# Epigenetic Repression of the NFATc1 Transcription Factor in Human Lymphomas

Dissertation for the completion of Doctorate degree in Natural Sciences at the Bayerische Julius-Maximilians-Universität Würzburg

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# Table of Contents


**1. Introduction.....7**

1.1. Epigenetic Regulation of Eukaryotic Gene Expression……..…7

*1.1.1. DNA methylation in mammalian cells……..……..7*

*1.1.2. Histone modifications in mammalian cells……..*16

1.2. Epigenetics and Cancer…….24

1.3. The family of NF-AT factors……...29

**2. Results.....32**

2.1. LOH analysis of the *nfatc1* region.....32

2.2. The *nfatc1* promoter hypermethylation in primary human lymphomas……..33

2.3. Inverse correlation between NFATc1 expression and promoter methylation...37

2.4. The *nfatc1* gene contains multiply Sp1/Sp3 binding sites……..……..39

2.5. The DNA methylation and Sp1/Sp3 binding……..….41

2.6. Histone modifications in the *nfatc1* promoter region in Hodgkin cell lines…....42

**3. Discussion.....45**

**4. Summary……50**

**5. Material and Methods.....…51**

5.1. Materials……51

*5.1.1. Instruments*……51

*5.1.2. General materials*……..…52

*5.1.3. Chemical reagents*……..……..…52

*5.1.4. Solutions and Buffers*……..…57

*5.1.5. Growth Medium*……69

5.2. Methodology……70

*5.2.1. Mammalian Cell Culture*……..…70

*5.2.2. DNA Methods*……..……71

*5.2.3. RNA Methods*……..……72

*5.2.4. Protein Methods*……75

*5.2.5. DNA/Protein Interaction Assay*……78

**6. References……82**

**7. Appendix……..103**

7.1. Professional profile……103

7.2. Publications .....104

# 1. INTRODUCTION

## 1.1 Epigenetic Regulation of Eukaryotic Gene Expression

Epigenetics is described as a mitotically and meiotically heritable influence on gene expression that is not accompanied by a change in DNA sequence (Li, 2002). Epigenetic regulation is associated with two major events. The first event is DNA methylation which was shown to correlate with transcription repression and gene silencing in a variety of species. The second epigenetic event is reflected by covalent histone modifications leading to chromatin remodeling. The great diversity of these modifications and their combinatorial nature led to the proposition of the so called “histone code” hypothesis (Jenuwein and Allis, 2001).

Beside gene expression regulation, epigenetic events are known to be involved in mammalian X-chromosome inactivation, imprinting, centromere inactivation, position effect variegation and viral protection.

### 1.1.1 DNA methylation in mammalian cells

#### 1.1.1.1 DNA methylation and the genome

DNA methylation in mammalian cells is a major post-replicational modification that occurs predominantly at the 5’-position of cytosine within the CpG dinucleotide or, in some rare cases, within CpNpG trinucleotides (Clark et al., 1995).

In general, the CpG dinucleotide is greatly under-represented in mammalian genomes: due to spontaneous deamination methyl-CpG often mutate to TpG and instead of expected frequency 0.08, the observed frequency is about 0.04 (Bird, 1980). But mammalian genomes contain short CpG-rich stretches of approximately one kilobase in length in which the density of CpGs is close to the theoretical value. These regions are called as CpG islands (Bird et al., 1985). Although they account for only about 1% of the genome and for 15% of the total genomic CpG sites, these islands contain 50%-70% of the unmethylated CpGs (Cooper and Krawczak, 1989; Antequera and Bird, 1993). Recent estimates suggest that there are at least 29,000 such regions in the human genome. Many of which surround the 5' ends of genes (Lander et al., 2001).

Whereas roughly about more than half of all CpGs remain unmethylated in normal adult cells the extent of DNA methylation greatly changes during mammalian development. Thus, the genomes of mature sperm and egg are highly methylated – comparable to methylation in somatic cells. Within hours after fertilization the male

genome is striped by an active demethylation process (Mayer et al., 2000; Oswald et al., 2000). In contrast, the maternal genome is only passively demethylated during subsequent cleavage divisions (Li, 2002). Finally, during a discrete phase of early development, methylation levels in the mouse decline sharply to about 30% of the typical somatic level (Monk et al., 1987; Kafri et al., 1992). Total methylation level is extended to normal during subsequent *de novo* methylation, but lately it tends to decrease during tissue differentiation (Ehrlich et al., 1982).

#### 1.1.1.2 DNA methylation machinery

DNA methylation in mammalian cells appears to be established by a complex interplay of at least three independently encoded DNA methyltransferases (DNMT's) which are classified into two groups: (1) the maintenance DNA methyltransferase (DNMT1) and (2) the *de novo* methyltransferases (DNMT3a, DNMT3b)(Fig. 1). DNMT1 is ubiquitously expressed and responsible for maintenance of methylation pattern during DNA replication by methylating hemi-methylated CpG sites. Dnmt3a and Dnmt3b are regulated during development and establish new DNA-methylation patterns. (Hsieh, 1999; Lyko et al., 1999; Okano et al., 1999). Dnmt2 is the smallest enzyme among the eukaryotic methyltransferases. It consists only of the catalytic part which is similar to prokaryotic cytosine-C5-methyltransferases. This enzyme was isolated recently when searching for DNMT family members; but the function of DNMT2 remains still unclear. It was shown that deletion of *dnmt2* gene has no obvious phenotype in mice (Okano et al., 1998b; Hermann et al., 2003). Therefore, despite all DNMT proteins share highly conserved catalytic COOH-terminal catalytic domains, these enzymes have different substrate specificities and are thought to have different methylation activities *in vivo.*

**DNMT1.** Up to date DNMT1 is known as the most abundant methyltransferase in mammalian somatic cells (Robertson et al., 1999). It was purified from murine erythroleukemia cells using chromatographic fractionation (Bestor and Ingram, 1983) and now it is found in most, if not all, mammalian cells (Trasler et al., 1996). According to *in vitro* studies, Dnmt1 has up to 40-fold preference for hemimethylated DNA substrates over unmethylated substrates, indicating that the main function of Dnmt1 is to maintain specific methylation patterns (Yoder et al., 1997a; Fatemi et al., 2001).

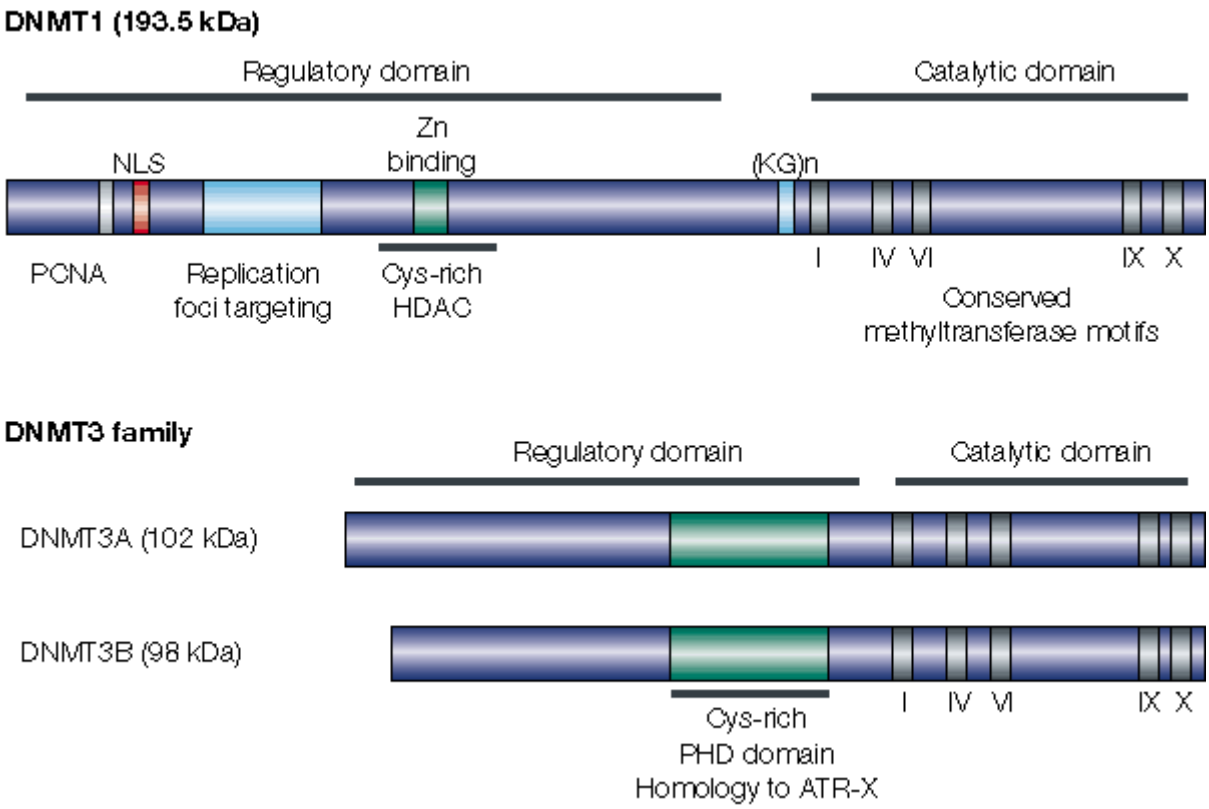


**Figure 1. Schematic structure of the three catalytically active DNA methyltransferases in mammals showing the N-terminal regulatory and C-terminal catalytic domains, and other regions with known functions.** The catalytic domains of the three enzymes are conserved, but there is little similarity between their N-terminal regulatory domains. PCNA, domain that interacts with proliferating cell nuclear antigen; NLS, nuclear localization signal; (KG)n, lysine-glycine repeat hinge region; HDAC, histone deacetylase interaction domain; PHD, plant homeodomain motif that shows homology to the *ATR-X* ($\alpha$-thalassaemia, mental retardation, X-linked) gene [adapted from (Robertson and Wolffe, 2000)].

Within the last decade, both murine and human DNMT1 have been cloned and characterized. Dnmt1 is a very large protein of 184 kDa of approximately 1620 amino acids long. The first N-terminal 1100 amino acids constitute the regulatory domain of the enzyme and contain a nuclear localization sequence (Bestor and Verdine, 1994), a replication *foci* targeting domain (Leonhardt et al., 1992), and a cysteine-rich $Zn^{2+}$ DNA binding domain (Bestor, 1992). This domain lacks any sequence specificity. It has been suggested that it determines the distance traversed between replication and methylation (Chuang et al., 1996).

Many proteins have been reported to interact with the N-terminal part of Dnmt1 in yeast two hybrid and/or interaction assays (Chuang et al., 1997; Robertson et al., 2000; Rountree et al., 2000; Zardo et al., 2002; Fuks et al., 2003; Margot et al., 2003; Liu and

Fisher, 2004). Its N-terminus seems to form a platform for the binding of proteins involved in chromatin condensation and gene regulation. The remaining C-terminus region contains all conserved motifs for catalysis and is similar to that of prokaryotic DNA (cytosine-5) methyltransferases (Bestor et al., 1988; Kumar et al., 1994). It also shows homology with the polybromo-1 protein from chicken (Bestor and Verdine, 1994) which plays a role in the transport of Dnmt1 to the replication *foci* as well (Liu et al., 1998).

The domains of DNMT1 are joined by Gly-Lys repeats and can be separated by V8 protease treatment from intact mouse DNMT1 (Bestor, 1992). However, expression of either N- or C-terminus has not yielded a catalytically active enzyme (Fatemi et al., 2001), suggesting that the interaction and interplay between the regions is essential for activity. Recently, it has been demonstrated that a mouse prokaryotic methyltransferase hybrid, DNMT1-HhaI, containing the intact N terminus of DNMT1 and most of the coding sequence of HhaI, has a 2.5-fold preference for hemimethylated DNA, whereas HhaI by itself has preference for unmethylated DNA (Pradhan and Roberts, 2000). This suggests that the N-terminus of DNMT1 is crucial for the discrimination between methylation of unmethylated and hemimethylated DNA.

The DNMT1 is expressed in the following isoforms: (1) short oocyte specific DNMT1o, (2) the somatic full-length form DNMT1s (Mertineit et al., 1998) and (3) a splice variant known as DNMT1b (Bonfils et al., 2000).

DNMT1o which lacks the 118 N-terminal amino acid residues is generated by a distinct promoter. It is the dominant DNMT1 isoform in mature oocytes and during early embryogenesis but it disappears soon after implantation (Mertineit et al., 1998; Ratnam et al., 2002). The majority of DNMT1o is localized in cytoplasm. It transiently relocates to the nucleus during the eight-cell stage suggesting that it is essential for the establishment of normal methylation patterns of imprinted genes (Cardoso and Leonhardt, 1999; Ng et al., 1999; Doherty et al., 2002). This suggestion is supported by data demonstrating that the inactivation of this oocyte-specific Dnmt1 inhibits the methylation and expression of imprinted genes during mouse embryogenesis, and murine embryos lacking functionally active DNMT1o die between E14 and E21 (Howell et al., 2001).

Despite transcripts for the somatic isoform of Dnmt1 are present within mouse oocytes and early embryos the somatic form of the protein is not produced at these stages. In contrast to the short isoform, DNMT1s is actively expressed after implantation and by day 7 all detectable Dnmt1 protein is the full-length form (Carlson et al., 1992;

Mertineit et al., 1998; Ratnam et al., 2002). All evidence suggests that the primary function of the Dnmt1s isoform is to maintain inherited methylation patterns, including genomic imprints, and methylation patterns established *de novo* in the early stages of postimplantation development (Li et al., 1992; Li et al., 1993).

As mentioned above, several reports have identified key cellular proteins interacting with DNMT1. The most notable are the proliferative cell nuclear antigen (PCNA) (Chuang et al., 1997), histone deacetylases (HDACs) (Fuks et al., 2000; Rountree et al., 2000), the retinoblastoma gene product (Rb) (Fuks et al., 2000; Robertson et al., 2000), DNMT3a and DNMT3b (Kim et al., 2002) and the histone methyltransferase SUV39H1 (Fuks et al., 2003). DNMT1 binds to PCNA in intact cells at the replication foci participating in methylation of newly duplicated DNA chains (Leonhardt et al., 1992; Chuang et al., 1997; Liu et al., 1998). Binding of HDAC1/2 and SUV39H1 suggests that DNMT1 could silence genes by recruiting chromatin-modifying enzymes. Using chromatographic fractionation techniques, it was established that the Rb-E2F1-HDAC1 complex is capable to repress promoters containing E2F binding sites in a methylation independent manner (Robertson et al., 2000).

It was also shown that MeCP2 forms complexes with hemimethylated as well as fully methylated DNA. Immunoprecipitated MeCP2 complexes show DNA methyltransferase activity to hemimethylated DNA suggesting that DNMT1 associates with MeCP2 for the maintenance methylation of DNA *in vivo* (Kimura and Shiota, 2003).

**DNMT2** is the smallest enzyme among the eukaryotic methyltransferases. It consists of 391 amino acids and bears only the catalytic part of the enzyme which is similar to prokaryotic cytosine-C5-methyltransferases (Yoder and Bestor, 1998).

The *dnmt2* gene appears to be well conserved among eukaryotes, not only in organisms whose genomes are methylated (i.e. in mammals, Arabidopsis thaliana, Xenopus laevis and Danio rerio), but also in organisms lacking detectable cytosine methylation, such as in *Schizosaccharomyces pombe* and in *Drosophila melanogaster*. It is ubiquitously expressed in multiple mRNA species in most human and mouse tissues. Adult patterns of Dnmt2 expression in human and mouse tissues are very similar to those of Dnmt1 (Okano et al., 1998b; Yoder and Bestor, 1998). But targeted deletion of DNMT2 in embryonic stem cells did not affect methylation of newly integrated retroviral DNA indicating DNMT2 to be non-essential for DNA methylation and development (Okano et al., 1998b). However, mouse and human Dnmt2 proteins could be trapped by 5-azacytidine, which indicates an as yet undetected Dnmt activity (Liu et al., 2003).

Hence, DNMT2 represents an enigmatic methyltransferase whose functional role remains to be demonstrated.

**DNMT3a and DNMT3b** were identified by homology to DNMT1. They are strongly expressed in ES cells, early embryos, and developing germ cells but expressed at low levels in differentiated somatic cells. The architecture of the DNMT3 enzyme family is similar to DNMT1 showing a large amino terminal regulatory region attached to a catalytic domain. But in contrast to DNMT1, these methyltransferases can methylate hemimethylated and unmethylated DNA as substrate at the same rate (Okano et al., 1998a; Chen et al., 2002). It was also shown that DNMT3a and DNMT3b are essential for *de novo* methylation in ES cells and postimplantation embryos, as well as for *de novo* methylation of imprinted genes in the germ cells (Okano et al., 1999; Hata et al., 2002). These data were also confirmed by analysis of animals deficient for one or both of DNMT3 enzymes (Li, 2002).

In general, DNMT3a and DNMT3b exhibit partially overlapping specificities *in vivo* with Dnmt3b having a preference for methylated minor satellite repeats. This observation is supported by the finding catalytic domain mutations in the human DNMT3b gene from ICF syndrome patients (Hansen et al., 1999; Xu et al., 1999). These patients show a significant loss of methylation in satellite 2 and 3 sequences adjacent to the centromere in chromosome 1, 9, and 16 resulting in chromosome instability (Jeanpierre et al., 1993; Ji et al., 1997).

**DNMT3L** is the third member of this family but has no catalytic activity. Instead it appears to bind to DNMT3a and DNMT3b and to regulate their function. Loss of DNMT3L from early germ cells caused meiotic failure in spermatocytes, which do not express DNMT3L. Whereas dispersed repeated sequences were found to be demethylated in mutant germ cells, tandem repeats in pericentric regions were methylated normally. This result indicates that the DNMT3L protein might have a function in the *de novo* methylation of dispersed repeated sequences in a premeiotic genome scanning process that occurs in male germ cells at about the time of birth (Bourc'his and Bestor, 2004). A broader role for DNMT3L in regulation of *de novo* methylation in somatic tissues has not yet been established so far.

As mentioned previously, the cooperativity between DNMT1 and DNMT3a or DNMT3b may underlie links between maintenance and *de novo* methylation. While DNMT1 is efficient at maintaining the DNA methylation patterns of sequences with low densities of CpG sites, there may be a requirement for DNMT3a and/or DNMT3b in the maintenance methylation of high-density CpG sequences (Liang et al., 2002).

Conversely, DNMT1 can also cooperate with Dnmt3a and/or Dnmt3b in *de novo* methylation. This may involve additive contributions of the low *de novo* methyltransferase activity of DNMT1, tight coordination of two or more Dnmt members in the same complex, and/or other undefined synergisms (Fatemi et al., 2002; Kim et al., 2002; Lorincz et al., 2002; Rhee et al., 2002; Datta et al., 2003). Cooperation between maintenance and *de novo* methylation is likely to play a key role in methylation spreading where incomplete or noncontiguous methylation of CpG sites is extended outward to yield longer stretches of contiguous methylated DNA sequence.

#### 1.1.1.3 Interpreting the DNA methylation signal

Apparently, there are two different ways in which gene expression could be repressed by DNA methylation. First, local cytosine methylation of a particular sequence can directly interfere with DNA recognition by specific transcription factors (Ohlsson et al., 2001; Takizawa et al., 2001). This effect was clearly demonstrated for the chromatin boundary element binding protein, CTCF whose interaction with DNA was shown to be blocked by specific CpG methylation allowing an enhancer to stimulate promoter activity across the inert boundary site. The switching of CTCF plays a critical role in imprinting of the Igf2 gene, which is expressed exclusively from the paternal allele during development (Bell et al., 1999; Hark et al., 2000; Ohlsson et al., 2001). In addition to the binding of CTCF, several other transcription factors are known to be blocked by CpG methylation, but the biological consequences of this process are unknown (Tate and Bird, 1993).

Second and, in general, a more common way to repress gene activity by DNA methylation is recruiting of methyl-CpG binding proteins such as MeCP2 (Lewis et al., 1992) and its relatives, the methyl-CpG binding domain proteins MBD1–MBD4 (Hendrich and Bird, 1998), as well as the unrelated protein Kaiso (Prokhortchouk et al., 2001).

**MeCP2** is a highly abundant methyl-CpG binding protein which binds selectively to a single symmetrically methylated CpG (Lewis et al., 1992). This protein is associated with chromosomes throughout the cell cycle, colocalizes with methyl CpG rich DNA (Lewis et al., 1992), and consists of two functional domains (Fig. 2). The highly conservative N-terminal domain (MBD) was shown to be sufficient to direct specific binding to methylated DNA (Nan et al., 1993). A second functional domain (TRD) is required for transcriptional repression *in vitro* and *in vivo* (Nan et al., 1997; Jones et al., 1998; Kaludov and Wolffe, 2000).

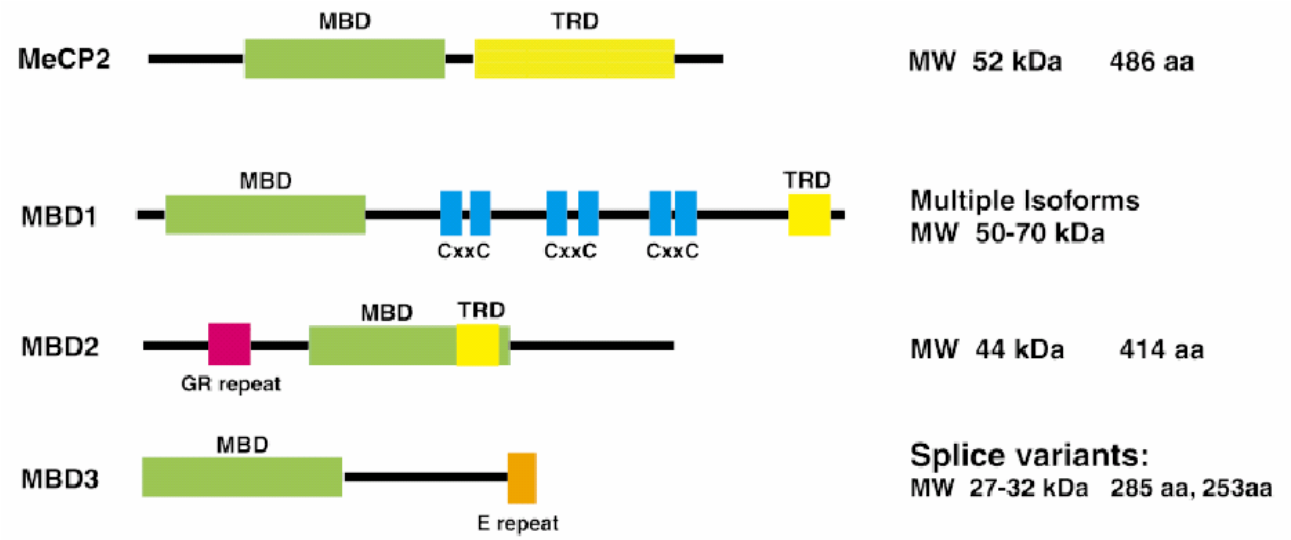


**Figure 2. Domain organization of MBD family members.** The various members of the MBD family implicated in transcriptional repression are depicted. For each protein, the methyl CpG-binding domain is depicted as a green box, the transcriptional repression domain (where identified) as a yellow box. The CXXCXXC motifs of MBD1 are shown as blue boxes and the GR repeats of MBD2 are depicted as a purple box. The acidic repeat at the carboxyl terminus is shown as orange box. (Wade, 2001a).

The ability of MeCP2 to form complexes with the transcriptional corepressor Sin3 and histone deacetylases in mammalian cells suggests that this protein could be involved in the assembly of a specialized chromatin state at methylated loci. Importantly, in both mammalian cells and Xenopus oocytes, artificial recruitment of the previously defined MeCP2 TRD to a promoter leads to transcriptional repression that is partially relieved by inhibitors of histone deacetylase. These findings led to the notion that MeCP2 might repress transcription of target genes within a chromatin infrastructure (Jones et al., 1998; Nan et al., 1998).

**MBD1** is the largest member of the family MBD proteins and contains two or three copies of a cysteine-rich motif (CXXC) that is also found in the DNA methyltransferase protein DNMT1, the histone methyltransferase MLL, and numerous other uncharacterized ESTs (Cross et al., 1997).

In contrast to other methyl-CpG binding proteins, MBD1 is able to repress transcription from both methylated and unmethylated promoters. MBD1 contains a powerful TRD with functions analogous to that of MeCP2 despite a lack of obvious sequence similarity. A proportion of its repression activity appears to rely upon recruitment of histone deacetylases (HDACs), although this activity is much less dependent upon HDAC1 and HDAC2 than is that of MeCP1 or MeCP2 implying that it is also not a component of the previously defined Mi-2/NuRD and Sin3 complexes (Cross et al., 1997; Fujita et al., 1999; Fujita et al., 2000; Ng et al., 2000).

In mammalian cells, MBD1 is localized throughout euchromatin with additional concentrations at a subset of pericentromeric regions on mitotic chromosomes (Fujita et al., 1999; Ng et al., 2000). It also has been detected at the methylated allele of an imprinted gene (Fournier et al., 2002). However, in general the physiological function of MBD1 still remains to be elucidated.

**MBD2** is another member of methyl-CpG binding proteins which plays an important role in silencing of methylated DNA. MBD2 binds methylated DNA in a manner very similar to the isolated MBD domain of MeCP2 and localizes to major satellite DNA when expressed in mouse cells as a chimeric MBD2-GFP protein (Hendrich and Bird, 1998).

The amino terminus of MBD2 contains an extensive stretch of alternating glycine and arginine residues (Fig. 2)(Hendrich and Bird, 1998) . Similar to MBD1 and MeCP2, MBD2 has a transcriptional repression domain identified in a tethering assay. Mapping of the domain of MBD2b (a version lacking the amino terminal 140 amino acids) required for transcriptional repression in this assay identified a small region that partially overlaps the MBD domain (Boeke et al., 2000).

Immunoprecipitation studies demonstrated that in mammalian cells MBD2 is physically associated with HDAC1 indicating that MBD2 could be the long-sought methyl CpG binding component of the MeCP1 complex (Ng et al., 1999). Furthermore, while MBD2 is associated with HDAC1 and with RbA p48/p46, coimmunoprecipitation analysis showed that it is not a component of the previously defined Sin3 and Mi-2/NURD complexes (Ng et al., 2000). However, a direct interaction of Sin3A with MBD2b in the region sufficient to direct transcriptional repression has also been described (Boeke et al., 2000).

**MBD3** is the smallest member of the MBD family, coding for a protein of approximately 30 kDa. In contrast to other MBD family members, mammalian MBD3 is unable to bind methylated DNA substrates. However, it maintains the same exon/intron organization as MBD2 (Hendrich et al., 1999a; Wade et al., 1999). The mRNA corresponding to MBD3 was detected in most of somatic tissues investigated and also in ES cells. (Hendrich and Bird, 1998).

Numerous laboratories have demonstrated MDB3 as a component of a multiprotein complex containing a chromatin remodeling ATPase, a HDAC and other proteins. This complex is termed the Mi-2 complex, the NuRDcomplex, or HDAC1 cII (Wade et al., 1998; Wade et al., 1999; Zhang et al., 1999; Humphrey et al., 2001).

These findings clearly indicate that MDB3 acts as a transcriptional corepressor maintaining transcriptionally silenced chromatin.

**MBD4** is the only member of the MBD family that is not associated with histone deacetylase activity and, therefore, it appears not to be involved in transcriptional repression.

MBD4 consists of two well-conserved, functional domains separated by a poorly conserved spacer region. The N-terminal MBD domain has the highest similarity to MeCP2 in the MBD family, and its C terminal domain shows homology to bacterial DNA repair enzymes (Hendrich et al., 1999b).

MBD4 expression was detected in most human tissues. In mouse MBD4 protein was found to be associated mostly with hypermethylated DNA sequences (Hendrich and Bird, 1998).

Although the MDB domain binds to symmetrically methylated CpG dinucleotides MDB4 has strong preference to 5-methyl CpG paired with TpG and exerts specific DNA N-glycosylase activity for these G:T mismatches. As this particular G:T mismatch is the expected product of deamination of a single 5-methyl-C, MBD4 has been designated as a repair enzyme specific for methylated DNA (Hendrich et al., 1999b; Petronzelli et al., 2000).

Initially, **Kaiso** was described as a novel member of a rapidly growing family of POZ-ZF transcription factors that include the Drosophila developmental regulators Tramtrak and Bric à brac, and the human oncoproteins BCL-6 and PLZF interacting specifically with the p120 protein in mammalian cells (Daniel and Reynolds, 1999).

Later, it was shown that despite the absence of a recognizable MBD motif, Kaiso clearly has the capacity to recognize selectively methyl CpG. Unlike other methyl CpG binding proteins, Kaiso binds to symmetrically methylated CpG dinucleotides using Zink-finger domains and exhibits methylation-dependent repression in transient transfection assays (Prokhortchouk et al., 2001).

Recently Kaiso was identified as a component of the N-CoR complex (Yoon et al., 2003) but the mechanism by which Kaiso mediates methylation-dependent repression still remains to be investigated.

### 1.1.2 Histone modifications in mammalian cells

As described above, methylation of cytosines can block binding of some transcription factors and create binding sites for methyl-CpG binding proteins which are in turn recruited in complexes that modify chromatin. Methyl-CpG binding proteins

therefore serve as a bridge between two major epigenetic events: DNA methylation and N-terminal histone modification named “histone code”. The growing number of core histone modifications include acetylation, methylation and ubiquitination of lysine (K) residues, phosphorylation of serine (S) and threonine (T) residues, and methylation of arginine (R) residues (Fig. 3).

#### 1.1.2.1 Histone acetylation

Histone acetylation is the most abundant and probably the best studied type of histone modification. Histone acetylation occurs at the ε–amino groups of evolutionarily conserved lysine residues located in the N-termini of histones. Despite all core histones are shown to be acetylated *in vivo,* modifications of histones H3 and H4 are, however, more extensively characterized than those of H2A and H2B. Important positions for acetylation are Lys9 and Lys14 in histone H3, and Lys5, Lys8, Lys12 and Lys16 in histone H4 (Bjerling et al., 2002).

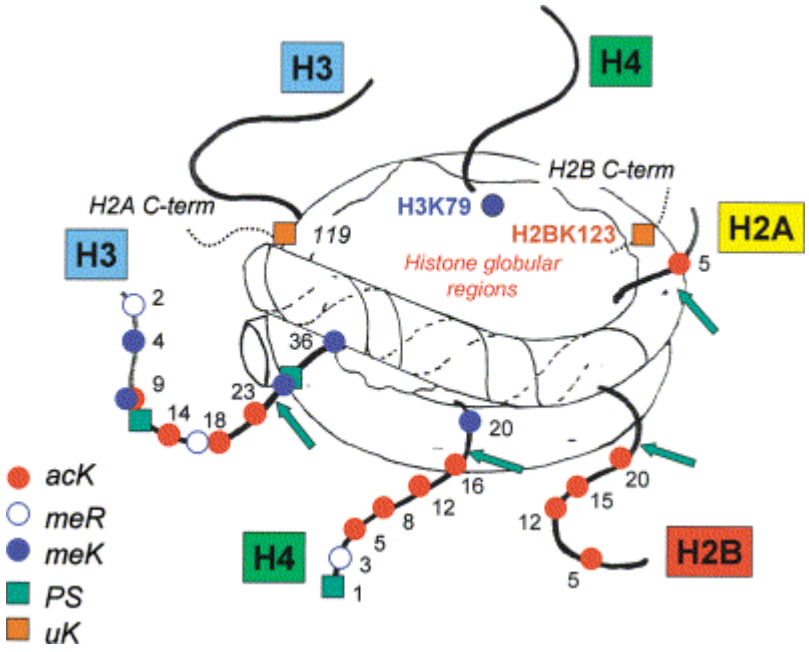


**Figure 3. Histone modifications on the nucleosome core particle.** The nucleosome core particle showing 6 of the 8 core histone N-terminal tail domains and 2 C-terminal tails. Sites of posttranslational modification are indicated by colored symbols that are defined in the key (lower left); *acK* - acetyl lysine; *meR* - methyl arginine; *meK* - methyl lysine; *PS* - phosphoryl serine; and *uK* - ubiquitinated lysine. Residue numbers are shown for each modification. Note that H3 lysine 9 can be either acetylated or methylated. The C-terminal tail domains of one H2A molecule and one H2B molecule are shown (dashed lines) with sites of ubiquitination at H2A lysine 119 (most common in mammals) and H2B lysine 123 (most common in yeast). Modifications are shown on only one of the two copies of histones H3 and H4 and only one tail is shown for H2A and H2B. Sites marked by green arrows are susceptible to cutting by trypsin in intact nucleosomes (Turner, 2002).

Steady-state levels of acetylation of the core histones result from the balance between the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Wade, 2001b).

A relationship between histone acetylation and transcriptional activation was first suggested more than 30 years ago (Allfrey, 1966). In general, increased levels of histone acetylation (hyperacetylation) are associated with increased transcriptional activity, whereas decreased levels of acetylation (hypoacetylation) are associated with repression of gene expression.

**HATs** can be divided into five families on the basis of a number of highly conserved structural motifs. These include the Gcn5-related acetyltransferases (GNATs); the MYST (for 'MOZ, Ybf2/Sas3, Sas2 and Tip60)-related HATs; p300/CBP HATs; the general transcription factor HATs, which include the TFIID subunit TAF250; and the nuclear hormone-related HATs SRC1 and ACTR (SRC3) (Carrozza et al., 2003).

Each of the HAT proteins that have been characterized *in vivo* is found to be associated with large multiprotein complexes that share some similarities in subunit composition. Numerous observations suggest that HAT substrate specificity is conferred through association with additional HAT complex subunits (Carrozza et al., 2003). A member of GNAT family, Gcn5, was shown to be catalytic subunit of the SAGA transcriptional activation complex (Grant et al., 1997) as well as part of the SALSA (Sterner et al., 2002) and SLIK complexes (Pray-Grant et al., 2002). In addition, the mammalian cell-cycle regulatory proteins, c-Myc and the E2F members, contact and recruit GNAT complexes through the TRRAP subunit (McMahon et al., 2000; Lang et al., 2001) suggesting that HAT complexes not only specify histone modification, but also transcriptional function in targeting these complexes to promoters. The human MYST family member HBO1 is the first HAT detected in the protein complex which binds DNA at the replication origin (Iizuka and Stillman, 1999).

**HDACs** superfamily members can be separated into three classes based on their homology to yeast histone deacetylases (Marks et al., 2003).

Class I HDACs share catalytic domain homology to a yeast HDAC called Rpd3 and include HDAC1, HDAC2, HDAC3 and HDAC8. All these HDACs are ubitiquitously expressed in many mammalian cell lines and tissues and localized preferentially in the nucleus (Thiagalingam et al., 2003). As a part of large protein complexes, class I HDACs play an important role in transcriptional regulation, cell cycle control and DNA repair. Thus, three protein complexes have been characterized that contain both

HDAC1 and HDAC2: Sin3, NuRD and Co-REST. The Sin3 complex and the NuRD complex consist of a core unit containing HDAC1, HDAC2, Rb-associated protein 48 (RbAp48, which binds histone H4 directly) and RbAp46. In addition to functioning through these complexes, HDAC1 and HDAC2 can also bind directly to DNA binding proteins such as YY1 (Yin and Yang 1, a cellular nuclear matrix regulatory protein), Rb binding protein-1 and Sp1 (de Ruijter et al., 2003). HDAC3 was shown to be associated with SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) and N-CoR (Kao et al., 2000; Bertos et al., 2001).

Class II HDACs were identified and isolated by homology to novel yeast HDAC Hda1. This class includes HDAC4, 5, 6 and 7. The HDAC9 and HDAC10 were also assigned as class II HDACs. These proteins spanning ~ 1000 amino acids are twice as large as class I HDACs and have their catalytic domain at the C-terminus. Exept for HDAC10 which contains an N-terminal catalytic domain and HDAC6 which has two catalytic domains also located in its N-terminal part (Thiagalingam et al., 2003). The highest levels of these HDACs were detected in heart, brain and skeletal muscle (Cress and Seto, 2000; Zhou et al., 2000). Only HDAC10 was shown to be a nuclear protein, others class II HDACs are located in cytoplasm and have been found to shuttle into the nucleus when they are needed (Thiagalingam et al., 2003). Like class I HDACs, these HDACs perform their function being recruited into large multiprotein complexes. HDAC4, HDAC5 and HDAC7 specifically interact through their N-terminal domain with the myogenic transcriptional factor MEF2 which is essential for muscle cell differentiation (Zhang et al., 2002). Binding of HDACs to MEF2 inhibits MEF2 activity and, therefore, blocks cell differentiation. This HDAC-MEF2 interaction could be disrupted by CaMK which induces the nuclear export of HDACs by phosphorylation (Lu et al., 2000). In association with HDAC3, HDAC4 and HDAC5 were reported to form a repressor complex with NCoR and SMRT (Grozinger et al., 1999; Huang et al., 2000). Recently, it was demonstrated that the catalytic domain of HDAC4 interacts with HDAC3 via NCoR/SMRT, and HDAC4 does not show any enzymatic activity in this complex (Fischle et al., 2002). These observations indicate that class II HDACs regulate transcription by bridging the enzymatically active SMRT/NCoR/HDAC3 complex and select transcription factors independently of any intrinsic HDAC activity.

The third class of human HDACs consists of homologues of yeast and mouse Sir2 protein and, therefore, its members are designed as sirtuins. There are seven sirtuins: SIRT1-7, and most of these proteins are relatively small (~ 300 amino acids) (Thiagalingam et al., 2003). The catalytic domain contains two CXXC motifs that

function as a zinc finger domain and at least one hydrophobic region that could function as a leucine zipper (Brachmann et al., 1995; Frye, 1999). The histone deacetylase activity of these enzymes is absolutely depended on NAD+ but in contrast to other HDACs, it's insensitive to trichostatin A (TSA) (Frye, 1999; Landry et al., 2000). Immunofluorescence studies has demonstrated that unlike yeast Sir2, human Sir2 does not localize in the nucleus.(Afshar and Murnane, 1999). Recently, Sirt1 protein was shown to regulate p53 acetylation and p53-dependent apoptosis indicating that the function of this enzyme is required for specific developmental processes (Cheng et al., 2003).

#### 1.1.2.2 Histone methylation

Although methylation of histones was firstly reported more than 40 years ago (Murray, 1964), direct evidence linking methylation and transcription was found only recently when the H3 arginine-specific histone methyltransferase (HMT) CARM1 was shown to interact and cooperate with the steroid-hormone-receptor coactivator GRIP-1 in transcriptional activation (Chen et al., 1999).

There are two types of histone methylation, targeting either arginine (R) or lysine (K) residues. The most frequent and well investigated modification is methylation on histones H3 (lysines 4, 9 and 27) and H4 (lysine 20 (Strahl et al., 1999; Grant, 2001)). This process is catalyzed by histone methyltransferases (HMTs), which use S-adenosylmethionine (SAM) as a cofactor, like HATs utilize acetyl-coenzyme A as a cofactor (Rice and Allis, 2001). Methylation of lysine differs from acetylation in several important aspects. First, the ε-amino group of a targeted lysine residue can accept one, two, or three methyl groups to form mono-, di-, or trimethylated products. Recent findings suggest that di- and trimethylation have different functions within the transcriptional context, although the significance and exact nature of any difference remains unknown (Turner, 2003). Second, whereas acetylation results in the neutralization of the positive charge on the ε-amino group, methylation leaves the charge intact. Third, many data suggest that methylation is relatively irreversible (Byvoet, 1972; Byvoet et al., 1972; Thomas et al., 1972), and no histone demethylases have yet been isolated. Thus, methylation may only be removed passively by dilution during replication, by exchange on chromatin, or alternatively by proteolytic cleavage of the protein portion carrying the methyl group (Ehrenhofer-Murray, 2004).

The mammalian **Suv39h** enzymes and their *S. pombe* homologue, Clr4, were the first HMTases identified (Rea et al., 2000). The conserved SET-domain of the Su(var)3-

9-related HMTases specifically catalyzes the methylation of H3-K9 creating a high-affinity binding site for the chromodomain of heterochromatin protein 1 (HP1) which is essential for the formation of transcriptionally silenced chromatin (Lachner and Jenuwein, 2002). H3-K9 methylation was reported to participate in transcriptional repression (Noma et al., 2001) and formation of highly condensed pericentromeric chromatin (Lachner and Jenuwein, 2002), as well as in X-chromosome inactivation (Boggs et al., 2002; Peters et al., 2002). Recently, methylation of H3-K27 and H4-K20 was also shown to be a mark for inactive chromatin (Sims et al., 2003).

In contrast to H3-K9 methylation, methylation of H3-K4 seems to play the opposite role: tri-methylated H3-K4 is associated with fully activated promoters (Santos-Rosa et al., 2002) and specifically impairs Suv39h1-mediatedmethylation at H3-K9 (Wang et al., 2001). Moreover, binding of the histone deacetylase NuRD repression complex to the H3 N-terminal tail is precluded by methylation at lysine 4, but not lysine 9 (Nishioka et al., 2002; Zegerman et al., 2002). Di-methylation of H3-K4 correlates with the basal transcription and appears to be global, mediating general mechanism of disrupting large-scale silencing (Ng et al., 2003). In human cells H3-K4 is methylated by Set1-like protein Set1/Ash2 which was found in a complex with the host cell coactivator C1 (HCF-1), a protein known to be associated with a Sin3 histone deacetylase complex containing HDAC1 and HDAC2. The association of HDACs with HCF-1 might indicate the removal of existing H3-K4 acetyl groups to prepare for Set1-mediated methylation because H3-K4 acetylation is thought to prevent methylation (Wysocka et al., 2003). However, it is unclear whether H3-K4 is acetylated in higher eukaryotes (Sims et al., 2003).

Aside from H3-K4 methylation, transcriptionally open chromatin is also methylated at the H3-K36 and H3-K79 positions (Sims et al., 2003). H3-K79 methylation is catalyzed by the histone methyltransferase disruptor of telomeric silencing 1 (**DOT1**) in yeast (Lacoste et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002) and DOT1-like protein (**DOT1L**) in humans (Feng et al., 2002). Mutation of K79 and mutations that abrogate DOT1 catalytic activity impair telomeric silencing (Lacoste et al., 2002; Ng et al., 2002) indicating that DOT1 mediates telomeric silencing via methylation of K79. Taken together in line with results that methylated H3-K79 is abundant in bulk yeast histones and is deficient in telomeres, it has been proposed that H3-K79 excludes silent information regulator (SIR) complexes, thus yielding sufficient amounts of SIR proteins to assemble repressive complexes at heterochromatic sites (van Leeuwen et al., 2002).

### 1.1.2.3 Histone phosphorylation

Histone phosphorylation involving Ser-10 of histone H3 and Ser-10 of histone H2B has also emerged as an important modification, both in transcriptional activation and in chromosome condensation during mitosis (Grant, 2001).

H3 phosphorylation at Ser-10 begins during prophase, with peak levels detected during metaphase, ultimately followed by a general decrease in the amount of phosphorylation during the progression through the cell cycle to telophase (Gurley et al., 1978). The same mitosis-specific phosphorylation was shown later for Ser-28 and Thr-11 (Goto et al., 1999; Preuss et al., 2003), but the functional relevance of these modifications still remains unclear. Detailed analysis of Ser-10 H3 phosphorylation revealed that it starts in the pericentric heterochromatin and spreads throughout the genome during the G2–M phase transition (Hendzel et al., 1997). This modification is necessary for the initiation of chromosome condensation (Van Hooser et al., 1998; Wei et al., 1998) but might not to be required for cell-cycle progression where phosphorylation of histone H2B could play a redundant role (Cheung et al., 2000a; Hsu et al., 2000).

Several proteins were identified as mitotic kinases of histone H3: the Ipl1/AIR-2 aurora kinase in *S. cerevisae* and *C. elegans* (Hsu et al., 2000), NIMA kinase in Aspergillus nidulans (De Souza et al., 2000) and multiple Ipl1 homologues in higher eukaryotes (Bischoff and Plowman, 1999; Giet and Prigent, 1999) as histone H3 mitotic kinases. The activity of these kinases is counterbalanced by type1 phosphatases (PP1) (Hsu et al., 2000; Murnion et al., 2001). Regulation of histone H3 phosphorylation via an interplay between the activities of protein kinases and phosphatases is thought to be a mechanism regulating the H3 phosphorylation and therefore promoting proper chromosomal condensation and segregation (Hans and Dimitrov, 2001).

Several observations demonstrated that Ser-10 phosphorylation plays also a role in transcriptional regulation. It was shown that rapid histone H3 phosphorylation correlates with the induction of immediate-early (IE) genes, including the proto-oncogenes *c-fos* and *c-jun*, by stimulating fibroblast cells with growth factors, phorbol esters, okadaic acid, protein synthesis inhibitors or inhibitors of protein phosphatases. Phosphorylation is targeted to a minute fraction of histone H3, which is also especially susceptible to hyperacetylation. Phosphorylation and acetylation of histone H3 occur on the same histone H3 tail on nucleosomes associated with active IE gene chromatin (Mahadevan et al., 1991; Clayton et al., 2000). Aditionaly, several HDACs have an increased HAT activity on serine 10-phosphorylated substrates while mutation of Ser-10

decreases activation of Gcn5-regulated genes (Cheung et al., 2000b; Lo et al., 2000) suggesting that histone methylation and phosporylation might be coupled during the activation of transcription via a MAP kinase-signaling cascade (Thomson et al., 1999).

#### 1.1.2.4 Histone ubiquitination

The three core histones H2A, H2B, H3 and the linker histone H1 were reported to be ubiquitinated in many eukaryotic species (Osley, 2004). The first histone found to be modified by ubiquitin was H2A. Ubiquitination affects 10-15% of H2A histone in higher eukaryotes (Jason et al., 2002; Osley, 2004). It was shown that ubiquitin is linked to the ε-amino group of K119 in H2A at the beginning of the C-terminus by an isopeptide bound forming a bifurcated structure (Fig. 3)(Jason et al., 2002).

Attachment of a ubiquitin molecule to the side chain of a lysine residue in the acceptor protein is a complex process involving multi-enzyme-catalyzed steps including enzymes designed as E1, E2 and E3. E1 initiates this cascade by ubiquitin activation which involves the formation of an ubiquitin-E1 thiol ester via ubiquitin adenylate intermediate. E1 transfers activated ubiquitin to the second enzyme, E2, also resulting in the formation of thiol ester intermediate. The conjugation of activated ubiquitin to the target protein requires full activity of the third protein in this cascade – an isopeptide ligase called E3 which binds both to E2 and the substrate (Jason et al., 2002; Zhang, 2003; Osley, 2004) .

Studies investigating the role of histone ubiquitination in transcription regulation suggest that this modification could have different effects on gene expression. Early observations detected positive correlations between transcriptional activation and ubiquitination. In Drosophila cells, approximately half of nucleosomes in actively transcribed *copia* and *hsp70* genes contained ubiquitinated histone H2A, in contrast to nontranscribed 1.688 satellite DNA where it was detected in less than one out of 25 nucleosomes (Levinger and Varshavsky, 1982). Polyubiquitilated histones H2A, H2A.Z, and H2B were also found in chromatin fractions enriched in transcriptionally active gene sequences of bovine thymus, in chicken erythrocytes, and Tetrahymena macro- and micronuclei (Nickel et al., 1989). Later, it was shown that in mammalian cells inhibitors of heterogeneous nuclear RNA synthesis can selectively reduce the level of ubiquitinated histone H2B, indicating that histone ubiquitination could be directly dependent on active transcription (Davie and Murphy, 1994). Studies in yeast demonstrated that substitution of the H2B ubiquitination site at K123 leads to impaired transcription of certain highly inducible genes. This gene-associated H2B ubiquitination

was shown to be transient, increasing early during activation, and then decreasing coincident with significant RNA accumulation (Henry et al., 2003).

Other data, however, show that histone ubiquitination could regulate gene expression in a negative fashion. Thus, ubiquitinated histones have been found in transcriptionally inactive compartments, such as in Tetrahymena micronuclei (Nickel et al., 1989) or the sex body of mouse spermatids (Baarends et al., 1999). In yeast, ubiquitin conjugase Rad6 was shown to be required for the repression of ARG1 gene (Turner et al., 2002) and for telomeric and HML silencing (Huang et al., 1997).

These controversial effects could possibly be explained by impact of histone ubiquitination on other histone modifications such as acetylation and methylation.

It was reported that the murine histone deacetylase 6 (mHDAC6) associates with two proteins implicated in ubiquitination, and that a zinc-finger domain located at the C-terminal of HDAC6 can directly bind to ubiquitin (Seigneurin-Berny et al., 2001). These findings suggest a potential link between histone ubiquitination and histone acetylation.

Positive effects of histone ubiquitination on transcription can be explained by observations that H3-K4 methylation requires functional Rad6 and intact H3-K123 (Dover et al., 2002; Sun and Allis, 2002). The same dependence was also demonstrated for H3-K36 methylation (Briggs et al., 2002) which is known to be associated with transcriptionally open chromatin (Sims et al., 2003). At the same time, histone H2B ubiquitination down-regulates H3-K36 methylation (Henry et al., 2003) elucidating the way in which it participates in transcriptional silencing.

## 1.2 Epigenetics and Cancer

According to the classical Knudson hypothesis, two hits are required for the tumor suppressor gene inactivation and subsequent development of malignant tumor. These hits were believed to be caused by such genetic disorders as intragenic single-point mutations or loss of chromosomal material (loss of heterozygosity (LOH) or homozygous deletion). Recently due to growing number of evidences suggesting the crucial role of epigenetic mechanisms in gene regulation, the Knudson model was expanded to include aberrant DNA methylation patterns and altered histone modifications (Fig. 4).

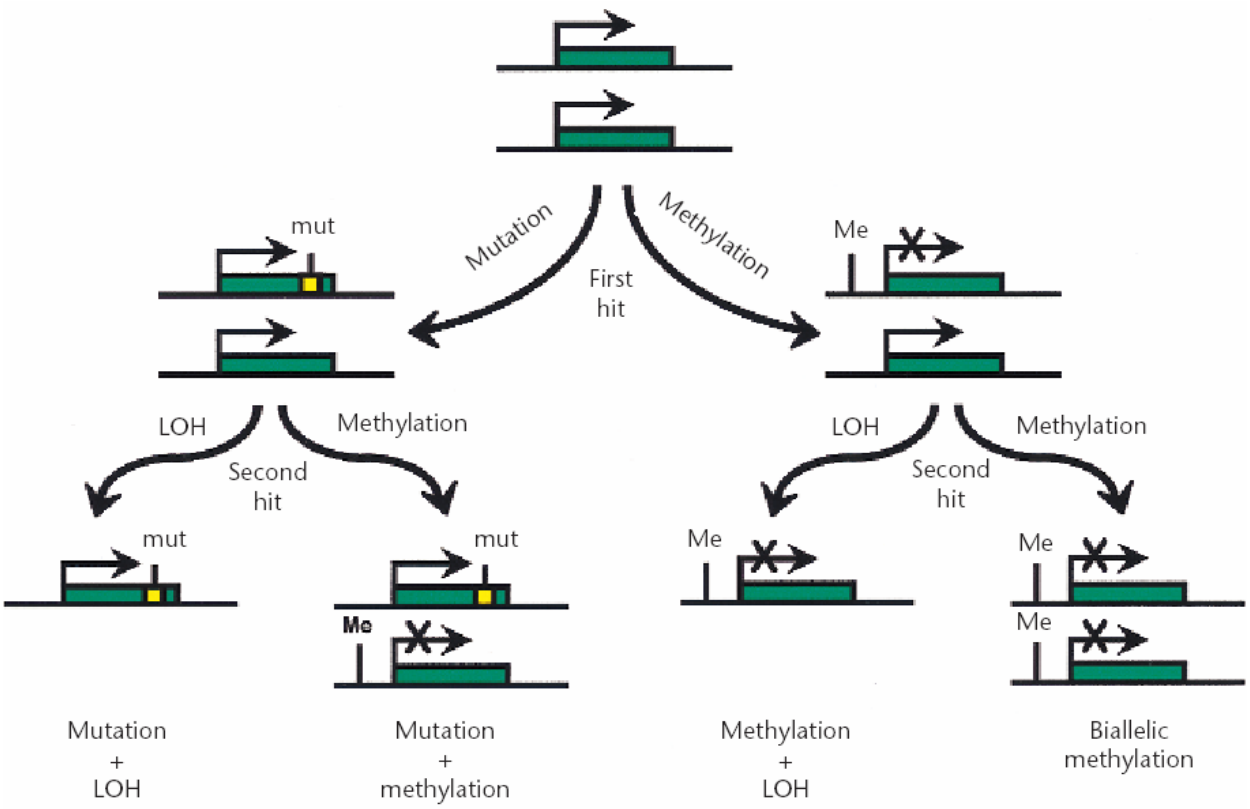


**Figure 4. Knudson's two-hit hypothesis revised.** Two active alleles of a tumor suppressor gene are indicated by the two green boxes shown at the top. The first step of gene inactivation is shown as a localized mutation on the left or by transcriptional repression by DNA methylation on the right. The second hit is shown by either LOH or transcriptional silencing (adopted from (Jones and Laird, 1999)).

### 1.2.1 DNA methylation in cancer

Global genomic DNA hypomethylation was one of the first epigenetic events associated with cancer cells. In comparison to normal tissue of the same origin, a reduction in the DNA methylation level has been found to be a hallmark of most tumors investigated (Gama-Sosa et al., 1983; Feinberg, 1988; Ehrlich, 2002). In some cases this hypomethylation can result in increased expression of the normally repressed transposons leading to potentially dangerous transposition events (Yoder et al., 1997b; Florl et al., 1999). Lack of DNA methylation may also cause genomic instability as it was shown in the hypomethylated genome of embryonic stem cells lacking Dnmt1 (Chen et al., 1998). This protective effect of DNA methylation might be explained by the observation that it is involved in control of homologous recombination. (Colot et al., 1996; Colot and Rossignol, 1999). Furthermore, the pericentromeric areas of human

chromosomes are particularly heavily methylated in constitutive heterochromatin. Loss of this pericentromeric methylation, either in a genetic disorder immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome, or by treatment with the demethylating agent 5-aza-2'-deoxycytidine is associated with increased chromosome translocation (Hernandez et al., 1997; Ji et al., 1997; Tuck-Muller et al., 2000).

In contrast to the global hypomethylation in tumors, which spans wide regions of the genome and occurs mainly at the sparsely distributed CpG dinucleotide sequences, hypermethylation is localized in dense CpG islands regions (Baylin and Herman, 2000; Szyf, 2003). To date, hypermethylation of CpG island has been shown to be associated with the silencing of a number of classic tumor suppressor genes. In fact, this aberrant silencing can disrupt the expression of genes involved in the fundamental pathways that lead to cancer (Table 1). Besides these classic tumor suppressor genes, there is an increasing list of genes whose corresponding CpG island shows aberrant methylation in cancer. The significance of inactivating some of these genes for tumor progression is unclear, but such events may simply reflect a methylation abnormality of cancer cells (Rountree et al., 2001).

**Table 1:** Fundamental pathways altered by DNA methylation in cancer.

| **Pathway affected** | **Genes silenced by CpG island methylation** |
|---|---|
| Cell cycle control | Rb, p16(INK4$\alpha$), p15, p14(ARF), p73 |
| DNA damage repair | MLH1, $O^6$MGMT, GST$\pi$, BRCA1 |
| Inhibiting apoptosis | DAP-kinase, Caspase-8, TMS1 |
| Invasion tumor architecture | E-cadherin, VHL, APC, LKB1, TIMP3, Thrombospondin1 |
| Growth factor response | ER, RAR$\beta$, Androgen Receptor, Endothelin B Receptor, RASSF1A |

Methylation of most of these genes has been recently reviewed in Baylin and Herman, (2000) and Rountree et al., (2001). References for the genes not covered by these reviews include Bianco et al. (2000), Conway et al. (2000), Dammann et al. (2000), Esteller et al. (2000), Esteller et al. (2000), Esteller et al. (2000), Esteller et al. (2000), Teitz et al. (2000) and Zheng et al. (2000)

A non-random character of aberrant CpG island hypermethylation was demonstrated by a global analysis of the methylation status of 1,184 unselected CpG islands in each of 98 primary human tumors using restriction landmark genomic scanning (RLGS). Identified patterns of methylation were shared within each tumor type

together with patterns and targets that displayed distinct tumor type specificity. Furthermore, methylation abnormalities were detectable in both low and high-grade malignancies, again supporting the revised Knudson model (Fig. 4)(Costello et al., 2000).

The mechanisms that produce aberrant DNA methylation leading to tumor development are not well understood yet.

One hypothesis proposed that several key enzymes that regulate DNA methylation, such as the DNMTs, are candidates to account for DNA methylation abnormalities in cancer. Overexpression of DNMTs is detected in different tumor types (Kautiainen and Jones, 1986; Issa et al., 1993; Belinsky et al., 1996; Melki et al., 1998; De Marzo et al., 1999; Robertson et al., 1999; Xie et al., 1999; Ahluwalia et al., 2001), and it was found that increased DNMT1 expression in normal cells can promote cellular transformation (Wu et al., 1993; Bakin and Curran, 1999) whereas genetic knock-down of DNMT1 l reversed tumorogenesis (Laird et al., 1995). Another potential factor, in conjunction with the modest increase in DNMT levels, is the deregulation or misdirecting of the enzymatic or properties of these enzymes. Alterations in DNMT function may explain both hypo- and hypermethylation (Dennis et al., 2001; Kishimoto et al., 2001; Lindroth et al., 2001) as well as cause shifts in DNA methylation patterns (Dennis et al., 2001).

A second event leading to CpG island hypermethylation could be the breakdown in the protection of these islands through a loss of specific protective elements. Candidate factors responsible for this protection are members of Sp family which have been found to prevent Aprt gene promoter region methylation (Brandeis et al., 1994; Macleod et al., 1994). However, direct evidence for this type of mechanism in cancer is still lacking.

Loss of DNA demethylase activity in cells was also hypothesized to be responsible for hypomethylation of DNA and induction of genes involved in invasion and metastasis of tumors. The first and only known human DNA demethylase has been identified in a human lung carcinoma cell line (Ramchandani et al., 1999). This demethylase has the potential to function as a repair enzyme with the ability to correct aberrantly methylated CpG sequences. The factors that control the template specificity of DNA demethylase have not been identified. Future research has to clarify its role in the regulation of cell growth and differentiation (Momparler, 2003).

Aberrant DNA methylation is closely related with another epigenetic event which can repress transcription of tumor suppressor genes. However, it is unclear whether

chromatin remodeling causes DNA methylation or histone modifications follow DNA methylation.

The hypothesis that DNA methylation is a primary event and modifications in chromatin structure are a secondary one is suggested by the discovery that the methylated DNA binding protein MeCP2 recruites corepressor complexes and HDACs to methylated promoters (Nan et al., 1997; Jones et al., 1998). Experiments with pharmacological inhibitors of both DNA methylation and histone deacetylation also support this observations. It was shown that 5-azacytidine (5-azaC), an inhibitor of DNA methylation, could activate a number of methylated tumor suppressor genes, while treatment with the HDAC inhibitor Trichotatin A (TSA) alone was insufficient to induce these genes (Cameron et al., 1999). At the same time treatment of tumor cells with 5-azaC reduces the level of methylated K9 in histone H3 and increases the level of methylated K-4 in histone H3 in the promoter region of genes silenced by aberrant DNA methylation (Nguyen et al., 2002).

Aberrant deacetylation of histones in nucleosomes caused by disruption of HAT or HDAC activity can be associated with the development of cancer, apart from any relationship to DNA methylation. Genes that encode these enzymes are translocated, amplified, overexpressed and/or mutated in various cancers of both hematological and epithelial origin (Cress and Seto, 2000; Mahlknecht and Hoelzer, 2000; Timmermann et al., 2001). For example, gene translocations in some types of leukemia can generate fusion proteins that recruit HDAC and bind to promoters to silence genes involved in differentiation (Johnstone, 2002). The importance of histone modifications in cancer generation is illustrated by the marked antitumor activity of different inhibitors of HDAC, both in animal models and in preliminary clinical trials (Marks et al., 2001; Johnstone, 2002).

The molecular mechanism of action of HDAC inhibitors is related to their activation of a subset of genes that can exert cell cycle arrest, differentiation or apoptosis in tumor cells. (Marks et al., 2001; Johnstone, 2002). Microarray analysis has shown that inhibitors of HDAC can reactivate a subset of genes, but not genes that contain hypermethylated CpG islands (Suzuki et al., 2002a).

## 1.3 The family of NF-AT factors

NF-AT (nuclear factor of activated T cells) was originally described as a putative transcription factor in nuclear protein extracts from activated Jurkat T cells binding to the human interleukin-2 (IL-2) promoter (Shaw et al., 1988). Soon afterwards, this factor was identified as a target for the immunosuppressants cyclosporin A (CsA) and FK506 (Emmel et al., 1989; Mattila et al., 1990; Randak et al., 1990; Brabletz et al., 1991) which are efficient inhibitors of T cell activation (Liu, 1993). Today, four closely related members of the NF-AT family have been cloned and characterized in detail (Figure 5). These four NF-AT proteins, designated NF-ATc1–c4, share a DNA binding domain of approximately 300 amino acid residues with 68–73% sequence homology between the various NFAT proteins.

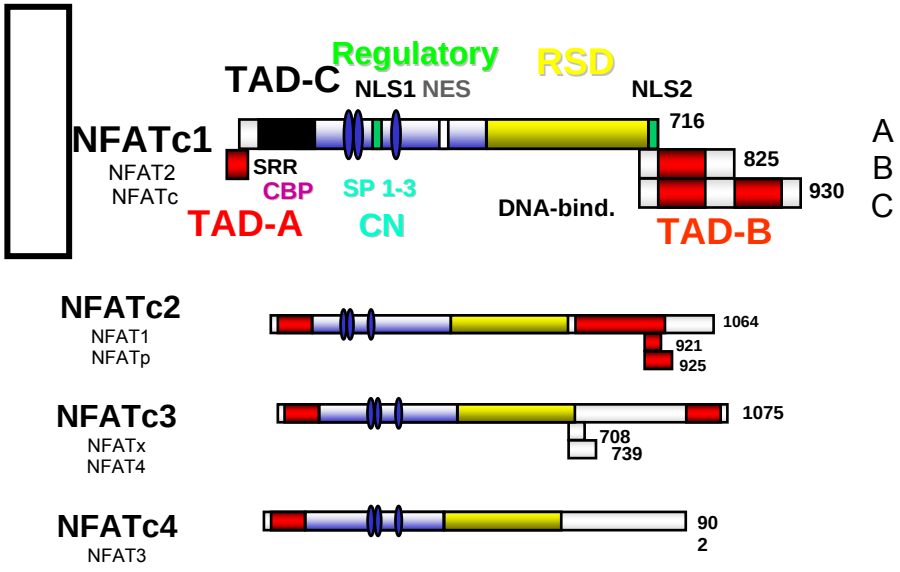


**Figure 5. Schematic structure of NF-AT factors.** The DNA binding regions of NF-ATs, the RSD, are shown as yellow boxes. The N- and C-terminal transactivation domains, TAD-A and TAD-B are drawn in red. Putative transactivation domain TAD-C is drawn in black. Further sequence motifs are shown for NF-AtTc1 only where TAD-B consists of two peptides which are separated by an inhibitory domain. For the regulatory domain located between TAD-A and the RSD, the position of the serine-rich region (SRR) and of SP motifs 1–3 are indicated. In addition, binding regions for the transcriptional co-factor CBP and the Ca2+-dependent phosphatase calcineurin as well as signals for nuclear localization (NLS) and nuclear export (NES) of NF-AT2 are shown.

Due to sequence similarities with the DNA binding (Rel) domain of Rel/NF-B factors which are reflected in a very similar architecture, the DNA binding domain of NFATs is

often designated as Rel similarity domain (RSD). Further common features of NF-AT family members are their strong N-terminal transactivation domain (Luo et al., 1996; Avots et al., 1999) and, in particular, their regulatory domains located between the N-terminal TAD and the RSD.

The nuclear translocation of genuine NFAT factors is regulated by the $Ca^{2+}$/calmodulin-dependent phosphatases calcineurin (Crabtree, 1999) , which binds to several sites within the regulatory region of NFATs and controls their nuclear import and export (Rao et al., 1997; Serfling et al., 2000).

NFATs have been shown to regulate the expression of multiple cytokines and other regulatory molecules, including interleukin-2 (IL-2), IL-3, IL-4, IL-5, GM-CSF, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, CD40 ligand (CD154), and CD95 ligand (FasL). Further studies have also demonstrated that NFAT proteins controll immune responses and homeostasis of B lymphocytes, NK cells, macrophages, mast cells, and eosinophils, among others (Rao et al., 1997).

Several lines of evidence suggest also a crucial role of NFATc1 in tumorogenesis.

Numerous findings demonstrating that both the CD95 ligand and TNF promoter contain multiple NFAT sites (Li-Weber et al., 1999; Falvo et al., 2000) suggest that NFATs are major regulators of activation-induced cell death (AICD) of lymphocytes and, therefore, NFATs might act as a tumor suppressor gene. This idea is supported by retroviral tagging experiments where the murine *nfatc1* and *nfatc3* loci, but not the *nfatc2* locus, were identified as common insertion sites (CIS) for oncogenic viruses (Suzuki et al., 2002b; Akagi et al., 2004). For the *nfatc1* gene, all retroviral insertions were found to be located either within or close to the *nfatc1* promoter or poly A site regions suggesting that retroviral insertions affect NFATc1 expression. Clonal insertion of the tumorigenic retrovirus SL3-3 (Sorensen et al., 1996) just behind the promoter region of *nfatc1* (Chuvpilo et al., 2002a) was found to be correlated with an inactivation of NFATc1 expression (Chuvpilo et al., unpubl. data).

However, contrary to their effect on pro-apoptotic genes, NFAT factors appear also to control the expression of several anti-apoptotic genes. Thus, NFATc1/A expression in BALM-14, a Burkitt lymphoma cell line, protected these cells against BCR-mediated AICD (Kondo et al., 2003). Moreover, transformation of pre-adipocytes by a constitutively active version of NFATc1 indicated that NFATc1 could be an oncogene for the development of human adipocytic and other tumors (Neal and Clipstone, 2003).

In light of these findings one may assume that the specific effect of individual NFAT proteins on apoptosis regulation might rely on their capability to control the expression of both pro- and anti-apoptotic genes at the appropriate stage of T cell differentiation and activation. Our data demonstrate that NFATc1 expression is suppressed by epigenetic mechanisms in the majority of anaplastic large cell lymphomas (ALCLs) and classical Hodgkin's lymphomas (cHLs) suggesting a tumor suppressor function for NFATc1, in particular by the expression of strongly indusibl short isoform NFATc1/A. However, it still remains to be shown whether the *nfatc1* gene might also act as an oncogene.

# 2. RESULTS

## *2.1 LOH analysis of nfatc1 gene region*

Immunohistochemical staining of NFATc1 expression detected negative or significantly decreased NFATc1 expression in all primary Reed-Stenberg cells (97%) and systemic ALCL (Akimzhanov et al., submitted). To investigate the possibility that Nfatc1 silencing is caused by genetic instability, we performed LOH analysis of the *nfatc1* locus. Within this locus there are 12 fragments containing simple CA/GT repeats within *nfatc1* gene which could be potentially used as microsatellite markers; 4 of them were found to be polymorphic and selected to detect LOH. These markers are located between exons 4 and 5 (GT6-I), exons 8 and 9 (GT11, CA8), exons 10 and 11 (CA6). Additionally, we used two microsatellite markers mentioned in the UniSTS database and corresponding to *nfatc1* gene region (D18S497 and D18S1221). We examined six patients diagnosed with lymphoma comparing in each case DNA samples isolated from normal and tumor tissues (Fugure 6)(Table 2). However, no LOH was detected suggesting that NFATc1 silencing is not associated with large genetic alterations within the locus.

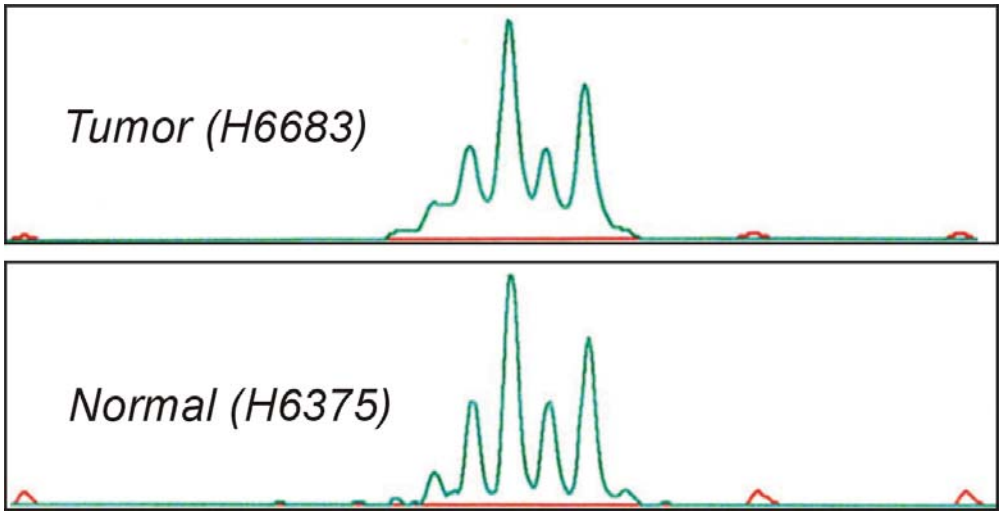


**Figure 6. LOH analysis of the *nfatc1* locus.** A representative fluorescent electropherogram for LOH are shown. Both tumor and normal tissue samples from the patient #3 (see Table 2) were analysed using the microsatellite marker D18S497.

**Table 2:** LOH analysis of the *nfatc1* locus.

| ***Patient #*** | *Year* | *Normal tissue* | *Tumor tissue* | *LOH* |
|---|---|---|---|---|
| **1** | 1990 | 17949 | 18797 | no |
| **2** | 1996 | 20382 | 19844 | no |
| **3** | 1997 | 6683 | 6375 | no |
| **4** | 1999 | 2516 | 5553 | no |
| **5** | 1995 | 1025 | 27651 | no |
| **6** | 1993-94 | 11012 | 9854 | no |

## 2.2 The *nfatc1* promoter hypermethylation in primary human lymphomas

Analysis of CpG dinucleotides distribution within the *nfatc1* gene revealed two CpG islands located over the P1 and P2 promoters (Figure 7)(Chuvpilo et al., 2002b). This prompted us to investigate the role of aberrant DNA methylation in suppression of NFATc1 in human lymphomas using bisulfite genomic sequencing. To correlate the methylation pattern of P1 and P2 promoters with loss of NFATc1 expression we isolated DNA from tumor tissues corresponding to ALCL, PTCL and enteropathy-type T-cell lymphomas. The DNA samples collected were then modified with Na-bisulfite which converts unmethylated but not methylated cytosine to thymidine residues (Frommer et al., 1992).

As shown in Figure 9 for a fragment of the *nfatc1* P1 promoter, CpG residues spanning the nucleotides from *–102 to –138* were found to be methylated in two systemic ALCL, three PTCL and two ETCL cases. In contrast to these lymphoid tissues, genomic P1 DNA from from Jurkat T cells which are known to express NFATc1 in response to TPA+Ionomycin (T+I) or $\alpha$CD3+$\alpha$CD28 stimulation (Chuvpilo et al., 1999) was completely unmethylated (Figure 9).

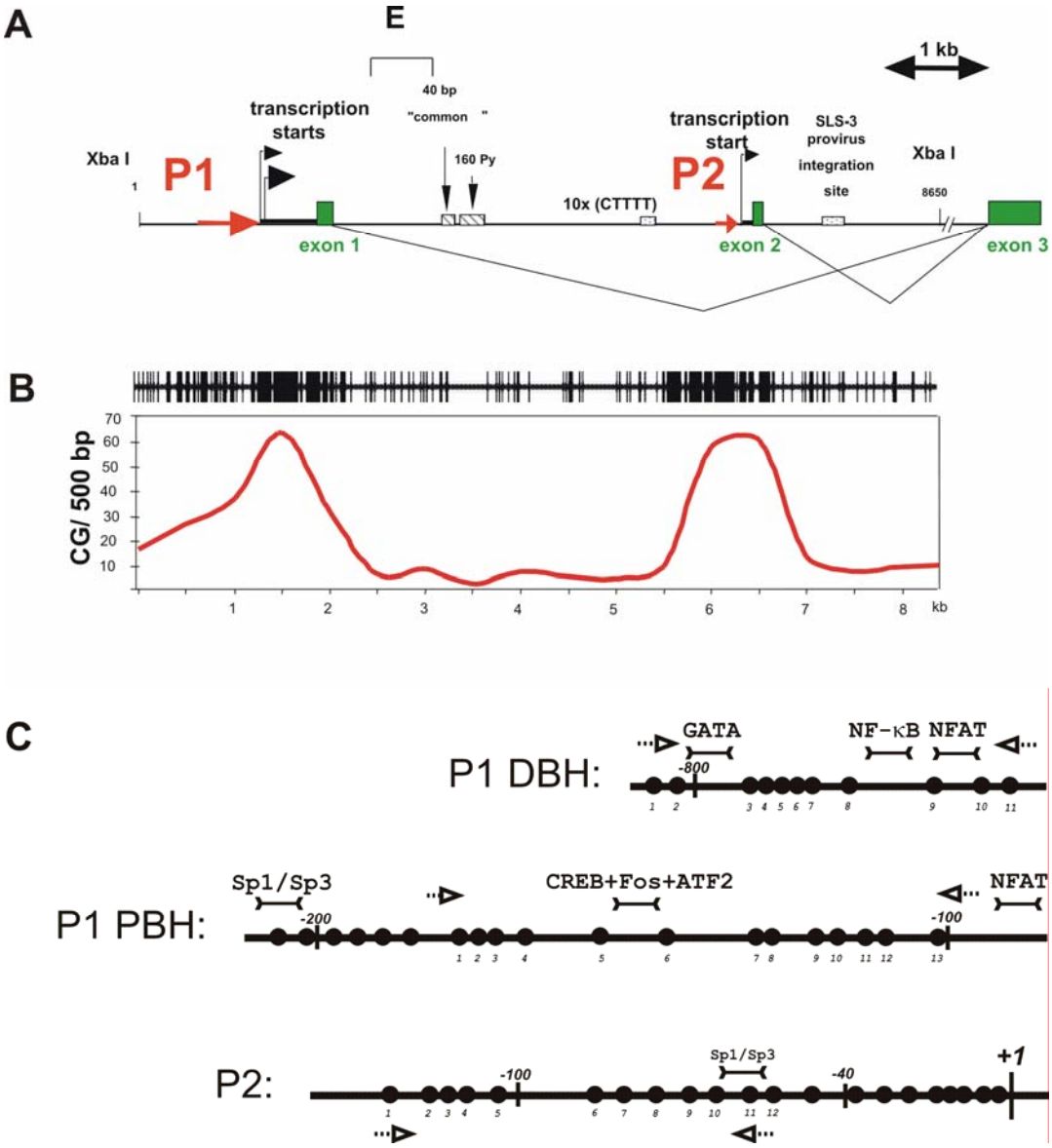


**Figure 7. (A) Structure of the 5` 8.65 Kb XbaI DNA fragment from the *nfatc1* loci harboring the two promoters P1 and P2.** The protein coding portions of exons 1 and 2 from the murine *nfatc1* gene are indicated by green boxes, and their 5′ untranslated mRNA regions are indicated by thick black bars. Red arrows before both 5′ mRNA regions indicate the promoters P1 and P2. Conspicuous sequence motifs are a stretch of 40 bp which is found in several other genes alternatively spliced at their 5′ ends (Schorpp et al., 1997), a stretch of 160 pyrimidines, ten copies of sequence CTTTT, and an integration sequence for the retrovirus SL3-3, a potent inducer of T cell lymphomas (Sorensen et al., 1996). **(B) Distribution of CpG residues within the promoter region.** Above, one vertical dash indicates one CpG residue. The graph below shows the distribution of 375 bp CpGs within 500 bp intervals of the XbaI fragment. **(C) Map of promoter regions used for bisulfite genomic sequencing.** The location of the transcription factors binding sites, transcriptional start sites and the binding sites of tagged primers are presented. Black points on the horizontal line indicate the sites of CpG dinucleotides. Distances are shown in bp relative to the transcriptional start sites. DBH: Distal Block of Homology, PBH: Proximal Block of Homology.

To compare the *nfatc1* methylation pattern in lymphoid and normal cells we purified genomic DNA from mouse embryonic stem cells, naïve and peripheral human T-cells and sequenced the P1 fragments using the same approach. As illustrated in Figure 8 for the P1 PBH, in contrast to tumors, P1 region in both naïve and peripheral T-cells cells was hypomethylated supporting the idea that NFATc1 silencing could be explained by repressive epigenetic changes. P1 DNA demethylation in mouse embryonic stem cells (line WW6) suggests that tissue specific *nfatc1* methylation pattern could be established at the early stage of mammalian development.

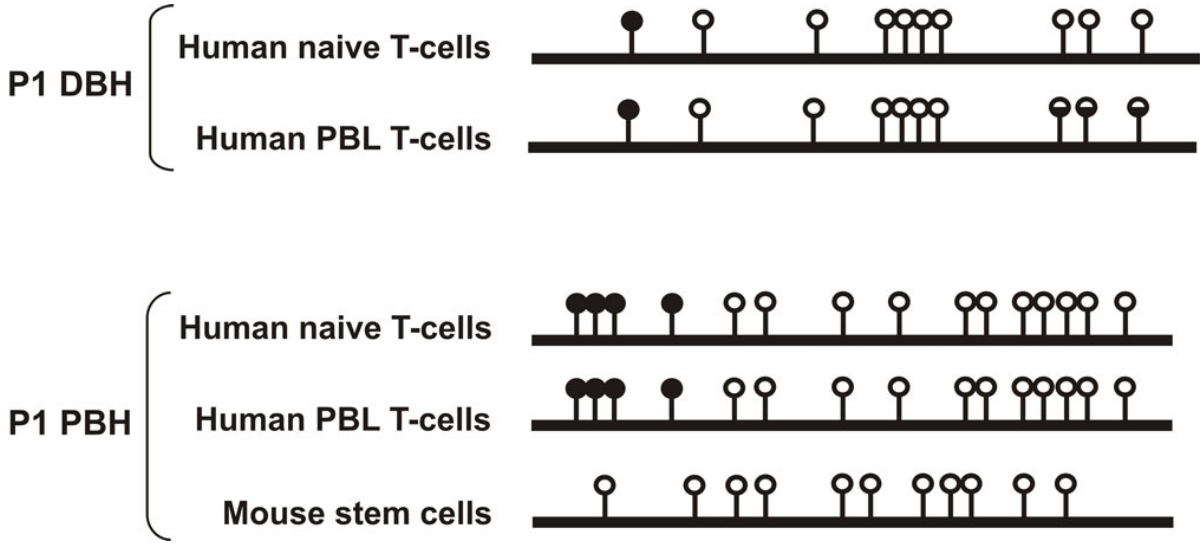


**Figure 8. NFATc1 methylation profile in primary cells.** A schematic representation of *nfatc1* P1 promoter region. Filled circles indicate DNA methylation, empty circles no methylation, and half-filled circles partial methylation. DNA samples isolated from mouse embryonic stem cells, human naïve and peripheral T cells were sequenced after Na-bisulfite modification for the detection of methylated CpG residues using primers directed against PBH P1 and DBH regions.

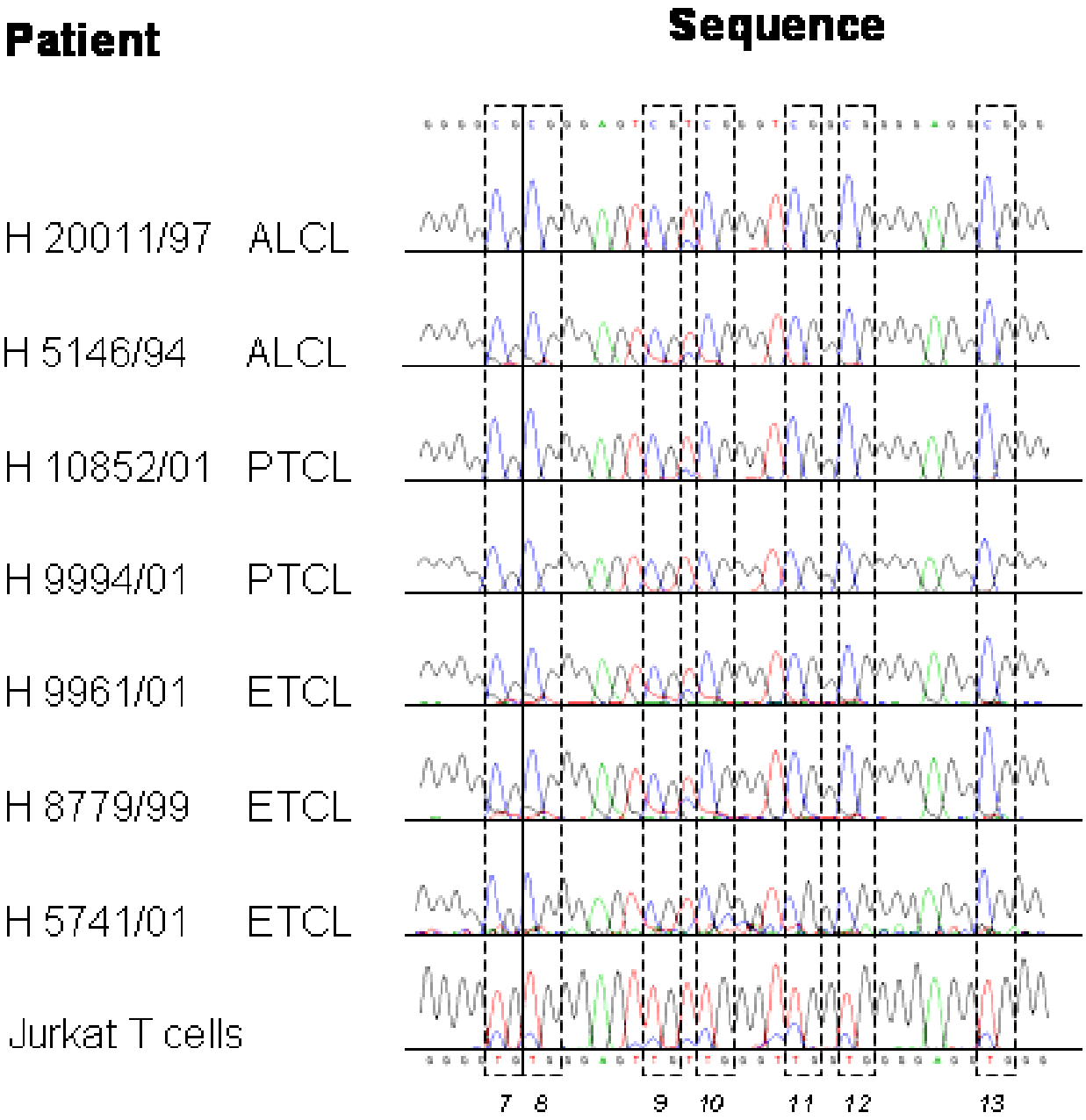


**Figure 9. The *nfatc1* promoter DNA is highly methylated in primary lymphoid tissues.** DNA samples from patients diagnosed with lymphoma were sequenced after Na-bisulfite modification for the detection of methylated CpG residues using primers directed against PBH P1 region. CpG dinucleotides are indicated with numbers as in Fig. 5. As control, the methylation profile of Jurkat T Cells is shown below.

## 2.3 Inverse correlation between NFATc1 expression and promoter methylation.

For the next studies we choose six Hodgkin's lymphoma cell lines, i.e. the lines L540, L591, L428, L1236, KMH2, HD-MyZ and, as controls, Jurkat T leukemic cells and DOHH2 B cell lymphoma cells. As it was mentioned above, *nfatc1* gene expression is regulated by two promoters which are highly conserved between mouse and human. First, we investigated *nfatc1* gene methylation pattern using primers directed against the distal block of homology located 800 kb upstream of the P1 promoter (Figure 7C). This fragment harbors three binding sites for GATA, NF-κB and NFAT transcription factors and seems to play an important role in *nfatc1* gene regulation (Chuvpilo et al., 2002a). Sequence analysis following Na-bisulfite modification of genomic DNA and PCR amplification demonstrated that the distal homology block of P1 is hypermethylated in L540 and L591 cells whereas in other cell lines this region remains to be unmethylated or methylated to a significantly less extent (Figure 10). To determine if the differential methylation correlates with differential expression we also performed western blot assays using NFATc1 specific antibodies. As shown in the right panel of Figure 10, L540 and L591 cells did not express any NFATc1 whereas the other four HL cell lines expressed low amounts of NFATc1 compared to Jurkat and DOHH2 cells which expressed either all three prominent NFATc1 isoforms (Jurkats) or predominantly the short isoform A (DOHH2), both before and after TPA/ionomycin (T/I) stimulation. Taken together these observations strongly suggest that DNA methylation is a mechanism responsible for repressing the *nfatc1* gene in different human lymphomas.

In further experiments we established the DNA methylation pattern for the two blocks of the P1 promoter and for the P2 promoter. A summary of P1 and P2 DNA methylation profile for L540 and L428 cells shown in Figure 11 clearly demonstrates that cells bear the same DNA methylation level between distinct parts of the *nfatc1* promoter region. Thus, both CpG islands associated with P1 and P2 promoters are involved in *nfatc1* gene regulation by DNA methylation.

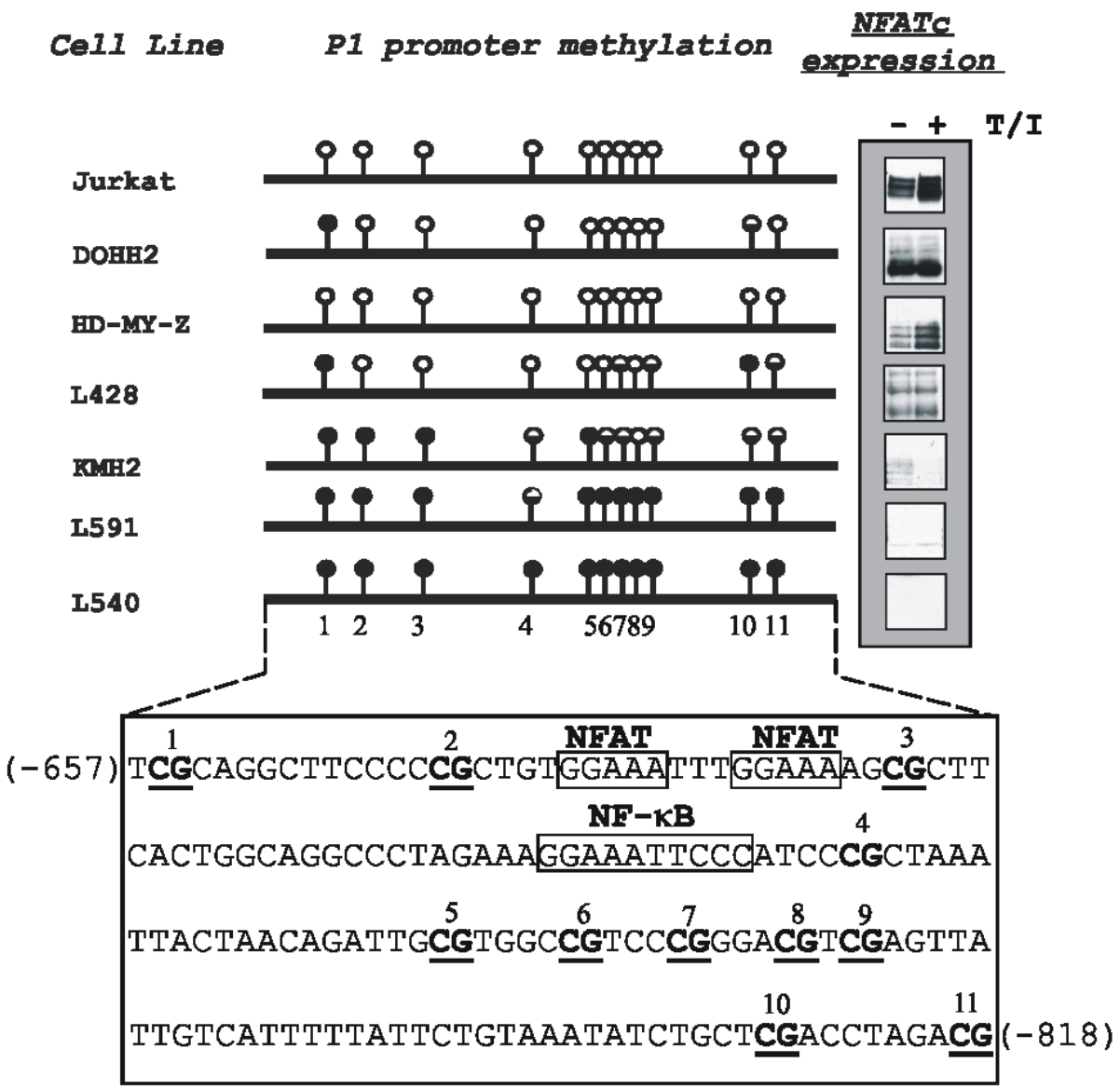


**Figure 10. Summary of DNA hyper-methylation of the distal block of homology of the P1 promoter in several Hodgkin's lymphoma cell lines, compared to Jurkat T cells and DOHH2 B cells.** Filled circles indicate DNA methylation, empty circles no methylation, and half-filled circles partial methylation. The DNA sequence spanning the nucleotides from position –657 to –818 which was investigated is shown below. Binding sites for NFAT and NF-κB transcription factors (Chuvpilo et al., 2002a) are boxed, and the CpG methylation sites are underlined and in bold type. NFATc1 protein expression of cell lines which were either non-induced (-) or induced by TPA/ionomycin (T/I) for 4 h is shown at the right side by Western immunoblots.

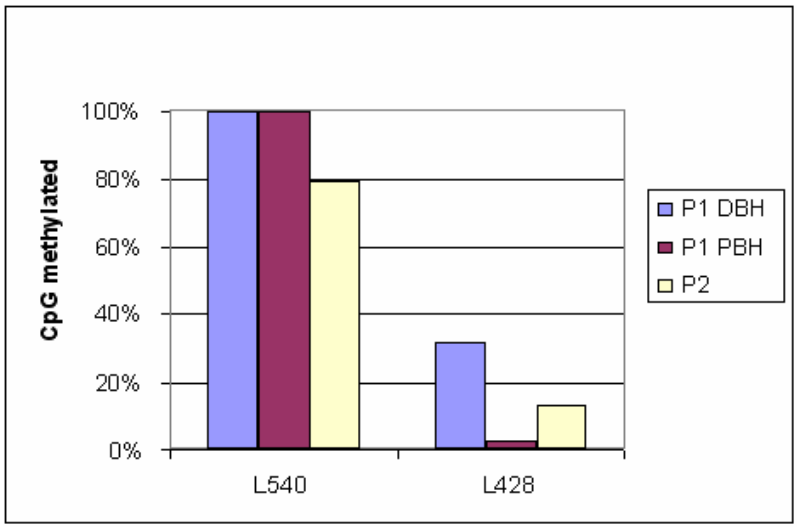


**Figure 11. DNA methylation pattern within the *nfatc1* promoter region.** Three different loci corresponding to nfatc1 promoter region in two HL cell lines were examined using bisulfite genomic sequencing.

## 2.4 The *nfatc1* gene contains multiple Sp1/Sp3 factor binding sites

Sequence analysis of the *nfatc1* gene revealed several putative Sp1/Sp3 transcription factors binding motifs localized within the *nfatc1* promoter region. To confirm the identity of these binding sites we carried out EMSA assays using radiolabeled double-stranded oligonucleotides representing sequences from nucleotide positions -40 to -73, -100 to -120 and -195 to -217 within P1 and position -50 to -70 within P2. The latter site contains two Sp binding motifs and was designed as $Sp1/3_{tand}$.

Figure 12 shows gel shift and competition assays for $Sp1/3_{-40-73}$ and consensus Sp1/Sp3 (cSp1/3) oligonucleotides using nuclear protein extracts prepared from EL-4 cells. Several DNA-protein complexes were observed but no differences were detected between unstimulated cells (lines 1, 11) and cells stimulated with TPA/Ionomycin (lines 2, 12).

The generation of two of these complexes were found to be competed by an excess of unlabeled consensus (lines 7 and 8) and $Sp1/3_{tand}$ (lines 5 and 6) oligonucleotides. In contrast, addition of mutated oligonucleotides containing a single nucleotide substitution within the binding core didn't prevent formation of these complexes (lines 9 and 10) indicating that the nucleotide mutated in this oligonucleotide was necessary for the binding of Sp factors.

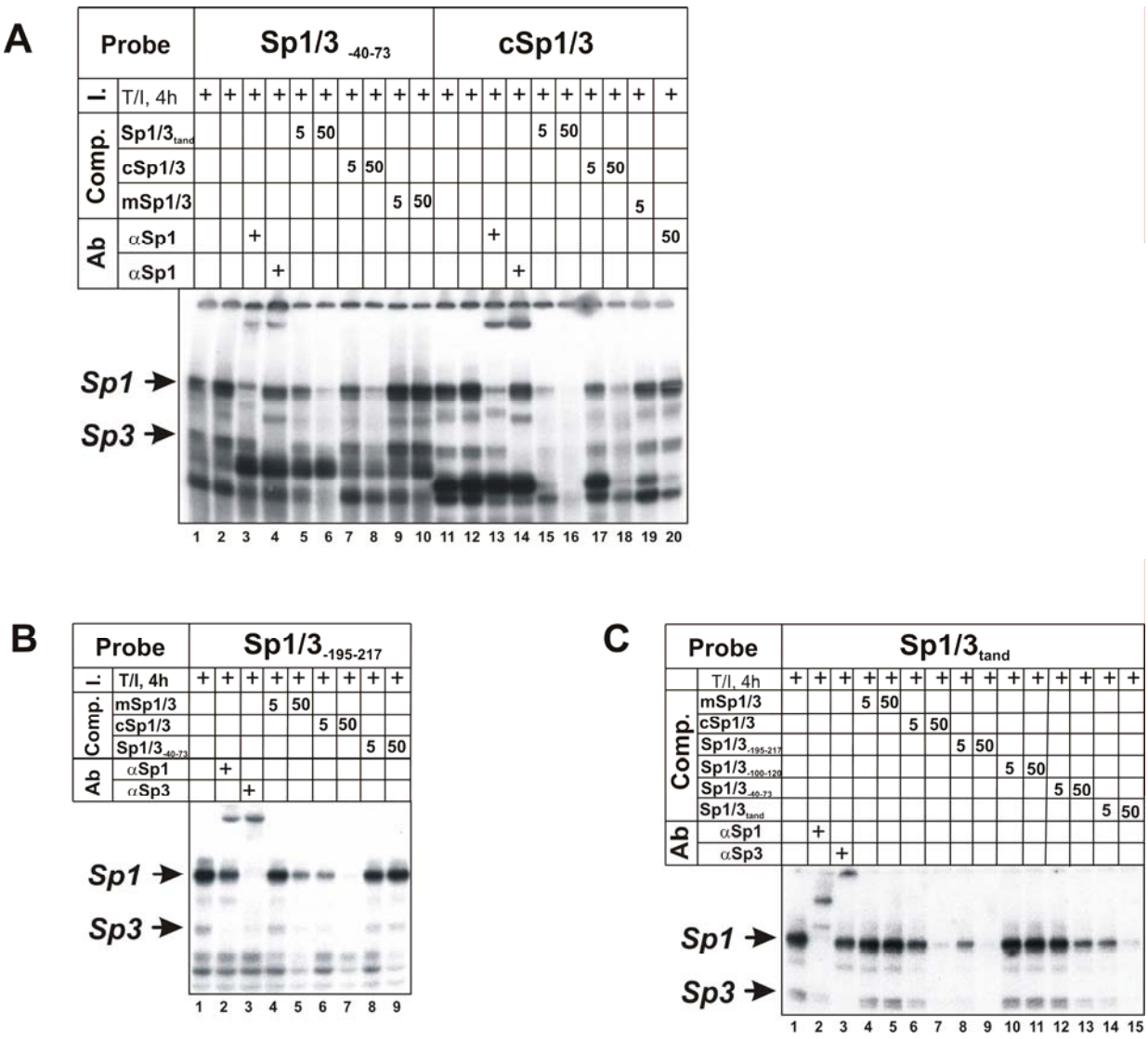


**Figure 12. Analysis of binding of transcription factors Sp1/Sp3 to the Sp1/3 binding sites assayed by EMSA.** Positions are shown relative to transcription start site of the *nfatc1* promoter. Black arrows indicate specific DNA-protein complexes with EL-4 nuclear protein extracts. **(**A) Binding of the probe corresponding to the Sp1/3 recognition binding site located at positions -40 to -73 of P1. (B) Binding of the probe corresponding to the Sp1/3 recognition binding site located at positions -195 to -217 of P1. (C) Binding of the probe corresponding to the Sp1/3 recognition binding site located at positions -50 to -70 of P2.

The specificity of indicated Sp1 and Sp3 complexes was confirmed by addition of antibodies specific for Sp1 and Sp3 which were both able to selectively affect gel migration pattern of these proteins forming a clear supershift. According to this assay, the Sp3 complex exhibits a faster mobility compared to Sp1 complex (lines 3,4 and 13,14).

Similarly, we found binding of Sp1 and Sp3 transcription factors to the $Sp1/3_{-95\ -217}$ as well as to the tandemly arranged Sp binding motifs in P2 (Figures 12, B and C). However, no complex formation was detected with a probe containing the $Sp1/3_{-100-120}$ site. Contrary to other sites, this oligonucleotide also failed to compete with the consensus Sp1 motif (Figure 12C, lines10 and 11). Taken together, these results indicate that Sp family members bind specifically to both P1 and P2 promoters and, therefore, it is very likely that they participate in the transcriptional regulation of *nfatc1* gene.

## 2.5 DNA methylation and Sp1/Sp3 binding

Previous studies demonstrated that at least one Sp binding site located in proximal homology block of P1 promoter is affected by DNA methylation in L540 and L591 cells. To investigate whether methylation of this site influences binding of Sp1 or Sp3 factors to these recognition elements additional EMSA assays were carried out using a probe methylated *in vitro*. For this experiment we selected the oligonucleotide bearing the Sp1/Sp3 binding site at positions -95 to -217 and containing five CpG dinucleotides. As described above, it was shown to interact with Sp1/Sp3 proteins with almost the same affinity as a consensus Sp binding site (Figure 13). Methyl sidegroups were added to all CpG dinucleotides within the oligonucleotide sequence including central CpG site within the binding core. As shown in Figure 13, DNA methylation did not abolish binding of Sp1/Sp3 factors although the “cold” methylated oligonucleotide competed for the factor binding less efficiently than an unmethylated probe.

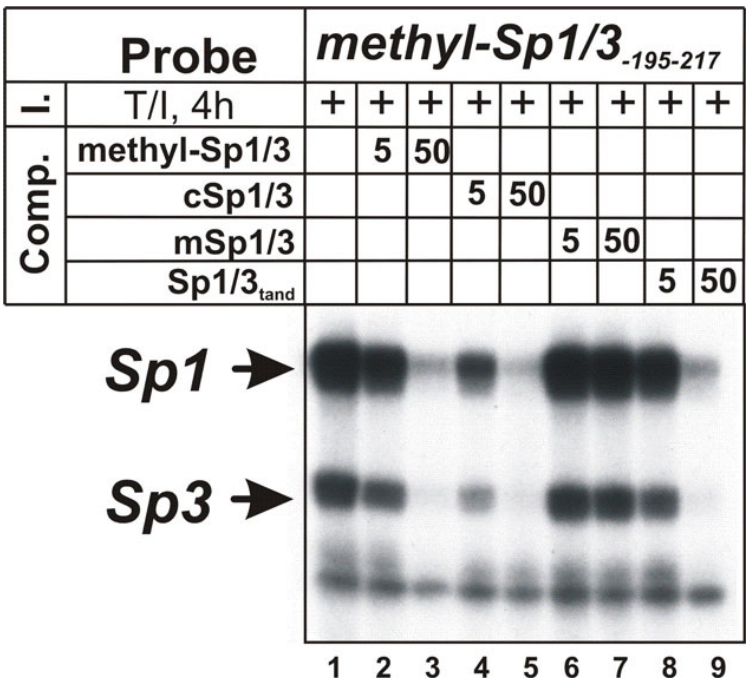


**Figure 13. Analysis of binding of Sp1/Sp3 to the methylated recognition Sp1-3-binding sites assayed by EMSA.** Five cytosines within CpG dinucleotides of a probe designed as methyl-Sp1/3$_{-195-217}$ corresponding to position -195 to -217 relative to transcription start site of the *nfatc1* promoter were methylated *in vitro*. Black arrows indicate specific Sp1/Sp3 complexes generated with nuclear protein extract from Jurkat T cells.

Finally, these results led us to conclude that DNA methylation itself can not directly interfere with DNA recognition by Sp1/Sp3 transcription factors and require additional repressive mechanism accounted for *nfatc1* transcription silencing.

## 2.6 Histone modifications of *nfatc1* promoter region in Hodgkin cell lines

To assess the histone modification status of the *nfatc1* gene, we performed chromatin immunoprecipitation (ChIP) analyses with antibodies raised against acetylated histone H4 and tri-methylated H3-K4 and H3-K9 histones. Whereas acetylated and tri-methylated H3-K4 histones are indicative for transcriptionally active chromatin, tri-methylated H3-K9 indicates the presence of transcriptionally inactive heterochromatin (Jenuwein and Allis, 2001; Lachner et al., 2003; Schotta et al., 2004). Three HL cell lines were selected to determine whether HL cells which do not express NFATc1 differ in histonemodification of their *nfatc1* promoter chromatin from those which do express NFATc1.

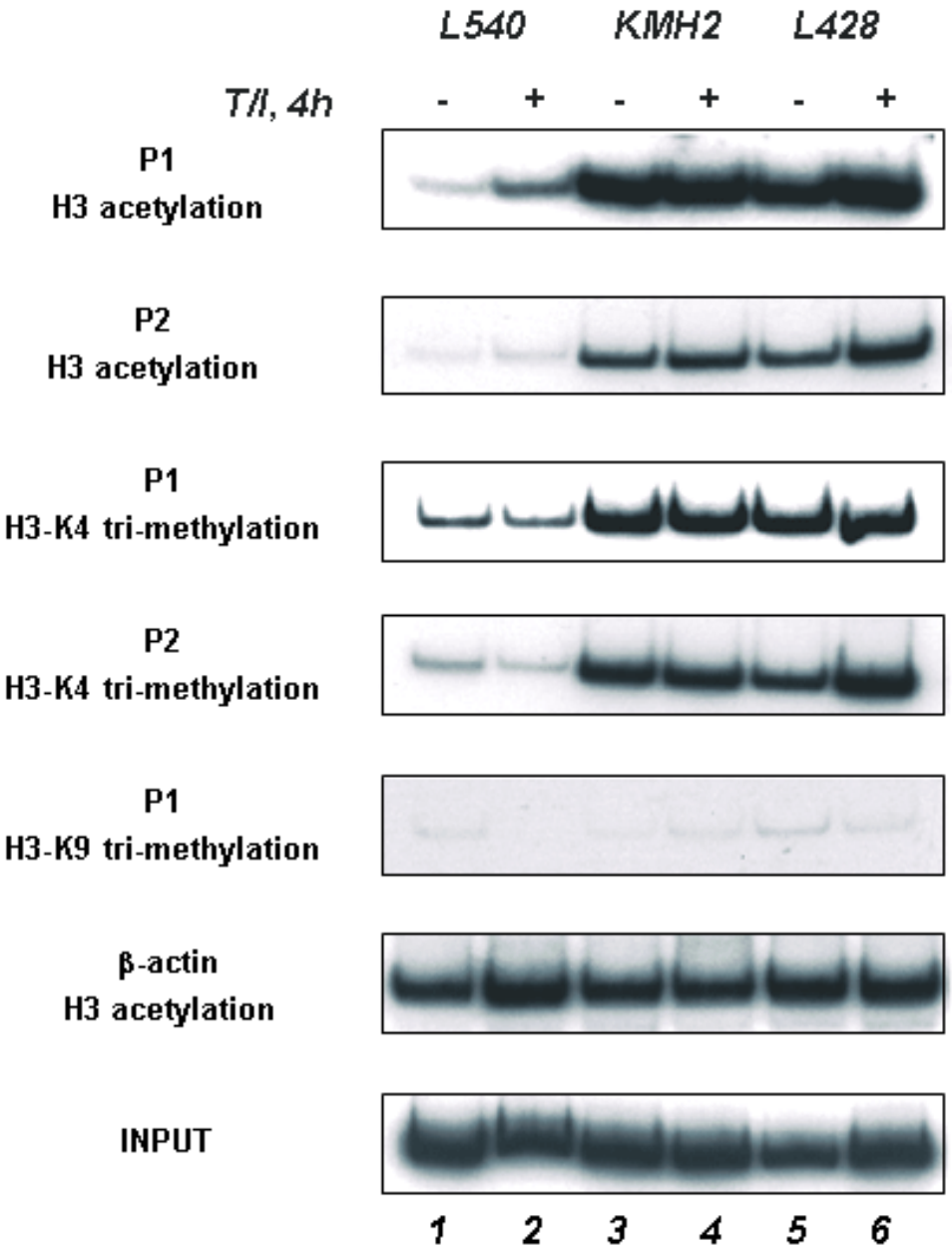


**Figure 14. Strong reduction in histone H3 acetylation and H3-K4 tri-methylation in the Hodgkin's cell line L540 in which NFATc1 expression is suppressed.** ChIP assays. The HL cell lines L540, KMH2 and L428, either non-induced- or induced by TPA/ionomycin for 4h (+) were cross-linked by formaldehyde, and their nuclei were isolated and sonicated. The resulting chromatin fragments were immunoprecipitated using antibodies specific for acetylated histone H3, tri-methylated H3-K4 or H3-K9. After DNA isolation, promoter DNAs were amplified by PCR using primers for the distal block of homology of the P1, P2 and ß-actin promoters, followed by agarose gel electrophoresis (Soutoglou and Talianidis, 2002).

As shown in Fig. 14, in L540 HL cells which do not express any NFATc1, both the P1 and P2 promoter regions contain much less acetylated H3 and tri-methylated H3-K4 histones than those in KMH2 and L428 cells which express various amounts of NFATc1 (Fig. 10). No differences were detected between these cell lines for the H3 acetylation at the ß-actin promoter. However, all ChIP assays using antibodies against tri-methylated H3-K9 resulted in very weak signals which did not reveal any consistent differences between these three HL cells lines.

# 3. DISCUSSION

Previously, it was shown by immunohistochemical stainings that NFATc1 expression is suppressed or strongly diminished in human anaplastic large cell lymphomas (ALCL) and classical Hodgkin's lymphomas (cHLs) (Akimzhanov et. al., unpublished). The inactivation of NFATc1 expression in these lymphomas could be due to several molecular mechanisms, including genetic instability of the *nfatc1* locus and repressive epigenetic changes, such as DNA methylation and histone modification.

**LOH analysis of *nfatc1* gene**

The detection of genomic instability is an important step in molecular analysis of tumorogenesis. Somatic alterations in microsatellite sequences have been termed "microsatellite instabilities" due to a deletion or an insertion of one or more repeat units. Microsatellite instability, referred to as replication error phenotype, is associated with defects in the DNA mismatch repair machinery (Fishel et al., 1993; Bronner et al., 1994). Analysis of the highly polymorphic microsatellite loci not only provides information about microsatellite instabilities, but also allows the detection of allelic deletion in tumor cells. Loss of heterozygosity can only be assayed in heterozygous loci. Tumor suppressor genes are generally inactivated by an intragenic mutation within one allele and the subsequent loss of the corresponding (wild) allele, resulting in a condition termed "loss of heterozygosity". Somatic DNA alterations leading to LOH are generally more complex and may include multifocal deletion, mitotic recombination or nondisjunctional chromosomal loss, as well as gene conversion and point mutation (Meuth, 1990). The frequent allelic loss at particular chromosomal regions in tumors indicates the presence of a tumor suppressor gene. Loss of heterozygosity and microsatellite instability are integral parts of neoplasmic progression. Analysis of this abnormality is of great significance for clinical diagnosis and is recommended in molecular screening for both inherited and sporadic neoplasms (Jass et al., 1995; Aaltonen et al., 1998).

Microsatellite instability has been described in a wide variety of malignancies. It was shown to be a characteristic feature of the malignant Hodgkin and Reed-Sternberg cells in classical Hodgkin's lymphoma and the lymphocytic and histiocytic cells in lymphocyte predominant Hodgkin's lymphoma as well as high-grade large B-cell lymphoma, cutaneous T-cell lymphoma, enteropathy-type T-cell lymphoma (Starostik et

al., 2000a; Starostik et al., 2000b; Re et al., 2002; Baumgartner et al., 2003; Rubben et al., 2004). However, data regarding the role of microsatellite instability in the pathogenesis of ALCL lymphoma are limited.

In our study, we have analyzed six well-characterized cases of systemic T-cell ALCL arising in immunocompetent patients for the presence of microsatellite instability. Genomic DNA isolated from paired normal and tumor tissue was analyzed at six microsatellite loci by polymerase chain reaction. We were unable to identify any loss of heterozygosity in our cases suggesting that abnormalities in the DNA mismatch repair system do not play a major role in the pathogenesis of most systemic ALCL. This finding suggests that according to expanded Knudson model, NFATc1 repression might be explained entirely by epigenetic mechanisms affecting both alleles of the gene (Fig. 4). Thus, this prompted us to focus in the next studies on epigenetic alterations of *nfatc1* promoter DNA in lymphoid tumors.

### *Nfatc1* promoter methylation

Aberrant methylation of promoter CpG islands is now well recognized as a mechanism accounted for the loss of tumor suppressor gene expression in human cancers, and the list of methylated genes identified in various cancer types continues to grow.

Sequence analysis of CpG dinucleotides distribution within *nfatc1* gene reveals two CpG islands which appear to be the targets for DNA methylation. This idea is supported by previous investigations using *in vitro* methylated P1 fragments. When the methylated and nonmethylated promoter fragments were tested in EL-4 cells, a 5-fold decrease in the Ionomycin+Fosculin-mediated activity was detected for methylated P1 DNA compared to nonmethylated control DNA (Chuvpilo et al., 2002b).

Both western blotting analysis (Chuvpilo et al., 1999) or immunohistochemical stainings (Akimzhanov et al., unpublished) indicate that NFATc1 is expressed at high levels in primary T cells, including those adjacent to transformed cells, suggesting that NFATc1 is highly expressed and not down-regulated in normal lymphoid tissues. These observations are further supported by experimental data of bisulphite genomic sequencing of naïve and peripheral T cells. The methylation pattern of P1 DNA clearly demonstrates that *nfatc1* promoter region is free of methylation at all steps of T cell differentiation (Figure 9). Analysis of *nfatc1* methylation profile in embryonic stem sells indicates in turn that this hypomethylated pattern could be established at the early stages of the development. On the other hand, published data suggest that de *novo* methylation of CpG islands leading to suppression of a target gene is a rare occurrence

in normal somatic tissues (Ehrlich et al., 1982) and changes in CpG islands methylation could be a tumor-specific event.

In our studies we have found that *nfatc1* promoter region is hypermethylated in a variety of primary lymphomas analyzed providing therefore a possible explanation for the observed loss of NFATc1 expression in human lymphomas. To our knowledge, this is the first study demonstrating aberrant methylation of the *nfatc1* gene in lymphoid cancers. This finding is likely to support a tumor suppressor role of NFATc1 discussed above.

Further experiments with Hodgkin lymphoma derived cell lines suggested an inverse correlation between *nfatc1* expression and promoter hypermethylation. From these, L540 and L591 cells which do not express any NFATc1 protein were found to be hypermethylated in *nfatc1* promoter region whereas in the other four NFATc1 expressing HL cell lines DNA of this region remained unmethylated (Figure 9). These results suggest that DNA methylation of the *nfatc1* region is indeed critical for NFATc1 transcription and plays an important role in NFATc1 inactivation.

**Sp family members are involved in *nfatrc1* transcriptional regulation.**

Sp1 and Sp3 belong to the superfamily of Sp $Zn^{2+}$-finger transcription factors (Marin et al., 1997; Suske, 1999). Both Sp1 and Sp3 are essential for survival as Sp1 knockout and Sp3 knockout mice died before or at birth (Marin et al., 1997; Bouwman et al., 2000). Sp1 is a transactivator/enhancer of gene transcription, but the role of Sp3 in gene regulation is less clear (Suske, 1999). Sp3 is homologous to Sp1 exhibiting similar affinities to Sp1 binding sites. While Sp3 may enhance Sp1-mediated transcription for a small number of genes, Sp3 may also suppress Sp1-mediated transcription in most genes that have been studied. It has been proposed that Sp3 competes with Sp1 for the binding to Sp1 motifs and thus serves as a negative regulator acting through Sp1 sites (Suske, 1999).

Sequence analysis of *nfatc1* gene revealed several putative binding motifs for Sp family of transcription factors. Subsequent EMSA assays confirmed *in vitro* binding of Sp1 and Sp3 factors to four binding sites located in the P1 and P2 promoters (Figure 10) suggesting that Sp proteins might be involved in the transcriptional regulation of *nfatc1* gene, probably regulating its basal activity.

Although a strong correlation between promoter methylation and gene silencing has extensively been demonstrated, the molecular mechanisms of this methylation-modulated gene inactivation remain unclear.

Two hypotheses have been proposed to explain transcriptional inactivation by promoter methylation. One explanation for methylation-induced gene repression emphasizes a direct influence of methylation on the binding of transcription factors. Since many mammalian transcription factors have CpG-rich binding sites in their DNA recognition element, methylation occurring at the CpG of the specific binding element may sterically interfere with binding of transcription factors to DNA, thus inhibiting transcription (Bird, 2002). For instance, a HpaII site (CCGG) is located in an element to which the transcription factor AP-2 binds. Methylation at this HpaII site (CCmGG) within the AP-2-binding site inhibits AP-2 binding and suppresses AP-2-regulated gene transcription in C6-glioma and CV-1 cells (Comb and Goodman, 1990). This direct effect of methylation on binding of transcription factors is also observed for other genes including the cyclic AMP (cAMP)/cAMP-responsive element (CRE, TGACGTCA) and the retinoblastoma binding factor 1 recognition sequence (AGCTGCCGCGGGCGGAAGT) (Iguchi-Ariga and Schaffner, 1989; Ohtani-Fujita et al., 1993). However, reports regarding the effect of methylation at Sp1-binding sites on Sp1 binding are conflicting. Several researchers have shown that methylation at Sp1-binding sites has no influence on Sp1 binding and gene expression (Holler et al., 1988; Ohtani-Fujita et al., 1993). In contrast, others have reported that methylated CpG dinucleotides variably interfere with Sp1 binding by 50 to 95% depending on the configuration of methylated cytosines within the consensus Sp1-binding element (Clark et al., 1997; Mancini et al., 1999). Taken together, which mechanism is predominant in the methylation-induced gene repression may be dependent on the cell type, the transcription factor, or the received stimuli.

To investigate whether methylation of the Sp1/Sp3 binding site influence binding of Sp1 or Sp3 to *nfatc1* gene we performed EMSAs using an *in vitro* methylated oligonucleotide corresponding to one of their recognition elements within the P1 promoter. This densely methylated probe contained five methyl-CpG sites located both within and outside the binding motif. As shown above in Figure 10, methylation of the Sp1 site resulted in only a very weak, if any decrease in Sp1/Sp3 binding as confirmed by competition assays. Therefore, it does not seem to be not involved in *nfatc1* repression. However, several studies published previously point to a potential role for Sp binding sites in maintaining the CpG island in unmethylated and transcriptionally active state (Brandeis et al., 1994; Macleod et al., 1994). Therefore, the role of Sp transcription factors in lymphoma tumorogenesis still remains to be elucidated.

**Chromatin remodelling within the *nfatc1* promoter region**

As it has been discussed above, aberrant CpG island DNA methylation is not an isolated phenomenon in the epigenetics of cancer progression because it occurs in a context of disturbance of chromatin status. To define a role of histone modification in NFATc1 silencing we have choosen three HL cell lines which previously have shown significant differences in *nfatc1* expression and methylation pattern. Expression analysis demonstrated that L428 and KMH2 cells produce different amounts of all three NFATc1 protein isoforms whereas no NFATc1 was detected in L540 cells. At the same time, L540 cells are highly methylated in both CpG islands in contrast to other cell lines selected for this experiment. To examine whether HL cells which do not express NFATc1 differ in modification of their *nfatc1* promoter chromatin from those which do express NFATc1 we performed ChIP assay using antibodies raised against acetylated histone H3, tri-methylated H3-K4 and H3-K9 histones.

A direct correlation between CpG methylation and histone acetylation is confirmed by the growing numbers of reports demonstrating that methyl-binding proteins are responsible for recruiting of histone deacetylase activity. Despite a relationship between histone H3-K4 tri-methylation and DNA methylation is not well elucidated yet, this histone modification is known as a hallmark for the transcriptionally active chromatin. Supporting these models, the P1 and P2 promoter regions in both L428 and KMH2 cells contained significantly increased levels of acetylated histone H3 as well as tri-methylated histone H3-K4 compared to L540 cells. Opposite to other histone modifications observed, H3-K9 methylation was reported to participate in transcriptional repression and formation of highly condensed pericentromeric chromatin creating a high-affinity binding site for the chromodomain of HP1(Boggs et al., 2002). However, in our studies we didn't detect any differences in H3-K9 tri-methylation associated with P1 or P2 promoters of *nfatc1* in all cell lines used for analysis. Taken together, these data led us to conclude that repression of the *nfatc1* promoter region results in a distinct deacetylation and decrease in H3-K4 tri-methylation but does not result in localization of the *nfatc1* locus to heterochromatic nuclear sites.

# 4. SUMMARY

We examined the regulation of NFATc1 in different lymphomas and observed an inversed correlation between the methylation status and expression of NFATc1. Our data demonstrate that aberrant DNA methylation associated with chromatin remodeling within *nfatc1* locus is a major mechanism for the repression of NFATc1 expression, suggesting that the DNA methylation-mediated transcriptional silencing of NFATc1 may be a critical event in the tumorogenesis of ALCLs and cHLs. Furthermore, the DNA methylation of human *nfatc1* promoter region could be used as a novel biomarker of tumor progression.

Our results indicate a close link between the loss of immunoreceptor signaling and NFATc1 expression in human lymphomas. For both ALCLs and cHLs, defects in immunoreceptor signaling have been described which result in a loss of receptor-mediated gene expression programs (Schwering et al., 2003; Bonzheim et al., 2004; Marafioti et al., 2004). In T cells, one indicator gene of these programs appears to be the *nfatc1* gene whose expression is controlled by TCR signals (Chuvpilo et al., 2002a). In contrast, in T cells NFATc1 expression is unaffected by TCR signals, and NFATc2 was found to be expressed at normal levels in ALCLs and cHLs (L.K., unpubl. data). Moreover, the activity of NF-κB factors which can bind to certain NFAT binding sites and share a distantly-related DNA binding domain with NFATs is strongly elevated in cHL cells (Bargou et al., 1997; Hinz et al., 2001; Hinz et al., 2002) suggesting that NFATs and NF-κBs exert very different effects on generation and maintenance of Hodgkin's lymhomas.

However, it should be mentioned that in Burkitt's and further B cell lymphomas in which NFATc1 proteins are strongly expressed and controlled by receptor signals (Kondo et al., 2003), they could exert a promoting function in tumor development. The genes of p53 family members p63 and p73 are prominent examples for mammalian genes whose products can act both as oncoproteins and tumor suppressor genes (Hibi et al., 2000; Stiewe and Putzer, 2002), and it is likely that more genes exist which encode both tumor suppressors and oncoproteins. It remains to be shown whether the *nfatc1* gene is one of them.

## Zusammenfassung

Wir haben die Regulation von NFATc1 in verschiedenen Lymphomen untersucht und beobachteten eine umgekehrte Korrelation zwischen dem Ausmaß an Methylierung und der Expression von NFATc1. Unsere Daten demonstrieren, dass eine aberrante DNA-Methylierung, die mit veränderter Chromatinstruktur innerhalb des nfatc1 Lokus assoziiert ist, der Hauptmechanismus für die Repression der NFATc1-Expression ist. Es wäre zu vermuten, dass die durch DNA-Methylierung verursachte transkriptionelle Abschaltung von NFATc1 der kritische Schritt bei der Tumorgenese von ALCLs und cHLs ist. Des weiteren könnte das Ausmaß der DNA-Methylierung in der humanen *nfatc1*-Promotorregion als neuer Biomarker für Tumorprogression genutzt werden.

Unsere Daten indizieren eine enge Verbindung zwischen dem Verlust von Immunrezeptorsignalen und der NFATc1-Expression in humanen Lymphomen. Für sowohl ALCLs als auch cHLs wurden Defekte in der Immunrezeptorsignalgebung beschrieben, welche sich im Verlust des Rezeptor vermittelten Genexpressionsprogramms niederschlagen (Schwering et al., 2003; Bonzheim et al., 2004; Marafioti et al., 2004). In T-Zellen scheint das *nfatc1*-Gen eins der Indikatorgene dieses Programms zu sein, dessen Expression durch TCR-Signale kontrolliert wird (Chuvpilo et al., 2002a). Im Gegensatz dazu bleibt die NFATc2-Expression in T-Zellen unbeeinflusst von TCR-Signalen, weshalb NFATc2 in ALCLs und cHLs auch in normalem Ausmaß exprimiert wird (L.K., unpubl. data). Andererseits ist die Aktivität der NF-κB-Faktoren, die auch an bestimmte NFAT-Bindungsstellen binden können und deren DNA-Bindungsdomäne entfernt mit der der NFATs verwandt ist, in cHL-Zellen stark erhöht (Bargou et al., 1997; Hinz et al., 2001; Hinz et al., 2002). Das lässt vermuten, dass NFATc1 und die NF-κB-Faktoren eine sehr unterschiedliche Rolle bei der Entstehung und dem Erhalt der Hodgkinlymphome spielen.

Es sollte aber erwähnt werden, dass in Burkitts und anderen B-Zelllymphomen, in denen NFATc1-Proteine stark exprimiert und darüber hinaus durch Rezeptorsignale kontrolliert sind (Kondo et al., 2003), diese eine Tumor fördernde Funktion ausüben könnten. Die Gene der p53-Familienmitglieder p63 und p73 sind prominente Beispiele für Säugergene, deren Produkte sowohl als Onkoproteine als auch als Tumorsuppressoren fungieren können (Hibi et al., 2000; Stiewe and Putzer, 2002), und es ist wahrscheinlich, dass es noch weitere Gene gibt, die beide Funktionen ausüben. Es wird zu zeigen sein, ob das *nfatc1*-Gen eins von ihnen ist.

# 5. MATERIALS AND METHODS

The methods described in this section are all based upon today's standard molecular and cellular biology techniques.

## 5.1 Materials

### 5.1.1 Instruments

| **Hardware** | **Manufactorer** |
|---|---|
| Autoclave | Stiefenhofer |
| Bacterial shaker | New Brunswick Scientific |
| Balance machine | Sartorius, Hartenstein |
| Cold centrifuge | Heraeus |
| DNA sequencer 373A | Perkin Elmer |
| FACScan™ | Becton Dickinson |
| Gel documentation system | Herolab |
| Gel camera | Stratagene, Hoefer |
| Gel dryer | H.Hölzel |
| Heating blocks | Hartenstein |
| Hybridization oven | Bachofer |
| Ice machines | Genheimer |
| Intensifying screen | DuPont |
| Laminar hoods | Heraeus, Gelaire |
| Light microscope | Olympus, Leica |
| Liquid nitrogen tank | Tec-lab |
| Luminometer | Berthold |
| Microliter pipettes | Eppendorf, Brand |
| Microcentrifuge | Eppendorf |
| Multichannel pipette | Eppendorf |
| Multi dispencer pipette | Eppendorf |
| PCR machine | Perkin Elmer, MWG |
| pH meter | Ingold, Hartenstein |
| Phosphoimager | Fujix BAS-2000 III, Fuji, |
| Quartz cuvettes | Hellma |
| Refrigerators (-20°C; -70°C) | Privileg, Bosch, Heraeus |
| Rotors (JA-10, JA-14) | Beckman |
| Scintillation counter | Canberra Packard |
| Shaking incubator | Hartenstein |
| Power supplier | Amersham Pharmacia |
| SDS-PAGE apparatus | BioRad |
| Spectrophotometer | Amersham Pharmacia |
| Ultracentrifuge | Beckman |
| UV lamp (UVT-20M) | Herolab |
| Vortexer | Hartenstein |
| Waterbath | Hartenstein |
| Water filtration unit (MilliQ Plus) | Millipore |
| Western blot apparatus | Hoefer |

### 5.1.2 General materials

| Reagent | Purchased from |
|---|---|
| Cell strainer (70 µM) | Falcon |
| 2 ml cryotubes | Greiner bio-one |
| Disposable needles, Cuvettes & Syringes | Hartenstein |
| Glasswares | Schott |
| Nitrocellulose membrane | Schleicher & Schuell |
| Polypropylene tubes | Greiner bio-one, Nunc |
| Parafilm | Hartenstein |
| Pipette tips | Eppendorf |
| Pipettes | Sarstedt |
| Röntgen film (13x18 cm, BioMax) | Kodak |
| Sterile filters (0.2 µM/ 0.45 µM) | Schleicher & Schuell |
| Tissue culture plates | Greiner bio-one, Falcon |
| Tissue culture flask (50, 250, 500 ml)) | Greiner bio-one |
| Tissue culture dish (60 mm, 90 mm) | Falcon, Greiner bio-one |
| Tubes (1.5 & 2 ml) | Sarstedt, Eppendorf |
| Whatman paper | Schleicher & Schuell |

### 5.1.3 Chemical reagents

| Reagent | Purchased from |
|---|---|
| Acetic Acid [$C_2H_4O_2$] | Carl Roth |
| Acrylamid solution | Carl Roth |
| AEBSF (Pefabloc SC) | Roche |
| Agar-Agar | Carl Roth |
| Agarose | Sigma-Aldrich |
| Ampicillin | Hoechst |
| APS | Merck Eurolab |
| ATP-disodiumsalt [$C_{10}H_{14}N_5O_{13}P_3Na_2$] | Sigma-Aldrich |
| ß-glycerophosphate [$C_3H_7O_6PNa_2$] | Carl Roth |
| ß-mercaptoethanol | Carl Roth |
| BioRad protein assay (5x Bradford reagent) | BioRad |
| Bisoprolol | Tocris |
| Boric Acid | Merck Eurolab |
| Bromophenol blue | Merck Eurolab |
| BSA Fraction V | Carl Roth |
| Butanol [$C_4H_{10}O$] | Carl Roth |
| Calcium chloride [$CaCl_2$] | Carl Roth |
| CD62L MACS beads | Miltenyl Biotech |
| Chloroform [$CHCl_3$] | Carl Roth |
| Citric acid [$C_6H_8O_7 \cdot H_2O$] | Carl Roth |
| Column for murine $CD4^+$ cell | CEDARLANE® |
| Coomassie brilliant blue R-250 | Roche Applied Science |

| | |
|---|---|
| Cyclosporin A [CsA] | Novartis Pharma |
| Cyclic AMP [cAMP] | Sigma |
| DEPC | Carl Roth |
| Diethanolamine | Roth |
| Disodiumhydrogenphosphate [$Na_2HPO_4 \cdot 7H_2O$] | Merck Eurolab |
| D-Luciferin [$C_{11}H_8N_2O_3S_2$] | AppliChem |
| DMEM | Gibco BRL |
| DMSO | Carl Roth |
| dNTPs | MBI-Fermentas |
| DTT | Carl Roth |
| ECL Chemiluminiscence Kit | Amersham, Roche |
| EDTA [$Na_2$ EDTA$\cdot 2H_2O$] | Carl Roth |
| EGTA | Sigma-Aldrich |
| Ethanol [$C_2H_5OH$] | Carl Roth |
| Ethidium Bromide [EtBr] | Sigma-Aldrich |
| Ferrous(II)sulfat [$FeSO_4$] | Carl Roth |
| Ferric(III)chloride Hexahydrat [$FeCl_3 \cdot 6H_2O$] | Carl Roth |
| FCS | Gibco BRL |
| Ficoll | Amersham Pharmacia |
| Formaldehyde [$CH_2O$] | Carl Roth |
| Forskolin | Calbiochem |
| Gel extraction kit (Jetsorb) | Genomed |
| Glutathione sepharose | Sigma-Aldrich |
| Glycerin (87%) | Carl Roth |
| Glycin [$C_2H_5NO_2$] | Merck Eurolab |
| H-89 | Sigma |
| HBSS | Gibco BRL |
| Hepes | Carl Roth, Gibco BRL |
| Hydrochinon | Carl Roth |
| Hydrochloric Acid [HCl] | Merck Eurolab |
| Hydrogen Peroxide [$H_2O_2$] | Carl Roth |
| 4-Hydroxytamoxifen [$C_{26}H_{29}NO_2$, *Z*-isomer] | Sigma-Aldrich |
| ICI-118 551 | Tocris |
| IL-4, murine, recombinant, *E.coli* | Dr. E. Schmitt, Mainz |
| Ionomycin | Sigma-Aldrich |
| IPTG | Boehringer-Ingelheim |
| Isoamylalcohol | Carl Roth |
| Isopropanol [2-Propanol, $C_3H_8O$] | Carl Roth |
| Isoproternol | Sigma |
| Leupeptin hydrochloride | Roche Applied Science |
| L-Glutamine | Gibco BRL |
| L-Glutathione [$M_r$: 307·3] | Serva |
| Lithium chloride [LiCl] | Sigma-Aldrich |
| Milk powder | Saliter |
| Magnesium acetate [$Mg(C_2H_3O_2)_2 \cdot 4H_2O$] | Sigma-Aldrich |
| Magnesium chloride [$MgCl_2$] | Carl Roth |
| Magnesium sulfate [$MgSO_4 \cdot 7H_2O$] | Carl Roth |
| Manganese chloride [$MnCl_2$] | Fluka |
| MEM [100x] | Gibco BRL |
| MES [$C_6H_{13}NO_4S$] | Sigma-Aldrich |
| Methanol [$CH_4O$] | Carl Roth |

| | |
|---|---|
| Metrizamide [$C_{18}H_{22}I_3N_3O_8$] | Serva |
| MOPS | Carl Roth |
| Paraformaldehyde | Merck Eurolab |
| PCR purification kit | Qiagen |
| PEG 4000 | NEB |
| Penicillin (10,000 IU/ml) | Hoechst |
| Phenol [$C_6H_6O$, TE equilibrated] | Carl Roth |
| PIPES | Serva |
| Plasmid-DNA Isolation kit (Maxi) | Macherey-Nagel, Qiagen |
| Plasmid DNA Isolation kit (Mini) | Genomed |
| PMSF | Serva |
| Poly dI/dC | Boehringer Ingelheim |
| Ponceau Red | Sigma Aldrich |
| Potassium acetate [$C_2H_3KO_2$] | Carl Roth |
| Potassium chloride [KCl] | Sigma-Aldrich |
| Potassium dihydrogen phosphate [$KH_2PO_4$] | Sigma-Aldrich |
| Potassium hydrogen phosphate [$KHPO_4$] | Sigma-Aldrich |
| Potassium hydroxide [KOH] | Carl Roth |
| Propidiumiodide (PI 1 mg/ ml $ddH_2O$) | Sigma Aldrich |
| Protease inhibitor tablet (complete mini) | Roche Applied Science |
| Protein-A/G sepharose | Santa Cruz |
| Radioactive nucleotides [$\gamma^{32}$P-ATP, $\alpha^{32}$P-dCTP, $\alpha^{32}$P-UTP] | Amersham Pharmacia |
| RNase Protection Assay Kit (RiboQuant) | BD Pharmingen |
| RPMI 1640 | Gibco BRL |
| Rubidium chloride [RbCl] | Carl Roth |
| Saponin | Sigma |
| SB 203580 | Sigma |
| Sodium acetate [$CH_3COONa \cdot 3H_2O$] | Merck Eurolab |
| Sodium azide [$NaN_3$] | Merck Eurolab |
| Sodium bisulfite | Sigma |
| Sodium carbonate [$Na_2CO_3$] | Carl Roth |
| Sodium chloride [NaCl] | Carl Roth |
| Sodium fluoride [NaF] | Sigma-Aldrich |
| Sodium hydrogen phosphate [$NaH_2PO_4 \cdot H_2O$] | Merck Eurolab |
| Sodium hydroxide [NaOH] | Carl Roth |
| Sodium orthovanadate [$Na_3VO_4$] | Fluka |
| Sodium pyruvate [$C_3O_3H_3Na$] | Gibco BRL (100 mM) |
| Sodium citrate [$C_6H_5Na_3O_7 \cdot 2H_2O$] | Carl Roth |
| SDS | Carl Roth |
| Sephadex G50 | Amersham Pharmacia |
| Sulfosalicylic acid | Sigma Aldrich |
| Tamoxifan | Sigma |
| TaqDyePrimer sequencing Kit | Perkin Elmer |
| TEMED | Carl Roth |
| TPA | Sigma Aldrich |
| Transfection reagents (SuperFect™, PolyFect™) | Qiagen |
| Trichloroacetic acid [$C_2HCl_3O_2$] | Sigma Aldrich |
| Tris | Carl Roth |
| Triton X-100 | Sigma Aldrich |
| Trizol reagent | Gibco BRL |
| Trypan blue 0.1% | Gibco BRL |

| | |
|---|---|
| Tween 20 | Carl Roth |
| Western blotting substrate (Lumi light) | Roche |
| X-VIVO 15 | BioWhittaker |
| Xylene cyanol FF | Serva |
| 2-YT Broth | GibcoBRL |
| Zeocin (100 mg/ml) | Invitrogen |

### *5.1.3.1 DNA size markers*

The GeneRuler 100 bp and 1 kb DNA size markers were procured from MBI-Fermentas. The size of fragments in markers was as following:

100 bp Marker 1,031 / 900 / 800 / 700 / 600 / 500 / 400 / 300 / 200 / 100 / 80

1 kb Marker 10,000 / 8,000 / 6,000 / 5,000 / 4,000 / 3,500 / 3,000 / 2,500 / 2,000 / 1,500 / 1,000 / 750 / 500 / 250

### *5.1.3.2 Protein standards*

The protein size marker BENCHMARK™ was procured from Gibco BRL.

### *5.1.3.3 Enzymes*

| | |
|---|---|
| All restriction endonucleases and modifying enzymes | MBI-Fermentas |
| SAWADY PWO DNA polymerase | Peqlab |
| Proteinase K and RNase [Ribonuclease] Type I-A | Sigma-Aldrich |

### *5.1.3.4 Antibodies for EMSA*

| Ag | Ab | Format | Clone | Manufacturer |
|---|---|---|---|---|
| Sp1 | Rat | Purified | TRFK5 | BD Pharmingen |
| Sp3 | Rat | Purified | TRFK4 | BD Pharmingen |

### *5.1.3.5 Antibodies for Western Blot*

| Ag | Ab | Format | Clone | Manufacturer |
|---|---|---|---|---|
| NFATc1 | Rabbit Anti-mouse | Purified | polyclonal5 | Immunoglobe |
| β-actine | Mouse | Purified | AC-15 | Sigma Aldrich |

### *5.1.3.6 Antibodies for ChIP*

| Ag | Ab | Format | Clone | Manufacturer |
|---|---|---|---|---|
| Acetylated H3 | Rabbit | Purified | polyclonal | Abcam Limited |
| Methylated H3-K | Rabbit | Purified | polyclonal | Abcam Limited |

| | | | | |
|---|---|---|---|---|
| Methylated H3-K9 | Rabbit | Purified | polyclonal | Abcam Limited |

### *5.1.3.7 Oligonucleotides and primers*

All oligonucleotides and primers were procured from MWG Biotech. The lyophilized oligos were dissolved in 10 mM Tris, pH 7.5 to a final concentration of 100 pmol/µl. For the sequencing reaction, lyophilized oligos were dissolved in 10 mM Tris, pH 7.5 to a final concentration of 10 pmol/µl. Olygonucleotides were stored at –20°C.

#### *5.1.3.7.1 Olygonucleotides for EMSA:*

| | |
|---|---|
| Sp1/3$_{-40-73}$ | TCCGAACTGGGCGGCAGAGTCG |
| Sp1/3$_{-100-120}$ | GCCGGCTCGGGCGGGCAGGGGC |
| Sp1/3$_{-tand}$ | GGTGACAGGGGCGGGACCGGGGCGGGAGGCCGG |
| Sp1/3$_{-195-217}$ | CGCGGGGAGGGGCGGGCGCTCGGCG |
| methyl-Sp1/3$_{-195-217}$ | ***C***G***C***GGGGAGGGG***C***GGG***C***GCT***C***GG***C***G |

#### *5.1.3.7.2 PCR primers for the amplification of microsatellite markers within the nfatc1 locus:*

| Marker | forvard primer | Reverse primer |
|---|---|---|
| ***GT6(I)*** | GCTGGCAACTGTGGCCAAACAG | CGCACAGCCACACTGGGAAATG |
| ***GT11*** | CATGCGTCCTGCTCCATTTGC | GATCAAGCCACTGGACTCCAGCC |
| ***CA8*** | CTAGTGTAGTCTCACCAACTGAG | CATATATCCACAATATAATGTAC |
| ***CA6*** | GAACTCAGTATTCATCTGAAATAG | GCTATTTGTTCTCTACATGTCTTG |

#### 5.1.3.7.3 *PCR-Primers for DNA methylation studies:*

| | |
|---|---|
| 1m_ad | AGAAAGTTYGGTATGTTGAAGTTATTATG |
| 1m_ar | ATATAAATAACCRAAAAACCTCCCCCCRTC |
| 1m_adn | GTTGAAGTTATTATGTAAAATYGTAGGTTT *(nested)* |
| 1m_arn | CRTCAAAAATCRCRCAATCRCRCCCATAAAAC *(nested)(seq)* |
| 2m_srn1 | CCRCRACCCTAAAACCTACRCRATAAC |
| 2m_sdn1 | GTTTTTAGGYGAGYGGTTGTYGYGGYG |

| | |
|---|---|
| 2m_srn2 | CRCRATAACTCCRAACCCTACCCRC *(nested) (seq)* |
| 2m_sdn2 | GGGYGTTYGGYGATTYGTTTTYGGG *(nested)* |
| 3m_sr | CRAACCRAAACCRAAACRAAAACCRAAATC |
| 3m_sdn | GATAYGAGTTTATTTAAAAATTYGTGTTYG |
| 3m_sdn | GATAYGAGTTTATTTAAAAATTYGTGTTYG *(nested)(seq)* |
| 3m_srn | CRAAAACCRAAATCRCRACCRCCAAAAATTC *(nested)* |

#### *5.1.3.7.4 PCR-Primers for ChIP-Assays:*

| | |
|---|---|
| d1_dir | GAGACGTGAGAGAGGAAAGTGTGAGTGG |
| d1_rev | GAAAGCCCGGCATGCTGAAGTCATTATG |
| d3_dir | CAGGGCACAAGAGGCCGGGGGAC |
| d3_rev | GTCGCGGCCGCCAGGGGTTC |

### 5.1.4 Solutions and Buffers

All chemicals of molecular biology research grade were procured from respective manufacturers and all solutions were prepared using pure distilled (Milli-Q grade) autoclaved water. Wherever necessary, solutions were sterile filtered or autoclaved.

*APS stock solution (10 %, 10 ml)*

| | |
|---|---|
| APS | 1 g |

*Blocking buffer for Western hybridization*

| | |
|---|---|
| Milk powder | 5 g |
| Dissolved in 100 ml of 1x TBS-T | |

*Blocking buffer for Western hybridization using phospho-specific antibodies*

| | |
|---|---|
| BSA fraction V | 5 g |
| Dissolved in 100 ml of 1x TBS-T | |

*Brefeldin A:* Dissolved in absolute ethanol at a concentration of 2 mg/ml.

*BSS (Balanced Salt Solution)*

Working solution: BSS I & BSS II (1:1) + dd $H_20$ [8:10]

*BSS I (10x, 1000 ml, Sterile filtered)*

| | |
|---|---|
| Glucose | 10.0 g |
| $KH_2PO_4$ | 0.6 g |
| $Na_2H\ PO_4.2H_20$ | 2.3 g |
| Phenol Red | 0.1g |

*BSS II (10x, 1000 ml, Sterile filtered)*

| | |
|---|---|
| $CaCl_2.\ 2H_20$ | 1.86 g |
| KCl | 4.0 g |
| NaCl | 80.0 g |
| $MgCl_2.6H_20$ | 2.0 g |
| $MgSO_4.7H_20$ | 2.0 g |

*Calcium chloride stock solution (1 M, 1000 ml)*

| | |
|---|---|
| $CaCl_2$ | 110.98 g |

*Coating buffer for anti-CD3 antibody* 0.05 M Tris of pH 9.5

*Colony hybridization solutions*

<u>CH solution I (always prepared fresh)</u>

| | |
|---|---|
| NaOH | 0.5 N |

<u>CH solution II</u>

| | |
|---|---|
| Tris- HCl (pH 7.5) | 0.2 M |
| NaCl | 1.0 M |

<u>CH solution III</u>

| | |
|---|---|
| SDS | 1% |
| EDTA | 1 mM |
| $Na_2HPO_4$ (pH 6.8) | 40 mM |

<u>CH pre-hybridization solution</u>

| | |
|---|---|
| SSC | 5x |
| SDS | 0.2% |

| | |
|---|---|
| Denhardt solution | 2x |
| Salmon sperm DNA | 100 μg/ml |

CH stop buffer

| | |
|---|---|
| Loading dye | 6x |
| SDS | 0.5% |
| EDTA | 50 mM |

CH washing buffer

| | |
|---|---|
| SSC | 2x |
| SDS | 0.2% |

*Coomassie blue solution (1000 ml)*

| | |
|---|---|
| Coomassie Brilliant Blue R-250 | 2.5 g |
| Methanol | 450 ml |
| Acetic Acid | 100 ml |

*DEPC-treated dd$H_2O$ (RNase-free, 1000 ml)*

| | |
|---|---|
| DEPC | 1 ml |

DEPC was mixed thoroughly, incubated overnight (~ 16 h) under hood at RT, autoclaved and stored at RT; all solutions and buffers for RNA work were prepared in DEPC-treated dd$H_2O$.

*Denaturing PAA-Gel for RNase Protection Assay (1000 ml; 6% gel solution)*

| | |
|---|---|
| Urea | 280.0 g (8M) |
| 30% Acryl-Bisacrylamide solution | 240 ml |
| 10x TBE buffer | 100 ml |

Composition of one gel (Polymerization takes approx. 1 -2 hours at RT)

| | |
|---|---|
| 6% gel solution | 30 ml |
| 10% APS | 300 µl |
| TEMED | 60 µl |

*DNA Electrophoresis Buffer (1000 ml)*

TAE (50x) 20 ml

The solution was supplemented with 0.25 ml EtBr stock solution per liter of TAE and boiled for dissolving the agarose.

*DNA gel composition*

| | 0.7% | 1.0 % | 2.0 % |
|---|---|---|---|
| Agarose | 1.05 g | 1.5 g | 3.0 g |
| 20x TAE | 7.5 ml | 7.5 ml | 7.5 ml |
| $ddH_2O$ | 142.5 ml | 142.5 ml | 142.5 ml |
| EtBr (5mg/ml) | 25 µl | 25 µl | 25 µl |

*DTT stock solution (1 M, 20 ml)*

DTT 3.09 g

DTT powder was dissolved in 10 mM sodium acetate (pH 5.2), aliquoted and frozen in -20°C.

*EDTA stock solution (0.5 M, 1000 ml)*

$Na_2$ EDTA·$2H_2O$ 186.1 g

pH of the solution was adjusted to 8.0 with 10 M NaOH (~ 50 ml); EDTA can be dissolved only in correct pH.

*EGTA stock solution (0.25 M, 1000 ml)*

EGTA 95 g

pH of the solution was adjusted to 8.0 with KOH; EGTA can be dissolved only in correct pH.

*EMSA Solutions*

<u>PAA gel for the preparation of radioactive DNA probes (50 ml, 12%)</u>

$ddH_2O$ 30 ml

30% acryl-bisacrylamide solution 15 ml

10x TBE buffer 5 ml

10% APS 300 µl

| | |
|---|---|
| TEMED | 60 µl |

Gel polymerization takes approx. 1 hour.

EMSA gel (6%, 100 ml)

| | |
|---|---|
| dd $H_2O$ | 70 ml |
| 30% acryl-bisacrylamide solution | 20 ml |
| 10x TBE buffer | 10 ml |
| 10% APS | 500 µl |
| TEMED | 50 µl |

EMSA binding buffer (3x, 50 ml)

| | |
|---|---|
| 1M Hepes/KOH (pH 7.9) | 3 ml |
| 1 M KCl | 7.5 ml |
| 0.5 M $Na_2EDTA{\cdot}2H_2O$ (pH 8.0) | 300 µl |
| 1 M DTT | 150 µl |
| Ficoll | 6 g |

Aliquotes were stored at -20°C and thawed before use on ice.

EMSA running buffer (1x, 1000 ml)

| | |
|---|---|
| TBE (10x) | 100 ml |

EMSA stop buffer

| | |
|---|---|
| SDS | 0.5 % |
| EDTA | 20 mM |
| Bromophenol blue | |

*Ethidium bromide stock solution (100 ml)*

| | |
|---|---|
| EtBr | 1 g |

The solution was stored at 4°C in a dark bottle.

*FACS buffer (stored at 4°C)*

| | |
|---|---|
| 10x PBS, pH 7.4 | 50 ml |
| Cell culture grade $H_20$ | 450 ml |
| 1.0 M Sodium azide | 0.5 ml |

2.5 g of BSA was layered on top of the liquid mixture, dissolved at RT without stirring, sterile filtered the mixture and stored at 4°C.

*Gel loading sample buffer, 6x (MBI Fermentas)*

| | |
|---|---|
| Glycerine | 60% |
| EDTA | 60 mM |
| Bromophenol blue | 0.09% |
| Xylene Cyanol FF | 0.09% |

*2x HBS*

| | |
|---|---|
| Hepes/KOH (pH 7.05) | 50 mM |
| KCl | 10 mM |
| Dextrose | 12 mM |
| NaCl | 280 mM |
| $Na_2HPO_4$ | 1.5 mM |

The solution was sterile filtered through 0.45 µ filter, aliquoted and stored at -20°C.

*HEPES/ KOH stock solution (1 M, 1000 ml)*

| | |
|---|---|
| HEPES | 238.33 g |

pH of the solution was adjusted to 7.2/ 7.4/ 7.9 with KOH.

*Luciferase harvesting buffer (50 ml)*

| | |
|---|---|
| 1.5 M Tris/HCl (pH 7.8) | 1.7 ml |
| 1 M MES | 2.5 ml |
| Triton X-100 | 50 µl |

The solution was freshly prepared and 50 µl DTT stock solution (1M) was added just before use.

*Luciferase assay buffer (50 ml)*

| | |
|---|---|
| 1.5 M Tris/HCl (pH 7.8) | 4.17 ml |
| 1 M MES | 6.25 ml |
| 1 M $Mg(C_2H_3O_2)_2.4H_2O$ | 1.25 ml |

The solution was freshly prepared and supplemented with very little ATP just before use.

*Luciferin solution for luciferase assay (100 ml)*

| | |
|---|---|
| Luciferin | 28 mg |
| 1 M $KHPO_4$ (pH 7.8) | 0.5 ml |

The solution was aliquoted and stored at -20°C.

*Metrizamide solution*

| | |
|---|---|
| Metrizamide | 14.5 g |
| Dendritic cell medium | 100 ml |

The solution was sterile filtered by passing through 20 µ filter, aliquoted and stored at -20°C.

*Northern blot agarose gel solution*

| | |
|---|---|
| Agarose | 1.5 g |
| $H_20$ | 70 ml |
| 10x MOPS | 10 ml |
| Formaldehyde | 18 ml |

*Northern blot pre-pre hybridization solution* 2x SSC +1% SDS

*Northern blot pre-hybridization solution*

ULTRAhyb™ (Ambion): ULTRAhyb contains 50% formamide. Exact composition of this commercial hybridization solution is not disclosed. Ambion claims that ULTRAhyb contains a unique blend of hybridization accelerators and blocking agents that greatly enhance the levels of hybridization so that signals that once took days to visualize become apparent in hours.

*Northern blot denaturation solution* 0.01 N NaOH + 3M NaCl

*Northern blot washing solution I (RT)* 2x SSC + 0.1% SDS

*Northern blot washing solution II* (68°C) 0.2x SSC + 0.1% SDS

*Nuclear and cytoplasmic extract preparation buffers*

Extraction buffer A (Hypotonic, 1000 ml)

| | |
|---|---|
| 1 M Hepes/KOH (pH 7.9) | 10 ml |
| 1 M KCl | 10 ml |
| 0.5 M $Na_2EDTA{\cdot}2H_2O$ (pH 8.0) | 200 µl |
| 0.25 M EGTA (pH 8.0) | 400 µl |

Solution was stored at 4°C. For 10 ml Buffer A, following inhibitors were added before experiment: 10 µl DTT stock solution (1M) and 50 µl AEBSF (0.2 M).

Extraction buffer C (High salt, 1000 ml)

| | |
|---|---|
| 1 M Hepes/KOH (pH 7.9) | 20 ml |
| 1 M KCl | 400 ml |
| 0.5 M $Na_2EDTA.2H_2O$ (pH 8.0) | 2 ml |
| 0.25 M EGTA (pH 8.0) | 4 ml |

Solution was stored at 4°C. For 10 ml Buffer C, the following inhibitors were added before experiment: 10 µl DTT-stock solution (1M), protease inhibitors [100 µl AEBSF (0.2 M), 10 µl leupeptin (2 mM) and 10 µl aprotinin (0.3 M)].

*PBS (10x, 1000 ml)*

| | |
|---|---|
| NaCl | 80 g |
| KCl | 2 g |
| $CaCl_2$ | 1 g |
| $MgCl_2$ | 1 g |
| $Na_2HPO_4{\cdot}7H_2O$ | 26.8 g |
| $KH_2PO_4$ | 2.4 g |

pH of the solution was adjusted to 7.4 with 1 N HCl.

*Phosphatase inhibitor (stock solution, final working concentration is indicated)*

Sodium Orthovanadate [$Na_3VO_4$ stock solution (0.2 M): 4 mg/ml in $H_2O$]:1 mM

pH of the solution was adjusted to 10.0 with 1 N NaOH or 1 N HCl (solution becomes yellow), boiled for 10 min at 100°C (solution becomes colorless), cooled to RT and subsequently pH was again adjusted to 10.0; this was repeated till solution becomes colorless at RT and pH gets stabilized at 10.0. Aliquots were stored at -20°C and just before use boiled for 5 min at 100°C and left at RT to cool down.

*Ponceau red solution (100 ml)*

| | |
|---|---|
| Ponceau red | 2.0 g |
| Trichloroacetic acid | 30.0 g |
| Sulfosalicylic acid | 30.0 g |

*Potassium chloride stock solution (1 M, 1000 ml)*

| | |
|---|---|
| KCl | 74.6 g |

*Potassium hydrogen phosphate stock solution (1 M, 1000 ml)*

| | |
|---|---|
| $KHPO_4$ | 135.1 g |

pH of the solution was adjusted to 7.8 with KOH.

*Potassium phosphate buffer (0.2 M, 1000 ml)*

| | |
|---|---|
| $KH_2PO_4$ | 27.2 g |

pH was adjusted to 7.0 with 1 M KOH.

*Protease inhibitors (final working concentration is indicated)*

| | |
|---|---|
| Aprotinin [Stock solution (0.3 M): 2 mg/ml $H_2O$] | 0.3 µM |
| Leupeptin [Stock solution (2 mM): 1 mg/ml $H_2O$] | 2 µM |
| AEBSF [Stock solution (0.2 M): 50 mg/ml $H_2O$] | 1 mM |

*RNA loading buffer (5x, 10 ml, stored at 4°C)*

| | |
|---|---|
| Saturated bromophenol blue solution | 16 µl |
| 500 mM EDTA, pH 8.0 | 80 µl |
| 37% (12.3 M) formaldehyde | 720 µl |
| 100% glycerol | 2 ml |
| Formamide | 3.084 ml |
| 10x RNA gel buffer | 4 ml |

*RNA gel buffer (10X, Stored in dark bottle at RT, 1000 ml)*

| | |
|---|---|
| MOPS (0.2M) | 41.85 g |
| NaOAc (0.051M) | 6.8 g |
| EDTA (0.01M) | 2.92 g |
| Adjust pH to 7.0 with NaOH (~15 ml) | |

*RNA gel running buffer (1x, 1000 ml in DEPC - treated $H_2O$)*

| | |
|---|---|
| 10x RNA gel buffer | 100 ml |
| 37% (12.3 M) formaldehyde | 20 ml |

*Saponin buffer (stored at 4°C)*

| | |
|---|---|
| Saponin | 1 ml |
| FCS | 10 ml |
| Na azide | 100 µl |
| PBS | 989 ml |

*SDS stock solution (10%, 1000 ml)*

| | |
|---|---|
| SDS | 100 g |

The solution was warmed to 70°C to dissolve and pH was set to 7.2 with 1 N HCl.

*SDS-PAGE sample buffer (4 x, 100 ml)*

| | |
|---|---|
| 1.5 M Tris/HCl (pH 6.8) | 20 ml |
| SDS | 2.4 g |
| Glycerine (87 %) | 50 ml |
| ß-mercaptoethanol | 25 ml |
| Bromphenol blue | 0.04% |

The solution was warmed to 70°C and stored at -20°C.

*SDS-PAGE running buffer (10x, 1000 ml)*

| | |
|---|---|
| Tris | 30.3 g |
| Glycin | 144.1 g |
| 10% SDS | 100 ml |

pH of the solution was adjusted to 8.5 with 1 N HCl.

*Sodium acetate stock solution (3 M, 1000 ml)*

| | |
|---|---|
| $CH_3COONa \cdot 3H_2O$ | 408.24 g |

pH of the solution was adjusted to 5.2 with concentrated acetic acid.

*Sodium chloride stock solution (5 M, 1000 ml)*

| | |
|---|---|
| NaCl | 292.22 g |

The solution was dissolved by heating to 60°C.

*Sodium hydroxide stock solution (10 M, 1000 ml)*

| | |
|---|---|
| NaOH | 400 g |

*Sodium phosphate buffer (0.2 M, 1000 ml)*

| | |
|---|---|
| $NaH_2PO_4 \cdot H_2O$ | 27.6 g |

*Stripping buffer for nitrocellulose membrane (1000 ml)*

| | |
|---|---|
| 1.5 M Tris/HCl (pH 6.8) | 41.7 ml |
| 10% SDS | 200 ml |

Before use, 100 ml buffer was supplemented with 700 µl ß-mercaptoethanol.

*TAE buffer (Tris/Acetate/EDTA, 50x, 1000 ml)*

| | |
|---|---|
| Tris | 242 g |
| 0.5 M $Na_2EDTA \cdot 2H_2O$ (pH 8.0) | 100 ml |
| Concentrated acetic acid | 57.1 ml |

*TBE buffer (Tris/Borate/EDTA, 10x, 1000 ml)*

| | |
|---|---|
| Tris | 108 g |
| Boric Acid | 55 g |
| 0.5 M $Na_2EDTA \cdot 2H_2O$ (pH 8.0) | 40 ml |

*TBS (20x, 1000 ml)*

| | |
|---|---|
| Tris | 121.0 g |
| NaCl | 175.2 g |
| KCl | 7.5 g |

pH of the solution was adjusted to 7.6 with 1M HCl (~ 10.2 ml).

*TBS/Tween (TBS-T, 1x, 1000 ml)*

| | |
|---|---|
| TBS (20x) | 50 ml |
| Tween 20 | 1 ml |

*TE buffer (Tris/EDTA, pH 8.0, 1000 ml)*

| | |
|---|---|
| 1.5 M Tris/HCl (pH 8.0) | 6.7 ml |
| 0.5 M EDTA (pH 8.0) | 0.2 ml |

*Transfer buffer for Western blot (1000 ml)*

| | |
|---|---|
| Glycine | 2.9 g |
| Tris | 5.8 g |
| 10% SDS | 3.7 ml |
| Methanol | 200 ml |

*Tris/ HCl stock solution (1.5 M, 1000 ml)*

| | |
|---|---|
| Tris | 181.7 g |

pH of the solution was adjusted to 6.8/ 7.5/ 7.8/ 8.0/ 8.8 with 1 N HCl.

*Whole cell extract preparation buffer (Kyriakis lysis buffer modified, 1000 ml)*

| | |
|---|---|
| 1 M Hepes/KOH (pH 7.4) | 20 ml |
| 0.25 M EGTA (pH 8.0) | 8 ml |
| NaF | 2.1 g |
| ß-Glycerophosphate | 10.8 g |
| Glycerine (87%) | 115 ml |
| Triton X-100 | 10 ml |
| $NaN_3$-solution (10%) | 4 ml |

The solution was stored at 4°C. For 10 ml $KLBM^+$ buffer, the following inhibitors were added before the experiment: 10 µl DTT-stock solution (1M), protease inhibitors [50 µl AEBSF (0.2 M), 10 µl leupeptin (2 mM) and 10 µl aprotinin (0.3 M)] and phosphatase inhibitor [50 µl Sodium orthovanadate (0.2 M)].

## 5.1.5 Growth Medium

### *5.1.5.1 Mammalian cell culture media*

*Suspension Cell line (Jurkat, EL-4, DOHH-2, and other lymphoid cells)*

| | |
|---|---|
| RPMI 1640 | 1000 ml |
| FCS (Jurkat, EL-4) | 5 % |
| FCS (DOHH2, L428, KMH2, L540, L591, L1236) | 10 % |
| L-Glutamin (200 mM) | 10 ml |
| Penicillin (10.000 IU/ ml), Streptomycinsulphate (10 mg/ ml) | 6 ml |
| β-Mercaptoethanol (50 mM) | 1 ml |

*Adherent cell line (293T)*

| | |
|---|---|
| DMEM | 500 ml |
| FCS | 10% |
| L-Glutamine (200 mM) | 5 ml |
| Penicillin (10,000 IU/ ml), Streptomycinsulphate (10 mg/ ml) | 3 ml |
| β-Mercaptoethanol (50 mM) | 500 µl |
| Sodium pyruvate (100 mM) | 5 ml |
| HEPES (1 M) | 5 ml |

## 5.2 Methodology

### 5.2.1 Mammalian Cell Culture

#### *5.2.1.1 Maintenance of cell lines*

All cell lines were handled under highly sterile condition in a laminar hood and cultivated in appropriate media supplemented with antibiotics. Cells were incubated at 37°C, 5 % $CO_2$ in humidified incubator. Suspension cell lines like EL-4 and Jurkat T cells were split every 3-4 days just by refreshing with complete RPMI medium to a small aliquot of cell suspension.

#### *5.2.1.2 Induction of cells*

The T lymphocytes were induced either by plate bound αCD3 Ab plus soluble αCD28 Ab or pharmacological agents TPA and ionomycin that activate Ras/Protein Kinase and calcium-dependent pathways, respectively, to mimic signal through the TCR.

TPA :20 ng/ml for stimulation of a stable cell line, 10 ng/ml for primary cells and EL-4 cells

Ionomycin :1 μM for stimulation of a stable cell line, 0.5 μM for primary cells and EL-4 cells

#### *5.2.1.3 Isolation of PBMC from human blood.*

50–60 ml of blood from normal healthy donors were collected in the presence of anticoagulant. The blood was diluted with equal volumes of PBS. An equal volume of diluted blood was overlaid on Ficoll-Paque-plus in a 1:1 ratio and centrifuged at 400 x g for 20–30 min at 18–20 °C. The leukocyte population was collected from the interface and washed with PBS several times to remove plasma and Ficoll. About 50 million washed cells in 10 ml of RPMI medium containing 10% FCS were plated in 100-mm culture dishes to allow monocytes to adhere on the surface of the dish for 2–3 h. The non-adherent cells (mainly lymphocyte population) were removed, washed with fresh medium, cultured in RPMI medium, and labeled as PBLC. Attached monocytes were washed twice with warm RPMI medium containing 10% FCS and allowed to remain in the dish overnight at 37 °C in 5% CO2. During this period the monocytes detach from the dish. They were collected and washed in fresh RPMI medium and labeled as PBMC.

## 5.2.2 DNA Methods

#### *5.2.2.1.1 Electrophoresis of DNA on agarose gels*

Double stranded DNA fragments with lengths between 0.1 kb and 10 kb can be separated according to their lengths on agarose gels. Agarose is added to 1x TAE to obtain a final concentration between 0.3-2%. Boil the suspension in the microwave until the agarose is completely solubilised. Allow the agarose to cool down to around 50°C before adding ethidium bromide up to 0.5 μg/ml and pour into the gel apparatus. Add DNA gel loading buffer to the DNA sample and apply on the gel. Electrophorese in 1x TAE buffer at 85 mA. The DNA can be visualised under UV-light.

#### *5.2.2.1.2 Isolation of DNA from agarose (QIAEX II agarose gel extraction protocol)*

This protocol is designed for the extraction of 40-bp to 50-kbp DNA fragments from 0.3-2% standard agarose gels in TAE or TBE buffer. DNA molecules are adsorbed to QIAEX II silica particles in the presence of high salt. All non-nucleic acid impurities such as agarose, proteins, salts, and ethidium bromide are removed during washing steps.

Excise the desired DNA band from the agarose gel under the UV light. Weigh the gel slice and add 3 volumes of Buffer QG to 1 volume of gel for DNA fragments 100-bp-4 kbp; for DNA fragments more than 4 kbp, add 2 volume of QG plus 2 volumes of $H_2O$. Resuspend QIAEA II by vortexing for 30 sec; add 10 μl (or 30 μl) of QIAEX II to the sample containing not more than 2 μg of DNA (between 2-10 μg). Incubate at 50°C for 10 min to solubilise the agarose and bind the DNA. Mix by vortexing every 2 min to keep QIAEX II in suspension. Centrifuge the sample for 30 sec and carefully remove supernatant with a pipette. Wash the pellet with 500 ml of Buffer QG and then twice with Buffer PE. Air-dry the pellet and elute the DNA in 10 mM Tris-HCl or $H_2O$ and resuspend the pellet by vortexing. Incubate at RT for 5 min (or at 50°C for 5 min) for DNA fragments not more than 4 kbp (for DNA fragments between 4-10 kb). Centrifuge for 30 sec and carefully pipette supernatant into a clean tube.

#### *5.2.2.1.3 Purification of DNA (QIAquick PCR purification kit)*

This protocol is designed to purify single- or double-stranded PCR products or DNA plasmids ranging from 100 bp to 10 kbp. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. The impurities are washed away and pure DNA is eluted with Tris buffer or $H_2O$.

Add 5 volume of buffer PB to 1 volume of the contaminants and mix. Place a QIAquick spin column in a 2 ml collection tube. Apply the mixed sample to the QIAquick column and centrifuge 30-60 sec. Discard flow-through and place QIAquick column back into the same collection tube. Add 0.75 ml washing buffer PE to column and centrifuge for 30-60 sec. Discard flow-through and place QIAquick column back into the same collection tube. Centrifuge column for an additional 1 min at maximum speed. Place QIAquick column in a clean 1.5 ml microfuge tube. Add 50 μl elution buffer EB or $H_2O$ to the centre of the QIAquick column and centrifuge for 1 min. Store the purified DNA at -20°C.

#### *5.2.2.1.4 Sodium bisulfite treatment of DNA*

Sodium bisulfite treatment was carried out essentially as described by (Clark et al., 1994) with minor modifications. Genomic DNA (10 mg) was denatured with 0.3 M NaOH and treated with 3.6 M sodium bisulfite (pH 5.0) at 55°C for 5-10 h. Bisulfite-treated DNA was purified by series of precipitations. Purified DNA samples were desulfonated with 0.3 M NaOH at room temperature, neutralized with ammonium acetate, ethanol precipitated, and resuspended in 30 μl of Tris-EDTA buffer.

### 5.2.3 RNA Methods

#### *5.2.3.1 RNA isolation from mammalian cells*

RNA isolation was done in a clean, ribonuclease-free environment. All glasswares were baked overnight at 180°C. Double distilled water and all solutions except Tris-containing solutions were treated with 0.1% DEPC solution, followed by autoclaving. Disposable plastic wares were used. Chloroform resistant plastic wares were rinsed in chloroform. Cuvette was washed every time with 0.1M NaOH and 1 mM EDTA, followed by washing with RNase-free water. Disposable gloves were worn all times during the experiment. Total

cellular RNA was isolated using TRIZOL reagent and the procedure was followed according to the instructions of manufacturer.

### *5.2.3.2 RNA electrophoresis in formaldehyde agarose gels*

Electrophoresis tank was cleaned with detergent solution (0.5% SDS), thoroughly rinsed with RNase-free water followed by ethanol and allowed to dry. The edges of the gel-tray were sealed with tape and an appropriate comb cleaned with 70% ethanol was placed. For RNA gel preparation, 1.2 – 1.5 g agarose was dissolved in 70 ml of DEPC - treated water by heating in a micro oven and cooled to approx. 60°C. RNA gel was prepared, by pouring agarose fluid in the gel tray and allowed to polymerize at RT. In the meanwhile, one volume of 5x RNA loading buffer was mixed with 4 volumes of RNA sample, briefly spinned down, incubated for 3-5 minutes at 65°C and chilled on ice. Samples were loaded into the wells of the polymerized agarose gel and electrophoresed at 5-7 V/cm in 1x RNA gel running buffer.

### *5.2.3.3 Ribonuclease protection assay*

The method can be used to quantitate RNAs, to map the positions of introns, and to identify the locations of 5´ and 3´ ends of mRNAs on cloned DNA templates. Preparations of RNA containing an mRNA of interest were hybridized to a radiolabeled single-stranded RNA probe. At the end of the reaction, a mixture of RNase A and RNase T1 was used to degrade unhybridized regions of the probe, and the surviving molecules are then separated by denaturing gel electrophoresis and visualized by autoradiography.

***Probe synthesis:*** Before the start of probe synthesis, the heating block was set at 37°C and the following reagents were brought to RT: α-$^{32}$P-UTP, GACU nucleotide pool, DTT, 5X transcription buffer and RPA template set. For each probe synthesis, following reagents from the kit were added (in order) to a 1.5 ml eppendorf tube, mixed by gentle pipetting or flicking, quickly spinned in a microfuge and incubated at 37°C for 1 hour:

1 µl RNasin®
1 µl GACU pool
2 µl DTT
4 µl 5X transcription buffer
1 µl RPA template set
5 µl [α-$^{32}$P]UTP
1 µl T7 RNA polymerase

The reaction was terminated by adding 2 µl of DNase, mixed by gentle flicking, quickly spinned in a microfuge and incubated at 37°C for 30 minutes. For separation of probes,

reaction of each sample mixed with the loading dye was directly loaded on the 6% polyacrylamide gel which was run at 40 mA until the first marker line reaches a marked line, usually 12 cm from the wells. The glass plates were removed carefully. The gel was covered with plastic foil and exposed for 1-2 min to X-ray film at RT. An autoradiogram band, corresponding to specific probe band was cut out with a sterile needle. The gel slice was trasferred to a new 1.5 ml eppendorf tube and homogenized with a paster pipette. 300 µl of 1M ammonium acetate was added and incubated for 1 hour at 60°C then spinned in a microfuge for 2 minutes at RT and transferred the upper aqueous phase to a new 1.5 ml tube. To precipitate RNA, 900 µl ice cold 100% ethanol and 10 µg of yeast tRNA were added, mixed by inverting the tube(s), incubated for 2-3 minutes on dry ice (or 30 minutes at -70°C) and centrifuged for 20 minutes at RT (or 15 minutes at 4°C). The supernatant was removed carefully and the pellet was dried for 5 to 10 minutes at RT. The RNA pellet was solubilized in 50-100 µl of hybridization buffer. 1 µl was taken for quantification in the scintillation counter. Usually maximum yield was expected in the range of 1-3 x $10^6$ Cherenkov counts/µl (measurement of cpm/µl without the presence of scintillation fluid) with an acceptable lower limit of 3 x 105 Cherenkov counts/µl. After quantification, this 1 µl probe was used as undigested probe upon dilution to 1:50 in the blue buffer. The probe was stored for no longer than 2-3 days at -20°C.

***RNA hybridization:*** The following things were arranged before the start of hybridization: heating block at 90°C, precipitated RNA of desired amount and hybridization oven at 56°C. For hybridization, 5 µg of target RNA was mixed with 10 µl of diluted probe in a 1.5 ml tube. The RNA was solubilized by gentle vortexing for 3-4 minutes followed by a quick spin in the microfuge. Samples were placed in a heating block pre-warmed to 90°C for 5-10 minutes (up to 30 minutes). The tubes were transferred to hybridization oven set at 56°C and incubated for 12-16 hours.

***RNase treatment:*** The heating block was turned to 30°C for 15 minutes prior to the RNase treatment. For 20 samples, RNase cocktail was prepared by mixing 2.5 ml RNase buffer and 6 µl of RNase A + T1 mix. Overnight samples were mixed with 100 µl of RNase cocktail, briefly centrifuged, and incubated for at least 45 minutes at 30°C.

***Proteinase K treatment:*** The heating block was turned to 37°C. For 20 samples, proteinase K cocktail was prepared by mixing 390 µl of proteinase K buffer, 30 µl of

proteinase K and 30 µl of yeast tRNA. After RNase treatment, 18 µl of proteinase K cocktail was added, mixed and incubated for 30 minutes at 37°C.

***Precipitation and electrophoresis of RNA:*** For the precipitation of dsRNA, 100 µl of ammonium acetate and 600 µl of isopropanol were mixed to each sample, incubated for 15 minutes at -20°C and centrifuged at RT for 15-30 minutes. The supernatant was removed and the pellet was dried at 95°C for 2-3 minutes. The pellet was dissolved in 4 µl loading buffer, denatured at 90°C for 3 minutes and transferred on ice. In the meanwhile, gel solution was prepared (for composition see 5.1.5). The polymerized gel was pre-run at 30 W for 1 hour in 1x TBE. Samples were loaded on the gel along with the undigested probe. After the blue dye reached 2/3$^{rd}$ of the length of gel, electrophoresis was stopped, the gel was dried in vacuum for 1 hour at 95°C and the autoradiogram was established by exposing the gel for 24-48 hours to X-ray film with an intensifying screen at -70°C.

## 5.2.4 Protein Methods

### *5.2.4.1 Preparation of protein extracts*

***Preparation of whole protein extract from mammalian cells:*** Cells were centrifuged (1200 rpm, 5 minutes, 4°C), washed with cold PBS (Without $Ca^{++}$ and $Mg^{++}$), resuspended in 1 ml of PBS (without Ca/Mg), transferred to 1.5 ml tubes and again pelleted (2000 rpm, 2 minutes, RT). The cell pellet was resuspended in cold whole cell extract preparation buffer supplemented with protease and phosphatase inhibitors ($KLBM^{+}$) (100 µl per 1 x $10^7$ cells). Cells were disrupted by two times freezing and thawing on dry ice or passing the cell suspension through 26G needle 10 times and incubated for further 10 minutes. The cell suspension was centrifuged for 30 minutes at 14,000 rpm, 4°C, and supernatant was saved as whole cell extract, which was stored for future use at -70°C. The protein concentration of the supernatant was determined by Bio-Rad protein assay.

***Preparation of nuclear and cytoplasmic protein extracts from mammalian cells:*** Cells were centrifuged at 1200 rpm for 5 min at RT. The cell pellet was resuspended in 1 ml cold PBS buffer (without $Ca^{++}$ and $Mg^{++}$), transferred to a 1.5 ml tube and again centrifuged at 2000 rpm for 2 min at RT to remove the supernatant. The pellet was resuspended in 200 µl to 1 ml of extraction buffer $A^{+}$ (100 µl per 1x$10^7$ cells) and incubated for 20-30 min at 4°C. Extraction buffer $A^{+}$ is a low salt buffer ($^{+}$ indicates that DTT and PMSF were added to buffer A), which allowed the cells to swell. To destroy the swollen cells, the solution was

passed 10 times through 1 ml syringe with 26G needle and centrifuged at 7,000 rpm for 2 minutes in the cold room. The supernatant contained cytosolic fraction and the pellet, which appeared transparent, contained nuclear fraction. The supernatant was transferred to a fresh tube and kept on ice. The pellet was washed with 800 µl extraction buffer $A^+$, centrifuged at 7,000 rpm for 2 minutes in the cold room and the pellet was resuspended in extraction buffer $C^+$ (leupeptin was added in addition to DTT and PMSF) by pipetting and vigorously mixing with brutal force, followed by vortexing the nuclear extract vigorously for 30 minutes in the cold room. Now, the suspension was centrifuged at 14,000 rpm for 30 minutes in the cold room and supernatant containing nuclear proteins was frozen in –70°C. The protein concentration of the supernatant was determined by Bio-Rad protein assay.

***Measurement of protein concentration (Bio-Rad protein assay):*** The Bio-Rad protein assay is based on the observation that when Coomassie Brilliant Blue G-250 binds to the protein, the absorbency maximum shifts from 450 nm to 595 nm. Equal volumes of cell lysate containing 1 - 20 µg of protein was added to 1 ml of diluted dye reagent (1:5 dilution of dye reagent concentrate in $ddH_2O$), mixed well and O.D value was measured at 595 nm. KLBM buffer was always included in the control as following: 2 µl of KLBM buffer + 998 µl of Bradford reagent. The O.D value of the sample was divided by 0.178 to determine the protein concentration.

### *5.2.4.2 Immunodetection*

***SDS-polyacrylamide gel preparation and electrophoresis:*** SDS-polyacrylamide gels were prepared in 8 cm x 10 cm x 1.5 mm mini gel format according to the standard Laemmli method (Laemmli, 1970). Separating or lower gel mix was prepared according to the volume required, poured in the gel apparatus, overlaid gently with 0.1 % SDS and allowed to polymerize at room temperature.

Table 5.2: Composition of protein gels (all numerical figures are in ml)

| | **Stacking gel, pH 6.8** | **Separating gel, pH 8.8** | | | |
|---|---|---|---|---|---|
| **Percentage of the gel** | **4%** | **8%** | **10%** | **12%** | **15%** |
| Distilled water | 6.8 | 5.8 | 5.0 | 4.1 | 2.85 |
| 1.5 M Tris-HCl, pH 8.8 | - | 3.125 | 3.125 | 3.125 | 3.125 |
| 1.5 M Tris-HCl, pH 6.8 | 1.25 | - | - | - | - |
| Acryl-/ Bisacrylamide (29% / 1% w/v) | 1.7 | 3.35 | 4.15 | 5.0 | 6.25 |

| | | | | | |
|---|---|---|---|---|---|
| 10% (w/v) SDS | 0.1 | 0.125 | 0.125 | 0.125 | 0.125 |
| 10% APS | 0.1 | 0.125 | 0.125 | 0.125 | 0.125 |
| TEMED | 0.01 | 0.01 | 0.005 | 0.005 | 0.005 |
| Total Volume | 10.0 | 12.5 | 12.5 | 12.5 | 12.5 |

After the separating gel was polymerized, the overlay was decanted and gently washed with distilled water. The stacking gel was poured, the comb was inserted and allowed to polymerize at RT. Requisite concentration of protein samples were mixed with 4x Laemmli buffer and denatured by heating at 95°C for 5 min, loaded in the wells of polymerized gel and electrophoresed at constant current, 25-30 mA per gel, in 1x SDS-PAGE running buffer.

***Western blotting and hybridization:*** The SDS-PAGE gel was electrotransferred onto nitrocellulose membrane at 40 mA overnight at 4°C. The air-dried membrane was incubated in a blocking solution (5% fat free milk in 1X TBS-T) for 30 minutes to 1 hour at RT. Membrane was directly incubated in primary antibody solution (1:2000 in blocking solution) for 2 to 3 hours at RT. After incubation, membrane was washed in 1X TBS-T for 1 x 20 minutes and 3 x 5 minutes each. Now, membrane was incubated in secondary antibody conjugate solution (1:2000 in blocking solution) for 1 to 2 hours at room temperature and washed in 1X TBS-T for 1 x 20 minutes and 3 x 5 minutes each. Colour was developed with ECL developing solution according to the instructions of the manufacturer (Amersham).

***Stripping off nitrocellulose membrane:*** Nitrocellulose membrane was stripped off the first antibody to detect the level of another protein by hybridization with another antibody. This was done by incubating the membrane in stripping buffer (pre-warmed to 60°C) and placing in a water bath set at 60°C for 30 min with shaking. Now the membrane was washed one time with dd$H_2O$ and three times with TBS/Tween for 5 min each. The membrane was ready for staining with another primary antibody.

## 5.2.5 DNA/Protein Interaction Assays

The Electrophoretic Mobility Shift Assay (EMSA) provides a simple and rapid method for detecting DNA-binding proteins *in vitro*. This method has widely been used in the study of sequence-specific DNA-binding proteins such as transcription factors. The assay is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The gel shift assay is performed by incubating a purified protein, or a complex mixture of proteins (such as nuclear or cell protein extract preparations), with a $^{32}P$ end-labeled DNA fragment containing the putative protein binding site. The reaction products are then analyzed on a non-denaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using unlabeled DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences.

### *5.2.5.1 Radioactive labeling and purification of DNA probe*

Oligonucleotides were dissolved in dd$H_2O$ to a final concentration, 100 pmol/µl. For each probe, 20 µl of sense and antisense oligonucleotides were mixed, vortexed, spinned them down briefly, and incubated in a thermoblock at 65°C until they reached this temperature. Now DNA was taken out from 65°C and let it cool down to RT for hybridization. Now the final volume obtained was 40 µl of 100 pmol/µl double stranded DNA. This was diluted to 20 ng/µl (for dsDNA of 25 nucleotides in length, 1pmol corresponds to 9ng, so 100 pmol/µl = 900 ng/µl and therefore dilution factor was 45x). All reactions were setup in tightly fitting screw cap tubes as following:

| | |
|---|---|
| dd $H_2O$ | 5.0 µl |
| 10x PNK buffer | 1.0 µl |
| ds-Oligo | 1.0 µl (40 ng) |
| $^{32}P$-γATP | 2.0 µl (40 mCi) |
| PNK | 1.0 µl |

The reaction mix was vortexed, spinned down and tubes were placed behind a radioactive shield for incubation at 37°C for 30 min (The incubation time of 30 minutes was strictly followed since the PNK enzyme has the property of removing phosphate group upon longer incubation). In the meanwhile, 12 % acrylamide gel was prepared for purification of labeled probe. After incubation, 5 µl of EMSA stop buffer was added to stop the reaction. Now the samples were loaded on the polymerized gel, run in 1x TBE at 20mA for 2 hours and the desired band was cut after exposing the gel to a film. The gel slice was mixed with 150 µl of 10 mM KCl and kept on rotation for O/N at 4°C. The radioactivity was measured

in 2 µl aliquot and the probe was diluted to 20,000 cpm / µl. If not used immediately, the radioactive probe was stored at -20°C for maximum of 3 weeks.

*5.2.5.2 Electrophoretic Mobility Shift Assay (EMSA)*

A 6 % polyacrylamide gel was poured and allowed to polymerize for 1 hour at RT. The polymerized gel was pre-run in 0.4x TBE at 20 mA (constant 200 V) till the power dropped down to 10A (usually for 2 hours) to let the salt run out off the gel. For an EMSA, nuclear proteins were used. The master mix for each sample was prepared as following:

| | |
|---|---|
| 3x binding buffer | 3.3 µl |
| poly dI/dC (1 µg/µl) | 0.7 µl |
| dd $H_2O$ minus nuclear extract and probe | up to 10.0 µl |

The master mix for all similar samples (i.e. same probe) was mixed with radioactive probe (~20,000 cpm for each sample), aliquoted into different tubes for different nuclear extracts, mixed with respective nuclear extracts (2.0 µg) and incubated for 20-30 min on ice. For adjusting the different concentrations of nuclear extracts to the final volume of 10 µl, the buffer C was used. For “Supershift assays”, 1 µl (1 µg/µl) of antibody solution against the transcription factor to be studied was added. An 100-fold excess of cold oligos were added for competition assays. For separation of complexes, 8 µl of each sample was directly loaded on the 6% polyacrylamide gel which was run at 220 V until the marker reaches a marked line, usually 13 cm from the wells. The glass plates were removed carefully and the gel was immersed in 10% acetic acid for 20 min to fix small fragments. A Whatman paper was placed on the gel and carefully separated from the glass plate. The gel was covered with plastic foil and dried for 60-120 min in a vacuum dryer. An autoradiogram was established by exposing the gel for 24 - 48 hours to X-ray film at -70°C with an intensifying screen.

**Chromatin Immunoprecipitation (ChIP)**

Chromatin is comprised of nucleosome subunits, each of which consists of DNA wound around eight histone proteins. Recent findings have revealed that large multiprotein complexes are often involved in regulatory processes taking place in the nucleus. The regulation of transcription in eukaryotic cells is critically dependent on the dynamic state of chromatin. Transcriptional activation and inactivation is intimately associated with either the relaxed or taut conformation of chromatin structure. To unravel the structure and dynamics of these nuclear protein complexes and determine their interactions with the

DNA template *in vivo*, many scientists use an assay known as the chromatin immunoprecipitation (ChIP) assay. The ChIP assay combines two straightforward steps — first, in vivo formaldehyde cross-linking of whole cells that freezes protein-protein and protein-DNA interactions, followed by immunoprecipitation of protein-DNA complexes with specific antibodies from sonicated extracts and PCR.

The cells ($2x10^5$/ml, 200ml) were grown in RPMI media and induced. Crosslinking was performed by adding 37% formaldehyd to the final concentration of 1% directly into the media and rocked for 5 min at room temperature. The cells were washed with ice-cold PBS/BSA and resuspended on ice for 30 min in 10 ml of swelling buffer, containing protease inhibitors, 1μg/ml leupeptin and 1mM PMSF. Following dounce homogenization, the nuclei were collected by 7x passing the cells through needle on ice and pelleted by centrifugation (2500 rpm for 5 min at 4°C, no brake). The nuclei were resuspended in 2 ml of sonification buffer, containing protease inhibitors as above and incubated on ice for 10 min. As an additional purification step we did three times freezing/thowing procedure on dry ice. The chromatin was sonicated to an average length of about 600 bp. Sonication conditions were: 0,5 sec/pulse max power, in 30 sec bursts followed by 1 min cooling on ice for a total sonication time of 2-2.5 min per sample. Debris was cleared by centrifugation at a max speed for 10 min at 4°C. The supernatant was transferred to a new tube. To reduce nonspecific backgroung, the sample was pre-cleared with 40-50 μl of a salmon sperm DNA/protein A agarose slurry and 20 μl rabbit antiserum for 2 hours at 4°C with rotation. The supernatant was divided into two fractions: one for a “no antibody control” and the second was immunoprecipitated with 3 μl antibodies overnight at 4°C. 30 A260 units of the pre-cleared chromatin we used for one immunoprecipitation. Immune complexes were collected with 50 μl of salmon sperm DNA/protein A agarose slurry for 1 hr 4°C with rotation. Then, beads were washed twice consecutively with 400 μl of each solution: sonification/ hight salt/ LiCl/ TE. Separate tips for each sample were used to avoid a contamination. The immunocomplexes were eluted with 250 μl of elution buffer for 15 min at 65°C with rotation. Beads were pelleted at a max speed for 3 min and supernatant was transferred to clean tubes. Elution step was repeated and both elutions were combined in the same tube.

Formaldehyde crosslinks were reversed by adding of 5 M NaCl to a final concentration of 0,3 M to the eluates and incubated 4-6 hours at 65°C with rotation.

After successive treatment with 1 μl of 20 μg/ml Proteinase K for one hour at 45°C, the samples were extracted with phenol-chloroform and precipitated with 1/10 V of AcONa pH 5.2 and 3 V of 100% ethanol at -20°C for 1 hour. Next, samples were centrifugated for 60 min at max speed and pellets were resuspended in 25-30 μl of Tris or water. 1 μl of the immunoprecipitated DNA and input DNA were analyzed by radiactive PCR using promoter specific primers. Amplifications were performed in the presence of $\alpha$-$^{32}$P dCTP, and PCR products were analyzed on a 5% polyacrylamide gel. To ensure that amounts of PCR products accurately reflected the amounts of template DNA, control PCR reactions (26,27,28,29,30,31 and 32 cycles) were performed.

# 6. REFERENCES


Aaltonen, L.A., Salovaara, R., Kristo, P., Canzian, F., Hemminki, A., et al.: Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med 338 (1998) 1481-7.

Afshar, G. and Murnane, J.P.: Characterization of a human gene with sequence homology to Saccharomyces cerevisiae SIR2. Gene 234 (1999) 161-8.

Ahluwalia, A., Hurteau, J.A., Bigsby, R.M. and Nephew, K.P.: DNA methylation in ovarian cancer. II. Expression of DNA methyltransferases in ovarian cancer cell lines and normal ovarian epithelial cells. Gynecol Oncol 82 (2001) 299-304.

Akagi, K., Suzuki, T., Stephens, R.M., Jenkins, N.A. and Copeland, N.G.: RTCGD: retroviral tagged cancer gene database. Nucleic Acids Res 32 Database issue (2004) D523-7.

Allfrey, V.G.: Structural modifications of histones and their possible role in the regulation of ribonucleic acid synthesis. Proc Can Cancer Conf 6 (1966) 313-35.

Antequera, F. and Bird, A.: Number of CpG islands and genes in human and mouse. Proc Natl Acad Sci U S A 90 (1993) 11995-9.

Avots, A., Buttmann, M., Chuvpilo, S., Escher, C., Smola, U., Bannister, A.J., Rapp, U.R., Kouzarides, T. and Serfling, E.: CBP/p300 integrates Raf/Rac-signaling pathways in the transcriptional induction of NF-ATc during T cell activation. Immunity 10 (1999) 515-24.

Baarends, W.M., Hoogerbrugge, J.W., Roest, H.P., Ooms, M., Vreeburg, J., Hoeijmakers, J.H. and Grootegoed, J.A.: Histone ubiquitination and chromatin remodeling in mouse spermatogenesis. Dev Biol 207 (1999) 322-33.

Bakin, A.V. and Curran, T.: Role of DNA 5-methylcytosine transferase in cell transformation by fos. Science 283 (1999) 387-90.

Bargou, R.C., Emmerich, F., Krappmann, D., Bommert, K., Mapara, M.Y., et al.: Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J Clin Invest 100 (1997) 2961-9.

Baumgartner, A.K., Zettl, A., Chott, A., Ott, G., Muller-Hermelink, H.K. and Starostik, P.: High frequency of genetic aberrations in enteropathy-type T-cell lymphoma. Lab Invest 83 (2003) 1509-16.

Baylin, S.B. and Herman, J.G.: DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet 16 (2000) 168-74.

Belinsky, S.A., Nikula, K.J., Baylin, S.B. and Issa, J.P.: Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. Proc Natl Acad Sci U S A 93 (1996) 4045-50.

Bell, A.C., West, A.G. and Felsenfeld, G.: The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. Cell 98 (1999) 387-96.

Bertos, N.R., Wang, A.H. and Yang, X.J.: Class II histone deacetylases: structure, function, and regulation. Biochem Cell Biol 79 (2001) 243-52.

Bestor, T., Laudano, A., Mattaliano, R. and Ingram, V.: Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. J Mol Biol 203 (1988) 971-83.

Bestor, T.H.: Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. Embo J 11 (1992) 2611-7.

Bestor, T.H. and Ingram, V.M.: Two DNA methyltransferases from murine erythroleukemia cells: purification, sequence specificity, and mode of interaction with DNA. Proc Natl Acad Sci U S A 80 (1983) 5559-63.

Bestor, T.H. and Verdine, G.L.: DNA methyltransferases. Curr Opin Cell Biol 6 (1994) 380-9.

Bird, A.: DNA methylation patterns and epigenetic memory. Genes Dev 16 (2002) 6-21.

Bird, A., Taggart, M., Frommer, M., Miller, O.J. and Macleod, D.: A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. Cell 40 (1985) 91-9.

Bird, A.P.: DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 8 (1980) 1499-504.

Bischoff, J.R. and Plowman, G.D.: The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. Trends Cell Biol 9 (1999) 454-9.

Bjerling, P., Silverstein, R.A., Thon, G., Caudy, A., Grewal, S. and Ekwall, K.: Functional divergence between histone deacetylases in fission yeast by distinct cellular localization and in vivo specificity. Mol Cell Biol 22 (2002) 2170-81.

Boeke, J., Ammerpohl, O., Kegel, S., Moehren, U. and Renkawitz, R.: The minimal repression domain of MBD2b overlaps with the methyl-CpG-binding domain and binds directly to Sin3A. J Biol Chem 275 (2000) 34963-7.

Boggs, B.A., Cheung, P., Heard, E., Spector, D.L., Chinault, A.C. and Allis, C.D.: Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes. Nat Genet 30 (2002) 73-6.

Bonfils, C., Beaulieu, N., Chan, E., Cotton-Montpetit, J. and MacLeod, A.R.: Characterization of the human DNA methyltransferase splice variant Dnmt1b. J Biol Chem 275 (2000) 10754-60.

Bonzheim, I., Geissinger, E., Roth, S., Zettl, A., Marx, A., Rosenwald, A., Muller-Hermelink, H.K. and Rudiger, T.: Anaplastic Large Cell Lymphomas lack the

expression of T-cell receptor molecules or molecules of proximal T-cell receptor signaling. Blood (2004).

Bourc'his, D. and Bestor, T.H.: Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature 431 (2004) 96-9.

Bouwman, P., Gollner, H., Elsasser, H.P., Eckhoff, G., Karis, A., Grosveld, F., Philipsen, S. and Suske, G.: Transcription factor Sp3 is essential for post-natal survival and late tooth development. Embo J 19 (2000) 655-61.

Brabletz, T., Pietrowski, I. and Serfling, E.: The immunosuppressives FK 506 and cyclosporin A inhibit the generation of protein factors binding to the two purine boxes of the interleukin 2 enhancer. Nucleic Acids Res 19 (1991) 61-7.

Brachmann, C.B., Sherman, J.M., Devine, S.E., Cameron, E.E., Pillus, L. and Boeke, J.D.: The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. Genes Dev 9 (1995) 2888-902.

Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A. and Cedar, H.: Sp1 elements protect a CpG island from de novo methylation. Nature 371 (1994) 435-8.

Briggs, S.D., Xiao, T., Sun, Z.W., Caldwell, J.A., Shabanowitz, J., Hunt, D.F., Allis, C.D. and Strahl, B.D.: Gene silencing: trans-histone regulatory pathway in chromatin. Nature 418 (2002) 498.

Bronner, C.E., Baker, S.M., Morrison, P.T., Warren, G., Smith, L.G., et al.: Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature 368 (1994) 258-61.

Byvoet, P.: In vivo turnover and distribution of radio-N-methyl in arginine-rich histones from rat tissues. Arch Biochem Biophys 152 (1972) 887-8.

Byvoet, P., Shepherd, G.R., Hardin, J.M. and Noland, B.J.: The distribution and turnover of labeled methyl groups in histone fractions of cultured mammalian cells. Arch Biochem Biophys 148 (1972) 558-67.

Cameron, E.E., Bachman, K.E., Myohanen, S., Herman, J.G. and Baylin, S.B.: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21 (1999) 103-7.

Cardoso, M.C. and Leonhardt, H.: DNA methyltransferase is actively retained in the cytoplasm during early development. J Cell Biol 147 (1999) 25-32.

Carlson, L.L., Page, A.W. and Bestor, T.H.: Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. Genes Dev 6 (1992) 2536-41.

Carrozza, M.J., Utley, R.T., Workman, J.L. and Cote, J.: The diverse functions of histone acetyltransferase complexes. Trends Genet 19 (2003) 321-9.

Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W. and Stallcup, M.R.: Regulation of transcription by a protein methyltransferase. Science 284 (1999) 2174-7.

Chen, R.Z., Pettersson, U., Beard, C., Jackson-Grusby, L. and Jaenisch, R.: DNA hypomethylation leads to elevated mutation rates. Nature 395 (1998) 89-93.

Chen, T., Ueda, Y., Xie, S. and Li, E.: A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. J Biol Chem 277 (2002) 38746-54.

Cheng, H.L., Mostoslavsky, R., Saito, S., Manis, J.P., Gu, Y., et al.: Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. Proc Natl Acad Sci U S A 100 (2003) 10794-9.

Cheung, P., Allis, C.D. and Sassone-Corsi, P.: Signaling to chromatin through histone modifications. Cell 103 (2000a) 263-71.

Cheung, P., Tanner, K.G., Cheung, W.L., Sassone-Corsi, P., Denu, J.M. and Allis, C.D.: Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. Mol Cell 5 (2000b) 905-15.

Chuang, L.S., Ian, H.I., Koh, T.W., Ng, H.H., Xu, G. and Li, B.F.: Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. Science 277 (1997) 1996-2000.

Chuang, L.S., Ng, H.H., Chia, J.N. and Li, B.F.: Characterisation of independent DNA and multiple Zn-binding domains at the N terminus of human DNA-(cytosine-5) methyltransferase: modulating the property of a DNA-binding domain by contiguous Zn-binding motifs. J Mol Biol 257 (1996) 935-48.

Chuvpilo, S., Jankevics, E., Tyrsin, D., Akimzhanov, A., Moroz, D., et al.: Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. Immunity 16 (2002a) 881-95.

Chuvpilo, S., Jankevics, E., Tyrsin, D., Akimzhanov, A., Moroz, D., et al.: Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. Immunity 16 (2002b) 881-95.

Chuvpilo, S., Zimmer, M., Kerstan, A., Glockner, J., Avots, A., et al.: Alternative polyadenylation events contribute to the induction of NF-ATc in effector T cells. Immunity 10 (1999) 261-9.

Clark, S.J., Harrison, J. and Frommer, M.: CpNpG methylation in mammalian cells. Nat Genet 10 (1995) 20-7.

Clark, S.J., Harrison, J. and Molloy, P.L.: Sp1 binding is inhibited by (m)Cp(m)CpG methylation. Gene 195 (1997) 67-71.

Clark, S.J., Harrison, J., Paul, C.L. and Frommer, M.: High sensitivity mapping of methylated cytosines. Nucleic Acids Res 22 (1994) 2990-7.

Clayton, A.L., Rose, S., Barratt, M.J. and Mahadevan, L.C.: Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation. Embo J 19 (2000) 3714-26.

Colot, V., Maloisel, L. and Rossignol, J.L.: Interchromosomal transfer of epigenetic states in Ascobolus: transfer of DNA methylation is mechanistically related to homologous recombination. Cell 86 (1996) 855-64.

Colot, V. and Rossignol, J.L.: Eukaryotic DNA methylation as an evolutionary device. Bioessays 21 (1999) 402-11.

Comb, M. and Goodman, H.M.: CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res 18 (1990) 3975-82.

Cooper, D.N. and Krawczak, M.: Cytosine methylation and the fate of CpG dinucleotides in vertebrate genomes. Hum Genet 83 (1989) 181-8.

Costello, J.F., Fruhwald, M.C., Smiraglia, D.J., Rush, L.J., Robertson, G.P., et al.: Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 24 (2000) 132-8.

Crabtree, G.R.: Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT. Cell 96 (1999) 611-4.

Cress, W.D. and Seto, E.: Histone deacetylases, transcriptional control, and cancer. J Cell Physiol 184 (2000) 1-16.

Cross, S.H., Meehan, R.R., Nan, X. and Bird, A.: A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. Nat Genet 16 (1997) 256-9.

Daniel, J.M. and Reynolds, A.B.: The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. Mol Cell Biol 19 (1999) 3614-23.

Datta, J., Ghoshal, K., Sharma, S.M., Tajima, S. and Jacob, S.T.: Biochemical fractionation reveals association of DNA methyltransferase (Dnmt) 3b with Dnmt1 and that of Dnmt 3a with a histone H3 methyltransferase and Hdac1. J Cell Biochem 88 (2003) 855-64.

Davie, J.R. and Murphy, L.C.: Inhibition of transcription selectively reduces the level of ubiquitinated histone H2B in chromatin. Biochem Biophys Res Commun 203 (1994) 344-50.

De Marzo, A.M., Marchi, V.L., Yang, E.S., Veeraswamy, R., Lin, X. and Nelson, W.G.: Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. Cancer Res 59 (1999) 3855-60.

de Ruijter, A.J., van Gennip, A.H., Caron, H.N., Kemp, S. and van Kuilenburg, A.B.: Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J 370 (2003) 737-49.

De Souza, C.P., Osmani, A.H., Wu, L.P., Spotts, J.L. and Osmani, S.A.: Mitotic histone H3 phosphorylation by the NIMA kinase in Aspergillus nidulans. Cell 102 (2000) 293-302.

Dennis, K., Fan, T., Geiman, T., Yan, Q. and Muegge, K.: Lsh, a member of the SNF2 family, is required for genome-wide methylation. Genes Dev 15 (2001) 2940-4.

Doherty, A.S., Bartolomei, M.S. and Schultz, R.M.: Regulation of stage-specific nuclear translocation of Dnmt1o during preimplantation mouse development. Dev Biol 242 (2002) 255-66.

Dover, J., Schneider, J., Tawiah-Boateng, M.A., Wood, A., Dean, K., Johnston, M. and Shilatifard, A.: Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. J Biol Chem 277 (2002) 28368-71.

Ehrenhofer-Murray, A.E.: Chromatin dynamics at DNA replication, transcription and repair. Eur J Biochem 271 (2004) 2335-49.

Ehrlich, M.: DNA methylation in cancer: too much, but also too little. Oncogene 21 (2002) 5400-13.

Ehrlich, M., Gama-Sosa, M.A., Huang, L.H., Midgett, R.M., Kuo, K.C., McCune, R.A. and Gehrke, C.: Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. Nucleic Acids Res 10 (1982) 2709-21.

Emmel, E.A., Verweij, C.L., Durand, D.B., Higgins, K.M., Lacy, E. and Crabtree, G.R.: Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. Science 246 (1989) 1617-20.

Falvo, J.V., Uglialoro, A.M., Brinkman, B.M., Merika, M., Parekh, B.S., et al.: Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter. Mol Cell Biol 20 (2000) 2239-47.

Fatemi, M., Hermann, A., Gowher, H. and Jeltsch, A.: Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. Eur J Biochem 269 (2002) 4981-4.

Fatemi, M., Hermann, A., Pradhan, S. and Jeltsch, A.: The activity of the murine DNA methyltransferase Dnmt1 is controlled by interaction of the catalytic domain with the N-terminal part of the enzyme leading to an allosteric activation of the enzyme after binding to methylated DNA. J Mol Biol 309 (2001) 1189-99.

Feinberg, A.P.: Alterations in DNA methylation in colorectal polyps and cancer. Prog Clin Biol Res 279 (1988) 309-17.

Feng, Q., Wang, H., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Struhl, K. and Zhang, Y.: Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. Curr Biol 12 (2002) 1052-8.

Fischle, W., Dequiedt, F., Hendzel, M.J., Guenther, M.G., Lazar, M.A., Voelter, W. and Verdin, E.: Enzymatic activity associated with class II HDACs is dependent on a

multiprotein complex containing HDAC3 and SMRT/N-CoR. Mol Cell 9 (2002) 45-57.

Fishel, R., Lescoe, M.K., Rao, M.R., Copeland, N.G., Jenkins, N.A., Garber, J., Kane, M. and Kolodner, R.: The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell 75 (1993) 1027-38.

Florl, A.R., Lower, R., Schmitz-Drager, B.J. and Schulz, W.A.: DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas. Br J Cancer 80 (1999) 1312-21.

Fournier, C., Goto, Y., Ballestar, E., Delaval, K., Hever, A.M., Esteller, M. and Feil, R.: Allele-specific histone lysine methylation marks regulatory regions at imprinted mouse genes. Embo J 21 (2002) 6560-70.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L. and Paul, C.L.: A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A 89 (1992) 1827-31.

Frye, R.A.: Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. Biochem Biophys Res Commun 260 (1999) 273-9.

Fujita, N., Shimotake, N., Ohki, I., Chiba, T., Saya, H., Shirakawa, M. and Nakao, M.: Mechanism of transcriptional regulation by methyl-CpG binding protein MBD1. Mol Cell Biol 20 (2000) 5107-18.

Fujita, N., Takebayashi, S., Okumura, K., Kudo, S., Chiba, T., Saya, H. and Nakao, M.: Methylation-mediated transcriptional silencing in euchromatin by methyl-CpG binding protein MBD1 isoforms. Mol Cell Biol 19 (1999) 6415-26.

Fuks, F., Burgers, W.A., Brehm, A., Hughes-Davies, L. and Kouzarides, T.: DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat Genet 24 (2000) 88-91.

Fuks, F., Hurd, P.J., Deplus, R. and Kouzarides, T.: The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. Nucleic Acids Res 31 (2003) 2305-12.

Gama-Sosa, M.A., Slagel, V.A., Trewyn, R.W., Oxenhandler, R., Kuo, K.C., Gehrke, C.W. and Ehrlich, M.: The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 11 (1983) 6883-94.

Giet, R. and Prigent, C.: Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. J Cell Sci 112 ( Pt 21) (1999) 3591-601.

Goto, H., Tomono, Y., Ajiro, K., Kosako, H., Fujita, M., et al.: Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation. J Biol Chem 274 (1999) 25543-9.

Grant, P.A.: A tale of histone modifications. Genome Biol 2 (2001) REVIEWS0003.

Grant, P.A., Duggan, L., Cote, J., Roberts, S.M., Brownell, J.E., et al.: Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes Dev 11 (1997) 1640-50.

Grozinger, C.M., Hassig, C.A. and Schreiber, S.L.: Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proc Natl Acad Sci U S A 96 (1999) 4868-73.

Gurley, L.R., D'Anna, J.A., Barham, S.S., Deaven, L.L. and Tobey, R.A.: Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. Eur J Biochem 84 (1978) 1-15.

Hans, F. and Dimitrov, S.: Histone H3 phosphorylation and cell division. Oncogene 20 (2001) 3021-7.

Hansen, R.S., Wijmenga, C., Luo, P., Stanek, A.M., Canfield, T.K., Weemaes, C.M. and Gartler, S.M.: The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. Proc Natl Acad Sci U S A 96 (1999) 14412-7.

Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M. and Tilghman, S.M.: CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature 405 (2000) 486-9.

Hata, K., Okano, M., Lei, H. and Li, E.: Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development 129 (2002) 1983-93.

Hendrich, B., Abbott, C., McQueen, H., Chambers, D., Cross, S. and Bird, A.: Genomic structure and chromosomal mapping of the murine and human Mbd1, Mbd2, Mbd3, and Mbd4 genes. Mamm Genome 10 (1999a) 906-12.

Hendrich, B. and Bird, A.: Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol Cell Biol 18 (1998) 6538-47.

Hendrich, B., Hardeland, U., Ng, H.H., Jiricny, J. and Bird, A.: The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites. Nature 401 (1999b) 301-4.

Hendzel, M.J., Wei, Y., Mancini, M.A., Van Hooser, A., Ranalli, T., Brinkley, B.R., Bazett-Jones, D.P. and Allis, C.D.: Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106 (1997) 348-60.

Henry, K.W., Wyce, A., Lo, W.S., Duggan, L.J., Emre, N.C., et al.: Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev 17 (2003) 2648-63.

Hermann, A., Schmitt, S. and Jeltsch, A.: The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity. J Biol Chem 278 (2003) 31717-21.

Hernandez, R., Frady, A., Zhang, X.Y., Varela, M. and Ehrlich, M.: Preferential induction of chromosome 1 multibranched figures and whole-arm deletions in a human pro-B cell line treated with 5-azacytidine or 5-azadeoxycytidine. Cytogenet Cell Genet 76 (1997) 196-201.

Hibi, K., Trink, B., Patturajan, M., Westra, W.H., Caballero, O.L., Hill, D.E., Ratovitski, E.A., Jen, J. and Sidransky, D.: AIS is an oncogene amplified in squamous cell carcinoma. Proc Natl Acad Sci U S A 97 (2000) 5462-7.

Hinz, M., Lemke, P., Anagnostopoulos, I., Hacker, C., Krappmann, D., et al.: Nuclear factor kappaB-dependent gene expression profiling of Hodgkin's disease tumor cells, pathogenetic significance, and link to constitutive signal transducer and activator of transcription 5a activity. J Exp Med 196 (2002) 605-17.

Hinz, M., Loser, P., Mathas, S., Krappmann, D., Dorken, B. and Scheidereit, C.: Constitutive NF-kappaB maintains high expression of a characteristic gene network, including CD40, CD86, and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. Blood 97 (2001) 2798-807.

Holler, M., Westin, G., Jiricny, J. and Schaffner, W.: Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev 2 (1988) 1127-35.

Howell, C.Y., Bestor, T.H., Ding, F., Latham, K.E., Mertineit, C., Trasler, J.M. and Chaillet, J.R.: Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. Cell 104 (2001) 829-38.

Hsieh, C.L.: In vivo activity of murine de novo methyltransferases, Dnmt3a and Dnmt3b. Mol Cell Biol 19 (1999) 8211-8.

Hsu, J.Y., Sun, Z.W., Li, X., Reuben, M., Tatchell, K., et al.: Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. Cell 102 (2000) 279-91.

Huang, E.Y., Zhang, J., Miska, E.A., Guenther, M.G., Kouzarides, T. and Lazar, M.A.: Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. Genes Dev 14 (2000) 45-54.

Huang, H., Kahana, A., Gottschling, D.E., Prakash, L. and Liebman, S.W.: The ubiquitin-conjugating enzyme Rad6 (Ubc2) is required for silencing in Saccharomyces cerevisiae. Mol Cell Biol 17 (1997) 6693-9.

Humphrey, G.W., Wang, Y., Russanova, V.R., Hirai, T., Qin, J., Nakatani, Y. and Howard, B.H.: Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. J Biol Chem 276 (2001) 6817-24.

Iguchi-Ariga, S.M. and Schaffner, W.: CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. Genes Dev 3 (1989) 612-9.

Iizuka, M. and Stillman, B.: Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. J Biol Chem 274 (1999) 23027-34.

Issa, J.P., Vertino, P.M., Wu, J., Sazawal, S., Celano, P., Nelkin, B.D., Hamilton, S.R. and Baylin, S.B.: Increased cytosine DNA-methyltransferase activity during colon cancer progression. J Natl Cancer Inst 85 (1993) 1235-40.

Jason, L.J., Moore, S.C., Lewis, J.D., Lindsey, G. and Ausio, J.: Histone ubiquitination: a tagging tail unfolds? Bioessays 24 (2002) 166-74.

Jass, J.R., Cottier, D.S., Jeevaratnam, P., Pokos, V., Holdaway, K.M., Bowden, M.L., Van de Water, N.S. and Browett, P.J.: Diagnostic use of microsatellite instability in hereditary non-polyposis colorectal cancer. Lancet 346 (1995) 1200-1.

Jeanpierre, M., Turleau, C., Aurias, A., Prieur, M., Ledeist, F., Fischer, A. and Viegas-Pequignot, E.: An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. Hum Mol Genet 2 (1993) 731-5.

Jenuwein, T. and Allis, C.D.: Translating the histone code. Science 293 (2001) 1074-80.

Ji, W., Hernandez, R., Zhang, X.Y., Qu, G.Z., Frady, A., Varela, M. and Ehrlich, M.: DNA demethylation and pericentromeric rearrangements of chromosome 1. Mutat Res 379 (1997) 33-41.

Johnstone, R.W.: Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov 1 (2002) 287-99.

Jones, P.A. and Laird, P.W.: Cancer epigenetics comes of age. Nat Genet 21 (1999) 163-7.

Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J. and Wolffe, A.P.: Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 19 (1998) 187-91.

Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H. and Razin, A.: Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev 6 (1992) 705-14.

Kaludov, N.K. and Wolffe, A.P.: MeCP2 driven transcriptional repression in vitro: selectivity for methylated DNA, action at a distance and contacts with the basal transcription machinery. Nucleic Acids Res 28 (2000) 1921-8.

Kao, H.Y., Downes, M., Ordentlich, P. and Evans, R.M.: Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes Dev 14 (2000) 55-66.

Kautiainen, T.L. and Jones, P.A.: DNA methyltransferase levels in tumorigenic and nontumorigenic cells in culture. J Biol Chem 261 (1986) 1594-8.

Kim, G.D., Ni, J., Kelesoglu, N., Roberts, R.J. and Pradhan, S.: Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. Embo J 21 (2002) 4183-95.

Kimura, H. and Shiota, K.: Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. J Biol Chem 278 (2003) 4806-12.

Kishimoto, N., Sakai, H., Jackson, J., Jacobsen, S.E., Meyerowitz, E.M., Dennis, E.S. and Finnegan, E.J.: Site specificity of the Arabidopsis METI DNA methyltransferase demonstrated through hypermethylation of the superman locus. Plant Mol Biol 46 (2001) 171-83.

Kondo, E., Harashima, A., Takabatake, T., Takahashi, H., Matsuo, Y., Yoshino, T., Orita, K. and Akagi, T.: NF-ATc2 induces apoptosis in Burkitt's lymphoma cells through signaling via the B cell antigen receptor. Eur J Immunol 33 (2003) 1-11.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R.J. and Wilson, G.G.: The DNA (cytosine-5) methyltransferases. Nucleic Acids Res 22 (1994) 1-10.

Lachner, M. and Jenuwein, T.: The many faces of histone lysine methylation. Curr Opin Cell Biol 14 (2002) 286-98.

Lachner, M., O'Sullivan, R.J. and Jenuwein, T.: An epigenetic road map for histone lysine methylation. J Cell Sci 116 (2003) 2117-24.

Lacoste, N., Utley, R.T., Hunter, J.M., Poirier, G.G. and Cote, J.: Disruptor of telomeric silencing-1 is a chromatin-specific histone H3 methyltransferase. J Biol Chem 277 (2002) 30421-4.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680-5.

Laird, P.W., Jackson-Grusby, L., Fazeli, A., Dickinson, S.L., Jung, W.E., Li, E., Weinberg, R.A. and Jaenisch, R.: Suppression of intestinal neoplasia by DNA hypomethylation. Cell 81 (1995) 197-205.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., et al.: Initial sequencing and analysis of the human genome, Nature, 2001, pp. 860-921.

Landry, J., Sutton, A., Tafrov, S.T., Heller, R.C., Stebbins, J., Pillus, L. and Sternglanz, R.: The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc Natl Acad Sci U S A 97 (2000) 5807-11.

Lang, S.E., McMahon, S.B., Cole, M.D. and Hearing, P.: E2F transcriptional activation requires TRRAP and GCN5 cofactors. J Biol Chem 276 (2001) 32627-34.

Leonhardt, H., Page, A.W., Weier, H.U. and Bestor, T.H.: A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell 71 (1992) 865-73.

Levinger, L. and Varshavsky, A.: Selective arrangement of ubiquitinated and D1 protein-containing nucleosomes within the Drosophila genome. Cell 28 (1982) 375-85.

Lewis, J.D., Meehan, R.R., Henzel, W.J., Maurer-Fogy, I., Jeppesen, P., Klein, F. and Bird, A.: Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell 69 (1992) 905-14.

Li, E.: Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3 (2002) 662-73.

Li, E., Beard, C. and Jaenisch, R.: Role for DNA methylation in genomic imprinting. Nature 366 (1993) 362-5.

Li, E., Bestor, T.H. and Jaenisch, R.: Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69 (1992) 915-26.

Liang, G., Chan, M.F., Tomigahara, Y., Tsai, Y.C., Gonzales, F.A., Li, E., Laird, P.W. and Jones, P.A.: Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Mol Cell Biol 22 (2002) 480-91.

Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S. and Jacobsen, S.E.: Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. Science 292 (2001) 2077-80.

Liu, J.: FK506 and cyclosporin, molecular probes for studying intracellular signal transduction. Immunol Today 14 (1993) 290-5.

Liu, K., Wang, Y.F., Cantemir, C. and Muller, M.T.: Endogenous assays of DNA methyltransferases: Evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo. Mol Cell Biol 23 (2003) 2709-19.

Liu, Y., Oakeley, E.J., Sun, L. and Jost, J.P.: Multiple domains are involved in the targeting of the mouse DNA methyltransferase to the DNA replication foci. Nucleic Acids Res 26 (1998) 1038-45.

Liu, Z. and Fisher, R.A.: RGS6 interacts with DMAP1 and DNMT1 and inhibits DMAP1 transcriptional repressor activity. J Biol Chem 279 (2004) 14120-8.

Li-Weber, M., Laur, O. and Krammer, P.H.: 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 29 (1999) 3017-27.

Lo, W.S., Trievel, R.C., Rojas, J.R., Duggan, L., Hsu, J.Y., Allis, C.D., Marmorstein, R. and Berger, S.L.: Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14. Mol Cell 5 (2000) 917-26.

Lorincz, M.C., Schubeler, D., Hutchinson, S.R., Dickerson, D.R. and Groudine, M.: DNA methylation density influences the stability of an epigenetic imprint and Dnmt3a/b-independent de novo methylation. Mol Cell Biol 22 (2002) 7572-80.

Lu, J., McKinsey, T.A., Nicol, R.L. and Olson, E.N.: Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. Proc Natl Acad Sci U S A 97 (2000) 4070-5.

Luo, C., Burgeon, E. and Rao, A.: Mechanisms of transactivation by nuclear factor of activated T cells-1. J Exp Med 184 (1996) 141-7.

Lyko, F., Ramsahoye, B.H., Kashevsky, H., Tudor, M., Mastrangelo, M.A., Orr-Weaver, T.L. and Jaenisch, R.: Mammalian (cytosine-5) methyltransferases cause genomic DNA methylation and lethality in Drosophila. Nat Genet 23 (1999) 363-6.

Macleod, D., Charlton, J., Mullins, J. and Bird, A.P.: Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev 8 (1994) 2282-92.

Mahadevan, L.C., Willis, A.C. and Barratt, M.J.: Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. Cell 65 (1991) 775-83.

Mahlknecht, U. and Hoelzer, D.: Histone acetylation modifiers in the pathogenesis of malignant disease. Mol Med 6 (2000) 623-44.

Mancini, D.N., Singh, S.M., Archer, T.K. and Rodenhiser, D.I.: Site-specific DNA methylation in the neurofibromatosis (NF1) promoter interferes with binding of CREB and SP1 transcription factors. Oncogene 18 (1999) 4108-19.

Marafioti, T., Pozzobon, M., Hansmann, M.L., Delsol, G., Pileri, S.A. and Mason, D.Y.: Expression of intracellular signaling molecules in classical and lymphocyte predominance Hodgkin disease. Blood 103 (2004) 188-93.

Margot, J.B., Ehrenhofer-Murray, A.E. and Leonhardt, H.: Interactions within the mammalian DNA methyltransferase family. BMC Mol Biol 4 (2003) 7.

Marin, M., Karis, A., Visser, P., Grosveld, F. and Philipsen, S.: Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Cell 89 (1997) 619-28.

Marks, P., Rifkind, R.A., Richon, V.M., Breslow, R., Miller, T. and Kelly, W.K.: Histone deacetylases and cancer: causes and therapies. Nat Rev Cancer 1 (2001) 194-202.

Marks, P.A., Miller, T. and Richon, V.M.: Histone deacetylases. Curr Opin Pharmacol 3 (2003) 344-51.

Mattila, P.S., Ullman, K.S., Fiering, S., Emmel, E.A., McCutcheon, M., Crabtree, G.R. and Herzenberg, L.A.: The actions of cyclosporin A and FK506 suggest a novel step in the activation of T lymphocytes. Embo J 9 (1990) 4425-33.

Mayer, W., Niveleau, A., Walter, J., Fundele, R. and Haaf, T.: Demethylation of the zygotic paternal genome. Nature 403 (2000) 501-2.

McMahon, S.B., Wood, M.A. and Cole, M.D.: The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. Mol Cell Biol 20 (2000) 556-62.

Melki, J.R., Warnecke, P., Vincent, P.C. and Clark, S.J.: Increased DNA methyltransferase expression in leukaemia. Leukemia 12 (1998) 311-6.

Mertineit, C., Yoder, J.A., Taketo, T., Laird, D.W., Trasler, J.M. and Bestor, T.H.: Sex-specific exons control DNA methyltransferase in mammalian germ cells. Development 125 (1998) 889-97.

Meuth, M.: The structure of mutation in mammalian cells. Biochim Biophys Acta 1032 (1990) 1-17.

Momparler, R.L.: Cancer epigenetics. Oncogene 22 (2003) 6479-83.

Monk, M., Boubelik, M. and Lehnert, S.: Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development 99 (1987) 371-82.

Murnion, M.E., Adams, R.R., Callister, D.M., Allis, C.D., Earnshaw, W.C. and Swedlow, J.R.: Chromatin-associated protein phosphatase 1 regulates aurora-B and histone H3 phosphorylation. J Biol Chem 276 (2001) 26656-65.

Murray, K.: The Occurrence of Epsilon-N-Methyl Lysine in Histones. Biochemistry 127 (1964) 10-5.

Nan, X., Campoy, F.J. and Bird, A.: MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. Cell 88 (1997) 471-81.

Nan, X., Meehan, R.R. and Bird, A.: Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. Nucleic Acids Res 21 (1993) 4886-92.

Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N. and Bird, A.: Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393 (1998) 386-9.

Neal, J.W. and Clipstone, N.A.: A Constitutively Active NFATc1 Mutant Induces a Transformed Phenotype in 3T3-L1 Fibroblasts. J Biol Chem 278 (2003) 17246-54.

Ng, H.H., Feng, Q., Wang, H., Erdjument-Bromage, H., Tempst, P., Zhang, Y. and Struhl, K.: Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. Genes Dev 16 (2002) 1518-27.

Ng, H.H., Jeppesen, P. and Bird, A.: Active repression of methylated genes by the chromosomal protein MBD1. Mol Cell Biol 20 (2000) 1394-406.

Ng, H.H., Robert, F., Young, R.A. and Struhl, K.: Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol Cell 11 (2003) 709-19.

Ng, H.H., Zhang, Y., Hendrich, B., Johnson, C.A., Turner, B.M., Erdjument-Bromage, H., Tempst, P., Reinberg, D. and Bird, A.: MBD2 is a transcriptional repressor

belonging to the MeCP1 histone deacetylase complex. Nat Genet 23 (1999) 58-61.

Nguyen, C.T., Weisenberger, D.J., Velicescu, M., Gonzales, F.A., Lin, J.C., Liang, G. and Jones, P.A.: Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. Cancer Res 62 (2002) 6456-61.

Nickel, B.E., Allis, C.D. and Davie, J.R.: Ubiquitinated histone H2B is preferentially located in transcriptionally active chromatin. Biochemistry 28 (1989) 958-63.

Nishioka, K., Chuikov, S., Sarma, K., Erdjument-Bromage, H., Allis, C.D., Tempst, P. and Reinberg, D.: Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation. Genes Dev 16 (2002) 479-89.

Noma, K., Allis, C.D. and Grewal, S.I.: Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science 293 (2001) 1150-5.

Ohlsson, R., Renkawitz, R. and Lobanenkov, V.: CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet 17 (2001) 520-7.

Ohtani-Fujita, N., Fujita, T., Aoike, A., Osifchin, N.E., Robbins, P.D. and Sakai, T.: CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. Oncogene 8 (1993) 1063-7.

Okano, M., Bell, D.W., Haber, D.A. and Li, E.: DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99 (1999) 247-57.

Okano, M., Xie, S. and Li, E.: Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 19 (1998a) 219-20.

Okano, M., Xie, S. and Li, E.: Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res 26 (1998b) 2536-40.

Osley, M.A.: H2B ubiquitylation: the end is in sight. Biochim Biophys Acta 1677 (2004) 74-8.

Oswald, J., Engemann, S., Lane, N., Mayer, W., Olek, A., Fundele, R., Dean, W., Reik, W. and Walter, J.: Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10 (2000) 475-8.

Peters, A.H., Mermoud, J.E., O'Carroll, D., Pagani, M., Schweizer, D., Brockdorff, N. and Jenuwein, T.: Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. Nat Genet 30 (2002) 77-80.

Petronzelli, F., Riccio, A., Markham, G.D., Seeholzer, S.H., Stoerker, J., Genuardi, M., Yeung, A.T., Matsumoto, Y. and Bellacosa, A.: Biphasic kinetics of the human

DNA repair protein MED1 (MBD4), a mismatch-specific DNA N-glycosylase. J Biol Chem 275 (2000) 32422-9.

Pradhan, S. and Roberts, R.J.: Hybrid mouse-prokaryotic DNA (cytosine-5) methyltransferases retain the specificity of the parental C-terminal domain. Embo J 19 (2000) 2103-14.

Pray-Grant, M.G., Schieltz, D., McMahon, S.J., Wood, J.M., Kennedy, E.L., Cook, R.G., Workman, J.L., Yates, J.R., 3rd and Grant, P.A.: The novel SLIK histone acetyltransferase complex functions in the yeast retrograde response pathway. Mol Cell Biol 22 (2002) 8774-86.

Preuss, U., Landsberg, G. and Scheidtmann, K.H.: Novel mitosis-specific phosphorylation of histone H3 at Thr11 mediated by Dlk/ZIP kinase. Nucleic Acids Res 31 (2003) 878-85.

Prokhortchouk, A., Hendrich, B., Jorgensen, H., Ruzov, A., Wilm, M., Georgiev, G., Bird, A. and Prokhortchouk, E.: The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. Genes Dev 15 (2001) 1613-8.

Ramchandani, S., Bhattacharya, S.K., Cervoni, N. and Szyf, M.: DNA methylation is a reversible biological signal. Proc Natl Acad Sci U S A 96 (1999) 6107-12.

Randak, C., Brabletz, T., Hergenrother, M., Sobotta, I. and Serfling, E.: Cyclosporin A suppresses the expression of the interleukin 2 gene by inhibiting the binding of lymphocyte-specific factors to the IL-2 enhancer. Embo J 9 (1990) 2529-36.

Rao, A., Luo, C. and Hogan, P.G.: Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 15 (1997) 707-47.

Ratnam, S., Mertineit, C., Ding, F., Howell, C.Y., Clarke, H.J., Bestor, T.H., Chaillet, J.R. and Trasler, J.M.: Dynamics of Dnmt1 methyltransferase expression and intracellular localization during oogenesis and preimplantation development. Dev Biol 245 (2002) 304-14.

Re, D., Zander, T., Diehl, V. and Wolf, J.: Genetic instability in Hodgkin's lymphoma. Ann Oncol 13 Suppl 1 (2002) 19-22.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., et al.: Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406 (2000) 593-9.

Rhee, I., Bachman, K.E., Park, B.H., Jair, K.W., Yen, R.W., et al.: DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416 (2002) 552-6.

Rice, J.C. and Allis, C.D.: Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr Opin Cell Biol 13 (2001) 263-73.

Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L. and Wolffe, A.P.: DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat Genet 25 (2000) 338-42.

Robertson, K.D., Uzvolgyi, E., Liang, G., Talmadge, C., Sumegi, J., Gonzales, F.A. and Jones, P.A.: The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res 27 (1999) 2291-8.

Robertson, K.D. and Wolffe, A.P.: DNA methylation in health and disease. Nat Rev Genet 1 (2000) 11-9.

Rountree, M.R., Bachman, K.E. and Baylin, S.B.: DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat Genet 25 (2000) 269-77.

Rountree, M.R., Bachman, K.E., Herman, J.G. and Baylin, S.B.: DNA methylation, chromatin inheritance, and cancer. Oncogene 20 (2001) 3156-65.

Rubben, A., Kempf, W., Kadin, M.E., Zimmermann, D.R. and Burg, G.: Multilineage progression of genetically unstable tumor subclones in cutaneous T-cell lymphoma. Exp Dermatol 13 (2004) 472-83.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J. and Kouzarides, T.: Active genes are tri-methylated at K4 of histone H3. Nature 419 (2002) 407-11.

Schorpp, M., Hofmann, M., Dear, T.N. and Boehm, T.: Characterization of mouse and human nude genes. Immunogenetics 46 (1997) 509-15.

Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupta, R., Reuter, G., Reinberg, D. and Jenuwein, T.: A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev 18 (2004) 1251-62.

Schwering, I., Brauninger, A., Klein, U., Jungnickel, B., Tinguely, M., et al.: Loss of the B-lineage-specific gene expression program in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. Blood 101 (2003) 1505-12.

Seigneurin-Berny, D., Verdel, A., Curtet, S., Lemercier, C., Garin, J., Rousseaux, S. and Khochbin, S.: Identification of components of the murine histone deacetylase 6 complex: link between acetylation and ubiquitination signaling pathways. Mol Cell Biol 21 (2001) 8035-44.

Serfling, E., Berberich-Siebelt, F., Chuvpilo, S., Jankevics, E., Klein-Hessling, S., Twardzik, T. and Avots, A.: The role of NF-AT transcription factors in T cell activation and differentiation. Biochim Biophys Acta 1498 (2000) 1-18.

Shaw, J.P., Utz, P.J., Durand, D.B., Toole, J.J., Emmel, E.A. and Crabtree, G.R.: Identification of a putative regulator of early T cell activation genes. Science 241 (1988) 202-5.

Sims, R.J., 3rd, Nishioka, K. and Reinberg, D.: Histone lysine methylation: a signature for chromatin function. Trends Genet 19 (2003) 629-39.

Sorensen, A.B., Duch, M., Amtoft, H.W., Jorgensen, P. and Pedersen, F.S.: Sequence tags of provirus integration sites in DNAs of tumors induced by the murine retrovirus SL3-3. J Virol 70 (1996) 4063-70.

Soutoglou, E. and Talianidis, I.: Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation. Science 295 (2002) 1901-4.

Starostik, P., Greiner, A., Schultz, A., Zettl, A., Peters, K., Rosenwald, A., Kolve, M. and Muller-Hermelink, H.K.: Genetic aberrations common in gastric high-grade large B-cell lymphoma. Blood 95 (2000a) 1180-7.

Starostik, P., Greiner, A., Schwarz, S., Patzner, J., Schultz, A. and Muller-Hermelink, H.K.: The role of microsatellite instability in gastric low- and high-grade lymphoma development. Am J Pathol 157 (2000b) 1129-36.

Sterner, D.E., Belotserkovskaya, R. and Berger, S.L.: SALSA, a variant of yeast SAGA, contains truncated Spt7, which correlates with activated transcription. Proc Natl Acad Sci U S A 99 (2002) 11622-7.

Stiewe, T. and Putzer, B.M.: Role of p73 in malignancy: tumor suppressor or oncogene? Cell Death Differ 9 (2002) 237-45.

Strahl, B.D., Ohba, R., Cook, R.G. and Allis, C.D.: Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena. Proc Natl Acad Sci U S A 96 (1999) 14967-72.

Sun, Z.W. and Allis, C.D.: Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature 418 (2002) 104-8.

Suske, G.: The Sp-family of transcription factors. Gene 238 (1999) 291-300.

Suzuki, H., Gabrielson, E., Chen, W., Anbazhagan, R., van Engeland, M., Weijenberg, M.P., Herman, J.G. and Baylin, S.B.: A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 31 (2002a) 141-9.

Suzuki, T., Shen, H., Akagi, K., Morse, H.C., Malley, J.D., Naiman, D.Q., Jenkins, N.A. and Copeland, N.G.: New genes involved in cancer identified by retroviral tagging. Nat Genet 32 (2002b) 166-74.

Szyf, M.: Targeting DNA methylation in cancer. Ageing Res Rev 2 (2003) 299-328.

Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M. and Taga, T.: DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell 1 (2001) 749-58.

Tate, P.H. and Bird, A.P.: Effects of DNA methylation on DNA-binding proteins and gene expression. Curr Opin Genet Dev 3 (1993) 226-31.

Thiagalingam, S., Cheng, K.H., Lee, H.J., Mineva, N., Thiagalingam, A. and Ponte, J.F.: Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci 983 (2003) 84-100.

Thomas, G., Lange, H.W. and Hempel, K.: [Relative stability of lysine-bound methyl groups in arginie-rich histones and their subfrations in Ehrlich ascites tumor cells in vitro]. Hoppe Seylers Z Physiol Chem 353 (1972) 1423-8.

Thomson, S., Mahadevan, L.C. and Clayton, A.L.: MAP kinase-mediated signalling to nucleosomes and immediate-early gene induction. Semin Cell Dev Biol 10 (1999) 205-14.

Timmermann, S., Lehrmann, H., Polesskaya, A. and Harel-Bellan, A.: Histone acetylation and disease. Cell Mol Life Sci 58 (2001) 728-36.

Trasler, J.M., Trasler, D.G., Bestor, T.H., Li, E. and Ghibu, F.: DNA methyltransferase in normal and Dnmtn/Dnmtn mouse embryos. Dev Dyn 206 (1996) 239-47.

Tuck-Muller, C.M., Narayan, A., Tsien, F., Smeets, D.F., Sawyer, J., Fiala, E.S., Sohn, O.S. and Ehrlich, M.: DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. Cytogenet Cell Genet 89 (2000) 121-8.

Turner, B.M.: Cellular memory and the histone code. Cell 111 (2002) 285-91.

Turner, B.M.: Memorable transcription. Nat Cell Biol 5 (2003) 390-3.

Turner, S.D., Ricci, A.R., Petropoulos, H., Genereaux, J., Skerjanc, I.S. and Brandl, C.J.: The E2 ubiquitin conjugase Rad6 is required for the ArgR/Mcm1 repression of ARG1 transcription. Mol Cell Biol 22 (2002) 4011-9.

Van Hooser, A., Goodrich, D.W., Allis, C.D., Brinkley, B.R. and Mancini, M.A.: Histone H3 phosphorylation is required for the initiation, but not maintenance, of mammalian chromosome condensation. J Cell Sci 111 ( Pt 23) (1998) 3497-506.

van Leeuwen, F., Gafken, P.R. and Gottschling, D.E.: Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell 109 (2002) 745-56.

Wade, P.A.: Methyl CpG-binding proteins and transcriptional repression. Bioessays 23 (2001a) 1131-7.

Wade, P.A.: Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. Hum Mol Genet 10 (2001b) 693-8.

Wade, P.A., Gegonne, A., Jones, P.L., Ballestar, E., Aubry, F. and Wolffe, A.P.: Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet 23 (1999) 62-6.

Wade, P.A., Jones, P.L., Vermaak, D. and Wolffe, A.P.: A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase. Curr Biol 8 (1998) 843-6.

Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Borchers, C., Tempst, P. and Zhang, Y.: Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. Mol Cell 8 (2001) 1207-17.

Wei, Y., Mizzen, C.A., Cook, R.G., Gorovsky, M.A. and Allis, C.D.: Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in Tetrahymena. Proc Natl Acad Sci U S A 95 (1998) 7480-4.

Wu, J., Issa, J.P., Herman, J., Bassett, D.E., Jr., Nelkin, B.D. and Baylin, S.B.: Expression of an exogenous eukaryotic DNA methyltransferase gene induces transformation of NIH 3T3 cells. Proc Natl Acad Sci U S A 90 (1993) 8891-5.

Wysocka, J., Myers, M.P., Laherty, C.D., Eisenman, R.N. and Herr, W.: Human Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3-K4 methyltransferase are tethered together selectively by the cell-proliferation factor HCF-1. Genes Dev 17 (2003) 896-911.

Xie, S., Wang, Z., Okano, M., Nogami, M., Li, Y., He, W.W., Okumura, K. and Li, E.: Cloning, expression and chromosome locations of the human DNMT3 gene family. Gene 236 (1999) 87-95.

Xu, G.L., Bestor, T.H., Bourc'his, D., Hsieh, C.L., Tommerup, N., et al.: Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402 (1999) 187-91.

Yoder, J.A. and Bestor, T.H.: A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. Hum Mol Genet 7 (1998) 279-84.

Yoder, J.A., Soman, N.S., Verdine, G.L. and Bestor, T.H.: DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. J Mol Biol 270 (1997a) 385-95.

Yoder, J.A., Walsh, C.P. and Bestor, T.H.: Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13 (1997b) 335-40.

Yoon, H.G., Chan, D.W., Reynolds, A.B., Qin, J. and Wong, J.: N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso. Mol Cell 12 (2003) 723-34.

Zardo, G., Reale, A., Passananti, C., Pradhan, S., Buontempo, S., De Matteis, G., Adams, R.L. and Caiafa, P.: Inhibition of poly(ADP-ribosyl)ation induces DNA hypermethylation: a possible molecular mechanism. Faseb J 16 (2002) 1319-21.

Zegerman, P., Canas, B., Pappin, D. and Kouzarides, T.: Histone H3 lysine 4 methylation disrupts binding of nucleosome remodeling and deacetylase (NuRD) repressor complex. J Biol Chem 277 (2002) 11621-4.

Zhang, C.L., McKinsey, T.A. and Olson, E.N.: Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation. Mol Cell Biol 22 (2002) 7302-12.

Zhang, Y.: Transcriptional regulation by histone ubiquitination and deubiquitination. Genes Dev 17 (2003) 2733-40.

Zhang, Y., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Bird, A. and Reinberg, D.: Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev 13 (1999) 1924-35.

Zhou, X., Richon, V.M., Rifkind, R.A. and Marks, P.A.: Identification of a transcriptional repressor related to the noncatalytic domain of histone deacetylases 4 and 5. Proc Natl Acad Sci U S A 97 (2000) 1056-61.

# 7. APPENDIX

## 7.1 Professional Profile

### *Name :* Askar M. Akimzhanov

### *Contact Adress*

*Institute Adress:*

Askar Akimzhanov
Universität Würzburg
Institut für Pathologie
Josef-Schneider-Str. 2
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### *Personal Information*

*Born* :01.05.1978

*Sex* :Male

*Civil status* :Single

*Nationality* :Russian Federation

### *Education*

M.Sc. Molecular Biology and Biochemistry, 1999

Department of Natural Sciences

Novosibirsk State University

Novosibirsk, Russia.

### *Research Experience*

| | |
|---|---|
| *Since March 2001:* | Graduate student at the Institute of Pathology, University of Würzburg, Germany. |
| *Sep. 1999-Feb. 2001:* | Graduate student at the Novosibirsk Institute of Bioorganic Chemistry, Novosibirsk State University, Russia. |
| *March 1997-June 1999:* | Undegraduate research, Novosibirsk Institute of Bioorganic Chemistry, Novosibirsk State University, Russia. |

**Technical Skills**

DNA isolation and sequencing, Southern blotting, DNA methylation analysis (bisulfite genomic sequencing), microsatellite analysis, RNA isolation, RNase protection assay, RT-PCR, Quantitative real-time RT-PCR, Western blotting, isolation of whole cell and nuclear extracts from cell cultures, cell culture manipulations, DNA-protein interaction assays (EMSA, Chromatin Immunoprecipitation).

## 7.2 Publications and Other Scientific Activities

### Publications

1. **Akimzhanov A.***, Krenacs L**.**, Bagdi E, Kondo E., Schlegel T., Chuvpilo S., Rüdiger T., Müller-Hermelink H.-K., Palmetshofer A., Serfling E. Epigenetic changes in *nfat1* promoter region reflect the suppression of NFATc1 in human lymphoid tumors defective in immunoreceptor signalling **(Submitted) (*The first two authors contributed equally to this work)**.

2. Chuvpilo S., Jankevics E., Tyrsin D., **Akimzhanov A.**, Moroz D., Jha M.K., Schulze-Luehrmann J., Santner-Nanan B., Feoktistova E., Konig T., Avots A., Schmitt E., Berberich-Siebelt F., Schimpl A., Serfling E. Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. **Immunity.** 2002 Jun;16(6)

3. Semenov DV, Kanyshkova TG, **Akimzhanov AM**, Buneva VN, Nevinsky GA. Interaction of human milk lactoferrin with ATP. **Biochemistry (Mosc)**. 1998 Aug;63(8).

4. Semenov DV, Kanyshkova TG, Kit YY, Khlimankov DY, **Akimzhanov AM**, Gorbunov DA, Buneva VN, Nevinsky GA. Human breast milk immunoglobulins G hydrolyze nucleotides. **Biochemistry (Mosc).** 1998 Aug;63(8).

## Oral Presentations

A. Akimzhanov, S. Chuvpilo, E. Jankevics, D. Tyrsin, D. Moroz, M.K. Jha, J. Schulze-Luehrmann, B. Santner-Nanan, E. Feoktistova, T. Konig, A. Avots, E. Schmitt, F. Berberich-Siebelt, A. Schimpl, E. Serfling. Autoregulation of NFATc1/A Expression Facilitates Effector T Cells to Escape from Rapid Apoptosis. *Gene Regulation in Lymphocyte Development; October 7-10, 2002 Santorini, Greece.*

## Poster Presentations

1. Akimzhanov A., Schlegel T., Serfling E. DNA and Histone Modifications are associated with NFATc1 gene silencing in Hodgkin's Lymphoma Cells. 7$^{th}$ *Joint Meeting of the Signal Transduction Society (STS); 8 - 10 November 2003, Weimer, Germany.*

2. A.M. Akimzhanov, D.V. Semenov, G.A. Nevinsky Comparison of milk protein kinase with secretory immunoglobulin A possessing protein kinase activity. *The Second International Conference on Bioinformatics of Genome Regulation and Structure; 7-11 August 2000, Novosibirsk, Russia.*