Tumorigenesis and Neoplastic Progression

Epigenetic Changes and Suppression of the Nuclear Factor of Activated T Cell 1 (NFATC1) Promoter in Human Lymphomas with Defects in Immunoreceptor Signaling

Askar Akimzhanov,* Laszlo Krenacs,[†]
Timm Schlegel,* Stefan Klein-Hessling,* Enikö,
Bagdi,[†] Eva Stelkovich,[†] Eisaku Kondo,[‡]
Sergei Chuvpilo,* Philipp Wilke*, Andris Avots,*
Stefan Gattenlöhner,*
Hans-Konrad Müller-Hermelink,*
Alois Palmethofer,* and Edgar Serfling*

From the Institute of Pathology,* University of Wuerzburg,
Wuerzburg, Germany; the Laboratory of Tumor Pathology and
Molecular Diagnostics,[†] Institute of Biotechnology, Bay Zoltan
Foundation for Applied Research, Szeged, Hungary; and the
Department of Pathology,[‡] Okayama University Graduate School
of Medicine, Dentistry, and Pharmaceutical Sciences,
Okayama, Japan

The nuclear factor of activated T cell 1 (Nfatc1) locus is a common insertion site for murine tumorigenic retroviruses, suggesting a role of transcription factor NFATc1 in lymphomagenesis. Although NFATc1 is expressed in most human primary lymphocytes and mature human T- and B-cell neoplasms, we show by histochemical stainings that NFATc1 expression is suppressed in anaplastic large cell lymphomas and classical Hodgkin's lymphomas (HLs). In HL cell lines, NFATc1 silencing correlated with a decrease in histone H3 acetylation, H3-K4 trimethylation, and Sp1 factor binding but with an increase in HP1 binding to the NFATC1 P1 promoter. Together with DNA hypermethylation of the NFATC1 P1 promoter, which we detected in all anaplastic large cell lymphoma and many HL lines, these observations reflect typical signs of transcriptional silencing. In several lymphoma lines, methylation of NFATC1 promoter DNA resulted in a "window of hypomethylation," which is flanked by Sp1-binding sites. Together with the under-representation of Sp1 at the NFATC1 P1 promoter in HL cells, this suggests that Sp1 factors can protect P1 DNA methylation in a directional manner. Blocking immunoreceptor signaling led to NFATC1 P1 pro-

motor silencing and to a decrease in H3 acetylation and H3-K4 methylation but not DNA methylation. This shows that histone modifications precede the DNA methylation in NFATC1 promoter silencing. (Am J Pathol 2008, 172:215–224; DOI: 10.2353/ajpath.2008.070294)

In lymphoid cells, the three nuclear factor of activated T-cell (NFATc) proteins NFATc1, -c2, and -c3 are expressed to regulate the transcription of numerous genes that control lymphocyte activation, differentiation, and apoptosis.^{1–3} The activity of NFATc factors is controlled by immunoreceptor signals that, in addition to other signaling events, lead to a rise in intracellular free Ca²⁺ and the subsequent activation of the Ca²⁺/calmodulin-dependent Ser/Thr-specific phosphatase calcineurin. Calcineurin binds to the regulatory region within the N-terminal half of NFATc proteins and removes most of numerous phospho-residues, thereby promoting the nuclear translocation, DNA binding, and transactivation of NFATc. In lymphocytes, NFATc factors control the transcription of numerous lymphokine genes, such as interleukin-2, interleukin-4, and interferon- γ genes, but also of genes that regulate proliferation and apoptosis. Examples of such genes are the *CDK4* gene and the *Fas ligand* gene, whose promoters are bound and controlled by NFATc factors.^{4–9} These and other experimental data suggested that, in addition to their role in normal lymphocyte development, NFATc factors, if aberrantly ex-

Supported by the Mildred Scheel Foundation for Cancer Research, the Deutsche Forschungsgemeinschaft, the Wilhelm Sander Foundation, the Alexander von Humboldt Foundation, and the Hungarian Scientific Research Fund (OTKA-T 046663 KON).

A.A., L.K., and T.S. contributed equally to this work.

Accepted for publication October 1, 2007.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Dr. Edgar Serfling, Department of Molecular Pathology, Institute of Pathology, Josef-Schneider-Strasse 2, D-97080 Wuerzburg, Germany. E-mail: serfling.e@mail.uni-wuerzburg.de.

pressed, might also be involved in the generation of lymphoid tumors (see also¹⁰).

Experimental evidence for this conclusion can be derived from retroviral tagging experiments in which the murine *Nfatc1* locus has been identified as a common insertion site for oncogenic viruses (see also the Mouse Retrovirus Tagged Cancer Gene Database at <http://RTCGD.ncifcrf.gov>).¹¹ In these studies, all retroviral insertions were found to be located close to either the two *Nfatc1* promoters or polyadenylation sites,¹² suggesting that retroviral insertions modulate NFATc1 expression. At similar positions, retroviral insertion sites have also been identified within the murine *Nfatc3* gene, and proviral insertion resulted in a loss or decrease of NFATc3 expression. Because infections of newborn mice with the T-cell lymphomagenic retrovirus SL3-3 MLV led to an acceleration of lymphoma generation in NFATc3-deficient mice compared with wild-type mice, we concluded that NFATc3 exerts a tumor suppressor-like activity for the generation of viral-induced lymphoid tumors.¹³ Investigations of NFATc2-deficient mice that develop chondrocytic neoplasms led to a similar conclusion, ie, NFATc2 can act like a tumor suppressor for the generation of chondrocytic malignancies.¹⁴

However, in other cell types and under other experimental settings, NFAT factors can also exert oncogenic activities. Transformation of pre-adipocytes by a constitutively active version of NFATc1/A showed that this NFAT protein acts as an oncogene for the development of human adipocytic and other tumors,¹⁵ whereas in human classical Hodgkin's lymphoma (cHL), NFATc1 was found to be suppressed.¹⁶ In a subset of large B-cell lymphomas, NFATc1 and c-Rel were detected to be expressed constitutively, to interact with each other, and to control synergistically the expression of CD40 ligand/CD154, thereby maintaining the survival of these aggressive lymphomas.¹⁷ In a similar way, NFATc1 and NFATc2 contribute to the constitutive expression of B-lymphocyte stimulator in large B-cell lymphomas and mantle cell lymphomas and support the survival and proliferation of these malignant B-cell lymphomas.¹⁸ NFATc1 was found to be overexpressed in human pancreatic tumors and to enhance *c-myc* expression, and inhibition of calcineurin/NFATc1 signaling resulted in attenuation of cell proliferation and anchorage-independent growth.¹⁹ Similar results on a constitutive activation of calcineurin have recently been reported for T-cell acute lymphoblastic leukemias.²⁰ Induced by integrin signals, NFATc2 (and NFAT5) promotes the invasion of human breast cancer cells²¹ by the induction of cyclooxygenase 2, which controls prostaglandin E2 synthesis.²² The NFATc2-induced motility and invasion of breast cancer cells can be blocked by Akt/PKB signals,²³ which were shown to suppress the activation of NFATcs in thymocytes and peripheral T cells.^{24,25} Taken together, these data indicate that alterations in calcineurin/NFATc signaling play an important role in human cancerogenesis, and because of their immunoreceptor-dependent activation, aberrant NFATc activity affects particularly the generation and maintenance of human lymphomas.

In this study, we have investigated the expression of NFATc1 (and NFATc2) in human lymphomas. By immunohistochemical staining, we found that NFATc1 expression is suppressed in human anaplastic large cell lymphomas (ALCLs) and cHLs. Both ALCL and cHL are lymphoma entities with a defective immunoreceptor signaling.^{26–28} The silencing of the *NFATC1* locus is correlated with low levels of histone H3 acetylation, H3-K4 trimethylation, and Sp1 factor binding in its P1 promoter region, which is part of a CpG island and controlled by numerous inducible transcription factors.²⁹ We also show here that hypermethylation of P1 promoter DNA correlates with the suppression of NFATc1 expression that, in part, can be released by inhibition of *de novo* DNA methylation by 5-aza-2'-deoxycytidine (5-aza-dC). On interruption of immunoreceptor signaling by cyclosporin A (CsA) in EL-4 thymoma cells that results in the suppression of NFATc1, but not NFATc2, we observed distinct changes in histone modification, whereas DNA methylation remained unaffected. These results suggest that defects in immunoreceptor signaling in ALCL and HL cells lead to a cascade of epigenetic changes that, finally, contribute to the stable silencing of the *NFATC1* promoter(s) in these human lymphomas.

Materials and Methods

Tissue Samples and Cells

Formalin-fixed, paraffin-embedded samples from 3 hyperplastic tonsils, 2 lymph nodes with nonspecific hyperplasia, and 226 malignant lymphoma cases were selected from the histopathology files of the Institute of Pathology, University of Wuerzburg; the Laboratory of Tumor Pathology and Molecular Diagnostics, Institute of Biotechnology, Bay Zoltán Foundation for Applied Research Szeged; and the Department of Pathology, Okayama University. All samples from patients were used following permission and informed consent by the patients. The list of malignant lymphoma cases that were investigated is given in Table 1. Murine EL-4 T lymphoma cells; human Jurkat leukemic cells; the human ALCL lines SR-786, SUP-M2, and KARPAS 299; and the human HL cell lines L428, L540, L1236, and KM-H2 were grown in RPMI containing 10% fetal calf serum to a density of 2×10^5 cells per milliliter and used in all biochemical assays.

Immunohistochemistry and Western Blots

For detection of NFATc1 expression, a mouse IgG1 monoclonal antibody was used recognizing amino acids 197 to 304 of human NFATc1 (clone 7A6; sc-7294; Santa Cruz Biotechnology, Santa Cruz, CA), common to all major NFATc1 isoforms.^{30,31} Immunohistochemical reactions were performed using a streptavidin-biotin-horseradish peroxidase complex method, following heat-induced antigen retrieval performed in 0.5 mol/L Tris buffer (pH 6.0) and pressure cooker. An immunoreaction was classified positive if proposed neoplastic cells revealed moderate to marked homogeneous (cytosolic) staining

Table 1. NFATc1 Expression in Malignant Lymphoma Cases as Detected by Immunohistochemistry

Cases studied (n)	NFATc1 expression [n (%)]			Overall low (– and ±) [n (%)]
	–	±	+	
Systemic ALCL (22)	17 (77)	5 (23)	0	22 (100)
ALK+ (10)	8 (80)	2 (20)	0	10 (100)
ALK– (12)	9 (75)	3 (25)	0	12 (100)
Cutaneous ALCL (11)	1 (10)	5 (45)	5 (45)	6 (55)
Other PTCL (49)	2 (4)	5 (10)	42 (86)	7 (14)
Unspecified (28)	2 (7)	3 (11)	23 (82)	5 (18)
AITL (10)	0	0	10 (100)	0
ETL (11)	0	2 (18)	9 (82)	2 (18)
Mature B-cell neoplasms (93)	11 (12)	3 (3)	79 (85)	14 (15)
B-CLL (6)	0	0	6 (100)	0
MCL (20)	0	0	20 (100)	0
FL (5)	0	0	5 (100)	0
MZL (9)	0	0	9 (100)	0
DLBCL (13)	0	0	13 (100)	0
Mediastinal (3)	0	0	3 (100)	0
Burkitt (18)	0	0	18 (100)	0
Plasmacytoma/myeloma (19)	11 (33)	3 (9)	5 (15)	14 (43)
Classical HL (38)	33 (87)	4 (10)	1 (3)	37 (97)
NLP HL (13)	0	1 (8)	12 (92)	1 (8)

AITL, angioimmunoblastic T cell lymphoma; ALK, anaplastic lymphoma kinase; B-CLL, B-cell chronic lymphoid leukemia; DLBCL, diffuse large B-cell lymphoma; ETL, enteropathy-type T-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; NLP HL, nodular lymphocyte predominance HL; PTCL, peripheral T-cell lymphoma.

for NFATc1. In some cases, moderate to marked NFATc1 immunostaining occurred in a proportion of the tumor cells, and those that showed staining in at least one-half of these cells were scored to be positive. For the exact immunolocalization of NFATc1 in plasma cells with higher sensitivity, NFATc1 staining was combined with an Ig light chain antibody (Ab) cocktail, using double immunofluorescent stains and confocal laser scanning microscopy in formalin-fixed, paraffin-embedded sections of hyperplastic tonsils.

Western blots were performed by fractionation of whole protein extracts from human and murine cell lines on 10% polyacrylamide gels followed by immunodetection using polyclonal Abs raised against the N-terminal halves of NFATc1 or c2, respectively (ImmunoGlobe, Grobostheim, Germany), or against Sp1.³² Equal protein loading of gels was determined by staining of filters with Ponceau red.

DNA Methylation Assays

CpG methylation studies of the human *NFATC1* promoter region were performed using the PCR-based sodium-bisulfite DNA modification procedure.³³ The PCR products were directly sequenced, and semiquantitative data on methylation degree (Figure 2B) were derived from the sequencing peaks. The sequences of the primers used in PCR amplifications for sequencing of bisulfite-modified DNA were as follows. P1 promoter, distal block of homology: forward, 5'-AACAAATAAACRCRTCCCCRAACCTC-CAC-3' (for primary, outer amplification), and reverse, 5'-GAAYGGGTTAGAYGGGAYGTTTGTGTTTAY-3' (for primary, outer amplification); forward, 5'-AAACRCRTC-CCRAACCTCCCCACRCCRACC-3' (nested), and reverse, 5'-GAYGGGAYGTTTGTGTTTAYGYGGGTGTTGG-3' (nested). P1 promoter, proximal block of homology: forward,

5'-CCRCRACCCTAAAACCTACRCRATAAC (for primary, outer amplification), and reverse, 5'-GTTTTAGGYGAG-YGGTTGTGYGGYG-3' (for primary, outer amplification); forward, 5'-CRCRATAACTCCRAACCTACCCRC-3' (nested), and reverse, 5'-GGGYGTTYGGYGATTGTTTGYGG-3' (nested).

Determination of gross DNA methylation in 5-aza-dC-treated HL cell lines (Supplemental Figure S1 at <http://ajp.amjpathol.org>) were performed by combined high-performance liquid chromatography/tandem mass spectroscopy assays.³⁴

Electrophoretic Mobility Shift Assays and Chromatin Immunoprecipitation Assays

Nuclear proteins from Jurkat T and HL cell lines were prepared and used in electrophoretic mobility shift assays (EMSA) as described previously.²⁹ For the detection of Sp1 binding to the P1 promoter, the following (double-stranded) oligonucleotides were incubated as radioactively labeled probes together with 5 µg of nuclear protein: –825 site, (–836) 5'-GGCCTCCCCA-CGCCGGCCCCCTGCCA-3' (–811); –585 site, (–594) 5'-CCCGCGCCCCCGCCCCCGCCCCCT-3' (–572); –210 site, (–220) 5'-CGCGGGGAGGGGCGGGCGCTCGGCG-3' (–196); –15 site, (–30) 5'-TCCGAACCTCGCCGGCG-GAGTCG-3' (–9). The nucleotides in bold correspond to the Sp1 core binding sequence; the underlined nucleotides correspond to the Sp1 consensus binding site.³⁵

For competition, a consensus Sp1 site oligonucleotide (cSp1/3) and a mutated version (mSp1/3) (sc-2502 and sc-2503, respectively) were purchased from Santa Cruz Biotechnology. In addition, a Sp1/3_{–210} site methylated in all CpG residues (methyl-Sp1/3; Figure 4C) and a tandemly arranged Sp1/Sp3 site (Sp1/3_{tn}; Figure 4B) from

the *NFATC1* P2 promoter were used. In supershift EMSAs, 1 μ l of polyclonal Ab raised against Sp1 or Sp3 (sc-644x; Santa Cruz Biotechnology) was added to the incubations.

Chromatin immunoprecipitation (ChIP) assays were performed as described previously.³⁶ Cells were cross-linked using formaldehyde, nuclei were isolated and sonicated, and DNA-protein complexes were immunoprecipitated after pre-clearing with protein-A sepharose blocked with salmon sperm DNA using anti-acetylated histone H3 Ab (06-599; Upstate, Lake Placid, NY), anti-trimethylated H3-K4 Ab (8580; Abcam, Cambridge, UK), anti-dimethylated H3-K9 (05-768; Upstate), anti-trimethylated H3-K9 (8898; Abcam), anti-HP1 (sc-28735; Santa Cruz Biotechnology), and anti-Sp1.³² After washing, elution, and reversion of cross-links, the DNA was isolated and used in PCRs that were performed with the following primers to amplify human or murine *NFATC1* promoters. Human *NFATC1* P1 promoter, distal block of homology: forward, 5'-GAGACGTGAGAGAGGAAAGTGTGAGTGG-3', and reverse, 5'-GAAAGCCCGCATGCTGAAGT-CATTATG-3'. Human *DHFR* promoter: forward, 5'-AACCT-CAGCGCTTCACCCAA-3', and reverse, 5'-CGCACGT-AGTAGGTTCTGTC-3'. Murine *Nfatc1* P1 promoter, distal block of homology: forward, 5'-GAAAAGGACTCCTGGGAA-3', and reverse, 5'-CAAAGACCCAGAGGAGGA-3'. Murine *Nfatc2* promoter: forward, 5'-GCTCTACCTTAGG-GACCA-3', and reverse, 5'-GGTTTGAATCCAGACT-AGGA-3'.

Real-Time PCR Assays

RNAs were extracted by TRIzol from cell lines treated with 5-aza-dC (1 to 5 μ mol/L) for 4 days. RNAs were reverse transcribed using the iScript cDNA synthesis kit of Bio-Rad (Hercules, CA). SYBR Green real-time PCR assays were performed with an ABI PRISM 7700 sequence detection system according to the protocol of ABgene (Hamburg, Germany). For detecting *NFATc1*-, *BOB.1/OBF1*-, *PLC γ 2*-, and *Syk*-specific RNAs, the following primers from Qiagen (Hilden, Germany) were used: Hs_ *NFATc1*-1-SG (QT00094157); Hs_ *POU2AF1*_1-SG (QT0.0001540), Hs_ *PLC γ 2* (QIT00050393), and Hs_ *SYK*_1 (QT00050043). β -Microglobulin primers were used as an internal standard.

Results

NFATc1 Expression Is Suppressed in ALCLs and cHLs

In immunohistochemical stainings of human hyperplastic tonsils and lymph nodes using a *NFATc1*-specific monoclonal antibody raised against all *NFATc1* isoforms (see Materials and Methods), we observed cytosolic staining of virtually all lymphoid T and B cells (with the exception of a faint or no nuclear staining in some germinal center B cells) (Supplemental Figure S2, A and B, at <http://ajp.amjpathol.org>). No staining was observed in endothelial cells and epithelial cells and, as shown in double stainings with monoclonal antibodies against Ig light chains, in

Figure 1. Immunohistochemical stainings of human lymphomas showing the loss of *NFATc1* expression in ALCL and cHL. **A** and **B**: Negative *NFATc1* immunostaining of tumor cells in anaplastic lymphoma kinase-positive (**A**) and anaplastic lymphoma kinase-negative (**B**) systemic ALCL cases. **C**: Enteropathy-type T-cell lymphoma with strong cytosolic staining of *NFATc1*. **D**: Strong homogenous cytoplasmic *NFATc1* positivity in a mantle B-cell lymphoma. **E**: Large Hodgkin's and Reed-Sternberg cells in a cHL demonstrating the loss of *NFATc1* expression. **F**: Nodular lymphocyte predominance Hodgkin's lymphoma showing a strong membranous *NFATc1* positivity. Therefore, in contrast to cHL, primary nodular lymphocyte predominance Hodgkin's lymphoma cells express *NFATc1*.

plasma B cells (Supplemental Figure S2, C and D). When we used the same Ab for immunostaining of a large panel of human lymphoid tumors, the cytoplasm of the majority of tumor cells was also positively stained (Table 1). Thus, a strong cytosolic expression of *NFATc1* was found in the majority of mature B-cell neoplasms: All cases of B-cell chronic lymphoid leukemia, mantle cell lymphoma, follicular lymphoma, diffuse large B-cell lymphoma, mediastinal large B-cell lymphoma, and Burkitt's lymphoma showed consistent *NFATc1* staining, and in the majority of Burkitt's lymphoma cells, a nuclear appearance of *NFATc1* was observed. In contrast to the loss of *NFATc1* expression in normal plasma cells, neoplastic plasma cells of 8 of 19 plasmacytoma/myeloma cases revealed a positive *NFATc1* immunostaining (Figure 1; Supplemental Figures S3 and S4 at <http://ajp.amjpathol.org>; Table 1).

However, in all systemic ALCL cases, immunostaining for *NFATc1* was negative or significantly weaker than in the intermingled small T lymphocytes, both for anaplastic lymphoma kinase-positive and -negative cases (Figure 1, A and B). The accompanying small T cells showed consistently high levels of *NFATc1* expression, favoring these cells as endogenous controls in all *NFATc1* stainings. In primary cutaneous ALCLs, 55% of the cases investigated showed a distinct decrease in or no *NFATc1* immunostaining (Table 1; Supplemental Figure S3A). In other peripheral T-cell lymphomas, the overwhelming majority

of cases exhibited a positive NFATc1 immunostaining (Table 1; Figure 1C; Supplemental Figure S3B).

Similar to most ALCL cases, cHL cases showed a conspicuous loss or strong decrease in NFATc1 staining in their large Hodgkin Reed-Sternberg (HRS) cells (Figure 1E), and in only one case (3%), a heterogeneous NFATc1 immunostaining of moderate intensity was detected in about 50% of the Hodgkin Reed-Sternberg cells. In contrast to cHLs, in nodular lymphocyte predominance Hodgkin's lymphoma, the tumor cells showed a strong, homogeneous NFATc1 positivity in the great majority of cases (Figure 1F; Table 1). These data show that like a number of other signaling molecules, NFATc1 expression is suppressed in ALCLs and cHLs, ie, in human lymphoid tumors with a defective immunoreceptor signaling.^{26–28}

NFATC1 P1 Promoter DNA Is Hypermethylated in ALCLs and Classical Hodgkin's Lymphoma Cells

The suppression of NFATc1 in these human lymphomas could be due to several molecular mechanisms, such as due to the genetic instability of the *NFATC1* locus or repressive epigenetic silencing, including DNA methylation. To show which mechanism(s) might be involved, we tested six microsatellite *NFATC1* markers with respect to a loss of heterozygosity in tumors from ALCL patients. However, in DNAs from six patients, we were unable to detect any loss of heterozygosity at the *NFATC1* locus. In addition, Southern blot assays using NFATC1-specific gene probes did not reveal gross alterations of the NFATC1 gene structure in DNA from several HL lines (data not shown). These negative findings prompted us to investigate DNA methylation of *NFATC1* promoter region in lymphoid tumors using PCR-based sequencing of genomic DNA on sodium-bisulfite modification.³³

The promoters P1 and P2 of the *NFATC1* gene spanning approximately 800 and 200 bp, respectively, are conserved between mouse and human and situated within CpG methylation islands (Figure 2A).²⁹ The inducible P1 promoter can be subdivided into two highly conserved regions of approximately 250 bp, which we designated as the proximal and distal block of homology.²⁹ Each block of homology contains multiple CpG dinucleotides, is flanked by Sp1/Sp3-like binding motifs, and harbors binding sites for several inducible transcription factors, such as for CREB/Fos/ATF-2 and NFAT factors and, in the distal block of homology, for nuclear factor- κ B (Figure 2A). Because we know that introducing mutations into the tandemly arranged NFAT-binding site within the distal block of homology abolished almost all inducible promoter activity,²⁹ we performed DNA methylation assays using PCR primers for the amplification of P1 DNA from positions –506 to –855.

In these assays, P1 DNA methylation was investigated in primary T cells from peripheral human blood; from Jurkat T leukemia cells; from the HL cell lines L428, L540, L1236, and KM-H2; and from the ALCL cell lines SR-786, SUP-M2, and KARPAS 299. Whereas in human primary

Figure 2. Hypermethylation of *NFATC1* P1 promoter DNA in HL and ALCL cell lines. **A:** Scheme of the human chromosomal *NFATC1* gene and its P1 promoter region. The inducible promoter P1 and the proximal polyA site that control NFATc1/αA expression¹² are shown in red, the constitutive promoter P2 and the distal polyA2 site are shown in green. Horizontal arrows indicate transcription starts directed by the promoters P1 and P2, respectively. RSD, Rel similarity domain. The organization of *NFATC1* gene in exons (Ex1, Ex2, etc.) is also indicated. Below, the organization of P1 promoter region is shown. CpG dinucleotides are marked by vertical dashes. The Sp1/Sp3 binding sites are shown as large black dots that flank the proximal and distal blocks of homology of P1 promoter region. Binding sites are indicated for inducible transcription factors within these blocks of homology. The binding of transcription factors to these sites has been shown in EMSAs (data not shown).²⁷ The major start site of transcription is indicated by +1. **B:** DNA methylation at the CpG dinucleotides of distal block of homology of P1 promoter in human peripheral blood lymphocytes (PBL), Jurkat T leukemia cells, in the HL cells lines KM-H2, L1236, L428, and L540 and the ALCL lines Sup-M2, SR-786, and Karpas 299. Filled boxes indicate full DNA methylation, empty boxes no methylation, and half-filled boxes partial methylation. The binding sites for Sp1/Sp3 transcription factors are indicated. **C:** Typical Western immunoblots for the detection of NFATc1 expression in peripheral blood lymphocytes, Jurkat, KM-H2, L428, and L540 HL cells. The cells were either noninduced (–) or induced by T+I (+) for 4 hours. Equal protein loading was checked by Ponceau staining of membranes. A, B, and C mark the position of NFATc1 isoforms in blot of peripheral blood lymphocytes. T+I, TPA (10 ng/ml) and ionomycin (0.5 μM) induction for 4 hours.

effector T cells, NFATc1 is fully expressed on T-cell activation,³¹ depending on the subline, NFATc1 is either inducibly²⁹ or constitutively expressed in Jurkat T cells, ie, NFATc1 expression does not show a marked increase on induction by T+I (Figure 2C). In the majority of tumor cells, ie, in all three ALCL lines and in the HL lines L428, L540, and L591, NFATc1 expression is suppressed, whereas in KM-H2 (and L1236) cells, a weak expression was observed (Figure 2C). This expression of NFATc1 agrees well with the methylation status of P1 promoter DNA: In peripheral blood lymphocyte T cells, the P1 DNA was free of methylation, whereas in Jurkat cells (showing

Figure 3. Re-expression of NFATc1, BOB.1/OBF1, PLCy2, and Syk RNAs on inhibition of DNA methylation by 5-aza-dC. KM-H2, SUP-M2, L428, or L540 cells were treated for 4 days with 1, 2.5, or 5 μmol/L 5-aza-dC, and the RNA of cells was isolated for the detection of specific RNA concentrations in real-time PCR assays (see details in Materials and Methods). The “fold change” refers to the expression of corresponding RNAs in nontreated cells. All measurements were performed as triplicates, and one typical experiment from two assays is shown.

constitutive expression), KM-H2 cells, and L1236 cells, 17 of central CpG residues were found to be free of methylation, and several flanking CpG residues appeared to be either partially or fully methylated. In contrast, in all ALCL cell lines and in the HL lines L428 and L540, all 29 CpG residues within the distal block of homology were fully methylated (Figure 2B). In agreement with published data on the DNA methylation of numerous other promoters,³⁷ these data suggest an inverse relationship between P1 promoter DNA methylation and NFATc1 expression in ALCL and HL cell lines.

To demonstrate whether in these tumor cell lines NFATC1 gene expression is suppressed by P1 DNA methylation, we treated ALCL (SUP-M2) and HL (KM-H2, L428, and L540) cells for 4 days with 1, 2.5, or 5 μmol/L 5-aza-dC. When we measured the RNA concentrations in 5-aza-dC-treated cells, we observed a distinct increase in RNA levels by real-time PCR assays. Whereas in SUP-M2 ALCLs and in KM-H2 and L428 HL cells, a 3.5- to 6-fold increase in NFATc1 RNA concentrations was detected, in L540 HL cells, a 13–25-fold increase was detected on 3 days of 5-aza-dC treatment. This is a distinctly stronger increase in RNA expression than that observed for the expression of *Bob.1* (*Pou2af1*) and *SYK* genes that are known to be controlled by DNA methylation (Figure 3).^{38,39} It shows that DNA methylation contributes to suppression of the NFATC1 locus both in ALCL and HL cells.

Binding of Sp1/Sp3 Transcription Factors Marks a “Window of Hypomethylation” within NFATC1 P1 DNA

The NFATC1 promoters bear several binding motifs for Sp1, a transcription factor that was described to interfere

with DNA methylation.^{40,41} Interestingly, these putative Sp1-binding sites appear to mark the borders of hypomethylated P1 DNA segments that we identified in Jurkat cells, KM-H2 HL cells, and L1236 HL cells (Figure 2B). To see whether these sites are bound by Sp1 in Jurkat and HL cells, we performed EMSAs using radioactively labeled probes, including the putative Sp1 sites around the positions –825 and –585. Incubation with nuclear proteins from Jurkat cells or HL cells resulted in the appearance of several retarded bands. Although factor binding to the distal –825 site appeared to be weaker than to the proximal –585 site, supershift assays with Abs raised against Sp1 and the related factor Sp3 indicate that Sp1 as well as Sp3 bind to both sites (Figure 4A, the shifted Sp1-Abs bands labeled by arrows in lanes 3 and 7 and the disappearance of Sp3 complex in lanes 4 and 8). This conclusion is supported by the efficient competition of factor binding using a 50 mol/L excess of an unlabeled Sp1 consensus binding site (Figure 4A, lanes 2 and 6).

Sp1/Sp3 binding could also be demonstrated for putative Sp1 sites around the positions –210 and –15 that flank the proximal block of P1 homology. EMSAs with a consensus Sp1 binding site (Sp1/3_{cons}) illustrate a very similar factor binding as to the –210 and –15 sites (Figure 4B). Competition with a 50-fold excess of an unlabeled tandemly arranged Sp1/Sp3 site from the P2 promoter (Sp1/3_{in}) or a Sp1/Sp3 consensus site (cSp1/3) led to suppression of Sp1 and Sp3 complex formation, whereas a mutated Sp1/Sp3 site (mSp1/3) was unable to compete (Figure 4B). Likewise, incubation with Abs specific for Sp1 or Sp3 resulted in the supershift of Sp1 or Sp3 complexes, respectively (Figure 4B, arrows). Moreover, methylation of DNA probe did not inhibit Sp1/Sp3 binding to the –210 site (Figure 4C), because it did not impair Sp1 binding to other sites.⁴² It is very likely that the Sp1/3 sites around the positions –210 and –15 also flank a window of 20 de-methylated CpG dinucleotides that is protected against DNA methylation.

When we compared factor binding to Sp1 sites in nuclear protein from Jurkat cells with that from HL cells, a weaker binding was detected in EMSAs using protein extracts from L428 and L540 HL cells compared with Jurkat cells (Figure 4A, compare lanes 11 to 16 with lanes 9 and 10). Whereas in immunoblots, somewhat less Sp1 protein was detected in HL cells than in Jurkat cells (Figure 4D), in ChIP assays, the P1 promoter appeared to be unbound by Sp1 in L540 HL cells but bound in Jurkat cells (Figure 4E). These findings suggest that the binding of Sp1 contributes to DNA methylation status and thereby to the activity of NFATC1 P1 promoter.

Epigenetic Histone Modifications Reflect the Activity of NFATC1 Promoter in Lymphoma Cells

We also questioned whether HL cells that do not express NFATc1 differ in histone modification of their NFATC1 promoter chromatin from those that do express NFATc1. To distinguish active from inactive chromatin sites, we used Abs raised against acetylated histone H3, trimethyl-

Figure 4. Binding sites of transcription factors Sp1 and Sp3 mark the borders of DNA methylation within the P1 *NFATC1* promoter. **A:** Factor binding to the two Sp1/Sp3 sites around the positions -585 and -825 that flank a "window of hypomethylation" (see Figure 2B) within the distal block of homology of the P1 promoter. In EMSAs, the binding of Sp1 and Sp3 in nuclear protein extracts from Jurkat cells is demonstrated by competition with a 50-fold excess of an unlabeled consensus Sp1-binding site (lanes 2 and 6) and by supershifts with Abs specific for Sp1 (lanes 3 and 7, labeled by arrows) and Sp3 (see lanes 4 and 8). In lanes 9 to 16, EMSAs were performed with nuclear protein from uninduced cells (–) or from Jurkat, KM-H2, L428, and L540 cells induced by T+I for 4 hours (+). **B:** Sp1/Sp3 binding to the -15 and -210 sites of P1 promoter in the proximal block of homology. Nuclear proteins from induced Jurkat cells were incubated with an oligonucleotide probe of site -15 (lanes 1 to 10), of site -210 (lanes 21 to 29) or a Sp1 consensus site (lanes 11 to 20). In supershift assays, Sp1- and Sp3-specific Abs (α Sp1 and α Sp3) were added as indicated. For competition, a 5- or 50-fold excess of unlabeled oligonucleotides of a tandemly arranged Sp1/3 site from the P2 promoter (Sp1/2_{in}), a consensus Sp1/Sp3 site (cSp1/3), a mutated Sp1/3 consensus site (mSp1/3), or a Sp1/3₋₁₅ site were added to the incubations as indicated. **C:** DNA methylation does not impair Sp1/Sp3 binding. A chemically synthesized -210 oligonucleotide probe methylated at all C residues (methyl-Sp1/3) was incubated with nuclear protein from Jurkat cells and assayed as in **A** and **B**. For competition, a 5- or 50-fold molar excess was added of cSp1/3, a consensus Sp1/Sp3 site, mSp1/3, a mutated Sp1/3 consensus site, and methyl-Sp1/3, a Sp1/3₋₂₁₀ site that was methylated at all Cs. **D:** Detection of Sp1 expression in Jurkat, KM-H2, L428, and L540 cells by Western blot immunoassays. Nuclear proteins were analyzed from uninduced cells (lanes 1, 3, 5, and 7) or from cell induced by T+I for 4 hours (lanes 2, 4, 6, and 8). Comparable protein loading of lanes of the gel was detected by staining of filters with Ponceau red. **E:** Detection of Sp1 binding to the P1 and DHFR promoters *in vivo* by ChIP assays. Nuclear proteins were analyzed from uninduced cells (lanes 1, 3, 5, and 7) or from cells induced as in **D** (lanes 2, 4, 6, and 8).

lated H3-K4, and di- and trimethylated H3-K9 in ChIP assays. Both acetylated and trimethylated H3-K4 histones are indicative for transcriptional active chromatin, whereas methylated H3-K9 is a property of inactive chromatin.^{43–45} As shown in Figure 5, A and B, in L540 HL cells that do not express NFATc1, the P1 region contains much less acetylated H3 than those in KM-H2 cells and Jurkat cells that express NFATc1, and trimethylated H3-K4 histones are under-represented at the P1 promoter in L540 cells compared with KM-H2 cells. In contrast, similar low levels of H3-K9 di- and trimethylation were observed for L540, KM-H2, and Jurkat cells, although HP1 protein that binds to the methylated H3-K9⁴⁶

was detected at the P1 promoter in L540 but not in Jurkat cells (Figure 5B). Contrary to P1 chromatin, almost identical levels of acetylated and methylated H3 histones and HP1 were detected at the β -actin and DHFR promoters in L540, KM-H2, and Jurkat cells (Figure 5).

To demonstrate a direct link between the loss of T cell receptor (TCR) signaling and epigenetic alterations at the NFATC1 P1 promoter, we blocked the expression of NFATc1 in murine EL-4 lymphoma cells by the immunosuppressant CsA and studied the modification of histones and methylation of DNA at the murine *Nfatc1* P1 promoter. As shown in Figure 6A, treatment of EL-4 cells for 2 days with low doses of CsA

Figure 5. Reduction of histone H3 acetylation, H3-K4 trimethylation, and Sp1 binding in the HL cell line L540 in which NFATc1 expression is suppressed. ChIP assays. Jurkat cells and the HL cell lines L540 and KIMH2, either noninduced (–) or induced by T+I for 4 hours (+) were cross-linked by formaldehyde, and their nuclei were isolated and sonicated. The resulting chromatin fragments were immunoprecipitated using Abs specific for acetylated histone H3, trimethylated H3-K4, di- and trimethylated H3-K9, HP1, or Sp1. After DNA isolation, promoter DNAs were amplified by PCR using primers for the distal block of homology of the P1, the β -actin, or the *DHFR* promoters followed by gel electrophoresis.

abrogated the induction of NFATc1 expression by T+I that mimic TCR signals in T cells. In contrast, no or a very weak suppressive effect of CsA treatment was observed on NFATc2 expression. At the P1 promoter, a distinct decrease was detected in histone H3 acetylation and H3-K4 trimethylation, whereas these histone modifications remained constant at the *Nfatc2* promoter chromatin. Under the same conditions, we also tested the DNA methylation status of *Nfatc1* promoter DNA, but we were unable to detect any methylation of CpG residues on CsA treatment of EL-4 cells at the P1 promoter (data not shown).

Discussion

The results of this study confirm and extend earlier experimental data of immunohistochemical stainings on the expression of NFATc1 in numerous human lymphomas and its suppression in Hodgkin Reed-Sternberg cells of cHLs.¹⁶ Our results suggest an association between the suppression of *NFATC1* gene in both cHLs and ALCLs and of inhibition of immunoreceptor signaling in these lymphoma entities. For cHLs as well as ALCLs, defects in immunoreceptor signaling have been described that result in a silencing of receptor-mediated gene expression program.^{26–28} One indicator gene of this induction program is the *NFATC1* gene, whose full, optimal expression in lymphocytes is controlled by immunoreceptor and co-receptor signals.¹²

We have shown here a close correlation between the DNA methylation of 5'-CpG island in the *NFATC1* P1 promoter region and silencing of P1 promoter activity. In ALCL and cHL cell lines that do not express NFATc1, all CpG dinucleotides within P1 appeared to be methylated, and treatment of these cells by 5-aza-dC led to a re-activation of NFATc1 expression. Although tumor cells are often characterized by a global hypomethylation, the 5'-CpG islands in the promoter region of many genes, in particular of tumor

Figure 6. Inhibition of T-cell receptor signaling by CsA results in a block of NFATc1 expression and decrease in histone H3 acetylation and H3-K4 trimethylation at the murine *Nfatc1*, but not *Nfatc2*, promoter. **A:** Western immunoblot assays of whole protein extracts from EL-4 T lymphoma cells pretreated with 100 ng/ml CsA for 48 hours followed by induction with T+I for 6 hours in the absence or presence of CsA. Polyclonal Abs raised against the N-terminal halves of either NFATc1 or c2 were used for immunodetection. **B:** ChIP assays using chromatin preparations from EL-4 cells that were treated as in A. In PCRs, primers for the detection of *Nfatc1* P1 or *Nfatc2* promoter DNAs were used (see Materials and Methods).

suppressor genes, are often hypermethylated, thereby silencing these genes in tumors.³⁷ DNA hypermethylation and transcriptional silencing have been described for the 5'-CpG islands of *RB1*, *VHL*, *CDKN2A* (*p14^{ARF}* and *p16^{INK4a}*), *MSX2*, and *BRCA1* genes,^{37,47} and the *NFATC1* gene is a further addition to this list.

In striking contrast to the silencing of NFATc1 expression, the *NFATC2* gene, which also bears a 5'-CpG island in its promoter region, is active in cHL cells. This leads to the question of why the CpG islands of closely related genes become either hypermethylated or remain hypomethylated in tumors. Typical examples of such tumor suppressor genes are the *BRCA1* and *MSX2* (*MLH/MSH*) genes: Although the 5'-CpG island of the *BRCA1* gene is hypermethylated in breast and ovarian tumors,⁴⁸ that of the *BRCA2* gene is not,⁴⁹ and although the 5'-CpG island of the *MSX2* (*MLH1*) gene is hypermethylated in colon, gastric, and endometrial tumors, those of *MSH2*, -3, and -6 genes are nonmethylated.⁵⁰ It is remarkable that CpG island hypermethylation frequently affects genes that are controlled by two different promoters, ie, by a strong one that directs the most prominent transcripts and a weaker one that directs minor transcripts. Examples are the *BRCA1*, *APC*, *CDKN2A* (*p14^{ARF}* and *p16^{INK4a}*), and several other genes (see³⁷ for a more detailed discussion), including the *NFATC1* gene. Although it is unknown why CpG islands of genes with two promoters are predominantly methylated, the recruitment of DNA methyltransferases (DNMTs) to promoters with a high CpG content (or other structural abnormalities) might be one explanation. The expression of both *DNMT1* and *DNMT3b* is

increased in several solid and hematological tumors,^{51,52} and high local concentrations of CpG, histone modifications, and/or the binding of heterochromatic proteins, eg, of HP1, might lead to the predominant recruitment of DNMTs to such chromosomal domains and genes.

Despite frequent hypermethylation of tumor suppressor genes controlled by two promoters, the complete silencing of the *NFATC1* gene by DNA hypermethylation in ALCL and HL tumor cells is a surprising finding. Because of the activity of the inducible promoter P1, the *NFATC1* gene is strongly induced on immunoreceptor and co-receptor stimulation at several stages of T-cell development.^{12,53} Under optimal conditions, this results in the massive synthesis of NFATc1/ α A, a relatively short NFATc1 protein that differs remarkably in its N-terminal peptide and short C terminus from NFATc2 and from NFATc1/ β C, whose synthesis is directed by the constitutive *NFATC1* promoter P2. Although NFATc2 and NFATc1/ β C exert a pro-apoptotic activity in T cells²⁹ and Burkitt's lymphoma B cells,⁵⁴ NFATc1/ α A acts in a strong anti-apoptotic manner on ectopic expression in NFATc1-deficient DT40 chicken B cells (E. Kondo, unpublished data). Together with other experimental data,^{15,19} this favors NFATc1/ α A as an oncogene for the generation of (certain) lymphoid tumors. Although the contribution of NFATc1 proteins to tumor generation has to be elucidated in detail, the hypermethylation of the *NFATC1* gene from which a protein with oncogenic properties is expressed casts doubt on the general view³⁷ that only tumor suppressor genes but no oncogenes are hypermethylated in their 5'-CpG islands.

A further finding of general interest might be the detection of a "window of hypermethylation" in the *NFATC1* P1 promoter (Figure 2). In several lymphoma cells in which NFATc1 is expressed at moderate levels, the binding sites for inducible transcription factors within P1 form such windows that are flanked by Sp1/Sp3 binding sites. Sp1/Sp3 sites are known to affect *de novo* DNA methylation,^{40,41} and it is likely that at the *NFATC1* P1 promoter they suppress DNA methylation in a directional manner. More experiments have to show whether Sp1, Sp3, or other factors associating with these two transcription factors inhibit the activity of DNMTs.

The data from this study allow a preliminary view on the molecular events that take place at the *NFATC1* P1 promoter region during the silencing of the *NFATC1* gene. They indicate that persistent immunoreceptor-mediated signals are necessary to keep the *NFATC1* locus open in lymphoid cells. In peripheral T lymphocytes, missing TCR signals lead at first to a drop in P1-directed transcripts (transcribing exon 1), whereas transcripts directed by P2 (transcribing exon 2) are continuously synthesized.⁵³ The complete interruption of TCR-mediated signals by CsA (Figure 6) shows that missing TCR signals lead to deacetylation and decrease of H3-K4 trimethylation at the P1 promoter. Subsequently, such repressive events might result in hypermethylation of promoter DNA and in complete repression of the *NFATC1* locus, as it occurs in ALCLs and cHLs.

It remains to be shown why the expression of NFATc1, including the synthesis of NFATc1/ α A, ie, a protein with

oncogenic properties, is switched off in cHL and other lymphoid tumors. In contrast, the synthesis of nuclear factor- κ B, which shares structural properties with NFATs,¹⁻³ and of AP-1, the most common partner of NFATs in activated T cells,⁵⁵ is constitutively switched on in Hodgkin's lymphoma. In other human lymphomas and tumors, the expression of NFATc1 is enhanced and appears to contribute to tumorigenesis (¹⁹; E. Kondo, unpublished data).

Acknowledgments

We are very much indebted to Doris Michel and Ilona Pietrowski for excellent technical assistance. For critical reading of the manuscript, we are indebted to Dr. Anneliese Schimpl. We thank Dr. G. Suske (Marburg) for kind gifts of polyclonal Sp1 and Sp3 antibodies. We are also indebted to Drs. A. Brink and H. Stopper (Wuerzburg) for the performance of gross DNA methylation assays.

References

1. Serfling E, Berberich-Siebelt F, Chuvpilo S, Jankevics E, Klein-Hessling S, Twardzik T, Avots A: The role of NF-AT transcription factors in T cell activation and differentiation. *Biochim Biophys Acta* 2000, 1498:1-18
2. Hogan PG, Chen L, Nardone J, Rao A: Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev* 2003, 17:2205-2232
3. Macian F: NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* 2005, 5:472-484
4. Baksh S, Widlund HR, Frazer-Abel AA, Du J, Fosmire S, Fisher DE, DeCaprio JA, Modiano JF, Burakoff SJ: NFATc2-mediated repression of cyclin-dependent kinase 4 expression. *Mol Cell* 2002, 10:1071-1081
5. Latinis KM, Norian LA, Eliason SL, Koretzky GA: Two NFAT transcription factor binding sites participate in the regulation of CD95 (Fas) ligand expression in activated human T cells. *J Biol Chem* 1997, 272:31427-31434
6. Xiao S, Matsui K, Fine A, Zhu B, Marshak-Rothstein A, Widom RL, Ju ST: FasL promoter activation by IL-2 through SP1 and NFAT but not Egr-2 and Egr-3. *Eur J Immunol* 1999, 29:3456-3465
7. Li-Weber M, Laur O, Krammer PH: Novel Egr/NF-AT composite sites mediate activation of the CD95 (APO-1/Fas) ligand promoter in response to T cell stimulation. *Eur J Immunol* 1999, 29:3017-3027
8. Lee MO, Kang HJ, Kim YM, Oum JH, Park J: Repression of FasL expression by retinoic acid involves a novel mechanism of inhibition of transactivation function of the nuclear factors of activated T-cells. *Eur J Biochem* 2002, 269:1162-1170
9. Rengarajan J, Mittelstadt PR, Mages HW, Gerth AJ, Kroczeck RA, Ashwell JD, Glimcher LH: Sequential involvement of NFAT and Egr transcription factors in FasL regulation. *Immunity* 2000, 12:293-300
10. Viola JP, Carvalho LD, Fonseca BP, Teixeira LK: NFAT transcription factors: from cell cycle to tumor development. *Braz J Med Biol Res* 2005, 38:335-344
11. Akagi K, Suzuki T, Stephens RM, Jenkins NA, Copeland NG: RTCDG: retroviral tagged cancer gene database. *Nucleic Acids Res* 2004, 32:D523-D527
12. Serfling E, Chuvpilo S, Liu J, Hofer T, Palmethofer A: NFATc1 autoregulation: a crucial step for cell-fate determination. *Trends Immunol* 2006, 27:461-469
13. Glud SZ, Sorensen AB, Andrusis M, Wang B, Kondo E, Jessen R, Krenacs L, Stelkovic E, Wabl M, Serfling E, Palmethofer A, Pedersen FS: A tumor-suppressor function for NFATc3 in T-cell lymphomagenesis by murine leukemia virus. *Blood* 2005, 106:3546-3552
14. Ranger AM, Gerstenfeld LC, Wang J, Kon T, Bae H, Gravallesse EM, Glimcher MJ, Glimcher LH: The nuclear factor of activated T cells (NFAT) transcription factor NFATp (NFATc2) is a repressor of chondrogenesis. *J Exp Med* 2000, 191:9-22

15. Neal JW, Clipstone NA: A constitutively active NFATc1 mutant induces a transformed phenotype in 3T3-L1 fibroblasts. *J Biol Chem* 2003, 278:17246–17254
16. Marafioti T, Pozzobon M, Hansmann ML, Ventura R, Pileri SA, Robertson H, Gesk S, Gaulard P, Barth TF, Du MQ, Leoncini L, Moller P, Natkunam Y, Siebert R, Mason DY: The NFATc1 transcription factor is widely expressed in white cells and translocates from the cytoplasm to the nucleus in a subset of human lymphomas. *Br J Haematol* 2005, 128:333–342
17. Pham LV, Tamayo AT, Yoshimura LC, Lin-Lee YC, Ford RJ: Constitutive NF- κ B and NFAT activation in aggressive B-cell lymphomas synergistically activates the CD154 gene and maintains lymphoma cell survival. *Blood* 2005, 106:3940–3947
18. Kawano S, Otsu K, Kuruma A, Shoji S, Yanagida E, Muto Y, Yoshikawa F, Hirayama Y, Mikoshiba K, Furuichi T: ATP autocrine/paracrine signaling induces calcium oscillations and NFAT activation in human mesenchymal stem cells. *Cell Calcium* 2006, 39:313–324
19. Buchholz M, Schatz A, Wagner M, Michl P, Linhart T, Adler G, Gress TM, Ellenrieder V: Overexpression of c-myc in pancreatic cancer caused by ectopic activation of NFATc1 and the Ca²⁺/calcineurin signaling pathway. *EMBO J* 2006, 25:3714–3724
20. Medyouf H, Alcalde H, Berthier C, Guillemin MC, Dos Santos NR, Janin A, Decaudin D, de The H, Ghysdael J: Targeting calcineurin activation as a therapeutic strategy for T-cell acute lymphoblastic leukemia. *Nat Med* 2007, 13:736–741
21. Jauliac S, Lopez-Rodriguez C, Shaw LM, Brown LF, Rao A, Toker A: The role of NFAT transcription factors in integrin-mediated carcinoma invasion. *Nat Cell Biol* 2002, 4:540–544
22. Yiu GK, Toker A: NFAT induces breast cancer cell invasion by promoting the induction of cyclooxygenase-2. *J Biol Chem* 2006, 281:12210–12217
23. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A: Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol Cell* 2005, 20:539–550
24. Patra AK, Na SY, Bommhardt U: Active protein kinase B regulates TCR responsiveness by modulating cytoplasmic-nuclear localization of NFAT and NF- κ B proteins. *J Immunol* 2004, 172:4812–4820
25. Patra AK, Drewes T, Engelmann S, Chuvpilo S, Kishi H, Hunig T, Serfling E, Bommhardt UH: PKB rescues calcineurin/NFAT-induced arrest of Rag expression and pre-T cell differentiation. *J Immunol* 2006, 177:4567–4576
26. Bonzheim I, Geissinger E, Roth S, Zettl A, Marx A, Rosenwald A, Muller-Hermelink HK, Rudiger T: Anaplastic large cell lymphomas lack the expression of T-cell receptor molecules or molecules of proximal T-cell receptor signaling. *Blood* 2004, 104:3358–3360
27. Schwering I, Brauninger A, Klein U, Jungnickel B, Tinguely M, Diehl V, Hansmann ML, Dalla-Favera R, Rajewsky K, Kuppers R: Loss of the B-lineage-specific gene expression program in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. *Blood* 2003, 101:1505–1512
28. Marafioti T, Pozzobon M, Hansmann ML, Gaulard P, Barth TF, Copie-Bergman C, Robertson H, Ventura R, Martin-Subero JI, Gascoyne RD, Pileri SA, Siebert R, Hsi ED, Natkunam Y, Moller P, Mason DY: Expression pattern of intracellular leukocyte-associated proteins in primary mediastinal B cell lymphoma. *Leukemia* 2005, 19:856–861
29. Chuvpilo S, Jankevics E, Tyrstin D, Akimzhanov A, Moroz D, Jha MK, Schulze-Luehrmann J, Santner-Nanan B, Feoktistova E, Konig T, Avots A, Schmitt E, Berberich-Siebelt F, Schimpl A, Serfling E: Auto-regulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. *Immunity* 2002, 16:881–895
30. Northrop JP, Ho SN, Chen L, Thomas DJ, Timmerman LA, Nolan GP, Admon A, Crabtree GR: NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature* 1994, 369:497–502
31. Chuvpilo S, Avots A, Berberich-Siebelt F, Glockner J, Fischer C, Kerstan A, Eschejir C, Inashkina I, Hlubek F, Jankevics E, Brabletz T, Serfling E: Multiple NF-ATc isoforms with individual transcriptional properties are synthesized in T lymphocytes. *J Immunol* 1999, 162:7294–7301
32. Hagen G, Muller S, Beato M, Suske G: Sp1-mediated transcriptional activation is repressed by Sp3. *EMBO J* 1994, 13:3843–3851
33. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL: A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci USA* 1992, 89:1827–1831
34. Fink K, Brink A, Vienken J, Heidland A, Stopper H: Homocysteine exerts genotoxic and antioxidative effects in vitro. *Toxicol In Vitro* 2007, 21:1402–1408
35. Kadonaga JT, Tjian R: Affinity purification of sequence-specific DNA binding proteins. *Proc Natl Acad Sci USA* 1986, 83:5889–5893
36. Soutoglou E, Talianidis I: Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation. *Science* 2002, 295:1901–1904
37. Esteller M: CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002, 21:5427–5440
38. Ushmorov A, Ritz O, Hummel M, Leithauser F, Moller P, Stein H, Wirth T: Epigenetic silencing of the immunoglobulin heavy-chain gene in classical Hodgkin lymphoma-derived cell lines contributes to the loss of immunoglobulin expression. *Blood* 2004, 104:3326–3334
39. Yuan Y, Mendez R, Sahin A, Dai JL: Hypermethylation leads to silencing of the SYK gene in human breast cancer. *Cancer Res* 2001, 61:5558–5561
40. Brandeis M, Frank D, Keshet I, Siegfried Z, Mendelsohn M, Nemes A, Temper V, Razin A, Cedar H: Sp1 elements protect a CpG island from de novo methylation. *Nature* 1994, 371:435–438
41. Macleod D, Charlton J, Mullins J, Bird AP: Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. *Genes Dev* 1994, 8:2282–2292
42. Höller M, Westin G, Jiricny J, Schaffner W: Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. *Genes Dev* 1988, 2:1127–1135
43. Lachner M, Sengupta R, Schotta G, Jenuwein T: Trilogies of histone lysine methylation as epigenetic landmarks of the eukaryotic genome. *Cold Spring Harb Symp Quant Biol* 2004, 69:209–218
44. Jenuwein T, Allis CD: Translating the histone code. *Science* 2001, 293:1074–1080
45. Schotta G, Lachner M, Peters AH, Jenuwein T: The indexing potential of histone lysine methylation. *Novartis Found Symp* 2004, 259:22–37; discussion 37–47, 163–169
46. Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T: A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev* 2004, 18:1251–1262
47. Esteller M, Fraga MF, Guo M, Garcia-Foncillas J, Hedenfalk I, Godwin AK, Trojan J, Vaur-Barriere C, Bignon YJ, Ramus S, Benitez J, Caldes T, Akiyama Y, Yuasa Y, Launonen V, Canal MJ, Rodriguez R, Capella G, Peinado MA, Borg A, Aaltonen LA, Ponder BA, Baylin SB, Herman JG: DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. *Hum Mol Genet* 2001, 10:3001–3007
48. Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, Bussaglia E, Prat J, Harkes IC, Repasky EA, Gabrielson E, Schutte M, Baylin SB, Herman JG: Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J Natl Cancer Inst* 2000, 92:564–569
49. Collins N, Wooster R, Stratton MR: Absence of methylation of CpG dinucleotides within the promoter of the breast cancer susceptibility gene BRCA2 in normal tissues and in breast and ovarian cancers. *Br J Cancer* 1997, 76:1150–1156
50. Esteller M, Catusas L, Matias-Guiu X, Mutter GL, Prat J, Baylin SB, Herman JG: hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. *Am J Pathol* 1999, 155:1767–1772
51. Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, Jones PA: The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res* 1999, 27:2291–2298
52. Mizuno S, Chijiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y, Sasaki H: Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood* 2001, 97:1172–1179
53. Serfling E, Berberich-Siebelt F, Avots A: NFAT in lymphocytes: a factor for all events? *Sci STKE* 2007, 2007:pe42
54. Kondo E, Harashima A, Takabatake T, Takahashi H, Matsuo Y, Yoshino T, Orita K, Akagi T: NF-ATc2 induces apoptosis in Burkitt's lymphoma cells through signaling via the B cell antigen receptor. *Eur J Immunol* 2003, 33:1–11
55. Macián F, Lopez-Rodriguez C, Rao A: Partners in transcription: nFAT and AP-1. *Oncogene* 2001, 20:2476–2489