

# Hypomethylation of CD30 CpG islands with aberrant JunB expression drives CD30 induction in Hodgkin lymphoma and anaplastic large cell lymphoma

Mariko Watanabe<sup>1,\*</sup>, Yuji Ogawa<sup>1,\*</sup>, Kinji Itoh<sup>2</sup>, Tukasa Koiwa<sup>3</sup>, Marshall E Kadin<sup>4</sup>, Toshiki Watanabe<sup>3</sup>, Isao Okayasu<sup>5</sup>, Masaaki Higashihara<sup>1</sup> and Ryouichi Horie<sup>1</sup>

High expression of CD30 and JunB is the hallmark of malignant cells in Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL). Ligand-independent signaling by CD30 induces JunB, which activates the CD30 promoter, stabilizing CD30 expression and supporting the survival of Hodgkin–Reed–Sternberg (H–RS) and ALCL cells. Here we show for the first time CpG islands encompassing 60 CpG dinucleotides, located in the core promoter, exon 1 and intron 1 of CD30 gene. Analysis of the methylation status of CD30 CpG islands in H–RS, ALCL and unrelated cell lines reveals an inverse relationship between the extent of CD30 CpG methylation and CD30 expression. CD30 CpG islands of H–RS and ALCL cell lines are rarely methylated. Methylation of the CD30 promoter decreases CD30 induction and JunB action on the demethylated CD30 promoter enhances CD30 induction. CD30 and JunB are strongly expressed in H–RS and ALCL cells, whereas they are not expressed in nonmalignant lymphocytes in which CD30 CpG islands are rarely methylated. We conclude that constitutive action of aberrantly expressed JunB on hypomethylated CD30 CpG islands of lymphocytes triggers CD30 induction and initiates activation of the JunB–CD30–JunB loop, essential to the pathogenesis of HL and ALCL.

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CD30 is a member of the tumor necrosis factor (TNF) receptor superfamily initially identified on the surface of Hodgkin and Reed–Sternberg (H–RS) cells of Hodgkin lymphoma (HL).<sup>1–3</sup> Immunohistochemical analysis of a large range of human tumors has shown that CD30 is overexpressed not only by H–RS cells, but also by a subset of diffuse large cell neoplasms with anaplastic features called anaplastic large cell lymphoma (ALCL).<sup>4,5</sup>

Overexpression of CD30 is a characteristic of H–RS and ALCL cells. Ligand-independent signals triggered by overexpressed CD30 induce activation of NF- $\kappa$ B and the extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) pathway, both of which contribute to tumorigenesis and maintenance of survival of H–RS and ALCL cells.<sup>6–9</sup>

Approximately 98% of H–RS cells are derived from B cells and the remaining 2% of H–RS cells appear to be derived from T cells.<sup>10,11</sup> Amplification of genomic DNA and complementary DNA (cDNA) derived from single H–RS cells indicates a germinal center B-cell origin of most H–RS cells. However, the origin of H–RS cells with T-cell phenotype and that of ALCL cells is entirely unknown. Despite lack of information about the cell origin in T-cell HL and ALCL with CD30 overexpression, it is assumed H–RS and ALCL cells are derived from normal lymphocytes, which lack CD30 expression at resting state. Consequently, understanding the mechanisms for constitutive overexpression of CD30 is important to understand the pathogenesis of HL and ALCL.

Cloning and characterization of the promoter region of the CD30 gene enabled us to demonstrate that the CD30

<sup>1</sup>Department of Hematology, School of Medicine, Kitasato University, Sagamihara, Kanagawa, Japan; <sup>2</sup>Department of Pathology, School of Medicine, Toho University, Ohta-ku, Tokyo, Japan; <sup>3</sup>Laboratory of Tumor Cell Biology, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Minato-ku, Tokyo, Japan; <sup>4</sup>Department of Dermatology and Skin Surgery, Roger Williams Medical Center, Providence, RI, USA and <sup>5</sup>Department of Pathology, School of Medicine, Kitasato University, Sagamihara, Kanagawa, Japan  
Correspondence: Dr R Horie, MD, PhD, Department of Hematology, School of Medicine, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan.  
E-mail: rhorie@med.kitasato-u.ac.jp

\*These authors contributed equally to this work.

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promoter is composed of a microsatellite sequence (MS) containing CCAT repeats and a core promoter with Sp-1-binding sites. The Sp-1 site at -45 to -39 within core promoter is responsible for basal promoter activity.<sup>12,13</sup> On the other hand, the CCAT motif repeated in the CD30 MS represses the core promoter activity of CD30. We found that action of JunB on the AP-1 site in the upstream region of the CD30 core promoter is responsible for strong activity of the CD30 promoter in H-RS and ALCL cells.<sup>14</sup> We also showed ligand-independent CD30-ERK-MAPK signals induce amplification of JunB, which acts on the CD30 promoter to stabilize overexpression of CD30 in H-RS and ALCL cells.<sup>9</sup>

Methylation of dinucleotide cytosine-guanosine motifs (CpG), especially in CpG islands located within promoter regions, is a common mechanism of gene regulation. Until now, there was no knowledge of the role of epigenetic regulation of CD30 gene expression. To learn about the role of epigenetic regulation in CD30 overexpression, we analyzed the CD30 gene and identified CpG islands encompassing 60 CpG dinucleotides (CD30 CpG islands) within the core promoter and exon 1 and intron 1 of the CD30 gene. We studied the contribution of the methylation status of CD30 CpG islands and JunB expression levels to CD30 overexpression in CD30-positive and -negative cell lines including H-RS cells, ALCL cells, as well as in normal lymphocytes and germinal center cells. We confirmed our findings in tissue sections of HL and ALCL and nonmalignant lymphoid tissues.

## MATERIALS AND METHODS

### Cell Cultures

K562, HEK293, Jurkat, HeLa, ML1, ML2, FL and A549 cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and Fujisaki Cell Biology Center (Okayama, Japan). ALCL cell lines (SUDHL1 and Karpas299) and H-RS cell lines (L428, KMH2, HDLM2, L540 and HDMYZ) were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Nonadherent cell lines were cultured in RPMI 1640 and adherent cells in Dulbecco's modified Eagle's medium (DMEM) with supplementation of recommended concentrations of fetal calf serum (FCS) and antibiotics. Normal lymphocytes were separated from peripheral blood of healthy volunteers by differential centrifugation through Lymphoprep (AXIS SHIELD PoC AS, Oslo, Norway). Cells were washed with phosphate-buffered saline (PBS(-)) before use.

### Northern Blotting

Northern blot analysis was carried out essentially as described.<sup>15</sup> Briefly, 1  $\mu$ g per lane of poly (A)-selected RNA was size-fractionated by 1% formalin agarose gel electrophoresis and subsequently blotted onto Hybond-C extra nitrocellulose membranes (Amersham Bioscience,

Piscataway, NJ, USA). Filters were hybridized in  $4 \times$  SSC,  $1 \times$  Denhardt's, 0.5% sodium dodecyl sulfate (SDS), 0.1 M NaPO<sub>4</sub> (pH 7.0), 10% Dextran Na at 65°C with  $1.0 \times 10^6$  cpm/ml of random prime-labeled probes. After washing to a final stringency of  $0.2 \times$  SSC and 0.1% SDS at 65°C, filters were exposed to XAR-5 films (Eastman Kodak, Rochester, NY, USA) at -80°C. RT-PCR-amplified fragments of human CD30 and human GAPDH were used as probes.

### Immunohistochemistry

Cultured cells were immunostained with antibodies for CD30 and JunB, and fluorescence signals were detected using confocal microscopy. Cells were first washed three times with PBS (-). Cytospin samples were prepared using  $5 \times 10^4$  cells and fixed with 100% methanol for 10 min at room temperature, then cells were washed three times in PBS (-). Samples were incubated with primary antibody at the concentration of 5  $\mu$ g/ml at 4°C for overnight and washed with PBS (-) three times. After incubation with fluorescence-labeled secondary antibody for 30 min at 37°C, samples were washed three times in PBS (-) and covered with a PermaFluor antifade reagent (Thermo Shandon, Co., Pittsburgh, PA, USA). Fluorescence signals were detected using confocal microscopy (Radiance, 2000) (Bio-Rad Laboratories, Hercules, CA, USA). Antibodies used were as follows; anti-CD30 mouse monoclonal antibody (Ber-H2) (DAKO Kyoto, Japan) and anti-JunB mouse monoclonal antibody (C-11) (Sata Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Immunostaining of JunB and CD30 was performed on paraffin-embedded specimens of normal peripheral blood mononuclear cell (PBMC) and tonsil as well as lymph nodes affected with HL or ALCL. Prior to incubation of the anti-JunB antibody (C-11) or anti-CD30 antibody (Ber-H2), a heat-induced antigen retrieval was performed. Immunodetection was carried out with biotinylated goat anti-mouse IgG, followed by peroxidase-labeled streptavidine (DAKO). HISTOFINE™ kit (Nichirei, Tokyo, Japan) was used to detect the color reaction for peroxidase, according to the manufacturer's instructions.

### Immunoblotting

Immunoblot analysis was carried out as described.<sup>9</sup> Antibodies used were as follows; anti-CD30 antibody (Ber-H2) (DAKO) and anti- $\alpha$  tubulin mouse monoclonal antibody (TU-02) (Santa Cruz). Alkaline phosphatase-conjugated secondary antibodies are as follows; anti-mouse IgG (H&L) antibody (Promega Madison, WI, USA) and anti-mouse IgM antibody (Santa Cruz).

### CpG Methylation Analysis of Cell Lines

Methylation of the cytosine residue of the CpG site was analyzed by the bisulfite genomic sequencing method with slight modifications.<sup>16</sup> Briefly, genomic DNA of cell culture samples was extracted by SDS/proteinase K digestion,

followed by standard phenol–chloroform extraction and ethanol precipitation. Five  $\mu\text{g}$  of genomic DNA was used for bisulfite treatment. The DNA sample in 0.3 N NaOH was heat denatured at 75°C for 20 min, followed by incubation at 55°C for 4 h in 4.8 M Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and 0.5 mM hydroquinone (both from Sigma, St Louis, MO, USA). The sample DNA was purified using the Wizard DNA Clean-Up system (Promega, Madison, WI, USA) and treated with 0.3 N NaOH at 37°C for 20 min. DNA was precipitated with ethanol and dissolved in 50  $\mu\text{l}$  of H<sub>2</sub>O, and 1/20 of this solution was subjected to PCR amplification using the AccuPrime *Taq* DNA Polymerase System (Invitrogen Granningen, Netherlands). PCR was conducted with a set of primers indicated in Figure 1c as follows: forward primer, 5'-TAAGGGTATGG GAGAAGGTTT-3'; reverse primer, 5'-CCTCCCACCTA TAAATACTAAC-3' (converted cytosine residues are written in bold letters). All primer sequences were devoid of CpG dinucleotides to avoid biased amplification of methylated alleles. The reaction condition was as follows: initial denature at 94°C for 2 min, 45 cycles of 94°C for 30 s, 55°C for 30 s, 68°C for 1 min and the final extension at 68°C for 7 min. Amplified and gel-purified PCR products were cloned into pGEM-T Easy vector (Promega), and nucleotide sequences of 10 or more clones were determined.

### Bisulfite DNA Sequencing Analysis

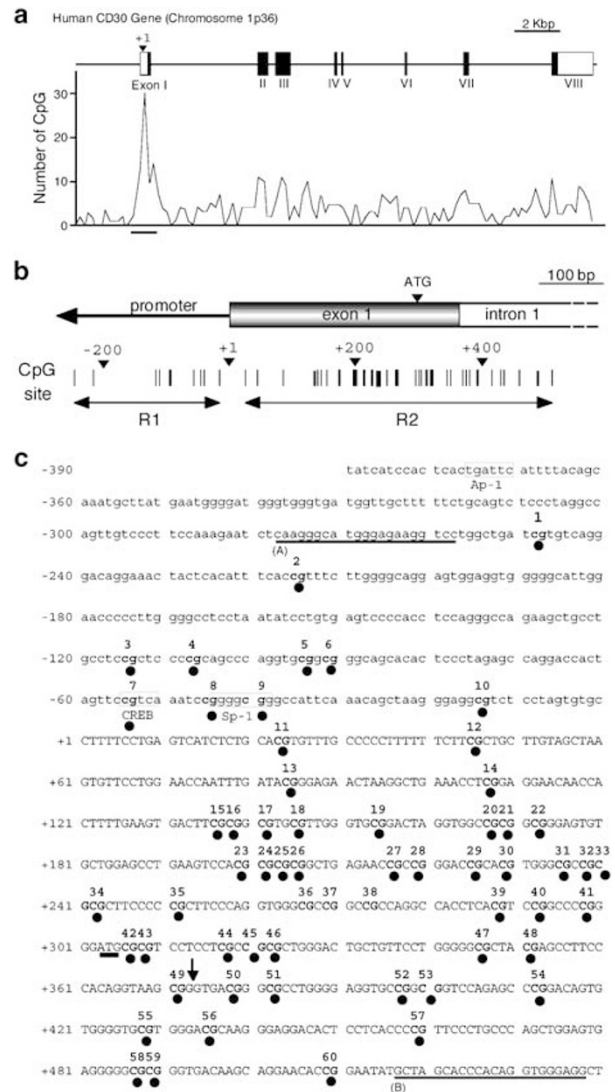
For DNA sequencing, we used an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Piscataway, NJ, USA).

### 5-Azacytidine Treatment

For 5-azacytidine (5-AzaC) (Sigma) treatment, HDMYZ, Jurkat, ML1, FL and HEK293 were seeded at a density of 10<sup>5</sup> cells/ml, and were treated with the demethylating agent 5-AzaC at 1 or 2  $\mu\text{M}$  for 72 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Then, 5-AzaC was removed and cells were cultured for 48 h.

### Reverse Transcriptase-PCR Analysis

Total RNA from culture cells was extracted using the QIAzol Lysis Reagent (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instruction. Each reaction mixture contained 2  $\mu\text{g}$  total RNA, 50 pmol oligo (dT) primer and 200 U SuperScript II reverse transcriptase (Invitrogen) in a 20  $\mu\text{l}$  cocktail. After denaturation of RNA at 70°C for 10 min, reaction mixtures were pre-incubated for 2 min at 42°C, then incubated at 42°C with reverse transcriptase for 50 min and finally denatured at 70°C for 15 min. PCR amplifications were performed using Takara Ex *Taq* DNA Polymerase System (Takara, Kyoto, Japan) according to the manufacturer's instruction. Each specific primers used were follows: CD30 forward, 5'-CAGCTGAGGAGTGGTGCCTCGG-3'; CD30 reverse, 5'-TCTGTCTCCTGCTCGGGGTAGTG-3';



**Figure 1** Map of CpG methylation sites in the promoter, exon 1 and intron 1 of the CD30; primer design for bisulfite sequencing. (a) The line graph indicates the number of CpG sites per 200 nucleotides in the CD30 gene. The region analyzed is indicated by a horizontal line. (b) Individual CpG sites analyzed are indicated as vertical lines and numbered from 1 to 60. Two clusters of CpG sites are indicated as R1 and R2. (c) Sequence of the CD30 genomic region analyzed. CpG sites are indicated by closed circles and numbered. The 5' transcription start site is designated as +1 and initiation codon (ATG) at +303 is underlined. The primers for PCR are underlined (a and b). The border between exon 1 and intron 1 is indicated by an arrow. Guanine at the nucleotide sequence +168 was reported as cytosine in the original report.<sup>31</sup> Potential transcription factor-binding sites are indicated.

$\beta$  actin forward, 5'-TCAGAAGGACTCCTATGTGG-3';  $\beta$  actin reverse, 5'-TCTCTTTGATGTACGCACG-3'.

### In Vitro Methylation and Reporter Gene Assays

Activities of the CD30 promoter were studied by transient reporter gene assays. CD30 genomic region (−272 ~ +531) or (−390 ~ +531) was amplified by PCR with Takara LA *Taq*

polymerase using genomic DNA extracted from KMH2 cells and introduced into an *MluI/HindIII* site of pGL3Basic vector (Promega). Constructs were methylated *in vitro* with CpG Methylase (S.sSI) as recommended by the manufacture (New England Biolabs, Ipswich, MA, USA). The methylation status was checked by digestion with restriction enzymes *HapII* and *HhaI*. Renilla luciferase expression vector driven by the herpes simplex virus thymidine kinase promoter, pRL-TK (Promega), was co-transfected to standardize transfection efficiency in each experiment. Luciferase activities were measured by Dual Luciferase assay kit (Promega). Transfection was carried out using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacture's instructions.

### Laser Capture Microdissection, DNA Extraction and Bisulfite Treatment of Clinical Samples

Formalin-fixed, paraffin-embedded sections were prepared from diagnostic biopsy samples of patients after informed consent. H-RS cells and ALCL cells, which were identified by staining with anti-CD30 antibody Ber-H2 (DAKO) and hematoxylin were microdissected using consecutive sections with modest staining by hematoxylin alone. Briefly, sections were dipped in xylene for 20 min, three changes of xylene for 20 s, three changes of 100% ethanol for 20 s, dipped in distilled water, three changes of 100% ethanol for 30 s and three changes of xylene for 20 s each. The sections were stained with hematoxylin lightly and after air-drying for 60 min, cells were microdissected using ARCTURUS LM200 Laser Capture Microdissection (LCM) unit (ARCTURUS, Mountain View, CA, USA) or Leica AS LMD microsystem (Leica Microsystems, Tokyo, Japan). DNA was extracted from microdissected samples after digestion with the PicoPure DNA Extraction Kit (ARCTURUS) followed by extraction with phenol-chloroform and precipitation with ethanol. Next, bisulfite-modified analysis was performed by MethylEasy DNA Bisulfite Modification Kit (Human Genetic Signatures Pty Ltd, Australia).

### Statistical Analysis

Differences between mean values were assessed by two-tailed *t*-test. A *P*-value <0.05 was considered to be statistically significant.

### Gene Bank Accession Number

The National Center for Biotechnology Information human genome sequence for CD30 is AJ 272029.

## RESULTS

### Identification of CpG Islands within the Core Promoter, First Exon and Intron of the CD30 Gene

Methylation of CpG islands located within the promoter region is one mechanism of epigenetic gene regulation. In order to address whether CD30 gene can be regulated epigenetically by CpG methylation, we analyzed the CpG sites in the 5'-region of CD30 gene. The analysis revealed

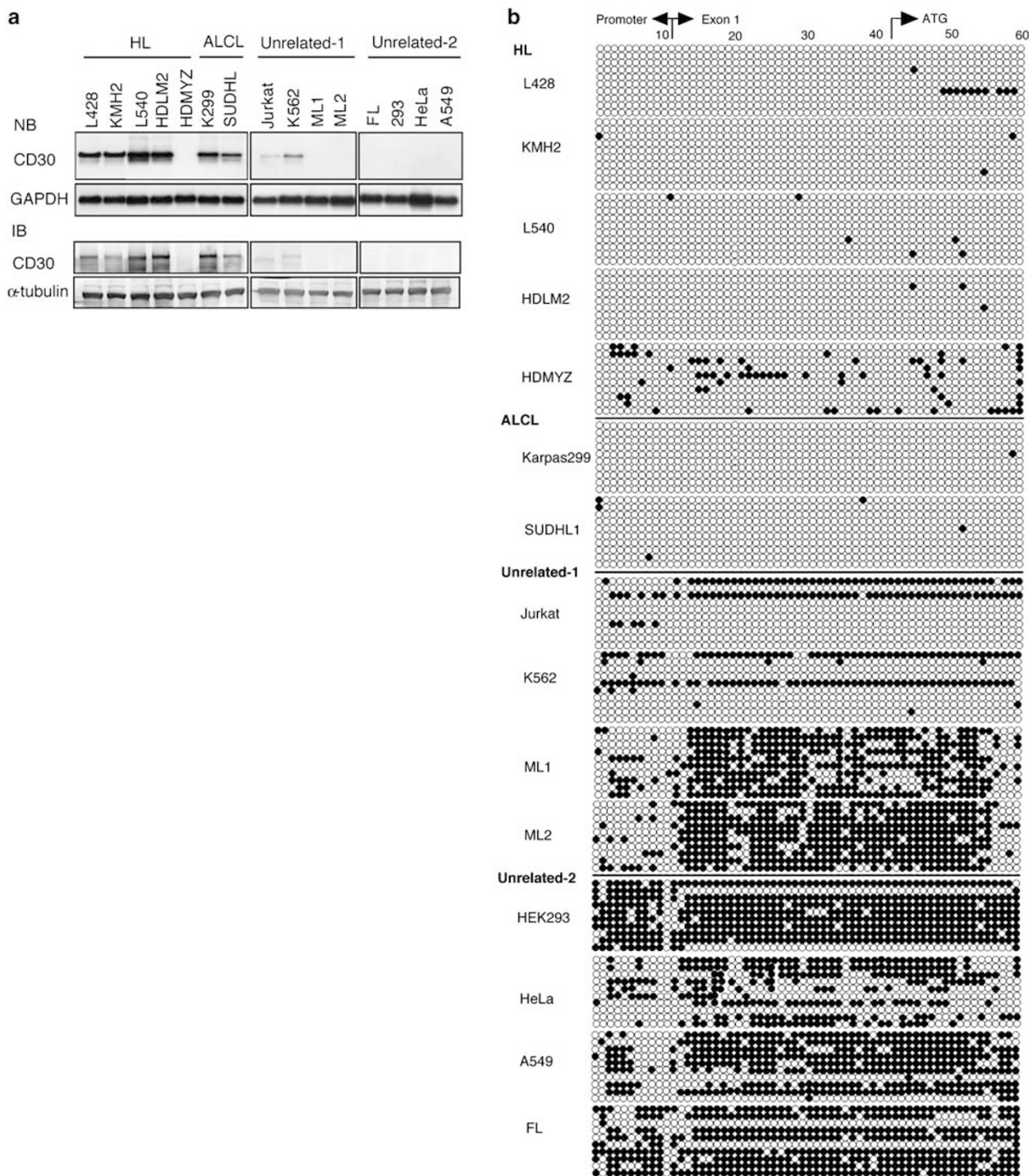
CpG-rich sequences encompassing 60 CpG dinucleotides expanding a region within the promoter, the first exon and the first intron of the CD30 gene (Figure 1a). We identified two CpG islands (island R1, -249 to -15; island R2, +23 to +509). We deduced that CpG dinucleotides, 1-10 (from -249~-15) are located within the core promoter region and CpG dinucleotides, 11-60 are located within the first exon and a part of the first intron of CD30 gene (from +23~+509) (Figure 1b). Primers designed for PCR to amplify the 816 bp sequence that encompasses these 60 CpG sites were underlined (A and B in Figure 1c). Bisulfite sequencing was used to study the methylation status of each CpG site within this region.

### Expression of CD30 mRNA and Proteins in H-RS, ALCL and Unrelated Cell Lines

We first examined the expression of CD30 mRNA and protein in H-RS cell lines (L428, KMH2, HDLM2, L540 and HDMYZ), ALCL cell lines (SUDHL1 and Karpas299) and unrelated cell lines used in this study. Unrelated cell lines examined include Jurkat (T-cell lymphoblastic lymphoma/leukemia), K562, ML1 and ML2 (all myeloid leukemia), HEK293 (embryonic kidney), FL (embryonal epithelium), HeLa (uterine cervical carcinoma) and A549 (lung adenocarcinoma). Among cell lines tested, all H-RS cell lines, except for HDMYZ, and all ALCL cell lines showed strong CD30 mRNA and protein expression. Among unrelated cell lines, Jurkat and K562 expressed lower levels of CD30 than H-RS or ALCL cells. Cell lines HeLa, ML1, ML2, HEK293, FL and A549 did not express CD30 (Figure 2a). These observations are in agreement with previous observations that CD30 is strongly expressed in H-RS and ALCL cells and absent or expressed at low levels in some hematopoietic cells, and not expressed in most nonhematopoietic cells.<sup>5</sup> Although HDMYZ is reported as a H-RS cell line, this cell line lacks several characteristics of H-RS cells including lack of CD30 expression.

### Methylation Status of CD30 CpG Islands in H-RS, ALCL and Unrelated Cell Lines

In order to map the methylation sites within the CD30 CpG islands, we treated DNA from H-RS cell lines, ALCL cell lines and unrelated cell lines with bisulfite, which chemically converts unmethylated cytosine to uracil, whereas it has no effect on methylated cytosine, that is in CpG. PCR amplification, cloning and sequencing of bisulfite DNA showed a specific methylation pattern of the analyzed 60 CpG sites in all cell lines (Figure 2b and Table 1). In general, methylation status could be classified into cell lines with rarely methylated CD30 CpG islands (L428, KMH2, L540, HDLM2, Karpas299 and SUDHL1), those with frequently methylated CD30 CpG islands (HeLa ML1, ML2, HEK293, FL and A549) and those with partially methylated CD30 CpG islands (HDMYZ, Jurkat and K562). Interestingly, all cell lines with rarely methylated CD30 CpG islands were derived from HL or



**Figure 2** The expression level of CD30 and the status of CD30 CpG island methylation in H-RS, ALCL and unrelated cell lines. **(a)** Northern blot analysis of CD30. One microgram of polyadenylic acid-selected RNA was subjected to analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control (top). Immunoblot analysis of CD30. Twenty micrograms of whole-cell lysates were subjected to analysis. Alpha tubulin served as a control (bottom). Cell lines used are indicated above the lanes. NB, northern blot analysis; IB, immunoblot analysis. **(b)** Results of bisulfite genomic sequencing coupled with TA cloning are shown. The methylation status of 10 clones for each sample is presented. Each circle represents a CpG site, a filled circle indicates methylation and an open circle indicates no methylation. Cell lines used are indicated on the left. The numbers described above correspond to those indicated in Figure 1.

ALCL, which are strongly CD30 positive. The percentage of CpG methylation in CD30 CpG islands from CD30-positive cells was very low (mean 5.3%) as compared with CD30-

negative cells (mean 55.1%) and statistically significant ( $P < 0.01$ ) (Table 1). These results indicate involvement of CpG methylation in the regulation of CD30 expression.

**Table 1 Methylation pattern of CD30 CpG islands**

Cell lines	CD30	Methylated CpG (%)		
		Total	R1	R2
L428	+	1.8	0.0	2.2
KMH2	+	0.5	1.0	0.4
L540	+	1.0	0.0	1.2
HDLM2	+	0.5	0.0	0.6
Karpas299	+	0.2	0.0	0.2
SUDHL1	+	0.8	3.0	0.4
Jurkat	+	17.7	12.0	18.8
K562	+	19.7	23.0	19.0
Mean		5.3	4.9	5.3
HDMYZ	–	11.8	13.0	11.6
ML1	–	58.9	21.0	66.2
ML2	–	66.3	17.0	76.2
A549	–	60.3	29.0	70.2
HeLa	–	42.8	22.0	47.0
HEK293	–	76.8	85.0	66.6
FL	–	68.8	62.0	75.2
Mean		55.1	35.6	59.0

+, positive; –, negative; total, R1+R2.

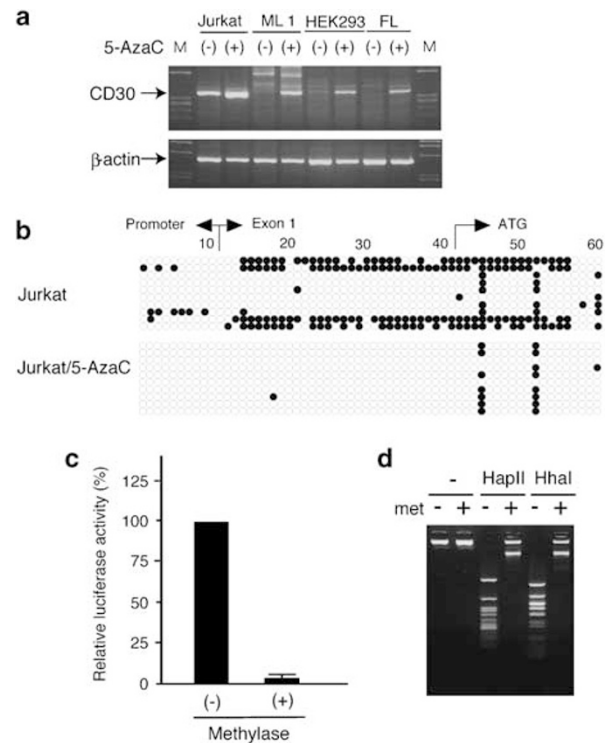
Methylated CpG (%) = (number of CpG sites methylated/total CpG sites) × 100.

The data presented are average of 10 clones analysed. R1 and R2 are indicated in Figure 1.

Among cell lines with frequently methylated CD30 CpG islands, relatively low frequency of methylation of R1 (promoter region) was found in ML1, ML2, HeLa and A549 (mean 22.3%), whereas methylation of R1 region frequency was high in HEK293 and FL (mean 73.5%) (Figure 2b and Table 1). Methylation frequency of CpG sites within R2 (intron 1 and exon1) is almost equally high in these six cell lines.

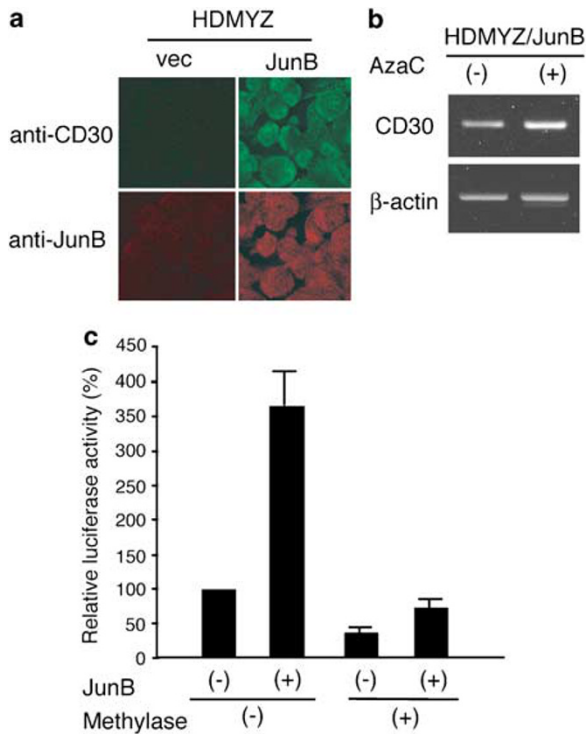
### Methylation of CD30 CpG Islands Decreases the Expression of CD30

To further examine whether methylation status of CD30 CpG islands affects the expression of CD30, we treated cell lines with modest CD30 expression (Jurkat) and cell lines without CD30 expression (ML1, HEK293 and FL) by 5-AzaC, which inhibits *de novo* and maintenance methylation. Treatment with 5-AzaC increased CD30 mRNA expression in cell lines with modest CD30 mRNA expression (Figure 3a). Notably, treatment with 5-AzaC induced CD30 mRNA expression in cell lines (ML1, HEK293 and FL) without constitutive CD30 expression (Figure 3a). Analysis of the methylation status of CD30 CpG islands in Jurkat revealed decreased CpG methylation levels after 5-AzaC treatment (Figure 3b). These



**Figure 3** Regulation of CD30 expression by methylation. (a) Induction of CD30 expression after demethylation by 5-AzaC. Cells were treated with 5-AzaC at 1 μM (Jurkat) or 2 μM (ML1, HEK293, and FL) for 72 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Then, 5-AzaC was removed and cells were cultured for 48 h. Using cDNA obtained from these cell lines, the difference in expression level of CD30 mRNA transcripts without (–) or with (+) 5-AzaC treatment was examined by RT-PCR. After 35 cycles of amplification, products were analyzed by 3% agarose gel electrophoresis. (b) Representative methylation patterns of CD30 CpG islands in Jurkat lymphoblastic cells before and after 5-Aza C treatment. (c) Effect of introduction of methylation in transcriptional control of the CD30 promoter. Reporter gene assays were carried out using Jurkat cells and reporter plasmid containing CD30 CpG islands (–272 to +531 in Figure 1c) treated with or without S.sSI methylase. Relative activity of CD30 promoter treated with S.sSI methylase is shown. Activity of untreated CD30 promoter served as a control, which was set to 100%. (d) Restriction analysis of the methylated reporter construct. The construct before and after methylation was digested with methylation-sensitive endonucleases (*HapII* and *HhaI*).

observations indicate that decreased methylation status triggers expression of CD30. To provide evidence for the direct effect of methylation on CD30 promoter activity, we performed a reporter gene assay with CD30 promoter constructs (nucleic acid position at –276 ~ +531 in Figure 1c) before and after *in vitro* methylation. Treatment with S.sSI methylase significantly reduced transcriptional activity of the CD30 promoter, suggesting that the introduction of methylation suppresses activity of the CD30 promoter (Figure 3c). Methylation of the construct was confirmed by enzyme restriction assay (Figure 3d). These observations indicate that methylation of CD30 CpG islands reduces the expression of CD30.



**Figure 4** Roles of JunB and methylation status of CD30 CpG islands in CD30 induction. **(a)** Induction of CD30 in HDMYZ cells with stably transduced JunB. HDMYZ cells ( $1 \times 10^7$ ) were transfected with 5  $\mu$ g of JunB expression vector or vacant vector using Lipofectoamine 2000 (Invitrogen) and selected by 3  $\mu$ g/ml of puromycin. Cells cloned were stained with antibodies for CD30 and JunB and analyzed by confocal microscopy. **(b)** Induction of CD30 expression after 5-AzaC treatment. HDMYZ cells constitutively overexpressing JunB were treated with 5-AzaC at 1  $\mu$ M for 72 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Then, 5-AzaC was removed and cells were cultured for 48 h. Using cDNA obtained from cells, the difference in expression level of CD30 mRNA transcripts without (-) or with (+) 5-AzaC treatment was examined by RT-PCR. After 35 cycles of amplification, products were analyzed by 3% agarose gel electrophoresis.  $\beta$ -Actin served as a control. **(c)** Effect of introduction of methylation in JunB-mediated induction of the CD30 promoter. Reporter gene assays were carried out using HDMYZ cells and 0.2  $\mu$ g of reporter plasmid containing CD30 CpG islands of CD30 gene (-390 ~ + 531 in Figure 1c) treated with or without S.ssi methylase. In total, 0.5  $\mu$ g of JunB or vacant vector was transfected based on the method described in the Materials and methods. Relative activity of CD30 promoter is shown. Activity of unmethylated CD30 promoter without JunB transfection served as a control, which was set to 100%.

### JunB Action on the Demethylated CD30 Promoter Enhances CD30 Induction

We recently reported that JunB plays an important role in CD30 induction.<sup>14</sup> Therefore, we examined the effect of CD30 CpG island methylation on JunB-mediated induction of the CD30 promoter. For this purpose we used HDMYZ cells, whose CD30 CpG islands are partially methylated. HDMYZ lacks CD30 expression (Figure 2a) and shows weak JunB expression (data not shown). We produced transformants of HDMYZ cells overexpressing JunB and three independent clones were examined for CD30 expression.

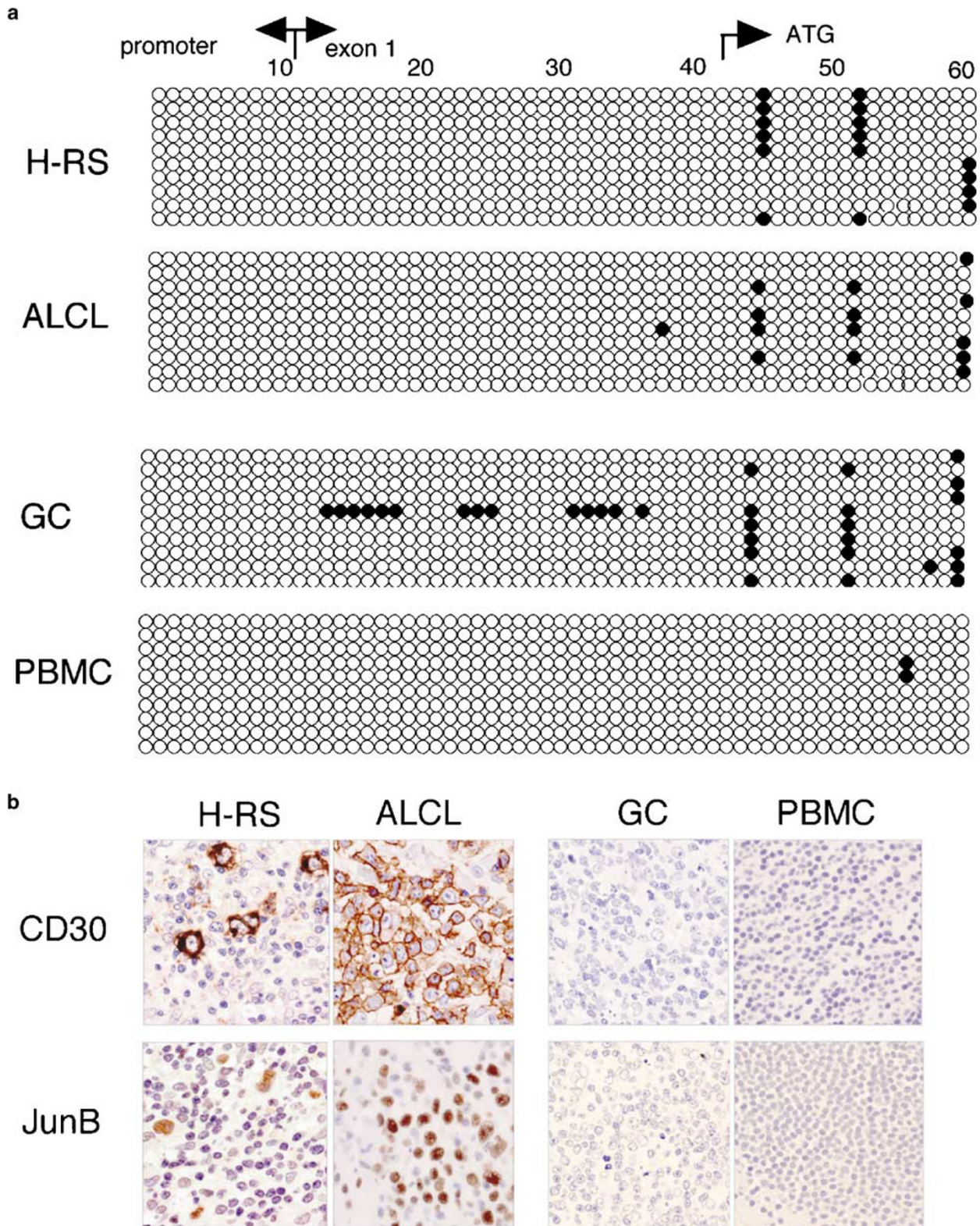
Constitutive overexpression of JunB in HDMYZ cells induced CD30, and 5-Aza C treatment of these clones enhanced CD30 expression. Representative results are shown in Figure 4a and b, respectively. The results indicate that JunB-mediated induction of CD30 is enhanced by reduced methylation status of the CD30 promoter. To confirm the above result, we performed a gene reporter assay with CD30 promoter constructs (nucleic acid position at -390 ~ + 531 in Figure 1c) before and after *in vitro* methylation. JunB-mediated CD30 promoter induction was abolished when the CD30 promoter treated with S.ssi methylase was used (Figure 4c). The result suggests that constitutive action of overexpressed JunB on reduced methylation of CD30 CpG islands enhances CD30 promoter induction.

### Status of CD30 CpG Island Methylation, JunB and CD30 Expression in H-RS Cells, ALCL Cells and Normal Lymphocytes

To obtain insight into CD30 induction during transformation of nonmalignant lymphocytes to H-RS and ALCL cells, we examined the methylation status of CD30 CpG islands in H-RS cells, ALCL cells and normal lymphocytes. For this purpose, H-RS cells, ALCL cells and nonmalignant germinal center (GC) cells were microdissected from tissue biopsy samples ( $n = 3$  for each). PBMC from healthy volunteers were also studied ( $n = 3$ ). We also examined the expression of CD30 and JunB in HL, ALCL, nonmalignant GC and PBMC by immunostaining ( $n = 5$  for each). CD30 CpG islands of H-RS cells, ALCL cells, GC cells and PBMC were rarely methylated. CD30 and JunB were strongly positive in H-RS and ALCL cells, whereas CD30 and JunB were negative in GC cells of tonsils and PBMC. Representative results are shown in Figure 5a and b. These results indicate that both JunB expression and hypomethylation of CpG islands are required for CD30 expression.

### DISCUSSION

Methylation of CpG islands located in gene promoter regions is thought to be one mechanism of gene regulation. For example, aberrant CpG methylation of the SHP-1 promoter occurs in the pathogenesis of both B- and T-cell lymphomas.<sup>17,18</sup> Therefore, we investigated the methylation status of the CD30 promoter, since CD30 overexpression is essential to the pathogenesis of HL and ALCL.<sup>6,9,19</sup> We identified CpG islands encompassing 60 CpG dinucleotides, located in the core promoter, exon 1 and intron 1 of CD30 gene. Our observations in various cell lines reveal that methylation of this region inversely correlates with expression of CD30, suggesting that the CpG methylation inhibits CD30 expression. This conclusion is supported by the results that treatment with demethylating agent 5-AzaC increases CD30 expression in cell lines with high CpG methylation, and CpG methylation of the CD30 promoter by S.ssi methylase decreases transcriptional activity of CD30 promoter.



**Figure 5** Status of CD30 CpG islands methylation and JunB expression in H-RS cells, ALCL cells and lymphocytes. **(a)** Analysis of H-RS cells, ALCL cells and GC cells of normal tonsils obtained by microdissection and PBMC by bisulfite genomic sequencing coupled with TA cloning. The methylation status of 10 clones for each sample is represented. Each circle represents a CpG site, a filled circle indicates methylation and an open circle indicates no methylation. Cells used are indicated on the left. The numbers above correspond to those indicated in Figure 1. **(b)** Expression of JunB and CD30 in H-RS cells, ALCL cells, GC cells and PBMC. Tissue samples (HL, ALCL and normal tonsil) and normal PBMC were stained with antibodies for CD30 (top) or JunB (bottom).



Analysis of the methylation pattern of cell lines with frequently methylated CD30 CpG islands (Figure 2b) indicates that low methylation frequency of CpG sites within R2 (exon 1 and intron 1) is significant for CD30 expression. On the other hand, the R1 (core promoter) contains GC box and CREB-binding motifs. These motifs in general initiate and promote transcription by recruiting general transcription factors and coactivators.<sup>20</sup> It has been demonstrated that DNA methylation can interfere with protein–DNA interactions, recruitment of histone deacetylases, and the induction of chromatin condensation necessary for gene inactivation. Several methyl-binding proteins can compete with transcription factors for the same sequences.<sup>21,22</sup> Therefore, it is likely that the methylation frequency of CpG sites within R1 is also critical for CD30 expression. In sum, low methylation frequency of CpG sites within both R1 and R2 regions appear to be necessary for CD30 induction.

We show that both hypomethylation of CD30 CpG islands and strong JunB expression are required for enhanced CD30 expression. We reported that CD30 overexpression in H–RS cells and ALCL cells is caused by strong AP-1 activity of JunB. Interaction of JunB with the AP-1 site in the CD30 promoter, which is situated upstream of the R1 region, drives CD30 expression.<sup>14</sup> However, JunB alone failed to recover activity of the CD30 promoter treated by S.sSI methylase (Figure 4c). JunB overexpression in cell lines with partial methylation could induce CD30 expression which was enhanced by 5-AzaC treatment (Figure 4a and b). Together, these results support the conclusion that both hypomethylation of CD30 CpG islands and JunB overexpression enhance CD30 expression.

Based on the results that the methylation status of CD30 CpG islands critically affect CD30 expression, we hypothesized that CD30 CpG islands in normal germinal center cells and peripheral lymphocytes are methylated and demethylation events directly trigger constitutive CD30 induction. However, our results show that CD30 CpG islands of normal lymphocytes are rarely methylated, suggesting that demethylation of CD30 CpG islands is not sufficient to trigger CD30 induction during lymphomagenesis. Instead, our current results indicate that aberrant induction of JunB at hypomethylated CD30 CpG islands triggers CD30 induction and initiates an autocrine loop of CD30-mediated activation of NF- $\kappa$ B and ERK1/2-MAPK in HL and ALCL.<sup>9</sup>

DNA hypomethylation and mismatch repair deficiency interact to cause invasive T- and B-cell lymphomas in mice.<sup>23</sup> The exact mechanism for lymphogenesis in these mice is unknown, although enhanced microsatellite instability is likely to be involved.<sup>24</sup> In the case of HL and ALCL, which are derived from B- and T lymphocytes, microsatellite instability is uncommon.<sup>25,26</sup> Instead, we found initiation and constitutive transcription of CD30 gene by aberrant JunB expression maintains hypomethylation of CD30 CpG islands and contributes to the transformation of lymphocytes to H–RS and ALCL cells. Previous reports support the notion

that constitutive transcription of genes inhibits the progression of their methylation.<sup>27–30</sup>

In conclusion, the results of this study indicate that the methylation status of CD30 CpG islands and expression level of JunB together are responsible for high CD30 expression. Combined with our recent findings, these results suggest that constitutive action of JunB on hypomethylated CD30 CpG islands in lymphocytes triggers CD30 induction and initiates high constitutive activity of a JunB–CD30–JunB loop, maintaining hypomethylation of CD30 CpG islands and contributing to the pathogenesis of HL and ALCL.

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