

**MOLECULAR MECHANISM OF HIV-1 TAT INDUCED NEURONAL
DYSFUNCTION**

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ABSTRACT

In the early years of the AIDS epidemic, being infected with the virus that causes the disease was considered a virtual death sentence. But with the development of highly active antiretroviral therapy (HAART), many infected with HIV-1 are living much longer. In fact, it is estimated that by 2015, about half of all HIV-positive individuals will be older than 50. Yet those over 50 also progress to AIDS faster than adults in their 20s or 30s. And those in the younger age bracket, even those responding well to antiretroviral therapy, still exhibit illnesses and clinical conditions commonly associated with older people, such as HIV-associated neurocognitive disorders (HAND), certain cancers, liver and bones diseases. For the most part, the reasons for this have remained a mystery. However, one may ask, how in the absence of circulating detected virus, viral proteins could cause this kind of damage. The answer is that eradication of latent viruses still unsuccessful and studies showed the persistence of HIV-1 in brain cells as well as the presence of viral proteins in CSF. This notion was supported by the compelling neuropathological data suggesting that the loss of Synaptic Plasticity occurs with the ongoing presence of virus and despite HAART. Clinically, these neuropathological data manifest by a gradual loss of working memory and learning disability, which promote alteration of synaptic plasticity that may manifest by symptoms similar to the ones observed in aged brain or what is called *PREMATURE BRAIN AGING*. Anatomically, working memory and learning ability functions are assured by neurons of the hippocampus, a brain area known-to-be affected by HIV-1 proteins. Mechanistically, several laboratories, including ours, demonstrated that viral proteins perform their functions through deregulation of several molecular pathways that can cause mitochondrial damage (such as depletion of mitochondrial calcium and release of ROS), inhibition of axonal transport leading to prevent neuronal communications or loss of long-term potentiation (LTP). Interestingly, CREB and BDNF

proteins have been shown to play an important role in this phenomenon directly or through its downstream target genes. In here, we examined the impact of HIV-1 Tat on CREB-BDNF pathway and whether Tat is using this pathway to cause neuronal deregulation.

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LIST OF ABBREVIATIONS

3'UTR	3'untranslated region
5-hmC	5-hydroxy methyl cytosine
5-mC	5-methyl cytosine
AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
Akt	Protein Kinase B
ANI	Asymptomatic neurocognitive impairment
AP-1	Activator protein 1
Arf	ADP-ribosylation factor
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BMEC	Brain Microvascular Endothelial Cells
c-ABL	Non-receptor tyrosine kinase
cART	Combination antiretroviral therapy
CCR5	C-C chemokine receptor type 5
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
ChIP	Chromatin Immunoprecipitation
CNS	Central nervous system
COX	Cytochrome c oxidase
CREB	cAMP response element-binding protein

CXCR4	Chemokine (C-X-C motif) receptor 4
DAPI	4',6-diamidino-2-phenylindole
DAT	Dopamine (DA) transporter
DEPC	Diethylpyrocarbonate
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
E2F3	E-box factor 3
EDTA	Ethylenediaminetetraacetic acid
EGR	Early growth response protein
ELISA	Enzyme linked immunosorbent assay
Erk	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GTP	Guanosine-5'-triphosphate
H3K4	Histone 3 lysine 4
HAD	HIV associated dementia
HAND	HIV-associated neurocognitive disorders
HIV	Human immunodeficiency virus
HIVE	HIV-encephalitis

HRP	Horseradish peroxidase
ICAM	Intercellular Adhesion Molecule 1
IL	Interleukin
IP	Immunoprecipitation
IRF	Interferon Regulatory Factor
JCV	John Cunningham virus
LDL	Low-density lipoprotein
LiCl	Lithium chloride
LPA	Lysophosphatidic acid
LRRK2	Leucine-rich repeat kinase 2
LTD	Long term depression
LTP	Long term potentiation
Luc	Luciferase
MAP-2	Microtubule-Associated Protein 2
MCP-1	Monocyte chemotactic protein
MeDIP	Methylated DNA Immunoprecipitation
miRNA	Micro RNA
MMP	Mitochondrial membrane permeabilization
MNI	Mild neurocognitive impairment
MOI	Multiplicity of infection
mRNA	Messenger RNA
MS	Multiple Sclerosis

mtDNA	Mitochondria DNA
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCL	Sodium chloride
NAPDH	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	Nuclear factor-kappa B
NMDAR	N-methyl-D-aspartate receptor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDGF-B	Platelet-derived growth factor subunit B
PIC	Pre-integration complex
PKC	Protein kinase C
PML	Progressive multifocal leukoencephalopathy
PNS	Peripheral nervous system
PSD-95	Postsynaptic density protein 95
PTP	Protein-tyrosine phosphatase
RA	Retinoic acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcription- polymerase chain reaction
RyR	Ryanodine receptor
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulfate
shRNA	Small hairpin RNA

siRNA	Small interfering RNA
SLNs	Solid lipid nanoparticles
snRNP	Small nuclear ribonucleic particles
SP1	Specificity protein 1
Tat	Transactivator of transcription
TET	Ten eleven translocation
TF	Transcription factors
TNF- α	Tumor necrosis factors alpha
TRAF3	TNF receptor associated factor
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UV	Ultra violet
XIAP	E3 ubiquitin-protein ligase
YAP	Yes associated protein

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involved in stabilizing the viral particle and transport of the viral genetic material into the nucleus of the host cell. CA protein is a structural protein responsible for the conical shape of the viral capsid, and the NC protein plays a key role in recognizing a specific stem loop at the 5' end of the HIV-RNA molecule, a necessary step that facilitates the incorporation of newly synthesized nucleic acid into the new virus.

The *Env* (envelope) gene codes for a 160 kDa precursor protein that requires glycosylation by the host cell machinery to be activated and transported to the cell membrane. It is cleaved into two separate proteins: gp41 and gp120. The gp41 protein contains the trans-membrane domain, and gp120 is positioned on the extracellular side of the cell membrane and on the newly formed virus after budding occurs. Gp120 subunit interacts with the CD4⁺ T-cell receptor, CCR5 and CXCR4, mediating the internalization of the virus (Capon and Ward 1991).

The *Pol* (polymerase) gene carries information for an additional three proteins: PRO (HIV-1 protease), responsible for cleaving the precursor polypeptides into mature viral proteins; IN (integrase), crucial in the process of integrating the viral DNA into the host cell genome; and RT (reverse transcriptase), the enzyme required for DNA reverse transcription from the HIV-RNA molecule. These peptides are released by protease (PRO) cleavage of the Gag-Pol fusion polypeptide, which is a product of a translational frame shift of the Gag mRNA (Parkin, Chamorro, and Varmus 1992; Jacks et al. 1988).

1.1.2 Auxiliary and Structural Genes

These genes code for Nef, Vpu, Vif, Vpr proteins (formerly known as accessory) as well as the transactivators Rev and Tat which are essential for the transcription of the HIV genome, and are also known as structural proteins.

Nef (Negative Factor) gene codes for the 27 kDa Nef protein. Nef is the first protein expressed in infected cells with detectable levels. It is believed that Nef plays a role as an inducer of HIV-1 infectivity, since there is an approximately 10-fold difference between HIV-1 strains containing Nef and HIV-1 strains that are lacking this gene. Nef is also linked to the down-regulation of surface CD4⁺ receptor (Kim et al. 1989; Miller et al. 1994).

Vpu (viral protein U) and **Vif** (viral infectivity factor) are genes that code for 14 and 23 kDa proteins respectively. Vpu is found in HIV-1 but not in HIV-2, and plays a role in the process of liberating the interaction between ENV (gp120-gp41) and internalized CD4⁺ receptor that can take place in the endoplasmic reticulum. (Willey et al. 1992).

Vif appears to interfere with the natural antiviral defenses of the infected cells, more specifically by inactivating APOBEC3G protein and preventing its integration into the virus. Recently, Vif has been shown to hijack CBF- β protein to degrade APOBEC3G and promote HIV-1 infection (Stefanie Jäger 2011).

Vpr (viral protein R) gene codes for a 14 kDa protein that is incorporated in the HIV virion. Vpr mediates the ability of HIV to infect non-dividing cells productively by enhancing the NLS (Nuclear Localization Signal) of the PIC (Pre-integration Complex) (Heinzinger et al. 1994). It also has the ability to induce cell cycle arrest *in vitro* by inhibiting the p34cdc2/cyclin B (Re et al. 1995). It has been suggested that Vpr is one of the viral proteins that promote neuronal dysfunction, and recently was shown to contribute in the development of HIV-Associated Neurocognitive Disorders (HAND) (Mukerjee et al. 2011; Jones et al. 2007).

The **Rev** (regulator of virion expression) gene codes for a 23 kDa protein that localizes within the nucleus of the host cell and regulates the export of un-spliced viral RNA from the nucleus to the cytoplasm. This is achieved by the binding of REV to a specific sequence at the

second intron of the viral RNA molecule known as RRE (Rev Response Element) (Malim et al. 1989).

The *Tat* (transactivator of transcription) gene codes for a 13 kDa protein and as its name suggests it is a key activator of HIV transcription. It is one of the first proteins to be expressed after infection occurs. Unlike typical transcription factors that are DNA binding proteins, Tat is a RNA binding protein that recognizes a specific sequence, TAR (Transactivator Response Element), from the HIV RNA molecule (Feng and Holland 1988). Tat is the protein responsible for the recruitment of the host positive transcription elongation factor b (P-TEFb) to an RNA hairpin formed at the 5'-end of nascent viral RNAs (TAR). P-TEFb is a complex composed of Cdk9 and cyclin T1 (CycT1) subunits that serve key roles in globally regulating RNA polymerase II dependent transcription. Tat mediated recruitment of P-TEFb drives the phosphorylation of the C-terminal domain (CTD) repeats of RNAP II by Cdk9. P-TEFb also exists in an inactive form, bound to the inhibitory 7SK snRNP complex, which dissociation is promoted by Tat in order to activate Cdk9. In addition, Tat has been linked to progressive neuronal loss and to the development of HAND (Chang, Mukerjee, et al. 2011; Li et al. 2009; Self et al. 2004). Although the neurotoxic effect of Tat in the central nervous system (CNS) has been studied extensively, the molecular mechanisms involved remain to be elucidated.

1.2 Mechanism of Infection

HIV-1 infects the CD4⁺ lymphocytes with high affinity, which eventually leads to the depletion of this subtype of immune cells and progression to AIDS. The physical interaction between the gp120 and the CD4⁺ receptor forces conformational change in the gp120 glycoprotein, which allows it to bind to specific cytokine receptors, CCR5 (receptor of choice for the R5 tropic virus) or CXCR4 (HIV-X4 tropic virus) (Arrildt KT 2012). The resulting

interaction leads to structural change in the gp41 protein which physically drives the fusion of the viral lipid layer with the host cell membrane (Kwong et al. 1998). Once inside the cell, the nucleocapsid containing the viral RNA disintegrates rapidly and releases the reverse transcription complex (viral RNA, RT, NC, MA, IN, Vpr, Nef, Vif) in the cytoplasm, where it attaches itself to actin microfilaments. Following the initiation of the reverse transcription, the PIC (pre-integration complex) is formed and translocated to the nucleus of the cell where the viral DNA is integrated into the host genome (Levin et al. 2010).

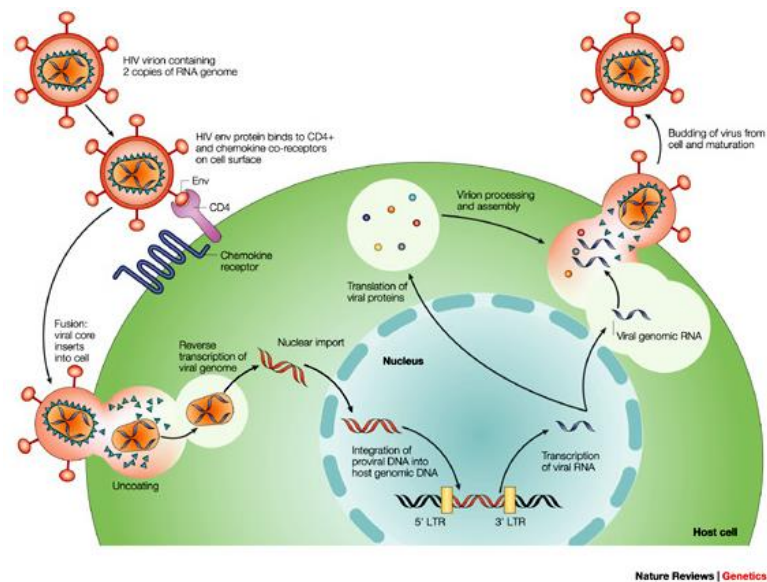


Figure 1.2 HIV Life Cycle (Rambaut et al. 2004).

After the initial infection, cells produce high amounts of viral particles that can infect healthy $CD4^+$, $CD8^+$ subgroup of lymphocytes, monocytes and macrophages. Infected immune cells migrate from the blood stream into various organ systems such as kidneys, muscles, digestive tract, CNS and PNS. This “deep” infiltration of the virus is associated with development of pathological conditions that are normally characteristic of the elderly. Such diseases include but are not limited to: cardiovascular diseases, malignancies, osteoporosis and HIV-Associated Neurocognitive Disorders (HAND) (Senior 2005).

1.3 HIV Associated Neurocognitive Disorders (HAND)

HIV infiltrates the brain soon after the initial infection. The initial “crossing” site of the virus is the Blood Brain Barrier (BBB). The BBB is composed of highly specialized monolayer of Brain Micro-Vascular Endothelial Cells (BMEC) lying on a relatively thick basal lamina. Astrocyte processes extend to the basal lamina and are in direct contact with it. They form a membrane structure that is supported by tight junctions between the cells. The integrity of the BBB is of key importance for the support of brain homeostasis, since it has a selective permeability. It is a physical barrier to pathogenic agents such as bacteria and viruses and to large hydrophilic molecules, but is readily permeable to other small or hydrophobic molecules such as O₂, hormones and CO₂ (Eugenin, Clements, et al. 2011). The exact mechanism used by the virus to cross the BBB remains unclear, however, several mechanisms have been proposed. The “Trojan Horse” mechanism is probably the most accepted, where infected immune cells from the blood stream can migrate through the BBB into the brain (Toborek et al. 2005). Further, in response to the viral infection, lymphocytes and macrophages can stimulate the production of pro-inflammatory cytokines such as TNF- α , IL-1, IL-6, which can affect the BBB adhesion molecules: ICAM-1, VCAM-1, E-selectin. This inflammatory process can lead to further activation, adhesion of immune cells, loosening of tight junctions, and cell death, followed by migration of infected microphages inside the brain (Persidsky et al. 1997).

Migrating macrophages and microglial cells located in the brain are the main reservoirs of infection in the CNS. A small population of astrocytes (around 5%) supports productive HIV infection and delivers constant cytotoxic stress to the BMEC, which can further damage the BBB.

Another proposed mechanism of infiltration of HIV-1 into the brain is the direct crossing of viral particles through the BMEC. Some studies suggest that viral gp120 is inducing active endocytosis and transport of HIV (Banks, Akerstrom, and Kastin 1998). An additional pathway suggests that HIV-1-infected and damaged endothelial cells leads to crossing of the virus into the brain, even though endothelial cells do not express the CD4 receptor. This mechanism is supported by *in vitro* observations that remain to be confirmed *in vivo* (Moses and Nelson 1994).

Viral factors are also able to modify the permeability of the BBB as well. For example, the viral protein Tat can affect the expression patterns of multiple adhesion molecules that are important for the integrity of the membrane and pro-inflammatory cytokines. Tat protein also accumulates in the brain, and it is directly linked to brain tissue damage in mouse models within hours of administration (Arese et al. 2001).

Despite the introduction of the combination antiretroviral therapy (cART), the prevalence of HIV-1 associated neurocognitive disorders remains high due to latent virus reactivation, inability of the drugs to efficiently cross the blood brain barrier, inflammation and neurotoxicity of the cART themselves. The lack of an effective biological test available for HAND requires the use of a neuropsychological approach (Marraa et al. 2009). This is achieved by introducing structured neurocognitive questions and tests, which are assessed and compared to a control group. Those domains allow physicians to diagnose the condition of the affected patients more accurately (Figure 3) (Woods et al. 2009).

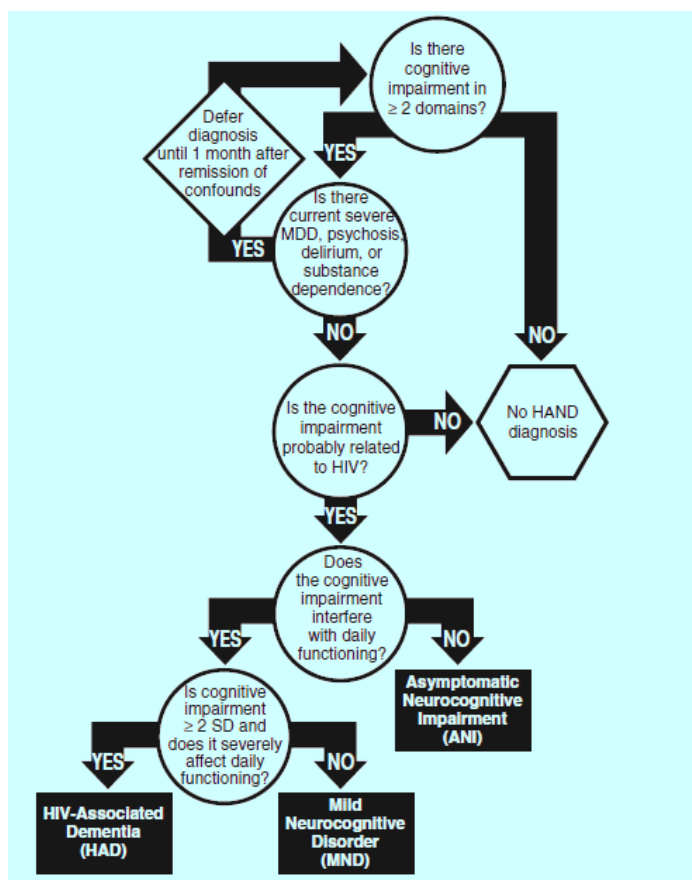


Figure 1.3 Diagnosis of HIV-Associated Neurocognitive Disorders (Woods et al. 2009).

Currently, HAND is divided into three major groups, depending on the degree of impairment of the individual. The severity of the disorder is diagnosed after the review of at least five neurocognitive tests of functions that are known to be impacted in HAND. Those tests include: executive functions, episodic memory, speed of information processing, motor skills, attention/working memory, language, and sensory-perception (Table1) (Valcour et al. 2011).

Table 1. HAND Nomenclature (Valcour et al. 2011).

Diagnostic entity	Cognitive performance	Functional performance
Normal cognition	Normal	Normal
Asymptomatic neurocognitive impairment	Acquired impairment in at least 2 cognitive domains (<1 SD)	Does not impact daily function
Mild neurocognitive disorder	Acquired impairment in at least two cognitive domains (<1 SD)	Interferes with daily function to at least a mild degree (eg, work inefficiency, reduced mental acuity)
HIV-associated dementia	Acquired impairment in at least 2 domains, typically in multiple domains with at least 2 domains with severe impairment (<2 SD)	Marked impact on daily function

The most severe form of HAND is HAD (HIV-associated dementia). It was observed in nearly 30% of the infected patients before the introduction of cART. Patients diagnosed with dementia show a severe impairment in at least two of the neurocognitive domains.

MND or Mild neurocognitive disorder is less severe than dementia but still impacts to some extent the quality of life of the infected person. It is diagnosed by a decline with SD >1 in at least two neurocognitive domains.

The third one is ANI (Asymptomatic Neurocognitive Impairment). ANI is identified under the same criteria as MND, but this condition does not have an impact on the daily activities of a person carrying HIV.

1.4 HIV-1 Tat and HAND

The Trans-activator of transcription (Tat) protein has been implicated in the pathophysiology of the neurocognitive deficits associated with HIV infection (Li et al. 2009). Although it is not the only viral factor contributing to this condition, it is the earliest protein, after Nef, to be produced by the pro-viral DNA in the infected cell.

Tat plays a role not only in the regulation of viral transcription, but may also, after being released from infected cells, interact and affect uninfected cells such as neurons causing cellular dysfunction (Ma and Nath 1997). Once in neurons, Tat can cause changes in host genes expression (Prendergast et al. 2002; Self et al. 2004; Singh et al. 2005; Mukerjee et al. 2008). More importantly, Tat production is not impacted by the use of antiretroviral drugs once the pro-viral DNA has been formed. Intra-cerebral injection of Tat can be lethal to mice within hours of injection (Sabatier et al. 1991). In adult animals Tat affects pre-attentive processes and spatial memory (Fitting, Booze, and Mactutus 2008). In Tat-transgenic mice, glial cell activation and neuronal loss are observed (Kim et al. 2003). Tat causes loss of selective populations of neurons *in vitro* as well as *in vivo* (Maragos et al. 2003). Brain regions particularly susceptible to Tat neurotoxicity include the striatum (Hayman et al. 1993), dentate gyrus, and the CA3 region of the hippocampus (Everall et al. 1995; Maragos et al. 2003) (Cheng et al. 1998). Tat also depolarizes the neuronal cell membrane when applied extracellularly, providing strong evidence for direct excitation of neurons on the cell surface (Cheng et al. 1998). Tat induces increases in levels of intracellular Ca^{2+} in neurons. This is followed by mitochondrial Ca^{2+} level elevation, generation of ROS, changes in the microRNA expression profile of the neurons (Chang, Mukerjee, et al. 2011), activation of caspases and apoptosis (Norman et al. 2007). All this supports the notion that HIV-Tat is a key contributor to HAND progression.

1.4.1 HIV-1 Tat and Microglia

Microglia is a special subtype of CNS immune cells. Unlike the neuronal cells and astrocytes which have neuroectoderm embryonic lineage, microglia cells share the same origin as macrophages and other hematopoietic cells (Perry, Nicoll, and Holmes 2010; Prinz et al.

2011). Previously the involvement of this type of cells in brain diseases was largely seen as secondary to its progression. Currently, more evidence suggests the leading role that microglia cells can play in brain pathologies including infections, facial nerve axotomy, Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), HAND and stroke (Kettenmann et al. 2011; Davoust et al. 2008; Ladeby et al. 2005). Microglia cells play a specific role in the progression of HAND. HIV Tat is shown to be cytotoxic and pro-inflammatory in the context of HAND (Minghetti et al. 2004). One of the physiological markers in advanced stages of HAND is microglial activation and multinuclear giant cells nodule formation. This can lead to changes in their immune effector functions, including phagocytosis and proinflammatory signaling pathways such as TNF-alpha and beta-chemokine production (Hahn et al. 2010; Kiebala et al. 2010). Recently, novel leucine-rich repeat kinase 2 (LRRK2) was identified as a potential pharmaceutical target for microglia activation inhibitor (Marker DF 2012). Protein-tyrosine phosphatase (PTP), CD45 is another promising molecule, since it is upstream from the proinflammatory intracellular signaling mediators (Jin et al. 2012). Additionally, IL-6 induction in microglia cells is NADPH dependent and reversible by the use of specific inhibitors (Turchan-Cholewo et al. 2009). This correlates with recent data showing increase in the release of glutamate, a possible explanation of the neuronal hyper excitability mediated toxicity (Gupta et al. 2010). Cautious optimism in alleviating HAND symptoms brings the fact that Ibudilast, a known non-selective cyclic AMP phosphodiesterase inhibitor that has recently shown promise as a treatment for neuropathic pain via its ability to attenuate glial cell activation also seems to attenuate HIV-Tat induced nuclear factor-kappa B (NF-κB) and TNF-alpha signaling activation (Kiebala et al. 2010; Kiebala and Maggirwar 2011). Interestingly Tat C but not Tat B was able to modulate the levels of tumor necrosis factor-receptor-associated factor 3 TRAF3 in a miR-32 dependent manner and can change the

downstream expression of IRF3 and IRF7 (Mishra, Chhatbar, and Singh 2012). The last might be an important insight, since both molecules are in the base immune activation in response to various stimuli.

1.4.2 HIV-1 Tat and Astrocytes

Most of the HIV Tat *in vivo* studies use mouse models that express Tat under the regulation of GFAP promoter in astrocytes. Unlike microglia cells, astrocytes rise from the same neuroectoderm embryonic lineage as neurons (Kriegstein and Alvarez-Buylla 2009). They are in direct contact with neuronal cells and play critical supportive role in maintaining their homeostasis. Additionally, astrocytes have mechanical and signaling function in the formation of the Blood Brain Barrier (BBB) (Ballabh, Braun, and Nedergaard 2004). Furthermore, astrocytes support productive HIV infection in the CNS, out of reach for almost all anti-retroviral treatments available today (Dunfee et al. 2006). Astrocytes are major contributor to the increased MCP-1 levels in the CNS in the context of HAND, Multiple sclerosis (MS) and other neurodegenerative conditions as well (Weiss et al. 1999; Mahad and Ransohoff 2003; Van der Voorn et al. 1999; Ransohoff et al. 1993). Studies have demonstrated the ability of HIV-Tat to induce MCP-1 in astrocytes through up-regulation of the (PDGF)- β (Bethel-Brown et al. 2012; Bethel-Brown et al. 2011). Wide range of transcription factors have been shown to be affected by Tat specifically. For example EGR-1 promoter is directly activated by Tat via specific serum response sequences within the promoter. This could be interpreted as probably one of the upstream molecular events that initiate Tat-induced astrocyte dysfunction and subsequent Tat neurotoxicity (Fan et al. 2011; Zou et al. 2010). Further, Akt and Erk1/2 survival signaling is affected which can be probably attributed to the up-regulation of MCP-1. Additionally, reports suggest that HIV-1 Tat was able to induce COX-2 and PGE2 synthesis in

astroglial cells through an NFAT/AP-1-dependent mechanism which is in unison with pro-inflammatory conditions in perivascular regions of the CNS, since this enzyme is expressed only in abnormal cellular environment such as cancer and inflammation (Wang YC; Blanco et al. 2008; Kim KN 2012). Another interesting finding demonstrates the importance of HIV Tat cysteine-rich domain in regulating wnt/ β -catenin signaling pathway. As a result wnt/ β -catenin cascade is silenced (Sharma A 2011; Henderson LJ 2012). This leads to abolishment of one of the natural HIV transcriptional suppression mechanisms. Moreover, there is a clear difference in the ability to modify this pathway between clade B and C Tat protein in a di-cysteine motif dependent manner.

Similar to microglia cells HIV Tat is associated with increased levels of nuclear factor-kappa B (NF- κ B) in astrocytes as well (Kiebala et al. 2010; Song et al. 2011), which in turn is linked to upregulation of adherence molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Astarci E; Zhong X 2012). Moreover, significant increases in TLR2 with reciprocal decreases in TLR9 expression in response to Tat are observed. This is usually associated with increase in nitric oxide levels (El-Hage et al. 2011). Interestingly, NADPH oxidase is responsible for HIV-1 Tat-induced generation of ROS and plays an important role in the up-regulation of adherence molecules such as VCAM-1/ICAM-1 (Song et al. 2011). This is important since increased levels of those molecules correlates with increased adhesion of immune cells. These results suggest that HIV-1 Tat disrupts the innate immune response of the central nervous system (CNS) which may lead to increased pathogenicity.

The cell death cascade also appears to be activated as data suggests that p73, member of the p53 family of proteins plays a suppressing role in HIV TAT acetylation by PCAF, a required

interaction for activation of HIV LTR promoter (Amini et al. 2005; Saunders et al. 2005). This appears to be mostly a defensive response to the HIV infection, since it does not necessarily leads to astrocytes apoptosis. However, Tat induction leads to variety of different inclusions characteristic of lysosomes, autophagic vacuoles, and lamellar bodies, which were typically present within distal cytoplasmic processes, (Fitting S 2012) which correlates with disrupted Long Term Potentiation (LTP) and memory formation in Tat transgenic Mice.

1.4.3 HIV-1 Tat and Oligodendrocytes

Oligodendrocytes are the myelin producing cells in the CNS. A single cell can maintain the myelin sheath of multiple neuronal cells, a key morphological characteristic for the proper function of the neuronal cell. Even though, this subtype of resident glial cells has not been shown to support any active HIV virus infection, their crucial role suggests that any disruption in their function can potentially contribute to HAND progression. Although the biological significance *in vivo* is yet to be determine, it has been demonstrated *in vitro* that oligodendrocytes are susceptible to the HIV-Tat protein in a Caspase-3 dependent manner (Hauser et al. 2009). Even though progressive multifocal leukoencephalopathy (PML) is evident in HIV infected individuals, reports appear to be as a result of a co-infection with a JC virus (Aksamit 2012; Datta et al. 2012). Recently, some evidence emerges that the cART treatment might be further contributing to this condition as well (Adachi et al. 2012; Naito et al. 2012). However, much more detailed studies are required to fully understand the effects of chronic HIV infection and the various viral factors on oligodendrocytes in the CNS.

1.4.4 HIV-1 Tat and Neuronal cells

Unlike microglia and astrocytes, neuronal cells does not support productive HIV infection, however they experience a severe cytotoxic stress in the form of viral proteins, pro-

inflammatory cytokines, disrupted BBB and cART (Biscione 2007; Giancola et al. 2006; Heaton et al. 2011; Heaton et al. 2010). Numerous studies have shown the deleterious effect that HIV Tat has in the hippocampal and subcortical and cerebellum areas in Tat transgenic mouse models (Chang, Mukerjee, et al. 2011; Fitting S 2012; Kim et al. 2003). Those pathophysiological changes are not only result of the deregulation of astrocytes and microglia as previously stated. HIV-Tat protein can directly affect the function and viability of neuronal cells as well. Two main theories emerge from the literature about the mechanism of direct Tat neurotoxicity: 1) HIV-1 Tat is able to induce changes in the neuronal cell homeostasis via extracellular signaling mechanism including receptors, changes in membrane permeability and composition. 2) Internalization of HIV-1 Tat protein leads to direct interaction with cellular factors involved with Ca⁺⁺ regulation, transcription and translation.

HIV-1 Tat is extensively studied in *in vitro* systems with apoptosis, impaired synaptic plasticity and Ca⁺⁺ influx appear to be the most cited results from those studies. Various mechanisms have been proposed to explain the dramatic effect that this protein has on neuronal cells. NMDAR receptor for example is often in the researcher's attention, because of its key importance in Ca⁺⁺ regulation and membrane polarization (Volianskis A), both affected in Tat treatment. Recent studies suggest that neuronal cell susceptibility relies on the expression levels of NMDAR. Not only that, but different subunits appear also to exacerbate the effect of Tat. For example rat hippocampal neurons appear less susceptible to Tat even though they highly express NMDAR receptor. This might be related to low levels of NR2A subunit (Eugenin, King, et al. 2011). Moreover, in differentiated human primary neurons, Tat induces phosphorylation at Tyr1184, 1325, and Tyr1425 the NR2A subunit (King et al. 2010). This further validates the important role of NMDAR receptor in understanding the mechanism of Tat induced neuronal deregulation.

Another example supporting the extracellular signaling theory is the growing evidence that indicates that HIV-1 Tat protein may affect the function of the dopamine transmission system. In turn, molecular components of dopamine neurotransmission may participate in a complex network of Tat-induced cell responses which result in neurodegeneration. It appears that Tat induced neurotoxicity has a reverse correlation with the D1 dopamine receptor expression levels and function. Higher levels of D1 will be related to lower apoptosis and blocked function of D1 to increased apoptosis rate in Tat treated cells (Silvers et al. 2007; Aksenov et al. 2008). Interestingly, Tat inhibits dopamine (DA) transporter (DAT) function through a PKC and trafficking-dependent mechanism and that Tat impacts the dopaminergic tone by regulating both DAT and vesicular monoamine transporter (VMAT2) proteins (Midde, Gomez, and Zhu 2012). Those changes, at least in DAT function are related to activation of ryanodine receptor (RyRs) via a calcium- and calpain-mediated process, and is independent of DAT protein synthesis, reinforcing the feasibility of RyR and GSK-3 β inhibition as clinical therapeutic approaches for HAND (Perry et al. 2010). However, the question to which specific RyRs are responsible for this signaling cascade is open for discussion. These findings further provide insight into understanding the mechanisms of HIV-1 viral protein-induced dysfunction of DA neurotransmission in HIV-infected patients.

In addition to cell surface receptor mediated signaling HIV-1 Tat has the unique property of entering the cell in a caveolar and lipid rafts dependent manner. This property of the Tat protein is widely used for mediating the delivery of large protein cargoes into cells when present in the extracellular milieu (Fittipaldi et al. 2004). However, it seems like that this is an understudied area in the subfield of Tat induced neurotoxicity. In BBB endothelial cells for example, Tat treatment leads to elevated GTP-RhoA levels and its downstream effectors, such as myosin phosphatase target subunit 1 and myosin light chain. In addition, Tat upregulated

expression and promoter activity of P-gp as well as its efflux function. Inhibition of the Rho signaling cascade effectively blocked P-gp overexpression at the level of promoter activity. Disruption of lipid rafts by depletion of membrane cholesterol by methyl-beta-cyclodextrin, but not caveolin-1 silencing, also abolished Tat-mediated RhoA activation and P-gp upregulation (Zhong, Hennig, and Toborek 2010). This shows the critical function of intact lipid rafts and the Rho signaling in HIV-1 Tat -mediated upregulation of P-gp at least in endothelial cells even though it is plausible that similar model might be applied in neuronal cells as well. Additional reports suggest that Tat uses numerous receptor mediated pathways including CD26, CXC chemokine receptor type 4(CXCR4), heparin sulphate proteoglycans and LDL (low-density lipoprotein) receptor-related proteins (Xiao et al. 2000; Chattopadhyay et al. 2008; Deshmane et al. 2011; Gutheil et al. 1994). This also suggests an active endocytosis mediated entry of HIV-1 Tat in neurons. Endocytosis is a fundamental function that plays critical role for the maintenance of neuronal function (Nixon and Cataldo 1995). Recently, the endolysosome pathway has been implicated in a variety of neurological disorders including AD (Alzheimer's disease), Parkinson's disease and HAND (Gelman et al. 2005; Nixon and Cataldo 2006). HIV-1 Tat can accumulate in endolysosomes, which leads to endolysosomes size increased, membrane integrity disruption, pH elevation, and autophagy inhibition. Once inside the cells, the protein can be released in the cytoplasm and translocated in the nucleus (Hui et al. 2012). Interestingly, HIV-Tat is associated with higher levels of nuclear and mitochondrial genomic DNA damage in the brain. High levels of nuclear and mtDNA 8-oxoG damage were identified in the cortex autopsy tissue of HAND patients. Increased accumulation of mtDNA mutations and depletion was also detected to occur in brain tissue in a subset of HAND (Zhang, Wang, et al. 2012). However, these results do not discriminate between different cell types in the brain and further validation is required. Additionally, HIV-1 Tat can cause a rapid dissipation of the

mitochondrial transmembrane potential, as well as cytochrome c release in isolated mitochondria. Pharmacological studies reveal that the Tat induced mitochondrial membrane permeabilization (MMP) is Bax/Bak, Bcl-2 and Bcl-XL independent and can be rescued by the anion-channel inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), but not by the ruthenium red, or ryanodine receptor blocker. Moreover, TAT is able to inhibit the cytochrome c oxidase (COX) activity in disrupted mitochondria making it the first viral protein to be a plausible COX inhibitor (Lecoeur et al. 2012). All this is an indicator that Tat can induce mitochondrial dysfunction in neurons independently from the Ca⁺⁺ influx and receptor mediated pathways previously described. Taking into account the central role that mitochondria have in neuronal function, it is easy to see why neuronal cells exhibit high susceptibility to HIV-1 Tat protein.

1.5 HIV-1 and MicroRNAs (miRNAs)

Over the last decade, small non-coding RNAs molecules (~ 20-30 nucleotides) have emerged as critical regulators in the expression and function of eukaryotic genomes. This regulation can occur at several levels of genome function, including chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability, and translation (Eacker, Dawson, and Dawson 2009; Sonntag 2010). The effects of small RNAs on gene expression and control are generally inhibitory, and the corresponding regulatory mechanisms are therefore collectively subsumed under the heading of RNA silencing. The central theme, in this regard, is that the small RNAs serve as specificity factors that direct effector proteins to target nucleic acid molecules via base-pairing interactions (Nelson, Wang, and Rajeev 2008). The two categories of small RNAs are the small interfering RNAs and microRNAs (miRNAs). miRNAs have been defined as regulators of endogenous genes. Involvement of miRNAs in

neurodegenerative diseases has been described and several of their target genes have been identified (Roszbach 2010).

miRNAs and siRNA are conserved in HIV-1-infected cells, however, their complete suppression is incompatible with cellular viability (Bartel 2004). Hence, virus-encoded RNAi-suppressors likely work modestly and in limited settings. Without the ability to fully suppress the cell's RNAi-restriction, HIV-1 can further ameliorate the cell's antiviral defense by mutating viral RNA-sequences to alter the sequence complementarily with cellular miRNAs. Indeed, there is evidence that selective and evasive nucleotide changes in HIV-1 sequences can be elicited rapidly by siRNA/shRNA induced RNAi (Muljo et al. 2005). Additionally, HIV-1 can reshape the infected cell's miRNA expression profile (Triboulet et al. 2007). One interpretation of this latter finding is that the virus has “learned” to repress the expression of virus-targeted miRNAs, while enhancing the expression of propitious miRNAs that upregulate protein factors that benefit HIV-1 replication (Das et al. 2004). This notion was supported by the identification of several human miRNAs (e.g. miR-28, -125b, -150, -223, and -382) that have the potential to target the 3'UTR of HIV-1 transcripts (Huang et al. 2007; Wang, Ye, et al. 2009) potentially rendering productive infection into latency (Houzet et al. 2008; Westerhout et al. 2005; Brass et al. 2008). Using computer-directed analyses, Bennasser and his colleagues found that HIV putatively encodes 5 candidate pre-miRNAs (Bennasser et al. 2004). Several miRNAs were also shown to affect HIV-1 gene expression and replication in vitro and in animal models (Klase et al. 2007). Reciprocally, HIV-1 was shown to affect the expression profile of several host miRNAs (Lama and Planelles 2007). Tat protein was also shown to deregulate expression levels of selected miRNAs, including the neuronal miR-128, in primary cortical neurons (Eletto et al. 2008). Interestingly, some targets of the upregulated miRNAs have been associated with neurological diseases. For instance, miR-378 has been shown to target CYP2E1, a cytochrome

p450 isoform whose polymorphism is associated with Parkinson's disease (Shahabi et al. 2009) and found tightly associated with dopamine-containing cells in the substantia nigra (Watts et al. 1998). Recently, it was reported that Mef2A induces the expression of miR-379-410 cluster in neurons and the expression of this cluster is important for dendritogenesis (Fiore et al. 2009). In addition our studies allowed the identification of 3 highly expressed miRNAs in neurons treated with Tat protein for 24hrs (miR-1, -7-1 and -34a) (Chang, Mukerjee, et al. 2011). All this suggests that miRNA expression patterns play a key role in HAND development since it has been demonstrated that neurons are targeted for injury during the onset and progression of HAND

1.6 HIV-1 and CREB/BDNF

CREB is a 43-kDa basic/leucine zipper transcription factor that is conserved and expressed in most tissues. CREB binds to the octanucleotide cAMP response element (CRE) TGANNTCA. The transcriptional function of CREB becomes activated after its phosphorylation on serine 133. CREB has been shown to be involved in neuronal functions through regulation of BDNF gene expression (Shaywitz and Greenberg 2003).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors. It plays important roles in regulating neurogenesis, synaptic plasticity, and neuronal survival-functions that are vital to learning, memory, and cognition (Zuccato and Cattaneo 2009). In the CNS, BDNF transcription is regulated by neuronal activity with high expression in the hippocampus and cortex. The BDNF gene contains multiple promoters that generate transcripts containing different non-coding exons spliced to a common single coding mRNA. Of the multiple BDNF alternative exons, transcription initiated from BDNF promoter IV is dramatically activated in neurons, after membrane depolarization and subsequent influx

of Ca^{++} (Chen et al. 2003). Abnormal BDNF transcription is associated with altered synaptic plasticity, neurodegenerative disorders, including Huntington, Alzheimer, and Parkinson diseases, as well as psychiatric disorders such as depression and schizophrenia, which could explain the disorders often observed in HIV+ patients.

Suppression of Long Term Potentiation (LTP) is a process which leads to the inevitable cognitive decline of the affected individuals. One of the key pathways involved in LTP initiation is CREB/BDNF. In the context of Tat induced neurotoxic stress, expression levels of miRNA-34a, miR-7 and 1 seem to be increased in primary neurons and in SH-SY5Y cells. Interestingly, miRNA-34a appears to have a reverse correlation with CREB levels, an important positive regulator of LTP. However, the exact mechanism how up regulation of miR-34a inhibits the levels of CREB is elusive, since CREB mRNA does not contain miR-34a binding sequences. Additionally, expression pattern of Brain derived Neurotropic Factor (BDNF) a direct transcriptional target of CREB, has been shown to be altered not only HIV mouse models and HAND human brain sections postmortem but in other neurological paradigms as well, which shows the fundamental significance of this pathway in neuronal cell survival and LTP. One possible explanation of this observation is the existence of an intermediate transcription factor that is under the direct regulation of miRNA-34a and is a positive regulator of CREB. It is known that CREB promoter contains several binding sites for CREB itself as a positive loophole regulator as well as few SP1 sites; however information about additional binding factors is limited. Bioinformatic analysis reveals an additional binding site for the E2F3 transcription factor, part of the E2F family of proteins. The E2F family consists of seven transcription factors with E2F-1 through 3 are known to be positive transcription regulators. Interestingly, miR-34a is able to silence the expression of E2F3 in the

context of prostate cancer and in tissue specific gene expression studies only two of the seven E2F family members are expressed in the CNS: E2F1 and E2F3.

1.7 HIV-1 and P73/P53/CDK9

P73 belongs to a family of p53-related transcription factors including p53, p73 and p63. The overall structure and sequence homology indicates that a p63/p73-like protogene is the ancestral gene, whereas p53 evolved later in higher organisms (Flores et al. 2002). p73 functions in a manner analogous to p53 by inducing tumor cell apoptosis and participating in the cell cycle checkpoint control through transactivating an overlapping set of p53/p73-target genes (Melino et al. 2003). In sharp contrast to p53, however, p73 is expressed as two NH₂-terminally distinct isoforms including transcriptionally active (TA) and transcriptionally inactive (Δ N) forms. Δ Np73, which has oncogenic potential, acts in a dominant negative manner against TAp73 as well as p53 (Rossi et al. 2005). The TA and the Δ N proteins are the predominant forms of p73 in human and mouse brain, respectively (Pozniak et al. 2000).

P73 is stabilized in response to a subset of DNA-damaging agents in a way that is distinct from that of p53, and exerts pro-apoptotic activity. Several lines of evidence suggest that p73 can induce tumor cell apoptosis in a p53-dependent and p53-independent manner (Yang et al. 2000). Alternative splicing of the human p73 gene generates at least 7 variants, α , β , γ , δ , ϵ , ζ and η (TA proteins) and at least 4 alternatively spliced N-terminal isoforms initiated at different ATG (Δ N proteins) (Grob et al. 2001; Moll and Slade 2004; Murray-Zmijewski, Lane, and Bourdon 2006). Genetic studies of p73 protein revealed three functional domains: transactivation domain (AA 1-54), DNA binding domain (AA 131-310), and oligomerization domain (AA 345-380), which are common to α and β isoforms of p73. The p73 gene is expressed in all normal tissues, although the expression is low. The importance of Δ Np73 in

this system is demonstrated by the fact that increasing $\Delta Np73$ levels alone is able to rescue sympathetic neurons from cell death. It should be noted that in neuroblastoma, $\Delta Np73$ failed to inhibit p73, however it did physically interact and inhibit p53. Overexpression of p73 induces transcription of p21WAF1 (Jost, Marin, and Kaelin 1997), and other genes involved in apoptosis. Similar to p53, p73 can be phosphorylated, acetylated, interact with mdm2 without promoting p73 degradation, and degraded by SUMO-1 (Fulco et al. 2003; Minty et al. 2000). Note that p53 cannot induce apoptosis in response to DNA damage without p73 (Yu et al. 2007). This illustrates that p73 is vital for p53-induced apoptosis and furthermore, that p73 itself is an important component of the tumor suppressor activity of p53. Interestingly, miR-34a expression is p53 dependent (Chang et al. 2007; Raver-Shapira et al. 2007). Even more importantly E2F3 was identified as a potent p53 suppressor (Ginsberg 2004).

It is demonstrated that in astrogloma cell line, HIV-Tat physically associates and induces the endogenous levels of p73, however, it inhibits its apoptotic activity and *vice versa* (Amini et al. 2005). Several lines of evidence indicate that stress-induced post-translational modifications of p73, such as phosphorylation and acetylation (Levrero et al. 1999), lead to a marked extension of its half-life. P73 stability is regulated at least in part by the proteasome-dependent degradation pathway, however posttranslational modifications of the protein can lead to further stabilization and accumulation. These results have given us the rationale for identifying the role of p73 along with its associated miR in Tat-induced neuronal cell death.

Interestingly CDK9 was shown to phosphorylate and stabilize p53 (Claudio et al. 2006). Further, CDK9 is associated with the molecular chaperone Hsp70 or a kinase-specific chaperone complex, Hsp90/Cdc37, to form two separate chaperone-cdk9 complexes (O'Keefe et al. 2000). These two complexes act sequentially to facilitate cdk9 folding/stabilization and

the production of the mature cdk9/CycT1 p-TEFb complex. Beside its interaction with CycT1, cdk9 interacts with three other cyclins, T2a, T2b and cyclin K (Fu et al. 1999; Napolitano et al. 1999). Each of the T-type cyclin/ckd9 complexes can phosphorylate the CTD of the large subunit of pol II, but only human (primate) CycT1/CDK9 complexes bind HIV-Tat and allow initiation of transcription. Thus cdk9 and CycT1, in addition to Tat, are key regulators of transcription of HIV-1 gene expression.

1.8 Experimental Objectives

In this study will investigate the underlying molecular mechanism of Tat induced neurite injury, neuronal cell death and the involvement of miRNAs. The proposed studies will provide better understanding for the overall role of HIV-Tat in HAND development and will investigate the role of miRNAs in the context of HIV-1 Tat induced neuronal dysfunction. For that pupouse we will focus on two specific pathways: CREB/BDNF and c-Abl/p73 in the context of neurite stability and neuronal dysfunction. Taken together, the information obtained here will open the door for future research and development of novel therapeutic strategies and disease biomarkers. The strategy to be used is illustrated in Figure 4.

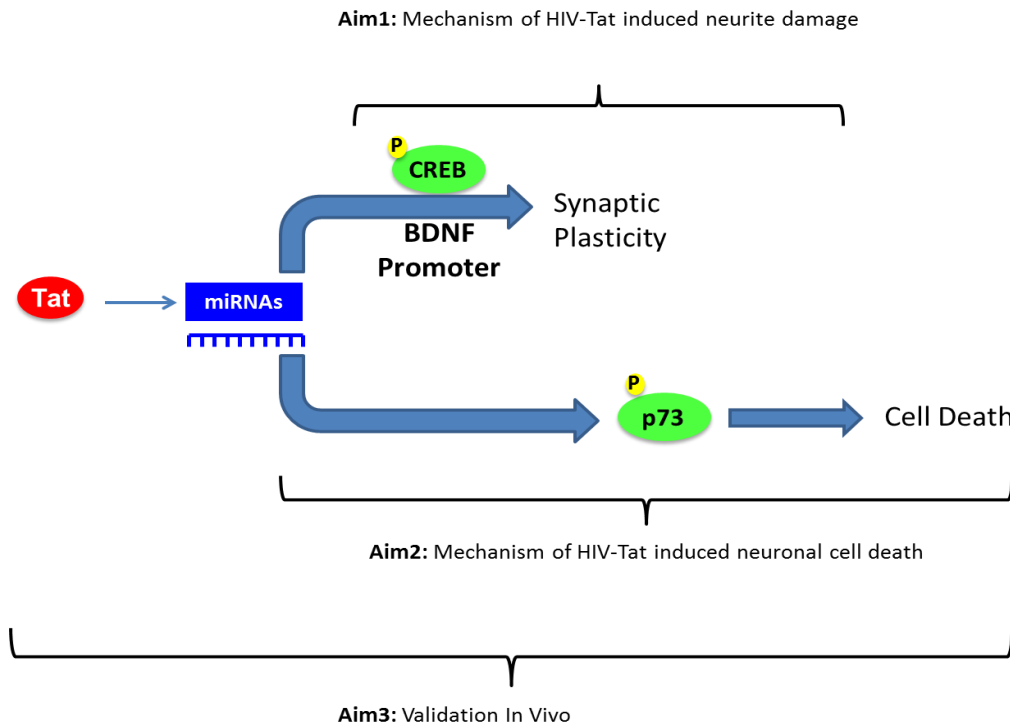


Figure 1.4 Schematic representation of the specific aims.

1.8.1 Specific Aim 1: Molecular mechanism of Tat-induced neurite injury.

The impact of miRNAs on synaptic plasticity is not well elucidated and their impact on functions of the cellular factors linked to development of HAND remains unclear. Therefore, in this aim, we will investigate this impact.

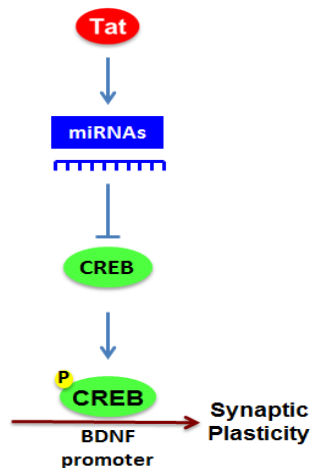


Figure 1.5 Schematic representation of specific aim 1

Approach: miRNAs, CREB, BDNF, and synaptic plasticity

Regulation of the BDNF promoter by phosphorylated CREB is important for synaptic plasticity. We recently demonstrated that CREB is a direct target of miR-34a. miR-34 is highly expressed in Tat-treated neurons (Chang, Mukerjee, et al. 2011). Therefore, we will examine the impact of miR-34a on CREB-regulation of BDNF promoter in Tat-treated neurons and the impact of miR-34a on synaptic plasticity as shown in the scheme.

1.8.2 Specific Aim 2: The role of p73/p53 proteins in Tat-induced loss of neuronal cell viability.

miRNA-34a is part of the cell death cascade regulated by p53. Both p53 and p73 have been shown to be upregulated in HIVE patients postmortem. More interestingly accumulation of p53 leads to a decrease rate of transcription of the HIV-1. In this aim we will investigate the possibility of a miRNA dependent mechanism of neuronal dysfunction.

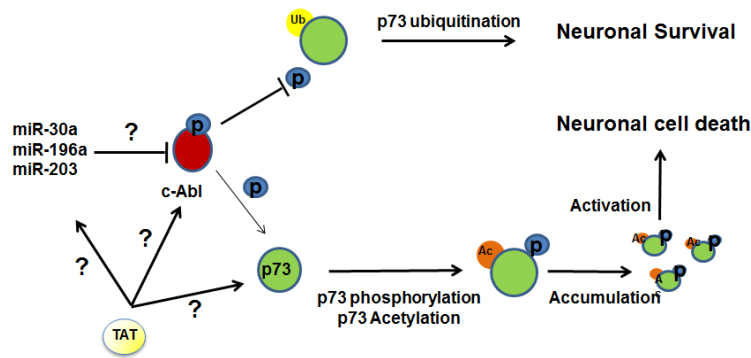


Figure 1.6 Schematic representation of specific aim 2

Approach A: miRNAs, p73 and neuronal viability

To better understand the potential role of p73 in HAND progression, a series of comprehensive molecular studies in neuronal cell cultures derived from the CNS will be performed that will focus on phosphorylation mechanism of Tyr99 residue of the p73 peptide. Phosphorylation of Tyr99 has long been associated with ROS stress and DNA damage, conditions that are characteristic for HIV-TAT induced neuronal stress.

Involvement of miRNAs in regulation of the p73 pro-apoptotic pathway will be investigated as well. The proteins identified to be involved in the regulation of the p73 pathway during Tat induced cell stress will be analyzed bioinformatically (TargetScan 6.2) to identify potential miRNAs seed sequences. Their biological relevance will be investigated by

introducing mimic or inhibitory miRNA molecules to the cells under HIV-1 Tat stress conditions.

Approach B: CDK9/p53 stability and HIV transcription

We previously described the existence of a functional and physical interplay between p53 and cdk9 that leads to accumulation and phosphorylation of p53 (Claudio et al. 2006). Accumulation of p53 affects transcriptional elongation of the HIV-1 LTR, therefore we sought to identify the mechanisms used by p53-cdk9 interaction leading to delaying the transcription of HIV-1. Identification of these factors and pathways will help in the design of new HIV-1 inhibitors

1.8.3 Specific Aim 3: Validation *In Vivo*.

In this aim, the data obtained from Aims 1 and 2 will be validated in Human Brain Tissue and Tat-transgenic mouse animal model using immunofluorescence and *in situ* hybridization techniques.

CHAPTER 2

HIV-1 TAT INDUCES AXONAL RETRACTION THROUGH INHIBITION OF E2F3

2.1 Introduction

One of the major issues associated with HIV-1 brain invasion is the increased incidence of neurite retraction (Gorantla, Poluektova, and Gendelman 2012) (del Palacio, Alvarez, and Munoz-Fernandez 2012). This event was observed even in the presence of the combination antiretroviral therapies (cART). Neurite retraction is the result of secondary complications such as HIV-associated dementia (HAD), mild neurocognitive disorder (MND), and asymptomatic neurocognitive impairment (ANI), with HAD being the most severe and ANI being asymptomatic but with pronounced physiological changes in the CNS (Valcour 2013). The exact molecular mechanisms leading to neurite retraction are not fully understood. Several reports indicated that HIV-infected macrophages and microglia produce neurotoxins (e.g. viral proteins) that have the ability to cause neuronal deregulation. Tat is among the released viral proteins that has been considered to be deleterious to neurons, however the mechanisms used by Tat to cause neurodegeneration remain unclear (Mayne et al. 2000; Chang, Mukerjee, et al. 2011).

The transactivator regulatory (Tat) protein has been implicated in the pathophysiology of the neurocognitive deficits associated with HIV infection (Li et al. 2009). This is the earliest protein to be produced by the pro-viral DNA in the infected cell. The protein not only drives the regulatory regions of the virus but may also be actively released from infected astrocytes and microglia cells and interacts with the cell surface receptors of neighboring uninfected neuronal cells in the brain leading to cellular dysfunction. It may also be taken up by these cells (Ensoli et al. 1993; Kolson et al. 1994; Ma and Nath 1997) and can activate a number of host

genes (Self et al. 2004; Prendergast et al. 2002; Singh et al. 2005; Mukerjee et al. 2008). The Tat protein is highly potent and has the unique ability to enter cells including neurons. Importantly, its production is not impacted by the use of antiretroviral drugs once the pro-viral DNA has been integrated within the host cell genome. Intracerebral injection of Tat can be lethal to mice within hours of injection (Sabatier et al. 1991). In adult animals Tat affects pre-attentive processes and spatial memory. In a Tat transgenic model there is marked glial cell activation and neuronal loss (Fitting, Booze, and Mactutus 2008). Tat causes loss of selective populations of neurons *in vitro* and *in vivo* (Maragos 2003; (Kim et al. 2003). Regions particularly susceptible to Tat neurotoxicity include the striatum, dentate gyrus, and the CA3 region of the hippocampus (Hayman et al. 1993; Everall et al. 1995). Further, neuropathological studies from patients with HIV infection show a preferential loss of neurons in the dentate gyrus and striatum (Cheng et al. 1998). Tat also depolarizes the neuronal cell membrane when applied extracellularly to outside-out membrane patches providing strong evidence for direct excitation of neurons on the cell surface. Tat induces dramatic increases in levels of intracellular Ca^{2+} in neurons followed by mitochondrial Ca^{2+} uptake, generation of ROS, activation of caspases, and eventually neuronal deregulation. These include alteration of synaptic plasticity and suppression of long-term potentiation (LTP) leading to premature brain aging. The exact molecular mechanisms used by Tat to perform these functions are not well understood and remain to be studied.

We recently demonstrated that neuronal deregulation in Tat-treated cells is microRNA-dependent (Chang, Mukerjee, et al. 2011). Using human neuron cells, we showed that Tat up-regulates the expression of miR-34a and down-regulates the expression levels of CREB and BDNF proteins, both factors play a key role in LTP (Kida 2012) (Zhao et al. 2013). In this regard, it has been shown that expression pattern of Brain derived Neurotropic Factor (BDNF)

a direct transcriptional target of CREB, has been altered not only in HIV mouse model and HAND human brain sections postmortem but in other neurological paradigms as well, which shows the fundamental significance of this pathway in neuronal cell survival and LTP (Edelmann E 2013). Intriguingly, neither protein (CREB or BDNF) is a direct target of miR-34a, which points to the existence of an intermediate transcription factor that is under the direct regulation of miRNA-34a and is a positive regulator of CREB. This could also mean that Tat decreases expression levels of these two proteins through an alternative mechanism that yet remains to be determined.

In this chapter, we showed that Tat is using miR-34a and its downstream target E2F3 to inhibit CREB and BDNF proteins functions. We also demonstrated, for the first time, that E2F3 protein is a positive regulator of the CREB promoter. Remarkably, this functional interplay between Tat, miR-34a and E2F3 was enough to cause alteration of synaptophysin distribution leading to neurites retraction and eventually to LTP inhibition.

2.2 Methods

Reagents (antibodies, primers and plasmids). Fluo Am Dyes were purchased from Invitrogen. Anti-E2F3, -HA, -BDNF and -CREB or H3K9 antibodies were purchased from Santa Cruz Biotechnologies and Cell Signaling, respectively. Purified recombinant Tat peptide was kindly received from the NIH AIDS Reagent Program. pcDNA3-E2F3-HA was kindly received from Dr. Joseph R. Nevins (Duke University). pGL3-BDNF4-Luc plasmid was obtained as follow: BDNF4 (1.3 kb) promoter sequence was synthetically generated by GenScript and received in a pUC57 backbone plasmid. The insert was released (*NcoI/SacI*) and inserted into the same sites into pGL3-Luciferase.

Cell Culture and treatments. SH-SY5Y neuroblastoma cells were maintained in F12 DMEM 50/50 supplemented with Sodium Pyruvate, Non-essential amino acids and 10% FBS final concentration. Cells were seeded in 50% confluence and differentiated with 10 μ M Retinoic Acid for at least 48 hours prior to Tat treatment (10ng/ml final concentration). Only SH-SY5Y cell in passage 25-35 were used.

Primary Cultures. Primary neuronal cultures were prepared as previously described (Niethammer et al. 2000) with minor modifications. Briefly, embryos were removed from mice at embryonic day 18. Separate the cortex and rinse in HBSS before digested in 0.125% trypsin. Triturate the digested cortex and dissociate into single cell suspension in culture medium (DMEM containing 10% FBS). Cell suspension was centrifuged at 200 g for 10 minutes and the pellet was gently re-suspended in culture medium. Pass the cell suspension through mesh #400 to remove the non-dispersed tissue and seed the cells at the density of 1.5x10⁵ cells/ml into 4-chamber slides coated with poly-D-lysine. Cultures were incubated in an incubator at 37°C in an atmosphere of 5% CO₂ in air. Change the medium into Neurobasal supplemented with 2% B27 and 0.5mM glutamine the next day. Change half of the medium every 3 days.

Chromatin Immunoprecipitation (ChIP). SH-SY5Y cells (10⁷) were transfected and/or treated with HIV-1 Tat protein and subsequently were fixed with 1% formaldehyde (final concentration) for 15 minutes, followed by 125mM glycine (final concentration) for 10 minutes at room temperature. Cells were then washed twice, incubated for 30 minutes on ice, and sonicated to shear the DNA. After sonication, the lysates were centrifuged and the supernatants were diluted. The chromatin was immunoprecipitated with anti-CREB, anti-E2F3, anti-H3K9 or anti-p53 antibodies. Antibodies were eluted from the immune complexes and cross-linking was reversed by heating at 65°C overnight. The following specific primers were used: +1

CREB site: sense 5'-agcctgccgtgtggtcat-3'; antisense 5'-tgaagctgggactcccccaacc-3'; +1600 CREB site: sense 5'-atagaagggcatgacacggga acc-3'; antisense 5'-ccgagggaaaacaaaacagcactcat-3'; -1600 CREB site: sense 5'-ccagggatacacagagaaacc-3'; antisense 5'-gtcagagtgggcatagatg-3'; BDNF4: sense 5'-tggggtgggggagtcaccag-3'; antisense 5'-accgtgctggc ctttcagc-3'; BDNF1: sense 5'-aggcatgacacgggaaccagact-3'; antisense 5'-agaggcctaggtcgggacaca-3'; miR-34a (-1500): sense 5'-aacatgggctcatcacagacacct-3'; antisense- 5'-aggtgcgtaacacatttgggcac-3'; miR-34a (+1500): sense 5'-taagtcaaaggccctgtgtttg-3'; antisense 5'-agctgcagtactgatgtgtctct-3'; MDM2: sense 5'-cagtaagcacc gacttgcttga-3'; antisense 5'-ttccgaagctggaatctgtgaggt-3'.

RNA isolation and quantitative real-time PCR. Total RNA was isolated by using TRIzol reagent following to the manufacturer's protocol. For mRNA quantification, cDNA was synthesized using SuperScript® VILO™ cDNA Synthesis Kit. Quantitative real-time PCR was performed in triplicate using Faststart universal SYBR green mix (Roche) on an Eco system (Illumina). The mRNA level was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The following human primers were used: miR-34a: Primer mix (Exiqon), GAPDH: sense 5'-tcgacagtcagccgcattctttt-3'; antisense 5'-accaaaccggtgactccgacct t-3'; CREB: sense 5'-aaagcagtgacggaggagcttga-3'; antisense 5'-ggctgggcttgaactgtcattt-3'; BDNF Exon 1: sense 5'-tggttctctgctctgctgtgcta-3'; antisense 5'-tccgaaatctcgggaaataggc a-3'; BDNF Exon 2: sense 5'-tagcgg ttaggctggaatagact-3'; BDNF Exon 1 and 2: antisense 5'-ggcagccttcatgcaaccaagta-3', BDNF Exon 3: sense 5'-cttagagggtcccgtttctcaa-3', BDNF Exon 4: sense 5'-gagcagctgccttgatggttactt-3', BDNF Exon 3 and 4: antisense 5'-aagccacctgtcctcggatgtt-3'.

Visualization and Measurement of Synaptophysin. Primary neuronal cells were cultured for 5 days before transduced with CellLight Synaptophysin-RFP BacMam at MOI of 5. Cells

were then cultured for additional 24 hours before Tat (10 ng/ml) protein was added. Images were acquired on live cells 24 hours post Tat treatment. Images were processed; synaptophysin vesicles number and size were quantified with Image J Software using the Compute Curvatures and Analyze particles plugins. Images from at least 10 different fields of two independent experiments were used for statistical analysis.

Live cell Imaging. Images of differentiated and Tat-treated SH-SY5Y cells grown in 100 cm² dishes were taken every 30 minutes for 96 hours using an EVOS AMD microscope (40x objective) installed inside the incubator. Images were analyzed using ImageJ software. Each image was then analyzed with the *compute curvature* plugin with Gaussian convolution sigma parameter set at 1, followed by a conversion to a binary image and finally surface area of the cells analyzed with the *measure* function. Surface area was expressed as a percentage covered surface per cell.

Fluo3 Calcium Indicator Assay. Fluo3 in 5mM working solution containing 0.02% Pluronic in DMSO was added to the differentiated and Tat-treated SH-SY5Y cell medium for final concentration of 5 μM for 30 minutes at 37°C. Cells were then washed 3 times with 1 ml of media. A confocal microscope equipped with live cell imaging setting was then used.

Immunohistochemistry. Frontal lobe brain tissues from HIV-positive patients with varying degrees of dementia, along with non-demented and HIV-negative controls were obtained from the national neuroAIDS tissue consortium (NNTC). The formalin-fixed and paraffin-embedded tissues were sectioned at 5 μm thickness and placed on electromagnetically charged glass slides. Sections were deparaffinized in xylene and re-hydrated through descending grades of alcohol up to water. Non-enzymatic antigen retrieval was performed in citrate buffer for 30 minutes at 95°C in a vacuum oven. Sections were then rinsed with PBS and

permeabilized in 0.2% Triton in PBS for 45 minutes at room temperature. Sections were rinsed again with PBS, and a blocking step was performed with normal BSA serum at room temperature in a humidified chamber for 2 hours. Primary antibodies were incubated overnight at 4°C and later for 1 hour at room temperature with fluorescently labeled secondary antibodies. The tissues were subsequently washed in PBS until finally mounted with DAPI containing medium (Vectashield).

Immunofluorescence. Cells were fixed for 3 minutes in 2% paraformaldehyde, rinsed with PBS and blocked with 1% BSA for 1 hour. A specific primary antibody (1:100 dilution) was incubated for 2 hours at room temperature (or overnight at 4°C), after which cells were incubated with a fluorescein-tagged secondary antibody for 1 hour at room temperature. Cells were then washed and mounted with DAPI containing medium. A LEICA EL6000 DMI3000 confocal microscope system was used with an UV laser (405 nm), an Argon laser (488nm wavelength), and a HeNe laser (543 and 633nm wavelength). Z-sections at the depth of 0.25-0.45 mm were generated. In some cases contrast and/or intensity was adjusted to improve comparison of different stains. When applied, these changes affected the entire panel.

In Situ Hybridization. miRCURY LNATM microRNA detection (FFPE), optimization kit 4, and hsa-miR-34a detection probe (3'-amino-labeled) were purchased from Exiqon. The experiment was performed as suggested by the manufacturer using mice brain tissues (parental and Tat transgenic) or human brain tissues (brain samples from HIV-1-infected patient with encephalitis and normal control brains were obtained from NNTC).

Western blot. Samples were lysed in RIPA buffer. Twenty micrograms of total extracts were subjected to Western blot analysis using specific antibodies as indicated. Densitometry analysis of the gels was carried out by using ImageJ software.

Statistics and Bioinformatics. Statistical analyses were used in all the experiments using Student's *t* tests or unbalanced analysis of variance. Furthermore, each experiment was repeated three times and the results were considered statistically significant if $p < 0.05$. All Results were expressed as mean \pm SD. Pubmed (<http://www.ncbi.nlm.nih.gov/pubmed/>) and Ensembl (<http://useast.ensembl.org/index.html>) were used to identify promoter and gene coding regions. ECR Genome Browser (<http://ecrbrowser.dcode.org/>) and Target Scan Human (http://www.targetscan.org/vert_61/docs/help.html) were used for transcription factors binding site and miRNAs prediction identification.

2.3 Results

HIV-1 Tat protein has been shown to be associated with neuronal dysfunction however, the exact mechanisms involved are not fully understood. In this regard, we previously demonstrated the ability of Tat to induce changes in miRNAs expression in neuronal cells leading to the deregulation of expression levels of several cell factors implicated in long term potentiation and depression (LTP and LTD) such as CREB and BDNF. In here, we aimed to decipher the mechanisms used by Tat to cause such a deregulation leading to neurites retraction by focusing on CREB-BDNF pathway.

HIV-Tat protein induces neurite retraction. First, we assessed the effect of extracellular Tat on calcium secretion status. Human neuronal cells, SH-SY5Y (1×10^5) grown in serum-free media, were treated with 10 nM (final concentration) of recombinant Tat protein for 30 minutes. The cells were incubated with Fluo-3 and intracellular calcium was measured every 3 seconds using confocal microscopy (Figure 1A). Within seconds, Tat addition led to a dramatic increase of intracellular calcium [Ca^{2+}]. A montage of calcium increase is displayed in Figure 1B.

Next, we examined whether Tat induced $[Ca^{2+}]$ spikes can lead to neurite damage. We recorded in live cell setting the ability of the cells to branch out and to communicate with other neurons. This was followed by measurements of the actual surface area covered by SH-SY5Y cells that were seeded in duplicate (mock untreated or treated with Tat) in approximately 40% confluences in differentiating medium. The cells were monitored for 20 hours prior to the addition of 10 ng/ml of recombinant Tat protein and then monitored for an additional 12 hours. Automatic bright field contrast-snap shot images were taken every 30 minutes to evaluate the status of neurite outgrowth progression. As shown in Figure 1C, measurement of the covered area revealed that addition of Tat resulted in neurite retraction and a decrease of the covered area that lasted until the 32nd hour followed by a modest recovery when compared to the mock untreated. The linear trend analysis (blue line) points to a negative slope effect of Tat protein on the ability of the cells to produce axons. An example of the dendritic alteration is shown in Figure 1D. Note that the measurement was done based on the average area covered per cell independently ability of Tat to cause cell death (Saunders et al. 2005; Sabatier et al. 1991).

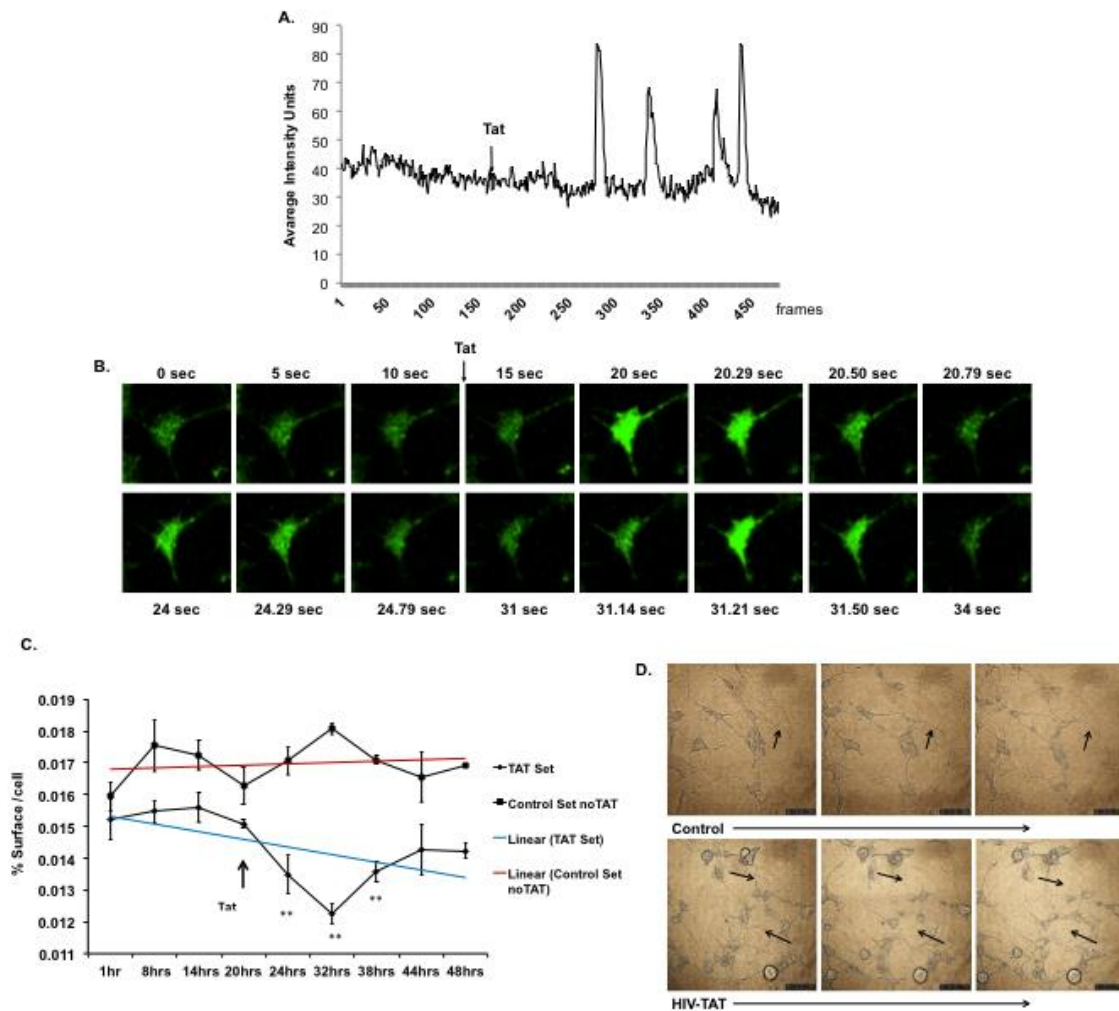


Figure 2.1 Tat promotes neurite retraction. **A.** Calcium levels in Tat-treated neurons. SH-SY5Y cells were incubated with Fluo-3 (Molecular Probes) and intracellular calcium (green) was measured every 3 seconds using confocal microscopy as previously described (Chang et al, 2011a). **B.** The figure represents a montage of images collected from cells at different time points. All measurements were performed using $n= 100$ and the results are statistically significant using Student's t test ($p<0.05$). **C.** SH-SY5Y cells were seeded at 40% confluence in 6 well plates with 10 μ M retinoic acid medium. Plates were placed on a live cell imaging station and bright field contrast images from the same field were acquired every 30 minutes. Images were analyzed with Image J software and the surface area covered by the cells

was normalized against the total number of the cells in each of the time points. The experiment was repeated with Tat supplemented medium added at 20th hour. **D.** Time lapse live cell imaging of differentiated cells with (bottom panels) or without (top panels) Tat protein in the medium. Arrows point to an apparent differences in the morphology of the cells. The experiment was repeated 3 times, the results are statistically significant using Student's *t* test (** $p < 0.005$) compared with the *Mock* control group

These results gave the rationale to examine the functional impact of this deregulation on cells and mainly on neurite stability. It has been shown that synaptogenesis and the stability of synapses are key players for proper neuronal function, signal transfer and LTP (Li et al. 2011). Therefore, we attempt to visualize and quantify the synaptic formation. Using the Cell Light transduction system (Life Technologies), we transduced an RFP tagged synaptophysin (MOI 5) into primary mouse neurons (mock, Tat-treated, transfected with E2F3 and/or treated with Tat). Note that synaptophysin visualization is used as a marker for synaptic structures (Dailey et al. 1994). Primary Mouse neurons and human differentiated SH-SY5Y cells did not exhibit morphological changes in response to the virus transduction. As shown in Figure 2A, quantification of synaptophysin puncta (red) corresponding to synaptic ending were significantly less in Tat-treated mouse neurons compared to mock treatment. Additionally, a clear and obvious morphological changes were observed in cells treated with Tat when compared to the mock untreated. Quantification of the positive puncta revealed significant decrease at 24 hours after Tat treatment (Figure 2B) without affecting the size of the synaptic vesicles (panel C).

To further confirm this observation, we examined the status of reelin protein (RELN) in Tat-treated cells. Reelin is a protein that regulates dendrite growth and controls cell-to-cell interactions during development (Dijkhuizen and Ghosh 2005). In adult brain, reelin modulates synaptic plasticity by enhancing the induction of the LTP (Weeber et al. 2002). Reelin loss or inhibition was shown to be associated with neurodegenerative diseases such as schizophrenia (Impagnatiello et al. 1998). Therefore, we measured expression of reelin in Tat-treated SH-SY5Y cells. Twenty-four hours post-treatment, RNA was collected and subjected to qPCR. As shown in Figure 2D, addition of Tat decreases the level of reelin by almost 50% when

compared to the mock untreated. These results confirmed our observation and hypothesis regarding the ability of Tat to cause neurite retraction.

HIV-1 Tat downregulates expression levels of CREB and BDNF. It has been shown that neurite retraction and regulation of LTP and LDP depend on the functions of several transcription factors such as BDNF and CREB proteins (Jan and Jan 2010). Further, we previously demonstrated that CREB protein levels are affected in both Tat treated cells as well as in Tat transgenic mouse brain (Chang, Mukerjee, et al. 2011). Note that BDNF is a downstream target of CREB with multiple alternative transcription start sites, which are ultimately spliced to a single functional mature BDNF protein and that exons I and IV of *bdnf* gene are transcriptional targets of CREB protein (Kwon et al. 2011) (Lesiak et al. 2013). To that end, using qPCR, we examined expression levels of BDNF transcripts in Tat treated SH-SY5Y cells. RT-PCR analysis revealed that only exon IV, but not exon I, of *bdnf* gene produced a transcript (Figure 2E). As expected, BDNF4 level decreases in Tat-treated cells at 24 hours, however, it recovers at 48 hours, which could be due to the half-life of Tat protein. These results are in agreement with axonal surface assay presented in Figure 1C.

Next, we examined the level of CREB mRNA in differentiated and treated SH-SY5Y cells. Similar to BDNF, CREB level decreases in Tat-treated cells at 24 hours, and recovers at 48 hours (Figure 2F). Taken together, these results suggest that Tat protein can promote neurite retraction through reduction of CREB and BDNF expression levels.

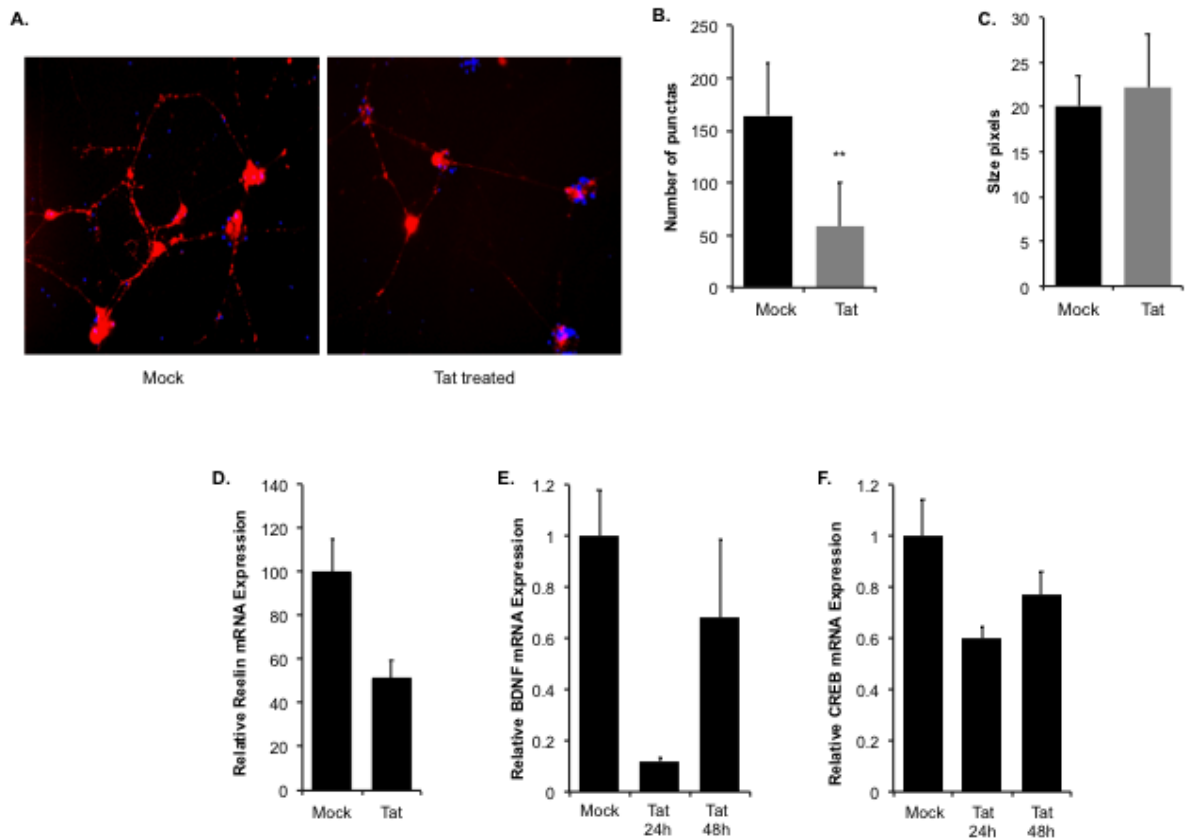


Figure 2.2 Tat alters the number and distribution of synaptophysin and expression levels of Reelin, CREB and BDNF. *A.* Representative images of synaptophysin vesicles distribution in mouse primary neurons \pm Tat protein. Synaptophysin was expressed using Cell Light Transduction at MOI 5. Tat protein or mock treatment were added 24 hours after the transduction. Images of live cells were acquired at 24 hours post-Tat addition. *B, C.* Synaptophysin vesicles number and size were assessed using Image J for each condition. Images from at least 10 different fields were taken for statistical analysis. *D, E and F.* Fold changes of 3 selected genes (Reelin, BDNF and CREB) involved in neurite retraction in Tat-treated SH-SY5Y cells compared to mock untreated obtained by qPCR. The experiment was repeated 3 times, the results are statistically significant using Student's *t* test (** $p < 0.005$)

compared with the *Mock* control group. Only 10 pg/ml of rTat were used for treatment. Reelin (*C*), BDNF (*D*) and CREB (*E*).

Tat reduces CREB expression through E2F3. Being a downstream target of CREB protein, it is normal to observe a reduction in BDNF level if CREB is inhibited. However, one may question the mechanisms involved in CREB inhibition. In this regard, we previously demonstrated that upregulation of miR-34a in Tat-treated SH-SY5Y leads to the inhibition of CREB mRNA and protein (Chang, Mukerjee, et al. 2011). It is well known that miRNAs exhibit their function through partial complementary sequences located at the 3' UTR of their target's mRNA. Those sequences are commonly referred to, as seeds and if miR-34a affects CREB expression then it is logical for CREB 3'-UTR to contain miR-34a seeds. Unfortunately, neither bioinformatics analysis of the CREB 3'-UTR mRNA (Figure 3A), or literature search revealed or described such site that could support our hypothesis.

Hence, we suspect the presence of an intermediate factor(s). To that end, we performed a CREB promoter analysis using ECR Browser for transcription factors binding sites. The analysis displayed three highly conservative binding sites for Sp1, Egr and E2F transcription factors (Figure 3A). Sp1 binding to the CREB promoter was previously described (Walker, Fucci, and Habener 1995), while EGR and E2F putative binding sites were only predicted. Further, TargetScan analysis of the 3'-UTR of the three transcription factors (Sp1, Egr and E2F) revealed that only E2F3 contains seed for miR-34a (Figure 3B). Furthermore, E2F3 has been shown to be a direct target of miR-34a and the functional relationship between E2F3 and miR-34a has been established (Welch, Chen, and Stallings 2007). Hence, we sought to examine whether decrease expression of CREB in Tat-treated cells is E2F3 and/or miR-34a dependent. To that end, we tested the impact of Tat protein on E2F1 (control) and E2F3 expressions in SH-SY5Y cells. Western blot analysis showed that addition of Tat led to a decrease in E2F3 expression (Figure 3C). Interestingly, expression level of E2F1 was too low to be detected or

affected by addition of Tat, which also confirm that the effect of Tat on E2F3 expression is specific. GAPDH was used as a control for equal protein loading.

To further confirm involvement of E2F3 in Tat-inducing CREB down-regulation, we performed a chromatin Immunoprecipitation (ChIP) assay to examine the ability of E2F3 to bind to the predicted region of the CREB promoter. SH-SY5Y cells were transfected with E2F3-HA tagged expression vector for 24 hours and then the cells were subjected to ChIP assay. In order to discriminate against any non-specific signal from the total E2F3 specific antibody, HA antibody was used. Several primers, covering 3 different regions from the CREB promoter as displayed in Figure 3D and described in the Methods section, were used. As expected, untransfected or Tat-treated cells exhibit low or no binding confirming the specificity of the antibody (Figure 3D). E2F3 was able to interact with the +1 region of the CREB promoter and not with the other two regions that do not contain E2F3 binding site. Efficiency of transfection was also controlled using GFP fluorescent plasmid (Figure 3D, inset).

Next, we sought to examine whether addition of E2F3 can rescue CREB. SH-SY5Y cells were transfected HA-E2F3 expression plasmid. Twenty-four hours later, the cells were treated with Tat protein for an additional 24 or 48 hours and then subjected to qPCR. As shown in Figure 3E, overexpression of E2F3 was able to modestly rescue CREB from being inhibited by Tat when compared to the mock untreated or to Tat-treated samples.

Functionally, overexpression of E2F3 led to a significantly higher covered axon surface area compared to the mock transfected Tat treated cells (panel F). Taken together, these results pointed to a significant role of E2F3 in CREB modification pathway.

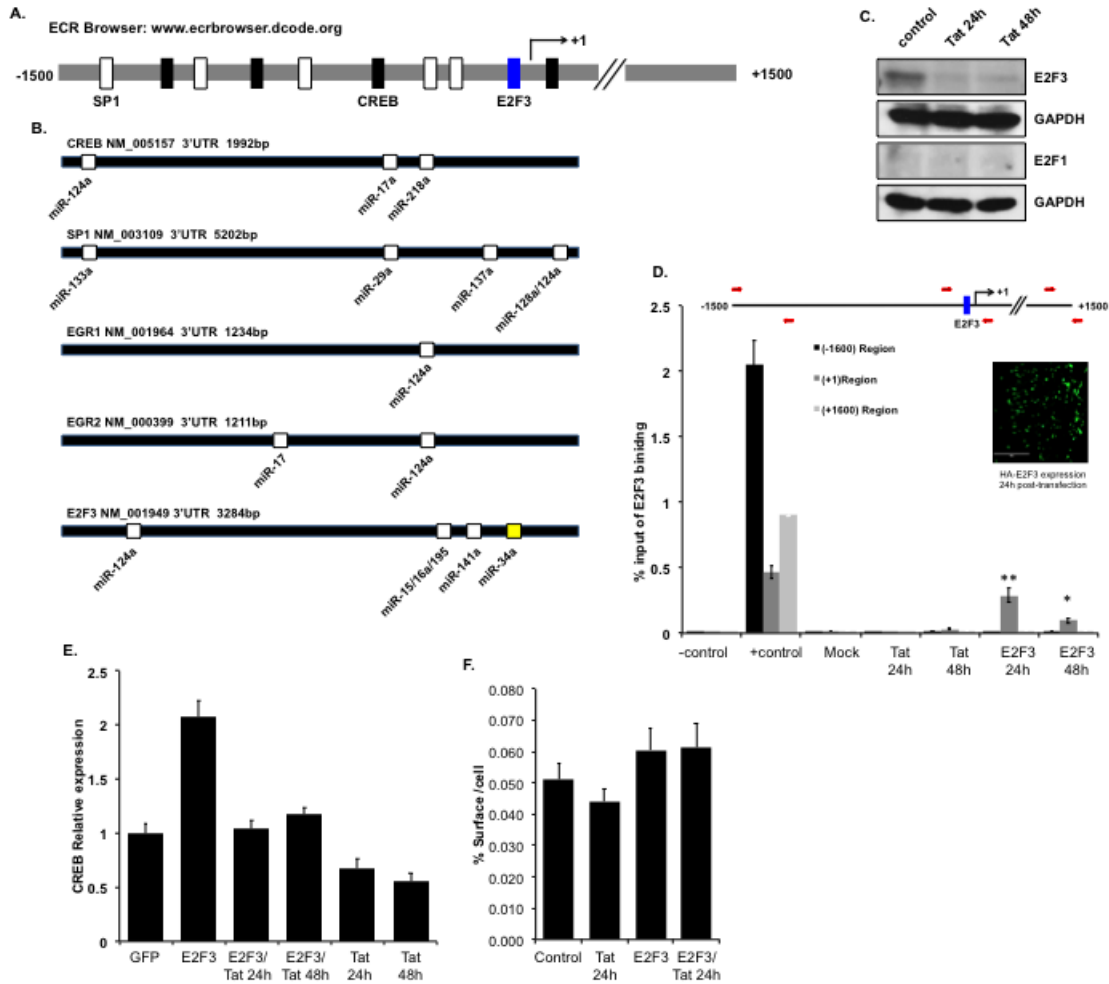


Figure 2.3 Identification of E2F3 as a new regulator of the CREB promoter. **A.** Schematic representation of the CREB promoter, the potential binding sites are also shown. Presence of binding sites was verified using the literature and ECR Browser analysis for highly conserved putative binding sequences. **B.** Schematic display of the 3'-UTR of known CREB, Sp1 or predicted Egr and E2F3 transcription factors mRNAs analyzed for potential miRNA-34a seeds (yellow box). **C.** Western blot analysis point to the ability of Tat to increase protein levels of E2F3 but not E2F1 in differentiated SH-SY5Y cells. Anti-E2F1, -E2F3, and -GAPDH antibodies were used as indicated. **D.** Schematic representation of the CREB promoter with the positions of the various primers used for ChIP assay. Binding of E2F3 to CREB DNA is also

shown. Extracts prepared from SH-SY5Y cells transfected with plasmid expressing HA-E2F3 expression plasmid and then treated with Tat protein were subjected to ChIP assay to evaluate the association of E2F3 with the CREB promoter DNA. Inset represents the transfection efficiency of the pcDNA-E2F3-HA plasmid at 24 hours post transfection. **E.** Fold changes of CREB gene mRNA in SH-SY5Y cells transfected with E2F3 expression plasmid \pm Tat protein compared to mock untreated as obtained by qPCR. The experiment was repeated 3 times, the results are statistically significant using Student's *t* test (* $p < 0.05$, ** $p < 0.005$) compared with the *Mock* control group. **F.** Following the same procedure as in Figure 1C, surface area measurements of cells transfected with E2F3 expression plasmid \pm Tat protein compared to the mock transfected and untreated.

Tat inhibits E2F3 through miR-34a. To further confirm that Tat deregulates neurite communication via up-regulation of miR-34a and down-regulation of E2F3, leading to inhibition of CREB-BDNF pathway, we sought to examine the exact role of miR-34a in this pathway. First, we performed an *in situ* hybridization assay using human brain cortical sections. As seen in Figure 4A, expression level of miR-34a is higher in the HIV encephalitis (HIVE) section compared to the control. In order to show that HIV-Tat protein is responsible for this increase, we also used Tat transgenic mouse model (Kim et al. 2003; Chang, Mukerjee, et al. 2011). Figure 4A (lower panels) displayed the abnormal expression levels of miR-34a in the hippocampus region of Tat-transgenic mice.

It is well known that miR-34a regulation is p53-dependent (Raver-Shapira et al. 2007). Further, we previously demonstrated the ability of HIV-1 Tat to up-regulate p53 protein (Mukerjee et al. 2008), we silence p53 gene and examined whether this inhibition affects miR-34a and/or CREB and BDNF expression levels. SH-SY5Y cells were transfected with small interference RNA directed against p53 gene (siRNA-p53) and then treated with 10 ng/ml of Tat protein for 24 hours. The cells were collected and subjected to quantitative PCR (qPCR) to examine expression level of miR-34a (Figure 4B) and/or of CREB and BDNF genes (Figure 4C). As shown in Figure 4 panels B and C, Tat decreases the levels of miR-34a (panel B) and of CREB and BDNF genes (panel C) in the presence of p53 but not in its absence when compare to the control. Efficiency of transfection and p53 silencing were verified using Western blot assay as shown in panel D.

We previously demonstrated that Tat protein could induce expression of p53 protein through induction of the p73 protein. We also showed that p53 is a downstream target of p73 and inhibition of p53 will not affect endogenous level of p73 (Mukerjee et al. 2008). Therefore,

as a control, we also examined the expression level of p73 protein using anti-p73 antibody (panel D). As seen in panel D, Tat induces endogenous level of p73 without been affected by siRNA-p53. Anti-GAPDH antibody was used to control equal protein loading.

On the other hand and to further validate the specificity of Tat on p53 and miR-34a and the downstream targets, we sought to examine whether addition of Tat enhances p53 binding to the miR-34a promoter (linc34a). Following the same procedure presented in Figure 3D, ChIP assay was performed using SH-SY5Y cells treated with Tat protein. As a positive control one set of cells was exposed to UV-light in order to induce endogenous p53. As shown in Figure 4E, Tat enhances p53 binding to the miR-34a promoter (+1500) and not (-1500). As expected, UV exposure increases the interaction of p53 to the +1500 region of the linc34a promoter. These results confirmed the specificity and the functional interplay between Tat, p53 and miR-34a.

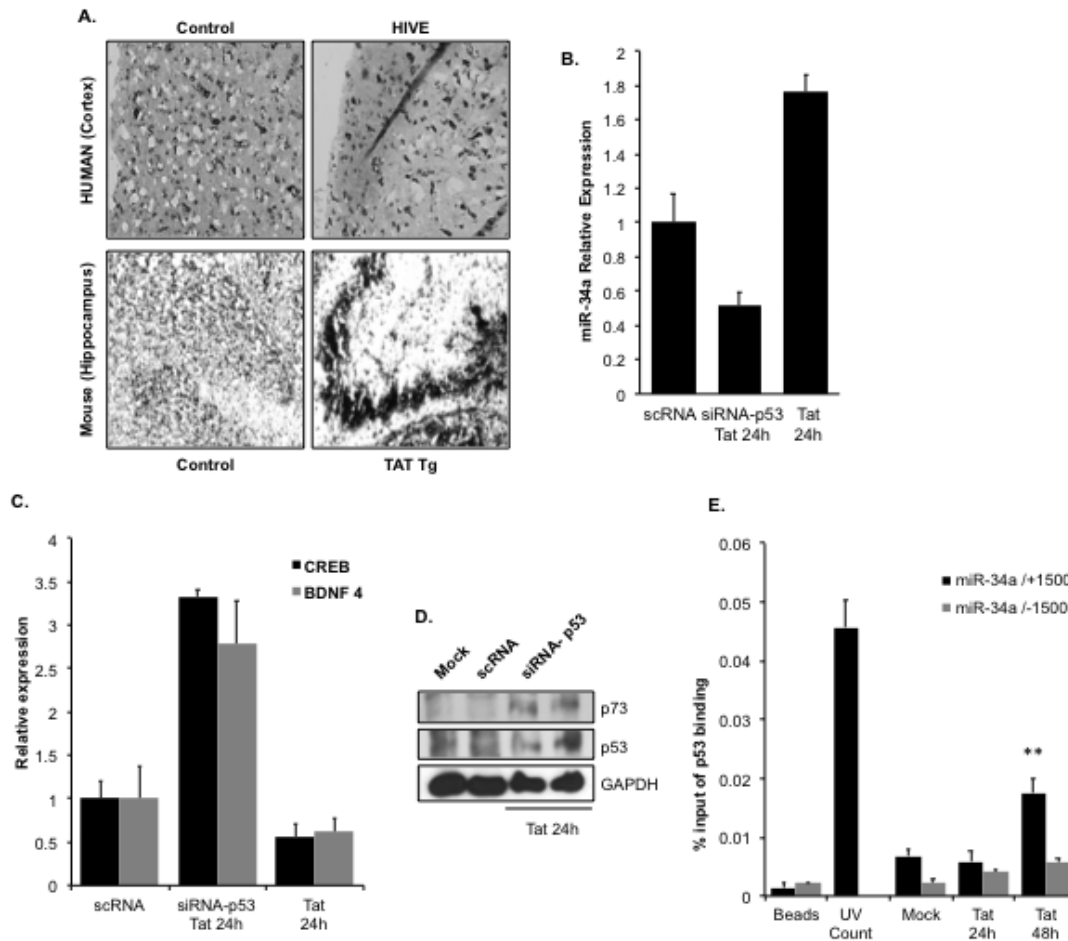


Figure 2.4 Tat suppresses E2F3 expression through induction of miR-34a. **A.** Distribution of miR-34a in the in human brain tissue of an HIVE patient (upper panels) and in Tat-transgenic mice (lower panels) as obtained by *in situ* hybridization assay. **B, C.** Fold changes of miR-34a (B) or CREB and BDNF (C) in SH-SY5Y cells transfected with small interference RNA directed against p53 gene (siRNA-p53) ± Tat protein compared to mock untreated obtained by qPCR. The experiment was repeated 3 times, the results are statistically significant using Student's *t* test (** $p < 0.005$) compared with the *Mock* control group. **D.** Western blot analysis point to the ability of Tat to increase proteins levels of p53 and p73. Tat failed to increase expression of p53 in the presence of siRNA-p53 but not of p73. Anti-p53, -

p73 or -GAPDH antibodies were used as indicated. Scramble RNA was used as a control. *E*. Extracts prepared from SH-SY5Y cells were subjected to ChIP assay to evaluate the association of p53 with the miR-34a promoter DNA (lnc34a) in the absence or presence of Tat protein. As a positive control, cells were also subjected to UV-light prior to the ChIP.

E2F3 overexpression promotes CREB binding to the BDNF promoter region. After establishing the relation between Tat and neurite retraction as well as the pathway and players involved, we sought to further examine the impact of E2F3 on CREB-BDNF association. As described above, BDNF gene contains multiple alternative transcription start sites defined as Exons (Timmusk and Metsis 1994; Timmusk et al. 1993) that are spliced together with a single protein coding exon. Each of the alternative transcription exon is under the regulation of promoter sequences and only exons I and IV contain CREB protein response elements (Tabuchi et al. 2002; Kwon et al. 2011; Lesiak et al. 2013), also shown by ECR Browser promoter sequence analysis (Figure 5A).

Hence, SH-SY5Y cells were transfected with E2F3 expression plasmids for 24 or 48 hours. The cells were then collected and subjected to ChIP assay using anti-CREB antibody to examine whether E2F3 overexpression strengthens CREB interaction to the BDNF promoter. As shown in Figure 5B, an increase in CREB binding to the BDNF promoter IV is observed in the presence of E2F3 when compared to the mock untransfected (panel B). These results confirm the important role of E2F3 in CREB regulation.

To further validate our observation and confirm the ability of E2F3 to drive BDNF-IV promoter activation through CREB, we performed a transient transfection assay. SH-SY5Y cells were transfected with the reporter plasmid BDNF-IV-Luc that contains three out the four CREB binding sites alone or in the presence of an increasing amount of E2F3 expression plasmids as marked. Note that Sp1 transcription factor increases CREB expression therefore Sp1 expression plasmid was also used as a positive control. The cells were collected at 48 hours after the transfection and subjected to luciferase assay. As shown in Figure 5C, E2F3

and/or Sp1 overexpression led to significant activation of the *bdnf* -IV promoter when compared to the mock transfected cells.

Finally, we measured the mRNA and proteins expression levels of CREB and BDNF in the presence of Tat and E2F3. SH-SY5Y cells were transfected with E2F3 expression plasmid and then treated with HIV-1 Tat protein for 24 hours. GFP plasmid was also used as a positive control. mRNA and total protein extracts were collected, and subjected to qPCR and Western blot analysis, respectively. As shown in Figure 5D and E, overexpression of E2F3 was enough to prevent inhibition of *bdnf*-IV and *creb* genes (panel D) when compared to the mock transfected/untreated or Tat-treated samples.

Using anti-E2F3, -CREB and -BDNF antibodies, similar results were obtained as revealed by Western blot analysis (panel E). Addition of Tat led to a decrease in the expression levels of E2F3, CREB and BDNF proteins (compare lanes 3 to lanes 1). However, Tat negative effect was significantly reversed in the presence of overexpressed E2F3 (compare lanes 2 to lanes 3 and 4). The percentage of increase or decrease of E2F3, CREB and BDNF expressions are also shown (panel E). Anti-GAPDH antibody was used to control equal protein loading. These results clearly demonstrated the link between Tat and neurite retraction. It is obvious that Tat is using miR-34a to decrease expression levels of E2F3 that leads to inhibition of CREB and BDNF, hence preventing neurite communications and eventually promoting neurocognitive disorders and premature brain aging.

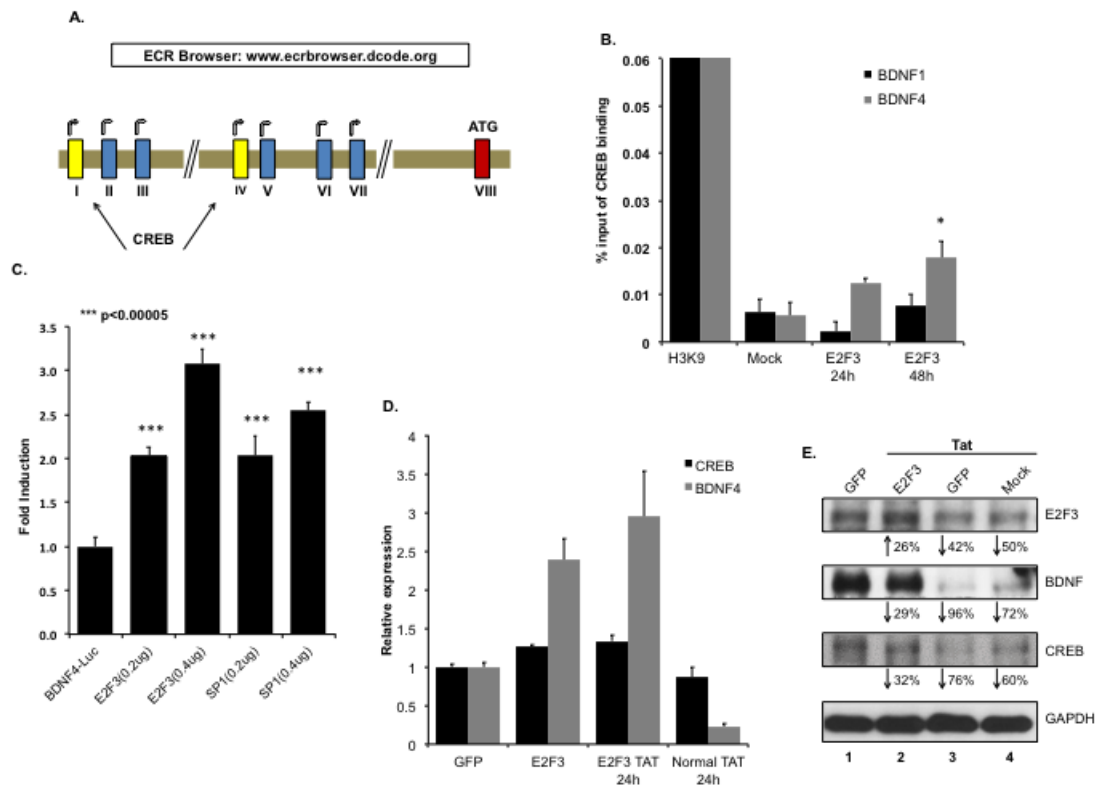


Figure 2.5 Effect of Tat and E2F3 on CREB-BDNF functional interplay. **A.** Schematic representation of the BDNF gene with all its exons. Only Exon I and IV (yellow) contain a CREB binding site. **B.** Extracts prepared from SH-SY5Y cells were subjected to ChIP assay to evaluate the association of CREB with the BDNF promoters I and IV in the presence of overexpressed E2F3. **C.** SH-SY5Y cells were transfected with 0.1 μ g of the BDNF IV-Luc full length along with an increasing concentration of E2F3 or Sp1 expression plasmids as indicated. The amount of DNA in each transfection mixture was normalized with pcDNA3. Luciferase activity was determined 48 hours after transfection. Results are displayed as histogram. **E.** Fold changes of CREB and BDNF in SH-SY5Y cells transfected E2F3 and then treated with Tat protein compared to mock untransfected/untreated obtained by qPCR. The experiment was repeated 3 times, the results are statistically significant using Student's *t* test ($p < 0.05$) compared with the *Mock* control group. **D.** Western blot analysis point to the ability of Tat to

alter proteins levels of E2F3, CREB and BDNF. Anti-E2F3, -CREB, -BDNF, or -GAPDH antibodies were used as indicated.

E2F3 and BDNF expression is reduced in HIVE brain. To further validate our *in vitro* data, we sought to examine expression levels of E2F3 and BDNF proteins *in vivo*, using human brain tissues prepared from mock non-infected or from HIV-1 infected patient with signs of encephalitis (HIVE). Note that we previously demonstrated that CREB expression level is reduced in Tat-transgenic mice (Chang, Mukerjee, et al. 2011). Using immunohistochemistry assay, we observe that expression level of E2F3 protein decreases in HIVE sections when compared to the mock uninfected (Figure 6A).

Distribution and subcellular localization of E2F3 and BDNF proteins were also examined by immunohistochemistry. As shown in panel B, in the uninfected section, E2F3 (green) was expressed ubiquitously with a perinuclear staining pattern as indicated by the lack of color shift in the DAPI (blue) staining (panel B), while in HIVE section, little or no E2F3 positive staining was observed. Similar results were obtained with BDNF (Figure 6B, BDNF panels). Note that the two proteins are localized in the cell compartment (merge panel). The experiment was repeated 3 times, the results are statistically significant using Student's *t* test (* $p < 0.05$, *** $p < 0.0005$) compared with the *Mock* control group.

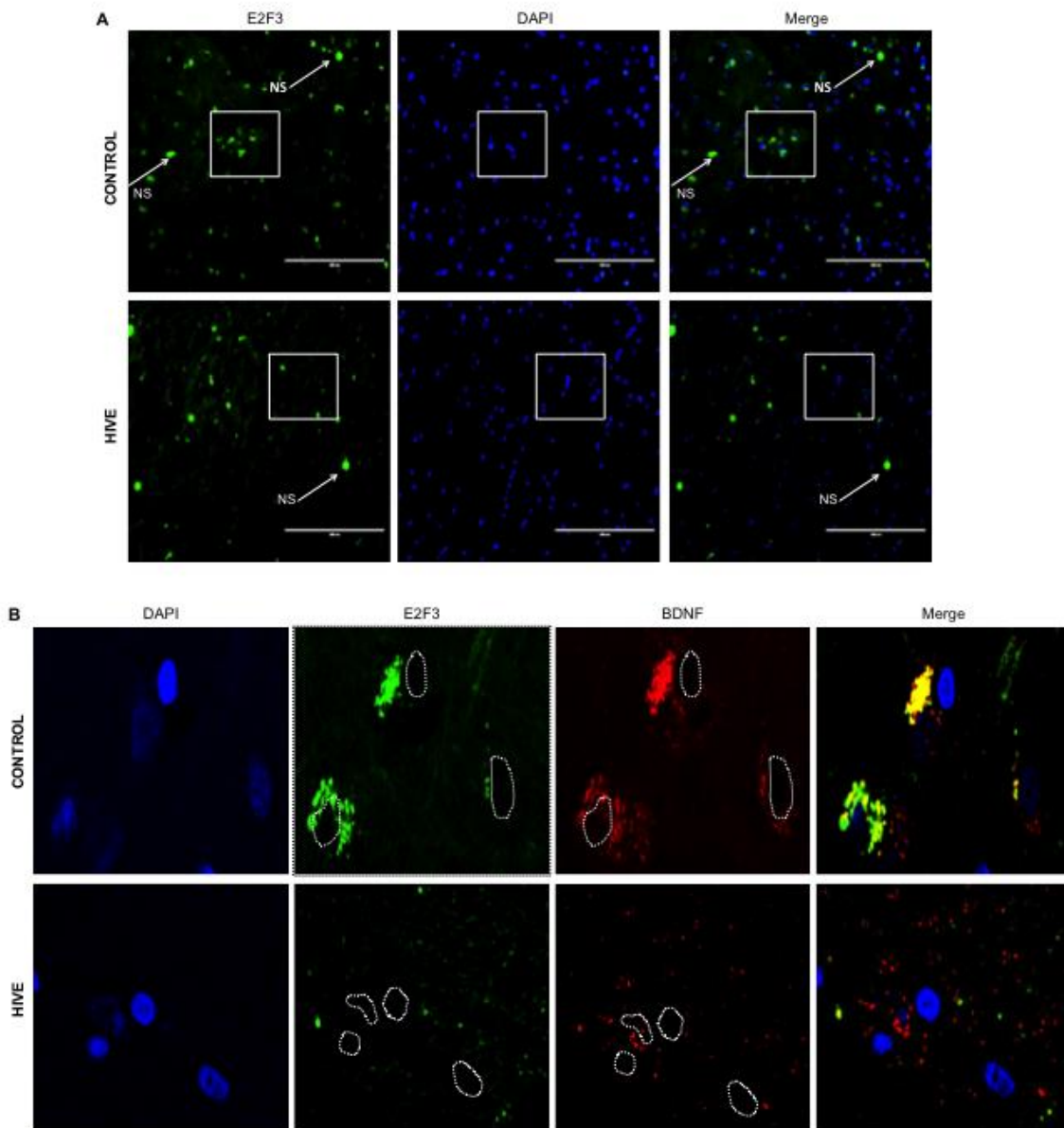


Figure 2.6 Immunohistochemistry assay depicting expression levels of E2F3 and BDNF protein. A and B. Distribution and expression level of E2F3 (green; panels *A* and *B*) and BDNF (red; panel *B*) proteins in human brain tissue of an HIVE patient compared to the mock patient obtained by immunohistochemistry assay.

E2F3 rescues Tat induced synaptophysin distribution alteration. Next, we examined whether overexpressed E2F3 can reverse the loss of synaptic endings. SH-SY5Y cells were transfected with E2F3 expression plasmids and then treated with Tat protein. As a control, pcDNA3 empty vector was also transfected. As shown in Figure 7A, the number of synaptophysin vesicles was higher in cells transfected with the empty vector, with E2F3 and in cells transfected with E2F3 and treated with Tat protein when compared to cells treated with Tat. Quantification and sizes of neurites (only) synaptophysin vesicles are presented in panels B and C, respectively. Further, quantification of the total number of vesicles (Figure 7D), which includes cell body and neurites synaptophysin vesicles revealed similar results as quantification of only neurites synaptophysin vesicles (Figure 7E). However, analysis of only neurites vesicles resulted in higher statistical significance. Taken together these results point to the ability of HIV-1 Tat to promote neurite injury through a mechanism that implicates miR-34a and several proteins mainly E2F3, the downstream target of miR-34a.

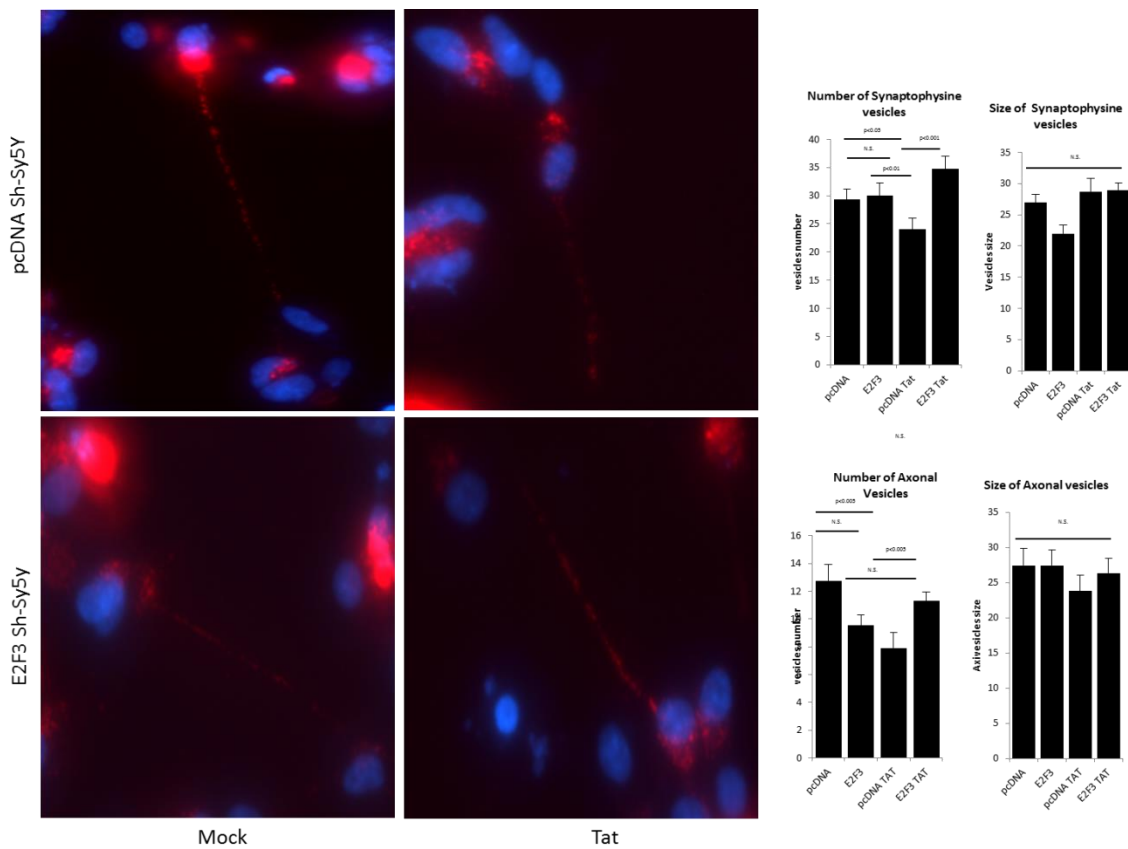


Figure 2.7 Distribution and quantification of Synaptophysin in E2F3-transfected cells.

A. Representative images of synaptophysin vesicles distribution in SH-SY5Y cells transfected with pcDNA3 empty vector or with E2F3 expression plasmid and then treated with Tat protein. Synaptophysin was expressed using Cell Light Transduction at MOI 5. Tat protein or mock treatment were added 24 hours after the transduction. Images of live cells were acquired at 24 hours post-Tat addition. Transfection was performed prior to synaptophysin transduction. **B-E.** Synaptophysin vesicles number and size were assessed using Image J for each condition. Images from at least 10 different fields were taken for statistical analysis. Error bars are presented as SEM. The experiment was repeated 3 times, the results are statistically significant using Student's *t* test (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$) compared with the *Mock* control group.

Discussion

Failure of the highly active antiretroviral therapy (HAART) to lower the incidence rates of HIV-associated neurocognitive disorders (HAND), gave the rationale to identify the molecular mechanisms involved. In this regard, it has been shown that the viral protein Tat is among the HIV-1 viral proteins that have the ability to induce neuronal cell dysfunction. Infected astrocytes and microglia cells have been shown to release Tat that could affect neuronal functions such as neuronal communication (Mukerjee et al. 2008; Norman et al. 2008; Norman et al. 2007). In here, we confirmed the negative role of Tat and its ability to promote neurite damage. We also identified the cellular factors and the pathway involved. Interestingly, Tat triggered an action potential signal leading to the induction of the p53 protein and its downstream microRNA target, miR-34a (Chang, Mukerjee, et al. 2011). Activation of miR-34a turns out to have a domino effect on several downstream direct and indirect targets such as E2F3, CREB and BDNF, all of which involved in neuronal communications (Figures 1-4). We also confirmed the deregulation of these factors in human brain tissues prepared from HIV-infected patient and in Tat-transgenic mice (Figures 4 and 6). These results pointed to the key role that miR-34a and its targets genes play in HIV-1 associated neurocognitive disorders.

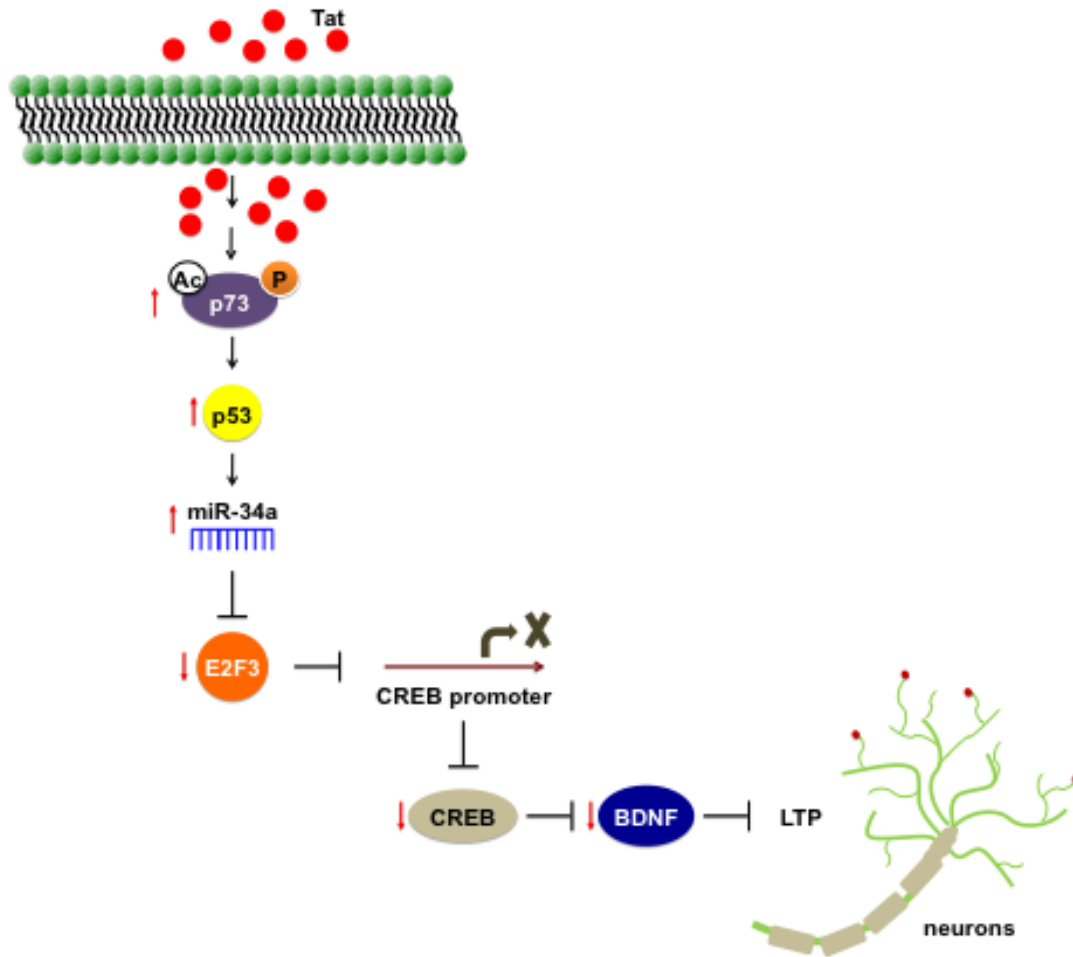


Figure 2.8 Pathway used by Tat to cause neuronal retraction. Schematic representation of the pathway used by Tat leading to neurite retraction. All the players are shown. (LTP= long term potentiation).

The relation between miR34a and neuronal deregulation is not without a precedent. It has been shown that an increase in miR-34a expression can alter hippocampal spinal morphology and function (Agostini, Tucci, Steinert, et al. 2011). Further, down-regulation of miR-34a was reported to have a positive impact on neuronal survival (Khanna et al. 2011). Furthermore, activation of miR-34a was shown to be associated with the down-regulation of 136 genes involved in cell motility, energy production and actin cytoskeleton organization, which indicates a critical role for miR-34a in neuronal precursor motility (Chang, Weng, et al. 2011). Recently, miR-34a was also shown to increase cortical neuronal vulnerability to injury. In this regard, Truettner and his colleagues found that expression levels of miR-34a increases after traumatic brain injury and inhibits Bcl-2 and XIAP, both anti-apoptotic proteins (Truettner, Motti, and Dietrich 2013). Additionally, miR-34a was found to be conserved between human, mouse and rat during neuronal development and to play a role in mouse NS cell differentiation (Aranha et al. 2010). Finally, increased miR-34a and its involvement in neurodegeneration are not limited to HIV-1. It has been shown that miR-34a is upregulated in Alzheimer's disease and in schizophrenia and has a negative role in both diseases (Wang, Liu, et al. 2009; Agostini, Tucci, Steinert, et al. 2011; Kim et al. 2010).

Additionally, it has been reported that E2F3 is a direct target of miR-34a (Reimer et al. 2011). Interestingly, E2F3 has been described to have a negative effect on the p53 expression (Aslanian et al. 2004). This observation confirmed the need of p53 to suppress E2F3 and to cause neuronal deregulation. These results further determine the major role that E2F3 might play in neuronal function in patients infected with HIV-1.

On the other hand, no reports describe a direct link between CREB and miR-34a. Further, in spite of the well-described relation between E2F family and CREB, the relation between

E2F3 and CREB has never been documented. In this regard, our data point to a new factor that could regulate the CREB promoter (Figure 3D) and deciphers the relation between CREB and miR-34a, a negative relation that led to neurites retraction and alteration of neuronal communication. Taken together, our results are considered a milestone that could partially explain the mechanisms leading to the development of neurocognitive disorders associated with HIV-1 infection.

It is noteworthy to mention that other HIV-1 proteins have been shown to cause neuronal damage such as gp120 and Vpr (Zhang, Wang, et al. 2012; Webber et al. 2013). However, the mechanisms used by these two proteins are not fully understood and remain to be identified. Nevertheless, all three HIV-1 proteins use a pathway(s) that involved BDNF protein, which point to the importance of BDNF and the need to prevent its inhibition.

Overall, neurite retraction is an important cellular function that assures neuronal communication and transfer of information and its alteration leads to neuronal degeneration. Induction of neurite retraction has been shown to be somehow a general phenomenon associated with neuronal dysfunction. In this regard, several cellular factors have been shown to trigger neurite retraction such as Rho, Wnt, accumulation of cytoplasmic calcium, and EP3. Therefore, it is necessary to investigate whether a common pathway exists between all these proteins and whether the function of E2F3 is altered in these pathways.

Further, neurite retraction observed by HIV-1 Tat (Figure 1) is not exclusive to HIV-1 and has been observed in other neurodegenerative diseases such Alzheimer and Parkinson. In this regard, it has been shown that lysophosphatidic acid (LPA) has the capability to induce neurite retraction and tau phosphorylation (Sayas et al. 1999). LPA was also found to alter CREB protein expression (Sun et al. 2011). Neurite retraction associated with Alzheimer's diseases was also noted in neuronal cells with increased PSD-95 protein or with reduced amount of

glyoxalase I activity (Kuhla et al. 2006; Leuba et al. 2008). In addition to HIV-1 and AD, neurite retraction was also described to be associated with Parkinson disease through LRRK2 protein (Plowey et al. 2008). Away from diseases, BDNF protein was found to inhibit hypoxia-induced neurite retraction by averting oxidative stress (Woronowicz et al. 2007).

In summary, our data point to the pathway and the cellular players used by HIV-1 Tat protein to cause neurite retraction and neuronal damage. However, this is the first report, to our knowledge, to show involvement of E2F3 protein in neurite retraction and the development of HIV-1 associated neurocognitive disorders. Therefore, our data can serve as the basis for the development of a new molecular approach that could prevent progression and development of neurocognitive disorders associated with HIV-1.

CHAPTER 3

INVOLVMENT OF MIR-196A IN HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

3.1 Introduction

The mechanisms and the molecules leading to the development of HIV-associated neurocognitive disorders have not been completely identified, however several reports point to the involvement of HIV-1 Tat protein (Zucchini et al. 2013; Fitting S 2012; Hahn et al. 2010).

The Trans-Activator of Transcription (Tat) regulatory protein has been implicated in the pathophysiology of the neurocognitive deficits associated with HIV infection (Chang, Mukerjee, et al. 2011; Arese et al. 2001). Tat protein drives the regulatory regions of the virus and may also be actively released from the cell and then interact with the cell surface receptors of other uninfected cells in the brain leading to cellular dysfunction (Rayne et al. 2010; Debaisieux et al. 2012). Tat protein can be taken up by uninfected cells including neurons and activates a number of host genes (Tryoen-Toth et al. 2013; Norman et al. 2007; Hui et al. 2012). In CNS, Tat has been shown to cause neuronal deregulation through a pathway that implicates p53 and p73 (Chang, Mukerjee, et al. 2011; Garden et al. 2004; Mukerjee et al. 2008). In animal model, intracerebral injection of Tat can cause death within hours of injection (Sabatier et al. 1991). In adult animals, Tat affects pre-attentive processes and spatial memory. Tat transgenic mice are marked by glial cell activation and neuronal loss (Carey et al. 2013; Li et al. 2004).

p73 belongs to the family of p53-related transcription factors including p53, p73 and p63. The overall structure and sequence homology indicates that a *p63/p73*-like proto-gene is the ancestral gene, whereas *p53* evolved later in higher organisms (Joerger et al. 2009; Melino et al. 2003; Flores et al. 2002). p73 functions in a manner analogous to p53 by inducing tumor cell apoptosis and participating in the cell cycle checkpoint control through activating an

overlapping set of p53/p73-target genes (Rossi et al. 2005). Genetic studies of p73 protein revealed three functional domains including transactivation, DNA binding and oligomerization domains. In sharp contrast to p53, p73 is expressed as two NH₂-terminally distinct isoforms including transcriptionally active (TA) and inactive (Δ N) forms. Although p73 expression is low, the p73 gene is expressed in all normal tissues. The TA and the Δ N proteins are the predominant forms of p73 in human and mouse brain, respectively (Grob et al. 2001; Moll and Slade 2004; Murray-Zmijewski, Lane, and Bourdon 2006). It should be noted that in neuroblastoma cells, Δ Np73 failed to inhibit p73 however, it did physically interact with and inhibited p53. p73 mimics p53 by being phosphorylated, in this regard, c-Abl was shown to phosphorylate p73.

In the present study, we examined the mechanisms used by Tat to induce p73 and the potential involvement of small non-coding RNA. These results are valuable to understand the mechanisms of neuronal deregulation associated with HIV-1-infection.

3.2 Methods

Tat protein. Recombinant full-length Tat protein prepared from HIV-1 clade B was obtained from the AIDS Reagent Program at National Institutes of Health.

Cell culture and HIV-1 Tat treatment. Human SH-SY5Y neuroblastoma cells were maintained in F12 DMEM 50/50 supplemented with Sodium Pyruvate, Non-essential amino acids and 10% FBS final concentration. Before each experiment cells were seeded in 50% confluence and differentiated with 10 μ M Retinoic Acid for at least 48 hours. Unless indicated otherwise, HIV-1 Tat supplemented medium (10 ng/ml final concentrations) was added and the cells were harvested at the specified time. Only SH-SY5Y cell in passage 25-35 were used.

Western blot analysis and Antibodies. Differentiated SH-SY5Y cells (1×10^6) were treated with Tat protein as indicated; harvested and re-suspended in RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton and 0.1% SDS). Western blotting (20 μ g/sample) was performed using cell lysate prepared 24 hours post-Tat-treatment as described (Sawaya et al. 1998). Anti-p73 (total and phosphorylated), -c-Abl, -p53, -cleaved capsase 3, -YAP, -Acetyl-Lysine, and -GAPDH antibodies were used. Anti-GAPDH and - β -actin were used as a control for equal protein loading.

Transfection Assays. SH-SY5Y cells were plated until reaching in 70-80% confluence in Opti-MEM serum free transfection medium. The 6 and 24 well Lipofectamine transfection protocols were followed. 0.5 μ g of the p73 promoter reporter plasmid was transfected alone or with 0.5 μ g of Sp1 expression plasmid for 24 hours prior to the addition of Tat protein. The medium was replaced, at 4 hours post-transfection, with media containing 10 μ M of retinoic acid (RA). Transfection was carried for 24 or 48 hours, then luciferase assays were performed as described (Mukerjee et al. 2008).

Immunohistochemistry. Frontal lobe brain tissues were received from the National NeuroAIDS Tissue Consortium (NNTC) bank and handled as described (Chang, Mukerjee, et al. 2011). Briefly, sections were permeabilized in 1% Triton in PBS for 15 minutes and quenched with 50 mM Ammonium Chloride. Sections were then rinsed with PBS, and a blocking step was performed with 1% BSA serum at room temperature for 2 hours. Primary antibodies (1:100 dilution) were incubated overnight at 4°C. After rinsing with PBS (x3), sections were incubated for 1 hour at room temperature with Alexa Fluor secondary antibodies at 1:100 dilution (Molecular probes). The tissue was subsequently washed in PBS until finally mounted with DAPI containing medium.

Immunofluorescence. Cells in culture were fixed for 3 minutes in 2% paraformaldehyde, rinsed with PBS and blocked with 1% BSA for 1 hour. Slides were incubated with primary antibody (1:100 dilution) for 1 hour at room temperature (or overnight at 4°C), then with a fluorescein-tagged Alexa Fluor secondary antibody for 1 hour at room temperature. Cells were then washed and mounted with DAPI containing medium. A LEICA EL6000 DMI3000 confocal microscope system was used with a UV laser (405 nM), an Argon laser (488nm wavelength), and a HeNe laser (543 and 633nm wavelength). Z-sections at the depth of 0.25-0.45 mm were generated. In some cases contrast and/or intensity were adjusted to improve comparison of different stains. When applied, these changes affected the entire panel.

Co-immunoprecipitation (Co-IP). Samples were lysed in non-denaturing lysis buffer containing protease and phosphatase inhibitors followed by Bradford Colorimetric Assay to determine the total amount of proteins. Total of 500 µg of protein was used for each IP reaction. IPs reactions were pre-cleared with 20 µl of Protein A- agarose beads for 30 minutes at 4°C, followed by the addition of 10 µg of primary antibody. Four hours later, 30µl of beads were added, the extracts were then incubated overnight at 4°C. Beads washed 5 times with PBS with protease/phosphatase inhibitors and re-suspended in 50 µl of RIPA buffer before being loaded on an SDS gel.

Quantitative PCR (qPCR). RNA was isolated using Trizol reagent. Briefly, cells were pelleted by centrifugation (300 x g for 5 minutes) and lysed with TRIZOL Reagent by repetitive pipetting. Five minutes later, 0.2 ml of chloroform/1 ml of TRIZOL was added. Samples were vortexed vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes. Following centrifugation at 12,000 x g for 15 minutes upper aqueous phase was carefully transferred without disturbing the interphase into fresh tube and mixed with 0.5 ml of

isopropyl alcohol /1ml of Trizol. After incubation at room temperature for additional 5 minutes, the samples were centrifuged for 10 minutes at 12000 g. RNA pellets were washed with 75% ethanol and dissolved in RNase free water containing DNase. Reverse transcription for miRNA detection was performed using 100 ng of purified RNA using the miRCURY LNA™ Universal RT microRNA PCR kit (Exiqon, cat# 203300). For gene expression, 1 µg of RNA was used with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, cat#11754-050).

Real Time PCR reaction was performed in 10 µl total volume of miRNA cDNA (1:80 final dilution with 5 µl of primers and enzyme containing SYBR Green master mix and PCR grade water for volume adjustment). Following primers were used: c-Abl sense 5'-acgtctgggcatttgagattgc-3', antisense 5'-tcagaggattccactgccaacat-3'; p73 sense 5'-tctggaaccagacagcacctactt-3', antisense 5'-tcagcagattgaactgggcatga-3'. miRNA-196a (cat# 204555) and miRNA-30a (cat#204791) primers were purchased from Exiqon and miRNA-196a mimic (cat# HMI0323) and miRNA-30a mimic (cat#HMI0454) primers were purchased from Sigma.

MTT Assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide). SH-SY5Y cells were seeded into 96-well plates at approximately 70% density per well (100 µl). The cells were then transfected with scRNA or mir-196a mimic for 24 hours before being treated with DMSO or Tat (10 ng/ml). Transfections were carried using Lipofectamine and modified for a 96 well format according to Invitrogen's recommendations. Cell Titer 96 Non-Radioactive Cell Proliferation Assay was used for the MTT assay (Promega), where at 3 hours before each of the desired time points, 20 µl of MTT solution (5 mg/ml in PBS) was added into each well and cells were incubated at 37°C for 3 hours. The medium was removed and 100 µl of DMSO was

added into each well. The plate was gently rotated on an orbital shaker for 10 minutes. The absorbance was detected at 570 nm with a micro-plate reader (Biotech ELx800)

Sequence-Specific Detection of 5-hydroxymethyl Cytosin. Genomic DNA was extracted from SH-SY5Y cells by incubating overnight in high salt lysis buffer with proteinase K followed by ethanol precipitation. The presence of 5-hmC at MspI restriction sites in the p73 promoter was determined using a Quest 5-hmC Detection Kit (Zymo Research) as described by the manufacturer using 500 ng genomic DNA. MspI cleaves DNA only when restriction-site cytosines are methylated or hydroxymethylated; 5-hmC specific glucosylation of residues results in protection from MspI cleavage. Consequently, the processed DNA was analyzed with qPCR using region specific primers. p73 promoter: sense 5'-agggccgggaggagacctt-3', antisense 5'-cctacctgccgtcgca-3'.

Statistics and Bioinformatics. Student's *t* tests or unbalanced analysis of variance was used. Furthermore, each experiment was repeated three times and the results were considered statistically significant if $p < 0.05$. All Results were expressed as mean \pm SD. Pubmed (<http://www.ncbi.nlm.nih.gov/pubmed/>), Ensembl (<http://useast.ensembl.org/index.html>) were used to identify promoter and gene coding regions and ECR Genome Browser (<http://ecrbrowser.dcode.org/>) and Target Scan Human (http://www.targetscan.org/vert_61/docs/help.html) were used for transcription factors binding site and miRNAs prediction identification.

3.3 Results

We previously demonstrated the presence of a functional and physical interaction between Tat and p73 proteins leading to neuronal deregulation (Mukerjee et al. 2008). In here, we aim to decipher the mechanisms involved and the potential role of small non-coding RNA.

Tat increases the levels of p73 protein. Accumulation of the p73 protein in the brain tissue of HIV-1 infected patients (Amini et al. 2005) gave the rationale to examine whether Tat protein plays a role in this induction. We performed an immunohistochemistry assay using human brain tissues to demonstrate the presence of Tat protein in these cells. As shown in Figure 1A, Tat was detected in reactive astrocytes and neurons (left and central panels) but not in the control (right panel).

Next, we investigate the presence of Tat and its effect on p73 protein in mice brain tissues prepared from the cortical area of control/parental or Tat-transgenic mice. As shown in Figure 1B, Tat increases the expression level of p73 protein (Alexa Fluor-488, *Green*) when compared to the control. Tat also promotes translocation of p73 to the nucleus of the cells (Merge panel). MAP-2 (Alexa Fluor-647, *Red*) was used to demonstrate the neuronal nature of the cells and DAPI (*blue*) was used to highlight the nuclei.

p73 protein accumulation was further validated in SH-SY5Y neuronal cells that were treated with 10 ng/ml of Tat protein for 24 and 48 hours. Twenty micrograms of extracts collected from these cells were subjected to Western blot analysis using anti-p73. As shown in Figure 1C, total levels of p73 increases in Tat treated extracts at 48 hours when compared to the mock. Anti- β -actin was used to demonstrate equal proteins loading.

As a positive control, one set of cells was exposed to UV light to induce endogenous p73. Differentiated SH-SY5Y cells were induced by UV and then treated with Tat protein as indicated. The extracts (20 μ g) were subjected to Western blot analysis using anti-p73 antibody (total). As expected, p73 expression increases in UV-treated cells and did increase further in UV-exposed cells and then Tat-treated (Figure 1D, lane 2). Interestingly, the additional band

was observed but was not as strong as in panel D and this may explained by the time of treatment (24 vs 48 hours).

Increase of the p73 expression and its activation are associated with its phosphorylation on tyrosin residue at position 99 (Flores et al. 2002). Furthermore, there is an apparent additional band at 48 hours indicated by an arrow in Figure 1C detected with total p73 antibody. All this suggests that there is a possible post-translation modification of the p73 protein. Hence, we examined whether addition of Tat leads to p73 phosphorylation at *Tyr99*. SH-SY5Y cells untreated or treated with 10 ng/ml of Tat protein for 48 hours were subjected to immunocytochemistry assay. As shown in panel E, An increased immunoreactivity of phosphorylated p73 (phosphor-Tyr 99) antibody was observed in these cells when compared to the mock treated. Phosphorylation of p73 at tyrosine residue 99 promotes its translocation to the nuclear matrix (Liu et al. 2011). Indeed, p73 subcellular localization is nuclear as shown in Figure 1E (p73 and Merge panels). Further, it has been describe that Tyrosine 99 phosphor-p73 might increase cell death via a pathway that implicates caspase-3 (Tsai and Yuan 2003). Therefore, we evaluated the expression levels of cleaved caspase-3 in these cells. Interestingly, induction of cleaved caspase-3 was observed in Tat-treated cells compared to the mock treated (panel E, caspase-3 panel).

To further confirm our observation, cells were counted and statistical analysis was used to demonstrate that Tat promotes significantly higher levels of phosphorylated p73 and cleaved casp-3 in these cells when compared to the Mock untreated (Figure 1F).

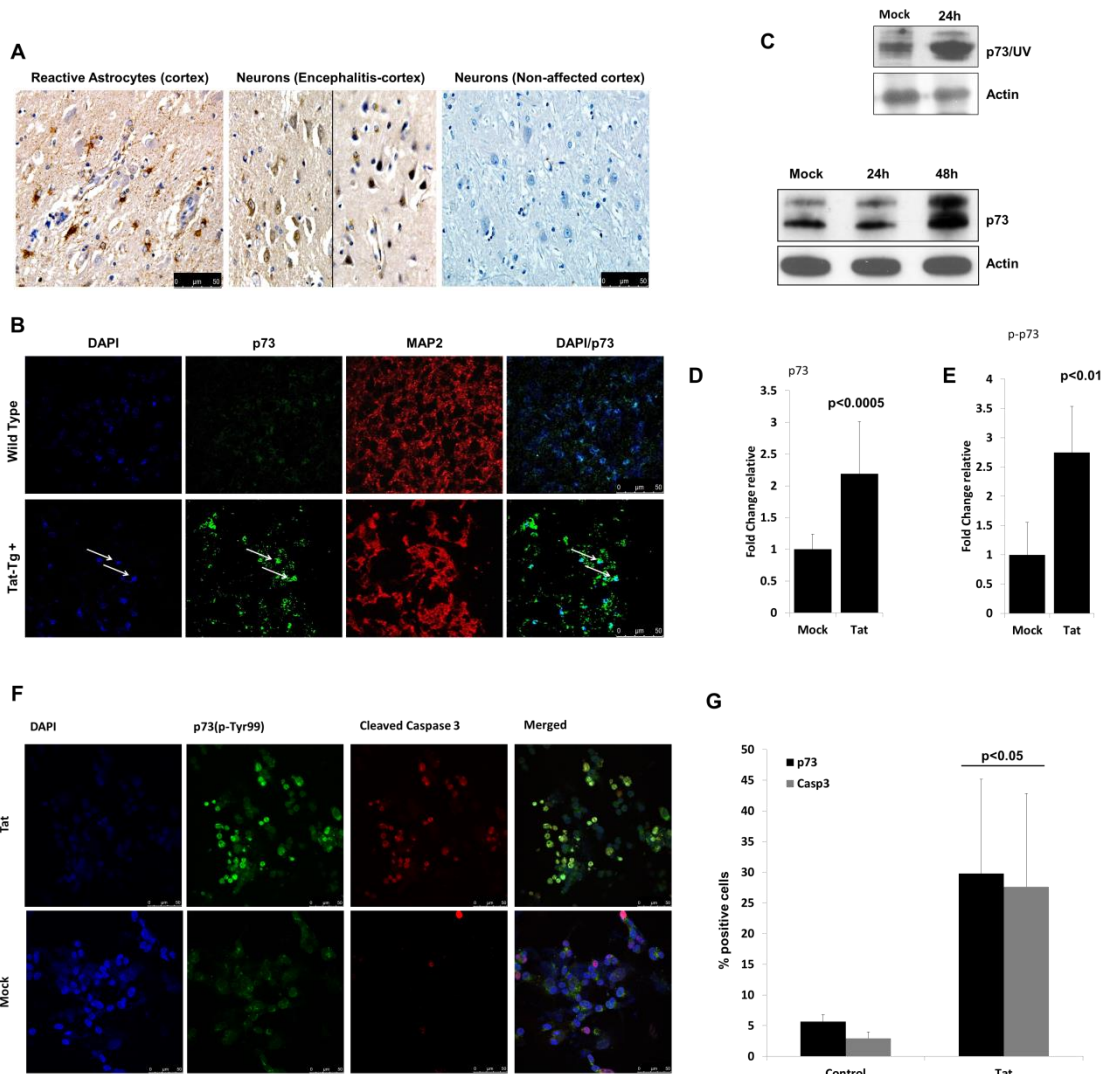


Figure 3.1 Tat increases the levels of p73 protein. (A) Immunohistochemistry displaying the accumulation of p53 in human cortical brain sections of an HIV patient with encephalitis (HIVE) when compared to the Mock non infected. Anti-p24 antibody was used to demonstrate the presence of HIV-1 proteins in these sections. (B) Immunofluorescence assay showing nuclear accumulation of p73 protein (arrow) in the brain (cortex) of Tat-transgenic mice when compared to the parental mice. Tat protein was induced with Doxycycline for 7 days after which brain tissues were harvested. (C, D) 20 μ g of protein extracts prepared from mock untreated or Tat-treated SH-SY5Y cells were subjected to Western blot analysis using anti-p73

(total) antibody (bottom band) in Tat-treated SH-SY5Y cells. Anti- β actin antibody was used as a control for equal protein loading. **(E)** Immunofluorescence in differentiated SH-SY5Y cells after Tat treatment using anti-phospho p73 (Tyr-99) and anti-Caspase 3 antibodies showed nuclear accumulation of phospho-p73 protein in Caspase 3 positive cells. **(F)** Quantification of the phospho-p73 and Caspase 3 positive cells from the experiment in panel E. Values represent the percentage of positive cells relative to the total number of cells in any given field counted (n=3). At least 70 cells in each field were counted.

Tat enhances the expression level of p73 through induction of c-Abl. Activation, phosphorylation and stabilization of p73 are c-Abl dependent (Codelia et al. 2010). This gave us the rationale to examine the status of c-Abl in Tat-treated SH-SY5Y cells and to test whether Tat is using this pathway to enhance the expression level of p73. To that end, SH-SY5Y cells (5×10^5) were treated with 10 ng/ml of Tat protein and harvested at different time point as indicated (Figure 2A). Total RNA was prepared from the cells and processed for quantitative PCR. The mRNA level of c-Abl increased in Tat-treated cells as of 1 hour when compared to the untreated control. Alternatively, we examined the expression level of c-Abl in these cells in presence of Tat. Protein extracts were prepared at the indicated times as indicated and subjected to Western blot analysis. As shown in Figure 2B, expression level of c-Abl increased as measured by Western blot in Tat-treated cells when compared to the control untreated. β -actin was used for equal protein loading (panel B). Interestingly, c-Abl expression decreased at 4 hours when compared to 2, 6 and 24 hours (panel B). This decrease in protein expression is not associated with decreased mRNA expression (compare 4 hours in panel A to panel B). Additional studies are needed to understand this phenomenon. As a positive control, induction of c-Abl was tested in UV-exposed cells and then treated with Tat protein (Figure 2B, lowers panels).

It is known that c-Abl phosphorylates p73 protein through their physical interaction, therefore, we sought to examine whether addition of Tat promotes c-Abl-p73 association. SH-SY5Y cells were treated with Tat protein for 24 hours. Extracts were prepared from these cells and subjected to immunoprecipitation using ant-p73 antibody followed by Western blot using anti-c-Abl antibody. As shown in panel C, Tat was able to bring the two proteins together. These results point to the ability of Tat to induce both c-Abl and p73 proteins and to promote their physical interaction.

Upon DNA damage, p73 is phosphorylated at Tyr99 by c-Abl and translocate to the nucleus and acetylated by p300 (Costanzo et al. 2002). Therefore, we sought to investigate whether Tat promotes p73 acetylation. SH-SY5Y cells were treated with 10 ng/ml of Tat protein for 24 and 48 hours, respectively. Protein extracts were then collected and subjected to immunoprecipitation using anti-p73 antibody followed by a Western blot analysis using anti-acetyl antibody. As a positive control, the cells were treated with 2 μ M of doxorubicin for 24 hours. Note that doxorubicin is a DNA damaging agent. As expected, p73 protein was acetylated in cells treated with doxorubicin when compared to the mock untreated (Figure 2D, compare lanes 1 and 2). Similarly, p73 protein was acetylated in Tat-treated cells and not in the mock untreated (compare lanes 4 and 5 to lane 3). These results further confirm the functional interaction between Tat and p73 protein.

It has been shown that c-Abl phosphorylates Yes associated protein (YAP) and promotes its interaction with the p73 protein leading to enhanced p73 apoptotic effect (30). Therefore, we sought to examine YAP status in Tat-treated cells. SH-SY5Y cells in duplicate were treated with Tat protein for 24 and 48 hours. The cells were collected where one set was subjected to Western blot analysis and the second to qPCR. As shown in Figure 2E and 2F, addition of Tat increase expression levels of phosphorylated YAP (protein and mRNA, respectively). This experiment confirmed induction of c-Abl and p73 in the presence of Tat through a mechanism that implicates YAP protein. However, one may ask, how Tat induces c-Abl?

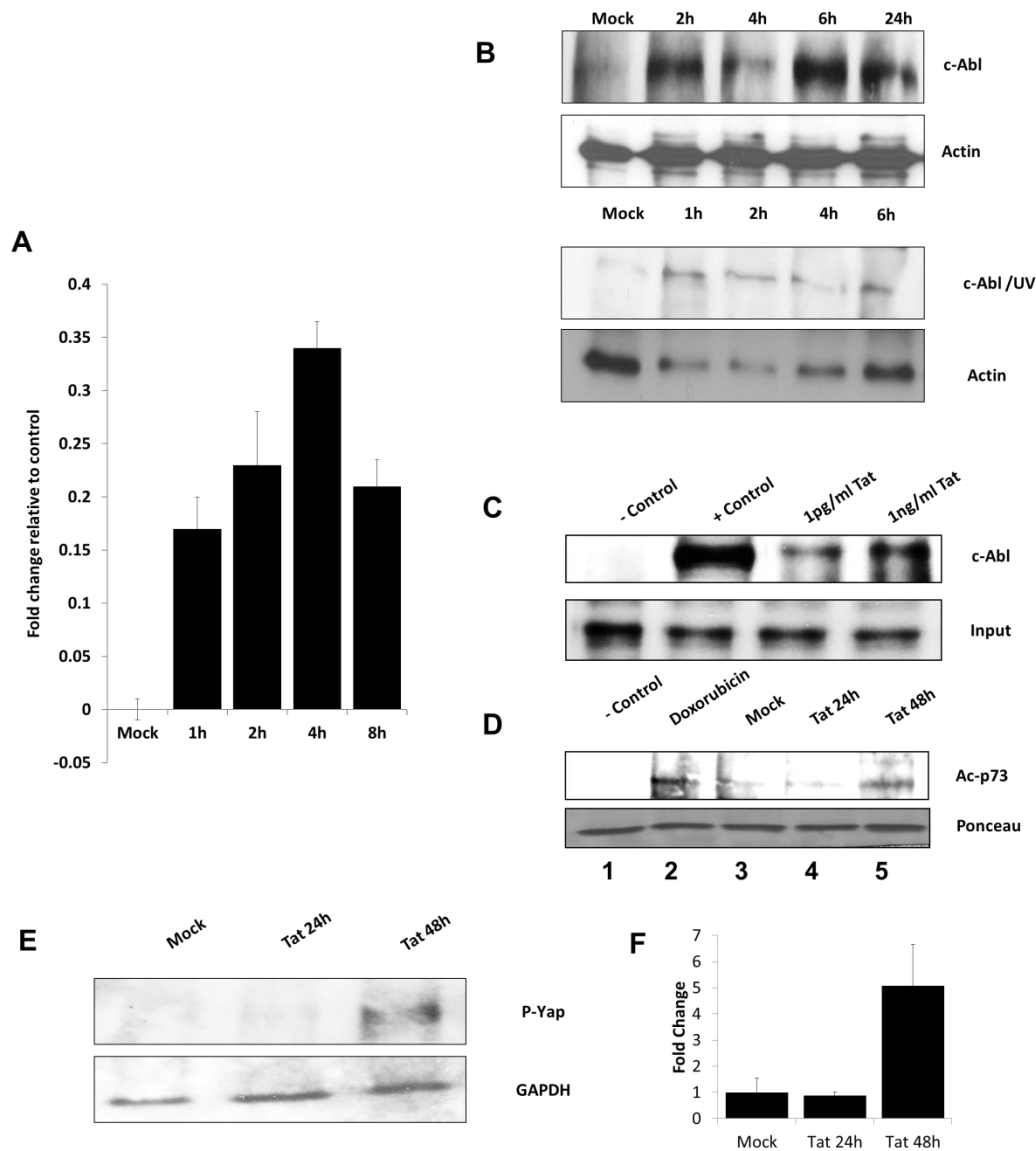


Figure 3.2 Tat increases expression levels of p73 protein through induction of c-Abl. (A) qPCR quantification of the c-Abl mRNA levels in Tat-treated SH-SY5Y cells. (B) Equal amounts of protein extracts (20 μ g/lane) were analyzed by Western blot using anti c-Abl antibody (Cell Signaling). Actin served as a loading control. (C) Immunoprecipitation analysis of lysates (500 μ g of total protein) prepared from SH-SY5Y cells treated with the indicated

amounts of Tat protein. Specific p73 antibody that could detect total p73 protein was used in the IP reaction. Elutes were analyzed by western blot with the c-Abl antibody. Interaction was indicative by the presence of c-Abl band in Tat-treated samples. UV irradiated cells lysate was used as a positive control. Lysates IPed only with beads (no antibody) were also used as a negative control. **(D)** Lysates prepared from SH-SY5Y cells differentiated \pm 10 ng/ml of Tat protein were subjected to IP reaction using p73 antibody (total p73). Elutes were analyzed by Western using anti Ac-Lys antibody. Doxorubicin was used as a DNA damaging agent at 2 μ M final concentration for 24 hours. Lysates IPed only with beads (no antibody) were used as a negative control. **(E, F)** Western blot and qPCR analysis display expression level of phosphorylated YAP protein and mRNA in Tat-treated cells as indicated.

Tat inhibition of miR-196 leads to induction of c-Abl and p73. Induction of c-Abl by Tat led us to examine the mechanisms involved. It is well known that c-Abl expression is regulated by several miRNA, such as miR-30a and miR-196a, as shown by *GeneCopoeia* (see also Figure 3A). miR-196a and miR-30a are bioinformatically predicted (TargetScan) to contain high probability target sequence within the 3'-UTR of the c-Abl mRNA. Therefore, we examined differential expressions of these miRNAs in Tat-treated cells. SH-SY5Y cells were treated with 10 ng/ml of Tat protein for 24 and 48 hours. The cells were collected and RNA was prepared from these cells and then subjected to quantitative PCR assay. Expression levels of miR-30a (Panel B) and miR-196a (Panel C) decrease in Tat-treated cells at 24 and 48 hours, respectively, when compared to the mock untreated.

In order to confirm the impact of these miRNAs on c-Abl, we performed a Western blot analysis. SH-SY5Y cells were transfected with mimic miR-30a or miR-196a for 24 hours prior to the addition of 10 ng/ml of Tat protein for an additional 24 hours. The cells were then washed and protein extracts were prepared and subjected to Western analysis using c-Abl antibody. As shown in Figure 3D, miR-30a failed to affect the levels of c-Abl, while a complete inhibition of c-Abl was observed in the presence of mimic miR-196a, leading to the conclusion that c-Abl is a direct target of miR-196a. GAPDH was used as a control for equal protein loading.

Next, we examined the impact of Tat protein on miR-196a in the presence of its mimic. Two sets of SH-SY5Y cells (one set for qPCR and the second for Western blot analysis) were transfected with miR-196a inhibitor, mimic miR-196a or with the scramble RNA for 24 hours, then treated with Tat protein for an additional 24 hours. As expected, the inhibitor decreases the expression level of miR-196a when compared to the mock as measured by qPCR as measured

by qPCR (Figure 3E). Tat protein failed to decrease miR-196a in the presence of the mimic. The scramble has a modest effect on the levels of miR-196a when compared to the mock (panel E). This experiment was repeated in SH-SY5Y cells transfected with miR-196a mimic only and then treated with Tat protein for 24 or 48 hours. Similar results were obtained as in panel E where Tat failed to inhibit miR-196a expression in the presence of mimic miR-196a (Figure 3F). As for the second set that was subjected to Western analysis, we observe that endogenous level of c-Abl decreases modestly in the presence of scramble RNA when compared to the control. Expression of c-Abl increases in cells transfected with miR-196a inhibitor and did not change in the presence of miR-196a mimic and Tat protein (panel G). Anti-GAPDH was used as a control for equal protein loading (GAPDH panel). Using the same samples to measure expression level of c-Abl by qPCR, we observe that Tat increases the level of c-Abl in the absence of miR-196a mimic, but not in the presence of mimic miR-196a (panel H).

To further validate our observations and the role of miR-196a, we performed a Western blot analysis. SH-SY5Y cells were treated with 10 ng/ml of Tat protein in the presence and absence of miR-196a mimic that was transfected 24 hours prior to the addition of Tat. Twenty-four hours later, the cells were washed and protein extracts were collected and subjected to Western analysis using anti-p73 (total and phosphor-Tyr99); anti-c-Abl; anti-p53 and anti-GAPDH. As shown in Figure 3I, Tat was able to increase expression levels of total and phosphorylated p73, c-Abl and p53 when compared to the mock untreated/ untransfected in the absence of miR-196a mimic but not in its presence. Anti-GAPDH was used as a control for equal protein loading (gapdh panel). These results demonstrated the dependability of Tat function on miR-196a expression to activate the p73/p53 pathway.

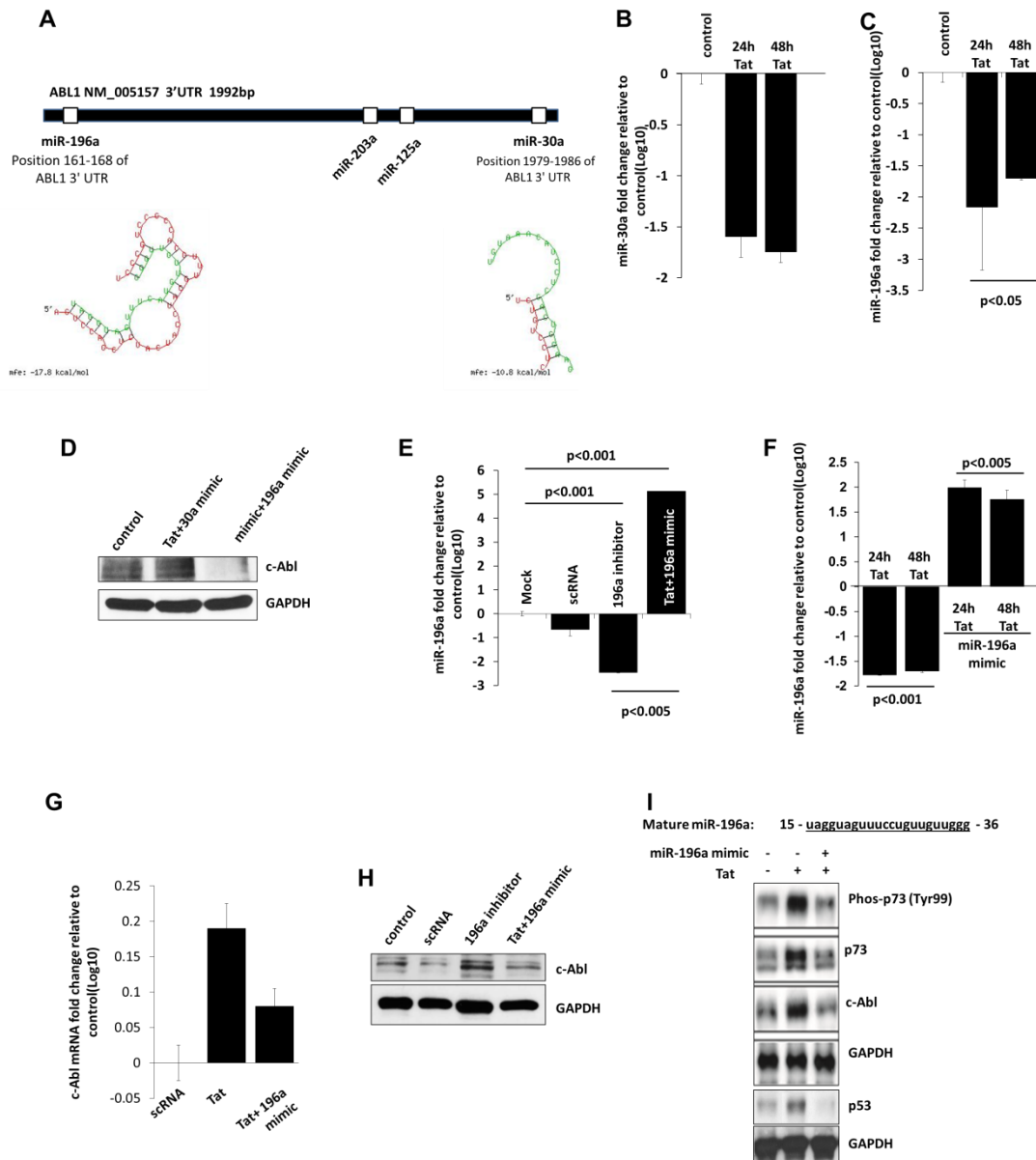
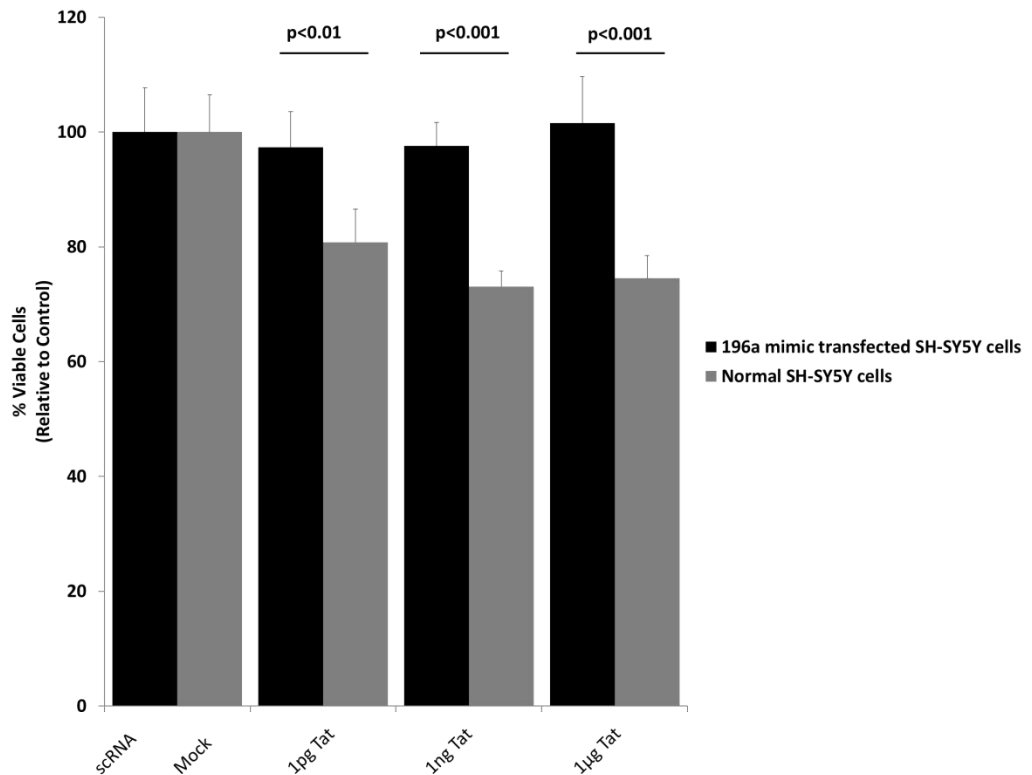


Figure 3.3 Expression levels of miR-196a, c-Abl and p73 in Tat-treated cells. (A) TargetScanHuman analysis of the c-Abl mRNA 3' untranslated region (UTR) for potential microRNA binding sites is displayed. (B, C) Extracts prepared from mock treated or Tat-treated SH-SY5Y cells were subjected to qPCR where the expression of miR-30a and miR-196a were determined. (D, E, F) SH-SY5Y cells were transfected with 100 pmols of 196a inhibitor molecule, miR-196a mimic and/or miR-30a for 24 hours and then treated with Tat protein for an additional 24 hours. Cells were harvested and subjected to Western blot analysis

to detect expression levels of c-Abl (**D**) or qPCR to measure expression levels of miR-196a (**E**, **F**). (**G**, **H**) Similarly, protein and mRNA levels of c-Abl were also measured in SH-SY5Y cells transfected with miR-196a mimic \pm Tat protein using qPCR (**H**) or Western blot (**G**). (**I**) Extracts prepared from SH-SY5Y cells transfected with miR-196a mimic \pm Tat protein were subjected to Western blot analysis using anti-p73 (total and phosphorylated), -c-Abl, -p53 and – GAPDH antibodies. Anti-GAPDH antibody was used as a control for equal protein loading.

Tat failed to cause neuronal death in the presence of miR-196a mimic. After the identification of the pathway used by Tat to cause neuronal damage and the mechanisms involved (miR-196a-c-Abl-YAP and p73), it was necessary to associate these mechanistic observations with function. To that end, SH-SY5Y cells were treated with increasing amount of Tat protein in the presence and absence of mimic miR-196a transfected 24 hours prior to the addition of Tat. As shown in Figure 4A, Tat causes significant neuronal death (10-30%) in the absence of mimic miR-196a but not in its presence when compared to the mock untreated but transfected with scrambled RNA (compare gray to black bars). An example of the cells was also shown in panel B. These results confirmed our observations and pointed to the major role played by miR-196a in order to prevent neuronal deregulation by neutralizing HIV-1 Tat protein function.

A



B

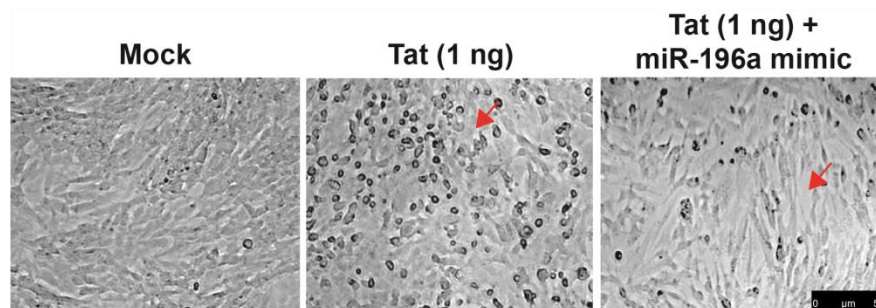


Figure 3.4 Cell viability in Tat-treated SH-SY5Y ± miR-196a mimic. (A) Histograms represent cell viability assay performed using differentiated SH-SY5Y cells transfected with scramble or miR-196a mimic and then treated with an increasing amount of Tat protein as indicated. Values are statistically significant and are presented as a percentage relative to the untreated control samples. The experiment was repeated 3 times. (B) An example of the cells is also displayed.

Tat induces p73 at transcriptional levels only in the presence of Sp1. Finally, we sought to examine whether, in addition to inducing p73 protein, Tat is able to activate p73 at transcriptional level. Note that the p73 promoter is regulated by several transcription factors mainly Sp1 (Figure 5A) (Logotheti et al. 2010; Sudhakar, Jain, and Swarup 2008), and its transcriptional activity heavily relies on the methylation enrichment of the CpG islands (Corn et al. 1999; Liu, Zhan, and Zheng 2008). Hence, it was interesting to test whether Tat could alleviate the methylation of the p73 promoter and renders it active by measuring the expression level of 5-hmC. The 5-hydroxymethylcytosine (5-hmC) is involved in the regulation of gene expression and prompt DNA methylation (Chouliaras et al. 2013). Interestingly, the highest level of this type of DNA modification is found in the CNS, suggesting a potentially crucial role in brain development and plasticity (Kriaucionis and Heintz 2009; Guo et al. 2011). Moreover, 5-hmC is directly linked to passive and active de-methylation of DNA sequences and positively correlated with gene expression in the human brain (Jin et al. 2011; Valinluck and Sowers 2007; Wu and Zhang 2011). To that end, SH-SY5Y cells were treated with Tat protein and then the level of 5-hmC was measured using qPCR to analyze the processed DNA using primers spanning the three known Sp1 binding sites (Figure 5A). As shown in Figure 5B, Tat increases the level of 5-hmC at 48 hours when compared to the control leading to the conclusion that addition of Tat protein indeed increase the 5-hmC levels at the Sp1 binding sites of the p73 promoter.

To validate this observation, we performed a transient transfection assay using 0.5 μ g of p73-luciferase reporter plasmid alone or in the presence of 0.5 μ g of Sp1 expression plasmid. Tat protein was added 24 hours post-transfection. As shown in Figure 5D, recombinant Tat enhances the transcriptional activity of the p73 promoter only in the presence of Sp1 but not in

its absence (compare lanes 3 and 4). These results suggest that although Tat could alleviate the methylation status of the p73 promoter, however it needs Sp1 to activate that promoter.

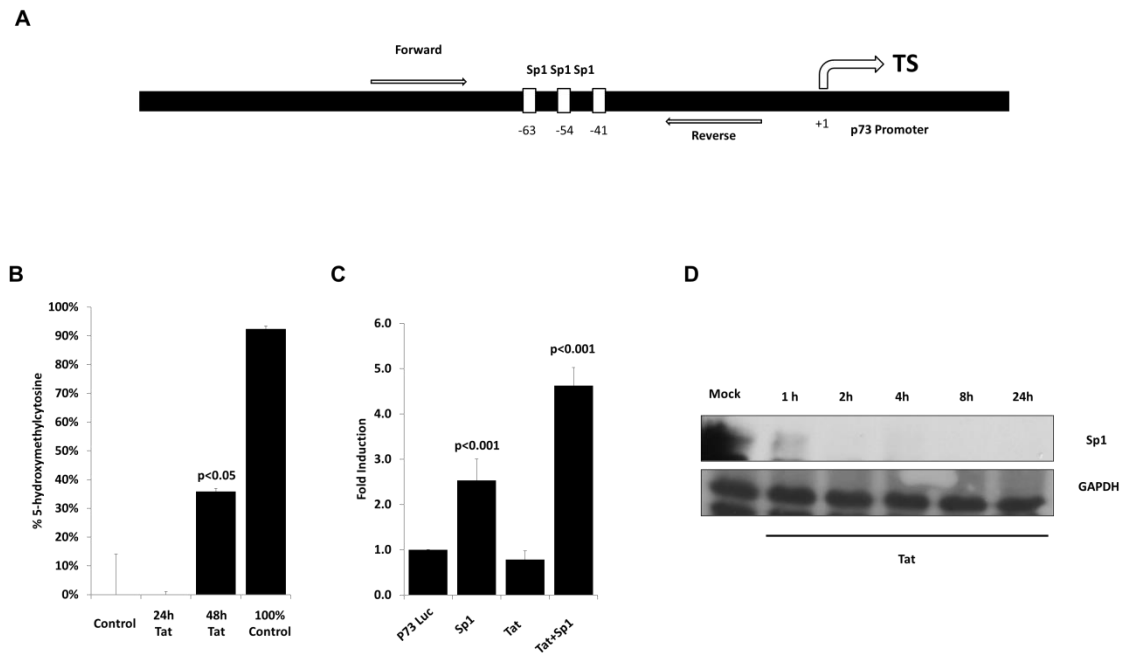


Figure 3.5 Tat affects the methylation status of the p73 promoter. (A) Schematic representation of the p73 proximal promoter region containing three Sp1 binding sites. The arrows represent the PCR primer set used to generate data in panel B. [TS = Transcription start site]. (B) Tat-treated SH-SY5Y cells were harvested at the indicated times for genomic DNA isolation. The DNA was processed using the Quest 5-hmC Detection Kit (Zymo Research). Processed DNA was analyzed by RT-PCR using primers spanning the Sp1 binding sites. Ct values were analyzed according to the manufacturer's protocols and presented as a percentage of relative methylation. (C) SH-SY5Y cells were transfected with the p73-luc reporter alone or in the presence of Sp1 expression plasmid and then treated with Tat protein. At 48 hours cells were harvested and subjected to one freeze-thaw cycle. Light detection readings are presented as a fold change relative to untreated samples. (D) Protein extracts from SH-SY5Y cells \pm Tat were analysed by Western using anti-Sp1 or anti-GAPDH (equal protein loading) antibodies.

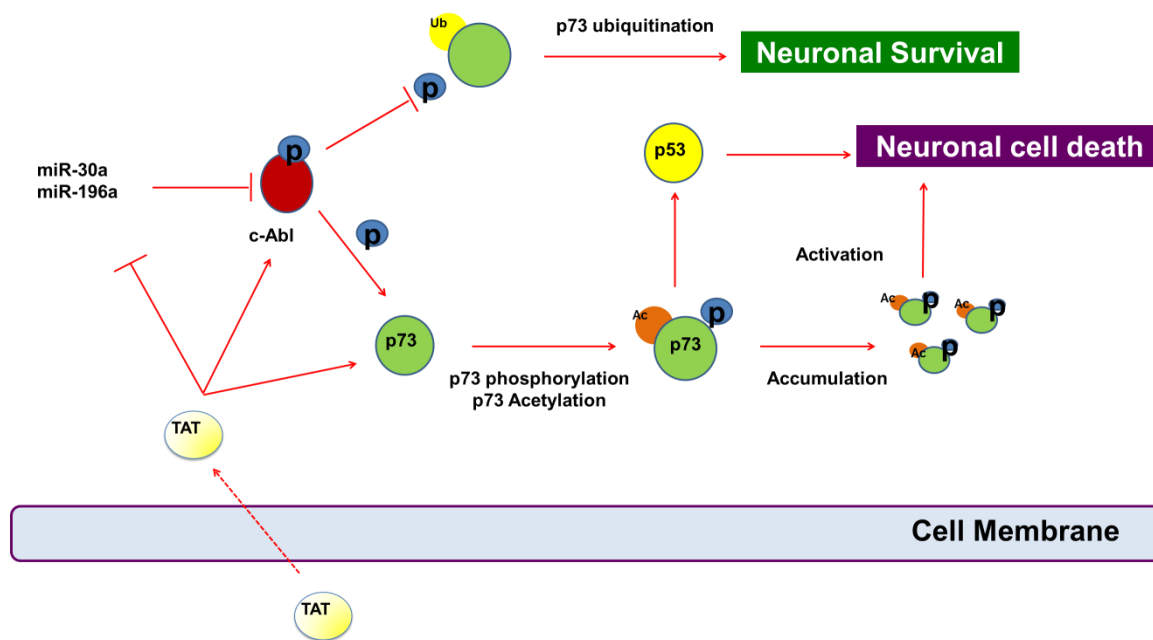


Figure 3.6 Schematic representation of Tat survival/death pathway. Schematic representation of the molecular mechanism used by HIV-1 Tat protein to deregulate the c-Abl-p73-p53 pathway leading to neuronal dysfunction and promoting HIV-1 associated neurocognitive disorders. miR-196a is a key player in this pathway and its function dictate the fate of the neurons.

3.4 Discussion

We previously demonstrated the involvement of p53 family members (p53 and p73) in neuronal deregulation in patients infected with HIV-1 (Mukerjee et al. 2010; Mukerjee et al. 2008). Further, in separate studies, we showed the link between these two proteins (p53 and p73) and HIV-1 in astrocytes (Amini et al. 2005; Bagashev et al. 2013; Saunders et al. 2005). Therefore, the relation between these two proteins and HIV-1 is not without a precedent and was established by several groups including ours, however, the exact molecular mechanisms leading to the activation of these two proteins and triggering their functions in neurons remain unclear.

In here, we deciphered some of these mechanisms used by HIV-1 Tat to activate the p53-p73 pathway leading to neuronal deregulation and eventually increase the incidence of HIV-1 associated neurocognitive disorders (HAND). Interestingly, we found that HIV-1 Tat activates the p73 pathway through a mechanism that involved small non-coding RNA (miRNA) such as miR-196a and miR-34a. In order to activate this pathway, Tat decreases the level of miR-196a leading to the upregulation of its target gene, c-Abl (Figures 2 and 3). Upregulation of c-Abl promotes physical interaction with p73 and its phosphorylation at tyrosine residue 99 (Figures 1-3). Once phosphorylated and the consequent acetylation, p73 changes its subcellular location, and becomes transcriptionally active (Figures 1 and 2) (Alvarez et al. 2004; Yuan et al. 1999). Interestingly, these events are reversed and eliminated in the presence of miR-196a mimic (Figure 3).

Dependence of HIV-1 proteins in general, and Tat in particular, on miRNA to perform their functions is not without a precedent. It has been shown that Tat deregulates miR-221/222 in order to induce expression of ICAM-1 protein, a phenomenon that could impact HIV-1 associated cardiomyopathy (Duan et al. 2013). Similarly, Mishra and Singh demonstrated that Tat modulates the expression of miR-101 to affect the function and expression level of VE-cadherin in human brain microvascular endothelial cells (Mishra and Singh 2013). At transcriptional level, it has been shown that Tat induces the levels of miR-34a, miR-182 and miR-217 in order to regulate the expression of the HIV-1 promoter (LTR) (Zhang, Chen, et al. 2012; Zhang, Wu, et al. 2012; Chen et al. 2013). In addition to human cells, dependence of Tat on miRNA was also shown in animal neurons (e.g. mice and macaque), where Tat was described to deregulate expression levels of miR-29b and miR-128a in order to perform its functions (Eletto et al. 2008; Hu et al. 2012). Taken together, these data corroborate with our

findings regarding the role of Tat in neurons and its association with miRNA (Chang, Mukerjee, et al. 2011) (Figures 2 and 3).

On a different note, several reports demonstrated the ability of Tat to cause neuronal death via a pathway that involved NMDA receptor (Haughey et al. 2001; Song et al. 2003; Eugenin et al. 2007; Aksenov et al. 2012). In our study, we demonstrated that the ability of Tat to cause neuronal death might be NMDA receptor-dependent; however this particular Tat function is not possible without the inhibition of miR-196a as shown in Figure 4. Further, in here we used recombinant Tat protein in small amount compared to other studies, which could also explain the modest cell death observed. Therefore, our data highlight the importance of microRNA not only as genes regulator but also as cell protector from death as previously demonstrated regarding the relation between Tat, p53 and miR-34a (Chang, Mukerjee, et al. 2011).

While, the functional association between miR-34a and p53 is well established, and any disruption of this association might affect the cell fate as demonstrated by several groups including ours (Raver-Shapira et al. 2007; Chang et al. 2007; Chang, Mukerjee, et al. 2011). In this regard, we previously demonstrated the importance and the role of miR-34a along with Tat protein neuronal deregulation (Chang, Mukerjee, et al. 2011). Further, the relation between miR-34a and p73 in neurons was recently studied where the authors demonstrated the dependence of p73 on miR-34a in neurodegenerative diseases such as Alzheimer's (Agostini, Tucci, Killick, et al. 2011). However, the functional relation between miR-196 and p53 family members (p53 or p73) or between miR-196a and c-Abl has never been studied or described. Therefore, our study is a milestone because it establishes the relation between miR-196a and HIV-associated neurocognitive disorders via a pathway that implicates c-Abl, p73 and p53 (Figures 2-4), and it also shed some light on the involvement of a new player in neuronal

regulation. Further, our study also links for the first time involvement of miR-196a in neuronal regulations, which makes miR-196a a novel target for therapeutic approach. Taken together, our data permit to draw a survival/death pathway that could be activated by HIV-1 Tat protein where miR-196a is the main player and its regulation can affect the cell fate (Figure 6). Hence, our data partially unveil the molecular mechanisms used by HIV-1 Tat that could lead to the development of neuronal deregulation. This last point remains to be validated in animal model where it can be associated with function and where the exact role of miR-196a as well as its downstream targets will be evaluated.

CHAPTER 4

CDK9 PHOSPHORILATES PIRH2 PROTEIN AND PREVENTS DEGRADATION OF P53 PROTEIN

4.1 Introduction

Cdk9, a 42-kDa protein, like many other cyclin-dependent kinases (CDKs), was identified during a cDNA screening intended to isolate novel regulators of the mammalian cell cycle (Grana et al. 1994). As no cyclin partner or cell cycle function was demonstrated at that time, cdk9 was temporarily designated PITALRE for its PSTAIRE-like sequence, a conserved motif found in CDC2 and related kinases (Bullrich et al. 1995). Cdk9 was shown to phosphorylate itself (Wei et al. 1998), as well as a variety of substrates *in vitro* and to be associated with various eukaryotic, yeast and viral proteins including the small nuclear 7SK snRNA (Nguyen et al. 2001), p53 (Claudio et al. 2006) and KSHV K-cyclin (Chang and Li 2008).

Wild-type p53 is expressed at low levels in most cells because of its short half-life under normal conditions. p53 levels are regulated in large part by the negative regulatory human homologue of the mdm2 protein, Hdm2. Mdm2 interacts with the N-terminal domain of p53, represses p53 transcriptional activity, mediates ubiquitination of p53 and targets it to the cytoplasm for proteasome-dependent degradation (Kubbutat, Jones, and Vousden 1997). Further, p53 can also be ubiquitinated and degraded by the COP1, ARF-BP, and/or Pirh2 proteins (Leng et al. 2003; Dornan et al. 2004; Chen et al. 2005).

Pirh2 is a gene regulated by p53 that encodes a RING-H2 domain-containing protein with intrinsic ubiquitin-protein ligase activity (Leng et al. 2003). Pirh2 physically interacts with p53 and promotes its ubiquitination. Expression of Pirh2 decreases the level of p53 protein and abrogation of endogenous Pirh2 expression increases the level of p53 (Jung, Liu, and Chen 2010). Furthermore, Pirh2 represses p53 functions including p53-dependent transactivation and

growth inhibition. Interestingly, phosphorylation of Pirh2 leads to its inactivation (Duan et al. 2007).

4.2 Methods

Plasmids. The pcDNA₃-cdk9, cdk9-dn, CMV-p53, pcDNA-Tat (101aa) and HIV_{JR-FL} have been previously described (Claudio et al. 2006; Sawaya et al. 1998; Sawaya et al. 2000). GST-Pirh2, wt and mutants, were previously described (Leng et al. 2003).

Cell Culture, and Transfection Assays. Human lung carcinoma (H1299), astrocytic (U-87MG) and microglial cell lines were maintained in DMEM + 10% FBS (Gibco), 100 units/ml penicillin, 50µg/ml streptomycin-G. Transfection was carried for 24hrs after which CAT assay was performed as previously described (Sawaya et al. 1996).

Cell Cycle Analysis. Human astrocytic cells, U-87MG, were maintained in serum-free media for 72hrs, prior to growth in DMEM+10% FBS. Cells were then transfected with p53 or cdk9 expression plasmids. To monitor for transfection efficiency and to select transfected cells for analysis, cells were co-transfected with EGFP-spectrin plasmid. At the indicated times, cells were processed for FACS analysis by fixing and staining them with propidium iodide to determine the DNA content and EGFP expression simultaneously. To distinguish between non- and transfected, cells were gated on a fluorescence-activated cell sorter, and the DNA profiles of both the EGFP-negative and the EGFP-positive population were determined.

Purification of Recombinant Proteins (GST). GST-Pirh2 (wt and mutants) fusion proteins were expressed and purified as described (Amini et al. 2005). The integrity and purity of the GST fusion proteins were analyzed by SDS-PAGE followed by Coomassie blue staining. Radiolabeled cdk9 protein was synthesized with TNT-coupled wheat germ extract system according to the manufacturer's recommendations (Promega).

In vitro Protein-Protein Interactions (GST Pull-Down Assay). Four microliter of [³⁵S] labeled and translated cdk9 protein was incubated with 5µg of GST or fusion proteins GST-Pirh2 coupled to glutathione sepharose beads in 300µl of Lysis Buffer 150 (LB 150) for 2hrs at 4°C with continuous rocking. After incubation, beads were pelleted and washed 5x with LB-150 buffer. Bound proteins were eluted with Laemmli sample buffer, heated to 95°C for 5min and separated by SDS-PAGE.

In vitro Kinase Assays. Kinase assays were performed essentially as described (Grana et al. 1994) with 0.5µg each of [³⁵S] IVT-cdk9 or cdk9-dn in 10µl reaction mixtures containing 50mM Tris-HCl (pH 7.5), 10mM MgCl₂, 1mM DTT, 100mg/ml BSA, 50mM ATP, and 2.5µCi of [γ -³²P] ATP. Reactions were incubated at 37°C for 30min, stopped with SDS loading dye, run on SDS-10% PAGE, fixed, stained, and then allowed to dry before autoradiography.

Immunoprecipitation and Western blotting. Cells were transfected with cdk9, mdm2, Pirh2, HIV-1 Tat or p53 expression plasmids. 24hrs post-transfection, 200 µg of cell extracts were immunoprecipitated with anti-p53, -cdk9, -Pirh2, -Tat, -pol II (Ser-2), pol II (Ser-5), or -CycT1 antibodies. Fifty micrograms of protein extracts were used for Western blot analysis as described (Mukerjee et al. 2010). Anti-mdm2 antibodies (4B2, 2A9, and 2A10) were a gift from Arnold J. Levine (Institute of Advanced Study, Princeton NJ) (Pauls et al. 2006). Anti-hsp70, and anti-Grb2 antibodies were used as a control for equal protein loading. Note that Pirh2 antibody (ab57152, Abcam) was used in all our experiments.

In vivo ubiquitination assay. U-87MG cells were transfected with plasmids encoding p53, cdk9, Pirh2, and Histidine-tagged ubiquitin (6xHis-Ub) either alone or in combination. Cells were collected 24hrs later, and lysates were subjected to Western blotting and

immunoprecipitation as described above. The total DNA level was kept constant by the addition of the empty vector.

RNA interference. 400nM of SmartPool small interfering RNA against Pirh2 (siRNA-Pirh2) (5'-ucaacuagaucgcuuuuuuu dTdT-3' and 5'-uucuccgaacgugucacgudTdT-3') were transfected into $\sim 1 \times 10^6$ U-87MG in serum free media alone or in the presence of 5 μ g of plasmids that express cdk9, p53, Pirh2, or 6xHis-Ub using RNAiFect transfection reagents (QIAGEN). Similarly, 200nM of non-specific siRNA or siRNA-cdk9 (50, 100 and 200nM) were transfected into microglial cells. Efficiency of transfection and the levels of the specific proteins were analyzed by Western blot assay (50 μ g of cell extracts) using Pirh2, Grb2 or GAPDH antibodies.

Immunocytochemistry. Immunocytochemistry was performed utilizing an Avidin-Biotin-Peroxidase kit, (Vectastain Elite ABC Peroxidase kit) as previously described (Deshmane et al. 2009). Primary antibodies were incubated overnight at room temperature (anti-Pirh2, or anti-cdk9 monoclonal antibodies). After rinsing with PBS, sections were incubated for one hour at room temperature with biotinylated anti-rabbit or anti-goat secondary antibodies. The tissue was subsequently incubated with Avidin-Biotin-Peroxidase complexes for 1hr at room temperature according and finally, the sections were developed with a diaminobenzidine substrate (Sigma Laboratories), counterstained with hematoxylin and coverslipped with Permount (Fisher Scientific).

Cell infection. Human U-937 cell line was maintained in RPMI + 10% FBS (Gibco), 100units/ml penicillin, 50 μ g/ml streptomycin-G. Cells in the log phase of growth were infected with JR-FL strain of HIV-1 as follows. 50ng of p24 containing virus stock were added to every 1×10^6 cells. Cells were incubated with virus stock in a small volume of serum free media for

2hrs at 37°C. The cells were then washed twice with PBS and a new fresh media containing 2% of FBS was added (0.5×10^6 cells /ml). The cells were also transfected with siRNA-Pirh2 or with CMV-Pirh2 as described (Deshmane et al. 2009). Cells were collected every alternate day for p24 ELISA test.

p24 ELISA. p24 antigens ELISA was performed as described (Sawaya et al. 2000). Each sample was assayed over a 10,000-fold range of dilution to ensure quantitation was based on an OD value within the linear range of the standards.

4.3 Results

We previously demonstrated that p53 protein inhibits the phosphorylation of the serine 2 residue of the carboxyl terminal domain (CTD) of polymerase II (Pol II) and stalls the transcriptional elongation (Claudio et al. 2006; Mukerjee et al. 2010). Hence we sought to unravel the mechanisms involved.

Cdk9 prevents p53-apoptotic capability in CNS-derived cells. Although not within the scope of this study, we sought to examine the effect of p53 accumulation on cell viability in the presence of overexpressed cdk9. Interestingly, accumulation of p53 and its inhibitory effect did not lead to cell death as shown in Figure 1A. At 22 and 40hrs, astrogloma cells transfected with p53 exhibit arrest in the G1/S checkpoint of the cell cycle (70.5%). p53 failed to arrest the cells in G1 in the presence of over expressed cdk9 (18.3% vs. 90.5%) (p53 and p53+cdk9 Panels at 40hrs). These data also suggest that accumulation of p53 in cdk9-transfected cells did not affect cell viability.

Ubiquitination and degradation of p53. Accumulation and activation of p53 gave us the rationale to investigate p53 ubiquitination status. U-87MG cells were transfected with pcDNA₃-6xHis-Ub, cdk9 and/or p53 expression plasmids using different combination. Cell extracts were

prepared at 24hrs where 200 μ g were incubated with anti-p53 and then analyzed by Western blot. As shown in Figure 1B, anti-His antibody reveals a major ubiquitination of p53 in p53/cdk9-transfected cells (lane 3) when compared to extracts prepared from cells transfected with only p53 plasmids (lane 2). No band(s) is/are observed in extracts prepared from mock cells (lane 1). The specificity of immunoprecipitation was examined when the cellular extracts were immunoprecipitated with beads only (no antibodies) and then subjected to Western analysis (lanes 4-6). Our data showed that p53 was ubiquitinated in extracts prepared from cdk9-transfected cells. Data from panel A showed that p53 failed to cause cell cycle arrest in cells where cdk9 was overexpressed. Our results corroborate with Goh *et al*, where they demonstrated that HIV-1 replication is more efficient if a cell cycle pause occurs (D'Orso and Frankel 2010). Hence, we concluded that a functional p53 (stalling transcriptional elongation) plays a role in viral replication as previously demonstrated (Pauls et al. 2006).

Inhibition of p53 is independent of Mdm2. Mdm2 is one of the factors that control p53 levels, hence, we sought to examine mdm2 function in cdk9-transfected cells. H1299 cells (p53^{-/-}) were transfected with 0.5 μ g of p53 and/or cdk9 expression plasmid for 24hrs. Note that mdm2 may undergo covalent modifications that reduces its function such as its phosphorylation on serine 395 (Maya et al. 2001). Western blot analysis reveals that the levels of mdm2 (total and phosphorylated) increase in the presence of p53 (Figure 1C, lane 2) and/or p53/cdk9 (lane 3) when compared to the mock untransfected (lane 1). Interestingly, mdm2 was phosphorylated on Serine 395 residue in H1299 transfected with cdk9 and/or p53 (lanes 2 and 3) as obtained with Mdm2 antibody, 2A10 that is specific for phosphorylated Ser-395 (Maya et al. 2001). Anti-mdm2 antibodies (4B2, and 2A9) were used as positive controls to determine the induction of mdm2 by p53 and/or cdk9. For equal protein loading, hsp70 antibody was used.

We then concluded that cdk9 promotes phosphorylation of mdm2, a phenomenon that might help preventing mdm2 from degrading p53.

In order to correlate protein expression with function, we performed transient transfection assay in H1299 cells using mdm2-CAT reporter plasmid transfected with p53 alone in the presence and absence of cdk9 expression plasmids. As shown in Figure 1D, upregulation of the mdm2 promoter increases in the presence of p53 and cdk9, which confirmed that increase in the expression level of p53 by cdk9 is associated and leads to the induction of p53 function also.

Can Pirh2 promote the Ubiquitination of p53? In addition to mdm2, Pirh2 protein has been shown to promote the ubiquitination of p53 (Brooks and Gu 2006; Brooks and Gu 2011). Therefore, we sought to examine whether cdk9 affects the status of Pirh2. H1299 cells were transfected with 0.5 μ g Tat and/or p53 expression plasmids as indicated. At 24hrs, the cells were collected and subjected to Western analysis using anti-Tat, -p53, -cdk9, -Pirh2, -CycT1, -Serine 2 and 5 of the CTD and anti-Grb2 antibodies. Overexpression of p53 led to an increase in the level of endogenous cdk9 (Figure 1D, lanes 2) when compared to the mock (lane 1). Interestingly, a decrease in the levels of endogenous Pirh2 was observed in p53-transfected cells (lane 2). As expected, p53 prevents the phosphorylation of Ser-2 but not Ser-5 of the CTD in these cells (lanes 2).

Phosphorylated Pirh2 is known to be inactive, therefore, we sought to examine whether cdk9 phosphorylates it. The cells were treated with Flavopiridol (cdk9 inhibitor) (Baumli et al. 2008). Interestingly, Pirh2 level increases while the level of p53 decreases (Panel E, lane 3). Presence of Tat was demonstrated in all lanes (lanes 1-3). For equal protein loading, Grb2 antibody was used. These results pointed to the ability of *i-* cdk9 to downregulate the level of

Pirh2; *ii*- p53 to escape the inhibitory effect of Pirh2; and *iii*- in addition to Calmodulin-dependent kinase II (CaMK II) (Duan et al. 2007), other kinases such as cdk9 have the ability to phosphorylate Pirh2.

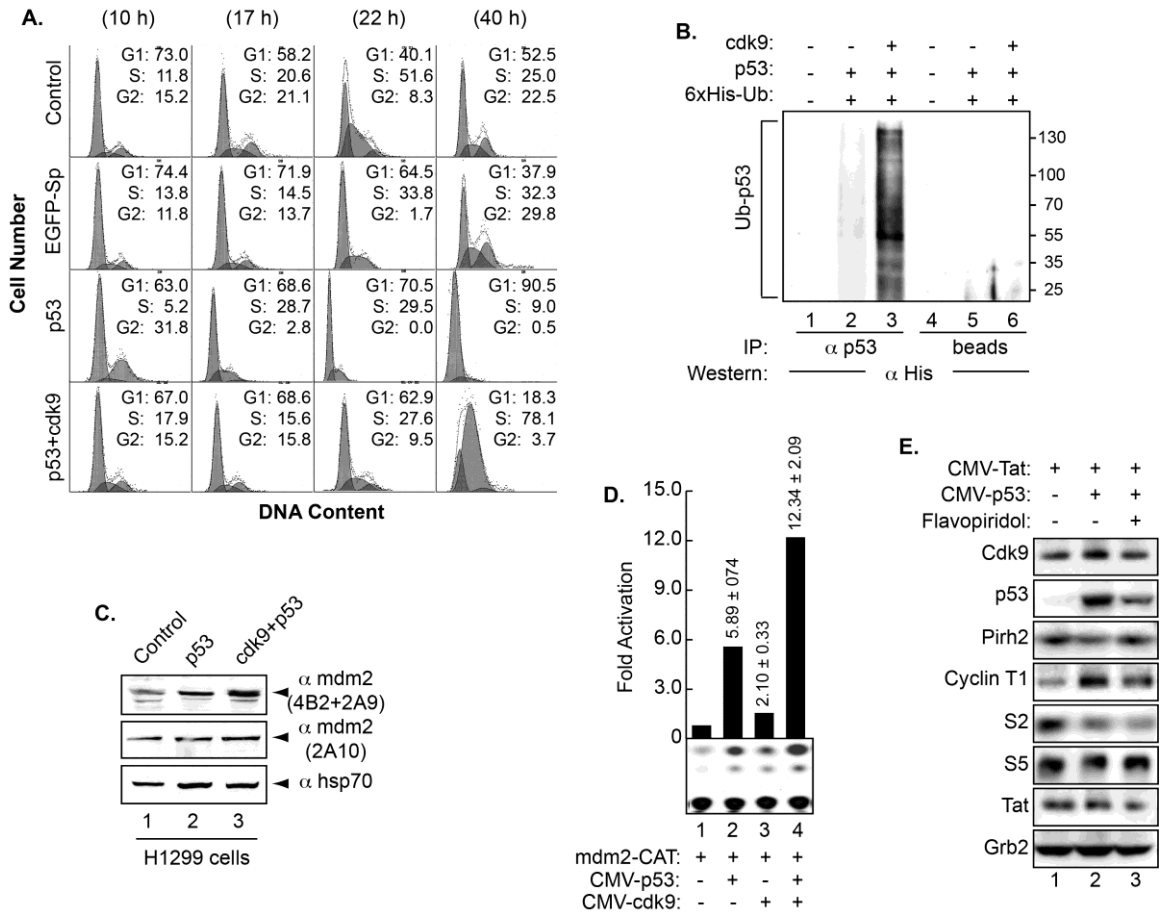


Figure 4.1 Status of p53 in cdk9-transfected cells and involvement of Pirh2. **A.** U-87MG cells were synchronized by serum starvation. The cells were transfected with plasmids expressing p53, or p53 and cdk9 together, along with a plasmid expressing EGFP-spectrin. At the indicated time, the cells were processed for the measurement of their DNA content and EGFP expression by FACS. The % of cells in G1, S and G2, are indicated in each window. **B.** U-87MG cells were transfected with 6xHis-Ubiquitin, p53, and cdk9. IP was with anti-p53

antibody (lanes 1-3) or beads alone (lanes 4-6) as indicated, followed by immunoblotting using anti-His antibody. **C.** Cdk9 phosphorylates serine 395 within mdm2. Extract prepared from H1299 cells (p53^{-/-}) were Western blotted with anti-mdm2. 4B2+2A9 recognize total mdm2, while 2A10 recognizes only mdm2 that is phosphorylated on serine 395. Anti-hsp70 was a loading control. **D.** H1299 were transfected with 0.1 µg of mdm2-CAT plasmid alone or in combination with 0.25 µg of p53 and/or cdk9 expression plasmids for 48hrs. The cells were lysed and subjected to CAT assay. **E.** Extract prepared from H1299 cells transfected or treated with 200 nM of Flavopiridol for 16 hours, prior to their transfection with Tat and p53, were Western blotted with anti-cdk9, -p53, -Pirh2, -Tat, -CycT1, -serine 2 and serine-5 (of the CTD) antibodies to detect the indicated proteins. Grb2 was use as a control for equal protein loading.

Cdk9 associates with Pirh2 protein in vitro and in vivo. Next, we sought to identify whether a physical interaction exists between cdk9 and Pirh2. Using immunoprecipitation/Western blot assays, we detect the formation of cdk9-Pirh2 complex. Briefly, the cells were transfected with Pirh2 (Figure 2, Panel A, lanes 4-6) or cdk9 (Panel B, lanes 4-6) expression plasmids. Total cell extracts were collected after 24hrs. 200 μ g of extracts were subjected to immunoprecipitation using anti-Pirh2 antibody (Panel A, lanes 3 and 6), or anti-cdk9 (Panel B, lanes 3 and 6) or with non-immune mouse serum (NIS) (Panels A and B, lanes 2 and 5) and then subjected to Western analysis using anti-cdk9 antibody (Panel A) or anti-Pirh2 (Panel B). In parallel, 50 μ g of extracts were directly Western blotted (lanes 1 and 4 of each Panel). This experiment demonstrated the ability of endogenous Pirh2 (lane 3) to interact with endogenous cdk9 (lane 3). Specificity of Pirh2: cdk9 interaction was confirmed using NIS. Interestingly, overexpression of Pirh2 induces endogenous level of cdk9 (Panel A, compare lanes 1 and 4). Reciprocally, a slight induction of endogenous Pirh2 was observed in cdk9-transfected cells (Panel B, compare lanes 1 and 4).

Mapping the cdk9-interacting domain of Pirh2. To determine the domain of Pirh2 involved in the interaction with cdk9, a series of GST-pull down assays were performed. Various N- and C-terminal Pirh2 deletion mutants were fused to GST, and incubated with purified recombinant cdk9. As shown in Figure 2D and E, deletion of the N- and C-terminal regions of Pirh2 up to residues 120 or 137, respectively, did not affect its ability to bind to cdk9. This suggests that amino acids 120-137 of Pirh2 are crucial for its association with cdk9. Panel E illustrates representative results from a GST pull-down assay obtained using *in vitro* translated cdk9. Panel F illustrates the size of GST (lane 2) or GST-Pirh2 deletion mutants (lanes 3-8), respectively. Interestingly, p53 and cdk9 proteins bind to the same domain within Pirh2, a phenomenon that may also explain Pirh2 inactivity (Leng et al. 2003). Although these

data demonstrated the presence of a physical interplay between cdk9 and Pirh2, we thought to examine whether a functional interaction exists.

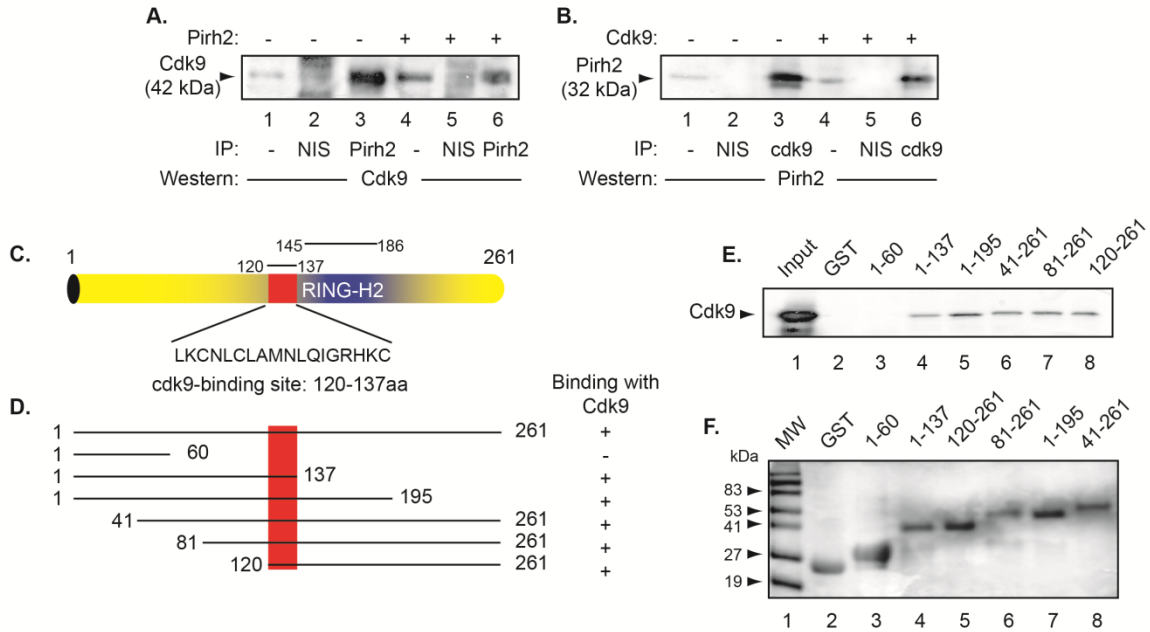


Figure 4.2 Association of cdk9 and Pirh2 proteins in vitro and in vivo. **A, B.** U-87MG cells were transfected with Pirh2 (Panel A lanes 4, 5, and 6) or with cdk9 (Panel B, lanes 4, 5, and 6). 200µg of cell extract were IPed with anti-Pirh2 (Panel A, lanes 3 and 6), or anti-cdk9 (Panel B, lanes 3 and 6), or mouse serum (Panels A and B, lanes 2 and 5). In parallel, 50µg of extracts were directly Western blotted (lanes 1, and 4 of each Panel). An arrow shows cdk9 or Pirh2. **C, D.** Identification of the Pirh2 domain that binds to cdk9. Schematic representation of the Pirh2 domains and its deletion mutants. Binding of cdk9 to various Pirh2 mutants are shown on the right. Strong (+), and none (-). **E.** A representative result from GST pull-down assays obtained with cdk9. Wild type Pirh2 or the indicated mutants fused to GST and immobilized on glutathione-sepharose were incubated with *in vitro* synthesized [³⁵S-methionine]-labeled cdk9 protein. Bound proteins were eluted and analyzed by SDS-PAGE. **F.**

A stained SDS gel showing the quality and sizes of GST or GST-Pirh2 deletion mutant fusion proteins.

Cdk9 phosphorylates Pirh2 and renders it inactive. Our data led us to examine whether association of cdk9-Pirh2 leads to Pirh2 phosphorylation as well as its inactivation. We then investigate the phosphorylation status of Pirh2 in cdk9-transfected cells. Microglial cells (Janabi et al. 1995) were transfected with an increasing amount of cdk9 expression plasmid (0.5, 1.0 and 2.5 μ g) for 24hrs. The cells were then collected and protein extracts were subjected to Western blot analysis. As expected, an increase in the levels of cdk9 and endogenous p53 proteins is observed (Figure 3A, lanes 1-4). Interestingly, increased amount of cdk9 led to a decrease in the levels of endogenous Pirh2 (Pirh2 Panel). Addition of cdk9 increases the endogenous levels of phosphorylated Pirh2 (pPirh2 Panel). Note that the extracts were incubated with anti-Ser/Thr antibody and then subjected to Western blot using anti-Pirh2 antibody. We also observe a slight induction of mdm2 endogenous levels in the presence of cdk9 (mdm2 Panel, lanes 3-4), which correlate with the data shown in Figure 1C. Grb2 was used as a control for equal protein loading. These results led us to conclude that phosphorylation of Pirh2 by cdk9 shorten its half-life which corroborate with previous data (Duan et al. 2007).

Next, we sought to eliminate the effect of cdk9, therefore microglial cells were transfected with increasing amount (50, 100 and 200nM) of small interference RNA directed against cdk9 (siRNA-cdk9). As shown in panel B, endogenous level of Pirh2 was not affected in cells transfected with scrambled/non specific siRNA (lane 2) when compared to the Mock/untransfected (lane 1). Endogenous level of Pirh2 increases in the absence of cdk9 (lane 5). GAPDH was used as a control for equal proteins loading. These results confirmed inhibition of Pirh2 by cdk9.

Phosphorylation of Pirh2 by cdk9 gave the rationale to further examine whether cdk9 phosphorylates Pirh2 directly and to identify the phosphorylated residue(s) within Pirh2. In order to eliminate other kinases, we performed *in vitro* kinase assay where we used GST-Pirh2 full length and deletion mutants. Cdk9 protein was purified using *in vitro* translation kit (IVT) (Claudio et al. 2006). Incubation of purified cdk9 with GST-Pirh2 (Full length and mutants) points to the ability of cdk9 to phosphorylate residues located between residues 195 and 261 within Pirh2 protein (Figures 3C and D) as demonstrated by Kinase assay. Note that GST-Pirh2 full length was used in all the lanes as internal positive control.

Pirh2 domain encompassing amino acids 195-261 contains 8 potential residues that can be phosphorylated (Serine [ser-S] and threonine [thr-T]) as shown in Figure 3E. Therefore, we sought to mutate all these residues to alanine (ala-A) and determine which residue is directly phosphorylated by cdk9. GST-Pirh2 (195-261) was used as a template where these mutations were introduced. Kinase assay points to the ability of cdk9 to phosphorylate Pirh2 on Ser-211 and Thr-217 residues (Figure 3F). Mutation of either residue prevented cdk9 from phosphorylating Pirh2. Full length Pirh2 was used as a control in all the lines (Panel F).

Phosphorylation of Pirh2 at Ser211/Thr217 by cdk9 enhances its self-ubiquitination. It has been shown that phosphorylation of Pirh2 by Calmodulin-dependent kinase II (CaMK-II) enhances its self-ubiquitination (Duan et al. 2007) . Therefore, we sought to investigate whether the same scenario is repeated in the presence of cdk9. Microglial cells were transfected with pcDNA3-6xHis-Ub, cdk9, GST-Pirh2 (full length or mutant S211/A-T217/A) expression plasmids as indicated. Cellular proteins were prepared after 24hrs, 200µg of extracts were incubated with anti-GST antibody and then analyzed by Western blot using anti-Pirh2 or anti-His antibodies. As shown in Figure 3G, phosphorylated full length Pirh2 was ubiquitinated

(lane 2) when compared to the mutant unphosphorylated (lane 3) or when compared to extracts prepared from cells transfected with only His-Ub plasmid (lane 1). In parallel, 50 μ g of extracts were directly Western blotted to examine the levels of endogenous and overexpressed proteins using anti-Pirh2, -cdk9, -GST and -Grb2 antibodies (Panel G). The slight decrease observed in the level of Pirh2 mutant (GST-Pirh2 panel, lane 3) may be due to the phosphorylation of GST-Pirh2 on different residues by other kinases. Grb2 was used to control equal proteins loading. These results further confirm that phosphorylated Pirh2 is self-ubiquitinated in cdk9-transfected cells.

Next, we examine the specificity of Pirh2 phosphorylation by cdk9. Microglia were transfected with cdk9 or cdk9 dominant mutant (cdk9-dn), which lacks the kinase activity . Protein extracts were collected and Western blotted to examine the levels of endogenous and/or overexpressed proteins using anti-Pirh2 (total and phosphorylated), -cdk9, -p53 and Grb2 antibodies. Pirh2 was phosphorylated in cells transfected with cdk9 but not with cdk9-dn (Panel H, compare lanes 2 and 3). The level of p53 decreases in cdk9-dn-transfected cells (lane 3). Grb2 was used to control equal proteins loading. These results further confirm the specificity of Pirh2 phosphorylation by cdk9.

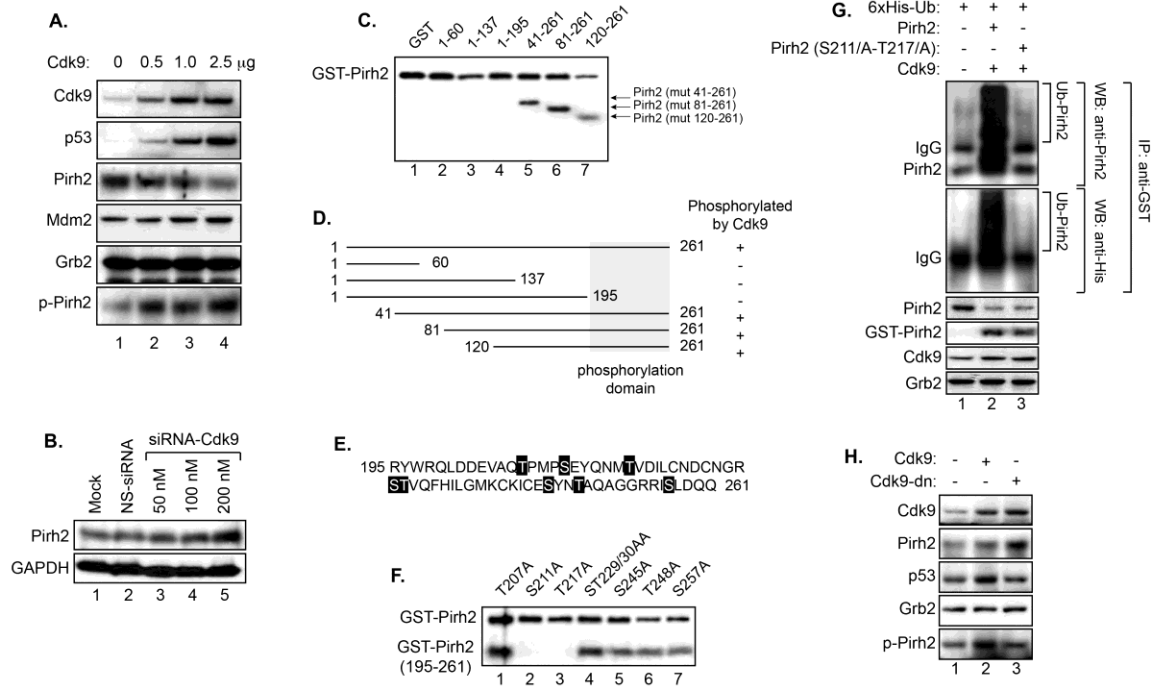


Figure 4.3 Phosphorylation of Pirh2 protein by cdk9 and identification of the phosphorylated residues. **A.** Extracts were prepared from human microglia transfected with an increasing concentration of cdk9 expression plasmid were Western blotted with anti-cdk9, -p53, -Pirh2 and mdm2 antibodies to detect the indicated proteins. Grb2 was used as a control for equal protein loading. 200 μ g of extracts were immunoprecipitated with anti-serine/threonine antibody and then subjected to Western blot analysis using anti-Pirh2 antibody as indicated. **B.** Microglial cells were transfected with non-specific siRNA or with an increasing amount of siRNA-Pirh2 (50, 100 and 200 nM). Lane 1 displays the levels of Pirh2 in extracts from untransfected cells lanes 2-5 display the level of Pirh2 in extracts transfected with siRNA-Pirh2. Anti-GAPDH antibody was used as a control for equal protein loading. **C.** Kinase assays were performed using IVT cdk9 (5 μ l) that were mixed with 1 μ g of GST (lane 1), GST-Pirh2 full length (lanes 2 - 7) together with GST-Pirh2 mutants as indicated and γ - 32 P-ATP. After 30 min at 37°C, Pirh2 phosphorylation was assessed by SDS-10% PAGE. The arrow shows the position of the GST-Pirh2 proteins. **D.** Identification of cdk9-domain within Pirh2. Schematic

representation of the Pirh2 domains and its deletion mutants. Phosphorylated Pirh2 mutants by cdk9 are shown on the right. Strong (+), and none (-). **E.** Amino acids sequence of the C-terminal of Pirh2 encompassing residues 195 to 261. Serine (S) and threonine (T) residues are marked by a black box. **F.** Kinase assays were performed using IVT cdk9 mixed with 1 μ g of GST-Pirh2 full length (lanes 1 - 7) together with GST-Pirh2 mutants (195-261) where serine and threonine were substituted with alanine (A) as indicated. Pirh2 phosphorylation was assessed by SDS-10% PAGE and the phosphorylated residues were identified (lanes 2 and 3). **G.** Microglial cells were transfected with 6xHis-Ubiquitin, GST-Pirh2 (Full length and mutant), and cdk9 as indicated. IP was with anti-GST antibody (lanes 1-3) followed by immunoblotting using anti-Pirh2 or -His antibodies. In parallel, extracts were Western blotted with anti-cdk9, -GST, -Pirh2, and -Grb2 antibodies to detect the indicated proteins. Grb2 was used as a control for equal protein loading. **H.** Protein extracts prepared from microglial cells were transfected with cdk9 or cdk9 dominant negative (cdk9-dn) expression plasmids were blotted with anti-cdk9, -p53, -Pirh2 and -Grb2 antibodies to detect the indicated proteins. Grb2 was used as a control for equal protein loading. In parallel, 200 μ g of extracts were immunoprecipitated with anti-serine/threonine antibody and then subjected to Western blot analysis using anti-Pirh2 antibody as indicated.

Induction of Pirh2 in HIV-1-infected cells. To further confirm this hypothesis regarding involvement of Pirh2 in HIV-1 replication, we analyzed the levels of and sub-cellular localization of Pirh2 and/or cdk9 by immuno-histochemistry. Consistent with our earlier data, Pirh2 and cdk9 were detected in microglia of an HIV encephalopathy case (Figure 4A). Both proteins were found in low levels in the cytoplasmic compartment of microglia in the normal

brain compared with a robust immunolabeling in the cytoplasm of infected microglia in the white matter of a case of HIV encephalopathy (overlay).

To further validate our observations, we performed an infection assay. U-937 cells were infected with JR-FL strain of HIV-1 as described in the Methods section. The cells were transfected with either siRNA-Pirh2 (to silence Pirh2) or with CMV-Pirh2 (to over express Pirh2 protein) 24hrs prior to the infection. Supernatant was collected every day from the cells for five days after which p24 ELISA test was performed to exam the infection efficiency. As shown in Figure 4B, addition of siRNA-Pirh2 delayed HIV-1 replication for 3 days only (green), a phenomenon that could be due to the inhibitory effect of p53 and its half life or to the capability of the virus to overcome p53 effect through an unknown, yet to be determined, pathway. Overexpression of Pirh2 did not affect viral replication and this could be due to the degradation of p53 by Pirh2 (blue). These results corroborate our data regarding decrease of Pirh2 and inhibition of HIV-1 transcription. siRNA-Pirh2 efficiency was validated by Western blot analysis using anti-Pirh2 antibody (Panel C).

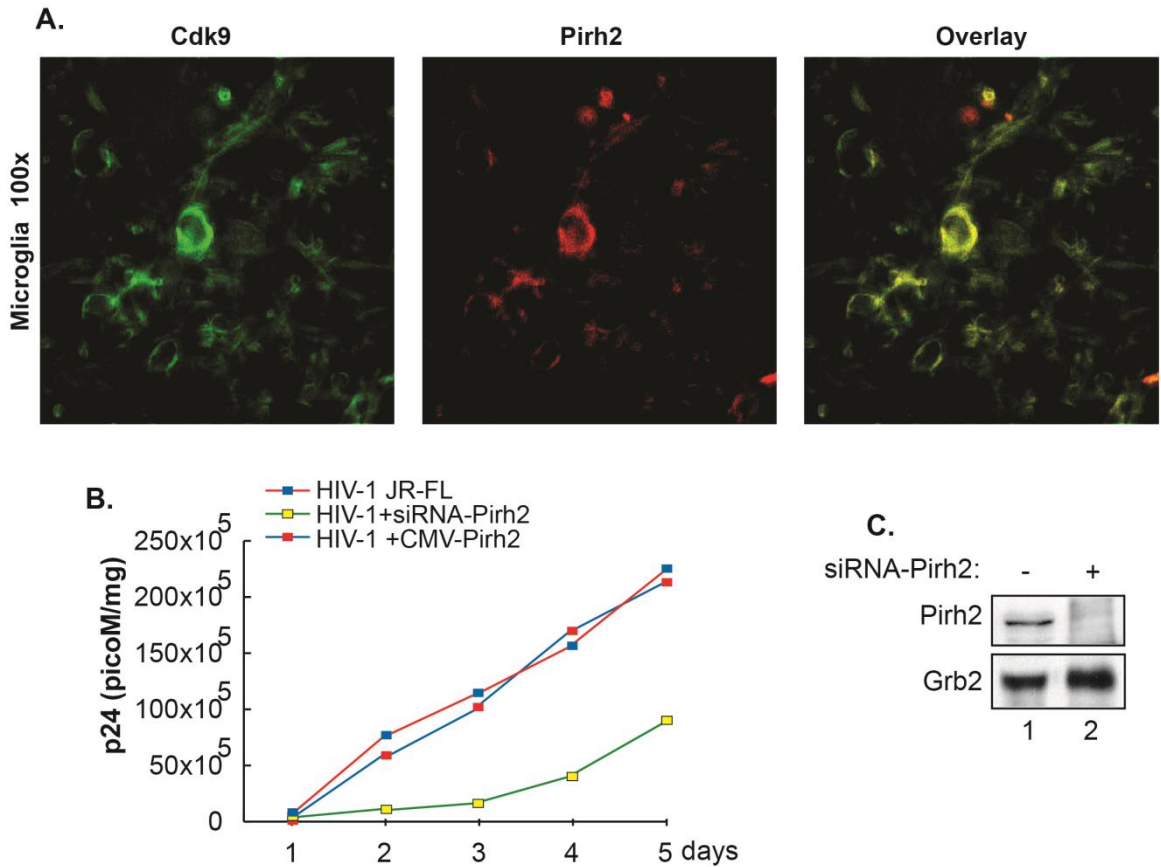


Figure 4.4 Detection of Pirh2 in Cells and its role in HIV-1 replication. **A.** Double labeling immunofluorescence demonstrates the co-localization of Pirh2 and Cdk9 in microglia in the white matter of a case of HIV encephalopathy (overlay). **B.** Histograms showing the amount of p24 protein measured in the supernatant of infected cells. As shown, demonstrated, addition of siRNA-Pirh2 delays HIV-1 replication in U-937 cells while overexpression promotes viral replication. **C.** Cells were transfected with siRNA-Pirh2. The efficiency of the transfection was investigated using extracts from untransfected (lane 1) or transfected (lane 2) cells that were blotted with anti-Pirh2. Grb2 was used as a control for equal protein loading.

4.4 Discussion

Mechanisms that could inhibit Pirh2. Several factors have been shown to affect Pirh2 stability such as Tip60 (Logan et al. 2004). Overexpression of TIP60 enhances Pirh2 protein stability and alters Pirh2 sub cellular localization. Interestingly, HIV-1 Tat was shown to physically interact with Tip60, to lower its level and to decrease Tip60 HAT activity (Col et al. 2005). This observation could also explain the inactivity of Pirh2 and the inhibitory effect of p53 in the presence of Tat protein in HIV-infected cells. In addition, Tip60 was shown to acetylate and activate p53 to prevent tumor growth (Sho et al. 2011) and inhibition of Tip60 can prevent acetylation of p53 as well as p53 activation and give place to tumor growth and cell proliferation. Note that acetylation of the C terminus of p53 prevents its degradation by ubiquitination as well its nuclear export.

In addition to the two factors described above, cdk9 may compete with p53 to interact to Pirh2 since both proteins bind to the same domain within Pirh2 protein (Figure 2). This could explain inactivation of Pirh2 but does not completely explain the inability of p53 to cause cells cycle arrest in cdk9-transfected cells. In this regard, it has been suggested, that cdk9 interacts with XPB, a subunit of TFIIH that is responsible for inhibition of CDK9 phosphorylation (Zhou et al. 2001). Release of cdk9 from this interaction was shown to be possible through association of p53 with XPB. In addition, association of cdk9 with CycT1, NELF and DSIF could explain its dissociation from Pirh2 and eventually inhibition of p53 and its ubiquitination by Pirh2.

Finally, it is well known that cdk9 is a subunit of the positive transcription elongation factor b (pTEFb) and that the recruitment of pTEFb to target genes requires de-ubiquitination of

H2Bub (Bres, Yoh, and Jones 2008). Therefore, it is possible that cdk9 associates with Pirh2 to block its ubiquitination activities.

Cdk9 phosphorylates several proteins. On the other hand, the ability of cdk9 to phosphorylate cellular factors other than NELF, DSIF and the CTD of pol-II is not without a precedent also. Others and we demonstrated that cdk9 phosphorylates p53 on serine 392 and renders it transcriptionally active (Claudio et al. 2006; Radhakrishnan and Gartel 2006). More recently, cdk9 has been shown to phosphorylate histone H1, a phenomenon that could regulate HIV-1 transcription (O'Brien et al. 2010). Therefore, it is not surprising for cdk9 to phosphorylate additional factors such as Pirh2. However, in most cases, cdk9 performs its functions through its interaction with CycT1 and the formation of the p-TEFb complex. Our data did not point to any involvement of CycT1 in Pirh2 phosphorylation and as shown in Figure 1, the level of CycT1 was not affected by the overexpression of Pirh2 or even its inhibition by siRNA-Pirh2.

In addition to Pirh2, cdk9 might use another pathway to deregulate and degrade p53 such as preventing its methylation. Since methylation is required for p53 stabilization (Chuikov et al. 2004), it is important to investigate whether cdk9 prevents this methylation. This is also important because methylation might interfere with Pirh2-mediated ubiquitination of the lysine residue at the C-terminus, which leads to subsequent p53 degradation and/or nuclear export.

In summary, our results provide an insight into the cellular function of cdk9 ranging from the ability of cdk9 to phosphorylate Pirh2, promoting Pirh2 self-ubiquitination and inhibition to negatively regulating p53 by promoting p53 degradation whether through Pirh2 or another pathway, indicates that cdk9, is involved in the master switch for cells undergo growth arrest or apoptosis versus proliferation and growth. It is also a key player in the regulation of HIV-1

gene expression and therefore, it is normal to suggest that phosphorylated Pirh2 will help keeping the negative effect of p53 and may be a novel target for the inhibition of HIV-1 gene expression and replication.

CHAPTER 5 CONCLUSION

The relation between HIV-1 and the development of neuronal disorders as well as the mechanisms involved remain to be determined. We aimed to decipher this relation by unraveling some of these mechanisms.

HIV-1 Tat protein has been described to have detrimental effect on neurons however the pathways used by Tat are unclear. In here, we confirmed the ability of HIV-1 Tat to cause neuronal deregulation. Using human primary cultures of neurons and neuronal cell line, we found that Tat functions depends on miRNAs, mainly miR-34a and miR-196a and their target genes (CREB, BDNF, p73, p53, and c-Abl). Tat level and functions were also validated using qPCR, Western blot, and immunohistochemical and *in situ* hybridization assays. As a result, we sought to investigate the functional significance of those miRNAs in regulating synaptic plasticity and cell viability.

Previously, Tat has been shown to cause neuronal dysfunction through a pathway that implicates TNF α and NF- κ B (New et al. 1998) or through its interaction with GSK-3 β (Maggirwar et al. 1999). Tat was also shown to deregulate neuronal calcium homeostasis, a phenomenon that leads to neuronal death (Brailoiu et al. 2006; Haughey et al. 1999; Kruman, Nath, and Mattson 1998). Further, Tat was described to promote cell death through mitochondrial hyperpolarization (Norman et al. 2007; Norman et al. 2008). In addition to the mentioned studies, Tat was shown to up-regulate the levels of p53 and p73, a phenomenon that also leads to loss of neuronal viability (Mukerjee et al. 2008).

Based on these studies, we sought to further investigate the role of Tat and its implication in neuronal deregulation. We hypothesize that HIV-1 Tat up-regulates p53 through a pathway that implicates down-regulation of miR-196a and activation of its downstream target, c-Abl. That

leads to phosphorylation and activation of p73, which in turn activates p53. Note that the relation between p73 and p53 was amply explored (Goldschneider et al. 2003; Jacobs, Kaplan, and Miller 2006; Miller, Pozniak, and Walsh 2000), however, the role and impact of miR-196a on this association has never been explored. Further, it has been shown that induction of oxidative stress leads to activation of c-Abl/p73 pathway (Klein et al. 2011). In this regard, several studies reported induction of oxidative stress pathway in Tat-treated human neurons (Shi et al. 1998), which explains our data regarding activation of the c-Abl and p73 proteins.

Interestingly, up-regulation of p53 led to a modest cell death and a significant neurite retraction. The lack of significant cell death could be due to the ability of Tat to physically interact with the N-terminal domain of p73 and prevented it from causing cell death (Amini et al. 2005). Further, it has been shown that c-Abl has the capability to phosphorylate pRB on serines residues S807/811, which will prevent neuronal death. Interestingly, pRB has been described to be phosphorylated on these specific residues in neurons isolated from HIV-1 infected patients (Akay et al. 2011).

Furthermore, global DNA methylation changes and methylation of p73 and p53 promoters could also provide an explanation for the lack of cell death. It has been shown that during aging phases tumor suppressor factors are methylated and unable to perform their apoptotic functions (Adams 2007; Ivanov and Adams 2011). Our unpublished data showed that Tat causes an increase in global DNA 5-hmC in SH-SY5Y cells and this was also confirmed *in vivo* in Tat-transgenic mice which corroborate with the literature. We are in the process of further exploring this avenue and examining the methylation status of the p53 promoter. Moreover, we were able to identify the factor(s) involved in p53-inhibition of transcriptional elongation. This p53 inhibitory effect prevents the phosphorylation of polymerase II on serine 2 of the carboxyl terminal domain (Claudio et al. 2006) that can stall the elongation. As a result, we showed that

cdk9 promotes the phosphorylation of mdm2 and Pirh2. Interestingly, cdk9 physically interacts with Pirh2 but not with mdm2. Interaction of cdk9 and Pirh2 led to the phosphorylation of two residues (S-211 and T-217) within Pirh2 protein. Finally, we showed that the level of Pirh2 is elevated in the brain of an HIV-1 patient. These results placed Pirh2 as a novel target for the inhibition of HIV-1 transcription and replication.

Similarly, small RNA in general and miRNAs in particular have been described to be involved in neuronal deregulation and in the development of neurodegenerative diseases. In this regard, miRNAs have been shown to be involved in psychiatric disorders (Zhou et al. 2009) such as schizophrenia and bipolar disorders (Kim et al. 2010). Furthermore, the link between miRNAs and neurodegenerative diseases such as Alzheimer, Huntington, and Parkinson diseases is becoming increasingly evident (Lau and de Strooper 2010; Bushati and Cohen 2008). miRNAs were also shown to be involved in the pathogenesis of neuroblastoma and astrocytoma (Moser and Fritzler 2010; Stallings 2009). Strikingly, miR-34a is among the miRNAs involved in these events. Identified by Welch *et al.* (Welch, Chen, and Stallings 2007), miR-34a was shown to be involved in cell cycle progression, cellular senescence, and apoptosis; however, it is unknown what all its targets are in mediating such functions. Among known miR-34a targets, *p53* and *SIRT1* genes were the most studied and shown to be involved in apoptosis or cell survival (Roos et al. 2005). miR-34a was recently shown to behave like a tumor suppressor in brain tumors and glioma stem cells (Guessous et al. 2010). In addition to its involvement in tumors and neurodegenerative diseases, miR-34a was also shown to play a role in psychiatric problems. In this regard, miR-34a was described to be one of the miRNAs involved in mood disorders (Zhou et al. 2009), schizophrenia, and bipolar disorders (Kim et al. 2010). miR-34a was also shown to be functionally linked to Bcl2 down-regulation by preventing the phosphorylation of CREB even though no miR-34a seeds exist in these genes

(Haughey et al. 1999), which corroborate our data. Finally, Tat mediated down-regulation of CREB has been shown to accompany the prevention of CREB phosphorylation through the PI3K pathway (Zauli et al. 2001).

Here we established the relation between the microRNAs (miR-34a and -196a), Tat, and neuronal regulation as demonstrated by Tat-dependent induction of miR-34a and indirectly the inhibition of miR-34a target genes by Tat. Such down-regulation of miR-34a target genes would likely lead to physiological changes in neurons that in turn would cause neuronal deregulation, neuronal loss, and eventually the development of HAND. These results further confirm our hypothesis regarding the involvement of miRNAs in neurodegenerative diseases, and further elucidation of Tat-induced gene regulation may provide novel explanation regarding the development of HAND.

Alternatively, our results raised a challenging question regarding the ability of HIV-1 proteins (*e.g.* Tat) to play a role in neuronal deregulation in the highly active antiretroviral therapy (HAART) era. The answer to this highly challenging question and the pathways involved in the development of HAND, besides the one studied here, remain unclear. Nevertheless, there are compelling neuropathological data showing that the HAND disease process occurs with the ongoing presence of virus, and despite the therapy, HAND remains very prevalent (Vivithanaporn et al. 2010). However, in a recent study, it was described that HIV-1 infects multipotent hematopoietic stem and progenitor cells. These cells allow the virus to hide and to be reactivated and re-infect additional cells even in the highly active antiretroviral therapy (HAART) era (Carter et al. 2011). Although the reasons for the reactivation of latent viruses are unclear, it was described that deregulation of miRNAs could lead to latent HIV reactivation (Han and Siliciano 2007). As a result, it is expected that viral

proteins released by latent reactivated viruses continue to play a role along with miRNAs in the development of HAND.

In this regard, an important question remains to be answered. How does Tat deregulate the miRNAs and what is/are the mechanism(s) involved? The answer to this question remains unclear. However, it is well described that HIV-1 benefits from deregulating the miRNAs of the host cell (Houzet et al. 2008). This theory is supported by the fact that miRNA-processing enzymes Drosha and Dicer are silenced to reduce generation in the cell of mature miRNAs (Houzet et al. 2008). This phenomenon can lead to a robust HIV-1 replication and preventing some miRNAs (*e.g.* miR-150 or miR-223) from silencing, reducing, or even delaying viral replication (Huang et al. 2007). This observation corroborates with the results published by Houzet *et al.* (Houzet et al. 2008) where they showed the down-regulation of these two miRNAs in T-cells. Furthermore, viruses such HIV-1 may use an alternative mechanism involving miRNAs to better replicate. For example, the HIV-1 RNA structure TAR has been reported to be processed by Dicer to release miRNAs that could be involved in chromatin remodeling. Because neurons do not support viral replication, the above-described mechanisms cannot explain the pathway used by Tat to deregulate the miRNAs, and therefore additional studies are required to decipher these mechanisms.

Another avenue that needs to be investigated in more details is the ability of viral infection to induce changes in fundamental epigenetic marker such as 5-hmC (5-hydroxymethylcytosine). Interestingly, the brain contains the highest levels of 5-hmC compared to other tissues and organs. This fact suggests that this DNA modification plays may play a key role in brain development and function. However, considering that several studies have shown how Tat can also affect signal transduction pathways, elucidating the exact pathway that

soluble Tat hijacks in neurons may prove useful in understanding HAND and may provide novel therapeutic targets.

In summary, although we demonstrate that Tat is able to affect neuronal dysfunction by altering miRNA expressions, the pathway used by Tat needs to be further evaluated. It may be that the increased expression of miR-34a may simply be the result of Ca²⁺ mobilization because this mobilization mediates numerous signaling pathways. To clarify this hypothesis, we examined the target genes involved in calcium homeostasis and deregulated in neurons in the presence of Tat (data not shown). Gene array data point to the ability of Tat to affect the expression levels (positively or negatively) of several genes involved in calcium homeostasis, further demonstrating the role Tat plays in altering homeostasis/mobilization of calcium in the cell (Chang, Mukerjee, et al. 2011). Interestingly, it has been shown that CaMK-II protein has the ability to phosphorylate Pirh2 and to renders it inactive (Duan et al. 2007). The authors also suggested that phosphorylation of Pirh2 by CaMK-II might create a balance between Pirh2-p53 relation that could help p53 stability and functions. These results led us to suggest the presence of a tight link between Tat calcium release and the development of HAND.

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APPENDIX

Table 2. Antibodies

Name	Company	Catalog number
Mouse-P73	Santa Cruz Bio	Sc-17823
Rabbit-pP73	Santa Cruz Bio	Sc-101769
Rabbit-pTyr	Santa Cruz Bio	Sc-18182
Goat-Map2	Santa Cruz Bio	Sc12012
Goat-ORAI	Santa Cruz Bio	Sc-74776
Rabbit-STIM1	Santa Cruz Bio	Sc-68897
Goat-SP1	Santa Cruz Bio	Sc-59X
Rabbit-BDNF	Santa Cruz Bio	Sc-546
Mouse-E2F3	Santa Cruz Bio	Sc-56665
Rabbit-cAbl	Santa Cruz Bio	Sc-131
Rabbit-DNMT 3a	Santa Cruz Bio	Sc-20703
Rabbit-DNMT 3b	Santa Cruz Bio	Sc-130740
Mouse-P53	Santa Cruz Bio	Sc-55476
Mouse-HA	Santa Cruz Bio	Sc-7392
Rabbit-TET1	Millipore	09-872

Rabbit-GAPDH	Cell Signaling	2118S
Rabbit-E2F1	Cell Signaling	3742S
Rabbit-CREB	Cell Signaling	9197S
Rabbit-DNMT1	Cell Signaling	5119S
Rabbit H3K9	Abcam	ab8898
5-hydroxymethylcytidine	Active Motive	39791

Table 3. Q-PCR primers 1

Name	Melting Temperature	Sequence
30a Meth For-1	59.0C ^o	GCAACTTGCGAACCGGGAAA
30a Meth Rev-1	60.2C ^o	TGACGACGAGTCGAGAAAGTCACC
30a Meth For-2	60.4C ^o	TGACTTTCTCGACTCGTCGTCAGC
30a Meth Rev-2	63.0C ^o	AAGCCCAGGTGCCCTAAACCACTT
GAPDH For	60.6C ^o	TCGACAGTCAGCCGCATCTTCTTT
GAPDH Rev	60.5C ^o	ACCAAATCCGTTGACTCCGACCTT
CREB1 For	60.2C ^o	AAAGCAGTGACGGAGGAGCTTGTA
CREB1 Rev	60.2C ^o	GGCTGGGCTTGAAGTGCATTTGT
34aMethFor1	62.7C ^o	TTTCAGGTGGAGGAGATGCCGCT
34aMethRev1	62.8C ^o	ATCTGCGTGGTCACCGAGAAGCA
pri-miR196aSet 1 For	60.0C ^o	CCAGTGGTCCCATTTACCAGATT
pri-miR196a Set 1 Rev	60.0C ^o	CAGGCAGTTTCTTGTTGCCGAGTT
pri-miR196a Set 2 For	60.0C ^o	AACTCGGCAACAAGAACTGCCTG
pri-miR196a Set 2 Rev	60.0C ^o	AGGTTGAGAGGACGGCATAAAGCA
CREB Promoter For +1	60.9C ^o	AGGTGTAGTTTGACGCGGTGTGTT

CREB Promoter Rev +1	60.2C ^o	TACAAGCTCCTCCGTCACTGCTTT
CREB ChIPFor(-1600)	60.2C ^o	TTGGAAGCAACTGTGGTCAATCGC
CREB ChIPRev(-1600)	60.0C ^o	TGGACAGTCTTTCATGTCCCACCT
CREB ChIPFor(+1600)	59.8C ^o	TTGAAGGACGTGCGGTATGACCTA
CREB ChIPRev(+1600)	59.1C ^o	AACCTTCAGAAGTTAGACGCCAGC
GAPDH ChIP For	61.5C ^o	TACTAGCGGTTTTACGGGCGCAC
GAPDH ChIP Rev	62.5C ^o	TCGAACAGGAGGAGCAGAGAGCG
BDNF4 ChIPFor(-300)	60.5C ^o	AGGGTCTTGGCTACAGGCAAATGA
BDNF4 ChIPRev(-300)	60.2C ^o	TAACGAACCAGGGCAGCCAAGATA
miR34a(-1500) For	60.5C ^o	AACATGGGCTCATCACAGACACCT
miR34a(-1500) Rev	60.1C ^o	AGGTGCGTAATCACATTTGGGCAC
miR34a(+1500) For	60.1C ^o	TAAGTGCAAAGGCCCTGTGTTTGG
miR34a (+1500) Rev	60.3C ^o	AGCTGCAGTACTGATGTGTGCTCT

Table 4. Q-PCR primers 2

Name	Company	Sequence
CDKNA1 For(-2kb)	60.3C ^o	TGCATGTGTGCTTGTGTGAGTGTG
CDKNA1 Rev(-2kb)	59.6C ^o	TAAGGGAGGACTTCTGCCCTGAAA
MDM2 promoter For	59.1C ^o	CAGGTAAGCACCGACTTGCTTGTA
MDM2 promoter Rev	60.1C ^o	TTCCGAAGCTGGAATCTGTGAGGT
BDNF1 promoter For	60.2C ^o	TGGTTCTTCTGCTCTGCTGTGCTA
BDNF1 promoter Rev	60.0C ^o	TCCGGAAATCTCGGGAAATAGGCA
miRNA 196a Primer Mix	58.0C ^o	Exiqon, Cat # 204386
miRNA 30a Primer Mix	58.0C ^o	Exiqon, Cat # 204791
miRNA 34a Primer Mix	58.0C ^o	Exiqon, Cat # 204486
BDNF Exon 1 For	60.9C ^o	TCTCCAGGACAGCAAAGGCACAAT
BDNF Exon 2 For	59.1C ^o	TAGCGGTGTAGGCTGGAATAGACT
BDNF Exon 1/2 Rev	60.2C ^o	GGCAGCCTTCATGCAACCAAAGTA
BDNF Exon 3 For	58.6C ^o	CTTAGAGGGTTCCCGCTTTCTCAA
BDNF Exon 4 For	59.5C ^o	GAGCAGCTGCCTTGATGGTACTT
BDNF Exon 3/4 Rev	60.8C ^o	AAGCCACCTTGTCTCGGATGTTT
cABL1 For	59.7C ^o	ACGTCTGGGCATTTGGAGTATTGC

cABL1 Rev	60.3C ^o	TCAGAGGGATTCCACTGCCAACAT
TA-p73 For	59.7C ^o	TCTGGAACCAGACAGCACCTACTT
TA-p73 Rev	60.7C ^o	TCAGCAGATTGAACTGGGCCATGA
P73 For	60.3C ^o	TTGAGGTCACCTTCCAGCAGTCCA
P73 Rev	60.0C ^o	TGCTCCGCTTTCTTGTA AACAGGC
P73 promoter For	62.8C ^o	AGGGCCGGGAGGAGACCTT
P73 promoter Rev	59.7 C ^o	CCTACCTGCCGTCGCA
