p-value less than 0.05.

(C) Percent infectivity of HIV-1_{IIIB} measured by infection of CEM-GFP in duplicate and flow cytometry, reported as the mean of the two technical replicates +/- standard deviation. A constant amount of Vif-proficient A200C HIV-1_{IIIB} molecular clone was co-transfected into a stable CBFβ knockdown HEK293T cell line with A3G in the presence of an increasing gradient of the indicated CBFβ complementation vector. Representative immunoblots of HA-tagged CBFβ variants, Vif, and A3G in cell lysates and of A3G in HIV-1 particles produced by those cells are shown with their respective tubulin (TUB) and p24 (CA) loading controls.

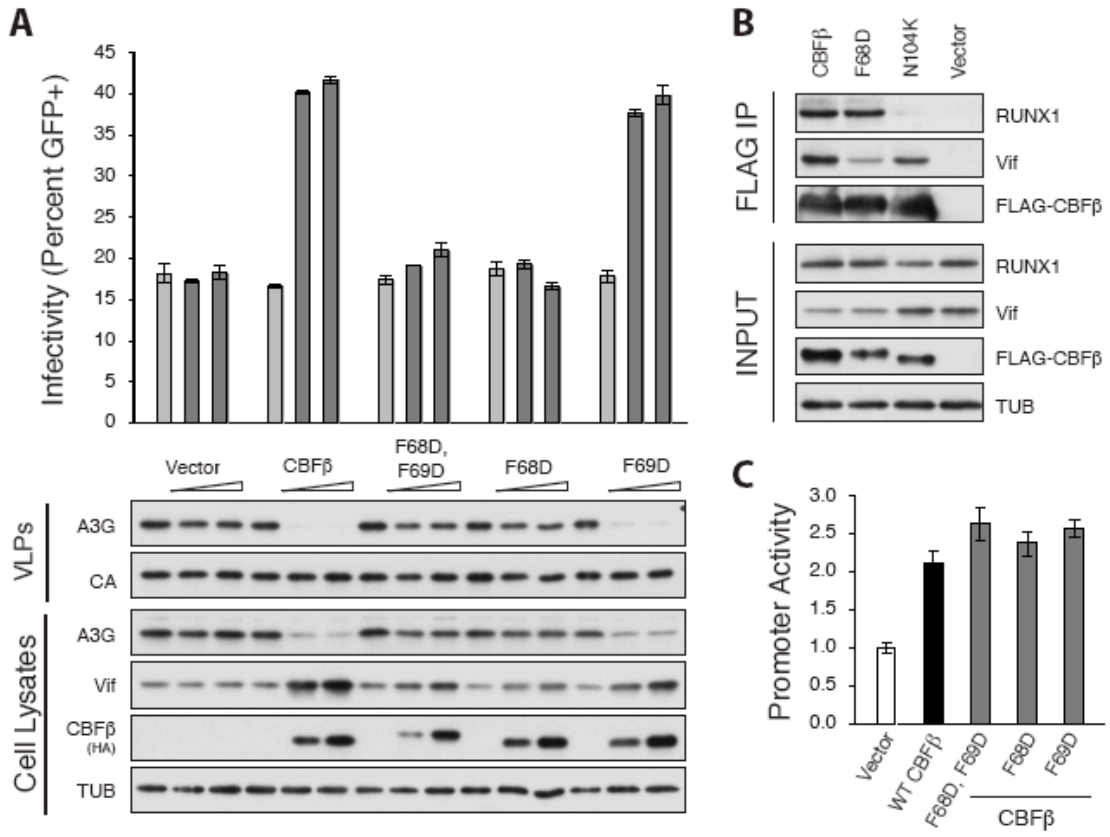

Figure 5-2. CBFβ F68D Disrupts the Interaction with HIV-1 Vif.

(A) Percent infectivity of HIV-1_{IIIB} measured by infection of CEM-GFP in duplicate and flow cytometry, reported as the mean of the two technical replicates +/- standard deviation. A constant amount of Vif-proficient A200C HIV-1_{IIIB} molecular clone was co-transfected into a stable CBFβ knockdown HEK293T cell line with A3G in the presence of an increasing gradient of the indicated CBFβ complementation vector. Representative immunoblots of HA-tagged CBFβ variants, Vif, and A3G in cell lysates and of A3G in HIV-1 particles produced by those cells are shown with their respective tubulin (TUB) and p24 (CA) loading controls.

(B) Immunoblots of FLAG-tagged CBFβ variants, Vif, and RUNX1 in cell lysates (Input) and after FLAG pull down (FLAG IP). Vif-proficient A200C HIV-1_{IIIB} molecular clone was co-transfected into a stable CBFβ knockdown HEK293T cell line with RUNX1 and the indicated FLAG-CBFβ expression vector. Tubulin (TUB) is the input loading control. Cells were treated with 2.5 mM MG132 for 16 hours prior to lysis to stabilize Vif in the absence of CBFβ.

(C) Activity of the *FOXP3* promoter reporter gauged by the activity of Firefly luciferase relative to the Renilla luciferase transfection control and reported as the mean +/- standard deviation of three independent biological replicates, normalized to the no CBFβ vector control (white bar). A constant amount of each luciferase construct was cotransfected with RUNX1 and either empty vector (white bar), HA-tagged CBFβ (black bar) or the indicated HA-CBFβ variant (gray bars) into a stable CBFβ knockdown HEK293T cell line.

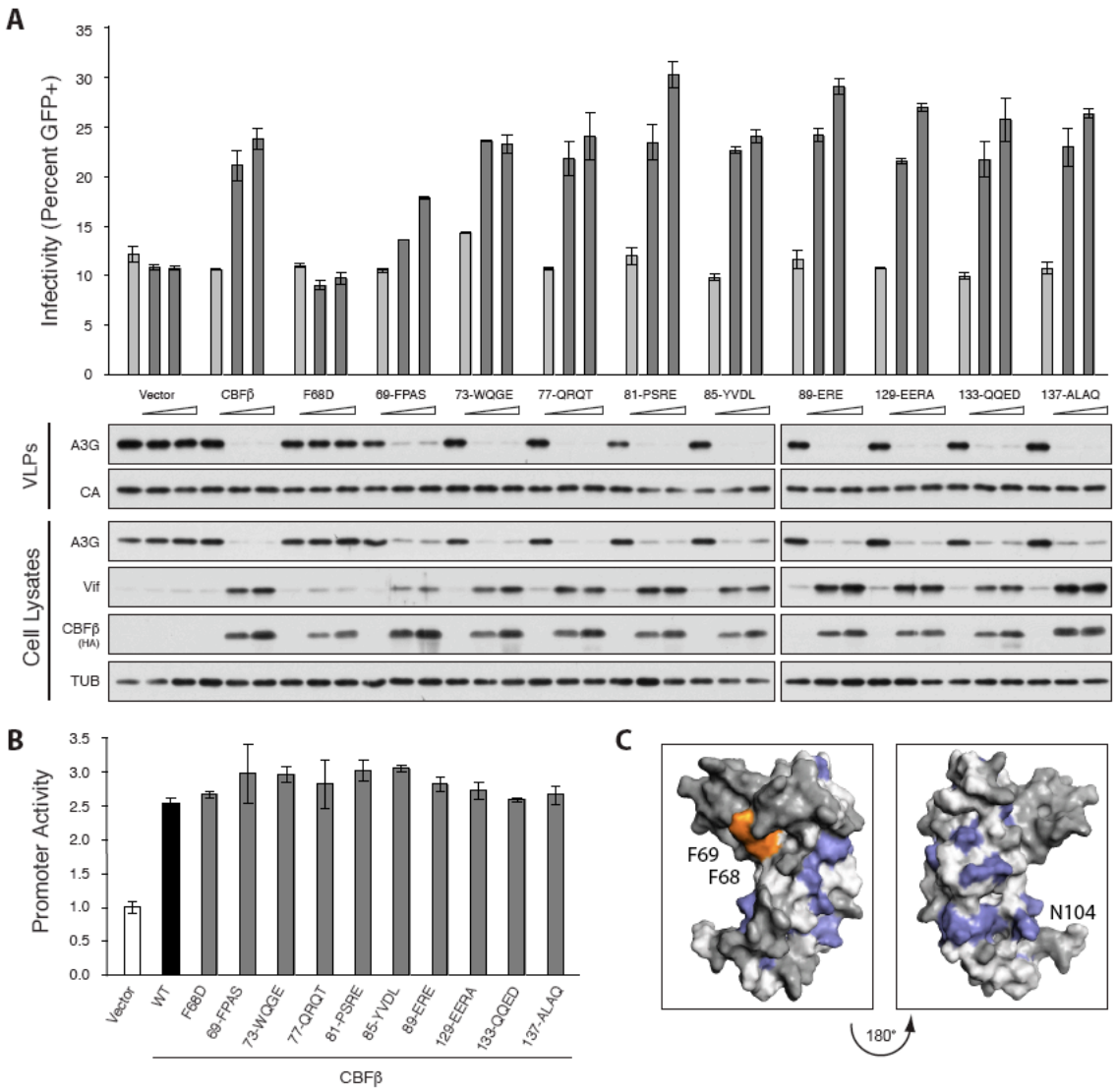

Figure 5-3. Alanine Scanning Fails to Reveal Additional Residues at the CBFβ/Vif Interface.

(A) Percent infectivity of HIV-1_{IIIB} measured by infection of CEM-GFP in duplicate and flow cytometry, reported as the mean of the two technical replicates +/- standard deviation. A constant amount of Vif-proficient A200C HIV-1_{IIIB} molecular clone was co-transfected into a stable CBFβ knockdown HEK293T cell line with A3G in the presence

of an increasing gradient of the indicated CBFβ complementation vector. Representative immunoblots of HA-tagged CBFβ variants, Vif, and A3G in cell lysates and of A3G in HIV-1 particles produced by those cells are shown with their respective tubulin (TUB) and p24 (CA) loading controls.

(B) Activity of the *FOXP3* promoter reporter gauged by the activity of Firefly luciferase relative to the Renilla luciferase transfection control and reported as the mean +/- standard deviation of three independent biological replicates, normalized to the no CBFβ vector control (white bar). A constant amount of each luciferase construct was cotransfected with RUNX1 and either empty vector (white bar), HA-tagged CBFβ (black bar) or the indicated HA-CBFβ variant (gray bars) into a stable CBFβ knockdown HEK293T cell line.

(C) CBFβ structural model depicting F68 (orange), residues that disrupted the RUNX1 heterodimer when altered (blue), and residues that had no impact on either the RUNX1 or Vif interaction when altered (dark gray). The remaining residues (light gray) were not altered in this study.

CBFβ Variant	Vif Stability	RUNX Activity
R3A	++	++
Q8R	++	+
R9E	++	++
E13K, E15K	++	++
F17D, F18D, R19E, K20E	++	-
R19E, K20E, R23D	++	++
E26K, K28E, Y29K	++	-
F32D, R35E	++	++
E38K, E39K	++	+
R40E, R43E	++	+
Q45K, N46K, C48K	++	++
R49E, R52E	++	++
D50, G51K	++	++
S53A, E54K	++	++
F57K	++	++
G61A	++	+
N63K	++	+
Q67A	++	++
F68D, F69D	-	++
F68D	-	++
F69D	+	++
R83E	++	++
Y94D	++	++
Y96D	++	++
Y98E	++	++
I102E	++	+
N104A	++	+
N104K	++	-
K111E, W113E	++	+
R118E	++	++
D120K	++	++
E126K, F127	++	-
E135R	++	+

'-' indicates no significant recovery in HIV infectivity or no significant RUNX reporter activity.
'+' indicates a partial recovery in HIV infectivity or RUNX reporter activity below wildtype.
'++' indicates recovery or activity at wildtype levels.
Significance determined by t-test, p-value < 0.05.

Table 5-1. Summary of CBFβ Amino Acid Substitutions and their Effects on Vif Stability and RUNX1 Function.

CHAPTER 6

Conclusions and Discussion – HIV-1 Vif, the Cellular Transcription Factor CBFβ, and the APOBEC3 Restriction Factors

FOREWORD

While there are a number of highly active antiretroviral drugs for the treatment of Human Immunodeficiency Virus (HIV), they are all expensive, invasive, susceptible to resistance, and none of them provide a cure. My thesis research has focused on identifying novel drug targets for therapeutics that may be used alone or in combination with existing programs to improve treatments and, ultimately, to provide an effective cure and control this epidemic. Though most current treatments curb viral replication through inhibition of an essential viral enzyme, an alternate strategy is to inhibit essential virus-host interactions. One of these potential targets is the interaction between the human APOBEC3 restriction factors and the HIV counteracting protein, Vif. Design of small molecule therapeutics that disrupt this interaction and free the antiviral APOBEC3 proteins has been hampered by an incomplete understanding of the Vif E3 ubiquitin ligase complex and conflicting reports as to which of the seven different APOBEC3 proteins may contribute to HIV restriction *in vivo*.

To definitively determine which APOBEC3 proteins contribute to HIV restriction and, thus, may be leveraged to combat HIV infection, my colleagues and I performed a comprehensive analysis of both seven-member human and rhesus macaque APOBEC3 families in T cells. Based on six criteria (expression, virion incorporation, HIV restriction, viral genome mutation, neutralization by Vif, and conservation in the rhesus macaque), we found that four APOBEC3 proteins, APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H, have the potential to be involved in HIV restriction *in vivo.* Small molecules that prevent Vif-mediated degradation of all four restrictive APOBEC3 proteins will likely be more effective than those that rescue any one alone.

To better understand the Vif E3 ligase complex responsible for degrading these four, restrictive APOBEC3 proteins, my collaborators and I performed extensive purification experiments with HIV Vif and discovered that Vif interacts with the cellular transcription factor CBFβ. We found that CBFβ not only allows for reconstitution of the Vif E3 ligase complex *in vitro*, but also stabilizes Vif *in vivo*, subsequently facilitating ligase assembly and allowing for the efficient degradation of APOBEC3G. Furthermore, this functional interaction is highly conserved, required to enhance the steady-state levels of Vif proteins from all tested varieties of HIV and required for the degradation of all restrictive human and rhesus APOBEC3 proteins by their respective lentiviral Vif proteins. Extensive mutagenesis of CBFβ allowed us to map the interaction with Vif to residue F68, a residue not required for the interaction between CBFβ and its normal transcription partner, RUNX. This indicates that Vif and RUNX need not be in direct competition for CBFβ, but it is still an open question as to whether Vif can alter the transcriptional program in infected cells.

Overall, my research shows that HIV is targeted by a swarm of APOBEC3 proteins, all of which are subverted by the viral Vif protein (**Figure 6-1**). For Vif to neutralize the APOBEC proteins, it must fist hijack the cellular protein, CBFβ. Disruption of this newly identified and highly conserved CBFβ-Vif interaction would release the multitude of restrictive APOBEC3 proteins and significantly inhibit HIV infection, making this interaction a promising new target for small molecule therapeutics.

The Restrictive APOBEC3 Repertoire

All seven human APOBEC3 cytosine deaminases have been implicated in Human Immunodeficiency Virus type 1 (HIV-1) restriction and hypermutation (reviewed in [9,12,13,106,183,254]). Proviral sequences from HIV-1 infected patients commonly bear two distinct guanine-to-adenine (G-to-A) hypermutation patterns, 5'GG-to-AG and 5'GA-to-AA[113,114,184,185]. APOBEC3G (A3G) displays an intrinsic preference for the second cytosine of 5'CC dinucleotides. Deamination of cytosine-to-uracil (C-to-U) at these positions in the HIV-1 minus strand during reverse transcription templates the insertion of adenine resulting in the hallmark 5'GG-to-AG mutation signature (*e.g.*[33,34,42,44,46,109]). All six other APOBEC3 family members display an intrinsic preference for 5'TC dinucleotides and it has therefore been unclear which of these proteins may act as a true HIV-1 restriction factor (*e.g.*[44,52,86,91,105,109,111,186]).

Towards determining which human APOBEC3 proteins may contribute to HIV-1 restriction and hypermutation *in vivo*, we performed a comprehensive, functional analysis of both seven-member human and rhesus macaque APOBEC3 repertoires[218]. Based on the well-characterized model of A3G restriction, I hypothesized that the other relevant APOBEC3 proteins should meet six criteria: 1) expression in $CD4^+$ T lymphocytes, 2) incorporation into Vif-deficient HIV virions in T cells, 3) restriction of Vif-deficient HIV in T cells, 4) neutralization by HIV Vif, 5) mutation of viral complementary DNA (cDNA) in a physiologically relevant spreading infection system, and 6) functional conservation with homologous proteins of rhesus macaque. Each human and rhesus APOBEC3 protein was either expressed stably in isogenic T cell lines or transiently in Human Embryonic Kidney (HEK)293T cells and challenged with Vif-proficient or Vif-

deficient HIV while APOBEC3 expression, packaging, viral infectivity, and proviral mutation were monitored. Based on the above criteria, our results implicate four APOBEC3 proteins, APOBEC3D (A3D), APOBEC3F (A3F), APOBEC3G (A3G), and APOBEC3H (A3H), in HIV-1 restriction and hypermutation.

While these studies demonstrated that A3D, A3F, A3G, and A3H are each *sufficient* to hypermutate and restrict Vif-deficient HIV-1, *i.e.* render a normally permissive T cell line nonpermissive for Vif-deficient HIV replication, they still relied on forced, exogenous expression. We verified that our stable cell lines all expressed each *APOBEC3* mRNA or protein in a near-physiologic range (*i.e.* near levels observed in primary $CD4^+$ T lymphocytes or in primary monocyte-derived macrophages) and performed careful titrations when expressing these proteins transiently, but did not show these proteins were *necessary* for hypermutation or restriction in a normally nonpermissive cell line. To address this concern, a fellow graduate student and I performed a series of gene knockout and knockdown experiments in a cell line that naturally expresses multiple APOBEC3 proteins in an attempt to render a normally nonpermissive T cell line permissive for Vif-deficient HIV replication[228].

Towards this end, we identified a nonpermissive, diploid T cell line expressing near-physiologic levels of A3D, A3F, and A3G. In this CEM2n model system, we used recombinant Adeno-Associated Virus (rAAV) mediated gene targeting to knockout A3G and A3F. A3G-null CEM2n cells were more permissive for Vif-deficient HIV-1 replication and also failed to induce 5'GG-to-AG mutations as expected. A3F-null cells were also more permissive than parental CEM2n cells for Vif-deficient HIV-1 replication, but retained the ability to impart 5'GA-to-AA mutations. Only when we used

A3D-specific short hairpin (sh)RNA to knockdown A3D expression in our A3F-null cells did we fully ablate 5'GA-to-AA mutations, indicating that A3D and A3F cooperate to inflict this hypermutation pattern as predicted from our previous work. Unfortunately, CEM2n cells do not express A3H, so we were unable to assess its effect on HIV-1 restriction and hypermutation in this system.

While the above studies clearly implicate a role for A3D, A3F, A3G, and, most likely, A3H in HIV-1 restriction and hypermutation[218,228], the relative importance of each is likely to vary based on cell type, expression level, haplotype, and viral strain. This is perhaps most vividly illustrated by recent studies with A3H. The ability of A3H to restrict HIV-1 is dependent upon its haplotype, of which at least seven are currently thought to be circulating in the human population[111,192,196,207-209]. While some haplotypes encode stable proteins and restrict Vif-deficient HIV-1 (*i.e.* haplotype II used in my studies above), several haplotypes fail to encode stable proteins or restrict[111,192,196,207-209]. Furthermore, the ability of different HIV strains to neutralize A3H differs with the encoded Vif allele, the neutralization capacity of which correlates with A3H haplotype[192,196,226,255]. Viral strains isolated from patients with a stable A3H haplotype typically display a capacity to neutralize A3H, likely indicating that stable A3H haplotypes exert a selective pressure on the virus to neutralize them *in vivo*. A3F and A3G, on the other hand, lack significant functional variation in the population and are neutralized by nearly all tested, circulating HIV-1 Vif alleles[226].

Finally, some consideration must be given to the cell type as each cell type will vary in respect to APOBEC3 expression profile and the regulatory environment within[181,182]. While the above studies focused on the major HIV-1 target cell, $CD4^+$ T

lymphocytes, HIV also infects other cell types including macrophages and dendritic cells, the APOBEC3 expression profiles of which remain unreported. As the regulation of APOBEC3 activity remains enigmatic, it is also difficult to predict the impact of different cellular environments on APOBEC3 restriction. As demonstrated above, APOBEC3B (A3B) exhibits vastly different behavior when it is expressed in a HEK293T line as opposed to a T cell line[218]. Some evidence suggests that APOBEC3A (A3A) may also possess restrictive activity when expressed endogenously in a myeloid cell as opposed to exogenously in a lymphoid cell[256-258]. Nevertheless, one would expect that if A3A were playing a significant role in HIV-1 restriction, that it would have selected for viruses with the capacity to neutralize A3A. While no such mechanism has been conclusively demonstrated, there has yet to be a study comparing the ability of myeloid versus lymphoid HIV strains to neutralize A3A or any of the other APOBEC3 proteins.

The overlapping ability of four APOBEC3 proteins to restrict and hypermutate HIV in $CD4^+$ T cells is extraordinary and it is yet unclear why this functional redundancy was originally selected for and subsequently maintained. One possibility is that this redundancy was selected for an additive effect, that is, the cumulative contribution of each APOBEC3 protein is required to reach the threshold for effective restriction of a given pathogen. While our data indicate that genetic ablation of one restrictive APOBEC3 protein in a nonpermissive cell can result in a partial recovery of viral infectivity, these experiments are carried out under high titer challenge[228]. Experiments knocking out or knocking down one or two APOBEC3 proteins does not appear to impact challenge with low titer virus in our more physiologic spreading infection assays, indicating that expression of just a single APOBEC3 protein at physiologic levels is

sufficient to restrict Vif-deficient HIV-1[228]. This is further validated by our single APOBEC3 expression experiments in permissive cells[218]. Thus, while all four do not seem to be required for an additive effect against HIV-1, they still may be required additively to restrict some other pathogen or parasitic genetic element.

Along those lines, an alternate reason for the maintenance of this redundancy may be selection for divergent function. While all four may contribute to HIV-1 restriction and hypermutation, they may each also be specialized to act against one or more different pathogens. In this sense, the APOBEC3 family may act as an innate immune network wherein overlapping subsets of proteins are specialized to restrict various exogenous pathogens and endogenous mobile elements. The maintenance of multiple genes with overlapping functions may more readily allow for the exploration of mutational space such that our innate defenses may adapt with relative rapidity to pathogen challenge. In support of this, each APOBEC3 protein is under positive selection[190,259,260], though it is unclear if this is due to multiple, independent pressures exerted on the divergent functions of individual proteins or due to shared pressures exerted on overlapping functions of subsets of proteins.

Finally, it must also be considered that this apparent redundancy may have been selected for some emergent property. For example, four slightly different, restrictive APOBEC3 proteins would more greatly limit the flexibility of a given pathogen to bypass or evolve resistance to them than would one protein alone and, in theory, form a better zoonotic barrier. Alternately, heterodimerization between the various APOBEC3 proteins may allow for combinatorial restriction whereby one partner facilitates improved incorporation or Vif evasion of the other thus resulting in synergistic inhibition of viral

replication. A better understanding of the APOBEC3 repertoire in other primates and mammals would aid in reconstructing the selective pressures that have sculpted the present day locus and would help to elucidate the genetic principles that underlie redundancy and innate restriction in mammals.

Comparative analysis between different APOBEC3 family members and their homologs has proved a powerful tool for mapping determinants of cellular localization, enzymatic activity, and protein-protein interactions[165,168,206,209,261,262]. For example, comparative studies between the human and African green monkey A3G proteins identified D128 as an important residue for interaction with the HIV Vif protein, an essential viral protein for neutralization of the APOBEC3 host defense[165-168]. Our studies with the human and rhesus repertoires revealed two salient differences between the two, both of which have since been molecularly explained.

One difference is that human A3B is able to restrict HIV-1 in HEK293T cells, but rhesus A3B is not. Using a series of chimeric human-rhesus A3B proteins, a colleague and I mapped the restrictive determinant to a single amino acid in the carboxy(C)-terminus at residue 316[263]. Human A3B D316 has HIV-1 restriction capability while rhesus A3B N316 does not and swapping these residues alters the restriction phenotypes respectively. We further demonstrated that this residue is essential for A3B enzymatic activity and maps to a region shown to be important for substrate recognition in other, related APOBEC family members. The second difference between the repertoires is that human A3D is only weakly restrictive while rhesus A3D is strongly restrictive. This is again due to a single amino acid difference in the C-terminus of the protein; human A3D C320 is only weakly restrictive while rhesus A3D Y320 is strongly restrictive and

swapping these residues alters the restriction phenotypes respectively[262]. These studies demonstrate the power of comparative analyses in molecular analysis, especially in repetitive gene families.

Overall, an important consideration from our systematic and comprehensive analysis of the human and rhesus APOBEC3 repertoires is that four proteins – A3D, A3F, A3G, and A3H – likely contribute to HIV restriction in T cells, not just one or two as previously inferred (**Figure 6-1**). While the relative importance of each will vary based on cell type, expression level, haplotype, and viral strain, all four proteins can contribute and may be leveraged by novel therapeutics to combat HIV infection. It is likely that small molecules that prevent Vif-mediated degradation of all four restrictive APOBEC3 proteins will be much more effective than those that rescue any single one (analogous to current combinatorial therapies). The functional overlap within the repertoire may be required for an additive effect, maintained through divergent functions, or allow for some emergent property; continued comparative analyses between repertoires of different species will be required to dissect its evolutionary history. Nevertheless, the strong parallels between the human and rhesus macaque APOBEC3 repertoires further suggest that the rhesus macaque may be a reasonable model system for testing such candidate pan-APOBEC3/Vif influencing compounds prior to human trials.

CBFβ as a new HIV Host Dependency Factor

Despite expressing four APOBEC3 antiviral restriction factors, HIV-1 is able to efficiently replicate in $CD4^+$ T lymphocytes and cause disease. HIV-1 neutralizes the restrictive APOBEC3 proteins by way of its accessory protein Vif, which acts as an

adaptor between the APOBEC3 proteins and an E3 ubiquitin ligase complex additionally composed of CULLIN5 (CUL5), ELONGINB (ELOB), ELONGINC (ELOC), and RBX2[16-23,138]. This E3 ubiquitin ligase complex serves to polyubiquitinate and target the APOBEC3 proteins for proteasomal degradation. While much effort has been made towards inhibiting this E3 ubiquitin ligase complex, and the Vif-APOBEC3 interaction in particular, little progress has been made. Part of the reason for this is that Vif has not been amenable to *in vitro* or structural studies, so all screening has been limited to low-throughput cell-based assays with random compound libraries. We hypothesized that the inability to purify Vif biochemically may be due to the lack of an essential and yet unidentified cofactor.

To address this possibility, our collaborators undertook an affinity purification-mass spectrometry (AP-MS) approach to identify Vif interacting factors in human HEK293T and Jurkat T cells[212,224]. Of the 24 Vif-interacting proteins that scored significantly in our pull downs based on specificity, reproducibility, and abundance, one protein came down in both cell types tested and in roughly stoichiometric ratios with the other known components of the E3 ligase complex, Core Binding Factor β (CBFβ). Further double affinity tag immunoprecipitation experiments with CBFβ, Vif, and other members of the E3 ligase complex confirmed CBFβ as a component of this complex and demonstrated that its recruitment to the complex is Vif-dependent. Indeed, simultaneous expression of CBFβ with Vif in *Escherichia (E.) coli* allowed for the purification and reconstitution of a stable E3 ubiquitin ligase complex with specific A3G polyubiquitination activity *in vitro*.

In vivo, we found that CBFβ was essential for HIV-1 Vif stability and/or E3 ligase assembly[224,229]. Stable knockdown of CBFβ resulted in lower steady-state levels of Vif and a diminished ability of Vif to degrade A3G, ultimately resulting in lower viral infectivity. Complementation with exogenous CBFβ recovered Vif steady-state levels, restored A3G-neutralization activity, and boosted viral infectivity. The HIV-1 Vif/CBFβ/APOBEC3 functional interplay is highly conserved as Vif proteins from multiple HIV-1 subtypes require cellular CBFβ for stability and for degradation of all Vif-sensitive, human APOBEC3 proteins[229]. Furthermore, Simian Immunodeficiency Virus of the rhesus macaque strain 239 (SIV_{mac239}) Vif requires CBFβ to degrade the Vif-sensitive APOBEC3 proteins of the rhesus macaque[229].

The current working model is that HIV-1 Vif hijacks cellular CBFβ to facilitate Vif folding and/or stability as well as nucleation of the APOBEC3-degrading E3 ubiquitin ligase complex[224]. While the details are not yet known, an overall mechanism is beginning to emerge. First of all, it is known that treatment with the proteasome inhibitor MG132 ablates the difference in Vif steady-state levels in the presence or absence of CBFβ[224]. Furthermore, treatment with MG132 results in the accumulation of higher molecular weight Vif species consistent with ubiquitination and this accumulation is more apparent in the absence of CBFβ[224]. This is consistent with a model in which CBFβ serves to prevent the proteasomal degradation of HIV-1 Vif.

Additionally, it is now clear that HIV-1 Vif likely exists in multiple independent complexes and may be playing additional, yet undescribed roles in the course of infection aside from its well-characterized role in APOBEC3 neutralization[158,162,163,212,224,264]. Therefore, the function of CBFβ may not be solely to increase Vif steady-state levels, but

may be more intimately intertwined with the specific assembly of the APOBEC3-degrading E3 ligase complex. In support of this, immunoprecipitation experiments with various E3 ligase components have demonstrated that in the absence of CBFβ, Vif is less able to associate with CUL5[249,265]. This is further supported by our *in vitro* work, which requires assembly of a Vif-CBFβ-ELOB-ELOC complex prior to association with CUL5 and RBX2[224,251]. It may be that the stabilizing interaction between CBFβ and Vif promotes the adoption of a particular confirmation that is required for CUL5 binding, though more structural information on Vif is likely to be required for this hypothesis to be tested.

The identification of CBFβ allows for *in vitro* purification and analysis of HIV-1 Vif for the first time, offering a potential route towards obtaining a high-resolution Vif structure as well as a broad view of the Vif E3 ligase architecture[224,251]. HIV-1 Vif is the last HIV protein for which there is no high-resolution structural data, and CBFβ is likely to be essential in obtaining this final piece. A high-resolution structure of APOBEC3C (A3C) has been reported recently allowing for the eventual possibility of a Vif E3 ligase/A3C substrate co-structure[151]. With structural information and a viable *in vitro* system for the study of complex activity, we can begin asking more detailed mechanistic questions about Vif E3 ligase assembly and the field can begin to move towards high throughput, *in vitro* screens for small molecules that disrupt ligase activity and may serve as plausible therapeutic agents in the future.

One major caveat towards designing small molecules that specifically disrupt the CBFβ-Vif interaction is the possibility that such a molecule may disrupt normal CBFβ function. CBFβ normally functions as a non-DNA-binding subunit of the core binding

factor family of transcription factors. CBFβ heterodimerizes with RUNX1, RUNX2, or RUNX3 (generally referred to as RUNX proteins) to activate or repress transcription at several loci important for hematopoiesis and osteogenesis (reviewed in[230-232]). Heterodimerization induces a conformational change in the RUNX proteins that renders them more stable and increases their DNA-binding affinity[235-239]. CBFβ is broadly expressed, especially in hematopoietic lineages, and localizes primarily to the cytoplasm until signaling cascades allow for its nuclear translocation[240,266,267] (reviewed in[230-232]).

To determine if CBFβ's interaction and function with the RUNX proteins could be genetically separated from its interaction and function with HIV-1 Vif, my colleagues and I carried out an extensive mutagenesis screen of surface residues on CBFβ[268]. We found that several variants had diminished capacities to activate transcription from RUNX1-dependent promoters, but all of these retained their full ability to interact with and stabilize HIV-1 Vif. Furthermore, we revealed a single amino acid substitution, F68D, that completely disrupted the ability of CBFβ to bind and stabilize HIV-1 Vif, but that had no impact on its ability to heterodimerize with RUNX1. These separation-of-function mutants demonstrate that cellular CBFβ uses genetically distinct surfaces to bind RUNX1 and Vif and that HIV-1 Vif is not a molecular mimic of the RUNX transcription factors. As they are genetically separable, these data suggest that it is plausible to design small molecule inhibitors of the CBFβ-Vif that do not interfere with the CBFβ-RUNX interaction.

The interaction between CBFβ and Vif also opens up the possibility that Vif itself may be altering the transcriptional activity at CBFβ-RUNX dependent promoters. As Vif and RUNX1 interact with CBFβ on genetically separable interfaces, they do not

necessarily need to be in direct competition for CBFβ. However, if CBFβ is limiting or if binding to one partner supersedes binding to the other, they may be in competition and Vif may alter the cellular transcriptional program. To test this hypothesis, a T cell model system for investigating the CBFβ-Vif interaction is preferable, but this has been complicated by the fact that CBFβ appears essential in T cells (data not shown). Careful genetic manipulation of both T cell lines and the virus will likely be necessary before this question can be answered satisfactorily.

Overall, our studies with HIV-1 Vif have identified a new host dependency factor, CBFβ, which HIV-1 Vif requires to assemble the APOBEC3-degrading E3 ligase complex. The functional interaction between Vif and CBFβ is highly conserved among all tested HIV-1 subtypes and is required for Vif to degrade the entire repertoire of restrictive APOBEC3 proteins. CBFβ normally heterodimerizes with the RUNX proteins to alter transcription and it is possible that Vif may disrupt this normal transcriptional program by hijacking CBFβ. Importantly, the interaction between CBFβ and Vif is genetically separable form the interaction between CBFβ and RUNX, opening up the possibility of targeting this interaction for chemical inhibition. Disruption of this newly identified and highly conserved CBFβ-Vif interaction would release the entire restrictive repertoire of APOBEC3 proteins and significantly inhibit HIV infection, making this interaction a promising new target for small molecule therapeutics. These efforts are likely to be greatly facilitated by emerging *in vitro* and structural studies with the HIV-1 Vif-CBFβ-E3 ligase complex.

Reigning in the burgeoning HIV epidemic will likely require a combination of public health measures, robust treatment options, preventative measures (pre-exposure

prophylaxis or protective vaccination), and, potentially, the development of an effective cure (*i.e.* latency purging drug regimens combined with antiretroviral therapy). All of these require a detailed knowledge of basic HIV biology and pathogenesis, many aspects of which are still being discovered. Efforts such as those detailed here to help identify relevant HIV restriction factors and key HIV host dependency factors are essential in moving towards this goal. Until such time as a viable preventative measure or effective cure becomes available, we must rely on the improvement of existing and on the development of novel antiviral therapies. This will require investigating new ways to inhibit viral replication, particularly through the under investigated route of disrupting essential virus-host interactions. The APOBEC3-Vif-CBFβ interplay is crucial for viral survival, highly conserved, presents unique targets for small molecules, and represents one of the top candidates for future therapeutic development.

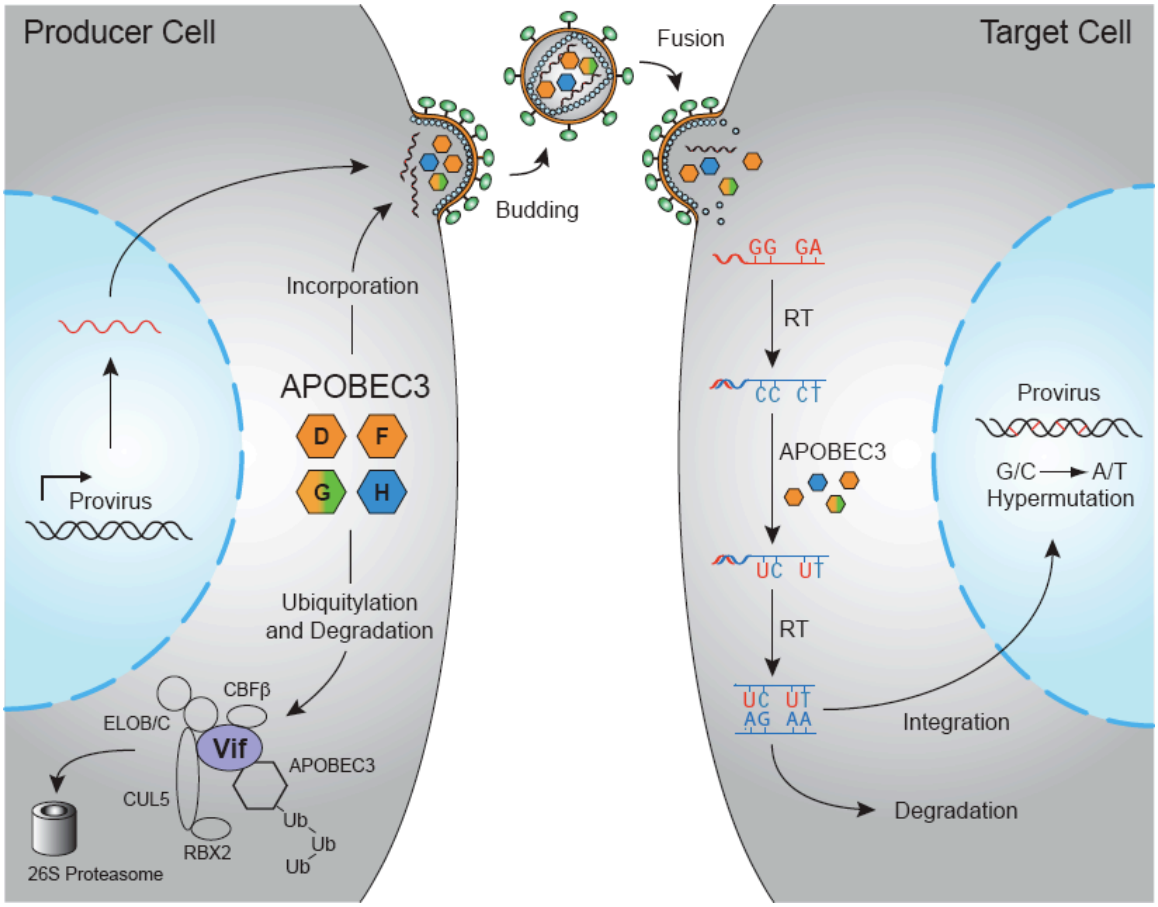

Figure 6-1. HIV Restriction by APOBEC3 proteins and the Vif Counter-Restriction Mechanism.

APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H can encapsidate into HIV virions and result in the deamination of cytosines-to-uracils (C-to-U) in viral cDNA upon initiation of reverse transcription in target cells. Uracil templates adenine upon second-strand synthesis resulting in guanine-to-adenine (G-to-A) mutations. The proviral cDNA is subsequently degraded or integrated, though many are rendered nonfunctional. HIV-1 Vif overcomes this restriction block in the producer cell by binding CFBβ and recruiting an E3 ubiquitin ligase complex to polyubiquitinate the APOBEC3 proteins, targeting them for degradation by the 26S proteasome. Model reprinted with permission from[11].

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