NEUROPILIN-1 IS UPREGULATED BY HTLV-1 bZIP FACTOR AND INHIBITS CELL-TO-CELL TRANSMISSION OF HTLV-1

by

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ABSTRACT

Human T-cell Leukemia Virus Type 1 (HTLV-1) is the etiologic agent of devastating diseases, including adult T-cell leukemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 relies heavily on cell-to-cell transmission as free virions are poorly infectious. Although cell-to-cell transmission is critical for efficient spread of HTLV-1, much is unknown about the impact of

extracellular proteins on viral transmission. Infection studies have been predominantly focused on HTLV-1 Transactivator protein (Tax), a viral protein with many roles in infection. HTLV-1 basic leucine zipper factor (HBZ) has recently been implicated infection, but relatively little is known about the role of HBZ in HTLV-1 viral spread. In this study, we found that HBZ upregulates expression of neuropilin-1 (NRP1). Neuropilin-1 is a ubiquitously expressed transmembrane receptor and an HTLV-1 receptor. HBZ is known to interact with a variety of cellular transcription factors, including AP-1 basic leucine zipper (bZIP) factors and cAMP response element binding protein (CBP)/p300 coactivator proteins. Our results indicate that HBZ interacts with certain AP-1 bZIP factors and CBP/p300 at a putative enhancer site downstream of *NRP1*. We propose a model in which HBZ upregulates NRP1 expression by forming an HBZ/AP-1 bZIP factor heterodimer, which interacts with the putative enhancer site with CBP/p300 coactivators and basal transcription machinery to upregulate expression of NRP1. Intriguingly, we discovered that NRP1 expression on HTLV-1-infected T-cells inhibits cell-to-cell transmission of HTLV-1. Furthermore, NRP1 expression does not alter virion release from infected cells, suggesting that NRP1 doesn't inhibit transmission through virion retention. We also provide evidence that NRP1 is incorporated into viral particles, resulting in a reduction in virion infectivity. Together, these results indicate that HBZ upregulates expression of NRP1, which reduces infection efficiency.

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LIST OF ABBREVIATIONS

AA	amino acid
ac	acetylation
AD	activation domain
aHSCT	allogenic hematopoietic stem cell transplantation
ATF	Activating transcription factor
ATL	adult T-cell leukemia
AZT	zidovudine
BR	basic region
bZIP	basic leucine zipper
CA	capsid
CBP	CREB-binding protein
CCR4	C-C motif chemokine receptor 4
CD	central domain
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CD25	cluster of differentiation 25, also known as IL2RA
CD70	cluster of differentiation 70
CD80	cluster of differentiation 80
CD150	cluster of differentiation 150, also known as SLAMF1
CNS	central nervous system
COL4	type IV Collagen
COL4A1	collagen alpha-1 (IV)
COL4A2	collagen alpha-2 (IV)
CREB	cAMP response element binding protein
CSF	cerebrospinal fluid
CTCL	cutaneous T-cell lymphoma
CTL	cytotoxic T-lymphocyte
CXCR3	C-X-C motif receptor 3

DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
ELISA	enzyme-linked Immunosorbent Assay
Env	HTLV-1 envelope glycoprotein
Foxp3	forkhead box P3
GFP	green fluorescent protein
GLUT1	glucose transporter-1
HAM/TSP	HTLV-1-assocaited myelopathy/tropical spastic paraparesis
HAT	histone acetyltransferase
HBZ	HTLV-1 basic leucine zipper factor
HSPG	heparin sulfate proteoglycans
HTLV-1	human T-cell Leukemia Virus type 1
HTLV-2	human T-cell Leukemia Virus type 2
HTLV-3	human T-cell Leukemia Virus type 3
HTLV-4	human T-cell Leukemia Virus type 4
ICAM-1	intracellular adhesion molecule 1
IN	integrase
IFN-α	interferon alpha
IFN-ɣ	interferon gamma
IL2RA	interleukin-2 receptor alpha chain
IS	immunological synapse
KIX	kinase-inducible interaction
LFA-1	lymphocyte function-associated antigen 1
LTR	long terminal repeat
MA	matrix
mRNA	messenger RNA
MTOC	microtubule organizing center
NAP1	nucleosome assembly protein 1
NC	nucleocapsid
NPC	nuclear pore complex

NRP1	neuropilin-1
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PIC	pre-integration complex
Pol	polymerase
Pro	protease
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
Rab5	Ras analog in brain-5
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RT	reverse transcriptase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
sgRNA	single guide RNA
sHBZ	spliced HBZ
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLAMF1	signaling lymphocytic activation molecule 1
SMAD3	SMAD family member 3
sMAF	small MAF
SNP	single nucleotide polymorphisms
Sp1	specificity protein 1
SU	surface subunit
TBP	TATA box-binding protein
Th1	T helper type 1
Tax	transactivator-X
TGF-β	transforming growth factor beta
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TM	transmembrane glycoprotein
TRE/TxRE	Tax-responsive element

TRE-1	Tax-responsive element-1, also known as vCRE
Treg	regulatory T-cell
TNF-α	tumor necrosis factor alpha
U3	unique/ Untranslated 3’
U5	unique/ Untranslated 5’
usHBZ	unspliced HBZ
VS	virological synapse
VSV	Vesicular stomatitis virus
VLP	virus-like particle
WT	wild-type
ZIP	leucine zipper
ZNF	zinc fingers

CHAPTER 1

An Introduction to Human T-cell Leukemia Virus

Discovery.

Human T-cell Leukemia Type 1 (HTLV-1) was first reported in 1980 following the isolation of this virus from T-cells collected from a patient with cutaneous T-cell lymphoma (CTCL) [1]. The first pathogenic human retrovirus to be identified, HTLV-1 is now known to be one of the most potent human oncoviruses and remains the main focus of HTLV research [2]. Since the isolation of HTLV-1, several other subtypes of HTLV have been discovered. HTLV-2 was isolated from a patient with hairy cell leukemia [3]. HTLV-2 is associated with increased cancer mortality, however, the association between HTLV-2 and cancer development is not well understood [4]. HTLV-2 has also been implicated in the development of neurological symptoms, however, a clear link between HTLV-2 and a characterized neurological disorder has not yet been described [5]. HTLV-3 and HTLV-4 were both identified in bushmeat hunters in Africa [6,7] and, to date, have not been implicated in the development of disease.

Epidemiology.

It is estimated that 5-10 million people worldwide are infected with HTLV-1[8]. Although HTLV-1 is widely distributed worldwide, it is considered endemic to certain regions including sub-Saharan Africa[9], South America, the Caribbean basin, northern Iran[10], Japan, and Central Australia[11-13]. The majority of HTLV-1-infected individuals remain asymptomatic for life but are still able to transmit the infection to others[14].

Transmission.

Interpersonal transmission of HTLV-1 can occur during transfusion of cellular blood products (through blood transfusion or IV drug use), sexual contact and during breastfeeding. Prolonged duration of breastfeeding and high maternal proviral load both contribute to likelihood of transmission from mother to infant during breastfeeding[15,16]. Breastfeeding remains a major route of transmission in certain populations with high infection rates due to a combination of factors, including limited access to HTLV-1 testing and infant formula. Due to increased blood screening methods to detect anti-HTLV-1 antibodies in blood donors, blood transfusions now pose a low risk of HTLV-1 transmission[14].

HTLV-1-Associated Diseases.

Approximately 3-5% of HTLV-1-infected individuals will develop an associated disease[8]. HTLV-1 is associated with a variety of pathologies, including leukemia, immune-mediated inflammatory diseases and increased susceptibility to certain bacterial pathogens. Disease outcomes associated with HTLV-1-infection are broad, which is thought to be due to differences in the host immune response, rather than HTLV-1 genotype differences[17].

HTLV-1 infection is associated with a number of immune-mediated inflammatory diseases, including HTLV-associated myelopathy/ tropical spastic paraparesis (HAM/TSP), uveitis[18,19], polymyositis[20], Sjogren's syndrome[21,22], sicca syndrome[23,24] and infective dermatitis[25]. Although HTLV-1 does not cause generalized immune suppression, as seen with HIV-1, HTLV-1 infection is associated with an increased susceptibility to certain bacterial pathogens including: *Mycobacterium tuberculosis*[26,27],

Staphylococcus aureus (infective dermatitis)[28], *Strongyloides stercoralis*[29] and *Sarcoptes scabiei*[30]. Adult T-cell leukemia/lymphoma (ATL), an aggressive leukemia, and HAM/TSP, a progressive neurodegenerative disease are the most common HTLV-1-associated pathologies.

Adult T-cell Leukemia.

HTLV-1 is described as one of the most potent oncoviruses with 3-5% of infected individuals developing Adult T-cell leukemia/lymphoma (ATL)[2,8]. Animal models have recapitulated the oncogenicity of two key HTLV-1 regulatory proteins, HTLV-1 basic leucine zipper factor (HBZ) and Transactivator-1 (Tax). HBZ and Tax will be discussed in greater detail in subsequent sections[31,32]. Shortly after the discovery of HTLV-1, it was determined that this retrovirus was the causative agent of ATL when T-cells isolated from an individual with ATL were found to contain the HTLV-1 genome[33].

ATL, a malignancy characterized by the aggressive proliferation of mature $CD4^+$ T-cells, develops in 3-5% of HTLV-1-infected individuals[34]. ATL typically occurs in individuals who were infected as infants through breastfeeding. The development of ATL typically occurs decades after initial infection, but median age-at-onset varies geographically[34]. In Japan, ATL is often diagnosed in individuals who are 60-70 years old[35]. In the United States, Europe, South America and Central America, the median age at diagnosis is 40-55 years[35-37]. In Brazil, pediatric cases of ATL have been identified[38].

ATL was once thought to be a cancer of HTLV-1-infected regulatory T-cells, however, recent studies have shown that HTLV-1 induces features of regulatory T-cells in conventional T-cells[39,40]. The HTLV-1 protein HBZ induces FoxP3 expression through

Smad3-dependent TGF-β signaling[32]. Indeed, one study found that in approximately 58% of ATL cases, FoxP3 expression was detectable in ATL cells[41].

The HTLV-1 genome has little sequence variability and the proviral sequence of asymptomatic individuals is not distinct from those of ATL or HAM/TSP patients, suggesting that the key determining factor(s) in the development of these diseases are due to differences in the hosts[17]. Chromosomal analysis identified chromosomal abnormalities in 96% of ATL patients tested[42]. Analysis of genetic mutations in ATL cells identified high integration of genetic abnormalities in the T-cell receptor (TCR)/NF-KB signaling pathway as the more predominant genetic mutation, seen in over 90% of cases[43]. T-cell receptor (TCR)/NF-KB signaling pathway related genetic mutations include: *PLCG1*, *PRKCB, CARD11, VAV1, IRF4* and *FYN*[43]. Mutations in other signaling factors (*STAT3, NOTCH1*), transcription factors (*IKZF2*, *TP53*, *GATA3*, and *IRF4*), epigenetic factors (TET2 and EP300), chemokine receptors (CCR3 and CCR7) and structural variants (CD274) were also identified in ATL cells[43,44].

ATL typically involves the bone marrow, skin, brain, blood and lymphoid organs. Based on circulating lymphocyte count, solid organ involvement and symptom severity, ATL is categorized into four subtypes: smoldering, chronic, acute, and lymphomatous[45] (**Table 1.1**). Prognosis and response to therapy vary drastically between the different ATL subtypes. For indolent ATL (smoldering and chronic), combination therapy using zidovudine (AZT) and interferon-alpha (IFN-α) can prolong survival[46]. The prognosis for aggressive ATL (acute and lymphomatous) is particularly poor, with death often occurring within a year of diagnosis[47,48]. The poor prognosis associated with aggressive ATL is due to a large tumor burden associated with multiorgan failure, hypercalcemia,

and/or frequent infections as the result of profound T-cell immune deficiency. Furthermore, aggressive ATL is often intrinsically resistant to chemotherapeutics[49]. Currently, allogenic hematopoietic stem cell transplantation (aHSCT) is the best treatment option for aggressive ATL, with one third of individuals achieving long-term survival[48,50]. However, aHSCT is not a feasible treatment option for many individuals[48]. Although aHSCT has been reported to be curative for approximately 35% of patients with aggressive ATL based on 3-year overall survival rates, aHSCT is also associated with transplant-related mortality in up to 40% of cases[48]. Therefore, Japanese guidelines suggest that aHSCT should only be considered after a first line of therapy has failed[48]. The combination of arsenic trioxide and IFN- α treatment has been found to induce cell cycle arrest and apoptosis in ATL cells[51] through proteasomal degradation of the HTLV-1 Tax protein and reversal of NF/kB activation[52,53]. More recent studies have found that arsenic trioxide in combination with low-dose AZT/ IFN-α may enhance long-term disease control with moderate side effects[54]. More recently, CCR4, a highly expressed chemokine receptor on ATL cells, has been targeted using the anti-CCR4 monoclonal antibody, mogamulizumab[55-57]. Mogamulizumab and lenalidomide, an immunomodulatory drug, have been approved for treatment of ATL in Japan[58-60].

HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis.

HTLV-1 is also the etiologic agent of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a disease impacting 0.25-3.8% of HTLV-1-infected individuals[61,62]. First described in 1969, HAM/TSP is a chronic, progressive neurodegenerative disorder of the central nervous system (CNS), which produces symptoms similar to those of multiple sclerosis (MS). HAM/TSP symptoms can vary

greatly, but the following five symptoms are typically present: lower limb stiffness and/or weakness, lumbar back pain, bladder dysfunction, bowel dysfunction and sexual dysfunction[63]. Mild cognitive impairment can also occur in HAM/TSP patients[64]. Symptoms of HAM/TSP usually begin during the fourth or fifth decade of life and 50% of inflicted individuals will become wheelchair-dependent within twenty years of symptom onset[65]. Individuals who are older at age of onset typically experience a more rapid progression of motor dysfunction[64,66].

Table 1.1. Classic characteristics of ATL.

	Acute	Lymphoma	Chronic	Smoldering
Circulating HTLV-1 antibodies	Yes	Yes	Yes	Yes
Circulating ATL cells	Yes	No	Yes	Yes
Lymphocyte count	Elevated	Normal	Elevated	Normal
Ca^{2+} level	High	High	Normal	Normal
Rash	Variable	Variable	Variable	Variable
Lymphadenopathy	Variable	Yes	Variable	No
Organomegaly	Variable	Variable	Mild	No
Skin and/or lung involvement	Variable	Variable	Variable	Variable
Bone marrow and/or spleen involvement	Variable	Variable	Variable	No
Bone, gastrointestinal and/or CNS involvement	Variable	Variable	No	No

Table 1.1 modified from previous studies [67-69]. Yes/No indicates whether or not a feature is characteristic of each subtype of ATL. Normal/Elevated/Low indicates the level of the indicated feature. Variable indicates that the presence/absence of a particular characteristic is not consistent between individuals with a particular ATL subtypes (*i.e.* variable between ATL cases of a particular subtype).

Individuals with HAM/TSP experience strong peripheral blood and CNS immune response to HTLV-1[70,71], with the proportion of HTLV-1-targeting cytotoxic T-lymphocytes (CTL) being higher in the cerebrospinal fluid (CSF) than peripheral blood mononuclear cells (PBMC) in HAM/TSP patients[71-74]. The immune response in HAM/TSP patients is severely skewed towards T helper type 1 cells (Th1), with infiltrating cells producing pro-inflammatory cytokines such as interferon- ɣ (IFN-ɣ), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)[75-78]. Furthermore, the infiltrating HTLV-1-infected T-cells express Th1 markers T-bet, INF-ɣ and the C-X-C motif receptor 3 (CXCR3)[79]. Together, this has led to the proposed model for HAM/TSP development in which CXCR3-expressing HTLV-1-infected T-cells cross the blood brain barrier, where they secrete IFN-ɣ. The secreted IFN-ɣ induces astrocytes to produce CXCL10, which induces migration of CXCR3+ inflammatory cells. Further production of IFN-ɣ by infiltrating cells continues the stimulation of astrocytes and continual infiltration of inflammatory cells into the CNS[80]. The resulting inflammatory response results in neuronal demyelination, known as “bystander damage”[81,82].

Other host immune factors, which are driven by genetics, are thought to impact the overall likelihood of HAM/TSP development. The specificity and efficiency of $CD8^+$ T-cells to HTLV-1-infected cells is determined by the HLA class 1 genotype of HTLV-1-infected individuals, which is turn determines how effectively the HTLV-1 proviral load is controlled. A significant reduction in proviral load and subsequent protective effects from HAM/TSP has been observed with the HLA class I genes, *HLA-A*01* (in Southern regions of Japan and Brazil) and *HLA-Cw*08* (in Southern regions of Japan)[83-85]. Intriguingly, stronger binding to an HBZ peptide was observed with *HLA-A*01* and *HLA-*

*Cw*08*, resulting in lower HTLV-1 proviral load and reduced HAM/TSP risk[86]. HLA alleles HLA-B*07 and HLA-B*5401 (HLA class I alleles) and HLA-DRB1*0101 (HLA class II allele) appear to be associated with increased susceptibility to HAM/TSP[83,84,87]. Furthermore, polymorphisms of various genes have been associated with altered risk of HAM/TSP development through analysis of single nucleotide polymorphisms (SNPs). Polymorphisms in the promoters of IL-10 (*IL10-592A),* stromal cell-derived factor 1 (SDF-1: *SDF1 + 801A*) and IL-15 (*IL-15 + 191C*) appear to be protective against HAM/TSP development, while polymorphisms in the promoter of IL-6 (IL6-634C) and TNF (TNF-863A) are detected at higher frequency in HAM/TSP patients[88-90].

Treatment options for HAM/TSP are limited. Due to a lack of effective treatment targeting the disease pathology, treatment protocols are typically directed at controlling pain, muscle spasms and urinary incontinence. Corticosteroid therapy is the most widely accepted HAM/TSP treatment; however, the benefits of this treatment are questionable[91]. Antiviral treatments, including combination therapies of the reverse transcriptase inhibitors zidovudine and lamivudine, have also been tested. However, these therapies did not reduce proviral load or improve symptoms[92,93]. Mogamulizumab, an anti-CCR4 antibody, has shown promise in the treatment of HAM/TSP. CD4+CCR4+ T-cells represent the main reservoir of HTLV-1[94]. In *in vitro* studies, mogamulizumab eradicated HTLV-1-infected T-cells in peripheral blood from HAM/TSP patients[79,95]. Furthermore, mogamulizumab has been found to reduce proviral load and improve neurological symptoms in patients with HAM/TSP in a recent clinical trial[96]. An additional, controlled trial focusing on mogamulizumab is currently underway in Japan (UMIN000019942).

Virion Structure.

HTLV-1 is an enveloped delta-retrovirus with a positive sense, single-stranded RNA genome. The HTLV-1 virion (**Figure 1.1**) is approximately 100nm in diameter and is composed of an outer envelope, which contains viral envelope protein (Env). Env contains two subunits: a transmembrane subunit (TM; gp21) and a surface subunit (SU; gp46). Within the envelope layer lies a layer of matrix protein (MA; p19) and the viral core. The viral core is composed of capsid protein (CA; p24) and contains two copies of the nucleocapsid protein (NC; p15)- studded 9-kb (+) ssRNA HTLV-1 genome, protease (PR), polymerase (Pol) and reverse transcriptase (RT).

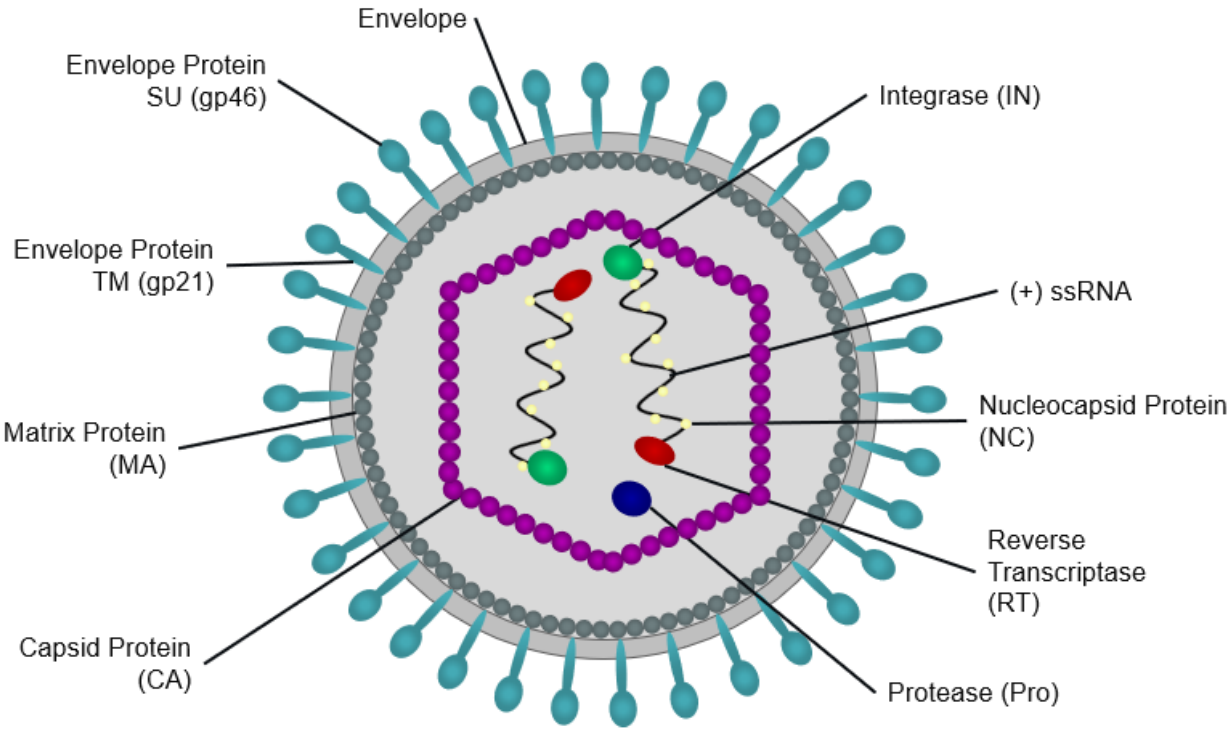

Figure 1.1. HTLV-1 virion structure. The HTLV-1 virion is composed of an envelope membrane, which contains viral envelope (Env) proteins. Env consists of a transmembrane component (TM) and surface subunit (SU). Beneath the envelope is a matrix (MA) layer and the viral core. The viral core is composed of capsid protein (CA), which contains two copies of the positive-sense (+) ssRNA viral genome, which is studded with nucleocapsid protein (NC), and the following viral enzymes: integrase (IN), reverse transcriptase (RT), and protease (PR).

HTLV-1 Cellular Tropism. HTLV-1 utilizes three receptors to infect cells: heparan sulfate proteoglycans (HSPG)[97,98], neuropilin-1 (NRP1)[99,100], and glucose transporter-1 (GLUT1)[101,102]. Due to the ubiquitous nature of these receptors, many different cell types are able to become infected by HTLV-1, including: CD4+ T-cells, CD8+ T-cells, endothelial cells, dendritic cells (DCs), B-cells, monocytes and macrophages[103-106]. However, HTLV-1 preferentially infects CD4+/CCR4+ cells and CD4+ T-cells account for over 90% of proviral load *in vivo*[107,108]. Interestingly, while CD4+ T-cells and CD8+ T-cells are similarly susceptible to HTLV-1, selective outgrowth of CD4+ T-cells, but not CD8+ T-cells, appears to contribute to the predominance of HTLV-1 infection of CD4+ T-cells[109].

The presence of an intragenic viral enhancer was recently discovered in the HTLV-1 provirus[110]. *In vitro* studies have demonstrated that this enhancer is important for chromatin openness, the induction of aberrant host gene transcription and regulation of viral gene transcription[110]. Studies using immortalized PBL cell lines established from HTLV-1 carrying the wild-type intragenic viral enhancer compared to a mutated intragenic viral enhancer have demonstrated that the wild-type form of this enhancer results in a T-cell population that is predominantly $CD3^+CD4^+$, rather than $CD3^+CD8^+$ as seen with cells infected by the virus containing the mutated enhancer, suggesting that this promoter contributes to the predominance of HTLV-1-infected $CD4^+$ T-cell *in vivo*[111].

De novo Infection and Persistence. HTLV-1 virions are rarely detectable in the serum of infected individuals[112] and only 1 in 10^5 viral particles produced by HTLV-1-infected lymphocytes are infectious[113]. There are multiple factors which appear to contribute to the negligible infectivity of cell-free HTLV-1 particles. Firstly, most viral particles

released into the supernatant of HTLV-1-infected cells have an incomplete capsid shell, indicating an issue with virion assembly[114]. Secondly, the infectious half-life of HTLV-1 virions at 37°C is approximately 36 minutes, which is notably lower than the 8.5 hour half-life of bovine leukemia virus at the same temperature[115]. The relatively low half-life of HTLV-1 is the result of the labile disulfide bonds between the SU and TM components of gp46[115].

Although HTLV-1 spread primarily occurs through cell-to-cell transmission, HTLV-1-infected T-cells and DCs can generate free virions[116,117]. Cell-free HTLV-1 virions can infect MDDCs and, to a lesser extent, T-cells, based on *in vitro* studies[117,118] **(Figure 1.2)**. Dendritic cells can either become productively infected by virions and infect other cells (cis-infection), or they can capture virions and transfer them to target cells (trans-infection)[119,120]. Given the primary routes of interpersonal HTLV-1 transmission in which epithelial barriers are exposed to HTLV-1, infection of dendritic cells has also been considered as an early step in infection. Although HTLV-1-infected T-cells are unable to cross the epithelial border or infect epithelial cells *in vitro*, epithelial cells are able to capture HTLV-1 virions from infected T-cells at their apical surface and release them at their basal surface through transcytosis[121]. Since free virions are rarely detected *in vivo*, this suggests that virions retained at the cell surface of T-cells may be moved across the epithelial layer through transcytosis, facilitating the infection of DCs, which are able to infect T-cells[117,119,120]. HTLV-1 infection may be spread from DCs to T-cells in secondary lymphoid organs, where surveilling T-cells actively interact with antigen presenting DCs. $CD4^+$ T-cells in secondary lymphoid tissues express higher levels of NRP1 than $CD4^+$ T-cells in peripheral blood[122]. Infected T-cells are then able to

transmit HTLV-1 to other T-cells[123]. Therefore, DCs are thought to represent an important HTLV-1 reservoir, promoting dissemination of HTLV-1 through clonally expanded CD4+ T-cells[119,124-126].

Transmission of HTLV-1 primarily occurs *via* cell-to-cell transmission through three non-exclusive mechanisms: the formation of virological synapses (VS), cellular conduits and transfer of the viral biofilm[127-130] **(Figure 1.2)**. The VS is formed when intercellular adhesion molecule-1 (ICAM-1) on the surface of an infected cell interacts with lymphocyte function associated antigen-1 (LFA-1) on a target cell. This interaction initiates a signaling event that results in polarization of the microtubule organization center (MTOC) towards the point of cell-cell contact and accumulation of HTLV-1 core protein complexes and genome at the point of contact[131,132]. The VS is reminiscent of an immunological synapse (IS), however, MTOC polarization during the IS occurs within a T-cell following interactions of the T-cell receptor with the major histocompatibility complex of an antigen-presenting cell[133]. Cellular conduits are filopodium-like protrusions that allow for transmission of HTLV-1 over a greater distance[129]. The viral biofilm is carbohydrate-rich, extracellular assembly composed of extracellular matrix proteins and linker proteins, including agrin, collagen, galectin-3, CD4, CD150 (also known as signaling lymphocytic activation molecule 1 [SLAMF1]), CD25 (also known as interleukin-2 receptor alpha chain [IL2RA]), CD70, and CD80[130,134]. It has been proposed that virions are retained within these assemblies, which are rapidly transferred to target cells upon contact[130]. Type IV collagen (COL4) expression specifically has been found to be activated in HTLV-1-infected T-cells. COL4 expression enhances HTLV-1 transmission through enhanced transfer of HTLV-1 virions from effector to

target cell, suggesting a model in which COL4 is important for tethering HTLV-1 virions to the cell surface, promoting efficient cell-to-cell transmission[135]. HTLV-1 persists throughout the lifetime of the host primarily through clonal expansion of infected T-cells, also known as mitotic viral spread[61,136]. However, a recent study determined that infectious viral spread through *de novo* infection is ongoing during HTLV-1 infection[137].

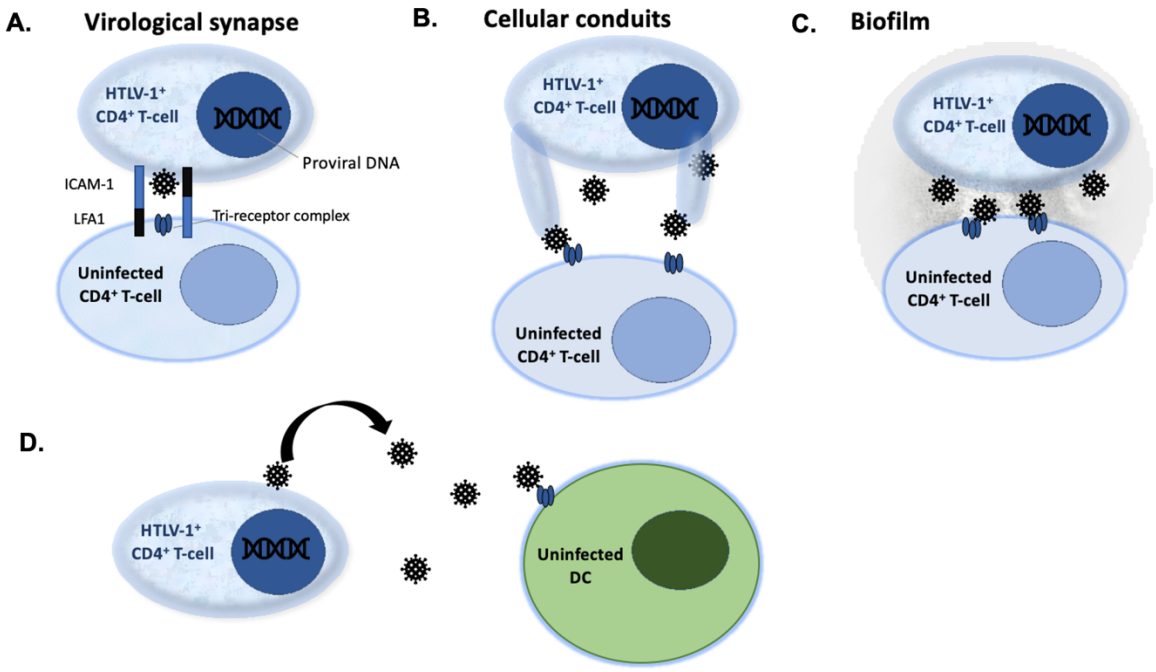

Figure 1.2. Mechanisms of HTLV-1 infectious spread. Infectious spread of HTLV-1 primarily occurs through cell-to-cell transmission between T-cells **(A-C)** or cell-free transmission to DCs **(D). (A)** The virological synapse (VS) is characterized by the interaction between ICAM-1 on an infected cell with LFA-1 on the surface of a target cell, creating a point of close cell-cell contact. The interaction between these receptors triggers polarization of the MTOC in the infected cell towards the VS followed by budding and release of viral particles. **(B)** Cellular conduits are filopodium-like projections that extend from an infected cell towards the target cell, allowing for transmission across a greater distance. **(C)** The carbohydrate-dense viral biofilm retains viral particles at the cell surface and potentially serves as a vehicle for viral particle transfer to target cells. **(D)** Cell-free HTLV-1 viral particles can productively infect MDDCs.

Virion Assembly, Budding and Maturation.

Given the limited research focusing on the generation of HTLV-1 virions, much of the current knowledge on retroviral assembly, budding and maturation is based on HIV-1 studies. However, it is known that HTLV-1 Rex exports viral mRNAs out of the nucleus through nuclear pore complexes (NPCs) and into the cytoplasm[138-141]. Following nuclear export, the Env precursor is synthesized in the endoplasmic reticulum and directed to the plasma membrane by the Golgi apparatus. During transport to the plasma membrane, host furin-like proteases cleave the envelope polyprotein precursor into SU (gp46) and TM (gp21)- the functional forms of the envelope glycoproteins. Covalent interactions maintain the association between the SU and TM subunits, which will be incorporated into the cell membrane[142].

The remaining steps required for the development of infectious retroviral particles are as follows[143]:

1) Assembly of Gag polyprotein at the plasma membrane, facilitating formation of the immature viral capsid. Interactions between Gag-retroviral RNA, Gag-Gag and Gag-membrane are essential for viral particle assembly and budding[144].
2) Budding of the immature capsid from the cell membrane, resulting in envelopment of the capsid. Unlike HIV-1, in which membrane binding of Gag is dependent upon phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2], there is no preferential binding of HTLV-1 Gag to PI(4,5)P2 for targeting of virion budding sites[145].
3) Release of the immature viral particle from the cell surface

4) Maturation of the viral particle. During virus budding and shortly after virus release, the viral protease is responsible for cleavage of Gag and Pol proteins, which is essential for virion maturation and the development of infectious particles[143,146,147].

HTLV-1 Receptors.

Three receptors are utilized by HTLV-1 to facilitate entry into target cells: heparan sulfate proteoglycans (HSPGs), neuropilin-1 (NRP1) and glucose transporter-1 (GLUT1)[148].

Heparan Sulfate Proteoglycans.

Heparan sulfate proteoglycans (HSPGs) are transmembrane proteins with negatively charged, long, linear heparan sulfate glycosaminoglycan chains covalently attached[149,150]. HSPGs have numerous capabilities, including functioning as: a receptor for proteases and protease inhibitors, a co-receptor for cell adhesion receptors and various tyrosine kinase-type growth factors and binding cytokines, chemokines, growth factors and morphogens to prevent proteolysis[149]. Interestingly, it has been demonstrated that HTLV-1 SU is able to bind to HSPGs, indicating that HSPGs function as a cellular attachment receptor for HTLV-1[97]. More recently, HSPGs have been demonstrated to be important for binding and internalization of HTLV-1 viral particles into CD4+ T-cells[98,151].

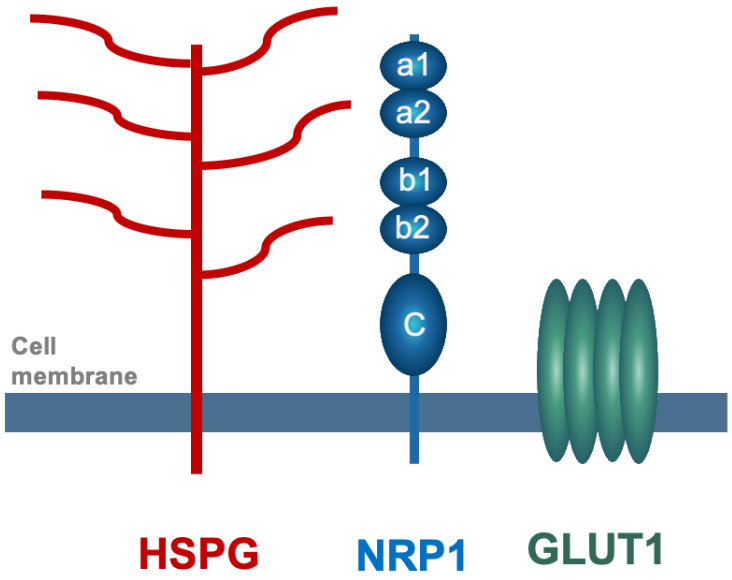

Figure 1.3. The HTLV-1 receptor complex. The HTLV-1 receptor complex includes HSPGs, NRP1 and GLUT-1. HSPGs are cell surface receptors composed of a core domain and long heparin glycosaminoglycan chains. NRP1 is a transmembrane glycoprotein composed of 5 extracellular domains. GLUT-1 is a multi-pass transmembrane protein.

Neuropilin-1.

The 120 kDa transmembrane glycoprotein, NRP1, is involved in a number of important cellular processes[152,153]. NRP1 is ubiquitously expressed in many cell types, particularly those within the central nervous system and vasculature systems[154,155]. Within the immune system, NRP1 expression is more restricted and tightly regulated and NRP1 is primarily expressed on DCs, monocytes, macrophages, natural killer cells and regulatory T-cells[156-161]. In regulatory T-cells (Tregs), which are important for maintaining immunological self-tolerance, NRP1 is involved in tolerance-mediated responses, transplantation acceptance, and driving tumor growth[162-165]. Overexpression of NRP1 on DCs and Tregs has been implicated in tumor development[165-167].

Structurally, NRP1 has five extracellular domains (a1, a2, b1, b2, and c), a transmembrane domain, and a short cytoplasmic domain[152] **(Figure 1.3)**. Independently, without the presence of a co-receptor, NRP1 is not known to possess intrinsic signaling capabilities, however, when functioning as a co-receptor, NRP1 is able to impart biological functions[153]. The most well studied NRP1 ligands are semaphorins and vascular endothelial growth factor (VEGF-A), which utilize plexin and VEGF receptor (VEGFR) as co-receptors, respectively[168,169].

NRP1 contains a short cytoplasmic domain which contains a conserved PDZ domain-binding (SEA) motif able to interact with PDZ containing proteins. Due to the small size of the NRP1 cytoplasmic domain, it was once thought that this domain was not involved in cell signaling[170]. However, it has since been indicated that the domain is important for signaling during two conditions in which NRP-1 functions as a co-receptor. Firstly, upon the interaction of the extracellular component of NRP1 with α5β1 integrin,

the NRP1 SEA motif is able to bind to synectin. Together, these interactions initiate a signaling event that results in the internalization of active α5β1 integrin in Ras analog in brain-5 (Rab5)-positive early endosomes[171]. Additionally, the interaction between the cytoplasmic domain of NRP1 and synectin also appear to be important for efficient complex formation between NRP1 and VEGFR-2[172].

The a1, a2 and b1 domains of NRP1 can interact with class II semaphorins (Sema3) and class IV semaphorins (Sema4)[170]. NRP1/Sema3 interactions are important for axonal guidance[173]. Sema4A/NRP1 interactions are involved in the stability and function of regulatory T-cells[163]. NRP1 has also been implicated in other immune processes, including the formation of the immunological synapse (IS). Interestingly, treatment of T-cells or DCs with anti-NRP1 antibody reduces duration of T-cell/DC contact, suggesting a role of homotypic NRP1 interactions between T-cells and DCs during the formation of the IS[174]. However, the specific NRP1 domain(s) involved in this process remains unknown[174].

Interactions between NRP1 and VEGF, mainly VEGF-A_{165}, involve vascular endothelial growth factor receptor (VEGFR)[153]. VEGF-A_{165} is secreted, rather than being sequestered in the extracellular matrix, and contains 7 coding exons (1-5, 7 and 8)[175]. VEGF-A_{165} interacts with b1 and b2 domains of NRP1[170]. The association of $VEGF_{165}$ with NRP1 can occur in either a HSPG-dependent or HSPG-independent manner[176-178]. In the presence of HSPG, the exon 7 domain of VEGF-A_{165} can bridge HSPG to the b domain of NRP1[178-180]. In the absence of HSPG, the exon 8 domain of VEGF-A_{165} facilitates binding of $VEGF_{165}$ to the b domain of NRP1.

In addition to functions in normal physiological processes, NRP1 also serves as a receptor for multiple viruses, including SARS-CoV-2 and HTLV-1[100,151,181,182]. Binding of the SARS-CoV-2 viral spike protein to the b1 domain of NRP1 induced a conformational change in the spike protein, facilitating cleavage of the spike protein by a furin protease into two polypeptides: Spike-1 (S1) and Spike-2 (S2)[183-185]. This process yields an Arg-Arg-Ala-Arg (RRAR) C-terminus sequence motif on S1 that conforms to the "C-end rule" (CendR)[186]. CendR facilitates internalization of particles displaying the C-terminal RRAR motif through NRP1-dependent endocytosis[186,187].

During HTLV-1 viral entry, HTLV-1 SU binds to the b domain of NRP1 through molecular mimicry of $VEGF_{165}$ exon 8[99]. Specifically, residues 85-94 and 304-312 of HTLV-1 SU bind to the b1 domain of NRP1[188]. Interestingly, the SU/NRP1 interaction is enhanced by HSPGs[99]. Unlike SARS-CoV-2, which exploits the CendR for NRP1-mediated endocytosis, HTLV-1 utilizes NRP1 as a binding factor and GLUT1 for internalization[99,189].

Glucose Transporter-1.

Glucose transporter-1 (GLUT-1) is a class I facultative glucose transporter composed of 12 hydrophobic transmembrane α-helices involved in the transport of glucose, galactose, mannose, glucosamine and ascorbic acid[190]. GLUT-1 a ubiquitous transporter and is expressed at very low levels in quiescent T-cells[191]. However, expression of this transporter is induced following activation of T-cells[191]. GLUT-1 was the first HTLV-1 receptor to be identified and is required for HTLV-1 infection of CD4+ T-cells[102,189,192]. Residues D106 and Y114 of SU have been demonstrated to be involved in GLUT-1 binding[192]. Based on retroviral studies, it's thought that the interaction

between HTLV-1 Env and GLUT1 initiates a conformational change in the SU-TM complex and that activates a fusion domain within the TM subunit, facilitating fusion of the viral and cell membranes[193-197].

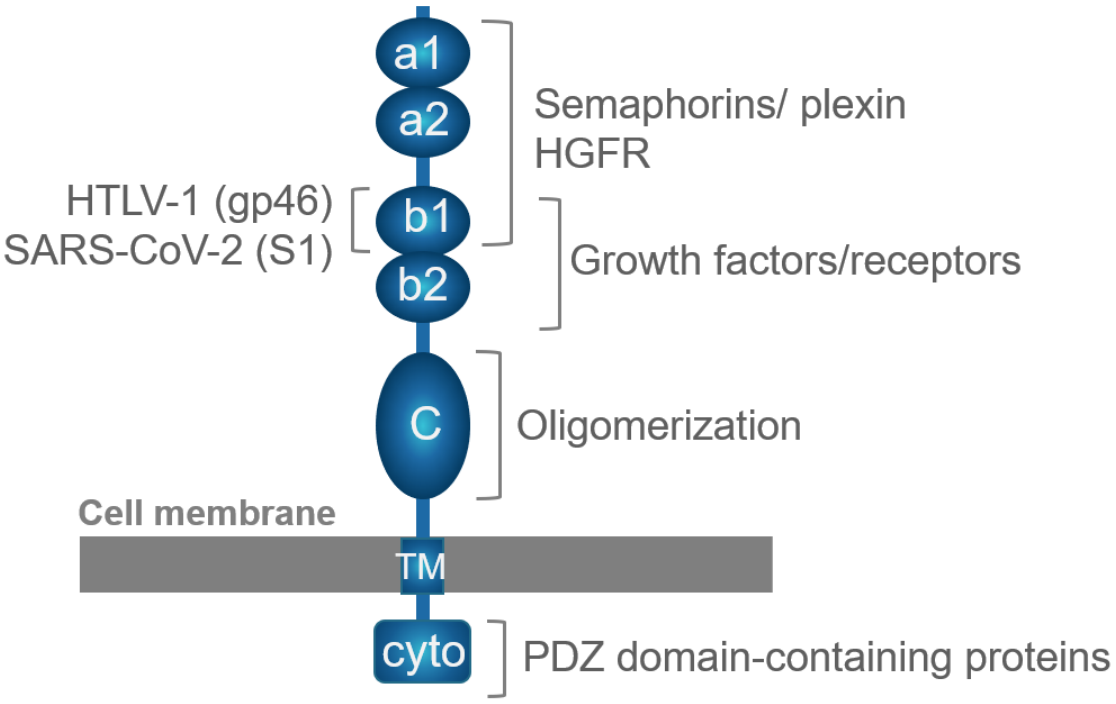

Figure 1.4. NRP1 structure and binding partners. NRP1 has 5 extracellular domains (a1, a2, b1, b2 and c), a transmembrane domain and a small cytoplasmic domain. Domains a1, a2 and b1 are important for binding of semaphorins, an interaction which utilizes plexin as a co-receptors. Domains b1 and b2 are important for binding of VEGF, which involves VEGFR as a co-receptor. The b1 domain also serves as a binding site for HTLV-1 gp46 and SARS-CoV-2 S1. The c domain is important for receptor oligomerization. NRP1 also contains a transmembrane (TM) domain and a cytoplasmic (ctyo) domain, which can interact with PDZ domain-containing proteins.

Retroviral Genome Integration.

After viral entry has occurred, the viral CA core, which contains two copies of the viral RNA genome, RT, IN, and PR is delivered to the cytoplasm where the positive sense ssRNA genome is reverse transcribed into a dsDNA intermediate by the viral reverse transcriptase and integrated into the host cell genome by the viral integrase[198]. Naturally infected T-cells typically contain one copy of the HTLV-1 provirus[199]. Viral integration was previously thought to occur at random sites within the host's genome, however, it is now known that integration is directed toward a nonpalindromic DNA motif[200] within transcriptionally active regions of the genome[201]. Furthermore, integration is more likely to occur near certain host genes, particularly *STAT1*, *HDAC6* and *TP53*[202]. HTLV-1 genome integration into acrocentric chromosomes 13, 14 and 15 is associated with improved survival of infected cells based on the frequency in which these integration sites were identified in HTLV-1-infected cells[199,203].

Following integration of the HTLV-1 genome into a host-cell chromosome, the HTLV-1 provirus is flanked by the 5' and 3' long terminal repeats (LTR) which contain bidirectional promoters to facilitate viral gene transcription[204-207] (**Figure 1.5**). Each LTR contains three regions: the unique 3' (U3), the repeated (R), and the unique 5' (U5)[208-210]. The U3 region of the 5' LTR contains a segment known as the Tax response element I (TRE-1)[204]. Although the TRE-1 contains three discontinuous base pair (bp) repeats, the middle TRE-1 repeat (TRE-1 II) is the most important for efficient transcription[204]. Each TRE-1 contains three conserved domains (A, B, and C)[209,211-213]. Of these conserved domains, the B domain is especially important for viral gene expression as it contains five of the eight base pairs that make up what is known as the

viral cAMP response element (vCRE), the location at which the HTLV-1 *trans*-activator protein (Tax) is able to initiate transcription from the viral promoter. Tax is able to utilize the B domain and either the A or C domain for transactivation[214].

Proviral Genome and Viral Proteins.

HTLV-1 is a complex retrovirus, indicating that it contains genes common to all retroviruses (*pro*, *pol*, *gag* and *env)* and genes encoding regulatory and accessory proteins within the 9 kilo base (kb) genome[215] **(Figure 1.5)**. The *pro* and *pol* genes encode the viral protease and reverse transcriptase, respectively. The *gag* gene encodes capsid, matrix and nucleocapsid (structural proteins). The *env* gene encodes the two components of the viral envelope protein: the transmembrane protein and surface glycoprotein.

Genes encoding regulatory and accessory proteins are located within the *pX* region, which has 6 open reading frames (ORF I-VI). p12, a protein which can be further processed to p8, is encoded in ORF-I[216]. p8 is important for infection as it has been observed to induce cellular conduit formation[127,129]. ORF-II encodes p13 and p30[216,217]. p8/12, p13 and p30 are expressed at low levels *in vivo* and have roles infectivity and persistence but are not essential for HTLV-1-infected cells[218-223].

Three alternatively spliced regulatory genes are located within the *pX* region: *rex* and *tax* on the plus-strand and *hbz* on the minus-strand[224]. ORF- III and ORF-IV encode Rex and Tax as the result of doubly spliced RNA[225]. *hbz*, the only viral gene in the antisense orientation, is transcribed from the 3' LTR and encodes HBZ[226-228].

HTLV-1 Trans-activator Protein.

Tax-Mediated Gene Expression and Oncogenesis.

Tax is a viral transcription factor that is indispensable for high-level expression of HTLV-1 genes[229-232]. Activation of the 5' LTR by Tax involves recruitment of cAMP-response element-binding protein (CREB) to the vCRE region of TRE-1. CREB can form homodimers or a CREB/ATF heterodimer through leucine zipper domain interactions[209,233,234]. Importantly, the phosphorylation of CREB at serine 133 enables its transcriptional activity[235]. The zinc-finger domain of Tax facilitates the formation of a Tax homodimer, which subsequently binds to the CREB dimer[209,233,234]. The Tax dimer associates with the G/C rich regions flanking the vCRE, stabilizing the complex, promoting enhanced binding specificity of CREB dimers and facilitating efficient proviral transcription from the 5' LTR[236-240].

Efficient sense viral transcription is also highly dependent upon CBP or p300 coactivator protein recruitment to the Tax/CREB complex[241-244]. CBP and p300 are paralogous proteins with several conserved domains, including: the SRC-interacting domain, two cysteine-histidine-rich domains (CH1 and CH3), and the KIX domain[245,246]. These domains serve as binding sites for a variety of cellular and viral transcription factors. Tax is able to interact with the CH1, the SRC-interacting and the KIX domains of CBP/p300 coactivators[241,247-251]. CBP/p300 coactivators are able to acetylate histone and non-histone substrates and promote chromatin remodeling to facilitate gene expression[252-255]. Nucleosome assembly protein 1 (NAP1), a histone chaperone molecule, has also been implicated in Tax-mediated chromatin remodeling[254].

Tax contributes to the oncogenesis of infected cells through various mechanisms. Tax activates the non-canonical NF-kB pathway, which contributes to cell survival and oncogenesis[255]. In addition to activating the NF-kB pathway, Tax interacts with host signaling proteins to facilitate persistent activation of this pathway[256-258]. However, hyper-activation of NF-kB signaling leads to cellular senescence[259,260]. Tax can also drive senescence through interactions with the deubiquitinase USP10 and subsequent reactive oxygen species (ROS) induction[261,262].

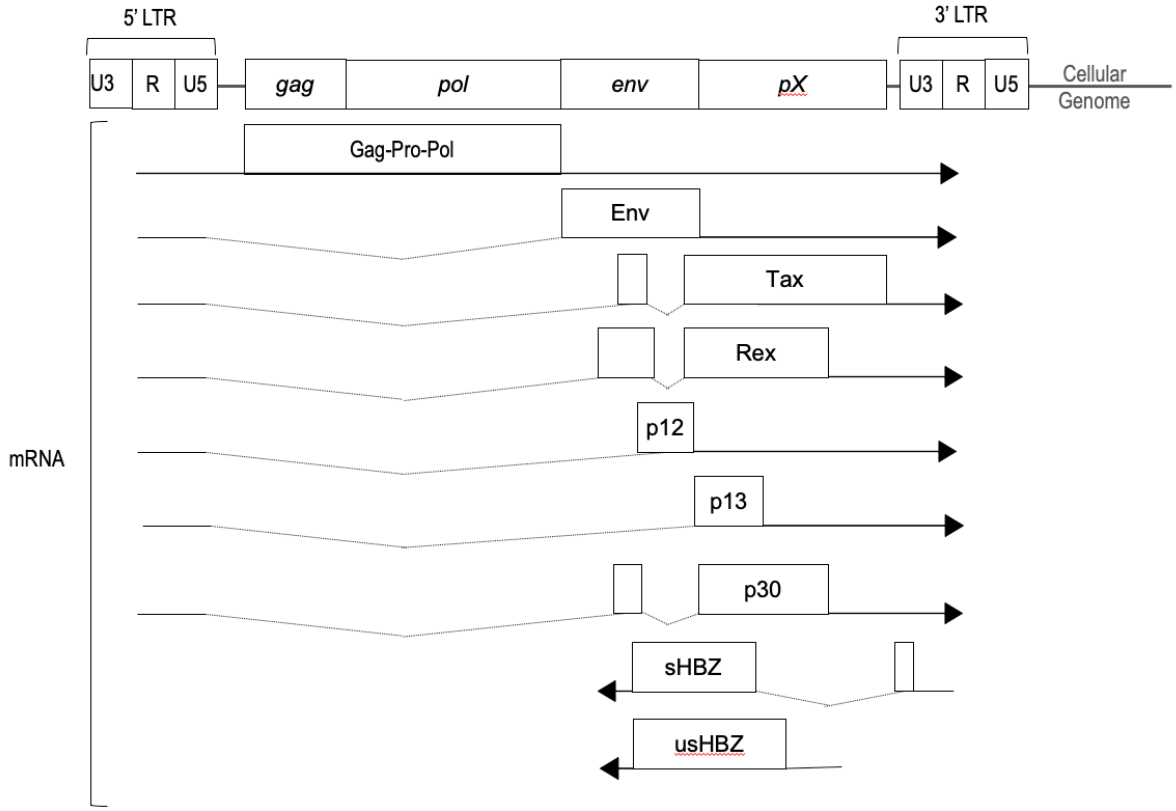

Figure 1.5. Schematic representation of the HTLV-1 provirus and mRNA transcripts. The integrated HTLV-1 genome is flanked by long terminal repeats (LTRs) at the 5' and 3' ends. The LTRs are the result of the integration process and contain untranslated regions (U3 and U5) and a repetitive region (R). Most viral genes, including tax, are expressed from the 5' LTR. Spliced HBZ (sHBZ) and unspliced HBZ (usHBZ) are expressed from the 3' LTR.

The Roles of Tax in Infectious Spread of HTLV-1.

Tax is a potent activator of numerous transcription pathways, affecting many cellular functions and products[248,263-270]. Tax is capable of deregulating over one hundred genes and is of particular importance in HTLV-1 pathogenesis, including cellular transformation and infection[271]. Tax has various functions that support spread of HTLV-1 infection, including *trans*-activation of proviral transcription through the 5' LTR promoter, which is required for virion production[229]. Tax also promotes infection by activating the signaling cascade that stimulates MTOC polarization during formation of the VS[272]. Cell-to-cell transmission of HTLV-1 through upregulation of components of the viral biofilm and other cellular factors would enhance infection, including: ICAM1, fascin, type IV collagen and gem (**Table 1.2)**

Tax is highly immunogenic and promotes an anti-HTLV-1 cytotoxic T-cell response[273-275]. Consequently, Tax expression is tightly controlled, resulting in intermittent expression throughout infection[276,277]. Tax expression is downregulated through deletion of the 5' LTR, methylation of the 5' LTR and non-sense mutations[278-284]. Interestingly, propagation of HTLV-1 continues throughout infection[201,285], even when Tax is not expressed[202], which may be supported by constitutive expression of HTLV-1 basic leucine zipper factor (HBZ)[286].

Table 1.2. Tax enhances infection through upregulation of certain cellular genes.

Cellular Gene	Protein Product	Proposed Role in Infection	Reference
CCL22	Chemokine ligand 22	Attraction of CCR4+ T-cells	107
COL4A1	Collagen 4 alpha 1	Biofilm component	130,135,287
COL4A2	Collagen 4 alpha 2	Biofilm component	130,135
FSCN-1	Fascin	Enhanced virion release and cell-to-cell transmission	288,289
LGALS3	Galectin-3	Biofilm component	130,290
GEM	GTP-binding mitogen-induced T-cell protein	Cytoskeleton remodeling	290,291
ICAM-1	Intracellular adhesion molecule-1	VS formation, MTOC polarization, and syncytium formation	292,293
TNFAIP2	M-sec	Enhanced membrane protrusions and Gag clustering	294
VCAM-1	Vascular cell adhesion molecule-1	Syncytium formation	295,296

HTLV-1 Basic Leucine Zipper Factor.

HBZ is constitutively expressed throughout HTLV-1 infection and impacts numerous cellular functions[297]. The *hbz* gene is transcribed from TATA-less bidirectional promoter in the 3' LTR that contains transcription start sites dispersed throughout the U5 and R regions of the 3' LTR[205,297]. Consistent with previous studies implicating specificity protein 1 (Sp1) in transcription from TATA-less promoters, Sp1 is important for *hbz* transcription[298,299]. Two isoforms of HBZ have been described: unspliced HBZ (usHBZ) and spliced HBZ (sHBZ) which are 209 and 206 amino acids (AA), respectively[205,300]. The AA sequences of the two HBZ isoforms only differ in a small region of the N-terminus and the domains involved in transcriptional regulation are highly conserved in both HBZ isoforms[205,300,301]. Spliced HBZ, the predominant isoform, has a longer half-life and more strongly suppresses Tax-mediated sense viral transcription[205,228,297,300].

HBZ protein contains an N-terminal activation domain (AD), two central basic regions, and a C-terminal basic leucine zipper domain (bZIP) (**Figure 1.6**)[226,227]. Based on the presence of nuclear localization signals within the basic regions of HBZ (two within the central domain and one within the bZIP domain) and the demonstrated presence of HBZ in the nucleus, HBZ is traditionally classified as a nuclear protein[226,302,303]. Due to the lack of a high-quality antibody targeting HBZ, HBZ localization has primarily been explored using cells transfected with tagged HBZ plasmids. Recent studies from one laboratory have indicated that HBZ is only present in the cytoplasm of asymptomatic carriers and HAM/TSP patients, while tumor cells from leukemic patients contain HBZ in the cytoplasm and the nucleus[304]. However, the HBZ

antibody generated and utilized by for the latter study has not been validated for immunofluorescence microscopy[305]. Furthermore, there is some discrepancy when comparing HBZ localization using GFP-tagged HBZ and the recently developed HBZ antibody (*i.e.* one study determined that HBZ is localized to the nucleus and the cytoplasm, while another study determined that HBZ is only localized to the nucleus)[305]. Given the complicating factor described above and the abundance of evidence supporting nuclear localization of HBZ, nuclear localization of HBZ is widely accepted.

Roles of HBZ in Gene Expression.

***Viral Gene Expression*.**

HBZ is able to downregulate sense proviral transcription through multiple mechanisms. The HBZ bZIP domain can interact with CREB to inhibit binding of CREB to vCREs[226,306]. The HBZ AD contains two LxxLL-like motifs, which interact directly with the kinase-inducible domain (KID) interacting domain (KIX) of CBP/p300[307]. Recruitment of CBP/p300 by HBZ through this interaction results in sequestration of these coactivators from Tax, ultimately down-regulating Tax-mediated proviral transcription **(Figure 1.7)**[306-308]. sHBZ represses Tax-mediated proviral transcription much more strongly than usHBZ[205]. sHBZ and Sp1, together, are able to have the opposite impact on the HBZ promoter, upregulating expression of HBZ **(Figure 1.7)**[205]. Furthermore, recent studies have demonstrated that HBZ RNA can also downregulate sense viral transcription by impacting the interaction of RNA polymerase II (RNAPII) with the 5' LTR through displacement the basal transcription initiator, TATA box-binding protein (TBP)[309].

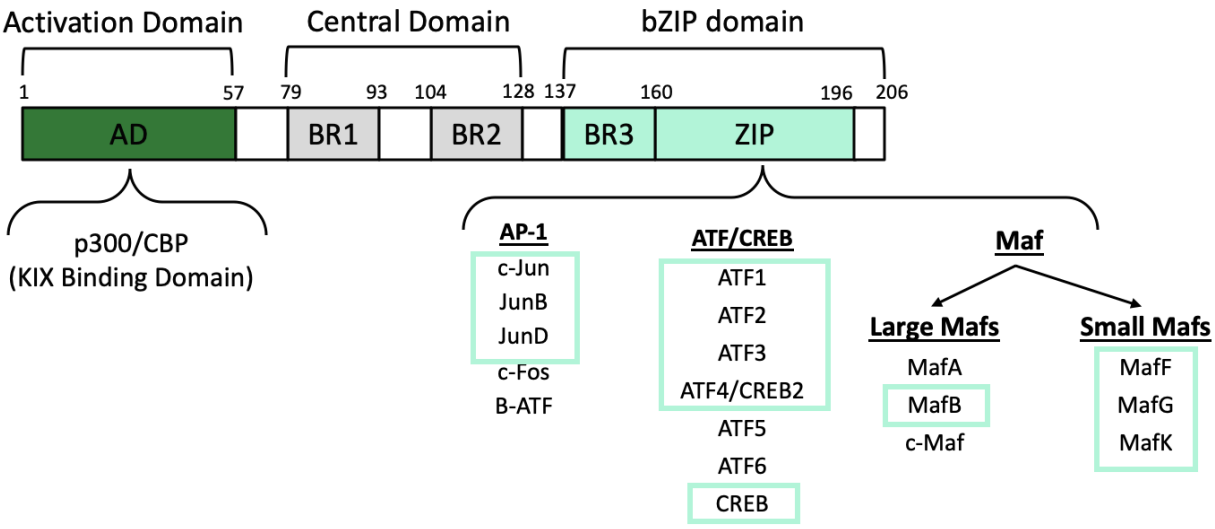

Figure 1.6. Schematic representation of the structure of HBZ and select HBZ binding partners. HBZ is a 206 amino acid protein that can be divided into three distinct domains: the activation domain (AD), the central domain, and the bZIP domain. The AD can interact with the KIX domain of p300/CBP. The central domain contains two basic regions and is involved in nuclear localization. The bZIP domain can interact with the HAT domain of p300/CBP and members of the AP-1, ATF/CREB, and Maf families of transcription factors (indicated by green boxes).

***Cellular Gene Expression*.**

HBZ can also interact with CBP/p300 to alter cellular gene expression. HBZ can bind to the HAT and C/H3 domains of CBP/p300, which results in a reduction in p53 acetylation and activity[310]. It has also been demonstrated that HBZ, through its N-terminal LxxLL-like motif, can form a ternary complex with p300 and SMAD family member 3 (SMAD3) to enhance activation of the TGF-β signaling, resulting in forkhead box P3 (Foxp3) expression[39]. Activation of TGF-β signaling and subsequent Foxp3 expression is advantageous for HTLV-1-infected T-cells as it promotes the conversion to regulatory T-cells, thereby promoting viral persistence[39].

The bZIP domain of bZIP transcription factors contains a basic region involved in DNA binding and a ZIP region that facilitates dimerization through coiled-coil interactions with similar domains of other bZIP factors. The basic region of HBZ, however, is atypical and lacks consensus amino acid motifs present in most bZIP factors that facilitate efficient DNA binding and DNA binding only occurs in rare situations. Although unable to form homodimers, HBZ can form heterodimers with certain members of the Jun, Maf and activating transcription factor (ATF)/CREB families of cellular bZIP factors **(Figure 1.6)**[311]. Interactions between c-Jun or JunB and unspliced HBZ often results in transcriptional repression through bZIP factor sequestration and proteosomal degradation, which effectively prevents c-Jun and JunB from activating transcription[302,311,312]. Recently, it was shown that HBZ splice variant 1 actually stabilizes c-Jun and JunB expression[313]. HBZ can activate gene expression by stimulating JunD-dependent transcription from an AP-1 consensus site[302]. HBZ-JunD heterodimers have also been shown to activate transcription of *hbz* and *hTERT* through

interactions with the *hbz* and *hTERT* promoters, respectively. This mechanism occurs through binding of an HBZ/JunD heterodimer to the transcription factor SP-1 when SP-1 is prebound to the DNA (**Figure 1.7**)[314-316]. HBZ can also heterodimerize with members of the Maf transcription factor family and ATF/CREB bZIP factors[226,306,311,317-319]. Although most of these interactions results in transcriptional repression, HBZ is able to activate transcription of certain genes. For example, HBZ can form dimers with small Mafs, which can bind MARE sequences within the *HMOX1* upstream enhancer site, resulting in activation of gene expression[317]. HBZ can also stimulate expression of the bZIP transcriptional factor, activating transcription factor (ATF)-like 3 (BATF3), which results in upregulation of BATF3 and subsequent regulation of downstream targets of BATF3[320]. Post-transcriptionally, HBZ impacts gene expression by altering mRNA splicing events[321,322].

It has been demonstrated that both HBZ mRNA and HBZ protein, the only viral protein constitutively expressed in ATL cells, are able to alter normal cellular functions[297]. HBZ RNA and protein can both induce C-C motif chemokine receptor 4 (CCR4), promoting T-cell migration and proliferation[323]. HBZ RNA and protein are also able to activate T-cell immunoreceptor with Ig and ITIM domains (TIGIT) expression, resulting in impaired anti-viral immunity[324]. However, the most well-known role of HBZ RNA and protein is in oncogenesis. HBZ RNA has been implicated in ATL as it promotes proliferation and inhibits apoptosis of T-cells[325]. Alternatively, HBZ protein increases apoptosis[297]. Further promoting oncogenesis, HBZ promotes genetic instability and interferes with normal DNA repair mechanisms[326,327]. Indeed, the bZIP domain of HBZ reduces non-homologous end joining repair of double-stranded DNA

breaks[326]. The oncogenic potential of HBZ has been further demonstrated using a mouse model, in which transgenic mice expressing HBZ in CD4+ T-cells developed T-cell lymphoma and inflammation[32].

The Roles of HBZ in Infectious Spread of HTLV-1.

Interestingly, *in vivo* analyses exploring the role of HBZ in infection using HTLV-1 molecular clones and rabbit model system identified a possible role of HBZ in infection[328]. The ability of HTLV-1 producer cell lines encoding wildtype HBZ, a severely truncated HBZ mutant or a C-terminal leucine zipper mutant to infect rabbits was assessed. Infection with virus from either HBZ mutant resulted in a significantly lower proviral load compared to infection from virus produced by wildtype HBZ-expressing cells. Furthermore, the similar trend exhibited from infection with virus from HBZ truncation mutant and leucine zipper mutant cells supports a potential role of the ZIP domain in HBZ-mediated enhancement of HTLV-1 infection[328]. A similar study was performed using rhesus macaques. Rhesus macaques were inoculated with HTLV-1 molecular clone-derived virus expressing an HBZ knockout through two point mutations. Interestingly, spontaneous partial or full seroconversion to wildtype was identified following inoculation[223]. Together, results of these *in vivo* studies support a potential role of HBZ in cell-to-cell transmission of HTLV-1.

Furthermore, our laboratory recently demonstrated *in vitro* that HBZ significantly enhances cell-to-cell transmission of HTLV-1[329]. We found that expression of HBZ in T-cells increases cellular aggregation, a phenotype attributed to elevated levels of ICAM-1 [329]. This is consistent with previous reports implicating ICAM-1 as an important adhesion molecule during formation of the virological synapse[292]. Additionally, we also

found that HBZ upregulates transcription of the *MYOF* gene, which encodes for myoferlin, a protein involved in vesicles trafficking[330]. Upregulation of myoferlin inhibits HTLV-1 envelope degradation and increases adhesion. Other genes reported to be involved in infection, including *NRP1*, *COL4A1* and *GEM* were also identified by our lab as potentially upregulated by HBZ. However, the possibility that *NRP1* could potentially be upregulated by HBZ was particularly intriguing as NRP1 is known to be important for infection of target cells. Until now a role for NRP1 on HTLV-1-infected cells has not been demonstrated. Given this, the focus of our study was to determine the implications of NRP1 expression on HTLV-1-infected T-cells, specifically in terms of viral infectivity.

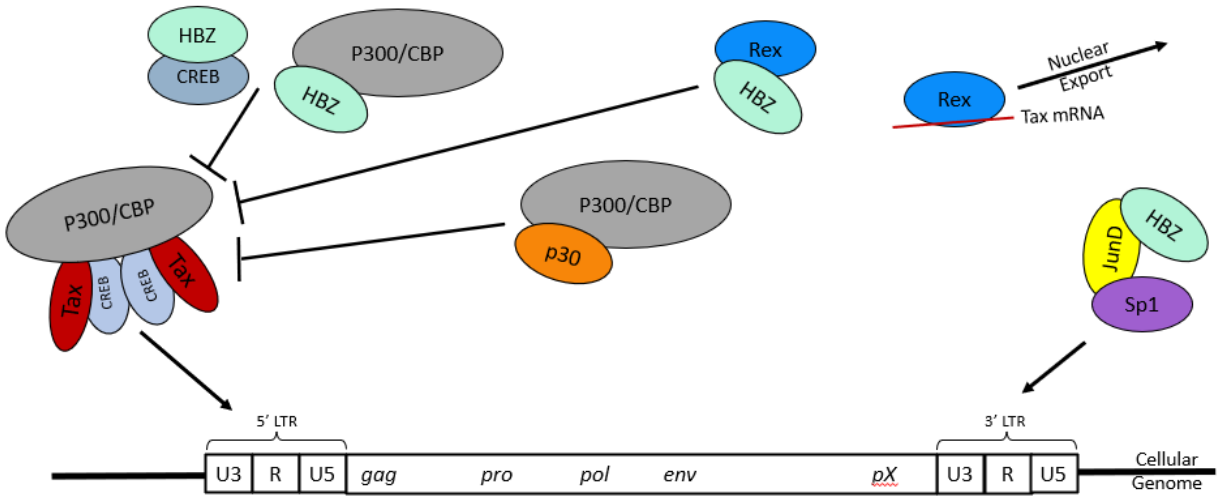

Figure 1.7. Schematic representation of HTLV-1 viral gene expression by Tax and HBZ. Tax forms complexes with CREC and p300/CBP, which can interact with the 5' LTR to activate sense viral transcription. HBZ can form a complex with Sp1 and JunD to activate antisense viral transcription from the 3' LTR. Tax mRNAs are exported from the nucleus by Rex. HBZ can down-regulate Tax-mediate proviral transcription by sequestering CREB, p300/CBP or Rex.

CHAPTER 2

Upregulation of Neuropilin-1 inhibits HTLV-1 infection.

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ABSTRACT

Infection with human T-cell leukemia virus type 1 (HTLV-1) can produce a spectrum of pathological effects ranging from inflammatory disorders to leukemia. *In vivo*, HTLV-1 predominantly infects $CD4^+$ T-cells. Infectious spread within this population involves the transfer of HTLV-1 virus particles from infected cells to target cells only upon cell-to-cell contact. The viral protein, HBZ, was found to enhance HTLV-1 infection through transcriptional activation of *ICAM1* and *MYOF*, two genes that facilitate viral infection. In this study, we found that HBZ upregulates transcription of *COL4A1*, *GEM* and *NRP1*. *COL4A1* and *GEM* are genes involved in viral infection, while *NRP1*, which encodes neuropilin-1 (Nrp1) serves as an HTLV-1 receptor on target cells but has no reported function on HTLV-1-infected cells. With a focus on Nrp1, cumulative results from chromatin immunoprecipitation assays and analyses of HBZ mutants support a model in which HBZ upregulates *NRP1* transcription by augmenting recruitment of Jun proteins to an enhancer downstream of the gene. Results from *in vitro* infection assays demonstrate that Nrp1 expressed on HTLV-1-infected cells inhibits viral infection. Nrp1 was found to be incorporated into HTLV-1 virions, and deletion of its ectodomain removed the inhibitory effect. These results suggest that inhibition of HTLV-1 infection by Nrp1 is caused by the ectodomain of Nrp1 extended from virus particles, which may inhibit binding of virus particles to target cells. While HBZ has been found to enhance HTLV-1 infection using cell-based models, there may be certain circumstances in which activation of Nrp1 expression negatively impacts viral infection, which is discussed.

INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is a complex retrovirus that primarily infects $CD4^+$ T-cells *in vivo*. Worldwide, 5-10 million people are estimated to be infected with HTLV-1 of which 5-10% will experience pathological effects associated with the viral infection[8,285]. Specifically, HTLV-1 is the etiologic agent of an often fatal form of leukemia designated adult T-cell leukemia (ATL) and, separately, a progressive inflammatory neurodegenerative disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)[80,331]. HTLV-1 infection is additionally associated with other inflammatory maladies that include infective dermatitis, uveitis, polymyositis, and Sjogren's syndrome[81].

Infectious spread of HTLV-1 within the T-cell population requires direct contact between HTLV-1-infected and target T-cells. Once cell-to-cell contact is established, virions are transferred to target cells through a virological synapse or through cellular conduits or from virions contained in an extracellular biofilm-like matrix that is released from the surface of the infected cell[127-130,289]. These infection mechanisms are not believed to be mutually exclusive. Subsequently, HTLV-1 virions bind to the cell surface and fuse with the plasma membrane through interactions of the surface unit (SU) of the HTLV-1 envelope protein with three receptors: heparin sulfate proteoglycans (HSPGs), neuropilin 1 (Nrp1) and glucose transporter 1 (Glut1)[97,100,189]. These receptors are believed to act in concert as a tri-receptor complex with HSPGs mediating initial virion attachment that, through interactions between HSPG and Nrp1, deliver the virion to Nrp1 to establish high-affinity binding. At this stage a conformational change in SU is believed to promote Glut1 binding, which induces fusion and entry[148].

Nrp1 displays a diverse array of coreceptor functions. It interacts with plexins to mediate class 3 Semaphorin signaling, which is involved in repulsive axon guidance[168,169]. It also functions as a coreceptor for multiple growth factor receptors involved in angiogenesis, specifically augmenting signaling activated by vascular endothelial growth factor 165, platelet-derived growth factor-B, hepatocyte growth factor and fibroblast growth factor[332-335]. In addition, Nrp1 enhances signaling through transforming growth factor β1 (TGF-β1)[336], which is associated with maintaining regulatory T-cells and, through a non-canonical signaling pathway, has also been implicated in cancer progression[362]. The composition of the extracellular region of Nrp1 is critical to the binding of this diverse set of signaling ligands. This region of the protein contains two tandem N-terminal CUB domains (a1 and a2) for Semaphorin binding followed by two tandem Factor V/VIII homology domains (b1 and b2) for growth factor binding followed by a membrane proximal MAM domain (c) that is proposed to position the other domains away from the membrane, allowing for an extended ectodomain[337,338].

The HTLV-1 protein, HTLV-1 basic leucine zipper (bZIP) factor (HBZ), regulates transcription through its capacity to interact with an array of cellular transcriptional regulators. HBZ contains an N-terminal activation domain with two LxxLL motifs that mediate high-affinity binding to the paralogous cellular coactivators p300 and CBP (interchangeably denoted p300/CBP)[307,308]. Within its C-terminal region, HBZ contains a leucine zipper (ZIP) domain that forms heterodimers with certain cellular bZIP factors, including Jun proteins, members of the maf family and certain members of the ATF/CREB family[226,302,306,311,317-319,339,340]. In addition, HBZ has been reported to interact

with other transcriptional regulators[341,342]. One consequence of its transcriptional regulator function is to activate cellular genes important for HTLV-1 infection. Specifically, HBZ upregulates expression of ICAM-1[329], which facilitates binding of infected cells to target cells and promotes formation of the virological synapse[131,289]. In addition, HBZ induces expression of myoferlin, which abrogates lysosomal-mediated degradation of the HTLV-1 envelope protein and promotes cell adhesion[330].

In this study, we provide evidence that HBZ increases expression of two other genes that contribute to HTLV-1 infection, *COL4A1* and *GEM*. Additionally, we found that HBZ increases expression of *NRP1*. While contributions of the former genes to viral infection have been investigated[135,291], no role for Nrp1 expressed on HTLV-1-infected cells has been reported, which prompted us to investigate Nrp1 further. We identified an enhancer downstream of the *NRP1* gene that was bound by HBZ, resulting in increased recruitment of Jun proteins and p300/CBP to this chromosomal region. Analysis of HBZ mutants and results from ChIP assays support a primary role for HBZ in increasing binding of Jun proteins to the enhancer. Unexpectedly, mutations in the LxxLL motifs of HBZ did not reduce *NRP1* transcription, indicating that HBZ is not directly involved in the recruitment of p300/CBP to this enhancer. In *in vitro* infection assays, Nrp1 inhibited infection, an effect that was associated with its incorporation into cell-free virions produced by HTLV-1-infected T-cell lines. Finally, using HEK293T cells, we found that inhibition of infection by Nrp1 was abolished by deletion of its extracellular domain, suggesting that the extended ectodomain of Nrp1 on HTLV-1 virions inhibits infection. Given the overall positive role of HBZ toward viral infection, we speculate that negative effects on infection caused by activation of Nrp1 might only arise during specific stages

of HTLV-1 pathogenesis, such as progression from an indolent to an aggressive form of ATL, which is discussed.

RESULTS

Genes involved in HTLV-1 infection are upregulated in HBZ-expressing cells. HBZ was shown to enhance HTLV-1 infection by activating transcription of *ICAM1* and *MYOF*[329,330]. Analysis of previous gene expression microarray data using HeLa cell clones lacking or expressing HBZ[343], revealed three additional genes potentially upregulated by HBZ that were previously reported to be involved in viral infection. These included *COL4A1*, *GEM*, and *NRP1*. *COL4A1* along with *COL4A2* encode collagen type IV alpha 1 and 2 chains, respectively, and are expressed in HTLV-1-infected T-cells[135]. COL4A1 and COL4A2 likely form the collagen matrix of biofilm-like viral assemblies on HTLV-1-infected cells that transfer virions to target cells during cell contact[130]. Gem is a GTP-binding protein with roles in signal transduction[344]. In HTLV-1-infected T-cells, Gem promotes formation of cell-to-cell conjugates, potentially increasing infection of target T-cells[291]. Lastly, *NRP1* encodes neuropilin-1 (Nrp1), which is the cellular receptor that interacts with the HTLV-1 envelope protein to facilitate stable binding of virions to target cells[99]. Using quantitative reverse transcriptase PCR (qRT-PCR), expression of all three genes was confirmed to be elevated in the HBZ-expressing cells (**Figures 2.1A, 2.1C and 2.1D**). Given that *COL4A1* and *COL4A2* are "head-to-head" genes, they are expected to share certain promoter elements, which led us to also analyze *COL4A2* expression. Expression of this gene was also slightly elevated in HBZ-expressing cells (**Figure 2.1B**). It is important to note that *COL4A1/A2*

expression is high in HeLa cells, which might partially mask levels of activation by HBZ. Regulation of *COL4A1* and *GEM* expression by HBZ was partly supported by *in silico* analysis of RNA-seq data from Nakagawa *et al.* who used CRISPR-Cas9 to disrupt the *hbz* gene in ATL cells[320] (**Supplemental Figure 1**). Furthermore, analysis of ChIP-seq data from the same study revealed peaks of HBZ-enrichment associated with each gene[320] (**Supplemental Figure 1**). The identification of upregulation of these genes is particularly interesting because HBZ generally downregulates expression of certain genes through sequestration of transcription factors[226,306,311,317-319].

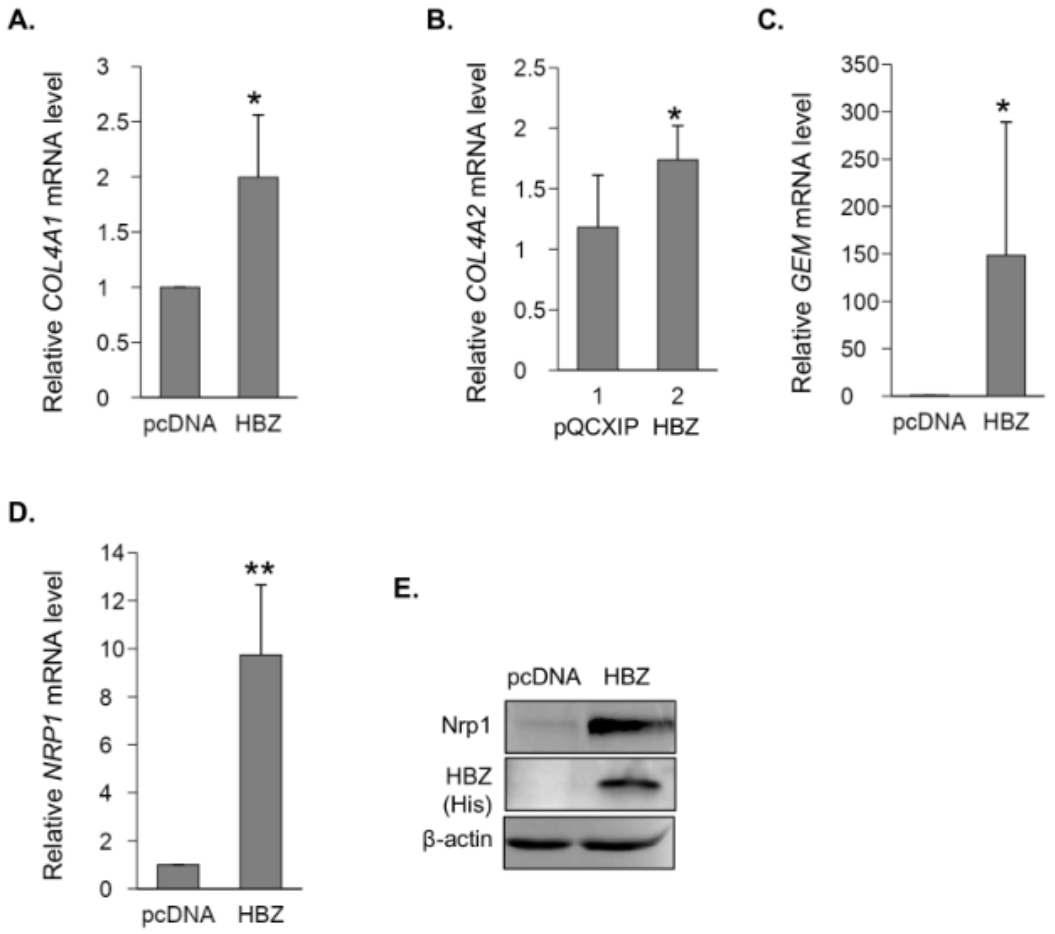

Figure 2.1. HBZ upregulates genes involved in HTLV-1 infection. (A) Relative *COL4A1* mRNA levels in HeLa clonal cell lines expressing wild-type HBZ (HBZ) or carrying an empty expression vector (pcDNA). The graph shows qRT-PCR results average from four independent experiments using one set of HeLa pcDNA and HeLa HBZ cells lines. **(B)** Relative *COL4A2* mRNA levels in HeLa cells transduced with lentiviral vectors to express wild-type HBZ (HBZ) or insert the empty expression vector (pQCXIP). The graph shows qRT-PCR results averaged from six independent transduction sets using one set of HeLa pcDNA and HeLa HBZ cells lines. **(C)** Relative *GEM* mRNA levels in HeLa clonal cell lines expressing wild-type HBZ (HBZ) or carrying an empty expression vector (pcDNA). The graph shows qRT-PCR results average from eight independent experiments using one set of HeLa pcDNA and HeLa HBZ cells lines. **(D)** Relative *NRP1* mRNA levels in HeLa clonal cell lines expressing wild-type HBZ (HBZ) or carrying an empty expression vector (pcDNA). The graph shows qRT-PCR results average from five independent experiments using one set of HeLa pcDNA and HeLa HBZ cells lines. For all graphs, HBZ values are normalized to that of the empty vector (set to 1), and error bars show standard deviations; *, $p<0.05$; **, $p<0.01$. **(E)** Nrp1 expression in empty vector (pcDNA) and HBZ-HeLa clones. Whole cell extracts

(40 μg for Nrp1 and β-actin, 75 μg for His) were analyzed by western blot using antibodies against Nrp1, HBZ (His) and β-actin. Credit: (A) (B) (C) (D) RNA, cDNA, qRT-PCR/data input - Kimson Hoang; Statistical analysis: Wesley Kendle; Graph creation: Wesley Kendle and Isabelle Lemasson. (B) Transduction: Nicholas Polakowski. (E) Western blot- Kimson Hoang.

Nrp1 expression is elevated in HTLV-1-infected T-cells lines and primary cells infected with HTLV-1. Roles for COL4A1, COL4A2 and Gem in HTLV-1 infection have been investigated. In contrast, in the context of expression by the infected cell, whether Nrp1 participates in viral infection is not known. This point led us to pursue further analyses of Nrp1. We first correlated higher mRNA levels with higher protein levels in HeLa cells expressing HBZ compared to cells carrying the empty vector (**Figure 2.1E**). HeLa cells, rather than HTLV-1-infected cells, were utilized to examine the relationship between HBZ and Nrp1 expression at the protein level because there currently isn't a quality HBZ antibody available and the use of HeLa cells expressing His-tagged HBZ allowed for identification of HBZ based on the presence of the His tag. In addition to HBZ, the HTLV-1 Tax protein is a transcriptional regulator[343]. However, unlike HBZ, expression of Tax in HeLa cells did not lead to an increase in the level of Nrp1 (**Supplemental Figure 2**). *In silico* analysis of the microarray data from the HBZ knockout cells of the Nakagawa *et al.* study[320] revealed reduced *NRP1* mRNA levels by each of the two *hbz*-targeted guide RNAs used in the two ATL cell lines tested (**Figure 2.2A).** Due to limitations of the data provided from the Nakagawa *et al* study[320] (ie. only relative expression values provided), statistical analysis could not be performed. Furthermore, sufficient control data was not available for these data, limiting the ability to draw conclusions from the data. Western blot analysis confirmed that Nrp1 is present in HTLV-1-infected T-cell lines, with the highest levels of the protein found in ATL-2 and MT-2 cells (**Figure 2.2B**). Importantly, flow cytometric analysis confirmed the presence of Nrp1 on the surface of infected cells (**Figure 2.2C**). qRT-PCR analysis also indicated that Nrp1 is expressed in recently established HTLV-1-immortalized clones from human

peripheral blood lymphocytes[111] (**Figure 2.2D**). Interestingly, some clones exhibited substantially higher levels of *NRP1* mRNA than ATL-2 cells. This comparison of recently established HTLV-1-immortilized clones and long-term HTLV-1 cell lines suggests that Nrp1 may be activated early in the course of infection, however, additional studies with appropriate statistical analysis are needed to further support these findings.

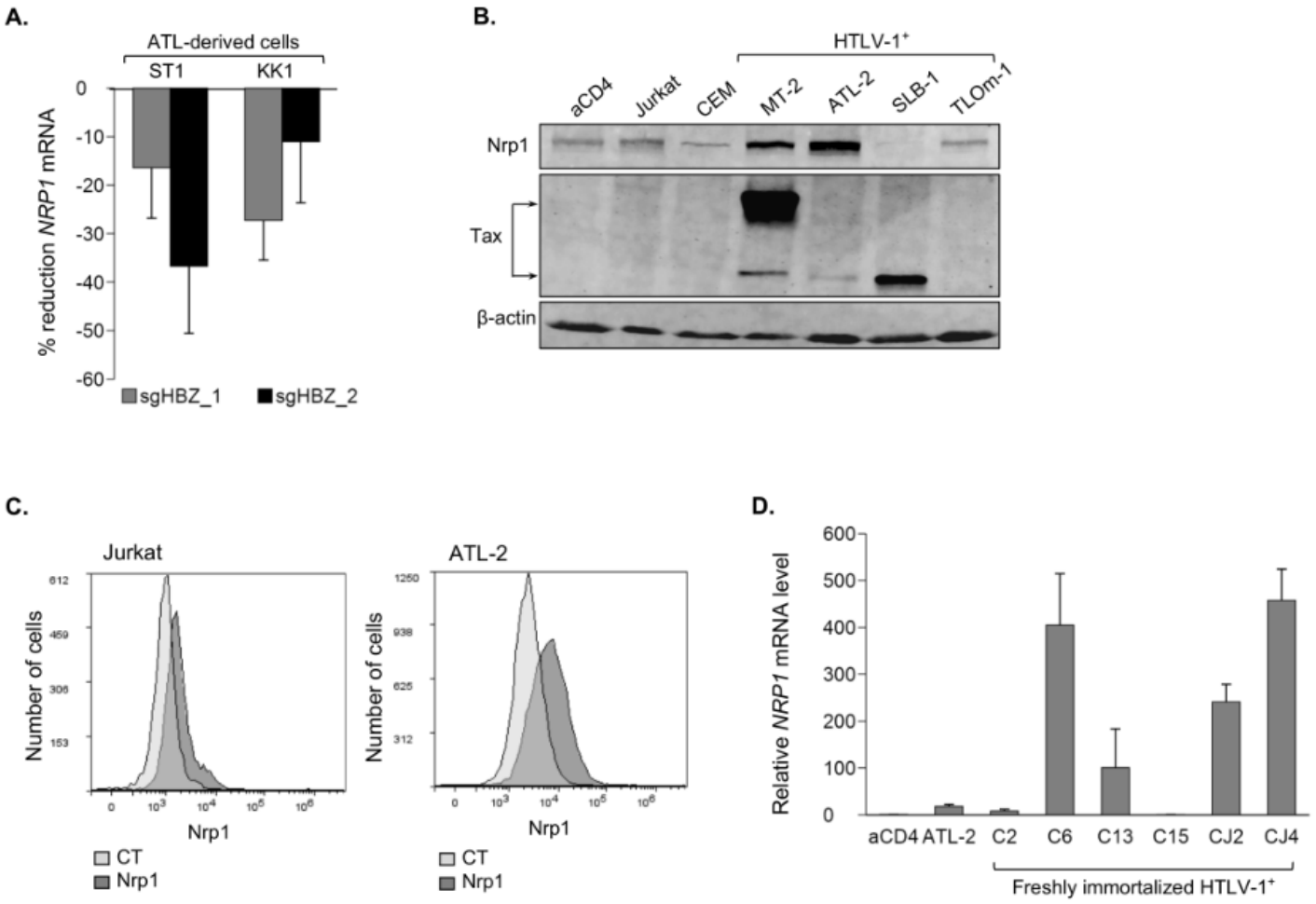

Figure 2.2. HBZ upregulates NRP1 expression. (A) Deletion of HBZ in ST1 and KK1 ATL-derived cells reduces *NRP1* expression. The graph was generated from published microarray data (GEO accession number GSE94409[320] and shows the percent reduction in *NRP1* transcript levels after inducing CRISPR/Cas9-mediated knockout of HBZ in the ATL-derived cell lines, ST1 and KK1, using two different guide RNAs (sgHBZ_1 and _2). Values are from day 8 post-induction except for sgHBZ_2 in KK1, which is the day 7 values (no day 8 data provided for this specimen). Data were obtained using GEO2R with calculations based on averaged values from the four array features probing for different regions of the *NRP1* transcript; **p*<0.05, ***p*<0.01. **(B)** Nrp1 expression in non-infected activated CD4$^+$ T-lymphocytes (aCD4) and T-cell lines. Whole cell extracts (45 µg for Nrp1 and β-actin, 50 µg for Tax) were analyzed by western blot using antibodies against Nrp1, Tax and β-actin. **(C)** Nrp1 expression on the cell surface of T-cell lines. Jurkat and ATL-2 cells were labeled with an Nrp1 antibody, fixed and analyzed by flow cytometry. Histograms are representative of three independent experiments and show relative cell surface labeling as follows: unlabeled

cells (CT, light grey) and Nrp1 antibody (dark gray). **(D)** Relative *NRP1* mRNA levels in HTLV-1-immortalized human T-cell clones recently established from peripheral blood lymphocytes (PBL). The graph shows qRT-PCR results averaged from three separate RNA extractions. Values were normalized to those for activated $CD4^+$ T-cells (set to 1). Error bars represent standard deviations. Credit: (A) *in situ* analysis (data collection and compilation): Wesley Kendle; graph creation: Wesley Kendle, Isabelle Lemasson (B) Western blot: Wesley Kendle (Nrp1 and β-actin) and Kimson Hoang (Tax). (C) Flow cytometry, data analysis, figure creation: Kimson Hoang and Isabelle Lemasson; (D) Cell lines provided by Amanda Panfil; RNA, cDNA, qRT-PCR/data input: Kimson Hoang; data compilation, statistical analysis: Wesley Kendle; Graph creation: Wesley Kendle, Isabelle Lemasson.

HBZ activates *NRP1* transcription from an enhancer downstream of the gene. To characterize the mechanism through which HBZ upregulates *NRP1* transcription, we first analyzed ChIP-seq data from the Nakagawa *et al.* study[320]. In the two cell lines examined in this study, a peak of HBZ-enrichment was identified approximately 50 kb downstream of *NRP1* (**Figure 2.3A** and data not shown). This site is also 176 kb upstream of *ITGB1* (**Supplemental Figure 3A**); however, expression of this gene is not affected by HBZ according to microarray data (data not shown). To test for HBZ-enrichment at this site in another HTLV-1-infected T-cell line, we performed ChIP assays using SLB-1 cells. SLB-1 cells were utilized as they express low levels of Nrp1 in hopes that the induction of additional HBZ expression via transduction with His-tagged HBZ would not induce Nrp1 expression to a toxic level in the cells. As antibodies against HBZ that are suitable for ChIP assays have not been developed, cells were transduced to express HBZ with a C-terminal 6xHis epitope tag for immunoprecipitation. Through this approach we observed significant enrichment of HBZ at the peak region identified in KK1 cells compared to a downstream off-target region (**Figure 2.3B**). These results support our analysis of data from the previous study, showing that HBZ is recruited to a chromosomal region downstream of the *NRP1* gene. A general analysis of this region using the UCSC genome browser revealed that it is denoted as an enhancer independent of HBZ and HTLV-1 infection[345,346]. Specifically, it shows hypersensitivity to DNase I and is flanked by peaks of histone H3 lysine 27 (H3K27ac; **Supplemental Figure 3**). Although our data suggests that the identified region approximately 50kB downstream of *NRP1* may serve as an *NRP1* enhancer based on recruitment of certain

transcription factors, including HBZ, further studies are needed to confirm that this region truly functions as an enhancer.

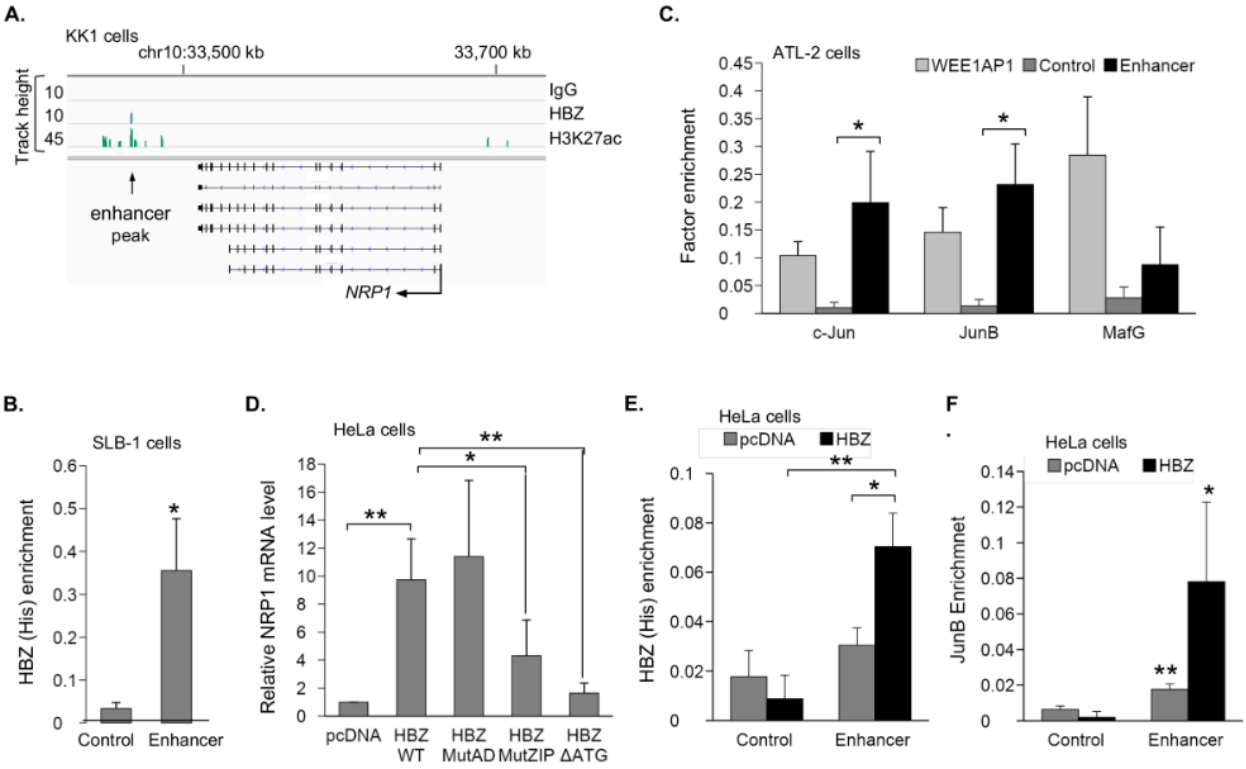

Figure 2.3. HBZ increases c-Jun and JunB recruitment to an enhancer downstream of the NRP1 gene. (A) HBZ associates with a chromosomal site (enhancer peak) approximately 200kb downstream of the *NRP1* transcription start site (indicated by the bent arrow). Peaks of enrichment for HBZ, H3K27ac and IgG (negative control) at the *NRP1* locus in KK1 cells are shown in the IGV Browser. Genomic coordinates are based on the NCBI36/hg18 assembly. Data were obtained from published ChIP-Seq data sets (GEO accession number GSE94732[320]. **(B)** HBZ binds to the enhancer region in SLB-1 cells. The graph shows levels of HBZ enrichment at the off-target control site and the enhancer region averaged from four independent ChIP assays using SLB-1 cells transduced to express HBZ with a C-terminal 6xHis tag. **(C)** c-Jun and JunB are enriched at the enhancer region in ATL-2 cells. The graph shows average levels of factor enrichment at the off-target control site, the enhancer region, and at the AP-1 site in the *WEE1* promoter (WEE1-AP1). Data are from four (c-Jun) and three (JunB and MafG) independent ChIP assays. **(D)** Relative *NRP1* mRNA levels in HeLa clonal cell lines expressing wild-type HBZ (HBZ-WT), the activation domain mutant (HBZ-MutAD), the leucine zipper domain mutant (HBZ-MutZIP), the translational-defective mutant (HBZ-ΔATG), or carrying the empty expression vector

(pcDNA). The graph shows qRT-PCR results average from five independent experiments, with values normalized to that for pcDNA (set to 1). **(E)** HBZ binds to the enhancer region in HeLa cells. The graph shows levels of HBZ enrichment at the off-target control site and the enhancer region averaged from three independent ChIP assays using HeLa cells expressing HBZ or carrying the empty vector (pcDNA). **(F)** JunB binds to the enhancer region in HeLa cells. The graph shows levels of HBZ enrichment at the off-target control site and the enhancer region averaged from three independent ChIP assays using HeLa cells expressing HBZ or carrying the empty vector (pcDNA). For all graphs, error bars show standard deviations; *, $p<0.05$, **, $p<0.01$. Credit: (A) Analysis and figure creation: Wesley Kendle; (B) ChIP method development / optimization and transduction: Nicholas Polakowski; ChIP: Wesley Kendle / Nick Polakowski; : RT-PCR/data input Kimson Hoang; Statistical analysis/graph generation: Wesley Kendle and Isabelle Lemasson (C) ChIP: Wesley Kendle; qRT-PCR/data input: Kimson Hoang; Statistical analysis/graph generation: Wesley Kendle and Isabelle Lemasson (D) RNA, cDNA, qRT-PCR/data input: Kimson Hoang and Erica Korleski; Statistical analysis and graph generation: Wesley Kendle and Isabelle Lemasson. (E) ChIP: Wesley Kendle / Nick Polakowski; qRT-PCR/data input: Kimson Hoang; Statistical analysis/graph generation: Wesley Kendle (F) ChIP: Wesley Kendle; qRT-PCR/data input: Kimson Hoang; Statistical analysis and graph generation: Wesley Kendle.

Recent evidence indicates that, through dimerization with small Mafs and Jun proteins, HBZ can associate with the DNA[311,317,330]. We therefore analyzed enrichment of these proteins at the downstream enhancer. ChIP analysis of ATL-2 cells revealed significant enrichment of c-Jun and JunB at this site compared to the off-target region (**Figure 2.3C**). Strikingly, the level of enrichment of these proteins at the enhancer matched that at the AP-1 site in the *WEE1* promoter, which served as the positive control. Consistent with these results, the DNA sequence of the enhancer region contained two full consensus AP-1 binding sites as well as several partial sites (**Supplemental Figure 3**). No significant enrichment was detected for the small Maf, MafG, at the enhancer (**Figure 2.3C**).

We then used HeLa clonal cell lines to expand on these observations. First, we compared *NRP1* mRNA levels in a clone expressing wild-type HBZ and two clones expressing mutant versions of the viral protein: HBZ-MutAD, which is defective for binding to p300/CBP, and HBZ-MutZIP, which is defective for binding to cellular bZIP factors[307,317]. In addition, we analyzed a start codon mutant (HBZΔATG) that is not translated into the viral protein[306]. Cell lines expressing either HBZ-MutZIP or HBZΔATG showed a significant reduction in *NRP1* mRNA levels compared to cells expressing wild-type HBZ, while no significant change was observed with HBZ-MutAD (**Figure 2.3D**). Using ChIP assays, we verified that HBZ was enriched at the *NRP1* enhancer in the HeLa cells expressing wild-type HBZ (**Figure 2.3E**). Lastly, using ChIP assays to compare the HeLa clones expressing HBZ and carrying the empty expression vector, we observed enrichment of JunB at the enhancer in the presence of HBZ

(**Figure 2.3F**). Together, these results indicate that HBZ activates *NRP1* transcription by forming heterodimers with Jun proteins on the enhancer.

Analysis of ChIP-seq data from the Nakagawa *et al.* study[320] also revealed peaks of H3K27ac at and around the *NRP1* enhancer (**Figure 2.3A**). This modification is generated by the KAT activity of p300 and CBP, suggesting the involvement of these coactivators in HBZ-mediated *NRP1* transcription. In the HeLa clones, ChIP assay results revealed that both p300 and CBP were enriched at the enhancer compared to at the off-target region with substantially greater coactivator enrichment in the presence of HBZ (**Figure 2.4A and 2.4B**). Consistent with this observation, siRNA-mediated knockdown of both coactivators reduced *NRP1* mRNA levels in both the HBZ-expressing and empty vector clones (**Figure 2.4C**). Knockdown of p300 and CBP was confirmed by western blot (**Figure 2.4D**). In ATL-2 cells, treatment with the p300/CBP KAT-specific inhibitor, A485, significantly reduced *NRP1* mRNA levels (**Figure 2.4E**). A similar effect of A485 was observed in a recently established HTLV-1-immortalized clone (**Figure 2.4F**). Together, these results indicate that HBZ upregulation of *NRP1* transcription is associated with enhanced recruitment of p300/CBP to the downstream enhancer.

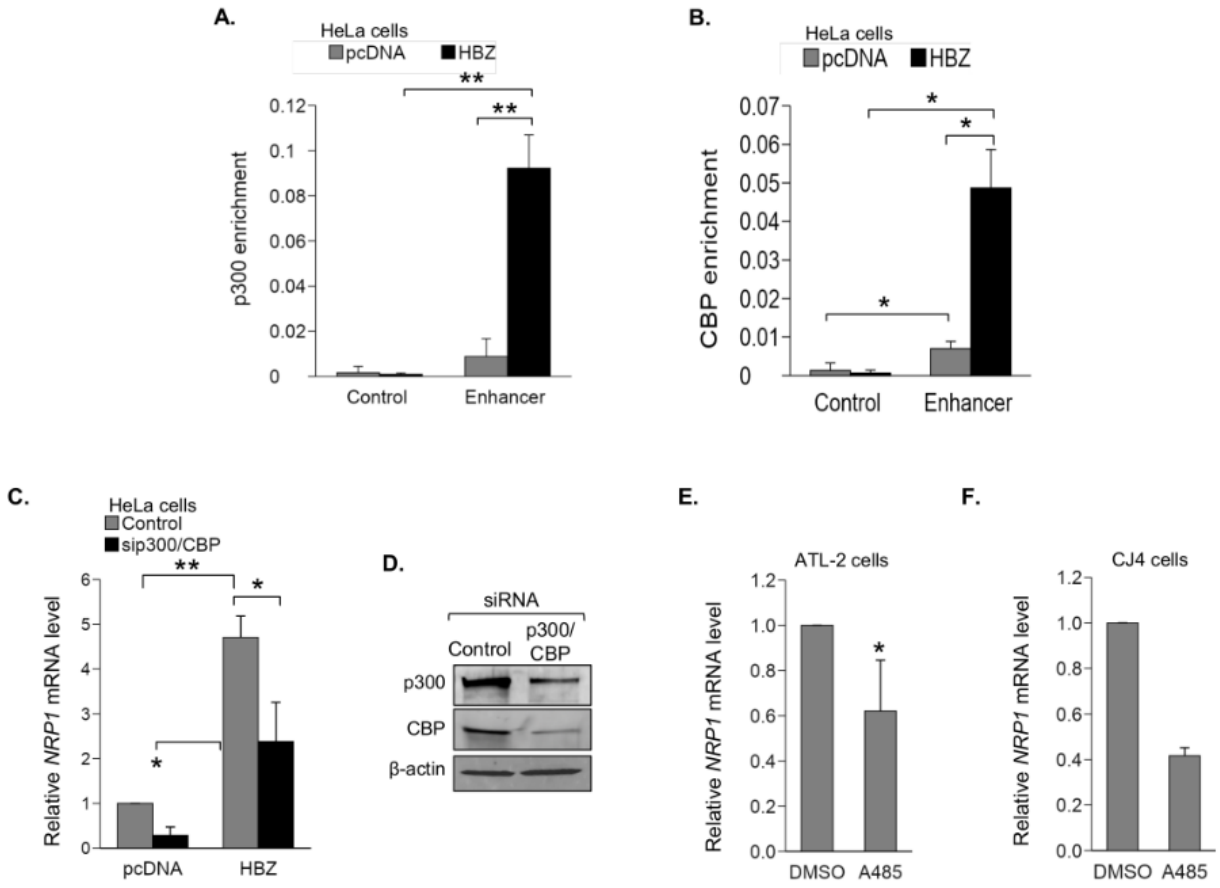

Figure 2.4. p300/CBP is recruited to the *NRP1* enhancer. p300 **(A)** and CBP **(B)** bind the *NRP1* enhancer region. Graphs show average values from three independent ChIP assays using empty vector (pcDNA) and HBZ-expressing HeLa cells. **(C)** siRNA-mediated depletion of p300 and CBP abrogates activation of *NRP1* transcription by HBZ. HeLa clonal cell lines expressing wild-type HBZ (HBZ) or carrying an empty expression vector (pcDNA) were transfected with an siRNA pool targeting p300 and CBP or a non-targeting siRNA pool (Control). The graph shows qRT-PCR results averaged from four independent transfection experiments with values normalized to those for the empty-vector clone (pcDNA) transfected with the non-targeting siRNA pool (set to 1). **(D)** siRNA-mediated depletion of p300 and CBP. HeLa cells were transfected with an siRNA pool targeting p300 and CBP (p300/CBP) or a non-targeting siRNA pool (Control). Whole cell extracts (15 µg for p300, 40 µg for CBP and β-actin) were analyzed by western blot using antibodies against p300, CBP and β-actin. Inhibition of p300/CBP KAT activity reduces *NRP1* transcription in **(E)** an HTLV-1-infected T-cell line (ATL-2) and **(F)** an HTLV-1-immortalized primary human T-cell clone (CJ4). Cells were treated with A485 (10 µM) or the carrier (DMSO) for 3h. Graphs show qRT-PCR results averaged from four (ATL-2 cells) and two (CJ4 cells) independent experiments with A485 values normalized to those for DMSO (set to 1). For all graphs, error bars show standard deviations; *, $p<0.05$; **, $p<0.01$. Credit: (A) ChIP: Nick Polakowski; qRT-

PCR/data input: Kimson Hoang; Statistical analysis and graph generation: Wesley Kendle; (B) ChIP: Nick Polakowski; qRT-PCR/data input: Kimson Hoang; Statistical analysis/Graph Generation: Wesley Kendle; (C) Transfection/RNA: Isabelle Lemasson; cDNA, qRT-PCR/data input: Kimson Hoang; Statistical analyis/graph generation: Wesley Kendle; (D) Whole cell extract: Isabelle Lemasson; Western blots: Wesley Kendle; (E) Cell treatment: Isabelle Lemasson; RNA, cDNA, qRT-PCR/data input: Kimson Hoang; Statistical analysis/graph generation: Wesley Kendle; (F) Cell treatment: Isabelle Lemasson; qRT-PCR/data input: Kimson Hoang; Statistical analysis/graph generation: Wesley Kendle.

Nrp1 expression in HTLV-1-infected T-cells inhibits HTLV-1 infection. We were interested in establishing whether Nrp1 expressed by HTLV-1-infected T-cells influenced HTLV-1 infection. To test this possibility, we first analyzed how knocking down Nrp1 expression in these cells influenced their ability to infect target reporter cells. In these experiments, we used MT-2 and ATL-2 cells based on their higher Nrp1 expression compared to SLB-1 cells. These two effector cell lines were transduced to express shRNA targeting the *NRP1* transcript (shNRP1) or, as a negative control, GFP (shGFP) and then co-cultured with Jurkat-pminLUC-vCRE reporter cells (**Figure 2.5A**). Following HTLV-1 infection, cells express the viral protein, Tax, which *trans*-activates the promoter driving luciferase expression in the Jurkat-pminLUC-vCRE cells[329]. C8166/45 cells were used as negative control effector cells, as they do not produce HTLV-1 virus particles due to defects in their proviruses[347]. In MT-2 cells, knockdown of Nrp1 led to a significant increase in HTLV-1 infection over that of shGFP-transduced cells (**Figure 2.5B**). Western blot analysis showed that levels of the HTLV-1 structural protein, $p19^{gag}$, and the HTLV-1 envelope surface unit (SU), gp46, were not affected by Nrp1 knockdown (**Figure 2.5C**). Furthermore, clarified culture supernatants contained similar levels of $p19^{gag}$, indicating that Nrp1 knockdown does not affect the production of HTLV-1 virus particles (**Figure 2.5D**). However, it's important to note that, while detecting p19 in the cell supernatant is widely accepted method to quantify viral particles, this method cannot differentiate between infectious and non-infectious virions. Furthermore, quantification of infectivity of cell-free virus from transduced HTLV-1-infected T-cells using cell-free infection assays is challenging due to insufficient

amounts of cell-free virus produced by these cells. Comparable results were obtained using transduced ATL-2 cells (**Figure 2.5E-5G**).

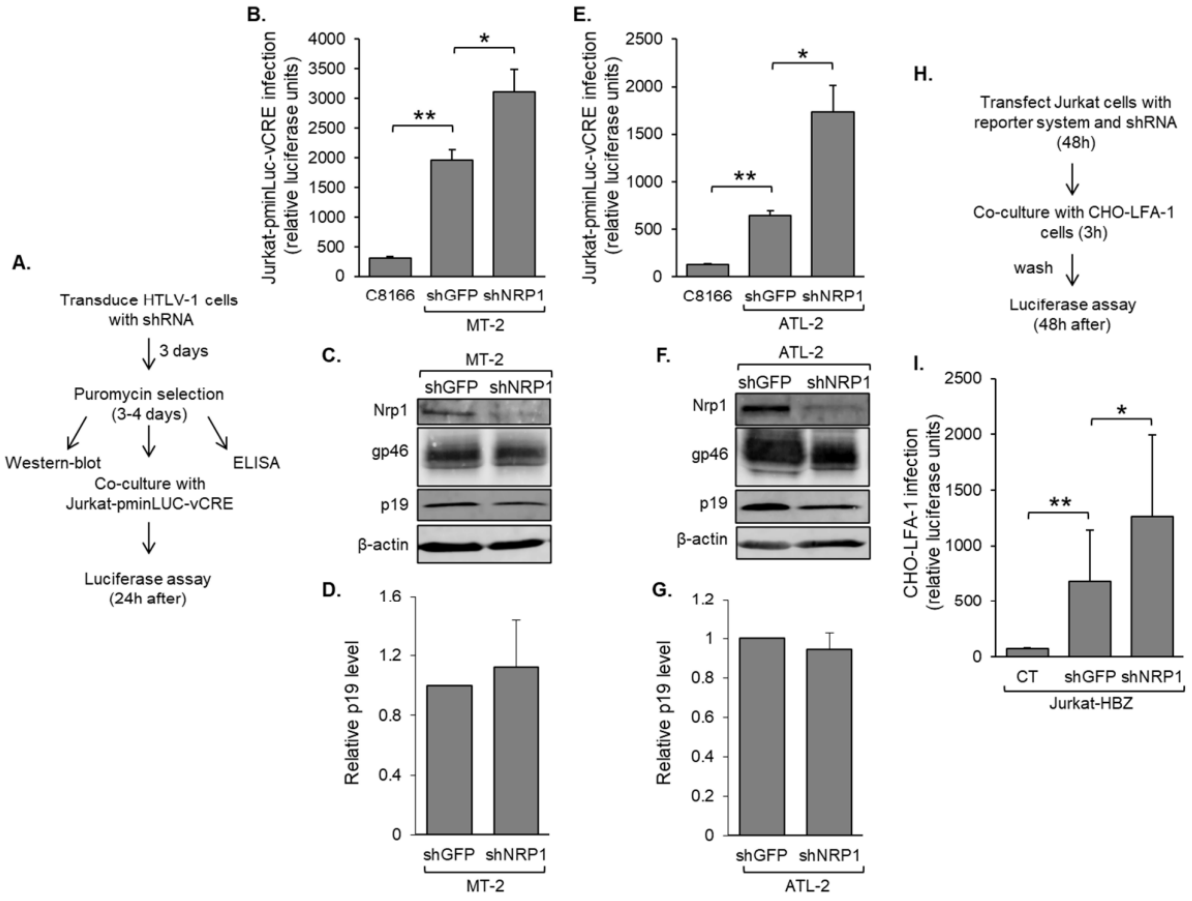

Figure 2.5. *NRP1* knock-down increases HTLV-1 infection. (A) The flow diagram shows the co-culture/infection assay procedure using HTLV-1-infected cells as donor cells and Jurkat-pminLUC-vCRE cells as target cells. **(B)** shRNA-mediated depletion of Nrp1 in MT-2 cells increases HTLV-1 infection. Jurkat-pminLUC-vCRE cells were co-cultured with MT-2 cells under puromycin selection following transduction with expression vectors for a negative control shRNA (shGFP) or an shRNA targeting the *NRP1* transcript (shNRP1), or co-cultured with non-infectious C8166/45 cells. The graph shows luciferase values averaged from three replicates of a single experiment and is representative of three independent experiments. **(C)** shRNA-mediated depletion of Nrp1 in MT-2 cells does not affect levels of gp46 (SU) and Gag p19. Whole cell extracts (50 μg for Nrp1 and β-actin; 15 μg for gp46 and Gag p19) were analyzed by western blot using antibodies against Nrp1, gp46, Gag p19 and β-actin. **(D)** shRNA-mediated depletion of Nrp1 in MT-2 cells does not affect levels of cell-free virus. Levels of Gag p19 in clarified culture media were measured by ELISA. The graph shows relative values averaged from two independent transduction experiments. **(E)** shRNA-mediated depletion of Nrp1 in ATL-2 cells increases HTLV-1 infection. Experiments were done as described in (B) above. The graph shows luciferase values averaged from three replicates of a single experiment and is representative of two independent experiments.

(F) shRNA-mediated depletion of Nrp1 in ATL-2 cells does not affect levels of gp46 (SU) and Gag p19. Western blots were done as described in (C) above. **(G)** shRNA-mediated depletion of Nrp1 in ATL-2 cells does not affect levels of cell-free virus. Experiments were done as described in (D) above. The graph shows values averaged from two independent transduction experiments. **(H)** The flow diagram shows the co-culture/infection assay procedure using Jurkat cells as donor cells and CHO-LFA-1 cells as target cells. **(I)** shRNA-mediated depletion of Nrp1 in Jurkat donor cells increases HTLV-1 infection. Jurkat cells were co-transfected with pcDNA3.1, pCRU5HT1-inLuc and pSG-Tax (no infection, CT) or pCMVHT1, pCRU5HT1-inLuc, pSG-Tax and the shGFP or shNRP1 vector, and cocultured with CHO-LFA1 cells. The graph shows luciferase values averaged from replicates from three independent experiments. For all graphs, error bars show standard deviations; *, $p<0.05$, **, $p<0.01$. Credit: (A) Method development and optimization (B) (E) Cell expansion, transfection, transduction, antibiotic selection, infection assay, cell lysate collection, Bradford assay, luciferase assay, graph generation, statistical analysis: Wesley Kendle; Graph modification: Isabelle Lemasson. optimization (C) (F) Cell expansion, transfection, transduction, antibiotic selection, whole cell extracts, Western blots, figure generation: Wesley Kendle; (D) (G) Cell expansion, transfection, transduction, antibiotic selection, supernatant collection, ELISA, graph generation, statistical analysis: Wesley Kendle; Graph modification: Isabelle Lemasson.

Additionally, we analyzed Jurkat cells stably expressing HBZ (Jurkat-HBZ) as effector cells using a single-cycle, replication-dependent luciferase infection assay[272]. We used these cells based on the strong luciferase signal they generate in target cells[329]. For these assays, Jurkat-HBZ cells were co-transfected with an HTLV-1 packaging vector (pCMVHT1M), the replication-dependent HTLV-1 reporter vector (pCRU5HT1-inLuc), and to increase infection efficiency, a Tax expression vector. To examine effects of Nrp1 in this system, cells were additionally co-transfected with the shRNA expression vectors targeting *NRP1* or GFP transcripts. Transfected cells were co-cultured with adherent CHO-LFA-1 target cells and then removed, and luciferase activity was measured in the CHO-LFA-1 cells (**Figure 2.5H**). As target cells, CHO-LFA-1 express lymphocyte function-associated antigen 1 (LFA-1), which binds ICAM-1 on effector cells to stabilize cell-cell contact and induces formation of a virological synapse from which infection occurs[127]. With this approach, we observed that Jurkat-HBZ cells co-transfected with the shRNA vector targeting *NRP1* produced a higher level of infection than cells co-transfected with the shGFP control vector (**Figure 2.5I**).

We performed reciprocal experiments using SLB-1 cells, which display low Nrp1 expression compared to ATL-2 and MT-2 cells. Cells were transduced with an Nrp1 expression vector or the empty expression vector and then co-cultured with Jurkat-pminLUC-vCRE reporter cells (**Figure 2.6A**). Western blot results confirmed higher Nrp1 expression in the cells transduced with the Nrp1 expression vector compared to those transduced with the empty vector (**Figure 2.6B**). Consistent with the knockdown experiments, $p19^{gag}$ and gp46 were not affected by the variations in the level of Nrp1. However, higher Nrp1 expression was associated with a significant decrease in HTLV-1

infection (**Figure 2.6C**). These and the Nrp1 knockdown results indicate that Nrp1 expressed by effector cells has an inhibitory role in HTLV-1 infection.

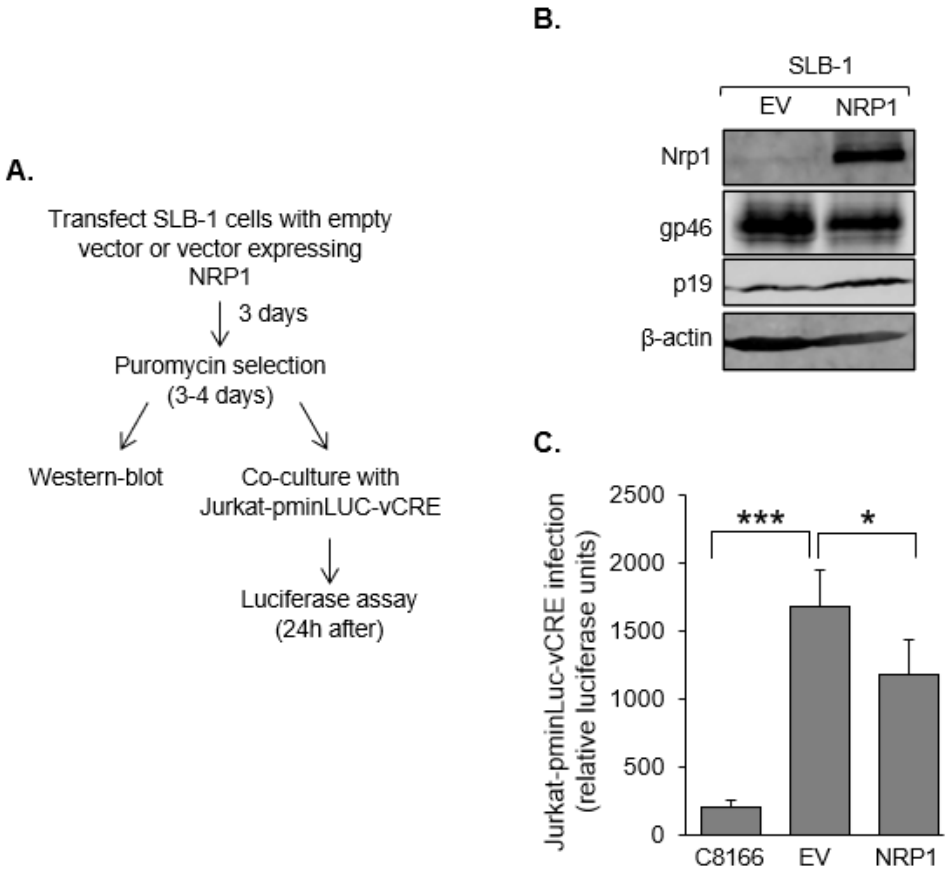

Figure 2.6. Overexpression of Nrp1 reduces infection. (A) The flow diagram shows the co-culture/infection assay procedure using HTLV-1-infected SLB-1 cells as donor cells and Jurkat-pminLUC-vCRE cells as target cells. SLB-1 cells were transduced with pLJM1-NRP1 (NRP1) or the pLJM1 empty vector (EV) and place under puromycin selection. **(B)** Nrp1 expression in transduced SLB-1 cells. Whole cell extracts (50 µg for Nrp1, Gag p19 and β-actin; 15 µg for gp46) were analyzed by western blot using antibodies against Nrp1, gp46, Gag p19 and β-actin. **(C)** Increased expression of Nrp1 in SLB-1 cells decreases HTLV-1 infection. Jurkat-pminLUC-vCRE cells were co-cultured with SLB-1 cells transduced with pLJM1-NRP1 (NRP1) or the pLJM1 empty vector (EV), or co-cultured with non-infectious C8166/45 cells. The graph shows luciferase values averaged from three replicates of each infection condition from a single experiment and is representative of three independent experiments. Error bars show standard deviations; *, $p<0.05$, ***, $p<0.001$). Credit: (A) Method development and optimization: Wesley Kendle; (B) Cell expansion, transfection, transduction, antibiotic selection, whole cell extracts, Western blots, figure generation: Wesley Kendle; (C) Cell expansion, transfection, transduction, antibiotic selection, infection assay, cell lysate collection, Bradford assay, luciferase assay, graph generation, statistical analysis: Wesley Kendle.

Nrp1 is incorporated into the viral particle. Consistent with our findings, a recent study demonstrated that, when expressed in effector cells, Nrp1 inhibits HIV infection[348]. This effect was found to be due to incorporation of Nrp1 into HIV virions, which led us to test whether Nrp1 is similarly incorporated into HTLV-1 virions. Western blot analysis revealed the possible presence of Nrp1 in cell-free HTLV-1 virions isolated by ultracentrifugation (**Figure 2.7A**). However, the ultracentrifugation method utilized cannot differentiate between protein associated with viral particles or exosomes. Given the extended extracellular structure of Nrp1 and its heavily glycosylated state, the authors of the previous study proposed that Nrp1 may sterically disrupt binding of HIV virions to target cells. To address this hypothesis in the context of HTLV-1, we analyzed an Nrp1 deletion mutant lacking most of its extracellular region (**Figure 2.7B and E**). HEK293T cells were transfected with the set of single-cycle, replication-dependent luciferase infection assay plasmids and co-transfected with an expression vector for full-length Nrp1 or the deletion mutant (**Figure 2.7C**). Subsequent analysis of luciferase activity from the cultures revealed a significant decrease in infection in cultures with ectopic expression of full-length Nrp1, while cultures with the deletion mutant showed no change in infection (**Figure 2.7D**). These results show that the extracellular region of Nrp1 is important for impairing infection and may be due to occlusion of virion-target cell interactions. However, these experiments could be better optimized to mitigate any potential impacts based on differential protein expression levels between the wild-type Nr1 and the mutant Nrp1.

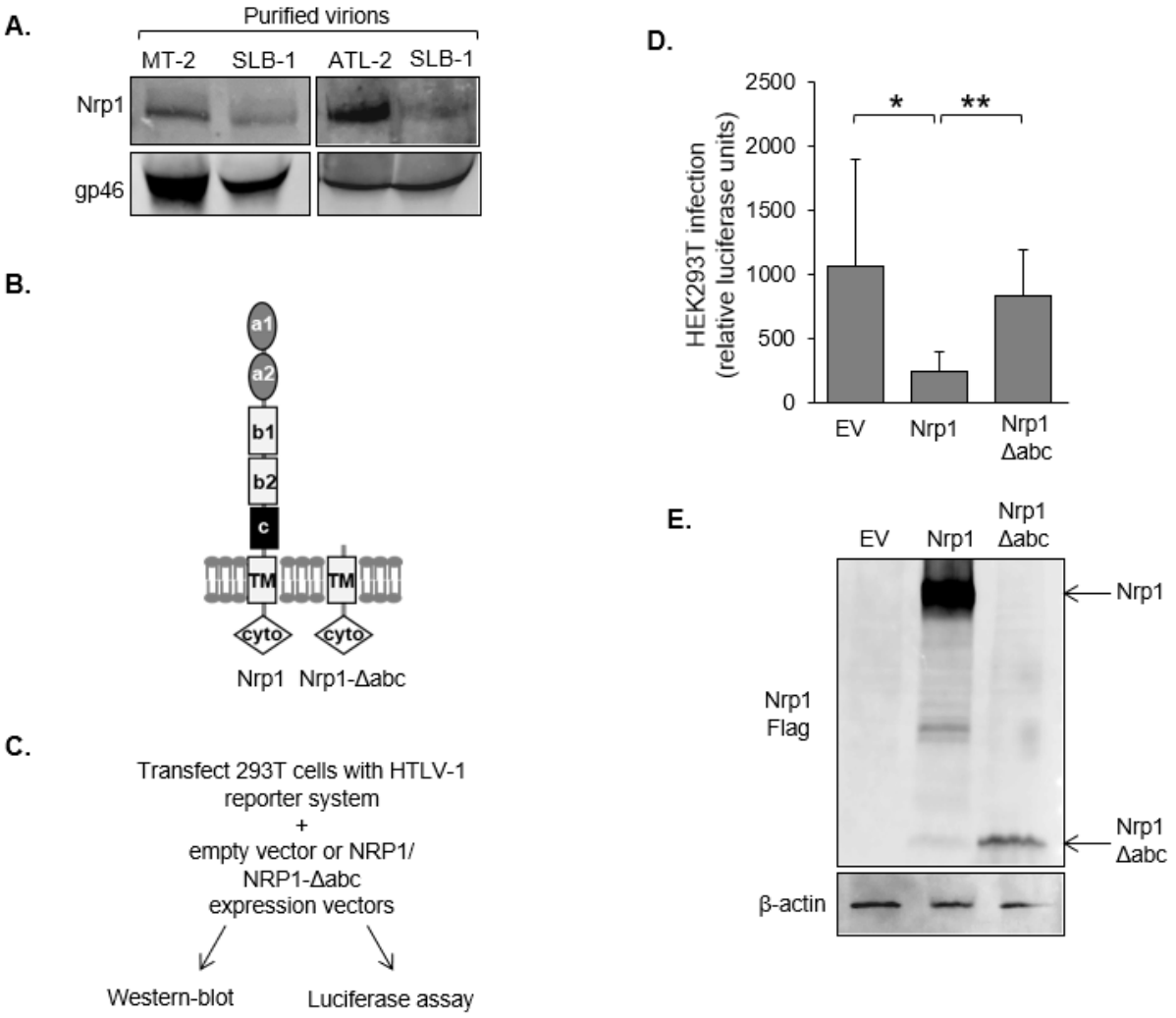

Figure 2.7. The ectodomain of Nrp1 is responsible for inhibition of HTLV-1 infection. (A) Nrp1 is incorporated into HTLV-1 virus particles. Culture media from MT-2, SLB-1 and ATL-2 cells were filtered, ultracentrifuged and analyzed by western blot using antibodies against Nrp1 and gp46. **(B)** The schematic shows full-length Nrp1 and the truncation mutant, Nrp1-Δabc. **(C)** The flow diagram shows the co-culture/infection assay procedure using HEK293T cells. **(D)** HTLV-1 infection is not inhibited by a Nrp1 truncation mutant lacking the ectodomain. HEK293T cells were co-transfected with pCMVHT1M, pCRU5HT1-inLuc and pQCXIP (EV), pQCXIP-NRP1 or pQCXIP-NRP1-Δabc. Luciferase assays were performed 48 h later. The graph shows luciferase values averaged from three independent experiments each performed in triplicate. Error bars show standard deviations; *, $p<0.05$; **, $p<0.01$. **(E)** Nrp1 expression in transfected HEK293T cells. Whole cell extracts (50 µg) were analyzed by western blot using antibodies against Nrp1 (Flag-tagged) and β-actin. Credit: (A) Method development and optimization: Wesley Kendle; Ultracentrifugation: Wesley Kendle (left), Kimson Hoang (right); Western blot: Wesley Kendle (left), Kimson Hoang (right). (B) (C) Figure

creation: Isabelle Lemasson, Kimson Hoang (D) Transfection, infection assay, graph
creation: Isabelle Lemasson, Kimson Hoang; (E) Transfection, Western blot, figure
creation: Isabelle Lemasson, Kimson Hoang.

DISCUSSION

HBZ was previously shown to enhance HTLV-1 infection by activating the expression of *ICAM1* and *MYOF*[329,330], and in this study we found that HBZ upregulates two additional cellular genes involved in infection, *COL4A1* and *GEM*[135,291]. Interestingly, apart from *MYOF*, these genes are also activated by the HTLV-1 encoded protein, Tax, which plays an essential role in HTLV-1 infection[289]. While the interplay between Tax and HBZ in infection has not been addressed, it is possible that both proteins act together to augment the expression of these genes. Alternatively, HBZ may play a supporting role to maintain some level of HTLV-1 infectivity when Tax expression switches to the off state. Indeed, in an HTLV-1-induced leukemic cell line, Tax expression was found to stochastically alternate between on and off states[276]. Moreover, when Tax is in the off state, virus particles may be retained on the surface of the cell in an extracellular matrix[130] and poised for infection. Finally, while mitotic expansion appears to be the primary mode of viral replication once the adaptive immune response is activated and a proviral set point is established, some infectious spread persists at this state[137,349,350].

In addition to these genes, HBZ upregulated expression of Nrp1, which on target cells, serves as the high-affinity binding receptor for HTLV-1 virions[148]. While the significance of *NRP1* expression by HTLV-1-infected T-cells has not been reported, *NRP1* expression was found to be upregulated in mouse primary $CD4^+$ T-cells transduced to express HBZ[324]. This observation prompted us to explore this gene further. We first analyzed how HBZ upregulates *NRP1* transcription. A peak of HBZ-enrichment was identified approximately 50 kb downstream of the gene. Interestingly,

independent of HBZ or HTLV-1 infection, cumulative data from multiple cell specimens show that this chromosomal region acts as an enhancer[346]. For example, it comprises a DNase I hypersensitive peak tightly flanked by peaks of histone H3 lysine 27 acetylation. These features are indicative of a nucleosome-free region bound by transcriptional regulators including p300/CBP that acetylates H3K27. The presence of p300 has been found to be a common feature of enhancers[351,352].

We speculate that HBZ primarily serves to increase association of Jun members (*i.e.*, c-Jun, JunB and JunD) with the enhancer. HBZ is known to form heterodimers with these factors through interactions between the leucine zipper (ZIP) domain of each protein[302,311,339], and mutations in the ZIP domain of HBZ that disrupt Jun protein-binding also abrogated *NRP1* transcription. The enhancer contains two consensus AP-1 binding sites as well as multiple partial sites. While heterodimers formed between HBZ and a Jun member may bind one or both consensus AP-1 sites, it is alternatively possible that such heterodimers target an AP-1 partial site. AP-1 transcription factors bind DNA through the basic region of the bZIP domain of each subunit[353], and in HBZ, this region lacks the conserved amino acid motifs involved in binding the AP-1 sequence. Therefore, in the context of an HBZ/Jun member heterodimer, the Jun member may contact an AP-1 half site while HBZ contacts an adjacent unrelated sequence. The observation that JunB is enriched at the enhancer in the absence of HBZ suggests that the cellular AP-1 factors are binding the consensus AP-1 sites.

It is possible that a second mechanism also contributes to the increased association of Jun members with the enhancer that involves increased abundance of these proteins in the presence of HBZ. We reported that the splice 1 variant of HBZ

(HBZ_{S1}), which was used in this study, stabilizes c-Jun and JunB by inhibiting their proteosomal degradation initiated by the E3 ubiquitin ligase, constitutive photomorphogenesis protein 1[313]. Of note, HBZ_{S1} is the most abundant variant in HTLV-1-infected T-cells[205,228,354,355].

In contrast to mutations in the ZIP domain, mutations in the AD of HBZ did not significantly affect transcription. This observation diverges from some previous results in which the AD has been shown to be central to transcriptional activation by HBZ[39,314,330,343]. While the AD appeared to be dispensable for activating *NRP1* transcription, both p300 and CBP displayed higher levels of association with the enhancer in the presence of HBZ. This observation might suggest that the increased association of Jun members with the enhancer augments recruitment of p300/CBP.

In addition to this proposed model, there are likely HBZ-independent mechanisms contributing to regulation of *NRP1* transcription in HTLV-1-infected T-cells. Indeed, there was a wide range of *NRP1* expression levels among the HTLV-1 T-cells lines and HTLV-1-immortalized clones we tested that did not necessarily reflect HBZ expression levels according to our previous results[330,343,356]. We cannot explain this variation in transcript and protein levels but suspect it may relate to genetic heterogeneity across the cell lines and clones.

In this study, we approached Nrp1 expression in HTLV-1-infected T-cells based on its role as the viral receptor that forms a high affinity interaction with SU. The ability of HBZ to increase expression of one of the HTLV-1 receptors appears to oppose conventional replication strategies used by some avian retroviruses and HIV. Expression of the viral receptor on cells infected with these retroviruses promotes

reinfection, leading to the accumulation of unintegrated DNA, which is cytopathic[357,358]. Through multiple virus-mediated mechanisms, HIV has been shown to generally prevent reinfection by eliminating CD4 from the surface of the infected cell[359]. While fusion and virus entry for HIV requires the chemokine receptors CCR5 or CXCR4[360], for HTLV-1 these processes are believed to require Glut1[148]. Interestingly, the HTLV-1 protein, Tax, was shown to reduce Glut1 at the cell surface by binding sorting nexin 27 (SNX27) and preventing SNX27 from trafficking Glut1 to the cell surface[361]. Therefore, HTLV-1 reinfection might be impaired by removal of Glut1 rather than Nrp1 from the plasma membrane.

We found that expression of Nrp1 on HTLV-1-infected T-cells and HTLV-1 producing cells was associated with decreased cell-to-cell viral infection without any significant effect on viral production. A similar observation was reported recently regarding HIV-infected cells of the monocyte lineage[348]. In this other study, Nrp-1 expressed by macrophages and dendritic cells was found to be packaged into the HIV virions produced by these cells, leading to reduced binding of these virions to target cells. The authors of this study speculated that the extended ectodomain of Nrp1 along with its heavily glycosylated state sterically inhibits attachment of virions to target cells. We similarly found that Nrp1 is incorporated into HTLV-1 virions, and consistent with the hypothesis of steric inhibition, an Nrp1 mutant lacking most of the ectodomain did not reduce viral infection. Therefore, Nrp1 might also reduce binding of HTLV-1 virions to target cells, however further investigation would be required to draw conclusions about the potential the impact of virion-incorporated Nrp1 on HTLV-1 infection.

The negative effect of Nrp1 on HTLV-1 infection appears to be outweighed by positive contributions from other HBZ-regulated genes, at least in the cell culture models we have tested[329,330]. It is possible that in some HTLV-1 carriers host genetic factors participate with HBZ to increase Nrp1 expression. Indeed, we found variability in *NRP1* transcript levels in the different HTLV-1-immortalized clones (from different donors) and, through analysis of GEO datasets, in CD4+ T-cells/PBMC from different HTLV-1 carriers (data not shown). Perhaps, Nrp1 might impact infection in cases where it is more highly expressed.

Finally, it is possible that Nrp1 contributes to other aspects of HTLV-1 biology not addressed in this study. One example involves the role of Nrp1 as a coreceptor for TGF-β receptor signaling[336,362], which is interesting considering that HBZ activates transcription through the downstream signaling effector, Smad3[39]. In addition, Nrp1 is capable of converting the latent form of TGF-β into the active form[336]. Therefore, increasing Nrp1 expression might represent a second mechanism by which HBZ enhances TGF-β signaling. This signaling pathway is implicated in establishing the regulatory T-cell-like phenotype documented for most HTLV-1 infected cells[363]. It would be interesting to investigate this, and other potential effects of Nrp-1 related to HTLV-1 infection and pathogenesis.

MATERIALS AND METHODS

Plasmids. pSG-Tax, pSG-Tax-His, pQC-HBZ-IP, pCMVHT1, pCRU5HT1-inLuc and pHCMV-G have been described[272,329,364-366]. pLJM1 was a gift from Joshua Mendell (Addgene plasmid # 91980)[367]. pUMVC was a gift from Bob Weinberg (Addgene plasmid # 8449)[368]. Nrp1 expression vectors were generated by cloning DYK-tagged *NRP1* from pcDNA3.1-C-(k)-NRP1-DYK (GenScript) into pQCXIP (Clontech) and pLJM1 at the BamH1 and EcoRI sites, respectively, using a Gibson Assembly Cloning Kit (New England Biolabs). shRNA vectors shGFP (SHC202) and shNRP1 [TRCN0000300917 (MT-2 transductions), TRCN0000322980 (Jurkat transfections and ATL-2 transductions)] were purchased from MilliporeSigma. pQCXIP-NRP1-TM was constructed by PCR-amplification of the transmembrane/cytoplasmic domain sequence, which was inserted into the PacI and EcoRI sites. pQCXIP-NRP1-Δabc was constructed by amplification of the signal peptide sequence, which was inserted into the BglII and MluI sites of pQCXIP-NRP1-TM (an MluI site had been added to the forward primer used to amplify the NRP-1-TM sequence).

Cell culture. Jurkat, CEM, C8166/45, MT-2, SLB-1 and ATL-2 cells were cultured in Isocove's modified Dulbecco medium (IMDM). Primary CD4+ lymphocytes, TL-Om1, and HTLV-1-immortalized lymphocyte clones[111] were cultured in Roswell Park Memorial Institute (RPMI) medium. HeLa and HeLa-HBZ clonal cell lines[343], CHO-LFA-1 clones[329], and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM). All cells were supplemented with 10% FBS or 10% FetalPlex (GeminiBio) and 2 mM L-glutamine, 100 U/mL penicillin, and 50 µg/mL streptomycin. Jurkat pminLuc-

viral CRE cells[329] and Jurkat-HBZ cells[329] were supplemented with 1.5 mg/mL of G418. HeLa and CHO clones were supplemented with 0.5 mg/mL of G418. Primary lymphocytes and lymphocyte clones were cultured with IL-2. Primary lymphocytes were activated in culture wells coated with anti-CD3 and anti-CD28 antibodies. Where indicated, cells were treated with 10 μM A-485 (MedChem Express) or DMSO for 3h.

Table 2.1 Cell lines used in this study.

Cell Line(s)	Transfected or Transduced	Vectors used:	Stable or Transient	Details
HeLa	Transfected	HBZ wild-type or mutant HBZ	Stable	Single, stable cell line used per HBZ condition (wild-type or mutant)
Jurkat	Transfected	shGFP (SHC202) or shNRP1 (TRCN0000322980)	Transient	Independent transfections
MT-2	Transduced	shGFP (SHC202) or shNRP1 (TRCN0000300917)	Transient (with short-term antibiotic selection)	Independent transductions
ATL-2	Transduced	shGFP (SHC202) or shNRP1 (TRCN0000322980)	Transient (with short-term antibiotic selection)	Independent transductions
SLB-1	Transduced	pLJM1- Empty or pLJM1-NRP1	Transient (with short-term antibiotic selection)	Independent transductions

RNA extraction, cDNA synthesis, and quantitative real-time PCR. RNA was isolated from cells using TRIzol Reagent (Invitrogen), and cDNA was synthesized with random hexamers using the iScript cDNA Synthesis Kit (Bio-Rad) or the Revert Aid kit (Thermo Fisher Scientific) as described by the manufacturers. Primer sequences are as follows:

UBE2D2, 5'- TGCCTGAGATTGCTCGGATCTACA- 3' and 5'-ACTTCTGAGTCCATTCCCGAGCTA -3'; COL4A1, 5'- TCTGGCTGTGGCAAATGT-3' and 5'- GGTAGTCCTGGTTCTCCAGTAT-3'; COL4A2, 5'- GCTTCTGGAAGGGCCAAT -3' and 5'- CACGGCACATCAAACTTCTTC -3'; GEM, 5'-AATGAATGGCTCCATGACCACTGC -3' and 5'- CTTGCAGTCAAACACCACTGCACA -3'; and NRP1c 5'- CAGAGCGCTCCCGCCTGAAC-3' and 5'-AAATGGCGCCCTGTGTCCCG-3'. Real-time PCR was performed using iTaq Universal Supermix (Bio-Rad) and a CFX Connect Real-Time PCR Detection System (Bio-Rad), and relative mRNA levels were determined as described[343]. Serial dilutions of an appropriate experimental sample were used to generate standard curves for all primer sets included on a PCR plate. From the compilation of all the standard curves for all primers and all PCR plates (analyses), including ChIP PCR plates, the amplification efficiencies ranged from 63.1-129% with correlation coefficients ranging from 0.935-0.999.

Western blot analysis. Cells were normalized to 5 x 10^5 cells/mL, cultured overnight, and harvested. Whole cell extracts were prepared, and western blotting was done as described[249]. Antibodies used were as follows: anti-His (ab9108), anti-Nrp1 (ab81321) and anti-MafG (ab154318) were purchased from Abcam; anti-β-actin clone C4 (MAB1501) was purchased EMD Millipore; anti-CBP (sc-1211), anti-p300 (sc 57865), anti-gp46 (sc 57865) and anti-p19 (sc 57870) was purchased from Santa Cruz; and anti-Tax (hybridoma 168B17-46-92) was obtained from NIH AIDS Research and Reagent Program. Blots were developed using Pierce ECL Plus (Thermo Fisher

Scientific) and scanned with a Typhoon RGB imager (Cytiva). Images were analyzed using ImageQuant TL v8.1 (GE Healthcare Lifesciences).

Flow cytometry. A total of 10^6 cells/labelling reaction was collected by centrifugation at 800 x g for 3 min at 4ºC, washed once in 2 mL of cold PBS/0.2% BSA (FACS buffer), and suspended in 50 µL of cold FACS buffer, to which 1 µg of anti-Nrp1 Alexa Fluor 647 (R&D Systems, FAB3870R) was added. Cells were labeled on ice for 1 h and then washed with 2 mL of FACS buffer. Cells were fixed with PBS/2% paraformaldehyde at 4°C for at least 30 m, suspended in 500 µL FACS buffer, and analyzed using a Cytek Aurora flow cytometer (Cytek Biosciences). Data were analyzed using FlowLogic Software.

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed using the ZymoSpin ChIP Kit (Zymo Research) according to the manufacturer's instructions with minor modifications. For p300 and CBP immunoprecipitations, chromatin was crosslinked using 10 mM disuccinimidyl glutarate (Thermo Scientific) for 45m and then crosslinked with formaldehyde; for all other immunoprecipitations, only formaldehyde was used. Crosslinked chromatin was sonicated using a Misonix Sonicator 4000 (20s pulse on, 30s pulse off, amplitude 40, 5m processing time). Each immunoprecipitation reaction contained 5 µg of antibody and 200 µg of crosslinked, sonicated chromatin. Antibodies used were as follows: anti-p300 (C-20, sc-585) from Santa Cruz Biotechnology; anti-CBP (D6C5, #7389), anti-JunB (C37F9, #3753) and anti-c-Jun (60A8, #9165) from Cell Signaling Technology; anti-MafG (ab154318) from Abcam.

HBZ was immunoprecipitated through its C-terminal 6xHis tag using an anti-6xHis antibody (Abcam, ab9108). Purified ChIP DNA was amplified in iTaq Universal Supermix (Bio-Rad) using a CFX Connect Real-Time PCR Detection System (Bio-Rad). Primer sequences are as follows: NRP1 HBZ peak 5'-GCCAGTTCAGTACCCAGTAATA -3' and 5'- CTGGAAATTAAGGTGGCTGTTT -3'; NRP1 off-target 5'-CTGAGACTTCTGGAGGCTAAAT-3' and 5'-GGTATCCCAAATTCCCAGAGT-3'; WEE1AP1 5'- CCAATCGGCTTATCGGCTTAT-3' and 5'-ACAGGAGCGTGTTTAGGTATTG -3'. Standard curves were generated for primer sets using 10-fold serial dilutions of each input DNA from the ChIP procedure and were included on each experimental plate. Enrichment values were quantified relative to the input as described[369,370].

Small RNA interference. The siGENOME SMART pool M-003486-04-0005 and M-003477-02-0005 were used to knock-down p300 and CBP respectively, while the siGENOME Non-Targeting siRNA pool#1 D-001206-13-05 was used as a control (Dharmacon). Cells were seeded to reach ~50% confluence on the day of transfection. Cells were transfected with 25 nM of siRNA using DharmaFECT 1 siRNA transfection reagent (Dharmacon) according to the manufacturer's instructions. The medium was changed 24 h after transfection, and cells were cultured for an additional 48 h prior to harvesting the cells.

Transfection and single-cycle, replication-dependent infection assays. Single-cycle, replication-dependent luciferase assays were performed using Jurkat-HBZ or

HEK293T cells as effector cells. Jurkat-HBZ cells (3 x 10^6) were electroporated with 4.5 µg of pCMVHT1[272] or pcDNA3.1 and 8 µg of pCRU5HT1-inLuc[272], 1.25 µg of pSG-Tax[365] and 1.25 µg of shRNA expression vector in 300 µL of RPMI/10 mM dextrose/0.1 mM dithiothreitol per 0.4 cm electroporation cuvette. Cells were exposed to a single exponential decay pulse of 200V/950µF. Forty-eight hours after electroporation, 5 x 10^5 transfected Jurkat-HBZ cells were co-cultured with 8 x 10^4 CHO-LFA-1 cells for 3h. Jurkat-HBZ cells were then removed, and the CHO-LFA-1 cells were washed four times with PBS. CHO-LFA-1 cells were cultured for an additional 48 hours, washed with PBS and lysed with Passive Lysis Buffer (Promega). HEK293T cells were plated at 2.4 x 10^5 cells/well in 24-well plates the day before transfection. Cells were transfected with 1.12 µg of pCMVHT1, 1.68 µg of pCRU5HT1-inLuc, and 1.2 µg of pQCXIP, pQCXIP-NRP1 or pQCXIP-NRP1-Δabc using TurboFect (ThermoFisher) as described by the manufacturer. The cells were washed with PBS and lysed with Passive Lysis Buffer (Promega) 48 hours later. Luciferase activity was measured using the luciferase assay system (Promega) and a GloMax 20/20 luminometer (Promega). Luminescence values were normalized to protein concentrations. HeLa cells were plated at 2.4 x 10^4 cells/well in a 6-well plate and cultured overnight. Cells were then transfected with 4 µg of pSG5 or pSG-Tax-His using TurboFect (ThermoFisher) as described by the manufacturer.

Retrovirus and Lentiviral transduction. For retroviral transfections, 2.5 x 10^6 HEK293T cells/10 cm dish were cultured overnight and then transfected with 8.4 µg pHCMV-G, 31.1 µg pUMVC and 50.5 µg pQCXIP or pQC-HBZ-IP using calcium phosphate. The medium was replaced with 9 mL/dish fresh medium 18h later. One day

later, the culture medium was passed through a 0.4 μm polyethersulfone (PES) filter and used to transduce HeLa cells in 6-well plates (1.5 mL viral supernatant/well); HeLa cells had been plated at 2.4 x 10^5 cells/well the previous day. Cells were placed under 2 μg/mL puromycin selection 48h later. Lentivirus transductions were done as described[330] but with the following modification: Media of transfected HEK293T cells were replaced with 10 mL IMDM supplement with 5% FBS to concentrate virus from viral supernatants using LentiX Concentrator (Takara). Cells were placed under puromycin selection (MT-2, 2μg/ml; SLB-1, 6μg/mL; ATL-2, 0.5μg/mL) three days later. Cells were processed for co-culture/infections, western blotting and/or ELISA following three to four days of antibiotic selection. Co-culture/infection assays were done as described[330].

Detection of virion and Gag p19 enzyme-linked immunosorbent assay (ELISA) in the culture medium. HTLV-1-infected T-cells, ATL-2, MT-2 and SLB-1, were cultured at 1 x 10^6 cells/mL overnight at 37°C. Supernatants were collected by centrifugation at 1300 RPM for 3 min at room temperature and filtered through a 0.2 μm PES filter to ensure complete removal of cells. Supernatants were centrifugated in a SW-40 Ti rotor (Beckman Coulter) at 20,000 RPM for 2 h at 4ºC. Concentrated virus was collected in 2x sodium dodecyl sulfate dye for western blot analysis. For ELISA detection, cells were equalized and cultured for 24h-48h. Supernatants were collected, filtered through 0.45μm PES filters, and virus was inactivated at 55ºC for 30 min. HTLV p19 Antigen ELISA (ZeptoMetrix) kit was used as described by the manufacturer. Absorbances were detected with an accuSkan FC (Fisher Scientific).

***In silico* analysis and statistical analysis.** Microarray data sets used in this study are available at NCBI Gene Expression Omnibus (GEO): GSE94409[320]. For each sample, probes corresponding to the *COL4A1*, *COL4A2*, *GEM* and *NRP1* transcripts in KK1 and ST1 cells infected with Ctrl, HBZ_1 or HBZ_2 sgRNAs were identified and GEO2R was used to obtain expression values. ChIP-Seq data sets from GEO accession number GSE94732[320] were analyzed using the Human Mar. 2006 (NCBI36/hg18) assembly with the IGV Browser[371]. Two-tailed Student's t-tests were used for two-group comparisons and significance was established at $p < 0.05$. For analysis of the ELISA data, average relative p19 values from multiple independent experiments were utilized to generate the figures.

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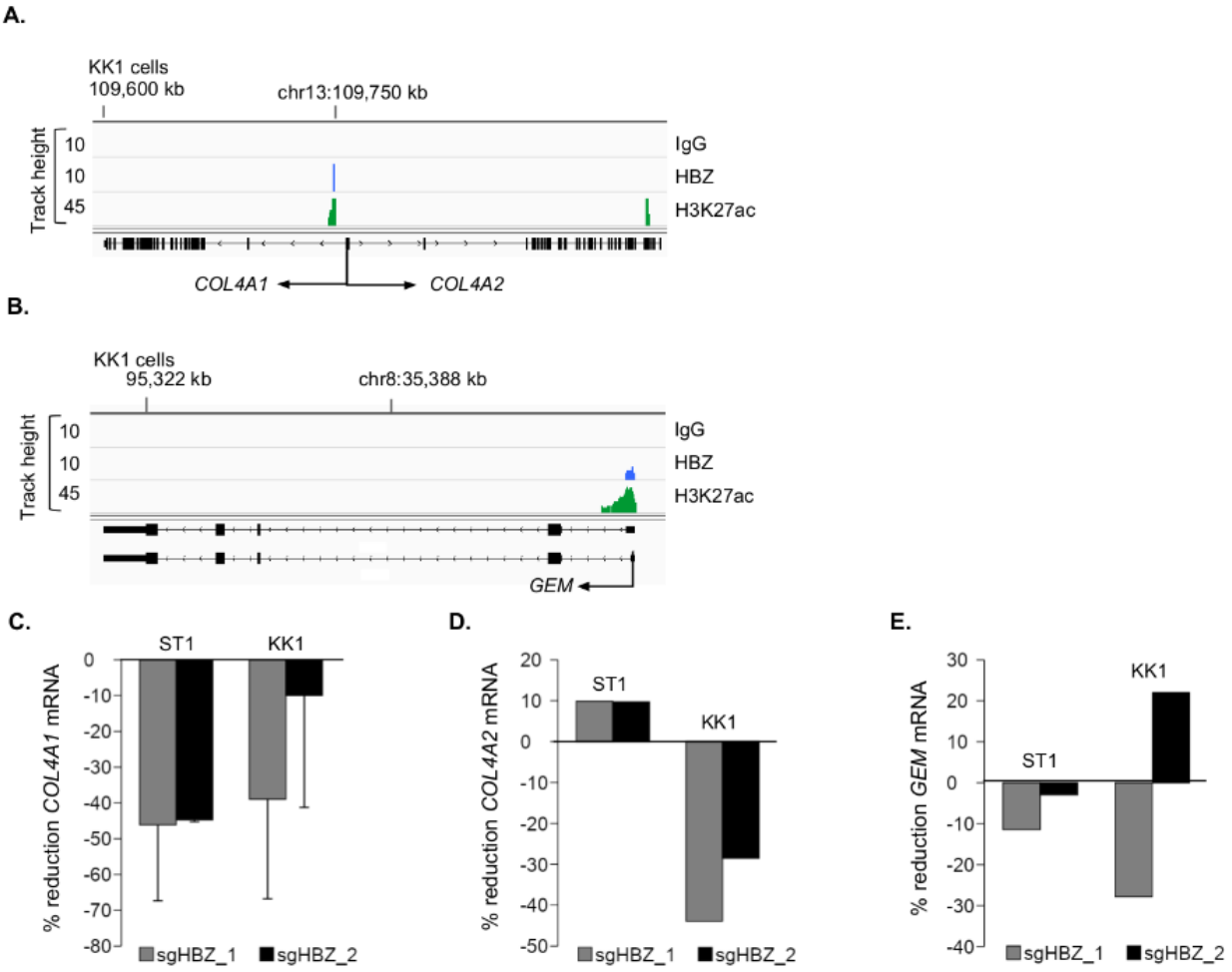
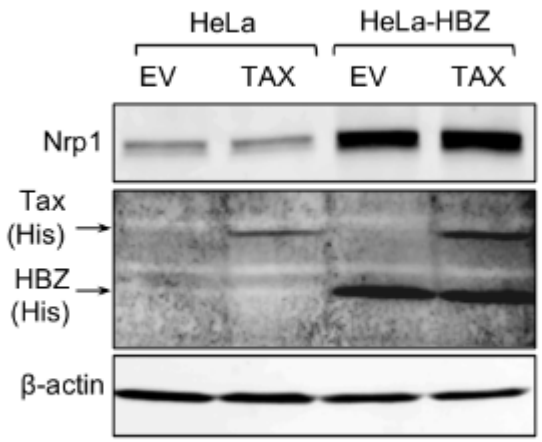
SUPPLEMENTAL FIGURES

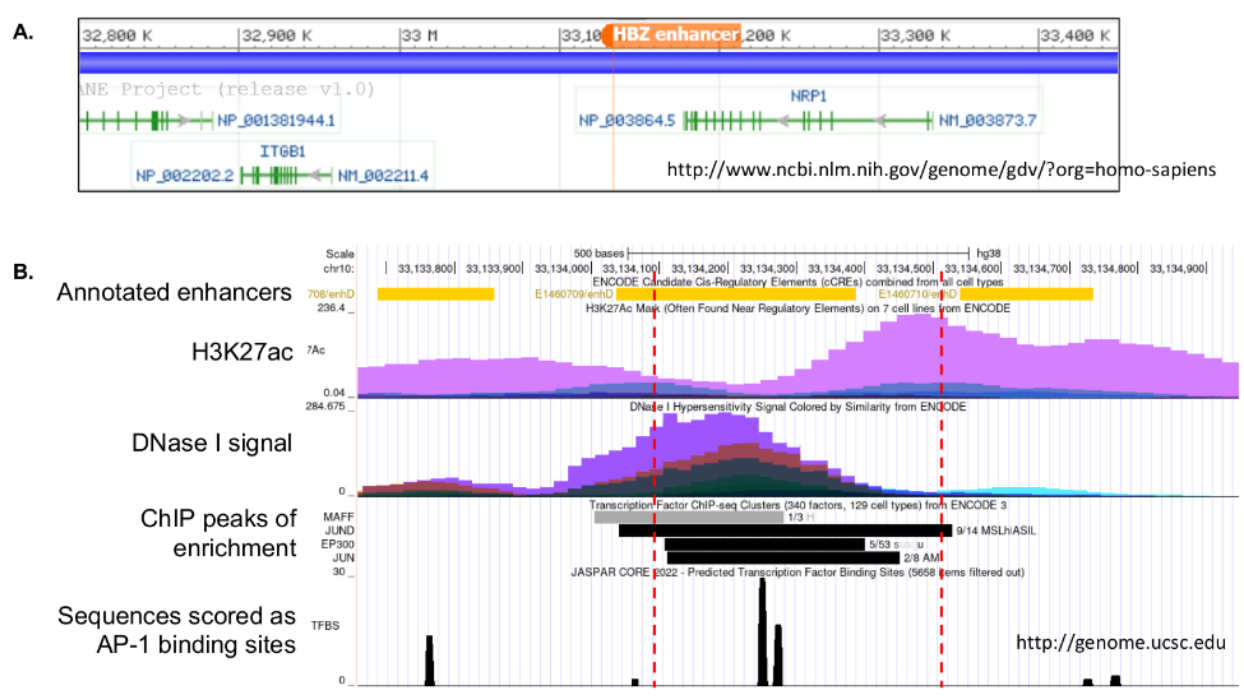


Supplemental Figure 1. HBZ activates *COL4A1*, *COL4A2* and *GEM* expression in ATL-derived cell lines. HBZ associates with chromosomal sites near the **(A)** *COL4A1/COL4A2* and **(B)** *GEM* transcription start sites (indicated by the arrows). ChIP-seq tracks for HBZ, H3K27ac, and negative control IgG are shown across the *NRP1* locus in KK1 cells using the IGV Browser. Genomic coordinates are based on the NCBI36/hg18 assembly. Data were obtained from published Data sets (GEO accession number GSE94732)[320]. Changes in **(C)** *COL4A1*, **(D)** *COL4A2* and **(E)** *GEM* expression following deletion of HBZ in ST1 and KK1 ATL-derived cell lines. Graphs were generated from published microarray data (GEO accession number GSE94409)[320] and show transcript levels after inducing CRISPR/Cas9-mediated knockout of HBZ, using two different guide RNAs (sgHBZ_1 and _2). Data are from day 8 post-induction except for sgHBZ_2 in KK1, which is the day 7 values (no day 8 data provided for this specimen). Values were obtained using GEO2R with calculations based on averaged values from the two array features for *COL4A1* and the single features for *COL4A2* and

GEM. Credit: (A) (B) Analysis and figure creation: Wesley Kendle; (C) Data compilation, data analysis and graph creation: Wesley Kendle; Graph update: Isabelle Lemasson. (D) (E) Data compilation, data analysis and graph creation- Wesley Kendle.



Supplemental Figure 2: Nrp1 expression in Tax transfected HeLa cells. HeLa cells were transfected with 4 µg of empty vector (EV) (pSG5) or pSG-Tax-6His (Tax) for 48h. Whole cell extracts (50 µg) were analyzed by western blot using antibodies against Nrp1, HBZ (6xHis epitope) and β-actin. Credit: Transfection, Western blot and figure creation: Isabelle Lemasson and Kimson Hoang.



Supplemental Figure 3. The site of the HBZ peak of enrichment **(A)** The site of the HBZ peak of enrichment, denoted in red and labeled HBZ enhancer, and neighboring genes (*ITGB1* and *NRP1*) are shown using the NCBI Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/?org=homo-sapiens). **(B)** The chromosomal features in and around the peak of HBZ-enrichment were derived from ENCODE data[346] and are shown using the UCSC Genome Browser[345,372]. The vertical hatched lines show the boundaries of the peak of HBZ enrichment. AP-1 binding site predictions are shown as peaks in the density graph[373]. **(C)** The DNA sequence corresponding to the HBZ peak encompasses bp 33,134,089-33,134,518 of chromosome 10 (GRCh38.p14 Primary Assembly). The bold sequences correspond to consensus AP-1 binding sites shown as the two peaks in panel B. Partial AP-1 binding sites are underlined. Credit: (A)(B) Analysis: Nicholas Polakowski; (C) Analysis: Wesley Kendle.

CHAPTER 3

New Understanding of the Role of HBZ and NRP1 in Cell-to-Cell Transmission of HTLV-1

Although the viral protein Tax has long since been considered the main viral protein involved in cell-to-cell transmission of HTLV-1, recent insights into the roles of HBZ during infection have expanded our understanding of the complex retrovirus HTLV-1 infection process. The goal of this research was to identify cellular genes upregulated by HBZ which impact viral transmission and to understand subsequent impacts on cell-to-cell transmission of HTLV-1.

HBZ alters expression of cell surface proteins involved in infection:

Regarding regulation of gene expression, HBZ often downregulates gene expression through sequestration of cellular bZIP factors[226,302,312]. However, a growing pool of knowledge about the ability of this intriguing viral protein to upregulate expression of certain cellular genes, accentuates how much is left to uncover about this bZIP factor. Indeed, HBZ is able to activate gene expression through dimerization with cellular AP-1 transcription factors, as is the case with the transcriptional activation of the human

telomerase gene, *hTERT*. Activation of *hTERT* gene expression by HBZ occurs through the formation of HBZ/JunD heterodimer and recruitment of this heterodimer to SP-1, a protein that binds directly to the DNA[314].

We have found that ICAM-1, an integral component of the virological synapse, expression is also enhanced in the presence of HBZ[329]. Enhanced ICAM-1 expression increases the efficiency of HTLV-1 cell-to-cell transmission, which we attributed to enhanced homotypic aggregation[329]. This is consistent with the cell-to-cell contact dependent nature of HTLV-1 transmission. Intriguingly, we have also found that HBZ upregulates COL4A1 and COL4A2 expression (**Figure 2.1**), two proteins which associate to form a heterotrimer in the extracellular matrix and enhance cell-to-cell transmission of HTLV-1. In this work, we found that HBZ upregulates expression of another cell surface receptor: NRP1.

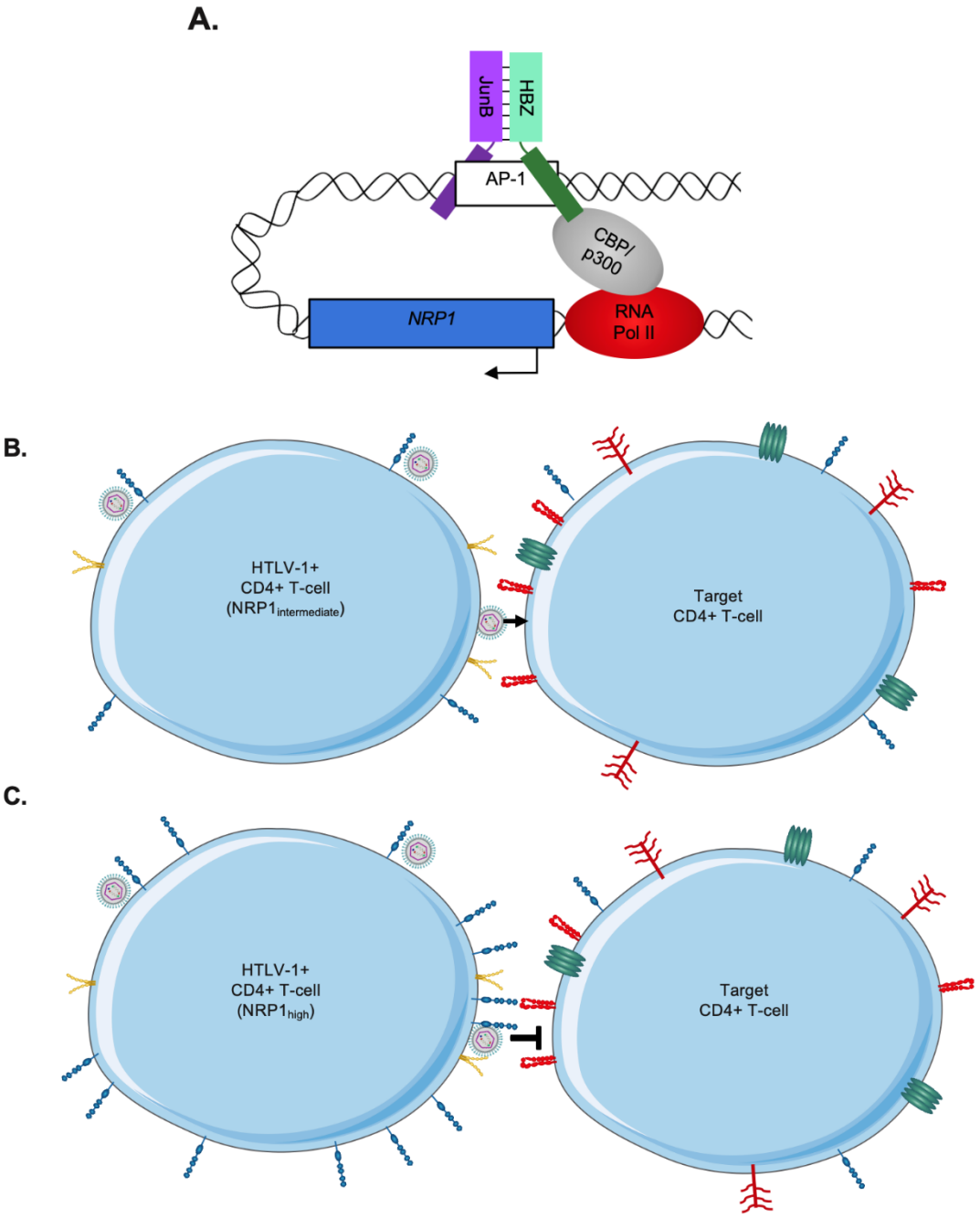
HBZ regulates NRP1 expression during HTLV-1 infection: We found that NRP1 expression is enhanced in the presence of HBZ. Since HBZ can interact with certain members of AP-1 family of transcription factors[302,311,312,314,339,374], we specifically assessed enrichment of HBZ and AP-1 bZIP factors at a downstream *NRP1* enhancer. HBZ, c-Jun and JunB were found to be enriched at a putative enhancer downstream of *NRP1* (**Figure 2.5**). Due to an atypical basic region, HBZ is generally unable to bind directly to DNA, however, it was recently discovered that HBZ/small Maf dimers are able to bind directly to DNA at MARE sites[311,317]. Based on our findings, we hypothesize that HBZ heterodimerizes with c-Jun or JunB and this heterodimer binds to the identified downstream *NRP1* enhancer site, either at one or both of the two identified AP-1 sequences or to the sequence flanking one of these AP-1 sites. It is also possible that

the HBZ heterodimer can bind to an AP-1 half site with a sequence similar to that of a complete AP-1 site. Due to an atypical basic region, it is possible that rather than bind perfectly to the AP-1 site, HBZ may bind in a unique way to the identified region. Further experimentation is needed to confirm the binding of an HBZ/AP-1 heterodimer to the identified sequence and to identify the specific nucleotides required for this binding. HBZ is also known to interact with p300/CBP coactivators proteins to alter normal cellular gene expression[39,307,308]. Interestingly, we also found that p300/CBP coactivators are enriched at the putative enhancer downstream of *NRP1* (**Figure 2.5**). Our results support a model where recruitment of an HBZ/AP-1 heterodimer (HBZ/JunB or HBZ/c-Jun) to the identified downstream promoter sequence, followed by recruitment of CBP/p300 coactivators and subsequent recruitment of basal transcription machinery, including RNA Pol II, to the *NRP1* transcription start site through a looping mechanism **(Figure 3.1)**. Based on this model, looping of the DNA occurs between transcription factors bound to the downstream *NRP1* enhancer and to the *NRP1* promoter. This mechanism brings the enhancer and promoter elements into close proximity, allowing the enhancer element to promote transcriptional activation. Alternatively, HBZ may also be contributing to upregulation of NRP1 through de-repression of *NRP1* transcription at the *NRP1* promoter. The *NRP1* promoter contains an SP1 binding site[375]. The *hTERT* promoter also contains an SP1 site which is important for transcriptional regulation[314]. In the absence of HBZ, the *hTERT* SP1 site is occupied by Sp1 in a complex with JunD and the transcriptional repressor menin[316]. In the presence of HBZ, Sp1/JunD/HBZ complexes activate transcription at the *hTERT* promoter, which also contains an Sp1 binding site[314]. Based on these data, it is possible that HBZ facilitates the upregulation

of NRP1 through a similar mechanism, by relieving menin-mediated repression of gene expression. It is important to note that not all HBZ-expressing cells overexpress NRP1 and this may be for a number of reasons. It is possible that differential NRP1 expression in HTLV-1-infected cells is due to genomic differences, including differences in host cell genome and viral integration sites, both of which can alter expression of cellular and viral genes and potentially lead to downstream impacts on NRP1 expression in the HTLV-1-infected T-cell.

NRP1 and HTLV-1 infection: As an HTLV-1 receptor, NRP1 has been the focus of numerous studies, primarily focusing on NRP1 expression on target cells. Indeed, human primary MDDCs are more susceptible to HTLV-1 infection than primary lymphocytes, which has been attributed to the higher NRP1 expression level on MDDCs than T- cells[117]. Furthermore, blocking of NRP1 on target cells with $VEGF_{165}$ reduces the susceptibility of cells to HTLV-1 infection[99].

Elevated NRP1 expression during HTLV-1 infection is unexpected because viral receptors are often downregulated during retroviral infection to prevent superinfection[376,377]. For example, Tax reduces GLUT1 expression during HTLV-1 infection[361]. Furthermore, during human immunodeficiency virus type 1 (HIV-1) infection CD4, CXCR4 and CCR-5 expression are downregulated[376,378-380]. Upregulation of NRP1 in infected T-cells suggests a beneficial role for this protein during HTLV-1 infection.


A.
JunB
HBZ
AP-1
CBP/
p300
NRP1
RNA
Pol II
B.
HTLV-1+
CD4+ T-cell
(NRP1intermediate)
Target
CD4+ T-cell
C.
HTLV-1+
CD4+ T-cell
(NRP1high)
Target
CD4+ T-cell

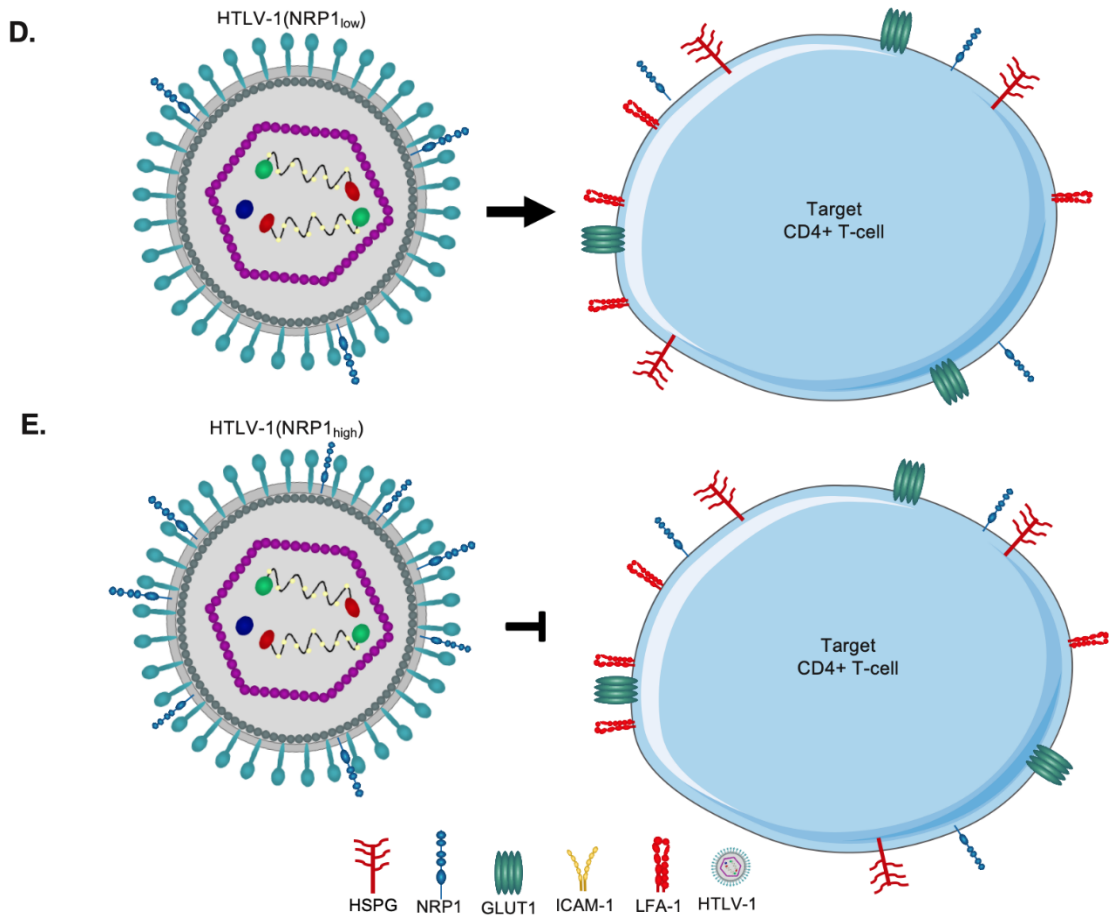


Figure 3.1. Proposed model of activation of NRP1 expression by HBZ and subsequent impact on HTLV-1 transmission. (**A)** An HBZ/JunB or HBZ/c-Jun heterodimer binds to the identified *NRP1* enhancer. CBP/p300 coactivator is recruited and interacts with HBZ. CBP/p300 recruits RNA Pol II, which activates transcription of *NRP1*. **(B-C)** Proposed model of the impact of NRP1 expression on HTLV-1-infected T-cells on the efficiency of cell-to-cell transmission of HTLV-1. Figure for illustrative purposes and is not to scale. (**B)** In HTLV-1-infected T-cells in which NRP1 expression is intermediately upregulated ($NRP1_{intermediate}$), ICAM-1 on the surface of the infected cell can successfully interact with LFA-1 on the surface of the target cell, resulting in the formation of the virological synapse and transfer of viral particles from the infected cell to the target CD4+ T-cell. **(C)** In HTLV-1-infected T-cells in which NRP1 expression is highly upregulated ($NRP1_{high}$), ICAM-1 on the surface of the infected cell can not successfully interact with LFA-1 on the surface of the target cell, reducing the likelihood of successful formation of the virological synapse and viral particle transfer from the infected cell to the target CD4+ T-cell. **(D-E)** Proposed alternative model of the impact of NRP1 expression on HTLV-1 transmission through altered interactions of the virion with the target cell. Figure for illustrative purposes and is not to scale. **(D)** HTLV-1 with low levels of NRP1 ($NRP1_{low}$) are able to more efficiently interact with target T-cells, resulting in increased likelihood of infection of target cells. **(E)** Higher levels of NRP1

($NRP1_{high}$) on HTLV-1 inhibits the ability of the virion to successfully infect target T-cells, possibly due to the glycosylation of NRP1.

Upon discovering that NRP1 is actually upregulated during HTLV-1 infection, we initially hypothesized that NRP1 would enhance infection through retention of virions at the cell surface or improved cell-to-cell adhesion. Indeed, NRP1-mediated homotypic aggregation between T-cells and DCs during the formation of the immunological synapse enhances the duration of this interaction[174]. However, our studies found that NRP1 on effector cells reduces cell-to-cell transmission of HTLV-1 (**Figure 2.7**), without impacting virion release from the cell surface (**Figure 2.8**). Also, our data indicates that the extracellular portion of NRP1 is important for hindering infection (**Figure 2.7**). Based on these findings, we propose a model in which the relatively large size of NRP1, combined with the expression level, inhibits cell-to-cell transmission of HTLV-1 by reducing the ability of ICAM-1 on the surface of the HTLV-1 infected cell and LFA-1 on the surface of the target cell to successfully interact and form the virological synapse, thereby blocking effective cell-to-cell contact, resulting in a reduction in infection when NRP1 is more highly expressed **(Figure 3.1. B-C)**. Interestingly, NRP1 has also been found to reduce the infection efficiency of HIV-1[348]. Indeed, NRP1 expression in MDDCs and DCs, both of which express high levels of NRP1 compared to many cell types, inhibits infectivity of the progeny virions[348]. NRP1 is incorporated into HIV-1 viral particles and inhibits the ability of the viral particles to attach to target CD4+ T-cells[348]. Wang *et al.* (2022) hypothesized that the inhibitory impact of NRP1 is because NRP1 is heavily glycosylated. As an alternative model, we hypothesize that virion-incorporated NRP1 hinders the ability of HTLV-1 to efficiently infect target cells **(Figure 3.1. D-E)**.

Based on these findings, it is reasonable to hypothesize that relatively high NRP1 expression on DCs may hinder HTLV-1 transmission to target CD4+ T-cells. Another

potential explanation for the reduction in infection with elevated NRP1 expression is that elevated cellular expression of NRP1 potentially leads to an increase in NRP1 in HTLV-1 progeny viruses which may hinder binding of HTLV-1 virions to target cells. Furthermore, NRP1 mediated inhibition of infection could potentially be the result of the large molecular size of NRP1 or the glycosylation of NRP1.

Additional potential roles for NRP1 during HTLV-1 infection: Our data indicates that elevated NRP1 expression in T-cells during HTLV-1 infection inhibits HTLV-1 infectivity. This is surprising given that HBZ has been shown to upregulate certain genes involved in infection[329,330]. However, it is possible that NRP1 has another role during HTLV-1 infection. One possibility is that upregulation of Nrp1 serves as an additional mechanism to prevent superinfection of the HTLV-1-infected cell. Indeed, HTLV-1 is known to downregulation GLUT1 expression likely to prevent superinfection[361]. NRP1 is known to be involved in angiogenesis, cell survival, cell migration and invasion[362]. NRP1 is often upregulated in tumor cells and NRP1 expression in cancer cells is correlated with likelihood of metastasis[381-384]. Based on this, it is possible that elevated NRP1 levels in HTLV-1-infected T-cells promote dissemination of ATL cells. This hypothesis could be tested using a xenograft mouse model in which xenograft mice are injected with ATL cells (from a single cell line) manipulated to express either relatively low or high amounts of NRP1[385]. Subsequent comparison of the organ and tissue infiltration of the ATL cells based on NRP1 expression levels would provide valuable insight into a potential novel role of NRP1 during HTLV-1 infection. If high NRP1 levels promote dissemination, xenograft mice infected with $NRP1_{high}$ ATL cells would experience more widespread dissemination of ATL cells. It is also possible that restriction of HTLV-1

infection is advantageous *in vivo* to restrict the number of infected cells synthesizing immunogenic proteins, limiting the immune response against HTLV-1.

Like NRP1, HBZ is also involved in cell migration and cell survival through the inhibition of apoptosis. HBZ attenuates FoxO3a function and alter its cellular localization, resulting in a suppression of apoptosis[386]. HBZ also induces CCR4 to promote T-cell migration[323]. Both of these functions of HBZ occur independently of NRP1. Therefore, because NRP1 also promotes cell migration and cell survival independently of the mechanisms driven directly by HBZ and HBZ drives NRP1 expression, it is possible that HBZ is able to promote cell survival and migration directly and indirectly.

In conclusion, the work presented here describes novel roles of HBZ and NRP1 in the HTLV-1 infection process. Our findings offer new insights into the differential roles of NRP1 during various stages of HTLV-1 infection. While NRP1 on a target cell enhances the likelihood of a cell becoming infected with HTLV-1, our study shows that NRP1 expression on the HTLV-1-infected T-cell hinders the cell-to-cell transmission of HTLV-1[99,100]. These findings illuminate the differing impacts of NRP1 at different points in the HTLV-1 infection process, an important distinction in the development of anti-HTLV-1 therapeutics. Indeed, since NRP1 on a target cell promotes infection, the development of prophylactic anti- HTLV-1 drugs involving a mechanism of action in which NRP1 is temporarily blocked or downregulated could be advantageous. Alternatively, for therapeutic anti-HTLV-1 drug development targeted at reducing viral spread, enhancing NRP1 expression in HTLV-1-infected cells could be advantageous.

ATTRIBUTIONS

Chapters 1-3 contain an adaptation of or reference results published in *Upregulation of Neuropilin-1 inhibits HTLV-1 infection* by Kendle *et al. Pathogens* **2023**, 12(6), 831; https://doi.org/10.3390/pathogens12060831, which is under a CC BY 4.0 license. Changes include rewriting of some of the sections and adjustments to certain figures.

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