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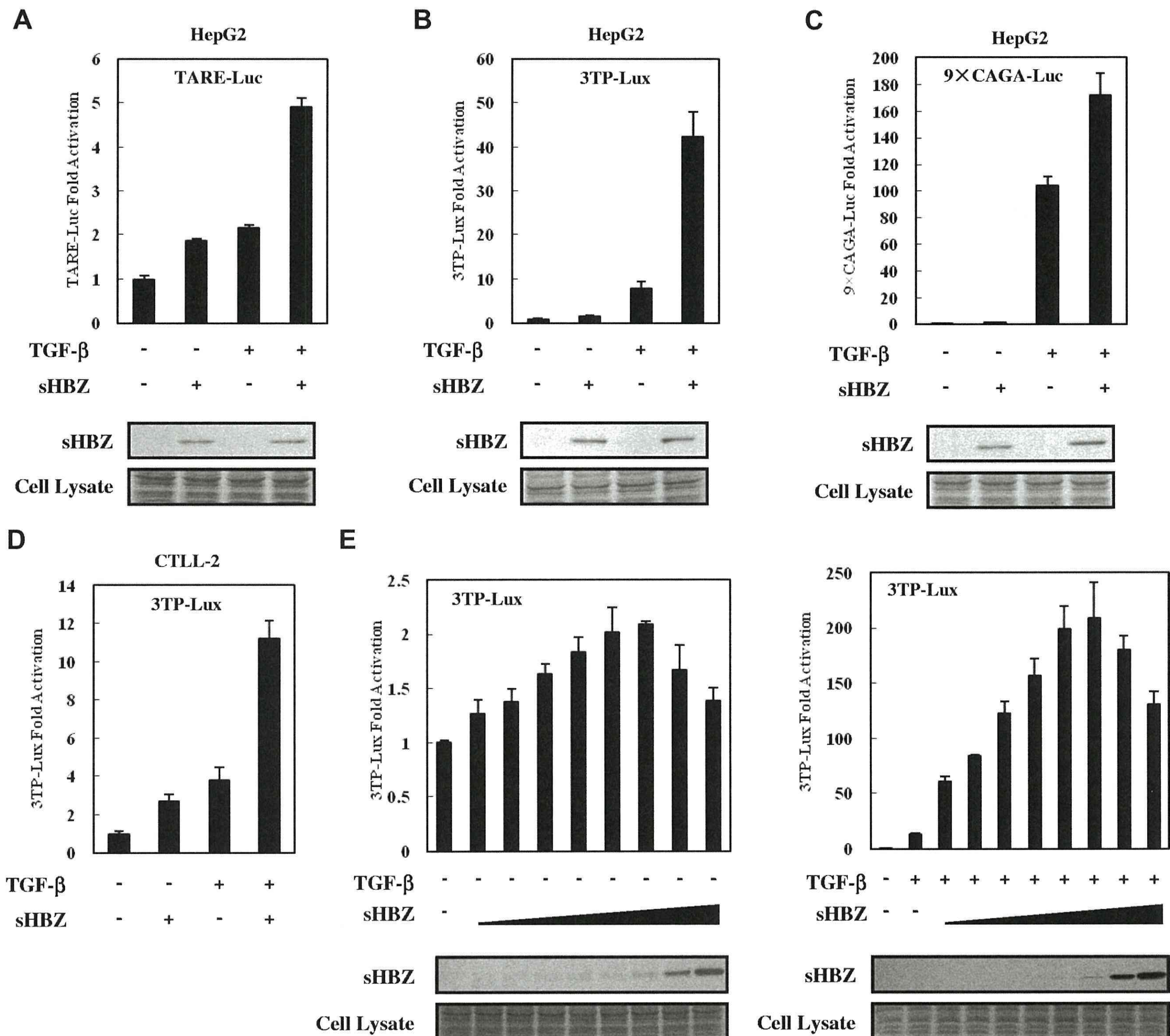

Figure 1. HBZ activated TGF-β signaling. In 12-well plates, HepG2 cells were cotransfected with 2 ng of phRL-TK, 0.5 μg of reporter plasmid TARE-Luc (A), 3TP-Lux (B), or 9 × CAGA-Luc (C), and 0.5 μg of pcDNA3.1-mycHis-sHBZ. At 24 hours after transfection, the cells were treated with TGF-β (10 ng/mL). After 24 hours, the cells were harvested and analyzed for luciferase activity. Expression of sHBZ was detected by Western blot (middle panel). Coomassie brilliant blue (CBB) staining was shown as the loading control (botom panel). (D) CTLL-2 cells were transfected with 3TP-Lux (2 μg), phRL-TK (10 ng), and pME18Sneo-sHBZ (0.4 μg) by electroporation. Luciferase activity was measured 24 hours after stimulation by TGF-β. (E) In 12-well plates, HepG2 cells were cotransfected with 3TP-Lux (0.5 μg), phRL-TK (2 ng), and pcDNA3.1-mycHis-sHBZ (0, 5, 10, 20, 50, 100, 200, 1000, and 4000 ng). At 24 hours after transfection, the cells were treated with or without TGF-β. After 24 hours, the cells were harvested and analyzed for luciferase activity. mycHis-sHBZ was detected by Western blot (middle panel). CBB staining was shown as the loading control (bottom panel).

Domains of HBZ responsible for enhancement of TGF-β–mediated transcription

Two major isoforms of the *HBZ* gene have been reported: spliced (*sHBZ*) and unspliced (us*HBZ*) *HBZ* (Figure 4A left panel). usHBZ caused a similar activation of TGF-β responses to that caused by sHBZ (Figure 4A right panel). We next evaluated the sHBZ deletion mutants shown in Figure 4B to determine which region of HBZ is responsible for activating TGF-β signaling. Three mutants (sHBZ-AD, sHBZ-ΔbZIP, and sHBZ-AD+bZIP) enhanced the response to TGF-β, whereas the sHBZ-bZIP mutant exhibited only suppressive activity (Figure 4C). The sHBZ-ΔbZIP mutant, which maintains the central domain (CD) and the activation domain (AD), had a dramatically reinforced activation capacity compared with sHBZ-AD; however, the central domain alone (sHBZ-ΔADΔbZIP mutant) did not influence TGF-β. The sHBZ-AD+bZIP mutant displayed a similar effect to that of wild-type sHBZ (Figure 1E; Figure 4C). These results indicate that the AD domain of HBZ is responsible for the activation of TGF-β signaling whereas bZIP domain shows a suppressive effect.

To determine the precise region within the AD domain involved in transcriptional activation, we performed reporter assays using sHBZ-ΔbZIP and its mutants (Figure 4D top panel). As illustrated in Figure 4D (middle panel), deletion of residues 20 to 38, which contains an LXXLL-like motif (LXXLL1), abrogated the enhancement of TGF-β signaling, whereas removal of the second LXXLL motif (LXXLL2) had no effect. Furthermore, we found that mutation of the LXXLL1 motif resulted in complete loss of activation. Because the LXXLL1 motif of HBZ is the major region where p300 binds,[35] this result further implicates p300 in the activation of TGF-β signaling by HBZ.

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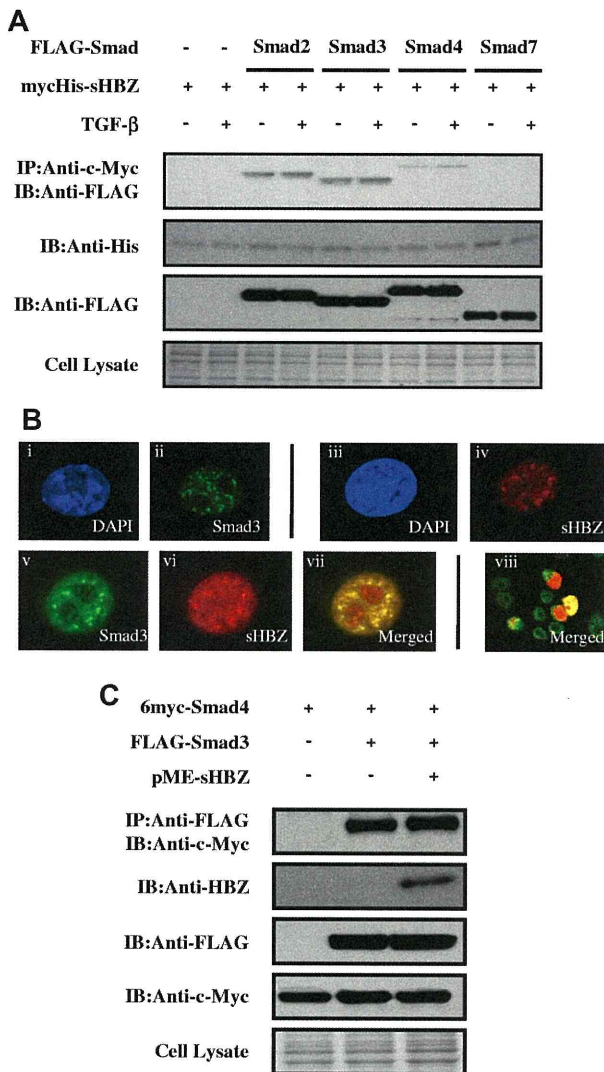

Figure 2. HBZ interacts with Smad proteins. (A) HBZ interacted with Smad proteins. COS7 cells were cotransfected with mycHis-sHBZ (6 μg) and FLAG-Smad2, Smad3, Smad4, and Smad7 (6 μg). At 24 hours after transfection, the cells were treated with or without TGF-β (5 ng/mL). Cell lysates were subjected to immunoprecipitation using anti-c-Myc followed by immunoblotting using anti-FLAG for detection of Smad proteins. (B) sHBZ colocalized with Smad3 in the cell nucleus. COS7 cells were transfected with mycHis-sHBZ (0.6 μg) together with (v-viii) or without (iii-iv) FLAG-Smad3 (0.4 μg). sHBZ was detected using anti-MYC Cy3 antibody (iv,vi). Smad3 was detected using anti-Flag-biotin and secondary streptavidin-Alexa-488 antibody (ii,v). The overlay of sHBZ and Smad3 is shown (vii-viii). DAPI (4,6-diamidino-2-phenylindole) was used to counterstain the nucleus (i,iii). (C) HBZ did not influence the Smad3/Smad4 interaction. COS7 cells were transfected with the indicated expression vectors (3 μg each). Cell lysates were subjected to immunoprecipitation using anti-FLAG followed by immunoblot (IB) using anti–c-Myc.

We mapped the region of HBZ interacting with Smad3 in more detail. As shown in Figure 4E, full-length sHBZ and 2 of its deletion mutants (sHBZ-ΔbZIP and sHBZ-AD+bZIP) associated with Smad3, wheres sHBZ-ΔAD, which lacks the AD domain, has no binding capability. sHBZ-ΔbZIP exhibited higher affinity for Smad3 than did full-length sHBZ. This result corroborates that of the luciferase assay in Figure 4C. To define which part of Smad3 binds HBZ, we performed a coimmunoprecipitation assay with Smad3 mutants in COS7 cells (Figure 4F). We found that Smad3 mutants without MH2 domain could not bind to sHBZ whereas only the MH2 domain of Smad3 physically interacted with sHBZ. Hence, the interaction with HBZ is mediated by the MH2 segment of Smad3 (Figure 4G).

Taken together, these observations demonstrate that HBZ enhances TGF-β signaling by physically associating with Smad3/p300 complexes via its AD domain.

Activation of TGF-β signaling is partially suppressed by high doses of HBZ via inhibition of AP-1

Previous studies have shown that Smads cooperate with AP-1 to mediate TGF-β–induced transcription.[36] In addition, HBZ has been reported to suppress AP-1 activity.[10] The observations shown in Figure 1E and Figure 4C prompted us to ask whether the activated TGF-β signaling was partially suppressed by higher HBZ expression levels because HBZ inhibited AP-1. A reporter assay showed that the suppression of TGF-β-mediated activation by high sHBZ doses (> 200 ng pcDNA3.1-mycHis-sHBZ plasmid DNA per well) was overcome by c-Fos, but not by enforced expression of Smad3, although overexpression of both Smad3 and c-Fos dramatically enhanced HBZ-mediated activation of TGF-β signaling (Figure 5A). To confirm this result, we performed a luciferase assay using a Luc vector, 9 × CAGA-Luc, which contains multiple CAGA sites but lacks AP-1 binding sites. As shown in Figure 5B, sHBZ persistently enhanced TGF-β–mediated activation of this reporter, even at high doses. In addition, we confirmed that the bZIP domain of spliced HBZ is required for the suppression of AP-1 signaling (Figure 5C). This result is consistent with the results of the luciferase assay (Figure 4C), in which sHBZ-ΔbZIP activation of TGF-β signaling was stronger than that of full-length sHBZ. These observations demonstrate that high levels of HBZ expression partially suppress TGF-β signaling by inhibiting AP-1 signaling and that the bZIP domain of HBZ is responsible for this suppressive activity.

Physiologic levels of HBZ overcome Tax-mediated suppression of TGF-β signaling

To rule out the possibility that the activated TGF-β signaling observed in this study was caused by overexpression of HBZ, we compared the expression level of HBZ protein in transfected HepG2 cells with those in ATL- and HTLV-1–associated cell lines. As shown in Figure 6A, HepG2 cells transfected with 200 ng pME18Sneo-sHBZ plasmid DNA per well on 12-well plates expressed sHBZ protein at levels equivalent or slightly higher than those in ATL- and HTLV-1–associated cell lines. This result suggested that sHBZ protein expressed at physiologic levels could therefore activate TGF-β–mediated signaling. Next, we examined the endogenous sHBZ and Smad3 protein complex. The expression of *Smad3* mRNA was variable in fresh ATL cells, ATL cell lines, and HTLV-1–associated cell lines (supplemental Figure 3). sHBZ was detected in the immunoprecipitate pulled down by a specific antibody against Smad3 (Figure 6B). These data further support the interaction between HBZ and Smad3 in HTLV-1–infected cells.

It has been reported that HTLV-1 Tax protein inhibits Smad-dependent TGF-β signaling.[18-20] Next, we performed a reporter assay to study the effect of HBZ on Tax-mediated suppression of TGF-β pathway. When coexpressed with Tax, sHBZ overcame Tax's repression of 3TP luciferase activity. Moreover, Tax had little effect on sHBZ-activated TGF-β signaling when equal amounts of sHBZ and Tax were expressed (Figure 6C).

HBZ enhances Foxp3 expression in naive T cells through Smad3

We next studied the effect of HBZ expression on transcription of TGF-β target genes. We expressed sHBZ in mouse naive T cells using a retrovirus vector. As shown in Figure 7A, expression of

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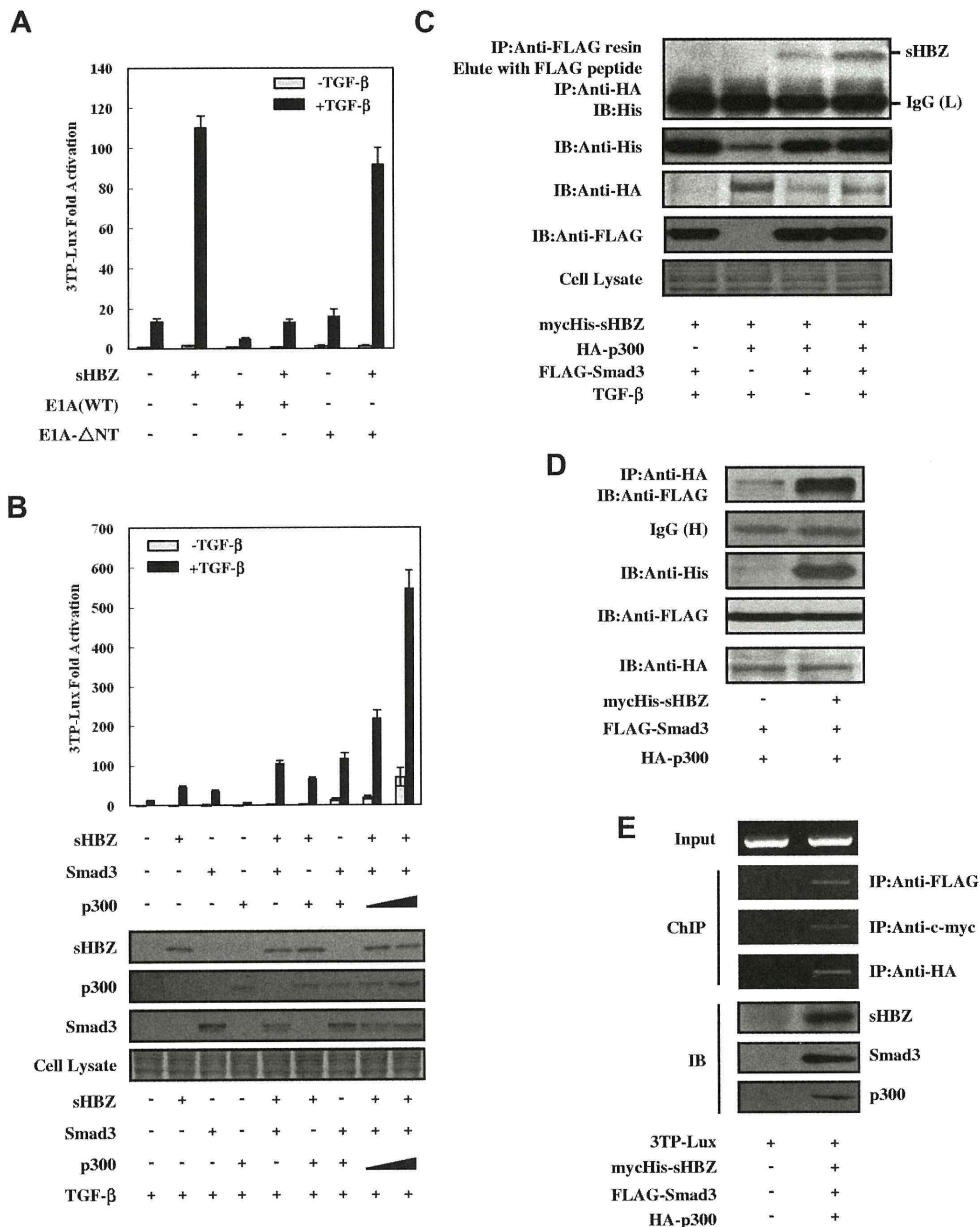

Figure 3. HBZ activates TGF-β signaling dependent on p300. (A) E1A repressed HBZ-induced activation of TGF-β. In 12-well plates, HepG2 cells were cotransfected with 3TP-Lux (0.5 μg), phRL-TK (2 ng), pME18Sneo-sHBZ (20 ng), and pCS2+-E1A or pCS2+-E1A-ΔNT (2 ng). Luciferase activity was measured 24 hours after stimulation by TGF-β (0, 10 ng/mL). (B) HBZ synergized with Smad3 and p300 to enhance TGF-β. HepG2 cells were cotransfected with 3TP-Lux (0.5 μg), phRL-TK (2 ng), pcDNA3.1-mycHis-sHBZ (200 ng), FLAG-Smad3 (50 ng), and pCMV-p300 (2, 5 μg). At 24 hours after transfection, the cells were treated with or without TGF-β (10 ng/mL). Luciferase activity was measured after 24 hours. Expression of sHBZ, Smad3, and p300 was detected by Western blot (middle panel). CBB staining was shown as the loading control (bottom panel). (C) HBZ, Smad3, and p300 could form a ternary complex. mycHis-sHBZ (4 μg), FLAG-Smad3 (4 μg), and HA-p300 (4 μg) were cotransfected into COS7 cells, which were subsequently treated with TGF-β (5 ng/mL). Ternary complexes were detected by sequential immunoprecipitation with anti-FLAG agarose affinity gel and anti-HA antibody, followed by immunoblotting with the His antibody. (D) HBZ enhanced the interaction between Smad3 and p300. COS7 cells were cotransfected with mycHis-sHBZ (4 μg), FLAG-Smad3 (4 μg), and HA-p300 (4 μg). Cell lysates (samples from the experiment of Figure 4E) were subjected to immunoprecipitation using anti-HA followed by immunoblotting with anti-FLAG. (E) sHBZ, Smad3, and p300 bind to the Smad-responsive promoter. After transfection with mycHis-sHBZ, FLAG-Smad3, and HA-p300, and treatment with 5 ng/mL of TGF-β for 24 hours, HepG2 cells were chromatin immunoprecipitated by each indicated antibody. The precipitated DNAs and 1% of the input cell lysates were amplified by the 3TP promoter specific primers. Expression of sHBZ, Smad3, and p300 was detected by Western blot (bottom panel).

sHBZ was associated with enhanced transcription of *Pdgfb*, *Sox4*, *Ctgf*, *Foxp3*, *Runx1*, and *Tsc22d1* genes and suppression of *Id2* gene; such effects were consistent with those by TGF-β. However, HBZ did not influence the level of *Cdkn1a*, *Cdkn2b*, and *Myc* gene transcripts. This result indicates that HBZ selectively modulates transcription of TGF-β target genes, such as TGF-β. HBZ does not interfere with the expression of genes associated with cell cycle and proliferation. To check whether HBZ could affect the cell growth via enhancing TGF-β signaling, we established stable sHBZ-expressing CTLL-2 cell lines. TGF-β suppressed proliferation of

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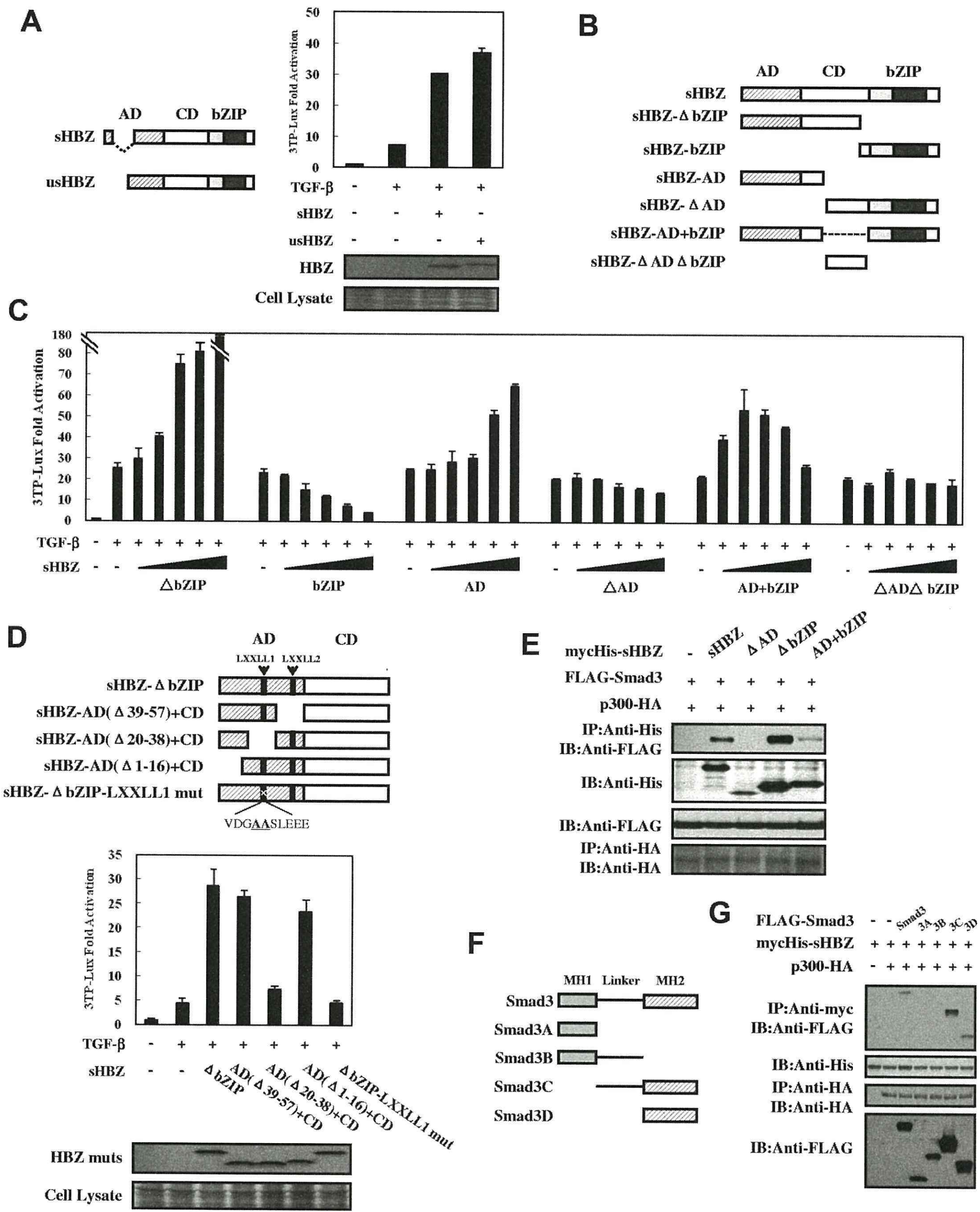

Figure 4. Domains of HBZ responsible for the activation of TGF-β signaling. (A) Comparison of the effect of sHBZ and usHBZ on TGF-β activation. (Left panel) Schematic diagram of sHBZ and usHBZ. (Right panel) HepG2 cells were cotransfected with 3TP-Lux (0.5 μg), phRL-TK (2 ng), pcDNA3.1-mycHis-sHBZ, or pcDNA3.1-mycHis-usHBZ (200 ng). Luciferase activity was measured 24 hours after 10 ng/mL TGF-β stimulation. mycHis tagged sHBZ and usHBZ were detected by Western blot (middle panel). CBB staining was shown as the loading control (bottom panel). (B) Schematic diagram of sHBZ and its mutants used in this study. Characteristic domains of sHBZ are indicated as follows: AD, CD, and basic leucine zipper domain (bZIP). (C) Analysis of sHBZ deletion mutants for their effect on TGF-β–mediated signaling. In 12-well plates, HepG2 cells were cotransfected with 3TP-Lux (0.5 μg), phRL-TK (2 ng), and pME18Sneo-sHBZ mutants (0, 5, 20, 100, 200, and 500 ng). Luciferase activity was measured 24 hours after stimulation by TGF-β (10 ng/mL). (D) The N-terminal LXXLL1 motif of HBZ is important in enhancing TGF-β–induced luciferase expression. (Top panel) Schema of sHBZ-ΔbZIP and its deletion mutants. The locations of the LXXLL motifs are indicated. The mutated residues in the LXXLL1 motif are in bold and underlined. (Middle panel) In 24-well plates, HepG2 cells were cotransfected with 3TP-Lux (0.25 μg), phRL-TK (1 ng), and mycHis-sHBZ-ΔbZIP or its mutants (1 μg). At 24 hours after transfection, the cells were stimulated with or without 10 ng/mL TGF-β. Cell lysates were subjected to luciferase assay 24 hours after stimulation. sHBZ-ΔbZIP and its mutants were detected by Western blot. CBB staining was shown as the loading control (bottom panel). (E) Determination of the region of HBZ responsible for the interaction with Smad3. COS7 cells were transfected with the indicated mycHis-sHBZ mutants along with the FLAG-Smad3 and HA-p300 vectors. Cell lysates were subjected to immunoprecipitation using anti–c-Myc followed by immunoblotting using anti-FLAG. (F) Schematic drawing of Smad3 and its deletion mutants. The locations of the MH1 domain, MH2 domain, and the linker domain are indicated. (G) Mapping the region of the Smad3 protein necessary for interaction with sHBZ. COS7 cells were transfected with HA-p300, mycHis-sHBZ, and full-length or mutant FLAG-Smad3. At 48 hours after transfection, total cell lysates were subjected to immunoprecipitation using anti–c-Myc followed by IB using anti-FLAG.

control CTLL-2 cells, whereas HBZ expressing CTLL-2 cells proliferated regardless of TGF-β (Figure 7B).

It has been reported that TGF-β signaling is critical for the development of $CD4^+CD25^+Foxp3^+$ regulatory T cells and that the binding of Smad3 to a specific enhancer region is required for activation of the *Foxp3* promoter.[21,29] As shown in Figure 7A, HBZ enhanced transcription of *Foxp3* gene induced by TGF-β. We therefore studied whether HBZ has any influence on the generation

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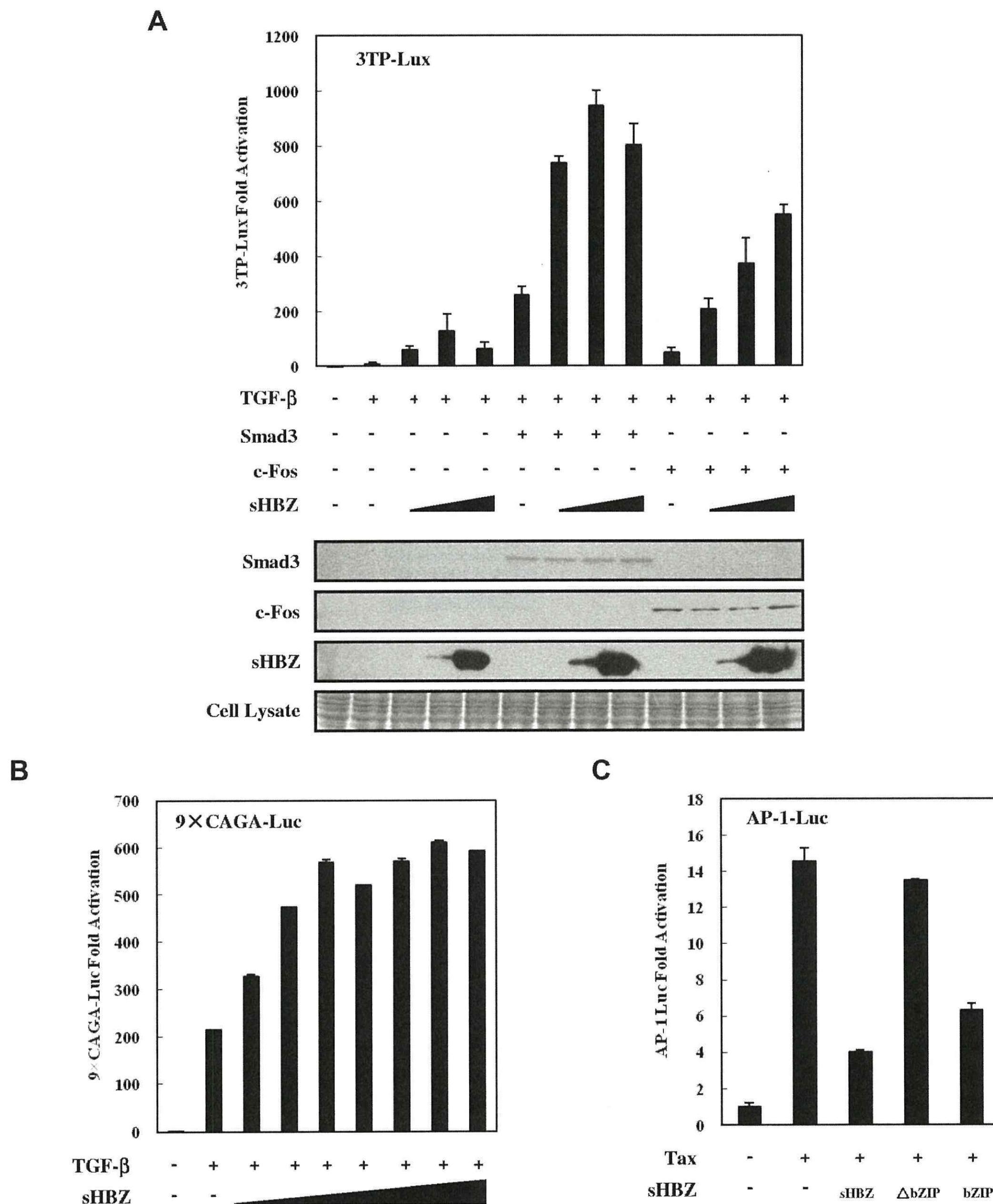

Figure 5. Higher expression of HBZ partially suppressed activation of TGF-β signaling via AP-1. (A) HepG2 cells were cotransfected with 3TP-Lux (0.5 μg), phRL-TK (2 ng), pCDNA3-c-Fos (0, 0.1 μg), FLAG-Smad3 (0, 0.1 μg), and pcDNA3.1-mycHis-sHBZ (0, 20, 200, and 4000 ng). At 24 hours after transfection, the cells were treated with or without TGF-β (10 ng/mL). After 24 hours, the cells were harvested, and luciferase activity was determined. Expression of sHBZ, Smad3, and c-Fos was detected by Western blot (middle panel). CBB staining was shown as the loading control (bottom panel). (B) HepG2 cells were cotransfected with 9 × CAGA-Luc (0.5 μg), phRL-TK (2 ng), and pME18Sneo-sHBZ (0, 2, 5, 10, 20, 50, 100, and 200 ng). Luciferase activity was measured 24 hours after 10 ng/mL TGF-β stimulation. (C) sHBZ inhibited AP-1 signaling via its bZIP domain. Jurkat cells were cotransfected with AP-1-Luc (1 μg), phRL-TK (10 ng), pCG-Tax (1 μg), and pME18Sneo-sHBZ mutants (1 μg). After 48 hours, luciferase activity was measured.

of Foxp3$^+$ T cells. Retrovirally expressed sHBZ protein increased the level of Foxp3 in conventional mouse CD4$^+$ T cells and also synergistically enhanced TGF-β–induced Foxp3 expression (Figure 7C,E). Treatment with SB431542, an inhibitor of the TGF-β type I receptor, did not change the induction of Foxp3 by sHBZ, whereas it completely blocked the TGF-β–induced Foxp3 expression (Figure 7C), indicating that an increase in TGF-β cytokine was not involved in this synergistic effect. sHBZ also induced FoxP3 expression in human naive T cells (Figure 7D).

Apart from Smad3, STAT5 is the main factor that sustains Foxp3 expression in both Treg and effector T cells.[37] Therefore, we next evaluated the effect of HBZ on STAT5 signaling. sHBZ was not capable of activating CA-STAT5a-mediated transcriptional activation of the Jγ1 promoter (supplemental Figure 4). Therefore, it is probable that HBZ induced the expression of Foxp3 through TGF-β/Smad3 responses. After treatment with SIS3, a specific inhibitor of Smad3, sHBZ-enhanced Foxp3 induction was reduced by > 70% (Figure 7E). Furthermore, the synergistic effect of sHBZ on TGF-β–induced Foxp3 expression was markedly inhibited by SIS3 (Figure 7E).

To further analyze the mechanism by which HBZ induces Foxp3 expression, we performed a reporter assay using the

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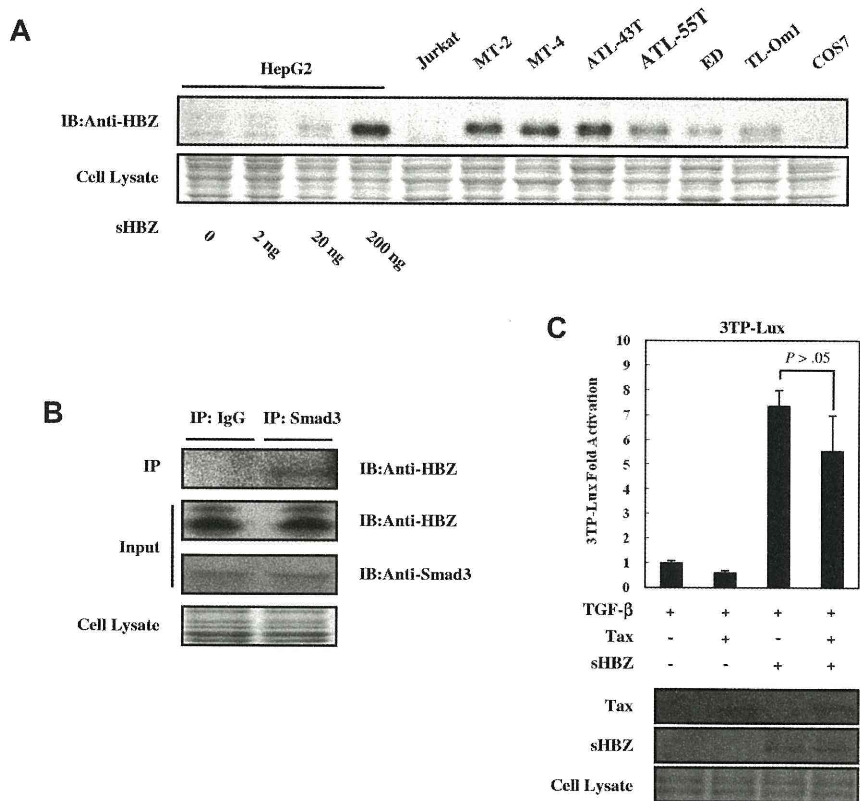

Figure 6. Physiologic level of HBZ overcame the Tax-mediated suppression of TGF-β signaling. (A) Comparing the level of HBZ protein in HBZ-transfected HepG2 cells with the level of HBZ in ATL and HTLV-1–immortalized cell lines. Total protein was extracted from sHBZ-transfected HepG2 cells (samples from supplemental Figure 1) and the indicated cell lines, and subjected to immunoblotting using HBZ antibody. (B) Endogenous HBZ interacted with Smad3. ATL-55T cells were treated with 10 ng/mL TGF-β. After 10 hours, whole cell lysate was subjected to immunoprecipitation with anti-Smad3 or control IgG, and immunoprecipitates were probed with anti-HBZ antibody. (C) HBZ overcame the repression of TGF-β signaling induced by Tax. In 12-well plates, HepG2 cells were cotransfected with 3TP-Lux (0.5 μg), phRL-TK (2 ng), pCG-Tax (0, 0.2 μg), and pcDNA3.1-mycHis-sHBZ (0, 0.2 μg). At 24 hours after transfection, the cells were treated with or without 10 ng/mL TGF-β. After 24 hours, the cells were harvested and analyzed for luciferase activity. sHBZ and Tax were detected by Western blot (middle panel). CBB staining was shown as the loading control (bottom panel).

enhancer and promoter of the mouse *Foxp3* gene. As shown in Figure 7F, sHBZ activated Foxp3-Luciferase expression but it failed to activate the *Foxp3* promoter alone, indicating that the *Foxp3* gene is regulated by HBZ through the enhancer. We next examined the contribution of Smad3 to HBZ-induced *Foxp3* enhancer activity. *Foxp3* reporter activity was reduced after mutation of the Smad-binding region of the enhancer, and treatment with SIS3 completely blocked the transactivation of *Foxp3* by sHBZ (Figure 7F). Furthermore, interaction of HBZ, Smad3, and p300 to human *FoxP3* enhancer was detected by ChIP assay in a HTLV-1–transformed cell line, MT-2 (Figure 7G). These results collectively indicate that the enhanced induction of Foxp3 expression by HBZ can be attributed, at least in part, to Smad3-dependent TGF-β signaling.

Discussion

Leukemic cells in most ATL cases, like Tregs, express CD4, and CD25. Foxp3 is a master regulator that controls the transcription of genes, which are critical for the suppressive function of Tregs. Two-thirds of ATL cases express FoxP3 in the tumor cells, indicating that such ATL cells are derived from Tregs.[23] Indeed, it has been reported that ATL cells have a suppressive effect on bystander $CD4^+$ T cells.[38] The proportion of HTLV-1 provirus is higher in $FoxP3^+$ Tregs than in uninfected cells.[24] Thus, these findings suggest that HTLV-1 infection increases virus-infected Tregs and finally transforms them. So far, the molecular mechanisms underlying the development of ATL Tregs have not been defined. TGF-β signaling has been implicated in both the development and function of Tregs,[21] and recently we reported that transgenic expression of the *HBZ* gene increased Tregs in vivo and HBZ enhanced transcription of the *Foxp3* gene.[26] Thus, HBZ induces Foxp3 expression and promotes Treg cell development in vivo. The present study links HBZ and TGF-β signaling by showing that HBZ induces Foxp3 expression via interaction with Smad3 and p300. Although HBZ impairs the suppressive function of Tregs to some extent, HBZ-expressing Tregs retain some suppressive functions.[26] Indeed, the immunodeficiency observed in ATL patients and HTLV-1 carriers might be attributable to the Treg phenotype induced by HBZ. The weak immunosuppressive potential retained by HTLV-1–infected Tregs may allow them to escape host immune attack, which possibly explains why HTLV-1 favors Tregs in vivo.

Several viruses have evolved distinct strategies to modulate TGF-β signaling using their own viral proteins. Examples include hepatitis B virus pX; hepatitis C virus core protein, NS3 and NS5; Kaposi sarcoma-associated herpesvirus K-bZIP; Epstein-Barr virus LMP1; and severe acute respiratory syndrome-associated coronavirus N protein.[39-44] Like HBZ, the HBV pX and severe acute respiratory syndrome N protein enhance the transcriptional responses of TGF-β. The common strategy used by viruses to modulate TGF-β signaling is the direct binding of viral proteins to Smad proteins. In this study, we demonstrated that the enhancement of the p300/Smad3 interaction by HBZ is critical for HBZ-induced TGF-β activation.

TGF-β exerts growth inhibitory effects, from which cancer cells usually escape during malignant progression. Accumulating evidence shows that TGF-β act as tumor suppressor at early stages of cancer development but can promote tumor progression at later stages of oncogenesis through tumor-cell-autonomous and host-tumor interactions. Like other cancer cells, HTLV-1–infected T cells are resistant to the growth-inhibitory effect of TGF-β.[45] As a mechanism of this resistance, Tax has been shown to inhibit TGF-β–mediated signaling, resulting in escape from the suppressive effect of TGF-β.[18-20] It is possible that Tax protein inhibits the

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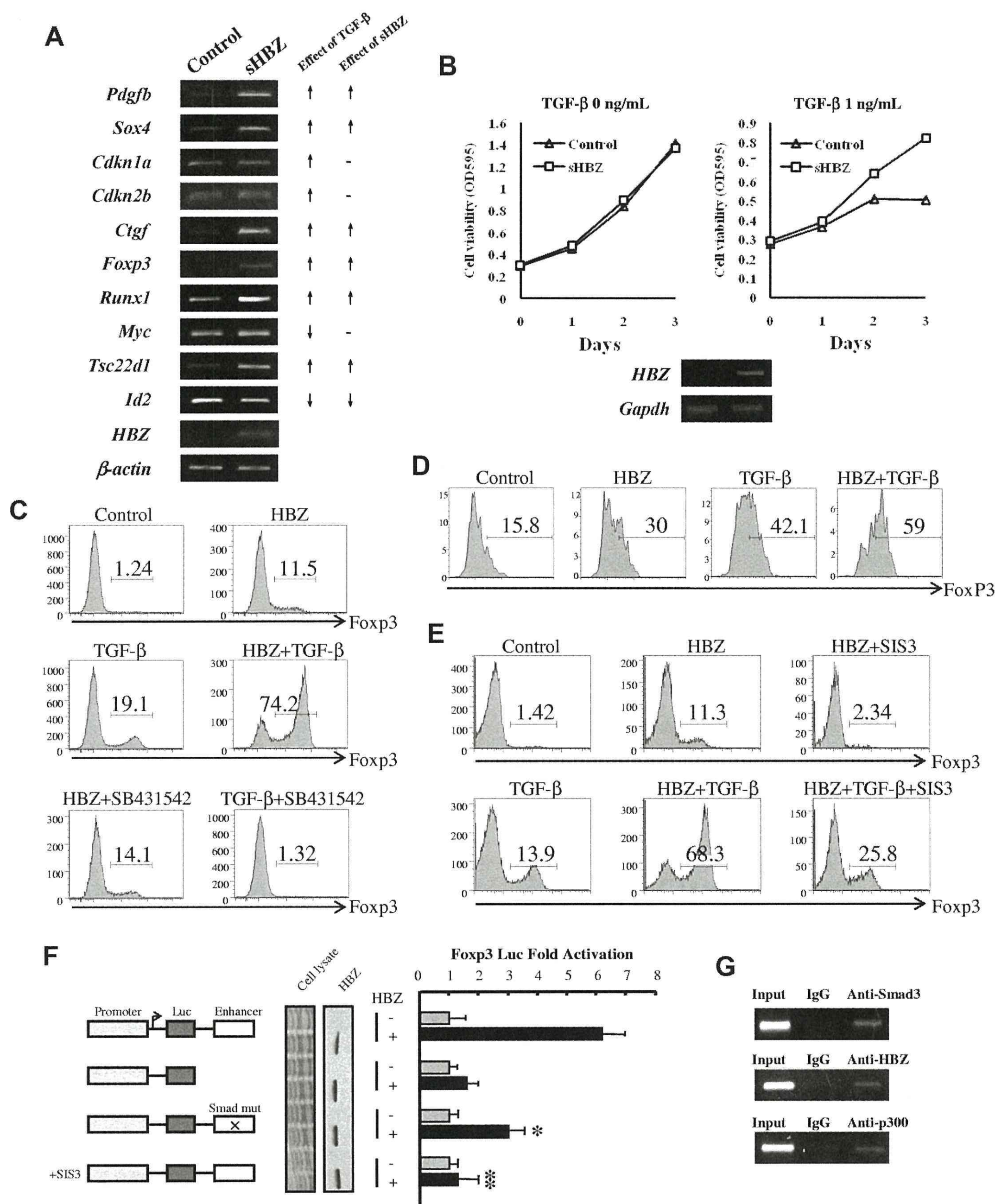

Figure 7. HBZ induced Foxp3 expression in naive T cells through Smad3. (A) HBZ modulated the expression of selected TGF-β target genes. (Left panel) Mouse naive T cells were transduced with pMXs-IG vector encoding sHBZ or empty vector. Forty-eight hours after viral infection, total RNA was extracted from sorted green fluorescent protein-positive cells. The level of *Pdgfb*, *Sox4*, *Cdkn1a*, *Cdkn2b*, *Ctgf*, *Foxp3*, *Runx1*, *Myc*, *Tsc22d1*, *Id2*, β-*actin*, and *HBZ* mRNA was analyzed by semiquantitative RT-PCR. (Right panel) Schema of the effect of TGF-β and sHBZ on TGF-β target gene transcription. ↑ indicates up-regulation; ↓, down-regulation; and -, no effect. (B) CTLL-2/pME18Sneo and CTLL-2/sHBZ, cells were plated in 96-well plates. Cells were treated with increasing concentrations of TGF-β for 72 hours. Proliferation of each cell was examined by methyl thiazolyl tetrazolium assay. Expression of *HBZ* was detected by RT-PCR (bottom panel). (C) SB431542, an inhibitor of the TGF-β receptor, could not inhibit the induction of Foxp3 by HBZ. Mouse $CD4^+CD25^-$ T cells were transduced with pGCDNsamI/N vector encoding sHBZ, or with empty vector. Three days after TGF-β (0.2 ng/mL) and SB431542 (5μM) treatment, cells were stained with anti-Foxp3 in addition to anti-NGFR and then analyzed by flow cytometry. Numbers indicate the percentage of Foxp3-positive cells among NGFR-positive cells. (D) HBZ induced FoxP3 in human naive T cells. Human $CD4^+CD25^-$ T cells were transfected with lentiviral vectors expressing sHBZ, or with empty vector. Two days after stimulating with TGF-β (0.1 ng/mL), cells were stained with antibodies for CD4, NGFR, and Foxp3 and then analyzed by flow cytometry. (E) SIS3 inhibited the HBZ-induced Foxp3 induction. Mouse $CD4^+CD25^-$ T cells were transduced with pGCDNsamI/N vector encoding sHBZ or empty vector. Fifteen hours after viral infection, SIS3 (5μM) and TGF-β (1 ng/mL) were added. Thirty-six hours after treatment, Foxp3 expression was detected by flow cytometry. Numbers indicate the percentage of Foxp3-positive cells among NGFR-positive cells. Representative data from 3 independent experiments are shown. (F) HBZ activated transcription of the *Foxp3* promoter through its Smad site of enhancer. EL4 cells were transfected with the Foxp3 reporter plasmid or its mutants with or without the sHBZ-expressing plasmid (pcDNA3.1-mycHis-sHBZ). Luciferase activity was measured 48 hours after stimulation by TGF-β. Expression of sHBZ was detected by Western blot. CBB staining was shown as the loading control. (G) HBZ formed complex with Smad3/p300 in *FoxP3* enhancer. MT-2 cells treated with 5 ng/mL of TGF-β for 2 hours, and chromatin immunoprecipitated by each indicated antibody. The precipitated DNAs and 1% of the input cell lysates were amplified by the specific primers for *FoxP3* enhancer.

growth suppressive effect of TGF-β in the early stage of ATL, whereas other mechanisms, which include increased expression of Smad7,[46] and aberrant expression of MEL1S,[27] suppress cytostatic effects of TGF-β/Smad signaling pathway when Tax expression is lost in the late stage. However, as shown in this study, HBZ-expressing CTLL-2 cells could proliferate in the presence of

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TGF-β. There are 2 possible scenarios. HBZ selectively modulates actions of TGF-β/Smad signaling pathway as shown in this study, which possibly does not influence transcription of TGF-β target genes associated with cell cycle and proliferation. Alternatively, because HBZ has growth-promoting activity,[7] it might counteract cytostatic activity of TGF-β, whereas other activities of TGF-β/Smad signaling pathway are enhanced by HBZ. It has been reported that tumors use the TGF-β/Smad system to induce gene responses that promote tumor growth, invasion, evasion of immune surveillance, and metastasis.[47] In HTLV-1, an important function of activated TGF-β/Smad pathway by HBZ is up-regulation of the *Foxp3* gene transcription and induction of Tregs. Further studies on TGF-β signaling in ATL are necessary to clarify its roles.

Dysregulation of TGF-β signaling has been reported in HTLV-1–associated HAM/TSP.[48] Expressions of TGF-β receptor II and Smad7 were suppressed in T cells of HAM/TSP patients, suggesting that these abnormalities are implicated in perturbed immune tolerance. As shown in Figure 6C, activation of TGF-β signaling by HBZ is more predominant than the suppressive effect by Tax, which might account for why more than half of ATL cases express FoxP3,[23] and the proportion of $FoxP3^+$ Tregs is higher in Tax^+ T cells derived from HTLV-1 carriers and HAM/TSP patients.[24] However, Smad7 expression is increased in ATL cells, indicating that TGF-β signaling is different between leukemic and carrier state. To elucidate the entire picture of TGF-β signaling in HTLV-1 infection and ATL needs further studies.

It has been reported that HBZ suppresses viral transcription from the HTLV-1 long terminal repeat by disturbing the interaction between Tax and p300/CBP, thereby displacing p300 from the viral promoter.[35] However, our report showed that the binding of HBZ with p300 stabilized the Smad3-p300 complex, resulting in TGF-β activation. HBZ competes with Tax for the same domain of p300, whereas HBZ and Smad3 have different p300 binding sites. We speculate that HBZ activation or repression of p300 transcriptional activation is dependent on the p300 binding partner. It is also probable that the function of the HBZ-p300 complex depends on the capacity of HBZ to recruit p300 onto the DNA element, a bimodal effect similar to that previously reported for the Tax-p300/CBP complex.[49] Consistent with our findings, a recent study reported that HBZ activated transcription of *DKK1* gene by interacting with the cellular coactivators p300 and CBP.[50]

In conclusion, we showed that HBZ enhanced TGF-β signaling by physically interacting with Smad3/p300, leading to the up-regulation of TGF-β target genes, including *Foxp3*. HTLV-1 may take advantage of this mechanism to elude host immune attack, allowing the infected cells to proliferate in vivo.

Acknowledgments

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Authorship

Contribution: T.Z., Y.S., T.I., and M.M. designed the research; T.Z., Y.S., K.S., and P.M. performed the research; P.L.G. and T.I. provided vital reagents; T.Z., Y.S., and M.M. analyzed the data; and T.Z., Y.S., P.L.G., T.I., and M.M. wrote the paper.

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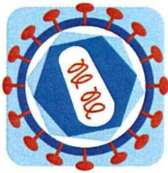

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RESEARCH

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ATF3, an HTLV-1 bZip factor binding protein, promotes proliferation of adult T-cell leukemia cells

Keita Hagiya[1], Jun-ichirou Yasunaga[1], Yorifumi Satou[1], Koichi Ohshima[2], Masao Matsuoka[1*]

Abstract

Background: Adult T-cell leukemia (ATL) is an aggressive malignancy of $CD4^+$ T-cells caused by human T-cell leukemia virus type 1 (HTLV-1). The *HTLV-1 bZIP factor* (*HBZ*) gene, which is encoded by the minus strand of the viral genome, is expressed as an antisense transcript in all ATL cases. By using yeast two-hybrid screening, we identified activating transcription factor 3 (ATF3) as an HBZ-interacting protein. ATF3 has been reported to be expressed in ATL cells, but its biological significance is not known.

Results: Immunoprecipitation analysis confirmed that ATF3 interacts with HBZ. Expression of ATF3 was upregulated in ATL cell lines and fresh ATL cases. Reporter assay revealed that ATF3 could interfere with the HTLV-1 Tax's transactivation of the 5' proviral long terminal repeat (LTR), doing so by affecting the ATF/CRE site, as well as HBZ. Suppressing ATF3 expression inhibited proliferation and strongly reduced the viability of ATL cells. As mechanisms of growth-promoting activity of ATF3, comparative expression profiling of ATF3 knockdown cells identified candidate genes that are critical for the cell cycle and cell death, including cell division cycle 2 (CDC2) and cyclin E2. ATF3 also enhanced p53 transcriptional activity, but this activity was suppressed by HBZ.

Conclusions: Thus, ATF3 expression has positive and negative effects on the proliferation and survival of ATL cells. HBZ impedes its negative effects, leaving ATF3 to promote proliferation of ATL cells via mechanisms including upregulation of CDC2 and cyclin E2. Both HBZ and ATF3 suppress Tax expression, which enables infected cells to escape the host immune system.

Background

Adult T-cell leukemia (ATL) is an aggressive $CD4^+$ T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1) [1-5]. In the plus strand of its genome, HTLV-1 encodes the regulatory proteins Tax and Rex and the accessory proteins p12, p30, and p13. The *HTLV-1 basic leucine zipper factor* (*HBZ*) gene is expressed as an antisense transcript. It has been reported that *HBZ* is consistently expressed and remains intact in all ATL cases and HTLV-1-infected individuals [6,7], where it promotes cell proliferation [6,8].

The *HBZ* gene is expressed as two isoforms: spliced HBZ (sHBZ) and unspliced HBZ (usHBZ) [9-12]. The expression of sHBZ in T-cells promotes T-cell proliferation whereas that of usHBZ does not [8,12]. HBZ was reported to repress Tax-mediated transactivation of viral transcription from the HTLV-1 promoter by dimerizing with transcription factors including cyclic AMP response element-binding protein 2 (CREB2), and members of the Jun family [10,13-16]. HBZ also promotes the degradation, directly and without ubiquitination, of some proteins that interact with HBZ [17]. Thus, HBZ interacts with host factors and modulates their function, which is likely to contribute to persistent infection of HTLV-1 *in vivo* and clonal expansion of infected cells.

Activating transcription factor 3 (ATF3) is a member of the ATF/cyclic AMP response element-binding (CRE) family of transcription factors [18]. *ATF3* is an adaptive response gene whose expression is regulated by changes in the extra- or intracellular environment. ATF3 activates signals including DNA damage [19], anoxia [20], hypoxia [21], and represses others, including inflammation [22].

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It can form homodimers or hetrodimers with other cellular bZIP transcription factors, including ATF2, c-Jun, JunB, and JunD, and exerts pleiotropic functions through ATF/CRE and AP-1 sites depending on cell type. It has also been pointed out that the *ATF3* gene has a potential dichotomous role in cancer development [23]: it has proapoptotic functions, like a tumor suppressor, but at the same time induces cell proliferation, like an oncogene. It has been reported as up-regulated in malignant breast cancer cells [23], Hodgkin cells [24], and prostate cancer cells [25] where it is associated with proliferation. Transgenic mice overexpressing ATF3 in basal epithelial cells develop basal cell carcinomas [26]. Up-regulation of ATF3 is also reported in ATL cells [27], yet the biological significance in ATL is not known. Moreover, the question of how ATF3 induces proliferation of cancer cells remains unsolved.

In the process of elucidating the function of sHBZ in T-cells [6,12,28], we identified ATF3 as a sHBZ-interacting protein. In this study, we characterized the role of ATF3 in ATL cells. ATF3 was constitutively expressed in ATL cell lines and fresh ATL cases. ATF3 could repress Tax-mediated transactivation through ATF/CRE sites. Expression of ATF3 was linked to proliferation of ATL cells via upregulation of cell cycle-associated genes and down-regulation of proapoptotic genes. Furthermore, while ATF3 alone enhanced p53 stability, and therefore activation; sHBZ inhibited this function.

Results

Identification of ATF3 as a sHBZ interacting protein

We employed a yeast two-hybrid system with sHBZ as bait, to identify potential binding partners for sHBZ. Human activated mononuclear cell RP1 libraries were used for this screening and several candidates were identified (data not shown). Among them, we focused on ATF3 for the following reasons: First, ATF3 was reported to play a role in both survival and proliferation of cancer cells [25,29-31]. Second, *ATF3* transcript is expressed in ATL cells [27] although little is known about the biological significance of this expression, in particular whether expression of ATF3 is associated with ATL cell proliferation [27]. Third, the relation between ATF3 and HTLV-1 viral transcription is unknown. Immunoprecipitation analysis demonstrated that sHBZ and ATF3 interacted when transfected in mammalian cells (Figure 1). By using a series of truncated proteins, we found that bZIP domains of both sHBZ and ATF3 are necessary for their interaction.

The ATF3 promoter is constitutively activated in ATL cell lines

Next, we checked the expression level of *ATF3* mRNA and protein in ATL cell lines. The *ATF3* gene has two promoters: a non-canonical alternative promoter P1 and the canonical promoter P2 (Figure 2A) [32,33]. RT-PCR analysis demonstrated that all ATL cell lines constitutively expressed the *ATF3* P1 and P2 transcripts (Figure 2B). ATF3 protein expression was also detected in all ATL cell lines (Figure 2B). In addition, all ATL cell lines expressed the *sHBZ* gene transcript while the *tax* gene was transcribed in only some ATL cell lines, consistent with earlier reports (Figure 2B) [6]. Although these data suggested that sHBZ expression might be associated with increased ATF3, ectopic expression of sHBZ did not induce *ATF3* gene transcription in Jurkat cells (data not shown). Immunohistochemical analysis of lymph nodes of ATL patients showed that lymphoma cells indeed expressed ATF3 (Figure 2C).

Suppressive effects of ATF3 on cellular and viral ATF/CRE sites

It has been reported that Tax activates the transcription of the plus strand of HTLV-1 as well as influencing host cellular gene transcription. Tax transcription of HTLV-1 genes depends on ATF/CRE-like sequences (viral CRE) in the U3 region of the HTLV-1 LTR [34,35]. ATF3, on the other hand, is reported to repress transcription from cellular ATF/CRE sites [36]. Based on these findings, we investigated whether ATF3 could influence Tax-mediated transcription. pCRE × 4-luc is a reporter construct containing a cellular ATF/CRE consensus sequence, while WT-luc contains ATF/CRE-like sequences from the HTLV-1 LTR. These plasmid DNAs were used to study the effect of ATF3 on transcription through cellular and viral CREs. Tax could activate the cellular and viral CRE reporters, but ATF3 by itself did not influence their activity (Figure 3A and 3B). ATF3 inhibited Tax-mediated transcription from ATF/CRE and viral CRE sites in a dose-dependent manner (Figure 3A and 3B). sHBZ also repressed Tax-mediated transcription, as reported previously [10]. When ATF3 and sHBZ were co-expressed, sHBZ did not inhibit the repressive function of ATF3. Next we checked the effect of ATF3 on Tax-mediated viral transcriptional activity. A reporter construct containing the entire HTLV-1 5'LTR was activated by Tax, as expected (Figure 3C). ATF3 repressed this transcription (Figure 3C). sHBZ also repressed Tax-mediated activation of this reporter, without interfering with the suppressive function of ATF3. These results suggest that ATF3 suppresses Tax-mediated ATF/CRE-dependent transcription both of cellular genes and the HTLV-1 LTR.

ATF3 has growth promoting activity in ATL cells

To investigate the functional significance of ATF3 expression in ATL cells, we transfected MT-4 and ED cells with lentiviral vectors expressing three different

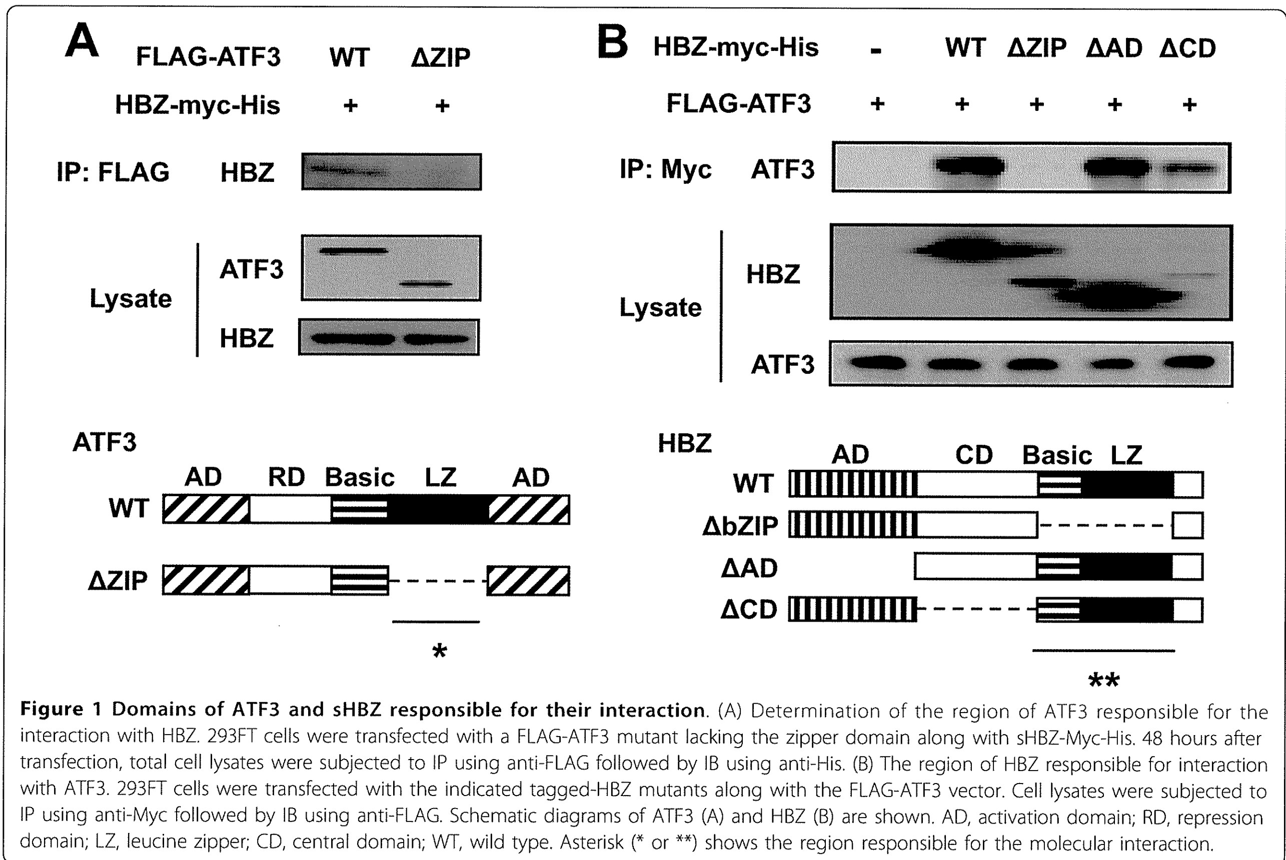

Figure 1 Domains of ATF3 and sHBZ responsible for their interaction. (A) Determination of the region of ATF3 responsible for the interaction with HBZ. 293FT cells were transfected with a FLAG-ATF3 mutant lacking the zipper domain along with sHBZ-Myc-His. 48 hours after transfection, total cell lysates were subjected to IP using anti-FLAG followed by IB using anti-His. (B) The region of HBZ responsible for interaction with ATF3. 293FT cells were transfected with the indicated tagged-HBZ mutants along with the FLAG-ATF3 vector. Cell lysates were subjected to IP using anti-Myc followed by IB using anti-FLAG. Schematic diagrams of ATF3 (A) and HBZ (B) are shown. AD, activation domain; RD, repression domain; LZ, leucine zipper; CD, central domain; WT, wild type. Asterisk (* or **) shows the region responsible for the molecular interaction.

ATF3-directed shRNAs. These shRNA expressions strongly suppressed ATF3 protein expression shown in Figure 4A. An MTT assay showed that knockdown (KD) of ATF3 resulted in reduced proliferation of both Tax expressing MT-4 cells and Tax non-expressing ED compared to control cells (Figure 4B). Cell cycle analysis revealed that the population of G1 cells increased, while the population of cells in S phase decreased in ATF3 KD MT-4 cells (Figure 4C). KD of ATF3, then, impaired the G1/S transition in MT-4 cells, and hence ATF3 expression in ATL cell lines was associated with their proliferation.

Transcriptional profile of ATF3 KD MT-4 cell

To find mechanisms by which ATF3 might increase proliferation, we performed oligonucleotide microarray analyses of ATF3-KD MT-4 cells and MT-4 cells transduced with a control vector. We compared the data from the negative control and ATF3-KD cells, and out of 18,400 transcripts, we first identified 2188 genes whose transcription changed more than two fold by KD. Of these, 1522 genes were up-regulated, and 658 down-regulated in ATF3-KD cells. Representative genes that were up-regulated or down-regulated by ATF3 are shown in Figure 5A and additional file 1.

We confirmed the expression of several up-regulated genes by RT-PCR to validate the results of the DNA microarray (Figure 5B). Suppressed expression of ATF3 increased the number of transcripts of proapoptotic genes, *Bim* and *Harakiri*. In contrast, cell division cycle 2 (CDC2) and cyclin E2 (CCNE2), which control the cell transition from G1 phase to S phase [37], were down-regulated in ATF3-KD cells. This is the first report that ATF3 affects the expression of these genes.

CDC2 is a direct target of ATF3

Since KD of ATF3 impairs the G1/S transition, we focused on *cdc2* and *ccne2* gene expression. Quantitative analysis by real-time PCR confirmed that transcription of both the *cdc2* and *ccne2* genes was down-regulated in ATF3 KD cells compared to control cells (Figure 5C). The *cdc2* gene expression was significantly decreased by KD of ATF3, so *cdc2* gene was chosen for further studies. To study whether the effect of ATF3 on the *cdc2* gene is direct or indirect, we investigated the binding of ATF3 to the promoter region of the *cdc2* gene (Figure 5D). This region contains two putative binding sites for ATF3, an AP-1 site near the transcription start site, and an ATF/CRE site farther 5'-ward (Figure 5D). A chromatin

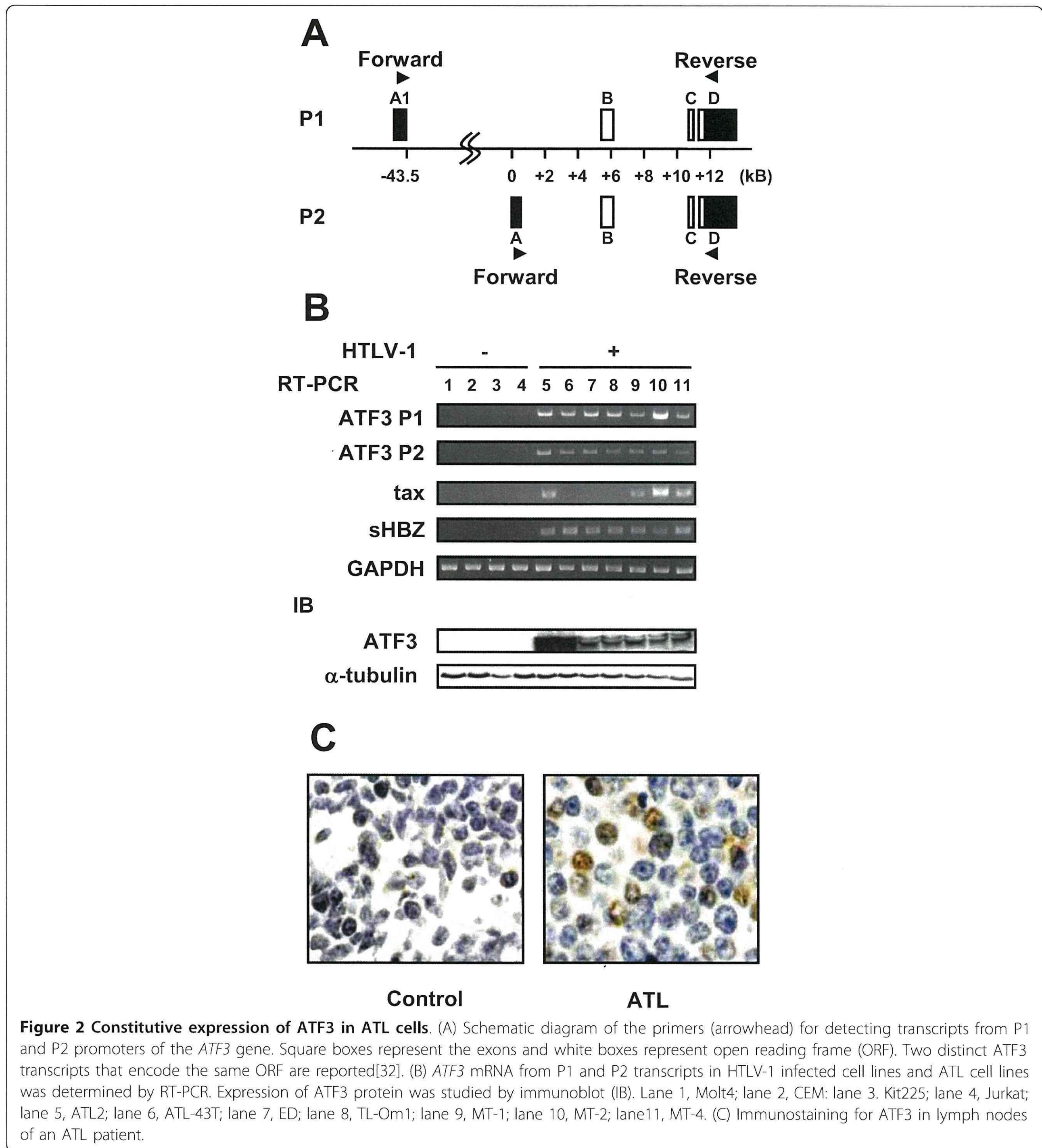

Figure 2 Constitutive expression of ATF3 in ATL cells. (A) Schematic diagram of the primers (arrowhead) for detecting transcripts from P1 and P2 promoters of the *ATF3* gene. Square boxes represent the exons and white boxes represent open reading frame (ORF). Two distinct ATF3 transcripts that encode the same ORF are reported[32]. (B) *ATF3* mRNA from P1 and P2 transcripts in HTLV-1 infected cell lines and ATL cell lines was determined by RT-PCR. Expression of ATF3 protein was studied by immunoblot (IB). Lane 1, Molt4; lane 2, CEM: lane 3. Kit225; lane 4, Jurkat; lane 5, ATL2; lane 6, ATL-43T; lane 7, ED; lane 8, TL-Om1; lane 9, MT-1; lane 10, MT-2; lane11, MT-4. (C) Immunostaining for ATF3 in lymph nodes of an ATL patient.

immunoprecipitation assay detected ATF3 bound to the proximal AP-1 site, but ATF3 bound to ATF site was non-specific (Figure 5E). Transient transfection of Jurkat T cells by electroporation with a vector expressing ATF3 up-regulated the expression of *cdc2* mRNA (Figure 5F). These results indicate that ATF3 directly activates transcription of the *cdc2* gene.

sHBZ inhibited the augmentation of p53 transcriptional activity by ATF3

In addition to its oncogenic function, ATF3 is also reported to augment transactivation of p53 responsive promoters in a non-small cell lung carcinoma cell line by protecting p53 from ubiquitin-associated degradation [31,38]. Expression of ATF3 in ATL cells therefore has

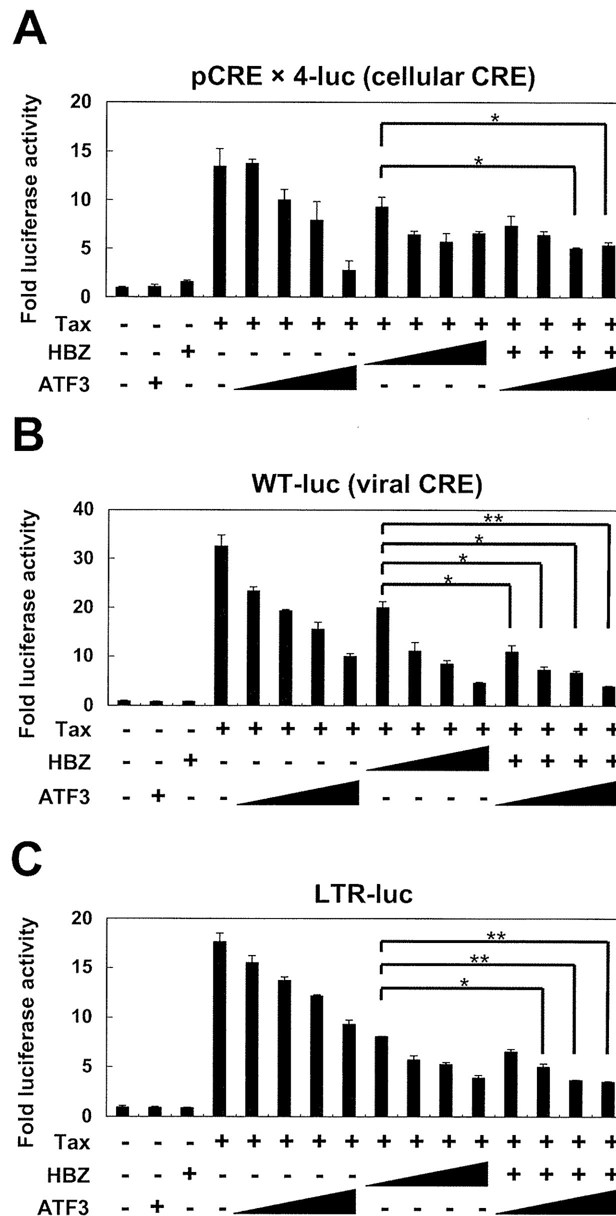

Figure 3 Suppressive effects of ATF3 on Tax-mediated transactivation through ATF/CRE sites. Jurkat cells were cotransfected with phRL-TK and expression vectors for ATF3, HBZ, and reporter plasmid pCRE × 4-luc (A), WT-luc (B), or LTR-luc (C) respectively. The total amount of DNA for transfection was equalized by adding empty vectors. After 24 hours, a dual luciferase reporter assay was performed as described in Materials and Methods. All the data are relative values of firefly luciferase normalized to Renilla luciferase and shown as a mean of a triplicate set of experiments (mean ± SD). $^{*}P$ <0.05; $^{**}P$ <0.01.

the potential to promote apoptosis through p53, since mutations of p53 are rare in ATL cases [39]. To explore this possibility, we checked the ability of ATF3 to augment p53 transcriptional activity in T-cells. A reporter assay showed that, as reported previously [31,38], ATF3 enhanced transcriptional activity of p53 in ZIP domain dependent manner (Figure 6A and 6B). sHBZ, though it had no influence on p53 transcriptional activity itself, suppressed the increased transcriptional activity of p53 by ATF3 (Figure 6A). Analyses using sHBZ deletion mutants showed that the bZIP domain and the central domain of sHBZ were responsible for the suppressive activity (Figure 6B). To investigate how sHBZ reduces ATF3's ability to enhance p53 transcriptional activity, immunoprecipitation analyses were performed (Figure 6C). ATF3 interacted with p53 but sHBZ reduced this interaction. Serial immunoprecipitation experiments demonstrated that sHBZ, ATF3 and p53 were present in

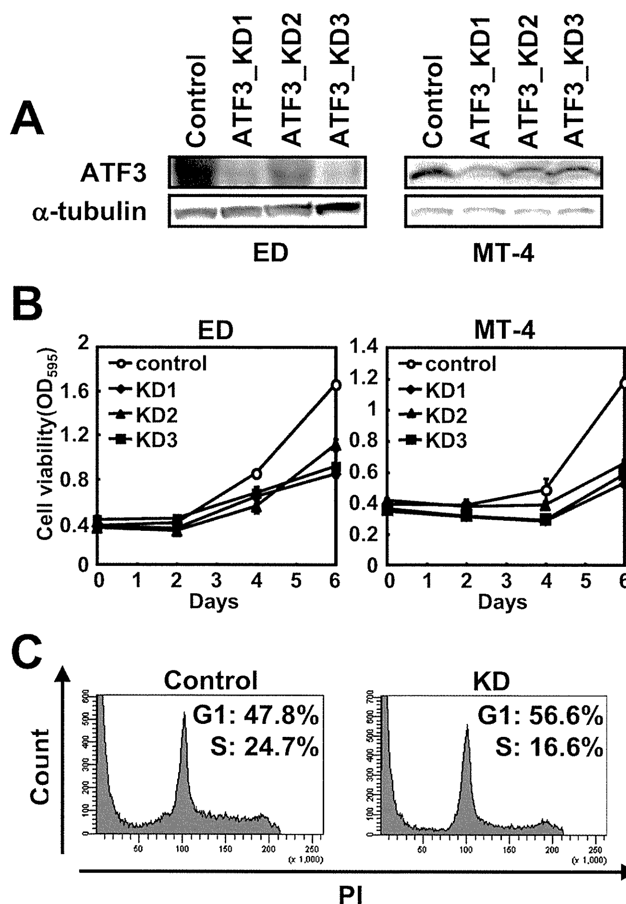

Figure 4 Knockdown of ATF3 by shRNA impairs proliferation of ATL and HTLV-1 infected cells. MT-4 and ED cells were transduced with lentivirus vector expressing control and ATF3-directed shRNA. (A) ATF3 protein was determined by immunoblot. (B) The cell growths of ATF3 knock-down ATL cells by shRNAs were measured by MTT assay. (C) The effect of ATF3 KD using ATF3_KD1 on cell cycle progression was analyzed by PI staining in MT-4 cells. Five days after infection, cells were analyzed by a flow cytometry as described in the Materials and Methods.

a complex together (Figure 6D). We propose that sHBZ binds directly to ATF3-p53 complexes; that this binding interferes, by unknown mechanisms, with ATF3 enhancement of p53 signaling; and that ATL cells expressing sHBZ can thereby escape the apoptosis that ATF3 expression might otherwise induce.

Discussion

In this study, a yeast two-hybrid system identified ATF3 as a binding partner of the HTLV-1 sHBZ protein. Aberrant expression of ATF3 has been reported in classical Hodgkin lymphoma (cHL) and malignant prostate cancer cell [24,25], where it is associated with increased proliferation. In addition, increased expression of ATF3 was also reported in ATL cases [27]. However, the mechanism by which ATF3 promotes proliferation of cancer cells remained unknown. In this study, we demonstrated that increased expression of ATF3 was linked to proliferation via enhanced transcription of the *cdc2* and *ccne2* genes, along with suppressed expression of proapoptotic factors including Harakiri, and Bim. ATF3 indeed bound to the promoter region of the *cdc2* gene and enhanced its transcription. Thus, ATF3 modulates transcription of cellular genes associated with proliferation and apoptosis.

ATF3 has been reported to act as transcriptional repressor of ATF/CRE sequences. In this study, we found that ATF3 suppressed activation, by the viral factor Tax, of transcription from CRE-like sequences in the 5'LTR. Tax, itself transcribed from the 5'LTR, is a major target of cytotoxic T-lymphocytes *in vivo* [40]. Therefore, suppression of *tax* gene transcription could benefit the survival of ATL cells, by allowing them to escape a cytotoxic T-lymphocyte response. In contrast to the *tax* gene, ATL cells need to express the *HBZ* gene transcripts for their proliferation [5]. HBZ is transcribed from the 3'LTR, and therefore unaffected by ATF3 suppression of the 5'LTR. By suppressing viral gene

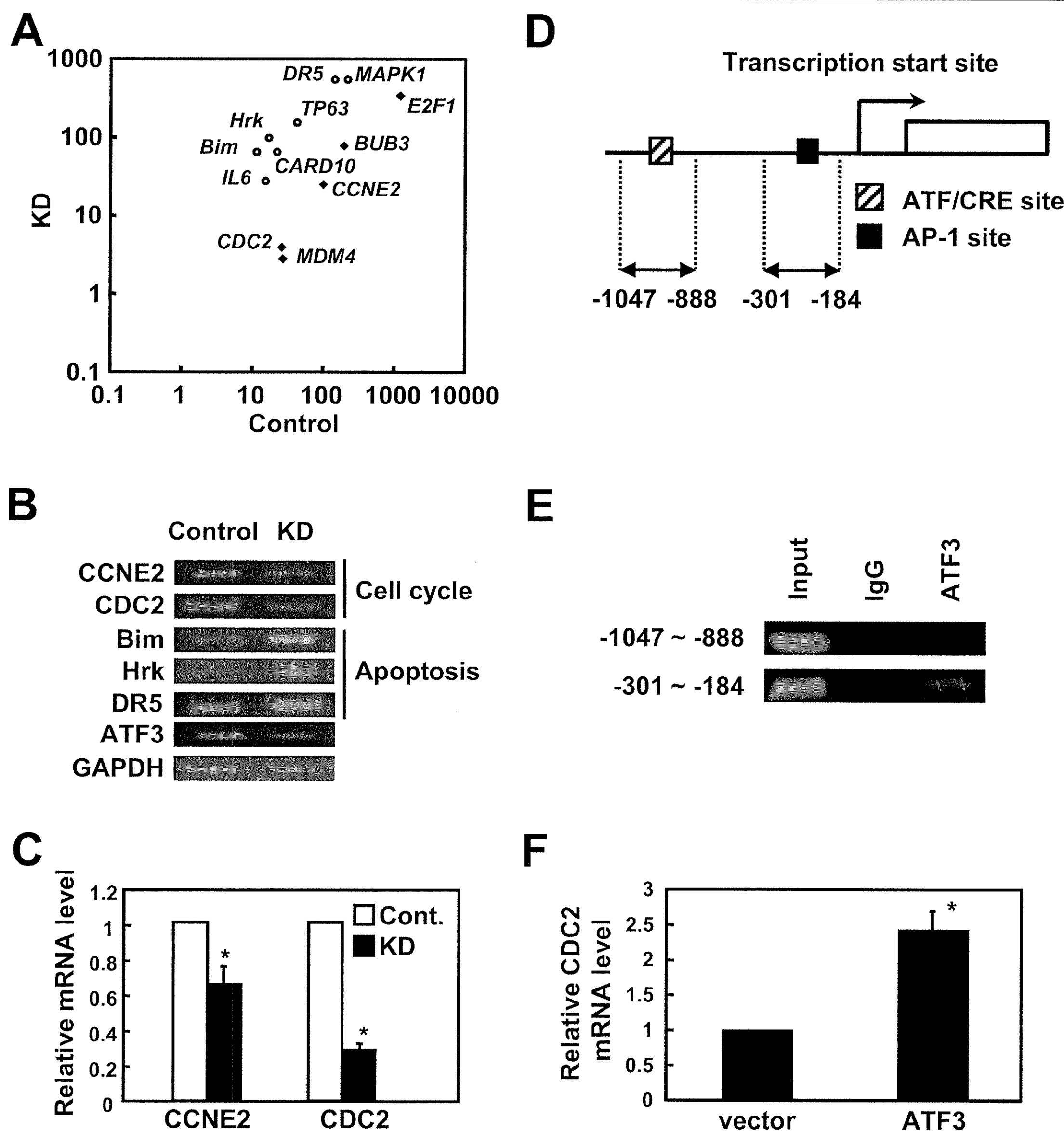

Figure 5 CDC2 is a direct target of ATF3. (A) The ratios of transcripts (Control/ATF3 KD populations) of 12 genes related to the cell cycle or apoptosis in the 2 groups are plotted. Open circles represent the up-regulated genes and black lozenges show the down-regulated genes. (B) The level of mRNA was studied by semi-quantitative RT-PCR to confirm the result of microarray analysis. (C) Control and ATF3 KD cells were analyzed by real-time PCR for the indicated mRNA. The expression level of control cells was defined as 1. Mean ± SD was based on results of three independent experiments ($P < 0.01$). (D) Schematic diagram of CDC2 primer used for ChIP assay. (E) 293FT cells were transfected with ATF3 expression vector. 48 hours after transfection, chromatin was prepared for a ChIP assay using an anti-ATF3 antibody. Anti-IgG was used as a negative control. (F) Jurkat cells were transiently transfected with ATF3 expression vector and CDC2 mRNA expression was measured by real-time PCR.

transcription through the 5'LTR, then, ATF3 modulates viral gene expression, favoring expression of the *HBZ* gene over the *tax* gene. Enforced expression of ATF3 in prostate cancer cells induces cell proliferation and accelerates progression from the G1- to S-phase of the cell cycle [25]. The same study also showed that KD of ATF3 expression decreased cells in S phase while it increased cells in G1 phase [25]. In addition, impaired G1/S transition in c-myc null cells was partially recovered by ATF3 expression [30], indicating the role of ATF3 in G1/S transition.

In this report, we present evidence that the expression of ATF3 is associated with G1/S progression via enhanced transcription of the *cdc2* and *ccne2* genes, and possibly others. In particular, ATF3 bound the CDC2 promoter directly. The *cdc2* gene plays a key role in the

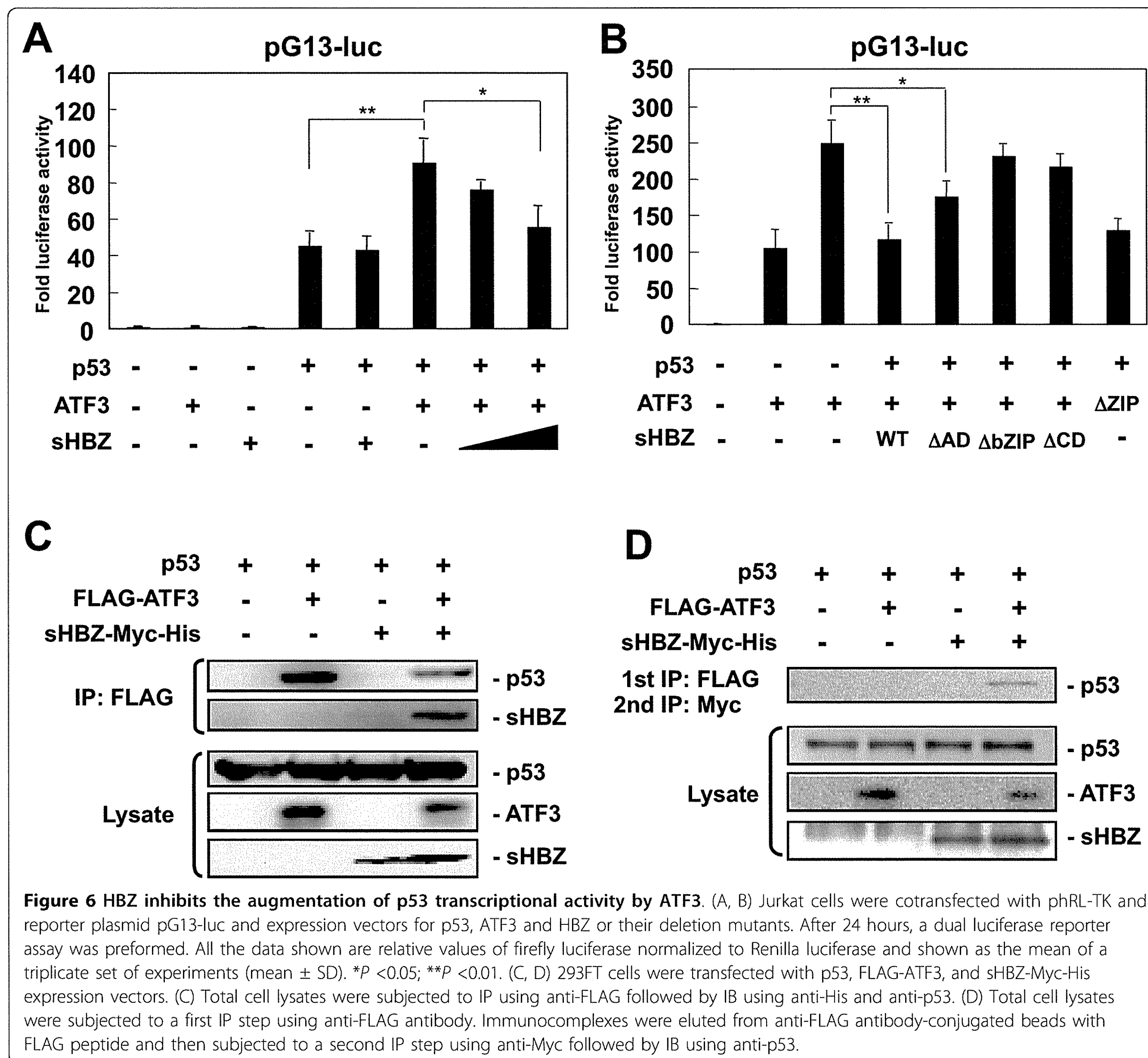

Figure 6 HBZ inhibits the augmentation of p53 transcriptional activity by ATF3. (A, B) Jurkat cells were cotransfected with phRL-TK and reporter plasmid pG13-luc and expression vectors for p53, ATF3 and HBZ or their deletion mutants. After 24 hours, a dual luciferase reporter assay was preformed. All the data shown are relative values of firefly luciferase normalized to Renilla luciferase and shown as the mean of a triplicate set of experiments (mean ± SD). **P* <0.05; ***P* <0.01. (C, D) 293FT cells were transfected with p53, FLAG-ATF3, and sHBZ-Myc-His expression vectors. (C) Total cell lysates were subjected to IP using anti-FLAG followed by IB using anti-His and anti-p53. (D) Total cell lysates were subjected to a first IP step using anti-FLAG antibody. Immunocomplexes were eluted from anti-FLAG antibody-conjugated beads with FLAG peptide and then subjected to a second IP step using anti-Myc followed by IB using anti-p53.

transition from the G1 phase to the S phase [41], and from the G2 phase to the M phase. The *ccne2* gene is reported to be highly expressed in a number of human primary tumors including breast, ovary, uterus, brain, and lung [42]. Our results now open the possibility that *ccne2*, as well as *cdc2*, may contribute to ATL as well.

Independent of its cell cycle-promoting function, ATF3 also acts like a tumor suppressor, enhancing p53 transcriptional activity by inhibiting its ubiquitin-mediated degradation [31,38]. ATF3 neither interferes with the p53-MDM2 interaction nor blocks the E3 ligase activity of MDM2, suggesting that binding of ATF3 to p53 likely induces a conformational change of p53 that inhibits ubiquitination [31,38]. Since *ATF3* is an adaptive response gene that responds to extra or intracellular changes, ATF3 stabilization of p53 counters cellular stress due to environmental insult and ensures genomic integrity [31,38]. Given that *p53* is mutated in only about 30% of ATL cases [43-45], and in fact the expression level of p53 protein increases in ATL cells [46], how is ATF3's p53-stabilizing activity consistent with the chromosome instability often observed in ATL cells [47]? In fact, post-translational inactivation of p53 is critical to understanding ATL development. A viral protein, Tax, can functionally inactivate p53 by competing for binding to E-box [48], as well as other mechanisms [49]. However, Tax is not expressed in many ATL cases, due to genetic and epigenetic changes of the HTLV-1 provirus [5,50], including nonsense mutations generated by APOBEC3G [51]. Mechanisms other than Tax must

therefore interfere with p53 signaling. As shown in this study, sHBZ binds to ATF3-p53 complexes. With these interactions, sHBZ reduces ATF3's ability to enhance p53 function. HTLV-1 is not unique in deploying viral proteins to perturb p53 function. The latency-associated nuclear antigen encoded by Kaposi's sarcoma-associated herpesvirus, for example, binds to von Hippel-Lindau factor and targets it for degradation[52]. The human papilloma virus-encoded E6 protein binds to the cellular E6-associated protein (E6AP), an ubiquitin ligase that targets p53 for destruction. In fact, this interaction is blocked by ATF3, revealing another way in which ATF3 reinforces p53 signaling [53].

In HTLV-1's case, sHBZ perturbs one ATF3 function - p53 stabilization - that might slow the proliferation of infected cells, while leaving other functions - promotion of G1/S transition, and repression of provirus transcription - unaffected. HTLV-1 reproduces mainly by promoting the clonal expansion of infected cells, rather than by producing new virus particles. As such, the potential benefits to the virus of modulating ATF3 function in this way are clear: ATF3, in combination with sHBZ, encourages infected cells to progress through the G1/S phase transition, unimpeded by a ATF3-p53 response, and free from detection by host immune cells that might recognize viral antigens transcribed from the 5'LTR.

Conclusions

This study reveals a role of ATF3 in regard to proliferation and viral gene transcription in ATL cells. The combined effects of ATF3 and sHBZ allow ATL cells to survive *in vivo*, and could be a target of therapy for this malignant disease.

Methods

Cell lines

All T-cell lines and ATL cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. 293FT cells were cultured in Dulbecco modified Eagle medium supplemented with 10% FBS and 500 μg/ml G418.

Yeast two-hybrid

A yeast two-hybrid screen was performed by Hybrigenics (http://www.hybrigenics.com) on a random-primed Leukocytes and Activated Mononuclear Cells cDNA library using HBZ as bait.

Plasmids

The ATF3 coding sequence was amplified by polymerase chain reaction (PCR) and was cloned into pCMV-Tag2 (Stratagene, La Jolla, CA), or pcDNA3 (Invitrogen, Carlsbad, CA). Expression vectors for sHBZ [28], its deletion mutants [28], reporter plasmids pWT-luc, pLTR-luc [34,35], and pG13-luc [54] were described previously. pCREx4-luc was purchased from Stratagene (La Jolla, CA). Luciferase assay was performed as described previously [12].

Knockdown analysis

Cells were infected with an shRNA lentiviral vector (Invitrogen) directed against ATF3. The following target sequence were chosen: ATF3_KD1 5'-GAGCTGAG-GTTTGCCATCC-3', ATF3_KD2 5'-GTGTATTGTC-CGGGCTCAG-3' and ATF3_KD3 5'-GAACGAGAA GCAGCATTTG-3' as described previously [24]. Control cells were infected with an shRNA retroviral vector expressing a nonsilencing construct provided also by Invitrogen that does not target any known vertebrate gene as described in manufacture's instruction.

Proliferation assay and cell cycle analysis

Cell viability was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [55]. In cell cycle analysis, after cell fixation with 70% ethanol, cells were suspended in 50 μg/ml Propidium Iodide solution containing 0.1 mg/ml RNase A and 0.05% Triton X-100 for 40 min at 37°C and were analyzed by flow cytometry.

Immunohistochemical analyses

The tissue specimens were obtained from human lymph nodes filed at the Department of Pathology at Kurume University. The study of clinical samples was approved by the local research ethics committee of the Kurume University. Tissue samples were fixed in 10% formalin in phosphate buffer and then embedded in paraffin and analyzed by immunohistochemical methods to determine ATF3 expression. Images were captured using a Provis AX80 microscope equipped with an OLYMPUS DP70 digital camera, and detected using a DP manager system (Olympus, Tokyo, Japan).

Electroporation

Electroporation was performed with Neon™ transfection system (Invitrogen). Electroporation parameters for Jurkat cell were those recommended by Invitrogen.

RNA isolation, Reverse transcriptase (RT)-PCR, real-time PCR

Total RNAs were extracted using TRIZOL (Invitrogen) according to the manufacturer's protocol. Primers for the *ATF3*, *HBZ*, and *tax* genes were described previously[6,32] The Power SYBR Green PCR Master Mix (Qiagen, Venlo, Netherlands) was used in real-time PCR analysis in triplicate with β-actin as an internal control. In general, the threshold cycle numbers for actin in different cells are very close, and the relative mRNA level

for the gene of interest is calculated as $2^{[Ct\ (actin)-Ct\ (gene)]}$, where Ct is threshold cycle number. Primers were 5'-TGGAAACCAGGAAGCCTAGC-3' (sense) and 5'-GAAATTCGTTTGGCTGGATCAT-3' (antisense) for CDC2; 5'-GAATGTCAAGACGAAGTA-3' (sense) and 5'-ATGAACATATCTGCTCTC-3' (antisense) for CCNE2.

Oligonucleotide microarray analysis

RNA processing and hybridization to U133 Plus 2.0 GeneChip microarrays were performed according to the manufacturer's protocol (Affimetrix, Santa Clara, CA). Data were analyzed with the GeneSpring GX 10 software (Agilent Technologies, Palo Alto, CA).

Immunoprecipitation (IP) and immunoblotting

Cell lysates were incubated with anti-His-Tag (PM002) (MBL, Nagoya, Japan), anti-c-myc (clone 9E10) and anti-FLAG M2 antibodies (Sigma-Aldrich, St Louis, MO) for 1 hour at 4°C, and immune complexes were incubated with protein G-sepharose (GE Healthcare, Little Chalfont, UK) for 1 hour at 4°C. The following antibodies were used for immunoblot: anti-ATF3 (Santa Cruz Biotechnologies, Santa Cruz, CA); anti-His-Tag (PM002) (MBL); anti-FLAG M2 and anti-p53-biotin (Sigma-Aldrich); peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG or streptavidin-biotinylated horseradish peroxidase complex (GE Healthcare). To detect ATF3 using anti-ATF3 antibody, Immuno-enhancer (Wako, Osaka, Japan) was used.

Serial IP

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail), and incubated for 1 hour at 4°C. For the first IP, after clarification by low-speed centrifugation, the supernatants were incubated with anti-FLAG M2 Affinity gel (Sigma-Aldrich) for 3 h at 4°C. The FLAG-agarose beads were then washed with lysis buffer and the bound proteins were eluted with FLAG elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.5 mg/ml FLAG peptide [Sigma]) for 1 hr at 4°C. For the second IP, after the FLAG-agarose beads were removed by centrifugation, the supernatants were incubated with anti-c-Myc for 1 hr at 4°C.

Additional material

Additional file 1: Figure S1. Identification of candidate genes regulated by ATF3 expression. Oligonucleotide microarray data for control and ATF3 KD MT-4 cells were subjected to cluster analysis with the GeneSpring GX 10 software. Each column represents expression level of a given gene. Red represents increased expression and green represents decreased expression relative to the normalized expression of the gene across all samples.

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Authors' contributions
This study was designed by KH and MM. Laboratory analysis was performed by KH. Data analysis was performed by KH, SY, YJ and MM. Samples and data were provided by OK. KH and MM wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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