Molecular and clinical dissection of infant acute lymphoblastic leukemia

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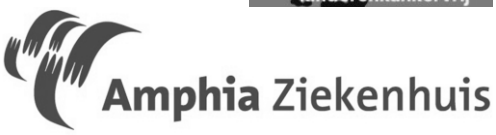

Moleculaire en klinische ontleding van acute lymfoblastische zuigelingenleukemie

Molecular and clinical dissection of infant acute lymphoblastic leukemia

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CHAPTER 1

General introduction

LEUKEMIA

Blood cell formation takes place in the bone marrow (hematopoiesis). Leukemia is cancer of the blood characterized by an abnormal growth of immature white blood cells in the bone marrow. Based on two characteristics, i.e. growth rate and immunophenotype, leukemia can be divided into several subtypes. Fast-growing, or *acute* leukemia, is the most common form of leukemia in children, whereas slow-growing, or *chronic* leukemia, is typically diagnosed in adults. Depending on the type of white blood cell that was subjected to malignant transformation, we distinguish between *lymphocytic* and *myeloid* leukemia. Lymphocytic leukemia arises in a type of white blood cell from which the lymphocytes are formed, while myeloid leukemia arises in white blood cells that develop into neutrophils, eosinophils, basophils, or macrophages. Due to the uncontrolled proliferation of the non-functional immature leukemic white blood cells (or blasts), the bone marrow becomes overgrown and the formation of healthy blood cells is strongly inhibited. In turn, the lack of healthy blood cells cause a substantial part of the symptoms that are associated with leukemia, including anemia, frequent infections and fevers, and bleedings or bruising. The non-functional leukemic blasts are released into the peripheral blood, from where they rapidly invade other organs such as the spleen, the liver, and occasionally the central nervous system. When left untreated, leukemia is typically fatal within weeks or months.

INFANT ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia (ALL) in infants (i.e. children below the age of 1 year) is rare, accounting for about 4% of all pediatric ALL cases. Compared with older children with ALL, infant ALL possesses unique clinical and biological features. The majority (~80%) of the infants diagnosed with ALL carry leukemia-specific chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene. [1, 2] As a result of such translocations, the N-terminal portion of the *MLL* gene becomes fused to the C-terminal region of one of its many translocation partner genes (Figure 1). [3] The hereby generated chimeric fusion genes are translated into MLL fusion proteins with pronounced transforming capacity. To date over 70 partners have been identified. [4]

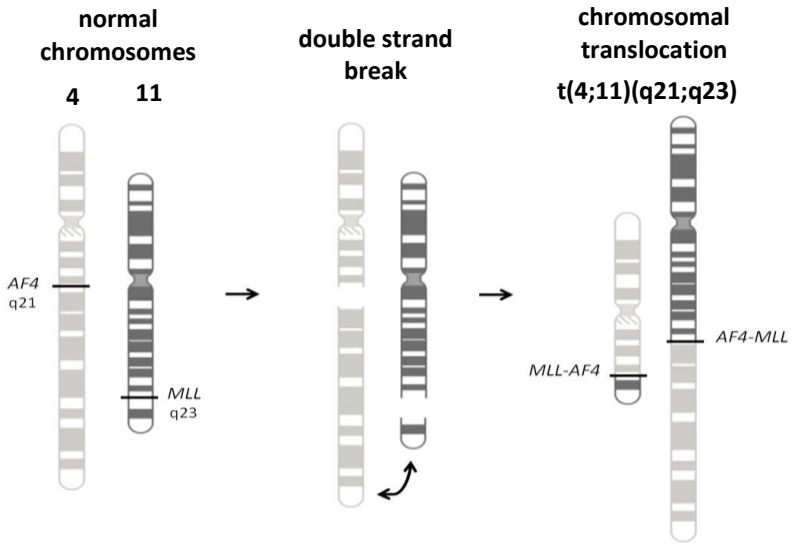

Figure 1. *MLL* translocation. Schematic representation of chromosomal translocation t(4;11)(q21;q23), fusing the N-terminal region of the *MLL* gene on chromosome 11 to the C-terminal region of the *AF4* gene on chromosome 4, and vice versa.

The most recurrent *MLL* translocations found among infant ALL patients are t(4;11), t(11;19), and t(9;11) [5, 6], giving rise to the fusion proteins MLL-AF4, MLL-ENL, and MLL-AF9, respectively (Figure 2). Infant ALL patients without a rearrangement of the *MLL* gene, are generally referred to as wild-type *MLL* infant ALL patients or *MLL* germline infant ALL patients.

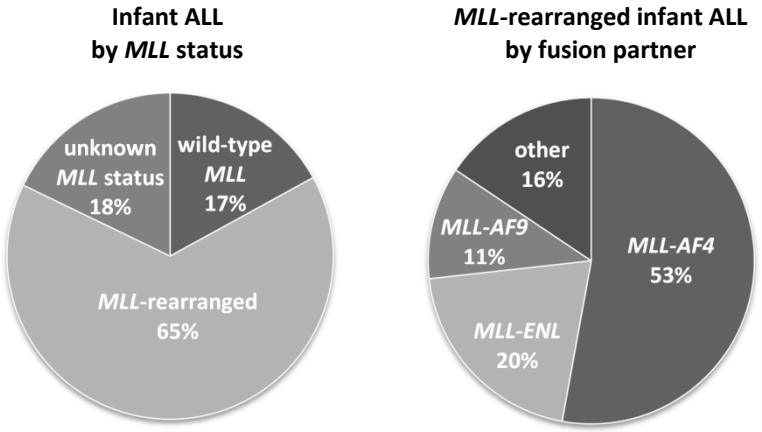

Figure 2. Frequencies of *MLL* rearrangements and wild-type *MLL* in infant ALL. Frequency of wild-type *MLL* and *MLL* rearrangement in infant ALL (left) and frequencies of fusion partner in *MLL*-rearranged infant ALL (right) according to the Interfant-99 study. [6]

While in the last decades event-free survival (EFS) chances for pediatric ALL patients have increased dramatically, nowadays exceeding 80%,[7] obtaining successful treatment results in infant ALL remains a major challenge. Although morphological complete remission is achieved in approximately 95% of the infant ALL patients, [8, 9] overall outcome in infant ALL is very poor due to an exceptionally

high relapse rate. To date, EFS rates for *MLL*-rearranged infant ALL approach 50%.[6] Apart from the presence of an *MLL* translocation (Figure 3), age <6 months, very high white blood cell counts at diagnosis, and a poor response to prednisone are the most important factors predicting a poor outcome in infant ALL. [6] Furthermore, minimal residual disease is an independent prognostic factor in infant ALL. Moreover, MDRD can be used for treatment intervention in infant ALL.[10] Neonatal leukemia (age <1 month) is very rare and generally assumed to be fatal. Except for case reports, there is no published data available concerning these neonates.

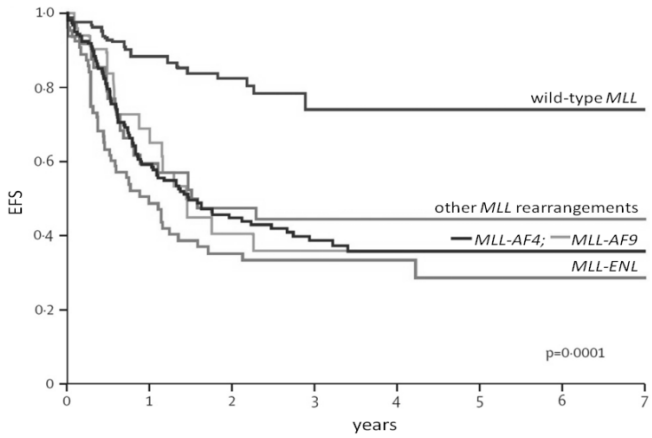

Figure 3. Clinical outcome of infant ALL. Event-free survival of infant ALL patients according to the status of *MLL* gene and type of MLL translocation.

TOWARDS TARGETED THERAPY IN INFANT ALL

Several studies demonstrated that *MLL*-rearranged ALL is characterized by a unique gene expression profile.[11, 12] Recently our laboratory published high-resolution gene expression profiling data showing that, apart from a fundamental signature shared by all *MLL*-rearranged infant ALL samples, each type of *MLL* translocation is associated with a translocation-specific gene expression signature as well. Furthermore, it was demonstrated that wild-type *MLL* infant ALL specifies a gene expression pattern that is different from both *MLL*-rearranged infant ALL and pediatric (non-infant) precursor BCP-ALL.[13] Although the large majority of patients achieves complete remission, approximately 63% relapses in the first year after achieving complete remission. More effective therapeutic strategies are therefore needed to improve prognosis. The unique gene expression profiles associated with *MLL*-rearranged infant ALL hold the potential to reveal novel genetic characteristics that may serve as therapeutic targets.

OUTLINE OF THIS THESIS

Chapter 2 is a review describing leukemia in neonates (infants <1 month), including neonatal acute myeloid leukemia (AML) and ALL. It covers biological and clinical aspects of this subgroup of infant leukemia patients and discusses future perspectives.

Neonatal –or congenital– ALL is rare and assumed inevitably fatal, but no series have been published on neonatal ALL except for case reports. **Chapter 3** reports the clinical features and outcome of neonatal ALL uniformly treated with curative intent according to the Interfant-99 protocol.

Infant ALL patients that do not harbor a rearrangement of the *MLL* gene (wild-type *MLL* infant ALL patients), carry a gene expression pattern that is different from both *MLL*-rearranged infant ALL and pediatric precursor BCP-ALL. These gene expression profiles are described in **chapter 4** and are the foundation of research described in the following chapters.

Chapter 5 addresses the thus far underreported group of wild-type *MLL* infant ALL patients. Although this group of patients generally does better than *MLL*-rearranged infant ALL patients in terms of survival, their prognosis is still worse compared with non-infant pediatric BCP-ALL patients. In order to find new openings for treatment optimization, in this chapter we clinically and molecularly characterize this wild-type *MLL* infant ALL patient group.

In order to facilitate the discovery of therapeutic targets in *MLL*-rearranged infant ALL, in **chapter 6** we report on gene expression profiling after repression of the MLL fusion protein. Herewith we identify genes readily responsive to the loss of the MLL fusion with the aim of discovering new therapeutic targets in *MLL*-rearranged infant ALL.

One of the genes responsive to the loss of the MLL fusion, and a gene recently identified to be transcriptionally driven by MLL fusion proteins is cyclin-dependent kinase 6 (*CDK6*). In **chapter 7** we validate *CDK6* as a direct target of the MLL-AF4 fusion protein and assess its role in the proliferative advantage of *MLL*-rearranged leukemia cells. We initiate investigation of the role of CDK6-inhibitor PD0332991 as a new treatment agent in *MLL*-rearranged ALL.

Differential gene expression analysis between infant ALL patients and pediatric (non-infant) BCP-ALL patients reveals *EID1* as specifically expressed in infant ALL which is discussed in **chapter 8**. To assess the potential of this gene as therapeutic target, we use lentiviral knockdown experiments to determine the consequences of loss of *EID1* on the growth and survival of *MLL*-rearranged leukemia cells.

Chapter 9 summarizes this thesis and comprises a general discussion and this thesis concludes with a Dutch summary in layman's terms in **chapter 10**.

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CHAPTER 2

Diagnosis and management of neonatal leukemia (*review*)

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SUMMARY

Leukemia in neonates (infants <1 month) is rare, whereby neonatal acute myeloid leukemia (AML) is more frequent than neonatal acute lymphoblastic leukemia (ALL). High mortality rates are observed, though AML has a better prognosis than ALL. Neonatal leukemia is typically presented with hepatosplenomegaly, leukemia cutis and/or hyperleucocytosis. Congenital infections should be ruled out before diagnosis. Rearrangement of the *MLL* gene is the most frequently occurring genetic aberration. Treatment includes intensive multi-agent chemotherapy, usually with age-related dose adjustments next to supportive care. Treatment intensification for ALL could be indicated in the future as the dismal prognosis is subject to high relapse rates in ALL.

INTRODUCTION

Neonatal – or congenital – leukemia is diagnosed in the first 30 days after birth. Estimated incidence of neonatal leukemia ranges from 1 to 5 per million live births. [1,2] Less than 1% of all childhood leukemia is diagnosed in neonates. [1] Two publications reviewing 117 patients and 145 patients respectively described a higher reported frequency of acute myeloid leukemia (AML) (56–64%) than acute lymphoblastic leukemia (ALL) (21–38%), plus a few cases of biphenotypic leukemia. [3,4]

Aside from trisomy 21, the most frequently occurring chromosomal aberration in both neonatal AML and ALL is a translocation involving the mixed lineage leukemia (*MLL*) gene, located on chromosomal band 11q23. Hereby the *MLL* gene breaks and fuses to one of its many translocation partners, forming a new fusion product. In normal embryogenesis *MLL* regulates gene expression in an epigenetic manner and is required for adequate numbers of hematopoietic progenitors and their proper differentiation.[5] In *MLL*-rearranged leukemia this program is deregulated, leading to an aberrant gene expression with upregulation of multiple oncogenes. [6] In earlier reports a frequency of *MLL* rearrangements in neonatal leukemia has been described of 30–65%, [3,4] but a more recent report in neonatal ALL describes a frequency of *MLL* rearrangements of up to 93%.[7] Very likely this difference is due to refinement of the technique in discovering *MLL* rearrangements over the last decades, like the introduction of the split-signal FISH methodology facilitating the identification of known and new translocations involving *MLL*.[14] Further, acute megakaryotic leukemia (AMKL) (FAB M7) in non-Down patients is strongly associated with t(1;22)(p13;q13), forming the fusion gene RBM15-MKL1. This rearrangement is present in about 70% of all infant AMKL

patients [8,9] and was reported in eight out of 13 non-Down AMKL patients reviewed by Isaacs.[4]

ETIOLOGY

The etiology of infant (<1 year of age) leukemia rather than neonatal leukemia has been studied. As the definition of congenital leukemia is arbitrary and due to the high incidence of *MLL* rearrangements in both congenital and infant leukemia, the etiology of these two groups will largely overlap and will here be discussed as one group.

The etiology of *MLL*-rearranged infant leukemia has been studied in depth over the last decades. The observation of infant twins with concordant leukemia who each share clonal *MLL* rearrangements in their leukemic cells provides unequivocal evidence that the leukemogenic event originates in utero.[10] In the same context it was demonstrated that *MLL-AF4* genomic fusion sequences are already present in the neonatal blood spots of Guthrie cards of infants later diagnosed with ALL, providing more profound evidence for prenatal initiation of the disease.[11] Associations between developing infant leukemia and maternal exposure to multiple toxins have been extensively explored, and demonstrated increased risk after maternal marijuana use and alcohol consumption, but not for instance cigarette smoking. [12,13] The observation that the topoisomerase-II inhibitor etoposide causes therapy-related *MLL*-rearranged AML[14] led to epidemiological and experimental studies of related dietary compounds called (bio)flavonoids. Flavonoids, present in citrus fruit, wine, tea and dark chocolate,

have been demonstrated in vitro to induce double-strand breaks of the *MLL* gene. A maternal diet high in flavonoids is therefore suspected to increase the risk of *MLL*-rearranged infant leukemia. Definite evidence for this hypothesis has not been provided, possibly due to the complexity of dietary research and the low incidence of *MLL*-rearranged infant leukemia. [15-17]

SIGNS AND SYMPTOMS

Clinical signs of neonatal leukemia vary a great deal among patients.

Characteristic clinical manifestations

Hepatosplenomegaly, a very frequently occurring symptom, can be found in around 80% of the patients. [3.18] Enlargement of the liver is found more often than an enlarged spleen. Enlarged lymph nodes are found in only one out of four patients. [3]

Leukemia cutis, which is described in around 60% of all patients, [3,4,18] are specific cutaneous leukemic infiltrates and usually appear as firm blue, red, or purple nodules in a generalized distribution. [19] Leukemia cutis is reported to be the initial presenting sign in about half of the neonatal cases. [4,20] It is a cause of the so-called 'blueberry muffin baby'.

The third clinical feature, hyperleucocytosis, is present in the majority of the patients. Forty-seven out of 55 (85%) ALL patients and 37 out of 72 (49%) of the AML patients had a white blood cell (WBC) count >50 × 109/l from the neonatal leukemia patient group reviewed by Isaacs.4 By contrast, infant AMKL patients with t(1;22) were described with a decreased leukocyte number, anemia and

thrombocytopenia.[21] A severe complication of hyperleucocytosis is the leucostasis syndrome, in which white cell plugs are formed in the microvasculature, leading to cardiac failure and respiratory and neurological problems. Neurological symptoms in the neonate as part of the leucostasis syndrome can be in the form of somnolence and coma, papilloedema, retinal vein distension and retinal haemorrhage.[22,23] A respiratory leucostasis manifests itself usually in the form of unspecific symptoms such as tachypnoea, dyspnoea, hypoxia, pulmonary infiltrates or respiratory failure.

Secondary clinical manifestations

Extramedullary infiltration of the leukemia and overgrowth of the bone marrow leading to anaemia, thrombocytopenia and/or neutropenia may cause various secondary clinical manifestations, e.g. increased bleeding tendency, infections and failure to thrive.

Diverse neurological findings such as cranial nerve palsies, seizures, and papilloedema are likely caused by infiltration into the central nervous system, which is present in more than a third of the patients. Meningeal leukemic infiltration is indicated by a bulging fontanel. Neurological findings may alternatively be explained by intracranial bleeding due to the thrombocytopenia, possibly also leading to a bulging fontanel, and secondary to infarction in the presence of leucostasis. Respiratory signs and symptoms may be caused by thrombocytopenia due to pulmonary hemorrhage, but can also be explained by leukemic infiltration leading to atelectasis or pneumonia due to neutropenia. Besides pneumonia, diverse infectious problems including sepsis occur in these neonates. Cardiac failure and non-immune hydrops in the newborn have been described due to severe anaemia. [24] Anaemia and thrombocytopenia can further

induce diverse signs such as pallor, failure to thrive, lethargy, melena, and bleeding tendencies.

Signs in utero

In-utero hepatosplenomegaly, hydrops and polyhydramnios can be detected by ultrasound. Fetoscopy and umbilical blood sampling can then establish the diagnosis of leukemia. When the leukemia leads to stillbirth, the placenta is enlarged and leukemic cells can be found in the extramedullary organs at autopsy. Isaacs[4] described five stillbirths among 145 diagnoses of neonatal leukemia, but the incidence should likely be estimated higher due to underreporting of stillbirth.

CYTOGENETICS

ALL

Morphologically three out of four patients with neonatal ALL have an immature B-cell phenotype without CD10 expression and with CD19 positivity.[3,17] T-Cell acute leukemia was described in only one case report. [25] Typical for neonatal (and infant) ALL is the coexpression of myeloid-associated antigens (e.g. CD33), suggesting origin in a stem cell not fully committed to the lymphoid differentiation. [4,26] A phenotypic switch from lymphoid to myeloid lineage (and vice versa) at relapse has been described repeatedly. [27-30] Ten out of 145 reviewed neonatal leukemia patients were described as biphenotypic.[4] In the great majority of these ALL patients t(4;11)(q21;q23) or t(11;19)(q23;p13) can be detected in the leukemic blasts.

AML

Around half of the reported neonatal AML patients have a monoblastic leukemia (FAB M5) which is therefore the most common form of myeloid leukemia in neonates. A myelomonocytic form (FAB M4) is present in around one out of five neonates presenting with AML.[4] Monoblastic and myelomonocytic leukemias are typified by positivity for CD13, CD14, CD15 and CD33. The most common chromosomal aberrations present in neonatal AML are t(11;19)(q23;p13) and t(9;11)(p21;q23). In the great majority of the AMKL (FAB M7) non-Down neonates t(1;22)(p13;q13) is present.

DIFFERENTIAL DIAGNOSIS

A true leukemia in neonates has to be distinguished both from other conditions such as transient myeloproliferative disorder (TMD); congenital infections, hypoxia, and haemolytic disease causing a leukaemoid reaction; and other congenital neoplasms such as neuroblastoma.

Although rare, several case reports have been published describing TMD in non-Down neonates. [31-37] A true differentiation from TMD can only be made in retrospect as, by definition, TMD remits spontaneously, generally in the first months of life. TMD usually shows characteristics of megakaryocytic differentiation under the light microscope and by flow cytometry. TMD is frequently presented by pronounced organomegaly. In TMD patients (including patients with trisomy 21) 20–30% develop into full-blown leukemia within three

years and this is typically of the acute megakaryocytic phenotype (M7),[38] with a specific genetic abnormality (GATA1 mutation).

A leukaemoid reaction is seen in the presence of congenital infections due to cytomegalovirus, syphilis, toxoplasmosis, rubella, listeria monocytogenes, herpes, and sepsis[39] but also after haemolytic disease and (birth-related) hypoxia. A leukaemoid reaction presents itself – like leukemia – with hyperleucocytosis and circulating blasts. Also hepatosplenomegaly and skin nodules (blueberry muffin baby) due to extramedullary haematopoiesis in the neonate is frequent with a leukaemoid reaction. However, the peripheral blood does not show the monoclonal cell population as is seen in a true leukemia. Further, in a leukaemoid reaction the bone marrow aspirate usually shows an increase of immature myeloid cells at different maturation stages and not the monoclonal leukemic population. Congenital infections usually come with intrauterine growth retardation and/or microcephaly and should be ruled out through serological tests. [39]

Other congenital neoplasms such as disseminated neuroblastoma and Langerhans histiocytosis can resemble congenital leukemia. Disseminated neuroblastoma can also present itself with hepatosplenomegaly and a blueberry muffin appearance. The bone marrow aspirate, however, will show no hyperleucocytosis and cell counts in peripheral blood are usually within normal ranges. A blueberry muffin appearance has a number of other differential diagnoses such as rhabdomyosarcoma and also Langerhans histiocytosis. Certain congenital infections and haemolytic disease can cause the dermal erythropoiesis underlying this particular appearance of the skin. A definite diagnosis can be made by performing a skin biopsy.

DIAGNOSIS

As soon as leukemia in the neonate is suspected, immediate referral to a paediatric oncologist/haematologist is required. Differential diagnoses such as congenital infections and haemolytic disease should be ruled out. A complete blood cell count including WBC differential count is required. Definite diagnosis can be made based on peripheral blood smear and bone marrow aspirate. Additionally laboratory assessment is needed to perform morphology, immunophenotyping and genotyping for full diagnosis and stratification of the leukemia (Box 1).

Complete blood cell count
Peripheral blood smear
Bone marrow aspirate with morphology, immunophenotyping and genotyping

Box 1. Key diagnostic tests for diagnosing leukemia

TREATMENT AND PROGNOSIS

When the diagnosis leukemia is established, an intensive multi-agent chemotherapeutic regimen should be started. There is no specific treatment protocol for the treatment of either neonatal ALL or neonatal AML. Neonatal ALL patients are usually treated as infant ALL patients with a chemotherapeutic regimen based on ALL principles combined with elements of AML treatment.

Treatment protocols include steroids, vincristine, l-asparaginase, 6-mercaptopurine and methotrexate together with anthracyclines and cytarabine. Neonatal AML patients are treated similarly to older AML patients with a chemotherapeutic regimen mainly based on cytarabine and anthracyclines. An overall survival of <10% is estimated for neonatal ALL from case reports published from 1970 to 2003. [3.4] In the more recent Interfant-99 study, 30 neonatal ALL patients were treated with a hybrid regimen combining ALL treatment with elements designed for treatment of AML, resulting in 17% long-term survival. [7] Relapse in neonatal ALL is particularly high; although in the Interfant-99 study 87% of neonates achieved a morphological complete remission, 73% of the patients relapsed. [7]

There is no report of neonatal AML patients treated on a uniform protocol but the survival is estimated at about 25%.[3.4] Relapse rate in AML was estimated at about 50%.[3] These numbers may be better nowadays as these case reports date back as far as the 1970s and the outcome of infant AML has improved considerably with intensive chemotherapy including repeated cycles of high-dose cytarabine and anthracyclines. [40,41] After age-related dosage adjustments, toxicities are considered manageable in neonates for AML and ALL. [7,41]

Next to chemotherapy, haematopoietic stem cell transplantation (HSCT) might be an additional treatment option. In paediatric AML it was recently shown that patients without *MLL* rearrangements did not benefit from transplantation. [42] Concerning the role of HSCT in the management of *MLL*-rearranged infant AML, contradictory results have been published; it was suggested that improved survival rates were achieved allocated to allogeneic stem cell transplantation in the AML-BFM 98 study, [42] whereas Balgobind et al. [43] in a much larger cohort showed that patients with *MLL*-rearranged AML did not benefit from allogenic

HSCT. In infant ALL, HSCT was reported to be a valuable option only in a small, group; namely *MLL*-rearranged ALL patients aged <6 months, either with poor response to steroids at day 8 or with leucocytes >300 × 10^9/l. [44] In infant ALL overall no added value of HSCT compared with chemotherapy alone has been shown. [45-47]

SUPPORTIVE CARE

During the initial period after diagnosis of leukemia, conservative measures are indicated such as correction of the electrolyte balance, hydration control, treatment of hyperuricaemia and coagulation imbalances in order to prevent serious complications. A more ambiguous topic in the initial supportive care of leukemia is rapid cytoreduction in order to prevent leucostasis syndrome. Whereas many practitioners order leukapheresis or exchange transfusion when WBC reaches 300 × 10^9/l, the actual therapeutic benefit of leukapheresis and exchange transfusion is unknown. [48-51] Besides WBC the clinical condition of the patient is important role in the decision-making for supportive care of the neonate with leukemia.

FUTURE PERSPECTIVES

The low survival rates for both neonatal ALL and AML warrant a decisive approach. Understandably there is a hesitation to treat these vulnerable patients,

but above all it is important to achieve and maintain complete remission for these neonates. New treatment strategies are urgently needed in order to increase current survival rates of neonatal leukemia. Data from the Interfant-99 study implied not that induction failure is causing the dismal prognosis of neonatal ALL but rather that the relapse rate is very high.[7] This advocates an early intensification of treatment. One possible strategy for intensification is reconsidering the dose reductions that are now common practice in the treatment of neonates due to fear of severe toxicity. Possibly these dose reductions contribute to the higher relapse rate in these young children, as the strongest predictor of relapse in patients with ALL is the administered treatment itself.[52] The limited number of pharmacokinetic studies in this population represent a challenge. Both the Interfant-99 study (infant ALL)[7] and the AML-BFM-98/-2004[41] applied age-related dose reductions and reported no excessive toxicity in neonates, which may allow dose intensification.

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CHAPTER 3

Outcome of congenital ALL treated on the Interfant-99 protocol

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ABSTRACT

Acute lymphoblastic leukemia (ALL) diagnosed in the first month of life (congenital ALL) is very rare. Although congenital ALL is often assumed to be fatal, no studies have been published on outcome except for case reports. The present study reports the outcome of thirty patients with congenital ALL treated with the uniform Interfant-99 protocol, a hybrid regimen combining ALL-treatment with elements designed for treatment of acute myeloid leukemia. Congenital ALL was characterized by a higher WBC-count and a strong trend for higher incidence of MLL-rearrangements and CD10-negative B-lineage ALL compared to older infants. Induction failure rate was 13% and not significantly different from that in older infants (7%, p=0.14) but relapse rate was significantly higher in congenital ALL patients (2-year cumulative incidence (SE) was 60.0 (9.3) vs 34.2 (2.3), p<0.001). Two-year EFS and survival of congenital ALL patients treated with this protocol was 20% (SE 9.1). Early death in CR and treatment delays due to toxicity were not different. The survival of 17% after last follow-up, combined with a toxicity profile comparable to that in older infants, justifies treating congenital ALL with curative intent.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) in infants (up to 1 year of age) is known to be biologically different from ALL in older children diagnosed with ALL. ALL in infants is more often associated with a higher tumor load at diagnosis[1,2], a rearrangement in the mixed lineage leukemia (MLL) gene and very immature B-cell phenotype (pro-B ALL) without CD10-expression.[1-3] Infant ALL cells are more resistant to several standard chemotherapeutic agents[3,4] and the disease is also characterized by a poorer prognosis compared to older children.[5-14]

Congenital ALL is diagnosed at birth or within the first month of life and is very rare. Although it is assumed to be inevitably fatal and toxicity of the chemotherapeutic agents in these very young infants is unclear, to the best of our knowledge no series have been published on congenital ALL except for case reports. Bresters et al[15] reviewed 24 patients with congenital ALL diagnosed over 25 years of time that were described in case reports: all patients died.

We recently reported the results of a large international collaborative trial, Interfant-99, in infants younger than 1 year with ALL.[14] Here we detail the outcome and characteristics of thirty patients with congenital ALL who received uniform therapy with curative intent.

PATIENTS AND METHODS

Patients

The Interfant-99 trial design, the inclusion criteria and recruitment methods have been published earlier.[14] Individual study groups obtained ethics approval from their own institutions. Of the 518 infants diagnosed with ALL, which account for approximately 3% of the ALL population, 35 patients were younger than 1 month (≤ 30 days), confirming the rarity of congenital ALL (about two children per every thousand with ALL). The present study reports on thirty cases were treated with Interfant-99 (Figure 1).

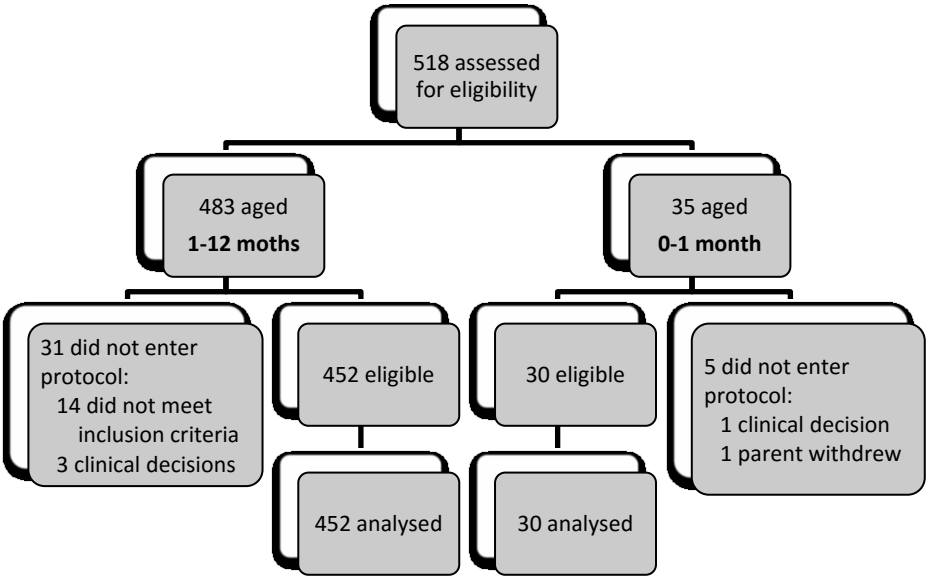

Figure 1. Trial profile

Procedures

Enrolled patients were stratified into standard-risk and high-risk groups on the basis of their response to one week of daily systemic prednisone (at a dose of 60 mg/m^2) and one intrathecal dose of methotrexate. Patients were classified as standard risk if their peripheral blood blast count was less than 1000 cells per µl at day 8 and high risk if the blast count was equal to or greater than 1000 per µl.[4,7] Patients were tested for MLL gene rearrangement with split-signal fluorescent in-situ hybridisation (FISH), polymerase chain reaction (PCR), or both. Absence of MLL rearrangement was defined as a negative split signal for FISH.

The Interfant-99 treatment was a hybrid regimen combining standard ALL treatment with elements designed for treatment of acute myeloid leukemia. The protocol consisted of multiagent phases of induction and consolidation chemotherapy, followed by maintenance treatment with antimetabolites.[16] Doses were adjusted according to patients' ages at the start of each treatment phase: children younger than 6 months were given two-thirds of the full dose and children of 6 to 12 months received three-fourths of the full dose. Total treatment duration was 104 weeks.

The induction phase consisted of a standard four-drug induction: dexamethasone, vincristine, daunorubicin, L-asparaginase (E. Coli) with the addition of low-dose cytarabine.[16] The MARAM phase was a consolidation course[17] that included high-dose cytarabine and high-dose methotrexate. OCTADD was a reinduction block derived from the consolidation phase of the Berlin-Frankfurt-Münster (BFM) trials for treatment of acute myeloid leukemia[18], except that prednisone was replaced by dexamethasone. Standard-risk patients were given a maintenance phase of oral 6-mercaptopurine and methotrexate, combined with pulses of dexamethasone and vincristine and intrathecal methotrexate with steroid in the

first three cycles. High-risk patients were given standard maintenance therapy intensified with pulses of cytarabine and etoposide in the first three cycles. Patients were randomly assigned to receiving an extra late intensification (VIMARAM) phase (similar to the MARAM maintenance block but with the addition of vincristine).[17] In case of availability of a suitable donor, high-risk patients were eligible for allogenic bone marrow transplantation. Only one congenital ALL patient underwent a bone marrow transplantation instead of maintenance therapy. For more details on the treatment protocol see Pieters et al, 2007.[14]

Statistical Analysis

The Interfant-99 trial database was used for analysis. The Fisher exact test was applied to investigate the association between age group (congenital ALL-patients versus older infants) and both patients' characteristics and rate of induction failures. The Wilcoxon test was used to compare durations of treatment phases between age groups. Endpoints were early death (during induction); resistance to induction (i.e., no complete remission (CR) at the end of the induction phase); relapse; death in CR; and second malignancy. Outcome measures were event-free survival (EFS), defined as the time from diagnosis to any one of the endpoints and survival, defined as time to death from any cause. Time was censored at the latest follow-up available, if no events were recorded. Follow-up was updated at December 2007. EFS and survival curves were computed with the Kaplan-Meier estimator and their standard error (SE) with the Greenwood formula. The log-rank test was used for univariate comparisons. A one-step Cox model was applied to estimate the hazard of relapse for congenital ALL as compared to older infants, adjusting for relevant factors. The probabilities of relapse and death in CR were

estimated by applying the cumulative incidence estimator which accounts for competing risks, and were compared according to Gray.[19] All tests were two-sided. SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), SAS 8.2 package (SAS Institute, Cary, NC, USA) and R statistical software (http://www.R-project.org) were used for data analysis.

RESULTS

Patient characteristics

35 Congenital ALL patients were eligible to enter the Interfant-99 protocol. Five patients did not enter due to various reasons (see figure 1), whom all died respectively at the day of diagnosis, two days after diagnosis, one month after diagnosis (two patients), and one patient who was treated died five months after diagnosis (two weeks after relapse). Table 1 shows the distribution of relevant characteristics in thirty patients with congenital ALL and in the remaining 452 infants treated with Interfant-99. The percentage of patients with a poor response to prednisone was not significantly different between the congenital ALL group (39%) and the older infants aged 1-12 months (30%) (p=0.29). Also sex and CNS-involvement did not differ between these two groups (p=0.71 and p=0.48, respectively). Congenital ALL cases more often presented with a high white blood cell (WBC) count (p=0.01): only 23% had a WBC count at diagnosis < 100 $x10^9$/l compared to 46% in the group aged 1-12 months. There was a trend towards a higher incidence of a CD10-negative, B-lineage immunophenotype in the congenital ALL group (76%) compared to the older patients (62%) (p=0.09) and for a higher incidence (p=0.09) of MLL-rearrangement in congenital ALL (93%) versus older patients (78%). The distribution of the different MLL fusion partners did not differ (p=0.53). In both age groups, t(4;11) was the most common type of translocation (congenital ALL 48% vs. 53% in older patients). A t(11;19) was found in 32% of congenital ALL patients, while this was seen in 19% of older infants.

	Enrolled patients		ZX	Standard-risk patients		High-risk patients	
Age at diagnosis, months	**0-1**	**1-12**		**0-1**	**1-12**	**0-1**	**1-12**
Total patients	30	452		17	307	12	139
Female sex	17 (57%)	234 (52%)	**0.71**	10 (59%)	161 (52%)	6 (50%)	71 (49%)
WBC count (cells per L)			**0.01**				
Less than $100x10^9$	7 (23%)	204 (46%)		7 (41%)	167 (55%)	0	35 (25%)
$100\text{-}300x10^9$	16 (53%)	123 (27%)		9 (53%)	91 (30%)	6 (50%)	32 (23%)
$> 300x10^9$	7 (23%)	121 (27%)		1 (6%)	48 (16%)	6 (50%)	72 (52%)
Not known	0	4		0	1	9	0
Immunophenotype			**0.09**				
B-lineage: CD10 positive	22 (76%)	251 (62%)		14 (88%)	168 (61%)	7 (58%)	80 (63%)
B-lineage: CD10 negative	6 (21%)	133 (33%)		2 (13%)	103 (37%)	4 (33%)	29 (23%)
B-lineage: CD10 unknown	1	37		1	25	0	12
Other	0	21 (5%)		0	5 (2%)	0	16 (11%)
AUL	1 (3%)	2 (0%)		0	1 (0%)	1 (8%)	1 (1%)
Not known	0	8		0	5	0	1
11q23 abnormalities			**0.09†**				
Not (fully) known	3	83		0	51	3	20
MLL germline	2 (7%)	80 (22%)		1 (6%)	59 (24%)	1 (11%)	21 (18%)
MLL rearrangement	25 (93%)	289 (78%)		16 (94%)	187 (76%)	8 (89%)	98 (82%)
t(4;11)	12 (48%)	154 (53%)		8 (50%)	101 (54%)	3 (38%)	50 (51%)
t(9;11)	1	34		1	25	0	8

	(4%)	(12%)		(6%)	(13%)		(8%)
t(11;19)	8 (32%)	56 (19%)		5 (31%)	34 (18%)	3 (38%)	22 (22%)
Other fusion partner‡	2 (8%)	23 (8%)		2 (13%)	12 (6%)	0	11 (11%)
Fusion partner unknown	2 (8%)	22 (8%)		0	15 (8%)	2 (25%)	7 (7%)
CNS involvement			**0.48**				
Yes	3 (10%)	41 (11%)		1 (8%)	25 (9%)	1 (13%)	14 (12%)
No	18 (90%)	347 (89%)		11 (92%)	244 (91%)	7 (88%)	102 (88%)
Not evaluable or not Known	9	64		5	38	4	31
***In vivo* prednisone response**			**0.29**				
Good response	17 (61%)	301 (70%)		16	300	1 (8%)	1 (1%)
Poor response	11 (39%)	127 (30%)		0	0	11 (92%)	127 (99%)
Not evaluable	0	6		0	2	0	4
Not known	2	18		1	5	0	7

Table 1. Patient characteristics by age group and risk group. All data are number (%). Percentages calculated as proportion of the known and evaluable data. AUL = acute undifferentiated leukemia. MLL = mixed lineage leukemia. CNS = central nervous system. □ Comparison (Fisher exact test) between congenital ALL patients and ALL patients aged 1-12 months. † p-value for association between age group and presence of MLL rearrangement. For MLL rearranged patients, distribution of translocation did not differ significantly (p=0.53) between age groups. ‡ The fusion partner of the MLL gene is defined, but differs from that in t(4;11), t(9;11) or t(11;19).

Outcome

Outcomes are described for 482 enrolled patients overall (thirty with congenital ALL, see Table 2), with a median follow-up time from diagnosis of 58 months (range 1-102). Nine patients were diagnosed at the age of 0-7 days and 21 patients at the age of 8-30 days. One congenital ALL patient (diagnosed at the age of 24 days) and two older patients died during the first week of prednisone treatment (and could therefore not be further stratified in the standard- or high-risk group). Two out of thirty congenital ALL patients (7%, diagnosed both at the age of 0-7 days) died during the induction phase whereas sixteen of 452 (4%) older patients died during induction. One congenital ALL patient did not achieve CR at the end of induction and died thereafter (age at diagnosis 0 days), while eleven patients were resistant among the older infants (eight died). The induction failure rate in congenital cases (13%) was not significantly different from that in older patients (7%, p=0.14).

Nineteen out of 26 (73%) congenital ALL patients had a relapse after achieving CR, while relapses in the infants of 1-12 months were 176/423 (42%) (p<0.001). All relapses in congenital ALL patients presented in the bone marrow and none were detected in the CNS. Ten relapses occurred within 6 months from CR, six between 7 and 12 months, and the remaining three after one year (of whom only one after the end of therapy, at 2.6 years). All relapsed patients died. The cumulative incidence (SE) of relapse in the congenital ALL patients was 60.0 (9.3) at two years, while the corresponding figure for older infants with ALL was 34.2 (2.3) (p<0.001). Results of the multivariable Cox model indicate that congenital ALL cases had a significantly higher risk of relapse than older infants (hazard ratio 2.4, 95% CI 1.5-3.9, p<0.001), even after adjusting for known prognostic factors (WBC

	Enrolled patients		p¨	Standard-risk patients		High-risk patients	
Age at diagnosis, months	**0-1**	**1-12**		**0-1**	**1-12**	**0-1**	**1-12**
Total patients	30	452		17	309	12	139
No CR	4 (13%)	29 (7%)	**0.14§**	2 (12%)	10 (3%)	1 (8%)	17 (12%)
Deaths in pre-phase	1 (3%)	2 (3%)		0	0	0	0
Deaths in induction	2 (7%)	16 (4%)		1 (6%)	8 (3%)	1 (8%)	8 (6%)
Resistants	1 (3%)	11 (2%)		1 (6%)	2 (1%)	0	9 (6%)
CR	26 (87%)	423 (93%)		15 (88%)	299 (97%)	11 (92%)	122 (88%)
Relapses	19 (73%)	176 (42%)	**<0.001**	10 (67%)	102 (33%)	9 (75%)	72 (51%)
Bone marrow	18 (95%)	124 (70%)		10 (100%)	67 (66%)	8 (89%)	56 (76%)
Bone marrow+ testis	1 (5%)	1 (1%)		10 (100%)	67 (66%)	8 (89%)	56 (76%)
Other	0	50 (13%)		0	34 (15%)	0	16 (10%)
SMN	0	1 (0%)		0	1 (0%)	0	0
Alive in CR	5 (17%)	223 (54%)		3 (18%)	180 (63%)	2 (17%)	43 (36%)
Deaths in CR	2 (8%)	23 (9%)	**0.7**	2 (40%)	16 (5%)	0	7 (6%)

Table 2. Outcome by age group and risk group. All data are number (%). SMN = secondary malignant neoplasm, CR = complete remission. * Unless mentioned otherwise, comparison of cumulative incidence of events in corresponding row(s), in congenital ALL versus ALL patients aged 1-12 months, † Comparison of complete remission rate in congenital ALL-patients vs. ALL-patients aged 1-12 months.

count at diagnosis, MLL gene rearrangement and response to prednisone) and other characteristics (sex, immunophenotype).

Two out of 26 (8%) congenital ALL patients in CR died from toxicity, one from a brain abscess and one due to septic complications (2 and 5 months from diagnosis, respectively). 23 deaths (9%) occurred in older ALL infants. The cumulative incidence (SE) of death in CR at two years was 6.7 (4.7) and 5.1 (1.0) in congenital ALL and older ALL infants, respectively (p=0.70).

Five patients (17%) with congenital ALL were still in CR at last follow-up, after 29 up to 103 months from diagnosis. No second malignancies were diagnosed among the congenital ALL-patients.

EFS and survival for congenital ALL were significantly lower than for older infants (Figure 2). Two-year EFS was 20.0% (SE 9.1, CI 2.2-37.8) and 54.2% (SE 2.4, 95%CI 49.5-58.9) and overall 2-year survival was 20.0% (SE 9.1, CI 2.2-37.8) and 66.4% (SE 2.4, 95%CI 62.1-70.7) for congenital ALL and older infants with ALL, respectively.

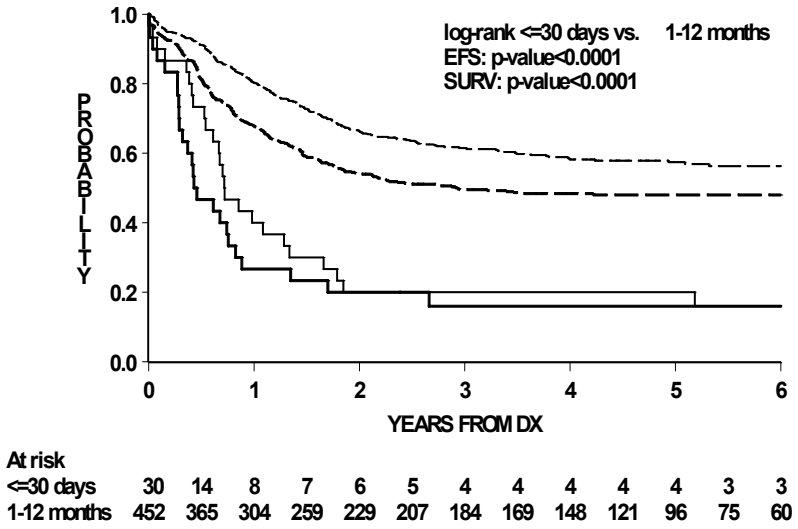

Figure 2. Outcome by age group in the Interfant-99 protocol. Log-rank test for difference in EFS and survival between congenital ALL patients and ALL patients 1 to 12 months of age. Continuous lines represent congenital ALL patients; dashed lines, ALL patients 1 to 12 months of age; thick lines (both groups), EFS; thin lines (both groups), survival.

As expected a treatment delay, as an indirect measure for toxicity, was observed in all phases of chemotherapy in congenital ALL patients as well as in older infants (see table 3). Nineteen congenital ALL patients and 335 infants aged 1-12 months of whom data were available had a median induction phase delay of 1 versus 0.3 weeks respectively. The consecutive phases, consolidation and reinduction were started with a median delay in congenital ALL patients of 2.1 weeks (1.8 in older infants), 1.6 weeks (2.1 in older infants) and 2.8 weeks (2.7 weeks in older infants). None of these differences in treatment delay between the two groups was statistically significant.

	Congenital ALL		Infant ALL		
Phase (planned duration)	Patients with available data	Actual duration (median)	Patients with available data	Actual duration (median)	P*
Induction (6)	19	7	335	6.3	0.22
Consolidation (6)	14	8.1	288	7.8	0.5
Reinduction (8)	11	9.6	209	10.1	0.26
Intensification (6)	4	8.8	69	8.7	0.84
Maintenance 1B (42)	4	42.7	98	41.9	0.55
Maintenance 1A (42)	2	44.4	14	43.1	-

Table 3. Treatment phase duration by age group. Patients in numbers. Durations in weeks. Infant ALL=aged 1-12 months. * Comparison of phase duration in congenital ALL-patients versus ALL-patients aged 1-12 months.

DISCUSSION

The limited, single case report based, literature to date suggested that congenital ALL was invariably fatal. The present study is the first that reports on a series of thirty patients with congenital ALL treated uniformly with curative intent. The Interfant database includes all diagnosed infants with ALL, even if they were not treated or not treated according to protocol. The present analysis demonstrates that the outcome, though worse than in older infants, is not inevitably fatal. 17% of the congenital ALL patients were still alive at last follow-up.

Resistance to prednisone prephase did not differ between the neonates and older infants. Neither was induction failure or mortality in CR significantly higher in the congenital ALL cases compared to older infants. Due to the small sample size, lack of significance in this study does not prove equivalence, therefore results should be interpreted with caution. However, patients with congenital ALL did have a significantly higher relapse rate. Congenital ALL was characterized by a significantly higher white blood cell count, a trend towards a higher incidence of MLL gene rearrangements and a CD10-negative B-lineage immunophenotype than ALL in older infants. These biological characteristics have all been associated with a dismal outcome, as also a very young age per se has been established as risk factor for an inferior outcome.[14] Nevertheless, a multivariate analysis showed that congenital ALL cases had a higher relapse rate also after adjustment of other risk factors than age. Possibly, due to fear for severe side-effects in these young patients, the reduced doses administered to these patients contribute to the higher relapse rate.

As there is evidence for a prenatal initiation of acute leukemia in young patients[20], we realize the definition of congenital ALL defined as diagnosed within the first 1st

month of life is arbitrary. The definition is based on previous literature (e.g. Bresters et al[15]), though the type of MLL-rearrangement shifts as infants are diagnosed later. 91% of infants younger than 6 months had MLL-rearrangements, compared with 66% of infants aged 6-12 months. Two-thirds of patients with t(4;11) or t(11;19) were younger than 6 months at diagnosis compared to one-third of patients with t(9;11).[14] A longer latency from initiation to diagnosis, and by this possibly a less aggressive form of leukemia, could be in part an explanation for the better outcome of the older infants with MLL-rearranged ALL.

Improvement of outcome for the congenital ALL patients is still urgently needed. New treatment strategies are under current investigation in order to improve outcome of infants with ALL. Unpublished observations of the Interfant-99 trial suggest that infants with MLL-rearrangements, younger age and a very high WBC might benefit from stem cell transplantation. In the current Interfant-06 study different early intensification strategies are studied in order to prevent early bone marrow relapses. New therapeutic targets have to be identified by unraveling the biology of MLL-rearranged ALL. Phase I/II trials with FLT3-inhibitors[21] are currently being initiated in infant MLL-rearranged ALL.

In conclusion, the current study shows a survival of 17% for congenital ALL and a toxicity profile comparable to that in older infants. This proves that congenital ALL is not invariably fatal and justifies treatment with curative intent. For now, the treatment to be used should be the same as for older infants but their poor outcome also necessitates the testing of newer approaches, such as new strategies to prevent early relapses as tested in the ongoing Interfant-06 and new targeted treatments as FLT3-inhibitors.

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CHAPTER 4

Gene expression profiling based dissection of *MLL* translocated and *MLL* germline acute lymphoblastic leukemia in infants

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ABSTRACT

Acute lymphoblastic leukemia (ALL) in infants (< 1 year) is characterized by a poor prognosis and a high incidence of MLL translocations. Several studies demonstrated the unique gene expression profile associated with MLL-rearranged ALL, but generally small cohorts were analyzed as uniform patient groups regardless of the type of MLL translocation, whereas the analysis of translocation negative infant ALL remained unacknowledged. Here we generated and analyzed primary infant ALL expression profiles (n=73) typified by translocations t(4;11), t(11;19), and t(9;11), or the absence of MLL translocations. Our data show that MLL germline infant ALL specifies a gene expression pattern that is different from both MLL-rearranged infant ALL and pediatric precursor BCP-ALL. Moreover, we demonstrate that, apart from a fundamental signature shared by all MLL-rearranged infant ALL samples, each type of MLL translocation is associated with a translocation-specific gene expression signature. Finally, we show the existence of 2 distinct subgroups among t(4;11)–positive infant ALL cases characterized by the absence or presence of HOXA expression, and that patients lacking HOXA expression are at extreme high risk of disease relapse. These gene expression profiles should provide important novel insights in the complex biology of MLL-rearranged infant ALL and boost our progress in finding novel therapeutic solutions.

INTRODUCTION

In recent years, genome-wide assessment of gene activity has proven to be of great value in tumor classification as well as in identifying unique gene expression signatures associated with drug response, prognosis, metastasis, angiogenesis, and tumorigenesis. In pediatric acute lymphoblastic leukemia (ALL), oligonucleotide microarray analyses have been shown to accurately predict 6 major prognostic and genetically distinct patient groups, including specific precursor B-cell lineage subtypes characterized by E2A- PBX1, BCR-ABL, TEL-AML1, and MLL translocations, or hyperdiploidy (> 50 chromosomes), and T-cell lineage ALL (T-ALL).[1-3] In addition, our laboratory recently identified a novel subgroup among children with genetically yet unclassified precursor B- ALL.[3] In other studies, we demonstrated how gene expression profiling can identify unique gene expression signatures associated with resistance to prednisone, vincristine, L-asparaginase, and daunorubicin in pediatric ALL.[4, 5] Moreover, these gene expression signatures appeared to be highly predictive for clinical outcome for the patients under investigation as well as in a completely independent patient cohort.[4]

Among the different genetic subgroups of pediatric ALL, MLL-rearranged ALL represents the most unfavorable type of leukemia and is most frequently diagnosed in infants (i.e., children younger than 1 year). In infant ALL, approximately 80% of the cases are typified by leukemia-specific chromosomal translocations involving the Mixed Lineage Leukemia (MLL) gene,[6] fusing the N-terminal portion of MLL to the C-terminal region of one of its many translocation partner genes. By far the most frequent MLL translocations found among infant ALL patients are t(4;11), t(11;19), and t(9;11),[7] giving rise to the fusion proteins

MLL-AF4, MLL-ENL, and MLL-AF9, respectively. These chimeric MLL fusion proteins exhibit pronounced transforming capacities[8] and independently contribute to an unfavorable prognosis.[7] To date, event-free survival rates for MLL-rearranged infant ALL range between 20% and 50%, depending on the treatment protocol.[7] Approximately 20% of the infant ALL patients carry germline (or wild-type) MLL genes, and nowadays have a far better prognosis with event-free survival chances of 75% to 95%.[7, 9]

Multiple microarray studies demonstrated that MLL translocations specify a distinct gene expression profile that is clearly distinguishable from other ALL subtypes and from acute myeloid leukemia (AML).[1-3, 10, 11] Moreover, Zangrando et al recently reported a gene expression signature commonly shared by MLL-rearranged ALL and AML patients, identifying deregulated genes specifically associated with the MLL translocation, irrespective of the type of leukemia.14 In most of these studies, however, rather small numbers of MLL-rearranged ALL samples were analyzed as a uniform patient group, regardless of the type of MLL translocation. Nevertheless, MLL-rearranged ALL may well represent heterogeneous biologic entities characterized by a fundamental gene expression profile shared by all patients despite the MLL fusion partner, whereas underlying expression signatures may discriminate between the different types of MLL translocations. To test this, we generated and analyzed gene expression profiles in a relatively large cohort of MLL-rearranged infant ALL samples, and indeed reveal the existence of specific gene expression signatures associated with the different MLL translocations frequently found in infant ALL. Furthermore, we sought to determine whether infant ALL patients carrying germline (or wild-type) MLL genes display gene expression profiles that resemble those of childhood ALL patients older than one year of age (non-infants), or whether these patients form yet

another genetically distinct ALL subgroup, and concluded the latter. Finally, we show that, among t(4;11)-positive infant ALL cases, 2 distinct subgroups can be identified based on the absence or presence of HOXA9, HOX10, HOXA7, HOXA5, and HOXA3 expression, and show dramatic differences in relapse-free survival.

METHODS

Patient samples

Bone marrow or peripheral blood samples from untreated infants (younger than 1 year) diagnosed with ALL were collected at the Erasmus MC–Sophia Children's Hospital and other institutes participating in the recently published international collaborative INTERFANT-99 treatment protocol.[7] Samples from pediatric ALL patients older than 1 year (i.e., non-infants) were selected from our cell bank. Absence of *MLL* rearrangement was defined as a negative split signal for FISH. For all primary patient samples used in this study, approval was obtained from the Erasmus MC Institutional Review Board, and authorization was acquired from the parents or legal guardians of the children via informed consent in accordance with the Declaration of Helsinki. Patient characteristics are listed in Supplemental material Chapter 4 Table 1.

Sample preparation

All samples were processed within 24 hours after sampling as described recently.[12] Briefly, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma), and non-leukemic cells were removed using immunomagnetic beads.[13] All leukemia samples used in this study contained more than 90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck)–stained cytospins.

Gene expression profiles

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified on a Nanodrop ND-1000

spectrophotometer (Isogen). The integrity of the extracted RNA was assessed on an Agilent 2100 Bioanalyzer (Agilent). 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) according to the manufacturer's guidelines. The infant ALL gene expression data presented in this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus[14] and is accessible via GEO Series accession number GSE19475. The gene expression data for the pediatric precursor BCP-ALL samples were deposited as GSE13351 as part of a recently published study.[3]

Quantitative real-time PCR analyses

Total RNA was extracted from a minimum of 5 X 106 leukemic cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The quality of the extracted RNA was assessed on 1% agarose gels. Extracted RNA was reverse transcribed as described before,[15] and the obtained cDNA was used to quantify mRNA expression of HOXA9, HOXA7, HOXA5, HOXA10, and HOXA3 relative to the housekeeping gene B2M using quantitative real-time polymerase chain reaction (PCR). For this, PCR products were amplified using the DyNAmo SYBR Green qPCR kit (Finnzymes) according to the manufacturer's recommendations, using SYBR Green as a fluorophore to detect transcripts on an ABI Prism 7900 sequence detection system (Applied Biosystems).

Statistical analyses

Raw array data were collectively normalized using variance-stabilizing normalization,[16] and differential gene expression was statistically evaluated using

linear models for microarray analyses.[17] Differences in gene expression were deemed significant at P values (adjusted for multiple testing according to the step-up procedure of Benjamini) of less than .01 (i.e., false discovery rate [FDR] < 0.01). All statistical analyses were performed in the statistical environment R using Bioconductor packages. Heatmaps were generated in GenePattern,[18] and graphical representations of principal component analyses (PCA) were produced using the GeneMath XT 1.6.1 software (Applied Maths).

As a measure of internal validation for the subtype-specific gene expression signatures, the global test[19] was applied to evaluate whether gene lists were significantly associated with a certain patient group. In all instances, the global test indicated that the expression of all selected probe sets was significantly associated with the corresponding patient group. To produce informative representations of discriminative probe sets, we chose to visualize the top 50 most significantly overexpressed probe sets for each subgroup in each comparison.

RESULTS

MLL-rearranged infant ALL versus pediatric precursor BCP-ALL: dataset validation

Nowadays, proper validation of gene expression profiling data is achieved either by a double-loop cross-validation procedure in which the sample population is divided into a training and a test set,[3] or by confirming differential gene expression in a truly independent patient cohort (e.g., Holleman et al[4]). However, infant ALL is a rare malignancy, and collecting an adequate number of samples to apply such validations remains difficult, even in our INTERFANT-99 patient cohort that currently represents the largest collection of infant ALL samples. Therefore, to avoid reduction of the sample size and maintain sufficient statistical power, we here adopted 2 recently published expression signatures that separate MLL-rearranged ALL from other ALL subtypes, based on which we used our samples as an independent patient cohort to validate the integrity of our dataset. The first signature was reported by Armstrong et al,[10] and represents 100 probe sets most significantly discerning between MLL-rearranged ALL (n = 17) and conventional precursor BCP-ALL samples. The second was published by Yeoh et al,[2] and composes 40 genes that distinguished pediatric MLL-rearranged ALL (n = 20) from all other known genetic subtypes of childhood ALL, including E2A-PBX1, BCR-ABL, and TEL-AML1 positive or hyperdiploid (> 50 chromosomes) BCP-ALL, and T-ALL. As both of these studies were performed on Affymetrix HU95A microarrays (containing 12 600 probe sets), we assessed the corresponding probe sets on the HU133plus2.0 arrays (containing 54 675 probe sets) and determined their discriminative capacity on our samples. For the MLL-rearranged ALL signature by Armstrong et al,[10] 97 probe sets (HU133plus2.0) could be identified to correspond with the 100 probe sets (HU95A) in the original signature. For the

signature reported by Yeoh et al,[2] all corresponding probe sets were found. Both signatures clearly separated our MLL-rearranged infant ALL patients (n = 59), consisting of t(4;11) (n = 29), t(11;19) (n = 22), and t(9;11)-positive (n = 8) cases, from our pediatric precursor BCP-ALL (n = 16) samples (Figure 1).

To exclude influences from subtype-specific gene expression signatures underlying pediatric ALL, we intentionally selected BCP-ALL samples from children older than one year of age that could not be assigned to any of the major genetic ALL subtypes. Of the 97 probe sets corresponding to the MLL-rearranged ALL signature by Armstrong et al,[10] 80 probe sets (82%) were significantly differentially expressed (FDR < 0.01) between our MLL-rearranged infant ALL and BCP-ALL samples. For the signature by Yeoh et al,[2] 32 of the 40 probe sets (80%) were expressed differentially (FDR < 0.01). Probe set identifications and descriptions, gene names, log-fold changes, and P values are listed in Supplemental material Chapter 4 Tables 2 and 3, respectively.

Given the superior number of probe sets on the HU133plus2.0 GeneChips used in the present study over the formerly used first-generation HU95A microarrays, we further explored whether this advantage results in a more pronounced class distinction than reported earlier. Comparing our gene expression profiles of MLL-rearranged infant ALL (n = 59) with those from pediatric BCP-ALL patients (n = 16), we found 14 246 of the 54 675 probe sets (26%) to be differentially expressed (FDR < 0.01), of which 6990 were up-regulated in MLL-rearranged infant ALL. Figure 2A shows a heatmap visualization of 100 probe sets most significantly up (n = 50) and down-regulated (n = 50) in MLL-rearranged infant ALL compared with pediatric precursor BCP-ALL. Probe set identifications and descriptions, gene names, log-fold changes, and P values are listed in Supplemental material Chapter 4 Table 4.

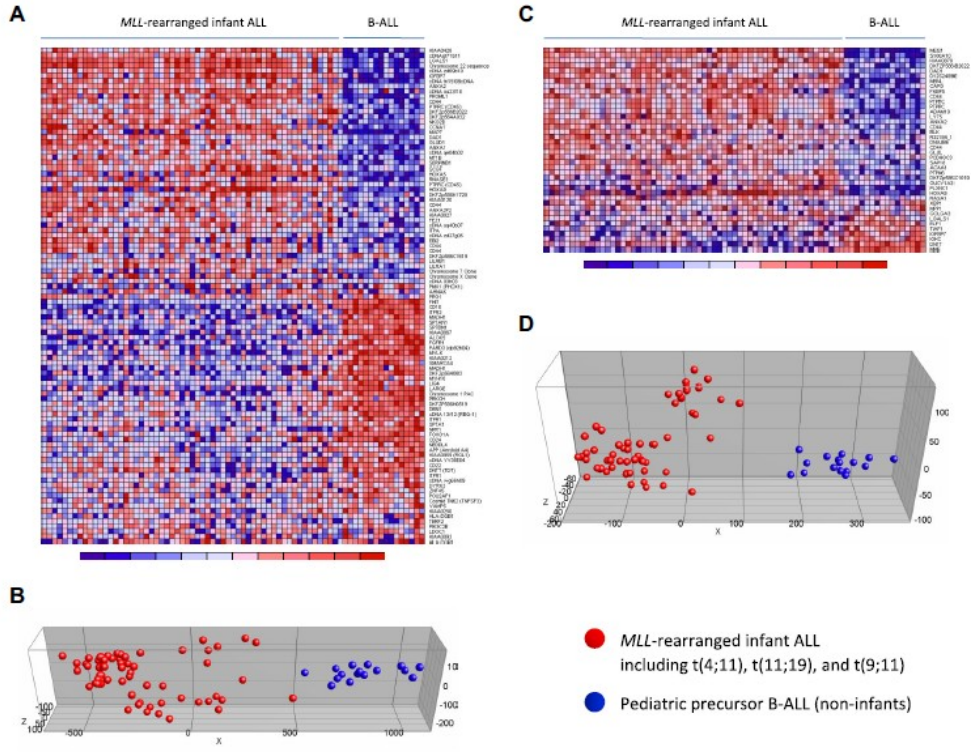

Figure 1. MLL-rearranged infant ALL versus pediatric precursor BCP-ALL (HU95A): dataset validation. Heatmaps separating our *MLL*-rearranged infant ALL (n = 59) from pediatric precursor BCP-ALL (n = 16) samples based on the *MLL*-rearranged ALL specific gene expression signatures (obtained on HU95A microarrays) published by Armstrong et al[10] (A) and Yeoh et al[2] (C). Columns represent patient samples, and rows represent the gene names corresponding to the probe sets. Normalized gene expression is depicted in red (high expression) or blue (low expression). (B,D) PCA for both signatures, respectively. Red dots indicate *MLL*-rearranged infant ALL samples (including t(4;11) (n = 29), t(11;19) (n = 22), and t(9;11)-positive (n = 8) cases), and blue dots represent pediatric precursor BCP-ALL cases (n = 16). Patient characteristics and detailed gene descriptions are listed in Supplemental Material Chapter 4 Tables 1, 2, and 3.

PCA revealed that using high-resolution HU133plus2.0 microarrays, by estimate covering the entire human genome, additional genes can be found that more clearly distinguish between MLL-rearranged ALL and conventional BCP-ALL than the signatures reported before (Figure 2B). For examples, probe sets corresponding to RLP38 (ribosomal protein L38), KCNK12 (potassium channel subfamily K member 12), and MDS027 (also known as HSPC300; hematopoietic stem/progenitor cell protein 300) are not present on HU95 microarrays but did appear among the 50 most significantly up-regulated genes in MLL-rearranged infant ALL samples in our HU133plus2.0- based data (Figure 2A). Of particular interest is the high-level expression of HSPC300, which was recently hypothesized to be associated with the metastatic potential of lung squamous cell carcinoma.25 As such, high level HSPC300 expression may well contribute to the aggressive nature of MLL-rearranged ALL and exemplifies how our HU133plus2.0-based gene expression profiles may further extend our insights in the biology of this malignancy.

Given the vast amount of probe sets significantly up- or down-regulated in MLL-rearranged infant ALL, we used high-level HSPC300 expression merely as an example of presumably many genes that have not been associated with MLL-rearranged ALL before.

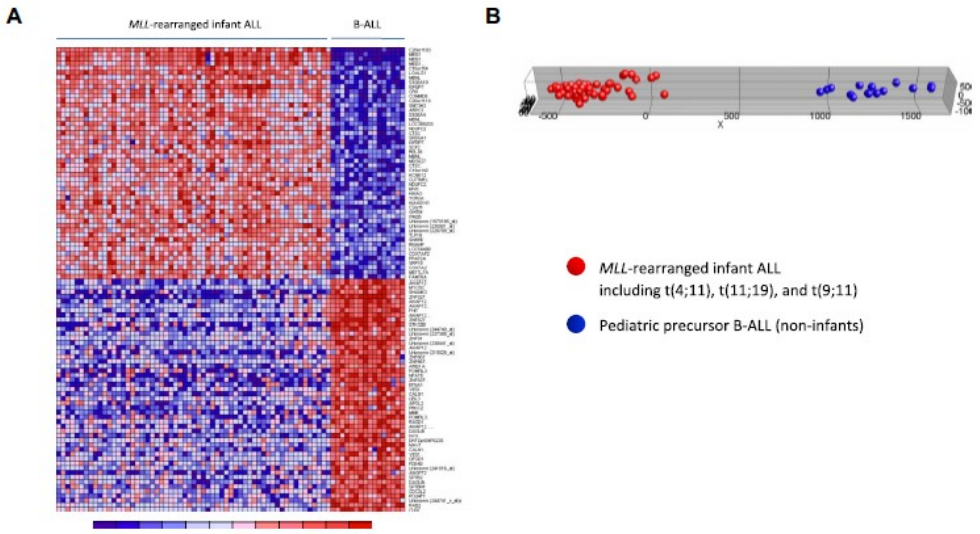

Figure 2. *MLL*-rearranged infant ALL versus pediatric precursor BCP-ALL. A. Heatmap showing the separation of *MLL*-rearranged infant ALL (n = 59) from pediatric precursor BCP-ALL (n = 16) samples based on the 100 probe sets most significantly discriminative between both patient groups as attained in our analyses using HU133plus2.0 GeneChips. Columns represent patient samples, and rows represent the gene names corresponding to the probe sets. Normalized gene expression is depicted in red (high expression) or blue (low expression). The top 50 probe sets are relatively overexpressed and the bottom 50 probe sets relatively underexpressed in *MLL*-rearranged infant ALL (which include t(4;11) (n = 29), t(11;19) (n = 22), and t(9;11)-positive (n = 8) cases). B. Graphic representation of PCA based on this gene expression signature, separating the *MLL*-rearranged infant ALL (red dots) from pediatric precursor BCP-ALL (blue dots) samples.

MLL germline infant ALL represents a unique subtype of childhood ALL

Next we asked whether infant ALL patients bearing germline MLL genes simply represent pediatric ALL patients of very young age (i.e., < 1 year) or whether these

patients compose an isolated ALL subgroup different from other known ALL subtypes. Therefore, we compared gene expression profiles of MLL germline infant ALL samples (n = 14) to those of the MLL-rearranged infant ALL (n = 59) and the pediatric precursor BCP-ALL samples (n = 16), lacking known genetic abnormalities. Initially, we performed a PCA, using all 54 675 probe sets present on the HU133plus2.0 GeneChip, without any selection. This unsupervised analysis roughly separated the germline MLL infant ALL samples from both the MLL-rearranged infant ALL and pediatric precursor BCP-ALL samples (Figure 3). Remarkably, the MLL germline infant ALL samples as a group clustered tightly to, but separately from, the MLL-rearranged infant ALL samples, and clearly away from the pediatric precursor BCP-ALL samples. Thus, apart from the presence of MLL translocations, young age (< 1 year), characteristically shared by all infants either carrying rearranged or germline MLL genes, also influenced this clustering (Figure 3).

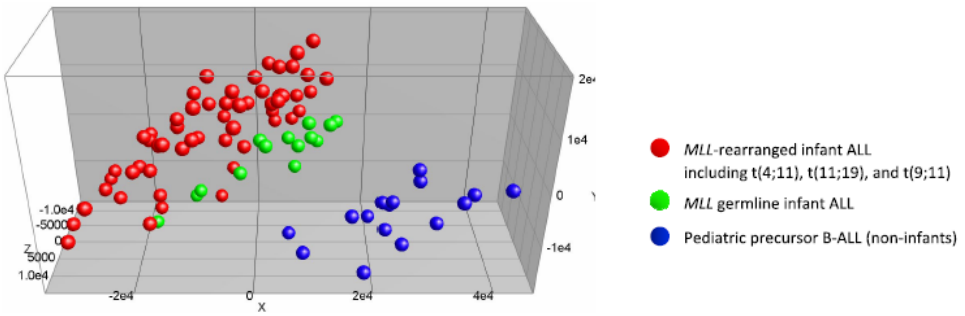

Figure 3. Unsupervised clustering analysis of *MLL*-rearranged infant ALL, *MLL* germline infant ALL, and pediatric precursor BCP-ALL. Completely unsupervised clustering analysis (PCA) of *MLL*-rearranged infant ALL (n = 59; red dots), *MLL* germline (wild-type *MLL*) infant ALL (n = 14; green dots), and pediatric precursor BCP-ALL (n = 16; blue dots) samples, using all 54 675 probe sets present on the HU133plus2.0 GeneChip.

Subsequently, to explore whether specific expression profiles could define these 3 patient groups more accurately, the 50 most significantly up-regulated probe sets for each group (compared with the other 2 groups combined) were selected. Differential expression of these most discriminative probe sets is visualized in a heatmap (Figure 4A). Probe set identifications and descriptions, gene names, log-fold changes, and P values are listed in supplemental Table 5. As expected and consistent with our unsupervised analysis (Figure 3), PCA showed that these 150 probe sets (almost) completely separated the MLL germline infant ALL samples from both the MLL-rearranged infant ALL and the pediatric precursor BCP-ALL samples (Figure 4B).

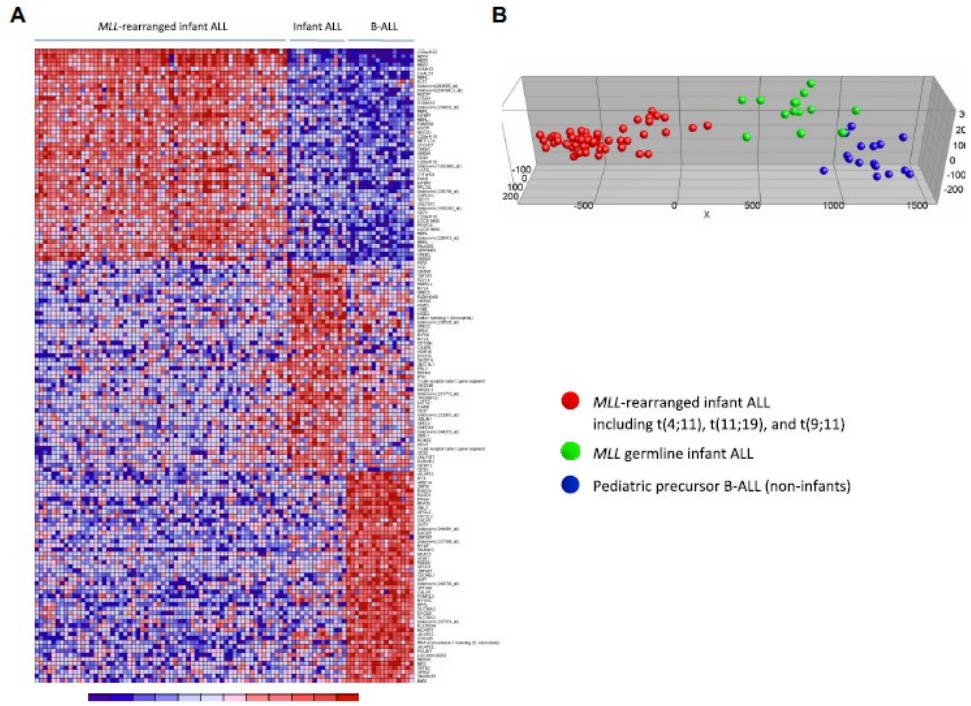

Figure 4. Supervised clustering analysis of *MLL*-rearranged infant ALL, *MLL* germline infant ALL, and pediatric precursor BCP-ALL. A. Heatmap visualizing differential gene expression separating *MLL* germline infant ALL (n = 14), from *MLL*-rearranged infant ALL (n = 59) and pediatric precursor BCP-ALL (n = 16) samples, based on the 50 most significantly up-regulated probe sets for each patient group (compared with the other patient groups combined). Columns represent patient samples, and rows represent the gene names corresponding to the probe sets. Normalized gene expression is depicted in red (high expression) or blue (low expression). B. Graphical representation of the supervised clustering of the samples based on this expression signature. Red dots indicate *MLL*-rearranged infant ALL; green dots, *MLL* germline infant ALL; and blue dots, the pediatric precursor BCP-ALL samples.

MLL translocation–specific GEP's among MLL-rearranged infant ALL patients

Accumulating evidence suggests that MLL translocations cause deregulated gene expression as a result of translocation-specific histone modifications, which may in part be influenced by the translocation partner gene.[31, 32] Therefore, we asked whether distinct gene expression profiles could be identified associated with the type of MLL translocation. For this we separated our MLL- rearranged infant ALL samples according to the type of translocation, i.e., t(4;11) (n = 29), t(11;19) (n = 22), or t(9;11) (n = 8), and determined the differentially expressed probe sets for each subgroup (compared with the other 2 subgroups combined). In total, 1229 probe sets were significantly differentially expressed between the 3 MLL-rearranged subgroups (FDR < 0.01). Figure 5A shows a heatmap visualizing the 50 most significantly up-regulated probe sets for each of the MLL-rearranged subgroups. Probe set identifications and descriptions, gene names, log-fold changes, and P values are listed in Supplemental material Chapter 4 Table 6. PCA showed that based on these 150 probe sets, t(4;11), t(11;19) and t(9;11)-positive infant ALL cases cluster completely separate form one another (Figure 5B).

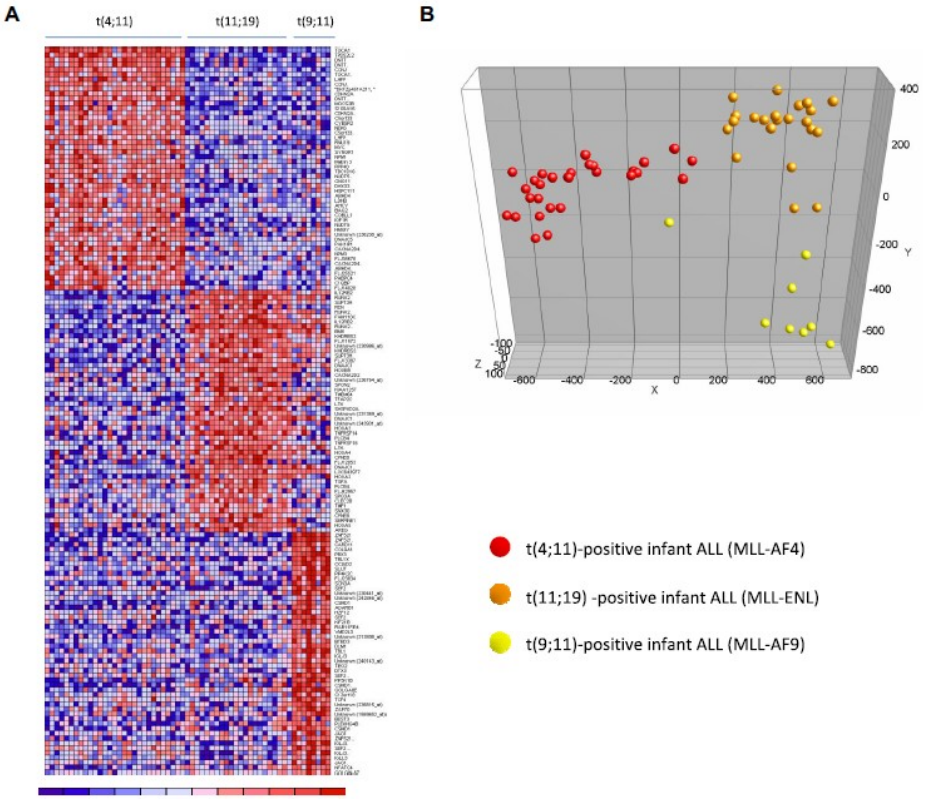

Figure 5. Gene expression–based separation of *MLL*-rearranged infant ALL subtypes. A. Heatmap demonstrating differential gene expression between t(4;11), t(11;19), and t(9;11)-positive *MLL*-r infant ALL samples, based on the 50 most significantly up-regulated probe sets for each patient group (compared with the other patient groups combined). Columns represent patient samples, and rows represent the gene names corresponding to the probe sets. Normalized gene expression is depicted in red (high expression) or blue (low expression). B. PCA plot clustering the t(4;11) (red dots), t(11;19) (orange dots), and t(9;11) (yellow dots) according to these 150 selected probe sets.

Subdivision of t(4;11)-positive infant ALL based on the presence or absence of HOXA expression

Finally, we asked whether gene expression profiles existed that subdivided MLL-rearranged infant ALL samples even among patients characterized by the same type of MLL translocation. Translocation t(4;11), giving rise to the MLL-AF4 fusion protein, is by far the most common MLL translocation among infant ALL patients (found in 50% of all cases).[7] As such, t(4;11)-positive infant ALL represents the largest subgroup of MLL-rearranged infant ALL cases in this study. Therefore, we particularly chose our t(4;11)-positive gene expression profiles to explore differential gene expression among t(4;11)-positive infant ALL cases. For this, the SD of the expression of each probe set was calculated among all t(4;11)-positive cases (n = 29), to identify probe sets with the largest variation, possibly indicating differential expression among these patients. Surprisingly, 6 probe sets corresponding to HOXA9, HOXA7, HOXA10, HOXA5, and HOXA3, appeared to display pronounced standard deviations, and consistently separated 2 sub- groups of t(4;11)-positive infant ALL samples uniformly characterized either by the presence (n = 13) or absence (n = 16) of HOXA expression (Figure 6 top panel). To validate these findings, quantitative reverse-transcribed PCR was applied to quantify HOXA9, HOXA7, HOXA10, HOXA5, and HOXA3 expression relative to the housekeeping gene B2M in primary t(4;11)-positive infant ALL samples characterized by either high (n = 5) or low (n = 5) HOXA expression (Figure 7). Adopting this separation, we compared the gene expression profiles and identified an additional 31 probe sets to be differently expressed between these subgroups (Figure 6 bottom panel). Several of these probe sets represented other homeobox genes, such as HOXA4, HOXB9, and IRXA1 (or IRX1) or denoted additional probe sets for HOXA10 and HOXA7. Probe set identifications and descriptions, gene names, log-fold changes, and P values are listed in Supplemental material Chapter 4 Table 7.

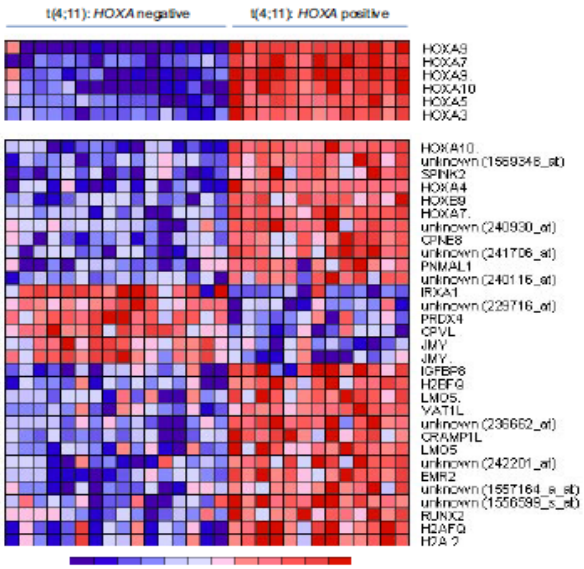

Figure 6. HOXA-based subclustering of t(4;11)-positive infant ALL samples. Heatmap visualizing 2 clusters among t(4;11)-positive infant ALL samples (n = 29) based on the present or absent of *HOXA9, HOXA10, HOXA7, HOXA5*, and *HOXA3* expression (upper panel). Apart from the 6 probe sets initially separating both patient groups, and additional 31 probe sets (lower panel) appeared to be significantly (FDR < 0.01) differentially expressed between *HOXA*-negative (n = 16) and *HOXA*-positive (n = 13) t(4;11)-positive infant ALL.

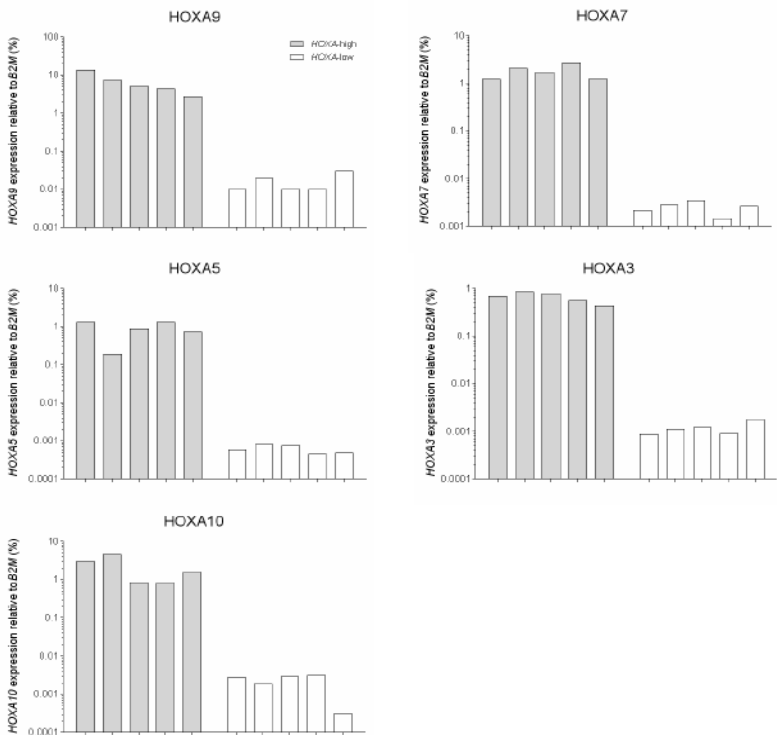

Figure 7. HOXA-based subclustering of t(4;11)-positive infant ALL samples. *HOXA9, HOXA10, HOXA7, HOXA5*, and *HOXA3* expression as determined by quantitative reverse-transcribed PCR analyses in t(4;11)-positive infant ALL samples characterized by high (n = 5) or low (n = 5) *HOXA* expression according to the microarray data.

Interestingly, the relapse-free survival varied significantly between both subgroups (P = .034), with t(4;11)-positive infant ALL patients negative for HOXA expression being at extreme high risk of disease relapse (Figure 8). The 1-year cumulative relapse incidence for HOXA-positive patients was 18.2% (± 12.3%) and for HOXA-negative patients 58.3% (± 15.4%). In a Cox model on the hazard of relapse, HOXA-negative t(4;11)-positive infant ALL patients had a significantly (P =

.036) 4.17-fold increased hazard ratio (95% confidence interval, 1.10-15.81) compared with HOXA- positive patients. However, as indicated by the relatively large 95% confidence interval, these findings should be interpreted with caution because of the small sample size. Nonetheless, a possible explanation for the pronounced difference in relapse-free survival between both t(4;11)-positive patient groups may lie in the genes that discriminate between them. For example, high-level PRDX4 (Peroxiredoxin 4) expression, such as that found in HOXA-negative t(4;11)-negative infant ALL samples (Figure 6), has been associated with metastasizing colon cancer.[21] In case PRDX4 also contributes to tumor progression and metastasis in MLL-rearranged ALL, up-regulated PRDX4 expression may contribute to the worse outcome of HOXA-negative t(4;11)-negative infant ALL patients compared with patients who do show HOXA expression.

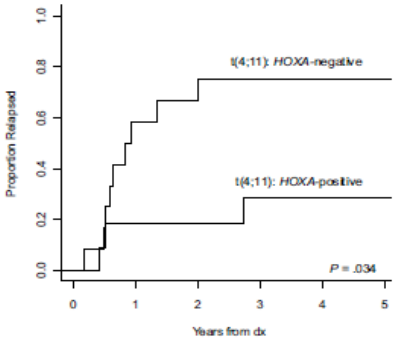

Figure 8. HOXA-based subclustering of t(4;11)-positive infant ALL samples. Relapse-free survival curves for *HOXA*-negative (n = 12) and *HOXA*-positive (n = 11) t(4;11)-positive infant ALL patients, demonstrating a significantly higher relapse incidence in t(4;11)-positive infant ALL patients lacking *HOXA* expression (*P* = .034). Because of a lack of data availability or exclusion of patients who died before entering the INTERFANT-99 treatment protocol, relapse-free survival could only be plotted for 23 of the 29 t(4;11)-positive infant ALL cases.

DISCUSSION

MLL-rearranged ALL samples display unique and ample deregulated expression profiles that are clearly distinguishable from profiles found in other specific ALL subtypes.[1-3, 10, 11] However, the number of *MLL*- rearranged infant ALL cases in these studies were small, inevitably leading to the analyses of these samples as a single patient group regardless of the type of *MLL* translocation. The most common *MLL* translocations among infant ALL patients are translocation t(4;11), t(11;19), and t(9;11), and the possible existence of specific gene expression profiles underlying these different *MLL* translocations re- mains unacknowledged. In addition, the aforementioned profiling studies made tremendous progress in classifying unique types of genetically distinct ALL subgroups, but infant ALL cases carrying germline *MLL* genes were never studied in these analyses. Therefore, the present study was designed to explore the possible existence of *MLL* translocation specific gene expression profiles, and evaluates how *MLL* germline infant ALL genetically relates to *MLL*-rearranged infant ALL and ALL in children older than 1 year.

Establishing the integrity of our data, we took 2 previously published gene expression profiles associated with *MLL*-rear- ranged ALL and applied these signatures to our *MLL*-rearranged infant ALL samples compared with pediatric precursor BCP-ALL samples. For both published signatures, approximately 80% of the probe sets in both of the signatures appeared significantly differentially expressed in our *MLL*-rearranged infant ALL samples, demonstrating that our dataset is consistent with other datasets reported earlier. The approximately 20% of the probe sets in both signatures that did not show differential expression in our samples may be explained by slight differences or biases in the composition of

the patient cohorts in which these signatures were originally identified. For example, the signature reported by Armstrong et al,[10] was based predominantly on t(4;11) and t(11;19)-positive cases, whereas no t(9;11)-positive cases were included. Moreover, this patient cohort also included *MLL*-rearranged ALL samples from children older than one year of age, as well as a few adult patients. Likewise, in the study of Yeoh et al,[2] the inclusion criteria of *MLL*-rearranged ALL samples were solely based on the presence of an *MLL* translocation regardless of age. Our *MLL*-rearranged ALL cohort consists entirely of infants younger than one year in which all 3 common *MLL* translocations found among infant ALL patients are represented.

Given the superior number of probe sets on the HU133plus2.0 GeneChips (used in the present study) over the first generation HU95A chips used in earlier studies,[2,12] we also compared *MLL*- rearranged infant ALL with *MLL* translocation-negative non-infant pediatric precursor BCP-ALL samples, based on our data. This comparison demonstrated that high-resolution HU133plus2.0 data are capable of separating these patient groups even more convincingly than already shown earlier and revealed differential expression of genes that have not been associated with *MLL*-rearranged ALL before, which may therefore provide further insights into this aggressive type of leukemia, on top of recent progress in understanding mechanism by which *MLL* fusions alter gene expression. The most important breakthrough in our comprehension of *MLL* translocation induced transformation has been the notion that, because of the loss of *MLL*-specific histone methyltransferase activity necessary for H3K4 methylation, MLL fusions recruit alternative histone methyltransferases (e.g., DOT1L) that subsequently establish H3K79 methylation. In turn, H3K79 methylation results in accessible chromatin at inappropriate loci, allowing the abnormal and presumably

pathogenic activation (expression) of associated genes.[22, 23] From this respect, MLL fusion proteins are often regarded as activating oncogenic molecules. In line with this assumption, we here show that approximately 7000 probe sets are significantly up-regulated in *MLL*-rearranged infant ALL compared with non-infant pediatric precursor BCP-ALL samples. On the other hand, we found an equal amount of probe sets to be significantly down-regulated in *MLL*-rearranged infant ALL, indicating that the considerably deregulated gene expression patterns in this disease are not necessarily characterized by an overrepresentation of activated genes but show that down-regulated gene expression is at least as common. In concordance with this, we recently found *MLL*-rearranged infant ALL samples to display vast amounts of genome-wide gene promoter methylation that appeared to be associated with the transcriptional silencing of the affected genes.[24] Thus, whereas the mechanisms by which MLL fusions activate gene expression are currently being elucidated, the mechanisms by which MLL fusions deactivate gene expression remain to be studied.

As infant ALL samples carrying germline *MLL* genes have not yet been properly analyzed as a single patient group, we compared gene expression profiles of these patients against *MLL*-rearranged infant ALL and non-infant pediatric precursor BCP-ALL profiles. A completely unsupervised clustering analysis revealed that *MLL* germline infant ALL resembles neither *MLL*-rearranged infant ALL nor pediatric precursor BCP-ALL lacking known genetic abnormalities. Based on this unsupervised analysis using all probe sets present on the HU133plus2.0 GeneChip, the *MLL* germline infant ALL samples seem more closely related to *MLL*-rearranged infant ALL samples (of the same age) than to the precursor ALL samples, also carrying germline *MLL* genes, derived from children older than one year. This finding possibly reflects the influences of very young age, at which ALL

(in the absence of *MLL* rearrangements) apparently develops after alternative mechanisms, giving rise to a characteristic gene expression profile. In other words, *MLL* germ- line infant ALL may represent a unique biologic entity. Alternatively, these patients could also display a gene expression profile. that is more similar than one of the established ALL subtypes not included in the present study.

Since the observation that *MLL*-rearranged ALL displays a highly characteristic gene expression profile,[10] scientists have been searching for the mechanisms driving deregulated transcription induced by the MLL fusion. As the *MLL* gene itself has specific histone methyltransferase activity,[25, 26] which is lost during fusion of *MLL* to one of its translocation partner genes, *MLL* translocations probably result in altered chromatin structures resulting from aberrant histone modifications. This may, to a large extent, explain the characteristic gene expression patterns uniformly associated with *MLL*-rearranged leukemia. However, influence of the translocation partner gene should not be ignored. A growing body of evidence implies that many of the MLL fusion partners are part of transcriptional regulation networks that also function through chromatin remodeling,[27] and not necessarily lead to similar changes. For instance, although the recruitment of the histone methyltransferase DOT1L has been well established for MLL-AF4 fusions, it is certainly not unthinkable that other MLL fusion partners recruit histone methyltransferases other than DOT1L, leading to alternative chromatin modifications. In any case, apart from basal deregulation of gene expression driven by the interruption of the *MLL* gene that is shared by all *MLL*-rearranged leukemias, the fusion partner seems to determine additional changes in gene expression characteristic for the type of *MLL* translocation. As shown in the present study, *MLL*-rearranged infant ALL samples carrying translocations t(4;11), t(11;19), or t(9;11) indeed display translocation specific

gene expression signatures that clearly separate these samples into 3 distinct patient groups. In line with these findings, we recently found that these different *MLL* translocations also specify distinct genome-wide promoter methylation patterns.[24] Hypothetically, these data may collectively imply that *MLL*- rearranged leukemias transform by dramatically changing epigenetic landscapes induced and guided by the type of MLL fusion protein, which initially triggers abnormal chromatin remodeling and subsequently alters genome-wide DNA methylation patterns and transcription, all in favor of the development of leukemia.

Finally, we asked whether distinct gene expression profiles could also be hidden among infant ALL patients carrying the same type of *MLL* translocation. Interestingly, we found the presence of 2 separate clusters among our t(4;11)-positive ALL samples, distinguishable by either the presence or absence of *HOXA9*, *HOXA7*, *HOXA10*, *HOXA5*, and *HOXA3* expression. Moreover, the separation of both t(4;11)-positive infant ALL subgroups was not based on moderate variations in *HOXA* expression but rather divided patients either firmly expressing or completely lacking *HOXA* gene expression. These findings confirm a similar observation recently reported by Trentin et al,[28] who showed that, based on the localization of the *MLL* breakpoints and the absence or presence of *AF4-MLL* (the reciprocal fusion transcript of *MLL- AF4*), and the presence or absence of *HOXA* expression, t(4;11)- positive ALL samples can be subdivided into 2 separate genetic subgroups. However, in contrast to the data from Trentin et al,[28] who identified hundreds of genes to be associated with either high or low *HOXA* expression, we only found 27 probe sets to significantly discriminate between t(4;11)-positive infant ALL patients expressing either high or low *HOXA* levels. Nevertheless, these findings are particularly remarkable, as *HOXA* overexpression is thought to be a hallmark of *MLL*-rearranged leukemias,[10, 29] and *HOXA9*

expression has recently been postulated to be required for leukemia survival in *MLL*-rearranged leukemia cell lines and primary *MLL*-rearranged AML samples.[30] Surprisingly, our data revealed that the absence of *HOXA* expression appears to be of significant clinical importance, as these patients are at extreme high risk of disease relapse, even within a patient group already characterized by a poor prognosis. Collectively, these observations challenge the dogma that *HOXA9* is consistently highly expressed in all *MLL*-rearranged leukemias, and demonstrate that HOXA9 is not per se required for the maintenance of *MLL*-rearranged infant ALL, as t(4;11)-positive infant ALL patients lacking *HOXA9* expression seem to be burdened by a more aggressive leukemia with a high risk of early relapse. Thus, in contrast to recent suggestions that suppression of HOXA9 may represent an attractive therapeutic approach in AML, targeting HOXA9 in t(4;11)- positive infant ALL appears not to be an option. Finally, these data clearly indicate that variations in gene expression patterns among *MLL*-rearranged infant ALL cases are not limited to the type of *MLL* translocation alone but continue to extend beyond translocation-specific subgroups, at least in case of translocation t(4;11).

Taken together, the present study demonstrates that the distinct gene expression profiles associated with *MLL*-rearranged infant ALL are more heterogeneous and complicated than ostensibly shown earlier and, to a certain extent, are dependent on the *MLL* translocation partner genes. In addition, based on our gene expression profiles, infant ALL patients lacking *MLL* translocations differ both from *MLL*-rearranged infant ALL and non-infant pediatric precursor BCP-ALL patients. The expression signatures reported here potentially constitute new and additional insights in the genetic makeup of both *MLL*-rearranged and *MLL* germline infant ALL. The work at hand now is to unravel the biologic meaning of these signatures and implement these novel pieces of the puzzle into currently ongoing studies on

the complex biology of this malignancy. Eventually, these profiles should reveal novel therapeutic targets, uncover yet unidentified regulators of leukemogenesis and leukemia maintenance, and perhaps may become useful in future gene expression-based classification of pediatric ALL.

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CHAPTER 5

Molecular genetic characterization of infant acute lymphoblastic leukemia carrying wild-type *MLL* genes

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ABSTRACT

Approximately 20% of all infant ALL cases carry wild-type (or germline) *MLL* genes. Wild-type *MLL* infant ALL patients are generally regarded as young pediatric precursor BCP-ALL patients, but extensive characterization of this specific patient group largely remains unacknowledged. We studied a relatively large cohort of 78 wild-type *MLL* infant ALL samples, using clinical parameters, array-comparative genomic hybridization analysis, gene expression profiling, multiplex ligation-dependent probe amplification, and conventional sequencing. Wild-type *MLL* infant ALL patients are generally characterized by a lower incidence of favourable prognostic factors than pediatric (non-infant) BCP-ALL patients, and patients at high risk of therapy failure typically display an immature pro-B immunophenotype or respond poorly to prednisone. Using gene expression profiling, we found *MEIS1* expression to additionally be highly predictive for clinical outcome in wild-type *MLL* infant ALL with a favourable prognosis in the wild-type *MLL* infants with low *MEIS1* expression (DFS 88%% versus 50%, p=0.01). Wild-type *MLL* infant ALL represents a highly heterogeneous patient group, which cannot be unified by one or a few known recurrent genomic aberrations. High-level *MEIS1* expression and an immature pro-B immunophenotype in high-risk wild-type *MLL* infant ALL patients shows parallel with the unfavourable prognosis of *MLL*-rearranged infant ALL patients.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) in infants (<1 year of age) is a rare but highly aggressive type of leukemia, typically characterised by the presence of *MLL*-rearrangements, occurring in ~80% of these patients.[1] The prognosis for *MLL*-rearranged infant ALL patients is highly unfavourable.[2] In contrast, infant ALL patients carrying wild-type (or germline) *MLL* genes fare significantly better, with reported event free survival (EFS) chances of 74%-95%.[1, 2]

For infant ALL in general, the strongest predictors of a poor outcome are the presence of *MLL* rearrangements and age <6 months, followed by high white blood cell (WBC) counts (>300x10^9/L) and poor *in vivo* prednisone responses.[1, 3]

Infant ALL patients not carrying leukemia-specific rearrangements of the *MLL* gene are thought to resemble B-cell precursor ALL in older children (>1 year of age). However, we recently showed that wild-type *MLL* infant ALL specifies a gene expression pattern that is different from both *MLL*-rearranged infant ALL as well as from pediatric non-infant precursor BCP-ALL.[4] In an unsupervised clustering analysis, wild-type *MLL* infant ALL samples even appeared more closely related to *MLL*-rearranged infant ALL than to pediatric precursor BCP-ALL cases. Thus, regardless of the *MLL* status, young age (<1 year) apparently specifies gene expression similarities that are likely to be important to the biology of these leukemias.

Pediatric (non-infant) precursor BCP-ALL can generally be subdivided based on gross chromosomal aberrations, including the chromosomal translocations t(12;21) (*ETV6-RUNX1*), t(9;22) (*BCR-ABL1*), t(1;19) (*E2A-PBX1*), translocations of the *MLL* gene, or hyperdiploidy (>50 chromosomes). All of these subtypes display distinct clinical outcomes and gene expression profiles.[3, 5, 6] De Lorenzo *et al* [7]

recently published cytogenetic data of wild-type *MLL* infant ALL patients from the Interfant-99 trial and Children's Oncology Group (COG-P9407), and demonstrated that infants without *MLL* translocations share the same cytogenetic abnormalities as older children with ALL, albeit with a different distribution: a lower incidence of the favourable abnormalities *ETV6-RUNX1* and high hyperdiploidy, and a higher incidence of unfavourable abnormalities, including *BCR-ABL1*.

Other recent advances in pediatric BCP-ALL revealed several additional and often prognostically relevant DNA copy-number alterations and mutations, including *IKZF1* and *PAX5* deletions,[8] deletions in the 9p21.1 locus including the cell cycle regulators *CDKN2A* and *CDKN2B*,[9] *ETV6* alterations,[10] *JAK2R683* mutations,[8, 11] *PAR1* deletions, and *CRLF2* rearrangements.[12] By contrast, MLL rearranged infant ALL carry few somatic changes (copy number abnormalities, loss of heterozygosity, or single nucleotide variants)[13, 14], indicating that only a very small number of mutations are necessary to generate infant MLL-leukemia. Yet, no specific data exists on the distribution of such copy-number variations, or mutations in infant ALL patients carrying wild-type *MLL* genes.

In the present study we extensively characterize a relatively large cohort of wild-type *MLL* infant ALL patients, all treated according to INTERFANT treatment protocols (i.e. Interfant-99 or Interfant-06). The results are compared to similar data obtained in *MLL*-rearranged infant ALL patients (enrolled in INTERFANT studies) and pediatric (non-infant) precursor BCP-ALL patients uniformly treated according to the Dutch Childhood Oncology Group (DCOG) ALL-10 protocol.

MATERIALS AND METHODS

Note: A more elaborate and detailed description of all experimental procedures and data analysis methods can be found in the Supplemental Material Chapter 5.

Patient samples and sample preparation

Bone marrow and peripheral blood samples from infants (<1 year of age) with newly diagnosed ALL were collected at institutes participating in the international collaborative Interfant-99[4] and Interfant-06 studies. Pediatric (non-infant) patient data was obtained from the Dutch Childhood Oncology Group (DCOG) treated according to the ALL-10 protocol. Leukemic samples preparation was essentially carried out as described before.[4]

Gene expression profiling data

The gene expression profiling (Affymetrix platform) data used in the present study has previously been published, [4] and has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus[15] and is accessible via GEO Series accession number GSE19475. Additional unpublished gene expression data can be found under GEO accession number GSE58565.

In vivo *and* in vitro *prednisone response*

In vivo prednisone responses were determined after seven days of prednisone monotherapy (including a single intrathecal dose of methotrexate), prior to the initiation of combination chemotherapy. Patients were defined as good

responders when <1000 leukemic blasts/µL were detectable in the peripheral blood. Patients still burdened with ≥1000 leukemic blasts/µL after prednisone monotherapy were defined as poor responders. *In vitro* response to prednisolone (the active metabolite of prednisone) was determined by 4-day MTT assay as previously described.[16]

Oligo array-CGH

Oligo array-CGH (array competitive genomic hybridization) analysis was performed using the human genome CGH Microarray 105k-A (Agilent Technologies, Palo Alto, CA) according to the manufacturer's protocol using a dye-swap experimental design to minimize false positive results, as previously described.[17]

Multiplex ligation-dependent probe amplification (MLPA) analysis and JAK2 mutations

MLPA was performed using the SALSA MLPA P335 ALL-IKZF1 probemix kit (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol. Mutations in JAK2 exon 16 were analyzed by polymerase chain reaction amplification and subsequent sequencing as described previously.[11]

RESULTS

Wild-type MLL infant ALL patients are characterized by favorable clinical parameters

Clinical parameters known to predict outcome in *MLL*-rearranged infant ALL were compared between infant ALL patients carrying wild-type *MLL* genes (n=78) and *MLL*-rearranged infant ALL cases (n=70), as well as between wild-type *MLL* infant ALL patients and pediatric (non-infant) ALL patients (n=484). The adverse prognostic factors analyzed included age <6 months, white blood cell (WBC) counts >300 x 10^9 leukemic cells/L, a pro-B (CD10-) immunophenotype, and a poor *in vivo* prednisone window response (Table 1). Compared with *MLL*-rearranged infant ALL cases, infant ALL patients carrying wild-type *MLL* genes were significantly more often diagnosed at age >6 months, presented with more favourable WBC counts, more mature (pre-B or common) immunophenotypes, and generally responded well to a 7-day window of prednisone monotherapy (Table 1). However, despite the fact that wild-type *MLL* infant ALL patients were characterized by favourable prognostic factors, these factors were less frequently present compared to pediatric (non-infant) ALL patients. For instance, only 2% of the pediatric ALL patients presented with a highly immature pro-B immunophenotype, compared to 11% of the wild-type *MLL* infant ALL patients ($p<0.0001$).

	Wild-type *MLL* infant ALL (n=78)	***MLL*-rearranged infant ALL (n=70)**	***p***	**Pediatric (non-infant) ALL (n=484)**	***P***
Sex			0.62		0.09
Male	36 (46%)	29 (41%)		274 (57%)	
Female	42 (54%)	41 (59%)		210 (43%)	
Age at diagnosis			<0.001		NA
< 6 months	18 (23%)	41 (59%)		NA	
> 6 months	60 (77%)	29 (41%)		NA	
WBC (cells/L)			<0.001		<0.001
< 100 x 10^9	52 (69%)	16 (24%)		389 (89%)	
100-300 x 10^9	19 (25%)	24 (36%)		38 (9%)	
> 300 x 10^9	6 (8%)	27 (40%)		11 (3%)	
not known	1	3		46	
Immunophenotype			<0.001		<0.001
pro-B cell	8 (11%)	52 (78%)		9 (2%)	
common B-cell	25 (33%)	3 (4%)		257 (54%)	
pre-B cell	29 (39%)	10 (15%)		132 (28%)	
T-lineage	9 (12%)	0		80 (17%)	
Other*	4 (5%)	2 (3%)		1 (0%)	
not known	3	3		5	
Prednisone response			0.009		0.21
good response	62 (85%)	39 (65%)		385 (90%)	
poor response	11 (15%)	21 (35%)		41 (10%)	
not known	5	9		58	

Table 1. Clinical characteristics and prognostic factors of wild-type *MLL* infant ALL patients. *Other immunophenotypes included both acute undifferentiated and biphenotypic leukemias. WBC= white blood cell, and NA= not applicable.

Poor prednisone responses and a pro-B phenotype predict outcome in wild-type MLL infant ALL

Next we assessed the prognostic relevance of the above described predictive parameters in terms of disease-free survival (DFS), overall survival (OS), and cumulative incidence of relapse (CIR) after 5 years from diagnosis in wild-type *MLL* infant ALL patients (n=76) for whom clinical follow-up data was available (Table 2). For the entire group of patients, outcome measures were: 0.71 DFS (SE=0.05), 0.82 OS (SE=0.05), and 0.22 CIR (SE=0.05). Neither age <6 months at diagnosis, nor WBC counts >300 x 10^9 leukemic cells/L were predictive for clinical outcome within this group. In contrast, a poor prednisone response was marginally associated with an inferior outcome, whereas an immature pro-B immunophenotype was highly predictive for a poor clinical outcome. The 5-year OS in the wild-type *MLL* infant ALL patients diagnosed with pro-B ALL was 0.14, whereas this was 0.92 and 0.93 in wild-type *MLL* infants diagnosed with common BCP-ALL and pre-B ALL respectively ($p<0.001$) (Table 2).

	n	5-year DFS	SE	P	5-year OS	SE	P	5-year CIR	SE	P
Age at diagnosis				0.75			0.49			0.32
< 6 months	17	76.5	10.3		76.5	10.3		11.8	8.1	
> 6 months	58	70.0	6.1		84.0	4.9		24.8	5.8	
WBC count (cells/L)				0.83			0.30			0.74
< $100x10^9$	49	72.6	6.5		87.2	4.9		25.4	6.4	
$100\text{-}300x10^9$	19	68.4	10.7		73.7	10.1		15.8	8.6	
> $300x10^9$	6	66.7	19.3		66.7	19.3		16.7	16.7	
Prednisone response				0.22			0.23			0.33
good response	61	75.1	5.6		85.1	4.6		18.3	5.1	
poor response	10	56.3	16.5		67.5	15.5		32.5	16.7	
Immunophenotype				0.003			<0.001			0.03
pro-B cell	7	14.3	18.7	<0.001*	14.3	18.7	<0.001*	57.1	22.7	0.02*
common B cell	25	66.3	9.8		92.0	5.4		33.7	10.1	
pre-B cell	28	85.3	6.8		92.7	5.0		7.3	5.1	
T-lineage	9	66.7	15.7		64.8	16.5		22.2	14.8	
Other	4	-	-		-	-		-	-	

Table 2. Univariate analysis of prognostic factors in wild-type *MLL* infant ALL patients. DFS: Disease event-free survival; OS: overall survival; CIR: cumulative incidence of relapse**.** *p-value for comparison of pro-B immunophenotype versus all other phenotypes.

MEIS1 expression is a strong prognostic factor in wild-type MLL infant ALL patients

In search for additional prognostic factors for wild-type *MLL* infant ALL, we applied Significance Analysis of Microarrays (SAM) to screen our gene expression profiles (Affymetrix HU133plus2.0 GeneChips) for genes predictive for clinical outcome. For 30 wild-type *MLL* infant ALL patients both gene expression profiles and clinical follow-up data was available. Interestingly, the level of *MEIS1* expression (Affymetrix probe set 242172_at) appeared highly predictive for clinical outcome. Patients expressing low levels (i.e. below the median of the

entire patient group) of *MEIS1* (n=16) had a superior outcome in terms of DFS (Figure 1A) and OS (Figure 1B), over patients expressing high levels (i.e. above the median of the entire patient group, n=14) of *MEIS1* (DFS 88% versus 50% respectively, p=0.01). Remarkably, differential gene expression analysis between patients with high *MEIS1*-expression (n=18) and patients with low *MEIS1*-expression (n=18), could not identify differentially expressed genes other than *MEIS1* itself. Unfortunately, due to the small number of events (n=8), multivariate analysis of *MEIS1* in combination with other known prognostic factors could not be performed. Distribution analysis of prognostic factors only showed a significant difference in distribution of immunophenotype with a more immature phenotype in the wild-type infant ALL patients with high-level expression (p=0.002) (Supplemental material Chapter 5 Table 2). Strikingly, high-level *MEIS1* expression had no prognostic value in pediatric (non-infant) ALL patients.

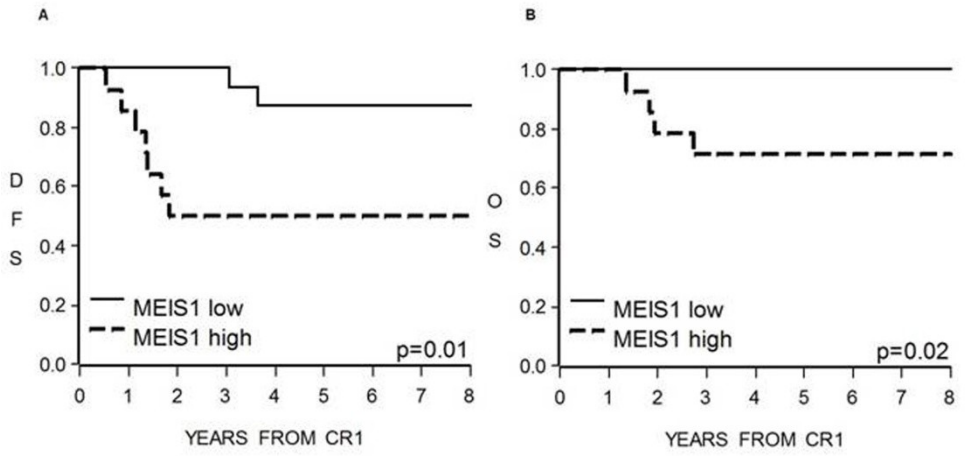

Figure 1. Survival in wild-type *MLL* infant ALL patients expressing low versus high levels of *MEIS1*. A. Disease-free survival (DFS) and B. overall survival (OS) for wild-type *MLL* infant ALL patients expressing low levels of *MEIS1* (below the median of the entire patient group, n=16; continuous line) and high levels of *MEIS1* (n=14; dotted lines).

Submicroscopic DNA copy-number variations (array-CGH analysis)

In order to detect submicroscopic deletions and amplifications in the DNA, we performed array-comparative genomics hybridization (array-CGH) on a cohort of wild-type *MLL* infant ALL patients (n=31) for whom genomic DNA was available (Supplemental material Chapter 5 Table 1). The results were compared with array-CGH data from pediatric (non-infant) BCP-ALL patients (n=113) (Table 3). Remarkably, copy-number variations were detected in 68% of wild-type *MLL* infant ALL patients which is significantly lower than in pediatric (non-infant) precursor BCP-ALL patients (95%) ($p<0.001$).

Furthermore we distinguished between numerical variations (i.e. loss or gain of whole chromosomes) and structural variations (i.e. partial deletions/amplifications, and translocations). The frequency of structural aberrations was much lower in wild-type *MLL* infants than in non-infant ALL patients (45% vs. 86%, respectively; $p<0.001$) (Table 3). The frequency of patients with numerical aberrations was higher among wild-type *MLL* infant ALL patients (23%) than in non-infant pediatric ALL patients (12%). This difference disappeared when correcting for the incidence of hyperdiploidy.

Several of the structural aberrations found among the wild-type *MLL* infant ALL patients included known oncogenes and/or tumor suppressors. In 6/28 (21%) of the wild-type *MLL* infants deletions of 9p21.3, containing the tumor suppressor genes *CDKN2A* and *CDKN2B,* were observed. Two of these patients carried homozygous deletions, while these deletions in the remaining four patients were heterozygous.

One wild-type *MLL* infant ALL patient with a numerically normal karyotype (so excluding hyperdiploid patients) carried an amplification of 17p13.1 containing *GAS7*, a gene known to be a translocation partner of *MLL*. Occasionally single and

unique wild-type *MLL* infant ALL patients were found to carry a deletion of 1p36.11 including the tumor suppressor gene *RUNX3*, of 7p22.3 to 7p15.3 including the *EVI1* gene at 7p21.2, or of 3q25.2 to 3q26.33 starting at the *MME* gene encoding human CD10 (Supplemental material Chapter 5 Table 1).

Finally, our cohort of wild-type *MLL* infant ALL patients included three patients diagnosed with T-ALL. One of these wild-type *MLL* infant T-ALL patients showed deletions of genes *SIL* and *PTEN*. Subsequent fluorescent in-situ hybridization (FISH) analysis demonstrated that this patient carried a *SIL-TAL1* fusion. Another wild-type *MLL* infant T-ALL patient had a deletion of 11q14.1 to 11q22.1, including the *PICALM (CALM)* gene.

	wild-type *MLL* infant ALL		pediatric non-infant BCP-ALL		
	all (n=31)	without HD (n=27)	all (n=113)	without HD (n=102)	B-others (n=39)
Type of aberration*					
No aberrations	10 (32%)	10 (37%)	6 (5%)	6 (6%)	2 (5%)
Numerical aberrations	7 (23%)	3 (11%)	13 (12%)	8 (8%)	4 (10%)
Structural aberrations	14 (45%)	14 (52%)	97 (86%)	96 (94%)	36 (92%)
Structural aberrations by oncogene/tumor suppressor gene and immunophenotype					
	BCP-ALL patients (n=28)[†]				
CDKN2A (9p21.3)					
homozygous deletion	2 (7%)	2 (8%)	12 (11%)	11 (11%)	8 (21%)
heterozygous deletion	4 (14%)	4 (16%)	21 (19%)	18 (18%)	9 (23%)
RUNX3 (1p36.11)					
Deletion	1 (4%)	1 (4%)	6 (5%)	3 (3%)	3 (8%)
GAS7 (17p13.1)					
Amplification	4 (13%)	1 (4%)	20 (18%)	11 (11%)	7 (18%)
Deletion	1 (4%)	1 (4%)	-	-	-
MME (3q25.2)					
Deletion	1 (4%)	1 (4%)	2 (2%)	1 (1%)	0
ETV1 (7p21.2)					
Deletion	1 (4%)	1 (4%)	5 (4%)	3 (3%)	1 (3%)
	T-ALL patients (n=3)			-	
STIL and *PTEN*[‡]					
Deletion	1 (33%)	NA	NA	NA	NA
PICALM (11q14.2)					
Deletion	1 (33%)	NA	NA	NA	NA

Table 3. Distribution of DNA copy-number variations in wild-type *MLL* infant ALL and pediatric non-infant precursor BCP-ALL patients. *As numerical and structural aberrations can occur combined in one patient, numbers mentioned don't necessarily add up to 100%. [†]BCP-ALL patients including patients with unknown immunophenotype. [‡]Fluorescent in-situ hybridization (FISH) confirmed a *SIL-TAL1* fusion caused by sub-deletion. HD = hyperdiploid karyotype.

Screening of B-cell development genes in wild-type MLL infant ALL

In pediatric (non-infant) precursor BCP-ALL, a number of genes involved in B-cell development are frequently altered, and several of these alterations (e.g. mutations, deletions and amplifications) are associated with high-risk ALL subtypes and implicated in clinical outcome.[3, 18, 19] We performed multiplex ligation-dependent amplification (MLPA) analysis using specific probes for single gene alterations in *CDKN2A*, *CDKN2B*, *IKZF1 PAX5*, *ETV6*, *BTG1*, *CRLF2*, and the Xp22.33 locus (Table 4). In none of the wild-type *MLL* infant ALL samples (n=32) tested, alterations of *IKZF1* were detected, whereas 17% of the pediatric non-infant BCP-ALL patients is known to carry a *IKZF1* deletion.[20] A deletion of *CDKN2A* and *CDKN2B* on 9p21.3 was found in six (19%) of the infants. For five of these infants array-CGH analysis was performed which showed the same results. In contrast, in 30% and 34% of the pediatric non-infant ALL samples deletions of *CDKN2A* and *CDKN2B* were found, respectively. In four wild-type *MLL* infant ALL patients deletions of *PAX5* were present together with *CDKN2A* and *CDKN2B*, whereas in two infants the *PAX5* deletion was the only observed abnormality as determined by MLPA. Interestingly, the frequency of *PAX5* deletions was significantly higher in wild-type *MLL* infant ALL patients (19%) than in the pediatric precursor BCP-ALL (6%) (p=0.02). The incidence of *ETV6* deletions was markedly lower in infants: 3% versus 11% of the pediatric precursor BCP-ALL patients.

Likewise, amplifications of *CRLF2*, as well as the Xp22.3 locus containing pseudoautosomal region 1 (*PAR1*), appeared to be rare events among wild-type *MLL* infant ALL patients (both occurring in 3% of the patients tested), whereas these lesions occur in 25% and 21% pediatric (non-infant) precursor BCP-ALL patients.

	wild-type *MLL* infant ALL n=32	pediatric non-infant ALL n=232
Deletions		
CDKN2A	6 (19%)	69 (30%)
CDKN2B	6 (19%)	78 (34%)
PAX5	6 (19%)	13 (6%)*
ETV6	1 (3%)	25 (11%)
BTG1	1 (3%)	0
Amplifications		
CRLF2	1 (3%)	59 (25%)†
Xp22.33	1 (3%)	48 (21%)*

Table 4. Multiplex ligation-dependent probe amplification (MLPA) analysis of genes associated with B-cell differentiation. Significant differences between wild-type *MLL* infant ALL and pediatric non-infant precursor BCP-ALL patients are indicated by * ($p<0.05$) and † ($p<0.01$) (Fisher exact test).

Finally, as *JAK2R683* mutations have been associated with high-risk pediatric BCP-ALL[8, 11], we screened for *JAK2R683* mutations in exon 16 by means of conventional PCR and subsequent sequencing analysis. No mutations in *JAK2R683* were detected in a total of 28 wild-type *MLL* infant ALL patient samples, whereas 6% of the *BCR-ABL1*-negative, high-risk pediatric ALL cases have been described to carry this specific mutation.[8]

DISCUSSION

In the present study we genetically characterized a large cohort of rare wild-type *MLL* infant ALL samples. The incidence of favorable clinical factors in wild-type *MLL* infant ALL turned out to be higher than in *MLL*-rearranged infant ALL, but lower than in pediatric precursor BCP-ALL. For instance, the incidence of a high WBC and the unfavorable pro-B (CD10-) immunophenotype are higher in *MLL*-rearranged infant ALL compared to that in wild-type *MLL* infant and pediatric ALL patients. A favorable *in vivo* response to prednisone was more often observed in wild-type *MLL* infant ALL patients when compared with *MLL*-rearranged infant ALL patients. In addition, we recently showed that wild-type *MLL* infant ALL patients have a low incidence of genetic abnormalities associated with a favorable prognosis (i.e., hyperdiploidy and *ETV6-RUNX1 (*or *TEL-AML1)* translocations),[7] whereas these abnormalities occur in about half of the non-infant pediatric precursor BCP-ALL patients.[21] These findings may contribute to the slightly worse outcome of wild-type *MLL* infant ALL patients compared with pediatric (non-infant) precursor BCP-ALL patients.

Gene expression analysis of wild-type *MLL* infant ALL revealed that the level of *MEIS1* expression was highly predictive for clinical outcome. In fact, none of the cases expressing relatively low levels of *MEIS1* experienced a 5-year disease-free survival of 88% and the 5-year overall survival rate for these patients appeared to be 100%. In contrast, for wild-type *MLL* infant ALL patients displaying relatively high levels of *MEIS1* expression the disease-free and overall survival rates were 50% and 71% respectively. This observation is in line with results of a study reported by the Children's Oncology Group (COG), showing that *MEIS1* expression was an independent predictor of clinical outcome in infant ALL including both

MLL-rearranged and wild-type *MLL* cases.[22] Interestingly, high-level expression of *MEIS1* is tightly associated with prognostically unfavorable *MLL*-rearranged leukemias[5, 23] and has been demonstrated to be important in oncogenicity.[24, 25] Hence, the prognostic relevance of *MEIS1* expression in wild-type *MLL* infant ALL patients may imply transformation events that to some extent resemble that of *MLL*-rearranged infant ALL cases. Furthermore, the strong influence on clinical outcome of *MEIS1* expression suggests that infant ALL expressing high levels of *MEIS1* represent a highly aggressive leukemia that require very few cooperative genetic lesion during leukemogenesis and/or leukemia maintenance. Taken together these data suggest that both *MLL*-rearranged infant ALL as well as wild-type *MLL* infant ALL patients may benefit from MEIS1 inhibition. The level of *MEIS1* expression had no prognostic significance in pediatric (non-infant) BCP-ALL.

To further characterize wild-type *MLL* infant ALL, we searched for submicroscopic copy-number variations. Although our unsupervised gene expression profiling-based clustering analysis recently indicated that these patients form a distinct entity distinguishable from both *MLL*-rearranged infant ALL and non-infant pediatric precursor BCP-ALL,[4] we could not identify any unifying factors characterizing this group of patients. Nonetheless, wild-type *MLL* infant ALL displayed a distinctive pattern of DNA copy-number variations and genetic abnormalities in genes associated with B-cell development. Compared with pediatric (non-infant) precursor BCP-ALL patients, wild-type *MLL* infant ALL patients less frequently harbor DNA copy-number variations. In approximately one third of the wild-type *MLL* infant ALL patients no copy-number alterations were detected in array-CGH. Similarly, the frequencies of deletions or amplifications specifically in B-cell development genes was much lower in wild-type *MLL* infant ALL compared with pediatric BCP-ALL patients, although higher

than in rearranged *MLL* infant ALL.[13, 14] Hypothetically, a longer latency time to diagnosis combined with an insufficient cellular repair mechanism in pediatric BCP-ALL might explain the higher frequency of structural aberrations in this group. One exception is *PAX5* gene deletions, which appeared in 19% of the infant ALL patients compared to 6% of pediatric BCP-ALL patients. We could not find an association between deletions of genes located on chromosome 9p and MEIS1-expression (Supplemental material Chapter 5 Table 2).

In conclusion, infant ALL patients carrying wild-type *MLL* genes form a distinct group from pediatric (non-infant) BCP-ALL patients, but is a very heterozygous patient group for which very few recurrent genetic abnormalities can be identified. The frequency of DNA copy number variations and molecular genetic lesions in genes involved in B-cell development are lower in wild-type *MLL* infant ALL compared to older children with ALL. Several poor clinical risk features are more frequent in wild-type *MLL* infant ALL. The strongest predictor of outcome in wild-type *MLL* infant ALL was the level of *MEIS1* expression, which may point to new opportunities for novel strategies in treating wild-type *MLL* infant ALL.

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CHAPTER 6

Identification of genes transcriptionally responsive to the loss of MLL fusions in *MLL*-rearranged ALL

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ABSTRACT

Introduction: *MLL*-rearranged acute lymphoblastic leukemia (ALL) in infants (<1 year) is characterized by high relapse rates and a dismal prognosis. To facilitate the discovery of novel therapeutic targets, we here searched for genes directly influenced by the repression of various *MLL* fusions.

Methods: For this, we performed gene expression profiling after siRNA-mediated repression of *MLL-AF4*, *MLL-ENL,* and *AF4-MLL* in *MLL*-rearranged ALL cell line models. The obtained results were compared with various already established gene signatures including those consisting of known MLL-AF4 target genes, or those associated with primary *MLL*-rearranged infant ALL samples.

Results: Genes that were down-regulated in response to the repression of *MLL-AF4* and *MLL-ENL* appeared characteristically expressed in primary *MLL*-rearranged infant ALL samples, and often represented known *MLL-AF4* targets genes. Genes that were up-regulated in response to the repression of *MLL-AF4* and *MLL-ENL* often represented genes typically silenced by promoter hypermethylation in *MLL*-rearranged infant ALL. Genes that were affected in response to the repression of *AF4-MLL* showed significant enrichment in gene expression profiles associated with *AF4-MLL* expressing t(4;11)+ infant ALL patient samples. *Conclusion:* We conclude that the here identified genes readily responsive to the loss of *MLL* fusion expression potentially represent attractive therapeutic targets and may provide additional insights in *MLL*-rearranged acute leukemias.

INTRODUCTION

A hallmark of acute lymphoblastic leukemia (ALL) in infants (<1 year of age) is a high incidence (~80%) of chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene [1, 2], in which the N-terminal portion of *MLL* fuses to the C-terminal region of one of its many translocation partner genes. [3] The most common *MLL* translocations found among infant ALL patients are t(4;11), t(11;19), and t(9;11), fusing *MLL* to *AF4*, *ENL* and *AF9*, respectively. [2, 4] *MLL*-rearranged infant ALL is associated with an adverse outcome, with event-free survival rates of only ~30-40%. [2]

MLL-rearranged ALL cells display unique gene expression profiles, consisting of overwhelming numbers of differentially transcribed genes [5, 6], which make it difficult to distinguish between the actual "drivers" of the leukemia from the so called "bystanders". Fortunately, recent advances allowed the identification of genes likely to be activated by MLL fusion proteins via the recruitment of DOT1L. [7-9] Yet, apart from MLL fusion driven activation of gene transcription, inactivation of transcription also plays an important role in *MLL*-rearranged ALL. We and others, recently demonstrated that *MLL*-rearranged infant ALL is characterized by unique patterns of gene promoter DNA hypermethylation, leading to transcriptional silencing of associated genes. [10, 11] To make matters even more complicated, more than half of the t(4;11)-positive ALL patients not only carry the *MLL-AF4* fusion transcript, but also express and translate the reciprocal *AF4-MLL* transcript, which has been proposed to substantially contribute, or even being essential, for leukemia development.[12, 13]

Here, we studied the direct transcriptional consequences of the loss of MLL fusion transcripts in order to identify potential target genes for therapeutic intervention.

For this, we performed gene expression profiling in *MLL*-rearranged ALL cell line models in which *MLL-AF4, AF4-MLL* or *MLL-ENL* expression was repressed by siRNA-mediated RNA interference. We postulate that genes directly responding to the loss of the MLL fusion represent important therapeutic targets and may provide additional insights into the actions of MLL fusion proteins.

METHODS

Note: More detailed descriptions of all experimental procedures and data analysis methods can be found in the Supplemental Material Chapter 6.

Cell line models

The BCP-ALL cell lines RS4;11 and SEMK2 both carry translocation t(4;11) generating the *MLL-AF4* and *AF4-MLL* fusion transcripts. KOPN-8 carries a t(11;19) translocation generating *MLL-ENL* transcripts. RS4;11 was established from the bone marrow of a 32-year-old woman [14], and was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). SEMK2 is a subclone of the SEM cell line, which was originally derived from a 5-year-old girl at relapse [15] and was kindly provided by Dr Scott Armstrong (Memorial Sloan Kettering Cancer Center, New York, USA). KOPN-8 was derived from a 3-month-old infant girl with B-cell precursor ALL and was purchased from DSMZ.[16]

siRNA-mediated RNA interference

Cells were transfected with siRNAs directed against *MLL-AF4* [17], *AF4-MLL* [13], or *MLL-ENL* (sense 5'-CCAAAAGAAAAGUCUGCCCAG-3; antisense 5'-CUGGGCAGACUUUUCUUUUGGUU-3'), using electroporation. Control cells were transfected with siRNAs against *AML1-MTG8* (*AGF1*) [18], a fusion transcript absent in both SEMK2 and RS4;11 cells. For the knock-down of *MLL-AF4* and *MLL-ENL*, cells were harvested after two days. For the *AF4-MLL* knock-down, cells were transfected with siRNAs a second time after two days of culturing, and eventually harvested at day 4. All experiments were performed at least three times.

RNA extraction

Total RNA was extracted from a minimum of $2x10^6$ cells using TRIzol reagent (Invitrogen, Life Technologies, Breda, The Netherlands) according to manufacturer's guidelines.

Gene expression profiling

Gene expression profiling was performed using HU133plus2.0 microarrays (Affymetrix) according to manufacturer's guidelines. Gene expression profiles for the primary infant ALL patients samples were generated and published previously [19].

RESULTS

Transcriptional consequences of MLL fusion knock-down

Compared to cells transfected with control siRNAs, *MLL-AF4* mRNA expression was reduced to 45% and 37% in the t(4;11)-positive ALL cell lines RS4;11 and SEMK2, respectively, upon transfection with siRNAs directed against *MLL-AF4*. Using siRNAs directed against *MLL-ENL*, the level of *MLL-ENL* expression in KOPN-8 cells was reduced to 5% (Figure 1A). Western blot analysis demonstrated a reduction of the MLL-AF4 protein expression (relative to control cells) of 28% and 52% in RS4;11 and SEMK2 cells, respectively (Figure 1B).

Figure 1. siRNA-mediated knock-down significantly decreases MLL fusion expression levels. A. mRNA expression levels of *MLL-ENL* (grey), wild-type *MLL* (white), and wild-type *ENL* (black) in KOPN-8 cells, or *MLL-AF4* (grey), wild-type *MLL* (white) and wild-type *AF4* (black) in RS4;11 and SEMK2 cells after transfection with active siRNA directed against the absent target *AML1/MTG8* (si*AGF1*), empty pulse (no siRNAs), and siRNAs directed against *MLL-ENL* and *MLL-AF4* respectively. Shown is the average mRNA expression of two experiments ± standard error of the mean. B. Protein expression levels of MLL-AF4 in RS4;11 and SEMK2 shown by western blot (left panel). The western blot was probed with antibodies against the N-terminus of MLL to detect MLL-AF4. Clathrin was used as a loading control. The graph (right panel) shows western blot quantification of MLL-AF4 protein expression relative to clathrin with the empty pulse control set at 100%.

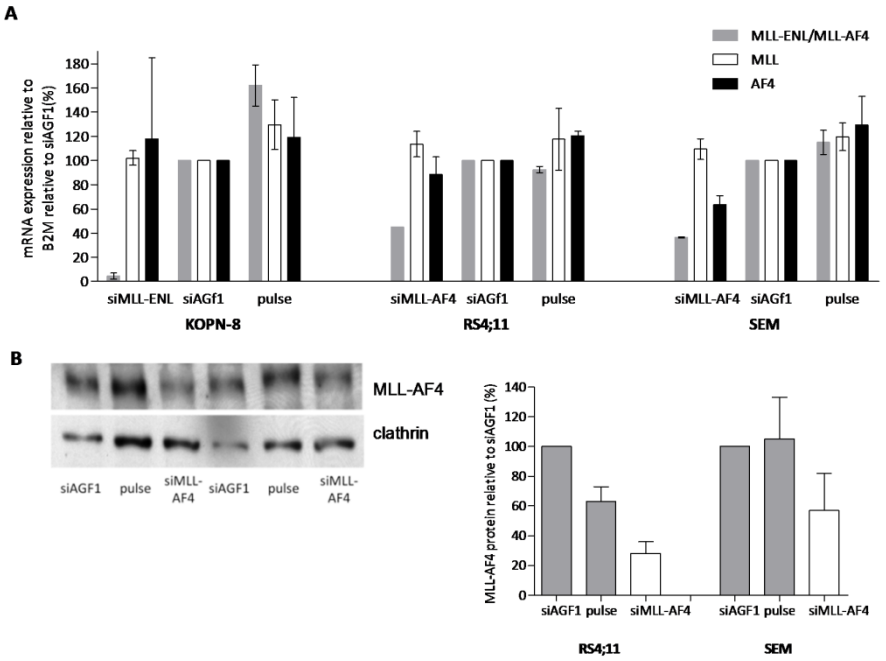

Figure 1. siRNA-mediated knock-down significantly decreases MLL fusion expression levels.

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Next, in order to identify genes directly responding to the loss of the MLL fusion, we generated gene expression profiles (HU133plus2.0 GeneChips, Affymetrix) in three independent experiments. Upon repression of *MLL-ENL* in KOPN-8 cells, significant differential expression was observed for 342 probe sets ($p<0.001$). Reduced expression of *MLL-AF4* resulted in significantly ($p<0.001$) altered expression of 26 probe sets in RS4;11 cells, and 145 probe sets in SEMK2 cells. Figure 2 shows heatmaps displaying the most significantly altered probe sets for all three cell lines.

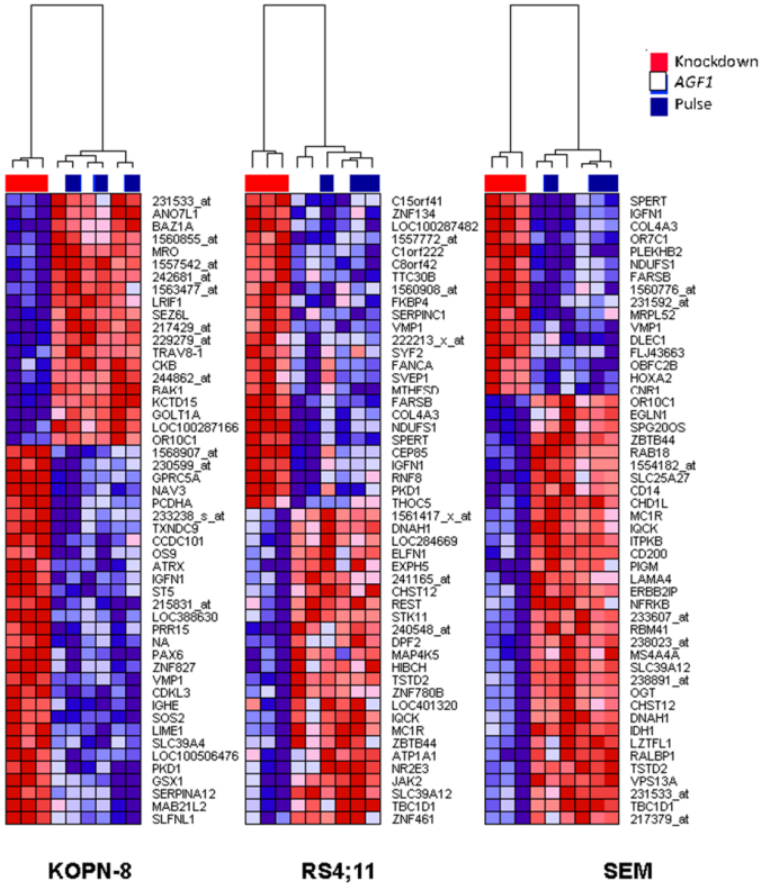

Figure 2. Differential gene expression in response to the repression of MLL-AF4 and MLL-ENL. Heatmap visualization of gene expression profiles of the top 50 most differentially expressed genes in response to *MLL-ENL* knock-down in KOPN-8 cells, and *MLL-AF4* knock-down in RS4;11 and SEMK2 cells, as compared to control cells transfected with si*AGF1* or electroporated in the absence of siRNAs. The presented data was derived from samples obtained from three independent experiments. Red depicts high expression, blue depicts low expression.

We searched for a core signature of genes consistently affected in all cell lines by performing a paired analysis of all samples in which the MLL fusion was suppressed (including SEMK2, RS4;11, and KOPN8), compared to all control samples, including cells transduced with control siRNAs directed against *AML1-MTG8* (*AFG1*), as well as control cells electroporated in the absence of siRNAs. Compared to cells transfected with control siRNAs, 101 probe sets appeared to be recurrently affected in all cell lines. Compared to cells only subjected to electroporation in the absence of siRNAs (i.e. pulse control), 86 probe sets were differentially expressed. Merging these analyses, we found 56 overlapping probe sets to be recurrently differentially expressed in all cell lines in which the MLL fusions were suppressed (Figure 3A). Hierarchical clustering analysis showed that these 56 probe sets effectively distinguished between cells in which the MLL fusion was knocked down and control samples, as separately shown for each cell line (Figure 3B). As these probe sets consistently responded to the loss of the MLL fusion, we postulate that this gene signature represents genes which are highly dependent on the presence of the MLL fusion, and as such may exert prominent functions in MLL fusion driven transformation. Probe set IDs, HGNC gene symbols, and log-fold changes of the obtained core signature consisting of the 56 probe sets are listed in the Supplemental material Chapter 6 (Table 1 and 2). HGNC gene symbols from these probe sets can also be found next to the heatmap in Figure 4.

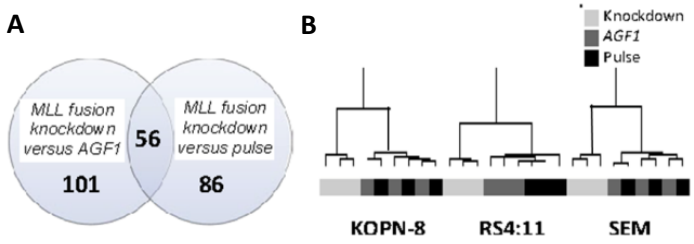

Figure 3. Differential gene expression in response to the repression of MLL-AF4 and MLL-ENL. A. Venn diagram showing the number of differentially expressed genes (p<0.001) in KOPN-8, RS4;11, and SEMK2 cells transfected with siRNAs against *MLL-ENL* and *MLL-AF4*, combined (*MLL* fusion knock-down, n=9) versus control cells transfected with si*AGF1* (*AGF1* control, n=9), versus control cells electroporated in the absence of siRNAs (pulse control n=9). All probe sets and gene symbols are listed in Table S1 and S2. B. Hierarchical clustering based on 56 differentially expressed probe sets recurrently affected in both KOPN-8, RS4;11, and SEMK2 cells upon *MLL* fusion knock-down (light grey), in control samples transfected with si*AGF1* (dark grey), and control samples electroporated in the absence of siRNAs (black).

Relevance of the MLL fusion knock-down signature

To explore the relevance of the obtained gene signature consisting of 56 probe sets responsive to the knock-down of the MLL fusion, we compared our signature to that of earlier published gene sets associated with *MLL*-rearranged ALL. The first signature, published by Guenther *et al* [8], contains 42 genes occupied by the MLL-AF4 fusion protein. The second gene set published by Krivtsov *et al* [7] consists of genes associated with MLL fusion mediated H3K79 dimethylation. Gene set enrichment analysis (GSEA) showed significant enrichment of these genes in our MLL fusion knock-down gene signature (normalized enrichment scores (NES) of

1.92 and 2.11 respectively; leading edge in Table S3 and S4 respectively) (Figure 3). These data indicate that some, but not all, genes transcriptionally activated by the MLL fusion gene itself, rapidly respond to the loss of the MLL fusion.

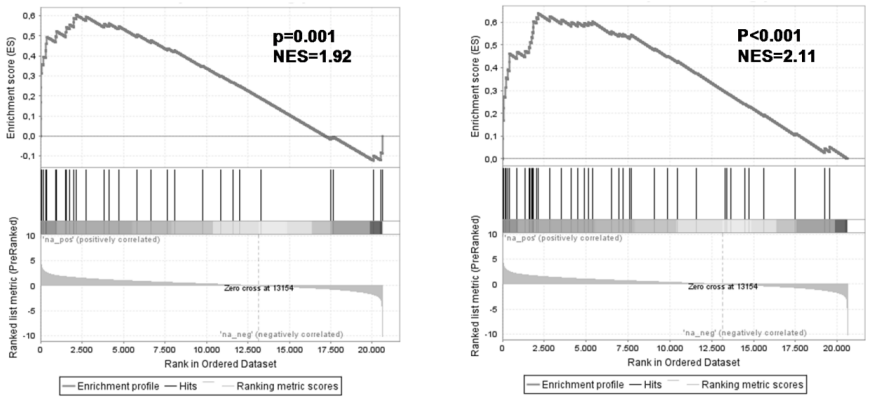

Figure 3. Genes responsive to the repression of MLL-ENL and MLL-AF4 often represent known MLL-AF4 target genes. Gene set enrichment analysis (GSEA) of the 56 differentially expressed probe sets recurrently affected in both KOPN-8, RS4;11, and SEMK2 cells upon *MLL* fusion knock-down in gene lists consisting of MLL-AF4 target genes as published by Guenther et al. (8) (left) and Krivtsov et al. (7) (right). NES=normalized enrichment score.

Moreover, using our core signature consisting of 56 probe sets associated with knock-down of the MLL fusion, we performed hierarchical clustering on gene expression profiling data generated on a large cohort of primary *MLL*-rearranged infant ALL (n=71), wild-type *MLL* pediatric precursor BCP-ALL (n=16), and wild-type *MLL* infant ALL (n=20) samples, as well as healthy bone marrow samples (n=13) as non-leukemic controls. Based on these 56 probe sets, *MLL*-rearranged

ALL samples could almost be flawlessly separated from ALL samples with wild-type *MLL* genes (Figure 4). These data imply that our MLL fusion knock-down signature represents genes highly characteristic for *MLL*-rearranged ALL. Similarly, gene set enrichment analysis (GSEA) showed strong enrichment in the *MLL*-rearranged patients of the 57 probe sets that are significantly lower expressed after MLL fusion knock-down (NES=1.95, p=0.002) (Figure 5, dataset in Table S5; leading edge in Table S6).

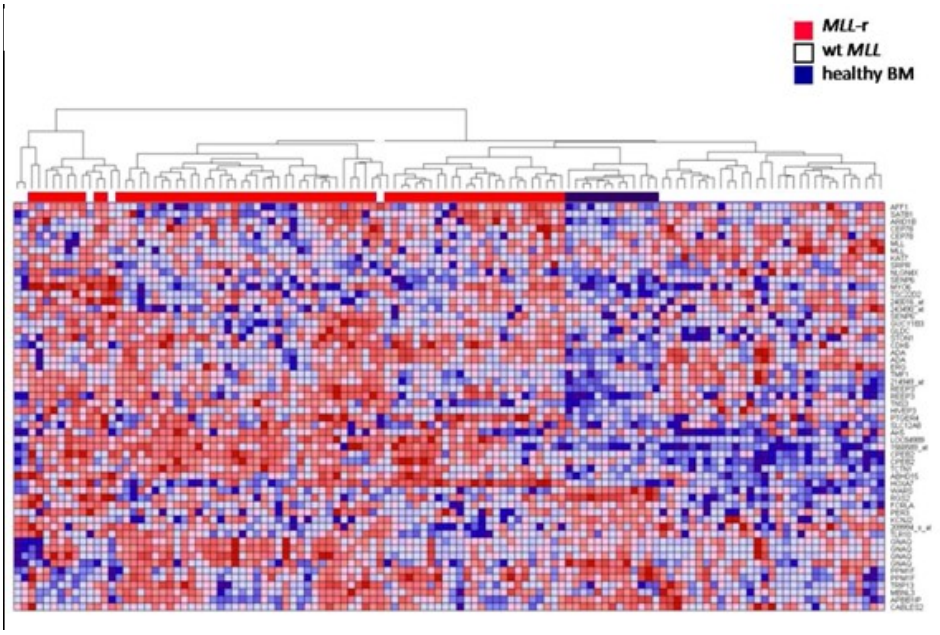

Figure 4. Genes responsive to the repression of MLL-ENL and MLL-AF4 accurately characterize primary *MLL*-rearranged infant ALL samples. Heatmap visualization and hierarchical clustering of primary *MLL*-rearranged infant ALL samples (*MLL*-r, red, n=71), wild-type *MLL* pediatric ALL samples (both infants (n=20) and children >1 year of age (n=16)) (wt *MLL*, blue, n=36), and whole bone marrow samples derived from healthy children (healthy BM, dark blue, n=13) based on the 56 differentially expressed probe sets recurrently affected in both KOPN-8, RS4;11, and SEMK2 cells upon *MLL* fusion knock-down. Up-regulated genes are depicted in red, down-regulated genes are depicted in blue.

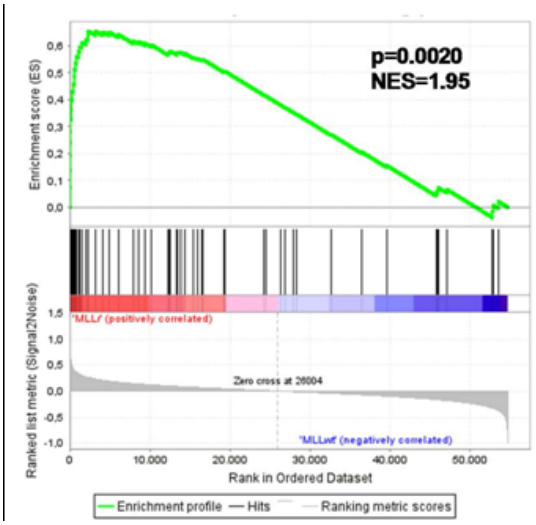

Figure 5. Gene set enrichment analysis of genes responsive to the repression of MLL-ENL and MLL-AF4. Gene set enrichment analysis (GSEA) of the 57 probe sets that are significantly lower expressed upon knock-down of the *MLL* fusion in gene expression profiles of *MLL*-rearranged patients. Probe sets and HGNC Gene Symbols of the gene set are listed in Table S5.

Pathway analysis using the database for annotation, visualization, and integrated discovery (DAVID) demonstrated a significant number of genes transcriptionally responsive to the loss of MLL fusion expression to be involved in the KEGG focal adhesion pathway (hsa04510, $p<0.0001$) and the KEGG small cell lung cancer pathway (hsa05222, $p=0.008$). Next, we used Ingenuity pathway analysis (IPA) to explore possible upstream regulators of the genes down-regulated upon the loss of MLL fusion expression. This revealed 30 potential regulators including 5 genes (i.e. *HOXA7*, *LIN28B*, *UPF1*, *EZH2*, and *MBD1*), and 25 miRNAs (see Table S7). Interestingly, the majority (i.e. 17 out of the 25) of the miRNAs that potentially

regulate the genes down-regulated upon *MLL* fusion knock-down, are predicted to target either *MLL* (i.e. *KMT2A*) or *AF4* (i.e. *AFF1*), or both. Hence, the genes observed to be transcriptionally responsive to the loss of *MLL* fusions, likely represent genes controlled by the MLL fusion itself.

Transcriptional consequences of AF4-MLL knock-down

Using siRNAs directed against *AF4-MLL* previously reported by Kumar *et al*. [13], we managed to reduce expression of *AF4-MLL* mRNA to ~40% in RS4;11 and ~53% in SEMK2 cells, as compared with cells transfected with control siRNAs (Figure 6A). In these experiments, the expression of wild-type *AF4* was not affected. However, despite numerous attempts, we were not able to prevent reduction of wild-type *MLL* expression to comparable levels of that of the *AF4-MLL* transcript (Figure 6A). Paired differential gene expression analysis between samples with a knock-down of *AF4-MLL* (n=6) and samples transfected with control siRNAs (n=6) revealed 80 differentially expressed probe sets ($p<0.001$). The same analysis comparing *AF4-MLL* knock-down samples with control cells electroporated in the absence of siRNAs (i.e. pulse control) (n=6), revealed 58 differentially expressed probe sets. A total of 36 overlapping probe sets (corresponding to 22 genes) were differentially expressed in both comparisons (Figure 6B). Probe set IDs, HGNC gene symbols, log-fold changes and p-values are listed in Table S8 and S9. Based on these 36 probe sets, hierarchical clustering could effectively separate *AF4-MLL* knock-down samples from control samples (Figure 6C). To validate the *AF4-MLL* knock-down signature, we compared our signature to a gene set published by Gaussmann *et al* consisting of *AF4-MLL* fusion target genes, [20] GSEA showed a significant enrichment of these *AF4-MLL* target genes in our *AF4-MLL* gene signature (NES=1.68, p=0.002) (Figure 7).

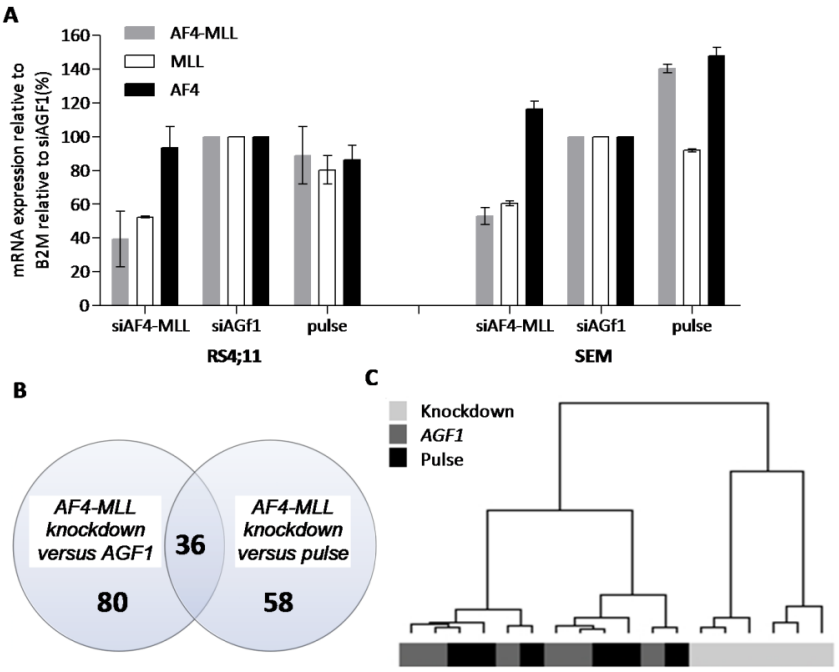

Figure 6. Differential gene expression in response to the repression of *AF4-MLL*. A. mRNA expression levels of *AF4-MLL* (grey), wild-type *MLL* (white), and wild-type *AF4* (black) in RS4;11 and SEMK2 cells after transfection with siRNAs directed against *AF4-MLL*, siRNAs against the leukemic fusion gene *AML1/MTG8* (si*AGF1*), or cells electroporated in the absence of siRNAs (pulse control). The average mRNA expression relative to the siAGF1 controls of two independent experiments ± standard error of the mean is shown. B. Venn diagram showing the number of differentially expressed genes ($p<0.001$) in RS4;11 and SEMK2 cells in which *AF4-MLL* was repressed (*AF4-MLL* knock-down, n=6), versus cells transfected with control siRNA (si*AGF1* control, n=6), and cells electroporated in the absence of siRNAs (pulse control, n=6). C. Hierarchical clustering based on 36 overlapping differentially expressed probe sets responsive to *AF4-MLL* repression in RS4;11 and SEMK2 (light grey), control samples transfected with si*AGF1* (dark grey), and pulse control samples (black).

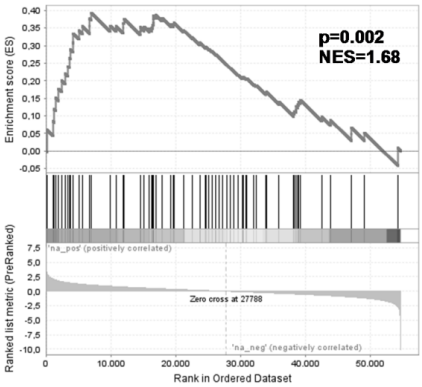

Figure 7. Differential gene expression in response to the repression of *AF4-MLL*. Gene set enrichment analysis (GSEA) of *AF4-MLL* associated transcription factors (Gaussmann *et al* [20]) in *AF4-MLL* knock-down ('na_neg') versus control samples ('na_pos').

Using our previously published gene expression profiling data, we compared t(4;11)-positive infant ALL samples which do and do not express the reciprocal *AF4-MLL* fusion product, as determined by PCR analysis. This comparison revealed 403 probe sets differentially expressed between both patient groups (p=0.01). Figure 8A shows a heatmap of the top 50 most significant differentially expressed probe sets. Based on these 50 probe sets, principal component analysis (PCA) showed a clear separation of both patient groups. Furthermore, we observed that the gene expression patterns of patients expressing the *AF4-MLL* fusion transcript were enriched for genes that were down-regulated after knock-down of the *AF4-MLL* in the cell line models RS4;11 and SEMK2 (GSEA; NES=1.67, p=0.02; leading edge in Table S12) (Figure 8B, upper panel). As a control we also analyzed enrichment of genes responsive to *AF4-MLL* knock-down in our *MLL-AF4* knock-

down signature, and found no significant enrichment (p=0.55) (Figure 8B, lower panel).

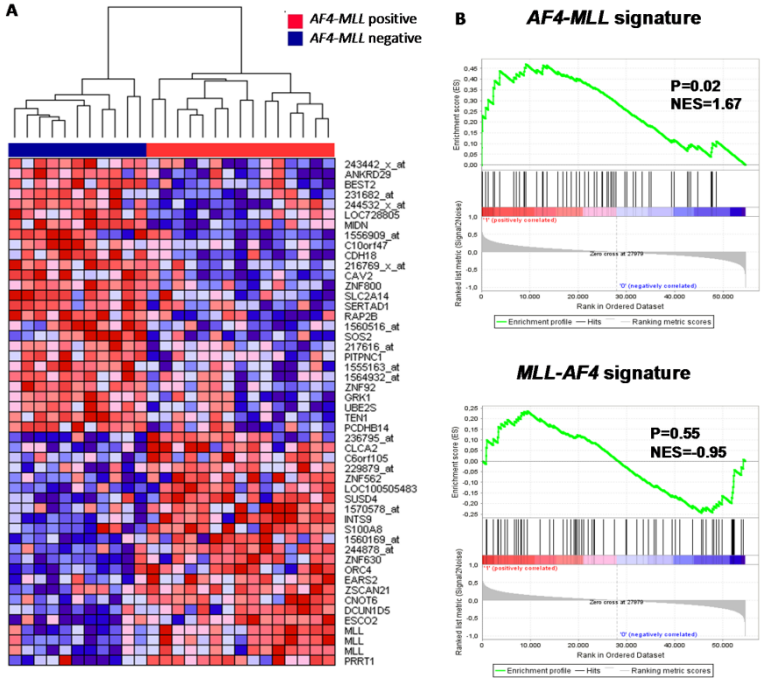

Figure 8. Genes responsive to *AF4-MLL* repression characterize *AF4-MLL* expressing (4;11)$^{+}$ infant ALL patients. A. Heatmap visualization and hierarchical clustering of primary t(4;11)+ infant ALL samples exhibiting *AF4-MLL* expression (n=15, red), or lacking *AF4-MLL* expression (n=11, blue), based on the top 50 most differentially expressed genes between both patient groups. Up-regulated genes are depicted in red, down-regulated genes are depicted in blue. B. Gene set enrichment analysis (GSEA) of AF4-MLL (upper panel) and MLL-AF4 (lower panel) target genes in *AF4-MLL* positive patients ('1') versus *AF4-MLL* negative t(4;11) patients ('0'). Probe sets and HGNC Gene Symbols are listed in Table S10 and S11.

Genes up-regulated after MLL fusion knock-down are enriched for hypermethylated promoter regions

Interestingly, while several known *MLL-AF4* target genes were down-regulated after the loss of the *MLL* fusion, a substantial proportion of genes in our *MLL* fusion knock-down signature were up-regulated (Figure 2). Per definition these genes are not directly regulated by the MLL fusion protein via H3K79 mediated transcription activation. Therefore, we explored an alternative mechanism of transcriptional regulation. We recently demonstrated that *MLL*-rearranged infant ALL is characterized by severe aberrant DNA hypermethylation, leading to transcriptional silencing of numerous genes. [10] Using 165 probe sets associated with gene promoter methylation in the majority of *MLL*-rearranged infant ALL patients, we applied GSEA on our *MLL* fusion knock-down signatures. GSEA demonstrated significant enrichment of these genes among the genes up-regulated after knock-down of *MLL-AF4* and *MLL-ENL* (NES=1.36, p=0.03; leading edge in Table S13) (Figure 9). These data suggest that there is an active interplay between MLL fusion proteins and DNA methylation patterns in *MLL*-rearranged ALL cells.

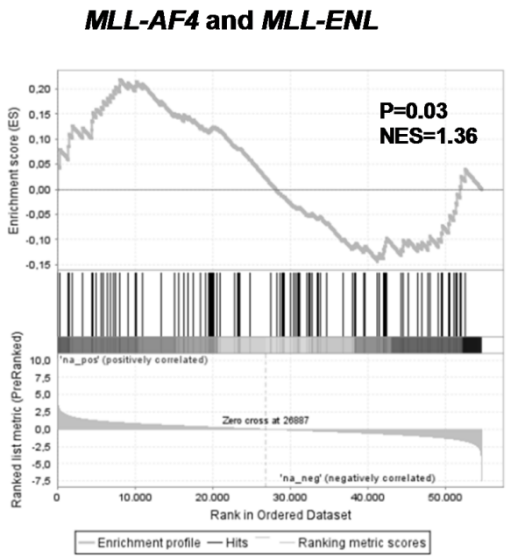

Figure 9. Genes up-regulated in response to *MLL-AF4* and *MLL-ENL* repression include genes normally silenced by promoter methylation in *MLL*-rearranged infant ALL. Gene set enrichment analysis (GSEA) of hypermethylated promoter regions in t(4;11) and t(11;19) patients (Stumpel *et al* [10]) in *MLL* fusion positive ('na_pos') versus *MLL* fusion negative ('na_neg') samples.

DISCUSSION

The here identified genes which are transcriptionally responsive to the repression of MLL-AF4 and MLL-ENL represent a rich source of potential therapeutic targets for *MLL*-rearranged acute leukemia. Apart from gene signatures associated with the loss of MLL-AF4 and MLL-ENL, we also identified genes responsive to the repression of AF4-MLL. As it has been suggested that the *AF4-MLL* oncogene could be indispensable for the initiation of t(4;11)$^{+}$ leukemias, our gene signatures associated with the presence and loss of *AF4-MLL* may well provide novel insights into the biology of this leukemia. Apart from genes down-regulated upon the loss of *MLL* fusions, we also identified a substantial number of genes which were up-regulated in response to *MLL* fusion repression. Although MLL fusion proteins activate a variety of target genes (represented in the datasets of Guenther [7, 8] and Krivtsov [7, 8]) by the recruitment of DOT1L and subsequent methylation of H3K79 [7, 8], these data suggest that the MLL fusion itself and/or its activated target genes also actively repress and activate other genes via alternative mechanisms. For instance, we found significant enrichment of the genes activated after knock-down of the *MLL* fusions in our previously published gene signatures associated with promoter hypermethylation in t(4;11)$^{+}$ and t(11;19)$^{+}$ infant ALL samples. [10] This underscores the importance of the role of DNA methylation in *MLL*-rearranged infant ALL, as it appears, to some extent, to be influenced by the presence of the MLL fusion.

Yet, the data presented here should be interpreted with caution as we were not able achieve complete repression of the *MLL* fusions, which may have affected the results. On the other hand, a full knock-down of the *MLL* fusion may not have provided better data per se, as that may have generated more non-specific effects

due to enhanced apoptosis induced by the loss of MLL-AF4. [13, 17] Other points of concern may be the slight down-regulation of wild-type *AF4* in SEMK2 cells transfected with siRNAs against *MLL-AF4*, as well as the fact that siRNAs directed against AF4-MLL also affected wild-type *MLL* expression. A possible explanation for this phenomenon is that *MLL* and *AF4* are downstream effectors of the fusion protein. However, we confirmed significant enrichment of differentially regulated genes upon repression of the different MLL fusions in recently published gene signatures consisting of MLL-AF4 target genes (7,8), and a gene signature associated with the loss of AF4-MLL. (17) Moreover, we also demonstrate that our identified genes accurately characterize *MLL*-rearranged ALL patient samples. Furthermore, Ingenuity pathway analysis (IPA) suggested that potential regulators of our gene signature consisting of genes transcriptionally responsive to the knock-down of MLL fusion genes, involve several miRNAs that supposedly target either *MLL* (i.e. KMT2A) and/or *AF4* (i.e. AFF1). This again implies that our gene signatures indeed consist of genes controlled by MLL fusion proteins. Therefore we believe that the obtained data is valid and informative, despite the limited levels of knock-down and the induced suppression of either AF4 or MLL.

Among the here observed genes that are down-regulated after knockdown of the MLL fusions we found several genes that potentially play important roles in leukemia maintenance and/or leukemogenesis. For instance, cyclin-dependent kinase 6 (*CDK6)*, which was identified as one of the *MLL* fusion target genes, as the genomic region encompassing *CDK6* revealed enhanced occupancy of both MLL-AF4 and H3K79 dimethylation.[8] We recently reported data showing the important role of CDK6 in the proliferation of *MLL*-rearranged ALL cells, demonstrating experimentally that inhibition of CDK6 readily induces impairment of leukemic cell proliferation. [21] Other genes in our core signature of genes

transcriptionally responsive to the loss of MLL fusion expression are potentially important in leukemogenesis. For example, high expression of the Ets family transcription factor ERG, which is down-regulated after MLL fusion knock-down, is associated with a poor clinical outcome in both acute myeloid leukemia (AML) and T-cell acute lymphoblastic leukemia (T-ALL). [22, 23] Moreover, enforced ERG expression induces both T-ALL and AML in murine models [24, 25], suggesting that ERG contributes to leukemia development. As the inhibition of ERG could possibly benefit the survival of *MLL*-rearranged ALL patients as well as patients suffering from AML and T-ALL, this gene represents an interesting candidate target gene for therapeutic intervention. More genes that are down-regulated after knockdown of the MLL fusions, and that have been associated with oncogenesis include SATB1 [26-28], KAT7 [29-31], ADA [32], PPM1F [33, 34], and HOXA7 [35].

Likewise, potential therapeutic targets can also be found among genes that are up-regulated upon knock-down of the MLL fusions. Among these genes we found regulator of G-protein signaling protein-2 (*RGS2*), which has been demonstrated to contribute to myeloid differentiation and its repression is considered to be an important event in leukemic transformation of FLT3-ITD$^+$ AML. [36] Moreover, RGS2 functions as a tumor suppressor in various human cancers. [37-39] Hypothetically, induction of this protein may suppress *MLL*-rearranged ALL progression.

In conclusion, we strongly believe that the genes identified in the present study represent genes which directly and readily respond to the loss of the MLL-AF4, MLL-ENL, or AF4-MLL, and that these genes potentially include attractive therapeutic targets and provide important insights into the biology underlying *MLL*-rearranged acute leukemias.

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CHAPTER 7

MLL fusion driven activation of CDK6 potentiates proliferation in MLL-rearranged infant ALL

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ABSTRACT

Acute lymphoblastic leukemia in infants (<1 year of age) is characterized by a high incidence of *MLL* rearrangements. Recently, direct targets of the MLL fusion protein have been identified. However, functional validation of the identified targets remained unacknowledged. In this study we identify *CDK6* as a direct target of the MLL fusion protein and an important player in the proliferation advantage of *MLL*-rearranged leukemia. *CDK6* mRNA was significantly higher expressed in *MLL*-rearranged infant ALL patients compared with *MLL* wild-type ALL patients ($p<0.001$). Decrease of *MLL-AF4* and *MLL-ENL* fusion mRNA expression by siRNAs resulted in down-regulation of *CDK6*, affirming a direct relationship between the presence of the *MLL* fusion and *CDK6* expression. Knockdown of *CDK6* itself significantly inhibited proliferation in the MLL-AF4 positive cell line SEM, whereas knockdown of the highly homologous gene *CDK4* had virtually no effect on the cell cycle. Furthermore, we show *in vitro* sensitivity of *MLL*-rearranged leukemia cell lines to the *CDK4*/6-inhibitor PD0332991, inducing a remarkable G1 arrest, and down-regulation of its downstream targets *pRB1* and *EZH2*. We therefore conclude that *CDK6* is indeed a direct target of MLL fusion proteins, playing an important role in the proliferation advantage of *MLL*-rearranged ALL cells.

INTRODUCTION

Obtaining successful treatment results in infant (<1 year of age) acute lymphoblastic leukemia (ALL) remains a major challenge in pediatric cancer. Infant ALL is characterized by chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene,[1] which occur in approximately 80% of the cases.[2] As a result of such translocations, the N-terminal portion of the *MLL* gene becomes fused to the C-terminal region of one of its many translocation partner genes.[3] The most recurrent *MLL* translocations found 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, respectively. To date, long term event-free survival (EFS) rates for *MLL*-rearranged infant ALL range between ~30% and 50%, depending on the treatment protocol.[2] In infant ALL, the presence of *MLL*-rearrangements, and young age are the strongest predictors of an unfavorable outcome.[2] Patients diagnosed with ALL below the age of 1 month have a 5-year overall survival of ~17%.[5]

MLL-rearranged ALL cells display unique gene expression signatures.[6, 7] Although this embodied an important finding, the overwhelming number of differentially expressed genes[8] made it difficult to distinguish between the actual "drivers" of the leukemia, and by-stander effects. Recently, however, the biology underlying MLL fusion driven regulation of gene expression became better understood. *MLL*, a histone methyltransferase, normally catalyzes trimethylation of the fourth residue of histone 3 (H3K4me3).[9, 10] In contrast, MLL fusion proteins have lost this ability.[11] Nonetheless, most of the recurrent MLL translocation partners, like AF4, ENL, and AF9, are able to connect to another histone methyltransferase, i.e. DOT1L. Consequently, MLL fusion proteins recruit DOT1L, which leads to dimethylation of lysine 79 on histone 3 (H3K79me2) instead of H3K4me3.[12-15] Both

H3K4me3 and H3K79me2 are associated with transcriptional activation.[16] Hence, genomic regions displaying aberrant H3K79me2 enrichment are prone to mark genes transcriptionally activated by the MLL fusion protein itself.[17] With this in mind, Guenther *et al* and Krivtsov *et al* independently identified signatures consisting of such genes, including for instance *MEIS1*, *FLT3*, and several *HOXA*-cluster genes.[17-19] Strikingly, based on H3K79 methylation profiles alone, *MLL*-rearranged ALL could be distinguished from other ALL subtypes lacking *MLL* rearrangements.[19] Thus, we now have access to smaller and more specific gene sets consisting of genes abnormally regulated by the *MLL* fusion protein itself. However, the question remains which of these genes contributed the most to leukemogenesis and/or leukemia maintenance. From this perspective, we here studied one of the identified MLL fusion target genes, i.e. *CDK6* (encoding human cyclin-dependent kinase 6).[18] Like its functional homologue CDK4, CDK6 is a serine/threonine kinase that is activated upon association with D-type cyclins (i.e. cyclin D1, D2 and D3) during the G1 phase of the cell cycle.[20] Activation of this complex leads to partial inactivation (by phosphorylation) of retinoblastoma protein 1 (RB1),[21] allowing progression to the S phase.[22] Activation of CDK4 and CDK6 can be prevented by complex formation with cyclin-dependent kinase inhibitors CDKN2A and CDKN2B. In human cancers, deregulation of the cell cycle is often mediated by alterations in CDK activity (including that of CDK4 and CDK6), inducing unscheduled proliferation as well as genomic and chromosomal instability.[23, 24] For instance, *CDK4* or *CDK6* overexpression has been demonstrated in several malignancies, including certain types of leukemia and lymphomas.[25]

As *CDK6*, but not *CDK4*, has been proposed as a direct target of MLL fusion proteins, we postulated that *MLL*-rearranged ALL cells may gain a proliferative advantage from MLL fusion driven up-regulation of *CDK6*. As a major complication

in studying MLL-rearranged ALL is that there are no human models recapitulating the presence of the MLL-AF4 fusion protein.[26-29] Therefore, we set out to explore to what extent *MLL*-rearranged ALL cells are dependent on CDK6 in terms of cell proliferation using the MLL-AF4 fusion protein positive cell line SEM.

MATERIALS AND METHODS

RNA extraction and cDNA synthesis

Total RNA was extracted from a minimum of $2x10^6$ cells using TRIzol reagent (Invitrogen, Life Technologies, Breda, The Netherlands) according to the manufacturer's guidelines. The quality of the extracted RNA was assessed on 1.5% agarose gels and cDNA was prepared for quantitative real-time PCR analysis as described earlier.[8]

Gene expression data

Bone marrow or peripheral blood samples were used from untreated infants (younger than 1 year) diagnosed with ALL included in the INTERFANT-99 treatment protocol.[2] For all primary patient samples used in this study, approval was obtained from the Erasmus MC Institutional Review Board, and authorization was acquired from the parents or legal guardians of the children via informed consent in accordance with the Declaration of Helsinki. All samples were processed within 24 hours after sampling as described recently.[30] All leukemia samples used in this study contained more than 90% leukemic cells. Total RNA was synthesized into biotinylated cRNA. Labeled cRNA was then fragmented and hybridized to HU133plus2.0 GeneChips (Affymetrix) according to the manufacturer's guidelines. The infant ALL gene expression data presented in this study have been deposited in National Center for Biotechnology Information Gene Expression Omnibus[31] and is accessible via GEO Series accession number GSE19475,[8] and the pediatric precursor BCP-ALL profiles were deposited as GSE13351.[32]

Quantitative real-time PCR analysis

CDK4, CDK6, MLL-AF4 and MLL-ENL mRNA expression levels were determined by quantitative real-time PCR analysis[33] using the DyNAmo SYBR Green qPCR kit (Finnzymes, #F-400) as described before. Oligonucleotide primers used for PCR amplification were purchased from Eurogentec. Primer sequences were as follows: CDK4 forward: 5'-TGGGCAGAAGTCTGTTTT-3' and reverse: 5'-GGAGGGGAATGTCATTAAG-3'; CDK6 forward: 5'-ACTGCCAAGAACTATGACTGT-3' and reverse: 5'-CTGCTGGGATTTGTTTTATT-3'; MLL-AF4 forward: 5'-CCCCGCCCAAGTATC-3', reverse: 5'-GGCGGCCATGAATG-3'; MLL-ENL forward: 5'-CCCCGCCCAAGTATC-3', reverse: 5'-GCTCGAAGTCTGAGTCTGA-3'. B2M was used as a reference gene: forward: 5'-GGAGCATTCAGACTTGTCTT-3', reverse: 5'-ATGCGGCATCTTCAAA-3'.

Cell culturing and in vitro sensitivity testing

The ALL cell lines SEM, BEL-1, RS4;11 (translocation t(4;11)-positive; generating fusion protein MLL-AF4), and KOPN-8 (translocation t(11;19)-positive generating the MLL-ENL fusion protein), were maintained as suspension cultures in RPMI 1640 with glutamax (Invitrogen, #61870036) supplemented with 10% (v/v) FCS and 2% penicillin/streptomycin/fungizone (PSF; Invitrogen, #15140-122) at 37°C in humidified air-containing 5% CO^2. In vitro cytotoxicity to PD0332991 (Pfizer) was determined by 4-day MTT assays as described before.[34] Drug exposure treatment was performed using 1 μM PD0332991 for 48 hours under standard culture conditions, unless mentioned otherwise.

ChIP and ChIP-seq procedure

ChIPs on SEM cells ($50x10^6$) were performed using the SimpleChIP Enzymatic Chromatin IP kit (Cell signaling technology, #9003) according to the manufacturer's instructions with minor modifications. The cells were fixed in RPMI medium containing 1% formaldehyde with gentle rotation for 10 minutes at room temperature and the reaction was stopped by glycine quenching (125mM final concentration). Nuclei were collected and digested with micrococcal nuclease (2µl, provided by the SimpleChIP kit) followed by 8´ of sonication (8 cycles of 30´´ of sonication and 30´´ without sonication) using a BioRuptor UCD-200 (Diagenode). Pull downs were performed on DNA fragments (ranging from 100 to 600 bp) using antibodies against N-terminal MLL (Bethyl laboratories inc., #A300-086A), C-terminal AF4 (AbCam. #ab60054), H3K4me3 (Millipore, #cs200554) and H3K79me2 (AbCam, #ab3594). ChIP-Seq samples were sequenced (36 bp reads) on the illumine GII platform and analyzed by NARWHAL.[35] The data was visualized using the UCSC genome browser (hg19).

DNA content and apoptosis measurement

DNA content (i.e. cell cycle distribution) measurement was done using the CycleTEST PLUS DNA Reagent Kit (BD Biosciences, #340242). $0.5x10^6$ cells were treated with 225 µl of solution A (containing trypsine) for 5' at room temperature, 175 µl of solution B (containing trypsine inhibitor) for 10' at room temperature, and 175 µl of solution C (containing propidium iodide (PI) and spermine tetrahydrochloride) for 15' on ice.

Induction of apoptosis was assessed by an Annexin V/PI assay. For this, $0.2x10^6$ cells were incubated in annexin binding buffer (10 mM HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$) and centrifuged at 1500 RPM at 4°C for 5'. Buffer was

removed and a solution of Annexin V (1:1000) and PI (2 µg/ml) (BD Biosciences, #556570) was added to the cell culture.

For both assays, detection of positive staining was performed on a FACSCalibur (Becton Dickinson) flow cytometer.

Transfection with siRNA

Leukemic cells ($4x10^6$) were transfected with specific siRNAs by electroporation in 4 mm electroporation cuvettes (Bio-Rad Laboratories, #1652081) containing 400 µL of RPMI medium supplemented with 10% fetal calf serum, in the presence of 10 µL of siRNAs (20 µM) directed against CDK4, CDK6 (ON-TARGET plus SMARTpool, Dharmacon), MLL-AF4, or AML1-MTG8 fusion protein (AGF1) as nonsense control (as described previously[36]), or in the presence of 50 µL of siRNAs (20 µM) directed against MLL-ENL: sense 5'-CCAAAAGAAAAGUCUGCCCAG-3; antisense 5'-CUGGGCAGACUUUUCUUUUGGUU-3'. For the latter experiment, control cells were transfected with 50 µL siAGF1 (20 µM). Electroporation was carried out applying a rectangular pulse of 300 V for 10 milliseconds, using a Gene Pulser MXcell Electroporation System (Bio-Rad Laboratories). After incubating for 15 minutes at room temperature, the cells were diluted to $1x10^6$ cells/ml and cultured under standard culture conditions. Transfection of siMLL-ENL and siMLL-AF4 was repeated after two days to reach maximum knock-down and cells were harvested and RNA isolated after 96 hours. Transfection experiments were repeated multiple times with similar results to those published in this manuscript.

Western blot

Cell pellets were snap-frozen in liquid nitrogen and stored at −80°C until further use. After thawing, cells were resuspended in 50 µL of lysis buffer containing: 5

mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate (Merck), 25 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM glycerolphosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 10 mM sodium fluoride (Sigma-Aldrich) and 50 µM of freshly prepared sodium pervanadate. Cells were lysed for 30' on ice. After lysing cells were centrifuged for 15' at 13.000 rpm and 4°C. Protein concentration was determined by BCA protein assay (Pierce Biotechnology, #23225) with different concentrations of bovine serum albumin as standards. Cell lysates containing 25 µg of protein were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell). Western Blots were probed with mouse anti-CDK4, anti-CDK6, anti-pRB1 S608 and anti-EZH2 (Cell Signaling, #2906, #3136, #2181, and #4905) and mouse anti-beta actin (as a control for equal loading) (Abcam) for 2 hours at room temperature. Next the western blots were incubated in fluorescently labeled secondary antibodies (LI-COR Biosciences) for 1 hour and subsequently detected using the Oddyssey Infrared Imaging System (LI-COR Biosciences).

RESULTS

The CDK6 locus is occupied by MLL and AF4, and associated with H3K79me2

Confirming recent observations[18, 19] we validated binding of the MLL-AF4 fusion protein at the genomic localization of *CDK6*. For this, ChIP-sequencing analyses was performed on chromatin precipitations (using antibodies against MLL, AF4, H3K4me3, and H3K79me2) obtained from MLL-AF4 positive SEM cells. We found pronounced co-occupancy of both the N-terminal domain of MLL and the C-terminal domain of AF4 at the transcriptional start site of *CDK6*. In contrast, at the *CDK4* locus, binding of MLL and AF4 appeared largely absent, suggesting MLL-AF4 occupancy at the *CDK6*, but not the *CDK4* locus (Figure 1, upper panel). Moreover, the presence of MLL and AF4 at the genomic *CDK6* locus appeared to be associated with both H3K4me3, as well as H3K79me2, whereas the *CDK4* locus did not seem to be marked by these histone modifications (Figure 1, lower panel). In addition, these histone marks were present on broad domains throughout CDK6. Similar spreading of both histone modifications at key leukemia and stem cell-associated genes has been described by Guenther et al.[18]

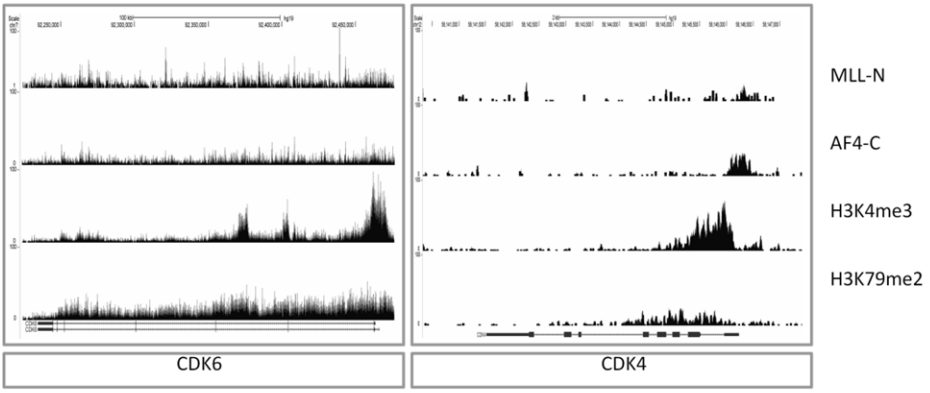

Figure 1. *CDK6* is marked by occupation of MLL, AF4, H3K79me2 and H3K4me3 in SEM. Graph visualizing number of annotated 36 base pair reads obtained from ChIP-seq analysis at the CDK6 locus (left) and CDK4 locus (right) using antibodies against the N-terminal domain of MLL and the C-terminal domain of AF4 (upper panel), H3K4 trimethylation and H3K79 dimethylation (lower panel) in MLL-AF4 positive cell line SEM. An additional 1kb upstream and downstream of CDK4 and an additional 10kb upstream and downstream of CDK6 is given.

Elevated CDK6 expression in MLL-rearranged infant ALL

Using our recently published gene expression profiling data (Affymetrix HU133plus2.0 GeneChips),[8] we evaluated *CDK6* and *CDK4* expression levels in *MLL*-rearranged infant ALL. *CDK6* appeared significantly higher expressed in the *MLL*-rearranged infant ALL patients when compared with infant ALL patients ($p=0.001$), pediatric non-infant (>1 year of age) precursor BCP-ALL patients ($p<0.0001$) carrying wild-type *MLL* genes, or healthy bone marrow samples ($p<0.0001$) (Figure 2A; left panel). All probe sets of *CDK6* had an FDR-adjusted p-value of 0.002 or smaller (Table 1).

ID	logFC	T	P.Value	adj.P.Val	B
224848_at	1.035591	7.564293	7.52E-12	3.29E-09	16.59254
243_g_at	0.546159	7.028207	1.22E-10	3.11E-08	13.91603
224847_at	0.892852	7.00689	1.36E-10	3.40E-08	13.81122
235287_at	1.072996	6.819905	3.53E-10	7.54E-08	12.89764
224851_at	0.961323	6.218592	6.99E-09	8.41E-07	10.03769
231198_at	0.566643	5.643918	1.07E-07	7.03E-06	7.433588
207143_at	0.573842	4.655778	8.15E-06	0.000215	3.320232
214160_at	0.438097	3.898921	0.000157	0.002164	0.547444

Table 1. All CDK6 probe sets significantly higher expressed in MLL-r infant ALL patients. Table representing all probe sets of CDK6 on the HGU133plus2.0 microarray with results from linear modelling for microarrays (LIMMA) searching for differentially expressed probe sets between MLL-rearranged infant ALL patients (n=68) and MLL wild-type samples (consisting of MLL wild-type infant ALL patients (n=18), pediatric (non-infant) BCP-ALL patients (n=16) and healthy bone marrow samples n=13). MLL-r = MLL-rearranged; ID = probe set; logFC = estimate of the log2-fold-change; T = moderated T-statistic; P.Value = raw p-value; adj.P.Val = FDR-adjusted p-value or q-value; B = log odds.

In contrast to *CDK6*, *CDK4* expression was not upregulated in *MLL*-rearranged infant ALL compared to infant ALL with wild-type MLL (Figure 2B; left panel). Among *MLL*-rearranged infant ALL patients, *CDK6* expression is higher than the expression of *CDK4* ($p<0.0001$) (Figure2A-B). The downstream CDK4/CDK6 target *RB1* also appeared significantly higher expressed in *MLL*-rearranged infant ALL patients when compared with pediatric precursor BCP-ALL patients and healthy bone marrow samples ($p<0.0001$). However, *RB1* expression levels in *MLL* wild-

type infant ALL patients appeared comparable to that of the *MLL*-rearranged infant ALL cases (Figure 2C).

Figure 2. CDK6 mRNA expression values are higher in MLL-rearranged infant ALL patients. Graphical representation of VSN-normalized expression values of CDK6 mRNA (224848_at) (A), CDK4 mRNA (202246_s_at) (B), and RB1 mRNA (203132_at) (C) from gene-expression profiling data (Affymetrix HU133plus2 GeneChips) in patient material. CDK6 is significantly higher expressed in MLL-rearranged infant ALL patients (MLL-r infant ALL, n=68) compared to MLL wild-type infant ALL (MLL wt infant ALL, n=18), pediatric (non-infant) precursor BCP-ALL patients (pre-BCP-ALL, n=16) and healthy bone marrow (healthy bone marrow, n=13). CDK4 is only significantly higher expressed in MLL-rearranged infant ALL patients compared to pediatric BCP-ALL patients. RB1 is significantly higher expressed compared to pediatric BCP-ALL patients and healthy bone marrow samples, but not compared to wild-type MLL infant ALL patients. The differences between patient groups were statistically analyzed using the Mann-Whitney U test. Probe sets with the largest difference between MLL-rearranged and MLL wild-type are shown in the graph; all probe sets of CDK6 had an FDR-adjusted p-value of 0.002 or smaller (Supplemental Table 1). Error bars represent the mean ± standard error of the mean.

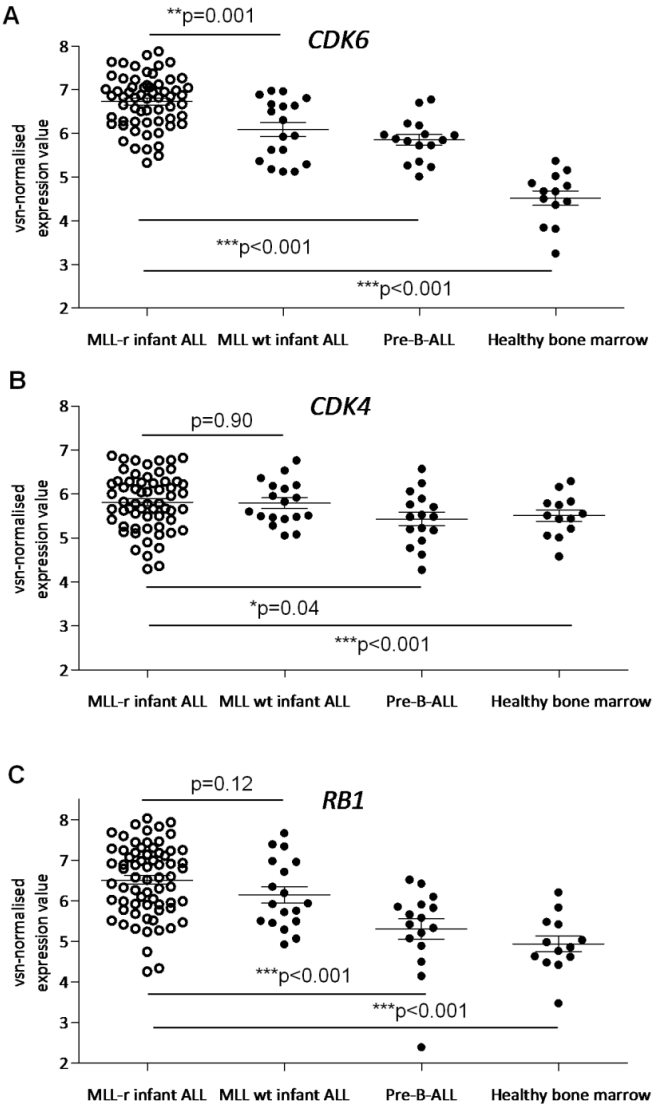

Figure 2. CDK6 mRNA expression values are higher in MLL-rearranged infant ALL patients.

WBC higher in patients with high CDK6 mRNA expression

Although the white blood cell (WBC) count at diagnosis obviously is subjected to many variations, this parameter may reflect some degree of leukemic cell proliferation and disease aggressiveness. For instance, among infant ALL patients, high WBC counts are highly characteristic and represents an independent predictor of an adverse outcome.[2] Thus, with WBC counts as a suggestive marker for leukemic blast proliferation, we determined whether high-level *CDK6* expression was associated with increased WBC at diagnosis. For this, we compared *MLL*-rearranged infant ALL patients with a "low" (< median) and "high" (≥ median) *CDK6* expression, and found that patients expressing high levels of *CDK6* presented with significantly higher WBC counts at diagnosis (p=0.027) (Figure 3, left panel). A similar association with the expression of *CDK4* was not observed (Figure 3, right panel).

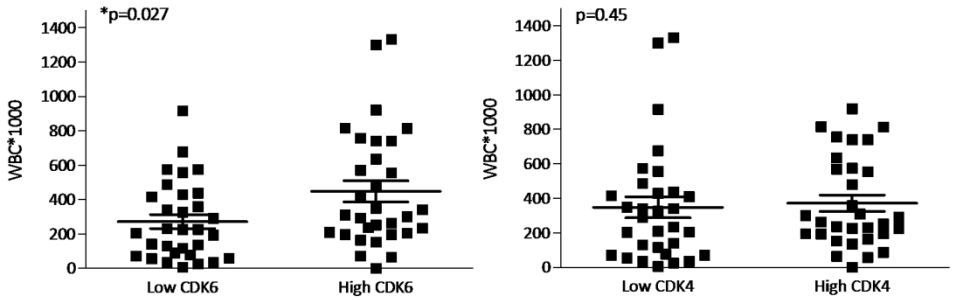

Figure 3. WBC count is higher in patients with high CDK6 mRNA expression. Graph shows white blood cell (WBC) count (X-axis) in MLL-rearranged patients with low (<median, n=31) and high (≥median, n=31) expression of CDK6 (left panel) and CDK4 (right panel). The difference between patient groups were statistically analyzed using the Mann-Whitney U test.

CDK6 expression is regulated by the MLL fusion

To confirm that *CDK6* indeed is regulated by the MLL fusion itself, we transfected the *MLL*-rearranged ALL cell lines KOPN-8 and SEM with siRNAs directed against *MLL-ENL* or *MLL-AF4* respectively. Transfection with si*MLL-ENL* in KOPN-8 resulted in a 92% decrease of the *MLL-ENL* transcript as compared with transfections with non-silencing siRNAs directed against *AGF1*. Similar experiments using si*MLL-AF4* in SEM cells showed a 67% reduction in *MLL-AF4* mRNA expression (Figure 4A). Knockdown of *MLL-ENL* and *MLL-AF4* led to pronounced decreases in *CDK6* expression in both KOPN-8 and SEM cells, whereas *CDK4* expression largely remained unaffected (Figure 4B). Similarly, upon knockdown of the MLL fusion, protein levels of CDK6 were affected to a greater extent than that of CDK4 (Figure 4C). Hence, these data are in line with our confirmatory ChIP-seq data (Figure 1), and clearly demonstrate that transcription of CDK6, but not CDK4, is driven by the MLL fusion itself.

Figure 4. CDK6 is downregulated after knockdown of MLL fusion by siRNA. Graphical representation of (A) expression values of MLL-ENL mRNA in KOPN-8 (left) and MLL-AF4 mRNA in SEM (right) after knockdown by siRNA of MLL-ENL and MLL-AF4 respectively (white bars) measured by RT-PCR relative to control siRNA AGF1 (siAGF1, grey bars); and (B) expression values of CDK6 mRNA (left) and CDK4 mRNA (right) in MLL fusion knockdown samples (white bars) measured by RT-PCR relative to control siAGF1 (grey bars). Expression of mRNA was measured relative to housekeeping gene B2M as loading control. Error bars represent the mean ± standard error of the mean. C. Protein levels of CDK4 and CDK6 after transfection with siRNA directed against MLL-ENL (right panel) and MLL-AF4 as shown by western blot (left panel) and graph visualizing quantification of western blot (right panel). β-actin is shown as loading control.

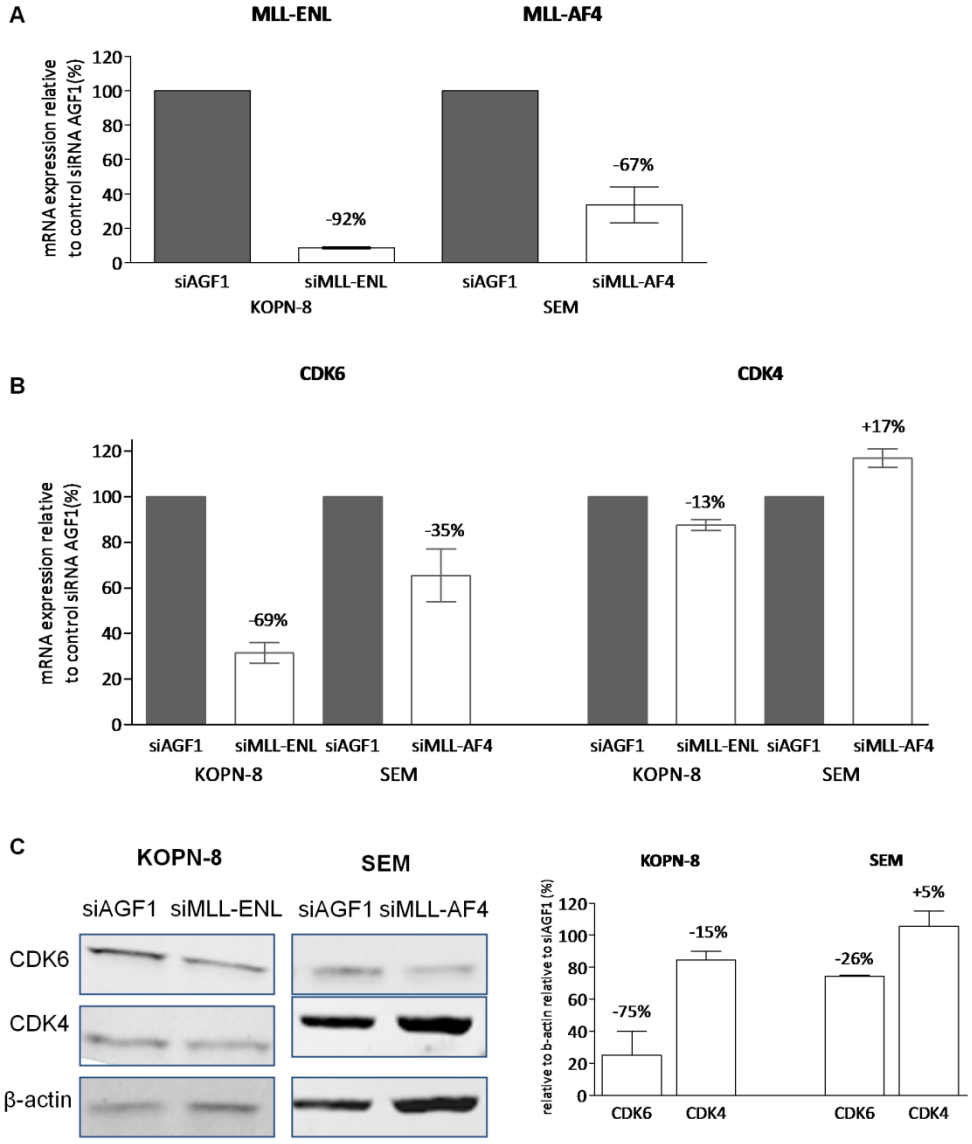

Figure 4. CDK6 is downregulated after knockdown of MLL fusion by siRNA.

CDK6 drives proliferation in MLL-rearranged ALL

To assess to what extent proliferation in *MLL*-rearranged ALL cells depends on the elevated expression of *CDK6*, we separately knocked down *CDK6* and *CDK4* in the t(4;11)-positive ALL cell line SEM. This led to the transient reduction of CDK6 and CDK4 protein expression to approximately 50% for 24 to 48 hours, whereupon protein expression was almost completely recovered (Figure 5A). Cell cycle analysis showed that suppression of CDK4 hardly affected proliferation. In contrast, knock-down of CDK6 led to marked increases in the number of cells present in the G0/G1 phase, at the expense of the percentage of cells in the S phase or G2/M phase (Figure 5B). Cell cycle analysis alternatively assessed by BrdU-staining at 48 hours after transfection, showed similar results with an increase in number of cells in the G1 phase and decrease of number of cells in the S phase after transfection with *CDK6*, while knockdown of *CDK4* practically had no effect on the cell cycle (Figure 5C). These data establish a role for CDK6, but not for its homologue CDK4, in the proliferation of *MLL*-rearranged ALL cells. Moreover, neither CDK4 nor CDK6 suppression affected cell viability as determined by Annexin V/PI-staining (Figure 5D).

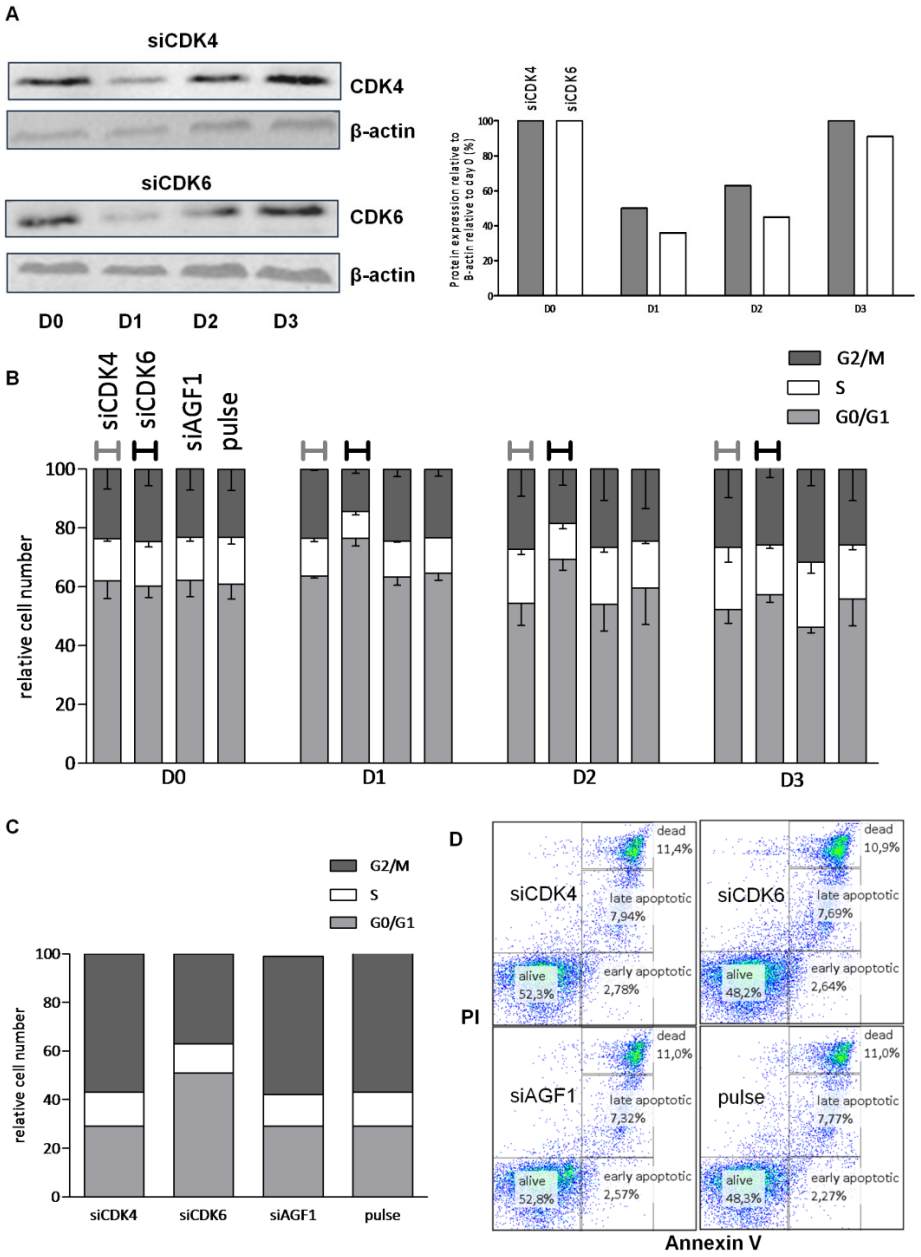

Figure 5. Proliferation of the *MLL*-rearranged cell line SEM depends on *CDK6* but not on *CDK4*.

Figure 5. Proliferation of the *MLL*-rearranged cell line SEM depends on *CDK6* but not on *CDK4*. A. Protein levels of CDK4 (upper panel) and CDK6 (lower panel) followed over three days (D0 to D3) after transfection with siRNA directed against CDK4 and CDK6 respectively as shown by western blot (left panel) and graphic visualization of quantification of western blot (right panel). β-actin is shown as loading control. B. Graph visualizing number of cells in the G0/G1 phase (grey), S phase (white) and G2/M phase (black) after knockdown by siRNA of CDK4 and CDK6 and transfection with siAGF1 and empty pulse control measured over four days (represented on the X-axis). Cell cycle phase assessment was done by DNA content measurement by means of proprium iodide staining quantified by flow cytometry. Bars represent mean - standard error of the mean. C. Graph visualizing cell cycle analysis results (G0/G1 phase, grey; S phase, white; G1/M phase, black) through bromodeoxyuridine (BrdU) staining quantified by flow cytometry at 48 hours after transfection with either siRNAs directed against CDK4, CDK6, nonsense target AGF1 and empty pulse in MLL-AF4 cell line SEM. D. Quantitative measurement of alive, early apoptotic, late apoptotic and dead cells 48 hours after transfection with siRNAs directed against CDK4 (upper left), CDK6 (upper right), nonsense target AGF1 (lower left) and empty pulse control (lower right) as measured by Annexin V (discriminating between alive and apoptotic/dead cells) on the X-axis and propidium iodide (PI, discriminating between apoptotic and dead cells) on the Y-axis.

In vitro sensitivity of MLL-rearranged ALL cell lines to PD0332991

Next we tested the *in vitro* sensitivity to the CDK4/CDK6 inhibitor PD0332991 of *MLL*-rearranged ALL cell lines SEM, KOPN-8, BEL-1, and RS4;11. All *MLL*-rearranged ALL cell lines tested appeared highly responsive to PD0332991 treatment, with IC_{50} values (i.e. the concentration required to inhibit 50% of the viable cells) ranging from ~0.08 – 8 μM (Figure 6).

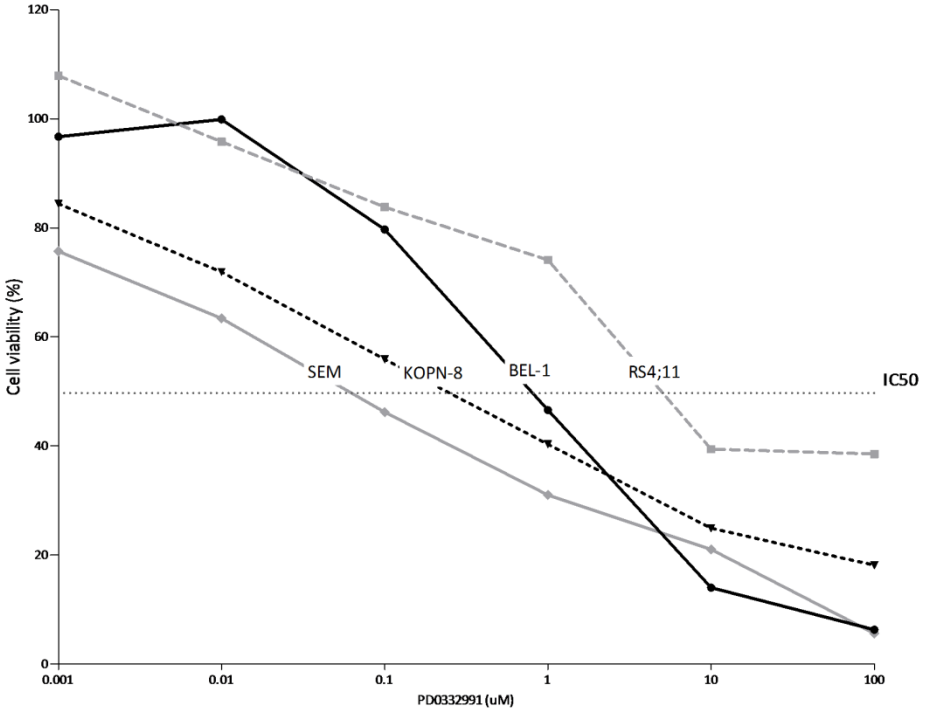

Figure 6. *In vitro* sensitivity of *MLL*-rearranged cell lines to *CDK4*/6-inhibitor PD0332991. Graph showing results of 4-day MTT assay with PD0332991 (in µM on X-axis) on MLL-rearranged ALL cell lines KOPN-8 (MLL-ENL), RS4;11, SEM and BEL-1 (MLL-AF4). Dotted line represents concentration needed to inhibit 50% of the viable cells (IC50).

Next we exposed t(4;11) SEM cells and t(11;19) KOPN-8 cells to 1 µM of PD0332991 for 48 hours. In SEM and KOPN-8 cells, this resulted in a considerable G1 arrest, almost doubling the percentage of cells residing in the G0/G1 phase, while the number of cells in the S phase dropped to less than 20% as compared to unexposed control cells (Figure 7). Likewise, the number of cells in G2/M phase was also markedly reduced.

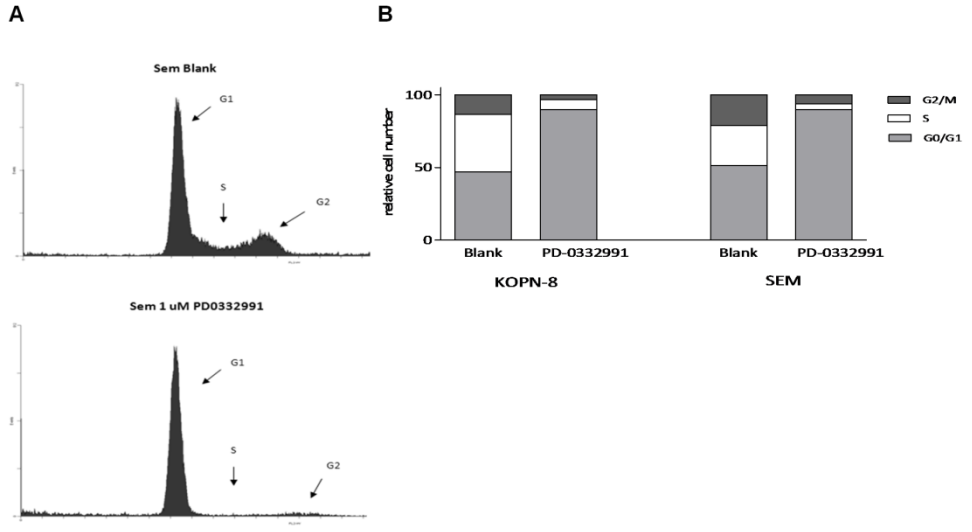

Figure 7. G1-arrest in MLL-rearranged ALL cell lines after PD0332991 treatment. A. Visualization of cell cycle phases as measured by DNA content through propidium iodide (PI) staining quantified by flow cytometry after exposure of MLL-AF4 positive cell line SEM to 1 µM PD0332991 (lower panel) and blank control (upper panel) for 48 hours. Cell cycle phases are pointed out by arrows in the graph. Both S phase and G2/M phase are significantly reduced, indicating a G1 arrest. B. Graphical representation of the number of cells that are in G0/G1 phase (grey), S phase (white) or G2/M phase (black) after treatment with 1 µM PD0332991 and blank control for 48 hours as measured by DNA content in KOPN-8 (left bars) and SEM (right bars). MLL-AF4 positive cell line SEM and MLL-ENL positive cell line KOPN-8 have a significant increase in number of cells in G0/G1 phase and decrease of cells in the S and G2/M phase.

PD0332991 inhibits downstream targets pRB1 and EZH2 in MLL-rearranged ALL cell lines

As pRB and EZH2 are downstream targets of CDK6, we determined protein levels of the pRB and EZH2 in the *MLL*-rearranged ALL cell lines SEM and KOPN-8 in the presence and absence of PD0332991 (1 μM). Exposure to PD0332991 did not affect CDK6 protein levels itself, which is hereditary to the working mechanism of PD0332991, but effectively suppressed RB1 phosphorylation and EZH2 expression in all three cell lines (Figure 8).

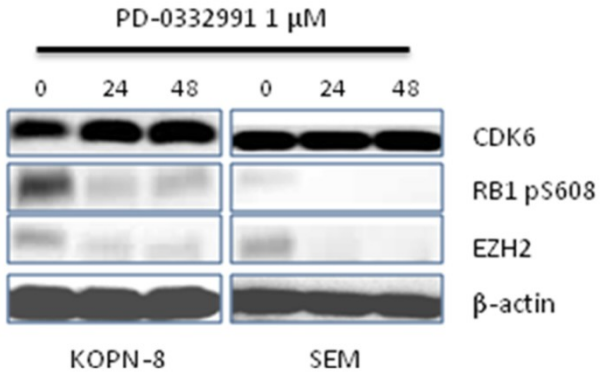

Figure 8. Treatment with *CDK4*/6-inhibitor PD0332991 affects downstream targets *p*RB and *EZH2*. Protein levels of CDK6, RB1 phosphorylation at serine 608 (pS608) and EZH2 in MLL-ENL positive cell line KOPN-8 and MLL-AF4 positive cell line SEM after 0 hours, 24 hours and 48 hours of treatment with 1 μM CDK4/6-inhibitor PD0332991 as shown by western blot. β-actin is shown as loading control.

DISCUSSION

We here confirm that, as proposed by previous reports, [18, 19] the cell cycle dependent kinase CDK6 represents a direct target of the MLL fusion protein in *MLL*-rearranged ALL. Furthermore, we show that the transcriptional activation of *CDK6* provides *MLL*-rearranged ALL cells with a proliferative advantage, which can effectively be counteracted by inhibiting CDK6. Hence, CDK6 may be an attractive therapeutic target for this aggressive type of leukemia, with the CDK4/CDK6 inhibitor PD0332991 as a potential active drug. Furthermore, this study demonstrates the importance of public databases (e.g. ChIP-seq and gene expression databases) for furthering research faster.

PD0332991 is a well-tolerated oral *CDK4*/6-inhibitor with myelosuppression[37] and neutropenia[38] as dose-limiting toxicities. Clinical benefit of PD0332991 has been shown with progression-free survival for over a year in 5 out of 17 mantle cell lymphoma patients[39], and partial response in patients with a progressive teratoma;[40] both tumors are characterized by high *CDK4* and *RB1* expression respectively. PD0332991 also significantly reduced tumor load in several Rb-positive human xenograft models *in vivo*.[41] In concordance with our results, Wang *et al*[42] showed that PD0332991 established a cell cycle block in AML cell line MV4;11, containing the MLL-AF4 fusion protein as well as an internal tandem duplication (ITD) of FLT3. They showed that CDK4/6 activation is a downstream effector of FLT3-ITD-mediated oncogenic pathways, and argue that sensitivity of MV4;11 to PD0332991 may be explained by activation of this pathway. Additionally we now argue that it is very well imaginable that MLL-AF4 is able to bind and upregulate CDK6 directly also in *MLL*-rearranged AML, and via this contributing to the sensitivity of MV4;11 to PD0332991. Further studies with

PD0332991 now are warranted to assess whether it can also effectively inhibit proliferation of *MLL*-rearranged ALL cells *in vivo*. Similarly to other inhibitors moving into the clinic, cautious testing of the effect on normal residual hematopoetic stem cells and progenitors is required due to the high bone marrow involvement of ALL.

Where CDK6 plays an important role in the proliferation of *MLL*-rearranged ALL, the highly homologous CDK4 does not seem to affect the proliferation to a great extent. Non-overlapping functional roles of CDK4 and CDK6 have been demonstrated earlier, and may be tissue-specific and depend on different complexes each kinase forms.[43-46] Also the present study suggests that CDK4 and CDK6 are not functionally redundant, with only CDK6 playing a critical role in the proliferation of *MLL*-rearranged ALL. Another possible explanation for a lack of effect on the proliferation after abrogation of CDK4 may lie in a compensatory mechanism, where the highly expressed CDK6 in *MLL*-rearranged ALL may possibly surmount the absence of CDK4.

Further, this study raises questions about the role of the polycomb group protein *EZH2* in *MLL*-rearranged leukemia. Although we demonstrate a significant decrease in the protein levels of *EZH2* and proliferation arrest after exposure to the *CDK4/6* inhibitor, recent studies in MLL-AF9 rearranged acute myeloid leukemia (AML) have shown that inactivation of this gene can compromise but not fully abrogate leukemic growth.[47, 48] This would suggest either the implication of other downstream targets of *CDK6* and the *RB1* gene, aspecific effects of PD0332991 or possibly a more important role of *EZH2* in *MLL*-rearranged ALL. Experiments using inactivation of *EZH2* in *MLL*-rearranged ALL could possibly shed more light on this specific issue.

There has been debate whether the “two hit” model – one hit activating an oncogene and one hit inactivating a tumor suppressor gene[49, 50] – applies to *MLL*-rearranged leukemia. Yu et al[51] already noted that the *MLL* rearrangement as a single event can result in two hits; an *MLL* fusion product with a partner gene could confer gain-of-function activity, and simultaneous *MLL* haploinsufficiency would contribute to the disordered cell fate of the leukemia. In this study we present an alternative way in which the *MLL* fusion confers gain-of-function activity; the *MLL* fusion upregulates expression of *CDK6* and by this drives the continued proliferation in *MLL*-rearranged leukemia.

In conclusion, *CDK6* is a direct target of the MLL fusion protein and plays an important role in the proliferation advantage of *MLL*-rearranged ALL cells. Additionally we show that the proliferation advantage of *MLL*-rearranged ALL gained by *CDK6* can be effectively targeted *in vitro* by the *CDK4/6* inhibitor PD0332991.

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CHAPTER 8

EID1 plays a critical role in the growth of *MLL*-rearranged ALL

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ABSTRACT

Introduction: Acute lymphoblastic leukemia in infants is characterized by a poor prognosis and a high incidence of *MLL* rearrangements. Infant ALL has been recognized to generate distinct gene expression profiles. In search for new treatment targets, we analyzed these gene expression signatures for differential expression. *Results:* EP300-interacting inhibitor of differentiation (*EID1*) mRNA was significantly higher expressed in infant ALL patients compared with pediatric (non-infant) BCP-ALL patients and healthy bone marrow samples ($p<0.001$). Subsequently, we performed shRNA mediated knockdown of *EID1* in human *MLL*-fusion gene leukemia cell line SEM, which resulted in reduced cell growth through both an induced G1-arrest and apoptosis. Gene expression profiling of cells transduced with *EID1* shRNA demonstrated reduced expression of E2F-driven genes, needed for cell cycle progression to the S-phase. Furthermore, EP300-associated factor *KAT2B* (a.k.a. *PCAF*) was downregulated upon knockdown of *EID1.* Both knockdown of *KAT2B* and *EP300* induced apoptosis in *MLL*-rearranged cell line SEM. *Results:* These results show that *EID1* expression is important for continued growth of *MLL*-rearranged ALL. Due to the critical role in growth and its specific expression in the leukemic blasts of infant ALL patients, *EID1* appears to be a good target for the development of new treatment strategies in infant ALL.

INTRODUCTION

Although overall survival among children diagnosed with acute lymphoblastic leukemia (ALL) nowadays exceeds 80% [1], the prognosis for infants (<1 year of age) with ALL remains dismal. [2] Infant ALL is characterized by chromosomal translocations of the *Mixed Lineage Leukemia* (*MLL*) gene, occurring in ~80% of the cases. In infant ALL, the most recurrent *MLL* translocation partner genes are *AF4*, *ENL*, and *AF9*. [2, 3] The prognosis for *MLL*-rearranged infant ALL patients (i.e. EFS rates of 30-40% [2]) is considerably worse than that for infants not carrying translocations of the *MLL* gene (EFS ~75% [2]). Hence, improved therapeutic strategies, especially for *MLL*-rearranged infant ALL patients, are urgently needed. Genome-wide transcriptome studies have shown that *MLL*-rearranged ALL patients display highly unique gene expression profiles. [4-6] In search of new and valid therapeutic targets we screened our gene expression profiling data. [6] In the present study we set out to investigate *EID1*, which we found highly and specifically expressed in infant ALL cells. The EID1 (EP300 interacting inhibitor of differentiation 1) protein binds and interacts with the histone acetyltransferase (HAT) EP300. In normal physiology, EP300 is widely expressed and involved in the regulation of many cellular processes, including cell growth, differentiation and apoptosis. [7-10] By blocking the HAT activity of EP300, EID1 is a potent inhibitor of differentiation. [11, 12] EID1 also binds the retinoblastoma (RB) protein. [11] In complex with members of the E2F family of DNA-binding transcription factors, RB represses the transcription of genes required for cell cycle progression, and as such represents an important gate keeper of the cell cycle. [13] Thus, binding of EID1 to both EP300 and RB suggests a link between tissue-specific differentiation and cell cycle exit, and has led to the assumption that EID1 confers inhibitory effects

on both cellular processes. [11, 12] High-level expression of EID1 in infant ALL cells may fulfill a crucial role in blocking differentiation and at the same time stimulate uncontrolled proliferation. If so, EID1 represents an attractive therapeutic target in infant ALL. In this study, we investigated to what extent *MLL*-rearranged ALL cells are dependent on the expression of EID1.

MATERIALS AND METHODS

Note: A more elaborate and detailed description of all experimental procedures and data analysis methods can be found in the Supplemental Material Chapter 8.

Gene expression profiling data

EID1 mRNA expression levels in primary samples from infants with ALL were derived from our previously published gene expression array data (HU133plus2.0 GeneChips; Affymetrix; probe set ID: 211698_at). [6] Raw array data were collectively normalized using variance-stabilizing normalization [14] and differential gene expression was statistically evaluated using linear models for microarray analyses (LIMMA). [15] Gene set enrichment analysis (GSEA) was performed using GSEA software. [16]

Quantitative real-time PCR analysis

Total RNA was extracted from a minimum of $2x10^6$ cells using TRIzol reagent (Invitrogen, Life Technologies, Breda, The Netherlands) according to the manufacturer's guidelines. The quality of the extracted RNA was assessed on 1.5% agarose gels, and cDNA was synthesized for quantitative real-time PCR analysis as described earlier. [6] *EID1* and *KAT2B* mRNA expression was measured by quantitative real-time PCR analysis using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) as described before. [17]

Cell line culturing and RNA interference

All knockdown experiments were performed in the leukemia cell line SEM, carrying the *MLL* translocation t(4;11), generating MLL-AF4 fusion proteins.

RNA interference was performed by means of lentiviral transduction. After 24 hours the DMEM medium was replaced with RPMI medium, and virus-containing medium was harvested 48 hours after transfection. Upon filtration through a 0.45-µm cellulose acetate filter, the virus stock was used to infect SEM cells. The pLKO.1 puromycin resistance marker allows selection for stably transduced cells. Puromycin (1 µg/ml) was added to the medium after 96 hours. Infected cells were analyzed for knockdown by RT-PCR and western blot analyses. All knockdown experiments were repeated at least two times.

Flow cytometry and cell cycle analysis

Induction of apoptosis was assessed by an Annexin V/PI assay. Cell cycle analysis was performed by BrdU labeling using the FITC BrdU Flow Kit (#559619, BD Pharmigen) according to manufacturer's protocol. Analysis of the flowcytometry data was performed using the FlowJo software version 7.6.5 (Tree Star).

Western blot analyses

Western Blots were probed with KAT2B rabbit monoclonal antibody (CellSignaling,) and beta actin mouse monoclonal antibody (as a control for equal loading) (Abcam) for 2 hours at room temperature. Next the western blots were incubated in fluorescently labeled secondary antibodies (LI-COR Biosciences) for 1 hour and subsequently detected using the Oddyssey Infrared Imaging System (LI-COR Biosciences).

RESULTS

High-level EID1 expression in infant ALL

Our recent gene expression profiling study revealed high-level expression of *EID1* (or *CRI1*) in *MLL*-rearranged infant ALL when compared with pediatric (>1 year of age) precursor B-cell ALL. [6] Further analysis showed that high-level expression of *EID1* was not exclusively restricted to *MLL*-rearranged infant ALL samples, but also appeared present in infant ALL cells bearing germline *MLL* genes. Compared with both pediatric precursor B-cell ALL (n=16) and healthy bone marrow samples (n=13), infant ALL cells (either with or without *MLL* translocations) significantly expressed higher levels of *EID1* (Figure 1A) (p<0.0001). Next, in order to validate these gene expression profiling data, we performed quantitative real-time PCR analysis. Again, *EID1* was significantly (p=0.002) higher expressed in primary *MLL*-rearranged infant ALL samples (n=6) as compared with samples from pediatric precursor B-cell ALL patients (n=6) (Figure 1B).

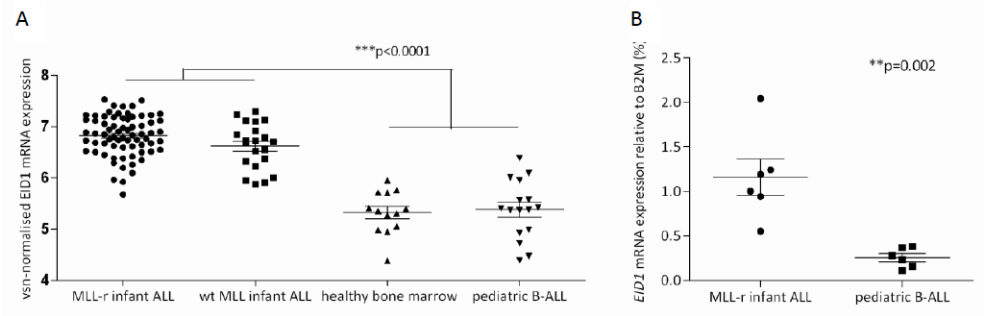

Figure 1. High expression of *EID1* in infant leukemia.

Figure 1. High expression of *EID1* in infant leukemia. A. Graphical representation of VSN-normalized expression values of *EID1* mRNA (211698_at) from gene-expression profiling data (Affymetrix HU133plus2 GeneChips) in patient material. *EID1* is significantly higher expressed in *MLL*-rearranged infant ALL patients (MLL-r infant ALL, n=68) and *MLL* wild-type infant ALL patients (MLL wt infant ALL, n=18) compared with healthy bone marrow (healthy bone marrow, n=13) and pediatric (non-infant) precursor BCP-ALL patients (pediatric BCP-ALL, n=16). The difference between patient groups was statistically analyzed using the Mann-Whitney *U* test. Probeset with the largest difference between *MLL*-rearranged and *MLL* wild-type are shown in the graph; all probesets of *EID1* had an FDR-adjusted p-value of <0.0001 (Supplementary material Chapter 8). B. Graphical representation of *EID1* mRNA expression in MLL-rearranged infant ALL (n=6) and pediatric (non-infant) precursor BCP-ALL patients (n=6) as determined by RT-PCR. Error bars represent the mean ± standard error of the mean.

Knockdown of EID1 induces cell cycle arrest and apoptosis in MLL-rearranged ALL

To determine whether *MLL*-rearranged ALL cells are dependent on high-level *EID1* expression, we performed *EID1* knockdown experiments in the MLL-AF4 positive ALL cell line SEM. SEM cells were transduced with shRNAs directed against *EID1* (sh*EID1*), or with non-silencing control shRNAs (sh*NSC*). Selection of cells stably expressing the shRNAs was established by exposure to puryomycin. Compared with the non-silencing control, transduction with sh*EID1* led to an 82% reduction in *EID1* expression three days after puromycin selection (Figure 2A). Knockdown of *EID1* resulted in marked decreases in the number of viable cells as determined by trypan blue dye exclusion staining (Figure 2B). To investigate the cause of this remarkable decline in cell viability, the effects of *EID1* knockdown were assessed by apoptosis and cell cycle analysis. Annexin V/propidium iodide (PI) staining

revealed a dramatic induction of apoptosis. Within two days after puromycin selection, virtually all cells challenged with shRNA against *EID1* were either apoptotic or necrotic (i.e. PI-positive) compared to the non-silencing control (Figure 3A). At the same time, BrdU/7-AAD staining showed an abrupt blocking cell cycle progression. After *EID1* knockdown, the percentage of SEM cells residing in the S phase was reduced to only 1% compared with ~23% in non-silencing control cells (Figure 3B). Collectively these data imply that *MLL*-rearranged ALL cells are highly dependent on high-level *EID1* expression.

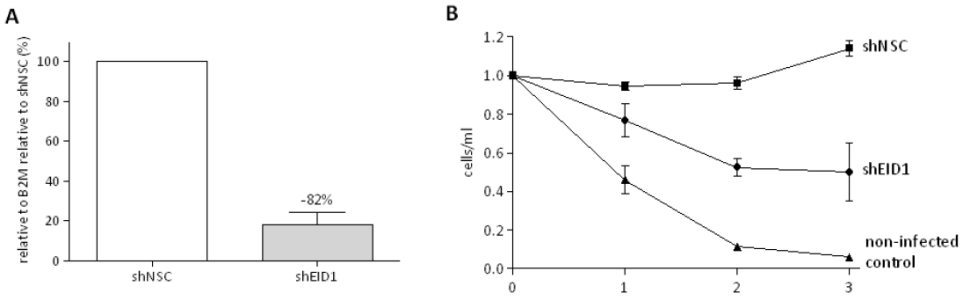

Figure 2. *EID1* knockdown inhibits growth in MLL-rearranged ALL. A. Expression values of *EID1* mRNA in SEM after knockdown by shRNA directed against *EID1* measured by RT-PCR relative to non-silencing control sh*NSC*. Expression of mRNA was measured relative to housekeeping gene *B2M* as loading control. B. Average live cell counts/ml (including cells undergoing apoptosis) determined over four days after puromycin selection in SEM cells transduced with *EID1* shRNA (sh*EID1*) or non-silencing control virus (sh*NSC*). Non-infected control: wild-type SEM cells without puromycin resistancy marker. Data represented are the means of three independent experiments. Error bars represent the standard error of the mean.

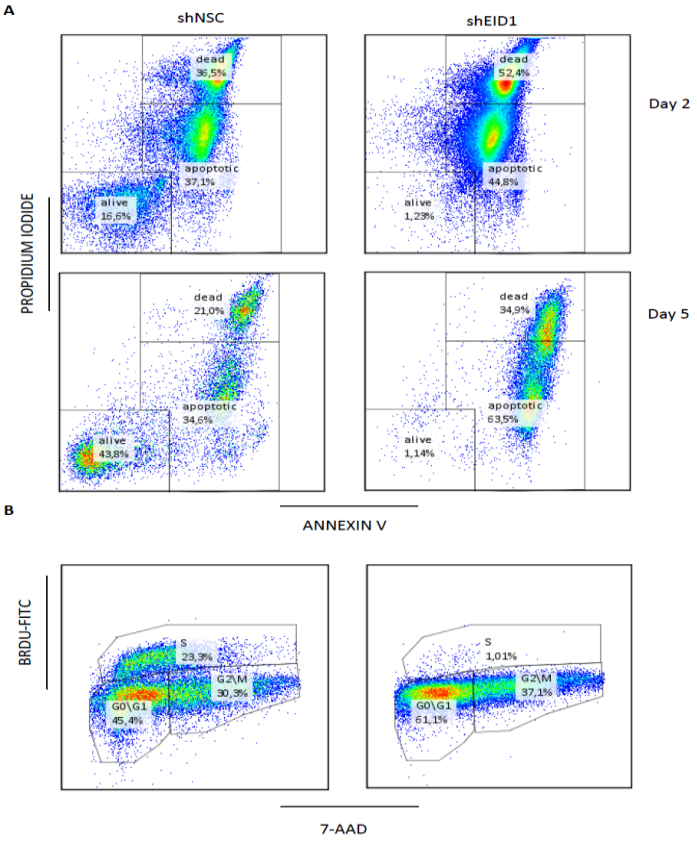

Figure 3. *EID1* knockdown induces apoptosis and cell-cycle arrest in MLL-rearranged ALL. A. Quantitative measurement of alive, apoptotic and dead transduced SEM cells two days (upper panel) and five days (lower panel) after puromycin selection with shRNAs directed against *EID1* (sh*EID1*, right) and non-silencing control (sh*NSC*, left) as measured by Annexin V on the X-axis and propidium iodide on the Y-axis. B. Cell cycle analysis indicating G0/G1-phase, S-phase and G2/M-phase of transduced SEM cells two days after puromycin selection with shRNAs directed against *EID1* and non-silencing control as measured by BrdU incorporation on the X-axis and 7-AAD (measuring total DNA content) on the Y-axis.

Transcriptional consequences of EID1 knockdown

To gain more insight into the effects of *EID1* repression in *MLL*-rearranged ALL cells, we performed genome-wide transcriptome analysis (using Affymetrix HGU133plus2.0 microarrays) in SEM cells transduced with sh*EID1* or non-silencing sh*NSC*. These experiments revealed differential expression ($p<0.001$) of 39 probe sets, corresponding to 26 genes, after *EID1* knockdown. Importantly, three of the probe sets corresponded to *EID1* itself, confirming successful repression of the target gene (Figure 4A). Moreover, 22 of the genes were down-regulated upon loss of *EID1* expression, whereas only four genes became up-regulated. Subsequently, we performed gene set enrichment analysis (GSEA) of gene expression in *EID1* knockdown samples versus control samples using gene sets that contain genes that share a transcription factor binding site as defined in the TRANSFAC database (C3: motif gene sets/TFT). In the downregulated genes, we found a significant enrichment of promoter regions containing the motif matched with E2F-related proteins, specifically E2F1 (normalized enrichment score (NES)=-1.77, q=0.01) (Figure 4B and Supplemental material Chapter 8).

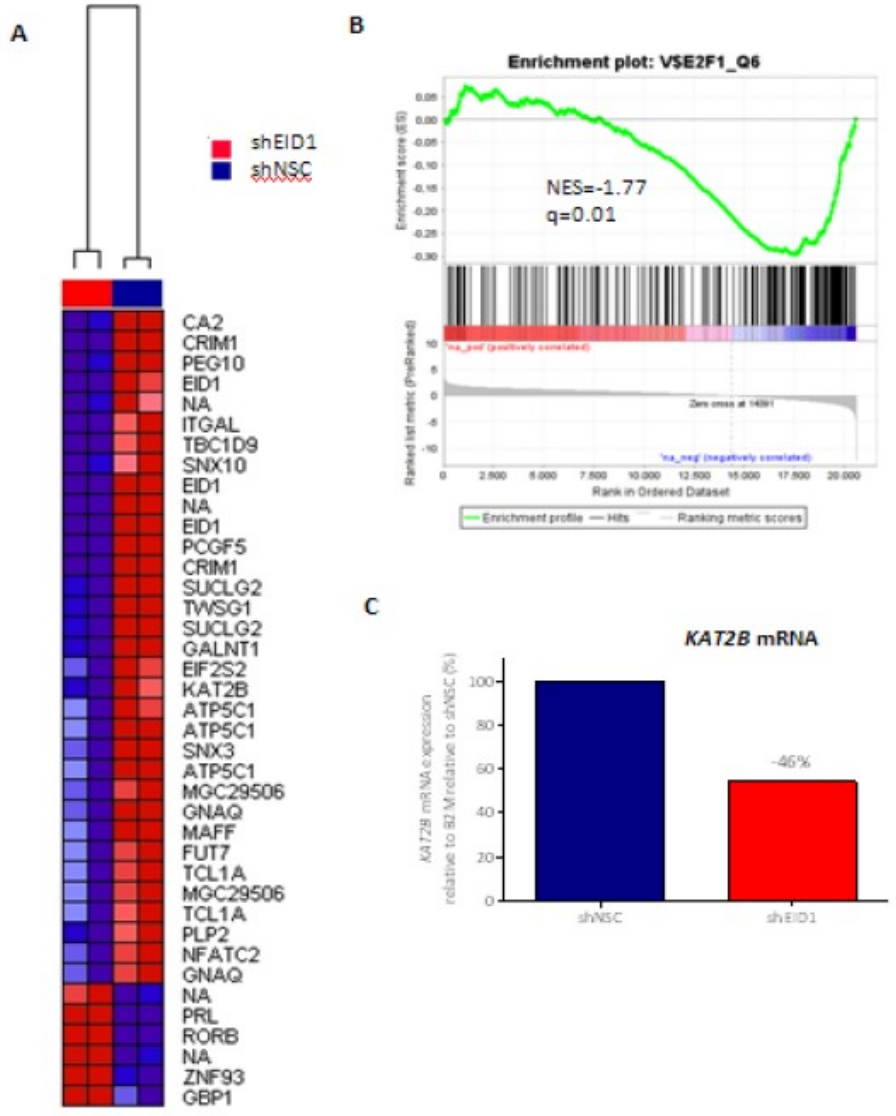

Figure 4. Gene expression profiling of *EID1* demonstrates downregulation of *KAT2B*.

Figure 4. Gene expression profiling of *EID1* demonstrates downregulation of *KAT2B*. A. Heatmap visualization of gene expression profiles (Affymetrix HGU133plus2.0 GeneChips) of *EID1* knockdown samples (n=2, red columns, shEID1) and non-silencing control samples (n=2, blue columns, shNSC) using 42 significantly different expressed probe sets (p<0.001). Upregulated genes are depicted in red, downregulated genes are depicted in blue. B. Gene set enrichment analysis (GSEA) of gene expression in *EID1* knockdown samples ('na_pos') versus control samples ('na_neg') using gene sets that contain genes that share a transcription factor binding site defined in the TRANSFAC database (C3: motif gene sets/TFT). Demonstrated is an enrichment of genes with promoter regions [-2kb,2kb] around transcription start site containing the motif TTTSGCGS which matches annotation for E2F1. The E2F-related gene set with the highest normalized enrichment score (NES) is shown here; out of 19 E2F-related gene sets analyzed 15 had nominal p-value <0.05 (Supplemental material Chapter 8). q = FDR-adjusted p-value. C. Expression values of *KAT2B* mRNA in SEM after knockdown by shRNA directed against *EID1* measured by RT-PCR relative to non-silencing control shNSC. Expression of mRNA was measured relative to housekeeping gene *B2M* as loading control.

Knockdowns of histone acetyltransferases KAT2B and EP300 induce apoptosis in MLL-rearranged ALL

Interestingly, one of the down-regulated genes in our *EID1* knockdown expression signature was *KAT2B* (or *PCAF*: EP300-associated factor), which like EP300 encodes a histone acetyltransferase important in regulating differentiation. Quantitative real-time PCR analysis confirmed *KAT2B* down-regulation in SEM cells transduced with sh*EID1* (Figure 4C). While *EID1* is believed to block differentiation by inhibiting EP300 activity, KAT2B seems to prevent differentiation via acetylation of RB. [18] On the other hand, Puri and co-workers [19]

demonstrated that the HAT activity of KAT2B, but not of EP300, is essential for muscle differentiation. Therefore, to elucidate the contribution of *KAT2B* down-regulation to the observed *EID1* knockdown phenotype in infant ALL, we assessed apoptosis induction and cell cycle distribution in *KAT2B* and *EP300* knockdown experiments. In comparison with the non-silencing control, transduction of MLL-AF4 positive SEM cells with sh*KAT2B* or sh*EP300* reduced the mRNA expression of these genes with 70% and 58% respectively (Figure 5A). Furthermore, Western blot analysis confirmed successful knockdown of KAT2B at the protein level (Figure 5B). Suppression of both KAT2B and EP300 reduced the number of viable cells as determined by trypan blue dye exclusion, but the most dramatic effects were observed after knockdown of EP300 (Figure 5C). In line with these findings, apoptosis induction and cell cycle inhibition were most prominently triggered in cells in which EP300 expression was reduced (Figure 5D and 5E). These data show that the loss of KAT2B to some extent contributes to the anti-leukemic phenotype of *EID1* inhibited SEM cells, but at the same time also imply that *MLL*-rearranged ALL cells are more dependent on the presence of EP300.

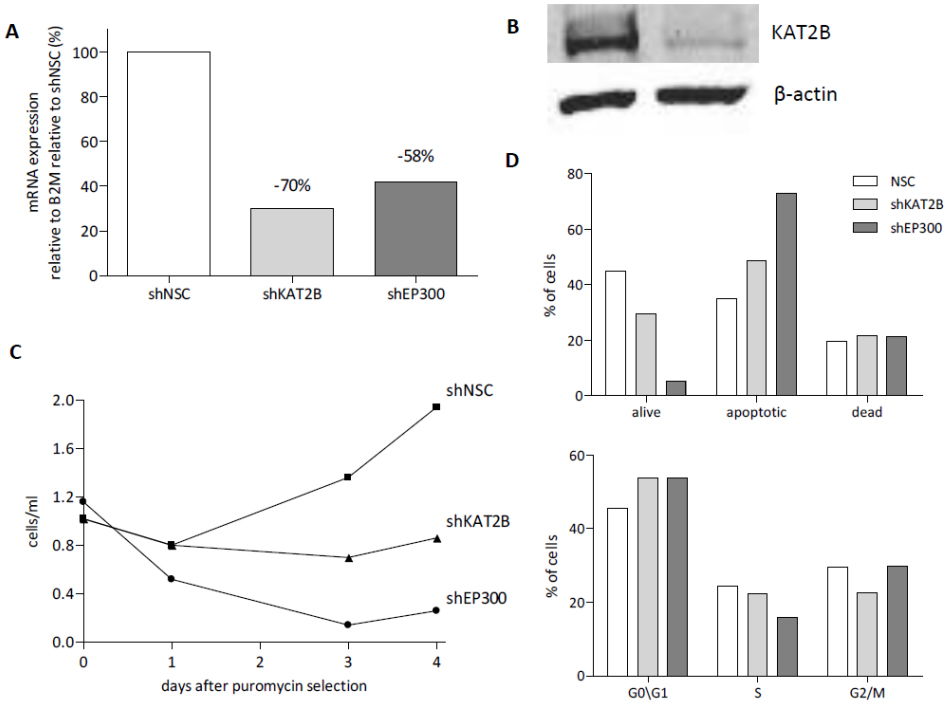

Figure 5. *KAT2B* and EP300 induce apoptosis in *MLL*-rearranged ALL. A. Expression of *KAT2B* mRNA (left, light grey) and *EP300* (right, dark grey) in SEM after knockdown by shRNA directed against *KAT2B* and *EP300* respectively, measured by RT-PCR relative to non-silencing control sh*NSC* with *B2M* as loading control. B. Protein levels of KAT2B in non-silencing control (sh*NSC*) and after knockdown by shRNA of *KAT2B* (sh*KAT2B*) as shown by western blot. β-actin is shown as loading control. C. Average live cell counts/ml determined over five days from puromycin selection in SEM cells transduced with *KAT2B* shRNA (sh*KAT2B*), *EP300* shRNA (sh*EP300*) or non-silencing control shRNA (sh*NSC*). D. Quantitative measurement of alive, apoptotic and dead transduced SEM cells five days after puromycin selection with shRNAs directed against *KAT2B* (shKAT2B, light grey), *EP300* (sh*EP300*, dark grey) and non-silencing control (sh*NSC*, white) as measured by Annexin V and PI, discriminating between apoptotic and dead cells.

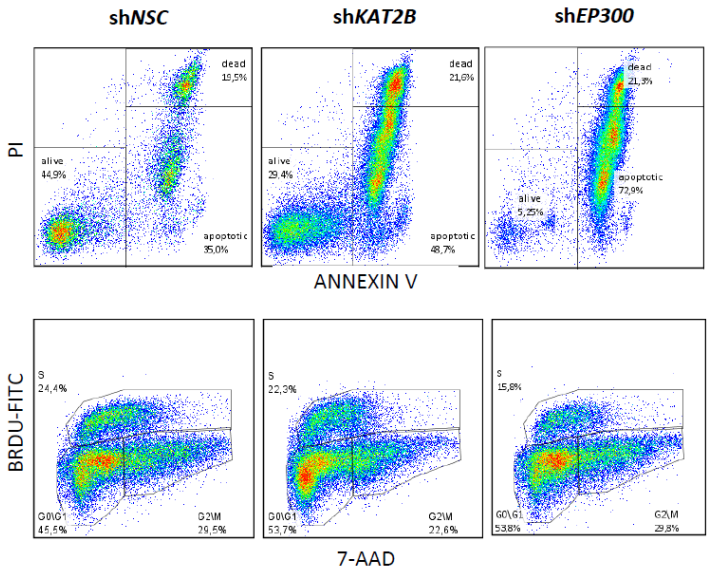

Figure 6. Cell cycle analysis after knockdown of KAT2B and EP300. Cell cycle analysis (gated on live cells) indicating G0/G1 phase, S phase and G2/M phase of transduced SEM cells two days after puromycin selection with shRNAs directed against *KAT2B* (shKAT2B, light grey), *EP300* (sh*EP300*, dark grey) and non-silencing control (sh*NSC*, white) as measured by BrdU incorporation on the X-axis and 7-AAD (measuring total DNA content) on the Y-axis.

DISCUSSION

We here show that among childhood ALL patients, *EID1* (EP300-interacting inhibitor of differentiation) is specifically expressed in patients <1 year of age (i.e. infants). Although the presence of *MLL* translocations is firmly associated with a poor clinical outcome and represents a hallmark of infant ALL, EID1 expression was not restricted to *MLL*-rearranged infant ALL alone. Infant ALL patients not carrying translocations of the *MLL* gene displayed *EID1* expression levels comparable to that of *MLL*-rearranged cases. Hence, elevated *EID1* expression cannot be ascribed to the presence of the MLL fusion protein, which was to be expected as MLL fusion proteins do not seem to bind to the genomic region where the *EID1* gene resides. [20, 21] Moreover, the consistent up-regulation of *EID1* in infant ALL can neither be explained by a commonly shared and specific immunophenotype. In contrast to CD10 positive common/precursor BCP-ALL cells from children older than 1 year of age, *MLL*-rearranged infant ALL cells usually display a pro-B (i.e. $CD10^-$) phenotype. However, like precursor BCP-ALL cells in older children, infant ALL cells lacking translocations of the *MLL* gene, often display common/pre-B ($CD10^+$) phenotypes. In line with this, throughout normal B-cell differentiation, no particular compartment seems to exhibit signs of differential expression of *EID1*. [22] Thus, at this point the etiology of high-level *EID1* expression in infant ALL remains obscure, yet as a therapeutic target its potential seems evident, as repression of EID1 immediately triggers leukemic cell death and cell cycle arrest.

Unfortunately, still little is known on the expression or role of EID1 in human disease. One study demonstrated an association of increased nuclear translocation of EID1 with the pathogenesis of Alzheimer's disease, [23] and another

study found a correlation of EID1 expression with the malignant potential of intraductal papillary mucinous neoplasms of the pancreas.[24] To the best of our knowledge, this is the first study reporting on *EID1* expression in hematological cell types or hematologic disorders.

Normally, EID1 protein is able to block the histone acetyltransferase activity of EP300, and with that inhibits differentiation [11, 12]. Overrepresentation of EID1 in infant ALL cells may therefore have contributed to a block in differentiation required for leukemic transformation. However, our data showed that *MLL*-rearranged ALL cells require EP300, as knockdown of this protein immediately triggers massive induction of leukemic cell death. Therefore, it seems unlikely that EID1 is inhibiting EP300 in *MLL*-rearranged ALL, as these cells apparently are highly dependent on functional EP300.

Apart from inhibitory actions on EP300, EID1 has also been reported to bind to retinoblastoma protein (RB), and with that inhibiting differentiation by preventing the activation of tissue-specific transcription.[11] To date it remains unclear whether RB binds to E2F and EID1 simultaneously, and thereby integrating cellular stimuli, or whether E2F and EID1 interact with RB separately.[25] Although our data is limited, the here observed down-regulation of E2F-regulated genes upon repression of EID1, at least suggests integrated actions of EID1 and E2F. Thus, in *MLL*-rearranged ALL cells, the function of EID1 as a regulator of E2F-related genes seems more important than the inhibition of the HAT activity of EP300. Yet, elucidating the exact function of EID1 in infant ALL cells using knock-down experiments will proof to be difficult due to the rapid and severe induction of leukemic cell death. Perhaps the introduction of high-level *EID1* expression in normal B-cell progenitors may be more informative. Nonetheless, our data indisputably demonstrate that *MLL*-rearranged ALL cells require EID1, and fail to

sustain viability when EID1 is repressed. Obviously, this makes EID1 an attractive therapeutic target in (*MLL*-rearranged) infant ALL, and warrants further studies exploring the possibility of developing small molecule inhibitors targeting EID1.

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CHAPTER 9

General discussion

SUMMARY AND GENERAL DISCUSSION

Survival rates for pediatric acute lymphoblastic leukemia (ALL) have greatly increased over the last decades, and nowadays approaches 90%.[1] Despite great efforts, the survival rates for infant (<1 year of age) acute lymphoblastic leukemia (ALL) to date is still poor with an overall survival of 55%.[2] Neonatal (<1 month of age) ALL and neonatal AML are rare. Etiology, symptoms, diagnosis, treatment and prognosis are extensively reviewed in **chapter 2**.

Chapter 3 elaborates on neonatal ALL. Analysis of thirty patients with neonatal ALL treated with the uniform Interfant-99 protocol shows that neonatal ALL was characterized by a significantly higher white blood cell count, a trend towards a higher incidence of *MLL* gene rearrangements, and a CD10-negative B-lineage (i.e. pro-B) immunophenotype than ALL in older infants. All of these factors are associated with a dismal outcome. A higher relapse rate in neonatal ALL was demonstrated, also after adjustment for these risk factors. Hypothetically, the high relapse rates can be explained by the administration of reduced chemotherapy dosages in these very young patients in the fear of severe side-effects. As pharmacokinetic studies in this particular population are very limited, determination of the right dosage represents a challenge. Both the Interfant-99 study[7] and the AML-BFM-98/-2004[41] apply dose reductions in their protocol. This study demonstrates an long term survival of 17%, and we therefore advocate treating neonatal ALL with curative intent. Early death in complete remission and treatment delays due to toxicity did not differ from that in older infant ALL patients, which may allow for dose intensification in neonates diagnosed with ALL.

Despite the strong association of the occurrence of *MLL* translocations in infant ALL patients, ~20% of all infant ALL cases carry wild-type (or germline) *MLL* genes. **Chapter 4** shows that wild-type *MLL* infant ALL carries a different gene expression profile from that of *MLL*-rearranged infant ALL as well as from pediatric precursor BCP-ALL. In the current Interfant-06 protocol, infant ALL patients carrying wild-type *MLL* genes are treated uniformly, stratified as a low-risk group. However, in **chapter 5** we demonstrate that these wild-type *MLL* infant ALL patients form a distinct but very heterozygous patient group for which very few recurrent genetic abnormalities can be identified. A subgroup of the wild-type *MLL* infant ALL patients shows homology with the high-risk *MLL* infant ALL patients with high-level expression of *MEIS1* and an immature pro-B phenotype. We show that high-level expression of *MEIS1* predicts poor outcome, and low-level expression of *MEIS1* predicted an EFS of 100% in wild-type *MLL* infant ALL patients. If this can be confirmed in an independent set of patients we suggest a novel risk stratification strategy in which the expression of *MEIS1* serves as a stratifier for this particular sub-group of patients. Currently, wild-type *MLL* infant ALL patients are by default treated as low risk infant ALL patients. Using *MEIS1* as a risk stratifier, treatment can potentially be more individualised with ultimately a lower toxicity profile for the good prognosis group and improved survival for the high-risk group with high-level expression of *MEIS1* using an intensified treatment protocol. Alternatively, improving survival for this particular group would involve better targeted treatment rather than more intensive treatment. MEIS1 has previously been demonstrated to be important in the oncogenicity of *MLL*-rearranged leukemia.[3, 4] The down-regulation of MEIS1 in leukemic cell lines impaired engraftment in NOD/SCID mice and reduced proliferation.[4, 5] These data suggest that both *MLL*-rearranged infant ALL as well as wild-type *MLL* infant ALL patients may benefit

from MEIS1 inhibition. To date there is no known effective inhibitor of MEIS1, and a differential gene expression analysis between patients with high *MEIS1*-expression and patients with low *MEIS1*-expression did not identify obvious downstream effectors that can be targeted. The discovery of an effective small molecule inhibitor of MEIS1 should therefore now be the next step studying the potency of targeting MEIS1.

Infant ALL is still in urgent need for more optimal, targeted treatment strategies. Despite an intensive 2-year treatment protocol for these very young children, overall survival of *MLL*-rearranged infant ALL is around 50%. Despite good initial responses to current therapies, high relapse rates in these infants contribute greatly to an overall poor prognosis.[2] In search for new targets for therapy, current literature focuses on the biology of MLL-fusion driven gene expression and direct MLL-fusion protein binding sites. We hypothesise that *MLL*-rearranged leukemia is not per se dependent solely on such direct targets. We earlier showed that that for example S100A8/S100A9, which is not a direct target of MLL-AF4, induces prednisolone resistance and S100A8/S100A9 inhibitors may improve outcome in *MLL*-rearranged ALL.[6] Also additional genetic events may be involved in the oncogenicity such as RAS mutations.[7] Indirect targets may further potentially contribute to the oncogenicity of *MLL*-rearranged leukemia. In search for new targets for therapy, we used gene expression profiling before and after RNA interference mediated knockdown of the *MLL*-fusion for the identification of a novel set of genes influenced by the presence of the MLL-fusion protein (**chapter 6**). Interestingly, the set of obtained genes that rapidly responded to the loss of the MLL fusion protein was enriched for hypermethylated promoter regions in t(4;11) and t(11;19) infant ALL patients. Our group earlier demonstrated

that aberrant DNA methylation is abundant in *MLL*-rearranged infant ALL.[8] The rapid activation of these genes after removal of the MLL-fusion protein suggests a direct role of the MLL-fusion protein in the inactivation of these genes through hypermethylation. Possibly, the inactivation of these genes is part of the leukemogenicity of the MLL-fusion. For example, NKX2 homeobox 1 (*NKX2.1)* is hypermethylated in the presence of the MLL-fusion protein and becomes upregulated after the removal of the MLL-fusion protein. *NKX2.1* is besides a known oncogene also a recognized suppressor of malignant progression.[9] The inactivation of *NKX2.1* by the MLL-fusion protein could possibly contribute to tumor progression. Reactivation of *NKX2.1* may contribute to suppression of tumor progression in *MLL*-rearranged leukemia. The various ways in which the MLL-fusion can exert its' leukemogenic gene expression other than through direct DNA binding thus generates interesting new treatment strategies to explore.

Similarly, we used the approach to reveal direct and indirect effects on gene expression through the AF4-MLL fusion protein. Besides the value of this gene set as source for discovering new targets for treatment of a subset of t(4;11) infant ALL patients, this particular set might also be of value shedding light on the role of the AF4-MLL protein in the initiation of t(4;11) infant ALL. It has been proposed that AF4-MLL is indispensable for the initiation of t(4;11) leukemias as the AF4-MLL protein was able to induce leukemia without the requirement of the reciprocal MLL-AF4 protein,[10] but this achievement has yet to be reproduced. Careful examination of this particular set of genes can possibly contribute to a better understanding of the biology underlying this protein.

An important note to make, when discussing the search of new treatment strategies in *MLL*-rearranged ALL, is that there is no good mouse model available. Despite various attempts by several laboratories, there are still no mice faithfully

imitating the origin of the disease. Results vary from no leukemogenesis at all[11] to a too long latency time[12] or a B-cell ALL phenotype that differs from the pro-B phenotype in *MLL*-rearranged ALL.[13, 14] Therefore we chose to identify the important players in *MLL*-rearranged ALL by the use of knockdown models and consecutive gene expression profiling. Nevertheless, we are aware of the outweighing results a proper mouse model would bring to this field.

Following through on the gene expression profiles derived from chapter 6, in **chapter 7** we validated the functional role of *CDK6*, one of the genes influenced by the presence of the MLL-fusion protein in *MLL*-rearranged ALL. As we show dependency of proliferation of *MLL*-rearranged ALL cell lines on CDK6, we suggest PD0332991, a CDK4/CDK6-inhibitor, as potential drug in the treatment of *MLL*-rearranged ALL. The next critical step in studying the role of CDK6 in *MLL*-rearranged ALL is testing the efficacy of PD0332991 in a xenograft mouse model of MLL-rearranged ALL before testing such a drug in the clinic. As PD0332991 induces a cell cycle arrest but not apoptosis, we propose testing this drug as part of chemotherapy regimen rather than as monotherapy. Due to the necessity of cell cycling for the cytotoxic effect of many of the chemotherapeutics used in ALL, this drug ideally should be combined with a drug able to induce cell death in non-cycling cells. Recently, it was reported that *CDK6* is also a critical effector of MLL fusions in acute myeloid leukemia and knockdown of CDK6 could successfully be mimicked with PD0332991.[15] Combined efforts of research in *MLL*-rearranged ALL and *MLL*-rearranged AML research would possibly fasten bringing this promising drug to the clinic.

Another interesting target for treatment in *MLL*-rearranged ALL is *EID1* as described in **chapter 8**. EID1 is highly expressed in both leukemic cells of *MLL*-

rearranged ALL patients and wild-type *MLL* infant ALL patients, and turns out to be critical for sustained growth in *MLL*-rearranged ALL cell lines. *EID1* therefore appears to be a good therapeutic target for the development of new treatment strategies in infant ALL, even though we have very little understanding of the working mechanism of EID1 in this type of leukemia.

Several initiatives are being deployed to search for new specific therapeutic compounds. A promising initiative is the Connectivity Map database,[16] (or cmap) which is a collection of genome-wide transcriptional expression data from four different cultured human cells treated with over 1300 bioactive small molecules. Through the search of common gene-expression changes, it enables the discovery of drug candidates. This technique can possibly form the hub in translational research between gene expression profiling and the discovery of drugs targeting genes of interest. The value of cmap was demonstrated by our group by the identification of PI3K inhibitors[17] and HDAC inhibitors[18] in infant acute lymphoblastic leukemia.

Alternatively, our group is currently applying a drug library-based identification of therapeutics that potentiate the treatment of MLL-rearranged infant acute lymphoblastic leukemia. This drug library consists of readily tested and approved therapeutics available in the clinic. By testing which of these therapeutics are able to effectively and specifically eliminate *MLL*-rearranged infant leukemic cells, we possibly find in a revolved manner the compounds that are activating or deactivating those genes responsible for the initiating and maintaining the leukemia. Moreover, this would reach the final goal of finding a targeted treatment for this poor-prognosis disease that is *MLL*-rearranged infant ALL.

In conclusion, infants suffering from acute lymphoblastic patients are in need for better treatment strategies. Gene expression profiling creates a powerful tool for screening for new treatment targets as shown in this thesis. Translation from the laboratory into the clinic remains a major challenge though various initiatives are undertaken to reduce the distance. Nevertheless continued research that bridges these potential treatment targets and a drug available in the clinic is still urgently needed.

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CHAPTER 10

Nederlandse samenvatting

List of publications

Curriculum vitae

PhD portfolio

Dankwoord

NEDERLANDSE SAMENVATTING VOOR DE NIET-INGEWIJDE

LEUKEMIE

Bloed wordt gemaakt in het beenmerg, dat zich bevindt in de lange pijpbeenderen van het lichaam. Een moederbloedcel begint te delen en bij elke deling veranderen de eigenschappen van de cel tot er geleidelijk verschillende soorten bloedcellen zijn, onder andere witte en rode bloedcellen. Als de bloedcellen rijp zijn, worden ze vanuit het beenmerg naar de bloedvaten getransporteerd. De rode bloedcellen zorgen dan bijvoorbeeld dat er zuurstof naar de weefsels en organen wordt gebracht en de witte bloedcellen zorgen voor de afweer van het lichaam tegen bacteriën en virussen. Soms ontstaan er bij deze delingen in het beenmerg fouten in het DNA, de code die de cel aanstuurt. Fouten in het DNA kunnen ervoor zorgen dat de bloedcel niet meer stopt met delen. Zo ontstaat leukemie, kanker van bloedcellen. Bij kinderen is er meestal sprake van een bepaald soort leukemie, zogenaamde acute lymfatische leukemie (ALL).

ZUIGELINGENLEUKEMIE

Heel soms komt acute leukemie ook voor bij kinderen jonger dan 1 jaar (ofwel zuigelingen), we spreken dan over zuigelingenleukemie. In Nederland zijn er ongeveer vier tot vijf kinderen per jaar die de diagnose zuigelingenleukemie krijgen. Zuigelingenleukemie is in veel opzichten anders dan de leukemie die

oudere kinderen krijgen. Zo overleeft slechts ongeveer de helft van de zuigelingen hun ziekte, terwijl dit ruim 80% is bij de kinderen met ALL ouder dan 1 jaar. Ook de fouten in het DNA van de leukemiecel zijn bij zuigelingen anders dan bij oudere kinderen. Zo is er bij ongeveer 80% van de zuigelingen met ALL sprake van een breuk in het zogenaamde *MLL*-gen, terwijl dit slechts bij 1% van de kinderen boven de 1 jaar het geval is. Een gen is een code in de cel die beschrijft hoe een eiwit voor de cel gemaakt moet worden. Als het gebroken *MLL*-gen aan een ander gen bindt, ontstaat er dus een foute code en daarmee een fout eiwit. Dit foute eiwit kan aanleiding geven tot een ongecontroleerde deling van de witte bloedcel en daarmee oorzaak zijn van leukemie. In zuigelingenleukemie zien we bijvoorbeeld het MLL-AF4 eiwit, waarbij een stuk van het gebroken *MLL*-gen vast is komen te zitten aan het *AF4*-gen. Er zijn sterke aanwijzingen dat dit *MLL-AF4* combinatie-gen de oorzaak is van de agressieve vorm van leukemie bij zuigelingen.

In een van de vijf zuigelingen komt zo'n fout MLL-eiwit echter niet voor. Er wordt vaak gedacht dat deze jonge kinderen een leukemie hebben die lijkt op die van oudere kinderen met leukemie, maar dit is nooit goed uitgezocht. Deze kinderen zonder fout MLL-eiwit hebben wel een betere overlevingskans van ongeveer 80%. Sommige zuigelingen krijgen al binnen een maand na de geboorte de diagnose leukemie, ook wel neonatale leukemie genoemd. Er wordt onder artsen vaak gedacht dat deze hele jonge kinderen altijd dood gaan en artsen zijn vaak bang om bij deze kinderen intensieve chemotherapie te geven. Echter, omdat het zo weinig voorkomt is eigenlijk niet goed bekend of en hoe deze kinderen behandeld moeten worden.

OP NAAR NIEUWE THERAPIE VOOR MLL-HERSCHIKTE ZUIGELINGENLEUKEMIE

We zoeken naar een therapie die beter de leukemiecellen doodt, zonder de gezonde cellen te beschadigen. Hiermee willen we de overlevingskansen van kinderen met de diagnose zuigelingenleukemie verbeteren.

Door speciale technieken te gebruiken in het laboratorium kunnen we zien hoe actief een gen in een cel is. Dit noemen we de genexpressie van de cel. Als we de genexpressie van leukemiecellen met een MLL herschikking vergelijken met leukemiecellen van oudere kinderen met ALL, met leukemiecellen van zuigelingen zonder MLL herschikking, en met gezonde beenmergcellen, zien we grote verschillen tussen welke genen aan en uit staan in de deze cellen. Door te vergelijken welke genen aan en uit staan in de leukemiecel met MLL herschikking, kunnen we beter begrijpen hoe de leukemie ontstaat en betere aanknopingspunten vinden om nieuwe en vooral meer specifieke behandelmethoden te ontwikkelen voor deze vorm van leukemie.

NEONATALE LEUKEMIE

Hoofdstuk 2 beschrijft wat er tot nu toe bekend is over neonatale leukemie. Er bestaat neonatale acute myeloide leukemie (AML) en neonatale ALL. Het verschil tussen myeloide en lymfatische leukemie zit hem in het type witte bloedcel dat ongecontroleerd is begonnen met delen en leukemiecel is geworden. Neonatale AML heeft een betere prognose dan neonatale ALL. De eerste symptomen van

neonatale leukemie zijn vaak een vergrote lever en milt, en huidafwijkingen. In het bloed worden grote hoeveelheden leukemiecellen gezien. Ook bij neonatale leukemie is er vaak sprake van een breuk in het *MLL*-gen. Het wordt behandeld met chemotherapie, maar vaak komen de leukemiecellen ondanks de therapie snel weer terug. **Hoofdstuk 3** gaat dieper in op neonatale acute lymfatische leukemie. Het beschrijft de data van een dertigtal kinderen met de diagnose ALL jonger dan 1 maand, welke zijn gevolgd en behandeld in de internationale Interfant-99 studie. We zagen dat 20% van deze kinderen met neonatale ALL na twee jaar na diagnose nog in leven was. Dit geeft voldoende reden om deze kinderen ondanks hun jonge leeftijd toch intensieve chemotherapie te geven.

GENEXPRESSIE IN ZUIGELINGENLEUKEMIE

Hoofdstuk 4 laat zien dat in de cellen van zuigelingen met een fout MLL-eiwit andere genen tot expressie komen dan in gezonde beenmergcellen, maar ook komen andere genen tot expressie vergeleken met leukemiecellen van oudere kinderen en leukemiecellen van zuigelingen zonder fout MLL-eiwit. De genen die aan en uit worden gezet in deze cellen dragen mogelijk bij aan het feit dat de cel niet meer kan stoppen met delen en/of ongevoelig zijn voor de huidige chemotherapie. Verder onderzoek naar deze genen moet helpen een betere therapie te vinden om kinderen met zuigelingenleukemie te genezen.

10.6 Zuigelingenleukemie zonder MLL-herschikking

Een op de vijf zuigelingen met leukemie, heeft geen foutief MLL-eiwit. Er wordt vaak gedacht dat de leukemie bij deze kinderen lijkt op de leukemie bij kinderen

ouder dan 1 jaar. In **hoofdstuk 5** laten we echter zien dat deze vorm van leukemie verschilt van de leukemie bij oudere kinderen en ook verschilt van zuigelingenleukemie met een foutief MLL-eiwit. Er zijn bijvoorbeeld minder vaak afwijkingen in het DNA bij deze kinderen dan bij oudere kinderen. Ook is minder goed te voorspellen bij welke van deze kinderen de leukemiecellen terugkomen na behandeling. Als een bepaald gen, genaamd *MEIS1*, erg actief is bij deze groep zuigelingen, komt de leukemie veel vaker terug na behandeling dan als dit gen minder actief is. Verder onderzoek naar dit gen kan wellicht bijdragen aan het vinden van een betere therapie voor deze kinderen.

DE CEL NA HET UITZETTEN VAN HET FOUTE MLL-GEN

In het laboratorium kunnen soms cellen afkomstig van patiënten ook buiten het lichaam gekweekt worden; gekweekte cellen worden ook wel cellijnen genoemd. In deze cellijnen kunnen we met behulp van virus als boodschapper van RNA naar de kern van de cel, genen aan en uit zetten. In **hoofdstuk 6** zetten we met behulp van zo´n virus met een specifiek stukje RNA in drie cellijnen het foute *MLL*-gen uit. Door hierna te bekijken welke genen aan- en uitgaan, hopen we te begrijpen hoe de *MLL* herschikking bijdraagt aan het ontstaan van de leukemie. Een voorbeeld van zo'n gen wat beïnvloedt wordt door het herschikte *MLL*-gen is cyclin-dependent kinase 6 (*CDK6*). Het CDK6-eiwit zorgt ervoor dat een cel kan blijven delen. In cellen met het foute MLL-eiwit staat *CDK6* hoog aan en als we de *MLL*-herschikking in de cellijnen uitzetten, gaat *CDK6* ook uit. Het lijkt er dus op dat het foute MLL-eiwit zorgt dat CDK6 aan en uit gaat. Met andere woorden, de *MLL*

herschikking lijkt het *CDK6* gen te gebruiken om ongecontroleerd te kunnen delen.

CDK6 IN MLL-HERSCHIKTE ZUIGELINGENLEUKEME

In **hoofdstuk 7** wordt dieper ingegaan op hoe CDK6 bijdraagt aan de leukemie in patiënten met een fout MLL-eiwit. In cellijnen met het foute MLL-eiwit hebben we het CDK6-gen uitgezet. Hierop gingen de cellen veel langzamer delen, hoewel het MLL-eiwit nog gewoon aanwezig was. Dit wijst erop dat het foute MLL-eiwit onder andere leukemie veroorzaakt door het CDK6-gen aan te zetten. Als we de cellijnen met het foute MLL-eiwit behandelden met een medicijn, genaamd PD0332991, dat in staat is het CDK6 eiwit te remmen, bleken deze veel minder snel te groeien. Verder onderzoek zal moeten laten zien of dit medicijn ook in muizen de groei van leukemie kan remmen. Als dat zo is, zou dat medicijn vervolgens in zuigelingen met leukemie onderzocht kunnen worden.

EID1 IN MLL-HERSCHIKTE ZUIGELINGENLEUKEMIE

Ook EID1 is een voorbeeld van een gen dat hoog aanstaat in zuigelingenleukemie, maar niet in oudere kinderen met ALL of in gezonde beenmergcellen. In **hoofdstuk 8** laten we zien dat als we het EID1-gen uitzetten in cellijnen, de leukemiecellen stoppen met delen en zelfs dood gaan. We denken daarom dat EID1 een belangrijke rol zou kunnen spelen in een nieuwe behandeling van

zuigelingenleukemie. Omdat EID1 een relatief onbekend eiwit is, hebben we (nog) geen medicijnen die EID1 kunnen remmen. We vinden daarom dat verder onderzoek naar dit eiwit erg belangrijk is.

CONCLUSIE

Concluderend is het nog steeds hard nodig dat er nieuwe behandelingen komen voor een betere overleving van zuigelingen met leukemie. Het bestuderen van welke genen aan en uit staan in zuigelingenleukemie heeft ons erg geholpen om te snappen welke genen belangrijk zijn voor de leukemie zoals blijkt uit dit proefschrift. Echter, het blijft erg moeilijk om de stap te maken van ontdekkingen in het laboratorium naar een werkzaam medicijn dat gegeven kan worden aan kinderen. Het blijft daarom belangrijk om verder onderzoek te doen dat deze afstand tussen het laboratorium en de kliniek overbrugt.

LIST OF PUBLICATIONS

1. van der Linden MH, Valsecchi MG, De Lorenzo P, Moricke A, Janka G, Leblanc TM, et al. Outcome of congenital acute lymphoblastic leukemia treated on the Interfant-99 protocol. Blood. 2009 Oct 29;114(18):3764-8.

2. Stam RW, Schneider P, Hagelstein JA, van der Linden MH, Stumpel DJ, de Menezes RX, et al. Gene expression profiling-based dissection of MLL translocated and MLL germline acute lymphoblastic leukemia in infants. Blood. 2010 Apr 8;115(14):2835-44.

3. van der Linden MH, Creemers S, Pieters R. Diagnosis and management of neonatal leukaemia. Seminars in fetal & neonatal medicine.2012 Aug;17(4):192-5.

4. van der Linden MH, Willekes M, van Roon E, Seslija L, Schneider P, Pieters R, Stam RW. MLL fusion driven activation of *CDK6* potentiates proliferation in *MLL*-rearranged infant ALL. Cell Cycle. 2014 Mar 1; 13(5): 834–844..

5. van der Linden MH, Seslija L, Schneider P, Driessen EM, Castro PG, Stumpel DJ, et al. Identification of genes transcriptionally responsive to the loss of MLL fusions in MLL-rearranged acute lymphoblastic leukemia. PloS one. 2015;10(3):e0120326.

6. van der Linden MH, Schneider P, Willekes M, Seslija L, Meijerink J, et al. Characterisation of wild-type *MLL* infant acute lymphoblastic leukemia reveals

high *MEIS1*-expression as most important prognostic factor. *Accepted for publication in Haematologica as letter to the editor.*

7. van der Linden MH, Elgerasma O, Rombout, S. Transvaginal ultrasound-guided thrombin injection for the treatment of secondary postpartum hemorrhage caused by a pseudoaneurysm of the uterine artery. *Invited case report for J Clin Case Rep.*

CURRICULUM VITAE

Marieke Hendrika van der Linden werd geboren op 26 augustus 1981 te Rotterdam. Zij behaalde haar diploma aan het Johan de Witt Gymnasium te Dordrecht in 1999. Hierna startte zij met de opleiding Bedrijfskunde aan de Erasmus Universiteit te Rotterdam, waar zij in 2004 in afstudeerde. Tevens behaalde zij in deze periode haar propedeuse Rechtsgeleerdheid. In 2005 werd zij toegelaten tot de studie Geneeskunde van het Erasmus MC, waarbij zij tevens werkzaam op het Trialbureau Interne Oncologie tot 2006. Zij deed vanaf 2006 afwisselend onderzoek op de afdeling Kinderoncologie en Immunologie. In 2007 startte zij met de master Clinical Research, in het kader waarvan zij een summer course volgde aan Harvard University te Boston, USA. Zij behaalde in 2010 zowel haar doctoraal Geneeskunde als haar master Clinical Research. In maart 2009 begon zij haar promotietraject op de afdeling Kinderoncologie, dat resulteerde in dit proefschrift, onder begeleiding van prof. dr. Rob Pieters and dr. Ronald Stam. Vanaf augustus 2012 volgde zij haar coschappen, die zij in augustus 2014 *cum laude* afsloot. Hierna is zij tot november 2015 werkzaam geweest als arts-assistent op de afdeling Gynaecologie van het Albert Schweitzer Ziekenhuis te Dordrecht. In december 2015 is zij begonnen met de opleiding tot gynaecoloog aan het Erasmus MC (opleider mw. dr. Ten Kate), waarvan de eerste twee jaar als AIOS zullen plaatsvinden in het Amphia ziekenhuis te Breda (opleider mw. dr. Dijksterhuis). Zij woont samen in Rotterdam met haar partner Tiede-Jan de Jong en dochters Saar (2011) en Sophie (2013).

PhD PORTFOLIO

Name PhD student:	Marieke Hendrika van der Linden
Erasmus MC Department:	Pediatric Oncology
Research School:	Molecular Medicine (MM)
PhD-period:	March 1st 2009-September 1st 2012
Promotor:	Prof. dr. R. Pieters
Co-promotor:	Dr. R.W. Stam

PhD training

Biomedical courses		
	Biomedical English Writing and Communication	2010
	Classical Methods for Data Analysis (NIHES)	2009
Specific courses		
	Micro-array Data Analysis using R & Bioconductor Intensive Course (LUMC)	2009
	Applied Bioinformatics (MM)	2009
	Basic and Translation Oncology (MM)	2009
	Biomedical Research Techniques	2009
	Browsing the genome with Ensembl	2010

Seminars and workshops		
	Annual Molecular Medicine Day, Erasmus MC	2009-2012
	Annual Pediatric Oncology Symposium, Erasmus MC	2009-2011
	Annual PhD Day, Erasmus MC	2009-2011
	Annual Pediatric Research Day, Erasmus MC	2009-2012
	KiKa Promovendi Dag	2011-2012
Presentations		
	See also '(Inter)national conferences'	
	8 oral presentations at the weekly Pediatric Research Meetings and Pediatric Oncology Research Meetings	2009-2012
	Oral presentation Molecular Medicine Day	2012
(Inter)national conferences		
	51st ASH Annual Meeting, New Orleans, USA (poster presentation)	2008
	AACR Cancer Genetics, Puerto Rico, USA (poster presentation)	2010
Teaching		
	Supervising Mariëlle Maas, HLO bachelor thesis, Avans Hogeschool Breda	2011

DANKWOORD

Zonder enige relevante pipetteerervaring, kwam ik als student in 2006 aan op het laboratorium Kindergeneeskunde om “wat onderzoek te doen”. Nu zes jaar later, ligt er een boekje vol met experimenten en analyses die ik niet had kunnen uitvoeren zonder de hulp van een heleboel mensen en instellingen.

Ik wil beginnen met het bedanken van alle (ouders van) patiëntjes die hebben meegewerkt aan dit onderzoek. Ik hoop dat jullie bijdrage snel zal helpen om de overleving van leukemie bij zuigelingen te verbeteren. Ook wil ik Stichting Kinderen Kankervrij (KiKa) bedanken voor de financiële ondersteuning van dit onderzoek. En KiKa zou niet bestaan zonder de vele mensen die zich belangeloos inspannen met als doel de overleving van kinderen met kanker te verbeteren, dank jullie allen!

Rob, toen ik bij jou kwam omdat ik als student Geneeskunde onderzoek wilde doen naast mijn studie, heb je me verteld dat ik het best ervaring kon opdoen op het laboratorium. Hoewel me dit lang niet zulk sexy onderzoek leek als het onderzoek in de kliniek, ben ik gaan kijken en wat was dat een goede keuze! Naast dat het lab inderdaad een hele goede leerschool is om de biologie en daarmee geneeskunde echt te leren begrijpen, heb ik ook ontzettend veel van jou geleerd. Op een heel relaxte wijze kon je altijd over alle uithoeken in mijn onderzoek meepraten en me af en toe terugroepen naar de rode draad. Het was jouw vraag aan het einde van elke bespreking “En wat ga je nu eerst doen?”, dat dit boekje er nu ligt en niet gestrand is in tal van onuitgewerkte ideeën.

Ronald, je bent begonnen me uit te leggen hoe ik een pipet moest vasthouden (niet met mijn wijsvinger bovenop de pipet dus), wat illustreert hoe bleu ik de eerste dag bij je aankwam. Met engelengeduld echter heb je altijd zowel mijn

biologische als technische vragen rustig weten te beantwoorden. Jouw kracht van duidelijk uitleggen komt naar voren in hoe je presenteert en schrijft, waarin ik enorm veel van je geleerd heb afgelopen jaren. Als mensen begrijpen wat ik probeer te zeggen in dit boekje, is dat grotendeels aan jou te danken!

Lidija, wat heb jij me veel geholpen in het begin. Altijd had je tijd om me bij de hand te nemen en de meest basale labtechnieken uit te leggen. Ik heb jou als collega en persoon altijd enorm gewaardeerd en vind het nog steeds jammer dat je weg bent gegaan, maar ben blij dat je het zo naar je zin hebt in je (inmiddels allang niet meer) nieuwe baan. Pauline, Merel en Sandra, als analisten van de Infant-groep staan jullie altijd klaar om vragen te beantwoorden of experimentjes uit te voeren voor ons, dank jullie!

Trudy, Marjolein en Iris, wat een leuke collega's leverden ze van de AML!

Emma, vanaf de keer dat we het in het elektroforesehok over apenliefde spraken, ben je mijn leukste collega ooit. Tijden lang is het zo geweest dat ik jou meer sprak dan Tiede in een week. Werkelijk bijna alles is over tafel geweest en meer dan eens hebben we (soms wel heel duistere) kanten ontdekt aan onszelf, waardoor ik ook mezelf beter heb leren kennen afgelopen jaren. Naast dat ik je (uiteraard) een enorm gezellige collega vind, vind ik je ook nog eens een heel goede onderzoeker met een hele kritische kijk op je data. Meer dan vanzelfsprekend behoor jij dan ook mijn paranimf te zijn vandaag!

MTQ, unique, magnifique en heel ludique (?), ik ben zo blij dat ik met jullie mijn studententijd heb mogen doen! Met deze periode toch maar mooi in de pocket, kan ik nu het serieuzere leven aan, waar onder andere het schrijven van dit boekje het resultaat van is. Dat we later als oude vrouwtjes maar weer aan het strand van Salou, Llorret da Mar of Miami (..misschien nu wel echt Florida ☺) mogen liggen!

Lieve familie, hoe truttig het dan ook moge klinken, ik voel me oprecht gezegend met een familie waarin het (meestal) enorm gezellig is, heftig gediscussieerd kan worden zonder ruzie te maken en waarin iedereen ook nog eens echt altijd voor elkaar klaar staat. Door jullie ben ik opgegroeid tot iemand die redelijk met twee benen op de grond staat, wat meer dan wie of wat dan ook ten grondslag ligt aan dit boekje. Lieve mama, zonder jou was ik nu nooit zo gelukkig geweest!

Lieve Tiede-Jan, hier dan het resultaat van waar ik zes jaar lang mee bezig ben geweest en waar jij de afgelopen drie jaar niet meer naar hebt mogen vragen. Hoewel dit boekje er vast ook wel had gekomen zonder jou, door jou is het leuk gebleven, ook als we tussendoor besluiten nog een huis te verbouwen (óns huis!) of na maanden slaaptekort door een nieuw klein meisje in ons leven. Samen kunnen wij alles! Lieve Saar en Sophie, jullie zijn met stip het beste wat ik ooit bereikt heb!

APPENDICES

SUPPLEMENTAL MATERIAL CHAPTER 4

Table 1. Patient characteristics

#	Chip name / color code	MLL translocation	age (months)	sex	Immuno-Phenotype
1	1488_4-11	t(4;11)	4.21	female	pro-B
2	1227_4-11	t(4;11)	10.28	female	pro-B
3	1442_4-11	t(4;11)	1.94	male	pro-B
4	VU9815_4-11	t(4;11)	3.22	male	pro-B
5	1587_4-11	t(4;11)	0.62	female	pro-B
6	1776_4-11	t(4;11)	0.66	female	pro-B
7	178_4-11	t(4;11)	5.59	male	pro-B
8	1817_4-11	t(4;11)	0.00		B-lineage not specified
9	1966_4-11	t(4;11)	1.91	female	pro-B
10	1977_4-11	t(4;11)	6.41	female	pro-B
11	1990_4-11	t(4;11)	6.44	male	pro-B
12	2582_4-11	t(4;11)	1.61	female	pro-B
13	2864_4-11	t(4;11)	0.79	female	pro-B
14	300_4-11	t(4;11)	6.83	female	B-lineage not specified
15	3218_4-11	t(4;11)	8.05	male	pre-B
16	3230_4-11	t(4;11)	5.91	female	pro-B
17	3595_4-11	t(4;11)	3.45	male	pro-B
18	3686_4-11	t(4;11)	1.87	female	pro-B
19	3814_4-11	t(4;11)	1.61	male	pro-B
20	385_4-11	t(4;11)	9.43	female	pro-B
21	3980_4-11	t(4;11)	3.09	female	pro-B
22	3939_4-11	t(4;11)	1.22	female	pro-B
23	4669_4-11	t(4;11)	0.00	female	pro-B
24	4190_4-11	t(4;11)	4.37	male	pro-B
25	4757_4-11	t(4;11)	0.00	female	pro-B
26	4773_4-11	t(4;11)	0.00	female	pro-B
27	929_4-11	t(4;11)	3.58	male	pro-B

28	788v_4-11	t(4;11)	9.43	female	pro-B
29	635_4-11	t(4;11)	2.83	male	pro-B
30	vr39_11-19	t(11;19)	7.72	male	pro-B
31	1037_11-19	t(11;19)	8.77	female	common
32	1060_11-19	t(11;19)	5.72	female	pre-B
33	1191_11-19	t(11;19)	9.10	male	pre-B
34	1225_11-19	t(11;19)	3.65	female	common
35	1679_11-19	t(11;19)	2.30	female	pro-B
36	1702_11-19	t(11;19)	11.01	male	pro-B
37	2009_11-19	t(11;19)	0.00	female	pro-B
38	2458_11-19	t(11;19)	6.01	female	pro-B
39	2346_11-19	t(11;19)	3.35	female	pro-B
40	2146_11-19	t(11;19)	5.65	female	pro-B
41	2571_11-19	t(11;19)	0.00	male	B-lineage not specified
42	3922_11-19	t(11;19)	4.14	male	common
43	3831_11-19	t(11;19)	2.00	female	pre-B
44	4483_11-19	t(11;19)	0.00	male	B-lineage not specified
45	54_11-19	t(11;19)	3.09	female	pro-B
46	474_11-19	t(11;19)	0.00	male	B-lineage not specified
47	668_11-19	t(11;19)	0.03	female	pro-B
48	618_11-19	t(11;19)	5.39	male	pro-B
49	711_11-19	t(11;19)	10.74	male	pro-B
50	743_11-19	t(11;19)	7.79	male	pre-B
51	888_11-19	t(11;19)	5.32	female	pro-B
52	148_9-11	t(9;11)	0.36	female	B-lineage not specified
53	1501_9-11	t(9;11)	10.25	female	pro-B
54	1656_9-11	t(9;11)	6.01	female	pre-B
55	2088_9-11	t(9;11)	11.66	male	pro-B
56	3921_9-11	t(9;11)	4.14	male	B-lineage not specified
57	4919_9-11	t(9;11)	0.00	male	B-lineage not specified

58	620_9-11	t(9;11)	9.82	female	pre-B
59	656_9-11	t(9;11)	4.21	male	pre-B
60	2249_germline	MLL germline	6.34	female	B-lineage not specified
61	2624_germline	MLL germline	11.33	female	pre-B
62	2807_germline	MLL germline	11.07	male	pre-B
63	3310_germline	MLL germline	0.00	male	B-lineage not specified
64	382_germline	MLL germline	0.00	female	B-lineage not specified
65	4159_germline	MLL germline	2.53	male	pre-B
66	4927_germline	MLL germline	0.00	male	B-lineage not specified
67	512_germline	MLL germline	5.49	male	pre-B
68	560_germline	MLL germline	5.98	male	pre-B
69	1093_germline	MLL germline	0.00	male	B-lineage not specified
70	682_germline	MLL germline	5.95	male	pre-B
71	824_germline	MLL germline	9.07	male	pro-B
72	943_germline	MLL germline	7.36	female	pre-B
73	927_germline	MLL germline	8.97	female	pre-B
74	3663_B-ALL	MLL germline	64.56	female	common
75	3665_B-ALL	MLL germline	182.4	male	pre-B
76	3700_B-ALL	MLL germline	44.04	female	pre-B
77	3708_B-ALL	MLL germline	52.32	male	pre-B
78	3716_B-ALL	MLL germline	26.88	male	common
79	3720_B-ALL	MLL germline	25.2	female	common
80	3738_B-ALL	MLL germline	65.76	female	common
81	3739_B-ALL	MLL germline	101.64	male	pre-B
82	3740_B-ALL	MLL germline	13.92	male	pre-B
83	3744_B-ALL	MLL germline	28.2	female	pre-B
84	3752_B-ALL	MLL germline	62.04	male	pre-B
85	3753_B-ALL	MLL germline	184.56	male	pre-B
86	3754_B-ALL	MLL germline	51.84	female	common
87	3769_B-ALL	MLL germline	92.28	male	common
88	3791_B-ALL	MLL germline	52.32	female	common

89	3799_B-ALL	MLL germline	58.32	male	pre-B

Table 2. Probe set IDs, gene names, log-fold changes and p-values, ALL samples

Gene name	HU133plus2.0 probe ID	logFC	adj. p-value (FDR<0.01)
CD10	203434_s_at	-3.49466	1.90E-13
DKFZP586H0519	203593_at	-1.7241	1.16E-06
CD24	209772_s_at	-1.87863	2.09E-05
DYRK3	210151_s_at	-0.72122	0.003989125
KIAA0867	211789_s_at	-1.92882	3.03E-11
ITPR1	211323_s_at	-1.09037	2.01E-06
DNTT (TDT)	210487_at	-2.11708	0.000680043
SPTA1	206937_at	-0.98777	2.62E-06
CD22	204581_at	-1.08489	0.000269759
cDNA 13f12 (RBQ-1)	205178_s_at	-0.89437	1.47E-06
DKFZp564I083	215164_at	-2.44457	1.40E-08
FOXO1A	202723_s_at	-1.26208	3.77E-06
FHIT	206492_at	-2.16086	3.53E-15
MYH10	212372_at	-0.99061	3.71E-08
SPTBN1	215918_s_at	-1.47501	2.21E-11
LIG4	206235_at	-1.20202	5.92E-08
NPR1	204648_at	-1.19589	3.08E-06
PRKCH	206099_at	-1.08029	1.12E-06
PARD3 (qb92h04)	210094_s_at	-1.58949	2.34E-10
KIAA0959 (RGL1)	209568_s_at	-0.73142	0.000176538
TERF2	203611_at	-0.24117	0.355355289
ITPR1	203710_at	-0.72207	0.001656473
PIK3C2B	204484_at	-0.22589	0.417294927
MYLK	202555_s_at	-2.00744	3.04E-10
SPTAN1	208611_s_at	-1.07247	5.45E-12
Cosmid TN62 (TNFSF3)	207339_s_at	-0.62413	0.043504
ITPR3	201188_s_at	-1.66796	8.32E-13
POU2AF1	205267_at	-0.69591	0.025510066
MADH1	210993_s_at	-3.36853	5.19E-12
SMARCA4	212520_s_at	-0.97677	1.02E-08
HLA-DQB1	209480_at	-0.3784	0.661531493
KIAA0250	201794_s_at	-0.18079	0.198515005
APP (Amyloid A4)	214953_s_at	-1.11575	6.60E-05
MADH1	227798_at	-2.93574	1.39E-08

LARGE	215543_s_at	-1.55638	3.05E-07
ZNF45	207304_at	-0.53137	0.014406606
DBN1	217025_s_at	-0.98332	1.32E-06
KIAA0212	203279_at	-0.79365	3.41E-09
ALOX5	204446_s_at	-2.23795	1.03E-10
KIAA0093	213012_at	-0.17579	0.524138429
Chromosome 1 PAC	207826_s_at	-2.72038	4.01E-07
cDNA wg66h09	207971_s_at	-0.40222	0.003024465
VAMP5	204929_s_at	-0.32312	0.10523592
cDNA YY38E04	213766_x_at	-0.99807	0.000217517
NEDDL4	212445_s_at	-0.79196	4.57E-05
LDOC1	204454_at	-0.22459	0.482524024
FGFR1	211535_s_at	-1.38079	1.57E-10
HLA-DQB1	211654_x_at	-0.3086	0.351706056
LGALS1	201105_at	3.410196	3.57E-16
cDNAqf71b11	200872_at	2.303216	3.76E-18
AHNAK	212992_at	0.194004	0.420820516
NKG2D	205821_at	1.639294	1.11E-08
CCNA1	205899_at	2.725713	1.18E-08
CD44	212063_at	1.177711	2.95E-10
PMX1 (PHOX1)	226695_at	-0.27321	0.260110668
KIAA0027	213395_at	0.894091	2.23E-05
cDNA tn15f08cDNA	203781_at	1.585328	2.67E-13
cDNA zd69b10	203186_s_at	1.812345	2.90E-15
HOXA9	214651_s_at	3.57721	4.00E-06
CD44	204490_s_at	1.006367	1.10E-05
CD44	204489_s_at	0.699904	0.00128117
Chromosome 7 Clone	213823_at	0.64659	0.029421521
ANXA1	201012_at	1.939072	4.42E-08
Chromosome X Clone	205504_at	0.384011	0.051790236
PTPRC (CD45)	212588_at	1.175792	2.97E-10
CD44	212014_x_at	0.648347	0.002602935
PTPRC (CD45)	207238_s_at	1.049341	1.73E-06
LILRB1	229937_x_at	0.746738	0.00668284
cDNA zd27g05	204122_at	1.016228	0.000274492
RNASE3	206851_at	2.042179	1.47E-06
SERPINB1	212268_at	1.194162	1.73E-07

PROML1	204304_s_at	3.376316	2.40E-10
Chromosome 22 sequence	201105_at	3.410196	3.57E-16
DKFZp586K1720	214752_x_at	0.779126	4.25E-06
cDNA oq40b07	209610_s_at	0.701223	0.000133648
KIAA0120	210978_s_at	0.644645	5.17E-06
EBI2	205419_at	1.427619	0.000940803
IGFBP7	201162_at	3.351629	4.32E-14
ANXA2	208816_x_at	1.012687	2.84E-12
DAD1	200046_at	1.674455	2.20E-08
SCGF	211709_s_at	1.640905	2.11E-07
cDNA qe04b02	203837_at	1.040053	8.32E-08
MAP7	202890_at	2.55388	2.07E-08
ITPA	209171_at	0.618455	0.000170319
PRG1	213496_at	0.017213	0.949528842
GLUD1	200947_s_at	0.631157	3.04E-08
FEZ1	203562_at	1.155458	7.96E-05
LILRA1	207872_s_at	0.545992	0.009583201
KIAA0428	201153_s_at	1.281491	7.30E-19
MT1B	217165_x_at	1.683759	1.18E-07
HOXA5	213844_at	3.198684	4.27E-07
DKFZp564A032	204502_at	1.666619	1.35E-09
cDNA 03h03	212859_x_at	0.635986	0.06471336
DKFZp586B2022	202720_at	1.664821	1.30E-09
cDNA ou23f10	205081_at	2.27626	6.92E-12
ANXA2P2	211241_at	0.828735	1.97E-05
DKFZp586C1619	232624_at	-0.37853	0.004537581

Table 3. Probe set IDs, gene names, log-fold changes and p-values, B-ALL samples

Gene name	U95A probe ID	HU133plus2 probe ID	logFC	adj. p-value (FDR<0.01)
MBNL	34306_at	201153_s_at	1.281491	7.30E-19
ADAM10	40797_at	202603_at	1.118213	2.77E-09
LGALS1	33412_at	228416_at	-0.07693	0.758135211
S100A10	39338_at	200872_at	2.303216	3.76E-18
IGFBP7	2062_at	204253_s_at	-0.84478	6.55E-05
PLXNC1	32193_at	206470_at	0.88019	0.000748791
PTPRC	40518_at	212588_at	1.175792	2.97E-10
D12S2489E	36777_at	205821_at	1.639294	1.11E-08
MPP1	32207_at	202974_at	-0.02838	0.907767139
SAP18	33859_at	208741_at	0.52054	0.005668646
CAPG	38391_at	201850_at	1.251809	8.46E-07
MEIS1	40763_at	204069_at	4.199016	7.99E-28
CD44	1126_at	212063_at	1.177711	2.95E-10
FKBP5	34721_at	224840_at	1.220177	0.000124693
HOXA9	37809_at	214651_s_at	3.57721	4.00E-06
GOLGA3	34861_at	226949_at	-0.05704	0.650787509
IGKC	38194_s_at	224795_x_at	-0.86087	0.000306183
PCDHGC3	657_at	215836_s_at	0.651789	0.00113155
GUCY1A3	36918_at	229530_at	0.665482	0.040991331
KIAA0878	32215_i_at	225202_at	1.710707	1.24E-07
LY75	38160_at	205668_at	1.12421	1.18E-05
DAD1	38413_at	200046_at	1.674455	2.20E-08
MME	1389_at	203434_s_at	-3.49466	1.90E-13
DNTT	34168_at	210487_at	-2.11708	0.000680043
CD44	2036_s_at	204490_s_at	1.006367	1.10E-05
GLUL	40522_at	215001_s_at	0.666713	0.000791835
BLK	854_at	206255_at	0.888812	4.53E-06
ELF1	40067_at	212420_at	-0.36957	0.078609658
XBP1	39756_g_at	200670_at	0.043623	0.808221545
TIAF1	36940_at	202039_at	-0.71642	8.71E-06
RASA1	36935_at	202677_at	0.276024	0.159642266
DKFZP586B2022	32134_at	202720_at	1.664821	1.30E-09
DKFZp586C1019	39379_at	212371_at	0.367991	0.006577485

CD44	40493_at	204489_s_at	0.699904	0.00128117
ANXA2	769_at	208816_x_at	1.012687	2.84E-12
ACAA1	40415_at	214274_s_at	0.50277	8.39E-05
R32184_1	35983_at	209461_x_at	0.898273	0.000886448
PTPRC	40519_at	212588_at	1.175792	2.97E-10
PTPN6	794_at	206687_s_at	0.490544	0.005199699
DNAJB6	41234_at	209015_s_at	0.700531	0.012168026

Table 4. Probe set IDs, gene names, log-fold changes and p-values corresponding to Fig. 2

Gene name	HU133plus2.0 probe ID	logFC	adj. p-value (FDR<0.01)
C20orf103	219463_at	5.086355852	5.19E-43
MEIS1	1559477_s_at	4.202263797	3.49E-27
MEIS1.	204069_at	4.199004835	3.65E-27
MEIS1..	242172_at	3.393844331	7.76E-25
C16orf54	1559584_a_at	1.871648724	7.74E-20
LGALS1	201105_at	3.479053576	5.94E-19
MBNL	201153_s_at	1.291895405	7.81E-19
S100A10	200872_at	2.302110256	2.88E-18
IGFBP7	201163_s_at	3.341882785	1.65E-17
CRI1	211698_at	1.464354162	1.74E-16
COMMD8	218351_at	1.952872849	2.81E-16
C20orf118	235529_x_at	2.289038674	1.05E-15
SMT3H2	213879_at	0.852744138	1.52E-15
ARPC3	208736_at	0.972323597	4.22E-15
S100A4	203186_s_at	1.823768435	4.29E-15
MBNL.	235879_at	1.359727641	4.29E-15
LOC389203	225014_at	1.287510226	1.17E-14
NDUFC2	206936_x_at	0.651835075	1.48E-14
CTSC	225647_s_at	1.899361672	4.34E-14
SRD5A1	204675_at	1.260205611	4.73E-14
IGFBP7.	201162_at	3.374287701	7.02E-14
SCP2	211733_x_at	1.251595913	7.31E-14
RPL38	202028_s_at	0.878550714	7.67E-14
MBNL..	235173_at	1.085835956	8.82E-14
MDS027	224575_at	0.992232272	9.30E-14
CTSC.	201487_at	2.080800234	1.03E-13
C19orf42	224717_s_at	0.927622926	1.03E-13
KCNK12	220448_at	2.193352061	1.18E-13
CLPTM1L	226935_s_at	0.769917526	1.30E-13
NDUFC2.	222521_x_at	0.890885477	1.32E-13
MVK	36907_at	0.570506672	1.42E-13
HAAO	205657_at	0.903401219	2.39E-13
TOR2A	227972_at	1.550603876	2.81E-13

KIAA0141	201978_s_at	0.710897378	3.37E-13
C2orf1	203781_at	1.590817778	4.33E-13
GHITM	209248_at	0.87751713	4.60E-13
ITM2B	217731_s_at	1.01223592	5.32E-13
Unknown (1570185_at)	1570185_at	0.759764607	5.36E-13
Unknown (235901_at)	235901_at	1.300217381	6.28E-13
Unknown (226789_at)	226789_at	1.580648571	6.85E-13
TLP19	223017_at	0.781353543	6.85E-13
SNRPE	215450_at	1.072435973	7.48E-13
RNAHP	213629_x_at	1.45165087	9.23E-13
LOC54499	208716_s_at	1.468452539	1.06E-12
COX7AP2	217249_x_at	0.906253199	1.08E-12
PPAP2A	210946_at	1.054668698	1.13E-12
SRP19	205335_s_at	0.817362746	1.42E-12
COX7A2	201597_at	0.801864974	1.47E-12
METTL7A	207761_s_at	1.687467961	1.48E-12
FAM78A	227002_at	1.35316581	1.48E-12
AKAP12	1555395_at	-2.643127448	1.06E-22
MYO5C	218966_at	-1.305441477	2.64E-21
SHANK3	227923_at	-3.76397613	5.00E-21
ZNF827	243618_s_at	-3.167649282	1.04E-19
AKAP12.	241679_at	-3.212610217	2.64E-18
AKAP12..	227530_at	-2.648595041	2.34E-17
FHIT	206492_at	-2.200485527	2.48E-17
AKAP12…	210517_s_at	-3.907135431	6.57E-17
ZNF827.	228046_at	-2.017206814	7.16E-17
STK32B	219686_at	-3.069238994	8.69E-17
Unknown (244740_at)	244740_at	-2.198463872	1.97E-16
Unknown (227388_at)	227388_at	-1.869299136	2.31E-16
ZNF91	206059_at	-1.411647206	2.66E-16
Unknown (230441_at)	230441_at	-1.927226199	8.06E-16
AKAP12….	227529_s_at	-2.416001719	9.12E-16
Unknown	215028_at	-2.511394633	2.20E-15

(215028_at)			
ZNF667	207120_at	-2.116303401	2.20E-15
ZNF667.	236635_at	-1.345140336	2.81E-15
ARID1A	210649_s_at	-1.165698911	4.68E-15
POMFIL3	224771_at	-2.240146612	6.24E-15
NFAT5	215092_s_at	-2.104686291	6.63E-15
ZNF827.	226764_at	-2.232241484	7.74E-15
EFNA1	202023_at	-2.094204389	9.10E-15
YES1	202932_at	-2.625443852	1.18E-14
CALN1	230698_at	-2.258640782	1.18E-14
UBL3	201534_s_at	-1.37815028	2.58E-14
APOL2	221013_s_at	-1.363346757	3.12E-14
PRKCZ	202178_at	-2.581065357	4.71E-14
MME	203434_s_at	-3.530878734	5.94E-14
POMFIL3.	224773_at	-2.552668536	9.41E-14
RASD1	223467_at	-2.102175015	9.42E-14
AKAP12…..	231067_s_at	-2.574167828	1.04E-13
DAGLB	225833_at	-1.365151723	1.04E-13
NYX	234496_x_at	-1.412919122	1.17E-13
DKFZp434P0235	231902_at	-1.370237301	2.13E-13
NAV1	224772_at	-2.360986107	2.15E-13
CALN1.	223885_at	-2.148017117	2.17E-13
YES1.	202933_s_at	-2.743102242	2.44E-13
GFOD1	219821_s_at	-1.658227745	3.02E-13
PDE4B	211302_s_at	-1.806891159	4.33E-13
Unknown (241816_at)	241816_at	-1.180486004	4.33E-13
ANGPT2	211148_s_at	-1.873862688	4.67E-13
SPTB2	212071_s_at	-1.426532627	4.67E-13
DAGLB.	225832_s_at	-1.098623437	5.14E-13
SPTBN1	200671_s_at	-2.343033058	5.36E-13
CDC2L2	215329_s_at	-0.968128295	6.55E-13
POU4F1	211341_at	-2.282947956	6.96E-13
Unknown (244741_s_at)	244741_s_at	-1.780574222	7.91E-13
RAB2	208730_x_at	-0.784504074	8.05E-13
CHD7	226123_at	-1.183746619	1.12E-12

Table 5. Probe set IDs, gene names, log-fold changes and p-values corresponding to Fig. 4

Gene name	HU133plus2.0 probe ID	logFC	adj. p-value (FDR<0.01)
C20orf103	219463_at	4.496028591	4.64E-36
MEIS1	204069_at	3.475319004	4.08E-22
MEIS1 .	1559477_s_at	3.452660911	1.02E-21
MEIS1 ..	242172_at	2.858976873	1.02E-21
KCNK12	220448_at	2.284088949	4.37E-19
LGALS1	201105_at	2.748692614	7.09E-17
MBNL	201153_s_at	1.03307555	6.19E-15
FLT3	206674_at	2.067097531	6.78E-15
Unknown(243605_at)	243605_at	1.982931386	9.46E-15
Unknown(204304_s_at)	204304_s_at	3.217498592	9.79E-15
NUDT7	228855_at	1.658193543	3.62E-14
CCNA1	205899_at	2.89291088	4.68E-14
S100A10	200872_at	1.723441249	7.98E-14
Unknown (234032_at)	234032_at	1.376165898	8.35E-14
MBNL.	235173_at	0.854609698	1.10E-13
IGFBP7	201163_s_at	2.475715661	1.11E-13
MBNL..	235879_at	1.197917647	1.11E-13
FAM78A	227002_at	1.125156839	4.55E-13
HSCP1	218217_at	1.160329207	5.99E-13
NKG2D	205821_at	1.652298539	9.59E-13
C20orf118	235529_x_at	1.660988391	1.07E-12
METTL7A	207761_s_at	1.401316307	1.21E-12
ZCCHC7	1555562_a_at	1.192352353	1.54E-12
CPEB2	226939_at	1.49657958	1.75E-12
GREM1	218468_s_at	2.773842337	1.85E-12
CD44	212063_at	1.123775805	1.97E-12
C20orf118.	1559883_s_at	1.367480064	2.41E-12
Unknown (1552665_at)	1552665_at	1.327997132	2.42E-12
VAT1L	226415_at	2.903226028	2.42E-12
C11orf24	52164_at	0.701723316	2.88E-12
PAN3	225563_at	0.997766635	3.42E-12

IGFBP7.	201162_at	2.640903861	3.97E-12
RPL33L	203781_at	1.183702192	7.00E-12
Unknown (226789_at)	226789_at	1.320430935	8.71E-12
CORO1C	222409_at	1.295663476	1.52E-11
TECT1	218584_at	0.947469049	1.79E-11
CKLFSF3	224733_at	0.958636382	1.82E-11
Unknown (1555392_at)	1555392_at	1.60386068	1.83E-11
CD72	215925_s_at	1.810496724	2.39E-11
C20orf118..	204502_at	1.499926812	2.49E-11
LOC219688	1559266_s_at	1.882003671	2.57E-11
PPAP2A	210946_at	0.8079595	3.01E-11
LOC219688.	1559265_at	1.647289119	3.14E-11
MBNL…	1558111_at	1.154555727	3.14E-11
Unknown (226413_at)	226413_at	0.646134752	3.27E-11
MBNL….	201152_s_at	0.920890371	3.53E-11
RNASE6	213566_at	2.231902816	5.09E-11
SERPINB1	213572_s_at	1.219791703	5.79E-11
CPEB2.	235479_at	1.207289953	6.80E-11
GREM1.	218469_at	2.625424931	6.83E-11
PTRF	208789_at	0.886125075	7.16E-05
SCD	200832_s_at	1.390183259	8.65E-05
CMTM7	226017_at	1.051200988	0.000235686
TNFSF3	207339_s_at	1.42215701	0.000299884
P2Y14	206637_at	1.36502195	0.000390702
HNRPLL	225386_s_at	1.30473207	0.000390702
MYL4	210395_x_at	0.797261557	0.000390702
SPEC2	224709_s_at	0.530992075	0.000390702
PLEKHG4B	230671_at	1.093216829	0.000426285
CMTM7.	235099_at	2.15475961	0.000436216
WSB2	201760_s_at	0.696565857	0.000605921
COBL	213050_at	0.964554956	0.001099439
WSB2.	213734_at	0.634303395	0.001446517
Deltex homolog 1 (Drosophila)	1559618_at	1.260813821	0.001524853
Unknown (228528_at)	228528_at	0.62742243	0.001834701

SPEC2.	1552613_s_at	0.655077446	0.001958731
NRN1	218625_at	1.524230398	0.002040378
ELFN2	1559072_a_at	1.004144111	0.002040378
MYL4.	216054_x_at	0.613076445	0.002208751
CD158K	207314_x_at	1.071722302	0.002468029
CASP8	213373_s_at	0.682993768	0.003067496
WDR38	243900_at	0.501769801	0.003067496
DOCK9	212538_at	1.051387026	0.004345185
SH2D1A	211209_x_at	0.872446985	0.005107091
SEC15L1	232599_at	0.768120099	0.005549215
PRL3	206574_s_at	1.376636931	0.005653317
REPIN1	219041_s_at	0.681680653	0.005754645
RYK	202853_s_at	1.061888889	0.006104664
T-cell receptor beta C gene segment	211796_s_at	1.211717656	0.006677468
SH2D4B	1563849_at	0.725902423	0.007191717
KIR2DL1	210890_x_at	0.530675354	0.007271407
Unknown (221773_at)	221773_at	1.207076779	0.00736279
TP53INP1A	225912_at	1.533384709	0.007655558
LZTS2	1555881_s_at	0.530522084	0.007902655
ICAM3	204949_at	0.99224382	0.008007863
CD27	206150_at	0.89864279	0.008180476
Unknown (232951_at)	232951_at	1.02446043	0.008478862
ABLIM1	200965_s_at	0.7503754	0.008661745
SPEC2..	234260_at	0.656905905	0.008848429
GNPDA1	202382_s_at	0.889163034	0.009258671
Unknown (244375_at)	244375_at	0.61879128	0.00946457
SPR-1	236265_at	0.70376123	0.0096611
HOXB2	205453_at	1.017011133	0.009766752
UEV3	1554396_at	0.652376127	0.010414273
T-cell receptor beta C gene segment.	213193_x_at	0.740538672	0.010733429
CD3Z	210031_at	1.132155362	0.011137508
CKLFSF7	1560754_at	0.819553574	0.012046886
PLEKHF2	218640_s_at	0.679038624	0.012046886
SH3P12	218087_s_at	0.992758199	0.012571455

CD3G	206804_at	0.837705322	0.012571455
AKAP12	1555395_at	2.445440415	5.25E-16
NYX	234496_x_at	1.435416127	1.91E-15
ARID1A	210649_s_at	1.121271433	1.91E-15
ZNF91	206059_at	1.331838524	1.13E-14
RAB2A	208730_x_at	0.786920002	1.65E-14
RASD1	223467_at	2.088478771	2.68E-14
EFNA1	202023_at	2.001846724	2.68E-14
NFAT5	215092_s_at	2.048052481	3.43E-14
UBL3	201534_s_at	1.363691523	3.43E-14
APOL2	221013_s_at	1.334369446	3.43E-14
CDC2L2	215329_s_at	0.985832306	3.43E-14
CALN1	230698_at	2.160466521	9.11E-14
GGT1	209918_at	0.954882312	1.11E-13
Unknown (244491_at)	244491_at	1.063219407	1.58E-13
DAGLB	225833_at	1.299517309	1.76E-13
ZNF667	207120_at	2.005419594	2.72E-13
Unknown (227388_at)	227388_at	1.740422312	2.72E-13
MYLIP	223129_x_at	1.054861704	5.20E-13
SHANK3	227923_at	3.384060651	5.27E-13
HDAC3	227510_x_at	1.170891013	5.70E-13
ACIN1	201715_s_at	0.657718319	5.70E-13
PDE4B	211302_s_at	1.777677119	6.59E-13
GFOD1	219821_s_at	1.565474067	6.59E-13
ZNF667.	236635_at	1.256605046	6.59E-13
CDCREL1	209768_s_at	1.273608094	9.19E-13
NXF1	208922_s_at	0.949875647	1.39E-12
Unknown (244793_at)	244793_at	2.002411851	1.69E-12
SPTAN1	208611_s_at	1.018334408	2.63E-12
CALN1.	223885_at	2.02908685	2.91E-12
POMFIL3	224771_at	2.098774158	2.93E-12
MYO5C	218966_at	1.151360846	3.38E-12
IER5L	226552_at	1.741002346	3.56E-12
SLC38A2	220924_s_at	0.953419074	3.56E-12
DAGLB.	225832_s_at	1.044825568	3.59E-12

SLC38A2.	218041_x_at	0.935145477	4.07E-12
Unknown (237374_at)	237374_at	1.772348178	4.08E-12
FLJ31614	1553185_at	1.391684664	4.33E-12
MCART1	219480_at	2.104674304	5.85E-12
AKAP12.	241679_at	2.897861416	7.32E-12
STK32B	219686_at	2.732787621	9.41E-12
RNA exonuclease 1 homolog (S. cerevisiae)	244281_at	1.000907484	9.98E-12
AKAP12..	227530_at	2.417079866	1.24E-11
POU4F1	211341_at	2.182371857	1.24E-11
LOC100128252	244740_at	2.008494047	1.24E-11
NEDD9	202150_s_at	1.540484513	1.49E-11
IER3	201631_s_at	1.761438273	1.54E-11
SPTB2	200671_s_at	2.197947386	1.95E-11
SFRS7	213649_at	0.984624698	1.97E-11
SMARCF1	212152_x_at	0.737661675	2.18E-11
EHD1	209038_s_at	1.626907923	2.32E-11

Table 6. Probe set IDs, gene names, log-fold changes and p-values corresponding to Fig. 5

Gene name	HU133plus2.0 probe ID	logFC	adj. p-value (FDR<0.01)
TOCA1	215017_s_at	2.559953	6.89E-15
TPD52L2	201379_s_at	1.488809	2.15E-13
DNTT	210487_at	3.063569	6.35E-10
DNTT.	1566363_at	2.572288	7.28E-10
CCNJ	229091_s_at	0.926719	1.43E-09
TOCA1.	242310_at	1.374711	3.44E-09
LHFP	218656_s_at	1.694537	7.26E-09
CCNJ.	219470_x_at	0.796031	7.26E-09
DKFZp451A211	1556114_a_at	1.285373	7.95E-09
CDKN2A	209644_x_at	0.923952	2.24E-08
DNTT..	1566362_at	1.746103	6.74E-08
MOCS2B	218212_s_at	1.363284	1.90E-07
S100A16	227998_at	1.278861	6.31E-07
CDKN2A.	207039_at	1.090366	1.45E-06
C5orf33	229299_at	1.090741	1.51E-06
CYB5R2	220230_s_at	1.498323	1.71E-06
NEK6	223158_s_at	0.806739	2.21E-06
C5orf33.	226946_at	1.080412	2.86E-06
LHFP.	232935_at	1.218228	4.48E-06
RNU19	1567681_at	0.885787	7.92E-06
MYC	202431_s_at	1.013794	8.47E-06
SYNGR1	210613_s_at	0.792152	8.47E-06
NPM1	221923_s_at	0.528623	1.12E-05
Matrin 3	242260_at	0.954129	1.49E-05
RPP40	213427_at	0.778875	1.63E-05
TBC1D16	222116_s_at	1.107452	2.23E-05
NUDT5 .	222824_at	0.842525	2.25E-05
GNG11	204115_at	1.534628	2.81E-05
DHX33	222875_at	0.606983	2.83E-05
HSPC111	203023_at	0.738846	2.84E-05
ABHD4	218581_at	0.970173	3.53E-05

LDHB	201030_x_at	0.499643	3.53E-05
AHCY	200903_s_at	0.615318	3.76E-05
BAG2	209406_at	0.818315	4.57E-05
COBLL1	203642_s_at	0.969948	4.62E-05
IGF1R	225330_at	0.74695	5.00E-05
NUDT5	223100_s_at	0.830156	5.53E-05
HMGIY	206074_s_at	0.674514	5.53E-05
Unknown (236238_at)	236238_at	1.062795	5.64E-05
DNAJC5	224612_s_at	0.545687	6.61E-05
PAK1IP1	218886_at	0.622538	7.12E-05
CACNA2D4.	228083_at	1.459348	8.65E-05
NPM3	205129_at	0.706398	8.70E-05
FLJ38678	228249_at	1.219178	0.000104108
CACNA2D4..	1552690_a_at	0.953812	0.000104108
ABHD4.	242023_at	1.289658	0.000109934
FLJ25521	1564151_at	0.809965	0.000125932
PABPC4	201064_s_at	0.52496	0.000125932
C1QBP	208910_s_at	0.621309	0.000131157
FLJ14028	224603_at	0.727887	0.000131732
IL12RB2	1560999_a_at	2.718085	2.81E-07
RUNX2	236859_at	2.629947	1.85E-05
SUPT3H	206506_s_at	1.27754	1.85E-05
REN	206367_at	1.393712	3.81E-05
RUNX2.	236858_s_at	1.994329	4.75E-05
FAM110C	226863_at	1.820986	4.82E-05
IL12RB2	206999_at	1.700688	4.82E-05
RUNX2	232231_at	2.324295	6.65E-05
BMI1	202265_at	1.278213	6.65E-05
KHDRBS3	209781_s_at	1.33512	9.59E-05
FLJ11572	232544_at	1.883434	0.000100591
Unknown (230986_at)	230986_at	1.618617	0.000112542
KHDRBS3	230249_at	1.244589	0.000159193
SUPT3H.	211106_at	1.022298	0.000319877

FLJ13387	1557285_at	1.715535	0.000371065
DNAJC1	242216_at	1.321202	0.000371065
HOXB9	216417_x_at	0.90293	0.000371065
CACNA2D2	204811_s_at	1.042558	0.000440239
Unknown (236764_at)	236764_at	1.031713	0.000440239
SPON2	218638_s_at	1.030903	0.000509256
KIAA1257	1554852_a_at	0.655182	0.000620548
TMEM64	225972_at	0.775037	0.000641599
TFAP2C	205286_at	1.44508	0.000672777
LTK	207106_s_at	0.833476	0.000672777
SH3PXD2A	224817_at	0.746945	0.000672777
Unknown (231369_at)	231369_at	0.567252	0.000672777
DNAJC1.	222621_at	0.977505	0.000737038
Unknown (243931_at)	243931_at	0.94868	0.000776962
HOXA3	235521_at	2.001119	0.000792966
TNFRSF14	209354_at	0.562151	0.000809727
PLCB4	203896_s_at	1.347276	0.000924533
TNFRSF18	224553_s_at	1.068249	0.000937889
LTK.	217184_s_at	0.930905	0.001145713
HOXA4	206289_at	1.382214	0.00115584
CPNE8	241706_at	1.237197	0.0012408
FLJ12053	233259_at	1.04071	0.0012408
DNAJC1..	222620_s_at	0.937347	0.0012408
LOC643977	235291_s_at	1.20954	0.001342176
HOXA2	214457_at	1.04513	0.001438474
TGFA	205016_at	1.038809	0.001495606
PLCB4.	203895_at	1.482765	0.001554123
FLJ42957	237591_at	0.635692	0.001554123
SPG3A	223340_at	0.999075	0.00160921
CLEC2B	209732_at	1.072006	0.001733017
TMF1	213024_at	0.566226	0.001841754
SNX30	226249_at	0.963997	0.001888555
CPNE8.	228365_at	1.372333	0.001898577

SERPINE1	1568765_at	1.101198	0.001899276
HOXA5	213844_at	2.250199	0.001956651
AREG	205239_at	1.908526	0.001985735
ZNF521	226677_at	4.473705	4.87E-22
ZNF521.	226676_at	4.180613	1.85E-21
CARD11	223514_at	1.969846	1.94E-09
COL9A1	243932_at	2.018196	1.11E-08
PBX3	204082_at	1.885843	2.09E-08
TBL1X	213400_s_at	2.677367	1.26E-07
OCIAD2	225314_at	1.47741	2.83E-07
SLU7	212592_at	3.915242	3.16E-07
PIP4K2C	218942_at	2.098933	4.54E-07
FLJ23834	235650_at	2.101898	5.38E-07
SCN3A	210432_s_at	2.920688	1.20E-06
SEF2	212387_at	1.872205	4.51E-06
Unknown (230441_at)	230441_at	1.178759	4.51E-06
Unknown (242846_at)	242846_at	1.346623	7.20E-06
CSMD1	241960_at	2.504962	7.39E-06
ADARB1	203865_s_at	2.530064	1.16E-05
HZF12	1552634_a_at	1.009475	1.16E-05
SEF2.	213891_s_at	2.138735	1.20E-05
KIF21B	204411_at	1.83281	1.20E-05
RAB11FIP4	224482_s_at	1.589028	1.20E-05
VMD2L3	1555492_a_at	1.47805	1.23E-05
Unknown (213808_at)	213808_at	1.87021	2.06E-05
BTBD3	202946_s_at	1.369698	2.12E-05
DLM1	242020_s_at	1.362838	2.14E-05
TBL1	201869_s_at	1.635954	2.17E-05
IGLJ3	215379_x_at	2.317115	2.73E-05
Unknown (240143_at)	240143_at	1.843791	4.81E-05
TIEG2	218486_at	1.910182	7.33E-05
DTX3	235721_at	1.611847	7.33E-05

SEF2..	212386_at	2.028266	7.86E-05
PIP5K1B	229116_at	1.614346	7.86E-05
CSMD1.	231223_at	2.998008	8.79E-05
GOLGA8E	213737_x_at	1.982831	8.99E-05
C13orf18	44790_s_at	2.412767	0.000103745
TCF4	222146_s_at	1.889432	0.000111127
Unknown (236815_at)	236815_at	1.478541	0.000150806
ZAP70	214032_at	1.105778	0.00015567
Unknown (1569652_at)	1569652_at	1.623276	0.000157557
BEST3	224520_s_at	1.75102	0.000174297
PLEKHG4B	236255_at	1.306582	0.000184462
CSMD1	1553405_a_at	2.094623	0.000190994
JAG1	209099_x_at	1.86105	0.000239029
ZNF521..	1561002_at	1.468551	0.000282193
IGLJ3.	209138_x_at	2.305006	0.000282473
SEF2…	212385_at	2.017211	0.000323629
IGLJ3..	214677_x_at	2.22554	0.000344195
IGLL3	215946_x_at	1.631147	0.000344195
JAG1.	216268_s_at	2.019343	0.000360234
NFATC4	213345_at	1.516531	0.000364207
GOLGIN-67	210425_x_at	1.19848	0.000421966

Table 7. Probe set IDs, gene names, log-fold changes and p-values corresponding to Fig. 6

Gene name	HU133plus2.0 probe ID	logFC	adj. p-value (FDR<0.01)
HOXA9	209905_at	-5.642306392	5.56E-15
HOXA7	235753_at	-2.867714705	1.71E-13
HOXA9	214651_s_at	-5.278173137	4.72E-13
HOXA10	213150_at	-4.405201631	1.62E-12
HOXA5	213844_at	-3.675623612	1.62E-12
HOXA3	235521_at	-2.918100349	1.62E-12
HOXA10	213147_at	-2.643718186	3.16E-10
HOXA4	206289_at	-1.933258806	2.24E-07
HOXA7	206847_s_at	-1.846205431	2.17E-06
unknown (1569348_at)	1569348_at	-1.463720025	4.60E-05
EMR2	207610_s_at	-1.198090592	4.81E-05
CPNE8	228365_at	-2.111623139	4.81E-05
unknown (24706_at)	241706_at	-1.836454698	6.47E-05
HOXB9	216417_x_at	-1.122875687	7.14E-05
VAT1L	226415_at	-2.023043589	0.000136835
unknown (236662_at)	236662_at	-1.394690852	0.000174828
SPINK2	206310_at	-2.356185682	0.000255723
IRXA1	230472_at	3.130165841	0.000255723
H2BFQ	202708_s_at	-1.446241383	0.00049311
CRAMP1L	225172_at	-0.811165772	0.000843264
LMO5	211126_s_at	-1.024684095	0.001376281
IGFBP8	209101_at	-2.268610841	0.001452582
unknown (229716_at)	229716_at	1.493845075	0.002288068
unknown (240930_at)	240930_at	-1.247027131	0.002288068
LMO5	207030_s_at	-1.30824137	0.002469476
unknown (1556599_s_at)	1556599_s_at	-1.971865713	0.002596639
CPVL	208146_s_at	1.669370028	0.003764845
JMY	226352_at	1.535434485	0.004418689
H2AFQ H2AFO)	218280_x_at	-1.305749006	0.004838001
JMY	241985_at	1.494108489	0.005213742
unknown (1557164_a_at)	1557164_a_at	-0.923353222	0.00526384

unknown (242201_at)	242201_at	-0.915141147	0.00526384
unknown (240116_at)	240116_at	-0.943012023	0.006252255
RUNX2	232231_at	-2.175688771	0.006822942
PRDX4	201923_at	1.302297581	0.007423354
H2A.2	214290_s_at	-1.096544544	0.008498623
PNMAL1	218824_at	-1.868179415	0.008498623

SUPPLEMENTAL MATERIAL CHAPTER 5

MATERIALS AND METHODS

Patient samples and sample preparation

Samples from pediatric ALL patients older than 1 year (i.e., non-infants) were selected from our cell bank present at the Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands. All samples were freshly processed within 24 hours after sampling as previously described. [1] Briefly, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (NycomedPharma), and non-leukemic cells were removed using immunomagnetic beads. [2] All leukemia samples used in this study contained more than 90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck)-stained cytospins.

RNA and DNA extraction

Total RNA and gDNA were extracted from a minimum of $5x10^6$ leukemic cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions with minor modifications. Quantification of DNA was performed using a spectrophotometer. Quantification and assessment of integrity of the extracted RNA was assessed using the Agilent 2100 Bio-analyzer (Agilent).

Gene expression profiling

Raw array data were collectively normalized using variance-stabilizing normalization [3] and additionally corrected for batch effects using ComBat[4]. Removal of batch effects was visually verified by unsupervised principle component analysis. Differential gene expression was statistically evaluated using linear models for microarray analyses. [5, 6]

In vitro prednisone response

The *in vitro* response to prednisolone (the active metabolite of prednisone) response was determined by 4-day MTT cytotoxicity assays as extensively described before.[7] Patients were deemed prednisolone sensitive in case the LC50 value (i.e. the concentration of prednisolone lethal to 50% of the leukemic cells) was <0.1 μg/mL, intermediate to prednisolone at LC50 values 0.1-150 μg/ml, and resistant to prednisolone at LC50 values >150 μg/ml.

Oligo array-CGH

Raw microarray image files were processed with Feature Extraction (Agilent Technologies, Santa Clara, CA). Results were analyzed using Agilent Genomic Workbench version 6.5 (Agilent Technologies, Santa Clara, CA).

Multiplex ligation-dependent probe amplification

Results were analyzed using GeneMarker 1.85 (SoftGenetics, State College, USA). Peak intensities of the probes in the patients were compared to peak intensities in healthy controls; a ratio >1·3 was defined as an amplification, a ratio <0.75 defined as a mono-allelic deletion, and a ratio <0.25 was defined as a bi-allelic

deletion. When multiple exons of a gene were screened, only the samples with deletion or amplification of all exons were considered to be altered.

JAK2 mutation screening

Purified polymerase chain reaction products of JAK2 exon 16 were bi-directionally sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems Inc., Foster City, CA, USA). The sequence data were assembled and analyzed for mutations using CLC Workbench version 3.5.1 (CLC Bio, Aarhus, Denmark).

TCF3-PBX1 PCR

TCF3-PBX1 was determined positive upon detection of a band after PCR using the following primers: forward: 5'-CACCAGCCTCATGCACAA-3', reverse: 5'-TCGCAGGAGATTCATCACG-3'.

Statistical analyses

Disease free survival (DFS) was calculated from date of first remission to the date of event which included relapse, death in complete remission, or second malignancy, whichever occurred first. Overall survival (OS) was calculated from the date of first remission to the date of death from any cause. Observations of patients were censored at the date of last contact when no events were observed. Follow-up was on December 31th 2009 for the Interfant-99 cohort, and on December 31th 2013 for the Interfant-06 cohort, with a median (interquartile range) follow-up of 7·0 years (5·1 – 8·0) and 4·6 years (3·8 – 5·5), respectively. The Kaplan-Meier method was used to estimate the probabilities of DFS and OS, with standard errors (SE) calculated according to Greenwood. Curves were compared using the log-rank

test. Cumulative incidence of relapse (CIR) were estimated adjusting for competing risks of death and second malignancy were statistically analyzed by the Gray test. We used the Fisher exact test to assess the association between patients' characteristics and cohorts. All tests were two-sided. Analyses were performed using SAS 9.2 (SAS institute, Cary, NC, USA) and R 3.1.0 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria) at the Interfant Trial Center. The significance analysis of microarray (SAM) [8] was used to identify probe sets significantly associated with EFS, score was calculated with 200 permutations. Gene set enrichment analysis (GSEA) was performed using GSEA software [9].

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Table 1. Summary of array-CGH data of 31 wild-type *MLL* infant ALL patients

Case (n)	Sex	Age (months)	Immuno-phenotype	Chromosome	Cytoband	Start	Stop	+/-
1	F	11	unknown	NO ABERRATIONS				
2	M	5	pre-B	7	p22·3 - p15·3	149068	20814521	-
				9	p24·3 - p13·2	193993	36847230	-
				9	p21·3	21827673	21998367	--
3	M	5	pre-B	9	p21·3 - p21·2	21482343	27254039	-
				16	p13·3	70150	4404795	+
				16	p13·3 - p13·2	5622326	8521959	-
4	F	6	T	NO ABERRATIONS				
5	M	8	pro-B	9	q31·1 - q34·3	105109369	140193874	+
				17	p13·3 - p13·1	28969	8074153	-
6	F	8	pre-B	14	complete chromosome			+
				22	complete chromosome			+
7	F	7	pre-B	NO ABERRATIONS				
8	M	6	unknown	1	complete chromosome			-
				2	complete chromosome			-
				4	complete chromosome			-
				9	complete chromosome			-
				13	complete chromosome			-
				15	complete chromosome			-
				16	complete chromosome			-
				18	complete chromosome			+
				19	complete chromosome			-
				20	complete chromosome			-
				X	complete chromosome			+
				Y	complete chromosome			-
9	F	7	pre-B	9	p24·3 - p21·2	152931	26455189	--
				9	p24·3 - p24·1	345879	6206019	-
				9	p24·1	6234464	6781084	--
				9	p24·1 - p23	6967740	9962217	-

				9	p23 - p22·3	10013642	14387386	--
				9	p22·3	14496495	16425025	-
				9	p22·3 - p22·2	16437991	18063498	--
				9	p22·2 - p21·3	18490144	21537536	-
				9	p21·3	21573783	22755422	--
				9	p21·3 - p21·2	22889384	26264718	-
				9	p21·2 - p11·1	27498421	47002387	-
				9	q12 - q21·11	67701166	72430420	-
				9	q21·13 - q21·2	78718532	80054660	-
				9	q22·1 - q22·32	90543552	98124268	-
				9	q33·1	119125575	120550685	-
				20	q11·21 - q13·33	31170057	62363774	-
10	F	6	T	1	p33	47475493	47540696	-
				10	q23·31	89615444	89666862	-
11	F	11	pre-B	9	p24·3 - p13·2	193993	37317972	-
				13	q14·11 - q34	40064678	114124062	-
12	F	9	unknown	NO ABERRATIONS				
13	M	7	common	4	complete chromosome			+
				6	complete chromosome			+
				10	complete chromosome			+
				14	complete chromosome			+
				17	complete chromosome			+
				18	complete chromosome			+
				21	complete chromosome			+
				X	complete chromosome			+
14	M	10	pre-B	NO ABERRATIONS				
15	M	0	unknown	1	p36·11	24235782	25193094	-
16	F	10	unknown	4	complete chromosome			+
				6	complete chromosome			+
				14	complete chromosome			+
				17	complete chromosome			+
				18	complete chromosome			+

				21	complete chromosome			+
17	M	2	pre-B	14	complete chromosome			+
				22	complete chromosome			+
18	F	5	unknown	22	q11·22	20730547	21443935	-
19	F	7	common	3	q25·2 - q26·33	156242699	180759981	-
20	M	10	Other	1	p35·3 - p35·2	29103090	31160825	-
				16	p11·2	30849299	33517567	-
				17	p13·1	9934363	10343321	+
				18	q21·32	55088482	55882821	+
21	F	10	pro-B	NO ABERRATIONS				
22	M	10	common	6	complete chromosome			+
				14	complete chromosome			+
				17	complete chromosome			+
				18	complete chromosome			+
				21	complete chromosome			+
23	F	9	common	9	p24·3 - p13·2	229226	36930463	-
24	M	10	T	11	q14·1 - q22·1	84284829	99548539	-
25	F	11	common	NO ABERRATIONS				
26	M	5	unknown	8	complete chromosome +			
				19	complete chromosome +			
27	F	9	common	NO ABERRATIONS				
28	F	5	common	1	complete q-arm			+
29	M	9	pre-B	NO ABERRATIONS				
30	F	11	common	9	p24·1 - p21·1	6631559	32777373	-
				9	p21·3	20375131	22638651	--

					9	p21·3	21399600	21490892	-
31	M	4	pro-B	NO ABERRATIONS					

Overview of aberrations found by array-CGH. Alterations in copy-number variation regions are omitted. + = amplification of one allele, - = loss of heterozygosity, -- = loss of both alleles. UK = unknown.

Table 2. Distribution of prognostic factors in low versus high *MEIS1* expressing wild-type infant ALL patients

	MEIS1 < median n=18	MEIS1 > median n=18	P*
Age at diagnosis			0·356
< 6 months	4 (22%)	6 (33%)	
> 6 months	14 (78%)	12 (67%)	
WBC count (cells/L)			0·168
< $100x10^9$	9 (60%)	9 (56%)	
$100\text{-}300x10^9$	2 (13%)	6 (38%)	
> $300x10^9$	4 (27%)	1 (6%)	
Prednisone response			0·327
good response	12 (92%)	11 (79%)	
poor response	1 (8%)	3 (21%)	
Immunophenotype			0·002
pro-B cell	0	3 (20%)	
common B cell	2 (15%)	8 (53%)	
pre-B cell	9 (69%)	1 (7%)	
T-lineage	2 (15%)	3 (20%)	

All data are number (%). *P-value comparing the distribution of poor prognostic factors between low-level expression of *MEIS1* (n=18) and high-level expression of *MEIS1* (n=18) in wild-type *MLL* infant ALL using the Fisher's Exact test.

Table 3. Analysis of chromosome 9p gene deletions in low versus high *MEIS1* expressing wild-type infant ALL patients.

	MEIS1-expression < median (n=13)	MEIS1-expression > median (n=11)	p-value*
Deletion			
CDKN2A	4 (31%)	2 (18%)	0·65
CDKN2B	3 (15%)	2 (18%)	1
PAX5	4 (31%)	2 (18%)	0·65
CDKN2A, CDKN2B and/or PAX5	6 (46%)	2 (18%)	0·21

All data are number (%). *P-value comparing the distribution of gene deletions between low-level expression of *MEIS1* (n=13) and high-level expression of *MEIS1* (n=11) in wild-type *MLL* infant ALL using the Pearson Chi-Square test.

SUPPLEMENTAL MATERIAL CHAPTER 6

METHODS AND MATERIALS

Cell culturing

Leukemia cell lines were maintained as suspension cultures in RPMI 1640 with glutamax (Invitrogen, Life Technologies) supplemented with 10% (v/v) FCS and 2% penicillin/streptomycin/fungizone (PSF; Invitrogen, Life Technologies) at 37°C in humidified air containing 5% CO_2.

Transfection with siRNA

$4x10^6$ cells were transfected by electroporation in 4 mm electroporation cuvettes (Bio-Rad Laboratories, Benicia, USA) 400 µL of RPMI medium plus 10% fetal calf serum together with 10 µL of esiRNA (20 µM) directed against *MLL-AF4 (si**MA6) (1), AML1-MTG8* fusion protein (si*AGF1*) as an active siRNA control which is non-silencing in this cellular context (as described previously (2)), *AF4-MLL* (as described previously (3)), or 50 µL siRNA (20 µM) directed against *MLL-ENL*: sense 5'-CCAAAAGAAAAGUCUGCCCAG-3; antisense 5'-CUGGGCAGACUUUUCUUUUGGUU-3' with 50 µL si*AGF1* (20 µM) as an active non-silencing control . siRNAs were purchased from Eurogentec (Seraing, Belgium). Electroporation was performed with the Gene Pulser MX cell Electroporation System (Bio-Rad Laboratories, Benicia, USA) with a rectangle pulse of 350 V for 10 milliseconds. After incubating for 15 minutes at room

temperature, the cells were diluted to $1x10^6$ cells/ml and cultured under standard culture conditions. Cells transfected with siRNAs directed against *MLL-AF4* and *MLL-ENL* and the relative controls were harvested after two days. Cells transfected with siRNAs directed against *AF4-MLL* and the relative controls were repeatedly transfected by electroporation after two days under the same conditions and harvested at day 4. All knock-down experiments were performed at least three times.

RNA extraction and cDNA synthesis

Total RNA was extracted from a minimum of $2x10^6$ cells using TRIzol reagent (Invitrogen, Life Technologies, Breda, The Netherlands) according to the manufacturer's guidelines. The quality of the extracted RNA was assessed on 1.5% agarose gels and cDNA was prepared for quantitative real-time PCR analysis as described earlier (4).

-PCR analysis

MLL-AF4, MLL-ENL and *AF4-MLL* mRNA expression was quantified by real-time PCR analysis using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) as described before (5). Oligonucleotide primers used for PCR amplification were purchased from Eurogentec (Seraing, Belgium). Primer sequences were as follows: *MLL-AF4* forward (*MLL* exon 8): 5'-CCCCGCCCAAGTATC-3', reverse (*AF4* exon 5): 5'-GGCGGCCATGAATG-3'; *MLL-ENL* forward (*MLL* exon 8): 5'-CCCCGCCCAAGTATC-3', reverse (*ENL* exon 7): 5'-GCTCGAAGTCTGAGTCTGA-3'; *AF4-MLL* forward (*AF4* exon 3): 5'-CAGGCCCCTAGTGAATC-3', reverse (*MLL* exon 12: 5'-TTTCGGCACTTATTACACTC-

3'; *MLL* forward (exon 9): 5'-GCAGGCACTTTGAACATC-3', reverse (exon 11): 5'-AAGGGCTCACAACAGACTT-3'; *AF4* forward (exon 3): 5'-AATCCCCTGAACTGAAAC-3', reverse (exon 6): 5'-TTTGGGTTACAGAACTGACA-3'; *ENL* forward (exon 6): 5'-CGGCCAAGGACAAGA-3', reverse (exon 7): 5'-ATGGCTCGAAGTCTGAGT-3'. *B2M* was used as a reference gene: forward: 5'-GGAGCATTCAGACTTGTCTT-3', reverse: 5'-ATGCGGCATCTTCAAA-3'. t(4;11)+ infant ALL patient samples were screened for the presence of *AF4-MLL* expression using PCR analysis with the following primer sequences: forward: 5'-CTCCCCTCAAAAAGTGTTGC-3' (*AF4* exon 3), reverse: 5'-CTTTGCCTGGAGTTGTGGAT-3' (*MLL* exon 13).

Western blot

The MLL–AF4 fusion protein was detected using medium-sized 5% polyacrylamide gels. Proteins are resolved at 60–80 volt for at least 10–12 hours at room temperature. The blotting procedure was performed overnight at 4°C on nitrocellulose membranes. Blots were incubated with mouse monoclonal anti-MLL^N/HRX (clone N4.4) (Upstate Biotechnology, Temecula, CA, USA #05–764) and anti-clathrin HC (clone TD.1) (Santa Cruz Biotechnology, Middlesex, UK #sc-12734) as a loading control. Visualization of the antibodies was done using standard procedures. Western blot procedure and quantification was performed twice.

Gene expression data

RNA was synthesized into biotinylated cRNA. Labeled cRNA was then fragmented and hybridized to HU133plus2.0 GeneChips (Affymetrix) according to the manufacturer's guidelines. Differential gene expression analysis was performed using linear modeling for microarray data (LIMMA) (6) and was

performed in the statistical environment R using Bioconductor packages. Heatmaps were generated in Genepattern using Pearson correlation for hierarchical clustering. (7) The pediatric precursor B-ALL samples were deposited as GSE13351 (8) as part of recently published studies in the National Center for Biotechnology Information Gene Expression Omnibus. (9) Gene set enrichment analysis (GSEA) was performed using GSEA software (10). GSEA on gene sets which resulted from paired analyses were done on pre-ranked lists. Pathway analysis was done using DAVID bioinformatics (11) and Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). Gene sets that are used throughout the manuscript other than previously published gene sets are listed in the Supplemental data.

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Table 1. Differentially expressed genes in response to the repression of MLL-AF4 and MLL-ENL as compared to the si*AGF1* control (n=101) or the pulse control (no siRNAs) (n=86) (Figure 2B)

101 probe sets: *MLL* fusion KD versus *AGF1* control				**86 probe sets: *MLL* fusion KD versus pulse control**			
Probe set	**HGNC Gene Symbol**	**logFC**	**P.Value**	**Probe set**	**HGNC Gene Symbol**	**logFC**	**P.Value**
225785_at	REEP3	1.13	8.84E-11	225785_at	REEP3	0.94	2,52E-08
1552665_at	LOC84989	0.95	8.30E-09	1552665_at	LOC84989	0.94	5,30E-08
202318_s_at	SENP6	0.72	1.24E-08	202318_s_at	SENP6	0.66	7,36E-08
200918_s_at	SRPR	-0.51	2.36E-07	1568589_at	NA	0.66	7,52E-08
1568589_at	NA	-0.66	2.59E-07	200918_s_at	SRPR	0.49	8,34E-08
204897_at	PTGER4	0.43	4.77E-07	202319_at	SENP6	0.49	7,96E-07
202319_at	SENP6	0.54	5.91E-07	203408_s_at	SATB1	-0.36	8,54E-07
221045_s_at	PER3	0.36	3.62E-06	228774_at	CEP78	-0.35	2,48E-06
203408_s_at	SATB1	-0.38	3.69E-06	240016_at	NA	0.26	2,54E-06
226796_at	ABHD15	-0.52	4.03E-06	212078_s_at	MLL	-0.38	2,64E-06
235479_at	CPEB2	-0.28	6.65E-06	226796_at	ABHD15	-0.49	2,70E-06
240016_at	NA	0.23	9.69E-06	204304_s_at	PROM1	-0.45	4,19E-06
37384_at	PPM1F	-0.28	1.37E-05	235479_at	CPEB2	-0.27	5,48E-06
228774_at	CEP78	-0.36	1.41E-05	1557985_s_at	CEP78	-0.30	9,22E-06
203753_at	TCF4	-0.37	1.42E-05	201924_at	AFF1	-0.42	9,71E-06
219874_at	SLC12A8	0.35	1.73E-05	214949_at	NA	-0.35	1,02E-05
224862_at	GNAQ	0.73	2.35E-05	204033_at	TRIP13	-0.34	1,32E-05
235016_at	REEP3	0.35	3.01E-05	235016_at	REEP3	0.38	1,33E-05
213541_s_at	ERG	-0.40	3.52E-05	224862_at	GNAQ	0.61	1,94E-05
203216_s_at	MYO6	-0.37	4.60E-05	219874_at	SLC12A8	0.30	2,01E-05
1557985_s_at	CEP78	-0.32	4.86E-05	226939_at	CPEB2	-0.47	2,06E-05
207143_at	CDK6	-0.37	5.14E-05	241926_s_at	ERG	-0.40	2,26E-05
203063_at	PPM1F	-0.24	5.76E-05	213541_s_at	ERG	-0.36	2,36E-05
214949_at	NA	-0.36	6.30E-05	225181_at	ARID1B	0.37	4,15E-05
235372_at	FCRLA	0.33	6.49E-05	204897_at	PTGER4	0.36	5,43E-05
223750_s_at	TLR10	0.24	6.68E-05	238767_at	NA	-0.24	7,14E-05

201924_at	AFF1	-0.37	7.12E-05	216705_s_at	ADA	-0.40	7,27E-05
208934_s_at	LGALS8	0.25	7.47E-05	210571_s_at	CMAHP	0.28	8,87E-05
1564776_at	NA	-0.30	7.69E-05	219563_at	LINC00341	-0.23	9,46E-05
204639_at	ADA	-0.42	1.04E-04	235753_at	HOXA7	-0.38	0,000105
213413_at	STON1	0.24	1.07E-04	37384_at	PPM1F	-0.25	0,00012
201889_at	FAM3C	0.34	1.16E-04	224861_at	GNAQ	0.52	0,000129
226939_at	CPEB2	-0.46	1.19E-04	1561707_at	LOC150185	0.24	0,000143
200049_at	KAT7	-0.31	1.24E-04	1567224_at	HMGA2	-0.24	0,000145
202615_at	GNAQ	0.57	1.32E-04	207819_s_at	ABCB4	0.20	0,000159
207966_s_at	GLG1	0.28	1.34E-04	230925_at	APBB1IP	-0.35	0,000165
225181_at	ARID1B	0.44	1.35E-04	207143_at	CDK6	-0.35	0,000179
235964_x_at	SAMHD1	0.43	1.50E-04	203216_s_at	MYO6	-0.33	0,000181
224861_at	GNAQ	0.57	1.59E-04	233931_at	NA	-0.30	0,00019
226004_at	CABLES2	-0.27	1.76E-04	212079_s_at	MLL	-0.32	0,000193
203817_at	GUCY1B3	-0.60	1.76E-04	204094_s_at	TSC22D2	-0.30	0,000205
230925_at	APBB1IP	-0.37	1.77E-04	226004_at	CABLES2	-0.25	0,000231
201859_at	SRGN	0.40	1.82E-04	204639_at	ADA	-0.36	0,000232
204304_s_at	PROM1	-0.43	1.83E-04	224863_at	GNAQ	0.43	0,00024
225406_at	TWSG1	0.30	1.91E-04	206847_s_at	HOXA7	-0.41	0,000243
214948_s_at	TMF1	-0.35	2.31E-04	235122_at	HIVEP3	0.32	0,000248
204033_at	TRIP13	-0.30	2.42E-04	218584_at	TCTN1	0.34	0,000276
201925_s_at	CD55	0.27	2.49E-04	217853_at	TNS3	0.42	0,000302
243490_at	NA	-0.42	2.62E-04	214948_s_at	TMF1	-0.33	0,000308
235529_x_at	SAMHD1	0.39	2.67E-04	235919_at	NA	-0.37	0,00031
210480_s_at	MYO6	-0.33	2.70E-04	202615_at	GNAQ	0.48	0,00032
211555_s_at	GUCY1B3	-0.49	2.76E-04	204621_s_at	NR4A2	0.16	0,000339
AFFX-M27830_M_at	NA	-0.31	2.80E-04	200629_at	WARS	0.31	0,000378
225355_at	NEURL1B	-0.25	2.91E-04	219497_s_at	BCL11A	0.22	0,000417
230281_at	C16orf46	0.22	3.04E-04	210432_s_at	SCN3A	0.36	0,00045
216705_s_at	ADA	-0.41	3.15E-04	232544_at	NA	-0.24	0,000451
218584_at	TCTN1	0.38	3.17E-04	1553145_at	FLJ39653	-0.32	0,000452
221933_at	NLGN4X	0.27	3.19E-04	224906_at	ANO6	-0.21	0,000456
235753_at	HOXA7	-0.36	3.20E-04	213413_at	STON1	0.24	0,000457
200629_at	WARS	0.30	3.37E-04	200049_at	KAT7	-0.26	0,000473
204836_at	GLDC	0.28	3.49E-04	220459_at	MCM3AP-AS1	-0.26	0,000491

202723_s_at	FOXO1	-0.31	3.69E-04	223750_s_at	TLR10	0.23	0,000527
202656_s_at	SERTAD2	-0.27	4.00E-04	205488_at	GZMA	0.33	0,000529
224882_at	ACSS1	-0.28	4.01E-04	211965_at	ZFP36L1	0.37	0,000553
203860_at	PCCA	0.20	4.27E-04	229838_at	NUCB2	0.22	0,000574
202388_at	RGS2	0.44	4.37E-04	225639_at	SKAP2	-0.34	0,000577
212080_at	MLL	-0.20	4.40E-04	229498_at	MBNL3	-0.32	0,000592
209994_s_at	NA	0.19	4.50E-04	225283_at	ARRDC4	0.17	0,000613
243001_at	RBFA	-0.34	4.60E-04	212045_at	GLG1	0.20	0,000623
213734_at	NA	0.26	4.81E-04	202388_at	RGS2	0.37	0,000624
224863_at	GNAQ	0.42	5.02E-04	203817_at	GUCY1B3	-0.54	0,000626
213704_at	RABGGTB	0.23	5.19E-04	209994_s_at	NA	0.19	0,000668
235400_at	FCRLA	0.30	5.38E-04	219498_s_at	BCL11A	-0.21	0,00067
235401_s_at	FCRLA	0.32	5.75E-04	211962_s_at	ZFP36L1	0.38	0,000685
230415_at	NA	-0.21	5.82E-04	206765_at	KCNJ2	0.50	0,000729
243879_at	NA	-0.25	5.90E-04	211991_s_at	HLA-DPA1	-0.47	0,000791
224567_x_at	MALAT1	0.43	5.94E-04	204836_at	GLDC	0.21	0,000796
206765_at	KCNJ2	0.47	5.95E-04	203063_at	PPM1F	-0.22	0,000801
235122_at	HIVEP3	0.35	5.97E-04	222862_s_at	AK5	0.24	0,000817
222862_s_at	AK5	0.23	6.24E-04	243490_at	NA	-0.36	0,000817
228496_s_at	CRIM1	0.27	6.28E-04	228886_at	LRRC27	0.16	0,000825
205006_s_at	NMT2	0.18	6.71E-04	235372_at	FCRLA	0.27	0,000827
236443_at	NA	-0.20	6.90E-04	235292_at	FLJ32255	0.25	0,000923
240236_at	STXBP5L	-0.22	7.00E-04	221933_at	NLGN4X	0.27	0,000954
231812_x_at	PHAX	0.19	7.18E-04	221045_s_at	PER3	0.25	0,000962
243769_at	NA	0.35	7.31E-04				
212078_s_at	MLL	-0.29	7.45E-04				
228377_at	KLHL14	0.27	7.48E-04				
232096_x_at	FOXP1-IT1	-0.23	7.78E-04				
224993_at	MLLT1	-0.22	7.85E-04				
209447_at	SYNE1	0.26	7.88E-04				
229594_at	SPTY2D1	0.19	8.09E-04				
224793_s_at	TGFBR1	0.34	8.53E-04				
237173_at	NA	-0.18	8.60E-04				
224699_s_at	ESYT2	-0.22	8.91E-04				
234723_x_at	NA	0.22	8.92E-04				
217853_at	TNS3	0.38	9.05E-04				

212079_s_at	MLL	-0.31	9.44E-04
242911_at	MED13L	-0.21	9.47E-04
226301_at	C6orf192	0.23	9.56E-04
228771_at	ADRBK2	0.21	9.71E-04

Table 2. Differentially expressed genes in response to the repression of MLL-AF4 and MLL-ENL as compared to the si*AGF1* control and the pulse control (no siRNAs) combined (n=56) (Figure 2B)

Probe set	HGNC Gene Symbol
1552665_at	LOC84989
1557985_s_at	CEP78
1568589_at	NA
200049_at	KAT7
200629_at	WARS
200918_s_at	SRPR
201924_at	AFF1
202318_s_at	SENP6
202319_at	SENP6
202388_at	RGS2
202615_at	GNAQ
203063_at	PPM1F
203216_s_at	MYO6
203408_s_at	SATB1
203817_at	GUCY1B3
204033_at	TRIP13
204094_s_at	TSC22D2
204639_at	ADA
204836_at	GLDC
204897_at	PTGER4
206765_at	KCNJ2
207143_at	CDK6
209994_s_at	NA
212078_s_at	MLL
212079_s_at	MLL
213413_at	STON1
213541_s_at	ERG
214948_s_at	TMF1
214949_at	NA
216705_s_at	ADA

217853_at	TNS3
218584_at	TCTN1
219874_at	SLC12A8
221045_s_at	PER3
221933_at	NLGN4X
222862_s_at	AK5
223750_s_at	TLR10
224861_at	GNAQ
224862_at	GNAQ
224863_at	GNAQ
225181_at	ARID1B
225785_at	REEP3
226004_at	CABLES2
226796_at	ABHD15
226939_at	CPEB2
228774_at	CEP78
229498_at	MBNL3
230925_at	APBB1IP
235016_at	REEP3
235122_at	HIVEP3
235372_at	FCRLA
235479_at	CPEB2
235753_at	HOXA7
240016_at	NA
243490_at	NA
37384_at	PPM1F

Table 3. Leading edge of GSEA comparing MLL-fusion knockdown samples versus control samples using MLL-AF4 target genes from Guenther *et al* (Figure 3, upper panel)

HGNC Gene Symbol
ERG
CDK6
PROM1
HOXA7
PPP2R5C
ZEB2
GALNT2
UBASH3B
HOXA10
TNRC18
SUPT3H
JMJD1C
CPNE8
ADAM10
BCL7A
MEIS1
TWIST1
SENP6

Table 4. Leading edge of GSEA comparing MLL-fusion knockdown samples versus control samples using MLL-AF4 target genes from Krivtsov *et al* (Figure 3, lower panel)

HGNC Gene Symbol
PROM1
HOXA7
CLEC14A
BCL2
SOCS2
RPL32
MAP3K5
HOXA10
HOXA6
CD93
FOSL2
ADCY9
CEBPA
ZNRF1
FUT4
MEIS1
C3orf65
HEXB
KCNK12
VLDLR
MMP17
MRM1
LCN8
GREM1
HTRA3
AKR7A2
SERPINB1
PCDHGC3
RUNX2

CDKN1A
NLRP3
CTGF
TMEM173

Table 5. Differentially lower expressed genes in response to the repression of MLL-AF4 and MLL-ENL as compared to the si*AGF1* control and the pulse control (no siRNAs) combined (n=57) (Figure 4B)

Probe set	HGNC Gene Symbol
1553145_at	FLJ39653
1557985_s_at	CEP78
1564776_at	NA
1567224_at	HMGA2
1568589_at	NA
200049_at	MYST2
200918_s_at	SRPR
201924_at	AFF1
202656_s_at	SERTAD2
203063_at	PPM1F
203216_s_at	MYO6
203408_s_at	SATB1
203753_at	TCF4
203817_at	GUCY1B3
204033_at	TRIP13
204082_at	PBX3
204094_s_at	TSC22D2
204304_s_at	PROM1
204639_at	ADA
206847_s_at	HOXA7
207143_at	CDK6
210480_s_at	MYO6
211555_s_at	GUCY1B3
211991_s_at	HLA-DPA1
212078_s_at	MLL
212079_s_at	MLL
212080_at	MLL
213541_s_at	ERG
214948_s_at	TMF1
214949_at	NA
216705_s_at	ADA

219498_s_at	BCL11A
219563_at	C14orf139
223840_s_at	SPATA9
224699_s_at	ESYT2
224882_at	ACSS1
224906_at	ANO6
225355_at	NEURL1B
226004_at	CABLES2
226796_at	ABHD15
226939_at	CPEB2
228774_at	CEP78
229498_at	NA
230415_at	NA
230925_at	APBB1IP
232544_at	NA
233931_at	NA
235479_at	CPEB2
235753_at	HOXA7
235919_at	NA
236443_at	NA
238767_at	NA
240236_at	STXBP5L
243001_at	C18orf22
243490_at	NA
243879_at	NA
37384_at	PPM1F

Table 6. Differentially expressed genes in response to the repression of MLL-AF4 and MLL-ENL as compared to the si*AGF1* control and the pulse control (no siRNAs) combined (n=36) (Figure 5C)

Probe set	HGNC Gene Symbol
1552726_at	ADAMTS17
204249_s_at	LMO2
208724_s_at	RAB1A
209789_at	CORO2B
211924_s_at	PLAUR
212080_at	MLL
212262_at	QKI
212636_at	QKI
212750_at	PPP1R16B
213708_s_at	MLX
214743_at	CUX1
214866_at	PLAUR
217910_x_at	MLX
219326_s_at	B3GNT2
222631_at	PI4K2B
222870_s_at	B3GNT2
222942_s_at	NA
222958_s_at	DEPDC1
223017_at	TXNDC12
223171_at	DYM
224967_at	UGCG
225935_at	CUX1
226297_at	HIPK3
226689_at	CISD2
226793_at	LINC00294
227069_at	CUX1
228008_at	NA
228094_at	AMICA1
228486_at	SLC44A1
232278_s_at	DEPDC1

233727_at	NA
235545_at	DEPDC1
236513_at	NA
238041_at	TCF12
239400_at	NA
41577_at	PPP1R16B

Table 7. Upstream regulators of the differentially lower expressed genes in response to the repression of MLL-AF4 and MLL-ENL.

Upstream Regulator	Molecule Type	p-value of overlap
miR-92a-3p	mature microrna	9.39E-08
mir-196	microrna	1.97E-06
miR-344d-3p	mature microrna	2.65E-06
miR-144-3p	mature microrna	6.57E-06
miR-153-3p	mature microrna	1.07E-05
miR-219a-5p	mature microrna	1.18E-05
miR-17-5p	mature microrna	1.48E-05
HOXA7	transcription regulator	1.76E-05
miR-196a-5p	mature microrna	4.21E-05
miR-137-3p	mature microrna	4.87E-05
MIRLET7	group	5.61E-05
mir-142	microrna	5.61E-05
miR-142-3p	mature microrna	6.91E-05
miR-148a-3p	mature microrna	7.82E-05
miR-590-3p	mature microrna	8.92E-05
LIN28B	other	1.34E-04
miR-3922-5p	mature microrna	1.63E-04
miR-154-5p	mature microrna	1.72E-04
miR-186-5p	mature microrna	1.90E-04
UPF1	enzyme	2.89E-04
miR-191-5p	mature microrna	3.79E-04
miR-448-3p	mature microrna	3.79E-04
miR-342-3p	mature microrna	4.49E-04
miR-9-5p	mature microrna	4.87E-04
miR-21-5p	mature microrna	5.42E-04
miR-155-5p	mature microrna	7.12E-04
EZH2	transcription regulator	8.23E-04
miR-200b-3p	mature microrna	8.47E-04
miR-128-3p	mature microrna	8.53E-04
MBD1	transcription regulator	9.26E-04

Table 8. Leading edge of GSEA comparing *MLL*-rearranged patients versus wild-type *MLL* patients using 57 MLL-AF4 target gene probe sets (Figure 4B)

Probe set	HGNC Gene Symbol
204304_s_at	PROM1
232544_at	IGFBP7
212080_at	MLL
235753_at	HOXA7
206847_s_at	HOXA7
236443_at	PAX5
1553145_at	FLJ39653
233931_at	ZFR
203753_at	TCF4
1564776_at	1564776_at
203216_s_at	MYO6
243001_at	C18orf22
1568589_at	REEP3
207143_at	CDK6
235479_at	CPEB2

Table 9. Differentially expressed genes in response to the repression of MLL-AF4 and MLL-ENL as compared to the si*AGF1* control (n=80) or the pulse control (no siRNAs) (n=58) (Figure 5B)

80 probe sets: ***AF4-MLL* KD versus *AGF1* control control**				**58 probe sets:** ***AF4-MLL* KD versus pulse**			
Probe set	**HGNC Gene Symbol**	**logFC**	**P.Value**	**Probe set**	**HGNC Gene Symbol**	**logFC**	**P.Value**
222631_at	PI4K2B	-0.58	3.21E-07	212636_at	QKI	-0.89	1.14E-06
212636_at	QKI	-0.87	5.29E-07	222631_at	PI4K2B	-0.64	2.31E-06
209312_x_at	NA	0.45	1.42E-06	226297_at	HIPK3	-0.72	3.91E-06
226297_at	HIPK3	-0.72	1.57E-06	225935_at	CUX1	-0.36	4.82E-06
228486_at	SLC44A1	-0.45	2.98E-06	204517_at	PPIC	0.41	1.35E-05
204670_x_at	NA	0.40	3.23E-06	222870_s_at	B3GNT2	-0.56	1.89E-05
228008_at	NA	-0.33	5.30E-06	219326_s_at	B3GNT2	-0.55	2.86E-05
208306_x_at	NA	0.39	6.16E-06	217910_x_at	MLX	-0.30	3.44E-05
219326_s_at	B3GNT2	-0.45	6.97E-06	212750_at	PPP1R16B	0.40	3.98E-05
213708_s_at	MLX	-0.36	8.64E-06	41577_at	PPP1R16B	0.42	4.59E-05
215193_x_at	NA	0.42	1.06E-05	213708_s_at	MLX	-0.35	5.09E-05
222870_s_at	B3GNT2	-0.60	1.14E-05	201858_s_at	SRGN	0.54	5.15E-05
223343_at	MS4A7	0.42	1.28E-05	223017_at	TXNDC12	-0.46	7.93E-05
222958_s_at	DEPDC1	-0.56	1.93E-05	238041_at	TCF12	-0.37	8.67E-05
41577_at	PPP1R16B	0.39	2.12E-05	205632_s_at	PIP5K1B	0.29	1.36E-04
208724_s_at	RAB1A	-0.35	2.60E-05	201859_at	SRGN	0.42	1.45E-04
226689_at	CISD2	-0.42	3.66E-05	222958_s_at	DEPDC1	-0.59	1.53E-04
217910_x_at	MLX	-0.27	5.38E-05	236513_at	NA	-0.31	1.55E-04
233727_at	NA	0.35	5.85E-05	204518_s_at	PPIC	0.32	1.60E-04
236513_at	NA	-0.30	5.87E-05	208724_s_at	RAB1A	-0.29	1.62E-04
204249_s_at	LMO2	0.45	6.07E-05	228486_at	SLC44A1	-0.51	1.95E-04
227699_at	C14orf149	-0.31	6.07E-05	229844_at	FOXP1	-0.27	3.02E-04
211990_at	HLA-DPA1	0.29	7.34E-05	226793_at	LINC00294	-0.25	3.30E-04
225935_at	CUX1	-0.29	7.47E-05	209789_at	CORO2B	-0.37	3.45E-04
211924_s_at	PLAUR	0.30	7.52E-05	214743_at	CUX1	-0.31	3.59E-04
212750_at	PPP1R16B	0.35	1.00E-04	233727_at	NA	0.30	3.74E-04

223047_at	CMTM6	-0.35	1.13E-04	232278_s_at	DEPDC1	-0.54	4.08E-04
214866_at	PLAUR	0.23	1.16E-04	209711_at	SLC35D1	-0.26	4.51E-04
217947_at	CMTM6	-0.36	1.38E-04	223171_at	DYM	-0.57	4.52E-04
229307_at	ANKRD28	0.32	1.64E-04	1552726_at	ADAMTS17	0.27	4.53E-04
226793_at	LINC00294	-0.27	1.65E-04	235545_at	DEPDC1	-0.58	4.70E-04
223171_at	DYM	-0.45	1.65E-04	203725_at	GADD45A	-0.29	4.83E-04
235534_at	NA	-0.24	1.69E-04	204891_s_at	LCK	-0.30	4.89E-04
223017_at	TXNDC12	-0.43	1.72E-04	203320_at	SH2B3	0.28	5.01E-04
211654_x_at	HLA-DQB1	0.32	1.82E-04	226689_at	CISD2	-0.31	5.19E-04
238759_at	CCDC88A	0.26	1.94E-04	212262_at	QKI	-0.23	5.40E-04
217974_at	TM7SF3	-0.31	2.22E-04	225262_at	FOSL2	0.23	5.47E-04
235545_at	DEPDC1	-0.54	2.24E-04	204249_s_at	LMO2	0.40	5.50E-04
211656_x_at	HLA-DQB1	0.36	2.31E-04	228094_at	AMICA1	0.27	5.59E-04
1553601_a_at	TMIE	0.25	2.35E-04	1554343_a_at	STAP1	0.29	5.66E-04
208894_at	HLA-DRA	0.38	2.37E-04	222942_s_at	NA	0.24	5.85E-04
212262_at	QKI	-0.27	2.53E-04	221786_at	C6orf120	0.24	5.97E-04
232278_s_at	DEPDC1	-0.48	2.75E-04	210845_s_at	PLAUR	0.27	6.66E-04
209789_at	CORO2B	-0.43	2.79E-04	214866_at	PLAUR	0.22	6.75E-04
211991_s_at	HLA-DPA1	0.29	2.87E-04	221595_at	C7orf64	-0.23	6.77E-04
239400_at	NA	0.27	2.94E-04	212080_at	MLL	-0.53	7.28E-04
234915_s_at	DENR	-0.26	2.95E-04	209712_at	SLC35D1	-0.29	7.34E-04
210982_s_at	HLA-DRA	0.40	3.00E-04	218446_s_at	FAM18B1	0.30	7.41E-04
242520_s_at	C1orf228	0.41	3.33E-04	228008_at	NA	-0.34	7.59E-04
222248_s_at	SIRT4	-0.19	3.48E-04	204174_at	ALOX5AP	0.33	8.10E-04
226981_at	MLL	-0.61	3.73E-04	224967_at	UGCG	0.39	8.13E-04
213975_s_at	LYZ	0.28	3.94E-04	218017_s_at	HGSNAT	-0.23	8.66E-04
224391_s_at	SIAE	0.32	4.07E-04	210752_s_at	MLX	-0.20	8.81E-04
203416_at	CD53	0.26	4.09E-04	210140_at	CST7	0.40	9.15E-04
222942_s_at	NA	0.22	4.11E-04	239400_at	NA	0.26	9.28E-04
223060_at	C14orf119	-0.27	4.25E-04	227069_at	CUX1	-0.17	9.41E-04
228094_at	AMICA1	0.26	4.58E-04	238873_at	NA	-0.18	9.70E-04
218865_at	MOSC1	-0.25	4.66E-04	211924_s_at	PLAUR	0.25	9.79E-04
228135_at	C1orf52	-0.51	5.01E-04				
207791_s_at	RAB1A	-0.32	5.14E-04				
226946_at	NADKD1	0.27	5.36E-04				
228783_at	BVES	-0.30	5.39E-04				
202804_at	ABCC1	0.36	5.55E-04				

227069_at	CUX1	-0.20	5.79E-04
212080_at	MLL	-0.39	6.24E-04
238041_at	TCF12	-0.31	6.33E-04
1554712_a_at	GLYATL2	0.25	6.39E-04
218668_s_at	RAP2C	-0.29	6.66E-04
224967_at	UGCG	0.32	6.79E-04
227819_at	LGR6	-0.31	7.29E-04
201778_s_at	KIAA0494	-0.34	7.33E-04
214743_at	CUX1	-0.29	7.54E-04
224691_at	UHMK1	-0.28	7.63E-04
228345_at	CHIC1	-0.34	8.10E-04
227809_at	ZC3H6	0.22	8.80E-04
204635_at	RPS6KA5	-0.18	8.92E-04
225784_s_at	ZC4H2	-0.20	9.03E-04
212771_at	FAM171A1	-0.19	9.42E-04
224818_at	SORT1	-0.29	9.63E-04

Table 10. Down-regulated genes in *AF4-MLL* signature (Figure 6B, upper panel)

Probe set	HGNC Gene Symbol
1557192_at	NA
201776_s_at	KIAA0494
201778_s_at	KIAA0494
203725_at	GADD45A
204045_at	TCEAL1
205771_s_at	AKAP7
206498_at	OCA2
207791_s_at	RAB1A
208724_s_at	RAB1A
209711_at	SLC35D1
209789_at	CORO2B
210752_s_at	MLX
212080_at	MLL
212262_at	QKI
212636_at	QKI
213227_at	PGRMC2
213708_s_at	MLX
214743_at	CUX1
215001_s_at	GLUL
217910_x_at	MLX
217947_at	CMTM6
217974_at	TM7SF3
217982_s_at	MORF4L1
218865_at	MOSC1
219326_s_at	B3GNT2
222631_at	PI4K2B
222870_s_at	B3GNT2
222958_s_at	DEPDC1
223017_at	TXNDC12
223047_at	CMTM6
223060_at	C14orf119
223171_at	DYM
224818_at	SORT1
225784_s_at	ZC4H2
225935_at	NA

226297_at	NA
226478_at	TM7SF3
226689_at	CISD2
226793_at	LOC283267
226868_at	GLT8D3
226980_at	DEPDC1B
226981_at	MLL
227069_at	NA
227699_at	C14orf149
228008_at	NA
228135_at	C1orf52
228345_at	CHIC1
228486_at	SLC44A1
228540_a]t	QKI
228783_at	BVES
229844_at	NA
231896_s_at	DENR
232278_s_at	DEPDC1
235545_at	DEPDC1
236513_at	NA
238041_at	NA
238756_at	GAS2L3
238873_at	NA

Table 11. Down-regulated genes in *MLL-AF4* signature (Figure 6B, lower panel)

Probe set	HGNC Gene Symbol
1557192_at	NA
201776_s_at	KIAA0494
201778_s_at	KIAA0494
203725_at	GADD45A
204045_at	TCEAL1
205771_s_at	AKAP7
206498_at	OCA2
207791_s_at	RAB1A
208724_s_at	RAB1A
209711_at	SLC35D1
209789_at	CORO2B
210752_s_at	MLX
212080_at	MLL
212262_at	QKI
212636_at	QKI
213227_at	PGRMC2
213708_s_at	MLX
214743_at	CUX1
215001_s_at	GLUL
217910_x_at	MLX
217947_at	CMTM6
217974_at	TM7SF3
217982_s_at	MORF4L1
218865_at	MOSC1
219326_s_at	B3GNT2
222631_at	PI4K2B
222870_s_at	B3GNT2
222958_s_at	DEPDC1
223017_at	TXNDC12
223047_at	CMTM6
223060_at	C14orf119
223171_at	DYM
224818_at	SORT1
225784_s_at	ZC4H2
225935_at	NA

226297_at	NA
226478_at	TM7SF3
226689_at	CISD2
226793_at	LOC283267
226868_at	GLT8D3
226980_at	DEPDC1B
226981_at	MLL
227069_at	NA
227699_at	C14orf149
228008_at	NA
228135_at	C1orf52
228345_at	CHIC1
228486_at	SLC44A1
228540_at	QKI
228783_at	BVES
229844_at	NA
231896_s_at	DENR
232278_s_at	DEPDC1
235545_at	DEPDC1
236513_at	NA
238041_at	NA
238756_at	GAS2L3
238873_at	NA

Table 12. Leading edge of GSEA comparing AF4-MLL positive patients versus AF4-MLL negative t(4;11) patients using 58 AF4-MLL target gene probe sets (Figure 6B, upper panel)

Probe set	HGNC Gene Symbol
226981_at	MLL
212080_at	MLL
206498_at	OCA2
217974_at	TM7SF3
222958_s_at	DEPDC1
228345_at	CHIC1
226868_at	GXYLT1
236513_at	PRELID2
238041_at	TCF12
222631_at	PI4K2B
226689_at	CISD2
210752_s_at	MLX
226980_at	DEPDC1B
227069_at	CUX1
223060_at	C14orf119

Table 13. Leading edge of GSEA comparing MLL fusion knockdown versus control samples using dataset from Stumpel *et al* (Figure 7)

Probe set	HGNC Gene Symbol
1553264_a_at	SYN1
1554112_a_at	ULK2
1554980_a_at	ATF3
1570071_at	MYO15A
202388_at	RGS2
203898_at	CRCP
204739_at	CENPC1
204924_at	TLR2
206299_at	FAM155B
206906_at	ICAM5
206908_s_at	CLDN11
207373_at	HOXD10
207692_s_at	ACAN
208605_s_at	NTRK1
208942_s_at	SEC62
209119_x_at	NR2F2
210051_at	RAPGEF3
210673_x_at	NKX2.1
211170_s_at	PDE10A
216971_s_at	PLEC
219527_at	MOSC2
220487_at	SNTG2
221015_s_at	CDADC1
221608_at	WNT6
222200_s_at	BSDC1
226178_at	SOCS4
227062_at	NEAT1
233052_at	DNAH8
233287_at	SLC6A17
234721_s_at	CYP26B1

SUPPLEMENTAL MATERIAL CHAPTER 8

MATERIALS AND METHODS

Gene expression profiling data

The gene expression profiling data was generated according to the manufacturer's guidelines, and deposited in the National Center for Biotechnology Information Gene Expression Omnibus [1] and is accessible via GEO Series accession number GSE19475.

Quantitative real-time PCR analysis

Oligonucleotide primers used for PCR amplification were purchased from Eurogentec (Seraing, Belgium). Primer sequences were as follows: *EID1* forward: 5'-GGGCGAGGAATTTGA-'3, reverse: 5'-CGGGTCTTCTCATAATG-3'; *KAT2B* forward: 5'-CTGCCAGCAAAAGAAG-3', reverse: 5'-GTCGCCTCATAACAGTGAA-3'; *EP300* forward: 5'-ACTTGGCACCTTTCTAGAGAATC-3', reverse: 5'-AGGACGGAAATGAACACTA-3'. *B2M* was used as a reference gene: forward: 5'-GGAGCATTCAGACTTGTCTT-3', reverse: 5'-ATGCGGCATCTTCAAA-3'.

Cell line culturing and RNA interference

SEM cells were maintained as a suspension culture in RPMI 1640 with glutamax (Invitrogen, Carlsbad, California) supplemented with 10% (v/v) FCS and 2% penicillin/streptomycin/fungizone (PSF; Invitrogen, Carlsbad, California) at 37°C in humidified air containing 5% CO_2.

RNA interference was performed by means of lentiviral transduction using pLKO.1 vectors expressing specific shRNA target sequences obtained from the MISSION TRC library (Sigma-Aldrich, St. Louis, USA). Target shRNA sequences were as follows: EID1: 5'-GAAGAAGCCGACAAGATGTTT-3', KAT2B: 5'-GCAGATACCAAACAAGTTTAT-3', and for EP300: 5'-CAATTCCGAGACATCTTGAGA-3'. As a control we used vectors expressing validated non-silencing control sequences (RHS1707; Open Biosystems, Thermo Scientific, Waltham, Massachusets, USA). Virus was produced by transient transfection of 293T human kidney cells with a mixture of psPAX-2 and pMD2G-VSVG.

Flow cytometry and cell cycle analysis

Induction of apoptosis was assessed by an Annexin V/PI assay. For this, $0.2x10^6$ cells were incubated in annexin binding buffer (10 mM HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$) and centrifuged at 1500 RPM at 4°C for 5'. Buffer was removed and a solution of Annexin V (1:1000) and PI (2 µg/ml) (BD Biosciences, San Jose, California, USA) was added to the cell culture, immediately followed by the measurement of Annexin V and PI positivity by flow cytometry using a FACS Calibur (Becton Dickinson, Franklin Lakes, New Jersey, USA).

Cell cycle analysis was performed by BrdU labeling using the FITC BrdU Flow Kit (#559619, BD Pharmigen, Franklin Lakes, New Jersey, USA) according to manufacturer's protocol. Briefly, 10 µM BrdU was added to $1x10^6$/ml cells, and incubated for 30' at 37◦C. Then, cells were fixated and permeabilized, refixated and treated with 300 µl of 300 µg/ml DNAse for 1 hr at 37◦C. Cells were incubated for 20' with anti-BrdU and taken up in 20 µl7-AAD solution and 1 ml staining buffer to identify early apoptotic cells. BrdU and 7-AAD positivity was determined by flowcytometer.

Analysis of the flowcytometry data was performed using the FlowJo software version 7.6.5 (Tree Star, Ashland, Oregon, USA).

Western blot analyses

Cell pellets were snap-frozen in liquid nitrogen and stored at −80°C until further use. After thawing, cells were resuspended in 50 µL of lysis buffer containing: 5 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate (Merck, Whitehouse Station, New Jersey, USA), 25 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM glycerolphosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 10 mM sodium fluoride (Sigma-Aldrich, St. Louis, USA) and 50 µM of freshly prepared sodium pervanadate. Next, cells lysis was allowed for 30' on ice, following centrifugation for 15' at 13.000 rpm and 4°C. Protein concentration was determined using the BCA protein assay (Pierce Biotechnology, Rockford, Illinois, USA) with varying concentrations of bovine serum alb umin as standards. Cell lysates containing 25 µg of protein were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Western Blots were probed with KAT2B rabbit monoclonal antibody (CellSignaling, Beverly, Massachusets, USA) and beta actin mouse monoclonal antibody (as a control for equal loading) (Abcam, Cambridge, UK) for 2 hours at room temperature. Next the western blots were incubated in fluorescently labeled secondary antibodies (LI-COR Biosciences, Lincoln, Nebraska, USA) for 1 hour and subsequently detected using the Oddyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska, USA).

References

1. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002;30(1):207-10.

Table 1. All *EID1* probe sets significantly higher expressed in *MLL*-rearranged infant ALL patients.

ID	logFC	T	P.Value	adj.P.Val	B
211698_at	1.473499	15.25835	5.38E-28	5.88E-24	52.89395
208669_s_at	1.001935	10.96376	6.63E-19	3.98E-16	32.49505
208670_s_at	1.13307	7.80794	5.52E-12	3.57E-10	16.90912

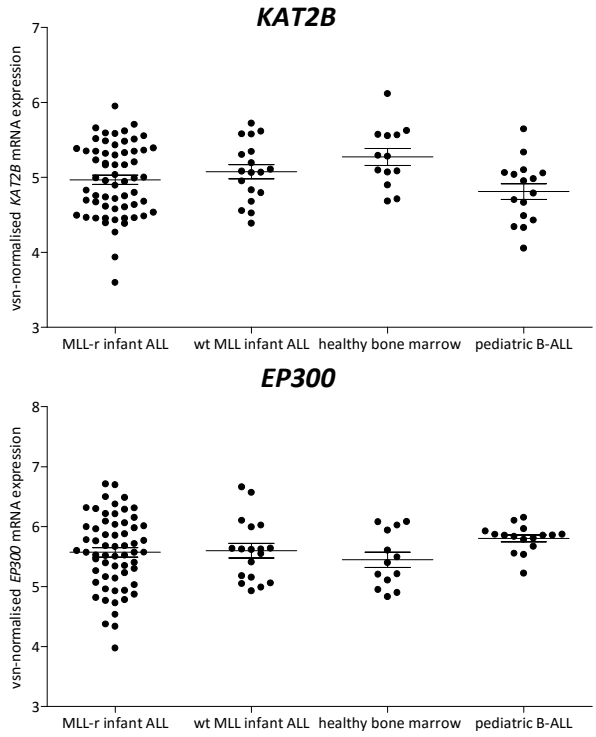
Table representing all probe sets of *EID1* on the HGU133plus2.0 microarray with results from linear modeling for microarrays (LIMMA) searching for differential expression between *MLL*-rearranged infant ALL patients (n=68) and MLL wild-type samples (consisting of MLL wild-type infant ALL patients (n=18), pediatric (non-infant) B-ALL patients (n=16) and healthy bone marrow samples n=13). ID = probe set; logFC = estimate of the log2-fold-change; T = moderated T-statistic; P.Value = raw p-value; adj.P.Val = FDR-adjusted p-value or q-value; B = log odds.

Table 2. E2F-related gene set enrichment analysis in *EID1* knockdown samples.

Name	Size	ES	NES	FDR q-value	Rank at max
V$E2F1_Q6	191	-0.297	-1.77628	0.012848	3022
V$E2F1DP1_01	195	-0.28545	-1.68798	0.012914	3022
V$E2F_02	195	-0.28042	-1.75679	0.0134	3022
V$E2F_Q6	192	-0.27967	-1.69279	0.013477	3022
V$E2F1_Q6_01	202	-0.2783	-1.66796	0.013483	3040
V$E2F1DP2_01	195	-0.28545	-1.71425	0.01448	3022
V$E2F4DP1_01	200	-0.28027	-1.69323	0.014855	3108
V$E2F4DP2_01	195	-0.28545	-1.71543	0.016372	3022
V$E2F1DP1RB_01	191	-0.26611	-1.61549	0.016612	3040
V$E2F_Q4	194	-0.27293	-1.623	0.017059	3022
V$E2F_Q6_01	191	-0.26039	-1.55002	0.026141	2675
V$E2F_03	194	-0.2425	-1.48024	0.039623	2675
V$E2F_Q4_01	191	-0.24272	-1.45996	0.041936	2986
V$E2F1_Q3	195	-0.22785	-1.34786	0.079357	2280
V$E2F_Q3	180	-0.21263	-1.26453	0.127975	2675
V$E2F1_Q4_01	184	-0.20863	-1.20229	0.188485	2986
V$E2F1_Q4	199	-0.19949	-1.19508	0.189354	2977
V$E2F_Q3_01	188	-0.19611	-1.1775	0.204105	2986
V$E2F1_Q3_01	208	-0.16036	-1.00128	0.612541	3017

Gene set enrichment analysis (GSEA) of gene expression in *EID1* knockdown samples versus control samples using gene sets that contain genes that share a transcription factor binding site defined in the TRANSFAC database (C3: motif gene sets/TFT). Demonstrated is an enrichment of genes with promoter regions [-2kb,2kb] around transcription start site containing motifs matched with E2F(-related) proteins. (N)ES = (normalized) enrichment score. FDR q-value = FDR-adjusted p-value.

Figure 1. *KAT2B* and *EP300* mRNA expression in infant ALL



Graphical representation of VSN-normalized expression values of *KAT2B* mRNA (203845_at) and *EP300* (202221_s_at) from gene-expression profiling data (Affymetrix HU133plus2 GeneChips) in patient material of *MLL*-rearranged infant ALL patients (MLL-r infant ALL, n=68)**,** *MLL* wild-type infant ALL patients (MLL wt infant ALL, n=18), healthy bone marrow (healthy bone marrow, n=13) and pediatric (non-infant) precursor B-ALL patients (pediatric B-ALL, n=16). Error bars represent the mean ± standard error of the mean.