APOBEC3 Transcriptional Regulation and HIV-1 Restriction in T Lymphocytes

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ABSTRACT

Human immunodeficiency virus type-1 (HIV-1), the etiologic agent of acquired immunodeficiency syndrome (AIDS), has caused one of the most widespread and devastating pandemics in human history, and continues to persist as a substantial burden on global healthcare, social and economic systems, despite significant advances in modern anti-retroviral therapies. HIV-1 primarily infects CD4+ T lymphocytes, and to a lesser degree, macrophages, monocytes and dendritic cells. In the absence of therapeutic intervention, HIV-1 infection results in the gradual depletion of CD4+ T cells, leading to a severely compromised immune response and increased susceptibility to a wide range of opportunistic infections and malignancies.

The innate immune response to HIV-1 infection in CD4+ T cells is mediated in part by members of the APOBEC3 family of DNA cytosine deaminases. In the absence of the viral Vif protein, multiple APOBEC3 enzymes can package into virions budding from an infected cell. Following virus entry into a new target cell, the APOBEC3 enzymes catalyze the deamination of cytosines to uracils in viral reverse transcription intermediates, resulting in mutations that can render viral gene products non-functional. The HIV-1 Vif protein counteracts the antiviral activity of the APOBEC3 enzymes by commandeering a cellular ubiquitin ligase comprised of CBF-β, ELOB, ELOC, CUL5 and RBX2, to polyubiquitylate the APOBEC3 enzymes and target them for proteasomal degradation. Thus, viral progeny are mostly protected from APOBEC3 mutagenesis.

Despite significant advances in understanding the mechanisms that govern APOBEC3-dependent HIV-1 restriction, as well as Vif-dependent counteraction of this

host defense, little is known about how these innate antiviral enzymes are regulated at the transcriptional level. The first part of this thesis identifies the CBF-β/RUNX transcription complex as a critical regulator of *APOBEC3* gene expression in CD4+ T cells (*APOBEC3C*, *APOBEC3D*, *APOBEC3F*, *APOBEC3G*, and *APOBEC3H*, but not *APOBEC3A* or *APOBEC3B*). This unexpected discovery suggests that HIV-1 Vif may employ a secondary mechanism in counteracting the host APOBEC3 defense by hijacking CBF-β from RUNX-associated transcription complexes to downregulate transcription of the *APOBEC3* genes themselves. Thus, Vif may disarm the host APOBEC3 response by targeting these enzymes for proteasomal degradation, while simultaneously interfering with their ongoing expression at the transcriptional level.

The seven membered *APOBEC3* gene family is highly polymorphic within the human population, and several common genetic variations manifest as clear biochemical phenotypes. The second part of this thesis focuses on a rare variant of APOBEC3C (S188I), which confers enhanced HIV-1 restriction activity in comparison to the predominant S188 variant, which has been largely disregarded as playing a role in innate immunity to HIV-1 in T cells. The studies within characterize the antiviral activity of this APOBEC3C variant in multiple CD4+ T cell lines, and ultimately demonstrate that the S188I polymorphism renders APOBEC3C capable of protecting cells against Vif-deficient virus replication. These findings provide an additional example of meaningful variation within the human APOBEC3 repertoire that may impact virus replication and transmission *in vivo*, and will likely be the subject of follow up in several large ongoing HIV-1 infected patient cohort studies.

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CHAPTER 1: INTRODUCTION – APOBEC3 INNATE IMMUNITY TO HIV-1 INFECTION IN CD4+ T CELLS

HIV-1 and the AIDS Pandemic

First isolated in 1983 [1], the causative agent of acquired immunodeficiency syndrome (AIDS), human immunodeficiency virus type-1 (HIV-1), has spawned one of the most widespread and devastating global pandemics in human history. Since its onset, nearly 70 million people have been infected with the virus, and approximately 38 million deaths have been attributed to AIDS-related disease [2]. Tremendous progress over the last two decades has been made toward understanding the molecular biology of the virus, and has led to the development of highly effective combination therapies that effectively suppress HIV-1 replication and largely prevent the progression of AIDS-related disease [3]. Despite these life-saving advances in treatment regimens, no currently available pharmacological intervention strategies offer a cure to this lifelong infection. Moreover, effective therapies have consistently proven difficult to administer effectively to particularly high-risk populations, such as those in sub-Saharan Africa where 68% of the global infections are estimated to reside [2].

HIV-1 primarily infects and replicates in cluster of differentiation-4 positive (CD4+) T cells, and to a lesser extent, monocytes, macrophages and dendritic cells. Replication of the virus *in vivo* is characterized by an initial acute phase in which viral load can exceed 10^6 RNA copies per milliliter of human plasma. This surge in viral replication results in the significant and rapid depletion of CD4+ T cells [4, 5]. Following the establishment of an adaptive immune response, generally about 6 weeks post infection, viral load is immunologically suppressed and CD4+ T cell numbers generally rebound modestly. The virus then enters a chronic, clinically latent stage characterized by

low-level viral replication, and the gradual, but steady depletion of CD4+ T cells. Left untreated, most patients develop AIDS (defined by a CD4+ T cell count of less than 200 cells/μL) and become highly susceptible to a number of other generally innocuous bacterial, viral and fungal pathogens [6, 7], as well as cancers [8-10], due to a severely compromised immune system.

HIV-1 is a member of the *lentivirus* genus, and of the larger *retroviridae* family of RNA viruses [11]. Like all retroviruses, HIV-1 is a positive sense, non-segmented virus that reverse-transcribes its RNA genome into a linear double-stranded DNA, which undergoes insertion into the host cell chromosomal DNA. In total, the HIV-1 genome is approximately 9.7 kb in size, and contains 3 overlapping reading frames. The prototypical retroviral genes *gag*, *pol* and *env* encode the structural, enzymatic and cell entry proteins, respectively (**Figure 1-1**). In addition to these core retroviral genes, HIV-1 harbors six accessory genes that are required for productive virus replication *in vivo*: *tat* and *rev* encode proteins that promote provirus transcription and mRNA export, respectively, and *vif*, *vpu*, *vpr* and *nef* that remodel the cellular environment to favor virus replication. These accessory genes are highly conserved across the primate-specific lineage of lentiviruses (HIVs and simian immunodeficiency viruses [SIVs]), with the exception of *vpu*, which is replaced by *vpx* in HIV-2 and the sooty mangabey (SIVsm) and rhesus macque-tropic (SIVmac) lentiviruses, and completely absent in related viruses that infect the African green monkey (SIVagm), sun-tailed monkey (SIVsun), L'hoest monkey (SIVloest), and mandril (SIVmnd) [12].

Innate Immunity to HIV through APOBEC

The mammalian response to viral infection is driven by both innate and adaptive arms of the immune system. Adaptive immunity to viruses is guided primarily by the presentation of virus-derived peptides on major histocompatibility complexes (MHCs) by antigen presenting cells, which facilitates the rapid selection and clonal expansion of antigen-specific B cell and T cells [13]. HIV-1 is able to circumvent clearance by these highly specific responses by impairing cellular immunity through the depletion of CD4+ T cells, and by acquiring immune-escape mutations that prevent the recognition of MHC-presented viral antigens by cytotoxic T lymphocytes (CTLs) or disrupt binding of high affinity neutralizing antibodies [14-16]. The innate immune response provides an additional barrier against viral infection, and is activated by the sensing of pathogen-associated molecular patterns (PAMPs), such as foreign nucleic acid in the case of HIV-1 [17], which in turn which drives the expression of antiviral genes including interferons and other pro-inflammatory [18]. A subset of these induced genes, termed restriction factors, have the capacity to block HIV-1 replication by directly impairing specific replication steps during the virus lifecycle [19].

The apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3 (APOBEC3; abbreviated herein as A3) family constitutes one arm of the innate restriction factor defense against HIV-1 infection, and can potently inhibit virus replication in a variety of cell types including CD4+ T cells [20-22]. Encoded by a tandem gene cluster on chromosome 22, the human A3 repertoire is comprised of 7 highly homologous enzymes [APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3C

(A3C), APOBEC3D (A3D), APOBEC3F (A3G), and APOBEC3H (A3H)] that catalyze the deamination of cytosine to uracil on single-stranded DNA substrates [20]. Mechanistically, these enzymes thwart HIV-1 replication by incorporating into assembling virions in a productively infected cell, and subsequently catalyzing the formation of uracil lesions in reverse transcription intermediates following virus entry in a new target cell **(Figure 1-2)**. These uracils template the erroneous incorporation of adenines instead of guanines during synthesis of the viral genomic strand, which manifest as missense and non-sense mutations that render viral gene products non-functional. While all seven human A3 proteins are enzymatically active, only a subset - A3D, A3F, A3G and A3H - have been shown to inhibit HIV-1 replication in the primary reservoir for virus replication, CD4+ T cells [23, 24]. Correspondingly, these restrictive A3 proteins are expressed at high levels in primary CD4+ T cells, and their expression is further upregulated in response to HIV-1 infection [23, 25].

Vif Counteracts Host A3 Restriction to Enable Lentivirus Replication

Despite the overlapping and potent antiviral activities of restrictive A3 enzymes, HIV-1 and related primate lentiviruses continue to persist as significant pathogens in their respective hosts, largely due to conservation of the small virally encoded protein viral infectivity factor (Vif). Vif is expressed upon viral infection and functions as a molecular adapter to recruit the restrictive A3 enzymes to a host cell Cullin-RING E3 ubiquitin ligase complex comprised of Elongin B (ELOB), Elongin C (ELOC), Cullin5 (CUL5), RING box protein 2 (RBX2), and core binding factor β (CBF-β) [26-28]. Assembly of

this full complex in turn triggers the polyubiquitylation of the A3 enzymes, which promotes their degradation through the cellular proteasome (**Figure 1-2**). Thus, by hijacking a cellular ubiquitin ligase, HIV-1 is able to efficiently deplete cellular A3 protein levels and largely prevent A3 incorporation into assembling progeny virions that would otherwise destroy the virus through lethal mutagenesis.

While the canonical host ubiquitin ligase scaffold proteins ELOB, ELOC, CUL5, and RBX2 were first reported as components of the Vif-A3 polyubiquitylation complex nearly 15 years ago [26], the role of CBF-β as an essential component in this complex was discovered only recently [27, 28]. In contrast to the other host proteins in this complex, CBF-β had not been associated with the Cullin-RING family of E3 ubiquitin ligases, but rather was known as an important transcriptional co-factor involved in development and immune cell function [29, 30]. CBF-β normally forms a heterodimer with the DNA-binding RUNX proteins (RUNX1, RUNX2 and RUNX3) to regulate the transcription of genes involved in T cell lineage commitment and immune function (through RUNX1 and RUNX3), and osteoblast development (through RUNX2) [29, 30]. A recent crystal structure of a portion of the HIV-1 Vif-associated ubiquitin ligase complex revealed that Vif forms an extensive direct interaction with CBF-β, and that this binding surface overlaps significantly with the RUNX binding site [31]. *In vitro* binding experiments utilizing recombinant Vif, CBF-β and RUNX1 proteins demonstrated that Vif can block the assembly CBF-β/RUNX complexes, raising the possibility that Vif may play an additional role in promoting HIV-1 infectivity by interfering with normal CBF-β/RUNX dependent gene expression [32]. In agreement with this model, Vif

overexpression in the Jurkat T cell line alters the expression of nearly 100 CBF-β/RUNX dependent genes [32], though the functional impact of these transcriptional effects on HIV-1 replication and pathogenesis have not been determined.

Comparative analyses of Vif proteins from divergent lentiviral species have revealed that the requirement for CBF-β as a co-factor in enabling host A3 counteraction by Vif is specific to primate lentiviruses (HIVs and SIVs) [33, 34], despite nearly absolute conservation of binding to other core ubiquitin ligase machinery (some non-primate Vif proteins utilize CUL2 and RBX1 instead of CUL5 and RBX1 [35]). Vif proteins encoded by other lentiviruses including feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), maedi visna virus (MVV), and caprine arthritis encephalitis virus (CAEV) remain capable of degrading their cognate host A3 proteins in the absence of cellular CBF-β, although MVV and CAEV Vif proteins uniquely require cyclophilin A (CypA) as a co-factor in trigger host A3 polyubiquitylation [35, 36]. The requirement for additional co-factors in the case of FIV and BIV Vif proteins is currently unknown.

Vif Utitizes Multiple Distinct Interaction Surfaces to Neutralize Divergent A3 Proteins

To successfully circumvent lethal mutagenesis by the human A3 repertoire, HIV-1 Vif must bind and trigger the polyubiquitylation of at least four restrictive A3 proteins: A3D, A3F, A3G, and A3H [23, 24]. Given the significant homology between these enzymes, one might anticipate that Vif would have evolved to bind a single conserved

structural motif found on all four of these restrictive A3 proteins (*e.g.* the enzyme active site). However, extensive mutagenesis studies with Vif demonstrate that this is clearly not the case, and that in fact, HIV-1 Vif has adapted to use at least three distinct binding surfaces to independently antagonize A3F, A3G, and A3H, respectively (the Vif-binding domains of A3C and A3D are nearly identical to A3F and are recognized by the same binding determinants on HIV-1 Vif [37]). For example, the conserved DRMR14-17 motif in Vif is specifically required for binding and degrading A3F [38], YRHHY40-44 for A3G [38], and F39/N48/GDAK60-63 for A3H [39-41] (**Figure 1-3A**).

The identification of separation-of-function Vif mutants has lead us to propose an evolutionary model to explain the emergence of multiple distinct A3 interaction determinant on Vif that we have termed the "Wobble Model" (first discussed in [42], reviewed in [43]) (**Figure 1-3B**). In this model, we postulate that an ancestral mammalian host possessing a relatively simple A3 repertoire (for example, a single restrictive A3 protein) that likely required only one high affinity interaction surface on its cognate ancestral lentiviral Vif protein to enable virus replication. Co-evolution between this ancestral lentivirus and the simultaneous divergence of host species likely gave rise to advantageous adaptations within the host A3 repertoire, such as gene duplications and the rapid selection of Vif-resistant A3 variants. Iterative host adaptations and rapid selection of compensatory mutations in Vif ultimately resulted in the emergence of distinct interaction determinants that enable binding and counter action of expanded repertoire of divergent A3 proteins, such as is the case in primates today. This model remains to be tested in a controlled experimental setting, though comparative analyses of divergent

primate lentiviral Vif proteins and their cognate A3 proteins have revealed numerous examples on both virus and host sides in which Vif-A3 interactions were disrupted, and subsequently restored by compensatory single amino acid changes as the virus transmitted between closely related host species (e.g. between non-human primates, and subsequently from chimpanzees to humans) [44, 45].

Regulation of *A3* Gene Expression in CD4+ T Cells

Members of the human *A3* gene family exhibit broad variation in expression patterns across different primary cell and tissue types [25, 46]. For example, *A3A* expression is restricted to the myeloid lineages of hematopoietic cells, including monocytes, macrophages and dendritic cells, where it is highly induced upon exposure to type I interferons (IFN), CpG DNA, and lipopolysaccharide [25, 46-48]. Moreover, *A3B* is uniquely regulated by protein kinase C (PKC) and the non-canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [49]. In contrast, the related family member *A3C* is expressed nearly ubiquitously in the vast majority of human tissues and cell types, as well as many common cell lines [25, 46, 50]. CD4+ T cells, the primary reservoir for HIV-1 replication, exhibit a distinct A3 expression profile that is characterized by high levels of *A3C*, *A3D*, *A3F*, *A3G* and *A3H* (with *A3C* and *A3G* being the highest), and very low to undetectable levels of *A3A* and *A3B*. Treatment of primary CD4+ T cells with common mitogens (e.g. phytohemaglutinin (PHA), interleukin 2 (IL-2), anti-CD3/CD28) *ex vivo* results in a modest increase in expression of *A3C*, *A3D*, *A3F*, *A3G*, and A3H [23, 25, 46], and highlight a positive correlation between

A3 gene expression and the T cell activation state. HIV-1 infection of activated primary CD4+ cells *in vitro* results in the further upregulation of *A3C*, *A3G*, *A3H*, and to a lesser extent, *A3D* and *A3F*, although this induction is time-dependent with peak transcript levels typically observed at 7 to 9 days post infection [23].

Currently, the molecular mechanisms that regulate the *A3* expression in CD4+ T cells at the transcriptional level are poorly understood. In stark contrast to myeloid cell types, the subset of *A3* genes expressed in CD4+ T cells are largely non-responsive to type I interferon treatment [25, 51], implying that there are cell type-specific mechanisms for *A3* gene regulation. Despite the apparent decoupling of the T cell-specific *A3* repertoire from the innate type I interferon response, a handful of other soluble signaling molecules including IL-2, IL-6, and IL-15 significantly induce *A3G* expression in primary CD4+ T cell cultures [51], though the effects of these cytokines on other *A3* genes have not been specifically examined. Gene expression studies in both the T cell line H9 and primary T cells have additionally shown that treatment with phorbyl myristate acetate (PMA), a broad activator of several PKC dependent signaling pathways, potentiates the upregulation of A3G, and can be blocked by small molecule inhibitors of the downstream mitogen-activated protein kinase (MAPK) signaling pathway [51, 52].

Certain transcription factors have been shown to be important for *A3* gene expression in a variety of T cell lines and primary T cells. For example, overexpression of T-box transcription factor 21 (T-bet), which directs CD4+ T cells into the type-1 T helper cell lineage (Th1), results in the upregulation of *A3F* and *A3G*. In contrast, overexpression of GATA transcription factor 3 (GATA3), which commits cells to a type-

2 T helper cell (Th2) phenotype, results in the downregulation of both *A3F* and *A3G* [53]. Because both T-bet and GATA3 influence the expression of numerous cellular genes, it remains unclear whether their role in modulating *A3* transcription is dependent on direct binding to *A3* gene promotors, or is rather a consequence of altering the expression of other upstream regulatory factors.

Specific interrogation of the *A3G* promoter region has implicated several additional factors in *A3G* transcription, including Sp1, Sp2, NFAT, and IRF-4 [54, 55]. Sp1 and Sp2 knockdown was sufficient to significantly reduce *A3G* promoter activity in a plasmid-based luciferase reporter assay, and direct binding of these proteins within the endogenous *A3G* promoter was confirmed by a series of electrophoretic mobility shift assays, suggesting that Sp1 and Sp3 are positive regulators of *A3G* transcription in CD4+ T cells [54]. NFAT and IRF-4 were also identified through predicted binding sites within the *A3G* promoter region, and their combined overexpression was sufficient induce endogenous A3G expression in multiple cell lines that normally harbor negligible levels of *A3G* transcript [55].

The recent discovery of the transcription factor CBF-β as an obligate co-factor to HIV and SIV Vif proteins raised the question of whether Vif may play an additional role in modulating cellular gene transcription by interfering with normal CBF-β/RUNX dimerization and function [28, 32, 33, 56]. Somewhat surprisingly, the CBF-β/RUNX transcription factor complex appears to be required for *A3D*, *A3F*, *A3G*, *A3H*, and to a lesser degree *A3C*, expression in both the H9 T cell line and primary CD4+ T cells [57] (see **Chapter 2** of this thesis). These recent insights suggest that primate lentiviruses may

have adapted Vif proteins that bind CBF-β to directly interfere with transcription of the restrictive *A3* genes, thereby enabling an additional A3 counter-defense beyond the canonical polyubiquitylation mechanism [43].

Natural Variation in the Human *A3* Gene Family and Implications for HIV-1 Restriction

Within the human population, the *A3* gene family exhibits considerable allelic variation. Several common polymorphisms have been associated with functional outcomes, including disease progression and impaired enzyme stability. *A3B* is functionally deleted in a large fraction of Southeast Asians, and has been associated with an increased risk of breast cancer in these populations [58-61]. A3H is the most polymorphic member of the *A3* gene family, and is represented by at least seven distinct haplotypes that vary considerably in protein stability and antiviral activity. A3H haplotypes II and V encode stable enzymes with potent restriction activity against HIV-1, whereas haplotypes I, III, IV, VI and VII code for largely unstable proteins that have little to no impact on HIV-1 replication [41, 62, 63]. Two rare A3D variants found primarily in African populations, R97C and R248K, also exhibit compromised HIV-1 restriction activity in cell culture based assays [64]. A common A3G variant, A3G H186R, also enriched in African populations, has been associated with accelerated progression to AIDS, though *in vitro* comparisons between the H186 and R186 variants have failed to reveal any appreciable differences in enzymatic activity [65]. Additional polymorphisms

in A3A, A3F, and A3G have been examined, though no functional variation has been ascertained [64].

A3C has been largely discounted as having any role in HIV-1 restriction, despite being expressed at high levels in primary CD4+ T cells and efficiently targeted for degradation by HIV-1 Vif. Recently, a rare A3C variant (S188I) was characterized as having significant HIV-1 restriction activity in a 293T-based single cycle infection system [66, 67], and has prompted a reexamination of what defines the "HIV-1 restrictive" A3 repertoire. These findings have now been validated in T cell-based infection systems, which demonstrated that expression of the A3C I188 variant is sufficient to block Vif-deficient HIV-1 replication, while the common S188 variant has comparably little restriction activity (see **Chapter 3** of this thesis).

The phenotypes associated with many common A3 variants found within the human population clearly demonstrate that some individuals are endowed with a more active and diverse A3 repertoire than others, though the overall impact of most of these polymorphisms on HIV-1 pathogenesis remains unknown. Different A3 repertoires presumably impose distinct selection pressures on the virus *in vivo*, and may force specific adaptations in Vif or elsewhere in the virus that confer resistance to the subset of A3 proteins that directly threaten virus replication in a respective human host. In support of this idea, African HIV-1 isolates more frequently encode Vif proteins that are capable of counteracting A3H, likely due to the increased representation of stable A3H haplotypes (II/V). In contrast, viruses isolated from populations enriched for unstable A3H haplotypes (I/III/IV/VI/VII) more frequently have Vif proteins that have lost

activity against A3H [39, 41, 63]. The future examination of large HIV-1 patient cohorts of known *A3* genotypes may provide important insights into the overall impact on of A3 variation of HIV-1 replication and potential disease outcomes.

FIGURES

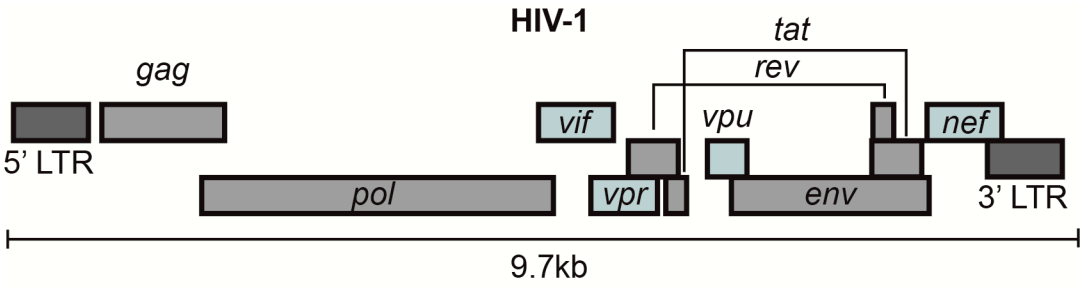

Figure 1-1. Schematic of the HIV-1 genome.

The complete HIV-1 is encoded on a 9.7kB non-segmented RNA, and contains three overlapping reading frames. The structural, enzymatic and cell entry proteins are encoded by *gag*, *pol* and *env* respectively. *Tat* and *rev* encode protein required for provirus transcription and mRNA export, respectively. *Vif*, *vpr*, *vpu* and *nef* function as critical remodelers of the cellular environment to enable productive HIV-1 replication. The coding genes are additionally flanked by long terminal repeat sequences on both 5' and 3' ends of the viral genome.

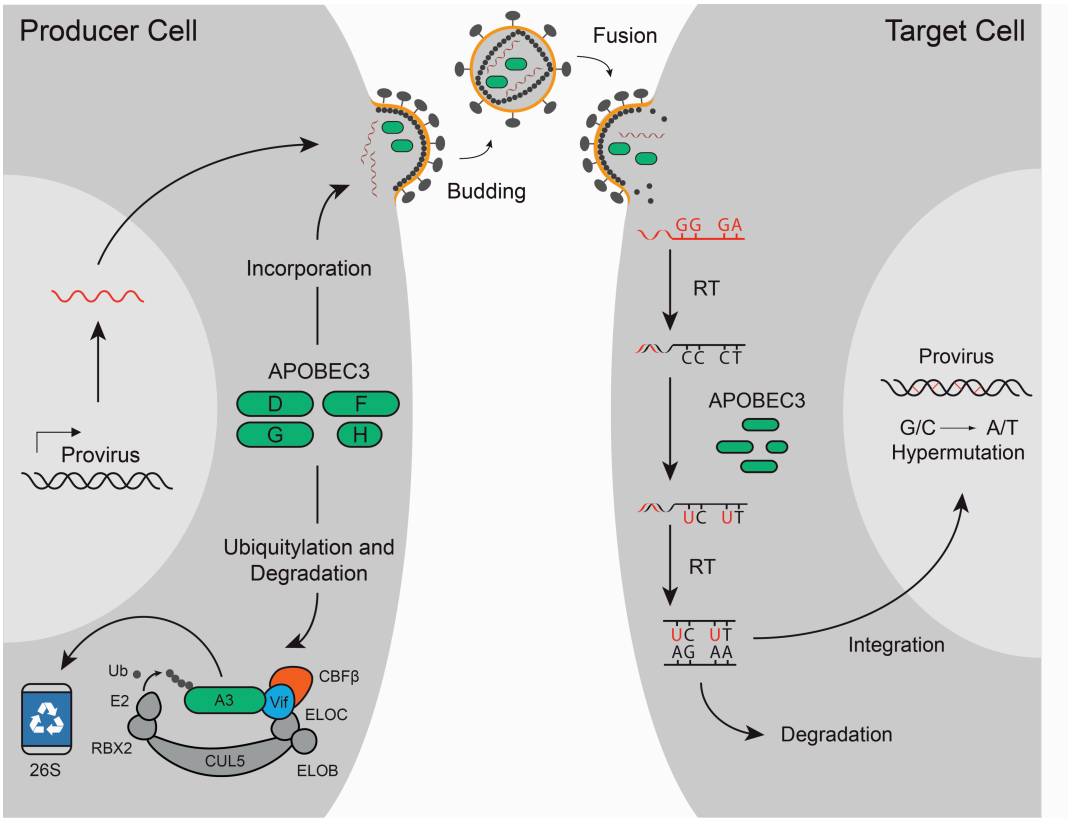

Figure 1-2. Model for HIV-1 restriction by A3 proteins.

In an HIV-1 infected CD4+ T cell, A3D, A3F, A3G and A3H have the capacity to package into HIV virions and catalyze the deamination of cytosine to uracils in viral cDNA replication intermediates upon the initiation of reverse transcription in a subsequently infected target cell. These uracil lesions template the incorporation of adenine upon complementary strand synthesis, resulting in guanine to adenine mutations that can destroy the coding capacity of the viral genome. HIV-1 Vif overcomes this antiviral mechanism by recruiting a cellular ubiquitin ligase complex to promote the polyubiquitylation and proteasomal degradation of A3 proteins.

This figure was adapted and modified from: Hultquist et al. (2011) J. Virol. 85:11220-34.

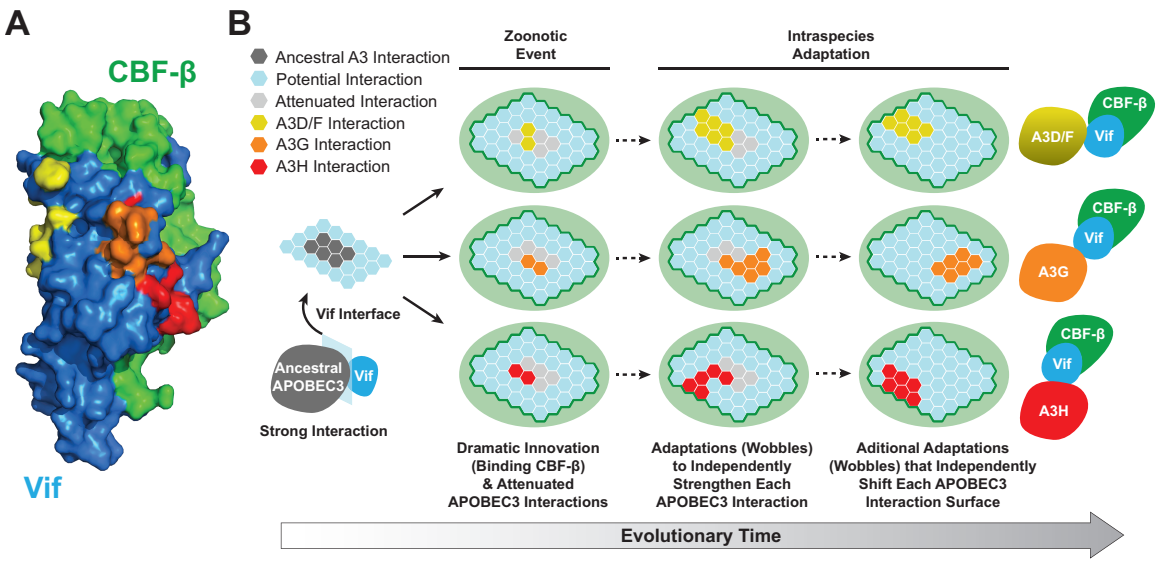

Figure 1-3. An evolutionary model to explain the dynamic nature of the interactions between the lentiviral Vif protein and host APOBEC3 enzymes.

(A) Space-filled representation of the Vif/CBF-β complex (PDB 4N9F). Yellow, orange, and red shading highlights Vif-binding surfaces unique to A3D/F, A3G, and A3H, respectively. The A3D and A3F interaction surfaces are largely overlapping due to high levels of homology caused by a recent gene duplication. (B) A schematic of the "Wobble Model" to explain adaptation of lentiviral Vif. Left to right: The ancestral Vif-APOBEC3 interaction is predicted to require a single interface. Virus transmission to a new host with a larger APOBEC3 repertoire requires a major adaptive change, such as the binding of CBF-β during viral zoonosis into primate species. This event most likely reshaped Vif and attenuated the Vif-APOBEC3 interaction. However, it provided a physical substrate for a series of rapid adaptations to independently strengthen each Vif-APOBEC3 interaction. Iterative adaptation over evolutionary time eventually manifested as the

present-day, largely non-overlapping surfaces of HIV-1 Vif that interact with A3D/F, A3G, and A3H independently.

This figure was adapted from: Reuben S. Harris and Brett D. Anderson. (2017) PLoS Pathogens 12: e1005958.

CHAPTER 2

TRANSCRIPTIONAL REGULATION OF APOBEC3 ANTIVIRAL IMMUNITY THROUGH THE CBF-B/RUNX AXIS

Adapted with permission from: Brett D. Anderson and Reuben S. Harris. (2015) Science Advances 1:e1500296

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INTRODUCTION

The overall innate immune response is composed of sensors, transducers, and effectors [68]. Sensors bind to pathogen-associated molecular patterns and relay signals to effector proteins to mediate clearance of sensed pathogens. The APOBEC3 family of DNA cytosine deaminases are vital innate immune effector proteins because they can be up-regulated by type I interferons and they afford protection against a wide range of DNA-based parasites including retroviruses, endogenous retrotransposons, and a variety of DNA viruses [20, 69-71]. Human cells can express up to seven distinct APOBEC3 enzymes (A/B/C/D/F/G/H), as well as more distantly related family members APOBEC1, AID, APOBEC2, and APOBEC4. The hallmark activity of most members of this family is single-stranded DNA cytosine-to-uracil deamination.

The AIDS virus HIV-1 is the most-studied APOBEC3-susceptible pathogen. A large number of studies using various model systems, including T cell lines and primary CD4 T lymphocytes, have combined to show that APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H have the potential to suppress HIV-1 replication (see recent publications [24, 41, 63, 72] and references therein). These enzymes block virus replication by interfering with reverse transcription and deaminating viral complementary DNA (cDNA) cytosines to uracils, which manifest as genomic strand guanine-to-adenine mutations. However, this potent antiviral mechanism is subverted by the viral infectivity factor (Vif), which nucleates the formation of an E3 ubiquitin ligase complex to promote their degradation (reviewed by [20, 69-71]).

HIV-1 Vif forms an APOBEC3 ubiquitin ligase complex by hijacking the transcriptional cofactor CBF-β to form a scaffold for recruitment of a cullin-RING ligase complex consisting of CUL5, ELOB, ELOC, and RBX2 [28, 56]. Vif itself forms the substrate recognition component that directly binds cellular APOBEC3 enzymes and targets them for polyubiquitination and degradation. The requirement for CBF-β is conserved for Vif function among different HIV-1 subtypes and simian immunodeficiency virus (SIV) isolates, which are primate- specific pathogens, but it is dispensable for nonprimate lentiviral Vif proteins to degrade the APOBEC3 of their host species (that is, BIV, MVV, CAEV, and FIV degrading bovine, ovine, caprine, and feline APOBEC3 proteins) [33, 34, 73-75]. The essential nature of the primate lentiviral Vif/CBF-β interaction is highlighted by the extensive interface evident in a recent crystal structure (4800 $Å^2$) [31]. CBF-β functions canonically as a cofactor to the RUNX family of transcription factor proteins to regulate the expression of a battery of genes involved in hematopoietic and bone cell development [29, 30]. The virus may therefore also exploit the Vif/CBF-β interaction to manipulate the T cell transcription program to somehow benefit virus replication. In support of this general idea, HIV-1 Vif overexpression in the APOBEC3-low Jurkat cell line has been shown to alter the expression of dozens of cellular genes that exhibit significant RUNX1 enrichment near their putative transcriptional start sites [32].

Surprisingly, during the course of interrogating CBF-β function in T cells, we discovered that this protein is actually a positive regulator of transcription of the restrictive *APOBEC3* genes themselves. Both knockdown and knockout experiments

demonstrate that CBF-β is required for *APOBEC3* gene expression. Complementation experiments with previously characterized CBF-β separation-of-function alleles show that the CBF-β interaction with RUNX proteins is required for *APOBEC3* transcription. These results suggest that Vif may hijack CBF-β for two major reasons: to form the canonical APOBEC3 degradation complex and to down-regulate the expression of the *APOBEC3* genes. In support of this idea, Vif-proficient HIV-1 replication is virtually unimpeded and Vif-deficient HIV-1 is partly recovered in CBF-β–depleted T cells, in both instances because the endogenous *APOBEC3* genes are now poorly expressed. This molecular "double whammy" is the first example to our knowledge whereby a viral protein hijacks a single host protein to leverage two distinct mechanisms to overcome a potent and multifaceted antiviral defense system.

RESULTS

CBF-β depletion in T cells results in down-regulation of multiple APOBEC3 genes

To study the role of CBF-β in T cells, we transduced the H9 T cell line with a short hairpin RNA (shRNA) construct to knock down *CBF*-β or with a nonspecific shRNA as a negative control. As expected from our previous studies, this procedure reduced *CBF*-β mRNA levels by >90% and caused a corresponding reduction in CBF-β protein levels (**Figure. 2-1, A and B**) [33, 56, 76]. However, unexpectedly, *CBF*-β depletion also resulted in a strong reduction in endogenous APOBEC3F and APOBEC3G protein levels (**Figure. 2-1B, and 2-S1A**). The RT-qPCR analyses revealed a corresponding decline in *APOBEC3G* mRNA levels, as well as large decreases in *APOBEC3C*, *APOBEC3D*, *APOBEC3F*, and *APOBEC3H* mRNA levels (**Figure 2-1C**).

CBF-β knock down in primary human CD4 T cells also caused reduced *APOBEC3D*, *APOBEC3F*, and *APOBEC3G* mRNA levels, as well as those of a positive control (*TBX21*) (**Figure 2-S2**) [32].

To rule out potential off-target effects of the shRNA-mediated knockdown approach, we used CRISPR/Cas9 [77] to disrupt exon 2 of the *CBF*-β gene (**Figure 2-1D**). *CBF*-β disruption did not appear to adversely affect the H9 T cell line, evidenced by high targeting frequencies (>30%) and by parental and null lines showing similar growth kinetics. *CBF*-β–null H9 derivatives also showed large reductions in *APOBEC3C*, *APOBEC3D*, *APOBEC3F*, *APOBEC3G*, and *APOBEC3H* mRNA and APOBEC3F and APOBEC3G protein levels in comparison to the parental cell line (**Figure 2-1, E and F, and 2-S1B**). Thus, both knockdown and knockout results combined to strongly implicate CBF-β as a positive transcriptional factor for *APOBEC3C*, *APOBEC3D*, *APOBEC3F*, *APOBEC3G*, and *APOBEC3H* expression in T cells.

The CBF-β/RUNX interaction is required for APOBEC3 transcriptional regulation

We next used CBF-β separation-of-function mutants [76, 78] and focused on *APOBEC3G* expression as a phenotypic readout to determine whether regulation of *APOBEC3* transcription is mediated through the canonical CBF-β/RUNX pathway. Specifically, we used CBF-β N104K, which disrupts the interaction with RUNX proteins but not Vif, and CBF-β F68D, which disrupts the interaction with Vif but not RUNX proteins (**Figure 2-2, A to C**). To confirm the monofunctionality of each of these mutants, we showed that N104K blocks RUNX1-dependent transcriptional activation of a *FOXP3* luciferase reporter construct but is still fully competent for Vif-mediated

degradation of APOBEC3G (**Figure 2, B, and C**) [76]. In contrast, F68D still retains the full capacity to promote RUNX1-dependent transcription, but has compromised Vif degradation function (**Figure 2-2, B, and C**).

Each CBF-β separation-of-function mutant was then used in a series of complementation experiments. Only CBF-β F68D, which still interacts with RUNX proteins, was able to complement the *CBF*-β knockdown and cause a restoration in cellular APOBEC3G protein levels (**Figure 2-2D**). The CBF-β N104K mutant, which is defective for both physical and functional interactions with RUNX proteins, failed to rescue APOBEC3G expression levels (**Figure 2-2D**). Together, these results demonstrate that CBF-β regulation of APOBEC3G expression requires a functional interaction with at least one RUNX transcription factor. CBF-β depletion renders H9 cells permissive for HIV-1 replication independent of Vif function.

All current models for HIV-1 Vif function predict that CBF-β depletion should lead to Vif instability and degradation, compromised Vif-E3 ubiquitin ligase function, and a diminished ability to counteract restriction by APOBEC3G and related APOBEC3 restriction factors [20, 69-71] In contrast, HIV-1 particles produced in CBF-β– depleted cells were not restricted and showed slightly higher infectivity in spreading infections in comparison to the same virus replicating in parallel in the H9 parental cell line (**Figure 2-S3**). The CBF-β knockdown was durable through the entire duration of two independent experiments as judged by immunoblotting. This counterintuitive phenotype was even clearer for Vif-deficient viral particles produced in CBF-β– depleted cells, because these retained about 60% of Vif-proficient HIV-1 infectivity (**Figure 2-3, A, and B**). In

contrast and as expected from many previous studies, Vif-deficient particles were poorly infectious after production in the multi-APOBEC3–expressing H9 cell line with endogenous CBF-β intact (**Figure 2-3B**). These observations are supported by immunoblots of cellular and viral particles showing, as above, that CBF-β is required to maintain endogenous APOBEC3G expression, and that Vif expression is diminished when CBF-β is depleted (**Figure 2-3C**).

DISCUSSION

The mechanisms regulating the *APOBEC3* antiviral state in T lymphocytes have remained poorly defined, despite extensive literature documenting the potent restriction activities of these enzymes (reviewed by [20, 69-71]). At the transcriptional level, it is clear that type I interferons can stimulate *APOBEC3* transcription, but this is strongly cell type–dependent. For instance, *APOBEC3A* is strongly up-regulated by interferon treatment of myeloid lineage cells but not T cells, and *APOBEC3G* has a strong interferon response in primary hepatocytes but minimal responsiveness in T lymphocytes [25, 46, 48, 79-81]. Additionally, overexpression of the transcription factors NFAT and IRF4 can induce *APOBEC3G* mRNA and protein expression in cell culture experiments, though the importance of these transcription factors in endogenous *APOBEC3G* regulation or in regulating other family members has not been addressed [55]. Recent work has also demonstrated specific up-regulation of APOBEC3B in multiple distinct human cancers, and the underlying mechanisms have yet to be defined except for human papillomavirus (HPV)–positive cancers where the virus itself promotes *APOBEC3B* transcriptional up-regulation and induces genomic mutagenesis as collateral damage [50,

82-85]. Despite obvious complexities with both specialized and general *APOBEC3* gene expression programs, a full understanding of these processes is likely to be important for future antiviral and anticancer strategies. Here, we show that the transcription cofactor CBF-β is a strong positive regulator of multiple *APOBEC3* genes in CD4+ T cells and that this regulation and the overall antiviral state are dependent on a functional interaction between CBF-β and the RUNX proteins (**Figure. 2-4A**). CBF-β depletion renders H9 cells permissive to Vif-deficient HIV-1 infection, demonstrating that CBF-β is critical for maintaining the overall APOBEC3 antiviral state in T lymphocytes. Our results suggest a model for virus-infected cells in which Vif hijacks CBF-β to counteract the APOBEC3 antiviral defense through a polyubiquitination mechanism while simultaneously down-regulating transcription of the *APOBEC3* genes themselves by precluding CBF-β/RUNX complex formation (**Figure. 2-4B**). This model provides a parsimonious and evolutionarily attractive explanation for why the earliest primate lentivirus, likely a simian tropic ancestor to modern HIV-1, adapted Vif to bind CBF-β from among a large pool of other cellular scaffolding possibilities. One can only imagine that the expanded APOBEC3 protein repertoire of higher primates posed too great of a restrictive barrier for an ancient lentiviral zoonosis, until the Vif protein adapted to use CBF-β and down-regulate this restriction mechanism at both the transcriptional and posttranslational levels. Because the APOBEC3 restriction factors are at least as ancient as placental mammals, all modern lentiviruses have a Vif protein to counteract restriction by cellular APOBEC3 enzymes [86]. It is likely that the ancestral APOBEC3 counteraction mechanism was CBF-β–independent and that adaption to bind CBF-β was necessary for lentiviral

invasion of the primate branches of the mammalian phylogenetic tree. This scenario is concordant with recent data demonstrating that the Vif proteins of nonprimate lentiviruses, FIV, BIV, MVV, and CAEV, function independently of CBF-β [34, 73-75] and with the fact that the mammalian hosts of these viruses have simpler APOBEC3 repertoires[20]. It is supported by the existence of many canonical RUNX-binding sites throughout the human *APOBEC3* locus (**Figure 2-S4**) and also by a previous study showing that Vif overexpression in the Jurkat T cell line could affect the transcriptional activity of dozens of RUNX-regulated genes (although *APOBEC3* expression was not altered possibly because basal mRNA levels are already too low in Jurkat) [32].

The results presented here are also encouraging from a drug development perspective because primate lentiviruses may be fully addicted to cellular CBF-β. In support of this idea, primate lentiviruses are largely confined to cell types of the hematopoietic compartment such as CD4 T lymphocytes that express this cellular protein. Moreover, an obligate relationship between Vif and CBF-β is evidenced by the extensive heterodimer interface (4800 $Å^2$), which would constrain Vif physically and evolutionarily and block it from interacting with many other cellular proteins. This is an attractive drug target because small molecules that disrupt the Vif/CBF-β interaction may preserve high levels of *APOBEC3* gene expression and correspondingly high levels of APOBEC3- mediated antiviral immunity. Because the APOBEC3 enzymes directly mutate viral cDNA and can do so at lethal levels, this potent antiviral response may be able to suppress virus replication indefinitely.

MATERIALS AND METHODS

Human cell lines

H9 and derivative cell lines were maintained in RPMI (HyClone) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin. 293 and derivative cell lines were maintained in Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin.

Human primary CD4 T cells

Human primary CD4 T cells were isolated from same-day draw peripheral whole blood (MemorialBloodCenter, Saint Paul, MN). Peripheral blood mononuclear cells were enriched by whole-blood fractionation over a Ficoll-Paque (GE Healthcare) gradient, and naïve CD4 T cells were subsequently purified by negative selection (CD4 T Cell Isolation Kit II, MiltenyiBiotec). CD4 T cells were stimulated with phytohemagglutinin (10 μg/ml) and interleukin-2 (IL-2) (20 U/ml) and maintained in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin, and IL-2 (20 U/ml).

Plasmid DNA transfection experiments

293T cells (abbreviated as 293 throughout the paper to avoid confusion with T cells and T lymphocytes) were transfected using Transit LTI (Mirus) according to the manufacturer's protocol. All downstream analyses were performed 48 hours after transfection.

Knockdown experiments

The *CBF*-β shRNA was derived by subcloning the short hairpin sequence from

the pGIPZ-based Open Biosystems catalog no. RHS4430-99161432 into the pLKO.1 puro vector (Addgene 8453) by direct ligation using Age I and Eco RI restriction sites (pGIPZ-based vectors showed poor infectivity in T cell lines). The shControl construct was obtained from Open Biosystems (catalog no. RHS4346). shRNA- expressing lentiviruses were prepared as described (10).

CRISPR/Cas9-mediated knockouts

Guide RNA sequences identical to *CBF*-β exon 2 were ligated into the lentiCRISPRv1 vector (Addgene #49535) according to accompanying Zhang llaboratory protocols [87]. Knockout derivative cell lines were generated by targeting the 5′-GGCCGTCGCGGCAGGCGTTC-3′ site in exon 2 of the *CBF*-β gene. This targeting construct was generated by annealing oligos 5′-CACCGGCCGTCGCGGCAGGCGTTCG-3′ and 5′-AAACGAACGCCTGCCGCGACGGCC-3′ and ligating the resulting double-stranded DNA into Bsm BI–digested lentiCRISPRv1. The transducing vector was produced by transfecting 293 cells with the lentiCRISPRv1-targeting construct along with pΔ-NRF (HIV-1 *gag*, *pol*, *rev*, *tat* genes) and pMDG (VSV-G) expression vectors. Virus-containing culture supernatants were filtered (0.45 μm) and concentrated by centrifugation (22,000*g*, 2 hours, 10°C), and viral pellets were resuspended in complete RPMI. H9 cells were incubated with Cas9/CRISPR-encoding lentiviruses for 48 hours and then placed under drug selection (puromycin, 1 μg/ml). Clones were generated by limiting dilution in 96-well U-bottom plates, followed by outgrowth and screening by immunoblotting and target DNA sequencing.

Immunoblotting experiments

Cell lysates prepared in 2.5x Laemmli sample buffer were separated by SDS–polyacrylamide gel electrophoresis and electrophoretically transferred to PVDF-FL membranes (Millipore). Membranes were blocked in 4% milk in phosphate-buffered saline (PBS) and incubated with primary antibodies diluted in 4% milk in PBS + 0.1% Tween 20. Secondary antibodies were diluted in 4% milk in PBS + 0.1% Tween 20 + 0.01% SDS. Membranes were imaged on a LI-COR Odyssey instrument. Primary antibodies used in these studies were rabbit anti-APOBEC3G [National Institutes of Health (NIH) ARRRP 10201 courtesy of J. Lingappa], rabbit anti-APOBEC3F (NIH ARRRP 11474 courtesy of M. Malim), rabbit anti– CBF-β (Epitomics), rabbit anti-HA (Cell Signaling), rabbit anti-FLAG (Sigma F7425), mouse anti–HIV- 1 p24/CA (NIH ARRRP 3537 courtesy of B. Chesebro and K. Wehrly), mouse anti–HIV-1 Vif (NIH ARRRP 6459 courtesy of M. Malim), and mouse anti-tubulin (Covance). Secondary antibodies used were IRDye 800CW goat anti-rabbit (LI-COR 827-08365) and Alexa Fluor 680 goat anti-mouse (Molecular Probes A-21057).

RNA isolation, cDNA synthesis, and RT-qPCR

RNA was extracted from cell lines and primary cells (RNeasy, Qiagen) and used as a template for random hexamer-primed cDNA synthesis (Transcriptor, Roche). Gene expression was analyzed on a Roche LightCycler 480 instrument as described (24). Primer and probe sequences are listed in table S1.

CBF-β complementation experiments

HA-tagged CBF-β variants were subcloned into the CSU6-IDR2 IRES-dsRed

lentiviral packaging vector (courtesy of W. Sundquist) and cotransfected into 293 cells along with pΔ-NRF (HIV-1 *gag-pol-rev-tat*) and pMDG (VSV-G) expression vectors. Forty-eight hours after transfection, culture supernatants were filtered (0.45 μm) and concentrated by centrifugation (22,000*g*, 2 hours, 10°C). Viral pellets were resuspended in complete RPMI and used to directly infect H9 cells. Transduced cell populations were assayed for dsRed expression by flow cytometry and analyzed by immunoblotting 72 hours after transduction.

Dual luciferase transcription reporter assays

Transcriptional reporter assays were performed in *CBF*-β knockdown 293 cells using a *FOXP3* promoter luciferase reporter construct as described (21).

HIV-1 single-cycle and spreading infectivity experiments

VSV-G pseudotyped HIV-1 was produced by cotransfecting 293 cells with HIV-1_{IIIB} A200C molecular clone derivatives (pIIIB or pIIIB Δ*vif*) and pMDG (VSV-G). Forty-eight hours after transfection, culture supernatants containing viruses were filtered (0.45 μm) and concentrated by centrifugation (22,000*g*, 2 hours, 10°C). Virus pellets were resuspended in complete RPMI, and viral titer was determined by serial dilution on CEM-GFP indicator cells. Viruses were added to H9 cultures (multiplicity of infection, 0.4), and cells were washed twice in PBS 12 hours after infection to remove any residual pseudotyped virus. Forty- eight hours later, cell-free supernatants containing progeny viruses were used to infect CEM-GFP reporter cells. HIV-1–spreading infections were performed as described [23].

ADDITIONAL CONTRIBUTIONS

We thank A. Haase, T. Ikeda, A. Land, C. Richards, N. Shaban, and P. Southern for helpful comments. LentiCRISPRv1 was a gift from F. Zhang. RUNX3 ChIP-seq data were obtained from the ENCODE consortium (ENCSR000BRI) and originally provided by R. Myers. Salary support for B.D.A. was provided in part by NIH T32 AI83196 and subsequently NIH T32 AI007313. This work was supported by grants to R.S.H. from the NIH (R01 AI064046 and P01 GM091743).

FIGURES

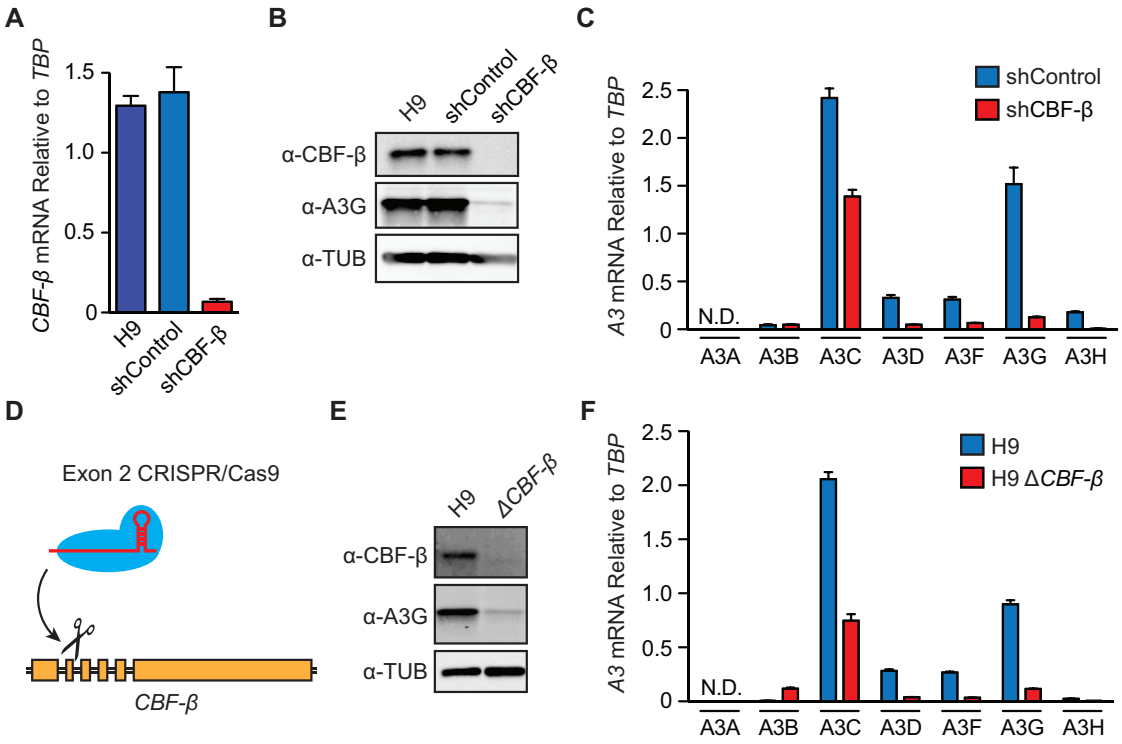
A
CBF-β mRNA Relative to TBP
1.5
1.0
0.5
0
H9
shControl
shCBF-β
B
H9
shControl
shCBF-β
α-CBF-β
α-A3G
α-TUB
C
A3 mRNA Relative to TBP
shControl
shCBF-β
2.5
2.0
1.5
1.0
0.5
0
N.D.
A3A
A3B
A3C
A3D
A3F
A3G
A3H
D
Exon 2 CRISPR/Cas9
CBF-β
E
H9
ΔCBF-β
α-CBF-β
α-A3G
α-TUB
F
A3 mRNA Relative to TBP
H9
H9 ΔCBF-β
2.5
2.0
1.5
1.0
0.5
0
N.D.
A3A
A3B
A3C
A3D
A3F
A3G
A3H

Figure 2-1. *CBF*-β knockdown and deletion decreases expression of APOBEC3 mRNAs and proteins.

(A) *CBF*-β mRNA levels relative to *TBP* in H9 and knockdown derivatives by real-time quantitative polymerase chain (*n* = 3 with mean ± SD shown). (B) Representative immunoblots of CBF-β and APOBEC3G protein levels in the parental H9 T cell line and shRNA-transduced pools. Tubulin (TUB) is a loading control. (C) RT-qPCR of *APOBEC3* mRNA levels relative to *TBP* in cells transduced with a control shRNA or a CBF-β–specific shRNA (*n* = 3 with mean ± SD shown; N.D., not detected). (D) Schematic of CRISPR/Cas9 disruption of *CBF*-β exon 2. (E) Representative immunoblots of CBF-β and APOBEC3G protein levels in H9 cells and a *CBF*-β knockout derivative. (F) RT-qPCR of *APOBEC3* mRNA levels relative to *TBP* in H9 cells and a *CBF*-β knockout derivative (*n* = 3 with mean ± SD shown).

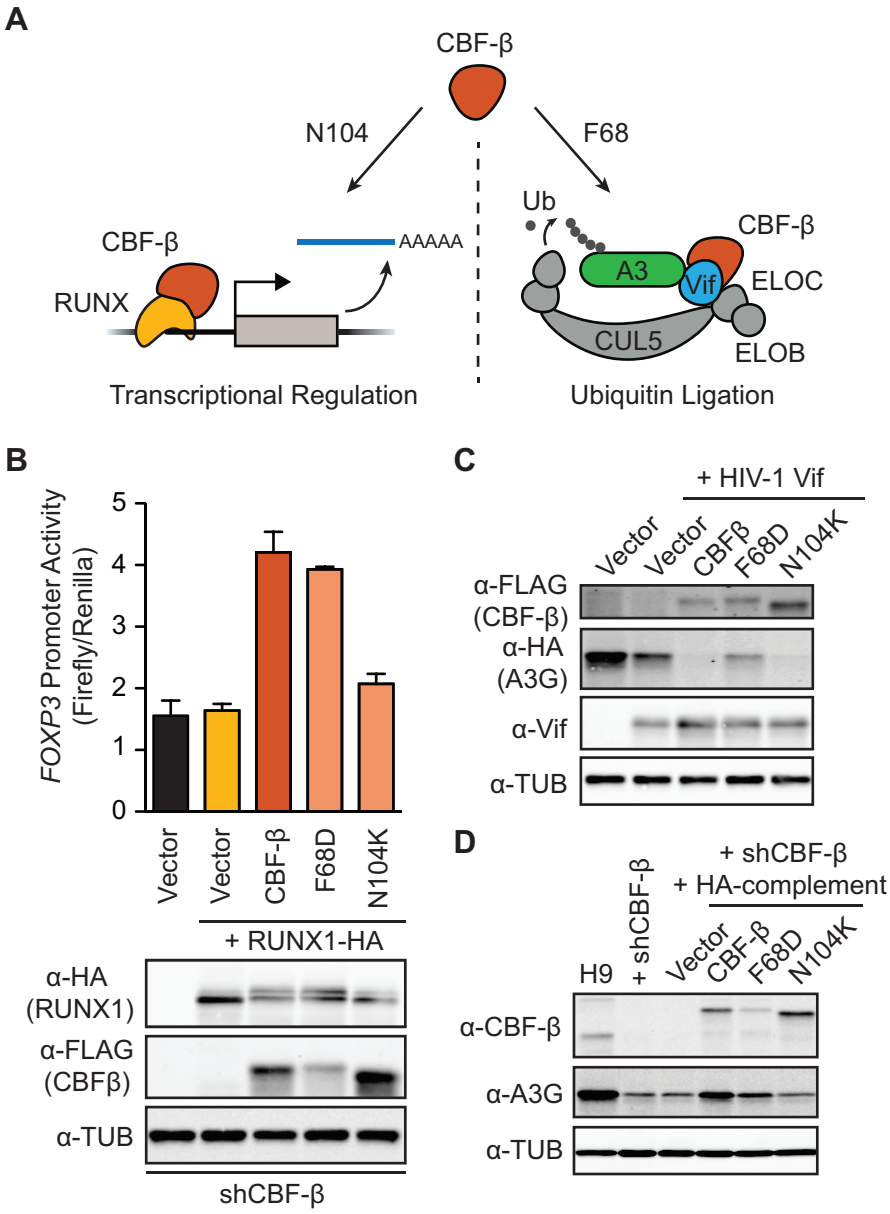
A
CBF-β
N104
F68
Ub
CBF-β
AAAAA
A3
Vif
ELOC
RUNX
CUL5
ELOB
Transcriptional Regulation
Ubiquitin Ligation
B
FOXP3 Promoter Activity (Firefly/Renilla)
0
1
2
3
4
5
Vector
Vector
CBF-β
F68D
N104K
+ RUNX1-HA
α-HA (RUNX1)
α-FLAG (CBFβ)
α-TUB
shCBF-β
C
+ HIV-1 Vif
Vector
Vector
CBFβ
F68D
N104K
α-FLAG (CBF-β)
α-HA (A3G)
α-Vif
α-TUB
D
+ shCBF-β
+ HA-complement
H9
+ shCBF-β
Vector
CBF-β
F68D
N104K
α-CBF-β
α-A3G
α-TUB

Figure 2-2. RUNX interaction is necessary to restore APOBEC3G expression in CBF-β–depleted cells.

(A) Schematic depicting established phenotypes of CBF-β separation-of-function mutants. Residue N104 is required for CBF-β transcription function with RUNX proteins, whereas F68 is required for Vif-E3 ligase-mediated degradation of APOBEC3 enzymes. (B) Histogram reporting *FOXP3* promoter activity as measured by firefly luciferase levels relative to a *Renilla* luciferase cotransfection control. The indicated CBF-β expression construct, RUNX1 expression construct, and appropriate empty vector controls were cotransfected with luciferase vectors into *CBF-β* knockdown 293 cells 48 hours before luciferase activity measurement (n = 3; mean ± SD shown). Representative immunoblots from a single experimental replicate are shown below. (C) Representative immunoblots showing Vif functionality [APOBEC3G-HA (hemagglutinin) degradation activity] in the presence of the indicated FLAG-CBF-β constructs 48 hours after transfection into *CBF*-β knockdown 293 cells. (D) Immunoblots showing the results of a representative complementation experiment using *CBF*-β knockdown H9 cells and the indicated HA-–CBF-β expression constructs or controls. APOBEC3G levels are low in the absence of CBF-β or in the presence of CBF-β N104K (even with higher expression levels relative to the other constructs). In contrast, APOBEC3G levels are restored by expressing wild-type CBF-β or the F68D mutant.

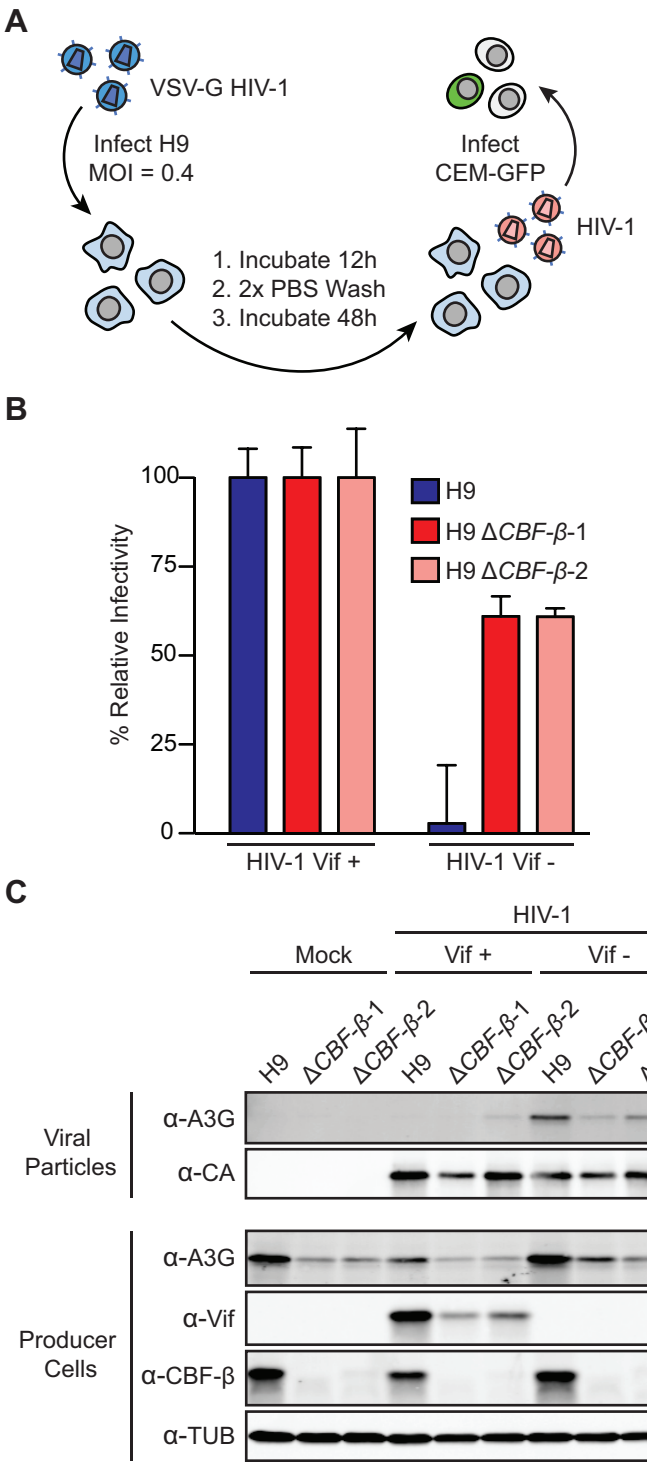
A
VSV-G HIV-1
Infect H9
MOI = 0.4
1. Incubate 12h
2. 2x PBS Wash
3. Incubate 48h
Infect
CEM-GFP
HIV-1
B
% Relative Infectivity
100
75
50
25
0
H9
H9 ΔCBF-β-1
H9 ΔCBF-β-2
HIV-1 Vif +
HIV-1 Vif -
C
HIV-1
Mock
Vif +
Vif -
H9
ΔCBF-β-1
ΔCBF-β-2
Viral
Particles
α-A3G
α-CA
Producer
Cells
α-A3G
α-Vif
α-CBF-β
α-TUB

Figure 2-3. *CBF*-β knockout protects HIV-1 from APOBEC3-mediated restriction. (A) Schematic of HIV-1 single- cycle infectivity assay. (B) Relative infectivity of Vif-proficient and Vif-deficient viruses produced in H9 cells or *CBF*-β knockout clones (n = 2; mean ± SD shown). Vif-proficient viral infectivity for each cell line is set to 100% to facilitate comparisons. (C) Representative immunoblots of relevant cellular and viral proteins from the experiment depicted in (B), as well as additional data from mock-treated cells.

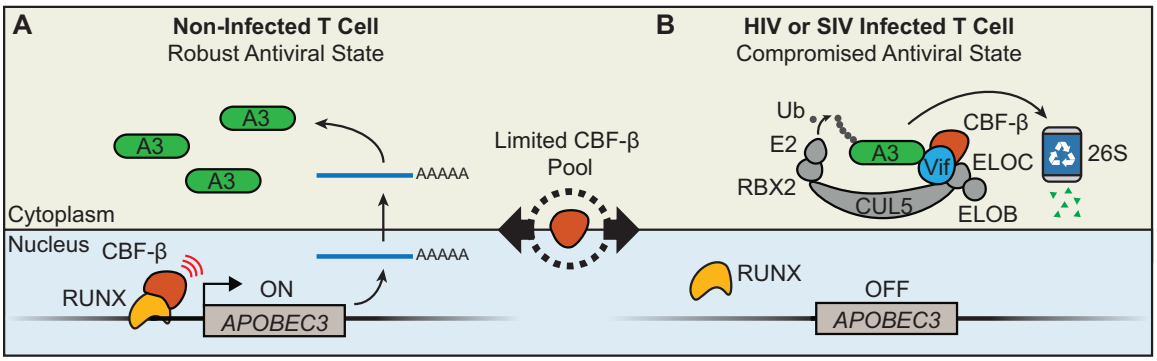

Figure 2-4. New models for APOBEC3-mediated antiviral state and Vif function.

(A) CBF-β/RUNX drives transcription of *APOBEC3* genes and maintains a robust antiviral state in the absence of HIV-1 infection in CD4+ T cells. (B) In HIV-1– or SIV–infected cells, Vif prevents CBF-β from binding RUNX transcription complexes to down-regulate *APOBEC3* gene transcription and simultaneously promote APOBEC3 protein polyubiquitination and proteasomal degradation.

Gene	mRNA NCBI Accession	5' Primer	3' Primer	UPL #	UPL Probe Sequence
APOBEC3A	NM_145699	gagaagggacaagcacatgg	tggatccatcaagtgtctgg	26	ctgggctg
APOBEC3B	NM_004900	gacccttggtccttcgac	gcacagccccaggagaag	1	cctggagc
APOBEC3C	NM_014508	agcgcttcagaaaagagtgg	aagtttcgttccgatcgttg	155	ttgccttc
APOBEC3D	NM_152426	acccaaacgtcagtcgaatc	cacatttctgcgtggttctc	51	ggcaggag
APOBEC3F	NM_145298	ccgtttggacgcaaagat	ccaggtgatctggaaacactt	27	gctgcctg
APOBEC3G	NM_021822	ccgaggacccgaaggttac	tccaacagtgctgaaattcg	79	ccaggagg
APOBEC3H	NM_181773	agctgtggccagaagcac	cggaatgtttcggctgtt	21	tggctctg
CBF-β	NM_001755	actggatggtatgggctgtc	aaggcctgttgtgctaatgc	18	tcctgctg
RUNX1	NM_001754	ccaaagagtgtggaattttggt	aaacagggcgagttgcat	55	ggagagga
RUNX2	NM_001024630	ggttaatctccgcaggtcac	tgcttgcagccttaaatgact	83	ggtggctg
RUNX3	NM_001031680	ggcctctccatgccttct	aggagggaagaaactacaaggac	38	ggaagcag
TBP	NM_003194	cccatgactcccatgacc	tttacaaccaagattcactgtgg	51	ggcaggag
Tbx21	NM_013351	tgtggtccaagtttaatcagca	tgacaggaatgggaacatcc	9	tggtgatg

Table 2-S1. Quantitative PCR Primer and Probe Information.

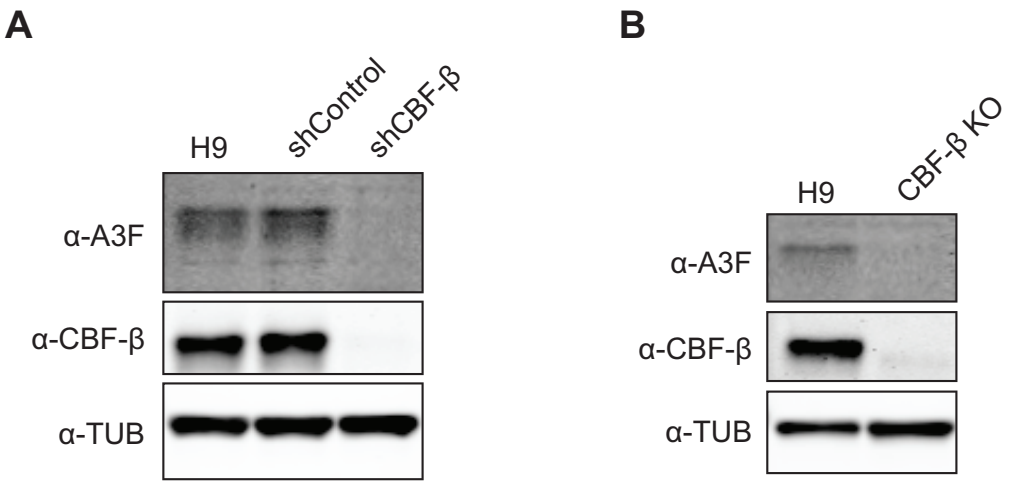

Figure 2-S1. CBF-β knockdown or knockout causes decreased A3F protein levels.

(A) Representative immunoblots of A3F and CBF-β in H9 cells following lentiviral transduction of a control or *CBF−β* specific shRNA construct. Tubulin (TUB) is shown below as a loading control.

(B) Immunoblots of A3F and CBF−β protein levels in parental H9 cells in comparison to a representative *CBF−β* null derivative. Tubulin (TUB) is shown below as a loading control.

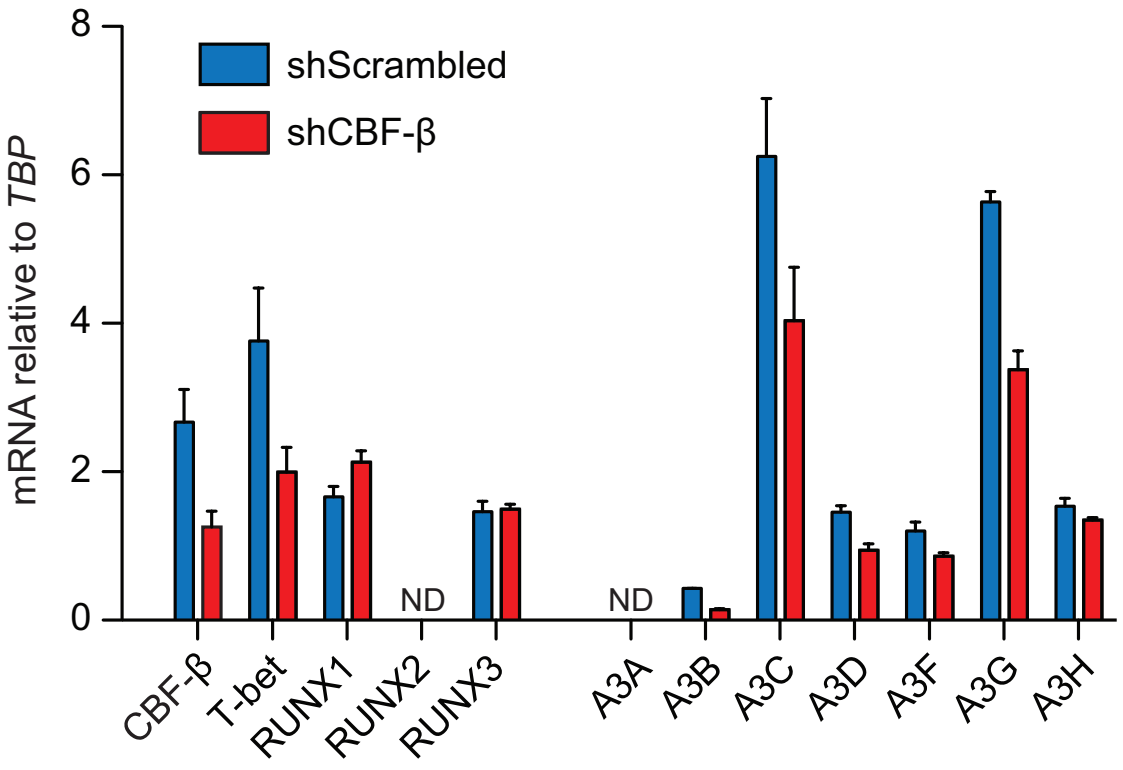

Figure 2-S2. *CBF-β* knockdown in primary human CD4+ T cells results in a comcomitant reduction in the mRNA levels of *APOBEC3D*, *APOBEC3F*, and *APOBEC3G*.

RT-qPCR of primary CD4+ T cells treated with shControl or shCBF-β expressing viruses. Expression analyses were carried out following drug selection with puromycin to enrich for transduced cells. Reported as the mean of 3 reactions ± s.d.. N.D. – not detected.

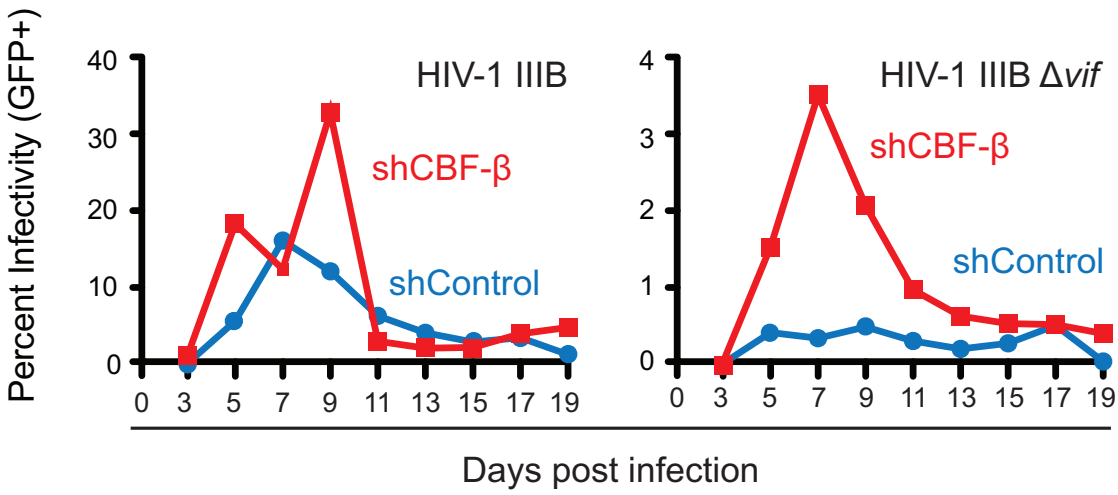

Figure 2-S3. *CBF-β* knockdown renders H9 cells more permissive to *vif*-deficient HIV-1 replication.

Spreading infection kinetics in H9 cultures treated with shControl or shCBF-β expressing viruses following puromycin selection. Infections were intitiated at MOI = 0.01. Culture infectivity was monitored over time by passaging viral supernatants on CEM-GFP reporter cells. Viral infectivity on CEM-GFP cells was quantified by flow cytometry 48h post-infection.

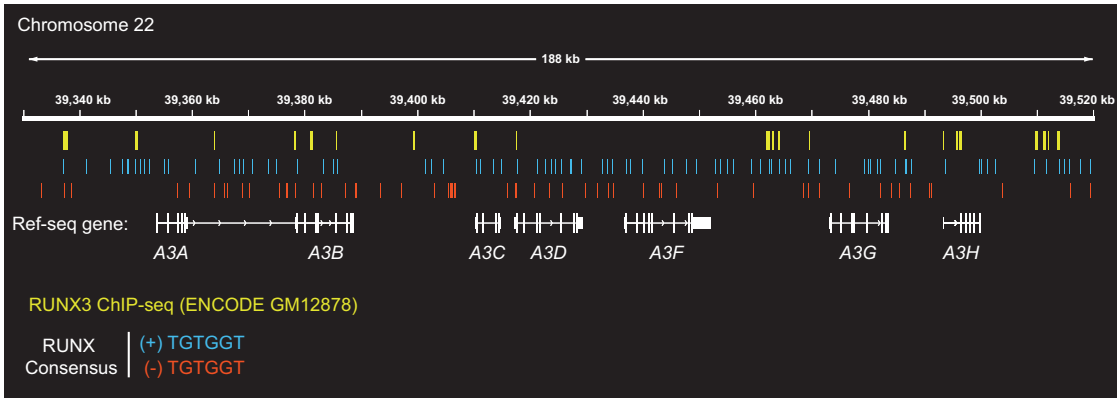

Figure 2-S4. Schematic of predicted and ChIP-validated RUNX binding sites within the human *APOBEC3* locus.

The human *APOBEC3* locus is depicted by a thick white line, and Ref-seq exons are shown below as white tics or boxes. The blue and the red tics represent consensus RUNX-binding sites predicted from the human genome DNA sequence. The yellow tics represent RUNX3-binding sites demonstrated by ENCODE ChIP-sequencing of the lymphoblastoid cell line GM12878 (ENCSR000BRI).

CHAPTER 3: NATURAL APOBEC3C VARIANTS ELICIT DIFFERENTIAL HIV-1 RESTRICTION ACTIVITY IN T CELL LINES

This chapter is adapted from a manuscript submitted for publication that is awaiting peer review: Brett D. Anderson, Amber St. Martin, William L. Brown, and Reuben S. Harris. In review, Retrovirology.

Authors' contributions: Conceived and designed the experiments: BDA, RSH. Performed the experiments: BDA. Analyzed the data: BDA, RSH. Contributed reagents: BDA, AS, WLB. Wrote the manuscript: BA, RSH.

INTRODUCTION

Retroviruses, including the AIDS virus human immunodeficiency virus type-1 (HIV-1), must evade destruction by an extensive array of antiviral host proteins known as restriction factors [88, 89]. The seven human APOBEC3 (A3) enzymes constitute an important arm of this innate network of restriction factors. These enzymes catalyze the deamination of cytosine to uracil in single-stranded (ss)DNA substrates, and APOBEC3D (A3D), APOBEC3F (A3F), APOBEC3G (A3G) and APOBEC3H (A3H) are known to contribute to HIV-1 restriction (reviewed by [22, 90]). These four enzymes package into budding virions and, following virus entry into a new target cell, catalyze the formation of uracil lesions in reverse-transcription intermediates. These uracils template the incorporation of adenines during synthesis of the viral genomic strand, resulting in G-to-A mutations.

HIV-1 and related lentiviruses avoid lethal levels of A3 induced mutation through the virion infectivity factor (Vif), which is a small viral protein required for virus infectivity in most cell types. Vif functions as a molecular adapter to recruit A3 enzymes to a host ubiquitin ligase comprised of CBF-β, CUL5, RBX2, ELOB, and ELOC for polyubiquitination and subsequent degradation by the proteasome [26, 28, 56]. HIV-1 Vif effectively triggers the degradation of human A3D, A3F, A3G and A3H, which would otherwise restrict virus replication [23]. Vif also targets human APOBEC3C (A3C) for degradation, despite this enzyme eliciting little Vif-deficient HIV-1 restriction activity [23, 37, 67, 91]. Moreover, human A3C is highly expressed in the primary cellular reservoir for HIV-1 replication, CD4+ T cells, and is upregulated upon HIV-1 infection

similar to the other restrictive A3 proteins [23]. Curiously, human A3C has also been shown to potently restrict a strain of simian immunodeficiency virus isolated from African Green Monkeys (SIVagm) in the absence of Vif in 293-based single cycle infection experiments [67, 92]. These observations indicate that human A3C is a *bona fide* retrovirus restriction enzyme and, further, that HIV-1 (or its SIV precursor from chimpanzees) may have recently evolved a Vif-independent mechanism for evading restriction by this enzyme.

The *A3* gene family exhibits significant variation within the human population [64]. First, a deletion spanning the entire *A3B* coding sequence occurs at 37% frequency worldwide with clear geographic biases [58, 59]. Second, *A3H* has at least 7 distinct haplotypes that exhibit drastically different expression levels and HIV-1 restriction phenotypes [41, 62, 93]. The most common A3H haplotype (hap I) is a poorly expressed protein with little to no Vif-deficient HIV-1 restriction activity, and it is found at a 48% frequency worldwide and implicated in cancer mutagenesis [59, 62, 93, 94]. In contrast, the next most common A3H haplotype (hap II) is well expressed and shows strong Vif-deficient HIV-1 restriction activity [59, 62, 93, 94]. Third, two recent reports have characterized a rare A3C-Ile188 variant (~2% global allele frequency) with enhanced restriction activity against Vif-deficient HIV-1 in a 293 based single cycle infection model in comparison to the predominant A3C-Ser188 enzyme [66, 67]. Biochemical studies have indicated that the enhanced restriction activity of A3C-Ile188 may be due to increased enzyme processivity for cytosine deamination and/or to an increased propensity to homodimerize [66, 67].

The impact of the rare A3C-Ile188 enzyme on HIV-1 restriction in T lymphocytes has yet to be studied. Here, using a combination of knockout and stable expression approaches in multiple T cell lines, we show that neither genetic deletion of *A3C* in the non-permissive CEM2n T cell line, nor complementation with natural A3C variants is sufficient to alter HIV-1 replication kinetics in the presence or absence of Vif. However, stable expression of A3C-Ile188 was able to restrict Vif-deficient virus replication in the permissive SupT11 T cell line in the absence of other A3 proteins. Taken together, these findings confirm prior reports identifying the rare Ile188 allele as a more restrictive form of A3C. Our studies underscore the possibility that this naturally occurring A3C variation may influence HIV-1 replication and pathogenesis *in vivo*.

METHODS

Cell lines

SupT11 [23], CEM2n [24] and derivative cell lines were maintained in RPMI (Hyclone) supplemented with 10% FBS (Thermo Fisher) and 1% penicillin/streptomycin. 293 cells were maintained in DMEM (Hyclone) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin. 293 cells were transfected with TransIT LTI (Mirus) according to the manufacture's protocol. All experiments were harvested 48 h post-transfection.

A3C expression plasmids

The C-terminally triple-HA tagged A3C construct in the pcDNA3.1(+) backbone has been described [23]. The N-terminally triple-HA tagged A3C constructs were ligated into the pcDNA3.1(+) backbone with a triple-HA epitope sequence between the *Nhe*I and

*Hind*III cloning sites. Untagged A3C constructs were assembled similarly into an epitope tag-free pcDNA3.1(+) backbone. Untagged A3C variants were subcloned into the CSU6-IDR2 IRES-eGFP lentiviral expression vector (previously described [57] but modified here to express eGFP instead of dsRed) for stable transduction experiments in CEM2n and SupT11 cells.

Immunoblots

Cell pellets were lysed directly in 2.5x Laemmli sample buffer, separated by 12.5% SDS-PAGE, and transferred to PVDF-FL membranes (Millipore). Membranes were blocked in 4% Milk in PBS and incubated with primary antibodies diluted in 4% Milk in PBS supplemented with 0.1% Tween20. Secondary antibodies were diluted in 4% Milk in PBS supplemented with 0.1% Tween20 and 0.01% SDS. Membranes were imaged on a Licor Odyssey instrument. Primary antibodies used in these studies were rabbit anti-A3C (Proteintech 10591-1-AP), rabbit anti-A3G (NIH AARP 10201 courtesy of J. Lingappa), rabbit anti-HA (Cell signaling C29F4), mouse anti-Tubulin (Sigma T5168), mouse anti-HIV-1 p24/CA (NIH ARRRP 3537 courtesy of B. Chesebro and K. Wehrly), and mouse anti-HIV-1 Vif (NIH AARRP 6459 courtesy of M. Malim). Secondary antibodies employed were IRdye 800CW goat anti-rabbit (Licor 827-08365) and Alexa Fluor 680 goat anti-mouse (Molecular Probes A-21057), except when detecting the anti-A3C antibody, which was probed with an anti-Rb HRP-conjugated secondary antibody (Bio Rad 1706515) and visualized using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher). The following A3C antibodies failed to detect overexpressed N-terminally tagged A3C variants by immunoblotting: rabbit anti-A3C (Abcam

ab181356), rabbit anti-A3C (Abcam ab209560), rabbit anti-A3C (LSBio LS-B14538), and mouse anti-A3C (Sigma SAB1403204).

HIV-1 single-cycle infectivity experiments

293-based HIV-1 single cycle infectivity assays were performed by co-transfecting 293 cells with an HIV-1_{IIIB} A200C *vif*-deficient molecular clone derivative (pIIIB Δ*vif*) [95, 96]. 48 h later, cell free supernatants containing progeny viruses were used to infect CEM-GFP reporter cells. Reporter cell infectivity was assayed 48 h post infection by flow cytometry.

Cas9-mediated knockouts

Guide RNA sequences homologous to *A3C* exon 3 were generated using the CRISPR Design Tool (Massachusetts Institute of Technology) and cloned into the lentiCRISPRv1 vector (Addgene #49535) according to the accompanying Zhang lab protocol [87]. Knockout derivative cell lines were generated by targeting the 5'GGGGCTCCGCAGCCTGAGTC-3' site in exon 3 of the *A3C* gene. This targeting construct was generated by annealing oligos 5'-ACACCGGGGCTCCGCAGCCTGAGTCG -3' and 5'-AAAACGACTCAGGCTGCGGAGCCCCG -3' and ligating the resulting dsDNA into BsmBI digested lentiCRISPRv1. 293 cells were transfected with the lentiCRISPRv1 targeting construct along with pΔ-NRF (HIV-1 *gag*, *pol*, *rev*, *tat* genes) and pMDG (VSV-G) expression vectors and 48 h later virus containing supernatants were filtered (0.45 μm) and concentrated by centrifugation (22,000 x g, 2 h, 10 °C). Viral pellets were resuspended in complete RPMI and incubated with cells for 48 h before being placed

under drug selection (1 μg/mL puromycin). Clonal populations were isolated by limiting dilution of the drug resistant cell pool and assayed for A3C expression by immunoblotting.

A3C complementation

CSU6-IDR2 IRES-eGFP vectors encoding untagged A3C variants were co-transfected into 293 cells with pΔ-NRF (HIV-1 *gag-pol-rev-tat*) and pMDG (VSV-G). 48 h post transfection, culture supernatants were filtered (0.45 μm) and concentrated by centrifugation (22,000 x g, 2 h, 10 °C). Viral pellets were resuspended in complete RPMI and used to directly infect SupT11 and CEM2n target cells. Transduced cell populations were assayed for GFP expression by flow cytometry and A3C expression was measured by immunoblotting 72 h post-transduction.

HIV-1 spreading infections

HIV-1 spreading infections were performed as described [23]. Briefly, replication-competent HIV-1 stocks were produced by transfecting 293 cells with HIV-1_{IIIB} A200C wild-type (pIIIB) or *vif*-deficient (pIIIB Δ*vif*) molecular clone derivatives [95, 96]. 48 h post-transfection, culture supernatants were filtered (0.45 μm), and viral titers were determined by serial titration on CEM-GFP reporter cells. CEM2n and SupT11 derivative cell lines were infected accordingly, and virus replication was monitored every other day (beginning on day 3 post-infection) by infecting CEM-GFP reporter cells with culture supernatants. Infected CEM-GFP cells were fixed at 48 h post-infection and assayed for GFP expression by flow cytometry on a Becton Dickinson FacsCanto II instrument.

RESULTS

Immunoblot comparisons of epitope-tagged and untagged A3C variants

The first study comparing A3C-Ser188 and A3C-Ile188 used a C-terminal HA tag and found much lower expression levels of the Ile188 variant [64]. A more recent report using C-terminally HA-tagged A3C variants in a different vector showed no significant expression difference [67]. Our results using different C-terminally triple-HA-tagged variants mirror the original report with the Ile188 variant expressing at lower levels (top blot, lanes 2/3 vs 4/5 in **Figure. 3-1A**). Another recent study used N-terminally HA-tagged variants and found no expression difference [66]. Our results with different N-terminally triple-HA-tagged A3C variants confirm this observation (top blot, lanes 6/7 vs 8/9 in **Figure. 3-1A**). We postulate that the observed instability of the Ile188 variant is due to an interaction with the C-terminal HA-epitope tag in some, but not all, vectors (*i.e.*, an epitope tag-associated artifact).

In an attempt to resolve this issue, we tested all available commercial anti-A3C antibodies in immunoblot experiments and found only one reagent that reproducibly detected both transfected and endogenous A3C (Proteintech #10591-1-AP; see validation in knockout studies below and Materials and Methods for a list of reagents that failed). This polyclonal antibody was raised against full-length A3C, and it confirmed a subset of the anti-epitope blots above and yielded an additional new consideration. First, it showed that the N-terminally HA-tagged A3C-Ser188 construct expresses much better than the reciprocal C-terminally HA-tagged construct (middle blot, lanes 6/7 vs 2/3 in **Figure 3-1A**). Second, it strongly detects HA-A3C-Ser188 but only weakly the Ile188 variant

(middle blot, lanes 6/7 vs 8/9 in **Figure 3-1A**). This expression difference was particularly clear for untagged A3C proteins (middle blot, lanes 10/11 vs 12/13 in **Figure 3-1A**). These data strongly indicate that the dominant antibody in the polyclonal mixture recognizes an epitope that includes Ser188. Consistent with this interpretation, a single amino acid substitution, Ser188 to Leu, also renders A3C undetectable in immunoblot experiments with this antibody (data not shown). These epitope tag and immunoblot limitations impose reasonable but significant constraints on studies with A3C.

Single cycle infectivity results using 293 cells

Because untagged constructs provide more accurate models for endogenous proteins and because untagged A3C-Ser188 displays visibly higher expression levels than both N- and C-terminal triple-HA-tagged derivatives, hereon only the untagged set of constructs is used in functional studies. In agreement with prior reports [66, 67], untagged A3C-Ser188 causes a modest 2-fold reduction in the infectivity of Vif-deficient HIV-1 in the 293-based single cycle infection system (**Figure 3-2**). A comparison of this A3C-Ser188 construct and a Glu68-to-Gln derivative indicated that effect is independent of the conserved catalytic glutamate. In contrast, A3C-Ile188 caused a larger 4-fold infectivity decline that at least partly required deaminase activity. An empty vector and an A3G expression vector served as negative and positive controls, respectively, for these transient expression studies.

A3C deletion and variant complementation does not alter HIV-1 replication kinetics in non-permissive CEM2n cells

Little is known about the role of endogenous A3C in HIV-1 restriction in T cells. Endogenous A3C is detected readily in non-permissive H9 and CEM2n cell lines, but not in the permissive SupT11 cell line using the commercial anti-A3C antibody described above (**Figure 3-3A**). Cas9-mediated genome editing was used to generate an *A3C* knockout derivative of the non-permissive CEM2n [24]. This cell line is homozygous for A3C-Ser188 (PCR sequencing results not shown). The parental CEM2n line was transduced with a vector expressing Cas9 and an *A3C*-specific guide RNA, and limiting dilution was used to isolate clones harboring no detectable A3C protein by immunoblot (*e.g.*, clone 1 in **Figure 3-3B**). To our knowledge, this is the first description of an A3C-null cell line. A3C-null clones show no overt phenotypes such as morphology or growth rates in comparison to non-targeted sister clones (data not shown).

Next, we used transduction to generate pools of CEM2n ΔA3C cells expressing A3C-Ser188, A3C-Ile188, or an empty vector as a negative control. Each construct also expressed eGFP to control for transduction efficiency (and to help mitigate the fact that the untagged A3C-Ile188 variant cannot be detected with available antibodies; **Figure 3-1**). In all instances, near complete transduction efficiency was confirmed by GFP+ flow cytometry (**Figure 3-4A**). Moreover, immunoblotting was able to show that complemented A3C-Ser188 expression levels were similar to endogenous levels normally found in the parental CEM2n line (**Figure 3-4B**). As explained above, protein-level expression of A3C-Ile188 could not be measured accurately due to Ser188 likely

being part of the main epitope recognized by the antibody. Nevertheless, these two variants are expected to express at similar levels because the vectors are otherwise isogenic, the Ser188 and Ile188 variants express similarly with N-terminal tags, and the eGFP levels of the complemented pools are almost indistinguishable (and results below demonstrate the functionality of the A3C-Ile188 construct).

To address the role of endogenous A3C (Ser188/Ser188) and to compare complemented A3C-Ser188 and A3C-Ile188 variants in parallel in HIV-1 spreading infections, transduced cell pools were infected with HIV-1_{IIIB} or a Vif-deficient derivative and virus replication was monitored over time. Neither the A3C deletion nor complementation with either A3C variant altered the kinetics of Vif-proficient virus replication with all CEM2n ΔA3C derivatives showing peak infectivity at 7 days post-infection (**Figure 3-4C**). In contrast, CEM2n ΔA3C derivatives remained fully non-permissive to Vif-deficient virus replication presumably due to expression of other endogenous restrictive A3 enzymes including A3D, A3F, A3G and A3H [23, 24] (**Figure 3-4D**). Vif-deficient virus replication in the permissive SupT11 cell line confirmed infectivity of the viral stock. Taken together, these results demonstrate that deletion of A3C is not sufficient to render CEM2n cells detectably permissive to Vif-deficient HIV-1 replication, and that complementation with A3C-Ser188 or A3C-Ile188 is unable to alter the kinetics of virus replication in the presence of Vif, potentially because both variants are targeted for degradation by Vif at similar efficiencies [67].

Stable of expression of A3C-Ile188 in SupT11 cells restricts Vif-deficient HIV-1 replication

The phenotypically redundant antiviral activities of A3D, A3F, A3G, and A3H make it challenging to study A3C activity in non-permissive T cell lines such as CEM2n. Therefore, permissive, non-A3 expressing T cell lines such as SupT11 provide robust systems for comparing the antiviral activities of single DNA deaminases [23, 40-42, 63, 97, 98]. Prior to knowledge of A3C-Ile188, we reported that that A3C-Ser188 is unable to restrict Vif-deficient HIV-1 when stably expressed in this system [23]. These studies were additionally limited by the utilization of a C-terminal triple-HA epitope tag, which we now appreciate is detrimental to A3C expression (**Figure 3-1A**). Therefore, the intrinsic restriction activities of untagged A3C-Ser188 and A3C-Ile188 were compared following stable transduction and expression in SupT11 cells. As above, efficient transduction was confirmed by GFP+ flow cytometry for all constructs (**Figure 3-4A**) and by immunoblotting for A3C-Ser188 (**Figure 3-4B**).

The resulting SupT11 pools were infected with three different MOI of Vif-deficient HIV-1 (0.01, 0.02, and 0.05) and virus replication kinetics were monitored over time. At the lowest initial MOI, A3C-Ile188 elicited strong suppression of virus replication in contrast to A3C-Ser188, which caused a modest but reproducible delay (**Figure 3-5C**). Interestingly, virus replication kinetics were delayed by A3C-Ile188 when an initial intermediate MOI was used, but they were unaffected when a higher MOI was used. In all instances, both A3C-Ser188 and A3C-Ile188 caused a modest suppression in overall infectious viral titers, in comparison to the vector control condition analyzed in

parallel (**Figure 3-5C**). Overall, despite not being able to visualize untagged A3C-Ile188 by immunoblotting, these results demonstrated that A3C-Ile188 is capable of Vif deficient HIV-1 restriction in the SupT11 T cell line and that A3C-Ser188 may also possess some (albeit weak) restriction activity.

DISCUSSION

The A3 DNA cytosine deaminase family imposes a significant barrier to productive HIV-1 infection, especially for viruses lacking Vif. Prior studies converged on A3D, A3F, A3G and A3H as the HIV-1 restrictive A3 repertoire in CD4+ T cells ([23, 24, 99, 100]; reviewed by [22, 69, 89]). Our current studies indicate that A3C-Ile188 has the potential to contribute to HIV-1 restriction in human T cells. Our studies confirm prior 293-based single cycle results [66, 67] and, importantly, extend this conclusion to the more physiologically relevant T cell line SupT11. Additional work will be needed to extend these findings to primary T lymphocytes, for instance, through simultaneous Cas9-mediated deletion of A3D/F/G/H *ex vivo*. Such studies would be facilitated by an anti-A3C monoclonal antibody that binds equally well to both naturally occurring A3C-Ser188 and A3C-Ile188 variants.

The seven gene human *A3* locus is variable in the human population, consistent with the overall function of the encoded enzymes in innate immunity and previously documented evidence for positive selection [67, 101-104]. Known variations include complete inactivation of *A3* family members (29.5 kb *A3B* deletion [58] and *A3H*-Δ*N15* [62]), hypomorphic alleles (*A3H-G105*; [62]), and hypermorphic alleles such as the A3C-

I188 variant (this study and prior work [66, 67]). Several other variants have been documented but, thus far, their functional relevance is less clear.

A3C is conserved in primates and phylogenetic comparisons show evidence for positive selection, including multiple amino acid substitutions within the predicted Vif-binding interface [37, 67, 105]. This suggests an ongoing conflict between lentiviruses with Vif and ancestral A3C enzymes. Consistent with this idea, cross species comparisons have shown restriction of SIVagm by human A3C [67, 92]. Taken together with the fact that HIV-1 Vif still degrades A3C and HIV-1 infection induces *A3C* expression, it is possible that A3C-Ser188 and A3C-Ile188 still impose some (albeit low) selective pressure on present day HIV-1 (or SIV in recent evolutionary history). Alternatively, A3D and/or A3F are still so similar to A3C that molecular mimicry within the Vif binding surface is sufficient to maintain Vif function against A3C.

A3C amino acid 188 is located in the C-terminal helix, which is physically separated from the Vif binding interface [37]. However, phylogenetic comparisons for this region are similarly interesting. Within the human A3 repertoire, most enzymes have Ile at the analogous structural position suggesting a need for greater restriction activity [67]. Sequence comparisons across divergent primate species indicate that Ile188 is the ancestral primate residue, occurring in divergent primate species including the orangutan, gibbon, siamang, and all sequenced Old World monkeys [67]. In contrast, Ser188 is found in humans and other hominids including chimpanzees, gorillas, and bonobos. Curiously, chimpanzee and gorilla A3C proteins have been shown to have HIV-1 restriction activity comparable to the human A3C-Ile188 variant, despite harboring Ser at

the analogous amino acid position, suggesting that additional amino acid differences between hominid species may play a role in defining intrinsic restriction activity [66]. *In vitro* biochemical purifications of these enzymes have revealed a correlation between self-dimerization of A3C in solution and HIV-1 restriction activity (observed for human A3C-Ile188, chimp A3C and gorilla A3C, but not human A3C-Ser188) [66]. The biochemical characterization of additional primate A3C proteins will be needed to test the broad relevance of dimerization in A3C-dependent restriction of primate lentiviruses, though these findings demonstrate that Ile188 is not the only determinant for enhanced restriction activity.

The overall contribution of A3C-Ile188 to HIV-1 replication *in vivo* remains unclear at this time, though relative comparisons in single cycle infection experiments suggest that its antiviral activity will pale in comparison to the potent restriction activity of A3G (*e.g.*, **Figure 3-2**). Nevertheless, these findings reveal an additional example of genetic variation within the human A3 repertoire that may influence HIV-1 evolvability and pathogenesis in the primary cellular reservoir, CD4+ T cells. Future studies leveraging large patient cohorts of known *A3C* genotypes, with due attention to other variations in *A3B* and *A3H*, may be able to address this possibility.

CONCLUSION

We confirm here that the rare human A3C-Ile188 variant exhibits enhanced restriction activity against Vif-deficient HIV-1 in a 293-based single cycle infection system. Using Cas9-mediated gene knockout in non-permissive CEM2n T cells, we

found that endogenous A3C is not required for HIV-1 restriction, most likely due to the dominant restriction activities of A3D, A3F, A3G and A3H. Complementation of A3C-deleted CEM2n resulted in a similar lack of change in virus replication kinetics. Stable expression of A3C-Ile188 was, however, able to convert normally permissive SupT11 T cells to a non-permissive phenotype with respect to Vif-deficient virus replication. Taken together, these findings reveal a role for A3C in HIV-1 restriction in T cells, and highlight an additional level of variation within the human *A3* gene locus that may play a role in HIV-1 adaptation and pathogenesis *in vivo*.

ADDITIONAL CONTRIBUTIONS

We thank Christopher Richards, Nadine Shaban, Terumasa Ikeda, Gabriel Starrett, and Diako Ebrahimi for helpful comments throughout this study and during preparation of the manuscript. This work was supported by NIAID R37 AI064046 and NCI R21 CA206309. BDA received salary support from NIAID F31 AI116305, and ASM from NSF GRFP 00039202.

FIGURES

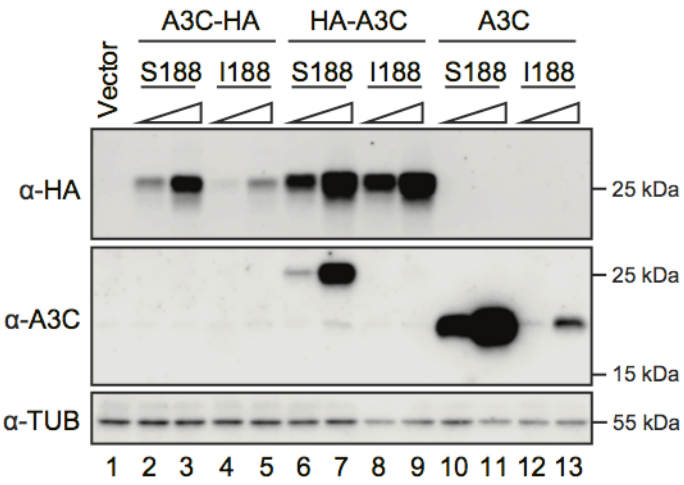

Figure 3-1. Expression of epitope-tagged and untagged A3C derivative constructs in 293 cells.

Immunoblots of 293 cells transfected with 100ng or 400ng of C-terminally tagged (A3C-HA), N-terminally tagged (HA-A3C), or untagged A3C variants using either an anti-HA or anti-A3C antibody for detection. Tubulin is used as a loading control.

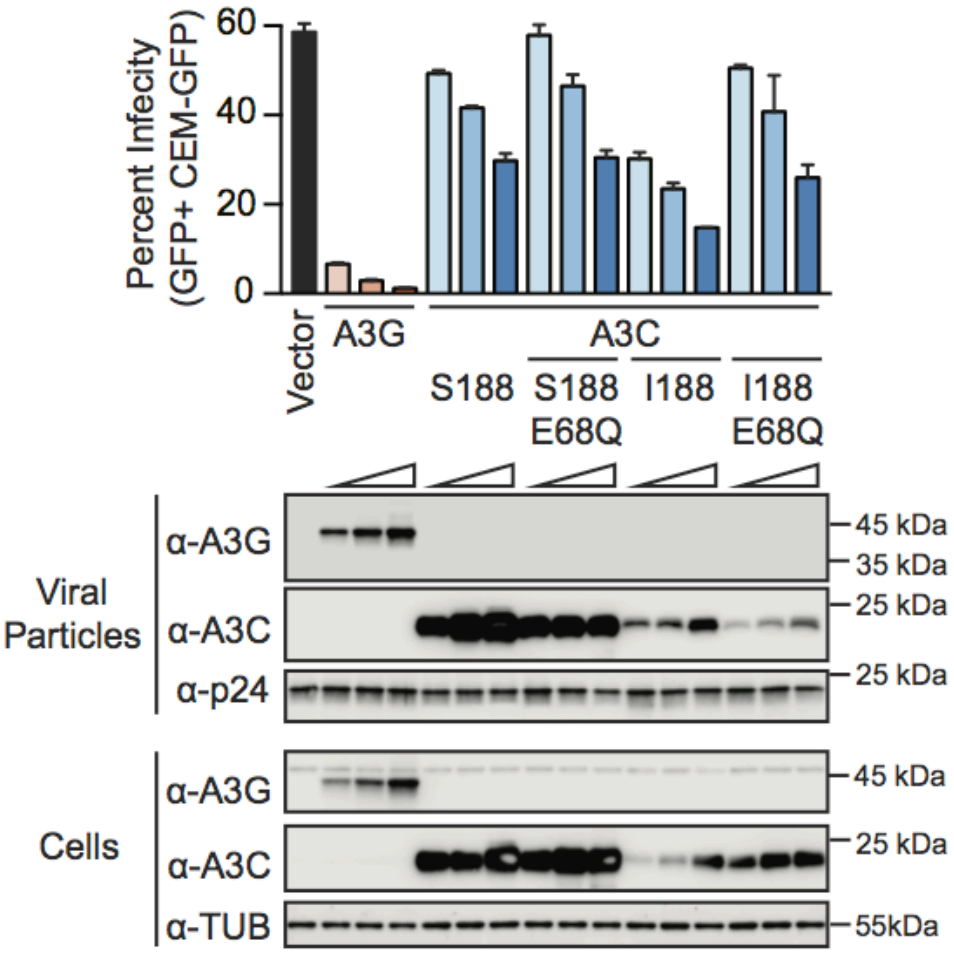

Figure 3-2. Untagged A3C-Ile188 exhibits enhanced HIV-1 restriction activity in 293 cells.

Single cycle infectivity of Vif-deficient HIV-1 viruses produced in the presence of untagged A3C-S188 or A3C-I188 with or catalytic mutant derivatives (E68Q). Immunoblots are shown below for viral particles (anti-A3G, anti-A3C, and anti-p24) and producer cells (anti-A3G, anti-A3C and anti-Tubulin).

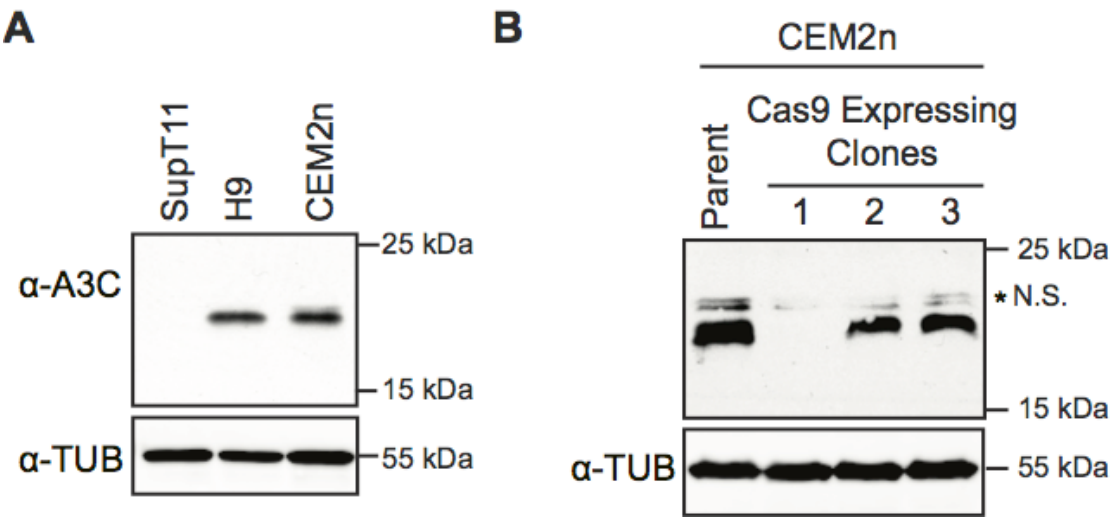

Figure 3-3. Endogenous detection and genetic deletion of A3C in CEM2n T cells.

(A) Immunoblots of endogenous A3C in SupT11, H9, and CEM2n cells. Tubulin is used as a loading control.

(B) Representative immunoblots of Cas9/gRNA-targeted CEM2n derivative clones. Tubulin is used as a loading control.

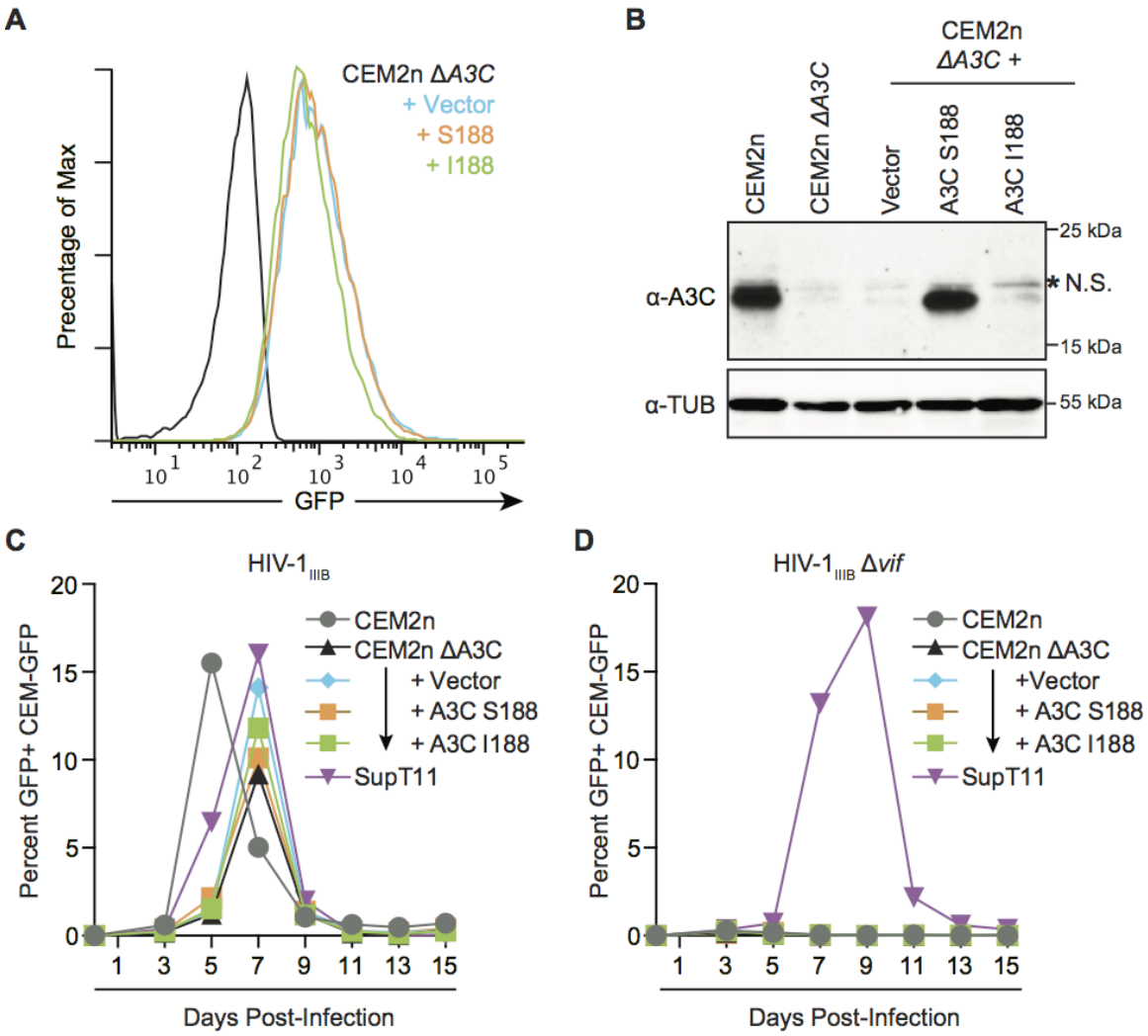
A
CEM2n ΔA3C
+ Vector
+ S188
+ I188
Precentage of Max
GFP
B
CEM2n
ΔA3C +
CEM2n
CEM2n ΔA3C
Vector
A3C S188
A3C I188
25 kDa
* N.S.
15 kDa
α-A3C
α-TUB
55 kDa
C
HIV-1IIIB
CEM2n
CEM2n ΔA3C
+ Vector
+ A3C S188
+ A3C I188
SupT11
Percent GFP+ CEM-GFP
Days Post-Infection
D
HIV-1IIIB Δvif
CEM2n
CEM2n ΔA3C
+Vector
+ A3C S188
+ A3C I188
SupT11
Percent GFP+ CEM-GFP
Days Post-Infection

Figure 3-4. HIV-1 replication phenotypes following A3C deletion and variant complementation in non-permissive CEM2n cells.

(A) Flow cytometry of CEM2n ΔA3C cell pools 72 h post-transduction with GFP-reporter complementation vectors.

(B) Immunoblots of A3C in the parental CEM2n line, CEM2n ΔA3C, and complemented CEM2n ΔA3C derivatives. Tubulin is used as a loading control.

(C) Spreading infection kinetics of Vif-proficient HIV-1 (initial MOI = 0.02) CEM2n ΔA3C cell pools. SupT11 cells are included as a permissive cell type control. Virus infectivity was determined by infection of CEM-GFP with culture supernatants followed by flow cytometry analysis 48 h later.

(D) Spreading infection kinetics of Vif-deficient HIV-1 (initial MOI = 0.02) CEM2n ΔA3C cell pools. SupT11 cells are included as a permissive cell type control. Virus infectivity was determined by infection of CEM-GFP with culture supernatants followed by flow cytometry analysis 48 h later.

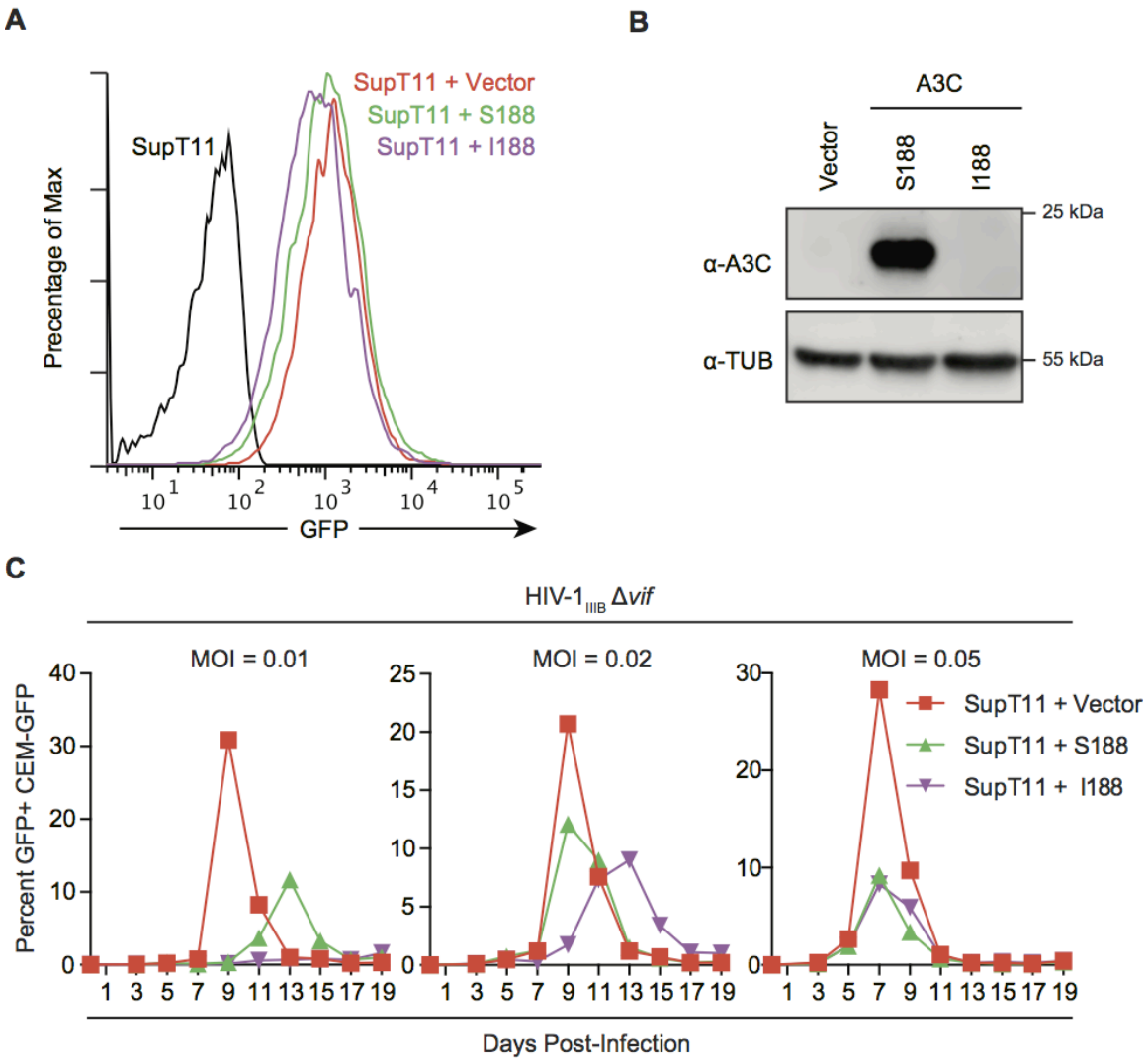
A
SupT11
SupT11 + Vector
SupT11 + S188
SupT11 + I188
Precentage of Max
GFP
B
A3C
Vector
S188
I188
25 kDa
α-A3C
α-TUB
55 kDa
C
HIV-1IIIB Δvif
MOI = 0.01
MOI = 0.02
MOI = 0.05
SupT11 + Vector
SupT11 + S188
SupT11 + I188
Percent GFP+ CEM-GFP
Days Post-Infection

Figure 3-5. Stable Expression of A3C I188 in SupT11 cells provides a partial block to Vif-deficient HIV-1 replication.

(A) Flow cytometry of SupT11 cell pools 72 h post transduction with GFP-reporter complementation vectors.

(B) Immunoblots of A3C in parental SupT11 cells and A3C complemented derivatives. Tubulin is used as a loading control.

(C) Spreading infection kinetics of Vif-deficient HIV-1 at increasing initial MOI on SupT11 derivatives transduced with an empty vector, A3C S188 or A3C I188. Virus infectivity was determined by infection of CEM-GFP with culture supernatants followed by flow cytometry analysis 48 h later.

CHAPTER 4

CONCLUSIONS AND DISCUSSIONS – APOBEC3 TRANSCRIPTIONAL REGULATION AND NATURAL VARIATION

APOBEC3 Transcriptional Regulation in CD4+ T Cells

Since the initial discoveries 15 years ago of A3G as a potent inhibitor of *vif*-deficient HIV-1 replication [106] and as a DNA cytosine deaminase [107], a tremendous amount of progress has been made in understanding the molecular biology behind both A3-dependent virus restriction and circumvention of the host A3 defense through the HIV-1 Vif protein (reviewed by [19, 70, 108-110]). However, the mechanisms that regulate *A3* gene expression in the context of an HIV-1 infection remain poorly understood. Following HIV-1 infection, *A3C*, *A3G*, *A3H*, and to a lesser degree *A3D* and *A3F*, are significantly upregulated, though the mechanisms that elicit this induction are not known [23]. *A3G* upregulation in CD4+ T cells has been observed following exposure to several soluble factors, including IL-2, IL-7, and IL-15 [51], though their specific relevance in the context of a productive HIV-1 infection remains unclear. Additionally, several transcription factors including NFAT, IRF-4 [54, 55], and Tbet [53] have been shown to elicit the upregulation of *A3G* (and *A3F* in the case of Tbet) in CD4+ T cells, though it is important to note that these studies relied on overexpression systems, and the impact of endogenous levels of these transcription factors on *A3* gene regulation remains to be determined.

A3 gene expression patterns vary widely between different hematopoietic cell types. For example, CD4+ T cells typically express significant levels of *A3C*, *A3D*, *A3F*, *A3G*, and *A3H* mRNA (with *A3C* and *A3G* being the highest), and almost no detectable *A3A* and *A3B* [23, 25]. *A3A* expression is largely confined to the myeloid lineage of cells [25, 46-48], and *A3B* expression is readily detectable in both myeloid cells and B cells

[46]. Of particular note, *A3B* upregulation has been recently observed in a variety of human cancer types, suggesting a potential involvement in somatic mutagenesis [49, 50, 82, 83, 85, 111-113]. Of all of the human *A3* genes, *A3C* appears to be the most ubiquitously expressed, and is found at significant levels cell lines and primary human tissues [25, 46, 50].

In addition to innate differences in basal level expression levels, distinct regulatory pathways can modulate expression of specific *A3* genes in a cell type specific manner. For example, *A3A* and *A3G* are highly induced by type I IFNs in myeloid cells and hepatocytes, respectively [25, 46-48]. Moreover, *A3B* alone is specifically upregulated by PMA treatment in a variety of normal and tumor derived epithelial cell lines in a manner that can be blocked by chemical inhibition of both PKC and non-canonical NF-κB signaling pathways [49]. Interestingly, PMA treatment stimulates *A3G* upregulation in multiple CD4+ T cell models and can be reversed by PKC inhibition, suggesting that there may be some general overlap in *A3* regulatory mechanisms between cell types despite inherent differences in the responses of specific *A3* genes.

The studies described in this thesis report the CBF-β/RUNX transcription complex as a novel mechanism for *A3* gene regulation in CD4+ T cells (**Chapter 2**; [57]). In both the non-permissive H9 T cell line, as well as primary CD4+ T cells, endogenous CBF-β depletion elicited dramatic reductions in basal level *A3C*, *A3D*, *A3F*, *A3G* and *A3H* gene expression. *A3* gene expression was only rescued by CBF-β variants competent for RUNX binding, indicating that the CBF-β/RUNX interaction is required for CBF-β-dependent regulation of *A3* genes. The requirement for specific RUNX

proteins was more difficult to determine as their knockdown consistently resulted in non-viable cell cultures. Despite this experimental limitation, one can assume that RUNX1 or RUNX3 (or both) are involved in *A3* regulation, as RUNX2 expression in largely confined to the bone compartment. Future studies will be required to determine whether CBF-β/RUNX complexes act directly on *A3* gene promoters to stimulate their transcription in T cells. In support of this idea, a RUNX3 ChIP-seq dataset shows significant RUNX3 enrichment within *A3* promoter regions [57]. Still, although a direct regulatory role is favored, it is conceivable that CBF-β/RUNX complexes may also be involved in regulating the expression of other transcription factors that are directly responsible for modulating *A3* transcription.

The fact that HIV-1 Vif requires CBF-β to trigger the polyubiquitylation and degradation of the restrictive A3 enzymes makes the discovery of CBF-β as a regulator of *A3* gene transcription particularly intriguing. Vif forms an extensive direct interaction with CBF-β that ultimately precludes the binding of RUNX proteins, and thus it would appear that Vif may have the capacity to directly interfere with *A3* gene transcription by impeding the assembly of CBF-β/RUNX transcription complexes. In support of this idea, overexpression of Vif in the semi-permissive Jurkat T cell line has been shown to alter the expression of nearly 100 CBF-β/RUNX-dependent genes, though similar experiments will have to be performed in more physiologically relevant cell lines and primary T lymphocytes for full validation. Nevertheless, it is clear that CBF-β/RUNX function is required for maintaining a strong A3 antiviral defense, and that depletion of CBF-β in H9 T cells is sufficient to render the culture permissive to Vif-deficient HIV-1 replication

[57]. Collectively, these observations suggest a model in which HIV-1 Vif hijacks cellular CBF-β to simultaneously promote the degradation of the A3 enzymes and the transcriptional repression of their cognate genes [57]. If true, this would represent a striking example of evolutionary economy, whereby the virus has commandeered a single host protein to counteract a potent antiviral defense through two distinct and highly complementary mechanisms.

The idea that HIV-1 may suppress *A3* gene transcription by usurping CBF-β from RUNX complexes plays into an emerging theme of viral gene products modulating host cell *A3* expression. For instance, *A3B* is upregulated in the vast majority of HPV-associated cervical, as well as head and neck cancers [82, 85, 114] . *A3B* upregulation is attributable to expression of the HPV E6 oncoprotein, which functionally inactivates the cellular tumor suppressor p53 [115, 116] and may serve to de-repress *A3B* transcription. Similar upregulation of *A3B* has been observed in response polyomavirus infection, and has been attributed to expression of the large T antigen [117]. It is presently unclear what evolutionary benefit to the virus would arise from triggering the upregulation of an antiviral protein such as A3B, though it is possible that this may be a mechanism for driving cellular transformation through stochastic A3B-driven mutagenesis of the host cell genome, which may in turn provide an environment more conducive to virus replication and dissemination. Alternatively, A3B may provide a source of sublethal mutation to the virus, ultimately promoting viral diversity and escape from destruction by the adaptive immune response.

While the discovery of CBF-β and the RUNX proteins as positive regulators of

A3 gene expression in CD4+ T cells provides several important insights into this innate antiviral defense, significant questions remain to be addressed. Namely, what impact does Vif have on *A3* mRNA expression during a replicating HIV-1 infection, and what is the relative importance of *A3* transcriptional repression compared to the canonical A3 polyubiquitylation mechanism? Furthermore, are CBF-β/RUNX involved in the temporal upregulation of multiple *A3* genes following HIV-1 infection, or do other pathways drive this induction? The studies presented in this thesis are likely to provide a foundation to address these questions and lead to a more comprehensive understanding of A3-dependent innate immunity to viral infections.

Natural APOBEC3 Variation

Susceptibility to viral infection and pathogenesis can be dramatically influenced by natural genetic variations within host species. One particularly illustrative example of variation in intraspecies genetic susceptibility to viral pathogenesis can be gleaned from attempts to control invasive rabbit species in Australia during the 1950s through the intentional release of myxoma virus. Exposure to myxoma virus causes mortality in rabbits, and was successful in reducing the invasive population by 85%. However, a subset of the rabbit population exhibited natural resistance to viral pathogenesis, and these control efforts have largely selected for the proliferation of resistant rabbits in subsequent decades [118, 119].

In the case of HIV-1, the only concrete example of natural resistance to infection is conferred by a rare 32 base pair deletion polymorphism in the *CCR5* gene (*CCR5 Δ32*), which encodes the co-receptor for R5-tropic HIV-1 strains [120, 121]. This deletion

results in a truncated CCR5 protein that fails to traffic to the cell surface, thus preventing infection by viruses that require CCR5 binding for cell entry (*CCR5 Δ32* patients remain susceptible to infection with X4-tropic and dual-tropic HIV-1 isolates that utilize CXCR4 as a co-receptor). The robustness of the *CCR5 Δ32* allele in conferring resistance to HIV-1 infection and disease progression has made *CCR5* a prime candidate for modification as part of autologous gene therapy efforts to establish resistance in HIV-1 patients. In support of this approach, disruption of the *CCR5* gene using sequence-specific zinc-finger nucleases in human hematopeotic stem cells prior to engraftment into NOD/SCID/IL2rγ deficient mice has been shown to rapidly populate animals with virus resistant human cells following HIV-1 challenge. Several additional genetic variations, most notably the HLA-B*57 allele, have been associated with elite controllers [122, 123]. None of these polymorphisms, however, have been shown to uniformly confer a disease resistance phenotype in infected patients.

The seven gene human *A3* locus is highly variable within the human population, and multiple common polymorphisms have been associated with disease outcomes. For example, the *A3B* deletion allele found primarily in Southeast Asian descendants has been associated with an increased risk of breast cancer [58-61], persistent hepatitis B infection [124], and increased susceptibility to falciparum malaria infection. Additionally, a missense polymorphism in A3G (H186R) has been linked to accelerated progression to AIDS in HIV-1 infected patients [65]. A3H, the most polymorphic member of the human *A3* gene family, is represented by at least seven haplotypes, only two of which encode stable proteins with significant restriction activity against HIV-1 (haplotypes II and V)

[41, 62, 63]. Stable A3H haplotypes have been associated with increased CD4+ T cell counts and lower viremia in HIV-1 infected patients [63]. Numerous other polymorphisms within the human *A3* gene repertoire have been reported, though the vast majority do not appear to influence protein stability or enzymatic activity, and have not been associated with disease progression or outcomes [64].

This thesis (**Chapter 3**) addresses a rare A3C polymorphism (S188I), which was shown recently to have significant restriction activity against HIV-1 in a 293T-based single cycle infection system [66, 67]. Several prior efforts aimed at defining the HIV-restrictive repertoire of A3 enzymes in CD4+ T cells had largely dismissed A3C as playing a significant role in innate immunity to HIV-1 infection [23, 92], though these studies only examined the predominant A3C Ser188 variant. More recent studies reporting enhanced HIV-1 restriction by the A3C Ile188 variant did not specifically examine the activity of this protein in a T cell model system [66, 67]. While this distinction may seem trivial, it is extremely important to validate A3 restriction activity in a T cell system. For instance, A3B potently restricts HIV-1 infection in 293T cells [125-128], but this enzyme has no discernable restriction activity in T cell lines [23]. To this end, our studies demonstrated that stable expression of the A3C Ile188 variant is indeed sufficient to slow *vif*-deficient HIV-1 replication in SupT11 T cells (which are naturally devoid of A3 expression), while the A3C Ser188 variant had comparably little impact on viral infectivity. We additionally deleted endogenous *A3C* in the non-permissive T cell line CEM2n, which normally expresses significant levels of *A3C*, *A3D*, *A3F* and *A3G*. Neither *A3C* deletion, nor subsequent complementation of *A3C* knockout cells with the

Ser188 or Ile188 variant had any impact on wildtype HIV-1 replication kinetics, and *vif*-deficient viruses were restricted regardless of A3C status (like due to other restrictive A3 enzymes). These observations in the A3C-null CEM2n derivative were consistent with prior single *A3* gene knockout studies on *A3F* and *A3G*, which resulted in similarly unchanged resistance phenotypes with respect to Vif-proficient HIV-1 replication [24], further emphasizing the level of functional redundancy in the human *A3* gene family. Taken together, these results demonstrate that the A3C Ile188 variant is capable of exerting significant HIV-1 restriction activity in T cells, and reveal an additional level of functional variation in the restrictive A3 repertoire.

While several common polymorphisms within the human *A3* gene family have clear biochemical consequences ranging from complete loss of expression (*A3B* deletion and A3H-ΔN15) to single amino acid substitutions that alter protein stability and activity (A3C, A3D, A3H), the overall impact of natural variation within the A3 enzyme family on HIV-1 replication and pathogenesis *in vivo* is not well understood (with the exception of the A3G H186R variant associated more rapid progression to AIDS [65]). Stable A3H haplotypes are disproportionately enriched in African populations, and consequently, African HIV-1 isolates more frequently encode Vif proteins that efficiently neutralize A3H [41]. Conversely, in populations where stable A3H alleles are less common, circulating HIV-1 isolates are less likely to encode Vif proteins with activity against A3H, implying that HIV-1 adapts to deal with the specific A3 repertoire of its current host [41]. These observations in population-specific host and virus genetics raise the question of whether a virus adapted to a host with a less restrictive A3 repertoire (e.g.

unstable A3H haplotypes or A3C Ser188) might be less likely to transmit into a host with more restrictive A3 enzymes (e.g. stable A3H haplotypes or A3C Ile188). *In vitro* experiments using primary T cell cultures have demonstrated that viruses encoding Vif proteins with attenuated activity against A3H replicate poorly in cells from donors with stable A3H haplotypes [41, 63], thus lending some credibility to the idea that A3 enzymes may act as potent barriers to productive infection in specific transmission situations (hypothesized originally in [41]) The existence of such transmission barriers *in vivo* has yet to be confirmed, but will likely be the subject of future studies.

Concluding Remarks

In the 15 years following their initial discovery as retrovirus restriction factors, the APOBEC3 family of DNA cytosine deaminases has continued to remain at the cutting edge of research in the area of innate immunity to viruses. While their role in the restriction of HIV-1 and other mammalian lentiviruses has become well established, several emerging lines of evidence have begun to implicate these enzymes in playing a role in the lifecycles of others viruses, including HBV [129-138], HPV [114, 139, 140], and multiple polyoma viruses [117]. Additionally, several A3 enzymes have been implicated in tumor mutagenesis and therapeutic resistance, often but not always in combination with virus infection [50, 82, 83, 112, 138, 141-146]. An better understanding of the molecular mechanisms by which diverse pathogens avoid destruction by the APOBEC3 restriction factors is likely to reveal novel targets for pharmacological intervention, and provide valuable insight into the mechanisms that regulate the cell-specific expression and antiviral activity of these enzymes. Similarly, a greater

mechanistic understanding of the natural variation within the *APOBEC3* gene repertoire will undoubtedly uncover additional genetic determinants that predispose or protect individuals from specific pathogens and malignancies. While the past decade and a half of research has offered solutions to many puzzles relating to the functions and malfunctions of these enzymes, it has also resulted an order of magnitude more new and exciting questions that currently remain unanswered. Given that, it is a virtual certainty that the next 15 years of research on the APOBEC3 enzymes will bring about even more exciting discoveries and surprises than the last.

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