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Identification of Potential Drug Targets Using Genomics and Proteomics: A Systems Approach

I. Chapter Overview

Current *human immunodeficiency virus 1* (HIV-1) antiviral therapies have proven to be insufficient and limited due to the ability of the virus to develop resistant mutants. Specific cellular targets are needed for the next generation of HIV-1 therapeutics. Emerging genomic and proteomic techniques have elucidated a myriad of cellular genes involved in viral replication representing a collection of possible new drug targets. Here, the cellular–viral interactions of viral proteins are introduced and expanded

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upon to elucidate phenotypic changes as well as variations in cellular gene expression levels induced by HIV-1 infection. The collective organization of data utilizing a systems approach allows for the mapping of complex pathways and interactions between otherwise unknown protein partners. In support of this approach, we examine the multitude of cellular changes related to the expression of Tat, Nef, and Gag. In closing, we demonstrate how counteracting specific effects of viral proteins can alter disease pathogenesis.

II. Introduction

The race to complete the human genome as well as the successful sequencing of the genomes of many other organisms has provided modern science with an array of tools that promise to facilitate advancements in many branches of biological research. Genomics is classified as the study of an organism's genome through the expression and function of its genes. This field of study is concerned with the systematic use of genome information, associated with other data and is made possible by a series of techniques related to identification of genes and the level of gene expression. Foremost among genomic techniques is the oligonucleotide microarray, which when applied to cDNA derived from RNA of a sample population allows for the assessment of the total level of mRNA transcripts in a cell. These levels are influenced by the rates of transcription and turnover of each mRNA sequence and are often indicative of changes in protein expression. When used comparatively, the oligonucleotide microarray allows changes in mRNA levels between samples, such as normal versus diseased, to be monitored in cells or tissues. Additionally, array technology has been used to identify polymorphisms in gene coding sequences, vouching for the specificity and sensitivity of this genomic approach. Adaptation of this technology to viral sequences allows for the monitoring of viral RNA levels with relative ease.

Following the central dogma of molecular biology, the field of genomics has progressed to its protein equivalent, proteomics. Proteomics is defined as the large-scale study of proteins and their interactions through structure and function, therefore defining an organism's proteome. The mechanism behind defining a proteome follows two basic steps: first, the separation of proteins from a complex protein mixture, followed by the identification/characterization of the single protein or complex based on comparison to a defined protein sequence database. These two aspects of protein identification are often performed through the utilization of front-end purification techniques such as liquid chromatography (LC) or 2D gel electrophoresis (2DGE) followed by mass spectrometry (MS). LC can be directly coupled to a mass spectrometer such that a complex protein mixture is infused into a high performance liquid chromatography (HPLC) system, separated based

on size, charge, or affinity into fractions that are directly injected into an MS system therefore reducing the complexity of the samples and allowing for the direct identification of the protein(s) of interest. Additionally, a 2DGE approach for front-end purification results in a visual separation of a mixture of proteins in two dimensions based on isoelectric point and mass. A comparative analysis of protein spots from 2D gels representing different cellular states provides insight into changes in protein expression levels. These protein spots can be excised from the 2D gel and submitted to an MS system for analysis. Proteins may be isolated based on tissue type, cell type, and organelle localization in order to monitor changes in protein levels at specific locations, which can give clues to alterations such as protein trafficking, expression, and localization. These standard proteomic techniques are adaptable to subdivisions of the proteome such as the interactome, glycome, metabalome.

The fusion of genomics and proteomics has led to an array of techniques for studying the overall state of RNA transcription and protein expression in a cell. Chromatin immunoprecipitation (ChIP), for example, allows DNA associated with a given protein to be captured and analyzed by polymerase chain reaction, sequencing of recovered DNA, or microarray. Initial screening by a genomic or proteomic technique may identify patterns that can be followed up by more directed analysis with another technique. Overall, the disciplines of genomics and proteomics allow for the rapid identification of the overall cellular environment and can be applied to identify proteins and processes involved in HIV-1 pathogenesis and replication as well as the identification of novel pathways. Many studies have been performed on HIV-1 using these genomic and proteomic approaches. In this chapter, we will discuss some of the work published in the field while providing examples of how these approaches may be integrated to yield a greater understanding of the way in which HIV-1 influences the host cell. Examination of these studies will assess the current state of genomics and proteomics as applied to the field of human retrovirology as well as to identify key points of interest for future studies.

III. Viral Targets

A. Viral Genomics

The HIV-1 genome includes nine viral genes. All the viral genes are expressed from a single promoter located in the viral long-terminal repeat (LTR) (Coffin *et al.*, 1997; Greene and Peterlin, 2002); three of these genes (*Gag*, *Pol*, and *Env*) are common to all lentiviruses and the other six (*Tat*, *Rev*, *Nef*, *Vpr*, *Vpu*, and *Vif*) are referred to as accessory genes. *Tat*, the viral transactivator, feeds back on the viral promoter and increases transcription (Bohan *et al.*, 1992; Feinberg *et al.*, 1991; Jeang *et al.*,

1999; Laspia *et al.*, 1989). Temporal regulation of protein expression is achieved through alternative splicing regulated by the viral protein Rev (Cullen, 1991; Fukumori *et al.*, 1999; Hope, 1997; Hope and Pomerantz, 1995). Rev serves to stabilize transcripts and leads to the production of singly spliced and finally unspliced messages. In this way, viral gene expression is temporally regulated such that each protein is produced as needed (Coffin *et al.*, 1997; Fukumori *et al.*, 1999; Greene and Peterlin, 2002).

Although the regulation of viral gene expression was elucidated before the advent of genomics, modern techniques can still be useful in studying viral gene expression. Recently, the development of viral gene arrays allows for the detection of genomic DNA or viral transcripts. These arrays are capable of detecting sequence from all the open reading frames of HIV-1, human T-cell leukemia virus types 1 and 2, *Hepatitis C virus*, Epstein-Barr virus, human herpesvirus 6A and 6B, poxviruses, Varicella-zoster virus, and Kaposi's sarcoma-associated herpesvirus (Cohrs *et al.*, 2006; Conejero-Goldberg *et al.*, 2005; Ghedin *et al.*, 2004; Kostrzynska and Bachand, 2006; Ryabinin *et al.*, 2006; Yuan *et al.*, 2006). Initial experiments show its utility in detecting expression of viral genes and in identifying proteins bound to the viral genome through ChIP–chip analysis (Ghedin *et al.*, 2004). Use of a viral chip will provide researchers with a new tool for diagnostics, examination of viral gene expression under different conditions, and the identification of the interplay between viruses in coinfection. Indeed, the viral array has been used to examine the effect of the CDK inhibitor Cyc202 on viral gene expression (Ghedin *et al.*, 2004). As pharmaceuticals targeting various stages of the viral life cycle within the infected cell become available, technologies such as the viral array will prove invaluable in examining their effects. Future work in this field will likely focus on examining changes in the expression level of specific viral genes in specific cell types and in response to therapies.

B. Current Therapies and Future Prospects

When administered properly, the currently available HIV medications are capable of controlling infection, they do not, however, represent a cure. While the drugs merely prevent infection of new cells, latently infected cells continue to produce varying levels of wild-type and mutant virus. If therapy is discontinued, long-lived reservoirs of infected cells are capable of producing infectious virus and continuing the progression to AIDS. Additionally, the currently available drugs specifically target viral proteins such that small polymorphisms largely affect the efficiency of drug action. The high rate of mutation caused by the viral reverse transcriptase enzyme, for example, and the large numbers of new virus produced during each round of infection allow the virus to select variants with mutations that make them resistant to currently available therapies.

Current therapies in HIV are insufficient due to their inability to cure the disease and the ability of the virus to become resistant to the treatment over time. New therapies must be developed that target new mechanisms important to the viral life cycle. HIV encodes only nine genes; a fact that forces the virus to interact with and subvert cellular processes for its own benefit. Identifying these interactions and their effect on both the virus and the cell will reveal a new range of targets for future therapeutics.

The use of broad and powerful approaches afforded to us by the omics age will enable identification of such targets. First and foremost to this approach is the understanding of the contact point between the virus and the cell—that is, the interaction between viral components and cellular components. It is this interaction that allows the virus to influence cellular processes. Second, we must understand the effect this interaction has on the system as a whole. Finally, we can map this interaction to specific pathways within the cell, thereby identifying a mechanism for the observed effect. Once a target has been discovered, a drug can be selected that counteracts this effect by either preventing the initial interaction or altering the affected pathway to counteract this interaction.

In this chapter, we will discuss the important aspects of understanding the virus effect on the host organism using a systems approach. A sampling of the many interactions that HIV has with its host will be incorporated with a discussion of their overall effect, followed by a look into the identification of potential drug targets. Tat, Nef, and Gag will be used as examples of how to follow the effect of one viral protein through the cellular system to a potential drug target. Instead of providing a broad picture of how modern proteomic and genomic approaches can be used to identify potential drug targets, we will provide specific examples throughout the chapter.

IV. Cellular/Viral Protein–Protein Interactions

The study of protein–protein interactions between HIV-1 and the host cell provides an important insight in the mechanisms that allow the virus to manipulate cellular activities and/or alter cell regulatory mechanisms for its benefit (Tasara *et al.*, 2001). In order for HIV-1 to efficiently replicate in a susceptible host, cellular proteins must first be incorporated into the virion. Throughout the viral life cycle, events such as the assembly of the preintegration complex (PIC), reverse transcription of the HIV-1 genome, viral gene expression, viral assembly, and budding are all the results of specific viral–host protein interactions (Bannwarth and Gatignol, 2005; Bieniasz *et al.*, 1999; Bryant and Ratner, 1990; Ciborowski and Gendelman, 2006; Freed, 2002; Hope, 1997; Misumi *et al.*, 2002).

Identification of cofactors involved in HIV-1 infection and/or replication can be accomplished based on the molecular interactions between viral

and cellular proteins (Tang, 2002). To date, interactions between viral and host cellular proteins have been identified and characterized using classical scientific approaches appropriate to the field of virology (Kellam, 2001). As the HIV-1 genome consists of only nine viral genes, the characterization of the interactome, the complete set of protein–protein interactions, is a tangible goal. Prior to the advent of the "-omics" world, viral–host interactions were investigated solely using established experimental biology techniques and research methods that should now complement postgenomic methods rather than being replaced (Kellam, 2001). A collection of HIV-1–host protein interactions are displayed in Table I; though not exhaustive, the list of interactions establishes the major proteins responsible for successful viral infection, replication, and immune suppression, among others.

Fortunately, virology has caught up to emerging sciences and as a result has incorporated genomic and proteomic techniques into the study of infectivity and therapeutics. The utilization of high throughput postgenomic research techniques such as gene expression microarrays and protein arrays, LC, yeast two-hybrid (Y2H) screens, and MS allows for the rapid detection of viral–host protein interactions and aids in the understanding and manipulation of the associated viral and cellular pathways (Kellam, 2001).

A. Cellular Protein Interactions of Tat

Tat has been known to not only stimulate the HIV LTR promoter but also modulate and induce cellular genes. Historically, the mechanism of action by Tat has been assigned to the level of initiation and elongation (Bohan *et al.*, 1992; Feinberg *et al.*, 1991; Kato *et al.*, 1992; Laspia *et al.*, 1989; Marciniak and Sharp, 1991; Marciniak *et al.*, 1990). The effect of Tat on preinitiation, initiation, and elongation has been observed through a number of biochemical interactions including physical binding to Sp1 (Chun *et al.*, 1998), stabilization of the TFIID/TFIIA complex on the HIV-1 TATA box (Kashanchi *et al.*, 1996), recruitment of a functional TBP or TFIID (Chiang and Roeder, 1995; Dal Monte *et al.*, 1997; Garcia-Martinez *et al.*, 1997; Kashanchi *et al.*, 1996; Roebuck *et al.*, 1997; Veschambre *et al.*, 1995), phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (RNA Pol II) by a number of kinases, including TFIIH (Blau *et al.*, 1996; Herrmann and Mancini, 2001; Parada and Roeder, 1996), and binding of Tat directly to RNA Pol II. In recent years, Tat has also been shown to bind a number of other factors regulating chromatin structure located at the HIV promoter and enzymes that phosphorylate the large subunit of RNA Pol II, resulting in efficient elongation of transcription. They include Tat/cyclin T/CDK9 and Tat/CBP/p300 (Bieniasz *et al.*, 1999; Deng *et al.*, 2000, 2001; Herrmann and Mancini, 2001).

Although much of the focus has been on Tat's ability to recruit the cyclin T/CDK9 complex [known as phosphorylated positive transcription

TABLE I Protein–Protein Interactions Between HIV-1 Viral Proteins and Their Cellular-Interacting Proteins[a]

Viral protein category	*HIV-1 virus protein*	*Cellular-interacting proteins*
Structural	Gag (p55)	
	p17—Matrix	BAF (Lin and Engelman, 2003; Mansharamani *et al.*, 2003), Calmodulin (Daube *et al.*, 1991; Radding *et al.*, 2000), HEED (Peytavi *et al.*, 1999), EF1α (Cimarelli and Luban, 1999), hIF2 (Wilson *et al.*, 1999), Actin (Bukrinskaya *et al.*, 1998)
	p24—Capsid	CypPA (Luban *et al.*, 1993)
	p7—Nucleocapsid	Actin (Cimarelli and Luban, 1999; Liu *et al.*, 1999)
	p6	Tsg101 (Garrus *et al.*, 2001; VerPlank *et al.*, 2001), AIP1 (Strack *et al.*, 2003)
	Pol	
	p51—Reverse transcriptase	β-Actin (Liu *et al.*, 1999)
	p31—Integrase	Importin/Karyopherin α/β (Gallay *et al.*, 1997; Hottiger and Nabel, 1998), INI1 (Gallay *et al.*, 1997), UDG (Yung *et al.*, 2001)
	Env (gp160)	ApoH (Willetts *et al.*, 1999)
	gp120	CD4 (McDougal *et al.*, 1986)
Accessory	Vpu (p16)	BTrCP (Margottin *et al.*, 1998), UBP (Callahan *et al.*, 1998), Fas (Casella *et al.*, 1999)
	Vpr (p12/p10)	TFIIB (Agostini *et al.*, 1996), Cyclin T1/CDK9 (Sawaya *et al.*, 2000), hVIP/mov34 (Mahalingam *et al.*, 1998), Karyopherin α (Gallay *et al.*, 1997), ANT (Jacotot *et al.*, 2001), HHR23A (Withers-Ward *et al.*, 1997), p300 (Felzien *et al.*, 1998), p53 (Sawaya *et al.*, 1998), UNG (Bouhamdan *et al.*, 1996), RIP/VprBP (Zhang *et al.*, 2001)
	Vif (p23)	Sp140 (Madani *et al.*, 2002), Vimentin (Karczewski and Strebel, 1996), Cul5 (Yu *et al.*, 2003), Elongin B/C (Yu *et al.*, 2003), Rbx1 (Yu *et al.*, 2003), CEM15/APOBEC3G (Sheehy *et al.*, 2003)
	Nef (p27/p25)	NBP1 (Lu *et al.*, 1998), Human thioesterase II (Watanabe *et al.*, 1997), CD4 (Grzesiek *et al.*, 1996), ASK1 (Geleziunas *et al.*, 2001; Peterlin and Trono, 2003), AP-1, 2, 3 (Coleman *et al.*, 2006; Craig *et al.*, 2000; Greenberg *et al.*, 1997; Schwartz *et al.*, 1996), MHC-I (Schwartz *et al.*, 1996; Williams *et al.*, 2002), PI3K (Wolf *et al.*, 2001), β-COP (Piguet *et al.*, 1999), PACS1 (Piguet *et al.*, 2000), Src family tyrosine kinases (Lee *et al.*, 1996), p53 (Greenway *et al.*, 2002)

(*continued*)

TABLE I (*continued*)

Viral protein category	*HIV-1 virus protein*	*Cellular-interacting proteins*
Regulatory	Tat (p16/p14)	TBP (Chiang and Roeder, 1995; Dal Monte *et al.*, 1997; Garcia-Martinez *et al.*, 1997; Kashanchi *et al.*, 1994; Roebuck *et al.*, 1997; Veschambre *et al.*, 1995), p32 (Fridell *et al.*, 1995), CBP/p300 (Col *et al.*, 2001; Deng *et al.*, 2000, 2001; Hottiger and Nabel, 1998; Kiernan *et al.*, 1999; Ott *et al.*, 1999), CAK/TFIIH (Blau *et al.*, 1996; Cujec *et al.*, 1997; Herrmann and Mancini, 2001; Parada and Roeder, 1996), Tip60 (Kamine *et al.*, 1996), P-TEFb (Chen *et al.*, 1999; de Falco and Giordano, 1998; Fujinaga *et al.*, 1998; Herrmann and Mancini, 2001; Karn, 1999; Majello *et al.*, 1999; Wei *et al.*, 1998), Sp1 (Chun *et al.*, 1998; Kamine *et al.*, 1991), BRG1 (Agbottah *et al.*, 2006; Mahmoudi *et al.*, 2006; Mohrmann *et al.*, 2004)
	Rev (p19)	p32 (Tange *et al.*, 1996), eIF-5A (Ruhl *et al.*, 1993), CRM1 (Neville *et al.*, 1997), hRIP/Rab (Farjot *et al.*, 1999), Importin-β (Truant and Cullen, 1999), B23 (Fankhauser *et al.*, 1991)

[a]Abbreviations: BAF, barrier-to-autointegration factor; HEED, human EED; EF1α, elongation factor-1α; hIF2, a human homologue of bacterial translation initiation factor 2; CyPA, cyclophilin A; Tsg101, tumor susceptibility gene 101; AIP1, actin-interacting protein 1; INI1, integrase interactor 1; RIP, receptor-interacting protein; NBP1, Nef-binding protein-1; ASK1, apoptosis signal-regulating kinase 1; AP, adaptor proteins; MHC, major histocompatibility complex; PI3K, phosphatidylinositol 3-kinase; PACS, phosphofurin acidic cluster sorting protein; P-EFBb, phosphorylated positive transcription elongation factor b.

elongation factor b (P-TEFb)], emerging work indicates the importance of Tat in recruiting other factors required for initiation and elongation. Several reports have indicated a role of Tat in associating with TBP and facilitating transcriptional initiation (Brady and Kashanchi, 2005; Chiang and Roeder, 1995; Kashanchi *et al.*, 1994; Raha *et al.*, 2005). Additionally, it has been shown that Tat associates with CDK2/cyclin E, promoting phosphorylation of Tat and the CTD of RNA Pol II (Ammosova *et al.*, 2006; Deng *et al.*, 2002).

1. Tat and Cyclin T/CDK9

Tat activates the HIV LTR by binding to TAR to recruit and activate cellular factors. TAR is a 59-residue RNA leader sequence that folds into a specific stem-loop structure (Bannwarth and Gatignol, 2005; Cullen, 1991; Garcia *et al.*, 1989). The sequence of the bulge (Cordingley *et al.*, 1990; Dingwall *et al.*, 1990; Roy *et al.*, 1990) and loop (Berkhout and Jeang, 1989; Feng and Holland, 1988; Selby *et al.*, 1989) of TAR is critical for Tat activation of the LTR. Tat binds to the bulge and recruits cellular factors that bind the loop, one of which, P-TEFb (Fig. 1C), a protein kinase composed of CDK9 and cyclin T1, is stimulated by Tat (Richter *et al.*, 2002; Wei *et al.*, 1998). Activation of P-TEFb results in hyperphosphorylation of the large subunit of the RNA Pol IICTD and activation of transcription elongation (Kim *et al.*, 2002).

CDK9 is analogous to a component of the P-TEFb, isolated from Drosophila, which stimulates promoter-paused RNA Pol II to enter into productive elongation (Chen *et al.*, 1999; de Falco and Giordano, 1998; Herrmann and Mancini, 2001; Karn, 1999; Majello *et al.*, 1999; Wei *et al.*, 1998). A histidine-rich stretch of cyclin T1, the other component of P-TEFb, binds to the CTD of RNA Pol II, which is required for the subsequent expression of full-length transcripts from target genes (Taube *et al.*, 2002). CDK9 phosphorylation is required for high-affinity binding of Tat/P-TEFb to TAR. Furthermore, P-TEFb phosphorylation regulates Tat transactivation *in vivo* (Fong and Zhou, 2000; Garber *et al.*, 2000; Zhou *et al.*, 2000). Other studies on P-TEFb-regulated transcription and the involvement of Tat reveal the involvement of multiple factors in transcriptional control. Other studies show the involvement, although not the requirement, of hSpt5 in Tat-mediated transcription (Winston, 2001; Wu-Baer *et al.*, 1998). Other studies have identified a cellular inhibitor of P-TEFb, called Hexim1 (Fraldi *et al.*, 2005; Schulte *et al.*, 2005), which inhibits P-TEFb in the presence of the 7SK small nuclear RNA (Chen *et al.*, 2004; Michels *et al.*, 2004). Interestingly, the ability of Hexim1 to inhibit Pol II transcription relies on the presence of a Tat-like arginine-rich motif (Yik *et al.*, 2004). These multiple levels of transcriptional control suggest that there are many nuances to Tat-induced transactivation that have not yet been discovered.

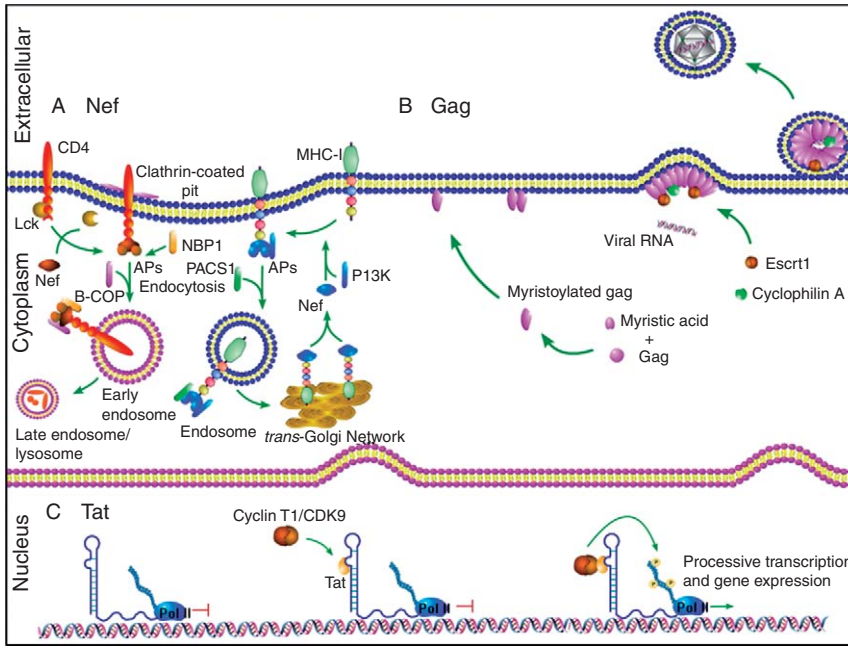

FIGURE 1 Alteration and subversion of cellular processes by *human immunodeficiency virus 1* (HIV-1). (A) Nef removes CD4 and major histocompatibility complex (MHC) from the cell surface by endocytosis and degradation. Nef competitively binds the C-terminal cytoplasmic tail of CD4, displacing bound Lck. AP allow binding of β-COP1, which binds to CD4-bound Nef, facilitated by binding of Nef-binding protein-1 (NBP1). β-COP1 then drives formation of the early endosome and finally the Nef complex dissociates from CD4 as it is further degraded in the lysosome. For MHC, binding of Nef to PI3K allows binding of Nef to MHC-I. The endocytosis of MHC-I is aided through the binding of phosphofurin acidic cluster sorting protein (PACS1). MHC-I is transported to the *trans*-Golgi Network where degradation of MHC and dissociation of Nef occur. (B) Coordinated modification of Gag followed by Gag–Gag and other cellular interactions leads to viral assembly and budding. Gag is myristoylated, which targets it to the cellular membrane. Once on the membrane, Gag interacts with other Gag molecules and begins to form the viral core. As this happens, Gag recruits viral RNA and the cellular genes cyclophilin A (CyPA) and Escrt1, which facilitate budding. (C) HIV-1 transcription is aided by Tat's ability to recruit cyclin T1/CDK9 [known as phosphorylated positive transcription elongation factor b (P-TEFb)], which in turn phosphorylates the C-terminal domain of RNA polymerase II (RNA Pol II). In the absence of Tat, transcription pauses between 50 and 100 nucleotides after transcription starts. In the presence of Tat, the TAR element recruits HIV-1 TAT, which recruits cyclin T1/CDK9, this in turn phosphorylates Pol II and leads to activated transcription.

2. *Tat and CBP/p300*

Examination of defective Tat transactivation in murine cells revealed a role for P-TEFb (Benkirane *et al.*, 1998; Bieniasz *et al.*, 1999; Fujinaga *et al.*, 1999; Ramanathan *et al.*, 1999). Additionally, studies of Tat transactivation

in murine cells revealed a role for p300 and PCAF. Tat has been shown to interact with the histone acetyltransferases (HAT) p300 and PCAF. Supporting evidence suggests that Tat-associated HAT activity is important for transactivation of integrated, but not unintegrated, HIV-1 (Deng *et al.*, 2000). Furthermore, it has been shown that the Tat–p300 interaction increases the HAT activity of p300 on histone H4, which is associated with nucleosomal DNA (Deng *et al.*, 2001). The association of Tat with p300 and PCAF may be critical for remodeling of the chromatin downstream of TAR to allow progressive transcription elongation. The acetyltransferase activity of p300 may also alter the activity of the viral Tat protein. p300 has been shown to acetylate lysine 50 in the TAR RNA-binding domain of Tat, while PCAF acetylates lysine 28 in the activation domain of Tat (Col *et al.*, 2001; Kiernan *et al.*, 1999; Ott *et al.*, 1999). Acetylation of lysine 28 by PCAF enhances Tat binding to the Tat-associated kinase, cdk9, while acetylation by p300 at lysine 50 of Tat promotes the dissociation of Tat from TAR RNA (Deng *et al.*, 2000; Kiernan *et al.*, 1999; Mujtaba *et al.*, 2002). Acetylation of lysines 28 and 50 of Tat has been demonstrated to be important for viral replication (Bres *et al.*, 2002; Deng *et al.*, 2000).

In support of the ability of acetylated Tat to increase transcription, several groups have demonstrated that SWI/SNF binds to acetylated Tat (Agbottah *et al.*, 2006; Mahmoudi *et al.*, 2006; Treand *et al.*, 2006). SWI/SNF is a chromatin remodeling complex that may be involved in the removal of nucleosomes at the HIV-1 LTR and activates an increase in viral transcription (Agbottah *et al.*, 2006). This recruitment of SWI/SNF by Tat has been reported to be regulated by the binding of acetylated Tat to Brm (Treand *et al.*, 2006) or BRG1 (Agbottah *et al.*, 2006; Mahmoudi *et al.*, 2006). This interaction was shown to be critical for achieving high levels of viral transcription.

B. Cellular Protein Interactions of Nef

The HIV-1 gene product Nef (negative factor) is a multifunctional protein that contributes to HIV pathogenesis through alteration of endocytosis, signal transduction via the downregulation of cell surface receptors, vesicular trafficking, and immune evasion; therefore, enhancing virion infectivity and viral production (Arold and Baur, 2001; Das and Jamel, 2005; Geyer *et al.*, 2001; O'Neill *et al.*, 2006; Roeth and Collins, 2006). Nef is a small protein of ~27 kDa that is expressed abundantly in the early stages of viral replication and regularly undergoes posttranslational modifications including phosphorylation and the irreversible myristoylation of its N-terminus; this localizes the protein to the cellular membrane (Arold and Baur, 2001; Bentham *et al.*, 2006; Geyer *et al.*, 2001; Harris, 1995). The structure of Nef includes a membrane anchoring region in addition to an unstructured

flexible loop providing an extensive surface area that is capable of undergoing important conformational changes (e.g., for transient binding) and is readily accessible for interactions (Arold and Baur, 2001; Geyer *et al.*, 2001). Nef has a positive effect on viral infection and replication by promoting the survival of infected cells through interaction with cellular proteins involved in both trafficking of cell surface receptors and signaling molecules (Fig. 2) (Arold and Baur, 2001; Das and Jamel, 2005).

1. Downregulation of CD4

CD4 is the primary host cell surface receptor required for T-lymphocyte ontogeny, activation of mature helper T lymphocytes, and serves as the primary receptor for HIV-1. Nef downregulates the transmembrane glycoprotein CD4 through the acceleration of endocytosis and lysosomal degradation in order to facilitate increased release and infectivity of virus particles as well as preventing superinfection (Arold and Baur, 2001; Das and Jamel, 2005; Geyer *et al.*, 2001; Watanabe *et al.*, 1997). Ironically, although CD4 is needed for viral infection, three of nine viral genes are involved in its removal and degradation shortly after infection (Bour *et al.*, 1999; Cortes *et al.*, 2002). Nef downregulation of CD4 prevents the formation of complexes between the HIV Env-encoded protein gp120 and CD4 (Arold and Baur, 2001; Benson *et al.*, 1993). It recruits additional adaptor proteins (APs) and cofactors in order to effectively internalize CD4. Nef directly binds the dileucine motif of the cytoplasmic tail of CD4 (Fig. 1A), therefore displacing the otherwise bound Src family tyrosine kinase Lck, and promotes the recruitment of AP-1, 2, 3 via the interaction of its μ2 subunit with Nef's own dileucine motif (Das and Jamel, 2005; Greenberg *et al.*, 1997; Mangasarian *et al.*, 1997; Piguet *et al.*, 1999). These AP complexes interact with cytosolic clathrin-coated pits that mediate transport between the *trans*-Golgi, endosomes, and lysosomes as well as vesicles that mediate endocytosis of CD4 (Craig *et al.*, 2000; Das and Jamel, 2005; Janvier *et al.*, 2001). The direct interaction of Nef and AP-2 is mediated and strengthened by the binding of Nef to Nef-binding protein-1 (NBP1) (Das and Jamel, 2005; Lu *et al.*, 1998). As a result, Nef targets these internalized CD4 molecules for degradation by both binding CD4 and recruiting β-COP, the β-subunit of COP1 coatomers in endosomes, for routing and shuttling of CD4 to lysosomes based on acidic residues (Piguet *et al.*, 1999). This degradation process is also facilitated by the binding of Nef to human thioesterase II protein that regulates the intracellular level of acyl-CoA therefore increasing the efficiency of protein myristoylation, which is critical for the membrane localization of Nef (Liu *et al.*, 1997; Watanabe *et al.*, 1997). These interactions occur quickly, as Nef, once localized to the cell membrane, is endocytosed with CD4 within minutes (Arold and Baur, 2001).

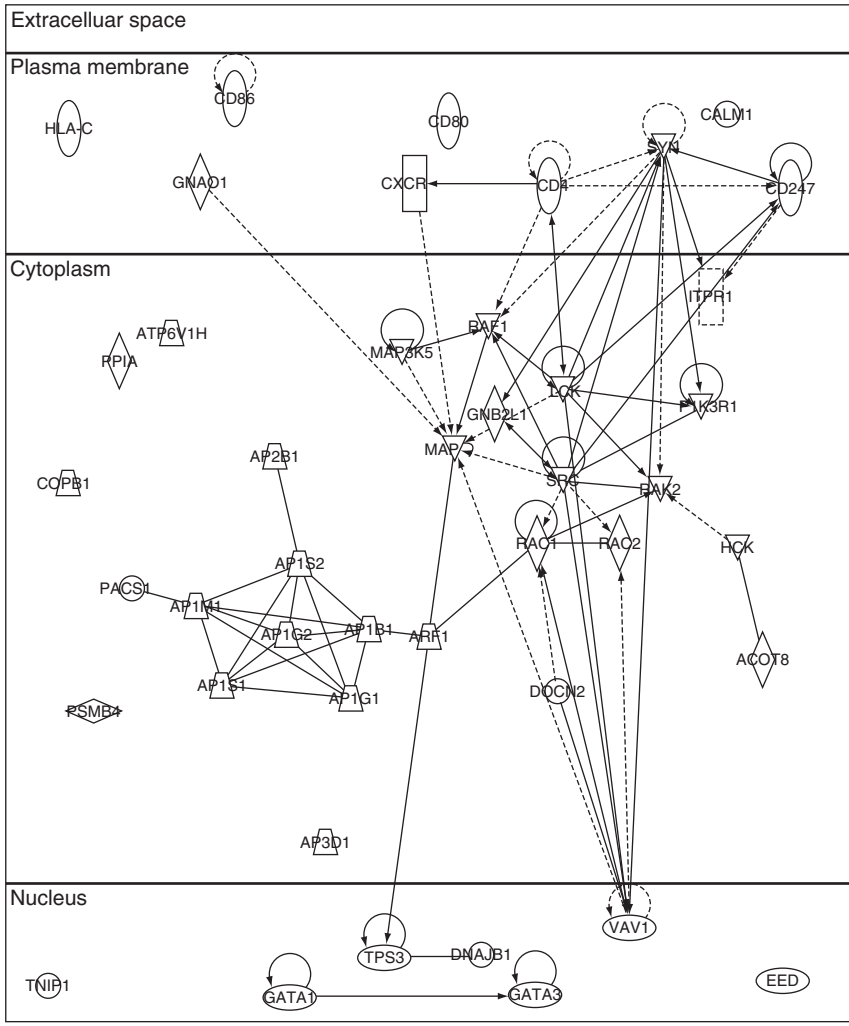

FIGURE 2 System diagram of Nef-interacting proteins. Ingenuity generated diagram showing the relationship between various Nef-binding proteins. The close interaction of these proteins suggests a coordinated effect by Nef upon the host cell. Shapes indicate different protein types; rectangle—cytokine, diamond—enzyme, and triangle—kinase. The character of the connecting line defines the interaction; solid line—direct interaction, dashed line—indirect interaction, arrow—acts upon, and no arrow—binding.

2. Downregulation of MHC-I

Mobilization of the host's adaptive immune response to a viral infection involves the presentation of viral peptides on the surface of infected cells by the major histocompatibility complex (MHC). The downregulation of

MHC-I by Nef assists HIV-1-infected cells in escaping the cytotoxic T-lymphocyte-mediated elimination of virus-infected cells (Das and Jamel, 2005; Geyer *et al.*, 2001). Nef increases the rate of internalization of cell surface MHC-I molecules through the endosomes (via AP-1 binding) followed by transportation to the *trans*-Golgi network, then finally to clathrin-containing vesicles (Fig. 1A) (Das and Jamel, 2005; Roeth *et al.*, 2004). Nef selectively downregulates the HLA-A and HLA-B MHC-I molecules through binding a unique cytoplasmic tyrosine kinase residue, as opposed to the HLA-C and HLA-E molecules, which are required for cellular protection from lysis by natural killer cells (Das and Jamel, 2005). The Nef shuttling effect on MHC-I to the *trans*-Golgi network is blocked by inhibitors of phosphatidylinositol 3-kinase (PI3K) through the additional binding of phosphofurin acidic cluster sorting protein (PACS1) (Das and Jamel, 2005; Peterlin and Trono, 2003; Piguet *et al.*, 2000; Swann, 2001; Wolf *et al.*, 2001). Nef, therefore, decreases the expression of MHC-I molecules by manipulating PACS1 that controls the endosomes-to-Golgi trafficking of furin and M6PR by bridging those molecules with the AP complex of endosomal clathrin-coated pits (Peterlin and Trono, 2003; Piguet *et al.*, 2000).

3. Nef and Apoptosis

Nef interferes with the Fas-FasL apoptotic pathway by inhibiting apoptosis signal-regulating kinase 1 (ASK1), a serine/threonine kinase significant in both the Fas and the tumor necrosis factor (TNF) signaling pathways (Geleziunas *et al.*, 2001; Peterlin and Trono, 2003). The binding of Nef to ASK1 inhibits both Fas- and TNF-α-mediated apoptosis (Geleziunas *et al.*, 2001; Peterlin and Trono, 2003). Additional Nef interactions affecting apoptosis include the binding and activation of PI3K, PI3K phosphorylates PAK, PAK phosphorylates Bad, resulting in the release of the antiapoptotic Bcl-2 or Bcl-X_L complex (Das and Jamel, 2005; Peterlin and Trono, 2003). Nef also interacts directly with tumor suppressor protein p53, decreasing its proapoptotic, transcriptional, and DNA-binding activities, and protecting HIV-1-infected cells from p53-mediated apoptosis (Das and Jamel, 2005; Greenway *et al.*, 2002). Blocking and interfering with these apoptotic pathways can prevent the premature death of an HIV-1-infected cell by virus-induced cytopathicity that leads to the completion of the viral replication cycle (Peterlin and Trono, 2003).

C. Cellular Protein Interactions of Gag

HIV-1 Gag drives the assembly and release of infectious viral particles during the final stage of viral replication (Freed, 1998; Gottlinger, 2001; Li and Wild, 2005). HIV-1 Gag is synthesized initially into a precursor polyprotein, $Pr55^{Gag}$, and is subsequently cleaved shortly after budding by

the HIV-1 protease into the mature Gag proteins: p17 matrix, p24 capsid, p7 nucleocapsid, and p6 (Freed, 1998). Maturation of the Gag proteins results in specific localization throughout the virion as well as major morphological transformation of the virion structure (Freed, 1998). The ability of Gag to interact with various cellular proteins is important to its function in both infection of a target cell and maturation of the virus (Fig. 3). HIV-1 Gag

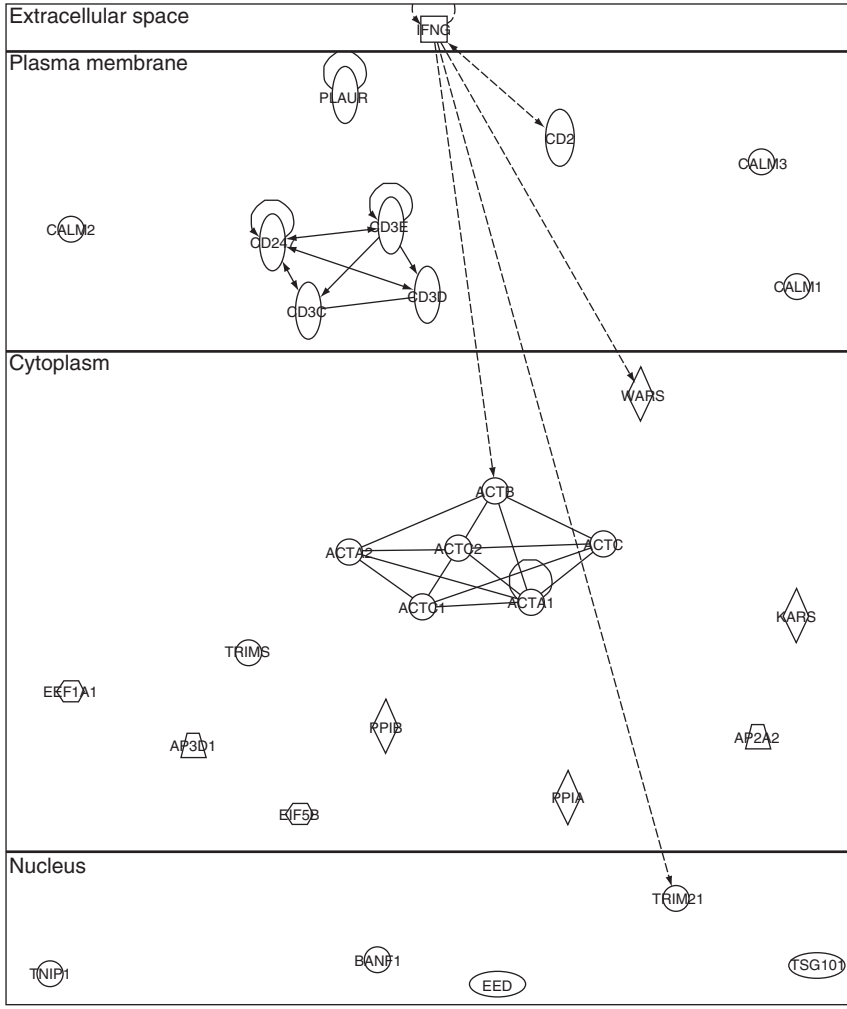

FIGURE 3 System diagram of Gag-interacting proteins. Ingenuity generated diagram showing the relationship between various Gag-binding proteins. Shapes indicate different protein types; rectangle—cytokine, diamond—enzyme, and triangle—kinase. The character of the connecting line defines the interaction; solid line—direct interaction, dashed line—indirect interaction, arrow—acts upon, and no arrow—binding.

proteins, in addition to integrase and viral nucleic acids, are incorporated into PICs along with cellular proteins in order to actively penetrate the nuclear membrane initiating infection (Sorin and Kalpana, 2006). Upon infection, the HIV-1 PICs rapidly associate with the cytoskeletal compartment through specific protein–protein interactions between viral factors and host cellular proteins (Tasara *et al.*, 2001).

1. p17 Matrix: Directing Assembly and Binding at Plasma Membrane

In mature virions, the matrix protein p17 is a 132-amino acid polypeptide derived from the N-terminus of the $Pr55^{Gag}$ precursor that forms a protective shell attached to the inner surface of the plasma membrane of the virus (Fiorentini *et al.*, 2006). The major function of p17 is to direct binding and assembly at the plasma membrane of the virion (Freed, 1998). Localization of p17 is determined via posttranslational modifications where myristoylation targets the plasma membrane and additional phosphorylation facilitates release from the membrane to associate with reverse transcription complexes (Bukrinskaya *et al.*, 1998). Calmodulin binding to the membrane-binding amphipathic region of p17 also regulates matrix localization mimicking the effect of proteolysis (Radding *et al.*, 2000). The matrix protein binds actin microfilaments of the host cell cytoskeleton after infection to activate reverse transcription (Bukrinskaya *et al.*, 1998; Tasara *et al.*, 2001). Barrier-to-autointegration factor proteins are also incorporated into HIV-1 virions and interact with p17 to aid in the transition from reverse transcription complex to PIC as well as to interact with viral DNA (Mansharamani *et al.*, 2003). Additional transcriptional regulation is seen through the binding of p17 to the human EED (HEED) protein, a homologue of the mouse gene *eed* family that have been reported to function as transcriptional repressors and gene silencers (Peytavi *et al.*, 1999). It is hypothesized that HIV-1 infection might deregulate silent cellular genes or that HEED might be involved in the docking of the PIC to specific host DNA insertion sites via p17 binding (Peytavi *et al.*, 1999). p17 alters translational efficiency as well as transcriptional via binding of elongation factor-1α, essential for the delivery of aminoacyl-tRNAs to ribosomes (Cimarelli and Luban, 1999). The accumulation of Gag p17 impairs translation and therefore serves to release viral RNA from polysomes (Cimarelli Luban, 1999). The direct binding of the human homologue (hIF2) of bacterial translation initiation factor 2 also has the potential to regulate viral translation (Wilson *et al.*, 1999).

2. p24 Capsid and p7 Nucleocapsid

The capsid protein p24, consisting of an N- and a C-terminal domain, condenses to form the conical core structure surrounding the viral genome (Endrich *et al.*, 1999). The predominant interaction of p24 is with cellular

protein cyclophilin A (CyPA), which characteristically has a peptidyl-prolyl *cis-trans* isomerase activity and interacts with cyclosporin A (Endrich *et al.*, 1999). The precise role of CyPA in the viral life cycle remains elusive; however, the knockdown of CyPA expression or the disruption of the p24–CyPA interaction results in reduced infectivity and prevents encapsidation into the virion (Sorin and Kalpana, 2006). Virions depleted of CyPA are blocked at early stages of reverse transcription, suggesting involvement in the early stages of infection (Sorin and Kalpana, 2006). The p7 nucleocapsid protein plays several important roles in the viral life cycle including virus assembly, viral genomic RNA encapsidation, primer tRNA placement, and enhancement of viral reverse transcription (Liu *et al.*, 1999). p7 can bind actin directly therefore associating the HIV-1 Gag protein with the cytoskeleton (Liu *et al.*, 1999).

3. *p6: Accessory Protein*

Efficient HIV release requires the *cis*-acting, tetrapeptide P(S/T)AP "late domain" (L domain) found in the p6 domain of Gag (von Schwedler *et al.*, 2003). The L domain mediates the detachment of the virion by recruiting host tumor susceptibility gene 101 (Tsg101), a component of the class E vacuolar protein sorting (Vps) machinery (Strack *et al.*, 2003), which is involved in regulation of intracellular trafficking, transcriptional regulation, and cell cycle control (VerPlank *et al.*, 2001). Binding of p6 to Tsg101 is required for the release of infectious HIV-1 (Fig. 1B) and facilitates budding by linking the p6 L domain to vacuolar sorting machinery (Garrus *et al.*, 2001). A second region within p6 contributes to the virus release function through the binding of host protein, ALG-2-interacting protein 1, serving as a component of the viral budding machinery (Strack *et al.*, 2003).

V. Viral-Induced Cellular Alterations

A. Tat: Effects on Cellular Transcription

The primary function of Tat is the transactivation of the viral LTR through recruitment of cellular factors to the viral promoter. By interacting with TAR, Tat recruits cyclin T1/CDK9 and activates Pol II-driven transcription (Fig. 1C). In addition, Tat interacts with CBP/p300 that remodels chromatin for more efficient transcription, the recruitment of which is regulated by the ability to bind to nascent RNA transcripts. Tat binding to cellular targets provides a mechanism with which the virus can alter the environment favorably. Given Tat's specific interactions with transcription factors such as Sp1, TBP, and P-TEFb, among others, it has the ability to alter gene expression at the transcriptional level; indeed, many studies have shown that Tat expression influences native cellular processes

(Chauhan *et al.*, 2003; Coiras *et al.*, 2006; de la Fuente *et al.*, 2002; Ensoli *et al.*, 1993; Izmailova *et al.*, 2003; Liang *et al.*, 2005; Nelson *et al.*, 2003; New *et al.*, 1997; Pocernich *et al.*, 2005; Viscidi *et al.*, 1989; Westendorp *et al.*, 1994). In order to further examine the role of Tat in regulation of cellular processes, several groups have chosen to use a proteomic or genomic approach.

Dendritic cells (DCs) are likely the first cells infected following mucosal exposure to HIV-1. Izmailova *et al.* (2003) examined the changes in Tat-expressing DCs using Affymetrix gene arrays. They found that Tat upregulated many interferon-inducible genes. Specifically, Tat upregulated the expression of interferon regulatory factor-7 and signal transducer and activator of transcription 1 (STAT1), both of which are transcriptional regulators of interferon response and may be responsible for upregulation of other IFN-inducible genes (Izmailova *et al.*, 2003). Additionally, four chemokines were shown to be upregulated in Tat-expressing DCs: human monokine induced by interferon-γ, monocyte chemoattractant protein-3, monocyte chemoattractant protein-2, and interferon-inducible protein-10. In support of these findings, the authors show that culture supernatant from Tat-expressing DCs induced monocyte and T-cell chemotaxis. Interestingly, despite these changes in function and chemokine production, DCs expressing Tat did not become activated or differentiate into mature DCs, as determined by detection of the cell surface markers CD40, CD80, CD83, CD86, and CD25 (Izmailova *et al.*, 2003).

In a similar study, the effects of Tat on $CD4^+$ T cells, the primary target of HIV-1, was examined using a proteomic approach (Coiras *et al.*, 2006). Opposing studies have shown that Tat can induce apoptosis in uninfected bystander T cells; however, Coiras *et al.* (2006) proved the opposite effect for Tat-expressing cells. In fact, Tat induced resistance to apoptosis mediated by tunicamycin in Jurkat T cells. The authors associate this effect with the observed downregulation of cytoskeletal proteins such as β-actin and β-tubulin. In the absence of β-actin and β-tubulin, there is a disruption in cytoskeletal arrangement, leading to the loss of acting depolymerization as mediated by apoptosis-inducing signals, thus altering a critical event in the apoptotic cascade. The authors also observed the downregulation of Annexin II and the Rac/Rho-GDI complex, which influences the fusion and internalization of an infecting virion, thereby reducing the rate of super infection.

Another study examined the effects of Tat expression in human astrocytes, as a means to examine processes important in HIV-1-associated dementia (Pocernich *et al.*, 2005). This study also observed downregulation of β-tubulin and components of the Rac/RHO-GDI complex. Other proteins downregulated in Tat-expressing astrocytes included protein phosphatase 2A (PP2A) inhibitor, heterogeneous nuclear ribonucleoprotein A1, and heat-shock protein 70 (HSP70), which all likely cause an increase in viral replication. PP2A augments Tat-regulated transcription (Ammosova *et al.*, 2005; Faulkner *et al.*, 2003), therefore a downregulation of PP2A inhibitor will

increase transcription. Heterogeneous nuclear ribonucleoprotein A1 may also downregulate Tat-regulated transcription or block splicing and production of the Tat protein. HSP70 downregulation, along with HSP32, protects the cell from stress-induced cell death. Finally, HSP70 has been shown to be involved in chaperoning and allow the correct folding of the cyclin T/CDK9 complex; suggesting that a downregulation of HSP70 may affect P-TEFb activity (O'Keeffe *et al.*, 2000).

Two papers published by de la Fuente *et al.* (2002) and Liang *et al.* (2005) examine the effect of Tat on cellular gene expression in T-cell lines, focusing specifically on latently infected cells as well as the differential effects of Tat at various stages of the cell cycle. Interestingly, the microarray experiments performed in these studies showed that ~66% of the differentially expressed genes were downregulated. de la Fuente *et al.* (2002) identified many genes differentially regulated in latently infected T cells as compared to uninfected cells. Clustering of the differentially expressed genes identified four distinct cellular networks: signal transduction, translation, cell cycle regulation, and chromatin remodeling. Overall, the observed changes suggest that the Tat-expressing cells are altered to express larger amounts of virus through chromatin remodeling and an increase in translational machinery. In addition, signal transduction and cell cycle machineries are altered in a way that favors proliferation and survival of the cell (Fig. 4).

A subsequent study by Liang *et al.* (2005) examined the Tat-dependent effects in different stages of the cell cycle in an attempt to identify therapeutic targets in the host cell genome. The authors observed many of the same differentially expressed genes as previous studies, however, choose to focus on a subset of genes whose upregulation due to Tat is independent of the cell cycle. The role of Rev-binding protein 2, Pou2Af1, cyclin A1, PPGB, EXT2, and HEXA was examined using small interfering RNAs (siRNA)-mediated knockdown. siRNA-mediated repression of these genes did not result in changes in the cell cycle or induction of apoptosis; however, knockdown of these genes resulted in a lower level of p24 Gag detectable in the cell culture media, suggesting a drop in viral replication (Liang *et al.*, 2005). This work showed evidence that viral replication can be blocked by targeting cellular proteins critical to viral replication.

Each of these studies outlines a cellular phenotype associated with Tat expression. An emerging pattern of Tat-influenced differentially expressed cellular genes provides insight into the effect of Tat expression. Tat functions through an upregulation of gene expression; however, 66% of cellular genes are downregulated in a Tat-expressing cell, likely through a secondary effect. For instance, Tat activates specific interferon response genes, prevents apoptotic signaling, triggers proliferation, promotes cellular chemotaxis, triggers release of chemoattractants, and prevents differentiation. When taken together, these changes indicate that Tat is providing a favorable environment for virus production. Resistance to apoptosis and the proliferative

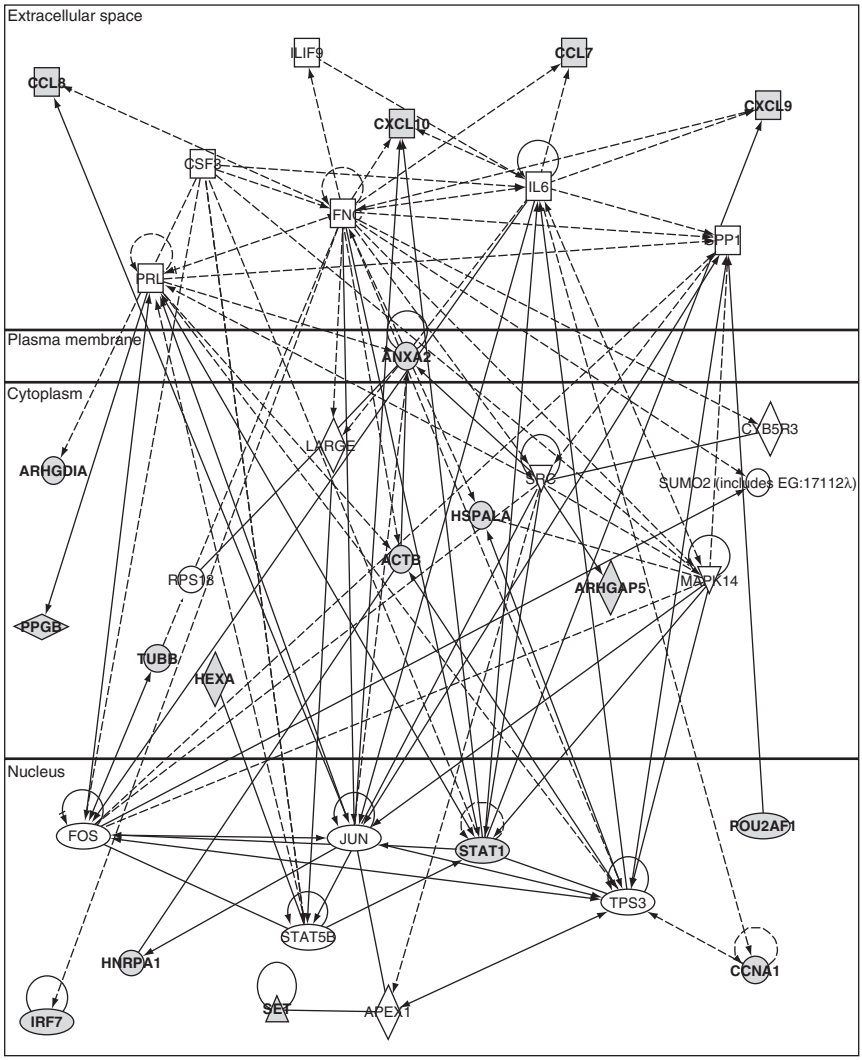

FIGURE 4 System diagram outlining Tat's effect on cellular proliferation. Ingenuity generated diagram showing the relationship between 18 genes differentially regulated by Tat (gray shaded nodes) and other genes relating to cellular proliferation. This diagram shows how alteration of expression of a few key genes can have a large effect on the cellular system. Of particular interest are the more important nodes such as STAT1, Annexin II, and Fos. Shapes indicate different protein types; rectangle—cytokine, diamond—enzyme, and triangle—kinase. The character of the connecting line defines the interaction; solid line—direct interaction, dashed line—indirect interaction, arrow—acts upon, and no arrow—binding.

signals produced by Tat ensure that viral replication and transcription continue. Meanwhile, the changes in chemokine expression and cellular migration ensure that target cells are available to be infected by the newly

produced virions. The function of Tat in altering the cellular environment provides a target for therapy.

Flavopiridol, originally developed as an anticancer agent, functions by blocking cyclin T1/CDK9 function. It is highly specific for cdk9 inhibition and can be used to block Tat transactivation of viral LTR at a very high therapeutic index (Chao *et al.*, 2000; de la Fuente *et al.*, 2003; Sedlacek, 2001). This ability to block Tat transactivation through P-TEFb may also allow the drug to prevent the alteration of cellular gene expression by Tat.

A study by Nelson *et al.* (2003) examines the ability of flavopiridol not only to block Tat transactivation of the viral LTR but also to diminish some of the pathogenic effects induced by HIV-1 gene expression. Using a murine model of HIV-1-associated nephropathy (HIVAN) that focuses on renal expression of full-length and spliced HIV-1 mRNAs from a Gag and Pol-deficient provirus, the authors examine the effects of flavopiridol treatment on viral gene expression, renal pathogenesis, cellular gene expression, and toxicity. Proviral gene expression was reduced in all flavopiridol-treated animals, but not in control animals. Several clinical parameters of HIVAN were also examined in flavopiridol-treated animals (Nelson *et al.*, 2003). The ratio of urine protein to creatinine levels normalized in flavopiridol-treated animals. Additionally, four serological abnormalities associated with HIVAN (albumin levels, blood urea nitrogen, cholesterol, and triglycerides) returned to normal after flavopiridol treatment. Liver pathology, as determined by histochemical analysis, also returned to normal in treated animals. These results were traceable to the gene expression level through microarray analysis. Microarrays indicated that genes differentially expressed in HIV-expressing liver were returned to levels comparable to healthy liver (Nelson *et al.*, 2003).

This study shows the feasibility of targeting cellular genes involved in viral replication (Nelson *et al.*, 2003). By approaching possible treatment options through inhibiting Tat transactivation, the authors have identified a way to both block viral replication and treat the pathogenic effects associated with infection. This approach may provide a perfect complement to the current therapies that prevent the infection of new cells. The use of flavopiridol will reduce the production of infectious virus, as well as deplete the effects of viral infection. When applied to the primary target of infection, the immune system, this approach may possibly prevent much of the immune dysregulation associated with HIV-1 infection.

The association of Tat with cyclin E/CDK2 and subsequent phosphorylation of RNA Pol II CTD and Tat suggest an important role cdk2 in HIV-1 transcription (Ammosova *et al.*, 2006; Deng *et al.*, 2002). As a direct extension of this work, two papers have been published that examine the use of Cyc202, a cyclin E/CDK2 inhibitor, in blocking HIV-1 replication (Agbottah *et al.*, 2005; Wang *et al.*, 2001). Previous work by these authors showed that p21/Waf1, a cellular inhibitor of cdk2, 3, 4, and 6, was downregulated in

HIV-1-infected cells (Clark *et al.*, 2000). They reasoned that HIV-1-infected cells might be more sensitive to a cdk inhibitor, such as Cyc202, than uninfected cells. The authors showed that Cyc202 both decreases the rate of HIV-1 transcription and causes selective apoptosis of infected cells. In this way, the authors have identified a viral-induced change (loss of p21/Waf1), observed a crucial interaction between virus and host (Tat/cyclin E/CDK2), and targeted this interaction with a specific drug.

B. Nef: Altering the Cell Surface

A microarray study examining the effects of Nef on T-cell activation has identified a number of cellular factors upregulated in Nef-expressing cells. Simmons *et al.* (2001) created a Jurkat T-cell line that expresses Nef under the control of the doxycycline transactivator. The authors identified a series of upregulated genes associated with T-cell activation; NFκB, Jun-D, cFos, TFIID, cdk9, and various Pol II subunits. Additionally, the study identified several genes upregulated by Nef that have been shown to be upregulated in infected primary cells such as HSP70. Overall, these changes are indicative of an activated T cell in which viral replication will be increased. Interestingly, these Nef-mediated changes required the presence of several T-cell receptor subunits, suggesting that this transcriptional profile was the result of Nef altering cell membrane associated factors and tuning the cell for maximum activation. Nef-expressing cells were also treated with the immunosuppressive drug cyclosporin A, which inhibited expression of genes upregulated by Nef, suggesting a role of NFAT and NFκB in the Nef-mediated alteration of the cellular activation (Simmons *et al.*, 2001).

A different study identified a role for Nef in altering cholesterol synthesis in the cell. Indeed, HIV-1 infection is inherently linked to cholesterol metabolism such that the virus buds from cholesterol-rich lipid rafts on the cell surface, as well as evidence that prolonged HIV disease is associated with a systemic lypodystrophy (van't Wout *et al.*, 2005). van't Wout *et al.* (2005) showed that HIV-1 infection mediates the upregulation of a panopoly of enzymes associated with cholesterol biosynthesis, including the enzyme involved in the rate-limiting step of cholesterol biosynthesis, HMGCR. This increase in factors necessary for cholesterol biosynthesis was not present in cells infected with a virus lacking Nef. Additionally, increased cholesterol production was detected in cells infected with wild-type, but not δ-Nef, viruses. This lack of cholesterol production was associated with a decrease in the infectivity of progeny virus, likely due to the absence of sufficient cholesterol in the lipid rafts.

A study by Janardhan *et al.* (2004) elucidates a possible mechanism for Nef's ability to alter cell signaling and trigger T-cell activation. This group used a Jurkat cell line expressing an HA and Flag-tagged Nef to identify Nef-interacting proteins. Nef associated proteins were then identified by MS

analysis. The most interesting finding was the association of Nef with the DOCK2/ELMO1 complex, which is necessary to Rac-induced signaling. Supporting data showed that Nef expression in resting T cells activates Rac signaling, which appears to reduce the T-cell activation threshold. In agreement with previous Nef studies, this chapter identifies a role for Nef in altering the membrane compartment of the cell and encouraging T-cell activation.

Although no drug currently exists to block Nef-mediated effects, the above studies suggest that Nef may provide a valuable therapeutic target. Indeed, Nef is dispensable for replication in tissue culture but is also needed for pathogenesis in native infection. This suggests a role for Nef in mediating the disease phenotype beyond its direct interaction with the virus. In support of Nef as a drug target, several groups have targeted siRNA against Nef and show reduction in viral replication—a result that agrees with studies that have used Nef-deficient viruses in their proteomic and genomic screening. The use of cyclosporin A reveals that it may be possible to counteract Nef-mediated changes in the cell by targeting naturally occurring signal cascades.

Nef directly manipulates the extracellular surface of the host plasma membrane in order to mediate optimal viral conditions for infection, budding, and release. The protein composition of the surface and cytoplasmic faces of the membrane directly influence the efficiency and success of any potential HIV-1 infection. The identification and characterization of membrane-associated proteins, therefore, would provide an accurate library of cell signaling receptors and would accentuate the infection and budding processes of HIV-1. Unfortunately, the development of such a membrane proteome encounters numerous difficulties in isolating membrane proteins away from other cellular organelles as well as from the membrane itself while still remaining intact. A study by Berro *et al.* (2007) has successfully identified and characterized the membrane proteome of HIV-1-infected cells using a biotin-directed affinity purification method. This study additionally utilized the identification of surface markers through comparative proteomics (2DGE and MALDI-TOF) between ACH2 HIV-1 latently infected T cells and its parental uninfected cell line CEM, which allowed for the characterization of the presence of differentially expressed proteins between these two states. Seventeen proteins were found to be differentially expressed on the membrane of ACH2 cells regulating cellular and viral pathways including cell survival, differentiation, apoptosis, adhesion, and migration. For example, the receptor-associated proteins that were identified as differentially expressed in ACH2 cells include Bruton's tyrosine kinase and the X-linked inhibitor of apoptosis. Both of these proteins are involved in antiapoptotic pathways and were found exclusively in infected cells, therefore it was hypothesized that infected cells upregulate antiapoptotic mechanisms to counterbalance virus-induced apoptosis and

maintain cell survival. Bruton's tyrosine kinase is a protein tyrosine kinase which, on activation, is translocated to the membrane where it mediates apoptosis through the activation of members of the antiapoptotic machinery, through the PI3K pathway, or through the NFκB pathway (Bajpai *et al.*, 2000; Berro *et al.*, 2007; Islam and Smith, 2000; Petro *et al.*, 2000, 2002). X-linked inhibitor of apoptosis inhibits caspases 3, 7, and 9 suppressing apoptosis indicating that during infection cells are dependent on antiapoptotic pathways to counter the apoptotic function of HIV-1 accessory protein (Berro *et al.*, 2007; Liston *et al.*, 1996; Schimmer *et al.*, 2004). Therefore, unique cell surface proteins present on HIV-1-infected cells could be utilized in antiviral therapy against an infection that would preferentially target a pathway vital for viral replication, but be dispensable for normal cellular function.

C. Gag: Using Cellular Factors to Facilitate Budding

Discrete functional domains within retroviral Gag proteins typically direct the association with plasma membranes, Gag–Gag dimerization, and the budding of virus from membranes driving the assembly and release of retroviral particles (Goila-Gaur *et al.*, 2003). L domains of Gag appear to promote viral budding by interacting with cellular host factors through binding of specific sequence motifs (Goila-Gaur *et al.*, 2003). These L domains have also been shown to associate with the cellular ubiquitination and endosomal sorting machinery of the host cell (Sorin and Kalpana, 2006). The identification of cellular host proteins critically involved in the cellular endosomal pathway that function late in the assembly/release pathway during the budding of virions from the plasma membrane through a combination of biochemical, virological, molecular, and structural approaches has provided insight into the manner in which viruses have evolved to exploit this cellular machinery (Freed, 2003).

The p6 domain of HIV-1 Gag contains a highly conserved PTAP motif that confers HIV-1 L domain activity and is critical for virus release (Goila-Gaur *et al.*, 2003; Reeves and Piefer, 2005). Lysine residues on HIV-1 p6 are monoubiquitinated serving to facilitate budding by targeting defective Gag molecules for proteolytic degradation, by preventing interference with viral budding, or to create docking sites for cellular proteins participating in viral budding (Garrus *et al.*, 2001). Monoubiquitination of proteins is also often a signal for sorting proteins from either the biosynthetic or the endocytic pathways to the multivesicular body and the lysosomes (Freed, 2003). As such, the human Tsg101, a ubiquitin conjugating E2 enzyme variant protein, binds to HIV-1 p6, shown through Y2H studies, indicating that a specific component of the cellular trafficking machinery is involved in retrovirus release (VerPlank *et al.*, 2001). Tsg101 is involved in regulation of intracellular trafficking, transcriptional regulation, and cell

cycle control and plays an essential role in forming and sorting protein cargo into the multivesicular body /late endosome (Freed, 2003; VerPlank *et al.*, 2001). Identification of a cleaved fragment of Tsg101 from a μRPLC-MS/MS analysis was published by Chertova *et al.* (2006). A proteomic analysis was performed to identify any and all cellular host proteins, which become incorporated into a budding endosomal virion (Chertova *et al.*, 2006). Serving as a method of both Tsg101 verification and proteomic analysis validation, the presence of Tsg101 in an endosomal virion confers confidence. Depleting Tsg101 or inhibiting endosomal trafficking also prevents HIV-1 release at a late stage, providing a direct correlation between lack of binding and function (Garrus *et al.*, 2001). Complementary siRNA studies have been performed to demonstrate the critical role of Tsg101 in the process of HIV-1 budding (Elbashir *et al.*, 2001; Garrus *et al.*, 2001; Tang, 2002). Garrus *et al.* (2001) designed a siRNA duplex homologous to a region of the Tsg101 coding sequence causing depletion of endogenous Tsg101 to undetectable levels. Depletion of Tsg101 using this method significantly reduced the release of virion-associated matrix and capsid proteins and reduced viral infectivity (Garrus *et al.*, 2001). An exogenous siRNA-resistant protein expression construct was introduced in Tsg101-depleted cells order and was shown to rescue viral release and infectivity (Garrus *et al.*, 2001; Tang, 2002). This assay validated the importance of the p6–Tsg101 interaction in HIV replication and due to no significant alteration of growth characteristics in Tsg101-depleted cells, it also demonstrated the importance of a potential target for novel antiviral agents (Tang, 2002). Unfortunately, any existing anti-budding treatments involving Tsg101 reduce viral infectivity significantly *in vitro*; however, encounter problems with cellular toxicity *in vivo* (Reeves and Piefer, 2005).

VI. Other Approaches

In addition to the typical proteomic or genomic approaches outlined above, which provide methods for broadly screening interactions, changes in transcription, and alterations in cellular proteins, the tools of the "omics" age may be utilized in other ways. These include examinations of the proteome of the virion, the identification of biomarkers, and the use of siRNA in treatment and screening.

A. Virion Proteome

Although standard proteomic approaches have been applied to monitor changes in the protein expression and localization in infected cells, these techniques may also be used to examine the content of the virion. As the virion buds from the cell, it incorporates cellular proteins from the plasma membrane and cytoplasm of the cell. These proteins may be incorporated at

random, based on the composition of the membrane and cytoplasm or they may be preferentially incorporated into the virion through associations with viral proteins. These proteins may be important to the viral life cycle and provide a target for future therapies.

Two notable studies examined the content of the virion using proteomic techniques (Chertova *et al.*, 2006; Saphire *et al.*, 2006). Both studies purified virion from infected cells and then identified virion-associated proteins by MS analysis. Chertova *et al.* (2006) evaluated the content of virions that bud internally in macrophages. In support of the validity of this method, they identified Tsg101 and actin-interacting protein 1, two cellular proteins whose interaction with Gag is necessary for virion budding. In this way, proteomic analysis of the virion may reveal the presence of proteins preferentially packaged during budding, which may be critical to viral budding, fusion, or integration.

The study by Saphire *et al.* (2006) examines the use of LC/tandem MS in identifying proteins associated with the virion. In validation of their technique, they successfully identified CyPA as a virion-associated protein. In addition, they identified CD48, a cellular complement determining factor, in the virion. This suggests a possible role for CD48 in avoiding complement mediated lysis. Interestingly, both of these groups identified histones within the virion. Although this might be due to cosedimentation of RNA-associated histones with the virion, this finding leads to the intriguing possibility that histones may be packaged with the virus. These studies outline the validity of using proteomic approaches in identifying virion components, but do not pursue the importance of their findings. However, future work in identifying proteins specifically incorporated into the virion may elucidate a new sphere of possible drug targets.

B. Biomarkers

A biomarker is a substance used to identify a particular biological state, especially a disease state such as cancer or infection. Although plasma viral load and antibody titer serve as efficient diagnostic markers in screening for infected individuals, there may be some value to identifying biomarkers associated with HIV-1 disease.

Small subsets of infected individuals, called long-term nonprogressors (LTNPs), naturally repress the virus (Guadalupe *et al.*, 2003; Paroli *et al.*, 2001; Valdez *et al.*, 2002). In these patients viral load becomes undetectable and CD4 T-cell counts are stable for 10 years or more. It is not currently well understood what mechanisms control viral replication in these individuals. Comparison of samples from LTNPs versus viremic patients may yield the discovery of biomarkers associated with nonprogression. Indeed, Sankaran *et al.* (2005) examined the gene expression profile of gut mucosal T cells in LTNPs as compared to patients with high viral load. They determined that

nonprogression was associated with a state of immune inactivation, which could potentially prevent the spread of virus. LTNPs represent an interesting subset of infected individuals. These patients are capable of living with the disease, in the absence of therapy, for long periods of time. Understanding the mechanisms and markers associated with LTNPs may yield clues as to how HIV-1 virulence is mediated. Studies such as these may help direct the focus of future studies concerned with controlling HIV-1 disease after infection.

A case study by Andersson *et al.* (2006) examines the value of using biomarkers to monitor the disease state in HIV-1-infected patients. They show that protein levels in the cerebrospinal fluid of infected patients can be used as a surrogate marker for HIV-1 associated dementia. Specifically, they show that treatment with highly active antiretroviral therapy reduces the levels of cerebrospinal fluid-associated NFL, Tau, and GFAP proteins, which have been previously associated with ongoing neural damage. Although studies such as these cannot be easily linked to therapy, biomarkers can be used to monitor the effectiveness of treatment and possibly provide clues to the mechanisms of disease pathogenesis.

C. RNA Interference

RNA interference (RNAi) is a recently discovered cellular pathway wherein short sequences of RNA can target genes for silencing through transcriptional repression, RNA cleavage, or translation inhibition (Agrawal *et al.*, 2003; Bartel, 2004; Hannon, 2002; Zamore and Haley, 2005). Study of the mechanisms of RNAi has made it possible to design siRNAs that will specifically silence a targeted gene. This discovery makes it possible to quickly develop siRNAs to alter the expression of a target gene. With this in mind, RNAi can be used to target the virus, cellular genes involved in viral replication, or target cellular genes to screen for involvement in RNAi.

A broad selection of studies have examined the efficacy of siRNA in treating HIV infection (Chang *et al.*, 2005; Colbere-Garapin *et al.*, 2005; Jacque *et al.*, 2002; Morris and Rossi, 2006b; Provost *et al.*, 2006; Suzuki *et al.*, 2005; Westerhout *et al.*, 2005). siRNA targeted against various sequences within the HIV genome have shown to be effective in suppressing viral replication (Jacque *et al.*, 2002; Morris and Rossi, 2006a,b). However, the high mutation rate of the virus allows the production of variants that are resistant to the administered siRNA. One strategy to overcome this is delivering siRNAs targeted to the wild-type sequence as well as the likely escape mutations (ter Brake and Berkhout, 2005). Unlike standard pharmaceuticals, the sequence specificity makes it possible to design siRNA to target any desired sequence. ter Brake *et al.* demonstrate that siRNA targeted against likely escape mutants can be effective. Various methods are being developed to allow delivery of siRNA to the appropriate target cell ranging

from lentiviral delivery to antibody-mediated targeting of the siRNA (Landen *et al.*, 2006; Morris and Rossi, 2006a; Oliveira *et al.*, 2006; Song *et al.*, 2005; Veldhoen *et al.*, 2006).

One unique approach to the problem of siRNA delivery is the use of lentiviral-mediated delivery of siRNA-expressing vectors into stem cells (Anderson *et al.*, 2006; Li *et al.*, 2005; Morris and Rossi, 2006a,b). Integration of an siRNA-expressing vector into a cell insures a stable and long-term suppression of a target gene. Combined with the ability of hematopoietic stem cells to develop into all of the HIV-1 target cells, it now becomes possible to engineer T cells and macrophages that carry specific siRNA. These vectors could be designed to target HIV-1 viral RNA, or downregulate expression of the appropriate coreceptor, thus preventing infection. These approaches may prove especially useful when applied to research in animals, especially the newly developed mouse model. In this new small animal model for HIV-1 infection, immune-deficient mice (Rag and common γ-chain knockout) are irradiated and then implanted with human $CD34^+$ hematopoietic stem cells (Baenziger *et al.*, 2006; Berges *et al.*, 2006; Watanabe *et al.*, 2006; Zhang *et al.*, 2006). Our laboratory has shown that these mice develop a human immune system, are capable of being infected with HIV-1, and have further shown the validity of using this model in evaluating drugs and small peptide therapeutics (unpublished data). This model could also be adapted to use stem cells carrying an anti-HIV siRNA or other siRNA of interest and be used to screen potential drugs and viral–cellular interactions.

The development of siRNA libraries allows for the rapid identification of genes important to HIV-1 infection (Nguyen *et al.*, 2006). Treatment of HIV-1 infected cells with a library of siRNA covering all known human genes, followed by screening for viral production, allows genes that influence HIV-1 infection to be identified. Works by several groups have shown these studies to be effective and have identified several genes involved in viral replication, including the group I PAK kinases; PAK1, PAK2, and PAK3 (Nguyen *et al.*, 2006). This provides a system wherein viral contact with cellular pathways can be identified without screening for interactions. Additionally, this approach allows the identification of genes that influence the virus but are not themselves differentially regulated by the virus.

VII. Conclusion

The disciplines of genomics and proteomics afford us a new way of examining HIV-1 infection, its interaction with cellular proteins, the changes the virus induces in the host cell, and the outcome of the course of the disease. By using a systems approach, in which information from

genomic, proteomic, and phenotypic studies is combined, new and more effective drug targets can be elucidated. Although extensive work has gone into identifying the interactions of viral proteins with the host cell, we are only now beginning to understand what these interactions entail and how they relate to specific stages of the viral life cycle. The work thus far performed on Tat, Nef, and Gag outlines how to apply an "omics" approach to understanding viral changes in the host cell network and how to counteract these changes using therapeutics. Future work involving rigorous systems biology approaches will undoubtedly uncover new targets for treatment of HIV/AIDS.

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