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Interactions Between HIV-1 TAT and Tumor Suppressor P53

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INTERACTIONS BETWEEN HIV-1 TAT AND TUMOR SUPPRESSOR P53

by

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DEDICATION

I dedicate this study to my dad, who would be the most proud; to my mom, who knows I couldn't have done it without her!; to my husband Chris, and my babies Lane and Elan, who can always make me smile. I love you all more than you know.

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ABSTRACT

The most well studied cancer-related gene in history, p53, is responsible for protecting the integrity of the genome. A variety of different stresses are detected by p53 and its regulators, allowing for numerous post-translational modifications that activate p53. After activation p53 can then regulate transcription of a myriad downstream targets, resulting in either cell cycle arrest or apoptosis. One of the stress signals that activates p53 is viral infection in part through the induction of type-1 interferon. However some viruses, like the DNA tumor viruses have evolved factors capable of binding to p53 and inhibiting its ability to regulate its downstream factors in order to avoid cell cycle arrest or apoptosis and complete the viral life cycle. The human immunodeficiency virus (HIV), probably the most notorious virus of our time because of our inability to completely eradicate it from a host, is capable of activating p53 in certain cell types upon infection. Several studies have reported on the functions of p53 during HIV infection, such as inhibiting full-length transcription of HIV, interaction with a number of HIV-encoded proteins such as the trans-activator of transcription (Tat), and eventual wide-spread apoptosis in T-cells, however these disparate roles are undoubtedly not the whole story. Our hypothesis is that an HIV factor, Tat is capable of mitigating the downstream effects of p53. To test this hypothesis we used U2-OS cells and created U2-OS cell lines that express Tat protein. These cells were then treated with the DNA damaging agents to activate p53. At various time post treatment, we measured promoter activity, mRNA and

protein expression of p53 and its targets p21 and bax. Without further investigation, at the present time we have no convincing evidence that Tat inhibits p53 function.

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CHAPTER 1

INTRODUCTION

1.1 CELLULAR ROLE OF p53

p53, a protein now famous for its function as a tumor suppressor, was discovered because of its binding with Simian vacuolating virus 40 (SV40) large tumor (T) antigen, a viral protein essential for initiation and maintenance of cellular transformation (Lane and Crawford 1979). First suspected of being an oncogene because of the ability of some p53 cDNA to transform normal cells to tumorigenic cells (Eliyahu, Raz et al. 1984; Parada, Land et al. 1984), these cDNA were eventually recognized as mutant forms, while wild-type p53 could prevent transformation (Finlay, Hinds et al. 1989). Several discoveries demonstrated the importance of p53 for tumor suppression. Mutations in p53 have been found in almost every type of cancer and at rates of up to 50% (Olivier, Hollstein et al. 2010), $p53^{-/-}$ mice were found to develop normally but acquire tumors very early (Donehower, Harvey et al. 1992) and mutant p53 was found at high levels in a wide range of human cancers (Bartek, Bartkova et al. 1991). Determining the role of wild-type p53 in maintaining genetic stability was becoming increasingly significant and when it was finally uncovered, p53 was dubbed the "guardian of the genome" (Lane 1992).

The most notable function of wild-type p53 is that of transcription factor. The p53 protein recognizes and binds to regions of DNA called response elements (REs) found in certain promoters (El-Deiry, Kern et al. 1992) and increases transcription of the

corresponding genes (Farmer, Bargonetti et al. 1992). The set of genes regulated will ultimately determine the fate of the cell. One possible cellular outcome is programmed cell death or apoptosis (Shaw, Bovey et al. 1992) and can be achieved through inducing a number of genes such as Bax, PUMA, Noxa, and Fas (Benchimol 2001) that are involved in permeablization of the mitochondrial membrane and the caspase cascade. In other circumstances a more appropriate state for the cell might be cell cycle arrest, either temporary or permanent arrest known as senescence. In these situations p53 moderates genes involved in cell cycle progression such as p21, a cyclin dependent kinase (CDK) inhibitor, or cdc25c that mediates initiation of mitosis, to stop advancement of the cell cycle (El-Deiry, Tokino et al. 1993; Clair, Giono et al. 2004) (Figure 1.1).

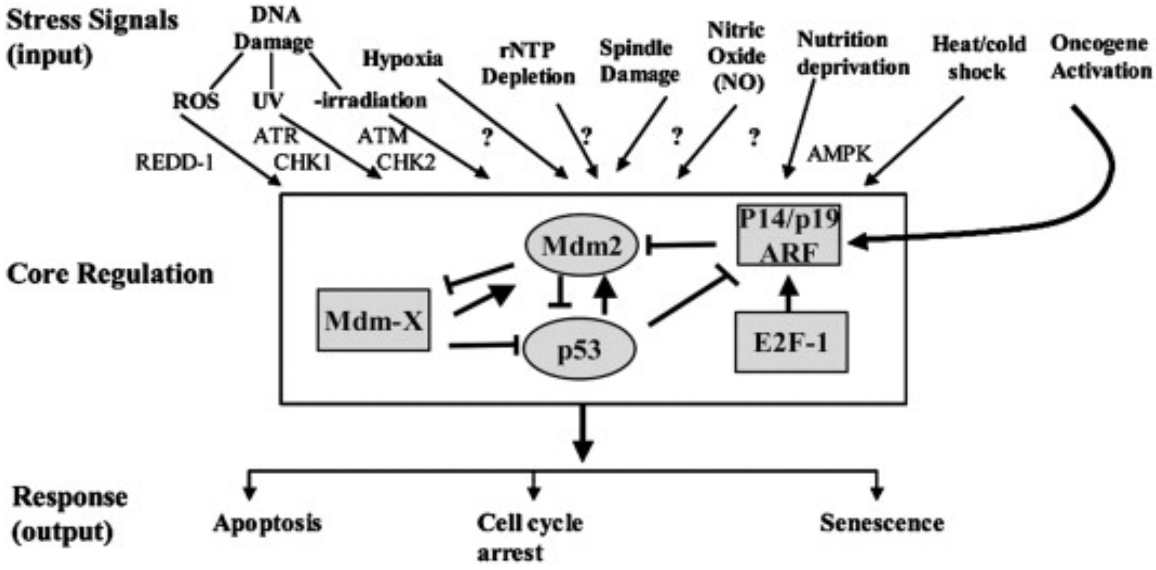


Figure 1.1 The p53 pathway. A diverse assortment of stress signals can be recognized by the p53 core regulatory molecules through post-translational modifications. Activation of p53 then leads to three possible responses, apoptosis, cell cycle arrest and senescence. (From Levine 2009).

The need for such disparate consequences stems from the ability of p53 to respond to numerous forms of cellular stress including, but not limited to, viral infection,

oncogenes, and genotoxic agents (Levine 1997). These stresses lead to a number of posttranslational modifications (PTMs) of the p53 protein that can cause its stabilization, localization to the nucleus, increased binding to the REs of its target genes, and moderate interactions with its binding partners (Gu and Zhu 2012). The types of PTM that the p53 protein can undergo include phosphorylation, methylation, glycosylation, and ubiquitylation to name a few (Meek and Anderson 2009). Specifically there are at least a handful of residues subject to acetylation on the p53 protein by more than one enzyme. Lysine 120 is acetylated by Tip60, a histone acetyltransferase, after DNA damage and while this alteration is necessary for induction of apoptosis it is not required for cell cycle arrest (Tang, Luo et al. 2006). While certain PTMs appear to work in an individual "switch-like" fashion, there is also evidence for cooperative and sequential effects (Gu and Zhu 2012). The type and intensity of the cellular stimuli induce different PTM patterns which function to fine-tune the p53 response for a particular outcome.

Activation of p53 by cellular stress is achieved in part by manipulating its interactions with its negative regulators, primarily murine double minute 2 (mdm2). Under normal cellular conditions p53 activity is kept in check by a number of negative regulators through strict control of protein levels and spatial separation from its targets. This is partially accomplished by complex formation with the mdm2 protein (Momand, Zambetti et al. 1992) which blocks the p53 transactivation domain (Lin, Chen et al. 1994) and acts as a ubiquitin ligase targeting p53 for degradation (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). Nuclear export of p53 through exposure of the nuclear export sequence (NES) or the NES of mdm2 in the mdm2 p53 complex (Roth, Dobbelstein et al. 1998; Stommel, Marchenko et al. 1999) helps regulate p53 activity by

physically separating p53 protein from its target binding sites. Mdm4, a protein similar to mdm2, is also necessary for regulation of p53, but in a manner nonoverlapping with that of mdm2 (Parant, Chavez-Reyes et al. 2001). Mdm2 and mdm4 are so imperative for controlling the activity of p53 that mouse embryos deficient in either do not survive to birth unless also deficient for p53 (de Oca Luna, Wagner et al. 1995; Jones, Roe et al. 1995; Migliorini, Denchi et al. 2002).

However when cells become stressed from DNA damage or other trauma the negative regulators of p53 are inhibited. For example during the DNA damage response to ionizing radiation mdm2 is inhibited from binding to p53 because of phosphorylation of p53 by ataxia telangiectasia mutated (ATM) (Kastan 2008). ATM simultaneously phosphorylates mdm2 and mdm4 hastening their rapid degradation (Chen, Gilkes et al. 2005; Stommel and Wahl 2005). This leads to a physical release of p53 protein from mdm2 and an accumulation of p53 protein and downstream activity. An alternate pathway to p53 activation through its negative regulator mdm2 is caused by oncogene activation. Stimulation of several oncogenes, including Ras and Myc, lead to an increase in arf protein (Palmero, Pantoja et al. 1998; Zindy, Eischen et al. 1998). Arf protein then binds mdm2 inhibiting its regulation of p53 and contributes to p53 accumulation and stimulation of its cellular effects (Pomerantz, Schreiber-Agus et al. 1998; Zhang, Xiong et al. 1998). Clearly inhibition and destabilization help uncouple p53 from its negative regulators through direct binding and PTM and contribute to the p53 stress response.

p53 is one of the most well studied regulators of genomic stability, especially in its role as a tumor suppressor. Even though it was first detected in complex with a viral

protein, the role of p53 in viral infections has not been as well characterized, particularly in relation to the most infamous virus of our time, HIV.

1.2 VIRAL INFECTION AND p53

After its initial discovery in SV40 infected cells, the p53 protein was shown to bind to the human Papilloma virus-16 and 18 E6 (Werness, Levine et al. 1990), and the Adenovirus E1b-55Kd protein (Sarnow, Ho et al. 1982), both viral proteins necessary for host cell transformation (Figure 1.2). Eventually the purpose of the interaction between the viral proteins and p53 became evident, to inactivate p53 and protect the infected cell from p53-induced cell cycle arrest and apoptosis (Levine 2009), mimicking the role of the cellular factor mdm2. The number of viral factors found to mitigate the activity of p53 either by direct interaction or by upstream or downstream effects is already substantial and continuing to grow (Sato and Tsurumi 2012). However the role of p53 in the innate antiviral response is still not completely elucidated. Interferons (IFNs) and other pro-inflammatory cytokines are central to the host response to viral infection. An intriguing topic of research considers the factors elicited during the IFN response and their interactions with p53.

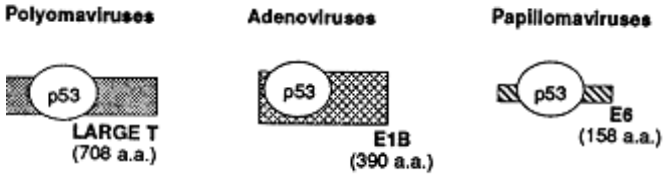


Figure 1.2 Binding of p53 by proteins from the DNA tumor viruses including the large T antigen, E1B protein, and E6 protein. (Adapted from Werness, Levine et al 1990)

The induction method of type 1 IFNs is dependent on the type of infecting virus and on cell type, and it involves multiple pathways for sensing infection (Perry, Chen et al. 2005). A cellular response to infection is triggered by conserved molecular motifs called pathogen-associated molecular patterns (PAMPs) that can be found on viral nucleic acids packaged in endosomes, cytoplasmic viral nucleic acids, and viral proteins (Randall and Goodbourn 2008). These triggers can be sensed through two distinct pathways, the extracytoplasmic and cytoplasmic both utilizing pattern recognition receptors (PRRs). The extracytoplasmic pathway consists of the family of Toll-like receptors (TLRs), membrane bound proteins that recognize PAMPs in the environment and recruit the corresponding signaling components and transcription factors using their cytoplasmic domains (Garcia-Sastre and Biron 2006). Certain host cell proteins in the cytoplasm, like the RNA helicases RIG-1 and mda5, have also been classified as PRRs since they have been demonstrated to recognize viral nucleic acids and stimulate production of IFNs (Garcia-Sastre and Biron 2006).

While differing combinations of PAMP and PRR may be responsible for activating unique pathways to IFN transcription, there seem to be a handful of common factors. Nuclear factor kappa-B (NF-κB) and members of the IFN regulatory factor (IRF) family must be translocated to the nucleus from the cytoplasm for transcription of the type 1 IFNs to occur (Randall and Goodbourn 2008). Type 1 IFNs are released from the cell where they can enhance the innate antiviral response, in both an autocrine and paracrine manner, by binding to type 1 IFN receptors (Perry, Chen et al. 2005).

Type 1 IFN receptors are transmembrane proteins consisting of two subunits IFN α/β receptor 1 (IFNAR1) and IFN α/β receptor 2 (IFNAR2) which dimerize upon IFN

type 1 binding (Stark, Kerr et al. 1998). This interaction activates two members of the the Janus family of tyrosine kinases, JAK-1 a nd TYK-2 which phosphorylate the signaling transducer and activator of transcription (STAT) 1 and 2 (Platanias and Fish 1999). The activated forms of STAT1 and 2 then complex with IRF9 to form IFN-stimulated gene factor 3 (ISGF3) that is transported to the nucleus and binds to DNA sequences containing IFN-stimulated response elements (ISREs) to upregulate transcription of IFN-stimulated genes (ISGs). Other IFN-activated STATs and STAT complexes are also reported to be capable of DNA binding and transcription activation (Garcia-Sastre and Biron 2006).

The p53 promoter contains sequences similar to ISREs capable of being bound by ISGF3. This helps explain the induction of p53 mRNA and protein upon treatment with IFN-α/β (Takaoka, Hayakawa et al. 2003). When this induction occurs early, less than 9 hours post treatment, it does not in itself result in activation of p53 or regulation of the downstream targets of p53. On the other hand prolonged exposure to IFN-β, 3-12 days, can lead to permanent cell cycle arrest at least partially due to PTM and activation of p53 (Moiseeva, Mallette et al. 2006).

However infection of MEFs with vesicular stomatitis virus (VSV) induces IFN-β, p53 with and without PTMs, and some downstream targets of p53 within 16 hours. At 24 hours post infection $p53^{-/-}$ MEFs show an increased resistance to apoptosis when compared to MEFs wild-type for p53. Also the viral yield in VSV infected MEFs was 30-fold greater when p53 was not present (Takaoka, Hayakawa et al. 2003). In fact "super p53" MEFs, generated from mice receiving an extra copy of p53, demonstrated even less tolerance for VSV replication as measured by viral yield, and showed increased

levels of PUMA and apoptotic cells (Munoz-Fontela, Angel Garcia et al. 2005). Infection with influenza virus also upregulates p53 protein and activity, cell type independently. There was a severe decrease in the viability of influenza virus infected cells when p53 was present and functional, and an increase in viral yield when p53 is inhibited (Turpin, Luke et al. 2005).

The actions of p53 may also be regulated by factors in the antiviral IFN response pathway other than IFN itself. Protein kinase R (PKR) is a type 1 IFN inducible target that binds and is activated by dsRNA from viral infections as well as cellular activators such as PACT (Patel and Sen 1998). PKR halts viral replication through phosphorylation of the cellular translation initiation faction eIF2 inhibiting viral protein synthesis (Colthurst, Campbell et al. 1987). PKR has also been reported to increase IFN production probably through the activation of NF-κB (Perry, Chen et al. 2005). The interactions of PKR with p53 have been implicated in the antiviral host response. PKR has been reported to bind and phosphorylate the p53 protein at Ser^{392}, and enhance phosphorylation of Ser^{18} increasing its transcription activity (Cuddihy, Li et al. 1999; Cuddihy, Wong et al. 1999). Another method for p53 upregulation by proteins involved in the IFN pathway concerns the ability of STAT1 to negatively regulate Mdm2 and physically interact with p53 in response to DNA damage leading to induction of certain pro-apoptotic p53 gene targets (Townsend, Scarabelli et al. 2004).

This evidence supports the idea that p53 plays a role in virus-induced senescence and apoptosis, and modulation of viral replication. Lastly there is evidence for p53 directly targeting several members of the type 1 IFN response for upregulation, including IRF9, TLR3, IRF5, and ISG15 (Rivas, Aaronson et al. 2010), increasing activity of the

IFN promoter ISRE (Turpin, Luke et al. 2005), and enhancing cellular release of IFN (Munoz-Fontela, Macip et al. 2008). This would seem to indicate the presence of a positive feedback loop involving p53 and the IFN-mediated antiviral response pathway (Figure 1.3). Therefore viral infections triggering an IFN response could also provoke p53 activation leading to cell cycle arrest, or apoptosis. Depending on the life cycle of the virus it might prove advantageous to evolve factors to mitigate the p53 response.

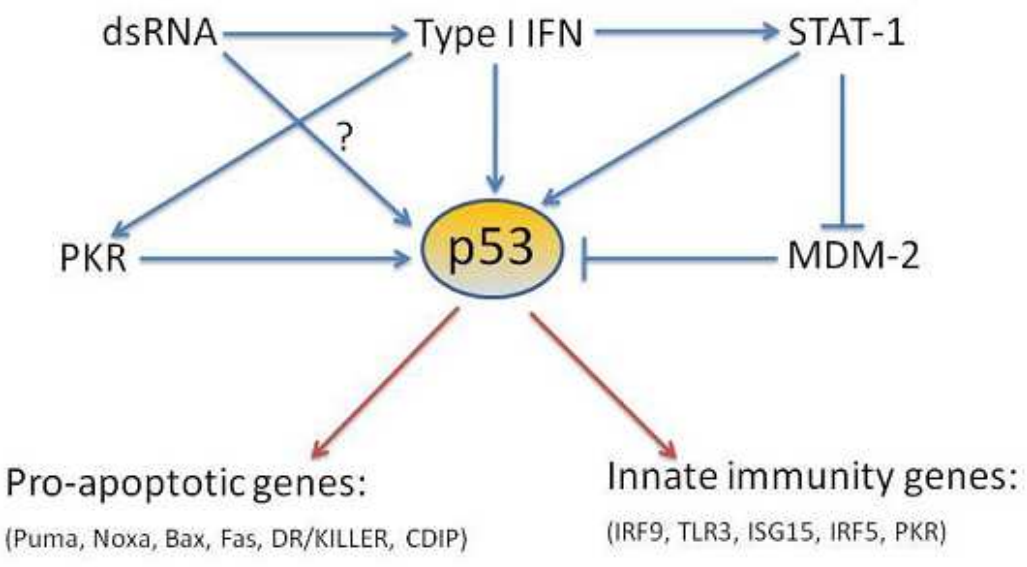


Figure 1.3 Feedback loop involving p53 and type 1 interferon. Viral infection leads to induction of type 1 IFN and p53 which results in induction of pro-apoptotic genes and genes involved in the innate immune response. (Rivas, Aaronson et al. 2010)

1.3 HUMAN IMMUNODEFIENCY VIRUS TYPE 1

Acquired immune deficiency syndrome (AIDS) is characterized by a set of symptoms, including recurrent infections, certain cancers, and low $CD4^+$ T cell counts, and develops as infection by human immunodeficiency virus (HIV) before progressing to

the final stage. The Centers for Disease Control and Prevention (CDC) estimates that 39 million people worldwide have died from AIDS since the epidemic began and another 35 million people are living with the virus as of 2013. Of those 35 million infected, only 12.9 million were believed to be receiving antiretroviral therapy (ART). Most antiretroviral drugs target the enzymes necessary for HIV infection and/or replication to occur. ART is highly effective especially when used in combination regimens. When three of more antiretroviral drugs are taken together it is called highly active antiretroviral therapy (HAART). Although HAART can efficiently minimize viral load, specific characteristics of HIV make it necessary to continue therapy indefinitely to maintain this effect. The complexity of the interactions between HIV and the innate immune system along with the propensity of HIV to rapid mutation have led to a challenging scientific and medical issue.

HIV is of the family *Retroviridae* because it contains RNA and reverse transcriptase and was further classified as a lentivirus due to its similarity in sequence and morphology to known members of the *Lentivirinae* genus (Gonda, Wong-Staal et al. 1985). Based on its genetic diversity, HIV was further divided into HIV-1 and HIV-2. HIV-2 however displays much lower transmission efficiency and is therefore mostly restricted to West Africa and is endemic as opposed to the pandemic spread seen by HIV-1 (Reeves and Doms 2002).

HIV-1 infection can be described in two stages; early and late. The early stage describes the process of the mature virus entering the cell and integrating its nuclear material with that of the host cell. The late stage describes the process of viral mRNA

and protein production, viral assembly and emergence of mature virions from the host cell.

The Early Phase

The HIV-1 envelope is a lipid bilayer derived from the host cell and containing projections formed from glycoproteins known as gp120 and gp41 encoded on the *env* gene (Muesing, Smith et al. 1985). HIV-1 infects mainly CD4+ T cells and macrophages by gp120 interaction with the cell surface receptor CD4 (Dalgleish, Beverley et al. 1984) and a chemokine co-receptors, like CCR5 (Wells, El Proudfoot et al. 1996). This leads to exposure of the gp41 fusion domain to the cell membrane and rearrangement of the viral envelope resulting in fusion of the viral and host cell membranes (Engelman and Cherepanov 2012) releasing the viral core to the interior of the host cell.

The core contains two copies of single stranded viral RNA tightly bound to *gag* encoded p7 nucleocapsid protein, and proteins necessary for viral replication, such as reverse transcriptase and integrase, surrounded by the mature capsid formed from *gag* encoded p24 (Fukui, Imura et al. 1993). Uncoating of the core disassembles the capsid shell (Ambrose and Aiken 2014) and likely triggers reverse transcription of viral RNA (Fitzon, Leschonsky et al. 2000).

The RNA is reverse transcribed into double stranded DNA by two *pol* gene products, p66 and p51, that form a heterodimer yielding the reverse transcriptase enzyme (Veronese, Copeland et al. 1986) capable of RNA to DNA polymerase, DNA to DNA polymerase and ribonuclease H functions. The double stranded DNA along with several viral proteins including integrase and Vpr form a pre-integration complex (PIC) (Bukrinsky, Sharova et al. 1993) and are transported to the nucleus in a manner that has

not yet been fully elucidated (De Rijck, Vandekerckhove et al. 2007). Once nuclear localization occurs a third product of the *pol* gene, integrase, cuts the host chromosomal DNA and joins the 3'end of the viral DNA to the cellular DNA (Bushman, Fujiwara et al. 1990). Host enzymes then complete the process joining the 5' ends of the viral DNA to the host DNA to establish a stable provirus.

The Late Phase

Transcription of the integrated provirus now proceeds to produce viral mRNA utilizing the host cellular machinery. The viral trans-activator of transcription (Tat) protein recruits the host factors P-TEFb, CDK9, and cyclin T1 to the viral transactivation response (TAR) element found at the 5' end of initiated viral transcripts to act as a promoter element (Fujinaga, Cujec et al. 1998), and stimulates CDK9-mediated phosphorylation of RNA polymerase II (Zhou, Halanski et al. 2000) greatly enhancing transcription elongation. While small viral mRNAs are easily exported from the nucleus, larger mRNAs require help from the viral regulator of expression, Rev. Multiple Rev molecules bind to the Rev response element (RRE) found in the *env* coding region of unspliced mRNA (Malim, Hauber et al. 1989) and the presence of a nuclear export signal (NES) facilitates egress of the Rev-bound unspliced mRNA from the nucleus (Fischer, Huber et al. 1995).

Translation of cytoplasmic, viral mRNA to protein necessary for virion assembly and budding also utilizes cellular machinery ie. the rough endoplasmic reticulum and cytosolic polysomes. Virion morphogenesis begins when the necessary factors, mainly viral genomic RNA, the Env proteins, and the Gag polyprotein, are transported to the plasma membrane. The different Gag polyprotein domains work in concert to complete

assembly. Multiple Gag polyproteins anchor by their MA domains collectively at specific regions of the plasma membrane. The CA domains interact to create a spherical Gag lattice that organizes the virion and distorts the membrane. The viral genomic RNA is secured on the NC domain while viral accessory proteins and factors in the ESCRT pathway are recruited and bound by the p6 region. The cellular ESCRT pathway is responsible for plasma membrane remodeling and scission and has been hijacked to complete budding of the new virion from the host cell. Maturation of the immature virion can occur concurrently or just after budding and relies on cleavage of the Gag polyproteins by the viral protease enzyme. Rearrangement of the fully processed proteins leads to a mature infectious particle (Sundquist and Kraeusslich 2012). These mature virions are now capable of infecting new cells.

While many HIV-1 infected $CD4^+$ T cells will play host to viral replication before undergoing apoptosis early during the innate immune response, this is not a universal outcome. Some infected cells enter a state known as latency. This is a reversible condition that exists when an infected cell is capable of but does not actively produce virus particles (Eisele and Siliciano 2012). This is not the principal means for HIV to evade the host immune response as it is in the *Herpesviridae* family of viruses (Perng and Jones 2009). The primary means of immune escape by HIV is rapid evolution (Borrow, Lewicki et al. 1997; Richman, Wrin et al. 2003). However with the success of HAART at diminishing active production of virus, the populations of latently infected cells known as reservoirs have taken on new importance and frustrated work towards a cure. Because latent cells maintain the ability to produce functional virus (Pantaleo, Graziosi et al.

1993; Chun, Stuyver et al. 1997), lapses in HAART lead to reactivation of latent cells and a rebound in viremia (Davey, Bhat et al. 1999).

Latent infections probably develop early after the initial host infection and can occur in both active and resting $CD4^+$ T cells (Chavez, Calvanese et al. 2015). Most of the active $CD4^+$ cells that eventually make up the latent pool were probably infected while transitioning to a resting state from an active state and were not productive. However there is new evidence that a small number of cells actively produce virus and, through an unknown process, revert to a resting state (Chavez, Calvanese et al. 2015). The mechanisms for producing latently infected cells are still unclear but there is evidence that location and orientation of viral genome integration may play a role. Integration has been reported to occur ~90% of the time at sites within genes that are actively transcribed in resting $CD4^+$ T cells (Han, Lassen et al. 2004). The many open questions still surrounding HIV mean that it remains an important and interesting area of research.

1.4 p53-MEDIATED RESPONSE TO HIV-1 INFECTION

"The immune system has only limited success in opposing infection by HIV" (Audige, Urosevic et al. 2006)

The p53 response to HIV-1 infection is likely multifaceted and dependent on a number of factors such as cell type and phase of infection. p53 has been implicated in mediating cell cycle arrest and apoptosis and also moderating transcription of the viral genome from the LTR through response to type 1 IFN, DNA damage and HIV encoded proteins. Again the pattern of PTM likely determines the subsequent actions of p53.

These actions may in part be hijacked and utilized by the virus for its own preferred outcome, be that viral replication, latency or apoptosis.

It is difficult to study the initial steps of HIV infection in humans, however attempts to illuminate this early stage using SIV infection in rhesus macaques offers some insight. Plasmacytoid dendritic cells (pDCs) are the initial predominant cell type to arrive at the site of infection (Kwa, Kannanganat et al. 2011). SIV/HIV then activates pDCs to produce chemokines and cytokines that attract other immune cells, such as CD4+ T-cells, leading to a robust and systemic infection (O'Brien, Manches et al. 2013).

While pDCs do not show a reduction in cell viability (Yonezawa, Morita et al. 2003) and are poor sources for viral replication after HIV-1 infection, they are the principal producers of type 1 IFN (Ferbas, Toso et al. 1994). Type 1 IFN is at least partially responsible for the upregulation of p53 mRNA and protein in HIV-1 infected CD4+ T cells (Imbeault, Ouellet et al. 2009; Imbeault, Lodge et al. 2009).

Another method of p53 activation following HIV-1 infection, involves the HIV-1 glycoproteins, both the Env complex, made up of gp120 and gp41, and soluble gp120. Infected cells expressing the Env complex on their surface are able to fuse with uninfected cells containing CD4 receptors and CXCR4 or CCR5 co-receptors, creating syncytia, and inducing phosphorylation of p53 and upregulation of p53 targets, such as PUMA, leading to mitochondrial membrane permeabilization and apoptosis (Genini, Sheeter et al. 2001; Perfettini, Castedo et al. 2005). This appears to be a major source of immune cell depletion in the progression of HIV-1/AIDS.

After p53 is activated it is capable of regulating transcription from promoter REs. Regulation of transcription in mammalian cells is one of the most important and well-

studied functions of p53, but p53 also has the ability to regulate transcription from viral promoters. The HIV-1 LTR has been shown to be yet another viral promoter that can be regulated by p53. Numerous groups have reported that wild-type p53 has an inhibitory effect on transcription from the HIV-1 LTR (Duan, Ozaki et al. 1994) while some mutant forms of p53 have been shown to increase transcript elongation (Subler, Martin et al. 1994). The mechanisms by which p53 regulates HIV-1 LTR transcription elongation are still being identified. Reports have been made of complex formation with TATA box binding protein (TBP) and Sp1 (Borellini and Glazer 1993; Chen, Farmer et al. 1993). More recently it was suggested that cdk9 was phosphorylating p53 along with Mdm2 thereby stabilizing p53 and leading to its accumulation, which in turn decreased transcriptional elongation from the HIV-1 LTR (Bagashev, Fan et al. 2013). Pirh2 was also reported to be phosphorylated by cdk9 which led to its inactivation (Bagashev, Fan et al. 2013). This is particularly interesting in light of the fact that the inhibitory effects of p53 on the HIV-1 LTR reported were transitory and transcription elongation was salvaged possibly by the activity of Pirh2 (Mukerjee, Claudio et al. 2010). It has also been noted that overexpression of p53 prevents phosphorylation of serine 2 in the carboxy terminal domain of RNA polymerase II which is necessary for efficient transcription elongation from the HIV-1 LTR (Mukerjee, Claudio et al. 2010). It is no surprise that the effect of p53 on HIV transcription has been and continues to be a topic of interest.

In addition to its trans-activating role, p53 has been found to interact with a couple of HIV-1 factors. For example HIV-1 Vpu has been reported to inhibit ubiquitination of p53 by β-TrcP promoting p53 stability and accumulation and ultimately

stimulating p53-mediated apoptosis in T cells (Verma, Ali et al. 2011). There is also evidence of p53 interacting with the Tat protein, but the mechanisms and effects of this complex are still being investigated.

1.5 p53 AND HIV-1 "TRANS-ACTIVATOR OF TRANSCRIPTION"

As was previously described, p53 is upregulated by several pathways perhaps cell type specifically after HIV-1 infection. For certain cell types the actions of p53 might be hijacked by the virus and utilized in a manner detrimental to the host, such as wide-spread apoptosis in T cells. Our interest was in determining whether or not the actions of p53 could be mitigated by the HIV-1 factor Tat, prolonging the cellular life span to allow for completion of the viral life cycle. This would be similar to the actions of the p53 responsive factors found in the small tumor viruses; SV40 T antigen, HPV E6, etc.

Numerous viral factors including some produced by HIV have been shown to interact with p53 and other players in the p53 pathway. One such factor is HIV-1 Tat. Most isolates of the HIV-1 virus produce Tat protein that consists of 101 amino acids. However a few isolates are known to produce a functional 86 amino acid version of the Tat protein. Tat was discovered because of its role as a trans-activating protein, responsible for enhancing transcription from the HIV-1 LTR. Eukaryotic RNA polymerase II is capable of forming short HIV-1 transcripts but is inefficient at transcription elongation. Tat binds both with the eukaryotic positive transcription elongation factor (P-TEFb), a complex of cyclin T1 and cyclin dependent kinase 9 (Cdk9), and the TAR stem-loop structure of newly synthesized HIV-1 mRNA. These associations lead to phosphorylation of RNA polymerase II by Cdk9 and initiate efficient

transcription of full length HIV-1 mRNAs. Tat also recruits other cellular proteins necessary for relieving repression of the HIV-1 LTR, such as the CREB-binding protein (CBP)/ p300 complex (Romani, Engelbrecht et al. 2010).

Tat has also been implicated in several other functions since its discovery, including regulation of gene expression from other viruses like polyomavirus BK (Gorrill, Feliciano et al. 2006) and from host cells leading to downstream effects including the inhibition of apoptosis (Zheng, Yang et al. 2007) and immune suppression (Gupta, Boppana et al. 2008). Tat has also been linked to the increase in cancers seen in AIDS patients (Nunnari, Smith et al. 2008), and to the onset of HIV-associated dementia caused by induction of neuronal apoptosis (Haughey and Mattson 2002). Tat is fast getting a reputation as a versatile protein with a full dance card, however its association with p53 has yet to be fully elucidated.

p53 has been reported to inhibit Tat transactivation of the HIV-1 LTR (Duan, Ozaki et al. 1994; Li, Wang et al. 1995) however Tat has also been demonstrated to suppress transcription from the p53 promoter (Li, Wang et al. 1995). These studies examining interaction between p53 and Tat have indicated binding does occur. Therefore a detailed examination of the structural and biophysical characteristics of the interaction was performed using peptide mapping, fluorescence anisotropy, and NMR spectroscopy. It was determined that amino acids 1-35 and 47-57 of Tat bind directly to the tetramerization domain of p53 which corresponds to residues 326-355 (Gabizon, Mor et al. 2008).

A few studies have attempted to determine the cellular ramifications of p53 and Tat binding. One such study found that the presence of Tat resulted in the loss of the

G_1/S checkpoint after DNA damage induced by gamma irradiation. The G_1/S checkpoint is regulated by the p53 target, p21, and the levels of p21 were found to be decreased when Tat was present. Because their results also indicated that wild-type Tat was able to inhibit p53 transcription activation while mutant Tat was not, it was tentatively suggested that the decrease in p21 and the failure of the G_1/S checkpoint was caused by inhibition of p53 transactivation (Clark, Santiago et al. 2000).

The objective of the studies presented here was to identify whether the presence of HIV-1 Tat in vitro would inhibit the ability of p53 to trans-activate its downstream targets, p21 and bax. This was carried out by introducing Tat into the U2-OS cell line either by transient or stable transfections and activating p53 with the DNA damaging agents Camptothecin, Cisplatin, Doxorubicin, and Etoposide. The effects on p21 and bax promoter activity, mRNA, and protein were studied using luciferase assays, qPCR, and immunoblotting, respectively. Because of the complex nature of HIV infection and its tendency towards latent infection which cannot at this point be eradicated even with HAART, elucidating the interactions between p53 and HIV factors like Tat is an important goal.

CHAPTER 2

MATERIALS AND METHODS

2.1 PREPARATION OF TAT HA EXPRESSION VECTOR AND CREATION OF TAT POSITIVE CELL LINES

In order to study the p53 response in the presence of the HIV-1 Tat protein, the pcDNA3 Tat HA expression vector (Addgene plasmid 14654) and the human osteosarcoma U2-OS cell line was used. The pcDNA3 Tat HA plasmid encodes a wild-type, 101 amino acid version of the HIV-1 Tat protein tagged with influenza hemagglutinin (HA). This vector also contains a selectable marker for neomycin resistance. The U2-OS cell line was chosen because it produces wild-type p53 and can be used to study the p53 DNA damage response pathway which includes upregulation of p21 and bax. The human osteosarcoma cell line U2-OS was grown in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 4.5 g/L glucose, 100 U/ml penicillin, 100 ug/ul streptomycin, and 2 nM L-glutamine.

U2-OS cells were grown to confluency and split 1.45 x 10^6 in 100 mm cell culture dishes. After 24 hours the cells were transfected with 2.5 ug and 5 ug of pcDNA3 Tat HA expression vector using TransFast Transfection Reagent (Promega) in a 1:1 charge ratio of TransFast reagent to DNA. Selection of cells positive for the pcDNA3 Tat HA

plasmid was achieved by addition of 200 ug/mL Geneticin G418 sulfate (GIBCO) to the growth medium. Clones were expanded, then PCR was used to test for the presence of the pcDNA3 Tat HA vector and immunoblotting with the HA probe (Santa Cruz Biotechnology) was used to test for the production of Tat HA protein. The U2-OS Tat HA cell lines were grown in DMEM with identical supplementation as the U2-OS cell line plus 200 ug/ml G418.

The pcDNA3 Tat HA expression vector, and the pTRE2 vector (Clontech), that contains the Tet-responsive promoter $P_{hCMV*-1}$, were digested with the restriction enzymes Not I and BamH I. The Tat HA fragment was gel purified and ligated to the pTRE2 plasmid and will herein be referred to as the "pTRE2 Tat HA" Tet-induced expression vector (Figure 2.1).

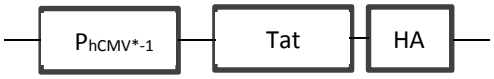


Figure 2.1 Segment of pTRE2 Tat HA vector.

The U2-OS Tet-On Cell Line (Clontech) expresses a tetracycline-regulated transactivator, rtTA, that in the presence of doxycycline binds to the Tet-responsive element (TRE) of the $P_{hCMV*-1}$ promoter. U2-OS Tet-On cells were grown to confluency and split 1.45 x 10^6 cells in 100 mm cell culture dishes. After 24 hours the cells were co-transfected with pTRE2 Tat HA vector and pSV2gpt vector using TransFast Transfection Reagent (Promega) in a 1:1 charge ratio of TransFast reagent to DNA. Cells positive for the pSV2gpt plasmid were selected by addition of the GPT selection mixture (section 2.2). Clones were expanded into 24 well plates and tested for the presence of Tat DNA

with PCR. Cell lines positive for Tat DNA were then tested for protein production with immunoblotting using an HA probe (Santa Cruz Biotechnology). The U2-OS Tet-On cell line was grown in DMEM with identical supplementation as the U2-OS cell line plus 200 ug/ml G418. The U2-OS Tet-On Tat cell line was grown in DMEM with identical supplementation as the U2-OS Tet-On cell line plus 15 ug/ml mycophenolic acid, 250 ug/ml xanthine, 15 ug/ml hypoxanthine, 2 ug/ml aminopterin, 10 ug/ml thymidine (GPT selection mixture). Four DNA damaging agents were used to induce a p53 response in all cell lines; 14 uM Camptothecin, 35 uM Cisplatin, 1 uM Doxorubicin, and 10 uM Etoposide (all Sigma).

2.2 PROTEIN EXTRACTION AND IMMUNOBLOTTING

Approximately 1 x 10^6 cells were plated on 100 mm cell culture dishes and after 24 hours were treated with DNA damaging agents. At the indicated time points the cells were washed with 5 mL PBS, harvested by scraping in 1 mL of PBS, and pelleted by centrifugation for 2 minutes. The supernatant was removed by aspiration and the pelleted cells were lysed using 50 uL of RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) with 10 uL/mL HALT Protease Inhibitor Cocktail that includes 1 mM AEBSF, 800 nM Aprotinin, 50 uM Bestatin, 15 uM E64, 20 uM Leupeptin, 10 uM Pepstatin A (Thermo Scientific) added, then sonicated. The protein concentration was determined using Bradford Protein Assay (Bio-Rad) with a UV-1700 PharmaSpec UV-VIS spectrophotometer (Shimadzu) reading at a wavelength of 595 nm. Protein lysates were stored at -70 ^{o}C.

Thirty ug of protein were mixed in a 1:1 ratio with Laemmli Sample Buffer (Bio-Rad) and denatured by boiling at 100 ^{o}C for 3 minutes. Samples were separated by SDS-PAGE using 12% SDS-polyacrylamide gels and 1X Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 200 V for 1 hour. Gels were rinsed in 1X TBST (10 mM Tris pH 8, 150 nM NaCl, 0.05% Tween-20) before protein was transferred by electrophoresis to Amersham Hybond-ECL nitrocellulose membrane (GE Healthcare) in protein transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 100 V for 1 hour. Membranes were rinsed in 1X TBST and incubated in 2% powdered milk in TBST overnight to block non-specific binding of antibodies.

Membranes were rinsed three times with 1X TBST, then probed by incubating for 1 hour with the recommended dilutions of the following mouse monoclonal antibodies: 1:2000 p53-421 (Calbiochem) with 1:2000 p53-DO1 (Calbiochem), 1:50 p21 (Santa Cruz Biotechnology), and 1:200 HA-probe (Santa Cruz Biotechnology). Membranes were washed once with 1X TBST for 15 minutes, then two more times for 5 minutes, before being incubated in anti-mouse IgG-peroxidase antibody (Sigma-Aldrich) diluted 1:2000010 for 1 hour. Membranes were again washed once for 15 minutes and three times for 5 minutes. All washes and incubations were performed on a shaker at room temperature.

Membranes were incubated in room temperature ECL Prime Western Blotting Detection Reagents 1 and 2 (GE Healthcare) mixed in a 1:1 ratio for 5 minutes. One of two imaging methods were utilized. In the first method a sheet of Kodak BioMax Light Film was placed over a membrane wrapped in Saran Plastic wrap and exposed until the

linear range was obtained. In the second method the membrane was imaged using the ImageQuant LAS 4000 system (GE Healthcare).

2.3 LUCIFERASE ASSAY

Approximately 5 x 10^4 cells were plated in each well of a 24-well plate. After 24 hours the cells were cotransfected with 100 ng/uL of a vector containing the firefly luciferase gene under the control of one of three promoters, p53, p21, or bax, along with 10 ng/uL of the pRL-CMV vector which contains the *Renilla* luciferase gene under the control of the CMV promoter. Twenty four hours after transfection some cells were treated with DNA damaging agents. At the indicated time, zero to twenty four hours post, the cells were harvested with 100 uL of Passive Lysis Buffer (Promega) incubated for 15 minutes at room temperature on the shaker. The experiments were run in duplicate or triplicate. The samples were analyzed using the Dual-Luciferase Reporter System (Promega). The luciferase readings were measured once with the Zylux FB12 luminometer. The ratio of Luciferase to Renilla was determined and averaged for each set of duplicates or triplicates and normalized to a control. Standard deviations were also determined for the duplicates or triplicates and normalized to the same control. If an experiment was replicated then the normalized means were averaged and standard deviations were calculated from the normalized means.

2.4 RNA PURIFICATION AND RT-qPCR

Approximately 1 x 10^6 cells were plated on 100 mm cell culture dishes and after 24 hours were treated with DNA damaging agents. At the indicated time points the cells

were washed with 5 mL PBS, harvested by scraping in 1 mL of PBS, and pelleted by centrifugation for 2 minutes. The supernatant was removed by aspiration and the RNA was extracted using the Total RNA Purification Kit (Norgen Biotek). The RNA concentration was determined using the Nanodrop 2000c (Thermo Scientific). cDNA was transcribed from 2 ug purified RNA using the random decamers and the Reverse Transcription System (Promega).

Amplification of the genes p53, p21, bax, and PUMA was achieved using primers specific for use with Real-Time qPCR (Table 2.1) and the 7300 Real-Time PCR System (Applied Biosystems) with a denaturation step at 95 ^{o}C for 10 minutes, followed by 40 cycles of denaturation at 95 ^{o}C for 15 seconds, primer annealing at 60 ^{o}C for 20 seconds, and primer extension at 72 ^{o}C for 40 seconds, and lastly a final extension at 72 ^{o}C for 5 minutes. The reactions were run in triplicate and error bars indicate that samples were run in multiple independent experiments. The housekeeping gene GAPDH was used as an internal control. Experimental genes were normalized to the housekeeping gene and analyzed using the $2^{-\Delta\Delta Ct}$ method.

Table 2.1 Forward and reverse primer sequences used for quantitative Real-Time PCR (Invitrogen).

Gene	Forward	Reverse
GAPDH	5′-ACATCGCTCAGACACCATG-3′	5′-TGTAGTTGAGGTCAATGAAGGG-3′
p53	5′-GCCATCTACAAGCAGTCACAG-3′	5′-TCATCCAAATACTCCACACGC-3′
p21	5′-TGTCACTGTCTTGTACCCTTG-3′	5′-GGCGTTTGGAGTGGTAGAA-3′
bax	5′-GACATGTTTTCTGACGGCAAC-3′	5′-AAGTCCAATGTCCAGCCC-3′
PUMA	5′-CGACCTCAACGCACAGTAC-3′	5′-CCTAATTGGGCTCCATCTCG-3′

CHAPTER 3

RESULTS

3.1 EVIDENCE OF BINDING BETWEEN P53 AND HIV-1 TAT

Due to the evidence in the literature indicating the possibility of an interaction between the tumor suppressor p53 and the HIV-1 Tat protein our lab decided to test for direct binding between the two purified proteins (Duan, Ozaki et al. 1994; Li, Wang et al. 1995; Clark, Santiago et al. 2000; Gabizon, Mor et al. 2008). We first purified p53 and HIV-1 Tat proteins by cloning cDNAs of Tat-101 and human wtp53 into the pET-52 vector. Ni-affinity chromatography was used to purify the His-tagged proteins from *E. coli*, followed by separation on SDS-polyacrylamide gel electrophoresis with Coomassie blue staining and immunoblotting with anti-p53 and anti-Tat antibodies (Figure 3.1).

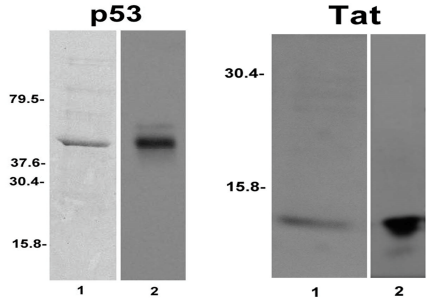


Figure 3.1 Purification of p53 and Tat proteins. His-tagged proteins were purified from *E. coli* by Ni-affinity chromatography, and assayed on SDS-PAGE either with Coomassie blue staining (lane 1) or immunoblotting with anti-p53 and anti-Tat antibodies (lane 2).

Once purified, Tat and p53 proteins demonstrated complex formation in vitro when co-immunoprecipitated with anti-p53 antibody and immunoblotted with anti-Tat antibody (Figure 3.2A). p53-Tat complexes were also co-immunoprecipitated from cell lysates of U2-OS cells transfected with the pcDNA3 Tat expression vector (Figure 3.2B). In collaboration with Dr. Yeh at the BRITE Institute in Durham, NC, complexes were also detected by florescence polarization assay between p53 and Tat peptides that correspond to published interaction sites, 1-35 and 47-57, when increasing the concentration of p53 (Figure 3.2C). Unlabeled Tat competes with FITC-Tat for binding with p53 (Figure 3.2D).

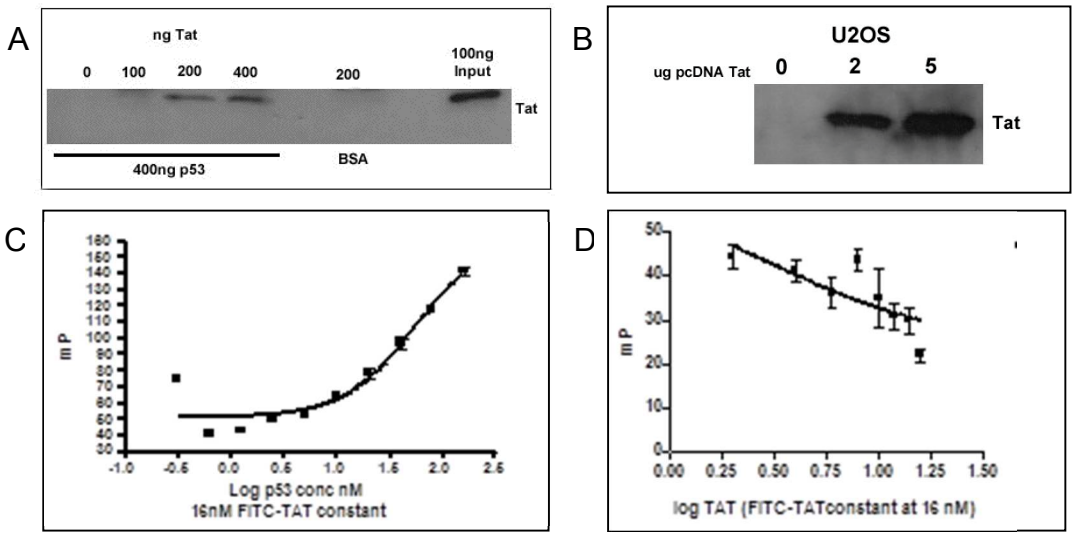


Figure 3.2 Binding of p53 and HIV-1 Tat proteins and peptides. A) Increasing amounts of purified HIV-1 Tat proteins were incubated with a constant amount of purified p53 protein, followed by incubation with anti-p53 antibody coupled to sephadex, then precipitated and analyzed by western blot using anti-Tat antibody. B) Increasing amounts of pcDNA3 Tat expression vector were transfected into U2-OS cells. Cell lysates were incubated with anti-p53 antibody coupled to sephadex, precipitated and analyzed by western blot using anti-Tat antibody. C) Increasing amounts of p53 were incubated with 16nM FITC-Tat and analyzed by florescence polarization assay. D) Increasing amounts of unlabeled Tat peptide added to 160nM p53 and 16nM FITC-Tat and analyzed by florescence polarization.

Considering the evidence that p53 and Tat are capable of binding, we were interested in determining whether this interaction might minimize the innate host p53 response to HIV infection in a manner similar to the interaction between p53 and the p53 inhibitors produced by the small tumor viruses, such as SV40. We tested whether the binding of p53 by Tat has a direct and negative effect on the ability of p53 to respond to DNA damage. Our model system used U2-OS cells, because they naturally express wild-type p53, which were manipulated to also express HIV-1 Tat. We used several DNA damaging agents to induce a p53 damage response in cells both expressing and not expressing HIV-1 Tat and studied the response of p53 through upregulation of its targets, p21 and bax.

3.2 EXPRESSION OF HIV-1 TAT IN U2-OS CELLS

We created three U2-OS cell lines containing wild-type p53 that would also express HIV-1 Tat. To create a cell line in which transcription of Tat could be induced, we transfected a plasmid containing the HIV-1 Tat HA gene under the control of an inducible pTRE2 promoter into Tet-On U2-OS cells. Individual clones were harvested and tested for the presence of the pTRE2 Tat HA expression vector using PCR (Figure 3.3). Positive clones were then used western blotting to test for the presence of the Tat HA protein before and after induction with 2 ug/ml doxycycline (Figure 3.4). We also transfected a pcDNA3 plasmid containing the same HIV-1 Tat gene under the control of a constitutively expressing CMV promoter into U2-OS cells and tested for the presence of the expression vector using PCR and for the HA tagged Tat protein using western

blotting (data not shown). Clones positive for Tat protein were expanded and used in immunoblotting, luciferase assays, and real-time PCR.

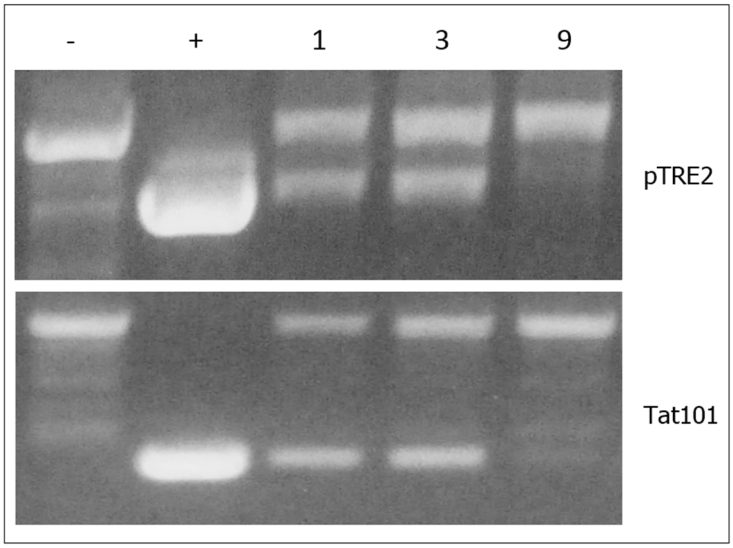


Figure 3.3 Presence of pTRE2 TatHA plasmid in transfected cells. Tet-On U2-OS cells were incubated with pTRE2 TatHA inducible expression vector, pSV2gpt selection vector, and Transfast transfection reagent. Clones were harvested and PCR was performed using primers for pTRE2 and Tat101. Cell lysates from untransfected cells (lane 1), pure plasmid (lane 2), clone 1 (lane 3), clone 3 (lane 4), and clone 9

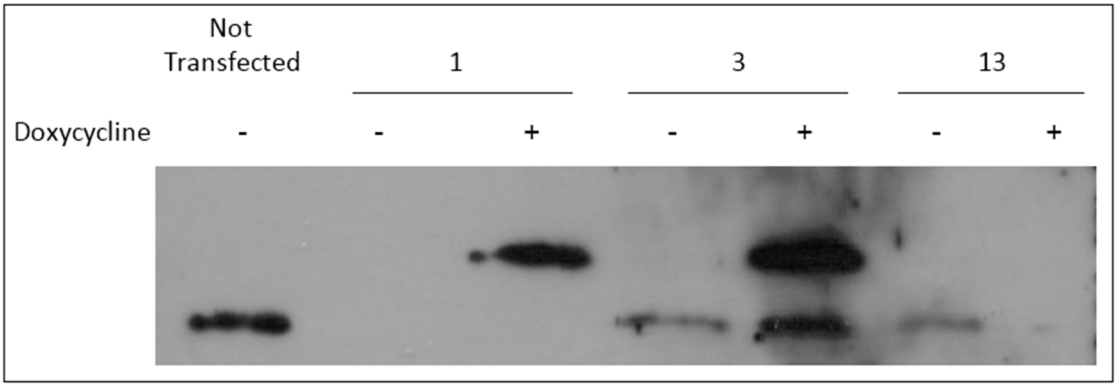


Figure 3.4 Presence of HA tagged Tat protein in transfected cells after addition of doxycycline. Clones were left untreated or exposed to 2 ug/mL doxycycline to induce expression of TatHA protein in positive cells. First lane contains lysate from untransfected U2-OS cells as a negative control.

3.3 CHARACTERIZATION OF THE EFFECTS OF DNA DAMAGING AGENTS ON P53, THE P53 TARGETS, P21 AND BAX, IN CELLS EXPRESSING HIV-1 TAT

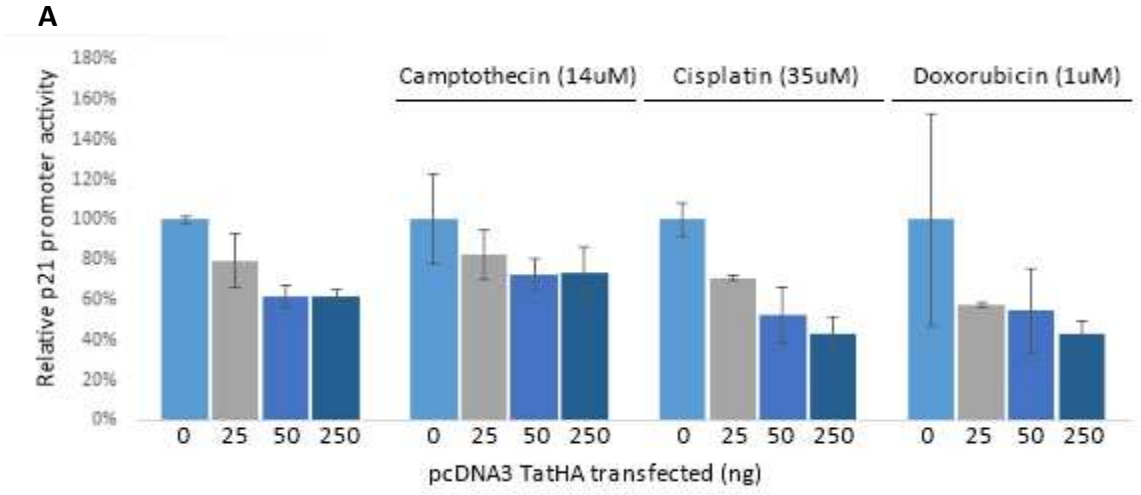
3.3.1 PROMOTER ACTIVITY (LUCIFERASE ASSAY)

In order to determine whether Tat was capable of inhibiting the p53 response to DNA damage, we began by studying the promoter activity of the p53 targets, p21 and bax, in the presence of increasing amounts of Tat using luciferase assays. Typically after exposure to DNA damage an increase in p21 and bax promoter activity is reported. If Tat is able to mitigate the p53 response we would expect to see a decrease in activity from the p21 and bax promoters following DNA damage when Tat is present.

U2-OS cells were co-transfected with vectors carrying the luciferase gene under the control of either the p21 or bax promoter along with either 0, 10, 25, 50, 100, or 250 ng of pcDNA3 HIV-1 Tat. A vector containing the Renilla-luciferase gene was also included and acted as a control for transfection efficiency. Twenty four hours post

transfection some of the cells were treated with DNA damaging agents to induce a p53 response. The DNA damaging agents used were 14 uM Camptothecin, 35 uM Cisplatin, 1 uM Doxorubicin, and 10 uM Etoposide. Luciferase and Renilla activity were measured 24 hours after the addition of DNA damaging agents to the cell culture media. The normalized luciferase reading indicate the relative promoter activity under the observed conditions.

The first set of experiments show a decrease in p21 promoter activity in the presence of Tat, as would be expected if Tat were able to inhibit p53 (Figure 3.5A). The same was true of bax promoter activity when Doxorubicin was added, however not when Camptothecin or Cisplatin were present in the culture media (Figure 3.5B). This decrease in activity was seen for both promoters even when no DNA damaging agent was used to induce p53, which might be explained by Tat decreasing even basal levels of activity. However from these experiments we are unable to determine whether each of the DNA damaging agents can cause an increase in activity from the p21 or bax promoters in the absence of Tat. While these results seem to support our hypothesis,



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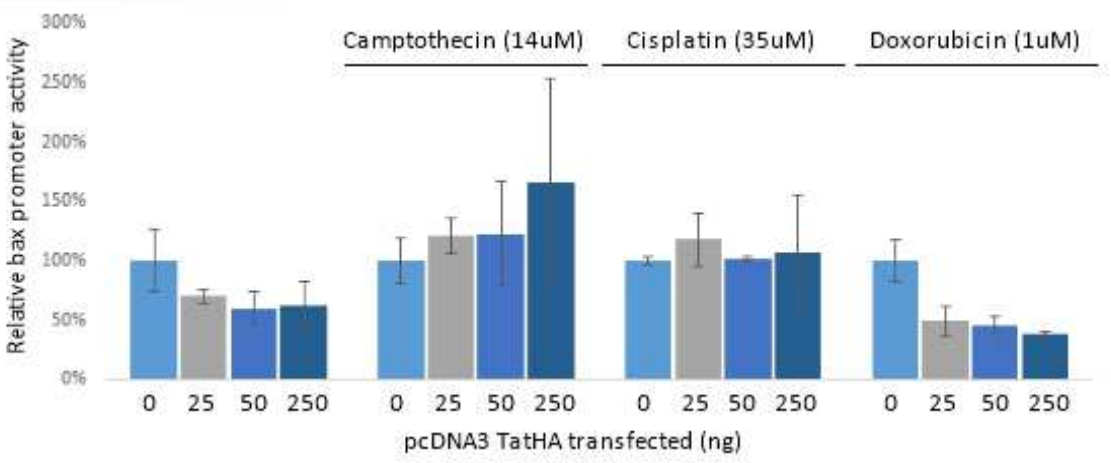


Figure 3.5 p21 and bax promoter activity after transfection with pcDNA3 TatHA and treatment with DNA damaging agents. U2-OS cells were transfected with pcDNA3 TatHA vector at 0, 25, 50, and 250 ng, and a luciferase vector controlled by either the p21 promoter (A) or the bax promoter (B). Twenty four hours later cells were either left untreated or treated with 14 uM Camptothecin, 35 uM Cisplatin, or 1 uM Doxorubicin. Error bars represent normalized standard deviations between duplicate samples.

there were some large standard deviations and small sample size, so we wanted to repeat the experiment and decided to focus on one DNA damaging agent, Doxorubicin.

Next we repeated the luciferase assay three times with duplicate samples of each condition, using only one DNA damaging agent, Doxorubicin. We also included transfections with two nonfunctioning Tat mutants that we did not expect to decrease p21 or bax promoter activity. The results fit our expectations in this regard since none of the data fit a trend in promoter activity decreasing as Tat concentration increases (Figure 3.6). However there was also no evidence of a decrease in p21 promoter activity in the presence of increasing amounts of full length Tat with p53 induction or without (Figure 3.6A). The trend of the data appears to show a decrease in the activity of the bax

promoter with increasing concentrations of Tat, however the large standard deviations cannot allow us to conclude that these data support our hypothesis.

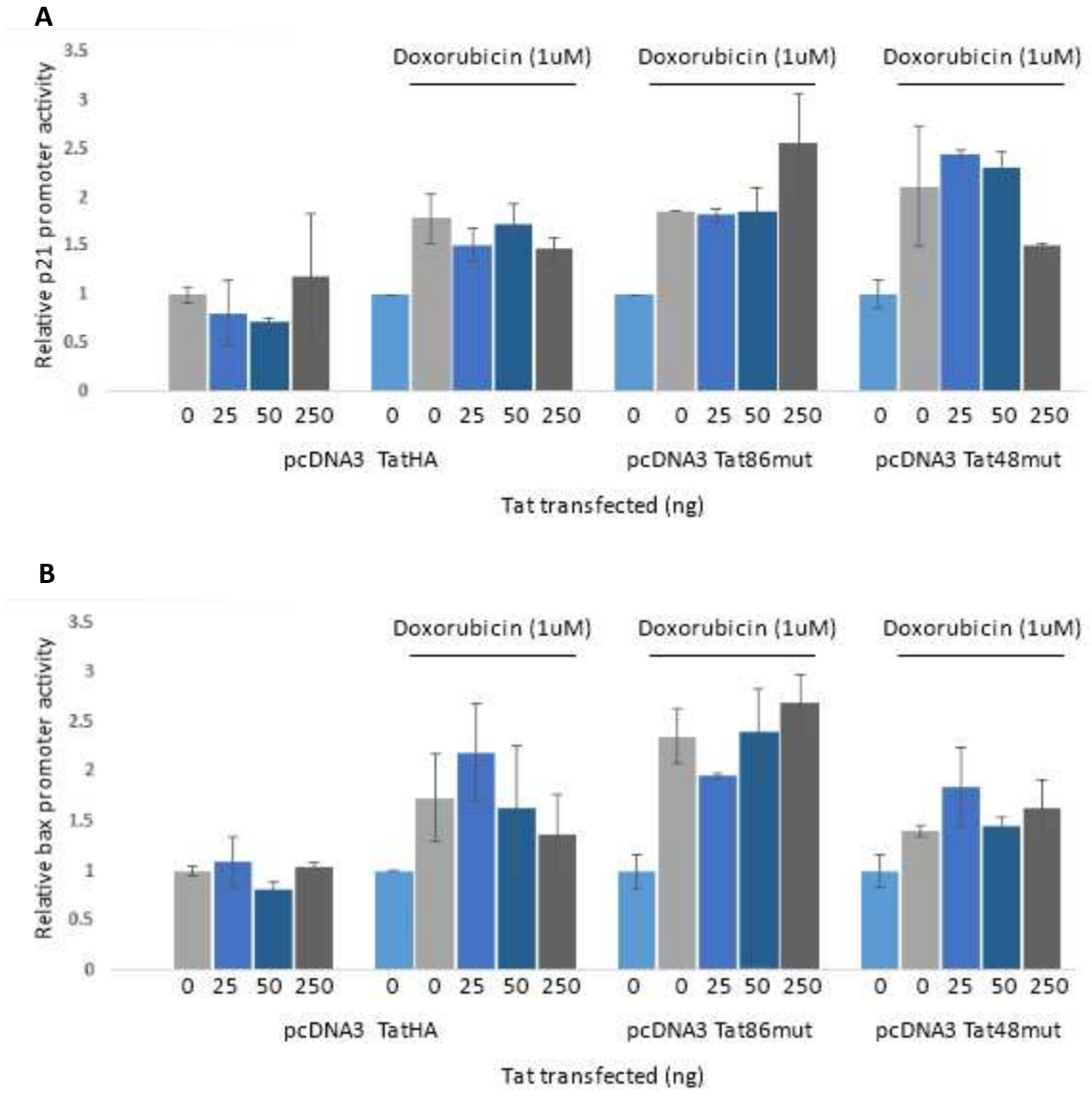
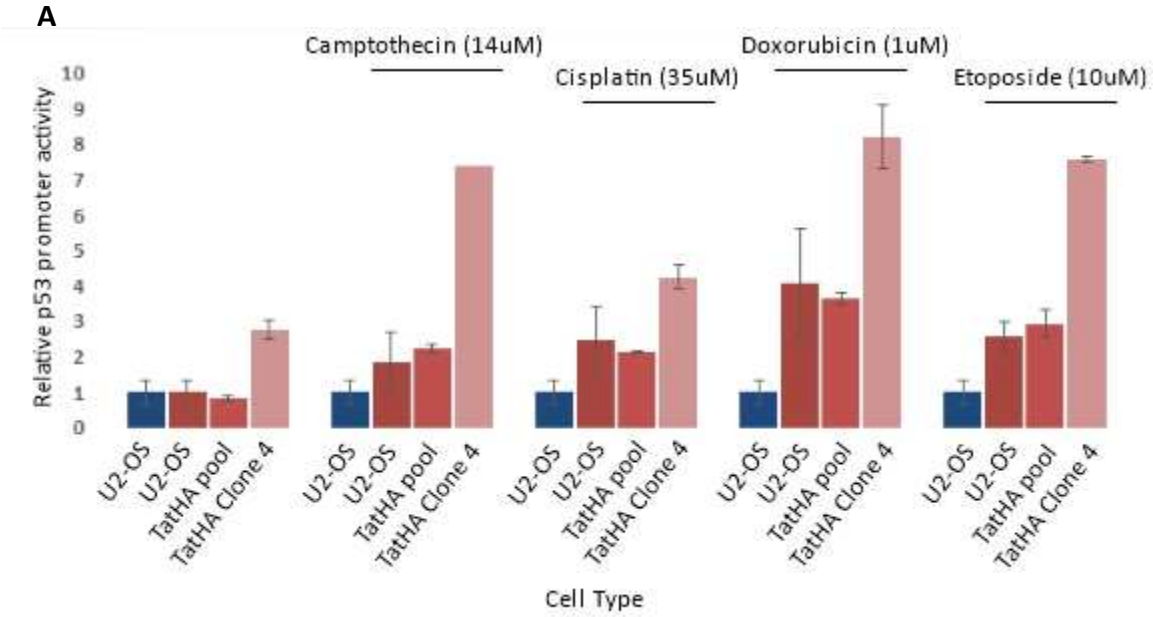


Figure 3.6 p21 and bax promoter activity after transfection with full-length TatHA, or Tat mutants and treatment with 1 uM Doxycyline. U2-OS cells were transfected with pcDNA3 TatHA or a mutant Tat vector at 0, 25, 50, or 250 ng, and a luciferase vector controlled by either the p21 promoter (A) or the bax promoter (B). Twenty four hours later cells were either left untreated or treated with 1 uM Doxorubicin. Error bars represent standard deviations between three sets of normalized averages.

Because we could not be sure of the actual concentration of Tat being produced in the transfected cells, we decided to repeat the experiment with the Tat producing cell lines U2-OS TatHA pool and U2-OS TatHA C4 (clone 4). We again tested the activity of the p53, p21, and bax promoters in the presence of Tat and with p53 induction from DNA damaging agents. U2-OS cells were used as a control. If Tat were able to block the downstream effects of p53 we would expect a decrease in p21 and bax activity in the Tat producing cell lines. Since the p53 promoter is not typically induced, we did not expect to see a difference in activity in the presence of Tat.

While the U2-OS TatHA pool response was similar to U2-OS and did not show a decrease in promoter activity when compared to the Tat free cells, we found it interesting that the U2-OS TatHA C4 cell line that constitutively expresses Tat protein, displayed higher baseline levels (without p53 protein induction through DNA damage) of activity from all three promoters; twice as high from the bax promoter (Figure 3.7C) and nearly three times higher activity from the p21 (Figure 3.7B) and p53 (Figure 3.7A) promoters.



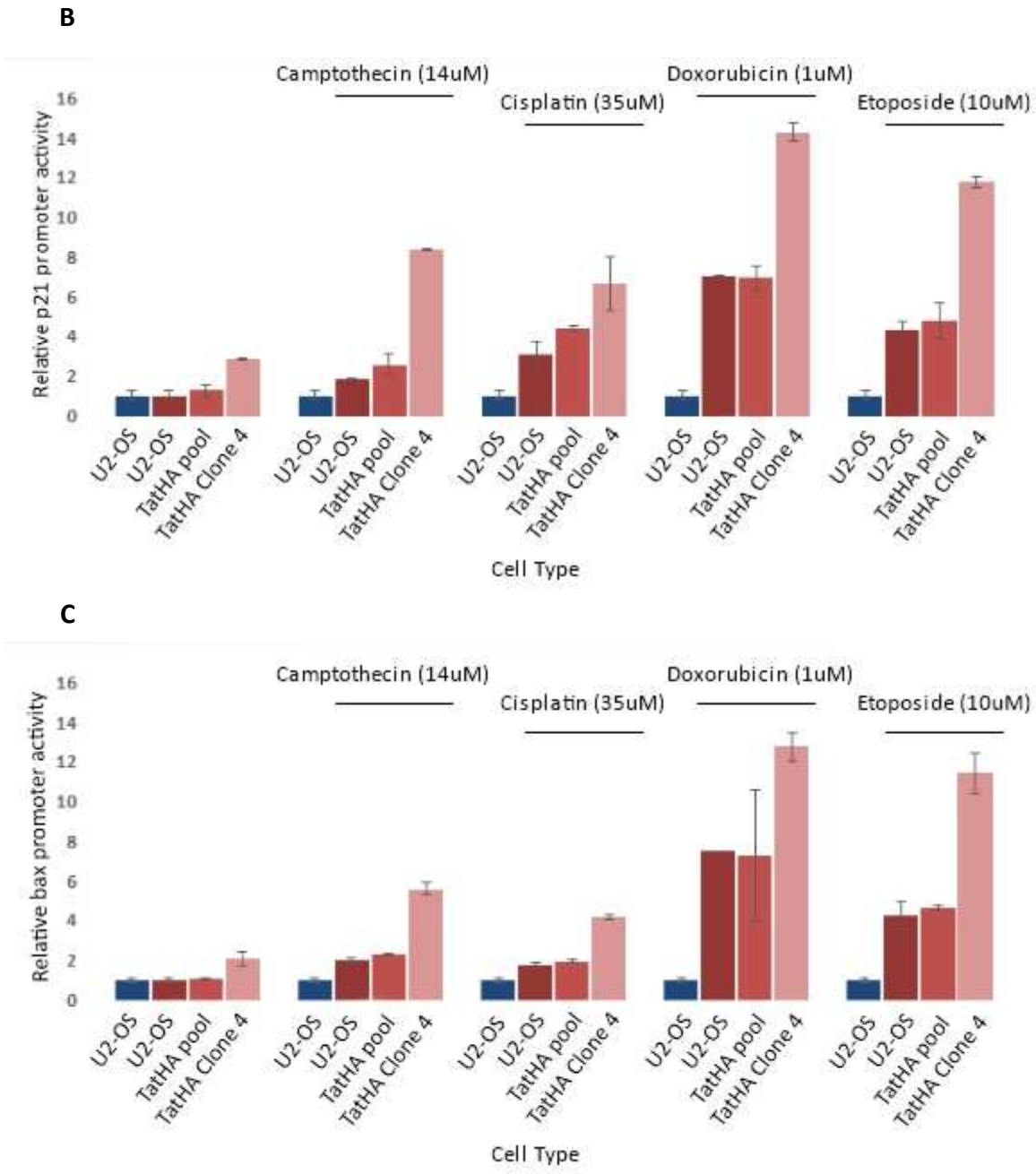


Figure 3.7 p53, p21, and bax promoter activity in U2-OS cells constitutively expressing HA tagged Tat and treated with DNA damaging agents. U2-OS cells and U2-OS cells constitutively expressing TatHA either from a pool or a cloned cell line where transfected with a luciferase vector controlled by a p53 promoter (A), a p21 promoter (B), or a bax promoter (C) and treated with either 14 uM Camptothecin, 35 uM Cisplatin, 1 uM Doxorubicin, and 10 uM Etoposide. Error bars represent the normalized standard deviation of duplicate samples.

There was also still an increase in activity from all three promoters after the addition of DNA damaging agents even in the cells expressing Tat protein. Therefore we again cannot conclude that this data supports our hypothesis that Tat inhibits the p53 DNA damage response.

As was previously discussed HIV-1 infection is complex and the factors involved change in response to numerous influences such as cell type and stage of infection. Due to the complicated nature of HIV-1 infection, we decided to look at p21 promoter activity at intervals over a 24 hour time frame in U2-OS cells and U2-OS TatHA C4 cells both untreated and exposed to the DNA damaging agent Cisplatin. Because the inhibition of p53 by Tat might logically be the most useful early during infection, in order to maximize the rate of infection and number of infected cells before being destroyed by apoptosis, we wanted to measure p21 promoter activity early following DNA damage. Given our hypothesis the anticipated outcome was little change in the p21 promoter activity over time when no DNA damaging agent is added, an increase in promoter activity in the U2-OS cell line upon addition of Cisplatin, and no change or a decrease in activity in the U2-OS Tat C4 cell line upon addition of Cisplatin.

The untreated U2-OS cells show an interesting increase in p21 promoter activity up to hour 8 and then a decrease to hour 24 (Figure 3.8). When the U2-OS cells were treated with the DNA damaging agent Cisplatin at 35 uM the activity of the p21 promoter does not increase from the baseline of 1.0 until hour 14 when it is 2.3 and remains at this higher level of activity at hour 24. The U2-OS cell line constitutively expressing Tat showed a 2 times greater baseline activity at 0 hours than did the U2-OS cell line. The untreated U2-OS TatHA C4 cell line also displayed some level of fluctuation during the

time course, most notably however was the drop in p21 promoter activity from 1.97 at 0 hours to 0.73 at 24 hours, which will be discussed in more detail in the following paragraph. While the U2-OS TatHA C4 cells that were treated with 35 uM Cisplatin did not exhibit this drop at the 24 hour time point, they also did not exhibit an increase in p21 promoter activity at any point over the baseline level of p21 promoter activity found in the Tat positive cell line that would indicate a p53 response to DNA damage similar to that seen in the U2-OS cell line exposed to Cisplatin. While these results do not support our hypothesis in the anticipated manner, they also do not suggest that Tat is a benign factor in U2-OS cells in the presence or absence of DNA damage.

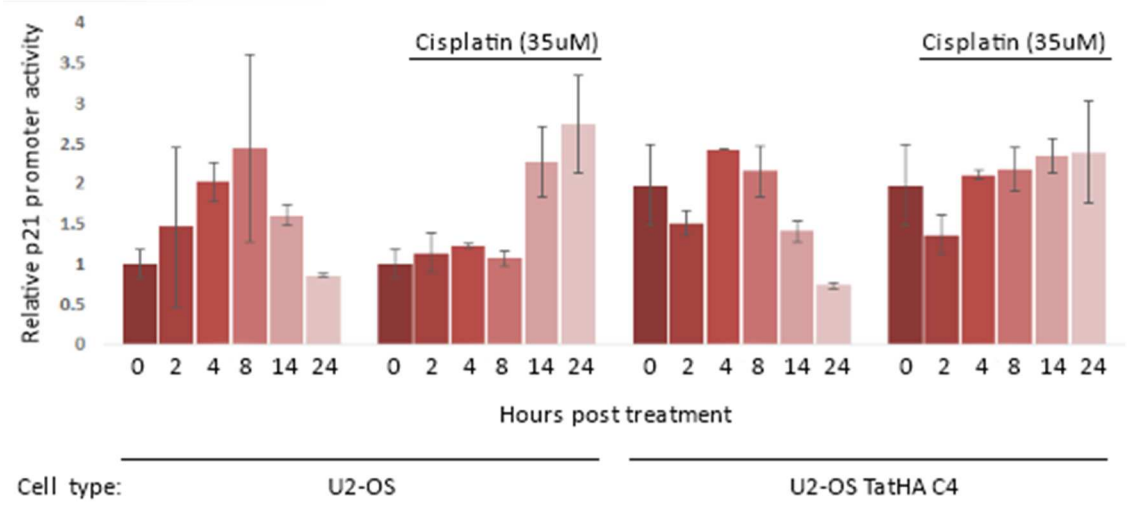


Figure 3.8 p21 promoter activity in U2-OS and TatHA Clone 4 cells, untreated and treated with Cisplatin over 24 hours. U2-OS cells and TatHA C4 cells were transfected with a luciferase vector controlled by a p21 promoter and treated with 35 uM Cisplatin. Cells were harvested 0, 2, 4, 8, 14, and 24 hours prior to treatment with Cisplatin. Error bars represent the normalized standard deviation of duplicate samples.

Moreover the drop in activity in the untreated Tat positive cell line at 24 hours was of particular interest because all of the control samples for the other experiments were harvested at time points that would be analogous to the 24 hour time point shown here. Therefore if this drastic decrease in activity seen at 24 hours was present in the other experiments, including qPCR and immunoblotting, it is possible that our control samples are deceptively low and that is why the experimental samples appear high in comparison. At the very least it illustrates a problem with collecting only one time point when utilizing a dynamic system.

However prior to the final luciferase assay in which we learned the importance of multiple time points, we performed several experiments to observe the effects downstream of promoter activity. We assayed mRNA levels and protein levels of p53 and the p53 targets, p21, and bax using qPCR and immunoblotting.

3.3.2 mRNA EXPRESSION (QUANTITATIVE REAL-TIME PCR)

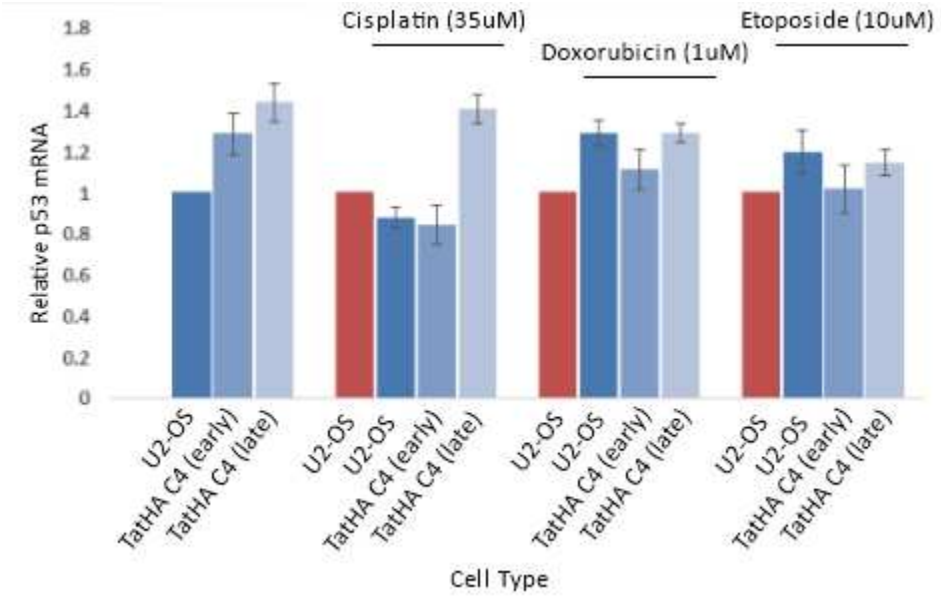
In order to compare the relative levels of mRNA in our experimental cell lines, the cells were either left untreated or treated with a DNA damaging agent and harvested after 24 hours. qPCR was used to amplify the genes p53, p21, and bax. Also due to a suspicion that we were losing expression of the Tat gene after several passages in the TatHA C4 clone we ran the experiment using cells that experienced a minimal number of passages, and cells that experienced double that number. The cells that underwent few passages are referred to as “early” while those that were grown in culture for a month before the experiment are referred to as “late”. If there were a loss of Tat expression occurring in these cells we would expect to see no difference in the control U2-OS cell

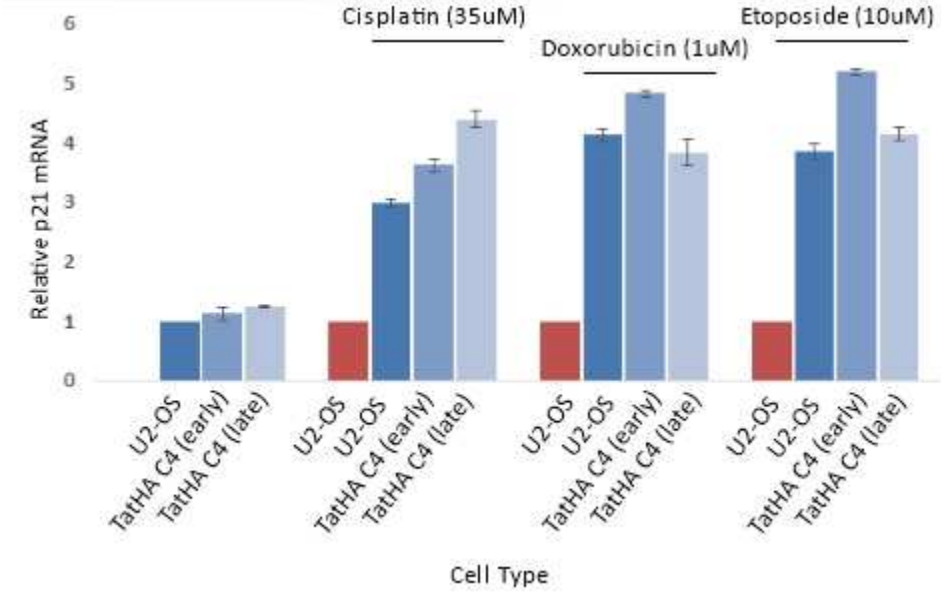
line and the "late" TatHA C4 cell line. A decrease in p21 and bax mRNA expression in the TatHA C4 cell lines after p53 induction via DNA damaging agents would support our hypothesis that Tat is able to mitigate p53 activation of these targets.

There were some differences in mRNA expression in all of the cell lines (Figure 3.9), but the data does not support our hypothesis since neither the "early" nor "late" cell lines exhibit a decrease in p21 and bax mRNA levels compared to the level in the U2-OS cells when induced by DNA damaging agents. The basal p21 mRNA expression level in the TatHA C4 cell line were not higher than the basal U2-OS level in the way that the promoter activity was. The bax and p53 basal mRNA levels were only slightly higher in the C4 cells compared to the basal level in the U2-OS cells, up 1.5 from 1.0.

The differences also do not clearly demonstrate a loss of Tat expression since the expression seen in the "late" TatHA C4 cell line does deviate from the expression of p21 and bax seen in the U2-OS cell line for except the p21 levels when treated with Doxorubicin and Etoposide(Figure 3.9B). Interestingly the mRNA expression in the "late" cells also deviates from mRNA expression in the "early" cells for reasons that are not entirely clear. It is possible that this is another artifact of only selecting one time point in a system that seems to naturally be quite volatile over time.

This experiment was repeated in the TatHA pool cell line and the pTRE2 TatHA inducible cell line with similar results (data not shown).


Cisplatin (35uM)
Etoposide (10uM)
Doxorubicin (1uM)
Relative p53 mRNA
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
U2-OS
U2-OS TatHA C4 (early)
U2-OS TatHA C4 (late)
Cell Type


Cisplatin (35uM)
Etoposide (10uM)
Doxorubicin (1uM)
Relative p21 mRNA
6
5
4
3
2
1
0
U2-OS
U2-OS TatHA C4 (early)
U2-OS TatHA C4 (late)
Cell Type

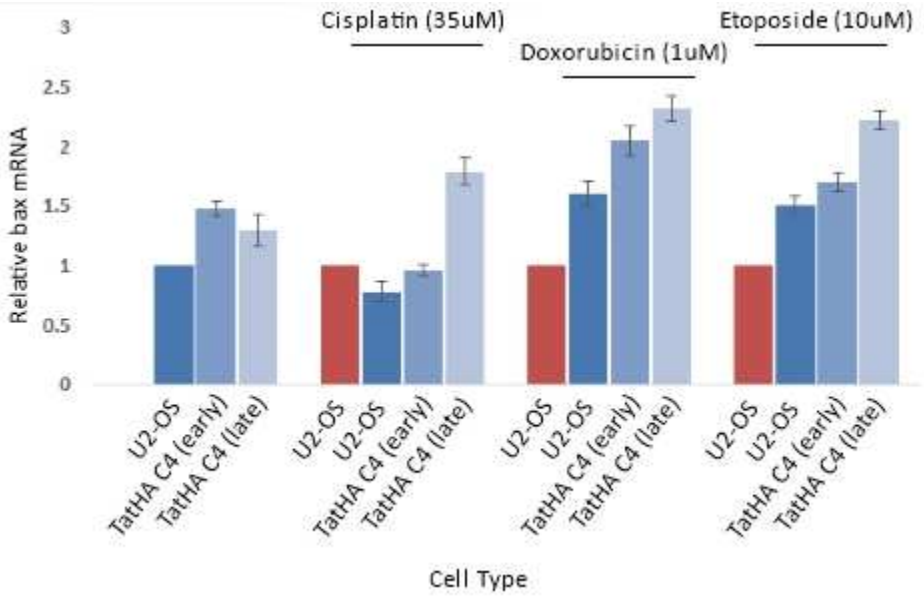


Figure 3.9 mRNA levels in U2-OS and TatHA Clone 4 cells before and after DNA damage. U2-OS and TatHA C4 "early" (few passages) and "late" cells were treated with DNA damaging agents and assayed by qPCR for p53 (A), p21 (B), and bax (C) mRNA expression.

3.3.3 PROTEIN EXPRESSION (IMMUNOBLOTTING)

We also examined p53 and p21 protein expression in the pTRE2 Tat HA inducible cell line, the Tat HA pool, the Tat HA C4 cell line, and the U2-OS cell line 24 hours after activation of p53 with DNA damaging agents. If our hypothesis was correct we expected to see a decrease in the p21 protein in the presence of Tat after activation of p53 with DNA damage. Based on our hypothesis we did not expect to see a change in p53 protein expression. Our initial blot using the inducible Tat HA cell line is not shown due to high background, but it appeared to show a slight decrease in p53. However when the experiment was repeated, the data showed no change in p53 or p21 protein expression when Tat was induced by the addition of doxycycline and p53 was activated by DNA

damaging agents (Figure 3.10). Expression of p21 protein after DNA damage in the Tat HA pool was also assayed and compared to the U2-OS cell line (Figure 3.11). In this case the results indicated that the levels of p21 protein were lower in the Tat positive cell line than in the U2-OS control cell line when p53 is activated by DNA damage. While this appears that Tat may be inhibiting p21 expression it is rather far downstream from the p53 trans-activating step which we hypothesized to be targeted. As discussed in the next section, overall we have not been able to clearly demonstrate an effect of Tat on p53 activity in this model system. There are numerous possibilities for these results aside from the possibility that there is no affect due to a Tat-p53 interaction. Considering the important role p53 has been shown to play in many viral infections, we find it unlikely that a viral protein that binds to p53 during infection has no effect on its activity.

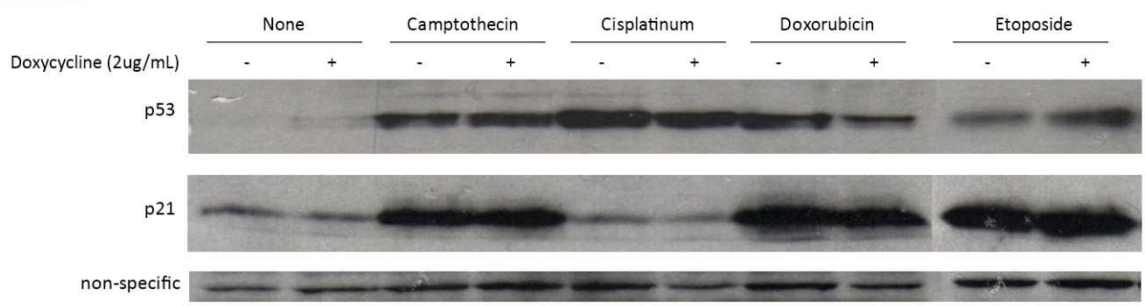


Figure 3.10 p53 and p21 protein expression in pTRE2 Tat HA inducible cell line. p53 and p21 protein levels with and without induction of Tat HA expression by doxycycline, and activation of p53 with 35 uM Camptothecin, 14 uM Cisplatin, 1 uM Doxorubicin, and 10 uM Etoposide. Showing non-specific band as loading control.

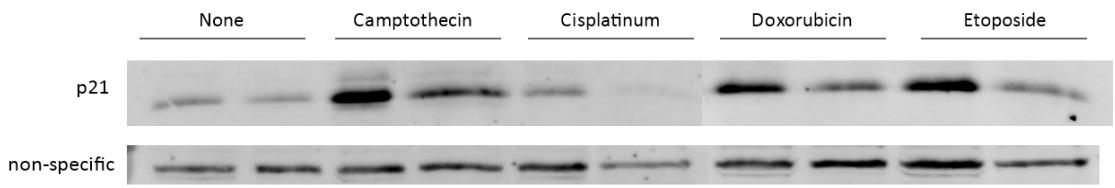


Figure 3.11 p21 protein expression in U2-OS cells and Tat HA pool. p21 protein level with and without presence of Tat HA, and activation of p53 with 35 uM Camptothecin, 14 uM Cisplatin, 1 uM Doxorubicin, and 10 uM Etoposide. Lanes 1, 3, 5, 7, and 9 are U2-OS cell line. Lanes 2, 4, 6, 8, 10 are Tat HA pool. Showing non-specific band as loading control.

CHAPTER 4

CONCLUSIONS

Our hypothesis that Tat could mitigate the ability of p53 to transactivate its downstream targets was based on our own preliminary data and published reports of evidence of binding between Tat and p53 (Clark, Santiago et al. 2000; Gabizon, Mor et al. 2008). There are also reports that Tat is able to regulate transcription of p53 while p53 can mitigate the trans-activating function of Tat (Duan, Ozaki et al. 1994; Li, Wang et al. 1995). Our hypothesis took into account that the small tumor viruses produce factors capable of binding and inhibiting p53 as a way of escaping cell cycle arrest and apoptosis in order to complete the viral life cycle.

Taken in concert our data do not indicate that the presence of Tat can inhibit the ability of p53 to induce the targets p21 and bax when p53 is induced by the DNA damaging agents 14 uM Camptothecin, 35 uM Cisplatin, 1 uM Doxorubicin, and 10 uM Etoposide in U2-OS cells. The results of our luciferase assays, immunoblots, and qPCR do not convincingly and repeatedly show a decrease in the promoter activity, mRNA, or protein of p21 or bax in the presence of Tat when treated with DNA damaging agents.

However we have evidence of a phenomenon in which the activity of the p21 promoter decreases drastically in the presence of Tat at 24 hours. We have also demonstrated that in U2-OS cells constitutively expressing Tat, the p53, p21, and bax promoters are all at least twice as active as in U2-OS cells not expressing Tat. While these results do not support our hypothesis directly they do provide an interesting area of

future research. We plan to further investigate the activity of the p53, p21, and bax promoters in the presence of the DNA damaging agents in the U2-OS and the U2-OS Tat C4 cell lines over a 24 hour timespan. Eventually we hope to expand that to examine the levels of mRNA and protein as well.

We also recognize that the system utilized in this study is imperfect for examining conditions in HIV infected cells. We are currently working on a method of inducing p53 using type-1 interferon instead of chemotherapeutic agents which will induce p53 PTMs and a host cellular response more similar to a cell that has been infected with HIV-1. The DNA damaging agents may be activating a p53 pathway that is unrelated to viral infection. When utilizing IFN to activate p53, we can monitor a p53 target specific to this pathway, IRF9 (Munoz-Fontela, Macip et al. 2008). Also instead of using osteosarcoma cells we think the most biologically relevant cell type for this study would be a T-cell line expressing wild-type p53 or primary T-cells. Our collaboration with the Gatignol HIV lab at McGill University in which infections with HIV are being performed will hopefully allow us to carry out analogous experiments in T-cell lines that have been infected with HIV-1. Although we have been unable to detect a consistent effect of Tat on p53 activity, there were still noteworthy differences in the Tat positive cells that make us eager to move forward in this area of research, exploring the interaction between the tumor suppressor, p53 and HIV-1 trans-activator of transcription, Tat.

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