INTERACTION BETWEEN HTLV-1 BASIC LEUCINE ZIPPER FACTOR (HBZ) AND
HOST CELL PROTEINS PROMOTES CELLULAR TRANSFORMATION

by

Stephanie Sanders

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Stephanie Sanders

Greenville, NC

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Approved by:

Dr. Isabelle Lemasson

Dept. Microbiology and Immunology, Brody School of Medicine

ABSTRACT

Human T-cell Leukemia Virus Type 1 (HTLV-1) is a complex retrovirus which preferentially infects CD4+ T-cells. Endemic to Japan, regions of South America, Africa, and the Caribbean, HTLV-1 is transmitted through breastfeeding, sexual contact, and contact with infected blood or blood products. In 5% of cases, HTLV-1 infection leads to Adult T-cell Leukemia (ATL) after a 40 to 60 year period of low viral activity. Diagnosis of ATL is equivalent to a death sentence as currently available treatments only serve to extend the life of patients by a few months.

HTLV-1 encodes unique regulatory and accessory proteins which are important for viral replication as well as modulating host cell activities. One of these proteins includes the HTLV-1 basic leucine zipper factor (HBZ). Clinical research reveals that HBZ is the only viral protein consistently expressed in patients with ATL, and *in vitro* transformation assays support that HBZ is important for cellular transformation. The exact mechanisms by which HBZ promotes cellular transformation are not known; therefore, understanding the molecular biology of HBZ and other proteins is important for developing effective treatments for ATL.

HBZ is a nuclear protein that has been shown to interact with cellular transcription factors in the CREB/ATF and AP-1 families, as well as with coactivators including p300/CBP. These interactions regulate or deregulate expression of host cell and proviral genes. One focus in our laboratory is to identify and characterize novel interactions between HBZ and cellular proteins. HBZ interactome studies performed in our laboratory reveal that HBZ interacts with the small Maf family of basic leucine zipper (bZIP) transcriptional regulators. Leucine zipper dimerization between small Mafs and other compatible bZIP proteins allows them to be recruited to Maf Responsive Elements (MAREs) on the genomic DNA as transcriptionally repressive

homodimers, or as transcriptionally active heterodimers. We found that interaction between HBZ

and small Mafs occurs directly through a dimerization of each protein's leucine zipper using *in*

vitro GST pulldown assays.

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Introduction

HTLV-1 is a complex retrovirus that preferentially infects CD4+ T-lymphocytes. Its genome is composed of a single positive sense strand of RNA which is reverse transcribed by a prepackaged enzyme Reverse Transcriptase into double stranded DNA. This DNA is incorporated into the host cell's DNA where it is known as the provirus and can be transcribed as part of the cell's normal functioning (Satou and Matsuoka, 2012). Endemic to Japan, regions of South America, Africa, and the Caribbean, the true prevalence of HTLV-1 is not known; however, there are an estimated 10-20 million infected individuals worldwide (Gessain and Cassar, 2012). HTLV-1 may be transmitted through breastfeeding, sexual contact, and contact with infected blood or blood products (Gessain and Cassar, 2012; Matsuoka, 2005). Since the virus does not spread using free virions, it must enter the cell via cell-to-cell contact. The GLUT-1 transporter, Heporan sulfate proteoglycan (HSPG) and neuropilin-1 (Nrp1) have all been shown to help facilitate the entry of the HTLV-1 virus (Matsuoka, 2005; Jones et. al., 2006; Ghez et al, 2006). An infected cell forms a viral synapse with a target cell through which virions flow into the target cell, spreading the infection (Matsuoka, 2005). Infection with HTLV-1 can lead to the development of HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), a neurodegenerative disorder, as well as Adult T-cell Leukemia (ATL) (Matsuoka, 2005).

In 5% of cases, HTLV-1 infection leads to ATL after a 40 to 60 year period of low viral activity during which infected individuals remain asymptomatic (Manns, Hisada, and Grenade, 1999). This is likely why the actual number of HTLV-1 infected individuals is under reported. As long as the individual remains asymptomatic, they have the potential to continue spreading the virus to others who, in turn, also may not know that they are infected. ATL is a non-Hodgkin

lymphoma in which leukemic cells are characterized by multiobulated "flower" cells and morphologically unique lymphocytes (Manns, Hisada and Grenade, 1999). Diagnosis of ATL is equivalent to a death sentence, as currently available treatments only serve to extend the life of patients by a few months. The average survival of a patient with ATL is 13 months under intense chemotherapy. This is due in part to the resistance of the virus to antiretroviral regimes and chemotherapy treatments, as well as immunodeficiency associated with the disease (Matsuoka, 2005). In order to develop more successful treatments, we must understand the molecular biology of HTLV-1 infection, replication, and transformation.

The structure of the HTLV-1 provirus (Fig 1) is very similar to other retroviruses, in that it possesses a 5' and 3' long terminal repeat (LTR) which contain promoter elements that regulate viral gene expression. Additionally, it encodes the classic *gag, pro, pol* and *env* genes which encode structural and replicative proteins (Matsuoka 2005). However, the provirus also contains a pX region, a characteristic unique to HTLV-1. This region encodes viral accessory and regulatory proteins like Transactivator X (Tax) and the HTLV-1 basic leucine zipper factor (HBZ). Tax is involved in viral replication and proliferation of infected cells by promoting transcription of viral proteins from the 5'-LTR (Matsuoka, 2005). Tax also interacts with cellular transcription factors like p53 in order to promote genetic instability and immortalization of infected T-cells. Taken together, Tax can be implicated in cellular immortalization and leukemogenesis following HTLV-1 infection (Matsuoka, 2005).

While *in vitro* data support that Tax is important for cellular transformation, *in vivo* data show that Tax alone may contribute to, but is not sufficient for leukemogenesis as it is a target for the human immune system, specifically cytotoxic T-cells (Matsuoka, 2005).
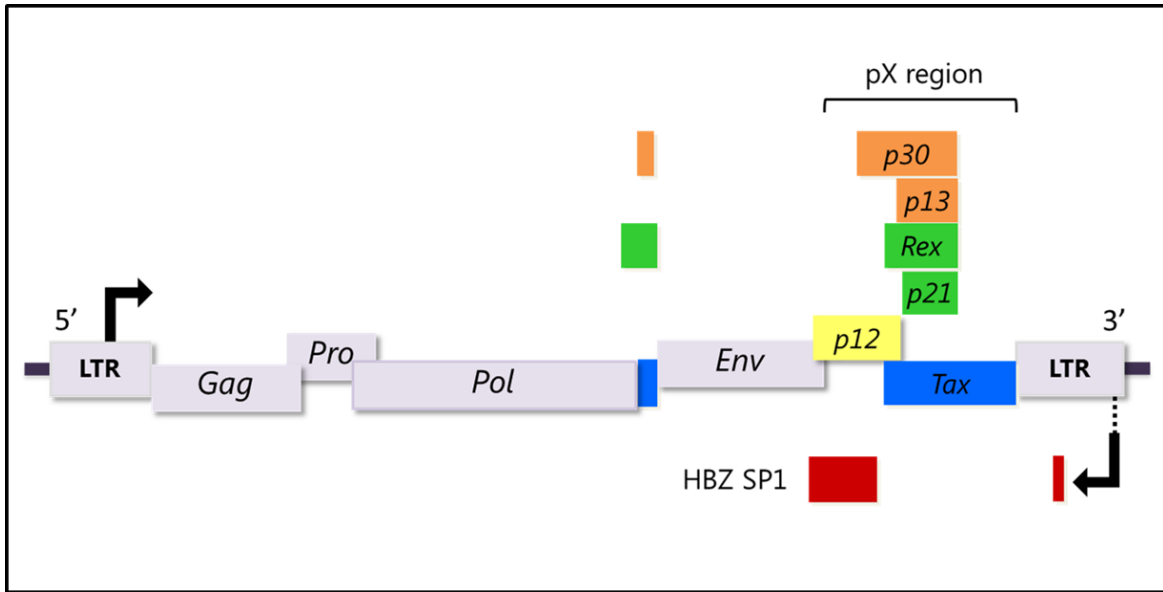
Figure 1: The HTLV-1 provirus

The provirus contains a 5' and 3' long terminal repeat (LTR) along with common viral genes encoding the Gag, Pro, Pol, and Env proteins. It also contains a pX region unique HTLV-1 in which accessory and regulatory proteins are encoded. HBZ is the only protein encoded on the antisense strand of the virus.

Research demonstrates that HBZ plays a role in immune evasion by reducing the expression of Tax (Gaudray, 2002). During viral replication, the survival of HBZ expressing cells is amplified and it has been found that these cells are more resistant to the immune response of the host (Satou and Matsuoka. 2012). It is important to note that there is an immune response to HBZ expressing cells, however, this response is much lower than the immune response to other HTLV-1 associated proteins such as Tax, perhaps due to HBZ's poor immunogenicity (Enose-Akahata et al. 2013). Interestingly, ATL infected cells also show dramatically decreased expression of the genes encoded by the 5' LTR including Tax with 60% of patients exhibiting no Tax expression at all (Matsuoka, 2005). Studies have shown that the expression of HBZ increases the infectivity of HTLV-1, the growth of the infected T-cells and the transformation of infected cells (Enose-Akahata et al. 2013). This suggests that HBZ is an important factor in the overall survival of HTLV-1, as well as the onset of ATL (Enose-Akahata et al, 2013; Satou and Matsuoka, 2012).

HBZ is encoded on the antisense strand of the provirus and contains an activation domain, a central domain, and a basic leucine zipper (bZIP) domain as shown in Figure 2 (Satou and Matsuoka, 2012). The activation domain, located at the N-terminal of HBZ, is used to recruit coactivators like p300/CBP to aid in the activation of transcription (Clerc, 2008). The bZIP domain contains a basic region responsible for binding DNA at specific sites, and an alpha helical leucine zipper motif characterized by heptad leucine repeats which facilitates dimerization with compatible bZIP factors. These interactions influence a variety of cellular processes, many of which are hypothesized to contribute to carcinogenesis.

Previous research in our laboratory used liquid chromatography and tandem mass spectrometry to identify novel binding partners of HBZ in order to identify new interactions that
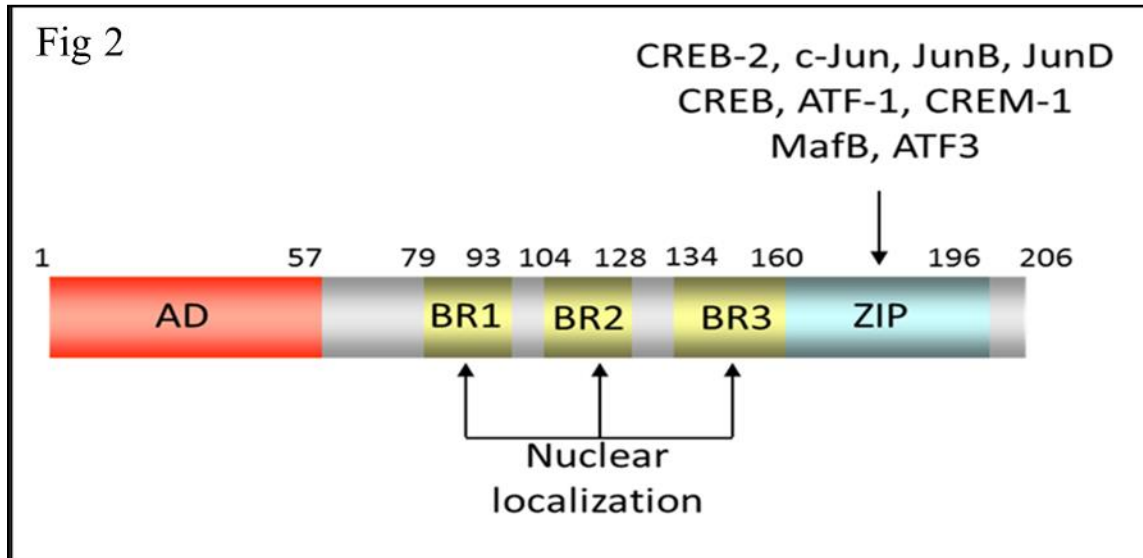
Figure 2: Basic structure of HBZ

HBZ contains an activation domain (AD) on its N-terminus (1-57) that is responsible for interacting with coactivators p300/CBP. Its C-terminal bZIP domain (160-206) is responsible for interactions with many other bZIP transcription factors including CREB-2, c-Jun, JunB, JunD, CREB, ATF-1, CREM-1, MafB, and ATF3. The basic regions (79-93, 104-128, 134-160) contained in the central region of HBZ facilitate nuclear localization of the protein.

may contribute to ATL. These binding partners, shown in Table 1, included known binding partners JunD, JunB, ATF-1, and CREB. HBZ forms complexes with these transcription factors and thereby interferes with host cell signaling pathways such as the AP-1 pathway (Satou and Matsuoka, 2012). This interaction frequently sequesters the transcription factors away from their DNA targets; such is the case with CREB, c- Jun, and ATF-1 (Lemasson, 2007; Clerc, 2009). In some cases, HBZ is found to use these binding partners to bind DNA and activate transcription of target genes, as in the case of JunD (Gazon, 2012).

Understanding the interactions between HBZ and host cell proteins will aid in elucidating the exact mechanisms of cellular transformation. In addition to known binding partners, HBZ interactome studies performed in our laboratory reveal that HBZ interacts with the small musculoaponeurotic fibrosarcoma (Maf) family of bZIP transcriptional regulators. The small Maf family consists of three functionally redundant proteins known as MafF, MafG, and MafK. They contain a well conserved basic region and leucine zipper domain; these domains facilitate DNA binding at Maf Responsive Elements (MAREs) and dimerization with some other bZIP containing proteins respectively (Motohashi et al, 1997). Members of the small Maf family do not have activation domains; therefore, small Maf dimers are found to repress transcriptional activity (Motohashi et al, 1997). Small Mafs are obligate binding partners of Cap'n'Collar proteins; this interaction leads to activation of genes involved in oxidative stress response and are required for the regulation of embryogenesis (Motohashi et al, 1997). An interaction between HBZ and small Maf transcription factors may implicate changes in the host cell antioxidant response pathway which could either promote cell survival or lead to increase DNA damage. Our project endeavors to confirm an interaction between HBZ and small Maf transcription factors, characterize this interaction as direct or indirect, and determine what region of HBZ

bZIP Transcription Factors	HAT Coactivators
Jun	p300
JunB	CBP
JunD	
ATF-1	
ATF-2	
ATF-7	
CREB-1	
CREM	
C/EBPG	

Table 1: Binding partners of HBZ

The table shows previously reported binding partners found by our laboratory using liquid chromatography and tandem mass spectrometry.

facilitates its interaction with small Mafs. We hypothesize that HBZ and small Maf proteins directly interact via hydrophobic interactions between the leucine zipper domains of each protein.

Materials and Methods

Co-Immunoprecipitation

HEK 293T cells were plated at 2 x 10^6 cells per plate 24 hours before they were transiently co-transfected with combinations of pCDNA-HBZ-myc-his, pCMV-MafG-flag, pCMV-MafK-flag. Transfections were performed with Turbofect (Thermo Fischer) according to standard protocol. Transfected cells were incubated at 37°C, 5% CO_2 for 48 hours before whole cell protein extracts were harvested in RIPA (50 mmol Tris HCl pH 8.0, 1% Triton X-100, 100 mmol NaCl, 1 mmol $MgCl_2$, phenylmethylsulfonyl fluorde, benzamidine, leupeptine, aprotinin) Protein concentrations were measured by Bradford protein assay.

Anti-flag (Sigma) and anti-myc resins were prepared and incubated with 300 μg total protein for 16 hours. Unbound protein was washed away with 5 washes in RIPA and protease inhibitors. Protein complexes were eluted with SDS dye and heat before being analyzed by Western blot. The blot was probed for both HBZ and Maf to determine if these proteins were found in the same complex.

Anti-myc resin was prepared by adding 10μL packed volume of protein G coupled agarose resin and incubating with 2μg anti-myc monoclonal antibody (Millipore) for 1 hour. The new resin was washed twice with RIPA plus protease inhibitors before use.

Cloning Recombinant Proteins

GST-tagged HBZ truncation mutants includes HBZ-L2C/L4C, GST-AD (1-57), GST-CD (58-129), and GST-bZIP (130-206). The appropriate truncated sequences were amplified by PCR from pcDNA HBZ-myc-his using the following primers: GST-AD (F 5' CGGATCCATG GCGGCCTCAGGGCTG 3'; R 5' CGAATTCTCACCTGCCGCGGCTTTCCTC 3'); GST-CD (F 5' CGGGATCCCTTCGACGGGGCCCTCCAG 3'; R 5' CGAATTCTCACTGCTTTCTCCG GGCAAC 3'); GST-bZIP (F 5' CGGGATCCGAAGAGCAGGAGCGCCGTG 3'; R 5' CGAAT TCTTATTGCAACCACATCGC 3') GST-HBZ-L2C/L4C (F 5' CGGATCCATGGCGGCCTCA GGGCTG 3'; R 5' CGAATTCTTATTGCAACCACATCGC 3'). Amplified sequences were ligated into the pGEX-4T-2 plasmid using BamHI and EcoRI sites, adding a glutathione S-transferase (GST) tag to the N-terminal end of each recombinant protein.

The GST-MafG plasmid was created by amplifying the MafG sequence from a template vector (DNASU Plasmid Repository) using the following primers: F 5' GAAGATCTGAATTC ATGACGACCCCCAAT 3'; R 5' CCCAAGCTTCAACGATCGGGCATC 3'. The MafG sequence was ligated into pGEX-4T-2 which was cut at BamHI and SmaI sites. All plasmids were validated through sequencing (Operon) to ensure that no mutations were inserted during the process.

Protein Purification

BL21 (DE3) pLysS chemically competent *Escherichia coli* cells were transformed with the pGEX plasmids described previously and individual colonies were selected and expanded. Growth of transformed cells was maintained with 100 µg/mL Ampicillin and 25 µg/mL Chloramphericol. Expression of recombinant proteins was induced in exponentially growing

cells with 0.4 mM IPTG for three hours. GST tagged proteins were purified from bacterial lysates with glutathione resin (Sigma).

GST Pulldown Assay

Glutathione beads were pre-bound with 50 pmol GST tagged recombinant proteins in 1X phosphate buffer saline (PBS) and rocked for 1 hour at 4°C. The pre-bound beads were washed twice with 1X PBS plus protease inhibitors. 30 pmol MafF was added in Pulldown Buffer (20 mM Hepes pH 7.9, 0.5 mM EDTA pH 8.0, 10% glycerol, 0.05% Nonidet P-40, 5 µM $ZnSO_4$, 2.5 mM $MgCl_2$, 50 mM KCl) with a protease inhibitor, PMSF and reducing agent dithiothreitol (DTT). Reactions were incubated overnight at 4°C. In experiments where DNA was included, 400 nM T-MARE sequence was added with MafF in Pulldown Buffer plus protease inhibitors before the beads were rocked overnight. Beads were washed 4 times with Pulldown Buffer plus protease inhibitors before protein complexes were eluted with SDS dye and heat, and analyzed by Western blot.

Results

HBZ is found in complex with the small Mafs

The first evidence obtained by our laboratory that small Mafs are novel binding partners of HBZ was derived from the liquid chromatography and tandem mass spectrometric analysis of HBZ-containing protein complexes. To confirm the validity of these reported interactions, we used co-immunoprecipitation experiments to test whether small Mafs can bind HBZ *in vitro*. When co-expressed, HBZ co-purified with MafG (Figure 3A lane 9) and MafK (Fig 3B lane 9) when HBZ-containing complexes were isolated by anti-myc immunoprecipitation. In the
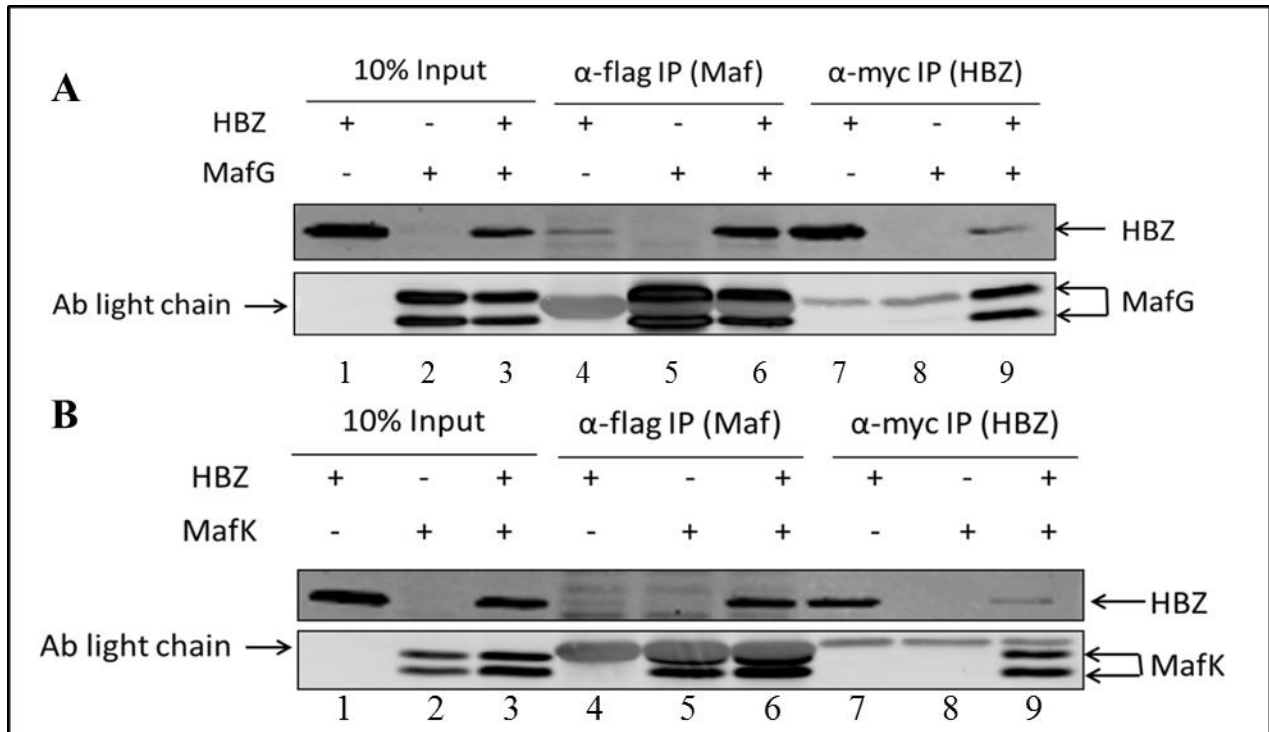
Figure 3: Co-Immunoprecipatation assays confirm that small Mafs interact with HBZ

A) HEK 293T cells were transiently co-transfected with HBZ-myc-his and MafG-flag. Whole cell protein extracts were then prepared for anti-flag and anti-myc immunoprecipitation. Eluted proteins were analyzed by western blot. The anti-flag immunoprecipitation was used to detect Maf containing complexes (A, B lanes 4-6) while the anti-myc immunoprecipitation was used to detect HBZ containing complexes (A, B lanes 7-9). In each experiment, 10% of the total protein extract was loaded for comparison (A, B lanes 1-3).

B) HEK 293T cells were transiently co-transfected with HBZ-myc-his and MafK-flag. Whole cell protein extracts were then prepared for anti-flag and anti-myc immunoprecipitation. Eluted proteins were analyzed by western blot. The anti-flag immunoprecipitation was used to detect Maf containing complexes (A, B lanes 4-6) while the anti-myc immunoprecipitation was used to detect HBZ containing complexes (A, B lanes 7-9). In each experiment, 10% of the total protein extract was loaded for comparison (A, B lanes 1-3).

reciprocal experiment, HBZ was co-purified with both MafG (Fig 3A lane 6) and MafK (Fig 3B lane 6). While this experiment confirms that HBZ and small Maf proteins are found in complex together, it does not confirm whether this is a direct interaction between HBZ and a small Maf. It also does not determine where on either protein the interaction takes place.

The interaction between HBZ and small Mafs is a direct dimerization of the leucine zipper

GST pulldown assays were performed to characterize HBZ's interaction with small Mafs, as well as to show which domain of HBZ facilitates the interaction. Because this assay uses specific purified proteins, as opposed to whole cell protein extracts, the interaction between two proteins can be more specifically characterized. A direct interaction is observed when both proteins of interest are found in the same lane on the western blot. Glutathione beads were pre-bound with GST (Fig 4B, lane 2), GST-HBZ (Fig 4B, lane 4), and GST-MafG (Fig 4B, lane 3). Purified MafF was incubated with the pre-bound resin. Eluted proteins were analyzed by Western blot and showed that MafF interacted with GST-HBZ (Fig 4B, lane 4), and GST-MafG (Fig 4B, lane 3) as a positive control. This result supports that the interaction between HBZ and small Mafs is direct.

To determine what domain of HBZ is responsible for this interaction, HBZ truncation mutants were added to the pulldown assays. Glutathione beads were prebound with GST (Fig 4C, lane 2), GST-HBZ (Fig 4C, lane 3), GST-bZIP (Fig 4C, lane 4), GST-AD (Fig 4C, lane 5), and GST-MafG (Fig 4C, lane 6). Purified MafF was incubated with this prebound resin. Eluted proteins were analyzed by Western blot which showed that the direct interaction between HBZ and MafF occurs at its bZIP domain (Fig 4C, lane 4). This result supports that the interaction is facilitated by the bZIP domain of HBZ.
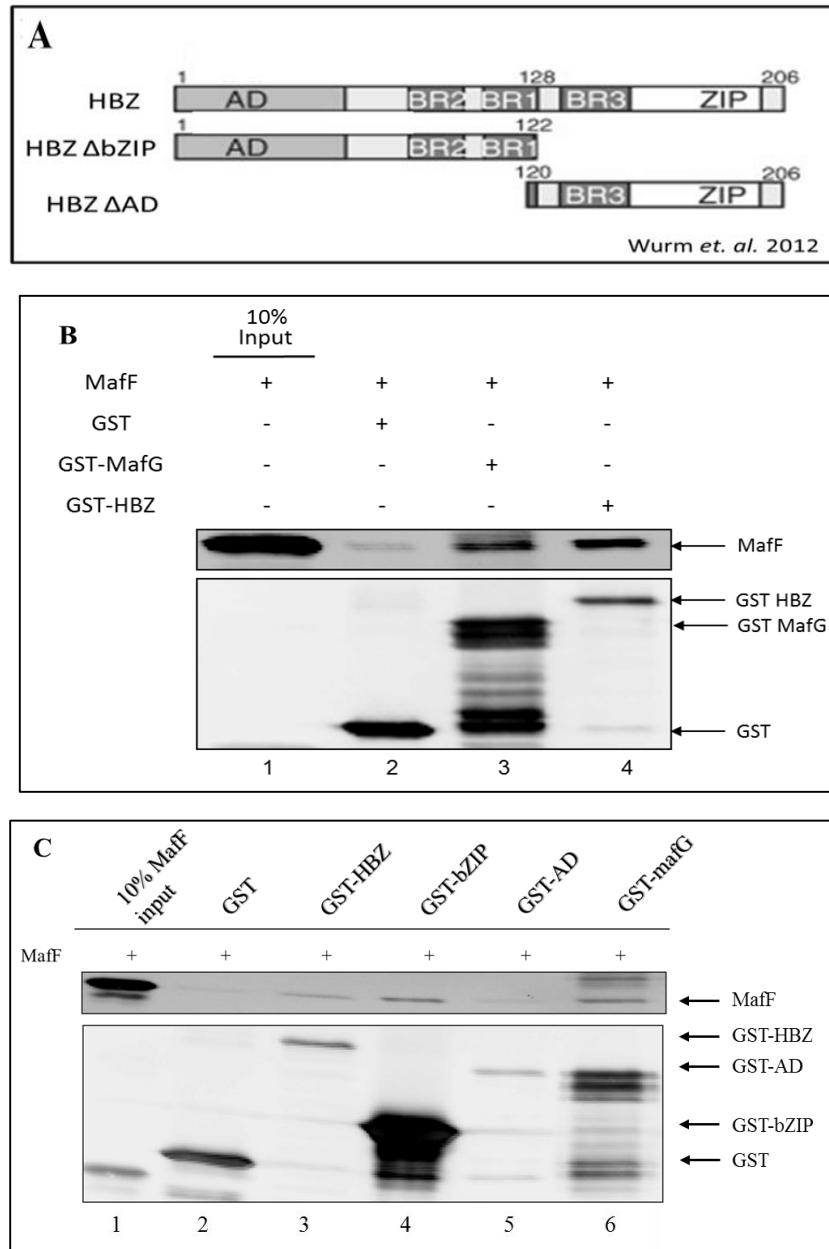
Figure 4: The interaction between HBZ and small Maf proteins is direct

A) GST pulldown assays were performed with full length and truncated GST-tagged HBZ.
B) 50 pmol GST tagged HBZ and MafG were prebound to glutathione beads with 1X PBS.
Beads were washed with 1X PBS before 30 pmol MafF was added in Pulldown Buffer plus
protease inhibitors. Protein complexes were eluted with SDS and heat before being separated by
SDS-PAGE and detected with Western blotting.
C) 50 pmol GST tagged proteins were prebound to glutathione beads with 1X PBS. Beads were
washed with 1X PBS before 30 pmol MafF was added in Pulldown Buffer plus protease
inhibitors. Protein complexes were eluted with SDS and heat before being separated by SDS-
PAGE and detected with Western blotting.

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MafF binding to GST-HBZ and its bZIP domain was weaker than expected, especially when compared to the 10% input lane. It has been reported in the literature that DNA can be used to help facilitate the interaction between proteins (Ramirez and Nyborg, 2007). For this reason, we hypothesized that adding MARE DNA to our GST pulldown assays would facilitate stronger binding between MafF and HBZ (Fig 5). Once again, GST (Fig 5, lane 2), GST-HBZ (Fig 5, lane 3), GST-bZIP (Fig 5, lane 4), GST-AD (Fig 5, lane 5), and GST-MafG (Fig 5, lane 6) were prebound to glutathione beads. The beads were then incubated with MafF and MARE DNA; proteins were eluted and analyzed by Western blot. The results show that MARE DNA does not facilitate the interaction between HBZ and small Maf proteins. The amount of MafF binding to HBZ (Fig 5, lane 3) and its bZIP domain (Fig 5, lane 4) appears to have decreased and background binding on the activation domain of HBZ is still present (Fig 5, lane 5).

Discussion

Liquid chromatography and tandem mass spectrometry was performed by our lab to reveal novel binding partners for HBZ in order to develop a better understanding of the molecular biology behind cellular transformation in HTLV-1 infected cells. The list of novel binding partners included small Maf transcriptional regulators. We used co-immunoprecipitation assays to confirm this interaction, and GST pulldown assays to characterize the interaction as direct or indirect.

Our results indicated that the interaction between small Mafs and HBZ is direct and occurs at the bZIP region on HBZ. This evidence supports that the interaction between HBZ and small Mafs occurs through leucine zipper dimerization. However, low levels of background
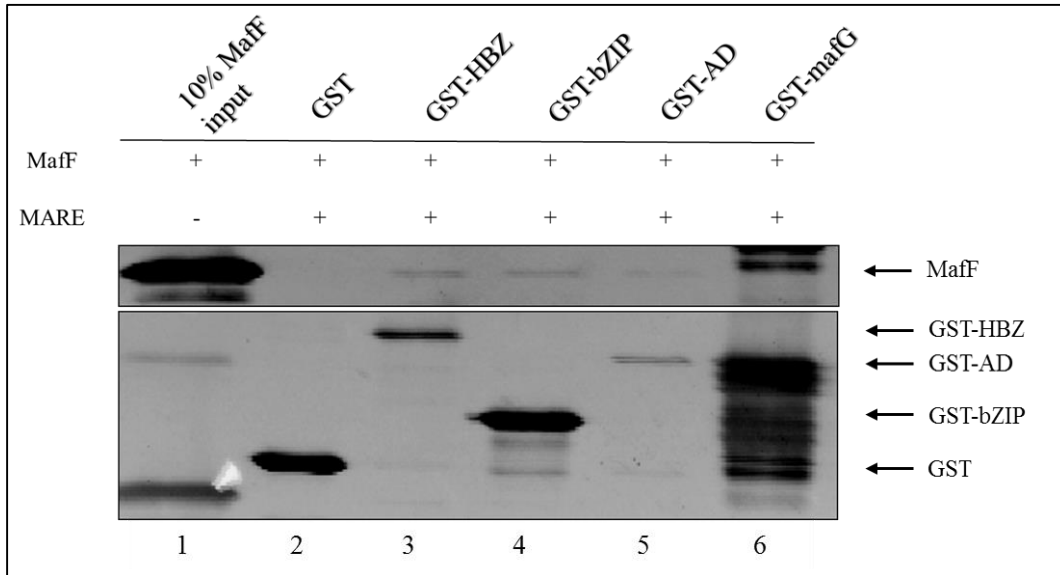
Figure 5: DNA does not facilitate the interaction between MafF and HBZ

50 pmol GST tagged proteins were prebound to glutathione beads with 1X PBS. Beads were washed with 1X PBS before 30 pmol MafF and 400 nM MARE DNA was added in Pulldown Buffer plus protease inhibitors. Protein complexes were eluted with SDS and heat before being separated by SDS-PAGE and detected with Western blotting.

binding was still observed when using the GST-AD mutant and the binding of MafF to the bZIP domain was not as strong as we expected. The HBZ truncation mutants used in these experiments contained pieces of the other regions of HBZ, specifically its central region (Fig 4A); this additional piece may be affecting our results. In order to achieve cleaner results, new HBZ mutants have been cloned (Fig 6). These mutants are further truncated to eliminate the portions of the central region that were previously included. We have also added a central region mutant to explore its role in the interaction between small Mafs and HBZ. A mutant in the leucine zipper domain has been cloned to further confirm our results by demonstrating that the interaction between HBZ and small Mafs depends on hydrophobic interactions between leucine residues in the leucine zipper. In addition, we plan to perform the reciprocal GST pulldown assays in which we will create point mutations in the leucine zipper of small Mafs. The reciprocal experiment will determine whether these mutations are sufficient to abolish the small Maf interaction with full length HBZ.

In addition to characterizing the interaction between HBZ and small Mafs, we are endeavoring to determine how this interaction could affect cellular pathways. Previous research has shown that many times, HBZ sequesters transcription factors away from the DNA, negatively affecting transcription; however, this is not always the case. In the case of JunD, HBZ is associated with transcriptional activation through an SP-1 dependent mechanism. In an effort to determine the transcriptional activity of the HBZ/small Maf complex, we plan to perform transcriptional reporter assays.

Small Mafs have been implicated in regulating the expression of oxidative stress response genes (Motohashi et al, 1997). These pathways are responsible for removing reactive oxygen species from the cell to prevent DNA damage and promote cell survival. The activation domain
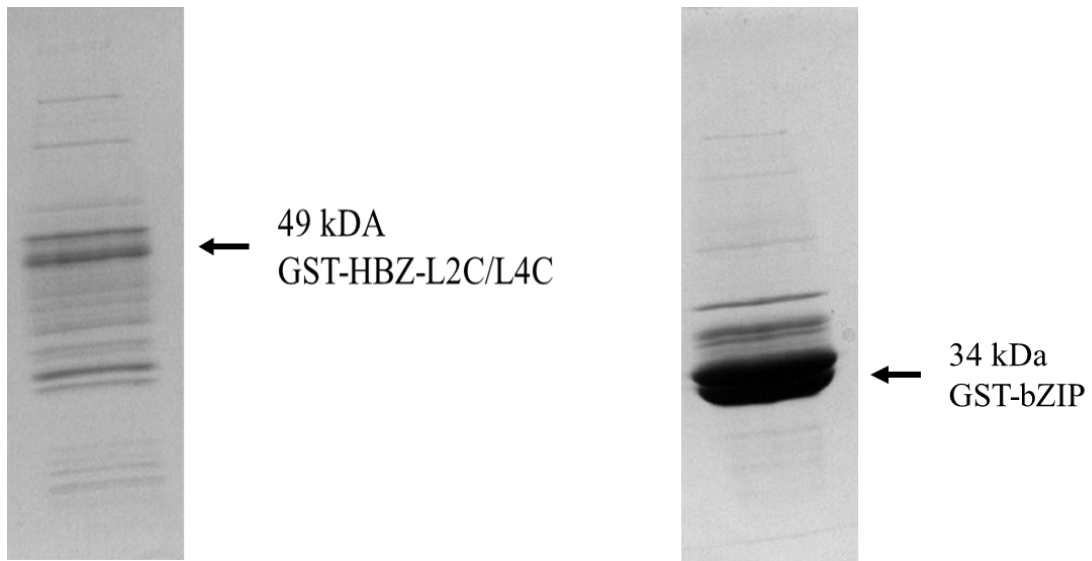
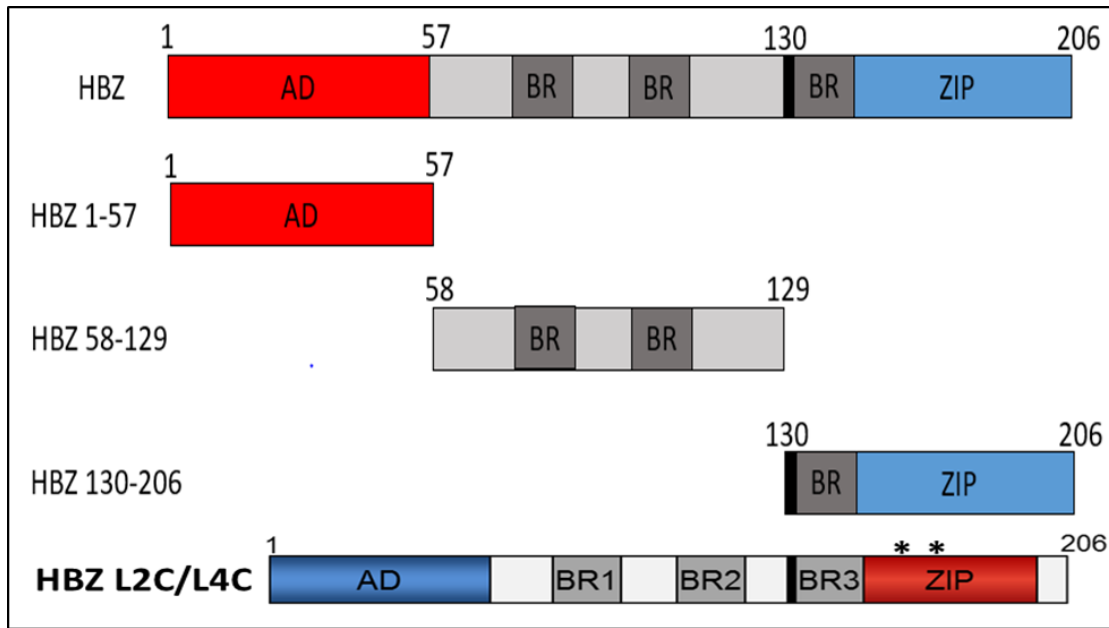**Figure 6: New GST tagged HBZ mutants**

New mutants were cloned into the pGEX-4T-2 plasmid. Plasmids were transformed into E. coli bacteria and induced with IPTG. Proteins were purified with glutathione resin and eluted with reduced glutathione.

of HBZ has been shown to interact with p300 and CBP, leading us to question whether small Mafs will also be found in this complex and whether HBZ will serve as a transcriptional activator that is no longer under the same regulatory constraints. If this is the case, it is possible that this complex will lead to a constitutive activation of oxidative stress response pathways which would pose a host of problems for the HTLV-1 infected cell.

The interaction between HBZ and small Maf proteins could be a factor that leads to cellular transformation regardless of whether or not it activates or represses transcription of genes related to small Mafs. The repression of these genes could allow reactive oxygen species to build up in the cell, leading to DNA damage and the introduction of new mutations, a common contributing factor in carcinogenesis. However, the activation of oxidative stress response pathways could promote cell survival and proliferation, resulting in increased populations of infected cells as well as drug resistance. We believe that ATL is the product of several survival pathways that have been altered, which could explain why it only affects 5% of HTLV-1 patients; an interaction with small Mafs could be a step in the disruption of a survival pathway.

Once the activities of the HBZ/small Maf interaction have been determined, we hope to identify pathway components that can serve as novel therapeutic targets in the attempt to create a more specific and highly efficacious treatment for HTLV-1 infected individuals and ATL patients. Since HBZ is an important protein for cellular transformation, continued research into its interactions with host cell proteins will allow us to elucidate how HBZ contributes to this process. We have taken another step towards that goal by defining an interaction with small Maf proteins. More research is needed to develop novel treatments and one day cure patients with ATL.

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