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Making Sense of Multifunctional Proteins: Human Immunodeficiency Virus Type 1 Accessory and Regulatory Proteins and Connections to Transcription

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Abstract

Viruses are completely dependent upon cellular machinery to support replication and have therefore developed strategies to co-opt cellular processes to optimize infection and counter host immune defenses. Many viruses, including human immunodeficiency virus type 1 (HIV-1), encode a relatively small number of genes. Viruses with limited genetic content often encode multifunctional proteins that function at multiple stages of the viral replication cycle. In this review, we discuss the functions of HIV-1 regulatory (Tat and Rev) and accessory (Vif, Vpr, Vpu, and Nef) proteins. Each of these proteins has a highly conserved primary activity; however, numerous additional activities have been attributed to these viral proteins. We explore the possibility that HIV-1 proteins leverage their multifunctional nature to alter host transcriptional networks to elicit a diverse set of cellular responses. Although these transcriptional effects appear to benefit the virus, it is not yet clear whether they are strongly selected for during viral evolution or are a ripple effect from the primary function. As our detailed knowledge of these viral proteins improves, we will undoubtedly uncover how the multifunctional nature of these HIV-1 regulatory and accessory proteins, and in particular their transcriptional functions, work to drive viral pathogenesis.

Keywords

human immunodeficiency virus; host-pathogen interactions; transcription; virus replication; multifunctional proteins

INTRODUCTION

A defining feature of retroviruses is the reverse transcription of an RNA genome into a DNA copy, which is subsequently integrated into the host cell genome. The family *Retroviridae* can be further subdivided at the genus level: *Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Lentivirus*, and *Spumavirus*. Gammaretroviruses, such as Moloney murine leukemia virus (M-MLV), and alpharetroviruses, such as avian sarcoma leukosis virus (ASLV), encode only the *gag, pol*, and *env* structural and enzymatic genes to complete their life cycle. In contrast, other species of retroviruses, such as human

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immunodeficiency virus type 1 (HIV-1) of the *Lentivirus*, encode several additional accessory and regulatory proteins to modulate host cell immune responses or control the expression of the viral genome. The accessory proteins of HIV-1 are Vif, Vpr, Vpu, and Nef and the regulatory proteins are Tat and Rev. Since the discovery in the early 1980s that HIV-1 is the causative agent of acquired immune deficiency syndrome (AIDS), intensive research has been dedicated to understanding how these viral proteins subvert the host cell machinery to support active replication. Generally, each of these proteins has a highly conserved function that is considered its primary activity. However, numerous additional activities have been attributed to these viral proteins (secondary activities), leading to the conclusion that the accessory and regulatory factors are highly multifunctional. In this review we summarize the primary, most conserved function of the accessory and regulatory proteins but also describe secondary activities attributed to these relatively small proteins. These secondary activities have not been conclusively proven in all cases and we do not exhaustively cover all reports in the literature, nor is it clear whether these functions simply reflect pleiotropic effects indirectly stemming from their primary functions. However, it is intriguing that many of the secondary functions alter either viral or cellular transcription, prompting us to explore the possibility that HIV-1 utilizes multifunctional proteins to rewire host transcription networks. By emphasizing this one function common to all the accessory and regulatory proteins (transcription), we illustrate how perturbations in diverse cellular pathways (i.e., the primary function of the proteins) can be funneled into a single type of secondary output. Although these transcriptional effects may benefit the virus in various ways, it is not yet clear whether they are strongly selected for during viral evolution. It will be important to explore separation-of-function mutants in these viral proteins to establish whether they truly encode multiple essential functions or whether the secondary transcriptional effects are entirely indirect. If the transcriptional responses are indeed a ripple effect from the primary function, these viral factors may still have opportunities to evolve and better exploit these secondary functions for virus replication given that HIV-1 was transmitted to its human host only approximately one hundred years ago (1).

Tat

Primary Function

Following HIV-1 integration into the host genome, RNA polymerase II (RNAP II) assembles at the viral promoter located in the 5′ long terminal repeat (LTR) and begins the process of transcribing viral RNA. The promoter contains *cis* elements for host transcription factors, including three SP1 sites and two nuclear factor κB (NF-κB) sites, which provide an entirely cell-intrinsic mechanism to activate transcription, especially in activated T cells targeted by HIV-1 where the NF-κB pathway is induced (2). However, despite this appropriation of cellular mechanisms to increase gene expression, transcription complexes that assemble at the HIV-1 promoter generate predominantly short, incomplete viral transcripts (3).

To bypass this block to transcription elongation, HIV-1 encodes its own transcription factor, Tat, which increases the processivity of RNAP II to generate full-length viral mRNAs (Figure 1) (4). Tat activates transcription by binding to a nascent, 5′ stem-loop RNA structure termed the transactivation response element (TAR) (5). A major host cofactor for

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Tat is cyclin T1 (CCNT1) (6), which, together with cyclin-dependent kinase 9 (CDK9), constitutes positive transcription elongation factor b (P-TEFb). This heterodimeric host kinase phosphorylates and activates paused RNAP II and regulates elongation at most cellular genes (7). The earliest models of Tat activity proposed that Tat recruited the P-TEFb complex to the nascent TAR RNA, which positioned the kinase in proximity to the stalled polymerase for phosphorylation-dependent activation (8). Interestingly, CCNT1 also contributes to RNA binding as it contacts bases in the TAR loop to achieve a high-affinity interaction (9). The Tat-P-TEFb complex potently stimulates viral gene expression, initiating the postintegration steps of the life cycle, which eventually leads to viral budding and the infection of new cells.

The majority of P-TEFb in the cell is sequestered in an RNA-based inactive complex termed the 7SK small nuclear ribonucleoprotein (snRNP) (10, 11) in which the HEXIM1 protein inhibits the kinase activity of CKD9 in an RNA-dependent manner (12). Consistent with the original model of Tat activity, HIV-1 replication (13, 14) or Tat expression alone (14) releases a significant fraction of P-TEFb from the inhibitory complex. This release is due to competitive binding between Tat and HEXIM1 for CCNT1, and the P-TEFb freed from the 7SK snRNP can then be delivered to a paused RNAP II at the viral promoter for activation.

However, 7SK snRNP–inhibited P-TEFb can be recruited to the HIV-1 promoter and associates with RNAP II (15), which provides a simple explanation for why RNAP II transcription is nonprocessive in its basal state. When Tat is synthesized, it can also bind the HIV-1 promoter together with the 7SK snRNP even in the absence of TAR, which demonstrates that the viral RNA is not necessary for recruitment of Tat or P-TEFb. Once the TAR RNA is transcribed by RNAP II, the proteins of the inhibitory 7SK snRNP are displaced from DNA-bound transcription complexes, which activate the kinase activity of P-TEFb to phosphorylate RNAP II. In addition, Tat forms a soluble Tat–7SK snRNA–P-TEFb complex upon ejection of HEXIM1 (16), which itself might be recruited to the viral promoter. TAR expression would then hand off Tat from the cellular RNA to the viral RNA, completing the displacement of the inhibitory complex. In addition to the 7SK snRNP, Tat binds a larger P-TEFb complex termed the superelongation complex (SEC) (16, 17). The 7SK snRNP and SEC are distinct complexes, although recent work has demonstrated that the AFF1 scaffold protein of the SEC is a ubiquitous P-TEFb partner and therefore a component of the 7SK snRNP (18). The SEC is required for full Tat activation and the AFF4 scaffold increases the affinity of Tat-P-TEFb for TAR by 30-fold, likely by limiting the flexibility of the Tat-TAR recognition motif (TRM) of CCNT1 (19). The extent of cross talk between the 7SK snRNP and the SEC is still uncertain; however, Tat clearly utilizes multiple host complexes to achieve potent transcriptional stimulation of the integrated provirus.

Secondary Function

In addition to activating the viral promoter, Tat alters the expression of cellular genes to facilitate viral replication. For example, HIV-1 infection or Tat expression increased the transcription and secretion of four chemokines (IP-10, HuMIG, MCP-2, and MCP-3) in immature dendritic cells (iDCs) (20), which then induced the chemotaxis of T cells and monocytes. The migration of these HIV-1 target cells was proposed as a means for the virus

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to amplify the infection. Despite the induction of chemokines, Tat did not cause generic iDC maturation, arguing for a specific cellular response. As Tat is localized to the nucleus, cytoplasm, and cellular membrane (21, 22), its effect on cellular transcription could be direct or indirect.

Several groups have demonstrated a direct effect of Tat on host transcription by localizing the viral transcription factor to cellular promoters. Chromatin immunoprecipitation (ChIP)-on-chip with a microarray chip containing human promoter sequences illustrated that Tat could bind approximately 450 promoters in Jurkat cells, although changes in the expression of these genes were not globally determined (23). However, Tat specifically bound the promoters and increased the expression of two regulatory proteins of the PP2A phosphatase, PPP2R1B and PPP2R5E. PP2A dephosphorylates FOXO3a, which then translocates to the nucleus to activate a proapoptotic pathway. HIV-1 infection can cause apoptosis in $CD4^+$ T cells (23), and specific knockdown of the regulatory phosphatase subunits prevented the observed Tat-dependent apoptosis in Jurkat cells. Therefore, Tat induction of PPP2R1B and PPP2R5E expression is a plausible mechanism for HIV-1-triggered apoptosis of infected cells. More recent Tat ChIP-sequencing and RNA-sequencing experiments in Jurkat cells have also uncovered 456 cellular genes that are bound by Tat and experience changes in gene expression; however, the overlap with the previously identified Tat gene targets was not evaluated (24). Some genes are stimulated, whereas others are downregulated, and regulation may occur via transcription initiation or elongation. TAR-like RNA structures were not identified on the nascent transcripts that might provide binding sites for Tat, but instead, ETS1, a T cell master transcription factor, was enriched near most Tat peaks (Figure 2). Indeed, Tat bound ETS1 and knockdown of ETS1 reduced Tat recruitment at target genes, suggesting that Tat affects host gene transcription through the ETS1 interaction. It is not yet clear how hijacking cellular transcription networks may benefit the virus or whether the interaction with ETS1 is under selective pressure.

Tat may also alter cellular transcription indirectly, as many genes that show changes in expression do not show corresponding physical enrichment of Tat (24). This may occur by altering the activity of other transcription factors, which consequently affect downstream gene expression. For example, Tat increases IRF7 and STAT1 expression (20), which regulates interferon-inducible genes. Tat may indirectly affect transcription of other genes through competition with protein-protein interactions. For example, in forming the Tat–P-TEFb complex for viral transcription, Tat buries 3,500 $Å^2$ of surface area on P-TEFb, the majority on the CCNT1 subunit (25). This interaction surface is competitive with several host proteins that control P-TEFb activity, including HEXIM1 (14), BRD4 (26), and CIITA (27), and indeed, Tat expression, even at low physiological levels during infection, releases a substantial amount of P-TEFb from the 7SK snRNP (13, 14). The pool of freed P-TEFb released from the inhibited 7SK complexes would then be available to activate cellular gene expression, either with or without Tat. At the CD69 promoter, for example, Tat binding increases P-TEFb recruitment and transcription and may require ETS1 (24). In another example, competition between Tat and CIITA for CCNT1 binding decreases the expression of major histocompatibility complex (MHC) class II genes and inhibits antigen presentation in macrophages, potentially reflecting another strategy for Tat to establish optimal replication conditions (27). Finally, BRD4 inhibitors activate Tat-dependent HIV-1

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transcription by increasing the pool of P-TEFb available for Tat, consistent with a competition model between Tat and BRD4 (28). These results suggest that Tat may alter many host P-TEFb regulatory pathways by titrating the elongation factor away from other binding partners.

Tat protein produced during infection can be secreted (22) and enter neighboring cells by endocytosis (29). Although the biological importance of extracellular Tat has not yet been conclusively established, the secreted Tat pool can activate T cells independently of antigen but not increase T cell proliferation. These activated T cells are then prone to infection, further amplifying viral replication (30, 31). This activity appears to be largely a transcriptional effect, as exogenous Tat deregulates 94 genes in primary T cells, leading to the secretion of interleukin 17 (IL-17) to generate a proinflammatory state suited to viral infection (30), although it is unclear whether this transcriptional effect is direct or indirect.

It will be interesting to determine whether the functions of Tat in viral and cellular transcription entirely overlap at the genetic level or whether certain amino acids in Tat specifically affect cellular transcription. In this regard, several amino acids in Tat that do not overlap with other protein reading frames in the virus are highly conserved in patients (Glu9, Pro10, Trp11, Gln17, Thr20, and Ala21) yet display neutral selection during replication competition experiments in Sup-T1 cells (32). Future work will reveal whether some of these residues have undergone positive selection for host transcription responses or other functions that are not recapitulated in tissue culture, or whether these residues function in a cell-type-specific manner.

Rev

Primary Function

After transcriptional activation by Tat, expression of the full-length viral transcripts is regulated by splicing and export. Complete cellular processing yields spliced RNAs that are exported from the nucleus through the canonical TAP pathway and results in translation of only three viral proteins: Tat, Rev, and Nef. Rev is then reimported into the nucleus through its nuclear localization sequence (NLS), where it directs the export of partially and fully unspliced messages that are translated into the remaining viral proteins and provides genomic RNA for packaging into budding virions (33). Rev, an RNA-binding protein similar to Tat, exports the intron-containing RNAs by binding specifically to the ~350-nucleotide, highly structured Rev response element (RRE) contained in the Env sequence (Figure 1) (34). The Rev NLS, which is an arginine-rich motif (ARM), doubles as its RNA-binding domain. Rev also encodes two hydrophobic oligomerization domains (ODs) and a leucine-containing nuclear export sequence (NES), which are critical for its export activity. Rev-mediated nuclear export is a multistep process that is initiated when the ARM contacts a high-affinity site in stem IIB of the RRE RNA (33). Rev then likely dimerizes on the RNA through the OD when a second Rev monomer contacts the adjacent stem IIABC in the RRE, followed by further Rev oligomerization on the RNA to yield an export-competent RNP containing 6–10 Rev subunits (35–39).

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The major host cofactor for Rev export is chromosome maintenance factor 1 (CRM1) (40). CRM1 normally exports cellular proteins that contain a leucine-rich NES and is not typically involved in mRNA export. Rev hijacks CRM1 for viral RNA export by functioning as an adaptor, wherein the Rev NES engages the host export factor and the ARM engages RRE-containing messages. Interestingly, recent electron microscopy reconstructions demonstrate that the export complex contains a dimer of CRM1 (41). Given that CRM1 had been shown to function only as a monomer for known cellular cargos, Rev-dependent CRM1 dimerization is likely a way to increase recognition of the low-affinity Rev NES sites. Assembly of this virus-host export complex results in robust cytoplasmic trafficking of intron-containing HIV-1 messages to complete the later stages of the virus life cycle. It is currently unclear whether the dimerization of CRM1 is unique to the Rev-RRE RNP or reflects a mode of binding of other host cargos. It also is not known whether Rev-RRE binding significantly alters the endogenous pool of CRM1 and thereby affects host RNA export or gene expression as a secondary consequence, or whether Rev at physiological concentrations binds and exports cellular messages that contain RNA structural elements similar to the RRE or other RNA features.

Secondary Function

Rev indirectly regulatesHIV-1 transcription in at least two ways. First, the fully spliced transcripts encoding Tat, Rev, and Nef lack the RRE; therefore, Rev export of RRE-containing messages decreases the accumulation of fully spliced messages. This posttranscriptional negative feedback loop decreases Tat protein levels (42). Second, Rev decreases Tat protein at a posttranslational step. In this mechanism, physiological expression of Rev during infection decreases the levels of the NQO1 protein, which is an inhibitor of the 20S core proteasome. Tat, an inherently unstructured protein, can be degraded by the 20S complex, and the Rev-dependent decrease in the inhibitory NQO1 protein activates the 20S proteasome to degrade Tat and decrease viral transcription (Figure 2) (43). Therefore, Rev inhibits Tat activity at posttranscriptional and posttranslational levels. Interestingly, several transcription factors, including p53 (44), are intrinsically disordered and are regulated by 20S proteasomal degradation. Future work may uncover that Rev also indirectly deregulates cellular transcription by inducing the degradation of transcription factors through NQO1 and the 20S proteasome.

Vif

Primary Function

Among the known lentiviral accessory proteins, Vif is found in all lentiviruses except equine infectious anemia virus (EIAV). The primary function of Vif is to counteract the antiviral effects of host APOBEC3 (A3) innate immune proteins, restriction factors that inhibit replication by inducing hypermutation of the viral genome (45–47). Vif antagonizes A3 by hijacking a cellular Cullin-RING ubiquitin ligase (CRL), resulting in the ubiquitination and subsequent targeting of A3 for proteasomal degradation (48–51). The HIV-1 Vif E3 ligase complex comprises CRL5, which includesCullin-5 (CUL5), elongin B (ELOB), elongin C (ELOC), and RING-box protein 2 (RBX2), as well as a noncanonical cofactor, core-binding factor beta subunit (CBFβ) (50–52). The recruitment of CBFβ to the Vif E3 ligase is

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surprising given that CBFβ is a transcription cofactor and not a component of a known cellular E3 ligase. Recruitment of CBFβ serves to stabilize HIV-1 Vif and is required for HIV-1 Vif-mediated A3 degradation activity in vivo (Figure 1) (50, 51, 53–55). More recent work has shown that CBFβ is required only for primate lentiviral infection, as it is dispensable for nonprimate Vif function (53, 56). These data suggest that there was an evolutionary pressure that promoted the acquisition of CBFβ to the primate Vif complex. A number of reviews cover the primary function of Vif to ubiquitinate APOBEC3 proteins (57–60); here, we discuss the role Vif has in altering the host transcriptome.

Secondary Function

CBFβ forms a heterodimer with members of the RUNX family of transcription factors, serving to both stabilize RUNX steady-state levels and enhance DNA-binding affinity to regulate the expression of a diverse set of genes (61, 62). Initial in vitro biochemical work established that Vif can outcompete RUNX for CBFβ binding, suggesting that the virus may utilize the Vif-CBFβ interaction to alter gene expression in infected T cells (54). Indeed, overexpression of HIV-1 Vif in permissive Jurkat T cells altered the expression patterns of a large number of genes that exhibit enriched RUNX1 binding sites (Figure 2) (54). Moreover, a pharmacological approach to inhibit RUNX1 demonstrated that RUNX1 and CBFβ play a role in reducing HIV-1 replication (63). Together, these data establish that the presence of Vif alters endogenous RUNX activity, potentially to the benefit of the virus.

More recent work by Anderson & Harris (64) presents an additional facet to the role the Vif-CBFβ interaction has in viral infectivity. In their efforts to investigate CBFβ function, they employed a separation-of-function mutant that would allow CBFβ to bind to either RUNX or Vif (64, 65). Using this separation-of-function CBFβ construct, they discovered that the CBFβ- RUNX interaction is required for APOBEC3 transcription. Reduction or ablation of CBFβ mRNA by RNA interference (RNAi) or CRISPRs reduced the expression of APOBEC3 (C, D, F, G, and H) mRNA, as detected by quantitative reverse transcription PCR in either $CD4^+$ T cell lines or primary $CD4^+$ T cells. Furthermore, an RNAi-resistant CBFβ complemented the CBFβ knockdown by increasing A3G protein expression levels, and this effect required interaction with the RUNX proteins. Importantly, ablation of CBFβ rendered nonpermissive H9 cells permissive to infection with a Vif-deficient HIV-1 virus, as the restrictive potential of APOBEC3 in these cells was almost completely suppressed (64). Additionally, these data might explain why primate Vif acquired the CBFβ interaction. It is tempting to speculate that the Vif-CBFβ interaction developed in an effort to allow Vif to disrupt the RUNX-mediated transcription of APOBEC3 proteins and thus counteract the APOBEC3 repertoire at the transcriptional and posttranslational levels. Although it is uncertain what driving force promoted the primate Vif-CBFβ interaction, together these findings highlight the large transcriptional changes that arose from Vif hijacking CBFβ and the significant implications this has on both the virus and the infected cell.

In addition to the transcriptional consequences of hijacking CBFβ, Vif remodels the cellular phosphoproteome during HIV-1 infection. A whole-cell proteomics study demonstrated that Vif was necessary and sufficient for the proteasomal degradation of the B56 family of regulatory subunits of the cellular phosphatase PP2A, and quantitative phosphoproteomics

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revealed Vif-dependent hyperphosphorylation of over 200 cellular proteins (66). Intriguingly, the ability of Vif to target PPP2R5 subunits is found in primate and nonprimate lentiviral lineages, suggesting that remodeling the cellular phosphoproteome is a conserved function of Vif. Although there is currently no direct link to transcription through PP2A degradation, the general importance of posttranslational modifications, especially phosphorylation, in regulating transcription is well-established. Therefore, future work will likely demonstrate that Vif-mediated remodeling of host phosphorylation will have major effects on cellular transcription.

Vpu

Primary Function

The *vpu* gene is found exclusively in HIV-1 and precursor simian immunodeficiency virus (SIV) strains and produces a small, transmembrane protein, Vpu, that is expressed late in the viral replication cycle (67–70). Initially, Vpu was observed to play a critical role in facilitating viral egress from the plasma membrane; however, these observations were cell type specific, suggesting the presence of a host restriction factor (67, 71). Almost 20 years after the discovery that Vpu promotes viral release, the host factor BST-2/Tetherin was identified as a target of Vpu (72, 73). The Vpu-mediated inhibition of BST-2/Tetherin is the most active area of current Vpu-related research; however, additional functions are attributed to this protein. These include primarily the downregulation of CD4 and MHC1 molecules, the inhibition of NF-κB activation, and the formation of a viroporin ion channel in the Golgi apparatus to alter membrane potential and possibly enhance virion release (74–78).

By removing membrane-bound host proteins that inhibit viral replication, particularly CD4 and BST-2/Tetherin (79), Vpu remodels the cell surface to carry out its functions. To downregulate CD4, Vpu targets newly synthesized CD4 while it is in the endoplasmic reticulum (ER), thus preventing it from trafficking to the plasma membrane (Figure 1). Mechanistically, Vpu does this by recruiting the CUL1–β-TrCP–Skp1–RBX1 E3 ligase complex to the ER, where it ubiquitinates newly synthesized CD4 molecules, leading to their retention in the ER (80). As a result, the ubiquitinated CD4 molecules are processed through the ER-associated degradation (ERAD) pathway and ultimately degraded by the proteasome (81, 82). In addition to functioning in the ER, Vpu acts at the plasma membrane to counteract the inhibitory role of BST-2. BST-2/Tetherin is a type II transmembrane protein that is thought to directly tether Vpu-deficient virions to the surface of infected cells (83, 84). Vpu decreases BST-2/Tetherin found on the cell surface by directly binding to it and inhibiting the recycling of internalized BST2 back to the plasma membrane (Figure 1) (73, 85, 86). Vpu can also ubiquitinate BST-2/Tetherin via the β-TrCP–Skp1–RBX1 E3 ligase complex, which destines BST-2/Tetherin for lysosomal degradation (87, 88). Lentiviruses neutralize host BST-2/Tetherin through multiple mechanisms; select SIV strains utilize Nef, HIV-2 utilizes its Env glycoprotein, and HIV-1 has evolved to utilize Vpu (89–96). That lentiviruses have evolved multiple ways to antagonize BST-2 emphasizes the importance of neutralizing this restriction factor for viral pathogenesis.

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Secondary Function

Vpu also influences immune signaling, particularly through deregulation of the NF-κB pathway. This is thought to occur through two distinct mechanisms. First, Vpu restricts NF-κB signaling by downregulating BST-2/Tetherin. In addition to its role in preventing viral budding, BST-2/Tetherin activates the NF-κB pathway (97–99). Therefore, Vpu-induced reduction of BST-2/Tetherin at the plasma membrane also dampens NF-κB signaling (Figure 2). Second, Vpu sequesters the F-box protein β-TrCP, which it uses to ubiquitinate BST-2/Tetherin. During the normal activation of the NF-κB pathway, the $SCF^{\beta\text{-TrCP}}$ ligase degrades the inhibitor of NF-κB (IκB), which allows the nuclear translocation of NF-κB. However, as noted above, Vpu, which contains a canonical DpSGxxpS phosphodegron bound by β-TrCP, also uses $SCF^{\beta\text{-TrCP}}$ to ubiquitinate BST-2/Tetherin. In this model, Vpu binding of $SCF^{\beta\text{-TrCP}}$ prevents the degradation of IκB, which further inhibits signaling through the NF-κB pathway (100, 101). These effects were also observed during infection, arguing against an overexpression artifact (100). A Vpu phosphodegron mutant that is deficient in binding to $SCF^{\beta\text{-TrCP}}$ (S52/56N) is unable to inhibit the NF-κB pathway (100, 102, 103), which supports a role for the ubiquitin ligase in Vpu antitranscriptional activity and is consistent with both models. In support of the sequestration model, Vpu expression also stabilizes a number of $SCF^{\beta\text{-TrCP}}$ substrates, including β-catenin. Importantly, this stabilization is lost with the Vpu S52/56N mutant.

Changes to NF-κB signaling likely has significant transcriptional consequences for the cell, because NF-κB is an important transcription factor that facilitates biological processes such as cell proliferation, cytokine production, and induction of apoptosis (104). The two-pronged deregulation of NF-κB signaling by Vpu also has similarities to the downregulation of A3G by Vif. In each case, a strong primary interaction (Vpu–β-TrCP or Vif–CBFβ) modulates two nodes of a pathway, with an ultimate effect on transcription. A simple model, then, for the pleiotropic effects of the HIV-1 regulatory and accessory proteins is that they target critical host proteins involved in many pathways, such that their deregulation leads to a multitude of effects in the cell.

Vpr

Primary Function

The Vpr protein is conserved across human and primate lentiviruses and is specifically incorporated into the viral particle by interactions with the p6 domain of Gag (105). Vpr has many reported functions, including LTR transactivation, nuclear import of the preintegration complex, cellular apoptosis, cell cycle arrest, and activation of the DNA damage response (106–111). The activation of the DNA damage response by Vpr is conserved across primate lentiviruses and is considered its primary function (112). The interaction of Vpr with $CRL4^{DCAF1}$ is required for many of these phenotypes (113–115). For example, knockdown of DCAF1 prevents Vpr-induced G_2 arrest, highlighting that the $CRL4^{DCAF1}$ ubiquitin ligase complex is essential for Vpr's primary function (Figure 1) (113, 114).

Extensive effort has been directed at identifying the cellular substrate of the Vpr-$CRL4^{DCAF1\text{-}Vpr}$ complex whose ubiquitination results in the DNA damage response and G_2

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arrest. Laguette et al. (116) recently proposed that aberrant activation of the SLX4 DNA damage response complex by Vpr induces cell cycle arrest. In this model, Vpr recruitment of DCAF1 and the kinase PLK1 to the MUS81-EME1 endonuclease of the SLX4 complex prematurely activates its nucleolytic activity, increasing FANCD2 foci and causing G_2 arrest. However, other work has shown that Vpr can induce cell cycle arrest even if SLX4 is knocked out by CRISPR (117). Another model of Vpr-induced cell cycle arrest proposes that Vpr binds the $CRL4^{DCAF1}$ ligase and sequesters it away from its normal substrates whose degradation is required for proper cell cycling. This model is supported by the fact that DDB1 knockdown alone causes cells to arrest in G_2 (115, 118) and DCAF1 knockdown causes G_1 and G_2 arrests (113). A recent crystal structure of UNG2-Vpr-DCAF1-DDB1 complex supports this model, highlighting that Vpr binds to DCAF1 on the typical DCAF substrate interaction surface, potentially occluding the recruitment of normal substrates (119). UNG2 appears to be a neo-substrate for DCAF1, as Vpr adapts UNG2 to the ligase complex, providing the entirety of the interaction surface. Chen et al. (120) have proposed that UNG2 contributes to the Vpr-mediated decrease in error rate of reverse transcription. One issue with the sequestration model is that a specific point mutant in Vpr (R80A) is unable to arrest cells in G_2 but maintains the interaction with DCAF1 (114, 116), suggesting that DCAF1 binding alone is not entirely sufficient to induce G_2 arrest.

Secondary Function

Despite several proposed models of activity, Vpr clearly induces the DNA damage response with subsequent G_2 arrest. Vpr can also modestly increase transcription from the HIV-1 LTR. It was originally assumed that this transcriptional activation was direct because Vpr interacts with cellular transcription factors, including SP1 (121), which provided a recruitment mechanism for LTR stimulation. Further, Vpr and Tat can interact with each other and with distinct regions of CycT1, resulting in synergistic activation of theHIV-1LTRthrough P-TEFb (122). However, other work has demonstrated that the transcriptional effect by Vpr appears to be largely indirect, as G_2 arrest alone induces expression from the LTR (Figure 2) (123, 124). Moreover, Vpr mutants that are unable to arrest cells in G_2, including R80A, are also unable to activate the LTR, whereas there is no correlation between Vpr nuclear import mutants and transactivation (124–126). Therefore, based on the mutant phenotypes, Vpr-mediated activation of the viral promoter may be solely an indirect effect. Given that Vpr increases the expression of numerous cellular genes (127), it will be interesting to determine whether this transcriptional effect is similarly due to activation of the DNA damage response and G_2 arrest. In addition, many human genes are cell cycle regulated (128) and could be affected by Vpr expression.

It is intriguing that Vif, Vpu, and Vpr all target host ubiquitin ligase complexes. Integration into the ubiquitin-proteasome pathway may generally result in pleiotropic effects, including changes in transcription, as these ligases are components of signaling pathways and generally have multiple substrates. As degrons for ubiquitin ligases are often short, unstructured peptides, it might be relatively easy for the accessory factors to rapidly evolve degron-mimics to hijack these complexes.

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Nef

Primary Function

Nef is a small viral accessory protein that is produced early in HIV-1 infection (129). Although not essential for viral replication in permissive cells, long-term infection with Nef-defective HIV-1 progresses to AIDS very slowly, if at all (130). Many functions have been credited to Nef, including modulating signaling by protein tyrosine kinases and downregulating CD4, MHC-I, BST2/Tetherin, and other cell surface receptors (92, 94, 96, 131"–135). Nef modulates host trafficking by binding to AP-1 and AP-2 clathrin adaptor complexes involved in coated vesicle budding, and the ESCRT machinery involved in degradative lysosomal sorting (136). MHC-I is rerouted from the *trans*-Golgi network to the endo-lysosomal system for degradation by Nef and AP-1 (137). Additionally, Nef and AP-2 bind CD4 at the plasma membrane, which triggers the recruitment of clathrin, budding, and eventual lysosomal destruction (Figure 1) (138). Membranous CD4 downregulation prevents superinfection and is thought to aid viral egress by preventing Env-CD4 interactions during budding (139). On the basis of recent findings, the mechanism by which Nef promotes HIV-1 infectivity may be mediated by the downregulation of the integral membrane protein, SERINC5 (140, 141). Little biochemical literature exists on SERINC5 and it is currently unclear whether SERINC5 binds Nef directly or via one or more additional factors, as AP-2 is essential for SERINC5 downregulation (140, 141). Nef-dependent downregulation of SERINC5 is proposed to be one of the most important contributions of Nef to infectivity by HIV-1 and is now a major focus of Nef-related research.

Secondary Function

Nef is the most abundantly expressed viral protein early in infection and can activate transcription through NF-κB or nuclear factor of activated T cells (NFAT) pathways (102, 142, 143). In contrast, Vpu is expressed late in viral infection and inhibits the NF-κB pathway. It is assumed that early stimulation of NF-κB by Nef helps initiate the potent positive transcriptional feedback loop of Tat. Using luciferase reporter assays to investigate the role Nef has in NFAT and NF-κB signaling, Wang et al. (142) found that Nef activation of an NFAT-luciferase reporter required myristoylation, suggesting an indirect effect on transcription through signaling events at the plasma membrane (Figure 2). Similarly, Nef expression alone did not increase NF-κB transcription but first required pathway stimulation, indicating that Nef regulates the response to stimulation (signal transduction) rather than directly activates NF-κB (144). Importantly, activation of the NF-κB pathway promotes transcription of viral genes, and in the case of HIV-1, binding of NF-κB p50–p65 heterodimers to the HIV-1 LTR is necessary for viral replication (145). Indeed, HIV-1 Nef enhances both LTR promoter activity and the transcription of HIV-1 provirus (102). Although it would appear that HIV-1 Nef exploits cellular signaling cascades to directly promote viral replication, it is possible that remodeling of host membrane proteins indirectly triggers cellular transcription. Mutational analyses are needed to better define how Nef is directly involved in deregulating cellular gene expression.

A more global assessment of Nef-induced changes to cellular transcription revealed that Nef induces a gene expression program that is highly similar to anti-CD3 T cell activation (146).

Much of this transcriptional induction required signaling through NF-κB or NFAT, supporting the earlier work on Nef function. Interestingly, several transcription factors that upregulate the LTR, including ETS1 and CDK9, were induced. CDK9 protein levels also increase upon Nef expression, and the Nef-dependent increase in LTR activation was sensitive to CDK9 inhibition. Furthermore, as highlighted above, ETS1 recruits Tat to many cellular genes for altered transcription (24). It is intriguing that Nef increases the expression of critical Tat host machinery and, in this way, acts to both increase viral transcription and further deregulate host transcription. Analogous to the Rev-stimulated degradation of Tat, the secondary effects of Nef provide a clear example of cross talk between the HIV-1 accessory and regulatory proteins to rewire the host for optimal infection.

CONCLUSION

Altogether, these examples highlight the multifunctional nature of HIV-1 regulatory and accessory proteins and emphasize a common ability to modulate cellular or viral transcription. Many viruses encode a relatively small number of genes and likely express multifunctional proteins that can alter host transcriptional networks. For example, adenovirus and human papillomavirus encode proteins that degrade p53 and alter p53-dependent transcription (147, 148); the Marburg virus VP24 protein disrupts the Keap1-Nrf2 interaction, which upregulates the transcription of cytoprotective genes (149); and HPV E7 and adenovirus E1A facilitate the expression of cell cycle–promoting genes to induce the transition from G_1 to S phase (150–152).

Intriguingly, theHIV-1 transcriptional effects that we describe here appear to benefit the virus, yet it is unclear whether these effects are selected for during viral evolution. As our understanding of HIV-1 evolution continues to improve, we now recognize that many factors influence viral fitness. These factors include (*a*) a condensed genome and overlapping open reading frames, which influence the adaptability of neighboring genes in a manner that helps purge unfit viruses (32); (*b*) the host-pathogen arms race of accessory protein adaptation to maintain restriction factor counteraction; and (*c*) the acquisition of new binding partners such as CBFβ to potentially aid Vif in antagonizing APOBECs at the transcriptional and posttranslational levels. By improving our knowledge of viral proteins and using technologies that will enable us to study virus-host systems, we will undoubtedly uncover how the complex, multifunctional nature of accessory and regulatory HIV-1 proteins, particularly their transcriptional functions, work in concert to drive viral pathogenesis. It will be interesting to uncover how much cross talk exists between the viral proteins and the extent to which they cooperate to rewire the cell. Similar to the Rev-mediated degradation of Tat or the Nef-induced expression of Tat cofactors, the viral accessory and regulatory factors might have unexpected overlapping functional circuitry, so it will be important to evaluate both virus-host and virus-virus interactions while teasing apart the discrete activities of these multifunctional proteins.

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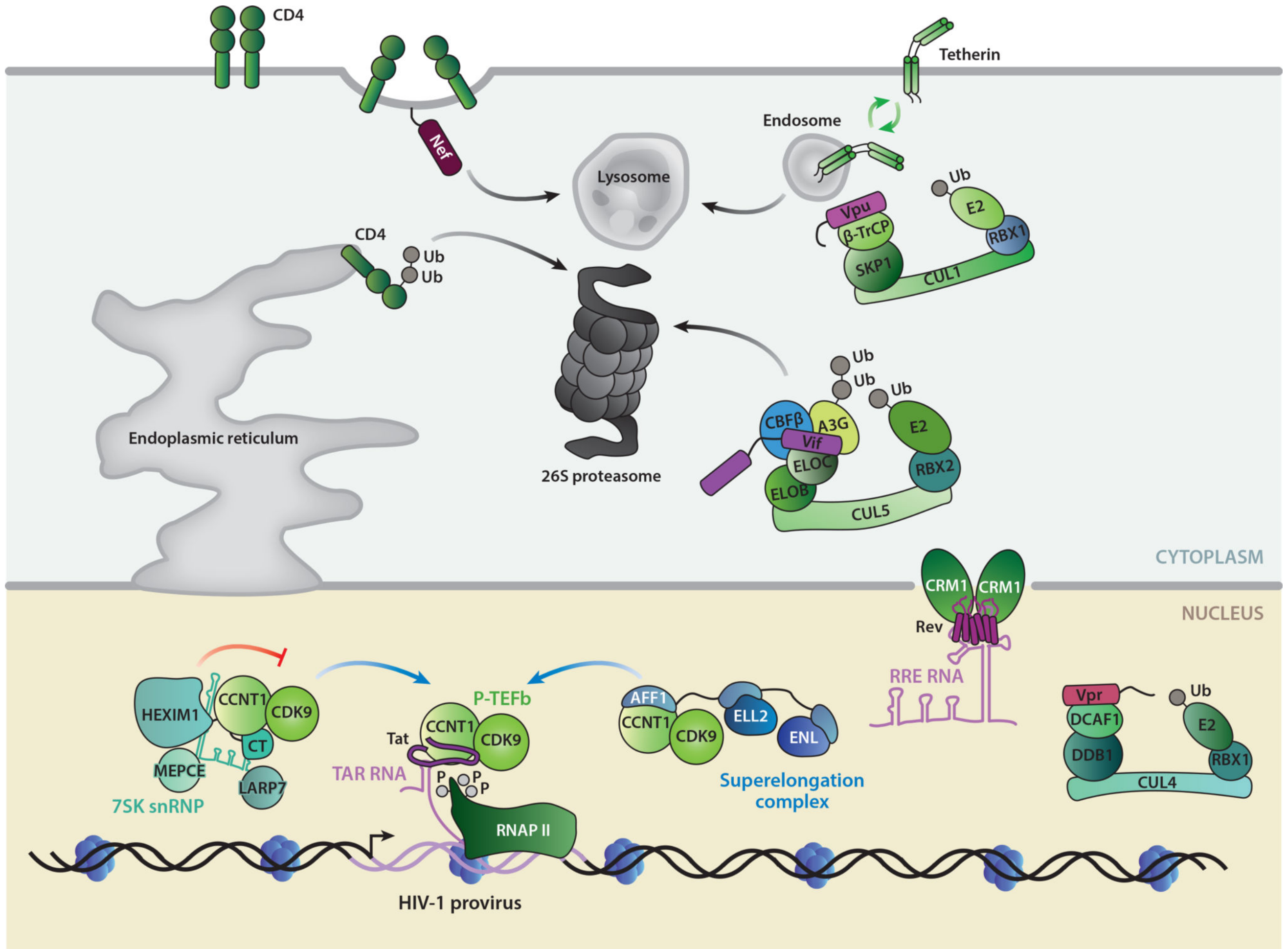

Figure 1.

Primary effects of HIV-1 accessory and regulatory proteins. Tat recruits P-TEFb to a paused RNAP II at the HIV-1 promoter in the nucleus to activate viral transcription. Tat utilizes P-TEFb from both the 7SK snRNP and the superelongation complex to activate transcription (indicated by *blue arrows*). Rev exports partially and fully unspliced viral messages from the nucleus to the cytoplasm. Vif, Vpr, and Vpu all utilize Cullin-RING ubiquitin (Ub) ligases for their primary activities of A3G degradation, DNA damage response/ G_2 arrest activation, and CD4/tetherin degradation, respectively. Nef induces the lysosomal degradation of CD4 at the plasma membrane. Gray arrows indicate the viral targeting of cellular substrates to the 26S proteasome or lysosome for degradation. Viral proteins are shaded in purple and hijacked host complexes in green and blue.

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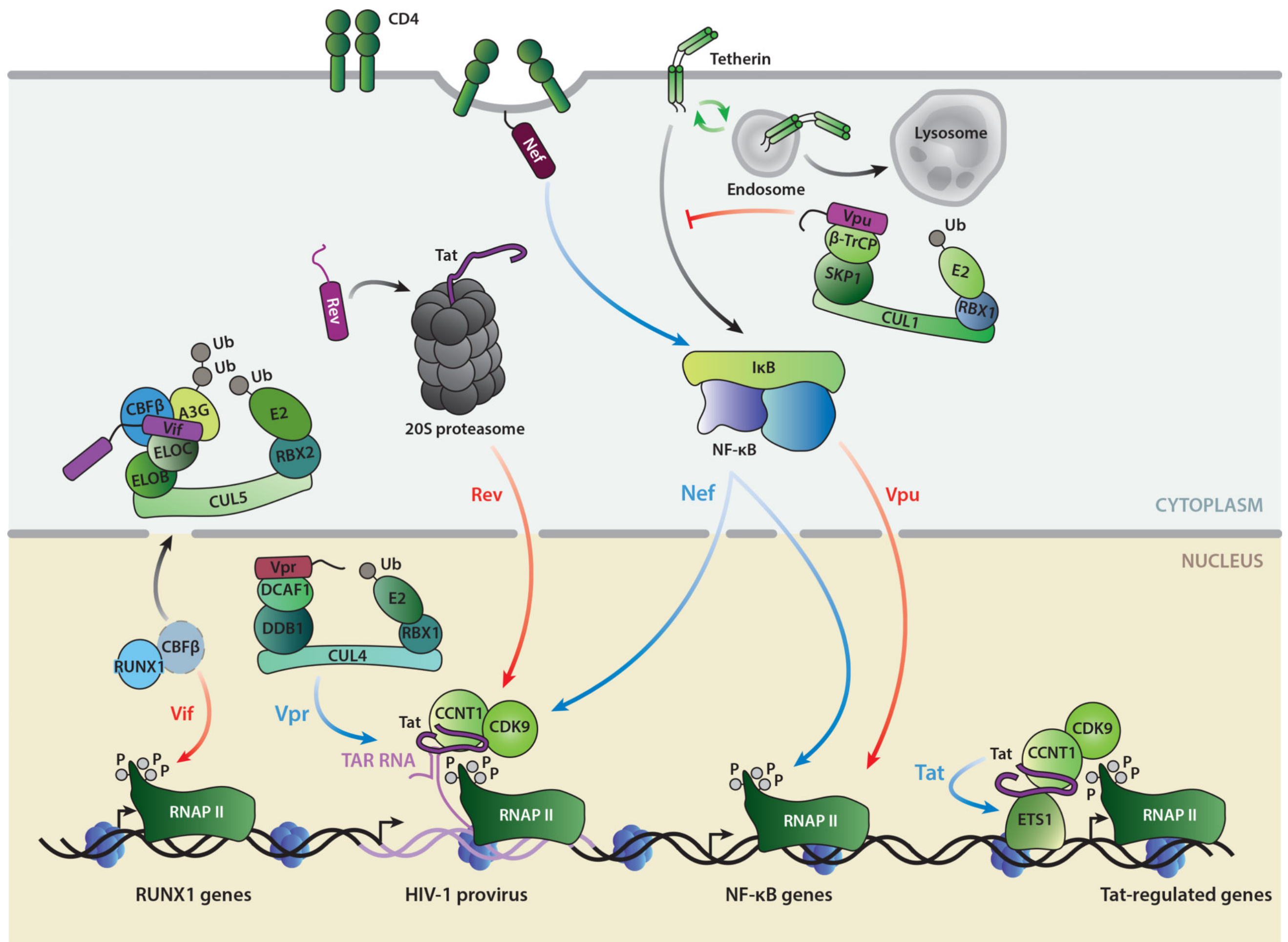

Figure 2.

Transcriptional effects of HIV-1 accessory and regulatory proteins. Tat directly deregulates multiple cellular genes through the ETS1 transcription factor. Rev-induced degradation of Tat through the 20S proteasome decreases HIV-1 transcription. Vif sequesters CBFβ from RUNX1 to decrease RUNX1-dependent transcription, including the A3G gene. Vpr DNA damage response activation and G_2 arrest activate HIV-1 transcription. Membrane-bound Nef modulates signaling pathways through NF-κB to activate both viral and cellular transcription. Vpu inhibits tetherin signaling through NF-κB to decrease transcription at NF-κB target genes. Blue arrows indicate positive effects on transcription, whereas red arrows indicate inhibitory effects.