HIV-1 Viral Infectivity Factor Recruitment of Cellular Protein Complexes to Counter Host Antiviral Response

By
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Human cytidine deaminase apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3) proteins (e.g. APOBEC3G) provide a potent host defense against HIV, in the absence of viral infectivity factor (Vif). These antiviral proteins induce nucleotide modifications, specifically converting cytosines to uracils in newly synthesized minus-strand viral DNA during the HIV reverse transcription process, ultimately resulting in abortive viral infection. HIV must overcome this and other host defenses to successfully replicate and spread. HIV-1 encodes the Vif protein, which binds and suppresses the antiviral effects of select APOBEC3 proteins by targeting them for degradation through the 26S proteasome enabling the virus to carry out a productive infection.

Our lab was the first to demonstrate that Vif recruits cellular Cullin5 (Cul5), Elongin B (Elo B), and Elongin C (Elo C) to form a viral E3 ubiquitin ligase to target APOBEC3 proteins for polyubiquitination and degradation. Recently, we reported the direct interaction between Vif and a previously unknown binding partner and regulator of Vif-induced APOBEC3 degradation, core binding factor beta (CBF-β).

This dissertation reports on the functional importance of the Vif:CBF-β interaction and characterizes this interaction using fluorescence imaging and functional assays in mammalian cells as well as in vitro over-expressed protein complex binding studies (Chapter 3). Next, the E3 ligase components that bind Vif have
long been thought to require residues in Vif’s C-terminus, exclusively. However, this report provides evidence that the Vif:Cul5 interaction also requires a motif in Vif’s N-terminus (Chapter 4). Recognizing the importance for these multiple points of interaction between Vif and the E3 ligase, a rapid in vitro fluorescence based inhibitor assay was developed to identify small-molecule inhibitors of the Vif:CBF-β and Vif:Cul5 interaction with the ultimate goal of developing novel anti-HIV therapeutic compounds (Chapter 5). Finally, this report discusses a novel role for Vif as a transcriptional enhancer when in complex with the heterodimeric transcription factor RUNX:CBF-β. The Vif:RUNX:CBF-β complex appears to up-regulate cytokines that may facilitate HIV replication (Chapter 6). The critical role that Vif plays during HIV infection demonstrates its potential utility as a novel HIV therapeutic target.

Advisor: Xiao-Fang Yu
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COMMON ABBREVIATIONS

HIV-1: Human immunodeficiency virus type 1; Vif: Viral infectivity factor; APOBEC3: apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3; Cul5: Cullin 5; Elo B/C: Elongin B/C; CBF-β: Core binding factor beta; RBX2: RING box protein 2; E2: ubiquitin conjugating enzyme; E3: ubiquitin ligase; HCCH: Vif zinc binding motif, $^{108}\text{Hx}_5-\text{Cx}_{17-18}-\text{Cx}_{3-5}-\text{H}^{139}$; SOCS Box: Suppressor of Cytokine Signaling box; B/C Box: Elongin B/C binding domain; FL: Full length; N: N-terminal protein domain; PBS: Phosphate-buffered saline, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
CHAPTER 1

INTRODUCTION TO RETROVIRAL RESTRICTION FACTORS AND HIV COUNTER MEASURES

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General Introduction

Human Immunodeficiency Virus (HIV) is a human viral pathogen that has had major global health and socio-economic impacts, particularly in the developing world[1]. Untreated HIV infection leads to the depletion of host helper CD4+ T-cells over time, loss of cell-mediated immunity and subsequent failure of the host immune system[2]. The dysfunction of the immune response leaves the human host vulnerable to a variety of life-threatening infections and cancers[2].

According to the most recent UNAIDS global AIDS report, an estimated 35.3 million people were living with HIV in 2012[1]. This estimate has increased from previous years due to a global increase in access to life-saving antiretroviral therapy[1]. Additionally, there were 2.3 million new HIV infections globally, demonstrating a 33% decline in the number of new infections from 3.4 million in 2001[1]. The number of AIDS deaths has also declined with 1.6 million AIDS deaths in 2012, down from 2.3 million in 2005[1]. Since the first clinical observation of the disorder in 1981[3] and subsequent reports describing the isolation of HIV in 1983[4], the scientific community has made many attempts to develop a preventive vaccine and curative treatment[5, 6]. Unfortunately, more than 30 years since its discovery, the virus continues to evade all therapeutic attempts at eliminating it completely. Yet, there have been a few recent and extraordinary cases of adult and child patients cured of HIV[7, 8], giving the medical community hope for eventually finding a cure for all patients. In addition,
the current combination anti-retroviral therapy regimen, highly active antiretroviral therapy (HAART), has been extremely effective at lowering viral loads to undetectable levels, substantially decreasing the number of new AIDS diagnoses as well as HIV new infections[1]. However, HAART does not completely eliminate the virus or restore health, contributing to novel sequelae referred to as non-AIDS morbidity[9, 10]. In addition, there are concerns that the current lines of treatment may be less effective in the future due to the high incidence of mutation-induced resistance[11-13]. In fact, it was previously estimated that over 75% of HIV-infected individuals in the United States carry viruses, which are resistant to one or more antiviral drugs[14]. The virus undergoes an error prone reverse transcription step during its life cycle, which contributes to a high mutation rate of the HIV-1 genome[15]. Combined with a high rate of HIV replication[16], a continued evolution of viral sub-species occurs leading to the development of drug resistant mutants. In order to develop new therapies that would potentially replace existing ones and ideally eliminate the virus completely from the host, additional basic scientific research is required to understand the detailed mechanism of how HIV infects, replicates, and evades both the immune system and current lines of therapeutic intervention. Our lab has contributed to this research priority by focusing our efforts on how select HIV accessory proteins counteract host restriction measures.
HIV Classification & Biology

HIV belongs to the family *Retroviridae* and the genus *Lentivirus*. The Latin prefix *lenti-* describes the viruses long clinical latency period between initial infection and progression to disease. HIV is classified into two major types, HIV type 1 (HIV-1) and HIV type 2 (HIV-2); each type is further divided into several groups and further into subtypes. HIV-1, group M is the more common and virulent form that is largely responsible for the global epidemic. HIV-2 is largely confined to parts of West Africa, due primarily to its poor capacity to transmit between hosts. Many mammalian species can be infected with related *lentiviruses* including: simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). This group of viruses contains two (2) copies of a single-stranded, positive sense RNA genome molecule enclosed in an enveloped icosahedral capsid-containing particle.

*Retroviridae* viruses belong to a family that includes a group of eukaryotic retro-transposable elements and differ from other families of virus that utilize their RNA genome for immediate viral replication. Instead, after infection, retroviruses reverse transcribe their RNA genomes into DNA intermediates by using the genome-encoded and virion-incorporated reverse transcriptase enzyme[17, 18]. The resulting proviral DNA genome is subsequently integrated into the host cell chromosome using another viral protein, integrase[19]. All retroviral genomes encode at least three key genes that comprise the core, structural, enzymatic, and surface components of the viral particle: group specific antigen (gag), polymerase (pol), and envelope (env). While retroviruses share genetic
similarities, there are differences among encoded small regulatory genes. In addition to the key retroviral genes, the HIV genome encodes six small non-structural/non-enzymatic genes that code for the following viral proteins: Trans-activator of transcription (Tat), Regulator of expression of virion proteins (Rev), Virion infectivity factor (Vif), Viral protein R (Vpr), Negative factor (Nef), and Viral protein U (Vpu) (HIV-1 encoded) or Viral protein X (Vpx) (HIV-2 encoded paralog of Vpr) (Table 1-1 and Figure 1-1). In addition, the HIV-1 Nef open reading frame does not overlap with Env, whereas HIV-2 Nef maintains an overlap with the C-terminal portion of Env. With the exception of regulatory proteins, Rev and Tat, the other proteins, termed accessory proteins, are not essential for HIV replication in certain in vitro cell types[20]. However, the accessory proteins are important for the pathogenesis, replication and spread of HIV during natural host infection[20].
<table>
<thead>
<tr>
<th><strong>HIV-1 Proteins</strong></th>
<th><strong>Type</strong></th>
<th><strong>Name and amino acid number</strong></th>
<th><strong>Function</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural</strong></td>
<td>Matrix, p17 (gag) (132 aa)</td>
<td>Targets Gag and Gag-Pol polyproteins to plasma membrane</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Capsid, p24 (gag) (231 aa)</td>
<td>Forms core of viral particle</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nucleocapsid, p7 (gag) (55 aa)</td>
<td>Binds single-stranded nucleic acids non-specifically protecting it from nucleases</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p6 (gag) (52 aa)</td>
<td>Mediates incorporation of Vpr during viral assembly</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Surface, gp120 (env) (483 aa)</td>
<td>Binds CD4 leading to Env structural changes facilitating co-receptor binding/viral entry</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transmembrane, gp41 (env) (345 aa)</td>
<td>Mediates fusion between host and viral membranes following receptor binding</td>
<td></td>
</tr>
<tr>
<td><strong>Enzymatic</strong></td>
<td>Protease (pol) (99 aa)</td>
<td>Cleaves polyproteins, gag and gag-pol to make an infectious particle</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse Transcriptase, p51 (pol) (440 aa)</td>
<td>Reverse transcription of the HIV genome</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Integrase (pol) (288 aa)</td>
<td>Removes two 3’ nucleotides from each strand of linear viral DNA, leaving CA-OH ends to facilitate HIV genome integration into host genome</td>
<td></td>
</tr>
<tr>
<td><strong>Accessory/regulatory</strong></td>
<td>Vif, p23 (192 aa)</td>
<td>Recruits host E3 ligase to degrade APOBEC3 proteins and prevent viral genome hypermutation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vpr, p15 (96 aa)</td>
<td>Transports viral genome from cytoplasm into nucleus, induces G2 arrest</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vpu, p16 (82 aa)</td>
<td>Promotes CD4 degradation when complexed with gp160 in E.R.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nef, p27 (206 aa)</td>
<td>Promotes CD4 and MHCI downregulation from the surface</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tat, p14 (86 aa)</td>
<td>Enhances transcription of proviral DNA at promoter by binding an RNA hairpin, transactivating response element</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev, p19 (116 aa)</td>
<td>Prevents viral mRNA splicing binding to RRE site located in env region</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-1. List of HIV-1 proteins (post-protease processing and their functions.)
**Figure 1-1. Genomic organization of primate lentiviruses.**

HIV-1/HIV-2/SIV genomes all encode *gag*, *pol*, and *env* genes. In addition, each lineage encodes a unique set of accessory genes. HIV-1, SIV<sub>cpz</sub>, and related viruses encode the *vpu* gene while HIV-2 and related viral lineages encode a unique *vpx* gene. SIV<sub>agm</sub> and related viruses lack both *vpx* and *vpu* genes.
HIV Transmission & Replication

Years of scientific research have led to a detailed understanding of how HIV is spread, its lifecycle within the host (Figure 1-2), and how the virus evades the immune system. Although current lines of treatment are very effective at reducing spread, HIV transmission prevention remains a global public health problem[1, 21]. Between 2001 and 2012, the estimated incidence of HIV decreased by 50% in 26 developing nations, yet 2.5 million people still became newly infected in 2011[1]. HIV spreads among humans using three major routes: across mucosal surfaces during sexual contact, mother-to-child transmission during pregnancy/delivery and breastfeeding, as well as percutaneous inoculation (e.g. intravenous drug use)[22]. Shortly after mucosal exposure, a very small number of transmitted/founder (T/F) viruses, viruses that initiate new host primary infection, establish an initial infection by attaching to and entering a target cell (i.e. CD4+ T-cells)[23]. The viral surface protein, GP120 requires an interaction with a host cell receptor, CD4[24], and co-receptor[25, 26], often CCR5[23], in order to attach and fuse with the host target cell. HIV transmission efficiency relies on a number of variables including: the inoculum concentration from the infected person (i.e. higher virus levels increase probability of infection), susceptibility factors of the exposed person (e.g. genital tract inflammation in uninfected individual and circumcision), and the virulence/hardiness of the founder virus[22].
HIV-1 entry occurs via fusion of the virus and host cell membrane, which is mediated by the viral gp41 trans-membrane protein[27]. Upon entry, the viral core is released into the cytoplasm followed by reverse transcription and the “uncoating” of the viral genetic material from the capsid. Following reverse transcription of the HIV RNA genome, the HIV pre-integration complex (PIC), a nucleoprotein complex consisting of the proviral DNA genome product, viral proteins (i.e. integrase, matrix, and Vpr), and host proteins is actively imported into the nucleus[28]. Importantly, nuclear import of HIV and other lentiviruses does not require nuclear envelope disruption[29]. Thus, HIV is free to infect non-dividing cells[30]. Nuclear import of the PIC is followed by the integration of proviral DNA into the host cell chromosome, mediated primarily by integrase[19]. Viral full length and truncated mRNAs are subsequently transcribed from the HIV long terminal repeat (LTR) promoter, processed and transported into the cytosol for translation into retroviral proteins. Soon after viral protein synthesis, viral structural proteins assemble at the plasma membrane in order to form budding particles. Upon budding from a producer cell, the virus finds a target cell to infect. After the primary host infection takes place, viral evolution leads to tropic variants that can use either CCR5 or CXCR4 co-receptors for subsequent infection of host CD4+ T-cells, macrophages or dendritic cells.

Depending on viral and host properties, progression from HIV to AIDS can be distinguished into three groups. Rapid progressors (RP) are individuals who progress to AIDS within 3-5 years after initial infection and who demonstrate a
rapid decline of CD4+ T-cell counts concurrently with increasing plasma viremia. Slow progressors (SP) progress over 10-15 years with declining CD4+ T-cells and increasing plasma viremia during that time. Long-term non-progressors (LTNP), can be infected for more than 20 years while maintaining >500 CD4+ T-cells/ml blood and low viral loads without using anti-retroviral drugs[31]. A subset of LTNPs called HIV (or elite) controllers is able to control viremia to undetectable levels [32]. An explanation for how these individuals effectively control viral levels could prove essential for developing an effective vaccine.
Figure 1-2. HIV-1 life cycle.

HIV-1 entry is mediated by binding to cell surface CD4 and co-receptors followed by virion-cell fusion. Virion uncoating occurs and reverse transcription proceeds in the cytoplasm. After provirus integration, viral transcription and translation lead to synthesis of viral proteins. Virion particles are assembled and bud from the plasma membrane. Source: National Institute of Allergy and Infectious Disease.
Host Immune Response to HIV Infection

The human immune system is a complex system that is divided into two primary response arms: the innate and adaptive immune responses. In addition, a distinct component of the immune system, termed intrinsic antiviral immunity, is comprised of host factors that directly act on viral pathogens to decrease replication[33]. The host innate immune response uses physical barriers and antigen-nonspecific defense mechanisms immediately or within several hours after microbe exposure in order to eliminate the invading organism and prevent infection. This initial host response uses germline-encoded pattern recognition receptors to mediate pathogen sensing of carbohydrate, lipid, and protein structures unique to the pathogen, to instruct the adaptive immune response. The adaptive immune response is the learned recognition of antigen-specific structures on pathogens that can lead to a future quicker and more effective response to subsequent infections by the same pathogen.

The innate immune system can be further subdivided into individual components: anatomical barriers and inflammatory response/component system. The skin and mucosal membranes are the first lines of defense against many external pathogens. The primary transmission route for HIV continues to be via virus exposure to mucosal surfaces during sexual contact[22]. During sexual intercourse, contaminated bodily fluids are exchanged between uninfected and infected partners[22]. The mucosa epithelial lining, vaginal pH, and mucosal secretions provide a semi-effective barrier against HIV infection, but the
effectiveness depends on the site, structural integrity, and inflammatory state of the membrane[34]. Beneath the layer(s) of mucosal lining are a variety of immune cells that are responsible for protecting the lining from invading microbes[22]. HIV can infect a subset of these immune cells. While the vaginal mucosa contains several layers of tissue, the mucosa lining the anus and rectum is only one cell thick and, thus more susceptible to HIV infection[22]. Moreover, if the mucosal lining is damaged prior to or during sexual intercourse, the risk of transmission increases[22].

Prior to responding, the innate system must sense the invading pathogen using receptors that bind pathogen-associated molecular patterns (PAMPs)[35]. Multiple innate immune cell types exist to combat invading microbes non-specifically by utilizing pattern recognition receptors (PRR)[35]. These cells include phagocytes (e.g. monocytes and macrophages) primed for antigen clearance, professional antigen-presenting cells (e.g. dendritic cells) aimed at capturing and presenting foreign material to adaptive immune cells for the induction of immunological memory, and cytolytic cells (e.g. natural killer cells and neutrophils) which target pathogen and infected cells for destruction[35]. Three classes of PRRs have been discovered in mammalian organisms: toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I)-like receptors (RLR), and nucleotide oligomerization domain (NOD)-like receptors (NLR)[35]. Activation of these receptors result in the induction of distinct responses that leads to the
release of a variety of cytokines, which create an unfavorable environment for the invading virus[35].

Similar to other single-stranded RNA viruses, HIV triggers innate immune receptors, particularly TLRs[35]. TLRs are type I transmembrane proteins that function as homodimers or heterodimers. Currently, there are 10 known functional human TLRs and upon stimulation TLRs activate signal transduction pathways, which induce dendritic cell maturation and cytokine production. TLR7 and TLR8 are triggers of HIV infection and result in the potent activation of dendritic cells and the release of type 1 interferons (IFN-α and IFN-β), tumor necrosis factor alpha (TNF-a), and interleukin 15 (IL-15)[35]. These cytokines play key roles in shutting down viral replication in infected cells, while also promoting the activation of the immune response[35]. Interestingly, HIV-1 infection elicits a more pronounced early inflammatory response compared to related pathogens, hepatitis B and C[35]. This observation has led some to speculate that this enhanced response may contribute to the immunopathology associated with early HIV infection[35]. While dendritic cells and other immune cells have been observed to be involved in the response against HIV, epidemiological studies have pointed to a central role for natural killer cells in containing the infection[35].

HIV infects CD4+ T-cells, one of the most important cell types that regulate the adaptive immune response[24]. CD4+ T-cells play multiple roles during viral
infection[34]. Thus, their dysregulation during HIV infection makes it possible for the virus to successfully overcome host anti-viral responses. A subset of CD4+ T-cells, helper CD4+ T-cells, are responsible for activating and maintaining both CD8+ T-cells and antibody-producing B-cells. Upon activation, these cells produce a variety of cytokines including: interleukin-2 (IL-2), interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α). A reduction in certain cytokines during HIV infection is indicative of the subsequent exhaustion of T-cells, where the number of T-cells produced to replace dying cells is reduced leading to a net loss in overall T-cells[34]. Furthermore, the dysfunctional response contributes to the impaired function of CD8+ T-cells[34]. Normally, CD8+ T-cells are responsible for killing infected cells in the body. During acute HIV infection, CD8+ T-cells are able to perform this task; however, as CD4+ T-cell function declines, CD8+ T-cells are unable to effectively control infected cells[34]. Interestingly, the immune systems of LTNPs are capable of maintaining CD4+ T-cell and CD8+ T-cell function[36]. Specifically, LTNP CD4+ T-cells may express and secrete more IL-2, IFN-γ, IL-7 and CCR7 compared to RP[36]. Thus, the long-term maintenance of immune memory in LTNPs may be due to sufficient IL-2 extracellular and CCR7 surface expression levels, which maintains the self-renewal and promotes circulation of CD4+ memory cells[36]. CD8+ T-cells in LTNPs have been shown to divide more efficiently during infection and have more effective function against HIV-specific antigens compared to RP CD8+ T-cells[36]. In addition, like CD4+ T-cells, LTNP CD8+ T-cells produce more IL-2 and IFN-γ compared to RP[36].
Intrinsic antiviral immunity is a special category because it combines concepts from both traditional branches of the immune system (i.e. adaptive and innate immunity)[33]. The innate immune system senses pathogens using PRRs, which sense PAMPS. This sensor triggers the expression of a variety of non-specific antimicrobial proteins. An adaptive immune response is later developed to target a specific invading microbe. On the other hand, the intrinsic antiviral immune system recognizes a specific virus or group of viruses and attenuates virus replication directly[33]. Similar to innate immunity, intrinsic immunity does not react any differently upon re-infection by the same virus[33]. Moreover, like adaptive immunity, intrinsic immunity targets a specific type or class of pathogens[33]. In contrast to the adaptive immune response, which involves somatic learning and memory, host intrinsic immunity factors are germline-encoded[33]. Importantly, proteins or restriction factors that make up the intrinsic immune system are constitutively expressed, although these factors can be further induced by viral infection[33]. Because HIV integrates its genome into the host genome, a system that can act quickly to shut down viral infection, following viral entry is critical to disrupting the lifecycle and limiting HIV replication. Intrinsic immunity is mediated by host factors with important roles in restricting HIV-1 replication, such as APOBEC3 family proteins, TRIM5α, Tetherin, and SAMDH1[33]. These restriction factors are also regulated by the type-1 interferon induced during innate PRR signaling[33]. HIV-1 counteracts some of these
restriction factors via accessory proteins and avoids up-regulation of the restriction factors by inhibiting interferon signaling pathways[33].
Host Intrinsic Restriction to Retroviruses and Viral Antagonism

As discussed above, HIV-1 replication can be immediately controlled in infected host cells by constitutively expressed intrinsic immune factors known collectively as ‘restriction factors’[33]. Several host restriction factors have been identified that combat the invasion of HIV-1 and other retroviruses[33]. Many of these proteins are further up-regulated upon interferon stimulation as part of an ‘antiviral state’ switch in the cell[33]. While many of these proteins have been discovered as factors that restrict invading microbes, it is likely that the role of these restriction factors extends beyond controlling foreign pathogens. Most, if not all, likely also have regulatory functions in uninfected cells to control endogenous mobile retroelements. Nonetheless, host restriction factors of retroviruses have been one of the most well-studied model systems (Table 1-2). Tripartite motif-containing protein 5α (TRIM5α), Tetherin/BST-2, and SAM domain and HD domain-containing protein 1 (SAMHD1), constitute specific restriction factors that prevent lentivirus cross-transmission among primates of different species[33]. On the other hand, some restriction factors aim to limit infection from species-specific retroviruses including: apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3) proteins[33].
<table>
<thead>
<tr>
<th>HIV-1 Host Restriction Factor</th>
<th>Function</th>
<th>Counteracting Viral Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOBE3G/F[37, 38]</td>
<td>Introduces G to A mutation and interferes with reverse transcription</td>
<td>Vif</td>
</tr>
<tr>
<td>TRIM5α[39]</td>
<td>Binds viral capsid and prevents viral uncoating as well as infection at or before reverse transcription; innate immune sensing of retroviral infection</td>
<td>Human TRIM5 is ineffective against HIV-1</td>
</tr>
<tr>
<td>Tetherin/BST2[40]</td>
<td>Restricts release of virions from the cell surface</td>
<td>HIV-1 Vpu, HIV-2 Env, SIV Nef</td>
</tr>
<tr>
<td>SAMHD1[41, 42]</td>
<td>Reduces dNTP pool required for cDNA synthesis</td>
<td>HIV-2/SIV&lt;sub&gt;mac&lt;/sub&gt; Vpx</td>
</tr>
<tr>
<td>MX2/MXB[43, 44]</td>
<td>Inhibits capsid-dependent nuclear import of viral protein-RNA complexes</td>
<td>unknown</td>
</tr>
</tbody>
</table>

Table 1-2. List of HIV-1 host restriction factors and viral counter proteins
FRIEND VIRUS SUSCEPTIBILITY (Fv)

One of the first restriction factors to be studied was discovered in mice, Friend virus susceptibility 1 (Fv1)[45]. This factor, along with a closely related factor (Fv4) controls the replication of Friend murine leukemia virus (F-MLV), a retrovirus that causes erythro-leukemia in susceptible strains of mice, as well as other murine leukemia viruses[46]. There are two primary alleles of the Fv1 gene, Fv1\textsuperscript{b} (in Balb/c mice) and Fv1\textsuperscript{n} (in NIH/swiss mice) that are distinguished by their ability to block infection by different strains of MLV[47]. N-tropic viruses replicate efficiently in Fv1\textsuperscript{n} mice, but inefficiently in Fv1\textsuperscript{b} mice[47]. Conversely, B-tropic viruses replicate efficiently in Fv1\textsuperscript{b} mice but not in Fv1\textsuperscript{n} mice[47]. Crossing mice with both Fv1\textsuperscript{n} and Fv1\textsuperscript{b} backgrounds confers offspring resistance to infection by N- or B-tropic viruses[47]. One amino acid (residue 110) in the capsid (CA) confers MLV tropism[48]. While the detailed mechanism of restriction is still unclear, Fv1 binds to the MLV-CA and is thought to restrict the pre-integration complex (PIC) from entering the nucleus[47].

Ecotropic MLVs interact with the cell surface receptor, murine cationic amino acid transporter (mCAT) to infect mice. Originally identified in Japanese wild mice, Fv4 is an MLV envelope protein mimic encoded in the genome of an endogenous MLV provirus and contributes to the restriction of ecotropic MLV[46]. Fv4 blocks virus entry by competing for cell surface receptor binding sites and confers very efficient resistance to infection[46].
TRIM5α and TRIMCYP

Interestingly, several human cell lines can restrict N-tropic MLV, yet do not express an ortholog to Fv1β. A factor previously named restriction factor 1 (Ref1) was determined to be responsible for the restriction of N-tropic MLV in human cells[39, 49]. Furthermore, non-human primates expressed a factor that restricted HIV-1 called lentivirus restriction factor 1 (Lv1)[49]. Importantly, both Ref1 and Lv1 were dominant protective factors that could be conferred to permissive cells by introducing their expression[49]. In 2004, Ref1 and Lv1 were determined to both be species-specific variants of TRIM5α[39, 49]. TRIM5α is a member of the tripartite-motif (TRIM) family that shares a common organization at the amino terminus, which contains a RING domain, a B-box domain and a coiled-coil domain as well as a C-terminal PRY-SPRY (B30.2) domain[33, 39].

All the domains of TRIM5α are required for retroviral restriction activity[50]. The B30.2/SPRY domain determines the specificity of capsid recognition and is known to bind to the viral capsid during the post viral entry phase and inhibit viral replication by inducing premature uncoating, causing impaired reverse transcription and nuclear import[50]. Interestingly, as a result of B30.2 domain capsid recognition specificity, human TRIM5α potently restricts MLV, yet does not inhibit HIV or simian immunodeficiency virus (SIV) from rhesus macaques (SIVmac); however, TRIM5α from rhesus macaques counters HIV but not SIV-mac[50]. Amino acid differences within the PRY-SPRY domain account for the
species-specific restriction profile of Trim5α[50]. In fact, a single amino acid change within the PRY-SPRY domain allows human Trim5α to restrict HIV-1[50].

In addition to blocking post-entry steps, TRIM5α also regulates signal transduction processes, which can promote innate immune signaling to respond to infection[50]. TRIM5α can activate AP-1 and NF-κB in HEK293T cells, but not type I interferons, by promoting the K-63 linked ubiquitination and activation of the kinase TAK1[50]. Furthermore, the B-box and coiled-coil domains are important for the high-order multimerization and low-order dimerization of Trim5α, both necessary for restriction[50].

Owl monkeys as well as several macaque species encode a TRIM5α variant called TRIMCyp, in which the TRIM5α B30.2/SPRY domain is replaced by cyclophilin-A due to alternative mRNA splicing[33, 50]. TRIM-Cyp also has restrictive activity against some retroviruses determined by cyclophilin A binding specificity[33, 50]. This example of convergent evolution surprisingly occurred independently at least twice in primate evolutionary history. In owl monkeys, TRIMCyp arose from a direct long interspersed element-1 (LINE-1) mediated retrotransposition of cyclophilin A into intron 7 of TRIM5[51]. Yet, in select macaques, cyclophilin A was introduced into the 3' UTR of TRIM5 along with a novel splice acceptor mutation, thus leading to TRIMCyp expression[51]. As with TRIM5α, minimal amino acid residue changes in the CypA domain allow rhesus
macaque TRIMCyp to restrict HIV-1[51]. Thus, species-specific viral recognition by Trim5α and TRIMCyp is dictated by a limited number of residues in the C-terminal domain[50].

**TETHERIN/BST-2**

Tetherin/BST-2/CD317/HM1.24 is a host restriction factor capable of inhibiting the release of a broad range of viruses from the plasma membrane of infected cells and is highly expressed in plasmacytoid dendritic cells and differentiated B-cells[40]. In addition to inhibiting the release of HIV particles, tetherin inhibits the release of filoviruses (e.g. Ebola and Marburg viruses), arenaviruses (e.g. Lassa virus), and other retroviruses including gammaretroviruses (e.g. MLV) and spumaretroviruses (foamy virus)[47, 52]. While cells that constitutively express high levels of tetherin (e.g. HeLa) are restrictive to viral budding, other cells can express low levels of tetherin (e.g. HEK293T) and permit budding[47]. However, tetherin can be upregulated by type I IFN[47, 52]. Thus, IFN-1 treatment reduces the permissiveness of HEK 293T cells to HIV-1 and Ebola virus, partly resulting from tetherin up-regulation[47, 52].

Tetherin is a type II single pass transmembrane protein divided into an amino-terminal cytoplasmic domain; a single pass transmembrane helix domain; an extracellular, predominantly alpha helical domain; and a carboxy-terminal glycosylphosphatidylinositol (GPI) membrane anchor[47]. Molecular structure studies have determined that tetherin forms a tetrameric structure via two coiled
coil-mediated parallel dimers[47]. In addition, the structure contains an anti-parallel four-helix bundle configuration, stabilized by three disulfide bridges, and allows for flexibility[47]. This flexibility may lend itself to interactions with other host proteins or viral proteins in order to fulfill its function[47, 52]. Studies support a mechanism whereby tetherin restricts viruses by “tethering” virions at the cell surface via insertion of the GPI anchor into the virion envelope or by dimerization of two tetherin molecules, one anchored at the host cell membrane and one anchored at the virion envelope[47, 52]. In order to promote endocytosis, the cytoplasmic domain binds to clathrin adaptors[47, 52]. Tethered virions are internalized via tetherin-clathrin mediated endocytosis and subsequently degraded within the endosome[47, 52]. It is the unique topology, and not the amino acid residues, that is responsible for tetherin’s function to tether virions to the cell surface[47, 52].

To inhibit this restriction, retroviruses have developed at least three countermeasures. The HIV-1 genome encodes the Vpu accessory protein, which directly binds to tetherin via their transmembrane domains to prevent the virion retention phenotype[40]. Vpu is a single pass type-I membrane protein containing an amino-terminal transmembrane domain and a carboxy-terminal cytoplasmic tail domain that is required for recruitment of the host cellular β-TrCP subunit of the Skp1-Cullin1-F-box ubiquitin ligase complex[47]. The Vpu-recruited ligase mediates the ubiquitination of tetherin at the lysine and serine/threonine residues in the cytoplasmic tail domain and subsequent lysosomal degradation[47].
Lysosomal degradation is mediated by hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), a component of the Endosomal Sorting Complexes Required for Transport (ESCRT-0) complex[47, 52].

In addition, SIV sooty mangabey (SIVsmm) and SIVmac both express a Nef protein that has the ability to counteract tetherin, displaying species specificity in their ability to counteract the host tetherin[47, 52]. Nef binds to the cytoplasmic tail domain of tetherin via its C-terminal domain and recruits the AP-2 clathrin adaptor complex in order to mediate internalization of cell-surface tetherin[47, 52]. Interestingly, the cytoplasmic tail of tetherin, which has been evolving under positive selection in primates, contains a motif necessary for SIVmac Nef interaction that is deleted in humans[47, 52]. Thus, human tetherin is resistant to Nef-mediated down-regulation[47, 52].

Finally, HIV-2 as well as SIVmac envelope both counter tetherin to promote virion particle release. Similar to SIV Nef and HIV-1 Vpu, HIV-2 and SIVmac Env proteins promote the cell surface down-regulation of tetherin[47, 52]. While the Env proteins promote the intracellular sequestration of tetherin, they do not mediate degradation of tetherin[47, 52]. The detailed mechanism that Env uses to antagonize tetherin remains unclear; however, reports suggest that the gp41 cytoplasmic tail of Env is required[47, 52]. In addition to retroviruses, tetherin can also be antagonized by other viral family proteins including: the Kaposi sarcoma-
associated herpesvirus (KSHV) K5 protein, which promotes the down-regulation and endosomal degradation of cell surface tetherin through the ESCRT-mediated pathway; the Ebola virus full-length glycoprotein (GP) can counter tetherin independent of protein down-regulation or surface expression and has the ability to antagonize multiple primate species’ tetherin[47, 52]. Given its broad anti-viral activity, other viruses have likely evolved novel mechanisms to inhibit tetherin. Indeed, anti-tetherin defense systems have been discovered to exist among a diverse group of viruses, which emphasizes the importance of tetherin to the immune system and the strong viral selective pressure it has imposed by its potent restriction[47, 52].

**SAMHD1**

SAMHD1 is a dGTP-activated deoxynucleoside triphosphate triphosphohydrolase (dNTPase)[42]. The host factor’s dNTPase activity has been linked to HIV/SIV restriction[42]. In addition, the restriction factor has been reported to inhibit retrotransposition of endogenous retroelement, LINE-1[53]. Recent structural data support a mechanism of dGTP-dependent tetramer formation, which requires the cooperation of three subunits and two dGTP/dATP molecules at each allosteric site[54]. SAMHD1 is highly expressed in myeloid cells where it reduces cellular deoxynucleotide pool levels[41, 42]. Thus, retrovirus reverse transcription is hampered due to the limited deoxynucleotide substrate availability[42]. Humans with missense mutations in SAMHD1 are associated with Aicardi Goutieres Syndrome (AGS), an autoimmune encephalitis disorder that mimics a state of viral infection leading to interferon production[55].
Due to the SAMHD1 gene mutations, AGS patients are prone to increased endogenous reverse transcription activity due to the reduced availability of deoxynucleotide pools[55].

In 2011, the accessory protein Vpx, not encoded in the HIV-1 genome, was demonstrated to be required for inhibiting SAMHD1 restrictive function allowing primate lentiviruses to efficiently infect monocytes, dendritic cells, and mature macrophages[41]. Vpx binds to SAMHD1 and mediates its degradation by the proteasome, thus relieving the suppression of deoxynucleotide pools and promoting viral reverse transcription[41]. Despite this important function, only two of the eight major lineages of primate lentiviruses encode Vpx: HIV-2/SIV<sub>sm</sub>/SIV red-capped mangabeys (SIV<sub>rcm</sub>). A paralogous accessory gene called Vpr, which causes cell cycle arrest, is encoded in all extant primate lentiviruses. Both Vpr and Vpx are incorporated in the virion core and bind to the Cul4 E3 ligase complex through interactions with DCAF1[56].

**MX2/MXB**

Within the past few months, yet another HIV-1 restriction factor was discovered that highly restricts infection in monocytic derived cells as well as T-cells[43, 44]. Myxovirus resistance 2 (MX2 also known as MXB) is an IFN-induced effector molecule and was reported to be a potent anti-HIV-1 restrictive factor[43, 44]. HIV-1 capsid is most likely the target of MX2 because modification of the HIV-1 CA can control HIV susceptibility to MX2[43, 44]. Furthermore, the block to
infection occurs at a late post-entry step, with both the nuclear accumulation and chromosomal integration of nascent viral complementary DNA suppressed[43, 44]. While MX2 was effective against all HIV-1 strains as well as SIV mandrill (SIV\textsubscript{man}), it was less effective against HIV-2, SIV\textsubscript{mac}, and SIV\textsubscript{agm}[43, 44]. The new factor also does not inhibit other retroviruses such as murine leukaemia virus or the orthomyxovirus influenza virus[43, 44]. Interestingly, human MX1 (also known as MXA), a closely related protein that has long been recognized as a broadly acting inhibitor of RNA and DNA viruses, including influenza virus, does not affect HIV-1[43, 44].

APOBEC3

One of the most well-studied restriction factors are members of a family of proteins known as APOBEC3[57]. APOBEC3G is the best-characterized APOBEC3 family member that functions to block the replication of a broad number of endogenous mobile elements and exogenous viral pathogens[57]. APOBEC3G as well as other APOBEC3 proteins can restrict HIV by deaminating viral cDNA cytosines to uracils causing detrimental genomic mutation that lead to an abortive infection[58]. The HIV-1 genome encodes the Vif protein to antagonize the restrictive function of APOBEC3 proteins that have anti-HIV activity[59]. Vif recruits a cellular E3 ubiquitin ligase similar to Vpx and Vpr in order to mediate APOBEC3 degradation[60]. In addition, multiple reports have suggested other mechanisms that Vif may use to circumvent the antiviral activities of the APOBEC3 proteins by several mechanisms including: 1) inhibition of APOBEC3G mRNA translation[61] and 2) promoting the formation of
high molecular weight APOBEC3 complexes[62]. My graduate thesis has focused on further understanding this restriction factor and HIV’s strategy to defend itself by recruiting a host E3 ubiquitin ligase. Prior to going into more detail about the Vif:APOBEC3 relationship, it is important to review what is currently known about cellular E3 ligases. I will conclude the Introduction with additional information about our current understanding of APOBEC3, Vif, and their association during HIV-1 infection of the cell.
CELLULAR E3 UBIQUITIN LIGASES

Ubiquitination is a process, which covalently attaches the 76 amino acid long ubiquitin protein chains to target proteins in order to regulate many cellular processes. The ubiquitination process consists of three primary steps. First, an ubiquitin-activating enzyme, E1, binds and activates ubiquitin in an ATP-dependent manner to transfer it to an ubiquitin-conjugating enzyme, E2[63, 64]. Next, E2 transfers ubiquitin to an ubiquitin ligase, E3[63, 64]. Finally, the E3 ligase mediates the covalent attachment of ubiquitin through its C-terminal glycine residue to a lysine residue on the target protein[63, 64]. In addition to the specific E3 ligase complex utilized, the regulatory effect of ubiquitination depends on ubiquitin chain length and which lysine (out of 7 total lysine residues) is used to covalently attach subsequent ubiquitins. For example, Lys 48-linked chains that are at least four subunits in length usually mark a protein for proteasomal degradation by the 26S proteasome, while Lys 63-linked chains can regulate endocytic trafficking of a protein[63, 64]. In addition, ubiquitination can mediate protein-protein interactions, cellular localization, DNA repair, and other cellular processes[63, 64].

Two distinct classes of E3 ubiquitin ligases are known to date: Homologous to E6-AP C-Terminus (HECT) and the RING (Really Interesting New Gene) finger families[64]. The RING finger E3 ligase family directly mediates ubiquitin transfer from the E2 to the substrate, while HECT family ligases form a covalent thio-
ester linkage intermediate with ubiquitin before transferring it to the target protein[64]. The RING finger E3 ubiquitin ligases act as a scaffold to orient the E2 conjugated with ubiquitin and the substrate such that the ubiquitin is efficiently transferred to the substrate[64]. The cullin-RING ubiquitin ligases (CRLs) are an example of the RING family E3 ligases[64]. CRLs assemble a multi-component protein complex composed of a cullin family member (e.g. Cul4), a RING finger protein (e.g. Rbx), an adaptor protein (e.g. DCAF1), and a substrate receptor (e.g. Vpx)[64]. As a complex, the CRL complex bridges the E2 with a substrate molecule (e.g. SAMHD1)[64].

Cullin proteins act as a scaffold that recruits the additional components of an E3 ligase complex as well as the E2 enzyme and target substrate[64]. Cullin proteins adopt an elongated conformation in which the N-terminus recruits the substrate receptor and adaptor proteins, while the RING protein binds at its C-terminus[64]. The substrate and ubiquitin are brought closely together for ligation upon neddylation, a process whereby NEDD8 is attached to target protein, of the Cullin C-terminus[64]. Six (6) closely related cullins and three atypical cullin family members are encoded in the human genome and each forms a unique E3 ligase complex[64]. The first crystal structure of a CRL, the Skp (adaptor protein), cullin1 (scaffold), F-box (substrate receptor) component complex (SCF) gave insight into the molecular architecture of the complex[65]. After modeling the complex with Rbx1 and the E2, UbcH7, the model suggested that there was a 50-80 Å gap between the substrate receptor and the E2 active site supporting the
concept that the E2 directly transfers ubiquitin to the substrate[65, 66]. While the
SCF complex has been the best-characterized CRL complex to date, very
recently the structure for a Cul5-based ankyrin SOCS box protein complex has
been solved[67]. The model developed confirms the likelihood that the E2 directly
transfers ubiquitin to the substrate; however, it also highlights the structural
diversity that allows CRLs to capture a variety of targets[67].

The diversity of adaptor proteins and substrate receptors lends itself to the ability
of E3 ligases to target a variety of target substrates. While Cul1 binds to an F-box
protein, Cul2 and Cul5 both assemble an ElonginC-Cullin-SOCS box (ECS) E3
ligase[64]. The ECS ligase interacts with the adaptor proteins, Elongin B and
Elongin C (Elo B/C) and a Suppressor of Cytokine Signaling (SOCS) box protein
as its substrate receptor[64]. SOCS box protein contain a conserved ~12 amino
acid sequence motif, (STP)LXXX(CSA)XXXϕ) termed the B/C box which
interacts with Elo C primarily; however, Elo B can make limited contact with
regions of the SOCS that extend outside of the B/C box[63]. The B/C box is
highly conserved among SOCS box proteins and demonstrates high structural
similarity[64].

While all known SOCS box proteins assemble with Elo B/C, most discriminate
between Cul2 and Cul5. For example, Cul5 can recruit SOC3, Elo B/C, and
Rbx1[68], while Cul2 can interact with von Hippel-Lindau protein (VHL), Elo B/C,
and Rbx1 to target Jak2 kinase and the hypoxia-inducible factor 1α (HIF1α)[69], respectively, for degradation by the proteasome. Motifs downstream of the SOCS box containing proteins have been defined to determine Cul2 and Cul5 selection[68]. SOCS box proteins that bind Cul5 contain the consensus sequence, termed the cullin box, $\phi XXLP\phi PXX\phi XX(Y/F)(L/I)$, where LP$\phi$P is especially important for selectivity[68]. SOCS box containing proteins that bind Cul2 contain another less defined cullin box consensus sequence that replaces the Cul5-specific LP$\phi$P sequence with LP, VP, or IP[68].
VIF AND APOBEC3

Vif is a 23kDa 192 amino acid highly basic HIV accessory protein expressed by all known lentiviruses (Figure 1-1), except equine infectious anemia virus and is known for the pathogenic replication of lentiviruses in vivo[70-72]. Early studies indicated that vif-deficient (Δvif) HIV-1 viruses were 10- to 1000-fold less infectious than their wild-type counterparts, confirming the importance of the accessory protein during infection[73]. While the full-length molecular structure of the protein has not been elucidated, multiple reports have contributed to a better understanding of Vif’s molecular architecture[74-77]. Vif has been described as a highly unstructured protein[78]; however, it may be this characteristic that allows the protein to interact with a variety of host cell proteins in order to function. Indeed, Vif’s C-terminus contains many functional motifs, including a SOCS box motif very similar to the cellular motif, that are critical to its ability to recruit a host E3 ligase in order to antagonize the anti-HIV-1 APOBEC3 family of proteins[37, 79].

The APOBEC family consists of 11 proteins (APOBEC1, APOBEC2, APOBEC3 (A, B, C, DE, F, G, and H), APOBEC4, and activation induced cytidine deaminase (AID) [80]. The family members all share a conserved, zinc-binding cytidine deaminase motif and mediate deamination at the C4 position of a cytosine base resulting in a uracil mutation[80]. Each APOBEC enzyme confers specificity for either DNA or RNA[80]. APOBEC3 proteins act on ssDNA intermediates and thus have broad antiviral activity[80]. In addition to HIV/SIV,
APOBEC3 can also affect the replication of MLV, HTLV, Rous sarcoma virus, hepatitis B virus, human papillomavirus, as well as the mobility of endogenous LTR and non-LTR retro-elements[81-86].

The APOBEC3 family was initially discovered in 2002 to exist on chromosome 22[87]. Prior to its discovery, Vif was known to be essential in select cells, termed ‘non-permissive’ (e.g. primary T cells, macrophages, and certain T-cell lines) for successive rounds of HIV replication, while other cells were ‘permissive’ (e.g. HEK293T, HeLa, and Cos-7 cell lines) to HIV replication, in the absence of Vif. Thus, researchers posited that the requirement for Vif was due to either the existence of an unknown restriction factor or the lack of an essential co-factor in non-permissive cells[88]. Experiments generating heterokaryon cells by combining the genetic material of both a permissive and non-permissive cell supported the existence of a restriction factor that could be counteracted by Vif[89].

Soon after the discovery of APOBEC3 proteins, APOBEC3G (previously referred to as CEM15) was reported to be a potent HIV restriction factor counteracted by Vif[37]. Later, additional APOBEC3 family members were reported to restrict HIV and be inhibited by Vif; however APOBEC3G and APOBEC3F appear to be the most potent in vivo[90-93]. In the absence of HIV-1 Vif, APOBEC3G is packaged into nascent virion particles and restricts the virus upon its next round of
infection\[37\] (\textbf{Figure 1-3}). APOBEC3G can induce hypermutations on the retroviral cDNA during reverse transcription leading to deleterious HIV genomic mutations\[94\]. Furthermore, there is evidence that APOBEC3G can also restrict HIV in a deaminase-independent fashion\[95, 96\]. Reports suggest that this restriction is due to APOBEC3G acting as a physical barrier to RT, preventing reverse transcription elongation\[97\]. Supporting this theory are reports that catalytically inactive mutants of APOBEC3G are capable of restricting HIV-1 and HTLV-1\[83, 98\].

\textbf{HIV Vif Recruitment of Host E3 ligase and APOBEC3}

In a producer cell, Vif mimics a cellular SOCS box protein to recruit the cellular E3 ubiquitin complex of Cullin5 (Cul5), ElonginB/ElonginC (Elo B/C), and RING box binding protein (Rbx) \[79, 99, 100\] . Vif was initially identified as a component of a Cul5-based E3 ligase through immunoprecipitation experiments using the cell lysate of HIV-1 infected cells, in which Vif was epitope-tagged with hemaglutinin (HA) \[79\] . To defend the virus against cellular APOBEC3, Vif also acts as a substrate receptor that recruits APOBEC3 molecules to the ligase to facilitate its ubiquitination and subsequent degradation by the 26S proteasome\[79\] (\textbf{Figure 1-4}). Our lab and another lab recently discovered that a host co-factor, core binding factor beta (CBF-\(\beta\)) also interacts with Vif\[101-103\]. Furthermore, this interaction between Vif:CBF-\(\beta\) is required for APOBEC3 ubiquitination and degradation (detailed in chapter 3) \[101, 102\] . As a result, APOBEC3G is not packaged into virion particles and virions can productively infect a subsequent target cell.
As mentioned earlier, the Vif C-terminus is responsible for many interactions with host proteins, especially for the recruitment of the E3 ligase[79, 104] (Figure 1-5). The C-terminal domain contains a highly conserved Elo C binding motif (144SLQYLA149) that closely resembles the canonical B/C box sequence[105, 106]. Early studies demonstrated that a SLQ – AAA Vif mutant lacks the ability to pull down Cul5 and Elo B/C[104]. This is supported by data, which confirms that the Vif's SOCS box mimics cellular SOCS box domains, structurally[76]. In addition, the Vif:Elo C binding is mediated by hydrophobic interactions similar to the interactions between cellular SOCS box motifs and Elo C[76]. Prior to the Vif motif structural data, co-immunoprecipitation studies revealed that a Vif L145A mutant was no longer capable of binding to Elo C and results in a 10,000 fold loss in affinity in isothermal titration calorimetry (ITC) experiments[99, 104, 107]. Structural data revealed that Vif residue L145 buries into a hydrophobic pocket on the surface of Elo C[76] (Figure 1-6). In addition, residue L148 also contributes to the binding between Vif and Elo C[76]. Furthermore, the interaction between Vif and Elo B/C is crucial for Vif to assemble with Cul5[108] (additional data presented in Chapter 3) and has recently been proposed to be important for efficient CBF-β binding[109].

Cul5 is the scaffold subunit of the E3 ligase and is required by Vif to ubiquitinate APOBEC3[79]. While the Vif B/C-box motif appears to be highly similar to the cellular B/C box, the interaction between Vif and Cul5 is primarily mediated through a largely novel motif[110]. Vif contains a degenerate cullin box.
downstream of the B/C box; Vif contains a $^{161}$PPLP$^{164}$ sequence motif, instead of the canonical LPΦP[99, 111]. It is unclear whether this 'cullin box' contributes to Cul5 binding or whether it is required for Vif multimerization. In order to recruit Cul5, Vif contains a novel zinc-binding $^{108}$HX$_5$Cys$X_{17-18}$Cys$X_{3-5}$H$^{139}$ (HCCH) domain upstream of the B/C box that mediates interaction between Vif and Cul5[106, 112, 113]. Early experiments showed that a C-terminal Vif truncated protein (residues 99-192) bound Cul5 with a 90nM affinity in the presence and absence of the cullin-box[107]. This affinity between Vif and Cul5 is similar to the affinity between cellular SOCS box proteins and Cul5 (affinities range from 10-1000nM) [114] (Table 1-3). Interestingly, a recent report using full length Vif demonstrated the binding affinity was actually much stronger (~5nM) [115]. We have confirmed this reported affinity and have further identified other Vif N-terminal domain residues that contribute to the affinity between Vif and Cul5 (detailed in chapter 4).

The Vif HCCH motif binds to zinc and is classified as a zinc finger protein[106, 113]. The zinc chelators N,N,N',N''-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) and Ethylenediaminetetraacetic acid (EDTA) are both effective at disrupting the interaction between Vif and Cul5[116]. Several reports have shown that mutations in the defined HCCH motif impair Vif function[79, 104, 110]. An early report demonstrated that the two histidine and two cysteine residues in the HCCH motif are required for Vif:Cul5 binding, as any single point mutation of these residues results in the inability for Vif to pull down Cul5[110, 117].
Furthermore, mutation of the conserved His and Cys residues of the HCCH motif in SIV$_{agm}$ Vif inhibits Cul5 binding[110]. In addition to the HCCH residues, hydrophobic residues (120IRKAL124) in the middle of the motif have also been demonstrated to affect the ability of Vif to bind Cul5 and are thought to form a hydrophobic interface with Cul5[117]. The C-terminal domain, including the HCCH motif, has been proposed to be unstructured in the absence of Cul5 and Elo B/C[78]. The structural flexibility of the HCCH motif may allow for greater flexibility when binding to Cul5, in the absence of a canonical cullin box. Furthermore, although the primary function of Vif appears to be to degrade APOBEC3 molecules, the structural flexibility in the C-terminus may also allow the Vif protein to interact with other host proteins for diverse purposes.

Unlike cellular SOCS box proteins, which contain a cullin box downstream of the B/C box and thought to control cullin selection, it is predicted that the Vif HCCH motif controls selection for Cul5 and not Cul2 or Cul7. In addition, Vif binds to the first cullin repeat of Cul5[113, 118]. A homology model of the Vif:Cul5:Elo B/C complex predicts that the Vif is oriented toward the loop 5 region of Cul5 (residues 118-134) [119]. This model is supported by pull down experiments, which demonstrate that a mutant Cul5 that lacks residues 119-139 does not interact with Vif:Elo B/C[118]. Taken together, Vif appears to bind Cul5 through the HCCH zinc-binding motif, which mediates selective binding to Cul5 over other cullin proteins.
In addition to the recruitment of the cellular Cul5-based E3 ligase, Vif must also recruit APOBEC3G/F to the viral E3 ligase in order to successfully degrade the anti-HIV factor[60, 120]. Several reports have contributed to the understanding of the Vif residues that are critical for mediating APOBEC3G/F degradation[121-130]. The Vif N-terminus region contains multiple discontinuous binding points for APOBEC3G/F. Species-specificity exists among APOBEC3G:Vif interactions; human APOBEC3G can be targeted by HIV-1 Vif but not by SIVagm Vif, while SIVagm APOBEC3G can be targeted by SIVagm Vif but not by HIV-1 Vif. Interestingly, species-specific Vif sensitivity is instructed by a single amino acid in APOBEC3G. The HIV-1 Vif residues $^{14}$DRMR$^{17}$ are thought to interact with APOBEC3G. If this region is replaced with SIV Vif residues $^{14}$SEXQ$^{17}$, the binding specificity is altered from human APOBEC3G to African green monkey APOBEC3G[128]. The two positively charged arginine residues in the HIV-1 Vif DRMR motif are thought to form an ionic interaction with a negatively charged aspartic acid residue (D128) of human APOBEC3G. In comparison, the SIV Vif glutamic acid residue (E15) is though to interact with the African green monkey APOBEC3G positively charged lysine residue (K128) [128]. Additional regions in the Vif N-terminus are required for APOBEC3G degradation – 21WXSLVK26, 40YRHHY44, 69YXXL72, and 81LGXGXSIEW89. While the 14DRMR17, 21WXSLVK26, 69YXXL72, and 81LGXGXSIEW89 regions are all important for APOBEC3F degradation, the 40YRHHY44 does not appear to be important. In addition, the first C-terminal domain region (171EDRW174) of Vif was also
shown to be important for APOBEC3G/F binding and degradation, the first demonstration of a region outside of the N-terminal domain being important for the recruitment of APOBEC3[130].

Our group recently identified a new Vif binding partner and regulator of the Vif mediated APOBEC3 function, called CBF-β[101, 131]. CBF-β forms a heterodimeric complex with a family of transcription factors, AML/RUNX, and is required to regulate the transcription of target genes by binding to the DNA sequence TGTGGT[132]. Two primary isoforms of CBF-β are ubiquitously expressed among human and mouse tissue: a 182 and 187 amino acid isoform[133]. The first 165 amino acids are identical for each isoform, while the remaining C-terminal amino acids are different[133]. Both CBF-β (182) and (187) form a complex with RUNX/CBFα family proteins at similar positions in the N-terminus (residues lie in two distinct areas) [133]. CBF-β serves as a non-DNA binding subunit of the heterodimer formed with RUNX/CBFα[132]. In the absence of stimulation, the CBF-β subunit is found in the cytoplasm associated with filamin A, an actin associating protein[134]. The three-dimensional structure of CBF-β (residues 1-141) alone and in complex with the runt domain has been determined[135-137]. Structure analyses revealed that CBF-β mediates a conformational change in CBF-α (the DNA-binding subunit) leading to increased binding within the major and minor grooves of DNA[137]. These complexes act as transcriptional activators as well as repressors and play important roles in the differentiation of diverse cell types, including T lymphocytes[138]. For example,
RUNX complexes can bind an interleukin 4 (IL-4) silencer, repressing the expression of IL4 in both naïve CD4+ T cells and Th1 cells[139]. In addition, RUNX proteins can silence CD4 expression during T cell development[140]. Interestingly, both of these regulatory activities are important to HIV-1 during infection.

While the host factor has important functions in the cell, CBF-β appears to also stabilize Vif in vivo and in vitro and allow Vif to efficiently recruit Cul5 to the Vif E3 ligase complex[77, 103, 115]. In addition, the protein is required for in vitro APOBEC3G ubiquitination[102, 131]. Additional studies are necessary to further understand the role of CBF-β in the Vif:E3 ligase assembly and eventual degradation of APOBEC3 proteins.
In the absence of Vif, APOBEC3 molecules can be incorporated into budding virions and prevent efficient reverse transcription as well as introduce C to U mutations via deamination. Mutated viral DNA can either be degraded or serve as the template for the transcription of a sense-strand DNA. Sense strand DNA contains G to A hypermutations that may generate premature stop codons or alternative mutations in the viral protein that hinders successful viral replication.

In the presence of Vif, APOBEC3 is degraded in the producer cell and is unable to be incorporated into budding virus. Source:[141]
Figure 1-4. Model of Vif-E3 ligase for APOBEC3 degradation

Vif recruits a cellular E3 ubiquitin ligase that consists of Cul5, Elo B/C, Rbx-2 as well as co-factor CBF-β and APOBEC3 molecules to mediate the polyubiquitination and proteasomal degradation of anti-HIV factor, APOBEC3.
**Figure 1-5. Schematic of Vif Interaction Domains**

Vif directly interacts with Cul5, Elongin B/C and co-factor CBF-β to form a functional E3 ligase complex. Both Cul5 and the Elongin B/C complex are known to bind in the C-terminal HCCH and B/C Box motif, respectively. Recent data supports the idea that Elongin B also makes contact with the PPLP motif in the C-terminus. Additionally, both CBF-β and APOBEC3 molecules bind discontinuous residues in the N-terminus.
Figure 1-6. Detailed Molecular interactions between HIV-1 Vif and Elo C.

HIV Vif B/C box (red) uses extensive hydrophobic interactions with Elo C (yellow) helices 3 and 4 during formation of the complex. Modified from source:[76]
<table>
<thead>
<tr>
<th>Syringe sample</th>
<th>Cell sample</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cul5-N</td>
<td>Vif C-term (95-192):Elo B/C</td>
<td>327 +/- 40[115]</td>
</tr>
<tr>
<td>Cul5-N</td>
<td>Vif C-term (100-192):Elo B/C</td>
<td>89 +/- 26[107]</td>
</tr>
<tr>
<td>Cul5-N</td>
<td>Vif FL:Elo B/C:CBF-β-FL</td>
<td>5 +/- 2[115]</td>
</tr>
<tr>
<td>Cul5-N</td>
<td>SOCS1:Elo B/C</td>
<td>1000 +/- 80[142]</td>
</tr>
<tr>
<td>Cul5-N</td>
<td>SOCS2:Elo B/C</td>
<td>9.5 +/- 3[142]</td>
</tr>
<tr>
<td>Cul5-N</td>
<td>SOCS3:Elo B/C</td>
<td>105 +/- 10[142]</td>
</tr>
<tr>
<td>Cul5-N</td>
<td>SOCS4:Elo B/C</td>
<td>10 +/- 2[142]</td>
</tr>
<tr>
<td>Cul5-N</td>
<td>SOCS5:Elo B/C</td>
<td>7 +/- 3[142]</td>
</tr>
<tr>
<td>Cul5-N</td>
<td>SOCS6:Elo B/C</td>
<td>23 +/- 10[142]</td>
</tr>
<tr>
<td>Cul5-N</td>
<td>SOCS7:Elo B/C</td>
<td>40 +/- 20[142]</td>
</tr>
</tbody>
</table>

Table 1-3. List of Cul5 and SOCS box containing protein binding affinities.
PROJECT GOALS AND AIMS

The primary goal of my thesis has been to further understand how Vif recruits the E3 ubiquitin ligase, including Cul5, Elo B/C, and CBF-β to target APOBEC3 proteins for ubiquitination and subsequent degradation. To overcome problems of stability and solubility, I focused on constructing multiple truncated Vif-expressing plasmids and co-expressed them with Vif binding partners CBF-β, Elo B/C, and Cul5. The complexes were used for in vitro purification, characterization, biochemical, and crystallization experiments. Furthermore, an in vitro high throughput small molecule inhibitor screen was developed to identify inhibitors of the interaction between Vif and Cul5 as well as Vif and CBF-β. In addition to in vitro experiments, we have also used in vivo experiments to determine the functional importance of the Vif:CBF-β interaction using fluorescence imaging and functional assays in mammalian cells.

Next, another project goal was to characterize an alternative function for Vif as a regulator of the RUNX:CBF-β complex. Again, using both in vivo as well as in vitro assays, we established a novel role for Vif as a transcriptional enhancer when in complex with the heterodimeric transcription factor RUNX:CBF-β. The Vif:RUNX:CBF-β complex appears to up-regulate cytokines that may facilitate HIV replication.

HIV-1 has evolved strategies to undermine many antiviral responses and as a consequence, successfully propagates in the specific host environment. One of
the most important aspects of HIV biology is our knowledge of the contacts that HIV viral proteins make with the host system. During the life cycle of the virus, viral proteins interact with several host proteins that either help or interfere with successful viral replication. In most infected hosts, the virus can use these multiple interactions in order to subvert the host immune response, providing an ideal environment for successful viral replication and survival. Further study of the evolved mechanisms that HIV uses to counteract the host immune response is necessary to develop novel strategies to completely eliminate the virus from the host. Likewise, understanding the host restriction factors that contribute to HIV restriction is equally critical to the development of curative therapies.
CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 *E. coli* Plasmid Constructs, Expression, Purification, and Protein Visualization

2.2 Mammalian Cell Plasmid Constructs, Transfection, Expression, Immunoprecipitation and Protein Analysis

2.3 Antibodies
**E. coli Plasmid Constructs, Expression, Purification, and Protein Visualization**

**Vector Constructs**

Duet vectors (EMD Millipore) were used primarily for overexpression experiments to facilitate the co-expression of multiple proteins within a single *E. coli* cell. Each Duet vector is designed for the cloning and co-expression of two target genes. All vectors contain two multiple cloning sites (MCS) each of which is preceded by a T7lac promoter for induction by isopropyl-D-thiogalactopyranoside (IPTG) and ribosome binding site (rbs). Multiple Duet vectors may be used together in compatible host strains to co-express up to 8 target proteins. A 6X-His-tag can be added to the N-terminus of the gene in MCS1 of each Duet vector. To avoid more than one protein having a His tag, the NcoI restriction site can be used to remove the tag from genes that are placed in MCS1. The following proteins were cloned into Duet vectors: Vif, CBF-β, Elo B/C, and Cul5. Using this system, we have successfully co-expressed up to five proteins to assemble a Vif-based E3 ligase complex, *in vitro*.

In addition, GST vectors (pGEX-6p-1, GE Healthcare) were used to facilitate solubility of tagged proteins. In addition, pGEX-6P-1 encodes the recognition sequence for site-specific cleavage by PreScission Protease between the GST domain and the multiple cloning site and a tac promoter for chemically inducible, high-level expression of GST-tagged recombinant proteins. Cul5 was cloned into the pGEX-6p-1 vector.

Vectors are described in Table 2-1.
**E. coli Vif and Binding Partner Constructs**

All Vif constructs encoded the Vif protein (Figure 2-1) of HIV-1 NL4-3 (Genbank accession number EU541617) with a single nucleotide silent mutation at position 84 from cytosine to thymine to facilitate the use of the restriction enzyme Ndel in cloning experiments. All clones were derived from full-length Vif192 in the pET21 vector, a gift from Drs. Rahul M. Kohli and James T. Stivers (Johns Hopkins School of Medicine).

Due to Vif insolubility, we developed multiple truncated Vif constructs and co-expressed them with constructs encoding Vif binding partners, CBF-β, Elo B/C, and Cul5. Truncated Vif176, Vif154, Vif140, and Vif110 were cloned into pET21 vector. In addition, full-length vif and truncated vif genes were cloned into MCS2 of the Duet vectors, pET-Duet1 and pRSF-Duet1 with Cul5 or CBF-β in MCS1, respectively.

Mouse CBF-β (residues 1–187, Genbank accession number NM022309) cDNA were cloned from plasmid containing CBF-β gene, a gift from Nancy A. Speck (University of Pennsylvania). Murine CBF-β isoform 1 (residues 1–187), human CBF-β isoform 2 (residues 1–182, Genbank accession number AK290462) and truncated human CBF-β (residues 1–140) human were cloned into pRSF-Duet with 6X-His-tag. Human Elongin B (Genbank accession number NM007108) and Elongin C (residues 17 to 112, Genbank accession number NM001204864) in
the pACYC-Duet plasmid (no 6X-His-tag) were a gift from Alex Bullock (University of Oxford, Oxford, United Kingdom). The N-terminal domain residues 1-393 with two point mutations, V341R and L345D for enhanced solubility) human cul5 gene (Genbank accession number NM003478) was cloned into both pET-Duet and pGEX-6p-1 Cul5-NTD.

Expression and Purification of Vif Complexes
For expression, plasmids containing Vif, Elo B/C, CBF-β, and Cul5 were co-transformed into Escherichia coli NiCo21(DE3) (NEB) cells. If cells were transformed with more than one plasmid, the cells were placed on ice for 20-30min followed by a heat shock step at 42C for 45sec and shaken for 2 hours at 37C before plating. To further enhance the success of multiple plasmid transformations, incubated transformed cells were centrifuged at 3000rpm and cell pellet was resuspended in 50-100uL of LB. Transformations were plated on Luria broth (LB) agar containing the vector specific antibiotic at half of the standard concentration for double and triple transformations (50ug/mL ampicillin (Amp), 17ug/mL chloramphenicol (Cam), 50ug/mL streptomycin (Strep), and/or 50ug/mL kanamycin (Kan) and incubated at 37C overnight.

Colonies were selected from transformation experiments and a starter culture was grown at 37C for 5-8hrs. To co-express proteins, LB broth with appropriate antibiotics were inoculated with starter culture and grown to an OD₆₀₀ of 0.7-1 at 37C. Protein expression was induced with 0.1-0.2mM IPTG overnight at 16C or
23°C by induction. Harvested cells were lysed in 1X phosphate-buffered saline (PBS) with 0.25mM TCEP and 30mM imidazole (for 6X-His-tag purification) by sonication and centrifuged at 10000rpm for 30 min. For solubility analysis, the supernatant was removed and the pellet resuspended to the original volume. For nickel affinity purification, the supernatant was transferred to Ni-NTA beads (Invitrogen), and the flowthrough was loaded onto Ni-NTA beads for two more passages. After washing with 1X PBS with TCEP and 40mM imidazole, protein complexes were eluted with 1X PBS with TCEP and 250mM imidazole. In some experiments, gel filtration was utilized to remove excess 6X-His-tagged CBF-β as well as *E. coli* protein trace contamination.

Cul5-NTD was cloned into both pET-Duet with Vif as well as in the pGEX-6p-1 vector for separate expression. The GST-Cul5-NTD (pGEX-6p-1) construct was expressed in *E. coli* NiCo21(DE3) cells overnight at 16°C or 23°C by induction with 0.1-0.2mM IPTG. Harvested cells were lysed by sonication in 1X PBS with 0.25mM TCEP then clarified by centrifugation at 10000rpm for 30 min. The supernatant was transferred to glutathione-Sepharose 4B beads (GE Healthcare) for glutathione S-transferase (GST) affinity chromatography. The GST tag was then removed using Prescission protease (GE Healthcare). Gel filtration chromatography was utilized for further purification. Purified GST-Cul5-NTD was incubated with purified Vif:Elo B/C:CBF-β complex at 4°C for 1hr prior to a subsequent Ni-affinity or GST-affinity purification step.
Each Vif complex or Cul5-NTD sample was concentrated to 250-400uL and loaded onto a Superdex 200 (10/300 GL) column (GE Healthcare) with a 500ml loop and run at a flow rate of 0.3 ml per min; the column was calibrated using vitamin B12 (1,370 Da), myoglobin (17,000 Da), ovalbumin (44,000 Da), gamma globulin (158,000 Da), and thyroglobulin (670,000 Da) as standards. The gel filtration buffer for Vif complexes and Cul5-NTD was composed of 1X PBS with TCEP (unless otherwise noted).

**SDS PAGE of Purified Proteins**

To analyze expression, solubility and binding of Vif along with binding partners, protein samples were separated by SDS-PAGE and visualized with coomassie staining and/or by western blotting/immunblotting with specific antibodies. For coomassie staining, gels were incubated in coomassie brilliant blue (CBB) R-250 (Sigma) solution (45% methanol, 45% dH$_2$O, 10% acetic acid, 0.1% CBB R-250) for 10-30min. Next, gels were washed in destaining solution (45% methanol, 45% dH$_2$O, 10% acetic acid) until desired band/background ratio was reached. For quicker analysis and enhanced sensitivity, gels immersed in coomassie stain were heated in the microwave for 30-40sec. In addition, to reduce destaining time, folded paper towels were included in the destaining solution.

For western blot, proteins were separated by SDS-PAGE, then transferred to
nitrocellulose membranes (Bio-Rad). After blocking with 1X PBS with 0.02% Tween 20 containing 5% BSA for 10min-1h at room temperature, membranes were incubated with a specific primary antibody (concentration ranged from 1:10-1:5000 depending on antibody sensitivity/specificity) overnight at 4C. After three 5-10min washes with 1X PBS with Tween 20, the membranes were stained with an appropriate alkaline phosphatase-conjugated secondary antibody (1:3,000, Sigma) for 1h at room temperature. After three washes with 1X PBS with Tween 20, the membranes were incubated with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro-blue tetrazolium (NBT) substrate (Sigma). The antibodies used in this study were specific for: Vif (the AIDS Research Reagents Program), CBF-β (Abcam), (Santa Cruz Biotechnology, Inc), Elo C (BD Transduction Laboratories), Alkaline Phosphatase-conjugated secondary mouse, goat and rabbit (Jackson Immunoresearch).
<table>
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<tr>
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<td>Ampicillin</td>
<td>50</td>
<td>pBR322</td>
</tr>
</tbody>
</table>

**Table 2-1 E. coli vectors**

Summary of properties of pGEX and Duet vectors used for single protein and multi-protein co-expression of Vif-E3 ligase complex components.
Figure 2-1. Sequence of HIV-1 Vif with secondary structure predictions.

The University College London PSIPRED server was used to predict Vif secondary structure.
Mammalian Cell Plasmid Constructs, Transfection, Expression, Immunoprecipitation and Protein Analysis

The primary vectors used in most experiments were CMV-promoter-driven constructs (e.g. pCDNA 3.1 and pCMV). In addition to a CMV promoter, the VR1012 vector also contains intron A to achieve greater levels of protein expression.

Vif constructs were either HXB2 (Genbank accession number K03455) expressed in VR1012 or a codon-optimized NL4.3 Vif (called HVif) expressed in pcDNA. HVif-pcDNA is a partially codon-optimized version of the native NL4.3 vif gene and was achieved by changing the first 84 vif codons to conform to the reported codon usage of highly expressed human genes. The HXB2 vif gene is inserted into the intron A-containing VR1012 vector. Both strategies for Vif expression achieve physiologically relevant levels of Vif in the cell. Additionally, fluorescent protein-tagged Vif (e.g. YFP-Vif and CFP-Vif) were constructed using the EYFP or ECFP contracts (Novagen). CBF-β isoform 2 was cloned into pcDNA 3.1, VR1012, as well as EYFP or ECFP constructs. The pcDNA-CBF-β was cloned with and without a myc or HA epitope tag.

Transfection, Expression, Immunoprecipitation, and Protein Analysis.

Plasmids were complexed with PEI-Max at a 2.5:1 ratio in Opti-Mem (Invitrogen) buffer for 30min. Cells were transfected with plasmid:PEI complexes and harvested 48 h later, washed with PBS and lysed in lysis buffer (50mM Tris,
75mM NaCl, 0.1% NP-40 and Complete Protease Inhibitor Cocktail Tablet (Roche) (pH 7.4)) at 4°C for 15min, followed by centrifugation at 10,000 × g for 20 min. HA immunoprecipitation was carried out by mixing soluble lysates of transfected cells from a 10cm dish with 40uL anti-HA affinity matrix beads (Roche) and incubating the mixture at 4°C for 3h. The samples were washed six times with wash buffer (20 mM Tris, 50 mM NaCl, 0.1 mM EDTA and 0.05% Tween-20 (pH 7.5)). Bound protein was eluted with elution buffer (100mM glycine-HCl, pH 2.5). The eluted protein was analyzed by SDS-PAGE and immunoblotting with appropriate antibodies.

Mammalian Cell Lines and Maintenance

293T and MAGI-CCR5 cells (AIDS Research and Reference Reagents Program, catalogue no. 3522) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) with 10% fetal bovine serum and penicillin/streptomycin (D-10 medium) and passaged when confluent.
Antibodies

The following antibodies were used: anti-HA (Covance, MMS-101R-1000), anti-Myc (Upstate, 05-724), anti-V5 (Invitrogen, R960-25), anti-β-actin (Sigma, A3853), anti-histone H3 (Genscript, A01502), anti-human ribosomal P antigens (Immunovision, HP0-0100), anti-HA antibody–agarose conjugate (Roche, 11815016001), anti-Elo B (Santa Cruz, sc-1558), anti-Elo C (BD Transduction Lab, SIII/P15, 610760), anti-GAPDH (Sigma, G8795), anti-CBF-β (Abcam, ab11921), anti-CUL5 (Santa Cruz, sc-13014), anti-p53 (Oncogene Research Products, OP03) and anti-GFP (Genscript, A01704); anti-CAp24 (catalogue no. 1513) and anti-Vif antibodies (catalogue no. 2221) were obtained from the AIDS Research and Reference Reagents Program.
CHAPTER 3

IDENTIFICATION OF NOVEL VIF BINDING PARTNER AND REGULATOR, CORE BINDING FACTOR BETA AND IN VITRO CHARACTERIZATION OF VIF:CBF-β:E3 LIGASE COMPLEXES


3.1 Introduction

3.1.1 Biochemical discovery of new Vif binding partner

3.1.2 Functional role in assisting Vif-induced APOBEC3 degradation

3.2 Methods

3.3 Vif:CBF-β localization in HEK293T Cells: Live cell fluorescence imaging
3.4 FRET assay confirms *in vivo* binding

3.5 CBF-β binds and increases Vif solubility *in vitro*

3.6 Attempts to crystallize Vif:CBF-β complexes with and without Cul5 and/or Elo B/C

3.7 Discussion
Introduction

Biochemical Discovery of New Vif Binding Partner

Over the years, multiple research groups have discovered a variety of interactions that are made between HIV and host proteins. A large-scale proteomics study, performed in 2011 resulted in one of the most comprehensive lists of interactions that each of the fifteen (15) proteolytically cleaved HIV proteins and three (3) HIV polyproteins, encoded by the less than 10kb genome, make with the host proteome[143]. The study identified over 500 physical interactions between HIV and host proteins[102, 143]. The resulting map of physical interactions between the HIV and host proteins contributes to understanding the molecular mechanisms that underlie HIV infection. In this study, the authors discovered a novel interaction between Vif and a host factor, CBF-β, that was responsible for Vif function.

At the same time, our lab independently discovered an additional interaction between Vif and the host factor, CBF-β[131]. With the intention of discovering novel cellular protein interactions with Vif, our lab constructed an infectious HIV-1 clone in which the end of the Vif gene was HA-tagged (HXB2-VifHA) [60, 131] . Next, we infected H9 cells with HXB2-VifHA or HXB2 (control) viruses and characterized the interaction of cellular proteins with Vif-HA by co-immunoprecipitation analysis[60, 131]. As mentioned before, HIV-1 Vif forms an E3 ubiquitin ligase with Cul5, Elo B/C, and Rbx to promote the polyubiquitination and degradation of various substrates, including APOBEC3 as well as Vif
Untagged Vif was not immunoprecipitated from control-infected H9 cells[60, 131]. Cul5 (84 kDa) was co-precipitated with Vif–HA and confirmed by immunoblotting with a Cul5-specific antibody; Elo B/C was also co-precipitated with Vif–HA[60, 131]. These proteins were not co-precipitated from control HIV-1-infected H9 cells. Surprisingly, an additional protein of ~22 kDa that had not been previously identified co-precipitated with Vif-HA[131] (Figure 3-1). This protein was identified by mass spectrometry to be CBF-β[131]. An interaction between HIV-1 Vif–HA and CBF-β was also detected in transfected HEK293T cells, indicating that the interaction between CBF-β and HIV-1 Vif can occur in the absence of other viral proteins[131].
Figure 3-1. Co-immunoprecipitation of Vif-HA and partner proteins

Immunoblot of precipitated samples from HIV-1- or HIV-1 Vif–HA- infected H9 cells using antibodies against CUL5, CBF-β, Elo B, Elo C or HA (to detect Vif–HA). CBF-β is a newly discovered Vif binding partner. Source: [101]
Functional role in assisting Vif-induced APOBEC3 degradation

To determine whether CBF-β was required for Vif mediated human APOBEC3G degradation, we successfully silenced expression of endogenous CBF-β by using RNAi directed against two regions in the CBF-β coding sequence[131]. When endogenous CBF-β was silenced, the ability of Vif to reduce the expression of APOBEC3G was blocked and NL4-3 viral infectivity was reduced[131] (Figure 3-2A). In addition, APOBEC3G was efficiently packaged into HIV-1 virions in CBF-β silenced cells[131]. In contrast, silencing CBF-β expression had no effect on the viral infectivity of NL4-3 or NL4-3ΔVif when APOBEC3G was absent, indicating that CBF-β regulates HIV-1 infectivity only in the presence of APOBEC3G[131] (Figure 3-2B). Additionally, HIV-1 Vif-mediated degradation, virion exclusion, and suppression of APOBEC3F also required CBF-β[131]. Because HIV-1 Vif mainly reduces the expression of APOBEC3G by inducing its degradation, these data indicated that CBF-β is required for the HIV-1 Vif-mediated degradation of APOBEC3G[131].

Next, we wanted to understand the role that CBF-β plays in the formation of the Vif:E3 ligase complex. First, CBF-β-myc was transfected into cells that endogenously express the components of the E3 ligase and a co-immunoprecipitation assay was performed. Results indicated that CBF-β-myc did not interact with these components alone[131]. Additionally, CBF-β does not interact directly with APOBEC3G and is not required for the Vif–APOBEC3G interaction[131]. Subsequently, an RNAi knockdown of endogenous CBF-β was
performed in HEK293T cells also transfected with Vif-HA[131]. Cul5 and Vif-HA were no longer able to interact in the absence of CBF-β[131]. However, Vif-HA and APOBEC3G retain the ability to bind with each other in the absence of CBF-β[131]. Therefore, CBF-β is unlikely to be an integral component of the Cul5–Elo B/C E3 complex under physiological conditions. Yet, CBF-β is required for Vif to bind Cul5 and form a complete E3 ligase complex. In this chapter, using cell imaging techniques, fractionation assays, and in vitro complex binding assays, further characterization of the Vif:CBF-β complex was accomplished.
Figure 3-2. Vif-mediated degradation of APOBEC3G requires CBF-β.

A. CBF-β is required for Vif-mediated degradation exclusion of APOBEC3G from virions. B. Viral infectivity was assessed by multinuclear activation of a galactosidase indicator (MAGI) assay. Error bars represent the standard deviations from triplicate wells. Source: [101]
Methods

Plasmid construction

CBF-β coding sequences were amplified by reverse transcription and PCR using mRNA samples from H9 cells with the following primers: forward 5’-
GCTAGCAAGATGCGCGCGTCGTG -3’, reverse 5’-
AAGCTTACTACAGATCTTCTTTGATAGTGTGTTTCGCTTTTGC -3’ containing NheI and HindIII sites. The PCR product was cloned into pcDNA3.1 to generate pCBFβ-myc. pCBFβΔN1, CBFβΔN2, CBFβΔN3, CBFβΔN4, CBFβΔN5 and CBFβΔN6 were made from pCBFβ-myc by site-directed mutagenesis and confirmed by DNA sequencing. The following expression vectors have been previously described 7, 20, 29, 30: VR1012, HIV-1 Vif expression vectors pVif-myc and pVif-HA, pAPOBEC3G-HA, pAPOBEC3G-myc, pUbiquitin-myc, pCul5-HA, pAPOBEC3F-V5, E4orf6-myc, SOCS3- HA, human p53, SIVmacVif-HA, SIVagmVif-myc, BIVVif-HA, HXB2Neo, and HXB2NeoVif-HA. pVifΔN-HA was constructed with the following primers: forward 5’- GTCGACATGGACCCTGAACTAGC -3’, reverse 5’-
GGAATTCCTACGCGTAATCTGGGACGTCGTAAGGGTAGTGTTCCATTCATTGTGGCT -3’ containing SalI and NotI sites, respectively, and a C-terminal HA tag. pNL4-3ΔVif and pcDNA-HVif were generous gifts from Dr. K. Strebel. pRUNX1-myc and p(CBF)4TKLuc were generous gifts from Dr. A. Friedman. NL43-ΔE-EGFP was a generous gift from Dr. R. Silicano. Plasmids pVifW11A, W21A, W38A, DR14/15AA, K22E, Y40A, RH41/42AA, T74A, E76A, R77A, and W79A were made from pVif-myc by site-directed mutagenesis and confirmed by
DNA sequencing. To generate yellow fluorescent protein (YFP) N-terminal epitope-tagged NL4-3 Vif, pcDNA-hVif was used to PCR-amplify the Vif coding region and cloned into the NotI and XbaI sites of pcDNA3-YFP (David T. Yue). To generate yellow or cyan fluorescent protein (Y/CFP) C-terminal epitope-tagged NL4-3 viral infectivity factor (Vif), pCDNA HVif wild-type was used to PCR amplify the vif coding region (primers -forward 5’-
GACGTGGAATTCCGCCGCCACCATGGAGAACCGGTGGC – 3’ and reverse 5’–
ATGGATCCCCGCCAGACCCCCCGGGTTGGTGCTC –3’) and cloned into the EcoR1 and BamHI sites of pEYFP-N1 or pECFP-N1 (Clontech). To generate cyan fluorescent protein (CFP) N-terminal epitope-tagged CBF-β, the CBF-β coding region was cloned into the NotI and XbaI sites of pcDNA3-CFP (David T. Yue). The YFP-CFP dimer construct was a generous gift from Dr. D.T. Yue and was generated by fusing CFP and YFP with a 21 amino acid linker, SRAQASNSAVDGTAGPGSIAT. The following CBFβ shRNA clones was obtained from Open Biosystems: TRCN0000016643, with mature sense sequence 5’- CGAGAGTATGTCGACTTAGAA-3’; TRCN0000016644, 5’-
CCGCGAGTGAGATTAAGTA-3’; TRCN0000016645, 5’-
GAAGATAGAGACAGGTCTCAT-3’; TRCN0000016646, 5’-
GCTGGCAGTAACTGGCAAGAA-3’; TRCN0000016647, 5’-
TGAGATTAAGTACACGGGCT-3’.

Antibodies and cell lines
The H9 human CD4+ T-cell line was maintained in RPMI-1640 medium (Invitrogen) with 10% fetal bovine serum and penicillin/streptomycin (R-10 medium). 293T and MAGI-CCR5 cells (AIDS Research and Reference Reagents Program, Cat #3522) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% fetal bovine serum and penicillin/streptomycin (D-10 medium) and passaged when confluent. The following antibodies were used: anti-HA (Covance, MMS-101R-1000), anti-myc (Upstate, 05-724), anti-V5 (Invitrogen, R960-25), anti-β-actin (Sigma, A3853), anti-histone H3 (Genscript, A01502), anti-human ribosomal P antigens (Immunovision, HP0-0100), anti-HA antibody-agarose conjugate (Roche, 11815016001), anti-Elo B (Santa Cruz, sc-1558), anti-Elo C (BD Transduction Lab, SIII/P15, 610760), anti-GAPDH (Sigma, G8795), anti-CBF-β (Abcam, ab11921), anti-Cul5 (Santa Cruz, sc-13014), anti-p53 (Oncogene Research Products, OP03), and anti-GFP (Genscript, A01704); anti-CAp24 (Cat #1513) and anti-Vif antibodies (Cat. #2221) were obtained from the AIDS Research and Reference Reagents Program.

RNA interference

RNAi against CBFβ was carried out using a pool of two duplexed short interfering RNAs (siRNAs) (Dharmacon): duplex 1, sense: 5'-CCAGCAGGAGGAUGCAUUUU, antisense: 5'-'PUUAUGCAUCCUCUGCGGUU; duplex 2, sense: 5'-GCAGGCAAGGUAUAUUUGAUU, antisense: 5'-'PUCAAUAUACCUGCGCCUGCUU. RNAi against Cul5 was carried out using a
pool of four duplexed short interfering RNAs (siRNAs) (Dharmacon): duplex 1, sense: 5'- GACACGACGUCUUAUAUUAU, antisense: 5'-
PUAAUAUAAGACGUCGUGUCUU; duplex 2, sense: 5'-
CGUCUAAUCUGUAAAGAAUU antisense: 5'-
PUUCUUAACACAGAUAGACGUU; duplex 3, sense: 5'-
GAUGAUACGGCUUUGCUAAU, antisense: 5'-PUUAGCAAAGCCGUAUCAUCU; duplex 4, sense: 5'-
GUUCAACUCGAUACUAUU, antisense: 5'-PUUAGUAUUCGUAG
UUGAACUU. 293T cells were transfected with the CBFβ or Cul5 siRNA pool at a total final concentration of 100nM using Lipofectamine 2000 (Invitrogen). The non-targeting siRNA no. 2 (Dharmacon) was used as a control. Protein expression was monitored by immunoblotting 2-3 days after transfection.

Identification of Vif-binding proteins

Vif-containing complexes were purified from HIV-1Vif-HA and control HIV-1 (HXB2Neo) infected H9 cells by immunoprecipitation and analyzed by SDS-PAGE. Gels were fixed in 50% methanol/10% acetic acid for 10 min, stained with mass spectrometry-compatible colloidal-Coomassie brilliant blue G- 250 (Bio-Rad 1610406) staining solution (20% methanol, 8% ammonium sulfate, 1.6% phosphoric acid, 0.08% Coomassie blue G-250) to detect protein bands, and de-stained with distilled water. Protein standards from Bio-Rad (Cat. #1610314) were used to estimate protein size. Protein bands of interest were cut out of the gel and rinsed twice with 50% methanol (HPLC grade). In-gel digestion was
performed on protein bands cut out of colloidal-Coomassie blue-stained SDS-polyacrylamide gels using sequencing-grade modified trypsin (Promega). Extracted peptides were co-crystallized in 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA) (10 mg/ml in 50% acetonitrile/0.3% TFA) and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry on a Voyager DE STR (Applied Biosystems, home.appliedbiosystems.com) using Voyager Instrument Control Panel (v5.1) and Data Explorer (v4.0) software. Data were acquired in reflector mode, and masses were externally calibrated using a standard peptide mixture to <50 ppm error. Proteins were identified by searching the acquired monoisotopic masses against the NCBI nonredundant or SwissProt databases using the MS-Fit search engine of ProteinProspector (prospector.ucsf.edu).

**APOBEC3G Ubiquitination Assay**

HEK293T cells were transfected with expression vectors encoding APOBEC3G-HA, HIV-1 Vif, and ubiquitin-myc, either individually or in combination. Cells were also transfected with control or CBFβ-specific siRNA. Transfected cells were treated with 10µM MG132 (proteasome inhibitor) for 16h, beginning at 24h after transfection, and then lysed in lysis buffer (50 mM Tris, pH 7.5, with 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10µM MG132, and complete protease inhibitor cocktail tablets), followed by centrifugation at 10,000xg for 30 min. Lysates were applied to anti-HA antibody-conjugated agarose beads (Roche) and incubated for 3h at 4°C. After incubation, the beads were washed six times
with washing buffer (20 mM Tris, pH 7.5, with 0.1M NaCl, 0.1 mM EDTA, and 0.05% Tween 20), then eluted with elution buffer (0.1 M glycine-HCl, pH 2.0) followed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-myc and anti-HA antibodies.

Live-cell Imaging and FRET assay

HEK293T cells were transfected with YFP-Vif (2 µg) and CFP-CBFβ (0.25 µg) using Lipofectamine 2000 (Invitrogen) in 6-well coverslip glass-bottom cell culture dishes (MatTek) at 37C with 5% CO2. 8-fold excess YFP-Vif was used due to low expression of YFP-Vif compared to CFP-CBF-β. YFP-CFP dimer (0.25 µg), co-transfection of YFP (0.25 µg) + CFP (0.25 µg), and CFP-CBFβ alone were used for a positive and negative FRET signal as imaging controls. Cells were visualized 24-36h after transfection using a Zeiss LSM510-Meta confocal imaging system equipped with four argon lasers (458, 477, 488, and 514 nm lines), two HeNe lasers (542 and 633 nm), and one diode laser (405 nm). YFP and CFP were excited using a 514 nm and 458 nm laser, respectively. Following excitation, channel mode images were collected using a long pass filter for CFP emission (LP 420) or band pass filter for YFP emission (BP 530-600). In addition, the channel mode detector gain was set so that neither CFP nor YFP images contained saturated pixels prior to bleaching. A total of 10 images (5 pre-bleach and 5 post-bleach) were acquired for each cell with a 63x objective with 2x zoom, and image analysis and manipulation was performed using Zeiss LSM imaging software. FRET analysis was performed using the acceptor photo-bleaching
technique: YFP or YFP-Vif was photo-bleached for approximately 5 minutes at 514 nm (100% laser power), and the average donor signal was evaluated before and after acceptor photobleaching. As a control, cells were also imaged under similar conditions, except the 514nm laser was set at 0% transmission to prevent acceptor photobleaching. Two experiments were performed for each bleached sample for a total of 10 cells each. One experiment was performed for each unbleached sample for a total of 4 cells each. The FRET efficiency % was calculated using the FRET macro of Zeiss LSM software. The following equation was employed: %E = (FDΔ/FDpost)*100. FDΔ: the change in donor (CFP) fluorescence intensity pre and post acceptor (YFP) bleaching; FDpost: donor fluorescence intensity post acceptor bleaching. The FRET macro of Zeiss LSM software also subtracts background when calculating FRET efficiency %.

**Gel Filtration Chromatography**

Each Vif complex and Cul5 sample was concentrated to 300ul and loaded onto a Superdex 200 (10/300 GL) column (GE Healthcare) with a 500ul loop and run at a flow rate of 0.3 ml per min; the column was calibrated using vitamin B12 (1,370 Da), myoglobin (17,000 Da), ovalbumin (44,000 Da), gamma globulin (158,000 Da), and thyroglobulin (670,000 Da) as standards. The gel filtration buffer for Vif:CBF-β was composed of 20 mM Tris- HCl pH 8.0, with 150 mM NaCl and 10% glycerol. The gel filtration buffer for Vif:CBF-β:Elo B/C, Vif:CBF-β:Elo B/C:Cul5, and Cul5 was 20 mM Tris-HCl, pH 8.0, with150 mM NaCl.

**Pull-down Analysis of the Vif:CBF-β Interaction**
For pull-down experiments analyzing the interactions between Vif and CBF-β, supernatant was incubated on Ni-NTA agarose for 30min at 4C. After incubation, the reaction mixtures were washed 10x with 1ml lysis buffer. The samples were then analyzed by SDS-PAGE and visualized with Coomassie staining or by immunoblotting with specific antibodies.

**Crystallization Studies**

All Vif complexes that were purified to homogeneity were further screened using a hanging drop vapor diffusion protocol. Sparse matrix conditions from different crystallization conditions test kits (Hampton Research) were used with 1-10mg/mL protein complex depending on solution behavior of the complex. Using the hanging drop method, we placed a small (1-2uL) droplet of the sample mixed with crystallization reagent on a siliconized glass cover slide inverted over the reservoir in vapor equilibration with the reagent. The drop loses water over time due to the difference in precipitant concentration between the drop and the reservoir. The drops were examined each day for the first week and once per week after the first week to determine whether crystals were present. The advantages of the hanging drop technique include the ease in viewing the drop through glass, reduced chance of crystals sticking to the hardware, and easy access to the drop.
**Vif:CBF-β localization in HEK293T Cells: Live cell fluorescence imaging**

One aim of my project was to further delineate where Vif and CBF-β associate in the cell as well as what type of association exists between the two cells (i.e. direct interaction or indirect association). Using a live cell imaging approach, we observed co-localization of yellow fluorescent protein (YFP)–Vif and cyan fluorescent protein (CFP)–CBF-β in transfected 293T cells. HEK293T cells were co-transfected with YFP-Vif (2 µg) and CFP-CBF-β (0.25 µg) in 6-well glass-bottom plates. Cells were imaged 24-36h post-transfection with a Zeiss LSM510 Meta confocal imaging system. Immunoblot analysis revealed the expression level and confirmed predicted molecular weight for each fluorescent-tagged protein. CFP-CBF-β was transfected at 1/8 the concentration compared to YFP-Vif due to the difference in expression levels.

Alone, both YFP-Vif and CFP-CBF-β appeared to localize in both the nucleus and the cytoplasm[131] (Figure 3-3 and 3-4). However, when co-expressed, both proteins appear to localize in the cytoplasm of both HEK293T cells as well as HeLa cells (Figure 3-5). HEK293T cell fractionation of untagged Vif and CBF-β confirmed the live cell imaging results (Figure 3-6). Surprisingly, YFP-tagged Vif appeared primarily in the nucleus in the presence and absence of over-expressed untagged and CFP-tagged CBF-β. It is unclear why there is a difference in the results between the two experimental techniques. However, it is possible that Vif-YFP may associate with the cell membrane inner leaflet, which our fractionation protocol did not separate the nuclear and membrane fractions.
Upon obtaining results that indicated there was a difference between how untagged and YFP-tagged Vif localize in the cell, we were curious whether the fluorescent protein fused Vif maintained its ability to degrade APOBEC3G. Both Vif constructs were overexpressed in cells with or without APOBEC3G and CBF-β. When compared, both unfused and fused Vif were capable of degrading APOBEC3G in HEK293T cells, while A YFP-CFP dimeric protein has no effect on APOBEC3G protein levels (Figure 3-7). Thus, neither the fused fluorescent protein nor the difference in localization seems to affect the ability for Vif to reduce the levels of APOBEC3G in the cell. Furthermore, YFP-Vif appears to localize to the cytoplasm when co-expressed with HA-tagged APOBEC3G. Interestingly, the appearance of small punctate bodies appears in the cytoplasm, possibly the site of Vif-associated APOBEC3G high molecular weight complexes. Reports have demonstrated that Vif promotes the formation of functionally inactive APOBEC3G high molecular weight ribonuclear complexes that may reach megadaltons in size, comprised of both cellular and/or viral RNA and proteins.
Figure 3-3. Live cell imaging of YFP-Vif in HEK293T cells.

HEK293T cells were transfected with YFP-Vif and imaged live using fluorescence confocal microscopy. YFP-Vif localizes to both the cytoplasm and nucleus of the cell.
Figure 3-4. Live cell imaging of CFP-CBF-β in HEK293T cells.

HEK293T cells were transfected with CFP-CBF-β and imaged live using fluorescence confocal microscopy. CFP-CBF-β localizes to both the cytoplasm and nucleus of the cell.
Figure 3-5. Live cell imaging of co-expressed YFP-Vif and CFP-CBF-β in HEK293T cells.

HEK293T cells were transfected with both CFP-CBF-β and YFP-Vif and imaged live using fluorescence confocal microscopy. Both proteins localize to primarily to the cytoplasm of the cell when co-expressed.
Figure 3-6. Subcellular fractionation of CFP-CBF-β and YFP-Vif in HEK293T cells.

Immunoblot of fractionated untagged Vif and YFP-Vif indicate that in the presence of CBF-β untagged Vif localizes to the cytoplasm; however, YFP-Vif localizes to both the cytoplasm and nucleus of the cell.
### Figure 3-7. YFP-Vif degrades APOBEC3G.

Comparing untagged and YFP-Vif, both proteins are able to degrade APOBEC3G in HEK293T cells. CBF-β enhances Vif-mediated degradation of APOBEC3G.
**FRET assay confirms *in vivo* binding**

Fluorescence Resonance Energy Transfer (FRET) acceptor photobleaching exploits the ability for an acceptor fluorophore (e.g. YFP) to quench a donor fluorophore (e.g. CFP) signal when in close proximity (~1-10 nm). When the acceptor fluorophore is bleached, the signal from the donor is increased if the two fluorophores are in close proximity. Thus, this technique can be used as a proxy for whether two proteins, which are fused to two FRET pairs (e.g. YFP and CFP), physically interact.

YFP and CFP-fused Vif and CBF-β constructs were created and introduced in HEK293T cells[131]. Twenty four (24) hours after transfection, cells were imaged by confocal fluorescence microscopy. The negative control (co-expressed CFP + YFP) had a very low FRET efficiency of approximately 1%. As expected, the calculated FRET efficiency for unbleached samples was small due to minimal change in donor signal intensity during unbleached acquisition of the collected images. In contrast, FRET acceptor photobleaching indicated a physical interaction between YFP-Vif and CFP-CBFβ, when co-expressed. CFP-CBFβ intensity increased after photobleaching YFP-Vif, indicating dequenching of the donor signal. After photobleaching, the FRET efficiency for the experimental sample was approximately 9.5% compared to the positive control efficiency (CFP-YFP dimer) 18.7%. The FRET efficiency for YFP-Vif and CFP-CBF-β is approximately half that of the positive control which may reflect an increased distance between YFP and CFP molecules when fused to Vif (**Figure 3-8**).
Figure 3-8. FRET efficiency for co-expressed YFP-Vif and CFP-CBF-β in HEK293T cells.

**FRET Efficiency % = \( \frac{F_{DΔ}}{F_{Dpost}} \) * 100**
CBF-β binds and increases Vif solubility *in vitro*

Structure-function examination of full-length Vif has been limited due to difficulty in obtaining suitable quantities of soluble full-length Vif protein[144-147]. Previously, in an attempt to overcome this limitation, a denaturing/refolding method was developed for purifying soluble recombinant Vif; however, this approach produced Vif protein that was prone to forming high molecular weight aggregates in solution[144]. Vif's tendency to aggregate and become insoluble has limited its structural characterization and functional analysis[146]. Co-expression of binding partners has been shown to improve the solubility and stability of various proteins[148]. We have determined that *in vitro* co-expression of Vif with Elo B/C and CBF-β can greatly improve the solubility of full-length Vif[103]. In addition, C-terminal truncated Vif mutants of up to 140 amino acids can still interact with CBF-β[103]. Both separately purified and co-expressed Cul5 (residues 1–393) readily interacts with this complex[103]. Vif:CBF-β-Elo B/C:Cul5 complexes purified by our strategy are not prone to aggregate and are therefore ideal for facilitating future structural and biochemical studies of Vif function and complex assembly.

CBF-β co-expression improves the solubility of Vif

To identify strategies that could result in the expression of large quantities of soluble full-length Vif recombinant proteins, we constructed various prokaryotic expression vectors for HIV-1 Vif and its co-factors. Recombinant Vif protein (residues 1–192) was efficiently expressed in *E. coli* BL21(DE3) but remained
predominantly insoluble as indicated by Coomassie staining. The Vif protein was also identified by immunoblotting using a Vif-specific antibody (Figure 3-9A). Co-expression with Elo B/C improved the solubility of Vif, but only to a limited extent. When Vif was co-expressed with CBF-β-140-His (residues 1–140 of CBF-β with six histidine residues at the N-terminus), the solubility of Vif improved significantly. Approximately 67% of the total Vif protein became soluble in the presence of CBF-β-140-His. Expressing CBF-β and Elo B/C together further enhanced the solubility of Vif. When Vif was co-expressed with CBF-β and Elo B/C, 90% of the Vif proteins became soluble (Figure 3-9B).

CBF-β interacts with Vif

The ability of CBF-β-140-His to increase the solubility of Vif suggests that there is an interaction between Vif and CBF-β-140-His. To determine whether Vif and CBF-β could interact directly, we attempted to co-precipitate Vif with CBF-β-140-His and found that Vif in the soluble fraction could be efficiently pulled down by the CBF-β-140-His on a nickel column. The presence of Vif and CBF-β-140-His in the soluble input fraction and the co-precipitated samples was confirmed by immunoblotting using a Vif- or CBF-β-specific antibody.

There are two major CBF-β isoforms that are highly conserved in mammals: isoform1 has 182 amino acids, while isoform 2 has a 187 amino acid sequence that is generated by alternative splicing[133]. The two isoforms differ in the last 22 amino acids. Human and mouse CBF-β differ by two amino acids (42 A/T and
Next, we asked whether the natural isoforms of CBF-β could interact with Vif and found that an interaction did indeed occur between HIV-1 Vif and isoform 1 CBF-β 182 as well as isoform 2 CBF-β 187 in co-precipitation experiments. To our knowledge, this is the first reported evidence of a direct interaction between HIV-1 Vif and various forms of CBF-β, in vitro. Our data also indicate that amino acids 1–140 of CBF-β are sufficient for HIV-1 Vif binding.

**Purified Vif:CBF-β:Elo B/C proteins form a stable monomeric complex**

Soluble Vif and CBF-β-140 complexes were purified by nickel affinity chromatography and analyzed by gel filtration using a Superdex200 10/300 GL size exclusion column. Gel filtration analysis suggested that Vif and CBF-β-140 formed a large aggregated complex of approximately 1000 kDa. Protein analysis by Coomassie staining of the peak fraction after separation by SDS-PAGE suggested a 1:1 ratio of Vif:CBF-β-140. Full length or truncated CBF-β were monomeric in solution. This observation supports previous findings that Vif directly interacts with CBF-β. Gel filtration analysis of purified Vif:CBF-β-140:Elo B/C revealed that the complex formed a homogeneous complex of 65–75 kDa. Protein analysis by Coomassie staining of the peak fraction indicated a 1:1:1:1 ratio of Vif:CBF-β-140:Elo B:Elo C or Vif:CBF-β-187:Elo B:Elo C. The calculated molecular weight of the monomeric Vif:CBF-β-140:Elo B/C complex (65 kDa) was in close agreement with our gel filtration results (~75 kDa) suggesting that Vif:CBF-β:Elo B/C complex is a monomeric complex in solution (Figure 3-10).
The stability of the purified Vif:CBF-β-140 complexes was low: at 4C, the complexes precipitated after only a few hours. After 16h at 4C, 50% of the Vif protein precipitated. More Vif protein than CBF-β-140 protein appeared in the precipitates, although the initial ratio of Vif and CBF-β was about 1:1. In contrast, the Vif:CBF-β-140:Elo B/C complexes were more stable: only a trace amount of Vif precipitated after 16h at 4C.

Previous studies have suggested that HIV-1 Vif can bind RNA[149]. We found that the Vif:CBF-β-140:Elo B/C complexes were resistant to RNase treatment. Purified Vif:CBF-β-140:Elo B/C complexes were untreated or treated with 40 mg/ml of RNase A and 20 U/ml RNase T1 at 37C for 4h. After buffer exchange, the treated samples were purified using nickel columns. RNase treatment did not affect the co-purification of Vif, Elo B, and Elo C with CBF-β-140-His when compared to the untreated sample. These data suggest that the Vif:CBF-β:Elo B/C complexes are not RNA-dependent. The OD 280/260 ratio in the peak fraction of the Vif:CBF-β-140:Elo B/C complexes also argued against the presence of RNA.

**Interaction of CBF-β with Vif truncation mutants**

We next asked which region of Vif was required for the interaction between Vif and CBF-β. Two truncated Vif mutants spanning residues 1–176 and 1–140 were constructed and co-expressed with CBF-β-140-His. Truncated Vif in the soluble fractions was analyzed by co-precipitation with CBF-β-140-His using nickel
beads. SDS-PAGE and Coomassie staining indicated that both truncated Vif176 and Vif140 co-precipitated with CBF-β-140-His; this finding was confirmed by immunoblotting with a Vif- or CBF-β-specific antibody. The pull-down fractions were further analyzed by size exclusion. Both Vif176-CBF-β-140 and Vif140-CBF-β-140 formed large aggregates. Peak fractions were analyzed by SDS-PAGE followed by Coomassie staining. Both Vif176-CBF-β-140 and Vif140-CBF-β-140 complexes showed a 1:1 ratio of Vif:CBF-β. These results suggested that N-terminal residues 1–140 of HIV-1 Vif are sufficient for CBF-β binding.

Vif:CBF-β:Elo B/C forms a complex with Cul5

Because binding to Cul5 is essential for Vif-mediated ubiquitination and degradation of target proteins such as APOBEC3G and APOBEC3F[60], we next determined whether these purified Vif:CBF-β-140:Elo B/C complexes could interact with Cul5. Vif:CBF-β-140:Elo B/C complexes and Cul5 NTD were purified separately. The purified Vif:CBF-β:Elo B/C complexes were mixed with purified Cul5 protein and subsequently analyzed by gel filtration. As compared to Vif:CBF-β-140:Elo B/C (blue line) and Cul5 (cyan line), the mixture (red line) had an earlier elution peak (Figure 3-10). This result suggested that the Vif:CBF-β-140:Elo B/C complex forms a complex with Cul5. SDS-PAGE analysis of the peak fractions further confirmed that Cul5 and Vif:CBF-β-140:Elo B/C formed a complex. Molecular weight analysis by gel filtration indicated that the molecular size of the Vif:CBF-β-140:Elo B/C:Cul5 complex was approximately 135 kDa, equal to the sum of Cul5 (~62 kDa) and Vif:CBF-β-140:Elo B/C (~75 kDa)
(Figure 3-10 and 3-11). Further analysis using affinity pull-down via His- tagged CBF-β confirmed the formation of Cul5:Vif:CBF-β-140:Elo B/C complexes. These Cul5:Vif:CBF-β-140:Elo B/C complexes were stable at 4C over 16h. In addition to separately expressing and purifying Cul5 and the Vif complex, we can co-express Cul5 with the Vif:CBF-β-140- Elo B/C to form a complex that includes Cul5.
Figure 3-9. Vif solubility in the presence of binding partners

Soluble Vif protein was obtained by co-expression with CBF-β and Elo B/C. Vif was untagged, while CBF-β was tagged with 6X His residues at the N-terminus. (A) Solubility of Vif alone and of co-expressed Vif:Elo B/C, Vif:CBF-β, and Vif:CBF-β:Elo B/C. Tot, total lysate; S, supernatant; P, pellet; CBB, Coomassie staining. (B) Quantification of Vif protein by immunoblotting in (B).
Figure 3-10. Size-exclusion chromatogram of Vif-E3 complexes.

Gel filtration profile of Vif:CBF-β:Elo B/C complexes (blue line), Vif:CBF-β:Elo B/C:Cul5 complexes (red line), and Cul5 (cyan line). The elution volume (Ve) in milliliters and molecular weights are indicated. The black line corresponds to the protein standards: 1, thyroglobulin (670,000 Da); 2, gamma globulin (158,000 Da); 3, ovalbumin (44,000 Da); 4, myoglobin (17,000 Da); 5, vitamin B12 (1,370 Da).
**Figure 3-11. Coomassie stain of purified Vif-E3 complex with molecular weight prediction**

Peak fractions of protein complexes were checked by SDS-PAGE with Coomassie staining (upper panel): Cul5N:Vif:CBF-β:Elo B/C complexes (lane 1), Vif:CBF-β:Elo B/C complexes (lane 2), and Cul5N (lane 3). The molecular sizes of these complexes (as compared to the molecular standards) are shown in the lower panel. Ve, elution volume; Vo, void volume.
Attempts to crystallize Vif:CBF-β complexes with and without Cul5 and/or Elo B/C

Despite the current knowledge of how Vif recruits the components of the E3 ubiquitin ligase, additional details are important for a more clear understanding of the assembly. It is well understood that Vif’s lack of structural integrity in the absence of binding partners contributes to the lack of a crystal structure for full-length Vif. To date, the only structural information available for Vif is the C-terminal truncated B/C box in complex with Elo B/C[76]. Additional knowledge about Vif binding partners is critical to overcoming the obstacle of obtaining a crystal structure. We have further characterized a previously unknown binding partner, CBF-β. In hopes that the addition of CBF-β to our Vif:E3 ligase complexes would stimulate growth, we screened all purified Vif complexes for crystallization. See Table 3-1 for a summary list of the concentrations and number of conditions screened for each purified complex. While a detailed screen of all Vif:CBF-β complexes has not been performed, to date no Vif complex crystals have been obtained using the conditions summarized in the table.
<table>
<thead>
<tr>
<th>Complex</th>
<th>Concentration (mg/mL)</th>
<th># of Conditions</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vif1-192:CBF-β:Elo B/C</td>
<td>5</td>
<td>200</td>
<td>Affinity, size exclusion</td>
</tr>
<tr>
<td>Vif1-176:CBF-β:Elo B/C</td>
<td>5</td>
<td>200</td>
<td>Affinity, size exclusion</td>
</tr>
<tr>
<td>Vif1-154:CBF-β:Elo B/C</td>
<td>5</td>
<td>200</td>
<td>Affinity, size exclusion</td>
</tr>
<tr>
<td>Vif1-140:CBF-β</td>
<td>5</td>
<td>200</td>
<td>Affinity</td>
</tr>
<tr>
<td>Vif1-126:CBF-β</td>
<td>5</td>
<td>200</td>
<td>Affinity, size exclusion</td>
</tr>
<tr>
<td>Vif1-192:CBF-β:Elo B/C:Cul5</td>
<td>5</td>
<td>300</td>
<td>Affinity, size exclusion</td>
</tr>
<tr>
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<td>5</td>
<td>300</td>
<td>Affinity, size exclusion</td>
</tr>
<tr>
<td>Vif1-192(M3A/L):CBF-β:Elo B/C:Cul5</td>
<td>5</td>
<td>100</td>
<td>Affinity, size exclusion</td>
</tr>
</tbody>
</table>

Table 3-1. Vif-E3 ligase complex crystallization conditions.
Discussion

Here, I have reported an enhanced understanding of the significance that the molecular interaction between viral Vif and host CBF-β plays during HIV-1 infection. First, our lab discovered that this viral-cellular protein interaction is required in order for Vif to mediate the polyubiquitination and subsequent proteasomal degradation of an HIV restriction factor, APOBEC3[101]. Next, I have demonstrated that this interaction is likely to take place in the cytoplasm of mammalian cells using both live cell confocal imaging and a detergent based subcellular fractionation protocol. Previously, Farrow et al. reported that Vif cytoplasmic localization is required for APOBEC3G neutralization and successful viral replication[150, 151]. In their study, they found that the Vif protein from an HIV-1 isolate, LTNP4, which grew poorly in parental T cell lines unless wild-type Vif was over-expressed, contained a naturally occurring mutational motif[150]. The motif was observed at amino acid positions 90 to 93, replacing RKRR with K KRK, a motif that commonly functions as a nuclear localization signal (NLS) in many viral and cellular systems[150]. Thus, our discoveries that CBF-β is required for Vif anti-APOBEC3 function and that the interaction between CBF-β and Vif contributes to Vif cytoplasmic localization provide further support for the importance of Vif cellular localization.

Additionally, I provide evidence that CBF-β directly interacts with Vif alone and in complex with E3 ligase components, in vivo and in vitro. Using a live cell imaging
approach, we observed co-localization of yellow fluorescent protein (YFP)–Vif and cyan fluorescent protein (CFP)–CBF-β in the cytoplasm of transfected HEK293T cells. In addition, we obtained a positive FRET signal from imaged cells co-expressing an YFP-fused Vif and CFP-fused CBF-β. Furthermore, prokaryotic over-expressed Vif proteins were found to be more soluble in the presence of its partner proteins, Elo B/C and CBF-β. In fact, co-expression of HIV-1 Vif with CBF-β in the absence of all other human factors increased Vif solubility in *E. coli*. Soluble Vif could be co-precipitated with both His-tagged full length or truncated (residues 1-140) CBF-β. In addition, Vif residues 1-140 are sufficient for interaction with CBF-β. Thus, we provide evidence that the B/C box and cullin box of Vif are not required for the direct interaction. In the absence of binding partners, previous research has suggested full length Vif appears to be unstructured and poorly soluble, *in vitro*[146]. Wolfe *et al.* were able to obtain soluble C-terminal domain fragments of Vif in complex with Elo B/C and Cul5[107]. Attempts at characterizing full length Vif in complex with Elo B/C and Cul5 were unsuccessful, suggesting that the N-terminus was responsible for Vif’s poor solubility, in the absence of N-terminal binding partners[107]. We have shown that CBF-β binds the N-terminal region of Vif, specifically requiring hydrophobic interactions at amino acids W21 and W38. We hypothesize that the exposure of the N-terminal hydrophobic surface may contribute to Vif insolubility when expressed alone. *In vivo*, CBF-β appears to be necessary for Vif:Cul5 binding, though CBF-β does not bind Cul5 directly. Thus, a possible role for CBF-
β would be to stabilize Vif structure and promote the assembly of the Vif:Cul5 E3 ubiquitin ligase complex.

Association of Vif with CBF-β alone, and especially in combination with Elo B/C, greatly increases the solubility of full-length Vif. We have shown that a stable complex containing Vif:CBF-β-140:Elo B/C can be purified in large quantities. More importantly, the Vif:CBF-β-140:Elo B/C complexes we produced could interact with purified Cul5 and form stable Cul5:Vif:CBF-β-140:Elo B/C complexes. This successful purification of monomeric Vif:E3 ligase complexes in high purity will greatly facilitate future biochemical studies, structural determination, and functional analyses in this field. In addition, our strategy for purifying Cul5:Vif:CBF-β-140:Elo B/C complexes may lead to useful screening approaches for identifying novel anti-HIV drug candidates.
CHAPTER 4

VIF N-TERMINAL MOTIF IS REQUIRED FOR CUL5 BINDING


4.1 Introduction

4.2 Methods

4.3 N-terminal motif in full length Vif is important for Cul5 interaction, in vitro

4.4 Vif N-terminal motif binds Cul5 in mammalian cells and is required for APOBEC3 degradation and restoring HIV infectivity

4.5 Assessing the binding affinities and thermodynamics of Vif (wild-type and point mutants): CBF-β:Elo B/C with Cul5 using isothermal titration calorimetry
4.6 Binding affinity between Cul5 and Vif N-terminal single point mutant is significantly reduced

4.7 Vif mutant complexes are structurally different from wild-type

4.8 Discussion
Introduction

In order to hijack the Cul5-E3 ligase complex, Vif mimics cellular protein motifs that are responsible for recruiting ubiquitin ligase components[104, 106, 112, 152, 153]. Many of these motifs have been found within Vif’s carboxyl terminus. Vif contains a conserved SOCS box domain, including the B/C Box motif (residues 144-155) and the cullin box (residues 158-173) [104, 152]. The B/C Box serves as the primary attachment point between Vif and Elo B/C[60, 104]. Structural and biophysical data indicates that there is a second weaker interaction between the semi-conserved Vif cullin box and Elo B/C, but this has been found not to be required for Elo B/C and Vif interaction[76, 108]. Rather, this weak interaction has been demonstrated to position the cullin box, particularly the PPLP motif, for Cul5-Vif interaction[76, 108]. Importantly, a single amino acid substitution in the highly conserved lentiviral Vif SOCS box reduces the ability of Vif to block virion packaging of APOBEC3G and to fully suppress the antiviral activity of APOBEC3G[104, 152]. In addition to the role of the cullin box, a Vif zinc binding domain, $^{108}Hx_5-Cx_{17-18}-Cx_{3-5}-H^{139}$ (HCCH), has been reported to mediate the primary interaction with Cul5[106, 110, 112, 113]. As expected, mutation of either the HCCH domain or the cullin box severely inhibits Cul5 binding and APOBEC3G degradation[76, 106, 108, 113, 116].

While the C-terminus has been implicated in binding several E3 ubiquitin ligase components, several discontinuous Vif residues in the amino-terminus have been reported to be necessary for APOBEC3G/F interaction[121, 123, 125, 126, 128,
However, reports have also demonstrated that the C-terminal cullin box mediates contact with APOBEC3G[124]. In addition, CBF-β binds an amino-terminal motif of Vif; Vif tryptophan residues 21 and 38 are key mediators of this interaction[101]. We have demonstrated that silencing CBF-β expression in mammalian cells severely suppresses Vif:Cul5 formation[101]. In addition, recombinant CBF-β increases Vif solubility, in vitro[103]. These two points suggest that CBF-β is important for the structural integrity of Vif molecules.

While both CBF-β and APOBEC3 proteins interact with Vif at the N-terminus, it is unclear whether Vif makes contacts with E3 ubiquitin ligase components in the N-terminus. Others and we have reported amino acids in the N-terminus that are important for APOBEC3G/F degradation, yet do not mediate direct interaction with APOBEC3G/F[121, 123]. We also showed that a N-terminal truncated (amino acids 99-192) Vif can not precipitate endogenous Cul5 in 293T cells[101], although others have demonstrated that a N-terminal truncated Vif has a high binding affinity for Cul5, in vitro[107]. However, a group recently reported Cul5 binds to full length Vif with greater affinity when compared to a C-terminal half fragment containing both the HCCH domain and cullin box[115]. Taken together, these reports suggest that Cul5 may interact with additional Vif residues in the N-terminus.

Alanine-scanning mutagenesis is a technique used to determine either the catalytic or functional role of protein residues. Alanine is used to replace an
amino acid residue of choice and essentially eliminates the side chain beyond
the β-carbon, yet does not typically alter the main-chain conformation (as can
glycine or proline). In addition, the alanine residue replacement does not impose
extreme electrostatic or steric effects. In this chapter, I use alanine scanning
mutagenesis extensively to delineate the interaction between Vif and Cul5.
Surprisingly, I find that Cul5 requires a Vif N-terminal motif (25VxHxMY30) for
efficient binding. More importantly, this motif is required specifically for Cul5, but
not Elo B/C or CBF-β, to interact with full length Vif in mammalian cells as well as
recombinant protein in solution. Using isothermal titration calorimetry, I
demonstrate that a single point N-terminal Vif mutant has a severely reduced
binding affinity for Cul5 compared to wild-type Vif. Vif N-terminal mutants that
disrupted Cul5 formation were also less efficient at degrading APOBEC3G/F and
restoring HIV infectivity in the presence of APOBEC3G. Although the Vif N-
terminal amino acids were necessary for Cul5 interaction, the mutation of each
residue to alanine induced a change in the secondary structure of the Vif:CBF-β-
140:Elo B/C complex as suggested by results from circular dichroism
spectroscopy and size-exclusion chromatography experiments. Thus, it is
unclear whether this motif is required for direct interaction or whether the motif is
required for the structural integrity of the C-terminal HCCH motif. Finally, we
provide clear in vitro evidence that full length wild-type Vif requires Elo B/C
bound at its B/C box in order to recruit Cul5.
Methods

Plasmid Construction

_E.coli constructs:_ Human Cul5 ([103]) and NL4.3 Vif coding sequences were cloned into the pET-Duet plasmid using 5’ Ncol / 3’ EcoRI (MCS1) and 5’Ndel / 3’ Xhol (MCS2) restriction sites, respectively to create Cul5/Vif pET-Duet constructs. All point mutants of Vif were created by site-directed mutagenesis.

Elongin B and Elongin C (residues 17 to 112) in the pACYC-Duet plasmid were a gift from Alex Bullock. The genes for Elongins B and C were subcloned into the pCDF-Duet vector. CBF-β isoform 2 (residues 1-182) and truncated CBF-β (residues 1-140) from human were cloned into MCS1 of pRSF-Duet ([103]) to create 6X histidine-tagged CBF-β-Full length(-FL) and CBF-β-N-terminus (-N). wild-type and mutants of Vif were subcloned into MCS2 of pRSF-Duet using 5’Ndel / 3’Xhol restriction sites with CBF-β to create his-CBF-β/Vif pRSF-Duet.

_Mammalian cell constructs:_ HVif-HA was constructed by PCR amplifying codon optimized Vif from pcDNA-HVif and cloning the product into VR1012 plasmid via EcoRI and BamHI restriction sites. The following primers were used to create HVifHA: forward 5’ – CTCTCTGAATTGAACTAGCTGGG – 3’ and reverse 5’ - ATGGATCCCTACGCTTAATCTGGGGACGTGTAAGGGTAGTGTCATCCATTG – 3’ (HA). To generate yellow fluorescent protein (YFP) epitope-tagged NL4-3 viral infectivity factor (Vif), pCDNA HVif wild-type and mutant constructs were used to PCR amplify the vif coding region and cloned into the BamH1 and EcoRI sites of pEYFP-N1.
Protein expression and purification

Plasmids were transformed or co-transformed into *E. coli* NiCo21(DE3) cells (New England Biolabs C2529H) according to manufacturer’s protocol. Cells were incubated for two hours at 37°C and plated on media with appropriate antibiotic selection marker. If more than one plasmid was transformed, cells were centrifuged at 5,000rpm for 5min and then all cells were plated. Plates were incubated at 37°C overnight and single colonies were chosen for protein production. Cells were grown to an OD of 0.8-1 at 37°C, cooled to 23°C, and induced overnight at 23°C with 0.2 mM isopropyl-D-thiogalactopyranoside (IPTG). Harvested cells containing his-CBF-β:Vif:Elo B/C complexes were lysed in lysis buffer (1X PBS, 0.25mM TCEP, 30mM imidazole), sonicated, and centrifuged at 10,000g for 20 min. Nickel affinity purification – soluble supernatant was added to Ni-NTA beads (Invitrogen) and incubated at room temperature for 2h. Beads with bound protein were washed 6X with wash buffer (1XPBS, 0.25mM TCEP, 40mM imidazole). Bound protein was eluted with elution buffer (1X PBS, 0.25mM TCEP and 250mM imidazole). Gel filtration using a Superdex 200 column (GE Healthcare) was utilized to remove trace contaminants. Harvested cells containing Cul5-NTD (residues 1 to 393 with two point mutations, V341R and L345D) were lysed in lysis buffer (1X PBS and 0.25mM TCEP). The supernatant was transferred to glutathione-Sepharose 4B beads (GE Healthcare) for purification. The GST tag was then removed using
PreScission protease (GE Healthcare) in lysis buffer at 4°C for 36h. Cul5-NTD was subsequently purified by gel filtration.

**Transfection, co-immunoprecipitation, and infectivity assay.**

HEK293T cells were maintained at 37°C, 5% CO2 in DMEM (Invitrogen, catalog 11995-073) with added 10% fetal bovine serum (Sigma, catalog F4135). Vif-HA and APOBEC3-V5 plasmids were complexed with PEI-Max at a 2.5:1 ratio in Opti-Mem (Invitrogen, catalog 31985-070) buffer for 30min. Cells were transfected with plasmid:PEI complexes and harvested 48 h later, washed with PBS and lysed in lysis buffer (50mM Tris, 75mM NaCl, 0.1% NP-40 and Complete Protease Inhibitor Cocktail Tablet (Roche, catalog 04693159001) (pH 7.4)) at 4°C for 15min, followed by centrifugation at 10,000 × g for 20 min. HA immunoprecipitation was carried out by mixing soluble lysates of transfected cells from a 10cm dish with 40uL anti-HA affinity matrix beads (Roche, catalog 11815016001) and incubating the mixture at 4°C for 3h. The samples were washed six times with wash buffer (20 mM Tris, 50 mM NaCl, 0.1 mM EDTA and 0.05% Tween-20 (pH 7.5)). Bound protein was eluted with elution buffer (100mM glycine-HCl, pH 2.5). The eluted protein was analyzed by SDS-PAGE and immunoblotting with appropriate antibodies. Infectivity (MAGI) assay was performed as previously described.

**Live Cell Confocal Imaging**
Plasmids (Vif-YFP 2ug and CBF-β 0.5ug) were transfected into 293T cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. Cells were visualized at 25°C using a Zeiss LSM510-Meta confocal imaging system equipped with four argon lasers (458, 477, 488, and 514 nm lines), two HeNe lasers (542 and 633 nm), and one diode laser (405 nm). All images were acquired with a 100X objective, and image analysis and manipulation was performed using Zen 2009 software.

**Immunoblot analysis**

Proteins were separated by SDS-PAGE, followed by transfer to nitrocellulose membrane (Bio-Rad). After blocking with PBS-buffered saline-Tween 20 containing 5% BSA for 20min at room temperature, membranes were incubated with a specific antibody overnight at 4°C. After three washes with PBS-buffered saline-Tween 20, the membranes were stained with an alkaline phosphatase-conjugated secondary antibody (1:3,000, Jackson Immunoresearch) for 2h at room temperature. After three washes with PBS-buffered saline-Tween 20, the membranes were incubated in development buffer containing 5-bromo-4-chloro-3′-indolyolphosphate (BCIP) and nitro-blue tetrazolium (NBT) substrate (Sigma). The antibodies used in this study were specific for: Vif (the AIDS Research Reagents Program, catalog 2221), Cul5 (Santa Cruz Biotechnology Inc., catalog sc-13014), CBF-β (Abcam, catalog ab11921), Elo B (Santa Cruz Biotechnology, Inc, catalog sc-11447), Elo C (BD Transduction Laboratories, catalog 610760), HA (Invitrogen, catalog 715500), and V5 (Invitrogen, catalog R96025).
Isothermal titration calorimetry

ITC experiments were performed using a VP-ITC microcalorimeter from MicroCal/GE Healthcare (Northampton, MA, USA). Each protein or protein complex was purified by a Glutathione or Ni-NTA affinity protocol followed by gel filtration in PBS, pH 7.4, with 0.25mM TCEP. Titrations were conducted by adding Cul5 in steps of 10µL every 300s to the calorimetric cell (volume ~1.4mL) containing wild-type or mutant Vif:CBF-β:Elo B/C complex. The concentrations of Cul5 and the Vif:CBF-β:Elo B/C complex were 25 and 2.5µM, respectively. Saturation was reached in 20-28 injections. All experiments were conducted at 25°C. The heat evolved upon each injection of Cul5 was obtained from the integral of the calorimetric signal. The heat associated with binding to Vif:CBF-β:Elo B/C complex in the cell was obtained by subtracting the heat of dilution from the heat of reaction. The individual heats were plotted against the molar ratio, and the enthalpy change (ΔH) and association constant (K_a = 1/K_d) were obtained by nonlinear regression of the data.

Circular Dichroism Spectroscopy

CD experiments were conducted using a Jasco J-710 spectropolarimeter. Wavelength scans were performed with 0.4 mg/mL Vif complexes prepared in buffer containing PBS with 0.25mM TCEP in a 0.1 cm cuvette in a water-jacketed cell. Spectra were averaged over 3 consecutive scans collected from 195 to 260
nm. The individual scans were recorded using a scan rate of 20 nm/min, a bandwidth of 1 nm and a response time of 2 s per point. Buffer scans were accumulated and subtracted from the sample scans and the mean residue ellipticity was computed. The temperature was kept constant at 25 °C. Spectral analysis was performed using the Dichroweb online analysis program. Initial and final wavelengths were 260 and 195 nm, respectively, in wavelength steps of 0.2 nm. Analysis was performed using the K2D algorithm.
**N-terminal motif in full length Vif is important for Cul5 interaction, in vitro.**

Others and we have attempted to produce stable complexes including full length Vif that are conducive to crystallization to obtain a high resolution structure for the Vif protein. Even with the addition of CBF-β, efforts to crystallize the complex have been futile. In order to obtain protein crystals, a homogenous and pure protein solution is required. Previous reports have highlighted the observation that multiple small species of Vif are expressed due to internal initiation at downstream initiation codons in the *vif* gene (Met8, Met16 and Met29) [155]. We have confirmed the presence of these smaller products and were curious whether modification of these methionines would increase the homogeneity of the Vif complex protein solution. To address this problem, we decided to construct Vif mutant plasmids in which either one or all methionines were replaced with alanine (M8A, M16A, M29A and Met8/16/29A).

First, we decided to confirm that each mutant was capable of forming a complex with CBF-β, Elo B/C and Cul5. Wild-type or mutant Vif was co-expressed with Cul5, Elo B/C, and 6XHis-tagged CBF-β (amino acids 1-140) in NiCo21(DE3) competent *E.coli* (NEB) at 23C with 0.2mM IPTG. After 24hrs, cells were harvested, lysed by sonication, and clarified by centrifugation. His-tagged CBF-β and interacting partners were pulled down from supernatants using Ni-NTA affinity beads (Qiagen). Both Vif M8A and M16A formed a complex with all proteins similar to wild-type. Interestingly, Vif M29A and M8/16/29A were both unable to stably associate with Cul5, yet still interacted with CBF-β and Elo B/C.
Based on band intensity, the calculated relative quantity of Cul5 pulled down by Vif mutants, M29A and M8/16/29A, were reduced by more than 60% and 80%, respectively.

We were surprised to observe a reduction in Cul5 binding upon mutation of a single amino acid in the N-terminus, but were curious whether additional amino acids surrounding Met29 were also capable of disrupting the Vif:Cul5 interaction (Figure 4-1). Indeed, both His27 and Tyr30 contributed to Vif:Cul5 interaction, in vitro, both reducing Cul5 binding by greater than 60%. Interestingly, His28 minimally contributed to the interaction between Vif and Cul5, only reducing Cul5 binding by approximately 20%. Taken together, the experimental evidence suggests that an N-terminal motif regulates the Vif:Cul5 interaction using recombinant protein. We wanted to further explore this possibility in mammalian cells.
Figure 4-1. Vif N-terminal amino acids are responsible for Cul5 interaction, *in vitro*.

While Vif wild-type and H28A mutant pull down Cul5 efficiently, H27A, M29A and Y30A mutants are unable to bind Cul5 efficiently.
Vif N-terminal motif binds Cul5 in mammalian cells and is required for APOBEC3 degradation and restoring HIV infectivity.

While we were confident of our experimental evidence demonstrating that there were amino acids in the Vif N-terminus that made contact with Cul5, in vitro, we wanted to explore the importance of this motif in mammalian cells. Interestingly, this motif resides in a predicted alpha helix. We decided to assess the importance of additional residues within this alpha helix in mediating APOBEC3 degradation. Several single point mutant constructs were created replacing amino acid residues 18-31 with alanine. Next, HEK 293T cells were co-transfected with plasmids containing either APOBEC3G or APOBEC3F with wild-type Vif, mutant Vif or empty vector. As expected, wild-type Vif efficiently mediated APOBEC3G as well as APOBEC3F degradation compared to control vector. Importantly, mutation of Vif residues, His27, Met29, and Tyr30 led to a reduced capacity for Vif to degrade both APOBEC3G and APOBEC3F. Vif mutants, L24A, V25A, and to a lesser extent I31A also inhibited Vif’s ability to degrade both APOBEC3G and APOBEC3F. In agreement with our prior observation that His28 was unimportant for Vif:Cul5 formation, the Vif-H28A mutant along with Vif-R23A were both capable of inducing APOBEC3G and APOBEC3F degradation similarly to wild-type Vif. Finally, lysine 22 and 26 were selectively important for APOBEC3G degradation.

To investigate which Vif N-terminal amino acids interact with Cul5, we immunoprecipitated HA-tagged single point mutants that were inefficient at
degrading both APOBEC3G and APOBEC3F. Vif wild-type and mutant plasmids were transfected into 293T cells. In order to enhance Vif:Cul5 binding as well as Vif and endogenous Cul5 expression, CBF-β was overexpressed and the proteasome inhibitor, MG132, was added to the culture media, respectively (Figure 4-2a). HA-tagged Vif along with bound proteins were immunoprecipitated using anti-HA affinity matrix beads. As expected, wild-type Vif co-precipitated with Cul5 as well as CBF-β and Elo B. Consistent with our previous, Vif residues, Trp21 and Trp38, were important for CBF-β as well as Cul5 binding. Also as expected, Vif mutant, H108A was incapable of binding Cul5, however maintained its ability to bind both Elo B/C and CBF-β. Vif mutants, H27A, M29A, and Y30A, but not H28A were unable to co-precipitate endogenous Cul5, consistent with in vitro results (Figure 4-2b). An additional Vif residue, Val25, also contributed to Cul5 binding. Interestingly, Vif L24A and I31A, which were unable to mediate both APOBEC3G and APOBEC3F degradation, still bound Cul5 similar to wild-type Vif. Furthermore, while a Vif double mutant V25H27A was unable to bind Cul5, it was still able to bind CBF-β and Elo B/C as well as localize to the cytoplasm of the cell similar to wild-type, suggesting that the N-terminal mutants are structurally stable and available for Cul5 cytoplasmic assembly.

Next, we investigated whether N-terminal mutants that were unable to degrade APOBEC3G were restricted in restoring HIV infectivity in the presence of APOBEC3G. 293T cells were transfected with Vif-deficient pNL4.3 plasmid along with plasmids overexpressing APOBEC3G and wild-type or N-terminal
mutant Vif proteins. Virus was harvested from the supernatant after 36h and
used to infect MAGI cells as previously described. As expected, Vif N-terminal
mutants had a reduced ability to restore HIV infectivity in the presence of
APOBEC3G (Figure 4-3). Single alanine mutants, V25A, H27A, M29A, and
Y30A were at least 40% less efficient compared to wild-type at restoring HIV
infectivity. Furthermore, double mutant V25H27A was as inefficient as the H108A
mutant compared to wild-type Vif.
Figure 4-2a. Select Vif N-terminal mutants have a reduced ability to bind Cul5 in HEK293T cells (cell lysate).

HA-tagged Vif wild-type and mutant proteins along with CBF-β were overexpressed in HEK 293T cells. Two days post-transfection, cells were lysed and cleared lysate was mixed with anti-HA matrix affinity beads for 4-8hrs. Incubated beads were washed several times followed by elution of bound proteins. Select Vif N-terminal mutants (V25A, H27A, M29A, and Y30A) that do not efficiently degrade APOBEC3G and APOBEC3F have a reduced ability to co-precipitate Cul5; however, CBF-β and Elo B/C can still bind Vif.
Figure 4-2b. Select Vif N-terminal mutants have a reduced ability to bind Cul5 in HEK293T cells (IP: HA).

HA-tagged Vif wild-type and mutant proteins along with CBF-β were overexpressed in HEK 293T cells. Two days post-transfection, cells were lysed and cleared lysate was mixed with anti-HA matrix affinity beads for 4-8hrs. Incubated beads were washed several times followed by elution of bound proteins. Select Vif N-terminal mutants (V25A, H27A, M29A, and Y30A) that do not efficiently degrade APOBEC3G and APOBEC3F have a reduced ability to co-precipitate Cul5; however, CBF-β and Elo B/C can still bind Vif.
Figure 4-3. Vif N-terminal mutants are inefficient at restoring HIV infectivity.

Vif wild-type and mutant containing virus were produced and used to infect MAGI cells. Infected cells were stained using X-gal. The histogram demonstrates that Cul5-binding deficient Vif mutants were inefficient at restoring HIV infectivity in the presence of APOBEC3G. Error bars represent the standard error from triplicate experiments. Capsid p24 levels are shown in the western blot.
Binding affinity between Cul5 and Vif N-terminal single point mutant is significantly reduced.

Recently, a group reported that the binding affinity between Cul5 and full length Vif complexed with Elo B/C and CBF-β was enhanced by nearly 80-fold compared to a N-terminal truncated Vif:Elo B/C complex[115]. Since we had observed reduced binding between Vif mutants and Cul5 by pull-down assays, we postulated that single point mutants in the N-terminus of Vif would reduce the binding affinity for Cul5. To test our hypothesis, we performed ITC experiments to directly measure the affinity between recombinant Cul5 and wild-type Vif as well as N- and C- terminal mutants (V25A and H108A) complexed with CBF-β and Elo B/C.

Vif complexes including his-tagged full length CBF-β and Elo B/C in addition to GST-Cul5 were separately overexpressed in E. coli and purified using affinity and size exclusion chromatography, as previously described. ITC experiments were employed to determine the binding thermodynamics between Vif wild-type and mutant complexes. Wild-type or mutant Vif:CBF-β:Elo B/C complexes in the calorimetric cell were titrated by stepwise additions of Cul5. Cul5 bound to the wild-type Vif:CBF-β:Elo B/C complex with an affinity of 5.9nM (Figure 4-4 and Table 4-1), which corresponded to change in Gibbs free energy of -11.2kcal/mol at 25C. The contributions from the enthalpy (ΔH) and entropy (-TΔS) to Gibbs free energy were -9.0 and -2.2 kcal/mol, respectively. The values are consistent with the previously published measurements by Salter et al. Additionally, Vif N-
terminal mutant complexes that were still capable of binding to Cul5 (i.e. L24A and H28A) bound to Cul5 with an affinity similar to wild-type.

Next, a change from valine to alanine at position 25 in the N-terminal mutant Vif V25A complex resulted in a loss of enthalpic interactions of -3 kcal/mol, which translates to a 90-fold loss in binding affinity (Kd = 511 nM). Furthermore, the N-terminal mutant Vif H27A complex was similar to the V25A complex with a binding affinity of 660 nM. Intriguingly, the Vif C-terminal point mutant (H108A), which is part of the reported HCCH motif, only reduced the binding affinity of Cul5 for Vif by 6-fold (Kd = 36 nM). The loss in enthalpic interactions were, in fact, larger for the binding to the H108A mutant (ΔH = -4.0 kcal/mol) but because the entropy contribution was more favorable (TΔS = -6.1 kcal/mol) and partially compensated the loss in enthalpy, the overall binding affinity was reduced to a lesser extent than for the binding to the V25A mutant (Table 4-1).
Figure 4-4. Representative isotherm for wild-type Vif and Cul5.

Isothermal titration calorimetric analyses of the interaction between Cul5 and Vif wild-type reveal a prototypical sigmoidal curve for high affinity protein interactions.
<table>
<thead>
<tr>
<th>Syringe sample</th>
<th>Cell sample</th>
<th>Kd (nM)</th>
<th>ΔG (kcal/mol)</th>
<th>ΔH (kcal/mol)</th>
<th>-TΔS (kcal/mol)</th>
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</thead>
<tbody>
<tr>
<td>Cul5-N</td>
<td>Vifwt:Elo</td>
<td>5.9 +/- 1.7</td>
<td>-11.2 +/- 0.2</td>
<td>-9.0 +/- 0.2</td>
<td>-2.2 +/- 0.1</td>
</tr>
<tr>
<td></td>
<td>B/C:CBF-β-FL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cul5-N</td>
<td>VifVal25Ala:</td>
<td>511 +/- 100</td>
<td>-8.6 +/- 0.25</td>
<td>-6 +/- 0.3</td>
<td>-2.6 +/- 0.2</td>
</tr>
<tr>
<td></td>
<td>Elo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B/C:CBF-β-FL</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cul5-N</td>
<td>VifHis108Ala:Elo</td>
<td>36 +/- 11</td>
<td>-10.1 +/- 0.1</td>
<td>-4 +/- 0.2</td>
<td>-6.1 +/- 0.4</td>
</tr>
<tr>
<td></td>
<td>B/C:CBF-β-FL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cul5-N</td>
<td>VifLeu24Ala:Elo</td>
<td>3.5 +/- 1.0</td>
<td>-11.5 +/- 0.2</td>
<td>-10.2 +/- 0.2</td>
<td>-1.3 +/- 0.1</td>
</tr>
<tr>
<td></td>
<td>B/C:CBF-β-FL</td>
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<td></td>
</tr>
<tr>
<td>Cul5-N</td>
<td>VifHis27Ala:Elo</td>
<td>660 +/- 130</td>
<td>-8.4 +/- 0.15</td>
<td>-4.9 +/- 0.3</td>
<td>-3.5 +/- 0.3</td>
</tr>
<tr>
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<td>B/C:CBF-β-FL</td>
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<tr>
<td>Cul5-N</td>
<td>VifHis28Ala:Elo</td>
<td>6.6 +/- 1.7</td>
<td>-11.2 +/- 0.2</td>
<td>-7.6 +/- 0.15</td>
<td>-3.6 +/- 0.15</td>
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<td></td>
<td>B/C:CBF-β-FL</td>
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</tbody>
</table>

Table 4-1. Recombinant Vif mutants have a lower Cul5 binding affinity compared to wild-type Vif using Isothermal titration calorimetry.

Table for Vif wild-type and mutants demonstrating a lower affinity between mutant Vif and Cul5 compared with Vif wild-type.
**Vif mutant complexes are structurally different from wild-type.**

To determine if the mutant complexes were structurally similar to wild-type Vif complexes, the complexes were all analyzed by circular dichroism spectroscopy. Vif complexes (0.4 mg/mL) were measured at room temperature in PBS with TCEP (0.25 mM). Subsequent analysis of the CD spectra with the Dichroweb[156, 157] analysis program suggested that all of the N-terminal mutant Vif complexes that don’t bind Cul5 (i.e. V25A, H27A, M29A, and Y30A) had a higher percentage of alpha helical structure and lower percentage of beta sheet structure (Figure 4-5 and Table 4-2). Surprisingly, the CD spectra for the Vif H108A C-terminal mutant complex suggested that our control mutant is also structurally different than wild-type. Furthermore, Vif complexes that did bind Cul5 were very similar in structure. Additionally, gel filtration chromatography results appear to be consistent with this change in secondary structure. All mutant complexes, which were unable to bind Cul5, eluted slower from a Superdex 200 (GE Healthcare) size-exclusion column.
Figure 4-5. CD spectroscopy analysis reveals that Vif mutant complexes are structurally different from wild-type.

Purified Vif complexes including 6X-His-CBF-β (residues 1-182) and Elo B/C were purified by nickel affinity and size exclusion chromatography. Each complex was analyzed by circular dichroism spectroscopy. CD spectra for Vif wild-type and mutant complexes showed a distinction in the minima at 208 and 222nm for mutant complexes that do not bind Cul5, suggesting these mutants have more alpha helical structure.
Table 4-2. CD spectroscopy analysis reveals that Vif mutant complexes are structurally different from wild-type.

Spectra analysis reveals differences between wild-type and mutant complex secondary structure and confirms that the mutants that do not bind Cul5 have a higher percentage of alpha helical structures; however, the percentage of beta-sheet structures is reduced.
Full length Vif requires Elo B/C binding for Vif:Cul5 formation.

Previous reports have suggested that Elo B/C is required for Vif:Cul5 formation[107, 108]. These reports have relied primarily on Vif point mutants in mammalian cells or recombinant fragments of Vif, due to the difficulty in expressing full length Vif in vitro in the absence of CBF-β. Since CBF-β greatly enhances Vif solubility, we can more easily explore how full length wild-type Vif interacts with partner proteins, in vitro. Recombinant Vif complexes were formed by co-transforming and expressing constructs containing genes for wild-type Vif (full length 1-192, 1-154, and 1-140) with his-tagged CBF-β, Elo B/C and Cul5 in E. coli. Complexes were purified from cell lysates using Ni-NTA affinity beads.

Both Vif full length and 1-154 fragment proteins were able to form a stable complex with CBF-β, Cul5, and Elo B/C. Each of the recombinant Vif constructs contained the B/C box motif; however, Vif 1-154 lacks the cullin box. Not surprisingly, while Vif 1-140, lacking the B/C box, was able to bind CBF-β, it was no longer able to associate with Elo B/C or Cul5 (Figure 4-6). Thus, while the cullin box is dispensable for Vif to bind Elo B/C and Cul5, the B/C Box is necessary. Furthermore, the presence of Elo B/C is not sufficient for Vif to bind Cul5 in the absence of the B/C box.

Next, we wanted to explicitly define whether the Vif:Cul5 formation requires only the presence of the B/C box or alternatively the presence of an Elo B/C bound B/C box. To address this question, full length Vif was co-expressed with CBF-β and Cul5 in the presence or absence of Elo B/C. Again, his-tagged CBF-β and
bound proteins were pulled down from cell lysates using Ni-NTA beads. While Cul5 was readily pulled down with Vif and CBF-β when Elo B/C was present, Cul5-Vif formation was severely inhibited in the absence of Elo B/C (Figure 4-7). Taken together, these data confirm the requirement for Elo B/C to bind the B/C box motif of full length Vif prior to Vif forming an interaction with Cul5.
Figure 4-6. Purification of full-length and truncated Vif-E3 ligase complexes.

Full length Vif and Vif 1-154 bind Cul5 and Elo B/C, but not Vif 1-140 which lacks the BC Box visualized by coomassie stain.
Figure 4-7. Purification of the Vif-E3 ligase complex in the presence and absence of Elo B/C.

Full length Vif does not bind Cul5 in the absence of Elo B/C, even with an intact B/C Box visualized by coomassie stain and western blot.
Discussion

Here, I report an enhanced understanding of the molecular interactions between the HIV-1 Vif protein and the host E3 ubiquitin ligase scaffold protein, Cul5 as well as Elo B/C. Previously, our group and others demonstrated the importance of Vif residues Val25, His27, and Tyr30 for APOBEC3 suppression[121, 123, 154]. For the first time, we demonstrate through multiple lines of experimental evidence that Met29 contributes to APOBEC3G/F suppression and that an N-terminal motif \( ^{25}\text{VxHxMY}^{30} \) in the Vif polypeptide is required for Cul5 interaction, thus providing a rationale for its essential role in suppressing APOBEC3 proteins.

In addition, we also further validate the obligatory role of Elo B/C bound to Vif’s B/C box for recruiting Cul5 to the Vif complex. Based on our previous and current data, the recruitment of Cul5 to the Vif E3 ubiquitin ligase can be summarized in the following steps: (1) full length Vif is unbound to Cul5 in the absence of CBF-β and Elo B/C; (2) Vif binds concurrently to CBF-β at its N-terminus and Elo B/C at its C-terminus, inducing structural changes at both termini; (3) once Vif is bound to both CBF-β and Elo B/C, Cul5 binds to Vif’s \( ^{25}\text{VxHxMY}^{30} \) N-terminal motif as well as amino acids within the HCCH domain and cullin box to assemble a functional ubiquitin ligase. We have demonstrated previously that Vif does not require Elo B/C or CBF-β in order to bind APOBEC3G[60, 101]. In addition, APOBEC3G binding is not necessary for full length Vif to bind Cul5[103]. Thus, Vif may recruit APOBEC3G prior to or after Cul5 incorporation.

The once difficult task of producing full-length soluble Vif for \textit{in vitro} experimental analyses have been overcome with the discovery of one of Vif’s binding partners,
We have exploited this recent finding in order to successfully co-express and purify recombinant full-length Vif with known interacting partners CBF-β, Elo B/C, and Cul5. Through pull-down assays, we initially observed a weak interaction between a Vif N-terminal methionine (Met29) mutant and Cul5. This mutant specifically reduced the interaction between Vif and Cul5, not affecting its interaction with other interacting partners, CBF-β and Elo B/C. Next, scanning alanine mutagenesis of surrounding Vif residues revealed that His27 and Tyr30 were specifically important for Vif:Cul5 interaction; mutation of His28 had a minimal effect on Vif:Cul5 formation. Furthermore, these Vif mutants were required for Cul5 interaction in mammalian cells as well as for efficient APOBEC3G/F degradation. Val25 was also found to be critical for association with Cul5.

Isothermal titration calorimetry analyses revealed that the affinity between full length wild-type Vif and Cul5 in the presence of CBF-β and Elo B/C (apparent Kd = 5.9nM) was similar with a variety of SOCSbox proteins:Elo B/C interaction (SOCS2, 4, 5, 6, 7:Elo B/C apparent Kd = 7-40 nM) [115, 142] . However, the Vif V25A mutant affinity for Cul5 was severely compromised (90-fold reduction) compared to wild-type Vif, while C-terminal mutant H108A reduced the affinity 6-fold. It is plausible that these residues affect Vif tertiary structure, thus affecting Vif:Cul5 interaction. However, we observed that both N- and C-terminal binding partners, CBF-β and Elo B/C still bind to all of these mutants, suggesting that the Vif single alanine point mutants remain in a structural state similar to the native
conformation. However, we cannot rule out that the C-terminal HCCH domain is specifically altered by changes to amino acids in the N-terminus of the full length Vif molecule. Indeed, we found that the structure of the protein complex does change when either N-terminal (i.e. V25A, H27A, M29A, and Y30A) or C-terminal (i.e. H108A) residues are altered to alanine using both circular dichroism spectroscopy and size-exclusion chromatography. Thus, the changes in N-terminal and C-terminal amino acids can alter the structure of Vif such that CBF-β and Elo B/C still bind, yet precludes Cul5 binding.

Previous reports suggested that the 23S/RLV25 motif was important for APOBEC3 suppression[60, 104, 121, 123]. Our results demonstrate that Leu24 and Val25 are most critical in this motif; Arg23 played no or a minor role in APOBEC3G/F suppression, respectively. Given that residue 23S/R is highly conserved between HIV and SIV molecules, it is unclear whether this residue plays a role independent of APOBEC3 degradation. In addition, Vif H108A bound to Cul5 with reduced binding affinity when compared to wild-type; however, the affinity was stronger than the Vif V25A mutant. Comparatively, single mutation of this histidine residue to alanine results in a marked decrease in Cul5 interaction by immunoprecipitation analysis in mammalian cells. Furthermore, reports have demonstrated that the Vif C-terminal domain is sufficient to recruit Cul5, although full length Vif had a greater binding affinity for Cul5 compared to C-terminal domain fragments[107, 115]. It is plausible that the V25A mutant complex fell apart during the ITC measurement; however, it appeared intact similar to wild-
type throughout the purification protocol. Next, while Vif L24A had a diminished ability to suppress both APOBEC3G/F, this mutant could still bind to Cul5 as well as CBF-β and Elo B/C. Dang et al. reported that mutation of this leucine residue also had no effect on APOBEC3G/F binding[123]. It is intriguing to think that there is a yet to be discovered Vif binding partner that interacts with this residue and is necessary for efficient APOBEC3G/F suppression.

His27/28 was previously reported to contribute to APOBEC3G suppression. Here we show that His27, but not His28, contributes to both APOBEC3G and APOBEC3F suppression as well as mediates contact with Cul5. It has not been ruled out whether His28 may play a role in mediating the interaction and suppression of other APOBEC3 proteins; however, previous reports have observed that Vif H28A mutant HIV clones grow as well as wild-type in the presence of APOBEC3G or APOBEC3F[127, 158]. Interestingly, we observed a mobility shift for both Vif H27A and H28A mutants in SDS-PAGE gels, yet the mutants migrate at different speeds. This observed phenomenon suggests that these two histidines may contribute to Vif folding and structure, at least in the presence of SDS.

Remarkably, the discovered Vif N-terminal motif that facilitates binding with Cul5 is in close proximity to amino acids that mediate binding to its substrate molecule, APOBEC3 as well as its regulator, CBF-β. APOBEC3G/F bind discontinuous motifs in the N-terminus and some reports have suggested that it
makes contact with a C-terminal motif. We have demonstrated previously that Trp21 and Trp38 are important for both CBF-β and Cul5 binding and we confirm the importance of these residues in this report[101]. Perhaps, the Vif N-terminal motif facilitates Cul5-E2 ubiquitin conjugation to APOBEC3 molecules by bringing the molecules in closer proximity. Tyr30 was previously suggested to be important for both RNA binding as well as APOBEC3G binding and suppression[121, 149]. In addition, substitution of histidine for tyrosine at position 30 in Vif was found to correlate with a reduced ability to transmit the virus from mother to child, supporting the importance of this residue for Vif function[159]. Tyr30 was critical for suppression of APOBEC3G, although we also observed reduced suppression of APOBEC3F. To our surprise, we observed that this residue also mediates Cul5 interaction. It is unclear whether APOBEC3F may also interact with this residue. However, it appears that this residue doesn’t entirely disrupt APOBEC3F interaction because we do not observe complete recovery of APOBEC3F levels as we do with APOBEC3G levels in the presence of this mutant compared to empty vector. How a single Vif residue interacts with both APOBEC3G and Cul5 is a mystery, although this is not the first time that a Vif residue has been implicated in binding more than one protein[124, 160]. In addition, we can appreciate that Vif is an extremely complex macromolecule and understanding precisely how it interacts with all of its partner host proteins will require solving its 3-dimensional crystal structure.
There are currently over 30 approved HIV drugs, which target only a few of the 15 HIV proteins encoded in its genome. However, problems related to drug failure, emergence of drug-resistant variants, and treatment-related adverse consequences persist. Thus, the expansion of anti-HIV therapies requires new targets. The discovery of novel inhibitors that combat additional HIV proteins depends upon the understanding of how viral proteins bind cellular factors to increase viral fitness. The precise role of each of the Vif domains in mediating interaction with cellular proteins requires further study. The insights to be gained from exploring these possible interfaces between Vif and the E3 ligase complex components have the potential to contribute to the design of novel HIV inhibitors.
CHAPTER 5

DESIGN AND OPTIMIZATION OF IN VITRO PRIMARY HIGH-THROUGHPUT SCREEN FOR SMALL MOLECULE INHIBITORS OF VIF:CBF-β AND/OR VIF CUL5 INTERACTIONS

5.1 Introduction

5.2 Design and proof of concept

5.2.1 Screening lead compounds that bind CBF-β and inhibit CBF-β:RUNX binding
**Introduction**

The intelligent design of novel inhibitors that combat additional HIV proteins depends upon the understanding of cellular factors that bind viral proteins. It has been established that Vif is essential to the production of infectious virions by associating with and degrading anti-HIV factor, APOBEC3G/F[161]. Understanding all protein interactions required for Vif-mediated degradation of this essential host factor is critical to the design of potential Vif inhibitors. While the interface between Vif and Elo B/C is conserved across SOCS box family members, the motifs responsible for Vif interaction with Cul5, APOBEC3, and CBF-β are all suspected to be distinct from cellular interaction motifs. The importance of the interactions that Vif makes with cellular proteins has been discussed in the preceding chapters. Therefore, these unique interfaces provide a potential avenue for novel anti-HIV therapeutics. We have successfully developed an *E. coli* over-expression system that combines the expression of multiple binding proteins to enhance the solubility of Vif in solution. Our ability to obtain Vif:E3 ligase complexes for in vitro studies gives us the unique opportunity to probe these complexes for small molecule inhibitors that disrupt these interfaces. These inhibitors may be developed into future anti-HIV drug therapies and/or be used as tools for *in vivo* and *in vitro* experiments.

Inhibitor screens have been developed to target the Vif:APOBEC3G interface; however, *in vitro* screens that target the Cul5-Vif and CBF-β-Vif interfaces have yet to be developed. Nathans *et al.* established a cell-based assay utilizing YFP
tagged APOBEC3G[162]. The authors monitored levels of YFP-APOBEC3G
during HIV infection in the presence and absence of Vif using a fluorescence
plate reader. YFP-APOBEC3G is expected to decrease in the presence of Vif;
however, the fluorescence should increase if the Vif:APOBEC3G interaction is
disrupted. Two compound inhibitors were identified that affect YFP-APOBEC3G
fluorescence in non-permissive cells. However, it is unclear whether these
inhibitors affect the interaction between Vif and APOBEC3G or whether the
inhibitors disrupt some other essential interaction. Recently, another group
established a structure-based virtual screening technique for identifying inhibitors
of the Vif –Elo C interaction[163]. The group utilized a previously constructed
three-dimensional Vif:Elo B/C homology model. The authors identified one
inhibitor that restricted APOBEC3G action against HIV-1 infection. However,
inhibitors targeting the Vif:Elo C interface may cause off-target effects due to the
conserved nature of the interaction between the two proteins; Elo C binds a
structurally similar B/C box in other cellular SOCS box proteins.

While the cell-based and structure-based established assays have the potential
for identifying inhibitors of Vif:APOBEC3G or Vif:Elo C interfaces, we can take
advantage of our ability to purify large amounts of the Vif:CBF-β:Elo B/C:Cul5
complexes to develop an in vitro assay to identify inhibitors that disrupt the
Vif:Cul5 and Vif:CBF-β interaction. One advantage of the in vitro system over
cell-based assays is its simplicity, time efficiency, and facile ability to scale up,
while the structure-based virtual screen can introduce many errors if the original
protein structure model is incorrect. False positive hits are common when using an in vivo system because inhibitors may disrupt an essential cellular function instead of the intentional target. The high frequency of false positive hits can be minimized using an in vitro assay by designing proper controls. I have developed a high-throughput drug screen assay, which can be used to identify small molecule inhibitors that disrupt the interaction between Vif and CBF-β as well as Vif and Cul5.

**Design and proof of concept**

Development of in vitro to screen for inhibitors of HIV-1 Vif and CBFβ as well as Vif and Cul5

A quantitative fluorescence-based assay was developed to permit sensitive high throughput screening of Vif inhibitors. Using a novel in vitro E. coli based strategy to purify soluble full-length Vif:CBF-β:Elo B/C:Cul5 complex, we are able to screen small molecule compounds from diverse compounds including: herbs, marine products, and microorganisms in addition to chemical libraries to determine inhibitors of Vif:CBF-β and Vif:Cul5 interactions using a fluorescent tagging approach (**Figure 5-1**).

We have already established that overexpressed Vif and CBF-β interact with each other in E. coli and can be highly purified by affinity tag purification protocols. Next, we created a fluorescently tagged (yellow fluorescent protein) full length Vif (Vif-YFP) that can be over-expressed in E. coli. Vif-YFP interacted
with 6x-histidine tagged CBFβ (his-CBFβ) and Elo B/C and the complex could be purified in large amounts using a Ni-NTA affinity purification scheme. As a control, Vif-YFP was shown not to bind to nickel beads alone.

To screen for small molecule inhibitors, we overexpressed Vif-YFP with his-CBFβ and Elo B/C in *E. coli* cells. Next, cells were lysed to liberate soluble Vif-YFP:his-CBF-β:Elo B/C complexes and purified using a Ni-NTA affinity matrix followed by gel filtration. Pre-purified complexes were applied to nickel-coated 96-well plates (Thermo Scientific). His-CBF-β bound to the nickel coated plates and interacting partner complex, Vif-YFP:Elo B/C bound as well via the his-CBF-β bridge. Next, the bound complex was incubated with a small selection of inhibitors for optimization; however, incubation with an infinite number of potential small molecule inhibitors can be performed in a 96 well format. After a designated amount of time (1-8hrs), the solution containing the inhibitor was removed and the plate wells were washed with buffer. To determine inhibitors that disrupt the Vif:CBF-β interaction, fluorescence levels were detected using a fluorescence plate reader. Comparing fluorescence levels of a negative control, inhibitors that disrupt the interaction between the two proteins will result in a reduced fluorescence reading.

An initial proof of concept experiment was performed to determine whether the fluorescence plate-based system would perform well. A benzodiazepine small molecule Ro5-3335 was recently reported to interact with RUNX1 and CBF-β
directly, repress RUNX1/CBF-β-dependent transactivation in reporter assays, and repress RUNX1-dependent hematopoiesis in zebrafish embryos[164]. Since Ro5-3335 is known to interact with CBF-β and disrupt the interaction with RUNX, we hypothesized that the small molecule may also be effective at disrupting the interaction between Vif and CBF-β. Plate bound Vif-YFP:hisCBF-β:Elo B/C complexes were incubated with various concentrations of Ro5-3335. While the positive control, 1% SDS, reduced the fluorescence signal for Vif-YFP, it did not reduce the level of his-CBF-β bound to the plate. As expected, the CBF-β-Elo B/C indirect association is also disrupted in the presence of 1% SDS. Unlike SDS, Ro5-3335 did not decrease the signal at the highest concentration used (50uM). The consistent levels of Vif-YFP before and after treatment with Ro5-3335 were confirmed by both a fluorescence reader (Figure 5-2a) and by SDS-PAGE/Coomassie stain (Figure 5-2b). Three additional allosteric inhibitors of the RUNX-CBF-β interaction were tested against the Vif:CBF-β interaction, but were equally ineffective at disrupting the interaction with Vif compared with Ro5-3335.

In addition to developing an assay to identify inhibitors of the interaction between Vif and CBF-β, we were also interested in developing a screen for testing the interaction between Vif and Cul5. An mCherry-tagged Cul5 (Cul5-mCherry) plasmid was constructed. Cul5-mCherry interacted with the Vif-YFP:hisCBF-β:Elo B/C complex. Small molecule inhibitors that disrupt the interaction between Vif and Cul5 will result in a reduced fluorescence signal. To determine whether an inhibitor is specific to the Vif:Cul5 interaction, instead of the Vif:CBF-β
interaction, two separate excitation/emission filter pairs must be used. For Vif-YFP, the 485/20nm excitation filter and 528/20nm emission filter can be successfully used to avoid fluorescence overlap with the Cul5-mCherry signal. For Cul5-mCherry, the 585/10nm excitation and 610/10nm emission filters can be used to avoid overlap with the Vif-YFP signal. We have confirmed that a positive control for disruption, 1% SDS, results in a reduced signal reading. However, additional inhibitors have not been tested.

We plan to use small molecule compounds from diverse compounds from herbs, marine products, and microorganisms in addition to chemical libraries from TimTek and the Johns Hopkins High throughput screen Center to determine inhibitors of Vif:CBF-β and Vif:Cul5 interactions using the developed fluorescent plate-based approach. We intend to employ both in-vitro (purified Vif:CBF-β:Cul5:Elo B/C protein complexes) based high-throughput assays for discovering new HIV-1 inhibitors. Potential inhibitors discovered using these assays will be further tested for efficacy.
Figure 5-1 Diagram of the *in vitro* high-throughput strategy to identify small molecule inhibitors of Vif:CBF-β interaction.
Figure 5-2a. R05-3335 does not disrupt Vif-YFP:his-CBF-β-141 interaction.

Fluorescence signal values were recorded for Vif:CBF-β interaction in Ni-NTA plates before and after incubation with R05-3335.
Figure 5-2b. R05-3335 does not disrupt Vif-YFP:his-CBF-β-141 interaction.

Coomassie stain of treated Vif:CBF-β complex reveals that R05-3335 does not disrupt the interaction between Vif and CBF-β.
CHAPTER 6

NOVEL FUNCTIONS OF VIF IN COMPLEX WITH CBF-β:RUNX


6.1 Introduction

6.2 Methods

6.3 Vif forms a complex with CBF-β:RUNX proteins in the nucleus

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6.6 Discussion
Introduction

One of the best studied roles for HIV-1 Vif has been its ability to recruit the Cul5:Elo B/C E3 ubiquitin ligase to induce degradation of anti-viral factor APOBEC3 proteins. As presented in the preceding chapters, we recently identified an additional host factor, CBF-β, which Vif recruits in order to regulate the degradation of anti-HIV APOBEC3 proteins. CBF-β is also a well-studied host protein that is important for many cellular immune processes when complexed with another family of host proteins, RUNX. CBF-β constitutively binds to and regulates the DNA binding capacities of RUNX family proteins [165], which are key transcription factors for lineage-specific gene expression in several major developmental pathways [166-169]. Humans express three RUNX genes: RUNX1, RUNX2 and RUNX3 [166, 167, 170]. Among them, RUNX1 is indispensable for definitive hematopoiesis[171] and its functional dysregulation leads to leukemia[172], while RUNX3 was shown to play important roles in thymopoiesis[167, 173]. Interestingly, both RUNX1 and RUNX3 are expressed at high levels in HIV-1 target cells, including CD4+ T cells and macrophages[166, 174]. Although it has been recently demonstrated that recombinant Vif does not form a complex with CBF-β and the RUNT domain of RUNX family proteins[77], it is unclear whether HIV-1 Vif associates with full length RUNX proteins and alters RUNX-mediated regulation of gene expression in infected cells. A previous graduate student, Anjie Zhen, made many initial experimental observations, which suggested that Vif and RUNX associated with each other and that Vif
regulated RUNX function in the cell. We collaborated on multiple aspects of this project and I will present some of our latest findings.
**Methods**

**Plasmids**

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3-ΔEnv-EGFP (Cat# 11100) from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano (Zhang, 2004 #231). CBF-β coding sequences were amplified by reverse transcription and PCR using mRNA samples from H9 cells with the following primers: forward 5’- GCTAGCAAGATGCCGCGCGTCGTG -3’, reverse 5’- AAGCTTACTACAGATCTTCTTCTGATATGAGTTTTTGTTCGGGTCTTGTTGTC TTCTTGCC -3’ containing NheI and HindIII sites. The PCR product was cloned into pcDNA3.1 to generate pCBFβ-myc. The following expression vectors have been previously described[60, 175, 176]: HIV-1 Vif expression vectors pVif-myc and pVif-HA in VR1012 vector, pNL4-3ΔVif and pcDNA-hVif were generous gifts from Dr. K. Strebel. Runx1A, Runx1B, Runx1C, Runx1C-myc were generous gifts from Dr. A. Friedman. Runx3-myc was generous gift from Dr. Nancy Speck. 3.3kb and 0.3kb mouse IL-2 promoter luciferase constructs were generous gift from Dr. J.D. Powell. Lentivirus plasmid Vif-IRES-GFP, Vif reverse-IRES-GFP and Vifmyc-IRES-GFP were constructed by subcloning codon optimized pcDNA-HVif (NIH AIDS Reagent Program, #10077) into pCCL plasmid via BamHI and Xbal restriction sites. Vif-reverse-IRES-GFP was subcloned with reverse orientation and does not translate into proper proteins. HVif-HA and myc-HVif-HA was constructed by PCR amplifying codon optimized Vif from pcDNA-HVif and subcloning into VR1012 plasmid via EcoRI and BamHI restriction sites. To
generate human IL-2 promoter luciferase, the human IL-2 promoter was PCR amplified from Jurkat cell genomic DNA (primers-forward 5'-TATGCAGGTACCTGTATGCAATTAGCTC – 3' and reverse 5'-TATGCACCTCGAGCATGTGGGGAGTTGAGG –3') and cloned into the Kpn1 and XhoI sites of pGL2 (Promega). To generate mCherry epitope-tagged RUNX1B, pCMV-RUNX1B was used to PCR amplify the RUNX1B coding region (primers-forward 5' – GACGTGAATTCTAGGCTTCAGACACTATATTG – 3' and reverse 5'– ATACCGGTCCGCCTGCAGACCCGCCGTAGGGCCGCCACACCGG –3') and cloned into the EcoRI and AgeI sites of pmCherry-N1 (Clontech).

**Antibodies and Cell Lines**

The Jurkat human CD4+ T-cell line was maintained in RPMI-1640 medium (Invitrogen) with 10% fetal bovine serum and penicillin/streptomycin (R-10 medium). HEK293T cells (AIDS Research and Reference Reagents Program, Cat #3522) were maintained in Dulbecco's modified Eagle's medium (DMEM, Life technology) with 10% fetal bovine serum and penicillin/streptomycin (D-10 medium) and passaged when confluent. The following antibodies were used: anti-HA (Covance, MMS-101R-1000), anti-myc (Upstate, 05-724), anti-β-actin MAb (Sigma, A3853), anti-histone H3 (Genscript, A01502), anti-GAPDH (Genscript, A00084), anti-HA antibody-agarose conjugate (Roche, 11815016001), mouse anti-CBFβ (Abcam, ab11921), anti-Runx1 (Santa Cruz sc-28669), anti-Runx3 (Abcam ab68938). Monoclonal anti-CAp24 (Cat #1513) and
rabbit anti-Vif antibodies (Cat. #2221) were obtained from the AIDS Research and Reference Reagents Program.

**Cytoplasmic and Nuclear Fractionation**

HEK293T cells were transfected with desired plasmids for 48 hours by lipofectamine 2000 (Life Technology) following manufacturer's instructions. Cells were then washed twice in PBS and lysed in 0.5% Triton in PBS for 15 min on ice. Samples were then centrifuged at 13000rpm for 10 minutes. Supernatants were harvested as the cytoplasmic fraction. Pellets were washed in lysis buffer and then lysed in 1XSDS loading buffer as the nuclear fraction. Cellular localization of Vif and RUNX proteins were then analyzed by western blot. Anti-histone H3 (Genscript, A01502), anti-GAPDH (Genscript, A00084) antibodies were used to detect endogenous histone H3 and GAPDH to indicate nuclear or cytoplasmic fraction, respectively.

**Quantitative real-time PCR (qRT-PCR)**

RNA obtained from cells were purified by Trizol (Life technology) according to the protocol and treated with DNase I by incubation in 10 µl of diethyl pyrocarbonate (DEPC)-treated water with 1x RQ1 RNase-Free DNase buffer, 1 µl of RQ1 RNase-free DNase (Promega) and 4 U of RNase inhibitor (New England Biolabs) for 30 min at 37°C. The DNase was inactivated by the addition of 1 µl RQ1 DNase stop solution and incubated at 65°C for 10 min. The RNA was reverse transcribed by using random primers and the Multiscribe reverse
transcriptase from the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. The cDNA was either undiluted or serially diluted in DEPC-treated water before input into the real-time reaction to ensure that the amplification was within the linear range of detection. The StepOne Real-Time PCR system (Applied Biosystems, Carlsbad, CA) was used for the qRT-PCR amplifications. The reactions were performed under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a dissociation protocol. Single peaks in the melting curve analysis indicated specific amplicons. The target sequences were amplified using the following primer pairs: GAPDH, forward (5'-GCAAATTCCATGGCACCCTG-3'), and reverse (5'-TCGCCCTTATTGATTTTGGG-3'); IL-2, forward (5'-TCACCAGGATGCTACATTGTCCTTAGACACTGA-3'), reverse (5'-GAGGTTTGAGTTTCTTCTTCTAGACACTGA-3'); 18S, forward (5'-GACACCATTGTTGCCACGAGT-3'); FoxP3, forward (5'-TCTGCACCTTCCCACATCC-3'), reverse (5'-GACACCATTGCTGAGGCTGAGG-3'); CyclinD, forward (5'-GGCGGAGGAGAACCAATCC-3'), reverse (5'-TGTGAGGCGGTAGTTCAGCAG-3'); 18S, forward (5'-GTAACCCGTTGAACCCCATT-3'), reverse (5'-CCATCCAATCGGTAGTAGCG); CD4, forward (5'-CCTGGTAGTAGCCTCAGT-3'), reverse (5'-AGACAGTGCATGTCCAGGTG-3'); CXCR4, forward (5'-TACACCAGGAAATGGGCTC-3'), reverse (5'-CATGCCACATCGCGTGAGT-3'); Tbet, forward (5'-TGGTGCTGCTGATCGCTGCTG-3'), reverse (5'-CCACTACGGAGTACGGGGA-3'); IL-17, forward (5'-TGGTGCTGCTGCTGCTGCTG-3'), reverse (5'-CGGGGAGTTCTTGTCTCCTG-3').
3'); CBF-β, forward (5'-GGATGGTATGGGCTGTCTGG-3'), reverse (5'
TCCTTCTCCGAGCCTCTTCA-3'); IFNg, forward (5'
CCAACGCAAAGCAATACATG-3'), reverse (5'-CGCTTCCCTGTTTTTAGCTGC
3'); GMCSF, forward (5'-TGATGGCCAGCCACTACAAG-3'), reverse (5'
CCAGCAGTCAAAGGGGATGA-3'); p21, forward (5'
CCTGTCACTGTCTTGTACCCCT-3'), reverse (5'-GCCTTTGGAGTGTTAGAAAT
3'); MCSFR, forward (5'-ATTCATCAACGGCTCTGGCA-3'), reverse (5'
AGGACCTCAGGGTGATGGTC-3'); IL-8, forward (5'
TCTGCAGCTCTGTGAAGG-3'), reverse (5'-TGGGTTGGAAAGGTTTGGAG
3'); IL-6, forward (5'-CCAGTACCCCCAGGAGAAGA-3'), reverse (5'
CTGAGATGCCGTCGAGGATG-3'); MIP1a, forward (5'
GGCCTCTCTGCAACCAGTTCT-3'), reverse (5'
GAATCTGCGGGGAGGTGTA-3'.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation were performed using SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) (Cell signaling #9002). Briefly, 10 million Jurkat cells were transduced with Vif-myc-IRES-GFP lentiviral vector. 2 days post transduction, cells were crosslinked with 1% formaldehyde for 15 minutes. Afterwards, the nucleus was isolated and chromatin was digested with microcococcal nuclease and sonication. Immunoprecipitation were carried overnight at 4C with either mouse IgG or anti-myc tag antibody (Millipore, 05-724) and protein G provided in the Kit. Afterwards, beads were washed with low
salt and high salt washing buffer and DNA were eluted with ChIP elution buffer. Reversal of crosslinks was carried out at 65C for 2 hours and DNA was purified using spin columns. DNA fragments were amplified by PCR with specific primers as follows: The human IL-2 promoter; forward: 5’-CTC TAG CTG ACATGT AAG AAG C-3’; reverse: 5’-CTA CAC TGA ACA TGT GAA TAG C-3’. The 5’ distal region of the human IL-2 gene; forward: 5’-TGA CAA GTG TGA ACC TGG AG-3’; reverse: 5’-GAG CAT ATC CAT CAT CTC AC-3’. To test if Vif affect RUNX1 DNA binding activity, 293T cells were transfected with RUNX1-myc, CBF-β-myc, MCSFR-luc and either Vif-IRES-GFP or VifR-IRES-GFP. 2 days after transfection, cells were crosslinked and ChIP were performed accordingly with anti-Runx1 antibody (Abcam, ChIP grade). DNA fragment were quantified by real time PCR using the following primers: GMCSF, forward: 5’-TTCCCATGTGTGGCTGATAA-3’, reverse: 5’-CTGTGTACTGGGCTCACTGG-3’; MCSF-R, forward: 5’-TGGGTTCAAGGCTTTGTTTT-3’; reverse: 5’-CAGTCTGATTGGCTGCTACC-3’.

**Primary CD4+ cells isolation and HIV-1 infection**

Human peripheral blood was obtained from anonymous, healthy adult donors in the form of leukopacks through the UCLA Center for AIDS Research Virology Core Laboratory. PBMCs from healthy blood donors were isolated by density gradient centrifugation from leukopacks using Ficoll-Paque PLUS (STEMCELL Technology). Primary CD4 T cells were isolated using CD4+ T Cell Isolation Kit II using autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, 10^7
isolated PBMCs were incubated at 4°C with 10ul biotin-antibody cocktail for 10 minutes in and then incubated with 20ul and anti-biotin microbeads in binding buffer for another 15 minutes (phosphate-buffered saline [PBS], pH 7.2; 0.5% bovine serum albumin; and 2 mM EDTA [ethylenediaminetetraacetic acid]). The labeled cells were then passed through a magnetic MS cell sorting (MACS) column, and negatively selected CD4 cells were eluted. All cells were cultured in RPMI medium supplemented with 10% heat inactivated fetal bovine serum, 20ug/ml Gentamicin and 2 mM L-glutamine.

To make virus for infection, HEK293FT cells were transfected with 30ug NL4-3ΔE-GFP or NL4-3ΔEΔVifGFP and 5ug pVSVG plasmid per 150 cm² flasks for 3 days by Lipofectamine 2000 (Life technology), supernatant were then harvested and virus were concentrated by ultracentrifuge at 20K for 2 hours using a 20% sucrose cushion. Viral pellet were then dissolved in 100ul PBS on ice for 1hours and tittered in Jurkat cells.

Purified CD4+ T cells were stimulated and propagated with 10ug/ml plate bound anti-CD3 (BD bioscience 555336) and 1ug/ml soluble anti-CD28 (BD bioscience 555725) for 2 days, and then were infected or mock infected with 2MOI purified viral stock for 2days. Cells were re-stimulated with PMA and ionomycin for 4 hours before immunostaining and flow analysis. Average percentage of IL-2, IL-4 and IFN-γ positive cells from mock or NL4-3ΔE-GFP or NL4-3ΔEΔVifGFP infected cells were compared and tested with one tailed paired t-test.
Lentivirus Transduction

HEK293T cells were transfected with 15ug pCCL-Vif-IRES-GFP or pCCL-Vif-IRES-GFP as well as 15ug p89.6 and 5ug pVSVG plasmids for 3 days per 150 cm$^2$ by Lipofectamine (Life technology). Supernatant were then purified and lentivirus were concentrated by ultracentrifugation at 28K for 1.5 hours using a 20% sucrose cushion. Virus pellet were then dissolved in 100ul PBS at 4 degree for 10 hours and tittered in Jurkat cells.

PBMCs from healthy blood donors were isolated by density gradient centrifugation from leukopacks using Ficoll-Paque PLUS (STEMCELL Technology. PBMCs were then stimulated with 10ug/ml plate bound anti-CD3 (BD bioscience 555336) and 1ug/ml soluble anti-CD28 (BD bioscience 555725) for 2 days, and then were infected or mock infected with 2 MOI purified lentivirus stock for 3 days. Cells were re-stimulated with PMA and ionomycin for 6 hours before immunostaining and flow analysis. Average relative ratio of IL-2 and IFN-$\gamma$ positive cells from Vif transduced cells compared to VifR transduced cells were tested with one sample t-test.

Macrophage Differentiation and Transduction

Monocytes were purified from healthy PBMCs through magnetic activated cell sorting through positive selection utilizing CD14 (Miltenyi Biotech). Purified monocytes were stimulated with 10ng/ml M-CSF in RPMI-1640 medium
containing 10% Fetal Calf Serum for 7 days. Cells were then washed and transfected with 2MOI VSVG pseudotyped Vif-IRES-GFP or control lentivirus Vif-reverse-IRES-GFP and cultured for 3 days in the absence of M-CSF before analyzed by flow cytometry. Relative MCSFR mean fluorescent intensity ratio of Vif transduced cells compared to VifR transduced cells were compared and tested with one sample t-test.

**Immunostaining and Flow Cytometric Analysis of Intracellular Cytokines**

Infected or control primary CD4+ cells or PBMCs were fixed and permeabilized using Cytofix/Cytoperm Fixation and Permeabilization solution (BD bioscience) following protocol. Cells were then stained with PerCP-Cy5.5 conjugated anti-IL-2 (BD biosciences), APC conjugated anti-IFN-γ (ebioscience), PE conjugated anti-IL-4 (BD biosciences) or PE conjugated anti-GMCSF (eBiosciences), eFlour anti-CD3 (ebio-science) and APC-H7 conjugated anti-CD4 (BD bioscience) in staining buffer for 30 minutes at 4°C and washed twice with 1X Perm/Wash TM solution prior to cytometric analysis. Transduced or control CD14+ cells were stained with PC7 conjugated anti-CD11c (ebiosciences), APC eflour780 conjugated anti-CD4 (ebiosciences), PE conjugated anti-MCSF (ebiosciences) and biotin-conjugated anti-MCSFR (ebiosciences) and secondary antibody APC conjugated anti-biotin. Immunostained samples were analyzed by using LSR II Digital Flow Cytometer (BD Bioscience). Data analyses were performed using FACSDiva Software (BD Bioscience) and flowjo.
**Reporter Gene Assay**

RUNX1, CBF-β, and Vif expression plasmids were co-transfected with reporter plasmids pMCSF-R-luc or pGM-CSF-luc using PEI-MAX transfection reagent. pGL4.70 was transfected together with the reporter plasmid as an internal control (for pGM-CSF-luc experiment only), and its activity was used to normalize transfection efficiencies. Twenty-four hours after transfection, the cells were harvested and analyzed using the Promega Dual luciferase reporter assay kit (according to manufacturer’s protocol) and a luminometer. Each assay was performed three times with triplicate samples.

**Chromatin fractionation**

Cells were lysed in triton lysis buffer for 15 minutes. Insoluble cell debris, including chromatin, was pelleted by centrifugation (2500g for 10 minutes). The supernatant was harvested and represented the soluble input control. Pellets were washed once with nuclease buffer and resuspended nuclease buffer containing 200 U/ml micrococcal nuclease (NEB). Pellets were incubated for 1h on ice and then centrifuged at 13000g for 20 minutes. The supernatant was harvested and represented the chromatin-bound fraction.

**Protein Expression and Purification**

Plasmids were transformed or co-transformed into *E. coli* NiCo21(DE3) cells (New England Biolabs C2529H) according to manufacturer’s protocol. Cells were incubated for two hours at 37°C and plated on media with appropriate antibiotic
selection marker. If more than one plasmid was transformed, cells were centrifuged at 5,000 rpm for 5 min and then all cells were plated. Plates were incubated at 37°C overnight and single colonies were chosen for protein production. Cells were grown to an OD of 0.8-1 at 37°C, cooled to 23°C, and induced overnight at 23°C with 0.2 mM isopropyl-D-thiogalactopyranoside (IPTG, corning cellgro 46-102-RF). Harvested cells containing GST-RUNX1A alone, full length CBF-β and full length Vif, or GST-RUNX1A, CBF-β and Vif were resuspended in lysis buffer (1X PBS, 0.25 mM TCEP (Hampton Research HR2-651), 5% glycerol), sonicated for 15 min, and centrifuged at 10,000 g for 20 min. Soluble supernatant was passed through a 5 mL glutathione sepharose 4B fast flow column (GE 17-5131) at a constant rate (2 mL/min) and subsequently washed with lysis buffer at 5 mL/min using a chromatography pump system (AKTA Explorer). Bound proteins were eluted with elution buffer (1X PBS, 0.25 mM TCEP, 5% glycerol, 10mM L-glutathione reduced (Sigma Aldrich G4251).

**CD4+ T-cell and Macrophage Infection for Microarray Analysis.**

Macrophage Infection: PBMCs were isolated from whole blood using Ficoll-Paque and resuspended in serum free RPMI (5% L-glu, 5% Penn/Strep), and placed into T75 flasks (6x10^6 cell/ml I think) for 3-4hrs at 37C. Monocytes will adhere during this time. Adherent cells were washed two times with PBS and media replaced with 10ml of RPMI, 10%FBS, 20% Human AB Serum, 5% L-glu, 5% Penn/Strep. Cells were incubated for 7 days at 37C, replacing the media
every 3-4 days. On day 7-10, infect with VSV-G pseudotyped HIV-1 Vif+ (NL4-3∆E-EGFP) and HIV-1 Vif- (NL4-3∆EΔVif EGFP) viruses.

Infected cells were sorted by GFP+ signal and RNA was extracted using Qiagen RNeasy kit. Extracted RNA was immediately taken to Johns Hopkins Medical Institute Deep Sequencing and Microarray Core Hybridization Facility for microarray analysis. RNA was hybridized to the Affymetrix Prime View Human Gene Expression Array as previously described. Expression data was pre-processed using the robust microarray (RMA) algorithm and subsequently analyzed using Spotfire DecisionSite software (TIBCO Software Inc., Palo Alto, CA). The microarray data were also analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems).
Vif forms a complex with CBF-β:RUNX proteins in the nucleus

Initially, we tested the ability of Vif to 1) disrupt the interaction between CBF-β and RUNX proteins; 2) degrade RUNX proteins; and 3) interfere with nuclear transport of RUNX proteins. To determine whether Vif disrupts the interaction between RUNX and CBF-β, we transfected HEK293T cells with myc-tagged RUNX1C expression vector and increasing amounts of a Vif expression plasmid and pulled down RUNX1C-myc with anti-myc antibody. Similar amounts of CBF-β were co-immunoprecipitated with RUNX1C-myc, despite exposure to increasing concentrations of Vif proteins. This result suggested that there was no binding competition between Vif and RUNX1C to CBF-β. However, co-precipitation of Vif with RUNX1C was detected, indicating that Vif and RUNX1C either interacted with each other or via a CBF-β bridge. Additionally, Vif could interact with other RUNX proteins including RUNX1A, RUNX1B, RUNX1C as well as RUNX3 (Figure 6-1). In addition to overexpressed RUNX, Vif also interacts with endogenous RUNX proteins during HIV-1 infection. To determine whether Vif, RUNX and CBF-β exist in a single complex, we performed serial immunoprecipitation first using anti-myc and then anti-HA antibody from cells transfected with RUNX1C-myc or HXB2Vif-HA or both plasmids. After serial immunoprecipitation, endogenous CBF-β was detected only in cells that express both RUNX1C-myc and HXB2Vif-HA. In addition, we overexpressed and purified recombinant Vif, CBF-β and GST-tagged RUNX1. While both CBF-β and Vif were detected in GST pull down fractions in the presence of GST-RUNX1, neither protein was detected in the elution sample in the absence of GST-
RUNX1. Together, these results provide strong evidence that Vif associates with RUNX/CBF-β proteins.

In contrast to Vif mediated degradation of select APOBEC3 proteins, co-expression of HIV-1 Vif with RUNX1C did not lead to the degradation of RUNX1C (Figure 6-1). Furthermore, HIV-1 Vif did not affect the expression of three overexpressed RUNX1 splice variants (RUNX1A, RUNX1B, RUNX1C) or two RUNX3 (p-RUNX3, d-RUNX3) isoforms. Endogenous RUNX1 or RUNX3 expression is also not altered during HIV-1 infection of Jurkat T cells. Additionally, RUNX1C remained exclusively nuclear in the presence of Vif, suggesting that Vif does not alter RUNX1C nuclear localization. In fact, Vif appeared to translocate to the nucleus in the presence of RUNX1C (Figure 6-2a). Increased nuclear presence of Vif-CFP was also observed by confocal microscopy comparing 293T cells that express Vif-CFP alone and cells that co-express Vif-CFP and RUNX1-mcherry (Figure 6-2b). Therefore, besides forming a viral E3 ligase with host factors Cul5, Elo B/C and CBF-β in the cytoplasm to promote the polyubiquitination of APOBEC3 proteins, Vif also appears to interact with the RUNX/CBF-β complex in the nucleus.
Figure 6-1. Vif associates with RUNX1 proteins.

Tagged Vif co-precipitates RUNX1B and RUNX1C from HEK293T cells co-expressed with each protein.
**Figure 6-2a.** Vif translocates to the nucleus in the presence of RUNX1.

Subcellular fractionation of Vif and RUNX reveals that when co-expressed in HEK293T cells, RUNX modifies the cellular localization of Vif.
Figure 6-2b. Vif translocates to the nucleus in the presence of RUNX1 and CBF-β.

Live cell imaging of HEK293T co-expressing fluorescent protein fused Vif and RUNX1 indicates that Vif localizes to the nucleus in the presence of RUNX; however, Vif appears in the cytoplasm in the presence of CBF-β alone.
Vif up-regulates CBF-β:RUNX controlled genes

Since RUNX proteins are key transcription factors involved in hematopoeisis and control expression of various cytokines in HIV-1 target cells, we were interested in determining if Vif modifies the RUNX transcriptional regulatory activity on target genes. For example, RUNX1 has been demonstrated to up-regulate Interleukin-2 (IL-2) as well as interferon-gamma (IFN-γ) in primary T cells and Jurkat cells[177]. RUNX3, which is up-regulated in differentiated Th1 cells, promotes IFN-γ while suppressing IL-4 production[178]. It was previously reported that productively infected, primary CD4+ T cells are hyper-responsive to T cell activation with anti-CD3/CD28 and produce elevated levels of many cytokines, including IL-2 and IFN-γ[179]. To determine if Vif is required for virus induced activation of RUNX target genes, we mock-infected or infected anti-CD3/CD28 stimulated primary CD4+ T cells from 4 healthy donors with equal amounts of VSV-G pseudotyped HIV-1 Vif+ (NL4-3ΔE-EGFP) and HIV-1Vif- (NL4-3ΔEΔVif EGFP) viruses. Two days after infection, cells were re-stimulated and stained for CD4 and intracellular IL-2, IFN-γ and IL-4. CD4+ cells infected with Vif+ virus produced higher levels of IL-2 (p<0.01) and IFN-γ (p<0.01) compared to mock infected cells. In comparison, ΔVif virus infected CD4+ cells showed significantly reduced IL-2 (p<0.01) and moderately decreased IFN-γ production (p=0.06) compared to Vif+ virus infected cells. No significant change of IL-4 production was observed between Vif+ and ΔVif viruses (p=0.33). To examine if Vif affects broad Runx gene transcription regulation, we sorted productive infected (GFP+) primary CD4 cells and measured the level of a panel...
of RUNX target genes by real time PCR (Figure 6-3). We found that in infected cells Vif altered a large number of RUNX target genes.

Several HIV-1 proteins, such as Nef and Tat, contribute to immune activation[180]. To determine whether Vif expression alone can modulate IL-2 and IFN-γ expression, we transduced PBMCs from 5 healthy donors with lentiviral vector expressing Vif-IRES-GFP or VifR-IRES-GFP (vif gene in reverse orientation and only expresses GFP) as a control. Two days after transduction, intracellular IL-2 and IFN-γ were analyzed for CD3+/CD4+/GFP+ T cells. Vif transduced CD4+ T cells expressed consistently higher levels of IL-2 (p<0.01) and IFN-γ (p=0.02) compared to cells transduced with the control vector.

To determine if Vif itself also affects global RUNX gene regulation, Jurkat cells were transduced with Vif expression vector (Vif-IRES-HSA) or a control vector (VifR-IRES-GFP) and were activated and analyzed by real-time PCR. Similar to what we have found in primary T cells with infection, we observed that Vif significantly increased a variety of RUNX-targeted genes. To test if Vif directly acts on RUNX target gene’s promoter, we co-transfected Jurkat cells with Vif or Vif21/38AA, which does not bind CBF-β, with human IL-2 promoter controlling a luciferase reporter gene. We found Vif, but not Vif21/38, activates IL-2 promoter activity (Figure 6-4). Vif also activates a murine IL-2 (mIL-2) promoter in a dose dependent manner, but has little effects on truncated mIL-2 promoter that does not contain RUNX binding sites. To test if Vif could physically associate with
RUNX DNA binding sites, we performed chromatin immunoprecipitation with cells transduced with a Vif-myc expressing lentivirus. We were also able to detect specific association of Vif with RUNX responsive DNA element on the IL-2 promoter but not the 5' distal region, demonstrating that Vif is directly involved in RUNX/CBF-β transcriptional regulation of the IL-2 promoter. To determine if Vif activation of RUNX target genes is dependent on RUNX, we knocked down RUNX1 expression by transducing Jurkat cells with RUNX1shRNA lentivirus. Three days following transduction, cells transduced with RUNX1shRNA lentivirus showed significantly reduced RUNX1 level compared to cells that were transduced with shRNA. Afterwards cells were transduced with either Vif expressing (Vif-IRES-HSA) or control lentivirus (VifR-IRES-HSA) and cultured for two days. Two days after Vif transduction, cells are activated and analyzed by real time PCR. The absence of RUNX1 significantly impaired Vif transactivation of many RUNX target genes, such as IL-2, IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF) and T-bet. On the other hand, Vif had limited effects on RUNX suppressed genes such as IL-4 and CD4. These results indicate that Vif transactivation of RUNX target genes is dependent on RUNX. Similarly, we found expression of CBF-β is also required for Vif activation of RUNX target genes.

In addition to regulation of T cell functions and differentiation, RUNX proteins are also crucial for myeloid cell differentiation[181]. For example, RUNX1 has been shown to up-regulate macrophage colony stimulating factor receptor (M-
CSFR)[182] and GM-CSF expression[183]. To investigate if Vif expression affects M-CSFR expression in primary macrophages, we purified monocytes from 3 healthy donors and differentiated them into macrophages. Afterwards, cells were then transduced with either the control or the Vif expressing vector for 3 days and subsequently analyzed by flow cytometry for M-CSFR expression. Macrophages transduced with Vif expressed higher levels of M-CSFR with about 2.5 fold increase in median fluorescent intensity for M-CSFR (p=0.02). Also, we found that macrophages infected with the Vif+ HIV-1 expressed higher levels of M-CSFR compared to cells that were infected with ΔVif virus. We have also observed that Vif elevates GM-CSF production in macrophages transduced with Vif expression vector.

To determine if Vif directly affects RUNX transactivation activity, we co-transfected HEK293T cells with M-CSFR promoter-driven luciferase reporter and a combination of vectors encoding Vif and RUNX1C. RUNX1C up-regulated M-CSFR promoter activity in a dose dependent manner and the addition of Vif significantly enhanced RUNX1C transcription activity. In the absence of RUNX1C, Vif had no detectable activity on the M-CSFR promoter. Consistent with our ex vivo data, we found that Vif also enhanced RUNX1C transactivation activity on GM-CSF promoter in a RUNX-dependent manner. To determine the potential mechanism of Vif effects on RUNX, we performed chromatin immunoprecipitation of RUNX1 in 293T cells co-transfected with RUNX1 and either Vif expressing (Vif-IRES-GFP) or control plasmid VifR-IRES-GFP. DNA
associated with RUNX1 was quantified by real time PCR with primers that amplify RUNX1 DNA binding sites. We found that Vif increased RUNX1 DNA binding activity to M-CSFR and GM-CSF promoter. In addition, we found increased association of RUNX1 with chromatin in the presence of Vif. The results suggest that Vif may potentiate RUNX transactivation by promoting its association with chromatin and recruitment to DNA binding sites.
Figure 6-3. Vif enhances the expression of RUNX target genes during infection.

GFP expressing cells that were productively infected with VSV-G pseudotyped NL4-3ΔE-GFP or NL4-3ΔEΔVif GFP were stimulated by PMA and ionomycin for 3 hours and sorted. cDNA were made from sorted cells and real time PCR were performed to measure a panel of RUNX target genes level. Relative fold increase comparing cells that were infected with Vif- to Vif+ virus were shown.
Figure 6-4. wild-type Vif enhances human IL-2 promoter activity.

IL-2 promoter luciferase activity was measured in the presence and absence of wild-type and W21/38A mutant Vif. While wild-type Vif enhanced human IL-2 promoter activity, the mutant Vif was unable to increase IL-2 promoter activity.
Microarray analysis of CBF-β:RUNX regulated genes from CD4+ T cells and Macrophages infected with wild-type and Vif-deficient HIV

In addition to real time PCR, we also wanted to confirm existing and explore additional RUNX genes that may be affected by the presence of Vif during infection. Again, we mock-infected or infected anti-CD3/CD28 stimulated primary CD4+ T cells from 4 healthy donors with equal amounts of VSV-G pseudotyped HIV-1 Vif+ (NL4-3ΔE-EGFP) and HIV-1Vif- (NL4-3ΔEΔVif EGFP) viruses. Two days after infection, cells were re-stimulated. Next, cells were sorted to enrich infected cells using the GFP+ signal, lysed and RNA extracted using the Qiagen RNeasy kit. Extracted RNA was immediately brought to Johns Hopkins Microarray Core Facility for processing and microarray analysis. RNA was hybridized to the Affymetrix Prime View Human Gene Expression Array as previously described. Expression data was pre-processed using the robust microarray (RMA) algorithm and subsequently analyzed using Spotfire DecisionSite software (TIBCO Software Inc., Palo Alto, CA). The microarray data were also analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems). Of more than 36,000 transcripts, Vif enhanced 46 unique genes (greater than 3 fold change) in HIV-1 infected cells compared to HIV-1 dVif infected cells. In addition, Vif down-regulated 15 unique genes (less than 3 fold change).

Surprisingly, many of the genes that were up-regulated by Vif using real-time PCR and promoter luciferase assays were not shown to be up-regulated using
the microarray approach. For example, IL-2, IFN-γ, and GM-CSF levels were unchanged in the presence or absence of Vif during HIV-1 infection of CD4+ T cells. Interestingly, another cytokine, IL-8 was up-regulated in the presence of Vif. Subsequent real time PCR analysis and an IL-8 promoter luciferase assay confirmed that Vif plays a role in up-regulating this gene in cells (Figure 6-5). Furthermore, RUNX family proteins have been demonstrated to regulate IL-8 levels. Thus, Vif may regulate this cytokine through RUNX.

While we were not able to confirm many of the previously discovered Vif:RUNX regulated genes that were discovered in the initial real time PCR and promoter assays, there were many interesting genes that have not been reported to be regulated by RUNX, but were altered by Vif expression. Gene ontology analysis revealed that the group of Vif-altered genes belonged to two major cellular pathways: androgen signaling and cell cycle regulation (Figure 6-6B). While the connection between Vif and androgen signaling is unclear, multiple reports have demonstrated Vif’s role in regulating the cell cycle [184, 185].

Next, monocyte derived macrophages were cultured for 7 days and subsequently infected with either VSV-G pseudotyped HIV-1 Vif+ (NL4-3ΔE-EGFP) or HIV-1Vif- (NL4-3ΔEΔVif EGFP) viruses (special thanks to Jason Huska for his contribution to these experiments). After 3 days, cells were harvested and separated by flow cytometry cell sorting to enrich for GFP+ infected cells. RNA extraction and subsequent processing by the microarray facility was similar to the
RNA extraction for CD4+ T cells. Again, we were unable to confirm differences between select RUNX regulated genes such as IL-2 and IFN-γ in the presence versus absence of Vif during HIV infection. However, in line with previous real time PCR and promoter assay results, we detected a 2-fold increase in GM-CSF levels in Vif+ HIV-1 infected cells compared to Vif-deficient infected cells. Furthermore, similar to CD4+ T-cells, IL-8 levels were elevated in the presence of Vif compared to HIV-1 infected cells deficient in Vif expression. An explanation for the difference between our real-time PCR results and microarray results may be due to signal saturation. Indeed, both IL-2 and IFN-γ both have signals at the top of the array’s dynamic range and are subject to possible compression.

**Microarray analysis of HIV differentially regulated host genes**

In addition to analyzing differences between gene expression in the presence or absence of Vif during HIV infection, we were also interested in host genes that were altered during HIV infection as compared to mock-infected cells. We focused our analysis specifically to the transcript level differences between Vif+ HIV infected cells and mock-infected cells. In CD4+ T-cells, 2090 host genes were either up-regulated or down-regulated (1.8 fold change) during HIV infection; while in macrophages, 2889 genes were differentially regulated. Comparing CD4+ T-cells and macrophages, HIV modulates 277 common cellular genes among the two cell types (**Figure 6-7a** and **b**). Gene ontology analysis revealed that genes that affect the cell cycle are highly enriched during HIV infection of both macrophages and CD4+ T-cells. In T-cells, the main pathways
affected by HIV infection include: GADD45, Pancreatic and cell cycle signaling (Figure 6-6A).

Interestingly, we found that HIV-1 infection enhanced the expression of a short isoform of RUNX1 (RUNX1A); however, infection did not differentially regulate the expression of longer isoforms of RUNX1 (RUNX1B and RUNX1C) or RUNX2 and RUNX3. Using three different oligonucleotide probe sets that are complimentary to nucleotide sequences present in RUNX1A, HIV-1 infection, in the presence of Vif, contributed to a 2-4 fold increase in RUNX1A expression. In addition, Vif expression appears to minimally enhance RUNX1A expression approximately 1.3-1.5 fold. Our data is consistent with a recent report, which also observed an increase specifically in RUNX1A expression in HIV-1 infected CD4+ T-cells [186]. In addition, the group also identified the enhanced expression of RUNX1 interaction partners, LEF1 and EVI5 [186]. We found that these two RUNX partners were also up-regulated in our samples (LEF1 – up 2.5 fold, EVI5 – up 1.5 fold).

We were also curious whether genes that code for restriction factors are differentially regulated during HIV infection. Surprisingly, most APOBEC3 proteins including APOBEC3G and 3F were unchanged during infection. Yet, APOBEC3H was up-regulated in both CD4+ T-cells (~3 fold change) and macrophages (~5 fold change) (Figure 6-8a and b). In addition, BST2/Tetherin and TRIM22 were both up-regulated during HIV infection in both cell types.
Contrastingly, TRIM5 was up-regulated in macrophages, but not in CD4+ T-cells; while SAMHD1 was up-regulated in CD4+ T-cells, but not in macrophages.
Figure 6-5. Vif enhances human IL-2 and IL-8 promoter activity in 293T cells.

IL-8 promoter luciferase activity was measured in the presence and absence of wild-type Vif. IL-8 promoter activity is enhanced in the presence of Vif; however less than IL-2 promoter activity.
Figure 6-6. Biological pathways affected during HIV infection and by Vif during HIV infection of CD4+T cells. A. Top 10 canonical pathways affected during HIV infection leading to differential expression of cellular genes. B. Top 10 canonical pathways affected specifically by Vif during HIV infection. Cutoff settings: Fold change was set at absolute value of 2 and p-value of 0.05.
Figure 6-7a. HIV up-regulation of host genes in macrophages and CD4+ T-cells.

Microarray analysis reveals that 154 shared genes within macrophages and CD4+ T-cells are up-regulated during HIV infection. Differentially regulated genes were up-regulated 2-fold with a p-value < 0.5.
Figure 6-7b. HIV down-regulation of host genes in macrophages and CD4+ T-cells.

Microarray analysis reveals that 123 shared genes within macrophages and CD4+ T-cells are down-regulated during HIV infection. Differentially regulated genes were down-regulated 2-fold with a p-value < 0.5.
Figure 6-8a. Differentially expressed host restriction factors in CD4+ T-cells during HIV infection.

Microarray analysis reveals that APOBEC3H is highly up-regulated during HIV infection and is the only APOBEC3 gene up-regulated under our specific conditions. Additional host restriction factors are minimally up-regulated.
Figure 6-8b. Differentially expressed host restriction factors in macrophages during HIV infection.

Microarray analysis reveals that APOBEC3H is highly up-regulated during HIV infection and is the only APOBEC3 gene up-regulated under our specific conditions. Additional host restriction factors are minimally up-regulated.
Discussion

Taken together, the experimental evidence demonstrates that Vif associates with and most likely functionally modifies the RUNX:CBF-β complex in the nucleus. This novel Vif function is distinct from its role as a viral CRL E3 ligase that targets APOBEC3 molecules in the cytoplasm. Rather than inducing degradation of RUNX proteins, Vif associates with the RUNX/CFB-β complex and translocates into the nucleus. Furthermore, Vif up-regulates several RUNX target genes, many of which are involved in immune activation such as IL-2, GM-CSF, IL-8, MIP1α and to a lesser extent, IFN-γ in virus infected CD4+ T cells. While we did not detect differences between the expressions of some of these genes by microarray analysis, it is possible that we need to optimize our cell culture stimulation and infection conditions. In order to increase the chance of detecting a difference between Vif+ compared to Vif-deficient cells, a reduced baseline expression levels of IL-2 and IFN-γ in CD4+ T-cells is desirable.

By increasing production of several pro-inflammatory cytokines levels, Vif may work in concert with other viral proteins to activate naïve and resting T cells and promote differentiation of monocytes into macrophages to increase the available viral target cells and facilitate viral replication and spread. Moreover, we found that Vif enhances activation at the M-CSFR promoter in a RUNX dependent manner leading to increased M-CSFR expression in macrophages. It has previously been shown that HIV-1 infection induces M-CSF production [187] to subvert apoptotic killing of HIV-1 infected macrophages via M-CSFR signaling.
Therefore, by enhancing the level of M-CSFR expression level in macrophages, Vif may also promote long-term survival of the infected cells. Furthermore, we found that Vif activation of RUNX target genes is dependent on RUNX activity. For Vif activated genes, Vif not only associates with RUNX DNA binding sites on the promoter but also promotes RUNX DNA binding and association with chromatin. In summary, HIV-1 Vif protein modifies RUNX:CBF-β transcriptional regulation on several target genes as a previously unknown strategy of HIV-1 to promote immune activation and viral persistence. Since RUNX proteins are key transcription factors during thymus differentiation and T helper cell differentiation, it would be intriguing to determine whether Vif can also affect multiple stages of T cell differentiation by modifying RUNX functions, contributing to the loss of T cell homeostasis and other aspects of HIV-1 pathology. The interaction between Vif and RUNX/CBF-β proteins may be a potential therapeutic target for controlling HIV-1 infection and viral mediated immunopathogenesis.
CHAPTER 7

FUTURE DIRECTIONS

7.1 Additional mapping of the Vif:Cul5 interface to obtain a more detailed view of N- and C-terminal binding motifs

7.2 Detailed mapping of the Vif:CBF-β interface

7.3 Further crystallization studies of Vif:E3 ligase complexes

7.4 Identification of small molecule inhibitors of Vif:CBF-β and Vif:Cul5 interaction
**Introduction**

Normal host protein-protein interactions and dynamics can be altered upon HIV infection resulting in new interactions between viral and host proteins, most likely to the virus' advantage. There are currently over 30 approved HIV drugs which target only a few of the 15 HIV proteins encoded in its genome. However, problems related to drug failure, emergence of drug-resistant variants, and treatment-related adverse consequences persist. Thus, the range of anti-HIV therapies requires expansion. The discovery of novel inhibitors that combat additional HIV proteins depends upon the identification and understanding of cellular factors that bind viral proteins.

**Additional mapping of the Vif:Cul5 interface to obtain a more detailed view of N- and C-terminal binding motifs**

Since we identified a novel N-terminal motif that is responsible for Cul5 interaction, we plan to study whether there are still additional as yet to be discovered regions of the Vif polypeptide that are required for Cul5 or even Elo B/C binding. The N-terminal motif spanning residues 25-30 that is required for Cul5 interaction is interesting because residues close to this region are also thought to interact with various host proteins, such as APOBEC3 and CBF-β. As many regions in the Vif N-terminus have been implicated in APOBEC3G degradation, these additional regions should be examined for CBF-β binding. The primary goal of mapping the required motifs for Vif:Cul5 interaction may yield
greater insight into the requirement and regulation of the Vif:Cul5 interaction for APOBEC3G/F degradation.

**Detailed mapping of the Vif:CBF-β interface**

Additionally, the identification of additional Vif motifs required for CBF-β binding are equally important for an enhanced understanding of the mechanism used to degrade APOBEC3G/F. Recently, our lab established a more detailed understanding of residues required for the interaction between Vif and CBF-β. First, in terms of CBF-β binding, the Vif N-terminus was very sensitive to deletions. We determined that Vif fragment 5-126 was sufficient to form a stable complex with CBF-β *in vitro*; however, removal of additional residues disrupted the interaction between the two proteins. Additionally, ionic interactions were not the main contributor to the interaction between Vif and CBF-β. Instead, hydrophobic interactions were important for maintaining the Vif:CBF-β complex, since it could be disrupted by non-ionic detergent. Site-directed mutagenesis of conserved hydrophobic amino acids revealed novel residues in Vif that were important for CBF-β binding and APOBEC3 inactivation. In particular, W5, W21, W38, W89, and F115 were shown to be required for the binding of Vif to CBFβ. Interestingly, residue F115 is positioned within the well-characterized HCCH domain (residues 108-139). Thus, the HCCH motif may have a dual role in recruiting both Cul5 and CBF-β.

**Further crystallization studies of Vif:E3 ligase complexes**


We have screened many Vif-E3 ligase complexes for crystallization; however, to date we have been unsuccessful in obtaining crystals for structure analysis. As we learn more about specific Vif regions required for ligase assembly, our Vif constructs can be modified to contain minimal binding domains of interest to reduce the inherent flexibility and increase the likelihood of crystallization. It is also possible to try a variety of crosslinking conditions with the Vif-E3 ligase complex in order to reduce the intrinsic flexibility of the complex. Additionally, more screening conditions should be tested with a variety of complex concentrations. Finally, we primarily used two purification steps (affinity and gel filtration) for isolating the Vif:E3 ligase complex; however, it is possible that utilizing a three step purification process that includes an ion exchange step may increase the likelihood of obtaining crystals.

During the writing of this thesis, a 3-dimensional crystallographic structure of the Vif-CBF-β-Cul5-Elo B/C complex was solved by another research group[188]. The Vif structure was comprised of 5 alpha helices and 6 beta strands, which formed an anti-parallel beta sheet. The interaction between Vif and CBF-β was quite extensive spanning most of the N-terminal portion of the Vif polypeptide. As expected and previously reported, the Vif BC box formed an alpha helix which interacted directly with two helices in Elo C. Furthermore, an alpha helix formed within the HCCH domain directly interacted with N-terminal Cul5 residues. All three partner proteins formed hydrophobic contacts with Vif hydrophobic surface residues, leaving the positively charged surface area solvent exposed.
Surprisingly, the residues that form the first N-terminal alpha helix and were reported in this thesis to be important for Vif-Cul5 interaction, do not contribute to the direct interaction between Vif and Cul5 in the reported crystal structure. In addition, helix 1 was also not involved in Elo B/C and CBF-β binding to Vif. Further investigation is warranted to understand how this helix contributes to either the overall Vif structure or recruitment of Cul5.

**Identification of small molecule inhibitors of Vif:CBF-β and Vif:Cul5 interaction**

We have developed a high-throughput small molecule inhibitor screen to identify compounds that inhibit the interaction between Vif and CBF-β as well as Vif and Cul5. The fluorescence plate-based screen was optimized using a small molecule inhibitor known to bind and disrupt the CBF-β:RUNX interaction. We plan to test diverse compounds from herbs, marine products, and microorganisms in addition to chemical libraries from TimTek and the Johns Hopkins High throughput screen Center to determine inhibitors of Vif:CBF-β and Vif:Cul5 interactions using the developed screen. To further develop initial screen hits, we plan to employ a cell-based two-hybrid screen (CheckMate/Flexi, Promega) using a luciferase reporter gene, which can be adapted for a high-throughput method for drug screen assays. CBF-β will be fused to a Gal4 protein DNA binding domain, while Vif will be fused to VP16 protein transcriptional activating domain. When the two proteins interact, the reporter gene will be expressed and its expression signal is recorded using a luminometer plate reader.
(Synergy HT, BioTek). In the presence of an inhibitor, the interaction between Vif and CBF-β will be disrupted and luciferase expression will be dramatically decreased. Additionally, we would like to develop assays that are intended to eliminate false-positive and non-specific inhibitors and allow prioritization of promising hits for further testing (selectivity and toxicity).
REFERENCES


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150. Farrow MA, Somasundaran M, Zhang C, Gabuzda D, Sullivan JL, Greenough TC: **Nuclear localization of HIV type 1 Vif isolated from a long-


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SUMMARY STATEMENT

I am an organized, independent, and self-motivated scientist with 10+ years of education and experience in the life sciences. I have a desire to utilize my technical knowledge in the life sciences to work as a scientist, analyst, and/or consultant where I can leverage my problem solving and communication skills.

EDUCATION

Doctor of Philosophy (PhD) in Molecular Microbiology & Immunology (February 2014), Johns Hopkins Bloomberg School of Public Health

Master of Science (MS) in Biotechnology (May 2008), Johns Hopkins University

Bachelor of Arts (BA) in Biology (May 2003), College of Charleston

EXPERIENCE (RESEARCH)

Researcher/PhD Student, HIV-Host Protein Interactions (August 2008-present)
Leading a project to understand the assembly of HIV-1 viral infectivity factor (Vif) with host E3 ligase components Cullin 5, Elongins B/C, and core binding factor
beta (CBF-β) that are responsible for the suppression of an anti-HIV host factor, APOBEC3.

• Developed high-throughput in vitro fluorescence-based assay to test library of small molecule inhibitors of the interaction between HIV-1 Vif and key host factors (CBF-β and Cullin 5)
• Developed assay to detect and visualize interaction between HIV-1 Vif and CBF-β in mammalian cells
• Wrote pre-doctoral research proposal and obtained NIH individual pre-doctoral fellowship - $85,000
• Wrote/co-authored Technology Transfer Seed Grant - $50,000
• Wrote/co-authored multiple peer reviewed scientific publications
• Presented (oral and poster) research at national and local scientific meetings
• Trained and managed 4 students (1 undergraduate, 3 graduate) to develop and execute a research project
• Manage lab resources inventory and ordering
• Meet with advisor 1-2x/week to discuss project progress

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, 21205
Thesis Advisor - Xiao-Fang Yu, MD, ScD.

Teaching Assistant, Pathogenesis of Bacterial Infections (October 2009-December 2009)
• Assisted course professor in planning and implementing course objectives.

• Led laboratory practicums as well as created and evaluated midterm and final exams.

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, 21205
Professor – Egbert Hoiczyk, PhD.

Researcher, Cancer Research Interns in Residence (May 2008-August 2008)
Led a project to validate adenoviral replication/host cell lysis in BALB-neuT murine tumor cell lines for future preclinical trial use in immune-competent murine models.
National Cancer Institute, National Institutes of Health, Bethesda, MD, 20892
Principal Investigator – John C. Morris, M.D.

Researcher, Post-Baccalaureate Research Education Program (June 2006-July 2008)
Part of a research team that sought to understand the effects of concentrated animal feeding operations on the environment as well as the effects of mercury on the immune system

• Co-author of 4 peer reviewed publications

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, 21205
Principal Investigator – Ellen K. Silbergeld, Ph.D.
EXPERIENCE (NON-RESEARCH)

Technology Transfer Analyst Intern (October 2012-present)

- Developed over 25 technical, commercial, and IP assessment reports (including prior art, patentability, and market analyses) for new technologies disclosed to JHTT
- Assist Licensing Associates with the development of commercialization strategies and intellectual property managers with IP filing strategies for JHU technologies
- Receive intensive training in the field of intellectual property management, patent prosecution, marketing, and technology transfer policy

Office of Technology Transfer, Johns Hopkins University, Baltimore, MD, 21201
Coordinator – Nakisha Holder, PhD.


Part of a team of education volunteers that taught biology and mathematics secondary school in various locations throughout the United Republic of Tanzania.

- Participated in 10-week intensive language (Swahili, 124 hours), cultural (62 hours), health/safety (22 hours), and technical training in education pedagogy and practice (117 hours)
- Developed curriculum and daily lesson plans for Mathematics (topics - algebra, trigonometry, calculus, basic statistics, and geometry) and
Biology (topics - nervous/endocrine systems, plant and animal regulation, Reproduction and meiosis, mitosis, evolution, and genetics) courses

• Led 20-28 forty minute course periods over the course of two years
• Developed and executed a 2-day health seminar for approximately 75 students and 2 teachers focusing on sexual responsibility and HIV/AIDS progression, symptoms and transference

Lindi, Tanzania, East Africa

Country Director – Marily Knieriemen

PUBLICATIONS

HIV-1 Vif Recruits Cul5 via a Novel N-terminal Motif to Suppress APOBEC3.  
Evans SL, Schön A, Gao Q, Han X, Zhou X, Freire E, and Yu XF.  

Evolutionarily Conserved Requirement for CBF-β in the Assembly of the HIV/SIV Vif-Cullin5-RING E3 Ubiquitin Ligase. Han X, Liang W, Hua D, Zhou X, Du J,  
January 2014.


* indicates collaborating first authors

**PRESENTATIONS**

Vif recruits Cul5 via a novel N-terminal motif to suppress APOBEC3.

**Evans SL**, Han X, Zhou X, Schon A, Freire E, and Yu XF.


Evolutionarily Conserved Requirement of CBF-β for the Assembly of HIV/SIV Vif Cul5 Cullin-Ring Ligase.


Association between HIV-1 Vif and Host CBF-β is Essential to Viral Evasion of APOBEC3 Restriction.


RELEVANT TECHNICAL SKILLS

- general molecular biology lab techniques: PCR, RT-PCR, site-directed mutagenesis, plasmid cloning and preparation, nucleic acid electrophoresis, SDS-PAGE, immunoblotting, immunoprecipitation
- general microbiology and mammalian cell culture: *E.coli* culture and transformation, cell culture (HeLa, HEK293T, various T-cell lines, primary lymphocytes, THP-1), primary lymphocyte isolation from blood samples, transient and stable transfection of mammalian cells, virus production, concentration (by ultracentrifugation) and cellular infection
- *in vitro* protein production and analysis: (*E. coli*) protein overexpression, protein purification (affinity, ion-exchange, and size exclusion) using fast protein liquid chromatography (FPLC), protein crystal preparation and screening, dynamic light scatter (DLS), isothermal titration calorimetry (ITC)
- flow cytometry
• promoter reporter gene assay (luciferase-based)
• microarray statistical data analysis
• live cell and fixed confocal fluorescence microscopy, live cell protein-protein interaction assay (FRET)

GRANTS, HONORS, AND PROFESSIONAL MEMBERSHIPS

Scientist Mentoring & Diversity Program (SMDP) for Biotechnology Scholar
2013-present

Intramural NIAID Research Opportunities (INRO) Scholar 2013-present

Ruth L. Kirschstein National Research Service Award 2011-2013
Individual Pre-doctoral Fellow (National Institutes of Health)

Johns Hopkins Biomedical Scholars Association 2009-present

Vice President – 2011-2012
Parliamentarian – 2010-2011

J Harold Drudge Scholar 2009-10