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LYMPHOID NEOPLASIA

SOX4 is a direct target gene of *FRA-2* and induces expression of *HDAC8* in adult T-cell leukemia/lymphoma

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Key Points

- SOX4 is consistently expressed in ATL, is involved in ATL cell growth, and induces genes such as GCRK, NAP1, and HDAC8 in ATL.
- FRA-2/JUND and SOX4 form an important oncogenic cascade in ATL, leading to upregulation of genes such as HDAC8.

Previously, we have shown that an AP-1 family member, *FRA-2*, is constitutively expressed in adult T-cell leukemia/lymphoma (ATL) and, together with *JUND*, upregulates *CCR4* and promotes ATL cell growth. Among the identified potential target genes of *FRA-2/JUND* was *SOX4*. Here, we examine the expression and function of *SOX4* in ATL. *SOX4* was indeed consistently expressed in primary ATL cells. *FRA-2/JUND* efficiently activated the *SOX4* promoter via an AP-1 site. Knockdown of *SOX4* expression by small interfering RNA (siRNA) strongly suppressed cell growth of ATL cell lines. Microarray analyses revealed that *SOX4* knockdown reduced the expression of genes such as germinal center kinase related (*GCKR*), NAK-associated protein 1 (*NAP1*), and histone deacetylase 8 (*HDAC8*). We confirmed consistent expression of *GCKR*, *NAP1*, and *HDAC8* in primary ATL cells. We also showed direct activation of the *HDAC8* promoter by *SOX4*. Furthermore, siRNA knockdown of *GCKR*, *NAP1*, and *HDAC8* each significantly suppressed cell growth of ATL cell lines. Taken together, we have revealed an important oncogenic cascade involving *FRA-2/JUND* and *SOX4* in ATL, which leads to the expression of genes such as *GCKR*, *NAP1*, and *HDAC8*. (*Blood*. 2013;121(18):3640-3649)

Introduction

Adult T-cell leukemia/lymphoma (ATL) is an aggressive mature $CD4^+$ T-cell malignancy etiologically associated with human T-lymphotropic virus type 1 (HTLV-1).[1] Previously, we and others have shown that most ATL cases strongly express CC chemokine receptor 4 (CCR4).[2-4] We have further shown that *FRA-2*, a member of the AP-1 family transcription factors, is constitutively expressed in ATL and—together with another AP-1 family member, *JUND*—induces *CCR4* expression and promotes cell growth in ATL.[5] Furthermore, *FRA-2/JUND* induces the expression of various genes including proto-oncogenes such as *c-MYB*, *MDM2*, and *BCL-6* in ATL.[5] We have obtained similar results with cutaneous T-cell lymphomas (CTCLs), which also frequently express CCR4, albeit less strongly than ATL.[6] These results suggest that *FRA-2/JUND* plays an important oncogenic role in mature T-cell malignancies expressing CCR4.

The list of potential target genes of *FRA-2/JUND* in ATL includes *SOX4*, a member of the Sry-related high mobility group box family of transcription factors.[5] *SOX4* is known to play important roles in various developmental processes including those that give rise to T cells and B cells.[7,8] *SOX4* is also frequently overexpressed in a variety of solid tumors and considered to be a potential oncogene.[9-12] We therefore examined the expression and function of *SOX4* in ATL. We demonstrated that *SOX4* is indeed consistently expressed in ATL cell lines and primary ATL cells. We confirmed that *FRA-2/JUND* directly activates the *SOX4* promoter via an AP-1 site. Furthermore, we demonstrated that *SOX4* is involved in ATL cell growth and upregulates the expression of genes such as germinal center kinase related (*GCKR*),[13] NAK-associated protein 1 (*NAP1*),[14] and histone deacetylase 8 (*HDAC8*)[15-17] in ATL. We confirmed direct activation of the *HDAC8* promoter by *SOX4*. Furthermore, we demonstrated that *GCKR*, *NAP1*, and *HDAC8* are each involved in ATL cell growth. Taken together, we have revealed an important oncogenic cascade involving *FRA-2/JUND* and *SOX4* in ATL, which leads to the expression of downstream effector genes such as *GCKR*, *NAP1*, and *HDAC8* in ATL.

Methods

Cells

All cell lines and primary cell samples used in this study were described previously.[2,5,18] Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples obtained from healthy adult donors and acute ATL patients with a high leukemia cell count (>90%) using Ficoll-Paque (Amersham Biosciences Corp). Normal $CD4^+$ T cells (purity, >96%) were further purified from PBMCs by negative selection using the IMagnet system (BD Pharmingen). Primary ATL cells and normal resting $CD4^+$ T cells were stored in aliquots at –80°C and used for experiments without culture. Activated $CD4^+$ T cells were prepared by culturing normal resting $CD4^+$ T cells in the presence of Dynabeads Human T-Activator CD3/CD28 (Life

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Technologies) and 100 U/mL recombinant IL-2 (Shionogi) for 12 days. A written informed consent was obtained from each donor in accordance with the Declaration of Helsinki. This study was approved by the ethics committee of the Kinki University Faculty of Medicine.

RT-PCR

Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed as described previously[5] using KOD FX DNA polymerase (TOYOBO, Osaka, Japan). In brief, the amplification conditions were denaturation at 94°C for 30 seconds (5 minutes for the first cycle), annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds (5 minutes for the last cycle) for 30 cycles for *FRA-2*, *JUNB*, *JUND*, *SOX4*, *GCKR*, *NAP1*, and *HDAC8*, and 25 cycles for *GAPDH*. Amplification products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The primers for *FRA-2*, *JUND*, *JUNB*, and *GAPDH* were described.[5] The primers for *SOX4*, *GCKR*, *NAP1*, and *HDAC8* are as follows: +5′-CACATCAAGCGACCCATGAAC-3′ (forward) and –5′-CCGGTACTTGTAGTCGGGGTAGT-3′ (reverse) for *SOX4*; +5′-TGAGGCAACGATGGAACAGTTAT-3′ (forward) and –5′-CATTTGTGGCAGCCTTTTGTATC-3′ (reverse) for *GCKR*; +5′-GGTTTGGAACAAGAGCTGGAACT-3′ (forward) and –5′-ACAGGCTTTCTTGATTGCAGTTG-3′ (reverse) for *NAP1*; +5′-GCACCATGGAGATGGTGTAGAAG-3′ (forward) and –5′-GACCACTGCTTTGGGATTAAAGG-3′ (reverse) for *HDAC8*.

Quantitative real-time PCR was performed as described previously.[5] The primers and fluorogenic probes used for *FRA-2*, *JUNB*, *JUND*, *GCKR*, *HDAC8*, and *GAPDH* were obtained from the TaqMan kit (Applied Biosystems). We synthesized primers and fluorogenic probes for *SOX4* and *NAP1* as follows: +5′-CATGTCCCTGGGCAGCTT-3′ (forward), –5′-AGCCGGGCTCGAAGTTAAAA-3′ (reverse), and 5′-TCGTCGGCGCTCGACCGG-3′ (probe) for *SOX4*; +5′-TGAACTACTAAGAAAACTGAAAACCTCAA-3′ (forward), –5′-TCCAAGGTCTTCACTGCATCCT-3′ (reverse), and 5′-TGCAATCAAGAAAGCCTGTGCCCCT-3′ (probe) for *NAP1*.

Immunoblot analysis

Whole-cell lysates were prepared using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific). Immunoblot analysis was performed as described previously.[19] The following antibodies were used: rabbit anti-SOX4 (Abcam), monoclonal anti–α-tubulin (clone DM1A; Sigma-Aldrich), horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG) (Merck Chemicals), and goat anti-mouse IgG (GE Healthcare). Bands were visualized using the ECL plus detection kit (GE Healthcare).

Immunologic staining

These were performed as described previously.[4] Briefly, cells were fixed, blocked, and reacted with rabbit anti-SOX4 (Abcam) or normal rabbit IgG (Beckman Coulter) and then with goat anti-rabbit IgG $F(ab')_2$–Alexa Fluor 546 (Invitrogen). After being counterstained with 4,6 diamidino-2-phenylindole (DAPI) (Invitrogen), cells were observed under a fluorescence microscope (BZ-8000; Keyence). Formalin-fixed paraffin-embedded biopsy tissues were obtained from archival tissue collections in the Department of Pathology, Kinki University Faculty of Medicine (ATL, n = 7; tonsillitis, n = 2). The tissue sections were heated 3 times for 5 minutes in target retrieval solution (DAKO) and treated with rabbit anti-SOX4 (Abcam) or normal rabbit IgG (DAKO) followed by biotin-labeled goat anti-rabbit IgG and the Vectastain ABC/HRP kit (Vector Laboratories). After the enzymatic development, sections were counterstained with Gill hematoxylin. Images were obtained using a Biozero BZ-8000 fluorescence microscope (objective, 40×) and BZ-II Analyzer software (Keyence).

Luciferase reporter assay

This assay was performed as described previously.[5] Briefly, the major transcriptional start sites (+1) of *SOX4* and *HDAC8* were determined by the method of rapid amplification of the complementary DNA 5′ end (data not shown). The promoter regions of *SOX4* (from –768 to +102 bp) and *HDAC8* (from –712 to +126 bp) were amplified from human genomic DNA using PCR with the following primers: +5′-CATGCTAGCGACCCTTGCTTGGTTAAGAGAGT-3′ (forward) and –5′-CATCTCGAGCAGGAGTTCCTCCAGTGCAGACT-3′ (reverse) for *SOX4*; +5′-CATCTCGAGCAGATTTGCCAGAGTAAATTATTGAGA-3′ (forward) and –5′-CATAAGCTTACCAGCGACTGCCCACTGT-3′ (reverse) for *HDAC8*. The amplification products were digested with *Nhe*I and *Xho*I for *SOX4* or *Xho*I and *Hin*dIII for *HDAC8*, and cloned into pGL3-basic luciferase reporter plasmid (Promega). Serial 5′-truncated promoter regions were similarly amplified using PCR with appropriate primers and cloned into pGL3. We also generated the *SOX4* promoter fragment (from –768 to +102 bp) with a mutated AP-1 site (from CATGAGGAAGC to CATTTGGACTC) and the *HDAC8* promoter fragment (from –712 to +126 bp) with a mutated SOX4 site (from AACAAGGA to ACCATGGA) using 2-step PCR with appropriate primers. The expression vectors for *c-FOS*, *FRA-2*, and *JUND* were described previously.[5] The expression vectors for the spliced form of HTLV-1 bZIP factor (*sHBZ*) and *SOX4* were generated as follows. We first constructed an expression vector, pIRES2/EGFP-EF1α, by inserting the EF1α promoter between the cytomegalovirus promoter and the multicloning sites of pIRES2/EGFP (Promega). *sHBZ* was amplified from an HTLV-1$^+$ T-cell line TCL-Kan using PCR with the following primers: +5′-CATGAATTCGGCGTGGATGGCGGCCTCAGGGCTGTTTC-3′ (forward) and –5′-CATGGATCCTTATTGCAACCACATCGCCTCCAGC-3′ (reverse). Amplification products were digested with *Eco*RII and *Bam*HI and cloned into pIRES2/EGFP-EF1α to make psHBZ-EGFP. Similarly, *SOX4* complementary DNA was amplified from an ATL cell line ST1 using PCR with the following primers: +5′-CATGAATTCGAGGCCATGGTGCAGCAA-3′ (forward) and –5′-CATGGATCCTCAGTAGGTGAAAACCAG-3′ (reverse). Amplification products were digested with *Eco*RII and *Bam*HI and cloned into pIRES2/EGFP-EF1α to make pSOX4-EGFP. The luciferase reporter gene driven by HTLV-1 long terminal repeat (LTR) (pGL3-HTLV-1-LTR) and the expression vector for HTLV-1 *Tax* (pHβPr.1-TAXMT2) were described previously.[20] Using DMRIE-C transfection reagent (Invitrogen), cells (3×10^5) were transfected with 1.5 μg of a reporter plasmid, 0.5 μg of an expression plasmid for a transcription factor, and 0.3 μg of pSV-β-galactosidase (Promega). After 24 hours, assays were performed using a luciferase assay kit (Promega). Data were normalized with β-galactosidase activity, which served as an internal control for transfection efficiency.

ChIP assay

This was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) and following the manufacturer's instructions. Briefly, cells (1×10^7) were cross-linked with 1% formaldehyde for 10 minutes at room temperature. Chromatins were digested to a size range between 150 and 900 bp using micrococcal nuclease. After brief sonication, cell lysates were obtained. The quality of chromatin samples was routinely checked by gel electrophoresis and ethidium bromide staining, which confirmed the amount and size range of chromatins. Chromatin samples were diluted 5-fold in chromatin immunoprecipitation (ChIP) buffer and incubated at 4°C overnight with 6 μg of anti-histone H3 (clone D2B12), a positive control provided in the kit (Cell Signaling Technology), anti–FRA-2 (sc-604), anti-JUND (sc-74), or normal rabbit IgG. Anti–FRA-2 and anti-JUND were obtained from Santa Cruz Biotechnology. Immune complexes were collected with protein G magnetic beads and incubated at 65°C for 2 hours to reverse the protein-DNA cross-links. DNAs were purified with a DNA-purification column. The AP-1 site of the *SOX4* promoter was quantified using real-time PCR with the following primers and fluorogenic probe: +5′-GCACGCGGAGATTATTATTGC-3′ (forward), –5′-AACGCTTCCTCATGCCAAAC-3′ (reverse), and 5′-TCGGGTTCCAAGCCAATGGGAA-3′ (probe).

Cell proliferation assay

Small interfering RNAs (siRNAs) targeting *FRA-2* (SI00420455), *JUNB* (SI03077445), *JUND* (SI00075985), and their control (1022064) were obtained from Qiagen. siRNAs targeting *SOX4* (HSS106819), *HDAC8*

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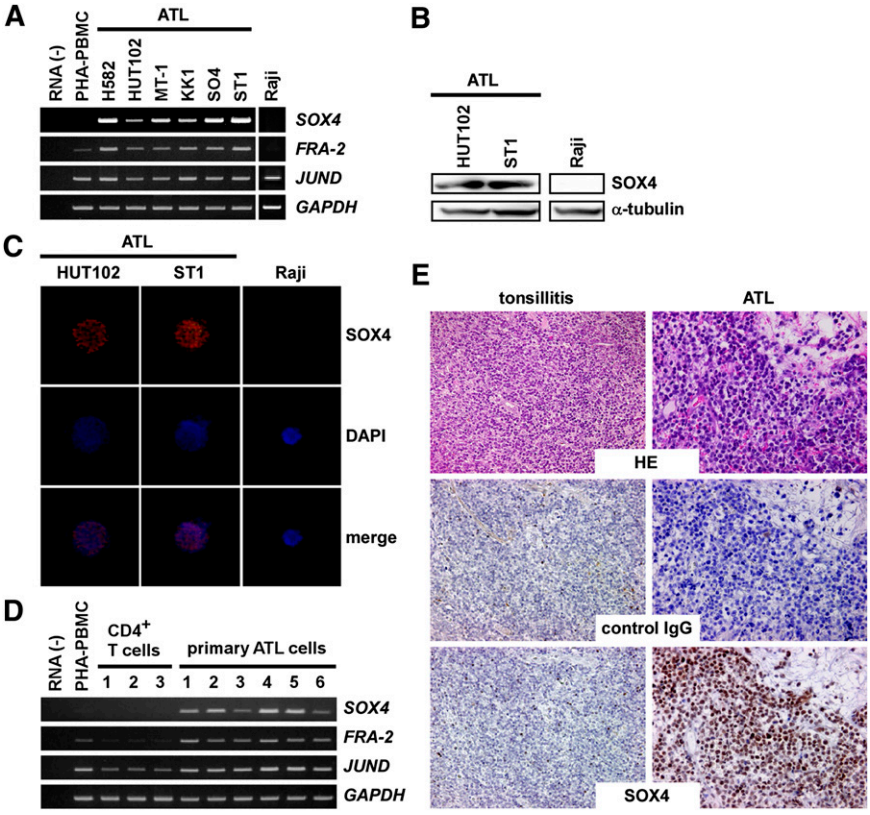


Figure 1. Expression of *SOX4* in ATL. (A) RT-PCR analysis of ATL cell lines. Semiquantitative RT-PCR was performed to analyze expression of *SOX4*, *FRA-2*, and *JUND* in 6 ATL cell lines. Normal PBMCs treated with PHA for 3 days (PHA-PBMC) and Raji, a Burkitt lymphoma cell line, were also included. *GAPDH* was used as a loading control. The representative results from 3 separate experiments are shown. (B) Immunoblot analysis. Cell extracts prepared from 2 ATL cell lines and Raji were subjected to immunoblot analysis. After electrophoresis and electrotransfer, blotted filters were reacted with anti-SOX4 or anti–α-tubulin. The representative results from 3 separate experiments are shown. (C) Immunocytochemistry. Two ATL cell lines and Raji were used. Cells were fixed and stained with anti-SOX4 or control IgG. Cells were counterstained with DAPI. The representative results from 3 separate experiments are shown. Original magnification, ×800. (D) RT-PCR analysis of primary cells. Semiquantitative RT-PCR was performed to analyze expression of *SOX4*, *FRA-2*, and *JUND* in normal $CD4^+$ T cells and primary ATL cells (>90% leukemia cells). *GAPDH* was used as a loading control. The representative results from 3 separate experiments are shown. (E) Immunohistochemistry. Tissue sections of inflamed tonsils (n = 2) and ATL skin lesions (n = 7) were stained with anti-SOX4 or control IgG. The representative results are shown. Original magnification, ×400. HE, hematoxylin-eosin; PHA, phytohemagglutinin.

(HSS183330), *GCKR* (HSS117367), *NAP1* (HSS127541), and their control (12935-145) were obtained from Invitrogen. Cells were transfected with siRNAs on Nucleofector (Lonza) using T solution and the O-17 program. The transfection efficiency of siRNAs was close to 95%, as determined using fluorescent siRNA. Cells overexpressing *SOX4* were isolated 2 days after transfection with pSOX4-EGFP through sorting enhanced green fluorescent protein (EGFP)–expressing cells on FACSVantage (Becton Dickinson). In some experiments, cells were treated with broad-spectrum histone deacetylase (HDAC) inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), which were purchased from Wako and Cayman Chemical, respectively. For cell proliferation assays, cells were seeded in a 96-well plate (0.5×10^4 cells per well) and viable cells were counted every 24 hours on FACSCalibur (Becton Dickinson) by gating out cells stained with propidium iodide.

Oligonucleotide microarray

Microarray analyses were performed using the Affymetrix GeneChip HG-U133 Plus 2.0 array (Affymetrix) as described previously.[5] Briefly, ST1 cells were transfected with control siRNA or *SOX4* siRNA as described in the previous section. After 48 hours, total RNAs were prepared and checked to be of good quality on Agilent 2100 Bioanalyzer (Agilent Technologies). All microarray data were submitted to Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo; accession no. GSE38021). The analysis was performed using BRB Array Tools software (version 3.3.0; http://linus.nci.nih.gov/BRB-ArrayTools.html) developed by Richard Simon and Amy Peng.

Statistical analysis

The Student *t* test was used to analyze differences between 2 groups. One-way analysis of variance with the Tukey post hoc test was used for multiple groups. All data were analyzed using GraphPad Prism (version 5.01; GraphPad Software). We considered $P < .05$ to be statistically significant.

Results

Expression of *SOX4* in ATL

We first examined expression of *SOX4* in ATL cell lines. As shown in Figure 1A, *SOX4* was consistently expressed in ATL cell lines together with *FRA-2* and *JUND*. Raji, a Burkitt lymphoma cell line, which was used as a negative control, expressed neither *FRA-2* nor *SOX4*. We also confirmed SOX4 protein expression in 2 ATL cell lines (HUT102, ST1) by immunoblotting, which detected a 47-kDa band (Figure 1B), and by immunocytochemistry, which detected nuclear staining (Figure 1C). We next examined *SOX4* expression in primary ATL samples. As shown in Figure 1D, PBMCs derived from acute type ATL patients (>90% leukemia cells) (n = 6) consistently expressed *SOX4*, *FRA-2*, and *JUND* at elevated levels (Figure 1D). In contrast, normal $CD4^+$ T cells derived from healthy adult donors (n = 3) expressed *SOX4* and *FRA-2* only at low levels. Quantitative PCR confirmed elevated expression of *SOX4* in ATL cell lines and primary ATL samples (supplemental Figure 1, available on the *Blood* website). Furthermore, immunohistochemical staining consistently revealed strong nuclear expression of SOX4 protein in infiltrating leukemic cells in skin lesions of ATL (n = 7) (Figure 1E). In contrast, anti-SOX4 hardly reacted specifically with normal tonsillar lymphocytes (n = 2) in comparison with control IgG

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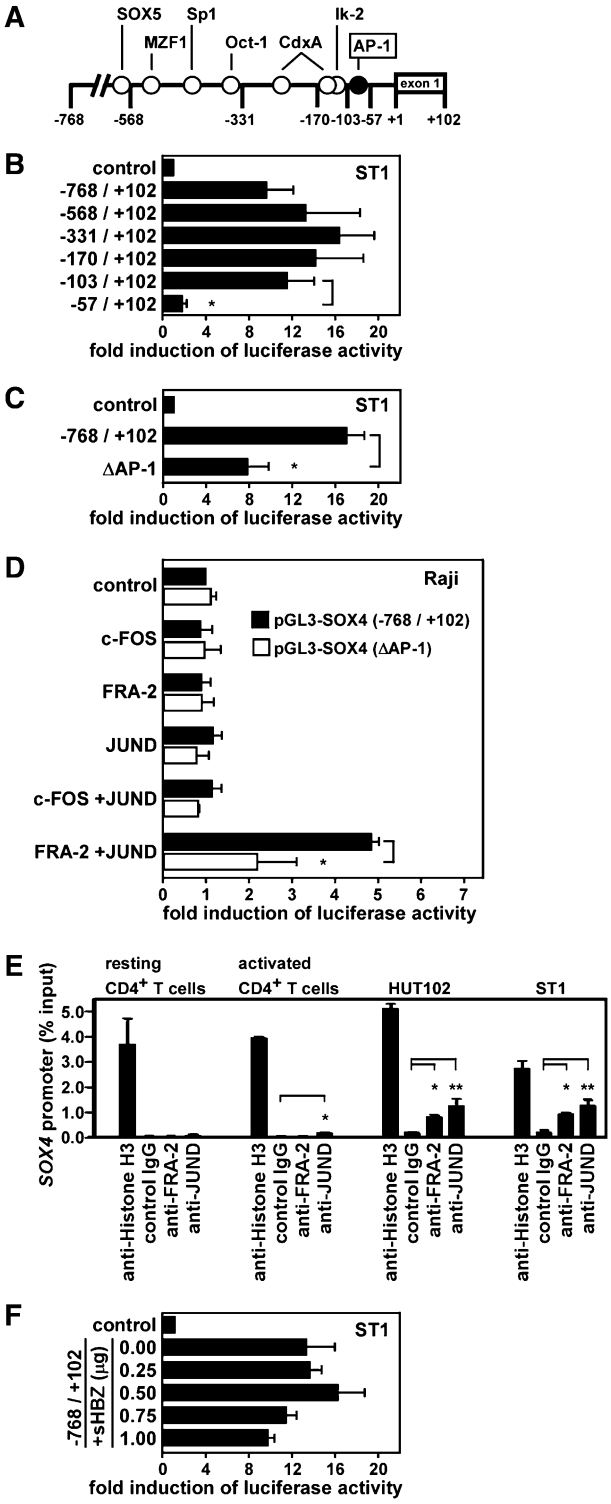


Figure 2. Direct activation of the *SOX4* promoter by *FRA-2* and *JUND*. (A) A schematic depiction of the *SOX4* promoter region from –768 to +102 bp with potential regulatory elements. (B) Deletion analysis. Luciferase reporter assays were performed using reporter constructs containing the *SOX4* promoter fragment from –768 to +102 bp and its successive 5′-truncated fragments. The region from –103 to –57 bp was found to be necessary and sufficient for the reporter gene expression in ST1 cells. Data are presented as mean ± SEM of 3 separate experiments. $*P < .05$. (C) Mutation analysis. Luciferase reporter assays were performed in ST1 cells using reporter constructs containing the *SOX4* promoter fragment from –768 to +102 bp with an AP-1 site at –92 to –82 bp being wild-type or mutated (ΔAP-1, from CATGAGAAGC to CATTGGACTC). Data are presented as mean ± SEM of 3 separate experiments. $*P < .05$. (D) Reconstitution experiment. Raji cells were cotransfected with pGL3-SOX4 (–768/+102) with the wild-type AP-1 site or mutated (ΔAP-1) and expression vectors for *c-FOS*, *FRA-2*, *JUND*, or control vector as indicated. After 24 hours, luciferase assays were performed in triplicate. Promoter activation was shown as fold induction of luciferase activity in cells transfected with indicated AP-1 member expression vectors vs cells transfected with the control vector. Transfection efficiency was normalized by β-galactosidase activity. Data are presented as mean ± SEM of 3 separate experiments. $*P < .05$. (E) ChIP assay. Chromatins prepared from normal resting and activated $CD4^+$ T cells and 2 ATL cell lines (HUT102 and ST1) were fragmented and immunoprecipitated with anti-histone H3 (positive control), anti-FRA-2, anti-JUND, or control IgG (negative control). Real-time PCR was performed to quantify the AP-1 site of the *SOX4* promoter in precipitated chromatin fragments relative to total input DNA (% input). Data are presented as mean ± SEM of 3 separate experiments. $*P < .05$; $**P < .01$. (F) Effect of HTLV-1 *HBZ* on the *SOX4* promoter. ST1 cells were cotransfected with pGL3-SOX4 (–768/+102) and an expression vector for the spliced form of *HBZ* (*sHBZ*) or control vector as indicated. After 24 hours, luciferase assays were performed in triplicate. Transfection efficiency was normalized by β-galactosidase activity. Data are presented as mean ± SEM of 3 separate experiments.

(Figure 1E). These results confirm that *SOX4* is consistently expressed at both messenger RNA (mRNA) and protein levels in ATL cells. Furthermore, strong nuclear staining of SOX4 is highly characteristic of ATL cells.

SOX4 is a direct target gene of *FRA-2*/*JUND*

We next addressed whether *SOX4* is a direct target gene of *FRA-2* and *JUND* in ATL. Using siRNA knockdown and quantitative PCR, we confirmed that *FRA-2* siRNA and *JUND* siRNA but not *JUNB* siRNA or control siRNA efficiently reduced *SOX4* mRNA in ATL cell lines (supplemental Figure 2). We therefore examined the transcriptional control of the *SOX4* promoter in ATL. Figure 2A shows the presence of potential *cis* elements in the *SOX4* promoter region from –768 to –1 bp relative to the transcriptional start site. We inserted the DNA fragment from –768 to +102 bp and its successive 5′-truncated fragments into a luciferase reporter plasmid pGL3-basic and transfected these constructs to an ATL cell line ST1. As shown in Figure 2B, a strong promoter activity was observed until the –103-bp fragment, while the –57-bp fragment had little promoter activity. Thus, a major *cis* element(s) of the *SOX4* promoter in ST1 cells was mapped within –103 to –57 bp. An AP-1 site was found in this region at –92 to –82 bp (Figure 2A). To directly address the role of this AP-1 site in the promoter activation, we generated a –768- to +102-bp construct with mutations in the AP-1 site (from CATGAGGAAGC to CATTTGGACTC). The *SOX4* promoter activity in ST1 cells was indeed significantly ($P < .05$) reduced by the mutations in the AP-1 site (Figure 2C). The partial reduction could be due to incomplete inactivation of the AP-1 element or to the presence of other regulatory element(s) in this region. We next performed a reconstitution experiment using Raji, a Burkitt lymphoma cell line, which did not express *SOX4* (Figure 1). Cells were cotransfected with the –768- to +102-bp construct of the *SOX4* promoter with the wild-type or mutated AP-1 site and the expression vectors for *c-FOS*, *FRA-2*, and *JUND*, singly or in combination. As shown in Figure 2D, only the combination of *FRA-2* and *JUND* activated the *SOX4* promoter with the wild-type AP-1 site in Raji, while the combination of *c-FOS* and *JUND* had no such effect. Furthermore, the activation of the *SOX4* promoter by *FRA-2* and *JUND* was significantly ($P < .05$) reduced by the mutations in the AP-1 site. Again, the reduction was partial. Given that there were no other potential AP-1 sites in the promoter region used (Figure 2A), this might further support the notion that the inactivation of the AP-1 element was incomplete. Taken together, *FRA-2*/*JUND* indeed directly activates the *SOX4* promoter at least partly via the AP-1 element at –92 to –82 bp.

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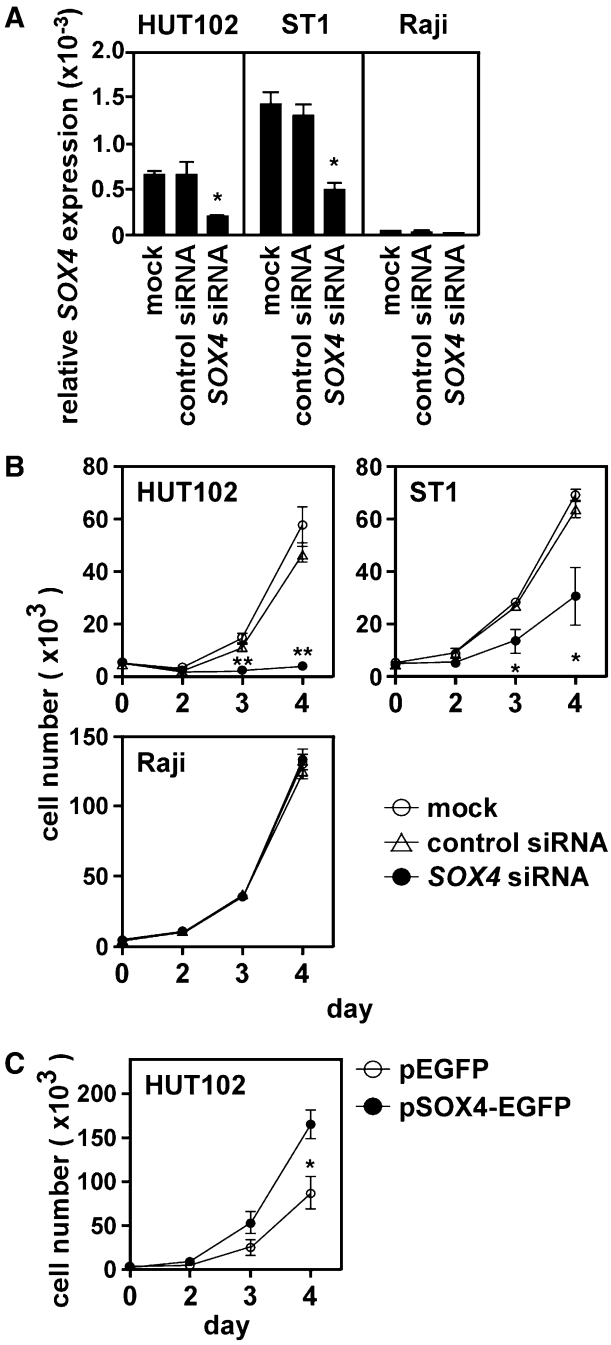


Figure 3. Role of *SOX4* in ATL cell growth. (A) Effect of siRNA knockdown on *SOX4* expression. Two ATL cell lines (HUT102 and ST1) and Raji were transfected with control siRNA or *SOX4* siRNA. After 48 hours, total RNA samples were prepared, and quantitative real-time PCR was performed. Data are shown as mean ± SEM of 3 separate experiments. $^*P < .05$. (B) Effect of *SOX4* siRNA on cell growth. Two ATL cell lines (HUT102 and ST1) and Raji were transfected with control siRNA or *SOX4* siRNA and cultured in a 96-well plate at 0.5×10^4 cells per well. At indicated time points, viable cells were counted on FACSCalibur by gating out cells stained with propidium iodide. Data are shown as mean ± SEM of 3 separate experiments. $^*P < .05$; $^{**}P < .01$. (C) Effect of *SOX4* overexpression on cell growth. HUT102 cells were transfected with the control EGFP vector or pSOX4-EGFP. After 48 hours, cells expressing EGFP were sorted and cultured in a 96-well plate at 0.5×10^4 cells per well. At indicated time points, viable cells were counted on FACSCalibur by gating out dead cells stained with propidium iodide. Data are presented as mean ± SEM of 3 separate experiments. $^*P < .05$.

To demonstrate the direct binding of FRA-2 and JUND to the AP-1 site of the *SOX4* promoter in ATL cells, we next performed ChIP assays. Chromatins were prepared from 2 ATL cell lines (HUT102 and ST1) and normal resting and activated $CD4^+$ T cells. The prepared chromatins were reacted with anti-histone H3 (positive control), anti-FRA-2, anti-JUND, or control IgG (negative control). Quantitative PCR was used to determine the amounts of DNA fragments containing the AP-1 site of the *SOX4* promoter in immune complexes. As shown in Figure 2E, while anti-histone H3 precipitated the chromatins containing the AP-1 site of the *SOX4* promoter from all samples with similar efficiencies, anti-FRA-2 and anti-JUND precipitated the chromatins containing the AP-1 site of the *SOX4* promoter from 2 ATL cell lines but not from normal resting or activated $CD4^+$ T cells. These results support the direct binding of FRA-2 and JUND to the AP-1 site of the *SOX4* promoter in ATL.

HTLV-1 bZIP factor (HBZ), which is encoded by the minus strand of HTLV-1 provirus, is consistently expressed in primary ATL cells and considered to play an important role in ATL oncogenesis.[21] Importantly, *HBZ* functions as a negative regulator of *Tax*-mediated HTLV-1 transcritpion.[21] Recently, *SOX4* was reported to be a possible downstream gene of *HBZ*.[22] We therefore examined the effect of the splice form of HBZ (sHBZ), which is the major *HBZ* transcript in ATL,[21] on the *SOX4* promoter. In Raji cells, coexpression of *sHBZ* did not activate the *SOX4* promoter at all but rather suppressed its activation by *FRA-2* and *JUND* (data not shown). Similarly, cotransfection of the *sHBZ* expression vector did not activate the *SOX4* promoter in ST1 cells but rather suppressed it at high plasmid doses (Figure 2F). In parallel, we confirmed that coexpression of *sHBZ* suppressed *Tax*-mediated activation of the HTLV-1 LTR promoter as reported previously (supplemental Figure 3).[21] Thus, the *sHBZ* expression vector used was functional. Given that HBZ was reported to form a heterodimer with JUND,[23] HBZ might compete with FRA-2 for JUND. Taken together, we concluded that *SOX4* is not a direct target gene of *HBZ*.

Role of *SOX4* in ATL cell growth

We next examined the role of *SOX4* in ATL cell growth. Quantitative PCR confirmed that *SOX4* siRNA but not control siRNA effectively reduced *SOX4* mRNA in 2 ATL cell lines (HUT102 and ST1) (Figure 3A). Under these conditions, *SOX4* siRNA but not control siRNA strongly suppressed cell growth of 2 ATL cell lines (Figure 3B). On the other hand, *SOX4* siRNA had no effect on the cell growth of Raji (Figure 3B), which did not express *SOX4* (Figure 1A). We next overexpressed *SOX4* in HUT102, which only moderately expressed *SOX4* (supplemental Figure 1). Overexpression of *SOX4* strongly enhanced cell growth of HUT102 (Figure 3C). Taken together, these results clearly demonstrated that *SOX4* is involved in ATL cell growth.

Identification of downstream target genes of *SOX4* in ATL

Given the strong effect of *SOX4* on cell growth of ATL cell lines (Figure 3), we sought to identify downstream target genes of *SOX4* in ATL. Using the Affymetrix high-density oligonucleotide microarray, we compared the gene expression profiles of ST1 cells transfected with control siRNA and *SOX4* siRNA. Table 1 summarizes the top 50 genes whose expression was reduced >2-fold by *SOX4* siRNA. To validate the microarray data, we selected 3 genes from Table 1 for further study; namely, *GCKR* (also called *MAP4K5*),[13] *NAP1* (also called *AZI2*),[14] and *HDAC8*.[15-17] These genes were chosen because they were likely to be important in ATL cell growth. As shown in Figure 4A, ATL cell lines and primary ATL samples consistently expressed *GCKR*, *NAP1*, and *HDAC8*. Quantitative PCR further confirmed elevated expression of *HDAC8* in

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Table 1. Identification of potential downstream target genes of *SOX4* in ATL

Name	Gene symbol	Fold change	Probe set
MARCKS-like protein 1 (MARCKS-related protein)	*MARCKSL1 (MRP)*	4.5	200644_at
Aquaporin 3	*AQP3*	4.3	203747_at
Chondroitin sulfate *N*-acetylgalactosaminyltransferase 2	*CSGALNACT2*	3.8	218871_x_at
Transmembrane emp24 protein transport domain containing 9	*TMED9*	3.3	208757_at
Dedicator of cytokinesis 11	*DOCK11*	3.3	238356_at
Poly (ADP-ribose) polymerase family, member 6	*PARP6*	3.3	234710_s_at
Wolf-Hirschhorn syndrome candidate 2	*WHSC2*	3.2	203112_s_at
AE-binding protein 2	*AEBP2*	3.2	225889_at
SEC16 homolog B (*S cerevisiae*)	*SEC16B*	3.0	1564423_a_at
Kelch-like 23 (*Drosophila*)	*KLHL23*	3.0	213610_s_at
Superoxide dismutase 2, mitochondrial	*SOD2*	2.9	221477_s_at
Heparan sulfate 2-*O*-sulfotransferase 1	*HS2ST1*	2.9	203285_s_at
Armadillo repeat containing, X-linked 4	*ARMCX4*	2.9	227444_at
Solute carrier family 4, sodium bicarbonate cotransporter, member 7	*SLC4A7*	2.8	209884_s_at
RAP1 interacting factor homolog (yeast)	*RIF1*	2.8	214700_x_at
ArfGAP with FG repeats 2	*AGFG2*	2.7	222126_at
AT-rich interactive domain 1B (SWI1-like)	*ARID1B*	2.7	225181_at
Calpain 1, (μ/I) large subunit	*CAPN1*	2.7	200752_s_at
Ligand of numb-protein × 2	*LNX2*	2.7	227569_at
Family with sequence similarity 189, member B	*FAM189B*	2.6	203550_s_at
Major histocompatibility complex, class II, DQ β 1	*HLA-DQB1*	2.6	211656_x_at
Tax1 (human T-cell leukemia virus type I) binding protein 3	*TAX1BP3*	2.6	215464_s_at
Mitogen-activated protein kinase kinase kinase kinase 5 (germinal center kinase related)	*MAP4K5 (GCKR)*	2.6	203552_at
RAB6A, member RAS oncogene family	*RAB6A*	2.6	201048_x_at
Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*); translocated to, 4	*MLLT4*	2.6	224685_at
Homeobox B7	*HOXB7*	2.6	204779_s_at
5-azacytidine induced 2 (NAK-associated protein 1)	*AZI2 (NAP1)*	2.6	227904_at
Histone deacetylase 8	*HDAC8*	2.6	223345_at
Hemoglobin, ε 1	*HBE1*	2.5	205919_at
fem-1 homolog c (C. elegans)	*FEM1C*	2.5	213341_at
COMM domain containing 5	*COMMD5*	2.5	224387_at
Solute carrier family 44, member 2	*SLC44A2*	2.5	224609_at
Matrin 3	*MATR3*	2.4	242260_at
Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	*TFPI*	2.4	210664_s_at
Protein phosphatase 1, catalytic subunit, β isozyme	*PPP1CB*	2.4	201408_at
β-1,3-glucuronyltransferase 3 (glucuronosyltransferase I)	*B3GAT3*	2.4	203452_at
Pyroglutamyl-peptidase I	*PGPEP1*	2.4	219891_at
ADP-ribosylation factor–like 1	*ARL1*	2.4	201657_at
Taxilin γ	*TXLNG*	2.4	219969_at
Acyl-CoA synthetase family member 3	*ACSF3*	2.4	1556552_a_at
Retinol dehydrogenase 11 (all-trans/9-cis/11-cis)	*RDH11*	2.4	217776_at
Ataxin 7–like 1	*ATXN7L1*	2.4	227732_at
Adaptor-related protein complex 3, δ 1 subunit	*AP3D1*	2.4	210974_s_at
Nucleoporin 160 kDa	*NUP160*	2.4	214963_at
Discs, large (*Drosophila*) homolog-associated protein 4	*DLGAP4*	2.4	202572_s_at
Heparan sulfate (glucosamine) 3-*O*-sulfotransferase 3B1	*HS3ST3B1*	2.4	1561908_a_at
AP2-associated kinase 1	*AAK1*	2.3	205434_s_at
Insulin-induced gene 1	*INSIG1*	2.3	201625_s_at
Calponin 2	*CNN2*	2.3	201605_x_at
Polymerase (RNA) mitochondrial (DNA directed)	*POLRMT*	2.3	203783_x_at

ATL cell lines and primary ATL samples (supplemental Figure 1). We also confirmed that *SOX4* siRNA but not control siRNA significantly reduced the expression of *GCKR*, *NAP1*, and *HDAC8* in 2 ATL cell lines (Figure 4B). These results in part validated our microarray data.

Direct activation of the *HDAC8* promoter by SOX4

We further examined direct activation of the *HDAC8* promoter by *SOX4*. Figure 5A depicts the promoter region of *HDAC8* with potential *cis* elements. We inserted the DNA fragment from –712 to +126 bp relative to the transcriptional start site and its successive 5′-truncated fragments into pGL3-basic and transfected these constructs to ST1 cells for luciferase reporter assays. The results mapped a major regulatory element within –358 to –173 bp (data not shown). A potential SOX4 element was present at –243 to –236 bp. To directly address the role of this SOX4 element, we generated the –712- to +126-bp construct with mutations in the SOX4 element (from AACAAGGA to ACCATGGA). As shown in Figure 5B, the *HDAC8* promoter activation in ST1 cells was significantly ($P < .05$) reduced by the mutations in the SOX4 element. Furthermore, cotransfection of the *SOX4* expression vector

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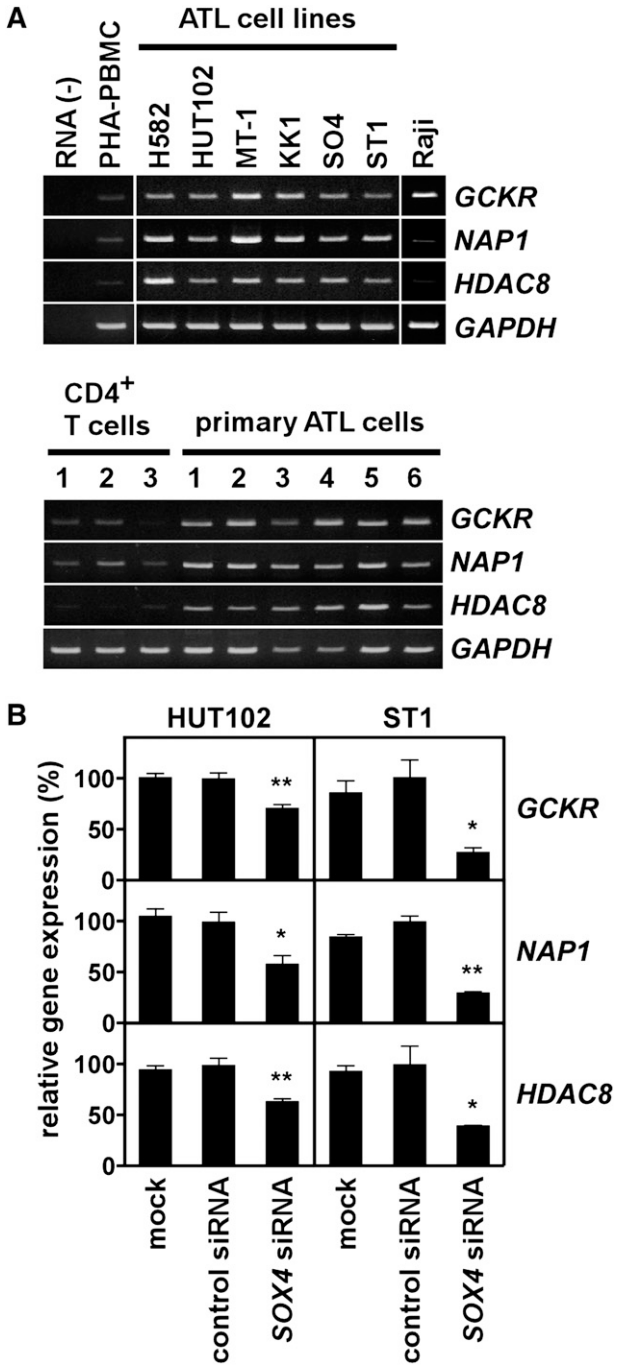


Figure 4. Expression of *SOX4* target genes in ATL. (A) Gene expression analysis. Semiquantitative RT-PCR was performed to analyze expression of *GCKR*, *NAP1*, and *HDAC8* in ATL cell lines and Raji (top panel), and in normal $CD4^+$ T cells and PBMCs from ATL patients (>90% leukemia cells) (bottom panel). PHA-PBMC, normal PBMCs treated with PHA for 3 days. *GAPDH* was used as a loading control. The representative results from 3 separate experiments are shown. (B) Effect of *SOX4* siRNA on target gene expression. Two ATL cell lines (HUT102 and ST1) were transfected with control siRNA or *SOX4* siRNA. After 48 hours, total RNA samples were prepared. Quantitative real-time PCR was performed for *GCKR*, *NAP1*, *HDAC8*, and *GAPDH*. The expression levels were normalized by *GAPDH*. Data are shown as mean ± SEM of 3 separate experiments. $^*P < .05$; $^{**}P < .01$.

in Raji cells efficiently activated the *HDAC8* promoter with the wild-type SOX4 element but not that with the mutated SOX4 element (Figure 5C). Taken together, *SOX4* indeed directly activates the *HDAC8* promoter at least partly via the SOX4 element at –243 to –236 bp.

Role of *HDAC8*, *GCKR*, and *NAP1* in ATL cell growth

Recently, Hasegawa et al reported that a pan-HDAC inhibitor LBH589 effectively suppressed cell growth in ATL cell lines and primary ATL cells.[24] Consistent with their report, broad-spectrum HDAC inhibitors TSA and SAHA efficiently suppressed cell growth in ATL cell lines (Figure 5D). In particular, ST1 cells were highly sensitive to the HDAC inhibitors. Furthermore, we demonstrated that *HDAC8* siRNA but not control siRNA significantly suppressed cell growth of ATL cell lines (Figure 5E). *HDAC8* siRNA had no such growth-suppressive effect on control Raji (Figure 5E). It was also notable that only partial reduction of *HDAC8* expression efficiently suppressed ATL cell growth. In particular, ST1 cells were highly sensitive to the effect of *HDAC8* knockdown. We further demonstrated that *GCKR* siRNA as well as *NAP1* siRNA significantly suppressed cell growth of 2 ATL cell lines but not that of Raji (supplemental Figure 4).

Discussion

SOX4 is a member of the SOX (Sry-related high mobility group box) family of transcription factors that share homology in their DNA-binding domain, the high mobility group box.[25] *SOX4* is known to be involved in various biological processes including T- and B-cell development.[7,8] Furthermore, overexpression of *SOX4* was reported in a wide range of human cancers including breast, brain, lung, pancreatic, salivary gland, prostate, bladder, and ovarian cancers.[9-12] The role of *SOX4* in tumors may depend on the cellular context. For example, in the case of bladder cancer, strong expression of *SOX4* significantly correlated with increased patient survival and overexpression of *SOX4* induced apoptosis in cancer cells.[9] Similarly, reduced expression of *SOX4* was significantly correlated with metastasis and poor prognosis of melanoma patients.[26] In sharp contrast, knockdown of *SOX4* induced apoptosis in prostate and adenoid cystic cancer cells,[10,27] and suppressed tumor growth and local metastasis in hepatocellular carcinoma.[28] Recently, Pan et al reported a DNA damage sensor function of *SOX4*.[29] They demonstrated that, upon DNA damage, *SOX4* was quickly induced in parallel with *p53* and promoted cell arrest and apoptosis in a *p53*-dependent manner.[29] Thus, the strong upregulation of *SOX4* in various solid tumors may in part reflect the increased DNA damages in such tumor cells. As for hematologic malignancies, *Sox4* was identified as the gene most frequently associated with retroviral integration sites in a large panel of mostly B- and some T-cell lymphomas induced by retroviral insertion mutagenesis in AKXD and NFS.V^+ mouse strains.[30] This suggests that *Sox4* is an important oncogene in B- and T-cell lymphomas. *Sox4* was also demonstrated to promote mouse myeloid leukemia development in cooperation with *Mef2c*[31] or in association with reduced expression of *Sfpi1*.[32] Consistently, a significant negative correlation was observed in the expression levels of *SOX4* and *PU.1* (the human counterpart of *Sfpi1*) in human acute myeloid leukemia.[32] Recently, a link between *SOX4* and ATL was also suggested. A set of genes including *SOX4* were shown to be up-regulated in the presence of HTLV-1 *bZIP* factor (*HBZ*),[22] which is considered to play an important role in ATL oncogenesis.[21] However, this study did not examine the mechanism of upregulation of *SOX4* by HBZ or the role of *SOX4* in ATL.

Previously, we have shown that the AP-1 family member *FRA-2* is constitutively expressed in ATL and together with

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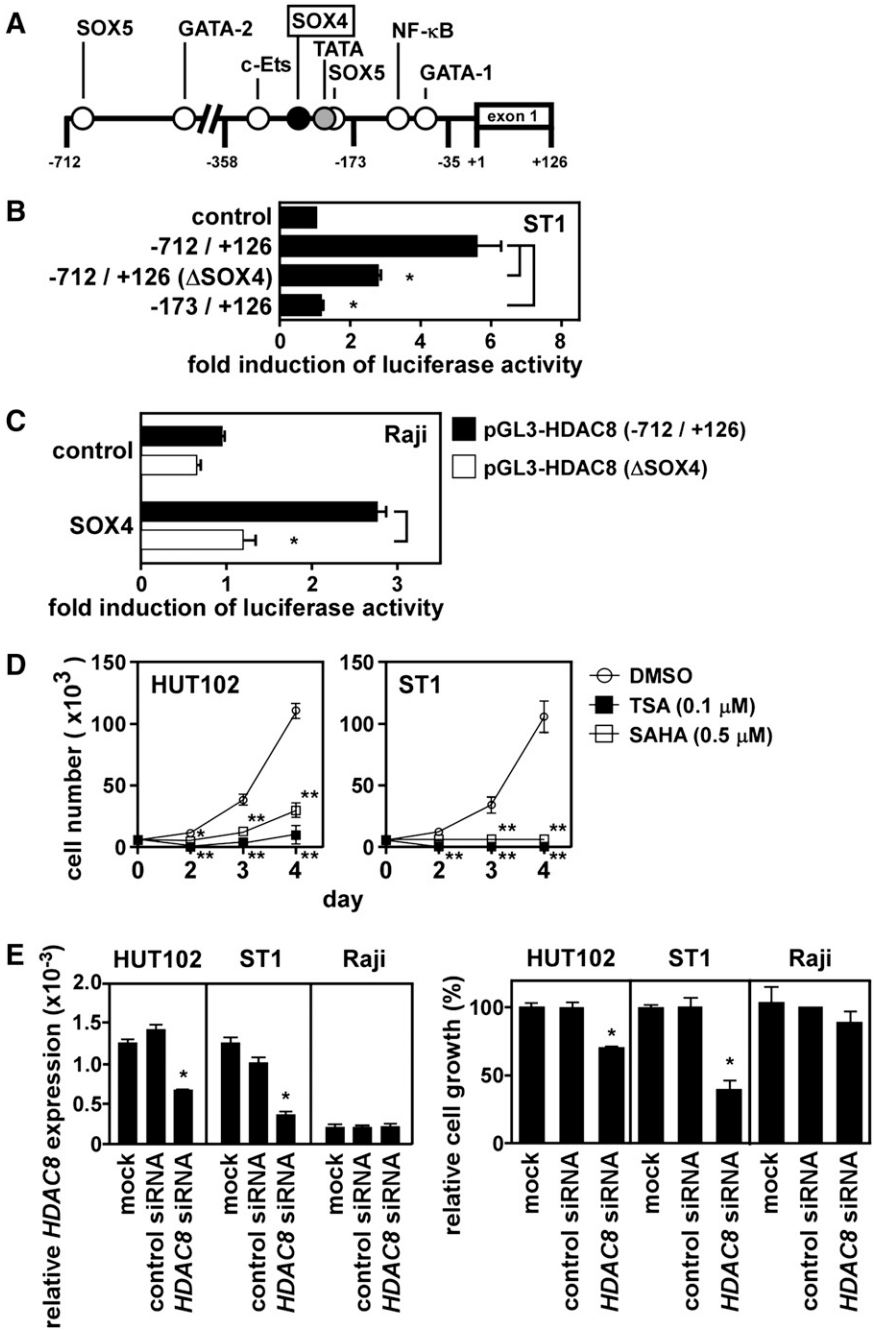


Figure 5. Direct activation of the *HDAC8* promoter by *SOX4*. (A) A schematic depiction of the *HDAC8* promoter region from –712 to +126 bp with potential *cis* regulatory elements. (B) Mutation analysis. Luciferase reporter assays were performed with the *HDAC8* promoter fragment from –712 to +126 bp with the SOX4 site at –243 to –236 bp being wild-type or mutated (ΔSOX4, from AACAAGGA to ACCATGGA). Data are presented as mean ± SEM of 3 separate experiments. *$P < .05$. (C) Reconstitution experiment. Raji cells were cotransfected with pGL3-HDAC8 (–712/+126) or pGL3-HDAC8 (–712/+126) ΔSOX4 and the *SOX4* expression vector or control expression vector as indicated. After 24 hours, luciferase assays were performed in triplicate. Promoter activation was expressed as fold induction of luciferase activity in cells transfected with the *SOX4* expression vector vs cells transfected with the control expression vector. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents mean ± SEM of 3 separate experiments. *$P < .05$. (D) Effect of broad-spectrum HDAC inhibitors on cell growth. ATL cell lines (HUT102 and ST1) were treated with TSA, SAHA, or vehicle (DMSO) and cultured in a 96-well plate at 0.5×10^4 cells per well. At indicated time points, viable cells were counted on FACSCalibur by gating out cells stained with propidium iodide. Data are shown as mean ± SEM of 3 separate experiments. *$P < .05$; **$P < .01$. (E) Effect of *HDAC8* siRNA on cell growth. Two ATL cell lines (HUT102 and ST1) and Raji were transfected with control siRNA or *HDAC8* siRNA. After 48 hours, quantitative PCR was performed for *HDAC8* and *GAPDH*. Levels of *HDAC8* expression were normalized by *GAPDH* (left). Cells were also cultured in a 96-well plate at 0.5×10^4 cells per well. After 72 hours, viable cells were counted on FACSCalibur by gating out cells stained with propidium iodide (right). Data are presented as mean ± SEM of 3 separate experiments. *$P < .05$.

JUND up-regulates the expression of *CCR4* and various other genes including several proto-oncogenes such as *c-MYB*, *MDM2*, and *BCL6*.[5] *SOX4* was also found among the potential downstream target genes of *FRA-2*/*JUND* in ATL.[5] In the present study, we have shown that (1) *SOX4* is indeed consistently expressed in primary blood-circulating as well as skin-infiltrating ATL cells (Figure 1), (2) *FRA-2*/*JUND* directly activates the *SOX4* promoter via an AP-1 site (Figure 2), (3) HTLV-1 *HBZ* has no positive effect on the *SOX4* promoter (Figure 2), and (4) *SOX4* is involved in ATL cell growth (Figure 3). It is also notable that *SOX4* is hardly expressed in normal $CD4^+$ T cells in the blood or normal lymphoid tissues (Figure 1). Thus, *SOX4* could be a novel diagnostic and therapeutic target molecule of ATL. Furthermore, by using an oligonucleotide microarray, we have identified potential downstream target genes of *SOX4* in ATL (Table 1).

Among the genes in Table 1, we have chosen *GCKR* (also called *MAP4K5*),[13] *NAP1* (also called *AZI2*)[14] and *HDAC8*[15-17] for further study. We focused on these genes because of their potential importance in ATL cell growth. We have confirmed their consistent expression in ATL cell lines and primary ATL samples (Figure 4). We have also shown direct activation of the *HDAC8* promoter by *SOX4* via a putative SOX4 site (Figure 5). We have further shown that *HDAC8*, *GCKR*, and *NAP1* are all involved in cell growth of ATL cell lines (Figure 5, supplemental Figure 3). Although various genes were reported as potential target genes of *SOX4* in bladder cancer,[9] adenoid cystic carcinoma,[27] and prostate cancer,[10,33] *GCKR*, *NAP1*, and *HDAC8* were not described in these previous studies and may thus be unique to ATL.

Mitogen-activated protein (MAP) kinases are activated in response to various cellular stimuli including cytokines and

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growth factors to mediate a wide range of cellular responses.[34] There are 3 major groups of MAP kinases: the p38 MAP kinase family, the extracellular signal-regulated kinase family, and the c-Jun NH_2-terminal kinase (JNK) family.[34] The activation pathways of MAP kinases are composed of 3-tiered kinase cascades: MAPKs, MAPKKs (MAP2Ks), and MAPKKKs (MAP3Ks).[34] MAP kinases are also known to be involved in the regulation of apoptosis and growth of hematologic malignancies.[35] Germinal center kinase (GCK) related (GCKR, also called MAP4K5) is a member of the GCK family proteins. GCKR was originally reported as a major mediator of tumor necrosis factor–induced activation of stress-activated protein kinase (also called JNK).[13] It has been shown that TRAF2 recruits and activates GCKR, which in turn activates stress-activated protein kinase/JNK.[36] *GCKR* was also reported to be an important mediator of cellular transformation induced by *Bcr-Abl*, the oncogene associated in Philadelphia chromosome–positive myelogenous leukemia.[37] Specifically, Bcr-Abl activates GCKR through Ras-dependent pathway to activate JNK.[37] In acute myeloid leukemia, constitutive activation of JNK has been shown to correlate with treatment failure and increased multidrug resistance.[38] Constitutive activation of JNK was also reported in ATL.[39] Thus, the enhanced expression of *GCKR* may play a role in the constitutive activation of JNK in ATL, which in turn may activate c-JUN and JUND,[40,41] leading to the constitutive activation of AP-1 in ATL.[42]

NAP1 (NAK-associated protein 1, also called AZI12) was initially identified as a protein interacting with NAK (IκB kinase–related kinase).[14] NAP1 activates NAK to protect cells from tumor necrosis factor-α–induced apoptosis.[14] NAP1 is also essential for double-stranded RNA–dependent production of interferon-β.[43] The enhanced expression of *NAP1* in ATL may thus play a role in the constitutive activation of nuclear factor–κB in ATL.[44]

HDACs are a group of enzymes whose primary functions are considered to be nucleosomal histone deacetylation, a major event that represses eukaryotic gene transcription. However, recent evidence has shown that HDACs are also involved in various cellular processes such as cell-cycle control, differentiation, and apoptosis by deacetylating a wide range of nonhistone proteins.[45] HDACs are divided into 2 major groups: the zinc-dependent classical HDACs comprising class I (HDACs 1, 2, 3, and 8), class II (HDACs 4, 5, 6, 7, 9, and 10), and class IV (HDAC11) and the zinc-independent, NAD-dependent class III sirtuin enzymes.[45] HDAC inhibitors have attracted much attention because of their potential application in cancer therapy.[46,47] *HDAC8* was originally cloned by 3 groups and shown to be expressed in various normal tissues and cancer cell lines with predominant nuclear localization in transfected cells.[15-17] Subsequently, however, HDAC8 was specifically observed in cells with smooth muscle differentiation in normal human tissues, colocalized with the smooth muscle α-actin in the cytosol, and shown to be essential for smooth muscle cell contractile capacity.[48,49] Importantly, an HDAC8-specific inhibitor, PCI-34051, was reported to induce caspase-dependent apoptosis through activation of phospholipase Cγ1 in T-cell lines derived from T-cell acute lymphoblastic leukemia or CTCLs but not in other hematopoietic or solid tumor cell lines.[50] The reason for such selective activity is not clear but may be related to the dominant expression of phospholipase Cγ1 in T cells.[50] Although PCI-34051 has not been tested for ATL, our present results strongly suggest that HDAC8-specific inhibitors such as PCI-34051 would be highly effective on ATL (Figure 5). In fact, a pan-HDAC inhibitor, LBH589, was already shown to have a potent antiproliferative effect on ATL.[24] We also confirmed that the broad-spectrum HDAC inhibitors TSA and SAHA efficiently suppressed ATL cell growth (Figure 5). The enhanced expression of *HDAC8* in ATL may in part account for the therapeutic effectiveness of HDAC inhibitors on ATL. The molecular function of *HDAC8* in ATL remains to be seen.

In conclusion, we have demonstrated an important oncogenic cascade involving *FRA-2*/*JUND* and *SOX4* in ATL, which induces target genes such as *HDAC8*, a promising molecular target of ATL and other T-cell malignancies.[50] The aberrant expression of *FRA-2* has also been frequently observed in CTCLs including anaplastic large-cell lymphoma.[6,51] Indeed, we have found consistent expression of *SOX4* and its downstream target genes such as *HDAC8* in clinical samples of CTCLs (T.H., T.N., Naoki Oiso, Akira Kawada, and O.Y., manuscript in preparation). Thus, there may be a common oncogenic cascade involving *FRA-2*/*JUND* and *SOX4* in mature T-cell malignancies such as ATL and CTCLs. The mechanism of aberrant expression of *FRA-2* in ATL and CTCLs remains to be elucidated.

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Authorship

Contribution: T.H., T.N., and O.Y. designed the research; T.H. and T.N. performed the research; T.A. and K.N. helped with the microarray analyses; and T.H. and O.Y. wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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