Epigenetics in *MLL*-rearranged Infant Acute Lymphoblastic Leukemia

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Epigenetics in *MLL*-rearranged Infant Acute Lymphoblastic Leukemia

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acute lymfatische leukemie bij zuigelingen

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"Do not go where the path may lead,
go instead where there is no path and leave a trail."

(Ralph Waldo Emerson)

I lovingly dedicate this thesis to my parents,
who supported me each step of the way

I also dedicate this thesis to the memory of Brian, whose
optimism, zest for life and inner strength
was an inspiration for all of us

“Our lost friends are not death, but gone before, advanced a stage of two upon that road which we must travel in the steps they trod.”

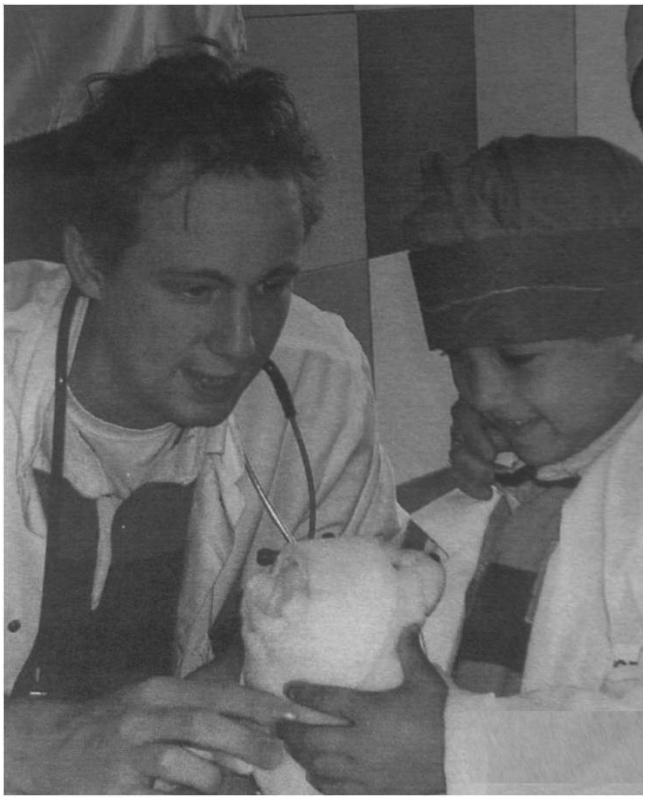
(Aristophanes)

IN MEMORIAM

Brian Robert Hermand van Westing

Bri/ Doc

September 27, 1978 — March 27, 2009



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PROLOGUE

"An understanding of biology is fundamental to understanding how disease works, and consequently how it can be diagnosed, treated and prevented."

(Adapted from McGavin and Zachary, 2007)

1

General Introduction, Aims and Scope of this Thesis.

A SHORT HISTORY

Travelling back in time

As far back as 400 B.C., the ancient Greek physician Hippocrates already postulated that the sanguine (from sanguis, Latin for "blood") was one of the four "humors" or basic body fluids.[1] Yet, the concept of a cellular composition of the blood remained unnoticed. In 1658 the Dutch natural scientist Jan Swammerdam was the first to observe red blood cells under the microscope,[2, 3] followed by a more precise description of these red corpuscles by his acquaintance Antoni van Leeuwenhoek who even approximated their size: "25.000 times smaller than a fine grain of sand" **(figure 1)**.[2, 4]

" I have diverse times endeavoured to see and to know what parts the Blood consists of; and at length I have observed, taking some Blood out of my own hand, that it consists of small round globules driven through a crystalline humidity or water: Yet, whether all Blood be such, I doubt. And exhibiting my Blood to myself in very small parcels, the globules yielded very little colour."
(The first Microscopical observations of Blood by Antoni van Leeuwenhoek in 1674)

These observed red corpuscles or erythrocytes remained the only known type of blood cells during the 150 years that followed, until in 1843 the French pathologist Gabriel Andral reported on the existence of clear white blood cells or leukocytes.[2, 5] Simultaneously, an English country practitioner named William Addison also witnessed these white cells, as well as observing a disease state with "pussy white blood cells" that seemed capable of passing through the walls of capillary vessels.[3, 6] Roughly around the same time the French physician Albert Donné also described a bizarre disease that he believed to be characterized by a maturation arrest of transitional blood cells.[7] Soon thereafter, in 1845, one of Donné's foreign students, John Hughes Bennett, provided a description of this disease which he named leukocythemia or

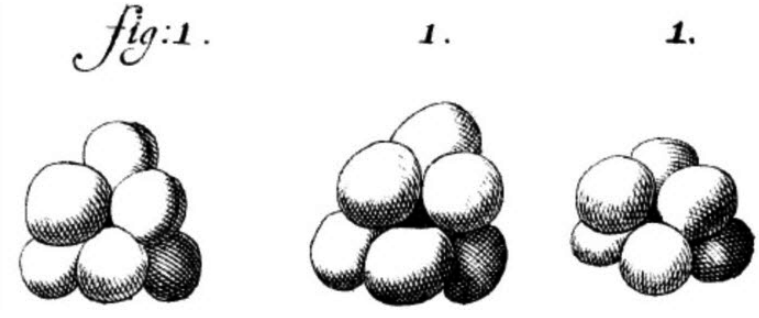

Figure 1. The first visualisations of blood cells.
A schematic drawing made by microscopist Antoni van Leeuwenhoek in 1695, and published in *Arcana Natura Detecta.*[4]

"white-cell blood".[2, 8] In that same year, Rudolf Virchow independently described a lethal disease characterized by enlargement of the spleen and an excess of immature cells in the blood.[9, 10] Without knowing, Addison, Donné, Bennett and Virchow all described the same disease. However, it was Virchow who finally designated the disease simply leukemia (Greek for "white blood"). Not much later, in 1879, Paul Ehrlich recognized six different types of white corpuscles in the blood,[1, 2, 11] a discovery that would pave the way for the in-depth investigation of the respective blood cells and their formation.

GENERAL INTRODUCTION

Tracing the journey of blood cell development

Blood cell formation or hematopoiesis embodies an intricate process in which a delicate balance between proliferation (cell growth), differentiation (cell specialization), maturation (cell development or ripening) and apoptosis (programmed cell death) is of vital importance. Therefore, this process is stringently controlled by so called cytokines, such as interleukins and colony-stimulating factors.[12, 13]

The dorsal aorta has emerged as the first autonomous site for definitive hematopoiesis during the second month of embryonic development.[14-16] From the seventh month of fetal development on, blood cells are formed in the fetal liver and spleen.[17, 18] In the last two months of fetal development, blood cells migrate to the bone marrow which becomes the main site of hematopoiesis, maintaining this function throughout our lives.

Within the bone marrow, self-renewing pluripotent hematopoietic stem cells are able to differentiate into multipotent progenitor cells, and subsequently into common myeloid or common lymphoid progenitors. In turn, these cells give rise to more committed but still immature progenitor cells which are able to differentiate along their respective lineages into several different types of mature and functional blood cells. The myeloid lineage comprises monocytes, granulocytes (further sub-divided into neutrophils, eosinophils, and basophils), erythrocytes, and thrombocytes, while the lymphoid lineage generates B and T lymphocytes and natural killer cells **(figure 2)**.[12, 18] Once blood cells are mature and fully functional, they become detached from the bone marrow and are released into the peripheral blood to become part of the blood circulation. T cells mature in the thymus, and the spleen serves as a reservoir for erythrocytes.

Red blood cells or erythrocytes represent the most abundant type of blood cells and are responsible for the distinctive red color of the blood. These specialized cells contain hemoglobin, and their main function is to bind oxygen and transport it throughout the body to the intended target tissues. The different types of white blood cells or leukocytes, such as monocytes, granulocytes and lymphocytes, fulfill important functions within the immune system, protecting the body against

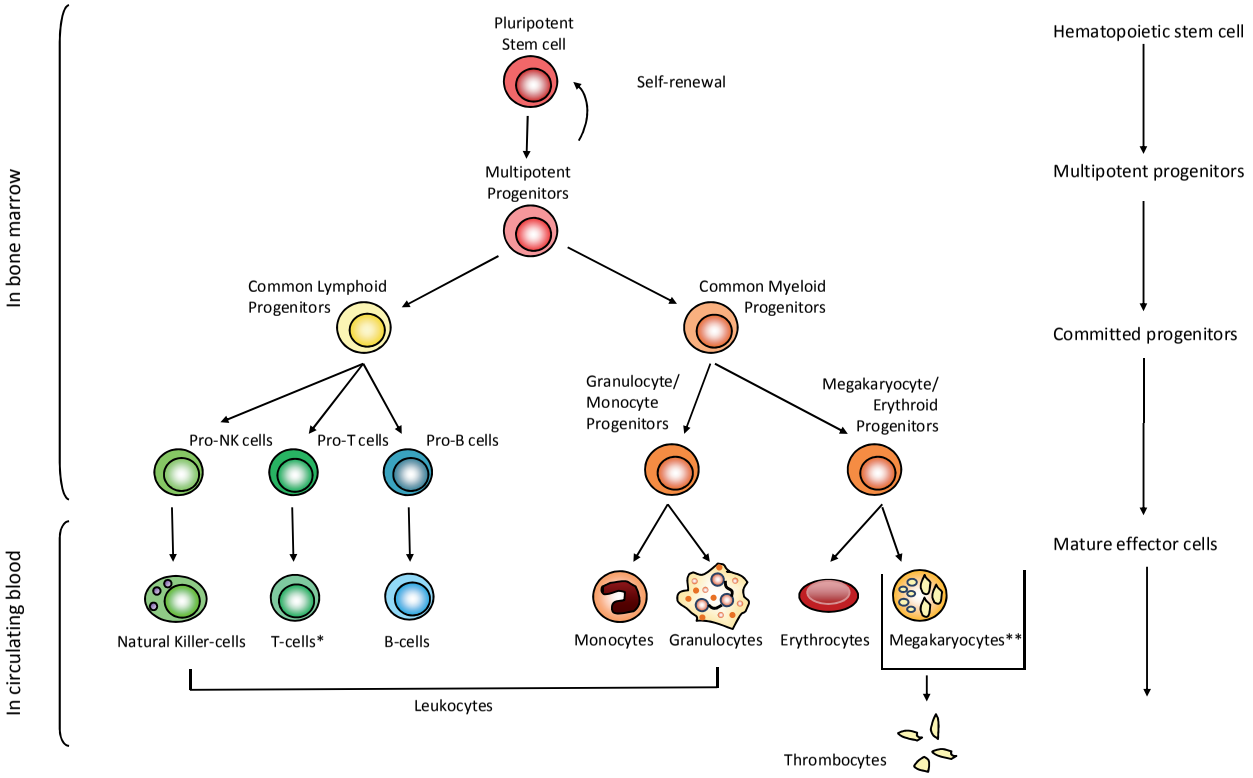

Figure 2. A schematic presentation of normal hematopoiesis.
In a nutshell, a self-renewing stem cell in the bone marrow produces multipotent progenitors that in turn generate committed progenitors and lineage-specific progenitors. Eventually mature blood cells will form that escape the bone marrow to circulate in the blood. *T-cells leave the bone marrow to undergo maturation in the thymus which accounts for their designation. **Megakaryocytes reside in the bone marrow and are not considered to be mature cells. NK = Natural Killer.

various pathogens (e.g. bacteria, viruses, or fungi), as well as targeting and eliminating dying and abnormal (including to cancerous) cells. Megakaryocytes form platelets or thrombocytes important for normal blood clotting in response to injuries, a process usually referred to as hemostasis.[18] In addition to being tightly controlled, hematopoiesis also constitutes a highly dynamic process which requires the replenishment of ~$7x10^9$ blood cells per kilogram body weight per day. When necessary, the production can be increased up to 5 - 10-fold, or readily shifted towards a preferred type of cell, to meet the body's requirements.

When blood cells go wrong or derail

Uncontrolled expansion of immature and non-functional leukocyte progenitor cells (termed blasts) evidently compromises hematopoiesis by disrupting the delicate balance between proliferation, differentiation, maturation, and apoptosis.[13] Progression of this process inevitably leads to leukemia development. As a consequence of abnormal accumulation of immature leukocytes, the production of healthy mature (and functional) blood cells is severely inhibited,[18] resulting in *anemia* (lack of blood) due to loss of erythrocytes, as well as bleeding and bruising due to loss of thrombocytes. Furthermore, depletion of functional white blood cells leads to an increased risk of infections. Moreover, leukemic cells start to circulate throughout the body via the peripheral blood stream and from there are able to infiltrate other organs such as the liver and the spleen, resulting into hepatomegaly (enlargement of the liver) and splenomegaly (enlargement of the spleen). Without adequate treatment, patients diagnosed with leukemia die from the malignancy.

Types of leukemia

As uncontrolled expansion or leukemia can occur in any of the distinct types of white blood cells at different stages during the maturation process, the term "leukemia" represents an umbrella term for a broad spectrum of hematological malignancies. Leukemia can be subdivided into *acute* and *chronic* forms. Acute leukemias are characterized by rapidly proliferating blasts of highly immature progenitors, and usually develop relatively fast. In contrast, chronic leukemias result from uninhibited proliferation of more mature, but still abnormal and non-functioning blood cells, and develop slowly. Leukemia can be further classed as myeloid (or myelogenous) and lymphoid (or lymphoblastic/ lymphocytic) leukemias, depending on the lineage of the progenitor cell affected. Whereas chronic myeloid leukemia (CML) and chronic lymphoblastic leukemia (CLL) are predominantly diagnosed in adults, acute leukemias, especially acute lymphoblastic leukemia (ALL), are most commonly found in children. Acute leukemia represents the most common type of pediatric cancer, accounting for ~30% of all childhood malignancies.[19] Among pediatric leukemia patients, acute myeloid leukemia (AML) is observed in ~15% of the cases, whereas the majority (>80%) is diagnosed with ALL.[20, 21]

Childhood acute lymphoblastic leukemia

ALL can be further subdivided by the type of lymphoblast affected. In children, ALL originating from immature B-cells is far more common (~85% of the cases) than ALL originating from immature T lymphocytes (~15% of the cases).[22]

The peak incidence of ALL is observed in children aged 2-5 years.[23] In general, pediatric leukemias originate *in utero* (during fetal development),[24] and are characterized by genetic abnormalities, such as balanced chromosomal translocations and numerical variations of the chromosomes.[25-27] Chromosomal abnormalities that are acquired before birth are considered to be the initiation step or "first oncogenic hit" for leukemia development. For transition to overt leukemia postnatal secondary genetic changes or so called "second hits" are necessary.[24]

The above mentioned age distribution is similar to that of many common childhood infectious diseases, such as respiratory infections, and therefore suggests an infectious etiology.[28] As a matter of fact, an abnormal immune response to common infections has emerged as a plausible mechanism contributing to the etiology.[28, 29] Besides infections in early childhood, environmental exposures, such as radiation and electromagnetic fields, have been put forward as possible causes of leukemia development in children.[30] However, these potential causes account for relatively few cases. Therefore as with many of the human cancers, the exact etiology remains obscure.

Childhood ALL can be subdivided according to different recurrent cytogenetic abnormalities such as copy number variations and chromosomal rearrangements. The most common subtypes are, for instance, presence of leukemic-specific hyperdiploidy (>50 chromosomes); the translocation t(12;21)(p13;q22) leading to the *TEL-AML1* gene fusion; the translocation t(9;22)(q34;q11) (also known as the Philadelphia chromosome), giving rise to *BCR-ABL* fusions; the t(1;19)(q23;p13.3) translocation generating *E2A-PBX* fusions, and varying translocations at chromosome arm 11q23 involving the *MLL* gene **(figure 3A)**.[20, 22, 27]

The World Health Organisations' (WHO) classification of childhood ALL is built from these genetic aberrations combined with an immunophenotypic classification based on the different maturation stages in B lymphocyte development **(table 1)**.[31]

Table 1. The WHO classification of childhood B cell ALL based on immunophenotype and genotype.[31]

Immunophenotype	CD19	CD10	cy μ*	sur μ**	Genotype
Pro B cell ALL	+	-	-	-	MLL-AF4, MLL-ENL
Common B cell ALL	+	+	-	-	TEL-AML, BCR-ABL
Pre B cell ALL	+	+	+	-	E2A-PBX
(Mature) B cell ALL	+	+	+	+	

* cy = cytoplasmic, ** sur = surface

Children diagnosed with ALL are treated with combination chemotherapy, which usually includes different classes of chemotherapeutic drugs such as glucocorticoids (prednisone or dexamethasone), vincristine, anthracyclines (e.g. daunorubicin or doxorubicin), L-asparaginase, methotrexate and 6-mercaptopurine.[31] Current treatment regimens for childhood ALL nowadays result in long-term survival in >85% of the patients **(figure 4A)**.[19, 23] Nonetheless, treatment success depends to some extent on the cytogenetic subgroup. For example, patients characterized by the *TEL-AML1* fusion or hyperdiploidy usually have superior outcomes over other ALL subtypes.[20] Although the tremendous improvement in the treatment of childhood ALL represents one of the most prominent success stories in pediatric oncology, small subsets of patients did not fully benefit from these developments and remain at high risk of treatment failure. Therefore, the ongoing challenge in pediatric oncology resides in improving prognosis for these children in particular.

Acute lymphoblastic leukemia in infants

In ~4% of all occurrences, ALL is diagnosed in an infant (i.e. a child < 1 year of age).[32] ALL in infants is one of the most aggressive subtypes of childhood leukemia.[32, 33] The overall survival rate for infant ALL patients remains at best ~50% **(figure 4A)**.[34] Infant ALL patients usually present with very high tumor loads,[31] reflected as exceedingly high white blood cell counts (median: $100x10^9$/liter), and massive hepato-splenomegaly often accompanied by central nervous system involvement.[32, 34] Biologically, infant ALL is characterized by a high incidence (~80% of the cases) of chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene on chromosome arm 11q23 **(figure 3B)**.[20, 32] The poor prognosis especially concerns the patients carrying *MLL* translocations **(figure 4B)**.[1]

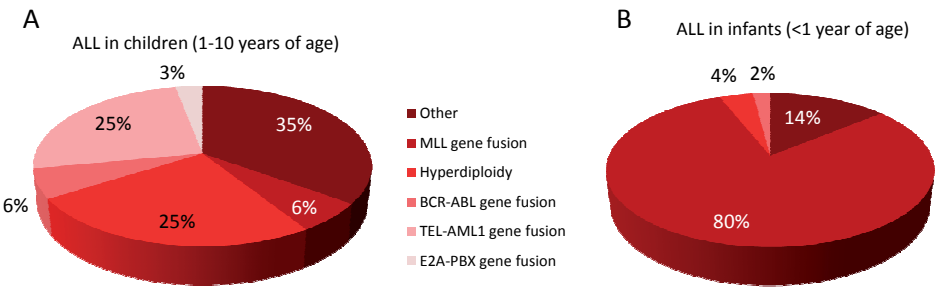

Figure 3. The most common (cyto)genetic subtypes of ALL in children (A) and in infants (B).

MLL-rearranged ALL in infants is characterized by unique gene expression profiles that are distinguishable from the profiles found in other types of leukemia.[35-38] These gene expression profiles are consistent with an early hematopoietic progenitor showing expression of multi-lineage markers.[35] In line with this *MLL*-rearranged infant ALL cells

often display a highly immature pro-B ($CD34^+$, $CD19^+$, $CD10^-$) immunophenotype,[39] still co-expressing specific myeloid surface markers such as CD15 and CD65. In contrast, ALL cells from infants or non-infant pediatric ALL patients carrying wild-type *MLL* genes usually show immunophenotypes of slightly more mature B-cells typified by CD10-positivity.[39] In addition to these differences, *MLL*-rearranged infant ALL cells frequently display resistance to glucocorticoids and L-asparaginase.[39] This phenomenon, together with the above described differences between infants and older children with ALL, already suggested that standard childhood ALL treatments are not sufficient or appropriate for infant ALL patients.

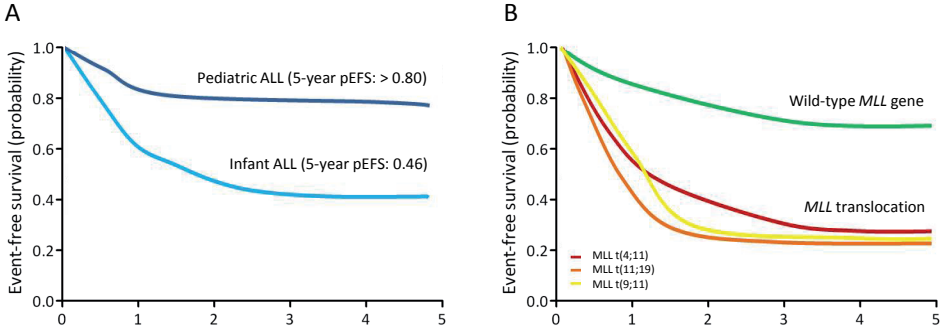

Figure 4. Event-free survival in childhood ALL.
A. Probability of event-free survival (pEFS) in infants and older children with ALL. **B.** pEFS in infant ALL divided according to the presence or absence of *MLL* gene rearrangements. These Kaplan Meier curves are based on the data obtained from the international collaborative INTERFANT-99 treatment protocol.[34]

Improved treatment for infant ALL patients

In 1999 a novel international treatment protocol specifically designed for the treatment of infant ALL was launched. This treatment protocol, designated INTERFANT-99[34], constitutes a hybrid protocol of the standard ALL protocol including elements from AML treatment regimens, especially the use of cytarabine (cytosine arabinoside, or Ara-C). Infant ALL cells appeared to be highly sensitive to this nucleoside analogue drug cytarabine,[40,41] probably caused by over-expression of the *human equilibrative nucleoside transporter 1* (*hENT1*), on which cytarabine is mainly dependent to permeate the membranes of leukemic cells.[42] Hence, the INTERFANT-99 protocol included sequential courses of low-dose and high-dose cytarabine throughout the duration of the entire treatment. The overall results from the INTERFANT-99 study showed that this approach succeeded in reaching long-term survival in >50% of the cases for the first time. Still, ~50% of the infant ALL patients are not cured, and further improvements are urgently needed. Hence, a firm understanding of the biology underlying this type of leukemia is of utmost importance in order to bring about the development of more effective, and above all, targeted therapies, especially for patients carrying *MLL* translocations.

Genetics: *MLL* gene rearrangements

The *MLL* translocation, rightfully denoted as a dangerous liaison,[43] was first detected and described by Rowley in 1991.[44] As a result of the chromosomal translocations involving chromosome 11q23, the N-terminal portion of the *MLL* gene becomes fused to the C-terminus of one of its many translocation partner genes derived from other chromosomes. The most frequent (~50% of the cases) *MLL* translocation found in infant ALL is t(4;11)(q21;q23) which fuses the *MLL* gene to the *AF4* gene on chromosome locus 4q21 resulting in the chimeric *MLL-AF4* fusion product **(figure 5)**.[39, 45] Other frequent *MLL* translocations in infant ALL are t(11;19)(q23;p13.3)[46] and t(9;11)(p21;q23)[47] generating the *MLL-ENL* and *MLL-AF9* fusion genes respectively.[34, 39] As the fusion of these genes occurs in-frame, the resulting chimeric genes are transcribed and translated to give rise to highly oncogenic fusion proteins.[43]

The most likely mechanism causing chromosomal translocations, such as the *MLL* translocation, is malfunctioning of DNA double strand repair leading to non-homologous end joining.[48, 49] In addition the presence of Alu elements, DNAse hypersensitive sites as well as topoisomerase II cleavage sites have been put forward.[50] *MLL* translocations usually have thought to be primary events. They arise prenatally during fetal development,[51] and these leukemia fusion genes can be found on archived blood spots (Guthrie cards) from childhood and especially infant leukemia patients.[52, 53] Interestingly, *MLL* translocations have been detected in bone marrow mesenchymal stem cells obtained from infants with t(4;11)-positive ALL suggesting the translocation occurs in an early mesodermal precursor to mesenchymal and hematopoietic lineages.[54] Remarkably, *MLL* translocations may only be oncogenic when they occur in hematopoietic cells.[55] For oncogenicity, mouse models demonstrated the requirement of a fusion partner, indicating that loss of MLL function on its own was not sufficient for transformation.[56] In addition, the presence of a wild-type MLL allele is needed for transformation.[57]

According to Knudson's two hit hypothesis,[58] two sequential events are necessary for cancer to develop. The *MLL* translocation is usually considered to be the initiating factor or first hit before leukemia development. As *MLL*-rearranged leukemias typically have a very short latency (< 1 year), there is dispute on whether the *MLL* translocation would be the only hit, or whether the translocations induce rapid acquisition of additional genetic aberrations after birth.[27] In this respect, elevated expression of *fms- like tyrosine kinase 3* (*FLT3)*[35] has been put forward as a possible second hit providing a proliferative and/or survival advantage.[59] *FLT3* mutations have been identified in 7-16% of the *MLL*-rearranged infant ALL cases.[60, 61] Still these mutations cannot explain the majority of *MLL*-rearranged infant ALL cases. It has to be noted that so far no specific deletions or amplifications/ copy number aberrations have been detected in *MLL*-rearranged ALLs.[62, 63]

Typically an inverse relation between chromosomal instability and epigenetic aberrations has been observed in cancers[64] including leukemias.[65] Therefore an alternative theory would be that the second hit is formed by an altered "epigenetic landscape" or so

called "epimutations", directed by the oncogenic MLL fusion. An adaptation or extension of Knudson's two hit hypothesis has already been proposed for epigenetic aberrations such as DNA methylation by Peter Jones and Peter Laird in 1999.[66]

In concordance with this the wild-type (normal) *MLL* gene encodes a so called epigenetic (see below) modulator, i.e. a histone 3 lysine 4 (H3K4) methyltransferase, tightly involved in transcriptional regulation during definitive hematopoiesis.[67] From this perspective, wild-type *MLL* ensures maintenance of activation of its many target genes, such as the homeobox cluster A (*HOXA*) genes.[68] Disruption of the *MLL* gene, e.g. by chromosomal translocations, severely compromises its function. In case of an *MLL* translocation generating an MLL fusion protein, MLL loses its so called methyltransferase domain and thus its ability to methylate H3K4. As the most frequent *MLL* fusion partner genes in infant ALL possess the ability to recruit another histone methyltransferase (i.e. DOT1L) the H3K4 methylating actions of MLL are replaced by H3K79 di-methylation catalyzed by DOT1L.[55, 69] In turn, recruitment of DOT1L leads to abnormal epigenetic regulation inducing unique gene-activation patterns, presumably promoting leukemia development.[35] It has been known for a long time that chromosomal translocations contribute to cancer development.[26, 43] More recently it became clear that abnormal epigenetic events also play important roles in the initiation and progression of human malignancies.[70-74] Hence, in infant ALL, chromosomal translocations of the *MLL* gene are likely to induce abnormal epigenetic lesions,[75] indicating that this malignancy has a strong epigenetic component.[76]

Epigenetics: the world beyond genetics

The term '*epi-genetics*' (επι comes from the Greek "beyond" or "above") was first coined in 1940 by the British geneticist Conrad Waddington. It describes the interactions of genes with their environment, which bring about their phenotype (i.e. the observable characteristics).[72, 77] Nowadays epigenetics is usually referred to as the study of heritable changes in gene activity without affecting the DNA nucleotide sequence itself.[72 78, 79]

In contrast to genetic aberrations, epigenetic modifications are attached to the DNA or the histones (proteins around which the DNA is wound to form chromosomes). The two best-known components of the epigenetic code constitute histone modifications (regulating chromatin densities and thereby the accessibility of underlying gene loci), and DNA methylation (regulating the accessibility of gene promoters) **(figure 6)**.[79, 80]

Therefore, both epigenetic mechanisms regulate gene behavior by either allowing or denying transcription factors access to the gene promoters. Not surprisingly, these components usually function cooperatively. When the chromatin adopts a "closed" (densely packed) conformation preventing transcription factors to bind to the associated loci, the promoters of the corresponding genes usually become highly methylated. As such, DNA methylation provides a "safety lock" ensuring that transcription of these genes is fully blocked. Likewise, in response to other histone modifications enforcing a more "open" structure of the chromatin, the transcriptional

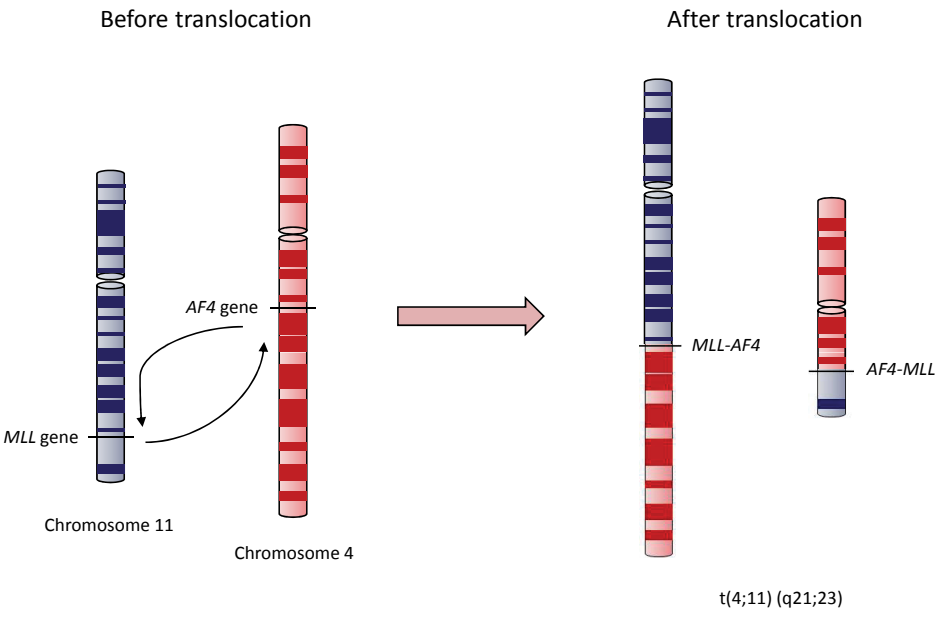

Figure 5. *MLL* gene rearrangement (schematic representation).
Chromosomal translocation t(4;11)(q21;q23) fusing the N-terminal region of the *MLL* gene on chromosome 11 to the C-terminal region of the *AF4* gene on chromosome 4 and vice versa. The resulting gene fusion is called *MLL-AF4*. *Adapted from http://www.erasmusmc.nl/alkg-cs/242905/243025/infants.*

machinery is allowed access to the corresponding genes. In that case, the methylation at gene promoters is usually reversed, allowing undisturbed transcription.

Regulation of histone modifications (i.e. chromatin structure) and DNA methylation constitutes a highly dynamic process, responsible for activating or deactivating proper gene expression throughout the lifespan of a cell, as well as according to its type and function. In cancer, the epigenetic landscape of tumor cells often becomes severely altered, supporting malignant transformation.[70]

Decoding the epigenetic language

Reading the histone code

Histones are bulky proteins in eukaryotic cell nuclei (around which the DNA is wound) that dynamically regulate the structure (density) of the chromatin (i.e. open and active versus closed and inactive). The behavior of the histones is regulated by the addition or removal of chemical modifications at the so called histone tails (see **figure 6**). Histone modifications include, amongst others, methylation, acetylation, ubiquitination, phosphorylation, and SUMOylation, and are placed or removed by a specialized group of proteins known as histone modifying enzymes.[70, 79, 81] For example, histone methylation is catalyzed by histone methyltransferases, while histone acetylation is

performed by histone acetyltransferases. Moreover, histone methyltransferases are being counteracted by histone demethylases, and histone acetyltransferases are being counteracted by histone deacetylases (HDACs). Some histone modifications advocate an open chromatin structure, while others plead for a closed configuration.

The histone methyltransferase MLL is of utmost importance in hematopoiesis, especially in the early phases of lymphocyte development. Mll-deficient cells in mice are unable to develop into lymphocytes and fail to contribute to fetal liver hematopoietic stem cell/progenitor populations.[67]. The replacement of H3K4 (see above) immediately affects hematopoiesis. In contrast, involvement of one of the *MLL* loci in a chromosomal translocation (leaving the remaining allele intact) is not lethal. Instead, early lymphocyte progenitors in which *MLL* rearrangements arise are effectively blocked in their differentiation and acquire the ability of uncontrolled cell proliferation, leading to leukemia.

Interpreting DNA methylation

DNA methylation or the addition of methyl-groups to certain cytosine nucleotides preceding guanine nucleotides (so called CpGs) in the transcription initiating promoter

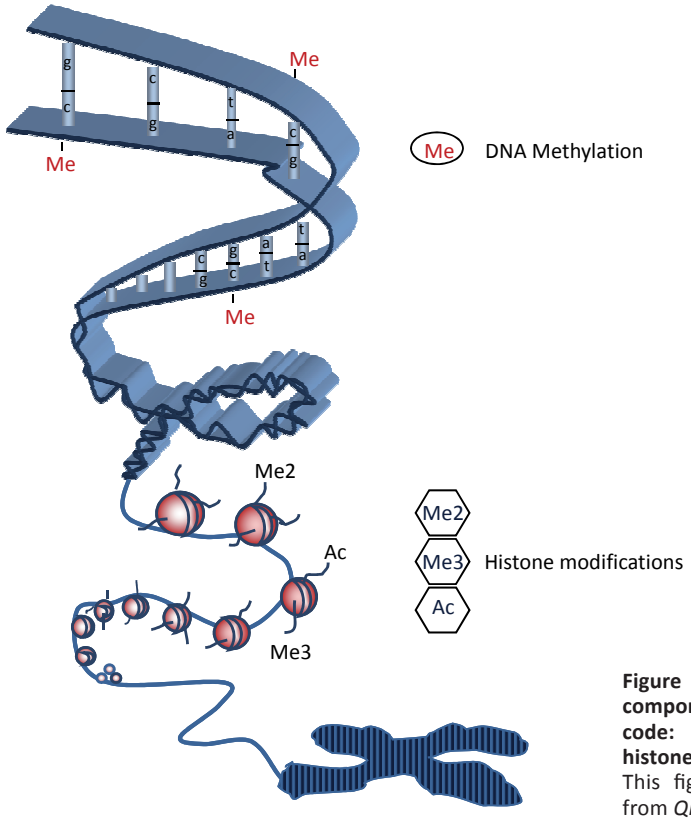

Figure 6. The two main components of the epigenetic code: DNA methylation and histone lysine tail modifications. This figure has been adapted from *Qiu, Nature, 2006*.[72]

regions of a gene, blocks the binding of the transcriptional machinery and results in gene inactivation. In turn, demethylation of such promoter regions restores gene activity.[82] The process of DNA methylation is facilitated by DNA methyltransferases (DNMTs) of which two types exist. *DNMT3A* and *DNMT3B* are responsible for *de novo* DNA methylation, while *DNMT1* ensures the maintenance of a certain methylation state over progressing cell divisions.[83]

Like chromatin remodeling, DNA methylation is a highly functional process in normal development.[8] Well-known examples of gene silencing as a consequence of DNA methylation are the inactivation of one of the X chromosomes in female individuals[84] and parental imprinting in which hypermethylation of one of the alleles of a gene leads to monoallelic expression.[85] In addition, as tissue-specific gene expression patterns are required for proper functioning of the cells in a certain tissue, genes that are not required in a specific type of cell usually become silenced by promoter DNA hypermethylation.

In (hematological) malignancies DNA methylation patterns often are substantially altered. Human cancers are frequently characterized by excessive amounts of promoter CpG hypermethylation and global hypomethylation in non-coding repetitive DNA regions **(figure 7)**.[86, 87] Promoter hypermethylation typically affects genes that normally confer protection against uncontrolled cell growth (for instance tumor suppressor genes). Genes that stimulate cancer development and progression (so called proto-oncogenes) and genes that confer resistance to apoptosis (encoding anti-apoptotic proteins) often become inappropriately activated by the loss of promoter methylation.[74] Evidently, abnormal distributions of DNA methylation patterns play an important role in cancer development.[66, 70, 88]

Epigenetic drugs: reversal of erroneous epigenetic lesions

In contrast to genetic abnormalities such as mutations, translocations, deletions, and duplications, aberrant epigenetic modifications are to some extent reversible. Currently, two types of epigenetic drugs have been developed, and widely studied in both *in vitro* and *in vivo* settings.

The first class of epigenetic drugs involves histone deacetylase (HDAC) inhibitors, targeting inappropriate histone deacetylation events.[89] As histone acetylation usually is associated with active chromatin states, inhibition of deacetylation by HDAC inhibitors is believed to reverse the closed chromatin conformation into a more open one, resulting in gene re-activation. Known types of HDAC inhibitors include hydroxamic acids, such as trichostatin A (TSA), vorinostat (SAHA), and panobinostat (LBH589); cyclic tetrapeptids, such as depsipeptide (FK228); benzamides, such as entinostat (MS-275), and aliphatic acids such as valproic acid (VPA). SAHA and FK228 have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of T-cell lymphomas. In addition, LBH589 is in phase III clinical trials for various types of cancers, and VPA and MS-275 are currently in phase II clinical trials for various malignancies, including leukemias.[89, 90]

The second class of epigenetic drugs involves demethylating agents designed to inhibit abnormal DNA methylation, and re-activate inappropriately silenced genes.[91] These demethylating agents typically represent cytidine analogs, such as 5-azacytidine (azacitidine)[92] and 5-azadeoxycytidine (decitabine).[93] Demethylating agents, or DNMT inhibitors, compete with normal cytidines for incorporation into the DNA. As this process requires DNA synthesis (and thus cell divisions), these drugs are especially effective in rapidly proliferating cells like, e.g., cancer cells. Once incorporated into the DNA, these cytidine analogs are recognized as normal cytidines and as such become subjected to methylation by DNMTs. However, during this process, demethylating cytosine analogs covalently and irreversibly bind to the DNMTs, whereas at normal cytosine residues the DNMTs detach after fulfilling their methyl-group donation. As a result demethylating cytosine analogs trap the DNMTs to the DNA, thereby causing cellular depletion of functional DNMTs, and impairing the ability of the cell to distribute its intended methylation over subsequent cell cycles.[94] Several clinical trials have demonstrated biological activity and clinical responses for both decitabine and azacitidine in adults diagnosed with myelodysplastic syndromes (MDS) or chronic myelomonocytic leukemia (CMML).[95-98] Recently, azacitidine and decitabine have been granted FDA approval for the treatment of MDS.[99]

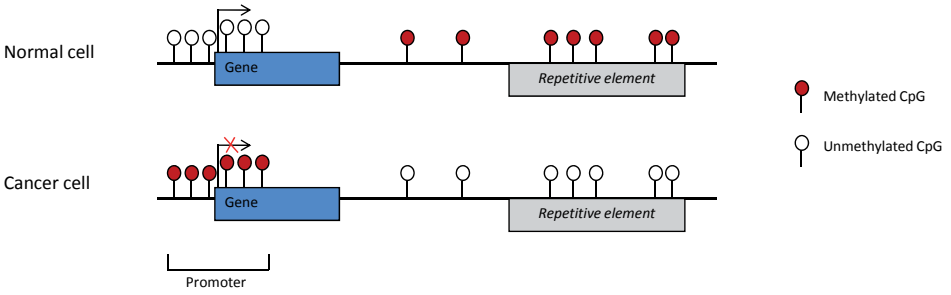

Figure 7. Altered methylation state in cancer cells (simplistic representation).
In normal cells CpG islands are protected from methylation. CpG sites away from transcription start sites or promoter regions and in repetitive elements are typically methylated. The situation is reversed in cancer, resulting in promoter hypermethylation and global hypomethylation. CpG islands flanking transcriptional start sites of some genes may become methylated. Repetitive elements become unmethylated. White lollipops show unmethylated cytosine nucleotides and red lollipops represent methylated cytosines.

AIMS

As described, *MLL*-rearranged ALL, affecting the majority of infant ALL patients, has been suggested to arise from abnormal epigenetic events induced by MLL fusion proteins. However, little was known on the role of DNA methylation in this malignancy. We hypothesized that the inappropriate inactivation or activation of a substantial number of genes, constituting the unique gene expression profiles observed in *MLL*-rearranged infant ALL,[35-38] might be associated with abnormal DNA methylation patterns **(figure 8)**.

Therefore this research project was designed to:

I. **Explore the *MLL*-specific DNA methylation pattern in infant ALL (i.e. the *MLL* methylome)**
II. **Investigate the *in vitro* potential of epigenetic drugs, such as demethylating agents, in *MLL*-rearranged infant ALL**

Since current therapies still fail in over 50% of the *MLL*-rearranged infant ALL cases, more effective treatment strategies for these very young children are urgently needed. Inhibiting aberrant DNA methylation may well represent an innovative therapeutic approach to improve prognosis of *MLL*-rearranged infant acute lymphoblastic leukemia.

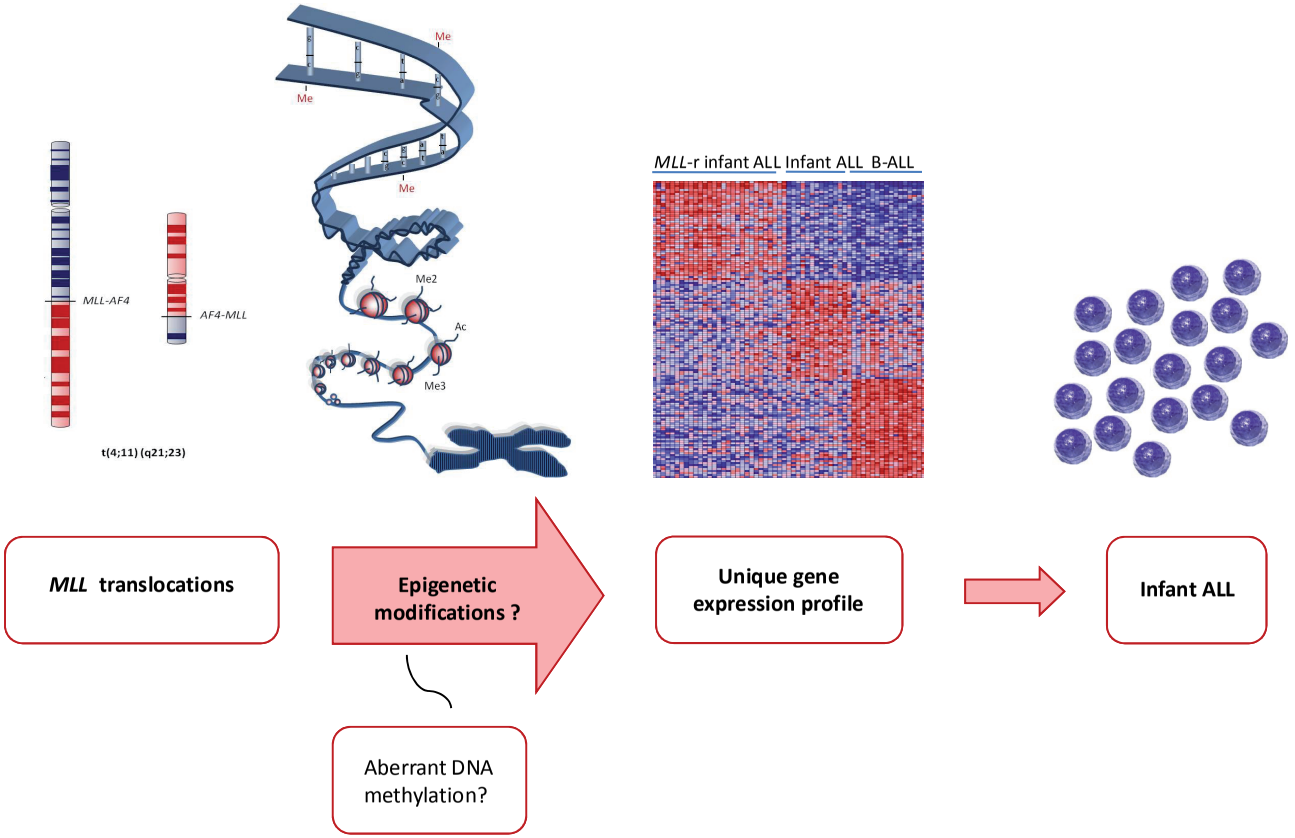

Figure 8. Hypotheses.
A diagrammatic impression of how hypotheses were formed before the initiation of this thesis project.

SCOPE

To achieve the goals mentioned in the previous paragraph, several studies have been performed sequentially. The different phases of the research conducted will be introduced in this section.

The work described in this thesis is divided into two parts. In **part I**, the role of aberrant DNA methylation in *MLL*-translocated infant ALL was explored.

In **chapter 2**, we started our journey into "the world beyond genetics" of infant ALL by a genome-wide screening. The DNA methylation patterns of 70 infants (<1 year of age) with ALL were characterized using the Differential Methylation Hybridization (DMH) technique on CpG microarrays and compared with DNA methylation in normal pediatric bone marrows. The relation between promoter DNA methylation and gene-expression was examined. This study provided the first insights into the *MLL* methylome. In addition, we investigated whether the degree of aberrant DNA methylation influenced clinical outcome. Using cell line models, we examined the potential of the demethylating agent zebularine both in terms of reversal of the aberrant DNA methylation signature, as well as the *in vitro* cytotoxicity in *MLL*-rearranged ALL cells.

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate the translation of over 60% of all human protein-coding genes[100] either via mRNA degradation and/or via translational repression. Increasing evidence has emerged that deregulated miRNA expression contributes to the development of various hematological malignancies.[101-103] In 2006, the first literature became available demonstrating that miRNA activity could also be regulated epigenetically.[104] Therefore, we set out to investigate whether the aberrant CpG island methylation found in infant ALL **(chapter 2)** also extended to miRNA loci. This hypothesis was confirmed by the observation that infant ALL patients could also be correctly separated according to their miRNA methylation patterns **(chapter 3.1)**. Encouraged by these data, we studied the aberrant CpG methylation at miRNA loci extensively for t(4;11)-positive infant ALL patients in **chapter 3.2**. We selected the miRNAs that were hypermethylated and down-regulated in patients compared with healthy pediatric bone marrows. We then selected the miRNAs that could be demethylated and re-activated after exposure to the demethylating agent zebularine. For these miRNAs, we looked into the potential target genes and examined whether aberrant methylation at these miRNA loci influenced clinical outcome. Although this study primarily investigated epigenetic regulation of miRNAs, the other side of the coin, where miRNAs themselves influence the epigenetic machinery, was taken up as well.

Typically, increased levels of gene promoter methylation in human cancers are accompanied by genome-wide hypomethylation of non-promoter CpGs.[86] This cancer-specific shift of methylation to gene promoters at the expense of global methylation has become a dogmatic event in current cancer epigenetics. In **chapter 4**, we set

out to investigate whether this phenomenon also occurs in *MLL*-rearranged infant ALL by analyzing the methylation status of the repetitive elements LINE-1, Alu and SAT-α. As repetitive elements reside throughout the genome, their methylation status is recognized as a surrogate marker for global methylation.[105] As the presence of a globally hypomethylated state could have consequences for the use of demethylating agents as potential therapeutic agents, investigating global hypomethylation in *MLL*-rearranged infant ALL was important.

In **part II** of this thesis, we focused on the potential of drugs targeting the epigenetic code in *MLL*-rearranged infant ALL.

Clofarabine is a next-generation nucleoside analogue drug showing promising results for refractory childhood leukemias.[106] Clofarabine has been designed to incorporate the favorable pharmacological characteristics of two drugs that are able to demethylate DNA. For that reason, we investigated whether *MLL*-translocated infant ALL cells were sensitive to clofarabine in **chapter 5**. In addition, we examined whether clofarabine worked synergistically with cytarabine, a major cytotoxic drug currently used in the treatment protocols for infants with ALL. Most importantly, we looked at the ability of clofarabine to demethylate DNA and re-express silenced genes.

When we examined the DNA methylation profiles obtained at diagnosis **(chapter 2)** more closely, we came across specific genes that appeared hypomethylated and highly expressed in *MLL*-rearranged infant ALL. Evidently, targeting hypomethylated genes is not as straight-forward as targeting hypermethylated genes. Therefore, in search for compounds capable of targeting these hypomethylated and highly expressed genes, we applied connectivity mapping on the obtained hypomethylation signature in **chapter 6**. The Connectivity Map is a large collection of gene expression data from cultured human cell lines treated with various compounds,[107] which enables the discovery of drugs that reverse the presented signature. Subsequently, we validated the compounds obtained from connectivity mapping both in terms of *in vitro* cytotoxicity on primary *MLL*-rearranged infant ALL cells, as well as down-regulation of the hypomethylated and highly expressed genes. As the compounds identified by the connectivity map were mostly epigenetic drugs, this study emphasized the need for incorporation of epigenetic drugs into the current treatment regimens for *MLL*-rearranged infant ALL.

In **chapter 7**, we discuss the findings presented in this thesis and provide directions for future research. This chapter essentially characterizes *MLL*-rearranged infant ALL as an epigenetic disease. The different components of the epigenetic code are interrogated with a focus on DNA methylation. In addition, the potential of epigenetic therapies, such as demethylating agents and HDAC inhibitors, for *MLL*-rearranged leukemias is addressed.

In **chapter 8**, we present a bullet point summary of the work described in the different chapters of this thesis. Furthermore, in **chapter 9**, a layman's summary is offered in Dutch.

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PART I:
ABERRANT DNA METHYLATION

"Genetics is the Master of the genome;
Epigenetics is the Mistress of the genome."

(Susan J. Clark, Special Conference on Cancer Epigenetics, American Association for Cancer Research, 2010)

2.

Specific Promoter Methylation Identifies Different Subgroups of *MLL*-rearranged Infant Acute Lymphoblastic Leukemia, Influences Clinical Outcome and Provides Therapeutic Options.

Dominique J.P.M. Stumpel, Pauline Schneider, Eddy H.J. van Roon, Judith M. Boer, Paola de Lorenzo, Maria G. Valsecchi, Renee X. de Menezes, Rob Pieters, and Ronald W. Stam

Blood 2009; *114(27): 5490-8*

ABSTRACT

Background

MLL-rearranged infant acute lymphoblastic leukemia (ALL) remains the most aggressive subtype of childhood leukemia, displaying a unique gene-expression profile.

Objective

Here we hypothesized that this characteristic gene-expression signature may have been established by potentially reversible epigenetic modifications.

Methods

We used Differential Methylation Hybridization (DMH) to explore the DNA methylation patterns underlying *MLL*-rearranged ALL in infants. The obtained results were correlated with gene-expression data to confirm gene silencing as a result of promoter hypermethylation.

Results

Distinct promoter CpG island methylation patterns separated different genetic subtypes of *MLL*-rearranged ALL in infants. *MLL* translocations t(4;11) and t(11;19) characterized extensively hypermethylated leukemias, whereas t(9;11)-positive infant ALL and infant ALL carrying wild-type *MLL* genes epigenetically resembled normal bone marrow. Furthermore, the degree of promoter hypermethylation among infant ALL patients carrying t(4;11) or t(11;19) appeared to influence relapse-free survival, with patients displaying accentuated methylation being at high relapse risk. Finally, we show that the demethylating agent zebularine reverses aberrant DNA methylation and effectively induces apoptosis in *MLL*-rearranged ALL cells.

Conclusions

These data suggest that aberrant DNA methylation occurs in the majority of *MLL*-rearranged infant ALL cases and guides clinical outcome. Therefore, inhibition of aberrant DNA methylation may be an important novel therapeutic strategy for *MLL*-rearranged ALL in infants.

INTRODUCTION

While long-term survival rates in childhood Acute Lymphoblastic Leukemia (ALL) exceed 80%,[1] the survival chances of infants (<1 year of age) still range between 20-50%.[2] Approximately 80% of infants with ALL carry chromosomal translocations involving the *MLL* gene,[3] fusing the N-terminal portion of the *MLL* gene to the C-terminal region of one of its translocation partner genes. The most frequent *MLL* translocations among infant ALL patients are t(4;11), t(11;19) and t(9;11),[2, 4] giving rise to the fusion proteins MLL-AF4, MLL-ENL and MLL-AF9. These chimeric MLL fusion proteins exhibit pronounced transforming capacities,[5] and independently contribute to an unfavorable prognosis.[2, 6]

As a member of the trithorax gene family, *MLL* is involved in transcriptional regulation.[7] Therefore, structural alterations of this gene may be expected to affect its function, presumably leading to transcriptional deregulation. Not surprisingly, recent gene expression profiling studies characterized *MLL*-rearranged ALL as a unique type of leukemia that is genetically clearly separable from other ALL subtypes.[8, 9] As epigenetic modifications affect gene expression patterns,[10] we hypothesized that the specific gene expression profiles associated with *MLL*-rearranged infant ALL may well be driven by epigenetic changes, which recently have been established to play important roles in the development and progression of leukemia.[11] The most widely studied epigenetic event in hematological malignancies constitutes transcriptional gene silencing by promoter CpG island hypermethylation.[11, 12] This phenomenon either leads directly to the silencing of tumor suppressor genes, or indirectly to up-regulation of other genes, when silencing of certain regulatory genes relaxes the suppression on their target genes. Hence, genome-wide promoter hypermethylation potentially results in abnormal gene expression profiles that favor malignant transformation. For example, we recently demonstrated that *FHIT*, a putative tumor suppressor gene, is characteristically silenced in *MLL*-rearranged infant ALL cells by CpG hypermethylation, and that re-expression of this gene induced apoptosis in these cells.[13]

Here we applied Differential Methylation Hybridization (DMH), an array-based technique that allows genome-wide screening of DNA methylation, using two different microarray platforms to explore the DNA methylation patterns underlying *MLL*-rearranged infant ALL. We show that different types of *MLL* translocations are associated with distinct patterns of DNA methylation, and we found that the degree of DNA methylation influences clinical outcome, identifying subgroups of *MLL*-rearranged infant ALL patients that may particularly benefit from therapeutic strategies containing demethylating agents.

MATERIAL AND METHODS

Patient samples

We studied 57 newly diagnosed infant ALL patients, enrolled in the international INTERFANT-99 treatment protocol[2] (patient characteristics listed in **Supplemental table 1**). Forty-four patients (77%) carried *MLL* translocations, and thirteen (23%) harbored untranslocated (wild-type) *MLL* genes. Among the *MLL* translocated patients, twenty-one were positive for t(4;11), seventeen for t(11;19) and six patients carried translocation t(9;11). Written informed consent and institutional review-board approval were obtained for all patients. Whole normal bone marrow samples obtained from eight non-leukemic pediatric patients were included as controls. Leukemic cell isolation and enrichment to achieve more than 90% leukemic blasts, as well as DNA and RNA extractions were performed as described before.[14]

Leukemia cell lines

RS4;11, SEMK2 and BEL-1 represent t(4;11)-positive precursor B-cell ALL cell lines. SEMK2 was originally derived from a 5-year-old girl at relapse[15] and was kindly provided by Dr. Scott Armstrong (Dana Farber Cancer Institute, Boston, Massachusetts, USA). BEL-1 was a generous gift from Dr. Ruoping Tang (University Laboratory, Paris, France).[16] RS4;11 was established from the bone marrow of a 32-year-old woman,[17] and was, like all other cell lines used in this study, purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). KOPN-8 harbors translocation t(11;19) and was derived from a 3-month-old infant girl with B-cell precursor ALL. REH and TOM-1 represent precursor B-lineage ALL cells exhibiting a TEL-AML1 fusion, and a Philadelphia chromosome, respectively. JURKAT and HSB2 both are T-lineage ALL cell lines, and Kasumi-1 and MV4;11 are AML cell lines. Kasumi-1 carries the t(8;21) AML1-ETO fusion gene, and MV4;11 harbors *MLL* translocation t(4;11). All cell lines were maintained as suspension cultures in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Integro), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.125 µg/ml fungizone (Invitrogen) at 37°C in humidified air containing 5% CO_2.

Differential Methylation Hybridization using CpG island microarrays

Differential Methylation Hybridization (DMH) was performed essentially as described by Yan et al. **(Supplemental methods).**[18, 19] DMH was applied on two different CpG island microarray platforms with limited overlap in CpG island probes. The first was the custom spotted 9K microarray chip developed by Huang and co-workers, containing 8,640 *Mse*I fragment probes.[18] In addition, we also used the first commercially available genome-wide CpG island microarrays (Agilent Technologies, Santa Clara, USA). These high-resolution microarrays contain 243,497 60-mer oligonucleotide probes, including 67,487 CpG island probes located in or near gene promoters. For the present study, only these probes located in gene promoters were used. Due to

restricted availability of patient material, DNA methylation profiling using the Agilent microarrays was performed in 49 of the 57 infant ALL patients.

Gene expression profiling using Affymetrix GeneChips

Gene expression profiles were generated for t(4;11)-positive (n=15) and t(11;19)-positive (n=14) infant ALL cases, using the same samples for which DNA methylation profiles were already produced on Agilent microarray chips. Expression profiles were also generated for whole healthy pediatric bone marrow samples, however, these did not correspond to the samples in which the DNA methylation patterns were determined. RNA processing, microarray hybridization (HU133 plus 2.0 Affymetrix GeneChips), and washing steps were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). The infant ALL gene expression data and DNA methylation data presented in this study have been deposited in the NCBI Gene Expression Omnibus and are accessible via the GEO Series accession number GSE18400.

In vitro cytotoxicity assay and exposure to zebularine

In vitro sensitivity of leukemia cell lines to the demethylating agent zebularine[20, 21] was determined by four-day 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT)-assays as described previously.[22] Zebularine was a generous gift from Dr. Victor E. Marquez (National Cancer Institute of Frederick, Frederick, Maryland, USA). To study the effects of demethylation on *MLL*-rearranged ALL cells, the cell lines SEMK2 and RS4;11 were cultured for 10 days in the presence or absence of 100 µM zebularine.

Statistical analyses

Normalization of the CpG island microarray data was performed using global locally weighted scatterplot smoothing (loess) normalization,[23] and differentially methylated CpG islands were identified using the linear models for microarray data (limma) package in the R statistical environment (R Development Core Team, 2007).[24] These models use an empirical Bayes approach to moderate the standard errors of the estimated standardized log-fold changes by borrowing information across genes. This results in more stable assumptions and enhanced power, especially when group sizes are small.[25] The resulting list of p-values was corrected for multiple testing by the false discovery rate (FDR) step-up procedure of Benjamini & Hochberg.[26] An FDR-adjusted p-value <0.01 was regarded significant. As a measure of internal validation for the subtype-specific methylation signatures, permutation testing (global test)[27] was applied to evaluate whether genes were significantly associated with a certain type of *MLL* translocation. For this, the tendency of repeated re-assignment of individual samples to their original cluster was assessed. As a default 10.000 permutations were used to calculate p-values.

Gene expression values were calculated using Affymetrix Microarray Analysis Suite® (MAS) 5.0.2 software. Unscaled expression signals were normalized using variance stabilization and normalization (vsn).[28]

Relapse-free survival was computed with the Kaplan Meier estimator. The duration of relapse-free survival was defined as the time from diagnosis until the date of leukemia relapse or the last follow-up. The probability of relapse in complete remission was estimated by applying the cumulative incidence estimator. The log-rank test was used to compare outcomes between different patient groups, and a one-step Cox model was applied to estimate the hazard of relapse for these patients, adjusting for already established risk stratification according to the international INTERFANT-06 treatment protocol. Briefly, patients were stratified as high-risk when aged <6 months (183 days) and displaying white blood cell counts (WBC) >300x10^9/L at diagnosis. Alternatively, patients were classified as medium-risk when aged >6 months at presentation, or aged <6 months with WBC <300x10^9 / L.

(Statistical) software

We used the statistical environment R (R Development Core Team, 2007) version 2.6.1 for the microarray analyses, including packages limma,[24] global test[27] and vsn.[28] Heatmaps were generated in GenePattern version 3.1.1 (Broad Institute, MIT, *http://genepattern.broad.mit.edu*), and PCA plots were produced using Genemath XT 1.6.1. software (Applied Maths, Inc., Austin TX, USA). SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for computation of survival statistics.

RESULTS

Unsupervised analysis based on DNA methylation patterns separates different infant ALL subtypes

Using Differential Methylation Hybridization (DMH) on two different microarray platforms, genome-wide promoter DNA methylation profiles were generated for infant ALL patients carrying *MLL* translocations t(4;11), t(11;19) or t(9;11), and infant ALL patients bearing wild-type *MLL* genes. To explore whether these samples showed leukemia-specific increases in promoter CpG island methylation, these profiles were compared with DNA methylation patterns obtained from bone marrow samples derived from healthy children. Initially, we performed a principal component analysis (PCA), using all CpG island probes present on each array without any selection. Based on the first three components of the PCA, which explain 41.8% (9K chip) and 32.2% (244K chip) of the total variance, the patient samples were visualized **(figure 1)**.

Interestingly, for both microarray platforms, this unsupervised analysis separated two major groups. Infant ALL samples that carry t(9;11) or wild-type *MLL* genes clustered together with normal bone marrow samples, whereas infant ALL samples carrying t(4;11) or t(11;19) clustered tightly together separately from the other samples. Although the cluster comprising t(9;11)-positive, untranslocated infant ALL samples and normal bone marrow samples appeared more heterogeneous, it has to be taken into account that this cluster consists of three different types of samples.

Moreover, epigenetic heterogeneity is already present among the normal bone marrow samples. Finally, we emphasize that this analysis is completely unguided.

Specific DNA methylation patterns further separate the different infant ALL subtypes

Subsequently, to explore whether specific DNA methylation profiles could define the genetic subgroups of infant ALL more accurately, the 20 most discriminative hypermethylated genes for each group (as compared with all other relevant subgroups combined) were selected. For both microarray platforms the gene names, log-fold changes, and p-values are listed in the Supplemental data **(Supplemental tables 2S and 3S)**. Permutation testing validated the robustness of the subtype-specific methylation signatures. Using the selected genes, we generated heatmaps in which both the genes and the samples were clustered hierarchically (Euclidean distance and complete linkage) **(figure 2A)**. This semi-supervised analysis revealed that *MLL* t(4;11) and t(11;19)-positive patients could clearly be separated from one another and from the other samples. In contrast, hypermethylated genes that unambiguously separate t(9;11)-positive samples from *MLL* wild-type (untranslocated) infant ALL samples, could not be identified. Moreover, the most significantly hypermethylated genes shared by t(9;11)-positive and wild-type *MLL* samples were also methylated in healthy bone marrow samples **(Supplemental figure 1S)**, and therefore likely reflect normal methylation in healthy hematopoietic cells. Importantly, these genes are hypomethylated in infant ALL harboring translocation t(4;11) or t(11;19).

Next, PCA was used to better visualize these different clusters, emphasizing the separation of the samples into the three expected groups characterized by t(4;11), t(11;19) or t(9;11) together with translocation-negative infant ALL **(figure 2B)**. When included, the normal bone marrow samples remained within the cluster comprising samples carrying t(9;11) or wild-type *MLL* genes **(figure 2C)**. In concordance with this, no significant aberrant DNA methylation could be detected in t(9;11)-positive or untranslocated (wild-type *MLL*) infant ALL, when separately compared with normal bone marrow.

Exclusion of non-leukemia-related epigenetic differences between subtypes of infant ALL

To test whether non-leukemia related epigenetic differences were present among the sample population, which may compromise our findings, we performed several comparisons deducting this possibility. Since leukemic samples were either obtained as peripheral blood or as bone marrow, we first compared DNA methylation patterns in bone marrow samples with samples derived from peripheral blood. No significant differences in promoter CpG island methylation were observed. From this, we concluded that samples of both sources could legitimately be used together in our analyses.

Next, and similarly, sex differences were assessed. A female predominance (62% vs. 38%) was identified among the *MLL*-rearranged infant ALL subgroups, whereas a

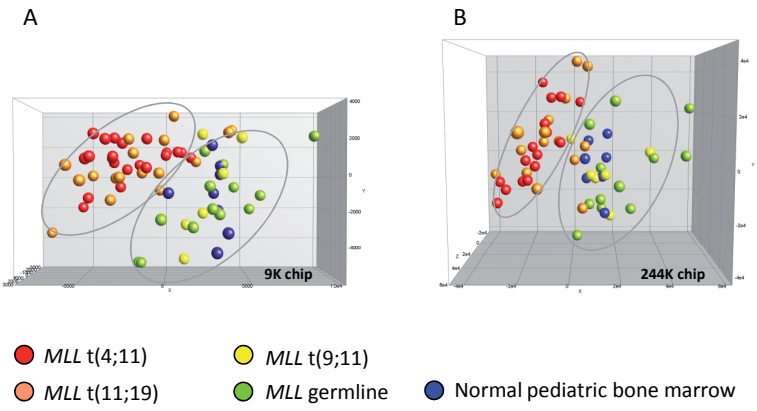

Figure 1. Unsupervised clustering analysis of DNA methylation in infant ALL.
Principal Component Analyses (PCA) of the CpG island methylation data from infant ALL patients and normal bone marrows using all probes present on each microarray platform. Each case is color-coded indicating the specific infant ALL subgroups. **A.** shows data from the custom spotted 9K CpG island microarray. t(4;11) (n=21; red), t(11;19) (n=17; orange), t(9;11) (n=6; yellow), *MLL* wild-type ALL (n=13, green) and normal bone marrow (n=8; blue). **B.** shows data from the commercially available 244K CpG island microarray (Agilent). t(4;11) (n=16; red), t(11;19) (n=15; orange), t(9;11) (n=6; yellow), *MLL* wild-type ALL (n=12; green) and normal bone marrow (n=7; blue). Due to restricted availability of patient material, Agilent DNA methylation profiles were generated for 49 infant ALL patients and 7 normal bone marrow samples.

male predominance (61% vs. 39%) was present in the translocation-negative (wild-type *MLL*) infant ALL group. 176 probes were differentially methylated between male and female patients (FDR<0.01) according to the Agilent microarray platform. The 121 probes corresponding to 75 genes, hypermethylated in females were all located on the X chromosome. Conversely, most of the 55 probes, corresponding to only 5 genes hypermethylated in male individuals were located on the Y chromosome. None of these genes appeared to interfere with the infant ALL subtype-specific methylation signatures.

In general, infant ALL represents a highly immature type of precursor B-cell leukemia with t(4;11)- and t(11;19)-positive infant ALL most often displaying the most immature phenotypes.[4] We therefore investigated whether the identified DNA methylation profiles reflected to some extent different maturation stages of early B-cell development. For this, we compared samples with a pro-B-cell ($CD34^+$, $CD19^+$, ($CD10^-$)) leukemia with the more mature pre-B-cell ($CD34^-$, $CD19^+$, $CyIg\mu^+$) leukemia within the group of samples carrying t(11;19), t(9;11) or wild-type *MLL* genes. Infant ALL samples carrying t(4;11) were excluded because these nearly all represented pro-B-cell leukemias and thus would strongly bias these analyses. No significant differences were found between pro-B and pre-B infant ALLs.

Finally, no significant differences in promoter CpG island methylation could be observed when DNA methylation patterns from infants diagnosed below 6 months of age were compared with those from infants diagnosed between 6 months and 1 year of age.

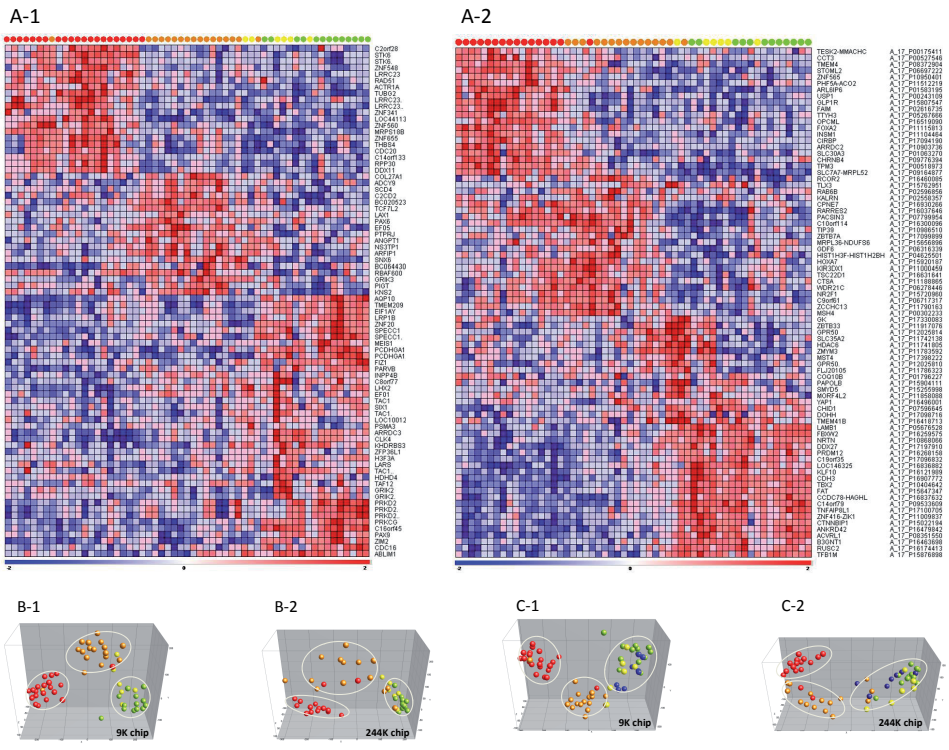

Figure 2. Infant ALL subtype-specific CpG island hypermethylation.
A1-2. Heatmaps showing the 20 most significantly hypermethylated probes for each infant ALL subtype. Columns represent patient samples and rows represent genes. Relative DNA methylation levels are shown in red (high) and blue (low). Genes and samples were ordered using hierarchical cluster analysis (Euclidean distance, complete linkage), and gene identifiers are listed at the right. **B1-2.** Principal component analyses (PCA) separating t(4;11) (red), t(11;19) (orange), t(9;11) (yellow) and *MLL* wild-type infant ALL (green). **C1-2.** shows the PCA when normal pediatric bone marrow samples (blue) are included in the analysis. **2-1.** shows data from the custom 9K CpG island microarray. **2-2.** shows data from the commercially available 244K CpG island microarray (Agilent).

Taken together, these comparisons indicate that the DNA methylation patterns as presented in this study represent leukemia-specific profiles that are unlikely to be influenced by differential CpG island methylation induced by non-leukemic factors.

Correlation between promoter methylation and gene expression

Given the aberrant methylation patterns in t(4;11)- and t(11;19)-positive infant ALL samples, we investigated the effects of promoter hypermethylation (Agilent platform) on gene expression (Affymetrix platform) of corresponding genes. Compared with normal bone marrow samples, infant ALL cells carrying t(4;11) displayed a total of 794 hypermethylated CpG island probes (FDR<0.01), and 75 probes were significantly hypermethylated in t(11;19)-positive infant ALL (FDR<0.01). From these analyses, the most significantly hypermethylated genes were selected. Gene names, log-fold

changes in methylation, and p-values for these genes are listed in the Supplemental data **(Supplemental tables 4S and 5S)**. Next, DNA methylation array data were compared with gene expression profiles from the same samples, and visualized as heatmaps and PCA plots **(figure 3)**. Promoter hypermethylation and down-regulated gene expression correlated for ~90-95% of the genes in both t(4;11)-positive and t(11;19)-positive infant ALL. However, for the remaining genes we observed the opposite; despite extensive hypermethylation, these genes were expressed higher in leukemic samples than in normal bone marrow.

The degree of methylation influences clinical outcome in *MLL*-rearranged infant ALL

In both the heatmaps and PCA plots that represent the most significantly hypermethylated genes among t(4;11)-positive and t(11;19)-positive infant ALL, a clustering of patient samples into two subgroups appeared. Ostensibly, one cluster represents patient samples that, at least for the selected genes, seem to be more densely hypermethylated than the samples in the other cluster **(figure 3)**. To better visualize this difference in degree of methylation we plotted the normalized methylation log-ratios of the genes. This semi-quantitative representation of the data indeed confirmed differences in the degree of methylation between both clusters **(Supplemental figure 2S).** To explore the clinical relevance of these subgroups, we computed risk of relapse statistics for these patient groups **(Supplemental figure 3S)**. Four patients received bone marrow transplantation (BMT) in complete remission. For these patients data were censored at BMT. One patient died before the start of treatment (referred to as early death), and was excluded from further analyses. Twelve out of 16 (75%) patients from the “heavily” methylated subgroup had a relapse after achieving complete remission, whereas among the “lightly” methylated patients, relapses occurred in 5/12 (42%) of the cases. The cumulative incidence of relapse at 1 year after diagnosis was significantly ($p<0.05$) different for the “heavily” and “lightly” methylated subgroups with incidences of 52.5 (SE: 13.7) and 35.7 (SE: 15.5) respectively. The number of patients that could be included in these analyses is not sufficient to evaluate the impact of the degree of DNA methylation adjusted for known prognostic factors (like age, white blood cell count and the *in vivo* response to prednisone) separately. Therefore, we used the INTERFANT-06 risk stratification which represents a combination of these factors.[2] Although these results must be interpreted with caution, the Cox regression model indicates that heavy methylation confers an increased risk of relapse (hazard ratio 5.77, 95% CI 1.57-21.2, $p=0.01$) **(Supplemental table 6S)**. The separate clustering of these two patient groups, however, did not appear in the gene expression profiles **(figure 3)**. This implies that the grouping of these patients and the observed variance in relapse-free survival rather reflects progressive accumulation of genome-wide methylation, than direct differences in gene expression. In line with this hypothesis we show that the division into “heavy”

and "light" methylation remains present when all significantly hypermethylated probes are used in a semi-quantitative representation **(Supplemental figure 2S)**.

MLL t(4;11)-positive cell lines as models for demethylation

DNA methylation patterns of two t(4;11)-positive precursor B-cell ALL cell lines (i.e. RS4;11 and SEMK2) were compared with the profiles from the t(4;11)-positive infant ALL samples. Nearly 50% of the 100 most significantly hypermethylated genes in t(4;11)-positive infant ALL were also hypermethylated in these cell lines **(figure 4A)**. Representing reasonable models for t(4;11)-positive infant ALL samples, we next studied the effects of demethylation on these genes by comparing DNA methylation profiles of these cell lines before and after a 10-day exposure to 100 μM of the demethylating agent zebularine. In the SEMK2 and RS4;11 cell lines, respectively 72% (33/46) and 59% (27/46) of the hypermethylated genes showed notable decreases in methylation upon exposure to zebularine. The genes display varying degrees of drug-induced demethylation **(figures 4B-C)**. For some of the genes the methylation status could be restored to nearly normal levels as observed in healthy hematopoietic cells.

Specific zebularine sensitivity in *MLL*-rearranged ALL cells

To further investigate the sensitivity of ALL cells to *in vitro* demethylation, cytotoxicity assays were performed using escalating dosages of zebularine. Also two AML cell lines were added to the data set. AML cells (with or without an *MLL* translocation) seem to be less sensitive to the demethylating agent zebularine than *MLL*-rearranged ALL cells, but the *MLL*-rearranged AML cell line MV4-11 does appear more sensitive than the t(8;21)-positive AML cell line Kasumi-1. Clearly, *MLL*-rearranged ALL cells were significantly more sensitive to zebularine than the other cell lines ($p<0.01$) **(figures 5A-B)**. As shown in figure 5B, on average the IC_{50} value (i.e. the concentration inhibitory to 50% of the cells) in *MLL*-rearranged ALL cells was ~50 μM, whereas zebularine failed to reach an IC_{50} value in other types of ALL cell lines.

DISCUSSION

We here present the first global view of the DNA methylome in infant *MLL*-rearranged Acute Lymphoblastic Leukemia (ALL). *MLL*-rearranged infant ALL represents an aggressive and difficult to treat type of leukemia characterized by a unique gene expression profile, that clearly separates this malignancy from other ALL subtypes.[8, 9] Since epigenetic modifications directly influence gene expression patterns,[10] we hypothesized that specific DNA methylation patterns may underlie the characteristic gene signature as observed for *MLL*-rearranged infant ALL. Our data largely support this hypothesis, as the majority of *MLL*-rearranged infant ALL cases (i.e. those characterized by t(4;11) or t(11;19)) represent hypermethylated leukemias, whereas t(9;11)-positive and *MLL* translocation-negative (wild-type *MLL*) infant ALL display DNA methylation patterns that closely resemble that of normal bone marrow.

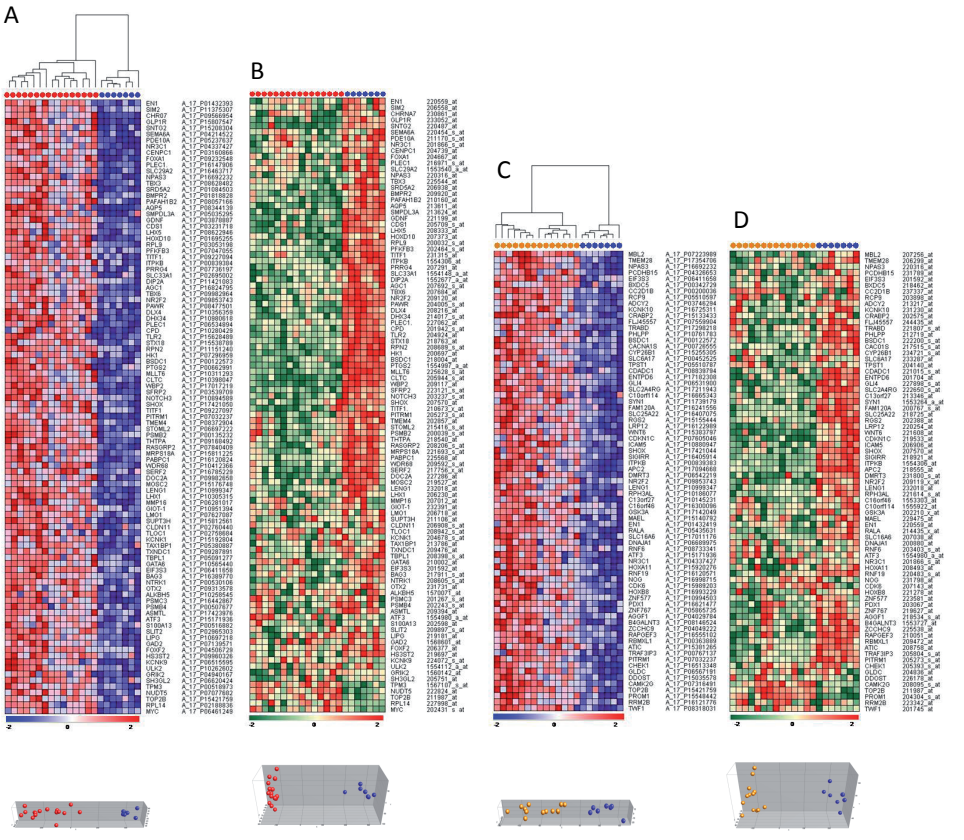

Figure 3. Correlation between CpG island methylation and gene expression.
A. Heatmap and PCA showing the most significantly hypermethylated genes in t(4;11)-positive infant ALL (red dots) compared with normal bone marrows (blue dots). Relative DNA methylation levels are shown in red (high) and blue (low). **B.** Heatmap and PCA showing the corresponding gene expression levels from the same genes and samples as presented in Figure 3A. Relative gene expression values are shown in red (high) and green (low). Similarly, the **C.** CpG methylation data and **D.** gene expression data are presented for t(11;19)-positive infant ALL samples (orange dots) as compared with normal bone marrows (blue dots).

Moreover, distinct leukemia-specific DNA methylation patterns could be identified for the different *MLL*-rearranged infant ALL subtypes as defined by the type of *MLL* translocation or absence of such translocations. Interference of non-leukemia related epigenetic differences in DNA methylation (such as age, sex-specific differences in methylation and differences related to B-cell maturation stages of leukemic cells) with our results could be excluded **(Supplemental tables 7S and 8S)**.

Thus, the presence as well as the patterns of aberrant DNA methylation in infant ALL appear, at least to some extent, dependent on the presence and type of *MLL* fusion, which may reflect a mechanism proposed recently.[29] Apart from DNA methylation, a second component of the epigenetic code involves histone modifications,[30] shaping

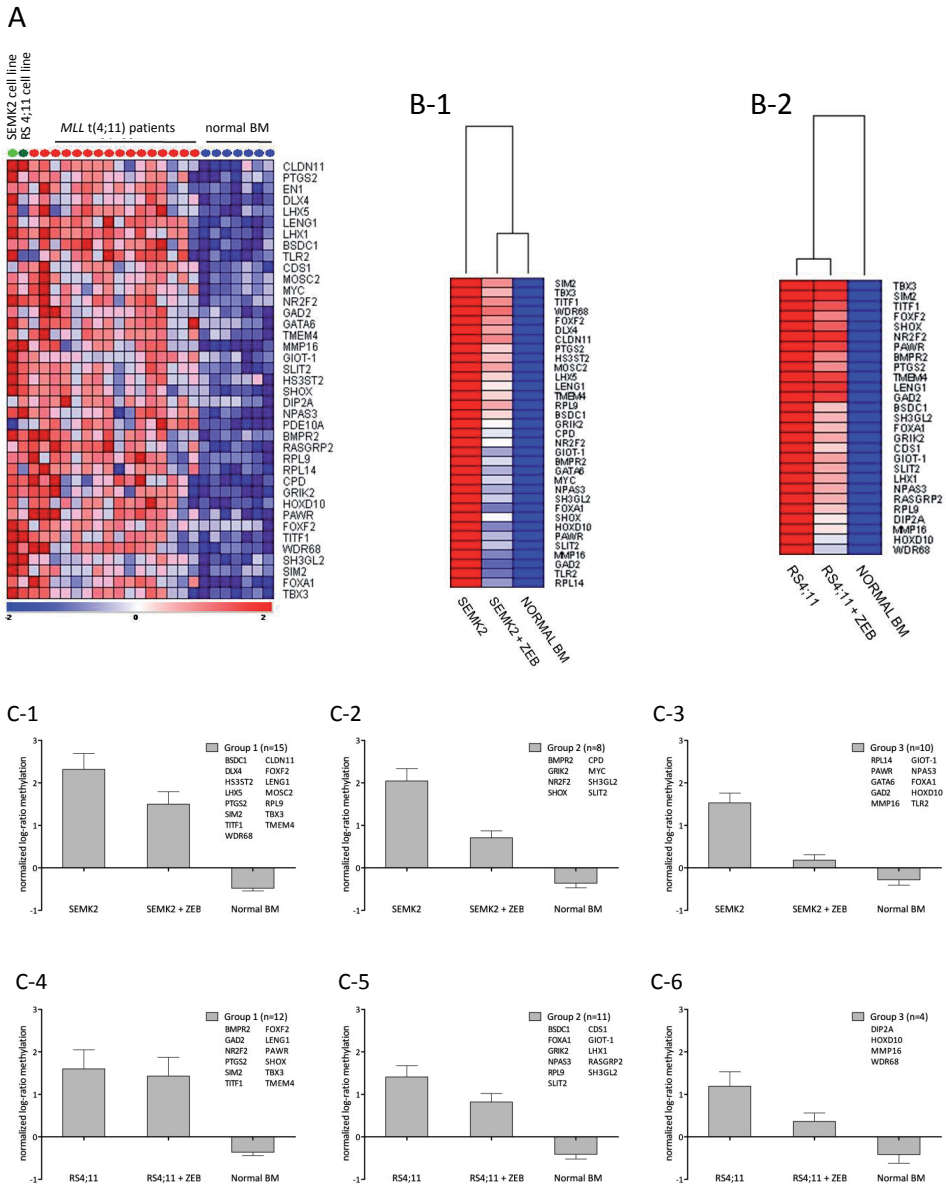

Figure 4. ALL cell lines as models for (de)methylation.
A. Heatmap showing methylation levels in the t(4;11)-positive B-ALL cell lines SEMK2 (light green dot) and RS4;11 (dark green dot) of genes most significantly methylated in t(4;11)-positive infant ALL patients (red dots) as compared with normal bone marrows (blue dots). **B.** Heatmaps showing methylation levels of these genes after exposure to zebularine. These methylation levels were compared with the average methylation levels as determined from normal bone marrow samples (n=7). **B1** SEMK2 cell line and **B2.** RS4;11 cell line **C1-6.** Graphs displaying the mean and the standard error of the mean (SEM) of changes in methylation levels after zebularine exposure. Genes were divided into three groups for each cell line according to the degree of responsiveness to zebularine. **C1-3.** SEMK2 cell line and **C4-6.** RS4;11 cell line.

the chromatin in an open (transcriptionally active) or closed (inactive) conformation. An inactive chromatin state is usually associated with hypermethylated CpG promoter regions, whereas active chromatin marks, such as H3K4 trimethylation and H3K79 dimethylation, denote unmethylated promoters, allowing transcription. Interestingly, the *MLL* gene itself has specific histone methyltransferase activity,[31, 32] which is lost during fusion of the *MLL* gene to one of its translocation partners. Therefore, MLL fusions can be expected to result in altered chromatin structures due to aberrant histone modifications. Recently, Krivtsov and Armstrong (2007) proposed that the recruitment of different histone methyltransferases by different MLL fusion proteins, may indeed result in inappropriate histone modifications directed by the *MLL* fusion partner.[29] Given the sound interplay between histone modification and CpG island methylation, this proposed influence of different *MLL* fusion genes on histone modifications, and the apparent influence of the *MLL* fusion partner on DNA methylation as shown in the present study, are presumably linked. In addition, the paper by Krivtsov et al. (2007),[29] as well as the study by Mueller and co-workers (2007)[33] demonstrated the recruitment of a transcriptional elongation complex to *MLL* target genes, resulting in gene activation (i.e. expression). These studies suggest that MLL fusion proteins trigger or maintain the leukemia by the activation of specific target genes. In contrast, our present study shows that, apart from specific gene activation, *MLL*-rearranged ALL is also characterized by severe gene inactivation, which may well be driven by the same MLL fusion. *MLL*-rearranged ALL cells typically mirror highly immature B-cells. Possibly, the MLL fusion ignores the activation of many genes that should have been activated (by wild-type *MLL*) at this stage of B-cell development, and necessary for proper differentiation towards mature and functional B-cells. This would suggest that our observed patterns of gross genome-wide DNA methylation is in favor of blocking B-cell differentiation, while simultaneously the MLL fusion activates several (proto-

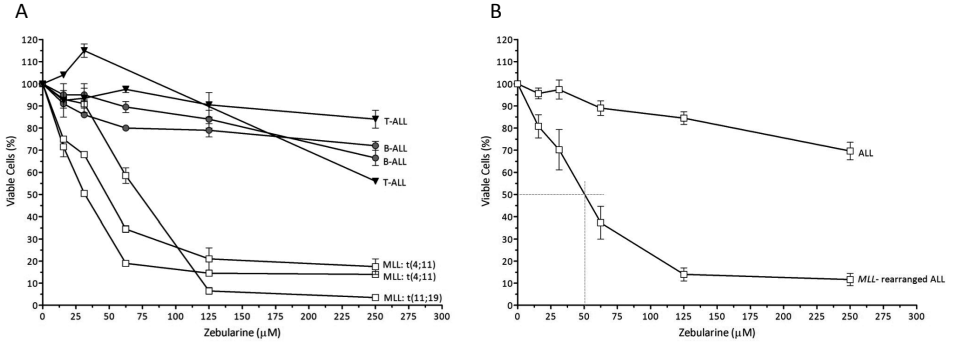

Figure 5. *In vitro* cytotoxicity to zebularine.
A. Dose-response curves showing the *in vitro* cytotoxic response to zebularine in individual leukemia cell lines with or without *MLL* rearrangements, or **B.** the mean cytotoxic response for *MLL*-rearranged ALL cell lines (n=4) and for the other ALL cell lines (n=4). Error bars represent standard error of the mean (SEM). The differences between the means of the groups were statistically analyzed using the 2-tailed Student *t* test ($p<0.01$ for each concentration used).

onco) genes in favor of uncontrolled cell proliferation and survival. Alternatively (or additionally), inappropriate activation of certain genes by the MLL fusion may in turn induce abnormal inactivation (silencing) of several other genes. However, these proposed mechanisms are highly speculative and remain to be confirmed.

Nonetheless, we can conclude from our data that MLL-AF4 and MLL-ENL represent MLL fusion proteins that both alter histone modifications that result in strongly altered DNA methylation patterns. The differences found in DNA methylation patterns between MLL-AF9 and MLL-ENL may then seem surprising given the apparent common mechanism of transformation involving the recruitment of *DOT1L* as put forward by others.[27,33] Surprisingly, the MLL-AF9 fusion did not lead to significant aberrant DNA methylation in infant ALL. This suggests that oncogenic transformation in t(4;11)- and t(11;19)-positive infant ALL patients may be facilitated or largely driven by gross epigenetic changes, whereas t(9;11)-positive infant ALL cells presumably transform via alternative mechanisms. In concordance with this is that t(9;11)-positive ALL patients characteristically seem to be different from other *MLL*-rearranged infant ALL patients. For example, t(9;11)-positive infant ALL is typically diagnosed at a later stage during infancy and is usually characterized by a more mature immunoglobulin gene rearrangement pattern (immunophenotype) than t(4;11)- and t(11;19)-positive infant ALL.[2, 4] On the other hand, no significant differences in survival exist between infant ALL patients carrying either t(4;11) or t(11;19) and patients with t(9;11).[2]

Studying the genes most significantly hypermethylated in t(4;11)- and t(11;19)-positive infant ALL samples, we found that the expression of the vast majority of these genes (~90-95%) was indeed down-regulated. Among the hypermethylated genes we found genes that were previously described to be silenced due to DNA hypermethylation in *MLL*-rearranged ALL, such as the tumor suppressor gene *FHIT*[13] and the *DLX3* gene,[34] demonstrating the integrity of our data. Moreover, most of these genes responded well to exposure to the demethylating agent zebularine in t(4;11)-positive cell line models. Among the most significantly hypermethylated genes for either t(4;11)-positive or t(11;19)-positive infant ALL, a limited overlap was observed. Nevertheless, global gene ontology analysis showed that most of the down-regulated genes in both subgroups are involved in transcriptional regulation **(Supplemental table 9S)**. This pronounced epigenetic deregulation of the transcriptional machinery may indeed have contributed to the unique gene expression profile characteristic for *MLL*-rearranged ALL.[8, 9] Yet, this would not be true for t(9;11)-positive infant ALL, as no aberrant promoter hypermethylation was observed in these samples. This apparent contradiction, however, is easily explained by the fact that most of the published *MLL*-specific gene expression signatures, including the signatures reported by Armstrong et al. (2002), are predominantly based on t(4;11)- and t(11;19)-positive samples.[8] Therefore, gene expression profiling studies including t(9;11)-positive infant ALL samples may well come to demonstrate that profiles associated with t(9;11) are different from those obtained in t(4;11)- and t(11;19)-positive samples.

Remarkably, about 5% of the most significantly hypermethylated genes in t(4;11)- and t(11;19)-positive infant ALL remained highly expressed. This observation

controverts the dogma that promoter methylation per definition induces suppression of gene expression. However, Weber and co-workers recently nuanced this dogma by elegantly demonstrating the influence of promoter CpG density on the ability to induce transcriptional repression.[35] Therefore, these methylated but highly expressed genes may well exhibit promoters containing weak CpG islands (i.e. a low or intermediate CpG density), which are unable to repress transcription even when methylated. Another possible explanation for this would again be the involvement of the MLL fusion protein, which may have induced activating histone modifications on otherwise inactive regions in the chromatin associated with promoter methylation. In turn, this newly acquired open chromatin state may have overruled the relatively weak DNA methylation, allowing transcription despite earlier established epigenetic silencing. If so, this group of genes may well represent potential therapeutic targets directly influenced by the MLL fusion itself.

Most of the genes that were methylated in t(9;11)-positive infant ALL and infant ALL carrying wild-type *MLL* genes were also methylated in normal bone marrow. Presumably, these represent genes that were already silenced in normal hematopoietic cells, but became hypomethylated in t(4;11) and t(11;19) positive infant ALL cells. Interestingly, among these were several genes with oncogenic potential, such as *CDH3*, *TBX2*, *ERCC1* and *NPR2* **(figures 2A-B)**, that have been reported to be involved in proliferation, tumor aggressiveness and prognosis in a wide range of human cancers.[36, 37] Among these hypomethylated genes also appeared the *HOXA9* gene which was previously described to be protected from methylation by the MLL-fusion itself.[38] Thus, the present study not only characterizes epigenetically down-regulated genes, but also identifies proto-oncogenes that may be inappropriately expressed in t(4;11)- and t(11;19)-positive *MLL*-rearranged ALL in infants. Obviously, such genes represent yet another set of candidate target genes for future therapeutic intervention.

Of main therapeutic interest is our finding that the degree of DNA methylation among t(4;11)- and t(11;19)-positive infant ALL patients is related to relapse-free survival, with patients presumably carrying heavily methylated genomes being at an increased risk of relapse. Therefore, these children in particular should be considered candidates for therapies including inhibitors of DNA methylation, especially since we here show that *MLL*-rearranged ALL cells are highly sensitive to zebularine *in vitro*. The authors believe that this increased sensitivity to demethylation is rather based on the presence of a general methylator phenotype (i.e. globally deregulated DNA methylation) than on the actual re-expresssion of a fixed number of hypermethylated genes. Apparently, genome-wide demethylation is sufficient to cause *MLL*-rearranged ALL cells to undergo apoptosis. This is in concordance with the identification of a heavily and a lightly methylated subgroup of *MLL*-rearranged infant ALL, which is also based on a widespread phenotype with more or less pronounced levels of DNA methylation that are in fact not visible at the gene-expression level. In conclusion, the findings presented here urgently require gene per gene validation studies and mandate additional studies using demethylating agents in the currently only available genuine mouse model for *MLL*-rearranged ALL, recently described by Krivtsov et al.[39]

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Supplemental information is available in the appendices.

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“Fingers are to the keys of a piano
as the epigenome is to the genome.”

Unknown

3

Aberrant Methylation at MicroRNA Loci

3.1.

Differential MicroRNA Methylation among Subtypes of *MLL*-rearranged Infant Acute Lymphoblastic Leukemia.

Dominique J.P.M. Stumpel, Rob Pieters and Ronald W. Stam

In a previous study we demonstrated the presence of distinct promoter methylation patterns among different subtypes of *MLL*-rearranged Acute Lymphoblastic Leukemia (ALL) in infants.[1] We showed that patients carrying the most frequently observed *MLL* translocations, i.e. t(4;11) or t(11;19), are burdened with severely methylated genomes whereas samples carrying t(9;11) as well as infant ALL patients not carrying *MLL* translocations essentially displayed methylation patterns that resembled healthy bone marrow. Recently, others independently confirmed hypermethylation in *MLL*-rearranged ALL.[2] Moreover, we showed that the degree or severity of methylation in t(4;11)- and t(11;19)-positive infant ALL appeared to be correlated with clinical outcome.[1] Encouraged by these observations we here asked whether interrogating aberrant methylation at microRNA loci would yield similar findings.

Using methylation-sensitive restriction enzyme-based Differential Methylation Hybridization (DMH) and 244K CpG island microarrays (Agilent Technologies, Santa Clara, USA),[1] we examined CpG methylation at all 1118 probes corresponding to miRNAs (n=113) present on these arrays. Unsupervised analysis using all probes readily separated the patient samples into two groups corresponding to t(4;11)- and t(11;19)-positive infant ALL on the one hand, and t(9;11)-positive and *MLL* translocation-negative infant ALL patients together with healthy bone marrows on the other hand **(figure 1A)**. A supervised analysis based on the most discriminative

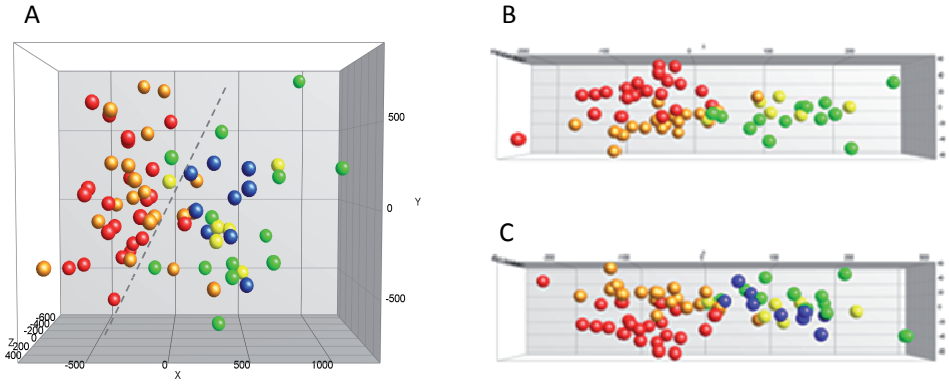

Figure 1. Principal component analyses of miRNA methylation patterns in infant ALL.
A. Unsupervised principal component analysis (PCA) based on the miRNA methylation data from infant ALL patients (n=62) and normal bone marrows (n=9) using all 1118 probes present on the 244K microarray platform (Agilent) corresponding to miRNAs (n=113). Each case is color-coded, indicating the specific infant ALL subgroups: t(4;11) (n= 23; red), t(11;19) (n=20; orange), t(9;11) (n= 6; yellow), untranslocated infant ALL (n=13, green), and healthy bone marrow (n=9; blue). **B.** Supervised principal component analysis based on 80 probes, including the 20 most significantly differentially methylated miRNA probes for each infant ALL subtype. Differentially methylated miRNA probes were identified by the use of the linear models for microarray data (limma) package in the R statistical environment (R Development Core Team, 2007). **C.** Principal component analysis when healthy bone marrows were included into the PCA plot.

probes appeared able to separate t(4;11)- and t(11;19)-positive samples from one another. However, t(9;11)-positive infant ALL samples consistently clustered together with untranslocated infant ALL samples and healthy bone marrow samples **(figures 1B-C)**. Strikingly, miRNA methylation-based clustering of these patient samples greatly resembled the clustering patterns based on protein-coding gene promoter methylation as obtained recently.[1]

Already in 2005, Lu and coworkers mentioned that classifying human cancers based on miRNA expression appeared to be more accurate than mRNA expression-based classification.[3] The fact that subtypes of *MLL*-rearranged infant ALL can be separated by differential miRNA methylation therefore likely mirrors aberrant miRNA expression among these samples. In turn, this would emphasize a strong link between CpG island methylation and miRNA silencing. Alternatively, the obtained miRNA patterns reflect the severity of abnormal genome-wide DNA methylation patterns in the t(4;11)- and t(11;19)-positive subtypes as reported before. Presumably, these explanations are not mutually exclusive.

MLL-rearranged leukemias are known for their unique gene expression profiles[4, 5] at least to some extent guided by deviant histone modifications resulting in the aberrant activation of numerous genes.[6] As we showed recently, this massive activation of genes is counterbalanced by vast numbers of genes being epigenetically silenced via CpG island methylation.[1] Here we demonstrate that abnormal genome-wide DNA methylation in t(4;11)- and t(11;19)-positive infant ALL also affects miRNAs, which may again favor gene activation. Hypothetically, miRNA silencing releases the suppressive actions on their target genes, allowing inappropriate gene expression of otherwise restrained transcripts. In other words, gene expression in *MLL*-rearranged leukemias is controlled by a multitude of regulatory mechanisms, all of which seemingly influencing one another. Although this adds yet another layer of complexity to the already complicated biology of this malignancy, these observations do mandate further studies exploring the involvement of miRNA methylation and expression in *MLL*-rearranged leukemias.

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3.2

Hypermethylation of Specific MicroRNA Genes in *MLL*-rearranged Infant Acute Lymphoblastic Leukemia: - Major Matters at a Micro Scale -

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ABSTRACT

Background

MLL-rearranged acute lymphoblastic leukemia (ALL) in infants (<1 year) is the most aggressive subtype of childhood leukemia. In order to develop more suitable treatment strategies, a firm understanding of the biology underlying this disease is of utmost importance. *MLL*-rearranged ALL displays a unique gene expression profile, partly explained by erroneous histone modifications. We recently showed that t(4;11)-positive infant ALL is also characterized by pronounced promoter CpG hypermethylation.

Objective

Here we investigated whether this widespread hypermethylation in *MLL*-rearranged infant ALL also affected microRNA (miRNA) expression.

Methods

We performed CpG methylation analyses at 122 miRNA loci using Differential Methylation Hybridization (DMH) and miRNA expression analyses using quantitative real-time PCR on primary t(4;11)-positive infant ALL samples (n= 22) and normal pediatric bone marrows (n= 7).

Results

We identified 11 miRNAs that were down-regulated in t(4;11)-positive infant ALL as a consequence of CpG hypermethylation. Seven of these miRNAs were re-activated after exposure to the demethylating agent zebularine. Five of these miRNAs are associated either with *MLL* or MLL fusions, and for miR-152 we found both *MLL* and *DNMT1* as potential targeted genes. Finally, a high degree of methylation of the miR-152 CpG island was strongly correlated with a poor clinical outcome.

Conclusions

Our data suggest that inhibitors of methylation have a potential beyond re-expression of hypermethylated protein-coding genes in t(4;11)-positive infant ALL. We here provide additional evidence that they should be tested for their efficacy in *MLL*-rearranged infant ALL in *in vivo* models.

INTRODUCTION

Hematological malignancies characterized by chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene are rare among children, and typically affect infants (<1 year of age).[1] Approximately 80% of infants diagnosed with acute lymphoblastic leukemia (ALL) carry such *MLL* rearrangements that independently contribute to disease aggressiveness.[2] The most frequent *MLL* translocation in infant ALL is t(4;11) fusing the N-terminus of the *MLL* gene to the C-terminus of transcription factor *AF4*, generating the oncogenic fusion protein MLL-AF4. Normally, wild-type MLL functions as an important epigenetic regulator: a histone methyltransferase facilitating histone 3 tri-methylation at lysine 4 (H3K4me3).[3] As a result of *MLL* translocations, one allele of the *MLL* gene becomes disrupted and its normal function is compromised by the loss of its methyltransferase domain. Instead, the MLL fusion proteins MLL-AF4, MLL-ENL, MLL-AF9, and MLL-AF10 recruit alternative histone methyltransferases, such as DOT1L, leading to histone 3 di-methylation at lysine 79 (H3K79me2).[4, 5] In turn, these illegitimate histone modifications lead to inappropriate activation of numerous genes, establishing a unique gene expression profile presumably favoring leukemia development.[6, 7]

Apart from gene activation guided by erroneous histone modifications, we recently showed that in *MLL*-rearranged infant ALL also numerous genes are inactivated or silenced as a consequence of DNA hypermethylation.[8] This is especially true for t(4;11)-positive infant ALL, in which the degree of genome-wide promoter hypermethylation appeared to be associated with an increased risk of relapse.[8] In the present study, we asked whether the wide-spread hypermethylation patterns as observed in *MLL*-rearranged infant ALL have also affected microRNA (miRNA) expression. Hypothetically, hypermethylation or silencing of miRNAs may successively lead to abnormally activated miRNA-targeted genes and hence, may further have contributed to the unique gene expression profile of *MLL*-rearranged infant ALL. To test this, we here studied the relation between two forces representing hallmark discoveries of contemporary biology: DNA methylation and miRNA expression.[9]

MiRNAs are small (~22-nucleotides) RNA molecules that regulate the translation of over 60% of all human protein-coding genes[10] via transcript destabilization and/or translational repression. Increasing evidence has emerged that deregulated miRNA expression contributes to the development of various hematological malignancies.[11-13] Aberrant expression of certain miRNAs has also been observed in leukemias characterized by *MLL* translocations. For instance, miR-196b was found to be over-expressed in *MLL*-rearranged infant ALL compared with other subtypes of childhood precursor B-ALL,[12] and appeared to participate in leukemogenesis by stimulating proliferation, while blocking differentiation in hematopoietic progenitor cells.[14] More recently, up-regulation of the entire miR-17-92 cluster was described in *MLL*-rearranged leukemias.[15] Strong binding of the MLL fusion protein to this miRNA cluster led to inhibition of apoptosis and promoted proliferation by regulation of the relevant target genes.

So far, most studies in *MLL*-rearranged leukemia focused on miRNA over-expression.[12, 15, 16] However, in a recent genome-wide miRNA study we found putative tumor suppressing miRNAs, such as let-7b and miR-708, to be down-regulated in *MLL*-rearranged ALL, and to be associated with oncogene up-regulation.[12] Therefore, the present study was designed to explore the silencing effects of genome-wide CpG island hypermethylation on miRNA expression, and the ability of the demethylating agent zebularine to restore miRNA activity.

MATERIAL AND METHODS

Patient samples

We studied 22 newly diagnosed infants (<1 year) with t(4;11)-positive ALL enrolled in the international INTERFANT-99 treatment protocol.[1] In order to make a valid comparison, wild-type *MLL* infant ALL samples (n=10) and non-*MLL* childhood precursor B-cell ALL samples (n=10) from our cell bank were added. Mononuclear cells were isolated from bone marrow samples using sucrose density centrifugation.[17] The percentage of leukemic cells was determined by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins, and in case blast percentages were below 90%, samples were enriched by elimination of non-malignant cells using immunomagnetic beads (Dynabeads, Dynal, ASA, Oslo, Norway) In the case of contaminating monocytes, the CD14 marker was used, CD15 in the case of myeloid cells and E-1 antigen in the case of erythroid cells.[18] DNA methylation microarrays and gene expression microarrays were performed for 22 infant ALL patients. In addition, CpG microarrays were performed for 10 wild-type *MLL* infant ALL samples and 10 non-*MLL* childhood precursor B-cell ALL samples. Due to restricted availability of patient material, miRNA expression assays were performed for only 5 infant ALL patients. The miRNA expression assay specific for miR-152 could only be performed for 13 infant ALL patients. Normal bone marrow samples obtained from seven non-leukemic pediatric patients were included as controls. All samples were collected after approval of the institutional review board and informed consent from parents or legal guardians.

Cell line culture and zebularine treatment

SEMK2 is subclone of the SEM cell line which is a t(4;11)-positive precursor B-ALL cell line derived from a 5-year old girl at relapse[19, 20] (kindly provided by Dr. Scott Armstrong (Dana Farber Cancer Institute, Boston, MA, USA)). The cell line was maintained as a suspension culture in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS) (Integro, Zaandam, the Netherlands), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.125 µg/ml fungizone (Invitrogen) at 37°C in humidified air containing 5% CO_2. Cells were exposed to 100 µM of the demethylating agent zebularine[21] for 3, 6 or 10 days, whereas control samples were unexposed. Cell viability was assessed by Annexin V staining determined by flow cytometry using a FACSCalibur (Becton Dickinson).

Isolation and purification of DNA and RNA

Genomic DNA and total cellular RNA were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The quality of the extracted DNA was assessed on 1.0% agarose gels, and the RNA integrity on a Agilent 2100 Bio-analyzer using RNA 6000 Nano Assay LabChips (Agilent, Amstelveen, the Netherlands). All RNA samples had an RNA Integrity Number (RIN) of ≥ 7.5.

Differential methylation hybridization for assessment of CpG island methylation

Extracted genomic DNA was used to assess the methylation status of CpG islands associated with miR-genes by the methylation-sensitive restriction enzyme-based Differential Methylation Hybridization (DMH) procedure using 244K CpG island microarrays (Agilent Technologies, Santa Clara, USA).[8] These high-resolution CpG microarrays contain 243,497 60-mer oligonucleotide probes, including 1118 probes corresponding to 122 miR-genes encoding 113 mature miRNAs. Some of the probes represent CpG islands (embedded in between different miR-genes) associated with 2 or 3 mature miRNAs belonging to the same miRNA cluster. A genomic DNA pool derived from healthy males (n=5) and females (n=5) (Promega Benelux BV, Leiden, the Netherlands) was used as a common reference. Labeling and hybridization procedures were performed as reported previously.[8] Detailed procedures are described in the **Supplemental methods**. Data extraction was performed using Agilent Feature Extraction 9.5.3 software, and unprocessed genome-wide DNA methylation data was deposited in the NCBI Gene Expression Omnibus under the GEO Series accession number GSE18400 as part of our recent study on DNA methylation patterns in *MLL*-rearranged infant ALL.[8] CpG island methylation data are presented as normalized log-ratios of patient signal divided by the common reference signal.

miRNA expression analyses

Expression levels of 365 miRNAs were determined by using the Taqman MicroRNA array-platform based on stem-loop quantitative real-time (RT)-PCR (Applied Biosystems, Foster City, CA, USA).[22] Reactions were performed according to the manufacturer's guidelines and the amplification of obtained cDNA was monitored on an ABI 7900HT Sequence Detection System (Applied Biosystems). MiRNA expression levels were determined in t(4;11)-positive infant ALL (n=5), and normal bone marrow (n=7) samples, as well as in zebularine-treated and untreated SEMK2 cells. An additional set of 13 t(4;11)-positive patients was examined for miR-152 expression by using a separate assay based on stem-loop quantitative RT-PCR specifically for miR-152. This assay was performed in duplicate for all patients. The mean of Ct-values for snoR-13 and -14 (Taqman MicroRNA array), and snoR-1 (miR-152 assay) was used as a reference for input RNA. The expression was calculated as a percentage of snoRNA: $2^{-\Delta Ct} \times 100$, in which ΔCt is equal to "Ct^{miRNA} minus $Ct^{control\ snoRNA}$". Hypermethylated miRNAs were considered re-expressed by demethylation when >2-fold increases in expression were observed for at least two time points (3, 6 or 10 days) of zebularine treatment.

Gene expression analyses using Affymetrix GeneChips

Affymetrix HGU133 plus 2.0 GeneChips (Santa Clara, CA, USA) were used to study mRNA expression levels of the potential miRNA target genes *DNMT1*, *MLL*, *ZEB2* and *HOXA3* in t(4;11)-positive infant ALL patients (n=22) and in normal bone marrow samples (n=7). Data were processed as described before[7] and raw infant ALL gene expression data was deposited in the NCBI Gene Expression Omnibus under the GEO Series accession number GSE 19475 as part of one of our recent studies.[7]

Quantitative real-time PCR analysis

Total RNA was reverse transcribed as described before[17] and the obtained cDNA was used to quantify *MLL, DNMT1*, *ZEB2* and *HOXA3* mRNA expression in cell line SEMK2, using quantitative real-time PCR analysis. *B2M,* encoding human *beta-2-Microglobulin*, was used as a housekeeping reference gene. All oligonucleotides were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CA) and primer combinations are listed in **Supplemental table 1S**. PCR products were amplified and stained using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) according to the manufacturer's recommendations and detected on an ABI 7900HT Sequence Detection System (Applied Biosystems).[7] Per experiment samples were analyzed in duplicate and all experiments were conducted twice. Highly expressed miRNA targeted genes were considered down-regulated by re-activation of the miRNA when mRNA expression was at least 2-fold decreased after zebularine treatment.

Statistical analyses

Normalization of the CpG island microarray data was performed using global locally weighted scatterplot smoothing (loess) normalization.[23] Differentially methylated CpG island loci and differentially expressed miRNAs were identified using linear models for microarray data (limma) as previously described.[8, 24] Differential methylation and differential expression was regarded significant at p-values (adjusted for multiple testing according to the step-up procedure of Benjamini and Hochberg[25]) of <0.05 (i.e. False Discovery Rate (FDR) <0.05). All statistical analyses were performed in the statistical environment R using Bioconductor packages (R Development Core Team, 2007). Heatmaps were generated in GenePattern version 3.1.2 and principal component plots were produced using Genemath XT 1.6.1. software (Applied Maths, Inc., Austin TX, USA).

The risk of relapse and the overall survival (OS) were computed with the Kaplan-Meier estimator. Overall survival was defined as the time from diagnosis until death or last follow-up. The log-rank test was used to compare outcomes between different patient groups. SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for computation of survival statistics.

RESULTS

CpG island hypermethylation at miRNA loci in t(4;11)-positive infant ALL

We previously showed that *MLL*-rearranged infant ALL patients carrying translocation t(4;11) exhibit severely hypermethylated genomes.[8] Here we investigated whether the genome-wide deregulation of DNA methylation also affected miRNA activity. For this, multiple datasets were successively analyzed following the flowchart depicted in **figure 1.** First, an unsupervised principal component analysis using all the 1118 oligonucleotide probes related to 122 miR-genes (encoding 113 mature miRNAs) present on the 244K CpG island microarray (Agilent) readily separated the data into two groups, corresponding to t(4;11)-positive infant ALL (n=22) and normal bone marrow samples (n=7) **(Supplemental figure 1S)**. Fifty-nine out of these 122 miR-genes encoding 55 mature miRNAs were significantly hypermethylated (FDR <0.05) in t(4;11)-positive infant ALL cases compared with normal bone marrows

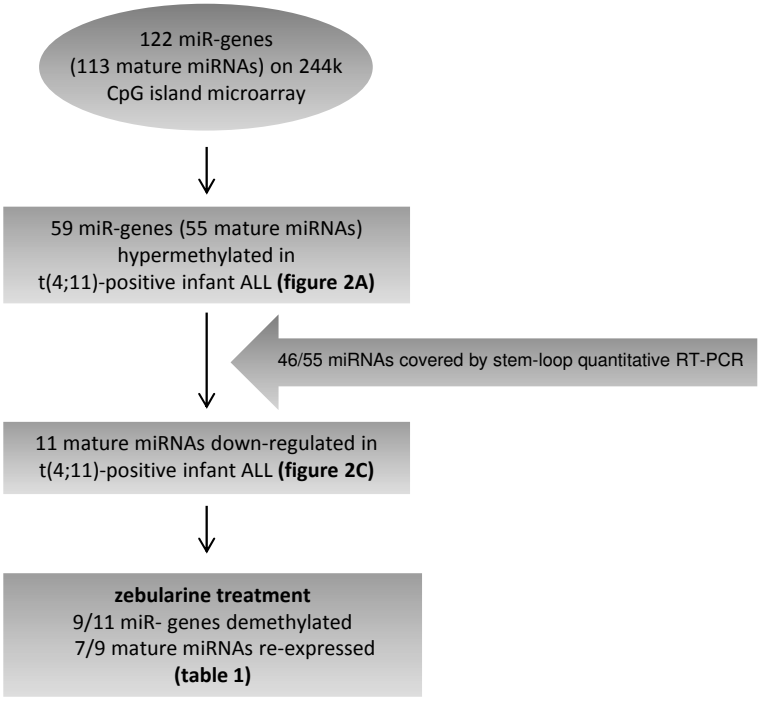


Figure 1. Flowchart depicting the sequential analyses performed in this study.
Several sequential analyses were performed on different data-sets, and miRNAs were selected for further evaluation based on the following criteria: hypermethylated miRNAs are transcriptionally silenced, and exposure to the DNA methyltransferase inhibitor zebularine results in demethylation and re-expression. Seven miRNAs met these criteria.

(figure 2A). Agilent probe IDs, log-fold changes in methylation and p-values are listed in **Supplemental table 2S**. As shown in **figure 2A**, among the t(4;11)-positive infant ALL samples two subgroups could be identified that either displayed heavy (n=7) or light (n=15) miR-gene methylation. While we observed a similar phenomenon in our study of the methylation patterns of protein-coding gene promoters,[8] the here presented separation between heavy and light miR-gene methylation is more pronounced **(figures 2A and 2B)**.

Down-regulated expression of hypermethylated miR-genes

Subsequently, miRNA expression was assessed in t(4;11)-positive infant ALL patients (n=5) and in normal bone marrow samples (n=7) by quantitative stem-loop-based RT-PCR. For 46 out of the 55 hypermethylated miRNAs the expression level could be evaluated, and 11 of these 46 mature miRNAs appeared to be consistently down-regulated in t(4;11)-positive infant ALL (FDR<0.05) **(figure 2C, Supplemental table 2S)**, presumably as a consequence of CpG island hypermethylation.[26] For some miRNAs, such as miR-148a, miR-503, and miR-432, >100-fold down-regulated expression was observed in t(4;11)-positive infant ALL as compared with normal bone marrow.

Responsiveness of miRNA loci to demethylation by zebularine

In t(4;11)-positive infant ALL, the expression of the majority of genes transcriptionally silenced by promoter hypermethylation could be re-activated by the DNA methyltransferase inhibitor zebularine.[8] We here examined the responsiveness of the silenced and hypermethylated miRNAs **(figure 2C)** to demethylation. For this, the t(4;11)-positive precursor B-ALL cell line SEMK2 (a subclone of the SEM cell line) was exposed to 100 µM of zebularine for 3, 6 or 10 days. At these consecutive time points both CpG island methylation and miRNA expression were determined. Nine miRNA loci could be demethylated by zebularine **(Supplemental figure 2SA)**, and for 7 of these, i.e. miR-200b, miR-200a, miR-429, miR-152, miR-10a, miR-503 and miR-432, expression was increased **(table 1, Supplemental figure 2SB)**. Interestingly, hypermethylation at these miR-gene loci turned out to be specific for t(4;11)-positive infant ALL when compared with wild-type *MLL* infant ALL samples (n=10) and non-*MLL* childhood ALL samples (n=10) **(figure 3)**.

Demethylation and re-expression of miRNA clusters

About 40% of all miRNAs are found to be organized in clusters on human chromosomes.[27] By definition, miRNAs encoded less than 3 kb apart from one another are considered to be clustered and often are collectively expressed at comparable levels.[9, 27] Five of the 7 miRNAs that were re-expressed upon demethylation appeared to be part of such miRNA clusters. For instance, miR-200b is part of a miRNA cluster on chromosome 1p36.33 further including miR-200a and miR-429 **(figure 4A)**. Together with miR-424, miR-503 forms a miRNA cluster on chromosome Xq26.2 **(figure 4B)**, and miR-432 is located within a large miRNA cluster on chromosome 14q.32.2 comprising miR-431,

miR-432, miR-433, miR-127 and miR-136 **(figure 4C)**. Interestingly, neighboring miRNAs belonging to the same cluster also seem to be affected in a similar fashion, albeit in some cases (e.g. miR-127 and miR-433) less significantly.

The two remaining miRNAs, miR-10a and miR-152, are both located on chromosome 17q21.32, however, the genomic distance between these miRNAs exceeds with >500kb the limit of 3 kb, and they should therefore be considered as individual entities.

Clinical relevance of hypermethylation at miRNA loci

Epigenetically regulated miRNAs embedded within or near methylated CpG islands have been linked before to clinical outcome in precursor B-ALL.[28] Here we present a similar finding in *MLL*-rearranged infant ALL involving miR-152. Although this miRNA locus is consistently methylated in all t(4;11)-positive infant ALL samples tested **(figure 1A)**, the magnitude of methylation and expression varied per patient **(figures 5A and 5B)**. Using the median log-ratio of miR-152 methylation as obtained from CpG microarray data **(figure 5A)** as the cut-off value, t(4;11)-positive patients were divided into 2 groups displaying either high or low degrees of methylation. The median miRNA expression was 2-fold lower in the group with a high degree of methylation (FDR<0.05) **(figure 5C)**. Furthermore, heavy methylation of the miR-152 CpG island was associated with an increased risk of relapse (p=0.0016) **(figure 5D)** and a reduced overall survival (p<0.001) **(figure 5E)**. The two known prognostic factors in infant ALL, i.e. age < 6 months and white blood cell (WBC) count > $300x10^9$/L,[1] did not differ significantly between the groups **(Supplemental table 3S)**. Although convincing, these results have to be interpreted with caution, given the low number of patients.

Potential target genes of miR-152

Encouraged by the clinical relevance of the methylation status of the miR-152 CpG island, we set out to identify possible miRNA targeted genes by using the algorithms TargetScan and Pictar[29] which base their prediction on sequence homology between miRNA and potential mRNA target. Both algorithms showed that DNA methyltransferase 1 (*DNMT1)*, also known as *CXXC9*, on chromosome 19p13.2 represents a possible target for miR-152 (probability of conserved targeting (P_{CT}, a measure for assessing the biological relevance of predicted miRNA–target interactions)[10]: 0.77). Moreover, *DNMT1* has recently been verified as a target of miR-152 in hepatobiliary cancer.[30] Based on known sequence similarity we investigated whether the *MLL* gene on chromosome 11q23, alias *CXXC7,* could be another miR-152 targeted gene. Interestingly, the seed sequence of miR-152 appeared entirely present in the 3' untranslated regions (UTR) of both *DNMT1* and *MLL* (P_{CT}: 0.86) **(figure 6A)**. Interestingly, miR-148, the other member of the highly conserved miR-148/152 family, which is also down-regulated due to CpG hypermethylation in t(4;11)-positive infant ALL **(figures 6B and 6C)**, has the same seed sequence as miR-152 and may therefore also target *DNMT1* and *MLL* **(figure 6A)**, which has already been proven for *DNMT1*.[30]

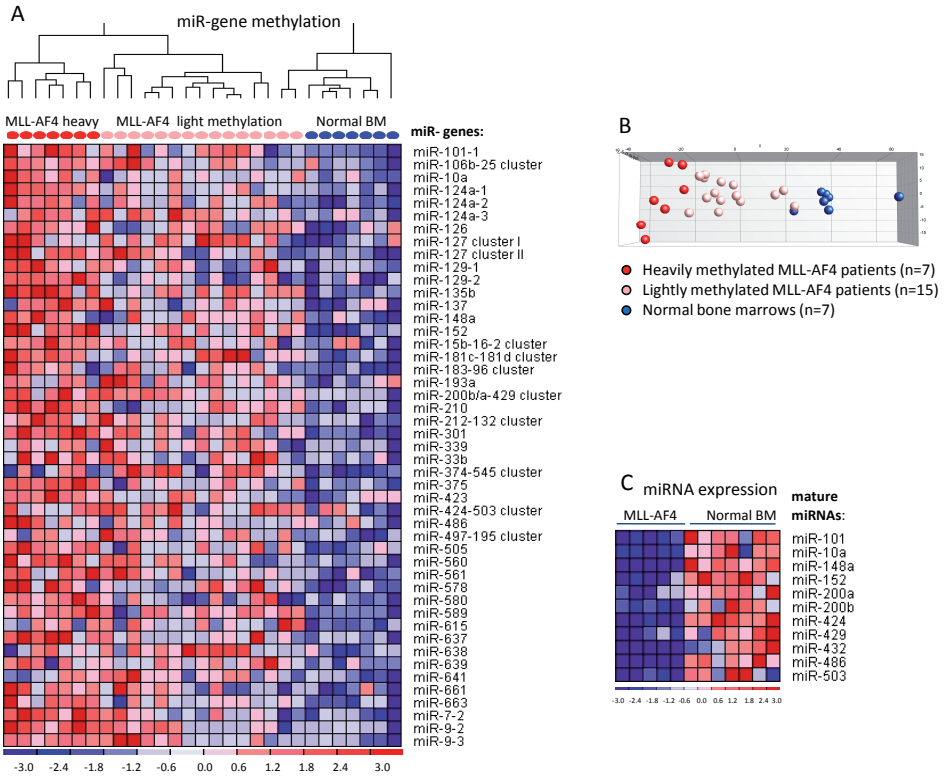


Figure 2. Hypermethylation and down-regulated expression of miRNAs in t(4;11)-positive infant ALL. **A.** Heatmap showing the 59 miR-genes (corresponding to 55 mature miRNAs) hypermethylated in t(4;11)-positive infant ALL (n=22) compared with healthy bone marrow (n=7) samples (FDR<0.05). Columns represent patient samples and rows represent miR-genes. Relative CpG methylation levels are shown in red (high) and blue (low). CpG probes located in close proximity to miRNA clusters are annotated as miRNA cluster. MiR-127 cluster I contains miR-433 and miR-127, and miR-127 cluster II relates to miR-432 and miR-136 **(figure 3C)**. Samples were ordered using hierarchical cluster analysis (Euclidean distance, complete linkage). **B.** Principal component analyses (PCA) using the 59 hypermethylated miR-genes separating heavily (red, n=7) and lightly (pink, n=15) methylated t(4;11)-positive infant ALL, and normal bone marrow samples (blue, n=7). Based on the first three components of the PCA explaining 62.5% of the total variance, the samples were visualized. **C.** Heatmap displaying the expression of miRNAs that were significantly down-regulated in t(4;11)-positive infant ALL samples (n=5) as compared with normal bone marrows (n=7) (FDR<0.05). Relative expression levels are shown in red (high) and blue (low). MiRNA expression profiling could only be performed for 5 patients due to restricted availability of patient material. Among the 5 patients were 3 lightly methylated patients and 2 heavily methylated patients. Similarly as observed in our previous study, the separation between heavily and lightly methylated patients is not reflected at the expression level.[8]

Table 1. Zebularine-induced demethylation and re-expression of silenced miRNAs.
Table depicts hypermethylated miRNAs that were transcriptionally silenced, but demethylated and re-expressed upon zebularine treatment. Expression levels were measured by quantitative RT-PCR, and fold-changes between t(4;11)-positive ALL and normal bone marrow (nBM), as well as expression levels in the SEMK2 cell line before (Z0) and after treatment (for 3 (Z3), 6 (Z6) or 10 (Z10) days) are shown. MiRNAs were considered re-expressed by zebularine when the expression was increased more than 2-fold for at least two time points during treatment.

miRNA	cluster	chromosomal location	t(4;11) vs nBM		re-expression
			fold-change in expression	FDR	Z0 Z3 Z6 Z10
miR-10a	-	17q21.32	-7	0.016	
miR-152	-	17q21.32	-13	0.001	
miR-200a	miR-200b/a-429	1p36.33	-3	0.042	
miR-200b			-4	0.016	
miR-429			-5	0.016	
miR-432	miR-127	14q32.2	-480	0.043	
miR-503	miR-424-503	Xq26.2	-218	0.036	

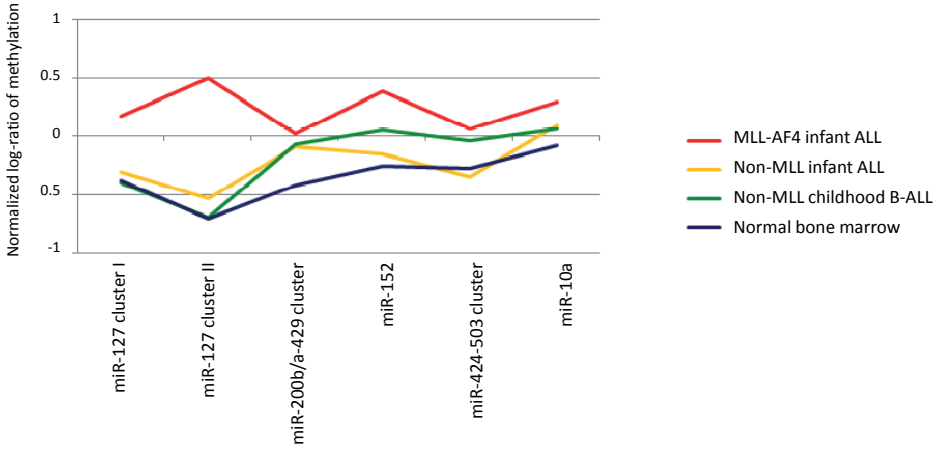
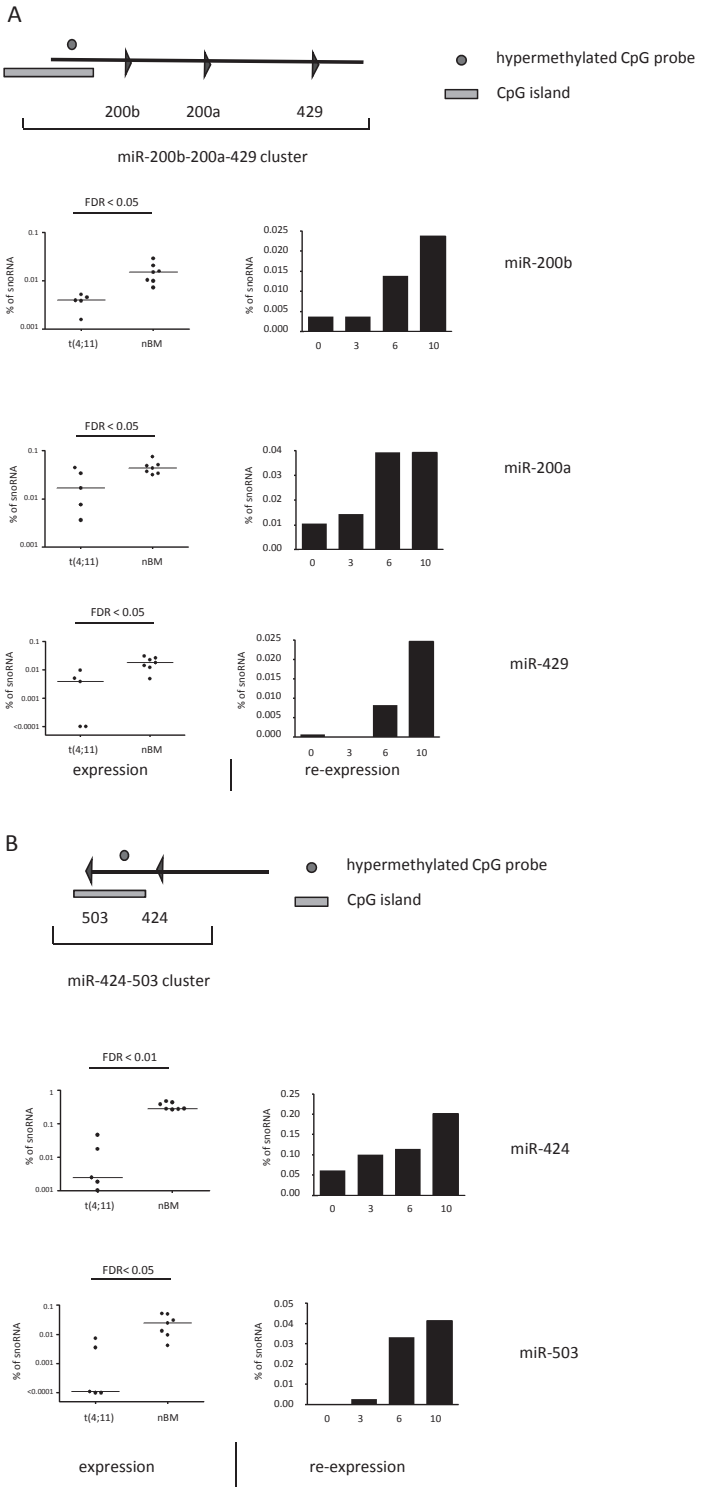


Figure 3. Methylation of selected miR-genes is specific for MLL-AF4-positive infant ALL.
Graphical representation of the methylation levels of the selected miRNA genes in t(4;11)-positive infant ALL samples (n=15, red line) along with the log-ratios of wild-type *MLL* infant ALL samples (n=10, yellow line) and non-*MLL* childhood ALL samples (n=10, green line) relative to normal pediatric bone marrow samples (n=7, blue line). CpG methylation data are presented as normalized log-ratios of patient signal divided by the common reference signal.

DNMT1 and *MLL* are highly expressed in t(4;11)-positive infant ALL patients **(figures 6D and 6E)** and demethylation of miR-152 by zebularine leading to up-regulation of miR-152 readily resulted in down-regulation of mRNA expression of these genes **(figures 6F and 6G)**. In contrast to its family member miR-152, miR-148 could not be re-activated after exposure to zebularine for 10 days.


A
hypermethylated CpG probe
CpG island
200b
200a
429
miR-200b-200a-429 cluster
FDR < 0.05
% of snoRNA
t(4;11)
nBM
0
3
6
10
miR-200b
miR-200a
miR-429
expression
re-expression
B
503
424
miR-424-503 cluster
FDR < 0.01
FDR< 0.05
miR-424
miR-503

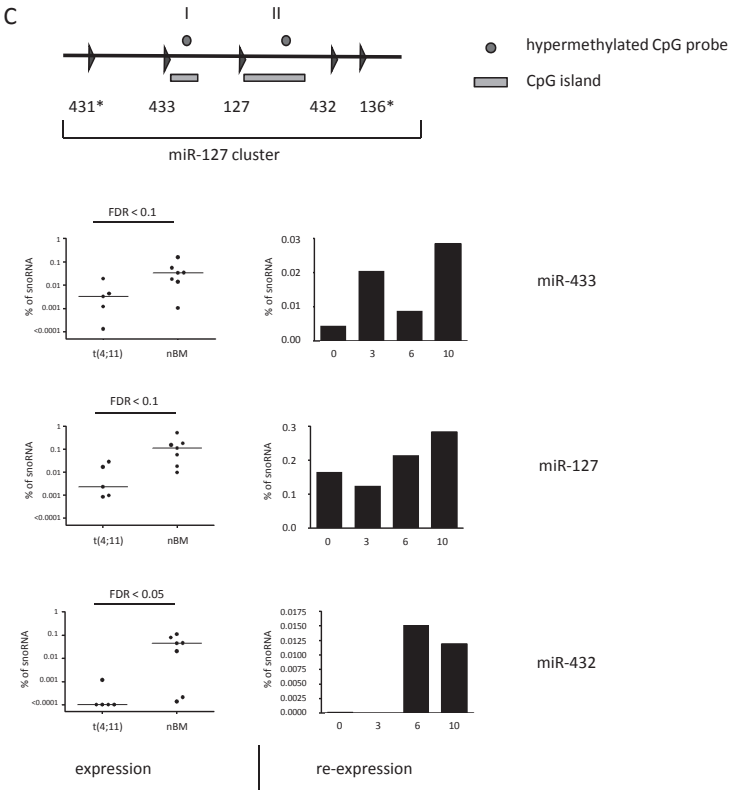


Figure 4. MiRNAs located in miRNA clusters.
A. Schematic representation of the positions of the different miRNAs belonging to the miR-200b-200a-429 cluster on human chromosome 1p36.33. **B**. Schematic representation of the positions of the miRNAs from the miR-424-503 cluster on human chromosome Xq26.2. **C**. Schematic representation of the positions of the different miRNAs belonging to the miR-127 cluster, miR-431, miR-433, miR-127, miR-432, and miR136, on human chromosome 14q32.2 MiRNAs indicated by an astrix (*) were not covered by RT-PCR primers. "I" and "II" denote miR-127 cluster I and II respectively as used in figure 1A. The locations of the miRNAs are indicated by triangles. The CpG island region is indicated as a grey bar, and the individual hypermethylated CpG island probes (Agilent microarrays) are depicted as circles. MiRNA expression levels were determined by quantitative RT-PCR in t(4;11)-positive infant ALL patients (n=5) and in the SEMK2 cell line in the presence and absence of zebularine for 3, 6 or 10 days. P-values are from the limma model (Supplemental table 2S).

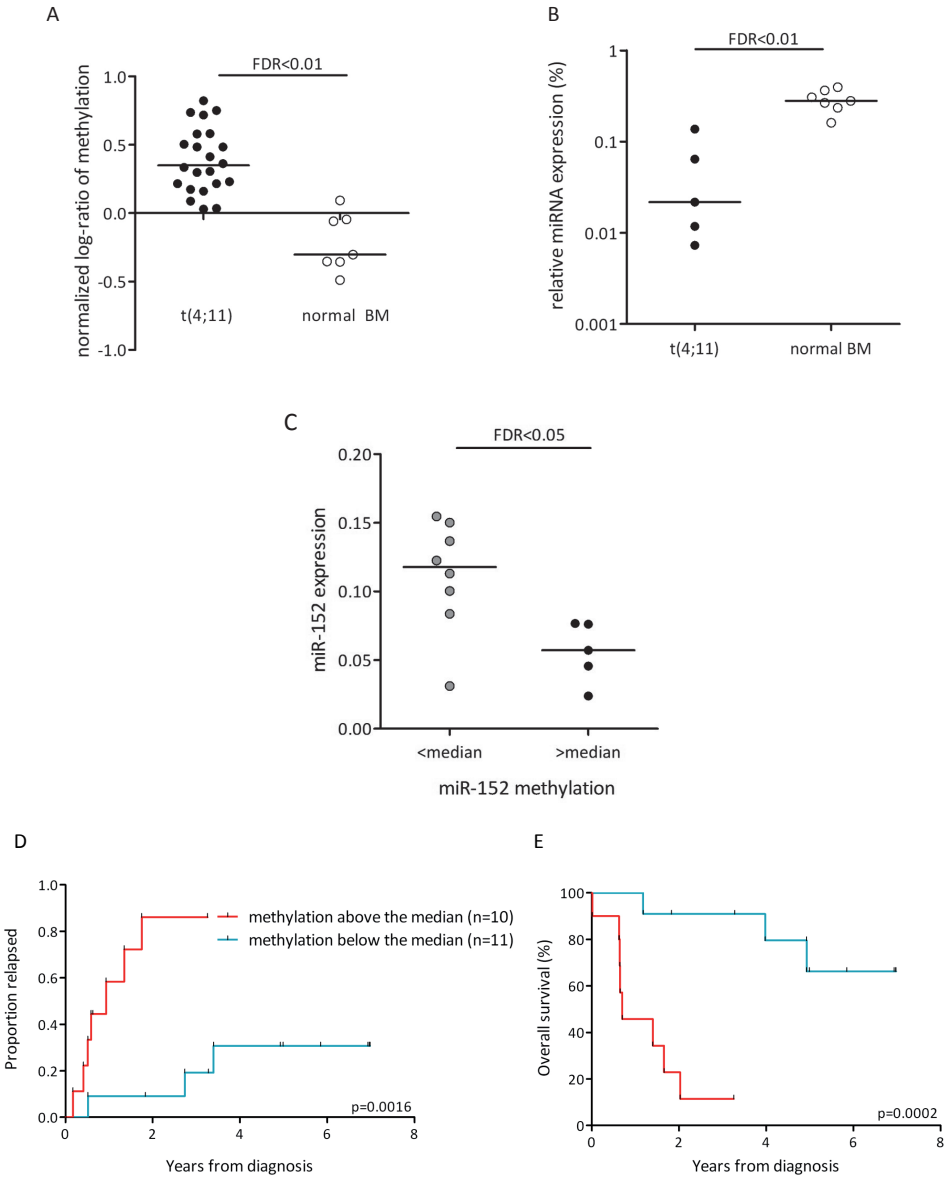


Figure 5. MiR-152 methylation, expression, and patient survival
A. Methylation levels of the miR-152 CpG island as obtained by differential methylation hybridization (DMH) presented as normalized log-ratios of methylation in t(4;11)-positive infant ALL (n=22) and normal bone marrow (n=7) samples. P-value is from the limma model. **B.** MiR-152 expression levels as determined by quantitative RT-PCR in t(4;11)-positive infant ALL patients (n=5) and normal bone marrows (n=7). P-value is from the limma analyses. **C.** MiR-152 expression in t(4;11)-positive infant ALL patients (n=13) as determined by using the miR-152 quantitative RT- PCR assay in patients with methylation below or above the median value. **D.** Risk of relapse as determined by the Kaplan Meier estimate and **E.** overall survival (OS) in t(4;11)-positive patients (n=21) divided by the median degree of DNA methylation **(figure 5A).** One patient died before start of treatment and was excluded from survival analyses (denoted early death). normal BM = normal bone marrow.

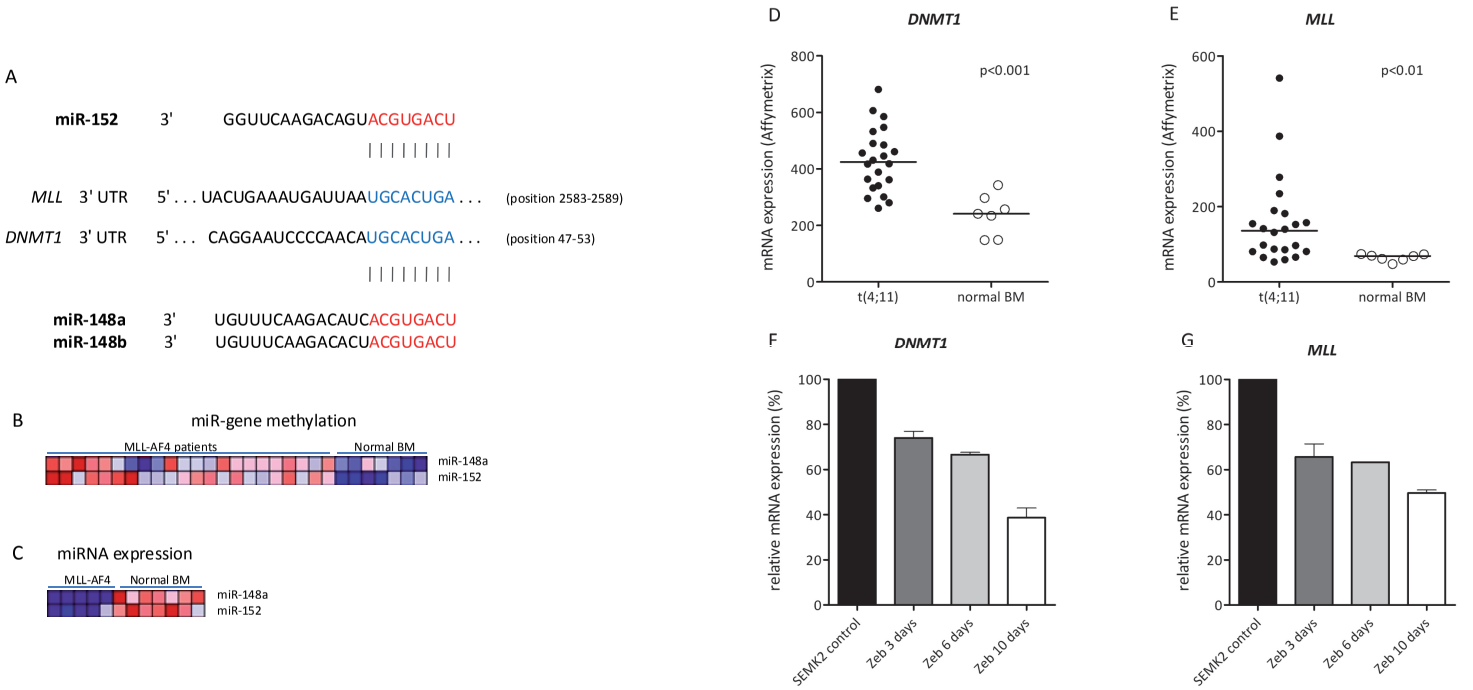


Figure 6. *MLL* and *DNMT1* as potential miR-152 and miR-148 targeted genes.
A. Putative miR-152 targeted genes were identified using TargetScan (*www.targetscan.org*) and Pictar.[29] MiRNA sequences were analyzed using miRbase[42] (*http://www.mirbase.org*) and detailed information on base pairing between miR-152 and its target sites in the 3'UTRs of the *DNMT1* and *MLL* genes was available through TargetScan. MiR-148, the other member of the broadly conserved miR-148/152 family, harbors a seed sequence identical to that of miR-152. **B.** Methylation values for miR-148 (CpG island microarray data) in t(4;11)-positive infant ALL patients (n=22) and normal bone marrow samples (n=7), and **C.** miR-148 expression data as obtained from quantitative RT-PCR for t(4;11)-positive infant ALL patients (n=5) and normal bone marrows (n=7). **D.** *DNMT1*, and **E.** *MLL* normalized mRNA expression levels as obtained from gene expression profiling (Affymetrix GeneChips) in t(4;11)-positive infant ALL patients (n=22) and normal bone marrow samples (n=7). P values are from limma models. **F.** *DNMT1,* and **G.** *MLL* mRNA expression levels relative to the housekeeping reference gene *B2M* were determined by RT-PCR in the t(4;11)-positive cell line SEMK2 before and after exposure to 100μM of zebularine for 3, 6 and 10 consecutive days. Expression levels in unexposed SEMK2 cells were set to 100%. Highly expressed miRNA targeted genes were considered down-regulated by re-activation of the miRNA when mRNA expression was at least 2-fold decreased after zebularine treatment.

ZEB2 and *HOXA3* as potential miRNA-target genes in t(4;11)-positive infant ALL

According to literature the *zinc finger E-box binding homeobox 2* (*ZEB2)* gene is the best-known validated target gene of the miR-200b/a-429 cluster[31] and the *homeobox A3* (*HOXA3)* gene has been described as a potential miR-10a targeted gene.[32] Both of these genes are known to be highly expressed in t(4;11)-positive infant ALL[5, 7] which was validated in our t(4;11)-positive infant ALL cohort (Affymetrix GeneChips) **(Supplemental figures 3SA and B)**. After zebularine treatment the miR 200b/a-429 cluster was up-regulated, as well as miR-10a **(table 1)**, and *ZEB2* expression was clearly down-regulated **(Supplemental figure 3SC)** whereas *HOXA3* expression was only marginally decreased **(Supplemental figure 3SD)**.

DISCUSSION

MLL-rearranged acute lymphoblastic leukemia (ALL) in infants represents a high-risk subtype of childhood leukemia characterized by a complex biology. Initiating transformation, the MLL fusion protein alters normal MLL histone methyltransferase activity and instead is guided by the fusion partner. In turn, these inappropriate histone modifications induce aberrant transcription of multiple genes, resulting in highly characteristic gene expression profiles.[6, 7] In contrast to this widespread gene activation, we recently demonstrated that vast numbers of genes are epigenetically silenced by promoter hypermethylation.[8] This was recently confirmed in an independent study by Schafer et al.[33] Genome-wide hypermethylation is especially apparent in t(4;11)-positive infant ALL and as shown in the present study extends its effects on miRNA expression.

Comparing t(4;11)-positive infant ALL with healthy bone marrow samples, a total of 59 out of 122 analyzed miR-genes were significantly hypermethylated. Seven of these miRNAs were selected for further evaluation, because they were transcriptionally silenced, and exposure to the DNA methyltransferase inhibitor zebularine resulted in their demethylation and re-expression: miR-200b, miR200a, miR429, miR-152, miR-10a, miR-503 and miR-432 **(table 1)**. Interestingly, some of these miRNAs have been described to function as tumor suppressing miRNAs. For instance, miR-432 is located within the large miR-127 cluster **(figure 4C)** which was previously shown to be silenced in various malignancies by CpG island hypermethylation and aberrant histone modifications.[9] In that study, re-expression of miR-127 resulted in down-regulation of the proto-oncogene B-cell CLL/lymphoma 6 (BCL6) at the protein level.[9] In addition, down-regulation of miR-200b in human cancers leads to disease progression via elevated expression of the two E-cadherin transcriptional repressors: *ZEB1* and *ZEB2*.[31] Importantly, *ZEB2* is highly expressed in t(4;11)-positive ALL as a consequence of MLL-AF4-driven histone modifications.[5] Our data demonstrate both *ZEB2* down-regulation **(Supplemental figure 3SC)** and re-activation of miR-200b **(Supplemental figure 2SB)** by zebularine suggesting that *MLL* translocation-induced transcription involves the concerted regulation of both chromatin structure and miRNA activity.

Another example of MLL fusion-driven miRNA regulation involves the polycistronic miR-424-503 cluster. In t(9;11)-positive myeloid leukemias miR-424 expression was repressed by the MLL-AF9 fusion protein.[34] We here show that the miR-424-503 cluster is down-regulated by CpG island hypermethylation in t(4;11)-positive ALL, possibly directed by the MLL-AF4 fusion. Likewise, miR-10a is down-regulated in *MLL*-rearranged acute myeloid leukemia (AML).[35] In line with these observations, we found miR-10a to be down-regulated in t(4;11)-positive infant ALL. Although miR-10a is located within the HOXB cluster, one of its putative targets is *HOXA3.*[32] In concordance with miR-10a silencing, *HOXA3* expression is elevated in t(4;11)-positive infant ALL patients **(Supplemental figure 3SB)**. However, re-activation of miR-10a by demethylation only marginally repressed *HOXA3* expression at the mRNA level, suggesting that miR-10a exerts its inhibition on putative target HOXA3 largely on the protein level. Alternatively, additional forces retain the expression of this gene.

Of high interest was that we identified the wild-type *MLL* gene as a potential target of miR-152. A recent report suggests that co-expression of wild-type *MLL* is required for MLL-AF9-induced leukemogenesis.[36] Whether wild-type *MLL* is also required for the development or maintenance of t(4;11)-positive leukemias remains uncertain. However, as we show here, epigenetic inactivation of miR-152 at least prevents the down-regulation of wild-type *MLL*. Treatment with the demethylating agent zebularine induced expression of miR-152 and down-regulation of wild-type *MLL* at the mRNA level. Moreover, the other miR-148/152 family member miR-148 utilizes the same "seed" sequence as miR-152 and therefore may also target the *MLL* gene **(figure 6A)**. MiR-148 is also down-regulated as a consequence of CpG island hypermethylation in t(4;11)-positive infant ALL **(figures 6B and 6C)**, which may have further obstructed down-regulation of wild-type *MLL*. In addition, in case of an *MLL*-translocation, the N-terminal region of the *MLL* gene is usually retained in the fusion protein, whereas the C-terminus containing the 3' UTR is lost. Therefore, miR-148/152 would not be able to target the leukemic MLL-AF4 fusion. However, in the majority of t(4;11)-positive ALL samples, also the reciprocal *AF4-MLL* transcript is present,[37] and from recently published mouse models it appears that the AF4-MLL fusion is essential for leukemic transformation.[38] Hypothetically, besides targeting wild-type *MLL,* miR-152 may target the oncogenic reciprocal *AF4-MLL* fusion product. The fact that the *AF4-MLL* transcript is detected in 80% of t(4;11)-positive ALL samples indicates that this reciprocal transcript may contribute to the leukemogenic transformation into a full-blown leukemia, but may not be essential for further maintenance of the leukemia.

Epigenetic silencing of miR-152 and possibly miR-148 may therefore have contributed to this process by facilitating AF4-MLL-driven induction of leukemic transformation. The selected miRNAs were not methylated in wild-type *MLL* infant ALL patients and non-*MLL* childhood ALL patients **(figure 3)**. In concordance with this, none of the selected miRNAs in this study was found to be associated with CpG island hypermethylation in adult and pediatric precursor B-cell ALL patients in a previous study[28] which provides additional evidence that the here identified miRNAs are specific for t(4;11)-positive ALL. MiR-152 has been described to be silenced due to CpG hypermethylation in cancer before.[39]

Apart from the associations of the selected miRNAs with *MLL* and MLL fusions, another notable feature stands out. The expression of at least three out of these miRNAs seems to be regulated by the genes executing DNA methylation: miR-200b, miR-200a and miR-10a were most significantly up-regulated in a Dnmt1^{-}/Dnmt3^{-} knock-out mouse model.[32] As DNMT1 and DNMT3 (DNA methyltransferase 1 and 3) facilitate maintenance and *de novo* DNA methylation respectively, expression of these miRNAs is evidently controlled by the epigenetic force of DNA methylation. MiR-152 appears to be an effector of the epigenetic machinery itself as one of its putative targets is *DNMT1.*[30] Therefore, miR-152 can be added to the growing list of so-called "epi-miRNAs", such as miR-29b and miR-290, which control the regulation of DNMTs.[40] MiR-148 has recently been added to this list of epigenetic effector miRNAs, because it targets DNMT3b.[41] As we have shown in the present study, the degree of miR-152 methylation varies among t(4;11)-positive infant ALL samples and is highly predictive for clinical outcome. Similarly, we recently found that the degree of genome-wide promoter methylation also varies among *MLL*-rearranged infant ALL and that patients with heavily methylated genomes are at extremely high risk of disease relapse.[8] Heavily methylated cases displayed a higher degree of methylation of the miR-152 CpG island **(Supplemental figure 4S)**. Thus, the varying levels of miR-152 methylation and *DNMT1* expression may determine the severity of genome-wide methylation. Zebularine is a cytidine analog that requires incorporation into the DNA where it covalently binds DNMT1, and protein levels of this protein will be reduced directly by this mechanism. Then, indirectly, through re-expression of miR-152, the conventional RNA silencing mechanism will down-regulate the expression of *DNMT1* even further. Zebularine-induced re-activation of miR-152 which targets *DNMT1* may thus enhance the efficiency of the compound and explain its recently demonstrated potential in *MLL*-rearranged infant ALL.[8] Until recently, it was believed that the demethylating actions of DNMT inhibitors, such as 5-azacytidine, decitabine (5-aza-deoxycytidine), and zebularine are limited to hypermethylated (tumor suppressor) genes. We here show that zebularine, through re-activation of important hypermethylated miRNAs, is able to down-regulate proto-oncogenes in *MLL*-rearranged infant ALL. Obviously, the stability of zebularine was a key factor in allowing the re-expression of silenced miRNAs genes which peaks after 10 days. This stresses the imperative need to test zebularine or comparable compounds in mouse models and subsequent clinical trials in infant leukemia.

Besides the previously published hypermethylation at protein-coding genes[8] we here showed that the aberrant DNA methylation in t(4;11)-positive infant ALL extended into methylation at miRNA loci. We identified miRNAs that were in-activated due to CpG island hypermethylation and the majority of these miRNAs has been found to be associated with the *MLL* gene or *MLL*-rearranged leukemia, implying an important role for the MLL-AF4 fusion in the deregulation of these miRNAs. We further show that the silenced status of miR-152 has impact on the survival of t(4;11)- positive infant ALL patients. This silenced status can be reversed by zebularine. All together, our study indicates that although miRNAs are small in size, they have major implications

in t(4;11)-positive infant ALL. This emphasizes the need for further investigation of demethylating agents, such as zebularine, to improve the treatment of these young patients with leukemia.

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Supplemental information is available in the appendices.

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“Experimental confirmation of a prediction is merely a measurement. An experiment disproving a prediction is a discovery”

(Enrico Fermi, Italian physicist, 1901-1954)

4

Absence of Global Hypomethylation in Promoter Hypermethylated *MLL*-rearranged Infant Acute Lymphoblastic Leukemia.

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ABSTRACT

Background

MLL-rearranged acute lymphoblastic leukemia (ALL) in infants remains the most aggressive type of childhood leukemia characterized by exceedingly high rates of early disease relapse. The majority of *MLL*-rearranged infant ALL cases display severe promoter CpG island hypermethylation which is related to clinical outcome. Compared with other ALL subtypes, *MLL*-rearranged ALL cells are highly sensitive to demethylating agents *in vitro*.

The general and dogmatic belief in cancer epigenetics dictates that gene promoter hypermethylation is accompanied by DNA hypomethylation in non-promoter regions of the genome. This global hypomethylation may hamper the use of demethylating agents.

Objective and methods

In this study, we examined global methylation densities in *MLL*-rearranged infant ALL (n=45) in comparison with wild-type *MLL* infant ALL (n=11), non-infant B- cell precursor ALL (n=11) and normal pediatric bone marrow (n=9) samples. We performed high-resolution bisulfite pyrosequencing to determine methylation levels at the repetitive elements LINE-1, Alu, and SAT-α. As an additional measure of global methylation levels we used the LUminometric Methylation Assay (LUMA).

Results

We found that *MLL*-rearranged infant ALL is not characterized by global hypomethylation, despite its characteristic promoter CpG hypermethylation patterns. Instead, we observed a moderate trend towards global hypermethylation and demonstrated that these methylated non-promoter sequences are responsive to demethylating agents.

Conclusions

MLL-rearranged infant ALL cells are characterized by an overall increase of genomic methylation. In addition, the methylation of both promoter and non-promoter is affected by demethylating agents. The lack of global hypomethylation in these cells is of additional therapeutic value, as it provides supplementary targets to tackle epigenetic deregulation.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children and is generally characterized by a favorable outcome with event-free survival (EFS) chances of >85%.[1] In ~4% of all cases, however, ALL is diagnosed in an infant (<1 year of age), and the prognosis for these very young children is notably inferior (EFS ~50%).[2, 3] Among childhood ALL patients, infant ALL represents a distinct clinical and biological entity. Clinically infant ALL patients characteristically present with exceedingly high white blood cell counts, hepatosplenomegaly, and often display central nervous system involvement.[2] Biologically infant ALL is characterized by a unique gene expression profile that is distinguishable from profiles obtained in older children with precursor B-cell ALL.[4, 5] Moreover, the majority of infants with ALL (~80%) carry balanced chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene on chromosome 11q23.[2] These *MLL* translocations represent independent predictors of a dismal clinical outcome,[3, 6] and consequently the EFS rate for *MLL*-rearranged infant ALL in particular remains at best ~40%.[3] The most prevalent *MLL* translocations in infant ALL include t(4;11), t(11;19) and t(9;11), fusing the N-terminus of *MLL* to the C-terminus of *AF4*, *ENL* and *AF9* respectively.[3, 7] In the case of these gene fusions, the *MLL* gene loses its 3'-terminal Suppressor of variegation 3-9, Enhancer of zeste, Trithorax (SET) domain. This domain exerts histone methyltransferase (HMT) activity responsible for the activating histone 3 lysine 4 trimethylation (H3K4me3) mark[8] that protects associated DNA stretches against methylation.[9, 10] Histone modifying protein complexes containing ALL-inducing MLL fusion proteins are able to recruit an alternative HMT, designated DOT1L.[11, 12] In contrast to full length MLL, DOT1L catalyzes dimethylation of histone 3 at lysine 79 (H3K79me2), and recruitment of this HMT by MLL fusion proteins thus establishes abnormal H3K79me2 histone marks.[11] Given the sound interplay between histone modifications (regulating chromatin conformations) and gene promoter DNA methylation,[13] *MLL* translocations are likely to provoke aberrant DNA methylation patterns.

Emphasizing this hypothesis, we and others recently demonstrated that the majority of *MLL*-rearranged infant ALL cases is characterized by severe promoter hypermethylation,[14, 15] in particular patients carrying t(4;11) or t(11;19).[15] These studies specifically focused on CpG island methylation at gene promoters. Here we present data evaluating DNA methylation in infant ALL on a more global level by studying the methylation status at several wide spread repetitive elements to determine whether the increase in promoter hypermethylation is accompanied by global hypomethylation as is typically found in numerous malignancies.[16-20]

Repetitive elements are interspersed repeated DNA sequences that originate from transposable elements,[21] or represent tandemly repeated simple sequences (satellite DNA). These elements comprise about 45% of the human genome,[22] and contain a substantial portion of its CpG islands. Therefore, methylation of repetitive elements is frequently used as a representative for global DNA methylation.[18, 23, 24] The most abundant retrotransposon sequences in the human genome include

the long interspersed element 1 (LINE-1) and the short interspersed element (SINE) Alu.[25] Centromeric satellite α (SAT-α) represents the largest of the tandem repeat sequences.[26] Whereas in normal tissues promoter CpG islands are typically unmethylated leading to active gene transcription,[27] repetitive DNA elements are usually silenced by methylation preventing them from being transcribed.[28, 29] The opposite is often observed in human malignancies, especially in solid tumors, where repetitive elements are frequently hypomethylated when high numbers of gene promoters become hypermethylated.[16-18, 20] Global hypomethylation is typically associated with chromosomal instability. In hematological malignancies this apparent shift in methylation has only been described for chronic myeloid leukemia (CML).[19, 30] Methylation status at repetitive elements has never been investigated in childhood hematological malignancies. The present study is the first to demonstrate that *MLL*-rearranged infant ALL lacks global hypomethylation despite severe promoter CpG hypermethylation. This is a finding with therapeutical consequences.

MATERIAL AND METHODS

Patient samples

We studied 56 newly diagnosed infant ALL patients (<1 year of age) enrolled in the INTERFANT-99 treatment protocol.[3] Clinical characteristics are listed in **table 1**. Samples were screened for the presence of *MLL* rearrangements using split-signal FISH analysis and the type of translocation was determined using Reverse Transcriptase (RT)-PCR. Twenty-one infants had *MLL* translocation t(4;11), eighteen t(11;19), and six t(9;11). In eleven infant ALL patients no *MLL* translocation could be detected and these were denoted as "wild-type *MLL*" patients. In addition 11 non-infant (>1 year of age) childhood precursor B-cell ALL cases collected at the Erasmus MC – Sophia Children's Hospital Rotterdam were studied. The genetic subtype of these patients was determined using FISH analysis, RT-PCR assays, or flow cytometry. Four cases were positive for the TEL-AML1 translocation, 3 cases were hyperdiploid, and 4 cases were designated "B-other" **(table 1)**. B-other cases were negative for TEL-AML1, MLL-AF4, BCR-ABL and hyperdiploidy. Leukemic cell isolation and enrichment to >90% of leukemic blasts were performed as described before.[31, 32] From 5 *MLL*-rearranged infant ALL patients remission material was available. In addition, normal bone marrow samples from non-leukemic children (n=9; including one infant) were used as controls **(table 1)**. Approval was obtained from the Erasmus MC Institutional Review Board, and informed consent was acquired from parents or legal guardians according to the Declaration of Helsinki.

Cell line models and exposure to demethylating agents

The t(4;11)-positive precursor B-ALL cell lines SEM and RS4;11 were purchased from DSMZ (Braunschweig, Germany). Both cell lines were maintained as suspension cultures in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen Lifesciences, Breda,

the Netherlands) supplemented with 10% fetal calf serum (FCS) (Integro, Zaandam, the Netherlands), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.125 µg/ml fungizone (Invitrogen Lifesciences, Breda, the Netherlands) at 37°C in humidified air containing 5% CO_2. These cell lines were exposed to the demethylating agents decitabine (0.5 µM; Sigma-Aldrich, Zwijndrecht, the Netherlands) or zebularine (100xµM; kindly provided by Dr. Victor E. Marquez, National Cancer Institute of Frederick, Frederick, Maryland, USA) for a time period of 10 days. Due to its relatively short half-life, decitabine had to be replenished every 48 hours. For the more stable compound zebularine this was not necessary. Cells were sampled after 0, 1, 3, 7, and 10 days of exposure to decitabine or zebularine. Cell viability was assessed by Annexin V staining determined by flow cytometry using a FACSCalibur (Becton Dickinson).

Table 1. Patient characteristics and controls.

Parameter	Value
Infant ALL patients, n	56
Median age, years (IQR)	0.46 (0.22-0.72)
Sex, n (%)	
Female	32 (57%)
Male	24 (43%)
Median white blood cell count (x10^9/l) (IQR)	291.50 (95.18-469.00)
Genetic subtype	
MLL t(4;11)	21 (38%)
MLL t(11;19)	18 (32%)
MLL t(9;11)	6 (11%)
Wild-type MLL	11 (20%)
Non-infant B cell precursor ALL patients, n	11
Median age, years (IQR)	6.12 (3.45-13.22)
Sex, n (%)	
Female	4 (36%)
Male	7 (64%)
Median white blood cell count (x10^9/l) (IQR)	36.23 (10.94-96.34)
Genetic subtype	
TEL-AML1	4 (36%)
Hyperdiploid	3 (27%)
B-other *	4 (36%)
Healthy pediatric bone marrows, n	9
Median age, years (IQR)	9.50 (5.39-15.36)
Sex, n (%)	
Female	4 (44%)
Male	5 (56%)

IQR = interquartile range: the 25th and the 75th percentiles are shown. *B-other childhood ALL cases are negative for BCR-ABL, TEL-AML1, MLL-AF4 and hyperdiploidy.

Isolation and purification of DNA and RNA

Genomic DNA was extracted using TRIzol reagent (Invitrogen Ltd, Paisly, UK) according to the manufacturer's protocol. For the LUminometric Methylation Assay (LUMA) DNA was isolated using the QIAamp DNA Mini Kit (Qiagen Benelux B.V., Venlo, the Netherlands). The quality of the extracted DNA was assessed on 1.5% agarose gels.

Pyrosequencing for quantitative CpG methylation analysis

Genomic DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research Corporation, CA, USA) according to the manufacturer's instructions. Bisulfite treatment of the DNA converts cytosine nucleotides to uracil, but leaves 5-methylcytosine residues untouched. This way discrimination against methylated or unmethylated cytosines is possible. Pyrosequencing is a "sequencing by synthesis" method based on the detection of pyrophosphate release upon nucleotide incorporation **(Supplemental methods)**. Through an enzymatic reaction visible light is generated in an amount proportional to the released pyrophosphate.[33] Approximately 20 ng of bisulfite converted DNA was amplified using bisulfite-specific primers for LINE-1, Alu or SAT-α. For LINE-1 the commercially available Pyromark LINE-1 assay (Qiagen Pyrosequencing Inc.) was used. For Alu and SAT-α the pyrosequencing assays as described by Bollati and coworkers[16, 34] were adopted and further optimized. Buffers and hotstart Taq polymerase used for PCR analysis were from the Qiagen Pyromark PCR kit (Qiagen Pyrosequencing, Inc.). The annealing temperatures for the Alu and SAT-α primers were 43°C and 50°C, respectively, in the presence of 2 or 1 mM $MgCl_2$. PCR products (antisense strand for LINE-1 and sense strands for Alu and SAT- α) were purified using Streptavidin Sepharose HP beads (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 M NaOH solution. Next, 0.3 µM of pyrosequencing primer was annealed to the purified single-stranded PCR product. All samples were analyzed in duplicate on a PyroMark MD system (Qiagen Pyrosequencing, Inc.). Subsequent quantification of methylation density was performed using the Pyro Q-CpG software version (Qiagen Pyrosequencing, Inc.). The degree of methylation is expressed as the number of methylated cytosine nucleotides divided by the amount of methylated and unmethylated cytosines x 100%. Before sequence analysis the completeness of bisulfite conversion was evaluated by analyzing cytosine – thymidine ratios in control sequences: non-CpG cytosines should be completely converted into thymidines. In all samples the percentage of non-converted cytosines was <3%.

LUminometric Methylation Assay (LUMA)

The LUminometric Methylation Assay (LUMA) was used as an additional measurement of global DNA methylation.[24, 35] LUMA is based on differential digestion by the methylation-sensitive restriction enzyme *Hpa*II (New England Biolabs, Ipswich, USA) and its methylation-insensitive isoschizomer *Msp*I (New England Biolabs, Ipswich, USA). In parallel a digestion with *Eco*RI (New England Biolabs, Ipswich, USA) was

performed to ensure equal DNA input. A polymerase extension assay was then carried out on a Pyromark MD System (Qiagen Pyrosequencing Inc.) in order to quantify global DNA methylation. The percentage of methylated DNA was expressed as 100 - (the ratio of (*Hpa*II/*Eco*RI and *Msp*I/*Eco*RI) x 100%). The assay was performed in duplicate.

Statistical analysis

Differences in repetitive element methylation between each patient group separately and normal bone marrows were evaluated using the Mann-Whitney *U* test (performed in SPSS 17.0 statistical software). Analyses were two-tailed, and differences were considered statistically significant at $p<0.05$. Graphpad Prism graphical software version 5 (La Jolla, CA, USA) was used for data visualization.

RESULTS

Absence of repetitive element hypomethylation in childhood ALL

High-resolution bisulfite pyrosequencing was used to quantify cytosine methylation densities in the retrotransposable elements LINE-1 and Alu, and at centromeric SAT-α sequences. For LINE-1 four CpG dinucleotides were studied, whereas for Alu and SAT-α three CpGs were interrogated. Initially, the levels of methylation per CpG dinucleotide were evaluated as the mean of each of the following patient groups consisting of *MLL*-rearranged infant ALL (n=45), wild-type *MLL* infant ALL (n=11) or non-infant pediatric precursor B-ALL (n=11) cases. The obtained results were compared with the levels of methylation in healthy pediatric bone marrow samples (n=9) (including one infant), and with bone marrow samples from *MLL*-rearranged infant ALL cases in remission (n=5). As shown in **figure 1**, no evidence was found for pronounced repetitive element hypomethylation in these leukemic samples, nor were any differences observed between the different types of leukemia. Moreover, for LINE-1 and SAT-α leukemic samples appeared to exhibit marginally higher levels of methylation compared with normal bone marrow samples and non-leukemic samples derived from patients with *MLL*-rearranged infant ALL at remission **(figure 1)**. A similar view is obtained when the mean levels of methylation over the different CpG sites are depicted per patient **(figure 2)**. Despite the fact that the variations in methylation between the groups are small, these differences do appear consistent **(figure 2)**, and in most instances the deviation from normal bone marrows reaches statistical significance **(table 2)**. Moreover, the levels of CpG dinucleotide methylation in the studied repetitive elements in remission samples are comparable to those measured in healthy bone marrow samples. This suggests that the observed increases in methylation in leukemic samples are, although small, leukemia-specific. In any case, neither *MLL*-rearranged infant ALL nor wild-type *MLL* pediatric ALL samples seem to display hypomethylation at these loci, in spite of the earlier observed hypermethylation at multiple gene promoters patterns in t(4;11)- and t(11;19)-positive infant ALL.[15]

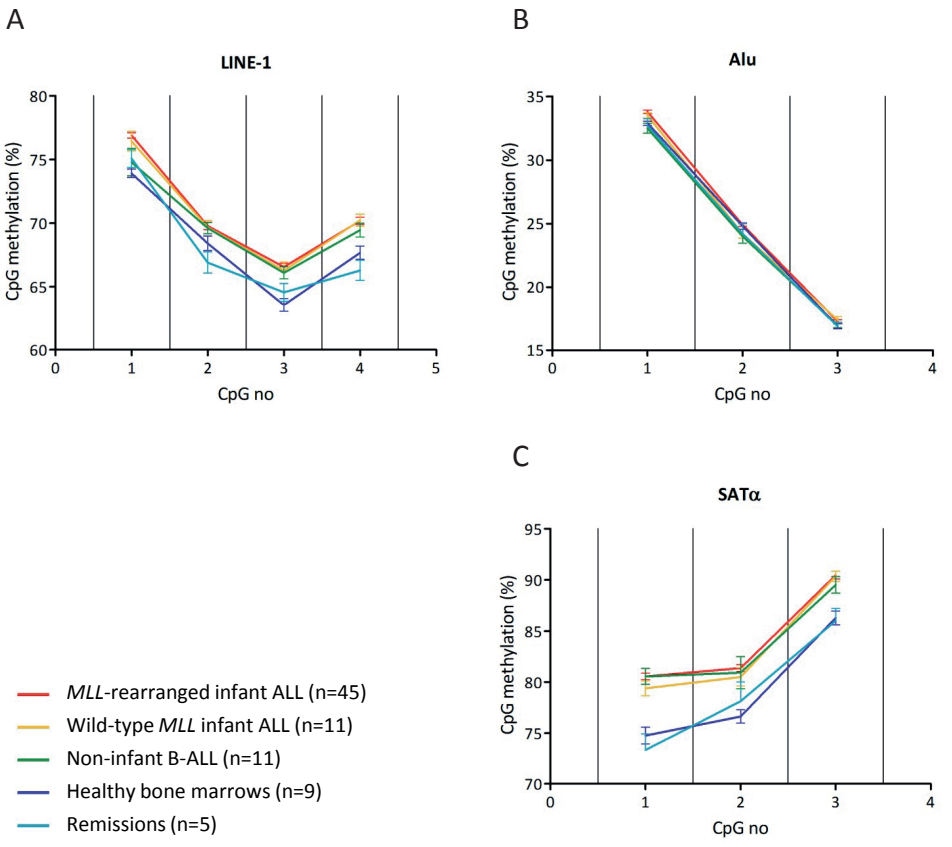


Figure 1. Mean repetitive element methylation per CpG site.
Quantitative methylation levels as determined by bisulfite pyrosequencing are visualized as the mean for each patient group per CpG in the repetitive elements **A.** LINE-1, **B.** Alu, and **C.** SAT-α. Means and standard errors of the mean (SEM) were calculated for each patient group and the healthy references: *MLL*-rearranged infant ALL (n=45, red line), wild-type *MLL* infant ALL (n=11, yellow line), non-*MLL* non-infant B-cell precursor ALL (n=11, green line), healthy bone marrows (n=9, blue line), and remissions (n=5, light blue line). "CpG no." = CpG number in the 'sequence to analyze'.

Genome-wide hypermethylation in *MLL*-rearranged infant ALL

Besides repetitive element methylation analyses, global CpG methylation was also estimated using the LUminometric Methylation Assay (LUMA), which is based on differential digestion between methylated and unmethylated cytosines at *Hpa*II recognition sites.[35] In contrast to repetitive elements that allow evaluation of non-promoter CpG islands dispersed throughout the genome but at specific non-coding loci, LUMA provides a global view of genome-wide CpG methylation without distinguishing between coding or non-coding sites. In concordance with the above described results on repetitive element methylation, infant ALL samples also displayed slightly increased levels of genome-wide methylation as estimated

Table 2. Differential quantitative DNA methylation levels of the repetitive elements LINE-1, Alu and SAT-α in childhood ALL.

Patients		LINE-1		Alu		SAT-α		LUMA	
	n	median (IQR)	p-value	median (IQR)	p-value	median (IQR)	p-value	median (IQR)	p-value
Infants									
MLL t(4;11)	21	71.19 (69.62-72.08)	0.00*	25.40 (24.95-25.80)	0.01*	83.87 (82.74-84.61)	0.00*	76.94 (71.92-79.04)	0.00*
MLL t(11;19)	18	70.55 (68.42-71.38)	0.01*	25.40 (24.95-26.03)	0.02*	83.39 (82.56-84.58)	0.00*	77.87 (75.13-79.55)	0.00*
MLL t(9;11)	6	70.99 (70.03-71.90)	0.00*	24.95 (24.55-25.30)	0.59	84.97 (81.56-86.78)	0.01*	74.50 (70.59-77.08)	0.02*
Wild-type MLL	11	70.86 (67.76-71.90)	0.09	24.90 (24.60-25.70)	0.25	83.41 (81.47-84.13)	0.00*	74.63 (71.89-79.20)	0.09
Non-infants	11	70.41 (68.55-71.08)	0.04*	24.40 (23.80-25.20)	0.30	82.66 (78.38-84.69)	0.05	69.49 (61.72-74.35)	0.20
Healthy bone marrows	9	68.17 (66.86-68.94)		24.80 (24.40-25.05)		78.81 (76.01-80.22)		68.59 (52.15-71.55)	

IQR = interquartile range: the 25th and the 75th percentiles are shown. P-values are from a Mann Whitney U test for differences between the leukemia subtypes and healthy bone marrows. Significant p-values are indicated with an astrix (*).

by LUMA compared with normal bone marrow samples **(figure 3)**. These differences reached statistical significance for all *MLL*-rearranged infant ALL subtypes **(table 2)**. The variation among healthy bone marrow samples (mostly derived from children older than 1 year of age) appeared fairly large, whereas the methylation levels of the samples from *MLL*-rearranged infant ALL patients during disease remission were markedly lower than any of the leukemic samples **(figure 3)**. These remission samples feasibly represent the most genuine controls for genome-wide methylation in *MLL*-rearranged infant ALL. In comparison with the methylation status in remission samples, elevated methylation in *MLL*-rearranged infant ALL is leukemia-specific. In contrast to the assessment of genome-wide methylation at repetitive elements, the levels of global methylation in *MLL*-rearranged infant ALL as estimated by LUMA may to some extent be influenced by severe gene promoter methylation that we and other observed earlier.[14, 15] Therefore these results may not be indicative of the true presence of genome-wide hypomethylation at non-promoter sequences, but do confirm that *MLL*-rearranged ALL cells indeed carry hypermethylated genomes, as shown before.[14, 15]

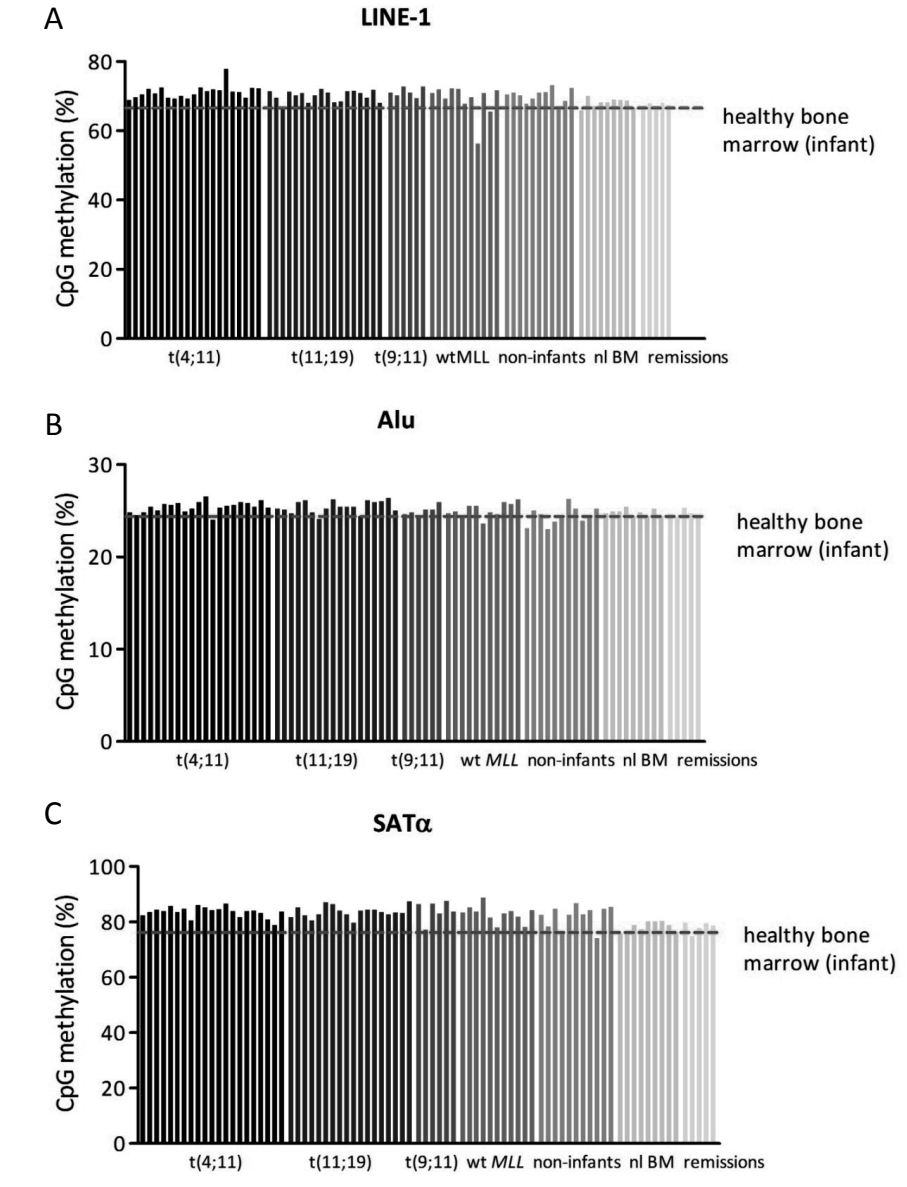


Figure 2. Mean repetitive element methylation per case.
Mean levels of CpG methylation as determined by bisulfite pyrosequencing calculated over 4 CpGs for LINE-1 **(A)** and 3 CpGs for Alu **(B)** and SAT- α **(C)**. The dashed line indicates the level of CpG methylation in healthy bone marrow obtained from an infant (< 1 year of age). "wt *MLL*" = wild-type *MLL* infant ALL and "nl BM" = healthy bone marrow.

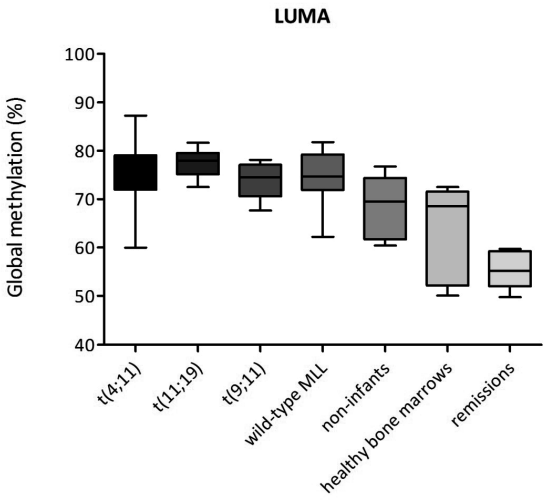


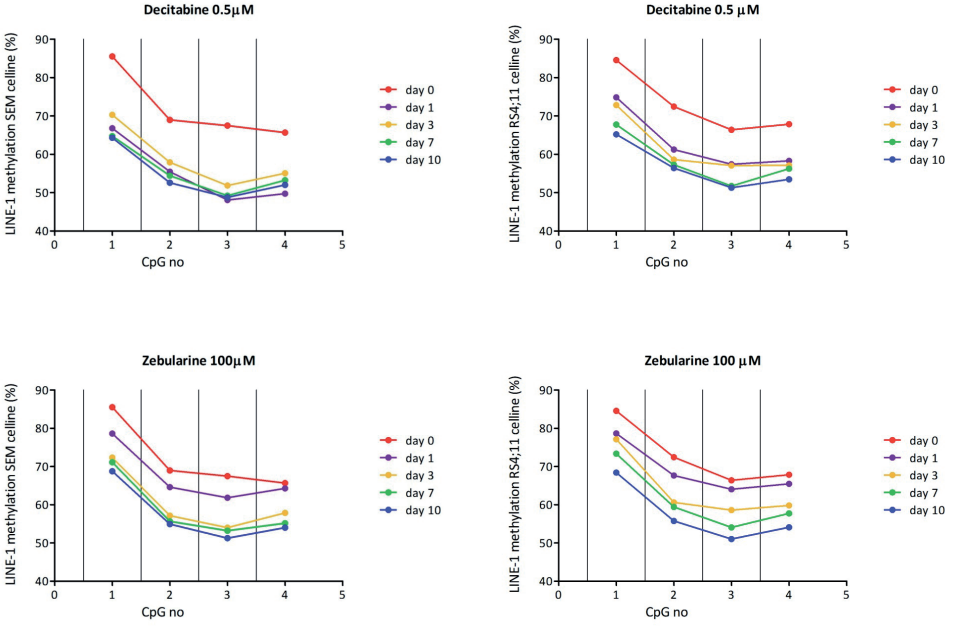
Figure 3. Global methylation as assessed by the LUminometric Methylation Assay (LUMA).
Box plots showing cytosine methylation interrogated at *Hpa*II recognition sites (including both promoter and non-promoter CpG islands) for t(4;11)-positive infant ALL patients (n=21), t(11;19)-positive infant ALL patients (n=18), t(9;11)-positive infant ALL patients (n=6), wild-type *MLL* infant ALL patients (n=11), non-infant childhood B-cell precursor ALL patients (n=11), healthy bone marrows (n=9), and remissions (n=5). The upper borders of the boxes represent the 75th percentiles, and the lower borders of the boxes represent the 25th percentiles. The horizontal lines inside the boxes represent the median methylation levels per patient group. Vertical lines outside the boxes indicate the ranges.

Response of repetitive element methylation to demethylating agents

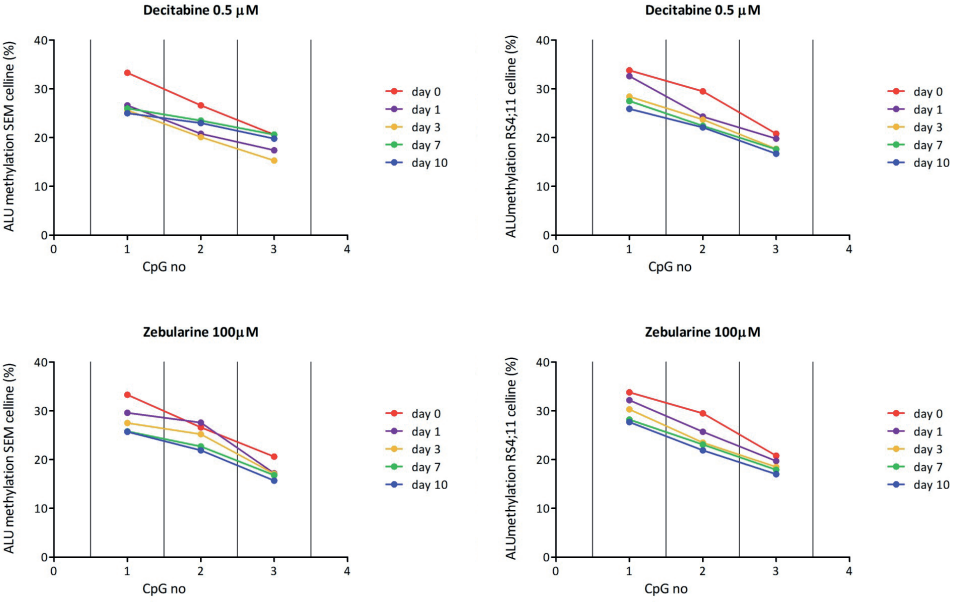
Previously, we showed that the expression of multiple genes silenced by abnormal promoter CpG hypermethylation in *MLL*-rearranged ALL cells could be restored to various extents in response to the DNA methyltransferase inhibitor zebularine.[15] Moreover, we demonstrated that *MLL*-rearranged ALL cells are particularly sensitive to this drug and suggested a possible role for demethylating agents in the treatment of *MLL*-rearranged infant ALL.[15] Similarly, others have shown that *MLL*-rearranged ALL cells are highly sensitive to decitabine.[14] To study the effects of such compounds on repetitive element methylation, we here evaluated the responsiveness of LINE-1, Alu, and SAT-α methylation to the DNA methyltransferase (DNMT) inhibitors decitabine and zebularine. For this, we used the t(4;11)-positive precursor B-ALL cell line models SEM and RS4;11 which were exposed separately to each compound for a duration of 10 days. Cell viability data are presented in **Supplemental figure 1S**.

As shown in **figure 4**, the methylation levels of all repetitive elements in these cell lines were comparable or slightly increased compared with those observed in t(4;11)-positive infant ALL patient samples. In both cell line models, repetitive element methylation was responsive to demethylation. In general, both decitabine and zebularine induced maximum demethylation at three days of exposure **(figure 4)**. Cell viability was not affected by decitabine after three days of exposure, whereas moderate induction of leukemic cell death was observed for zebularine **(Supplemental figure 1S)**. Neither compound induced massive hypomethylation at the interrogated CpGs, but rather seems to restore the methylation status of the repetitive elements in leukemic cells to the levels observed in healthy bone marrows samples.

A


Decitabine 0.5µM
LINE-1 methylation SEM celline (%)
CpG no
day 0
day 1
day 3
day 7
day 10
Decitabine 0.5 µM
LINE-1 methylation RS4;11 celline (%)
Zebularine 100µM
Zebularine 100 µM

B


Decitabine 0.5 µM
ALU methylation SEM celline (%)
ALUmethylation RS4;11 celline (%)
CpG no
day 0
day 1
day 3
day 7
day 10
Zebularine 100µM

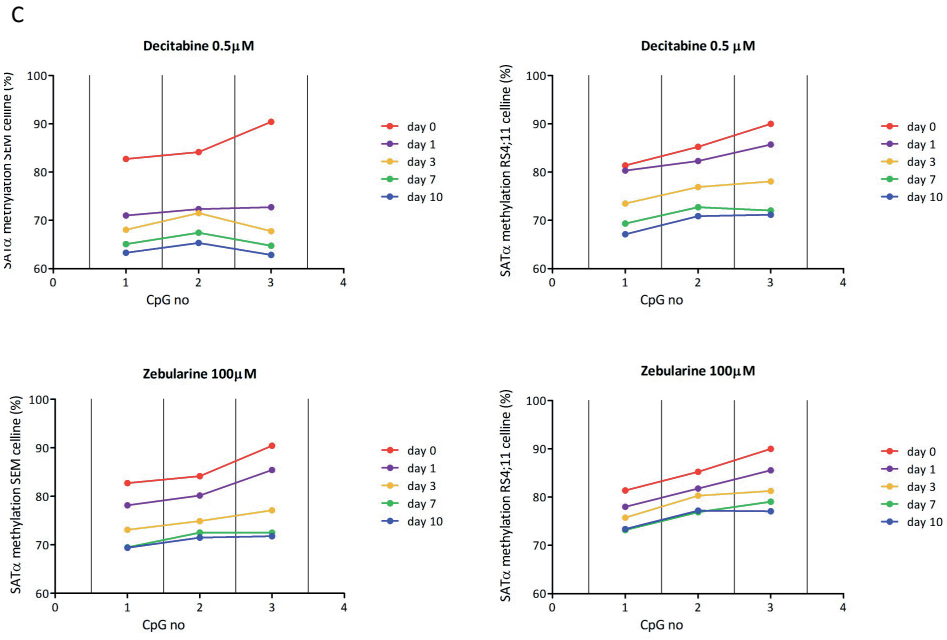


Figure 4. Demethylation of repetitive elements.
Responsiveness of repetitive element methylation to demethylation by decitabine (0.5 uM) and zebularine (100 uM) in the t(4;11)-positive cell line models SEM and RS4;11 after 0 (red line), 1 (purple line), 3 (yellow line), 7 (green line), and 10 days (blue line). **A.** LINE-1, **B.** Alu, and **C.** SAT- α.

DISCUSSION

Human cancer cells frequently display promoter CpG island hypermethylation resulting in down-regulation or silencing of associated genes.[27] Typically, increased levels of gene promoter methylation are accompanied by genome-wide hypomethylation of non-promoter CpGs leading to chromosomal instability.[17] Due to recurrent observations of this kind, of which numerous examples can be found in literature,[16-20] the cancer-specific shift of methylation to gene promoters at the expense of global methylation has become a dogmatic belief in current cancer epigenetics. In the present study, we investigated whether this phenomenon also occurs in *MLL*-rearranged infant ALL by analyzing the methylation status of the repetitive elements LINE-1, Alu and SAT-α. Methylation at these repetitive elements accounts for more than half of all cytosine methylation in the human genome[36] and has been validated to symbolize global methylation.[18, 23, 24] As an independent method of genome-wide methylation, the restriction-enzyme-based LUMA assay[24, 35] was used, which estimates methylation at *Hpa*II recognition sites. As approximately 50% of the *Hpa*II sequences are located within transposable elements and ~22% are located within gene promoter CpG islands,[37] LUMA provides additional information on the genome-wide methylation status.

In spite of previously found severe gene promoter hypermethylation,[14, 15] the here presented results show no evidence for genome-wide hypomethylation at non-promoter sequences in *MLL*-rearranged infant ALL. Instead, *MLL*-rearranged infant ALL cells appeared to be slightly more methylated at the studied repetitive elements than healthy bone marrows and cells obtained at disease remission, although the differences are subtle. Our LUMA results confirmed this observation by showing that *MLL*-rearranged infant ALL cells contained the highest levels of genome-wide DNA methylation (when both promoter and non-promoter CpG sites are analyzed). Although the lack of global hypomethylation in *MLL*-rearranged infant ALL may seem paradoxical, the literature shows that there are more exceptions to the general belief.[38, 39] Likewise, Kroeger and co-workers demonstrated accentuated methylation levels at several gene promoters in acute myeloid leukemia (AML) during relapse when compared with diagnostic material. Instead of an expected trend towards global hypomethylation (assessed by LINE-1 repetitive element methylation) they found higher levels of LINE-1 methylation in AML samples when compared with normal peripheral blood and observed slight increases in LINE-1 methylation during relapse.[38] Thus, including our present data, the mutual occurrence of both promoter and global hypermethylation may be a recurrent feature in hematological malignancies. Interestingly, the opposite has also been observed. For instance, Kaneda *et al*. found a positive correlation between promoter hypomethylation and global hypomethylation in human gastric cancers.[40] Taken together these findings demonstrate that aberrant changes in gene promoter methylation not necessarily provoke opposing changes in genome-wide or non-promoter CpG methylation.

The lack of global hypomethylation, despite substantial promoter hypermethylation, in *MLL*-rearranged infant ALL may reflect the unique development of this malignancy. Global hypomethylation is usually associated with genomic instability, allowing additionally acquired genetic hits to propel a pre-malignant cell into a fully transformed state.[41] Our findings that *MLL*-rearranged infant ALL cells remain globally methylated while numerous gene promoters become hypermethylated[15] may explain that, thus far, no copy number variations have been discovered in this type of leukemia.[42, 43] In contrast, *MLL*-rearranged leukemias seem entirely dependent on a drastically changing epigenetic landscape driven by abnormal histone modifications[44] and subsequent aberrant promoter methylation patterns.[15]

MLL-rearranged infant ALL seems to represent a highly methylated subtype of leukemia characterized by hypermethylation at specific gene promoter CpG islands, as well as globally at non-promoter sequences. We recently showed that exposure of *MLL*-rearranged ALL cells to the demethylating agent zebularine leads to re-activation of genes silenced by promoter hypermetylation.[15] Moreover, compared with other ALL subtypes, *MLL*-rearranged ALL cells appear to be highly sensitive to this drug, effectively eliminating the vast majority of leukemic cells *in vitro*.[15] Here we demonstrate that the observed methylation at several repetitive elements in *MLL*-rearranged ALL cells is also responsive to demethylation by DNMT inhibitors, such as decitabine and zebularine. Decitabine is known to induce demethylation of

repetitive elements.[45] However, for the more stable compound zebularine this has not been shown before.

MLL-rearranged infant ALL cells are characterized by an overall methylated genomic state and both promoter and non-promoter methylation respond to demethylating agents. Concerning possible drug targets, we postulate that the lack of global hypomethylation in these cells is of additional therapeutic value providing supplementary targets to tackle epigenetic deregulation. We remain confident that the implementation of demethylating therapies in current treatment protocols may be highly beneficial for *MLL*-rearranged infant ALL patients.

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Supplemental information is available in the appendices.

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PART II:
THERAPEUTIC IMPLICATIONS

“Men love to wonder, and that is the seed of science.”

(Ralph Waldo Emerson)

5

The Potential of Clofarabine in *MLL*-rearranged Infant Acute Lymphoblastic Leukemia.

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Submitted for publication

ABSTRACT

Background

MLL-rearranged acute lymphoblastic leukemia (ALL) in infants is the most difficult-to-treat type of childhood ALL displaying a chemotherapy-resistant phenotype and unique histone modifications, gene expression signatures, and DNA methylation patterns.

Despite their chemotherapy-resistant character, *MLL*-rearranged infant ALL cells respond remarkably well to nucleoside analogue drugs *in vitro*, such as cytarabine and cladribine, and to the demethylating agents decitabine and zebularine as measured by cytotoxicity assays. These observations led to the inclusion of cytarabine into the treatment regimens currently used for infants with ALL. However, survival chances for infants with *MLL*-rearranged ALL do still not exceed 30-40%.

Objective

Here we explored the potential of the novel nucleoside analogue clofarabine for the treatment of *MLL*-rearranged infant ALL.

Results

Compared with other nucleoside analogues, clofarabine effectively targeted primary *MLL*-rearranged infant ALL cells at the lowest concentrations, with median LC_{50} values of ~25 nM. Interestingly, clofarabine displayed synergistic cytotoxic effects in combination with cytarabine. Furthermore, at concentrations of 5-10 nM clofarabine induced demethylation of the promoter region of the tumor suppressor gene *FHIT*, a gene typically hypermethylated in *MLL*-rearranged ALL. Demethylation of the *FHIT* promoter region was accompanied by subtle re-expression of this gene both at the mRNA and at the protein level.

Conclusions

Taken together, we conclude that the addition of clofarabine to chemotherapy protocols for treatment of *MLL*-rearranged infant ALL patients may further improve prognosis for these very young children.

INTRODUCTION

Survival chances for children with acute lymphoblastic leukemia (ALL) have improved tremendously over the past decades.[1] Nonetheless, the prognosis for infants (<1 year of age) with ALL remains dismal.[2, 3] Infant ALL represents a highly aggressive type of leukemia characterized by chromosomal translocations involving the *MLL* gene (~80% of the cases)[4, 5] and typically presents with hepatosplenomegaly, exceedingly high leukocyte counts, and often shows central nervous system involvement.[6] Moreover, infant ALL cells are usually resistant to multiple chemotherapeutic drugs currently used in pediatric ALL treatment regimens, especially to glucocorticoids (such as prednisone and dexamethasone) and L-asparaginase.[7, 8] However, infant ALL cells have proven to be highly sensitive to the nucleoside analogue cytarabine (i.e. cytosine arabinoside, or ara-C),[7, 8] which appeared to be associated with elevated expression of the human *equilibrative nucleoside transporter 1* (h*ENT1*) on which cytosines are mainly dependent to permeate the cell membrane.[9] Based on these findings, a unique infant ALL treatment protocol (INTERFANT-99) was designed, implementing varying dosages of cytarabine throughout the treatment courses of a standard childhood ALL treatment regimen.[3] The INTERFANT-99 treatment protocol appeared successful, achieving long-term event-free survival in 47% of the infant ALL cases and realizing superior treatment results over earlier attempts exploring therapy intensification.[3]

Recently, we demonstrated that *MLL*-rearranged infant ALL is characterized by increased levels of DNA methylation at numerous gene promoters leading to suppressed expression of associated genes.[10] Furthermore, this study showed that the degree of promoter methylation is associated with the risk of disease relapse.[10] Interestingly, hypermethylated *MLL*-rearranged ALL cells appeared highly responsive to so-called demethylating agents (such as decitabine and zebularine).[10, 11] Like cytarabine, decitabine and zebularine are cytidine analogues, but in contrast to cytarabine, which was originally designed to inhibit DNA synthesis (**figure 1A**), these agents were specifically developed to inhibit DNA methylation. Demethylating cytidine analogues exert their actions by competing with normal cytosines for incorporation into the DNA. Once incorporated, these analogues are able to covalently bind, and thereby trap, DNA methyltransferases (DNMTs) during their donation of methyl groups on receiving cytidines (**figure 1A**). As a consequence, the cell becomes depleted from functional DNMTs and loses its ability to methylate the DNA during subsequent cell cycles.[12] Presumably, the sensitivity of *MLL*-rearranged ALL cells to demethylating cytosine analogues can be ascribed to the aberrant DNA methylation patterns recently found in this type of leukemia,[10, 11] but may certainly be enhanced by the elevated expression of h*ENT1* characteristically observed in infant ALL.[9] Thus, demethylating cytosine analogues embody promising candidates for the treatment of *MLL*-rearranged ALL in infants. Unfortunately, despite several clinical trials demonstrating biological activity and clinical responses for both decitabine and azacitidine in adults diagnosed with myelodysplastic syndromes (MDS) or chronic myelomonocytic leukemia (CMML),[13, 14] clinical results in general remain somewhat disappointing.[15,16]

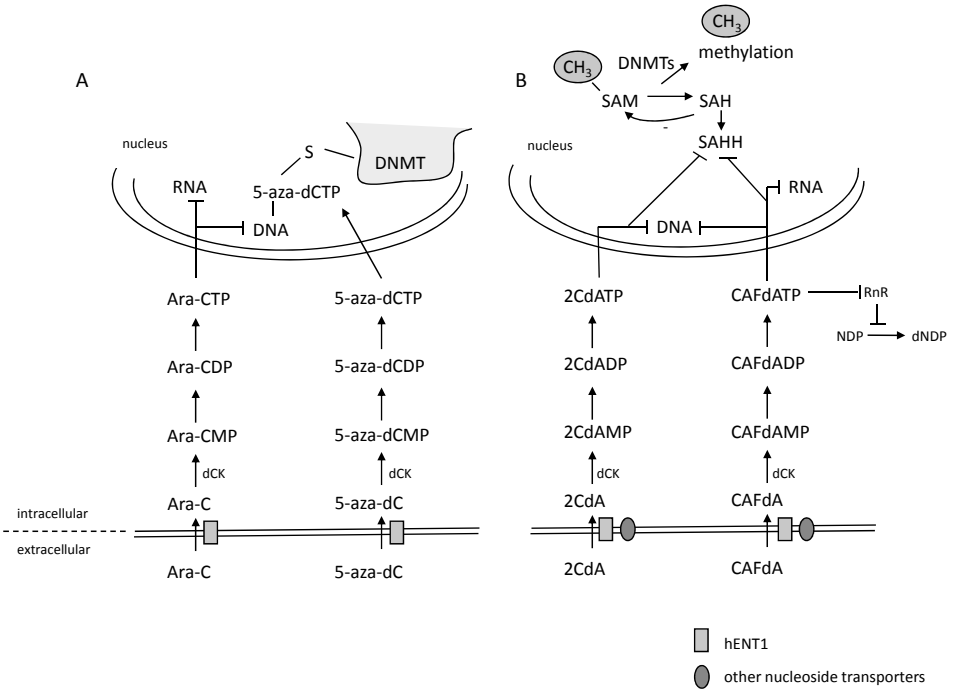

Figure 1. Mechanisms of action of the different nucleoside analogue drugs.
A. The cytosine analogues cytarabine (Ara-C), as well as the demethylating agents decitabine (5-aza-dC) and zebularine, mainly enter the cell via the human equilibrative nucleoside transporter 1 (hENT1). Inside the cell these pro-drugs are sequentially phosphorylated into active nucleoside tri-phosphates (dNTPs) which compete with normal cytidines for incorporation into the DNA (or RNA) during DNA (and RNA) synthesis. As such, cytarabine blocks RNA and DNA synthesis leading to apoptosis. In contrast, the demethylating agents decitabine and zebularine do not inhibit DNA synthesis, but covalently bind DNA methyltransferases (DNMTs) during methylation events. As a result, DNMTs become trapped onto the DNA, depleting the cell from functional DNMTs and impairing the ability of the cell to distribute methylation in subsequent cell cycles. **B.** The adenosine analogues cladribine (2CdA) and clofarabine (CAFdA), comparable to cytosine analogues, require triple phosphorylations to become effective and incorporated into the DNA. Apart from inhibition of DNA synthesis, cladribine, and presumably clofarabine as well, block DNA methylation by inhibition of S-Adenosyl Homocysteine Hydrolase (SAHH), which hydrolyses S-Adenosyl Homocysteine (SAH). The subsequent accumulation of SAH production impairs the formation of the methyl-donor S-Adenosyl Methionine (SAM) (which is used as a methyl-pool by DNMTs), and thereby inhibits DNA methylation. Finally, clofarabine also inhibits ribonucleotide reductase (RnR), blocking the formation of normal dNTPs, and as such eliminating the competition for incorporation into the DNA. Abbreviations used: Ara-C = cytarabine, 5-aza-dC = decitabine, 2CdA = cladribine, and CAFdA = clofarabine.

Apart from cytosine analogues, infant ALL cells also appeared to respond remarkably well to another nucleoside analogue, cladribine.[7, 8, 17] Although cladribine represents an adenosine analogue and as such lacks the ability to bind DNMTs, it has been reported to possess methylation-inhibiting properties via an alternative mechanism involving the inhibition of S-Adenosyl Homocysteine Hydrolase (SAHH) (**figure 1B**).[18]

As a consequence, the amount of intracellular deoxynucleoside triphosphates available for DNA replication becomes impaired which leads to apoptosis in rapidly dividing cells. Due to its resistance to inactivation by deamination or phosphorolysis, clofarabine is more stable than its predecessors.[19] In addition, clofarabine was shown to inhibit DNA methylation in lymphoma cells,[20] presumably through a mechanism comparable to that observed for cladribine (**figure 1B**). A recent study demonstrated that clofarabine also induces down-regulation of *DNA methyltransferase 1* (*DNMT1)* at the mRNA level in chronic myelogenous leukemia (CML) cells.[21]

While clofarabine has proven activity in the treatment of refractory and relapsed childhood ALL,[22 23, 24] we postulate that this agent may be particularly suitable for the treatment of infants with ALL, especially in patients carrying *MLL* translocations and hypermethylated genomes. Therefore, we here compared the cytotoxic effects of clofarabine and other nucleoside analogues on primary *MLL*-rearranged infant ALL cells, explored possible synergistic effects between clofarabine and cytarabine, and evaluated the potential of clofarabine to inhibit DNA methylation.

MATERIAL AND METHODS

Patient samples and leukemic cell isolation

In this study, primary patient samples were used from both infant (<1 year of age) (n=10) and pediatric non-infant (>1 year of age) (n=10) precursor B-ALL patients. All infant ALL cases were enrolled in the international INTERFANT-99 treatment study,[3] and all non-infant pediatric precursor B-ALL samples were derived from the Erasmus MC - Sophia Children's Hospital, Rotterdam, the Netherlands. Infant ALL samples were selected for the presence of t(4;11) which is the most common *MLL* translocation found among infant ALL patients.[3] Positivity for t(4;11) was assessed by split-signal FISH and RT-PCR analysis. Informed consent was obtained from the parents or legal guardians according to the recommendations of the Helsinki declaration and approved by the Institutional Review Board of the Erasmus University Medical Center.

Primary bone marrow or peripheral blood samples were obtained before treatment, and mononuclear cells were isolated using sucrose density-gradient centrifugation (density 1.077 g/ml; Lymphoprep, Nycomed Pharma, Oslo, Norway) within 24 hours of sampling. Cells were resuspended in RPMI 1640 medium (Invitrogen Life Technologies, Breda, the Netherlands) supplemented with 20% fetal calf serum (FCS; Integro, Zaandam, the Netherlands), 2 mM L-glutamine, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL sodium selenite (ITS media supplement; Sigma, St Louis MO, USA), 200 µg/mL gentamycin (Invitrogen Life Technologies, Breda, the Netherlands), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.125 µg/mL fungizone (Invitrogen Life Technologies, Breda, the Netherlands). When necessary, contaminating non-leukemic cells were removed using immunomagnetic beads (DynaBeads, Dynal Inc., Oslo, Norway) as previously described.[25] As a result, all leukemic samples used in this study contained more than 90% of leukemic blasts.

Leukemia cell lines

The cell lines SEM and RS4;11 were used as models for *MLL*-rearranged B-ALL. Both cell lines carry translocation t(4;11). SEM was originally derived from a 5-year-old girl at relapse[26] and RS4;11 was established from the bone marrow of a 32-year-old woman in first relapse.[27] The cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained as suspension cultures in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen Life Technologies, Breda, the Netherlands) supplemented with 10% FCS (Integro, Zaandam, the Netherlands), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.125 µg/mL fungizone (Invitrogen Life Technologies, Breda, the Netherlands) at 37°C in humidified air containing 5% CO_2.

In vitro cytotoxicity assays and nucleoside analogue exposure

The *in vitro* sensitivity of leukemia cells to decitabine (Sigma-Aldrich, Zwijndrecht, the Netherlands), zebularine (kindly provided by Dr. Victor E. Marquez, National Cancer Institute of Frederick, Frederick, Maryland, USA), cladribine (Sigma-Aldrich, Zwijndrecht, the Netherlands), cytarabine (Cytosar, Pharmacia BV, Woerden, the Netherlands) and clofarabine (Genzyme Europe, Naarden, the Netherlands) was determined by 4-day MTT-assays as described previously.[28] Briefly, leukemic cells were cultured in the absence or presence of varying concentrations of the above mentioned nucleoside analogues for four days, and drug sensitivity was expressed as the LC_{50} value (i.e. the concentration of nucleoside analogue drug lethal to 50% of the leukemic cells) or, in the case of cell lines, as the IC_{50} value (i.e. the concentration of nucleoside analogue drug inhibitory to 50% of the leukemic cells). Each experiment was performed in duplicate and carried out at least twice.

The effects of the hENT1 inhibitor NBMPR (*S*-(4-Nitrobenzyl)-6-thioinosine (NBMPR, Sigma-Aldrich, Zwijndrecht, the Netherlands) on clofarabine and cytarabine cytotoxicity were evaluated by performing standard MTT assays in the presence of 3 mM of NMBPR, both in the control cells (untreated cells) and in cells exposed to clofarabine or cytarabine.

In order to investigate whether the combination of cytarabine and clofarabine evoked either antagonistic or synergistic cytotoxic effects, cytarabine cytotoxicity (4-day MTT assays) was determined in the t(4;11)-positive cell line SEM in the absence and presence of 0.5 nM or 10 nM clofarabine. For this, cells were pre-incubated with 0.5 nM or 10 nM clofarabine prior to the actual MTT assay for cytarabine (during which clofarabine remained present throughout the entire experiment). Likewise, clofarabine cytotoxicity was determined in the absence and presence of 25 nM or 50 nM cytarabine. For all concentrations of cytarabine and clofarabine treatment, a hypothetical maximum additive effect was calculated using the following equation AxB/100, in which A and B indicate survival values with single agents. In case the actual product of cell viability of combined cytarabine/clofarabine treatment reflected the calculated value, the effects were considered additive. When the actual value was lower than the calculated value, the effect was deemed synergistic.

Isolation and purification of DNA and RNA

Genomic DNA and total cellular RNA were extracted from a minimum of $5x10^6$ cells using the DNeasy Blood and Tissue Kit and the RNeasy mini Kit (Qiagen Benelux BV, Venlo, the Netherlands) according to the manufacturer's protocol. The quality of the extracted DNA was assessed on 1.5% agarose gels, and the RNA integrity was determined using RNA 6000 Nano Assay LabChips on the Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, USA).

Pyrosequencing for quantitative CpG island methylation analysis

Genomic DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research Corporation, CA, USA) according to the manufacturer's instructions. Bisulfite treatment of the DNA converts non-methylated cytosine bases to uracil (which is replaced by thymine during subsequent PCR cycles), but does not affect 5-methylcytosine residues, allowing discrimination between methylated or unmethylated cytosines.[29] To quantitatively determine the levels of methylation at given loci, we subsequently applied pyrosequencing **(Supplemental methods)**.

Approximately 20 ng of bisulfite converted DNA was amplified using bisulfite-specific primers flanking the CpG island within the promoter of the *Fragile Histidine Triad (FHIT)* gene. For this, primers were designed using the Pyrosequencing Assay Design Software (Qiagen Pyrosequencing Inc.), and the sequences were as follows, forward primer: 5'-*GGGGAGGTAAGTTTAAGTGGAATATT*-3', (biotinylated) reverse primer: 5'-*ATCCCCACCCTAAAACCCTC*-3'. The PCR product amplified using this primer pair includes five separate CpGs within the *FHIT* promoter sequence. Amplification was performed in the presence of 4 mM of $MgCl_2$ using a touchdown PCR with the annealing temperature decreasing from 71 ºC to 64 ºC over 14 cycles of annealing for 1 minute, and subsequent denaturation for 15 seconds at 95ºC. The PCR was completed by 30 cycles of annealing at 64 ºC for 1 minute, and denaturation for 15 seconds at 95ºC. Buffers and hotstart Taq polymerase used for PCR were obtained from the Qiagen Pyromark PCR kit (Qiagen Pyrosequencing, Inc.). Amplified PCR products were initially analyzed on 2% agarose gels. Then, PCR products were denatured and antisense strands (containing biotin labels) were bound to Streptavidin Sepharose HP (Amersham Biosciences). Immobilized PCR strands coupled to the Sepharose beads were purified, washed, and denatured using a 0.2 M NaOH solution. Next, 0.3 µM of pyrosequencing primer (5'-*AAGTTTAAGTGGAATATTGT*-3') was annealed to the purified single-stranded PCR product and the sequencing reaction was performed in duplicate and analyzed on a PyroMark MD system (Qiagen Pyrosequencing, Inc.). Confirmation of complete bisulfite conversion was assessed by a cytosine/thymine control that was integrated in the *FHIT* CpG island assay. Subsequent quantification of methylation density on selected CpGs was performed using the Pyro Q-CpG software (Qiagen Pyrosequencing, Inc.). The degree of methylation in the *FHIT* CpG sequence was determined from the ratio of thymine and cytosine nucleotides and is presented as the number of methylated cytosine nucleotides divided by the amount of methylated and unmethylated cytosines x 100%.

Quantitative real-time PCR analysis

Total RNA was reverse transcribed as described previously[9] and the obtained cDNA was used to quantify mRNA expression using quantitative real-time PCR analysis as described elsewhere.[30] All oligonucleotides were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CA). Primer combinations used for transcript amplification of the *FHIT* target gene as well as the housekeeping reference gene *GAPDH* (encoding *glyceraldehyde-3-phosphate dehydrogenase*) have earlier been published.[31] PCR products were amplified using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) according to the manufacturer's recommendations, using SYBR Green as a fluorophore to detect amplified transcripts. Per experiment samples were analyzed in duplicate and all experiments were conducted twice.

Western blotting

Whole cell protein lysates containing 25 µg of protein were resolved on 10% polyacrylamide gels topped with 4% stacking gels and subsequently transferred to nitrocellulose membranes (Schleichler & Schuell, Dassel, Germany). The membranes were then probed with the following antibodies: rabbit polyclonal anti-FHIT (Millipore, Billerica, MA, USA, #07-172), or rabbit polyclonal anti-DNMT1 (New England Biolabs, Ipswich, MA, USA, #M0231S). Anti-beta-Actin mouse monoclonal antibodies (Abcam, Cambridge, USA, #ab6276) were used to detect beta-Actin and confirm equal loading in all lanes. Upon incubation (protected from light) with infrared-labeled secondary antibodies (IRDye 800CW goat-anti-rabbit antibody (#926-32211, LI-COR, Lincoln, NE) and IRDye 680 goat-anti-mouse antibody (#926-32220, LI-COR, Lincoln, NE)) in 5% milk, the membranes were washed in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (Merck Schuchardt OHG, Hohenbrunn, Germany). Finally, the membranes were scanned using an Odyssey Infrared Imaging System (LI-COR Inc., Lincoln, NE, USA), and protein expression was quantified using the Odyssey software.

Statistical analysis

Differences in LC_{50} values between *MLL*-rearranged infant precursor B- ALL and non-infant pediatric precursor B-ALL samples were evaluated using two-tailed Mann-Whitney *U* tests (performed in SPSS 17.0 statistical software) and considered statistically significant at p-values <0.05. Graphpad Prism graphical software version 5 (La Jolla, CA, USA) was used for data visualizations.

RESULTS

Infant ALL cells are highly sensitive to nucleoside analogues, including clofarabine

Although *MLL*-rearranged infant ALL cells usually are resistant to multiple chemotherapeutic drugs, these cells often are highly sensitive to nucleoside analogue

drugs including cytarabine and cladribine,[7-9] and the demethylating cytosine analogues zebularine[10] and decitabine.[11] Here we explored the potential of the new-generation nucleoside analogue clofarabine in *MLL*-rearranged infant ALL. **Figure 2** shows the chemical structures of the cytosine analogues cytarabine, decitabine and zebularine, and the adenosine analogues cladribine and clofarabine. The difference between normal cytidines and cytarabine is the inversion of 2'hydroxyl groups. Decitabine lacks the 2' hydroxyl group and carries an additional nitrogen in its base. Compared with normal adenosine, cladribine carries a chloro-group in its base. Clofarabine has an additional chloro-group and an additional fluor group present in its ribose structure.

We compared the effects of these nucleoside analogues on cell death and proliferation in the *MLL*-rearranged ALL cell lines SEM and RS4;11. As shown in **figure 3**, effective concentrations are convincingly lower for both adenosine analogues as compared with the cytosine analogues. The lowest IC_{50} values were observed for clofarabine, with inhibiting concentrations of 3.5 nM and 5 nM for RS4;11 and SEM respectively **(figure 3)**.

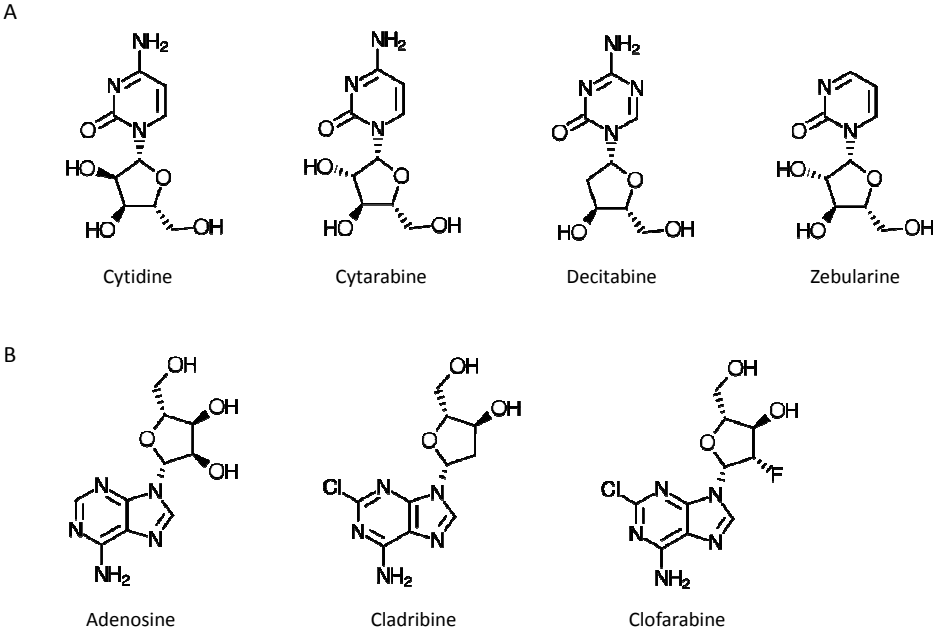

Figure 2. Chemical structures of the different nucleoside analogue drugs.
A. Shows the chemical structures of cytidine and the cytidine analogue drugs cytarabine, decitabine and zebularine. The difference between normal cytidines and cytarabine is the inversion of 2'hydroxyl groups from a *trans* position in cytidine to a *cis* configuration in cytarabine. Decitabine lacks the 2' hydroxyl group and carries an additional nitrogen in its base. Zebularine essentially shares its structure with cytarabine, but lacks the amino-group in its base. **B.** Shows the chemical structures of adenosine and the adenosine analogues cladribine and clofarabine. Compared with normal adenosine, cladribine carries a chloro-group in its base. Apart from the chloro-group, clofarabine has an additional fluor group present in its ribose structure.

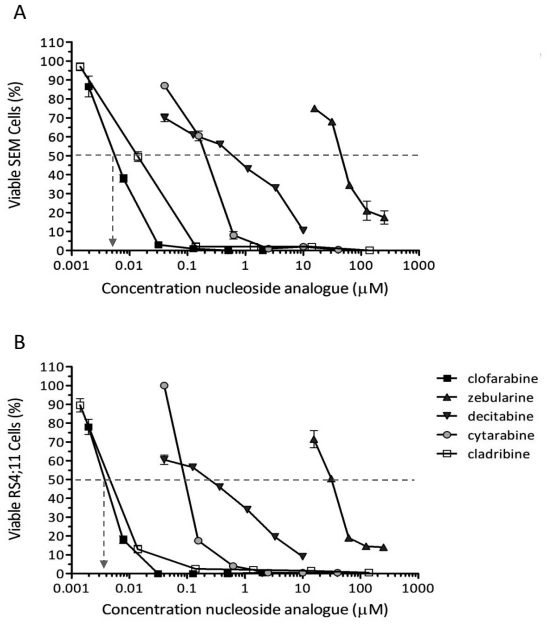

Figure 3. *In vitro* cytotoxicity of *MLL*-rearranged ALL cell lines to different nucleoside analogue drugs.
Dose-response curves showing the *in vitro* cytotoxic response (as determined by 4-day MTT assays) to the nucleoside analogue drugs clofarabine, cladribine, cytarabine, decitabine, and zebularine in the cell lines **A**. SEM, and **B**. RS4;11. Error bars represent standard errors of the mean (SEM). The IC_{50} values (concentrations inhibitory to 50% of the cells) for clofarabine are indicated by grey arrows.

Next, we compared the LC_{50} values of cytarabine, cladribine, and clofarabine in primary samples from either *MLL*-rearranged infant ALL patients (n=10), all carrying translocation t(4;11), and non-infant pediatric precursor B-ALL patients (n=10). Unfortunately, the demethylating agents zebularine and decitabine could not be tested on primary patient material, as these compounds require several cell divisions to become effective, and primary ALL cells generally stop proliferating once outside the patient's body. Although the other nucleoside analogues also require cell cycles to become incorporated into the genomic DNA, multiple studies have demonstrated that *in vitro* cytarabine and cladribine cytotoxicity can be induced in non-dividing patient cells,[9, 32] for example by inhibition of RNA synthesis.[32] As expected from earlier studies, *MLL*-rearranged infant ALL cells are significantly more sensitive to cytarabine (~2-fold difference, *p=0.001*) **(figure 4A)** and cladribine (1.4-fold difference, *p=0.005*) **(figure 4B)** compared with non-infant pediatric precursor B-ALL cells. Interestingly, *MLL*-rearranged infant ALL cells also appeared marginally (although not significantly) more sensitive to clofarabine (1.7-fold difference, *p=0.075*) **(figure 4C)**. Clofarabine effectively induced leukemic cell death in primary ALL samples at average LC_{50} values as low as 15 nM - 30 nM, while similar effects for cytarabine required ~1 mM - 2.5 mM **(figure 4)**.

Sensitivity to clofarabine is not dependent on the *hENT1* transporter

In a previous study, we investigated the mechanism underlying cytarabine sensitivity in *MLL*-rearranged infant ALL cells and found that these cells express significantly

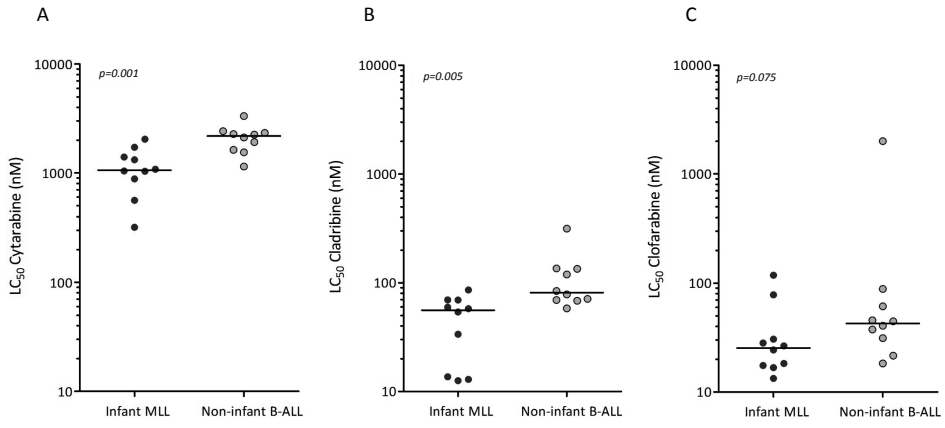

Figure 4. *In vitro* nucleoside analogue drug sensitivity of primary ALL cells.
Comparison of the *in vitro* drug response (as determined by 4-day MTT assays) in primary *MLL*-rearranged infant ALL (n=10) and non-infant pediatric precursor B-ALL (n=10) samples for the nucleoside analogue drugs **A.** cytarabine, **B.** cladribine, and **C.** clofarabine. Drug sensitivity is expressed as LC_{50} values (concentrations lethal to 50% of the cells). The lines indicate the median LC_{50} values in each patient group, and differences in drug response were statistically evaluated by Mann-Whitney U tests.

higher levels of the human *equilibrative nucleoside transporter 1* (h*ENT1*),[9] on which cytarabine is mainly dependent to permeate the cell membrane.[33] Therefore, we asked whether clofarabine sensitivity may also be dependent on h*ENT1* by measuring the effects of the hENT1 inhibitor NBMPR (*S*-(4-Nitrobenzyl)-6-thioinosine) (which significantly inhibits cytarabine influx and toxicity at 1 mM - 3 mM[33]) on clofarabine cytotoxicity. We corrected for cytotoxicity induced by NBMPR itself. As shown in **figure 5A**, 3 mM of NBMPR markedly inhibited the cytotoxic effects for cytarabine in the t(4;11)-positive ALL cell line SEM, whereas the effects on clofarabine cytotoxicity were far less pronounced **(figure 5B)**. Next, we evaluated the effects of NBMPR on cytarabine and clofarabine cytotoxicity in two t(4;11)-positive infant ALL patient samples. These experiments showed that blocking hENT1 using NBMPR reduces the cytoxic effects of cytarabine **(figures 5C-D)**, but does not affect the *in vitro* responses to clofarabine **(figures 5E-F)**.

Additive/synergistic cytotoxic effects for clofarabine and cytarabine in *MLL*-rearranged ALL

The first observation that infant ALL cells are highly sensitive to cytarabine[7] led to the successful implementation of cytarabine courses in the infant ALL treatment protocol INTERFANT-99.[3] However, with a new nucleoside analogue such as clofarabine already available for clinical testing and capable of targeting leukemic cells at nanomolar concentrations, important questions should be asked: Should cytarabine be replaced by clofarabine, or would *MLL*-rearranged infant ALL patients further benefit from

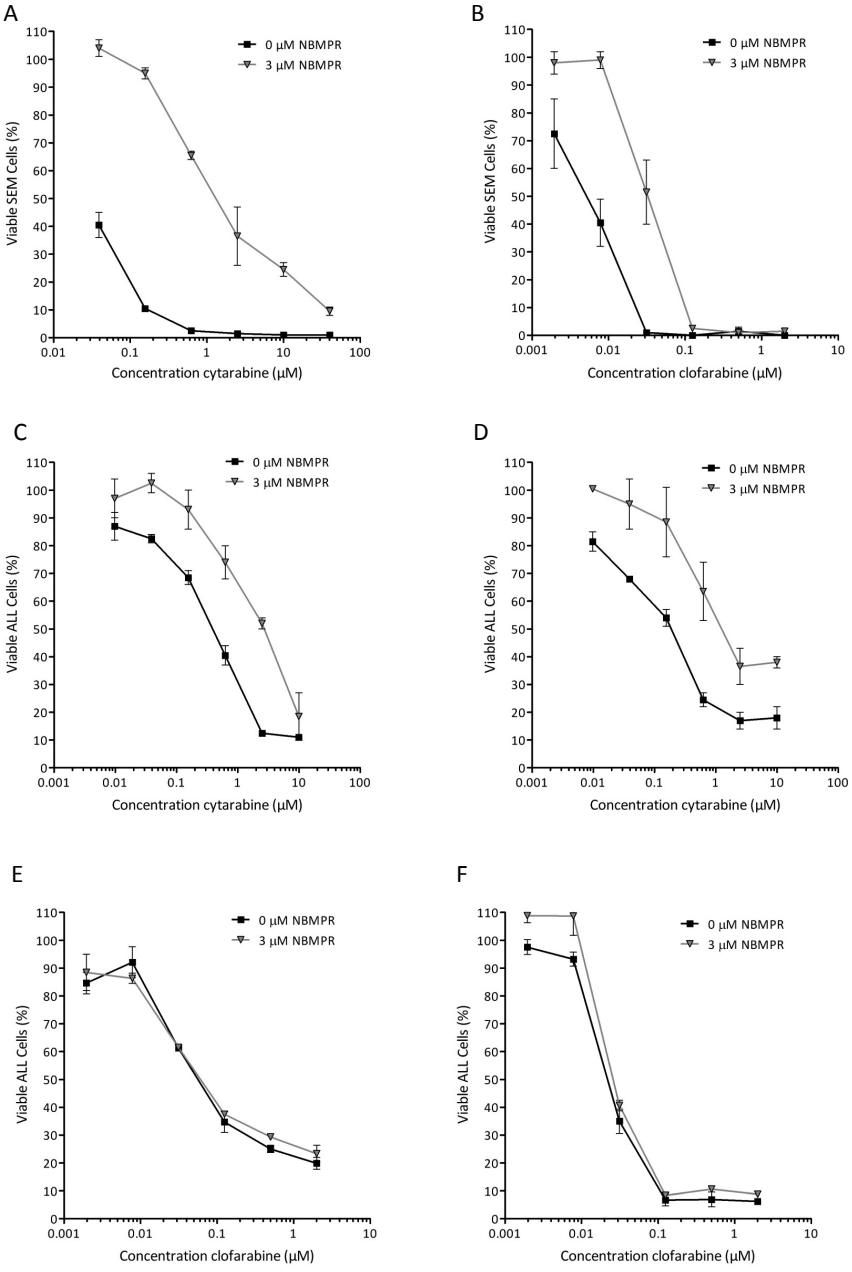

Figure 5. Effects of NBMPR on cytarabine and clofarabine cytotoxicity in *MLL*-rearranged ALL cells. The *MLL*-rearranged ALL cell line SEM was pre-incubated for 1 hour with or without 3 mM of the pharmacological hENT1-inhibitor NBMPR prior to determining **A.** cytarabine, and **B.** clofarabine cytotoxicity using 4-day MTT assays. (NBMPR remained present throughout the experiment). Similarly, **C-D.** cytarabine and **E-F.** clofarabine cytotoxicity was determined in two primary t(4;11)-positive infant ALL patient samples in the absence and presence of NBMPR (3 mM).

the addition of clofarabine to existing treatment protocols? Interestingly, synergy between clofarabine and cytarabine has been described both *in vitro* and *in vivo*.[34, 35]

To determine whether these nucleoside analogues also work synergistically in *MLL*-rearranged ALL, we tested cytarabine cytotoxicity in the presence of 5 nM or 10 nM of clofarabine, as well as clofarabine cytotoxicity in the presence of 25 nM or 50 nM of cytarabine in the t(4;11)-positive cell line SEM. As shown in **figures 6A** and **6B**, the cytotoxic effects of both drugs are markedly larger in each others' presence, especially at low dosages. However, true synergy exists when the cytotoxic effects of both agents tested simultaneously exceed the combined effect of both agents tested individually. As shown in **figure 6C**, strong synergistic effects were observed at 5 nM and 10 nM of clofarabine in combination with 50 nM of cytarabine, but only in case cells were pre-incubated with cytarabine (also see: material and methods). Four days of culturing SEM cells with a combination of 50 nM cytarabine/ 5 nM clofarabine and 50 nM cytarabine/ 10 nM clofarabine resulted in viable cell counts of ~33% and ~25% respectively. The calculated product derived from the effects of both agents when tested separately predicted viable cell counts for the combination of both drugs of ~80% and ~55%, respectively. However, the actual combinations of 50 nM cytarabine/ 5 nM clofarabine and 50 nM cytarabine/ 10 nM clofarabine resulted in viable cell counts of ~33% and ~25%, respectively. Yet, when the same combinations of clofarabine and cytarabine were tested in which clofarabine was added first (1 hour pre-incubation), the synergistic effects were either absent or only marginally detectable **(figure 6C)**.

Inhibition of DNA methylation by clofarabine

Clofarabine is thought to inhibit DNA methylation through either inhibition of S-Adenosyl Homocysteine Hydrolase (SAHH) as shown for cladribine,[18] or suppression of *DNA methyltransferase 1* (*DNMT1)* expression.[21] The demethylating cytosine analogues zebularine and decitabine inhibit DNA methylation by replacing normal cytosines in the genomic DNA where they covalently bind and trap DNMTs, thereby impairing the ability of the cell to methylate CpGs in consecutive cell cycles.[12]

Depletion of cellular DNMT is therefore commonly used as a reliable read-out for demethylation.[36] Depletion of functional DNMT1 usually is already observed after 24 hours of cell exposure to 0.5 mM of decitabine or 100 mM of zebularine **(figures 7A and 7B)**. In contrast, the DNMT1 binding capacity of both agents appeared to be largely diminished at nanomolar concentrations **(figures 7A and 7B)**. As clofarabine appeared effective at 5 nM - 10 nM, we assessed its ability to deplete DNMT1 at these concentrations. As shown in **figure 7C**, DNMT1 protein expression seems very moderately reduced after 7-10 days, but certainly not to the extent that inhibition of methylation as a result of DNMT1 depletion may be expected.

Although direct DNMT1 depletion by clofarabine appears an unlikely mechanism by which this agent inhibits DNA methylation, clofarabine-induced demethylation remains possible through alternative actions. Therefore, we further investigated demethylation as a measure of re-expression of the *FHIT* gene, known to be silenced

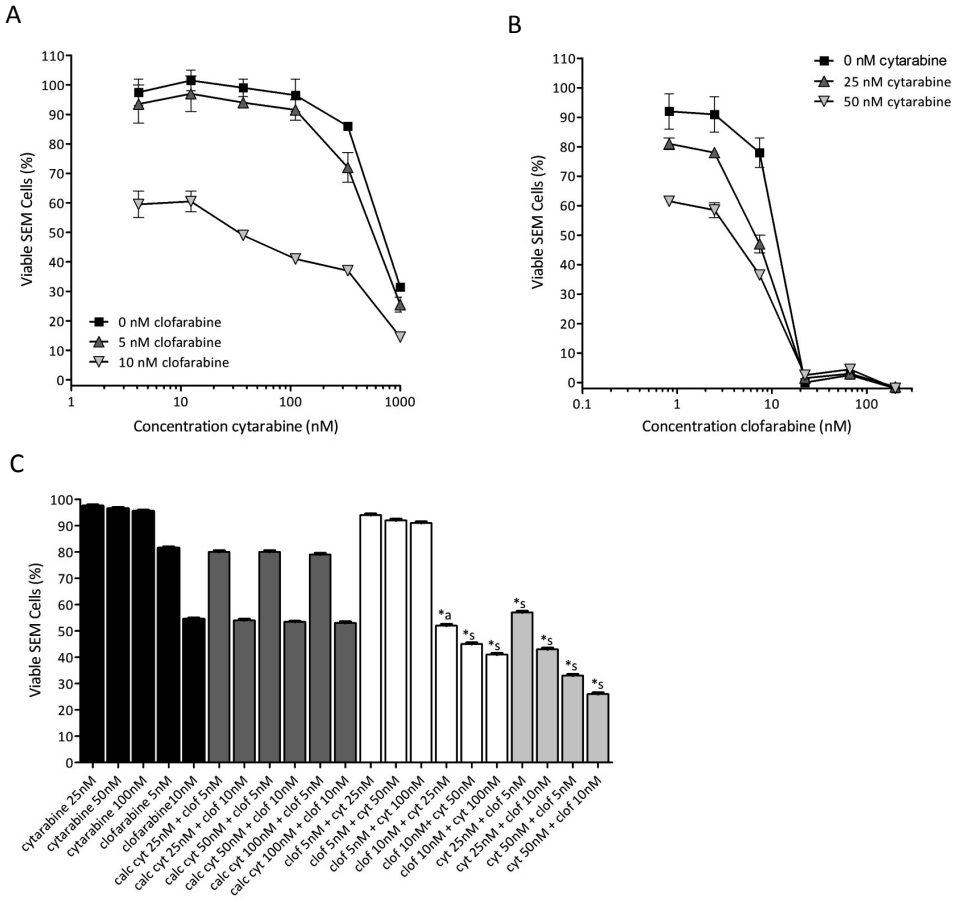

Figure 6. Evaluation of synergy between cytarabine and clofarabine cytotoxicity in the cell line SEM. **A.** The *MLL*-rearranged ALL cell line SEM was pre-incubated for 1 hour in the absence or presence of either 5 nM or 10 nM clofarabine before initiating 4-day MTT assays to determine cytarabine cytotoxicity. (clofarabine remained present throughout the entire experiment). **B.** Likewise, SEM cells were pre-incubated for 1 hour in the absence or presence of either 25 nM or 50 nM cytarabine before 4-day MTT assays were performed using low concentrations of clofarabine. (cytarabine remained present throughout the entire experiment). **C**. Shows the leukemic cell viability after 4-day exposures to cytarabine and/or clofarabine. Black bars indicate SEM cell viabilities in response to cytarabine or clofarabine alone at indicated concentrations. Dark grey bars represent the hypothetical additive effects of the cytarabine and clofarabine combined as predicted from the actual exposures of these agents individually (see: black bars). White and light grey bars show the cell viabilities of the actual combined exposures of cytarabine and clofrabine tested simultaneously. Cytarabine is abbreviated as “cyt”, and clofarabine as “clof”. Calculated is abbreviated as “calc”. Additive effects are indicated by: *a, and synergistic effects are marked by: *s.

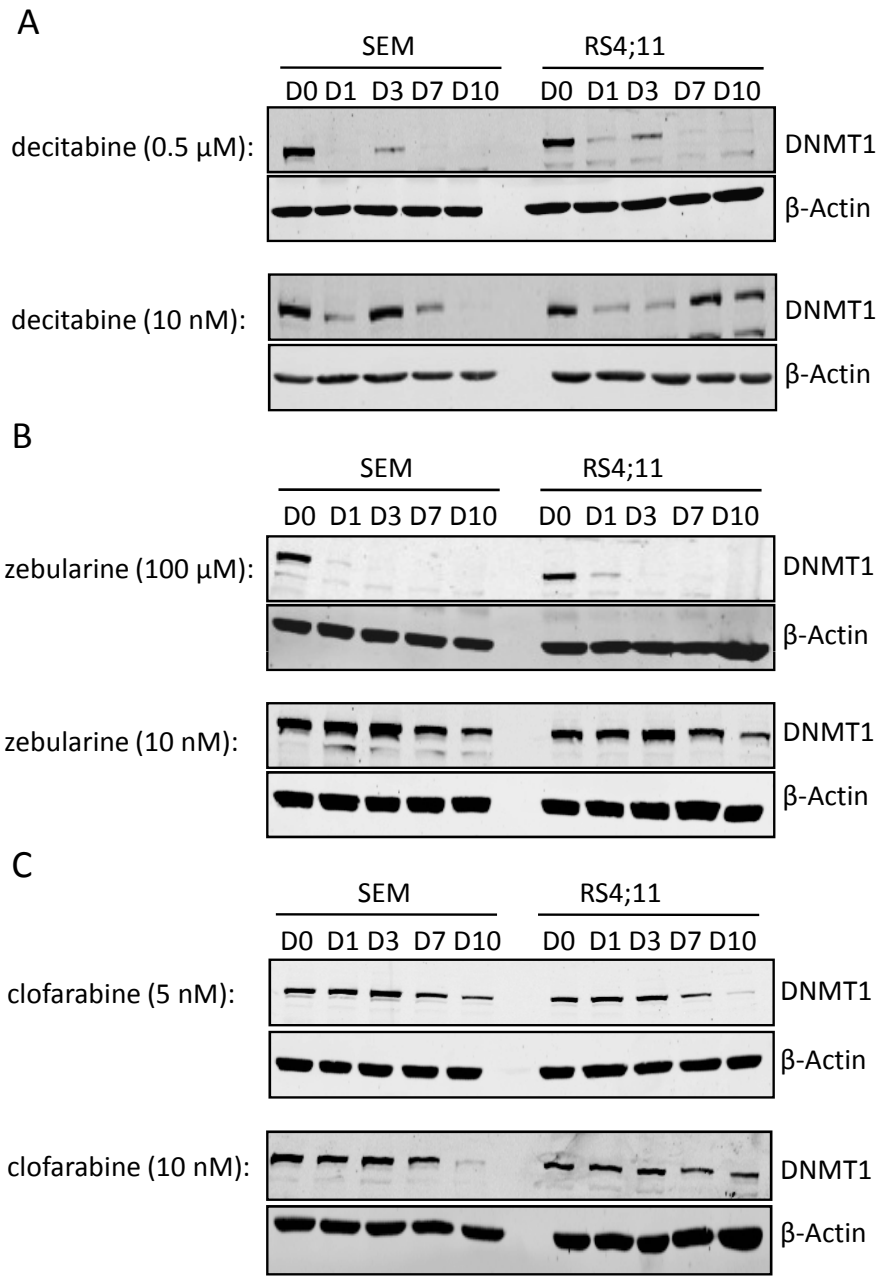

Figure 7. Cellular DNMT1 depletion induced by nucleoside analogues.
Western blot analysis demonstrating protein expression of DNMT1 in the cell lines SEM and RS4;11 in the absence or presence of the demethylating cytidine analogues **A.** decitabine and **B.** zebularine, and for the adenosine analogue **C.** clofarabine at indicated concentrations and progressing exposure times (expressed in days). Beta-actin was used as a control for equal loading.

by promoter methylation in *MLL*-rearranged ALL cells,[31] in response to clofarabine exposure. Using a quantitative bisulfite pyrosequencing assay specifically designed for the *FHIT* promoter CpG island, we quantitatively analyzed the level of *FHIT* promoter CpG methylation in SEM cells in the absence and presence of decitabine (0.5 μM), zebularine (100 μM) and clofarabine (5 nM or 10 nM). As expected, both the demethylating agents decitabine and zebularine induced pronounced demethylation of 3 out of the 5 interrogated CpG loci within the *FHIT* promoter at concentrations of 0.5 μM and 100 μM respectively **(figure 8A)**. At a concentration of 10 nM neither decitabine nor zebularine appeared capable of demethylating the *FHIT* promoter, although a modest and temporarily response was observed for 10 nM of decitabine after 24 hours of exposure **(figure 8A)**. Interestingly, 5 nM or 10 nM of clofarabine induced demethylation of the same CpGs to a comparable extent as 0.5 μM decitabine and 100 μM zebularine, demonstrating that clofafrabine indeed is capable of CpG demethylation at nanomolar concentrations **(figure 8A)**.

Next, we investigated whether demethylation of these *FHIT* promoter CpGs as induced by decitabine, zebularine and clofarabine also led to the re-activation of mRNA and protein expression. As shown in **figure 8B**, SEM cells exposed to 0.5 μM decitabine or 100 μM zebularine showed 3- to 4-fold increases in *FHIT* mRNA expression at day 7. Clofarabine (5 nM or 10 nM) showed a >2-fold increase in *FHIT* expression at the mRNA level **(figure 8B)**. At the protein level, FHIT was most convincingly re-expressed in response to 0.5 μM decitabine and 100 μM zebularine. At nanomolar concentrations these agents did not show re-activation of FHIT protein expression. In contrast, 5 nM or 10 nM of clofarabine induced subtle but detectable re-expression of the FHIT protein **(figure 8C)**.

DISCUSSION

While the pathophysiology underlying *MLL*-rearranged infant ALL is slowly being unraveled, it remains the genetic subtype of ALL with the worst clinical outcome. We here showed that clofarabine, a next generation adenosine analogue, is capable of demethylating aberrant gene promoter methylation in *MLL*-rearranged infant ALL cells. This confirms earlier observations by Zhang *et al*. who demonstrated that clofarabine displays demethylating properties in lymphoma cells.[22] Inhibition of aberrant DNA methylation may become very important in the treatment of *MLL*-rearranged infant ALL, as we recently showed that the majority of these patients display abnormal genome-wide DNA methylation patterns.[10] While the clinical effectiveness of the more conventional demethylating cytosine analogues such as decitabine and zebularine is not fully convincing,[15,16] clofarabine may provide an alternative. Especially since we show here that primary *MLL*-rearranged infant ALL cells are highly sensitive to clofarabine *in vitro*, evoking cell death in 50% of the leukemic cells at concentrations as low as ~25 nM. At the same time, our present study showed that 5 nM - 10 nM of clofarabine seems sufficient to trigger gene promoter demethylation and re-expression

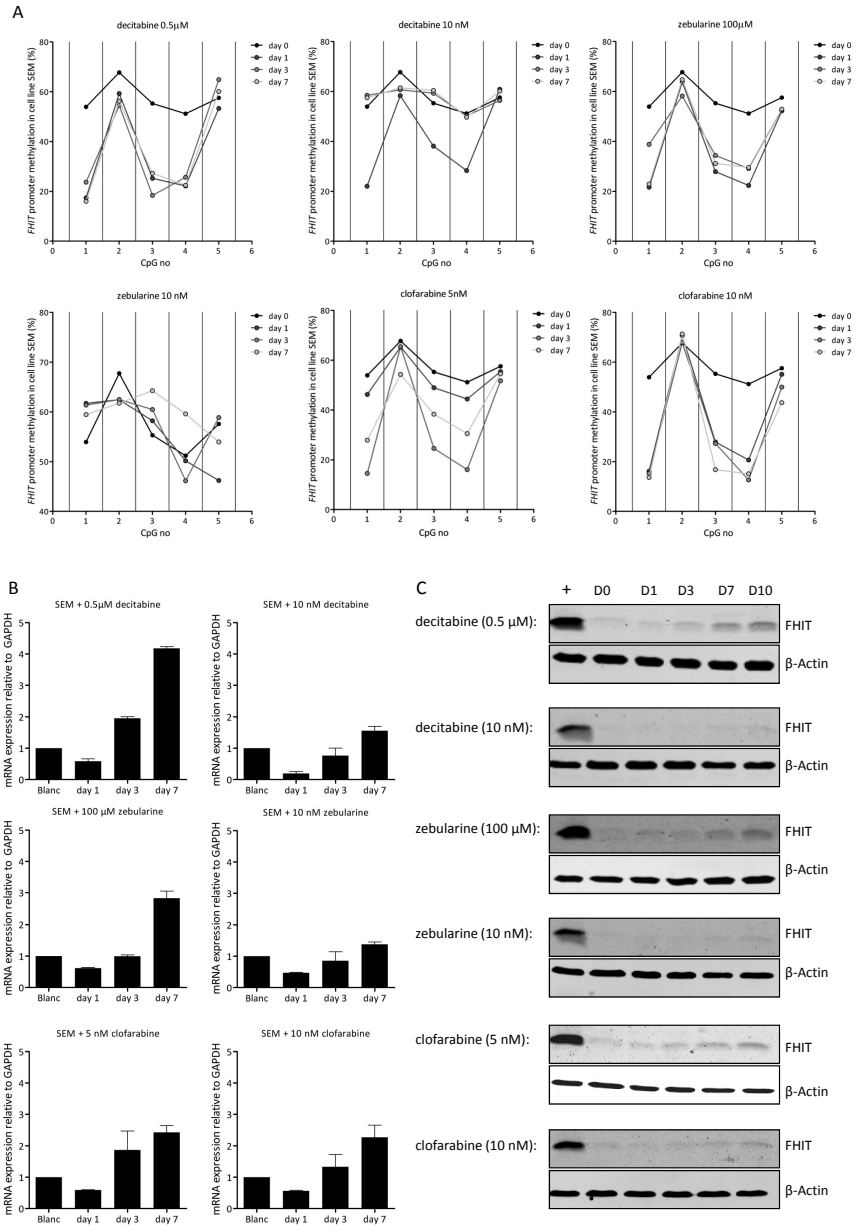

Figure 8. Demethylation and re-expression of the *FHIT* gene and protein by nucleoside analogues. **A.** Sensitivity of five individual CpGs within the *FHIT* gene promoter to demethylation by indicated nucleoside analogues at depicted concentrations as determined in the *MLL*-rearranged ALL cell line SEM. The progression of CpG demethylation was assessed at day 0 (untreated; black lines), at day 3 (dark gray lines), and day 7 (light gray lines). **B.** Quantitative Real-time PCR analysis showing the relative mRNA expression levels of the *FHIT* gene in the cell line SEM before and after exposure to the different nucleoside analogues decitabine, zebularine and clofarabine at indicated concentrations and time points. **C.** FHIT protein expression levels in the *MLL*-rearranged ALL cell line SEM in the absence or presence of decitabine, zebularine, or clofarabine at indicated concentrations and exposure periods. Beta-actin was used as a control for equal loading.

of the affected gene. Although the demethylating effects of clofarabine appear modest in comparison with the demethylating agents decitabine and zebularine, it must be noted that the performed experiments involved a single dose of each agent. In order to be clinically effective, demethylating agents generally require the administration of multiple doses over relatively long periods of time. Therefore, the demethylating properties of clofarabine may well be more pronounced at repeated or continued administrations. Nonetheless, whether demethylation by clofarabine would actually contribute to the remarkably good cytotoxic response as here observed in primary *MLL*-rearranged infant ALL cells *in vitro* remains to be confirmed. Yet, the sensitivity of this aggressive type of leukemia to clofarabine is promising and encouraging in terms of clinical implementation of this drug in *MLL*-rearranged infant ALL treatment regimes. In fact, a recent study using clofarabine in heavily pretreated pediatric ALL patients included five *MLL*-rearranged infant ALL cases of which three patients achieved complete remission on clofarabine treatment.[23]

Another important finding in our study is the observation that clofarabine and cytarabine have a synergistic cytotoxic effect on *MLL*-rearranged ALL cells. These data are in concordance with previously published data on myeloid leukemia cells,[34] although the concentrations used in our study were significantly lower. From this point of view, addition of clofarabine to cytarabine-based treatment protocols may seem a highly attractive treatment option, especially since cytarabine has been successfully included in the treatment of infant ALL.[3] Addition of clofarabine, effectively targeting *MLL*-rearranged ALL cells as a single agent at nanomolar concentrations, may not only be very beneficial, it may also allow the decrease of cytarabine dosages without losing its required cytotoxic effects. In a clinical setting, the combined use of clofarabine and low-dose cytarabine has already proven effective in adult and pediatric leukemias without *MLL* translocations.[35, 37]

In conclusion, clofarabine has significant cytotoxic effects on *MLL*-rearranged ALL cells from infants. In addition, it has demethylating effects on these cells. Pre-incubation and co-incubation with cytarabine induced synergistic cytotoxicity with clofarabine. Based upon these data, we propose a clinical study using clofarabine in *MLL*-rearranged infant ALL.

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Supplemental information is available in the appendices.

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“The cause is hidden; the effect is visible to all.”

(Publius Ovidius Naso)

6

Connectivity Mapping Identifies HDAC Inhibitors for the Treatment of t(4;11)-positive Infant Acute Lymphoblastic Leukemia.

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Leukemia 2012*; 26(4): 682-92*

ABSTRACT

Background

MLL-rearranged infant acute lymphoblastic leukemia (ALL) is an aggressive subtype of childhood leukemia characterized by a unique gene expression profile. We uncovered that the activation of particular (proto-onco)genes is mediated by promoter hypomethylation.

Objective and methods

In search for therapeutic agents capable of targeting these potential cancer-promoting genes, we applied connectivity mapping on the gene expression signature based on the genes most significantly hypomethylated in t(4;11)-positive infant ALL as compared with healthy bone marrows.

Results

This analysis revealed histone deacetylase (HDAC) inhibitors as suitable candidates to reverse the unfavorable gene signature. We show that HDAC inhibitors effectively induce leukemic cell death in t(4;11)-positive primary infant ALL cells, accompanied by down-regulation of *MYC*, *SET*, *RUNX1*, *RAN* as well as the *MLL-AF4* fusion product. Furthermore, DNA methylation was restored after HDAC inhibitor exposure.

Conclusions

Our data underline the essential role for epigenetic deregulation in *MLL*-rearranged ALL. Furthermore, we show for the first time that connectivity mapping can indirectly be applied on DNA methylation patterns, providing a rationale for HDAC inhibition in t(4;11)-positive leukemias. Given the presented potential of HDAC inhibitors to target important proto-oncogenes, including the leukemia-specific MLL fusion *in vitro*, these agents should urgently be tested in *in vivo* models and subsequent clinical trials.

INTRODUCTION

To date, *MLL*-rearranged infant acute lymphoblastic leukemia (ALL) remains the most aggressive type of childhood leukemia characterized by a high rate of early relapses and a grim prognosis.[1,2] This type of leukemia arises from balanced chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL)* gene[3] that appear to develop *in utero.*[4] Hence, *MLL*-rearranged ALL is typically diagnosed in infants at or shortly after birth. The most common *MLL* translocation among infant ALL patients, occurring in about 50% of the cases, is t(4;11) generating the fusion product *MLL-AF4*[1,5] as well as, in most instances, the reciprocal fusion product *AF4-MLL.*[6]

Given the importance of the *MLL* gene in regulating transcription during definitive hematopoiesis,[7,8] interruptions of *MLL* lead to abnormal gene expression patterns that presumably favor leukemia development.[9,10] These unique gene expression profiles are to some extent mediated by leukemia-specific histone modifications, such as histone 3 lysine 79 dimethylation (H3K79me2), established via recruitment of DOT1L by MLL fusion partners.[11] In addition, we recently demonstrated that t(4;11)-positive infant ALL is further characterized by the presence of distinct genome-wide DNA methylation patterns, displaying hypermethylation at multiple gene promoters leading to transcriptional silencing of the associated genes.[12] In the present study, we revisited our DNA methylation profiles and found that, besides vast amounts of hypermethylated genes, a number of genes appeared to be inaccurately hypomethylated. As similar analyses in healthy bone marrow samples revealed that these genes normally remain methylated and suppressed during hematopoiesis, hypomethylation of these genes in t(4;11)-positive infant ALL seems to represent leukemia-specific deregulation of transcription. Moreover, these genes also appeared methylated in pediatric ALL (both infants and older children) lacking translocations of the *MLL* gene, at levels nearly comparable to normal bone marrow samples, suggesting that hypomethylation of these genes is t(4;11)-specific.

Given this specificity and the fact that multiple hypomethylated genes represented several renowned proto-oncogenes, including several MLL-AF4 target genes,[13,14] we asked whether targeting these genes would have therapeutic potential. In search for compounds capable of targeting various hypomethylated genes at once, we here applied connectivity mapping[15] on a gene expression signature corresponding to the genes most significantly hypomethylated and consequently transcriptionally activated in t(4;11)-positive infant ALL. These analyses and additional experiments showed that histone deacetylase (HDAC) inhibitors effectively target t(4;11)-positive ALL cells, mediated or accompanied by down-regulation of several proto-oncogenes, including the MLL-AF4 fusion product itself.

MATERIAL AND METHODS

Patient samples

We studied 15 newly diagnosed t(4;11)-positive infant ALL patients (<1 year of age) enrolled in the INTERFANT-99 study **(Supplemental table 1S)**.[1] Whole normal bone marrow samples

(n=7) derived from non-leukemic children were used as controls throughout the study. In some instances, pediatric ALL samples (both infants and non-infants) were included as controls to demonstrate t(4;11)-specificity of the hypomethylation. Leukemic cell isolation and enrichment, as well as DNA and RNA extractions were performed as described before[16] without any alterations. Approval for this study and the use of patient material was obtained from the Erasmus MC Institutional Review Board, and informed consent was acquired from parents or legal guardians according to the Declaration of Helsinki.

t(4;11)-positive ALL cell line models

The t(4;11)-positive precursor B-ALL cell lines SEM and RS4;11 were purchased from DSMZ (Braunschweig, Germany). Both cell lines were maintained as suspension cultures in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS) (Integro, Zaandam, the Netherlands), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.125 µg/ml fungizone (Invitrogen) at 37°C in humidified air containing 5% CO_2.

HDAC inhibitor exposures and MTT assays

HDAC inhibitor exposure experiments were performed by culturing the cell lines SEM and RS4;11 in the presence of 1 µM of Trichostatin A (TSA, Sigma-Aldrich, Zwijndrecht, the Netherlands), 10 µM of Vorinostat (SAHA, Sigma-Aldrich, Zwijndrecht, the Netherlands), 500 nM of Panobinostat (LBH589, Novartis Oncology, Cambridge, MA), 10 mM of Valproic Acid (VPA, Sigma-Aldrich, Zwijndrecht, the Netherlands), 10 ng/ml of Romidepsin (FK228, Celgene Corporation, San Diego, CA) and 10 µM of MS-275 (Sigma-Aldrich, Zwijndrecht, the Netherlands). Cells were sampled after 6, 24 and 48 hours of exposure and cell viability was assessed using the trypan blue dye exclusion method. All drug exposures were repeated twice. *In vitro* HDAC inhibitor cytotoxicity was assessed by four-day MTT assays as described earlier.[17] In each cytotoxicity experiment samples were analyzed in duplicate.

Differential Methylation Hybridization using CpG island microarrays

Methylation-sensitive restriction enzyme-based Differential Methylation Hybridization (DMH) was performed using 500 ng genomic DNA and genome-wide CpG island microarrays (Agilent Technologies, Santa Clara, CA, USA) as previously described.[12] Detailed procedures are described in the **Supplemental methods**. The genome-wide DNA methylation data has been deposited in the NCBI Gene Expression Omnibus[18] under GEO Series accession number GSE18400 as part of our recently published paper on DNA methylation patterns in *MLL*-rearranged infant ALL.[12]

Gene expression profiling using Affymetrix GeneChips

Gene expression profiles were generated for t(4;11)-positive infant ALL cases (n=15) and healthy pediatric bone marrow samples (n=7), using the same samples for which DNA methylation profiles were produced. The exact methodology is described elsewhere.[10] Briefly, high-quality RNA was reverse transcribed using T7-linked oligo-dT primers, and the

obtained cDNA was used as a template to synthesize biotinylated cRNA. Labeled cRNA was then fragmented and hybridized to HU133plus2.0 GeneChips (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines. Gene expression values were calculated using Affymetrix Microarray Analysis Suite® (MAS) 5.0.2 software. The gene expression data was deposited in the NCBI Gene Expression Omnibus[18] under GEO Series accession number GSE 19475 as part of a large gene expression profiling study in infant ALL.[10]

Connectivity map analysis for compound searching

The Connectivity Map (cmap) represents a large collection of gene expression profiles generated in various human cancer cell lines exposed to a broad spectrum of FDA-approved compounds[15,19] (*http://www.broadinstitute.org/cmap/*). Using a gene set enrichment metric according to the Kolmogorov-Smirnov statistic, cmap analysis provides a ranked order of individual treatment instances based on their similarity to a given gene expression profile (or query signature). In the present study, the query signature represents a list of genes that appeared consistently highly expressed as a result of promoter hypomethylation in our selected cohort of t(4;11)-positive infant ALL samples (n=15). The output of the cmap analysis consists of a list of small-molecule compounds with an assigned gene enrichment metric: the connectivity score. This relative score represents the correlation between the query signature and one of the gene expression profiles of one of the cell lines treated with one of the compounds (as compared with untreated controls). For each cell line separately treated with each compound, connectivity scores are generated, which comprise both an up-score and a down-score. The down-score (a value between -1 and 1) determines the *in silico* potential of the associated compound to reverse the query signature under interrogation. Thus, in the present study, a high negative down-score indicates that the corresponding compound is likely to induce down-regulation of multiple genes in our query signature. Based on the highest negative down-scores, candidate agents were selected for further study.

Quantitative real-time PCR analysis

Total RNA was reverse transcribed and the obtained cDNA was used to quantify mRNA expression by quantitative real-time PCR analysis using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) as previously described.[20] Primer combinations used for transcript amplification of selected target genes as well as the housekeeping reference gene *beta-2-Microglobulin* (*B2M)* are listed in **Supplemental table 2S**. Per experiment samples were analyzed in duplicate and all experiments were conducted twice.

Western blot analysis

Western blot analysis for MYC, RUNX1, RAN and SET was performed as described elsewhere[16] using a Mini-Trans-blot system (Bio-Rad Life Science Group, Veenendaal, the Netherlands) and the following antibodies: mouse monoclonal anti-c-MYC (Merck, Darmstadt, Germany, #OP30), rabbit polyclonal anti-RUNX1 (Cell Signaling Technology

Inc., Danvers, MA, #4334), rabbit polyclonal anti-RAN (Cell Signaling Technology Inc., Danvers, MA, #4462). Mouse monoclonal anti-SET was kindly provided by Professor Kyosuke Nagata (Tsukuba, Japan). Blots were re-probed with an anti-Actin mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO, #A2547) to confirm equal loading in each lane. For the detection of the MLL-AF4 fusion protein, western blot conditions were slightly different than described above. Given the predicted size of the MLL-AF4 protein of ~270 kDa, medium sized 5% polyacrylamide gels were required. Proteins were resolved at 60-80 Volt for at least 10-12 hours at room temperature. For effective transfer of large proteins to nitrocellulose membranes, the blotting procedure was performed overnight at 4ºC. Blots were then probed with mouse monoclonal anti- MLL^N/HRX (clone N4.4) (Upstate Biotechnology, Temecula, CA, #05-764) and the MLL-AF4 protein was visualized using standard procedures.[16] The membrane was re-probed with anti-Clathrin HC (clone TD.1) (Santa Cruz Biotechnology, Middlesex UK #sc-12734) as a loading control. All Western Blot analyses were performed at least twice.

Statistical analysis

Data processing and analysis of microarray data were performed as previously described.[10,12,21] A p-value of <0.01, corrected for multiple testing by the false discovery rate (FDR) step-up procedure of Benjamini & Hochberg,[22] was regarded significant. All analyses were performed in R using Bioconductor packages (R Development Core Team, 2007). Heatmaps were generated in GenePattern version 3.1.1.[23]

Differences in proto-oncogene mRNA expression and differences in mean cytotoxic response were evaluated using the Mann-Whitney *U* test. Analyses were two-tailed, and differences were considered statistically significant at p<0.01.

Risk of relapse was computed with the Kaplan Meier estimator. Time to relapse was defined as the time from diagnosis until the date of leukemia relapse or the last follow-up. The log-rank test was used to compare outcomes between different patient groups. SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for computation of survival statistics.

RESULTS

High expression of hypomethylated proto-oncogenes in t(4;11)-positive infant ALL

Recently, we showed that t(4;11)-positive infant ALL is characterized by severe promoter hypermethylation,[12] which has been independently confirmed by others.[24] However, in our genome-wide DNA methylation analysis the promoters of a subset of protein-coding genes appeared to be significantly hypomethylated in t(4;11)-positive infant ALL (n=15) as compared with normal pediatric bone marrows (n=7). Among the most significantly hypomethylated genes (top 200, FDR<0.01), 36 were consistently highly expressed in t(4;11)-positive infant ALL samples, but not in healthy pediatric

bone marrows, as determined by gene expression profiling **(figures 1A and B) (Supplemental table 3S)**. Moreover, hypomethylation of these genes appeared to be specific for t(4;11)-positive infant ALL when compared with wild-type *MLL* infant ALL cases (n=10) and non-*MLL* childhood B-ALL cases (n=10). **(figure 1C)**. In conformity with this observation, the t(4;11)-positive ALL cell lines SEM and RS4;11 both uniformly showed hypomethylation and high-level expression for the entire gene list

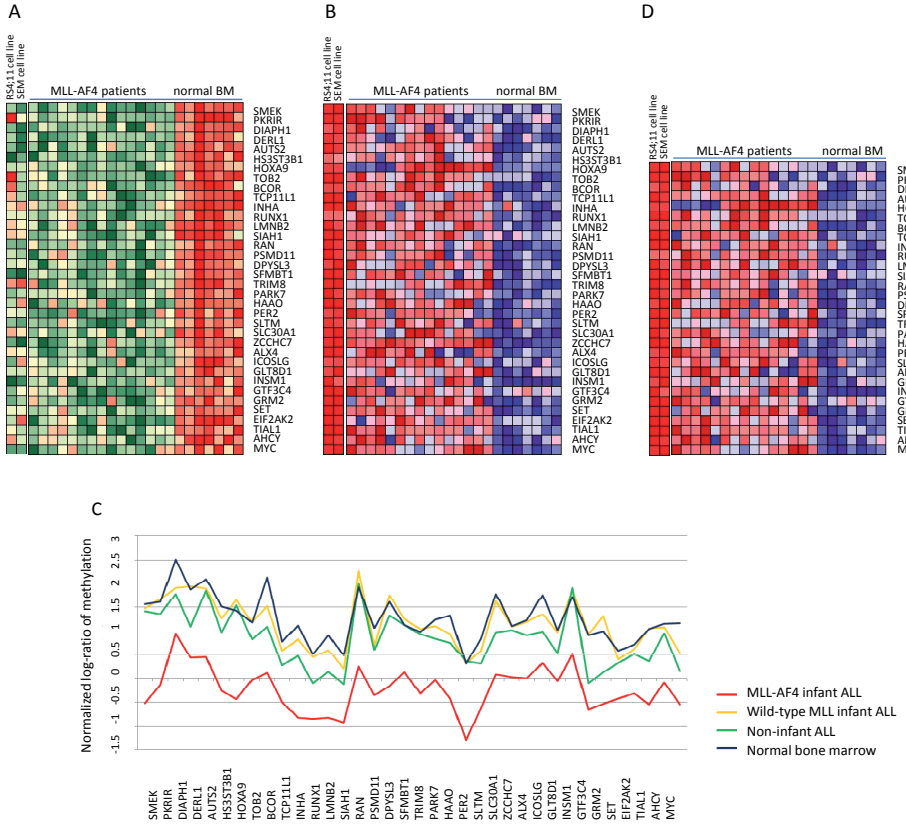


Figure 1. Heatmap visualization of gene hypomethylation and expression in t(4;11)-positive infant ALL.
A. Heatmap displaying the 36 genes most significantly hypomethylated in t(4;11)-positive infant ALL (n=15) as compared with normal bone marrows (n=7). Columns represent patient samples and rows represent genes. Relative DNA methylation levels are shown in red (high) and green (low). **B.** Heatmap showing the gene expression profiles (Affymetrix HGU133plus2.0 GeneChips) for the same patients and the same genes for which DNA methylation profiles were presented in **figure A**. Relative gene expression values are shown in red (high) and blue (low). **C.** Graphical representation of the of 36 selected hypomethylated CpG loci in primary t(4;11)-positive infant ALL samples (n=15) along with the log-ratios of wild-type *MLL* infant ALL samples (n=10) and non-*MLL* childhood ALL samples (n=10) relative to normal pediatric bone marrow samples (n=7). CpG methylation data are presented as normalized log-ratios of patient signal divided by the common reference signal. **D.** Gene expression profiles after elimination of 6 probe sets not present on Affymetrix HGU133A microarrays, based on which the connectivity map was established.

defined in t(4;11)-positive infant ALL patient samples **(figures 1A and 1B)**. Using the DAVID gene ontology database,[25] we found this gene list to be enriched for proto-oncogenes (p=0.009) and genes commonly involved in chromosomal translocations in leukemia (p=0.001) (DAVID enrichment score: 1.84) **(Supplemental figure 1S)**.

Focussing on the genes described in literature before as potential oncogenes in various malignancies, i.e. *MYC*, *HOXA9*, *SET*, *RUNX1*, *RAN*, *PARK7*, *DIAPH1* and *SFMBT1*, quantitative real-time PCR analysis was used to confirm high-level expression. The expression of all genes was significantly (p<0.01) higher in t(4;11)-positive infant ALL samples (n=15) than in pediatric whole normal bone marrow samples (n=7) **(figure 2)**. Moreover, these genes were also down-regulated in healthy $CD19^+$ B cells, indicating that epigenetic silencing of these proto-oncogenes is not only common throughout the normal hematopoietic system in general, but also applies to B-cell development in particular. One exception was the *Scm-like with four mbt domains 1* gene (*SFMBT1)* that appeared to be highly expressed in normal $CD19^+$ B cells despite down-regulation in whole bone marrow samples **(figure 2)**. Therefore, *SFMBT1* was excluded from further analyses.

Connectivity map analysis on a t(4;11)-positive hypomethylation-based gene expression profile

Effectively treating *MLL*-rearranged infant ALL remains one of the major challenges in pediatric hemato-oncology.[26] Therefore, we postulated that the identified list of hypomethylated and highly expressed genes **(figure 1)**, including several known proto-oncogenes, may represent potential targets for therapeutic intervention. As currently no straight-forward approaches have been identified to target hypomethylated genes, we set out to indirectly apply connectivity mapping[15,19,27] on hypomethylated genes originally identified by high-throughput DNA methylation profiling **(figure 1)**. The gene expression signatures available in the connectivity map (cmap) are based on the earlier Affymetrix HGU133A platform, whereas our gene expression data was generated on extended HGU133plus2.0 microarrays, which contain considerably more probe sets. Therefore, it was necessary to eliminate six genes from our original signature due to absence of corresponding probe sets on HGU133A microarrays. Using the remaining expression profile **(figure 1D)** as a query signature, cmap analysis was performed. Surprisingly, cmap analysis predicted that the compounds potentially suitable for the purpose of reversing (or down-regulating) this hypomethylation-based gene signature, predominantly involved histone deacetylase (HDAC) inhibitors, including repetitive entries for Trichostatin A (TSA), Vorinostat (or SAHA), Valproic Acid (VPA), and MS-275 **(Supplemental table 4S, Supplemental figure 2S)**.

HDAC inhibitors effectively target t(4;11)-positive ALL cells

As with most high-throughput analyses using vast data sets, connectivity mapping should at best be considered a hypothesis-generating tool, which requires additional validation. Therefore, we assessed *in vitro* cytotoxicity in t(4;11)-positive ALL cells induced by these compounds using four-day MTT assays. For this, six different HDAC

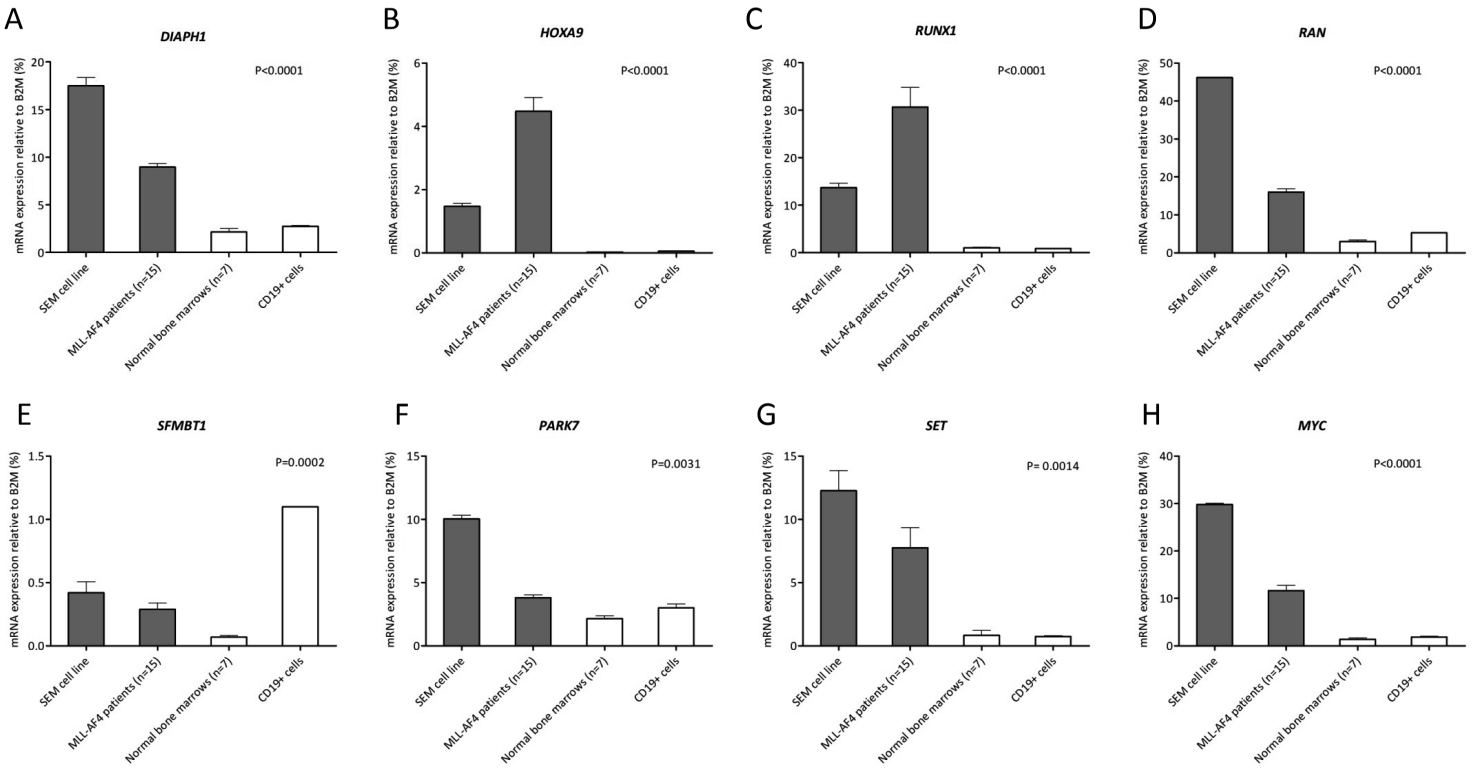


Figure 2. Relative mRNA expression of hypomethylated proto-oncogenes in t(4;11)-positive infant ALL.
Real-time PCR analysis showing the mRNA expression levels of the hypomethylated proto-oncogenes **A.** *DIAPH1*, **B.** *HOXA9*, **C.** *RUNX1*, **D.** *RAN*, **E.** *SFMBT1*, **F.** *PARK7*, **G.** *SET* and **H.** *MYC* relative to the housekeeping gene *B2M*. For each gene, relative expression levels were determined in the t(4;11)-positive cell line SEM, primary t(4;11)-positive infant ALL patient samples (n=15), normal bone marrow samples (n=7) and $CD19^+$ B cells. The p-values correspond to Mann-Whitney *U* tests comparing the expression levels in t(4;11)-positive infant ALL with the levels observed in normal bone marrow samples. Error bars represent standard errors of the mean (SEM).

inhibitors were selected, including the compounds proposed by cmap analysis: TSA, SAHA, VPA, and MS-275, and two additional HDAC inhibitors Panobinostat (LBH589)[28] and Romidepsin (FK228).[29] All HDAC inhibitors were able to eliminate the vast majority of primary t(4;11)-positive infant ALL cells, as well as t(4;11)-positive ALL cell lines **(figure 3)**. Primary cells from precursor B-ALL patients without *MLL* translocations (n=6) were generally less responsive to these agents, especially in case of TSA, LBH589 and FK228. In most instances, normal bone marrow samples (n=7) were largely unaffected (in terms of cytotoxicity) or at least demonstrated markedly reduced responsiveness to these drugs **(figure 3)**.

HDAC inhibitors repress activated proto-oncogene expression in t(4;11)-positive ALL cells

Although HDAC inhibitors effectively induced leukemic cell death in t(4;11)-positive ALL, the question remains whether these effects are indeed mediated or accompanied by down-regulation of the genes highly expressed and characterized by promoter hypomethylation. To study this, we exposed the t(4;11)-positive ALL cell lines SEM and RS4;11 to various concentrations of TSA, SAHA, VPA, MS-275, FK228 and LBH589 for 6, 24 or 48 hours. During these exposures, cell viability was monitored by trypan blue exclusion **(Supplemental figure 3S)**, and mRNA expression was determined using quantitative RT-PCR. Already after 6 hours of exposure, at which point no cytotoxicity was yet observed in neither cell line model, *RAN*, *SET*, and *MYC* mRNA expression was notably down-regulated to levels comparable with normal bone marrow samples **(figure 4)**. After 24 hours of exposure *RUNX1* was also substantially down-regulated in both SEM and RS4;11, whereas *HOXA9* and *PARK7* mRNA expression remained largely unaffected, or was even increased in response to certain HDAC inhibitors. Remarkably, *DIAPH1* was severely down-regulated by all HDAC inhibitors in SEM but not in RS4;11 cells **(figure 4)**.

Based on its high specificity in targeting t(4;11)-positive ALL cells **(figure 3)** and effective down-regulation of multiple proto-oncogenes at the mRNA level **(figure 4)**, the HDAC inhibitor LBH589 was selected for further evaluation. Western blot analysis on SEM and RS4;11 cells showed that both MYC and RUNX1 protein expression was substantially down-regulated by LBH589 within 24 hours of exposure **(figure 5A)**. In contrast, the RAN and SET protein expression levels were hardly affected.

High expression of proto-oncogenes increases risk of relapse

Next, in order to further investigate the clinical relevance of high-level expression of the selected proto-oncogenes, we computed survival statistics based on the relative expression obtained from quantitative RT-PCR analysis in a larger group of t(4;11)-positive infant ALL patients (n=28). The median expression levels were used as cut-off values to divide patients into groups characterized by either high or low proto-oncogene expression. Here we focused on the genes most responsive to HDAC inhibition at the mRNA level, i.e. *RAN*, *RUNX1*, *SET* and *MYC*. Elevated expression of each gene separately

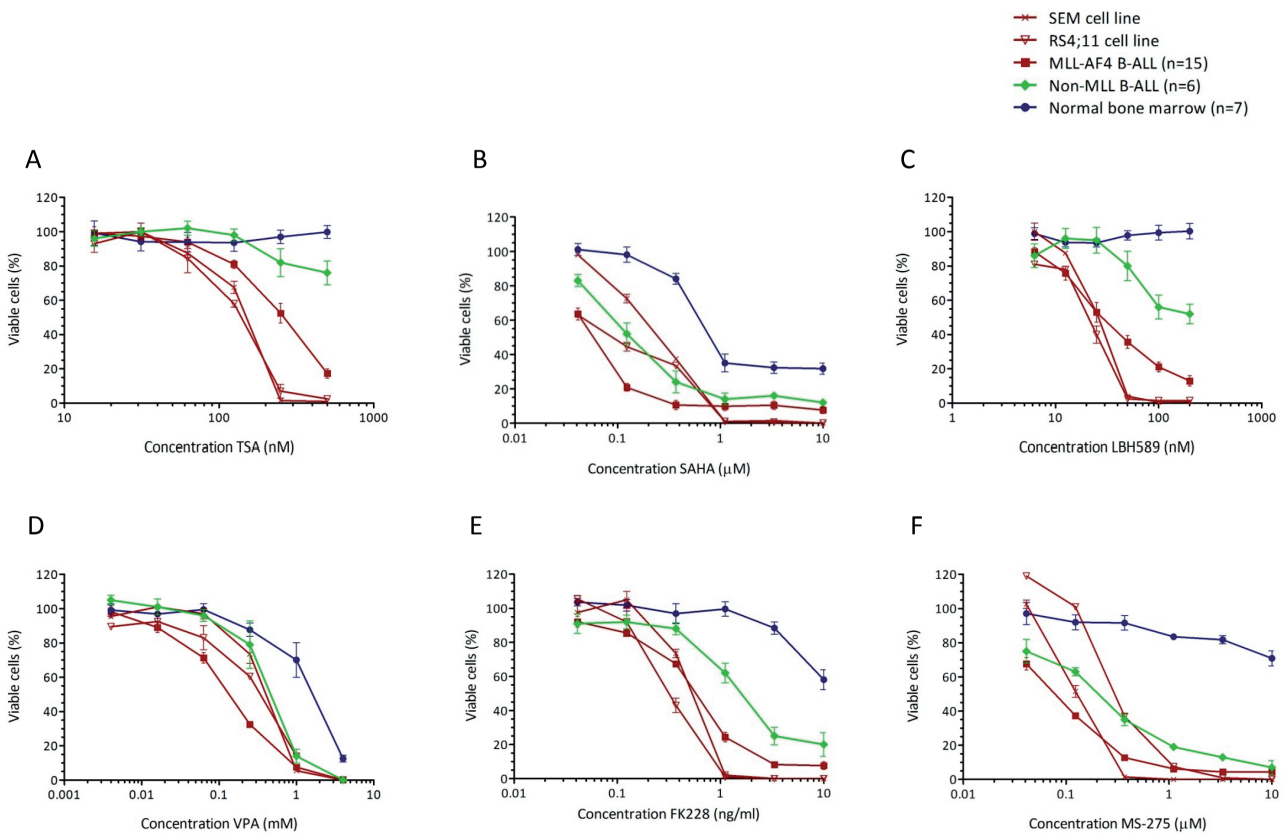
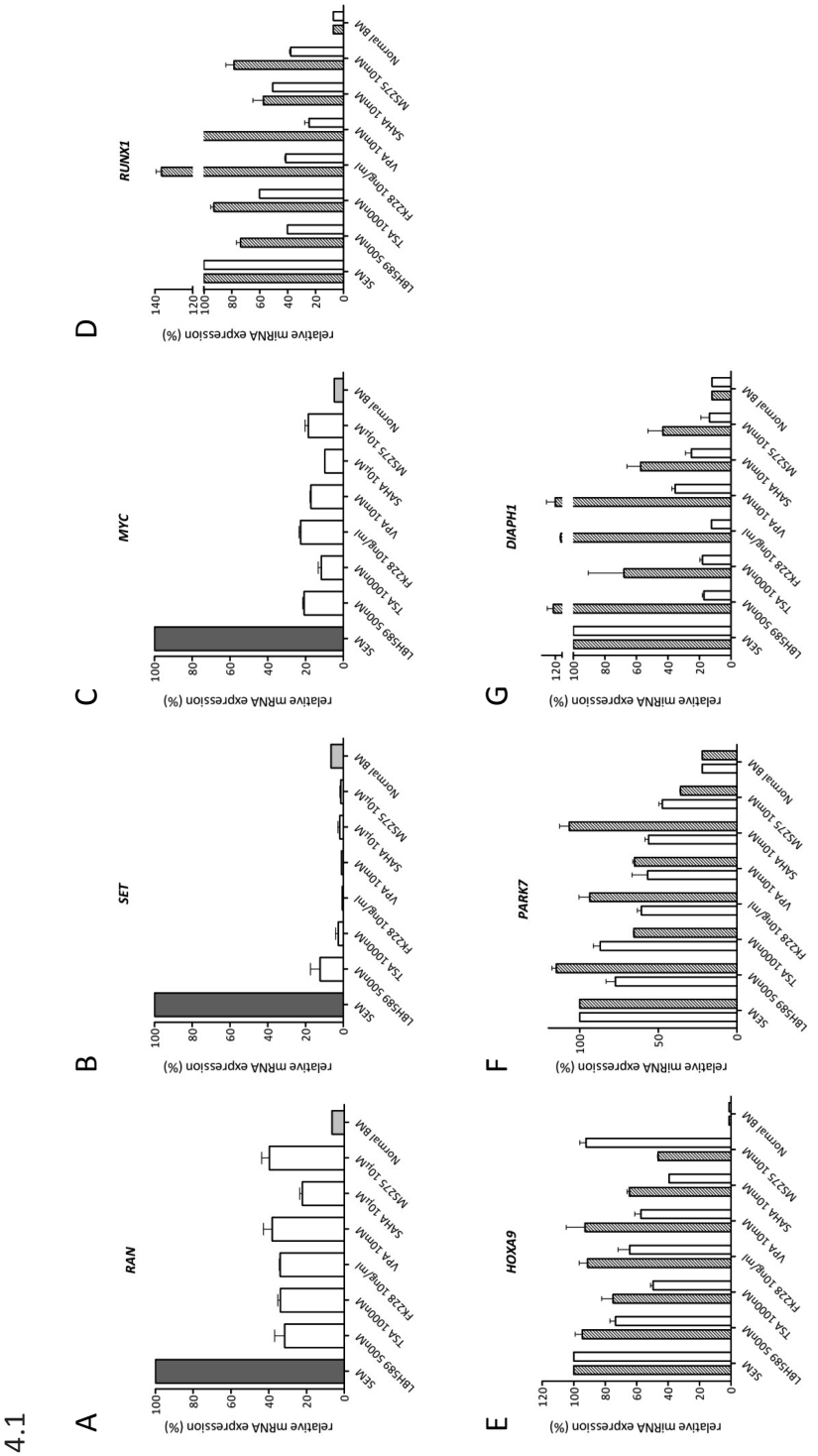


Figure 3. ***In vitro*** **cytotoxicity of t(4;11)-positive ALL cells to different HDAC inhibitors.**
Dose-response curves showing the mean *in vitro* cytotoxic response to HDAC inhibitors in the cell line SEM, the cell line RS4;11, t(4;11)-positive infant ALL patients (n=15), non-*MLL* childhood ALL patients (n=6) and normal bone marrows (n=7) **A.** TSA, **B.** SAHA, **C.** LBH589, **D.** VPA, **E.** FK228, **F.** MS-275. Error bars represent standard errors of the mean (SEM). Differences in mean cytotoxicity between patient cells and normal bone marrow cells were statistically analyzed using the Mann-Whitney *U* test and differences were considered statistically significant at $p<0.01$.

4.1


A
RAN
relative mRNA expression (%)
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C
MYC
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E
HOXA9
relative miRNA expression (%)
F
PARK7
relative miRNA expression (%)
G
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MS275 10mM
Normal BM

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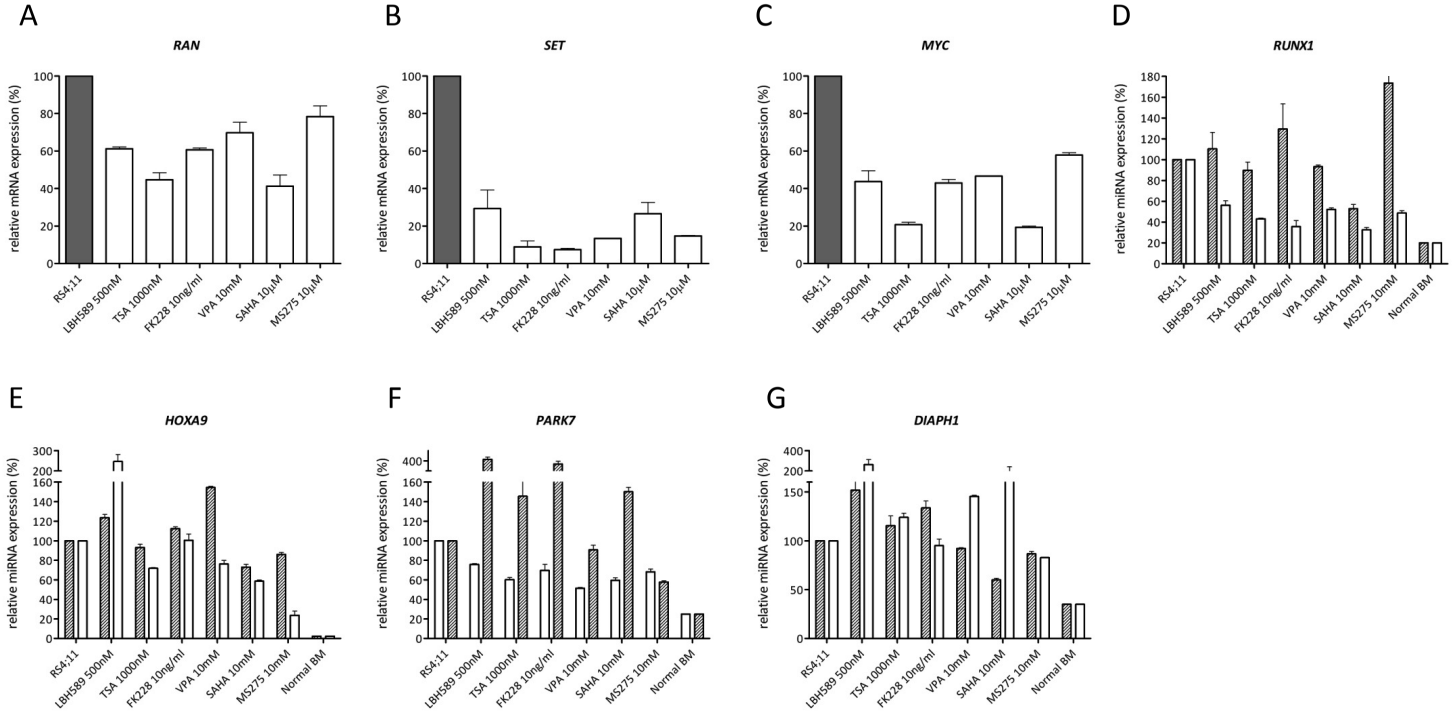

Figure 4. Relative proto-oncogene mRNA expression after exposure to HDAC inhibitors
mRNA expression levels of the proto-oncogenes **A.** *RAN*, **B.** *SET*, **C.** *MYC*, **D.** *RUNX1*, **E.** *HOXA9*, **F.** *PARK7* and **G.** *DIAPH1* relative to the housekeeping gene *B2M*. mRNA expression levels were determined in the t(4;11)-positive cell lines SEM and RS4;11 exposed to different concentrations of the six HDAC inhibitors TSA (1 µM), SAHA (10 µM), LBH589 (500 nM), VPA (10 mM), FK228 (10 ng/ml) and MS-275 (10 µM) for 6 hours. For *RUNX1*, *PARK7*, *DIAPH1* and *HOXA9* data after 24 hours exposure are presented as well (hatch fill). Expression levels in SEM or RS4;11 were set to 100%. **4-1.** the SEM cell line, **4-2.** the RS4;11 cell line.

barely had any influence on the risk of relapse **(Supplemental figures 4SA-D)**. However, patients who showed high expression (above the median value from RT-PCR) for 3 or 4 of the proto-oncogenes (n=7) had a significantly increased relapse risk **(figure 5B)**. Consequently, simultaneous down-regulation of 3 or more of these proto-oncogenes, as achieved by for instance the HDAC inhibitors TSA, SAHA and LBH589, may well be beneficial for patients otherwise at an extremely high risk of disease relapse. However, due to the low number of patients that could be included in these analyses, these observations must be interpreted with caution or at best be considered preliminary.

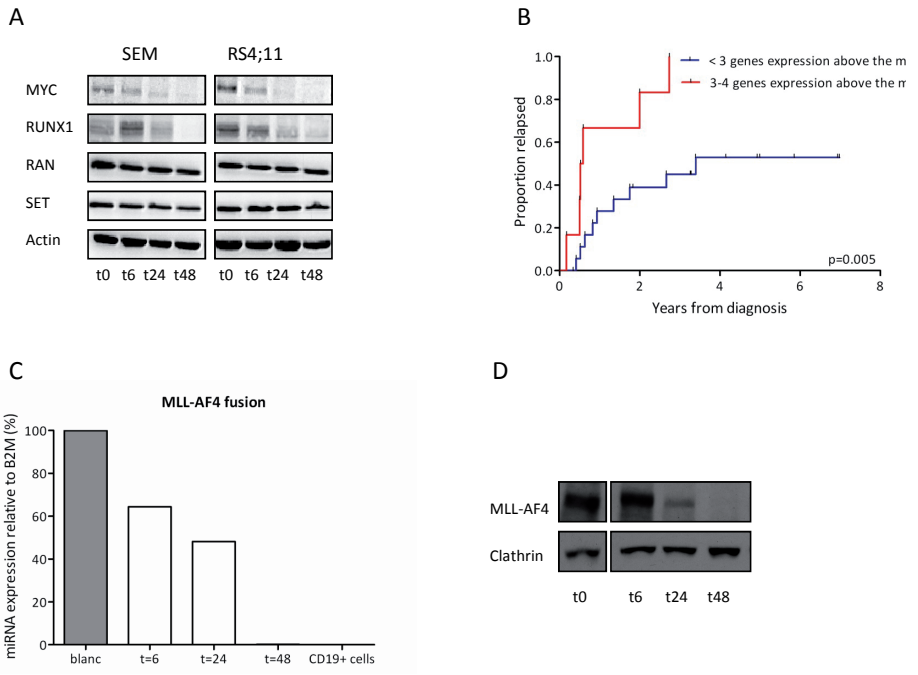

Figure 5. Proto-oncogene encoded protein down-regulation and MLL-AF4 fusion degradation induced by the HDAC inhibitor LBH589.
A. Western blot analysis of RUNX1, RAN, SET and MYC in the t(4;11)-positive ALL cell line models SEM and RS4;11 exposed to 500 nM of LBH589 for 6, 24 or 48 hours. Actin was used as a control for equal loading. **B.** Proto-oncogene dependent risk of relapse in t(4;11)-positive infant ALL. For the proto-oncogenes *RAN*, *RUNX1*, *MYC* and *SET* mRNA levels were determined using quantitative real-time PCR analysis in an extended cohort of primary t(4;11)-positive infant ALL samples (n=28). Based on the median expression levels, the patients were divided into two groups for which the risk of relapse was computed. One group of patients displaying high-level expression of 3 or 4 of the proto-oncogenes (n=7), and the other group showing high-level expression of <3 proto-oncogenes (n=21). The proportion of relapsed patients, as computed with the Kaplan Meier estimator, is presented on the Y-axis and the follow-up time (in years) is presented on the X-axis. The log-rank test was used to compare outcomes between different patient groups. **C.** mRNA expression levels of the *MLL-AF4* fusion gene relative to the housekeeping gene *B2M* as determined by quantitative RT-PCR analyses. mRNA expression levels were measured in the t(4;11)-positive cell line SEM exposed to LBH589 (500 nM) for 0, 6, 24 or 48 hours and in $CD19^+$ B cells. Expression levels in the unexposed SEM cell line were set to 100%. **D.** Western blot analysis of the MLL-AF4 fusion protein in SEM cells exposed to 500 nM of LBH589 for 6, 24 or 48 hours, using Clathrin as a loading control.

LBH589 down-regulates *MLL-AF4*

Noteworthy, when focusing on the genes most responsive to HDAC inhibition both at the mRNA and protein level, both the MYC and the RUNX1 proto-oncogene have been proposed as potential targets of the MLL-AF4 fusion.[13,14] Since it has been reported that HDAC inhibitors are able to abolish the oncogenic fusion proteins AML-ETO and PML-RARα,[30] we here asked whether LBH589 was able to degrade the MLL-AF4 fusion. If so, the here presented results may need to be interpreted from a different angle as well: do HDAC inhibitors directly down-regulate the genes under investigation, or is the suppression of the studied proto-oncogenes a direct consequence of the succumbed expression of MLL-AF4 fusion protein? Interestingly, both at the mRNA and protein level, LBH589 completely neutralized the expression of the MLL-AF4 fusion within 48 hours of exposure **(figures 5C and 5D)**.

Increased levels of methylation at proto-oncogene promoters after LBH589 exposure

The identification of the potential of HDAC inhibitors to target aberrantly activated genes in t(4;11)-positive infant ALL was based on specific hypomethylation at the promoters of these genes. As several of these genes were indeed down-regulated upon exposure to HDAC inhibitors, a final question remains whether transcriptional inactivation of these genes was established by restoration of promoter methylation. To answer this question, we generated genome-wide DNA methylation profiles for the t(4;11)-positive ALL cell line SEM exposed to 500 nM of LBH589 for 0 (controls), 6, 24 or 48 hours. Interestingly, upon LBH589 exposure, DNA methylation levels were indeed increased at the promoters of the selected proto-oncogenes (i.e. *DIAPH1*, *HOXA9*, *RUNX1*, *RAN*, *PARK7*, *SET*, and *MYC*), although the extent of augmented promoter methylation varied for each of the proto-oncogenes **(figure 6)**. Nonetheless, promoter CpG methylation of the housekeeping reference gene *B2M* appeared completely unaltered **(figure 6H)**, to some extent suggesting that LBH589 exposure truly targeted the methylation status of the gene promoters under investigation. However, it should be taken into account that not the methylation status per se was directly targeted by LBH589, but that restored methylation at these gene promoters rather reflects more non-specific effects. For example, HDAC inhibitor treatment may have specifically killed cells displaying hypomethylation at these loci, while subpopulations of cells bearing methylated gene promoters were more or less spared.

DISCUSSION

Approximately 80% of infants (< 1 year of age) diagnosed with acute lymphoblastic leukemia (ALL) carry leukemia-specific balanced chromosomal translocations involving the *MLL* gene, which represent strong predictors of an adverse outcome[31] and specify unique gene expression profiles.[9,10] Wild-type MLL functions as an epigenetic regulator of transcription through histone methyltransferase activity[32] and is essential for normal

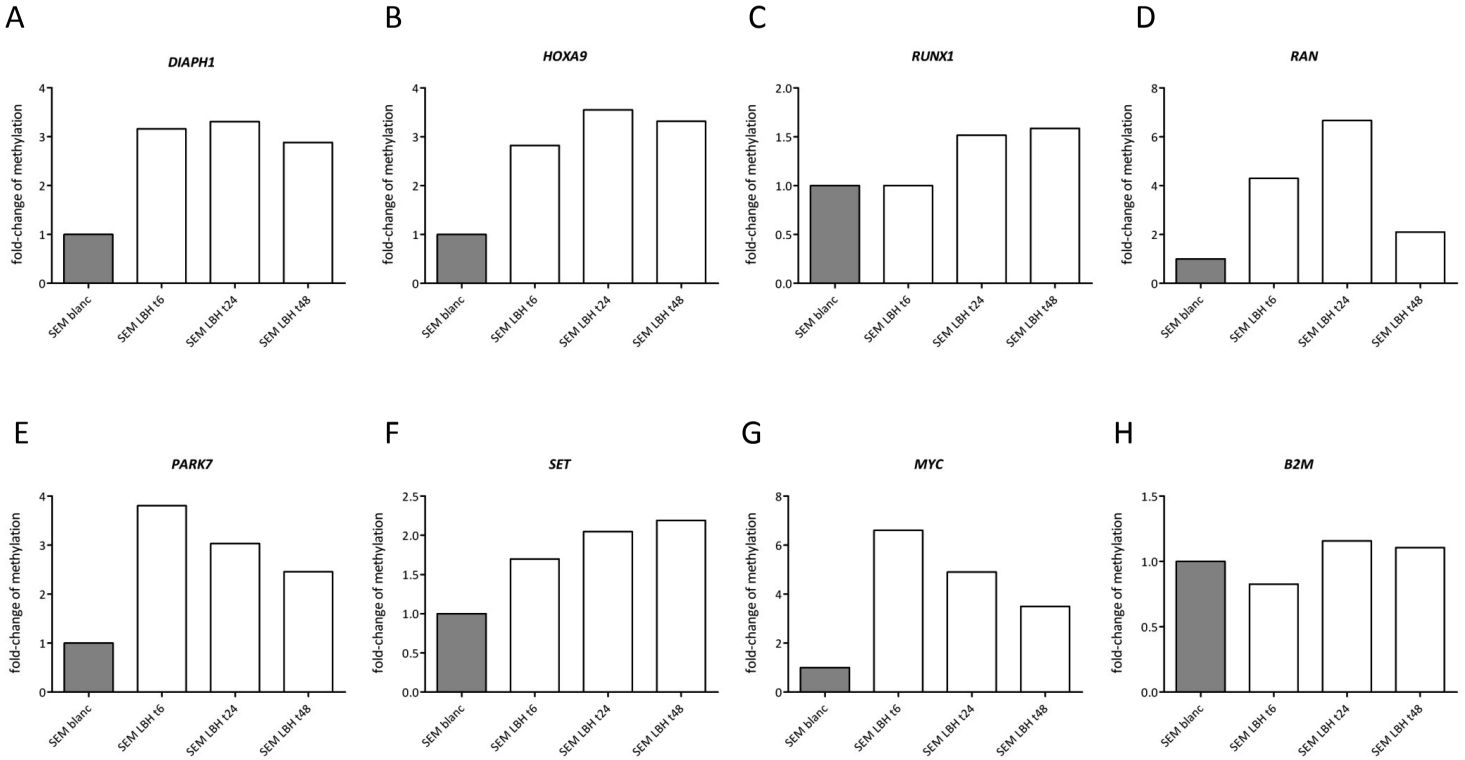

Figure 6. Increased methylation at specific gene promoters after exposure to LBH589.
DNA methylation levels from array-based DNA methylation profiling were determined in the t(4;11)-positive cell line SEM exposed for 6, 24 or 48 hours to HDAC inhibitor LBH589 (500 nM). Methylation levels of the proto-oncogenes **A.** *DIAPH1*, **B.** *HOXA9*, **C.** *RUNX1*, **D.** *RAN*, **E.** *PARK7*, **F.** *SET,* **G.** *MYC* and **H.** *B2M* relative to the unexposed cell line (SEM blanc) are presented. Fold-change of methylation is presented on the Y-axis and methylation levels in SEM blanc were set to 1.

hematopoiesis.[7] Specifically, wild-type MLL methylates histone 3 at lysine 4 (H3K4), which embodies an evolutionary conserved histone mark associated with primed gene activation. Reinsuring activated transcription, H3K4 methylation induces an open or active chromatin state and protects associated loci against DNA methylation at gene promoters to prevent associated genes from transcriptional silencing. Thus, in hematopoietic cells, abrogation of the normal function of *MLL* on one of the alleles, for example by chromosomal translocations, potentially leads to an impaired ability to effectuate H3K4 methylation.[14] Despite this apparent loss-of-function, it should be noted that the second *MLL* allele remains unaffected. Partially losing the ability to methylate H3K4, MLL fusions are now known to recruit an alternative histone methyltransferase, i.e. DOT1L, mediating H3K79 methylation. Consequently, H3K79 methylation introduces an additional active histone mark that allows the activation of otherwise inactive genes, presumably in favor of leukemia development.[13,14] Thus, abnormally over-expressed genes among the gene expression signatures associated with *MLL*-rearranged leukemias[9] can in part be explained by this mechanism.

Nevertheless, apart from vast amounts of genes characteristically over-expressed in *MLL*-rearranged infant ALL, at least equal numbers of genes are under-expressed or inactivated.[10] Recently, we demonstrated that one mechanism responsible for gene inactivation in *MLL*-rearranged infant ALL involves epigenetic silencing through gene promoter hypermethylation.[12] This latter is especially true in patients carrying the most common *MLL* translocation t(4;11), producing the MLL-AF4 fusion protein, and as recently reported, in most instances also the reciprocal fusion product AF4-MLL.[6] In the present study, we revisited our genome-wide DNA methylation profiles[12] and found that in t(4;11)-positive infant ALL numerous genes are also hypomethylated and consequently highly expressed. As these genes remained hypermethylated and silenced in normal hematopoietic cells, the loss of suppressive promoter methylation and consequent activation of transcription of these genes in t(4;11)-positive ALL may well have been involved in the transformation process. Supporting this assumption, these genes frequently involve potential proto-oncogenes, like *MYC,*[33] *HOXA9,*[34] *SET,*[35] *RUNX1,*[36] *RAN,*[37] *PARK7,*[38,39] and *DIAPH1.*[40] Moreover, several of the hypomethylated genes that we identified in this study, i.e. *ZCCHC7, HOXA9, and MYC* have all been shown to be activated by the MLL-AF4 fusion itself via H3K79 methylation through the recruitment of DOT1L.[13,14] This observation indisputably illustrates interactions between DNA methylation and histone modification in regulating gene expression. Apparently, MLL fusion protein-driven H3K79 methylation, leading to an open and active chromatin state, is frequently accompanied by the release of transcriptional suppressive promoter methylation at associated genes. From this perspective, our finding that HDAC inhibitors appeared to be particularly suitable to down-regulate aberrantly hypomethylated and highly expressed proto-oncogenes in t(4;11)-positive ALL cells seems rather unexpected and even counterintuitive. HDAC inhibitors are believed to induce transcriptional reactivation of dormant gene loci by suppressing histone deacetylases and allowing active chromatin states. At the same time, the full spectrum of the effects of these compounds, which often are non-specific and

off-target, and the exact mechanisms through which these agents act remain poorly understood. Although the here presented effects of various HDAC inhibitors on a specific set of hypomethylated proto-oncogenes seem to fall into the category of non-specific, yet highly favorable drug actions, this may be debated. Although off-target actions of HDAC inhibitors in general may have been demonstrated, the fact that the six different HDAC inhibitors displaying diverse chemical structures and belonging to different inhibitory classes, show similar results, strongly argues against unspecific effects. In other words, HDAC inhibitor-induced down-regulation of hypomethylated proto-oncogenes in *MLL*-rearranged ALL may very well involve specific drug actions, albeit through mechanisms not yet understood. Nonetheless, although the transcriptional suppressive actions of various HDAC inhibitors remain counterintuitive and difficult to explain, the clinical relevance of the presented data should not be ignored. In 4-day MTT assays all HDAC inhibitors tested (almost) completely eradicated the entire population of leukemic cells both in proliferating t(4;11)-positive cell line models, as well as in non-dividing t(4;11)-positive infant ALL patient samples. Although HDAC inhibitors are known to induce cell cycle arrest, the observed down-regulation of proto-oncogene expression is unlikely to be a result of this phenomenon as the cytotoxic responses in primary t(4;11)-positive infant ALL cells, which fail to proliferate *in vitro*, are comparable to those obtained in proliferating cell lines. Nor can the down-regulation of the proto-oncogenes by HDAC inhibition immediately be ascribed to the induction of leukemic cell death as suppression of proto-oncogene expression (as predicted by cmap analyses) is observed within 6 to 24 hours while the first overt signs of leukemic cell death are typically seen at 48 hours.

Perhaps a better explanation for the down-regulation of this specific set of hypomethylated proto-oncogenes primarily involves the degradation of the MLL-AF4 fusion product. Possibly, the hypomethylated and transcriptionally activated status of most (if not all) of the proto-oncogenes is more or less dependent on the MLL-AF4 fusion protein itself through the recruitment of DOT1L and subsequent methylation of H3K79. For instance, *HOXA9*, *MYC* and *ZCCHC7* have been proposed as potential MLL-AF4 target genes.[13,14] Direct down-regulation of the MLL-AF4 fusion by HDAC inhibition may thus have led to impaired H3K79 methylation, rapidly resulting in the inactivation of these specific proto-oncogenes as well. Yet, not all of the genes in our hypomethylated signature represent known MLL-AF4 target genes. This suggests that the down-regulation of these genes may not have been influenced by the degradation of MLL-AF4 directly, but may have been repressed indirectly as a result of direct suppression of their regulators. In any case, the possible involvement of MLL-AF4 degradation in down-regulating hypomethylated proto-oncogene expression immediately imposes another question: how does HDAC inhibition mediate the neutralization of the MLL fusion? Obviously, this phenomenon again is counterintuitive and a possible mechanism remains obscure, although important clues may already have been published. For examples, Xia et al. (2003), showed that the MLL repression domain, which is retained in MLL fusions and postulated to be required for transforming activity, interacts with histone deacetylase 1 (HDAC1) and partially mediates its activity.[41] Thus HDAC inhibition may well disrupt this

interaction and compromise vital functions of the MLL fusion, or perhaps its stability. On the other hand, this may explain degradation of MLL-AF4 at the protein level, but not its suppression at the mRNA level.

Whether or not proto-oncogene down-regulation by HDAC inhibitors is directly mediated by degradation of the MLL fusion or involves other (yet to be elucidated) mechanisms, the expression of several of the proto-oncogenes may have important clinical implications. Of specific interest are the proto-oncogenes that did display rapid responses at the mRNA level, i.e. *RAN*, *SET*, *MYC* and *RUNX1*, and in case of MYC and RUNX1, at the protein level as well. In contrast to *MYC*, which seems to promote transformation predominantly by stimulating cell proliferation,[33] the *RUNX1* proto-oncogene seems to affect differentiation. *RUNX1* is believed to be required in development during the endothelial to hematopoietic cell transition only, and not thereafter.[42] However, to date this has only been shown in mice, and a similar dependence in humans remains to be established. Nonetheless, it may be plausible that *RUNX1* expression could not have been repressed during lymphoid differentiation in t(4;11)-positive infant ALL cells. The fact that t(4;11)-positive ALL blasts characteristically resemble highly immature CD10-negative precursor B cells implies that the inability to down-regulate *RUNX1* at this stage may indeed have blocked differentiation. Moreover, amplification and over-expression of *RUNX1* has been associated with a poor prognosis in childhood precursor B-cell ALL in general,[43] further emphasizing its potential oncogenic role in developing B cells. Interestingly, it has been proposed that full oncogenic transformation by *RUNX* genes can only be accomplished in close collaboration with genes that rescue cell proliferation, such as *MYC*.[36] Therefore, the concerted up-regulation of both *MYC* and *RUNX1* in t(4;11)-positive infant ALL may not at all be a coincidence.

Through the identification of high-level expression of this particular set of known proto-oncogenes and the above suggested link between MYC and RUNX1, we here obviously touch upon the principle of 'oncogene cooperation' already described by Weinberg and coworkers in 1985.[44] Reflecting such a mechanism of oncogene cooperation in t(4;11)-positive ALL is our data showing an extremely high risk of relapse in patients firmly over-expressing three or more of the hypomethylated proto-oncogenes**.** However, these data must be interpreted cautiously because a multivariate analysis could not be performed due to low patient numbers. Targeting these proto-oncogenes, for example using HDAC inhibitors, may thus be a vital step in taming the aggressiveness of this malignancy. Yet, the keynote of this study should unquestionably be the opportunity for therapeutic intervention targeting the MLL-AF4 fusion itself. Given the tremendous cytotoxic effects of all HDAC inhibitors tested on primary t(4;11)-positive infant ALL samples *in vitro*, these data demand further studies evaluating HDAC inhibition in *in vivo* models[6] and subsequently in a clinical setting. Emphasizing this statement, most of the HDAC inhibitor concentrations used in our 4-day MTT assays that appeared sufficient to eliminate the vast majority of t(4;11)-positive infant ALL cells *in vitro*, resemble clinically achievable and tolerable plasma concentrations in various clinical trials. For example, in our *in vitro* data 0.5 - 1 μM of Vorinostat (SAHA) showed maximum cytotoxic effects in t(4;11)-positive infant

ALL cells, whereas plasma concentrations of 2.5 μM are easily exceeded in patients with relapsed lymphoma intravenously or orally treated with 75 – 900 mg/m^2 daily.[45] Similarly, in a phase II trial in patients with T-cell lymphoma treated with Romidespin (FK288), plasma concentrations were achieved ranging from 300 – 400 ng/mL[46] while the present study shows that concentrations of 1 – 10 ng/mL are sufficient to kill the majority of t(4;11)-positive ALL cells *in vitro*. Finally, plasma levels in patients with refractory hematologic malignancies intravenously treated with LBH589 easily reached 120 – 560 ng/mL[47] while in our *in vitro* data LBH589 concentrations of 20 – 50 ng/mL seem more than adequate. In addition, and further accentuating the potential of HDAC inhibition in *MLL*-rearranged leukemia, Burbury and co-workers recently published a case report in which they describe the successful treatment of a 60 year old patient suffering from *MLL*-rearranged leukemia who sustained a complete cytogenetic response to single-agent HDAC inhibitor treatment.[48] Based on the present observations demonstrating the potential of HDAC inhibitors, and recent findings from our laboratory showing the potential of demethylating agents,[12] we conclude that *MLL*-rearranged infant ALL patients would greatly benefit from treatment regimens including (combinations of) various epigenetic drugs.

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Supplemental information is available in the appendices.

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EPILOGUE

"The known is finite, the unknown infinite; intellectually we stand on an island in the midst of an illimitable ocean of inexplicability. Our business in every generation is to reclaim a little more land, to add something to the extent and the solidity of our possession."

(Thomas H. Huxley, On the reception of the origin of species, 1887)

7

MLL-rearranged Infant Acute Lymphoblastic Leukemia: An Epigenetic Disease which Should Be Treated Accordingly.

General Discussion and Future Perspectives

Dominique J.P.M. Stumpel, Rob Pieters, and Ronald W. Stam

INTRODUCTION

MLL-translocated leukemia from a clinical perspective

Translocations involving the *MLL* (*Mixed Lineage Leukemia)* gene are recurring chromosomal aberrations in hematological malignancies.[1] *MLL* rearrangements characterize aggressive leukemias and are predominantly found in infants (< 1 year of age) diagnosed with acute lymphoblastic leukemia (ALL) at or shortly after birth where they constitute up to 80% of the cases.[2, 3] They are found less frequently in older children and adults with ALL accounting for only 1% and 10% of the cases, respectively.[4, 5] In addition, they are present in about 15% of the childhood acute myeloid leukemia (AML) cases.[6, 7] Although survival rates for children with ALL have increased tremendously over the past decades,[8, 9] infants with *MLL*-rearranged ALL are still trailing behind with an event-free survival (EFS) rate of only 35-40%.[10] Presence of a rearrangement of the *MLL* gene alone is an independent predictor of a poor clinical outcome in infant ALL.[10, 11] Therefore, adequately treating *MLL*-rearranged infant ALL remains one of the major challenges in pediatric hemato-oncology.[12-15]

Aberrant epigenetic regulation due to MLL translocations

As wild-type MLL functions as a histone modifying enzyme playing an important role in early B-cell development,[16-18] researchers began focusing on deregulated epigenetics, possibly induced by the compromised function of MLL due to its genomic rearrangement.

At the C-terminus of the *MLL* gene resides a highly conserved SET (Suppressor of variegation 3-9, Enhancer of zeste, Trithorax) domain **(figure 1A)**, which possesses histone 3 lysine 4 (H3K4) methyltransferase activity. H3K4 trimethylation (H3K4me3) represents a histone mark associated with transcriptional initiation/ activation.[18, 19] In the case of an *MLL* translocation, the *MLL* gene loses its SET domain-containing C-terminus **(figure 1B)** and hereby its histone methyltransferase activity.[19] At first sight, this may suggest that the genetic basis of MLL fusion-driven leukemias consists of this apparent loss-of-function. However, the actual consequence of an *MLL* translocation is not only a loss, but rather a gain-of-function. Recent data on MLL translocations which fuse the *MLL* gene to the respective fusion partners *AF4*, *ENL*, and *AF9* **(figure 1B)** has shown that the loss of H3K4me3 activity is compensated by the recruitment of another histone methyltransferase named DOT1L (Disruptor Of Telomeric silencing 1-Like).[20-23] In contrast to the SET domain of the *MLL* gene, DOT1L catalyzes dimethylation on histone 3 at lysine 79 (H3K79me2). H3K79me2 represents a histone mark associated with transcriptional elongation; aberrant occupancy across gene loci results in inappropriate transcription of many genes encoding, e.g., transcription factors, chromatin remodelers and signaling molecules which play central roles in hematopoietic stem cell identity and self renewal.[24, 25] Collectively, these data suggest that MLL fusion proteins directly activate hematopoietic stem cell-like gene expression programs, that are also typically found in the leukemic stem

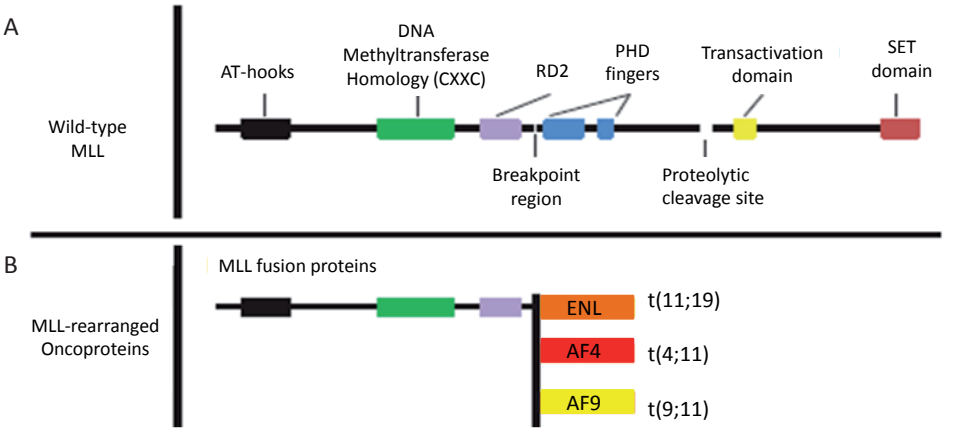


Figure 1. A schematic representation of MLL (A) and MLL-translocated fusion proteins (B).
A. The *MLL* gene (also referred to as *ALL1* or *HRX*) is the human homologue of the *Drosophila melanogaster* trithorax gene. The *MLL* gene comprises 37 exons and translates into a large 3968 amino acid protein.[27] At the very amino-terminal (N-terminal) end MLL contains 3 DNA binding AT hook domains that bind to cruciform DNA.[28] In addition, the N-terminal of MLL encloses a repressive domain with two subunits. Transcriptional repressor domain 1 is formed by a CxxC zinc-finger motif, which shows strong homology to the DNA methyltransferase 1 (DNMT1) (therefore also known as the MT homology domain).[29] The CxxC domain in MLL discriminates against DNA methylation by specifically binding to unmethylated CpG dinucleotides.[30] Transcriptional repressor domain 2 (RD2) consists of multiple plant homology domains (PHD fingers) that are often referred to as co-repressors,[31] because they can bind to histone deacetylases (HDACs) 1 and 2. Further to the carboxy-terminal (C-terminal) end is the transcriptional activation domain that can recruit a histone acetyltransferase [32] and controls the binding of proteins to the repressive domain. At the outermost C-terminal of MLL the highly conserved Suppressor of variegation 3-9, Enhancer of zeste, Trithorax (SET) domain is located which possesses histone 3 lysine 4 (H3K4) methyltransferase activity responsible for the histone 3 lysine 4 trimethylation (H3K4me3) mark associated with transcriptional initiation/activation.[18, 19] The different domains of MLL all contribute to the ultimate function of this master regulatory protein: maintaining pre-activated promoters in an permissive transcriptional state by changing the chromatin conformation.[30]
B. In the case of an *MLL* translocation, chromosome 11 breaks at chromosome arm 11q23 within a 8.3kb breakpoint cluster region in the *MLL* gene. Breakpoints typically occur between exons 8 and 13, but the exact location of the breakpoint varies among the different translocation partners and different types or lineages of leukemia.[33] The N-terminal of MLL then fuses to the C-terminal of one of over 65 translocation partners[33, 34] (see also: Atlas of Genetics and Cytogenetics in Oncology and Haematology last updated on 02/2010). MLL fusions consistently result in the deletion of PHD fingers.[35] In addition, the C-terminal SET domain, having histone methyltransferase (HMT) activity, is inevitably lost.[18] Research on *MLL*-rearranged leukemias is complicated because many fusion partners can be involved.[33] We focused on the most prevalent nuclear translocation partners occurring in greater than 80% of the *MLL*-rearranged infant leukemia cases: *AF4* (4q21), *ENL* (19p13.3), and *AF9* (9p23) leading to the expression of the fusion products *MLL-AF4*,[36] *MLL-ENL* [27] and *MLL-AF937* respectively.[55]
(Figure has been adapted from Tan et al., 2010)[38]

cell.[26] Emphasizing the importance of the abnormally acquired H3K79me2 histone mark, it was recently shown that *MLL*-rearranged leukemia is dependent on DOT1L,[39] and that mutations disrupting the interaction between MLL fusion proteins and DOT1L abolish leukemic transformation.[22]

Largely based on the results of the above described studies, *MLL*-rearranged leukemia is now increasingly recognized as an epigenetic malignancy.[20, 40, 41] However, while most of the research on epigenetic deregulation in *MLL*-rearranged leukemia has focused on aberrant histone modifications,[13, 20, 42-47] the role of DNA methylation remains rather underexplored. **Therefore, the research project described in this thesis aimed to study aberrant DNA methylation in *MLL*-rearranged infant ALL.**

Chapter 1 of this thesis (General Introduction) provides the background of pediatric ALL and *MLL*-rearranged infant ALL in particular. In addition, the field of epigenetics is introduced. **In the present chapter, the results of various studies (corresponding to the other chapters in this thesis) on DNA methylation in this aggressive subtype of childhood leukemia are discussed.**

ABERRANT PROMOTER DNA HYPERMETHYLATION IN MLL-REARRANGED INFANT ALL

Deregulated DNA methylation at gene promoters, leading to transcriptional silencing of the associated gene, is frequently observed in human cancers, especially in solid tumors.[48] Yet, aberrant gene promoter DNA methylation is becoming increasingly recognized as an important factor contributing to leukemogenesis.[49-51] Recent studies have demonstrated that (childhood) leukemias can be accurately classified based on their unique promoter DNA methylation patterns.[52-54] Although a true CpG island methylator phenotype (CIMP) associated with clinical outcome has only been identified in solid tumors, heavily methylated hematological malignancies have been characterized.[55, 56] Generally, adult leukemias tend to be more severely hypermethylated than childhood leukemias,[57] which may reflect that DNA methylation accumulates during life.

Since *MLL*-rearranged leukemias appear to be driven by MLL fusion-induced activation of certain gene expression profiles (see above), a role for aberrant DNA methylation in this particular type of leukemia may seem counterintuitive. However, gene expression profiling in *MLL*-rearranged infant ALL demonstrated that apart from vast numbers of genes that are uniquely and abnormally activated, at least similar numbers of genes are inappropriately down-regulated.[58] Thus, aberrantly acquired activating histone marks may not be the sole epigenetic force at work in *MLL*-rearranged ALL cells. In fact, subtle signs of an important role for DNA hypermethylation in *MLL*-rearranged leukemias have been published before. For instance, Gutierrez *et al*. studied several genes frequently methylated in human cancers and found that *MLL*-rearranged ALL cells displayed DNA hypermethylation at the promoters of these genes more frequently than other subtypes of childhood ALL.[59] In addition, the *distal-less homeobox 3* (*DLX3*) gene appeared to

be hypermethylated and down-regulated in pediatric MLL-AF4-positive ALL samples, but not in pediatric ALL samples carrying the more common TEL-AML1 translocation.[60] We previously demonstrated that promoter hypermethylation of the tumor suppressor gene *Fragile Histidine Triad* (*FHIT)* occurs in the vast majority of *MLL*-rearranged infant ALL cases, and far less frequently in other ALL subtypes.[61]

Encouraged by these observations, we performed the first genome-wide array-based DNA methylation profiling study in a large cohort of *MLL*-rearranged infant ALL patients (n=62) **(chapter 2)**.[62] We demonstrated that the majority of *MLL*-rearranged infant ALL cases, i.e. those characterized by translocation t(4;11) or t(11;19), were indeed characterized by severe promoter DNA hypermethylation. Moreover, a high degree of hypermethylation seemed to be associated with a worse clinical outcome.[62] In contrast, infant ALL patients carrying translocation t(9;11) or patients carrying wild-type *MLL* genes (no *MLL* translocation) displayed DNA methylation patterns comparable to those of healthy bone marrow samples. Our observations were independently confirmed by others, albeit in a rather small cohort of only five *MLL*-rearranged infant ALL cases.[63] The translocation-specific DNA methylation patterns still need to be validated in an independent cohort of *MLL*-rearranged infant ALL patients. More recently, a large classifying study in childhood ALL again confirmed that *MLL*-rearranged ALL is severely hypermethylated in comparison with other pediatric ALL subtypes.[52] To gain more insights into the *MLL* methylome, collaborative studies should be initiated comparing the DNA methylation patterns obtained from infants with *MLL*-rearranged ALL with those obtained from older children (> 1 year) and adults with *MLL*-translocated leukemias.

In our DNA methylation profiling study, t(9;11)-positive infant ALL cases resembled normal bone marrows.[62] While t(9;11)-positive ALL occurs at low frequencies, the MLL-AF9 fusion far more often leads to myeloid leukemia, and as such represents the most frequent *MLL* translocation in pediatric AML. In contrast to *MLL*-rearranged ALL, aberrant DNA methylation indeed appears less obvious in *MLL*-rearranged AML.[64, 65] In fact, compared with childhood AML samples without *MLL*-translocations, pediatric *MLL*-rearranged AML cells tend to be hypomethylated instead.[64] This suggests that the origin of distinct methylation patterns may in part be due to tissue-specific factors, and may not be solely related to the presence of an MLL fusion.[64] However, the introduction of the MLL-AF9 fusion protein in primary human hematopoietic stem/progenitor cells revealed the induction of DNA methylation patterns comparable to those in primary MLL-AF9-positive AML samples.[66] In contrast, other fusion genes, such as AML1-ETO, failed to reproduce the epigenetic signature observed in the patients.[66] Thus, the presence of *MLL* rearrangements is to some extent responsible for the induction of leukemia-specific DNA methylation patterns. In support of this, DNA methylation normally accumulates during life. The fact that *MLL*-rearranged ALL cells in infants (<1 year of age) are already exhibiting highly methylated genomes again suggests a leukemia-specific role for DNA methylation in MLL fusion-driven ALL. Whether this holds true for *MLL*-rearranged infant AML should be investigated in future studies.

ABERRANT MIRNA METHYLATION IN MLL-REARRANGED INFANT ALL

MicroRNAs (miRNAs) are small non-coding RNAs (about 21-25 nucleotides in length), that regulate the translation of ~60% of all human protein-coding genes via transcript destabilization and/or translational repression.[67, 68] High expression of a certain miRNA suppresses its target mRNA/protein. It has been proposed that, in addition to histone modifications and DNA methylation, miRNA-mediated regulation represents the third component of the epigenetic code.[69, 70] Since miRNAs are encoded by the genome, they are also subjected to epigenetic regulation (DNA methylation and histone modificaton) themselves.[71, 72]

Human cancers can be accurately classified based on their miRNA expression profiles.[73, 74] Increasing evidence has emerged that deregulated miRNA expression is involved in the development of certain leukemias, including *MLL*-rearranged ALL.[74-78] For instance, miR-196b was found to be over-expressed in *MLL*-rearranged infant ALL compared with other subtypes of childhood precursor B-ALL,[76] and appeared to participate in leukemogenesis by stimulating proliferation while blocking differentiation in hematopoietic progenitor cells.[79, 80] More recently, up-regulation of the entire miR-17-92 cluster was described in *MLL*-rearranged leukemias.[81] Strong binding of the MLL fusion protein to this miRNA cluster led to inhibition of apoptosis and promoted proliferation by regulation of the relevant target genes.

Most studies in *MLL*-rearranged leukemia research have focused on miRNA over-expression.[76, 81, 82] However, in a recent genome-wide miRNA profiling study, putative tumor suppressing miRNAs, such as let-7b and miR-708, were found to be down-regulated in *MLL*-rearranged infant ALL, and associated with oncogene up-regulation.[76] Altered miRNA expression can be caused by genetic mechanisms such as deletions, mutations and amplifications, or by deregulation of transcription factors. Recently, it became clear that miRNAs can also be regulated epigenetically.[71, 83] Hypermethylation of CpG islands related to miRNAs leads to silencing of miRNA expression and up-regulation of miRNA target genes.[71, 72]

Interestingly, unsupervised principal component analysis of patient samples based on methylation at miRNA loci mirrored analysis observed earlier for promoter DNA methylation **(figure 2)**: Infant ALL cases carrying *MLL* translocation t(9;11) or wild-type *MLL* genes displayed miRNA methylation patterns which resembled those of normal pediatric bone marrow samples. In contrast, t(4;11)- and t(11;19)-positive infant ALL samples clearly seem to harbor abnormal miRNA methylation patterns **(chapter 3.1)**.

In **chapter 3.2**, we explored the effects of miRNA CpG hypermethylation on miRNA expression in t(4;11)-positive infant ALL. We identified 11 miRNAs that were significantly down-regulated as a consequence of hypermethylation. Seven miRNAs, including miR-10a, miR-152, miR-200a, miR-220b, miR-429, miR-432, and miR-503, were re-expressed after treatment with the demethylating agent zebularine.[84] Five of these miRNAs appeared to be associated with either *MLL* or MLL fusions.

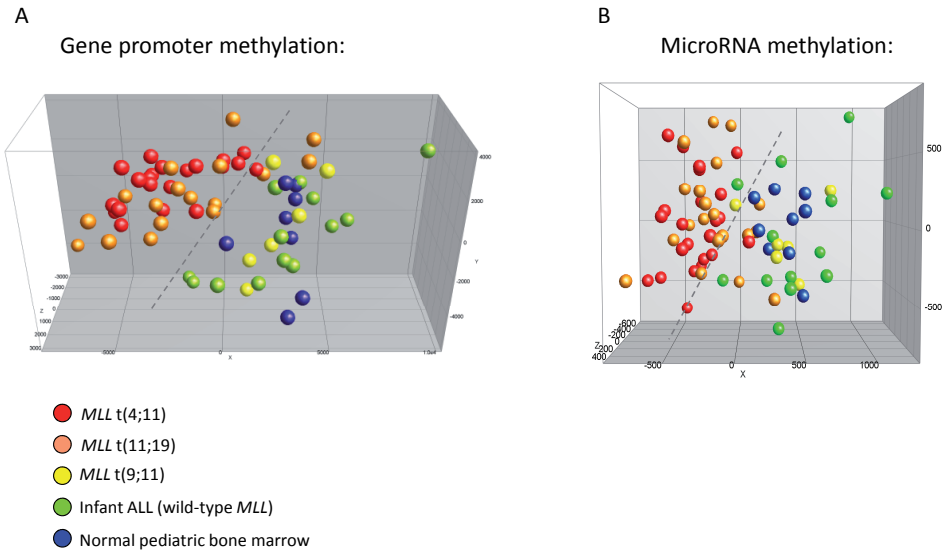


Figure 2. Principal component analyses of gene promoter methylation (A) and miRNA methylation (B) in infant ALL.
Unsupervised principal component analyses (PCA) based on the CpG array data (244K microarray platform, Agilent) from infant ALL patients (n=62) and normal bone marrows (n=9). Each case is color-coded, indicating the specific infant ALL subgroups: t(4;11) (n= 23; red), t(11;19) (n=20; orange), t(9;11) (n= 6; yellow), untranslocated infant ALL (n=13, green), and healthy bone marrow (n=9; blue). For the PCA presented in **figure B** all 1118 probes present on the 244K microarray corresponding to miRNAs (n=113) were selected.

The expression of putative targets of miR-10a, such as *HOXA3*, is elevated in *MLL*-rearranged infant ALL patients. In addition, *ZEB2*, a target of the MLL-AF4 fusion,[24] is under control of the miR-220b/a-429 cluster.[85] Another example of MLL fusion-driven miRNA regulation involves the polycistronic miR-424-503 cluster. In t(9;11)-positive myeloid leukemias, miR-424 expression was repressed by the MLL-AF9 fusion protein.[86] We showed that the miR-424-503 cluster was down-regulated by CpG island hypermethylation in t(4;11)-positive ALL, possibly directed by the MLL-AF4 fusion **(chapter 3.2)**. The miR-152 sequence, which is also hypermethylated in t(4;11)-positive infant ALL, is thought to normally target *DNA methyltransferase 1 (DNMT1)* and wild-type *MLL*. Interestingly, heavily methylated cases presented a higher level of methylation of the miR-152 locus. This might imply that the level of miR-152 methylation and concomitant *DNMT1* expression may determine the observed severity of DNA hypermethylation **(chapter 2)**. Moreover, comparable to the degree of gene promoter hypermethylation, the level of miR-152 methylation appeared predictive for clinical outcome.[62, 84] Therefore, it is important to unravel the role of miRNA 152 in *MLL*-rearranged infant ALL, for example by re-introducing specifically miRNA 152 expression into the leukemic cells and by further identifying and validating its target genes.

Very recently, it was shown that the fusion protein MLL-AF4 is regulated by miR-143. This miRNA is aberrantly and preferentially methylated in MLL-AF4 positive ALL cells, and its re-expression induces apoptosis and inhibits proliferation of leukemic cells.[87] This indicates that inhibition of miR-143 due to inappropriate methylation provides a proliferative advantage to MLL-AF4-positive ALL cells. Evidently, in *MLL*-rearranged ALL, miRNAs work both as targets and as effectors of the epigenetic machinery.[88]

GLOBAL DNA METHYLATION IN MLL-REARRANGED INFANT ALL

While we clearly established the presence of severe gene promoter hypermethylation in the majority of *MLL*-rearranged infant ALL patients,[62] we next studied DNA methylation in infant ALL on a more global genomic level **(chapter 4)**. In human cancers, a high degree of aberrant gene promoter hypermethylation is often accompanied by loss of methylation (or hypomethylation) in non-promoter DNA regions.[89-92] We quantitatively evaluated the levels of methylation at three types of repetitive elements. Repetitive elements are interspersed repeated DNA sequences that originate from transposable elements,[93] or represent tandemly repeated simple sequences (satellite DNA). These elements comprise about 45% of the human genome,[94] and contain a substantial portion of its CpG islands. Therefore, methylation at repetitive elements is frequently used as a surrogate marker for global DNA methylation.[91, 95, 96] As shown in **chapter 4**, despite severe promoter hypermethylation, we found no evidence for genome-wide hypomethylation at non-promoter sequences in *MLL*-rearranged infant ALL. Global hypomethylation is usually associated with genomic instability, allowing additionally acquired genetic hits to propel a pre-malignant cell into a fully transformed state.[97] Therefore, our findings may explain why additional genetic lesions (e.g copy number variations) have not been discovered in *MLL*-rearranged ALL.[98, 99] Literature shows that there are more exceptions to the central dogma that global hypomethylation accompanies promoter hypermethylation, especially among hematopoietic malignancies.[95, 100, 101] Thus, including our data presented in **chapter 4**, the mutual occurrence of both promoter hypermethylation and global hypermethylation may well be a recurrent feature in some hematological malignancies. This finding should not hamper the use of demethylating agents in therapeutic strategies for these diseases.

ABERRANT GENE PROMOTER HYPOMETHYLATION IN MLL-REARRANGED INFANT ALL

Apart from severe promoter hypermethylation, our genome-wide DNA methylation analysis **(chapter 2)** also revealed genes that are abnormally hypomethylated in t(4;11)-positive infant ALL patients.[62] The promoters of these genes are methylated in healthy bone marrow samples, as well as in t(9;11)-positive and *MLL* germline infant ALL cases **(chapter 2)**. Therefore, in **chapter 6** we explored aberrant hypomethylation of these

specific genes in t(4;11)-positive infant ALL. The hypomethylated and highly expressed genes in t(4;11)-positive infant ALL appeared enriched for proto-oncogenes, including *MYC*, *ZCCHC7, HOXA9*, *RUNX1*, *SET* and *RAN*.[102] Interestingly, several of these genes were previously shown to be activated by the MLL-AF4 fusion itself via aberrant H3K79 dimethylation and recruitment of DOT1L.[24, 103] This may not be surprising, as the CxxC domain of the MLL protein (and MLL fusion proteins) **(figure 1)** specifically targets non-methylated CpG dinucleotides.[104] The *HOXA9* gene best illustrates target recognition of MLL through binding to the CxxC domain. When MLL fusion proteins bind to specific unmethylated CpGs within the *HOXA9* locus, these CpG sequences become protected from DNA methylation. In addition, this correlates with low levels of the repressing histone mark H3K9me3, thus allowing for active transcription of *HOXA9*.[105] In case the DNA binding function of the MLL CxxC domain is disrupted, the *HOXA9* locus becomes methylated, the repressive H3K9me3 histone mark is enhanced, and the *HOXA9* gene becomes transcriptionally silenced.[105] Furthermore, the DNA binding function of the CxxC domain is required for leukemic transformation.[104, 105] These observations illustrate close interactions between DNA methylation and histone modification in regulating gene expression in *MLL*-rearranged leukemia. Our identification of hypomethylated proto-oncogenes, incuding *HOXA9*, that were recognized earlier as enriched for H3K79 dimethylation induced by aberrant DOT1L recruitment mediated by MLL fusion proteins, is in good concordance with this. Whether inappropriate histone modifications are involved at the other aberrantly methylated gene promoters remains to be investigated in future studies.

With severe promoter DNA hypermethylation on the one hand, and abnormal hypomethylation of crucial MLL fusion targets on the other, important questions remain to be answered. For instance, which of the two features is more important for MLL fusion-driven leukemogenesis? Is one of the two a consequence of the other? Or do both processes act in concert and are equally as important? In a recent paper, Bueno *et al*.[106] showed that introducing the MLL-AF4 fusion protein into human embryonic stem cells readily triggered the expression of known MLL fusion target genes,[24, 103] including genes we identified to be hypomethylated in infant ALL samples typified by the presence of MLL-AF4 **(chapter 6)**. This implies that hypomethylation of MLL fusion target genes represents an early and possibly crucial event for MLL fusion-driven leukemogenesis. For genes which are characteristically hypermethylated and silenced in *MLL*-rearranged infant ALL, the mechanism is less obvious. Unpublished data obtained from ongoing studies showed that MLL-AF4 knock-down in leukemia cell lines does not directly lead to vast changes in the DNA methylation status of hypermethylated genes *[personal communication with Prof. Olaf Heidenreich, Northern Institute for Cancer Research, Newcastle University, United Kingdom]*. This may suggest that once promoter hypermethylation is established in these cells, possibly as a consequence of an *MLL* translocation, methylation becomes more or less fixed. An alternative explanation may be that the observed patterns of promoter hypermethylation **(chapter 2)** are, at least to a great extent, not directly associated with the absence or presence of the MLL fusion protein. Yet, our data clearly showed

significant differences in promoter hypermethylation between *MLL*-rearranged infant ALL cases and infant ALL samples lacking translocations of the *MLL* gene **(chapter 2)**.

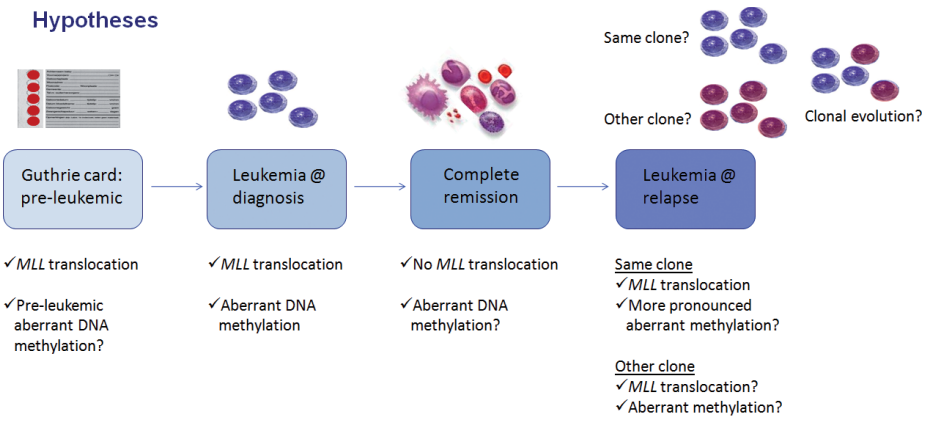
Another way to investigate whether increased aberrant promoter methylation is induced by *MLL* translocations themselves and embodies an important mechanism by which *MLL* rearrangements favor leukemic transformation would be to examine DNA methylation in blood cells obtained from the newborn. Since *MLL* translocations can already be detected in neonatal blood spots (on guthrie cards) from children who develop *MLL*-rearranged ALL within the first year of age,[107, 108] pre-leukemic signs of elevated abnormal promoter methylation might be detectable at this stage. Therefore, we have initiated studies to explore whether abnormal promoter methylation is already detectable at a pre-leukemic state in *MLL*-rearranged infant ALL, and to identify the initial (epi)genetic alterations preceding full leukemic transformation. Detection of pre-leukemic aberrant promoter methylation might provide opportunities for therapeutic intervention at a pre-leukemic stage in the future. In addition, methylation status of relapse cases will be examined. Since we found a reduced relapse-free survival for heavily methylated subgroups of infant ALL,[62] relapses originating from the same leukemic clone are expected to display pronounced and progressed aberrant DNA methylation levels.

EPIGENETIC THERAPIES FOR MLL-REARRANGED ALL

In contrast to genetic abnormalities, epigenetic aberrations are to some extent reversible by epigenetic drugs. Currently, two types of epigenetic drugs have been developed and widely studied both in *in vitro* as well as *in vivo* settings.

I. Histone modifying drugs

The first class of epigenetic drugs involves agents targeting histone modifying enzymes, of which histone deacetylase (HDAC) inhibitors represent the most common compounds.[109] HDAC inhibitors cause histones to become hyperacetylated which is usually related to an open chromatin structure resulting in gene activation. The best-known HDAC inhibitors include vorinostat (SAHA), panobinostat (LBH589), and valproic acid (VPA). SAHA has been granted approval from the U.S. Food and Drug Administration (FDA) for T-cell lymphomas. In addition, LBH589 is in phase III clinical trials for various types of cancers, and VPA is currently in phase II clinical trials for various cancers, including leukemias.[110] Although HDAC inhibitors are considered highly unspecific drugs as they target dynamic chromatin remodelling processes which makes it difficult to predict which genes are eventually influenced, these agents generally only affect <2% of all genes.[111] In addition, apart from “specific” actions actually targeting HDACs, most HDAC inhibitors also seem to exert more “off-target” non-histone effects. Nonetheless, although HDAC inhibitor treatment can by no means be considered targeted therapy, the therapeutic potential of these



compounds in a broad spectrum of human cancers is promising. In addition, it should be taken into account that chemotherapy in general is highly unspecific.

The rationale for using HDAC inhibitors in the treatment of human leukemias carrying chromosomal translocation AML1-ETO has already been established and proposed.[112, 113] Moreover, HDAC inhibitors already demonstrated strong cytotoxic effects in various leukemia cell lines, including models of *MLL*-rearranged acute leukemia.[114, 115] As shown in **chapter 6** of this thesis, we demonstrated that primary *MLL*-rearranged infant ALL samples are highly sensitive *in vitro* to various HDAC inhibitors.[102] Pan-HDAC inhibitors, such as LBH589, seemed to have the strongest leukemia-specific effects: this agent almost entirely eradicated all t(4;11)-positive infant ALL cells *in vitro*, while normal bone marrow cells appeared highly resistant and hardly affected.[102, 116] Although HDAC inhibitors were originally developed to re-activate genes silenced by suppressive histone marks, we showed that LBH589 effectively silenced several of the aberrantly hypomethylated and highly expressed proto-oncogenes, as well as the MLL-AF4 fusion protein (both at the mRNA and protein level) **(chapter 6)**.[102] The finding that HDAC inhibitors, and especially LBH589, are able to degrade the oncogenic MLL fusion protein[102] is of particular interest. Fusion degradation seems to occur before leukemic cell death. Thus by treating *MLL*-rearranged cells with LBH589, the consequences of the MLL translocation are blocked. Hence, LBH589 may well represent an ideal drug for the treatment of *MLL*-rearranged infant ALL. The enormous potential of LBH589 in eliminating *MLL*-rearranged infant ALL cells *in vitro*, and the successful use of this compound in various phase I/II trials in patients with refractory hematologic disorders, warrants clinical trials in (relapsed) *MLL*-rearranged ALL cases. In support of this, Burbury and co-workers recently published a case report describing the successful treatment of a 60-year old patient suffering from *MLL*-rearranged leukemia who sustained a complete cytogenetic response to single-agent HDAC inhibitor treatment.[117]

At the time we published our LBH589 data,[102] another research group reported on the development of a specific DOT1L inhibitor which selectively kills *MLL*-rearranged ALL cells by disrupting the interactions between MLL fusion proteins and DOT1L and eliminating H3K79 dimethylation.[118] However, this first specific DOT1L inhibitor is still in its developmental phase, and improved versions of the compound, following extensive *in vitro* and *in vivo* testing, are required. Therefore, it may well take several years before the first DOT1L inhibitors can be tested in phase I/II trials, and until then, the clinical applicability of these compounds remains uncertain. In contrast, multiple clinical trials using LBH589 are already in progress, and results are promising. Therefore, we are currently testing LBH589 in xenograft mouse models of *MLL*-rearranged ALL to evaluate its potential *in vivo*, and provide sufficient rationale to test this agent in clinical trials. Perhaps, future treatment regimens for *MLL*-rearranged acute leukemias will either include LBH589 or a DOT1L inhibitor, or even a combination of both agents.

II. DNA methyltransferase inhibitors

The second class of epigenetic drugs is represented by demethylating agents which are compounds that can inhibit DNA methylation resulting in the re-expression of previously hypermethylated and silenced genes.[119] The most commonly used demethylating agents are 5-azacytidine (azacitidine),[120] 5-azadeoxycytidine (decitabine),[121] and the more stable zebularine.[122] In **chapter 2**, we show that hypermethylated *MLL*-rearranged ALL cells are highly responsive to demethylating agents *in vitro*, re-activating numerous hypermethylated genes.[62] Moreover, massive re-activation of silenced genes appeared to be accompanied by growth inhibition of >80% of the leukemic cells.[62, 63] Besides targeting hypermethylated and silenced genes, we also demonstrated that zebularine reactivates important miRNAs and, through that, down-regulates certain proto-oncogenes **(chapter 3.2)**. Thus, demethylating cytosine analogues constitute promising candidates for the treatment of *MLL*-rearranged ALL in infants.

However, despite several clinical trials demonstrating biological activity and clinical responses for both decitabine and azacitidine in adults diagnosed with myelodysplastic syndromes (MDS) or chronic myelomonocytic leukemia (CMML),[123 126] results are somewhat disappointing, mainly due to pharmacological instability of the compounds leading to side-effects.[127, 128] These observations warrant the development of more stable demethylating agents, or the identification of alternative drugs with similar properties.

In **chapter 5**, we show that clofarabine, an adenosine analogue currently very promising for the treatment of different types of leukemia,[129-131] also possesses demethylating properties. In contrast to decitabine and zebularine, which typically inhibit DNA methylation at micromolar concentrations, clofarabine was able to inhibit methylation at concentrations as low as 5-10 nM. Besides, primary *MLL*-rearranged infant ALL cells appeared highly sensitive to clofarabine *in vitro*, on average killing

50% of the leukemia cells at a concentration of ~25 nM **(chapter 5)**. As a result of its demethylating activity, clofarabine may provide beneficial effects especially in *MLL*-rearranged infant ALL. On top of this, clofarabine works synergistically with cytarabine, a drug already implemented into the specific treatment protocols for infant ALL. Thus, while the development of more stable demethylating cytosine analogues awaits an important breakthrough, clofarabine may well serve as an attractive substitute. We therefore propose a clinical study using clofarabine in *MLL*-rearranged infant ALL.

III. Epigenetic combination therapy

Demethylating agents often work synergistically with HDAC inhibitors. Recently, it was shown that clofarabine also works synergistically with HDAC inhibitors.[132] Therefore, the effects of combinations of HDAC inhibitors and demethylating agents, including clofarabine, should be tested on *MLL*-rearranged infant ALL cells, both *in vitro* and *in vivo*. In addition, epigenetic drugs are known to increase cytotoxicity of other drugs. Therefore, it is worth testing whether the addition of epigenetic drugs to chemotherapeutics currently used in the treatment of infant ALL, for instance prednisone, influences the cytotoxicity induced by these drugs.

CONCLUDING REMARKS

Contributions from our laboratory (described in this thesis) and other research groups world-wide established that *MLL*-rearranged ALL results from multiple epigenetic hits. On the one hand, MLL fusion proteins induce aberrant histone modifications which in turn appear to trigger inappropriate gene activation accompanied by promoter hypomethylation on specific genes targeted by MLL fusions. On the other hand, *MLL*-rearranged ALL cells seem to harbor vast numbers of abnormally hypermethylated gene promoters, leading to transcriptional silencing of the affected genes. As described, it remains uncertain whether promoter hypermethylation is a direct consequence of the genesis of MLL fusions. Ongoing collaborative studies may eventually answer this question as well as other lingering questions. The field of epigenetics is currently moving forward at high speed, which will make the road ahead even more challenging. Nevertheless, the collective notion that *MLL*-rearranged ALL originates from epigenetic deregulation, and the subsequent observations that this type of leukemia responds remarkably well to epigenetic drugs, may lead to improved treatments in the near future.

Although the biological insights in *MLL*-rearranged ALL have rapidly progressed in the last 10 years, the work is far from completed. We conclude through the studies in this thesis that *MLL*-rearranged infant ALL is an epigenetic malignancy which should be treated accordingly.

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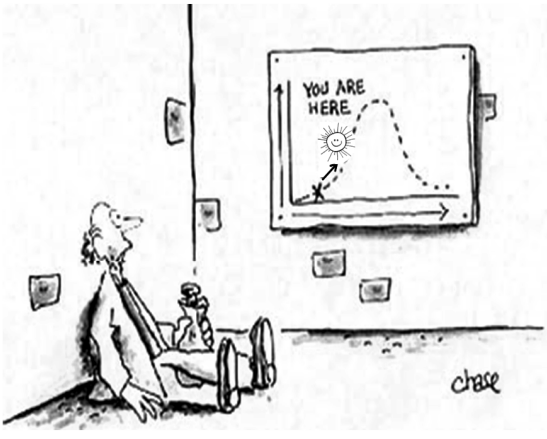

"Still round the corner there may wait,
a new road or a secret gate."

(John R. R. Tolkien)

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“J'ai voulu essayer de faire la synthèse
des connaissances que j’ai acquises.”

(Odile Schweisguth, pioneer of cancer treatment for children, 1979)

8

Bullet Point Summary

Chapter 2: The first insights into the *MLL* methylome

- Unique DNA methylation patterns separate the different genetic subtypes of infant acute lymphoblastic leukemia (ALL)
- The majority of *MLL*-rearranged infant ALL, i.e. the cases harboring t(4;11) or t(11;19), is characterized by aberrant promoter DNA hypermethylation leading to silencing of numerous genes
- A high degree of aberrant DNA methylation confers an increased risk of relapse
- The demethylating agent zebularine reverses the aberrant DNA methylation to some extent and effectively induces apoptosis in *MLL*-rearranged ALL cells

***MLL*-rearranged infant ALL is a hypermethylated subtype of childhood leukemia which is *in vitro* sensitive to demethylation.**

Chapter 3: Aberrant DNA methylation at miRNA loci

- Aberrant DNA methylation in *MLL*-rearranged infant ALL also encompasses aberrant methylation at miRNA loci leading to inactivation of miRNAs and inappropriate activation of miRNA target genes
- The majority of miRNAs silenced due to CpG hypermethylation is associated with the *MLL* gene or MLL-fusion proteins
- Zebularine re-activates hypermethylated miRNA genes in t(4;11)-positive infant ALL
- Heavy methylation of miRNA 152 is strongly correlated with an increased risk of relapse, and both *MLL* and *DNMT1* are potential target genes of miRNA 152

Aberrant methylation in t(4;11)-positive infant ALL is extended to aberrant methylation at miRNA loci. By re-activation of hypermethylated miRNAs, zebularine down-regulates proto-oncogenes in *MLL*-rearranged infant ALL. In addition to being epigenetically regulated, miRNAs can target the epigenetic machinery in *MLL*-rearranged infant ALL.

Chapter 4: Global DNA methylation

- *MLL*-rearranged infant ALL is not characterized by global hypomethylation, despite severe promoter CpG hypermethylation
- Repetitive elements tend to be hypermethylated in *MLL*-rearranged infant ALL
- The repetitive elements LINE-1, Alu and SATα are sensitive to demethylation by decitabine and zebularine

***MLL*-rearranged infant ALL is characterized by an overall increase in genomic methylation which may provide an explanation for the absence of additional genetic aberrations in this disease. In addition, it provides supplementary targets for future epigenetic therapies.**

Chapter 5: The potential of clofarabine

- Primary *MLL*-rearranged infant ALL cells are highly sensitive to nanomolar concentrations of clofarabine *in vitro*
- *MLL*-rearranged infant ALL cells tend to be more responsive to clofarabine *in vitro* than non-infant pre-B ALL cells
- Clofarabine is not dependent on the human equilibrative nucleoside transporter 1 (hENT1) for membrane permeability
- Clofarabine works synergistically with cytarabine in inducing apoptosis in *MLL*-rearranged ALL cells
- Clofarabine causes demethylation and re-expression of the hypermethylated tumor suppressor gene *Fragile Histidine Triad* (*FHIT*) in *MLL*-rearranged ALL cells

Clofarabine is an epigenetic-acting drug. The addition of clofarabine to current chemotherapy protocols for *MLL*-rearranged infant ALL may improve prognosis for these very young patients. Therefore, we suggest the initiation of a clinical study using clofarabine in *MLL*-rearranged infant ALL.

Chapter 6: Targeting specific hypomethylated genes

- The activation of particular (proto-onco)genes in t(4;11)-positive infant ALL is mediated by promoter hypomethylation
- The connectivity map reveals HDAC inhibitors as suitable candidates to reverse high expression of (proto-onco)genes in *MLL*-rearranged infant ALL
- HDAC inhibitors effectively induce leukemic cell death in t(4;11)-positive primary infant ALL cells, which is accompanied by down-regulation of highly expressed (proto-onco)genes
- LBH589 down-regulates the MLL-AF4 fusion, both at the mRNA as well as at the protein level
- After HDAC inhibitor exposure DNA methylation levels are restored in *MLL*-rearranged ALL cells

t(4;11)-positive infant ALL cells are sensitive to different classes of HDAC inhibitors *in vitro*, which may in part be due to down-regulation of the MLL-AF4 fusion.

We conclude that *MLL*-rearranged infant ALL patients may benefit from treatment regimens including epigenetic drugs, such as demethylating agents and HDAC inhibitors.

“Enthousiasme, creativiteit en nieuwsgierigheid zijn onmisbaar voor een onderzoeker, maar het kunnen overbrengen van zijn/haar onderzoeksideeën aan een wijder publiek is wat werkelijk bijdraagt aan het vooruit brengen van de wetenschap.”

(Geïnspireerd door Yvonne C.M. Staal, 2007)

9

Layman's Summary in Dutch

Nederlandse samenvatting voor niet-ingewijden

INLEIDING

Bloed

Het bloed dat door de bloedvaten van het menselijk lichaam stroomt, bestaat voor ongeveer de helft uit een gelige vloeistof (het bloedplasma) met daarin verschillende soorten bloedcellen. De rode bloedcellen (erythrocyten) zijn het sterkst vertegenwoordigd en het hemoglobine in deze rode bloedcellen geeft het bloed de karakteristieke rode kleur. Verder onderscheiden we nog witte bloedcellen (leukocyten) en bloedplaatjes (thrombocyten). De rode bloedcellen zorgen voor zuurstoftransport door het lichaam, terwijl de bloedplaatjes voor de stolling van het bloed zorgen. De witte bloedcellen zijn belangrijk voor de afweer tegen ziekten. Zij kunnen verder onderverdeeld worden in lymfatische cellen (B- en T-lymfocyten) en niet-lymfatische oftewel myeloide cellen.

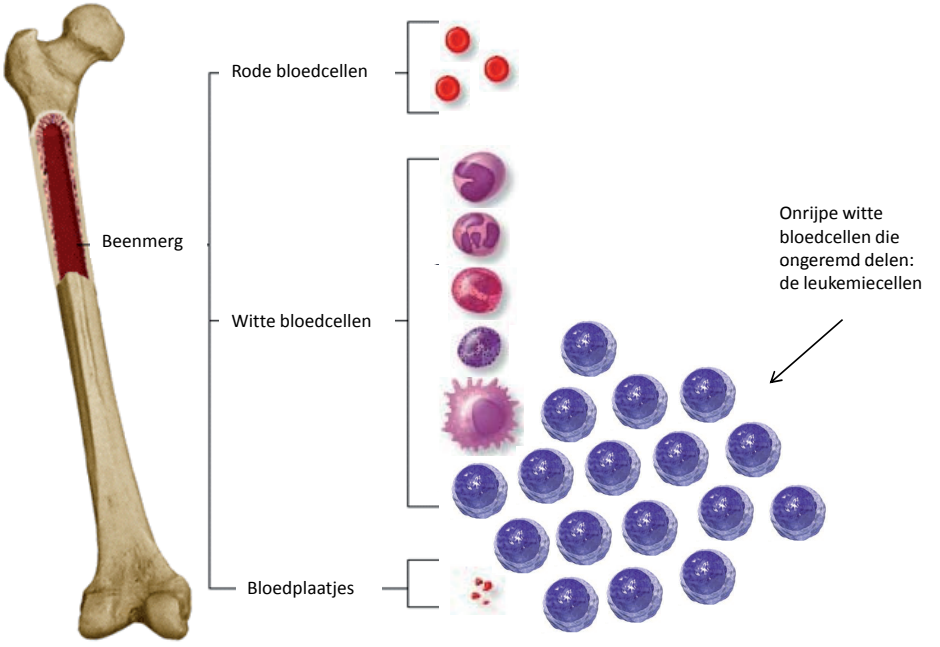
Bloedcellen hebben een beperkte levensduur. Om die reden moeten er voortdurend nieuwe bloedcellen worden aangemaakt. De vorming van nieuwe bloedcellen (hematopoiese) vindt plaats in het beenmerg dat zich in het binnenste van de botten bevindt **(figuur 1)**. Alle verschillende soorten bloedcellen ontstaan uit één gemeenschappelijke voorloper cel, de hematopoietische stamcel. Onder invloed van complexe regelmechanismen ontwikkelen zich uit onrijpe, nog niet functionele stamcellen rijpe en functionele bloedcellen. Wanneer de bloedcellen volledig zijn uitgerijpt en functioneel zijn, worden deze afgegeven aan het bloed.

Wat is leukemie?

Leukemie ontstaat wanneer onrijpe voorlopers van de witte bloedcellen zich ongecontroleerd beginnen te vermenigvuldigen **(figuur 1)**. De opeenstapeling van leukemiecellen in het beenmerg zal op den duur ten koste gaan van de aanmaak van gezonde functionele bloedcellen. Als gevolg hiervan zullen er te weinig rode bloedcellen aangemaakt kunnen worden, hetgeen leidt tot bloedarmoede (anemie). Verder ontstaat er een tekort aan bloedplaatjes in het bloed wat zal leiden tot bloedingen en blauwe plekken. Tot slot leidt de uitval van gezonde witte bloedcellen tot een minder goede afweer en een verhoogd risico op infecties. Wanneer alle ruimte in het beenmerg is ingenomen door de leukemiecellen, kunnen deze leukemiecellen zich via het bloed door het lichaam verspreiden en zo andere organen, zoals de lever en de milt, infiltreren. Zonder adequate behandeling met chemotherapie is leukemie dodelijk.

Verschillende soorten leukemie

Leukemie kan in elk van de verschillende typen witte bloedcellen ontstaan. Dit betekent dat leukemie eigenlijk een verzamelnaam is voor een breed spectrum aan soorten kanker van de bloedcellen. Zo kan leukemie ontstaan in een myeloide of een lymfatische cel, hetgeen zal leiden tot respectievelijk myeloide of lymfatische leukemie. Verder kan leukemie onderverdeeld worden in “acute” of “chronische” vormen. Acute leukemie wordt gekenmerkt door een snelle, agressieve uitbreiding

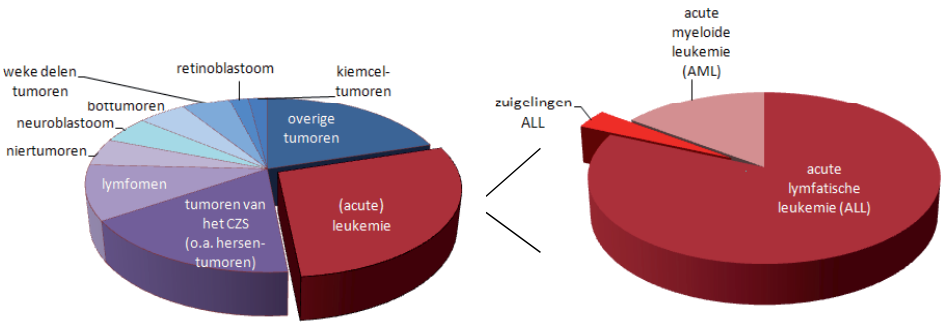


Figuur 1. Bloedcelvorming en het ontstaan van leukemie. (Sterk vereenvoudigde weergave)
Rode bloedcellen, witte bloedcellen en voorlopers van bloedplaatjes worden in het beenmerg in het binnenste van de botten gevormd. Wanneer onrijpe witte bloedcellen ongecontroleerd gaan delen, ontstaat er leukemie (Grieks voor “wit bloed”). De leukemiecellen verdringen op den duur de gezonde bloedcellen in het beenmerg.

van onrijpe voorloper cellen. Chronische leukemie ontwikkelt zich over het algemeen wat trager. Chronische myeloide leukemie en chronische lymfatische leukemie komen met name voor bij volwassenen. Acute leukemie wordt met name gediagnosticeerd bij kinderen. Acute myeloide leukemie komt slechts voor bij 15% van de kinderen met leukemie. De meerderheid van de kinderen met leukemie (ongeveer 85%) heeft acute lymfatische leukemie **(figuur 2)**.

Leukemie bij kinderen

Acute leukemie is de meest voorkomende kanker op de kinderleeftijd **(figuur 2)**. In Nederland worden jaarlijks ongeveer 150 kinderen gediagnosticeerd met leukemie. De meerderheid van deze kinderen heeft acute lymfatische leukemie (ALL). Hier kan weer een onderverdeling worden gemaakt tussen verschillende typen ALL. Lymfatische leukemie kan namelijk ontstaan in onrijpe T-lymfocyten of onrijpe B-lymfocyten. ALL voortkomend uit een onrijpe B-lymfocyt komt veel vaker voor dan ALL uitgaande van een onrijpe T-cel. De piekleeftijd van ALL bij kinderen is 2-5 jaar. Middels intensieve chemotherapie geneest ongeveer 85% van alle kinderen met ALL. Ondanks deze goede resultaten, zijn er helaas nog altijd kinderen met ALL die hun ziekte niet overleven.



Figuur 2. Verdeling van kanker op de kinderleeftijd in Nederland.
Cirkeldiagram links: van alle kinderen (0-18 jaar) met kanker wordt bij de meesten leukemie vastgesteld. Cirkeldiagram rechts: acute lymfatische leukemie (ALL) is het meest voorkomende type leukemie bij kinderen. In 4% van de gevallen van ALL bij kinderen wordt de leukemie gediagnosticeerd bij een zuigeling (jonger dan 1 jaar). (*Gegevens verkregen uit de Basisregistratie van de Stichting Kinderoncologie Nederland (SKION), 2009*) Afkorting: CZS = centraal zenuwstelsel.

Leukemie bij zuigelingen

ALL bij zuigelingen (kinderen jonger dan 1 jaar) is de meest agressieve vorm van leukemie op de kinderleeftijd. In tegenstelling tot de overlevingskansen voor kinderen ouder dan 1 jaar, zijn de overlevingskansen voor zuigelingen met ALL slechts ongeveer 50%. Hoewel het met behulp van de huidige chemotherapie doorgaans wel lukt om ogenschijnlijk alle leukemiecellen in het lichaam uit te roeien, komt de leukemie bij zuigelingen vaak binnen een jaar na diagnose weer terug. De terugkerende leukemiecellen zijn dan meestal niet gevoelig meer voor de standaard chemotherapie. Het feit dat de leukemie bij zuigelingen zo dikwijls terugkomt, suggereert dat enkele leukemiecellen de behandeling met chemotherapie hebben overleefd. In feite betekent dit dat de huidige chemotherapie voor zuigelingen met ALL niet toereikend is. Er zullen spoedig meer adequate medicijnen gevonden moeten worden die beter in staat zijn alle leukemiecellen te vernietigen om zo de kans op het terugkeren van de leukemie te verkleinen.

Genetica: het *MLL* gen en MLL afwijkingen

Alle cellen in het menselijk lichaam bevatten een kern waarin zich het DNA bevindt **(figuur 3)**. Het DNA vormt de blauwdruk van alle processen in de lichaamscellen. Het beschikt daarvoor over ongeveer 30.000 verschillende genen, waarin stukjes informatie worden bewaard. Zo zijn er genen die bepalen welke kleur ogen we hebben, of bijvoorbeeld hoe lang we worden.

Het *MLL* gen is essentieel voor de aanmaak van gezonde bloedcellen. Dit is onder andere gebleken uit studies waarbij in muizen opzettelijk het *MLL* gen is uitgeschakeld. Deze muizen vertonen ernstige defecten in de bloedcelvorming en zijn bovendien niet levensvatbaar. Bij zuigelingen met ALL zien we zeer frequent (bij ongeveer 80%

van de patiënten) afwijkingen in het *MLL* gen. Hierbij wordt niet het gehele *MLL* gen geïnactiveerd, maar is het gen wel dusdanig aangetast dat het een deel van zijn functie verliest. Deze afwijkingen vormen de basis voor het ontstaan van ALL bij zuigelingen. De defecten in het *MLL* gen komen uitsluitend voor in de leukemiecellen, en niet in de gezonde cellen van de patiënt. Wanneer deze afwijkingen ook in gezonde cellen zouden voorkomen, zou dit wijzen op een erfelijke aandoening. De afwijkingen van het *MLL* gen daarentegen, ontstaan tijdens het ontwikkelingsproces van het kind. Verscheidene studies hebben aangetoond dat de defecten van het *MLL* gen tijdens de zwangerschap ontstaan in de witte bloedcellen van het nog ongeboren kind. Zuigelingen met ALL worden dan ook doorgaans geboren met leukemie, hoewel dat bij de geboorte lang niet altijd meteen merkbaar is. Wel is het zo dat leukemiecellen met een afwijking van het *MLL* gen zich zeer agressief ontwikkelen. Dit leidt ertoe dat de leukemie zich vrijwel altijd binnen het eerste levensjaar openbaart. Het hebben van leukemie-specifieke *MLL* afwijkingen is geassocieerd met een slechte prognose. De overlevingskansen voor zuigelingen met leukemie met *MLL* afwijkingen liggen rond de 30-40%.

Defecten in het *MLL* gen worden zeer weinig gevonden bij oudere kinderen met leukemie. Dit heeft waarschijnlijk alles te maken met het agressieve karakter van deze vorm van leukemie: De aanwezigheid van *MLL* afwijkingen maakt dat de leukemie zich zo snel ontwikkelt en zich dus bijna altijd zal openbaren binnen het eerste levensjaar. Bij oudere kinderen spelen dan ook heel andere afwijkingen een rol in het ontstaan van de leukemie. Met recht kan dus gezegd worden dat leukemie bij zuigelingen met afwijkingen aan het *MLL* gen (we noemen dit *MLL*-herschikte leukemie bij zuigelingen) een uniek type leukemie vertegenwoordigt. Om die reden zal de ziekte ook anders behandeld moeten worden dan andere soorten leukemie bij kinderen.

Epigenetica

Op de voorkant van dit proefschrift is de metamorfose van rups naar vlinder afgebeeld als voorbeeld van epigenetische regulatie. De rups en de vlinder hebben namelijk precies dezelfde genen, echter door epigenetische regulatie worden er heel andere genen aan- en uitgezet, waardoor een rups in een vlinder verandert. Verstoorde epigenetische regulatie speelt een rol bij de transformatie van gezonde witte bloedcellen naar kwaadaardige leukemiecellen.

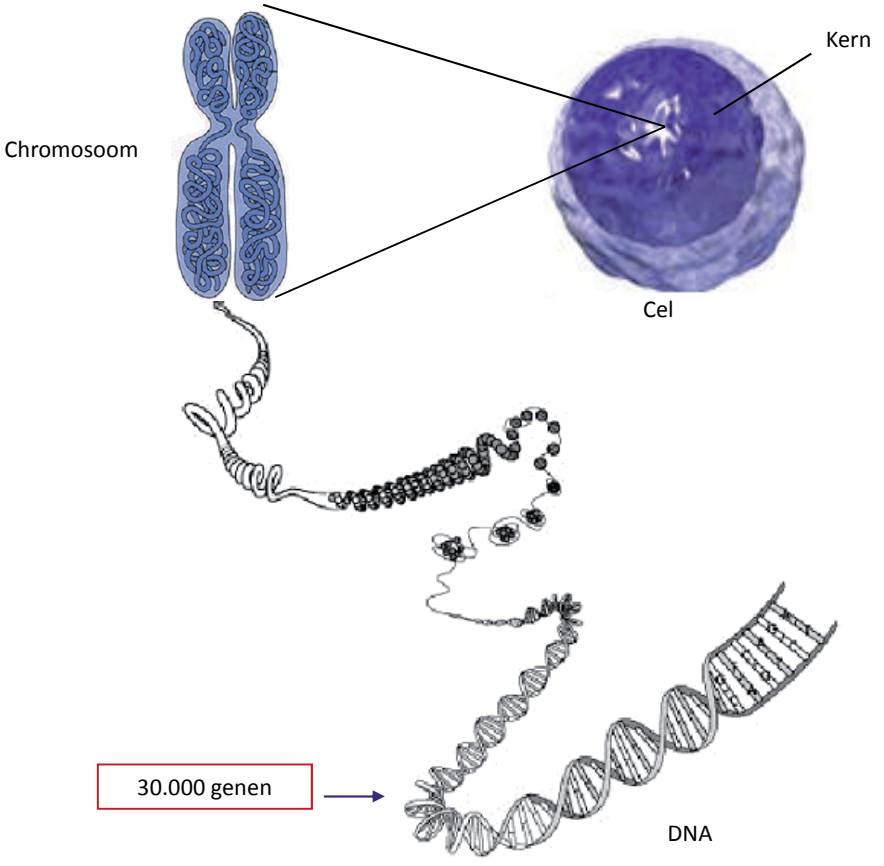
Zoals alle cellen in ons lichaam, gebruiken witte bloedcellen vernuftige en complexe regulatiesystemen om hun specifieke functie uit te kunnen voeren. Zoals beschreven bevat elke lichaamscel ongeveer 30.000 genen **(figuur 3)**. Toch bestaat er geen enkele cel die daadwerkelijk al deze genen gebruikt. Elk type cel heeft zijn eigen functie in ons lichaam en gebruikt een deel van de 30.000 genen om deze functie invulling te geven. Genen die nodig zijn voor het goed functioneren van bijvoorbeeld spiercellen zullen veelal overbodig zijn voor het goed functioneren van huidcellen. Wanneer cellen de verkeerde genen gaan gebruiken, gaat dit ten koste van hun functie. Het is dus van het grootste belang dat elke cel correct gebruik maakt van juist die set genen

die past bij de specifieke functie die de cel dient uit te voeren. Om dit te bereiken hebben cellen regulatiemechanismen tot hun beschikking welke ervoor zorgen dat uitsluitend de juiste genen worden geactiveerd. De epigenetica omvat de studie naar een dergelijk regulatiesysteem. Epigenetische regulatie zorgt ervoor dat een gen al dan niet geactiveerd kan worden. Genen die overbodig zijn voor een bepaald celtype zullen door epigenetische veranderingen in het DNA worden afgesloten en daardoor inactief worden. Het afsluiten van deze genen komt tot stand door een tweetal acties. Ten eerste wordt op plaatsen in het DNA waar genen liggen die niet nodig zijn voor de functie van een bepaalde cel het DNA zeer compact ineen gewonden **(figuur 4)**. Dit voorkomt dat zogenaamde transcriptie factoren (eiwitten in de cel die tot doel hebben genen te activeren) geen toegang meer hebben tot deze genen. Als extra slot op de deur wordt het de transcriptie factoren onmogelijk gemaakt zich te kunnen binden aan het begin van deze genen (waar de transcriptie factoren zich verzamelen om een gen te activeren). Het eerstgenoemde proces heeft te maken met de flexibele structuur van ons DNA. Voor een goede structuur wikkelt het DNA zich om zeer grote eiwitten (de histonen) welke bepalen waar het DNA dient te worden gesloten, en waar het DNA een wat "lossere" structuur aan mag nemen waardoor de genen op die plaatsen in ons DNA toegankelijk worden. De keuze van de histonen om het DNA een "open" of "gesloten" structuur te verschaffen, komt tot stand door subtiele signalen die aan de histonen worden meegegeven. Het tweede epigenetische proces waarbij het begin van een gen nog eens extra wordt geblokkeerd, vindt plaats aan het DNA zelf. Rond het begin van genen die door de desbetreffende cel als onnodig worden beschouwd, plaatst de cel massaal kleine chemische structuren (de zogenaamde methyl groepen) juist op die plaatsen waar transcriptie factoren willen binden. De aanwezigheid van deze methyl groepen verhindert vervolgens dat de transcriptie factoren het gen kunnen activeren **(figuur 4)**. We spreken dan van een gen dat door methylatie is geïnactiveerd.

Het is niet ongebruikelijk dat cellen in de loop van hun bestaan hun functie moeten aanpassen of flexibel moeten kunnen reageren op externe factoren welke minimale aanpassingen in functioneren vereisen. De epigenetische regulatie van genen is dan ook nooit definitief, maar flexibel en omkeerbaar. Indien nodig kunnen de methyl groepen aan het begin van afgesloten genen weer worden verwijderd en kunnen de histonen een meer "open" structuur aannemen. Hierdoor worden eerder geïnactiveerde genen weer actief gemaakt.

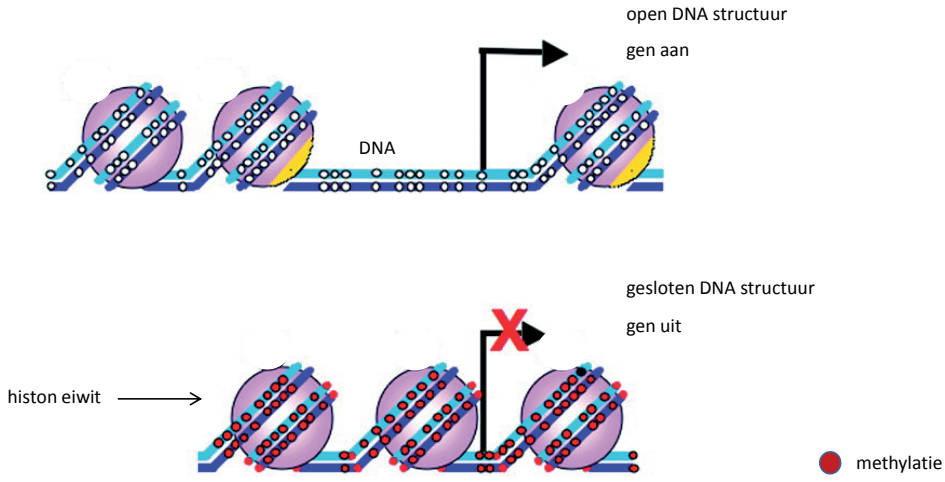
Epigenetica en leukemie (Doel van het onderzoek)

Bij kanker is de epigenetische regulatie vaak verstoord. Dikwijls wordt er een teveel aan methylatie gevonden wat leidt tot het inactiveren van genen die ons normaliter tegen het ontstaan van kanker beschermen (zogenaamde tumor onderdrukkende genen). Tegelijkertijd blijkt er juist methylatie te ontbreken op genen die de ontwikkeling van kanker vergemakkelijken (de zogenaamde kanker stimulerende genen). Doorgaans liggen er abnormale histon structuren ten grondslag aan het foutief plaatsen van methyl groepen aan het begin van de desbetreffende genen.



Figuur 3. Het DNA en genen.

Zoals gezegd is het *MLL* gen belangrijk voor de ontwikkeling van witte bloedcellen. *MLL* zorgt voor een juiste ontwikkeling van bloedcellen door tijdens de uitrijping van een bloedcel op tijd de epigenetische regulatie te beïnvloeden. Wanneer in het geval van *MLL*-herschikte leukemie bij zuigelingen de functie van het *MLL* gen is aangedaan, kan verwacht worden dat de epigenetische regulatie van de ontwikkeling van gezonde witte bloedcellen ontspoort. Hoewel dit voor de hand lijkt te liggen, was er nog maar erg weinig bekend over met name de DNA methylatie in *MLL*-herschikte leukemie. Het doel van het in dit proefschrift beschreven onderzoek was dan ook het karakteriseren van specifieke DNA methylatie patronen in *MLL*-herschikte ALL bij zuigelingen. Tevens is getracht te onderzoeken of bepaalde medicijnen die ingrijpen op epigenetische processen *MLL*-herschikte ALL cellen kunnen doden.



Figuur 4. Epigenetische regulatie.
Het DNA zit om histon eiwitten gewonden. Bovenste figuur: open DNA structuur waarbij een gen actief is. Onderste figuur: gesloten DNA structuur met als extra "slot" methylatie op het DNA (rode cirkels), zodat een gen geïnactiveerd wordt.

RESULTATEN

Overmatige methylatie aan kanker onderdrukkende genen

In **hoofdstuk 2** hebben we de mate van methylatie aan het begin van alle genen in het DNA in kaart gebracht in de leukemiecellen van zuigelingen met *MLL*-herschikte ALL. Om goed te kunnen onderscheiden of de methylatie op bepaalde genen "normaal" dan wel "afwijkend" was, is er telkens een vergelijking gemaakt met witte bloedcellen uit het beenmerg van gezonde kinderen. We vonden dat de leukemiecellen van zuigelingen met *MLL*-herschikte leukemie worden gekenmerkt door teveel methylatie aan het begin van talloze genen. Voorts bleek dat, zoals verwacht, deze methylatie leidde tot een verminderde werking of zelfs de totale uitschakeling van deze genen. Veel van de genen welke in de leukemiecellen door methylatie hun activiteit hadden verloren, bleken genen te zijn die normaliter een kanker onderdrukkende werking hebben. Vanzelfsprekend speelt het uitvallen van dit soort genen een rol bij de ontwikkeling van leukemie. Dit bleek tevens uit onze observatie dat de mate van methylatie zoals gevonden in de leukemiecellen van zuigelingen met *MLL*-herschikte ALL, het verloop van de ziekte voorspelt. Kinderen bij wie de leukemiecellen ernstig afwijkende methylatie vertoonden, bleken een groot risico te lopen op het terugkeren van de leukemie en het falen van de behandeling. Keerzijde van deze verontrustende bevinding was dat deze resultaten ook een mogelijke nieuwe behandeling aan het licht brachten. Er bestaan namelijk medicijnen welke afwijkende methylatie ongedaan maken. We hebben aan kunnen tonen dat deze zogenaamde demethylerende

middelen de foutieve methylatie inderdaad ongedaan maakten. De leukemiecellen bleken afhankelijk te zijn van de verstoorde methylatie om te kunnen functioneren. Ze konden niet meer overleven na toediening van de demethylerende medicijnen. Daarentegen bleken leukemiecellen van een ander leukemie type, zonder afwijkingen in het *MLL* gen en zonder verstoorde methylatie patronen, nauwelijks te reageren op deze medicijnen. Dit maakt dat demethylerende middelen interessante kandidaten zijn voor een verbeterde therapie voor specifiek zuigelingen met *MLL*-herschikte leukemie. Echter, voordat deze medicijnen kunnen worden toegevoegd aan de bestaande chemotherapeutica, zullen er extra experimenten moeten worden uitgevoerd. Hoewel demethylerende middelen in staat zijn om in het laboratorium leukemiecellen te doden, moet nog blijken of deze middelen dat ook kunnen in het lichaam. Om dit te onderzoeken worden er momenteel experimenten uitgevoerd waarbij muizen worden ingespoten met leukemiecellen van zuigelingen met *MLL*-herschikte ALL, welke vervolgens worden behandeld met deze demethylerende middelen. Wanneer deze experimenten laten zien dat de demethylerende medicijnen ook in staat zijn de leukemiecellen aan te pakken in de muis, zouden deze middelen gebruikt kunnen gaan worden bij de behandeling van kinderen met deze agressieve vorm van leukemie. Echter, zo ver is het nog niet. De tot nu toe beschikbare demethylerende medicijnen zijn instabiel gebleken wat kan leiden tot ongewenste bijwerkingen. Verder hebben klinische testen bij volwassenen met kanker uitgewezen dat deze middelen lang niet altijd zo effectief zijn als gehoopt. Toch staat de ontwikkeling van nieuwe medicijnen niet stil. Zo is er niet lang geleden een nieuw middel gelanceerd dat zeer adequaat blijkt te werken in de behandeling van kinderen met verscheidene soorten leukemie. Een eerdere publicatie over dit medicijn, genaamd clofarabine, suggereerde dat dit middel mogelijk demethylerende eigenschappen zou bezitten. In **hoofdstuk 5** hebben we onderzocht of dit daadwerkelijk het geval was, en of clofarabine daarmee een versterkt anti-leukemisch effect vertoont in *MLL*-herschikte ALL. Uit onze studies bleek dat *MLL*-herschikte zuigelingenleukemiecellen inderdaad zeer gevoelig zijn voor clofarabine, en dat het medicijn tevens een demethylerende werking heeft. Clofarabine lijkt dus een veelbelovend medicijn te zijn voor verbeterde behandeling van zuigelingen met *MLL*-herschikte leukemie.

Afwezigheid van methylatie aan kanker stimulerende genen

Toen we de DNA methylatie patronen beter bekeken, bleek er meer aan de hand dan uitsluitend overmatige methylatie aan genen die de ontwikkeling van kanker onderdrukken. Zoals beschreven in **hoofdstuk 6** zagen we dat op genen die in staat zijn de ontwikkeling van kanker te stimuleren de methylatie juist was verdwenen. Het wegvallen van de methylatie op deze genen bleek tot gevolg te hebben dat deze genen zeer actief waren in de leukemiecellen van zuigelingen met *MLL*-herschikte ALL. De abnormale activatie van deze genen in de leukemiecellen van zuigelingen met *MLL*-herschikte ALL lijkt dus betrokken bij het ontstaan van de leukemie. Ook hier bleek te gelden dat naar mate er meer van dit soort kanker stimulerende genen ten

onrechte geactiveerd bleken te zijn, de kans op het falen van de behandeling groter was. Interessant genoeg bleken er medicijnen te bestaan welke de ontbrekende methylatie aan de kanker stimulerende genen konden herstellen. In tegenstelling tot de demethylerende middelen werken deze middelen (de zogenaamde HDAC remmers) niet direct op het DNA, maar dereguleren deze medicijnen foutieve histon configuraties. Met andere woorden, deze HDAC remmers brengen veranderingen aan in de "open" en "gesloten" gebieden in het DNA, door de activiteit van de histonen te beïnvloeden. Onder invloed van deze medicijnen zagen we dat foutief geactiveerde kankergenen werden uitgeschakeld door vernieuwde methylatie aan het begin van deze genen. Veel belangrijker echter was de observatie dat deze middelen effectief in staat bleken *MLL*-herschikte ALL cellen te doden. Sterker nog, deze HDAC remmers bleken in staat om het afwijkende *MLL* gen, zo karakteristiek voor deze vorm van leukemie, uit te schakelen. Net als de demethylerende middelen worden er momenteel ook verscheidene HDAC remmers getest in eerder beschreven muizen studies. Mogelijk dat een combinatie van dit soort middelen in de toekomst bij zal dragen aan een verbeterde prognose voor zuigelingen met *MLL*-herschikte leukemie.

Meer gevolgen van verstoorde methylatie

Normaal gesproken gaat in kanker cellen een overmaat aan methylatie aan het begin van bepaalde genen ten koste van de methylatie elders in het DNA. Echter, in **hoofdstuk 4** laten we zien dat dit niet het geval is in *MLL*-herschikte ALL bij zuigelingen. Hoewel er overduidelijk te veel methylatie aan talloze genen werd waargenomen, bleek de methylatie in de rest van het DNA nog keurig in tact. Ogenschijnlijk lijkt dit niet zo heel belangrijk, maar dit opmerkelijke fenomeen bleek verstrekkende gevolgen te hebben. Recent is namelijk een nieuw "systeem" ontdekt dat bijdraagt aan de regulatie van de 30.000 genen in onze cellen. Het bleek dat in de gebieden in het DNA waar geen "echte" genen liggen, zich kleine gen-achtige structuren ophouden. Deze zogenaamde microRNAs kunnen niet worden gezien als genen, daar zij zelf geen "boodschap" of "informatie" verspreiden in de cel. Wat deze microRNAs wel doen, is de boodschap van bepaalde genen verstoren, en daarmee normaliter bijdragen aan de correcte regulatie van de 30.000 genen in de cel. In **hoofdstuk 3** laten we zien dat de hardnekkige methylatie in het DNA op plaatsen waar geen genen liggen (zoals gevonden in **hoofdstuk 4**) tot gevolg heeft dat sommige van deze microRNAs hun werk niet kunnen doen. Blijkbaar is de methylatie van een microRNA afdoende om het te inactiveren. Wanneer microRNAs inactief zijn, verliezen zij de grip op de genen die zij normaliter onderdrukken of verstoren. De activiteit van deze genen kan vervolgens afwijkend worden. Zo zorgt methylatie aan het begin van daadwerkelijke genen er dus voor dat die genen uit komen te staan, maar zorgt methylatie aan microRNAs ervoor dat er andere genen juist geactiveerd worden. Het zal niet verrassend zijn dat de ten onrechte geactiveerde genen als gevolg van microRNA methylatie genen bleken te zijn welke weer een stimulerende werking bleken te hebben op de ontwikkeling van leukemie.

CONCLUSIES EN TOEKOMSTPERSPECTIEVEN

Al het hierboven en in dit proefschrift beschreven werk laat zien dat *MLL*-herschikte ALL bij zuigelingen een type leukemie is dat wordt gekenmerkt door een zeer sterk ontregelde epigenetische regulatie. Het is dan ook niet verwonderlijk dat juist medicijnen welke ingrijpen op epigenetische processen in de cel in staat zijn deze soort leukemiecellen zeer effectief te doden. Hoewel de rol van epigenetische ontregeling in kwaadaardige ziekten al in 1983 werd ontdekt, heeft het onderzoeksveld dat zich bezighoudt met dit fenomeen lang in de schaduw gestaan van de klassieke genetica van kanker. Echter, in de afgelopen jaren is wereldwijd het besef gegroeid dat de epigenetica in veel gevallen lang open liggende vragen kon beantwoorden. Ook de notie dat *MLL*-herschikte ALL met recht een epigenetische ziekte kan worden genoemd is niet onopgemerkt gebleven. Het mag dan ook als een positieve ontwikkeling worden beschouwd dat veel onderzoekers welke hun aandacht richten op *MLL*-herschikte leukemie zich meer en meer verdiepen in de epigenetica. Maandelijks verschijnen er nieuwe publicaties waarin onderzoekers nieuwe epigenetische bevindingen beschrijven welke betrokken zijn bij het ontstaan van deze vorm van leukemie. Tevens zet dit veel onderzoekers aan om ook eventuele epigenetische misstappen te verkennen in andere typen leukemie. Daarnaast heeft de ontwikkeling van zogenaamde epigenetische medicijnen een vogelvlucht genomen, met als gevolg dat deze middelen steeds specifieker en verfijnder worden. Hoewel deze medicijnen (nog) niet voor alle vormen van kanker heilzaam zullen zijn, laten de onderzoeksresultaten beschreven in dit proefschrift zien dat zuigelingen met *MLL*-herschikte ALL wellicht gebaat kunnen zijn bij behandeling met epigenetische medicijnen.

De metamorfose van rups naar vlinder op de voorkant van dit proefschrift symboliseert ook zuigelingen met leukemie die na verbeterde behandeling (mogelijk met epigenetische medicijnen) hun vleugels weer wijd uit kunnen slaan om vrij te vliegen en hun dromen waar te maken.

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Abbreviations

2-CdA	= 2-Chlorodeoxyadenosine (Cladribine)
5-aza-dC	= 5-Aza- 2'-deoxycytidine (Decitabine)
ALL	= Acute lymphoblastic leukemia
AML	= Acute myeloid leukemia
Ara-C	= Cytarabine
Array CGH	= Array Comparative Genomic Hybridization
BM	= Bone marrow
BMT	= Bone marrow transplantation
CAFdA	= 2-Chloro-9-(2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl)adenine (Clofarabine)
CD	= Cluster of differentiation
cDNA	= copy DNA
ChIP	= Chromatin immunoprecipitation
CLL	= Chronic lymphoblastic leukemia
CMAP	= Connectivity map
CML	= Chronic myeloid leukemia
CMML	= Chronic myelomonocytic leukemia
CNS	= Central nervous system
CpG	= cytosine – phosphate – guanine
CR	= Complete remission
DAVID	= Database for Annotation, Visualization, and Integrated Discovery
DMH	= Differential methylation hybridization
DNA	= Deoxyribonucleic acid
DNMT	= DNA methyltransferase
DOT1L	= DOT1-like histone H3 methyltransferase
EFS	= Event-free survival
FACS	= Fluorescence-activated cell sorting
FCS	= Fetal calf serum
FDA	= Food and Drug Administration
FDR	= False discovery rate
FHIT	= *fragile histidine triad* gene
FISH	= Fluorescent in situ hybridization
FK228	= Romidepsin
GEO	= Gene Expression Omnibus
GEP	= Gene expression profiling
HAT	= Histone acetyltransferase
H3K4me3	= Histone 3 lysine 4 trimethylation
H3K79me2	= Histone 3 lysine 79 dimethylation
HDAC	= Histone deacetylase
hENT1	= human equilibrative nucleoside transporter
HMT	= Histone methyltransferase
HDM	= Histone demethylase
HOXA	= Homeobox cluster A
HSC	= Hematopoietic stem cell

HSCT = Hematopoietic stem cell transplantation
IC_{50} = Concentration inhibitory to 50% of the cells
LBH589 = Panobinostat
LC_{50} = Concentration lethal to 50% of the cells
limma = linear models for microarray analysis
LINE-1 = Long interspersed element 1
loess = locally weighted scatterplot smoothing
LUMA = LUminometric Methylation Assay
MDS = Myelodysplastic syndrome
miR-gene = microRNA gene
miRNA = microRNA
MLL = Mixed lineage leukemia
mRNA = messenger RNA
MS275 = Entinostat
MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide
NBMPR = *S*-(4-Nitrobenzyl)-6-thioinosine
NK = Natural killer
OS = Overall survival
PBMC = Peripheral blood mononuclear cells
PBS = Phosphate buffered saline
PCA = Principal component analysis
PCR = Polymerase chain reaction
pEFS = Probability of event-free survival
PHD finger = Plant homeo domain finger
PSQ = Pyrosequencing
RFS = Relapse-free survival
RNA = Ribonucleic acid
RT-PCR = Reverse transcriptase polymerase chain reaction
SAH = S-Adenosyl Homocysteine
SAHA = Suberoylanilide hydroxamic acid (Vorinostat)
SAHH = S-Adenosyl Homocysteine Hydrolase
SAM = S-Adenosyl Methionine
SATα = Satellite α
SEM = Standard error of the mean
SET = Suppressor of variegation 3-9, Enhancer of zeste, Trithorax
SINE = Short interspersed element
TSA = Trichostatin A
TSG = Tumor suppressor gene
UTR = Untranslated region
VPA = Valproic acid
VSN = variance stabilizing normalization
WBC = White blood cell
WHO = World Health Organization
WT = Wild-type

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“I not only use all the brains that I have,
but all that I can borrow”

(Woodrow T. Wilson (1856-1924), 28th President of the USA)

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"Ab Jove principum.''
"Laat ons met het voornaamste beginnen."
(Virgilius)

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Paranimfen

Pauline Schneider, lieve **Pauline**, ik had het geluk dat jij mij de eerste maanden in het lab op sleeptouw nam.

"Whatever you do, I'll do it too.
Show me everything and tell me how!"
(Phil Collins)

Daarnaast was het een luxe dat jij bij grote experimenten mee pipetteerde. Zoals jij weet, vond ik het de eerste jaren lastig om werk uit handen te geven, maar later was ik dolblij met jouw hulp (en jouw FHIT blotjes waren gewoon mooier)! Jij bent inmiddels hoofdanaliste geworden, hebt samen met Marien drie prachtige kinderen gekregen en toch weet jij altijd tijd vrij te maken voor experimenten aan zuigelingen leukemie. Petje af hoe jij dit voor elkaar krijgt! Ronald is de drijvende kracht achter onze onderzoeksgroep, maar jouw onvoorwaardelijke steun aan zijn zijde is daarbij onmisbaar. Pauline, ik vind het een grote eer dat jij naast mij zal staan als paranimf. Dankjewel voor jouw geweldige hulp en jouw lieve steun, zowel tijdens het onderzoek als in de aanloop naar de promotie plechtigheid! Zonder jouw hulp was dit boekje er niet geweest.

Patricia Garrido Castro, dear **Patricia**, we met when you came over to stay at my place for your job interview in 2010. From the minute we met, I knew we were sharing the same passion for the MLL research. Of course you got the job and now you are starring as a post-doc in the infant leukemia research group. I really appreciated your wise words and the experience you shared on how to finish a thesis. Also your English proficiency and your great writing style were very helpful. Above all, I would like to thank you for our wonderful friendship and for the warm and caring person you are! It's a great honour for me to have you by my side as a "paranimf". Now that the thesis has been finished there will be a lot more time for "wijntjes aan de Kralingse Plas".

Collega's Zuigelingen Leukemie Onderzoeksgroep

In 2006 startten wij met drie personen, maar al snel groeide de "infant group" uit tot een grote groep enthousiaste mensen.

"Someone who walks alone may reach his destination first,
but someone who is accompanied by a team will certainly travel much farther."

Lieve **Jill**, in 2007 begon jij als tweede promovenda in onze groep. Jij verbaasde ons met jouw handigheid in het lab en jij groeide uit tot een van de meest behulpzame collega's. Daarnaast hebben wij mogen genieten van de kookkunsten van Léon en jou. Dankjewel voor jouw lieve steun in de laatste fase van mijn promotie en veel succes met jouw laatste loodjes (wordt een mooi boekje!) en daarna met jouw verdere carrière als post-doc. **Lidija**, in 2008 kwam jij als analiste de groep versterken. Als ik jou vroeg om een experiment te doen, was het in no time zorgvuldig uitgevoerd. Dankjewel voor jouw fijne en vaak snelle hulp. Inmiddels heb jij jouw nieuwe uitdaging gevonden in een andere baan waarmee ik jou veel succes wens. **Emma** en **Marieke**, beiden als student "blijven hangen" in de infant group. Jullie mooie projecten zijn alweer een heel eind op weg. Veel succes met (de afronding van) jullie onderzoek en ongetwijfeld tot ziens daarna in de kindergeneeskunde. Beste **Jean**, jij werd analist in ons lab en ik leerde jou pyrosequencen. Dat was een hele uitdaging. Jij verbaasde mij toen jij vertelde dat pipetteren "niet echt jouw ding" was. Dankjewel dat jij toch heel wat platen gepyrosequenced hebt. Ik ben blij dat jij nu een werkplek hebt gevonden met minder pipetteerwerk, waar jij beter op jouw plaats bent. Beste

Robert, vaak spreken bio-informatici “een andere taal” dan biologen. Jij begreep echter onze biologische vraagstellingen direct en wist ze om te zetten in ingewikkelde bio-informatische analyses. Al vóór jouw afstuderen was jij van ongekende waarde voor onze groep. Daarnaast was het heel leuk en leerzaam om met jou samen te werken. Heel veel succes met jouw Master-opleiding en wellicht tot ziens daarna? Beste **Eddy**, wat was ik blij met jouw hulp toen ik als onervaren AIO maanden lang elke dag naar Leiden moest voor “arrays”. Jouw relaxedheid en ervaring brachten deze stresskip tot rust en jij ontwikkelde je tot een ware meester in het troubleshooten (en dat was hard nodig). Daarnaast genoot ik van de gezelligheid tijdens de thee met jou, Matt, Ivo en “the other guys” in het LGTC. Ik vind het leuk om te zien hoe jij nu - inmiddels in Rotterdam - verder werkt om de epigenetische geheimen van leukemie bij zuigelingen te ontrafelen. Heel veel succes met jouw post-doc carrière! De nieuwe analisten **Sandra** en **Merel**. Dear Sandra, we only met a couple of times, but when my thesis was approved you came to me, and you gave me a big hug. This really touched me! I know you became an important technician for the infant leukemia research group. Good luck with your further work. Beste **Merel**, jij bent er nog niet zo lang, maar ik heb gehoord dat jij je in rap tempo ontwikkelt tot een heel goede analiste. Heel veel succes met jouw werk.

“In learning we teach, and in teaching we learn.”
(Phil Collins)

Studenten

De geneeskunde studentes **Pauline** en **Sandra**. Samen probeerden wij nieuwe technieken op te zetten en daarvoor werd ons geduld ernstig op de proef gesteld. Hopelijk hebben jullie er net zoveel van geleerd als ik. Dankjulliewel voor de tomeloze inzet en gezelligheid en veel succes met jullie medische carrières! De Junior Med School studenten en jonge talenten **Sara** en **Sid**. Jullie zaten pas in vijf VWO toen jullie stage kwamen lopen in het lab: voor ons alle drie een uitdaging! In no time begrepen jullie wat epigenetica inhield en konden jullie zelfstandig experimenten uitvoeren. Jullie werk haalde de krant. Wat was ik trots! Leuk om te zien dat jullie nu – als geneeskundestudenten – nog steeds enthousiast zijn voor het onderzoek. Lieve **Lindsey**, jij kwam als eerste Junior Med School student in ons lab. Jij was weliswaar niet mijn studente, maar het was een genot om jou dingen te leren. In jouw bescheidenheid ben jij een veelbelovend talent. De andere studenten die stage liepen in de infant group: **Nannet**, **Isabelle** en **Sander**, dank voor jullie gezelligheid en veel succes met jullie medische en biologische carrières. **Nannet**, tot ziens als collega kinderarts-in-spe, ik heb daar vertrouwen in.

Collega’s Laboratorium Kindergeneeskunde

Ik had het geluk als promovenda deel uit te mogen maken van een groot en gezellig onderzoekslab, het lab KG, waar ik de wetenschap in al haar facetten mocht ervaren. Dat onderzoekers creatief zijn, bleek uit de jaarlijkse labdagen en kerstvieringen.

Verkleed als Griekse goden en godinnen zongen wij een lied, en in Schotse rokjes speelden wij Highland Games. Daarnaast had ik nooit gedacht dat ik ooit James Bond ("Jane Do") zou vertolken met Susan, Theo en Ad (die daarmee de prijs voor de beste mannelijke hoofdrol won) als tegenspelers. Hilarische momenten!

Binnen het lab kindergeneeskunde kon je altijd een fijn plekje vinden om te pipetteren. Het pyrosequencen vond plaats in "Marcel's lab". Beste **Marcel**, **Emiel**, **Kees** en **Sylvia E.**, dank voor jullie gezelligheid en het aanhoren van alle pyrosequence frustraties. **Emiel** en **Kees**, ik vond leuk dat jullie als eerste dankbaar gebruik hebben kunnen maken van de pyrosequencer. **Emiel**, succes met de afronding van jouw proefschrift en tot ziens als collega arts-assistent kindergeneeskunde. Wanneer het Oncolab te druk werd, was de blothoek in "het grote lab*" de aangewezen plek om verder te pipetteren.* Beste **Rolien**, **Ad**, **Anita**, **Dicky**, **Janneke B., Lilian**, **Lisette**, **Theo**, en **Ytje**, dank voor jullie gezelschap en alle praktische tips (waaronder Theo's memorial blotbakje). Veel succes bij alles wat jullie doen.

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De (oud-)postdocs **Astrid**, **Esther**, **Kirsten**, **Leila**, **Lieke**, **Mirjam**, **Maria**, **Marion**, **Peng**, **Sabine**, en **Sylvia B.**. Samen dragen jullie een schat aan ervaring bij: van stromale cellen tot muizen en zebravisjes. Dank voor het meedenken tijdens de research besprekingen en succes met jullie eigen carrières (veelal op nieuwe werkplekken). **Sabine** en **Sylvia B.**: tot ziens in Utrecht.

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"Our greatest glory is not in never falling,
but in getting up every time we do."
(Confucius)

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Kinderoncologisch Centrum Rotterdam

De kinder(hemato-)oncologen: **Andrica**, **Auke**, **Erna**, **Inge A.**, **Inge vd S.**, **Marry**, **Max**, **Marion**, **Michel** en **Roel**. Jullie deden altijd je best om tussen jullie poli's door bij onze onderzoeksbesprekingen te zijn. Beste **Andrica**, wij deelden een kamer tijdens mijn eerste ASH en wij mochten samen naar de ASPO Masterclass. Ik heb er bewondering voor hoe jij jouw onderzoek naar MDS weet te combineren met jouw werk als kinderoncologe. Succes met de laatste loodjes van jouw promotie. Beste **Auke**, na mijn presentaties stond jij meestal enthousiast op en kwam jij mee denken en goede suggesties doen. Dit heb ik enorm gewaardeerd! Jij focust je nu op de kliniek, maar jij hebt nog steeds een groot onderzoekshart. Dankjewel voor jouw belangstelling! **Inge A.**, dankjewel voor al jouw leuke reacties na mijn eerste presentaties voor de kliniek. **Inge vd S.**, wat was ik blij dat ik de werkzaamheden voor het kurenprogramma aan jou mocht overdragen, zodat ik me weer fulltime op het onderzoek kon richten. Ik hoop dat hier inmiddels een structurele oplossing voor gevonden is. Beste **Marry** en **Michel**, ik heb er bewondering voor hoe jullie het begeleiden van een grote onderzoeksgroep combineren met een drukke kliniek. Ik vind het heel leuk om te zien hoe de technieken waar ik de afgelopen jaren aan gewerkt heb nu dankbaar gebruikt worden door de promovendi in jullie groep. **Marry**, dankjewel voor jouw interesse in mij en in ons onderzoek. **Michel**, man van de klinische trials, ik hoop dat er nog veel discussies volgen over hoe wij epigenetische middelen in de kliniek gaan krijgen voor zuigelingen met leukemie.

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Molecular Haematology and Cancer Biology Unit, University College London

Dear **Dr. O. Williams**, dear Owen, I had the privilege to come to London to learn about the MLL westerns. Thank you for having me in your laboratory! Your research group is unique for its enthusiasm, expertise and its international mix of people. Dear **Hikari**, you were the best teacher I could wish for: very friendly, quick and efficient. Good luck with the preparations for your upcoming viva. Dear **Jasper**, **Vanessa**, **Maurizio** and **Lu**, thank you for your hospitality and your help during my stay in London.

Collega's Wilhelmina Kinderziekenhuis Utrecht

Vanaf januari 2011 combineerde ik het afronden van mijn proefschrift met het werken als arts-assistent kindergeneeskunde in het WKZ te Utrecht. Dat was een hele uitdaging.

"There and back again…"
(J.R.R. Tolkien, the Hobbit)

Mijn eerste stappen terug in de kliniek zette ik op de afdeling Neonatologie van het WKZ. Hier leerde ik weer hoe fantastisch leuk het is om dokter te zijn. Heel veel dank aan alle neonatologen, fellows, physician assistants en verpleegkundigen van de NICU, high care en medium care voor de begeleiding van deze onervaren arts-assistent. Mijn speciale dank gaat uit naar de toenmalige dokters van de medium care waar ik mijn vertrouwen terug kreeg: **Hens**, **Marja** en **Malgosia**. Daarbij waren **Willem** als coach en later ook **Petra** als mentor onmisbaar. Dankjewel Heike, mijn assistent-mentor, voor jouw lieve steun. Na mijn tijd op de neonatologie heb ik overwegend dienstenblokken gedaan in het WKZ. Veel dank aan alle kinderartsen en collega-assistenten dat jullie mij met mijn beperkte ervaring door die dienstenblokken heen gesleept hebben. Het was een leuke, maar pittige tijd, waarin ik heel snel ongelooflijk

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Collega's Meander Medisch Centrum Amersfoort

Vanaf januari 2012 volgde ik de opleiding tot kinderarts in het Meander Medisch Centrum te Amersfoort. Na een hersenschudding en een auto-ongeluk werd het een rare start. Veel dank aan alle kinderartsen en arts-assistenten kindergeneeskunde voor hun steun tijdens mijn herstel. In het bijzonder mijn opleider, **Dr. P.H.G. Hogeman**, beste Paul, jij bent niet alleen een heel goede kinderarts, maar ik heb jou mogen leren kennen als een opleider met hart voor zijn mensen. Heel veel dank voor jouw betrokkenheid. Beste **David** en **Nicole**, die eerste week op de pediatrie was fantastisch (dank aan **Nanda** voor het inwerken!). Ik kijk uit naar mijn resterende tijd in het Meander.

Familie en vrienden

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Het proofreading team: **Charlotte**, **Eddy**, **Ilse-Marije**, **Marloes v. K.**, **Pauline**, **Patricia**, **Rianne**, **Susan**, **Stephen**, **Swie ien**, en **Wietske.** Dank voor jullie kritische oog!

"A friend may well be reckoned the masterpiece of nature."
(Ralph Waldo Emerson)

Vriendinnetjes uit de brugklas in Maastricht: **Marijne** en **Zita**. **Zita**, jij ging mij voor als promovenda en ik had de eer om als paranimf aan jouw zijde te staan. Onze reis naar Maleisië was veel te kort, maar lang genoeg om het onderzoek even helemaal te vergeten. Lieve **Marijne**, wij werden allebei dokter en konden veel met elkaar delen. Ook al lukte het mij niet om jou op te zoeken in Zuid-Afrika, jouw steun tijdens de laatste loodjes van mijn proefschrift was er niet minder om. Dankjewel voor jouw betrokkenheid! Het vriendengroepje uit de middelbare schooltijd: **Dennis & Judith** met **Sam** en **Isabel**, **Twan**, **Bjorn & Patrick**, **Ralf & Charlotte** en **Linda & Richard**, dankjulliewel voor de heerlijke wintersport vakantie en alle gezellige etentjes tijdens die toch wel heel intensieve jaren. Lieve **Twan**, dank voor al jouw bemoedigende WhatsAppjes in de aanloop naar mijn promotie. Meiden van het University College: **Anika**, **Jessica**, **Marijn**, **Marlous**, **Sandra** en **Wietske**. Op UC thee bij Dawson's Creek aan de Kromhoutweg, en daarna heeft ieder zijn eigen pad gekozen. **Marijn**

en **Sandra**, jullie schreven allebei al een prachtig proefschrift. **Jessica**, voor jou nu ook de laatste loodjes vergelijkbaar met het afronden van een proefschrift (en wat hadden wij een productief dagje met sushi en een wijntje als beloning!). De etentjes samen blijven hartstikke leuk. Lieve **Wietske**, dankjewel voor al jouw interesse in hoe het met mij gaat en in wat ik doe! Mijn lieve huisgenootjes van de Albatros: **Merel**, **Berber** en **Marloes R.**. Dank voor alle gezellige drankjes en etentjes! Lieve **Merel**, ook jij promoveerde al en stuurde mij vanuit Noorwegen lange mails met tips voor de afronding. Dankjewel voor het meedenken en meeleven! Heel leuk dat jij straks met jullie nieuwe wereldburger weer even in Nederland bent. Lieve **Berber**, jouw lieve gebaren op moeilijke momenten wisten mij steeds weer te raken. Jij wist weinig van geneeskunde of promoties, maar jij kon je inleven als geen ander. Dankjewel voor de speciale persoon die jij bent! IFMSA vriendinnetjes **Janneke**, **Sophie**, **Marloes v.K.** en **Marije**. Ik geniet enorm van onze bijkletsmomentjes (al schiet die MCW video er meestal bij in). Het weekendje bij Sophie in Kopenhagen was leuk! Dankjulliewel ook voor jullie steun tijdens mijn herstel na het auto-ongeluk. Lieve **Marloes**, jij zat zelf met een voet in het gips en daarnaast leefde jij ook nog eens met mij mee. Jij bent een kanjer! Lieve **Marije**, wat was dat weekendje uitwaaien op Ameland heerlijk. Dankjewel voor onze bijzondere vriendschap. Ik kijk uit naar onze reis door Nieuw Zeeland. **Ursul**, dank voor jouw steun tijdens een voor jou heel zware periode. En als laatste geneeskundevriendinnetjes **Elsbeth**, **Ilse-Marije**, **Rianne** en **Swie Ien**. Dank voor alle gezellige wellness bezoekjes, high teas en weekendjes weg! Lieve **Ilse-Marije** en **Rianne**, onze vriendschap begon als “witkoppies” in Zuid-Afrika. Wat ben ik blij dat wij destijds die uitdaging aangegaan zijn. Lieve **Ilse-Marije**, jij bent ook een duizendpoot. Jouw succesvolle medische carrière combineer jij met een bijna even succesvolle hardloopcarrière. Daar heb ik veel bewondering voor. Heel veel succes met jouw promotie onderzoek. Jij bent een heel fijne vriendin die er altijd voor mij is! Lieve **Rianne**, als eerste van ons werd jij specialist, petje af! De manier waarop jij voor jouw vriendinnen klaar staat is bewonderenswaardig. Toen ik in Utrecht ging werken, richtte jij meteen een kamer voor mij in. Zonder jouw lieve steun was ik die eerste maanden in de kliniek niet doorgekomen. Dankjewel voor al jouw wijze raad en onze fijne vriendschap. Lieve **Elsbeth**, met jouw directheid en nuchterheid weet jij als geen ander tot mij door te dringen. Op “retraite” in Lekkerkerk bij **Oliver**, kleine **Lysander** en jou kwam ik pas echt to rust. En terwijl ik de laatste hand leg aan dit dankwoord, is jouw prachtige dochter Fenneke geboren. Dankjewel voor jouw onvoorwaardelijke steun en vriendschap. Lieve **Swie Ien**, wat is het heerlijk om een vriendinnetje te hebben dat alles zo goed begrijpt als jij. Misschien wel omdat wij zo op elkaar lijken. Of het nu een cursus gebarentaal, een balletje golf, een weekendje winkelen in Antwerpen of een dagje thermen is, ik krijg enorm veel energie van de tijd die wij samen doorbrengen. Dank voor de lieve zorgzaamheid van **Stephen** en jou tijdens mijn herstel. Er gaat nu weer meer tijd komen om samen leuke uitstapjes te maken!

Lieve **Brian**, het blijft raar dat jij er niet meer bent. Jij was een maatje voor mij en terwijl jij vocht tegen jouw ziekte was jij er altijd voor anderen. Jij ging altijd een beetje met mij mee op congres, zodat ik daar niet alleen nieuws over kinderleukemie

op kon snuiven, maar ook de nieuwste therapiemogelijkheden voor jouw tumor op kon sporen. Jij was positief tot het laatste moment en bent daarmee een voorbeeld geweest voor velen!

"You'll be in my heart, always…"
(Phil Collins)

Mijn lieve nichtje **Inge**, jouw interesse in wat ik doe en hoe het met mij gaat is onaflaatbaar. Allebei genieten wij van het werken met kinderen en inmiddels ben jij ook heel succesvol met jouw eigen bedrijfje. Ik ben trots op jou! Lieve **tante Hanny**, dit is wat jij schreef toen ik jou vroeg om de lekensamenvatting te lezen: "hoi Domieniek, op spelling vindt ik leuk om te doen. Natuurluk kan ik inhaudelijk niets voor jouw beteekenen. Durv jij het aan? Stuur maar!" Natuurlijk had ik het volste vertrouwen in jou. Met groot gemak heb jij die laatste spel- en typefouten uit de Nederlandse samenvatting gevist. Veel dank voor jouw snelle hulp! Lieve **oom Mathé**, jij zette een verkregen handicap om in een nieuw talent: jij ontwikkelde je tot kunstenaar. Dankjewel voor het ontwerpen van de afbeeldingen op de voorkant van mijn proefschrift! De vlinder en de rupsen zijn slechts een heel bescheiden voorbeeld van jouw grote schilderstalent.

Lieve **papa en mama**, dit proefschrift is aan jullie opgedragen. Wat jullie voor mij betekenen is niet in woorden uit te drukken.

"On ne voit bien qu'avec le coeur. L'essentiel est invisible pour les yeux."
(Antoine de Saint-Exupérie, Le Petit Prince)

Dankjewel dat jullie er werkelijk altijd voor mij zijn en mij steunen in alles wat ik doe! Dat jullie zo trots op mij zijn, maakt mij gelukkiger dan welke prestatie ook, maar vergeet niet dat ik ook heel trots ben op jullie. Het enthousiasme en de passie waarmee ik in het leven sta en de dingen doe die ik doe, heb ik van jullie mee gekregen. Ik houd ongelooflijk veel van jullie!!

“Laetum celebremus honorem!”
“Dit blijde eerbewijs zullen wij feestelijk vieren!”

(Virgilius)

“I am just trying to do things that are interesting for me.”

(Phil Collins)

ABOUT THE AUTHOR

LIST OF PUBLICATIONS

PHD PORTFOLIO

CURRICULUM VITAE (ENGLISH)

CURRICULUM VITAE (DUTCH)

LIST OF PUBLICATIONS

1. **Stumpel DJPM**, Schneider P, van Roon EHJ, Boer JM, de Lorenzo P, Valsecchi MG, de Menezes RX, Pieters R, Stam RW. Specific promoter methylation identifies different subgroups of *MLL*-rearranged infant acute lymphoblastic leukemia, influences clinical outcome, and provides therapeutic options. *Blood 2009; 114: 5490-8.*

2. Stam RW, Schneider P, Spijkers-Hagelstein JAP, van der Linden MH, **Stumpel DJPM**, de Menezes RX, de Lorenzo P, Valsecchi MG, Pieters R. Gene expression profiling-based dissection of *MLL*-translocated and *MLL* germline acute lymphoblastic leukemia in infants. *Blood 2010; 115: 2835-44.*

3. Schotte D, Lange-Turenhout EAM, **Stumpel DJPM**, Stam RW, Buijs-Gladdines J, Meijerink JPP, Pieters R, den Boer ML. Expression of miR-196b is not exclusively *MLL*-driven but especially linked to activation of *HOXA* genes in pediatric acute lymphoblastic leukemia. *Haematologica 2010; 95: 1675-82.*

4. **Stumpel DJPM***, Schotte D*, Lange-Turenhout EAM, Schneider P, Seslija L, de Menezes RX, Marquez VE, Pieters R, den Boer ML*, Stam RW*. Methylation of specific microRNAs in *MLL*-rearranged infant acute lymphoblastic leukemia: Major matters at a micro scale. *Leukemia 2011; 25: 429-39*. *shared first and last authorship.

5. **Stumpel DJPM,** Schneider P, Seslija L, Osaki H, Williams O, Pieters R, Stam RW. Connectivity mapping identifies HDAC inhibitors as suitable candidates for the treatment of t(4;11)-positive acute lymphoblastic leukemia. *Leukemia 2012; 26: 682-92.*

6. Kandimalla R, van Tilborg AAG, Kompier LC, **Stumpel DJPM**, Stam RW, Bangma CH, Zwarthoff EC. Genome-wide analysis of CpG island methylation in bladder cancer identified TBX2,TBX3, GATA2 and ZIC4 as pTa-specific prognostic markers. *European Urology 2012; 61: 1245-56.*

7. **Stumpel DJPM**, Kieven J, Schneider P, van Roon EHJ, Pieters R, Stam RW. Absence of global hypomethylation in promoter hypermethylated *MLL*-rearranged infant acute lymphoblastic leukemia. *European Journal of Cancer 2012, accepted for publication*.

8. Duijkers FAM, de Menezes RX, Beumer IJ, **Stumpel DJPM**, Admiraal P, Pieters R, Meijerink JPP, van Noesel MM. Nanomolar treatment with epigenetic drug combination induces genome-wide methylation and expression alterations in neuro-ectodermal cell lines. *Submitted.*

9. **Stumpel DJPM**, Schneider P, Pieters R, Stam RW. The potential of clofarabine in *MLL*-rearranged infant acute lymphoblastic leukemia. *Submitted.*

10. **Stumpel DJPM**, Pieters R, Stam RW. *MLL*-rearranged acute lymphoblastic leukemia in infants: an epigenetic disease which should be treated accordingly. *Submitted.*

“Live as if you were to die tomorrow,
and learn as if you were to live forever.“

(Mahatma Ghandi)

Erasmus MC
University Medical Center Rotterdam

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student:	Dominique J.P.M. Stumpel	PhD time:	2006-2010
Erasmus MC Department:	Pediatric Oncology-Hematology	Promotor:	Prof. dr. Rob Pieters
Research School:	Molecular Medicine	Co-promotor:	Dr. Ronald W. Stam

1. PhD TRAINING

	Year	Workload (ECTS)
General PhD Courses		
- Biomedical English Writing and Communication, Erasmus Medical Center, Rotterdam, the Netherlands, Feb-April 2008	2008	4
- Classical Methods for Data Analysis, Netherlands Institute for Health Sciences, Rotterdam, the Netherlands, Sept-Oct 2008	2008	5.7
Specific Masterclasses, Courses and Workshops		
- Masterclass by Prof. dr. B.J. Prakken and Prof. dr. H.J. Verkade, Day for Young Investigators, Pediatric Association of the Netherlands (NVK), Veldhoven, the Netherlands, Nov 2010	2010	0.2
- Second Weekend Workshop on Grant Writing and Successful Team Building, Training Upcoming Leaders in Pediatric Sciences (TULIPS), Bergen aan Zee, the Netherlands, April 2010	2010	1.0
- Amsterdam School of Pediatric Oncology (ASPO) Masterclass Pediatric Oncology, Schiermonnikoog, the Netherlands *(oral presentation),* Jan 2009	2009	2.0
- Applied Bioinformatics, Postgraduate School Molecular Medicine, Erasmus Medical Center, Rotterdam, the Netherlands, Sept 2008	2008	0.2
- MGC MicroArray Analysis Course, Medical Genetic Center, Erasmus Medical Center Rotterdam, the Netherlands, May 2007	2007	1.4
- European Hematology Association (EHA) Scientific Workshop on the role of epigenetics in haematological malignancies, Cannes, France, Feb 2007	2007	1.0
- Array Data Analysis using R and Bioconductor, Leiden Genome Technology Center, Leiden, the Netherlands, Jan 2007	2007	1.4
- Basic and Translational Oncology, Postgraduate School Molecular Medicine, Erasmus Medical Center, Rotterdam, the Netherlands, Nov 2006	2006	1.4
- Grow PhD course Epigenetics, School for Oncology and Developmental Biology, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, the Netherlands *(oral presentation + poster presentation),* Oct 2006	2006	2.0
- Biomedical Research Techniques, Postgraduate School Molecular Medicine, Erasmus Medical Center, Rotterdam, the Netherlands, Sept 2006	2006	1.4

	Year	Workload (ECTS)
(Inter)national conferences and presentations		
- American Society of Hematology (ASH) Annual Meeting 2010, Orlando, Florida, Dec 2010, *2 poster presentations* (Abstract Achievement Award)	2010	2
- 32nd Annual Conference of the Pediatric Association of the Netherlands (NVK), Veldhoven, the Netherlands, Nov 2010, *2 oral presentations* (Maarten Kappelle Award and runner-up Young Investigator Award)	2010	2
- Symposium on Translational Oncology, Dutch Cancer Association (KWF), Ede, the Netherlands, Sept 2010, *poster presentation*	2010	0.6
- Center for Pediatric Oncology Rotterdam (KOCR), 12th annual symposium "Who Cares", Rotterdam, the Netherlands, June 2010, *oral presentation*	2010	1.2
- Molecular Medicine Day, Postgraduate School Molecular Medicine, Erasmus Medical Center, Rotterdam, Feb 2010, *poster presentation* (3rd Prize Publication Award)	2010	0.6
- American Association for Cancer Research (AACR), Special Conference on Cancer Epigenetics, San Juan, Puerto Rico, Jan 2010, *oral presentation*	2010	2.0
- American Society of Hematology (ASH) Annual Meeting 2009, New Orleans, Louisiana, Dec 2009	2009	1.0
- Research Day Pediatrics, Sophia Children's Hospital, Rotterdam, the Netherlands, Nov 2009, *poster presentation* (Poster Award)	2009	0.6
- 41st Conference of International Society of Pediatric Oncology (SIOP), São Paulo, Brazil, Oct 2009*, session chairing and oral presentation* (Schweisguth Prize)	2009	2.0
- Keystone Symposium on Epigenetics, Development and Human Disease, Breckenridge, CO, Jan 2009, *poster presentation*	2009	1.6
- American Society of Hematology (ASH) Annual Meeting 2008, San Francisco, CA, Dec 2008, *oral presentation* (Abstract Achievement Award)	2008	2.0
- Research Day Pediatrics, Sophia Children's Hospital, Rotterdam, the Netherlands, Nov 2008, *oral presentation*	2008	1.2
- Day for Young Investigators, Pediatric Association of the Netherlands (NVK), Veldhoven, the Netherlands, Nov 2008, *poster presentation* (Scientific Poster Award)	2008	0.6
- 40th Conference of International Society of Pediatric Oncology (SIOP), Berlin, Oct 2008, *oral presentation*	2008	2.0
- 13th European Hematology Association (EHA) Congress, Copenhagen, Denmark, June 2008, *oral presentation* (International Travel Award)	2008	2.0
- American Association for Cancer Research (AACR), Special Conference on Cancer Epigenetics, Boston, MA, May 2008, *poster presentation* (Scholar-in-Training Award)	2008	1.6
- British Society of Hematology (BSH) 48th Annual Meeting 2008, Glasgow, Scotland, April 2008, *poster presentation*	2008	1.6
- Molecular Medicine Day, Postgraduate School Molecular Medicine, Erasmus Medical Center, Rotterdam, Feb 2008, *oral presentation*	2008	1.2
- American Society of Hematology (ASH) Annual Meeting 2007, Atlanta, Georgia, Dec 2007, *poster presentation*	2007	1.6
- Second bi-annual symposium on DNA methylation and cancer, Epiphamy Symposia on Translational Cancer Epigenetics, Gent, Belgium, Oct 2007	2007	0.2
- ZONMW Genetics Retreat, Kerkrade, the Netherlands, March 2007	2007	0.6

	Year	Workload (ECTS)
Other presentations		
- "Helpen Helpt" presentation for parents from children diagnosed with cancer, Feb 2009	2009	1.2
- Presentation for the Scientific Advisory Council of the Sophia Foundation, Sophia Children's Hospital Rotterdam, the Netherlands, Nov 2008 (Jan Molenaar Award)	2008	1.2
- Grand Round, presentation, Sophia Children's Hospital, Rotterdam, the Netherlands, January 2008	2008	1.0
- Research Meetings Laboratory of Pediatrics/ Pediatric Oncology/ Hematology (8 informal presentations)	2006-2010	1.6
Other experiences		
- Visiting researcher University College London (UCL), Institute of Child Health and Great Ormond Street Hospital for Children, London, United Kingdom, Department of Molecular Haematology and Cancer Biology (supervision: Dr. Owen Williams), Nov 2010		

2. TEACHING

	Year	Workload (ECTS)
- Lecturing 2nd year Medical Students (Minor Oncology)	2010	2.0
- Supervising practicals, Tutoring 4th year Medical Student (Pauline van der Burg) Validation and characterization of three hypermethylated putative tumor suppressor genes in *MLL*-rearranged infant acute lymphoblastic leukemia: *PAX5*, *SPARC* and *T-STAR*, *4- month internship*	2007	4.0
- Supervising Master's theses Master of Clinical Research Student (Sandra Zinkweg) Hypermethylation of the putative tumor suppressor genes C*DKN1C*, *PTGS2*, *PHF2* and *MXI1* in *MLL*-rearranged infant acute lymphoblastic leukemia, *10- month internship*	2008	10.0
- Other Junior Med School Students (5th grade high-school students) (Sid Morsink and Sara Creemers), Methylation of tumor suppressor genes in acute lymphoblastic leukemia in infants. *2 2-week internships*	2008	2.0

3. EXTRACURRICULAR

	Year	Workload (ECTS)
- Management of the program for Chemotherapy prescriptions/ Chemotherapy committee, Sophia Children's Hospital, Rotterdam, the Netherlands	2008-2009	1-2 days a week
- Inventor of the project "**The teddy-bear doctor, your best friend**", Sophia Children's Hospital, Rotterdam, the Netherlands	2007-2008	

"Project designed for (severely) sick children, to let them forget about their own disease for a moment while they focus on their sick teddy- bear being friendly and professionally treated by Teddy Bear Doctors (3rd and 4th year Medical Students specifically trained for the job by a pedagogist, a child psychologist and a pediatrician)."

The project was initiated at the Department of Pediatric Oncology in March 2008, and officially presented at the "Patient Prominent Day" at the Erasmus MC- Sophia Children's Hospital, Rotterdam on November 30th 2010.

The project has become an official project of the International Federation of Medical Students' Associations – the Netherlands (IFMSA-NL). As such, the project has now been realized in the Academic Medical Centers of among others Groningen, Nijmegen, and Utrecht.

http://www.ifmsa.nl/projecten/berendokter/berendokter.html

“Do what you believe is great work,
and the only way to do great work is to love what you do.”

(Steve Jobs)

CURRICULUM VITAE (ENGLISH)

Dominique Stumpel was born on June 15th 1980 in Breda, and she grew up in the south of the Netherlands. From 1992-1998, she attended grammar school at the Porta Mosana College in Maastricht and Pleincollege Eckart in Eindhoven, sequentially. In 1999, Dominique passed her propedeutic exam in Health Sciences at Maastricht University after which she enrolled at University College Utrecht, the Honours College of Utrecht University, where she got her pre-medical Bachelor of Science degree in 2001 *with honours*. Thereafter, Dominique entered a 5-year accelerated program at the Medical School of Utrecht University.

During medical school, Dominique completed clinical rotations in Ga Rankuwa, South Africa (pediatric dermatology) and in Assiut, Egypt (pediatric hematology). Furthermore, she was an active board member of the International Federation of Medical Students' Associations (IFMSA)-Utrecht. During her senior clinical internship at the Wilhelmina Children's Hospital in Utrecht (supervision: dr. Joost Frenkel and drs. Martine den Otter), Dominique became interested in pediatric hemato-oncology. In the final year of medical school, she investigated the molecular mechanisms underlying the pre-malignant hematological disorder severe congenital neutropenia (supervision: Prof. dr. Paul Coffer and dr. Miranda Buitenhuis). It was during this time in the lab that Dominique became highly enthusiastic for translational research.

On August 26th 2006, Dominique graduated from medical school, and on September 1st 2006 she started as the first Ph.D.- student in the Infant Leukemia Research Group at the Department of Pediatric Oncology/Hematology at the Erasmus MC-Sophia Children's Hospital in Rotterdam (supervision: Prof. dr. Rob Pieters and dr. Ronald Stam). During her time as a Ph.D.- student, Dominique presented her work at many international conferences. In addition, she spent some time at the Molecular Haematology and Cancer Biology Unit of the University College London - Institute of

Child Health in London, United Kingdom (supervision: dr. Owen Williams). The research focusing on epigenetic aberrations in *MLL*-rearranged infant ALL evidently became one of her passions.

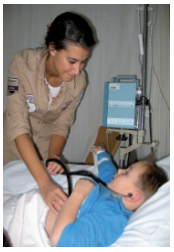
Work described in this thesis has been awarded several prizes among which the Jan Molenaar Award from the Sophia Foundation Scientific Board (2008), the Schweisguth Prize from the International Society of Pediatric Oncology (SIOP) (2009), the Maarten Kappelle Award and the Young Investigator Award (runner up) from the Paediatric Association of the Netherlands (NvK) (2010), and the first Tom Voûte Young Investigator Award from the Dutch Childhood Oncology Group (SKION) (2011).

In January 2011, Dominique started to work as a pediatric resident in the Wilhelmina Children's Hospital in Utrecht, headed by Prof. dr. Edward Nieuwenhuis and dr. Joost Frenkel. From January 2012 on, Dominique is continuing her pediatric residency in the Meander Medical Center in Amersfoort under supervision of dr. Paul Hogeman. Her goal is to become a pediatric hemato-oncologist.

Dominique lives in Rotterdam. She likes to play tennis, travel the world, and learn foreign languages.

“Kies een baan waarvan je houdt,
en je hoeft geen dag meer te werken.”

(Confucius)



CURRICULUM VITAE (NEDERLANDS)

Dominique Stumpel werd op 15 juni 1980 geboren te Breda. Van 1992-1998 volgde zij het gymnasium aan het Porta Mosana College te Maastricht en Pleincollege Eckart te Eindhoven. Na de middelbare school studeerde Dominique een jaar Gezondheidswetenschappen aan de Universiteit Maastricht en behaalde haar propedeuse. In 1999 werd zij toegelaten aan het University College Utrecht, het Honourscollege van de Universiteit Utrecht, waar zij in 2001 haar Bachelor of Science graad behaalde (*with honours*). Hierna startte Dominique een 5-jarig versneld traject aan de Medische Faculteit van de Universiteit Utrecht.

Tijdens haar geneeskunde opleiding volgde Dominique keuzecoschappen in Ga Rankuwa, Zuid-Afrika (Kinderdermatologie) en in Assiut, Egypte (Kinderhematologie). Daarnaast was zij een actief bestuurslid van de International Federation of Medical Students' Associations – Utrecht (IFMSA-Utrecht). Tijdens haar semi-artsstage in het Wilhelmina Kinderziekenhuis te Utrecht (supervisie: Dr. Joost Frenkel en Drs. Martine den Otter) raakte Dominique geïnteresseerd in de kinderhemato-oncologie. Deze interesse resulteerde in 5 maanden onderzoek naar ernstige aangeboren neutropenie in het Moleculaire Immunologie Laboratorium van het Universitair Medisch Centrum Utrecht (supervisie: Prof. Dr. Paul Coffer en Dr. Miranda Buitenhuis). Gedurende deze tijd in het lab raakte Dominique enthousiast voor translationeel onderzoek.

Op 26 augustus 2006 behaalde Dominique haar artsenbul, en op 1 september 2006 startte zij als eerste promovenda in de Zuigelingen Leukemie Onderzoeksgroep van de afdeling Kinderhemato-oncologie aan het Erasmus MC- Sophia Kinderziekenhuis te Rotterdam (supervisie: Prof. Dr. Rob Pieters en Dr. Ronald Stam). Dominique presenteerde haar onderzoeksresultaten op talloze internationale congressen. Ook heeft zij enige tijd ervaring opgedaan bij de Molecular Haematology and Cancer Biology Unit van het University College London – Institute of Child Health in Londen (supervisie: Dr. Owen Williams). Het onderzoek naar epigenetische afwijkingen bij *MLL*-herschikte leukemie bij zuigelingen werd een van haar passies.

Werk beschreven in dit proefschrift is bekroond met meerdere prijzen, waaronder de Jan Molenaar Prijs van de Sophia Stichting voor Wetenschappelijk Onderzoek (SSWO) (2008), de Schweisguth Prijs van de International Society of Pediatric Oncology (SIOP) (2009), de Maarten Kappelle Prijs en de Prijs voor de Jonge Onderzoeker van de Nederlandse Vereniging voor Kindergeneeskunde (NvK) (2010), en de eerste Tom Voûte Young Investigator Award van de Stichting Kinderoncologie Nederland (SKION) (2011).

In januari 2011 is Dominique na 4 jaar onderzoek teruggekeerd in de kliniek als arts-assistent kindergeneeskunde in het Wilhelmina Kinderziekenhuis te Utrecht (hoofd: Prof. Dr. Edward Nieuwenhuis, opleider: Dr. Joost Frenkel). Sinds januari 2012 vervolgt zij haar opleiding tot kinderarts in het Meander Medisch Centrum te Amersfoort (opleider: Dr. Paul Hogeman). Haar doel is een kinderhemato-oncologe te worden die kliniek combineert met translationeel onderzoek.

Dominique woont in Rotterdam. In haar vrije tijd tennist zij, en geniet zij ervan met vrienden verre reizen te maken en vreemde talen te leren.

APPENDICES

APPENDIX A. SUPPLEMENTAL METHODS

APPENDIX B. SUPPLEMENTAL FIGURES AND TABLES

APPENDIX A. SUPPLEMENTAL METHODS

CHAPTERS 2, 3 and 6

Differential Methylation Hybridization using CpG island microarrays

Differential Methylation Hybridization (DMH) was performed as described by Yan et al.[1, 2] Briefly, 0.5 ug of high-quality genomic DNA was digested using the restriction enzyme *Mse*I (New England Biolabs). Next, unphosphorylated linkers were ligated to the digested fragments, which were then sequentially digested with two methylation-sensitive restriction enzymes (*Bst*UI and *Hpa*II, New England Biolabs). These second digestions eliminate unmethylated fragments, enriching the samples for methylated sequences. The digested linker-ligated DNA was then used as a template for polymerase chain reaction (PCR) amplification (20 cycles of 97°C for 1 min and 72°C for 3 min, final extension at 72°C for 10 min), generating methylated amplicons. Using the BioPrime Array-CGH Genomic Labeling kit (Invitrogen, Carlsbad, USA), amino-allyl dUTPs were incorporated into the amplicons, allowing the amplicons to be labeled with the fluorescent dyes Cy5 (patient samples) and Cy3 (common reference samples). The common reference for all samples was a commercially available genomic DNA pool derived from five healthy males and five healthy females (Promega Benelux BV, Leiden, the Netherlands). Hybridization and washing were performed according to DeRisi[3] for the custom 9K microarray chips, and for the 244K microarray chips (Agilent), the Agilent ChIP-on-chip protocol version 9.0 was used. Hybridized slides were scanned with a 2565 AA DNA microarray scanner (Agilent Technologies), and the acquired images were analyzed using the GenePix Pro 6.0 software or the Agilent Feature Extraction 9.5.3 software.

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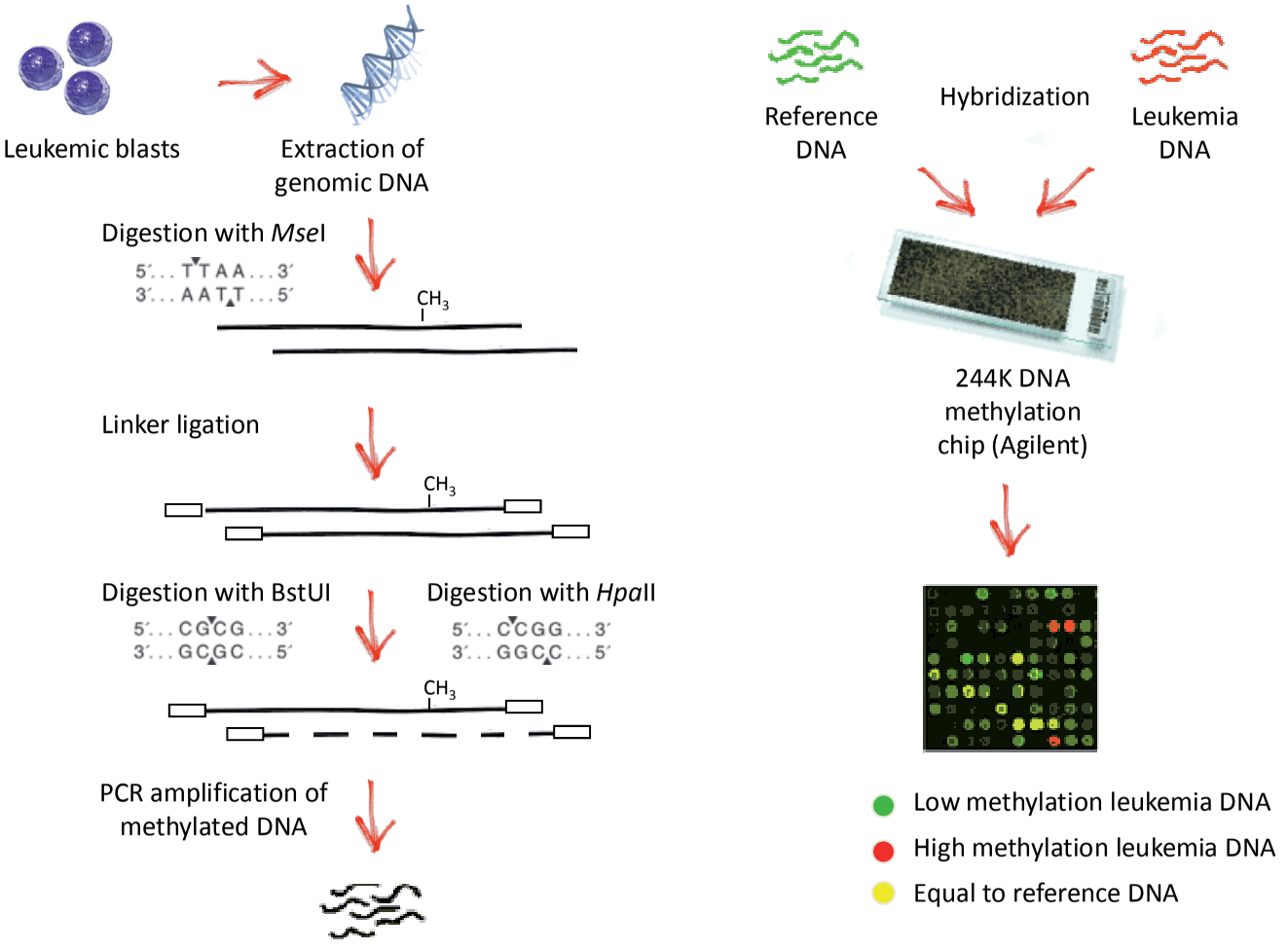
2. Yan PS, Potter D, Deatherage DE, Huang TH, Lin S. Differential methylation hybridization: profiling DNA methylation with a high-density CpG island microarray. *Methods Mol Biol.* 2009;507:89-106.
3. Huang TH, Perry MR, Laux DE. Methylation profiling of CpG islands in human breast cancer cells. *Hum Mol Genet.* Mar 1999;8(3):459-470.

Supplemental figure (next page): Outline of the differential methylation hybridization (DMH) technique.


Leukemic blasts
Extraction of genomic DNA
Digestion with MseI
5′...TTAA...3′
3′...AATT...5′
CH3
Linker ligation
CH3
Digestion with BstUI
5′...CGCG...3′
3′...GCGC...5′
Digestion with HpaII
5′...CCGG...3′
3′...GGCC...5′
CH3
PCR amplification of methylated DNA
Reference DNA
Hybridization
Leukemia DNA
244K DNA methylation chip (Agilent)
Low methylation leukemia DNA
High methylation leukemia DNA
Equal to reference DNA

CHAPTERS 4 and 5

Bisulfite pyrosequencing for quantitative DNA methylation analysis

Genomic DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research Corporation, CA, USA) according to the manufacturer's instructions. Bisulfite treatment of the DNA converts cytosine nucleotides to uracil (which is replaced by thymine during subsequent PCR cycles), but leaves 5-methylcytosine residues untouched. This way, discrimination against methylated or unmethylated cytosines is possible.[4] Subsequently, pyrosequencing was performed. Pyrosequencing is a "sequencing by synthesis" method based on the detection of pyrophosphate release upon nucleotide incorporation **(Supplemental figure A)**. Through an enzymatic reaction visible light is generated in an amount proportional to the released pyrophosphate.[4] Essentially, this technique allows sequencing of a single DNA strand by base per base synthesis of the complementary strand, while monitoring which nucleotide was actually added. As such, pyrosequencing can be used to quantitatively assess the level of methylation in a bisulfate treated DNA strand by comparing the cytosine/thymine ratios incorporated at selected CpG sites.[5]

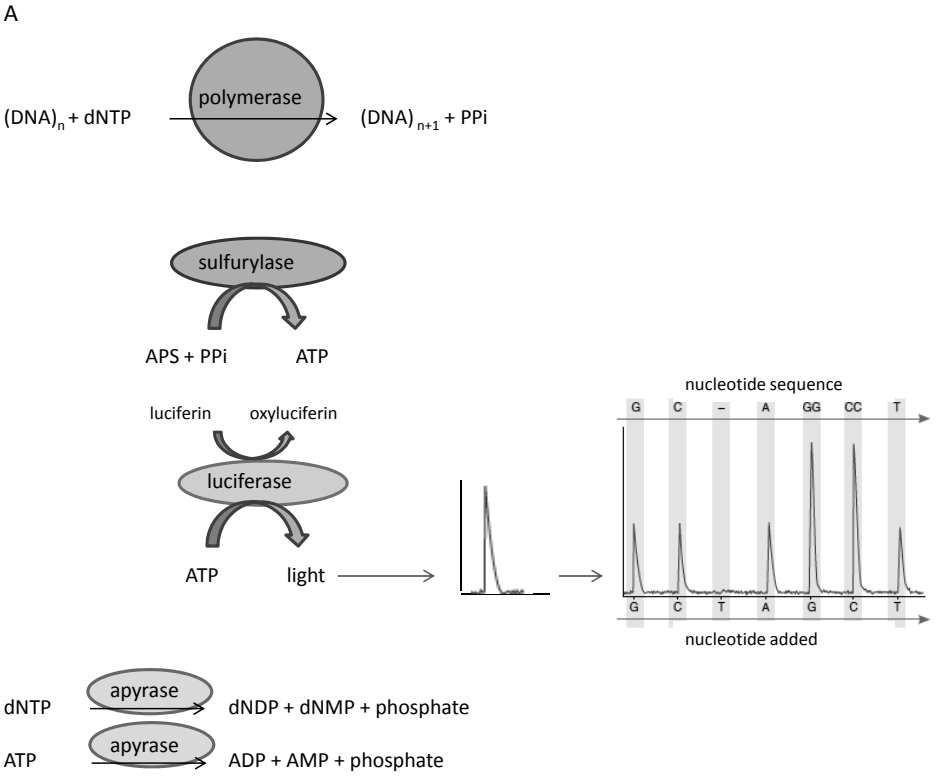
Approximately 20 ng of bisulfite converted DNA was amplified using bisulfite-specific primers. PCR products were purified using Streptavidin Sepharose HP beads (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 M NaOH solution. Next, 0.3 µM of pyrosequencing primer was annealed to the purified single-stranded PCR product. Sequencing reactions were performed on a PyroMark MD system (Qiagen Pyrosequencing, Inc.). Quantification of methylation density was performed using the Pyro Q-CpG software version 1.0.6. (Qiagen Pyrosequencing, Inc.). The degree of methylation was determined from the ratio of thymine and cytosine nucleotides and was expressed as the number of methylated cytosine nucleotides divided by the amount of methylated and unmethylated cytosines x 100% **(Supplemental figure B)**. Confirmation of complete bisulfite conversion was assessed by a cytosine/thymine control integrated into the assays used **(Supplemental figure B)**.

REFERENCES

4. Tost J, Gut IG. DNA methylation analysis by pyrosequencing. Nat Protoc. 2007;2(9):2265-2275.
5. Dejeux E, El abdalaoui H, Gut IG, Tost J. Identification and quantification of differentially methylated loci by the pyrosequencing technology. Methods Mol Biol. 2009;507:189-205

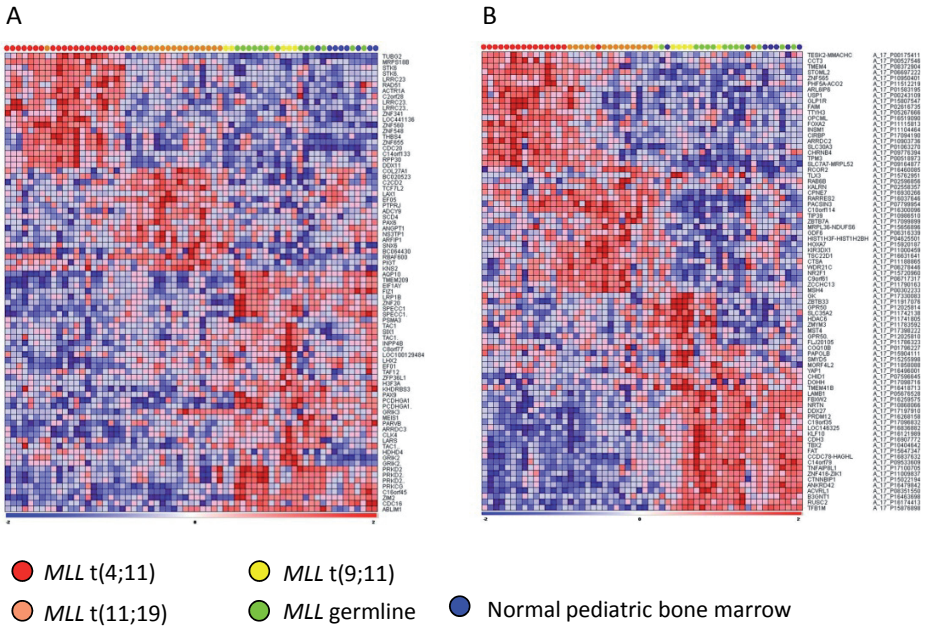
Supplemental figure (next page): Mechanism of pyrosequencing.
A. In short, a sequencing primer is hybridized to a single-stranded DNA template and subsequently incubated with the enzymes DNA polymerase, adenosine triphosphate (ATP) sulfurylase, luciferase, apyrase and the substrates adenosine 5´ phosphosulfate (APS) and luciferin. Deoxyribonucleotide triphosphates (dNTPs) are added, one at a time, to the pyrosequencing reaction. Upon the incorporation of a nucleotide, pyrophosphate (PPi) is released in an amount proportional to the amount of incorporated nucleotides. ATP sulfurylase then quantitatively converts PPi to ATP. In the presence of ATP light is produced by the luciferase-catalyzed reaction. This light is detected

by a charge coupled device (CCD) camera and integrated into a so-called pyrogram as a peak. The apyrase enzyme degrades unincorporated dNTPs as well as ATP excess. As the process continues, the nucleotide sequence of the complementary DNA strand can be inferred from the signal peaks in the pyrogram. **B.** Pyrogram. The degree of methylation at each CpG site (determined from the ratio of thymine and cytosine nucleotides) is expressed as a percentage (gray areas). Cytosine/thymine controls for completeness of bisulfite conversion are highlighted in light gray.
Remaining abbreviations used: dNDP = deoxyribonucleotide diphosphate, dNMP = deoxyribonucleotide monophosphate, ADP = adenosine diphosphate, and AMP = adenosine monophosphate.

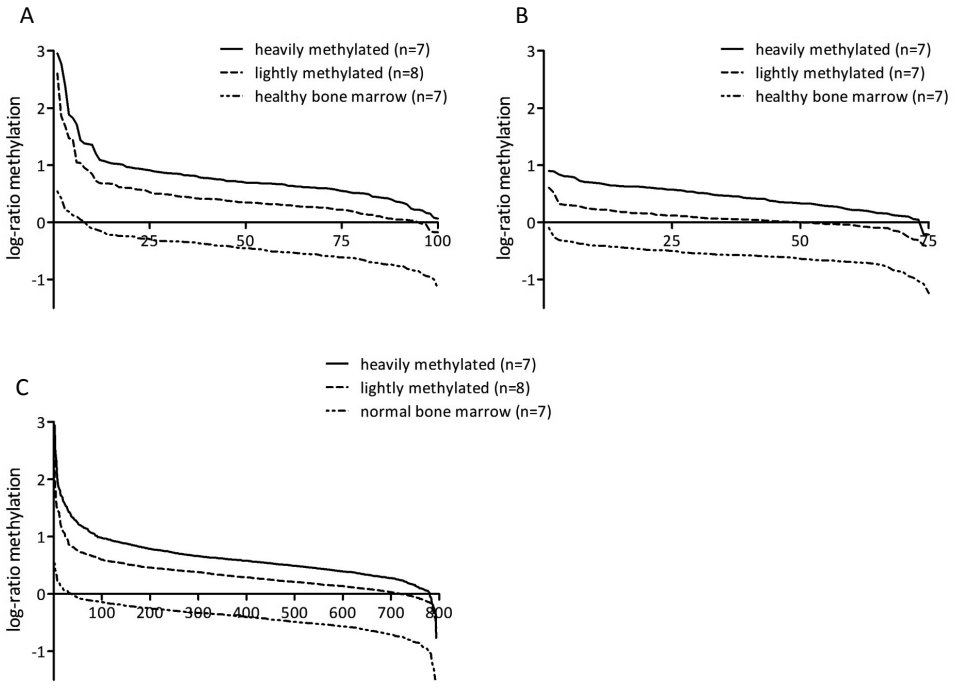


APPENDIX B. SUPPLEMENTAL FIGURES AND TABLES

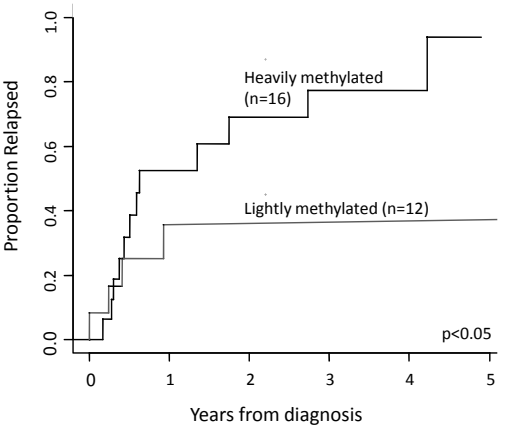
CHAPTER 2



Supplemental figure 1S. Heatmaps showing the top 20 most significantly hypermethylated probes for each infant ALL subtype (compared with the other subgroups). Additionally, normal bone marrow samples were added to the heatmaps.
Columns represent patient samples and rows represent genes. Relative DNA methylation levels are then shown in red (high) and blue (low). Genes and samples were ordered using hierarchical cluster analysis (Euclidean distance, complete linkage) and gene identifiers are listed at the right. Samples are color-coded to indicate the genetic subtype of infant ALL: t(4;11) (red), t(11;19) (orange), t(9;11) (yellow), infant ALL with wild-type *MLL* genes (green) and normal bone marrow (blue). **A.** Data are shown for the 9K chip and **B**. the 244K chip (Agilent). Due to restricted availability of patient material, Agilent DNA methylation profiles were generated for 49 infant ALL patients (t(4;11)-positive (n=16), t(11;19)-positive (n=15), t(9;11)-positive (n=6), and *MLL* wild-type (n=12)) and 7 normal bone marrows. The 20 most significantly hypermethylated genes shared by t(9;11)-positive and wild-type *MLL* samples were also methylated in normal bone marrow samples, implying that these genes are normally methylated in healthy hematopoietic cells.



Supplemental figure 2S. Semi-quantitative representation of the degree of methylation.
Visualization of the normalized and sorted log-ratios (patient signal divided by common reference signal) of methylation (Y-axis) for the most significantly hypermethylated genes (X-axis) in **A.** t(4;11) and **B.** t(11;19)-positive infant ALL patients. Different patient groups represent either heavily or lightly methylated clusters **(figure 3)**. **C.** Visualization of the normalized and sorted log-ratios of methylation for all significantly hypermethylated genes in t(4;11)-positive infant ALL patients, compared with normal bone marrow samples. These data demonstrate that the observed separation of two patient groups with varying degrees of methylation as observed among both t(4;11) and t(11;19)- positive infant ALL, is not restricted to the most significantly methylated genes. As shown here, these differences remain present when all hypermethylated probes (n=794) are used for t(4;11) positive patients.



Supplemental figure 3S. Relapse-free survival in t(4;11)-positive and t(11;19)-positive infant ALL patients divided by the degree of DNA methylation (Supplemental figure 2S), based on hierarchical clustering as shown in figure 3.
t(4;11)-positive and t(11;19)-positive patients were combined. Risk of relapse is presented on the Y-axis and the time of follow-up (in years) is presented on the X-axis. The p-value is from a log-rank test. Risk of relapse is significantly increased in the heavily methylated subgroup of infant ALL.

Supplemental table 1S. Patient characteristics.

Patient number	Age (months)	Sex	Type of *MLL* translocation	Immuno-phenotype	9K chip	Agilent 244K chip*	Affyme-trix genechip**
1	9.4	Female	t(4;11)	pro-B	v		
2	2.8	Male	t(4;11)	pro-B	v	v	N
3	9.4	Female	t(4;11)	pro-B	v	v	N
4	11.0	Female	t(4;11)	pre-B	v		
5	3.6	Male	t(4;11)	pro-B	v	v	N
6	6.6	Female	t(4;11)	pro-B	v	v	N
7	1.9	Male	t(4;11)	pro-B	v		
8	4.2	Female	t(4;11)	pro-B	v	v	
9	0.6	Female	t(4;11)	pro-B	v	v	N
10	0.7	Female	t(4;11)	pro-B	v	v	N
11	1.9	Female	t(4;11)	pro-B	v		
12	6.4	Female	t(4;11)	pro-B	v	v	N
13	6.4	Male	t(4;11)	pro-B	v	v	N
14	1.6	Female	t(4;11)	pro-B	v		
15	8.0	Male	t(4;11)	pre-B	v	v	N
16	5.9	Female	t(4;11)	pro-B	v	v	N
17	3.4	Male	t(4;11)	pro-B	v	v	N
18	1.6	Male	t(4;11)	pro-B	v	v	N
19	1.2	Female	t(4;11)	pro-B	v	v	N
20	3.1	Female	t(4;11)	pro-B	v	v	N
21	3.2	Male	t(4;11)	pro-B	v	v	N
22	7.7	Male	t(11;19)	pro-B	v	v	N
23	3.1	Female	t(11;19)	pro-B	v	v	N
24	5.4	Male	t(11;19)	pro-B	v	v	N
25	0.0	Female	t(11;19)	pro-B	v	v	N
26	10.7	Male	t(11;19)	pro-B	v	v	N
27	7.8	Male	t(11;19)	pre-B	v		
28	5.3	Female	t(11;19)	pro-B	v	v	N
29	8.8	Female	t(11;19)	common B	v	v	N
30	5.7	Female	t(11;19)	pre-B	v		
31	9.1	Male	t(11;19)	pre-B	v	v	N
32	11.0	Male	t(11;19)	pro-B	v	v	
33	0.0	Female	t(11;19)	pro-B	v	v	N
34	5.7	Female	t(11;19)	pro-B	v	v	N
35	3.4	Female	t(11;19)	pro-B	v	v	N
36	6.0	Female	t(11;19)	pro-B	v	v	N
37	2.0	Female	t(11;19)	pre-B	v	v	N
38	4.1	Male	t(11;19)	common B	v	v	N
39	0.4	Female	t(9;11)	unspecified B	v	v	
40	9.8	Female	t(9;11)	pre-B	v	v	
41	4.2	Male	t(9;11)	pre-B	v	v	
42	10.3	Female	t(9;11)	pro-B	v	v	

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Patient number	Age (months)	Sex	Type of *MLL* translocation	Immuno-phenotype	9K chip	Agilent 244K chip*	Affyme-trix genechip**
43	6.0	Female	t(9;11)	pre-B	v	v	
44	11.7	Male	t(9;11)	pro-B	v	v	
45	5.5	Male	Untranslocated	pre-B	v	v	
46	6.0	Male	Untranslocated	pre-B	v	v	
47	5.9	Male	Untranslocated	pre-B	v	v	
48	11.8	Female	Untranslocated	pro-B	v	v	
49	9.1	Male	Untranslocated	pro-B	v	v	
50	9.0	Female	Untranslocated	pre-B	v	v	
51	7.4	Female	Untranslocated	pre-B	v	v	
52	11.7	Female	Untranslocated	common B	v	v	
53	4.2	Male	Untranslocated	pro-B	v	v	
54	11.1	Male	Untranslocated	pre-B	v	v	
55	0.0	Male	Untranslocated	common B	v		
56	11.1	Female	Untranslocated	common B	v	v	
57	2.5	Male	Untranslocated	pre-B	v	v	

* Due to restricted availability of patient material, only 49 infant ALL patients could be hybridized to the 244K CpG island microarray.
** For the same reason only 15 t(4;11)-positive and 14 t(11;19)-positive patients were hybridized to the Affymetrix GeneChip.

(**Supplemental tables 2S – 5S:** available online at http://bloodjournal.hematologylibrary.org/ content/114/27/5490/suppl/DC1)

Supplemental table 6S. Results of the Cox regression model. t(4;11)-positive and t(11;19)-positive infant ALL patients combined were classified as heavily methylated (n=16) or lightly methylated (n=12) according to the degree of DNA methylation based on hierarchical clustering of methylation patterns as shown in figure 3. Patients were stratified as high-risk when aged <6 months (183 days) and displaying white blood cell counts (WBC) >300x10^9/L at diagnosis. Alternatively, patients were classified as medium-risk when aged >6 months at presentation, or aged <6 months with WBC <300x10^9 / L.

	HR (95% CI)	p-value
Interfant-06 medium-risk	1	
Interfant-06 high-risk	7.30 (2.12 – 25.1)	0.002
Lightly methylated	1	
Heavily methylated	5.77 (1.57 – 21.2)	0.01

(**Supplemental tables 7S – 8S:** available online at http://bloodjournal.hematologylibrary.org/ content/114/27/5490/suppl/DC1)

Supplemental table 9S. The different gene ontology clusters as created by the web-based tool Profcom based on the top 100 and top 75 of genes most significantly hypermethylated in t(4;11)- and t(11;19)-positive infant ALL (Supplemental tables 4S and 5S).

t(4;11)			
CLUSTER 1	**CLUSTER 2**	**CLUSTER 3**	**CLUSTER 4**
sequence-specific DNA binding	**transcription factor activity**	**RNA polymerase II transcription factor activity**	**protein binding**
HOXD10	HOXD10	HOXD10	HOXD10
FOXA1	FOXA1	TBPL1	HK1
NR2F2	NR2F2	TBX3	TBPL1
TBX3	TBX3	SUPT3H	TLR2
NR3C1	NR3C1	FOXF2	GAD2
DLX4	DLX4	LHX1	MLLT6
LHX5	LHX5		PSMC3
EN1	MYC		NR3C1
OTX2	EN1		CENPC1
SHOX	OTX2		SMPDL3A
GATA6	SHOX		PABPC1
ATF3	LMO1		NUDT5
FOXF2	TBX6		SIM2
TITF1	ATF3		MYC
LHX1	FOXF2		OTX2
	TITF1		WDR68
	LHX1		BMPR2
			TMEM4
			CDS1
			RPL14
			CLTC
			GATA6
			PTGS2
			EIF3S3
			WBP2
			NTRK1
			SFRP2
			TPM3
			TITF1
			NOTCH3
			RPN2
			SNTG2
			BAG3
			SEMA6A
			LIPG
			SLIT2

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t(11;19)	
CLUSTER 1	**CLUSTER 2**
sequence-specific DNA binding	**transcription factor activity**
NR2F2	NR2F2
NR3C1	NR3C1
EN1	EN1
HOXB8	HOXB8
HOXA11	HOXA11
SHOX	SHOX
ATF3	PDX1
PDX1	SLC2A4RG
	DMRT3
	ATF3

CHAPTER 3

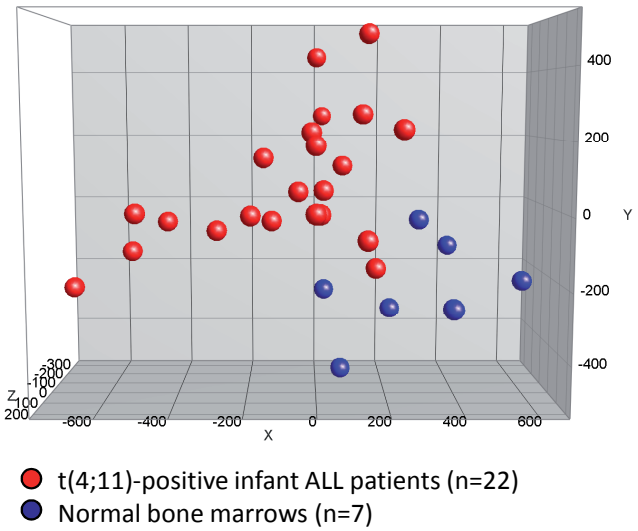
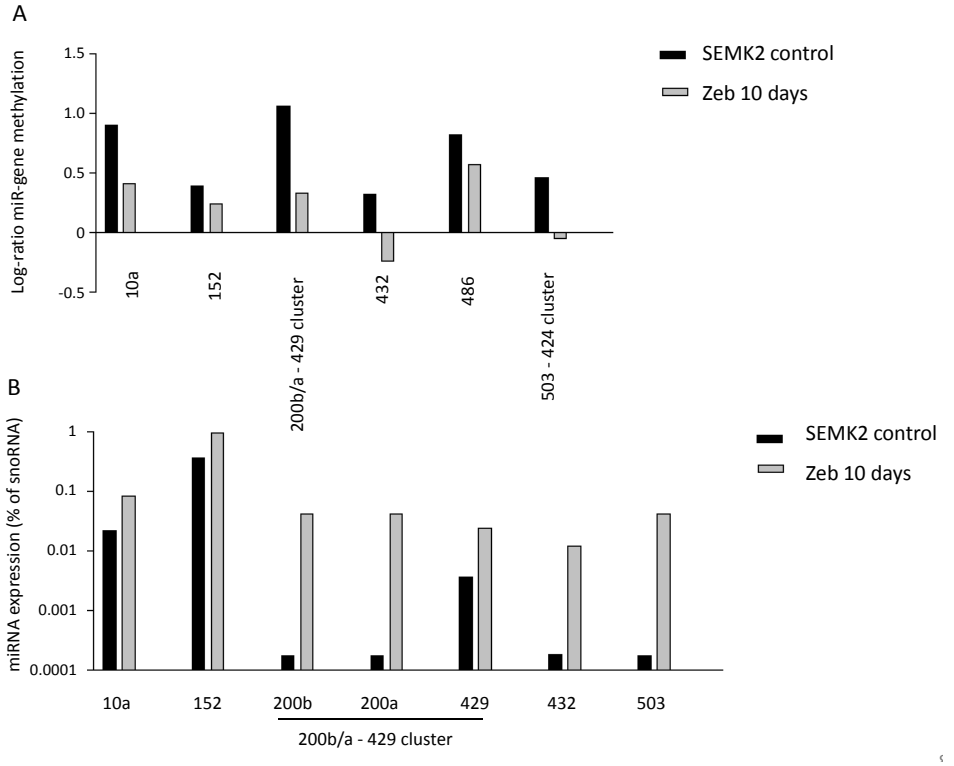
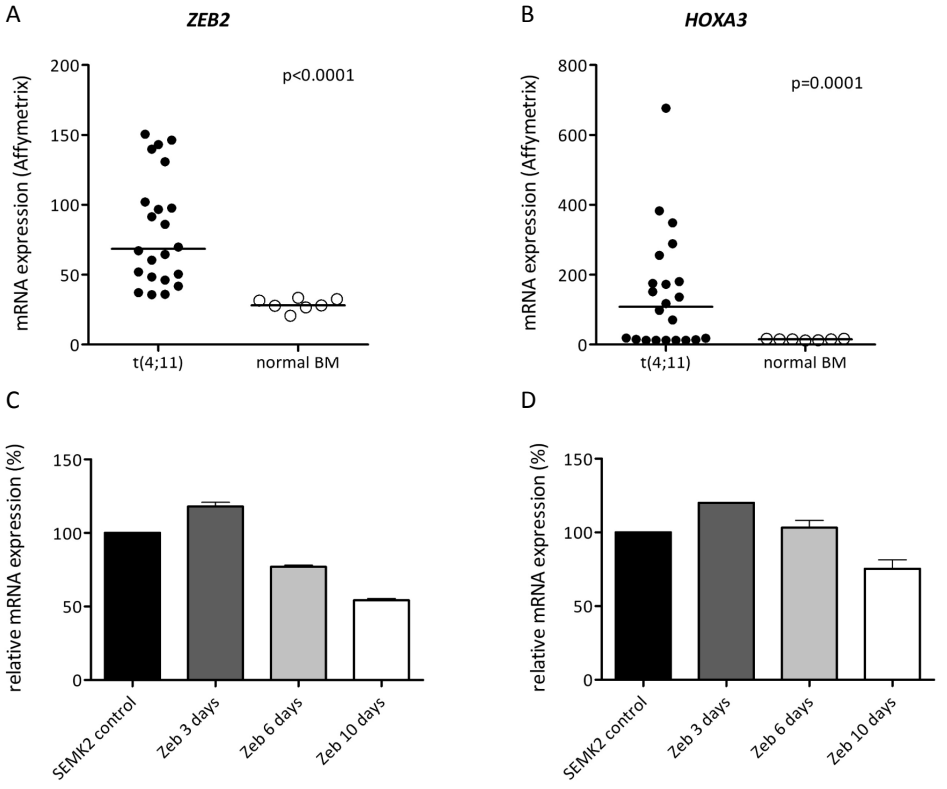


Figure 1S. Unsupervised clustering analysis of t(4;11)-positive ALL infants based on methylation data of 122 miR-genes

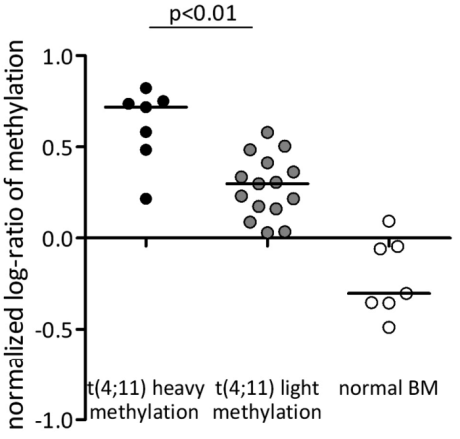
Principal Component Analyses (PCA) of the miRNA methylation data from t(4;11)-positive infant ALL patients (n=22) and normal bone marrows (n=7) using all 1118 probes related to 122 miR-genes present on the Agilent 244K CpG island microarray platform. Based on the first three components of the PCA explaining 43.6% of the total variance, the samples were visualized. Each case is color-coded indicating t(4;11)-positive infant ALL (red) and normal bone marrow (blue). The infant ALL patients (n=2) clustering together with the normal bone marrow samples showed little aberrant DNA methylation and had a very good clinical outcome.



Supplemental figure 2S. De-methylation and re-expression of miRNAs upon zebularine exposure. **A.** Graphs displaying the changes in methylation levels after 10 days of zebularine exposure. The normalized log-ratio of methylation is presented on the Y-axis and the different miR-genes are presented on the X-axis. **B.** Graphs displaying the changes in miRNA expression levels after 10 days of zebularine exposure. The relative expression is presented as the percentage of snoRNA expression on the Y-axis and the different miRNAs are depicted on the X-axis.



Supplemental figure 3S. *ZEB2* and *HOXA3* mRNA expression.
A. *ZEB2*, and **B.** *HOXA3* normalized mRNA expression levels as obtained from gene expression profiling (Affymetrix GeneChips) in t(4;11)-positive infant ALL patients (n=22) and normal bone marrow samples (n=7). *HOXA3* expression levels were derived from the 235521_at probeset present on Affymetrix U133 plus 2.0 gene arrays for which we previously demonstrated that levels determined by this probeset and real-time quantitative PCR were correlated. P values are from limma models. **C.** *ZEB2,* and **D.** *HOXA3* mRNA expression levels relative to the housekeeping reference gene *B2M* were determined by RT-PCR in the t(4;11)-positive cell line SEMK2 before and after exposure for 3, 6 and 10 consecutive days to 100 µM of zebularine. Expression levels in unexposed SEMK2 cells were set to 100%.



Supplemental figure 4S. Higher degree of methylation of the miR-152 CpG island in heavily methylated patients than in lightly methylated t(4;11)-positive infant ALL patients. Methylation levels of the miR-152 CpG island as obtained by differential methylation hybridization (DMH) presented as normalized log-ratios of methylation in heavily methylated t(4;11)-positive infant ALL patients (n=7), lightly methylated t(4;11)-positive infant ALL patients (n=15) and normal bone marrows (n=7). P-value is from the limma model.

Supplemental table 1S. Primer combinations used for quantitative RT-PCR.
All oligonucleotides were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CA).

Target gene	Sequence
DNMT1	
Forward	5'-TTGGGATGGAATTGGTAG-3'
Reverse	5'-GGCAGGGACACACAATA-3'
MLL	
Forward	5'-GGACCGCCAAGAAAAG-3'
Reverse	5'-GTTTCGGCACTTATTACACTC-3'
ZEB2	
Forward	5'-GCGGCATATGGTGACACACAA-3'
Reverse	5'-CATTTGAACTTGCGATTACCTGC-3'
HOXA3	
Forward	5'-CAACCCTACCCCTGCCAAC-3'
Reverse	5'-TGCTTTGTGTTTTGTCGAGA-3'
B2M	
Forward	5'-GGAGCATTCAGACTTGTTT-3'
Reverse	5'-ATGCGGCATCTTCAAA-3'

Supplemental table 2S. The 59 miR-genes most significantly hypermethylated in t(4;11)-positive infant ALL (compared with normal bone marrow) are listed in order of decreasing statistical significance.
The Agilent probe ID (Probe Name), miRNA gene name, log- fold change in CpG island methylation and p–value adjusted for multiple testing are shown (limma analyses). The log-fold change in expression as determined by RT-PCR is added for the corresponding miRNA as well as the adjusted p-value for differential expression (limma model). NA: not available.

Probe Name	miRNA gene	log- fold change in methylation	adjusted p- value of methylation	log- fold change in expression	adjusted p- value of expression
A_17_P09820223	miR-9-3	1.1621	0.0000	-0.0525	0.1006
A_17_P17000586	miR-301	0.7558	0.0000	-0.5153	0.2661
A_17_P10346983	miR-152	0.6060	0.0003	-0.2380	0.0010
A_17_P17111452	miR-181d	1.0587	0.0003	31.3055	0.0016
	miR-181c	1.0587	0.0003	0.1522	0.0023
A_17_P11989588	miR-505	0.6910	0.0006	0.0000	0.4905
A_17_P15166264	miR-135b	0.5656	0.0007	-0.0713	0.1799
A_17_P05278265	miR-589	0.7055	0.0007	-0.0338	0.0429
A_17_P05778799	miR-96	0.7064	0.0012	0.0071	0.0802
	miR-183	0.7064	0.0012	0.0397	0.1594
A_17_P09516785	miR-127	1.0599	0.0023	-0.1382	0.1086
	miR-433	1.0599	0.0023	-0.0386	0.1186
A_17_P04083798	miR-9-2	0.6522	0.0028	-0.0525	0.1006
A_17_P15007642	miR-200b	0.5753	0.0034	-0.0116	0.0160
	miR-429	0.5753	0.0034	-0.0147	0.0160
	miR-200a	0.5753	0.0034	-0.0242	0.0420
A_17_P10192468	miR-132	0.8544	0.0034	0.0257	0.4882
	miR-212	0.8544	0.0034	NA	NA
A_17_P17358557	miR-545	1.1325	0.0034	0.0582	0.0802
	miR-374	1.1325	0.0034	3.6490	0.0909
A_17_P03592449	miR-578	0.7838	0.0034	-0.0001	0.4882
A_17_P16067209	miR-124a-1	0.6597	0.0041	0.0004	0.4243
A_17_P09516794	miR-432	0.7068	0.0063	-0.0419	0.0429
	miR-136	0.7068	0.0063	NA	NA
A_17_P15068078	miR-101-1	0.6607	0.0067	-1.2285	0.0016
A_17_P01480873	miR-560	0.6413	0.0068	NA	NA
A_17_P05646114	miR-106b	0.7146	0.0068	9.3420	0.1086
	miR-25	0.7146	0.0068	-1.0491	0.4882
A_17_P17184825	miR-663	0.5223	0.0069	NA	NA
A_17_P06173939	miR-124a-2	0.6283	0.0084	0.0004	0.4243
A_17_P08362701	miR-615	0.7699	0.0095	-0.0001	0.4882
A_17_P10862114	miR-637	0.6331	0.0096	NA	NA
A_17_P01753106	miR-561	0.5896	0.0111	NA	NA
A_17_P00403562	miR-137	0.6254	0.0116	-0.0182	0.1034
A_17_P16406397	miR-210	0.5137	0.0120	0.6889	0.0160
A_17_P05772882	miR-129-1	0.5285	0.0124	-0.0001	0.4885

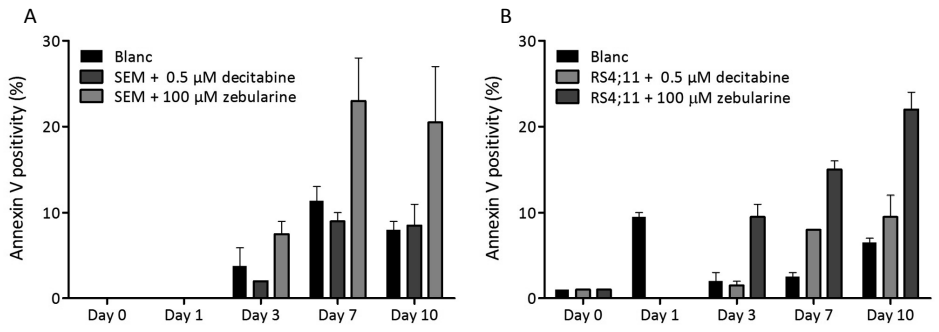
continued on the next page

Probe Name	miRNA gene	log- fold change in methylation	adjusted p- value of methylation	log- fold change in expression	adjusted p- value of expression
A_17_P10892647	miR-639	0.5292	0.0142	-0.0472	0.2716
A_17_P10211701	miR-497	0.6410	0.0147	-0.0042	0.2228
	miR-195	0.6410	0.0147	0.8791	0.3075
A_17_P09816920	miR-7-2	0.4566	0.0153	-0.0469	0.4882
A_17_P17107988	miR-638	0.7190	0.0204	NA	NA
A_17_P17210135	miR-124a-3	0.5492	0.0207	0.0004	0.4243
A_17_P07783270	miR-129-2	0.4978	0.0238	-0.0001	0.4885
A_17_P10961588	miR-641	0.7286	0.0254	NA	NA
A_17_P17399717	miR-424	0.4283	0.0271	-0.3243	0.0001
	miR-503	0.4283	0.0271	-0.0239	0.0358
A_17_P16277676	miR-126	0.4711	0.0279	0.3704	0.4890
A_17_P10283987	miR-193a	0.4757	0.0297	0.0112	0.4890
A_17_P16089634	miR-486	0.4128	0.0371	-16.9978	0.0046
A_17_P03871852	miR-580	0.3397	0.0381	0.0059	0.1133
A_17_P15383995	miR-375	0.3665	0.0391	-0.0299	0.3007
A_17_P10256737	miR-33b	0.5584	0.0391	NA	NA
A_17_P16993147	miR-10a	0.3933	0.0414	-0.1282	0.0160
A_17_P16967199	miR-423	0.5478	0.0429	0.5759	0.1010
A_17_P02716595	miR-15b	0.3254	0.0475	-5.5780	0.3847
	miR-16-1	0.3254	0.0475	7.0656	0.4890
A_17_P16147946	miR-661	0.3674	0.0481	-0.0001	0.4882
A_17_P15919150	miR-148a	0.3412	0.0490	-8.9739	0.0010
A_17_P05261048	miR-339	0.4310	0.0495	-0.0360	0.1942

Supplemental table 3S. Median values and ranges of white blood cell (WBC) count and age in the group with low miR-152 methylation (n=11) and the group with high miR-152 methylation (n=11).
P-values are from a t-test.

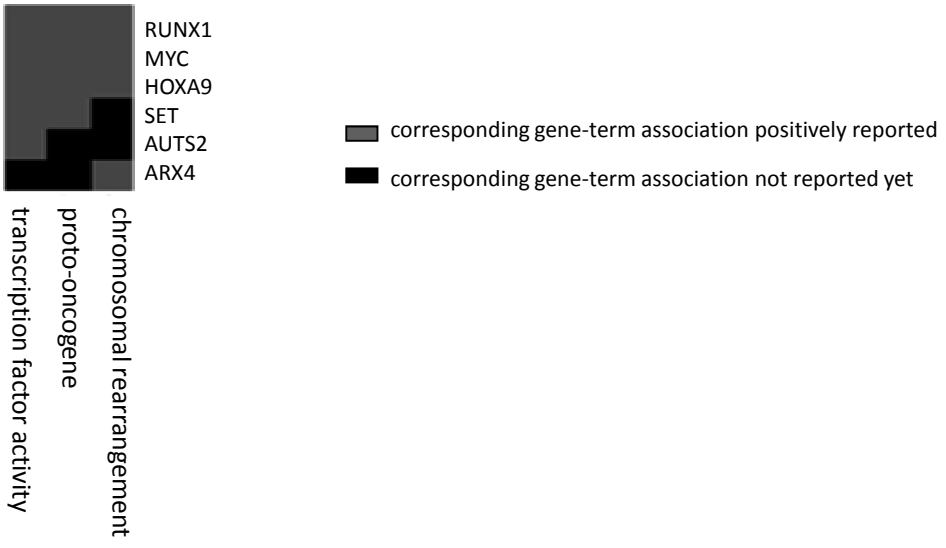
	Low miR-152 methylation (n=11)	High miR-152 methylation (n=11)	Significance
Median WBC count in cells/µL (range)	291000 (34000-816000)	309000 (25000-74000)	p=0.88
Median age in months (range)	6.5 (0.4-11.2)	3.4 (0.6-6.7)	p=0.11

CHAPTER 4

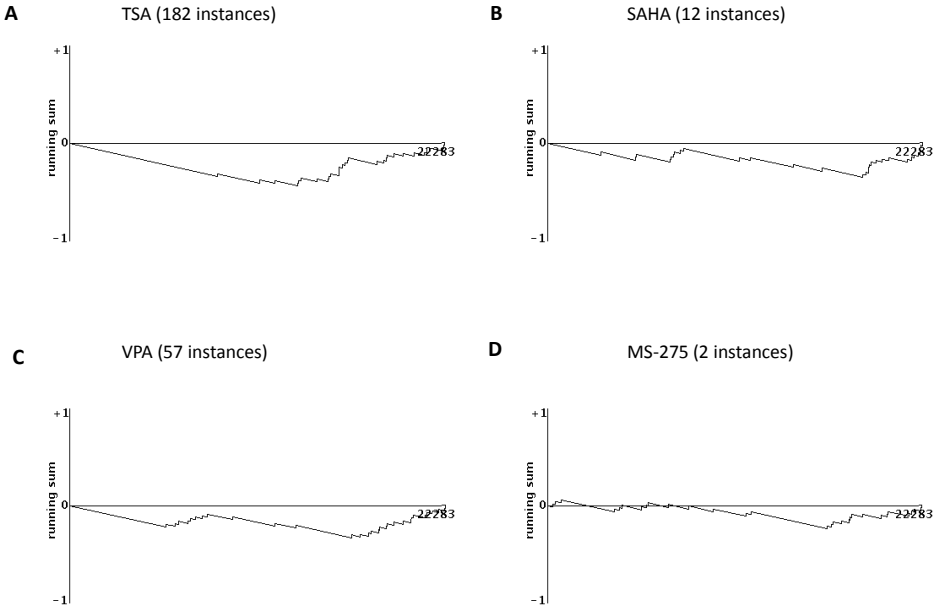


Supplemental figure 1S. Cell viability.
The t(4;11)-positive cell lines SEM and RS4;11 were exposed to the demethylating agents decitabine (0.5 uM) and zebularine (100 uM) for 10 days. Cells were sampled after 0, 1, 3, 7, and 10 days. At these time points cell death was measured by Annexin V staining determined by flow cytometry. **A.** cell death in the SEM cell line and **B.** cell death in the RS4;11 cell line.

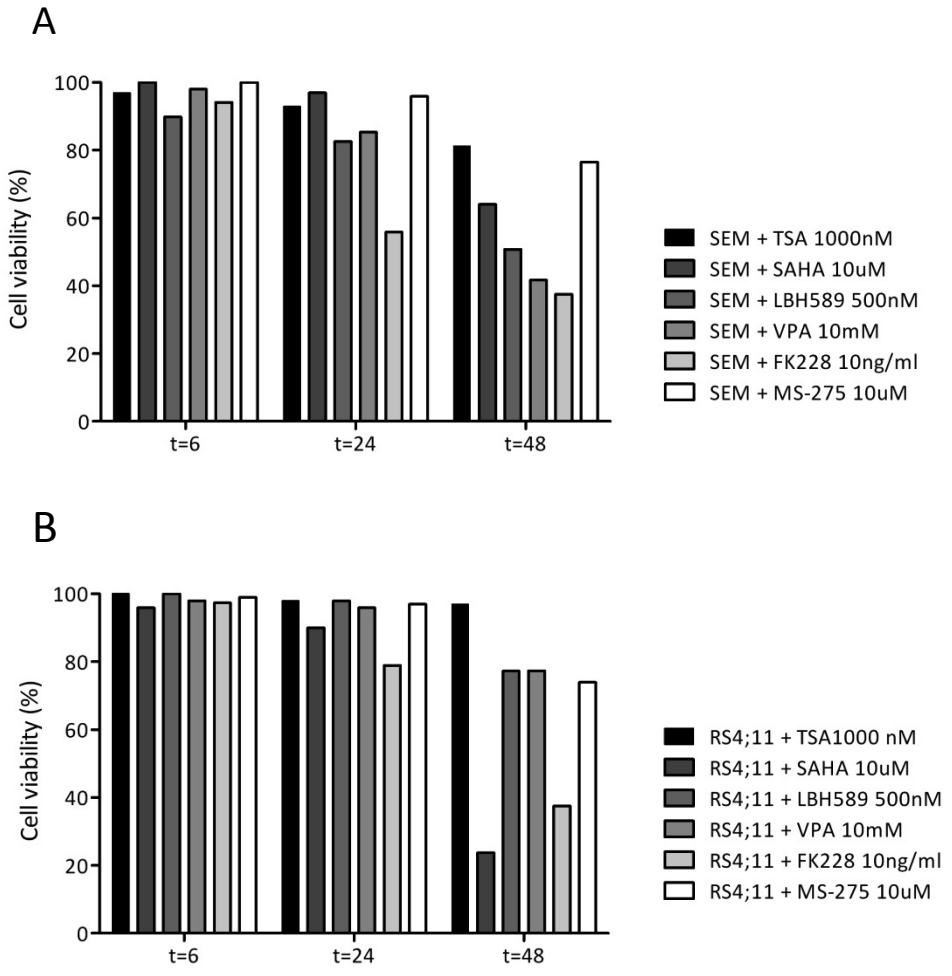
CHAPTER 6



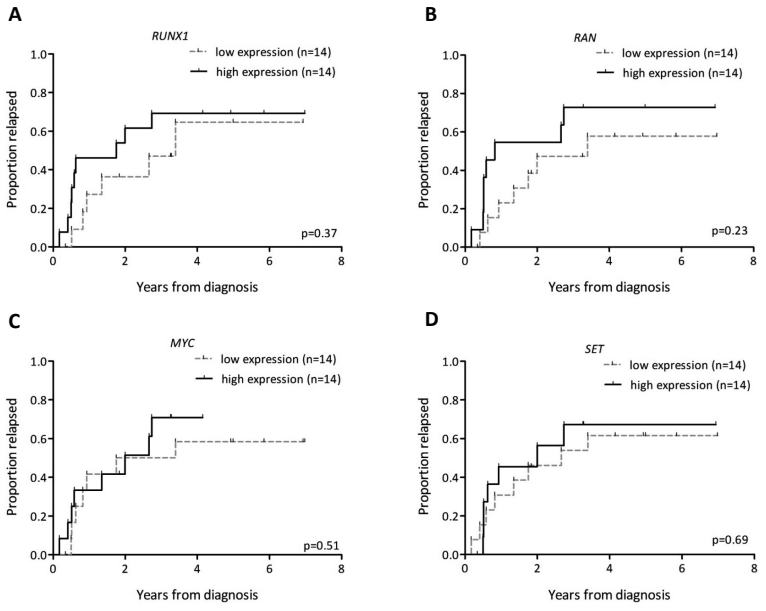
Supplemental figure 1S. Results obtained from the DAVID gene ontology database.
Our gene list (36 genes) was enriched for proto-oncogenes (*HOXA9*, *MYC*, *RUNX1*, *SET*) and genes commonly involved in chromosomal translocations in leukemia (*RUNX1*, *HOXA9*, *MYC*, *SET*, *AUTS2*). DAVID gene ontology database is available at: http://david.abcc.ncifcrf.gov/home.jsp.



Supplemental figure 2S. Results obtained from the connectivity map.
The graphs present the enrichment of the infant ALL gene signature for the most significant HDAC inhibitor instances (treatment-control pairs). For each probe set a value is assigned and the amplitude corresponds to the individual response of each probeset. High amplitudes represent a stronger response of more genes to the specific HDAC inhibitor. Negative values indicate negative correlations of the treatment instances with the gene signature. **A.** TSA, **B.** SAHA, **C.** VPA and **D.** MS-275.



Supplemental figure 3S. Cell viability counts.
The percentage of cell viability as measured by trypan blue exclusion is presented at three time points (after 6 hours, 24 hours and 48 hours) for the different exposures to the HDAC inhibitors TSA (1µM), SAHA (10µM), LBH589 (500nM), VPA (10mM), FK228 (10ng/ml) and MS-275 (10µM). **A.** SEM cell line, **B.** RS4;11 cell line. Indicated values represent means of duplicate cell countings.



Supplemental figure 4S. Risk of relapse in t(4;11)-positive infant ALL.
For the proto-oncogenes *RAN*, *RUNX1*, *MYC* and *SET* mRNA levels were determined using quantitative real-time PCR analysis in an extended cohort of primary t(4;11)-positive infant ALL samples (n=28). Based on the median expression levels, the patients were divided into two groups for which the risk of relapse was computed. **A.** *RUNX1*, **B.** *RAN*, **C.** *MYC*, **D.** *SET.* The proportion of relapsed patients, as computed with the Kaplan Meier estimator, is presented on the Y-axis and the follow-up time (in years) is presented on the X-axis. The log-rank test was used to compare outcomes between different patient groups. SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for computation of survival statistics. Due to the small number of patients, these data should be interpreted with caution.

Supplemental table 1S. Patient characteristics.

Patient number	Age (months)	Sex	Type of *MLL* translocation	Immunophenotype
1	9.4	Female	t(4;11)	pro-B/CD10-
2	2.8	Male	t(4;11)	pro-B/CD10-
3	9.4	Female	t(4;11)	pro-B/CD10-
4	11.0	Female	t(4;11)	pro-B/CD10-
5	3.6	Male	t(4;11)	pro-B/CD10-
6	6.6	Female	t(4;11)	pro-B/CD10-
7	1.9	Male	t(4;11)	pro-B/CD10-
8	4.2	Female	t(4;11)	pro-B/CD10-
9	0.6	Female	t(4;11)	pro-B/CD10-
10	0.7	Female	t(4;11)	pro-B/CD10-
11	1.9	Female	t(4;11)	pro-B/CD10-
12	6.4	Female	t(4;11)	pro-B/CD10-
13	6.4	Male	t(4;11)	pro-B/CD10-
14	1.6	Female	t(4;11)	pro-B/CD10-
15	8.0	Male	t(4;11)	pro-B/CD10-

Supplemental table 2S. Primer sequences used for quantitative real-time PCR analysis.
All oligonucleotides were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CA) and ordered from Eurogentec (Liege, Belgium).

Target gene	Sequence
DIAPH1	
Forward	5'-ATCCCACAGCACAGTCAT-3'
Reverse	5'-GGGTTGTTGTTGAGAGACA-3'
SFMBT1	
Forward	5'-GAGCTGCCTCAATGTGTAG-3'
Reverse	5'-GACAGCATTCCAGTTTGATAC-5'
RAN	
Forward	5'-TGGCAACAAAGTGGATATTA-3'
Reverse	5'-CGGGAGAGCAGTTGTCT-3'
PARK7	
Forward	5'-GTTCGCTCTAAACAAAACAGT-3'
Reverse	5'-TAGGCTGAGAAATCTCTGTGT-3'
HOXA9	
Forward	5'-CACGCTTGACACTCACACT-3'
Reverse	5'-CAGGGTCTGGTGTTTTGTA-3'
MYC	
Forward	5'-CGTCCTCGGATTCTC-3'
Reverse	5'-GCTGCGTAGTTGTGCTG-3'
SET	
Forward	5'-TTCCCGATATGGATGATG-3'
Reverse	5'-CCCCCCAAATAAATTGAG-3'
RUNX1	
Forward	5'-GACAGCCCCACCTTCC-3'
Reverse	5'-CCACTTCGACCGACAA-3'
MLL-AF4	
Forward	5'-GGACCGCCAAGAAAAG-3'
Reverse	3'-CTGGGGTTTGTTCACTGT-3'
B2M	
Forward	5'- GGAGCATTCAGACTTGTTT-3'
Reverse	5'- ATGCGGCATCTTCAAA-3'

Supplemental table 3S. The 36 genes most significantly hypomethylated in t(4;11)-positive infant ALL (compared with normal bone marrows) are listed in order of decreasing statistical significance.
The Agilent probe name, Affymetrix HGU133plus2.0 probe name, Entrez Gene ID, gene name, log- fold change of methylation and p–values adjusted for multiple testing are shown (limma analyses in R).

Agilent Probe Name	Affymetrix Probe Name	Gene ID	Gene Name	Log fold change methylation	Adjusted p value methylation	Adjusted p value expression
A_17_ P16727446	220368_s_at	SMEK1	SMEK homolog 1, suppressor of mek1	-2.0083	8.89E-11	0.0022
A_17_ P16475281	209323_at	PRKRIR	protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of	-1.7327	1.05E-08	0.0733
A_17_ P15747745	209190_s_at	DIAPH1	diaphanous homolog 1	-1.6503	2.00E-08	0.0208
A_17_ P06441385	222543_at	DERL1	Der1-like domain family, member 1	-1.5355	2.42E-08	0.0119
A_17_ P05519645	212599_at	AUTS2	Autism susceptibility candidate 2	-1.7367	2.96E-08	1.37E-05
A_17_ P10244001	227361_at	HS3ST3B1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	-1.7318	2.76E-09	0.0024
A_17_ P15920204	214651_s_at	HOXA9	homeobox A9	-1.7832	5.65E-08	0.0215
A_17_ P11512146	221496_s_at	TOB2	transducer of ERBB2, 2	-1.2234	8.92E-08	6.30E-05
A_17_ P11713320	219433_at	BCOR	BCL6 co-repressor	-2.0421	2.42E-08	8.03E-06
A_17_ P16433711	205797_s_at	TCP11L1	t-complex 11 (mouse)-like 1	-1.3046	1.26E-07	0.0007
A_17_ P15384777	210141_s_at	INHA	Inhibin, alpha	-1.8534	8.92E-08	0.0052
A_17_ P17233302	210365_at	RUNX1	runt-related transcription factor 1	-1.5102	1.30E-07	2.00E-05
A_17_ P10857565	218188_s_at	LMNB2	lamin B2	-1.6474	1.02E-07	2.82E-05
A_17_ P16893168	202980_s_at	SIAH1	seven in absentia homolog 1	-1.3724	1.58E-07	0.0154
A_17_ P16604897	200750_s_at	RAN	RAN, member RAS oncogene family	-1.7018	1.21E-07	3.54E-05
A_17_ P16970587	208777_s_at	PSMD11	proteasome 26S subunit, non-ATPase, 11	-1.3275	1.90E-07	0.0018

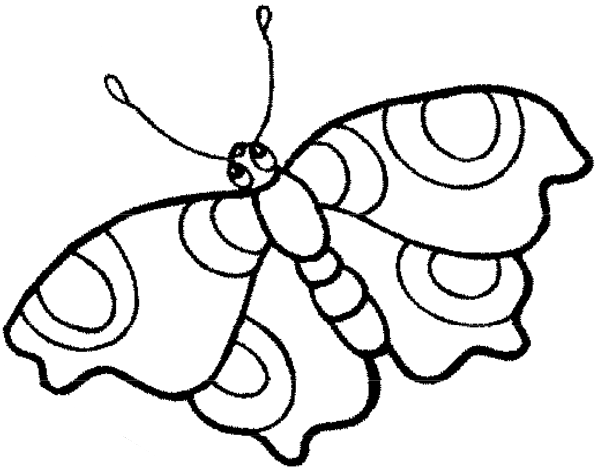
A_17_P15750644	201431_s_at	DPYSL3	Dihydropyrimidinase-like 3	-1.9369	1.58E-07	0.0002
A_17_P15439580	213370_s_at	SFMBT1	Scm-like with four mbt domains 1	-1.0876	3.87E-07	0.0085
A_17_P16379748	221012_s_at	TRIM8	tripartite motif-containing 8	-1.2759	2.47E-07	0.0001
A_17_P15020491	200006_at	PARK7	Parkinson disease 7	-1.4313	2.47E-07	0.0048
A_17_P01134075	205657_at	HAAO	3-hydroxyanthranilate 3,4-dioxygenase	-1.5989	2.47E-07	0.0009
A_17_P15398519	205251_at	PER2	period homolog 2	-1.4592	3.22E-07	0.0002
A_17_P16794555	217828_at	SLTM	SAFB-like, transcription modulator	-1.4051	3.53E-07	0.0052
A_17_P00775037	228181_at	SLC30A1	solute carrier family 30, member 10	-1.7559	2.47E-07	0.0002
A_17_P16175760	226496_at	ZCCHC7	zinc finger, CCHC domain containing 7	-1.1562	5.02E-07	5.45E-12
A_17_P16440138	208330_at	ALX4	aristaless-like homeobox 4	-1.225	5.02E-07	0.0164
A_17_P11411430	228976_at	ICOSLG	inducible T-cell co-stimulator ligand	-1.3840	3.93E-07	0.0002
A_17_P02241304	218147_s_at	GLT8D1	glycosyltransferase 8 domain containing 1	-1.1984	6.75E-07	0.0007
A_17_P11104475	206502_s_at	INSM1	Insulinoma-associated 1	-1.2634	4.70E-07	0.0002
A_17_P16270082	219198_at	GTF3C4	General transcription factor IIIC, polypeptide 4	-1.4536	4.70E-07	0.2063
A_17_P15438081	208465_at	GRM2	glutamate receptor, metabotropic 2	-1.4313	4.70E-07	0.0012
A_17_P16266012	200631_s_at	SET	SET translocation	-1.0590	1.69E-06	2.27E-05
A_17_P15234061	242898_at	EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	-0.9454	1.54E-06	0.0092
A_17_P07522030	202405_at	TIAL1	TIA1 cytotoxic granule-associated RNA binding protein-like 1	-1.7435	3.87E-07	0.0002
A_17_P11140901	200903_s_at	AHCY	S-adenosylhomocysteine hydrolase	-1.3563	1.09E-06	0.0011
A_17_P06461251	202431_s_at	MYC	myc myelocytomatosis viral oncogene homolog (avian)	-1.6514	1.44E-06	2.23E-05

Supplemental table 4S. Results obtained from the connectivity map.
The compounds are ranked according to their negative down-score. The down-score is a value between -1 and 1 that in our case represents the absolute enrichment of the interrogated gene signature in a given instance. A high negative down-score indicates that the corresponding compound is likely to induce down-regulation of the query signature. Candidate agents for further study were essentially selected based on these highest negative down-scores. Subsequently a permuted analysis was performed to correct for the possibility that the enrichment of all instances of each compound from the list would be observed merely by chance. Permutation testing was significant for TSA (p=0.00, specificity = 0.73), SAHA (p=0.04, specificity = 0.71) and MS-275 (p=0.04, specificity = 0.19), but not for VPA.

Compound name	Dose	Cell line	Ranking	Down Score
trichostatin A	100 nM	MCF7	1	-0.508
trichostatin A	100 nM	MCF7	2	-0.438
trichostatin A	1 µM	PC3	3	-0.434
trichostatin A	1 µM	MCF7	4	-0.419
trichostatin A	100 nM	MCF7	5	-0.419
trichostatin A	100 nM	MCF7	7	-0.414
trichostatin A	100 nM	MCF7	8	-0.414
trichostatin A	100 nM	MCF7	9	-0.413
trichostatin A	100 nM	MCF7	10	-0.408
trichostatin A	100 nM	MCF7	11	-0.407
trichostatin A	100 nM	MCF7	12	-0.406
vorinostat	10 µM	MCF7	13	-0.405
trichostatin A	100 nM	PC3	14	-0.405
trichostatin A	1 µM	MCF7	16	-0.399
trichostatin A	100 nM	MCF7	17	-0.399
trichostatin A	1 µM	MCF7	18	-0.397
trichostatin A	100 nM	MCF7	20	-0.392
trichostatin A	100 nM	HL60	21	-0.390
trichostatin A	1 µM	MCF7	22	-0.390
trichostatin A	1 µM	PC3	23	-0.389
trichostatin A	1 µM	MCF7	24	-0.388
trichostatin A	1 µM	MCF7	25	-0.386
trichostatin A	100 nM	PC3	26	-0.386
valproic acid	10 mM	HL60	27	-0.383
trichostatin A	1 µM	MCF7	29	-0.381
trichostatin A	100 nM	MCF7	30	-0.381
trichostatin A	1 µM	MCF7	31	-0.380
trichostatin A	1 µM	MCF7	32	-0.378
trichostatin A	100 nM	HL60	33	-0.377
trichostatin A	1 µM	MCF7	34	-0.376
valproic acid	10 mM	MCF7	35	-0.375
trichostatin A	100 nM	MCF7	36	-0.374
vorinostat	10 µM	MCF7	37	-0.373
trichostatin A	100 nM	MCF7	38	-0.372
trichostatin A	100 nM	MCF7	39	-0.371
trichostatin A	1 µM	PC3	41	-0.370

continued on the next page

Compound name	Dose	Cell line	Ranking	Down Score
trichostatin A	1 µM	MCF7	42	-0.369
trichostatin A	100 nM	MCF7	43	-0.368
trichostatin A	1 µM	PC3	44	-0.367
trichostatin A	100 nM	MCF7	48	-0.366
trichostatin A	100 nM	MCF7	49	-0.365
vorinostat	10 µM	MCF7	51	-0.364
trichostatin A	100 nM	MCF7	52	-0.364
trichostatin A	100 nM	MCF7	53	-0.363
trichostatin A	100 nM	PC3	54	-0.363
trichostatin A	100 nM	MCF7	55	-0.363
trichostatin A	1 µM	MCF7	56	-0.363
HC toxin	100 nM	MCF7	182	-0.314
MS-275	10 µM	PC3	376	-0.277
MS-275	10 µM	PC3	474	-0.267



“What the caterpillar calls the end,
the rest of the world calls a butterfly”

(Lao Tsu)