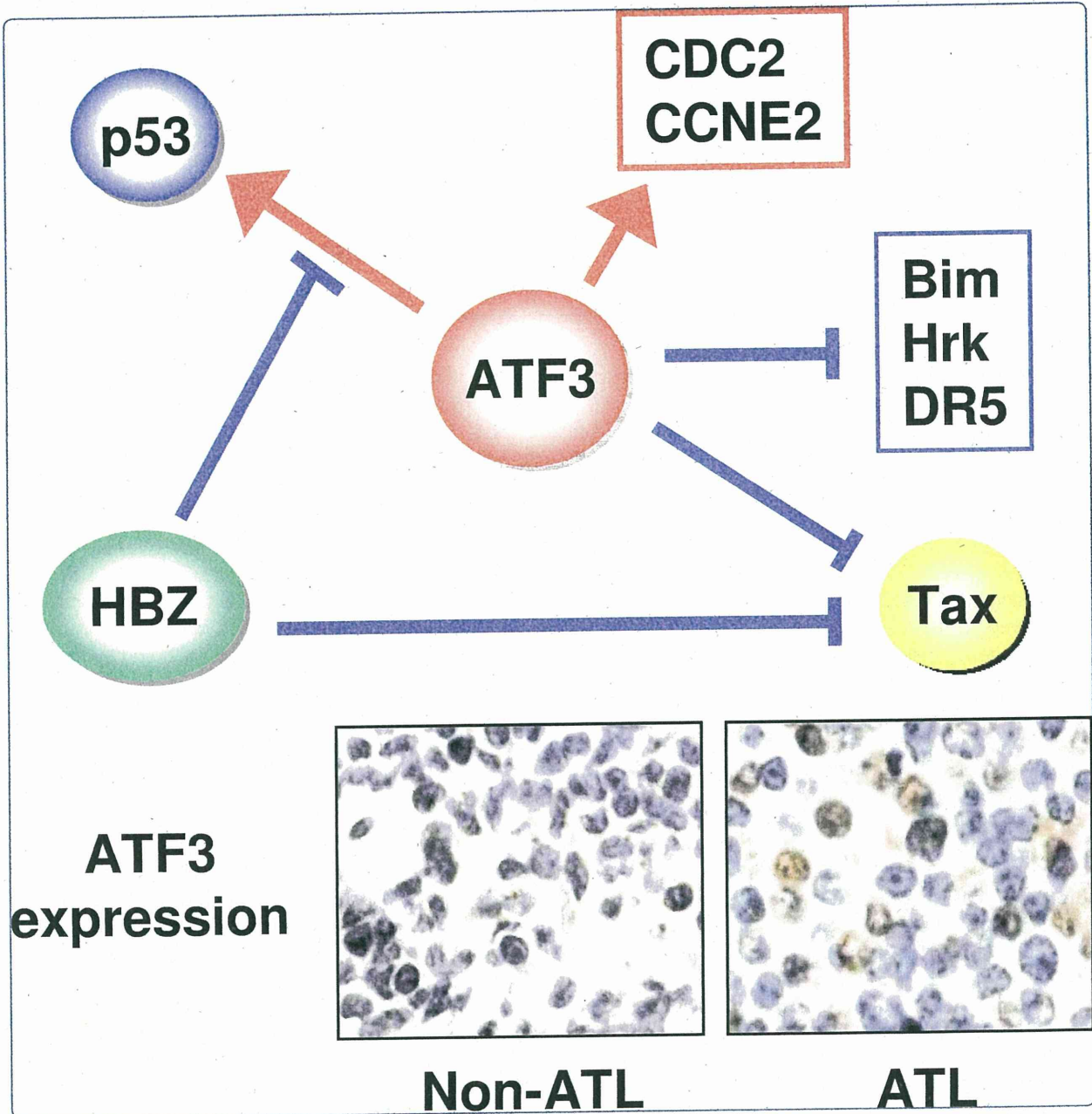
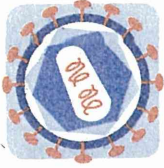


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ATF3, an HTLV-1 bZip factor binding protein, promotes proliferation of adult T-cell leukemia cells

Hagiya *et al.*



RESEARCH

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

Keita Hagiya¹, Jun-ichirou Yasunaga¹, Yorifumi Satou¹, Koichi Ohshima², 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 heterodimers 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

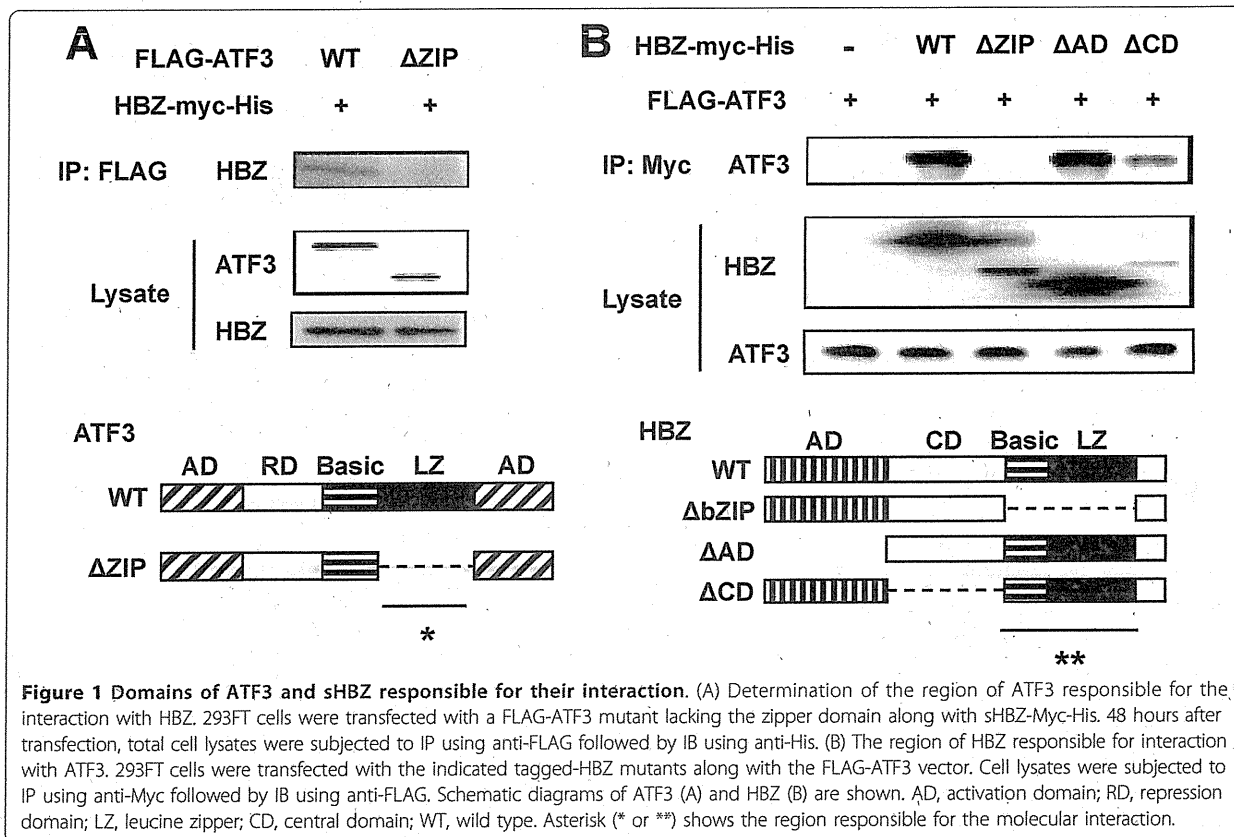
promoters: a non-canonical alternative promoter P1 and the canonical promoter P2 (Figure 2A) [32,33]. RT-PCR analysis demonstrated that all ATL cell lines constitutively expressed the *ATF3* P1 and P2 transcripts (Figure 2B). ATF3 protein expression was also detected in all ATL cell lines (Figure 2B). 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



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.

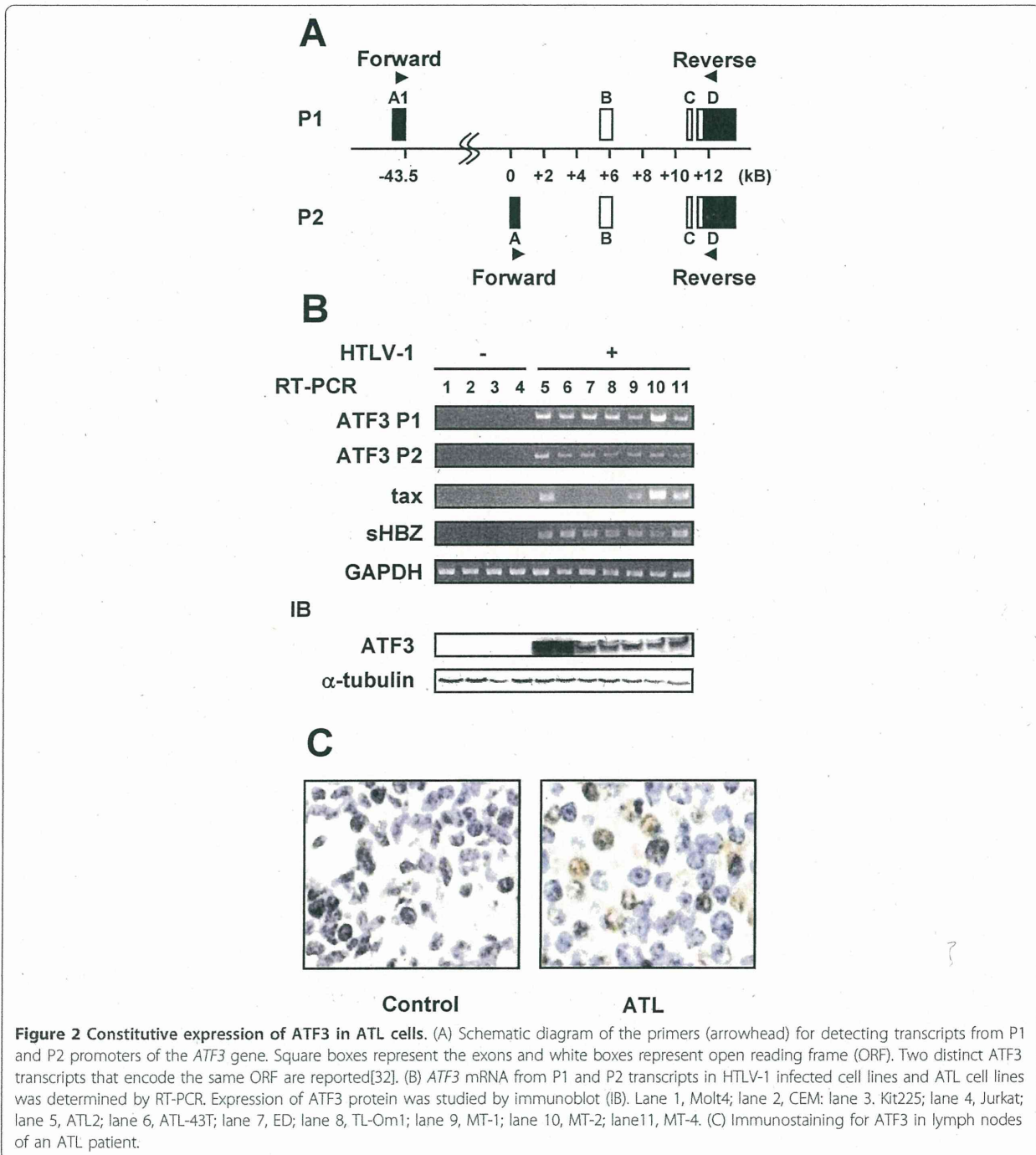
Transcriptional profile of ATF3 KD MT-4 cell

To find mechanisms by which ATF3 might increase proliferation, we performed oligonucleotide microarray analyses of ATF3-KD MT-4 cells and MT-4 cells transfected 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



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

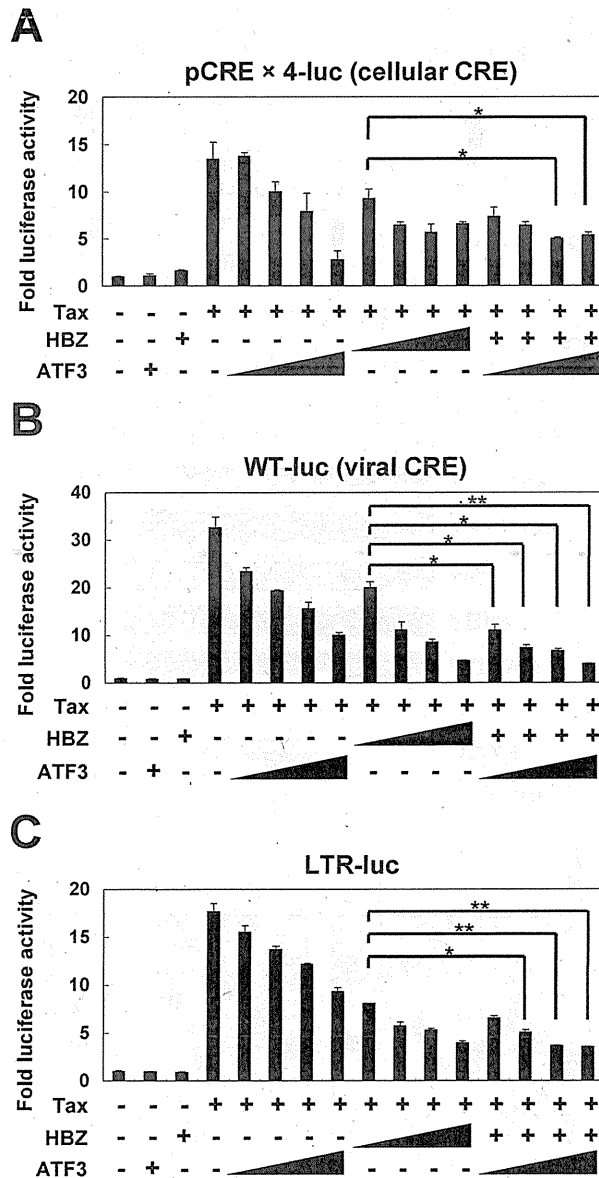


Figure 3 Suppressive effects of ATF3 on Tax-mediated transactivation through ATF/CRE sites. Jurkat cells were cotransfected with pRL-TK and expression vectors for ATF3, HBZ, and reporter plasmid pCRE × 4-luc (A), WT-luc (B), or LTR-luc (C) respectively. The total amount of DNA for transfection was equalized 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

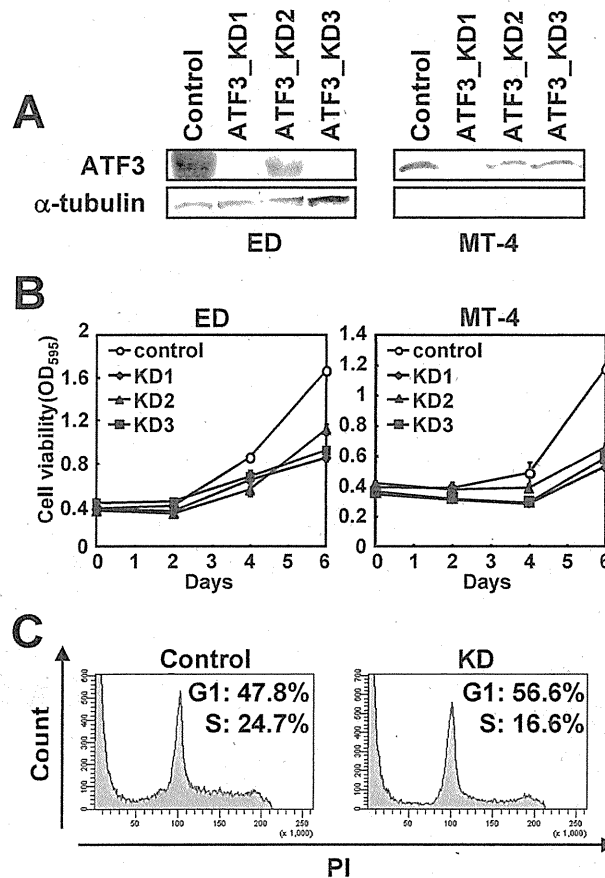


Figure 4 Knockdown of ATF3 by shRNA impairs proliferation of ATL and HTLV-1 infected cells. MT-4 and ED cells were transduced with lentivirus vector expressing control and ATF3-directed shRNA. (A) ATF3 protein was determined by immunoblot. (B) The cell growths of ATF3 knock-down ATL cells by shRNAs were 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.

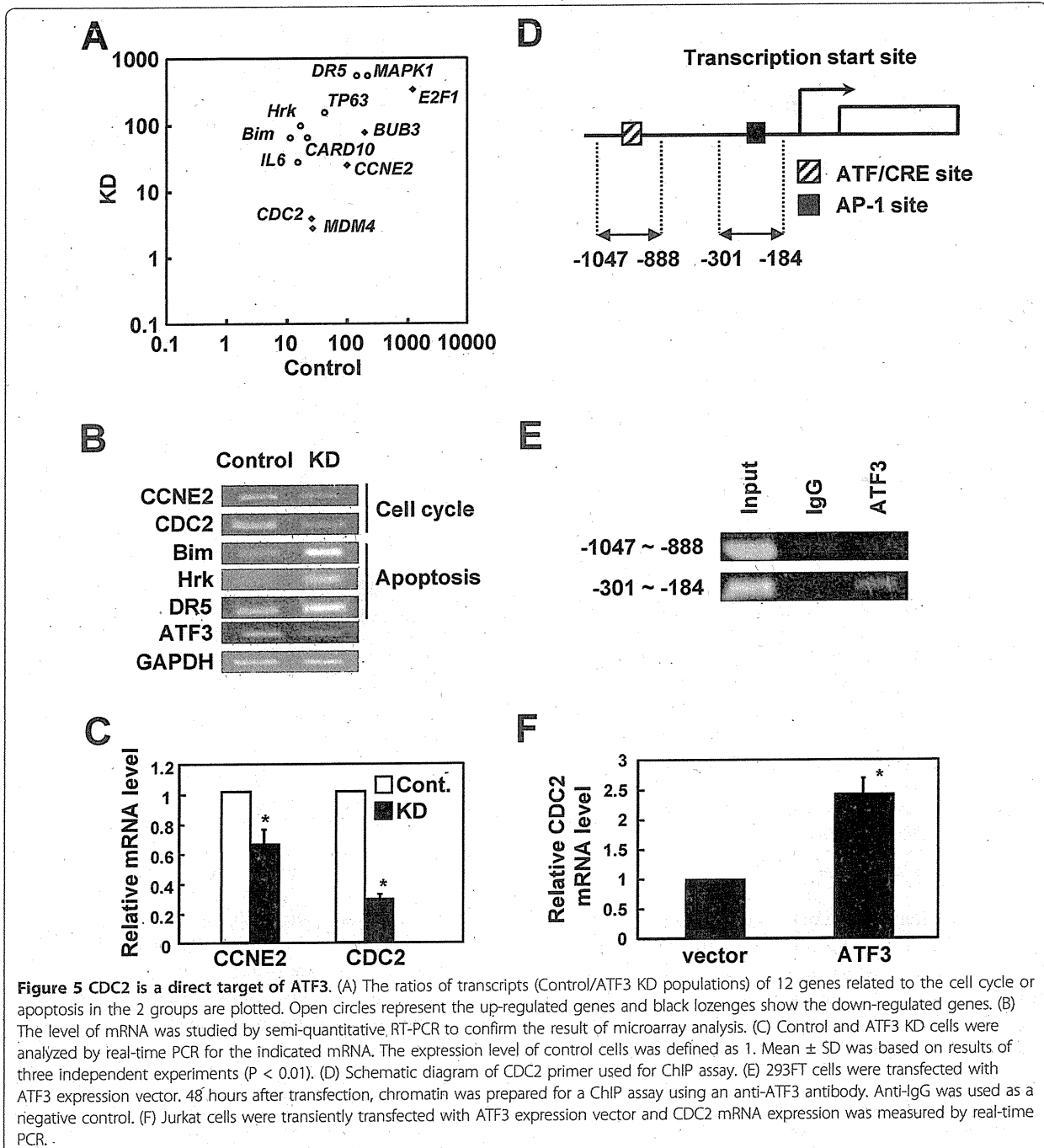
a complex together (Figure 6D). We propose that sHBZ binds directly to ATF3-p53 complexes; that this binding interferes, by unknown mechanisms, with ATF3 enhancement of p53 signaling; and that ATL cells expressing sHBZ can thereby escape the apoptosis that ATF3 expression might otherwise induce.

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 H \ddot{a} rakiri, 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.

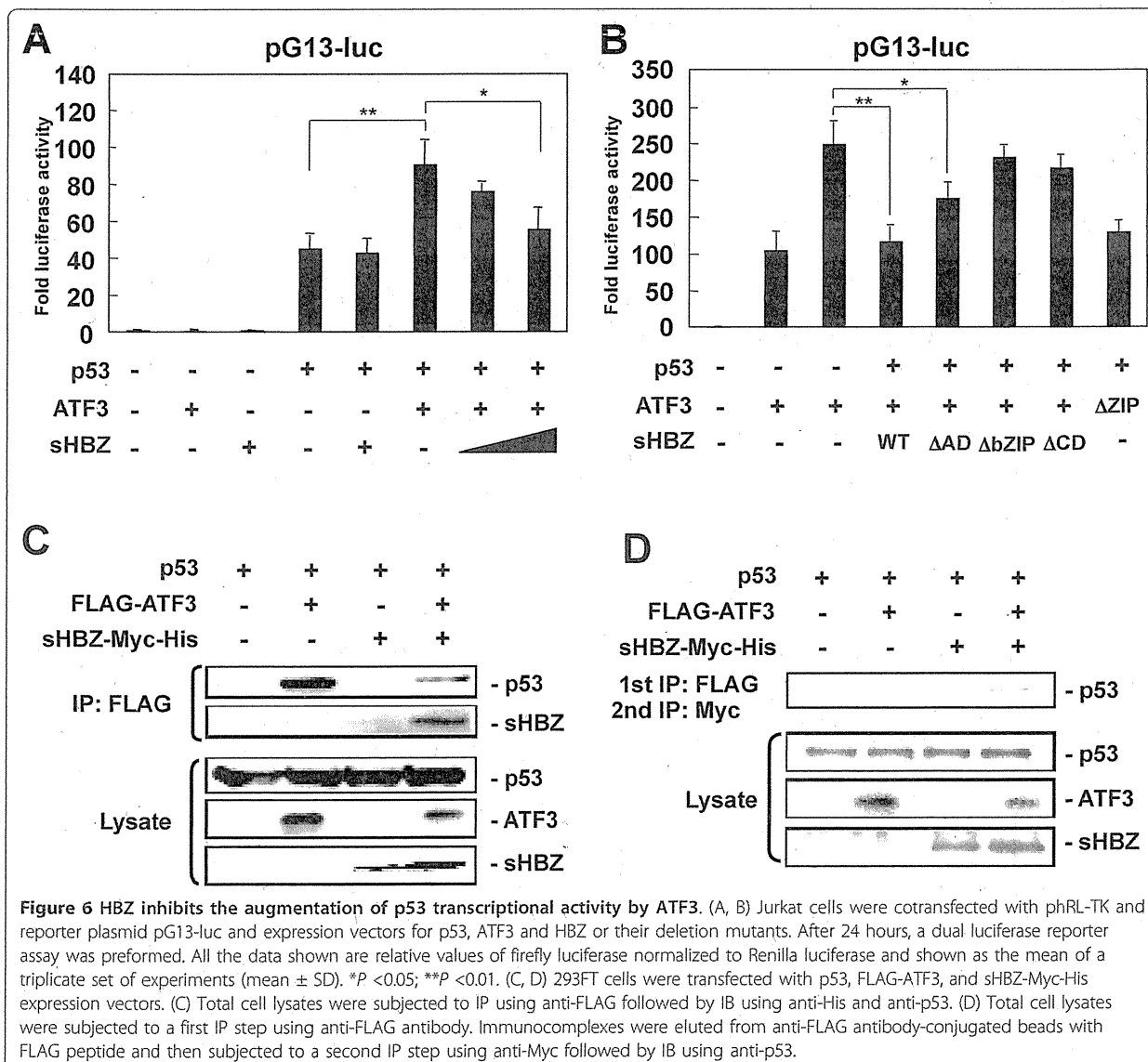
ATF3 has been reported to act as transcriptional repressor of ATF/CRE sequences. In this study, we found that ATF3 suppressed activation, by the viral factor Tax, of transcription from CRE-like sequences in the 5'LTR. Tax, itself transcribed from the 5'LTR, is a major target of cytotoxic T-lymphocytes *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



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



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 GCAGCATTG-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 $2^{[Ct(\text{actin})-Ct(\text{gene})]}$, 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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**Ubiquitin-Specific Peptidase 20 Targets
TRAF6 and Human T Cell Leukemia Virus
Type 1 Tax To Negatively Regulate NF- κ B
Signaling**

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Ubiquitin-Specific Peptidase 20 Targets TRAF6 and Human T Cell Leukemia Virus Type 1 Tax To Negatively Regulate NF- κ B Signaling[∇]

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NF- κ B plays a key role in innate and acquired immunity. Its activity is regulated through intricate signaling networks. Persistent or excessive activation of NF- κ B induces diseases, such as autoimmune disorders and malignant neoplasms. Infection by human T cell leukemia virus type 1 (HTLV-1) causes a fatal hematopoietic malignancy termed adult T cell leukemia (ATL). The HTLV-1 viral oncoprotein Tax functions pivotally in leukemogenesis through its potent activation of NF- κ B. Recent findings suggest that protein ubiquitination is crucial for proper regulation of NF- κ B signaling and for Tax activity. Here, we report that ubiquitin-specific peptidase USP20 deubiquitinates TRAF6 and Tax and suppresses interleukin 1 β (IL-1 β)- and Tax-induced NF- κ B activation. Our results point to USP20 as a key negative regulator of Tax-induced NF- κ B signaling.

Protein ubiquitination is an essential posttranslational modification that is implicated in many biological processes (14, 52). Ubiquitin is a small protein composed of 76 amino acids. It contains 7 lysine residues (K6, K11, K27, K29, K33, K48, and K63). Multiple ubiquitin monomers can become covalently linked, and polyubiquitin molecules linked through the lysine 48 residue (K48) are known to modulate protein degradation. In contrast, polyubiquitin molecules linked through the lysine 63 (K63) residue do not induce degradation but influence protein localization, protein-protein interaction, protein functional activation, and other activities (14, 44). The addition of ubiquitin to or the removal of ubiquitin from protein substrates can reversibly and dynamically change protein functions, and these reactions are executed by ubiquitin ligases and deubiquitinases (44). Accordingly, the ubiquitination process is mediated by the serial actions of E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase (14, 52), and deubiquitination is through deubiquitinating enzymes (DUBs) that directly remove ubiquitin molecules from their substrates (32). Based on sequence analyses, approximately 90 DUB genes have been identified in the human genome. These DUBs are divided into 5 subclasses according to their protein sequences: the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), Machado-Joseph disease protein domain proteases (MJDs), ovarian tumor proteases (OTUs), and JAMM motif proteases (32). The specificities and actions of these various deubiquitinases remain to be fully characterized.

In mammals, NF- κ B signaling plays key roles in inflammation, cell proliferation, and apoptosis (36). Like phosphorylation, ubiquitination is an important posttranslational modifi-

cation that can regulate NF- κ B activity (43). For example, the ubiquitination of a regulatory subunit in the IKK complex, IKK γ which is also known as NEMO, has a central role in signal transduction. It has been shown that K63-linked linear conjugation of ubiquitin to IKK γ positively regulates NF- κ B signaling (47, 53). In addition, the ubiquitination of tumor necrosis factor receptor-associated factors (TRAFs) is also important for IKK activation. TRAF6 is an E3 ubiquitin ligase, and it performs self-ubiquitination through K63-linked chains upon cellular activation through Toll-like receptors (TLRs) and cytokine receptors (8). Moreover, in the canonical NF- κ B pathway, I κ B α , which sequesters NF- κ B proteins in the cytoplasm in an inactive state, is conjugated with K48-linked polyubiquitin chains and is proteasomally degraded when cells are stimulated to activate NF- κ B (4). Similarly, cellular activation induces the ubiquitination and proteosomal processing of NF- κ B2 p100 to p52, allowing NF- κ B RelB/p52 dimers to translocate into the nucleus.

The role of DUBs in the NF- κ B pathway has also been studied. For example, the familial cylindromatosis tumor suppressor CYLD is one of the DUBs that have been found to suppress NF- κ B activity. CYLD has been shown to bind IKK γ and to reduce the ubiquitination of TRAF2, TRAF6, and IKK γ (3, 24, 48). A20 is a second well-studied DUB that negatively regulates NF- κ B activation by reducing the ubiquitination of TRAF2, TRAF6, and RIP1 (17, 20, 41). A20 has dual activities in ubiquitination and deubiquitination. Hence, A20 with TAX1BP1 as a cofactor promotes the cleavage of K63-linked polyubiquitin chains on RIP1, and A20 with E3 ligase Itch can conjugate K48-linked chains on RIP for proteosomal degradation (39, 51). Recently, it was also found that A20 can inhibit the ubiquitination of TRAF2 and TRAF6 by dissociating complexes composed of TRAFs and E2 ubiquitin-conjugating enzymes (41).

Infection by human T cell leukemia virus type 1 (HTLV-1) causes a fatal hematopoietic malignancy, adult T cell leukemia

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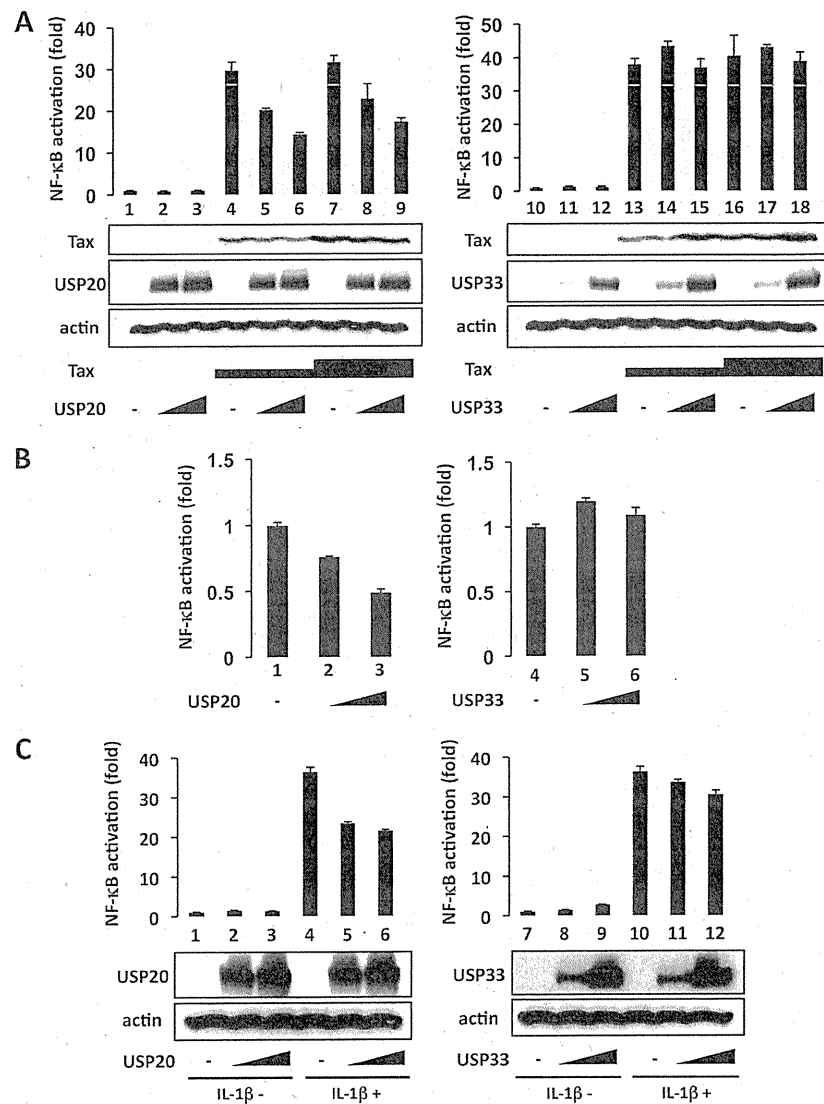


FIG. 1. Expression of USP20 suppresses HTLV-1 Tax- and IL-1 β -induced NF- κ B activation. (A) HEK293T cells were transfected with a Tax-expressing plasmid, Hpx-Tax (0.05 μ g in lanes 4 to 6 and 13 to 15; 0.15 μ g in lanes 7 to 9 and 16 to 18), and κ B-luc reporter (0.05 μ g) with pcDNA3-USP20 (0.5 μ g in lanes 2, 5, and 8; 1 μ g in lanes 3, 6, and 9) or pCMV-USP33 (0.5 μ g in lanes 11, 14, and 17; 1 μ g in lanes 12, 15, and 18). In each case, a pCMV- β -Gal plasmid (0.01 μ g) was included, and β -Gal values were used to normalize for transfection efficiency. Immunoblotting (lower panels) was done to confirm the expression of transfected and control (actin) protein using the indicated antibodies. (B) ATL2 cells were transfected with κ B-luc reporter (1 μ g) with pcDNA3-USP20 (2 μ g in lane 2; 4 μ g in lane 3) or pCMV-USP33 (2 μ g in lane 5; 4 μ g in lane 6). pCMV- β -Gal (0.2 μ g) was included in each transfection as an internal normalization control. (C) HEK293T cells were transfected with κ B-luc reporter (0.05 μ g) with pcDNA3-USP20 (0.5 μ g in lanes 2 and 5; 1 μ g in lanes 3 and 6) or pCMV-USP33 (0.5 μ g in lanes 8 and 11; 1 μ g in lanes 9 and 12) and were treated with recombinant IL-1 β (10 ng/ml) for 8 h (lanes 4 to 6 and 10 to 12). pCMV- β -Gal (0.01 μ g) was included in each transfection as an internal control. Immunoblotting was performed to confirm the expression of transfected and control (actin) protein using the indicated antibodies. Total amounts of transfected DNA were equalized in each sample by the addition of vector DNA. Cell lysates were assayed for luciferase. The results from three independent experiments are shown as average values \pm standard deviations (SD).

(ATL) (29), and one of its key regulatory proteins, Tax, plays important roles in viral pathogenesis (2, 10, 11, 26, 37). Tax (29) can potently activate NF- κ B through both the canonical and noncanonical pathways (15, 42, 45). Tax was recently found to inhibit A20 function by disrupting its interaction with TAX1BP1 and Itch (39). Currently, how other DUBs may contribute to Tax-induced NF- κ B signaling has not been studied. Here, we report on the characterization of USP20 for its regulation of Tax- and TRAF6-mediated activation of NF- κ B.

MATERIALS AND METHODS

Cells and reagents. Human embryonic kidney cell line HEK293T and human cervical cancer cell line HeLa were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and antibiotics. Human T cell lines MT1, MT2, MT4, ATL2, Jurkat, CEM, and H9 were maintained in RPMI 1640 with 10% FBS. Recombinant human interleukin-1 β (IL-1 β) (Peprotech) was used.

Plasmids and siRNAs. The κ B-luc reporter plasmid was described previously (19). Human USP20 coding sequences were amplified by PCR and subcloned into the pcDNA3 (Invitrogen) and the pCAG-HA (50) expression vectors. A

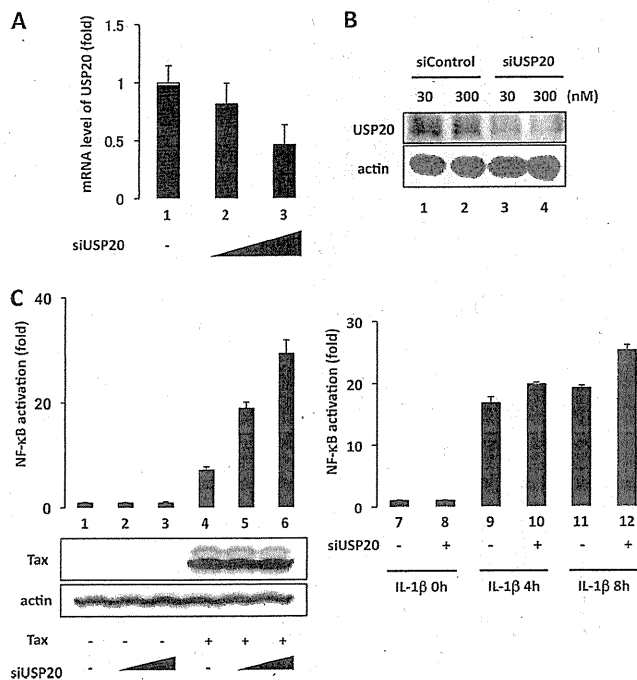


FIG. 2. Knockdown of cell-endogenous USP20 enhances HTLV-1 Tax- and IL-1 β -induced NF- κ B activation. (A) Sequence-specific siRNA was used to knock down USP20. The knockdown of cell-endogenous USP20 by siRNA was confirmed with real-time RT-PCR. USP20-specific siRNAs at 20 nM (bar 2) or 40 nM (bar 3) were transfected into HEK293T cells, from which RNA was extracted. USP20 mRNA levels were quantified by real time RT-PCR. (B) The competent knockdown of cell-endogenous USP20 by siRNA was checked by immunoblotting. USP20-specific siRNAs at 30 nM (lane 3) or 300 nM (lane 4) were transfected into Jurkat cells, and cell-endogenous USP20 protein was assayed by immunoblotting. (C) HEK293T cells were transfected with Hpx-Tax (bars 4 to 6) or treated with IL-1 β (50 ng/ml) at the indicated times (bars 9 to 12) with or without the cotransfection of siRNA for USP20 (20 nM in lanes 2 and 5; 40 nM in lanes 3, 6, 8, 10, and 12) (0 h of IL-1 β means that no treatment occurred). Total amounts of transfected DNAs and siRNAs were equalized by the addition of either vector DNA or control siRNA, respectively. Cell lysates were assayed for luciferase. Immunoblotting was done to confirm the expression of transfected and control (actin) protein using the indicated antibodies. The results from three independent experiments are shown as average values \pm SD.

human USP33 cDNA clone (I.M.A.G.E. 3874822) was purchased from ATCC, and the coding region was inserted into pCAG-HA. For ectopic expression of Tax, Hpx-Tax (18) and pCAG-Flag-Tax were used. Flag-TRAF6 has been described elsewhere (20). The pcDNA-HA-ubiquitin expression vector was used in immunoblotting experiments to detect ubiquitinated Tax and TRAF6. For the knockdown of USP20, synthesized small interfering RNAs (siRNAs) (Qiagen) were used. The target sequence of USP20 is 5'-TCGAGTGACACGGATGAG AAA-3'. Sequence-nonspecific siRNA was purchased from Qiagen and used as a negative control.

Antibodies. Mouse monoclonal anti-Tax (NIH AIDS Research and Reference Reagent Program) was used to detect Tax protein in immunoblotting. Anti-Flag monoclonal antibody (M2; Sigma), anti-Flag polyclonal antibody (Sigma), anti-hemagglutinin (anti-HA) monoclonal antibody (HA-7; Sigma), anti-HA polyclonal antibody (Sigma), anti-USP20 polyclonal antibody (Bethyl Laboratories), anti-USP33 polyclonal antibody (Bethyl Laboratories), anti-tubulin monoclonal antibody (DM1A; Sigma), and anti-actin monoclonal antibody (AC-40; Sigma) were purchased.

Luciferase assay. Expression plasmids or siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen). Cells were transfected with Tax and USP expression vectors or were transfected with siRNAs first, followed 48 h later with

κ B-Luc and pCMV- β -galactosidase (pCMV- β -Gal) plasmids. At 24 h after transfection of the reporters, cell lysates were subjected to luciferase assay. Total amounts of DNA and RNA to be transfected were adjusted by the addition of empty vectors or nonspecific siRNAs. To detect luciferase and β -Gal activity, luciferase substrate (Promega) and the Galacto-Star assay system (Applied Biosystems) were used. Relative values of luciferase activity were calculated using β -Gal activity as an internal control for transfection.

Coimmunoprecipitation assay. At 48 h after transfection, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES, 0.3% NP-40, 150 mM NaCl, 2 mM EDTA, 20 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 0.5 mM dithiothreitol [DTT], and 1 \times protease inhibitor cocktail from Roche). Cell extracts were subjected to immunoprecipitation with anti-Flag or anti-HA antibodies, and the captured proteins were detected by immunoblotting using the indicated antibodies.

Immunofluorescence. Cells were cultured on glass coverslips, and fixed in 4% paraformaldehyde at 24 h after transfection. After blocking of nonspecific reactions with 1% bovine serum albumin (BSA), cells were then incubated with the indicated primary antibodies, followed by a subsequent incubation with the secondary antibodies conjugated with Alexa Fluor 488 or 594 (Molecular Probes). DNA was counterstained with 0.1 μ g/ml Hoechst 33342. Coverslips were mounted in Prolong Antifade (Molecular Probes), and cells were visualized with a Leica TCS SP2 confocal microscope.

Real-time PCR. USP20 transcripts were quantified by real-time PCR. The sequences of the primers for USP20 were 5'-TCACAGAAGTCCACGAGAC G-3' (sense) and 5'-TTGTCCTTCCCCTTGACGAA-3' (antisense). To compare the relative expression levels between samples, we quantified mRNA levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal control using the primers 5'-GCTCACTGGCATGGCCITCCGTGT-3' (sense) and 5'-TGGAGGAGTGGGTGCTGCTGTGA-3' (antisense). Power SYBR green PCR Master Mix (Applied Biosystems) was used for preparation of the PCR mixtures, and the reaction was carried out with an Applied Biosystems 7500 real-time PCR system. All samples were measured in triplicate and analyzed with $\Delta\Delta C_T$ method.

Cell proliferation assay. ATL2 cells were transfected with USP20, USP33, or control expression vector using Amara Nucleofector II (Human T Cell Nucleofector kit, protocol X-001). Using a green fluorescent protein (GFP)-expressing plasmid, we analyzed the transfection efficiency of ATL2 cells with this method and found that routinely ~60% of cells were visibly GFP positive at 48 h after transfection. Cell proliferation was evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

RESULTS

USP20 suppresses HTLV-1 Tax- and cytokine-induced NF- κ B activation. Activation of NF- κ B contributes to cellular transformation by HTLV-1, and Tax is a potent activator of NF- κ B (15, 29, 42). Protein ubiquitination plays an important role in signal transduction; indeed, individual NF- κ B pathway constituents are frequently ubiquitinated when cells are stimulated (4, 14, 36). We reasoned that to maintain homeostasis, *de novo*-ubiquitinated proteins must eventually be deubiquitinated. To identify the cellular DUBs that might affect Tax-induced NF- κ B activation, we tested 12 discrete DUBs and found that USP20 can suppress Tax-induced luciferase expression in a dose-dependent manner (Fig. 1A, lanes 1 to 9). By comparison, a related DUB, USP33 (and 10 other DUBs [data not shown]), failed to influence NF- κ B activation by Tax (Fig. 1A, lanes 10 to 18). Next, we checked the activities of USP20 and USP33 in HTLV-1 transformed ATL2 leukemic cells which were directly derived from the leukemic cells of an ATL patient (46). We found that USP20, but not USP33, inhibited NF- κ B activity in ATL2 cells (Fig. 1B). Collectively, the results suggest that the NF- κ B activating pathways in HEK293T (Fig. 1A) and ATL2 (Fig. 1B) cells are both negatively regulated by USP20.

In order to characterize the roles of USP20 and USP33 in regard to other activators of NF- κ B, we investigated their

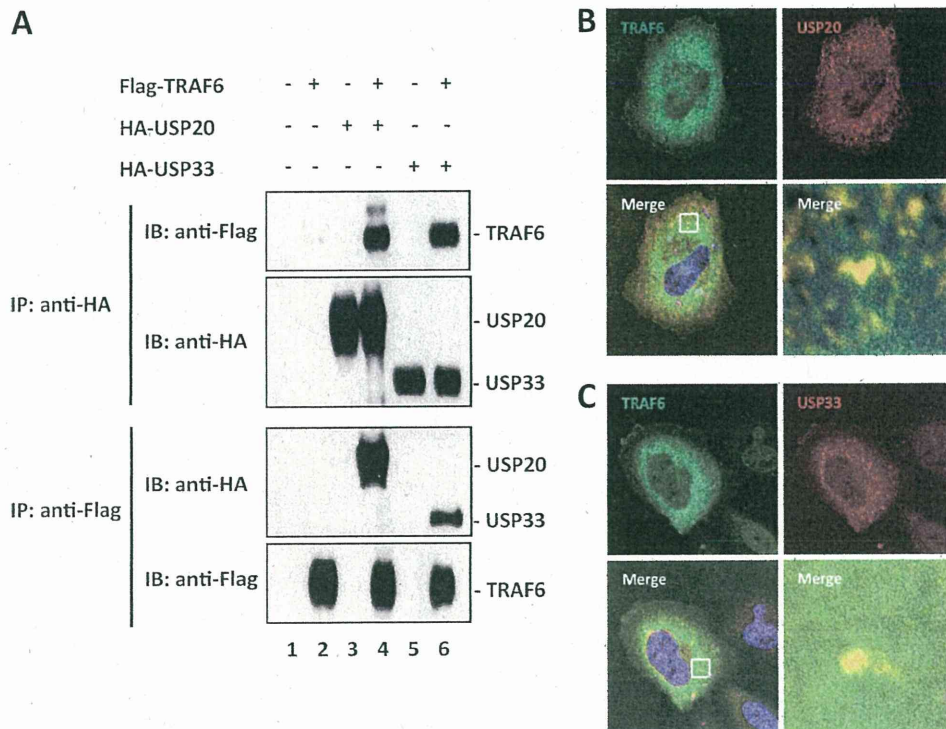


FIG. 3. TRAF6 interacts with USP20 and USP33. (A) HEK293T cells were transfected with the indicated plasmids, and immunoprecipitation was done with either anti-Flag or anti-HA antibody as indicated. The immunoprecipitates and total cell extracts were resolved by SDS-PAGE and immunoblotted. The blots were probed with either anti-Flag or anti-HA. (B and C) Flag-TRAF6 plasmid with either HA-USP20 or HA-USP33 was cotransfected into HeLa cells. Subcellular localizations of TRAF6, USP20, or USP33 were visualized with anti-Flag (green) or anti-HA (red). The nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole) (blue). The higher magnification of the "Merge" is shown in the lower right panels of panels B and C.

effects on IL-1 β -mediated activation. As shown in Fig. 1C, NF- κ B activation by IL-1 β was suppressed when either USP20 (Fig. 1C, lanes 5 and 6) or USP33 (Fig. 1C, lanes 11 and 12) was expressed in HEK293T cells. These findings suggest that both USP20 and USP33 can negatively regulate IL-1 β -mediated NF- κ B signaling, while USP20, but not USP33, can modulate Tax-induced NF- κ B activation (Fig. 1A).

To check for physiological relevance and to rule out that the results were simply from nonphysiological overexpression, we knocked down cell-endogenous USP20 in HEK293T cells using siRNAs. Using real-time reverse transcription-PCR (RT-PCR), we documented an siRNA dose-dependent knockdown of USP20 mRNA in HEK293T (Fig. 2A, bars 2 and 3). To check the knockdown effect of the siRNA at the protein level, we transfected the siRNA into Jurkat cells and confirmed by immunoblotting that USP20 expression was reduced as expected (Fig. 2B). Next, at 48 h after USP20 siRNA transfection into HEK293T cells, we transfected a κ B-luc reporter plasmid and checked for its induction by Tax or IL-1 β . With either Tax (Fig. 2C, compare bar 4 to bars 5 and 6) or IL-1 β (Fig. 2C, compare bar 9 to 10 and bar 11 to 12), the activity of the κ B-luc reporter was higher in USP20-knocked-down cells than in control siRNA-treated cells. Collectively, the findings in Fig. 1 and 2 are consistent with USP20 serving to negatively modulate cellular NF- κ B activity.

TRAF6 interacts with USP20 and USP33. To our knowledge, the above observations on USP20 and USP33 represent

the first demonstration of their effects on NF- κ B signaling. We were thus interested in searching for potential USP20/33 cellular substrates among known NF- κ B-signaling proteins. TRAF6 is an NF- κ B-signaling protein that has been reported to be regulated by ubiquitination (8). We next asked if TRAF6 would interact with USP20 or USP33 in coimmunoprecipitations. We transfected Flag-tagged TRAF6 and either HA-tagged USP20 or HA-tagged USP33 into HEK293T cells. Cell lysates were immunoprecipitated using anti-Flag or anti-HA, and the presence of HA- or Flag-tagged proteins in the respective immunoprecipitates was assessed by immunoblotting. As shown in Fig. 3A, TRAF6 bound USP20 and USP33. As a control, we checked if another NF- κ B-signaling protein also known to be ubiquitinated, IKK γ (47, 53), would interact with USP20 or USP33; however, the latter interaction was not observed (data not shown).

USP20 and USP33 have been reported to localize in perinuclear regions and in small vesicles throughout the cytoplasm (1, 7), and TRAF6 has been shown to locate diffusely in the cytoplasm (49). Next, we immunostained HeLa cells transfected with Flag-TRAF6 and either HA-USP20 or HA-USP33 (Fig. 3B and C). Although not all TRAF6 and USP20/USP33 colocalized together, we did observe partial colocalization of these proteins in the cytoplasm (see yellow costaining in magnified views of the "Merge" panels in Fig. 3B and C), consistent with the potential for intracellular protein-protein interaction.

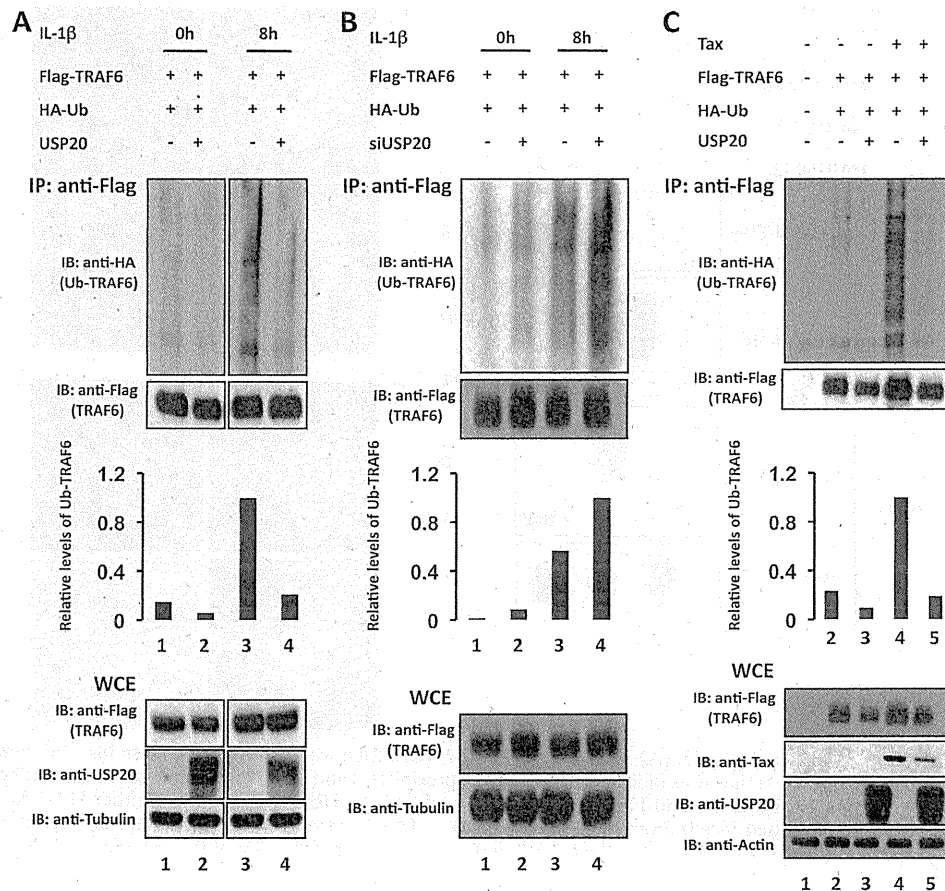


FIG. 4. IL-1 β - or Tax-induced ubiquitinated TRAF6 is deubiquitinated by USP20. (A) HEK293T cells were transfected as indicated with Flag-TRAF6, HA-Ub, and USP20. Cells without IL-1 β treatment (lanes 1 and 2) and cells treated with IL-1 β for 8 h (lanes 3 and 4) were subjected to immunoprecipitation (IP) followed by immunoblotting (IB) to detect ubiquitinated TRAF6 (anti-HA; top panel). WCE, whole-cell extract. (B) HEK293T cells were transfected as indicated with Flag-TRAF6, HA-Ub, and siRNA specific for USP20. Cells without treatment (0 h; lanes 1 and 2) and cells treated with IL-1 β for 8 h (lanes 3 and 4) were immunoprecipitated and detected by immunoblotting for ubiquitinated TRAF6 (anti-HA; top panel). (C) HEK293T cells were transfected as indicated with Tax, Flag-TRAF6, HA-Ub, and USP20 (lanes 2 to 5). Cell lysates were immunoprecipitated and immunoblotted for detection of ubiquitinated TRAF6 (anti-HA; top panel). Total amounts of transfected DNA or transfected siRNA were equalized by the addition of empty vector or control siRNA, respectively. Immunoprecipitations were performed using anti-Flag antibody. Ubiquitinated proteins were detected by immunoblotting with anti-HA antibody. Anti-Flag (for TRAF6), anti-USP20, anti-Tax, anti-tubulin, and anti-actin antibodies were used to verify the respective proteins in immunoblottings. To normalize the amount of ubiquitinated TRAF6, signals of ubiquitinated TRAF6 and total TRAF6 in the immunoblots were quantified by densitometry, and relative levels of normalized ubiquitinated TRAF6 were calculated. The results are graphed in the middle of each panel.

TRAF6 is a substrate of USP20. Previously, USP20 was reported to interact with HIF-1 α (27), type2 iodothyronine deiodinase (7), and β 2 adrenergic receptor (β 2AR) (1). Our finding of an interaction between USP20 and TRAF6 prompted us to ask if TRAF6 might be a functional USP20 substrate. We therefore checked for the effect of USP20 on IL-1 β -induced ubiquitination of TRAF6. We compared the levels of ubiquitinated TRAF6 in the absence or presence of transfected USP20 (Fig. 4A). IL-1 β treatment of cells for 8 h increased the amount of ubiquitinated TRAF6 (Fig. 4A, compare lane 1 to 3), and this increase was diminished by the expression of USP20 (Fig. 4A, compare lane 3 to 4). In addition, siRNA knockdown of cell-endogenous USP20 enhanced the level of ubiquitinated TRAF6 in IL-1 β -treated cells (Fig. 4B, compare lane 3 to 4). Altogether the results support an interpretation that USP20 promotes the deubiquitination of IL-1 β -induced ubiquitinated TRAF6.

In our experiments, Tax expression also increased intracellular ubiquitinated TRAF6, as reported previously (Fig. 4C, compare lane 2 to 4) (12). The Tax-induced ubiquitination of TRAF6 was also sensitive to deubiquitination by USP20 (Fig. 4C, compare lane 4 to 5). Thus, TRAF6 appears to be a *bona fide* USP20 substrate, since USP20 can efficiently deubiquitinate otherwise ubiquitinated TRAF6 that is induced by diverse stimuli such as Tax and IL-1 β .

Ubiquitinated Tax is a substrate for USP20. Ubiquitination, sumoylation, acetylation, and phosphorylation of Tax have been shown to influence its transcriptional function (5, 9, 22, 23, 25, 28, 34, 35, 40). Tax ubiquitination has been reported to be required for its activation of NF- κ B, and it has been found that the Tax-polyubiquitin chains are predominantly K63 linked (40). Because it was observed that USP20 expression inhibited Tax-induced NF- κ B activation (Fig. 1A), we wondered if this finding might also have re-

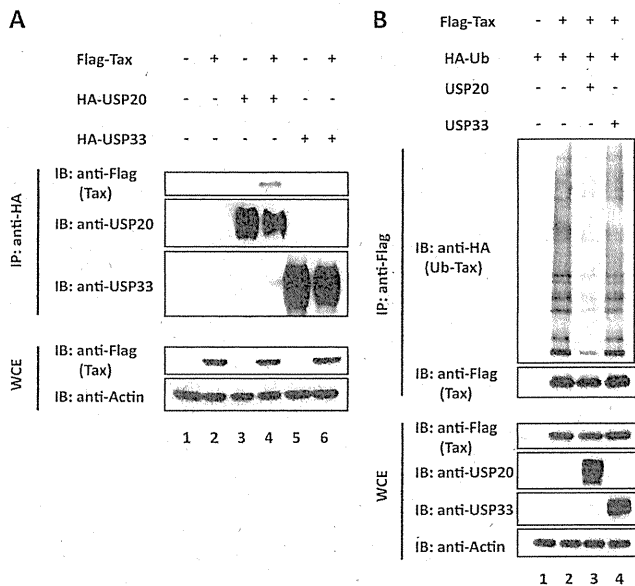


FIG. 5. USP20, but not USP33, deubiquitinates Tax. (A) Tax coimmunoprecipitates with USP20 but not USP33. The indicated plasmids were transfected into HEK293T cells and immunoprecipitated using anti-HA for HA-USP20 (USP20; lanes 3 and 4) or HA-USP33 (USP33; lanes 5 and 6). The immunoprecipitates were blotted and probed with anti-USP20 or anti-USP33. The coimmunoprecipitated Flag-Tax was verified by immunoblotting with anti-Flag. WCE, whole-cell extract. (B) Flag-Tax and HA-Ub as indicated were cotransfected into HEK293T cells with USP20 (lane 3) or USP33 (lane 4). Immunoprecipitation was performed with anti-Flag (top panels). Ubiquitinated Tax (lanes 2, 3, and 4; top panel) was detected using anti-HA in immunoblotting of anti-Flag immunoprecipitates. The amount of ubiquitinated Tax was reduced by the cotransfection of USP20 (lane 3). Amounts of Tax, USP20, USP33, and actin in the whole-cell lysates (WCE) are verified in the bottom panels by immunoblotting.

sulted from USP20-mediated deubiquitination of ubiquitinated Tax.

To check the potential interaction between Tax and either USP20 or USP33, we performed coimmunoprecipitation assays using cells transfected with Flag-tagged Tax and either HA-USP20 or HA-USP33. USP20 coimmunoprecipitated with Tax (Fig. 5A, top panel, lane 4), while USP33 did not distinctly coimmunoprecipitate with Tax (Fig. 5A, top panel, lane 6). Next, we asked how USP20 expression might affect the intracellular level of ubiquitinated Tax. Expression of transfected USP20 (Fig. 5B, compare lane 2 to 3), but not USP33 (Fig. 5B, compare lane 2 to 4), indeed reduced the abundance of ubiquitinated Tax in HEK293T cells. These results agree with the findings in Fig. 1A that USP20, but not USP33, inhibited Tax-induced NF- κ B activation.

USP20 levels are frequently low in HTLV-1-transformed cells, and overexpression of USP20 suppresses cellular proliferation. Many ATL cell lines that express Tax are activated for NF- κ B (30). If ubiquitinated Tax is needed to activate NF- κ B, then one prediction is that the level of USP20 in many ATL cells should be low. To examine this issue, the cell-endogenous levels of USP20 transcripts in several HTLV-1-positive or -negative T cell lines were compared by real-time RT-PCR. USP20 mRNA levels in HTLV-1-transformed Tax-expressing cell lines MT1, MT2, MT4, and ATL2 were indeed significantly

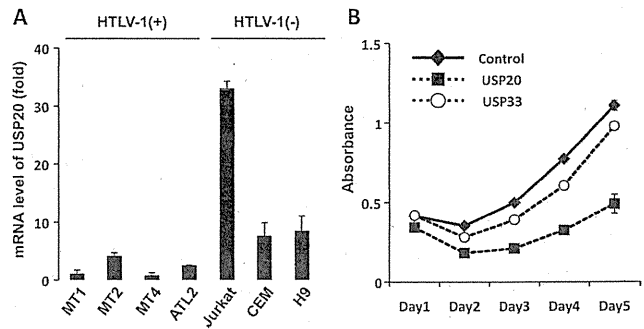


FIG. 6. USP20 is frequently expressed at a reduced level in HTLV-1-transformed cells. (A) USP20 transcripts in the indicated T cell lines were quantified by real-time RT-PCR. Compared to HTLV-1-negative cell lines (Jurkat, CEM, and H9), the HTLV-1 positive cell lines (MT1, MT2, MT4, and ATL2) have much reduced amounts of USP20 transcripts. (B) Ectopic expression of USP20 suppresses cell proliferation. ATL2 cells were transfected with USP20, USP33, or control plasmid, and cell proliferation was evaluated by MTT assay. Based on use of a GFP expression plasmid, the transfection efficiency of ATL2 cells was determined to be slightly greater than 60%.

lower than those in HTLV-1-negative cell lines Jurkat, CEM, and H9 (Fig. 6A). While we do not fully understand the mechanisms for why USP20 expression is reduced in HTLV-1 cells, our preliminary evidence suggests the involvement of epigenetic regulatory mechanisms, including DNA methylation and histone acetylation (data not shown).

Because NF- κ B is a prosurvival and proliferative factor in HTLV-1 cells, we next wondered if the overexpression of USP20, which negatively regulates NF- κ B activity, would inhibit the proliferation of these cells. We transfected USP20, USP33, or a control GFP plasmid into ATL2 cells. Based on the visualization of GFP, our ATL2 transfection efficiency slightly exceeded 60% (data not shown). We then assessed the proliferation of USP20- or USP33-transfected ATL2 cells. As shown in Fig. 6B, ATL2 cells transfected with USP20 proliferated distinguishably slower than GFP- or USP33-transfected ATL2 cells. While there are many ways to interpret this result, one explanation consistent with our other current findings is that the "transfected" USP20 deubiquitinated TRAF6 and/or Tax in ATL2 cells, reducing their NF- κ B activity and thus resulting in the slower cell growth (Fig. 6B).

DISCUSSION

NF- κ B activation is an important host immune response triggered by pathogens. However, an excessive or a prolonged response can evoke autoimmune disorder, septic shock, neoplastic diseases, and death (36). For optimal homeostasis, positive and negative mechanisms serve to balance ambient NF- κ B activity. Current evidence suggests that protein ubiquitination and deubiquitination may provide such a balancing process (43).

In T cells, TRAF6 is ubiquitinated upon receptor stimulation; the ubiquitinated TRAF6 positively regulates NF- κ B signal transduction (8, 47, 53). Here we show that two ubiquitin-specific peptidases, USP20 and USP33, can negatively regulate TRAF-NF- κ B signaling by targeting TRAF6 for deubiquitination. Other DUBs such as A20 and CYLD can also target