Identification of cooperating genetic events in acute leukemia

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SUMMARY

The genetic alterations associated with acute leukemia can be divided into two functional groups. The class I mutations enhance cellular proliferation and survival by constitutive activation of mainly protein tyrosine kinases signaling pathways. In contrast, the class II mutations frequently involve transcriptional regulators of normal hematopoietic differentiation, and result in a block of hematopoietic cell maturation and/or aberrant self-renewal capacity. However, expression of the class II mutations in the murine hematopoietic system often leads to myelodysplasic changes and acute leukemia after a long latency, suggesting that collaboration of additional genetic alterations might be required.

To identify potential cooperating genetic events facilitating induction of acute leukemia by the class II mutations, we modeled acute myelogenous leukemia (AML) in mouse by retrovirally expressing the *MLL/ENL* and *MOZ/TIF2* fusion genes in the bone marrow. The genetic background of leukemic mice (F1 hybrid mice resulting from crossbreeding the FVB/N and 129/s1 strains) allowed us to perform a genome-wide polymorphism analysis screening for loss of heterozygosity (LOH) that is frequently found in blasts from AML patients. However, a simple sequence length polymorphism (SSLP) based allelotyping as well as a mouse 5K single nucleotide polymorphism (SNP) array analysis did not showed any LOH, indicating that large scale of LOH might be a rare event in our mouse leukemia models.

Recent studies have shown that genomic insertion of retrovirus could influence the expression of adjacent genes and therefore contribute to oncogenic transformation of hematopoietic cells. The retroviral integration tagging approach has been widely used for seeking new proto-oncogenes or tumor suppressor genes, and especially for identifying collaborative events in tumor models that already harbored an initiating oncogenic event. To test the hypothesis that the integration of the provirus could act as cooperating events in our mouse leukemia models, we characterized the retroviral integration sites from 21 leukemic mice induced by retroviral expression of *MLL/ENL* and *MOZ/TIF2* fusion genes. Sixty-six integration flanking genes were identified, and most of them have been previously linked to tumorigenesis. Further determination of

their expression levels demonstrated that integration flanking genes like *Tcf7*, *Tnfrsf1*, *Mn1* and *Lhx2* were up-regulated, whereas *Pur*α, *Ppp2r5c*, *Runx3*, *Socs1* and *Prdm2* were down-regulated. Interestingly, in a MLL/ENL leukemic mouse carrying the integration adjacent to the *meningioma 1* (*Mn1*) gene, the clone harboring the *Mn1* integration prevailed over other co-existing clones harboring different integrations. Moreover, an *in vitro* cellular proliferation assay showed that the overexpression of MN1 significantly enhanced proliferation and self-renewal capacity of primary bone marrow cells. These findings suggested that MN1 possesses leukemic transforming potential and might functionally collaborate with the MLL/ENL fusion in the development of the acute leukemia.

In order to experimentally address this hypothesis, we performed a series of bone marrow transplant experiments. Indeed, co-expression of MN1 with MLL/ENL enhanced *in vivo* disease development, and resulted in a significantly reduced latency for induction of an aggressive acute leukemia than expression of MN1 or MLL/ENL alone. In addition, co-expression of MN1 increased the granulocyte-macrophage progenitor (GMP) cell population expressing Gr1/Mac1, Cd34 and c-Kit with leukemia-initiating properties as shown in secondary transplantation experiments. As MN1 has been previously proposed to exert its function as a transcriptional co-activator, we also aimed to identify the potential downstream target genes by transient MN1 expression in primary bone marrow cells. Gene expression profiling experiments revealed a series of genes with known roles in normal or malignant hematopoiesis such as CD34, FLT3, HLF, and DLK1 that were upregulated in MN1 overexpressing murine leukemias, as well as pediatric acute leukemias with high MN1 levels.

We also determined the MN1 levels in a large panel of pediatric acute leukemias. High MN1 expression levels were observed in 50 of 87 samples: high MN1 levels were found in a large proportion of B-cell ALL cases and in most infant leukemias that carry MLL fusions and are of B-cell origin. Additionally, siRNA-mediated MN1 knockdown resulted in cell cycle arrest and impaired clonogenic growth of human leukemia cell lines with high MN1 levels but not in cells with low (or undetectable) MN1 levels, suggesting the aberrant expression of MN1 contributes to malignant

cellular proliferation, and the inhibition of MN1 could represent a new therapeutic approach.

Taken together, while searching for cooperative genetic alterations in murine leukemias, we found that elevated levels of *MN1* oncogene can act as a functional collaborator in MLL/ENL (and probably other class II mutations) induced leukemia through a distinct genetic program that increases the leukemia stem cell pool. In addition, we also demonstrated for the first time that high MN1 levels are found in a significant fraction of childhood acute leukemias, and important for proliferation of the leukemic cells.

INTRODUCTION

1. Leukemia is a malignant disease in the blood system

Leukemia is defined as cancer of the blood forming system and characterized by abnormal proliferation of leukocytes (white blood cells). The disease is grouped into different clinical and pathological entities. Acute leukemia is characterized by a rapid increase of immature blood cells that do not perform the normal function, whereas in chronic leukemia, abnormal blood cells are excessively produced, although they are terminally differentiated and can still execute their normal function. Depending on which lineage is affected, leukemia is subdivided into myeloid leukemia involving the granulocytic, monocytic, erythroid or megakaryocytic cell lineage, or lymphocytic leukemia involving the B-cell, T-cell or natural killer (NK)-cell lineage. Based on these classifications, four main leukemia categories are defined, namely acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL). ALL occurs most commonly in children and is the most common type of leukemia in young children, whereas CLL most often affects adults over the age of 55. AML and CML both also occur more frequently in adults than in children [1].

The standard treatment regimen for most forms of leukemia includes chemotherapy with combination of different anti-cancer drugs depending on the leukemia subtype. Allogeneic or autologous bone marrow transplantation in combination with polychemotherapy can lead to cure for some patients, however, a large proportion of adult patients with acute leukemia face relapse and ultimately die from their disease. Intensive research efforts are ongoing to identify new compounds that are able to selectively target the leukemic cells. The small molecule protein kinase inhibitors such as imatinib (Gleevec®) is successfully used to treat patients of chronic phase CML with a five-year overall survival rate of 90% [2]. In AML, initial remissions can be achieved in over 70% of patients with conventional chemotherapy, however the five-year survival rate is below 40% and most patients relapse within 5 years despite continued treatment [3]. To develop more effective targeted therapeutics, the better understanding of molecular basis of the disease is necessary.

2. Leukemia as stem cell disease

In the hematopoietic system, stem cells and lineage marker negative progenitors account for less than 0.01-0.05% of the cells in bone marrow, and are divided into three different populations according to their ability to self-renew. Long-term hematopoietic stem cells (LT-HSCs) give rise to short-term HSCs, which in turn give rise to more restricted progenitors. During this process, the cells gradually lose their self-renewing potential and become mitotically more active. LT-HSCs have an indefinite propagation potential, and generate all lineages of mature cells through consecutive differentiation. In contrast, there is basically no detectable self-renewal potential in restricted progenitors, which can only give rise to single or oligo-lineages of hematopoietic cells [4].

Biological studies on acute myeloid leukemia (AML) have demonstrated that only 0.1% to 1% of the AML cells have the capacity to initiate the disease when injected into severe combined immunodeficient (SCID) mice [5,6]. These findings suggested that similar to normal hematopoiesis, leukemia is also a mixture of cells with different phenotypic characteristics as well as proliferative and self-renewal potentials. A small number of stem cells can restore themselves and maintain the disease, whereas the majority of leukemia cells are in a more "mature" state, unable to initiate the disease. In other words, “leukemic stem cells” (LSCs) seem to undergo an aberrant and poorly regulated process of hematopoiesis that is somehow analogous to the self-renewal and differentiation of normal stem cells [7].

The first experimental evidence suggesting the existence of LSCs came from observations made almost 40 years ago: only 1 out of 10,000-100’000 mouse myeloma cells obtained from mouse ascites were able to form colonies in semi-solid medium [8]. Similarly, human leukemia cells from AML patients also formed colonies at very low frequency suggesting the presence of a small number of self-renewing cells within the bulk of leukemic blasts [9,10].

The gold standard for identifying leukemic stem cells is initiating the disease by xenotransplantation of human leukemic blasts in severe immunodeficient recipients, usually non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. In

most human AML subtypes (with the exception of acute promyelocytic leukemia), the leukemia stem cells seem to be in the $CD34^{+}CD38^{-}$ or $CD34^{+}CD38^{+/low}$ cell fraction [5]. Similarly, experiments using a xenograft model of chronic phase of human chronic myeloid leukemia demonstrated that LSCs of the chronic-phase CML also resided in $CD34^{+}CD38^{-}$ compartment [11].

3. Therapeutic targeting of leukemia stem cells

A high frequency of disease relapse suggests that conventional chemotherapeutic regimes only eradicate proliferating leukemic bulk cells, but fail to efficiently target leukemia stem cells. Several studies suggested that similar to normal HSCs, the majority of LSCs remain silent in G_0 phase of cell cycle which makes this malignant population refractory to standard chemotherapy that generally hits actively dividing cells. LSCs also possess another HSC-like property, they also express high levels of ATP-associated transporter proteins, which contribute to multidrug resistance and survival from chemotherapy [12 13].

Currently, the rising research efforts were exerted on developing therapeutic strategies to selectively impairing the proliferation, survival or self-renewal of the LSCs while protecting normal HSCs. Such strategies could work through knockdown of genes that are essential regulators of leukemia stem cells self-renewing, although unfortunately, most genes contributing to LSCs function seem also to play important roles in their normal counterparts. Additionally, the surface makers predominantly expressed on LSCs could also help to selectively target LSCs [7].

There is increasing knowledge about the signaling pathways that are involved in proliferation and self-renewal of LSCs. It has been shown that aberrant NF-kappaB and PI3K signaling might control survival and maintenance of LSCs [14,15]. NF-kappaB is a transcription factor normally activated by inflammatory stimuli and during lymphoid development. Recent experiments showed that normal human $CD34^{+}$ progenitor cells do not express NF-kappaB, but NF-kappaB activity was found in AML stem cells. A novel functional NF-kappa B inhibitor (through blocking the upstream activator IKK) parthenolide induced selective apoptosis in AML stem cells

associated with p53 activation and increased reactive oxygen species (ROS) production. These studies demonstrated a paradigm that aberrant expression of NF-kappaB in LSCs could provide a unique therapeutic target for LSCs [14,16].

The lipid kinase phosphoinositide 3-kinase (PI3K)-AKT/PKB-mammalian target of rapamycin (mTOR) signaling pathway may present another therapeutic target for LSCs. PI3K activity was necessary for the survival of human AML blasts and treatment with pharmacologic inhibitors of PI3K or mTOR, combined with standard induction chemotherapy, induced apoptosis in AML cells and decreased the abundance of LSCs [15,17]. The phosphatase and tensin homologue (PTEN) protein functions as a negative regulator of PI3K-AKT/PKB. In a mouse model with conditional PTEN deficiency, PTEN ablation in the hematopoietic system initially impaired the maintenance of HSCs and led to development of a myeloproliferative disease that progressed to acute leukemia within weeks. The treatment with rapamycin, an inhibitor of mTOR, substantially blocked the generation or maintenance of LSCs and restored normal HSC function. Thus, based on differential dependence of PTEN, treatment with rapamycin could preferentially eradicate LSCs, and restore normal HSCs on the other hand [18,19]. As aberrant PI3K-AKT/PKB signaling is able to activate NF-kappaB, combining inhibitors of PI3K or mTOR with NF-kappaB inhibitors might have additive effects for elimination of LSCs. Several clinical trials are currently ongoing to explore the safety and efficacy of inhibitors of PI3K-AKT/PKB and NF-kappa B for the therapy of acute leukemia [20].

One promising approach for eliminating LSCs would be to turn-off critical LSC regulatory genes by using the RNA interference technology. By comparing gene expression profile of HSCs and committed progenitors with LSCs in a model of leukemia induced by retroviral expression of an *MLL/AF9* fusion oncogene, a small subset of LSC self-renewal associated genes was identified, including the *HOXA* cluster genes and the transcription factor *MEF2C*. Suppression of MEF2C expression by short hairpin shRNA not only reduced the colony-forming activity, but also strikingly impaired the development of leukemia *in vivo* when transplanting into secondary recipient mice [21]. Once the major obstacle of efficient *in vivo* delivery of interfering RNAs is solved, due to its high specificity this approach might be very promising [22].

Another potential strategy for selective LSC eradication might be monoclonal antibody based therapeutics specifically targeting LSC cell surface antigens. These antibodies could be engineered to deliver toxic anti-leukemia drugs, whose effects are limited to leukemia stem cells, with less or no effect on healthy cells. Several studies have suggested that LSCs exhibited some surface antigens that were different from normal HSCs. LSCs isolated from AML patients generally lacked expression of Thy-1 (CD90) [23] and c-Kit (CD117) [24] but expressed interleukin (IL)-3 receptor α (CD123) on the cell surface [25] and a novel antigen C-type lectin-like molecule-1 (CLL-1) was present exclusively on leukemic but not normal $CD34^{+}CD38^{-}$ cells [26,27], suggesting they would be rational cell-surface targets. Indeed, binding of CD123 with a diptheria toxin–IL-3 fusion protein selectively diminished LSCs *in vitro* and also impaired the AML engraftment of NOD/SCID mice [25 28]. Additionally, CD44, an adhesion molecule involved in a variety of cellular activity including organogenesis, cell homing and migration, has emerged recently as another promising target. Anti-CD44 monoclonal antibodies were able to induce differentiation and apoptosis of AML blasts and abolish leukemic repopulation in NOD/SCID mice through impairing the homing ability and viability of LSCs. These studies indicated that the interaction between LSC and stem cell niches is a prerequisite for maintenance of LSCs and provides a convincing direction for therapeutic intervention [29 30]. LSC-specific antibodies could also be conjugated with siRNAs which knock down crucial genes for the survival of leukemia stem cells. More interestingly, it has been shown that an antibody based siRNA delivery approach might reduce toxicity and increase specificity compared to conjugating with a cytotoxic agent [31].

With the advent of new technologies, like next generation sequencing, high-throughput genetic and epigenetic screening for LSC specific programs, we might be soon able to obtain a better understanding of its biology. Further biochemical and genetic studies of human patient samples and mouse leukemia models will provide essential clues for viable targets of specific therapeutic intervention. Based on it, a successful ‘precision bombing’ will be performed to eliminate LSCs and prevent relapse of the disease.

4. Leukemia as the product of functionally collaborating genetic lesions

LSCs share many functional and phenotypic similarities with HSCs, such as self-renewal potential and the maintenance of a quiescent stage. It has been rationally postulated that LSCs arise from HSCs as the result of accumulation of oncogenic mutations, based on the observation that stem cells persist for long periods and undergo a number of cell divisions increasing the likelihood to obtain the minimum number of mutations necessary for malignant transformation [5,6,32]. Alternatively, LSCs may also result from more differentiated progenitor cells that have reacquired the capacity for self-renewal and accumulated additional mutations for malignant transformation. [33,34]

The intensive molecular investigation over the past two decades has shed new light on the pathogenesis of hematological malignancies. A large number of recurrent genetic lesions have been identified to be associated with different subtypes of leukemias. Conventional cytogenetic analysis initially uncovered the presence of chromosomal translocations, large deletions and inversions. Improved molecular tools then allowed identification of smaller mutations like point mutation, microdeletions that are often associated with normal karyotypes. Several biological assays have been developed over the years to examine the *in vitro* transforming potential of leukemia-associated genetic lesions. In addition, *in vivo* oncogenic activity of an increasing number of leukemia-associated mutations has been demonstrated in well-designed animal models. Based on their cellular activity, it has been proposed to classify leukemia-associated genetic alterations into two functional groups. The class I mutations generally consist of genetic lesions that activate signal transduction pathways conferring cell survival and proliferation advantage, whereas the class II mutations comprise mutations that alter a transcriptional program essential for normal hematopoietic differentiation resulting in aberrant self-renewal and maturation arrest (**Fig. I**) [35,36].

The class I mutations

The class I mutations increase proliferation and survival potential of hematopoietic

stem and progenitor cells, often through constitutive activation of protein tyrosine kinases (PTKs) but normally do not affect cellular differentiation. Examples include gain-of-function mutations of ABL, JAK2, FLT3, PDGFR, KIT, activating mutation of the RAS family members, as well as loss-of-function alterations of NF1 or PTPN11 [37]. Activating JAK2 mutations are found in chronic myeloproliferative neoplasms and in more than 95% of patients with polycythemia vera (PV). Activating mutations of FLT3, KIT and RAS are present in more than 50% of AML patients. FLT3 is the most commonly mutated gene in approximately one third of AML. In 20%–25% of cases of AML, internal tandem duplications (ITD) in the juxtamembrane domain of FLT3 results in loss of an autoinhibitory domain leading to constitutive activation. Other FLT3 mutations consist of substitutions, small deletions, or insertions within the activation loop of the second kinase domain found in 5% to 10% of AML patients. The overall consequence of these FLT3 mutations is ligand-independent receptor dimerization and/or constitutive activation of its tyrosine kinase activity, leading to uncontrolled activation of several downstream signaling pathways, such as RAS, MAPK, and STAT5 pathways. The RAS/MAPK signal transduction pathway is a critical regulator of proliferation and survival of hematopoietic progenitors. Leukemic blasts from about 40% of AML patients showed constitutive activation of RAS signaling [38]. However, expression of most class I mutations in murine bone marrow generally leads to a lethal myeloproliferative disease (MPD), but not acute leukemia. In addition, most of these disorders such as the FLT3-ITD induced MPD are not transplantable into secondary recipient mice suggesting that the class I mutations do not confer self-renewal potential to the transformed cells [37].

The class II mutations

The class II mutations impair differentiation and augment self-renewal properties of hematopoietic progenitors by modulation of chromatin remodeling and recruitment of aberrant co-activator or -repressor complexes, while having modest effects on cell proliferation or survival. The class II mutations comprise gene rearrangements affecting transcriptional regulators such as the core-binding factor CBF, CEBPα, RARα, and MLL, and components of the transcriptional activation complex such as CBP, MOZ, and TIF2, which are all critical regulators for the differentiation program

of hematopoietic stem and progenitor cells. The core-binding factor (CBF) is a heterodimeric transcription factor composed by the AML1 (also known as RUNX1) and the CBFβ subunits, which are both essential for normal hematopoiesis [39]. Thus, disruptions of the AML1/CBFβ complex might cause differentiation arrest and subsequent leukemic transformation. Indeed, CBF is the target of multiple chromosomal translocations and mutations. Among them, the most frequent and extensively studied are the AML1/ETO (RUNX1/ETO) resulting from t(8;21) and CBFB/SMMHC from inv(16), which are present in approximately 10% and 5% of AML cases, respectively. The chromosomal rearrangements involving retinoic acid receptor alpha (RARα) on chromosome 15 are exclusively associated with acute promyelocytic leukemia (APL). APL-associated gene rearrangements are characterized by fusing RARα to several different partner genes, of which the most common fusion is PML/RARα resulting from t(15;17). PML/RARα inhibits the function of RARα by a dominant negative effect through recruiting the co-repressor complex, in a manner similar to the AML1/ETO, CBFB/SMMHC and ETV6/ AML1 fusions [38]. The MOZ/TIF2 fusion is generated by chromosomal rearrangement inv(8)(p11q13) associated with AML. MOZ (monocytic leukemia zinc finger) is a MYST family histone acetyltransferase (HAT), and the C2HC zinc finger nucleosome binding motif of MOZ is essential for leukemic transforming potential of the MOZ/TIF2 fusion, whereas HAT activity of MOZ is dispensable. TIF2 (transcription intermediary factor-2) belongs to the p160 nuclear receptor transcriptional coactivator family (NRCoAs) that interacts with CREB binding protein (CBP), which is also critical for transformation activity of MOZ/TIF2 [40]. A large number of studies have demonstrated that retroviral or transgenic expression of the class II mutations often led to development of myelodysplastic syndrome or AML-like phenotype in the mouse. However, the requirement of a relatively long latency for the onset of the disease suggested that the class II mutations might be essential but rather not sufficient for induction of an acute leukemia phenotype [38].

The class II mutations also play an important role in aberrant activation of self-renewal property of LSCs. Retroviral expression of a MLL/ENL fusion gene (a class II mutation associated with AML and ALL) in purified HSCs, or well defined (by expression of distinct surface markers) progenitor cells such as common myeloid

progenitors (CMPs) or granulocyte–macrophage progenitors (GMPs) led to immortalization *in vitro* and rapid onset of transplantable AMLs with almost identical phenotypes [34]. Similarly, CMP and GMP transduced with other class II mutations such as the MOZ/TIF2 or MLL/AF9 fusions (but not the class I mutation BCR/ABL) also initiated AML in recipient mice, suggesting human AML might also arise from committed myeloid progenitors on which self-renewal capacity was conferred by the aberrant transcription activity of those fusion genes [21,41]. Impressively, the LSCs in this set of the class II mutations induced murine leukemias exhibited a GMP-like immunophenotype, lacking expression of lineage markers, but highly expressing the stem or early progenitor markers such as CD34, c-Kit, and the Fcγ receptor II and III (CD16/CD32) [21,34,41]. The secondary transplantation of limiting dose of flowcytometer-sorted cells led to the estimation that 0.6–0.8% of the cells in bone marrow or spleen are LSCs [21,34,41]. However, this concept was recently challenged by the work of Cleary M.L. and colleagues who demonstrated that a high percentage of colony-forming cells (CFCs) from the bone marrow and spleen of leukemic mice can also initiate the disease in secondary recipients. These clonogenic cells were more frequent, accounting for 25%-30% of leukemic blasts, and expressed more mature myeloid lineage-specific markers [42]. Further evidence also arose from observations made with a transgenic B-cell lymphoma model. In this model, malignant cells transferred the disease to non-irradiated histocompatible recipients at a very high frequency (at least 1 in 10), supporting an idea that the analysis based on limiting-dilution transplantation experiments in immunodeficient animals might underestimate the actual LSC frequency [43].

The identity of LSCs might be also dependent on the type of the class II mutations as shown in a murine model of CALM (clathrin assembly lymphoid myeloid leukemia)/AF10 (ALL fused gene from chromosome 10) fusion positive AML. In contrast to acute leukemia induced by MLL/AF9 or MOZ/TIF2, the leukemia propagating cells had the phenotype of early B-lymphoid progenitors ($B220^{+}CD11b^{-}Gr\text{-}1^{-}$) with clonal immunoglobulin heavy-chain gene rearrangements. Similar to the murine model, CALM/AF10-positive AML patients also showed a population of CD45RA (the human homolog of B220) positive leukemic cells displaying clonal *Ig* DH-JH rearrangements that were able to form CFU-blast colonies *in vitro* [44]. Furthermore, the LSCs in PML/RARα-induced APL seem to more likely

reside in $CD34^{-}CD38^{+}$ than $CD34^{+}CD38^{-}$ compartment (as shown in MLL/AF9 leukemia) [45]. These observations probably reveal a truth that the different origins of LSCs and the distinct class II mutations underlying leukemic transformation lead to the diversity of LSC phenotype, which would make a universal targeted therapy approach for all subtypes of leukemia rather unlikely.

Functional cooperation of the class I and class II mutations in acute leukemia

Clinical and experimental data support the idea that acute leukemic blasts harbor more than one recurring mutations, strongly suggesting a multi-hit model for leukemogenesis. Recent studies have shown that although some class II mutations, such as the *MLL/AF4* or *TEL/AML1* fusion genes arise *in utero*, the protracted postnatal latency for the onset of leukemia is still observed in patients, indicating additional secondary genetic alterations were required to develop the leukemic phenotype [46,47]. A recent study on a monochorionic twin pair, one with frank leukemia and one in pre-leukemic stage, further demonstrated that the *TEL/AML1* fusion gene can act as a first-hit mutation and additional hits would have impact on progression of leukemia [48]. The genetic analysis of progression from chronic myelogenous leukemia (CML) to acute myelogenous leukemia (AML) also provided evidence for multi-step pathogenesis of acute leukemia. Several cases were reported as evidence, in which progression of chronic myeloproliferative disorders induced by the class I mutations such as BCR/ABL or TEL/PDGFRβ fusions to AML was associated with the acquisition of the class II mutations such as AML1/EVI1, AML1/ETO or NUP98/HOXA9 fusion. It strongly suggests that the class I mutations can act in concert with the class II mutations to cause AML [35]. In a recent comprehensive mutational study including 144 *de novo* diagnosed AML cases, 103 of 165 identified mutations were overlapped with other mutations, mostly consisting of the class I and class II mutations [49].

Generally, the mutations in the same group rarely occurred in the same AML patient. In contrast, the mutations from different complementation groups were frequently found in the same patient. In PML/RARα positive acute promyelocytic leukemia

(APL), more than 30% patients also harbor FLT3 activating mutations [37]. Experimental observations from murine models of APL provided solid evidence for functional cooperation between PML/RARα and activating FLT3 mutations. Expression of PML/RARα from the cathepsin G promoter in transgenic mice resulted in APL-like disease with a long latency (more than 6 months) and incomplete penetrance (~15%–30%) [37]. However, the retroviral expression of FLT3-ITD in bone marrow of these PML/RARα transgenic mice induced APL after a shorter latency and complete penetrance, suggesting that FLT3 mutations function as an additional hit and accelerator of the disease progression [50]. The functional cooperation between the class I and class II mutations have also been demonstrated in other mouse leukemia models. Expression of the BCR/ABL fusion causes a fatal myeloproliferative syndrome, however, co-expression of NUP98/HOXA9 with BCR/ABL rapidly induced a fatal AML phenotype that was in concordance with the observation of progression of CML chronic phase to blast crisis in patients [51]. Two of the most frequently gene rearrangements associated with AML, AML1/ETO and FLT3 length mutation, are insufficient to cause leukemia in animal models on their own. Nevertheless, retroviral expression of both of mutations together efficiently induced acute myeloid leukemia in mice, further supporting the concept of oncogenic collaboration between complementation mutations in leukemic transformation of hematopoietic progenitors [52]. Moreover, direct functional collaboration of the activating FLT3-ITD mutation with the MLL/AF9 was recently demonstrated in the bone marrow transplantation leukemia model [53]. Interestingly, the mutation of the p53 tumor suppressor gene seems to occur in conjunction with both of the class I and class II mutations and associated with multi-lineage dysplasia and a complex karyotype, indicating p53 mutations might present a functionally distinct class of mutation [49].

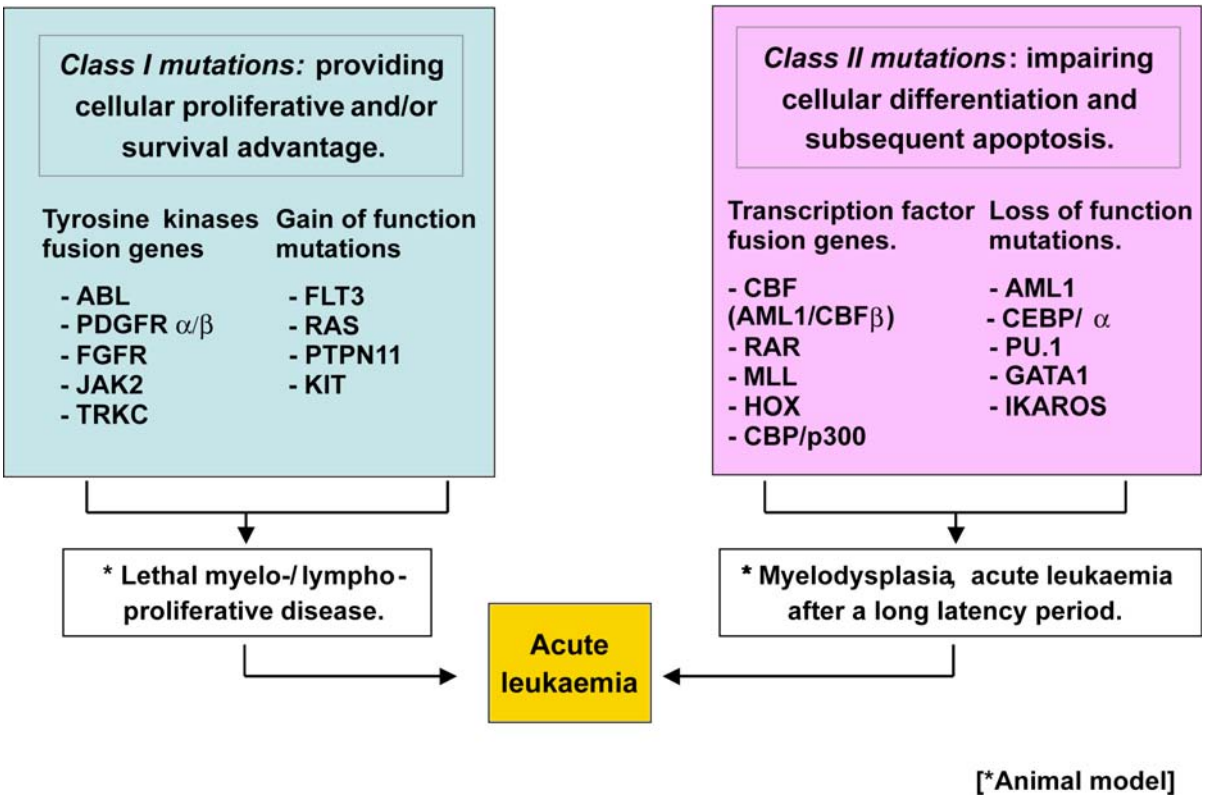


Fig. I. A functional collaboration model of genetic alterations leading to acute leukemia. In acute leukemia, genetic alterations are distinguished as the class I and class II mutations. The class I mutations usually affect kinases and other signaling proteins, which provide proliferative and/or survival advantage and induce a myeloproliferative disease rather than frank leukemia in mouse models. The class II mutations that alter transcription activities of common transcription factors impair differentiation and could result in a myelodysplastic syndrome-like disease or acute leukemia after a long latency in mouse models. Co-expression of class I with class II mutations in mice rapidly induces an acute leukemia phenotype.

5. Mixed lineage leukemia (MLL) gene in acute leukemia

Among the most common genes affected by the leukemogenic class II mutations is the *MLL* (Mixed Lineage Leukemia or Myeloid/Lymphoid Leukemia) gene on chromosome 11q23, also known as *ALL-1*, *HTRX* or *HRX*, which is frequently involved in chromosomal translocations. These translocations result in novel chimeric proteins containing the amino terminus of MLL fused in-fame with over 50 distinct partner proteins [54,55]. MLL gene rearrangements are observed in about 80 % of infant leukemia, 10% of childhood leukemia patients older that 1 year of age, and

approximately 5-10% of adult acute leukemias of either lymphoid or myeloid lineage derivation. In addition to spontaneously arising leukemia, MLL fusions are also found in patients with therapy-related leukemias occurring after treatment with inhibitors of topoisomerase II, which account for 5-10% of MLL associated leukemias. Generally, chromosomal abnormalities involving MLL gene are indicators of poor prognosis of the disease [56,57].

Function and structure of MLL

The *MLL* gene is located on chromosome 11q23.3 and consists of 37 exons spanning over 92kb. The around 11.9kb transcript encodes a 430kDa protein comprising 3969 amino acids, which is able to bind DNA and methylate histone H3 lysine 4 (H3K4) through its SET domain. In Drosophila, the trithorax protein, the homologue of MLL positively regulates the expression of target genes including multiple *homeodomain* (*Hox*) genes through H3K4 methylation [58]. Similarly, in mice the homozygous deletion of the *MLL* gene is lethal due to disturbed Hox-mediated body plan formation, indicating MLL maintained *Hox* gene expression is crucial for embryonic development. MLL-deficient hematopoietic progenitor cells from yolk-sac and fetal liver showed impaired colony forming and proliferation activities, suggesting MLL also plays an important role in hematopoiesis [59]. Furthermore, conditional ablation in adult mice demonstrated that MLL is critical to maintain adult hematopoietic stem cells [60].

The mature MLL protein consists of two subunits, N-terminal MLL (300/320kDa) and C-terminal MLL (180kDa), which are produced through proteolytic cleavage by taspase1 and noncovalently associated through the FYRN and FYRC domains forming a stable complex [61]. In the N terminus of MLL, three short AT-hook motifs are supposed to mediate binding to the minor groove of AT-rich DNA. Two speckled nuclear localization sites (SNL1 and SNL2) are found immediately C-terminal to the AT-hooks followed by a transcriptional repression domain (TRD) including two functional subunits, RD1 and RD2, which are retained in all MLL fusions and required for transformation by all MLL fusion proteins. RD1 contains the DNA methyltransferase (DMT) homology domain including the CXXC zinc finger domain

that recruits the polycomb repressor proteins HPC2 and BMI-1, and the corepressor CTBP [62]. RD2 mediates repression through recruitment of histone deacetylases HDAC1 and HDAC2 [62]. Adjacent localizes a plant homology domain (PHD) zinc-finger motif that interacts with the nuclear cyclophilin, CYP33 (which negatively regulates *HOXC8* and *HOXC9* transcription). Downstream of two taspase cleavage sites is the transcriptional activation (TA) domain, which recruits the transcriptional co-activator CBP (CREB-binding protein). The binding of MLL to promoters of *HOX* genes is in connection with CBP mediated acetylation of H3 and H4. Near the C-terminus of MLL is a SET (Su(var)3-9, enchancer-of-zeste, trithorax) domain that possesses histone H3 lysine 4 (H3K4) methyltransferase activity (**Fig. II**). The SET domain can interact with SWI/SNF chromatin remodeling complex and might play a direct role in the transcriptional activation of *HOX* genes [63,64].

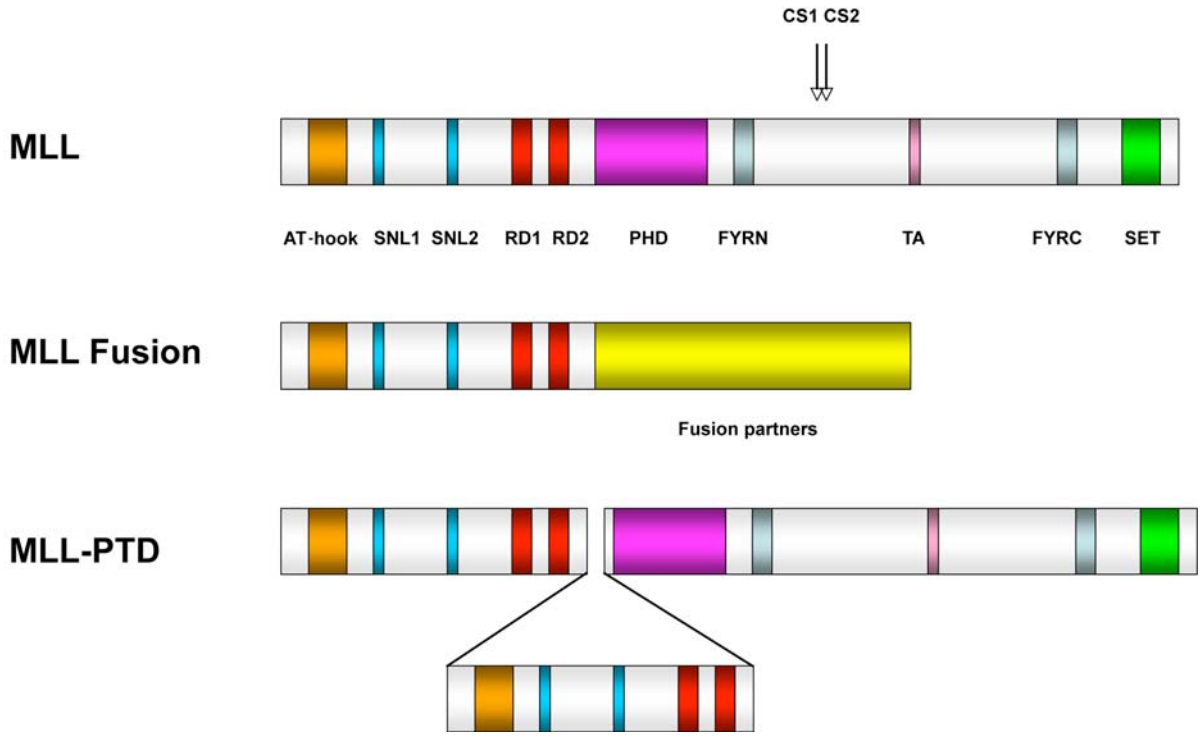


Fig. II. Schematic structure of the MLL protein and MLL fusions.

Putative functional domains are presented as colored boxes and labeled as follows: AT-hook, AT-hook DNA binding motifs; SNL1/2, speckled nuclear localization site1/2; RD, transcriptional repression domain; PHD, plant homology domain; TA, transcriptional activation domain; SET, Su(var)3-9, enchancer-of-zeste, trithorax domain; CS, cleavage site; MLL–PTD, MLL- partial tandem duplication.

MLL chromosomal abnormality and leukemia

The recurrent chromosomal translocations result in the first 8–13 exons of MLL in-frame fused with a variety of partner genes. More than 50 MLL fusion partner genes have been identified by cytogenetic and molecular genetic analysis, however only a few MLL fusions have been functionally studied.

According to differences in cellular location and putative function, some of MLL rearrangements can be classified into groups (**Table. I**) [58]. The first group is comprised of fusion partner genes encoding the nuclear DNA-binding proteins AF4 (ALL1 fused gene from chromosome 4), AF9, AF10, ENL (eleven nineteen leukemia gene) and ELL (eleven nineteen lysine-rich leukemia gene), which all together account for more than 80% of *MLL* translocation in leukemia patients [54,55].

The second group of MLL fusions involves cytoplasmic proteins such as GAS7, EEN, AF1p or Eps15, AF6 and AFX, found in more than 10% MLL rearranged leukemias. The common feature of this group of protein is coiled-coil oligomerization domains that are important for their transformation potential [65]. The third group of fusion partners includes septins (SEPT2, SEPT5, SEPT6, SEPT9 and SEPT11), which are cytoplasmic proteins playing a role in mitosis and cytoskeletal structure [66]. Another group of fusion partner proteins are histone acetyltransferases p300 and CBP. MLL is fused with these proteins retaining histone acetyltransferase activity, although TA domain, which mediates interaction of p300/CBP with wildtype MLL, does not exist in MLL fusions [67,68]. These two groups of MLL fusions are only observed in 2% of MLL associated leukemias. The last group contains only MLL–partial tandem duplication (MLL–PTD). MLL–PTD results from a unique MLL rearrangement, in which a variable number of exons 5 to 12 are duplicated and inserted before exon 11 or 12. MLL–PTD is found in 4-7% normal karyotype AML patients and also often associated with trisomy 11 abnormality [69].

Table I. Classification of MLL fusions (adapted from ref. 58)

	Putative function	Chr.	Fusion partner	Frequency in MLL associated leukaemia
Group 1	Nuclear, putative DNA-binding proteins	4q21 9p23 19p13.3 10p12 19p13.1	AF4 AF9 ENL AF10 ELL	>80%
Group 2	Cytoplasm, presence of coiled-coil oligomerization domain	1q32 17p13 19p13 6q27 Xq13	EPS15 GAS7 EEN AF6 AFX	>10%
Group 3	Cytoplasm, septin family, interact with cytoskeletal filaments, have a role in mitosis	Xq22 22q11 Xq24 17q25 4q21	SEPT2 SEPT5 SEPT6 SEPT9 SEPT11	>1%
Group 4	Nuclear, histone acetyltransferases	16q13 22q13	CBP P300	>1%
Group 5	MLL partial tandem duplication	11q23	N/A	4–7% of all AML with normal karyotyp

Since *HOX* genes are critical effectors of MLL in regulating embryonic development and hematopoiesis, they seems also to be important regulators of the leukemogenic activity of MLL fusions. Upregulation of *HOX* gene expression is a common feature of MLL rearranged leukemias [57]. The mice transplanted of Hoxa9 deficient bone marrow cells retrovirally expressing the MLL/ENL fusion failed to develop leukemia [70], suggesting an absolute requirement of Hoxa9 for the MLL/ENL induced AML. However, Hoxa9 seemed to be dispensable for leukemia induction by the MLL/AF9 fusion as demonstrated in a knock-in mouse leukemia model [71]. For MLL fusion proteins containing oligomerization domains such as leucine zippers and α-helical coiled-coil domains, formation of stable form of proteins by oligomerization may be crucial constituent of MLL rearrangement associated leukemogenesis. The development of leukemia in mice expressing N-terminal MLL fused with beta-galactosidase indicated that oligomerization mediated by beta-galactosidase may confer leukemogenic properties to MLL [72]. There is accumulating evidence linking the oncogenic activity of many MLL fusion partners to aberrant transcriptional regulation through chromatin remodeling. The transformation of myeloid progenitors by MLL/CBP involves epigenetic regulation of chromatin accessibility surrounding regulatory regions within MLL target genes [73]. Binding of DOT1L histone

methyltransferase, which methylates lysine 79 residues in histone H3 (H3K79), with MLL fusion partner AF10 is essential for MLL/AF10 leukemic activity and suppression of DOT1L abolishes MLL/AF10 mediated transformation of hematopoietic progenitors [74]. Interestingly, knockdown of DOT1L also suppressed leukemia induced by a knocked-in MLL/AF4 allele [75]. The oncogenic activity of MLL/ENL may also implicate histone modification, since MLL/ENL increases H3K79 levels on *HOXA9* and *MEIS1* promoters [76]. The methylation of H3K79 has been shown to be a mark of positive transcriptional regulation, and the replacement of H3K4 activity in wild-type MLL with H3K79 activity in the MLL fusion complex might represent an unknown mechanism of aberrant transcriptional regulation [77]. These studies suggested that detailed elucidation of the role of MLL in normal and malignant hematopoiesis might result in developing new principles for targeted therapeutic intervention [78].

MATERIALS AND METHODS

Cell lines: The following human leukemia cell lines were analyzed: MV4;11, acute myeloid leukemia (MLL/AF4+, FLT3-ITD+), MOLM13, acute myeloid leukemia (MLL/AF9+); EOL1, acute eosinophilic leukemia (MLL-PTD); THP1, acute monocytic leukemia (MLL/AF9+); KOCL44, acute lymphoblastic leukemia (MLL/ENL+), SEM, acute lymphoblastic leukemia (MLL/AF4+), KOPN8, acute lymphoblastic leukemia (MLL/ENL+), RS4;11, acute lymphoblastic leukemia (MLL/AF4+) and HL-60, acute myeloid leukemia. All cells were kept in RPMI 1640 with Glutamine (Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum and penicillin/streptomycin at 37°C.

Construction of recombinant retroviral vectors: Full-length human 5'-FLAG-tagged MN1 cDNA was excised (*SacI-HindIII*) from *pCMVTag2B-MN1* (a kind gift from Dr. Paul MacDonald, Cleveland, Ohio) and transferred into the *pSL1180* (*SacI-SmaI*) cloning vector and further subcloned into *pMSCV-IRES/EYFP*. The MLL/ENL cDNA (a kind gift from Dr. Robert Slany, Erlangen, Germany) was subcloned from *pMSCV-pgk/neo* into *pMSCV-IRES/EGFP* or *pMSCV-pgk/puro* using a unique *EcoRI* site. All expression plasmids were verified by extensive restriction digests, and by partial sequencing.

Bone marrow infections & transplantation: Bone marrow cells were harvested from 6- to 10-week-old [FVB/Nx129/s1]F1 mice 4 days after i.p. injection of 5-fluorouracil (5-FU) 150 mg/kg (Sigma), and were cultured for 24 hours in RPMI 1640 supplemented with 10% fetal bovine serum, 10ng/ml of human interleukin-6 (IL-6), 6ng/ml of murine interleukin-3 (IL-3) and 100ng/ml of murine stem cell factor (mSCF) (PeproTech EC, London, UK). HEK293T cells were transiently co-transfected with *pMSCV* retroviral vector and a packaging vector (*pIK6*), and virus containing supernatants were collected after 48h and concentrated by 60 min of centrifugation at 14000 rpm, 4°C. Retroviral infections were performed by spinoculation of the cells (2500rpm, 90min, 30°C) with retroviral supernatant on two consecutive days. $1x10^6$ transduced bone marrow cells were injected into the tail vein of lethally irradiated (^{60}Co, 950rad) syngenic recipients. For secondary transplantation, the limiting doses

of leukemic blasts (from $5x10^5$ to 500) from primary mice were transplanted into sublethally irradiated (450rad) recipients by tail vein injection.

Simple sequence length polymorphism (SSLP) based genotyping analysis: 52 SSLP / Microsatellite marks were used for PCR analysis of 19 mouse autosomes (2-3 widely spaced markers for each autosome). Genomic DNA isolated from leukemia mice (F1 hybrid mice, one allele from FVB/N and another one from 129/s1) was PCR amplified using dye-labeled primers for microsatellite markers. The PCR condition is at 94ºC for 15 sec, 55ºC for 15 sec, 72ºC for 30 sec for 10 cycles, 89ºC for 15 sec, 55º for 15 sec and 72ºC for 30 sec for 20 cycles. The PCR products were analyzed using ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Genotypes were scored using the Genemapper software package version 3.5 and loss of heterozygosity (LOH) was determined by measuring the intensity of two alleles (Applied Biosystems, Foster City, CA, USA).

Serial replating assay: 10^4 transduced bone marrow cells were plated in 1ml of methylcellulose culture (Stem Alpha.mIE, Stem Alpha, France) containing IL-3, IL-6 and mSCF. The number of colonies was counted after 7 days. Colonies were harvested, and 10^4 cells were replated in methylcellulose for 4 rounds.

Analysis of transplanted mice: After red cell lysis peripheral blood and bone marrow cells were counted and analyzed by using a flow cytometer (Cyan II, Becton Dickinson): single cell suspensions were stained with phycoerythrin (PE), or allophycocyanin (APC) fluorochromes-labeled c-Kit, Sca1, Gr1, Mac1, B220 and CD34 monoclonal antibodies (all from Pharmingen, San Diego, CA). Morphological histopathological analysis of peripheral blood, bone marrow, liver and spleen was performed using standard procedures.

Retroviral integration cloning by splinkerette PCR: Genomic DNA isolated from bone marrow or spleen cells of leukemic mice was digested with *NlaIII* or *MseI* for 12-16 hours, and ligated to the splinkerette linker overnight. The nested PCR was performed by using splinkerette linker-specific primers and primers recognizing the long terminal repeat of *pMSCV* [79,80]. Amplicons from the second PCR were

separated on 2% agarose gel, purified by gel purification kit (Qiagen, Hilden, Germany) and subcloned into *pCR 2.1-TOPO* vector (Invitrogen, Carlsbad, CA) prior to sequencing, or directly sequenced by using the BigDye Terminator v3.1 chemistry and ABI 3130 DNA genetic analyzer (Applied Biosystems, Foster City, CA). Obtained sequences were analyzed by using BLAST against the National Center for Biotechnology Information (NCBI) mouse genome database.

siRNA knockdown experiments: a) Lentiviral delivered MN1 siRNA: The MN1 specific shRNA or scramble shRNA lentiviral plasmids (*pLKO.1-puro*) were purchased from Sigma (St Louis, MO). Lentiviral vector packaging was performed according to the calciumphosphate method. Briefly, shRNA lentiviral plasmid was mixed with envelope plasmid *pMD2G*, packaging plasmid *pMDLg/pRRE* and Rev-expression plasmid *pRSV-Rev*, and 0.5M $CaCl_2$ was added. The mixture was added drop-wise to 2X BES while vortexing at full speed. After 20 minutes incubation, the precipitate was slowly added to the 293T cell monolayer. Lentiviral supernatants were harvested after 48 hours by spinning at 2500 rpm and filtering through 0.45 m filter. Lentivirus were further concentrated by spinning through Vivaspin 20 concentrator (Sartorius Bioloabs, Goettingen, Germany) at 4000rpm 4°C for 30 minutes, and stored at -80°C. **b) Retroviral siRNA knockdown of potential MN1 targets:** 2 cell lines established by serial replating *in vitro* were grown in RPMI1640 with growth-factors (IL3, IL6, mSCF) and were transduced with a retrovirus (*pSM2*) expressing miR-siRNA targeting murine gene coding for FLT3 (V2MM_48079, Open Biosystems), or CD34 (V2MM_26807). Transduced cells were selected in puromycin (2.5-5 µg/ml) and growth and survival were analyzed in liquid cultures for 5 days and methylcellulose for 10 days. Given results are representative for two independent experiments in duplicate.

Immunofluorescence: Cells were first fixed with 4% PFA and then cytospined and immunolocalization were performed. Cells were permeabilized with 0.5% Triton X-100 in PBS, treated with RNase and blocking was done in 0.1% Tween-20/PBS supplemented with 1% BSA. The MN1 primary antibody (a kind gift from M. Meesters & E. Zwarthoff, Rotterdam, The Netherlands) was added for 1 hour incubation at room temperature, slides were washed and incubated in the dark for one hour with the Alexa Fluor 488 goat anti-mouse antibody (Invitrogen, Carlsbad, CA). PI was

used for nuclei staining. Slides were washed and mounted with Fluorsafe Reagent (Calbiochem, San Jose, CA). Confocal microscopy was carried on a LSM 510 laser-scanning microscope (Zeiss, Oberkochen, Germany).

FACS sorting of Granulocyte Macrophage Progenitors (GMPs): The isolated bone marrow cells were first stained with the lineage cocktail which contains antibodies specific for the following mouse lineage markers: biotinylated rat anti-mouse CD5, CD11b, CD45R/B220, Ly-6G (Gr-1), and Ter119 (MAGM209, R&D Systems). Then cells were stained with a streptavidin Pacific Blue-conjugated (Invitrogen), a PE-Cy5-conjugated anti-mouse IL-7Rα (A7R34, eBioscience), a PE-conjugated anti-mouse FcγRII/III (93, eBioscience), an Alexa Fluor 647-conjugated anti-mouse CD34 (RAM34, BD Pharmigen), an APC-conjugated anti-mouse c-Kit (2B8, BD Pharmigen), and a PE-Cy7-conjugated anti-mouse Sca-1 (E13-161.7, Biolegend) monoclonal antibody. Granulocyte Macrophage Progenitors (GMPs) were FACS sorted as IL-7Rα$^-$ Lin$^-$ Sca-1$^-$ c-Kit$^+$ CD34$^+$ FcγRII/III^{hi} as described previously.[21]

Quantitative RT-PCR: Target validation was performed in triplicate by real-time PCR with SYBR-green on an ABI prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). For each target the results were normalized to GAPDH and given as ΔΔCt values normalized to MOCK-infected (*MSCV-IRES/EYFP*) bone marrow cells. The results are represented as the mean plus or minus standard deviations of 3 independent experiments. The detailed information about oligonucleotide primers is given in **Table 5**.

Gene-expression profiling: In three independent experiments bone marrow cells were transduced with *MSCV-MN1-IRES/EYFP* or empty vector. Seventy-two hours after transduction, EYFP-positive cells were FACS-sorted and RNA was isolated by ion-exchange chromatography with RNAmini (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA target was synthesized, fragmented, biotin-labeled using the WT Target Labeling and Control Reagents (Affymetrix, cat. # 900652, Santa Clara, USA) starting from 200ng total RNA, according to the procedure described in the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay Manual, Version 4. The cDNA was fragmented and the resulting

fragments of approximately 40-70 nucleotides were monitored with the Bioanalyzer using the RNA Nano 6000 Chip (Agilent, Palo Alto, USA). The hybridization cocktail containing fragmented biotin-labeled target DNA at a final concentration of 25ng/ l was transferred into GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix, cat. # 901168) and incubated at 45°C on a rotator in a hybridization oven 640 (Affymetrix) for 17 hours at 60rpm. The arrays were washed and stained on a Fluidics Station 450 (Affymetrix) by using the Hybridisation Wash and Stain Kit using the Fluidics Procedure FS450_0007. The GeneChips were processed with an Affymetrix GeneChip Scanner 3000 7G. DAT image files of the microarrays were generated using Affymetrix GeneChip Command Console (AGCC, version 0.0.0.676). All statistical analysis was performed using GeneSpring GX software (Agilent, Palo Alto, USA). Genes were considered as significant whenever the fold change was superior to 1.5 and the p-value <0.05. The data discussed in this publication have been deposited in NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and will be accessible through GEO Series accession number GSE13189.

Patient samples: Under informed consent by the guardians, seventy-three children diagnosed between August 2002 and April 2005 of ALL and fourteen of AML, according to the conventional FAB (French American British) and immunological criteria, were included in this study. 9 patients that were diagnosed of ALL were below one year of age. 78 were aged 1 to 15 years (median age 5.1 years). On the basis of the immunophenotype, sixty-four ALL patients were classified as prepre-B (n=10), cALL (n=38), pre-B (n=9), pro B-ALL (n=2), and T-ALL (n=5). Chromosome analysis from bone marrow cells was performed according to standard laboratory techniques. Definition of a clone and karyotype designation was according to ISCN 1995. Mononuclear cells were isolated using Ficoll-Hypaque (Pharmacia-LKB, Uppsala, Sweden) density gradient centrifugation (BM-MNC). RNA was extracted from BM-MNC at diagnosis and cDNA was synthesized by using random hexamers. RT-PCR for *TEL/AML1, BCR/ABL, MLL/AF4*, *E2A/PBX1, AML1/ETO, CBFbeta/MYH11* fusion genes was performed as previously described [81]. *FLT3* length mutations were determined by sequencing as previously described [82].

RESEARCH BACKGROUND

Expression of the class II mutations such as MLL fusions or MOZ/TIF2 in the murine bone marrow is able to induce an acute leukemia phenotype that closely resembles the human disease in many aspects. In most studies, the transplanted bone marrow cells have been transduced with a retrovirus expressing the respective leukemogenic fusion oncogene. A leukemic phenotype will develop after a latency period of couples of weeks or months. Interestingly, clonality analysis provides evidence that the leukemic blasts are composed of one (clonal) or only a few clones (oligoclonal) suggesting that additional genetic alterations might have been necessary for induction of the full blown disease phenotype. We therefore asked what could be the nature of potential functionally collaborating genetic events. In order to identify such genetic events for leukemogenesis of AML induced by class II mutations, we used the bone marrow reconstitution assay to model MLL/ENL and MOZ/TIF2 induced AML in the mouse. As shown previously, MLL/ENL and MOZ/TIF2 lead to the onset of AML after a latency of 3-4 months [40,58]. As more than 90% of the spleen and bone marrow cells are replaced by leukemic blasts in these mice, we were able to isolate genetic material (DNA, RNA) from a large number of tumor cells (**Fig. 1A**).

The homozygous gene mutations at distinct loci such as *WT1*, *FLT3*, *CEBPA*, and *AML1* were identified in AML patients with uniparental disomy (UPD). Those genes are well known as the targets of mutation in AML, and substitution of wild type allele with mutant allele caused by loss of heterozygosity (LOH) could act as the second hit that would contribute to leukemic transformation [83]. Therefore, we first planned to perform a genome-wide polymorphism analysis to determine if UPD is also frequently associated with leukemic mice. However, by applying a microsatellite PCR-based screen as well as a low resolution single nucleotide polymorphism (SNP) array approach we were not able to identify additional alterations in 21 murine leukemias induced by MLL/ENL or MOZ/TIF2.

Since in our model, a retroviral system was applied to express *MLL/ENL* or *MOZ/TIF2* fusion gene in mice, we wondered whether the deregulation of genes surrounding proviral insertions could act as collaborating genetic hit and cloned

retroviral integration sites in murine leukemias. Retroviral integration cloning strategy is a widely applied strategy for identifying oncogenes or tumor suppressor genes involved in cancer. The gene adjacent to a provirus can be activated by proviral enhancer or promoter insertion as well as post-transcription dysregulation, or inactivated by disruption of transcription [84,85]. A cell carrying such retroviral integration can acquire growth advantage and will be clonally selected to expand out. In such a case, those retroviral insertions may also serve as additional hits for multi-step progression of leukemia driven by the initial mutations [86].

The publication of the mouse genome sequence greatly advanced the large scale screening for novel oncogenes or tumor suppressor genes through the isolation of proviral insertion sites in the inbred mouse strains (such as AKXD and NFS.V^{+}) that produce high level of endogenous murine leukemia viruses (MuLVs), or the strains with deficiency of certain tumor suppressor genes were infected with replication competent retrovirus, such as murine moloney leukemia viruses (MuMoLVs). The experimental data from such screens demonstrated that the genes deregulated by retroviral integration could synergize with initial hits, by activation of oncogenes *c-Myc* or inactivation of tumor suppressor gene *Cdkn2a*, and induced leukemia or lymphoma in mice [87-90].

There is increasing evidence that the transduction of replication defective retrovirus (as applied in our models for transduction of the leukemia-initiating oncogene) may also contribute to tumorigenesis through retroviral integration mutagenesis. *Evi1* was identified as a (proto) oncogene by transducing mouse bone marrow cells with replication-incompetent murine stem cell virus (MSCV) expressing only neo, since the insertions of MSCV at 5' of the *Evi1* gene led to overexpression of Evi1 and immortalization of immature myeloid progenitors [79]. The advantage of this strategy is that it simplifies the identification of cooperating events through selection, because cell populations transformed are often clonal, and replication-defective viral vectors already contain an oncogene as the first ''hit'' [80,91]. Using a splinkerette-PCR approach, we identified 66 integration flanking genes from 21 murine leukemias induced by MLL/ENL (n=10) or MOZ/TIF2 (n=11). We validated the impact of proviral integration on expression of these genes by quantitative RT-PCR. Among the

positively validated genes, we identified *meningioma 1* (*MN1*) as a potentially collaborating oncogene.

The *meningioma 1* (*MN1*) gene was first identified as the target of a sporadic balanced chromosomal translocation in a patient with meningioma [92]. The absence of MN1 expression in the index patients has led to the suggestion that MN1 is a candidate tumor suppressor gene. Several studies have proposed that MN1 presumably acts as a transcriptional cofactor most probably through interaction with other transcriptional regulators such as p300/CBP or RXR/RAR [93,94]. MN1 was first linked to human leukemia after the cloning of the balanced chromosomal translocation t(12;22)(p13;q12) found in patients with AML, myelodysplasia (MDS) or CML. This translocation leads to the expression of a MN1/TEL fusion that consists of almost the entire open reading frame (ORF) of MN1 fused with the DNA binding moiety of ETV6 [95]. Expression of MN1/TEL in the mouse hematopoietic system by a conditional knock-in strategy resulted in the formation of T-cell lymphomas as well as AML after a long latency suggesting that MN1/TEL, like MLL fusions, is essential but not sufficient to induce the disease [96-98]. Gene expression profiling studies of a large number of human leukemia samples demonstrated that MN1 is deregulated in cases with alterations at 3q26 leading to EVI1 overexpression. In addition, elevated MN1 has been associated with the presence of inv16 leading to a CBFβ/MYH11 fusion [99]. During the time of our study, functional studies have demonstrated that overexpression of MN1 alone is able to induce an AML phenotype in mice [100,101]. Furthermore, high MN1 expression was shown to have negative prognostic impact in AML, especially in the absence of common karyotype abnormalities [102].

RESULTS

1. A mouse model for class II mutation induced acute leukemia

In order to identify and biologically characterize any cooperating genetic event for the development of AML induced by class II mutations, I first established mouse models of two well-characterized class II fusion oncogenes *MLL/ENL* and *MOZ/TIF2* known to induce acute leukemia when expressed in the mouse bone marrow [40,55]. Similar to previous studies, transplantation of bone marrow cells retrovirally expressing MLL/ENL or MOZ/TIF2 led to induction of acute leukemia phenotype in lethally irradiated syngeneic mice after a latency of 2-4 months (**Fig. 1 A&B**). The disease was characterized by high white blood counts, hepatomegaly, splenomegaly, lymphadenopathy and extensive bone marrow and organ infiltration, reflecting clinical features of AML patients associated with these two fusions (**Fig. 1C**).

2. Screening for loss of heterozygosity (LOH) in class II mutation mediated murine leukemias

Since the homozygous (point) mutations in known tumor suppressor or oncogenes resulting from loss of heterozygosity (LOH) has been frequently observed in human AML, we wondered whether this mechanism could provide collaborating genetic hits in our murine leukemia models. I therefore performed a simple sequence length polymorphism (SSLP) based genotyping analysis to screen for LOH in MLL/ENL or MOZ/TIF2 induced leukemia in mice ([FVB/N x 129/s1] F1). The F1 offspring used in this study were derived from FVB/N strain crossed with the 129/s1 strain, and contain alleles from different parental origins that can be distinguished by using distinct polymorphic markers. First I examined all 19 mouse autosomes with selected 52 microsatellite markers known to be polymorphic for both strains, representing 2-3 widely distributed microsatellie markers per chromosome (**Fig. 1D**). To increase the density of polymorphic markers, we also applied mouse mapping 5K single nucleotide polymorphism (SNP) array, whose mapping resolution is narrowed to a 1cM (~2Mb) region, and the median number of informative markers is 1,500 SNPs

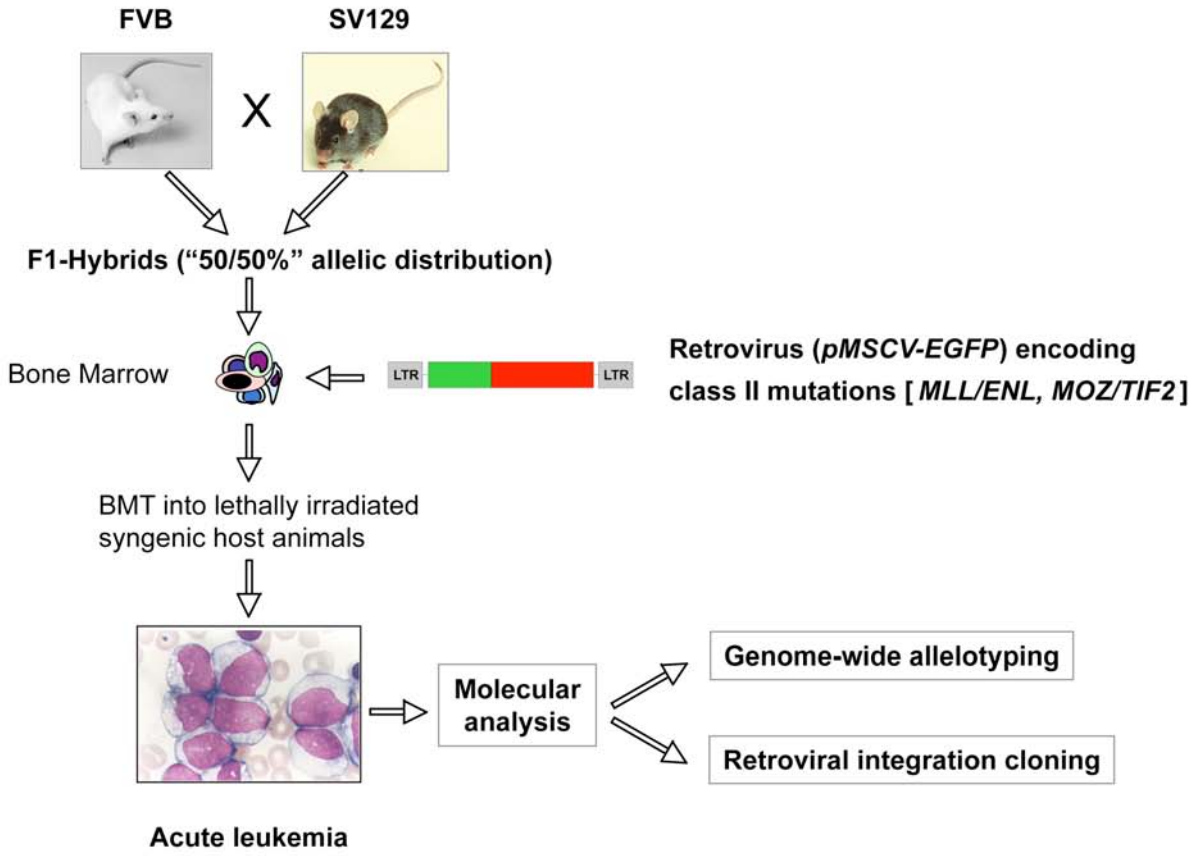
across two strains (performed by an external collaborator) (**Fig. 1E**). However, no region of LOH was found in both of the assays from all 21 mice (**Fig. 1F**), suggesting LOH might be rare events in murine leukemias induced by transplantation of bone marrow retrovirally expressing MLL/ENL or MOZ/TIF2. It is possible that LOH resulted from gene conversion occurring in very small regions of chromosomes would be overseen due to the limited density of our screens. Nevertheless, since LOH in human leukemia often involves large stretches of DNA, our analysis suggests that the large scale of LOH may be not a common event in MLL/ENL or MOZ/TIF2 induced acute leukemia in mice.

Fig.1. Mice developed acute leukemia after transplanted with bone marrow expressing MLL/ENL or MOZ/TIF2

A) Schematic illustration of the experiment. **B**) The transplantation of MLL/ENL or MOZ/TIF2 transduced bone marrow cells induced acute leukemia in recipient mice after the median latency of 64 days or 103 days respectively. **C**) MLL/ENL or MOZ/TIF2 induced murine leukemias were characterized as extensive infiltration of leukemic blasts in spleen, liver, skeletal muscle, kidney and lung. **D**) The microsatellite markers used for the genome-wide allelotyping. **E**) The mapping panel of Affymetrix GeneChip Mouse Mapping 5K SNP Kit. **F**) The PCR products amplified using microsatellite markers are separated by capillary electrophoresis and read by a DNA sequencer. The height of each major peak in electrogram indicates the amount of PCR products of each allele. The equal height of two peaks means no LOH occurs. One result was taken as a representative.

Fig. 1.

A


FVB
SV129
X
F1-Hybrids ("50/50%" allelic distribution)
Bone Marrow
LTR
LTR
Retrovirus (pMSCV-EGFP) encoding
class II mutations [MLL/ENL, MOZ/TIF2]
BMT into lethally irradiated
syngenic host animals
Molecular analysis
Genome-wide allelotyping
Retroviral integration cloning
Acute leukemia

B

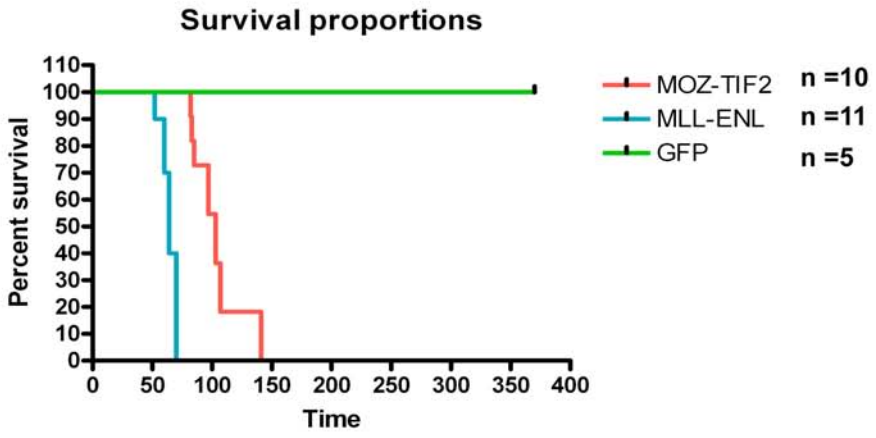

Survival proportions
Percent survival
110
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350
400
Time
MOZ-TIF2
MLL-ENL
GFP
n =10
n =11
n =5

Fig. 1.

C

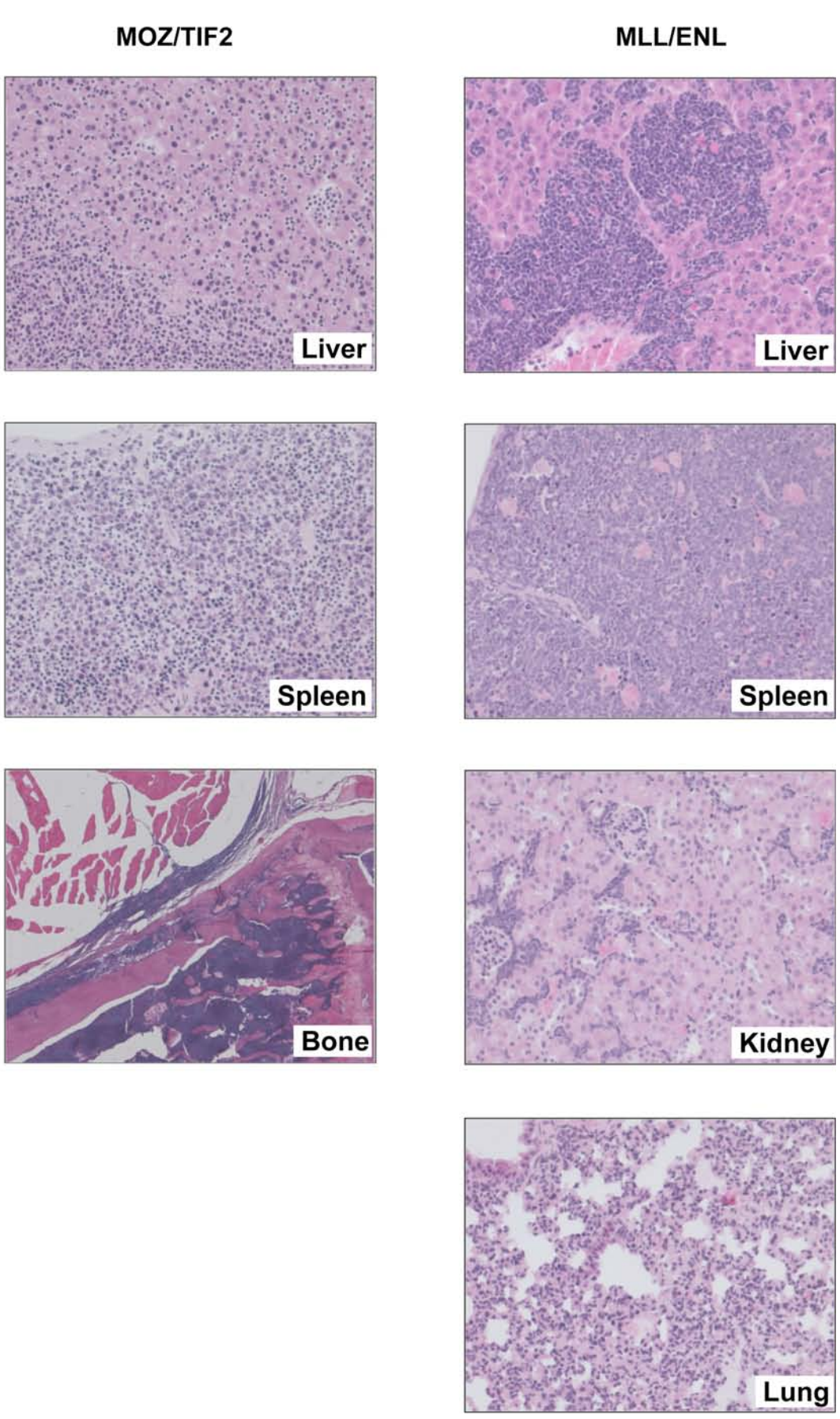
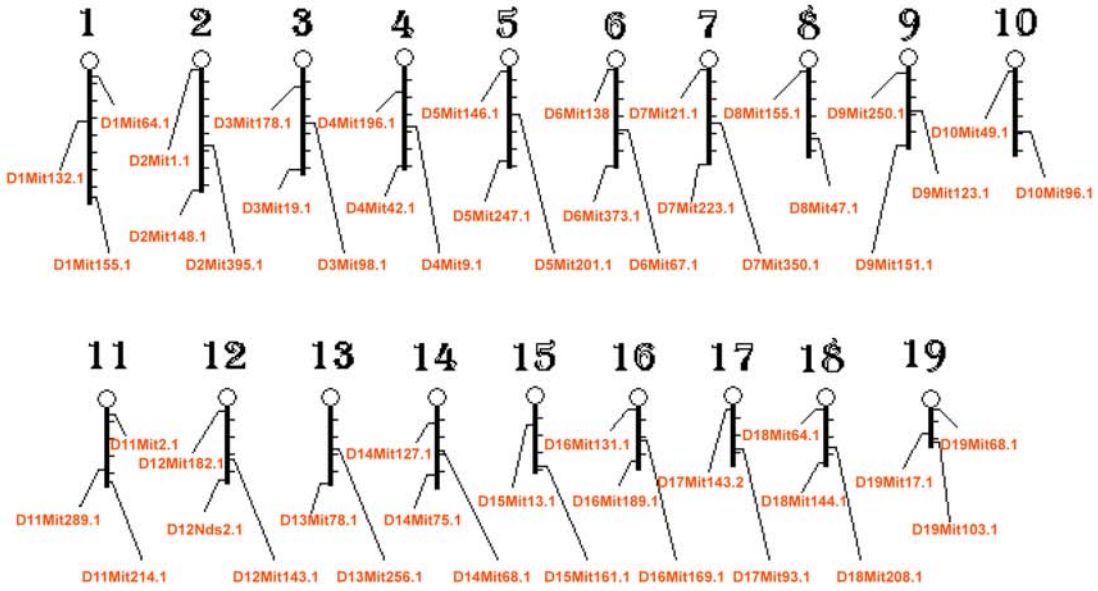


Fig. 1.

D


1
2
3
4
5
6
7
8
9
10
D1Mit64.1
D1Mit132.1
D1Mit155.1
D2Mit1.1
D2Mit148.1
D2Mit395.1
D3Mit178.1
D3Mit19.1
D3Mit98.1
D4Mit196.1
D4Mit42.1
D4Mit9.1
D5Mit146.1
D5Mit247.1
D5Mit201.1
D6Mit138
D6Mit373.1
D6Mit67.1
D7Mit21.1
D7Mit223.1
D7Mit350.1
D8Mit155.1
D8Mit47.1
D9Mit250.1
D9Mit123.1
D9Mit151.1
D10Mit49.1
D10Mit96.1
11
12
13
14
15
16
17
18
19
D11Mit2.1
D11Mit289.1
D11Mit214.1
D12Mit182.1
D12Nds2.1
D12Mit143.1
D13Mit78.1
D13Mit256.1
D14Mit127.1
D14Mit75.1
D14Mit68.1
D15Mit13.1
D15Mit161.1
D16Mit131.1
D16Mit189.1
D16Mit169.1
D17Mit143.2
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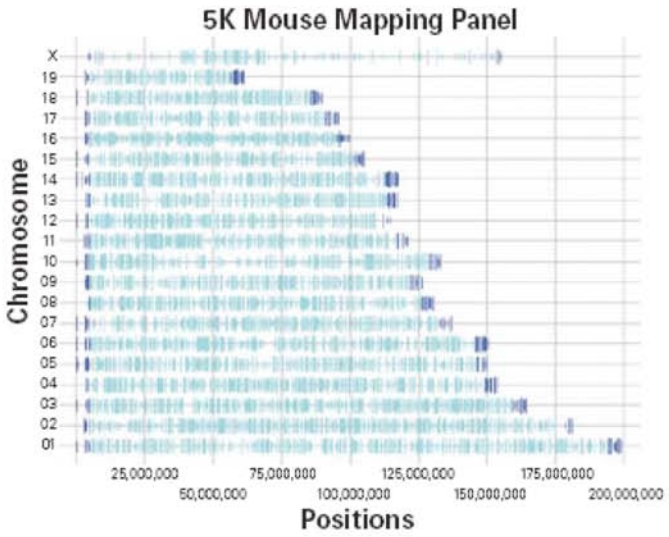
D18Mit144.1
D18Mit208.1
D19Mit17.1
D19Mit68.1
D19Mit103.1

E


5K Mouse Mapping Panel
Chromosome
X
19
18
17
16
15
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12
11
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09
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01
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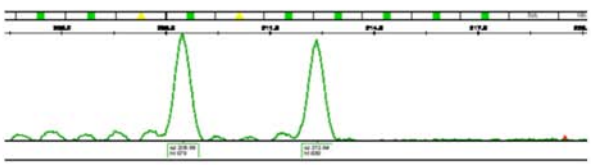
200,000,000
Positions

F



3. Hunting potential cooperating genetic events through retroviral insertion sites cloning

To gain more information about potential collaborative genetic events in the class II mutation mediated leukemogenesis, I analyzed retroviral integration sites from 10 MLL/ENL and 11 MOZ/TIF2 mice using a splinkerette PCR technique. The assay was performed with DNA from leukemic blasts from each animal in two independent experiments. The visualized PCR products were purified and sequenced, and the integration site nearest 5' and 3' annotated genes were determined (**Fig. 2A**). In total, I was able to identify 66 flanking genes from 21 leukemic animals and among them, 21 genes were common integration sites (CIS) flanking genes (**Table 1&2**). CISs mean the certain genomic regions that are targeted by retroviral integration in more than one tumor and are thus likely to encode a disease gene [103].

To confirm that genes flanking proviral integrations might be dysregulated, we examined the expression levels of some genes that have been previously linked to cancer by quantitative RT-PCR analysis. As shown in **Fig. 2B**, quantitative RT-PCR analysis revealed that *Tcf7*, *Tnfrsf1*, *Mn1*, *Lhx2* were all up-regulated, and *Pur-alpha*, *Ppp2r5c*, *Runx3*, *Socs1* as well as *Prdm2* were down-regulated in the animals carrying the respective integration when comparing with healthy controls or other leukemic mice without the corresponding integration.

PRDM2 is a member of PR domain-containing protein family, and encoded by retinoblastoma protein-interacting zinc finger gene (*RIZ*). PRDM is a family of transcriptional regulators that modulate cell differentiation, growth and apoptosis. A common feature of the *PRDM* gene family is that they can produce 2 types of transcripts that differ in the presence or absence of the PR domain (the PRDI-BF1-RIZ1 homologous region). Noteworthily, the products keeping PR-domain have anti-tumorgenic potential and are often down-regulated in cancer cells, whereas the products without PR-domain may act as oncogene and are over-expressed in tumor tissue [104]. EVi1 and PRDM16 also belong to PRDM family. Neal Copeland's group has shown that in 37 murine hematopoietic cell lines immortalized by an "empty" (carrying no transgene) MSCV retrovirus (that is used here), 7 lines contained MSCV

integrations in the first 2 intron of *Evi1* and another 13 lines contained integrations in the first intron of *Prdm16*, which promoted expression of transcript variants lacking PR-domain. The *PRDM2/RIZ* gene produces two mRNA and protein products through alternative promoters, *RIZ1* that contains the PR domain with methyltransferase activity, and *RIZ2* that lacks this domain. *RIZ1*, but not *RIZ2*, has been shown to have tumor suppressor activity, and altered *RIZ1/RIZ2* expression has been found in some human acute myeloid/lymphoid leukemias [105].

When we performed PCR using primers located in the 6th exon of *Prdm2*, the observed mRNA level of *Prdm2* were remarkably high, which was totally contrary to result using primers covering the region of PR-domain (**Fig. 2C**). Since exon 6 is located downstream of PR-domain, our results indicated that the insertion of retrovirus disrupted production of transcripts of *Riz1*, but led to accumulation of *Riz2* products, which might contribute to oncogenic expansion of MOZ/TIF2 transduced cells. The microsatellite PCR analysis of *Prdm2* surrounding regions showed that wild type allele was not replaced by proviral integrated allele, suggesting the DNA methylation of promoter region may be the reason leading to inactivation of expression of *Prdm2* in wild type allele.

Many of the genes flanking MLL/ENL or MOZ/TIF2 carrying proviral integrations have been functionally linked to cancer biology. In order to test their leukemic transforming potentials *in vitro*, we cloned the cDNAs of some of these target genes including PRDM2, LHX2, FLT3L, BCOR, SEI1, SEI3 and MN1 into MSCV virus and overexpressed them in murine bone marrow cells. We also tested their impact on cellular self-renewal capacity in serial replating assays. As shown in **Fig. 2 D&E**, among these 7 genes, MN1 overexpression significantly enhanced cellular growth of bone marrow cells and increased the resistance to deprivation of cytokines in medium. Moreover, only overexpression of MN1 resulted in increased serial replating capcity and still formed colonies in the third round of replating. These observations suggested that deregulated expression of MN1 might act as a collaborating genetic event in MLL/ENL mediated leukemogenesis.

Fig. 2.

A

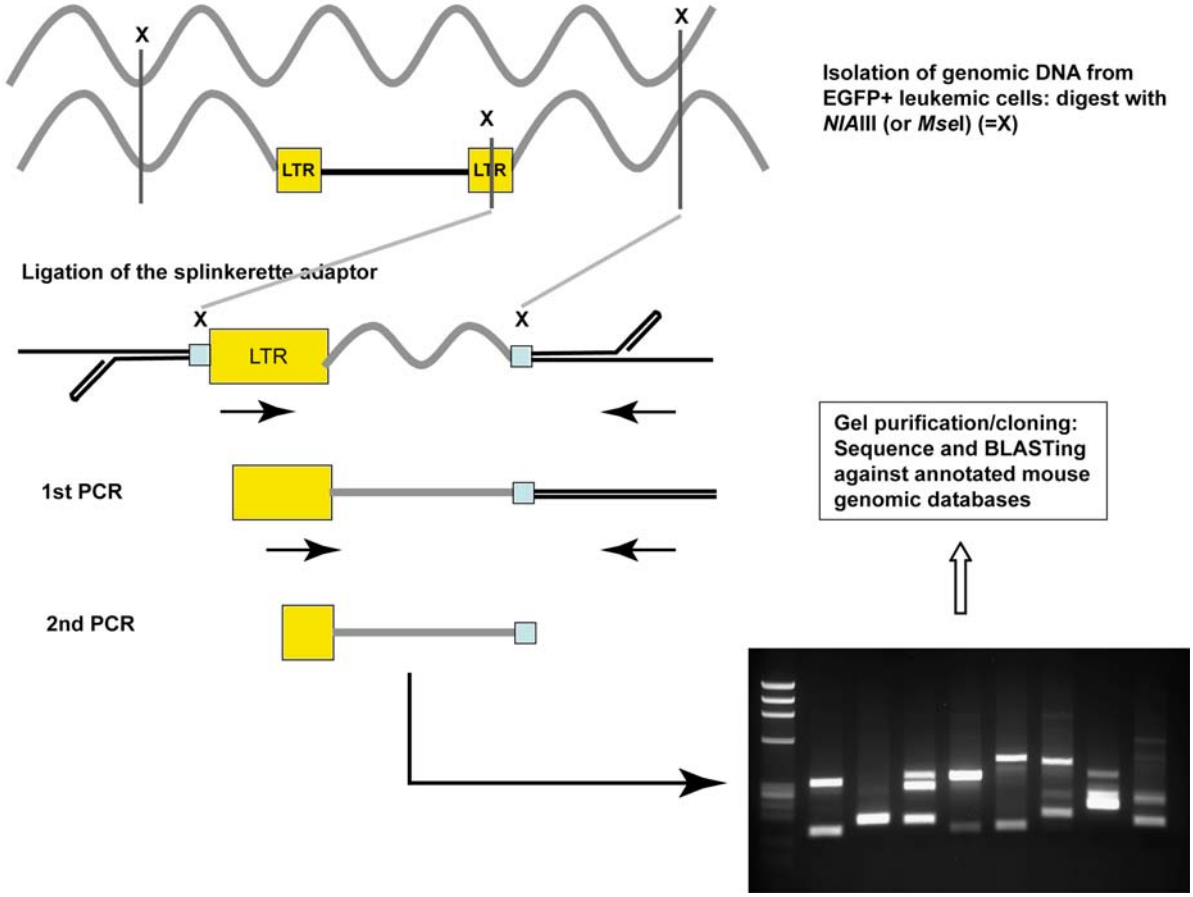

X
X
X
LTR
LTR
Isolation of genomic DNA from EGFP+ leukemic cells: digest with NlAIII (or MseI) (=X)
Ligation of the splinkerette adaptor
X
X
LTR
1st PCR
2nd PCR
Gel purification/cloning: Sequence and BLASTing against annotated mouse genomic databases

Fig. 2.

B

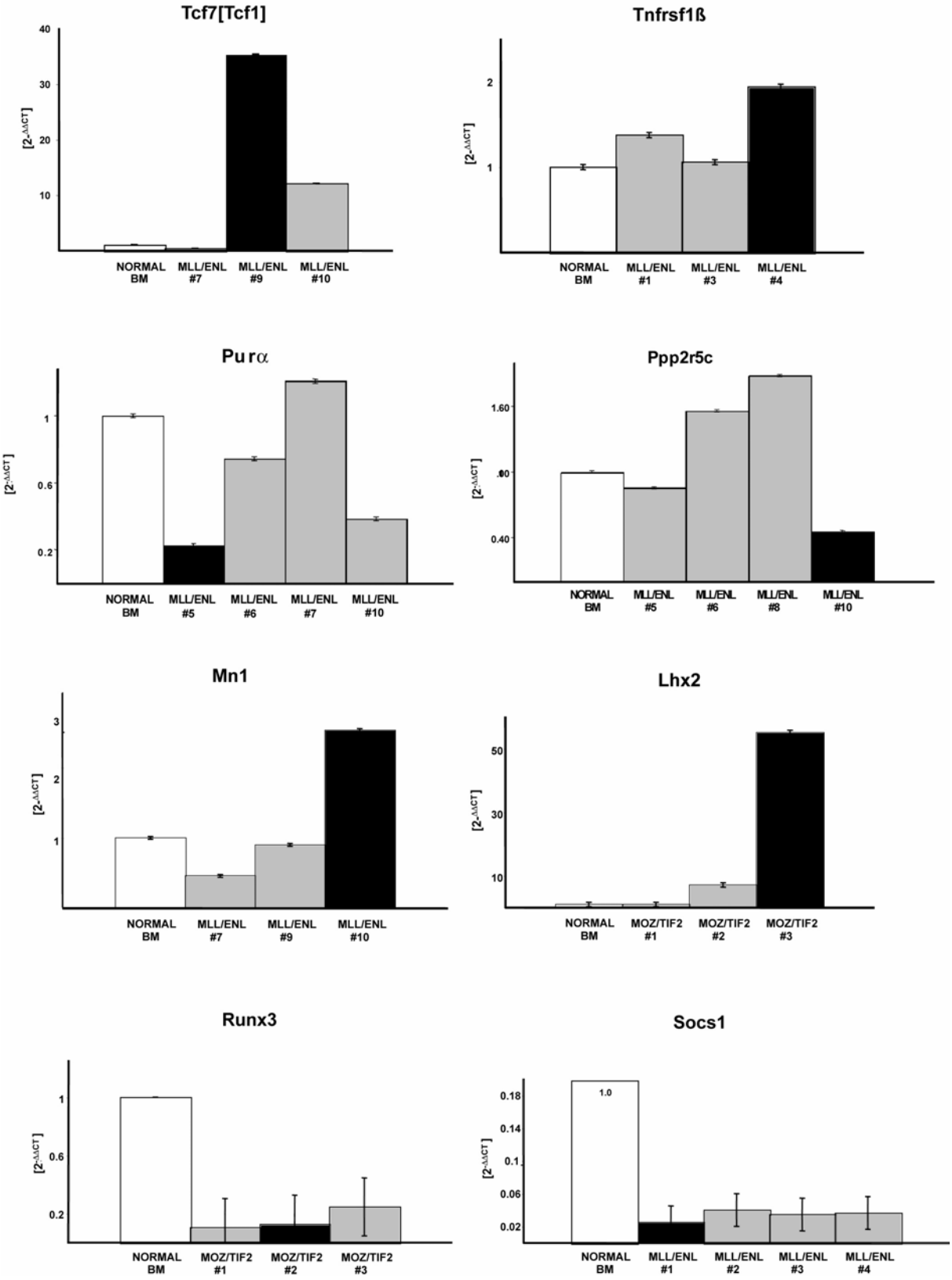
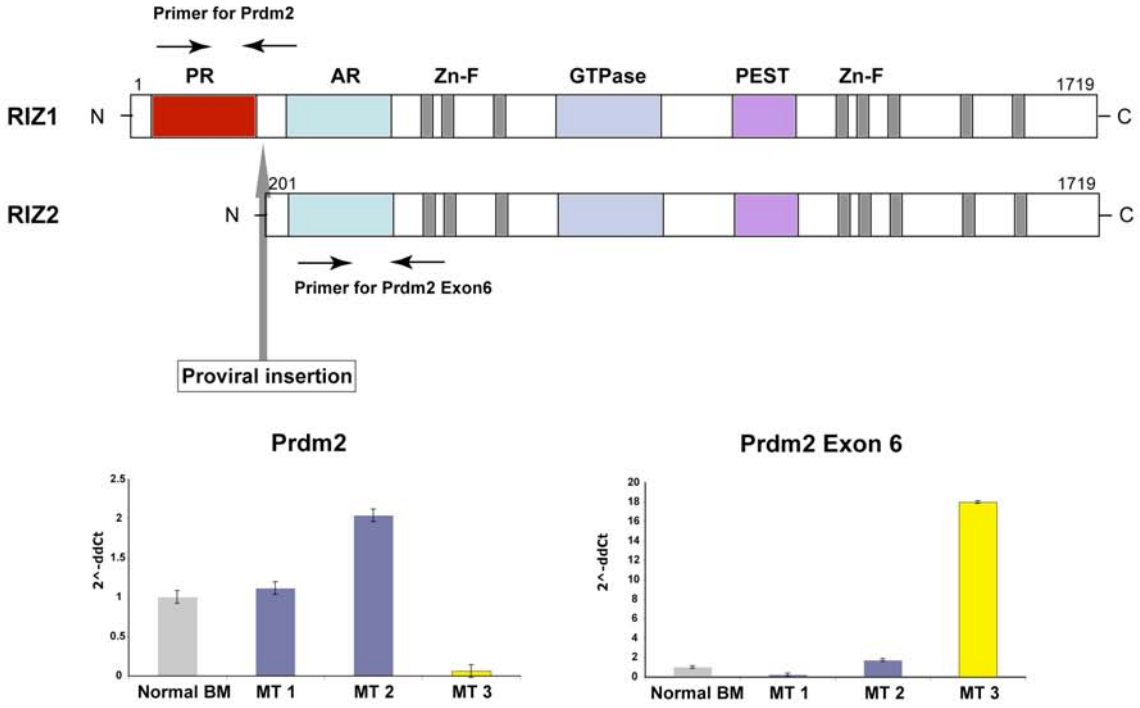
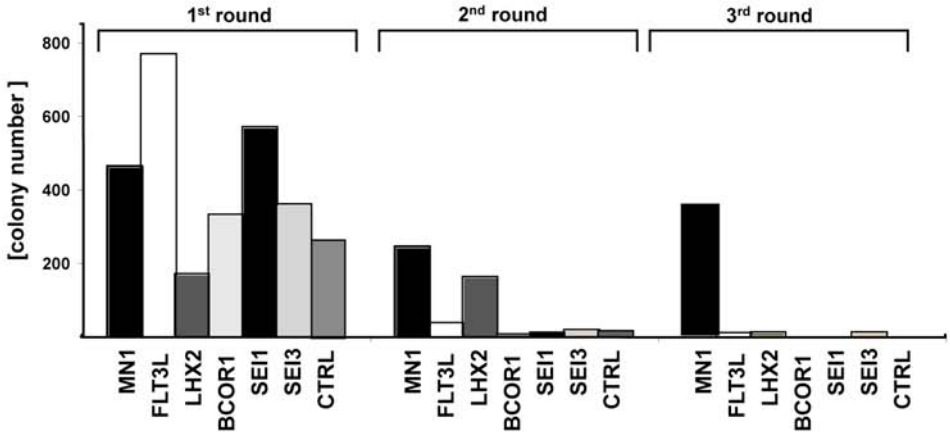

Tcf7[Tcf1]
[2-ΔΔCT]
40
30
20
10
NORMAL BM
MLL/ENL #7
MLL/ENL #9
MLL/ENL #10
Tnfrsf1ß
[2-ΔΔCT]
2
1
NORMAL BM
MLL/ENL #1
MLL/ENL #3
MLL/ENL #4
Pu rα
[2-ΔΔCT]
1
0.6
0.2
NORMAL BM
MLL/ENL #5
MLL/ENL #6
MLL/ENL #7
MLL/ENL #10
Ppp2r5c
[2-ΔΔCT]
1.60
.80
0.40
NORMAL BM
MLL/ENL #5
MLL/ENL #6
MLL/ENL #8
MLL/ENL #10
Mn1
[2-ΔΔCT]
3
2
1
NORMAL BM
MLL/ENL #7
MLL/ENL #9
MLL/ENL #10
Lhx2
[2-ΔΔCT]
50
30
10
NORMAL BM
MOZ/TIF2 #1
MOZ/TIF2 #2
MOZ/TIF2 #3
Runx3
[2-ΔΔCT]
1
0.6
0.2
NORMAL BM
MOZ/TIF2 #1
MOZ/TIF2 #2
MOZ/TIF2 #3
Socs1
[2-ΔΔCT]
1.0
0.18
0.14
0.1
0.06
0.02
NORMAL BM
MLL/ENL #1
MLL/ENL #2
MLL/ENL #3
MLL/ENL #4

Fig. 2.

C


Primer for Prdm2
PR
AR
Zn-F
GTPase
PEST
Zn-F
1
1719
RIZ1
N
C
201
1719
RIZ2
N
C
Primer for Prdm2 Exon6
Proviral insertion
Prdm2
2^-ddCt
Normal BM
MT 1
MT 2
MT 3
Prdm2 Exon 6
2^-ddCt
Normal BM
MT 1
MT 2
MT 3

D


1st round
2nd round
3rd round
[colony number]
800
600
400
200
MN1
FLT3L
LHX2
BCOR1
SEI1
SEI3
CTRL

E

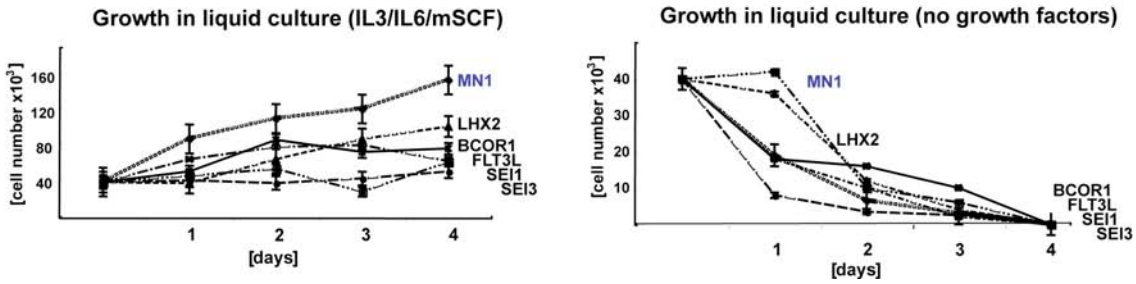

Growth in liquid culture (IL3/IL6/mSCF)
[cell number x10^3]
160
120
80
40
MN1
LHX2
BCOR1
FLT3L
SEI1
SEI3
1
2
3
4
[days]
Growth in liquid culture (no growth factors)
[cell number x10^3]
40
30
20
10
MN1
LHX2
BCOR1
FLT3L
SEI1
SEI3
1
2
3
4
[days]

Table 1. Retroviral integration sites cloned from murine leukemia induced by MLL/ENL

Mouse No.	Gene symbol	Full Name	(Predicted) gene function	Location and distance for retroviral integration	Orientation	Mouse chr.	Human chr.	CIS**
* ME 1 I	Rps6kb1	ribosomal protein S6 kinase polypeptide 1	protein serine/threonine kinase	Downstream 5659 bp	Opposite	4	17q23.1	N/A
	Hmgn2	high mobility group nucleosomal binding domain 2	DNA binding protein	Upstream 76653 bp	Opposite	4	1p36.1	N/A
II	Supt4h2	suppressor of Ty 4 homolog 2	transcription factor	Downstream 6797 bp	Opposite	11	17q21-q23	N/A
	Bzrap1	benzodiazapine receptor associated protein 1	benzodiazepine receptor binding	Upstream 29292 bp	Opposite	11	17q22-q23	N/A
III	Clec16a	C-type lectin domain family 16, member A	sugar binding	Downstream 28879 bp	same	16	16p13.13	1
	Socs1	suppressor of cytokine signaling 1	negetive regulator of JAK-STAT signaling	Upstream 13327 bp	Opposite	16	16p13.13	N/A
ME 2	Saps1	SAPS domain family, member 1	regulation of phosphoprotein phosphatase activity	Downstream 5254 bp	same	7	19q13.42	2
	Hspbp1	SPA) binding protein, cytoplasmic cochaperone 1	hsp70-interacting protein	Upstream 8554 bp	same	7	19q13.42	N/A
ME 3 I	Flt3l	FMS-like tyrosine kinase 3 ligand	ligand of receptor protein tyrosine kinase	Downstream 541 bp	Opposite	7	19q13.3	N/A
	Aldh16a1	aldehyde dehydrogenase 16 family, member A1	oxidoreductase activity	Upstream 5308 bp	Opposite	7	19q13.33	3
II	Cd83	CD83 antigen	adhesion receptor	Downstream 633289 bp	same	13	6p23	1
	Jarid2	jumonji protein	DNA binding protein	Upstream 293541 bp	same	13	6p24-p23	N/A
III	Clrn3	clarin 3	transmembrane protein	Downstream 55148 bp	Opposite	7	10q26.2	4
	Ptpre	protein tyrosine phosphatase, receptor type, E	protein tyrosine phosphatase	Upstream 60092 bp	same	7	10q26	4
IV	Ralgps2	Ral GEF with PH domain and SH3 binding motif 2	guanyl-nucleotide exchange factor	Downstream 73087 bp	Opposite	1	1q25.2	N/A
	Angptl1	angiopoietin-like 1	receptor protein tyrosine kinase signaling pathway	Downstream 126043 bp	same	1	1q25.2	N/A
	1700057K13Rik	hypothetical protein LOC73435	Unknown	Upstream 112647 bp	Opposite	1	1q25.2	N/A
ME 4 I	Hsp90aa1	heat shock protein 1, alpha	molecular chaperone	Downstream 4932 bp	Opposite	12	14q32.33	N/A
	Wdr20a	WD repeat domain 20	Unknown	Upstream 37074 bp	same	12	N/A	N/A
II	Mid1ip1	Mid1 interacting protein	negative regulator of microtubule depolymerization	Downstream 940212 bp	Opposite	X	Xp11.4	N/A
	Bcor	BCL-6 interacting corepressor isoform a	transcription repressor	Upstream 371669 bp	same	X	Xp21.2-p11.4	9
III	Tnfrsf1b	tumor necrosis factor receptor superfamily, member 1b	tumor necrosis factor receptor	1st intron	same	4	1p36.3-p36.2	1
IV	Itm2b	integral membrane protein 2B	induction of apoptosis	Downstream 10455 bp	Opposite	14	13q14.3	N/A
	Med4	vitamin D receptor interacting protein	transcription regulator	Upstream 113900 bp	same	14	13q14.2	N/A
ME 5 I	Ly86	lymphocyte antigen 86	immune response	Downstream 204132 bp	same	13	6p25.1	2
	Rreb1	ras responsive element binding protein 1 isoform 2	transcription regulator	Upstream 271015 bp	same	13	6p25	9
II	Psd2	pleckstrin and Sec7 domain containing 2	ARF guanyl-nucleotide exchange factor	Downstream 181588 bp	Opposite	18	5q31	1
	Pura	purine rich element binding protein A	DNA binding protein	Upstream 92667 bp	Opposite	18	5q31.3	1
ME 6 I	Rbks	ribokinase	ribose kinase	7th intron	same	5	2p23.3	N/A
II	Retn	resistin	hormone	Downstream 5657 bp	same	8	19p13.2	N/A
	1810033B17Rik	hypothetical protein LOC69189	Unknown	Upstream 2515 bp	same	8	19p13.2	N/A
III	Noxa1	NADPH oxidase activator 1	superoxide-generating NADPH oxidase activator	Downstream 69311 bp	same	2	9q34.3	N/A
	Nrarp	Notch-regulated ankyrin repeat protein	Notch signaling pathway	Upstream 16770 bp	Opposite	2	9q34.3	N/A
ME 7	Malat1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	prognostic parameter for nonsmall cell lung cancer	Downstream 5581 bp	same	19	11q13.1	N/A
	Frmd8	FERM domain containing 8	cytoskeleton	Upstream 49265 bp	same	19	11q13	3
ME 8	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	MAPK signaling pathway	Downstream 9864 bp	Opposite	10	19p13.3	1
	Gng7	guanine nucleotide binding protein (G protein), gamma 7 subunit	G-protein coupled receptor signaling pathway	Upstream 9958 bp	same	10	19p13.3	N/A
ME 9 I	Slc38a2	solute carrier family 38, member 2	amino acid and ion transport	Downstream 61937 bp	Opposite	15	12q	6
	Slc38a4	solute carrier family 38, member 4	amino acid and ion transport	Upstream 236288 bp	Opposite	15	12q13	N/A
II	Tcf7	transcription factor 7, T-cell specific	WNT signaling pathway	1st intron	same	11	5q31.1	N/A
ME 10 I	Mgat5	mannoside acetylglucosaminyltransferase 5	acetylglucosaminyltransferase	1st intron	same	1	2q21	1
II	LOC629605	hypothetical protein LOC629605	Unknown	Downstream 8254 bp	same	12	N/A	N/A
	Ppp2r5c	protein phosphatase 2, regulatory subunit B (B56), gamma isoform	protein phosphatase type 2A regulator	Upstream 26735 bp	same	12	14q32	3
III	Socs7	suppressor of cytokine signaling 7	negative regulation of insulin receptor signaling pathway	Downstream 12212 bp	Opposite	11	17q12	N/A
	LOC665512	similar to Rho GTPase activating protein 21	Unknown	Upstream 38114 bp	Opposite	11	N/A	N/A
IV	Mn1	meningioma 1	oncogene	Downstream 99834 bp	same	5	22q11	8
	C130026L21Rik	hypothetical protein LOC330164	Unknown	Upstream 27121 bp	same	5	N/A	N/A

* ME = Murine leukemia induced by overexpression of MLL/ENL. Roman Numerals mean different insertion sites identified from the same leumemia mouse.
** CIS = Common Integration Sites. Numbers indicate hits of the same integration flanking gene found in RTCGD database (http://rtcgd.abcc.ncifcrf.gov/).
N/A = not applicable.

Table 2. Retroviral integration sites cloned from murine leukemia induced by MOZ/TIF2

Mouse No.	Gene symbol	Full Name	(Predicted) gene function	Location and distance for retroviral integration	Orientation	Mouse chr.	Human chr.	CIS **
* MT 1	Cd244	CD244 natural killer cell receptor 2B4	signal transduction	1st intron	Opposite	1	1q23.3	1
MT 2	Il5	interleukin 5	cytokine	Downstream 39482 bp	same	11	5q31.1	5
	Irf1	interferon regulatory factor 1	transcription factor	Upstream 7766 bp	same	11	5q31.1	5
MT 3 I	Syf2	SYF2 homolog, RNA splicing factor	mRNA processing	Downstream 59425 bp	same	4	1p36.11	1
	Runx3	runt related transcription factor 3	transcription factor	Upstream 121925 bp	same	4	1p36	8
II	Prdm2	PR domain containing 2, with ZNF domain isoform 2	transcription regulator	4th intron	same	4	1p36.21	N/A
III	Lhx2	LIM homeobox protein 2	transcription factor	Downstream 113762 bp	Opposite	2	9q33-q34.1	1
	Nek6	NIMA (never in mitosis gene a)-related expressed kinase 6	protein serine/threonine kinase	Upstream 67647 bp	Opposite	2	9q33.3-q34.11	1
IV	2610034B18Rik	RIKEN cDNA 2610034B18 gene	Unknown	Downstream 132724 bp	Opposite	7	15q26.1	N/A
	Zfp710	zinc finger protein 710/5 primer UTR	transcription regulator	Upstream 13110 bp	same	7	15q26.1	2
MT 4	D130011D22Rik	RIKEN cDNA E030049G20 gene	Unknown	Downstream 715076 bp	Opposite	1	2q21.2	N/A
	Mgat5	mannoside acetylglucosaminyltransferase 5	acetylglucosaminyltransferase	Upstream 63030 bp	same	1	2q21	1
MT 5 I	Ppp2r3c	protein phosphatase 2, regulatory subunit B", gamma	protein phosphatase type 2A regulator	1st intron	Opposite	12	14q13.2	N/A
II	EG665466	predicted gene, EG665466	Unknown	1st intron	same	14	N/A	N/A
MT 6	Rps24	ribosomal protein S24 isoform 3	cytosolic small ribosomal subunit	Downstream 671073 bp	same	14	10 q22-q23	1
	D930049A15Rik	RIKEN cDNA I920194N01gene	Unknown	Upstream 295648 bp	same	14	N/A	N/A
MT 7 I	Cebpb	CCAAT/enhancer binding protein beta	transcription factor	Downstream 116678 bp	Opposite	2	20q13.1	23
	Ptpn1	protein tyrosine phosphatase, non-receptor type 1	protein tyrosine phosphatase	Upstream 128167 bp	Opposite	2	20q13.1-q13.2	1
II	Tmed10	transmembrane emp24-like trafficking protein 10	intracellular protein transport	1st intron	same	12	14q24.3	3
MT 8	Mcl1	myeloid cell leukemia sequence 1	Apoptosis regulator	Downstream 12361 bp	same	3	1q21	4
	Adamtsl4	ADAMTS-like 4	Apoptosis regulator	Upstream 3512 bp	Opposite	3	1q21.2	N/A
MT 9	Ly86	lymphocyte antigen 86	immune response	Downstream 323166 bp	same	13	6p25.1	2
	Rreb1	ras responsive element binding protein 1 isoform 2	transcription regulator	Upstream 151900 bp	same	13	6p25	9
MT 10	Zfp1	zinc finger protein 1	transcription regulator	Upstream 7325 bp / 5' UTR	Opposite	8	16q23.1	1
MT 11 I	Sertad3	SERTA domain containing 3	transcription regulator	Downstream 2047 bp	Opposite	7	19q13.2	N/A
	Sertad1	SERTA domain containing 1	transcription regulator	Upstream 10223 bp	Opposite	7	19q13.1-q13.2	N/A
II	Crtap	cartilage associated protein	spermatogenesis	Downstream 9990 bp	same	9	3p22.3	N/A
	Glb1	galactosidase, beta 1	beta-galactosidase	Upstream 525 bp	Opposite	9	3p21.33	2

* MT = Murine leukemia induced by overexpression of MOZ/TIF2. Roman Numerals mean different insertion sites identified from the same leumemia mouse.

** CIS = Common Integration Sites. Numbers indicate hits of the same integration flanking gene found in RTCGD database (http://rtcgd.abcc.ncifcrf.gov/).

N/A = not applicable.

Fig.2. Identification of cooperating oncogenes by cloning of retroviral insertion sites in MLL/ENL and MOZ/TIF2 murine leukemias.

A) Schematic illustration of the splinkerette PCR strategy. **B**) mRNA expression levels of retroviral integration flanking genes in MLL/ENL and MOZ/TIF2 leukemic mice. Relative expression levels of target genes were normalized to GAPDH expression in the same sample, calibrated to gene expression in normal murine BM and given as ΔΔCt value. Black bars represent the leukemic mice carrying corresponding integrations, and grey bars represent irrelevant leukemic mice. **C**) Insertion of retrovirus in *Prdm2* gene interrupted expression of *Riz1*, but promoted production of *Riz2* that lacks PR-domain. Murine leukemia MT3 harbors a retroviral integration site in *Prdm2* gene. **D**) Serial replating assay of murine bone marrow cells overexpressing retroviral integration target genes in methylcellulose. **E**) Growth curve of murine bone marrow cells overexpressing retroviral integration target genes in liquid culture with or without cytokines. MT means murine leukemia induced by MOZ/TIF2.

4. *Meningioma 1* (*MN1*) as a potential cooperating oncogene in MLL/ENL induced murine leukemia

In the MLL/ENL leukemia mouse no.10 (ME10), I found the proviral insertion sites adjacent to several proto-oncogenes or tumor suppressor genes including *Mgat5*, *Socs7*, *Ppp2r5c* and *Mn1*. To validate their potential impact, I firstly compared the expression levels of those genes in ME10 with 5 other MLL/ENL leukemias and a control sample pooled from the bone marrow from 5 normal mice. The expression of *Socs7* and *Ppp2r5c* was lower in ME10 than normal and other leukemic mice, whereas *Mn1* expression was significantly up-regulated by the MSCV-MLL/ENL provirus insertion (**Fig. 3 A&B)**. During our investigation the *Mn1* locus has been reported as a recurrent integration site in mouse leukemia models induced by reconstitution of bone marrow retrovirally expressing leukemogenic oncogenes (mutant *AML1*, *NUP98/HOX*) that might be functionally linked to MLL fusions [106,107]. We therefore focused on MN1 for further studies.

The clonal contribution of the MN1 targeting integration was determined by plating leukemic blasts from this respective MLL/ENL leukemia (ME10) in semi-solid medium and determined the integration sites by PCR. The integration near *Mn1* locus was predominant and could be detected in 12 out of 20 colonies, 3 colonies harbored the

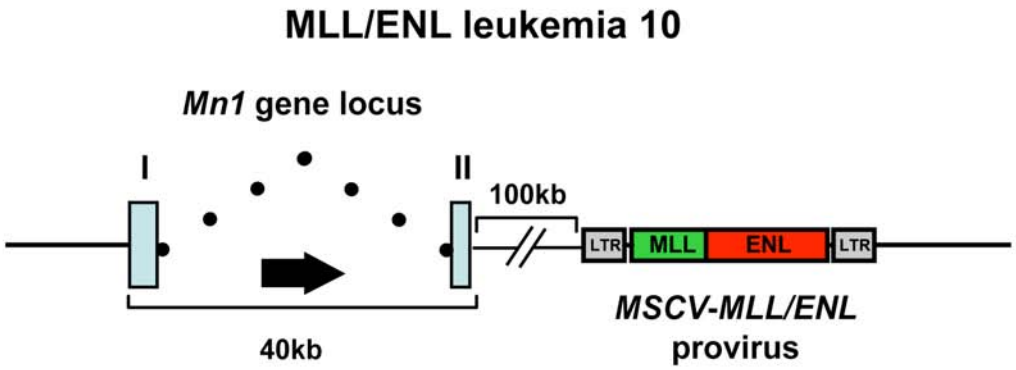
integration targeting *Mgat5*, and 5 had two insertions targeting *Socs7* and *Ppp2r5c* (**Fig. 3C**). Interestingly, after a second round of plating, an increase of colonies with the *Mn1* integration to 14 out of 20 was found. This observation suggested that overexpression of MN1 by the proviral integration supports the self-renewal of the respective clones. Retroviral expression of MGAT5 or know-down of PP2R5C by siRNA did result in aberrant self-renewal and growth advantage in liquid cultures as shown in MN1 transduced cells (**Fig. 3D&E**). These observations suggested that MN1 has oncogenic activity in primary bone marrow cells and might functionally collaborate with the MLL/ENL fusion in the development of the acute leukemia phenotype.

Fig.3. *MN1* acts as a putative cooperative oncogene in MLL/ENL induced mouse leukemia

A) Schematic representation of the integration sites of the *MSCV-MLL/ENL* provirus adjacent to the MN1 gene locus. **B**) mRNA expression levels of genes in the proximity of retroviral integration in 5 MLL/ENL leukemia mice. Relative expression levels of target genes were normalized to GAPDH expression in the same sample, calibrated to gene expression in normal murine BM and given as ΔΔCt value (* means not applicable). **C**) Clonal composition of the proviral integrations in “ME 10”. 20 colonies were analyzed by integration specific PCR. MN1 was the most predominant integration present. **D & E**) Overexpression of MN1 led to enhanced self renewal activity as shown in serial replating assays (**D**) and cellular proliferation in liquid cultures (**E**).

Fig. 3

A


MLL/ENL leukemia 10
Mn1 gene locus
I
II
100kb
LTR
MLL
ENL
LTR
MSCV-MLL/ENL
provirus
40kb

B

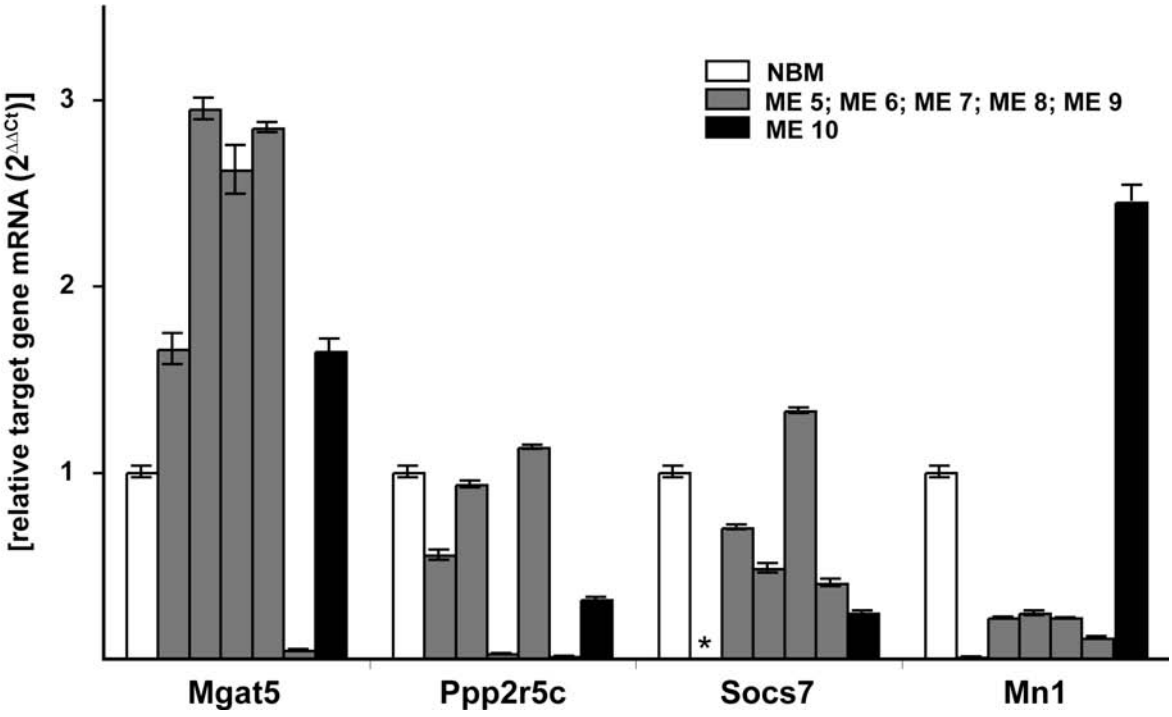
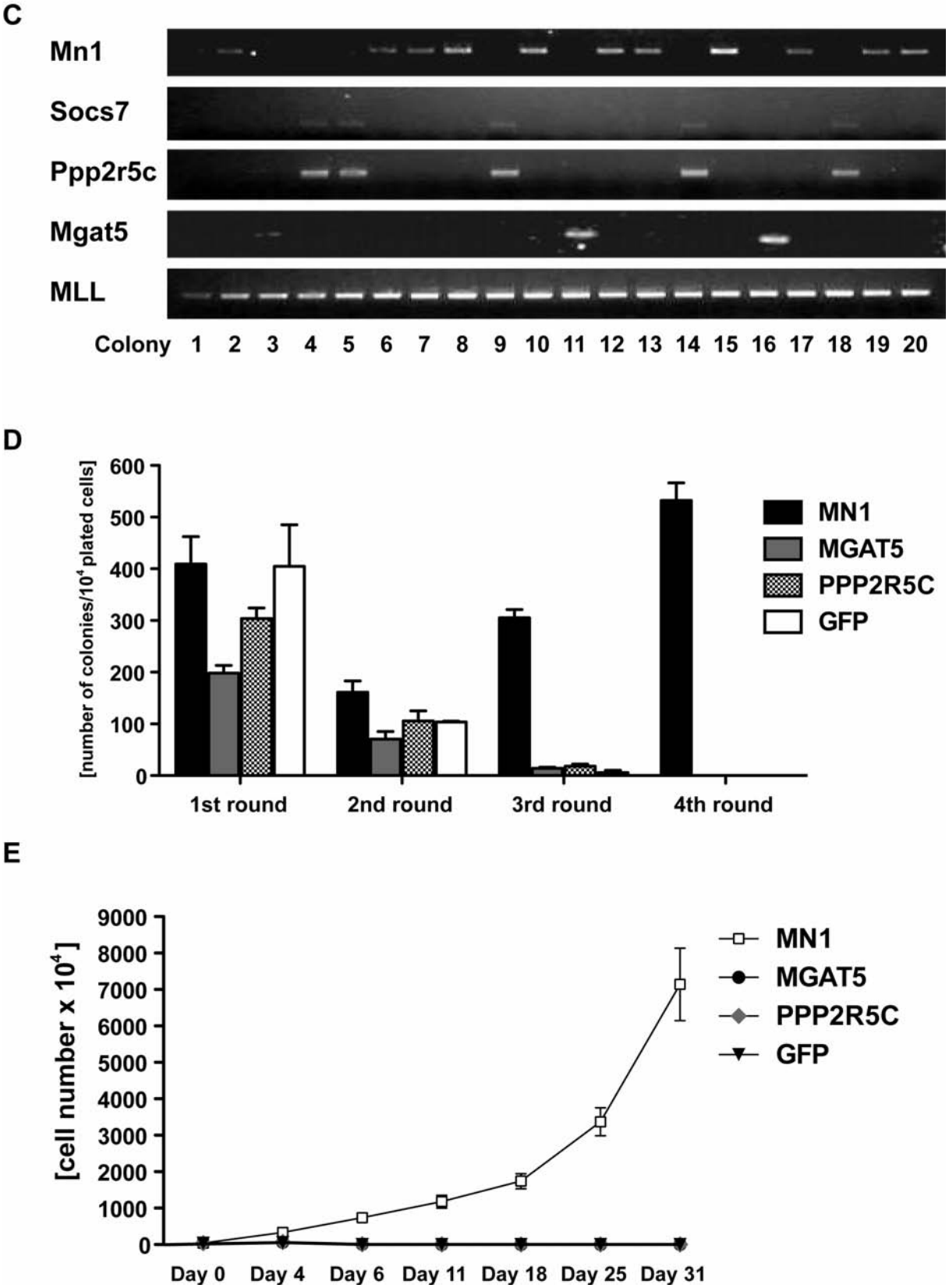

NBM
ME 5; ME 6; ME 7; ME 8; ME 9
ME 10
[relative target gene mRNA (2^ΔΔCt)]
3
2
1
*
Mgat5
Ppp2r5c
Socs7
Mn1

Fig. 3


C
Mn1
Socs7
Ppp2r5c
Mgat5
MLL
Colony 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
D
[number of colonies/10^4 plated cells]
600
500
400
300
200
100
0
1st round
2nd round
3rd round
4th round
MN1
MGAT5
PPP2R5C
GFP
E
[cell number x 10^4]
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Day 0
Day 4
Day 6
Day 11
Day 18
Day 25
Day 31
MN1
MGAT5
PPP2R5C
GFP

5. Elevated MN1 expression levels cooperate with MLL/ENL in murine leukemogenesis

In order to experimentally address the hypothesis that MN1 might cooperate with MLL/ENL in murine leukemia, I performed a series of bone marrow transplant experiments. Hereby, I reconstituted lethally irradiated mice with bone marrow transduced with retrovirus expressing MLL/ENL, MN1 or both. For co-expression bone marrow cells were first transduced with the virus encoding MLL/ENL followed by a second transduction with the MN1 expressing virus shortly after. Mice reconstituted with MLL/ENL (n=10) expressing bone marrow all developed lethal acute leukemia after a median latency of 64 days (**Fig. 4A**). Analogous to previous studies, all mice with MLL/ENL induced leukemia strongly mimicked the human disease with elevated white blood cells, and infiltration in hematopoietic organs such as liver, spleen and bone marrow (**Table 3**). Reconstitution with MN1 expressing bone marrow led to the development of an acute leukemia phenotype after a median latency of 100 days. The disease was characterized by elevated white blood cells count and the presence of leukemic blasts in liver, spleen, and bone marrow. Leukemic blasts from 5 mice analyzed expressed c-Kit and Gr1/Mac1 but no expression of markers like B220, TER119 or CD3 was observed (**Fig. 4B**). Interestingly, in some cases the MN1 containing provirus was inserted near the MN1 locus most probably resulting in further up-regulation of MN1 expression (**Table 4**).

In contrast to MLL/ENL or MN1 alone, reconstitution of bone marrow co-transduced with virus encoding MLL/ENL and MN1 induced an aggressive AML-like disease after a significantly shorter median latency of only 35 days post transplantation (**Fig. 4A**). The disease was characterized by very high white blood cell counts and excessive infiltration in multiple organs such as bone marrow, spleen, liver, kidney or lung (**Table 3, Fig. 4C**). Leukemic cells expressed both MLL/ENL and human MN1 as determined by RT-PCR (**Fig. 4D**). Leukemic blasts from MLL/ENL + MN1 disease expressed higher levels of c-Kit and CD34, but decreased levels of Gr1/Mac1 as compared to blasts from MLL/ENL induced disease (**Fig. 4B**). These *in vivo* observations suggested that MN1 could act as functional collaborator to MLL/ENL in murine leukemogenesis. No change in the latency of the disease development was

observed when bone marrow was co-transduced with MLL/ENL virus and an empty control virus (*MSCV-EGFP*), and such collaboration has not been observed between MOZ/TIF2 and LHX2 that was found to be up-regulated by the integration of the MOZ/TIF2 expressing provirus (data not shown).

Leukemic blasts overexpressing MLL/ENL and MN1 showed an increased clonogenic growth potential in methylcellulose cultures when compared to leukemic cells induced by MLL/ENL or MN1 alone (**Fig. 5A**). To address the *in vivo* significance of this observation, I performed a series of secondary transplantation experiments. As shown in **Fig. 5B**, transplantation of 5 $x10^5$ MLL/ENL leukemic blasts into sublethally irradiated recipients resulted in AML after a median latency of 36 days. In contrast, transplantation of the same number of MLL/ENL + MN1 leukemic cells resulted in AML after a significantly reduced latency (19 days, p=0.005). Further transplantations of dilutions of leukemic blasts showed that in contrast to MLL/ENL + MN1 leukemia, 2000 MLL/ENL blasts were not sufficient to induce the disease in all the recipients. These experiments provided strong evidence that MN1 could functionally collaborate with MLL/ENL and suggested that MN1 overexpression might lead to expansion of a leukemia-initiating cell population.

Fig.4. MN1 cooperates with MLL/ENL in murine leukemogenesis

A) Co-expression of MN1 and MLL/ENL induced acute leukemia in mice after a significantly shorter latency (median latency: 35 days) than MLL/ENL (64 days) or MN1 alone (100 days) ($p< 0.001$). **B**) Expression of cellular surface markers in murine acute leukemia induced by MLL/ENL, MN1 and MLL/ENL + MN1 was analyzed by flow cytometry. MLL/ENL + MN1 expressing leukemic blasts are characterized by high levels of Gr1/Mac1, c-kit and CD34. **C**) Histological analysis showed extensive infiltration of leukemic blasts in multiple organs including spleen (top panel), liver (middle panel), and skeletal muscle (lower panel) of mice transduced with MN1 or MLL/ENL + MN1. **D**) mRNA expression of MLL/ENL and MN1 in transplanted mice as shown by RT-PCR using primers covering the MLL/ENL breakpoint (upper row), human MN1 (middle row) and GAPDH (lower row). Two from each type of mice were taken as representatives.

Fig. 4

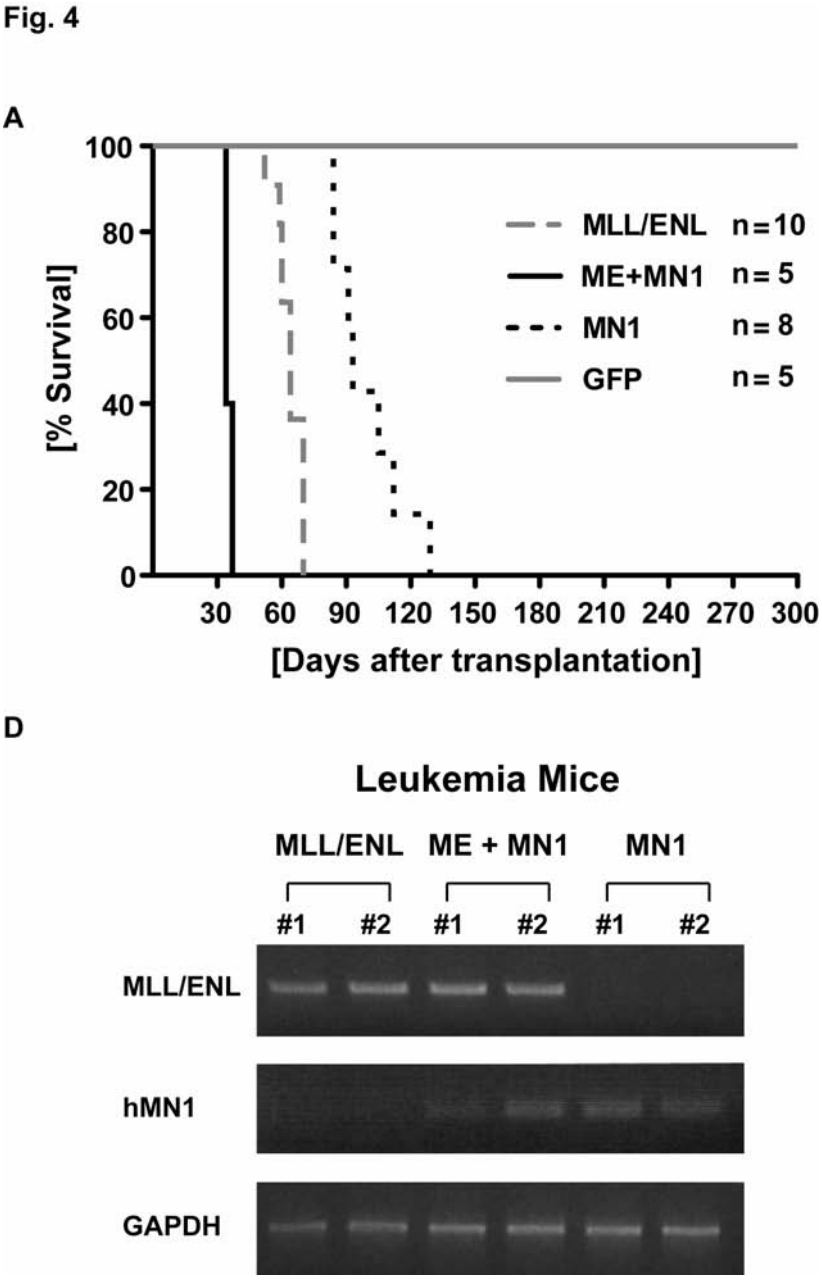


Table 3. Characteristics of murine leukemias induced by expression of MLL-ENL, MN1, and MLL-ENL + MN1

Mouse	Latency (days)	Spleen (mg)	Liver (mg)	WBC (10^9/L)	RBC (10^{12}/L)
MLL/ENL (n=10)	64 ± 5	629 ± 132	2239 ± 267	221.4 ± 121.3	8.2 ± 2.53
MN1 (n=8)	100 ± 13.3	340 ± 153	1314 ± 286	77.6 ± 25.5	8.35 ± 1.2
ME+MN1 (n=5)	35 ± 1.5	834 ± 259.2	1830 ± 300	106.55 ± 20.04	6.6 ± 1.42

Values shown as mean ± standard deviation. WBC, white blood cells; RBC, Red blood cells.

Fig. 4

B

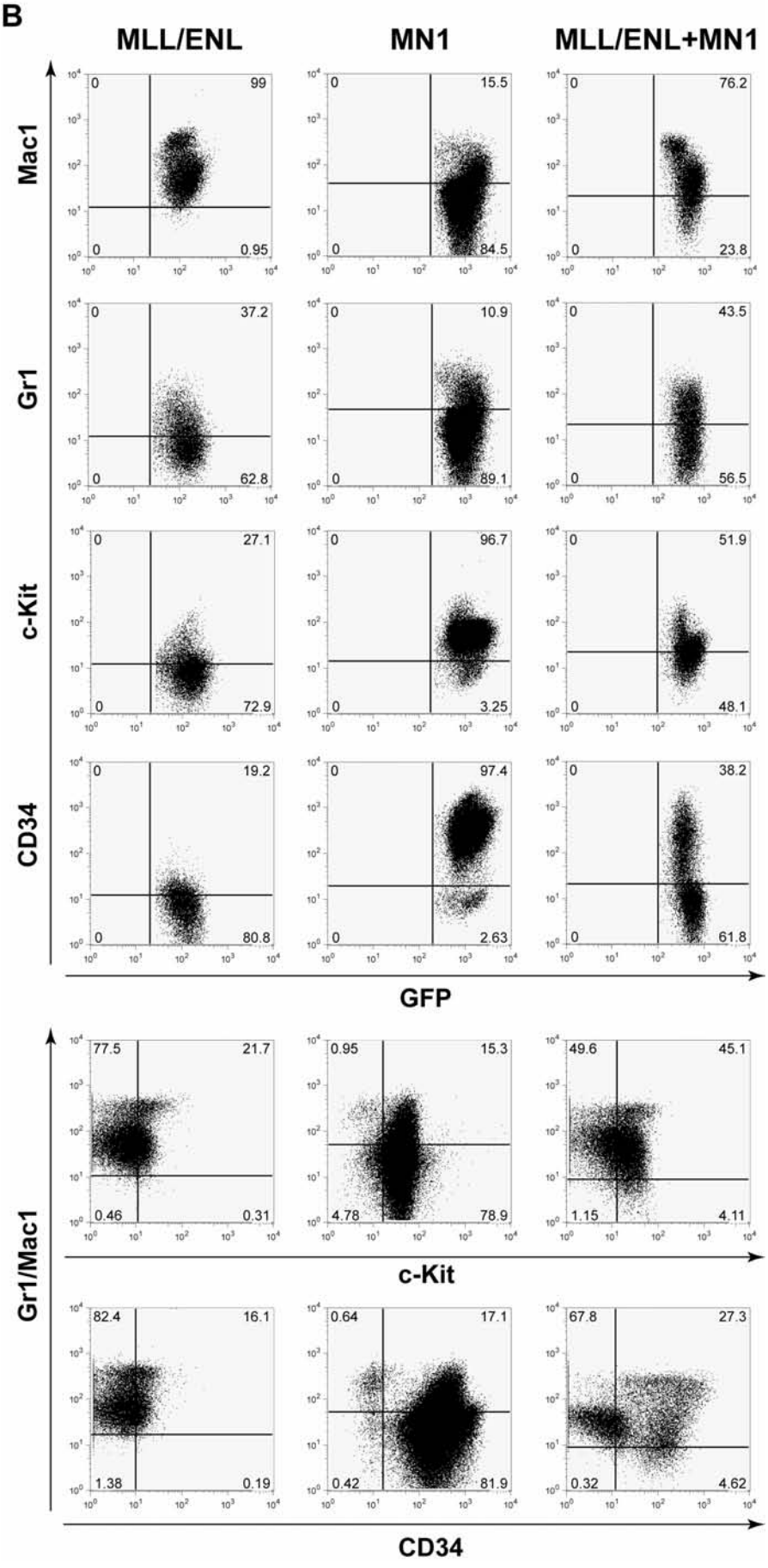

MLL/ENL
MN1
MLL/ENL+MN1
Mac1
Gr1
c-Kit
CD34
GFP
0
99
0.95
15.5
84.5
76.2
23.8
37.2
62.8
10.9
89.1
43.5
56.5
27.1
72.9
96.7
3.25
51.9
48.1
19.2
80.8
97.4
2.63
38.2
61.8
Gr1/Mac1
c-Kit
CD34
77.5
21.7
0.46
0.31
0.95
15.3
4.78
78.9
49.6
45.1
1.15
4.11
82.4
16.1
1.38
0.19
0.64
17.1
0.42
81.9
67.8
27.3
0.32
4.62

Fig. 4

C

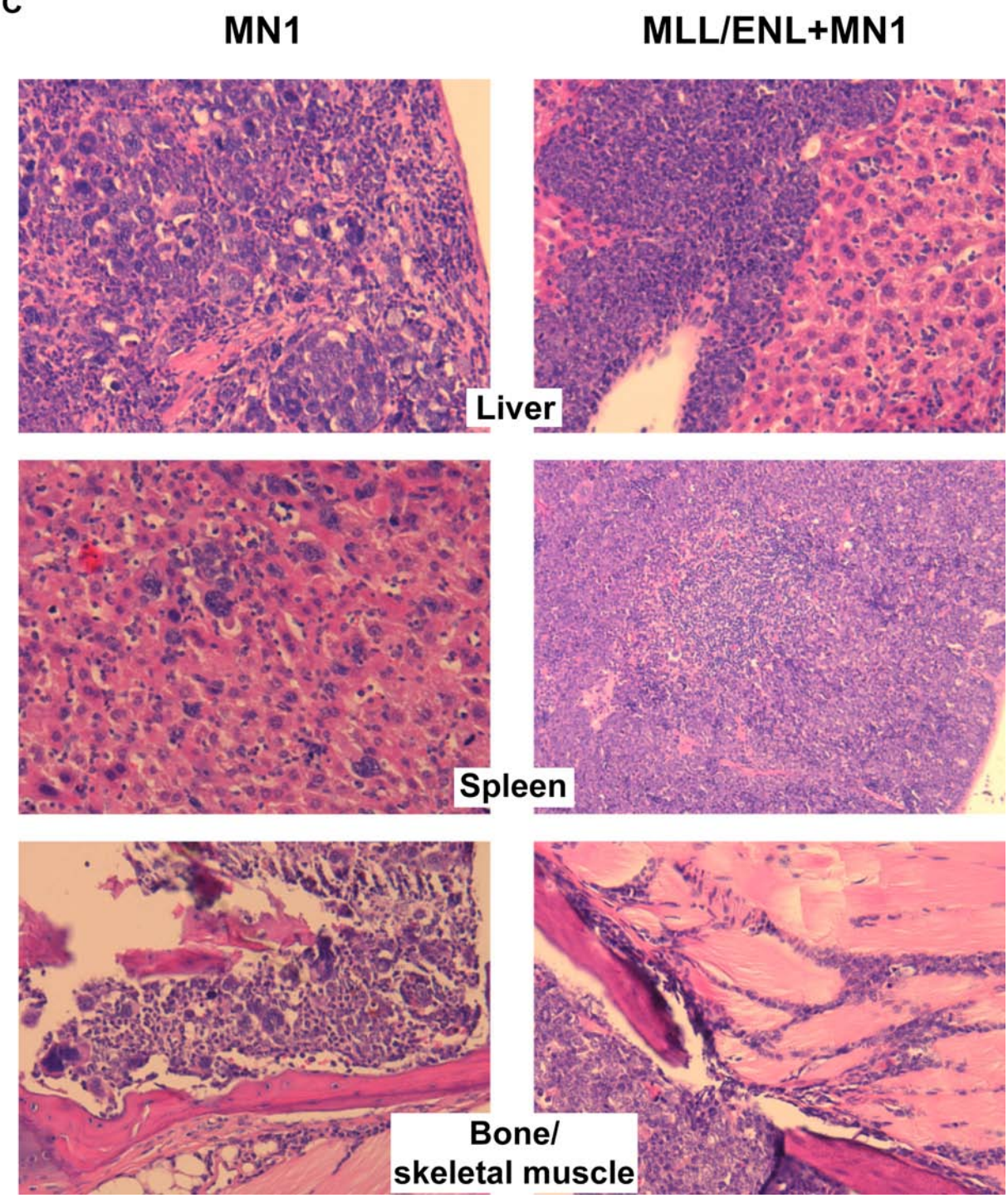


Table 4. Retroviral integration sites cloned from murine leukemia induced by MN1 or MLL/ENL+ MN1

Mouse No.	Gene symbol	Full Name	(Predicted) gene function	Location and distance for retroviral integration	Orientation	Mouse chr.	Human chr.	CIS***
* MN1	Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	oncogene	Downstream 112301 bp	same	6	12p12	8
	LOC546917	RIKEN cDNA 4933403M22	Unknown	Upstream 93731 bp	same	6	N/A	N/A
MN2 I	Ciapin1	cytokine induced apoptosis inhibitor 1	anti-apoptosis	Downstream 6165 bp	same	8	16q13-q21	N/A
	Coq9	coenzyme Q9 homolog	ubiquinone biosynthesis	Upstream 13140 bp	opposite	8	16q13	N/A
II	Vps37b	vacuolar protein sorting 37B	protein transport	1st intron	opposite	5	12q24	1
III	Mn1	meningioma 1	oncogene	Downstream 55820 bp	same	5	22q11	8
	Rncr2	retinal noncoding RNA 2	component of the nuclear matrix	Upstream 711126 bp	opposite	N/A	N/A	N/A
MN4	LOC628951	hypothetical protein LOC628951	Unknown	Downstream 11214 bp	opposite	16	N/A	N/A
	Cd47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer	cell adhesion	Upstream 150367 bp	opposite	16	3q13	2
MN5 I	Mgat5	mannoside acetylglucosaminyltransferase 5	acetylglucosaminyltransferase	2nd intron	opposite	1	2q21	1
II	Mtap7	microtubule-associated protein 7	cytoskeletal regulatory protein binding	1st intron	opposite	10	6q23	N/A
MN6 I	Lpgat1	lysophosphatidylglycerol acyltransferase 1	acyltransferase	Downstream 24861 bp	opposite	1	1q32	N/A
	Nek2	NIMA (never in mitosis gene a)-related expressed kinase 2	protein kinase	Upstream 18112 bp	opposite	1	1q32-q41	N/A
II	Lass6	longevity assurance homolog 6	ceramide biosynthesis	3rd intron	same	2	2q24	1
III	Lsg1	large subunit GTPase 1 homolog	GTP-binding protein	Downsream 195591 bp	opposite	16	3q29	N/A
	Centb2	centaurin, beta 2	GTPase activator	Upstream 351294 bp	opposite	16	3q29	1
MN7 I	Il17ra	interleukin 17 receptor	interleukin receptor	1st intron	opposite	6	22q11	N/A
II	1300014I06Rik	hypothetical protein LOC66895	phosphoinositide binding	Downstream 40192 bp	opposite	13	6p25	1
	Prpf4b	PRP4 pre-mRNA processing factor 4 homolog B	mRNA processing	Upstream 455671 bp	same	13	6p25	N/A
III	Ehd1	EH-domain containing 1	endocytosis	Downstream 3524 bp	same	19	11q13	N/A
	Cdc42bpg	CDC42 binding protein kinase gamma (DMPK-like)	protein kinase	Upstream 4562 bp	same	19	11q13	N/A
IV	LOC675933	similar to AT rich interactive domain 1A (Swi1 like)	Unknown	Downstream 59827 bp	opposite	4	N/A	N/A
	Rps6ka1	ribosomal protein S6 kinase polypeptide 1	protein kinase	Upstream 29556 bp	opposite	4	1p	4
**MEM1	Vav3	vav 3 oncogene isoform 1	guanyl-nucleotide exchange factor	2nd intron	Same	3	1p13	1
MEM2	Atp10a	ATPase, class V, type 10A	ATPase activity	1st intron	Opposite	7	15q11	N/A
MEM3	Ncam1	neural cell adhesion molecule 1 isoform 1	vascular endothelial growth factor receptor	4th intron	Opposite	9	11q23	N/A
MEM4 I	Sp2	Sp2 transcription factor isoform 1	transcription factor	1st intron	Same	11	17q21	1
II	Entpd7	ectonucleoside triphosphate diphosphohydrolase 7	nucleoside-di/triphosphatase	4th intron	Same	19	10q23-q24	1
MEM5 I	5830416P10Rik	hypothetical protein LOC381232	Unknown	Downstream 10640 bp	Opposite	19	N/A	N/A
	Dusp5	similar to dual specificity phosphatase 5	tyrosine/serine/threonine phosphatase	Upstream 74139 bp	Same		10q25	N/A
II	Prkar2b	protein kinase, cAMP dependent regulatory, type II beta	cAMP binding	2nd intron	Same	12	7q22	1

* MN = Murine leukemia induced by overexpression of MN1. ** MEM = Murine leukemia induced by co-expression of MLL/ENL with MN1. Roman Numerals mean different insertion sites identified from the same leumemia mouse.
*** CIS = Common Integration Sites. Numbers indicate hits of the same integration flanking gene found in RTCGD database (http://rtcgd.abcc.ncifcrf.gov/).
N/A = not applicable.

Previous studies suggested that MLL fusion genes not only transform hematopoietic stem cells, but are also able to block differentiation and to provide aberrant self-renewal to committed hematopoietic progenitor cells including granulocyte macrophage progenitors (GMPs), common myeloid progenitors (CMPs) and/or common lymphoid progenitors (CLPs) [34,108]. The significantly shorter latency in conjunction with a more immature, “stem-cell like” phenotype leukemia suggested that overexpression of MN1 might lead to an expansion of the MLL targeted leukemic progenitor population. It’s worth noting that in normal bone marrow the highest levels of MN1 expression were found in GMPs proposed to be potential targets of MLL fusion genes [100]. Therefore, we first compared the GMP populations in leukemic mice induced by MLL/ENL, MN1 or MLL/ENL + MN1. As shown in **Fig. 5C**, MLL/ENL + MN1 leukemia was characterized by a significant expansion of the GMP population as 20 times more than MLL/ENL.

In order to test the possibility that co-expression of MN1 expression increased the leukemic stem cell compartment, we isolated GMPs from MLL/ENL and MLL/ENL + MN1 leukemic mice and transplanted identical numbers of Leukemic-GMPs (L-GMPs) into sublethally irradiated hosts. As shown in **Fig. 5D,** transfer of 1000 L-GMPs from MN1 + MLL/ENL leukemia reproduced the leukemic phenotype in all recipients after a median latency of 34 days. In contrast, an identical number of L-GMPs from MLL/ENL leukemia reproduced the disease only in some animals after a significantly prolonged latency period of 42 days ($P < 0.05$). These experiments strongly support the idea of functional collaboration of MN1 with MLL/ENL at the level of GMP cell population with leukemia-initiating potential.

Fig. 5.

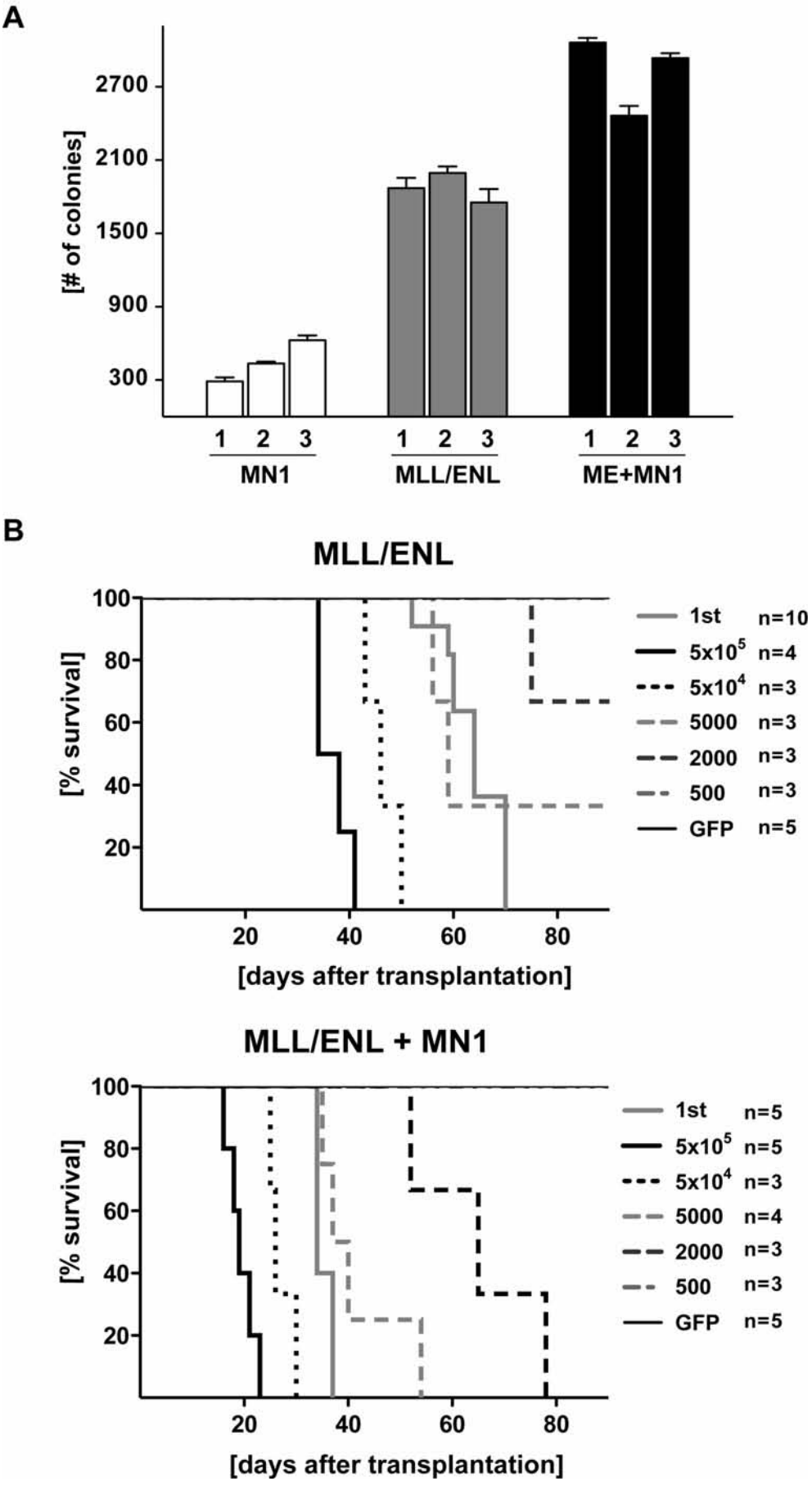
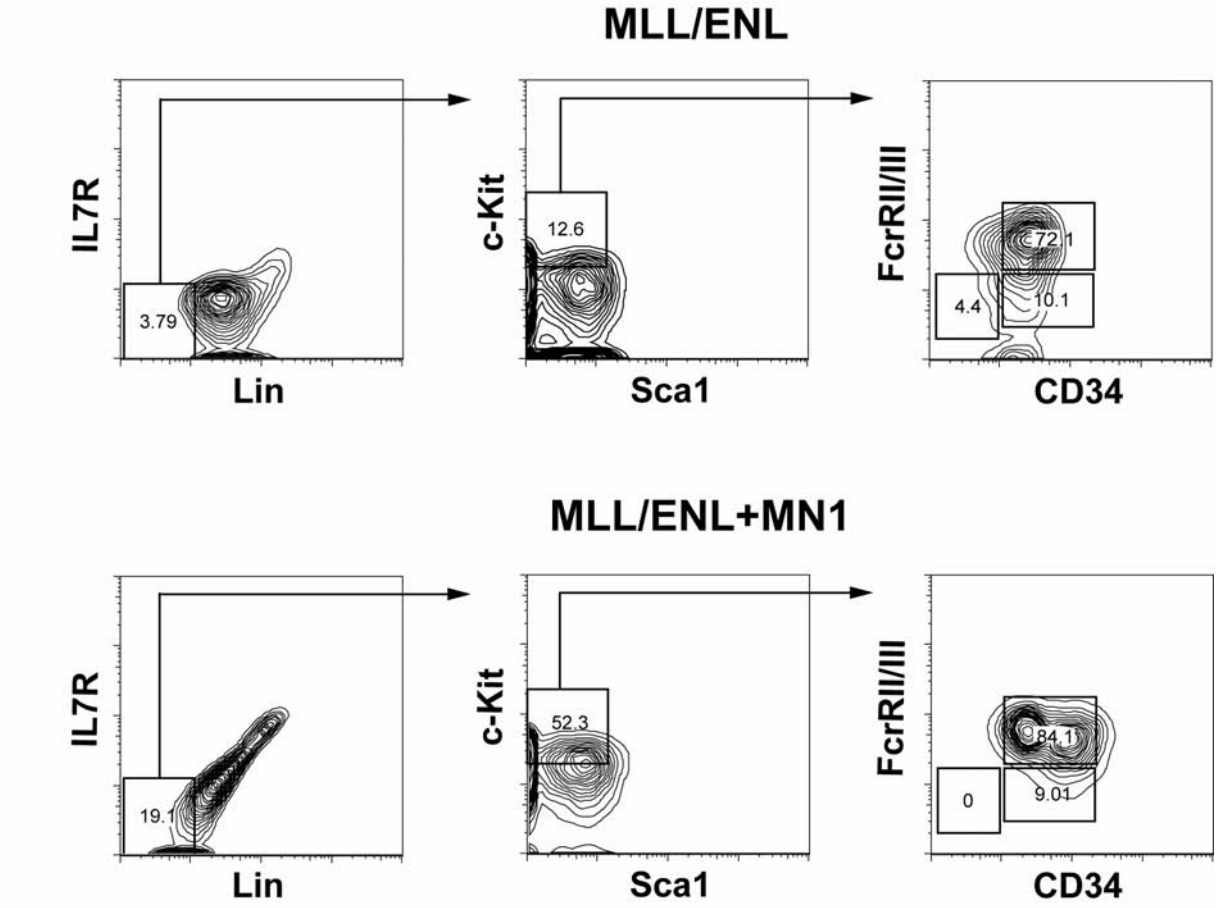

A
[# of colonies]
2700
2100
1500
900
300
1 2 3
MN1
1 2 3
MLL/ENL
1 2 3
ME+MN1
B
MLL/ENL
[% survival]
100
80
60
40
20
20
40
60
80
[days after transplantation]
1st n=10
5x10^5 n=4
5x10^4 n=3
5000 n=3
2000 n=3
500 n=3
GFP n=5
MLL/ENL + MN1
[% survival]
100
80
60
40
20
20
40
60
80
[days after transplantation]
1st n=5
5x10^5 n=5
5x10^4 n=3
5000 n=4
2000 n=3
500 n=3
GFP n=5

Fig. 5.

C


MLL/ENL
IL7R
3.79
Lin
c-Kit
12.6
Sca1
FcrRII/III
72.1
4.4
10.1
CD34
MLL/ENL+MN1
IL7R
19.1
Lin
c-Kit
52.3
Sca1
FcrRII/III
84.1
0
9.01
CD34

D

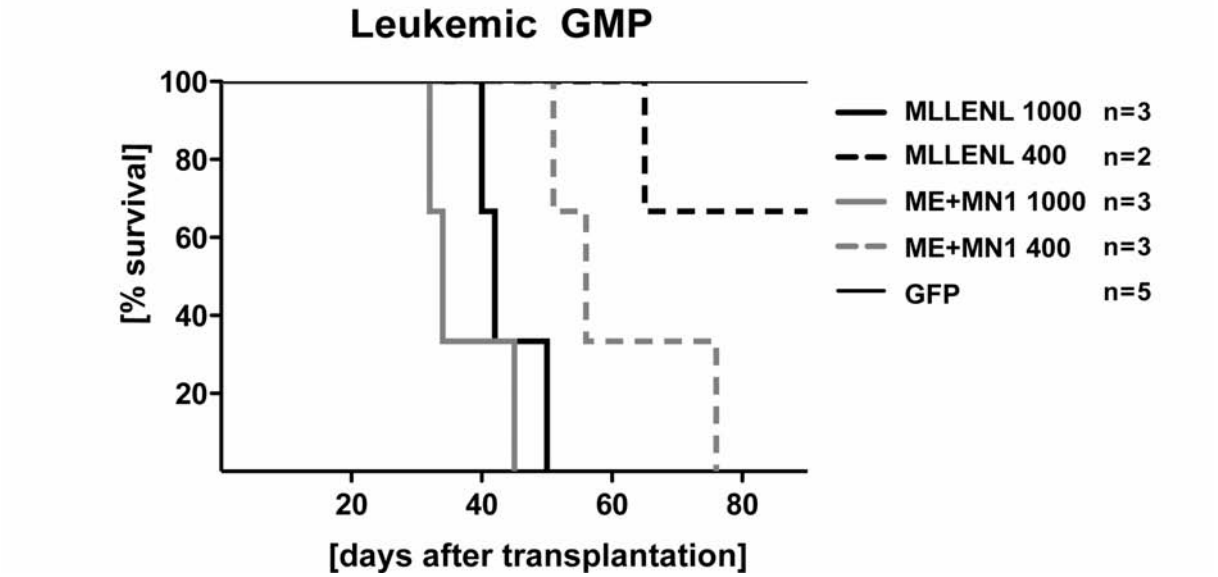

Leukemic GMP
[% survival]
100
80
60
40
20
20
40
60
80
[days after transplantation]
MLLENL 1000 n=3
MLLENL 400 n=2
ME+MN1 1000 n=3
ME+MN1 400 n=3
GFP n=5

Fig.5. Co-expression of MN1 with MLL/ENL increases the leukemia initiating-cell pool
A) Increased colony formation in methylcellulose of blasts from leukemic mice induced by the co-expression of MN1 with MLL/ENL as compared to MLL/ENL or MN1 alone. Error bars represent standard deviations of three independent experiments. **B**) Secondary transplantation with limiting dose of blasts from MLL/ENL or MLL/ENL+ MN1 leukemia mice. **C**) Increased leukemic GMP (L-GMP) population in MLL/ENL + MN1 leukemic mice as detected by flow cytometry and was gated as $IL7R^-$, Lin^-, c-Kit^{high}, $Sca1^-$, FcγRII/III^{high} and $CD34^+$ fraction. The frequency of L-GMPs in MLL/ENL + MN1 mice is about 8.4% (19.1% x 52.3% x 84.1%), whereas it is about 20 times lower (0.34%) in MLL/ENL mice. **D**) Recipient mice were transplanted with GMP population isolated from MLL/ENL or MLL/ENL+ MN1 leukemia mice. Median latency for disease development after transplant of 1000 MLL/ENL GMPs was 42 days; and 34 days for 1000 MLL/ENL + MN1 GMPs ($p< 0.05$).

6. Determination of presumptive MN1 targets in hematopoietic system

As MN1 has been proposed to act primarily as a transcriptional co-regulator in hematopoietic cells we sought to obtain some insights about the MN1-regulated oncogenic gene expression program. Thus, we compared the gene expression profiles of primary murine bone marrow cells transiently overexpressing MN1 (*MSCV-MN1-IRES/EYFP*) with cells transduced with a control vector (*MSCV-IRES/EYFP*) by using oligonucleotide arrays (**Fig. 6A**). As shown in **Fig. 6B** and **Table 6**, transient over-expression of MN1 resulted in ≥1.5 fold up-regulation of 831 genes and down-regulation of 268 genes ($p<0.05$). As we asked for putative targets that might be involved in the leukemogenic activity of MN1, we selected genes that were more than two-fold up-regulated and previously functionally linked to malignant hematopoiesis and validated their expression levels by quantitative PCR analysis. DLK1, FLT3, CD34, ANGPT2, MAP2K1IP1, HLF, PRNP, and MEIS1 were expressed at higher levels, and LIF and CRISP1 at lower levels in MN1 expressing cells as compared to mock-transduced mouse bone marrow (**Fig. 6C**). We next determined the expression levels of these genes in blasts from mouse leukemias induced by MN1, MLL/ENL or co-expression of MLL/ENL and MN1. In most murine samples, elevated expression levels of DLK1, CD34, FLT3, HLF were significantly associated with the presence of elevated expression of MN1 (**Fig. 6D**).

Fig. 6.

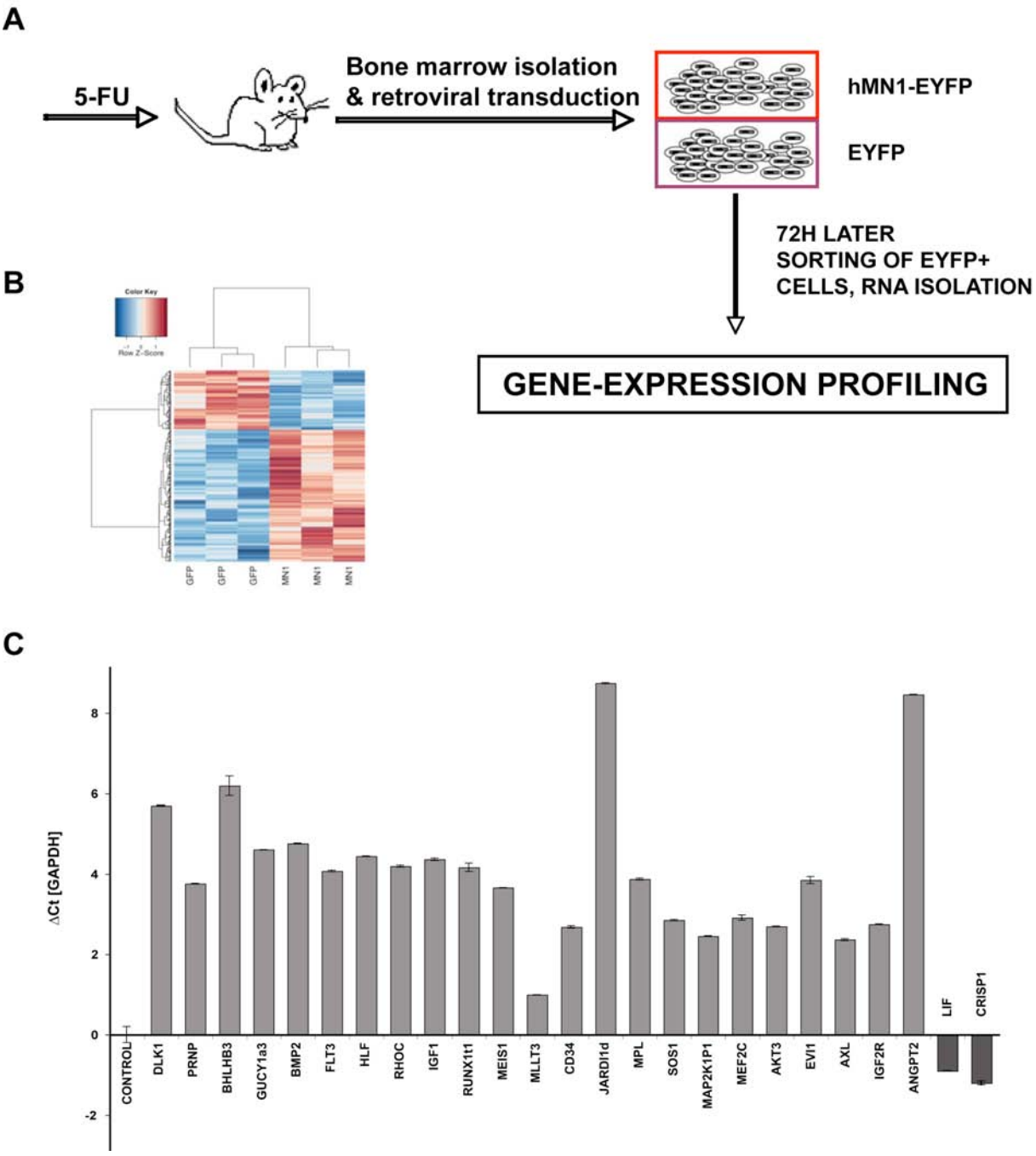

A
5-FU
Bone marrow isolation
& retroviral transduction
hMN1-EYFP
EYFP
72H LATER
SORTING OF EYFP+
CELLS, RNA ISOLATION
GENE-EXPRESSION PROFILING
B
Color Key
Row Z-Score
GFP
GFP
GFP
MN1
MN1
MN1
C
ΔCt [GAPDH]
8
6
4
2
0
-2
CONTROL
DLK1
PRNP
BHLHB3
GUCY1a3
BMP2
FLT3
HLF
RHOC
IGF1
RUNX1t1
MEIS1
MLLT3
CD34
JARID1d
MPL
SOS1
MAP2K1P1
MEF2C
AKT3
EVI1
AXL
IGF2R
ANGPT2
LIF
CRISP1

Fig. 6.

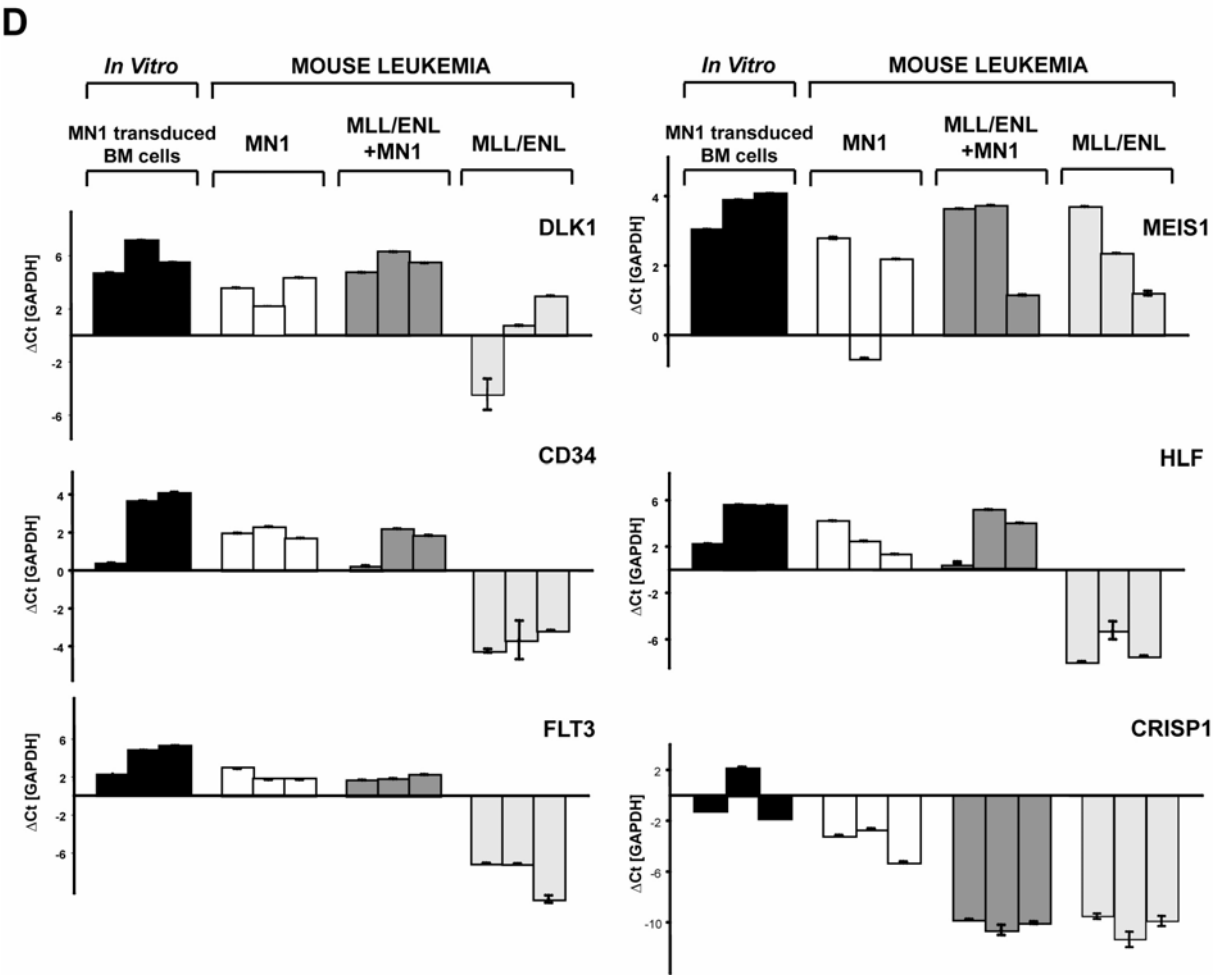


Fig.6. Searching for putative MN1 target genes in murine bone marrow cells

A) Schematic illustration of the experiment. **B**) The differential expression patterns between bone marrow progenitor cells transiently expressing human MN1/EYFP and cells transiently expressing EYFP are visualized as a heat map. Gene expression signature of bone marrow cells 72 hours after transduction with MN1-expressing retrovirus was identified by unsupervised analysis using the hierarchic clustering method. **C**) Quantitative RT-PCR-based validation of the up- and down-regulated expression of selected genes upon transient MN1 overexpression. A complete list of up- and down-regulated genes is given in **Table 6. D**) Quantitative RT-PCR analysis of the expression of selected genes in murine blasts from leukemia induced by expression of MN1, MLL/ENL or MLL/ENL and MN1. The expression levels of target genes were normalized to GAPDH, and given as ΔCt value relative to MOCK (*MSCV-EYFP*) transduced control cells (*in vitro*) or bone marrow from mice transplanted with MOCK-transduced cells (*in vivo*).

In order to validate these putative MN1 target genes in human leukemia, we compared their expression in a set of pediatric acute leukemia cases (including 7 B-ALL, 6 AML, 8 infant leukemias, 6 T-ALL) with elevated expression of MN1 versus those with low or absent MN1 expression levels (as found in normal bone marrow). As shown in **Fig. 7A**, we found remarkably higher expression levels of CD34 ($p=0.0021$) and FLT3 ($p=0.03$) in a cohort of pediatric leukemias with significantly elevated MN1 levels ($p<0.0001$) than in cases with low or undetectable MN1 levels. Expression of DLK1 and HLF1 correlated with MN1 in certain cases but was not significant as a cohort (data not shown). In contrast to the murine samples (**Fig. 6D**), no association of MEIS1 with elevated MN1 levels was observed (**Fig. 7A**).

To further investigate the significance of CD34 and FLT3 up-regulation in MN1 overexpressing cells, we reduced expression of these genes by the respective siRNAs in 2 cells lines immortalized by MN1 or MLL/ENL. Flow cytometric analysis 72 hours post transduction showed a 50-60% reduction of CD34 and FLT3 surface expression by siRNA mediated knockdown (**Fig. 7B**). Decreased levels of CD34 or FLT3 resulted in a significant reduction of colony formation of MN1 expressing cells, whereas knockdown of CD34 had no impact on the clonogenic growth of MLL/ENL expressing cells (**Fig. 7C**). In liquid culture, under continuous selection, we could only observe growth reduction of MN1 but not of MLL/ENL cells (**Fig. 7D**). These results suggest that CD34 and FLT3 could be important mediators of the leukemogenic activity of MN1.

Fig.7. Expression of putative MN1 target genes in pediatric acute leukemia

A) Quantitative RT-PCR analysis of expression of selected targets in pediatric leukemia cases with high or low MN1 expression, as well as in different types of childhood leukemia including B-ALL (n=7), Infant ALL (n=8), AML (n=6) and T-ALL (n=6). The expression levels of target genes were normalized to GAPDH, and given as ΔCt value relative to healthy controls (bone marrow). **B**) siRNA-mediated knockdown of CD34 and FLT3 in murine hematopoietic progenitor cells immortalized by MN1 or MLL/ENL overexpression as assessed by flow cytometry. **C & D**) Impact of siRNA-mediated knockdown of CD34 and FLT3 in MN1 and MLL/ENL immortalized cells on clonogenic growth in methylcelluose (**C**) and proliferation in liquid cultures (**D**).

Fig. 7.

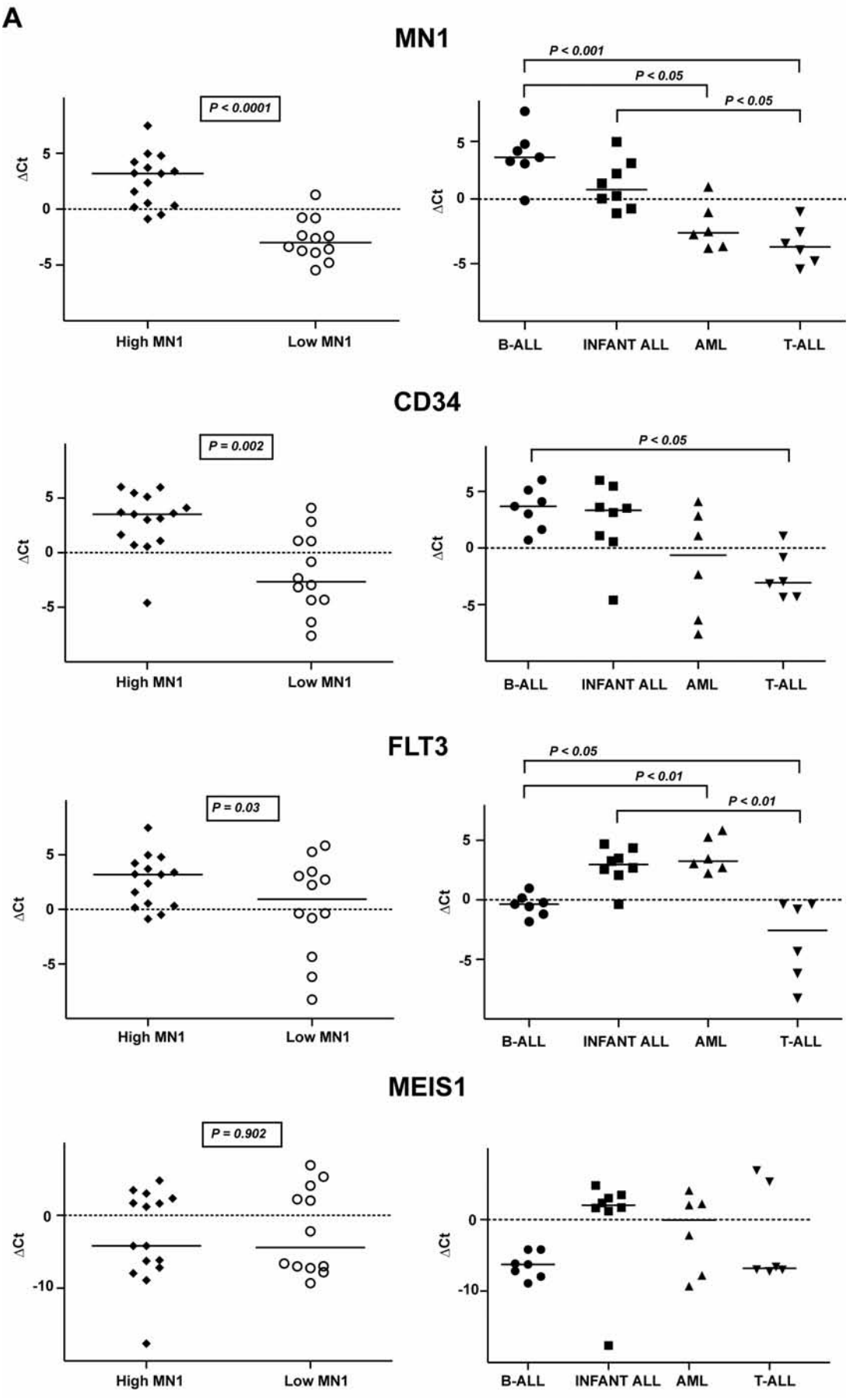
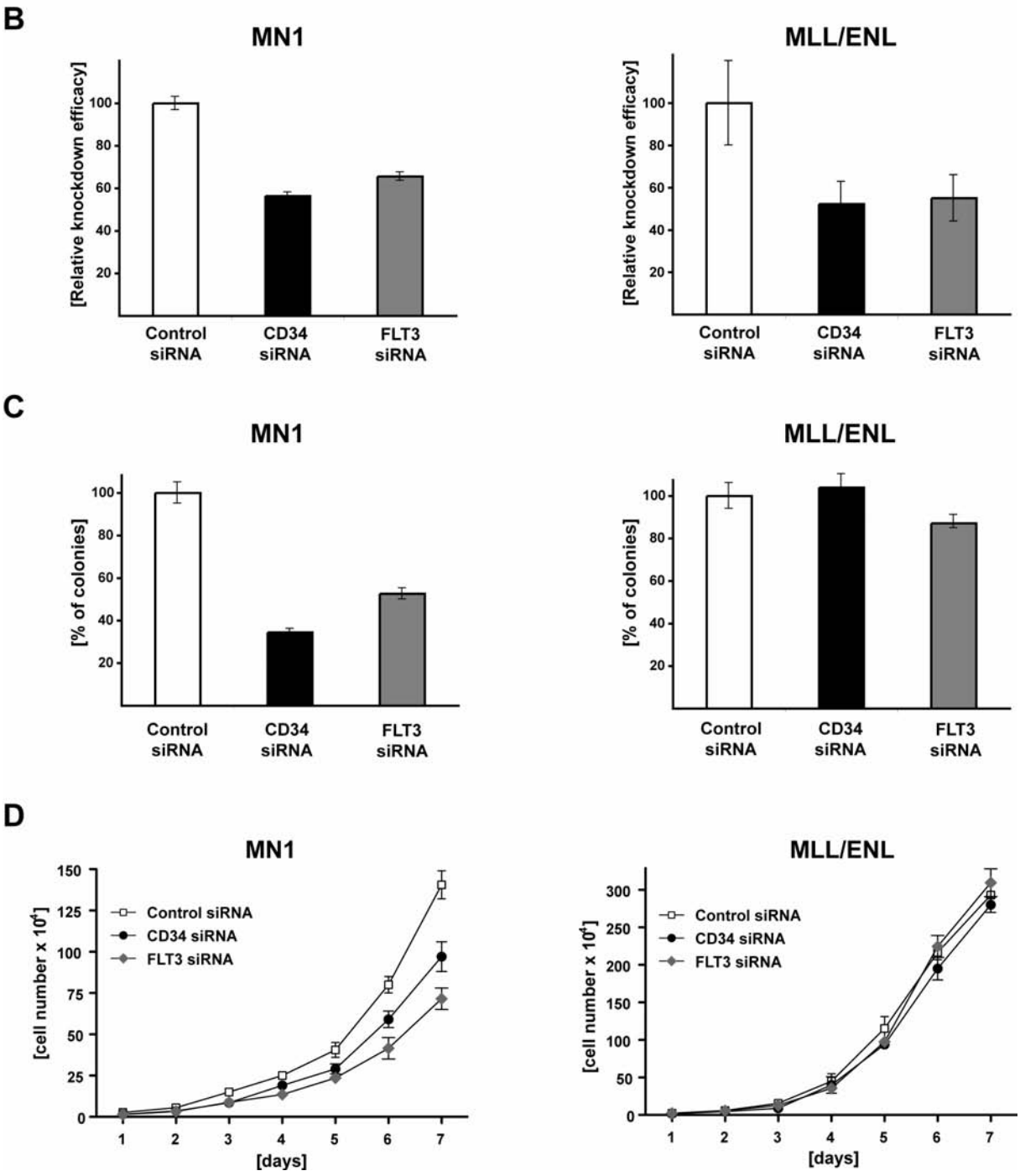

A
MN1
P < 0.0001
ΔCt
5
0
-5
High MN1
Low MN1
P < 0.001
P < 0.05
P < 0.05
B-ALL
INFANT ALL
AML
T-ALL
CD34
P = 0.002
P < 0.05
FLT3
P = 0.03
P < 0.05
P < 0.01
P < 0.01
MEIS1
P = 0.902
-10

Fig. 7.


B
MN1
[Relative knockdown efficacy]
100
80
60
40
20
Control siRNA
CD34 siRNA
FLT3 siRNA
MLL/ENL
[Relative knockdown efficacy]
100
80
60
40
20
Control siRNA
CD34 siRNA
FLT3 siRNA
C
MN1
[% of colonies]
100
80
60
40
20
Control siRNA
CD34 siRNA
FLT3 siRNA
MLL/ENL
[% of colonies]
100
80
60
40
20
Control siRNA
CD34 siRNA
FLT3 siRNA
D
MN1
[cell number x 10⁴]
150
125
100
75
50
25
0
Control siRNA
CD34 siRNA
FLT3 siRNA
1
2
3
4
5
6
7
[days]
MLL/ENL
[cell number x 10⁴]
300
250
200
150
100
50
0
Control siRNA
CD34 siRNA
FLT3 siRNA
1
2
3
4
5
6
7
[days]

7. MN1 expression in pediatric acute leukemia

Since the role of MN1 in pediatric leukemia has never been addressed, we determined the MN1 mRNA expression levels in a panel of 64 patients with acute lymphoblastic leukemia (ALL) including 10 prepre-B-ALL, 38 common ALL (cALL), 9 pre-B-ALL, 2 pro-B-ALL, and 5 T-ALL cases. No significant MN1 expression could be detected in T-ALL cases. Compared to the normal bone marrow, slightly elevated levels were seen in the pre-B-ALL samples. Interestingly, several cases of the more immature B-cell ALL showed significantly elevated levels of MN1 expression even higher than the ME-1 cell line carrying an inv16 previously shown to overexpress MN1 [100]. Surprisingly, the highest MN1 expression levels were observed in ALL cases originating from early B-cell precursors (**Fig. 8A**). MN1 expression was also determined in a group of 14 childhood AML cases. Significantly elevated MN1 levels were found in 3 patients (**Fig. 8B**). No correlation with the presence of FLT3-ITD mutations was observed in this small group of patients. We also analyzed MN1 expression in a panel of 9 cases of infant acute leukemia associated with MLL translocations. Interestingly, all cases expressed higher MN1 levels than a pooled sample of 5 normal bone marrows (**Fig. 8C**). Our results showed that, in pediatric leukemia, elevated MN1 expression is not limited to disease of the myeloid lineage, and that increased MN1 expression is not limited to cases carrying inv16, but is also found in leukemia harboring *MLL* fusion genes.

Fig.8. MN1 expression in pediatric acute leukemia

A) MN1 mRNA expression in childhood ALL samples assessed by quantitative RT-PCR. **B**) MN1 mRNA expression in childhood AML samples detected by quantitative RT-PCR. **C**) MN1 mRNA expression in infant leukemia samples with different MLL translocations detected by quantitative RT-PCR. Expression levels in patient samples are depicted as fold change calibrated to average MN1 expression in 5 pooled normal human BM samples.

Fig. 8.

A

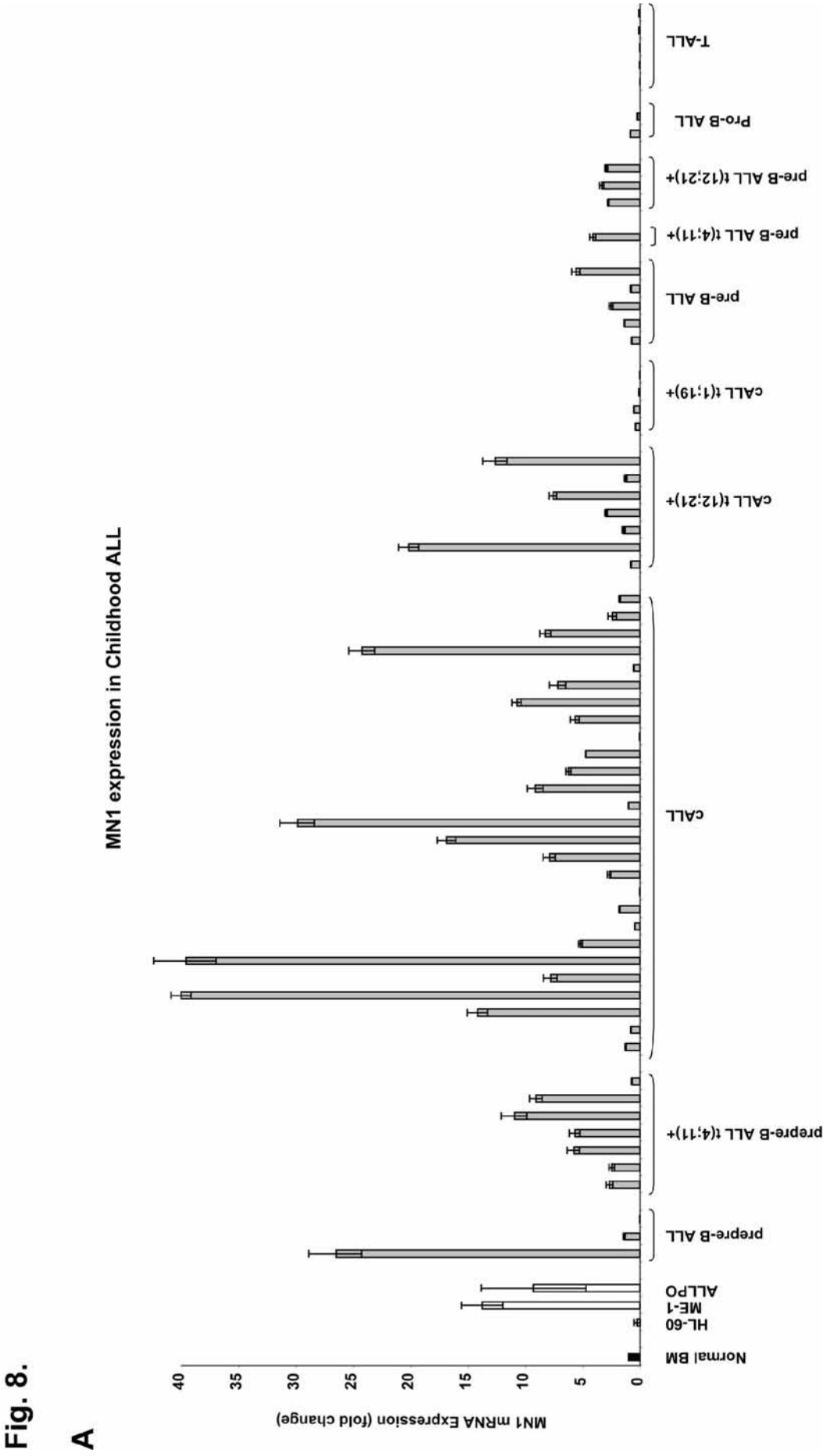
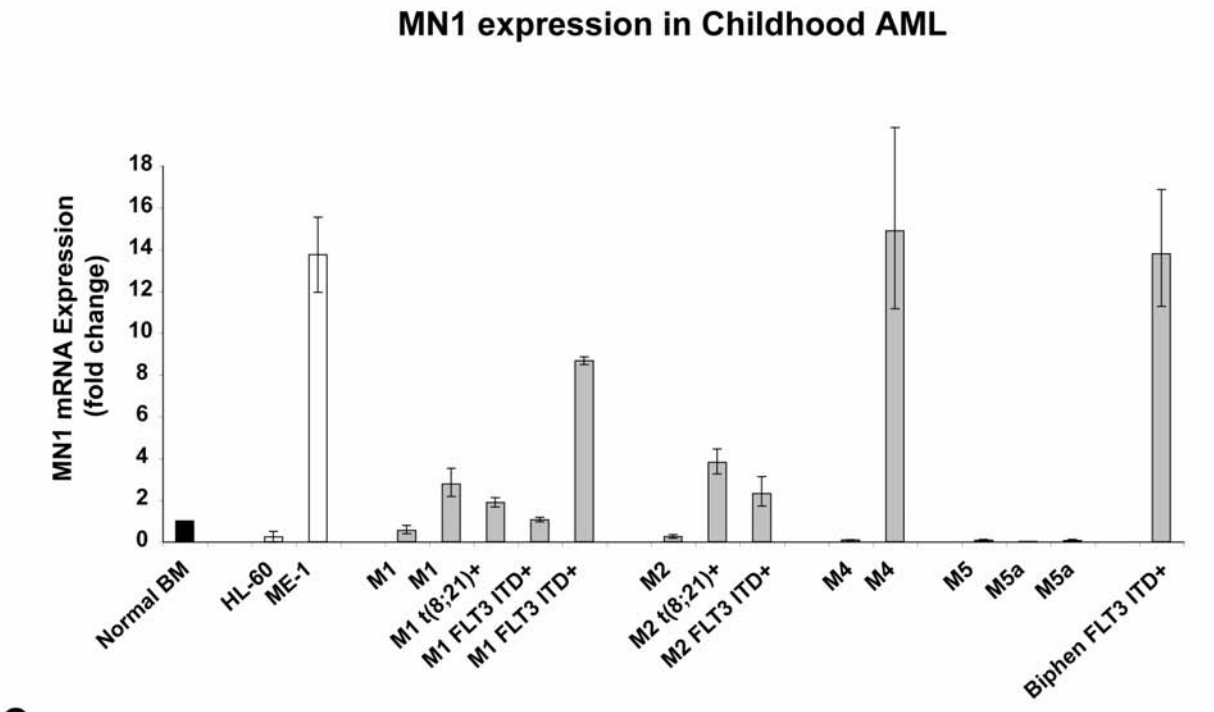
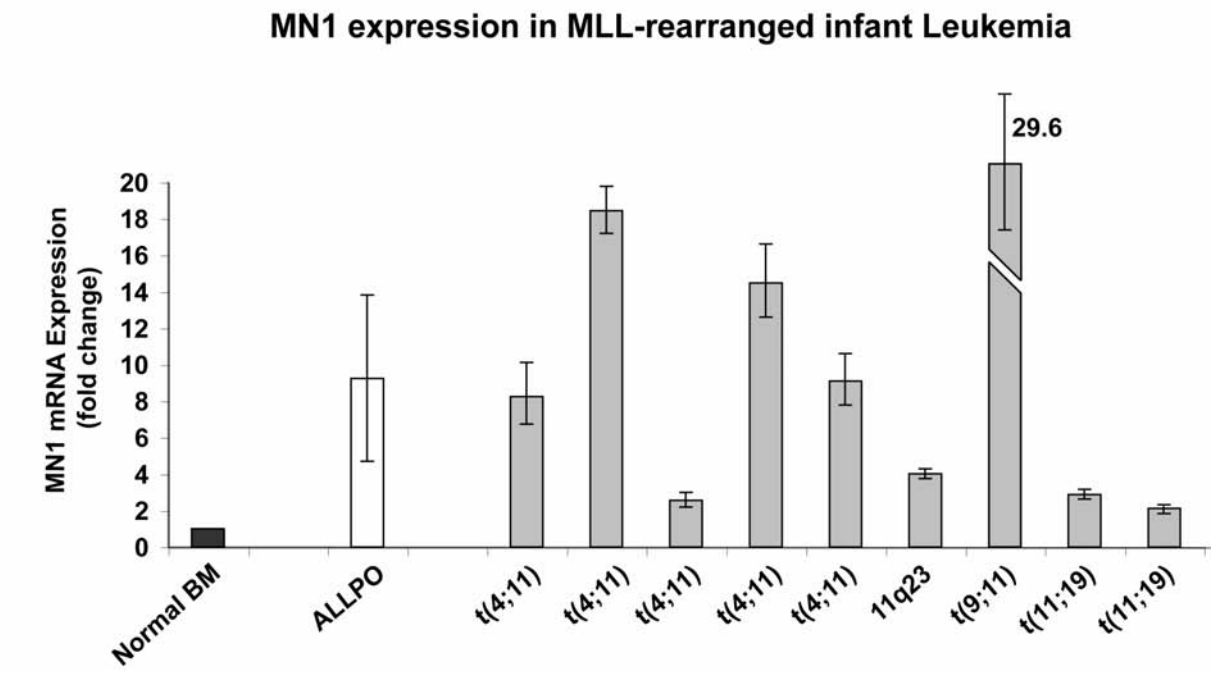

MN1 expression in Childhood ALL
MN1 mRNA Expression (fold change)
0
5
10
15
20
25
30
35
40
Normal BM
HL-60
ME-1
ALLPO
prepre-B ALL
prepre-B ALL t(4;11)+
cALL
cALL t(12;21)+
cALL t(1;19)+
pre-B ALL
pre-B ALL t(4;11)+
pre-B ALL t(12;21)+
Pro-B ALL
T-ALL

Fig. 8.

B


MN1 expression in Childhood AML
MN1 mRNA Expression (fold change)
0
2
4
6
8
10
12
14
16
18
Normal BM
HL-60
ME-1
M1
M1
M1 t(8;21)+
M1 FLT3 ITD+
M1 FLT3 ITD+
M2
M2 t(8;21)+
M2 FLT3 ITD+
M4
M4
M5
M5a
M5a
Biphen FLT3 ITD+

C

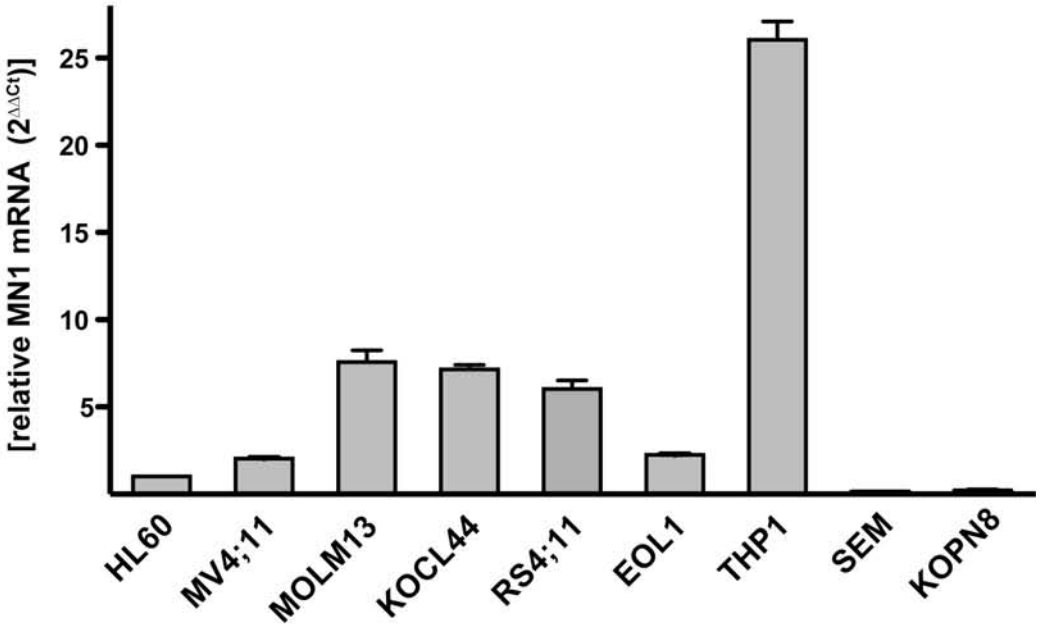

MN1 expression in MLL-rearranged infant Leukemia
MN1 mRNA Expression (fold change)
29.6
0
2
4
6
8
10
12
14
16
18
20
Normal BM
ALLPO
t(4;11)
t(4;11)
t(4;11)
t(4;11)
t(4;11)
11q23
t(9;11)
t(11;19)
t(11;19)

8. RNA interference-mediated knockdown of MN1 expression impairs the growth of human acute leukemia cells

Although elevated MN1 expression has been reported to provide negative prognostic information in adult AML cases[102], the contribution of MN1 to the biology of any leukemic cell clones remained unclear. To functionally study the role of MN1 in human leukemia, we first determined MN1 expression levels in a panel of acute leukemia cell lines (HL60, MV4;11, MOLM13, KOCL44, RS4;11, SEM, KOPN8, EOL-1, THP1*).* As shown in **Fig. 9A**, MN1 expression was detected in THP1, MOLM13, KOCL44, RS4;11 but not all MLL fusion positive cell lines. Findings on the mRNA level were also reflected on the protein levels as shown by immunofluorescence staining of MN1 in MOLM13 and SEM cells (**Fig. 9B**). To study the role of elevated MN1 expression for maintenance of the malignant cell phenotype, we transduced the cells with lentivirus expressing either MN1-specific or scramble siRNAs. A series of control experiments showed that four cell lines of the panel (HL60, MOLM13, RS4;11, THP1) could be efficiently lentivirally transduced (not shown). Expression of MN1 siRNAs resulted in a reduction of MN1 expression of >50% in HL60, MOLM13 and THP1 and 25-30% reduction in RS4;11 (**Fig. 9C**). RNAi-mediated knockdown of MN1 expression resulted also in a significant decrease of clonogenic growth (in methylcellulose) of THP1 and MOLM13 and to a lesser extent of RS4;11 (**Fig. 9D**). Likewise, decreased growth potential of the cell lines expressing MN1 siRNAs was also observed in liquid cultures (**Fig. 9E**). The growth of HL-60 cells that express very low levels of MN1 was not affected by MN1 knockdown. Cell cycle analysis of THP1 and MOLM13 cells showed that MN1-knockdown led to a significant increase of cells in G_0/G_1 associated with a reduction of cells in G_2/M and S phase (**Fig. 9F**). The observed reduced growth rate was not associated with an increase of apoptotic cell death determined by flow cytometric analysis (**Fig. 9G**). Taken together, these results demonstrated for the first time that increased MN1 expression provides a positive growth signal to human leukemic cells.

Fig. 9.

A


[relative MN1 mRNA (2^ΔΔCt)]
25
20
15
10
5
HL60
MV4;11
MOLM13
KOCL44
RS4;11
EOL1
THP1
SEM
KOPN8

B

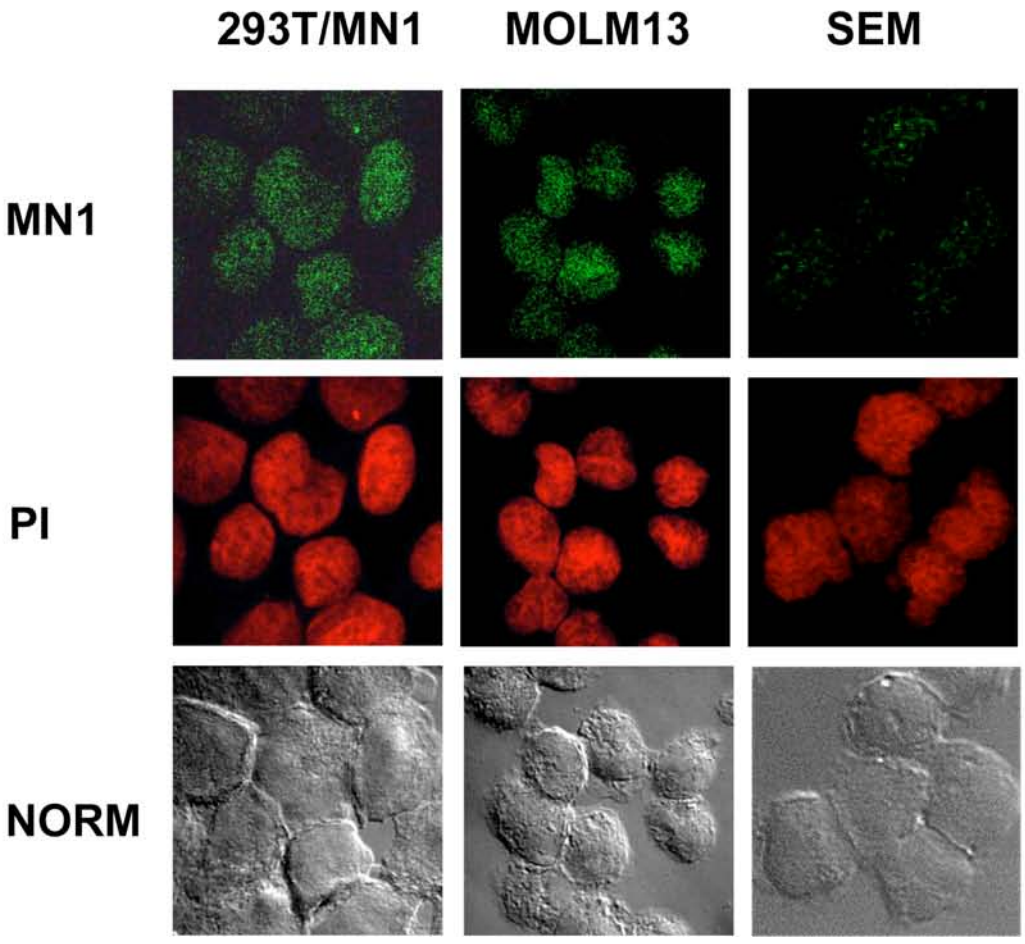

293T/MN1
MOLM13
SEM
MN1
PI
NORM

Fig. 9.

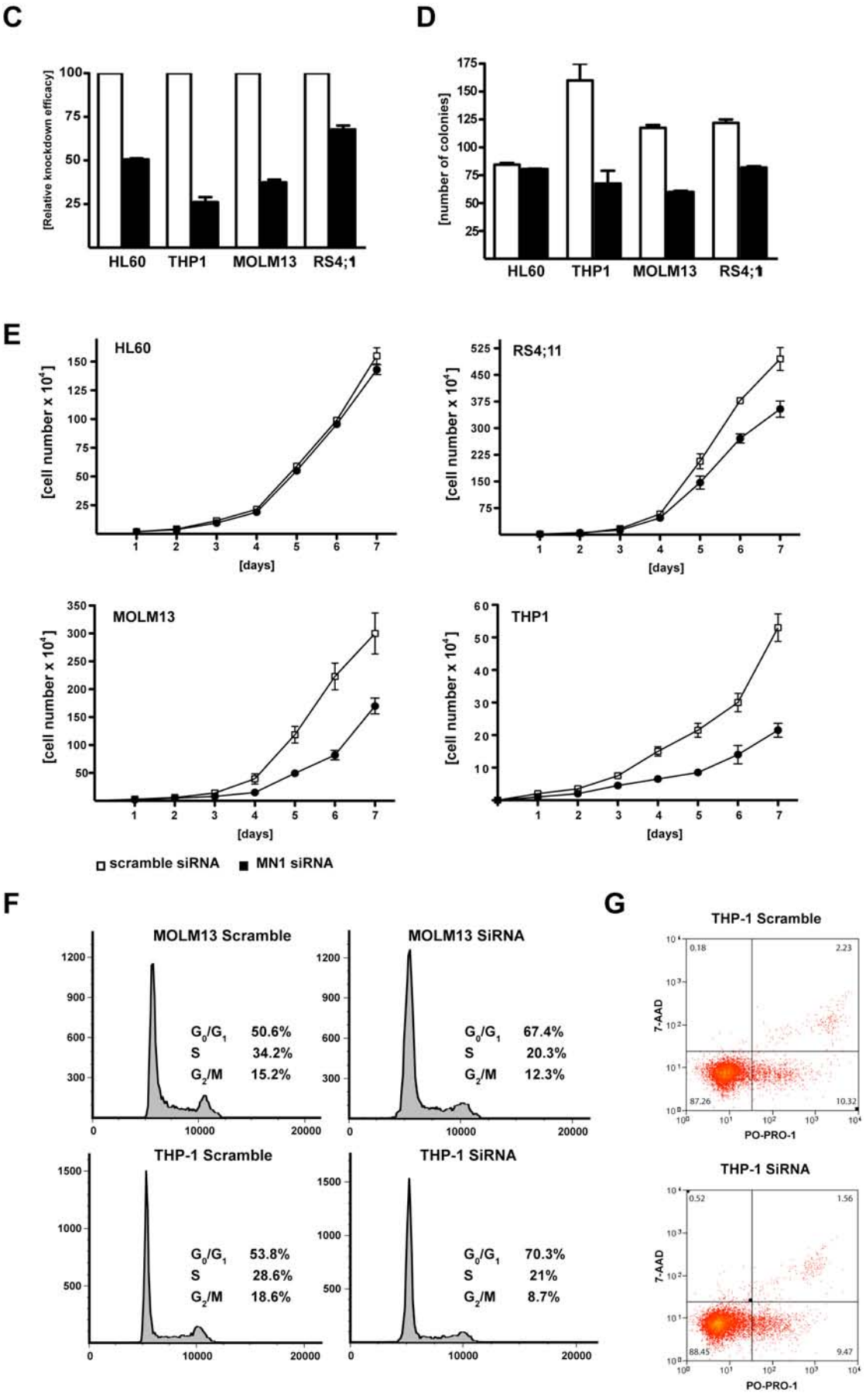

C
[Relative knockdown efficacy]
HL60
THP1
MOLM13
RS4;1
D
[number of colonies]
HL60
THP1
MOLM13
RS4;1
E
HL60
RS4;11
MOLM13
THP1
[cell number x 10⁴]
[days]
scramble siRNA
MN1 siRNA
F
MOLM13 Scramble
MOLM13 SiRNA
THP-1 Scramble
THP-1 SiRNA
G0/G1 50.6%
S 34.2%
G2/M 15.2%
G0/G1 67.4%
S 20.3%
G2/M 12.3%
G0/G1 53.8%
S 28.6%
G2/M 18.6%
G0/G1 70.3%
S 21%
G2/M 8.7%
G
THP-1 Scramble
THP-1 SiRNA
7-AAD
PO-PRO-1
0.18
2.23
87.26
10.32
0.52
1.56
88.45
9.47

Fig.9. Knockdown of MN1 expression impairs the growth of human acute leukemia cells with elevated MN1 levels

A) MN1 mRNA expression level in human acute leukemia cell lines determined by quantitative RT-PCR. Relative MN1 expression was normalized to GAPDH expression in the same sample, and shown as ΔΔCt value using HL-60 cells as a calibrator. **B**) Detection of MN1 protein expression in cell lines by immunofluorescence staining (Objective 40x/1.30). (MN1= MN1 recognized by MN1-specific antibody bound with green fluorescence labeled secondary antibody. PI= nuclear staining by propidium iodide, NORM = phase contrast) **C**) Efficacy of the lentiviral-mediated MN1 knockdown by human MN1 specific siRNA assessed by quantitative RT-PCR. **D**) MN1 knockdown impaired clonogenic growth (in methylcellulose) of THP1, MOLM13 and RS4;11 cell lines expressing high levels of MN1 but not HL-60 expressing very low levels of MN1. **E**) MN1 knockdown impaired proliferation of THP1, MOLM13 and RS4;11 cell lines but not HL-60 in liquid culture. **F**) Cell cycle analysis revealed that MN1 knockdown resulted in increased cells in G_0/G_1 phase and less cells in S and G_2/M phase. **G**) THP-1 cells were infected with lentivirus expressing MN1 specific siRNA or scramble siRNA and then stained with PO-PRO-1 and 7-AAD dyes. Apoptotic cells were indicated as PO-PRO-1 positive and 7-AAD negative cell populations by flow cytometric analysis. Results shown in C-E are compared to lentiviral infection with a scramble siRNA control. Open bars= scramble siRNA; black bars= MN1 specific siRNA.

Table 5. Oligonucleotide primers and resulting products used for RT-PCR analysis.

MOUSE Primers

	Forward 5'-3'	Reverse 5'-3'	Amplicon size (bp)
DLK1	CCCAGGTGAGCTTCGAGTG	GGAGAGGGGTACTCTTGTTGAG	215
FLT3	CCGCGAATGCACCAAGCTGT	GGTCCTGTTTGTGGAGTAGGT	210
PRNP	ATGGCGAACCTTGGCTACTG	CCTGACGTGGGTAACGGTTG	156
HLF	GACAGCTCCCCTTGAACCC	CTGCTGCTCTCATCGTCCA	150
MEIS1	GCAAAGTATGCCAGGGGAGTA	TCCTGTGTTAAGAACCGAGGG	234
ANGPT2	CAGCCACGGTCAACAACTC	CTTCTTTACGGATAGCAACCGAG	122
MAP2K1P1	ATGGCGGATGATCTAAAGCGA	GGCACTGTCGTTAGCCACT	119
IGF1	CTGGACCAGAGACCCTTTGC	GGACGGGGACTTCTGAGTCTT	268
CD34	AAGGCTGGGTGAAGACCCTTA	TGAATGGCCGTTTCTGGAAGT	156
RUNX1T1	ATGCCTGATCGTACCGAGAAG	GTCGTTGGCGTAAATGAGCTG	142
CRISP1	AAAGCAACCTGATAGTGTGGTC	AGGTGTGTATAGCCTTCCTTGA	162
LIF	ATTGTGCCCTTACTGCTGCTG	GCCAGTTGATTCTTGATCTGGT	139
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG	150
MN1	TGGTGGAGATGAGGACAAGA	CTTGGGGTCACCATCTGTG	163

HUMAN Primers

	Foward 5' - 3'	Reverse 5' - 3'	Amplicon size (bp)
DLK1	CTTTCGGCCACAGCACCTAT	GGTTCTCCACAGAGTCCGT	166
FLT3	TAAGCACAGCTCCCTGAATTG	GGAACGCTCTCAGATATGCAG	249
HLF	CCCTCGGTCATGGACCTCA	ACTTGGTGTATTGCGGTTTGC	126
MEIS1	GATATAGCCGTGTTCGCCAAA	CGGTGGCAGAAATTGTCACAT	158
ANGPT2	TCATGGAAAACAACACTCAGTGG	CGTCTGGTTCTGTACTGCATT	107
MAP2K1P1	ATGGCGGATGACCTAAAGCG	GGAAAGTCCAAGTTTGCTTCCT	198
CD34	CAACACCTAGTACCCTTGGAAGT	ACTGTCGTTTCTGTGATGTTTGT	88
MN1	GAAGGCCAAACCCCAGAAC	GATGCTGAGGCCTTGTTTGC	69
GAPDH	GTGGTCTCCCTGACTTTCAACAGC	ATGAGGTCCACCACCTGCTTGCTG	149
MLL/ENL	GCAAACAGAAAAAAGTGGCTCCCCG	ACCATCCAGTCGTGAGTGAACCCCT	373

Table 6. ≥1.5 up- or down-regulated genes (p<0.05) upon transient (72h) overexpression of MN1 in primary murine bone marrow cells as assessed by gene expression profiling.

refseq	Gene symbol	Fold change	Regu-lation	refseq	Gene symbol	Fold change	Regu-lation
NM_001002927	Penk1	8.89	up	NM_028493	Rhobtb3	2.69	up
NM_029529	Slc35d3	5.56	up	NM_008587	Mertk	2.68	up
NM_007993	Fbn1	5.29	up	NM_053110	Gpnmb	2.68	up
NM_173024	Serpina3b	5.12	up	NM_133222	Eltd1	2.68	up
NM_017469.3	Gucy1b3	4.83	up	XM_619639|XM_984786	Tns1	2.66	up
NM_010052	Dlk1	4.66	up	NM_139197	Gbgt1	2.65	up
NM_175406	Atp6v0d2	4.64	up	NM_009822	Runx1t1	2.64	up
NM_010050	Dio2	4.43	up	NM_019971	Pdgfc	2.63	up
NM_001007460	Zdhhc23	4.32	up	NM_030209	Crispld2	2.63	up
NM_011329	Ccl1	3.93	up	NM_016846	Rgl1	2.62	up
NR_001586|NR_003596	Speer1-ps	3.93	up		Folr2	2.62	up
NM_021443	Ccl8	3.88	up	NM_010329	Pdpn	2.60	up
NM_011170	Prnp|Prnd	3.76	up	NM_010605	Kcnj5	2.59	up
NM_024469	Bhlhb3	3.75	up	NM_008372	Il7r	2.59	up
NM_021896	Gucy1a3	3.70	up	NM_001081298	Lphn2	2.59	up
NM_007553	Bmp2	3.69	up	NM_025638	Gdpd1	2.58	up
	EG545728	3.58	up	NM_011520	Sdc3	2.56	up
NM_172589	Lhfpl2	3.56	up	NM_201518	Flrt2	2.55	up
NR_003596|NR_001586	Grm3|LOC623849	3.53	up	NM_008620	Gbp4	2.55	up
	D14Ertd668e	3.51	up	NM_007802	Ctsk	2.54	up
NM_011426	Siglec1	3.50	up	NM_007643	Cd36	2.54	up
NM_138741	Sdpr	3.45	up	NM_008885	Pmp22	2.53	up
NM_013703	Vldlr	3.43	up	NM_011333	Ccl2	2.52	up
NM_026037|NM_001083341	Mboat2	3.42	up	NM_001081298	Lphn2	2.51	up
NM_012008	Ddx3y	3.42	up	NM_009696	Apoe	2.50	up
NM_080555	Ppap2b	3.37	up	NM_008719	Npas2	2.49	up
NM_133974	Cdcp1	3.36	up	NM_028199	Plxdc1	2.49	up
NM_010612	Kdr	3.35	up	NM_023785	Ppbp	2.48	up
NM_001002268	Gpr126	3.34	up	NM_007405	Adcy6	2.47	up
NM_007572	C1qa	3.24	up	NM_177698|NM_030263	Psd3	2.47	up
NM_031159	Apobec1	3.23	up	NM_013612	Slc11a1	2.47	up
NM_019511	Ramp3	3.16	up	NM_026346	Fbxo32	2.47	up
	AI427515	3.13	up	NM_010008	Cyp2j6	2.46	up
NM_153170	Slc36a2	3.11	up	NM_153422	Pde5a	2.45	up
NM_007574	C1qc	3.08	up	NM_001081298	Lphn2	2.44	up
NM_172563	Hlf	3.05	up	NM_001033476	Ahnak2	2.44	up
NM_010229	Flt3	3.02	up	NM_009255	Serpine2	2.43	up
NM_001081235	Mn1	2.99	up	NR_003568	Gpr137b-ps	2.42	up
NM_012011	Eif2s3y	2.99	up		Fabp5	2.40	up
NM_007484	Rhoc	2.94	up	NM_133754	Fblim1	2.39	up
NM_010512|NM_184052	Igf1	2.92	up		Mmrn1	2.39	up
	Slamf7	2.92	up	NM_010634	Fabp5	2.39	up
NM_011175	Lgmn	2.91	up	NM_008512	Lrp1	2.39	up
NM_015800	Crim1	2.89	up	NM_134158	Cd300d|Cd300a	2.38	up
	LOC626578	2.89	up	NM_009484	Uty	2.38	up
	Robo3	2.85	up	NM_008881	Plxna1	2.38	up
NM_008198	Cfb	2.79	up	NM_009984	Ctsl	2.38	up
NM_145459	Zfp503	2.78	up	NM_013654	Ccl7	2.37	up
NM_013454	Abca1	2.78	up	NM_001081298	Lphn2	2.37	up
NM_008534	Ly9	2.77	up	NM_009777	C1qb	2.37	up
NM_022420	Gprc5b	2.76	up	NM_029509	5830443L24Rik	2.36	up
NM_028122	Slc14a1	2.75	up	NM_008594|NM_001045489	Mfge8	2.35	up
NM_133733	9030425E11Rik	2.74	up	NM_145437	4732429D16Rik	2.34	up
NM_024406	Fabp4	2.72	up	NM_016900	Cav2	2.33	up
	Fer1l3	2.72	up	NM_027763	Treml1	2.32	up
NM_207676|NM_207675	Cadm1	2.71	up	NM_010186	Fcgr1	2.31	up
NM_011407	Slfn1	2.30	up	NM_178695	Prrg4	2.31	up
NM_001081298	Lphn2	2.28	up	NM_008209	Mr1	2.31	up
NM_009141	Cxcl5	2.28	up	NM_009763	Bst1	2.30	up
NM_025629	Adamtsl5	2.25	up	NM_021436	Tmeff1	2.07	up
NM_019932	Cxcl4	2.25	up	NM_020561	Smpdl3a	2.07	up
NM_009917	Ccr5	2.24	up	NM_011125	Pltp	2.06	up
NM_009917	Ccr5	2.24	up	NM_007417	Adra2a	2.06	up
NM_009345|NM_001043228	Dntt	2.24	up	NM_053014	Agpat3	2.06	up
NM_013754	Insl6	2.22	up	NM_007388	Acp5	2.05	up
NM_010172	F7	2.22	up	NM_008509	Lpl	2.05	up
NM_021412	Mmp19	2.22	up	NM_011716	Wfs1	2.05	up
NM_145509	5430435G22Rik	2.22	up		EG432555	2.05	up
NM_011410	Slfn4	2.21	up		C130060K24Rik	2.05	up
NM_026018	Pdzk1ip1	2.21	up	NM_019919|NM_206958	Ltbp1	2.04	up
NM_008149	Gpam	2.21	up	NM_008528	Blnk	2.04	up
NM_011595	Timp3	2.21	up	NM_144783	Wt1	2.04	up
NM_133871	Ifi44	2.20	up	NM_033144	septin 8	2.04	up
NM_133987	Slc6a8	2.20	up	NM_172786	Il20ra	2.04	up
	Tmem86a	2.19	up	NM_007932	Eng	2.04	up
NM_010867|NM_001083934	Myom1	2.19	up	NM_011854	Oasl2	2.04	up
NM_001039676	Slc39a2	2.19	up	NM_030066	Armcx1	2.04	up
NM_010745	Ly86	2.18	up	NM_172203	Nox1	2.03	up
NM_011519	Sdc1	2.18	up	NM_133485	Ppp1r14c	2.03	up
NM_009899	Clca1	2.18	up	NM_008317	Hyal1	2.02	up
XM_619639|XM_984786	Tns1	2.18	up	NM_020026	B3galnt1	2.02	up
NM_001081957	OTTMUSG000000971	2.17	up	NM_175029	Atg4c	2.02	up
	Tmem140	2.17	up	NM_010789	Meis1	2.02	up
NM_183029	Igf2bp2	2.17	up	NM_031999	Gpr137b	2.01	up
	Plxnb2	2.15	up	NM_007440	Alox12	2.01	up
NM_175155	Sash1	2.15	up	NM_029499	Ms4a4c	2.01	up
NM_023516	2310016C08Rik	2.15	up	NM_008047	Fstl1	2.01	up
NM_032400	Sucnr1	2.15	up	NM_013875	Pde7b	2.01	up
NM_144559	Fcgr4	2.15	up	NM_205820	Tlr13	2.01	up
	Tmem106a	2.14	up	NM_145629	Pls3	2.01	up

NM_172603	Phf11	2.14	up	NM_054048	Rcor2	2.00	up
NM_026793	Myct1	2.13	up	NM_021506	Sh3rf1	2.00	up
NM_177379	Grit	2.13	up	NM_175472	Zcchc11	2.00	up
NM_013492	Clu	2.13	up	NM_152803	Hpse	2.00	up
NM_010863	Myo1b	2.12	up	XM_357051|XM_908720	Gm1276	1.99	up
NM_021460	Lipa	2.12	up	NM_001081298	Lphn2	1.99	up
NM_007945	Eps8	2.11	up	NM_021334	Itgax	1.99	up
NM_019521	Gas6	2.11	up	NM_016850	Irf7	1.99	up
NM_011303	Dhrs3	2.11	up	NM_007901	Edg1	1.99	up
NM_027102	Esam1	2.10	up	NM_172742	Mtmr10	1.99	up
NM_009254	Serpinb6a	2.10	up		H28	1.99	up
		2.10	up	NM_008057	Fzd7	1.99	up
NM_011395	Slc22a3	2.10	up	NM_175181|NM_001083810	2600010E01Rik	1.98	up
		2.09	up	NM_001025395|NM_009271	Src	1.98	up
NM_013838	Trpc6	2.09	up	NR_003508|NM_013606	Mx2	1.98	up
NM_183201	Slfn5	2.09	up	NM_031195	Msr1	1.97	up
	Med12l	2.09	up	NM_173740	Maoa	1.97	up
NM_199146	AI451617	2.09	up	NM_013690	Tek	1.97	up
NM_146028	Stac2	2.08	up	NM_010658	Mafb	1.97	up
NM_001081160	Mdga1	2.08	up	NM_207708|NM_009303	Syngr1	1.97	up
	Sbf2	2.08	up	NM_198028	Serpinb10	1.97	up
NM_133914|NM_001039103	Rasa4	2.08	up	NM_172647	F11r	1.97	up
NM_172133	Centa2	2.07	up	NM_007578	Cacna1a	1.96	up
NM_011594	Timp2	1.94	up	NM_011331	Ccl12	1.96	up
NM_029653|NM_134062	Dapk1	1.93	up	NM_177909	Slc9a9	1.96	up
NM_008709	Mycn	1.93	up	NM_010162	Ext1	1.95	up
NM_009333	Tcf7l2	1.93	up	NM_011451|NM_025367	Sphk1	1.95	up
NM_001033126	Cd27	1.93	up	NM_013730	Slamf1	1.95	up
NM_019976	Psrc1	1.93	up	NM_173006	Pon3	1.94	up
NM_001077404	Nrp2	1.93	up	NM_027326|NM_029931	Mllt3	1.94	up
NM_018779	Pde3a	1.92	up	NM_009593	Abcg1	1.94	up
NM_023118|NM_001037905	Dab2	1.92	up	NM_181071	Tanc2	1.84	up
NM_009943	Cox6a2	1.92	up	NM_009726	Atp7a	1.84	up
NM_177644	Rasal2	1.92	up	NM_030113	Arhgap10	1.84	up
NM_020258	Slc37a2	1.92	up	NM_133212	Tlr8	1.84	up
	EG240327	1.92	up	NM_181071	Tanc2	1.84	up
NM_001004157	Scarf1	1.92	up	NM_001081746	EG665378	1.84	up
XM_973954	RP23-330D3.3	1.92	up	NM_020270	Scamp5	1.84	up
	AW112010	1.92	up	NM_145226	Oas3|Oit3	1.83	up
NM_021356	Gab1	1.92	up	NM_008328	Ifi203	1.83	up
NM_007803	Cttn	1.91	up	NM_007408	Adfp	1.82	up
NM_029023	Scpep1	1.91	up	NM_008329|NM_001033450	Ifi204|Mnda	1.82	up
NM_011361	Sgk	1.91	up	NM_010575	Itga2b	1.82	up
NM_011817	Gadd45g	1.91	up	NM_008332	Ifit2	1.82	up
NM_008148	Gp5	1.90	up	NM_207217	Itfg3	1.82	up
NM_009853	Cd68	1.90	up	NM_029730	Mospd2	1.82	up
NM_133654	Cd34	1.90	up	NM_021281	Ctss	1.82	up
NM_011708	Vwf	1.89	up	NM_028248	Tmem87b	1.82	up
NM_172777	BC057170	1.89	up	NM_030601	Clca2	1.82	up
NM_009250	Serpini1	1.89	up	NM_007801	Ctsh	1.81	up
NM_011026	P2rx4	1.89	up	NM_008204	H2-M2	1.81	up
NM_027758	Tbc1d9	1.89	up	NM_025331	Gng11	1.81	up
NM_010549|NM_010550	Il11ra1|Il11ra2	1.89	up	NM_009231	Sos1	1.81	up
NM_011173	Pros1	1.88	up	NM_145516	Plekhb2	1.81	up
NM_133211	Tlr7	1.88	up	NM_009404	Tnfsf9	1.80	up
NM_010823	Mpl	1.88	up	NM_145838	St8sia6	1.80	up
NM_011419	Jarid1d	1.88	up	NM_008327|NM_011940	Ifi202b	1.80	up
NM_172867	Zfp462	1.88	up	NM_027678|NM_172642	Zranb3	1.80	up
NM_009599	Ache	1.88	up	NM_011158	Prkar2b	1.80	up
	EG665378	1.88	up	NM_007798	Ctsb	1.80	up
NM_194464	Mrvi1	1.88	up	NM_133167	Parvb	1.80	up
	Pyhin1	1.87	up	NM_029495|NM_178825	Epsti1	1.79	up
NM_021893	Cd274	1.87	up	NM_010821	Mpeg1	1.79	up
NM_010550	Il11ra2	1.87	up		4930506M07Rik	1.79	up
NM_021792	Iigp1	1.86	up	NM_008605	Mmp12	1.79	up
NM_010549	Il11ra1|Il11ra2	1.86	up	NM_138719|NM_010313	Gnb5	1.79	up
NM_031254	Trem2	1.86	up	NM_027836|NM_001025610	Ms4a7	1.79	up
NM_011074	Pftk1	1.86	up		AI427122	1.79	up
NM_011866	Pde10a	1.86	up	NM_030710	Slamf6	1.79	up
NM_130449	Colec12	1.86	up	NM_008624	Mras	1.79	up
NM_008012	Akr1b8	1.85	up	NM_009779	C3ar1	1.78	up
	AW548124	1.85	up	NM_133187	1110032E23Rik	1.78	up
	Etv1	1.85	up	NM_007515	Slc7a3	1.78	up
NM_018762	Gp9	1.85	up	NM_026835	Ms4a6d	1.78	up
NM_177167	Ppm1e	1.85	up	NM_175476|NM_001037727	Arhgap25	1.78	up
NM_027356	2700038N03Rik	1.85	up	NM_008357	Il15	1.78	up
NM_001045530	Ccnjl	1.85	up	NM_029068	Snx16	1.78	up
NM_016667	Sntb1	1.85	up	NM_025282	Mef2c	1.77	up
NM_178797	Mlstd1	1.84	up	NM_152804	Plk2	1.77	up
NM_026792	Agpat5	1.84	up	NM_144865	Reep2	1.77	up
NM_026470	Spata6	1.76	up	NM_011459	Serpinb8	1.77	up
NM_144538	Rab3il1	1.76	up	NM_001013366	Wdr91	1.77	up
NM_019920	Map2k1ip1	1.76	up	NM_013587	Lrpap1	1.77	up
	AU017193	1.76	up	NM_019413	Robo1	1.76	up
NM_028270	Aldh1b1	1.76	up	NM_025926|NM_027287	Dnajb4	1.76	up
NM_008625	Mrc1	1.75	up	NM_007904	Ednrb	1.76	up
XM_205829|XM_914710	Tm4sf19	1.75	up	NM_172637	Hectd2	1.76	up
NM_021515	Ak1	1.75	up	NM_013825	Ly75	1.76	up
NM_053273	Ttyh2	1.75	up	NM_024217	Cmtm3	1.69	up
NM_009624	Adcy9	1.75	up	NM_008398	Itga7	1.69	up
NM_001003955|NM_177466	Rab11fip5	1.75	up	NM_008630	Mt2	1.69	up
NM_011360	Sgce	1.75	up	NM_176073|NM_018755	Pgcp	1.69	up
NM_011183	Psen2	1.75	up	NM_027144	Arhgef12	1.69	up
NM_011785	Akt3	1.75	up	NM_153778	Atoh8	1.69	up
NM_011197	Ptgfrn	1.75	up	NM_009842	Cd151	1.69	up
NM_019920	Map2k1ip1	1.74	up		Gm129	1.69	up
NM_198095	Bst2	1.74	up	NM_020577	As3mt	1.69	up
NM_001039692|NM_029277	Arhgap12	1.74	up	NM_011504	Stxbp3a	1.69	up
NM_019455	Ptgds2	1.74	up	NM_145501	Pi4k2a	1.69	up
NM_027760	Rassf8	1.74	up	NM_009101	Rras	1.69	up
NM_018797	Plxnc1	1.74	up	NM_008768	Orm1	1.68	up

	B430306N03Rik	1.74	up	NM_008720	Npc1	1.68	up
NM_029001	Elovl7	1.74	up	NM_010548	Il10	1.68	up
NM_008873	Plau	1.74	up	NM_010045	Darc	1.68	up
NM_175540	Eda2r	1.73	up	NM_009808	Casp12	1.68	up
NM_029394	Snx24	1.73	up	NM_175034	Slc24a5	1.68	up
NM_001013833|NM_011160	Prkg1	1.73	up	NM_023063	Lima1	1.68	up
NM_026013|NM_001025582	Tmem77	1.73	up	NM_008102	Gch1	1.68	up
NM_009686	Apbb2	1.73	up		Stox2	1.68	up
NM_053180	Ccrk	1.73	up	NM_028030	Rbpms2	1.68	up
NM_007378	Abca4	1.72	up	NM_133990	Il13ra1	1.67	up
NM_172648	Ifi205	1.72	up	NM_026082	Dock7	1.67	up
NM_008876	Pld2	1.72	up	NM_146073	Zdhhc14	1.67	up
NM_028981|NM_001083616	Cacna1d	1.72	up	NM_010279	Gfra1	1.67	up
NM_139300	Mylk	1.71	up	NM_010515	Igf2r	1.67	up
NM_001005341	Ypel2	1.71	up	NM_133969|NM_008455	Cyp4v3|Klkb1	1.67	up
	Tmem65	1.71	up	NM_001004363	Nuak1	1.67	up
NM_011073	Prf1	1.71	up	NM_173028	Vps13a	1.67	up
NM_008367	Il2ra	1.71	up	NM_029364	Gns	1.67	up
NM_008443	Kif3a	1.70	up	NM_080708	Bmp2k	1.67	up
	AW555464	1.70	up	NM_001033291	Usp40	1.67	up
NM_013471	Anxa4	1.70	up	NM_020578	Ehd3	1.67	up
NM_001077364|NM_010286	Tsc22d3	1.70	up	NM_010587	Itsn1	1.67	up
NM_009465	Axl	1.70	up	NM_018784	St3gal6	1.67	up
NM_010397|NM_010399	H2-T22|H2-T9	1.70	up	NM_009929	Col18a1	1.67	up
NM_010687	Large	1.70	up	NM_013835	Trove2	1.66	up
NM_007963	Evi1	1.70	up	NM_023158	Cxcl16	1.66	up
NM_010421	Hexa	1.70	up	NM_028060	Slc35f2	1.66	up
NM_008624	Mras	1.70	up	NM_009368	Tgfb3	1.66	up
NM_001042614|NM_009155	Sepp1	1.70	up	NM_144520	Sec14l2	1.66	up
NM_172773	Slc17a5	1.70	up	NM_022029	Nrgn	1.66	up
NM_133859	Olfml3	1.70	up	NM_013790|NM_176839	Abcc5	1.66	up
NM_181542|NM_181545	Slfn10|Slfn8	1.70	up	NM_173440	Nrip1	1.66	up
NM_007837	Ddit3	1.69	up	NM_138756	Slc25a36	1.66	up
NM_010738	Ly6a	1.65	up	NM_146206	Tpcn2	1.65	up
NM_001029990	Mett11d1	1.65	up	NM_172753	4732435N03Rik	1.65	up
NM_144517	Tbc1d19	1.65	up	NM_052976	Ophn1	1.65	up
NM_011116	Pld3	1.65	up	NM_025446	Aig1	1.65	up
NM_023044	Slc15a3	1.65	up	NM_011391	Slc16a7	1.61	up
	BC013481	1.65	up	NM_008608	Mmp14	1.61	up
	Tbrg3	1.65	up	NM_011150	Lgals3bp	1.61	up
NM_008397	Itga6	1.65	up	NM_010818	Cd200	1.61	up
NM_181547	Nostrin	1.65	up	NM_008121	Gja5	1.61	up
	Phc3	1.65	up	NM_080466	Kcnn3	1.61	up
NM_033320	Glce	1.65	up	NM_011584	Nr1d2	1.61	up
NM_175512	Dhrs9	1.65	up	NM_008813	Enpp1	1.61	up
NM_183016	Cdc42bpb	1.64	up	NM_199448	Fez2	1.61	up
NM_207648	H2-Q6|H2-Q8	1.64	up	NM_013683	Tap1	1.61	up
NM_172289	Slc36a4	1.64	up	NM_023386	Rtp4	1.60	up
NM_023124	H2-Q8	1.64	up	NM_010560	Il6st	1.60	up
NM_007387	Acp2	1.64	up	NM_021297	Tlr4	1.60	up
NM_207530	Osbpl1a	1.64	up	NM_013748	Clnk	1.60	up
NM_016923	Ly96	1.64	up		8030451K01Rik	1.60	up
XM_889589|XM_909359	EG625046	1.64	up	NM_011961	Plod2	1.60	up
NM_016769	Smad3	1.64	up	NM_029898	Ankrd55	1.60	up
NM_009707|NM_178754	Arhgap6	1.64	up		A230067G21Rik	1.60	up
NM_001033245	Hk3	1.64	up	NM_022890	Cldn12	1.60	up
NM_026283	Samd8	1.63	up	NM_028787	Slc35f5	1.60	up
NM_175093	Trib3	1.63	up		EG665378	1.60	up
NM_025911	Ccdc91	1.63	up	NM_008533	Cd180	1.60	up
NM_016764	Prdx4	1.63	up	NM_016921	Tcirg1	1.59	up
NM_172468	Snx30	1.63	up	NM_019587	Plxnb3	1.59	up
NM_023320	Plekho1	1.63	up	NM_010819	Clec4d	1.59	up
NM_146122	Dennd1a	1.63	up	NM_001039039	Kctd21	1.59	up
NM_008986	Ptrf	1.63	up	NM_001007578	Armcx6	1.59	up
NM_010554	Il1a	1.63	up	NM_020509	Retnla	1.59	up
NM_146126	Sord	1.63	up	NM_133792	Lypla3	1.59	up
NM_024223	Crip2	1.63	up	NM_010683	Lamc1	1.59	up
NM_172883	Mfsd7	1.63	up	NM_053082	Tspan4	1.59	up
NM_172475	Frmd4a	1.63	up	NM_013839	Nr1h3	1.59	up
	Slc44a1	1.62	up		AY223547	1.59	up
NM_001033550	Lrrc8b	1.62	up	NM_175160	Zdhhc1	1.59	up
NM_009295	Stxbp1	1.62	up	NM_173379	Leprel1	1.59	up
NM_001025250|NM_009505	Vegfa	1.62	up	NM_025332	Gtpbp8	1.58	up
NM_013792	Naglu	1.62	up	NM_013761	Srr	1.58	up
NM_009019	Rag1	1.62	up	XM_905096	Irgb10	1.58	up
XM_001475971|XR_032907	LOC100041211	1.62	up	NM_010840	Mthfr	1.58	up
NM_023065	Ifi30	1.62	up		Cdc42bpa	1.58	up
NM_019778	Zbtb20	1.62	up	NM_010496	Id2	1.58	up
NM_008325	Idua	1.62	up	NM_009912	Ccr1	1.58	up
NM_009655	Alcam	1.62	up	NM_133891	Slc44a1	1.58	up
NM_145530	Rhov	1.62	up	NM_009052	Bex1	1.58	up
NM_028916	Efhc2	1.62	up	XM_915205|XM_985548	Syngap1	1.58	up
NM_054040	Tulp4	1.62	up	NM_028460|NM_152799	3110045G13Rik	1.58	up
XM_001480292	LOC100048461	1.62	up	NM_178772	Aadacl1	1.58	up
NM_025449	Nicn1	1.62	up		BC031353	1.58	up
NM_145134	Spsb4	1.62	up	NM_133977	Trf	1.58	up
NM_023409	Npc2	1.58	up	XM_001479889	Cd59a	1.58	up
NM_007715	Clock	1.58	up	NM_010501	Ifit3	1.58	up
NM_027209	Ms4a6b	1.58	up	NM_013692	Klf10	1.58	up
NM_134042	Aldh6a1	1.58	up	NM_007483	Rhob	1.58	up
NM_009706	Arhgap5	1.58	up	NM_030150	Dhx58	1.58	up
NM_011906	Gpr175	1.58	up	NM_023873	Cep70	1.55	up
NM_011347	Selp	1.58	up	NM_007897	Ebf1	1.55	up
NM_008635	Mtap7	1.57	up	NM_008732	Slc11a2	1.55	up
NM_018882	Gpr56	1.57	up	NM_008007	Fgf3	1.55	up
NM_019990	Stard10	1.57	up	NM_145987	Tmem82	1.55	up
NM_023655	Trim29	1.57	up		EG209380	1.55	up
NM_146025	Samd14	1.57	up	NM_027109	Dnase1l1	1.55	up
NM_019734	Asah1	1.57	up	NM_001009935|NM_023719	Txnip	1.55	up
NM_028238	Rab38	1.57	up	NM_008477	Ktn1	1.55	up
NM_053090	Drctnnb1a	1.57	up	NM_026674	Aph1c	1.55	up

NM_173370	Cds1	1.57	up	NM_178119|NM_001037136	Centg2	1.55	up
XM_001472523|XM_485967	EG434215	1.57	up	XM_483890|XM_906658	Trdn	1.55	up
NM_153103	Kif1c	1.57	up	NM_011068	Pex11a	1.55	up
NM_021414	4631427C17Rik	1.57	up	NM_178667	Tfdp2	1.54	up
NM_025760	Ptplad2	1.57	up	NM_028894	Lonrf3	1.54	up
NM_144912	Rad9b	1.57	up	NM_012056	Fkbp9	1.54	up
NM_012000	Cln8	1.57	up	NM_176841	Ccdc88a	1.54	up
NM_019580	Mir16	1.57	up	NM_013750	Phlda3	1.54	up
	EG665378	1.57	up	XM_893468|XM_911460	EG628705	1.54	up
NM_198862	Nlgn2	1.57	up	NM_026719	Lmbrd1	1.54	up
NM_172772	B230380D07Rik	1.57	up	NM_007395	Acvr1b	1.54	up
	Nlrc5	1.56	up		LOC435793	1.54	up
NM_025359	Tspan13	1.56	up	NM_145220	Appl2	1.54	up
NM_001081347	Rhobtb1	1.56	up	NM_007482	Arg1	1.54	up
NM_172477	Dennd2a	1.56	up	NM_016863	Fkbp1b	1.54	up
NM_013489	Cd84	1.56	up	NM_008737	Nrp1	1.54	up
NM_181545	Slfn8	1.56	up	NM_172708	Dok7	1.54	up
NM_178253	Klhdc1	1.56	up	NM_027340	Lipn	1.54	up
NM_009230	Soat1	1.56	up	XR_033405|XR_033767	LOC546714	1.54	up
NM_007514|NM_001044740	Slc7a2	1.56	up	NM_009452	Tnfsf4	1.54	up
	Disp1	1.56	up	NM_008161|NM_001083929	Gpx3	1.54	up
NM_019677	Plcb1	1.56	up	NM_021273	Ckb	1.54	up
NM_139140	Spats2	1.56	up	NM_010705	Lgals3	1.53	up
XM_147850|XM_001476299	BC030046	1.56	up	NM_013826	Mocs2	1.53	up
NM_144552	Stxbp6	1.56	up	NM_177178	Lmbrd2	1.53	up
NM_008598	Mgmt	1.56	up	NM_172051	Tmcc3	1.53	up
NM_153508	Clstn3	1.56	up	NM_008859	Prkcq	1.53	up
NM_172267	Phyhd1|Lrrc8a	1.56	up	NM_001037713	Fbxo39	1.53	up
NM_011890	Sgcb	1.56	up		Cmah	1.53	up
	Nbeal1	1.56	up	NM_172893	Parp12	1.53	up
NM_019432	Tmem37	1.56	up	NM_009099	Trim30	1.53	up
NM_009517	Zmat3	1.56	up		Zmym6	1.53	up
NM_001045513	Raph1	1.55	up	NM_001081260	Tnks1bp1	1.53	up
NM_147220	Abca9	1.55	up	NM_001001979	Megf10	1.53	up
NM_181848	Optn	1.55	up	NM_016965	Nckap1	1.53	up
NM_175465	Sestd1	1.55	up	NM_144731	Galnt7	1.53	up
NM_011913	Best1	1.55	up	NM_146001	Hip1	1.53	up
NM_026163	Pkp2	1.55	up	NM_145538	ENSMUSG00000058405	1.53	up
NM_007426	Angpt2	1.55	up	XM_981204|XM_917816	Myo9a	1.53	up
NM_028523	Dcbld2	1.55	up		1700081L11Rik	1.53	up
NM_178653	Sccpdh	1.55	up	NM_018804	Syt11	1.53	up
NM_028207	Dusp3	1.53	up	XM_001477846	LOC672511	1.51	up
NM_011309	S100a1	1.53	up	NM_007975	F2rl3	1.51	up
NM_011920	Abcg2	1.53	up	NM_172153|NM_178142	Lcorl	1.51	up
NM_026418	Rgs10	1.53	up	NM_010395	H2-T10	1.51	up
NM_145926	Mgat4b	1.52	up	NM_008311	Htr2b	1.51	up
NM_018819	Brp44l	1.52	up	NM_010145	Ephx1	1.51	up
NM_012048	Polk	1.52	up	NM_178704	Dpy19l3	1.51	up
NM_027154	Tmbim1	1.52	up	NM_144923	Blvrb	1.51	up
NM_013515	Stom	1.52	up	NM_011505	Stxbp4	1.51	up
	Tmem163	1.52	up	NM_177386	Sfmbt2	1.51	up
NM_025935	Tbc1d7	1.52	up	NM_019832	Gkap1	1.51	up
NM_025522	Dhrs7	1.52	up	NM_027764	Rcbtb1	1.51	up
NM_011305	Rxra	1.52	up	NM_177378	Rnf150	1.51	up
NM_010326	Gp1ba	1.52	up	NM_153103	Kif1c	1.51	up
NM_013646	Rora	1.52	up	NM_029091	Klc4	1.51	up
NM_153543	Aldh1l2	1.52	up	NM_013509	Eno2	1.51	up
NM_016780	Itgb3	1.52	up	NM_177861	Tmem67	1.51	up
NM_001079901	Repin1	1.52	up	NM_011852	Oas1g	1.51	up
NM_183355|NM_008783	Pbx1	1.52	up	NM_001082553|NM_030554	Rab27b	1.51	up
NM_016982	Vpreb1	1.52	up	NM_175489|NM_001003717	Osbpl8	1.51	up
NM_007644	Scarb2	1.52	up	NM_001079824	Hnrph3	1.51	up
NM_010171	F3	1.52	up	NM_016811	Dgka	1.50	up
XM_917816|XM_981204	Myo9a	1.52	up	NM_178728	AB112350	1.50	up
NM_009906	Tpp1	1.52	up	NM_145984	Prepl	1.50	up
	Disp1	1.52	up	NM_008235	Hes1	1.50	up
NM_010870	Naip5	1.52	up	NM_026298	Ift172	1.50	up
NM_010806	Mllt4	1.52	up	NM_019581	Gtpbp2	1.50	up
NM_177662	Ctso	1.52	up	NM_018861	Slc1a4	1.50	up
	AU023871	1.51	up	NM_019578	Extl1	1.50	up
NM_025975	Dynlt3	1.51	up	NM_013529	Gfpt2	1.50	up
NM_029655	Snx7	1.51	up	NM_013784	Pign	1.50	up
	BC022687	1.51	up	NM_026316	Aldh3b1	1.50	up
NM_027491	Rragd	1.51	up		Hrasls3	1.50	up
NM_152895	Jarid1b	1.51	up	NM_178729	Fbxl5	1.50	up
NM_020610	Nrip3	2.21	down	NM_010190	Fcnb	3.98	down
NM_181596	Retnlg	2.20	down	NM_009660	Alox15	3.97	down
NM_178200	Hist1h2bm	2.17	down	NM_001083955	Hba-a2	3.34	down
NM_001033632	Ifitm6	2.17	down	NM_010518	Igfbp5	3.33	down
NM_011061	Padi4	2.16	down	NM_008218	Hba-a1	3.26	down
	Mrgpra2	2.15	down	NM_025288	Stfa3	3.12	down
NM_009114	S100a9	2.13	down	NM_133246	Ms4a3	3.12	down
NM_001083957|NM_009799	Car1	2.12	down	NM_053111|NM_017385	Ear6|Ear7	3.11	down
NM_145968	Tagap|Tagap1	2.11	down	NM_001082546	BC100530|Stfa1	3.08	down
NM_009382	Thy1	2.11	down	NM_008220|NM_016956	Hbb-b1|Hbb-b2	3.07	down
NM_010215	Il4i1	2.11	down	NM_009127	Scd1	3.04	down
NM_007675	Ceacam10	2.10	down	NM_010720	Lipg	2.99	down
NM_001082545	Stfa2	2.08	down	NM_021274	Cxcl10	2.89	down
NM_007695	Chi3l1	2.07	down	NM_008920	Prg2	2.88	down
NM_024444	Cyp4f18	2.07	down	NM_015779	Ela2	2.84	down
NM_017370	Hp	2.05	down	NM_007894	Ear1	2.74	down
NM_008522	Ltf	2.05	down	NM_178372	Prss34	2.60	down
NM_001042710	Prssl1	2.04	down	NM_011332	Ccl17	2.58	down
NM_019957	Dnase2b	2.04	down	NM_009921	Camp	2.52	down
NM_183370|NM_183368	Sytl3	2.03	down	NM_001082545|NM_173869	Stfa2|Stfa2l1	2.51	down
NM_023049	Asb2	2.03	down	NM_013542	Gzmb	2.49	down
NM_009712	Arsb	2.03	down	NM_009846	Cd24a	2.49	down
NM_016917	Slc40a1	2.02	down	NM_153511	Il1f9	2.48	down
NM_008501|NM_001039537	Lif	2.01	down		Mrgpra6	2.47	down
NM_010700	Ldlr	2.00	down	NM_011090|NM_011091	Pira3|Pira4|Pira11	2.46	down
NM_008489	Lbp	1.99	down	NM_009616	Adam19	2.45	down

NM_009132	Scin	1.99	down	NM_013650	S100a8	2.45	down
NM_026862	Cd177	1.99	down	NM_011178	Prtn3	2.41	down
NM_153546	Mboat1	1.98	down	NM_007946	Epx	2.40	down
NM_021407	Trem3	1.98	down	NM_030720	Gpr84	2.37	down
NM_031168	Il6	1.98	down	NM_009402	Pglyrp1	2.37	down
NM_139200	Pscdbp	1.98	down		BC018473	2.36	down
NM_153101	Mrgpra2	1.97	down	NM_016914	Prg3	2.34	down
NM_009122	Satb1	1.97	down	NM_009864	Cdh1	2.30	down
NM_001004159	Clec4b2	1.96	down	NM_001038604|NM_021364	Clec5a	2.30	down
NM_013599	Mmp9	1.95	down	NM_009137	Ccl22	2.29	down
NM_207131	Cebpe	1.93	down	NM_026414	Asprv1	2.26	down
NM_175664	Hist1h2bb	1.93	down	NM_031181	Siglece	2.24	down
NM_177390	Myo1d	1.93	down	NM_029796	Lrg1	2.24	down
NM_007984	Fscn1	1.93	down	NM_011269	Rhag	1.84	down
NM_008491	Lcn2	1.93	down	NM_001076679	LOC751864	1.83	down
NM_007895|NM_017388	Ear2|Ear3	1.92	down	NM_134469	Fdps	1.83	down
NM_001013365	Osm	1.92	down	NM_021283	Il4	1.83	down
NM_001082543	Stfa1	1.91	down	NM_009384	Tiam1	1.83	down
NM_178259	Abca13	1.90	down	NM_213614|NM_001001999	Sept5|Gp1bb	1.83	down
NM_010206|NM_001079908	Fgfr1	1.90	down	NM_177390	Myo1d	1.82	down
NM_001082542	EG408196	1.90	down	NM_008355	Il13	1.82	down
NM_010482	Htr1b	1.89	down	NM_011436	Sorl1	1.81	down
NM_020034	Hist1h1b	1.89	down	NM_026515	2810417H13Rik	1.81	down
NM_010517	Igfbp4	1.89	down	NM_001099217	Ly6c2	1.80	down
NM_007895	Ear2|Ear3	1.89	down	NM_022032	Perp	1.80	down
NM_009769	Klf5	1.88	down	NM_001030294	Olfm4	1.80	down
NM_013698	Txk	1.88	down	NM_009712	Arsb	1.79	down
NM_010427	Hgf	1.87	down	NM_016674	Cldn1	1.78	down
NM_009638	Crisp1	1.87	down	NM_008518	Ltb	1.78	down
NM_010824	Mpo	1.86	down	NM_053112|NM_007895	Ear10|Ear2|Ear12	1.78	down
NM_175660	Hist1h2ab	1.85	down	NM_178197	Hist1h2bh	1.78	down
NM_172659	Slc2a6	1.66	down	NM_053272	Dhcr24	1.77	down
NM_023734	Pi16	1.66	down	NM_019577	Ccl24	1.77	down
NM_153094|NM_181064	Klrb1f	1.65	down	NM_011957	Creb3l1	1.76	down
NM_145149	Rasgrp4	1.65	down	NM_172694	Megf9	1.76	down
NM_011311	S100a4	1.65	down	NM_011926|NM_001039185	Ceacam1	1.76	down
NM_017461	Sepl1	1.64	down	NM_020010	Cyp51	1.76	down
NM_028808	P2ry13	1.64	down	NM_008288|NM_001044751	Hsd11b1	1.76	down
NM_009135	Scn7a	1.64	down		Tcrg-V2|Tcrg-V1	1.76	down
NM_010555	Il1r2	1.64	down	NM_024204	Ankrd22	1.76	down
NM_178202|NM_178199	Hist1h2bp	1.63	down	NM_001005510	Syne2	1.76	down
NM_178202|NM_178201	Hist1h2bp	1.63	down	NM_053112|NM_007895	Ear10|Ear2|Ear12	1.75	down
NM_001033922|NM_172623	Treml4	1.63	down	NM_007719	Ccr7	1.74	down
NM_177733	E2f2	1.63	down	NM_134066	Akr1c18	1.73	down
NM_011782	Adamts5	1.62	down	NM_177448	Mogat2	1.73	down
XM_001472239	LOC433368	1.62	down	NM_009477	Upp1	1.73	down
NM_007670	Cdkn2b	1.62	down	NM_023396	Rprm	1.72	down
	Ibrdc3	1.62	down	NM_030728	9930013L23Rik	1.72	down
NM_026384	Dgat2	1.62	down	NM_183249	1100001G20Rik	1.71	down
NM_172435	P2ry10	1.62	down	NM_007950	Ereg	1.71	down
NM_025831	1300014I06Rik	1.62	down	NM_011808|NM_001038642	Ets1	1.71	down
NM_013521	Fpr1	1.62	down	NM_008405	Itgb2l	1.70	down
NM_033596	Hist2h4	1.61	down	NM_007799	Ctse	1.70	down
NM_178202|NM_178198	Hist1h2bp	1.61	down	NM_175666	Hist2h2bb	1.70	down
	Kynu	1.61	down	NM_011950	Mapk13	1.70	down
	Sprr2a	1.61	down	NM_016672	Ddc	1.69	down
NM_008230	Hdc	1.60	down	NM_021406	Trem1	1.69	down
NM_207680|NM_207681	Bcl2l11	1.60	down	NM_011198	Ptgs2	1.68	down
XR_033339	LOC100042191	1.59	down	NM_010741	Ly6c1	1.68	down
NM_020559	Alas1	1.59	down	NM_178202|NM_178195	Hist1h2bp	1.68	down
NM_001079686|NM_153399	Syne1	1.59	down	NM_175535	Arhgap20	1.68	down
NM_138648	Olr1	1.59	down	NM_178202|NM_175665	Hist1h2bp	1.68	down
	Tomm40	1.59	down	XR_033108	LOC100041907	1.68	down
	6530402F18Rik	1.58	down	NM_030609	Hist1h1a	1.67	down
NM_178183	Hist1h2ak	1.58	down	NM_008812	Padi2	1.67	down
NR_002895	Pwcr1	1.58	down	NM_013596	Mc5r	1.66	down
NR_002895	Pwcr1	1.58	down	NM_010104	Edn1	1.66	down
NR_002895	Pwcr1	1.58	down	NM_153526	Insig1	1.66	down
NR_002895	Pwcr1	1.58	down	NM_016693	Map3k6	1.66	down
NM_175652	Hist4h4	1.58	down	NR_002895	Pwcr1	1.57	down
NR_002895	Pwcr1	1.58	down	NR_002895	Pwcr1	1.56	down
NR_002895	Pwcr1	1.58	down	NM_007664	Cdh2	1.56	down
NR_002895	Pwcr1	1.57	down		Ncf1	1.56	down
NM_172496	Cobl	1.57	down	NM_019984	Tgm1	1.56	down
NR_002895	Pwcr1	1.57	down	NR_002895	Pwcr1	1.56	down
NR_002895	Pwcr1	1.57	down	NR_002895	Pwcr1	1.56	down
	Pwcr1	1.57	down	NR_002895	Pwcr1	1.56	down
NM_201367	Gpr176	1.57	down		BC029169	1.56	down
NM_008572	Mcpt8	1.57	down	NM_013566	Itgb7	1.56	down
NR_002895	Pwcr1	1.57	down	NM_133809	Kmo	1.56	down
NM_009104	Rrm2	1.57	down	NM_007800	Ctsg	1.56	down
	Lsm5	1.57	down	NM_015736	Galnt3	1.55	down
NM_001081302	Trio	1.57	down	NM_011346	Sell	1.55	down
NM_009811	Casp6	1.53	down	NR_002895	Pwcr1	1.55	down
NM_025436	Sc4mol	1.52	down	NM_019391	Lsp1	1.55	down
NM_001097979|NM_178202	RP23-38E20.1	1.52	down		D8Ertd82e	1.55	down
NM_001097979|NM_178202	RP23-38E20.1	1.52	down	NM_019395	Fbp1	1.55	down
NM_145581	Siglec5	1.52	down	NM_011770	Ikzf2	1.55	down
NM_009911	Cxcr4	1.51	down	NM_178165|NM_153090	Fcrl1	1.54	down
NM_145942	Hmgcs1	1.51	down	NM_139198	Plac8	1.54	down
NM_175663	Hist1h2ba	1.51	down	NM_010553	Il18rap	1.54	down
NM_013458	Add2	1.51	down	NM_008046	Fst	1.54	down
NM_178593|NM_001038846	Rcsd1	1.51	down	NM_017464	Nedd9	1.54	down
NM_173733	Suox	1.50	down	NM_007575	Ciita	1.54	down
NM_010583	Itk	1.50	down	NM_008517	Lta4h	1.53	down
NM_026439	Ccdc80	1.50	down	NM_001081445|NM_010875	Ncam1	1.53	down
NM_024245	Kif23	1.50	down	NM_010931	Uhrf1	1.53	down

DISCUSSION

Several studies have suggested that retroviral expression of class II mutations such as *MLL* fusion genes (e.g. *MLL/AF9*, *MLL/ENL*, *MLL/GAS7*) or *MOZ/TIF2* in the murine hematopoietic system is necessary but rather not sufficient to induce an acute myeloid leukemia (AML) that recapitulates the human disease [21,34,40,42,109]. However until today whether secondary genetic alterations occur and what could be their nature remained unknown. In human AML, several small alterations such as point mutations (e.g. in the *FLT3* or *WT1* genes) can often be found in a homozygous state as a consequence of uniparental disomy (UPD) or loss of heterozygosity (LOH). By modeling the class II mutation mediated AML in a F1-hypbrid strain (FVB/N x 129/s1), we aimed to identify LOH of loci that could carry functionally collaborating mutated oncogenes or tumor suppressor genes. However, our genome-wide haplotype analysis using microsatellite marker based genotyping and SNP arrays did not reveal any large regions of LOH in 21 mouse leukemias induced by MLL/ENL or MOZ/TIF2. It is highly likely that the resolution of our technology with only about 1500 polymorphic markers throughout the 19 murine chromosomes was too low. Nevertheless, at the time of the analysis there was no commercial platform available that would have allowed increasing the sensitivity of our analysis. New technologies like high-throughput parallel sequencing will allow screening the whole genome of a leukemic cell for genetic lesions such as fusion genes, small mutations as well as epigenetic modification in a single analysis [110,111]. Using this technology, Ley and co-workers recently characterized an acute myeloid leukaemia genome of an AML patient that showed no gross chromosomal alterations in normal karyotyping. Strikingly, mutations in only 8 genes were found: 1 was the recurrent FLT3-ITD mutation combined with single mutations in 7 genes with unknown biological consequence, however, none of these 7 mutations were found in 100 unrelated AML patients [112].

Recent studies have shown that the retroviral insertion mutagenesis of replication defective retrovirus may be sufficient to immortalize hematopoietic progenitors and could favor the driver mutations in oncogenic transformation [79,80]. In our experiment, we used the replication-defective Murine Stem Cell Virus (MSCV) to express the

class II mutations (MLL/ENL or MOZ/TIF2) together with an internal ribosomal entry site (IRES)-regulated EGFP marker [79]. Thus, the integration of retrovirus could theoretically also act as the key cooperating event in these mouse leukemia models. Using a PCR based retroviral integration cloning strategy, we were able to clone 42 integrations with 66 integration flanking genes in 2 murine leukemia models. It has been previously shown that oncogenic genes identified by retroviral insertion mutagenesis are more frequently differentially expressed in human acute leukemia than randomly selected genes or genes located more distantly from provirus integration [85,113]. Interestingly, some genes flanking the integration sites such as *SOCS1*, *JARID2*, *PTPRE*, *HSP90*, *GADD45*, *MGAT5*, *PP2R5C*, *LHX2* or *MN1* have been previously linked to carcinogenesis [114-120].

The suppressor of cytokine signaling (SOCS)-1, a negative regulator of the Janus family tyrosine kinases (JAKs), has been reported to be hypermethylated in its promoter region in the majority of primary AML cases studied, which led to decreased expression and may contributed to malignant transformation [117]. HSP90 (heat-shock 90-kd protein 1, alpha) is a molecular chaperone that plays a key role in the conformational maturation of oncogenic signaling proteins, including HER2/ERBB2, AKT, RAF1, BCR/ABL, and mutated p53. In tumor cells, HSP90 complexes keep in an activated, high-affinity conformation that facilitates malignant progression [114]. High levels of *LHX2* (*LIM-homeobox* gene) expression are frequently observed in CML patients, regardless of disease status. *LHX2* is located in the same region as the reciprocal translocation that generates the BCR/ABL chimera, and it is silenced in normal bone marrow due to DNA methylation. However, in CML cells both alleles of *LHX2* locus were heavily hypomethylated, suggesting that the transcriptional activation of LHX2 in CML likely results from a *cis*-acting effect, rather than a *trans*-acting effect of the BCR/ABL fusion protein [121]. In the mouse model, the expression of Lhx2 in murine bone marrow led to immortalized hematopoietic cells with multipotent differentiation potential, and transplantation of these cells in irradiated recipients caused a chronic myeloproliferative disorder [115,116].

The integration near the *Mn1* locus resulted in significantly increased levels of mRNA expression of *Mn1* in the MLL/ENL expressing leukemic blasts. As these blasts carried several integrations, the integration targeting *Mn1* was clonally selected as

shown by PCR analysis of single clones grown in methylcellulose (**Fig. 3**). The *Mn1* gene locus has been previously reported as the recurrent integration site in mouse models of human hematological malignancies induced by retroviral oncogene expression such as NUP98/HOXD13 fusion or mutated AML1 [106,107]. Several recent studies suggested functional links between MLL, CBF and EVI1. EVI1 seems to be a downstream target of MLL/ENL and NUP98/HOX mediated transformation [122-125]. In addition, direct interactions between MLL and AML1 seem to provide epigenetic regulation of gene expression in leukemic cells [126]. Although the exact molecular mechanisms remain to be elucidated, a positive role of MN1 in a putative pathway involving CBF and EVI1 blocking differentiation and providing aberrant self-renewal could explain why a clone with the proviral integration mediated elevated MN1 expression would be selected in presence of the MLL/ENL fusion.

As MN1 showed *in vitro* and *in vivo* leukemogenic potential (**Fig. 3**)[100,101], retroviral integration-mediated MN1 overexpression in MLL/ENL induced leukemia suggested functional cooperation. Indeed, co-expression of MN1 with MLL/ENL resulted in the induction of an aggressive AML-like phenotype after a significantly reduced latency period compared to MLL/ENL or MN1 alone (**Fig. 4**). Previous studies have proposed that in addition to hematopoietic stem cells (HSCs), committed progenitor cells such as common myeloid or lymphoid progenitors (CMPs, CLPs) or even granulocyte-macrophage progenitors (GMPs) can also be targets of MLL/ENL, MLL/AF9, MLL/AF4 or MOZ/TIF2 induced transformation [21,41,108]. Interestingly, in normal mice the highest levels of MN1 were found in GMPs [100]. Detailed comparison of the MLL/ENL induced leukemia with the disease induced by expression of MLL/ENL and MN1 suggests that co-expression could lead to an expansion of the GMP population. Increased clonogenic activity of these cells further suggests that MN1 also regulates genes implicated in self-renewal. Accelerated induction of a leukemic phenotype in secondary transplants further supports the idea that GMPs represent a prominent disease initiating cell pool (also often referred as leukemia stem cells) in MLL/ENL+MN1 leukemia. Indeed, reconstitution of sorted GMP cells from MLL/ENL and MLL/ENL+MN1 leukemias into secondary recipients fully supported this idea (**Fig. 5D**). Further studies are needed to determine whether elevated MN1 levels would also increase the pool of leukemia-initiating cells in leukemias harboring other

MLL fusions, or other alterations previously linked to elevated MN1 levels like inv16, and alterations of EVI1 that are often associated with MN1 overexpression [99,100].

In adult *de novo* AML without detectable cytogenetic abnormalities, MN1 overexpression was associated with poor response to therapy, a higher disease relapse rate and shorter overall survival [102]. These clinical observations as well as our experimental work suggest that elevated MN1 expression might be a general signature for an expanded leukemic stem cell population. However, the nature of the disease-initiating stem cell in *MLL* fusion genes induced murine leukemia is still a matter of debate: both, expression of Gr1/Mac1$^+$ and c-Kit$^+$ covering 25% of the blasts, as well as lineage-negative (less than 1% of leukemic blasts) Leukemic-HSCs, L-GMPs, L-CLPs have been proposed to act as leukemia stem cells [21,34,42,108]. Nonetheless, independently of the chosen phenotypic markers, we observed a significant increase in potential leukemic stem cell pool when MN1 was co-expressed with MLL/ENL as illustrated by secondary transplantation experiments (**Fig. 5**).

Our results suggest functional cooperation of MN1 with MLL fusions in leukemogenesis. However, not all studied MLL fusion positive human leukemia cell lines expressed elevated MN1 levels (**Fig. 9A**). In addition, our own and other's gene expression profiling studies of experimental models of MLL leukemias never revealed MN1 as being a primary up-regulated target (unpublished observation) [42,108]. These observations raised the possibility that the MN1 expression status in a leukemic blast might be dependent on the maturation grade of the cell that was initially targeted by the MLL fusion and/or on a currently unknown mechanism that would maintain the MN1 levels high. Interestingly, an unusual methylation pattern of the MN1 promoter region was recently reported in leukemic cells, suggesting that epigenetic mechanisms might be needed to sustain the elevated MN1 expression levels in cell transformed by oncogenes such as MLL fusions [127].

It has been demonstrated that MN1 primarily acts as transcriptional co-regulator [93]. Transient overexpression of MN1 is associated with significant changes in the gene expression programs involving a large number of genes as shown recently in a human leukemia cell line (U937) [128]. In our model, overexpression of MN1 in primary bone marrow cells resulted in up-regulation of a set of genes that have been

associated with normal and malignant hematopoiesis (**Fig. 6, Table 6**). Moreover, some of these genes like FLT3, CD34, DLK1, MEIS1, and HLF were also up-regulated in blasts from murine leukemias induced by MN1 or MLL/ENL + MN1 expression. These MN1 regulated genes such as FLT3 are well known to be up-regulated and functionally involved in various human and mouse leukemias mediated by different fusion genes. Expression levels of FLT3 have a negative prognostic impact on MLL fusion positive pediatric leukemias and FLT3 has been validated as a potential therapeutic target [129-131]. Interestingly, FLT3, DLK1 and HLF seem to be deregulated upon aberrant MEIS1 or HOXA activity, but we did not found any *HOXA* gene that were up-regulated upon transient MN1 overexpression. Nevertheless, blasts from leukemias induced by MN1 or MLL/ENL + MN1 expressed significantly elevated levels of several genes of the *HOXA* cluster (*HOXA5*, *HOXA7*, *HOXA9* and *HOXA10*) suggesting a regulation through additional genetic hits (data not shown).

Strikingly, transient and stable expression of MN1 was always associated with increased CD34 expression (**FIG. 6**). Although the surface glycophosphoprotein CD34 is widely used as marker for early lympho-hematopoietic stem and progenitor cells, its biologic function remains not well understood [132]. Impaired clonogenic growth of MN1 transformed progenitor cells by CD34 siRNA mediated knockdown (**Fig. 7C & D**) suggests that CD34 could play an active role in MN1 mediated transformation. Moreover, a significant correlation of MN1 expression with CD34 levels was not only seen in the mouse leukemia model but also in cases of B-cell ALL or infant leukemias as a small cohort of primary pediatric leukemias (**Fig. 7**). These observations are currently being validated in a larger cohort of pediatric acute leukemia patients.

By exploring the role of MN1 in pediatric leukemia, we found significantly elevated levels of MN1 mRNA in a large fraction of B-cell ALL and infant leukemia cases (**Fig. 8**). Previous studies in adult AML have demonstrated deregulated expression of MN1 being associated with either alteration of 3q26 (EVI1) or inv16 (CBFβ) [99,100]. In adult AML with normal cytogenetics, increased levels of MN1 were proposed to be a negative prognostic factor [102]. In our pediatric cohort of leukemia patients, increased MN1 expression was often associated with the presence of MLL fusions. However, the group of patients with normal cytogenetics and elevated MN1 levels was too

small in our cohort to draw any conclusion about MN1 as a sole prognostic biomarker in pediatric leukemia.

In contrast to adults, most cases of pediatric leukemia with significantly elevated MN1 levels are of B-cell origin independently of whether or not they were carrying an MLL fusion. In mouse models, expression of MN1 always resulted in the generation of myeloid disorders [100,101]. Further work will be necessary to clarify this discrepancy. Nevertheless, we were able to find increased expression of MN1 target genes (CD34, FLT3) not only in the murine system, but also in most human cases with elevated MN1 (**Figs. 6D & 7A**).

Forced siRNA-mediated down-regulation of MN1 expression in cells that overexpress MN1 impaired growth in methylcellulose (colony formation) as well as in liquid culture. MN1 knockdown was associated with cell cycle arrest in G_0/G_1 phase (**Fig. 9**). These results showed, for the first time, that aberrant MN1 expression could be an important factor for proliferation of leukemic cells. Although early cell loss upon antibiotic selection cannot be excluded in our experiments, we did not observe any significant apoptotic cell death or signs of cellular senescence upon MN1 knockdown (data not shown). As leukemic and normal hematopoietic cells expressing low levels of MN1 were not affected by siRNA-mediated knockdown, selective inhibition of MN1 in leukemia initiating cells might be possible without affecting normal tissue. Future structure-function studies might be able to provide essential clues for therapeutic targeting of the aberrant MN1 activity found in a significant portion of pediatric acute leukemia patients.

The observations from my PhD work suggest that MN1 is a functional collaborator to MLL/ENL (and probably also other leukemogenic class II mutations) by increasing the compartment of leukemic stem cells through a distinct genetic program. Furthermore, I have identified some potential downstream target genes of MN1 that might be executors of its pro-leukemic activity. Although we are just at the beginning of understanding the role of MN1 in normal and malignant hematopoiesis, my work showed for the first time that elevated MN1 levels support proliferation of human acute leukemia cells suggesting that targeting aberrant MN1 activity could open new therapeutic avenues.

PERSPECTIVE

In our cohort of pediatric leukemia patients, we unexpectedly found significantly elevated MN1 expression levels in immature B-cell ALL and infant leukemias. As MN1 has never been studied in the B-cell lineage, the role of MN1 in the normal and malignant B-cells remains to be elucidated. The currently used bone marrow transduction & transplantation model of murine leukemia introduces a bias for developing myeloid malignancies due to the culture conditions (IL-3, IL-6 and mSCF) used to stimulate growth of the cells for efficient transduciton. In order to address the consequence of elevated MN1 levels in lymphoid cells, we therefore plan to transduce enriched lymphoid progenitors with the MN1 retrovirus in the medium that favors lymphoid cell growth (containing IL-7, FL and IL-11) before transplantation. These experiments could shed more light on the role of MN1 in lymphoblastic leukemia.

In addition, we will also determine the MN1 expression in normal B-cell precursors ranging from CLPs to pre-B, pro-B and mature B cells. Regulated MN1 expression during B-cell differentiation would suggest that this gene might play a role in normal B-cell development. Moreover, conditional ablation of MN1 expression in the lymphoid lineage would provide a perfect system to explore MN1 in normal and malignant B-cells. There are several laboratories currently aiming to develop conditional MN1 knockout animals.

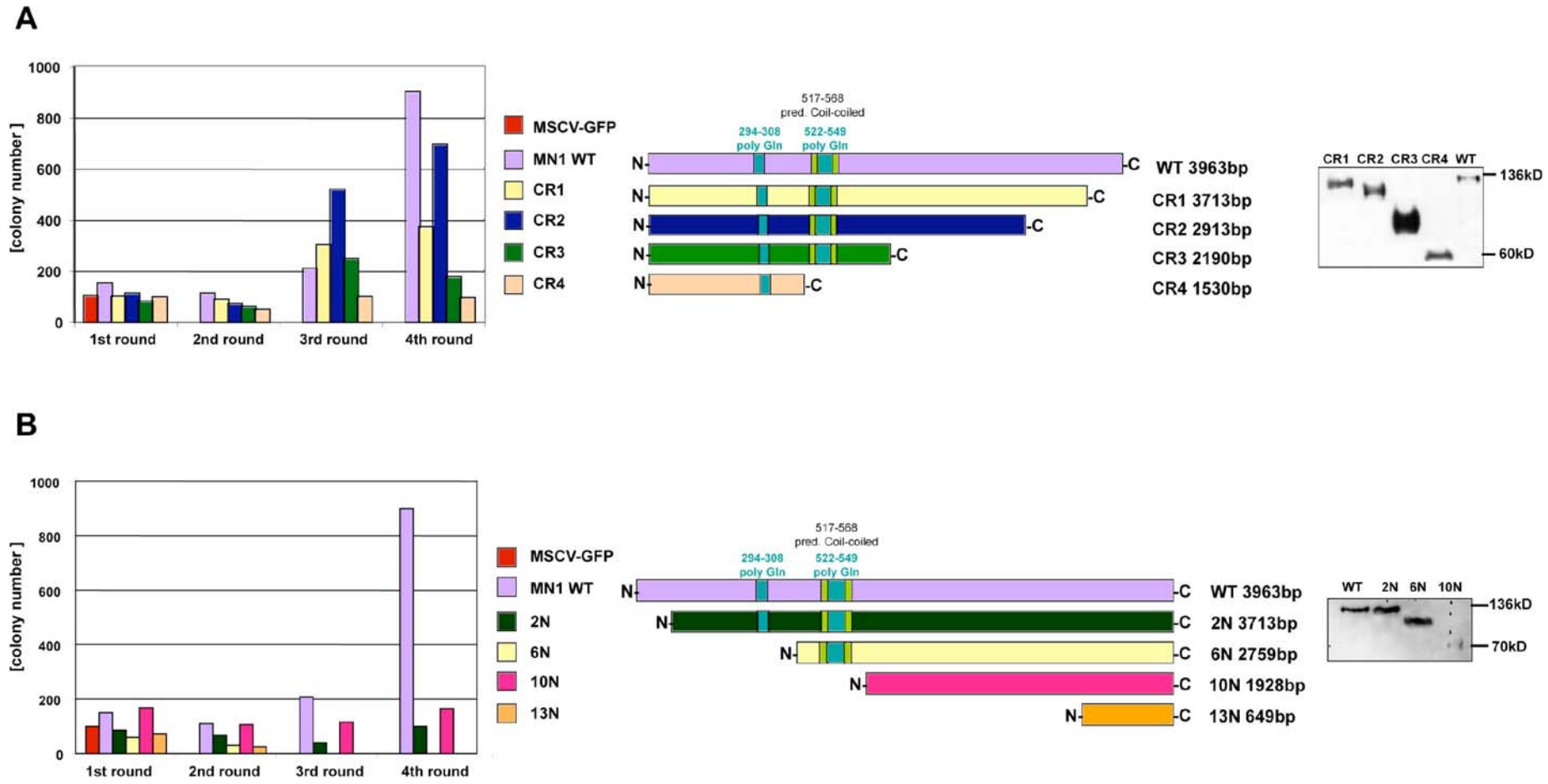
To further study the role of MN1 in B-cell ALL, we are currently determining MN1 expression levels in a larger panel of human ALL cell lines. Following the same strategy used for AML cells, we will use lentivirally expressed siRNAs to analyze the effects of MN1 knockdown on growth and survival of these cells (**Fig. 9**). We have also cloned the full-length MN1 cDNA in lentivirus to overexpress MN1 in cells with low or undetectable levels of expression of endogenous MN1. Subsequent gene expression profiling studies in B-ALL cells upon MN1 knockdown or overexpression should provide more information about the role of this oncogene in B-cell leukemia. The extracted putative MN1 target genes will be further validated in a large panel of B-cell ALL patient samples.

Several reports have recently suggested that MN1 might act as a transcriptional co-activator although it may not bind DNA directly. The so far most investigated substrate supporting this hypothesis is that a complex formed by MN1 and the co-activators p300 or RAC3 that synergistically activated retinoic-acid receptor (RAR/RXR) regulated transcription [94,133]. In addition, MN1 also indirectly interacted with the vitamin D receptor via currently unknown co-activators [134]. However, how does MN1 exert its oncogenic transforming potential in hematopoietic cells remains unclear. Although the gene expression profiling results of ours (**Fig. 6, Table 6**) and others [128,135] revealed some transcriptional factors that were regulated by transient expression of MN1, the underlying mechanisms remain to be identified. A protein-protein interaction screen would illustrate the potential transcription factors or co-activators that might physically interact with MN1, and further experiments such as chomatin-immunoprecipitation analysis could provide important insights about which genes are responsible for the self-renewal and proliferation of hematopoietic cells regulated by MN1. A previous study has suggested that the very N-terminal region of MN1 is important for its transactivation activity, however, a detailed structure-function analysis of MN1 is missing [94]. Therefore, we have started to clone and express a series of N- and C-terminal MN1 deletion mutants (**Fig. 10**). Preliminary results indicated that the N-terminal region of MN1 seems to be critical for its transforming activity. Further functional characterization of MN1 should elucidate the molecular mechanisms underlying its oncogenic potential, and facilitate future therapeutic targeting of the aberrant MN1 activity in leukemia patients.

Fig. 10. *In vitro* transforming activity of MN1 deletion mutants

The C-terminal (**A**) and N-terminal (**B**) MN1 deletion mutants were generated as depicted and cloned into *MSCV-IRES-GFP* vector. CR stands for C-terminal and N stands for N-terminal MN1 deletion mutants, and the size of deletion mutants is indicated. The expression of HA-tagged MN1 deletion mutants in 293T cells was detected by Westernblot analysis using anti-HA antibody. The murine bone marrow cells were transduced with MSCV retrovirus expressing different MN1 deletion mutants, and then replated in methylcellulose up to 4 rounds.

Fig. 10.


A
[colony number]
1000
800
600
400
200
0
1st round
2nd round
3rd round
4th round
MSCV-GFP
MN1 WT
CR1
CR2
CR3
CR4
517-568
pred. Coil-coiled
294-308
poly Gln
522-549
poly Gln
N-
-C
WT 3963bp
CR1 3713bp
CR2 2913bp
CR3 2190bp
CR4 1530bp
CR1 CR2 CR3 CR4 WT
136kD
60kD
B
[colony number]
1000
800
600
400
200
0
1st round
2nd round
3rd round
4th round
MSCV-GFP
MN1 WT
2N
6N
10N
13N
517-568
pred. Coil-coiled
294-308
poly Gln
522-549
poly Gln
N-
-C
WT 3963bp
2N 3713bp
6N 2759bp
10N 1928bp
13N 649bp
WT 2N 6N 10N
136kD
70kD

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09/1993---07/1998 **Bachelor of Medicine**, North China Coal Medical College, China

WORKING EXPERIENCE

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CONGRESS

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Oral Presentation: Meningeoma 1 (MN1) in childhood acute leukemia. 76th Annual Meeting of the Swiss Society of Internal Medicine, Lausanne, Switzerland. 23 May 2008.

PUBLICATIONS

1. **Liu T**, Jankovic D, Brault L, et al. Functional characterization of high levels of meningioma 1 as collaborating oncogene in acute leukemia. (Submitted)
2. Jankovic D, Gorello P, **Liu T**, et al. Leukemogenic mechanisms and targets of a NUP98/HHEX fusion in acute myeloid leukemia. Blood. 2008 Jun 15;111(12):5672-82.

AWARDS

12/2008 American Society of Hematology 2008 Travel Award