EPIGENETIC SILENCING OF THE PROAPOPTOTIC GENE BIM IN ANAPLASTIC LARGE CELL LYMPHOMA

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Abstract

BIM is a proapoptotic member of the Bcl-2 family. The BIM promoter can be the target of epigenetic silencing in various types of cancer. Here, we investigated the epigenetic status of BIM locus in NPM-ALK+ Anaplastic Large Cell Lymophoma (ALCL) cell lines and in lymph node biopsies form NPM-ALK+ ALCL patients. In all the cell lines tested, the BIM 5'UTR was densely methylated. Conversely, only very limited evidence of methylated lymphocytes from healthy donors. Treatment with the demethylating agent 5-azacytidine led to 5'UTR demethylation and BIM upregulation. By Chromatin Immunoprecipitation experiments, we also showed that BIM silencing occurs through recruitment of MeCP2 and of the SIN3a/Histone Deacetylase (HDAC) 1/2 co-repressor complex. BIM downregulation is associated with protection from apoptosis. Treatment with the deacetylase inhibitor Trichostatin-A restores the acetylation, upregulates BIM expression and induces massive cell death. Finally, the recruitment of the MeCP2/SIN3/HDAC1-2 silencing complex relies on BIM CpG methylation. Demethylation of BIM CpG island with 5-azacytidine leads to the detachment of the MeCP2 corepressor complex and to the reacetylation of the histone tails.

Crizotinib is a selective ATP-competitive small-molecule inhibitor of both ALK tyrosine kinases and their oncogenic variants (e.g. mutations, fusion proteins) and c-MET/ HGF receptor which are implicated in the progression of several cancers.

In this study we also demonstrated that NPM-ALK however, is not directly involved in determining the epigenetic status of Bim locus in ALCL NPM-ALK+. This study shows that, the epigenetic therapy such as demethylating agents and HDACi, in association with ALK inhibitor, Crizotinib, treatment could act synergistically, inducing massive apoptosis in ALCL by reactivating BIM expression and by NPM/ALK inactivation respectively.

Introduction

Apoptosis

Apoptosis (Fig. 1) is the genetically programmed process of cell death by which redundant or unwanted cells are eliminated, and which anticancer agents exploit to kill cancer cells. Defective apoptosis leads to cancer cells gaining oncogenic properties, such as extended cell lifespan, genetic mutations, cell proliferation even under cytotoxic condition, or resistance to chemotherapy, which result in eventual treatment failure [1-4]. Mammals have two distinct apoptosis signalling pathways. One is the 'extrinsic' (or 'death receptor') pathway which is activated by stimulation of death receptors on cell surfaces by their cognate ligands, such as TNF, Fas, or TRAIL, and results in caspase-8 activation. The other is the 'intrinsic' (or 'mitochondrial') apoptosis pathway which is regulated by the interplay of anti- and pro-apoptotic members of the Bcl-2 family. The latter pathway converges upon mitochondria, where signals result in mitochondrial outer membrane permeabilization (MOMP) and the release of pro-apoptotic factors, such as cytochrome *c*, AIF, or Diablo/Smac, which trigger activation of caspase-dependent and/or -independent cell demolition processes [5-8]. While numerous studies conducted during the past couple of decades have demonstrated the involvement of anti-apoptotic Bcl-2 family proteins, such as Bcl-2, Bcl-XL, or Mcl-, in disease development and resistance to therapy in hematologic malignancies, more recent studies have focused on the essential roles of BH3-only proteins, which are pro-apoptotic members of the Bcl-2 family of proteins, in disease development and therapy-induced tumor cell killing in

hematologic malignancies. The loss of function of BH3-only proteins promotes the development and proliferation of leukemias and lymphomas, particularly in the context of activation of an oncogene (*e.g.*, *c-myc*, *ras*, *etc.*), or functional deficiency of other tumor suppressor genes (loss of *p53*, *etc.*) [9, 10]. Moreover, the proper functioning of certain BH3-only proteins is essential for apoptotic initiation by anticancer treatments [11, 12].

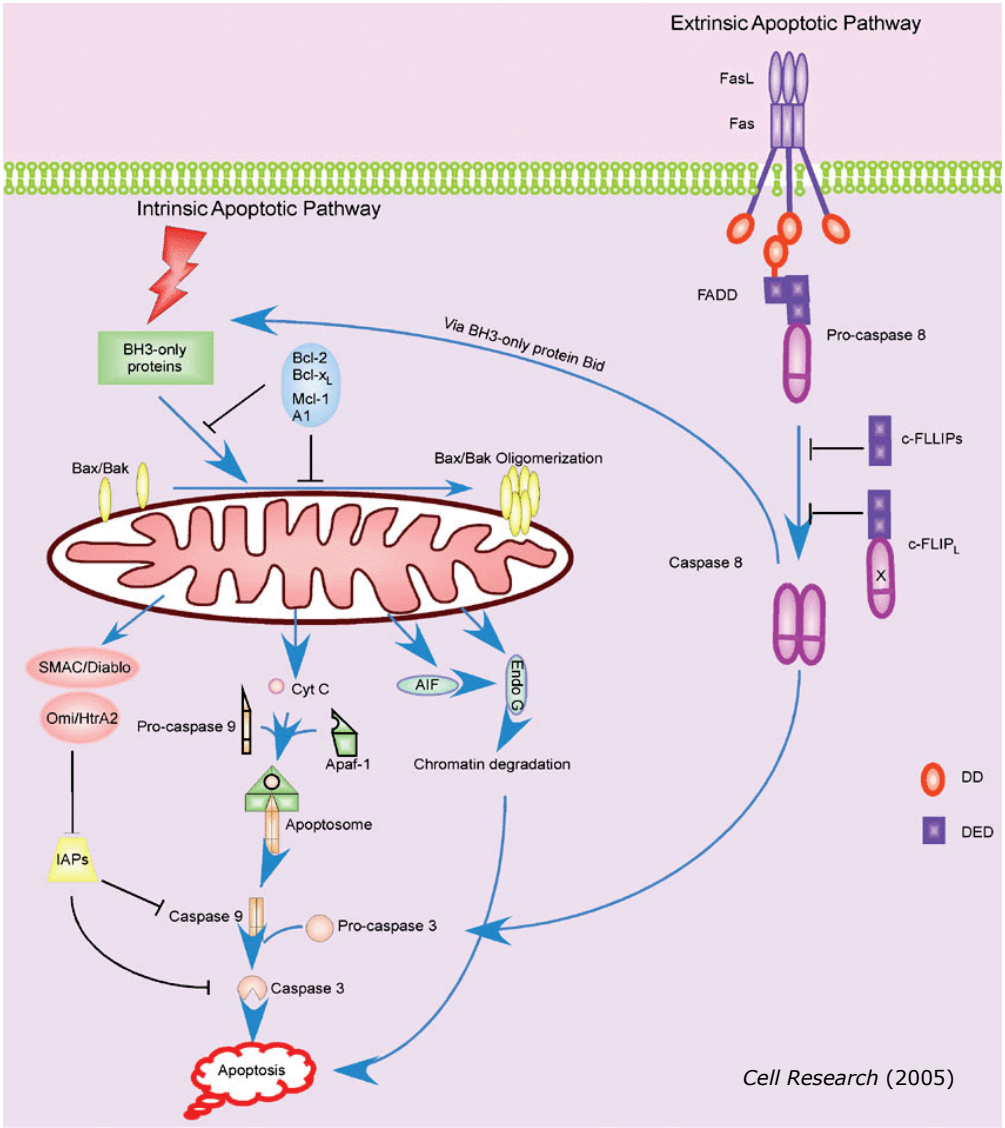

Fig. 1 Apoptotic pathways. Two major pathways lead to apoptosis: the intrinsic cell death pathway controlled by Bcl-2 family members and the extrinsic cell death pathway controlled by death receptor signaling. (adaptated from "Zhung N. The role of apoptosis in the development and function of T lymphocytes, 2005, Cell research 15: 751")

Bcl-2 family proteins

The Bcl-2 family includes at least 20 proteins that share between one and four conserved regions, designated “Bcl-2 homology domains (BH1, 2, 3 and BH4)”, and are classified into anti-apoptotic and pro-apoptotic members (Fig. 2). Anti-apoptotic members, comprising Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1 and Bcl-B, share four (or three in Mcl-1) regions of the BH domains. Pro-apoptotic proteins are further subdivided into two groups, Bax/Bak-like proteins and BH3-only proteins. Bax/Bak-like proteins include Bax, Bak, and Bok with three BH domains (BH1-3), and Bcl-XS with two BH domains (BH3 and BH4). BH3-only proteins share only the 9–16 amino acids BH3 region. Mammals have at least eight BH3-only proteins, including Bim (Bcl-2-interacting mediator of cell death, also known as Bod), Bad (Bcl-2-anagonist of cell death), Bik (Bcl-2-interacting killer, also known as Nbk or Blk), Bid (BH3-interacting domain death agonist), Hrk (Harakiri, also knownas DP5), Noxa, Puma (p53-upregulated modulator of apoptosis, also known as Bbc3) and Bmf (Bcl-2 modifying factor). Different members of this subgroup are induced and/or activated in response to different death stimuli, and these members can neutralize anti-apoptotic Bcl-2 family members [3, 13] (Fig. 3). The mechanisms by which BH3-only proteins, Bax/Bak like proteins and pro-survival Bcl-2 family members interact to mediate cell life and death decisions remain to be fully identified, so that this discussion will be limited to the most likely model for the interaction among anti- and pro-apoptotic Bcl-2 family proteins [11, 14] (Fig. 3). In healthy cells, Bax preferentially localizes to the

cytosol or loosely attaches to membranes in the form of monomers, while Bak localizes at the outer membrane of mitochondria and endoplasmic reticulum (ER). Anti-apoptotic Bcl-2 family members bind and inhibit Bax and Bak to prevent their oligomerization on the mitochondrial outer membrane, while BH3-only proteins remain inactive as a result of transcriptional repression or post-translational modification [13, 15, 16]. Enforced expression of BH3-only proteins does not induce apoptosis in Bax/Bak double-deficient cells, indicating that BH3-only proteins initiate apoptosis signaling, while Bax/Bak play a critical role downstream in apoptosis signaling [17, 18]. Upon activation of a death stimulus, BH3-only proteins are induced and/or activated transcriptionally and/or post-translationally, and bind to anti-apoptotic Bcl-2 proteins due to their stronger binding affinities, thereby liberating both Bax and Bak. All these molecular interactions of BH3-only proteins require an intact BH3 domain that forms an amphipathic α-helix for insertion into the hydrophobic groove formed by the BH1, BH2, and BH3 regions on the surface of the anti-apoptotic Bcl-2 family members [19]. BH3-only proteins are also conformationally modified to facilitate removal from anti-apoptotic relatives or their concentration is increased, leading to excess “free” BH3-only proteins. Individual BH3-only proteins differ markedly in their ability to bind to different anti-apoptotic Bcl-2 relatives [20-22]. Bim and Puma are so called “promiscuous” BH3-only proteins, because they bind to all anti-apoptotic Bcl-2 family members with high affinity. Other BH3-only proteins are more selective. Truncated Bid (tBid) binds with high affinity to Bcl-XL, Bcl-w, Mcl-1 and A1, but weakly to Bcl-2. Bik and Hrk bind to

Bcl-XL, Bclw and Mcl-1, but poorly to Bcl-2 or A1, Bad and Bmf bind strongly with Bcl-2, Bcl-XL, and Bcl-w, and Noxa binds only with high affinity to Mcl-1 and A1 [17, 20-22]. An important finding from cotransfection assays is that BH3-only proteins with different selective binding patterns (*e.g.*, Bad plus Noxa) are synergized in apoptosis induction. This clearly indicates that BH3-only proteins can cooperate with each other to induce apoptosis. Bim, Bid and Puma have been reported to bind directly to Bax and Bak to activate their pro-apoptotic function, but this has only been observed in detergents and has not yet been shown to occur under physiological conditions [21-23]. Nevertheless, it is clear that released Bax and Bak form oligomers on the mitochondrial outer membrane and then trigger MOMP, which results in the release of mitochondrial pro-apoptotic proteins. They may also perturb the ER/nuclear envelope to stimulate caspase-dependent and/or -independent cell demolition. Thus, the induction and/or the activation of BH3-only proteins is the first step for the initiation of apoptosis in response to cytotoxic stimuli, and the balance between BH3-only proteins and anti-apoptotic Bcl-2 family members is the determinant of cell fate resulting from most cytotoxic treatments.

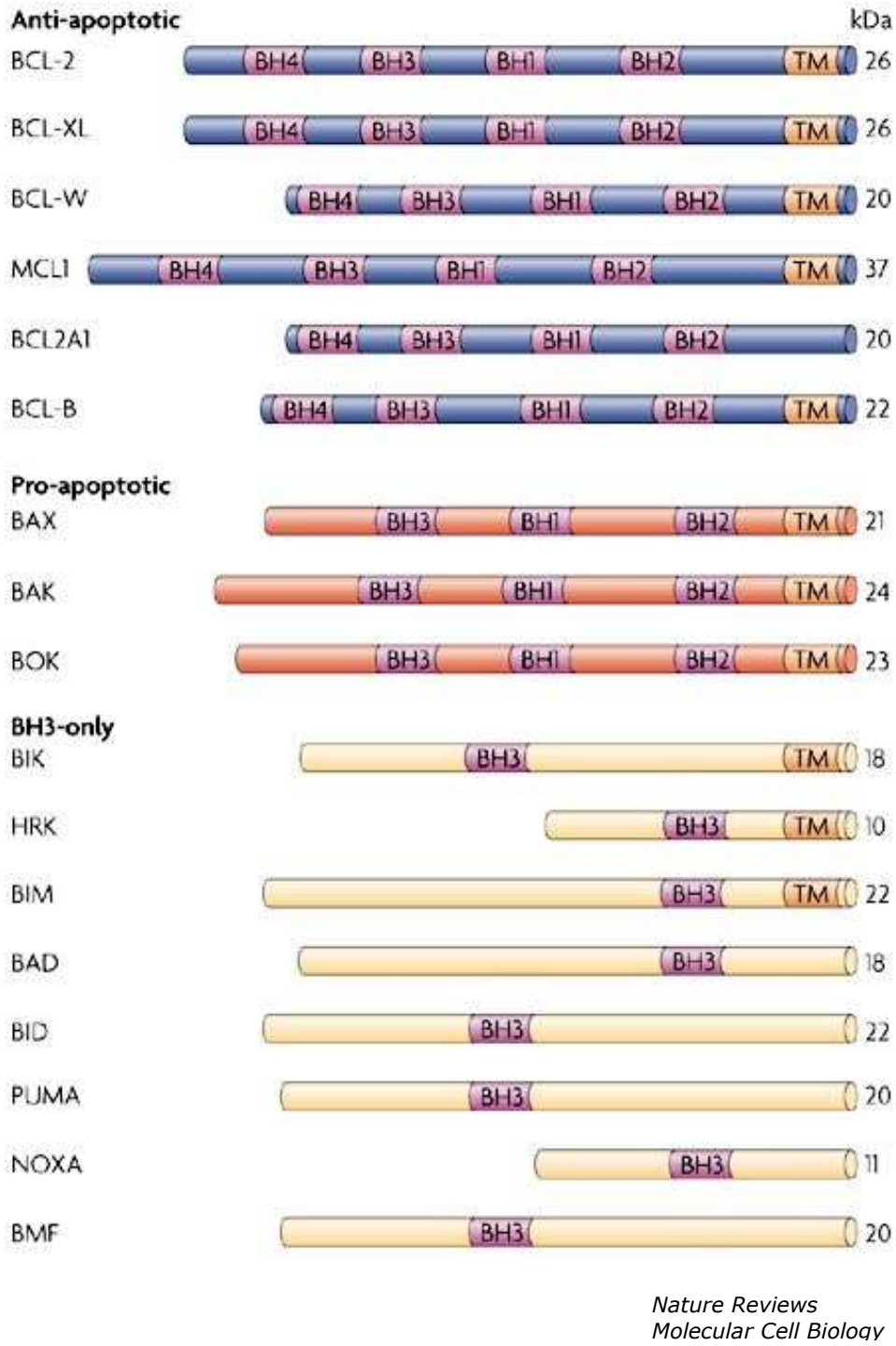

Fig. 2 B-cell lymphoma-2 (BCL-2)-family proteins Schematic representation of the mammalian BCL-2 family members: pro-survival and pro-apoptotic members. (Adapted from "Rebecca C. Apoptosis controlled demolition at the cellular level, 2008, Nature Reviews Molecullar Cell Biology, 9: 234")

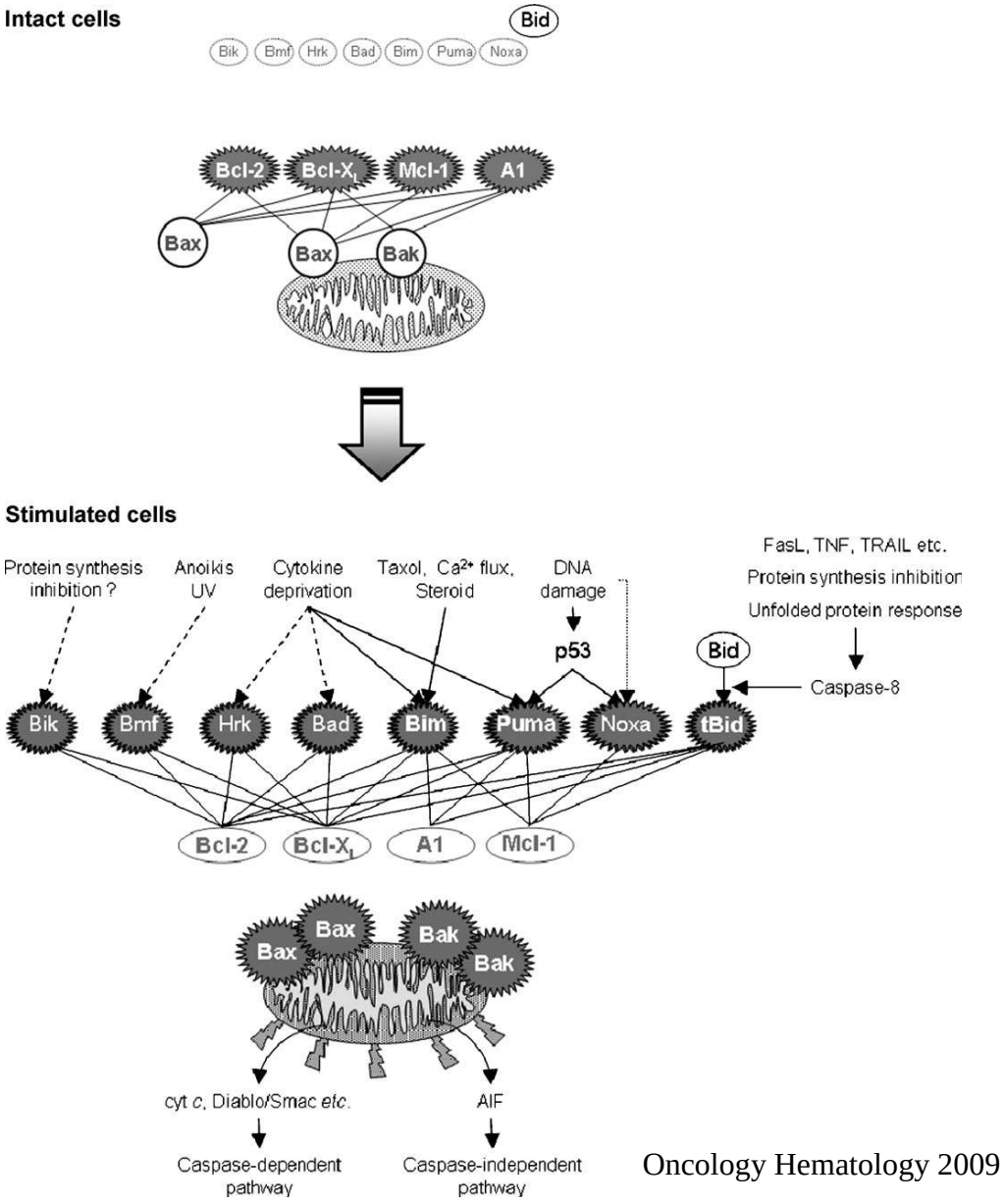

Fig. 3 Apoptosis-initiating molecular cascades of the Bcl-2 family of proteins under cellular stress conditions. Apoptosis is initiated by the activation of certain sets of BH3-only proteins according to the nature of the death stimuli. The activated BH3-only proteins in turn bind and inactive anti-apoptotic Bcl-2 proteins, and subsequently liberate and activate Bax/Bak on mitochondria. (adaptated from "Kuroda J. Involvement of BH3-only proteins in hematologic malignancies,2009,Oncology Hematology, 71: 91").

The BH3-only protein BIM

BIM is a pro-apoptotic member of the BCL-2 family. It plays a crucial role in the control of lymphocyte apoptosis [24, 25]. Alternative splicing of BIM mRNA produces at least twelve isoforms, but three are predominant: BIMEL (extra long), BIML (long) and BIMS (short). Several experiments

showed that BIM is expressed in lymphoid cells, myeloid cells, epithelial cells, germ-line cells and some populations of neuronal cells. In these tissues, BIMEL is the most abundantly expressed isoform [26].

BIMS has a stronger pro-apoptotic activity than the largest forms BIMEL and BIML [27]. This is probably due to the presence of a short peptide motif in BIMEL and BIML that allows their sequestration to the microtubule-associated dynein motor complex through binding to dynein-L-chain LC8, thus inhibiting their activity [28]. This short peptide motif is absent in BIMS. In response to apoptotic stimuli, BIMEL and BIML are released from microtubules and transported to the mitochondria where they inhibit anti-apoptotic proteins such as BCL-2 [28]. It has been demonstrated that all these three isoforms bind strongly to the BCL-2 pro-survival members; an interaction between BIM with either BAX and BAK [29, 30] has also been reported.

Role of BIM in the regulation of apoptosis

Studies with knockout mice have demonstrated that BIM plays an essential function in the control of haematopoiesis and in particular in lymphocyte development. BIM-/- mice have an increase of B cells, T cells, macrophages and granulocytes in their haematopoietic organs [24, 31]. Lymphoid cells and granulocytes derived from these mice were shown to be highly resistant to apoptosis induced by cytokine deprivation, and in particular it has been shown that BIM is required for both IL-2 and IL-7 withdrawal induced apoptosis [24, 31]. The role of BIM in lymphocyte maturation and haematopoietic system homeostasis is emphasized by its

function in apoptosis of autoreactive B and T cells. Its absence is in fact associated with the appearance of autoimmune diseases [24, 25, 32]. BIM has also a crucial role in T-lymphocyte death during the shutdown of an immune response. In fact it has been demonstrated that T cells from BIM-/- mice, activated either in vivo or in culture, survive much longer than wild-type T cells, and that during the termination of an immune response, T-lymphocyte death is initiated by BIM and requires the pro-apoptotic relatives BAX and BAK [7, 33].

Role of BIM in tumorigenesis and in the cytotoxic response

BIM deficiency may promote oncogenesis by favoring cell survival. Pertinent to this, it has been shown that 17% of human mantle cell lymphomas display homozygous deletion of the human BIM locus [34]. Recent studies demonstrate that BIM is an unusual tumour suppressor gene because it is haploinsufficient. The loss of a single allele results in an acceleration of lymphomagenesis induced by the expression of an Eμ-MYC transgene in B cells, with the resulting tumours retaining the single wild-type BIM allele [32]. Bim deficiency was shown to induce follicular lymphoma and to accelerate *myc*-induced lymphomagenesis in a mouse model [9, 32]. Moreover, the *bim* promoter is reportedly methylated in certain leukemias and lymphomas, and this methylation is associated with low levels of Bim expression and relative resistance to apoptotic stimuli (Piazza et al. 2005). In addition to genetic/epigenetic loss, cancer cells acquire constitutive active Ras/Erk and PI3K/Akt, which can promote

proteasomal degradation of Bim and repress *bim* transcription, respectively. This is the result of abnormal constitutive upregulation of tyrosine kinase activities, such as Bcr-Abl in chronic myelogenous leukemia (CML), in-frame internal tandem duplications in FLT3 receptor (FLT3-ITD) in acute myelogenous leukemia (AML), or NPM/ALK fusion protein by t(2;5)(p23;q35) in anaplastic large-cell lymphoma [35]. Recently, JNK-mediated Bim repression has been linked to chemoresistance in T-acute lymphoblastic leukemia (ALL) [36], while the Epstein-Barr virus oncoproteins EBNA3A and EBNA3C cooperatively downregulate Bim in Burkitt lymphoma [37].

It has been demonstrated that Bim is critical for apoptosis by various kinds of chemotherapeutics (Fig. 4). Thymocytes and pre-B cells derived from BIM-/- mice show significant resistance to apoptosis induced by irradiation with γ-rays or by treatment with glucocorticoids [24, 31]. It has in fact been shown that BIM is a key molecule in glucocorticoid-induced apoptosis, but the machinism by which BIM is activated is not clear [38]. The role of BIM in the cytotoxic response is emphasized also by the fact that, together with the BH3-only protein PUMA, BIM is essential for chemotherapeutic and γ-ray induced killing of non-malignant lymphocytes and myeloid cells, indicating that it could have a crucial role in the immunodeficiency that often results as a side-effect of anticancer therapy [7].The blockade of Bcr-Abl signaling by tyrosine kinase inhibitors (TKIs), such as imatinib, nilotinib, or INNO-406, or by 17-AAG, an inhibitor for heat shock protein (HSP)-90, induces Bim in Bcr-Abl-positive leukemic cells [39-43]. The blockade of Bcr-Abl signaling concurrently activates

other BH3-only proteins, such as Bmf, Bad, or Bik, and their concerted function is essential for apoptosis by Bcr-Abl shutdown. In this context, Bim is central, since the expression levels of Bim appear to determine the degree of cell killing by Bcr-Abl signaling shutdown [39]. Cooperation between Bim and Bad was also observed during apoptosis induced by dexamethasone plus the PI3K inhibitor LY294002 in a follicular lymphoma cell line [44]. Furthermore, cooperation of Bim and Noxa has been detected in apoptosis induced by seliciclib, a cyclin-dependent kinase inhibitor, in B-chronic lymphocytic leukemia (CLL) [45]. In myeloma cells, Bim is negatively regulated by IL-6 stimulation, and dissociation of Bim from Mcl-1 is the prerequisite for melphalan-induced apoptosis [46-48]. Bim is also crucial for apoptosis by the multikinase inhibitor sorafenib in AML [49], by the MEK-1/2 inhibitor in myeloma [50]. Certain apoptotic stimuli, such as the microtubule inhibitor paclitaxel, disrupt interactions between LC8 and the dynein motor complex, thereby liberating Bim [16, 28, 51]. Proteasome inhibitors such as bortezomib cause accumulation of Bim protein by suppressing its proteasomal degradation [40, 41]. However, apoptosis induced by proteasome inhibitors is not significantly affected by Bcl-2 overexpression or knockdown of Bim, and several reports support the involvement of both the intrinsic and the extrinsic pathway in proteasome inhibitor-induced apoptosis [40, 52, 53]. The role of Bim as a primary initiator in apoptosis by proteasome inhibitors remains controversial.

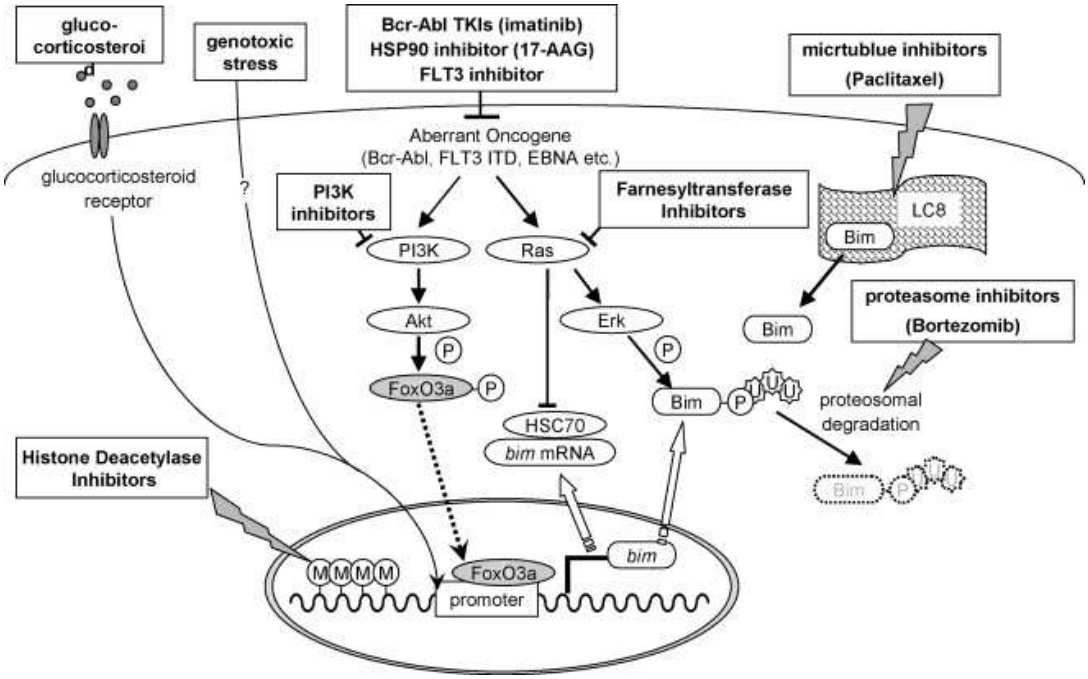

Fig. 4 Regulation of Bim expression and chemotherapy. The activity of FoxO3a, the most extensively characterized transcription factor for *bim*, is regulated by the PI3K/Akt pathway. Erk-I/II represses the pro-apoptotic activity of Bim by means of phosphorylation, thereby targeting this protein for ubiquitination and proteasomal degradation. Ras also dissociates heat shock cognate (HSC) 70 from *bim* mRNA, and promotes its degradation by ribonuclease. Hyper-methylation around the promoter region of the *bim* gene has been identified in specific cancers. M: methylation, P: phosphorylation, U: ubiquitination. (adaptated from "Kuroda J. Involvement of BH3-only proteins in hematologic malignancies,2009,Oncology Hematology, 71: 93")

Transcriptional and post-translational regulation of BIM activity

The regulation of BIM activity is not completely defined, but several mechanisms have been proposed. In haematopoietic cells, cytokine deprivation can activate the pro-apoptotic activity of BIM through at least two different pathways (Fig. 5).

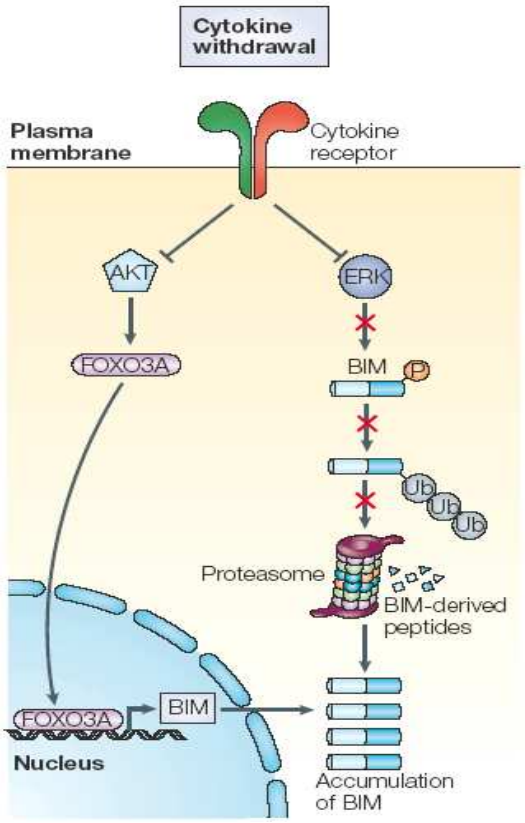

Fig. 5: Mechanisms for BIM activation in response to cytokine withdrawal in haematopietic cells (adapted from "Strasser A., The role of the BH3-only proteins in the immune system, 2005, Nature Reviews, 5: 194").

In myeloid cells exposed to cytokines withdrawal, it has been shown that the transcription factor Forkhead-box-03A (FOXO3A; FKHRL1) can up-regulate BIM mRNA transcription. In the presence of growth factors, the activity of FOXO3A is suppressed through phosphorylation mediated by AKT, while loss of cytokine stimulation causes AKT inactivation which in turn leads to activation of FOXO3A and to an increase in the level of BIM mRNA [54]. Recent study also disclosed cytokine-mediated post-transcriptional regulation of Bim, namely, the binding of heat shock cognate protein (HSC70), a chaperon protein which stabilizes *bim* mRNA, is regulated by cytokine-dependent association of cochaperones with

HSC70. By mediating Ras pathway, cytokines, such as IL-3, induces the expression of cochaperones, Bag-4 and Chip, those dissociate HSC70 from *bim* mRNA, and then allows *bim* mRNA for the substrate of ribonuclease [55]. The presence of growth factors can also inhibit BIM activity by a post-translational regulation. In fact it has been shown that in the presence of cytokines, BIM can be phosphorylated by the ERK kinase, which targets BIM for ubiquitylation and subsequent proteasomal degradation. In contrast, cytokine deprivation leads to the inactivation of ERK causing loss of BIM phosphorylation [56-58] and thus potentiating its pro-apoptotic activity.

Therefore, inhibitors of the Ras/Erk pathway and the proteasome are capable of accumulating BimEL and BimL [56].

In neuronal cells it has been reported that trophic factors deprivation causes BIM activation through the JUN-amino-terminal-kinase (JNK) [59]. JNKs can regulate the pro-apoptotic activity of BIM both transcriptionally, through activation of the JNK-activated transcription factor JUN, and post-translationally, through the phosphorylation of BimEL at Ser65.

BIM protein is also regulated post-translationally by sequestration to cytoskeletal structure inside the cells. As stated before, in non apoptotic cells BIMEL and BIML are sequestered to the microtubular dynein motor complex through direct interaction with the dineyn light chain LC-8. BIMS does not bind to LC-8, thus explaining the greater pro-apoptotic activity of this isoform. Certain stress conditions enable the release of BIMEL and BIML from the cytoskeletal, thus inducing their pro-apoptotic functions

[28]. In haematopoietic cells, BIM transcriptional regulation appears to be an important mechanism for regulating its function. In fact, during apoptosis of autoreactive lymphocytes, increased levels of BIM mRNA and protein have been observed, but little is known about the mechanisms and the pathways that are involved in this activation [25, 32].

Role of apoptosis in the development and functioning of the immune system

Regulation and coordination of the balance between cell survival and cell death is important for the proper functioning of the haematopoietic system. Different studies demonstrated the importance of apoptosis in the maintenance of cell number and in the removal of useless or autoreactive cells [60]. For example, in the lymphoid organs the maintenance of immune tolerance is dependent upon apoptosis induction of autoreactive T and B lymphocytes: defective elimination of these cells results in autoimmune diseases. Granulocytes undergo constitutive apoptosis in physiological conditions and are removed by macrophages as intact apoptotic cells. The phagocytosis of intact cells is important because granulocytes contain proteolytic enzymes and bactericidal factors and need to be removed prior to their disintegration. Defects in granulocyte apoptosis could lead to the leakage of their content out of the cell, thus prolonging inflammation and causing tissues damage.

Mice lacking Fas [61] and FasL [62] show an excess of lymphocytes, proving the importance of Fas-mediated apoptosis in haematopoietic system homeostasis. Furthermore, lymphoid and myeloid accumulation

has been observed in the presence of abnormalities in the Bcl-2 signalling pathway after expression of a Bcl-2 transgene [63-65] or depletion of the proapoptotic protein Bim [24]. Conversely, loss of Bcl-2 protein causes reduced survival of either lymphoid or myeloid cells [31, 66, 67] leading to immunodeficiency.

From a clinical point of view alterations in the regulation of apoptosis have been implicated in different haematopoietic disorders including myelodysplastic syndrome (MDS), severe aplastic anaemia (SAA) and uncontrolled proliferation leading to leukaemia [68].

Deregulation of apoptosis in the immune system and leukaemogenesis

As described previously, apoptosis is a physiological process for removing useless or harmful cells and plays an essential role for the normal development and function of the haematopoietic system. Consequently, alterations in apoptosis lead to imbalances between proliferation and cell death, violating tissue homeostasis and leading to proliferative or degenerative diseases.

Almost all the forms of cancer are characterized by defects in programmed cell death. Although more than 100 distinct types of cancer have been studied, all of them share essential alterations that characterize the tumour phenotype: self-sufficiency in growth signal, insensitivity to growth-inhibitory signals, limitless replicative potential, sustained angiogenesis, defective differentiation, ability to invade tissues and evasion of programmed cell death [4]. All tumours develop from single cells that

begin to proliferate abnormally. The development of cancer is thought to proceed via a multistep process in which cells become malignant through a progressive series of mutations that confer growth advantage on normal cells. This model is an extended form of the Knudson hypothesis that was first formulated by A. Knudson in the 1971 [69]. Nowadays it is known that most cancers affect the human population with an age dependent incidence, confirming the necessity of multiple genetic alterations in each cell for the malignant transformation [70].

The contribution of defective programmed cell death to tumour development was first established in lymphoma by the identification of the Bcl-2 proto-oncogene at the chromosomal breakpoint of the t(14;18) translocation [71] and confirmed by the description of deregulation in the expression of anti or pro-apoptotic members of the Bcl-2 family in haematological malignancies [72]. It is known that the Bcl-2 gene is involved in chromosomal translocations in over half of non Hodgkin's lymphoma [73] and its over-expression in the absence of DNA alterations has been associated with poor prognosis in some non Hodgkin's lymphoma [74]. Moreover, elevations in Bcl-2 protein levels are commonly found in other haematopoietic malignancies such as multiple myeloma (MM), chronic lymphocytic leukaemia (CLL), acute lymphocytic leukaemia (ALL) and acute myeloid leukaemia (AML) [75]. Higher levels of Mcl-1, a member of the Bcl-2 family, have been associated with failure to achieve a complete remission in CLL [76] and inactivating-mutations in the pro-apoptotic gene Bax have been observed in various haematopoietic malignancies [77].

Failure in the apoptotic pathways promotes cell accumulation by slowing the normal rate of cell turnover and induces a survival advantage of neoplastic clones relative to their normal counterpart. Defects in programmed cell death contribute to tumour progression also creating a permissive environment for genetic instability and allowing the accumulation of alterations. Furthermore, deregulation of apoptosis plays a major role in the protection from chemotherapeutic treatments [78-81]. In fact, since one of the main aims of cancer therapy is to kill tumour cells through induction of apoptosis, alterations and defects in apoptosis programmes can confer resistance to cytotoxic therapies [72].

Cancer cells could acquire resistance to programmed cell death through a variety of strategies that finally lead to the loss of function of pro-apoptotic molecules and/or to the activation of proto-oncogenes in order to overcome physiological growth inhibitory signals. It is already well defined that at the molecular level these strategies are mediated through the development of genetic and epigenetic abnormalities in cancer cells.

Epigenetic mechanisms

Nucleosomal histones: substrates for epigenetic modification

The organization of DNA into higher order structures, or nucleosomes, is a central component to epigenetic gene regulation. Each nucleosome, which represents the basic repeating unit of chromatin, consists of 147 bp of DNA

wrapped around a core of eight histones including two molecules each of H2A, H2B, H3 and H4 [82]. Individual nucleosomes are joined to each other by the linker histone H1 and a short length of DNA (~200±40 bp) to yield the 10 nm fiber, which may be further compacted into a helical structure referred to as a 30 nm fiber via interactions between the more variable, flexible histone tails, which protrude from the nucleosomal disk. The concept of a 'histone code' was proposed following the discovery of specific post-translational covalent modifications of these histone tails by acetylation, methylation, phosphorylation, glycosylation, SUMOylation and ubiquitylation. Such modifications act in a concerted manner to induce structural changes in the chromatin fiber and to regulate the accessibility of transcription factors to gene regulatory sequences, ADP ribosylate affecting gene expression [83]. There are a vast number of potential combinations of chromatin modifications that can be displayed by histones but several generalizations can be made. Transcribed genes may be present in nucleosome-free regions that are highly accessible to transcription factors, or in regions of chromatin that tend to be hyperacetylated through the action of histone acetyltransferases (HATs). By contrast, heterochromatic regions, generally silent in terms of gene expression, tend to be hypoacetylated through the action of histone deacetylases (HDACs) and methylated on cytosine-phosphate-guanine (CpG) dinucleotides by DNA methyltransferases (DNMTs). Acetylation of histones can change the physicochemical properties of these proteins, interfering with the electrostatic attraction between positively charged histones and negatively charged DNA. Furthermore, specific histone tail

modifications offer binding sites for the recruitment of other chromatin modification machinery. For example, specific histone tail residue methylation events may be associated with gene activation and others with gene repression (Fig. 6). We will describe with more details two main epigenetic mechanisms: methylation of CpG island and acetylation of histone tails.

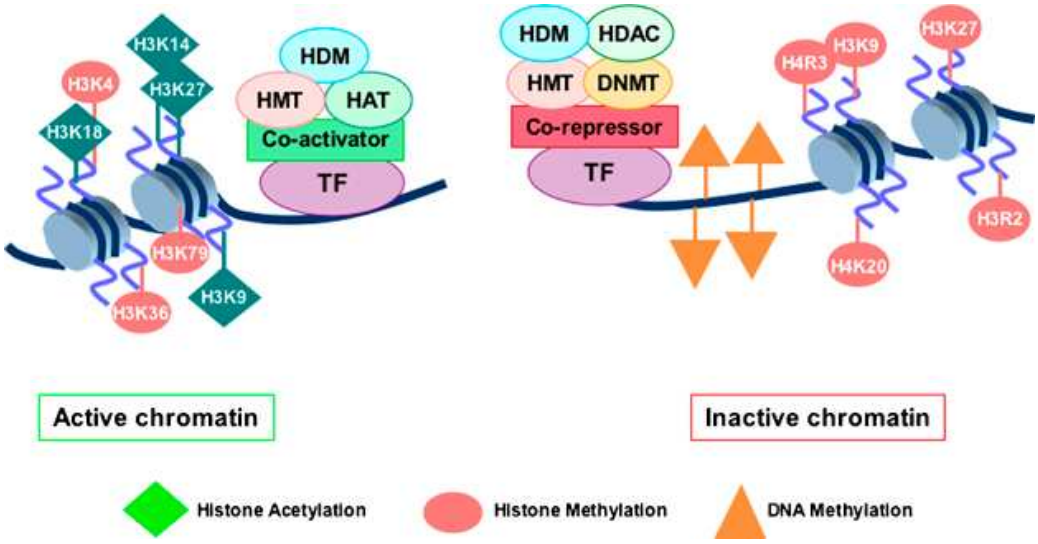

Fig. 6 Sequence-specific transcription factors act as docking molecules for the recruitment of DNA and histone-modifying activities to target gene promoters. Active transcription is associated with hyperacetylation and methylation of H3K4, H3K79 and H3K36 residues in promoter regions, whereas gene repression is associated with DNA methylation, hypoacetylation and methylation of H3K9, H3K27 and H4K20 residues. These modifications are mediated by chromatin-modifying enzymes including DNA methyltransferases (DNMTs), histone acetyltransferases (HATs)/histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone demethylases (HDMs). (adaptated from Rice KL. Epigenetic regulation of normal and malignant hematopoiesis, 2007, Oncogene, 26: 6698)

DNA methylation: a mark of stable gene silencing

DNA methylation involves the addition of a methyl group at position C5 of the cytidine ring in the context of a CpG dinucleotide, and is catalysed by a family of DNMTs (Fig. 7) including DNMT1, which preferentially targets hemi-methylated DNA and is required for 'maintenance' methylation during DNA replication; and DNMT3A and DNMT3B which are required

for *de novo* methylation [84]. In the mammalian genome, the distribution of CpG dinucleotides, which are predominantly methylated, is statistically underrepresented (~ 1 CpG per 100 bp), however the dinucleotide frequency occurs at near-expected levels in the promoters of an estimated 60% of human genes (~ 1 CpG per 10 bp), where cytosines are typically hypomethylated [85]. This enrichment of CpG dinucleotides in gene promoters is likely the result of spontaneous deamination of methylated cytosines to thymidine in nonregulatory sequences and these CpG-rich regions are typically referred to as 'CpG islands'. The regulation of gene expression by DNA methylation of target gene promoters is crucial for the control of several developmental processes including X inactivation [86], genomic imprinting [87], embryonic *Hox* gene patterning [87] and hematopoiesis. DNA methylation patterns are perturbed in many human cancers and typically involve regional hypermethylation of CpG islands affecting tumor suppressor genes, for example, $p15^{INK4b}$ (*CDKN2B*) and $p16^{INK4a}$ (*CDKN2A*), which are silenced in lymphoid and myeloid malignancies that occur within an overall setting of genome-wide DNA hypomethylation, which has been linked to genomic instability [88]. The identification of promoter hypermethylation 'signatures' linked to certain epithelial tumors and leukemia subtypes suggests that there is interplay between transcription factors and DNA methylation complexes regulating normal cellular differentiation that are awry in cancer cells [89-91]. DNA methylation of CpG islands is associated with transcriptionally silent chromatin, however whether DNA methylation induces transcriptional silencing *per se*, or functions as a stabilizer of silencing, remains

enigmatic. Although the exact hierarchy of events is unclear, the net effect of DNA methylation is local histone deacetylation and a closed chromatin configuration resulting in gene repression via two mechanisms. First, the presence of methyl groups acts to repel the binding of specific transcription factors, for example, in the case of the murine *H19*/*Igf2* imprinting control region on chromosome 7–69.09cM, which acts as a transcriptional insulator. Specifically, methylation of CpG dinucleotides on the paternal allele blocks binding of CTCF and allows a downstream enhancer to activate *Igf2* expression via a looping mechanism [92, 93]. Second, DNA methylation leads to the recruitment of methyl-CpG-binding domain (MBD) proteins which include five members: MeCP2, MBD1, MBD2, MBD3 and MBD4 [94]. These proteins interact with cytosine methyl groups within the major groove of DNA and through their interactions with DNMTs, specific transcription factors and chromatin-modifying enzymes are capable of repressing gene transcription. For example, MeCP2 has been shown to exist in a complex with the transcriptional co-repressor Sin3A and HDACs [95], and repressed the ability of PU.1 to activate transcription through its cognate-binding site [96-98]. The epigenetic control of the human α- and β-globin genes during erythropoiesis is considered a paradigm for differentiation-induced methylation changes during normal hematopoiesis. During erythropoiesis, the maturation of erythrocytes is associated with increased expression of α - and β-globin genes, which is required to synthesize large amounts of hemoglobin (~ 3 x 10^8 molecules per cell). The β-globin locus is located on chromosome 11 and consists of five genes ε, Gγ, Aγ, δ and β which are

under the regulation of a locus control region, located 6–22 kb upstream of the ε-globin gene [97]. In non-erythroid cells, these genes exist in a methylated, transcriptionally silent state. During erythroid differentiation, however, individual genes within the β-globin locus corresponding to embryonic (ε), fetal (Gγ Aγ) and adult (δ, β) stages of erythropoiesis are expressed in a sequential fashion, such that embryonic/fetal genes are ultimately silenced and adult genes are activated. The reactivation of fetal γ-globin in patients with sickle cell disease using the HDAC inhibitor (HDACI) sodium phenylbutyrate [99] and the DNMT inhibitor (DNMTI) 5-aza-2'-deoxycytidine (5Aza-dC) [100] demonstrated the therapeutic potential of reversing such epigenetic marks, and set the scene for its application in hematological malignancies. The mechanism of reactivation using 5Aza-dC is related to the depletion of functional DNMTs which become bound in a complex with 5Aza-dC-incorporated DNA, however the ability of 5Aza-dC to selectively degrade DNMT1 has also been reported [101]. The use of DNMTIs in combination with HDACIs has also been successfully used to reactivate silenced, hypermethylated tumor suppressor genes in human cancer cell lines. Studies by Cameron *et* al. (1999) [102] demonstrated that administration of trichostatin A (TSA) following treatment with low doses of 5Aza-dC, synergistically reactivated the expression of *MLH1*, *TIMP3*, $p15^{INK4b}$ and $p16^{INK4}$ in tumor cells [102]. Combinatorial treatment of mice with 5Aza-dC and sodium phenylbutyrate was also able to significantly reduce lung tumor development initiated by a tobacco-specific carcinogen in mice [103]. Since then, clinical trials involving the sequential administration of 5-azacytidine and sodium

phenylbutyrate in patients with myelodysplastic syndrome or acute myeloid leukemia (AML) have been conducted. These studies demonstrated an enhanced clinical response rate that was associated with demethylation of $p15^{INK4b}$ and acetylation of histones H3 and H4 [104]. Interestingly, induction of histone acetylation was observed before HDACI was administered, and although the mechanism by which 5Aza-dC results in histone acetylation is unknown, it appears that CpG island methylation is the dominant epigenetic mark responsible for stable gene silencing. Studies of the globin gene locus have also provided insights into potentially new mechanisms of epigenetic regulation. For example, the initial activation of embryonic/fetal genes is thought to be a result of promoter demethylation, as opposed to *de novo* methylation in adults, since differentiation of HSCs derived from either baboon fetal liver (FL) and adult bone marrow (ABM) into mature erythroblasts is accompanied by a progressive decrease in γ-globin promoter methylation and the concomitant activation of transcription in both FL and ABM [105]. These results suggest the existence of a DNA demethylase activity to counterbalance the repression mediated by DNMTs, and would also explain the active demethylation of the paternal genome that is observed shortly after fertilization [106]. The identification of ROS1 in Arabidopsis, which has DNA glycosylase/lyase activity specific for methylated substrates, and whose mutation is associated with DNA hypermethylation and gene silencing, provides evidence for such an activity [107]. Alternatively, demethylation may occur with replication of DNA during differentiation and failure to remethylate daughter strands. The

significance of a DNA demethylase, however, and other as yet undiscovered epigenetic marks/modifying proteins, lies in the ability to target the aberrant forms of these activities in a more specific manner. Similar to the globin locus, the expression of specific transcription factors required for the differentiation of other hematopoietic lineages is regulated by promoter methylation, and as such, these genes also represent potential targets for disruption in hematological malignancies. For example, PU.1 (SPI1) is highly expressed in HSCs and differentiated B cells, but not in T cells, correlating with the methylation status of the *PU.1* 5'UTR, which is hypermethylated in CD4+ and CD8+ cells [108]. PU.1 overexpression has been linked to peripheral T-cell lymphoma [109], and mice with deletion of a regulatory element upstream of *PU.1* developed AML [108, 110]. Furthermore, hypomethylation of *PU.1* was observed in patients with diffuse large B-cell lymphoma compared to normal lymph nodes, highlighting the requirement for tight epigenetic control of PU.1 in normal hematopoiesis [108]. Differential methylation of regulatory elements controlling the expression of other lineage-determining transcription factors has also been observed, including *GATA3*, which displays reduced methylation in naive and memory CD4+ cells compared to CD34+, CD8+, T and B cells, correlating with its known role in maturation of single-positive CD4 cells. As another example, *TCF7* and *Etv5* display higher methylation in B and T memory cells compared to naive counterparts [108]. The provenance of the aberrant methylation of specific target genes in the cancer cell remains to be fully elucidated. One source may be the aberrant expression of DNMTs normally responsible for the restricted

wave of methylation during blood development. Indeed the overexpression of DNMT1 and 3B, in addition to members of the methyl-CpG-binding proteins, has been reported in numerous malignancies including ovarian [111], breast [112], prostate [113] and lung cancers [114]. Furthermore, studies by Ostler *et al.* (2007) [115] reveal that truncated DNMT3B proteins deficient in the C-terminal catalytic domain are expressed in numerous cancer cell lines and primary acute leukemias. Overexpression of the most frequently expressed aberrant transcript, *DNMT3B7* in 293 cells, led to alterations in gene-expression patterns which corresponded with DNA methylation at CpG islands of these promoters, further supporting the role of DNMTs in the abnormal patterns of methylation observed in cancer cells [115]. Aberrant gene methylation in leukemia may also arise by the recruitment of DNMTs and associated chromatin-modifying proteins by cell type-specific transcription factors, which are commonly dysregulated in hematological malignancies including PML-RARα [116] and RUNX1/MTG8 [117]. The identification of such complexes and their specific target genes is likely to provide important insights into methylation-induced silencing in leukemic cells.

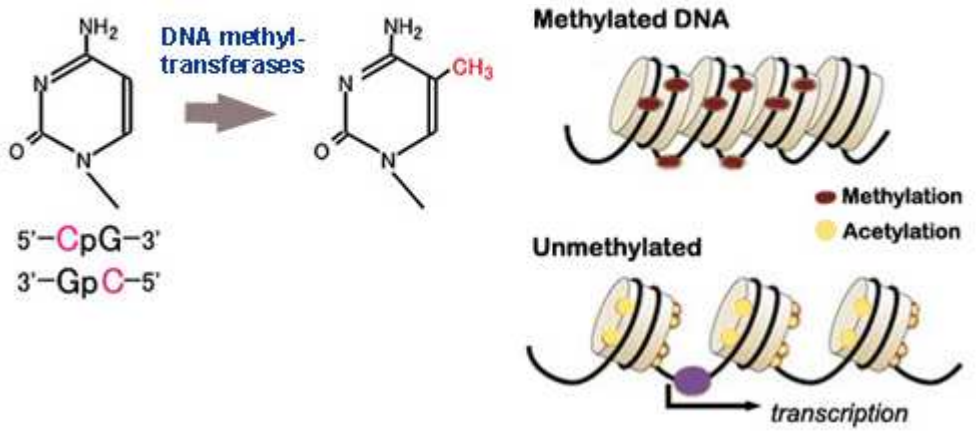

Fig. 7 DNA Methylation. Methylation by DNA methyltransferases at CpG islands represses gene transcription (Adapted from Taylor SM, 2006, Cellscience review, vol2 n.3)

Histone acetyltransferases and histone deacetylases: roles in normal hematopoiesis and leukemia

The acetylation of core histone tails in relation to gene expression has been extensively studied and is regulated by the opposing activities of HATs, which catalyse the transfer of acetyl groups from acetyl-CoA to lysine residues of target proteins, and HDACs, which catalyse the removal of acetyl groups. The ability of histone acetylation to regulate gene expression occurs via the direct effect of this modification on higher order chromatin structure, which serves to neutralize the charge between histone tails and the DNA backbone, and also by serving as a docking site for bromodomain-containing regulatory factors. In general, hyperacetylation of histones is associated with structurally 'open' chromatin and gene transcription, whereas histone deacetylation is linked to gene repression and/or heterochromatin formation [118] (Fig. 8). HATs can be divided into

three groups on the basis of their catalytic domains and comprise GNATs (Gcn5 *N*-acetyltransferases) which include Gcn5, p300/CBP-associated factor (PCAF), Elp3, Hat1, Hpa2 and Nut1 members; MYSTs, which include MOZ, MORF, Ybf2/Sas3, Sas2, HBO1 and Tip60 members; and p300/CBP (cAMP response element-binding (CREB) protein) [119]. These enzymes are recruited to target promoters by cell-specific transcription factors or chromatin-binding subunits such as bromodomain-containing proteins, or may even directly bind DNA, as in the case of activating transcription factor-2 [120]. HDACs can also be divided into categories on the basis of sequence and domain similarity, and include Class I HDACs (HDAC1–3 and HDAC8), which possess homology to yeast Rpd3 and are localized to the nucleus; Class II HDACs (HDAC4–7, HDAC9 and 10), which display similarity to the deacetylase domain of yeast Hda1 and travel between the nucleus and the cytoplasm; Class III HDACs, which consist of the silent information regulator (SIR2) family of nicotinamide adenine dinucleotide-dependent deacetylases (SIRT1-8) and Class IV HDACs (HDAC11) [121, 122]. Like HATs, HDACs function within the context of a multiprotein complex that includes DNA-binding transcriptional factors/unliganded nuclear receptors and co-repressor proteins such as NcoR, SMRT, Sin3a and NURD [123]. In addition to regulating transcription by affecting chromatin structure, HATs and HDACs are also capable of indirectly affecting gene expression by modifying non-histone substrates [121]. The acetylation of specific lysine residues of transcription factors has been shown to affect the subcellular localization, DNA binding, transcriptional activity, protein–protein interactions and stability of

several key transcription factors including p53, STAT3, RUNX1 and ETS, and not surprisingly, alterations in HAT/HDAC activity are linked to multiple cancers [124]. During hematopoiesis, lineage-restricted transcription factors regulate specific gene-expression patterns by recruiting HAT or HDAC complexes to the promoters of target genes [125]. For example, during erythropoiesis, erythroid-specific transcription factors including GATA-1, which is essential for red blood cell maturation and survival, directly recruit HAT-containing complexes to the β-globin locus to stimulate transcriptional activation. Specifically, GATA-1 recruits CBP to the β-globin gene locus, resulting in the acetylation of histones H3 and H4, and facilitating high-globin gene expression [126]. GATA-1 itself is also acetylated on conserved lysine residues by CBP, and although the effect of this modification remains controversial, the net result is enhanced transcriptional activity [127, 128]. In leukemia, the ectopic expression of wild-type (for example, TAL1/stem cell leukemia (SCL), BCL6) or chimeric transcription factors (for example, RUNX1-MTG8, TEL-AML1, PML-RARα and PLZF-RARα) results in the aberrant recruitment of histone-modifying activities to target genes that play important roles in cell cycle control and differentiation. TAL1/SCL, first identified by its translocation in T-cell acute lymphoblastic leukemia (T-ALL) [129] is a member of the basic helix–loop–helix (bHLH) transcription factors. TAL1/SCL is essential for the development of erythroid and megakaryocytic lineages, while negatively affecting myeloid differentiation. TAL1/SCL binds E-box motifs as a heterodimer with other bHLH proteins including E12, E47, HEB and E2-2, and is capable of activating and repressing transcription

depending on the specific association with co-activator or co-repressor complexes. For example, acetylation of TAL1/SCL by the co-activators p300 and the PCAF is linked to increased transcriptional activation and differentiation of murine erythroleukemia (MEL) cells in culture [130, 131], while the association with a co-repressor complex including mSin3A and HDAC1 in MEL and human T-ALL cells was linked to transcriptional repression and inhibition of erythroid differentiation [132]. The association between TAL1/SCL and mSin3A/HDAC1 declined upon MEL differentiation, suggesting that mSin3A and HDAC1 may inhibit the ability of TAL1/SCL to potentiate erythropoiesis and highlighting a possible mechanism for SCL-induced leukemogenesis [132]. Indeed, overexpression of Tal1/Scl in an E2A or HEB heterozygous background induced thymocyte differentiation arrest that was linked to the depletion of E47/HEB heterodimer and recruitment mSin3A/HDAC1 to the CD4 enhancer [133]. These tumors were hypersensitive to HDAC inhibitors, consistent with the notion that leukemogenesis by ectopically expressed TAL1/SCL was mediated by aberrant gene repression due to recruitment of co-repressor complexes. Translocations affecting HATs have also been implicated in tumorigenesis. For example, the rare translocations t(8;16)(p11;p13) and t(10;16)(q22;p13) fuse the MOZ (MYST3) and the MORF HATs with CBP in AML [134, 135]. In the case of the t(10;16)(q22;p13) translocation, the generation of MORF-CBP, which harbors the zinc-fingers, nuclear localization signals (NLS) and HAT domain of MORF, and the RARα-binding domain, CREB-binding domain, bromodomain and HAT domain of CBP, is thought to promote aberrant

patterns of acetylation; however since both reciprocal fusion proteins are expressed, the leukemogenic potential of these fusion proteins is unclear. Irrespective of the exact mechanism, the expression of these fusion proteins is associated with the loss of monoacetylated H4K16, which was recently identified as a common mark of cancer transformation [136]. The mixed lineage leukemia (MLL) gene is also involved in a translocation involving CBP, and the resultant MLL-CBP fusion has been shown to require both the CBP bromodomain and HAT domain for leukemic transformation [137]. These findings highlight the importance of HATs and HDACs in regulating genome-wide and loci-specific chromatin structure.

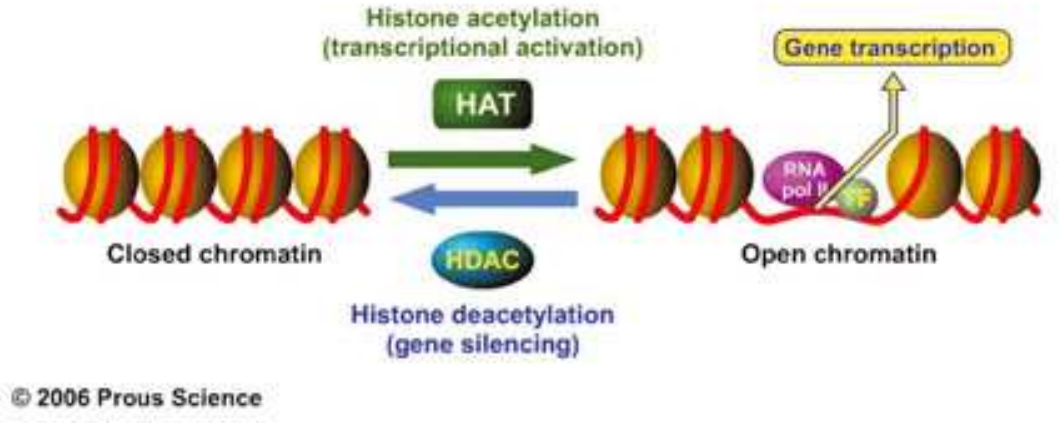

Fig 8 HAT and HDAC regulate gene transcription modifying chromatin structure (adapted from McIntyre J. Combination therapy with valproic acid acid in cancer: Initial clinical approach, 2007, Drug of the future, 32(1):45)

Epigenetic drugs in clinical trials for haematological Malignancies

HDAC inhibitors

HDAC inhibitors are a diverse group of compounds able to induce histone hyperacetylation and changes in a large set of proteins [121].

Several HDAC inhibitors (HDACis) exhibit impressive anti-tumour activity potentiated by little toxicity in *in vitro*, *ex vivo* and *in vivo* models and are now involved in clinical trials as monotherapies as well as in combination with other drugs [138]. Several classes of HDACis have been identified including short-fatty acids (such as butyric acid), hydroxamic acids (such as suberoylanilide hydroxamic acid, SAHA and trichostatin A, TSA), cyclic tetrapeptides and benzamides (such as MS-275). While it is well established that an important component of the action of HDACis is the induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, many sets of data point to the exciting potential of HDACis with selective anti-cancer activity but indicate the involvement of different molecular pathways. It has become quite clear that modulation of gene expression by HDAC inhibitors is not sufficient to describe their effects. The extensive research involving HDAC inhibitors has shown that they have effects in addition to the modulation of gene expression through chromatin remodelling (Fig. 9). Therefore, focusing on a unique target is likely to be inadequate in explaining the actions of HDAC inhibitors. Moreover, HDAC inhibitors can induce acetylation of protein targets, including transcription factors and heat shock proteins, which may contribute to apoptosis. The effects of

HDAC inhibitors on the extrinsic (TRAIL, Fas and death receptors) [139, 140] and intrinsic (mitochondrial) death signalling pathway [141] have been extensively studied. The reported changes differed according to the tumour and cell type. Moreover, in leukaemia cells, HDAC inhibitor induced apoptosis was caspase dependent [139, 140] whereas in myeloma cells it was independent of caspases [142]. Other possible mechanisms for HDAC inhibitor apoptosis, such as nuclear factor-kB activation, generation of ceramide, and modulation of heat shock proteins, have also been described [143].

Despite efforts still needing to be made in the understanding of the anticancer mechanism(s) of HDAC inhibitors, many clinical trials at present are actually based on their use against cancer. SAHA (vorinostat, Zolinza®; Merck) was approved by the US FDA in October 2006 for the treatment of cutaneous manifestations in patients with cutaneous T-cell lymphoma (CTCL) who have progressive, persistent or recurrent disease on or following two systemic therapies. Thereby, SAHA is the first of a new class of anticancer agents (<http://www.fda.gov/cder/foi/label/2006/021991lbl> *2006*). Apart from CTCL, vorinostat alone or more frequently in combination, is being used in clinical trials for many solid and haematological cancers, including pancreatic, breast, several types of leukaemia and lymphoma, thyroid, multiple myeloma, mesotelioma, NSCLC and colon cancer [121, 138, 144]. Moreover, vorinostat is also being applied in trials for pre-neoplastic manifestations such as MDS or myeloproliferative disorders. Apart from SAHA, a number of hydroxamates are being used in phase I–III clinical

trials as anticancer agents including the indolylethylamino-methylcinnamyl hydroxyamides LAQ-824 and LBH-589 (panobinostat) [145, 146]. However, whereas the development of LAQ-824 was terminated due to the possibility of toxicity problems, panobinostat is currently involved in a phase II/III clinical trial for chronic myeloid leukaemia (CML), refractory CTCL and multiple myeloma (MM). Panobinostat has been reported as being highly active against HDAC class I and IIa (IC_{50}s= 14 to 3 nM), but less potent against HDAC6 and HDAC8 [147]. Belinostat (formerly PXD-101) is another cinnamyl hydroxamate which inhibits both class I and class II HDACs in the submicromolar range. Belinostat is being used in phase II clinical trials for haematological malignancies as well as for many solid tumours [148, 149]. The cyclic peptide romidepsin (formerly known as FK-228) is involved in clinical trials for CML, AML and CTCL [150-152]. The benzamide MS-275 has been shown to induce apoptosis of acute myeloid, B-chronic lymphocytic leukaemia cells, Jurkat lymphoblastic T cells and prostate cancer cells [139, 153, 154]. This HDACi is used in clinical trials in combination with other anti-tumour agents [153, 155]. MGCD-0103 is a more recent benzamide developed by MethylGene. MGCD-0103 behaves as a HDAC1/2-selective HDACi [147] and is currently being used in phase II clinical trials for the treatment of haematological malignancies and in phase I/II trials for solid tumours [156].

Although it is tempting to speculate that HDACis kill cells by causing cell death or differentiation, their pleiotropic actions suggest that this is an oversimplification. For example, attention has been recently focused on

the ability of HDAC inhibitors to act as 'protein acetylases' altering the tubulin deacetylases (such as HDAC6), and interfering with the function of chaperone proteins [157]. The diverse mechanism(s) of HDACi action suggest that the simple acetylation of histones might not be the optimal tool to determine their activity, given that histone acetylation might be necessary, but not sufficient, for response. An improved understanding of the mechanism of action of HDAC inhibitors will be crucial for the rational design of future combination trials. A good deal of research interest concerns the issue of whether HDAC inhibitors will work more effectively when used in combination with existing anti-tumour drugs. Apparently, the synergistic effects generated from the combined use of HDACis and chemotherapy will be one of the hot issues in cancer therapy research [158]. Indeed, there is currently considerable interest in the development of regimens combining HDACis with other targeted agents that can enhance HDACi lethality, such as cyclin-dependent kinase inhibitors, Hsp90 antagonists, proteasome inhibitors and tyrosine kinase inhibitors [159-161]. Whether this approach will lead to improved antitumour action and selectivity is likely to be answered in the next few years.

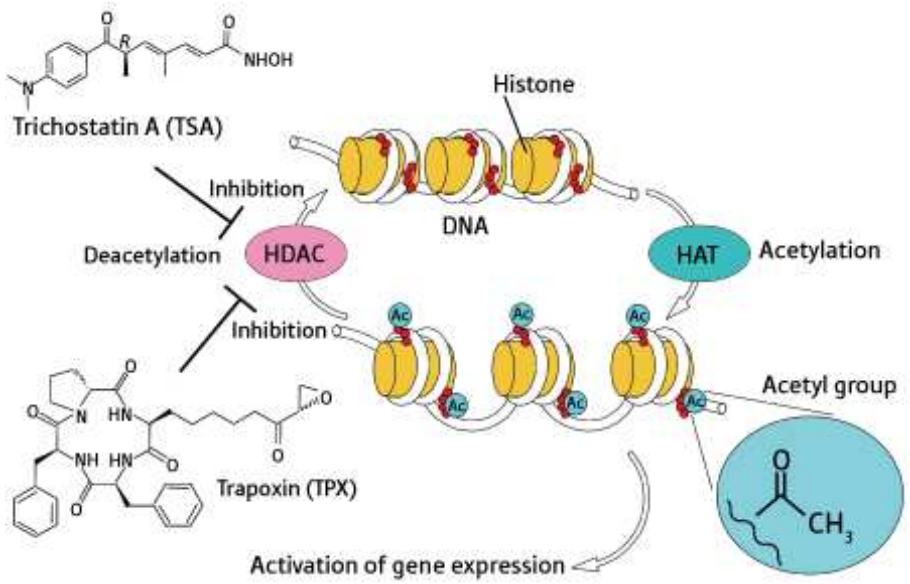

Fig 9 HDAC inhibitors induce hyperacetylation of histones promoting gene expression through chromatin remodelling.

DNA demethylating agents

The term DNA demethylating agents refers to compounds able to induce transient DNA hypomethylation. DNA methylation is the addition of a methyl group to a CpG site [162]. These sites bunch together in areas known as 'CpG islands' frequently in the proximity of gene promoters. DNA methylation, both aberrant and physiologic, can result in gene silencing and in the corresponding gene inactivation, due to either mutations or deletions, of tumour suppressor genes [163].

Actually, two hypomethylating agents are approved and widely used: 5-azacitidine [164] and 5-aza-2'-deoxycitidine (decitabine) [165]. These two drugs have significant activity in patients with higher risk myelodysplastic syndromes (MDS). Decitabine is a nucleoside analogue that indirectly inhibits DNMTs, thus resulting in global hypomethylation [166]. The finding of aberrant DNA methylation as a critical event in cancer, and the reversion by hypomethylating agents, has led to an increasing interest in

performing clinical trials with these compounds in MDS and acute myelogenous leukaemia (AML). Some concerns regarding the use of DNMT inhibitors have been raised as a result of the finding of genome instability in mice with reduced DNA methylation levels due to prolonged demethylating treatment [167], even if those results are not directly comparable to the transient DNMT inhibition applied in cancer patients. Initially, decitabine was used in several clinical trials in AMLs and higher risk MDS and some chronic myelogenous leukaemias (CML). In the initial studies, decitabine resulted in better responses compared with supportive care. Although the results of these initial studies are of great relevance, alternative schedules that did not require hospital admission have been tested [168]. The main toxicities were myelosuppression and its complications. In all cases the treatment with decitabine was associated with improved survival despite the fact that complete remissions were lower when compared with chemotherapy. This is probably due to the low induction of mortality observed. This notion represents a change in practice, as we may agree to a lower response rate if mortality is significantly lower and translates into better survival. 5-Azacitidine is the other nucleoside analogue, structurally related to decitabine. In contrast to decitabine, 5-azacitidine is a ribose structure that is incorporated into RNA and requires the activity of ribonucleotide reductase (RNR) to be incorporated into DNA and to exert its hypomethylating effect (Fig.10). Recent studies have reported a significant effect on survival in patients that received 5-azacitidine versus other treatments [169]. Interestingly,

patients with alterations of chromosome 7 derived the most benefit: clearly, these evidences will need further study.

From a mechanistic point of view, the most widely studied combination includes hypomethylating drugs and HDAC inhibitors [102]. Several studies have been reported with the combination of 5-azacitidine or decitabine with valproic acid (VPA) [170]. These studies with VPA have been mainly phase I or II in AMLs and higher risk MDS. The relative weakness of VPA as a HDACi supports the notion that replacing VPA by more active HDAC inhibitors may result in better clinical combinations. Such studies are ongoing, using either 5-azacitidine or decitabine with several HDAC inhibitors. One such study is the combination of 5-azacitidine and MGCD0103, a class 1 HDAC inhibitor with activity in AML and MDS [170-172].

Other nucleoside analogues, such as zebularine, have been developed to overcome the instability of decitabine and 5-azacitidine [173]. Unfortunately, zebularine has limited oral bioavailability in a pharmacokinetic study, and it is not clear whether active concentrations can be clinically achieved with oral administration [174]. Recently, additional compounds, such as the local anaesthetic procaine (and its derivative procainamide) and the main polyphenol compound in green tea, EGCG ((–)-epigallocatechin-3-gallate), have been shown to inhibit DNMTs and some of them are currently in phase I trials. Moreover, psammaplins have shown both DNMT and HDAC activity inhibition which might enable a combinatorial inhibition by using a single drug [175]. Finally, RG108 is the first rationally designed DNMT1 inhibitor with demethylating activity

both in *in vivo* and *in vitro* models [176]. Unfortunately, its activity seems too weak to have any potential in clinical use.

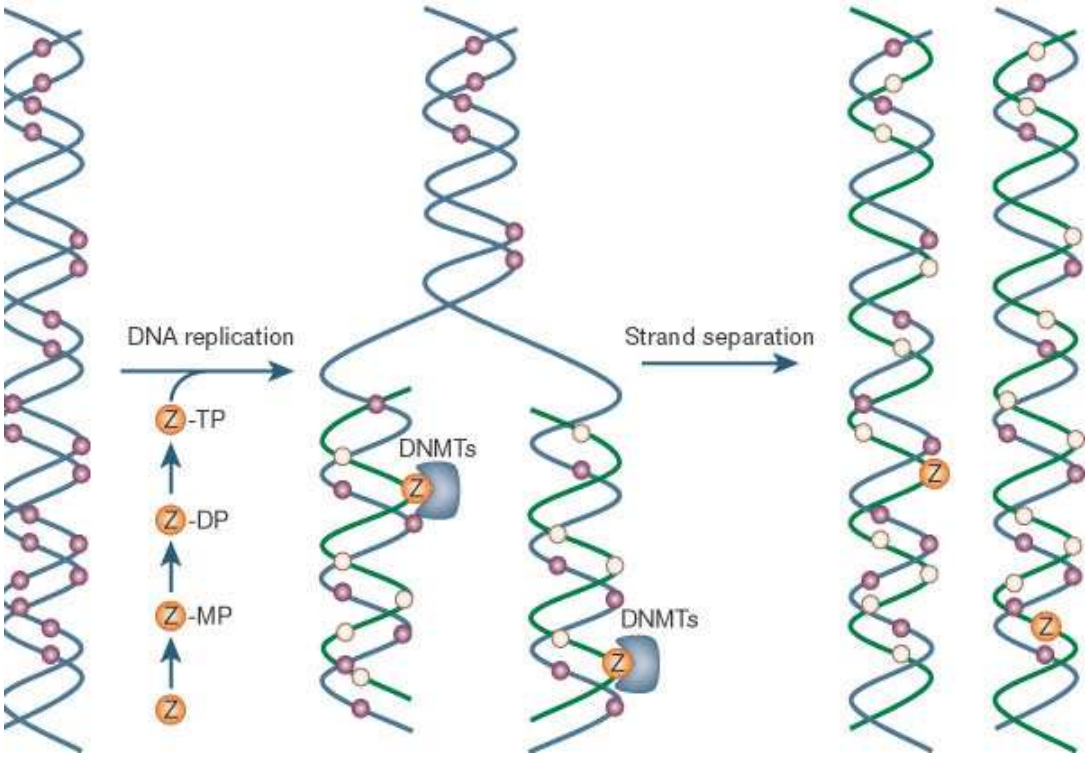

Fig 10 Deoxynucleoside analogues such as 5-aza-2'-deoxycytidine (depicted by Z) are converted into the triphosphate inside S-phase cells and are incorporated in place of cytosine into DNA. Ribonucleosides such as 5-azacytidine or zebularine are reduced at the diphosphate level by ribonucleotide reductase for incorporation (not shown). Once in DNA, the fraudulent bases form covalent bonds with DNA methyltransferases (DNMTs), resulting in the depletion of active enzymes and the demethylation of DNA. Pink circles, methylated CpG; cream circles, unmethylated CpG.
(adaptated from Egger G. Mechanism of action of nucleoside analogue inhibitors, 2004, Nature, 429:460)

Epigenetic alterations in haematological malignancies.

Conceptually, there are two main classes of epigenetic alterations that are potentially found in tumour cells: those due to the direct action of the triggering transforming event, and those subsequent to the transformation process itself. In this introduction we will focus an important and well

known epigenetic alteration found in acute promyelocytic leukaemia (APL).

Acute promyelocytic leukaemia (APL)

Retinoic acid receptors (RARs) are the main effectors of the signalling network instigated by retinoic acid (RA), and are important modulators of several cell processes, including haematopoietic differentiation [177]. RARs are transcription factors that bind specific response elements and repress transcription of target genes in the absence of a ligand, and activate transcription when they become bound by RA: the ligand triggers a conformational change in the receptor, leading to a switch in the association with transcriptional cofactors: repressive (such as HDACs) in the unbound state, co-activating in the presence of RA [178]. APL is caused in 100% of cases by RAR translocations, yielding different RAR fusion proteins (in >90% cases, the fusion partner is *PML,* and the fusion protein is PML-RAR) [178]. PML-RAR binds DNA with an altered specificity, expanding the repertoire of potential target genes for the fusion protein [179]. Additionally, physiological concentrations of RA are not able to induce the switch in the association with transcriptional coregulatory complexes, and PML-RAR maintains a repressive chromatin structure at target genes in the presence of RA [180]. There is a complex network of PML-RAR associated chromatin remodelling complexes: class I HDACs as part of Sin3 and NURD complexes, DNA methyl-transferases and factors able to bind methylated DNA (MBDs), and histone methyltransferases [116, 181-183] (Fig. 11). In several model systems (mainly at the cellular

level), recruitment of those activities has been shown to be required for the altered biological properties of the fusion protein [116, 181-183]. These studies have led to the proposal of a general model where the effect of the fusion protein is to 'freeze' the chromatin structure of target genes in a strongly repressive conformation, resembling heterochromatin [121]. This structure is refractory to physiological stimuli that occur during haematopoietic differentiation and that require activation of PML-RAR target genes, thus resulting in a block of differentiation [121].

Unfortunately, however, several bits of information are missing: for example, only a few studies are starting to address, systematically, chromatin changes induced by PML-RAR expression at target genes: most of the studies mentioned above make use of only one (the RARß promoter) or a few RAR target genes as exemplifying the entire picture, which is certainly not the case. Reassuringly, recent analyses confirm the ability of the fusion protein to recruit HDACs and cause histone deacetylation and histone methylation (histone H3K9 trimethylation) of target genes [184]. One important element to be considered is that the use of artificial cell models, although of clear help in the design of models and hypothetical mechanisms of action, is not sufficient to fully validate the proposed mechanism of action: mechanistical studies have to be performed in 'real', patient-derived cells, and – whenever possible – *in vivo* (through the use of animal models).

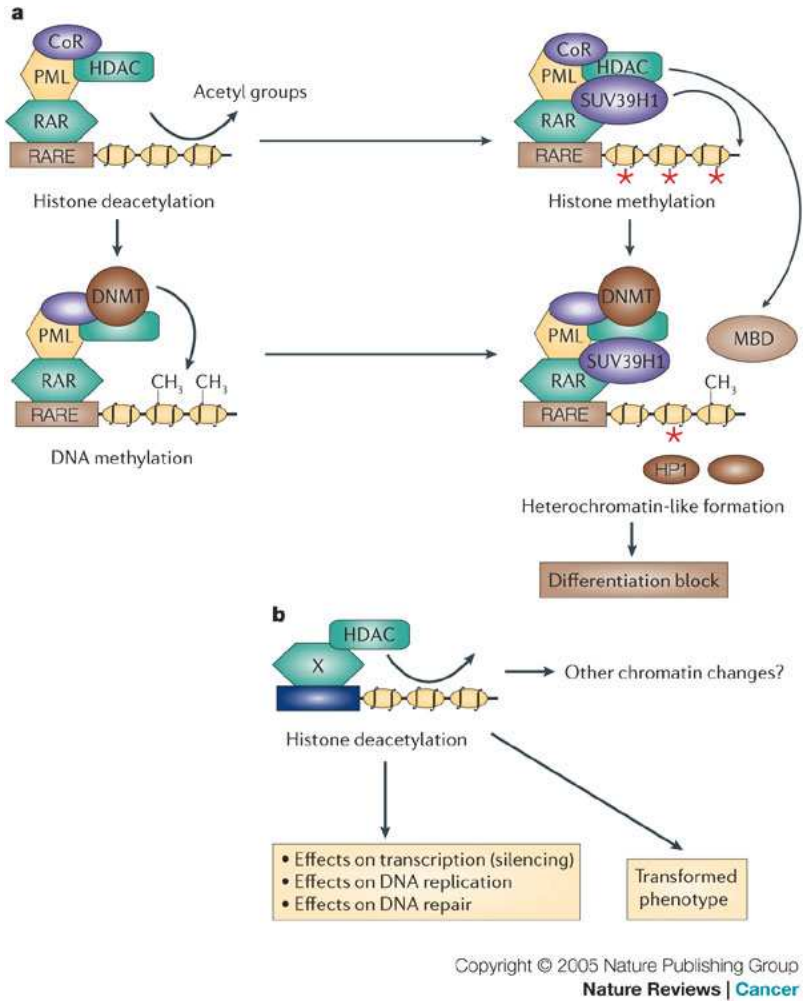

Fig. 11 A model for the deregulated action of HDACs on chromatin in APL and in other cancer cells. (adaptated from Minucci S. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer, 2006, 6:43)

Anaplastic large-cell lymphoma (ALCL)

Anaplastic large-cell lymphoma (ALCL) is a relatively uncommon tumor. It was first recognized by Stein et al. [185] in 1985, who reported the consistent expression of the Ki-1 antigen (later designated CD30) in tumors with frequent cohesive proliferation of large pleomorphic cells. Most of these tumors were labeled "histiocytic malignancies" [185]. The Ki-

1 monoclonal antibody was originally described by the same group and was used to identify a novel antigen in the Hodgkin lymphoma cell line L428 [186]. Subsequent immunophenotyping and gene rearrangement studies showed that the vast majority of ALCL tumors are derived from lymphoid cells of T or null immunophenotype [187]. Histologically, several ALCL variants have been described. Of these variants, the common, lymphohistiocytic, and small-cell are the most frequently encountered. The "horseshoe" or "wreath" cell is considered the cytologic hallmark of this disease [188]. ALCL occurs as two distinct clinical entities, as a widespread systemic disease, or as a localized cutaneous disease. Systemic ALCL comprises 2% to 8% of non-Hodgkin lymphomas in adults and 10% to 15% of these lymphomas in children [189]. The frequency of ALCL increases to 30% to 40% of non-Hodgkin lymphomas in children when only cases with large-cell morphology are included

ALK

The recognition of t(2;5)(p23;q35) established the molecular definition of a subset of ALCL tumors that harbors this translocation [190-192]. In 1994, two independent groups cloned the genes involved in this translocation and illustrated the fusion of the nucleophosmin (*NPM*) gene on chromosome 5q35 to the previously unidentified gene anaplastic lymphoma kinase (*ALK*) gene on 2p23 [193, 194]. This chromosomal translocation leads to the generation of the chimeric protein NPM-ALK. Identifying these tumors with this translocation became clinically feasible after the production of antibodies that specifically interact with chimeric

NPM-ALK and full-length ALK proteins [195]. Tumors harboring t(2;5)(p23;q35) and expressing ALK soon became a distinct clinicopathologic entity known as ALK-positive (ALK^+) ALCL, which is included in the current World Health Organization classification system . Several other chromosomal aberrations involving *ALK* were subsequently identified, including t(1;2)(q21;p23) [chimeric protein TPM3-ALK], inv2(p23q35) [ATIC-ALK], t(2;3)(p23;q21) [TFG-ALK], t(2;17)(p23;q23) [CLTC-ALK], t(2;19)(p23;q13.1) [TPM4-ALK], and t(2;X)(p23;q11-12) [MSN-ALK] [196]. Immunohistochemical staining has shown that ALK expression is both cytoplasmic and nuclear in tumors with t(2;5)(p23;q35) but is strictly cytoplasmic in most of the other variants [197]. All the *ALK* chromosomal aberrations lead to the expression and constitutive activation of ALK. This transmembrane receptor tyrosine kinase belongs to the insulin receptor superfamily. ALK expression in humans is normally limited to cells of neural origin. In mice embryos, it is widely expressed in the nervous system, but its expression level decreases significantly at birth [198, 199]. In *Drosophila melanogaster*, ALK is widely expressed in the gut [200]. These observations suggest that ALK plays an important role in the development of these systems. Full-length ALK has been detected in neuroblastoma and rhabdomyosarcoma [201, 202]. Full-length ALK protein expression has been described not only in nonhematologic tumors but also in rare cases of diffuse large B-cell lymphoma [203]. Whereas full-length ALK is not detected in normal hematopoietic cells, low levels of *NPM-ALK* and *ATIC-ALK* fusion gene mRNA have been identified in these cells [204, 205]. The expression of ALK in hematologic neoplasms is

largely limited to ALCL tumors of T-cell or null-cell immunophenotype, in which 40% to 60% of these tumors express ALK [201]. In 80% of these cases, ALK expression results from t(2;5)(p23;q35). Rare cases of large B-cell lymphoma have been found to have *ALK* rearrangements [206, 207]. *ALK* rearrangements and CLTC-ALK, TPM3-ALK, and TPM4-ALK chimeric proteins have also been detected in inflammatory myofibroblastic tumors and neuroblastoma [208, 209]. How ALK is physiologically activated is not completely known. In *D melanogaster*, ALK is activated by the Jelly belly (Jeb) protein, and the Jeb/ALK pathway appears to play an important role in the development of the gut system [210, 211]. In humans, the heparin-binding growth factors pleiotrophin and midkine have been reported to be the ligands binding and activating ALK [212, 213], but this idea is still controversial [214]. ALK^+ ALCL tends to affect children and young adults and has a striking male predominance. The majority of ALK^+ ALCL cases present as advanced stage (III or IV) systemic disease with generalized lymphadenopathy and extranodal involvement; particularly of the skin and soft tissue [215]. Compared with ALK-negative ALCL, ALK^+ ALCL demonstrates a significantly favorable prognosis [216].

NPM-ALK

To understand the oncogenic role of NPM-ALK, we need to understand the physiologic functions of the ALK and NPM proteins. We discussed the physiologic functions of ALK in the previous section. In contrast to ALK, NPM is a ubiquitously expressed RNA-binding nucleolar phosphoprotein [217]. Its physiologic functions include the shuttling of ribonucleoproteins

between the nucleus and cytoplasm. NPM carries the oligomerizing motif that drives the homodimerization of NPM-ALK, which leads to the constitutive activation of ALK tyrosine kinase [218]. Numerous studies have proven that NPM-ALK is oncogenic, and its transforming ability has been repeatedly demonstrated in vitro [218, 219]. Transgenic mouse models have shown that the enforced expression of NPM-ALK leads to the development of malignant lymphoma. A significant number of these lymphomas were of plasmablastic or B-cell immunoblastic morphology and phenotype, even when NPM-ALK expression was driven by T-cell–associated antigen promoters [220-225]. Furthermore, several of these lymphomas were restricted to the mediastinum and they lacked CD30 antigen expression. These important observations indicate that despite the universally accepted role of NPM-ALK in promoting lymphomagenesis, it is probably not the only factor that configures the known morphologic, immunophenotypic, and clinical features of NPM-ALK–expressing ALCL as we know it in humans. Most probably, NPM-ALK interacts with other biologic and molecular factors to result in the development of NPM-ALK–expressing lymphoma in humans with its characteristic features. NPM-ALK has been shown to interact with a wide range of oncogenic molecules (Fig. 10).

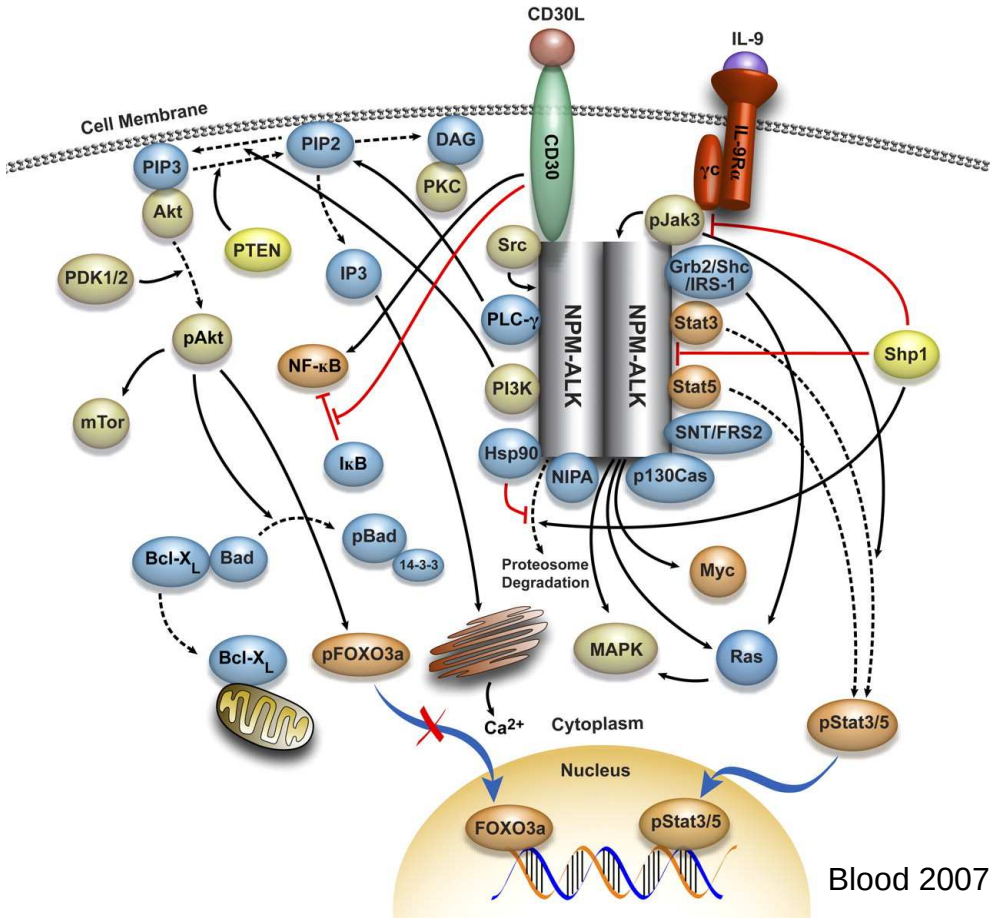

Fig.10 Molecular network interacting with NPM-ALK. A complex network of protein kinases, protein phosphatases, transcription factors, apoptosis and cell-cycle regulators, adaptor proteins, and other molecules has been proposed to interact with NPM-ALK. (adaptated from Amin HM. Pathobiology of ALK+ anaplastic large-cell lymphoma, 2007, Blood, 110: 2261)

Oncogenic signalling of NPM-ALK-fusion proteins in ALK+ ALCL

The oncogenicity of NPM-ALK fusion protein and other chimeras derived from rearrangements involving ALK gene in ALCL is due to the constitutive activation of the ALK catalytic domain, resulting in enhanced cell proliferation and survival. ALK-mediated transformation occurs through the activation of specific downstream molecules and pathways (Fig. 1), among which the Ras-ERK pathway seems to be essential for ALCL proliferation [196, 226], whereas the JAK3-STAT3 and PI3K-Akt

pathways are probably more important for cell survival [227, 228]. Constitutively phosphorylated NPM-ALK tyrosine residues act as docking sites for several downstream adaptors with SRC homology 2 (SH2) or phosphotyrosinebinding domains, such as SRC, SH2 domain-containing transforming protein (SHC), insulin receptor substrate 1 (IRS1) and growth factor receptor-bound protein 2 (GRB2). These molecules mediate the activation of the Ras-ERK pathway and then promote mitogenic stimuli responsible for lymphomagenesis [226]. Another molecule recently described to be involved in the NPM-ALK mediated transformation through Ras-ERK activation is the tyrosine phosphatase SHP2, also known as PTPN11 [229]. Anti-apoptotic signals in ALK+ ALCL are mostly mediated by the JAK-STAT and PI3K-Akt pathways. STAT3 has a central role in ALK-mediated tumourigenesis, as shown by both in vitro and in vivo studies [228, 230], and can be phosphorylated directly by NPM-ALK or through the activation of JAK3. STAT3 activation results in increased transcription of antiapoptotic factors and cell-cycle regulators such as BCLXL, survivin, cyclin D3, C/EBPb and myeloid cell leukaemia 1 (MCL1) [228, 231, 232]. PI3K is directly activated by NPM-ALK and phosphorylates its downstream effectors AKT1 and AKT2 [227]. The consequence of AKT activation is the inhibition of forkhead box O3A (FOXO3A), leading to upregulation of cyclin D2 and downregulation of FOXO3A-mediated transcription of genes that promote apoptosis and cell cycle arrest, such as BIM and p27 [233]. AKTalso functions as an activator of the mammalian target of rapamicin (mTOR), another molecule involved in cell survival regulation, and as an inhibitor of other pro-apoptotic

proteins such as BCL2-antagonist of cell death (BAD). It finally increases proteasomal degradation of p27 by phosphorylation [234, 235]. PI3K inhibitors cause apoptosis in NPM-ALK expressing lymphoma cells [227, 236], underlying the importance of the PI3K-Akt pathway in the control of cell survival in ALK+ ALCL. It has been recently shown that NPM-ALK is also able to phosphorylate JUN N-terminal kinase (JNK), hence inducing JUN activation and finally enhancing the activity of the transcription factor AP1. The result is the downregulation of p21 and the upregulation of cyclin D3 and cyclin A, responsible for cell cycle progression [237]. Another pathway involved in ALK-mediated lymphomagenesis seems to be the phospholipase C-γ (PLC-γ) pathway. PLC-γ probably binds directly to NPM-ALK through its SH2 domain, and the increased intracellular levels of Ca2þ generated by PLC-γ activation stimulate PKC, a kinase that affects the Ras-ERK pathway.

Aim

The Bim protein (Bcl-2 interacting mediator of cell death) is a pro-apoptotic BH3-only, Bcl-2-family member that appears to be a uniquely important tumour suppressor in the development of B and T lymphocytes. It regulates apoptosis during lymphocyte development by binding and inactivating prosurvival members of the Bcl-2 family and binding and activating the pro-apoptotic family member Bax [7, 238]. The role of Bim in lymphomagenesis came sharply into focus when it was discovered that in Em-Myc transgenic mice constitutively expressing Myc in B cells, loss of even a single Bim allele significantly accelerated lymphoma development and revealed Bim as a haploinsufficient tumor suppressor. Several studies have demonstrated that BIM activity can be controlled through transcriptional and post-translational mechanisms [25, 32, 54, 56-59, 239]. The Bim 5' regulatory region has a very large CpG-island that could be subject to transcriptional control. The predicted CpG-island is 6718 bp long and contains 595 CpG dinucleotides [240]. In the literature there are data about the role of epigenetic modifications in the regulation of BIM expression in hematologic malignancies. Epigenetic downregulation of Bim expression is associated with reduced optimal response to imatinib treatment in chronic myeloid leukemia [241] and with glucocorticoid poor-responsive pediatric acute lymphoblastic leukemia [242]; furthermore the proapoptotic *BIM* gene presented homozygous deletion in mantle cell lymphoma and promoter hypermethylation in Burkitt lymphoma [243].

The aim of this project will be to investigate if NPM-ALK+ ALCL (Anaplastic Large Cell Lymphoma), can escape the pro-apoptotic activity of BIM by epigenetic changes on its promoter region.

The methylation status of BIM promoter will be analyzed in neoplastic haematological cell lines, NPM-ALK+ and NPM-ALK- (negative control) cells, by bisulphite modification clonal sequencing analysis (BMCSA) and Real-Time Methylated-DNA immunoprecipitation (RT-MeDIP). Furthermore, the acetylation status of histone H3 will be evaluated in order to assess the linkage between BIM promoter hypermethylation and chromatin condensation. The role of BIM epigenetic modifications in the progression of tumours will be investigated through *in vitro* experiments in haematological cell lines and analyzing the presence of BIM promoter hypermethylation in *in vivo* tumoral samples. The majority of ALCL carries the translocation t(2;5) that encodes for the oncogenic tyrosine kinase NPM-ALK, fundamental for survival, proliferation, and migration of transformed T cells.

NPM-ALK protein is shown involved in epigenetic silencing, through methylation of CpG sites, of key molecules involved in the propagation of the TCR signaling. [244]. Therefore we will perform experiments to investigate if the oncogenic tyrosine kinase NPM-ALK could act in epigenetic regulation of Bim locus.

Materials and Methods

Patients samples

All the patients analyzed signed a written informed consent.

Cell lines

The cell lines NPM-ALK+: SU-DHL-1, SUP-M2, Karpas-299. The cell lines NPM-ALK- : K-562, LAMA-84-S. Inducible short hairpin RNAs (shRNA) cells: SU-DHL1 cells transduced with lentiviruses expressing an inducible shALK and an shCTRL (negative control of shRNA) were obtained from Chiarle's group of research. These shRNA cells were treated with 1μg/mL doxycycline for 96 h before the experiments. All these cell lines were cultured in RPMI-1640 (Bio WhittakerTM, Cambrex Biosciences, New Jersey, USA) supplemented with 10% fetal bovine serum (Bio WhittakerTM, Cambrex Biosciences, New Jersey, USA), 100Units/mL penicillin, 100μg/mL gentamycin and 2mM L-glutamine (Bio WhittakerTM, Cambrex Biosciences, New Jersey, USA). The cells were cultured in a humidified incubator at 37°C in an atmospohere of 5% CO_2.

5-Azacytidine, Tricostatin-A, NPM-ALK inhibitor treatments

10^6 cells for each cell line were seeded and treated with and without 1μM 5-Azacytidine (AzaC) (Sigma, St. Louis, MO, USA), 500nM Tricostatin-A (TSA) (Sigma, St. Louis, MO, USA) or NPM-ALK inhibitor 1.2μM. After 5 days , 3 days, 5/15 hours of treatment respectively with AzaC, TSA, or NPM-ALK inhibitor, cells were analyzed.

DNA modification by Sodium Bisulfite/ Hydroquinone reaction

DNA was extracted using TRIzol LS reagent (Invitrogen, Carlsbard, CA, USA) according to the manufacturer instructions . 2µg of genomic DNA was digested with BamHI (New England Biolabs, Ipswick, MA, USA) at 37°C. The reaction was stopped after 6 hours incubating the sample at 70°C for 5 minutes. Digested DNA was denatured with 0.3M final concentration of NaOH at 37°C for 15 minutes. Then Sodium Bisulfite (3M final concentration) and freshly prepared Hydroquinone (50mM final concentration) (Sigma, St.Louis, MO, USA) were added. The samples were overlaid with mineral oil and incubated in the dark at 50°C for 15 hours. The bisulfite-treated DNA was purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), eluted in 105µL of 1mM Tris-HCl pH 8 and incubated with 11µL of 3M NaOH at 37°C for 20 minutes. The DNA was precipitated with ammonium acetate (0,7M final concentration), 1µg of tRNA and 100% ethanol at -20°C for at least 4 hours. The pellet was washed with 70% ethanol and resuspended in 100µL of 1mM Tris-HCl pH 8. Bisulfite-treated DNA was stored at - 20° C until use.

Amplification, cloning and sequencing

5µL of bisulfite-treated DNA was amplified with a Nested-PCR protocol. The reactions were performed in 50µL volume containing GeneAmp PCR Buffer (Applied Biosystems, New Jersey, USA), 200µM each dNTPs, 2.5U Amplitaq (Applied Biosystems, New Jersey, USA) and 0.4µM of each primer. The primers used for the reaction were

Bim-CG-Ext-For 5'GTGTGTATTTTAGAGAAGTT3' and

Bim-CG-Ext-Rev 5'CCTTCAA AATTACCTTATAAC3' for the first PCR,

Bim-CG-For 5'GTTAGATTTTTTTTAGATTTGTTG3' and

Bim-CG-Rev 5'CAAACTA CAATTATCTACCTTC3' for the nested PCR.

PCR amplifications were carried out on a Mastercycler Personel (Eppendorf, Hamburg, Germany). The cycling conditions for amplification were, for the first round of PCR: 5 minutes at 94°C , 5 cycles of 1 minute at 94°C, 2 minutes at 48°C and 3 minutes at 72°C, 25 cycles of 30 seconds at 94°C, 2 minutes at 48°C and 90 seconds at 72°C with a final extension step of 10 minutes at 72°C. For the nested PCR: 2 minutes at 92°C, 30 cycles of 30 seconds at 92°C, 30 seconds at 48°C, 80 seconds at 72°C and a final step of 7 minutes at 72°C. PCR products were separated by electrophoresis on agarose gel, purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned using the TOPO cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA). At least 6 colonies for each sample were analyzed. Mini-preps were prepared using GenElute Plasmid Miniprep Kit (Sigma, St. Louis, MO, USA). The MWG-Biotech (MWG-Biotech AG, Ebersberg, DE) performed all DNA sequencing. Sequence analysis and alignments were performed using Vector NTI 7.0 (Invitrogen, Carlsbad, CA, USA). This analysis, defined as Bisulphite Modification Clonal Sequencing Analysis (BMCSA), allows the evaluation of the methylation status of 19 CpG sites sited from nucleotide -504 to +64 from the ATG start site of BIM gene.

TaqMan Real Time PCR

cDNA was synthesized from 20ng of total RNA, using 'TaqMan Reverse Transcription Reagents' (Applied Biosystems, Foster City, CA, USA). Quantitative Real Time RT-PCR was performed using 'TaqMan Universal PCR Master Mix' (Applied Biosystems, Foster City, CA, USA) on a '7900HT Sequence Detection System' (Applied Biosystems, Foster City, CA, USA) under standard conditions. All the Real Time RT-PCR experiments were performed in triplicate. mRNA of the housekeeping β-glucoronidase gene (GUS) was used as an internal reference. The sequences of BIM and GUS probes were respectively:

BimEx4_RevProbe 5'FAM-CCGCAACTCTTGGGCGATCCATATCTCTC TAMRA3' and

GUS-probe 5'FAM-CCAGCACTCTCGTCGGTGACTGTTCA-TAMRA3'.

The forward and reverse primers for Bim and GUS were respectively:

BimEx8_Rev 5'GGTGGTCTTCGGCTGCTTGG3' and

BimEx4_For 5'TTCCATGAGGCAGGCTGAAC3',

GUS-for 5'GAAAATATGTGGTTGGAGAGCTCATT3' and

GUS-rev 5'CCGAGTGAAGATCCCCTTTTTA3'.

The Real Time RT-PCR system was designed to obtain a 140bp fragment for Bim (amplifying all the 3 major splicing variants of Bim) and a 101bp fragment for GUS.

Western blot analysis

Rabbit anti-Bim and anti-Actin monoclonal antibodies were purchased from Sigma-Aldrich (Sigma, MO, Saint Louis, USA). Protein lysates,

prepared as reported above, were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Biorad Laboratories, Palm Springs, USA) and blotted onto Hybond ECL nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) following standard protocol. Proteins were visualized by chemiluminescence (Super Signal; Pierce, Rockford, IL, USA) with Image Station 440 CF (Eastman Kodak Company, NY, USA).

Methylated DNA Immunoprecipitation (MeDIP)

10µg of genomic DNA extracted as reported above were sonicated using a Labsonic BBraun sonicator. The protocol for the sonication step was optimized for the different cell lines analyzed. The average size of the sheared chromatin was ~500bp. 1µg of sonicated DNA was immunoprecipitated overnight at 4°C using 30µg of the polyclonal 5-Methyl-Cytosine antibody ab1884 (Abcam, Cambridge, UK) in an immunoprecipitation buffer containing 0.01% SDS, 1% Triton-X100, 1.1mM EDTA, 15mM Tris-HCl, 150mM NaCl, 7mM NaOH. The mixture was then incubated with 60µL of Salmon Sperm DNA/Protein A agarose (Upstate, Lake Placid, USA) for 1 hour at 4°C. The immunocomplex was then washed once with 1ml of solution 1 (0.1% SDS, 1% Triton X-100, 2mM EDTA, 10mM Tris-HCl, 150mM NaCl; pH 8,1), once with 1 ml of solution 2 (0.1% SDS, 1% Triton X-100, 2mM EDTA, 10mM Tris-HCl, 500mM NaCl; pH 8.1), once with 1 ml of solution 3 (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl; pH 8.1), and twice with solution 4 (10mM Tris-HCl, 1mM EDTA pH 8). The immunoprecipitated

DNA was then eluted incubating the samples twice with 250µL of freshly prepared Elution Buffer (1% SDS, 0.1M $NaHCO_3$) at room temperature for 15 minutes. The DNA was purified using QiaQuick PCR purification kit (Qiagen, Hilden, Germany) following standard protocol. The purified DNA was subsequently quantified using SyBr Green Real-Time PCR.

SyBr Green Real-Time PCR

Real-Time PCR was performed using the "SYBR GreenER qPCR SuperMix for ABI PRISM Master Mix" (Invitrogen, Carlsbard, CA, USA) on a "7900HT Sequence Detection System" (Applied Biosystems, Foster City, CA, USA) under standard conditions. 13ng of INPUT DNA (not immunoprecipitated DNA) and a corresponding amount of immunoprecipitated DNA were used. All the analyses were executed in triplicate. To assess the reproducibility of data, MeDIP and Real-Time were performed at least three times for every cell line. The resulting values were standardized accordingly with Weber et al. [245]. using GAPDH as a reference gene and further normalized for the number of CpG sites of each amplicon. The primers used for the analysis were:

Bim_mDip1Fw 5'ACTCGGTGAAGGATGATGCC3' and

Bim_mDIP1Rev 5'CACACCCGTTAGAGCTTGGC3';

Bim_mDIP1.5Fw 5'AGGAACAGACGACAAGAAATAG3' and

Bim_mDIP1.5Rev 5'GGACAC GGCTAAGTAGACTC3';

Bim_mDip2bisFw 5'GGGAGGCTAGGGTACACTTCGG3' and

Bim_mDip2bisRev 5'GCTCCTACGCCCAATCACTGC3';

Bim_mDip3bisFw 5'AAGTCCTGCTTTGTCTCCAG3' and

Bim_mDip3bisRev 5'AAGGCGAGGCGATTGTTGAC3';

Bim_mDip4Fw 5'GCCTGCAATCG CTGCATCTG3' and

Bim_mDip4Rev 5'GTCAACAGCTTGCGGAACTGG3';

GAPDH_SyBr3_For 5'TGCTTCTCTGCTGTAGGCTC3' and

GAPDH_SyBr3_Rev 5'AGCGTGTCCATAGGGTGCCA3'.

All the primers were used at 160nM final concentration and were tested for the absence of primer-dimers and aspecific products using a negative first-derivative melting curve analysis as well as direct visualization on agarose gel stained with Ethidium Bromide.

Methylation Specific PCR (MSP)

5µL of bisulfite-treated DNA was amplified with a Nested-PCR protocol. The reactions were performed in 50µL volume containing GeneAmp PCR Buffer (Applied Biosystems, New Jersey, USA), 200µM each dNTPs, 2.5U Amplitaq (Applied Biosystems, New Jersey, USA). The primers used for the first reaction were:

BIM-MSPext-For ($_{5'}$GGATTGGGTTTGGGGATGGTTT$_{3'}$) and

BIM-MSPext-Rev ($_{5'}$ATCCCACAAACCCTCCCCTCAA$_{3'}$).

The product of the first reaction was diluted 1:10 and 1µL of the dilution was used as template for the nested PCR, using the same enzyme. The primers used for the detection of the unmethylated and methylated DNA were respectively:

BIM-MSP-Uint-For ($_{5'}$TTTTTGATGAAGTGGTAGTT$_{3'}$) and

BIM-MSP-Uint-Rev ($_{5'}$AACAAAACCCAAAACTCAAA$_{3'}$);

BIM-MSP-Mint-For ($_{5'}$TTTTCGACGAAGCGGTAGTC$_{3'}$) and

BIM-MSP-Mint-Rev ($_{5'}$AACAAAACCCGAAACTCGAA$_{3'}$).

The cycling conditions for the first round of PCR were,: 7 min at 95°C , 5 cycles of 1' at 95°C, 2' at 58°C and 3' at 72°C, 25 cycles of 30s at 95°C, 2' at 58°C and 90 s at 72°C with a final extension step of 5' at 72°C. For the nested PCR: 7' at 95°C, 35 cycles of 30s at 95°C, 30s at 40°C, 1' at 72°C and a final step of 7' at 72°C.

Chromatin Immunoprecipitation (ChIP)

Two samples of 1.2 x10^6 cells for each cell line were incubated with formaldehyde 37% (Sigma, St.Louis, MO, USA) in RPMI medium (to 1% final concentration) for 10 minutes at room temperature on a platform rocker, to allow the crosslinkage between DNA and proteins. The crosslinking was then quenched by adding glycine to 125mM final concentration. The samples were centrifuged and the pellet washed twice with PBS added of 1mM phenylmethylsulfonyl fluoride, 1µg/ml aprotinin, 1µg/ml pepstatin. After removing the supernatant, cells were incubated with lysis buffer (10mM Tris-HCl pH 7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 0.1% SDS, 1% Triton X-100, 10% Glycerol, 1mM phenylmethylsulfonyl fluoride, 60µg/ml aprotinin, 10µg/ml leupeptin, 1µg/ml pepstatin) for 10 minutes on ice. DNA was sonicated using a Labsonic BBraun sonicator, obtaining an average size of the sheared chromatin of ~500bp. The protocol for the sonication step was optimized for the different cell lines analyzed. 200µL of the sonicated lysates were centrifuged for 10 minutes at 4°C and then resuspended in 1.8ml of ChIP dilution buffer (1% SDS, 0.1M NaHCO3, 1mM phenylmethylsulfonyl

fluoride, 1.5µg/ml aprotinin, 1µg/ml pepstatin). 50µL of each sample were stored at -20°C (INPUT). 80µL of Salmon Sperm DNA/ProteinA agarose (Upstate, Lake Placid, USA) were added and subsequently samples were incubated for 30 minutes at 4°C with rotation. The pellet was removed after centrifugation at 2000 rpm for 1 minute. One of the two samples was incubated with 2µL of α-acetylated histoneH3 antibody (Upstate, Millipore Corporation, MA, USA) at 4°C overnight (IgH3); the other one (no Ig) was incubated at 4°C under the same conditions but without antibody. The immunocomplex were then washed once with 1ml of solution 1 (pH 8.1) (0.1% SDS, 1% Triton X-100, 2mM EDTA, 10mM Tris-HCl, 150mM NaCl), once with 1 ml of solution 2 (pH 8.1) (0.1% SDS, 1% Triton X-100, 2mM EDTA, 10mM Tris-HCl, 500mM NaCl), once with 1ml of solution 3 (pH 8.1) (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl), and twice with solution 4 (pH 8) (10mM Tris-HCl, 1mM EDTA). The immunoprecipitated DNA was then eluted incubating the samples twice with 250µL of freshly prepared Elution Buffer (1% SDS, 0.1M $NaHCO_3$) at room temperature for 15 minutes. All the samples (INPUT, Ig-H3, no-Ig) were incubated with 0.2M final concentration NaCl for 4 hours at 65°C. The DNA was purified using QiaQuick PCR purification kit (Qiagen, Hilden, Germany). The purified DNA was subsequently amplified with Fast-start Taq-Polymerase (Roche Applied Science, Germany) following the standard protocol. The primers used for the reactions were:

Bim-ChIP-Int-For 5'GCCTGCAATCGCTGCATCTG3' and

Bim-ChIP-Int-Rev 5'GTCAACAGCTTGCGGAACTGG3' for BIM amplification; GAPDH_ChIP_For 5'CCCAACTTTCCCGCCTCTC3' and

GAPDH_ChIP_Rev 5'CAGCCGCCTGGTTCAACTG3' for GAPDH amplification. Densitometric analyses were performed using Kodak 1D 3.5 Image System (Perkin Elmer, USA).

Apoptosis detection

Quantification of apoptotic cells was performed by TUNEL assay using DeadEndTM Fluorometric TUNEL System (Promega, Madison, Wi, USA). $4x10^6$ cells were washed with PBS 1x, fixed with 1% methanol-free formaldehyde for 20 minutes on ice and resuspended with 70% ethanol. After 4 hors at -20°C, cells were incubated with fluorescein-12-dUTP in the presence of Terminal Deoxynucleotidyl Transferase for 1 hour at 37°C in the dark. Cells were washed with PBS and incubated with RNaseA (50µg/ml) and Propidium Iodide (5µg/ml) (Sigma, St.Louis, MO, USA) for 30 minutes at room temperature. Flow-cytometry analysis was performed on a Becton Dickinson FACSort by CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain view, CA, USA).

Immunohistochemistry

Representative formalin-fixed paraffin-embedded tissue blocks were sectioned (2 micron thickness). Sections were dewaxed in xylene before rehydration through graded alcohol to water. Antigen retrieval was performed in autoclave at 95° for 6 minutes in citrate buffer 0.05M. The p80 polyclonal serum (Sanbio, Uden, The Netherlands) was used at a

dilution of 1:10. A streptavidin–biotin system (Dako, Glostrup, Denmark) with development in amino-ethil-carbazol was applied.

Results

Identification of BIM 5'UTR methylation in NPM-ALK+ cells.

We analyzed the methylation status of 19 CpG sites in the 5'UTR of BIM locus, in the human NPM-ALK+ ALCL cell lines SU-DHL-1, KARPAS-299 and SUP-M2 and in the NPM-ALK- Chronic Myeloid Leukemia (CML) cell line LAMA-84 as a negative control, using a bisulfite clonal sequencing (BCS) technique. Globally, we identified a homogeneous, very high level of methylation (93.2%) in all the NPM-ALK+ cell lines (Fig. 1, Tab 1). In the SU-DHL-1 cell line, all the 19 CpG sites were found to be methylated. As expected, only a limited evidence of CpG methylation (26,1%) could be detected in the NPM-ALK- LAMA-84 cell line (Fig. 1). This correlated with an almost complete silencing of BIM expression in all the NPM-ALK+ cell lines but not in LAMA-84, as assessed by quantitative PCR (Q-PCR; Fig. 2) (p=0.0054) and western blot (Fig. 7 lower panel).

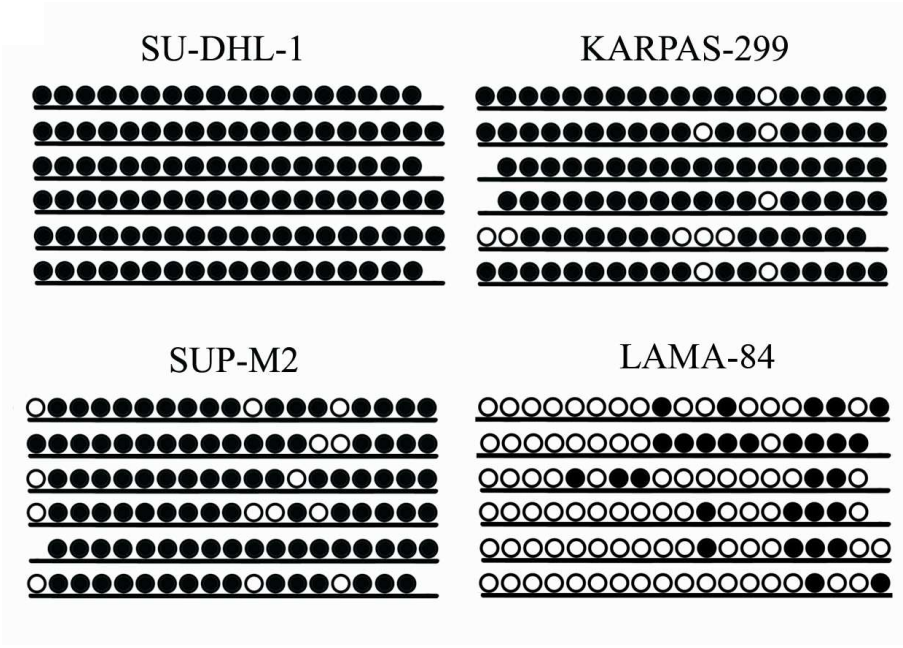

Fig. 1 Methylation pattern of Bim 5'UTR in NPM-ALK+ cell lines and in the NPM-ALK- Chronic Myeloid Leukemia (CML) cell line LAMA-84 as a negative control. Black bullets represent methylatd CpG sites; white bullets unmethylated sites. Horizontal bullet series represent sequential CpG sites; vertical series represent different clones from the same cell line.

CELL LINE	CELL TYPE	FEATURES	METHYLATION
SU-DHL-1	**Lymphoid**	**NPM-ALK+ Anaplastic large cell lymphoma (ALCL)**	**100%**
SUP-M2	**Lymphoid**	**NPM-ALK+ (ALCL)**	**87.6%** ± 7.3%
KARPAS-299	**Lymphoid**	**NPM-ALK+ (ALCL)**	**90.7%** ± 8.9%
LAMA-84	**Myeloid**	**BCR-ABL+ CML/Blast crisis**	**28.6%** ± 19.2%

Tab 1 Features of the ALCL cell lines and of the LAMA-84 (control cell line) and corresponding level (in percentage) of BIM promoter methylation analyzed by BMCSA (Fig.1).

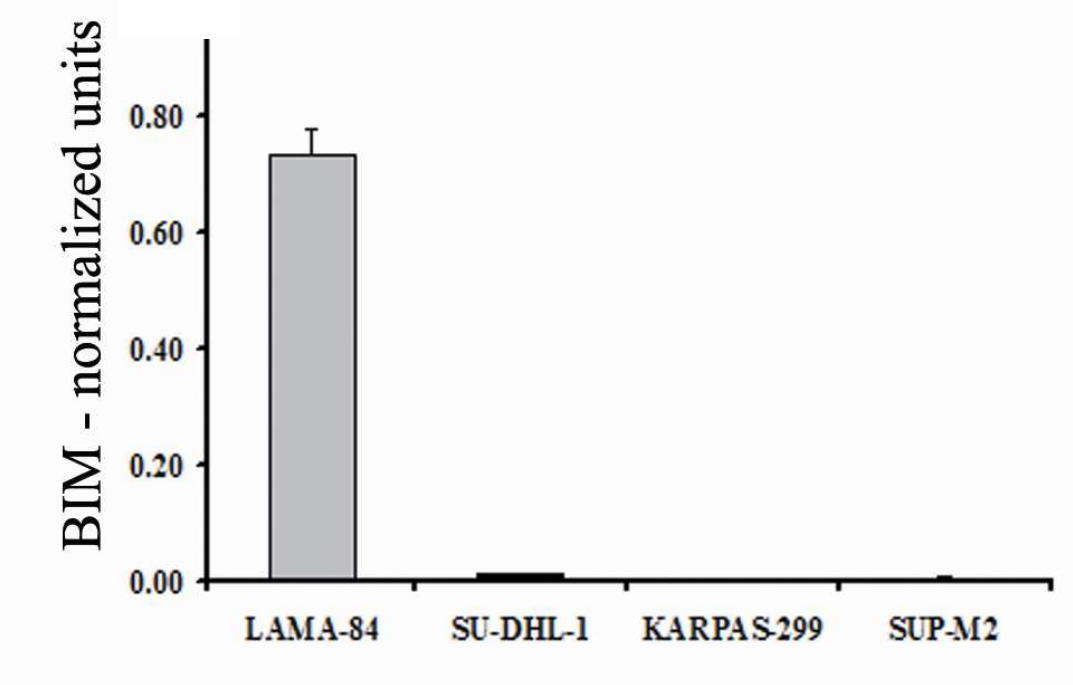

Fig. 2 Bim mRNA levels in NPM-ALK+ cell lines and in LAMA-84 cell line. The analysis was performed using Real-Time RT-PCR. The error bars represent the standard deviation of three different replicates.

To assess whether BIM methylation was present also in NPM-ALK+ ALCL *in vivo*, biological samples taken from lymph nodes of 6 patients affected by a NPM-ALK+ ALCL were analyzed using Methylation Specific PCR (MSP). Despite the likely presence of a mixed population of neoplastic and

normal cells in lymph nodes samples, as shown by immunohistochemistry for samples 1 and 2 (Fig. 4 upper panel), evidence for BIM methylation was shown in 5 cases (Fig. 3). Conversely, no evidence of BIM methylation could be found in lymphocytes from healthy donors (Fig. 3; controls #1 and #2). To further characterize BIM methylation, two ALCL samples (sample #1 and #2) and lymphocytes from 2 healthy donors (control #1 and #2) were also analyzed by BCS (Fig. 4, lower panel), confirming the presence of dense methylation in 2/5 clones in ALCL #1 and #2.

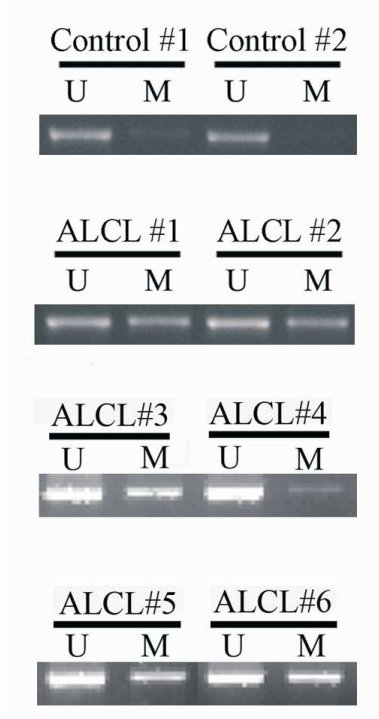

Fig. 3 Methylation status of Bim 5'UTR of ALCL samples and of lymphocytes from healthy donors using MSP. U represents unmethylatedspecific PCR and M methylation-specific PCR.

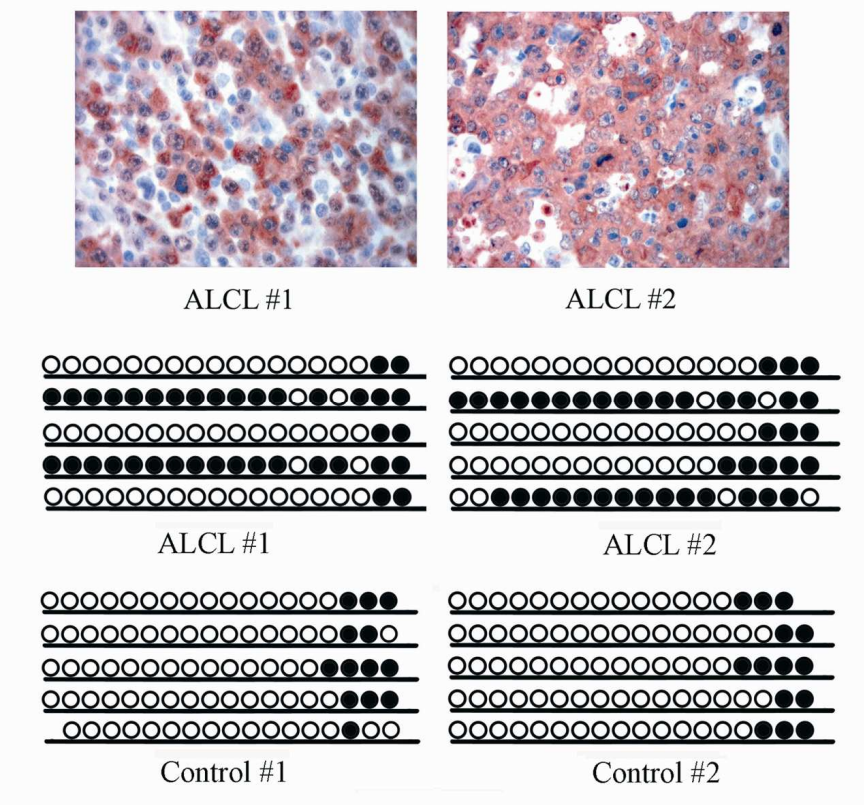

Fig 4 Upper panel: Immunoreactivity of neoplastic cells for anti-ALK p80 antibody (red) in lymph nodes of two NPM-ALK+ ALCL patients.
Lower panel: Corresponding methylation status of Bim 5'UTR (ALCL #1 and #2), compared with the methylation status of lymphocytes from two healthy donors (Control #1 and #2) using MSP.

To further analyze the methylation pattern of the whole CpG island, we carried out Real-Time Methylated DNA Immunoprecipitation (RT-MeDIP) profiling on SU-DHL-1 (NPM-ALK+) and LAMA-84 (NPM-ALK-) cell lines as a negative control. In SU-DHL-1, the analysis revealed a high enrichment for methylated DNA in the whole 5'UTR and in the first intron (Fig. 5, regions 4 and 5), while 5' from the transcription start site (Fig. 5, regions 1, 2 and 3) the enrichment level quickly decreased to levels similar to the control. As expected, no significant enrichment was detected in LAMA-84.

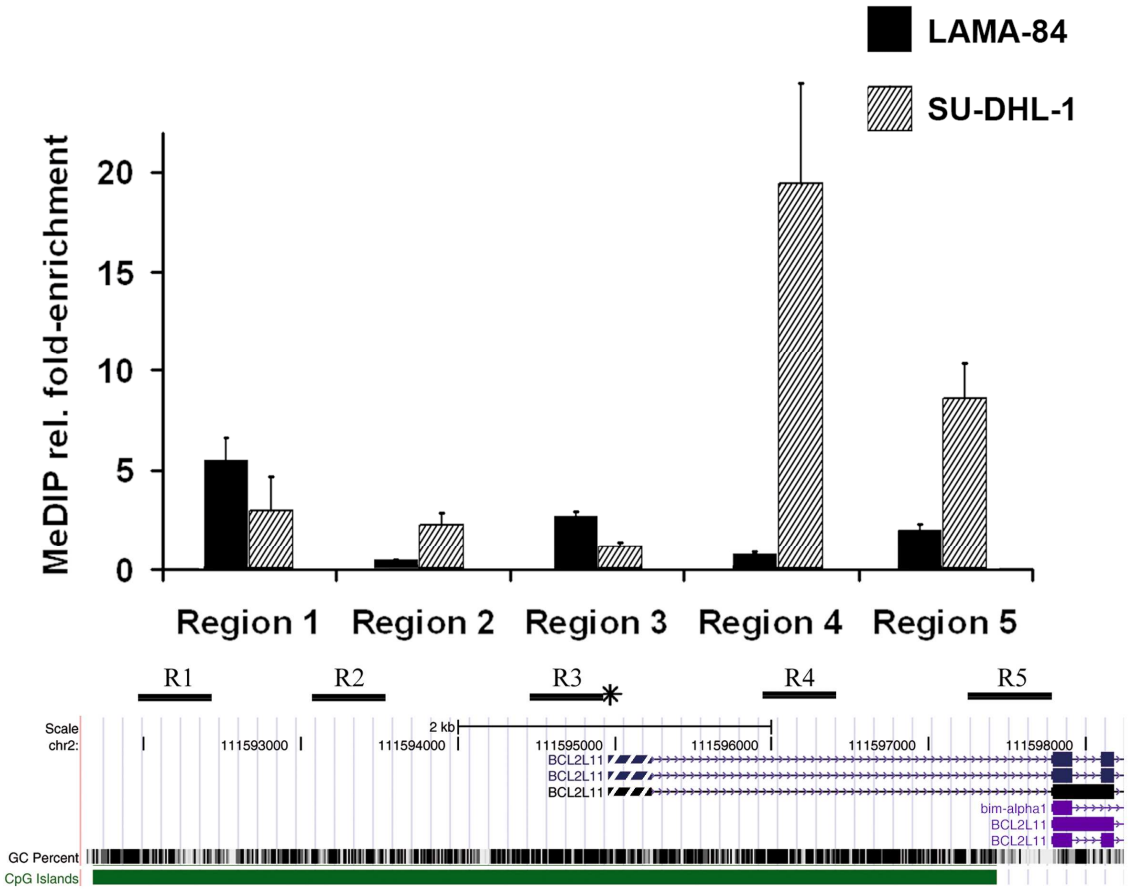

Fig. 5 RT-MeDIP analysis. RT-MeDIP for SUDHL1 and LAMA-84 is shown. The histogram bars represent the relative enrichment for methylated DNA in five different regions of Bim locus.
In the lower panel the schematic structure of Bim genomic locus is schown. The amplified regions are shown as thick black lines and labeled as regions 1-5. The asterisk indicates the first (5') transcription start site.

Treatment with demethylating agents leads to BIM upregulation and induction of apoptosis.

To characterize the contribution of BIM promoter methylation to gene silencing, the SU-DHL-1 and LAMA-84 cell lines were treated with the demethylating agent 5-azacytidine (AZA). Following treatment, metylation decreased from 100% to 0% in SU-DHL-1, while BIM epigenetic status was minimally affected in LAMA-84 (Fig. 6). Change in the methylation pattern of BIM was associated with BIM upregulation at mRNA (7.7 fold;

Fig. 7, upper panel) protein levels (Fig. 7, lower panel) and with induction of apoptosis (fig. 8) in SU-DHL-1 cell line, whereas, as expected, treatment with AZA was unable to induce BIM upregulation in LAMA-84 cells (Fig. 7).

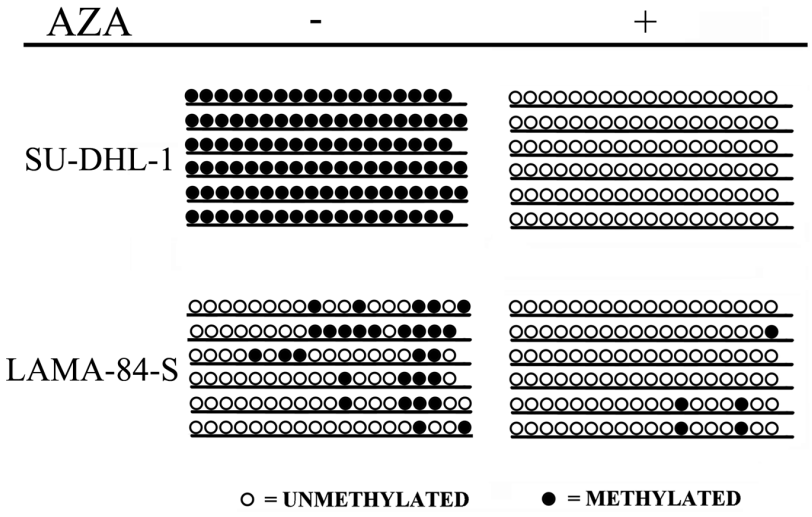

Fig. 6 Methylation Pattern of Bim 5'UTR in SU-DHL-1 and in LAMA-84 following treatment with AZA. Following treatment with 1μM AZA for five days, Bim 5'UTR methylation was evaluated in NPM-ALK+ SU-DHL-1 and in NPM-ALK- LAMA-84 cell lines. Horizontal bullet series represent sequential CpG sites; vertical series represent different clones from the same cell line.

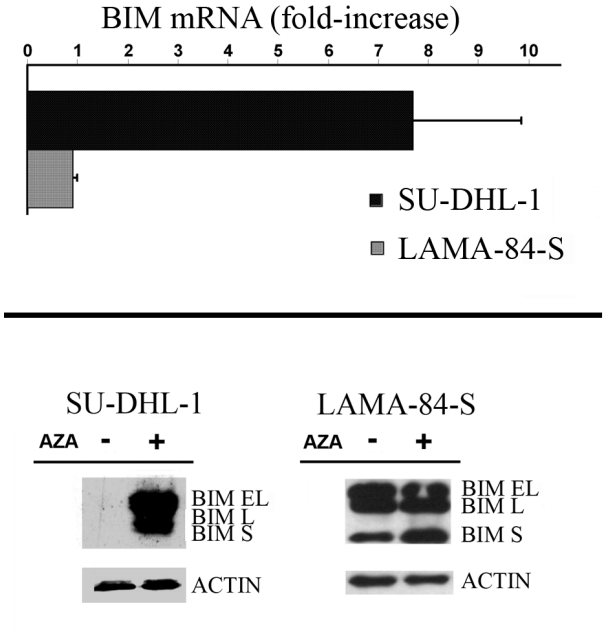

Fig. 7 Bim expression in SU-DHL-1 and LAMA-84 cell lines untreated and treated with AZA 1μM.
Upper panel: Bim mRNA expression (fold increase) of SU-DHL-1 and LAMA-84 cell lines, evaluated by Real-Time RT PCR after treatment with AZA 1μM.
Lower panel: Western Blot analysis of Bim expression in SU-DHL-1 and LAMA-84 cell lines untreated (-) and treated (+).

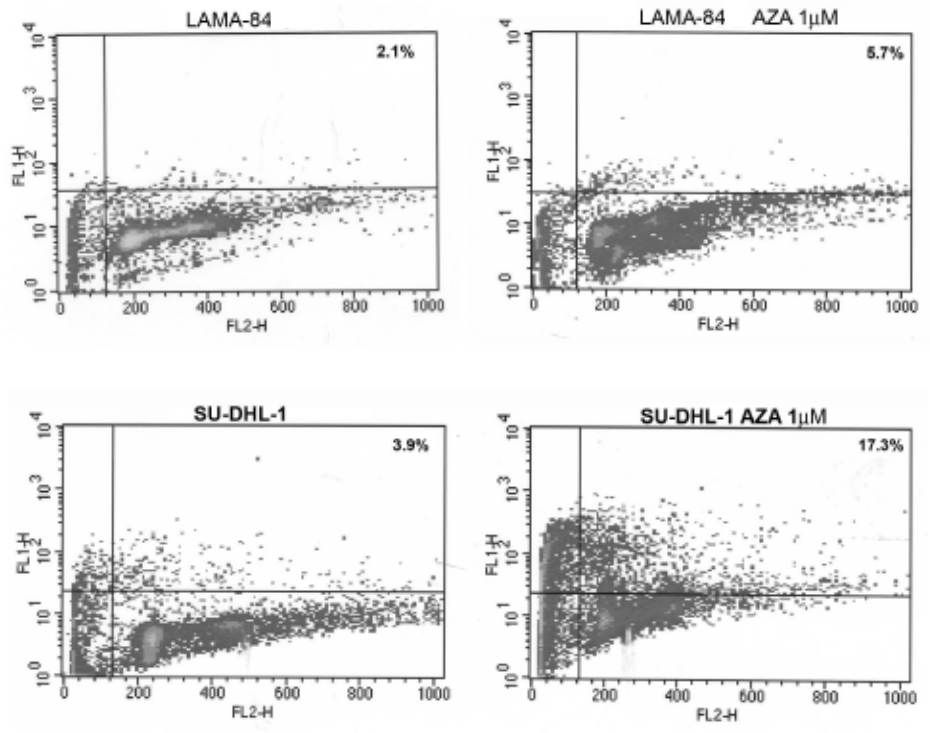

Fig. 8 Induction of apoptosis in SU-DHL-1 treated with AZA 1μM. SU-DHL-1 and LAMA-84 cell lines were treated with AZA 1μM for five days (right) and the induction of apoptosis was analyzed by TUNEL ASSAY (apoptotic cells in the upper-right square). The results were compared with the apoptotic status of untreated cells (left).

BIM is silenced through histone H3 deacetylation and chromatin condensation

To assess whether deacetylation of histone tails and chromatin condensation are involved in BIM epigenetic silencing, we analyzed the acetylation status of histone H3 tails at the BIM locus in the NPM-ALK+ KARPAS-299, SU-DHL-1, SUP-M2, and in the NPM-ALK- LAMA-84 cell lines. ChIP analysis showed that histone H3 tails at the BIM locus are strongly deacetylated in all the NPM-ALK+ cell lines (Fig. 9). Conversely, abundant histone H3 tail acetylation was apparent in LAMA-84 cells (Fig. 9).

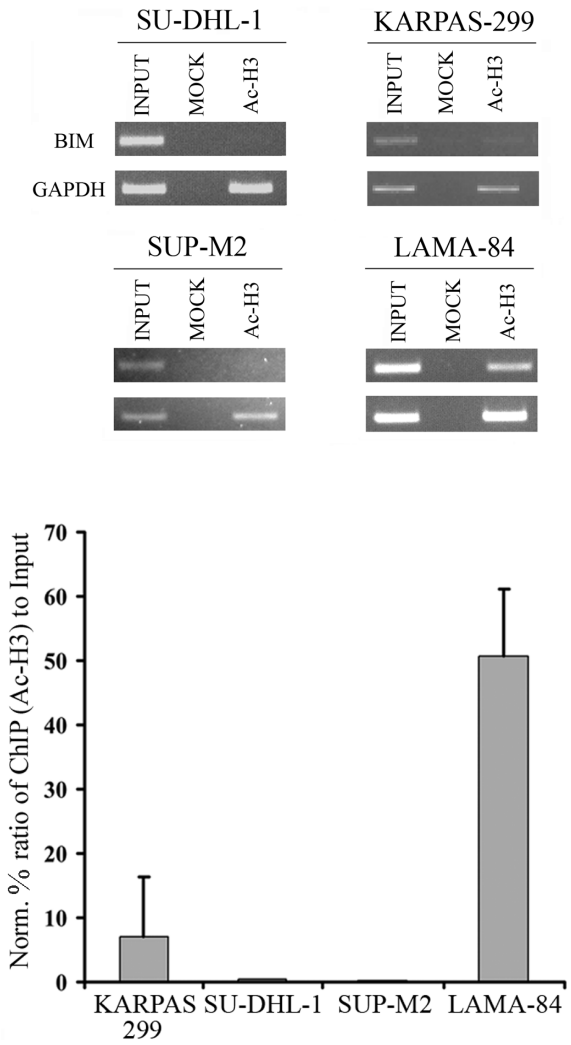

Fig. 9 Acetylation status of histone H3 tails at the Bim locus.
Upper panel: α – Acetylated-H3-ChIP analysis on the NPM-ALK+ cell lines: KARPAS-299, SU-DHL-1, SUP-M2; and on the LAMA-84 as negative control. Immunoprecipitates (Ac-H3) were subjected to PCR with a primer-pair specific for Bim and with another pair specific for GAPDH, as a positive control. PCR reactions were performed also using total chromatin input (INPUT) as template.
Lower panel: Densitometric analysis of Acetylated-H3 enrichment of Bim locus in the cell lines tested with ChIP.

To characterize the contribution of histone tail deacetylation to BIM silencing, SU-DHL-1 cells were treated with 500nM Trichostatin A (TSA), a potent inhibitor of class I and II histone deacetylases (HDACs), for three days. ChIP analysis following treatment with TSA confirmed the reacetylation of H3 tails (Fig. 10 upper panel). We subsequently analyzed

the effect of histone reacetylation on BIM expression by Q-PCR. Treatment with TSA enhanced BIM expression 21.6-fold (Fig. 10 lower panel).

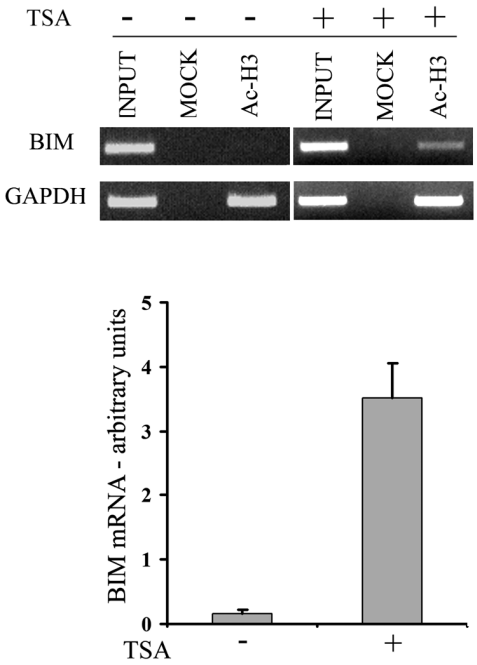

Fig. 10 Acetylation status of histone H3 tails at the Bim locus. After treatment with TSA.
Upper panel: α – Acetylated-H3-ChIP analysis on the NPM-ALK+ SU-DHL-1 untreated (-) and treated (+) with TSA 500nM for three days.
Lower panel: Bim expression in SU-DHL-1 untreated (-) and treated (+) with TSA 500nM for three days detected by Real-Time RT PCR.

The upregulation of BIM in SU-DHL-1 cell line was accompanied by the induction of massive apoptosis, as assessed by TUNEL assay (Fig. 11).

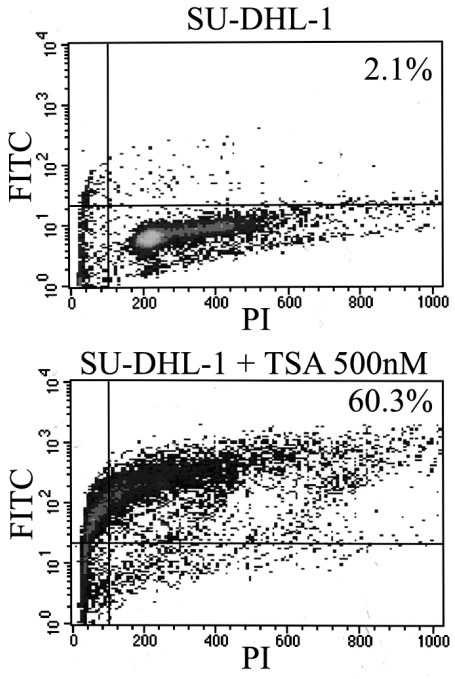

Fig. 11 Induction of apoptosis in SU-DHL-1 treated with TSA 500nM for three days (lower panel); the induction of apoptosis was analyzed by TUNEL ASSAY (apoptotic cells in the upper-right square). The result were compared with the apoptotic status of untreated cells (upper panel).

In the NPM-ALK- LAMA-84, in which no upregulation of BIM following treatment with TSA could be demonstrated (Fig. 12 upper panel), only a very limited pro-apoptotic effect was detected (Fig. 12 lower panel).

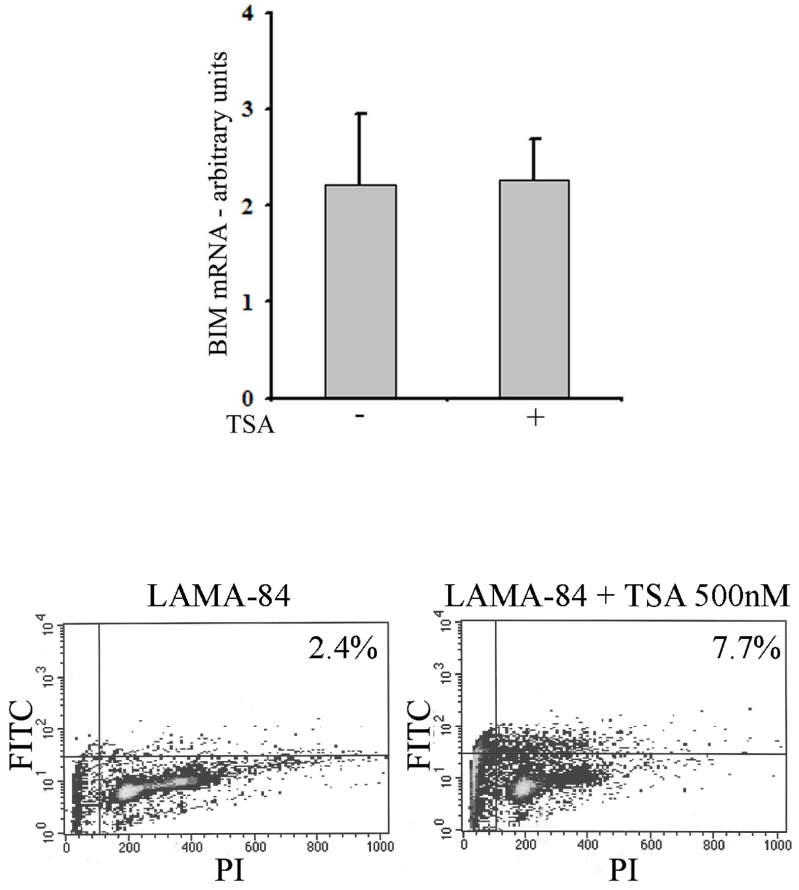

Fig. 12 TSA effect on Bim expression and on apoptosis induction in LAMA-84 cells. TSA doesn't induce Bim expression and a massive apoptosis in the negative control cell line.

BIM deacetylation occurs through recruitment of a MeCP2-SIN3 methyl binding protein/corepressor complex

The finding that treatment with HDAC inhibitors (HDACi) upregulates BIM, suggests that histone tail deacetylation plays an important role in BIM silencing. It is known that class I HDACs, most notably HDAC1, 2, and 3, are frequently associated with gene silencing in human cancer [246-248]. Due to the lack of DNA binding domains in HDACs, deacetylases require the presence of MBPs and corepressor complexes to properly bind methylated DNA and deacetylate histone tails.

HDAC1 and 2 are commonly found as a part of two multiprotein corepressor complexes: SIN3 and NuRD [249, 250]. Conversely, HDAC3 typically associates with the NCoR/SMRT complex [251], which is known to play a crucial role in hormone receptor signalling [252]. To study the involvement of specific HDACs in BIM silencing, we performed ChIP analyses with antibodies directed against HDAC1, 2 and 3 on SU-DHL-1 and we analyzed the immunoprecipitates by Q-PCR. As shown in Fig. 13a, HDAC1 and 2, but not HDAC3, associate with the methylated CpG island at the BIM genomic locus.

The identification of HDAC1 and 2 as a part of the BIM silencing machinery was suggestive for the involvement of SIN3 or NuRD corepressor complexes. To test this hypothesis, we performed ChIPs against MeCP2, a MBP that recruits SIN3 [253] and, less commonly, NCoR [254], and against MBD3, which is a core subunit of the multiprotein NuRD complex [250]. To confirm the absence of complexes involving HDAC3, we also set-up ChIPs against NCoR, which is part of the NCoR/SMRT complex and against Kaiso, a BTB/poxvirus - zinc finger family member able to bind NCoR [255]. As expected, the only significant association was found for MeCP2 (Fig. 13b), thus strengthening the hypothesis that silencing of BIM occurs through recruitment of a MeCP2-SIN3 methyl binding protein/corepressor complex (Fig. 14).

It is known that MeCP2 is able to bind to methylated and also to unmethylated DNA *in vitro*, albeit with different specificity and affinity [256]. To verify if the recruitment of MeCP2 was dependent on BIM 5'UTR methylation, we tested the binding of MeCP2 to the BIM locus with and

without pretreatment with AZA 1μM. Notably, the treatment with the demethylating agent was able to completely disrupt the association of MeCP2 to the BIM locus, thus indicating that MeCP2 acts through a methylation-dependent mechanism (Fig 13c). Interestingly, the disruption of the MeCP2-SIN3 methyl binding protein/corepressor complex following treatment with AZA was associated with a prompt reacetylation of the BIM locus (Fig 13d).

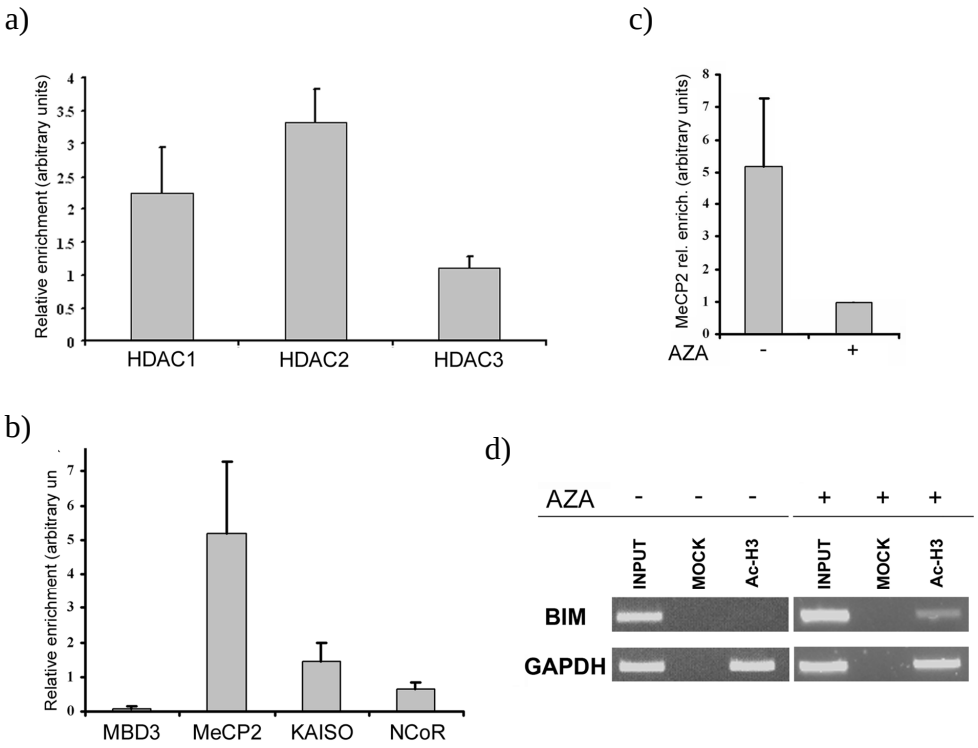

Fig. 13 Identification of the repressor complex associated with the silencing of Bim. a) ChIP analyses using α-HDAC1, 2 and 3 antibodies were performed in SU-DHL-1 cells in order to identify the HDACs involved in Bim silencing. The immunoprecipitates were analyzed using Real-Time PCR. The error bars represent the standard deviation of three replicates. b) Characterization of the repressor complex involved in Bim silencing with a second round of ChIP experiments using α-MBD3, MeCP2, KAISO and NcoR antibodies. The immunoprecipitates were analyzed using Real-Time PCR. c) To verify if the recruitment of MeCP2 was dependent on methylation of Bim locus, α-MeCP2 ChIP analysis was performed in untreated cells (-) and in cells treated with AZA 1μM (+) and immunoprecipitates were then analyzed
using Real-Time PCR. d) The effect of a demethylating agent on the acetylation status of histone H3 tails at Bim locus was investigated with α-Acetylated-H3 ChIP analysis in SU-DHL-1 in absence (-) and in presence (+) of AZA 1μM. Immunoprecipitates (Ac-H3) were subjected to PCR with a primer-pair specific for Bim and with another pair specific for GAPDH, as a positive control. PCR reactions were performed also using total chromatin input (INPUT) as template.

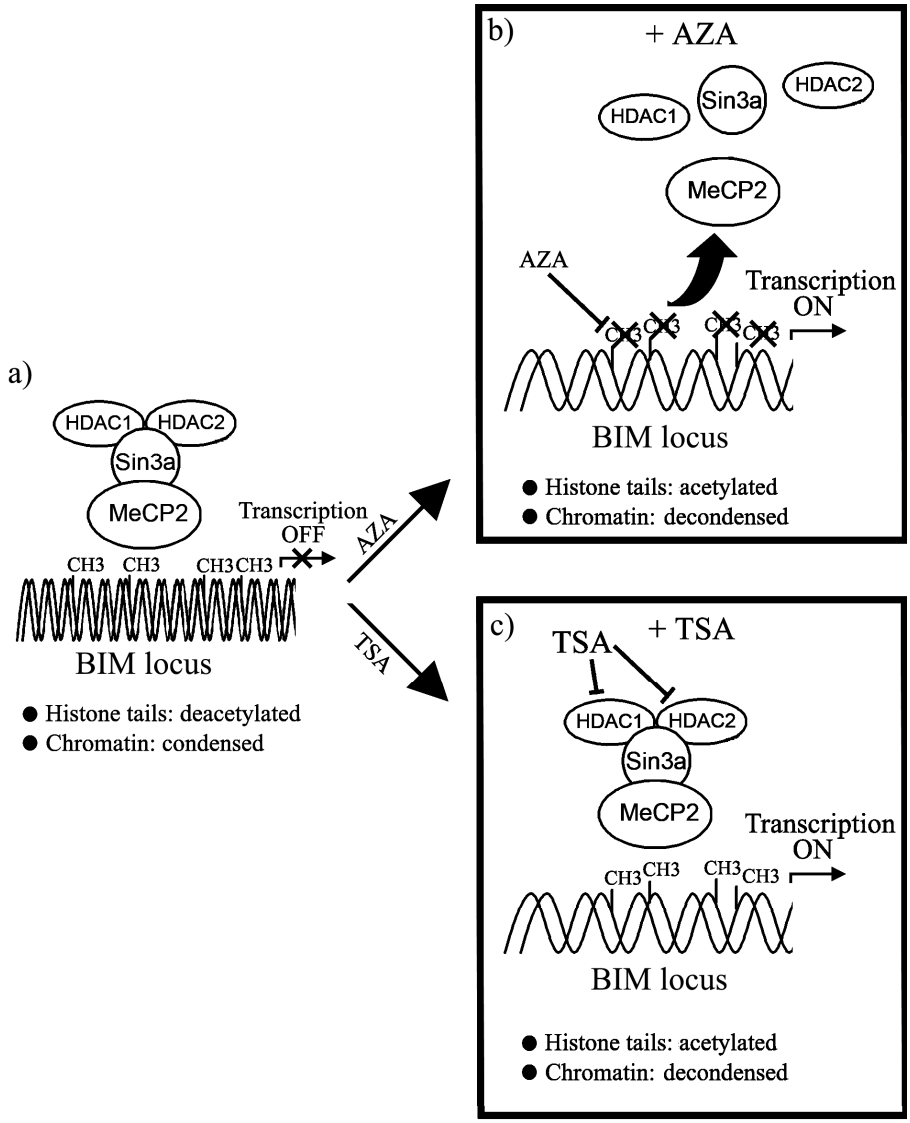

Fig. 14 Hypotesis of molecular mechanism of Bim silencing by recruitment of MeCP2-SIN3 methyl binding protein/corepressor complex

NPM-ALK inhibition isn't able to revert Bim epigenetic silencing.

We assessed if NPM-ALK fusion protein is involved in epigenetic downregulation of Bim.

The treatment with an NPM-ALK inhibitor as well as the silencing of NPM-ALK in SU-DHL-1 cell line with an inducible siRNA are able to only partially reactivate Bim expression, 4 and 2.22 fold respectively (Fig. 15)

compared Bim upregulation induced with TSA 500nM treatment (21.6 fold, Fig 10 lower panel). However, neither the inhibitor nor the inducible siRNA were able to revert Bim epigenetic status (Fig. 16). This suggests NPM-ALK is not directly responsible for the maintenance of Bim epigenetic status.

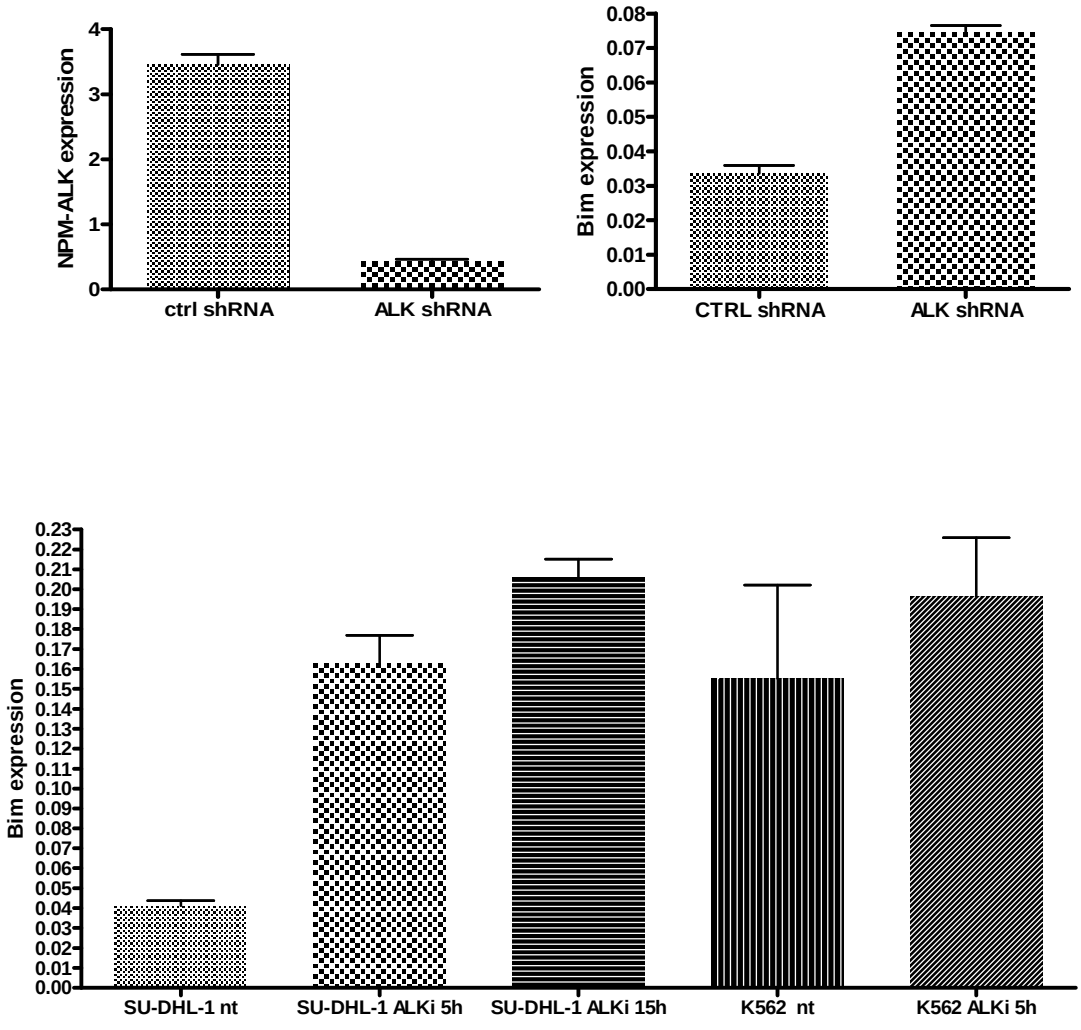

Fig. 15 Upper panel: NPM-ALK mRNA expression (left), Bim mRNA expression (right) in SU-DHL-1 ShALK and ShCTRL cells. shRNA cells were treated with 1 μg/mL doxycycline for 96 h before the mRNA expression analysis.
Lower Panel: Bim expression in SU-DHL-1 (left) and in K562 negative control cells (right), untreated (NT) and treated cells with NPM-ALK inhibitor.

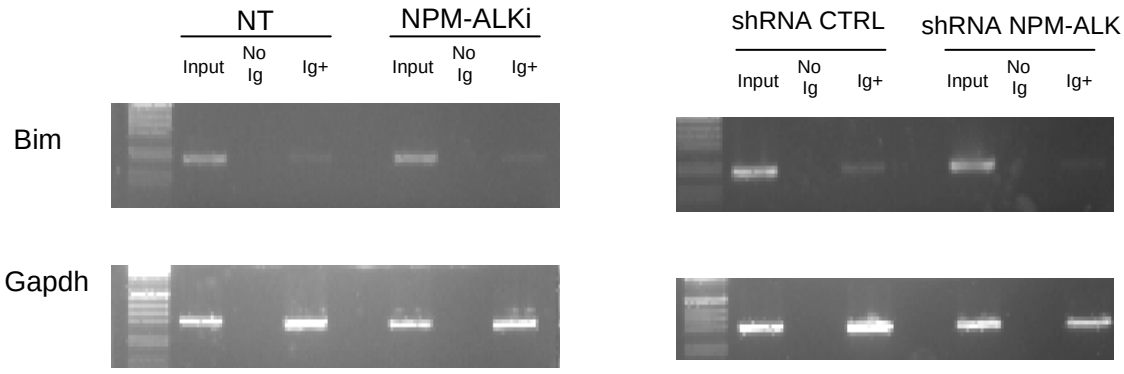

Fig. 16 Status of histone H3 tails in NPM-ALK silenced cells. Status of histone H3 tails at the Bim locus in SU-DHL-1 untreated (NT) and treated with NPM-ALK inhibitor (NPM-ALKi) 1.2 μM for 5 hours (left), in shALK and shCTRL SU-DHL-1 cells treated with 1 μg/mL doxycycline for 96 h before ChIP assay (right).

Discussion

The BH3-only, Bcl-2 family member BIM has recently emerged as one of the most potent proapoptotic factors involved in the homeostasis of the hematopoietic system [24, 257, 258]. Several mechanisms of BIM regulation have been described both at the transcriptional and post-transcriptional levels [54, 56-58, 239, 259, 260]. Additionally, recent studies showed that BIM expression can be silenced by epigenetic mechanisms in various types of cancers: in a subset of CML patients [241] , in a large part of renal cell carcinoma samples [261], in a high percentage of Burkitt Lymphomas [262] and in EBV-infected B cells [240].

Here we showed for the first time the existence of a BIM epigenetic silencing mechanism in ALCL. Methylation profiling by RT-MeDIP revealed that methylation is clustered within BIM 5'UTR, at least in SU-DHL-1 (Fig. 5). This finding suggests that the 5'UTR of BIM promoter may have an important role in controlling the expression of the gene. It is well known that silencing of a gene through epigenetic mechanisms is a complex phenomenon [262, 263]. Commonly, methylation of the promoter or the 5'UTR of a gene is just the first step of a long series of events finally leading to gene silencing. Epigenetic silencing can be triggered by CpG methylation through two main mechanisms: first, DNA methylation can repress transcription by directly impairing the binding of transcriptional activators to cognate DNA sequences [264, 265] second, methylated DNA binding proteins (MDB) can bind to methylated DNA sequences, thus recruiting multiprotein corepressor complexes carrying deacetylase activity. These complexes silence gene expression by triggering histone tail deacetylation and chromatin condensation [266]. Using ChIP experiments

we demonstrated the involvement of histone H3 tail deacetylation, a well-known marker of chromatin condensation [267], in BIM silencing (Fig. 9). To further characterize the molecular events leading to chromatin condensation, we performed ChIP experiments using anti HDAC1, HDAC2, HDAC3, MeCP2, MBD3, Kaiso, and NCoR antibodies, demonstrating that HDAC1, HDAC2 and MeCP2 but not HDAC3, MBD3, Kaiso and NCoR, are involved in BIM silencing (Fig. 13 a,b). HDAC1 and 2 are usually found as part of two corepressor complexes: SIN3 and NuRD. However, the identification of MeCP2, a MDB commonly associated with the SIN3 complex, strongly suggests the involvement of the latter at the BIM locus. This hypothesis is further supported by the absence of MBD3, which is part of the core NuRD complex, even if its constitutive presence in NuRD corepressor complex has been questioned [268]. The finding that treatment with demethylating agents is able to completely revert the binding of MeCP2 to the BIM locus (Fig. 13c) suggests that BIM silencing occurs through the following steps: CpG methylation -> binding of MeCP2 to methylated CpGs -> recruitment of SIN3 corepressor complex -> deacetylation of histone tails -> chromatin condensation (Fig. 14a). The evidence that a deacetylating complex plays a functional role in BIM downregulation is supported by the fact that treatment with HDACi is able to restore BIM expression (Fig. 14c). Interestingly, the detachment of MeCP2 following treatment with deacetylating agents is also associated with histone tail reacetylation (Fig. 13d, Fig. 14b), suggesting the presence of a yet unidentified acetylating complex acting in competition with MeCP2/SIN3.

Furthermore, we hypothesized the oncogenic tyrosine kinase NPM-ALK may be involved in epigenetic silencing of the tumor suppressor gene Bim in ALCL. In the literature there data about NPM-ALK is capable of inducing epigenetic silencing, through DNA methylation, of a tumor suppressor gene like STAT5A [269] or of molecules of TCR signalling [244] via STAT3. We performed experiments to investigate if NPM-ALK may have a role in epigenetic status of Bim locus in ALCL. We analyzed, by ChIP, chromatin compaction of Bim promoter in NPM-ALK silenced cells, with shRNA, or in ALCL cells treated with a specific inhibitor of NPM-ALK.

We obtained same results in both cases, we didn't detect chromatin decondensation; Bim locus is still deacetylated after silencing or inhibition of NPM-ALK (Fig. 16). Supporting this observation is the following result: Bim mRNA upregulation, after NPM-ALK silencing or inhibition, is lower (2.22 - 4 fold respectively) (Fig. 15) than that induced with HDACi (TSA) treatment (21.6 fold) (Fig. 10) These findings indicate NPM-ALK is not directly responsible for the maintenance of Bim epigenetic status .

While the epigenetic silencing of BIM may play a crucial role in protecting ALCL cells from apoptosis, other mechanisms leading to the downregulation of BIM have previously been identified. It is known that the transcription factor FoxO3a is able to directly activate the expression of BIM via its binding site on BIM promoter [270, 271]. This mechanism of regulation can be inhibited by phosphorylation of FoxO3a, which results in the exclusion of the transcription factor from the nucleus and thus in its functional inactivation [270, 271]. Notably, this mechanism was

demonstrated in NPM-ALK positive cell lines [35], suggesting that the impairment of the BIM pathway is an important step in leukemogenesis. Therefore, at least in ALCL cells, two different mechanisms, phosphorylation of FoxO3a through the activation of the PI3K-AKT pathway and BIM epigenetic silencing, may converge to inactivate the proapoptotic signal of BIM, thus protecting the cells from apoptosis. These findings reinforce the idea that BIM plays a major role in the surveillance against tumorigenesis and that the discovery and description of the mechanisms by which BIM, as well as other oncosuppressors, are inactivated in specific types of cancers may be of great importance in order to define evidence-based cancer treatment protocols. Specifically, this study demonstrates that demethylating drugs and HDACi significantly upregulate BIM expression and induce massive cell death in ALCL cell lines and suggests that the use of epigenetic modulators in combination with specific inhibitor of NPM-ALK could act synergistically and may play an important role in the treatment of ALCL.

Bibliography

1. Brown, J.M. and L.D. Attardi, *The role of apoptosis in cancer development and treatment response.* Nat Rev Cancer, 2005. **5**(3): p. 231-7.

2. Brunner, T. and C. Mueller, *Apoptosis in disease: about shortage and excess.* Essays Biochem, 2003. **39**: p. 119-30.

3. Cory, S. and J.M. Adams, *The Bcl2 family: regulators of the cellular life-or-death switch.* Nat Rev Cancer, 2002. **2**(9): p. 647-56.

4. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

5. Adams, J.M., *Ways of dying: multiple pathways to apoptosis.* Genes Dev, 2003. **17**(20): p. 2481-95.

6. Strasser, A., L. O'Connor, and V.M. Dixit, *Apoptosis signaling.* Annu Rev Biochem, 2000. **69**: p. 217-45.

7. Strasser, A., *The role of BH3-only proteins in the immune system.* Nat Rev Immunol, 2005. **5**(3): p. 189-200.

8. Green, D.R. and G. Kroemer, *The pathophysiology of mitochondrial cell death.* Science, 2004. **305**(5684): p. 626-9.

9. Egle, A., et al., *Bim is a suppressor of Myc-induced mouse B cell leukemia.* Proc Natl Acad Sci U S A, 2004. **101**(16): p. 6164-9.

10. Hemann, M.T., et al., *Suppression of tumorigenesis by the p53 target PUMA.* Proc Natl Acad Sci U S A, 2004. **101**(25): p. 9333-8.

11. Adams, J.M. and S. Cory, *The Bcl-2 apoptotic switch in cancer development and therapy.* Oncogene, 2007. **26**(9): p. 1324-37.

12. Cory, S. and J.M. Adams, *Killing cancer cells by flipping the Bcl-2/Bax switch.* Cancer Cell, 2005. **8**(1): p. 5-6.

13. Huang, D.C. and A. Strasser, *BH3-Only proteins-essential initiators of apoptotic cell death.* Cell, 2000. **103**(6): p. 839-42.

14. Willis, S.N., et al., *Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak.* Science, 2007. **315**(5813): p. 856-9.

15. Czabotar, P.E., et al., *Structural insights into the degradation of Mcl-1 induced by BH3 domains.* Proc Natl Acad Sci U S A, 2007. **104**(15): p. 6217-22.

16. Puthalakath, H. and A. Strasser, *Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins.* Cell Death Differ, 2002. **9**(5): p. 505-12.

17. Kuwana, T., et al., *BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly.* Mol Cell, 2005. **17**(4): p. 525-35.

18. Zong, W.X., et al., *BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak.* Genes Dev, 2001. **15**(12): p. 1481-6.

19. Petros, A.M., et al., *Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies.* Protein Sci, 2000. **9**(12): p. 2528-34.

20. Chen, L., et al., *Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function.* Mol Cell, 2005. **17**(3): p. 393-403.

21. Certo, M., et al., *Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members.* Cancer Cell, 2006. **9**(5): p. 351-65.

22. Letai, A., et al., *Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics.* Cancer Cell, 2002. **2**(3): p. 183-92.

23. Kim, H., et al., *Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies.* Nat Cell Biol, 2006. **8**(12): p. 1348-58.

24. Bouillet, P., et al., *Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity.* Science, 1999. **286**(5445): p. 1735-8.

25. Bouillet, P., et al., *BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes.* Nature, 2002. **415**(6874): p. 922-6.

26. O'Reilly, L.A., et al., *The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells.* Am J Pathol, 2000. **157**(2): p. 449-61.

27. O'Connor, L., et al., *Bim: a novel member of the Bcl-2 family that promotes apoptosis.* Embo J, 1998. **17**(2): p. 384-95.

28. Puthalakath, H., et al., *The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex.* Mol Cell, 1999. **3**(3): p. 287-96.

29. Harada, H., et al., *Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity.* Proc Natl Acad Sci U S A, 2004. **101**(43): p. 15313-7.

30. Marani, M., et al., *Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis.* Mol Cell Biol, 2002. **22**(11): p. 3577-89.

31. Villunger, A., et al., *Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival.* Blood, 2003. **101**(6): p. 2393-400.

32. Enders, A., et al., *Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells.* J Exp Med, 2003. **198**(7): p. 1119-26.

33. Pellegrini, M., et al., *Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 14175-80.

34. Tagawa, H., et al., *Genome-wide array-based CGH for mantle cell lymphoma: identification of homozygous deletions of the proapoptotic gene BIM.* Oncogene, 2005. **24**(8): p. 1348-58.

35. Gu, T.L., et al., *NPM-ALK fusion kinase of anaplastic large-cell lymphoma regulates survival and proliferative signaling through modulation of FOXO3a.* Blood, 2004. **103**(12): p. 4622-9.

36. Leung, K.T., et al., *Activation of the JNK pathway promotes phosphorylation and degradation of BimEL--a novel mechanism of chemoresistance in T-cell acute lymphoblastic leukemia.* Carcinogenesis, 2008. **29**(3): p. 544-51.

37. Anderton, E., et al., *Two Epstein-Barr virus (EBV) oncoproteins cooperate to repress expression of the proapoptotic tumour-suppressor Bim: clues to the pathogenesis of Burkitt's lymphoma.* Oncogene, 2008. **27**(4): p. 421-33.

38. Zhang, L. and P.A. Insel, *The pro-apoptotic protein Bim is a convergence point for cAMP/protein kinase A- and glucocorticoid-promoted apoptosis of lymphoid cells.* J Biol Chem, 2004. **279**(20): p. 20858-65.

39. Kuroda, J., et al., *Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic.* Proc Natl Acad Sci U S A, 2006. **103**(40): p. 14907-12.

40. Kuroda, J., et al., *Apoptosis-based dual molecular targeting by INNO-406, a second-generation Bcr-Abl inhibitor, and ABT-737, an inhibitor of antiapoptotic Bcl-2 proteins, against Bcr-Abl-positive leukemia.* Cell Death Differ, 2007. **14**(9): p. 1667-77.

41. Aichberger, K.J., et al., *Low-level expression of proapoptotic Bcl-2-interacting mediator in leukemic cells in patients with chronic myeloid leukemia: role of BCR/ABL, characterization of underlying signaling pathways, and reexpression by novel pharmacologic compounds.* Cancer Res, 2005. **65**(20): p. 9436-44.

42. Kuribara, R., et al., *Roles of Bim in apoptosis of normal and Bcr-Abl-expressing hematopoietic progenitors.* Mol Cell Biol, 2004. **24**(14): p. 6172-83.

43. Belloc, F., et al., *Imatinib and nilotinib induce apoptosis of chronic myeloid leukemia cells through a Bim-dependant pathway modulated by cytokines.* Cancer Biol Ther, 2007. **6**(6): p. 912-9.

44. Nuutinen, U., et al., *Inhibition of PI3-kinase-Akt pathway enhances dexamethasone-induced apoptosis in a human follicular lymphoma cell line.* Exp Cell Res, 2006. **312**(3): p. 322-30.

45. Hallaert, D.Y., et al., *Crosstalk among Bcl-2 family members in B-CLL: seliciclib acts via the Mcl-1/Noxa axis and gradual exhaustion of Bcl-2 protection.* Cell Death Differ, 2007. **14**(11): p. 1958-67.

46. Hideshima, T., et al., *Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma.* Oncogene, 2001. **20**(42): p. 5991-6000.

47. Gomez-Bougie, P., R. Bataille, and M. Amiot, *The imbalance between Bim and Mcl-1 expression controls the survival of human myeloma cells.* Eur J Immunol, 2004. **34**(11): p. 3156-64.

48. Gomez-Bougie, P., et al., *Melphalan-induced apoptosis in multiple myeloma cells is associated with a cleavage of Mcl-1 and Bim and a decrease in the Mcl-1/Bim complex.* Oncogene, 2005. **24**(54): p. 8076-9.

49. Zhang, W., et al., *Sorafenib induces apoptosis of AML cells via Bim-mediated activation of the intrinsic apoptotic pathway.* Leukemia, 2008. **22**(4): p. 808-18.

50. Pei, X.Y., et al., *MEK1/2 inhibitors potentiate UCN-01 lethality in human multiple myeloma cells through a Bim-dependent mechanism.* Blood, 2007. **110**(6): p. 2092-101.

51. Puthalakath, H., et al., *Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis.* Science, 2001. **293**(5536): p. 1829-32.

52. Servida, F., et al., *Sensitivity of human multiple myelomas and myeloid leukemias to the proteasome inhibitor I.* Leukemia, 2005. **19**(12): p. 2324-31.

53. Yeung, B.H., D.C. Huang, and F.A. Sinicrope, *PS-341 (bortezomib) induces lysosomal cathepsin B release and a caspase-2-dependent mitochondrial permeabilization and apoptosis in human pancreatic cancer cells.* J Biol Chem, 2006. **281**(17): p. 11923-32.

54. Dijkers, P.F., et al., *Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1*. Curr Biol, 2000. **10**(19): p. 1201-4.

55. Matsui, H., H. Asou, and T. Inaba, *Cytokines direct the regulation of Bim mRNA stability by heat-shock cognate protein 70.* Mol Cell, 2007. **25**(1): p. 99-112.

56. Ley, R., et al., *Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim.* J Biol Chem, 2003. **278**(21): p. 18811-6.

57. Akiyama, T., et al., *Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim.* Embo J, 2003. **22**(24): p. 6653-64.

58. Luciano, F., et al., *Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function.* Oncogene, 2003. **22**(43): p. 6785-93.

59. Putcha, G.V., et al., *Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis.* Neuron, 2001. **29**(3): p. 615-28.

60. Marsden, V.S. and A. Strasser, *Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more.* Annu Rev Immunol, 2003. **21**: p. 71-105.

61. Watanabe-Fukunaga, R., et al., *Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis.* Nature, 1992. **356**(6367): p. 314-7.

62. Takahashi, T., et al., *Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand.* Cell, 1994. **76**(6): p. 969-76.

63. McDonnell, T.J., et al., *bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation.* Cell, 1989. **57**(1): p. 79-88.

64. Strasser, A., et al., *Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease.* Proc Natl Acad Sci U S A, 1991. **88**(19): p. 8661-5.

65. Ogilvy, S., et al., *Constitutive Bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 14943-8.

66. Veis, D.J., et al., *Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair.* Cell, 1993. **75**(2): p. 229-40.

67. Nakayama, K., et al., *Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3700-4.

68. Wickremasinghe, R.G. and A.V. Hoffbrand, *Biochemical and genetic control of apoptosis: relevance to normal hematopoiesis and hematological malignancies.* Blood, 1999. **93**(11): p. 3587-600.

69. Knudson, A.G., Jr., *Mutation and cancer: statistical study of retinoblastoma.* Proc Natl Acad Sci U S A, 1971. **68**(4): p. 820-3.

70. Renan, M.J., *How many mutations are required for tumorigenesis? Implications from human cancer data.* Mol Carcinog, 1993. **7**(3): p. 139-46.

71. Tsujimoto, Y., et al., *Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation.* Science, 1984. **226**(4678): p. 1097-9.

72. Debatin, K.M., K. Stahnke, and S. Fulda, *Apoptosis in hematological disorders.* Semin Cancer Biol, 2003. **13**(2): p. 149-58.

73. Weiss, L.M., et al., *Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas.* N Engl J Med, 1987. **317**(19): p. 1185-9.

74. Hermine, O., et al., *Prognostic significance of bcl-2 protein expression in aggressive non-Hodgkin's lymphoma. Groupe d'Etude des Lymphomes de l'Adulte (GELA).* Blood, 1996. **87**(1): p. 265-72.

75. Kitada, S., et al., *Dysregulation of apoptosis genes in hematopoietic malignancies.* Oncogene, 2002. **21**(21): p. 3459-74.

76. Kitada, S., et al., *Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses.* Blood, 1998. **91**(9): p. 3379-89.

77. Brimmell, M., et al., *BAX frameshift mutations in cell lines derived from human haemopoietic malignancies are associated with resistance to apoptosis and microsatellite instability.* Oncogene, 1998. **16**(14): p. 1803-12.

78. Johnstone, R.W., A.A. Ruefli, and S.W. Lowe, *Apoptosis: a link between cancer genetics and chemotherapy.* Cell, 2002. **108**(2): p. 153-64.

79. Herr, I. and K.M. Debatin, *Cellular stress response and apoptosis in cancer therapy.* Blood, 2001. **98**(9): p. 2603-14.

80. Evan, G.I. and K.H. Vousden, *Proliferation, cell cycle and apoptosis in cancer.* Nature, 2001. **411**(6835): p. 342-8.

81. Kaufmann, S.H. and W.C. Earnshaw, *Induction of apoptosis by cancer chemotherapy.* Exp Cell Res, 2000. **256**(1): p. 42-9.

82. Luger, K., et al., *Crystal structure of the nucleosome core particle at 2.8 A resolution.* Nature, 1997. **389**(6648): p. 251-60.

83. Jenuwein, T. and C.D. Allis, *Translating the histone code.* Science, 2001. **293**(5532): p. 1074-80.

84. Okano, M., S. Xie, and E. Li, *Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases.* Nat Genet, 1998. **19**(3): p. 219-20.

85. Antequera, F. and A. Bird, *Number of CpG islands and genes in human and mouse.* Proc Natl Acad Sci U S A, 1993. **90**(24): p. 11995-9.

86. Goto, T. and M. Monk, *Regulation of X-chromosome inactivation in development in mice and humans.* Microbiol Mol Biol Rev, 1998. **62**(2): p. 362-78.

87. Terranova, R., et al., *Histone and DNA methylation defects at Hox genes in mice expressing a SET domain-truncated form of Mll.* Proc Natl Acad Sci U S A, 2006. **103**(17): p. 6629-34.

88. Galm, O., J.G. Herman, and S.B. Baylin, *The fundamental role of epigenetics in hematopoietic malignancies.* Blood Rev, 2006. **20**(1): p. 1-13.

89. Esteller, M., *Profiling aberrant DNA methylation in hematologic neoplasms: a view from the tip of the iceberg.* Clin Immunol, 2003. **109**(1): p. 80-8.

90. van Doorn, R., et al., *Epigenetic profiling of cutaneous T-cell lymphoma: promoter hypermethylation of multiple tumor suppressor genes including BCL7a, PTPRG, and p73.* J Clin Oncol, 2005. **23**(17): p. 3886-96.

91. Shames, D.S., et al., *A genome-wide screen for promoter methylation in lung cancer identifies novel methylation markers for multiple malignancies.* PLoS Med, 2006. **3**(12): p. e486.

92. Bell, A.C. and G. Felsenfeld, *Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene.* Nature, 2000. **405**(6785): p. 482-5.

93. Yoon, Y.S., et al., *Analysis of the H19ICR insulator.* Mol Cell Biol, 2007. **27**(9): p. 3499-510.

94. Fatemi, M. and P.A. Wade, *MBD family proteins: reading the epigenetic code.* J Cell Sci, 2006. **119**(Pt 15): p. 3033-7.

95. Nan, X., et al., *Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex.* Nature, 1998. **393**(6683): p. 386-9.

96. Bird, A., *DNA methylation patterns and epigenetic memory.* Genes Dev, 2002. **16**(1): p. 6-21.

97. Levings, P.P. and J. Bungert, *The human beta-globin locus control region.* Eur J Biochem, 2002. **269**(6): p. 1589-99.

98. Suzuki, M., et al., *Direct association between PU.1 and MeCP2 that recruits mSin3A-HDAC complex for PU.1-mediated transcriptional repression.* Oncogene, 2003. **22**(54): p. 8688-98.

99. Dover, G.J., S. Brusilow, and D. Samid, *Increased fetal hemoglobin in patients receiving sodium 4-phenylbutyrate.* N Engl J Med, 1992. **327**(8): p. 569-70.

100. Sauntharajah, Y. and J. DeSimone, *Clinical studies with fetal hemoglobin-enhancing agents in sickle cell disease.* Semin Hematol, 2004. **41**(4 Suppl 6): p. 11-6.

101. Ghoshal, K., et al., *5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal.* Mol Cell Biol, 2005. **25**(11): p. 4727-41.

102. Cameron, E.E., et al., *Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer.* Nat Genet, 1999. **21**(1): p. 103-7.

103. Belinsky, S.A., et al., *Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer.* Cancer Res, 2003. **63**(21): p. 7089-93.

104. Gore, S.D., et al., *Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms.* Cancer Res, 2006. **66**(12): p. 6361-9.

105. Singh, M., et al., *The gamma-globin gene promoter progressively demethylates as the hematopoietic stem progenitor cells differentiate along the erythroid lineage in baboon fetal liver and adult bone marrow.* Exp Hematol, 2007. **35**(1): p. 48-55.

106. Kishigami, S., et al., *Epigenetic abnormalities of the mouse paternal zygotic genome associated with microinsemination of round spermatids.* Dev Biol, 2006. **289**(1): p. 195-205.

107. Kapoor, A., F. Agius, and J.K. Zhu, *Preventing transcriptional gene silencing by active DNA demethylation.* FEBS Lett, 2005. **579**(26): p. 5889-98.

108. Ivascu, C., et al., *DNA methylation profiling of transcription factor genes in normal lymphocyte development and lymphomas.* Int J Biochem Cell Biol, 2007. **39**(7-8): p. 1523-38.

109. Mahadevan, D., et al., *Transcript profiling in peripheral T-cell lymphoma, not otherwise specified, and diffuse large B-cell lymphoma identifies distinct tumor profile signatures.* Mol Cancer Ther, 2005. **4**(12): p. 1867-79.

110. Rosenbauer, F., et al., *Lymphoid cell growth and transformation are suppressed by a key regulatory element of the gene encoding PU.1.* Nat Genet, 2006. **38**(1): p. 27-37.

111. Ahluwalia, A., et al., *DNA methylation in ovarian cancer. II. Expression of DNA methyltransferases in ovarian cancer cell lines and normal ovarian epithelial cells.* Gynecol Oncol, 2001. **82**(2): p. 299-304.

112. Butcher, D.T. and D.I. Rodenhiser, *Epigenetic inactivation of BRCA1 is associated with aberrant expression of CTCF and DNA methyltransferase (DNMT3B) in some sporadic breast tumours.* Eur J Cancer, 2007. **43**(1): p. 210-9.

113. Patra, S.K., et al., *DNA methyltransferase and demethylase in human prostate cancer.* Mol Carcinog, 2002. **33**(3): p. 163-71.

114. Lin, R.K., et al., *Alteration of DNA methyltransferases contributes to 5'CpG methylation and poor prognosis in lung cancer.* Lung Cancer, 2007. **55**(2): p. 205-13.

115. Ostler, K.R., et al., *Cancer cells express aberrant DNMT3B transcripts encoding truncated proteins.* Oncogene, 2007. **26**(38): p. 5553-63.

116. Di Croce, L., et al., *Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor.* Science, 2002. **295**(5557): p. 1079-82.

117. Liu, S., et al., *Interplay of RUNX1/MTG8 and DNA methyltransferase 1 in acute myeloid leukemia.* Cancer Res, 2005. **65**(4): p. 1277-84.

118. Verdone, L., et al., *Histone acetylation in gene regulation.* Brief Funct Genomic Proteomic, 2006. **5**(3): p. 209-21.

119. Lee, K.K. and J.L. Workman, *Histone acetyltransferase complexes: one size doesn't fit all.* Nat Rev Mol Cell Biol, 2007. **8**(4): p. 284-95.

120. Kawasaki, H., et al., *ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation.* Nature, 2000. **405**(6783): p. 195-200.

121. Minucci, S. and P.G. Pelicci, *Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer.* Nat Rev Cancer, 2006. **6**(1): p. 38-51.

122. Ouaissi, M. and A. Ouaissi, *Histone deacetylase enzymes as potential drug targets in cancer and parasitic diseases.* J Biomed Biotechnol, 2006. **2006**(2): p. 13474.

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

124. Bruserud, O., et al., *Protein lysine acetylation in normal and leukaemic haematopoiesis: HDACs as possible therapeutic targets in adult AML.* Expert Opin Ther Targets, 2006. **10**(1): p. 51-68.

125. Huo, X. and J. Zhang, *Important roles of reversible acetylation in the function of hematopoietic transcription factors.* J Cell Mol Med, 2005. **9**(1): p. 103-12.

126. Letting, D.L., et al., *Formation of a tissue-specific histone acetylation pattern by the hematopoietic transcription factor GATA-1.* Mol Cell Biol, 2003. **23**(4): p. 1334-40.

127. Boyes, J., et al., *Regulation of activity of the transcription factor GATA-1 by acetylation.* Nature, 1998. **396**(6711): p. 594-8.

128. Hung, H.L., et al., *CREB-Binding protein acetylates hematopoietic transcription factor GATA-1 at functionally important sites.* Mol Cell Biol, 1999. **19**(5): p. 3496-505.

129. Begley, C.G., et al., *Chromosomal translocation in a human leukemic stem-cell line disrupts the T-cell antigen receptor delta-chain diversity region and results in a previously unreported fusion transcript.* Proc Natl Acad Sci U S A, 1989. **86**(6): p. 2031-5.

130. Huang, S., et al., *p300 functions as a transcriptional coactivator for the TAL1/SCL oncoprotein.* Oncogene, 1999. **18**(35): p. 4958-67.

131. Huang, S., et al., *P/CAF-mediated acetylation regulates the function of the basic helix-loop-helix transcription factor TAL1/SCL.* Embo J, 2000. **19**(24): p. 6792-803.

132. Huang, S. and S.J. Brandt, *mSin3A regulates murine erythroleukemia cell differentiation through association with the TAL1 (or SCL) transcription factor.* Mol Cell Biol, 2000. **20**(6): p. 2248-59.

133. O'Neil, J., et al., *TAL1/SCL induces leukemia by inhibiting the transcriptional activity of E47/HEB.* Cancer Cell, 2004. **5**(6): p. 587-96.

134. Panagopoulos, I., et al., *Fusion of the MORF and CBP genes in acute myeloid leukemia with the t(10;16)(q22;p13).* Hum Mol Genet, 2001. **10**(4): p. 395-404.

135. Rozman, M., et al., *Type I MOZ/CBP (MYST3/CREBBP) is the most common chimeric transcript in acute myeloid leukemia with t(8;16)(p11;p13) translocation.* Genes Chromosomes Cancer, 2004. **40**(2): p. 140-5.

136. Fraga, M.F. and M. Esteller, *Towards the human cancer epigenome: a first draft of histone modifications.* Cell Cycle, 2005. **4**(10): p. 1377-81.

137. Santillan, D.A., et al., *Bromodomain and histone acetyltransferase domain specificities control mixed lineage leukemia phenotype.* Cancer Res, 2006. **66**(20): p. 10032-9.

138. Bolden, J.E., M.J. Peart, and R.W. Johnstone, *Anticancer activities of histone deacetylase inhibitors.* Nat Rev Drug Discov, 2006. **5**(9): p. 769-84.

139. Nebbioso, A., et al., *Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells.* Nat Med, 2005. **11**(1): p. 77-84.

140. Insinga, A., et al., *Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway.* Nat Med, 2005. **11**(1): p. 71-6.

141. Lindemann, R.K., et al., *Analysis of the apoptotic and therapeutic activities of histone deacetylase inhibitors by using a mouse model of B cell lymphoma.* Proc Natl Acad Sci U S A, 2007. **104**(19): p. 8071-6.

142. Fandy, T.E., et al., *Interactive effects of HDAC inhibitors and TRAIL on apoptosis are associated with changes in mitochondrial functions and*

expressions of cell cycle regulatory genes in multiple myeloma. Neoplasia, 2005. **7**(7): p. 646-57.

143. Rosato, R.R. and S. Grant, *Histone deacetylase inhibitors: insights into mechanisms of lethality.* Expert Opin Ther Targets, 2005. **9**(4): p. 809-24.

144. Mai, A. and L. Altucci, *Epi-drugs to fight cancer: from chemistry to cancer treatment, the road ahead.* Int J Biochem Cell Biol, 2009. **41**(1): p. 199-213.

145. Remiszewski, S.W., et al., *N-hydroxy-3-phenyl-2-propenamides as novel inhibitors of human histone deacetylase with in vivo antitumor activity: discovery of (2E)-N-hydroxy-3-[4-[[(2-hydroxyethyl)[2-(1H-indol-3-yl)ethyl]amino]methyl]phenyl]-2-propenamide (NVP-LAQ824).* J Med Chem, 2003. **46**(21): p. 4609-24.

146. Arts, J., et al., *R306465 is a novel potent inhibitor of class I histone deacetylases with broad-spectrum antitumoral activity against solid and haematological malignancies.* Br J Cancer, 2007. **97**(10): p. 1344-53.

147. Khan, N., et al., *Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors.* Biochem J, 2008. **409**(2): p. 581-9.

148. Steele, N.L., et al., *A phase 1 pharmacokinetic and pharmacodynamic study of the histone deacetylase inhibitor belinostat in patients with advanced solid tumors.* Clin Cancer Res, 2008. **14**(3): p. 804-10.

149. Qian, X., et al., *Activity of the histone deacetylase inhibitor belinostat (PXD101) in preclinical models of prostate cancer.* Int J Cancer, 2008. **122**(6): p. 1400-10.

150. Piekarz, R.L., et al., *Inhibitor of histone deacetylation, depsipeptide (FR901228), in the treatment of peripheral and cutaneous T-cell lymphoma: a case report.* Blood, 2001. **98**(9): p. 2865-8.

151. Piekarz, R.L., et al., *Cardiac studies in patients treated with depsipeptide, FK228, in a phase II trial for T-cell lymphoma.* Clin Cancer Res, 2006. **12**(12): p. 3762-73.

152. Byrd, J.C., et al., *A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia.* Blood, 2005. **105**(3): p. 959-67.

153. Lucas, D.M., et al., *The histone deacetylase inhibitor MS-275 induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells.* Leukemia, 2004. **18**(7): p. 1207-14.

154. Qian, D.Z., et al., *Antitumor activity of the histone deacetylase inhibitor MS-275 in prostate cancer models.* Prostate, 2007. **67**(11): p. 1182-93.

155. Hess-Stumpp, H., et al., *MS-275, a potent orally available inhibitor of histone deacetylases--the development of an anticancer agent.* Int J Biochem Cell Biol, 2007. **39**(7-8): p. 1388-405.

156. Moradei, O.M., et al., *Novel aminophenyl benzamide-type histone deacetylase inhibitors with enhanced potency and selectivity.* J Med Chem, 2007. **50**(23): p. 5543-6.

157. Bali, P., et al., *Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors.* J Biol Chem, 2005. **280**(29): p. 26729-34.

158. Carew, J.S., F.J. Giles, and S.T. Nawrocki, *Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy.* Cancer Lett, 2008. **269**(1): p. 7-17.

159. George, P., et al., *Combination of the histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC cells and AML cells with activating mutation of FLT-3.* Blood, 2005. **105**(4): p. 1768-76.

160. Nawrocki, S.T., et al., *Myc regulates aggresome formation, the induction of Noxa, and apoptosis in response to the combination of bortezomib and SAHA.* Blood, 2008. **112**(7): p. 2917-26.

161. Heider, U., et al., *Synergistic interaction of the histone deacetylase inhibitor SAHA with the proteasome inhibitor bortezomib in mantle cell lymphoma.* Eur J Haematol, 2008. **80**(2): p. 133-42.

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

163. Jones, P.A. and S.B. Baylin, *The epigenomics of cancer.* Cell, 2007. **128**(4): p. 683-92.

164. Silverman, L.R., et al., *Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B.* J Clin Oncol, 2002. **20**(10): p. 2429-40.

165. Kantarjian, H.M., et al., *Results of decitabine (5-aza-2'deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia.* Cancer, 2003. **98**(3): p. 522-8.

166. Yang, A.S., et al., *DNA methylation changes after 5-aza-2'-deoxycytidine therapy in patients with leukemia.* Cancer Res, 2006. **66**(10): p. 5495-503.

167. Gaudet, F., et al., *Induction of tumors in mice by genomic hypomethylation.* Science, 2003. **300**(5618): p. 489-92.

168. Kantarjian, H.M., et al., *Survival advantage with decitabine versus intensive chemotherapy in patients with higher risk myelodysplastic syndrome: comparison with historical experience.* Cancer, 2007. **109**(6): p. 1133-7.

169. Fenaux, P., et al., *Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study.* Lancet Oncol, 2009. **10**(3): p. 223-32.

170. Soriano, A.O., et al., *Safety and clinical activity of the combination of 5-azacytidine, valproic acid, and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome.* Blood, 2007. **110**(7): p. 2302-8.

171. Garcia-Manero, G., et al., *Phase 1 study of the oral isotype specific histone deacetylase inhibitor MGCD0103 in leukemia.* Blood, 2008. **112**(4): p. 981-9.

172. Jain, N., A. Rossi, and G. Garcia-Manero, *Epigenetic therapy of leukemia: An update.* Int J Biochem Cell Biol, 2009. **41**(1): p. 72-80.

173. Zhou, L., et al., *Zebularine: a novel DNA methylation inhibitor that forms a covalent complex with DNA methyltransferases.* J Mol Biol, 2002. **321**(4): p. 591-9.

174. Holleran, J.L., et al., *Plasma pharmacokinetics, oral bioavailability, and interspecies scaling of the DNA methyltransferase inhibitor, zebularine.* Clin Cancer Res, 2005. **11**(10): p. 3862-8.

175. Pina, I.C., et al., *Psammaplins from the sponge Pseudoceratina purpurea: inhibition of both histone deacetylase and DNA methyltransferase.* J Org Chem, 2003. **68**(10): p. 3866-73.

176. Brueckner, B., et al., *Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases.* Cancer Res, 2005. **65**(14): p. 6305-11.

177. Collins, S.J., *Retinoic acid receptors, hematopoiesis and leukemogenesis.* Curr Opin Hematol, 2008. **15**(4): p. 346-51.

178. Minucci, S. and P.G. Pelicci, *Retinoid receptors in health and disease: co-regulators and the chromatin connection.* Semin Cell Dev Biol, 1999. **10**(2): p. 215-25.

179. Kamashev, D., D. Vitoux, and H. De The, *PML-RARA-RXR oligomers mediate retinoid and rexinoid/cAMP cross-talk in acute promyelocytic leukemia cell differentiation.* J Exp Med, 2004. **199**(8): p. 1163-74.

180. Minucci, S., et al., *Histone deacetylases: a common molecular target for differentiation treatment of acute myeloid leukemias?* Oncogene, 2001. **20**(24): p. 3110-5.

181. Carbone, R., et al., *Recruitment of the histone methyltransferase SUV39H1 and its role in the oncogenic properties of the leukemia-associated PML-retinoic acid receptor fusion protein.* Mol Cell Biol, 2006. **26**(4): p. 1288-96.

182. Morey, L., et al., *MBD3, a component of the NuRD complex, facilitates chromatin alteration and deposition of epigenetic marks.* Mol Cell Biol, 2008. **28**(19): p. 5912-23.

183. Villa, R., et al., *Role of the polycomb repressive complex 2 in acute promyelocytic leukemia.* Cancer Cell, 2007. **11**(6): p. 513-25.

184. Hoemme, C., et al., *Chromatin modifications induced by PML-RARalpha repress critical targets in leukemogenesis as analyzed by ChIP-Chip.* Blood, 2008. **111**(5): p. 2887-95.

185. Stein, H., et al., *The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells.* Blood, 1985. **66**(4): p. 848-58.

186. Schwab, U., et al., *Production of a monoclonal antibody specific for Hodgkin and Sternberg-Reed cells of Hodgkin's disease and a subset of normal lymphoid cells.* Nature, 1982. **299**(5878): p. 65-7.

187. Herbst, H., et al., *Immunoglobulin and T-cell receptor gene rearrangements in Hodgkin's disease and Ki-1-positive anaplastic large cell lymphoma: dissociation between phenotype and genotype.* Leuk Res, 1989. **13**(2): p. 103-16.

188. Stein, H., et al., *CD30(+) anaplastic large cell lymphoma: a review of its histopathologic, genetic, and clinical features.* Blood, 2000. **96**(12): p. 3681-95.

189. Kadin, M.E., et al., *Childhood Ki-1 lymphoma presenting with skin lesions and peripheral lymphadenopathy.* Blood, 1986. **68**(5): p. 1042-9.

190. Morgan, R., et al., *Chromosome 5q35 breakpoint in malignant histiocytosis.* N Engl J Med, 1986. **314**(20): p. 1322.

191. Rimokh, R., et al., *A translocation involving a specific breakpoint (q35) on chromosome 5 is characteristic of anaplastic large cell lymphoma ('Ki-1 lymphoma').* Br J Haematol, 1989. **71**(1): p. 31-6.

192. Le Beau, M.M., et al., *The t(2;5)(p23;q35): a recurring chromosomal abnormality in Ki-1-positive anaplastic large cell lymphoma.* Leukemia, 1989. **3**(12): p. 866-70.

193. Morris, S.W., et al., *Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma.* Science, 1994. **263**(5151): p. 1281-4.

194. Shiota, M., et al., *Hyperphosphorylation of a novel 80 kDa protein-tyrosine kinase similar to Ltk in a human Ki-1 lymphoma cell line, AMS3.* Oncogene, 1994. **9**(6): p. 1567-74.

195. Shiota, M., et al., *Diagnosis of t(2;5)(p23;q35)-associated Ki-1 lymphoma with immunohistochemistry.* Blood, 1994. **84**(11): p. 3648-52.

196. Pulford, K., S.W. Morris, and F. Turturro, *Anaplastic lymphoma kinase proteins in growth control and cancer.* J Cell Physiol, 2004. **199**(3): p. 330-58.

197. Falini, B., et al., *Lymphomas expressing ALK fusion protein(s) other than NPM-ALK.* Blood, 1999. **94**(10): p. 3509-15.

198. Iwahara, T., et al., *Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system.* Oncogene, 1997. **14**(4): p. 439-49.

199. Morris, S.W., et al., *ALK, the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK).* Oncogene, 1997. **14**(18): p. 2175-88.

200. Loren, C.E., et al., *A crucial role for the Anaplastic lymphoma kinase receptor tyrosine kinase in gut development in Drosophila melanogaster.* EMBO Rep, 2003. **4**(8): p. 781-6.

201. Falini, B., et al., *ALK expression defines a distinct group of T/null lymphomas ("ALK lymphomas") with a wide morphological spectrum.* Am J Pathol, 1998. **153**(3): p. 875-86.

202. Lamant, L., et al., *Expression of the ALK tyrosine kinase gene in neuroblastoma.* Am J Pathol, 2000. **156**(5): p. 1711-21.

203. Delsol, G., et al., *A new subtype of large B-cell lymphoma expressing the ALK kinase and lacking the 2; 5 translocation.* Blood, 1997. **89**(5): p. 1483-90.

204. Trumper, L., et al., *Detection of the t(2;5)-associated NPM/ALK fusion cDNA in peripheral blood cells of healthy individuals.* Br J Haematol, 1998. **103**(4): p. 1138-44.

205. Maes, B., et al., *The NPM-ALK and the ATIC-ALK fusion genes can be detected in non-neoplastic cells.* Am J Pathol, 2001. **158**(6): p. 2185-93.

206. Gascoyne, R.D., et al., *ALK-positive diffuse large B-cell lymphoma is associated with Clathrin-ALK rearrangements: report of 6 cases.* Blood, 2003. **102**(7): p. 2568-73.

207. Onciu, M., et al., *ALK-positive plasmablastic B-cell lymphoma with expression of the NPM-ALK fusion transcript: report of 2 cases.* Blood, 2003. **102**(7): p. 2642-4.

208. Griffin, C.A., et al., *Recurrent involvement of 2p23 in inflammatory myofibroblastic tumors.* Cancer Res, 1999. **59**(12): p. 2776-80.

209. Lawrence, B., et al., *TPM3-ALK and TPM4-ALK oncogenes in inflammatory myofibroblastic tumors.* Am J Pathol, 2000. **157**(2): p. 377-84.

210. Lee, H.H., et al., *Jelly belly protein activates the receptor tyrosine kinase Alk to specify visceral muscle pioneers.* Nature, 2003. **425**(6957): p. 507-12.

211. Englund, C., et al., *Jeb signals through the Alk receptor tyrosine kinase to drive visceral muscle fusion.* Nature, 2003. **425**(6957): p. 512-6.

212. Stoica, G.E., et al., *Identification of anaplastic lymphoma kinase as a receptor for the growth factor pleiotrophin.* J Biol Chem, 2001. **276**(20): p. 16772-9.

213. Stoica, G.E., et al., *Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types.* J Biol Chem, 2002. **277**(39): p. 35990-8.

214. Moog-Lutz, C., et al., *Activation and inhibition of anaplastic lymphoma kinase receptor tyrosine kinase by monoclonal antibodies and absence of agonist activity of pleiotrophin.* J Biol Chem, 2005. **280**(28): p. 26039-48.

215. Gascoyne, R.D., et al., *Prognostic significance of anaplastic lymphoma kinase (ALK) protein expression in adults with anaplastic large cell lymphoma.* Blood, 1999. **93**(11): p. 3913-21.

216. Falini, B., et al., *ALK+ lymphoma: clinico-pathological findings and outcome.* Blood, 1999. **93**(8): p. 2697-706.

217. Borer, R.A., et al., *Major nucleolar proteins shuttle between nucleus and cytoplasm.* Cell, 1989. **56**(3): p. 379-90.

218. Bischof, D., et al., *Role of the nucleophosmin (NPM) portion of the non-Hodgkin's lymphoma-associated NPM-anaplastic lymphoma kinase fusion protein in oncogenesis.* Mol Cell Biol, 1997. **17**(4): p. 2312-25.

219. Bai, R.Y., et al., *Nucleophosmin-anaplastic lymphoma kinase of large-cell anaplastic lymphoma is a constitutively active tyrosine kinase that utilizes phospholipase C-gamma to mediate its mitogenicity.* Mol Cell Biol, 1998. **18**(12): p. 6951-61.

220. Turner, S.D., et al., *CD2 promoter regulated nucleophosmin-anaplastic lymphoma kinase in transgenic mice causes B lymphoid malignancy.* Anticancer Res, 2006. **26**(5A): p. 3275-9.

221. Kuefer, M.U., et al., *Retrovirus-mediated gene transfer of NPM-ALK causes lymphoid malignancy in mice.* Blood, 1997. **90**(8): p. 2901-10.

222. Miething, C., et al., *The oncogenic fusion protein nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) induces two distinct malignant phenotypes in a murine retroviral transplantation model.* Oncogene, 2003. **22**(30): p. 4642-7.

223. Chiarle, R., et al., *NPM-ALK transgenic mice spontaneously develop T-cell lymphomas and plasma cell tumors.* Blood, 2003. **101**(5): p. 1919-27.

224. Lange, K., et al., *Overexpression of NPM-ALK induces different types of malignant lymphomas in IL-9 transgenic mice.* Oncogene, 2003. **22**(4): p. 517-27.

225. Turner, S.D., et al., *Vav-promoter regulated oncogenic fusion protein NPM-ALK in transgenic mice causes B-cell lymphomas with hyperactive Jun kinase.* Oncogene, 2003. **22**(49): p. 7750-61.

226. Fujimoto, J., et al., *Characterization of the transforming activity of p80, a hyperphosphorylated protein in a Ki-1 lymphoma cell line with chromosomal translocation t(2;5).* Proc Natl Acad Sci U S A, 1996. **93**(9): p. 4181-6.

227. Bai, R.Y., et al., *Nucleophosmin-anaplastic lymphoma kinase associated with anaplastic large-cell lymphoma activates the phosphatidylinositol 3-kinase/Akt antiapoptotic signaling pathway.* Blood, 2000. **96**(13): p. 4319-27.

228. Zamo, A., et al., *Anaplastic lymphoma kinase (ALK) activates Stat3 and protects hematopoietic cells from cell death.* Oncogene, 2002. **21**(7): p. 1038-47.

229. Voena, C., et al., *The tyrosine phosphatase Shp2 interacts with NPM-ALK and regulates anaplastic lymphoma cell growth and migration.* Cancer Res, 2007. **67**(9): p. 4278-86.

230. Chiarle, R., et al., *Stat3 is required for ALK-mediated lymphomagenesis and provides a possible therapeutic target.* Nat Med, 2005. **11**(6): p. 623-9.

231. Coluccia, A.M., et al., *Bcl-XL down-regulation suppresses the tumorigenic potential of NPM/ALK in vitro and in vivo.* Blood, 2004. **103**(7): p. 2787-94.

232. Piva, R., et al., *Functional validation of the anaplastic lymphoma kinase signature identifies CEBPB and BCL2A1 as critical target genes.* J Clin Invest, 2006. **116**(12): p. 3171-82.

233. Tran, H., et al., *The many forks in FOXO's road.* Sci STKE, 2003. **2003**(172): p. RE5.

234. Rassidakis, G.Z., et al., *Inhibition of Akt increases p27Kip1 levels and induces cell cycle arrest in anaplastic large cell lymphoma.* Blood, 2005. **105**(2): p. 827-9.

235. Slupianek, A. and T. Skorski, *NPM/ALK downregulates p27Kip1 in a PI-3K-dependent manner.* Exp Hematol, 2004. **32**(12): p. 1265-71.

236. Slupianek, A., et al., *Role of phosphatidylinositol 3-kinase-Akt pathway in nucleophosmin/anaplastic lymphoma kinase-mediated lymphomagenesis.* Cancer Res, 2001. **61**(5): p. 2194-9.

237. Leventaki, V., et al., *NPM-ALK oncogenic kinase promotes cell-cycle progression through activation of JNK/cJun signaling in anaplastic large-cell lymphoma.* Blood, 2007. **110**(5): p. 1621-30.

238. Gavathiotis, E., et al., *BAX activation is initiated at a novel interaction site.* Nature, 2008. **455**(7216): p. 1076-81.

239. Putcha, G.V., et al., *JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis.* Neuron, 2003. **38**(6): p. 899-914.

240. Paschos, K., et al., *Epstein-barr virus latency in B cells leads to epigenetic repression and CpG methylation of the tumour suppressor gene Bim.* PLoS Pathog, 2009. **5**(6): p. e1000492.

241. San Jose-Eneriz, E., et al., *Epigenetic down-regulation of BIM expression is associated with reduced optimal responses to imatinib treatment in chronic myeloid leukaemia.* Eur J Cancer, 2009. **45**(10): p. 1877-89.

242. Bachmann, P.S., et al., *Epigenetic silencing of BIM in glucocorticoid poor-responsive pediatric acute lymphoblastic leukemia, and its reversal by histone deacetylase inhibition.* Blood.

243. Mestre-Escorihuela, C., et al., *Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas.* Blood, 2007. **109**(1): p. 271-80.

244. Ambrogio, C., et al., *NPM-ALK oncogenic tyrosine kinase controls T-cell identity by transcriptional regulation and epigenetic silencing in lymphoma cells.* Cancer Res, 2009. **69**(22): p. 8611-9.

245. Weber, M., et al., *Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells.* Nat Genet, 2005. **37**(8): p. 853-62.

246. Senese, S., et al., *Role for histone deacetylase 1 in human tumor cell proliferation.* Mol Cell Biol, 2007. **27**(13): p. 4784-95.

247. Laird, P.W., *Cancer epigenetics.* Hum Mol Genet, 2005. **14 Spec No 1**: p. R65-76.

248. Brown, R. and G. Strathdee, *Epigenomics and epigenetic therapy of cancer.* Trends Mol Med, 2002. **8**(4 Suppl): p. S43-8.

249. Laherty, C.D., et al., *Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression.* Cell, 1997. **89**(3): p. 349-56.

250. Zhang, Y., et al., *Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation.* Genes Dev, 1999. **13**(15): p. 1924-35.

251. Li, J., et al., *Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3.* Embo J, 2000. **19**(16): p. 4342-50.

252. Horlein, A.J., et al., *Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor.* Nature, 1995. **377**(6548): p. 397-404.

253. Jones, P.L., et al., *Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription.* Nat Genet, 1998. **19**(2): p. 187-91.

254. Kokura, K., et al., *The Ski protein family is required for MeCP2-mediated transcriptional repression.* J Biol Chem, 2001. **276**(36): p. 34115-21.

255. Yoon, H.G., et al., *N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso.* Mol Cell, 2003. **12**(3): p. 723-34.

256. Fraga, M.F., et al., *The affinity of different MBD proteins for a specific methylated locus depends on their intrinsic binding properties.* Nucleic Acids Res, 2003. **31**(6): p. 1765-74.

257. Bouillet, P., et al., *Gene structure alternative splicing, and chromosomal localization of pro-apoptotic Bcl-2 relative Bim.* Mamm Genome, 2001. **12**(2): p. 163-8.

258. Hildeman, D.A., et al., *Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim.* Immunity, 2002. **16**(6): p. 759-67.

259. Shinjyo, T., et al., *Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signaling in murine hematopoietic progenitors.* Mol Cell Biol, 2001. **21**(3): p. 854-64.

260. Biswas, S.C. and L.A. Greene, *Nerve growth factor (NGF) down-regulates the Bcl-2 homology 3 (BH3) domain-only protein Bim and suppresses its proapoptotic activity by phosphorylation.* J Biol Chem, 2002. **277**(51): p. 49511-6.

261. Zantl, N., et al., *Frequent loss of expression of the pro-apoptotic protein Bim in renal cell carcinoma: evidence for contribution to apoptosis resistance.* Oncogene, 2007. **26**(49): p. 7038-48.

262. Mestre-Escorihuela, C., et al., *Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas.* Blood., 2007. **109**(1): p. 271-80. Epub 2006 Sep 7.

263. Ting, A.H., K.M. McGarvey, and S.B. Baylin, *The cancer epigenome--components and functional correlates.* Genes Dev., 2006. **20**(23): p. 3215-31.

264. Watt, F. and P.L. Molloy, *Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter.* Genes Dev., 1988. **2**(9): p. 1136-43.

265. Tate, P.H. and A.P. Bird, *Effects of DNA methylation on DNA-binding proteins and gene expression.* Curr Opin Genet Dev., 1993. **3**(2): p. 226-31.

266. Klose, R.J. and A.P. Bird, *Genomic DNA methylation: the mark and its mediators.* Trends Biochem Sci., 2006. **31**(2): p. 89-97. Epub 2006 Jan 5.

267. Shahbazian, M.D. and M. Grunstein, *Functions of site-specific histone acetylation and deacetylation.* Annu Rev Biochem., 2007. **76**: p. 75-100.

268. Le Guezennec, X., et al., *MBD2/NuRD and MBD3/NuRD, two distinct complexes with different biochemical and functional properties.* Mol Cell Biol, 2006. **26**(3): p. 843-51.

269. Zhang, Q., et al., *STAT5A is epigenetically silenced by the tyrosine kinase NPM1-ALK and acts as a tumor suppressor by reciprocally inhibiting NPM1-ALK expression.* Nat Med, 2007. **13**(11): p. 1341-8.

270. Essafi, A., et al., *Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells.* Oncogene, 2005. **24**(14): p. 2317-29.

271. Stahl, M., et al., *The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2.* J Immunol, 2002. **168**(10): p. 5024-31.