EXPERT REVIEWS

Cell-type-specific proteome and interactome: using HIV-1 Tat as a test case

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HIV-1 is a small retrovirus that wreaks havoc on the human immune system. It is a puzzle to the scientific community how a virus that encodes only nine proteins can take complete control of its host and redirect the cell to complete replication or maintain latency when necessary. One way to explain the control elicited by HIV-1 is through numerous protein partners that exist between viral and host proteins, allowing HIV-1 to be intimately involved in virtually every aspect of cellular biology. In addition, we postulate that the complexity exerted by HIV-1 can not merely be explained by the large number of protein–protein interactions documented in the literature but, rather, cell-type-specific interactions and post-translational modifications of viral proteins must be taken into account. We use HIV-1 Tat and its influence on viral transcription as an example of cell-type-specific complexity. The influence of post-translational modifications (acetylation and methylation), as well as subcellular localization on Tat binding partners, is also discussed.

Keywords: epigenetics • HIV-1 • interactome • proteomics • Tat • transcription

Compared with large DNA viruses that contain more than 100 open reading frames, HIV-1 is a relatively simple RNA virus that elicits a profound change in its cellular host, including a complex interaction network that exists between HIV-1 proteins and cellular factors. The HIV-1 Human Protein Interaction Database, has catalogued 2589 interactions (out of 5×10^5 proteins in a cell) between HIV-1 proteins and cellular factors [201], with 834 being direct and 1755 being indirect [1]. This begs the question of how to reconcile this number of protein interactions in terms of HIV-1 biology and pathogenesis. We propose that one way to understand the complexity of these interactions is in a cell-type-specific manner. We suggest that cell-type differences, such as T cells, monocytes/macrophages and glial cells, and their inherent differential expression of common cellular proteins, plays a role in variability seen in global proteomic analyses that otherwise seek to establish an overall proteome of HIV-1 viral protein interactions with host cellular proteins. The protein–protein interactions of the well-known viral transactivator, Tat, will be examined in a cell-type-specific context, which can be used to further elicit characterization of epigenetic variability, especially during different stages of the viral lifecycle.

HIV-1 tropism

HIV-1 is capable of infecting $CD4^+$ T cells, monocytes, macrophages, dendritic cells, astrocytes and microglial cells [2]. $CD4^+$ T cells account for the largest portion of infected cells early in infection; however, once a patient progresses to AIDS, this cell population is severely diminished due to massive apoptosis. However, a latently infected pool of $CD4^+$ T cells remains through infection that are resistant to highly active antiretroviral therapy. Cells of the monocyte/macrophage lineage serve as an important viral reservoir for HIV-1, as viral replication is maintained at persistent level without the induction of apoptosis [3–7]. HIV-1 infection of CNS cells as well as viral toxins (Tat and gp120) released from infected cells, results in neurological complications, such as neurocognitive impairment and HIV-1-associated dementia, which is collectively referred to as NeuroAIDS [8–10].

As HIV-1 infects a diverse set of cells, the virus has adapted multiple mechanisms to allow survival in different host environments. For example,

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HIV-1 entry is mediated by the interaction between Env and the CD4 receptor, but HIV-1 can utilize either CCR5 or CXCR4 as coreceptors. However, HIV-1 has even been shown to enter astrocytes independent of CD4, as astrocytes do not express CD4, and CD4-blocking antibodies did not inhibit viral entry [11–13]. In T cells, the budding and assembly of the mature HIV-1 virions occurs almost exclusively at the cell surface of the plasma membrane of infected cells, whereas, in macrophages, this event occurs primarily within intracellular compartments known as multivesicular bodies (MVBs) [14]. Although these structures contain the late endosome protein CD36, these organelles appear to be composed of internalized plasma membrane domains [15]. In addition, HIV-1 replication in T cells is dependent on the cellular activation status, but macrophages can be infected regardless of the state of DNA synthesis [16]. Finally, HIV-1 transcription is differentially regulated in macrophages, T cells and microglial cells, with some cellular factors such as GATA-binding protein 3, v-ets erythroblastosis virus E26 oncogene homolog 1, lymphoid enhancer-binding factor 1 and nuclear factor of activated T cells, being important for transcription in T cells [17,18], while CCAAT/enhancer-binding proteins (C/EBPs) are critical for transcription in macrophages [19,20]. In regards to monocyte/macrophage replication, the viral protein, Vpr, influences viral transcription through binding to the coactivator, p300 [21] and through binding to cyclin T1 and Tat [22]. However, Vpr aids in viral replication in monocytes/macrophages, but not in nondividing $CD4^+$ T cells [23].

While HIV-1 has evolved to replicate efficiently in the aforementioned cells, it lacks this ability in monocytes, at least *in vitro*. Monocytes have been shown to have post-entry restriction of viral replication *in vitro* through various mechanisms, including impaired HIV-1 reverse transcription, defective nuclear import and integration of viral DNA. More recently, viral transcription has been shown to be inhibited in monocytes [24]. As monocytes differentiate into macrophages, cellular transcription is greatly altered and these defects in viral replication are no longer present, indicating that monocytes do not contain the necessary cellular factors to support HIV-1 replication.

Tat

Tat is the HIV-1 viral transactivator and, thus, essential for viral replication. To date, Tat has been shown to interact with 841 cellular proteins (264 direct interactions) [1], most of which have implications in transcriptional regulation. However, there are additional interactions that may account for the ability of Tat to influence other cellular processes, such as splicing, mRNA capping, proliferation, chromatin remodeling, nucleic acid chaperoning, RNA interference, apoptosis and proteasomal degradation [25–31]. Tat can also be secreted by infected cells and influence neighboring uninfected cells, increasing HIV-1 pathogenesis [9,10]. This role of Tat is particularly relevant in the CNS, where microglial cells are stimulated by Tat to produce proinflammatory cytokines and other neurotoxic molecules, contributing to HIV-1-associated dementia [9,32].

Given the possible wide array of cellular events that are influenced by Tat, it stands to reason that the 'interactome' of Tat is essential for allowing Tat to selectively influence cellular pathways at the appropriate time in specific cell types. Along these lines, we have performed preliminary experiments to address this hypothesis. Pull-down assays were performed utilizing a biotin–Tat peptide incubated with cell lysates from various different cell types (Figure 1). We used a number of cell extracts from epithelial cells (293T kidney, HeLa cervical carcinoma), T cells (CEMs and Jurkats), monocytes (THP1 and U937), glioblastoma cells (U87MG), HIV-1-infected cells (ACH2: infected CEMs; HLM1: infected HeLa's; J1.1: infected Jurkats; U1: infected U937s) and human T-lymphotropic virus-1 infected cells (C81 T cells) in the binding assays. Following overnight incubation, bound complexes were washed with high salt and the remaining proteins were used for western blot analysis or total protein staining. Figure 1A demonstrates, through western blot analysis, the ability of the biotin–Tat peptide to bind to two proteins involved in transcription, RNA helicase activity (RHA) and the chromatin remodeling complex (Ini1) when using 293T cell extracts. This apparent binding was not seen with a peptide that corresponded to p50 of the NF-κB protein. Figure 1B shows a more comprehensive set of pull-down assays with the biotin–Tat peptide when using extracts from 12 different cell types. Interestingly, Tat bound to RHA when using all cell extracts except the two monocytic lines (U937 uninfected and U1 HIV-1 infected cells). There were also lower levels of binding of RHA to Tat in C81 HTLV-1 infected cells. A similar pattern of binding was also seen with Ini1, as well as RNA polymerase II (Pol II). Importantly, these cell lines all contained endogenous RHA, Ini1 and Pol II (input lanes).

We further examined Tat binding to CDK2 and CDK9 in 293T, U937 and U87MG cells. CDK2 and CDK9 associate with Tat in 293T cells, but this association was not observed in U937 or U87MG cells (Figure 1C). The lack of CDK2 and CDK9 binding to Tat in monocytes and microglial cells could contribute to the HIV-1 transcriptional restriction that has been documented in monocytes and U87MG cells [24,33]. It is also important to note that the lack of detectable CDK9 (or lower levels) in U937 cells (Figure 1B, lane 3), may account for the lack of Tat/CDK9 binding in these cells.

Finally, when assaying for any visible binding partners of Tat in 293T, monocyte or glioblastoma cells, we found a very interesting set of interactions. Figure 1D shows that Tat binds to many partners in 293T cells (a cell type that is commonly used by many in the field for its ease of transfection) as with monocytes or glioblastoma cells. The bottom band in the last three lanes is the Tat peptide band. Collectively, these data strongly suggest that there are key differences between Tat binding partners in T cells, monocytes and astrocytes, which may contribute to Tat's classical role in transcription of the viral long terminal repeat (LTR) and its other documented roles, including activation of cellular genes, alteration of signal transduction in cytoplasm and its secretory activities.

Tat in transcription

It is well accepted that Tat plays a critical role in transcription elongation. While HIV-1 transcription can be initiated in the absence of Tat, it occurs at very low levels and results in short abortive transcripts. In the presence of Tat, HIV-1 transcription is increased

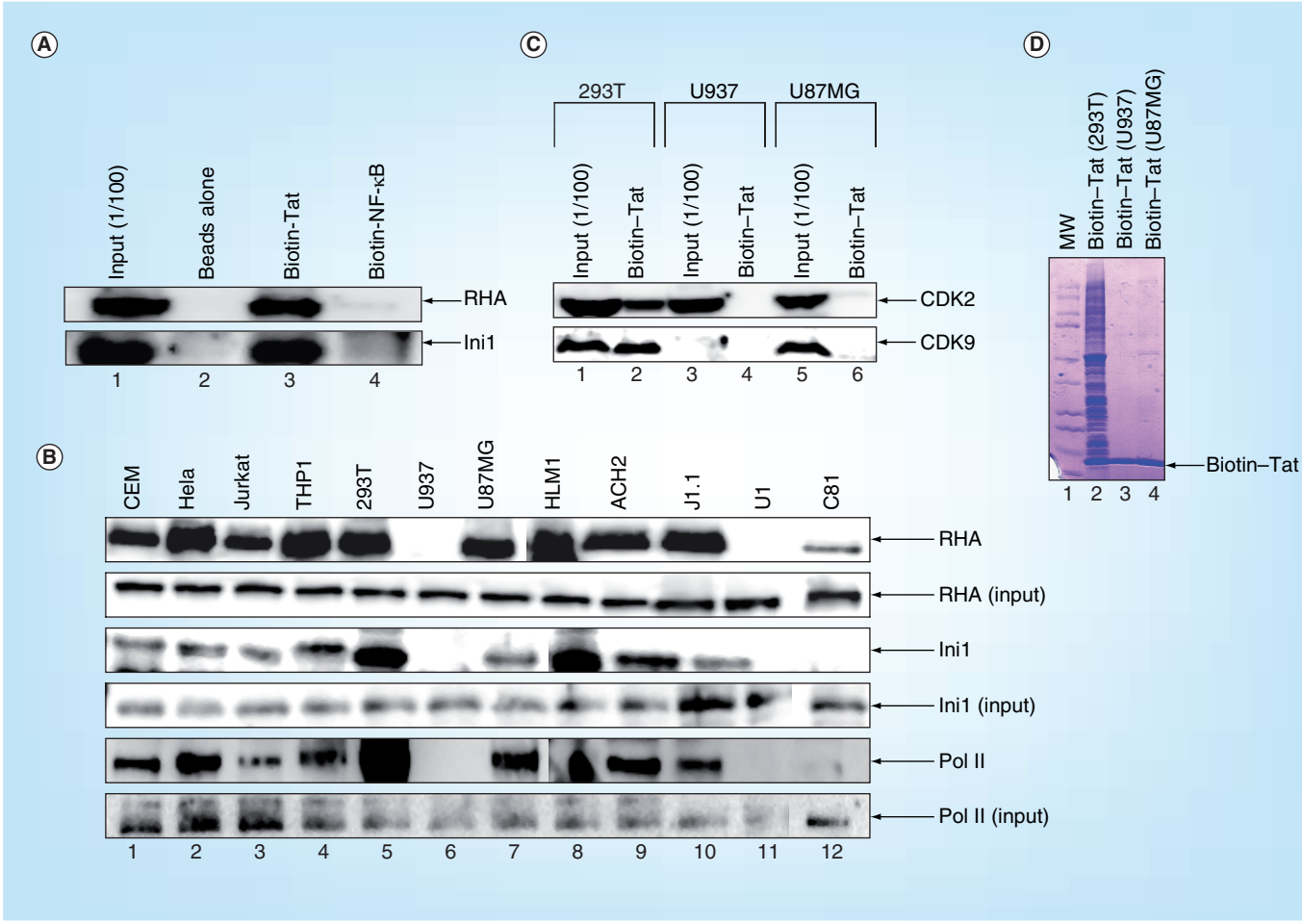

Figure 1. Tat binding partners vary depending on the cell type used. (A) Biotin–Tat peptide (50 µg, from aa 36–51), Biotin-NF-κB (p50) peptide (50 µg, from amino acids 426–446), or streptavidin beads alone were incubated overnight at 4°C with whole-cell extracts (1 mg) from 293T cells. Pulldowns were washed twice with 1 ml of TNE 600 + 0.1% NP-40, once with TNE 50 + 0.1% NP-40 and subsequently analyzed by SDS-PAGE (4–20%) and western blotted for RHA and chromatin remodeling complex. Lane 1 represents one out of 100 of the input for the binding assay. Similar pulldown experiments have previously been described [25]. **(B)** Biotin–Tat peptide was incubated with 12 different cell extracts (1 mg each) and processed as described in panel A. Pulldowns were western blotted for presence of RHA, chromatin remodeling complex and Pol II. **(C)** Biotin–Tat peptide was incubated with 293T, U937 or U87MG cell extracts (1 mg) and processed. Pulldowns were western blotted for presence of CDK2 and CDK9. **(D)** Commassie-stained gel of Biotin–Tat peptide pulldowns from 293T, U937 or U87MG cells (1/100 of beads after pulldown and washes). The bottom band in lanes 2–4 is the biotin–Tat peptide band.
Pol: RNA polymerase II; RHA: RNA helicase activity.

several hundred fold. Tat interacts with the *cis*-acting RNA element, TAR, which is located at the 5′ end of all viral transcripts (nt +1 to +59) [34,35]. The positioning of TAR downstream of typical initiation binding sites indicated that the interaction between Tat and TAR is important for elongation rather than initiation. Further support for Tat's role in elongation came from findings that Tat binds to the positive transcriptional elongation factor b (p-TEFb), resulting in increased Pol II C-terminal domain (CTD) phosphorylation and increased processivity of Pol II [36–46]. p-TEFb also phosphorylates the elongation factors, SPT5 and Tat-SF1, in a Tat-dependent manner [47], contributing to elongation efficiency.

The p-TEFb construct is a multiprotein complex that exists in two main forms, a small complex containing CDK9 and its cyclin partner (cyclin T1, T2a, T2b or K), and a large complex that also contains hexamethylene bisacetamide-induced protein 1 (HEXIM1) and 7SK small nuclear RNA (snRNA) [48–51]. The bromodomain protein, Brd4, enhances p-TEFb activity and recruitment to promoters and likewise has also been shown to be a component of the small p-TEFb complex [52]. However, in HIV-1-infected cells, Brd4 is not required for p-TEFb activity as Tat can substitute for Brd4 [52]. By contrast, the large p-TEFb complex has weak kinase activity and lacks the ability to activate HIV-1 transactivation *in vitro,* due to binding of HEXIM1 and 7SK snRNA [48,50,53,54]. In HIV-1-infected cells, cyclin T1/CDK9 is released from the large complex through competition of HEXIM1/cyclin T1 binding by Tat [55]. The small p-TEFb complex can take multiple forms owing to four possible cyclins and two subunits of CDK9 [56,57]. However, neither cyclin T2a nor T2b can support

HIV-1 transcription [44,58]. In addition, a recent study showed that cyclin K does not bind to HEXIM1 and that overexpression of cyclin K results in decrease HIV-1 replication [59]. It had been predicted that cyclin K would not support Tat transactivation owing to the lack of Cys261, which is critical for the zinc-dependent interaction between cyclin T1 and Tat [60]. Thus, the ability of p-TEFb to positively influence Tat transactivation is not merely determined by small versus large complex, but also is influenced by which cyclin is present in a cell.

It was originally thought that Tat plays little or no role in transcription initiation as HIV-1 transcription has an early Tat-independent phase and a late Tat-dependent phase [61]. However, Tat interacts with a number of general transcription factors that form the preinitiation complex (PIC) [61], including TATA binding protein (TBP) [62–65], TFIIH [37,40] and Pol II [39,66]. Although p-TEFb has been termed the positive transcriptional-elongation complex, it also has been shown to be a component of the PIC [67]. Both p-TEFb and TFIIH are important for the phosphorylation of Pol II CTD. However, TFIIH functions mainly during initiation, being released at the beginning of elongation, while p-TEFb maintains association with the elongation complex [47,67]. In addition, p-TEFb is important for the recruitment of a unique transcriptional complex to the LTR, which contains TBP, TFIIIB, mediator and Pol II, but not TBP-associated factors (TAFs) [65]. The TATA box is critical for p-TEFb-dependent transcription, as HIV-1 TATA box mutants are not activated by GAL4-CDK9 [68]. Tat also interacts with site-specific transcription factors important for LTR transcription, such as Sp1 [69], and NF-κB, as well as transcription coactivators, CBP/p300 and p/CAF. The interaction of Tat helps to stabilize the initiation complex through facilitating CBP/p300 binding to TFIIB and TBP, which is induced through a conformational change in CBP/p300 [70].

Although HIV-1 viral transcription is most efficient in a Tat-dependent manner, there is some degree of variability in transcriptional efficiency, as seen across different HIV-1 clades. Viral isolates can be classified based on differences in genomic sequences that impart changes in viral pathogenicity and replication. The most prevalent and almost exclusively studied HIV-1 isolate is clade B; however, the study of the genetic recombination and variation is important in understanding how the virus sequence evolves and mutates. In terms of viral transcription and replication, there appears to be a significant amount of sequence variation in the LTR promoter region amongst clades B, C and E, especially in the TATA box, the NF-κB enhancer region and the TAR structure [71]. Variation in these regions results in changes in the efficiency of Tat to bind to the viral DNA/RNA and, consequently, changes in transcriptional initiation. For example, Tat from the clade E virus has the highest degree of transactivation. Variation in these regions results in changes in the efficiency of Tat to bind to the viral DNA/RNA and, consequently, changes in transcriptional initiation (i.e., Tat from the clade E virus has the highest degree of transactivation).

Another way that Tat influences transcription is through chromatin remodeling [72,73]. This is especially evident on integrated promoters that contain a fully chromatinized promoter, versus transient transfected plasmids, which only have a partial reconstituted chromatin promoter. Tat binding to p300 induces its acetyltransferase activity, resulting in increased acetylation of histone H4 lysines 8, 12 and 16 [74]. The acetylation of Tat itself by p300 at lysine 50 in the highly conserved TAR RNA-binding domain assists in the dissociation of cyclinT1 from the Tat/cyclinT1/TAR complex [75]. This acetylation event results in the progression of viral transcription from the initiation complex to elongation also by transferring Tat onto the active RNA Pol II [75]. Acetylated Tat also interacts with the chromatin-modifying complex SWI/SNF, resulting in increased transcriptional elongation and chromatin remodeling through removal of Nuc-1 [76]. The importance of SWI/SNF is further demonstrated as the knockdown of BRG1 and Ini1 results in decreased Tat-dependent transcription [77]. In addition, Tat binds to the chromatin remodeler, Ini1, and this interaction is important for viral transcription [78]. The removal of post-translational modifications by specific enzymes is equally as important in Tat's activity in transcriptional regulation. For instance, the deacetylation of Tat by human sirtuin 1 allows for the recycling of unmodified Tat back to the HIV-1 promoter and allows for further transactivation events, including the availability of human sirtuin 1 as a transcriptional cofactor [79]. Collectively, HIV-1 transcription is a complex process that utilizes numerous cellular factors to proceed in a coordinated fashion and Tat may play multiple roles at various stages of transcription depending on its modifications (i.e., acetylation or methylation) and binding partners.

Tat & T cells

The HIV-1 virus primarily infects $CD4^+$ T cells in a susceptible host through the binding of cell surface-expressed CD4 molecules with the virion envelope glycoprotein Env. This binding is most often mediated by the cell surface coreceptors CCR5, CXCR4 and CCR3 [80]; however, several other minor coreceptors have been characterized, such as CCR2B [81] and CCR8 [80,82], which assist in the virion and cellular membrane fusion, leading to entry of the virus into the cell. Once the proviral DNA is integrated into the genome of the T cell, the established infection can lead to production of mature virions at a high enough rate to induce cell death or the infected cell is recognized and killed by cytotoxic T lymphocytes (CTLs), all of which leads to the depletion of $CD4^+$ T cells within the infected individual. As the primary target and reservoir for HIV-1 infection, model *in vitro* T cell lines are an obvious necessity for experimental studies.

A stable viral infection within a T cell can vary in terms of the degree of viral latency, that is, the state of production of new viral particles. HIV-1 can establish itself in long-lived cellular reservoirs, where only low levels of viral production are present and do not elicit an immune response. For infected T cells particularly, these cells can revert from an activated to a quiescent state, where no additional viral particles are produced; however, the proviruses can be reactivated in these resting T cells as a result of cellular signaling and other stimuli. Current treatments and therapies are unable to fully deplete the integrated provirus

contained within the resting T-cell viral reservoirs, mainly due to the inability to preferentially target these cells. Consequently, model *in vitro* systems of HIV-1 viral T cell infection vary based on the state of infection. The commonly used T-cell line, CEM, and its HIV-1-infected daughter cell line, ACH-2, differ only by the presence of a single copy of proviral DNA per cell [83]. Similarly, the lymphocytic Jurkat E6.1 cell line and its chronically infected derivative J1.1 differ by the stable infection of J1.1 with the LAI strain of HIV-1 [84]. While much has been learned regarding latency utilizing these cell lines, they are chronically infected rather than latently infected, due to the low level of virus production occurring in the absence of stimulation.

A number of models that more accurately represent latency have now been developed. The Verdin group developed the J-Lat latency model where Jurkat cells were infected with a HIV-1 vector carrying the green fluorescent protein (GFP) [85]. Latently infected cells were selected that were initially GFP-negative, but then became GFP-positive following stimulation with TNF-α. These clones have been used for a number of important studies on HIV-1 latency [77,86]. Another latency model by the Karn group utilizes Jurkat cells infected with a VSV-G pseudotyped lentivirus carrying a GFP-reporter and *Tat* gene [87]. These cells spontaneous lose HIV-1 gene expression and enter latency, but can be stimulated with TNF-α. Swiggard *et al.* demonstrated that HIV-1 can infect resting T cells and that a small percentage of these are latently infected, thus, providing another model for HIV-1 latency *in vitro* [88]. Finally, when SupT1 T cells were infected with the doxycycline-inducible HIV-rtTA virus, the majority of clones displayed latent infection, that were responsive to TNF-α, genistein, 5-azacytidine, but not PMA or IL-2 [89]. These newer models may provide a more faithful depiction of latency that can be assayed for cellular changes induced by latent HIV-1 infection.

The introduction of the viral protein Tat into infected T cells results in global cellular signaling changes, as well as changes in gene and protein expression levels. The majority of Tat proteomic studies, to date, have been performed within the context of T cells owing to their ease of use experimentally, as well as their significance to *in vivo* infections. Ringrose *et al.* have shown that infection of the PM1 T-cell line with viral stocks of the HIV-1 LAI clone results in the differential expression of cellular proteins involved in metabolism, apoptosis, stress responses, cell cycle, signal transduction, cell growth and division, homeostasis and motility, to name a few [90]. Additionally, the recent identification of over 3200 differentially expressed proteins in exogenously infected CEM cells and the mapping of these proteins to integral cellular pathways modified by HIV-1 infection, such as ubiquitination, nucleocytoplasmic transport, cell cycle progression, control of CDKs and their associated cyclins, and changes in metabolic pathways, is a testament of the wealth of information that can be obtained through these model systems [91].

The current model *in vitro* latently infected T-cell systems have sufficed for a functionally repetitive and consistent source of HIV-1 infection data; however, there is still no substitute for an *in vivo* model of latency. The closest mimicry of an *in vivo* system can be obtained in primary $CD4^+$ T cells, where additional studies involving course of infection and eventual establishment of latency of human cells in culture for a short period of time can provide an insight into Tat and its role in a primary infection.

Tat & monocytes/macrophages

Recently, viral transcription has been shown to be inhibited in monocytes [24]. Diminished transcription may be partially decreased owing to low to undetectable levels of cyclin T1 in undifferentiated monocytes [92]. However, Dong *et al.* showed that overexpression of cyclin T1 levels did not rescue HIV-1 transcription [24], indicating that there are additional factors missing from primary monocytes that are necessary for transcription. In fact, heterokaryons between monocytes and 293T cells restored LTR transcription. Thr186 of CDK9 was also shown to be phosphorylated following primary monocyte differentiation induced by M-CSF, while levels of total CDK9 remained unchanged [24]. Interestingly, we did not observe CDK9 expression in U937 cells (Figure 1C); however, this difference may be owing to the use of primary cells [24] versus transformed cells and/or the subunit of CDK9 detected. Phosphorylation of Thr186 is an event that has been demonstrated to increase its binding to the inhibitory 7SK snRNA and is caused by a novel HeLa nuclear kinase [93,94]. This phosphorylation site is found within the conserved T-loop of CDK9 and is needed to activate the kinase function of p-TEFb by allowing substrate access to the catalytic core [93]. CDK9 can be dephosphorylated by PP1α, where the kinase then interacts with Brd4 and is recruited to the PIC [94]. The dephosphorylation of CDK9 prior to its interaction with the HIV-1 PIC appears to be important to HIV-1 transcription activation [95]. This regulation of CDK9 and its involvement with p-TEFb in the context of Tat appears to be variable among cell types. However, there are studies indicating that the large p-TEFb complex increases during activation of PBLs when viral transcription is enhanced [96]. This information, combined with the large number of proteins that can constitute the p-TEFb complex, indicates that regulation and organization of p-TEFb is yet to be fully characterized in various cell lines or primary cells.

There are numerous site-specific transcription factors that can regulate LTR transcription, including Sp1, NF-κB, nuclear factor of activated T cells, lymphoid enhancer-binding factor 1/TCF-1α, C/EBP, CREB, USF, Ets, AP-1, YY1/LSF and p53 [97]. Some of these factors are cell-type specific and stimulus specific. The most clear-cut example is C/EBP that is important for LTR transcription in monocytes/macrophages but not in $CD4^+$ T cells [98]. The LTR contains two C/EBP binding sites between positions 170–110 [99], which are adjacent to two NF-κB binding sites. A recent study has indicated that C/EBPβ interacts with Tat, CDK9 and cyclin T1 [100–102]. The interaction of C/EBPβ with CDK9 is necessary for C/EBPβ binding to Tat and cyclin T1, as CDK9 immunodepletion altered the Tat/C/EBPβ and C/EBPβ/cyclin T1 interactions [100]. CDK9 phosphorylates C/EBPβ; however, the functional consequences of this phosphorylation are yet to be determined [100].

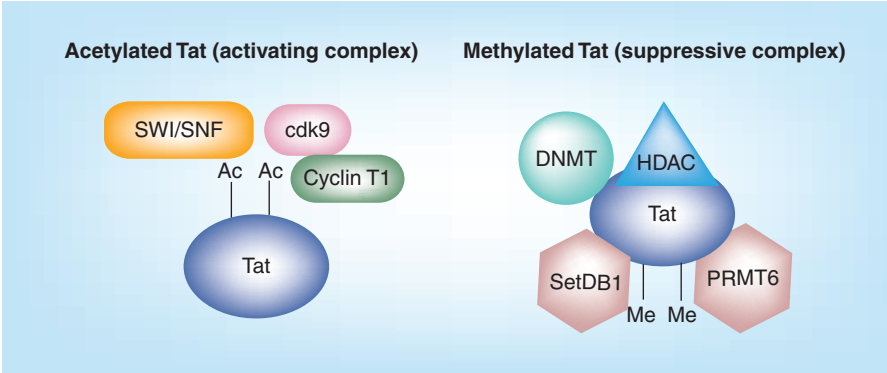

Figure 2. Post-translational modification influence Tat binding partners.
Acetylation and methylation are two possible post-translational modifications of Tat that influence protein partner interactions. The acetylation of Tat at multiple residues (K28, K50 and K51) is important for binding to key transcriptional (CDK9/cyclin T1) and chromatin remodeling components (SWI/SNF) to allow phosphorylation of Pol II CTD, dissociation of Tat from the TAR RNA structure, and consequently stimulation of transcriptional elongation. Methylated Tat (R52, R53, K50, and K51) recruits protein methyltransferases (PRMT6 and SETDB1) and HDAC/DNMT forming a repression complex resulting in a decrease in viral transactivation.
DNMT: DNA methyltransferase; HDAC: Histone deacetylase; PRMT6: Protein arginine methyltransferase 6; SETDB1: SET domain, bifurcated 1; SNF: Sucrose nonfermentable; SWI: Switch.

Tat & epigenetics

HIV-1 Tat has been extensively shown to be subject to post-translational modifications by host cellular enzymes, including phosphorylation, acetylation and methylation [25,103–105]. The addition of each modification results in significant changes in Tat's transactivation function, as well as radically different protein-binding partners, particularly seen in the opposing effects of Tat acetylation and methylation. Although unmodified Tat is able to stimulate transcriptional initiation through the recruitment of general host cell transcription factors, such as TFIIB and TFIIH [106], the acetylation of Tat at residues K28 and K50 by the histone acetyltransferases (HATs) pCAF and p300, respectively, is critical for the dramatic increase in transactivation of the viral LTR in integrated HIV-1 through the binding of the TAR regulatory element [70,74,107,108]. The acetylation of Tat at K28 results in increased affinity for p-TEFb, which works to hyperphosphorylate the CTD of Pol II, stimulating elongation of the viral transcript (Figure 2) [107,109]. Acetylated Tat is critical for the transactivation of the viral transcript, a function that is integrally linked to the chromatin state near the integrated provirus. Therefore, acetylated Tat has also been shown to interact with the SWI/SNF family of chromatin remodeling complexes, resulting in an increase in viral transcription [76,77,110]. Indeed, the association of Tat with the HATs p300 and hGCN5 results in the acetylation of K50 and K51, and the subsequent dissociation of Tat from the TAR RNA structure, thereby allowing transcriptional elongation to continue. While acetylated Tat is necessary for the activation of viral transcription, methylated Tat has been shown to assist in the suppression of viral transcription through the possible recruitment of protein and DNA methyltransferases, histone deacetylases (HDACs) and other proteins forming a repression complex and a decrease in viral transactivation [104,105,111]. The addition of methyl groups to lysine residues at positions 50 and 51 can be seen as a competitive event, where suppression is accomplished through the addition of methyl groups and activation through the addition of acetyl groups. Clearly, the proteome of acetylated Tat cellular binding partners is radically different from the proteome of methylated Tat cellular binding partners [25,72,105]; however, it is possible that both activation and repressive complexes are present at any given time within an infected cell. The state of the viral LTR transactivation can be directly correlated to the epigenetic profile of Tat; therefore, the presence or absence of post-translational modifications on both endogenously expressed Tat and recombinant/purified Tat proteins and peptides used in proteomic profiling experiments may play a significant role in the Tat interactome. The importance of the epigenetic state of both invading viral proteins, as well as the characterization of normal cellular proteins is often overlooked in global proteomic analyses of protein-binding partners and the example of HIV-1's viral protein Tat, clearly shows different protein binding partners, as well as opposing functions in an infected cell. Future proteomic studies will have to take into consideration the presence or absence of post-translational modifications (acetylation [25], methylation [104,105,111], phosphorylation [112] and ubiquitination [103]) in the particular cellular or disease state being studied.

Tat in subcellular organization

A potential pitfall during global proteomic analyses is the subcellular localization of the protein and interactions of interest. Although the characterization of protein-binding partners through a whole cellular lysate approach is appealing to identify all of the binding partners available, it is important to keep in mind whether the protein partners of interest would come into close proximity *in vivo*. In addition to making sense in terms of biological function, the analysis of smaller organelles or subcellular compartments is advantageous for the simplification of experimental procedures and data analysis. For HIV-1, numerous proteomic studies have been performed to this effect in an attempt to characterize the membrane proteome of a HIV-1-infected cell as well as the protein profile composition of a HIV-1 virion [113–115].

Keeping with the context of the Tat proteome, it is critical to understand that Tat is primarily a nuclear-bound protein, although it can be found cytoplasmically either prior to integration of the provirus or during budding vesicle formation. A recent study has specifically investigated the *in vitro* Tat nuclear interactome in T cells through an affinity chromatography

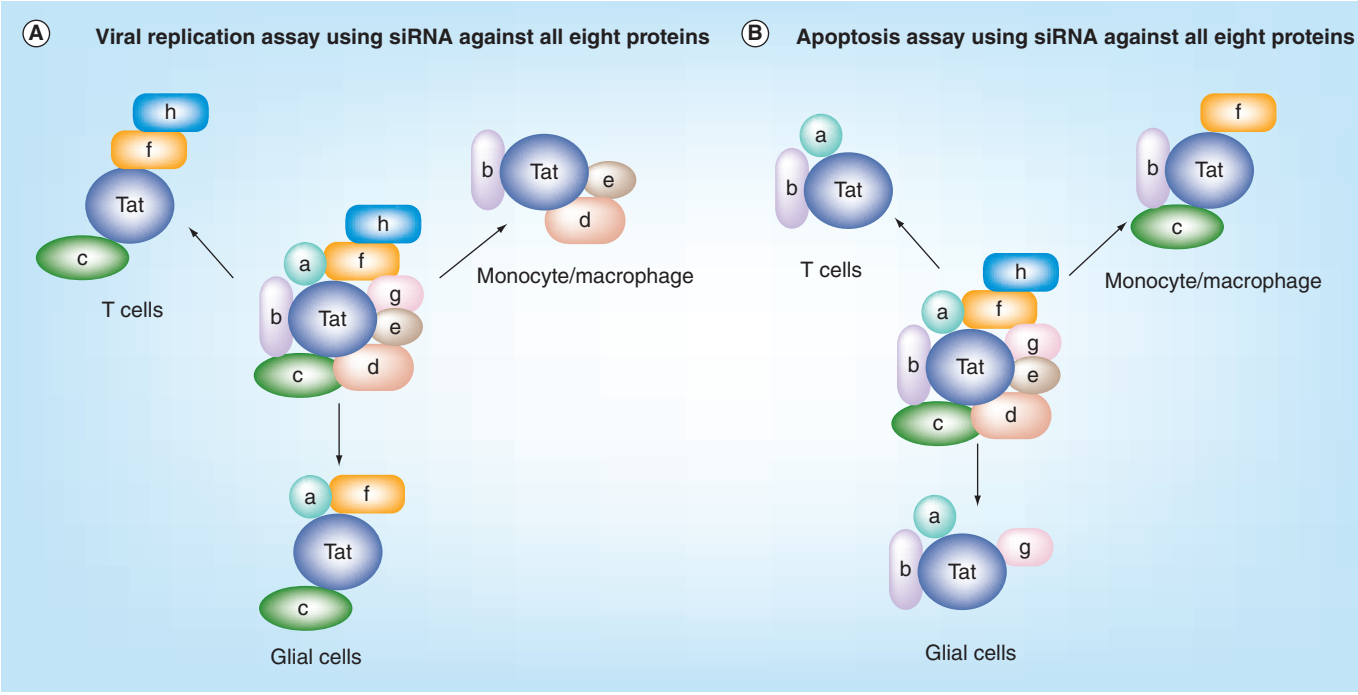

Figure 3. Cell-type- and assay-specific Tat-binding partners. A hypothetical model where Tat binds to numerous proteins from whole-cell extracts (the initial Tat proteome; i.e., the middle Tat diagram with eight partners). These interactions are predicted to be different depending on the cell type (T cell, monocyte/macrophage or glial cell). The significance of Tat-binding partners for specific functions of the virus can be detected through different functional assays performed in the presence of siRNA specific for the Tat-binding partners. For instance, **(A)** depicts possible different binding partners that influence viral replication in different cell types as identified through an siRNA screening assay and **(B)** depicts a different set of binding partners that may be important for apoptosis in the three HIV-1-infected cell types.

followed by a mass spectrometry approach [116]. The authors prepared nuclear extracts from the Jurkat T-cell line and allowed the extract to interact with immobilized GST-Tat, washed the nonspecific binding partners away and retained the Tat-interacting protein complexes. The GST-Tat-bound complexes were then separated on a gel, isolated, extracted and subjected to liquid chromotography tandem mass spectrometry for identification. This study identified 183 potential Tat binding partners within the nucleus of an uninfected T cell, 90% of which have not been previously characterized as interacting with Tat. As could be predicted, the majority of the identified proteins are involved in transcription and RNA processing, based on gene ontology assignments, with the rest of the proteins distributed equally amongst cellular functions, such as translation, cell cycle, DNA replication and signal transduction. This type of study represents how proteomic analyses can be performed on a comprehensive, functionally relevant platform, to not only identify every binding partner available but retain the biological significance. In contrast to a large-scale analysis that identifies thousands of potential binding partners and/or differentially expressed due to infection, the interactome of subcellular compartments can allow for the further validity of the binding partner on a reasonable experimental scale. It would be interesting to reassess the functionality and significance assigned to binding partners in a large-scale study to see if the function correlates to the location of the protein of interest in a cell.

Expert commentary

Published data to date suggest that the characterization of protein–protein interactions between HIV-1 viral proteins and host cellular proteins is dependent on too many variables to accurately assess valid binding partners, as well as to assign relevant biological function. Inconsistencies in these large datasets can predict a shift in the viral research field from characterizing the viral proteome to that of the viral interactome. Furthermore, cell types, viral and host post-translational modifications, and their possible compartmentalization are a few examples of what many in the field may have to consider in order to discern a meaningful set of complexes related to viral pathology.

Five-year view

The availability and ease of global proteomic studies have increased dramatically over the past couple of years. As with the standardization of the microarray, the overall study of global protein expression changes within a particular cell type or disease state is no longer sufficient to provide an insight into changes within a proteome. It is now necessary to relate the changes seen through a combination of experimental data and *in silico* prediction to functionality and biological relevance within the system being studied. Indeed, this holds true for the study of viral infections within a host cell, particularly complicated with a unique and multifaceted virus, such as HIV-1. Compared to other disease states, such as diabetes or cancer, viral infections can induce two

different areas of proteomic alterations, those that are related to the host and those that are related to the virus itself. In the context of the host, the proteome changes need to be implicitly focused based on cell type, T cell, monocyte/macrophage, glial cell lines or primary cells. Owing to fundamental differences in these cell types, the virus will interact and manipulate the cellular protein expression and interaction in dramatically different ways. This could be a potentially important venue when studying pediatric AIDS versus adult AIDS, where virus replication is much more pronounced and more difficult to control in the former.

Future studies will also need to take into consideration the choice of assay for the confirmation and validation of protein–protein interactions found through proteomic analysis. Confirmatory experiments are now common place for proteins identified through proteomic techniques; however, the choice of assay can play a significant role in the further downstream identification of protein–protein interactions. Figure 3 depicts the differences in protein–protein interactions that can be identified when a cell line is used in two different assays: viral replication (Figure 3A) and apoptosis (Figure 3B), and explains that the identification of the interaction of Tat with multiple cellular proteins, when confirmed through a functional assay, can result in the downstream identification of only some of the Tat binding partners. Again, these types of interactions can vary dramatically between cell types, whose differences can also affect the efficiency and reproducibility of an assay.

Future studies of viral protein interactomes should be performed with endogenously expressed, but not overexpressed, viral proteins. Proteomic outcomes will vary significantly if a protein is overexpressed or if recombinant tagged viral proteins are used for *in vitro* binding assays. The overexpression of viral proteins may lead to the identification of protein interactions that are only present in the excess of viral protein and, therefore, not biologically significant. In addition, recombinant proteins that are expressed in a bacterial system do not exhibit the same post-translational modifications and, possibly, do not have the correct secondary or tertiary protein structure, both of which can dramatically affect the protein–protein interactions that are detected.

Viral clade-specific proteomes will be necessary to investigate the protein–protein interaction differences between different HIV-1 clade isolates. Indeed, it has already been shown that amino acid sequence variation between Tat proteins of different clades can lead to the presence of more lysine residues and, consequently, the possibility of acetylation of these residues, thereby increasing the activation of the viral LTR [25]. The importance of post-translational modifications to the transcriptional state of a virally infected cell can no longer be ignored.

Finally, the cell-specific proteome, as discussed, could have implications for therapeutic selection. Along these lines, we have previously shown that a CDK inhibitor (Cyc202) inhibited HIV-1 at a much lower concentration in T cells as with monocytes/macrophages [117]. Results in Figure 1 show a possible mechanism where CDK2 or CDK9 binds to Tat from some but not all cell types, thereby influencing susceptibility to a given inhibitor. Therefore, to have optimal inhibitory results *in vivo*, it may be best to therapeutically target multiple viral protein interactions (i.e., use of multiple CDK inhibitors) at lower concentrations. Alternatively, the use of a pan CDK inhibitor could be beneficial. Future studies should, therefore, determine the benefit of targeting multiple viral–host protein interactions for therapeutics applications.

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Key issues

- Differences in HIV-1-infected cell types imparts variability in global protein expression.
- As the primary reservoir for HIV-1 infection, T-cell lines and *in vitro* models of infection are necessary for HIV-1 experimental studies.
- HIV-1 viral transcription is inhibited in monocytes; however, upon differentiation to macrophages, the cells are susceptible and serve as an important viral reservoir.
- HIV-1 and its viral transactivator, Tat, can interact directly or indirectly with over 800 cellular proteins to alter the host cell's transcriptional machinery.
- Incubation of biotin-conjugated Tat peptide corresponding to the core domain can preferentially bind and pull down RNA helicase activity and Ini1 cellular proteins in 293T kidney epithelia whole-cell extracts.
- Whole-cell extracts from 12 different cell lines were screened for biotin–Tat peptide-specific binding to RHA, Ini1 and Pol II and consistently exhibited lower levels of binding in U937 uninfected and U1 HIV-1 monocytic cell lines, as well as C81 human T-lymphotropic virus-1-infected T cells.
- Association of CDK2 and CDK9 with the biotin–Tat peptide was not observed in U937 monocytic or U87MG glioblastoma cells.
- Tat binds to many partners in the easily transfectable cell line 293Ts as with monocytes or glioblastoma cells.
- The establishment of an accurate pathogenic and latent model of HIV-1 infection in model cell lines is still a challenge in an *in vitro* system.
- The state of HIV-1 viral transactivation can be directly correlated to the epigenetic profile of Tat at a given cellular state.
- Subcellular fractionation within proteomic studies is a critical front-end purification strategy that keeps protein–protein interactions within the scope of naturally occurring cellular localization.

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Website

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