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Effects of p21 on HIV transcription

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EFFECT OF P21 ON HIV TRANSCRIPTION

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ABSTRACT

HIV represents a global health problem. The phenomenon of HIV latency presents challenges in treating and curing HIV infection. Understanding the mechanisms behind HIV latency may provide the route to cure HIV. In a subset of elite controllers, elevated levels of p21 provide resistance to HIV replication by inhibiting transcription. I attempted to understand the mechanisms by overexpressing p21 in HEK 293T cells.

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INTRODUCTION

Scope and Scale of HIV Infection

Since its discovery in the late 1970s, the human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) have presented a serious and growing medical and social problem. The HIV virus infects cells of the immune system, and most importantly CD4+ T-cells. Though initial infection presents as flu-like symptoms that soon resolve in a matter of weeks, with a significant drop in the amount of virus present in the blood, the virus continues to infect and kill CD4+ T-cells. This period between the end of the initial flu-like symptoms and the development of AIDS is known as clinical latency, and without treatment, it can last for as long as decade. Eventually, the fall in CD4+ T-cell count leads to immune dysfunction and the increased susceptibility to infection that is characteristic of AIDS. Once HIV infection has progressed to AIDS, life expectancy drops to three years. If a deadly opportunistic infection takes hold, this drops to one year without treatment [1].

Just as important as the seriousness of the disease is how widespread HIV infection has become. In 2014, 36.9 million people were infected with HIV, with 2.1 million new infections in 2013 and 1.5 million deaths. The burden of the disease is especially apparent in sub-Saharan Africa, with approximately 25.8 million people infected in 2014, which despite reductions still has the highest rate of new infections and deaths due to HIV. Worldwide, many of those previously and newly infected with HIV live in low-income nations with poor access to health care, including southeast Asia, which also saw a rise in the number of new infections. It is estimated that 51% of all

people infected worldwide do not know that they are HIV positive, and only 40% are on ART, despite the significant increases in lifespan and quality of life it offers. While considerable gains have been made in reducing the rate at which HIV infection spreads, there is still a great deal of work to be done in educating people about HIV, improving access to ART, and working to improve our understanding of HIV, especially in creating a vaccine for HIV, treating, and even curing the disease [2]. To this date, there is no cure for HIV infection, and individuals must remain on anti-retroviral therapy (ART) for the rest of their lives to prevent progression of the disease, presenting a significant burden on health care systems and difficulties in maintaining continuity of treatment.

HIV Lifecycle

HIV is a retrovirus that primarily infects CD4+ T-cells and macrophages, and successful entry, replication, and release of mature viral particles depends on a wide variety of host and viral factors. HIV enters cells by binding receptors present on the surface of cells. Most important in infecting CD4+ T-cells, HIV's primary target, is the CD4 or T-cell receptor, a multi-subunit transmembrane protein that normally interacts with major histocompatibility complex II on antigen presenting cells. This receptor interacts with a glycoprotein envelope spike known as Env, which is composed of the surface glycoprotein gp120 and the transmembrane glycoprotein gp41. The gp120 domain mediates the interaction with the CD4 receptor, which then bends, allowing the env protein to interact with chemokine receptors on the surface of the cell. Depending on the strain of HIV, this is either the CCR5 or the CXCR4 receptor [3].

This interaction brings the virus in close proximity with the plasma membrane of

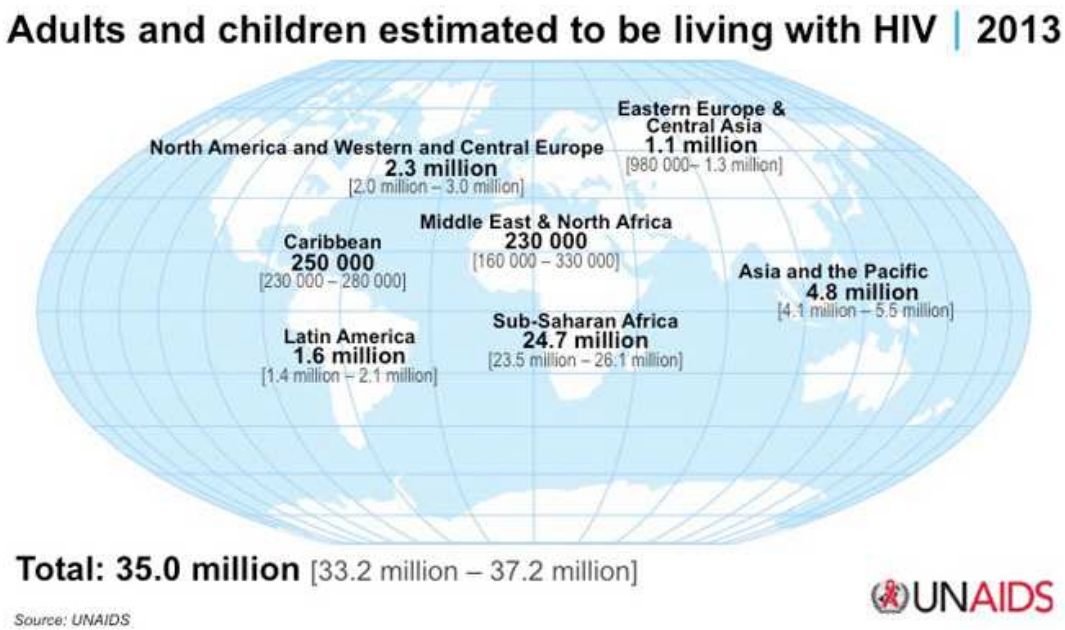

Figure 1HIV prevalence worldwide (Source: UNAIDS)

the cell, where gp120 and gp41 undergo conformational changes. This permits fusion of the virus's envelope with the cell membrane, releasing the viral capsule into the cytoplasm [3]. The presence of these receptors on other cell types allows HIV to infect them as well, in particular macrophages [4].

Following fusion and release of the viral capsule into the cytoplasm, the capsid proteins that compose the capsule uncoat, releasing the two single-stranded RNA molecules that compose the virus's genome into the cell. The viral proteins reverse transcriptase and integrase are also released into the cytoplasm. Reverse transcriptase begins reverse transcribing the RNA strands, using them as a template to form a minus sense DNA strand. The RNA template strand is then degraded, its nucleotides used to form the primer for synthesis of the positive sense DNA strand. The viral capsid proteins play an important role in the reverse transcription process, as evidenced by mutations in the protein that result in impaired reverse transcription. However, the process by which the viral capsule uncoats and forms first the reverse transcription complex, then the preintegration complex are presently poorly understood [3]. The completed, double stranded DNA reverse transcript is imported into the nucleus, where it is spliced into the host genome via integrase [4]. While integrase is capable of integrating the viral reverse transcript into the host genome on its own, there are a number of factors that can enhance its function. For example, the host protein LEDGF/p75 appears to play a role in localizing integrase to the nucleus, and if overexpressed, the integrase binding domain of the protein can inhibit HIV infection, showing that LEDGF/p75 can have significant effects on HIV infection [3].

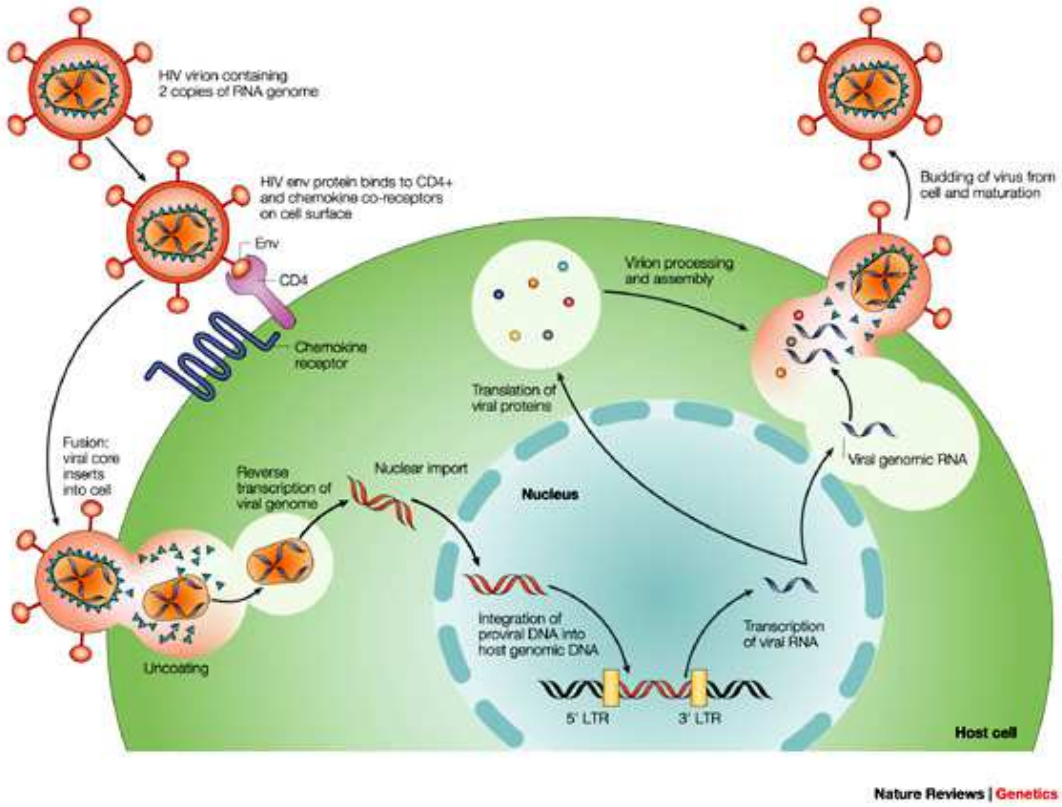

Figure 2 A summary of the HIV lifecycle and various inhibitors (Source: Laskey & Siliciano, Nature Reviews Microbiology 12, 772-780)

Once integrated, the HIV provirus is transcribed, a process reliant on a combination of host factors and viral products. One of the important early products of viral transcription and translation is the protein Tat, which acts to further promote viral transcription by binding to the TAR loop of the RNA transcript. This recruits transcription factors, including P-TEF-b, to the long terminal repeat (LTR) region of the provirus, promoting transcription [5]. A number of host cell factors influence HIV transcription, and these can vary between cell type. In particular, the CD4+ T-cell subtypes T_H1 and T_H2 differ in their expression of the proteins T-bet and GATA-3. These are key regulatory factors of the differentiation of T_H1 and T_H2 cells, with T-bet promoting the T_H1 phenotype and GATA-3 the T_H2 phenotype. While T-bet has not been shown to directly influence HIV transcription, it antagonizes the effects of GATA-3, which has an affinity for the LTR segment of the HIV provirus and promotes transcription. In addition, T_H2 have higher levels of c-Maf, another promoter of T_H2 differentiation and, like GATA-3, it binds to the LTR, promoting HIV transcription in cooperation with NF-κB and Nuclear Factor of Activated T-cells (NFAT) [3].

Other major products of viral translation include Rev, Gag, Gag-Pol, and Env. Rev promotes the export of unspliced viral transcripts from the nucleus by binding to the Rev-responsive element of the RNA transcript. Gag and Gag-Pol are polyproteins that contain a number of important viral products important for viral particle assembly and maturation. Gag coordinates the assembly of viral particles at the cell membrane, including its protein components and two unspliced viral RNA transcripts. Gag is also cleaved to form the capsid, nucleocapsid, matrix, and p6 proteins. 2400-5000 molecules

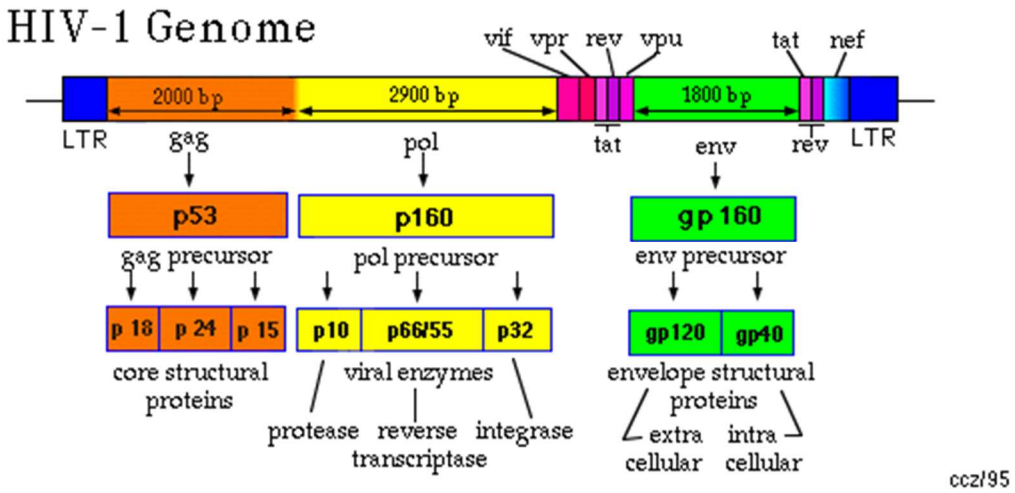

Figure 3 Components of the HIV genome and protein products (Source: http://www.yale.edu/bio243/HIV/genome.html)

of Gag form the immature viral capsule, which are then leaved to capsid, which forms the viral capsule in mature viral particles. Nucleocapsid, meanwhile associates with the RNA genome via zinc finger domains and is essential for viral encapsidation and infectivity. The matrix domain of Gag is essential for correctly localizing Gag to the cell membrane, without which release of viral particles is significantly impaired. Matrix also plays a key role in correct assembly of the virus, as it recruits Gag and Env glycoproteins to the inner leaflet of the cell membrane [3].

Gag-Pol contains many important viral proteins, most notably the HIV protease, which cleaves Gag and Gag-Pol following viral particle release to produce mature particles. Gag-Pol also releases reverse transcriptase and integrase. Env produces the gp160 protein, which is cleaved during Golgi trafficking to gp120 and gp41, and allows the virus to bind to its target receptors, a necessary step for infection. Once the viral proteins, viral RNA, and a number of host proteins are assembled at the cell membrane, the p6 domain of Gag hijacks the host cell's endosomal sorting machinery to facilitate budding and release of the viral particle from the cell. viral infectivity factor (vif), which greatly increases the infectivity of the viral particles by inhibiting APOBEC3G, protecting the viral RNA genome from suffering hypermutation. Several other viral proteins include viral protein U (vpU), which stimulates release of budded viral particles from the cell surface and proteasomal degradation of CD4, negative factor (Nef), which downregulates host expression of CD4 and major histocompatibility complex (MHC) I, and viral protein R (vpr), which induces cell-cycle arrest [3].

During budding and viral particle release, viral protease cleaves the Gag and Gag-

Pol proteins into their component domains, releasing capsid, nucleocapsid, matrix, and p6, allowing the particle to mature. Concurrently with release, the virus takes a portion of the host cell's membrane that has been coated with the Env glycoproteins with it, forming the viral envelope. Once released and mature, the HIV particle is then able to infect new cells, repeating the process [3].

Anti-retroviral Therapies and Restriction Factors of HIV

Anti-retroviral therapies control HIV by targeting important steps in its life cycle. Reverse transcriptase inhibitors block the conversion of viral RNA to DNA. Integrase inhibitors prevent the reverse transcribed DNA from being integrated into the host genome, while protease inhibitors stop the cleavage of viral proteins and maturation into an infectious viral particle. Most anti-retroviral therapies use two nucleoside reverse transcriptase inhibitors in combination with a non-nucleoside reverse transcriptase inhibitor, a protease inhibitor, or an integrase inhibitor [4]. Entry inhibitors like T20 and maraviroc act by targeting gp41 and acting as an antagonist to CCR5 respectively, preventing the virus from successfully fusing with the target cell [3].

Potential targets for future therapies include various restriction factors in host cells and the viral proteins that have evolved to counter them. Restriction factors are endogenous proteins that work to inhibit HIV infection and replication, acting on a number of steps in the life cycle and by various mechanisms. APOBEC3 DNA deaminases catalyze the conversion of cytosine to uracil in DNA, leading to hypermutation, especially guanine to adenine mutations, and defects in progeny viruses. APOBEC3G is also capable of incorporating into the viral capsule, allowing it to act in

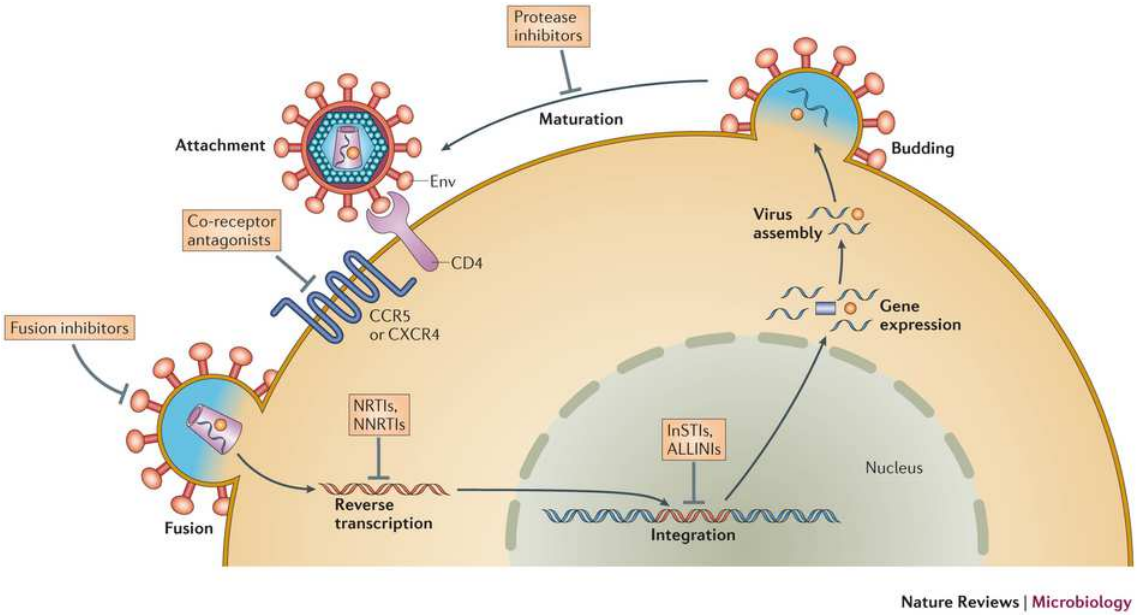

Figure 4 Schematic overview of various mechanisms of ATR drugs (Source:

the virus and in subsequently infected cells. The viral protein Vif counteracts the action of APOBEC3G, reducing its activity and permitting viral replication. However, Vif does not completely inhibit APOBEC3G, and the mutation it induces contributes to HIV's ability to evade the immune system by mutating its surface antigens. Drugs that act on APOCBEC3G or interfere with the Vif-APOCBEC3G could serve as treatments by increasing APOCBEC3G activity to the point where it interferes with viral replication. Alternatively, they could restrict APOBEC3G activity further, removing the genetic diversity it needs for successful immune evasion. [6]

Another restriction factor, tetherin, acts by interfering with the release of mature viral particles. Tetherin is a transmembrane protein with cytoplasmic and extracellular domains, with a glycosylphosphatidylinositol anchor at the end of the extracellular domain. As viral particles attempt to bud, the anchor is incorporated into the viral envelope, physically tying it to the cell membrane. Captured viral particles are then digested in lysosomes. The viral protein Vpu interferes with the ability of tetherin to interact with budding viral particles, directly inhibiting its activity. Vpu then recruits host cell factors that either sequester tetherin within the cell or target it for degradation. Vpu also has the effect of downregulating tetherin expression, further reducing its ability to prevent viral release. Certain strains of HIV-2 also have Vpu-like activity in their Env glycoproteins. Env does not lead to degradation of tetherin, but it does lead to sequestration of tetherin within intracellular vesicles. Drugs that counteract Vpu activity could interfere with viral replication, but as Vpu-deficient HIV is still able to replicate, their efficacy may be limited [6].

A third restriction factor, expressed primarily in macrophages and dendritic cells, is SAMHD1, a dNTP hydrolase. SAMHD1 is believed to interfere with viral replication by depleting intracellular reserves of deoxynucleoside triphosphates, starving reverse transcriptase of the material it needs to make the DNA reverse transcript of the viral genome. HIV-1 lacks a countermeasure to this, leading to the resistance of macrophages and dendritic cells to HIV-1 infection. However, HIV-2 possesses a protein called Vpx, which targets SAMHD1 for degradation and facilitates viral reverse transcription. It is thought that HIV-2's less pathogenic nature may be due to increasing myeloid cell infection, which in turn reduces immune activation and CD4+ T-cell turnover. If so, inhibitors of SAMHD1 may prove to be a viable therapy [6].

TRIM5, a member of a class of proteins with a common tripartite motif, is associated with antiviral activity in some of its isoforms. In particular, TRIM5α provides resistance against a variety of retroviruses and lentiviruses, but provides only weak protection against HIV-1. TRIM5α acts by binding to viral capsid proteins and inducing premature disassembly of the viral capsule before reverse transcription can occur. However, the exact mechanism remains unclear. There have been a number of studies on human TRIM5α variations, but the results have been mixed or unclear. Still, there is considerable interest in TRIM5α's potential as an antiviral treatment target. As it acts at a post-entry but pre-integration step, it can prevent HIV from integrating into the host genome, potentially allowing for a more effective treatment [7].

Current ART is highly successful, greatly increasing survival and slowing disease progression. After treatment with ART, the number of viral copies in blood plasma drops

below the clinical level of detection of 50 copies / mL, allowing the immune system to rebuild [8]. The use of multiple drugs in combination for ART is required to avoid drug resistance developing. Since HIV mutates so rapidly, it can readily develop resistance to any one drug, but the combination of drugs administered together can disrupt its ability to replicate before it develops effective resistance. Still, drug resistance remains a major problem for long term ART. Additionally, drug toxicity is a serious concern, as is patient adherence. Given the need for lifelong treatment, resistance, toxicity, and adherence are significant obstacles [3].

Unfortunately, ART also does not completely prevent progression to AIDS, and while it does slow the disease, maintaining continuity of care is very important. Any interruption in treatment can lead to rapid HIV rebound, bringing viral load up to pre-treatment levels. This problem is particularly important in sub-Saharan Africa, where access to treatment is not readily available and ensuring compliance is difficult. In addition, ART cannot cure the disease, necessitating life time therapy and compounding the problems of side effects, compliance, and drug resistance [4]. The development of an effective cure for HIV is therefore an important task for further research.

While ART allows much of the actively replicating virus to be cleared, a significant population of quiescent virus remains, and since ART is specific for disrupting the replication of HIV, it has no effect on this quiescent population. When ART is withdrawn, the latent HIV population transitions to an actively replicating state, leading to a sharp rise in viral load and to disease progression [4].

HIV Latency

The latent reservoir is defined as the population of cells that have the HIV provirus incorporated into their DNA, but are not actively transcribing the virus. Even in patients on ART with undetectably low viral loads (less than 50 per mL), replication competent virus can be isolated, demonstrating the presence of latently infected cells [9]. Additionally, peripheral blood mononuclear cells show low but stable levels of integrated HIV DNA in their genome [10]. Clinically, the most important features of the latent reservoir are its resistance to current therapies and its longevity. As mentioned, ART specifically interferes with the steps of HIV replication, and has little effect on this latent population. If this population were cleared quickly, this would present no difficulty, but cells can spend long periods in the latent state, with half-life of 44 months or longer. This provides a population of cells that can readily replenish the actively replicating population, resulting in disease rebound. [11] In addition, even if treatment begins promptly after infection, the latent reservoir establishes itself quickly, necessitating life-long treatment to control the disease [8].

This latently infected population of cells includes quiescent memory T cells, macrophages, and potentially hematopoietic stem cells. CD4+ memory T-cells may represent a significant part of the cellular reservoir, as activated T-cells can become infected with HIV, then return to a quiescent memory state. Since memory T-cells do not produce much virus while quiescent, they are both resistant to current treatments and provide a long-lived reservoir for HIV [11]. Quiescent T-cells are resistant to HIV infection due to low pools of available nucleotides, inhibiting HIV reverse transcription,

and the presence of the RNA-editing enzyme APOBEC3G, which causes hypermutation during reverse transcription. However, T-cells that were infected while active can return to a quiescent state, which inhibits HIV expression but also prevents current anti-retroviral drugs from acting on the virus, which rely on disrupting the steps of active replication [8]. Other T-cell populations express factors that restrict transcription of HIV, such as Bcl-11b, a transcriptional repressor that inhibits HIV transcription by recruiting HDAC1, HDAC2, and SUV39H1 to deacetylate and methylate the Nuc-1 histone in the LTR region. Additionally, it facilitates binding of heterochromatin protein 1, which promotes formation of heterochromatin at the LTR region. Bcl-11b also inhibits the activity of Tat by binding to it and redirecting it to heterochromatic region. However, whether this factor contributes to latency is not yet known [3].

Another population of T-cells that contributes to the latent reservoir are T-memory stem cells. Compared to other T-memory cell populations, T-memory stem cells show higher HIV-DNA per cell, with sequences that are conserved even after four to eight years of ART treatment. T-memory stem cells may therefore contribute significantly to both the latent reservoir and persistence of HIV infection [12].

The physiological composition of the latent reservoir remains unclear, however, but is believed to include anatomical reservoirs such as lymph nodes and other lymphatic tissues. Lymph nodes provide conditions that facilitate HIV replication, given the large number of susceptible cells present in them and the amount of direct cell-cell contact that occurs as part of normal immune surveillance and activation. In particular, lymphatic tissue associated with gut epithelia may contribute significantly to reservoir, as CD4+ T-

cells are extensively depleted in the first weeks after infection. Those that remain revert to a resting state, and persist even after ART. In contrast to the T-cell populations in the blood, most viral DNA and RNA detected in gut lymphatic tissue appear to be in effector memory T-cells rather than central memory and naïve T-cells. This shows that conditions in different anatomical compartments may shape the dynamics of HIV infection significantly. The exact role of gut lymphatic tissue as an HIV compartment is still a matter of debate. However, it does show signs of exchange with the blood compartment, and the gut may not be an isolated compartment of infection. The presence of HIV in the gut also complicates treatment, as drug penetration and metabolism varies significantly along the length of the digestive tract [12].

The latent reservoir may also include privileged compartments such as the central nervous system, where HIV infection is known to occur and is difficult to treat due to the blood-brain barrier [8]. The presence of HIV in the central nervous system is of great concern clinically, as AIDS-related neurocognitive disorders remain a common complication of chronic HIV infection. The important population of infected cells in the CNS consists primarily of macrophages. The four main varieties present are meningeal macrophages, macrophages of the choroid plexus, perivascular macrophages, and microglial macrophages. Perivascular and microglial macrophages are also significant contributers to neurological damage in HIV infection by maintaining an inflammatory state and releasing metalloproteinases and reactive oxygen species. Astrocytes are another cell population in the CNS capable of sustaining low-level HIV replication, contributing to the reservoir of virus present there. HIV is generally compartmentalized

in the CNS, with infected cells generally remaining within it. However, a high viral load in the CNS contributes to neurocognitive symptoms, potentially progressing to HIV-associated dementia [12].

Determining the size of the reservoir is a significant challenge in current research. Two means of potentially measuring it are polymerase-chain reaction based (PCR) methods and the viral outgrowth assay (VOA). The VOA has been the gold standard in research, and works by measuring the amount of virus produced after CD4+ T-cells have been exposed to phytohemaglutinin, a potent activator of CD4+ T-cells that works in an antigen-independent manner. Later, PCR methods were developed, and while they directly measure the amount of proviral DNA present, their results correlate poorly with those of VOA experiments. One apparent reason for the discrepancy is that PCR methods detect both replication-competent and non-induced viruses, while VOA only detects replication-competent virus. In addition, some of the non-induced proviruses are still replication competent and could be reactivated in further rounds, complicating the measurements of the inducible reservoir. [11] This disagreement contributes to the difficulty in measuring the size of the reservoir, which in turn makes determining the composition of the latent reservoir more difficult.

Mechanisms of HIV Latency

The exact mechanism by which HIV enters and leaves the latent state is unclear, and there are several mechanisms that might contribute to inhibition of transcription and latency. Understanding the molecular details of these mechanisms could provide targets for therapies that reactivate the latent reservoir, which in turn could allow for the

elimination of the latent reservoir and a cure for HIV infection.

While the most important mechanisms that establish latency in patients have yet to be determined, transcription of the HIV provirus is a rate-limiting step in viral replication. Availability of host factors such as Ap1, Sp1, and NF-κB is required for effective transcription. The p65/p50 heterodimer commonly referred to as NF-κB has been shown to be a key activator of HIV transcription. When the host cell is activated, NF-κB is translocated to the nucleus, where it promotes transcription of HIV by binding to sites in the LTR region of the HIV provirus [13] Additionally, NF- κB displaces histone deacetylase (HDAC) enzymes, contributing to an open, transcriptionally permissive chromatin state [8]. Conversely, interaction with IκB can sequester NF-κB in the cytoplasm, limiting the amount of active NF-κB in the nucleus and HIV provirus transcription. This inhibition is reinforced by p50-p50 homodimers recruiting histone deacetylase complexes, converting chromatin to a repressed state. Other inducible transcription factors that play a role in transactivating the HIV LTR region include C/EBP, NFAT, Ets/PU.1, and TCF/LEF-1. Inhibitory factors include LSF-1, YY-1, c-Myc, CTIP-2/Bcll11b, CBF-1, FBI-1 and ligand-activated nuclear receptors [13].

The viral protein Tat also plays an important role in transcription by overcoming a transcriptional block. Without Tat, viral transcription is initiated, but stalls around the +59 position, where NELF and DSIF inhibit further transcription. However, this is long enough for the stem-and-loop structure of the trans-activation-responsive region (TAR) to form, which binds Tat. Tat then recruits the host factor P-TEF-b, which in turn phosphorylates NELF, releasing its transcriptional block. P-TEF-b also phosphorylates

DSIF, converting it into a positive transcription factor. RNA polymerase II is phosphorylated at several sites as well, increasing its processivity and thereby increasing transcription. Recent evidence also shows that Tat recruits other factors like PCAF, which acetylates Tat and enhances its ability to recruit P-TEF-b. Without Tat, HIV shows much reduced transcription, and the absence of P-TEF-b in quiescent T-cells contributes to their low virus production [8].

For HIV to be actively transcribed, the site of integration must be permissive for transcription, which in turn is dependent on the organization of the chromatin that surrounds it. Histones are structural proteins that contribute significantly to this organization, with positively-charged, lysine-rich tails that associate directly with DNA. The degree of acetylation on these tails controls how closely they associate with DNA, with enzymes such as HATs and HDACs mediate histone acetylation and deacetylation, respectively. Acetylated histones cause chromatin to assume a more open conformation, permitting greater transcription. Conversely, deacetylation leads to a more closed conformation, which prevents key factors from binding and/or restricting the processiveness of RNA polymerase II. The HIV LTR segment has two nucleosomes closely associated with it: nuc-0, which is positioned at the 5' end of the LTR, and nuc-1, which is positioned closely to the transcription start site. Tat, in addition to recruiting P-TEFb to the LTR, facilitates the assembly of HAT complexes at the LTR, promoting an open conformation. The transcription factors NF-κB, NFAT, and C/BEPβ also recruit histone acetyltransferases, promoting a transcription-permissible state. Countering the effect of these factors are SWI/SNF complexes and methyltransferases. The BAF variant

of SWI/SNF contributes to the establishment and maintenance of HIV latency. The methyltransferases Suv39H1, Zeste 2, and heterochromatin protein 1 are implicated in mediating the deacetylation and trimethylation of nuc-1, contributing to HIV repression [13].

The importance of histones in chromatin organization have led to HDAC inhibitors being proposed as a potential means to eliminate the latent reservoir. In particular, the HIV provirus has a histone just downstream of the transcription start site, potentially interfering with transcription. Promoting acetylation of this histone could therefore promote HIV transcription. HDAC inhibitors have been shown to promote HIV transcription in vitro, but have to date failed to show significant reductions in the latent reservoir, and remain an area of active research. [14]

A third mechanism that can inhibit HIV transcription is transcriptional interference, which is defined as the suppression of one transcription unit by another neighboring cis-element. Transcriptional interference can occur when the HIV provirus has integrated into an actively transcribed gene, which happens with a large fraction of non-induced proviruses [13]. As the host genes are transcribed at moderate levels, simple availability of transcription factors and accessibility of the transcription site cannot be factors in silencing HIV [15]. The endogenous host gene may compete with HIV for transcription factors, reducing the rate at which it is transcribed. Alternatively, the RNA polymerase complexes transcribing the gene may collide with those transcribing HIV, interrupting transcription and contributing to HIV silencing [13].

Once transcription has been initiated, the next major checkpoint is elongation.

RNAP II associated protein, Gdown1, competes with TFIIF for RNA polymerase II, promoting assembly of a paused RNA polymerase complex, thereby inhibiting transcription. Supporting evidence for the importance of RNA polymerase pausing in controlling transcription comes from genome-wide mapping across multiple species, where 20-30% of genes have enriched RNAP II density at the 5' end of the gene, regardless of whether the gene was or was not actively transcribed. Among the factors needed to promote efficient elongation is P-TEF-b, which alleviates the elongation block by phosphorylating elements of the inhibited transcription complex. Nelf and DRB sensitivity-inducing factor are among these elements. When not phosphorylated, they inhibit RNA elongation, possibly by inhibiting extrusion of the transcript from the transcription complex. The importance of P-TEF-b is underscored by the function of the viral protein Tat, which recruits P-TEF-b to the paused transcription complex as its primary function. The Tat-P-TEF-b complex then phosphorylates RNA polymerase II, Nelf, and DSIR, inducing RNA polymerase II transcription and elongation Nelf is an important inhibitor of HIV transcription, as it is capable of binding to the TAR segment of the HIV transcript and inhibiting Tat transactivation of the HIV provirus. Additionally, if Nelf's concentration is depleted, histone acetylation and displacement of nucleosomes occurs, suggesting a link between transcription elongation and chromatin remodeling [13].

Elite Control of HIV Infection

Regulation of transcription is important in a group of HIV-positive patients known as elite controllers or long term nonprogressors, along with other mechanisms.

Clinically, elite controllers are those patients who maintain a high T cell count despite having detectable viral load. Specifically, elite controllers are defined as having fewer than 50 copies/mL of virus while not receiving antiretroviral treatment for at least one year. These patients represent a heterogeneous population, with a variety of mechanisms that could have a role in inhibiting progression of HIV to AIDS [16].

Some of these patients may be infected with a defective strain of HIV that is less infectious or replication-deficient [3]. Mutations or deletions in nef or the LTR region of the HIV genome can impair HIV replication, though there is evidence that infection with such crippled viruses can still lead to disease progression. However, cultures taken from elite controllers show few abnormalities in the HIV genome, suggesting that infection with defective virus is not a major contributor to long term non-progression [10]. This is promising, as it also suggests that other mechanisms of long-term nonprogression, dependent on host factors rather than viral factors, contribute more to inhibiting disease progression than chance mutations.

CD8+ T-cells can contribute to long term non-progression by controlling and eliminating infected cells. Part of this activity is mediated by direct cytotoxic activity, but much of it is through secretion of antiviral compounds. In some elite controller cohorts, CD8+ T-cells show a strong response to viral antigens, potentially allowing them to clear infected cells more effectively. CD8+ T-cells isolated from these patients show an ability to potently inhibit HIV infection by killing infected cells, even without exogenous stimulation. Interestingly, HIV-specific, interferon-γ secreting CD8+ T-cell counts are high in elite controllers, while in normal patients high CD8+ T-cell counts correlate with

high viral load and disease progression. Also interesting is that these cells are active in the absence of detectable viral antigen, which would seem to suggest that they should be inactive. Another activation marker, HLA-DR, is present in CD8+ T-cells isolated from elite controllers, and its presence is correlated with viremia in normal patients. CD8+ T-cells taken from elite controllers also show an ability to effect a multifunctional response, including degranulation and secretion of cytokines and chemokines. These properties suggest that CD8+ T-cells in some elite controllers may able to mount and sustain an effective response to HIV infection [10].

There are several proposed mechanisms for this effective response, which may each contribute to control of HIV infection. The cytotoxic T-lymphocytes (CTL) initially responsible for controlling the virus may exhibit a broad reactivity with variants of that virus via efficient crossreactivity. This prevents viral escape by mutation of surface antigens. Alternatively, new CTL variants may arise following viral mutation that are reactive to the new viral antigens. This process is usually impaired in progressor patients, but is effective in elite controllers. Additionally, the B27 and B57 alleles of the HLA gene may play a role in this response, as several studies have suggested that they target epitopes located within structurally important regions of viral proteins, where variations would come at the cost of viral fitness. Supporting this are studies that show an association between the breadth of the Gag-specific response and lower levels of plasma viremia [10].

Natural killer cells may also play a role in elite control of HIV infection, as highly inhibitory alleles of the KIR3DL1 receptor in the presence of its Bw4-80I ligand have a

protective association. This may be due to engagement of the activating KIR3DS1 receptor and loss of the signal mediated by highly inhibitory KIR3DL1 alleles, promoting activation of NK cells. Antibodies may play a role in elite control of HIV infection, but studies have been inconclusive. Some patients show higher levels of antibodies with broader activity against HIV, while others have lower levels. However, other antibody dependent mechanisms, such as antibody dependent cellular cytotoxicity may play a role, and further investigation is needed [10].

Other elite controllers show mutations that make the target cells harder for the virus to enter, like a 32 base pair deletion in the *CCR5* gene known as Δ32 [10]. Individuals homozygous for the Δ32 mutation show no infection with R5-tropic strains of HIV alone as they do not express the CCR5 receptor at the cell surface. When these individuals are infected with HIV, it is with an R4-tropic strain alone or an R5-strain in combination with an R4-tropic strain, demonstrating the importance of the receptors in HIV infection. In heterozygous individuals, the Δ32 mutation is correlated with lower viral loads, though not necessarily delayed disease progression [7].

Clinically, the Δ32 mutation of the CCR5 receptor is significant as it was involved in the only known case where HIV infection was entirely eliminated. Known as the Berlin patient, they have remained HIV-negative following a peripheral blood stem cell transplant from a Δ32-homozygous donor. Prior to the transplant, the patient's HIV infection had been successfully controlled by ART, and when discontinued in preparation for the transplant, the patient experienced a rebound to 6.9×10^6 HIV RNA copies/mL. ART was re-established until the day before the procedure. Following the transplant, the

Berlin patient remained HIV-negative even without ART, with no detectable levels of virus present. Subsequent attempts to replicate the result have failed, leaving its potential as a cure in question [17]. However, the Berlin patient has also sparked research into eliminating CCR5 expression in a patient's own T-cells via gene therapy [4].

Ligands for chemokine receptors also play a role in resistance to infection and disease progression. The chemokines MIP-1α and MIP-1β are produced by a variety of cells, including macrophages, NK cells, fibroblasts, and T-cells, and bind strongly to CCR5. Once bound, they desensitize CCR5 to HIV interactions. Patients with high levels of these chemokines show resistance to HIV progression, while patients with AIDS tend to have low levels. Additionally, an isoform of MIP-1α known as MIP-1αP can physically block entry of HIV. The copy number of the gene that encodes MIP-1αP varies between populations and individuals within those populations. Absolute copy count is less important than how it compares to others in that population, with higher copy numbers than average of the CCL3L1 gene that encodes MIP-1αP conferring resistance to HIV and lower than average counts with susceptibility [7].

Another CCR5 ligand, CCL5, is also associated with resistance to HIV infection. The CCL5 chemokine, encoded by the RANTES gene, inhibits the recycling of internalized CCR5 receptors to the cell surface, in turn suppressing infection by R5-tropic strains of HIV. Increased levels of RANTES expression are associated with delayed disease progression and slowed T-cell count reduction, but increased disease transmission. However, studies into the effects of different RANTES alleles have shown mixed results. Stromal cell-derived factor 1 (SDF-1) is a CXCR4 ligand and a potent

entry inhibitor for R4-tropic strains of HIV. Homozygotes for the SDF1-3`A allele show slower disease progression, but other studies involving heterozygotes have shown no correlation between the allele and disease progression. In general, the effect of receptor and ligand variations in inhibiting HIV infection suggests that interfering with receptor interactions could be a viable means of treating the disease. However, while studies with allosteric inhibitors of CCR5-HIV interactions have shown them to be effective in preventing HIV entry, they also have the undesired side effect of increased CCR5 activation [7].

Physiology of p21

In a particular subset of elite controllers, CD4+ T-cells have elevated levels of the cyclin-dependent kinase inhibitor protein p21, a trait that appears to confer resistance to HIV infection. As a number of cyclin-dependent kinases are linked to promotion of HIV reverse transcription, including CDK2 and CDK9, inhibition of them would limit HIV replication. In normal physiology, p21 plays a variety of roles in the cell, including proliferation, differentiation, migration, senescence, apoptosis, and DNA repair. p21 also plays a role in oncogenesis, both in promoting and inhibiting the process. The effect of p21 depends on its localization within the cell, in particular whether it is located in the nucleus or within the cytoplasm [18].

Within the nucleus, p21 acts a p53-dependent cell cycle inhibitor, contributing to blocks at several points in the cell cycle. At the G_1 and S phase checkpoints, p21 acts by binding and inhibiting activity of the E-CDK2 and A-CDK2 complexes. p21 contains three sites that are able to bind to CDK2: the N-terminal region site (K-site), Cy1 at the

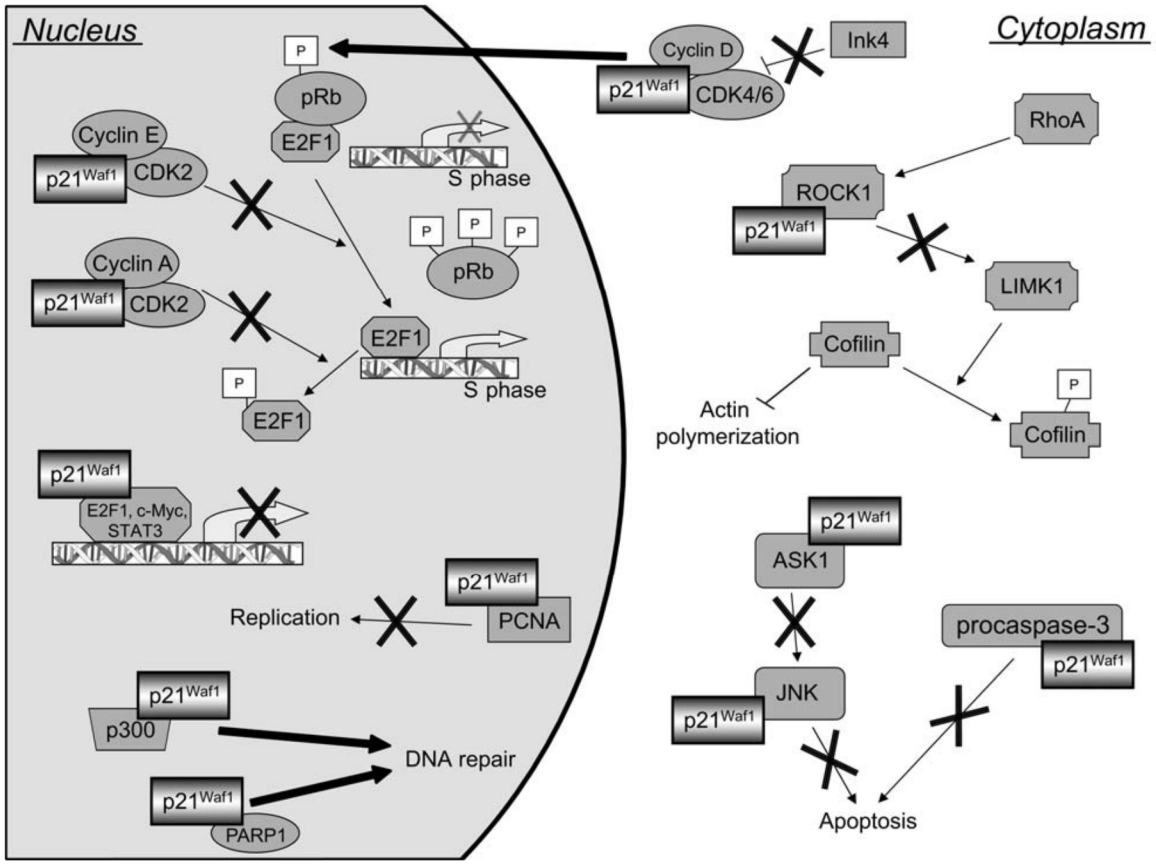

Figure 5 p21 acts on many cellular proteins, inhibiting and contributing to cell proliferation depending on whether it is localized to the cytoplasm or the nucleus.[18]

N-terminus, and Cy2 at the C-terminus. p21 binds to CDK2 by first interacting with a cyclin, then binding to CDK2 via the K-site. This shields the Thr160 residue of CDK2, preventing activation by CAK kinase. Additionally, the Cy1 site of p21 blocks the Thr14 and Thr15 residues of CDK2, preventing dephosphorylation of those sites by Cdc25A phosphatase. However, once CDK2 has been dephosphorylated at Thr14 and Thr15, p21 cannot inhibit its activity. While p21 also acts at the G_2 and M phase checkpoints, its exact mechanism is not yet known. However, it is possible that it contributes to those checkpoints by repressing the transcription of Emi1, which is an inhibitor of the APC/C ubiquitin ligase complex. APC/C then induces degradation of A- and B-type cyclins, leading to arrest in the G_2 checkpoint. Additionally, a site on p21's C-terminus is able to interact with the PCNA subunit of DNA-polymerase δ, inhibiting DNA replication and cell proliferation [18].

When present in the cytoplasm, p21 appears to have the opposite effect, where it promotes rather than inhibits cell proliferation and oncogenesis. In humans, relocation of p21 to the cytoplasm is linked to Akt/PKB kinase phosphorylation of the Thr145 residue, which is located near the nuclear localization sequence. This phosphorylation and relocation is accompanied by a loss of p21's anti-proliferative activity. Additionally, a number of mouse-knockout models show that certain cell types, including mouse embryonic fibroblasts, cannot be transformed in the absence of p21. Mice without p21 show a decreased rate of incidence of some tumor types, including ionizing radiation induced thymus lymphomas and PDGF-induced gliomas. Another mouse line engineered to overexpress the Thr145 phosphorylated form of p21 in mammary epithelium had

accelerated tumor expression and promoted lung metastasis compared to wild-type mice, providing further evidence of p21's carcinogenic role when localized to the cytoplasm. In humans, overexpression of p21 or its localization in the cytoplasm are correlated with poor prognosis in malignant tumors of skin, breast, prostate, ovary, cervix, and brain [18].

p21's carcinogenic activity is mediated through interaction with D-CDK4/6 in the cytoplasm. p21 binds to D-CDK4/6 via the Cy1 site, and contributes to the assembly of D-CDK4 complexes, possibly by counteracting the effects of Ink4 family inhibitors. Additionally, p21 binding to D-CDK4/6 contributes to p21 sequestration in the cytoplasm, preventing it from exerting its inhibitory effect on E- and A-CDK2 complexes in the nucleus.

Cytoplasmic p21 also has a role in cytoskeleton reorganization and cell migration, potentially contributing to metastatic behavior. The C-terminus of p21 is capable of uncoupling the αvβ3 integrins from focal contacts, inhibiting melanoma cell flattening. p21 interacts with various regulators of cytoskeleton organization, including Rho-associated kinase 1. p21 inactivates Rho-associated kinase 1, leading to inhibition of the RhoA-ROCK-LIMK1-cofilin cascade, leading to a decrease in focal point adhesion and actin polymerization. This suggests that it plays a role in metastasis, as these are required for cell migration. Cytoplasmic p21 contributes to inhibition of apoptosis by binding and inactivating a number of preapoptotic proteins. p21 inhibits a stress-induced apoptotic pathway by inhibiting the JNK family MAP kinases and their upstream activator MAPKKK ASK. p21 also binds to procaspase-3, preventing its processing to caspase-3

and subsequent Fas-mediated apoptosis. These provide protection against programmed cell-death, consequently promoting oncogenesis [18].

p21 and Elite Controllers

A study by Leng et al investigated the mechanisms by which the heightened expression of p21 provides resistance to HIV infection, showing that it inhibits a number of steps in the HIV life cycle. Leng et al showed that inhibition of CDK2 activity did indeed limit HIV-1 reverse transcription, resulting in lower levels of early, intermediate, and late reverse transcript products. Their work also showed that CDK2's effect was mediated through phosphorylation of viral reverse transcriptase, promoting its activity. When the threonine residue that CDK2 phosphorylated was replaced by alanine, the HIV variant showed markedly reduced replication efficacy. They then examined the stability of phosphorylated and unphosphorylated reverse transcriptase against proteolysis. The phosphorylated form showed increased resistance to proteolysis, suggesting that CDK2's contribution to HIV reverse transcription may be attributed to protecting reverse transcription from degradation. Leng et al also showed that silencing expression of p21 by p21-specific siRNA reduced the levels of p21 expression. Additionally, silencing p21 led to increased expression of HIV-1 in CD4+ T-cells isolated from elite controllers, supporting its role in restricting HIV-1 infection [19].

A paper by Chen et al studied the effects of p21 on other steps of the HIV life cycle. Their experiments showed that the early steps of viral infection, including reverse transcription and integration are inhibited in CD4+ T-cells taken from elite controllers. Additionally, they confirmed that CD4+ T-cells taken from some elite controllers showed

elevated levels of p21. Chen et al. then confirmed that elevated levels of p21 are correlated with resistance to HIV infection in CD4+ T-cells, as siRNA silencing of p21 infection led to increased susceptibility to infection. The activity of p21 in HIV resistance was not shown to be correlated with T-cell activation. Chen et al showed that elevated levels inhibit reverse transcription of HIV and transcription of the HIV provirus to mRNA. By comparing the levels of viral transcripts and late reverse transcripts, they showed that the effect of p21 on transcription is independent of its effect on reverse transcription. However, p21's effect on reverse transcription and integration were not shown to be independent of each other [20].

To determine the mechanism by which p21 inhibited transcription, Chen et al silenced p21 expression with siRNA, and found that CDK9 showed a stronger ability to phosphorylate RNA polymerase II, which is known to be a key step in promoting HIV transcription. Additionally, they showed that inhibiting p21 expression led to a 2-fold increase in the number of proximal and distal reverse transcripts present in infected CD4+ T-cells, providing further evidence that p21 plays a role in controlling HIV-1 infection [20].

Based on these observations from Chen et al, we hypothesized that p21 directly represses HIV transcription. To determine the biochemical mechanisms that cause p21-induced repression, 293T cells were transfected with p21 and pNL-43, an HIV clone, then harvested and assayed for HIV expression. Surprisingly, instead of inhibiting HIV expression, the overexpression of p21 led to increased expression of HIV.

METHODS

Transfection Protocol

Human embryonic kidney 293T cells (originally obtained from ATC) were plated in 6-well plates at 5-7.5x10^5 cells per well and incubated in 10%FBS DMEM with 1x P/S/L-Glu overnight at 37 C to reach a confluency of 60-70%. The transfection cocktail for each well consisted of 2 μg/mL of pNL43-Luc plasmid, 2 μg/mL of either p21 plasmid (experimental) or Fugw-Vec (control), 60 μL 2M $CaCl_2$, 500 μL HBS, and sterile water to bring the volume to 1 mL per well. The reagents were warmed to room temperature and mixed, starting with the sterile water and $CaCl_2$. The plasmid DNA was added and mixed by tapping. HBS was added dropwise, and the mixture aerated by pipetting. The solution was allowed to incubate for 20 minutes at room temperature, then aerated briefly. 1 mL of transfection solution was added to each well. The cells were incubated with the transfection reagent for 1 hour at 37 C. The transfection reagent was then removed and fresh media. The cells were then incubated overnight and harvested 24 hours post transfection.

Western Blot

Whole cell lysates were prepared by harvesting cells from one well (approximately 1 million cells). The cells were lysed with 100 μL of NP40 lysis buffer spiked with Calbiochem Cocktail set III protease inhibitor (1:1,000) and PMSF (1:100). The lysate was spun down at 15,000 RPM for 5 min. The supernatant was collected. 8 μL of sample was mixed with 2 μL of DTT and 8 μL of 2x SDS-loading dye for each lane, then heated at 99 C for 5 min. The samples were flash centrifuged and loaded into a 15

well, 10% polyacrylamide gel. 10 µL of EZ-run protein ladder was used as the size standard. The samples were transferred to Pall polyvinylidene difluoride membrane in transfer buffer via overnight protocol. The transfer buffer was 2.9g/L glycine, 5.8 g/L Tris Base, 0.37 g/L SDS, and 20% methanol in water. The membrane was blocked with 5% Carnation nonfat milk for one hour. The membrane was probed with anti-GAG, anti-p21, anti-beta actin (mouse IgG) and anti-GAPDH (rabbit IgG). The secondary antibodies were goat anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase. Membranes incubated with GE Healthcare ECL Prime Western Blot Detection Reagent for five min. Films exposed and developed to detect proteins.

p24 ELISA

Microtest 96 well ELISA plates, Clear (BD Falcon ref# 353279) were used. 4 µL of human polyclonal HIV immunoglobulin (AIDS reagent catalogue #3957) were diluted in 10.5 mL PBS. 100 µL of the diluted solution was added to each well of the plate. The plate was incubated at 37 C for 6 hours. The plate was washed four times with 200 µL of PBS per well. The plate was blocked with 200 µL of 5% FBS/PBS per well. The plate was sealed and incubated at 37 C for 1 hour. The plate was washed four times with 200 µL of 1x PBS, 0.2% Tween wash buffer per well. A standard curve was prepared with Perkin Elmer p24 standard via serial dilutions ranging from 4 ng/mL to 0 ng/mL, decreasing by 2x per dilution. The samples (supernatant from transfection wells) were diluted with 1x PBS, 10% FBS, 0.5% TritonX-100 diluent buffer. 200 µL of sample was loaded in each well. The plate was sealed with plastic and incubated at 37 C for 2 hours. The plate was washed four times with 200 µL of wash buffer per well. The primary

antibody (HIV-1 p24 Hybridoma (183-412-5c) from AIDS reagents) was prepared by diluting 420 μL of antibody in 10.5 mL of diluent buffer. 100 μL of primary antibody was added to each well and the plate was sealed and incubated at 37 C for 1 hour. The plate was washed four times with 200 μL of wash buffer. The secondary antibody (anti-mouse IgG HRP) was prepared by diluting 1 μL of antibody in 30 mL of diluent buffer. 100 μL of secondary was added to each well. The plate was sealed and incubated at 37 C for 1 hour. The plate was washed four times with 200 μL of wash buffer per well. TZM Peroxidase Substrate was warmed to room temperature, equal parts of solutions A and B mixed, and 100 μL of substrate mixture added to each well to develop the plate. The plate was incubated at room temperature until the lowest standard began to change colors (about 15-20 minutes) with 100 μL of 2M sulfuric acid per well. The plate was read with Biotek Synergy HT using Softmax software.

Flow Cytometry

Cells were harvested from one well (approximately 1 million cells). The cells were gated with forward and side scatter to identify the cells of interest. The cells within the gated population were assessed for green fluorescent protein expression through fluorescin isothiocyanate fluorescence. Transfected cells were compared against untransfected cells to determine transfection efficiency.

Luciferase

Cell lysates were prepared by collecting cells from one well (approximately 1 million cells) and lysed with 30 μL of Promega lysis buffer. The lysates were spun down at 15,000 rpm for 30 seconds. 20 μL of supernatant was placed in one well of a 96 well

plate. 100 μL of Promega luciferase substrate was added to the well. The luminescence was measured in Biotek Synergy HT using Softmax software.

RESULTS

In a paper by Chen et al, it was shown that CD4+ T-cells from a set of elite controllers had elevated levels of p21, and that these cells repressed HIV replication partly by restricting the transcription of the HIV provirus. To test the effect that heightened levels of p21 has on HIV transcription, HEK 293T cells were transfected with pNL43-LUC and either p21 or a Fuw-vector control. pNL43-LUC is an HIV clone that lacks Env, rendering it replication incompetent. In the place of Env is Luc, a gene that encodes for the luciferase protein. Since Luc is under the control of the HIV proviral transcriptional elements, expression of luciferase is correlated with HIV transcription. The results of the transfection were characterized through a luciferase assay, p24 ELISA, Western blotting, and flow cytometry.

In each transfection experiment, three wells of Fugw transfected cells and three wells of p21 transfected cells were collected for a luciferase assay. The results showed that there was a significant increase in relative light unit counts in the p21 transfected cells as opposed to the Fugw transfected cells, with an increase of 3-17 fold in the p21 transfected cells. This indicates that overexpression of p21 in HEK 293T cells promotes HIV transcription.

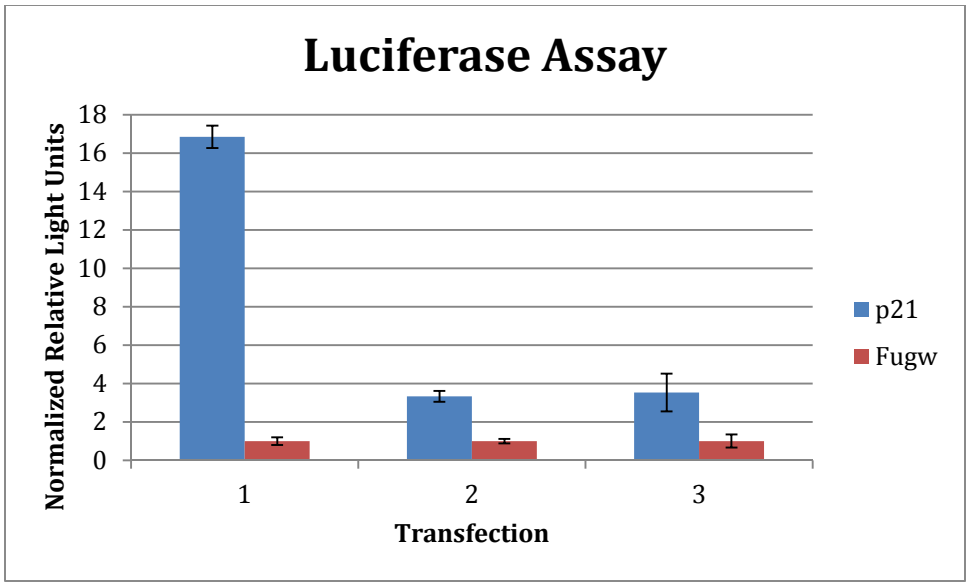

Figure 6. HEK 293T cells were transfected with pNL43-LUC and either a p21 plasmid or the Fugw vector control. Cells were incubated for 24 hours, then harvested. Each bar represents the average of three wells. The values were normalized to the average of the Fugw control. The p21 results show a clear increase in luciferase activity compared to the Fugw controls.

To determine whether luciferase correlated with the release of virus-like particles, HIV p24 Gag was measured in the samples collected from the transfection experiments. Supernatants were collected from three p21 transfected wells and three Fugw transfected wells diluted at 10, 100x, and 1000x dilution and assessed by p24 ELISA. Data are shown in Table 1 and 2 below. Although in general the samples did not yield detectable p24, p24 was more strongly expressed in p21-transfected samples. For example, the p21 transfected samples from transfections 2 and 4 showed measurable levels of p24, while the Fugw transfected samples fell below the limits of detection. Samples from transfections 2 and 4 were run through a second p24 ELISA undiluted and at 10x dilution. The undiluted p21 samples were above the range of the standard, while the corresponding Fugw sample remained in range. Comparison of the 10x diluted samples showed an approximately 20x and 10x increase in p24 levels. These data are consistent with the luciferase data above that indicate that p21 is enhancing HIV transcription.

Table 1. p24 ELISA (100x and 1000x dilutions)

Transfection (dilution)	1	2	3	4
Fugw (100x)	ND	ND	ND	ND
p21 (100x)	ND	45.78 ng/mL	ND	110.18 ng/mL
Fugw(1000x)	ND	ND	ND	ND
P21 (1000x)	ND	ND	ND	53.29 ng/mL

Table 2. p24 ELISA (Undiluted and 10x dilution, one sample)

Transfection (dilution)	2	4
Fugw(Undiluted)	1.465538	2.193739
P21(Undiluted)	Out of Range	Out of Range
Fugw(10x)	1.12589	3.208384
P21 (10x)	23.52277	30.77791

To confirm the effectiveness of transfection, immunoblots were performed for

p21, which was being overexpressed in the transfection. Signals were only detected in samples from the first transfection experiment and only with cells that had been transfected with the p21 plasmid. Since p21-transfected cells consistently showed higher levels of luciferase and p24, it is likely that the subsequent experiments had elevated levels of p21 that were below the level of detection. The samples were also probed for Flag, a tag added to p21 in the plasmid used to distinguish it from endogenous p21. No signal was detected, suggesting that there might be a problem with the tag or the antibody used. Beta actin and GAPDH were probed for to establish an endogenous control. Beta actin only gave weak signals. GAPDH gave consistent strong signals in all samples, and shows that even numbers of cells were collected in each experiment, though the number of cells may have differed between experiments. Representative exposures are shown.

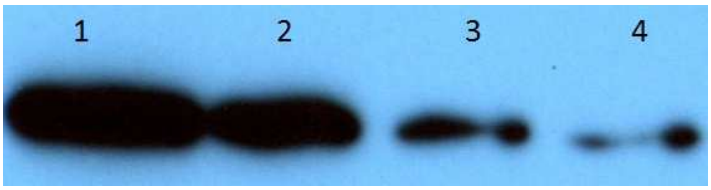

Figure 7. Western blot with samples from: 1, First Fugw transfection, 2. First p21 transfection, 3. Fourth Fugw Transfection, 4. Fourth p21 transfection. Primary: Anti-GAPDH 1:20,000 5% Non-fat milk at room temperature for 1 hour, 45 minutes. Secondary: anti-Rabbit-HRP 1:10,000 5% Non-fat milk at room temperature for 1 hour. Demonstrates the sample size is roughly equivalent in each set of cells harvested.

Figure 8. Western blot with samples from first Fugw transfection, first p21 transfection, second Fugw transfection, second p21 transfection. Primary: Anti-p21 1:10,000 5% NFM at room temperature for 1 hour. Secondary: anti-mouse-HRP 1:20,000 5% Non-fat milk at room temperature for 1 hour. The only signal visible is from the first p21 transfection, a result that was consistent across all Western blots.

To provide another assessment of transfection efficiency, one set of samples was run through flow cytometry. The results are shown in Figure 4. To identify live HEK 293T cells, the signals were gated with forward and side scatter. Fluorescin isothiocyanate fluorescence was used to identify cells that had been transfected. The rate of transfection was relatively low, with about 25% of Fugw transfected cells and 5% of p21 transfected cells showing significant expression. The low rate of transfection in the p21 transfected cells may account for the difficulties in detecting p21 in the immunoblots.

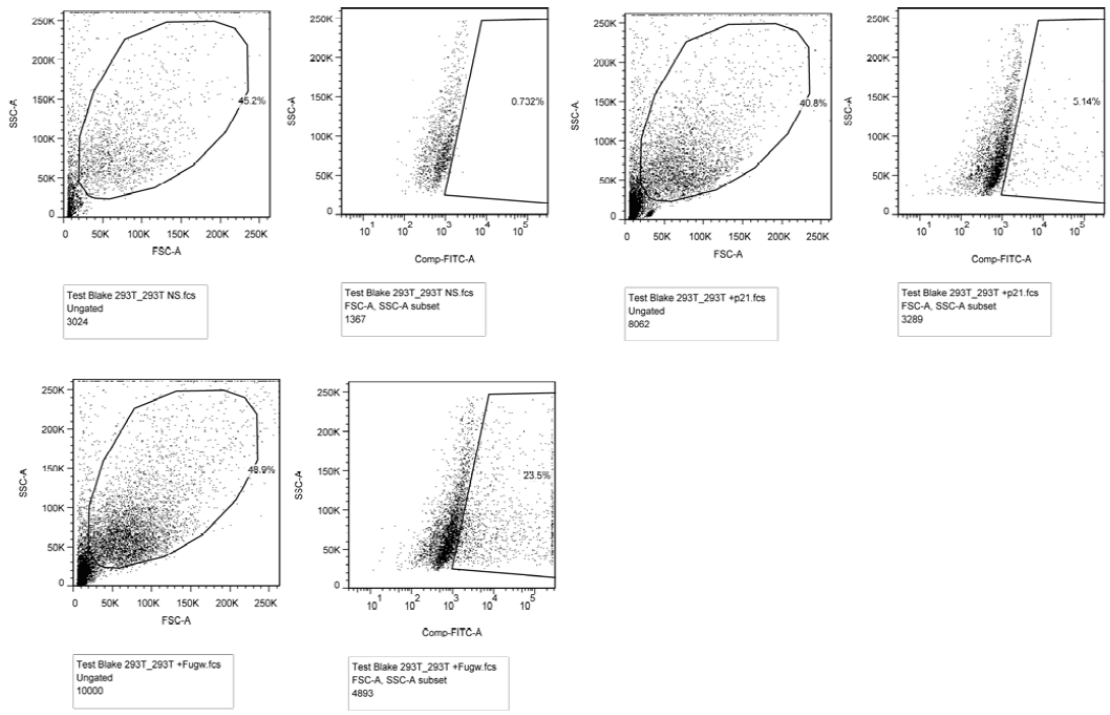

Figure 9. Flow cytometry. From left to right, first row: No sample (ungated); No sample (FSC-A, SSC-A gated, high FITC population); p21 transfected sample (ungated); p21 transfected sample (FSC-A, SSC-A gated, high FITC population). Second row: Fugw transfected sample (ungated); Fugw transfected sample (FSC-A, SSC-A gated, high FITC population). There is a 5.14% transfection rate in p21 transfected cells, and 25.5% in Fugw transfected cells.

DISCUSSION

Previous studies by Chen et al had shown that in a certain subset of elite controllers, high levels of p21 provided a repressive effect for HIV transcription. To explore the mechanisms behind this repression, I transfected HEK 293T cells to overexpress p21. Instead of a repressive effect, I observed a 3-fold increase or more in the p21 transfected cells compared to the controls. This suggests that overexpression of p21 in HEK 293T cells activates rather than represses HIV.

I encountered a number of technical difficulties during these experiments, especially with the Western blots. The failure to detect p21 in the later experiments could be due to a relatively low level of expression that was below the threshold of detection. This in turn could be attributed to a low level of transfection. The flow cytometry data shows that only about 5% of the cells transfected with p21 showed expression of the green fluorescent reporter protein, consistent with a low level of transfection in those cells.

From these results, I conclude that p21 has a strong role in increasing the expression of pNL-43 in 293T cells. The difference from prior results can be attributed to a number of reasons. First, the cells used are 293T cells, an immortalized human embryonic kidney cell line. Repeating these experiments in primary T-cells may show the opposite effect. Second, the conditions of these experiments were designed to overexpress of p21, well above the physiological levels of the protein. Since p21 was only detected in cells that had been transfected with the p21 plasmid, it's likely that it was present in higher concentrations than is typical for the cell type. This high level may

lead to the opposite of p21's effects at lower levels. The difficulty in detecting p21 in subsequent experiments, however, means that more sensitive experiments would be needed to confirm this.

Going forward, more experiments should be conducted to verify the results and explore the mechanism of how p21 affects HIV expression. In particular, the transfection experiment should be repeated in a T-cell line, like Jurkat T cells or even primary T cells. This would show if the increase in HIV expression is particular to 293T cells, or if it also occurs in T-cells. If the results repeat in T cells, or if they instead show a decrease in HIV expression, experiments should be conducted to determine the mechanism by which p21 affects HIV expression.

These results also suggest a potential course for clinical research. If p21 increases the levels of HIV expression in T cells when present in high concentration, therapies that target p21 expression could provide a way to eliminate the latent HIV reservoir in patients. By inducing HIV expression, the latent cells can be targeted and cleared as has been suggested by recent shock and kill strategies targeting chromatin structure [21].

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CURRICULUM VITAE