

Review Article

Regulation of HTLV-1 transformation

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Human T-cell leukemia virus type 1 (HTLV-1) is the only identified oncogenic human retrovirus. HTLV-1 infects approximately 5–10 million people worldwide and is the infectious cause of adult T-cell leukemia/lymphoma (ATL) and several chronic inflammatory diseases, including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), dermatitis, and uveitis. Unlike other oncogenic retroviruses, HTLV-1 does not capture a cellular proto-oncogene or induce proviral insertional mutagenesis. HTLV-1 is a trans-activating retrovirus and encodes accessory proteins that induce cellular transformation over an extended period of time, upwards of several years to decades. Inarguably the most important viral accessory protein involved in transformation is Tax. Tax is a multifunctional protein that regulates several different pathways and cellular processes. This single viral protein is able to modulate viral gene expression, activate NF- κ B signaling pathways, deregulate the cell cycle, disrupt apoptosis, and induce genomic instability. The summation of these processes results in cellular transformation and virus-mediated oncogenesis. Interestingly, HTLV-1 also encodes a protein called Hbz from the antisense strand of the proviral genome that counters many Tax functions in the infected cell, such as Tax-mediated viral transcription and NF- κ B activation. However, Hbz also promotes cellular proliferation, inhibits apoptosis, and disrupts genomic integrity. In addition to viral proteins, there are other cellular factors such as MEF-2, superoxide-generating NADPH oxidase 5- α (Nox5 α), and PDLIM2 which have been shown to be critical for HTLV-1-mediated T-cell transformation. This review will highlight the important viral and cellular factors involved in HTLV-1 transformation and the available *in vitro* and *in vivo* tools used to study this complex process.

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a complex δ retrovirus within the *Orthoretrovirinae* subfamily. Isolated over 40 years ago from a patient with cutaneous T-cell lymphoma, HTLV-1 was the first discovered human retrovirus [1]. Currently, an estimated 5–10 million people worldwide are infected with HTLV-1, with pockets of endemic infection in Africa, South America, the Caribbean, Southwestern Japan, and the Pacific islands [2]. Large portions of the world lack epidemiological data regarding HTLV-1 infection; therefore, the actual number of HTLV-1-infected individuals is likely much higher. This blood-borne pathogen has the potential to induce adult T-cell leukemia (ATL), an extremely aggressive CD4⁺ T-cell malignancy [1,3], HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a progressive neurodegenerative disease [4,5], and several other inflammatory diseases (uveitis, keratitis, dermatitis, and conjunctivitis) [6]. Approximately 5–10% of HTLV-1-infected patients will develop disease over the course of their lifetime. Unlike many other oncogenic retroviruses, HTLV-1 does not capture a proto-oncogene or induce proviral insertional mutagenesis. Instead, HTLV-1 is a trans-activating retrovirus and encodes accessory proteins that induce cellular transformation over an extended period of time. Therefore, in addition to lower disease penetrance, HTLV-1-mediated disease is unique due to its extensive clinical latency period upwards of several decades. This prolonged clinical latency period is heavily reliant

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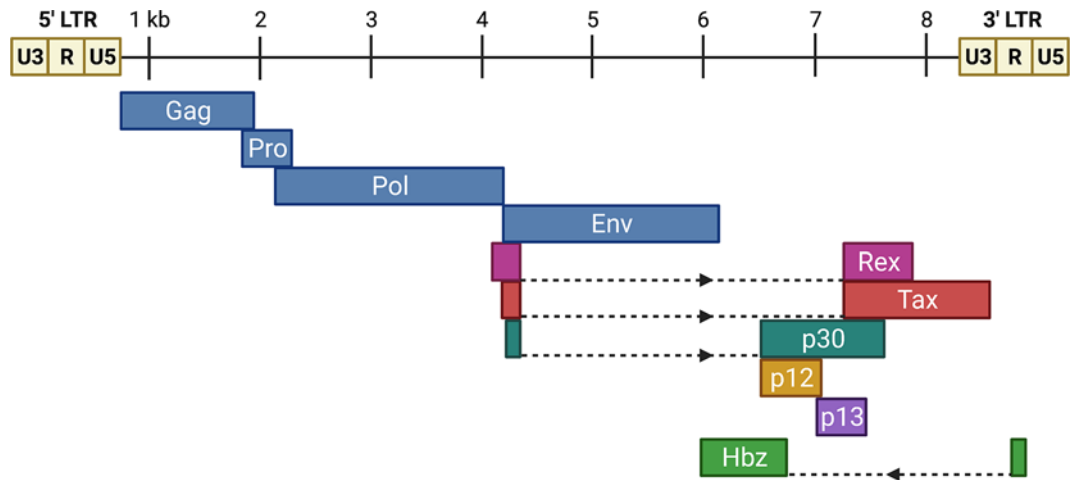


Figure 1. The HTLV-1 proviral genome

The structural and enzymatic proteins (Gag, Pro, Pol, and Env), in addition to five viral accessory proteins (Tax, Rex, p12, p13, and p30), are encoded by sense transcripts derived from the 5' LTR promoter. Hbz is the only viral accessory/regulatory protein encoded by an antisense transcript derived from a 3' LTR promoter.

on the transient expression of the viral regulatory protein, Tax. Although Tax and a subset of HTLV-1 accessory proteins have been directly implicated in the capacity of HTLV-1 to transform cells, the precise mechanisms of this process still remain largely unknown.

HTLV-1 has several routes of transmission, including breastfeeding, sexual intercourse, and exposure to infected blood products such as through blood transfusions or sharing of needles. Mother-to-child transmission through breastfeeding is typically the most common transmission route within endemic regions [7]. While most viruses are capable of infecting target cells through cell-free virions, transmission of HTLV-1 is primarily dependent on cell-to-cell transmission [8]. This phenomenon is partly due to the severely limited capacity of cell-free HTLV-1 virions within the blood to infect most cell types [9]. Interestingly, an *in vitro* study has suggested that transmission of HTLV-1 through a route such as breastfeeding requires transcytosis of free infectious HTLV-1 virions through the epithelial barrier [10]. These virions could subsequently infect dendritic cells (DCs), which are able to spread the HTLV-1 virions to healthy T cells [11]. Among the T-cell population, CD4⁺ T cells are the primary and preferential target cell of HTLV-1 infection, with CD8⁺ T cells constituting roughly 5% of the total infected cells [12,13]. During the acute stage of HTLV-1 infection, cell-to-cell transmission is prevalent resulting in efficient spread of the virus with limited immune detection. In addition to being hypothetically passaged to healthy T cells via infected antigen-presenting DCs, HTLV-1 virions can also be transmitted to adjacent cells through the following mechanisms: establishment of cellular conduits, formation of a virological synapse (VS), or through extracellular viral assemblies [14].

The HTLV-1 viral genome consists of a relatively small positive-sense RNA genome of approximately 9 kb in size. Two copies are packaged in the virus particle. Upon entry into a host cell, the ssRNA genome is reverse transcribed into dsDNA and integrated into the host genome. The integrated dsDNA form of the retroviral genome is termed the provirus. The viral genome is flanked by long terminal repeats (LTRs) at both the 5' and 3' ends. These direct repeats consist of three regions: the unique 3' (U3), the repeated (R), and the unique 5' (U5) regions. The LTRs also contain important elements necessary for viral transcription, polyadenylation, and integration. Like all retroviruses, HTLV-1 contains the standard structural and enzymatic genes (*gag*, *pro*, *pol*, and *env*) essential for viral replication (Figure 1). However, HTLV-1 is a complex retrovirus and also encodes accessory genes that contribute to the biology of the virus, of which a subset is critical to the pathogenesis. With the exception of one gene (*Hbz*), all the HTLV-1 genes are encoded from the sense strand of the proviral genome. One of the most critical accessory genes for HTLV-1 is *Tax*. Tax protein is required for transformation and functions as a transcription factor to enhance viral transcription from the promoter in the 5' LTR [15–18]. Conversely, the Hbz protein, encoded from the antisense genome strand counteracts many Tax activities, including transcriptional activation of the 5' LTR promoter [19–21]. Together, Tax and Hbz are essential for the ability of the virus to establish persistent infection and induce transformation of target T cells. This review will focus on both the viral factors (Tax, Hbz) and cellular players involved in HTLV-1-mediated cellular transformation, and the molecular tools and animal models currently available to study this dynamic process.

Table 1 Interacting partners of Tax

Interacting partner/pathway	Downstream effect	References
CREB/AFT/p300	Transactivation of the 5' LTR promoter and viral mRNA transcription	[28–31]
HDAC1	Prevents HDAC1's interaction with the 5' LTR, promoting viral transcription	[32]
SUV39H1	Recruits SUV39H1 to the promoter region, repressing viral transcription	[33]
DROSHA	Targets DROSHA for proteasomal degradation, increasing viral transcription	[36]
NEMO	Induces aberrant NF-κB activation	[38]
IκB protein	Targets IκB for proteasomal degradation, promoting NF-κB activation	[39]
p105	Targets p105 for proteasomal degradation, promoting NF-κB activation	[39]
NF-κB proteins	Promotes dimerization of NF-κB proteins to enhance their transcriptional potential	[40]
TAB2	Stimulates the IKK complex and induces aberrant NF-κB activation	[41]
RFN8	Stimulates the IKK complex and induces aberrant NF-κB activation	[42]
PP2A	Inactivates PP2A, promoting NF-κB activation	[43]
A20	Inactivates A20, promoting NF-κB activation	[44]
TRAF6	Promotes the stability of MCL-1, preventing apoptosis induction	[50]
p53	Indirect inhibition deregulates the cell cycle and promotes T-cell immortalization	[55]
Ras proteins/mTOR pathway	Tax-mediated activation promotes accelerated cell proliferation	[56,57]
MDAC1	Sequesters MDAC1, causing sustained DNA damage and increased mutations	[61,62]
USP10	Causes induction of reactive oxygen species	[66]

Abbreviations: AFT, activating transcription factor; A20, ubiquitin-editing enzyme A20; CREB, cyclic AMP response element-binding protein; DROSHA, Drosha ribonuclease III; HDAC1, histone deacetylase 1; IKK, IκB kinase; MCL-1, myeloid cell factor-1; MDAC1, mediator of DNA damage checkpoint 1; NEMO, NF-κB essential modulator; PP2A, protein phosphatase 2A; RFN8, ring finger protein 8; TAB2, TAK1-binding protein 2; TRAF6, TNF receptor-associated factor 6; USP10, ubiquitin-specific peptidase 10.

Viral proteins relevant to HTLV-1-mediated transformation

Of all the HTLV-1 proteins, Tax is the most important for driving HTLV-1-mediated T-cell transformation and its transformation capacity has been well demonstrated experimentally. Previous studies have shown that Tax plays a major role in the processes of HTLV-1-mediated T-cell immortalization of primary human lymphocytes, colony formation in rodent fibroblasts, and tumorigenesis in a variety of transgenic (Tg) mouse models [22–25]. As a virally encoded oncoprotein, Tax has extensive involvement in a multitude of different pathways related to the immortalization, gene expression, and pathogenesis of HTLV-1. The multifunctionality of Tax throughout these respective processes is derived from its capability to directly interact with a wide array of proteins, including transcription factors, cell signaling proteins, cell cycle regulators, apoptotic proteins, and DNA damage response factors (Table 1). These interactions favor transformation and pathogenesis by modulating viral gene expression, deregulating the cell cycle, disrupting the apoptotic response, and reducing genomic stability.

One of the key roles of Tax is transactivation of the promoter located in the 5' LTR. Tax-mediated viral transcription drives synthesis of all plus strand gene products including the structural and enzymatic viral proteins, in addition to regulating its own expression. Tax mediates viral transcription through three discontinuous G/C-rich 21-base pair repeats, known as Tax responsive elements (TREs), located within the U3 region of the 5' LTR [26,27]. These G/C-rich regions are directly adjacent to cyclic AMP response elements (CREs) and influence transcription when bound to transcription factors such as CRE-binding protein (CREB)/activating transcription factor (ATF) [28–30]. Tax does not directly bind DNA, but instead recruits transcription factors (CREB) to the CRE. This ultimately leads to a nucleoprotein complex that strongly favors recruitment of additional transcriptional co-activators and histone acetylases, such as CREB-binding protein (CBP) and p300. This multiprotein complex consisting of Tax, CREB/ATF transcription factors, and CRE/p300 histone acetylases leads to potent transactivation of the 5' LTR promoter and viral mRNA transcription [31]. Tax also regulates HTLV-1 gene transcription through binding to repressive epigenetic proteins such as histone deacetylase 1 (HDAC1) and the histone lysine methyltransferase suppressor of variegation 3-9 homolog 1 (SUV39H1) [32,33]. Tax has the ability to either promote viral transcription by directly preventing HDAC1 interaction with the viral promoter or repress viral transcription through the active recruitment of SUV39H1 to the promoter region. Another mechanism of Tax-mediated regulation of viral replication and gene transcription is the dysregulation of cellular miRNA expression, which has been frequently identified across HTLV-1-infected T-cell

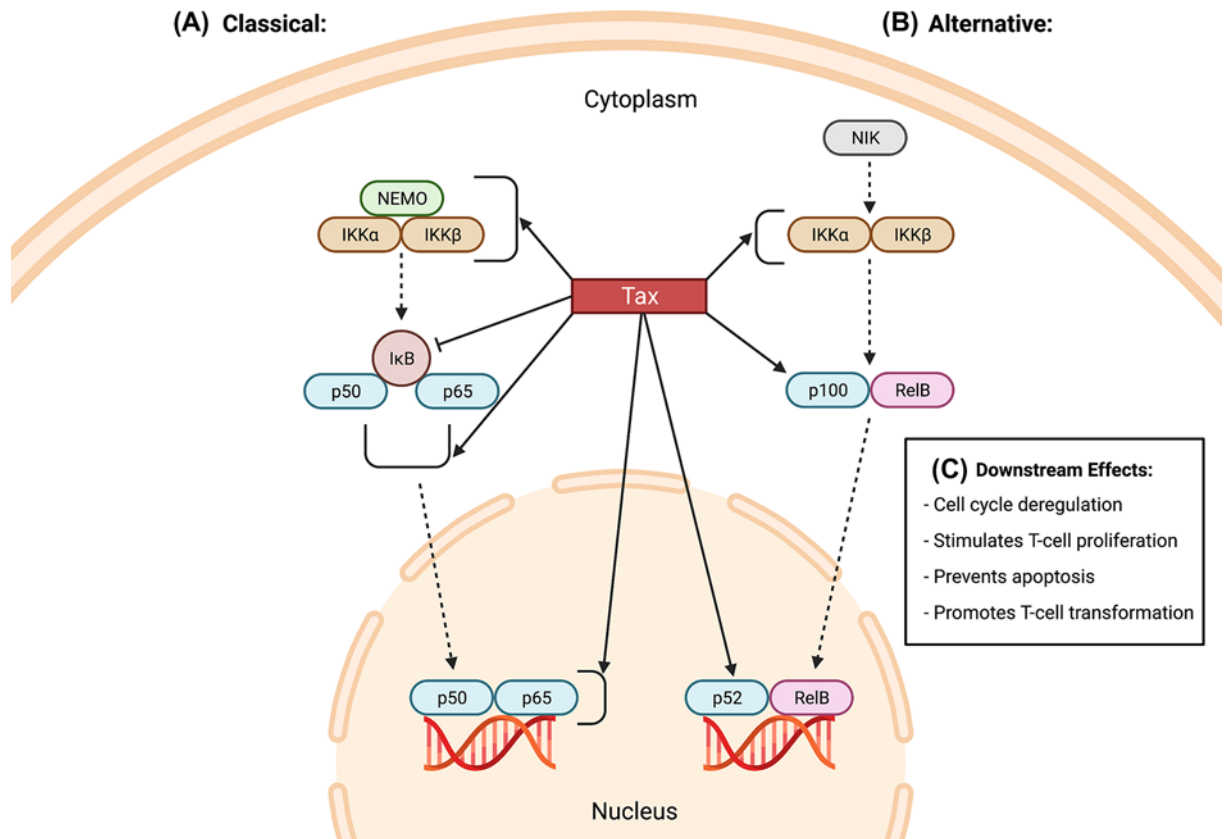


Figure 2. Tax-mediated activation of the NF- κ B pathway

HTLV-1-infected cells utilize Tax to stimulate both the classical (A) and alternative (B) NF- κ B pathways. (A) Tax interacts with NEMO to facilitate recruitment of the IKK subunits α and β , leading to activation of the IKK complex. Tax can also interact with these IKK subunits directly to promote their dimerization. The activated IKK complex then phosphorylates I κ B, subsequently releasing p50/p65 and permitting their translocation to the nucleus. Tax can also bind to p50/p65 subunits in the nucleus to induce dimer formation and recruit co-activators such as CBP/p300. (B) The alternative pathway depends on phosphorylation of the IKK complex by NIK. Once activated, the IKK complex then phosphorylates p100/RelB, stimulating proteasomal processing of p100 to p52. Tax accelerates this process by recruiting IKK subunit α to p100, thereby allowing p52/RelB to translocate to the nucleus. Similar to the classical pathway, Tax may additionally promote the dimerization and DNA binding activity of nuclear p52. (C) Tax-mediated activation of these respective NF- κ B pathways leads to several downstream cellular effects, including deregulation of the cell cycle, stimulation of T-cell proliferation, and increased expression of anti-apoptotic proteins, ultimately promoting viral transformation of HTLV-1-infected cells.

lines and ATL patient samples [34,35]. For the purposes of increasing viral transcription, Tax can disrupt miRNA machinery by targeting Drosha ribonuclease III (DROSHA) for proteasomal degradation [36].

In addition to regulating viral gene expression, Tax also has a strong influence over several cell signaling pathways such as NF- κ B (Figure 2). This activity of Tax is particularly relevant given that the NF- κ B transcription factor family plays several critical roles in the processes of apoptosis, cell proliferation, oncogenesis, and immune response development. Activation and nuclear translocation of cytoplasmic NF- κ B proteins is dependent on the phosphorylation and proteasomal degradation of I κ B proteins, which are commonly bound to NF- κ B dimers and thus masking their nuclear localization signal [37]. Tax induces aberrant NF- κ B activation by interacting with the NF- κ B essential modulator (NEMO) on the I κ B kinase (IKK) complex, subsequently promoting this kinase to actively phosphorylate I κ B proteins bound to NF- κ B dimers [38]. Alternatively, Tax can also directly bind to I κ B proteins, such as I κ B α and p105, to disrupt their NF- κ B binding interactions and target them for proteasomal degradation [39]. Tax also has the capacity to enhance the transcriptional potential of NF- κ B proteins by actively promoting their homo- or hetero-dimerization through direct interactions with their respective Rel homology domains [40]. In order to ensure persistent activation of NF- κ B within the cell, Tax is able to interact with a wide subset of proteins involved in

the regulation of NF- κ B. For example, Tax can interact with TAK1-binding protein 2 (TAB2) and Ring Finger Protein 8 (RFN8), two unique proteins involved in the downstream stimulation of the IKK complex [41,42]. Tax also binds to and inactivates negative regulators of the NF- κ B signaling pathway, such as the serine/threonine protein phosphatase PP2A and the ubiquitin-editing enzyme A20 [43,44]. In summation, Tax possesses a substantial array of different mechanisms to trigger and maintain the persistent activation of NF- κ B signaling within HTLV-1-infected cells. Given that chronic activation of NF- κ B has been shown to be critical for the immortalization and transformation of Tax-expressing cells, HTLV-1 transformed cell lines, and ATL patient samples alike, the NF- κ B pathway represents a key mechanism for Tax to influence the transformation of HTLV-1-infected cells [45,46].

Persistent activation of NF- κ B results in stimulation of growth factors, cytokines, and proto-oncogenes—all of which influence the host cell cycle. NF- κ B signaling also has the ability to activate expression of anti-apoptotic proteins. Not surprisingly, Tax-mediated activation of NF- κ B has been reported to enhance expression of the anti-apoptotic proteins X-linked inhibitor of apoptosis (XIAP), survivin, and Bcl-xL [47–49]. Tax can also protect HTLV-1-infected cells from apoptosis through other mechanisms, such as interactions with TRAF6 to promote the stability of myeloid cell factor-1 (MCL-1), an anti-apoptotic protein of the BCL-2 family [50]. Although Tax typically functions to increase the expression of anti-apoptotic proteins, studies have demonstrated that overexpression of Tax may result in the up-regulation of pro-apoptotic proteins [51]. Tax overexpression in the CD4⁺ T-cell line Jurkat leads to enhanced expression of the pro-apoptotic ligands CD95L, TNF- α , and TNF-related apoptosis inducing ligand (TRAIL), ultimately leading to widespread cell death [52–54]. In addition to inhibiting apoptosis, Tax also functions to regulate and stimulate T-cell proliferation. Tax-mediated inhibition of the tumor suppressor p53 is one of the most prominent methods utilized to deregulate the cell cycle and promote HTLV-1-mediated T-cell immortalization. Tax inhibits p53 indirectly through activation of either NF- κ B or CREB cell signaling pathways [55]. In addition, Tax promotes accelerated cell proliferation through the selective activation of Ras proteins and the mTOR signaling pathway [56,57]. Tax can also increase cell proliferation through NF- κ B-mediated induction of oncogenesis-related cellular miRNAs, miR-146a and miR-155 [58,59]. By enhancing the capacity of the cell to proliferate while simultaneously up-regulating the expression of anti-apoptotic proteins, Tax is effectively able to drive the immortalization and transformation process in HTLV-1-infected T cells.

Through modulation of several proteins critical for proper cell cycle regulation, Tax can also promote the transformation of HTLV-1-infected T cells by preventing the recognition and repair of DNA damage. The ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases exert cell cycle delay in response to compromised genomic integrity such as DNA damage or stalled replication forks [60]. Tax prevents the activation of ATM through sequestration of mediator of DNA damage checkpoint 1 (MDAC1), subsequently resulting in sustained DNA damage and a higher genomic mutation frequency [61,62]. Through its interactions with the CREB/ATF transcription factors, Tax can also decrease cyclin A promoter expression. Given that cyclin A kinase activity is vital for maintaining the proper levels of DNA replication during S phase, Tax-mediated suppression of this promoter greatly enhances the rate of genetic instability and DNA damage [63,64]. In addition to blocking DNA repair mechanisms across several phases of the cell cycle, Tax also has the ability to directly induce DNA damage through the induction of reactive oxygen species (ROS) intermediates and nitric oxide (NO). Previous studies found that primary human CD4⁺ T cells transduced with various Tax-expressing retroviral vectors produced ROS as a direct consequence of Tax expression, subsequently causing DNA damage and the initiation of cell senescence [65]. The interaction between Tax and the deubiquitinase USP10 is one of the molecular mechanisms that leads to Tax-mediated induction of ROS [66]. The ability of Tax to induce both DNA damage and prevent genomic repair mechanisms during the cell cycle enhances the oncogenic and transformative properties of Tax.

Although the expression of Tax alone is sufficient to promote tumorigenesis in Tg mouse models *in vivo*, Tax by itself is not able to efficiently transform primary human cells *in vitro* [24,67,68]. Therefore, the transformation of HTLV-1-infected T cells is dependent on other viral proteins in addition to Tax. A promoter on the antisense strand in the 3' LTR of the proviral genome drives expression of Hbz, the only viral protein derived from an antisense transcript in HTLV-1. A critical function of Hbz is to inhibit Tax-mediated activation of viral transcription within the nucleus of infected cells [69]. Hbz interacts with essential Tax transcription factors (ATF-1, CREB, JunB, JunD, and c-Jun) via its basic leucine zipper domain and sequesters them away from DNA, thus inhibiting Tax-mediated transcription [70]. In addition to suppressing activation of the 5' LTR promoter, Hbz also has the capacity to antagonize other roles of Tax, such as stimulation of the classical NF- κ B pathway. Through the antagonization and degradation of the NF- κ B component p65, Hbz is able to suppress Tax-driven activation of the classical NF- κ B pathway, which consequently results in the attenuation of the cellular senescence response [71]. Decreasing the activity of Tax is hypothesized to serve an important role in the survival of HTLV-1-infected cells and the controlled proliferation and clonal expansion of HTLV-1-infected CD4⁺ T cells is reliant on the Hbz-mediated down-regulation of Tax and

NF- κ B expression. Given the pathogenic and transformative properties of Tax, Hbz-mediated inhibition of this viral transcriptional activator additionally functions as a means to regulate the transformation process. However, loss of Hbz does not affect HTLV-1-mediated T-cell immortalization in cell culture [72]. Interestingly, the absence of Hbz does affect efficient viral infectivity and persistence in a rabbit model of infection [72]. These studies demonstrate that Hbz is critical in the efficient establishment and maintenance of chronic viral infections.

Hbz can also interact with the AP1 superfamily proteins ATF3 and JunD [73–75]. ATF3 is a transcription factor and also activates the tumor suppressor p53. Hbz interferes with the p53-enhancing function of ATF3 and enhances JunD transcriptional activity to promote proliferation of T cells [74,76,77]. Somewhat surprisingly, recent advances in the field have shown that *hbz* mRNA is also able to support cell proliferation, although the precise mechanism(s) remain unclear [78]. Like Tax, Hbz also has the ability to promote genetic instability, a significant factor driving HTLV-1-mediated oncogenesis. Hbz has been previously reported to increase the expression of miR-17 and miR-21, two unique oncogenic miRNAs that down-regulate the OBFC2A DNA-damage factor, subsequently leading to a higher propensity for DNA damage [79]. Additional *in vitro* studies with murine cell lines have demonstrated that the independent expression of Hbz significantly promotes soft agar cell proliferation and colony formation [76]. Altogether, Hbz functions as a key viral protein for both the regulation and promotion of HTLV-1-mediated T-cell transformation. Similar to Tax, Hbz is also able to inhibit apoptosis of infected cells. Bim, a pro-apoptotic gene, is suppressed by Hbz expression, while knockdown of Hbz increases Bim expression [80]. Further investigation of the mechanism behind the Hbz/Bim relationship found that Hbz disrupts the localization and function of FoxO3a, a critical activator of Bim and also Fas ligand (FasL).

Similar to Hbz, many of the remaining viral accessory proteins of HTLV-1 also play a key role in regulating Tax activity and the transformation potential of virally infected T cells [81]. The viral protein p30 is characterized as a negative post-transcriptional regulator of viral gene expression and Tax activity; p30 selectively binds to *tax/rex* mRNA transcripts and prevents their export from the nucleus [82]. Furthermore, p30 has been shown to prevent Tax-mediated transactivation of the 5'LTR promoter by interacting with CBP/p300 [83]. Previous studies have additionally indicated that p30 has the capacity to dampen Tax-mediated NF- κ B activation by destabilizing the p65 subunit [84]. Despite the ability of p30 to antagonize Tax, this viral protein can also promote T-cell transformation. Previous *in vitro* studies using immortalized human fibroblasts demonstrated that p30 can enhance viral transformation when coexpressed with Myc [85]. An additional viral accessory gene called p13 also disrupts the interaction between Tax and CBP/p300, resulting in the down-regulation of viral transcription [86]. p13 has also been implicated in elevated levels of intracellular ROS, subsequently enhancing the potential for DNA damage and viral transformation [87,88]. In contrast with p30 and p13, expression of the viral accessory protein p12 is typically not involved with suppression of Tax activity. Contrary, this viral protein enhances the potential for CD4⁺ T-cell proliferation through activation of nuclear factor of activated T cells (NFAT) and signal transducers and activators of transcription-5 (STAT-5) signaling pathways [89–91]. Several studies have also shown p12 is able to enhance viral infectivity and immune evasion [91–93].

Tax is the major driver of 5' viral transcription and thus has tight control over its own expression. In addition, Tax regulates cellular epigenetic proteins and miRNA machinery which influences the immortalization process. Several viral accessory proteins, including Hbz, function to regulate Tax activity, viral gene expression, and T-cell transformation through a variety of different mechanisms.

Cellular factors relevant to HTLV-1-mediated transformation

Transformation of HTLV-1-infected T cells is mediated by a wide variety of both viral and cellular proteins. Although viral proteins such as Tax and Hbz play an important role in T-cell transformation, there are several additional cellular factors that contribute to this process (Table 2). These cellular factors become increasingly relevant later during viral infection, as Tax expression is typically silenced in the majority of ATL patient samples [94]. Furthermore, a long clinical latency period suggests there are additional genetic and/or epigenetic events which occur to induce oncogenesis [95]. An integrated molecular study with whole-genome, exome, transcriptome, and targeted sequencing of 426 ATL cases found that despite the absence of Tax, a number of new somatic alterations were identified, and these alterations were found in T-cell receptor (TCR) and NF- κ B signaling [96]. These pathways have a significant overlap with the Tax interactome despite the absence of Tax in these patient samples.

Notably, a study by Shigemura et al. revealed that superoxide-generating NADPH oxidase 5- α (Nox5 α) is a cellular protein that is required for the maintenance of HTLV-1-transformed T cells [97]. This study demonstrated that Nox5 α is up-regulated in both ATL patient samples and HTLV-1 transformed cell lines such as Hut-102, MT1, MT2, and MT4. Furthermore, siRNA-mediated silencing of Nox5 α in MT1 and MT2 cell lines resulted in reduced T-cell

Table 2 Cellular factors involved in HTLV-1 transformation

Cellular factor	Transformation target/effect	References
Nox5 α	Up-regulation helps maintain the survival, proliferation, migration, and oncogenic potential of HTLV-1-transformed cells	[93]
MEF-2	Colocalizes with Tax to stabilize its interaction with CREB, causing increased viral transcription	[95]
PDLIM2	Targets Tax for proteasomal degradation, decreasing the transformation capacity of infected cells	[96]
PTHrP	Up-regulation maintains the transformation of infected cells	[98,99]
PRMT5	Up-regulation promotes the survival and transformation of infected cells	[100]
miR-93	Targets p21, MICB, and TP53INP1; increases the oncogenic potential and immune evasion of infected cells	[102–104]
miR-130b	Targets TP53INP1; maintains the survival of infected cells	[104]
miR-150	Functions as a tumor suppressor by repressing the proliferation of transformed cells	[105]

Abbreviations: MEF-2, myocyte enhancer factor-2; MICB, major histocompatibility complex class I chain-related B; Nox5 α , superoxide-generating NADPH oxidase 5- α ; PDLIM2, PDZ and LIM domain 2; PRMT5, protein arginine methyltransferase 5; PTHrP, parathyroid hormone-related protein; TP53INP1, tumor protein 53-induced nuclear protein 1.

proliferation and migration, in addition to increased apoptosis. Through the use of NOG mice transplanted with siRNA-transfected MT2 cells, the present study also illustrated that *in vivo* down-regulation of Nox5 α led to an observable decrease in the tumor growth rate of HTLV-1 tumor transplant mice. In summation, expression of Nox5 α is critical for maintaining the survival, proliferation, migration, and oncogenic potential of HTLV-1 transformed cells, and therefore serves as a key cellular factor in promoting the transformation phenotype of HTLV-1-infected T cells [97].

Several cellular proteins have been shown to be involved in HTLV-1-mediated T-cell transformation by interfering with the activity and expression of Tax. A study by Jain et al. demonstrated that myocyte enhancer factor-2 (MEF-2), a transcription factor for Interleukin 2 (IL-2) produced during peripheral T-cell activation, is substantially up-regulated within HTLV-1-infected cells and ATL patient samples [98,99]. Through shRNA-mediated knockdown within an *in vitro* co-culture system, MEF-2 was found to be essential for the transformation of CD4⁺ T cells by HTLV-1. Furthermore, direct inhibition of MEF-2 activity and expression resulted in decreased viral replication and transactivation of the 5' LTR promoter, which was attributed to the propensity of MEF-2 to colocalize with Tax and stabilize its interaction with CREB. Therefore, MEF-2 is not only required for viral transformation but is also critical for the ability of Tax to efficiently promote viral gene expression from the 5' LTR promoter [99]. In contrast with MEF-2, the ubiquitin E3 ligase, PDLIM2, functions to repress the transformation of HTLV-1-infected T cells by directly targeting Tax for proteasomal degradation at the nuclear matrix [100]. This role of PDLIM2 in HTLV-1 transformation was revealed in a study by Yan et al., which characterized PDLIM2 mRNA and protein to be highly expressed in HTLV-1-negative Jurkat cells and significantly down-regulated in HTLV-1-transformed T-cell lines such as C8166, MT4, Hut-102, and SLB-1. Co-expression of PDLIM2 and Tax in HTLV-1-transformed cell lines led to a dose-dependent decrease in Tax-mediated NF- κ B activation and viral transcription, while PDLIM2 knockout reversed this effect. In addition, artificial up-regulation of PDLIM2 in Tax-expressing Rat-1 fibroblasts suppressed the growth of these cells in soft agar, suggesting that PDLIM2 plays an important role in reducing the capacity of HTLV-1-infected cells to transform and proliferate *in vitro* [100]. Expression of PDLIM2 also had a significant impact *in vivo* since severe combined immunodeficiency (SCID) mice transplanted with C8166 and MT4 cells exhibited no tumorigenesis when co-expressed with both PDLIM2 and Tax [100]. Regulation of PDLIM2 has been strongly suggested to be mediated by the DNMT1 and DNMT3b methyltransferases, which are both significantly up-regulated in a multitude of different HTLV-1-transformed T-cell lines [101]. Altogether, MEF-2 and PDLIM2 are cellular proteins that function to either enhance or repress the transforming capacity of HTLV-1-infected cells by modulating the activity of Tax.

Another notable transforming factor for HTLV-1 is parathyroid hormone-related protein (PTHrP), which is constitutively expressed in HTLV-1 carriers and highly up-regulated during HTLV-1-mediated transformation of T cells [102,103]. Compared with an established HTLV-1 cell line such as MT2, the promoter of PTHrP was preferentially activated within recently immortalized HTLV-1 cell lines. In addition, the transient expression of Tax was not correlated with the expression pattern of PTHrP, indicating that this cellular protein likely plays a role in T-cell transformation independent of Tax activity [102]. Similar to PTHrP, expression of protein arginine methyltransferase 5 (PRMT5) has also been implicated in HTLV-1-mediated T-cell transformation. PRMT5 was up-regulated in both HTLV-1 transformed cell lines, ATL-derived cell lines, and during HTLV-1-mediated T-cell transformation *in vitro* [104]. This protein is also essential for the survival of HTLV-1 transformed cells since enzymatic inhibition of PRMT5 activity

results in significant dose-dependent toxicity in HTLV-1-transformed cell lines such as SLB-1 and Hut-102 [104]. Although the precise mechanisms of how PTHrP and PRMT5 directly contribute to the viral transformation process have yet to be fully elucidated, the elevated expression levels of these cellular factors within immortalized cell lines strongly suggest that they play a unique role.

miRNAs have also been shown to play many diverse and notable roles in the process of HTLV-1-mediated T-cell transformation. A review by Ruggero et al. outlines a large array of different miRNAs that have been reported to be up-regulated in HTLV-1-transformed T-cell lines and ATL-derived patient cell lines [105]. As described previously in this review, several miRNAs are completely dependent on the expression of specific viral proteins to promote cellular transformation, such as miR-155 with Tax and miR-21 with Hbz [58,79]. However, not all cellular miRNAs which affect HTLV-1-mediated transformation are controlled or regulated by viral gene products. For example, up-regulation of miR-93 in HTLV-1-transformed and ATL-derived cell lines has been linked to the expression of the E2F1 transcription factor. The oncogenic potential of miR-93 is derived from its tendency to target key cell cycle regulators such as p21 [106]. miR-93 can also aid in viral immune evasion by significantly down-regulating the expression of histocompatibility complex class I chain-related B (MICB), which serves as a major ligand for natural killer (NK) cells and CD8⁺ T cells [107]. In addition, miR-93 has been shown to function alongside miR-130b to specifically target tumor protein 53-induced nuclear protein 1 (TP53INP1), a tumor suppressor protein that exhibits relatively minimal expression in HTLV-1-transformed and ATL-derived cell lines. Antagonization of miR-93 and miR-130b in the HTLV-1-transformed cell line MT4 was reported to substantially reduce cell viability, demonstrating the importance of these miRNAs for the survival of HTLV-1-transformed cells [108]. However, not all up-regulated miRNAs in ATL patient lines have been reported to enhance the transformed phenotype. Shown to be up-regulated in ATL patient samples but down-regulated in HTLV-1-transformed cell lines, miR-150 has been reported to repress the proliferation of transformed cells by functioning as a tumor suppressor [109]. Cellular factors and miRNAs have a wide variety of diverse functions that can serve to either promote or suppress HTLV-1-mediated T-cell transformation independent of Tax or Hbz.

***In vitro* and *in vivo* models useful for transformation studies**

HTLV-1 is able to transform primary CD4⁺ T cells *in vitro* using a co-culture immortalization assay [110]. The use of this assay requires freshly isolated peripheral blood mononuclear cells (PBMCs) from a healthy donor co-cultivated with an HTLV-1 producer cell line (Figure 3). A viral producer cell line is essential as cell-free infection by HTLV-1 is extremely inefficient and successful infection requires the co-cultivation of infected cells with naïve target cells. The HTLV-1 producer cell line is typically subjected to lethal irradiation immediately prior to co-culture. This ensures that virions are successfully transmitted to target cells and producer cells are eliminated from the long-term co-culture. Initiation of transformation becomes apparent within 5–6 weeks following co-culture as detected by expansion of CD4⁺ T cells from the PBMCs mixed cell population. This type of assay allows for not only the study of early viral infection events, but also the ability of HTLV-1 to immortalize a target cell. Cell viability, cell number, and the number of transformed wells can be measured at weekly time points to assess the immortalization process. This type of assay has been useful for studying the function and biochemistry of individual viral proteins and protein modifications and their role in transformation [72,111–113]. Not only is this assay cost effective, but it is readily available for many labs with access to cell culture resources. The limitation of this type of study is the absence of a functional immune response and therefore selective pressure. Indeed, viral genes which have been shown to be essential for viral persistence and establishment of infection *in vivo*, such as Hbz, are not required for transformation *in vitro* [72,111].

Mouse models have provided a useful tool for studying cellular and viral factors required for development and maintenance of HTLV-1-mediated transformation. Some of the earliest mouse studies within the HTLV-1 field involved the use of Tax Tg mice [114–116]. These initial studies were useful for understanding the role of Tax and Tax-mediated disturbance of normal lymphocyte function. However, these early Tax Tg mouse models had tissue-specific expression patterns uncharacteristic of HTLV-1-infected patients. In an effort to restrict Tax expression to the lymphoid cell compartment, subsequent studies used alternative promoters to drive Tax expression. Tax driven by the granzyme B (Gzb) promoter was able to target expression to the mature T-cell compartment [24]. The Gzb-Tax Tg mice developed large granular lymphocytic leukemia/lymphoma and also spontaneously developed hypercalcemia, high frequency osteolytic bone metastases, and enhanced osteoclast activity—all symptoms frequently found in ATL patients. Subsequent studies have used other promoters (CD3- promoter–enhancer sequence, Lck proximal promoter) to drive Tax expression or coupled Tax Tg with LTR-luc Tg mice to monitor inflammation and lymphoma development using non-invasive bioluminescent imaging [25,68,117–119]. Shortly after the discovery of Hbz, Tg mice expressing Hbz in CD4⁺ T cells were developed [120]. Hbz Tg mice developed T-cell lymphomas and system

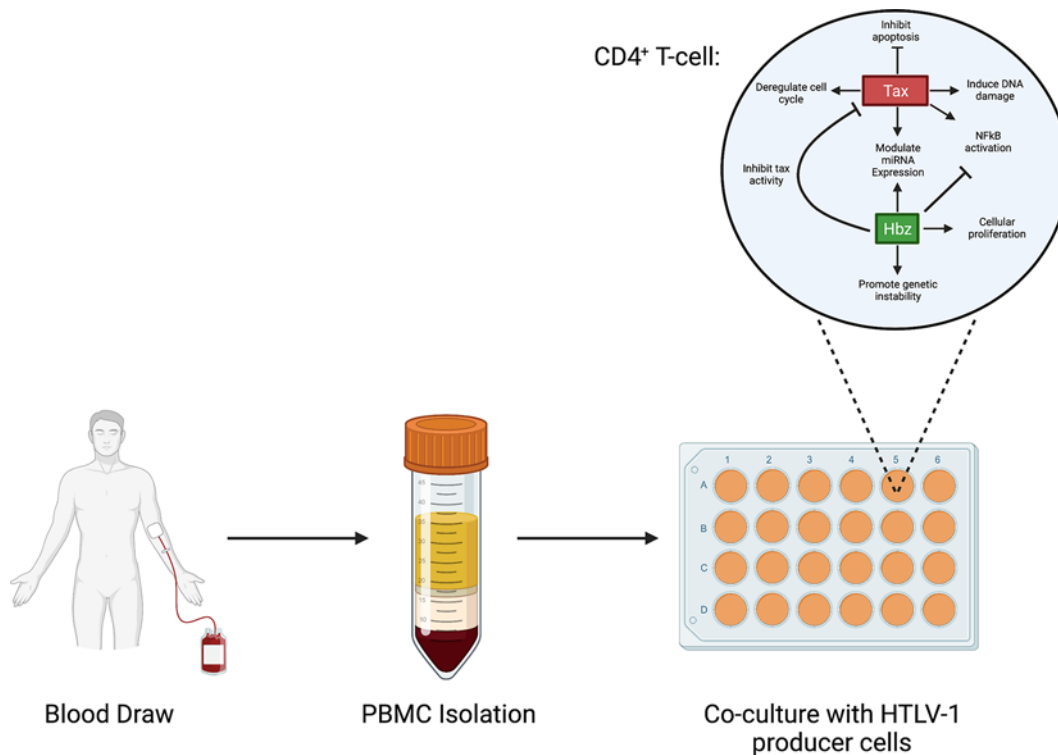


Figure 3. HTLV-1 co-culture immortalization assay

Human PBMCs are obtained from a healthy blood donor and immediately isolated through Ficoll density gradient separation. Freshly isolated PBMCs are subsequently co-cultured with lethally irradiated virus producer cells where CD4⁺ T cells will become infected with HTLV-1. Within the infected cell, Tax and Hbz viral proteins are expressed. Over a period of several weeks, these viral proteins play a prominent role in HTLV-1-mediated T-cell transformation by influencing a multitude of different cellular functions, as depicted above.

inflammation along with elevated levels of CD4⁺ Foxp3⁺ T(reg) cells and effector/memory CD4⁺ T cells. Tg mice have been especially useful for establishing the role of Tax and Hbz in transformation and oncogenesis. These mice also provide a valuable tool to test various future treatments, particularly those targeted at Tax or Hbz, and to investigate additional cellular factors involved in transformation and disease development.

In the early nineties, SCID mice were used to successfully model the proliferative and tumorigenic potential of ATL cells [121]. Of note, these mice were also treated with an anti-asialo GM-1 antibody to abolish NK cell activity and enhance the growth of lymphoma. Subsequent studies found that tumors developed in SCID mice injected with peripheral blood lymphocytes (PBLs) from ATL patients, but not with PBLs generated by *in vitro* co-culture immortalization assays [122]. Taken together, these data suggest that the genetic environment plays an important role in tumorigenesis and that cells from a non-leukemic origin have not gathered the essential genetic alterations needed to reach the full tumorigenic potential *in vivo*. NOD/SCID γc (null), or NOG mice, have immunological dysfunctions of T, B, and NK cells. Successful transplantation of various HTLV-1-transformed cell lines (SLB-1, Hut-102, and TL-om1) into these mice has been beneficial for examining viral-induced cell proliferation and tumorigenesis [123]. For example, SLB-1 cells with shRNA-mediated knockdown of Hbz had significantly reduced tumor burden and cell proliferation in NOG mice compared with wildtype SLB-1 cells, thus demonstrating the proliferative capacity of Hbz *in vivo* [124]. Like Tg mice, NOG mice represent an *in vivo* model for studying HTLV-1-mediated transformation and the development of novel drugs and targets to reduce disease burden.

In efforts to develop an improved model of HTLV-1 infection and disease progression, 'humanized immune system' (HIS) mouse models were developed. NSG mice, or NOD-SCID/ γ (null) (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) mice, lack mature T, B, and NK cells, are deficient in multiple cytokine signaling pathways, and have many defects in innate immunity. HIS mice are created by injecting human umbilical-cord stem cells (CD34⁺) into the livers of immunodeficient neonatal NSG mice, resulting in the development of human lymphocytes that appear phenotypically normal but cannot mount an adaptive immune response [125,126]. HIS mice inoculated with HTLV-1 consistently

reproduce the three key stages of HTLV-1-induced tumorigenesis in a very compact time frame (approximately 4–5 weeks): (1) persistent infection, (2) chronic proliferation of CD4⁺ T cells, and (3) development of lymphoproliferative disease. A recent study using these mice found that the envelope protein of HTLV-1 is important to induce CD4⁺ T-cell proliferation (vs. CD8⁺ T-cell proliferation), suggesting that T-cell transformation *in vivo* may also be influenced by the Env protein of the virus [125]. The ability to incite disease in this model allows for the study of T-cell transformation and tumorigenesis, as well as interaction of tumor cells with the microenvironment. Specific hurdles in the use of HIS mice are the cost associated with generating the mice, the maintenance, and care, but also the lack of a functional immune system. Recently, a novel HTLV-1-infected humanized mouse model was generated through intra-bone marrow injection of human CD133⁺ stem cells into NOG mice (IBMI-huNOG mice) [127]. After HTLV-1 infection, an increase in CD4⁺ T cells was observed in the periphery and atypical lymphocytes which represent ATL-specific flower cells were observed after 4–5 months. Proliferation and proviral load were also detected in CD4⁺ T cells. Importantly, the authors also report the presence of an HTLV-1-specific adaptive immune response in these animals. While technically challenging to create, this model will undoubtedly be useful for investigating *in vivo* mechanisms of transformation and disease development, as well as drug and vaccine candidates.

Recently, a Tg *Drosophila melanogaster* model expressing Tax in the compound eye and plasmatocytes (a leukocyte-like cell) has been described [128]. Tax expression induces a disruption of crystalline array of the ommatidia and an increase in plasmatocyte proliferation, demonstrating that Tax has transforming potential in the fly model. Not surprisingly, the induction of this eye phenotype is primarily dependent on the *Drosophila* homolog of IKK γ /NEMO. Conversely, an Hbz transgenic *Drosophila* model neither induces transformation nor NF- κ B activation *in vivo* [129]. Instead, Hbz overexpression prevents Tax-mediated NF- κ B activation and rescues the Tax-induced transformation phenotype. *Drosophila* models possess several advantages including fast generation time, high numbers of progeny, and several genetic screens (near complete collection of mutants and RNAi lines). In addition, several key signaling pathways are highly conserved between mammals and flies. However, while initial findings support this model system, any results from this model still require further validation in mammalian systems.

A key to the HTLV-1 transformation process is establishment of viral infection and viral persistence. HTLV-1 infection of rabbits mimics early infection in humans. New Zealand white rabbits inoculated with HTLV-1 become persistently infected [72,113,130]. The early rabbit humoral antibody responses against Gag and Env mimic asymptomatic early viral infection in humans. While these animals do not develop disease, they do recapitulate viral persistence (i.e., long-term viral latency) and enable the study of early viral infection events. An obvious limitation with the rabbit model is that it is not a disease model. Nevertheless, this animal model is invaluable for measuring early viral infection events and persistence in the presence of a functional immune system—factors which enable viral transformation.

Conclusions

HTLV-1 is a transactivating retrovirus that has the potential to induce ATL and/or various inflammatory diseases following an extensive clinical latency period of up to several decades. Given the nature of ATL to be an extremely aggressive and chemotherapy resistant CD4⁺ T-cell malignancy that is often fatal, there is an urgent need to understand the viral and cellular mechanisms that regulate or influence HTLV-1-mediated T-cell transformation. Serving as the primary driving force for viral transformation, the HTLV-1-encoded Tax oncoprotein is critical for viral gene expression, immortalization, and pathogenesis. Through its ability to directly interact with a multitude of different cellular proteins and pathways, Tax can transform infected T cells by modulating viral gene expression at the 5' LTR promoter, deregulating the cell cycle, disrupting the apoptotic response, and decreasing genomic stability. Many of these processes are heavily dependent on Tax-mediated activation of NF- κ B. Antagonization of Tax-driven NF- κ B induction and viral transcription is mediated by the viral protein Hbz. Derived from the anti-sense strand of the proviral genome, Hbz is required for the controlled proliferation and clonal expansion of HTLV-1-infected CD4⁺ T cells. Hbz can also promote the transformation of infected T cells by preventing apoptosis and reducing genomic stability. Tax and Hbz are not the only viral accessory proteins that influence the transformation of HTLV-1, as p12, p13, and p30 have individually been shown to play unique roles that contribute to this dynamic process. In addition to viral proteins encoded by HTLV-1, a multitude of cellular factors have also been illustrated to either promote (Nox5 α and MEF-2) or repress (PDLIM2) the transformation of HTLV-1-infected cells.

Molecular tools such as co-culture immortalization assays and *in vivo* models including rabbits, mice, and *Drosophila* have been critical in our understanding of this oncogenic virus. The role of Tax and Hbz in the transformation process is undisputed, however several unknowns remain: (1) How important are other viral proteins in

the transformation process? (2) What is the timing and expression level of Tax and Hbz required for efficient transformation? (3) Which cellular factors are critical for regulating Tax/Hbz? and (4) Which cellular factors are required to push an immortalized cell to oncogenesis/a diseased state? Since the discovery of HTLV-1 over 40 years ago, there has been considerable progress made towards understanding the viral transformation process. As the molecular tools and models improve, we will undoubtedly continue to refine our understanding of the dynamic HTLV-1-mediated cellular transformation process.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ATF, activating transcription factor; ATL, adult T-cell leukemia/lymphoma; ATM, ataxia-telangiectasia mutated; CBP/CREB, CREB-binding protein; CRE, cyclic AMP response element; HDAC1, histone deacetylase 1; HIS, humanized immune system; IKK, I κ B kinase; LTR, long terminal repeat; NEMO, NF- κ B essential modulator; NK, natural killer; NO, nitric oxide; Nox5 α , NAPDH oxidase 5- α ; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; PTHrP, parathyroid hormone-related protein; ROS, reactive oxygen species; SCID, severe combined immunodeficiency; SUV39H1, suppressor of variegation 3-9 homolog 1; Tg, transgenic.

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