

ABSTRACT

The complex retrovirus human T-cell leukemia virus type I (HTLV-1) is the causative agent of adult T-cell leukemia (ATL). Deregulation of cellular transcription is thought to be a key event in virally-mediated T-cell transformation. The HTLV-1 basic leucine zipper factor (HBZ) originates from the 3' long terminal repeat (LTR) and is transcribed from the sense DNA strand relative to other viral genes. HBZ is the only viral gene known to be consistently expressed in ATL cells. HBZ is able to repress HTLV-1 transcription, in part, by binding to the homologous cellular coactivators p300 and CREB-binding protein (CBP). HBZ binds the kinase-inducible interaction (KIX) domain of p300/CBP via two $\phi XX\phi\phi$ motifs in the activation domain of HBZ (HBZ-AD). In this study, it was determined that HBZ-AD binds with high affinity to the KIX domain of p300/CBP with an apparent equilibrium dissociation constant (K_d) of 3.2 ± 0.5 nM. The KIX domain contains two binding surfaces that are differentially targeted by various transcriptional regulatory proteins. One surface of KIX can bind the activation domain of the mixed lineage leukemia (MLL-AD) protein and the HTLV-1 transactivator Tax, among others. The other surface of KIX can contact the cellular transcription factors c-Myb or cyclic AMP (cAMP) response element binding protein (CREB). HBZ-AD was determined to bind the MLL-AD-binding surface of KIX and to compete with MLL-AD, Tax, and p53 for KIX binding. HBZ-AD was additionally found to significantly enhance the binding of the activation domain of c-Myb (c-Myb-AD) and CREB to KIX. HBZ was also shown to inhibit transcriptional activation mediated by MLL-AD and enhance activation mediated by c-Myb-AD. These results indicate that HBZ is able to alter gene expression by competing with one group of transcription factors for occupancy of KIX while enhancing the binding of other factors to a different surface of KIX.

MOLECULAR CHARACTERIZATION OF THE ACTIVATION DOMAIN
OF THE HTLV-1 HBZ PROTEIN AND ITS INTERACTION WITH THE KIX DOMAIN
OF THE CELLULAR TRANSCRIPTIONAL COACTIVATORS p300 AND CBP

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DEDICATION

To my father, Barry Dean Cook, 1941-2005.

*His steadfast assumption that I could achieve my highest goals
enabled me in ways I may never fully realize.*

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TABLE OF CONTENTS

LIST OF TABLES	viii
LIST OF FIGURES.....	ix
LIST OF ABBREVIATIONS	xii
CHAPTER 1: REVIEW OF THE LITERATURE	1
Introduction.....	1
HTLV-1: Overview and Epidemiological Data	4
Discovery.....	4
Epidemiology of HTLV-1	4
Transmission and Tropism of HTLV-1.....	5
HTLV-1 Persistence.....	6
ATL.....	7
Diagnostic Criteria and Symptoms of ATL.....	7
ATL Treatment.....	8
Vaccine Development	8
HTLV-1 Molecular Characterization	9
HTLV-1 Genome and Integration.....	9
Transactivation of HTLV-1 Genes.....	10
HTLV-1 Accessory Genes.....	11
Tax.....	11
The Pleiotropic Functions of Tax.....	11
The Oncogenic Potential of Tax.....	12

A Tax Reassessment.....	13
Re-Evaluating the Immortalizing Capability of Tax.....	14
Tax Silencing in ATL.....	15
<i>Ex Vivo</i> Cell Culture.....	15
Discovery of HBZ.....	16
HBZ Expression in ATL Cells	16
Antisense Transcripts in HTLV-2, HTLV-3, and HTLV-4	17
Transcription of HBZ.....	17
HBZ Isoforms.....	17
HBZ Promoter	18
Functions of HBZ.....	19
HBZ Protein Domains	19
HBZ Represses Expression of HTLV-1 Genes.....	19
HBZ Affects the Function of Multiple Cellular bZIP Proteins	23
HBZ Inhibits the Classical NF- κ B Pathway	24
HBZ Induces Proliferation of T Cells.....	25
HBZ RNA.....	25
HBZ RNA Affects Proliferation of T Cells.....	25
HBZ RNA Affects Transcription.....	26
HTLV-1 microRNA.....	26
HBZ Animal Studies.....	27
Rabbits	27
NOD/SCID/IL-2R γ^{null} Mice	28

Transgenic Mice	28
Macaques.....	29
New Theory of ATL Development.....	29
Cellular Transcriptional Coactivators p300 and CBP	31
Functions of p300/CBP.....	31
The KIX Domain of p300/CBP.....	34
The Two Surfaces of KIX.....	34
Consensus Binding Motif on Proteins Contacting the KIX Domain.....	35
Cooperative Binding to the KIX Domain.....	36
HBZ Binds the KIX Domain of p300/CBP	36
Summary of Findings.....	37
CHAPTER 2: DATA.....	39
Introduction.....	39
HBZ Binds with High Affinity to the KIX Domain of p300/CBP	39
Two ϕ XX $\phi\phi$ Motifs in HBZ-AD Mediate Interaction with KIX	47
HBZ-AD is Predicted to Form Two Amphipathic α Helices.....	53
Stoichiometry of the HBZ-AD•KIX Complex is 1:1.....	53
KIX Mutagenesis Indicates HBZ-AD Binds the MLL-AD-Binding Surface.....	61
HBZ-AD Competes with MLL-AD for Binding to KIX	67
HBZ-AD Competes with p53 for Binding to KIX	72
HBZ-AD Competes with Tax for Binding to KIX.....	75
HBZ-AD Fails to Compete with c-Jun for Binding to KIX.....	75
c-Jun Requires KIX Residues Necessary for c-Myb-AD/pKID Binding.....	80

Mutations Target a Novel Putative Binding Site on KIX	83
HBZ-AD Enhances the Binding of c-Myb-AD to KIX	84
HBZ-AD Enhances the Binding of Unphosphorylated CREB to KIX	90
Full-Length HBZ-Sp1 Fails to Inhibit Binding of pCREB or CREB to a CRE.....	99
Docking Programs Place Predicted HBZ-AD Structure at MLL-AD-Binding Site	102
CHAPTER 3: DISCUSSION	107
Model of HBZ-AD Binding to KIX.....	108
HBZ-AD Tertiary Structure and Anomalous SDS-PAGE.....	109
Flexibility of the MLL-AD-Binding Surface	110
Two Models Proposed for HBZ-AD Binding to KIX.....	111
Variation in Effects of HBZ-AD and MLL-AD on pCREB Binding to KIX.....	112
Protein Length and Phosphorylation may Mediate HBZ•CREB Interactions	113
A Model of Transcriptional Deregulation by HBZ.....	115
Oncogenic Potential of Deregulated MLL, c-Myb, CREB, p53 and p300/CBP	116
Summary	118
CHAPTER 4: MATERIALS AND METHODS	119
Bacterial Expression Vectors and Cloning.....	119
Expression and Purification of Recombinant Proteins	120
Electrophoretic Mobility Shift Assays and Apparent K_d Analysis	121
Crosslinking Assays	123
GST Pull-Down Assays.....	123
Cell Culture, Transient Cotransfection Assays and Luciferase Assays	124
REFERENCES	126

LIST OF TABLES

Table 1.	Summary of Probabilities of α -Helical Formation in HBZ-AD	54
Table 2.	Summary of GST-KIX Mutants	65
Table 3.	K _d Values of KIX-Binding Proteins	96

LIST OF FIGURES

Figure 1.	Schematic representation of the HTLV-1 genome, mRNA and proteins	2
Figure 2.	Schematic representation of HBZ-Sp1	20
Figure 3.	Schematic representations of p300/CBP and KIX.....	32
	A. Schematic representation of p300/CBP	
	B. Schematic representation of KIX	
	C. KIX folds into a three-helical bundle	
Figure 4.	HBZ-AD binds the KIX domain of p300/CBP with high affinity	40
	A. EMSA determination of apparent K_d for Gal4DBD-HBZ-AD•GST-KIX	
	B. Binding curve for Gal4DBD-HBZ-AD•GST-KIX	
	C. Coomassie-stained gel shows purified proteins for K_d reactions	
Figure 5.	Controls indicate Gal4DBD-HBZ-AD interacts specifically with GST-KIX...	43
Figure 6.	Full-length HBZ-Sp1 binds the KIX domain with high affinity.....	45
	A. EMSA determination of apparent K_d for Gal4DBD-HBZ-Sp1•GST-KIX	
	B. Binding curve for Gal4DBD-HBZ-Sp1•GST-KIX	
Figure 7.	Gal4DBD-HBZ-AD binds with high affinity to full-length p300.....	48
Figure 8.	Two $\phi XX\phi\phi$ motifs in HBZ-AD mediate binding to GST-KIX.....	50
	A. Sequences of HBZ-AD _{wt} and mutated $\phi XX\phi\phi$ motifs	
	B. HBZ-AD's $\phi XX\phi\phi$ motifs contribute unequally to GST-KIX binding	
Figure 9.	HBZ-AD is predicted to form an amphipathic α -helical hairpin.....	55
	A. Helical wheel analysis shows HBZ-AD's α helices are amphipathic	
	B. HBZ-AD is predicted to form a hairpin structure	

Figure 10.	Stoichiometry of the HBZ-AD•KIX complex is 1:1.....	58
	A. BS ³ crosslinking assay	
	B. Coomassie-stained gels show purified proteins for BS ³ assay	
Figure 11.	HBZ-AD binds the MLL-AD-binding surface of GST-KIX.....	62
	A. Locations of mutated KIX residues are indicated	
	B. HBZ-AD binds the MLL-AD-binding surface of GST-KIX	
	C. Coomassie-stained gels show purified GST and GST-KIX proteins	
Figure 12.	HBZ-AD competes with MLL-AD for binding to GST-KIX.....	68
Figure 13.	Full-length HBZ-Sp1 represses transcriptional activity of MLL in cells	70
	A. HBZ-Sp1 represses activity of Gal4DBD-MLL-AD	
	B. HBZ-Sp1 represses activation of MLL-responsive <i>Hox-c8</i> promoter	
Figure 14.	HBZ-AD competes with p53 for binding to GST-KIX.....	73
Figure 15.	HBZ-AD competes with Tax for binding to GST-KIX	76
Figure 16.	HBZ-AD fails to compete with c-Jun for binding to GST-KIX	78
	A. HBZ-AD does not compete with c-Jun for binding to GST-KIX	
	B. HBZ-AD enhances binding of c-Jun to GST-KIX	
Figure 17.	c-Jun requires KIX residues necessary for c-Myb-AD and pKID binding	81
	A. c-Jun exhibits unexpected binding pattern to GST-KIX mutants	
	B. Locations of mutated GST-KIX residues are indicated	
	C. KIX residues shifted in NMR are dispensable for c-Jun binding	
Figure 18.	HBZ-AD enhances binding of c-Myb-AD to GST-KIX	85
	A. HBZ-AD enhances binding of c-Myb-AD to GST-KIX	
	B. Full-length GST-HBZ-Sp1 does not interact with c-Myb-AD	

Figure 19.	HBZ-Mut-AD3 does not enhance binding of c-Myb-AD to GST-KIX.....	88
Figure 20.	Full-length HBZ-Sp1 enhances activity of c-Myb-AD in cells	91
Figure 21.	HBZ-AD does not enhance binding of pCREB to GST-KIX.....	93
Figure 22.	HBZ-AD enhances binding of unphosphorylated CREB to GST-KIX.....	97
	A. HBZ-AD enhances binding of CREB to GST-KIX	
	B. HBZ-AD does not interact with GST-CREB	
Figure 23.	Full-length GST-HBZ-Sp1 fails to inhibit pCREB or CREB binding to CRE	100
	A. Full-length GST-HBZ-Sp1 does not inhibit binding of pCREB to CRE	
	B. Full-length GST-HBZ-Sp1 shifts the unphosphorylated CREB•CRE complex	
Figure 24.	HADDOCK docks HBZ-AD at the MLL-AD-binding surface of KIX.....	104

LIST OF ABBREVIATIONS

A	adenine
AATF-1	apoptosis antagonizing transcription factor-1
AD	activation domain
AMP	adenosine monophosphate
AP-1	activator protein 1
ATCC	American type culture collection
ATF-1, -2, -3, -7	activating transcription factor-1, -2, -3, or -7
ATF-4	activating transcription factor-4 (AKA CREB-2)
ATL	adult T-cell leukemia
ATP	adenosine triphosphate
AZT	Zidovudine
B cells	bursa/bone marrow-derived cells
Bax	Bcl-2-associated X protein
Bcl-X	B-cell lymphoma 2-related gene
Br	bromodomain
BR1, 2, or 3	basic region 1, 2, or 3
BS ³	bis(sulfosuccinimidyl)suberate
bZIP	basic leucine zipper
C	cytosine
C2	carboxyl-terminal region 2
cAMP	cyclic adenosine monophosphate
CAPRI	critical assessment of predicted interactions
CBP	CREB-binding protein
CD	cluster of differentiation
CDK4	cyclin dependent kinase 4
CEBPG	CCAAT/enhancer-binding protein gamma
CH1, 2, or 3	cysteine/histidine-rich domain 1, 2, or 3
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
CREB-2	cyclic AMP response element binding protein-2 (AKA ATF-4)
CREM	cyclic AMP-responsive element modulator
CTL	cytotoxic T lymphocyte
D	aspartic acid
DBD	DNA-binding domain
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ΔG	delta G (change in Gibbs free energy)
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
<i>env</i>	<i>envelope</i>
FOXO3	forkhead box O3
G	glycine; guanine
G1 phase	gap 1 phase

G2 phase	gap 2 phase
<i>gag</i>	<i>group antigens</i>
Gal4DBD	Gal4-DNA binding domain
GST	glutathione S-transferase
HA	hemagglutinin
HAM/TSP	HTLV-associated myelopathy/tropical spastic paraparesis
HAT	histone acetyltransferase
HBZ	HTLV-1 basic leucine zipper factor
HBZ-AD	HBZ activation domain
HBZ-Mut-AD1	HBZ-AD mutant 24-VDGLL-28 → 24-VDGAA-28
HBZ-Mut-AD2	HBZ-AD mutant 44-LDGLL-48 → 44-LDGAA-48
HBZ-Mut-AD3	HBZ-AD mutant 24-VDGLL-28/44-LDGLL-48 → 24-VDGAA-28/44-LDGAA-48
HBZ-Sp1	HBZ major splice variant
HBZ-Sp2	HBZ minor splice variant
HDAC	histone deacetylase
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HM	HEPES/MgCl ₂
<i>hox</i>	homeobox containing gene
hTERT	human telomerase reverse transcriptase
HTLV-1, -2, -3, -4	human T-cell leukemia virus type 1, 2, 3, or 4
IBiD	interferon response factor-binding domain
IFN-α	interferon-alpha
IL-2Rα	interleukin-2 receptor alpha
IL-2γ	interleukin-2 receptor gamma
kcal	kilocalorie
KCl	potassium chloride
K _d	equilibrium dissociation constant
kDA	kilodalton
KID	kinase inducible domain
KIX	kinase-inducible interaction
L	leucine
Lck	lymphocyte-specific protein tyrosine kinase
LTR	long terminal repeat
M phase	mitosis phase
Mad1	mitotic arrest-deficient 1
MARE	MAF recognition element
MgCl ₂	magnesium chloride
MHC-1	major histocompatibility complex class I
MLL	mixed lineage leukemia
MLL-AD	mixed lineage leukemia activation domain
μM	micromolar
mM	millimolar
mol	mole
mRNA	messenger RNA

NFAT	nuclear factor of activated T cells
NF- κ B	nuclear factor kappa-light-chain enhancer of activated B cells
ng	nanogram
Ni-NTA	nickel-nitrilotriacetic acid
NK	natural killer
nM	nanomolar
NMR	nuclear magnetic resonance
NOD/SCID	nonobese diabetic/severe combined immunodeficiency
NOG	NOD/SCID/IL-2R γ^{null}
Nonidet P40	Tergitol-type nonylphenoxypolyethoxylethanol
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pCREB	phosphorylated cyclic AMP response element binding protein
pdb	protein data bank
pKID	phosphorylated kinase inducible domain
pM	picomolar
<i>pol</i>	<i>reverse transcriptase</i>
ϕ	phi, standing here for hydrophobic amino acids
R	gas constant
Rb	retinoblastoma protein
RNA	ribonucleic acid
RNAi	RNA interference
S phase	synthesis phase
S.E.	standard error
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	small (or short) hairpin RNA
siRNA	small (or short) interfering RNA
SREBP	sterol-responsive element binding protein
SRF	serum response factor
STLV-1, -2, -3	simian T-cell leukemia virus type 1, 2, or 3
T	thymine; temperature
T cells	thymus-derived cells
TAZ2	transcriptional-adaptor zinc-finger-2 domain
TM	Tris/MgCl ₂
Tris	tris(hydroxymethyl)aminomethane
TxRE	Tax responsive element
UAS	upstream activating sequences
usHBZ	unspliced HBZ
V	valine
vCRE	viral cyclic AMP response element
WB	Western blot
wt	wild type
X	any amino acid
ZIP	leucine zipper
ZnSO ₄	zinc sulfate
ZZ	zinc-binding domain near the dystrophin WW domain

CHAPTER 1: REVIEW OF THE LITERATURE

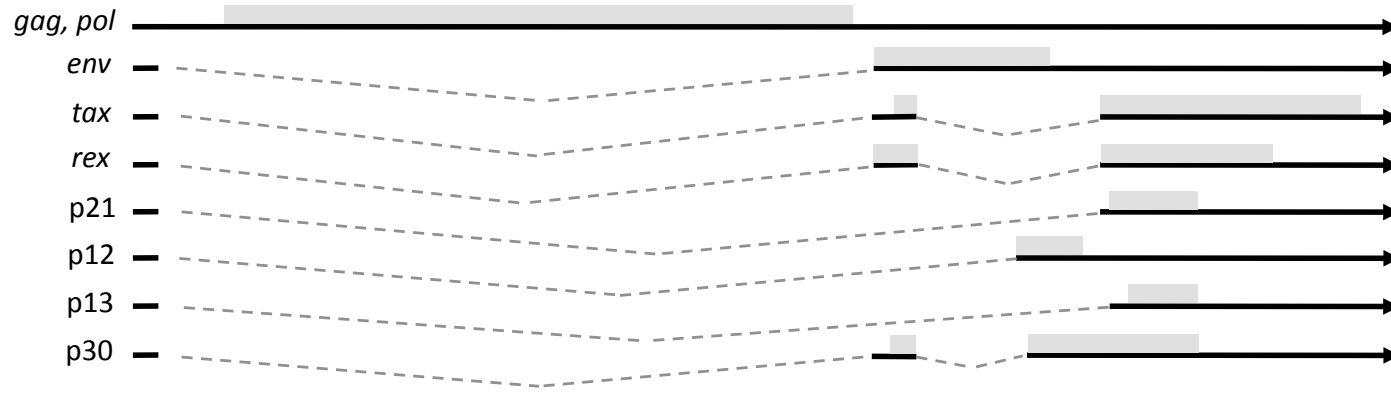
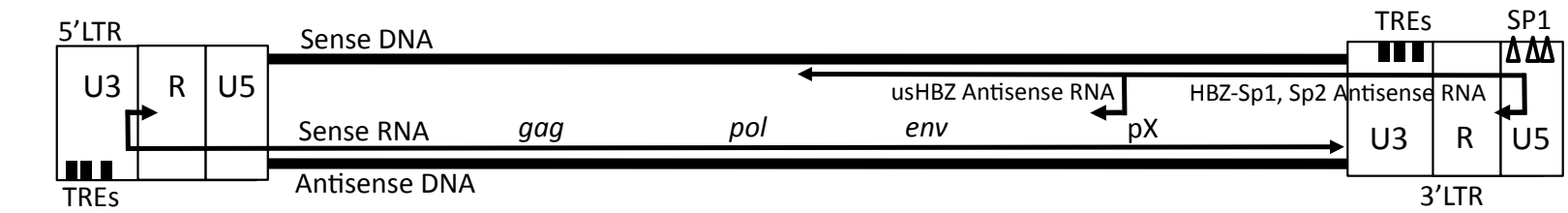
Introduction

Human T-cell leukemia/lymphoma virus type 1 (HTLV-1) is a human retrovirus associated with several distinct disorders, including adult T-cell leukemia/lymphoma (ATL) and two identical neurodegenerative conditions referred to as HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1]. HTLV-1, identified in the early 1980s, was the first human oncogenic retrovirus discovered [2-6]. Much research since then has sought to elucidate the mechanisms of HTLV-1-mediated ATL as well as HAM/TSP. The focus of this literature review is mainly on the role of HTLV-1 in the pathogenesis of ATL.

An HTLV-1 transcript, originating in the 3' long terminal repeat (LTR) and transcribed from the sense DNA strand relative to other viral genes, was reported in 1989 (Figure 1) [7]. This gene, HTLV-1 basic leucine zipper factor (HBZ), was further characterized in 2002 [8]. HBZ, unlike the viral transactivator Tax and other viral proteins, is expressed in almost all ATL cases [9, 10]. Based on these and other findings, it is now proposed that HBZ, along with Tax, plays a significant role in the transformation of infected T cells as well as in the maintenance of the leukemic phenotype.

Efforts are currently focused on determining the functions of HBZ in the persistence and transformation of infected T cells during the development of ATL. HBZ has been shown to down-regulate expression of HTLV-1 genes [8, 11-13] as well as significantly deregulate over 1,400 cellular genes [9]. Deregulation of cellular transcription is considered to be a key event in oncogenesis, and I have shown that a potential mechanism of HBZ-mediated deregulation occurs via dual effects of HBZ on the kinase-inducible

Figure 1. Schematic representation shows the HTLV-1 genome, messenger RNA (mRNA) and proteins. Bold black lines denote sense and antisense strand HTLV-1 genomic DNA flanked by 5' and 3' LTRs. Thin black lines indicate the direction of RNA synthesis. HBZ antisense mRNA is transcribed from the DNA sense strand (relative to other viral genes). Tax responsive elements are shown as black rectangles; Sp1 transcription factor binding sites are shown as triangles. Spliced mRNA is shown as black lines, gray boxes represent translated proteins, and dashed lines indicate spliced regions. Variations in N-termini of HBZ isoforms are shown.



MVNFVSA GLFRCLPYSCPED
 MAAS GLFRCLPYSCPED
 -TSRVRSVESRSL GLFRCLPYSCPED

usHBZ
 HBZ-Sp1
 HBZ-Sp2

usHBZ
 hbz-Sp1
 hbz-Sp2

interaction (KIX) domain of the homologous cellular transcriptional coactivators p300 and CREB-binding protein (CBP). HBZ competes with one group of proteins for binding to a particular KIX surface while enhancing the binding of two other proteins at a different surface of KIX. HBZ has previously been shown to compete with Tax for KIX binding, and I have shown that HBZ also competes with the cellular transcriptional regulator mixed lineage leukemia (MLL) protein and the cellular transcription factor p53 for occupancy of KIX. In contrast, HBZ enhances the binding of two other cellular transcription factors, c-Myb and the cyclic AMP (cAMP) response element binding protein (CREB), to a different surface on KIX.

HTLV-1: Overview and Epidemiological Data

Discovery

HTLV-1 was originally isolated and identified from ATL cell lines by two groups working independently in the US and Japan [2-6]. The virus was causally linked to the development of ATL in the mid 1980s [14]. Four HTLV isotypes have now been identified, including HTLV-2 [2], HTLV-3 [15], and HTLV-4 [16, 17]. Only HTLV-1 is associated with ATL [18]. HTLV-1 and the other HTLV isotypes are members of deltaretroviridae, a family that also includes bovine leukemia virus and the simian T-cell leukemia viruses STLV-1, -2, and -3 [19, 20]. In addition to causing ATL, HTLV-1 is also the pathogenic agent of HAM/TSP [18, 21, 22]. HTLV-1 is also associated with several other disorders [18] including uveitis [23], arthropathy [24], and infective dermatitis [25].

Epidemiology of HTLV-1

Current reviews cite that approximately 10-20 million people worldwide are infected with HTLV-1, but this estimate, originally cited in 1996 [26, 27], is based on data

that are now over 25 years old [28]. In addition, those data were derived mainly from serological screening of blood donors, a population thought to under-represent groups at high risk for HTLV-1 infection [18, 28]. Although a study in 2009 found there were insufficient published data to estimate a more current global prevalence [28], it is known that HTLV-1 infection is most endemic in Japan [18], with a current prevalence as high as 36.4 % in Tsushima and 17 % in Okinawa [28]. Other areas with significant HTLV-1 prevalence include some Caribbean islands, regions in sub-Saharan Africa, and certain parts of South America [18].

Transmission and Tropism of HTLV-1

HTLV-1 is spread through breastfeeding, sexual intercourse, intravenous (IV) drug use, and, prior to blood bank screening, by IV blood transfusion [18]. The virus is thought to spread mainly via cell-to-cell contact, since free virus particles are not efficiently infective [1, 18]. Persistence in the host is thought to be mainly sustained through virally-induced proliferation of infected cells [29, 30]. Persistence via cell proliferation is thought to account for the relative stability of the HTLV-1 genome compared with that of HIV, since HTLV-1 is replicated by the host cell machinery during the process of mitotic division rather than by the error-prone activity of reverse transcriptase [29]. It was recently found, however, that cell-free virus could infect myeloid and plasmacytoid dendritic cells, which were then able to infect CD4⁺ T cells, the exclusive cell type found to be monoclonally expanded in ATL [31, 32]. In a related study using Rhesus macaques infected with HTLV-1, results linked the ability of HTLV-1 to replicate in dendritic cells with its ability to maintain infection in the host, leading to speculation that infection of dendritic cells may be required for persistent infection in primates [33]. In addition to dendritic cells, HTLV-1 can also

infect other cell types including CD8+ T cells, B cells, monocytes and macrophages [34-41]. Notably, recent studies have also shown that HTLV-1 can infect hematopoietic stem cells [42-47].

HTLV-1 Persistence

Most people infected with HTLV-1 remain asymptomatic carriers during their lifetime [48]. ATL develops in approximately only 6.6 % of males and 2.1 % of females infected with HTLV-1 [48, 49]. HAM/TSP occurs in approximately 1 in 1,464 of HTLV-1-positive individuals, with a male:female ratio of 1:2.9 [50]. It is thought that HTLV-1 remains latent for decades prior to the onset of ATL. This is based on the assumption that most ATL patients were infected via breastfeeding [51] and the observation that ATL does not typically occur until mid- to late life [18, 48]. There is currently some controversy as to whether the virus is truly latent during this time or if a low level of viral transcription occurs [29]. Supporting the latter, there is evidence of a chronic cytotoxic T-lymphocyte (CTL) response to HTLV-1 antigens in some, but not all, infected carriers [29], which also suggests that expression of HTLV-1 may vary among individuals. A humoral antibody response is thought to occur in all infected individuals, although antibodies to Tax occur in 71 % - 93 % of HAM/TSP patients but in only 4 % - 31 % of ATL patients, compared to 27 % - 37 % of asymptomatic carriers [29, 52, 53].

The occurrence of HAM/TSP [54, 55] or ATL is almost always mutually exclusive [50, 56, 57], and it has been hypothesized that individual differences in parameters such as mode of infection, human leukocyte antigen (HLA) haplotype, and initial viral load may predispose certain individuals toward the development of one disease or the other [56].

ATL

ATL is defined as the monoclonal expansion of a single HTLV-1 infected CD4+ T cell [32, 48], although a proliferating oligoclonal population has been shown to precede development of monoclonal ATL [58]. The vast majority of ATL cases accordingly occurs in HTLV-1-infected hosts, although there have been limited reports of cases appearing to be ATL without apparent evidence of HTLV-1 infection [59, 60]. Interestingly, there have been multiple cases of ATL reported in which the complete provirus was not intact, and at least one study has correlated the presence of fragmented provirus inserts retaining the pX region of the genome with the development of ATL [61-64]. The age of onset is correlated with geographical location and ranges from 40 to 60 years of age [18, 48]. As mentioned previously, it is thought that the virus remains latent for many years prior to the onset of disease. Reports vary widely, however, on the estimated time of latency, ranging from 20 to 60 years [48, 65-69].

Diagnostic Criteria and Symptoms of ATL

Four subtypes of ATL exist, including acute, lymphomatous, smoldering and chronic. Acute and lymphomatous types progress rapidly, usually resulting in death within one year from diagnosis, whereas the chronic and smoldering types have a slower clinical course [48, 69]. A hallmark feature of ATL is the presence of T lymphocytes with hyper-lobulated nuclei referred to as “flower cells” [69]. Another diagnostic criterion for ATL is the presence of a single band in a Southern blot using digested genomic DNA and an HTLV-1 DNA probe, indicating monoclonal integration of the provirus in leukemic cells [69]. Additionally, the presence of serum antibodies to HTLV-1 antigens also indicates HTLV-1 infection [69]. Symptoms of ATL include lymphadenopathy, skin lesions,

hepatosplenomegaly and hypercalcemia [69]. Causes of death are most often pulmonary complications, opportunistic infections resulting from an immunocompromised state, or sepsis [67, 69].

ATL Treatment

Several treatment strategies exist for ATL, including intensive chemotherapy, antiretroviral therapy, the use of monoclonal antibodies to target malignant cell surface antigens, and allogeneic bone marrow transplantation [69, 70]. Chemotherapy has had limited effectiveness due to the resistance of ATL cells [48, 69, 71]. Trials using a combined antiretroviral therapy of Zidovudine (AZT) and interferon (IFN)- α appears to slightly lengthen the mean survival time for patients with acute, chronic and smoldering ATL [70]. Clinical trials are currently evaluating the effectiveness of monoclonal antibodies against two cell-surface markers expressed on ATL cells: the chemokine receptor CCR4 and the antigen CD25 (IL-2 receptor α) [70]. Allogeneic bone marrow transplantation has shown promise in extending the mean survival time of patients and is thought to be a potential cure for ATL, although graft versus host disease continues to be a significant issue [69, 70, 72, 73]. Other therapies currently being investigated include, among others, inhibitors of NF- κ B [70] and histone deacetylases (HDACs) [70, 74], arsenic trioxide, retinoid derivatives, the immunomodulatory drug Revlimid, and additional monoclonal antibodies to the ATL cell-surface antigens CD2, CD52, CD71, and CD30 [70].

Vaccine Development

Since Tax is considered the dominant immunogenic HTLV-1 protein, vaccine development has previously focused on Tax-specific CTLs [70]. Multivalent Tax peptide vaccines have been shown to produce Tax-specific CTLs in a mouse model [75, 76].

Although such a vaccine may be beneficial for patients expressing Tax, Tax expression is absent in the majority of ATL cases. It was proposed that HBZ, which has been shown to be consistently expressed, may be a promising target for immunotherapy [77]. An initial effort to isolate a high-affinity HBZ-specific CTL clone capable of lysing ATL cells was unsuccessful [77]. However, a new study has shown that the CTL clone isolated in that study is able to effectively lyse HTLV-1-infected cells from asymptomatic carriers and HAM/TSP patients [78]. Furthermore, analysis of 432 HTLV-1-infected individuals showed that those with HLA class I alleles that bind HBZ peptides exhibit significantly less proviral load and are more likely to remain asymptomatic than those whose HLA class I alleles do not bind HBZ [78]. Surprisingly, it was found that CD8⁺ cells specific for HBZ, not Tax, were most effective at controlling HTLV-1 [78]. This was unexpected given that Tax is more immunogenic than HBZ and had previously been thought to be the best target for a potential vaccine [78]. The study concluded that the ability of an individual's HLA class I alleles to recognize HBZ was a central determinant in their ability to generate a protective CD8⁺ response against HTLV-1 infection.

HTLV-1 Molecular Characterization

HTLV-1 Genome and Integration

The HTLV-1 genome is typical of complex retroviruses, with a 5' and 3' LTR flanking the *group antigens (gag)*, *reverse transcriptase (pol)*, and *envelope (env)* genes in addition to a pX region from which the regulatory and accessory genes of HTLV-1 are expressed (Figure 1) [48, 79]. The virus is thought to enter cells via a viral synapse formed between two cells [80, 81]. Once inside the host cell, the HTLV-1 RNA genome is reverse transcribed into DNA by reverse transcriptase and integrates into the host genome [82]. Reports vary

regarding the random nature of integration by HTLV-1, but it has recently been found that integration at active areas of transcription may favor the development of HTLV-1-mediated pathology [83-87].

Transactivation of HTLV-1 Genes

Once integrated into the host genome, HTLV-1 utilizes the cellular transcriptional machinery to express viral genes [88]. Tax, arising from a doubly spliced transcript, is the HTLV-1 transactivator [89-92]. The HTLV-1 promoter is located in the U3 region of the 5' LTR and minimally consists of three unequally spaced Tax-responsive elements (TxREs) (Figure 1) [93, 94]. The TxREs contain an imperfect cAMP response element (CRE) referred to as the viral CRE (vCRE) [95] that has been reported to interact weakly with various members of the activating transcription factor (ATF) and CREB family of transcription factors that includes: ATF-1, ATF-2, and CREB [96-98]; CREB-2 [99, 100]; and cAMP-responsive element modulator (CREM) [101]. In addition, the vCREs are reported to interact with c-Jun and c-Fos, two members of the activator protein 1 (AP-1) family [102]. Binding of ATF/CREB members to the vCREs is stabilized by Tax, which recognizes GC-rich areas flanking the vCREs [97, 98, 103, 104]. It is thought that basal expression levels of HTLV-1 induced by the transcription factors Sp1 [105], serum response factor (SRF) [106] and possibly c-Jun [102] produce Tax, which then forms a complex with ATF/CREB at the viral promoter [98, 103, 107-111]. Formation of this complex then leads to recruitment of the transcriptional coactivators p300 or CBP, resulting in activated viral transcription [112-115]. This process involves direct interactions between the KIX domain of the coactivators and Tax as well as between KIX and CREB [112-114, 116].

HTLV-1 Accessory Genes

In addition to Tax, HTLV-1 encodes another regulatory gene, Rex [117, 118], which has been shown to repress viral transcription [32, 119-121]. HTLV-1 also encodes several accessory genes from its pX region, including p12, p13, p21, and p30 [122-124]. These genes have been found to function in the establishment of persistent infection, facilitation of cell survival, and down-regulation of immune function [48, 122-124]. The accessory protein p12 has been shown to activate the nuclear factor of activated T cells (NFAT) [125] and enhance proliferation of T cells as well as suppress expression of the major histocompatibility complex class I (MHC-I) [48, 126]. Functions of p30 include translational repression of Rex and Tax transcripts and also repression of HTLV-1 transcription through interaction with the KIX domain of p300/CBP [48, 127].

Tax

The Pleiotropic Functions of Tax

Tax became the focal point in the search for an oncogenic mechanism after it was found to transactivate the HTLV-1 genome as well as several cellular genes [32]. Many studies since then, most of which have utilized Tax overexpression assays, have revealed that Tax can inhibit the activity of tumor suppressors, cause abnormal progression of the cell cycle, repress DNA repair, inhibit apoptosis and deregulate several transcriptional pathways including those of the nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B), CREB/ATF, AP-1, and SRF [32, 128-130].

Tax is thought to inhibit the activity of the tumor suppressor p53 by down-regulating p53-dependent transcription through competition with p53 for binding to the coactivator p300 [131, 132]. Tax also is reported to inactivate the tumor suppressor

retinoblastoma (Rb) protein via activation of the cyclin dependent kinase 4 (CDK4), allowing progression from the gap 1 (G1) phase to the synthesis (S) phase of the cell cycle [133]. In addition, Tax allows transition from the gap 2 (G2) phase to the mitosis (M) phase through its repression of the mitotic arrest-deficient 1 (Mad1) protein [134]. Tax compromises DNA repair by down-regulating the expression of DNA polymerase β [135] and DNA topoisomerase I [136]. Effects on apoptosis are reported to occur via Tax-mediated activation of NF- κ B [137, 138] and the B-cell lymphoma 2-related gene Bcl-X [139] and through repression of the Bcl-2-associated X protein (Bax) [140]. Tax also up-regulates expression of interleukin-2 receptor alpha (IL-2R α) through the NF- κ B pathway [141-143], but this was later found to be insufficient for T-cell growth due to the requirement for both alpha and beta subunits for IL-2R function [32].

The deregulation of cellular transcription by Tax can, in general, be categorized as up-regulation of pathways that promote cellular growth and down-regulation of genes that suppress growth [32]. Microarray data have shown that Tax-mediated deregulation extends to approximately 300 cellular genes [144]. However, despite the accumulated data showing Tax can produce a complex web of cellular dysfunction, no defined mechanism of oncogenesis has emerged [145].

The Oncogenic Potential of Tax

Initial evidence that Tax possesses oncogenic potential came from studies showing it can, when co-transfected with the *ras* oncogene, transform rodent embryo fibroblasts and induce tumors in nude mice, although neither *tax* nor *ras* alone could transform or induce tumors [146, 147]. Tax was shown to transform the already-immortalized rodent fibroblast line Rat-1 [146] and induce growth in the NIH 3T3 rodent cell line [148]. In

addition, numerous transgenic mice studies have shown that a variety of tumor types result from the expression of *tax* under various promoters [145, 149-154], although it was not until recently that a phenotype similar to ATL was achieved by expression of *tax* under control of the lymphocyte-specific protein tyrosine kinase (Lck) promoter [155, 156]. This and an earlier study showing that *tax* expression under control of the granzyme B promoter could induce lymphocytic leukemia [157] were the two models confirming that Tax alone was capable of leukemic and lymphomic transformation of T cells in mice [158].

Studies using primary human thymocytes showed that retroviral vectors expressing *tax* or the entire *pX* region of HTLV-1 could infect and immortalize primary human T cells [159, 160]. Immortalization of human primary cells refers to the process by which cultured cells are induced to a state of indefinite proliferation in the presence of IL-2, whereas oncogenic transformation of human primary cells refers to a state of IL-2-independent growth. Importantly, Tax alone has never been shown to transform human primary cells. Furthermore, the degree to which the effects of Tax summarized here recapitulate its *in vivo* function in humans during the development of ATL appears to be the subject of a growing controversy [161, 162].

A Tax Reassessment

Recent discoveries in comparative cancer biology impact many of the conclusions drawn from the above studies and add to the mounting argument against Tax as the sole mediator of oncogenesis [162-165]. For example, it has recently been shown that normal rodent cells can be transformed by perturbing two different signaling pathways, but transformation of analogous human cells requires perturbation of six pathways [162]. The early *in vitro* experiments in which *tax* alone, without *ras*, was unable to transform primary

rodent cells, which require disruption of only two pathways, cast doubt on the ability of *tax* alone to transform human cells, which require disruption in six pathways [161]. Since then, expression of *tax* under the Lck and granzyme B promoters has successfully induced leukemia and lymphoma in mice, but given the differences in susceptibility to oncogenic perturbations between mice and humans, these studies do not conclusively indicate that *tax* alone could cause ATL in humans [162, 165].

Re-Evaluating the Immortalizing Capability of Tax

The experiments indicating that Tax could immortalize primary human thymocytes relied on the transduction of *tax* using retroviral delivery vectors expressing part or all of their own genomes in addition to *tax* [159, 160]. In one case, the vector used encoded the entire *pX* region of HTLV-1 containing the complete 3' LTR, known now to include *hbx* and its promoter [160]. Based on the possibility that expression of these additional genes influenced results, a recent study re-evaluated the ability of Tax to immortalize primary human cells using a retroviral vector expressing no gene other than *tax* [161]. Surprisingly, they found that Tax possesses poor oncogenic activity in human cells and is rarely capable of immortalization. Furthermore, the only immortalized line produced in the study was thought to have resulted in part from random genetic events.

Regarding experiments in which Tax was shown to transform the already immortalized Rat-1 rodent fibroblasts, it is noteworthy to point out that, being immortalized, these cells had already undergone premalignant alterations allowing them to proliferate indefinitely in culture [164]. The premalignant condition of the cells combined with the finding that only two pathways need be disrupted for rodent cell transformation may limit the significance of these studies [161].

Also relevant to interpretation of these and other functional studies with Tax is the possibility that effects caused by Tax alone may not occur in the context of the complete HTLV-1 provirus. For example, it has been established that Tax promotes transition of the cell cycle from G1 to S phase [133]. However, it was recently found that infection with the complete HTLV-1 provirus arrests cells in G1, preventing transition to S phase [166].

Tax Silencing in ATL

One of the most difficult observations to explain regarding the idea that Tax is the sole mediator of oncogenesis is the lack of *tax* expression in the majority of ATL cases. In a study analyzing 41 ATL patient samples, Tax expression was found in only 34 % [167]. Studies before the use of the polymerase chain reaction (PCR) could not detect RNA for *tax* or any other viral gene in freshly isolated ATL cells [168-171]. A later study found via PCR that *tax* RNA was detected in fresh ATL cells but at 10^5 - 10^6 times less compared to an ATL cell line [172]. Subsequent studies have shown that in those cases of ATL in which *tax* is expressed, levels are significantly less than those found in asymptomatic carriers or HAM/TSP patients [167, 173].

Three mechanisms have so far been identified to account for the low or absent levels of *tax* expression in ATL patients: (1) deletion of the 5' LTR, which contains the viral promoter [63, 174]; (2) methylation of the 5' LTR at CpG sites [167, 175, 176]; and (3) mutations in the *tax* gene [145, 167, 177, 178].

Ex Vivo Cell Culture

It is interesting to note that fresh ATL cells cultured *ex vivo* for a period of days, presumably with an intact 5' LTR, have been shown to resume expression of viral RNA and antigens, including Tax [168-170, 179]. These culture-induced changes of the

transcriptional program of cells removed from their *in vivo* environment underscore concerns that immortalization or transformation in cell culture does not accurately reflect the process of *in vivo* oncogenesis [164]. A striking example is the ability of HTLV-2, which is not associated with ATL, to fully transform human primary CD8 T-lymphocytes to an IL-2 independent state in a Tax-dependent manner [180]. This finding is relevant when considering the significance of immortalization assays, as it indicates that the ability of a given gene or construct to immortalize or transform primary human T cells in culture may not be a valid indicator of its oncogenic properties *in vivo*, and perhaps vice versa.

Discovery of HBZ

A transcript arising from the 3' LTR of HTLV-1 and transcribed from the sense DNA strand (the strand complementary to that used to transcribe other viral genes) was identified as early as 1989 (Figure 1) [7]. The gene was not characterized, however, until 2002, when the protein was found to bind the bZIP domain of CREB-2 (ATF-4) in a yeast two-hybrid screen [8]. Since that time, much progress has been made toward elucidating the structure and functions of HBZ.

HBZ Expression in ATL Cells

Unlike Tax, HBZ expression is detected in essentially all ATL cases tested [9, 10]. In one study, HBZ-Sp1 mRNA was found in all but 5 of 72 samples tested, the exceptions being 3 asymptomatic carriers, one ATL patient and one ATL cell line, MT-1s, a line derived from the MT-1 line after many passages [10]. Of note, HBZ has been reported to be the only viral gene expressed in some cases of ATL [178]. In addition, the promoter of HBZ is located in the 3' LTR, which, in contrast to the 5' LTR, has been found intact in all cases of ATL tested [181, 182]. Moreover, a recent study showed that among 60 ATL cases, genetic alterations

leading to frame shifts or inappropriate stop codons were found to frequently occur in all known HTLV-1 genes except *hbz*, reportedly due to the action of a human cytidine deaminase that targets only one strand of viral DNA during reverse transcription [181]. Taken together, these findings, each derived from analysis of ATL patient samples, suggest a role for HBZ in the pathogenesis of ATL. In addition, expression levels of HBZ have been correlated with the severity of HAM/TSP, suggesting it may also contribute to the pathology of this disorder [183].

Antisense Transcripts in HTLV-2, HTLV-3, and HTLV-4

It was recently found that HTLV-2 encodes a gene analogous to HBZ [184]. This gene, the antisense protein of HTLV-2 (APH-2), lacks the basic leucine zipper domain of HBZ although it retains the ability to suppress Tax-2 mediated transcription [184]. In addition, HTLV-3 [185] and HTLV-4 [186] also have been reported to encode bZIP proteins on their minus strands. The following sections outline the major findings regarding HTLV-1 HBZ thus far.

Transcription of HBZ

HBZ Isoforms

Three isoforms of HBZ have been identified and termed unspliced (usHBZ), major spliced (HBZ-Sp1), and minor spliced (HBZ-Sp2) [9, 187, 188]. The amino acid sequences of usHBZ and HBZ-Sp1 are identical except for the first few amino acids at their N-termini (Figure 1) [188]. HBZ-Sp1 is translated into a 206 amino acid protein, while usHBZ results in 209 amino acids [188]. HBZ-Sp-2 encodes a 216-amino acid protein with approximately 14 amino acids at the N-terminus that differ from those of both usHBZ and HBZ-Sp1 [189]. HBZ-Sp1 mRNA has been reported to be the most abundant isoform detected in cells from

ATL patients and asymptomatic HTLV-1 carriers as well as in select HTLV-1 cell lines [10, 189]. In one study, the level of HBZ-Sp1 mRNA was approximately 4-fold higher than the level of usHBZ mRNA in each of the 54 patient samples tested [10]. The half-life of the HBZ-Sp1 protein also has been found to be longer than that of usHBZ in transiently transfected 293FT cells [182]. However, one study showed that mRNA levels of the usHBZ and HBZ-Sp1 were roughly equivalent in cells from ATL patients as well as in tested ATL cell lines, although HBZ-Sp1 resulted in more protein expression [188].

HBZ Promoter

The promoters of usHBZ and the spliced HBZ isoforms have been found to be TATA-less, resulting in characteristically scattered transcriptional start sites [9, 182]. The start sites of HBZ-Sp1 lie in the U5 and R regions of the 3' LTR [9], while the start sites of usHBZ are localized to the pX region of the genome [182]. There is no in-frame AUG initiator for HBZ Sp2 [187]. Analyses of the HBZ-Sp1 promoter have identified binding sites for the transcription factors Sp1 (not to be confused with the abbreviation for the major splice variant HBZ-Sp1), CREB, Tax, GATA-2 and AP-1 [182]. Further analysis showed that the transcription factor Sp1 binds the HBZ-Sp1 promoter at three sites and, based on its role in the regulation of housekeeping genes, is suggested to maintain constant levels of HBZ-Sp1 expression [182]. Although Tax also was found to activate the 3' LTR [190], its effects are not as strong as those on the 5' LTR [182]. Also, since HBZ expression is thought to remain consistent despite an absence of Tax expression in most ATL cells, the *in vivo* effect of Tax on HBZ expression may be limited [182]. Interestingly, it was found that the ability of Tax to induce expression of HBZ from the 3' LTR was dependent on the site of integration into the host genome [190].

Functions of HBZ

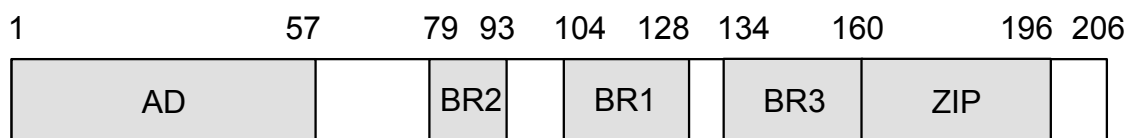
HBZ Protein Domains

HBZ consists of an N-terminus activation domain (AD), a central domain containing two basic regions (BR2 and BR1), and a basic leucine zipper (bZIP) that incorporates a third basic region (BR3) with a noncharacteristic DNA binding sequence upstream of the C-terminal leucine zipper (Figure 2) [8, 191-194]. Since the differences between usHBZ and the splice variants occur only at the N-terminus, the basic regions as well as the bZIP domains of all isoforms are the same. usHBZ has been shown to localize to the nucleus in a speckled pattern and to associate with heterochromatin when transfected into COS cells [8, 191]. This process was dependent on at least two intact basic regions in HBZ [191]. HBZ-Sp1, in addition to localizing to the nucleus in a speckled granular pattern, was also found in nucleoli [188]. Since usHBZ does not localize to nucleoli, this suggests a distinct function for each isoform [188]. Consistent with the nuclear localization of both usHBZ and HBZ-Sp1, microarray data have shown that HBZ deregulates the transcription of over 1,400 genes: 687 genes up-regulated by > 2 fold and 719 genes down-regulated by > 2 fold [9].

HBZ Represses Expression of HTLV-1 Genes

Transcription of HTLV-1 genes from the 5' LTR promoter is activated by the formation of a complex at the promoter consisting of Tax, an ATF/CREB family member, and p300/CBP. HBZ has been shown to down-regulate expression of HTLV-1 genes from the 5' LTR promoter in part through its association with ATF/CREB family members via interactions between their bZIP domains [8, 11, 13]. HBZ was reported to down-regulate expression of HTLV-1 genes via an interaction with CREB-2 [8]. It was further shown that HBZ was able to form a heterodimer with CREB-2 and prevent association of CREB-2 with

Figure 2. A schematic representation of HBZ-Sp1 shows the N-terminal activation domain (AD), three basic regions (BR2, BR1, and BR3) and the C-terminal leucine zipper (ZIP).



the vCREs at the 5' viral promoter, disrupting the CREB-2/Tax/coactivator complex required to activate transcription of viral genes [8]. HBZ was similarly shown to inhibit viral transcription through an interaction with the bZIP domain of CREB [13]. HBZ has also been shown to repress HTLV-1 transcription mediated by c-Jun, which is thought to produce basal-level expression from the 5' LTR [11].

It was subsequently found that HBZ could also down-regulate expression of HTLV-1 genes through its AD via an interaction with the KIX domain of p300/CBP [195]. This interaction with the KIX domain was found to be dependent on two ϕ XX $\phi\phi$ motifs in HBZ's AD, where ϕ is a hydrophobic amino acid and X is any amino acid. Consistent with these findings that HBZ down-regulates transcription of viral genes, a human lymphoblastoid B-cell line, 729-6, stably transfected with the ACH molecular clone of HTLV-1, showed lower levels of the HTLV-1 gag protein p19 than deletion mutants of the ACH clone not expressing HBZ [196]. Gag p19 is considered a reporter for the expression of all HTLV-1 genes, thus indicating that the absence of HBZ allowed for an increase in the expression of other viral proteins. A finding inconsistent with these is that when HBZ expression was knocked down using small hairpin RNA (shRNA) in the HTLV-1-infected cell line SLB-1, known to produce significant levels of HBZ, the expression of p19 remained unaffected [197].

In contrast to the ability of HBZ to down-regulate expression of HTLV-1 genes through its AD, it was recently shown that the AD of HBZ also mediates up-regulation of *Dkk1*, a gene that contributes to the development of bone lesions in multiple myeloma and may possibly function in the development of similar lesions found in ATL patients [198]. Interestingly, the data strongly suggested that up-regulation of *Dkk1* by HBZ occurred through an interaction between HBZ-AD and the KIX domain of p300/CBP [198].

HBZ Affects the Function of Multiple Cellular bZIP Proteins

The bZIP domain of HBZ has been found to interact with the bZIP domains of multiple cellular proteins that so far include: CREB [13]; CREB-2 [8]; ATF-1 [13, 199, 200]; ATF-2, ATF-3, and ATF-7 [200]; CREM-1a [13]; c-Jun and JunB [11]; JunD [201]; MAF, MAFB and MAFG [194]; and CCAAT/enhancer-binding protein gamma (CEBPG), CREBZF and c-Fos [194]. It was previously reported, however, that full-length usHBZ does not interact with c-Fos [202]. The bZIPs of HBZ were also reported to self-associate [194], but a previous study showed that full-length HBZ was unable to form dimers with the bZIP portion alone of HBZ [11]. Heterodimerization between the bZIP of HBZ and the bZIPs of some cellular proteins has been shown to prevent DNA binding by those cellular bZIPs, which include CREB [13], CREB-2 [8], ATF-2 and CEBPG [194]. Full-length usHBZ has been shown to inhibit the DNA-binding of c-Jun [11, 202] and JunB [11]. In contrast, it was concluded that heterodimers formed between the bZIP of HBZ and the bZIPs of either MAFB or MAFG retain the ability to bind a consensus MAF recognition element (MARE) [194]. Another study similarly showed that the bZIP of HBZ binds to full-length MAFB and enhances binding to a MARE sequence, but it was also found that full-length usHBZ not only prevents MAFB from binding the MARE sequence but also induces proteasomal degradation of MAFB [203]. Both the N- and C-termini of usHBZ were required for the degradation of MAFB.

An additional mechanism of HBZ-induced repression of c-Jun activity occurs via proteasomal degradation mediated by usHBZ, and this activity also is dependent on both the N- and C-termini of usHBZ but independent of ubiquitination [199, 202]. Notably, HBZ-Sp1 was less effective than usHBZ in promoting degradation of c-Jun [199]. Another study

using the major splice variant HBZ-Sp1 found that sequestration of c-Jun into inactive nuclear bodies was an additional and more dominant mechanism of HBZ-mediated repression of c-Jun activity than inhibition of DNA binding [12]. HBZ-Sp1 also sequesters JunB into nuclear bodies, representing an additional mechanism of HBZ-mediated repression for this factor [204]. In addition to c-Jun and MAFB degradation, full-length usHBZ has also been found to promote proteasomal degradation of JunB, JunD, ATF-4 and ATF-2, but not CREB or ATF-1 [199]. It was determined that the N-terminus of usHBZ interacts with the 26S proteasome, suggesting that HBZ tethers certain of its bZIP partners to the proteasome for degradation [199]. Interestingly, HBZ-Sp1 did not interact with the 26S proteasome as efficiently as usHBZ [199].

In contrast to HBZ's repressive effects on the above CREB/ATF and AP-1 family members, the activity of JunD is enhanced through its dimerization with usHBZ, which occurs in a manner dependent on the AD of HBZ [201]. A later study utilizing the major splice variant HBZ-Sp1 found that heterodimers between JunD and HBZ increase the expression of the human telomerase reverse transcriptase (hTERT) catalytic subunit gene. This activation was dependent on both the AD and bZIP domains of HBZ and occurred through interactions with the transcription factor Sp1 at the hTERT promoter, not via direct DNA binding by JunD or HBZ [205]. The dependence on the AD of HBZ in each of these cases suggests that variations in the N-termini of usHBZ and HBZ-Sp1 were insignificant in the findings.

HBZ Inhibits the Classical NF- κ B Pathway

While Tax activates both the classical and alternative NF- κ B pathways, HBZ has been shown to inhibit the classical pathway by decreasing the DNA binding of p65 and also

by inducing ubiquitin-dependent proteasomal degradation of p65 [206]. As with c-Jun and MAFB, the proteasomal degradation of p65 was found to depend on both the AD and bZIP domains of HBZ.

HBZ Induces Proliferation of T Cells

Multiple lines of evidence indicate that HBZ induces cell proliferation [9, 182, 197]. Two of these studies have examined the effects of short hairpin RNAs (shRNAs) against HBZ [9, 197]. Expression of HBZ was knocked down in the HTLV-1 transformed cell line SLB-1, resulting in significant suppression of T-cell proliferation [197]. In addition, small interfering RNAs (siRNAs) directed against HBZ in the HTLV-1-transformed MT-1 cell line also suppressed cell proliferation [9]. It was further determined that the proliferative function of HBZ, when stably transfected into the IL-2 dependent human T-cell line Kit 225, was due to HBZ RNA rather than protein [9].

HBZ RNA

HBZ RNA Affects Proliferation of T Cells

The conclusion that HBZ RNA rather than protein induced proliferation of T cells was based on experiments in which either HBZ protein synthesis was blocked while leaving HBZ RNA intact, or HBZ protein synthesis was allowed but RNA secondary structure was perturbed [9]. When the ATG initiation codon in an HBZ construct was mutated to TTG to block protein synthesis while leaving all other RNA intact, it was found that this mutant retained the ability to induce proliferation of cells [9]. A Western blot confirming that this mutant did not produce protein was published in a later study [182]. However, the possibility that translation of HBZ could have initiated at an alternative downstream ATG resulting in the ZIP domain was not addressed. This possibility bears on interpretation of

the Western blot, since a truncated HBZ would not have migrated at the expected position in the gel. Experiments showing the role of RNA in proliferation were based on silent mutations intended to disrupt the RNA secondary structure while leaving the amino acid sequence intact. These constructs failed to induce proliferation, leading to the conclusion that HBZ RNA rather than protein promotes cellular proliferation [9].

HBZ RNA Affects Transcription

The effects of HBZ RNA on cellular transcription also have been evaluated. Based on the same system described above to differentiate between HBZ protein and HBZ RNA functions, HBZ vectors encoding either wild type or the TTG mutant were used in microarray analyses to determine their effects on transcription [9]. It was determined that E2F1 as well as several of its target genes were significantly up-regulated by HBZ RNA [9]. Increased expression of E2F1 is associated with oncogenesis [207], and although Tax is known to up-regulate this gene [208, 209], overexpression of E2F1 has been found in ATL cell lines not expressing Tax [9]. This has led to the suggestion that HBZ RNA may up-regulate E2F1 expression in these cells.

HTLV-1 microRNA

A predicted RNA stem loop structure at the N-terminus of HBZ-Sp1 was determined to be critical for HBZ's RNA activity [9]. Also supporting a role for the N-terminus in the ability of HBZ to promote T-cell proliferation was the finding that usHBZ, which contains a different N-terminus, did not produce the same proliferative response as HBZ-Sp1 [182]. Since microRNAs also form stem loop structures, oligonucleotides from the *hbz* region predicted to form the RNA stem loop were used in a Northern blot analysis but failed to identify complementary microRNA [9]. A different study identified 10 sequences within

HTLV-1 using computational analysis that could potentially form microRNAs, four of which mapped to the minus strand [210]. Although one of the 10 predicted sequences was found within a small RNA library of MT-2 cells, no other experimental evidence supports the production of microRNA by HTLV-1. Infection with HTLV-1, however, has been found to alter the profile of cellular microRNAs that control functions such as cell survival, proliferation and immune surveillance [210-214]. In addition, although portions of HBZ RNA are complementary to transcript segments from other HTLV-1 genes, including *tax*, it has been concluded that HBZ-mediated repression of *gag*, *pol* or *tax/rex* does not occur through RNA interference (RNAi) [145, 196].

These studies have suggested a bimodal function of HBZ in which HBZ RNA induces cellular proliferation and HBZ protein represses expression of HTLV-1 genes [48, 178]. Further efforts are needed to distinguish functions of HBZ that are due to RNA versus those due to protein [145].

HBZ Animal Studies

Rabbits

Rabbits can easily be infected with HTLV-1 but do not typically develop symptoms of ATL [158, 215]. They have been used to study aspects of HTLV-1 infection such as transmission, infectivity and immune response. In one recent study, rabbits infected with either the wild type ACH HTLV-1 molecular clone or an HBZ deletion mutant (HTLV-1 Δ HBZ) were measured for proviral load and antibody production over an eight-week period [196]. Infection with wild type HTLV-1 produced a significantly higher proviral load than HTLV-1 Δ HBZ. Antibody production was first measured two weeks post infection, at which time wild type HTLV-1 showed a lower titer than HTLV-1 Δ HBZ. Subsequent titers at

two-week intervals showed that both wild type HTLV-1 and HTLV-1ΔHBZ continuously climbed, with wild type maintaining slightly higher levels overall than HTLV-1ΔHBZ. The interpretation of these data was that HBZ enhances infectivity and persistence. A related study obtained similar results in rabbits, showing that the absence of HBZ resulted in reduced proviral loads [33].

NOD/SCID/IL-2R γ ^{null} Mice

NOD/SCID/IL-2R γ ^{null} (NOG) mice are critically impaired in immune function. They lack B- and T-cell development and natural killer (NK)-cell activity, are deficient in interferon- γ production, and carry a mutation in the IL-2R γ gene. They therefore provide a useful model for ATL cancer cell engraftments [158]. A recent study assessing the effects of HBZ on cell proliferation *in vivo* utilized NOG mice subcutaneously injected with the SLB-1 ATL cell line expressing either wild type levels of HBZ or knocked down via stably transduced shRNA vectors [197]. All mice developed tumors, but the HBZ knockdown group exhibited significantly less cell proliferation, tumor growth and organ infiltration, indicating that HBZ functions in cell proliferation in NOG mice.

Transgenic Mice

HBZ was expressed in transgenic mice in CD4⁺ T cells under the mouse CD4 promoter/enhancer [9]. No difference was found in thymocyte subpopulations between the control mice and those expressing HBZ. However, the number of splenic CD4⁺ T cells in the HBZ expressing mice was slightly increased, suggesting that HBZ promoted proliferation of CD4⁺ cells.

Macaques

Rhesus macaques and other nonhuman primates can be infected with HTLV-1, and some studies have shown that they, unlike rabbits, are capable of developing symptoms associated with various HTLV-1-related disorders [33]. HBZ knockout studies have shown that HBZ contributes to HTLV-1 infectivity in macaques, although p12 and p30 were found to be more critical than HBZ for this function [33]. HBZ expression was knocked out in the macaques by creating a single nucleotide mutation resulting in a stop codon. Three out of 4 animals injected with the HBZ-knockout virus recovered the wild-type form, two fully sero-converted, and two only partially sero-converted. These results suggest a function for HBZ in the infectivity of macaques and possibly other primates [33].

New Theory of ATL Development

The findings that HBZ is consistently expressed in essentially all ATL cells and that it functions in cell proliferation, infectivity and persistence has led to a new working model of ATL development in which HBZ is thought to play a significant role [216]. Since Tax is not expressed in the majority of ATL cases, it is thought to exert its effects during the early stages of viral infection prior to the development of ATL. During this time, it is hypothesized that Tax induces premalignant changes in the cell, while HBZ influences the later course of infection. It is suggested that HBZ represses the expression of immunogenic viral proteins, allowing infected cells to escape immune detection. In addition, HBZ is thought to drive the proliferation of infected, premalignant cells, thereby maintaining a population that can undergo further oncogenic alterations ultimately resulting in ATL.

Consistent with this proposed two-step hypothesis are quantitative expression profiles of ATL cells and kinetic analyses of *in vivo* HTLV-1 gene expression upon infection

[10, 217]. These studies showed that Tax and other viral genes are expressed early in infection and that HBZ expression peaks over time while Tax expression diminishes.

This two-step model also provides a possible explanation for the recent finding that HBZ, not Tax, appears to be a critical target for a successful CD8⁺ response against HTLV-1 infection [78]. Cells with premalignant alterations caused by Tax early in infection might only evade detection by the immune system through the expression of HBZ, which could down-regulate the highly immunogenic Tax. If an individual does not have CD8⁺ T cells capable of detecting HBZ, the infected cells could then be driven to proliferate through the action of HBZ, leading to the second phase of oncogenesis [78].

Although HBZ was found to be dispensable for IL-2 dependent immortalization of human peripheral blood mononuclear cells (PBMCs) (containing a mixed population of mononuclear cells) by the ACH HTLV-1 molecular clone [193, 196], this does not rule out a possible transforming function of HBZ in T cells *in vivo*. As discussed earlier, *in vitro* immortalization does not recapitulate *in vivo* transformation. Additional events are believed to occur *in vivo* that culminate in complete transformation and the development of ATL. It is also noteworthy that none of the infectious molecular clones of HTLV-1 has been reported to transform human PBMCs *in vitro* to an IL-2-independent state, although we know that HTLV-1 is the causative agent of ATL *in vivo* [218-223].

Interestingly, HBZ protein has not been shown to be expressed from an HTLV-1 molecular clone in human PBMCs although it was reported [217]. In addition, HBZ protein is undetectable in infected rabbit PBMCs [217]. Although HBZ protein is found in most HTLV-1 cell lines, its presence in human patient samples has not been observed via Western blot though the presence of RNA has been consistently observed [9, 10, 188, 189].

A new study, however, has shown evidence of *in vivo* HBZ protein expression based on the presence of HBZ-specific CD8⁺ T cells in 31 % of 45 tested asymptomatic carriers [78]. Since it is well-documented that the transcription of HTLV-1 genes changes *ex vivo* [168-170, 179], it is reasonable to suggest that differences may exist in the expression of HBZ in *ex vivo* PBMCs compared to cells infected *in vivo*. Pertinent to this is the observation that HBZ RNA was expressed at significantly higher levels in ATL patient cells and ATL cell lines relative to asymptomatic carriers [10]. These data suggest that an *in vivo* event may be required for increased expression of HBZ. If so, the absence of this event could preclude observation of HBZ expression in *ex vivo* PBMCs as well as any potential transforming functions of HBZ.

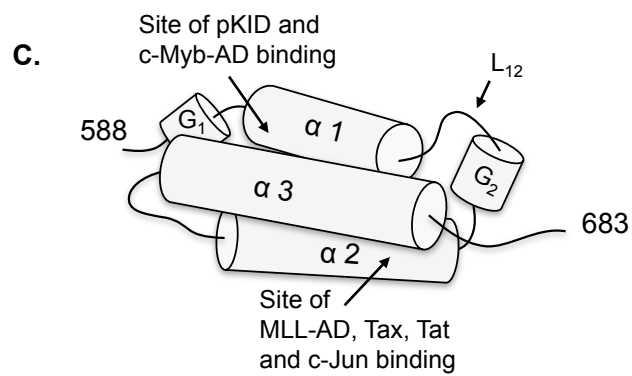
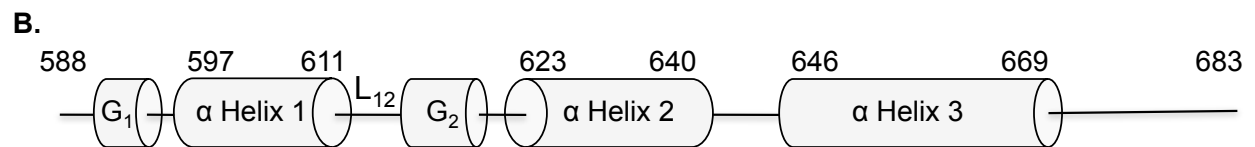
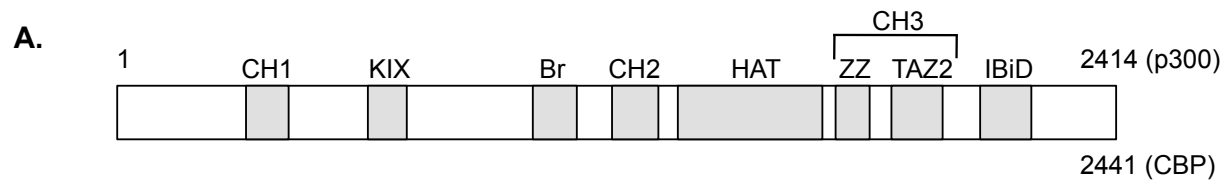
Cellular Transcriptional Coactivators p300 and CBP

The homologous cellular coactivators p300 and CBP are both large, multidomain proteins (Figure 3A). They are used by HTLV-1 to activate transcription of viral genes, but they are also required for the transcription of many cellular genes. Many transcription factors bind to p300/CBP, localizing the coactivators to promoters of specific genes [224-226]. Although p300 and CBP share many overlapping functions, they also have non-redundant roles [227, 228]. Dysfunction of these proteins has been implicated in numerous diseases, including cancer [225, 226, 229, 230]. Efforts are currently under way to develop drugs targeting interactions between various transcription factors and p300/CBP [229, 231-237].

Functions of p300/CBP

Once at a promoter, p300/CBP facilitate activated transcription in several ways. Each coactivator contains a histone acetyltransferase (HAT) domain that acetylates specific

Figure 3. Schematic representations of p300/CBP and KIX. **(A)** A schematic shows the principle domains of p300 and CBP, including the cysteine/histidine-rich domains (CH1 CH2 and CH3), the kinase-inducible interaction (KIX) domain, bromodomain (Br), histone acetyltransferase domain (HAT), zinc-binding domain near the dystrophin WW domain (ZZ), the transcriptional-adaptor zinc-finger-2 domain (TAZ2) and the interferon response factor-binding domain (IBiD). **(B)** The KIX domain consists of three amphipathic α helices, represented as long cylinders, and two short 3_{10} helices, G_1 and G_2 . L_{12} denotes the loop connecting α helix 1 to G_2 . **(C)** KIX folds into a three-helical bundle with a hydrophobic core, creating binding sites for a variety of transcription factors. The binding sites for pKID of pCREB, Tax, the HIV transcription factor Tat, c-Jun, and the minimal activation domains of c-Myb (c-Myb-AD) and MLL (MLL-AD) are indicated.



lysine residues on histones, leading to the opening of chromatin structure associated with activated transcription [224, 226]. Secondly, the coactivators serve as a support framework to connect and localize multiple proteins at a promoter [224, 226]. For example, they link specific transcription factors at a promoter to the cell's general transcriptional machinery as well as recruit additional transcriptional regulatory proteins [224, 226]. And thirdly, p300/CBP can modulate the activity of certain transcription factors through acetylation [224, 226]. In all, p300/CBP are known to bind over 400 proteins and have a central role in the regulation of cellular transcription [227].

The KIX Domain of p300/CBP

The KIX domain is highly conserved [238, 239] and 86 % homologous between p300 and CBP [224]. KIX has been shown to bind more than a dozen transcription factors and is thus critical for the regulation of many genes [225, 240]. KIX consists of amino acids 566-652 of p300 and 586-672 of CBP [241] and folds into a bundle of three amphipathic alpha helices with a hydrophobic core (Figures 3B, 3C) [239]. This core is enclosed at the N-terminus by a small 3_{10} G₁ helix and at the C-terminus by the 3_{10} G₂ helix.

The Two Surfaces of KIX

The nuclear magnetic resonance (NMR) solution structures of the KIX domain bound to various proteins have identified two distinct binding surfaces on KIX (Figure 3C) [239, 241, 242]. The first binding surface is a shallow groove formed between α helix 1 and α helix 3 [239]. Two transcription factors that bind this first KIX surface are phosphorylated CREB (pCREB) [239] and c-Myb [242]. The second binding surface is a larger groove between α helices 2 and 3. This surface incorporates residues from the C-terminus of α 1 as well as the L₁₂ loop and G₂ helix that connect α 1 and α 2 [241, 242].

A greater variety of transcription factors has been found to bind this surface, presumably due to a higher degree of flexibility in this area that can accommodate more variation in ligand structure [241]. The transcription factors that reportedly bind this second surface include among others MLL [241, 243, 244], c-Jun [245], the human immunodeficiency virus (HIV) transcription factor Tat [246], and the HTLV-1 transcription factor Tax [247] (Figure 3C). Recent NMR data show that p53 [248] and the forkhead box member FOXO3 can contact either KIX surface [249].

Consensus Binding Motif of Proteins Contacting the KIX Domain

Two common features have emerged among the more well-studied transcription factors known to bind the KIX domain. First, regardless of which KIX surface they bind, their binding regions have been shown to contain a conserved $\phi XX\phi\phi$ motif, with ϕ denoting a hydrophobic residue and X denoting any residue [248]. These include p53 [248], MLL [241, 243], c-Myb [241, 242], pCREB [239], E2A [250], FOXO3 [249], c-Jun [245, 248], Tax [247], and the sterol-responsive element binding protein (SREBP) [251].

NMR and other structural analyses show that a second common feature of these transcription factors is the folding of the amino acids encompassing a $\phi XX\phi\phi$ motif into an amphipathic α -helical secondary structure, either in solution or upon binding to KIX. NMR solution structures of KIX in complexes with small α -helical portions of the activation domains of various proteins show the $\phi XX\phi\phi$ motif within an α helix making contacts with KIX. NMR-derived structures exist for KIX in complex with α -helical portions of: (1) the phosphorylated kinase inducible domain (pKID; aa 101-160 used for NMR) of pCREB [239]; (2) the minimal activation domain of c-Myb (c-Myb-AD; aa 291-315 used for NMR) [242]; and (3) the minimal activation domain of MLL (MLL-AD; aa 2842-2869 used for NMR) with

c-Myb-AD (aa 291-315 used for NMR) [241]. Structural studies indicate that the activation domain of p53 (p53-AD) [248] and FOXO3 [249] also bind KIX via amphipathic α helices containing $\phi XX\phi\phi$ motifs. The activation domain of p53 contains two amphipathic α helices, each of which contains a $\phi XX\phi\phi$ motif, and it has been shown that each helix can simultaneously and cooperatively bind either surface of KIX [248]. Data suggest that c-Jun [245], Tax [247] and Tat [246] also assume a helical structure upon KIX binding.

Cooperative Binding to the KIX Domain

It has also been shown that two different proteins can simultaneously and cooperatively bind to the KIX domain [241, 243, 244, 252]. The interaction of KIX with pCREB induces positive cooperativity into the binding of RbAp48 to KIX [252]. The interaction between KIX and MLL-AD leads to a 2-fold increase in the affinity of KIX for either pKID or c-Myb-AD [244]. The NMR solution structure of KIX in complex with MLL-AD and c-Myb-AD shows that MLL-AD induces conformational changes in KIX in the regions of L₁₂, G₂ and α 3, which enhance electrostatic contacts between c-Myb-AD and KIX [241]. Although MLL-AD also induces positive cooperative binding of pKID to KIX, it was inconclusive from the NMR data whether the cooperativity arose from allosteric modification of KIX or through direct contacts between MLL-AD and pKID [241].

HBZ Binds the KIX Domain of p300/CBP

Our lab has shown that HBZ-AD binds the KIX domain in a manner dependent on two $\phi XX\phi\phi$ motifs [195]. It was also shown that HBZ competes with Tax for KIX-binding and that this interaction represses activation of HTLV-1 transcription from the 5' LTR [195]. It is hypothesized that the interaction between HBZ and KIX may also affect the transcription of cellular genes whose promoters are controlled by transcription factors that

interact with KIX, potentially contributing to the development of ATL. The studies contained herein extend the characterization of the interaction between HBZ-AD and KIX and show the effects of HBZ-AD on the binding of a select group of transcriptional regulatory proteins to the KIX domain.

Summary of Findings

HBZ-AD is shown to exhibit a strong affinity for the KIX domain with an apparent equilibrium dissociation constant (K_d) of 3.2 ± 0.5 nM, which is approximately three orders of magnitude stronger than the reported K_d s of MLL-AD and c-Myb-AD for KIX [244]. It is shown that each of HBZ-AD's two $\phi XX\phi\phi$ motifs contributes significantly but unequally to the affinity for KIX. It is also shown that HBZ-AD is predicted to form two amphipathic α helices, each containing one of the identified $\phi XX\phi\phi$ motifs. Despite the presence of more than one consensus KIX-binding motif in HBZ-AD, the stoichiometry of the HBZ-AD•KIX complex is found to be 1:1. Using site-directed mutagenesis to compromise one or the other of two well-characterized binding surfaces of KIX, it is determined that HBZ-AD binds the MLL-AD-binding surface of KIX and effectively competes for KIX with three proteins previously reported to bind this surface: MLL-AD, Tax and p53. Corresponding to HBZ-AD's ability to compete with MLL-AD for KIX *in vitro*, transcriptional activation assays show that HBZ represses the activity of MLL in cells. Surprisingly, HBZ-AD failed to compete with c-Jun for binding to KIX and appears instead to slightly enhance its affinity. In addition, HBZ-AD, like MLL-AD, is found to enhance the binding of c-Myb-AD to KIX. Transcriptional activation assays also showed a corresponding increase in c-Myb-AD activity in cells in response to HBZ. Interestingly, HBZ-AD was not found to increase the binding of pCREB to KIX but significantly enhances the binding of unphosphorylated CREB to KIX. Pertinent to

the possible physiological relevance of this finding is the additional finding that full-length HBZ appears to interact with unphosphorylated CREB and enhance binding to a consensus CRE. In addition, it is found that full-length HBZ is unable to inhibit full-length pCREB from binding the CRE. Taken together, these data show that the interaction between HBZ-AD and KIX provides a possible mechanism of HBZ-mediated deregulation of cellular transcription. Additionally, analysis of the *in vitro* data suggests that HBZ-AD may represent a novel mode of binding to KIX, which might provide useful information in the development of small-molecule drugs to modulate the activity of p300/CBP at certain promoters.

CHAPTER 2: DATA

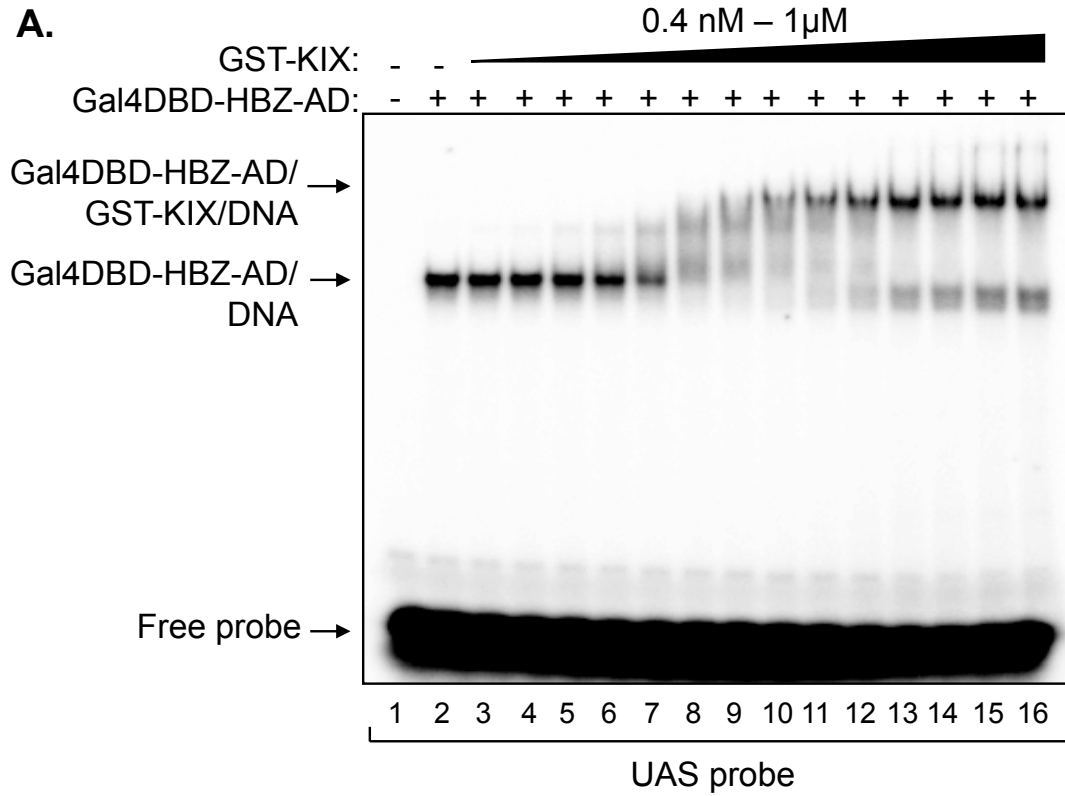
Introduction

Our lab has previously reported that two $\phi XX\phi\phi$ motifs in HBZ-AD mediate binding to the KIX domain of p300/CBP [195]. We also showed that endogenous HBZ and p300 could be co-immunoprecipitated from HTLV-1 infected cell lines, indicating the possibility of an interaction between HBZ and p300/CBP in T cells of infected individuals [195]. The studies contained herein further characterize the HBZ-AD•KIX interaction. HBZ-Sp1 (full-length or the AD) was the HBZ isoform used in these experiments.

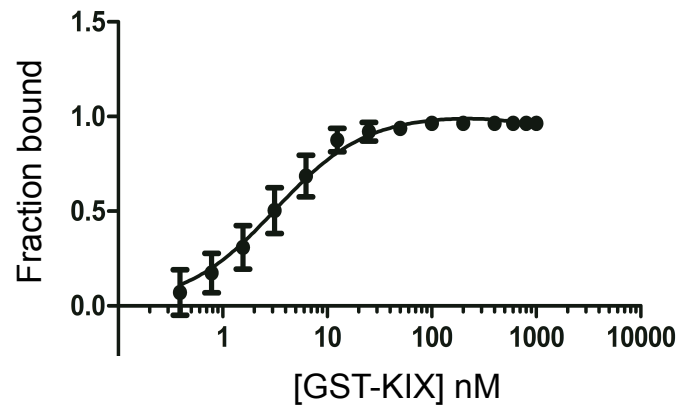
HBZ Binds with High Affinity to the KIX Domain of p300/CBP

In order to gauge the potential of HBZ to disrupt the functions of cellular transcription factors whose activities depend on interactions with KIX, we designed an electrophoretic mobility shift assay (EMSA) to calculate the apparent K_d of the interaction between HBZ-AD (aa 1-77) and KIX (aa 588-683 of CBP). Observing protein-protein interactions with an EMSA requires that one of proteins binds DNA, since visualization of the bands relies on a signal obtained from labeled DNA, in this case by [^{32}P]. Since neither KIX nor HBZ binds DNA directly, the DNA binding domain of the yeast transcription factor Gal4 (Gal4DBD, aa 1-147) was fused to the N-terminus of HBZ-AD. This Gal4DBD-HBZ-AD fusion protein was then able to bind a [^{32}P]-labeled DNA probe containing the upstream activating sequences (UAS) recognized by Gal4DBD, thereby indirectly tethering a [^{32}P]-probe to HBZ-AD. GST-KIX was titrated into binding reactions containing Gal4DBD-HBZ-AD, the [^{32}P]-labeled DNA probe was added, and reactions were then incubated to reach equilibrium. Complexes were separated by native gel electrophoresis (Figure 4A), and phosphorimaging analysis was used to quantify the disappearance of the Gal4DBD-HBZ-AD

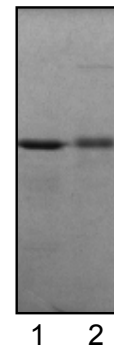
Figure 4. HBZ-AD binds the KIX domain of p300/CBP with high affinity. **(A)** The apparent K_d for the Gal4DBD-HBZ-AD•GST-KIX interaction was determined using EMSAs. Reactions contained UAS probe (2.5 nM), Gal4DBD-HBZ-AD (1 nM, lanes 2-16) and increasing concentrations of GST-KIX (0.4, 0.8, 1.6, 3.2, 6.25, 12.5, 25, 50, 100, 200, 400, 600, 800, and 1000 nM; lanes 3-16). **(B)** The binding curve, generated by nonlinear regression, yields an apparent K_d of 3.2 ± 0.5 nM determined by quantification of three independent EMSAs. The graph shows the fraction of Gal4DBD-HBZ-AD bound versus the concentration of GST-KIX (nM). **(C)** A Coomassie-stained gel shows purified GST-KIX (lane 1) and Gal4DBD-HBZ-AD (lane 2).



B.



C.



band as it became shifted by GST-KIX to form the Gal4DBD-HBZ-AD•GST-KIX complex. Nonlinear regression analysis of quantified bands from three independent experiments showed that 50 % of Gal4DBD-HBZ-AD was shifted at an average GST-KIX concentration of 3.2 ± 0.5 nM, by definition the K_d (Figure 4B). A Coomassie-stained gel with purified Gal4DBD-HBZ-AD and GST-KIX is shown in Figure 4C.

Control reactions to verify the specificity of these interactions are shown in Figure 5. Lane 2 shows the [³²P]-DNA•Gal4DBD-HBZ-AD complex. This DNA•protein complex was supershifted with an antibody recognizing the DBD of Gal4 (lane 3). No supershift was detected with GST alone (lane 4), demonstrating that the GST portion of GST-KIX is not involved in the interaction. The GST-tagged carboxyl-terminal region 2 (GST-C2) and GST-CH1, two domains of p300/CBP that do not interact with HBZ [195], also did not supershift Gal4DBD-HBZ-AD (lanes 5 and 6). GST-KIX (aa 588-683) efficiently supershifted Gal4-HBZ-AD and was confirmed to be associated with the complex by addition of an antibody directed against GST, which further retarded migration of the complex (lanes 7 and 8). Finally, GST-KIX alone did not bind the probe (lane 9).

To confirm that the AD of HBZ remains accessible in the context of the full-length HBZ-Sp1 isoform (aa 1-206), we constructed the fusion protein Gal4DBD-HBZ-Sp1. As before, GST-KIX was titrated into binding reactions, the [³²P]-labeled DNA probe was added, and reactions were incubated to reach equilibrium. Complexes were separated by native gel electrophoresis (Figure 6A), and phosphorimaging analysis was used to quantify the disappearance of the Gal4DBD-HBZ-Sp1 band as it became shifted by GST-KIX to form the Gal4DBD-HBZ-Sp1•GST-KIX complex. The binding curve shows that Gal4DBD-HBZ-Sp1 has a comparable affinity for GST-KIX (Figure 6B), with an average of three independent

Figure 5. Control reactions indicate that Gal4DBD-HBZ-AD interacts specifically with GST-KIX. EMSA reactions contained the UAS DNA probe (5 nM) and, where indicated, Gal4DBD-HBZ-AD (1 nM). Reactions were supplemented with either anti-Gal4DBD antibody (5 ng), GST (50 nM), GST-C2 (50 nM), GST-CH1 (50 nM), GST-KIX (50 nM) or anti-GST antibody (3.1 µg) as indicated.

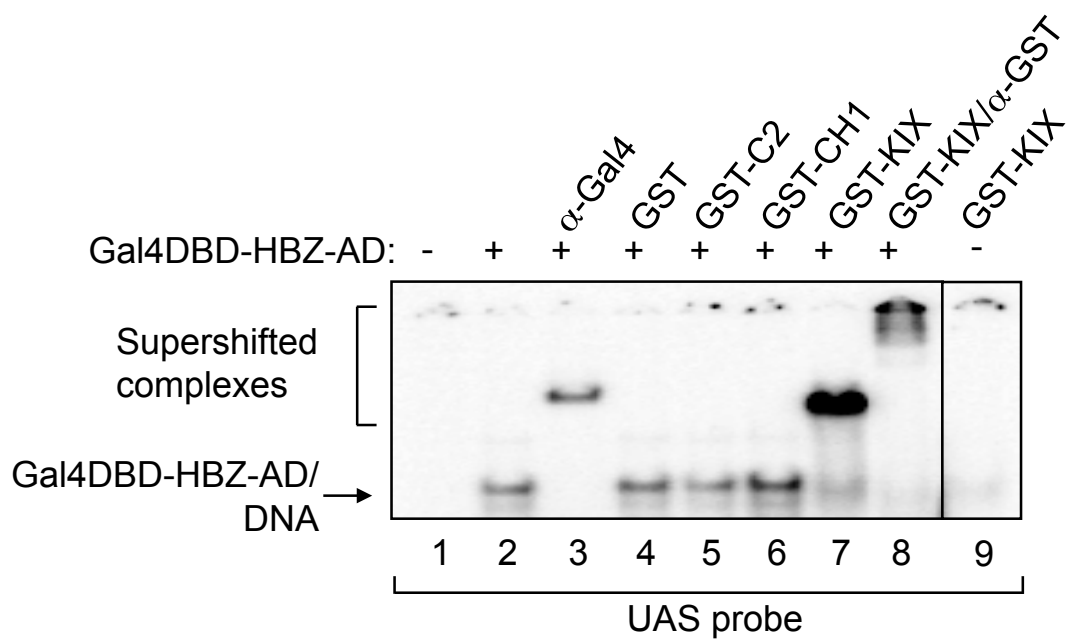
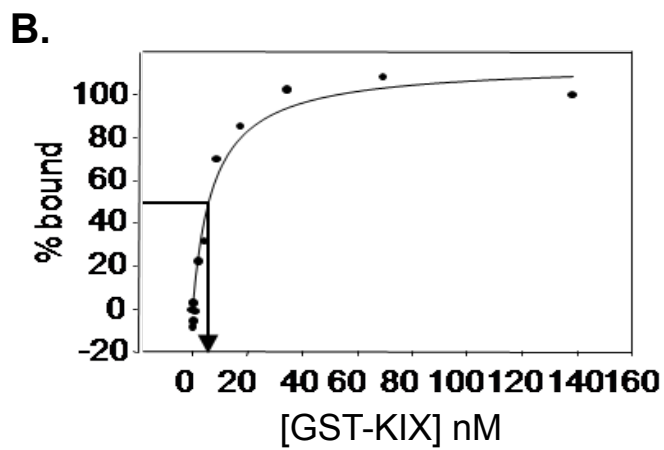
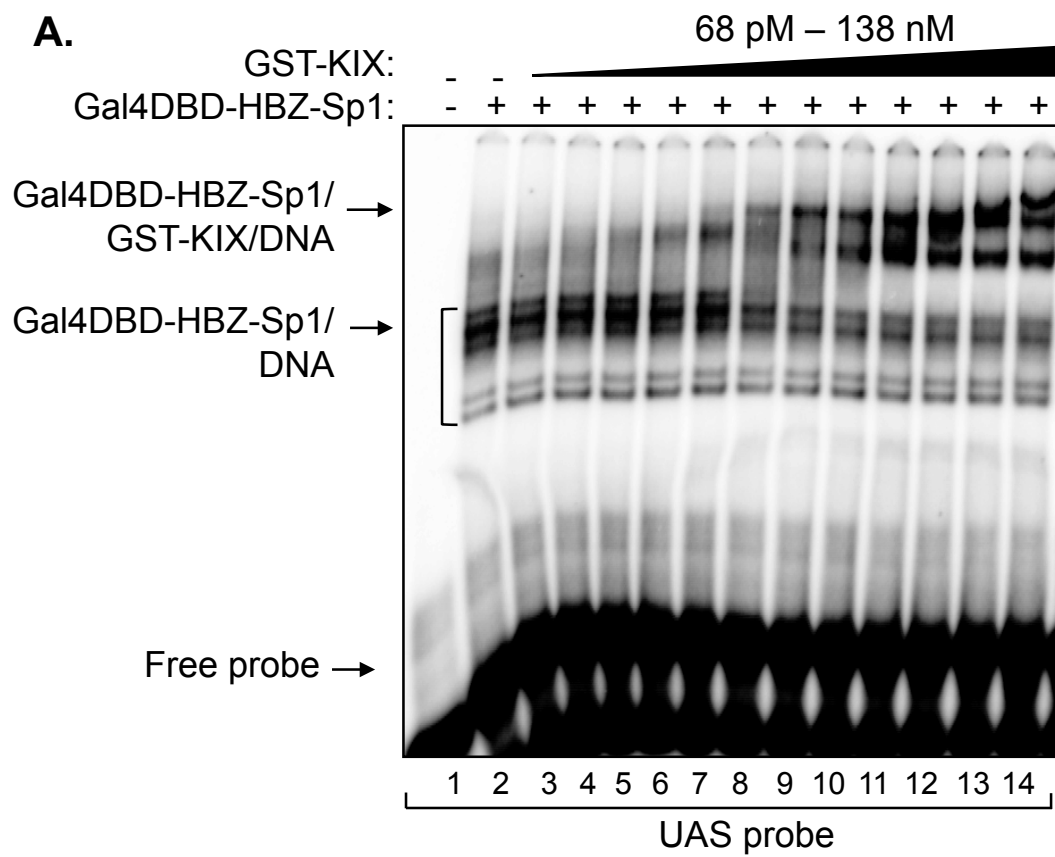


Figure 6. Full-length HBZ-Sp1 binds the KIX domain of p300/CBP with high affinity. **(A)** The apparent K_d for the Gal4DBD-HBZ-Sp1•GST-KIX interaction was determined using EMSAs. Reactions contained UAS probe (3.5 nM), Gal4DBD-HBZ-Sp1 (1.35 nM, lanes 2-14) and increasing concentrations of GST-KIX (68 pM, 135 pM, 270 pM, 540 pM, 1 nM, 2 nM, 4 nM, 8 nM, 16 nM, 35 nM, 70 nM, and 140 nM; lanes 3-14). **(B)** Binding curve, generated by nonlinear regression, shows the fraction of Gal4DBD-HBZ-Sp1 bound versus the concentration of GST-KIX (nM). An average of three independent experiments yielded an apparent K_d of 7.5 ± 1.7 nM.



experiments yielding an apparent K_d of 7.5 ± 1.7 nM. Multiple bands are observed in Figure 6A for unshifted Gal4DBD-HBZ-Sp1. These bands are presumably due to degradation or early termination translation products present in the purified Gal4DBD-HBZ-Sp1 protein. HBZ-AD on the other hand could be purified with comparative ease and with significantly higher yields from *E. coli*. Since HBZ-AD bound KIX with an affinity similar to that of HBZ-Sp1, HBZ-AD was used for the majority of subsequent experiments.

The KIX domain, which interacts with many transcription factors, has been shown to fold and function independently of other domains in p300/CBP [239, 253, 254]. To confirm that KIX similarly remains accessible for binding by HBZ-AD within full-length p300, an EMSA was performed using p300 instead of GST-KIX (Figure 7). A similar affinity was observed, with Gal4DBD-HBZ-AD almost completely shifted between 0.625 nM and 3 nM of p300 (lanes 8 and 9). In control reactions, p300 alone did not bind the probe (lane 12).

Two ϕ XX $\phi\phi$ Motifs in HBZ-AD Mediate the High-Affinity Interaction with KIX

It was previously shown that HBZ contains two ϕ XX $\phi\phi$ motifs, 24-VDGLL-28 and 44-LDGLL-48, and that these motifs mediate the high-affinity binding between HBZ and KIX [195]. It should be noted that HBZ-AD contains additional potential delineations of ϕ XX $\phi\phi$ motifs, which are indicated with dashed arrows in Figure 8A. This is not unusual, as other KIX-binding proteins including MLL, pKID, p53 and Tax also contain more than one potential delineation of a ϕ XX $\phi\phi$ motif. The 24-VDGLL-28 and 44-LDGLL-48 motifs were chosen for further analysis in this study based on the previous findings that mutations in these motifs adversely affect the binding of HBZ to KIX. No mutational analysis of other HBZ ϕ XX $\phi\phi$ motifs has been done.

Figure 7. Gal4DBD-HBZ-AD binds with high affinity to full-length p300. EMSA reactions contained UAS probe (2.5 nM), Gal4DBD-HBZ-AD (2.5 nM, lanes 2-10) and increasing concentrations of p300 (0.2 pM, 1 pM, 5 pM, 25 pM, 125 pM, 625 pM, 3 nM, and 15.6 nM; lanes 3-10). Lane 11 contained Gal4DBD-HBZ-Mut-AD3 (2.5 nM) and p300 (3 nM); lane 12 contained only p300 (15.6 nM) and the UAS probe. Lanes shown are from the same gel; vertical lines show where the gel was cropped.

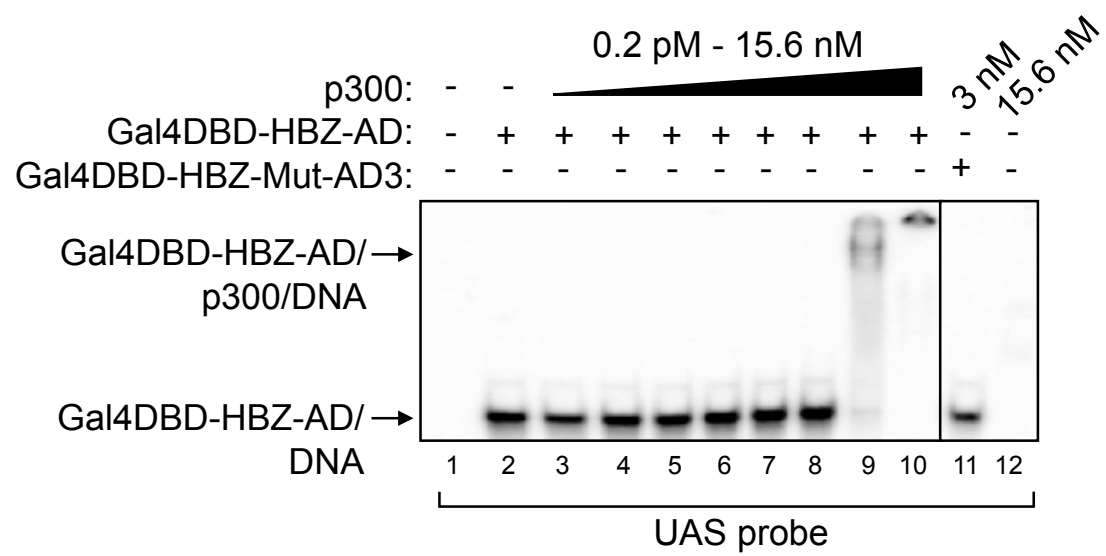


Figure 8. Two $\phi XX\phi\phi$ motifs in HBZ-AD mediate binding to GST-KIX. **(A)** Amino acids 16 to 51 of HBZ-Sp1 are shown. Indicated in bold are the two currently delineated $\phi XX\phi\phi$ motifs in the wild-type (wt) sequence and amino acid substitutions in HBZ-Mut-AD1, HBZ-Mut-AD2 and HBZ-Mut-AD3. Sequences predicted to form amphipathic α helices are underlined. Dashed arrows indicate other potential delineations of $\phi XX\phi\phi$ motifs. **(B).** Each $\phi XX\phi\phi$ motif in Gal4DBD-HBZ-AD contributes unequally to the affinity for GST-KIX. EMSA reactions contained UAS probe (3.25 nM) and 1nM Gal4-HBZ-AD wt, Gal4-HBZ-Mut-AD1, Gal4-HBZ-Mut-AD2 or Gal4-HBZ-Mut-AD3 as indicated. GST-KIX was titrated into reactions (62.5 nM, 125 nM, 250 nM, 500 nM, 1 μ M, and 2 μ M) as indicated.

A.

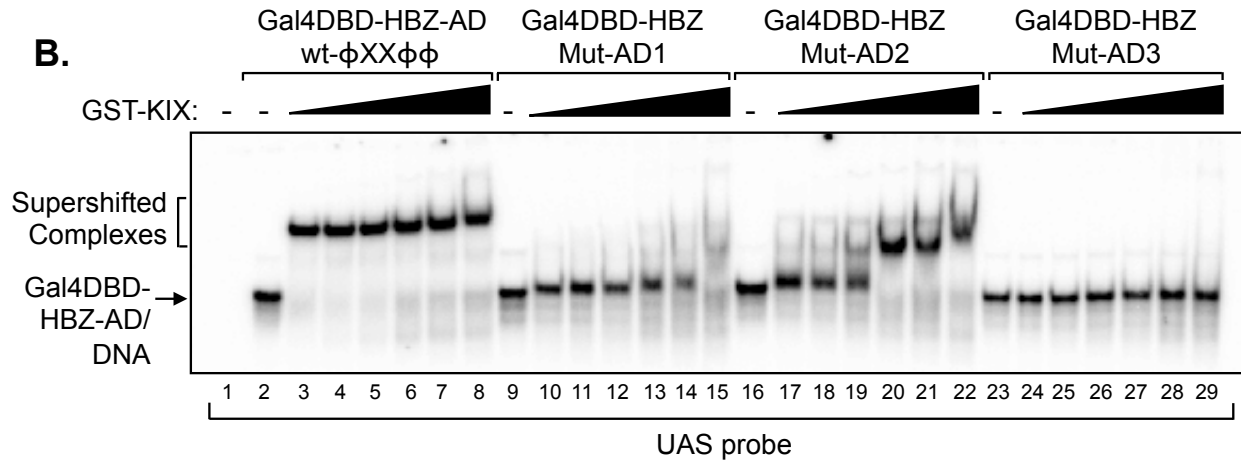
HBZ-AD wt ¹⁶ EDLLVEELVDGLLSLEEEELKDKEEEKAVLDGLLSLE ⁵¹

HBZ-Mut-AD1 EDLLVEELVDGAASLEEEELKDKEEEKAVLDGLLSLE

HBZ-Mut-AD2 EDLLVEELVDGLLSLEEEELKDKEEEKAVLDGAASLE

HBZ-Mut-AD3 EDLLVEELVDGAASLEEEELKDKEEEKAVLDGAASLE

B.



To determine the relative contribution of HBZ-AD's 24-VDGLL-28 and 44-LDGLL-48 motifs to KIX binding, three Gal4DBD-HBZ-AD mutant constructs were created (Figure 8A). Gal4DBD-HBZ-Mut-AD1 contains mutations in the first motif (24-VDGLL-28 to 24-VDGAA-28). Gal4DBD-HBZ-Mut-AD2 contains mutations in the second motif (44-LDGLL-48 to 44-LDGAA-48). Gal4DBD-HBZ-Mut-AD3 carries both of these mutations.

The affinity of each Gal4DBD-HBZ-AD mutant for GST-KIX was determined with EMSAs (Figure 8B). Equivalent titrations of GST-KIX show that mutations in the first motif decrease the affinity for KIX more than mutations in the second motif. The apparent K_d of Gal4DBD-HBZ-Mut-AD1 is approximately 800 nM, compared to 180 nM for Gal4DBD-HBZ-Mut-AD2. The third mutant, Gal4DBD-HBZ-Mut-AD3, was not shifted at the concentrations of GST-KIX used in these experiments, and it also failed to interact with full-length p300 at a concentration that shifted Gal4DBD-HBZ-AD wt (Figure 7, lane 11).

The K_d values correspond to changes in Gibbs free energy of -8.17 kcal/mol for Gal4DBD-HBZ-Mut-AD1 and -9.04 kcal/mol for Gal4DBD-HBZ-Mut-AD2, compared to -11.43 kcal/mol for Gal4-HBZ-AD_{wt}. The combined cost of both mutations is 5.65 kcal/mol. Although it was not determined what portion of this energy cost was due to changes in the folding equilibrium of each mutant, a net loss of 49.43 % of the total free energy resulted from mutations in the $\phi XX\phi\phi$ motifs. This indicates that the high-affinity interaction between HBZ-AD and GST-KIX is dependent on these motifs, whether through direct contacts with KIX, stabilization of intramolecular contacts within HBZ-AD, or a combination of both.

HBZ-AD is Predicted to Form Two Amphipathic α Helices

NMR solution structures of KIX bound to various proteins show in each case that KIX is contacted predominantly via residues in a consensus $\phi XX\phi\phi$ motif contained within an amphipathic α helix on the interacting protein [239, 241, 242]. Analysis of the HBZ-AD amino acid sequence with four different secondary structure prediction programs indicate that HBZ-AD forms two α helices, each containing one of the delineated $\phi XX\phi\phi$ motifs examined in this study (I-TASSER [255], NetSurfP [256], PSIPRED [257], and InterProScan via SWISS-MOD [258]). Table 1 summarizes the probabilities of α -helical formation for amino acids 16-57 in HBZ-AD. A break between the two helices is predicted to occur at residues 34-36. Helical wheel analysis further indicated that each of HBZ-AD's predicted α helices is amphipathic (Figure 9A) (analysis (<http://rzlab.ucr.edu/>; EMBOSS Pepwheel at <http://www.tcd.org/progs/pepwheel.php>)). The residues predicted to form amphipathic α helices are also underlined in Figure 8A.

In these respects, the $\phi XX\phi\phi$ motifs in HBZ-AD appear similar to those of other KIX-binding proteins. Notably, HBZ-AD's first predicted α helix forms a hydrophobic face longer than those of either pKID or the activation domains of c-Myb [238], SREBP [251], or MLL. Interestingly, however, tertiary structure computations show the two predicted α helices of HBZ-AD form a helix-loop-helix hairpin tertiary structure (Figures 9B, 24) [255]. If correct, this could represent a departure from previously characterized modes of KIX binding.

Stoichiometry of the HBZ-AD•KIX Complex is 1:1

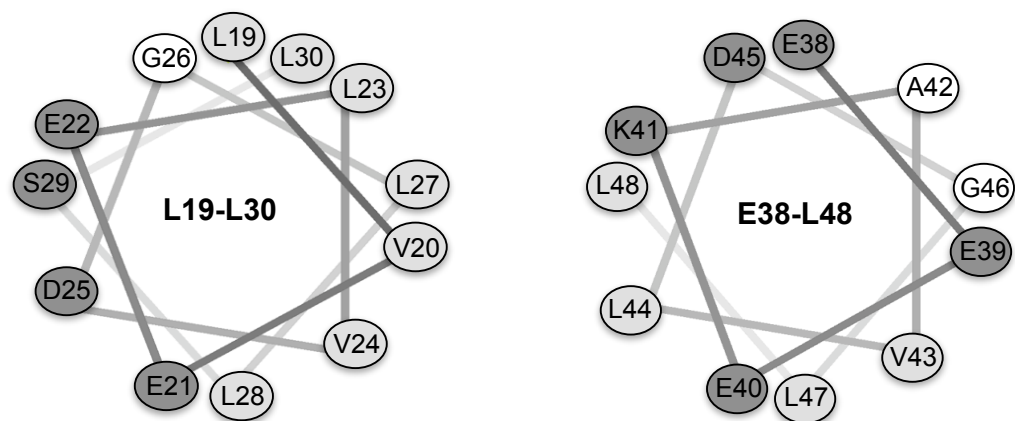
NMR solution structures of KIX in complex with c-Myb-AD or MLL-AD show that each of these proteins binds KIX via one amphipathic α helix containing a $\phi XX\phi\phi$ motif

Table 1. Summary of the Probabilities of α -Helical Formation for HBZ-AD. Probability values (on a scale of 0 – 1) were obtained from secondary structure prediction programs I-TASSER, NetSurfP, PSIPRED, and InterProScan (through SWISS-MOD). Amino acids outside the range of those displayed did not have significant probability of helical formation. Amino acids in bold would form amphipathic α helices.

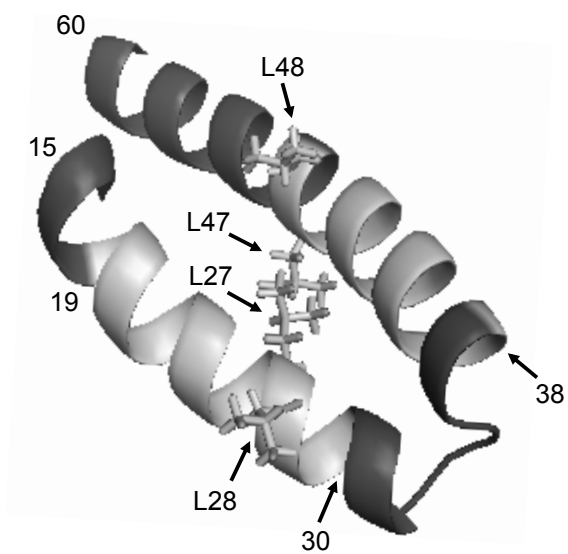
Amino Acid	E	D	L	L	V	E	E	L	V	D	G	L	L	S	L	E	E	E	L	K	D
Amino Acid #	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
I-TASSER	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.7	0.4	0.4	0.6	0.7	0.7	0.7	0.8	0.8	0.7	0.6	0.2	0.2
NetSurfP	0.4	0.6	0.7	0.8	0.9	1	1	1	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.6	0.4	0.4
PSIPRED	0.6	0.8	0.9	0.8	0.9	0.9	0.8	0.8	0.7	0.7	0.7	0.7	0.6	0.6	0.7	0.7	0.8	0.7	0.6	0.4	0.4
SWISS-MOD	0.8	0.7	0.7	0.8	0.9	1	1	1	1	1	0.9	1	0.9	0.8	0.8	0.9	1	0.9	0.8	0.5	0.4
AVERAGE	0.7	0.8	0.8	0.8	0.9	0.9	0.9	0.9	0.8	0.7	0.7	0.8	0.8	0.7	0.8	0.8	0.9	0.8	0.6	0.4	0.4
Amino Acid	K	E	E	E	K	A	V	L	D	G	L	L	S	L	E	E	E	S	R	G	R
Amino Acid #	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
I-TASSER	0.7	0.8	0.8	0.9	0.9	0.9	0.9	0.9	0.8	0.7	0.7	0	0	0.3	0	0.6	0.6	0.4	0.3	0.7	0.6
NetSurfP	0.7	0.8	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.7	0.6	0.6	0.4	0.5	0.7	0.8	0.7	0.6	0.3	0.1	0.1
PSIPRED	0.8	0.8	0.8	0.8	0.9	0.9	0.9	0.8	0.8	0.7	0.7	0.5	0.1	0.1	0.4	0.8	0.8	0.8	0.5	0.1	0.1
SWISS-MOD	0.6	0.8	0.9	0.9	1	1	1	1	0.9	0.9	0.9	0.9	0.8	0.6	0.6	0.7	0.6	0.5	0.4	0.2	0.2
AVERAGE	0.7	0.8	0.8	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.7	0.5	0.3	0.4	0.4	0.7	0.7	0.6	0.4	0.3	0.3

Figure 9. HBZ-AD is predicted to form an amphipathic α -helical hairpin. **(A)** Helical wheels show the amphipathic nature of HBZ-AD's predicted α helices. Uncharged, hydrophobic, and charged amino acids are indicated by unshaded, lightly shaded, and darkly shaded circles, respectively. **(B)** The tertiary structure of HBZ-AD is predicted to exist as an α -helical hairpin. The amphipathic portion of each predicted α helix is more lightly shaded than the remainder of the hairpin structure. Leucines 27 and 47 are predicted to make intramolecular contacts within the hairpin structure of HBZ-AD, while leucines 28 and 48 are exposed on the exterior.

A.



B.



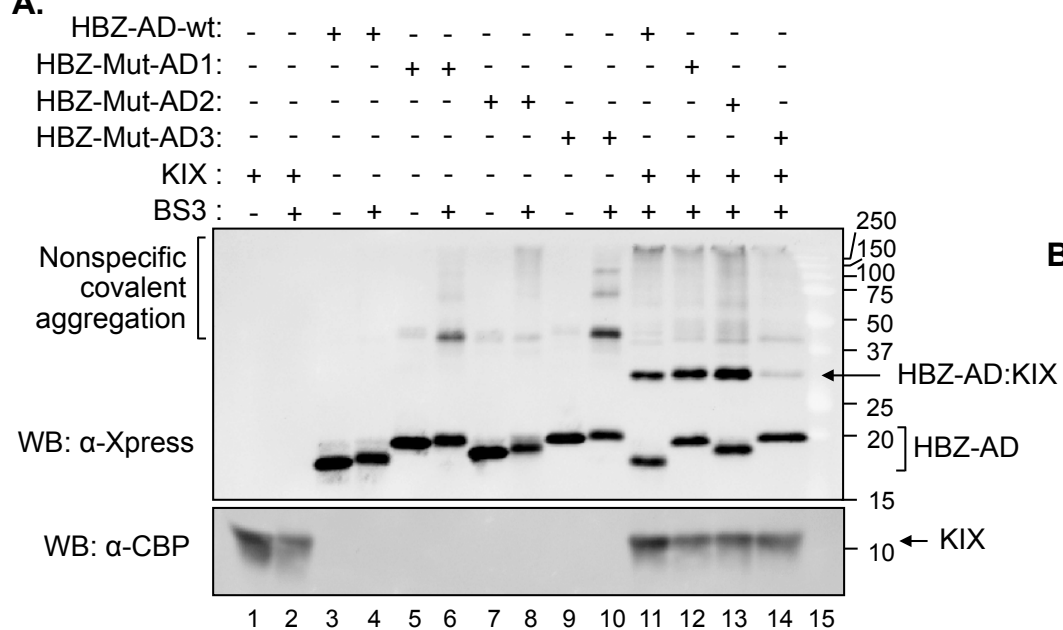
[241, 242]. pKID, on the other hand, binds KIX via two α helices, but importantly, the helix containing the $\phi XX\phi\phi$ motif as well as phosphorylated serine 133 mediates the majority of contacts with KIX [239]. Each of these proteins is thought to bind KIX with a 1:1 stoichiometry, but pKID is reportedly able to nonspecifically bind various other surfaces of KIX *in vitro* as it transitions into the bound state at its cognate site [259]. c-Myb was similarly reported to weakly bind the MLL-AD-binding site of KIX [248].

To determine if HBZ-AD, which contains two predicted amphipathic α helices with $\phi XX\phi\phi$ motifs, might simultaneously bind two molecules of KIX, we performed crosslinking assays using an excess of untagged KIX (aa 587-679). Untagged KIX was incubated with either HBZ-AD wild type or HBZ-Mut-AD1, -AD2, or -AD3 and the crosslinking reagent bis(sulfosuccinimidyl)suberate (BS³). BS³ crosslinks primary amine groups in close proximity to each other, creating covalent bonds between interacting protein species. The resulting covalently-linked complex is resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the stoichiometry is deduced based on the apparent molecular weight of the complex compared to the additive molecular weights of each individual protein (Figure 10A).

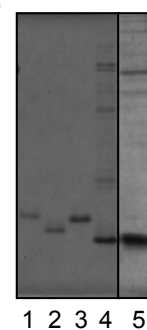
Untagged KIX alone migrated at 12 kDa (lane 1). In the absence of KIX, the HBZ-AD polypeptides were detected between 18-20 kDa (lanes 3-10). Although each HBZ-AD construct consists of amino acids 1-77, verified by DNA sequencing, it is evident that their migration in SDS-PAGE is not equivalent. This phenomenon was reported previously in a study using a panel of polypeptides with α -helical hairpin structure and is thought to arise from a complex relationship between SDS-binding, hydrophobicity, and robustness of tertiary structure [260]. These variations are visible only for smaller molecular weight

Figure 10. Stoichiometry of the HBZ-AD•KIX complex is 1:1. **(A)** Binding reactions contained 750 nM HBZ-AD wt, HBZ-Mut-AD1, HBZ-Mut-AD2 or HBZ-Mut-AD3 as indicated. Reactions additionally contained untagged KIX (27 μ M) and/or BS³ crosslinking reagent (1.8 mM) as indicated. Proteins were resolved with SDS-PAGE and detected by Western blot (WB) using the antibodies indicated. Protein markers were resolved in lane 15, and molecular weights are shown in kilodaltons. **(B)** Coomassie-stained gels show purified HBZ-Mut-AD3 (lane 1), HBZ-Mut-AD2 (lane 2), HBZ-Mut-AD1 (lane 3), HBZ-AD_{wt} (lane 4), and KIX (lane 5).

A.



B.



proteins due to the logarithmic dependence of band mobilities in SDS-PAGE [260]. This is clearly evident in the BS³ gel, where the 2-3 kDa variations are observed only for the HBZ-AD constructs and not for the higher molecular weight HBZ-AD•KIX complexes. Weak non-specific aggregation was observed with each HBZ-AD construct in the presence of BS³ (lanes 4, 6, 8, and 10). However, in the presence of KIX, the specificity of the HBZ•KIX interaction abrogated these effects (lanes 11-14). HBZ-AD wt incubated with untagged KIX and BS³ resulted in a covalently-linked complex formed between KIX and HBZ-AD wt that migrated at ~ 31 kDa, consistent with a 1:1 stoichiometry (lane 11).

As expected given the concentrations of proteins used in this assay, HBZ-Mut-AD1 and HBZ-Mut-AD2 also formed complexes with KIX (lanes 12 and 14), consistent with the EMSA data indicating nanomolar affinities for KIX for each of these constructs. Apropos to this observation, the band intensities for the complexes formed between KIX and HBZ-AD wt, HBZ-Mut-AD1, and HBZ-Mut-AD2 in this assay do not, as expected, reflect the relative differences in affinity of each HBZ-AD construct for KIX. Since each of these constructs exhibits nanomolar affinity for KIX and the concentration of KIX used in these assays was greater than nanomolar, a significant amount of complex was formed in each case. Also, although K_d values in the nanomolar range would predict that essentially all the HBZ-AD present in these reactions would bind KIX, the amount of complex preserved by crosslinking depends entirely on the amount of BS³ used, which must be balanced to optimize adequate complex formation for visualization while minimizing non-specific aggregation. The Coomassie-stained gels in Figure 10B show the purified HBZ-AD and KIX proteins used for the BS³ crosslinking assays.

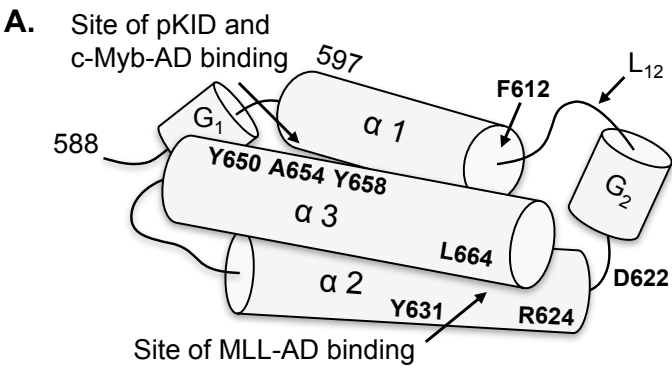
Results from the BS³ crosslinking experiments indicate that HBZ-AD binds only one molecule of KIX. The possibility that KIX could bind 2 molecules of HBZ-AD was ruled out in experiments determining the effects of HBZ-AD on the binding of c-Myb-AD and CREB to KIX, discussed in subsequent sections. Together, these data confirm that the HBZ-AD•KIX complex exists in a 1:1 stoichiometric ratio.

KIX Mutagenesis Indicates that HBZ-AD Binds the MLL-AD-Binding Surface of KIX

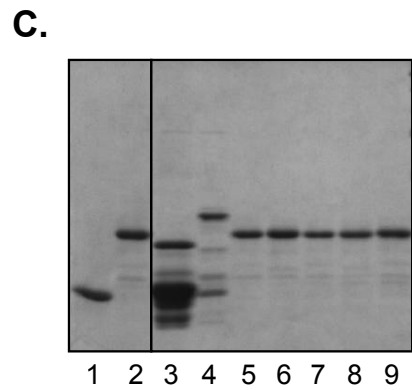
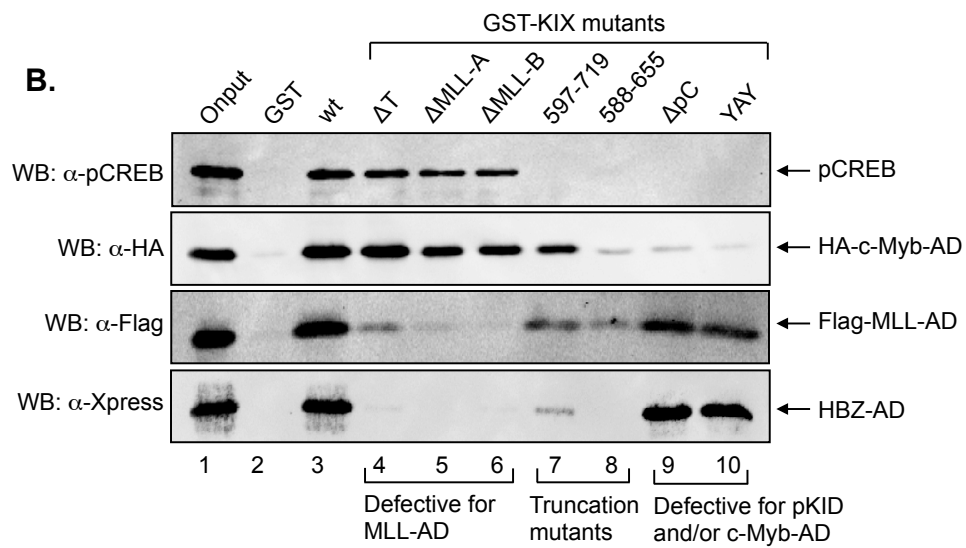
NMR studies have demonstrated that the KIX domain of p300/CPB contains two distinct binding surfaces (Figure 11A). The first binding surface is a shallow hydrophobic groove formed between α helices 1 and 3 and binds pKID and c-Myb-AD. The second binding surface is a larger groove located between α helices 2 and 3. This second surface incorporates residues from the C-terminus of α helix 1 as well as the L₁₂ loop and the small 3₁₀ helix G₂ that connect α helices 1 and 2. This surface of KIX is thought to be more accommodating to a larger variety of proteins, including MLL-AD [241] and Tax [261, 262], although to date only MLL-AD has been conclusively shown via an NMR solution structure to bind this surface of KIX [241]. Based on the finding that HBZ competes with Tax for KIX binding [195] and that Tax is thought to bind the same surface of KIX as MLL-AD, it was hypothesized that HBZ-AD would also bind the MLL-AD-binding surface of KIX.

The first experimental strategy to determine which surface of KIX is bound by HBZ-AD utilized a panel of GST-KIX mutants with disruptions in either the pKID/c-Myb-AD-binding surface of KIX or the MLL-AD-binding surface (Figure 11A). These mutants were then characterized for binding to pCREB, c-Myb-AD, MLL-AD and HBZ-AD using GST pull-down assays. All GST-KIX point mutations were introduced into GST-KIX₅₈₈₋₆₈₃. Three mutants were expected to be defective for MLL-AD binding: GST-KIX Δ T, GST-KIX Δ MLL-A, and

Figure 11. HBZ-AD binds the MLL-AD-binding surface of GST-KIX. **(A)** Approximate locations of mutated KIX residues are indicated on the KIX schematic and summarized in the table. **(B)** Mutations in the MLL-AD-binding surface of KIX disrupt HBZ-AD binding in GST pull-down assays. Binding reactions for pCREB, Flag-MLL-AD and HBZ-AD contained 500 nM GST, GST-KIX_{wt} (aa 588-683) or GST-KIX mutants Δ T (F612A, D622A, R624A), Δ MLL-A (Y631A, L664A), Δ MLL-B (D622A, R624A, Y631A, L664A), GST-KIX 597-719, GST-KIX 588-655, Δ pC (Y658A), or YAY (Y650A, A654Q, Y658A) as indicated. Reactions for HA-c-Myb-AD contained 350 nM GST or GST-KIX fusion protein. pCREB (6.5 nM), HA-c-Myb-AD (5 μ M), Flag-MLL-AD (83 μ M) or HBZ-AD (150 nM) were added to reactions. Fractions of onputs are shown in lane 1: pCREB (100 %), HA-c-Myb-AD (0.2 %), Flag-MLL-AD (0.1 %), and HBZ-AD (80 %). Bound proteins were resolved with SDS-PAGE and detected by Western blot (WB) using the antibodies indicated. **(C)** Coomassie-stained gels show purified GST, GST-KIX_{wt}, 588-655, 597-719, Δ pC, Δ T, Δ MLL-A, Δ MLL-B, and YAY (lanes 1-9, respectively).



GST-KIX Point Mutants	
ΔT	F612A, D622A, R624A
$\Delta MLL-A$	Y631A, L664A
$\Delta MLL-B$	D622A, R624A, Y631A, L664A
ΔpC	Y658A
YAY	Y650A, A654Q, Y658A



GST-KIX Δ MLL-B. GST-KIX Δ T has been previously characterized to be defective for Tax binding and so was expected to also be defective for MLL-AD binding based on reports that Tax binds the same surface of KIX as MLL-AD [261]. GST-KIX Δ MLL-A and GST-KIX Δ MLL-B were designed to selectively disrupt MLL-AD binding based on data from the NMR solution structure of KIX in complex with MLL-AD and c-Myb-AD [241] as well as from NMR chemical shift data from KIX bound to either MLL-AD or Tax [244, 247]. Two mutants, GST-KIX Δ pCREB and GST-KIX Δ YAY, were expected to be selectively defective for pCREB and c-Myb-AD binding based on previous findings [238, 239, 242, 250, 261, 263-265]. And finally, two truncation mutants, GST-KIX₅₉₇₋₇₁₉ [132, 266] and GST-KIX₅₈₈₋₆₅₅ [132, 267], were not expected to be competent for binding at either surface based on previous characterizations. For a detailed description of each mutant including the rationale for design and previous characterizations, see Table 2.

Figure 11B shows results from GST pull-down assays using the GST-KIX mutants in binding reactions with either pCREB, c-Myb-AD (aa 186-325), MLL-AD (aa 2829-2883) or HBZ-AD. All purified proteins bound specifically to GST-KIX_{wt} (lane 3) as evidenced by the lack of interaction with GST alone (lane 2). As expected, pCREB and c-Myb-AD bound GST-KIX Δ T (lane 4), GST-KIX Δ MLL-A (lane 5) and GST-KIX Δ MLL-B (lane 6), which are each defective for MLL-AD-binding. HBZ-AD, like MLL-AD, also failed to bind these mutants. In contrast, GST-KIX Δ pC (lane 9) and GST-KIX Δ YAY (lane 10), which are specifically defective for pCREB and c-Myb-AD-binding but retain the ability to bind MLL-AD, also bound HBZ-AD. All proteins except c-Myb-AD exhibited weak or undetectable binding to GST-KIX₅₉₇₋₇₁₉ (lane 7). The difference in c-Myb-AD and pCREB binding to this mutant is potentially due to their different mechanisms of binding and the different KIX residues contacted within the

Table 2. Summary of GST-KIX mutants

GST-KIX mutant	Expected Result and Rationale for Mutation
ΔT F612A D622A R624A	Previously characterized to reduce Tax binding by approximately two thirds without significantly affecting pCREB binding [261]. Expected to diminish MLL-AD binding to KIX based on previous reports that Tax binds the same surface of KIX as MLL-AD [247, 261]. NMR chemical shift data indicated that KIX residues F612, D622 and R624 were largely affected by MLL binding [244]. The NMR solution structure of the KIX•MLL-AD•c-Myb-AD complex subsequently confirmed that KIX F612 and R624 make direct contacts with MLL-AD, but direct contact was not observed between MLL-AD and KIX D622 [241]. KIX residues I611, F612, Y631, R624 and L664 form the hydrophobic groove into which MLL-AD binds. (NMR chemical shift data for KIX bound to Tax indicated that Tax shifted KIX residues F612 and D622 but not R624 [247].)
ΔMLLA Y631A L664A	Expected to disrupt MLL-AD binding based on the NMR solution structure of the KIX•MLL-AD•c-Myb-AD complex showing that MLL-AD makes direct contacts with KIX residues Y631 and L664 [241]. KIX Y631 and L664 help form the hydrophobic groove into which MLL-AD binds.
ΔMLLB D622A R624A Y631A L664A	Expected to disrupt MLL-AD binding. Consists of the same mutations carried by GST-KIX _{ΔMLL-A} plus two additional ones, D622A and R624A, from GST-KIX _{ΔT} . MLL-AD and Tax have been reported to bind the same surface of KIX [261] but may bind via different KIX residues based on comparisons of NMR chemical shift data for KIX bound to Tax [247] versus KIX residues contacted by MLL-AD in the NMR solution structure of the KIX•MLL-AD•c-Myb-AD complex [241]. Thus, in efforts to affect a putative HBZ binding site, three mutations targeting MLL-AD binding were combined with D622, which was shown via NMR to be shifted upon Tax binding [247] but not contacted by MLL-AD [241].
ΔpC Y658A	Previously characterized to be defective for pCREB binding [239, 261] without affecting Tax binding [261]. KIX residue Y658 interacts with phosphorylated serine 133 in pCREB and is required for the high-affinity interaction between pCREB and KIX [264, 265]. This mutant is also expected to disrupt c-Myb-AD binding based on the finding that three c-Myb-AD residues contact Y658 [242]. The KIX mutation Y658F was shown to increase the K _d of c-Myb-AD binding by approximately 4 fold over that for wild-type KIX [238].
YAY Y650A A654Q Y658A	Previously characterized to be defective for c-Myb-AD binding [250, 263]. The Y658A mutation is also expected to disrupt pCREB binding, and, in addition, Y650 and A654 also are involved in pCREB binding [238, 239]. The NMR solution structure of KIX with c-Myb-AD showed that all three of these KIX residues interact with c-Myb-AD [242]. As stated above, a Y658F mutation was found to increase the K _d of c-Myb-AD binding to KIX by approximately 4 fold [238].
597-719	Previously characterized to be defective for Tax, pCREB and p53 binding [132, 266]. Expected to be defective for both pCREB and MLL-AD binding based on

	previous reports that Tax binds the MLL-AD-binding surface of KIX [261]. It has been hypothesized that deletion of residues N-terminal to KIX Q597 results in decreased stability of the KIX 3-helical bundle due to lost enclosure of the hydrophobic core at the N-terminus of the KIX structure [266].
588-655	Previously characterized to be defective for Tax, c-Jun and p53 binding [132, 267]. A similar GST-KIX mutant, 588-665, was found to be defective for Tax binding [266]. Expected to be defective for most interactions. This mutant lacks approximately half of α helix 3, which includes residues involved in the binding of pCREB (I657, Y658) [238, 239, 265], c-Myb-AD (I657, Y658, K662) [242] and MLL-AD (K656, I660, L664, R668) [241].
Δc-JunA L620A	Expected to disrupt c-Jun and Tax binding based on NMR data indicating that both c-Jun and Tax shift L620 upon KIX binding [245, 247].
Δc-JunB E626A V629A	Expected to disrupt c-Jun and Tax binding based on NMR data indicating that both c-Jun and Tax shift E626 and V629 upon KIX binding [245, 247].

same general binding surface [239, 242]. Finally, GST-KIX₅₈₈₋₆₅₅ (lane 8), which is structurally disrupted in both binding surfaces, did not interact with any of the proteins. Overall, the pattern that emerges from the GST-KIX mutagenesis data indicates that HBZ-AD contacts only the MLL-AD-binding surface of KIX. Coomassie-stained gels with purified GST and each GST-KIX protein are shown in Figure 11C.

HBZ-AD Competes with MLL-AD for Binding to KIX

The mutagenesis data indicated that HBZ-AD interacts with the MLL-AD-binding surface of KIX and suggested that HBZ-AD, like Tax, would compete with MLL-AD for binding to KIX. Thus the second experimental strategy to identify the HBZ-AD binding site on KIX was competition binding assays. GST pull-down assays were performed in which HBZ-AD was titrated into reactions containing MLL-AD and GST-KIX (Figure 12, lanes 4-8). HBZ-AD effectively displaced MLL-AD from GST-KIX at a concentration 15 fold less than the concentration of MLL-AD (lane 7), further demonstrating the high affinity of HBZ-AD for KIX. Neither protein bound GST alone (lanes 1 and 2).

To determine whether full-length HBZ-Sp1 affects the activity of MLL-AD in cells, luciferase assays were performed using Jurkat T cells. Cells were co-transfected with a luciferase reporter plasmid containing five consensus binding sequences for Gal4DBD (pGalTK-Luc) [268] and an expression plasmid for MLL-AD (aa 2829 to 2883) [243] fused to Gal4DBD. A previous study correlated transcriptional activation of this reporter with interaction between MLL-AD and endogenous p300/CBP and recruitment of the coactivators to the promoter [243]. Consistent with this, significant luciferase activity was induced by the expression of Gal4DBD-MLL-AD compared to Gal4DBD alone (Figure 13A, lanes 2 and 3). Additional co-transfection of a plasmid for full-length HBZ-Sp1 [195] led to

Figure 12. HBZ-AD competes with Flag-MLL-AD for binding to GST-KIX. Competition binding reactions contained GST-KIX (500 nM) and Flag-MLL-AD (15 μ M) (lanes 3-8) with increasing concentrations of HBZ-AD (50 nM, 100 nM, 200 nM, 1 μ M, and 5 μ M; lanes 4-8). Control binding reactions contained GST (500 nM) with HBZ-AD (5 μ M, lane 1) or Flag-MLL-AD (15 μ M, lane 2). Fractions of HBZ-AD (2 % of maximum) and Flag-MLL-AD (0.5 %) onputs are shown in lanes 9 and 10, respectively. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibodies indicated.

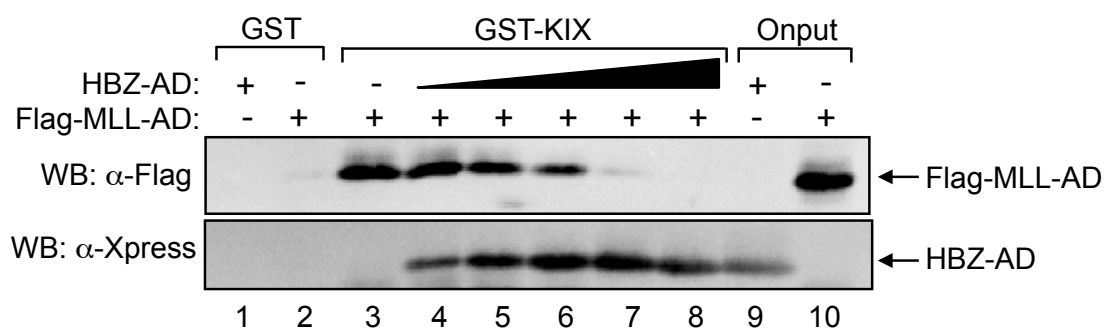
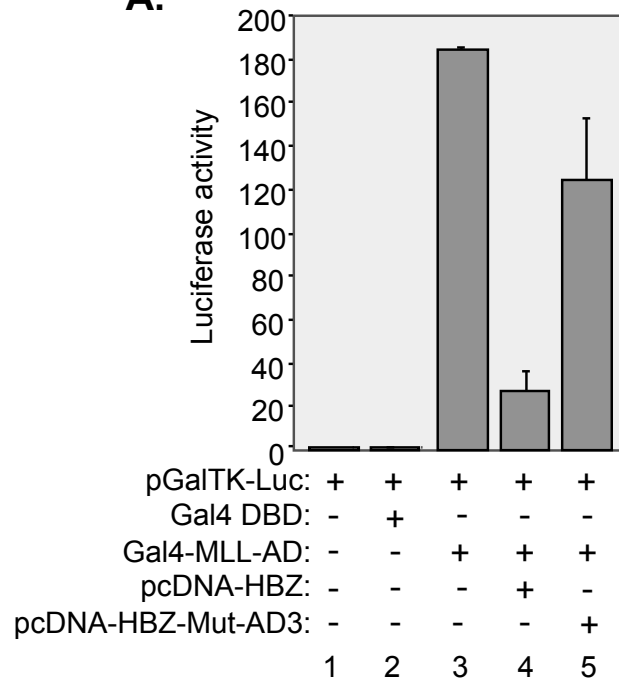
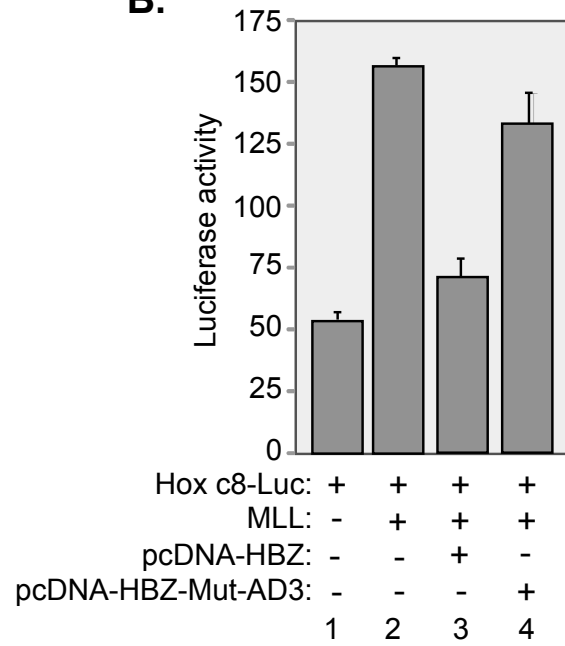


Figure 13. Full-length HBZ-Sp1 represses transcriptional activity of MLL in cells. **(A)**

HBZ-Sp1 represses activity of Gal4DBD-MLL-AD. Jurkat cells were transfected with pGalTK-Luc (100 ng) alone or in combination with Gal4DBD (50 ng), Gal4DBD-MLL-AD (50 ng), pcDNA-HBZ (800 ng) or pcDNA-HBZ-Mut-AD3 (800 ng) as indicated. Luciferase assays were performed 24 hours after transfection. The reported values are the average luminescence $\pm$ S.E. from one experiment performed in duplicate and are representative of three independent experiments. Luciferase activity was normalized to Renilla luciferase from the herpes simplex virus thymidine kinase promoter (pRL-TK, Promega). **(B)** HBZ-Sp1 represses activation of the MLL-responsive *Hox-c8* promoter in cells. 293T/T7 cells were transfected with *Hox c8*-Luc (200 ng) alone or in combination with Flag-MLL (100 ng), pcDNA-HBZ (700 ng) or pcDNA-HBZ-Mut-AD3 (700 ng) as indicated. Luciferase assays were performed 24 hours after transfection. The reported values are the average luminescence $\pm$ S.E. from one experiment performed in triplicate and are representative of three independent experiments. Luciferase activity was normalized to Renilla luciferase from the herpes simplex virus thymidine kinase promoter (pRL-TK, Promega).

A.**B.**

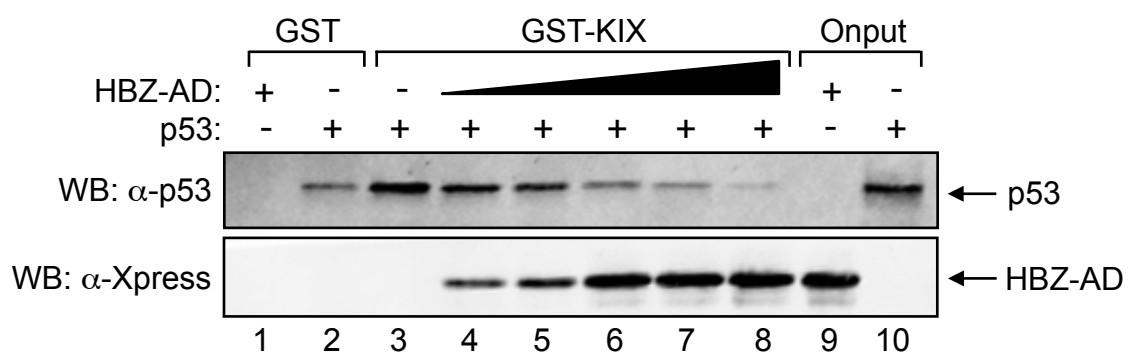
a greater than 6-fold reduction in luciferase activity (lane 4). In contrast, only a slight decrease in luciferase activity (<2-fold) was obtained by expression of full-length HBZ-Mut-AD3, which carries the same mutations in its $\phi XX\phi\phi$ motifs as the recombinant purified HBZ-Mut-AD3 used for the *in vitro* studies (lane 5) [195]. These results indicate that HBZ's effect on the activity of MLL-AD in cells is dependent on the $\phi XX\phi\phi$ motifs.

To determine whether HBZ affects the activity of MLL at a natural promoter, luciferase assays utilizing a reporter with the MLL-responsive *Hox c8* promoter [269] were performed as above. Consistent with results from Gal4DBD-MLL-AD, HBZ repressed MLL-AD-mediated activation from the *Hox c8* promoter while the HBZ double mutant did not (Figure 13B). These results suggest that HBZ competes effectively with MLL-AD for KIX-binding in cells and show that these effects are dependent on HBZ-AD's $\phi XX\phi\phi$ motifs.

HBZ-AD Competes with p53 for Binding to KIX

The tumor suppressor p53 has been found to interact with KIX [132]. A recent study showed that p53-AD contains two amphipathic α helices, each containing at least one $\phi XX\phi\phi$ motif, and that each of these helices can bind a different surface of KIX simultaneously and synergistically [248]. To determine the effect of HBZ-AD on this interaction, competition GST pull-down assays were performed in which HBZ-AD was titrated into binding reactions containing p53 and GST-KIX (Figure 14). HBZ-AD effectively displaced p53 from GST-KIX (lanes 4-8). HBZ-AD did not bind GST (lane 1), but some binding to GST was observed for p53 (lane 2). Overall, this result suggests that disrupting the interaction of one p53 helix from the MLL-AD-binding surface of KIX is sufficient to destabilize the interaction between p53 and KIX. Of note, the published K_d for the

Figure 14. HBZ-AD competes with p53 for binding to GST-KIX. Competition binding reactions contained GST-KIX (500 nM) and p53 (1.5 nM) (lanes 3-8) with increasing concentrations of HBZ-AD (50 nM, 100 nM, 500 nM, 1 μ M, and 5 μ M; lanes 4-8). Control binding reactions contained GST (500 nM) with HBZ-AD (5 μ M, lane 1) or p53 (1.5 nM, lane 2). Fractions of HBZ-AD (2 % of maximum) and p53 (133 %) onputs are shown in lanes 9 and 10, respectively. Bound proteins were resolved with SDS-PAGE and analyzed with Western blot (WB) using the antibodies indicated.



interaction between KIX and p53-AD of $19 \pm 5 \mu\text{M}$ [248] would indicate that much more p53 and/or GST-KIX would have been needed to reach binding conditions than the concentrations used in this assay. Initially, higher concentrations were used but resulted in over-saturation of the signal obtained in Western blot development. It is not known why concentrations below the reported K_d value retained the ability to interact with KIX in this assay.

HBZ-AD Competes with Tax for Binding to KIX

It has previously been reported that Tax binds the same KIX surface as MLL-AD [261] and that HBZ competes with Tax for KIX binding [195]. To confirm the ability of HBZ-AD to compete with Tax for binding to KIX, competition GST pull-down assays were performed with Tax, GST-KIX (Figure 15, lanes 3-8) and increasing amounts of HBZ-AD (lanes 4-8). As expected, HBZ-AD effectively removed Tax from GST-KIX. GST did not bind HBZ-AD (lane 1), but some background was observed with Tax (lane 2).

HBZ-AD Fails to Compete with c-Jun for Binding to KIX

It was expected that HBZ-AD would similarly compete with c-Jun for KIX binding, since MLL-AD, Tax and c-Jun are all thought to bind the same surface of KIX [245, 261, 267]. To determine the effect of HBZ-AD on this interaction, competition GST pull-down assays were performed in which GST-KIX and c-Jun (Figure 16A, lanes 3-7) were incubated with increasing concentrations of HBZ-AD (lanes 4-7). Unexpectedly, HBZ-AD failed to compete with c-Jun for GST-KIX binding and appeared instead to slightly enhance the interaction. The assays were repeated with slightly different conditions in Figure 16B in which c-Jun was incubated with GST-KIX in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of HBZ-AD. Two different concentrations of c-Jun were tested (lanes 3-4, lanes 5-6).

Figure 15. HBZ-AD competes with Tax for binding to GST-KIX. Competition binding reactions contained GST-KIX (500 nM) and Tax (1.7 μ M) (lanes 3-8) with increasing concentrations of HBZ-AD (100 nM, 250 nM, 500 nM, 1 μ M, and 5 μ M; lanes 4-8). Control binding reactions contained GST (500 nM) with HBZ-AD (5 μ M, lane 1) or Tax (1.7 μ M, lane 2). Fractions of HBZ-AD (1.5 % of maximum) and Tax (7 %) onputs are shown in lanes 9 and 10, respectively. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibodies indicated.

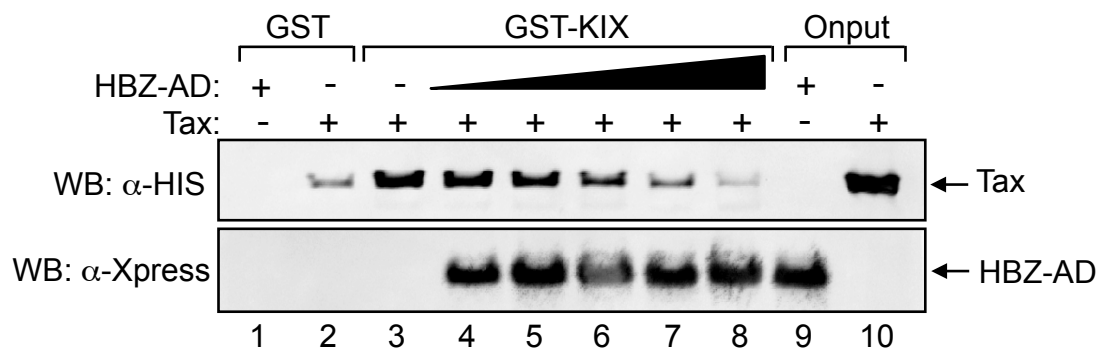
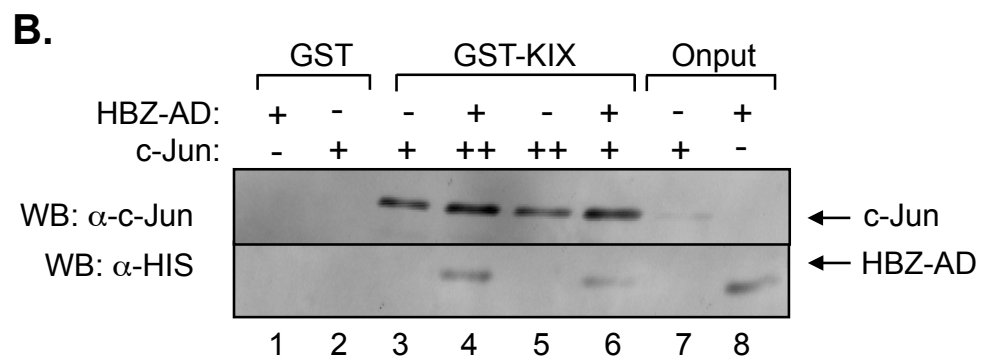
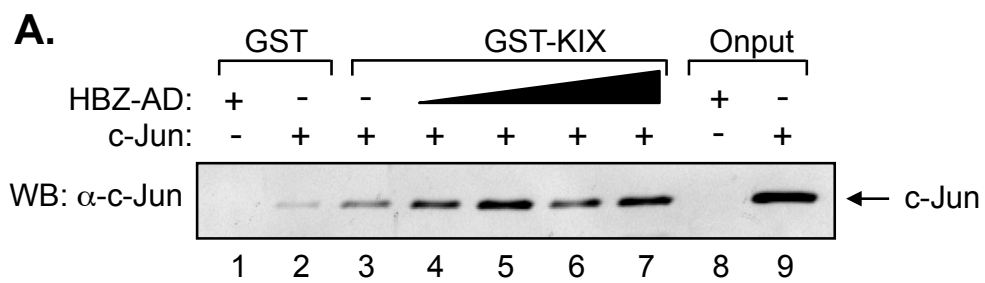


Figure 16. HBZ-AD fails to compete with c-Jun for binding to GST-KIX. **(A)** Competition binding reactions contained GST-KIX (100 nM) and c-Jun (250 nM) (lanes 3-7) with increasing concentrations of HBZ-AD (100 nM, 250 nM, 500 nM, and 1 μ M; lanes 4-7). Control binding reactions contained GST (100 nM) with HBZ-AD (1 μ M, lane 1) or c-Jun (250 nM, lane 2). A fraction of c-Jun (15 %) onput is shown in lane 10. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibody indicated. **(B)** HBZ-AD enhances the binding of c-Jun to KIX. Binding reactions contained GST-KIX (250 nM) and two different concentrations of c-Jun (100 nM in lanes 3-4; 200 nM in lanes 5-6) in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of 250 nM HBZ-AD. Control reactions contained GST (250 nM) with HBZ-AD (250 nM, lane 1) or c-Jun (200 nM, lane 2). Fractions of c-Jun (9 % of maximum) and HBZ-AD (75 %) onputs are shown in lanes 7 and 8 respectively. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibodies indicated.

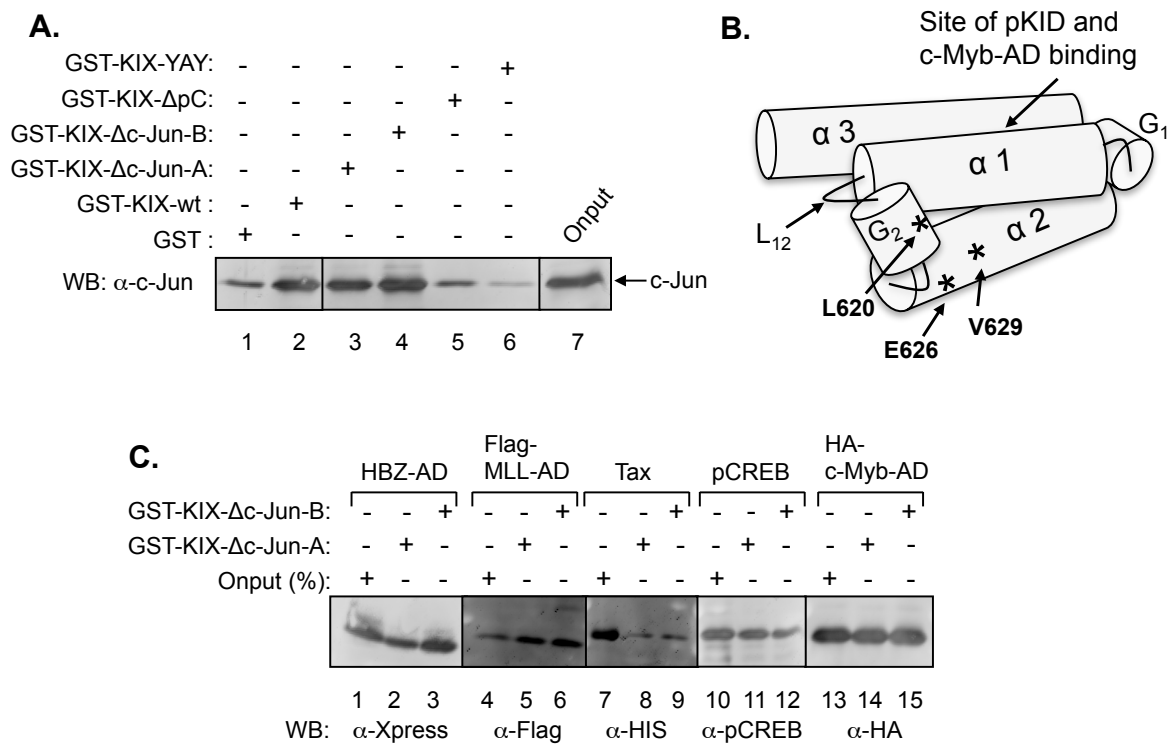


Although not dramatic, reactions containing HBZ-AD appear to bind slightly more c-Jun. GST did not bind HBZ-AD or c-Jun. Since c-Jun does not directly interact with HBZ-AD (personal communication, Dr. Lemasson, unpublished data), these findings suggest that the enhanced binding of c-Jun to KIX may be due to effects of HBZ-AD on KIX. The significantly lower band intensities seen on the membrane in Figure 16B, including the c-Jun positive control, are potentially due to a number of experimental factors such as reagent degradation, inefficient transfer of proteins to the membrane, or variations in antibody dilutions. The HBZ-AD bands appear significantly more faint in this figure than in others due to the use of a histidine antibody to detect the 6xHis tag on HBZ-AD rather than the antibody for the Xpress epitope used in other experiments.

c-Jun Binding Requires KIX Residues Necessary for c-Myb-AD and pKID Binding

Results from GST-KIX mutagenesis data unexpectedly showed that c-Jun is defective for binding to GST-KIX Δ PC and GST-KIX Δ YAY compared to GST-KIX_{wt}, indicating that residues involved in the binding of c-Myb-AD and/or pKID are also important for binding by c-Jun (Figure 17A, lanes 2, 5-6). Some binding of c-Jun to GST was noted in the control reaction, but significantly more c-Jun bound GST-KIX_{wt}, indicating that c-Jun specifically binds KIX. These findings, combined with the inability of HBZ-AD to compete with c-Jun for binding to KIX and the HBZ-AD-mediated enhanced binding of c-Jun to KIX, suggest that the binding site for c-Jun does not overlap with the HBZ-AD binding site. These findings also would be consistent with HBZ-AD-mediated allosteric modulation of KIX as a mechanism of increased binding of c-Jun, as opposed to a direct interaction between c-Jun and HBZ-AD.

Figure 17. c-Jun requires KIX residues necessary for c-Myb-AD and pKID binding. **(A)** c-Jun exhibits an unexpected binding pattern to GST-KIX mutants. Reactions contained c-Jun (175 nM) and 500 nM GST, GST-KIX_{wt} or GST-KIX mutants Δc-Jun-A (L620A), Δc-Jun-B (E626A, V629A), ΔpC (Y658A) or YAY (Y650A, A654Q, Y658A) as indicated. A fraction of c-Jun output (17 %) is shown in lane 7. Bound proteins were resolved with SDS-PAGE and detected by Western blot (WB) using the indicated antibody. Lanes shown are from the same membrane; vertical lines show where the membrane was cropped. **(B)** Locations of mutated KIX residues are indicated with an asterisk. **(C)** KIX residues identified to be strongly shifted upon c-Jun and Tax binding are dispensable for binding in GST pull-downs. Binding reactions contained 500 nM of the GST-KIX mutants Δc-Jun-A (L620A) or Δc-Jun-B (E626A, V629A) as indicated, with HBZ-AD (150 nM, lanes 2-3), Flag-MLL-AD (35 μM, lanes 5-6), Tax (1.7 μM, lanes 8-9), pCREB (7 nM, lanes 11-12), or HA-c-Myb-AD (5 μM, lanes 14-15). Onput fractions of HBZ-AD (40 %, lane 1), Flag-MLL-AD (0.3 %, lane 4), Tax (6 %, lane 7), pCREB (100 %, lane 10), or HA-c-Myb-AD (0.1 %, lane 13) are shown. Bound proteins were resolved with SDS-PAGE and detected by Western blot (WB) using the indicated antibodies.



Mutations Targeting a Novel Putative KIX Binding Site Fail to Affect Interactions

A review of NMR chemical shift data in the literature showed that upon binding to KIX, Tax and c-Jun shift many of the same KIX residues [245, 247]. Interestingly, many of these residues are clustered on a surface of KIX distinct from residues contacted by MLL-AD, pKID or c-Myb-AD in NMR solution structures [239, 241, 242]. It has been suggested that this area of KIX may be an as-yet uncharacterized binding surface [270].

To test this hypothesis, two additional GST-KIX mutants were designed targeting three of the KIX residues exhibiting significant chemical shifts upon c-Jun and Tax binding: L620, E626, and V629 (Figure 17B). These mutants, GST-KIX $_{\Delta\text{c-Jun-A}}$ (L620A) and GST-KIX $_{\Delta\text{c-Jun-B}}$ (E626A, V629A), were used in GST pull-down experiments with c-Jun (Figure 17A, lanes 3-4) and found to bind c-Jun with the same affinity as GST-KIX $_{\text{wt}}$ (Figure 17A, lanes 2-4). All additional proteins tested thus far also bound these GST-KIX mutants (Figure 17C), including HBZ-AD (lanes 2-3), Flag-MLL-AD (lanes 5-6), Tax (lanes 8-9), pCREB (lanes 11-12), and HA-c-Myb-AD (lanes 14-15).

While side-by-side comparisons with GST-KIX $_{\text{wt}}$ and a GST control would be needed to definitely rule out potential variations in binding to these mutants, the preliminary results shown here suggest that despite significant NMR chemical shifts, neither of the mutated residues appears critical to interactions between GST-KIX and c-Jun or Tax in GST pull-downs. Although the Tax band intensities in Figure 17C are weak (lanes 8-9), this is not unusual for Tax, as it has been the author's observation that the interaction between Tax and KIX is unstable and sensitive to wash times in GST pull-downs. The wash times in this experiment were significantly longer than those needed to optimize Tax-binding, as in Figure 15.

A potential explanation for these results is that c-Jun and/or Tax may shift KIX residues distal to the actual site of interaction. KIX is known to be allosterically modified by various ligands, indicating global conformational changes upon ligand binding. Therefore, the shifts observed in KIX upon c-Jun and Tax binding may be due to allosteric conformational changes in KIX rather than direct interactions. Supporting this possibility, several KIX residues that were shifted upon MLL-AD binding, including L620, were not subsequently found to make contacts with MLL-AD in the NMR solution structure [241, 244].

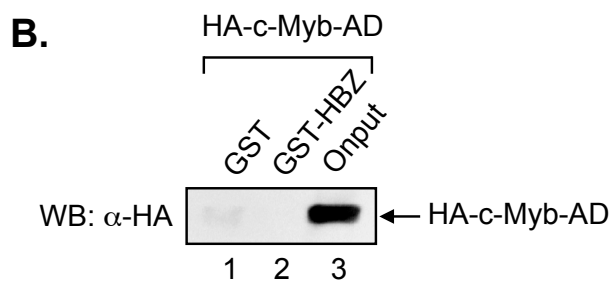
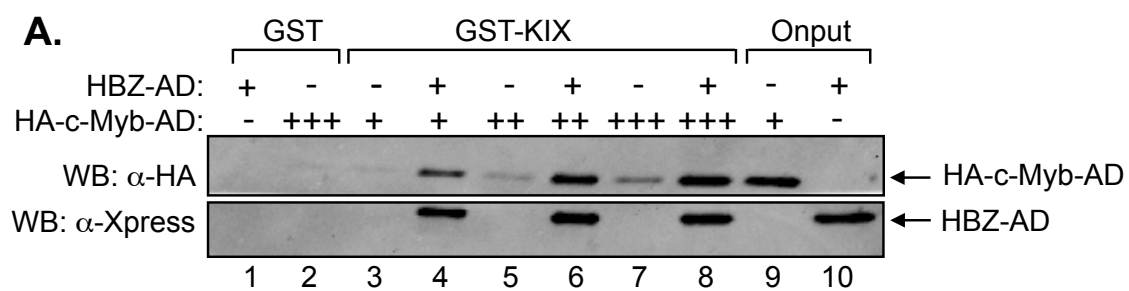
HBZ-AD Enhances the Binding of c-Myb-AD to KIX

The interaction of MLL-AD with KIX was found to induce positive cooperativity into the binding of pCREB [243], pKID [244] and c-Myb-AD [241] to their binding sites on the opposite surface of KIX (22,25,26). To determine whether HBZ-AD similarly enhanced the binding of c-Myb-AD and pCREB to KIX, GST pull-down assays were performed.

To test the effects of HBZ-AD on c-Myb-AD-binding, GST-KIX was incubated with c-Myb-AD in the absence or presence of a concentration of HBZ-AD sufficient to saturate KIX (Figure 18A, lanes 3-8). Three different concentrations of c-Myb-AD were tested in this manner (lanes 3-4, 5-6, and 7-8). In reactions containing HBZ-AD, significantly more c-Myb-AD bound to GST-KIX (lanes 4, 6 and 8). Quantification of the signals obtained by Western blot analysis (lanes 5 and 6 compared) shows a 6-fold increase in c-Myb-AD-binding in the presence of HBZ-AD.

To confirm that this effect was not due to a direct interaction between HBZ-AD and c-Myb-AD, a GST pull-down assay was performed using the maximum concentration of c-Myb-AD from Figure 18A with the same concentration of GST-HBZ as that of HBZ-AD

Figure 18. HBZ-AD enhances the binding of HA-c-Myb-AD to GST-KIX. **(A)** Binding reactions assess the effect of HBZ-AD on c-Myb-AD binding to GST-KIX (250 nM, lanes 3-8) at three different concentrations of HA-c-Myb-AD (0.1 μ M, lanes 3-4; 0.25 μ M, lanes 5-6; 0.5 μ M, lanes 7-8). Lanes 4, 6 and 8 contained 7.5 μ M HBZ-AD. Control reactions contained GST (250 nM) with HBZ-AD (7.5 μ M, lane 1) or HA-c-Myb-AD (0.5 μ M, lane 2) as indicated. Onput fractions of HA-c-Myb-AD (1 % of maximum) and HBZ-AD (0.8 %) are shown in lanes 9 and 10, respectively. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibodies indicated. **(B)** GST-HBZ-Sp1 does not interact with HA-c-Myb-AD in GST pull-down assays. Binding reactions contained HA-c-Myb-AD (0.5 μ M) and GST (7.5 μ M, lane 1) or GST-HBZ (7.5 μ M, lane 2). A fraction of the onput of HA-c-Myb-AD (1 %) is shown in lane 3. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibody indicated.



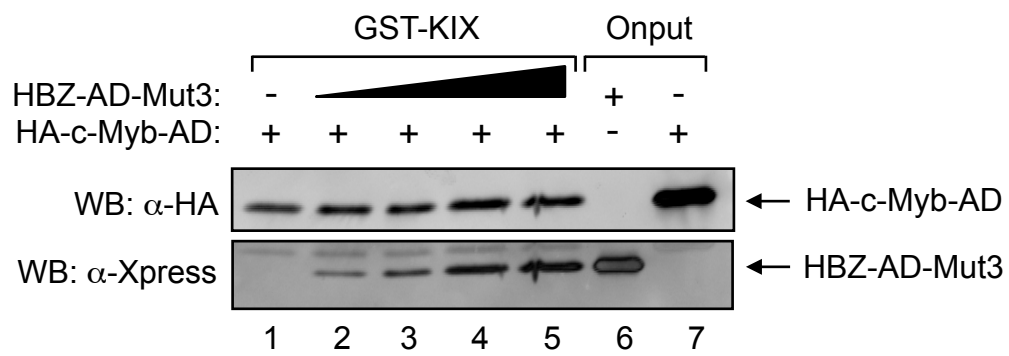
in Figure 18A. No interaction was observed between GST-HBZ and c-Myb-AD (Figure 18B, lane 2), suggesting that the increased binding of c-Myb-AD to GST-KIX was due to HBZ-AD-mediated effects on KIX.

HBZ-Mut-AD3 failed to enhance the binding of c-Myb-AD to KIX, despite retaining some ability at the high concentrations used in these experiments to bind GST-KIX (Figure 19). This finding suggests that while residues outside the currently delineated $\phi XX\phi\phi$ motifs on HBZ-AD may make contacts with KIX, the enhanced binding of c-Myb-AD to KIX appears to be mediated through one or both of HBZ-AD's $\phi XX\phi\phi$ motifs.

Interestingly, the interaction of MLL-AD with KIX also has been shown to involve residues outside its $\phi XX\phi\phi$ motif [243]. Notably however, the NMR solution structure of KIX with MLL-AD shows that three hydrophobic residues within MLL-AD's $\phi XX\phi\phi$ motif makes extensive contacts with KIX precisely in the region subsequently identified as the site of allosteric modification induced by MLL-AD [241, 271]. Together, these results strongly suggest that HBZ-AD, like MLL-AD, induces positive cooperativity into the binding of c-Myb-AD to KIX via allosteric changes in KIX mediated by one or both of HBZ-AD's $\phi XX\phi\phi$ motifs.

In addition, the finding that HBZ-AD increases the binding of c-Myb-AD to KIX is the first of three results (the other two are discussed in the subsequent section) indicating that KIX binds only one molecule of HBZ-AD. At the saturating concentrations of HBZ-AD used assessing HBZ-AD's effect on the binding of c-Myb-AD to KIX, HBZ-AD was not found to bind the pKID/c-Myb-AD-binding surface of KIX, as evidenced by its failure to compete with c-Myb-AD for binding to KIX. On the contrary, HBZ-AD increases the binding of c-Myb-AD to KIX, a finding not compatible with HBZ-AD also binding this surface of KIX.

Figure 19. HBZ-AD-Mut3 does not enhance binding of HA-c-Myb-AD to GST-KIX. Binding reactions contained GST-KIX (250 nM) and HA-c-Myb-AD (150 nM) (lanes 1-5) with increasing concentrations of HBZ-Mut-AD3 (500 nM, 1 μ M, 2.5 μ M, and 5 μ M; lanes 2-5). Onput fractions of HBZ-Mut-AD3 (2 % of maximum) and HA-c-Myb-AD (3 %) are shown in lanes 6 and 7, respectively. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibodies indicated.



To determine whether full-length HBZ-Sp1 affects the activity of c-Myb-AD in cells, the same luciferase assay system described for Figure 13A was used. A fusion construct of c-Myb-AD (aa 186-325) and Gal4DBD [238] was co-transfected into Jurkat T cells with an expression plasmid for GalTK-Luc. As reported for Gal4DBD-MLL-AD, activation of this reporter has been correlated with an interaction between Gal4DBD-c-Myb-AD and endogenous p300/CBP [238]. As expected, we found that expression of Gal4DBD-c-Myb-AD resulted in strong luciferase activity, while Gal4DBD alone did not (Figure 20, lanes 2 and 3). In contrast to HBZ's effects on the activity of Gal4DBD-MLL-AD, HBZ augmented Gal4DBD-c-Myb-AD activation of the luciferase reporter by more than 2 fold (lane 4), suggesting that HBZ enhances the interaction between c-Myb-AD and p300/CBP in cells. HBZ-Mut-AD3 failed to significantly enhance Gal4DBD-c-Myb-AD transcriptional activity (lane 5), again indicating that the effect is mediated by HBZ-AD's $\phi XX\phi\phi$ motifs.

HBZ-AD Enhances the Binding of Unphosphorylated CREB to KIX

Since MLL-AD was previously reported to induce positive cooperativity into the binding of both pCREB/pKID and c-Myb-AD to KIX, GST pull-downs were performed to assess whether HBZ-AD likewise increased the binding of pCREB to GST-KIX. Unlike the results obtained with c-Myb-AD, HBZ-AD did not increase the amount of pCREB bound to KIX (Figure 21, lanes 3 and 4). This result does, however, provide additional support for a 1:1 stoichiometric ratio for the HBZ-AD•KIX complex. That HBZ-AD failed to compete with pCREB for KIX binding even at the high concentration of HBZ-AD used in this assay again indicates that HBZ-AD does not bind the pKID/c-Myb-AD-binding surface of KIX.

The binding of CREB to KIX is controlled by CREB phosphorylation on serine 133. The reported K_d for the interaction between KIX and pKID is 0.7 μ M, compared to 108 μ M

Figure 20. Full-length HBZ-Sp1 enhances the transcriptional activity of c-Myb-AD in cells. Jurkat cells were transfected with pGalTK-Luc (100 ng, lanes 1-5) alone or in combination with Gal4-DBD (50 ng, lane 2), Gal4-c-Myb-AD (50 ng, lanes 3-5), pcDNA-HBZ (100 ng, lane 4) or pcDNA-HBZ-Mut-AD3 (100 ng, lane 5) as indicated. Luciferase assays were performed 24 hours after transfection. The reported values are the average luminescence $\pm$ S.E. from one experiment performed in duplicate and are representative of three independent experiments. Luciferase activity was normalized to Renilla luciferase from the herpes simplex virus thymidine kinase promoter (pRL-TK, Promega).

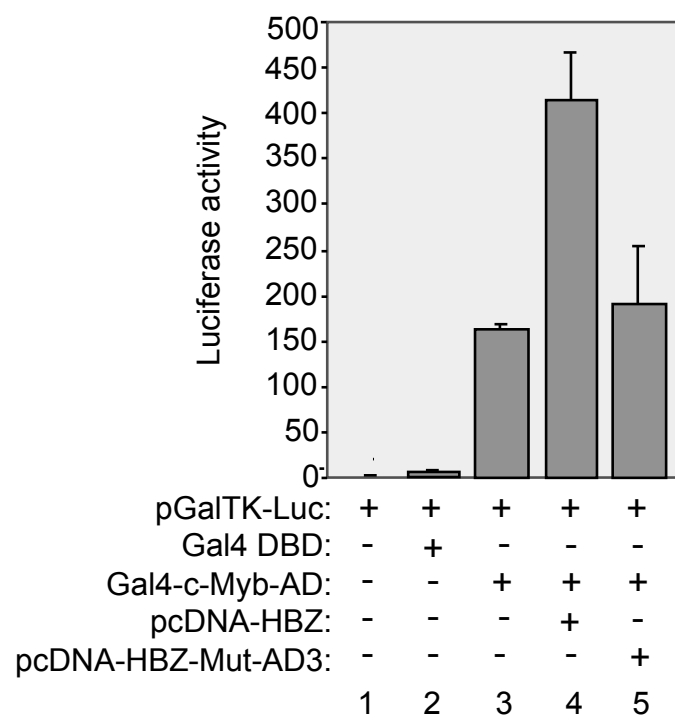
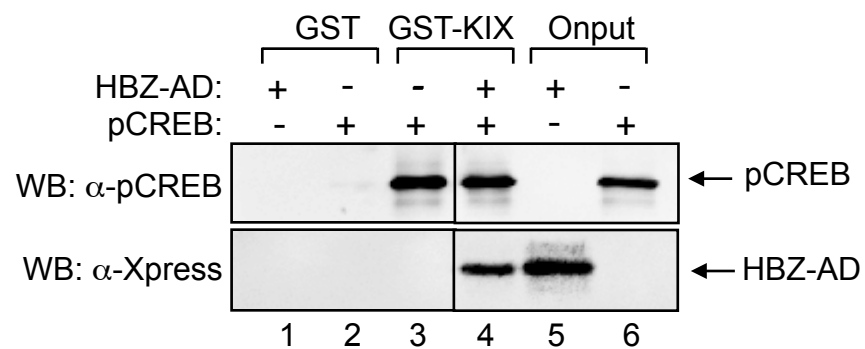


Figure 21. HBZ-AD does not enhance binding of pCREB to GST-KIX. Binding reactions contained GST-KIX (250 nM, lanes 3-4) and pCREB (25 nM, lanes 3-4) in the absence (lane 3) or presence of HBZ-AD (20 μ M, lane 4). Control reactions contained GST (250 nM) with HBZ-AD (20 μ M, lane 1) or pCREB (25 nM, lane 2). Onput fractions of HBZ-AD (0.5 %) and pCREB (50 %) are shown in lanes 5 and 6, respectively. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibodies indicated. Lanes shown are from the same membrane; vertical lines show where the membrane was cropped.



for unphosphorylated KID (Table 3) [265]. The possibility was considered, then, that HBZ-AD might stabilize the interaction between CREB and KIX via effects that are irrelevant or undetectable in the context of the high-affinity interaction between pCREB and KIX.

This hypothesis was tested using the same experimental procedure used to test the effects of HBZ-AD on c-Myb-AD. Three different concentrations of unphosphorylated CREB were incubated with GST-KIX in the absence or presence of saturating amounts of HBZ-AD (Figure 22A). As expected, very little CREB bound to GST-KIX in the absence of HBZ-AD (lanes 3, 5 and 7). However, in the presence of HBZ-AD, significantly more CREB remained bound (lanes 4, 6 and 8). Quantification of the signals obtained by Western blot analysis (lanes 5 and 6 compared) shows a 6.5-fold increased in CREB-binding in the presence of HBZ-AD, again strongly suggestive of HBZ-AD-mediated positive cooperativity.

Although the bZIP domains of HBZ and CREB have previously been shown to interact, an HBZ mutant lacking its bZIP did not interact with the CREB bZIP [13]. To determine if HBZ-AD would interact with full-length CREB, GST pull-downs using GST-CREB at a concentration equivalent to the maximum concentration of CREB used above was incubated with the same concentration of HBZ-AD used above. The results (Figure 22B) confirm that HBZ-AD does not interact with full-length CREB, again suggesting that HBZ-AD mediates enhanced binding to this surface of KIX via allosteric modulation. This result also provides one additional confirmation that the stoichiometric ratio of the HBZ-AD•KIX complex is 1:1, as evidenced by the failure of HBZ-AD even at high concentrations to compete with CREB for its binding site on KIX.

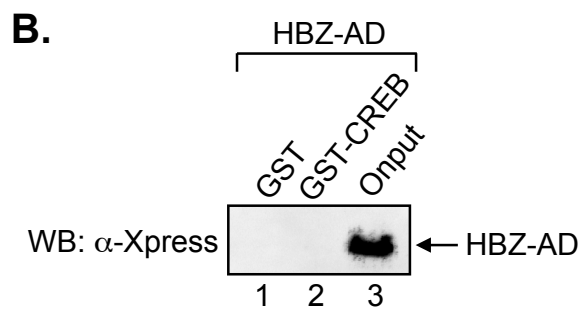
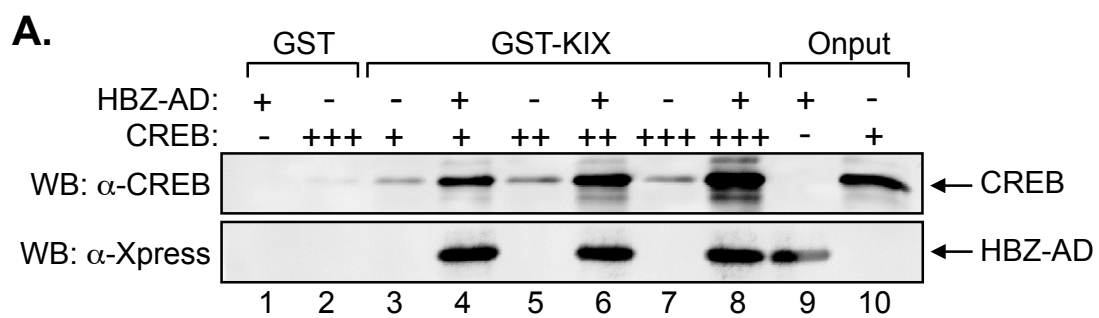
Table 3. K_d Values for KIX-Binding Proteins

Equilibrium Dissociation Constants (K_d , μM) for Activation Domains
of Cellular Proteins Binding to the KIX Domain of p300/CBP

pKID (pCREB) (aa 101-160)	0.7 ± 0.1 [265]
unphosphorylated KID (aa 101-160)	108 ± 5 [265]
MLL activation domain (aa 2840-2858)	2.8 ± 0.4 [244]
c-Myb activation domain (aa 291-315)	10.0 ± 2 [244]
p53 activation domain (aa 1-61)	19 ± 5 [248]

Figure 22. HBZ-AD enhances the binding of unphosphorylated CREB to GST-KIX. **(A)**

Binding reactions assessing the effect of HBZ-AD on CREB contained GST-KIX (500 nM, lanes 3-8) and three different concentrations of CREB (50 nM, lanes 3-4; 100 nM, lanes 5-6; 200 nM, lanes 7-8). Lanes 4, 6 and 8 contained 5 μ M HBZ-AD. Control reactions contained GST (500 nM) with HBZ-AD (5 μ M, lane 1) or CREB (200 nM, lane 2). Onput fractions of HBZ-AD (2 %) and CREB (12 %) are shown in lanes 9 and 10, respectively. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibodies indicated. **(B)** HBZ-AD does not bind GST-CREB in GST pull-down assays. Binding reactions contained 5 μ M HBZ-AD with either GST (200 nM, lane 1) or GST-CREB (200 nM, lane 2). A fraction of the onput of HBZ-AD (2 %) is shown in lane 3. Bound proteins were resolved with SDS-PAGE and analyzed by Western blot (WB) using the antibody indicated.



Full-Length HBZ-Sp1 Fails to Inhibit Binding of Full-Length pCREB or CREB to a CRE

Previous reports showed that the bZIP domain of HBZ interacts with the bZIP domain of CREB [13, 200] and prevents the bZIP of CREB from binding the consensus CRE [13]. This raised the question of what the physiological relevance might be of HBZ-AD-induced enhanced binding between CREB and KIX, since removal of CREB from the DNA would conceivably negate effects of enhanced interactions between CREB and p300/CBP.

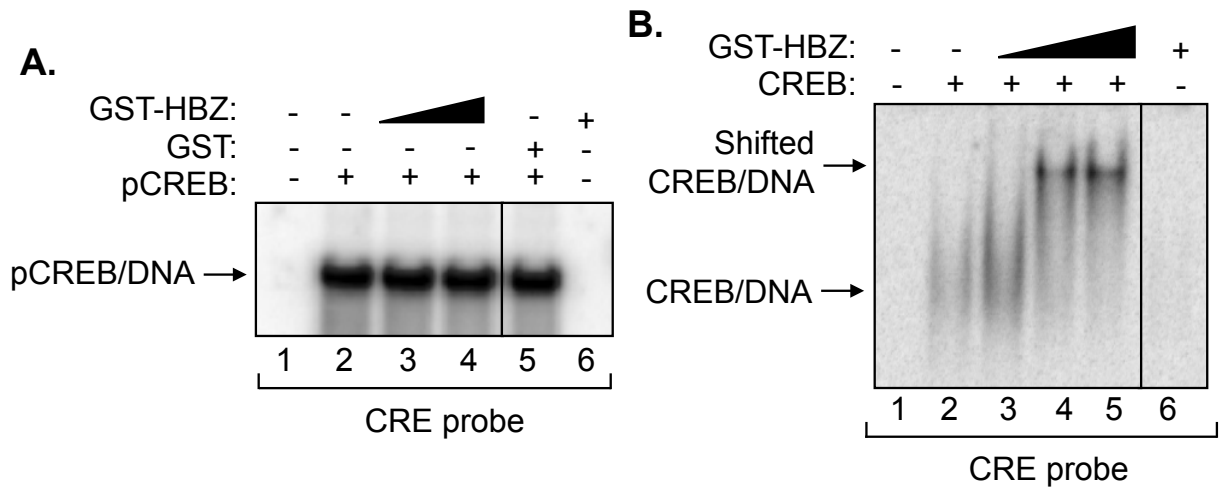
To determine the effect of full-length HBZ-Sp1 on interactions between pCREB or CREB with the consensus cellular CRE, EMSAs were performed. Reactions in Figure 23A contained pCREB with increasing amounts of GST-HBZ-Sp1 with the CRE (lanes 2-4). Full-length GST-HBZ failed to inhibit binding of pCREB to the CRE in these reactions, indicating the possibility that in the context of full-length HBZ and pCREB in the presence of the CRE, interactions between their bZIPs do not occur. GST also had no effect (lane 5), and GST-HBZ alone did not bind the CRE probe (lane 6).

The same experiment was conducted using unphosphorylated CREB instead of pCREB (Figure 23B). GST-HBZ was similarly unable to inhibit binding of unphosphorylated CREB to the CRE and unexpectedly appeared to stabilize and shift the CREB•CRE complex (lanes 3-5), possibly indicating that full-length HBZ can form heterodimers with unphosphorylated CREB that remain capable of binding the CRE.

Since it was previously reported that pre-incubation of CREB-bZIP with the vCRE prevented removal by HBZ-bZIP [13], it should be noted that neither CREB nor pCREB was pre-incubated with the CRE probe for the EMSAs shown in Figure 23. Rather, all proteins were incubated together prior to the addition of the CRE probe. Thus, the failure of GST-

Figure 23. Full-length GST-HBZ-Sp1 fails to inhibit binding of pCREB or CREB to a CRE.

(A) GST-HBZ-Sp1 does not inhibit binding of pCREB to CRE. EMSA reactions contained the CRE probe (1 nM), pCREB (1.5 nM, lanes 2-5) and increasing concentrations of GST-HBZ (38 nM and 150 nM; lanes 3-4). Control reactions contained pCREB with GST (150 nM, lane 5) and GST-HBZ alone (150 nM, lane 6) with the CRE probe. Lanes shown are from the same gel; vertical lines show where the gel was cropped. **(B)** GST-HBZ-Sp1 shifts the unphosphorylated CREB•CRE complex. EMSA reactions contained the CRE probe (1 nM), CREB (1.5 nM, lanes 2-5) and increasing concentrations of GST-HBZ (38 nM, 150 nM, and 750 nM; lanes 3-5). Lane 6 contains GST-HBZ alone (750 nM) with the CRE probe. Lanes shown are from the same gel; vertical lines show where the gel was cropped.



HBZ to inhibit binding of pCREB or CREB to the CRE cannot be attributed to pre-incubation before the addition of GST-HBZ.

Docking Programs Place Predicted HBZ-AD Structure at MLL-AD-Binding Surface of KIX

Computational docking programs to predict sites of protein-protein interactions have improved in reliability and accuracy in recent years, and many are available as online servers for use by non-experts [272, 273]. Of these, ClusPro [274-277], ZDOCK [278] and HADDOCK [279, 280] have been rated the most reliable [272, 281]. Results from the latest critical assessment of predicted interactions (CAPRI) indicate that the ClusPro webserver correctly predicted 5 of 13 targets, while HADDOCK's server correctly predicted 4 of 13 [272, 281]. All three programs allow input of experimental data, such as the identification of residues known to be involved in a protein-protein interaction based on mutagenesis studies [272].

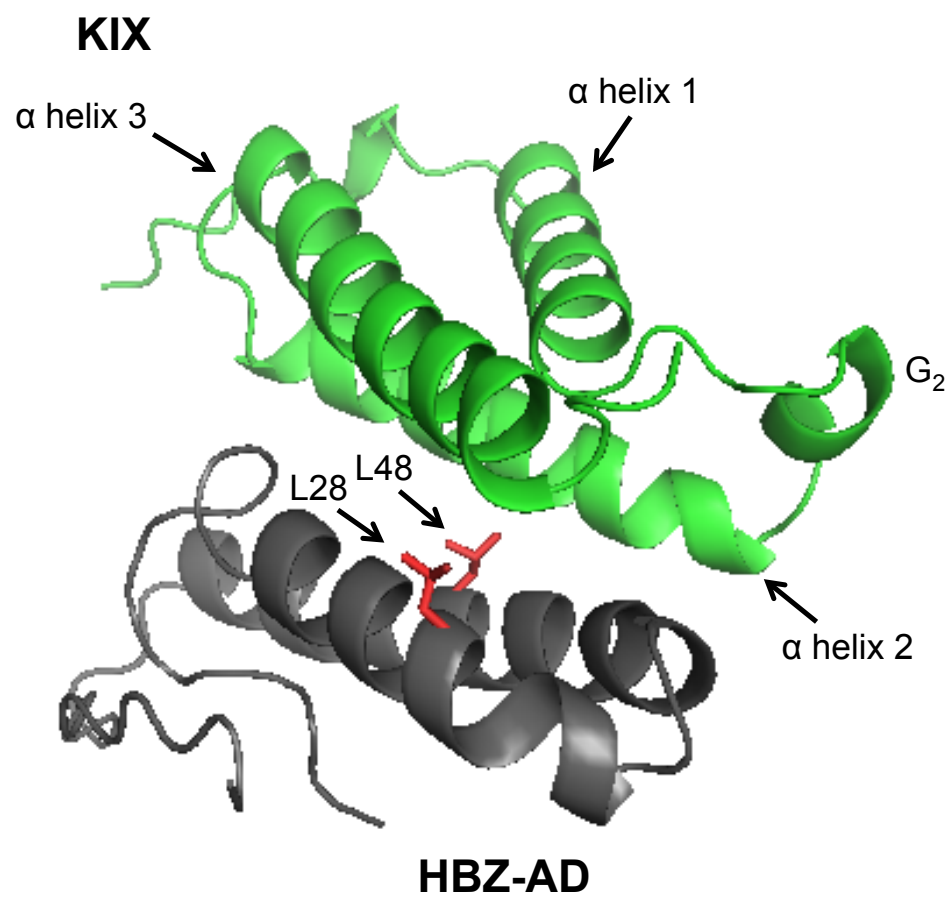
Each of these programs requires 3D structures from either NMR or crystal structures or computational predictions derived from amino acid sequences. The predicted 3D structure of HBZ-AD was obtained from the amino acid sequence using I-TASSER [255]. The NMR solution structure of KIX in a complex with c-Myb-AD and MLL-AD (pdb file 2agh) [241] is available from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (<http://www.pdb.org/pdb/home/home.do>). The c-Myb-AD and MLL-AD molecules were deleted from the 2agh pdb file, leaving only KIX. The HBZ-AD and KIX 3D structures were then submitted to the online docking servers ClusPro, ClusPro2, HADDOCK, HEX, PatchDock and ZDOCK. All submissions except to HADDOCK were made without input regarding experimental data.

The HADDOCK webserver requires specification of residues thought to be involved in binding. HBZ-AD residues were selected based on results with the mutated $\phi XX\phi\phi$ motifs showing that leucines 27, 28, 47 and 48 are critical to the interaction with KIX. Of these residues, only L28 and L48 were specified for docking based on the I-TASSER predicted 3D structure of HBZ-AD showing that residues L27 and L47 are directed toward the interior of the hairpin structure and are therefore unlikely to be available for KIX binding (Figure 9B). Based on the GST-KIX mutagenesis data, KIX amino acids Y631 and L664 were selected as possible interacting residues.

The top-scoring results from all programs placed HBZ-AD at or near the MLL-AD binding surface of KIX, and none of the top-scoring models placed HBZ-AD at or near the pKID/c-Myb-AD binding surface. All programs except HADDOCK predicted that HBZ-AD would bind KIX via only one of its two predicted α helices. HADDOCK results were returned in ranked clusters, with four ranked models per cluster. Models in the top-scoring HADDOCK cluster also docked HBZ-AD via one helix, but the second-scoring cluster placed HBZ-AD at the MLL-AD-binding surface of KIX via interactions involving both of HBZ-AD's predicted α helices (Figure 24).

It may be significant that the NMR structure of KIX used for docking was originally obtained from KIX in a complex with c-Myb-AD and MLL-AD, which were deleted from the file, leaving only the KIX structure. Although the MLL-AD-binding surface of KIX is thought to have significant plasticity, KIX bound to the single helix of MLL-AD would have been in the particular conformation specific for MLL-AD binding, which would be expected to be

Figure 24. HADDOCK docks HBZ-AD at the MLL-AD-binding surface of KIX via contacts made by both of HBZ-AD's $\phi XX\phi\phi$ motifs. HBZ-AD is shown in gray, with the side chains of leucines 28 and 48 shown in red. Leucines 27 and 47, which were predicted by I-TASSER to make intramolecular contacts within the HBZ-AD hairpin tertiary structure, are not visible at this orientation.



more closed than a potential conformation accommodating two helices. HADDOCK, unlike the other programs, incorporates side-chain and backbone flexibility, allowing for some degree of conformational change upon ligand docking [272, 282]. This feature may partially explain why this was the only program that returned a model utilizing both of HBZ-AD's predicted α helices.

In addition, the scoring differences between HADDOCK's first and second clusters arose mainly from increased restraint violations in the second cluster despite a larger buried surface area, which is considered the best predictor of a favorable interface. It may be possible that in an experimental rather than predictive model, the MLL-AD-binding surface of KIX would flex more upon HBZ-AD binding than permitted in the HADDOCK algorithm, leading to accommodation of both HBZ-AD's α helices and the maximum buried surface area.

CHAPTER 3: DISCUSSION

HBZ-AD has been shown in these studies to have a strong affinity for the KIX domain of the transcriptional coactivators p300/CBP, with an apparent K_d of 3.2 ± 0.5 nM. Over a dozen different transcriptional regulatory proteins have been found to bind the KIX domain [240], and the reported K_d values of proteins whose interactions with KIX have been quantified are in the micromolar range (Table 3). These include pKID [265], p53-AD [248], c-Myb-AD and MLL-AD [244]. Although it is not possible to quantitatively compare these values since they were derived using different methodologies in different labs, they suggest that HBZ-AD would be an effective competitor with transcription factors binding the same surface of KIX. In addition, the high affinity of HBZ-AD for KIX also indicates it would be a potent allosteric agent capable of affecting the other surface of KIX.

The KIX domain has been well-characterized by NMR to have two distinct binding surfaces. NMR structures show one surface of KIX is contacted by pKID [239] and c-Myb-AD [241, 242], while the other surface is contacted by MLL-AD [241]. A consensus $\phi XX\phi\phi$ motif occurring within an amphipathic α helix in each of these proteins has been shown to mediate critical interactions with KIX regardless of which KIX surface is contacted. The motif is similarly present in other KIX-binding proteins, including E2A [250], FOXO3 [249], c-Jun [245, 248], Tax [247], and SREBP [251]. HBZ-AD contains two predicted amphipathic α helices, each containing at least one delineated $\phi XX\phi\phi$ motif. Though the presence of more than one consensus motif in HBZ-AD suggested the possibility that HBZ-AD might simultaneously bind more than one molecule of KIX, the experimental evidence indicates that HBZ-AD binds only the MLL-AD-binding surface of KIX with a 1:1 stoichiometry.

Model of HBZ-AD Binding to KIX

The two $\phi XX\phi\phi$ motifs in HBZ-AD investigated in this study were each found to contribute significantly but unequally to interactions with KIX. Mutations in the 24-VDGLL-28 motif resulted in a 267-fold increase in the apparent K_d relative to wild type compared to only a 60-fold increase resulting from mutations in the 44-LDGLL-48 motif.

Potentially significant to the different contributions of each examined $\phi XX\phi\phi$ motif in HBZ-AD, the first predicted helix has more turns of unbroken amphipathicity than the second (Figure 9A), and the α -helical content for the second helix is predicted to fall sharply beginning around residues 47-48 (Table 1). An alternative delineation of a $\phi XX\phi\phi$ motif in the second helix (3'-47-LGDLV-43-5') includes residues V43 and L44, both of which lie well within the range of predicted α -helical formation (Figure 8A, Table 1). It may therefore be possible that this alternative motif mediates contacts with KIX to a greater degree than the 44-LDGLL-48 motif and that mutations of V43 and L44 would have resulted in a loss of binding more equivalent to that observed for mutations in the 24-VDGLL-28 motif.

The loss of affinity between KIX and the HBZ-AD mutants could arise from disrupted contacts directly between HBZ-AD's $\phi XX\phi\phi$ motifs and KIX or from effects on HBZ-AD folding. Although more experiments would be required to differentiate the portion of overall energetic costs associated with each possibility, the list of proteins known to bind KIX directly via $\phi XX\phi\phi$ motifs within amphipathic α helices strongly suggests disrupted direct contacts between KIX and HBZ-AD's $\phi XX\phi\phi$ motifs. Nevertheless, data from the BS³ assay, discussed below, indicate that HBZ-AD's $\phi XX\phi\phi$ motifs may also make intramolecular contacts within HBZ-AD, supporting a hypothesis that these motifs function

not only to mediate interactions with KIX but also to stabilize the secondary and/or tertiary structure of HBZ-AD.

HBZ-AD Tertiary Structure and Anomalous SDS-PAGE

HBZ-AD is predicted to form a helix-coil-helix hairpin tertiary structure (Figures 9B and 24). Experimental evidence supporting this prediction came unexpectedly from observations that the HBZ-AD mutants migrated anomalously in SDS-PAGE (Figure 10). This phenomenon has been shown to arise specifically in mutated hairpin peptides whose wild-type tertiary structure is particularly resistant to denaturation by SDS [260]. Although the details remain unclear, it is thought that mutations of key residues within hairpin peptides allow for greater access to the interior of the hairpin by SDS. This alters the degree of denaturation by SDS, resulting in anomalous migration in SDS-PAGE. Given that this phenomenon has been proposed for use as a screening tool to identify proteins with robust tertiary structure [260], it seems unlikely that HBZ-AD would exhibit such behavior in the absence of hairpin structure [260]. If correct, this represents a departure from the observed trend that KIX-binding proteins exist as intrinsically disordered proteins that fold upon binding KIX into an α -helical structure [253].

It is not known whether putative alterations in the tertiary structures of the HBZ-AD mutants reach a threshold of functional relevance in a physiological buffer in the absence of SDS. It may be possible that variations in structural integrity only become evident under the denaturing pressure of SDS. It is interesting, however, that the increasing degree of anomalous migration by each HBZ-AD mutant corresponds to its increasing loss of KIX-binding ability. The order of migration in SDS-PAGE, from fastest to slowest, is HBZ-AD_{wt}, HBZ-Mut-AD2, HBZ-Mut-AD1, and HBZ-Mut-AD3 (Figure 10A). This ranking directly

corresponds to the progressive loss of KIX-affinity by HBZ-Mut-AD2, HBZ-Mut-AD1, and HBZ-Mut-AD3. These findings suggest that disrupted intramolecular contacts, appearing to occur to the largest degree in HBZ-Mut-AD3, followed by HBZ-Mut-AD1 and HBZ-Mut-AD2, may in part account for the corresponding loss of KIX-binding activity by each mutant.

Flexibility of the MLL-AD-Binding Surface Accommodates Structural Variation

The MLL-AD-binding surface of KIX contacted by HBZ-AD has been shown to exhibit significant flexibility, presumably in order to accommodate a variety of different ligands [241]. It has been suggested that the shape of the MLL-AD-binding surface may in fact be determined by the bound ligand [241]. Evidence supporting this hypothesis comes not only from NMR studies [241] but also from reports showing that different ligands interacting with the MLL-AD-binding surface of KIX produce dramatically different allosteric effects in the opposite surface of KIX. For example, the binding of MLL-AD induces positive cooperativity into the binding of either pCREB or c-Myb-AD at the other surface of KIX. However, the small-molecule antagonist KG-501, which also binds the MLL-AD-binding surface of KIX, decreases the ability of pCREB to bind KIX at the other surface [232]. These different allosteric effects produced in the pCREB/c-Myb-AD-binding surface, both arising from ligand binding at the MLL-AD-binding surface, imply that the mode of binding at the MLL-AD-binding surface varies depending on the size or shape of the bound ligand.

Given this observation of binding-site flexibility, it may be possible that both of HBZ-AD's predicted helices contact the MLL-AD-binding surface. The docking program HADDOCK, which incorporates backbone and side-chain flexibility into its algorithm, returned a cluster of second-place models that docked HBZ-AD at the MLL-AD-binding

surface of KIX through interactions involving both of HBZ-AD's predicted helices (Figure 24). It is interesting to note that these models scored the highest on the amount of buried surface area in the bound state, which is considered to be the best indicator of a favorable interface, but had higher restraint violations than the first-place models predicting contact through only one helix. Had the algorithm been run with a KIX molecule not pre-formed to the single MLL-AD helix, the restraint violations may not have been a factor, possibly placing the cluster from which the model in Figure 24 was taken in first place.

Two Models Proposed for HBZ-AD Binding to KIX

Assuming a hairpin structure for HBZ-AD that is dependent on one or both of HBZ-AD's $\phi XX\phi\phi$ motifs, two potential models of KIX binding may be suggested given the following three observations: (1) experimental evidence indicating that both of HBZ-AD's $\phi XX\phi\phi$ motifs contribute to KIX binding; (2) NMR data showing that pKID, c-Myb-AD and MLL-AD each binds KIX via *direct* contacts through a $\phi XX\phi\phi$ motif within an amphipathic α helix; and (3) $\phi XX\phi\phi$ motifs occurring within amphipathic α helices in other proteins that interact with KIX.

The first model proposes that HBZ-AD makes direct contacts with KIX via only one $\phi XX\phi\phi$ motif, while residues in the other or both motifs stabilize intramolecular contacts within HBZ-AD. Secondly, HBZ-AD may contact KIX via both $\phi XX\phi\phi$ motifs, while one or both motifs also participate in intramolecular contacts within HBZ-AD. This second model is supported by the computational predicted tertiary structure of HBZ-AD and docking results showing that L28 and L48 mediate contacts with KIX (Figure 24), while L27 and L47 make intramolecular contacts within HBZ-AD's predicted hairpin structure (Figure 9B). If both of HBZ-AD's $\phi XX\phi\phi$ motifs, occurring on separate predicted helices, make

direct contacts with KIX, this would represent a departure from the mode of binding observed via NMR for MLL-AD in which contact occurs via only one helix.

To summarize, HBZ-AD represents a potentially novel mode of binding in which a putative hairpin structure contacts KIX, either through one or two amphipathic α helices, both of which contain a $\phi XX\phi\phi$ motif. One of the challenges to developing small-molecule inhibitors to block the interactions of transcription factors with p300/CBP is the moderate affinity typically achieved for their target sites, usually in the micromolar range [283]. The uncommonly strong affinity between HBZ-AD and KIX may originate from a previously uncharacterized mode of binding, which makes HBZ an attractive candidate for further structural studies.

Variation in Effects of HBZ-AD and MLL-AD on pCREB Binding to KIX

HBZ-AD was shown here to significantly enhance the binding of c-Myb-AD and unphosphorylated CREB to KIX. HBZ-AD also appeared to slightly enhance binding of c-Jun to KIX. Interestingly, although MLL-AD induces positive cooperativity into pKID and pCREB binding, HBZ-AD does not appear to enhance the binding of pCREB to KIX. It is not known whether MLL-AD induces cooperativity into the binding of unphosphorylated CREB. It should be noted however that though MLL-AD was confirmed to induce positive cooperativity into the binding of c-Myb-AD to KIX via allosteric changes in KIX, it could not be ruled out that the positive cooperativity induced into the binding of pKID to KIX arose from direct interactions between MLL-AD and pKID [241].

Making the assumption of allostery as the mechanism of MLL-AD-mediated positive cooperativity in pKID binding, one explanation for HBZ-AD's lack of effect on pCREB binding may lie in the previously discussed observation that different ligands at the MLL-

AD-binding surface can produce different allosteric changes in KIX. Thus, it would be feasible for HBZ-AD to bind the MLL-AD-binding surface of KIX yet not produce the same effect on pCREB binding as MLL-AD. Stabilization of the unphosphorylated CREB•KIX complex by HBZ-AD may occur via residues that become irrelevant in the context of CREB phosphorylation at serine 133 and its high-affinity interaction with tyrosine 658 on KIX.

Protein Length and Phosphorylation may Mediate Binding between HBZ and CREB

Previous findings showed that the bZIP of HBZ removes the bZIP of CREB from a cellular CRE *in vitro* [13]. However, full-length HBZ appears unable to remove full-length phosphorylated CREB from a CRE (Figure 23A). Variation in experimental procedure may explain the differing results. Reactions for the full-length proteins were incubated approximately two hours compared to 30 minutes for the bZIP proteins. It has been reported that DNA is both required for and cooperatively induces homodimerization of CREB as well as pCREB [284, 285]. If a pCREB•HBZ heterodimer forms prior to addition of DNA, a longer incubation time may allow full dissociation of putative pCREB•HBZ heterodimers, freeing pCREB to bind the CRE. Thus, the repressive effects of HBZ may be more visible with shorter incubation times. Once bound to the CRE, the affinity of the pCREB homodimer•CRE complex is reportedly in the low nanomolar range [285], so it may be unlikely that HBZ would reassociate with pCREB. However, it should also be noted that 100 times more HBZ was used relative to pCREB in reactions with the full-length proteins, while only 15 times more HBZ bZIP relative to CREB bZIP was used. Unless incubation time is critical, reactions with more HBZ would have been expected to show more, not less, repression, assuming the absence of a competing self-associating HBZ reaction.

Possibly relevant to this discussion are reports, though conflicting, that phosphorylation of CREB on serine 133 enhances its DNA-binding ability [285-287]. There are also reports that phosphorylation changes CREB's conformation and may influence dimer partner specificity [285-289]. The bZIP portion of CREB does not include serine 133 and would thus not be subject to potential alterations and selectivity that may be induced by phosphorylation. The results in Figure 23B however cannot be attributed to alterations due to phosphorylation, nor does it appear that the affinity of unphosphorylated CREB for the CRE disallowed an interaction with HBZ. Perhaps in the context of full-length proteins, additional contacts stabilize interactions of a CREB•HBZ heterodimer with a consensus CRE.

Interestingly, full-length HBZ was able to repress expression of a CRE reporter in forskolin-activated CEM T cells [13]. Forskolin increases cAMP levels in cells, which induces CREB phosphorylation by protein kinase A [290]. This would suggest that full-length HBZ transfected into cells is expressed at levels sufficient to remove full-length pCREB homodimers from a CRE reporter. Forskolin however has also been shown to up-regulate mRNA of several AP-1 family members [291-293]. AP-1 and other CREB/ATF members, as well as interfamily heterodimers, have been shown to bind cellular CREs with high affinity [294-300]. Moreover, a recent study has shown that members of different human bZIP families, including AP-1 and CREB/ATF, that were previously thought not to interact are capable of forming heterodimers [298, 300]. Given that AP-1 can bind [294-300] and activate [301-305] CREs and that HBZ has been shown to represses the activity of some AP-1 members and remove them from DNA [11, 202], it is conceivable that HBZ repression of the CRE reporter could have occurred via more than one mechanism.

A Model of Transcriptional Deregulation by HBZ

Transcriptional regulation involves a complex series of events including cell signaling, post-translational modifications, chromatin remodeling and assembly of multi-protein complexes. The cellular co-activators p300/CBP are pivotal in the assembly and control of these processes and have been found to interact with over 400 different proteins [224, 227, 240]. The KIX domain of p300/CBP has been shown to bind at least a dozen different transcription factors [224, 240].

The activation domain of HBZ has been shown in the present study to bind the MLL-AD-binding surface of KIX with high affinity and to compete with MLL-AD, Tax, and p53 for KIX binding. Given these results, it is likely that HBZ-AD similarly competes with other transcription factors binding this surface. On the other hand, HBZ-AD has been shown to enhance the binding of both c-Myb-AD and CREB to the other surface of KIX.

A previous report has indicated that HBZ both up- and down-regulates the expression of many genes [9]. The twofold effects of HBZ-AD on KIX suggest a model in which HBZ-AD can mediate either up- or down-regulation of cellular transcription. According to this model, up-regulation would occur when HBZ-AD enhances interactions between KIX and the transcription factors c-Myb or CREB, as well as other factors capable of binding this surface of KIX. This would increase the presence of p300/CBP at promoters controlled by these transcription factors, leading to increased transcription. In the case of down-regulation, HBZ-AD would compete with transcription factors that bind the MLL-AD-binding surface of KIX, thereby reducing the presence of p300/CBP at promoters controlled by those factors and inhibiting transcription of those genes. Therefore, through its

interaction with KIX, HBZ-AD could either increase or decrease the presence of p300/CBP at given promoters, resulting in an increase or decrease in the expression of given genes.

Oncogenic Potential of Deregulated MLL, c-Myb, CREB, p53, and p300/CBP

HBZ RNA has been found in essentially all ATL patient samples tested to date, in contrast with other viral genes, including Tax, which often are not expressed [145, 178, 193]. This finding has led to the hypothesis that HBZ is crucial for the transformation of infected cells and maintenance of the leukemic phenotype [145, 178, 193]. The exact mechanisms of leukemogenesis by HTLV-1 have not been determined, but it is thought that deregulation of cellular transcription plays a major role.

HBZ-AD was shown in this study to differentially affect interactions between KIX and the cellular proteins MLL-AD, p53, c-Jun, c-Myb and CREB. Deregulation of each of these proteins, as well as deregulated p300/CBP activity, has been associated with oncogenic potential. Both MLL and p300/CBP are directly involved in the remodeling of chromatin [224, 240, 306, 307], and their dysfunction has been associated with multiple hematological malignancies [230, 307]. The role of p53 as a tumor suppressor is well-documented [308]. Aberrant CREB activity has also been correlated with the development of various cancers including leukemia [309, 310]. The ability of HBZ-AD to enhance the binding of unphosphorylated CREB to KIX could potentially activate CREB-responsive genes in the absence of cAMP signaling. Importantly, the ability of HBZ to dimerize with unphosphorylated CREB and bind a cellular CRE may potentiate this effect. On the other hand, although dysfunction in c-Jun has been linked to oncogenesis [311, 312], an enhanced interaction between c-Jun and KIX mediated by HBZ-AD may be of little physiological consequence given that full-length HBZ has also been shown to down-regulate the activity

of c-Jun in cells and to remove c-Jun homodimers from the AP-1 response element *in vitro* [11, 202]. It may be possible, however, that HBZ is unable to remove c-Jun heterodimers from DNA, which may provide an opportunity for HBZ-AD to deregulate c-Jun activity via its effects on KIX.

Dysfunction in c-Myb activity has been implicated in the development of numerous cancers, including T-cell acute lymphoblastic leukemia [313]. Expression of c-Myb is thought to be restricted mainly to hematopoietic progenitor cells but can be up-regulated in activated T cells [314]. These expression patterns are interesting in light of recent data suggesting that HTLV-1 infected hematopoietic stem cells may play a role in the development of ATL [44, 46, 47, 65, 315] as well as numerous studies showing that HTLV-1 infection activates mature CD4⁺ T cells [316, 317]. C-Myb regulates proliferation and differentiation in hematopoietic stem cells via interactions with p300 [318], and studies in mice have shown that deficiencies in c-Myb lead to decreased numbers of differentiated CD4⁺ T cells [314]. Since the monoclonal expansion seen in ATL occurs almost exclusively in CD4⁺ T cells, it is interesting to speculate whether HBZ in an HTLV-1 infected hematopoietic stem cell could amplify c-Myb function by enhancing interactions with p300, thereby skewing differentiation toward CD4⁺ T cells. On the other hand, Tax- or immunological-mediated activation of a mature HTLV-1-infected CD4⁺ T cells may initiate up-regulation of c-Myb expression [314], which could then be enhanced by HBZ. It has been found that increased c-Myb expression contributes to T-cell transformation [313], therefore intensified c-Myb activity induced by HBZ-AD might contribute to transformation of T cells in ATL.

Summary

The data presented in this study have provided a potential molecular mechanism to help explain HBZ-mediated deregulation of cellular transcription in HTLV-1 infection. They have additionally raised the interesting possibility of a novel mode of binding to KIX, which might be further explored for the design of small-molecule inhibitors for p300/CBP. And finally, they have added another entry to the growing repertoire of proteins known to modulate interactions between transcriptional regulatory proteins and the KIX domain of p300/CBP.

CHAPTER 4: MATERIALS AND METHODS

Bacterial Expression Vectors and Cloning

Full-length HBZ-Sp1 (aa 1-206) was PCR-amplified from pcDNA3.1-MycHis-HBZ [195] and cloned into the EcoRI and XbaI sites of pSG424 [319], containing the Gal4-DBD (aa 1-147), to create the fusion protein Gal4-DBD-HBZ (pSG424-HBZ). pRSET-Gal4-DBD-HBZ was constructed by amplifying Gal4-DBD-HBZ from pSG424-HBZ and cloning the product into the BamHI and NcoI sites of pRSET-A (Invitrogen). pRSET-Gal4-DBD-HBZ-AD was constructed by amplifying Gal4-DBD-HBZ-AD (aa 1-77) from pRSET-Gal4-DBD-HBZ and cloning the product into the BamHI and NcoI sites of pREST-A. Two clones of pRSET-Gal4-HBZ-AD were used: the one used in Figure 4A did not have any mutations, while the one used in Figure 8B had an A to T substitution at aa 3 of HBZ. The affinities of both constructs for GST-KIX were tested and found to be the same. pRSET-Gal4-DBD-HBZ-AD mutants were constructed by amplifying HBZ-AD from the HBZ mutant templates with L27A/L28A, L47A/L48A, or L27A/L28A/L47A/L48 substitutions (pcDNA3.1-MycHis-HBZ) [195]. Products were cloned into the EcoRI site of pRSET-Gal4-DBD-HBZ-AD after removal of the HBZ-AD wt sequence by digestion with EcoRI. pRSET-HBZ-AD was constructed by amplifying HBZ-AD from pRSET-Gal4-DBD-HBZ and cloning the product into the EcoRI site of pRSET-B (Invitrogen). pRSET-HBZ-AD mutants were constructed by cloning the above HBZ-AD mutant PCR products into the EcoRI site of pRSET-B. *Escherichia coli* expression plasmids for untagged KIX (aa 587-679, from mouse CBP) [320], GST-CH1 (aa 302-451, from mouse CBP), GST-C2 (aa 2221-2441, from mouse CBP), GST-KIX (aa 588-683, from mouse CBP) and GST-KIX deletion mutants (aa 588-655 and 597-719, from mouse CBP) were previously described [266, 267]. GST-KIX 588-683 point mutants Δ T (F612A, D622A,

R624A), Δ MLL-A (Y631A, L664A), Δ MLL-B (D622A, R624A, Y631A, L664A), Δ pC (Y658A), and YAY (Y650A, A654Q, Y658A) were made using the QuikChange II site-directed mutagenesis kit (Stratagene) with the GST-KIX expression plasmid template. GST-HBZ and GST-CREB *E. coli* expression plasmids were described previously [195, 321]. The *E. coli* expression plasmid for CREB was described previously [103]. The *E. coli* expression plasmids for Tax and p53 were described previously [132, 267]. pRSET-Flag-MLL-AD (aa 2829-2883) was constructed by PCR-amplifying MLL-AD from pF-MLL [269] with the forward primer containing the Flag sequence, and cloning the product into the BamHI and EcoRI sites of pRSET-A. HA-c-Myb-AD (aa 186-325) was constructed by amplifying c-Myb-AD from pM2 Gal4-c-Myb (aa 186-325) [238] with the forward primer containing the HA sequence, and cloning the product into the BamHI and EcoRI sites of pRSET-A. All plasmids were sequenced. The original HA-c-Myb-AD construct has a V to A substitution at aa 267, which is outside the KIX-binding domain and does not contribute to the interaction [322].

Expression and Purification of Recombinant Proteins

E. coli BL21(DE3) pLysS cells (Agilent Technologies) were used for expression of Gal4-DBD-HBZ-AD wt and mutants, HBZ-AD wt and mutants, HA-c-Myb-AD, GST-CREB, GST-KIX, untagged KIX, CREB, p53 and Tax. BL21-codon plus (DE3) cells (Agilent Technologies) were used for expression of Gal4-DBD-HBZ, Flag-MLL-AD and GST-HBZ. The LB medium for HBZ-AD and Flag-MLL-AD cultures was supplemented with 1 % glucose. Expression and purification of GST fusion proteins were performed as described previously [112], except that proteins were dialyzed in HM 0.1 buffer (50 mM HEPES [pH 7.9], 100 mM KCl, 12.5 mM MgCl₂, 1 mM EDTA, 20 % [vol/vol] glycerol, 0.025 % [vol/vol] Tween 20 and 1 mM dithiothreitol [DTT]) overnight at 4°C. Dialyzed proteins were aliquoted and

stored at -80°C. All 6XHis-tagged proteins except Tax and p53 were purified using Ni-NTA agarose (QIAGEN) as described by the manufacturer and subsequently supplemented with 15 % glycerol and 5 mM DTT, aliquoted and stored at -80°C. Tax and p53 were eluted with a gradient and dialyzed overnight at 4°C against TM 0.1 (50 mM Tris [pH 7.9], 100mM KCl, 12.5 mM MgCl₂, 1 mM EDTA [pH 8.0], 20 % [vol/vol] glycerol, 0.025 % [vol/vol] Nonidet P40 and 1 mM DTT), aliquoted and stored at -80°C. Full-length p300 was expressed from a recombinant baculovirus in Sf9 cells and purified as previously described [323]. Untagged KIX was purified using Q-sepharose and SP-sepharose ion exchange columns (Amersham) as described previously [261]. CREB was purified as described previously [324] with additional heparin-agarose chromatography [325] and dialyzed overnight at 4°C against TM 0.1 with 1 mM DTT. Purity and concentration of proteins were assessed by SDS-PAGE using BSA for standard curves. Protein gels were stained with SYPRO Ruby protein gel stain 4 (Invitrogen) or Coomassie Brilliant Blue. CREB (1 µM) was phosphorylated using 50 units of the catalytic subunit of protein kinase A (P2645, Sigma) in 25 mM potassium phosphate buffer [pH 6.6] containing 20 µM adenosine triphosphate (ATP) and 5 mM MgCl₂ at 30°C. C-Jun was purchased from Promega.

Electrophoretic Mobility Shift Assays (EMSA) and Apparent K_d Analysis

Reactions (20 µl) contained 0.5x TM 0.1, 5 µM ZnSO₄, 5 mM DTT, and concentrations of protein and ³²P-end-labeled DNA probe indicated in the figure legends. The DNA probe recognized by Gal4DBD contained the UAS TTTGCATCGGAGGACTGTCCTCCGATGC. The consensus CRE probe sequence was CCTAGCCTGACGTCAGAGAGAG. Antibodies used to supershift complexes were anti-GST (G7781, Sigma) and anti-Gal4-DBD (sc-510, Santa Cruz). Reactions were incubated at 20°C for 2 hours to ensure that equilibrium binding

conditions were reached and then resolved on a non-denaturing polyacrylamide gel containing 40 mM Tris [pH 8.5] and 306 mM glycine with, in some gels, 0.04 % [vol/vol] Nonidet P40. Gels ranged in acrylamide concentration from 5-7 % (49:1 bis) and were electrophoresed at 230V at 4°C for 2-3 hours in 40 mM Tris [pH 8.5] and 306 mM glycine supplemented in some runs with 0.004 % [vol/vol] Nonidet P40. Following electrophoresis, gels were dried and visualized by PhosphorImager analysis with a Typhoon 9410 (GE Healthcare). Band intensities were quantified using ImageQuant TL (GE Healthcare).

The apparent K_d was calculated according to the equation $K_d = [\text{Gal4-DBD-HBZ-AD}]_{\text{free}}[\text{GST-KIX}]_{\text{free}}/[\text{Gal4-DBD-HBZ-AD} \bullet \text{GST-KIX}]$. This equation becomes simplified when 50 % of the Gal4-DBD-HBZ-AD band is shifted by GST-KIX. At this point, the two terms $[\text{Gal4-DBD-HBZ-AD}]_{\text{free}}$ and $[\text{Gal4-DBD-HBZ-AD} \bullet \text{GST-KIX}]$ are equal and cancel out, and the value of $[\text{GST-KIX}]_{\text{free}}$ is by definition the K_d . Since the concentration of free GST-KIX is not known in the gel, the value for $[\text{GST-KIX}]_{\text{total}}$, which is known, is substituted. This substitution is valid if, when 50 % of Gal4-DBD-HBZ-AD is shifted by GST-KIX, the amount of GST-KIX bound in the Gal4-HBZ-AD $\bullet$ GST-KIX complex is negligible compared to the total amount of GST-KIX present in the reaction. To accomplish this, the concentration of Gal4-DBD-HBZ-AD in the experiment ideally should have been approximately 10 fold less than the value of the K_d . However, due to the very high affinity of Gal4-DBD-HBZ-AD for GST-KIX, a concentration of Gal4-DBD-HBZ-AD 10 fold less than the K_d was not visible. Therefore, the most correct interpretation of the data for the Gal4-DBD-HBZ-AD_{wt} interaction with GST-KIX is that the K_d is somewhat less than the calculated 3.2 ± 0.5 nM. The fraction bound was calculated based on the disappearance of Gal4-HBZ-AD as it

became shifted by GST-KIX by the formula: $1 - ([\text{Gal4-DBD-HBZ-AD}]_{\text{free}} / [\text{Gal4-DBD-HBZ-AD}]_{\text{total}})$. It is thought that quantification based on the disappearance of bands rather than on the appearance of the shifted bands is more accurate [326]. This is due to the observation that dissociation of the shifted complex may occur during electrophoresis, and smearing of upper bands may lead to difficulties in quantification [326]. After quantification, values were fit to a hyperbolic one-site binding model with nonlinear regression using Prism® (GraphPad Software). Gibbs free-energy values were calculated from the K_d values using the equation $\Delta G = -RT \ln(1/K_d)$, where $R = 0.001987 \text{ kcal mol}^{-1} \text{ K}^{-1}$, and $T = 293 \text{ K}$ (20°C).

Crosslinking Assays

Binding reactions (15 μl) for bis[sulfosuccinimidyl] suberate (BS^3 , Pierce) crosslinking assays were performed in 0.25x HM 0.1 buffer, with concentrations of recombinant proteins indicated in the figure legend. Reactions were incubated for approximately 2 hours at 20°C without BS^3 , then 1.8 mM BS^3 was added, and reactions were further incubated at room temperature for 30 minutes. Crosslinking was quenched by addition of Tris (pH 7.5) to a final concentration of 50 mM followed by incubation at room temperature for 15 minutes. Proteins were resolved by SDS-PAGE and analyzed by Western blot analysis using the anti-Xpress antibody (R910-25, Invitrogen) to detect the Xpress epitope for pRSET vectors (HBZ-AD) and anti-CBP (sc-1211, Santa Cruz) to detect untagged KIX. Blots were developed using Enhanced Chemiluminescence (ECL) Plus (GE Healthcare) and visualized with a Typhoon 9410 (GE Healthcare).

GST Pull-Down Assays

GST fusion proteins (concentrations indicated in the figure legends) were bound at

4°C for 1 hour to 20 µl of glutathione-agarose beads that were equilibrated with 0.5x Superdex buffer (12.5 mM HEPES [pH 7.9], 75 mM KCl, 6.25 mM MgCl₂, 5 µM ZnSO₄, 20 % [vol/vol] glycerol, 0.05 % [vol/vol] Nonidet P40, 1 mM EDTA and 1 mM DTT). Beads were then washed twice with 500 µl 0.5x Superdex buffer, combined with the second protein (concentrations indicated in the figure legends) in a final volume of 50 µl 0.5x Superdex buffer for all reactions except those with MLL-AD and Tax, which were incubated in JM buffer (20 mM HEPES [pH 7.9], 0.5 mM EDTA, 10 % glycerol, 0.05 % Nonidet P40, 5 µM ZnSO₄, 2.5 mM MgCl₂, 25 mM KCl and 1 mM DTT). Reactions were incubated at 4°C overnight, and beads were then washed four times with 500 µl 0.5x Superdex buffer (or JM buffer for MLL-AD and Tax reactions). Proteins retained on the beads were resolved by SDS-PAGE and detected by Western blot analysis using the following antibodies: anti-Xpress (R910-25) from Invitrogen; anti-CREB-1 (sc-186), anti-pCREB-1 (sc-7978), anti-p53 (DO-1), and anti-HIS (H-15) from Santa-Cruz; anti-Flag M2 (F3165), anti-HA (H3663), and anti-GST (G7781) from Sigma. Blots were developed using ECL Plus chemiluminescence (GE Healthcare) and visualized with a Typhoon 9410 (GE Healthcare).

Cell Culture, Transient Cotransfection Assays and Luciferase Assays

Jurkat cells and 293T/17 cells (ATCC) were cultured in Iscove's modified Dulbecco's medium supplemented with 10 % fetal bovine serum, glutamine, and penicillin/streptomycin. For transient cotransfection assays, cells were plated at 4x10⁵ cells per well for Jurkat and 6x10⁴ cells per well for 293T/17 cells. Jurkat and 293T/17 cells were transfected with Lipofectamine (Invitrogen) or TurboFect (MBI), respectively, and a constant amount of DNA (1 µg). After 24 hours, the cells were harvested and lysed, and luciferase activity was measured using the Dual-Luciferase reporter assay system

(Promega) with a Turner Designs model TD 20-e luminometer. Luciferase activity was normalized to Renilla luciferase from the herpes simplex virus thymidine kinase promoter (pRL-TK, Promega). Mammalian expression plasmids pGal-TK-Luc [268], Gal4-c-Myb (aa 186-325) [238], pBXG1-MLL-AD (aa 2829 to 2883) [243], *Hox c8*-Luc (*Hox c8* promoter) and pF-MLL (full-length MLL) [269] were previously described.

REFERENCES

1. Satou, Y. and M. Matsuoka, *HTLV-1 and the host immune system: how the virus disrupts immune regulation, leading to HTLV-1 associated diseases*. J Clin Exp Hematop, 2010. **50**(1): p. 1-8.
2. Gallo, R.C., *History of the discoveries of the first human retroviruses: HTLV-1 and HTLV-2*. Oncogene, 2005. **24**(39): p. 5926-30.
3. Hinuma, Y., et al., *Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera*. Proc. Natl. Acad. Sci. USA, 1981. **78**(10): p. 6476-6480.
4. Poiesz, B.J., et al., *Detection and isolation of type C retrovirus particle from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma*. Proc. Natl. Acad. Sci. USA, 1980. **77**: p. 7415-7419.
5. Takatsuki, K., *Discovery of adult T-cell leukemia*. Retrovirology, 2005. **2**: p. 16.
6. Yoshida, M., I. Miyoshi, and Y. Hinuma, *Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease*. Proc. Natl. Acad. Sci. USA, 1982. **79**(6): p. 2031-2035.
7. Larocca, D., et al., *Human T-cell leukemia virus minus strand transcription in infected T-cells*. Biochem Biophys Res Commun, 1989. **163**(2): p. 1006-13.
8. Gaudray, G., et al., *The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription*. J. Virol., 2002. **76**(24): p. 12813-12822.
9. Satou, Y., et al., *HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells*. Proc Natl Acad Sci U S A, 2006. **103**(3): p. 720-5.

10. Usui, T., et al., *Characteristic expression of HTLV-1 basic zipper factor (HBZ) transcripts in HTLV-1 provirus-positive cells*. Retrovirology, 2008. **5**: p. 34.
11. Basbous, J., et al., *The HBZ factor of human T-cell leukemia virus type I dimerizes with transcription factors JunB and c-Jun and modulates their transcriptional activity*. J. Biol. Chem., 2003. **278**(44): p. 43620-46327.
12. Clerc, I., et al., *Propensity for HBZ-SP1 isoform of HTLV-I to inhibit c-Jun activity correlates with sequestration of c-Jun into nuclear bodies rather than inhibition of its DNA-binding activity*. Virology, 2009. **391**(2): p. 195-202.
13. Lemasson, I., et al., *Human T-cell leukemia virus type 1 (HTLV-1) bZIP protein interacts with the cellular transcription factor CREB to inhibit HTLV-1 transcription*. J Virol, 2007. **81**(4): p. 1543-53.
14. Yoshida, M., et al., *Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease*. Proc. Natl. Acad. Sci. USA, 1984. **81**(8): p. 2534-2537.
15. Calattini, S., et al., *Discovery of a new human T-cell lymphotropic virus (HTLV-3) in Central Africa*. Retrovirology, 2005. **2**: p. 30.
16. Mahieux, R. and A. Gessain, *The human HTLV-3 and HTLV-4 retroviruses: new members of the HTLV family*. Pathol Biol (Paris), 2009. **57**(2): p. 161-6.
17. Wolfe, N.D., et al., *Emergence of unique primate T-lymphotropic viruses among central African bushmeat hunters*. Proc Natl Acad Sci U S A, 2005. **102**(22): p. 7994-9.
18. Goncalves, D.U., et al., *Epidemiology, treatment, and prevention of human T-cell leukemia virus type 1-associated diseases*. Clin Microbiol Rev, 2010. **23**(3): p. 577-89.

19. Burmeister, T., *Oncogenic retroviruses in animals and humans*. Rev. Med. Virol., 2001. **11**(6): p. 369-380.
20. Maeda, N., H. Fan, and Y. Yoshikai, *Oncogenesis by retroviruses: old and new paradigms*. Rev Med Virol, 2008. **18**(6): p. 387-405.
21. Goncalves, D.U., et al., *HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) inflammatory network*. Inflamm Allergy Drug Targets, 2008. **7**(2): p. 98-107.
22. Ohshima, K., *Pathological features of diseases associated with human T-cell leukemia virus type I*. Cancer Sci, 2007. **98**(6): p. 772-8.
23. Mochizuki, M., et al., *Human T lymphotropic virus type 1 uveitis*. Br J Ophthalmol, 1994. **78**(2): p. 149-54.
24. Nishioka, K., T. Sumida, and T. Hasunuma, *Human T lymphotropic virus type I in arthropathy and autoimmune disorders*. Arthritis Rheum, 1996. **39**(8): p. 1410-8.
25. La Grenade, L., R.A. Schwartz, and C.K. Janniger, *Childhood dermatitis in the tropics: with special emphasis on infective dermatitis, a marker for infection with human T-cell leukemia virus-I*. Cutis, 1996. **58**(2): p. 115-8.
26. Bomford, R., M. Kazanji, and G. De The, *Vaccine against human T cell leukemia-lymphoma virus type I: progress and prospects*. AIDS Res Hum Retroviruses, 1996. **12**(5): p. 403-5.
27. de The, G. and R. Bomford, *An HTLV-I vaccine: why, how, for whom?* AIDS Res Hum Retroviruses, 1993. **9**(5): p. 381-6.
28. Hlela, C., et al., *The prevalence of human T-cell lymphotropic virus type 1 in the general population is unknown*. AIDS Rev, 2009. **11**(4): p. 205-14.

29. Asquith, B. and C.R. Bangham, *How does HTLV-I persist despite a strong cell-mediated immune response?* Trends Immunol, 2008. **29**(1): p. 4-11.
30. Boxus, M. and L. Willems, *Mechanisms of HTLV-1 persistence and transformation.* Br J Cancer, 2009. **101**(9): p. 1497-501.
31. Jones, K.S., et al., *Cell-free HTLV-1 infects dendritic cells leading to transmission and transformation of CD4(+) T cells.* Nat Med, 2008. **14**(4): p. 429-36.
32. Yoshida, M., *Molecular approach to human leukemia: isolation and characterization of the first human retrovirus HTLV-1 and its impact on tumorigenesis in adult T-cell leukemia.* Proc Jpn Acad Ser B Phys Biol Sci, 2010. **86**(2): p. 117-30.
33. Valeri, V.W., et al., *Requirement of the human T-cell leukemia virus p12 and p30 genes for infectivity of human dendritic cells and macaques but not rabbits.* Blood, 2010.
34. Clapham, R., et al., *Productive infection and cell-free transmission of human T-cell leukemia virus in a nonlymphoid cell line.* Science, 1983. **222**: p. 1125-1127.
35. Etoh, K., et al., *Persistent clonal proliferation of human T-lymphotropic virus type I-infected cells in vivo.* Cancer Res, 1997. **57**(21): p. 4862-7.
36. Ho, D.D., T.R. Rota, and M.S. Hirsch, *Infection of human endothelial cells by human T-lymphotropic virus type I.* Proc Natl Acad Sci U S A, 1984. **81**(23): p. 7588-90.
37. Hoffman, P.M., et al., *Human T-cell leukemia virus type I infection of monocytes and microglial cells in primary human cultures.* Proc Natl Acad Sci U S A, 1992. **89**(24): p. 11784-8.
38. Hoxie, J.A., D.M. Matthews, and D.B. Cines, *Infection of human endothelial cells by human T-cell leukemia virus type I.* Proc Natl Acad Sci U S A, 1984. **81**(23): p. 7591-5.

39. Koralnik, I.J., et al., *In vitro* infection of human macrophages by human T-cell leukemia/lymphotropic virus type I (HTLV-I). *AIDS Res Hum Retroviruses*, 1992. **8**(11): p. 1845-9.
40. Koyanagi, Y., et al., *In vivo* infection of human T-cell leukemia virus type I in non-T cells. *Virology*, 1993. **196**(1): p. 25-33.
41. Richardson, J.H., et al., *In vivo* cellular tropism of human T-cell leukemia virus type 1. *J Virol*, 1990. **64**(11): p. 5682-7.
42. Banerjee, P., et al., *Hematopoietic stem cells and retroviral infection*. *Retrovirology*, 2010. **7**: p. 8.
43. Banerjee, P., et al., *Human T-cell lymphotropic virus type 1 infection of CD34+ hematopoietic progenitor cells induces cell cycle arrest by modulation of p21(cip1/waf1) and survivin*. *Stem Cells*, 2008. **26**(12): p. 3047-58.
44. Feuer, G., et al., *Human T-cell leukemia virus infection of human hematopoietic progenitor cells: maintenance of virus infection during differentiation in vitro and in vivo*. *J Virol*, 1996. **70**(6): p. 4038-44.
45. Grant, C., et al., *Human T cell leukemia virus type I and neurologic disease: events in bone marrow, peripheral blood, and central nervous system during normal immune surveillance and neuroinflammation*. *J Cell Physiol*, 2002. **190**(2): p. 133-59.
46. Wencker, M., et al., *Human T-cell leukemia virus type 1 Tax protein down-regulates pre-T-cell receptor alpha gene transcription in human immature thymocytes*. *J Virol*, 2007. **81**(1): p. 301-8.
47. Yamazaki, J., et al., *Identification of cancer stem cells in a Tax-transgenic (Tax-Tg) mouse model of adult T-cell leukemia/lymphoma*. *Blood*, 2009. **114**(13): p. 2709-20.

48. Matsuoka, M. and K.T. Jeang, *Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation*. Nat Rev Cancer, 2007. **7**(4): p. 270-80.
49. Arisawa, K., et al., *Evaluation of adult T-cell leukemia/lymphoma incidence and its impact on non-Hodgkin lymphoma incidence in southwestern Japan*. Int J Cancer, 2000. **85**(3): p. 319-24.
50. Uchiyama, T., *Human T cell leukemia virus type I (HTLV-I) and human diseases*. Annu Rev Immunol, 1997. **15**: p. 15-37.
51. Chen, Y.X., et al., *Molecular detection of pre-ATL state among healthy HTLV-1 carriers in an endemic area of Japan*. Int J Cancer, 1995. **60**(6): p. 798-801.
52. Kira, J., et al., *Antibody titers to HTLV-I-p40tax protein and gag-env hybrid protein in HTLV-I-associated myelopathy/tropical spastic paraparesis: correlation with increased HTLV-I proviral DNA load*. J Neurol Sci, 1992. **107**(1): p. 98-104.
53. Saito, M., *Immunogenetics and the Pathological Mechanisms of Human T-Cell Leukemia VirusType 1- (HTLV-1-)Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)*. Interdiscip Perspect Infect Dis, 2010. **2010**: p. 478461.
54. Levin, M.C. and S. Jacobson, *HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP): a chronic progressive neurologic disease associated with immunologically mediated damage to the central nervous system*. J. Neurovirol., 1997. **3**(2): p. 126-140.
55. Osame, M., et al., *HTLV-I associated myelopathy, a new clinical entity*. Lancet, 1986. **1**(8488): p. 1031-2.
56. Barmak, K., et al., *Human T cell leukemia virus type I-induced disease: pathways to cancer and neurodegeneration*. Virology, 2003. **9**(5): p. 522-529.

57. Cooper, S.A., M.S. van der Loeff, and G.P. Taylor, *The neurology of HTLV-1 infection*. Pract Neurol, 2009. **9**(1): p. 16-26.
58. Okayama, A., et al., *Role of HTLV-1 proviral DNA load and clonality in the development of adult T-cell leukemia/lymphoma in asymptomatic carriers*. Int J Cancer, 2004. **110**(4): p. 621-5.
59. Nakase, K., et al., *HTLV-1 unrelated adult T-cell leukemia/lymphoma with unique phenotype and karyotype*. Am J Hematol, 2000. **64**(1): p. 64-6.
60. Shimoyama, M., et al., *Adult T-cell leukemia/lymphoma not associated with human T-cell leukemia virus type I*. Proc Natl Acad Sci U S A, 1986. **83**(12): p. 4524-8.
61. Konishi, H., N. Kobayashi, and M. Hatanaka, *Defective human T-cell leukemia virus in adult T-cell leukemia patients*. Mol Biol Med, 1984. **2**(4): p. 273-83.
62. Renjifo, B., et al., *Human T cell leukemia virus type I (HTLV-I) molecular genotypes and disease outcome*. J Acquir Immune Defic Syndr Hum Retrovirol, 1996. **13 Suppl 1**: p. S146-53.
63. Tamiya, S., et al., *Two types of defective human T-lymphotropic virus type I provirus in adult T-cell leukemia*. Blood, 1996. **88**(8): p. 3065-73.
64. Yang, Y.C., et al., *Human T-lymphotropic virus type I genome in adult T-cell leukemia/lymphoma*. Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi, 1991. **24**(1): p. 84-94.
65. Banerjee, P., et al., *Adult T-cell leukemia/lymphoma development in HTLV-1-infected humanized SCID mice*. Blood, 2010. **115**(13): p. 2640-8.
66. Hanchard, B., *Adult T-cell leukemia/lymphoma in Jamaica: 1986-1995*. J Acquir Immune Defic Syndr Hum Retrovirol, 1996. **13 Suppl 1**: p. S20-5.

67. Proietti, F.A., et al., *Global epidemiology of HTLV-I infection and associated diseases*. Oncogene, 2005. **24**(39): p. 6058-6068.
68. Yasunaga, J. and M. Matsuoka, *Leukemogenesis of adult T-cell leukemia*. Int J Hematol, 2003. **78**(4): p. 312-20.
69. Yasunaga, J. and M. Matsuoka, *Human T-cell leukemia virus type I induces adult T-cell leukemia: from clinical aspects to molecular mechanisms*. Cancer Control, 2007. **14**(2): p. 133-40.
70. Uozumi, K., *Treatment of adult T-cell leukemia*. J Clin Exp Hematop, 2010. **50**(1): p. 9-25.
71. Tsukasaki, K., et al., *VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801*. J Clin Oncol, 2007. **25**(34): p. 5458-64.
72. Choi, I., et al., *Long-term outcomes after hematopoietic SCT for adult T-cell leukemia/lymphoma: results of prospective trials*. Bone Marrow Transplant, 2010.
73. Hishizawa, M., et al., *Transplantation of allogeneic hematopoietic stem cells for adult T-cell leukemia: a nationwide retrospective study*. Blood, 2010. **116**(8): p. 1369-76.
74. Mori, N., et al., *Apoptosis induced by the histone deacetylase inhibitor FR901228 in human T-cell leukemia virus type 1-infected T-cell lines and primary adult T-cell leukemia cells*. J Virol, 2004. **78**(9): p. 4582-90.
75. Sundaram, R., et al., *A novel multivalent human CTL peptide construct elicits robust cellular immune responses in HLA-A*0201 transgenic mice: implications for HTLV-1 vaccine design*. Vaccine, 2003. **21**(21-22): p. 2767-81.

76. Sundaram, R., et al., *Protective efficacy of multiepitope human leukocyte antigen-A*0201 restricted cytotoxic T-lymphocyte peptide construct against challenge with human T-cell lymphotropic virus type 1 Tax recombinant vaccinia virus*. J Acquir Immune Defic Syndr, 2004. **37**(3): p. 1329-39.
77. Suemori, K., et al., *HBZ is an immunogenic protein but not a target antigen for HTLV-1-specific cytotoxic T lymphocytes*. J Gen Virol, 2009.
78. Macnamara, A., et al., *HLA class I binding of HBZ determines outcome in HTLV-1 infection*. PLoS Pathog, 2010. **6**(9).
79. Albrecht, B. and M.D. Lairmore, *Critical role of human T-lymphotropic virus type 1 accessory proteins in viral replication and pathogenesis*. Microbiol Mol Biol Rev, 2002. **66**(3): p. 396-406, table of contents.
80. Bangham, C.R., *The immune control and cell-to-cell spread of human T-lymphotropic virus type 1*. J Gen Virol, 2003. **84**(Pt 12): p. 3177-89.
81. Majorovits, E., et al., *Human T-lymphotropic virus-1 visualized at the virological synapse by electron tomography*. PLoS One, 2008. **3**(5): p. e2251.
82. Yoshida, M., *Discovery of HTLV-1, the first human retrovirus, its unique regulatory mechanisms, and insights into pathogenesis*. Oncogene, 2005. **24**(39): p. 5931-7.
83. Derse, D., et al., *Human T-cell leukemia virus type 1 integration target sites in the human genome: comparison with those of other retroviruses*. J Virol, 2007. **81**(12): p. 6731-41.
84. Doi, K., et al., *Preferential selection of human T-cell leukemia virus type I provirus integration sites in leukemic versus carrier states*. Blood, 2005. **106**(3): p. 1048-53.

85. Hanai, S., et al., *Integration of human T-cell leukemia virus type 1 in genes of leukemia cells of patients with adult T-cell leukemia*. Cancer Sci, 2004. **95**(4): p. 306-10.
86. Meekings, K.N., et al., *HTLV-1 integration into transcriptionally active genomic regions is associated with proviral expression and with HAM/TSP*. PLoS Pathog, 2008. **4**(3): p. e1000027.
87. Salcedo-Cifuentes, M., et al., *[Clonal expansion and genomic characterization of the human T-cell lymphotropic virus type I during the integration process in adult T-cell leukemia/lymphoma]*. Biomedica, 2009. **29**(2): p. 218-31.
88. Lenzmeier, B.A. and J.K. Nyborg, *Molecular mechanisms of viral transcription and cellular deregulation associated with the HTLV-I Tax protein*. Gene Ther. Mol. Biol, 1999. **3**: p. 327-345.
89. Franklin, A.A., and J. K. Nyborg, *Mechanisms of Tax regulation of Human T-Cell Leukemia Virus Type I gene expression*. J. Biomed Sci., 1995. **2**: p. 17-29.
90. Fujisawa, J., et al., *Functional activation of the long terminal repeat of human T-cell leukemia virus type I by a trans-acting factor*. Proc. Natl. Acad. Sci. USA, 1985. **82**(8): p. 2277-2281.
91. Seiki, M., et al., *The p40x of human T-cell leukemia virus type I is a trans-acting activator of viral gene transcription*. Jpn J Cancer Res, 1985. **76**(12): p. 1127-31.
92. Sodroski, J.G., C.A. Rosen, and W.A. Haseltine, *Trans-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells*. Science, 1984. **225**(4660): p. 381-5.
93. Fujisawa, J.I., et al., *A transcriptional enhancer sequence of HTLV-1 is responsible for transactivation mediated by p40^x of HTLV-1*. EMBO J, 1986. **5**: p. 713-718.

94. Shimotohno, K., et al., *Requirement of multiple copies of a 21-nucleotide sequence in the U3 regions of human T-cell leukemia virus type I and type II long terminal repeats for trans-acting activation of transcription*. Proc Natl Acad Sci U S A, 1986. **83**(21): p. 8112-6.
95. Jeang, K.T., et al., *Characterization of cellular factors that interact with the human T-cell leukemia virus type I p40x-responsive 21-base-pair sequence*. J Virol, 1988. **62**(12): p. 4499-509.
96. Franklin, A.A., et al., *Transactivation by the human T-cell leukemia virus Tax protein is mediated through enhanced binding of activating transcription factor-2 (ATF-2) ATF-2 response and cAMP element-binding protein (CREB)*. J Biol Chem, 1993. **268**(28): p. 21225-31.
97. Yin, M.J. and R.B. Gaynor, *Complex formation between CREB and Tax enhances the binding affinity of CREB for the human T-cell leukemia virus type 1 21-base-pair repeats*. Mol. Cell. Biol., 1996. **16**(6): p. 3156-3168.
98. Zhao, L.J. and C.Z. Giam, *Human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator, Tax, enhances CREB binding to HTLV-I 21-base-pair repeats by protein-protein interaction*. Proc. Natl. Acad. Sci. USA, 1992. **89**(15): p. 7070-7074.
99. Gachon, F., et al., *CREB-2, a cellular CRE-dependent transcription repressor, functions in association with Tax as an activator of the human T-cell leukemia virus type 1 promoter*. J. Virol., 1998. **72**(10): p. 8332-8337.
100. Reddy, T.R., et al., *Functional interaction of the HTLV-1 transactivator Tax with activating transcription factor-4 (ATF4)*. Oncogene, 1997. **14**(23): p. 2785-2792.

101. Suzuki, T., et al., *The trans-activator tax of human T-cell leukemia virus type 1 (HTLV-1) interacts with cAMP-responsive element (CRE) binding and CRE modulator proteins that bind to the 21-base-pair enhancer of HTLV-1*. Proc Natl Acad Sci U S A, 1993. **90**(2): p. 610-4.
102. Jeang, K.T., et al., *Induction of the HTLV-I LTR by Jun occurs through the Tax-responsive 21-bp elements*. Virology, 1991. **181**: p. 218-227.
103. Franklin, A.A., et al., *Transactivation by the human T-cell leukemia virus Tax protein is mediated through enhanced binding of activating transcription factor-2 (ATF-2) ATF-2 response and cAMP element-binding protein (CREB)*. J. Biol. Chem., 1993. **268**(28): p. 21225-21231.
104. Lenzmeier, B.A., H.A. Giebler, and J.K. Nyborg, *Human T-cell leukemia virus type 1 Tax requires direct access to DNA for recruitment of CREB binding protein to the viral promoter*. Mol. Cell. Biol., 1998. **18**(2): p. 721-731.
105. Barnhart, M.K., L.M. Connor, and S.J. Marriott, *Function of the human T-cell leukemia virus type 1 21-base-pair repeats in basal transcription*. J. Virol., 1997. **71**(1): p. 337-344.
106. Wycuff, D.R., H.L. Yanites, and S.J. Marriott, *Identification of a functional serum response element in the HTLV-I LTR*. Virology, 2004. **324**(2): p. 540-53.
107. Armstrong, A., et al., *Pleiotropic effect of the Human T-cell Leukemia Virus Tax protein on the DNA binding activity of eucary-otic transcription factors*. Proc Natl Acad Sci USA, 1993. **90**: p. 7303-7307.

108. Brady, J., K.-T. Jeang, J. Duvall and G. Khoury, *Identification of p40^x-responsive regulatory se-quences within the Human T-cell Leukemia Virus Type I long terminal repeat*. *J. Virol*, 1987. **61**: p. 2175-2181.
109. Felber, B.K., et al., *The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats*. *Science*, 1985. **229**(4714): p. 675-679.
110. Rosen, C.A., J.G. Sodroski, and W.A. Haseltine, *Location of cis-acting regulatory sequences in the human T-cell leukemia virus type I long terminal repeat*. *Proc. Natl. Acad. Sci. USA*, 1985. **82**: p. 6502-6506.
111. Suzuki, T., et al., *The trans-activator tax of human T-cell leukemia virus type 1 (HTLV-1) interacts with cAMP-responsive element (CRE) binding and CRE modulator proteins that bind to the 21-base-pair enhancer of HTLV-1*. *Proc Natl Acad Sci U S A*, 1993. **90**(2): p. 610-614.
112. Giebler, H.A., et al., *Anchoring of CREB binding protein to the human T-cell leukemia virus type 1 promoter: a molecular mechanism of Tax transactivation*. *Mol Cell Biol*, 1997. **17**(9): p. 5156-64.
113. Harrod, R., et al., *An exposed KID-like domain in human T-cell lymphotropic virus type 1 Tax is responsible for the recruitment of coactivators CBP/p300*. *Mol. Cell. Biol.*, 1998. **18**(9): p. 5052-5061.
114. Kwok, R.P., et al., *Control of cAMP-regulated enhancers by the viral transactivator Tax through CREB and the co-activator CBP*. *Nature*, 1996. **380**(6575): p. 642-646.
115. Lemasson, I., et al., *Transcription factor binding and histone modifications on the integrated proviral promoter in HTLV-I-infected T-cells*. *J Biol Chem*, 2002. **277**(51): p. 49459-49465.

116. Geiger, T.R., et al., *The human T-cell leukemia virus type 1 tax protein confers CBP/p300 recruitment and transcriptional activation properties to phosphorylated CREB*. Mol Cell Biol, 2008. **28**(4): p. 1383-92.
117. Kashanchi, F. and J.N. Brady, *Transcriptional and post-transcriptional gene regulation of HTLV-1*. Oncogene, 2005. **24**(39): p. 5938-51.
118. Younis, I. and P.L. Green, *The human T-cell leukemia virus Rex protein*. Front Biosci, 2005. **10**: p. 431-45.
119. Hidaka M, I.J., Yoshida M, Seiki M., *Post-transcriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory pro-teins*. EMBO J, 1988. **7**: p. 519-523.
120. Inoue, J.I., M. Yoshida, and M. Seiki, *Transcriptional (p40^x) and post-transcriptional (p27^{x-III}) reg-ulators are required for the expression and replication of human T-cell leukemia virus type I genes*. Proc Natl Acad Sci USA, 1987. **84**: p. 3653-3657.
121. Yoshida, M., *Expression of the HTLV-1 genome and its association with a unique T-cell malignancy*. Biochim Biophys Acta, 1987. **907**(2): p. 145-61.
122. Albrecht, B. and M.D. Lairmore, *Critical role of human T-lymphotropic virus type 1 accessory proteins in viral replication and pathogenesis*. Microbiol Mol Biol Rev, 2002. **66**(3): p. 396-406, table of contents.
123. Bindhu, M., A. Nair, and M.D. Lairmore, *Role of accessory proteins of HTLV-1 in viral replication, T cell activation, and cellular gene expression*. Front Biosci, 2004. **9**: p. 2556-76.
124. Nicot, C., et al., *Human T-cell leukemia/lymphoma virus type 1 nonstructural genes and their functions*. Oncogene, 2005. **24**(39): p. 6026-34.

125. Albrecht, B., et al., *Activation of nuclear factor of activated T cells by human T-lymphotropic virus type 1 accessory protein p12(I)*. J Virol, 2002. **76**(7): p. 3493-501.
126. Johnson, J.M., et al., *Free major histocompatibility complex class I heavy chain is preferentially targeted for degradation by human T-cell leukemia/lymphotropic virus type 1 p12(I) protein*. J Virol, 2001. **75**(13): p. 6086-94.
127. Zhang, W., et al., *Human T-lymphotropic virus type 1 p30(II) regulates gene transcription by binding CREB binding protein/p300*. J Virol, 2001. **75**(20): p. 9885-95.
128. Giam, C.Z. and K.T. Jeang, *HTLV-1 Tax and adult T-cell leukemia*. Front Biosci, 2007. **12**: p. 1496-507.
129. Grassmann, R., M. Aboud, and K.T. Jeang, *Molecular mechanisms of cellular transformation by HTLV-1 Tax*. Oncogene, 2005. **24**(39): p. 5976-85.
130. Hall, W.W. and M. Fujii, *Deregulation of cell-signaling pathways in HTLV-1 infection*. Oncogene, 2005. **24**(39): p. 5965-75.
131. Suzuki, T., M. Uchida-Toita, and M. Yoshida, *Tax protein of HTLV-1 inhibits CBP/p300-mediated transcription by interfering with recruitment of CBP/p300 onto DNA element of E-box or p53 binding site*. Oncogene, 1999. **18**(28): p. 4137-43.
132. Van Orden, K., et al., *Binding of p53 to the KIX domain of CREB binding protein. A potential link to human T-cell leukemia virus, type I-associated leukemogenesis*. J. Biol. Chem., 1999. **274**(37): p. 26321-26328.
133. Suzuki, T., et al., *HTLV-1 Tax protein interacts with cyclin-dependent kinase inhibitor p16INK4A and counteracts its inhibitory activity towards CDK4*. EMBO J, 1996. **15**(7): p. 1607-1614.

134. Jin, D.Y., F. Spencer, and K.T. Jeang, *Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1*. Cell, 1998. **93**(1): p. 81-91.
135. Jeang, K.T., et al., *HTLV-I trans-activator protein, tax, is a trans-repressor of the human beta-polymerase gene*. Science, 1990. **247**(4946): p. 1082-4.
136. Suzuki, T., et al., *HTLV-1 tax oncoprotein binds to DNA topoisomerase I and inhibits its catalytic activity*. Virology, 2000. **270**(2): p. 291-8.
137. Hirai, H., et al., *Transcriptional activator Tax of HTLV-1 binds to the NF-kappa B precursor p105*. Oncogene, 1992. **7**(9): p. 1737-42.
138. Suzuki, T., et al., *Tax protein of HTLV-1 destabilizes the complexes of NF-kappa B and I kappa B-alpha and induces nuclear translocation of NF-kappa B for transcriptional activation*. Oncogene, 1995. **10**(6): p. 1199-207.
139. Tsukahara, T., et al., *Induction of Bcl-x(L) expression by human T-cell leukemia virus type 1 Tax through NF-kappaB in apoptosis-resistant T-cell transfectants with Tax*. J Virol, 1999. **73**(10): p. 7981-7.
140. Brauweiler, A., et al., *Repression of bax gene expression by the HTLV-1 Tax protein: implications for suppression of apoptosis in virally infected cells*. Virology, 1997. **231**(1): p. 135-140.
141. Inoue, J., M. Seiki, T. Taniguchi, S. Tsuru, and M. Yoshida., *Induction of interleukin 2 receptor gene expression by p40^x encoded by human T-cell leukemia virus type I*. EMBO J, 1986. **5**: p. 2883-2888.
142. Maruyama M, S.H., Haraa H, Hatakeyama M, Seiki M, Fujita T, Inoue J, Yoshida M, Taniguchi T, *Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-I encoded p40^x and T3/Ti complex triggering*. Cell, 1987. **48**: p. 343-350.

143. Yoshida, M., et al., *Mechanism of the gene expression of HTLV-I and its association with ATL*. AIDS Res, 1986. **2 Suppl 1**: p. S71-8.
144. Ng, P.W., et al., *Genome-wide expression changes induced by HTLV-1 Tax: evidence for MLK-3 mixed lineage kinase involvement in Tax-mediated NF-kappaB activation*. Oncogene, 2001. **20**(33): p. 4484-4496.
145. Mesnard, J.M., B. Barbeau, and C. Devaux, *HBZ, a new important player in the mystery of Adult-T- cell leukemia*. Blood, 2006.
146. Matsumoto, K., et al., *Human T-cell leukemia virus type 1 Tax protein transforms rat fibroblasts via two distinct pathways*. J Virol, 1997. **71**(6): p. 4445-51.
147. Pozzatti, R., J. Vogel, and G. Jay, *The human T-lymphotropic virus type I tax gene can cooperate with the ras oncogene to induce neoplastic transformation of cells*. Mol Cell Biol, 1990. **10**(1): p. 413-7.
148. Tanaka, A., et al., *Oncogenic transformation by the tax gene of human T-cell leukemia virus type I in vitro*. Proc Natl Acad Sci U S A, 1990. **87**(3): p. 1071-5.
149. Gao, L., et al., *HTLV-1 Tax transgenic mice develop spontaneous osteolytic bone metastases prevented by osteoclast inhibition*. Blood, 2005. **106**(13): p. 4294-302.
150. Grossman, W.J., et al., *Development of leukemia in mice transgenic for the tax gene of human T-cell leukemia virus type I*. Proc Natl Acad Sci U S A, 1995. **92**(4): p. 1057-61.
151. Hall, A.P., et al., *Tumours derived from HTLV-I tax transgenic mice are characterized by enhanced levels of apoptosis and oncogene expression*. J Pathol, 1998. **186**(2): p. 209-14.
152. Hinrichs, S.H., et al., *A transgenic mouse model for human neurofibromatosis*. Science, 1987. **237**(4820): p. 1340-3.

153. Nerenberg, M., et al., *The tat gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice*. Science, 1987. **237**(4820): p. 1324-9.
154. Ruddle, N.H., et al., *Mice transgenic for HTLV-I LTR-tax exhibit tax expression in bone, skeletal alterations, and high bone turnover*. Virology, 1993. **197**(1): p. 196-204.
155. Hasegawa, H., et al., *Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I*. Nat Med, 2006. **12**(4): p. 466-72.
156. Ohsugi, T., et al., *The Tax protein of HTLV-1 promotes oncogenesis in not only immature T cells but also mature T cells*. Nat Med, 2007. **13**(5): p. 527-8.
157. Grossman, W.J., et al., *Development of leukemia in mice transgenic for the tax gene of human T- cell leukemia virus type I*. Proc. Natl. Acad. Sci. USA, 1995. **92**(4): p. 1057-1061.
158. Zimmerman, B., S. Niewiesk, and M.D. Lairmore, *Mouse models of human T lymphotropic virus type-1-associated adult T-cell leukemia/lymphoma*. Vet Pathol, 2010. **47**(4): p. 677-89.
159. Akagi, T., H. Ono, and K. Shimotohno, *Characterization of T cells immortalized by Tax1 of human T-cell leukemia virus type 1*. Blood, 1995. **86**(11): p. 4243-9.
160. Grassmann, R., et al., *Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a Herpesvirus saimiri vector*. Proc Natl Acad Sci U S A, 1989. **86**(9): p. 3351-5.
161. Bellon, M., et al., *HTLV-I Tax-dependent and -independent events associated with immortalization of human primary T lymphocytes*. Blood, 2010. **115**(12): p. 2441-8.
162. Rangarajan, A., et al., *Species- and cell type-specific requirements for cellular transformation*. Cancer Cell, 2004. **6**(2): p. 171-83.

163. Hahn, W.C. and R.A. Weinberg, *Rules for making human tumor cells*. N Engl J Med, 2002. **347**(20): p. 1593-603.
164. Hahn, W.C. and R.A. Weinberg, *Modelling the molecular circuitry of cancer*. Nat Rev Cancer, 2002. **2**(5): p. 331-41.
165. Rangarajan, A. and R.A. Weinberg, *Opinion: Comparative biology of mouse versus human cells: modelling human cancer in mice*. Nat Rev Cancer, 2003. **3**(12): p. 952-9.
166. Liu, M., et al., *Human T-cell leukemia virus type 1 infection leads to arrest in the G1 phase of the cell cycle*. J Virol, 2008. **82**(17): p. 8442-55.
167. Takeda, S., et al., *Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells*. Int J Cancer, 2004. **109**(4): p. 559-67.
168. Clarke, M.F., et al., *Methylation of human T-cell leukemia virus proviral DNA and viral RNA expression in short- and long-term cultures of infected cells*. Virology, 1984. **135**(1): p. 97-104.
169. Kitamura, T., et al., *Methylation pattern of human T-cell leukemia virus in vivo and in vitro: pX and LTR regions are hypomethylated in vivo*. Int J Cancer, 1985. **35**(5): p. 629-35.
170. Kiyokawa, T., et al., *p27x-III and p21x-III, proteins encoded by the pX sequence of human T-cell leukemia virus type I*. Proc. Natl. Acad. Sci. USA, 1985. **82**(24): p. 8359-8363.
171. Yoshida, M. and M. Seiki, *Recent advances in the molecular biology of HTLV-1: trans-activation of viral and cellular genes*. Annu Rev Immunol, 1987. **5**: p. 541-59.
172. Kinoshita, T., et al., *Detection of mRNA for the tax1/rex1 gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell*

- leukemia patients and viral carriers by using the polymerase chain reaction. Proc Natl Acad Sci U S A*, 1989. **86**(14): p. 5620-4.
173. Furukawa, Y., et al., *Human T-cell leukemia virus type-1 (HTLV-1) Tax is expressed at the same level in infected cells of HTLV-1-associated myelopathy or tropical spastic paraparesis patients as in asymptomatic carriers but at a lower level in adult T-cell leukemia cells. Blood*, 1995. **85**(7): p. 1865-70.
 174. Miyazaki, M., et al., *Preferential selection of human T-cell leukemia virus type 1 provirus lacking the 5' long terminal repeat during oncogenesis. J Virol*, 2007. **81**(11): p. 5714-23.
 175. Koiwa, T., et al., *5'-long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus in vitro and in vivo. J Virol*, 2002. **76**(18): p. 9389-97.
 176. Taniguchi, Y., et al., *Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms. Retrovirology*, 2005. **2**: p. 64.
 177. Furukawa, Y., et al., *Existence of escape mutant in HTLV-I tax during the development of adult T-cell leukemia. Blood*, 2001. **97**(4): p. 987-93.
 178. Matsuoka, M. and P.L. Green, *The HBZ gene, a key player in HTLV-1 pathogenesis. Retrovirology*, 2009. **6**: p. 71.
 179. Asquith, B., et al., *In vivo T lymphocyte dynamics in humans and the impact of human T-lymphotropic virus 1 infection. Proc Natl Acad Sci U S A*, 2007. **104**(19): p. 8035-40.
 180. Ross, T.M., S.M. Pettiford, and P.L. Green, *The tax gene of human T-cell leukemia virus type 2 is essential for transformation of human T lymphocytes. J Virol*, 1996. **70**(8): p. 5194-202.

181. Fan, J., et al., *APOBEC3G generates nonsense mutations in human T-cell leukemia virus type 1 proviral genomes in vivo*. J Virol, 2010. **84**(14): p. 7278-87.
182. Yoshida, M., et al., *Transcriptional control of spliced and unspliced human T-cell leukemia virus type 1 bZIP factor (HBZ) gene*. J Virol, 2008. **82**(19): p. 9359-68.
183. Saito, M., et al., *In vivo expression of the HBZ gene of HTLV-1 correlates with proviral load, inflammatory markers and disease severity in HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP)*. Retrovirology, 2009. **6**: p. 19.
184. Halin, M., et al., *Human T-cell leukemia virus type 2 produces a spliced antisense transcript encoding a protein that lacks a classic bZIP domain but still inhibits Tax2-mediated transcription*. Blood, 2009. **114**(12): p. 2427-38.
185. Calattini, S., et al., *Human T-cell lymphotropic virus type 3: complete nucleotide sequence and characterization of the human tax3 protein*. J Virol, 2006. **80**(19): p. 9876-88.
186. Switzer, W.M., et al., *Ancient, independent evolution and distinct molecular features of the novel human T-lymphotropic virus type 4*. Retrovirology, 2009. **6**: p. 9.
187. Cavanagh, M.H., et al., *HTLV-I antisense transcripts initiating in the 3'LTR are alternatively spliced and polyadenylated*. Retrovirology, 2006. **3**: p. 15.
188. Murata, K., et al., *A novel alternative splicing isoform of human T-cell leukemia virus type 1 bZIP factor (HBZ-SI) targets distinct subnuclear localization*. J Virol, 2006. **80**(5): p. 2495-505.
189. Cavanagh, M., et al., *HTLV-I antisense transcripts initiate in the 3'LTR and are alternatively spliced and polyadenylated*. Retrovirology, 2006. **3**: p. 15.

190. Landry, S., et al., *Upregulation of human T-cell leukemia virus type 1 antisense transcription by the viral tax protein*. J Virol, 2009. **83**(4): p. 2048-54.
191. Hivin, P., et al., *Nuclear localization of HTLV-1 bZIP factor (HBZ) is mediated by three distinct motifs*. J Cell Sci, 2005. **118**(Pt 7): p. 1355-62.
192. Hivin, P., et al., *A modified version of a Fos-associated cluster in HBZ affects Jun transcriptional potency*. Nucleic Acids Res, 2006. **34**(9): p. 2761-72.
193. Matsuoka, M., *HTLV-1 bZIP factor gene: Its roles in HTLV-1 pathogenesis*. Mol Aspects Med, 2010.
194. Reinke, A.W., G. Grigoryan, and A.E. Keating, *Identification of bZIP interaction partners of viral proteins HBZ, MEQ, BZLF1, and K-bZIP using coiled-coil arrays*. Biochemistry, 2010. **49**(9): p. 1985-1997.
195. Clerc, I., et al., *An interaction between the human T cell leukemia virus type 1 basic leucine zipper factor (HBZ) and the KIX domain of p300/CBP contributes to the down-regulation of tax-dependent viral transcription by HBZ*. J Biol Chem, 2008. **283**(35): p. 23903-13.
196. Arnold, J., et al., *Enhancement of infectivity and persistence in vivo by HBZ, a natural antisense coded protein of HTLV-1*. Blood, 2006. **107**(10): p. 3976-3982.
197. Arnold, J., et al., *Human T-cell leukemia virus type-1 antisense-encoded gene, Hbz, promotes T-lymphocyte proliferation*. Blood, 2008. **112**(9): p. 3788-97.
198. Polakowski, N., et al., *Expression of a protein involved in bone resorption, Dkk1, is activated by HTLV-1 bZIP factor through its activation domain*. Retrovirology., 2010. **7**: p. 61.

199. Isono, O., et al., *Human T-cell leukemia virus type 1 HBZ protein bypasses the targeting function of ubiquitination*. J Biol Chem, 2008. **283**(49): p. 34273-82.
200. Reinke, A.W., G. Grigoryan, and A.E. Keating, *Identification of bZIP interaction partners of viral proteins HBZ, MEQ, BZLF1, and K-bZIP using coiled-coil arrays*. Biochemistry, 2010. **49**(9): p. 1985-97.
201. Thebault, S., et al., *HBZ interacts with JunD and stimulates its transcriptional activity*. FEBS Lett, 2004. **562**(1-3): p. 165-170.
202. Matsumoto, J., et al., *HTLV-1 HBZ suppresses AP-1 activity by impairing both the DNA-binding ability and the stability of c-Jun protein*. Oncogene, 2005. **24**(6): p. 1001-1010.
203. Ohshima, T., et al., *HTLV-1 basic leucine-zipper factor, HBZ, interacts with MafB and suppresses transcription through a Maf recognition element*. J Cell Biochem, 2010. **111**(1): p. 187-94.
204. Hivin, P., et al., *The HBZ-SP1 isoform of human T-cell leukemia virus type I represses JunB activity by sequestration into nuclear bodies*. Retrovirology, 2007. **4**: p. 14.
205. Kuhlmann, A.S., et al., *HTLV-1 HBZ cooperates with JunD to enhance transcription of the human telomerase reverse transcriptase gene (hTERT)*. Retrovirology, 2007. **4**: p. 92.
206. Zhao, T., et al., *Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB*. Blood, 2009. **113**(12): p. 2755-64.
207. Pierce, A.M., et al., *Increased E2F1 activity induces skin tumors in mice heterozygous and nullizygous for p53*. Proc Natl Acad Sci U S A, 1998. **95**(15): p. 8858-63.

208. Iwanaga, R., et al., *Molecular mechanism of cell cycle progression induced by the oncogene product Tax of human T-cell leukemia virus type I*. *Oncogene*, 2001. **20**(17): p. 2055-67.
209. Lemasson, I., et al., *Activation of E2F-mediated Transcription by Human T-cell Leukemia Virus Type I Tax Protein in a p16(INK4A)-negative T-cell Line*. *J. Biol. Chem.*, 1998. **273**: p. 23598-23604.
210. Ruggero, K., et al., *Role of microRNAs in HTLV-1 infection and transformation*. *Mol Aspects Med*, 2010.
211. Bellon, M., et al., *Deregulation of microRNA involved in hematopoiesis and the immune response in HTLV-I adult T-cell leukemia*. *Blood*, 2009. **113**(20): p. 4914-7.
212. Pichler, K., G. Schneider, and R. Grassmann, *MicroRNA miR-146a and further oncogenesis-related cellular microRNAs are dysregulated in HTLV-1-transformed T lymphocytes*. *Retrovirology*, 2008. **5**: p. 100.
213. Tomita, M., Y. Tanaka, and N. Mori, *MicroRNA miR-146a is induced by HTLV-1 tax and increases the growth of HTLV-1-infected T-cells*. *Int J Cancer*, 2009.
214. Yeung, M.L., et al., *Roles for microRNAs, miR-93 and miR-130b, and tumor protein 53-induced nuclear protein 1 tumor suppressor in cell growth dysregulation by human T-cell lymphotropic virus 1*. *Cancer Res*, 2008. **68**(21): p. 8976-85.
215. Lairmore, M.D., L. Silverman, and L. Ratner, *Animal models for human T-lymphotropic virus type 1 (HTLV-1) infection and transformation*. *Oncogene*, 2005. **24**(39): p. 6005-15.
216. Barbeau, B. and J.M. Mesnard, *Does the HBZ gene represent a new potential target for the treatment of adult T-cell leukemia?* *Int Rev Immunol*, 2007. **26**(5-6): p. 283-304.

217. Li, M., et al., *Kinetic analysis of human T-cell leukemia virus type 1 gene expression in cell culture and infected animals*. J Virol, 2009. **83**(8): p. 3788-97.
218. Anderson, M.D., et al., *Transformation studies with a human T-cell leukemia virus type 1 molecular clone*. J Virol Methods, 2004. **116**(2): p. 195-202.
219. Collins, N.D., et al., *In vitro CD4+ lymphocyte transformation and infection in a rabbit model with a molecular clone of human T-cell lymphotropic virus type 1*. J Virol, 1996. **70**(10): p. 7241-6.
220. Derse, D., et al., *Examining the molecular genetics of HTLV-I with an infectious molecular clone of the virus and permissive cell culture systems*. J Acquir Immune Defic Syndr Hum Retrovirol, 1996. **12**(1): p. 1-5.
221. Liu, Y., et al., *Engraftment and tumorigenesis of HTLV-1 transformed T cell lines in SCID/bg and NOD/SCID mice*. Leuk Res, 2002. **26**(6): p. 561-7.
222. Robek, M.D. and L. Ratner, *Immortalization of CD4(+) and CD8(+) T lymphocytes by human T-cell leukemia virus type 1 Tax mutants expressed in a functional molecular clone*. J Virol, 1999. **73**(6): p. 4856-65.
223. Zhao, T.M., et al., *Characterization of an infectious molecular clone of human T-cell leukemia virus type I*. J Virol, 1995. **69**(4): p. 2024-30.
224. Chan, H.M. and N.B. La Thangue, *p300/CBP proteins: HATs for transcriptional bridges and scaffolds*. J. Cell. Sci., 2001. **114**(Pt 13): p. 2363-2373.
225. Goodman, R.H. and S. Smolik, *CBP/p300 in cell growth, transformation, and development*. Genes Dev., 2000. **14**(13): p. 1553-1577.
226. Kalkhoven, E., *CBP and p300: HATs for different occasions*. Biochem Pharmacol, 2004. **68**(6): p. 1145-55.

227. Bedford, D.C., et al., *Target gene context influences the transcriptional requirement for the KAT3 family of CBP and p300 histone acetyltransferases*. Epigenetics, 2010. **5**(1): p. 9-15.
228. Ramos, Y.F., et al., *Genome-wide assessment of differential roles for p300 and CBP in transcription regulation*. Nucleic Acids Res, 2010. **38**(16): p. 5396-408.
229. Dekker, F.J. and H.J. Haisma, *Histone acetyl transferases as emerging drug targets*. Drug Discov Today, 2009. **14**(19-20): p. 942-8.
230. Iyer, N.G., H. Ozdag, and C. Caldas, *p300/CBP and cancer*. Oncogene, 2004. **23**(24): p. 4225-31.
231. Bates, C.A., W.C. Pomerantz, and A.K. Mapp, *Transcriptional tools: Small molecules for modulating CBP KIX-dependent transcriptional activators*. Biopolymers, 2010.
232. Best, J.L., et al., *Identification of small-molecule antagonists that inhibit an activator: coactivator interaction*. Proc Natl Acad Sci U S A, 2004. **101**(51): p. 17622-7.
233. Buhrlage, S.J., et al., *Amphipathic small molecules mimic the binding mode and function of endogenous transcription factors*. ACS Chem Biol, 2009. **4**(5): p. 335-44.
234. Frangioni, J.V., et al., *Minimal activators that bind to the KIX domain of p300/CBP identified by phage display screening*. Nat Biotechnol, 2000. **18**(10): p. 1080-5.
235. Lee, L.W. and A.K. Mapp, *Transcriptional switches: chemical approaches to gene regulation*. J Biol Chem, 2010. **285**(15): p. 11033-8.
236. Li, B.X. and X. Xiao, *Discovery of a small-molecule inhibitor of the KIX-KID interaction*. Chembiochem, 2009. **10**(17): p. 2721-4.

237. Volkman, H.M., S.E. Rutledge, and A. Schepartz, *Binding mode and transcriptional activation potential of high affinity ligands for the CBP KIX domain*. J Am Chem Soc, 2005. **127**(13): p. 4649-58.
238. Parker, D., et al., *Role of secondary structure in discrimination between constitutive and inducible activators*. Mol Cell Biol, 1999. **19**(8): p. 5601-7.
239. Radhakrishnan, I., et al., *Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions*. Cell, 1997. **91**(6): p. 741-52.
240. Lee, L.W. and A.K. Mapp, *Transcriptional switches: chemical approaches to gene regulation*. J Biol Chem, 2010. **285**(15): p. 11033-11038.
241. De Guzman, R.N., et al., *Structural basis for cooperative transcription factor binding to the CBP coactivator*. J Mol Biol, 2006. **355**(5): p. 1005-13.
242. Zor, T., et al., *Solution structure of the KIX domain of CBP bound to the transactivation domain of c-Myb*. J Mol Biol, 2004. **337**(3): p. 521-34.
243. Ernst, P., et al., *MLL and CREB bind cooperatively to the nuclear coactivator CREB-binding protein*. Mol Cell Biol, 2001. **21**(7): p. 2249-58.
244. Goto, N.K., et al., *Cooperativity in transcription factor binding to the coactivator CREB-binding protein (CBP). The mixed lineage leukemia protein (MLL) activation domain binds to an allosteric site on the KIX domain*. J Biol Chem, 2002. **277**(45): p. 43168-74.
245. Campbell, K.M. and K.J. Lumb, *Structurally distinct modes of recognition of the KIX domain of CBP by Jun and CREB*. Biochemistry, 2002. **41**(47): p. 13956-64.

246. Vendel, A.C. and K.J. Lumb, *NMR mapping of the HIV-1 Tat interaction surface of the KIX domain of the human coactivator CBP*. *Biochemistry*, 2004. **43**(4): p. 904-8.
247. Vendel, A.C., S.J. McBryant, and K.J. Lumb, *KIX-mediated assembly of the CBP-CREB-HTLV-1 tax coactivator-activator complex*. *Biochemistry*, 2003. **42**(43): p. 12481-7.
248. Lee, C.W., et al., *Mapping the Interactions of the p53 Transactivation Domain with the KIX Domain of CBP (dagger)*. *Biochemistry*, 2009.
249. Wang, F., et al., *Synergistic interplay between promoter recognition and CBP/p300 coactivator recruitment by FOXO3a*. *ACS Chem Biol*, 2009. **4**(12): p. 1017-27.
250. Bayly, R., et al., *Critical role for a single leucine residue in leukemia induction by E2A-PBX1*. *Mol Cell Biol*, 2006. **26**(17): p. 6442-52.
251. Liu, Y.P., C.W. Chang, and K.Y. Chang, *Mutational analysis of the KIX domain of CBP reveals residues critical for SREBP binding*. *FEBS Lett*, 2003. **554**(3): p. 403-9.
252. Zhang, Q., N. Vo, and R.H. Goodman, *Histone binding protein RbAp48 interacts with a complex of CREB binding protein and phosphorylated CREB*. *Mol Cell Biol*, 2000. **20**(14): p. 4970-8.
253. Dyson, H.J. and P.E. Wright, *Intrinsically unstructured proteins and their functions*. *Nat Rev Mol Cell Biol*, 2005. **6**(3): p. 197-208.
254. Radhakrishnan, I., et al., *Structural analyses of CREB-CBP transcriptional activator-coactivator complexes by NMR spectroscopy: implications for mapping the boundaries of structural domains*. *J Mol Biol*, 1999. **287**(5): p. 859-65.
255. Roy, A., A. Kucukural, and Y. Zhang, *I-TASSER: a unified platform for automated protein structure and function prediction*. *Nat Protoc*, 2010. **5**(4): p. 725-38.

256. Petersen, B., et al., *A generic method for assignment of reliability scores applied to solvent accessibility predictions*. BMC Struct Biol, 2009. **9**: p. 51.
257. Buchan, D.W., et al., *Protein annotation and modelling servers at University College London*. Nucleic Acids Res, 2010.
258. Mulder, N.J., et al., *The InterPro Database, 2003 brings increased coverage and new features*. Nucleic Acids Res, 2003. **31**(1): p. 315-8.
259. Sugase, K., H.J. Dyson, and P.E. Wright, *Mechanism of coupled folding and binding of an intrinsically disordered protein*. Nature, 2007. **447**(7147): p. 1021-5.
260. Rath, A., et al., *Detergent binding explains anomalous SDS-PAGE migration of membrane proteins*. Proc Natl Acad Sci U S A, 2009. **106**(6): p. 1760-5.
261. Ramirez, J.A. and J.K. Nyborg, *Molecular characterization of HTLV-1 Tax interaction with the KIX domain of CBP/p300*. J Mol Biol, 2007. **372**(4): p. 958-69.
262. Vendel, A.C. and K.J. Lumb, *Molecular recognition of the human coactivator CBP by the HIV-1 transcriptional activator Tat*. Biochemistry, 2003. **42**(4): p. 910-6.
263. Kasper, L.H., et al., *A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis*. Nature, 2002. **419**(6908): p. 738-43.
264. Parker, D., et al., *Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism*. Mol. Cell. Biol., 1996. **16**(2): p. 694-703.
265. Zor, T., et al., *Roles of phosphorylation and helix propensity in the binding of the KIX domain of CREB-binding protein by constitutive (c-Myb) and inducible (CREB) activators*. J Biol Chem, 2002. **277**(44): p. 42241-8.

266. Yan, J.P., et al., *Molecular interactions between the coactivator CBP and the human T-cell leukemia virus Tax protein*. J. Mol. Biol., 1998. **281**(3): p. 395-400.
267. Van Orden, K., et al., *Binding of the human T-cell leukemia virus Tax protein to the coactivator CBP interferes with CBP-mediated transcriptional control*. Oncogene, 1999. **18**(25): p. 3766-72.
268. Dobner, T., et al., *Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor*. Science, 1996. **272**(5267): p. 1470-3.
269. Milne, T.A., et al., *MLL targets SET domain methyltransferase activity to Hox gene promoters*. Mol Cell, 2002. **10**(5): p. 1107-17.
270. del Sol, A., et al., *The origin of allosteric functional modulation: multiple pre-existing pathways*. Structure, 2009. **17**(8): p. 1042-50.
271. Bruschweiler, S., et al., *Direct observation of the dynamic process underlying allosteric signal transmission*. J Am Chem Soc, 2009. **131**(8): p. 3063-8.
272. Moreira, I.S., P.A. Fernandes, and M.J. Ramos, *Protein-protein docking dealing with the unknown*. J Comput Chem, 2010. **31**(2): p. 317-42.
273. Ritchie, D.W., *Recent progress and future directions in protein-protein docking*. Curr Protein Pept Sci, 2008. **9**(1): p. 1-15.
274. Comeau, S.R., et al., *ClusPro: a fully automated algorithm for protein-protein docking*. Nucleic Acids Res, 2004. **32**(Web Server issue): p. W96-9.
275. Comeau, S.R., et al., *ClusPro: an automated docking and discrimination method for the prediction of protein complexes*. Bioinformatics, 2004. **20**(1): p. 45-50.
276. Comeau, S.R., et al., *ClusPro: performance in CAPRI rounds 6-11 and the new server*. Proteins, 2007. **69**(4): p. 781-5.

277. Kozakov, D., et al., *PIPER: an FFT-based protein docking program with pairwise potentials*. Proteins, 2006. **65**(2): p. 392-406.
278. Chen, R., L. Li, and Z. Weng, *ZDOCK: an initial-stage protein-docking algorithm*. Proteins, 2003. **52**(1): p. 80-7.
279. de Vries, S.J., et al., *HADDOCK versus HADDOCK: new features and performance of HADDOCK2.0 on the CAPRI targets*. Proteins, 2007. **69**(4): p. 726-33.
280. Dominguez, C., R. Boelens, and A.M. Bonvin, *HADDOCK: a protein-protein docking approach based on biochemical or biophysical information*. J Am Chem Soc, 2003. **125**(7): p. 1731-7.
281. Lensink, M.F. and S.J. Wodak, *Docking and scoring protein interactions: CAPRI 2009*. Proteins, 2010. **78**(15): p. 3073-84.
282. de Vries, S.J., et al., *Strengths and weaknesses of data-driven docking in critical assessment of prediction of interactions*. Proteins, 2010. **78**(15): p. 3242-9.
283. Minter, A.R., B.B. Brennan, and A.K. Mapp, *A small molecule transcriptional activation domain*. J Am Chem Soc, 2004. **126**(34): p. 10504-5.
284. Shaywitz, A.J. and M.E. Greenberg, *CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals*. Annu. Rev. Biochem., 1999. **68**: p. 821-861.
285. Wu, X., et al., *cAMP response element-binding protein monomers cooperatively assemble to form dimers on DNA*. J Biol Chem, 1998. **273**(33): p. 20820-7.
286. Bullock, B.P. and J.F. Habener, *Phosphorylation of the cAMP response element binding protein CREB by cAMP-dependent protein kinase A and glycogen synthase kinase-3*

- alters DNA-binding affinity, conformation, and increases net charge. Biochemistry, 1998. 37(11): p. 3795-809.*
287. Nichols, M., et al., *Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. EMBO J, 1992. 11(9): p. 3337-46.*
 288. Sakamoto, M.K., et al., *Developmental profiles of phosphorylated and unphosphorylated CREBs in murine calvarial MC3T3-E1 cells. J Biochem, 1998. 123(3): p. 399-407.*
 289. Sharma, N., D.I. Lopez, and J.K. Nyborg, *DNA binding and phosphorylation induce conformational alterations in the kinase-inducible domain of CREB. Implications for the mechanism of transcription function. J Biol Chem, 2007. 282(27): p. 19872-83.*
 290. Montminy, M.R. and L.M. Bilezikjian, *Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature, 1987. 328(6126): p. 175-8.*
 291. Kimura, E., et al., *Induction of FOS and JUN proteins by adrenocorticotropin and phorbol ester but not by 3',5'-cyclic adenosine monophosphate derivatives. Mol Endocrinol, 1993. 7(11): p. 1463-71.*
 292. Pirson, I. and J.E. Dumont, *Jun B expression is regulated differently by three mitogenic pathways in thyrocytes. Exp Cell Res, 1994. 214(2): p. 561-9.*
 293. Suh, H.W., et al., *Regulation of c-fos and c-jun gene expression by lipopolysaccharide and cytokines in primary cultured astrocytes: effect of PKA and PKC pathways. Arch Pharm Res, 2004. 27(4): p. 396-401.*
 294. Benbrook, D.M. and N.C. Jones, *Heterodimer formation between CREB and JUN proteins. Oncogene, 1990. 5(3): p. 295-302.*

295. van Dam, H. and M. Castellazzi, *Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis*. Oncogene, 2001. **20**(19): p. 2453-2464.
296. Hai, T. and T. Curran, *Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity*. Proc Natl Acad Sci U S A, 1991. **88**(9): p. 3720-4.
297. Ivashkiv, L.B., et al., *mXBP/CRE-BP2 and c-Jun form a complex which binds to the cyclic AMP, but not to the 12-O-tetradecanoylphorbol-13-acetate, response element*. Mol Cell Biol, 1990. **10**(4): p. 1609-21.
298. Newman, J.R. and A.E. Keating, *Comprehensive identification of human bZIP interactions with coiled-coil arrays*. Science, 2003. **300**(5628): p. 2097-101.
299. Ryseck, R.P. and R. Bravo, *c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins*. Oncogene, 1991. **6**(4): p. 533-42.
300. Vinson, C., A. Acharya, and E.J. Taparowsky, *Deciphering B-ZIP transcription factor interactions in vitro and in vivo*. Biochim Biophys Acta, 2006. **1759**(1-2): p. 4-12.
301. de Groot, R.P. and P. Sassone-Corsi, *Activation of Jun/AP-1 by protein kinase A*. Oncogene, 1992. **7**(11): p. 2281-6.
302. Galien, R., R. Emanoil-Ravier, and G. Mercier, *Differential effects of c-jun and CREB on c-AMP response element activation by Ha-ras*. Oncogene, 1994. **9**(4): p. 1101-8.
303. Rutberg, S.E., et al., *CRE DNA binding proteins bind to the AP-1 target sequence and suppress AP-1 transcriptional activity in mouse keratinocytes*. Oncogene, 1999. **18**(8): p. 1569-79.

304. Sassone-Corsi, P., L.J. Ransone, and I.M. Verma, *Cross-talk in signal transduction: TPA-inducible factor jun/AP-1 activates cAMP-responsive enhancer elements.* Oncogene, 1990. **5**(3): p. 427-31.
305. Sharma, S.C. and J.S. Richards, *Regulation of AP1 (Jun/Fos) factor expression and activation in ovarian granulosa cells. Relation of JunD and Fra2 to terminal differentiation.* J Biol Chem, 2000. **275**(43): p. 33718-28.
306. Shilatifard, A., *Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression.* Annu Rev Biochem, 2006. **75**: p. 243-69.
307. Slany, R.K., *The molecular biology of mixed lineage leukemia.* Haematologica, 2009. **94**(7): p. 984-93.
308. Vogelstein, B., D. Lane, and A.J. Levine, *Surfing the p53 network.* Nature, 2000. **408**(6810): p. 307-10.
309. Shankar, D.B., J.C. Cheng, and K.M. Sakamoto, *Role of cyclic AMP response element binding protein in human leukemias.* Cancer, 2005. **104**(9): p. 1819-24.
310. Shankar, D.B., et al., *The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia.* Cancer Cell, 2005. **7**(4): p. 351-62.
311. van Dam, H. and M. Castellazzi, *Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis.* Oncogene, 2001. **20**(19): p. 2453-64.
312. Vogt, P.K., *Jun, the oncoprotein.* Oncogene, 2001. **20**(19): p. 2365-77.
313. O'Neil, J. and A.T. Look, *Mechanisms of transcription factor deregulation in lymphoid cell transformation.* Oncogene, 2007. **26**(47): p. 6838-49.

314. Greig, K.T., S. Carotta, and S.L. Nutt, *Critical roles for c-Myb in hematopoietic progenitor cells*. Semin Immunol, 2008. **20**(4): p. 247-56.
315. Grant, C., et al., *Human T cell leukemia virus type I and neurologic disease: events in bone marrow, peripheral blood, and central nervous system during normal immune surveillance and neuroinflammation*. J. Cell. Physiol., 2002. **190**(2): p. 133-159.
316. Hollsberg, P., *Mechanisms of T-cell activation by human T-cell lymphotropic virus type I*. Microbiol Mol Biol Rev, 1999. **63**(2): p. 308-33.
317. Swaims, A.Y., et al., *Immune activation induces immortalization of HTLV-1 LTR-Tax transgenic CD4+ T-cells*. Blood, 2010.
318. Sandberg, M.L., et al., *c-Myb and p300 regulate hematopoietic stem cell proliferation and differentiation*. Dev Cell, 2005. **8**(2): p. 153-66.
319. Sadowski, I. and M. Ptashne, *A vector for expressing GAL4(1-147) fusions in mammalian cells*. Nucleic Acids Res, 1989. **17**(18): p. 7539.
320. Mestas, S.P. and K.J. Lumb, *Electrostatic contribution of phosphorylation to the stability of the CREB-CBP activator-coactivator complex*. Nat Struct Biol, 1999. **6**(7): p. 613-4.
321. Harrod, R., et al., *An exposed KID-like domain in human T-cell lymphotropic virus type 1 Tax is responsible for the recruitment of coactivators CBP/p300*. Mol Cell Biol, 1998. **18**(9): p. 5052-61.
322. Wilczek, C., et al., *Myb-induced chromatin remodeling at a dual enhancer/promoter element involves non-coding rna transcription and is disrupted by oncogenic mutations of v-myb*. J Biol Chem, 2009. **284**(51): p. 35314-24.

323. Kraus, W.L., E.T. Manning, and J.T. Kadonaga, *Biochemical analysis of distinct activation functions in p300 that enhance transcription initiation with chromatin templates*. Mol. Cell. Biol., 1999. **19**(12): p. 8123-8135.
324. Hoeffler, J.P., J. W. Lustbader and C.-Y. Chen, *Identification of multiple nuclear factors that inter-act with Cyclic Adenosine 3',5'-Monophosphate Response Element-Binding Protein and Activating Transcription Factor-2 by protein-protein interactions*. Mol. Endocrin, 1991. **5**: p. 256-266.
325. Lopez, D.I., J.E. Mick, and J.K. Nyborg, *Purification of CREB to apparent homogeneity: removal of truncation products and contaminating nucleic acid*. Protein Expr Purif, 2007. **55**(2): p. 406-18.
326. Carey, J., *Gel retardation*. Methods Enzymol, 1991. **208**: p. 103-17.