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# HTLV-1 bZIP Factor Suppresses Apoptosis by Attenuating the Function of FoxO3a and Altering Its Localization

Azusa Tanaka-Nakanishi, Jun-ichirou Yasunaga, Ken Takai, and Masao Matsuoka

## Abstract

As the infectious agent causing human adult T-cell leukemia (ATL), the human T-cell leukemia virus type 1 (HTLV-1) virus spreads *in vivo* primarily by cell-to-cell transmission. However, the factors that determine its transmission efficiency are not fully understood. The viral genome encodes the HTLV-1 bZIP factor (HBZ), which is expressed in all ATL cases and is known to promote T-cell proliferation. In this study, we investigated the hypothesis that HBZ also influences the survival of T cells. Through analyzing the transcriptional profile of HBZ-expressing cells, we learned that HBZ suppressed transcription of the proapoptotic gene *Bim* (*Bcl2l11*) and that HBZ-expressing cells were resistant to activation-induced apoptosis. Mechanistic investigations into how HBZ suppresses *Bim* expression revealed that HBZ perturbs the localization and function of FoxO3a, a critical transcriptional activator of the genes encoding Bim and also Fas ligand (FasL). By interacting with FoxO3a, HBZ not only attenuated DNA binding by FoxO3a but also sequestered the inactive form of FoxO3a in the nucleus. In a similar manner, HBZ also inhibited *FasL* transcription induced by T-cell activation. Further study of ATL cells identified other Bim perturbations by HBZ, including at the level of epigenetic alteration, histone modification in the promoter region of the Bim gene. Collectively, our results indicated that HBZ impairs transcription of the *Bim* and *FasL* genes by disrupting FoxO3a function, broadening understanding of how HBZ acts to promote proliferation of HTLV-1–infected T cells by blocking their apoptosis. *Cancer Res; 74(1); 188–200. ©2013 AACR.*

## Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is estimated to infect 10 to 20 million people in the world (1). This virus causes not only a neoplastic disease of $CD4^+$ T cells, adult T-cell leukemia (ATL), but also chronic inflammatory diseases of the central nervous system, lung, or skin (2). HTLV-1 can be transmitted efficiently in a cell-to-cell fashion (3, 4), whereas free virus shows poor infectivity (5, 6), and virions are not detected in infected individuals. To increase the number of infected cells and facilitate transmission, HTLV-1 increases its copy number primarily by triggering the proliferation of infected cells, replicating within the host genome instead of undergoing viral replication (7, 8). Thus, HTLV-1 promotes proliferation and suppresses apoptosis of infected cells via complex interactions of viral proteins with host factors.

Among the viral genes encoded in HTLV-1, the *tax* gene has been extensively studied. Tax can activate various signal pathways like NF-κB, AP-1, and SRF (9). However, Tax expression is frequently undetectable in ATL cases. Importantly, the nonsense mutations in the *tax* gene are often observed in not only ATL cases but also infected cells of asymptomatic HTLV-1 carriers (10). These findings suggest that other mechanisms suppress the apoptosis of HTLV-1–infected cells in the absence of Tax expression (2). We have reported that the *HTLV-1 bZIP factor (HBZ)* gene is expressed in all ATL cases (11). Furthermore, HBZ promotes the proliferation of T cells and induces development of T-cell lymphomas and inflammatory diseases in transgenic mice (12). Therefore, we speculated that HBZ might also influence apoptosis.

There are two major pathways for apoptosis: the extrinsic and intrinsic apoptotic pathways, which are mediated by Fas and Bim, respectively (13). ATL cells are known to express high levels of Fas antigen, and are susceptible to Fas-mediated signaling (14). However, FasL expression is suppressed in ATL cells by silencing of the *early growth response 3* (*EGR3*) gene transcription, a phenomenon that enables ATL cells to escape activation-induced cell death (15). In addition, Tax increases expression of c-FLIP, which confers resistance to Fas-mediated apoptosis (16, 17). Furthermore, activation of NF-κB by Tax also enables HTLV-1–infected cells to be resistant to apoptosis (18). To date, the effects of HTLV-1 infection on Bim-mediated apoptosis remain unknown.

In this study, we analyzed transcriptional changes induced by HBZ expression in T cells, and found that transcription of a proapoptotic gene, *Bim*, was hindered by HBZ. This suppression led to decreased activation-induced cell death. We found that HBZ suppressed *Bim* transcription by targeting FoxO3a, a critical transcription factor for the *Bim* and *FasL* gene. In some

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ATL cell lines and ATL cases, the *Bim* gene transcription was also silenced by epigenetic mechanisms, but this phenomenon seemed to be secondary to HBZ-mediated suppression of transcription. Thus, it is suggested that HBZ suppresses both intrinsic and extrinsic apoptotic pathways and contributes to the proliferation of ATL cells.

## Materials and Methods

### Cell lines and clinical samples

HTLV-1 immortalized cell lines (MT-4), ATL cell lines (ED, TL-Om1, and MT-1), T-cell lines not infected with HTLV-1 (Jurkat, SupT1, and CCRF-CEM) were cultured in RPMI 1640 medium supplemented with 10% FBS and antibiotics at 37°C under a 5% $CO_2$ atmosphere. Jurkat cells stably expressing spliced form of HBZ (sHBZ), Jurkat-HBZ, were maintained as described previously (19). To construct CCRF-CEM cells stably expressing HBZ, CEM-HBZ, the coding sequence of HBZ was subcloned into pME18Sneo vector and then the expression vector or its empty vector were transfected into CCRF-CEM cells by using Neon (Invitrogen) according to the manufacturer's instructions. Stable transfectants were selected in G418 (1 mg/mL). 293T cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and antibiotics and when 293FT cells were cultured, 500 μg/mL G418 was added. Fas-blocking antibody was purchased from Alexis.

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of Kyoto University (G204). All patients provided written informed consent for the collection of samples and subsequent analysis.

### Plasmid constructs

Wild-type form of FoxO3a was generated by PCR amplification using Jurkat cDNA library and constitutively active form of FoxO3a (FoxO3aAAA) was also generated by PCR amplification with mutated primers (20). These PCR fragments were then subcloned into pCMV-Tag2B vector and pIRES-hrGFP-1a (Stratagene). The vectors encoding the myc-His-tagged form of HBZ and its mutants used in this study have been described previously (19, 21). We modified pLKO.1-EGFP vector for delivery of anti-FoxO3 short hairpin RNAs (shRNA) to Jurkat, Jurkat-control, and Jurkat-HBZ. shRNA sequence used was 5′-GCACAACCTGTCCTGCATAG-3′. The 6xDBE-Luc construct that contains six FOXO-binding sites known as DAF-16 binding elements (DBE) was kindly provided by Dr. Furuyama (Kagawa Prefectural University of Health Sciences, Kagawa, Japan) and the backbone of this vector was pGL3-basic (Promega; ref. 22).

### Luciferase assay

Jurkat cells were transfected with 0.2 μg/well of luciferase reporter plasmid, 1 ng/well of *Renilla* luciferase control vector (phRL-TK), 0.2 μg/well of FoxO3aAAA expression plasmid or its empty vector, and 0.6 μg/well of HBZ expression plasmid or its empty vector with caspase inhibitor Z-VAD-FMK (MBL). Plasmids were transfected using Neon (Invitrogen) according to the manufacturer's instructions. After 24 hours, cells were collected and luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega). Relative luciferase activity was calculated as the ratio of firefly to *Renilla* luciferase activity. Three independent experiments, each with triplicate transfections, were performed and typical results are shown.

### Microarray analysis

Jurkat-control and Jurkat-HBZ were stimulated with phorbol myristate acetate (PMA; 50 ng/mL) and ionomycin (Io; 1 μg/mL) for 9 hours. After the stimulation, cells were collected and total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. We then digested DNA using deoxyribonuclease I (Invitrogen) and cleaned up RNA using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. We then synthesized cDNA and performed microarray processing according to the GeneChip Expression Analysis Technical Manual (Affymetrix). All data were analyzed by using GeneSpring GX (Agilent Technologies). The microarray data related to this article have been submitted to the Gene Expression Omnibus under the accession number GSE48029.

### Immunofluorescence analysis

293FT cells were transfected with expression vectors using Lipofectamine LTX (Invitrogen) or TransIT (TaKaRa). Twenty-four hours after transfection, cells were reseeded on the poly-L-lysine–coated glass (Matsunami Glass Ind., Ltd.) or poly-D-lysine (Sigma)–coated glass. Twenty-four hours after the reseeding, cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.2% Triton X-100 for 15 minutes, and blocked by incubation in 5% BSA/PBS for 30 minutes. For immunostaining, the cells were incubated with anti-Foxo3a, anti-p-Foxo3a (Cell Signaling Technology), Cy3-conjugated anti-c-Myc (Sigma) or biotinylated anti-FLAG (Sigma) antibodies for 1 hour or in case of observation of endogenous expression, cells were incubated overnight at 4°C. Primary antibodies were visualized by incubating the cells with Alexa-Fluor 488–conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (Invitrogen) or AlexaFluor 488–conjugated streptavidin (Invitrogen). Nuclei were stained and mounted with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). To concentrate nonadherent cells onto a microscope slide, CytoFuge (StatSpin) was used. Fixation and blocking were performed as described earlier.

### Assessment of apoptosis

Apoptotic cells were routinely identified by Annexin V-APC (eBioscience) or phycoerythrin (PE) or fluorescein isothiocyanate (FITC; BioVision) -staining according to the manufacturer's instructions and analyzed with a flow cytometer (BD FACSCanto II; BD Biosciences). Data files were analyzed by using FlowJo software (TreeStar).

### Real-time PCR

Total RNA was isolated for the analysis using TRIzol reagent. RNA was treated with DNase I to eliminate the genomic DNA. Reverse transcription was performed using random primer and SuperScript III Reverse Transcriptase (Invitrogen). $CD25^-$ $CD4^+$ cells from healthy donor were obtained by using human

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CD4 T Lymphocyte Enrichment kit (BD Pharmingen). Then, cells were stimulated with PMA/Io for 9 hours, RNA was isolated, and reverse transcription was performed as described earlier. cDNA products were analyzed by real-time PCR using the Taqman Universal PCR Master Mix (PE Applied Biosystems) or FastStart Universal SYBR Green Master (Roche) and Applied Biosystems StepOnePlus Real-Time PCR System according to the manufacturer's instructions. Specific primers and Taqman probes for the *Bim* gene, *FasL* gene, and *GAPDH* internal control gene were purchased from Applied Biosystems. Primer sequences for the *HBZ* gene and *GAPDH* gene used for the evaluation of the knockdown efficiency in MT-1 cells have been described previously (11, 23). Primer sequences for the *HBZ* gene used for another experiment to evaluate the HBZ expression in Jurkat-HBZ, MT-1, TL-Om1, and ED cells were 5′-ATGGCGGCCTCAGGGCTGTT-3′ and 5′-GCGGCTTTCCTCTTCTAAGG-3′. Primer sequences for the *FoxO3a* gene used were 5′-ACAAACGGCTCACTCTGTCCCAG-3′ and 5′-AGCTCTTGCCAGTTCCCTCATTCTG-3′. All amplifications were conducted in triplicates. The relative quantification was calculated according to the method described in Applied Biosystems ABI prism 7700 SDS User Bulletin #2.

**Chromatin immunoprecipitation analysis**

Chromatin immunoprecipitation (ChIP) assay was performed according to the protocol recommended by Millipore. Cells were fixed with 1% formaldehyde for 10 minutes at room temperature, washed twice with ice-cold PBS, treated with SDS-lysis buffer (1% SDS, 50 mmol/L EDTA, and 200 mmol/L Tris-HCl) for 10 minutes on ice and then sonicated. Thereafter, the DNA/protein complexes were immunoprecipitated with antibodies specific for acetylated-Histone H3, acetylated-Histone H4, dimethylated-Histone H3 (Lys4), RNA polymerase II clone CTD4H8 (Millipore), trimethylated-Histone H3 (Lys27), anti-trimethyl-Histone H3 (Lys9) antibodies (Cell Signaling Technology), or normal rabbit IgG (Santa Cruz Biotechnology) overnight at 4°C. Immune complexes were collected with salmon sperm DNA-protein A and G Sepharose slurry, washed, and eluted with freshly prepared elution buffer (1% SDS, 100 mmol/L $NaHCO_3$). Protein-DNA complexes were de-cross-linked at 65°C for 4 hours. DNA was purified and subjected to real-time PCR for quantification of the target fragments. Sequences for the primer set are described previously (24, 25). For the evaluation of binding of FoxO3a to the FOXO-binding sites, 293T cells were transfected with 5 μg of 6xDBE-Luc construct, 5 μg of FoxO3aAAA expression plasmid together with or without 5 μg of HBZ plasmid using TransIT in 10-cm dishes. Anti-FLAG (Sigma) antibody was used for the immunoprecipitation. Primers used were 5′-AGTGCAGGTGCCAGAACATT-3′ and 5′-GCCTTATGCAGTTGCTCTCC-3′, which were constructed inside of the pGL3-basic vector. For the evaluation of the DNA-binding capacity of FoxO3a with or without HBZ, expression vectors for the HA-tagged FoxO3a and Flag-tagged HBZ were transiently cotransfected into 293T cells using the TransIT reagent. Twenty-four hours after the transfection, cells were collected and chromatin immunoprecipitation assay was performed as described earlier. For the immunoprecipitation, anti-HA (Sigma) antibody was used. Primers used for *Bim* gene promoter were 5′-CCACCACTTGATTCTTGCAG-3′ and 5′-TCCAGCGCTAGTCTTCCTTC-3′, which were constructed to contain the FOXO-binding sequence located in intron1. Primers used for *FasL* gene promoter were 5′-ACGATAGCACCACTGCACTCC-3′ and 5′-GGCTGCAAACCAGTGGAAC-3′, which were also constructed to contain the three FOXO-binding sequences.

Individual PCRs were carried out in triplicate to control for PCR variation and mean $C_t$ values were collected. Fold difference of the antibody-bound fraction (IP) versus a fixed amount of input (In) was calculated as

$$\mathrm{IP/In} = 2^{-\Delta\Delta C_t} = 2^{-\{C_t(\mathrm{IP}) - C_t(\mathrm{In})\}}$$

Then, the fold difference value for a target antibody (t) was subtracted by the nonspecific value derived from mouse or rabbit IgG ($t_0$):

$$(\mathrm{IP/In})^t - (\mathrm{IP/In})^{t_0}$$

**Bisulfite genomic sequencing**

Sodium bisulfite treatment of genomic DNA was performed as described previously (26). DNA regions were amplified using bisulfite-treated genomic DNA by nested PCR. To amplify promoter region (promoter 1) of *Bim*, primers used in the first PCR were 5′-TTTAGAGGGAGGAGAGTTTAAAG-3′ and 5′-CCCTACAACCCAACTCTAACTA-3′. Primers for the second PCR were 5′-AGGGTATAGTGAGAGCGTAGG-3′ and 5′-CAACTCTAACTAACGACCCC-3′. For promoter, two primers used in the first PCR were 5′-GTGTGATTGTTTTTTGAGGG-3′ and 5′-AAAATACCCCCAAACAAAATAC-3′. Primers for the second PCR were 5′-GCGGATTTAGTTGTAGATTTTG-3′ and 5′-ACTCTTTACCCAAAACAAACTTC-3′. PCR products were purified, cloned into pGEM-T Easy vector (Promega), and sequenced using the ABI PRISM 3130 Genetic Analyzer. For CpG methylation analysis, Web-based bisulfite sequencing analysis tool called QUMA (quantification tool for methylation analysis) was used (27).

**Coimmunoprecipitation assay, analysis of the p-FoxO3a localization, and immunoblotting**

Expression vectors for the relevant genes were transiently cotransfected into 293T cells using the TransIT reagent. Forty-eight hours later, cells were collected and coimmunoprecipitation assays were performed as described previously (28). For the analysis of the p-FoxO3a localization, nuclear and cytoplasmic proteins were extracted using Nuclear Complex Co-IP Kit (Active Motif). The proteins were subjected to SDS-PAGE analysis followed by immunoblotting with various antibodies. Antibodies used were anti-p-FoxO3a, anti-α-tubulin (Sigma), anti-FLAG, anti-HA (Sigma), and anti-His (Marine Biological Laboratory).

**Lentiviral vector construction and transfection of the recombinant lentivirus**

Lentiviral vector expressing shRNA against HBZ was constructed and recombinant lentivirus was infected as described previously (11). When more than 90% of cells expressed

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enhanced green fluorescent protein (EGFP), the *HBZ* and *Bim* gene expressions were analyzed by real-time PCR.

## Results

### The *Bim* gene transcription is suppressed in HBZ-expressing Jurkat and CCRF-CEM cells

To determine the effects of HBZ on gene expression, we first performed microarray analysis. Jurkat cells with or without expression of spliced form of HBZ (Jurkat-HBZ and Jurkat-control, respectively) were stimulated with PMA and Io for 9 hours. Gene expression profiles were then analyzed by DNA microarray. Table 1 shows the apoptosis-associated genes that were downregulated or upregulated in stimulated Jurkat-HBZ cells. Transcription of the *Bim* gene was prominently downregulated in HBZ-expressing Jurkat cells. To confirm the effect of HBZ on the *Bim* gene expression, we evaluated *Bim* mRNA levels in Jurkat-control and Jurkat-HBZ cells with or without PMA/Io stimulation using real-time PCR. As reported in the previous studies showing that treatment by PMA/Io or other stimulators induced *Bim* expression (29, 30), the *Bim* mRNA level of stimulated Jurkat-control cells was three-times higher than that of unstimulated cells, but that of Jurkat-HBZ cells did not change after stimulation (Fig. 1A). Similarly, increased *Bim* transcription by stimulation was also inhibited by HBZ in CCRF-CEM cells (Fig. 1A).

### HBZ inhibits apoptosis

It has been reported that Bim plays an important role in activation-induced cell death and T-cell homeostasis (31). Because the earlier data demonstrated that HBZ inhibits stimulation-induced *Bim* expression, we next investigated whether HBZ inhibits apoptosis in response to PMA/Io stimulation. To test this, Jurkat-control and Jurkat-HBZ were each incubated with or without PMA/Io for 9 hours, and then apoptosis was measured using Annexin V. The percentages of apoptotic cells in Jurkat-control and Jurkat-HBZ were 40.2% and 15% respectively, indicating that HBZ suppressed activation-induced apoptosis (Fig. 1B). We also treated cells with doxorubicin and found that HBZ slightly inhibited doxorubicin-induced apoptosis (Supplementary Fig. S1). Fas-mediated apoptotic pathway might be involved in antiapoptotic effect by HBZ. To assess the effect of Fas-mediated signaling on the activation-induced apoptosis, cells were also treated with or without Fas-blocking antibody (0.5 μg/mL) 30 minutes before the PMA/Io stimulation. The percentage of apoptotic cells without Fas-blocking antibody in Jurkat-control and Jurkat-HBZ were 36.9% and 22.4%, respectively. When cells were treated with Fas-blocking antibody, the percentage of apoptotic cells reduced and those were 24% and 13.2% in Jurkat-control and Jurkat-HBZ, respectively (Fig. 1C). Thus, Fas-blocking antibody partially inhibited apoptosis in Jurkat-HBZ, which indicates that Fas-mediated signals are also implicated in activation-induced cell death. Indeed, we found that the transcription level of *FasL* was suppressed in stimulated Jurkat-HBZ and CEM-HBZ cells compared with Jurkat-control and CEM-control cells (Fig. 1D), suggesting that downregulation of FasL by HBZ was also associated with inhibition of apoptosis.

**Table 1.** Apoptosis-associated genes that are upregulated or downregulated by HBZ

| Gene | Fold change | Gene ontology |
|---|---|---|
| API5 | 2.18 | Antiapoptosis |
| BCL2L11 (Bim) | −9.93 | Induction of apoptosis |
| CARD11 | 2.87 | Regulation of apoptosis |
| CASP1 | 2.97 | Apoptosis |
| CD28 | 4.60 | Positive regulation of antiapoptosis |
| COP1 | 9.41 | Regulation of apoptosis |
| DEDD2 | 2.01 | Induction of apoptosis via death domain receptors |
| DYRK2 | 2.16 | Induction of apoptosis |
| GZMB | −5.90 | Apoptosis |
| HIPK2 | 2.19 | Induction of apoptosis by intracellular signals |
| NLRP1 | 3.08 | Induction of apoptosis |
| PI3KR2 | −2.68 | Negative regulation of antiapoptosis |
| PLEKHF1 | 2.99 | Induction of apoptosis |
| PRDX2 | −2.10 | Antiapoptosis |
| PRF1 | 3.95 | Virus-infected cell apoptosis |
| RFFL | 2.20 | Apoptosis |
| SPHK1 | −4.26 | Antiapoptosis |
| TNFRSF9 | −2.61 | Induction of apoptosis |
| TP53INP1 | 2.32 | Apoptosis |
| VEGFA | −6.96 | Negative regulation of apoptosis |

NOTE: The table shows a list of apoptosis-associated genes that were downregulated or upregulated (by more than 2-fold) in stimulated Jurkat-HBZ cells identified by microarray analysis.

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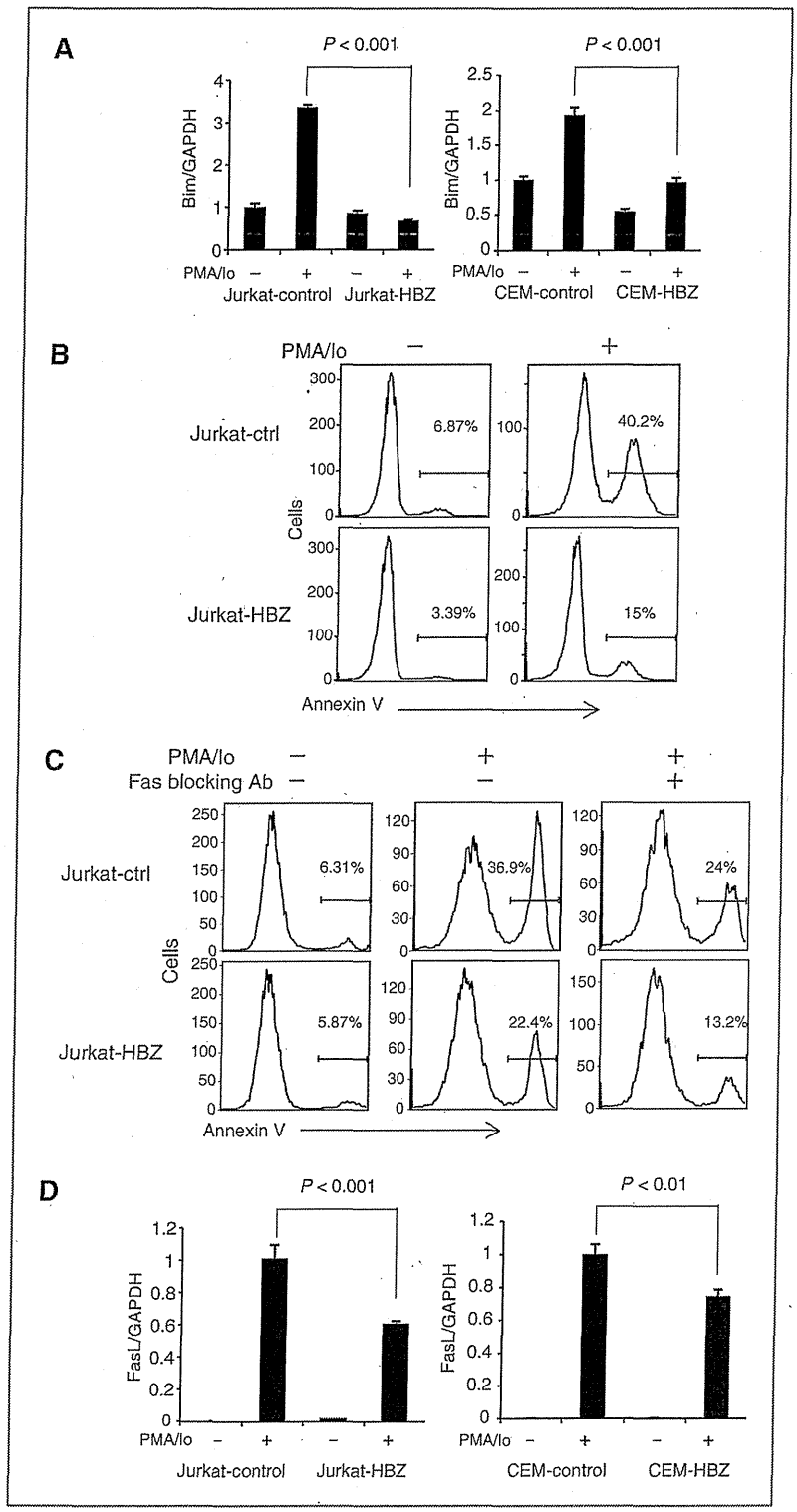


Figure 1. HBZ suppresses the transcription of the *Bim* and *FasL* genes and consequently stimulation-induced apoptosis. A, comparison of the *Bim* mRNA expression in the Jurkat-control, Jurkat-HBZ, CEM-control, and CEM-HBZ cells with or without PMA/Io stimulation by real-time PCR. B, Jurkat-control and Jurkat-HBZ were stimulated with PMA/Io for 9 hours and stained with Annexin V. Percentage of apoptotic cells was determined by flow cytometry. C, Jurkat-control and Jurkat-HBZ were treated with *Fas-blocking antibody for 30 minutes and* then stimulated with PMA/Io for 9 hours. Percentages of apoptotic cells were monitored by flow cytometry. D, comparison of the *FasL* mRNA transcription in the Jurkat-control, Jurkat-HBZ, CEM-control, and CEM-HBZ cells with or without PMA/Io stimulation by real-time PCR. Error bars, standard deviation. Statistical differences are calculated by Student *t* test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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**HBZ suppresses *Bim* expression through attenuation of FoxO3a**

We analyzed how HBZ suppresses the expression of *Bim* and *FasL*. It has been reported that a forkhead factor, FoxO3a, and p73 are important for the transcription of *Bim* and *FasL* (32, 33). FoxO3a and other FOXO family members are phosphorylated by protein kinases such as Akt or SGK on highly conserved serine and threonine residues (especially Thr32, Ser253, and Ser315 in FoxO3a), resulting in impaired DNA-binding activity and increased binding to the chaperone protein, 14-3-3 (20, 34, 35). Newly formed 14-3-3-FOXO complexes are then exported from the nucleus, thereby inhibiting FOXO-dependent transcription of key target genes such as *Bim*, *FasL*, and *TRAIL* (36).

First, we investigated whether FoxO3a is implicated for the activation induced cell death. As shown in Supplementary Fig. S2, the knockdown of FoxO3a resulted in the decreased apoptotic rate in Jurkat-control cells ($P < 0.05$). Furthermore, inhibition of Foxo3a did not influence activation-induced cell death in Jurkat-HBZ cells, suggesting the inhibitory effect of HBZ on Foxo3a function. To investigate whether HBZ affects FoxO3a function, Jurkat cells were transiently transfected with a plasmid expressing FoxO3aAAA, the constitutive active mutant of FoxO3a, which is no longer phosphorylated by Akt and is localized in the nucleus. The FoxO3aAAA was expressed together with hrGFP using an internal ribosome entry site (IRES; FoxO3aAAA-IREShrGFP). Jurkat cells were transiently transfected with full-length HBZ or its mutants. HBZ has three domains, an activation domain (AD), a central domain (CD), and a basic leucine zipper domain (bZIP; ref. 12). In this study, the deletion mutants (HBZ-ΔAD, HBZ-ΔbZIP, and HBZ-ΔCD) were used. The percentage of FoxO3aAAA induced apoptotic cells in the absence of HBZ was 69.6% while it was suppressed by HBZ (40.6%; $P < 0.001$; Fig. 2A). We also found that an HBZ mutant without activation domain lacks the activity to inhibit FoxO3aAAA-induced apoptosis (Fig. 2A), indicating the significance of activation domain in suppression of FoxO3a-mediated apoptosis. It has been reported that LXXLL motif in FoxO3a binds to its coactivator CBP/p300 (37). Similarly, HBZ has LXXLL-like motifs located in the $NH_2$-terminal region, which bind to KIX domain of CBP/p300 (38). We speculated that the LXXLL-like motifs of HBZ might affect FoxO3aAAA function through KIX domain of CBP/p300. An HBZ mutant, which has substitutions in 27th and 28th residues (LL to AA) of LXXLL-like motif, lack the activity to suppress FoxO3aAAA-mediated apoptosis (Fig. 2B), indicating that LXXLL-like motif of HBZ is critical for suppression of FoxO3a-mediated apoptosis.

Next, we analyzed the effect of HBZ on a FoxO3a responsive reporter. As shown in Fig. 2C, HBZ suppressed FoxO3a-mediated transcriptional activity ($P < 0.01$). To check whether HBZ inhibits DNA binding of FoxO3a, 293T cells were transiently transfected with FoxO3aAAA and FoxO3a reporter, 6xDBE-Luc, together with or without HBZ. The interaction of FoxO3aAAA to FOXO-binding sites was analyzed by ChIP assay. As shown in Figure 2D, the interaction of FoxO3aAAA to the FOXO-binding sites was interfered by HBZ, suggesting that HBZ inhibits FoxO3a-mediated apoptosis through suppression of the DNA binding of FoxO3a. To clarify the mechanism of HBZ-mediated FoxO3a inhibition, we examined interaction between HBZ and FoxO3a by the immunoprecipitation assay. It showed that HBZ interacted with FoxO3a (Fig. 2E and F). Experiments with FoxO3a deletion mutant revealed that HBZ interacted with the forkhead domain of FoxO3a (Fig. 2E). Analysis using HBZ deletion mutants showed that the central domain of HBZ interacted with FoxO3a (Fig. 2F).

**HBZ inhibits nuclear export of phosphorylated form of FoxO3a**

Next, we investigated the effect of HBZ on FoxO3a localization by confocal microscopy. We cotransfected 293FT cells with a plasmid expressing human wild-type FoxO3a (FoxO3aWT) protein and an HBZ-expressing plasmid. Consistent with previous reports, FoxO3a remained mainly in cytoplasm when cells were cotransfected with empty vector (Fig. 3A; refs. 20, 34). However, when it was expressed along with HBZ, FoxO3a was localized in both nucleus and cytoplasm (Fig. 3A). To determine whether mislocalized FoxO3a is phosphorylated (pFoxO3a) or not, we used anti-pFoxO3a antibody. Figure 3B and C demonstrated that nuclear-localized FoxO3a was phosphorylated in HBZ-expressing cells. Thereafter, we analyzed the localization of endogenous FoxO3a in HeLa and an ATL cell line, MT-1. Although pFoxO3a was localized widely both in cytoplasm and nucleus in HeLa cells, most pFoxO3a was localized in the nucleus in MT-1 (Fig. 3D), suggesting that endogenous HBZ inhibits the extranuclear translocation of pFoxO3a in this cell line. From the study of crystal structure of the human FoxO3a-DBD/DNA complex, it has been reported that phosphorylation at Ser253 causes a decrease on the DNA-binding ability (39). Abnormal localization of phosphorylated FoxO3a by HBZ might interfere the function of unphosphorylated FoxO3a in the nucleus. The abnormal localization of pFoxO3a prompted us to investigate whether HBZ bound to 14-3-3 along with FoxO3a, as 14-3-3 is a chaperon protein involved in nuclear–cytoplasm shuttling of FOXO family. As shown in Figure 3E, HBZ, FoxO3a, and 14-3-3 form a ternary complex. However, the binding of FoxO3a and 14-3-3 was not affected by HBZ (result of IP with anti-FLAG antibody and detected with anti-HA antibody).

As another possible mechanism for downregulation of *Bim* and *FasL*, we compared the transcription level of *p73* in Jurkat cells with and without HBZ expression. Activation of HBZ-expressing cells reduced transcription of *p73*, but the expression level of *p73* was variable among ATL cell lines (Supplementary Fig. S3A and S3B). We conclude that p73 is not responsible for suppression of *Bim* expression in ATL cells.

**Bim expression is suppressed in both ATL cell lines and ATL cases**

HBZ has been shown to suppress Bim expression through two different mechanisms as revealed in this study. To analyze Bim expression in ATL cells, we studied *Bim* mRNA levels in non-ATL cell lines and ATL cell lines with or without PMA/Io stimulation, and found that the *Bim* gene transcript was upregulated in Jurkat and CCRF-CEM cells, but not in SupT1 after activation. However, *Bim* transcripts were not increased

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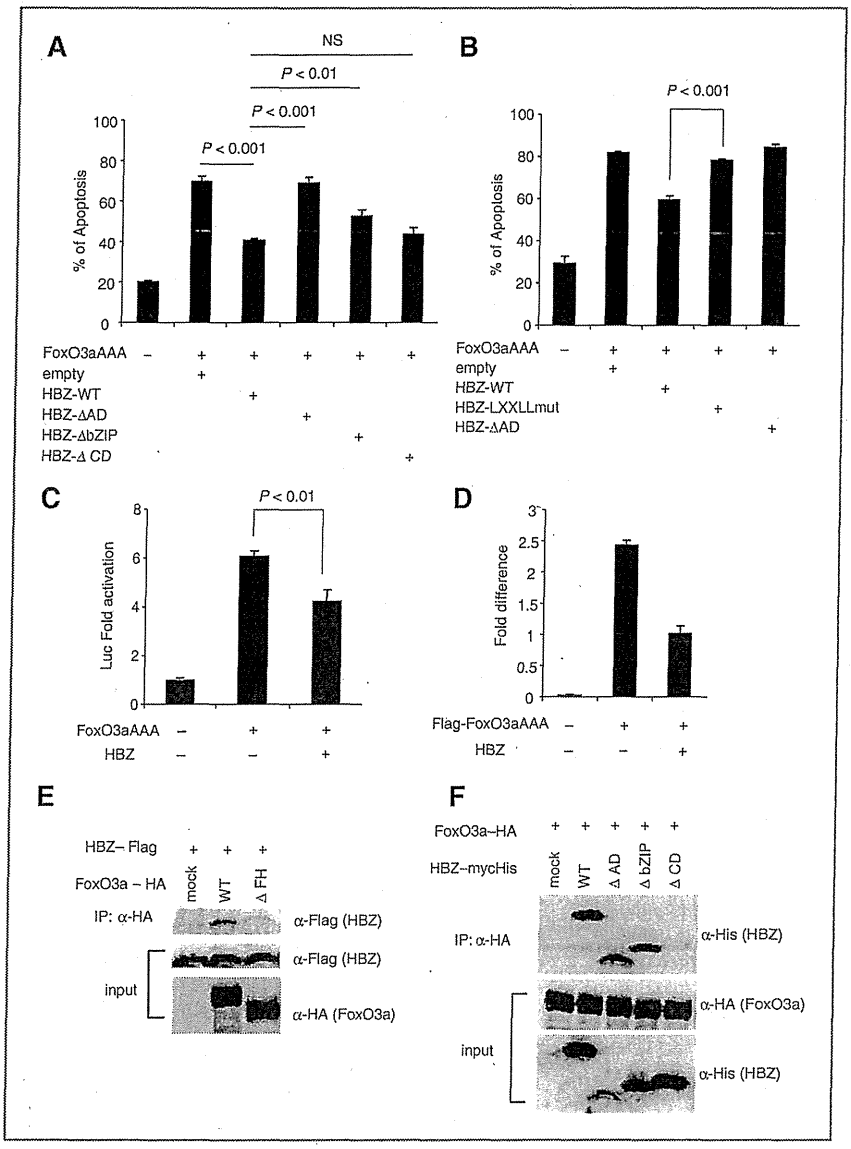


Figure 2. HBZ attenuates function of Foxo3a by physical interaction. A, Jurkat cells were transfected with FoxO3aAAA-expressing vector, a constitutively active form, by using Neon with or without HBZ or its mutants. Twenty-four hours after transfection, cells were stained with Annexin V and analyzed by flow cytometry ($n = 3$). B, Jurkat cells were transfected with FoxO3aAAA-expressing vector together with HBZ or its mutants by using Neon. Cells were stained with Annexin V and analyzed by flow cytometry ($n = 3$). Data are representative of three independent experiments. C, reporter construct containing the 6xDBE and FoxO3aAAA-expressing vector was transiently transfected with or without HBZ into Jurkat cells in the presence of Z-VAD-FMK and luciferase activities were measured. D, 293T cells were transfected with 6xDBE-Luc construct and Flag-tagged FoxO3aAAA expression vector together with or without HBZ expression vector. Cells were immunoprecipitated with anti-FLAG antibody and quantified by real-time PCR. Three independent ChIP experiments were done and representative data are shown. Error bars, experimental variation. E and F, the expression vectors of the indicated proteins were cotransfected into 293T cells, and their interactions were analyzed by immunoprecipitation assay. Data are representative of three independent experiments. *Statistical differences are* calculated by Student *t* test.

in all stimulated ATL cell lines (Fig. 4A). The *Bim* gene transcript was also downregulated in primary ATL cells (Fig. 4B) compared with resting peripheral blood mononuclear cells (PBMC) and phytohemagglutinin (PHA)-stimulated T cells. We also stimulated primary ATL cells and normal $CD4^+$ T cells with PMA/Io. The *Bim* gene transcription was quite low in primary ATL sample compared with normal $CD4^+$ T cells even though the cells were stimulated with PMA/Io (Fig. 4C). To confirm HBZ expression in representative ATL cell lines, we quantified the level of the *HBZ* mRNA transcription in Jurkat-HBZ, CEM-HBZ, MT-1, ED, and TL-Om1 by real-time PCR and confirmed that HBZ is expressed in these ATL cell lines (Supplementary Fig. S4). Microarray data, obtained from Gene Expression Omnibus (GEO), show that both *Bim* and *FasL* transcription levels are lower in ATL cases than healthy donors (accession number: GSE33615; Supplementary Fig. S5), supporting our data that *Bim* expression was suppressed in ATL cells.

### *Bim* expression is silenced by epigenetic mechanisms

Because the *Bim* gene transcription was severely suppressed in ATL cells, we investigated the epigenetic status (DNA methylation and histone modification) of the promoter region of the *Bim* gene in ATL cells. A previous study showed that the 0.8 kb region immediately upstream of exon 1 contains the important elements for the control of *Bim* expression

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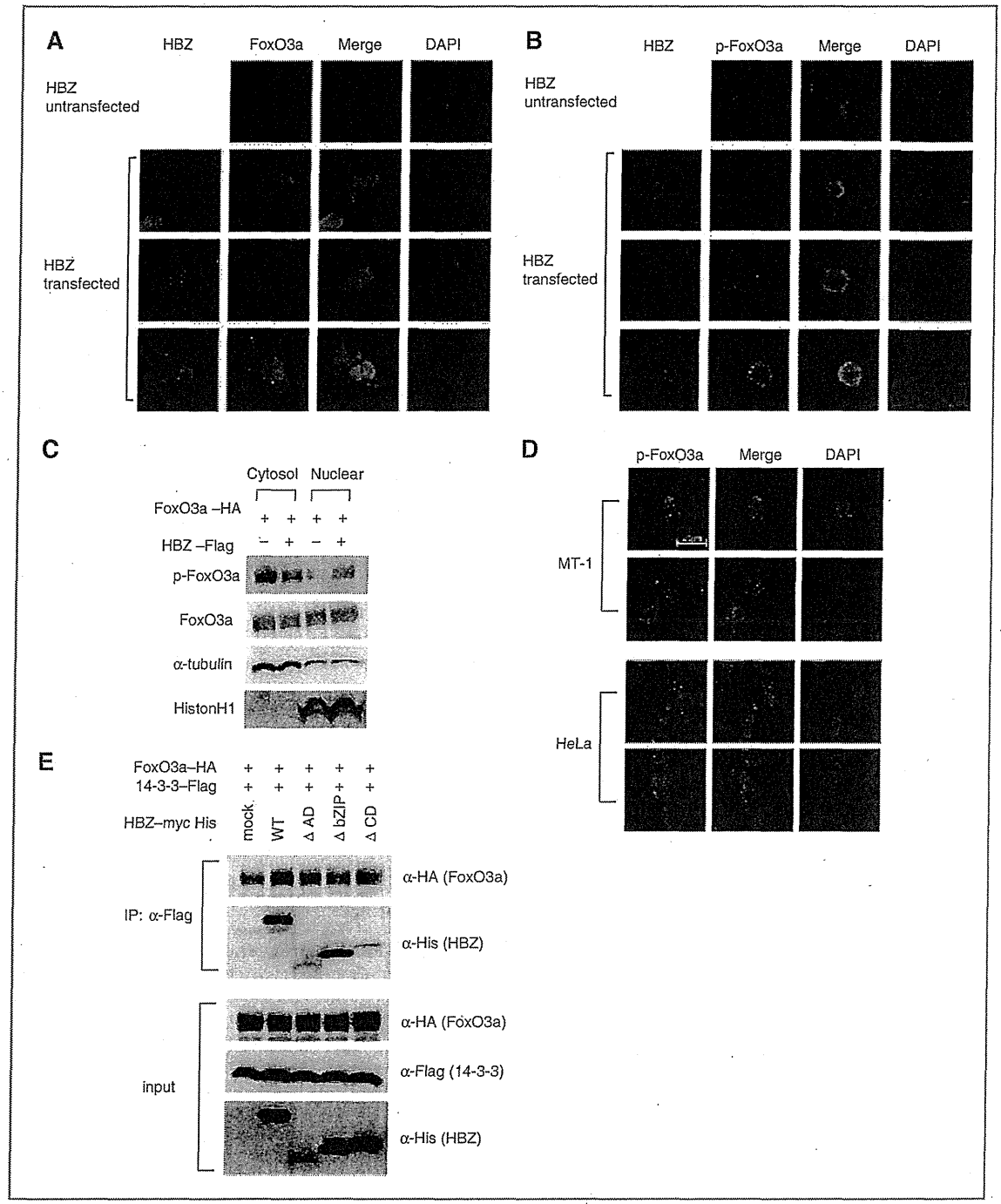


Figure 3. HBZ interferes with normal localization of FoxO3a by forming a ternary complex with FoxO3a and 14-3-3. 293FT cells were transfected with FoxO3aWT-Flag together with or without mycHis-HBZ. A and B, FoxO3a was detected using anti-FLAG-biotin and secondary Streptavidin-Alexa 488 (A), and p-FoxO3a was detected using anti-p-FoxO3a (ser253) and secondary anti-rabbit IgG-Alexa 488 antibody (B). DAPI was used to counterstain the nucleus. C, 293FT cells were transfected with HA-tagged FoxO3aWT together with or without Flag-tagged HBZ. Cytoplasmatic and nuclear fractions were extracted and p-FoxO3a was detected by Western-blotting. D, endogenous localizations of p-FoxO3a (ser253) in HeLa and MT-1 cells were examined using anti-p-FoxO3a. E, the interactions among HBZ, FoxO3a, and 14-3-3 were analyzed by immunoprecipitation.

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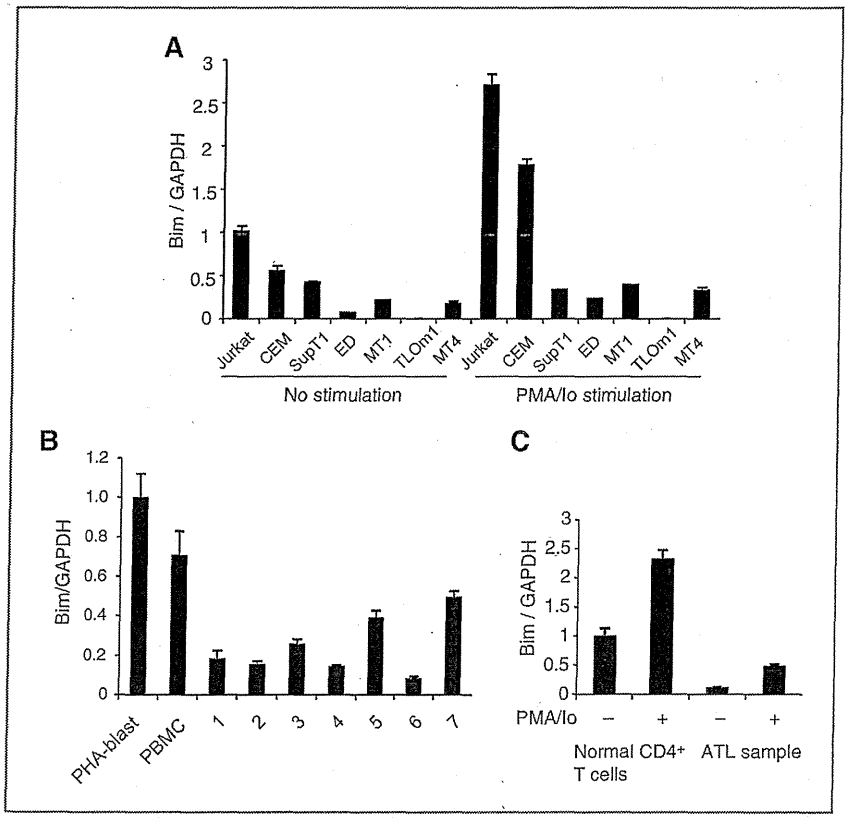


Figure 4. Bim expression is also suppressed in ATL cell lines and ATL cases. Comparison of the *Bim* mRNA expression in non-ATL cell lines and ATL cell lines with or without PMA/Io stimulation (A) and in PBMCs and PHA-blasts from healthy donor samples and fresh ATL samples (B) by real-time PCR. C, comparison of the *Bim* mRNA expression in healthy donor sample and ATL fresh sample with or without PMA/Io stimulation.

(promoter 1). The *Bim* promoter does not contain a TATA or CAAT box and has the characteristics of a "TATA-less" promoter (40). In addition, the alternative promoter has been reported to exist in intron 1 (promoter 2; refs. 41, 42). These two promoter regions are highly GC-rich and contain the binding sites for several transcription factors, including FoxO3a. To determine whether CpG sites in these *Bim* gene promoter regions are methylated in ATL cell lines, their methylation status was analyzed by bisulfite-mediated methylcytosine mapping (Supplementary Fig. S6A and S6B). The promoter 1 of *Bim* was hypermethylated in two ATL cell lines (ED and TL-Om1) and ATL case 1, whereas this region was not so methylated in MT-1 cells and two ATL cases. On the other hand, the promoter 2 was heavily methylated in two ATL cell lines (TL-Om1 and MT-1) and ATL case 1 and partially methylated in Jurkat cells (Supplementary Fig. S6B). These results suggest that in some cases, heavily methylated CpG sites of promoter 1 and 2 are associated with silencing of *Bim* transcription but these methylations can not account for suppressed *Bim* expression in all ATL cell lines and ATL cases.

Therefore, we next focused on the histone modification in the promoter region of *Bim*. It is well known that deacetylation of the histones are also common features of cancer, which results in transcriptional silencing of tumor suppressor genes (43). First, we analyzed the histone H3 and H4 acetylation and H3K4 trimethylation, which are all permissive marks (44), in promoter 1 of Jurkat, MT-1, and TL-Om1 cells. Contrary to our speculation, neither H3, H4 acetylation, nor H3K4 trimethylation differed between MT-1 and Jurkat cells (Supplementary Fig. S7). We next analyzed the histone modification status in promoter 2. As shown in Figure 5A, MT-1 and TL-Om1 cells exhibited decreased level of histone H3 acetylation and H3K4 trimethylation but not histone H4 acetylation. Because methylation of DNA is often preceded by dimethylation of H3K9 or trimethylation of H3K27 (both repressive marks) in oncogenesis (44), we asked whether there were differences in these epigenetic chromatin marks on the *Bim* gene promoter in ATL cell lines. TL-Om1 cells exhibited upregulated level of H3K9 dimethylation and H3K27 trimethylation compared with Jurkat cells (Fig. 5B and C), whereas MT-1 exhibited a little upregulated level of H3K27 trimethylation (Fig. 5C) in the promoter 2. These data suggest that histone modifications of promoter 2 are critical for the suppressed *Bim* gene transcription. We also performed ChIP analysis using anti-RNA polymerase II antibody (Fig. 5D) and revealed that Pol II binding was decreased in MT-1 and TL-Om1 cells, confirming suppressed transcription of the *Bim* gene. To further investigate the mechanisms involved in FoxO3a-mediated *Bim* gene transcription in the promoter 2, we transfected HA-tagged FoxO3a expression vector together with or without a HBZ expression vector into 293T cells and immunoprecipitated with anti-HA antibody. Then, the DNA-binding capacity of FoxO3a was

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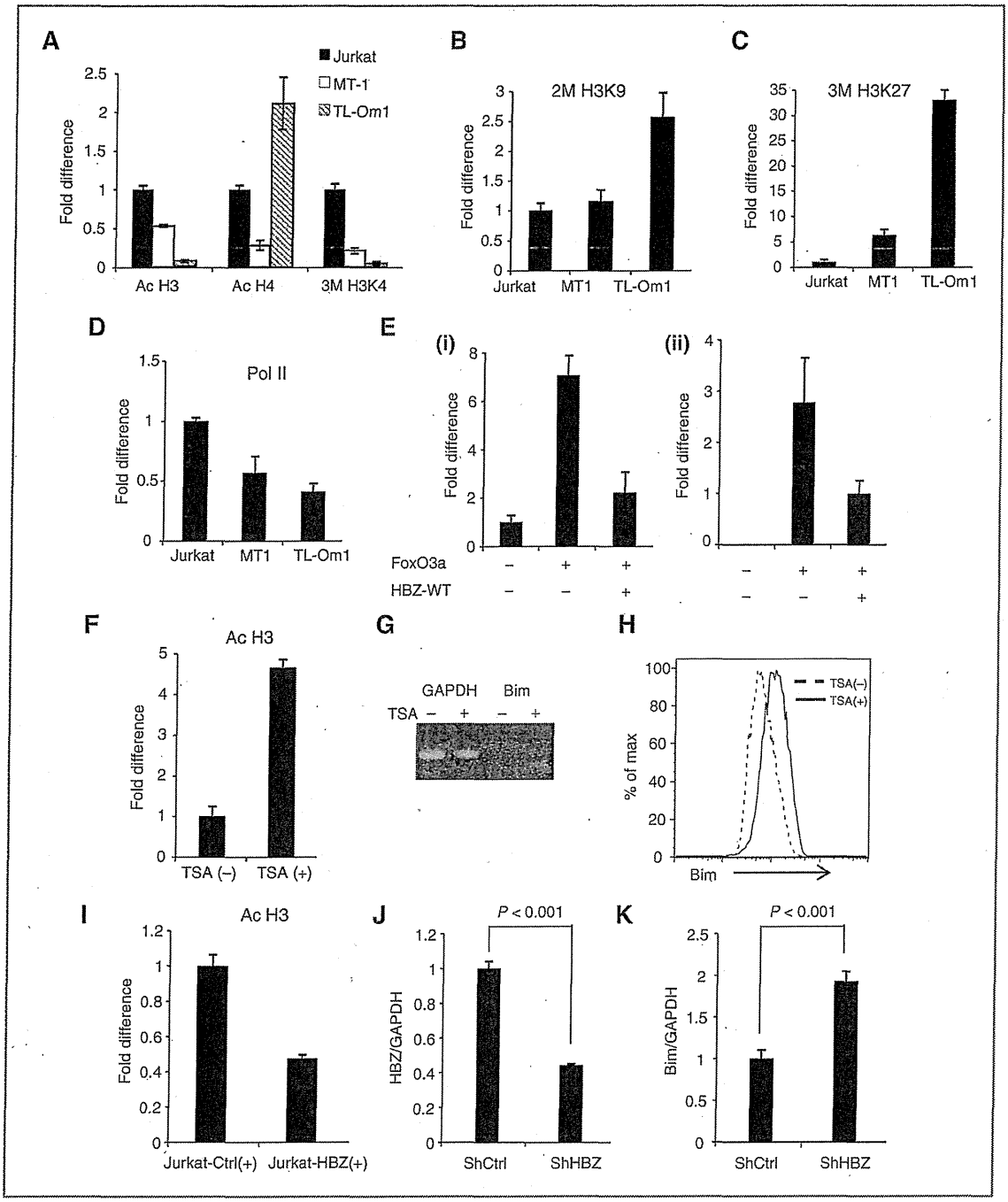


Figure 5. Epigenetic status of the promoter regions of the *Bim* gene. A–C, fold difference of acetylated histone H3, acetylated histone H4, trimethylated H3K4, dimethylated H3K9, or trimethylated H3K27; the data from Jurkat cells were arbitrarily set as 1.0. D, quantitative ChIP assay using RNA polymerase II (Pol II) antibody in Jurkat, MT-1, and TL-Om1 cells. E, 293T cells were transfected with HA-tagged FoxO3a expression vector together with or without HBZ expression vector. Cells were immunoprecipitated with anti-HA antibody and DNA-binding ability at promoter 2 was quantified by real-time PCR. F, fold difference of acetylated histone H3 in MT-1 cells, which were treated with or without 0.4 mmol/L TSA for 15 hours. The data from MT-1 cells without TSA treatment were arbitrarily set as 1.0. G and H, MT-1 cells were treated with 0.4 mmol/L TSA for 15 hours and *Bim* expression level was analyzed by quantitative real-time PCR and flow cytometry. I, fold difference of acetylated histone H3 in the Bim promoter in the Jurkat-control and Jurkat-HBZ cells 9 hours after the stimulation with PMA/Io. J, HBZ transcript in shRNA transfectant of MT-1 was quantified by real-time PCR. K, comparison of the *Bim* mRNA expression in control MT-1 cells and HBZ-KD MT-1 cells. Error bars, experimental variation. The data shown are representative of two or three independent experiments. Statistical differences are calculated by Student *t* test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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quantified by real-time PCR. Figure 5E shows that HBZ attenuated the DNA-binding capacity of FoxO3a in the promoter 2 of *Bim* (i) and *FasL* promoter (ii), suggesting that the suppressed binding of FoxO3a to the promoter regions leads to inhibition of the *Bim* and *FasL* genes transcription by HBZ.

Next, we treated MT-1 cells with trichostatin A (TSA), a cell-permeable chemical inhibitor of class I/II histone deacetylases (HDAC). Treatment of TSA resulted in a clear upregulation of acetylation of histone H3 (Fig. 5F) followed by Bim expression both at the mRNA (Fig. 5G) and protein levels (Fig. 5H), indicating that histone modification is associated with suppressed *Bim* transcription in MT-1. We also performed ChIP assay using Jurkat-control and Jurkat-HBZ cells, which were stimulated with PMA and ionomycin for 9 hours, and found that acetylation of histone H3 decreased in Jurkat-HBZ cells (Fig. 5I), suggesting that HBZ is implicated in histone deacetylation in T cells. To verify whether HBZ inhibits transcription of the *Bim* gene, we suppressed the *HBZ* gene transcription by shRNA as reported previously (11). Efficiencies of lentivirus vector transduction, which were determined by EGFP expression, were 90.5% and 90.3% for control MT-1 cells and HBZ-knockdown MT-1 cells, respectively. Suppressed HBZ expression led to increase the *Bim* gene transcription (Fig. 5J and K), indicating that HBZ expression is linked to suppression of *Bim* expression in ATL cells.

## Discussion

Human immunodeficiency virus type 1 (HIV-1) replicates vigorously and the generated virus infects target cells *in vivo*. Unlike HIV-1, HTLV-1 induces proliferation to increase the number of infected cells, as this virus is transmitted primarily by cell-to-cell contact (5). Therefore, HTLV-1–encoded proteins promote proliferation of infected cells and inhibit their apoptosis, resulting in an increased number of infected cells *in vivo* (2). In this study, we show that HBZ inhibits both the intrinsic and extrinsic apoptotic pathways via targeting FoxO3a, which leads to suppressed transcriptions of *Bim* and *FasL*. We demonstrated two mechanisms for perturbation of FoxO3a by HBZ: interaction of HBZ with FoxO3a and interference of nuclear export of phosphorylated FoxO3a. HBZ suppresses DNA-binding ability of active form of FoxO3a through interaction between central domain of HBZ and forkhead domain of FoxO3a. In addition, LXXLL-like motif of HBZ is implicated in inhibition of FoxO3a-mediated apoptosis, suggesting that HBZ interferes in interaction of CBP/p300 and FoxO3a. Furthermore, HBZ retains inactive form of FoxO3a in the nucleus through interaction with 14-3-3, leading to transcriptional repression of the target genes. Interestingly, accumulation of phosphorylated form of FoxO3a in the nucleus has been observed in HIV Vpr-expressing cells, which might be implicated in HIV-mediated resistance against insulin (28). Thus, FoxO3a is a target of both human retroviruses.

In this study, we showed that central domain of HBZ interacts with FoxO3a while LXXLL-like motif in activation domain of HBZ is responsible for suppressed apoptosis. LXXLL-like motif of HBZ has been reported to interact with KIX domain of p300 (38). The central domain of HBZ interacts with the forkhead domain of FoxO3a, which binds to the target sequence (35). This is the mechanism how HBZ inhibits DNA binding of FoxO3a. However, inhibitory effect of HBZ on apoptosis largely depends on LXXLL-like motif of activation domain (Fig. 2A and B). FoxO3a is also reported to interact with KIX domain of CBP/p300 (37). Forkhead domain of FoxO3a intramolecularly interacts with its conserved regions (CR) 3, and binding of forkhead domain to DNA releases CR3, allowing it to bind KIX of CBP/p300 (45). These findings suggest that HBZ interferes in the complex interaction between FoxO3a and CBP/p300, which is likely important to induce apoptosis.

It has been reported that Bim has a tumor-suppressor function in various cancers. Hemizygous loss of the *Bim* gene promoted development of B-cell leukemia in Myc-transgenic mice in which c-myc expression was driven by the immunoglobulin gene intron-enhancer (46). Insulin-like growth factor 1 (IGF-1), an important growth factor for myeloma cells, has been reported to suppress Bim expression by epigenetic and posttranslational mechanisms (25). In Epstein–Barr virus–infected B cells, *Bim* transcription is silenced by DNA methylation of the *Bim* gene promoter (47). Thus, impaired expression of Bim is associated with the various cancers, including the virus-related malignancies. FoxO3a is also a target of oncogenesis. BCR-ABL induces phosphorylation of FoxO3a, which leads to suppressed expression of Bim in $Ph1^+$ chronic myelogenous leukemia cells (32). In breast cancer, IκB kinase interacts with, phosphorylates FoxO3a, which causes proteolysis of FoxO3a (48). In this study, we revealed that HBZ hinders nuclear export of phosphorylated FoxO3a, and impairs function of FoxO3a likely through interaction of FoxO3a and p300. Thus, suppressed Bim and FasL expression through inhibition of FoxO3a by HBZ is a new mechanism for oncogenesis.

Besides FoxO3a perturbation by HBZ, we also have identified the epigenetic aberrations in the promoter region of the *Bim* gene in ATL cells, and found that Bim expression is suppressed by DNA methylation and histone modification. ATL cell lines exhibited upregulated level of H3K27 trimethylation in the promoter regions of *Bim*. It has been reported that enhancer of zeste (EZH) 2, a methyltransferase and component of the polycomb repressive complex 2, expression is increased in ATL cell lines (42). Because EZH2 plays an essential role in the epigenetic maintenance of H3K27 trimethylation, upregulated H3K27 trimethylation of the *Bim* gene promoter might be associated with increased expression of EZH2 in ATL cells. In addition, HBZ seems to be associated with histone deacetylation in MT-1 cells. According to the previous studies, it is known that both HBZ and FoxO3a bind to the histone acetyltransferase p300/CBP through the LXXLL motif (38). In this study, we found that the same motif is important for FoxO3a suppression and resulting inhibition of apoptosis. It is likely that HBZ decreases histone acetylation level on Bim promoter through the interaction with FoxO3a and dissociation of p300/CBP from the promoter. In addition to histone modifications, hypermethylation of CpGs in Bim promoter was observed in some ATL cells. These epigenetic aberrations likely occur as the secondary changes following long-time silencing of *Bim* by HBZ, although further investigations will be required.

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In this study, we demonstrated that HBZ suppresses activation-induced apoptosis by downregulation of proapoptotic genes, *Bim* and *FasL*. HBZ perturbs the function of FoxO3a by interaction, and induces epigenetic aberrations in the promoter region of the *Bim* gene. It has been shown that HBZ induces not only cancer but also inflammation *in vivo*. Because inflammatory diseases are essentially caused by failure to negatively regulate unnecessary immune responses by apoptosis, suppression of apoptosis by HBZ might be associated with HTLV-1–induced inflammation as well. Collectively, HBZ-mediated inhibition of apoptosis is likely implicated in both neoplastic and inflammatory diseases caused by HTLV-1.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** A. Tanaka-Nakanishi, J. Yasunaga, M. Matsuoka
**Development of methodology:** A. Tanaka-Nakanishi, M. Matsuoka
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** A. Tanaka-Nakanishi, K. Takai
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A. Tanaka-Nakanishi, J. Yasunaga, K. Takai, M. Matsuoka
**Writing, review, and/or revision of the manuscript:** A. Tanaka-Nakanishi, J. Yasunaga, M. Matsuoka
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M. Matsuoka
**Study supervision:** M. Matsuoka

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# Characterization of simian T-cell leukemia virus type 1 in naturally infected Japanese macaques as a model of HTLV-1 infection

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## Abstract

**Background:** Human T-cell leukemia virus type 1 (HTLV-1) causes chronic infection leading to development of adult T-cell leukemia (ATL) and inflammatory diseases. Non-human primates infected with simian T-cell leukemia virus type 1 (STLV-1) are considered to constitute a suitable animal model for HTLV-1 research. However, the function of the regulatory and accessory genes of STLV-1 has not been analyzed in detail. In this study, STLV-1 in naturally infected Japanese macaques was analyzed.

**Results:** We identified spliced transcripts of STLV-1 corresponding to HTLV-1 *tax* and HTLV-1 bZIP factor (*HBZ*). STLV-1 Tax activated the NFAT, AP-1 and NF-κB signaling pathways, whereas STLV-1 bZIP factor (SBZ) suppressed them. Conversely, SBZ enhanced TGF-β signaling and induced Foxp3 expression. Furthermore, STLV-1 Tax activated the canonical Wnt pathway while SBZ suppressed it. STLV-1 Tax enhanced the viral promoter activity while SBZ suppressed its activation. Then we addressed the clonal proliferation of STLV-1$^+$ cells by massively sequencing the provirus integration sites. Some clones proliferated distinctively in monkeys with higher STLV-1 proviral loads. Notably, one of the monkeys surveyed in this study developed T-cell lymphoma in the brain; STLV-1 provirus was integrated in the lymphoma cell genome. When anti-CCR4 antibody, mogamulizumab, was administered into STLV-1-infected monkeys, the proviral load decreased dramatically within 2 weeks. We observed that some abundant clones recovered after discontinuation of mogamulizumab administration.

**Conclusions:** STLV-1 Tax and SBZ have functions similar to those of their counterparts in HTLV-1. This study demonstrates that Japanese macaques naturally infected with STLV-1 resemble HTLV-1 carriers and are a suitable model for the investigation of persistent HTLV-1 infection and asymptomatic HTLV-1 carrier state. Using these animals, we verified that mogamulizumab, which is currently used as a drug for relapsed ATL, is also effective in reducing the proviral load in asymptomatic individuals.

**Keywords:** Simian T-cell leukemia virus, Human T-cell leukemia virus, Tax, HBZ

## Background

Human T-cell leukemia virus type 1 (HTLV-1) was the first human retrovirus found to cause a neoplastic disease, adult T-cell leukemia (ATL) [1,2]. Approximately 10 million people worldwide are estimated to be infected with this virus. HTLV-1 is endemic in specific areas including south-western Japan, Central and South America, the Caribbean, and intertropical Africa [3]. Most HTLV-1 carriers remain asymptomatic through their lives and only a small fraction of them develop ATL, a leukemia of HTLV-1-infected CD4$^+$ T cells, after a long latent period [4]. This virus also causes inflammatory disorders such as HTLV-1-associated myelopathy/tropic spastic paraparesis (HAM/TSP) [5,6] and uveitis [7].

The reason why most HTLV-1 carriers do not develop ATL is partly explained by the immune response of cytotoxic T cells (CTLs) against HTLV-1 proteins [8]. Immunosuppressive conditions, particularly following organ or bone

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marrow transplantation, can induce the development of ATL [9,10], indicating that the host immune system usually prevents the development of ATL. Two HTLV-1 proteins, Tax and HTLV-1 bZIP factor (HBZ), are thought to promote the proliferation of infected cells and ATL cells [4,11]. Tax is highly immunogenic to CTLs and the infected cells expressing Tax are kept to a small number [12]. Recently, it has been reported that CTLs to HBZ play a critical role in determining proviral load in carriers [13].

Animal models that are relevant to the human immune system are required for scientists to investigate how the immune response controls the proliferation of infected cells and viral replication *in vivo*. Old World monkeys are frequently infected with simian T-cell leukemia virus type 1 (STLV-1), which is closely related to HTLV-1 [14]. Like HTLV-1 infection, clonal proliferation of STLV-1-infected cells was detected by inverse PCR [15]. Furthermore, STLV-1 also leads to the development of lymphoproliferative diseases [16,17]. Based on these observations, it has been proposed that STLV-1-infected non-human primates may constitute a suitable animal model for HTLV-1 research. However, a detailed characterization of STLV-1 infection in non-human primates has not been achieved.

In the present study, Japanese macaques naturally infected with STLV-1 were investigated. We first identified the STLV-1 bZIP factor (SBZ) gene as an antisense transcript of STLV-1 similar to HBZ. Molecular analyses showed that STLV-1 Tax and SBZ have activities on various transcriptional pathways similar to those of HTLV-1 Tax and HBZ. Furthermore, we observed clonal proliferation of STLV-1-infected cells. Finally, anti-CCR4 antibody, which is currently used to treat ATL patients, was administered into STLV-1-infected Japanese macaques, and we found that this reduced the proviral load *in vivo*, indicating that anti-CCR4 antibody is effective for treatment of HTLV-1-associated inflammatory diseases. These results suggest that Japanese macaques naturally infected with STLV-1 show characteristics that correlate closely with those of HTLV-1 carriers and may therefore serve as a suitable animal model for the analysis of persistent HTLV-1 infection and HTLV-1 carrier state.

## Results

### Seroprevalence and proviral load of STLV-1 in Japanese macaques

To identify STLV-1-infected monkeys, we screened plasma samples for antibody against viral STLV-1 antigens by particle-agglutination test. Out of 533 Japanese macaques examined, 320 (60%) were seropositive, while only one rhesus macaque out of 163 (0.6%) was seropositive (Figure 1A). Proviral load in white blood cells was measured by quantitative real-time PCR for 115 seropositive Japanese macaques. Proviral load ranged from 0.001% to over 10% (Figure 1B). Since the DNA samples used in the above experiment were obtained from total white blood cells including granulocytes, these data likely underestimate proviral load of PBMCs.

### Functional similarity of STLV-1 Tax and STLV-1 bZIP factor to their counterparts in HTLV-1

Analysis of the STLV-1 pX region suggests the presence of *tax* coding gene and an antisense transcript in the minus strand of STLV-1 similar to *HBZ*. In order to examine if STLV-1 *tax* and *SBZ* genes are transcribed and processed to be mature mRNAs in STLV-1-infected PBMCs, STLV-1 *tax* and *SBZ* transcripts were amplified by RT-PCR using the primers flanking the putative splicing site (Figure 2). The length of the amplified fragments was comparable to that of the corresponding HTLV-1 transcripts, which are approximately 240 bp for *tax* and 310 bp for *HBZ*. We further verified that STLV-1 *tax* and *SBZ* transcripts are spliced at exactly the same location as HTLV-1 *tax* and spliced form of *HBZ* [11,18], respectively (Figure 2). To investigate the molecular functions of STLV-1 Tax and SBZ, we cloned the coding sequences of those proteins from the STLV-1 provirus in a Japanese macaque (Mf-5). Approximately 91% of the coding sequence of *tax* was identical in HTLV-1 (ATK) and Japanese macaque STLV-1, and 82% in *HBZ* (ATK) and Japanese macaque *SBZ*. Phylogenetic analyses show that Japanese macaque STLV-1 *env* in this study is close to Melanesian subtype C [5] (Additional file 1). Therefore, the STLV-1 protein sequences were aligned with HTLV-1 prototype ATK (subtype A) as well as Mel5

A

| | Total number of macaques examined | Antibody-positive | Positivity |
|---|---|---|---|
| Japanese macaques | 533 | 320 | 60% |
| rhesus macaques | 163 | 1 | 0.6% |

B

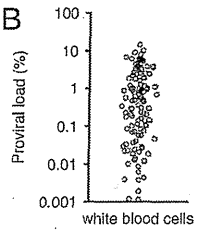


**Figure 1 STLV-1 infection in Japanese and rhesus macaques.** **(A)** STLV-1 seropositivity in Japanese macaques and rhesus macaques screened in this study is shown. **(B)** STLV-1 proviral load (percentage) in white blood cells of Japanese macaques is shown.

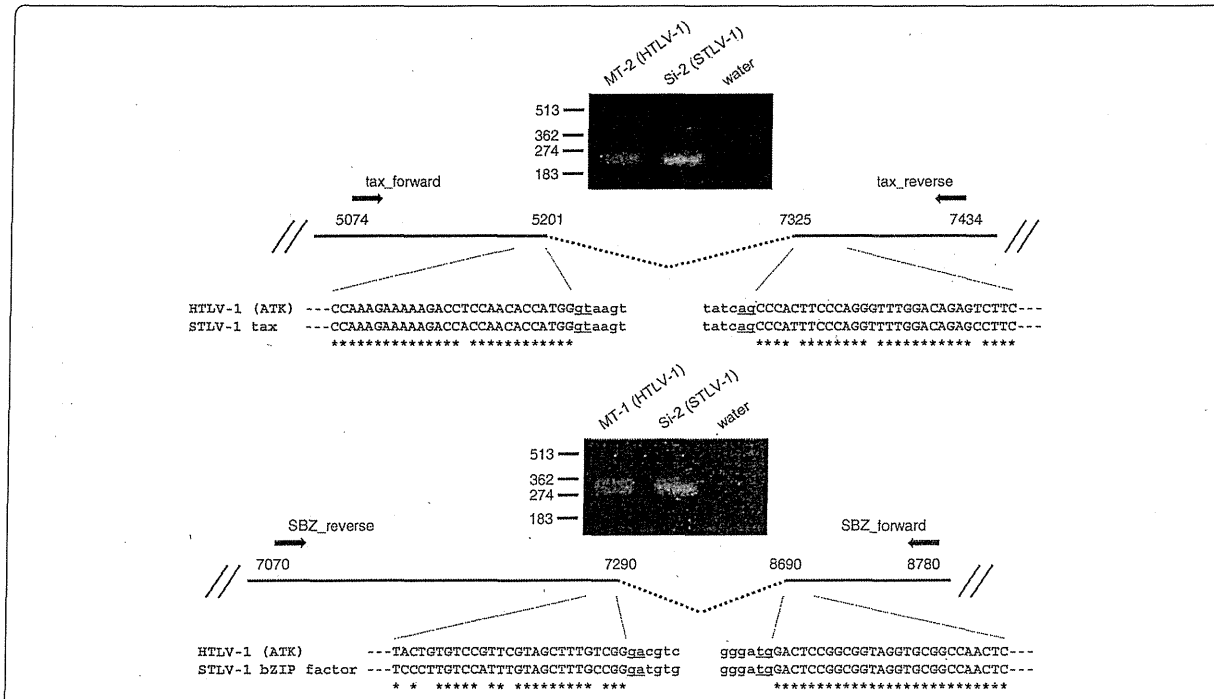


**Figure 2 Detection of STLV-1 *tax* and *STLV-1 bZIP factor* (*SBZ*) transcripts and their splicing junctions.** STLV-1 *tax* and *SBZ* transcripts were amplified by RT-PCR using the primers flanking the putative splicing site. The bands of the amplified fragments are shown together with the corresponding transcript of HTLV-1 in the images of agarose gel stained with ethidium bromide. Numbers in the scheme indicate the nucleotide positions of HTLV-1 ATK provirus. Sequences of the amplified STLV-1 *tax* and *SBZ* transcripts are represented with uppercase letters and aligned with a reference sequence of HTLV-1 (ATK). The lowercase letters represent the intron region of HTLV-1 or STLV-1 provirus.

(subtype C) for comparison, and presented in Figure 3. Approximately 93% of the STLV-1 Tax amino acid sequence was identical to that of HTLV-1 Tax (Figure 3A) and approximately 73% of the amino acid sequence of SBZ was identical to that of HBZ (Figure 3B). Notably, SBZ has some insertions and deletions, resulting in an excess of three amino acids compared with HBZ.

It was previously shown that HTLV-1 Tax activates the NF-κB, NFAT and AP-1 pathways [19,20], whereas HBZ suppresses them [21]. The effect of STLV-1 Tax on these


**Figure 3 Comparison of the amino acid sequences of STLV-1 Tax and SBZ with those of HTLV-1 Tax and HBZ.** Amino acid sequences of STLV-1 Tax **(A)** and SBZ **(B)** derived from an STLV-1$^+$ Japanese macaque (Mf-5) are compared respectively with those of HTLV-1 Tax and HBZ from two isolates. Asterisk represents the termination codon. Accession number: [GenBank:J02029] (ATK) and [GenBank:L02534] (Mel5).

pathways was analyzed using luciferase assays. We found that, like HTLV-1 Tax, STLV-1 Tax activated these pathways (Figure 4A). Conversely, SBZ suppressed these pathways when they were activated by phorbol myristate acetate and ionomycin (NFAT and AP-1) or HTLV-1 Tax (NF-κB) (Figure 4B).

Recently, our group reported that HBZ enhances TGF-β signaling via interaction with Smad2/3 and p300, thus inducing the expression of Foxp3 *in vitro* [22]. The analysis of HBZ transgenic mice further demonstrated an increase in Foxp3$^+$ T cells [23]. Therefore, we investigated whether SBZ also enhances TGF-β signaling. We found that SBZ enhanced signaling by the TGF-β pathway, while STLV-1 Tax suppressed it (Figure 4C). Like HBZ, expression of SBZ in mouse naïve CD4$^+$ T cells induced expression of Foxp3, and this expression was significantly enhanced by TGF-β (Figure 5). Thus, SBZ, like its counterpart HBZ, activates the TGF-β/Smad pathway and induces Foxp3 expression in CD4$^+$ T cells.

Next we studied STLV-1 Tax and SBZ for their capability to regulate the canonical Wnt pathway in the manner we recently reported for HTLV-1 Tax and HBZ [24]. STLV-1 Tax, like HTLV-1 Tax, elevated the activity of luciferase regulated by the promoter responsive to TCF/LEF in the presence of Dvl2 and DAPLE (Figure 4D). In contrast, when SBZ was co-expressed with Tax, luciferase activity was

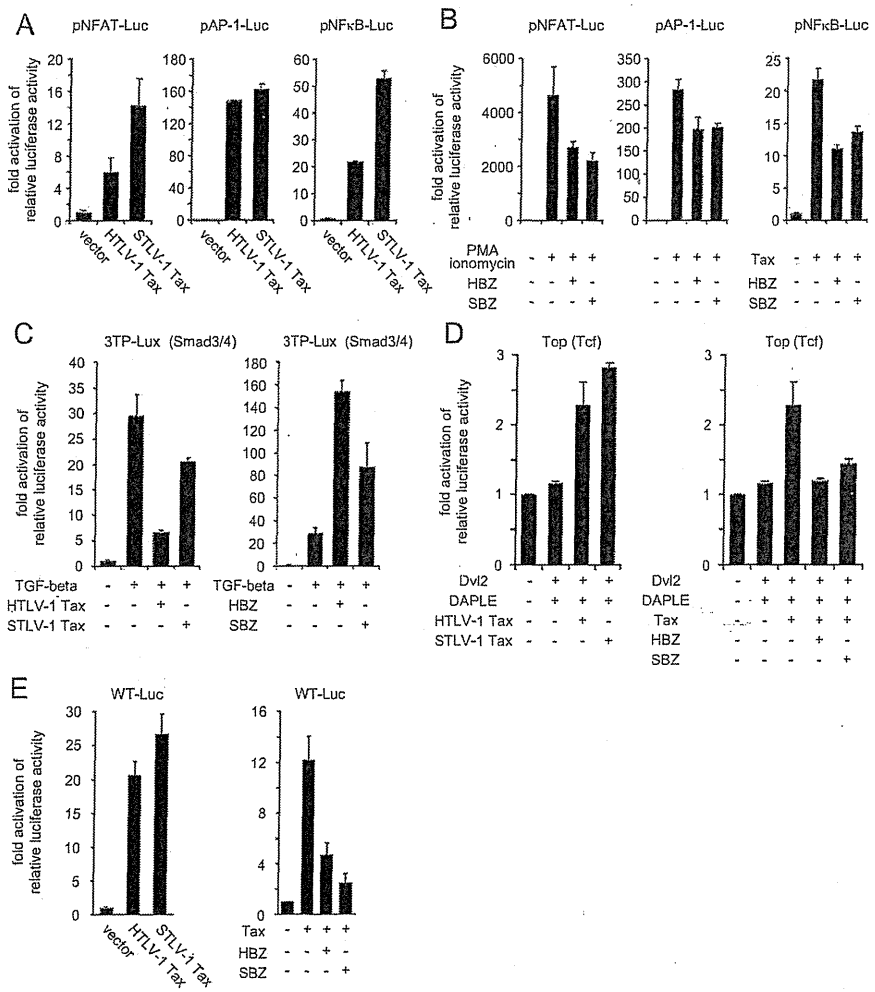


**Figure 4 Effects of STLV-1 Tax and SBZ on various signaling pathways.** Effects of HTLV-1 Tax or STLV-1 Tax **(A)**, and HBZ or SBZ **(B)** were analyzed using reporter plasmids for the NFAT, AP-1 and NF-κB pathways in Jurkat cells. **(C)** The effects of HTLV-1 Tax or STLV-1 Tax (left) and HBZ or SBZ (right) on the TGF-β signaling pathway were analyzed in HepG2 cells using the reporter plasmid 3TP-Lux, which contains the responsive element to Smad3/4. **(D)** The effects of HTLV-1 Tax or STLV-1 Tax (left) and HBZ or SBZ (right) on relative luciferase activity driven by TCF-responsive elements were analyzed using Jurkat cells. **(E)** The effects of HTLV-1 Tax or STLV-1 Tax (left) and HBZ or SBZ (right) on relative luciferase activity driven by viral LTR were analyzed using Jurkat cells. Firefly luciferase activity was normalized to that of Renilla luciferase and represented as fold activation compared to the relevant control. The data represent mean and standard deviation.

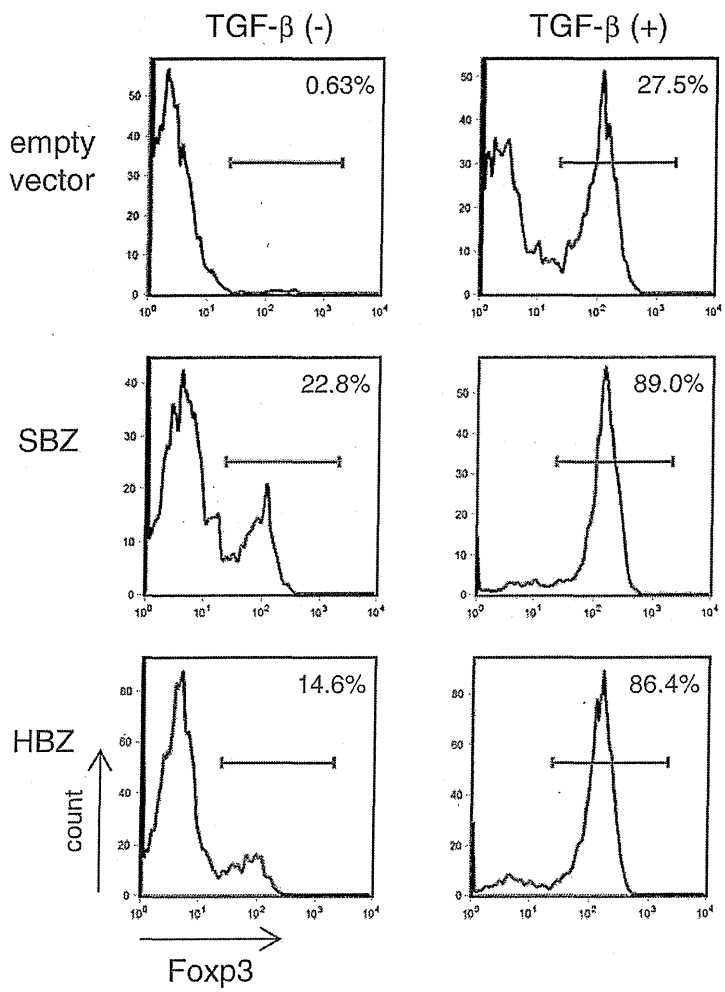


**Figure 5 Flow cytometric analyses of Foxp3 induction by SBZ.** SBZ or HBZ transduced mouse T cells that were positive for the transduction marker were analyzed for Foxp3 expression. The percentage of cells positive for Foxp3 is shown in each histogram. Each experiment was done at least in triplicate, and representative results are shown.

suppressed (Figure 4D). These results demonstrate that like their counterparts in HTLV-1, STLV-1 Tax activates the canonical Wnt pathway while SBZ suppresses it.

Lastly, regulation of viral promoter activity by STLV-1 Tax and SBZ was examined since it is known that HTLV-1 Tax activates the viral transcription from the 5' long terminal repeat (LTR) of the provirus while HBZ suppresses it. As presented in Figure 4E, STLV-1 Tax activated transcription of WT-Luc while SBZ suppressed it in Jurkat cells. It is consistent with functions of HTLV-1 Tax and HBZ.

### Clonal proliferation of STLV-1-infected cells in Japanese macaques

Clonal proliferation of HTLV-1-infected cells has been demonstrated by inverse PCR and next generation sequencing methods [25-27]. We analyzed the clonality of STLV-1-infected cells in seropositive Japanese macaques by identifying the genomic sequences adjacent to the 3' LTR. Briefly, genomic DNAs of monkey PBMCs were sheared by sonication and the integration sites of the provirus adjacent to the viral 3' LTR were amplified by linker-mediated PCR. Thereafter, we massively sequenced the integration sites and analyzed the abundance of each clones according to the method reported by Gillet et al. [27]. The detailed information on the deep sequencing is described in Additional file 2. The clonality of STLV-1-infected cells in three monkeys is shown in Figure 6A. Proviral load is represented as the percentage of STLV-1-infected cells in PBMCs. In monkeys with lower proviral load, a few major clones, together with many minor ones, were observed in Mf-1. Some clones proliferated in Mf-2 (Figure 6A, left

and middle). On the other hand, another monkey, Mf-3, which had higher proviral load (17%), possessed two major STLV-1-infected clones (Figure 6A, right). To study which cell types are infected by STLV-1, Tax expression in PBMCs obtained from one monkey (Mf-4) was analyzed by flow cytometry. The Tax-expressing cells were largely found to be $CD4^+$ T cells, as is the case with HTLV-1 infection in humans (Figure 6B).

### STLV-1-associated T-cell lymphoma in a Japanese macaque

A monkey (Mf-4) developed anorexia and had paralysis of the lower limbs. This monkey had high proviral load (53%) in PBMCs. We suspected that this monkey has developed a disease similar to HAM/TSP because paralysis of the lower limbs is one of the major symptoms of HAM/TSP patients. Magnetic resonance imaging (MRI) revealed a high intensity lesion in the brain on a T2-weighted image (Figure 6C). Pathological analysis showed that this tumor was a lymphoma with atypical morphology, and by immunohistochemical methods, it was found that these cells were $CD3^+$ $CD4^+$ (Figure 6D). In contrast, no obvious demyelination was observed in the spinal cord. Thus, this monkey was diagnosed with T-cell lymphoma in the brain rather than the disease like HAM/TSP. In this monkey, some major clones had proliferated in peripheral blood (Figure 6E, left). We found that the major clones in peripheral blood were also detected in the brain lesion (Figure 6E, right). These observations demonstrate that STLV-1 causes lymphoma in Japanese macaques. Notably, one of the major clones in the brain, which had its provirus integration site in chromosome 13, was not detected in PBMCs. This was confirmed by conventional PCR using the primers for the 3'LTR and the host genome proximal to the integration site (Figure 6F). Moreover, a clone with the integration site in chromosome 18 was also detected only in the brain lesion. These tumor-specific

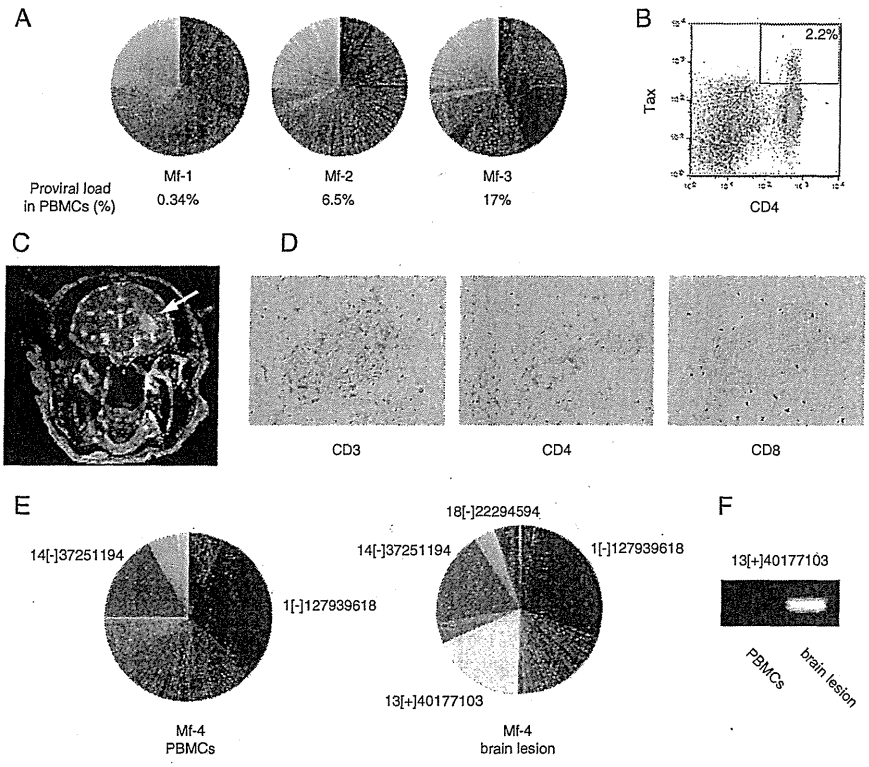


**Figure 6 Clonal proliferation of STLV-1-infected cells and lymphomatous lesion in the STLV-1-infected Japanese macaque. (A)** The relative frequency of $STLV\text{-}1^+$ clones in three monkeys (Mf-1, Mf-2 and Mf-3) is presented. Each area in the pie charts represents the proportion of provirus in a separate clone (identified by its unique integration site). **(B)** Flow cytometric analysis of PBMCs from an STLV-1-infected monkey shows that Tax-expressing cells are positive for CD4. **(C)** Magnetic resonance imaging of the brain of monkey Mf-4. The lesion is indicated by the white arrow. **(D)** Immunohistochemical analyses show that lymphoma cells are positive for CD3 and CD4. **(E)** Relative abundance of $STLV\text{-}1^+$ clones identified by unique integration sites of the provirus in PBMCs (left) and in the brain lesion (right) of Mf-4. Some of the abundant clones that are observed both in PBMCs and the brain lesion are painted in the same color in the two pie charts. **(F)** $STLV\text{-}1^+$ abundant clone 13[+] 40177103 is detected in the brain lesion by using the primers for 3' LTR and the genomic region, but not in PBMCs.