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Human T-cell leukemia virus type 1: replication, proliferation and propagation by Tax and HTLV-1 bZIP factor

Short title: Replication of human T-cell leukemia virus type 1

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Abstract

Human T-cell leukemia virus type 1 (HTLV-1) spreads primarily by cell-to-cell transmission. Therefore, HTLV-1 promotes the proliferation of infected cells to facilitate transmission. In HTLV-1 infected individuals, the provirus is present mainly in effector/memory T cells and Foxp3+ T cells. Recent study suggests that this immunophenotype is acquired by infected cells through the function of HTLV-1 bZIP factor (HBZ). Tax, which is encoded by the plus strand, is critical for viral replication and *de novo* infection, while HBZ, encoded by the minus strand, is important for proliferation of infected cells. Importantly, HBZ and Tax have opposing functions in most transcription pathways. HBZ and Tax cooperate in elaborate ways to permit viral replication, proliferation of infected cells and propagation of the virus.

Introduction

Transmission of human T-cell leukemia virus type 1 (HTLV-1) is confined to three routes; mother-to-infant, sexual parenteral transmission, and blood transfusion/needle sharing [1]. A striking feature of this virus is that HTLV-1 is transmitted primarily in a cell-to-cell fashion, and infectivity of free virions is very poor. In contrast, another human retrovirus, human immunodeficiency virus (HIV), transmits by both cell-free and cell-to-cell contact. The transmission of HTLV-1 requires living infected cells in breast milk, semen and blood products. To facilitate its transmission, this virus increases the number of infected cells *in vivo* by stimulating their proliferation.

HTLV-1 was discovered in 1980 as the first human retrovirus [2,3]. Thereafter, this virus was found to be linked with a human disease, adult T-cell leukemia (ATL) [4]. Subsequently it was found that this virus also causes another disease, HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), as well as HTLV-1 uveitis, infective dermatitis, and myopathy [1]. These diseases are thought to be associated with the fact that infected host immune cells proliferate *in vivo*. In this review, we summarize recent findings on the replication of HTLV-1, the proliferation of infected cells, and HTLV-1 propagation -- matters which are closely related for this virus.

Virus entry and cell-to-cell transmission

Unlike HIV, HTLV-1 can infect a variety of cells; its receptor is thought to be a commonly expressed molecule [5]. It has been reported that HTLV-1 envelope protein

interacts with three cellular molecules, heparan sulfate proteoglycan (HSPG) [6], neuropilin-1 [7], and a glucose transporter, GLUT1 [8], for entry into cells. Conformational changes of the complex consisting of the HTLV-1 virion and these molecules are thought to occur sequentially during the entry step. First, the HTLV-1 envelope attaches to HSPG, and it then forms complexes with neuropilin-1, which results in stabilization of the complex. Thereafter, GLUT1 is associated with the complex, and finally triggers the fusion process necessary to viral entry [9].

In vitro experiments showed that free virions had poor infectivity, while co-culture of uninfected cells with HTLV-1 infected cells easily established HTLV-1 infected cells [10]. It has been reported that cell-mediated infection of HTLV-1 is 10000 times more efficient than cell-free infection, while cell-to-cell infection by HIV-1 is only twice as efficient as cell-free infection [11]. Two models for the mechanism of cell-to-cell infection by HTLV-1 have been proposed: 1) virological synapse [12] and 2) biofilm [13], and 3) cellular conduits [14]. HTLV-1 infected cells form a virological synapse with uninfected cells; ICAM-1 and LFA-1 are implicated in this synapse formation. Tax is also implicated, specifically in microtubule reorientation [15]. Indeed, Tax enhances cell-to-cell infection [11]. On the other hand, there is evidence to support the biofilm model as well. HTLV-1-infected T cells retain viral particles with virally-induced extracellular matrix components, including collagen, agrin, tetherin and galectin-3 [13]. By cell contact, these viral assemblies adhere to other cells, resulting in infection with HTLV-1.

An increased number of infected cells augments the chances of transmission. Indeed,

for mother-to-infant transmission, it has been reported that infants have higher chances of getting infected from mothers with higher proviral loads [16].

Transmission of HTLV-1

HTLV-1 can be transmitted by breast-feeding, sexual contact and blood transfusion. This transmission requires living infected cells, since this virus transmits mainly by cell-to-cell contact (Figure 1). Therefore HTLV-1 infected cells are hypothesized to have attributes that promote their entry into breast milk and semen. It has been reported that breast milk contains T-cells, most of which are effector/memory T cells expressing LFA1 and ICAM-1 [17], and HTLV-1 provirus has been detected in such effector/memory T cells [18]. These findings suggest that HTLV-1 may confer a phenotype to infected cells that facilitates their entry into breast milk. What component of HTLV-1 confers this effector/memory phenotype to HTLV-1 infected cells? Transgenic expression of HBZ in CD4+ T cells increased the number of effector/memory T cells and regulatory T cells, while transgenic mice expressing Tax had no change in the phenotype of CD4+ T cells [19]. This clearly demonstrates that the immunophenotypes of ATL cells and HTLV-1 infected cells are conferred by HBZ, not by Tax. This conferred phenotype, which involves high levels of expression of adhesion molecules, enables HTLV-1 infected cells to enter into breast milk and semen (Figure 1).

Next, the virus must override epithelial barriers. How does HTLV-1 cross the

alimentary tract? Recently, it has been shown that free infectious HTLV-1 virions could cross the epithelial barrier via a transcytosis mechanism [20]. HTLV-1 virions could then infect human dendritic cells (DCs) that exist in the epithelial barrier [21]. Infected DCs likely migrate to draining lymph nodes and then form virological synapses with T cells (Figure 1). It is difficult to infect T cells by free virus *in vitro*. However, free virus can infect DCs, and infected DCs can propagate HTLV-1 infection, suggesting that DCs are the spreader of this virus *in vivo* [21]. Expression of adhesion and co-stimulatory molecules is critical for immunological synapses between T cells and DCs [22]. Thus, the immunophenotypes (effector/memory T cells, regulatory T cells, and enhanced expression of adhesion molecules) conferred by HBZ are critical for the further spread of this virus *in vivo*. Thus, using HBZ, HTLV-1 induces infected cells to acquire certain immunophenotypes that facilitate its entry into the body and its subsequent spread within the body.

Clonal proliferation of HTLV-1 infected cells

After infection, HTLV-1 spreads by cell-to-cell infection and DC mediated infection. This *de novo* infection of cells is thought to form a pool of infected cells at an early phase of infection. In an experiment using immunodeficient mice with human lymphocytes, administration of reverse transcriptase inhibitors, tenofovir disoproxil fumarate (TDF) or azidothymidine (AZT) beginning after one week of infection could neither block nor decrease proviral load of HTLV-1, while TDF or AZT could block infection when they were injected at the same time of infection [23]. These results

suggest that a pool of HTLV-1 infected clones is generated at very early phase of infection, and after that time, clonal proliferation of infected cells is predominant. This notion is also supported by clinical findings that reverse transcriptase inhibitors or integrase inhibitors did not alter proviral load in HTLV-1 infected individuals [24,25].

After this early stage of de novo infection, HTLV-1 infected clones are subject to selection by both host immunological attack and viral gene expression. In seroconvertors, the clonality of HTLV-1 infected cells was not stable at an early phase, but then stabilized at the chronic carrier state phase [26], indicating that HTLV-1 infected clones are selected at early phase of infection, and then, selected clones survive *in vivo*.

Since the HTLV-1 provirus integrates at random sites within the host genome, the clonality of HTLV-1 infected cells can be analyzed by studying these integration sites. Inverse PCR has been used to identify the integration sites and determine the clonality of infected cells [27,28]. Recently, high-throughput sequencing has been shown to be capable of detailed analysis of clonality [29]. It is well known that HAM/TSP patients possess higher proviral loads compared with asymptomatic carriers. Analysis of clonality using high-throughput sequencing revealed that the abundance of each clone did not differ, but the number of different clones increased in HAM/TSP patients compared with asymptomatic carriers [29]. In contrast, the abundance of certain clones increased in patients coinfected with HTLV-1 and strongyloides, and in infective dermatitis patients with HTLV-1 infection (IDH patients) [30]. It is noteworthy that ATL develops relatively frequently in IDH patients and HTLV-1 carriers coinfected

with strongyloides, while the occurrence of ATL is not so frequent in HAM/TSP patients [31]. Thus the enhanced abundance of clones and increased cell division might promote the development of ATL.

What drives cell division of HTLV-1 infected cells? HBZ is ubiquitously expressed in ATL cells and HTLV-1 infected cells *in vivo*, and promotes their proliferation [32]. In addition, Tax enhances mitogenic antigen-receptor signals [33,34]. The details of the mechanisms by which HBZ and Tax stimulate cell proliferation are complex and fascinating. In fact, HBZ and Tax have opposite effects on most signaling pathways [35] (Figure 2). For example, Tax activates the AP-1, NFAT, and CREB pathways while HBZ suppresses them [36,37]. Conversely, Tax inhibits TGF-β/Smad pathway whereas HBZ activates it [38]. Tax activates both the canonical and non-canonical NF-κB pathways [39]. HBZ inhibits only the canonical NF-κB pathway by interacting with p65. Expression of Tax promotes cell proliferation and simultaneously induces cellular senescence by induction of p21 and p27. HBZ prevents Tax induced cellular senescence by inhibiting p65 [40]. Thus, the elaborate interactions of various signaling pathways with Tax and HBZ control the proliferation of HTLV-1 infected cells. In addition to this relationship between HBZ and Tax, it has been reported that HBZ mRNA has growth-promoting activity [32] , indicating another complex connection of HBZ as RNA and protein.

Furthermore, we have reported that HBZ suppresses the canonical Wnt pathway by inhibiting DNA binding by TCF-1/LEF-1 transcription factors, while Tax activates canonical Wnt signaling [41]. In contrast, HBZ enhances the transcription of Wnt 5a,

which is a ligand for the non-canonical Wnt pathway. The canonical Wnt pathway is predominant during the development of T cells in the thymus, while non-canonical Wnt signaling is activated in peripheral T cells. These findings suggest that HBZ modulates the intra-cellular environment of peripheral T cells, which are natural target of this virus.

Control of transcription of viral genes

The HTLV-1 provirus encodes the regulatory genes (*tax* and *rex*) and the accessory genes (*p12*, *p13*, *p30*, and *HBZ*) in pX region; these genes regulate viral replication and the proliferation of infected cells [1]. For their transcription, the LTRs at each end of the provirus are used as promoters: the 5'LTR and 3'LTR control the transcription of the viral genes encoded in the plus and minus strands of the provirus, respectively (Figure 3). Since the plus strand of the provirus encodes all structural proteins and the viral genomic RNA, 5'LTR-mediated transcription is required for viral replication and transmission. Tax is a potent activator of viral transcription through the 5'LTR. Tax does not bind to DNA, but activates the transcription of target genes by recruiting various transcription factors and modifying the epigenetic status of promoter regions [42].The association between Tax and CREB is critical for viral gene transcription. There are three 21-bp repeat elements, called Tax-responsive elements (TREs), located in 5'LTR, and the Tax-CREB complex recruits several histone acetyltransferaeses including CREB biding protein (CBP), p300, and p300/CBP-associated factor (PCAF) to the LTR, resulting in induction of viral expression. In addition to Tax, some cellular

signaling machinery can enhance the activity of the 5'LTR. It has been shown that immune stimulation via T-cell receptor signaling activates the 5'LTR [34,43]. Another study showed that apoptotic signals induced viral transcription [44]. 5'LTR activation by these signals might be advantageous to efficient viral transmission and to viral "escape" from a dying host cell.

Importantly, viral replication is actually suppressed *in vivo* [45], while viral antigens including Tax are quickly expressed in infected cells after they are transferred to *ex vivo* culture [46]. Host immune surveillance eliminates infected cells by targeting viral antigens. Among viral proteins, Tax is a major target of cytotoxic T-cells (CTLs) [47]. It is well known that removal of CD8+ T-cells from PBMC allows infected cells to express Tax in the *ex vivo* cell culture [45], suggesting the presence of immune pressure against Tax *in vivo*. In addition, it was shown that, even in immunodeficient animal models, viral transcription from 5'LTR was suppressed, indicating that other mechanisms are involved in the silencing [48]. HTLV-1 can suppress its replication by its own proteins; p30 and HBZ are known to counteract Tax by competing for the binding to CREB, resulting in suppression of HTLV-1 replication [49]. p30 also inhibits the nuclear export of tax/rex mRNA [50]. Epigenetic changes, such as DNA methylation and histone modifications, are also involved in the silencing of HTLV-1. HTLV-1 differs from HIV in this respect. The LTR of HIV contains few CpG sites, while there are DNase hyper-sensitive regions, which explains the resistance of the HIV LTR to silencing [51,52]. On the other hand, the HTLV-1 LTR has many CpG sites, suggesting that HTLV-1 is susceptible to gene silencing mediated by DNA methylation.

CpG methylation in the HTLV-1 provirus is observed in HTLV-1 carriers, and methylation tends to increase and to spread toward the 5'LTR during disease progression [53]. Indeed, Tax expression is frequently missing in ATL cells by epigenetic silencing of the 5'LTR as well as by genetic destruction of the 5'LTR or the *tax* gene [54,55]. Destruction of Tax expression enables ATL cells to escape from Tax-specific CTLs. Recently, it was reported that a histone deacetylase inhibitor, valproate (VPA), enhanced the expression of Tax and Gag in cultured HTLV-1-infected cells from asymptomatic carriers and HAM/TSP patients, suggesting that viral expression is suppressed by epigenetic mechanisms even in the carrier state [56].

The 3'LTR functions as a promoter of the minus strand of the provirus [57]. It has been shown that the 3'LTR is conserved in all cases and CpGs are hypomethylated, suggesting that transcription through the 3'LTR is required for infected cells [53,58]. The *HBZ* gene is encoded in the minus strand, and alternative splicing makes the splice variants, the spliced and unspliced isoforms [59,60]. The spliced *HBZ* gene is transcribed from the 3'LTR, and the SP1 binding elements in 3'LTR are important for its transcription [57]. SP1 is a transcription factor ubiquitously expressed in a variety of cells, a fact which corresponds to the finding that HBZ is constitutively expressed in all ATL cases and HTLV-1 infected individuals [61]. It was also reported that SP1 forms a complex with HBZ and JunD and enhances the promoter activity of HBZ [62], suggesting that SP1 is a key transcription factor for the activity of the 3'LTR. Interestingly, it was shown that Tax positively regulates 3'LTR activity [57], although the significance of this observation remains unclear. Further studies need to be

conducted for us to fully understand the regulation of transcription via the 3'LTR.

The host immune system and proliferation of infected cells

After infection, provirus load (the number of infected cells) and clonality are determined by the balance between viral gene expression and the host immune response [63]. As described, Tax is highly immunogenic, while the immunogenicity of HBZ protein is very low [64]. However, provirus load is well correlated with the immune response to HBZ; a low immune response to HBZ is associated with a high provirus load in HTLV-1 infected individuals. It is thought that HTLV-1 evolves to reduce the immunogenicity of HBZ, which is constitutively expressed and critical for the proliferation of infected cells. Conversely, HTLV-1 infected cells express Tax more transiently. Tax is important for viral replication and *de novo* infection by HTLV-1. However, due to the high immunogenicity of Tax, HTLV-1 suppresses Tax expression *in vivo* by elaborately regulated mechanisms.

Conclusion

HTLV-1 evolved to propagate by cell-to-cell transmission. Therefore, this virus induces the proliferation of infected cells while under the pressure of host immune system. To this end, Tax and HBZ cooperate with each other in complicated ways to permit viral replication and promote the proliferation of infected cells. These phenomena are closely associated with the pathogenesis of this virus.

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*****This publication shows that a histone deacetylation inhibitor, valproate (VPA), enhances expression of Tax and Gag in cultured cells from HTLV-1-infected individuals. In contrast, HBZ expresssion is suppressed by VPA, suggesting that histone deacetylase(s) is involved in viral replication and cellular proliferation. VPA also induces apoptosis in HTLV-1-infected cells.

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******This publication first reported the presence of cytotoxic T lymphocytes to HBZ, and suggests the importance of immune response against HBZ to pathogenesis of HTLV-1.

Figure legends

Figure 1. Transmission and *de novo* infection with HTLV-1

HTLV-1 is transmitted via breast feeding, sexual intercourse, and blood transfusion. For any of these routes, living infected cells are essential. HTLV-1 infected cells have the immunophenotypes of effector/memory T cells or regulatory T cells. These cells tend to enter breast milk. HTLV-1 enters into the alimentary tract by transcytosis, and infects dendritic cells. Infected DCs transmit virus to uninfected T cells via virological synapses. Then infected T cells expand clonally *in vivo*.

Figure 2. Opposite functions of Tax and HBZ

Tax and HBZ have opposite functions in many signaling pathways. Tax activates the CREB pathway by recruiting CREB and CBP/p300 to the promoters of target genes, whereas HBZ also interacts with the same proteins, suppressing Tax-mediated transcription. Tax activates both the classical and the alternative NF-κB pathways, and HBZ selectively suppresses classical signaling by targeting p65. Tax activates PI3K and induces the transcription of AP-1 target genes. HBZ negatively regulates this pathway by its inhibitory interactions with c-Jun and JunB through their bZIP domains. Tax forms a complex with DAPLE and DVL, and activates the canonical Wnt pathway. HBZ interacts with LEF-1/TCF-1 at point further downstream in this pathway and suppresses the transcription of the target genes. Tax has a negative effect on the TGF-β/SMAD pathway; however, HBZ activates it by interacting with SMAD2/3 and

recruiting CBP/p300 to the promoters of the target genes.

Figure 3. Transcriptional control of HTLV-1 provirus

The 5'LTR is a promoter and enhancer of the plus strand transcripts that encode the viral genomic RNA, the structural proteins (Gag, Pol, and Env), and the regulatory/accessory proteins (Tax, Rex, p12, p13, and p30). Transcription via the 5'LTR is induced by recruiting the Tax-CREB-CBP/p300 complex to TREs in U3 region of 5'LTR, whereas the other viral factors (HBZ and p30) and epigenetic modifications on the 5'LTR suppress it. Some extrinsic factors are also associated with the activity of 5'LTR. In contrast, the 3'LTR is constitutively activated, and recruitment of SP-1 to its binding elements in U5 of the 3'LTR is important for 3'LTR activity. HBZ is encoded in the minus strand, and the HBZ-JunD complex enhances the transcriptional function of SP-1 on the 3'LTR.

Figure 1

Breast feeding

HTLV-1 infected cells
Effector/memory T cells, LFA-1+, ICAM-1+

HTLV-1

Transcytosis

HTLV-1

Infected dendritic cells

Clonal proliferation of HTLV-1 infected cells

Virological synapse

HTLV-1

Lymphatic organs

Figure 2

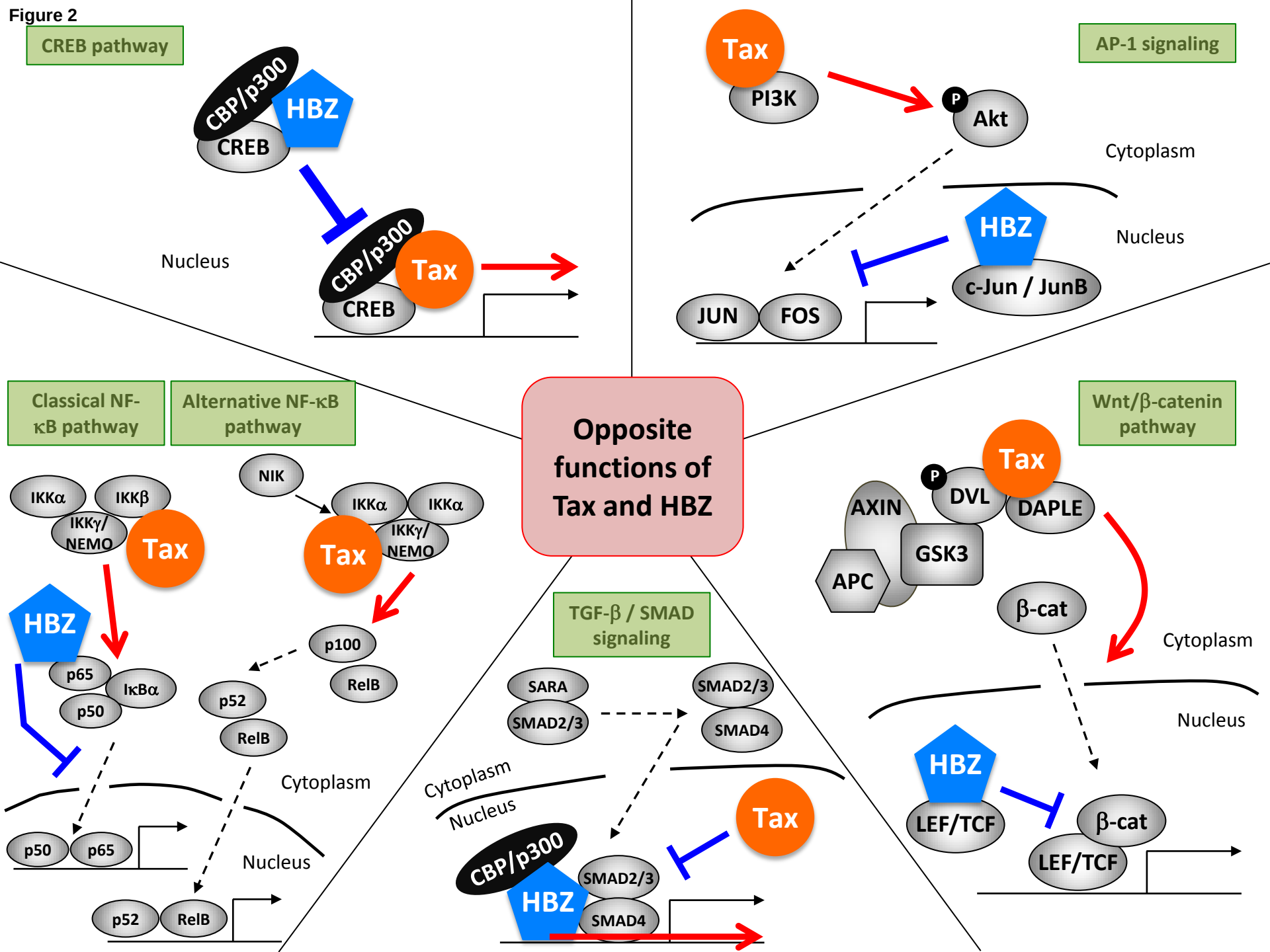
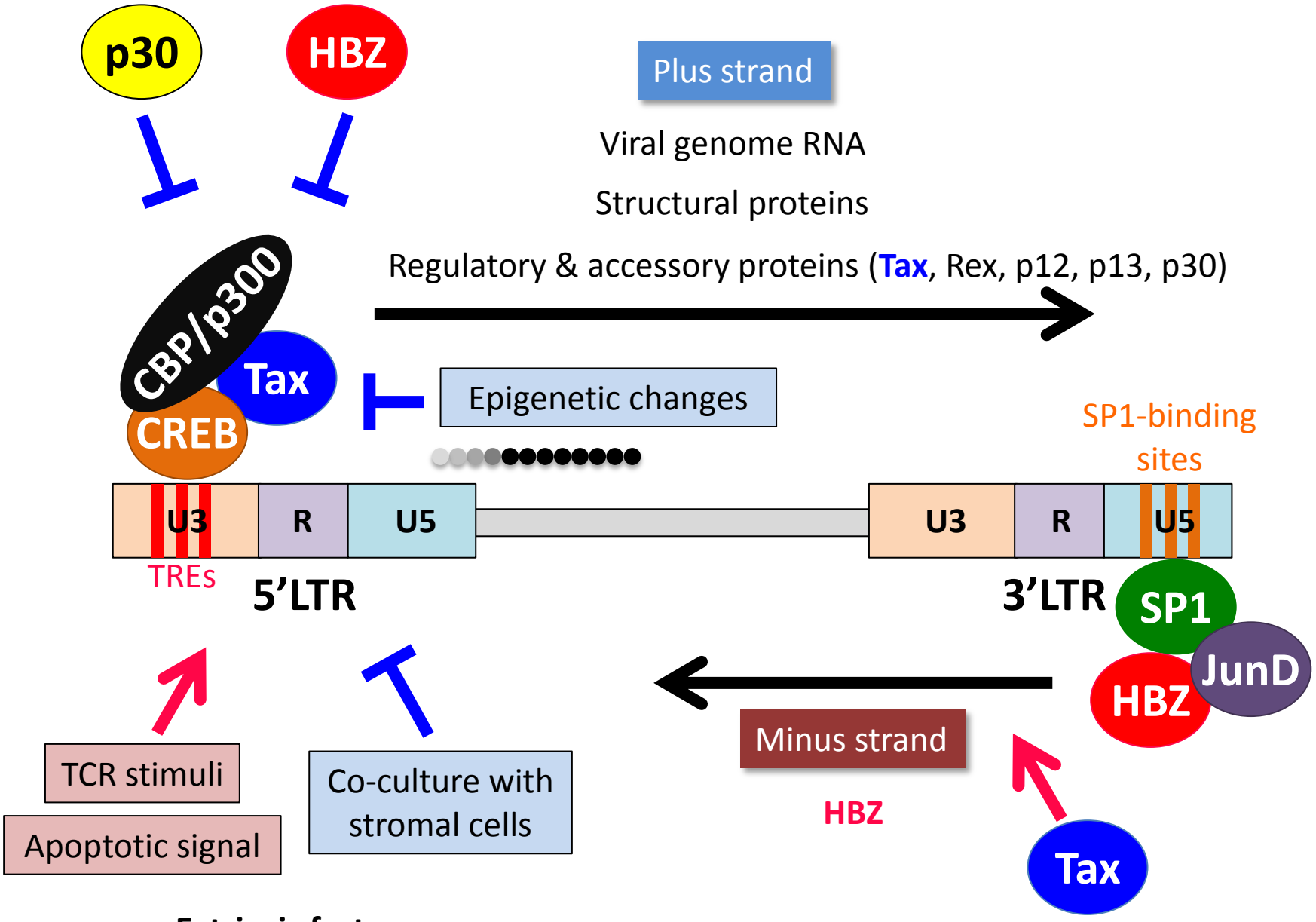

CREB pathway
CBP/p300
HBZ
CREB
Nucleus
CBP/p300
Tax
CREB
AP-1 signaling
Tax
PI3K
P
Akt
Cytoplasm
HBZ
Nucleus
c-Jun / JunB
JUN
FOS
Opposite functions of Tax and HBZ
Classical NF-κB pathway
Alternative NF-κB pathway
IKKα
IKKβ
IKKγ/ NEMO
Tax
HBZ
p65
IκBα
p50
Cytoplasm
p50
p65
Nucleus
NIK
IKKα
IKKα
IKKγ/ NEMO
Tax
p100
RelB
p52
RelB
p52
RelB
TGF-β / SMAD signaling
SARA
SMAD2/3
SMAD2/3
SMAD4
Cytoplasm
Nucleus
Tax
CBP/p300
HBZ
SMAD2/3
SMAD4
Wnt/β-catenin pathway
Tax
P
DVL
DAPLE
AXIN
GSK3
APC
β-cat
Cytoplasm
Nucleus
HBZ
LEF/TCF
β-cat
LEF/TCF

Figure 3


p30
HBZ
Plus strand
Viral genome RNA
Structural proteins
Regulatory & accessory proteins (Tax, Rex, p12, p13, p30)
CBP/p300
Tax
CREB
Epigenetic changes
SP1-binding sites
U3
R
U5
U3
R
U5
TREs
5'LTR
3'LTR
SP1
JunD
HBZ
Minus strand
HBZ
Tax
TCR stimuli
Apoptotic signal
Co-culture with stromal cells
Extrinsic factors