Identification, Function and clinical Relevance of mouse myeloid leukemia Genes

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Identificatie, functie en klinische relevantie van muizen myeloïde leukemiegenen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens het besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 27 april 2005 om 13.45 uur

door

Stefanus Joseph Erkeland

geboren te Goirle

Promotiecommissie

Promotors	Prof.dr. I.P. Touw Prof.dr. B. Löwenberg
Overige leden	Dr. R. Delwel Prof.dr. R. Kanaar Prof.dr. M. van Lohuizen

The work described in this thesis was performed at the Institue of Hematology, Erasmus Medical Center Rotterdam, The Netherlands. This work was supported by the Dutch Cancer Society "Koningin Wilhelmina Fonds". Financial support from the Dutch Cancer Society (KWF) for printing of this thesis is gratefully acknowledged.

Cover by Onno Roovers, Sabine Erkeland-Willemen and Stefan Erkeland

Printed by [Optima] Grafische Communicatie, Rotterdam

The Blind Men and the Elephant

It was six men of Indostan, to learning much inclined,
who went to see the elephant (Though all of them were blind),
that each by observation, might satisfy his mind.

The first approached the elephant, and, happening to fall,
against his broad and sturdy side, at once began to bawl:
"God bless me! but the elephant, is nothing but a wall!"

The second feeling of the tusk, cried: "Ho! what have we here,
so very round and smooth and sharp? To me tis mighty clear,
this wonder of an elephant, is very like a spear!"

The third approached the animal, and, happening to take,
the squirming trunk within his hands, "I see," quoth he,
the elephant is very like a snake!"

The fourth reached out his eager hand, and felt about the knee:
"What most this wondrous beast is like, is mighty plain," quoth he;
"Tis clear enough the elephant is very like a tree."

The fifth, who chanced to touch the ear, Said; "E'en the blindest man
can tell what this resembles most; Deny the fact who can,
This marvel of an elephant, is very like a fan!"

The sixth no sooner had begun, about the beast to grope,
than, seizing on the swinging tail, that fell within his scope,
"I see," quothe he, "the elephant is very like a rope!"

And so these men of Indostan, disputed loud and long,
each in his own opinion, exceeding stiff and strong,
Though each was partly in the right, and all were in the wrong!

So, oft in theologic wars, the disputants, I ween,
tread on in utter ignorance, of what each other mean,
and prate about the elephant, not one of them has seen!

John Godfrey Saxe (1816 - 1887)

Contents

CHAPTER 1

Introduction

1.1 Normal hematopoiesis

Hematopoiesis is the tightly regulated process of proliferation and differentiation of hematopoietic precursors towards mature blood cells (Fig. 1.1). During fetal development, primitive erythrocytes arise from the extra-embryonic yolk-sac [92]. The earliest definitive hematopoiesis including differentiation towards lymphoid, myeloid and erythroid lineages takes place in the paraortic splanchnopleura (PAS), aorta-gonad-mesonephros (AGM) region and liver in fetal stages [34, 35]. In contrast, in adult vertebrates hematopoiesis occurs mainly in the bone marrow (BM) [35].

All blood cells arise from common pluripotent precursors, the hematopoietic stem cells (HSC). HSCs have the ability of multi-lineage differentiation and sustained self-renewal [18, 121, 131]. HSCs are mainly present in the BM but are also found at low numbers in liver, spleen and perhaps in other organs as well [53, 121]. The estimated amount of HSCs is less than 0.005% of all hematopoietic cells in the BM [82]. Normally, HSCs cycle with an average turnover time of 30 days in mice but this process is enhanced in response to hematological stress, like bleeding and infection [121]. Intriguingly, a single mouse HSC can reconstitute the entire hematopoiesis for a normal lifespan of the animal [61, 91]. HSCs give rise to at least eight major hematopoietic lineages in vivo via multilineage and lineage restricted hematopoietic progenitor cells (HPCs) (Fig. 1.1) [114, 121]. HPCs have potential to differentiate into limited amount of mature cells of either lymphoid or myeloid lineages. The earliest myeloid precursor gives rise to neutrophils/granulocytes, erythrocytes, monocytes/macrophages and megakaryocytes/platelets and is called colony forming unit mixed granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) (Fig. 1.1). The lymphoid stem cell or common lymphoid precursor (CLP) gives rise to B- and T-lymphocytes (Fig. 1.1). In contrast to HSCs, mature blood cells have a finite lifespan ranging from 1 day for human neutrophils up to 120 days for erythrocytes [121]. The turnover of human hematopoietic cells is approximately 1 trillion per day, including 200 billion erythrocytes and 70 billion neutrophils [88].

The hematopoietic microenvironment is crucial for regulation of growth, differentiation, and survival of progenitor cells and tightly controls maintenance of normal blood cell numbers under steady state conditions and hematopoietic stress situations [18, 121]. The hematopoietic microenvironment consists of a heterogeneous population of hematopoietic and non-hematopoietic stroma cells [25, 37]. These stroma cells, including fibroblasts, endothelial cells, macrophages, adipocytes and osteogenic precursors produce a complex cocktail of extracellular matrix molecules and a variety of collagens and provide a source of many hematopoietic cytokines such as GM-CSF, G-CSF and SCF. The hematopoietic microenvironment provides the optimal niche for interaction of progenitor cells with these cytokines [55]. Furthermore, it stimulates interaction between hematopoietic cells and stroma cells via cell adhesion molecules such as integrins, selectins, and mucins and ligands on extracellular matrix components, including heparin sulfates, chemokines, collagens, laminin, thrombospondin-1 and fibronectin. Adherence of cells to these elements triggers a variety of signaling pathways, changes pH and calcium and lipids levels, regulates gene expression and cell cycle events and modulates growth factor signaling cascades including PI-3-kinase and Akt [37, 114].

Cytokines are essential regulators of normal hematopoiesis [55]. Cytokines that

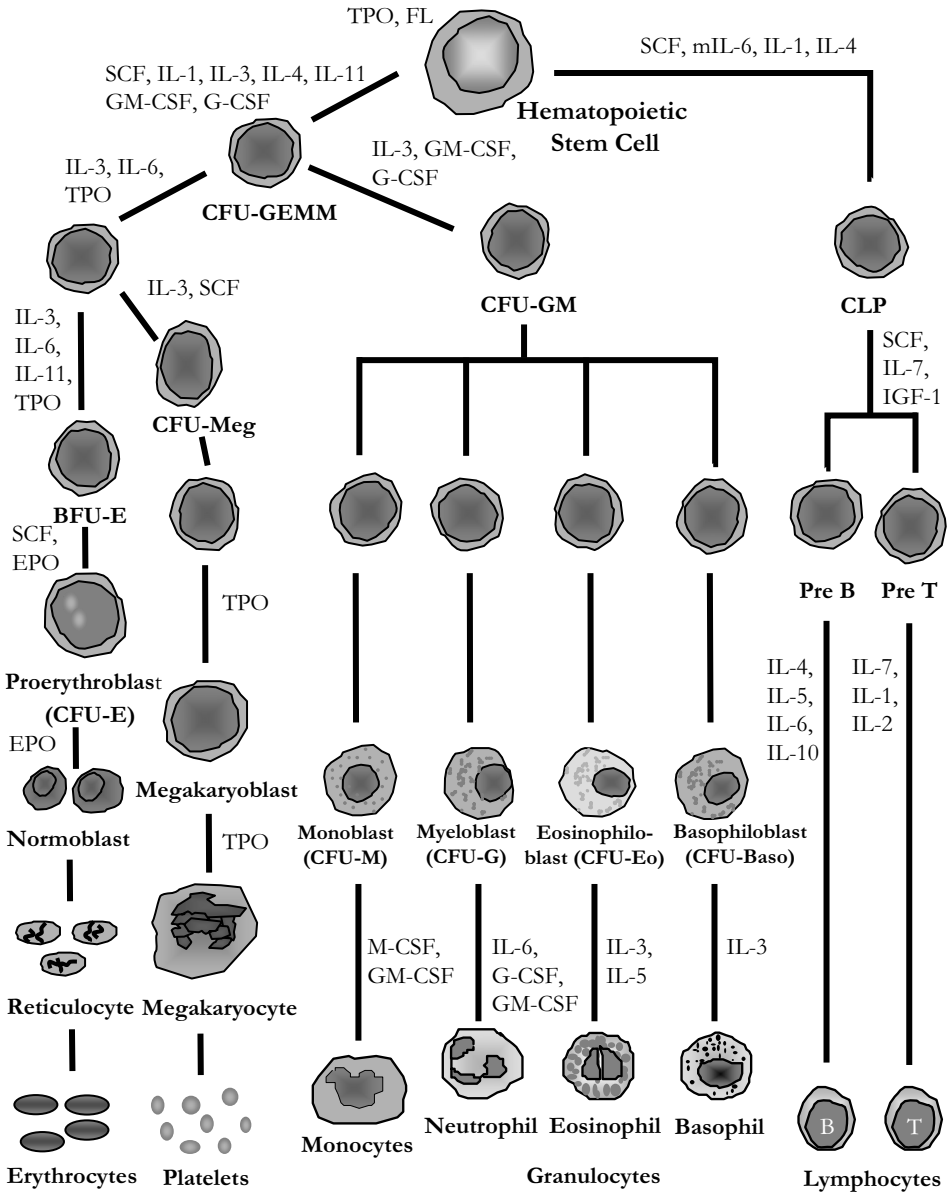

Figure 1.1 Schematic representation of hematopoiesis
Different types of mature blood cells arise from the hematopoietic stem cell. This complex process is directed by various cytokines of which only the major factors are depicted.

regulate early restriction of differentiation are e.g., stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), IL-3 and granulocyte (G)-CSF (Fig. 1.1). Molecules such as erythropoietin (EPO), thrombopoietin (TPO) and IL-5 are crucial for terminal differentiation initiation (Fig. 1.1) and some cytokines, such as thombopoietin (TPO), interleukin-3 (IL-3) and GM-CSF act on both primitive and committed hematopoietic cells. Cytokines bind particular receptors and initiate specific intracellular signals [55]. Based on structural homologies, most of these receptors belong to the cytokine receptor class I super family [8]. This family is characterized by four highly conserved cysteine residues and a tryptophan-serine repeat (WSXWS) in the extra cellular domain and both motifs are located within the cytokine receptor homology (CRH) region [8]. Some examples of cytokine receptors are interleukin-2 (IL2)-, IL3-, IL4-, IL5-, IL6-, IL7-, GM-CSF-, G-CSF- and LIF-receptors.

1.2 Leukemia

Leukemia is characterized by the uncontrolled clonal outgrowth of malignant blood cells, which overgrow normal blood cells and interfere with their function. Clinical symptoms of leukemia include infections due to granulocytopenia, anemia and bleeding caused by thrombocytopenia [76]. Leukemia can be of lymphoid or myeloid origin and is further divided in chronic and acute forms. Chronic forms of leukemias such as chronic lymphoid leukemia (CLL) and chronic myeloid leukemia (CML) are characterized by the accumulation of more mature blood cell types, whereas in acute forms of leukemias, acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) immature malignant hematopoietic cells accumulate. The incidence of leukemia in the Netherlands is for adolescent males 7.7 and for females 4.8 cases per 10^5 [112, 127]. Since the research described in this thesis is mainly focused on molecular abnormalities in AML, a brief introduction of the biological features of this type of leukemia is given.

Acute myeloid leukemia (AML) is characterized by block in differentiation and uncontrolled self-renewal capacity of early myeloid progenitors, resulting in increased number of immature nonfunctional blasts in the BM [37, 74, 76, 95]. In general, age is a strong prognostic factor in AML [74]. AML is a heterogeneous disease with variable responses to treatment [37, 75, 77]. Approximately 75% of young and middle-aged adults (< 60 years old) respond to chemotherapy and 35-40% survives for 5 years or more [74]. In contrast, older patients (>60 years) have a treatment response probability of 45-55% and less than 10% of these patients survive for 5 years or more [74]. Typical symptoms of AML are e.g., fatigue and hemorrhage (due to decrease in red cells), fever and infections (caused by non-functional white blood cells), pallor and dyspnea [76]. Leukemic blasts may infiltrate in various tissues, including liver, spleen, skin, lymph nodes, bone, gingival, and central nervous system [76]. Different morphological characteristics of AML can be identified by Wright-Giemsa staining of peripheral blood and BM preparations. The French-American-Britisch (FAB) group developed a method of AML classification based on morphological characteristics and divided leukemia in nine distinct subtypes [12, 13] and (Table 1) [76]. Several additional FAB subtype markers can be identified with

Table 1. The French-American-British (FAB) classification of AML and associated genetic abnormalities [76]

FAB subtype	Common name (% of cases)	Associated translocations and rearrangements (% of cases)	Genes involved
M0	Acute myeloblastic leukemia with minimal differentiation (3%)	inv(3q26) and t(3;3) (1%)	EVI1
M1	Acute myeloblastic leukemia without maturation (15-20%)		
M2	Acute myeloblastic leukemia with maturation (25-30%)	t(8;21) (40%), t(6;9)(1%)	AML-ETO, DEK-CAN
M3	Acute promyelocytic leukemia (5-10%)	t(15;17) (98%), t(11;17) (1%), t(5;17) (1%)	PML-RARα, PLZF-RARα, NPM-RARα
M4	Acute myelomonocytic leukemia (20%)	11q23 (20%), inv(3q26), t(3;3)(3%), t(6;9)(1%)	MLL, DEK-CAN, EVI1
M4eo	Acute myelomonocytic leukemia with abnormal eosinophils (5-10%)	inv(16), t(16;16)(80%)	CBFβ-MYH11
M5	Acute monocytic leukemia (2-9%)	11q23 (20%), t(8;16)(2%)	MLL, MOZ-CBP
M6	Erythroleukemia (3-5%)		
M7	Acute megakaryocytic leukemia (3-12%)	t(1;22) (5%)	unknown

immunological and/or cytochemical methods [76, 120]. Gene expression profiling has further highlighted the heterogeneous nature of human AML and has led to the identification of leukemia subgroups based on distinctive gene expression signatures [105, 126].

Some AML subtypes are associated with specific chromosomal translocations or rearrangements [73, 74]. It is generally accepted that genes implicated in particular chromosomal aberrations play a crucial role in the pathogenesis of the different subtypes of leukemia [73]. Importantly, specific cytogenetic abnormalities and gene mutations rather than cytological FAB classes are major determinants of therapeutic response [40, 41]. For instance, AML with translocations t(8;21), inv(16) and t(15;17) are characterized by a relatively good prognosis (favorable risk), whereas patients with t(6;9), 11q23 abnormalities, monosomies of chromosomes 7 (-7) or 5 (-5), or a complex karyotype (defined as 3 or more distinct cytogenetic aberrations) all have a poor prognosis (unfavorable risk) [73, 74, 76, 90, 95]. Mutations in the genes encoding the transcription factor CCAAT/enhancer binding protein alpha (CEBPA) and the FMS-like tyrosine kinase3 (FLT3) have also been associated with favorable and adverse prognosis, respectively [6, 38, 68, 100]. However, many patients exhibit cytogenetic and molecular indicators that do not belong to these two risk groups and are defined

Table 2. Recurrent chromosomal translocations in human AML [29]

Translocation	Fusion gene
A. Core binding factor (CBF)	
t(8;21)(q22;q22)	AML1/ETO
t(3;21)(q26;q22)	AML1/EVI1
inv(16)(p11;q22)	CBFβ/SMMHC
t(12;21)(p13;q22)	TEL/AML1
B. Retinoic acid receptor α (RARα)	
t(15;17)(q22;q11)	PML/RARα
t(5;17)(q31;q11)	NPM/RARα
t(11;17)(p13;q11)	PLZF/RARα
C. HOX genes	
t(7;11)(p15;p15)	NUP98/HOXA9
t(2;11)(q31;p15)	NUP98/HOXD13
t(12;13)(p13;q12)	TEL/CDX2
D. ETS genes	
t(12;22)(p13;q11)	MNI/TEL
t(16;21)(p11;q22)	TLS/ERG
E. Histone acetyl transferases/co-activators	
t(8;16)(p11;p13)	MOZ/CBP
t(11;16)(q23;p13)	MLL/CBP
t(11;22)(q23;q13)	MLL/p300
inv(8)	MOZ/TIF2
F. MLL	
t(1;11)(p32;q23)	AF1p/MLL
t(4;11)(q21;q23)	AF4/MLL
t(6;11)(q27;q23)	AF6/MLL
t(10;11)(p12;q23)	AF10/MLL
t(11;5)(q23;q31)	MLL/AF5
t(11;19)(q23;p13.1)	MLL/ELL
+11	

as intermediate risk [74, 76].
Most frequent target genes of translocations found in AML are transcription factors or other genes involved in transcriptional regulation (Table 2) [29, 73, 84]. Transcription factors involved in chromosomal translocations have in common that they are often conserved in evolution and crucial for normal hematopoiesis [29, 73]. In most cases, chromosomal translocation generates aberrant fusion proteins that have the potential to interfere with normal hematopoiesis [29, 73]. Some common abnormalities in AML and their characterization in mouse models will be discussed in more detail.

1.3 Mouse models for acute myeloid leukemia

To investigate the importance of the identified chromosomal aberrations for a particular human leukemia subtype, mouse models are being generated. The hematopoietic cells of the generated mouse models express the fusion protein or are deficient for the involved gene of interest. Using these mouse models, the role of the aberrant fusion proteins and/or inactivity of gene function in the onset of mouse leukemia can be investigated in vivo. The most relevant models for clinical AML will be introduced.

1.3.1 Translocations involving MLL

Translocations involving 11q23 occur in about 5% of AML patients and up to 10% of patients with acute lymphoid leukemia (ALL) [28]. Notably, 11q23 translocations occur in approximately 80% of AML and ALL patients under 1 year of age [106]. In more than 95% of cases, the gene at breakpoint 11q23 is the mixed-lineage leukemia gene (MLL, also called ALL-1, Htrx or HRX). Internal self-duplications of the MLL-gene involving exons 2 through exons 6 or 8 often associated with trisomy 11 are also found AML patients, indicating a major role for MLL in this disease [21, 109]. The MLL gene is approximately 110 kb and encodes an 11.7-kb transcript and a 431-kd protein containing three AT hooks and several domains involved in both transcriptional repression and activation. Currently, up to 40 different MLL translocations have been identified of which at least 19 fusions have been cloned and found to be in-frame. All MLL aberrations are clustered within a major breakpoint cluster region (BCR), which is a region of 8.3 kb including exons 5 through exon 11 [106, 124, 141]. The MLL part includes AT Hooks (DNA binding), and transcriptional repression domains (RD1 and RD2) [28]. Although only few of the MLL fusion partners share common domains, most of these genes are involved in either signaling or transcriptional regulation [3]. Some examples of MLL fusion partners are p300, CBP, eleven nineteen lysine-rich leukemia gene (ELL), eleven nineteen-leukemia gene (ENL) and ALL1 fused (AF) gene from chromosome 4, 9 and 10 (AF4, AF9, AF10). An important question is how MLL aberrations cause transformation of hematopoietic progenitor cells. MLL gene inactivation in mice showed that MLL is a pivotal regulator of hematopoiesis and crucial for embryonic development [49]. MLL is expressed in T, and B lymphocytes, and myeloid cells but not in erythroid cells [49]. MLL is crucial for normal hematopoietic progenitor

proliferation, survival and differentiation [49] and implicated in HOX gene regulation [28], suggesting that inactivation of MLL caused by translocation is the major oncogenic event. However, MLL null cells do not show an extended cell growth, which argues against this hypothesis [49]. Multiple studies have shown the relevance of MLL fusion partners in leukemogenesis. For example, MLL-ELL and MLL-ENL fusion proteins are capable to transform primitive myeloid progenitors and oncogenic capacities of these fusions are dependent on the transactivation domain of MLL fusion partners [31, 113]. In addition, knock-in mice, in which AF9 sequences are integrated in the MLL gene by homologues recombination corresponding to the human fusion, exhibit an early myelo-proliferative phenotype followed by a late acute myeloid leukemia [26]. Lastly, a mouse model that expresses the MLL-ENL fusion in the normal genomic context under endogenous transcriptional control shows myeloid leukemias with a rapid onset [36]. It is shown in Chapter 2 that the ELL gene is a target for common virus integration in Gr-1.4-induced myeloid leukemia. This finding strongly suggests that dysregulation of ELL itself, i.e., independent of the fusion partner MLL, can contribute to development of leukemia.

1.3.2 Translocations involving core binding factor (CBF)

The chromosomal aberration t(8;21) is found almost exclusively in AML FAB M2 classified patients (Table 1). In this translocation the 5' part of the AML1 (core binding factor (CBF)α, RUNX1) gene located on chromosome 21 is fused in-frame to almost the complete ETO gene [96, 106]. This fusion product contains the AML1 runt homology domain, which is a DNA binding domain, whereas ETO exchanges the normal AML1 transactivation domain [96]. The translocation by itself causes three potential "hits" caused by haploinsufficiency of AML1 and ETO and generation of abnormal AML1-ETO fusion protein.
The AML1 protein heterodimerizes with core binding factor β (CBFβ) and forms a transcriptional complex critical for activation of multiple genes involved in normal hematopoiesis, such as e.g., myeloproxidase, neutrophil elastase, IL-3, GM-CSF, CSF-1 and T-cell receptor chains [86, 96]. The phenotype of AML1 and CBFβ knock-out mice is an early embryonic death with characteristic haemorrhages in the central nervous system and lack of definitive hematopoiesis, demonstrating the importance of this transcriptional complex for normal development and blood cell formation [89, 129]. The AML/ETO fusion protein functions as a strong transcriptional repressor by interacting with nuclear hormone corepressor (N-Cor), mSin3A or mSin3B and histone deacetylases [48] and dominantly interferes with normal AML1 function [67]. AML1-ETO "knock-in" mice, in which AML1-ETO fusion is expressed from the endogenous AML1 promoter, reveal an identical phenotype as AML1 and CBFβ deficient mouse models. In addition, AML1-ETO inhibits auto-activation of C/EBPα expression and down regulates C/EBPα-dependent activation of myeloid promoters, thereby interfering with myeloid differentiation. Other fusion partners of AML1 are EVI1, EAP and MDS1. These proteins all contain the AML1 runt domain at the N-terminus, but differ at the C-terminal region and are thought to have the same dominant-negative inhibitory function on CBF function [142]. Although these results underscore the major role of AML1 disruption in leukemia, the fusion

partner may also influence the leukemic outcome. For example, AML-EVI1 fusion protein is able to transform Rat1 fibroblasts [64], acts as an inhibitor of TGF-β signaling by interfering with Smad3 activity and blocks TGFβ-induced growth inhibition of myeloid cells [63]. In various hematopoietic cell lines, including 32D, K562, MEL and U937, AML1-ETO induces a block in myeloid and erythroid differentiation [96]. However, conditional expression of AML1-ETO in mice is insufficient to induce leukemia by itself [50]. Treatment of these mice with chemical mutagen ENU induces malignant proliferation of myeloblasts, indicating contribution of AML1-ETO fusion protein in immortalization of myeloid progenitors [50]. It was recently found in primary hematopoietic cells that an activated TEL/PDGFβ receptor tyrosine kinase strongly cooperates with AML1-ETO in myeloid leukemia with a rapid disease onset and mortality [42]. These results suggest that receptor tyrosine kinases, including the frequently mutated FLT3 and c-Kit, might cooperate with AML1-ETO in leukemogenesis.
CBFβ is involved in translocation inv(16), often found in M4Eo leukemias, which fuses CBFβ to the smooth muscle myosin heavy chain (SMMHC) gene. Like AML1-ETO, the CBFβ-SMMHC fusion product is a dominant negative inhibitor of CBF. Notably, CBFβ/SMMHC knock in mice model show a similar phenotype as AML-ETO knock in, AML1 and CBFβ deficient models [23, 129], suggesting a high overlap in the transforming mechanisms of different CBF translocations due to disruption of CBF function.

1.3.3 Translocation involving RARα

Approximately 5-10% of AML cases are classified as FAB-M3. This type of leukemia is characterized by block in differentiation and uncontrolled accumulation at the promyelocytic stage and is therefore also referred to as acute promyelocytic leukemia (APL) (Table 1) [39]. APL is associated with chromosomal translocations involving the retinoic acid receptor alpha (RARα) gene located on chromosome 17q21 fused to distinct genes including promyelocytic leukemia (PML, 15q22), promyelocytic leukemia zinc finger (PLZF, 11q23), signal transducer and activator of transcription 5b (STAT5b, 17q11), nuclear mitotic apparatus protein (NUMA, 11q13) and nucleophosmin (NPM, 5q32) [39, 103, 140]. Except for the STAT5b translocation, these translocations give rise to two reciprocal fusion products [45].
Retinoids are vitamin-A derived hormones and activate two families of receptors, retinoic acid receptors (RARs), and retinoid X receptors (RXRs) [7]. These receptors belong to the steroid/thyroid hormone receptor super family and are all ligand induced transcriptional regulators [7]. The RAR family consists of three members RARα, RARβ and RARγ, which all bind to all-trans-retinoic acid and 9-cis-retinoic acid. The RXR family includes RXRα, RXRβ and RXRγ and binds to 9-cis-retinoic acid. RARs can form homodimers and heterodimers with RXRs and bind to specific DNA sequences, resulting in activation or repression of target genes [7]. RARα deficient mice die before the age of 2 months, suggesting roles for RARα in homeostatic processes [78]. These mice do not show compensatory increased levels of two related family members RARβ and RARγ [78]. However, antisense RARα transgenic mice have 30% to 80% reduced RARα protein levels in various tissues

but do demonstrate increased RARβ and RARγ levels [81]. More than 40% of homozygous transgenic mice developed different types of lymphomas with a latency of 2 to 18 months, indicating that RARα deregulation plays a role in oncogenesis [81].
The most common fusion partner of RARα is PML (98% of cases) and this protein is localized within nuclear body structures (PML bodies), functions as a growth suppressor and exhibit pro-apoptotic activities [140]. The fusion protein PML-RARα disrupts both PML and RARα dependent pathways [71, 97]. In contrast to wild-type RARα, the different fusion products act predominantly as transcriptional repressors through recruitment of nuclear co-repressor/HDAC complexes and DNA methyltransferases, resulting in a differentiation block at promyelocytic stage [71, 97, 140]. The function of ATRA is in part due to release of the co-repressor complex after binding of ATRA to the RARα part of the fusion, thereby reactivate normal gene expression [103, 104, 140]. Interestingly, transgenic mice expressing PML-RARα under control of human cathepsin G promoter show a myeloproliferative syndrome, but only 15%-20% of the mice develop an AML with a differentiation block at promyelocytic stage [43, 47]. Co-expression of the reciprocal fusion product RARα-PML resulted in an increased probability of APL development but did not shorten latency, suggesting that additional genetic lesions are still needed for transformation [98]. However, mice expressing the PML-RARα fusion under control of endogeneous murine cathepsin G (so-called knock-in mouse model) resulted in very low PML-RARα expression levels but high incidence of APL of more then 90% with latencies of 6-14 months [130]. This result indicates a crucial role for this fusion protein in APL [130]. However, the long latency suggests that additional leukemic events are required for full leukemia progression [130]. Several other mouse models for APL have been previously characterized, including e.g., PLZF-, NUMA-, and NPM-RARα [24, 45, 93, 99, 118]. The hCG-NUMA-RARα transgenic mice have a phenotype that is very similar to the disease in patients in whom a NUMA-RARα is identified [118], whereas NPM-RARα transgenic mice showed a spectrum of phenotypes from typical APL to CML [24]. The hCG-PLZF-RARα transgenic mice developed a CML-like leukemia rather than APL [46]. Strickingly, PLZF-RARα/RARα-PLZF double transgenic mice developed leukemia with classical APL features and are resistant to retinoic acid treatment as observed in human t(11;17) APL [45, 93].
PLZF protein has recently been implicated as a regulator of stem cell renewal [20, 27, 60]. Due to translocation, the ability of PLZF to regulate stem cell maintenance may be perturbed in t(11;17) APL. We found that the PLZF gene is a target for common virus integration in Gr-1.4-induced myeloid leukemia (Chapter 2). These findings suggest that perturbed PLZF expression, independent of the fusion partner RARα, can contribute to development of leukemia.

1.3.4 FLT3 mutations

The gene encoding FMS-like receptor tyrosine kinase (FLT3) is expressed in normal BM cells but exclusively in the early progenitors, including $CD34^+$ cells with high levels of c-kit [38]. This receptor belongs to the receptor tyrosine kinase (RTK) type III subfamily, which also incude c-kit, c-Fms and PDGF receptors based on the

common interrupted tyrosine kinase domain [38, 68]. Targeted disruption of the FLT3 gene in mice demonstrated a pivotal role for FLT3 in development of stem cells and B-cells [80]. FLT3 is highly expressed in the majority of AML patients including all FAB subtypes [38] and is the most frequent mutated gene in hematological malignancies [117]. Patients exhibiting mutations in the gene encoding FLT3 most often have a poor prognosis [38]. FLT3 activating mutations have been found in 1-3% of patients with ALL, 5-10% with myelodysplasia and in approximately 30% of AML patients [117]. The most common mutations in the FLT3 gene are internal tandem duplications (ITDs) in the juxtamembrane domain of the receptor [38, 68]. These ITDs are all in-frame and results in an elongated juxtamembrane domain. In addition, mutations in the FLT3 kinase domain at Asp835 have been found in approximately 7% of AML patients [1, 135]. FLT3 mutations are associated with constitutive activation of FLT3, resulting in enhanced phosphorylation of downstream targets such as STAT, AKT pathways and RAS/mitogen activated protein (MAP) kinases, which are involved in proliferation, differentiation and survival [38, 117].

FLT3 mutations cause growth factor independence and transformation of Ba/F3 and 32D cells [144]. In 32D cells, expression of mutated FLT3 blocks G-CSF-induced differentiation and suppresses expression of myeloid specific transcription factors through down modulation of C/EBPα [143, 144]. However, exogenous expression of FLT3/ITDs into mouse BM cells resulted in a myeloproliferative phenotype in-vivo but was insufficient to develop leukemia [59], indicating that additional cooperating events are necessary for full oncogenic transformation. Notably, FLT3 mutations are associated with for instance leukocytosis, high blast counts, t(15;17), t(6;9) and aberrations involving MLL [70, 117, 123]. In addition, PML/RARα and FLT3-ITD were shown to collaborate in APL-like disease induction with a short latency in mice [58]. FLT3 mutations are interesting targets for treatment of AML [116]. FLT3 specific drugs inhibit FLT3 activation but has minor effects on other tyrosine kinases in in vitro and in vivo models for FLT3-activated leukemia [19, 68]. Some compounds such as CEP701, PKC412 and SU5416 are in extensive clinical investigations [19, 68]. However, FLT3 inhibition as a single agent in AML treatment is unlikely to be effective and more research for optimal combination of FLT3 inhibitor and chemotherapy is required [68].

1.4 Retroviral insertional mutagenesis

Cloning of breakpoints at chromosomal translocations is a useful method to identify the transforming molecular mechanisms implicated in AML. However, it is now generally accepted that leukemogenesis is a multistep process, requiring defects in multiple genes. In support of this, mice harboring AML-derived fusion genes such as t(15;17), t(8;21) and inv(16) or mutations in FLT3 usually develop leukemia only after a long latency and with a low frequency, indicating that additional as yet undefined events are necessary to fully transform hematopoietic progenitors into AML cells [50, 56, 62, 130]. In addition, approximately 19% of AML patients lack any recognized (cyto) genetic abnormalities and the disease mechanisms in these patients are entirely unknown [73].

Retroviral insertional mutagenesis in mice has been used for identification of genes involved in different types of cancer such as several hematological neoplasms [57, 132], mammary carcinomas [87, 122, 128], and brain tumors [108]. The recently published mouse genome sequence and development of fast PCR and sequence protocols has made this strategy the most powerful tool to discover novel genes involved in acute myeloid leukemia.

Oncogenic retroviruses can be divided in two major categories namely, acute-transforming and slow-transforming retroviruses. The acute transforming retroviruses induce polyclonal tumors with a short latency of approximately 2 to 3 weeks after infection of the host and are able to transform cells in vitro [57]. The oncogenic activity of these viruses is dependent on the transforming sequences present in the viral genome. These defective viruses have exchanged viral sequences by recombination with cellular proto-oncogenes. Examples of acute transforming tumor viruses are avian myeloblastosis virus (AMV) containing the viral (v)-myb gene, derived from cellular (c)-myb [72], avian MC29 virus containing v-myc [2], and Abelson murine leukemia virus (A-MLV) expressing v-abl [111]. Slow-transforming retroviruses induce mono- or oligoclonal tumors in mice with latencies of at least 3 months and do not efficiently transform cells in vitro [57, 132]. These viruses do not contain proto-oncogenes but cause transformation as result of proviral integration in the host genome (Fig. 2), which is part of their normal life cycle. In this process, retroviral long terminal repeats (LTRs) influence normal gene expression pattern in a cell. This transforming process is called "retroviral insertional mutagenesis" [32, 57, 132] (Fig. 2).

Proto-oncogenes can be activated by a retrovirus due to promoter insertion (Fig. 2.1). In this situation, the provirus integrates in the promoter region or more upstream of a target gene and activates expression by its LTR promoter sequences [32, 57, 132]. As a consequence, the virus disrupts normal gene regulation, which often results in enhanced transcript levels of the target gene. Some well-documented examples are the ecotropic virus integration site 1 (Evi-1) [51, 52], Friend leukemia integration-1 (Fli-1) [9, 10, 125], c-myc [17, 33, 101, 110] and c-myb [132-134]. When the provirus integrates in the opposite orientation near a target gene, it may increase expression by the promoter enhancing activity of viral LTR sequences (Fig. 2.2). Virus integration in the 3' untranslated region (UTR) can increase gene expression by enhancing the stability of the mRNA due to premature termination and loss of instable sequences or rapid degradation mechanisms (Fig. 2.2) [57, 132].

Viral integration in a gene may also result in gene inactivation (Fig. 2.3) [32, 57, 132]. Examples of identified tumor suppressor genes are p53 [11, 14] and neurofibromatosis type 1 (Nf1) [66]. However, in some cases, intragenic integration results in expression of truncated protein with altered function. For instance, integration in the c-myb locus results in either carboxyl C- or amino N-terminal inactivating truncation [132, 133]. Another example is the Notch-1 gene, where virus sequences truncate the Notch-1 receptor, resulting in expression of a constitutive active form of the receptor [136].

Slow transforming retroviruses induce different types of cancer, including various hematological neoplasms [57, 132], mammary carcinomas [87, 122, 128], and brain tumors [108] when injected into different inbred strains of mice. To investigate the pathogenesis of different types of leukemia, multiple murine leukemia viruses (MLVs) are used in the laboratory such as CasBrE, Friend MLV and Moloney-MLV (Mo-MLV).

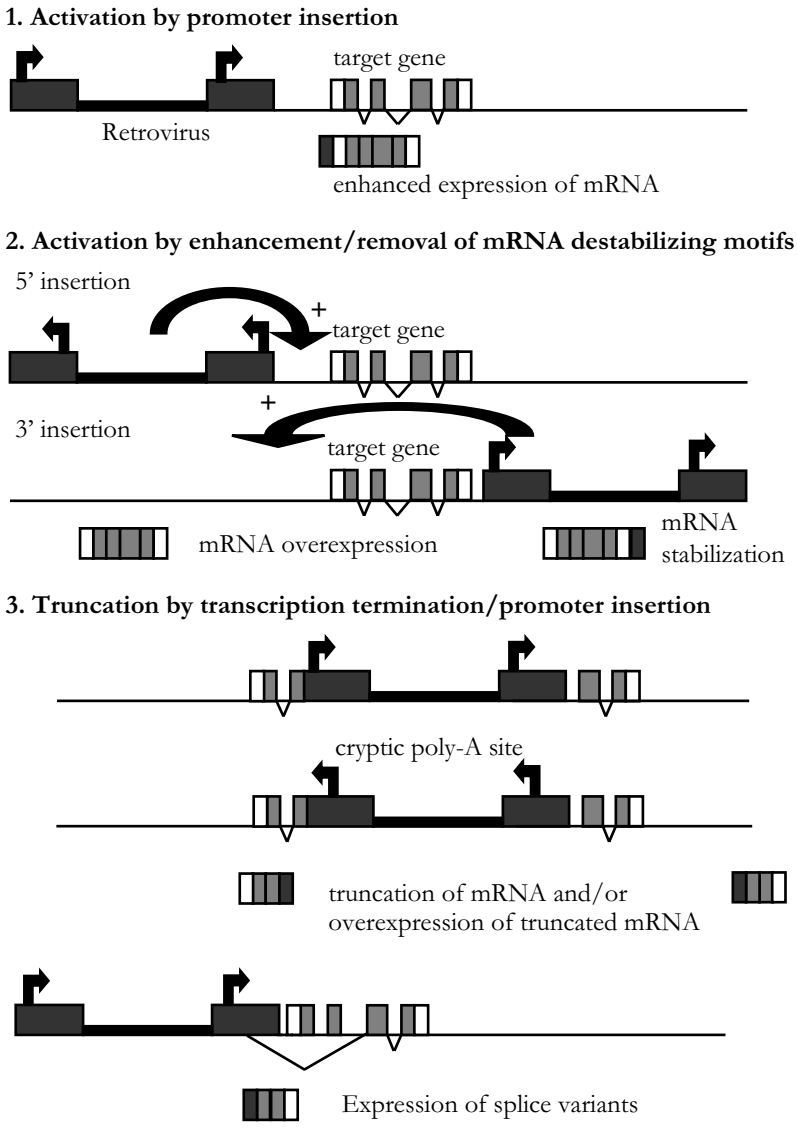

Figure 2. Mechanisms of retroviral insertional mutagenesis [57, 132]
The gray boxes represent protein-coding regions and untranslated regions are depicted as white boxes.
1. The virus causes enhanced mRNA expression by promoter insertion.
2. Enhancer sequences located in the LTR of the retrovirus increase expression of the wild type mRNA. Virus insertion in the 3' UTR may result in enhancement of mRNA expression by removing destabilizing sequences.
3. Viral insertion in a gene causes gene disruption, expression of transforming truncated protein or splice variants.

Table 3. Models of MLV induced disease [65]

Strain	Virus	Leukemia Phenotype	Incidence of AML %
LC wild mice, NIH Swiss	LC-MLV	Stem	30
C57BL/6	Rauscher-MLV	Granulocytic	70
ICR/Ha	Rich-MLV + Thymectomy	Myeloblasic	12
C3H	Gross-MLV + Thymectomy	Granulocytic	~20
NFS	CasBrM-MLV	Granulocytic	~25
DBA/2	Friend-MLV	Myeloblastic	66
C57BL/6	Friend-MLV	Granulocytic	20
BXH2	B ecotropic MLV	Myelomonocytic	100
NIH Swiss	Py101-M-MLV	Myelomonocytic	~50
BALB/c, NFS	Graffi-MLV	Myeloblastic	100
BALB/c	Moloney-MLV + Pristane	Promonocytic	100
NIH Swiss	SRS 19-6 MLV	Myeloblastic	25
NIH Swiss	CasBR-E MLV	Myeloid	~50

Different MLVs can be used to study distinct leukemia types (Table 2) [132]. For instance, CasBrE MLV infection of NIH Swiss mice results in ~50% myeloid leukemias [14-16, 65], whereas AKXD mice, which produce endogenous MLV, induces predominantly B- and T-cell leukemias (Table 3) [44, 65, 69, 85, 119].

Leukemogenicity of different MLVs is mainly dependent on the viral transcriptional control region (U3 region) located within the LTR [115]. At this site multiple nuclear factors can bind that influence virus replication and cell type specific transcription inducing activity in cells [4, 5, 94, 137-139]. For instance, the T Lymphoma-inducing reagents Mo-MLV, SL3-3, and MCF13 show the highest transcriptional activity in T-cells, whereas erythroleukemia-inducing Friend virus is more active in erythroid cells [57, 132].

When multiple insertions occur within a certain genomic locus of approximately 100 kb in independent tumors, these sites are referred to as common virus integration site (CIS) [54]. CIS-flanking genes are generally being considered as bonafide disease genes. However, as will be discussed in Chapter 2 and 3, genes located in virus integration sites thus far found only once are frequently also genes that are suspicious for a role in leukemia.

Retroviral insertional mutagenesis may also be used for identification of genes that cooperate in tumorgenesis with known genetic defects. In these screens, oncogene cooperation is accompanied by an earlier onset of disease in case of the mutant

mice when compared to wild-type littermates. Cooperating genes are identified for e.g., p27Kip1 [54] and Cdkn2a [79] tumor suppressor genes and for inv(16) translocation that creates the fusion oncogene CBFβ-MYH11 [22]. Insertional mutagenesis can even be used for identification of downstream targets, a parallel pathway or related disease genes of a certain proto-oncogene as shown for the kinase Pim1 [83].

1.5 Graffi-1.4 MLV

Studies described in this thesis are mainly focused on myeloid leukemias induced by Graffi-1.4 MLV (Gr-1.4). Gr-1.4 is an ecotropic and NB tropic retrovirus and induces mainly myeloid leukemias in multiple host strains such as FVB/N, BALB/c and NFS mice [35, 107]. This viral strain was purified from the Gr-virus complex, which consisted of three major viral clones Gr-1.2, Gr-1.4 and Gr-2.5 [102]. The main difference between the three Graffi virus clones is presences of a 60 bp repeat duplication in the U3 region of Gr-1.2 and Gr-2.5 LTR that is not present in the GV-1.4 [102]. Only Gr-1.2 and Gr-1.4 clones produced infectious retrovirus and induce myeloid leukemia in 100% of BALB/c and NFS mice. Interestingly, the latency of Gr-1.4 injected animals was much longer when compared to Gr-1.2 injected animals [102]. Mice from both panels started to die 120 days after injection. However, 50% of the Gr-1.2 and Gr-1.4 treated animals died after 155 days and 220 days respectively. Comparison of Gr-1.2 and Gr-1.4 LTRs shows difference of ~20% higher transcriptional activity of Gr-1.2 U3 region in various cell lines in a broad range of cell types [5]. These data indicate that the LTR U3 region is critical for transforming inducing potential of Gr-1.4. The Gr-1.4 U3 region contains a TATA and CAAT box and multiple cis acting regions that play a major role in viral cell specificity. Several transcription factors are shown to bind Gr-1.4 LTR sequences, such as members of the GATA family of transcription factors GATA1, GATA2 and GATA3 and core binding factor family members (CBFα, β) [4, 5]. In addition, Gr-1.4 LTR contains putative sites for Yin Yang 1 (YY1) and members of b-HLH family AFL-1 and AFL-2, but regulatory roles of these factors on Gr-1.4 LTR driven transcription are not demonstrated yet [5]. It was found that tumors induced by Gr-1.4 show relatively low percentage of tumors with rearrangements in known oncogenes such as c-myc, Fli-1 and Spi-1, indicating that other genes are involved in these myeloid leukemias [30].

1.6 Outline of this thesis

Leukemogenesis is a multistep process and various genetic defects are required for full oncogenic transformation of hematopoietic progenitors. Accordingly, mice harboring fusion genes derived from t(15;17), t(8;21) and inv(16) develop leukemia only after long latencies and with low frequencies [50, 56, 62, 130]. In addition, AML patients exhibiting chromosomal fusions frequently carry additional mutations in the receptor tyrosine kinases KIT or FLT3 [58, 59], but other acquired molecular mutations are likely to be present of which many still remain to be disclosed. Moreover, approximately 50% of AML patients do not present with known abnormalities [76].

The first aim of the work presented in this thesis was to identify novel genes involved in pathogenesis of AML. To this end, FVB/N mice were inoculated with the myeloid leukemia inducing virus Gr-1.4 MLV and a panel of myeloid leukemias was generated. In chapter 2, phenotypic characteristics of the obtained Gr-1.4 induced mouse leukemias, PCR methods used for identification of viral tagged sequences and the genes identified in this screen are presented.
The second aim was to extract critical disease genes involved in human leukemia out of the excessive data flow coming from retroviral mutagenesis screens. In this study expression profiling of AML patient samples [105, 126] and genes identified by retroviral tagging (chapter 2 and http://RTCGD.ncifcrf.gov) were combined. This analysis showed that genes close to or in VIS have a significantly increased probability to be differentially expressed in subclasses of adult AML compared to random chosen genes or genes more up- and downstream of VIS. The identified oncogenic networks and several lists of disease genes related to distinct clusters of AML patients are described in chapter 3.
The third aim was to investigate the function of some of the novel potential disease genes and related networks in mouse and human myeloid leukemia. Chapter 4 deals with investigations on the Yin-Yang 1 (YY1) gene. All VISs found in this locus were located in the YY1 promoter. YY1 promoter deregulation caused by viral insertions and the consequence of increased YY1 levels for proliferation, survival and myeloid differentiation of myeloid progenitor cells are described. Chapter 5 describes Gr-1.4 insertions found in the gene encoding WD40 and SOCS Box-2 (WSB-2). The function of this protein in signal transduction from the granulocyte colony-stimulated factor receptor was investigated. In chapter 6, virus integrations in the gene encoding thioredoxin interacting protein (Txnip) and their possible consequences for leukemia are described. Finally, the results described in these studies are more generally discussed in Chapter 7.

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CHAPTER 2

Large-scale identification of disease genes involved in AML

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Journal of Virology, 2004; 100:4154-4161

Abstract

Acute myeloid leukemia (AML) is a heterogeneous group of diseases in which chromosomal aberrations, small insertions or deletions, or point mutations in certain genes have profound consequences for prognosis. However, the majority of AML patients present without currently known genetic defects. Retroviral insertion mutagenesis in mice has become a powerful tool for identifying new disease genes involved in the pathogenesis of leukemia and lymphoma. Here we have used the Graffi-1.4 strain of murine leukemia virus, which causes predominantly AML, in a screen to identify novel genes involved in the pathogenesis of this disease. We report 79 candidate disease genes in common integration sites (CISs) and 15 genes whose family members previously were found to be affected in other studies. The majority of the identified sequences (60%) were not found in lymphomas and monocytic leukemias in previous screens, suggesting a specific involvement in AML. Although most of the virus integrations occurred in or near the 5' or 3' ends of the genes, suggesting deregulation of gene expression as a consequence of virus integration, 18 CISs were located exclusively within the genes, conceivably causing gene disruption.

Introduction

Acute myeloid leukemia (AML) is characterized by a block in myeloid differentiation that results in the accumulation of leukemic myeloid cells in the bone marrow and peripheral blood. For AML cases with specific chromosomal translocations, the identification and functional characterization of fusion genes located at translocation breakpoints have resulted in the discovery of pathways involved in leukemic transformation [34]. However, it also has become clear that these defects themselves are not sufficient to cause acute leukemia, supporting the theory that the disruption of multiple regulatory mechanisms is required to fully transform hematopoietic stem and progenitor cells toward AML. At present, cytogenetic parameters are used successfully in clinics for risk stratification of leukemia. For instance, AML cases with t(8;21), t(15;17), and inv(16) chromosomal abnormalities are classified as low risk, whereas cases with 3q26, 5q, and 7q abnormalities generally are classified as high risk, with unfavorable treatment outcomes resulting in early relapse and decreased overall survival [34, 47]. Importantly, for the majority of AML cases (±60%), tentatively classified as intermediate risk, the genetic parameters predictive for therapy outcome have not been identified [14, 15]. Furthermore, in the approximately 20 to 40% of AML patients without chromosomal abnormalities, the molecular pathogenesis remains entirely unknown [33].

Retroviral insertion mutagenesis in mice has become a powerful and rapid method for the identification of new genes involved in cancer [23]. This approach has benefitted greatly from both human and mouse genome programs and the recently developed genome database search programs [20, 29]. Studies aimed at finding novel genes involved in leukemia thus far have been carried out with virus strains that have a propensity to induce lymphoid malignancies or myelomonocytic tumors [21, 26, 32, 37, 44, 54]. To focus this strategy on myeloid leukemia, we have used the Graffi-1.4 (Gr-1.4)

virus, which has been demonstrated to induce leukemia with predominantly myeloid or mixed-lineage early hematopoietic phenotypes [9,48]. As with the Moloney and Cas-Br-M viruses used in previous studies, the Gr-1.4 virus does not contain oncogenic sequences but can deregulate or disrupt gene expression due to proviral integration. Recently, Erkeland SJ, et al., reported on a new Gr-1.4 virus common integration site (CIS) that is located in the Yin Yang 1 (YY1) promoter and that causes the deregulation of YY1 gene expression, resulting in defective myeloid differentiation [9]. Here we report 94 candidate leukemia disease genes that are targeted by the Gr-1.4 virus. While some of the affected genes in the Gr-1.4 virus-induced leukemia overlapped with those identified in lymphoma screens, suggesting a more general involvement in leukemogenesis and lymphogenesis, the majority of integrations appeared to affect novel genes that may be more specifically involved in the development of myeloid malignancies.

Material and methods

MuLV Gr-1.4-induced leukemia

Newborn mice were injected subcutaneously with 100 µl of cell culture supernatant from murine leukemia virus (MuLV) Gr-1.4-producing NIH 3T3 cells (a gift from E. Rassart, Department des Sciences Biologiques, Universite du Quebec a` Montreal, Montreal, Quebec, Canada). Mice were treated and analyzed for the development of leukemia as previously described [9]. Chromosomal DNA was isolated from leukemic cells for PCR-based screening [9].

Cytological analysis and immunophenotyping of leukemic cells

For morphological analysis, blood smears and cytospin preparations were fixed in methanol, stained with May-Grünwald-Giemsa stained, and examined by using an Axioscope microscope (Carl Zeiss BV, Weesp, The Netherlands). Single-cell suspensions of different organs were analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson and Co., Mountain View, Ca.). Cells were labeled as described previously [25] with the following rat monoclonal antibodies: ER-MP54, ER-MP58, M1/70 (Mac-1), F4/80, RB68C5 (GR-1), ER-MP21 (transferrin receptor), TER119 (glycophorin A), 59-AD2.2 (Thy-1), KT3 (CD3), RA3 6B2 (B220), and E13 161-7 (Sca1). Immunodetection was performed with goat anti-rat antibodies coupled to fluorescein isothiocyanate (Nordic, Tilburg, The Netherlands).

Inverse PCR of MuLV Gr-1.4-induced leukemia

The inverse PCR strategy was recently described in detail [9]. Briefly, genomic DNA from primary tumors was digested with HhaI (CGCG). After circularization by ligation (rapid ligation kit; Roche Diagnostics, Mannheim, Germany), an initial PCR was performed with MuLV Gr-1.4 (long terminal repeat [LTR])-specific primers L1 (5'-TGCAAGATGGCGTTACTGTAGCTAG-3') and L2 (5'-CCAGGTTGCCCC-AAAGACCTG-3'). Cycling conditions were 1 min at 94°C, 1 min at 62°C, and 3 min at 72°C for 30 cycles. For the second, nested PCR, primers L1N (5'-AGCCTTATGGTGGGGTCTTTC-3') and L2N (5'-AAAGACCTGAAACGACC-

TTGC-3') (15 cycles) were used. The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 10 pmol of each primer, and 2.5 U of Taq polymerase (Pharmacia, Uppsala, Sweden). The PCR fragments were analyzed on a 1% agarose gel.

Detection of virus integration by a specific nested PCR

To determine the localization of the Gr-1.4 provirus in specific virus-targeted genes in an extended panel of leukemias, a nested PCR was performed with DNA from primary tumors. For the first PCR, virus integration site locus-specific primers X1 and X2 were used in combination with MuLV Gr-1.4 LTR-specific primers L1 and L2 (Fig. 1). Cycling conditions were 1 min at 94°C, 1 min at 62°C, and 3 min at 72°C for 30 cycles. For the second PCR, nested virus integration site-specific primers X1N and X2N were used in combination with nested LTR-specific primers L1N and L2N under the same conditions (Fig. 1). The PCR products obtained were analyzed by Southern blotting. To verify the correct nature of the amplified bands, the blots were hybridized with radiolabeled gene-specific probes P1 and P2 (Fig. 1) at 45°C in Church buffer (0.5 M phosphate buffer [pH 7.2], 7% [wt/vol] sodium

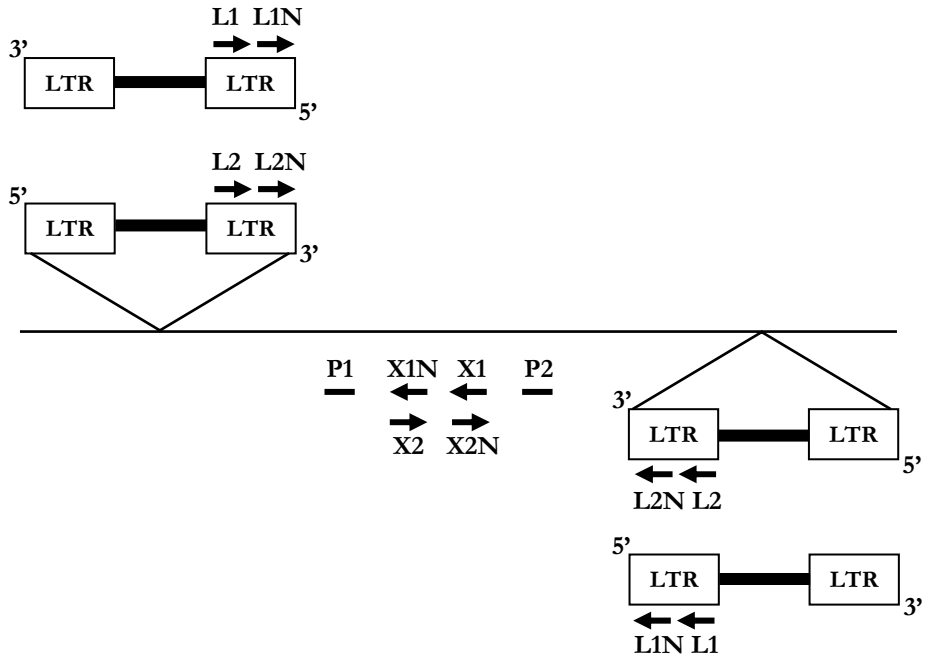

Figure 1. Directed PCR of chromosomal DNA to determine the commonality of virus integration site (VISs) identified by inverse PCR

Primers X1, X1N, X2 and X2N were designed in a locus that was identified as a virus integration by inverse PCR. To amplify flanking genomic sequences and to determine the localization and orientation of the integrated provirus, four different nested PCRs were performed. First, primers X1 and X2 were combined with Graffi-1.4 MuLV LTR primers L1 and L2. These products were amplified by a nested PCR, with primers X1N and X2N in combination with L1N and L2N. The specificity of the amplified bands was checked by Southern blot analysis with probes P1 and P2.

dodecyl sulfate, 10 mM EDTA) overnight. Signals were visualized by autoradiography according to standard procedures [34a].

Nucleotide sequence analysis

PCR products were sequenced by using an ABI 3100 sequencer (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) with MuLV Gr-1.4-specific forward primer L1. Virus flanking genomic sequences were analyzed by using GenBank (National Center for Biotechnology Information), a Celera discovery system (Celera Genomics, Rockville, Md.) [19, 29], and Ensembl (Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, United Kingdom) [20].

Results

Gr-1.4-induced leukemias

Eighty-nine newborn FVB/N mice were inoculated with Gr-1.4. When moribund, mice were sacrificed, and hematopoietic organs were isolated. Six mice died without signs of leukemia and were excluded from further investigation. Standard blood cell analysis was performed, and values were compared with the mean value for 10 normal FVB/N mice [24]. Most of the leukemic mice had increased numbers of peripheral white blood cells and decreased numbers of platelets and red blood cells compared to normal controls (data not shown). Blast percentages in the bone marrow ranged from 24 to 90%, with an average of 48%. Leukemic cells from 76 mice were immunophenotyped. The major immunophenotypic features of these leukemias are given in Table 1. Based on these criteria, the leukemias were classified

Table1. Gr-1.4-induced leukemias[a]

	Leukemia type	Immunophenotype	No. of leukemias
I	T-lymphoid Markers	MP21$^+$, CD3$^+$, Thy1$^+$	2
II	Mixed lymphoid, erythroid, myeloid differentiation markers	Gr1$^+$, F4/80$^+$, Mac1$^+$, Imm$^+$, CD3$^+$, B220$^+$, (*gcsfr*$^+$), (Ter119$^+$)	12
III	Myeloid differentiation Markers	Imm$^+$, MP21$^+$, (F4/80$^+$,Gr1$^+$, B220$^+$, Mac-1$^+$, *gcsfr*$^+$)	43
IV	myelo-monocytic blasts	Imm$^+$, Gr1$^-$, *gcsfr*, (F4/80$^+$),(Mac1$^+$), (B220$^+$)	15
V	Erythroid	Ter119$^+$, MP21$^+$ (Sca1$^+$)	4
Total			76

[a] All tumors were tested with the markers: MP21, MP58, CD3, Thy1, Gr1, F4/80, Mac1, Sca1, B220, Ter119 and gcsfr. Markers that were consistently negative are not shown, unless they were informative for discrimination between the myeloid subtypes III and IV. Imm$^+$ indicates positive staining for immature hematopoietic cell markers Sca1$^+$, MP58$^+$, MP54$^+$, (Thy1$^+$). Markers in parenthesis were not present in all individual tumors.

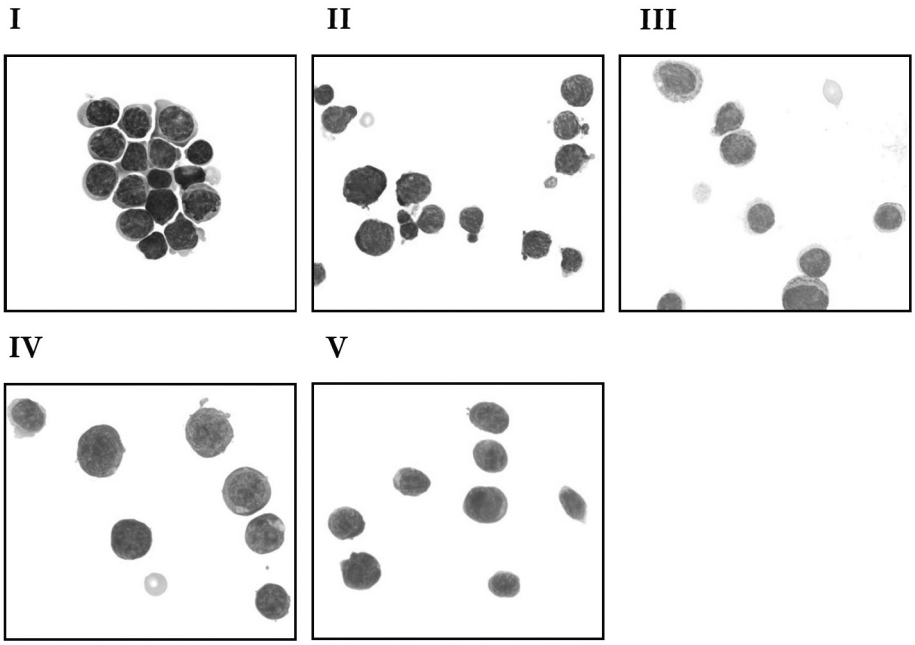

Figure 2. Morphological features of MuLV Gr-1.4-induced leukemia
Micrographs were taken with a Zeiss Axioscope microscope (magnification, 1.000). Morphological subtypes were lymphoblasts (I); mixed erythroblasts, myeloblasts, and lymphoblasts (II); myeloblasts (III); myelomonoblasts (IV); and erythroblasts (V).

as T-lymphoid; mixed lymphoid, erythroid, and myeloid; myeloid; myelomonocytic; or erythroid. Fifty-nine leukemias were analyzed morphologically. Representative examples are shown in Fig. 2 (and Suppl. Fig. 2). Almost all of the mice showed splenomegaly, with 25% showing thymus enlargement, 20% showing lymph node enlargement, and 55% showing liver involvement. In 5% of the mice, leukemic cells were present in the bone marrow and blood, without overt peripheral organ involvement.

Virus integration sites in Gr-1.4-induced leukemias

PCR analysis in conjunction with database searches identified a total of 94 different virus integration sites out of 69 tumors, 38 of which were found in earlier screens. Examples of this latter group of CISs are p53 [39, 41], Notch-1 [12, 13, 31], Evi-1 [38], NF1 (Evi-2) [5], Lck-1 [1, 11, 35], Pim-1 [50], HoxA9 (Evi-6) [43], Fli-1 [4, 8], and N-Myc [18, 51]. Notably, 79 of the 94 integrations were CISs, directly implicating the affected genes in leukemic transformation. The remaining 15 were included in this report because family members closely related to these genes were found in other studies. Fifty-six of the identified sequences were mapped near or in novel candidate leukemia genes. The products of the affected genes have been classified as receptors and signaling molecules (Table 2), as regulators of transcription (Table 3), and as having regulatory roles in other pathways (e.g., DNA stability and proteasomal targeting) or unknown functions (Table 4).

Table 2. Gr-1.4-MuLV integration sites in receptors and signaling-related molecules

Gene [a]	Virus integration site localization	Database [b]	Tumors [c]	type of leukemia [d]	Chromosome		Graffi unique CIS (x) or source [e]	Family member identified in other studies	unique database identifier
					Mouse	Human			
Il-2Rα	7 kb upstream of gene	C, E	1#	IV	2A1	10p15.1	RTCGD		NM_008367
Il-2Rβ	500 bp upstream of gene	C, E	1	III	15E2	22q13.1	RTCGD		NM_008368
Notch-1	exon12, intron14	C, E, N	2	II	2A3	9q34.3	RTCGD, [31]	Notch-2	NM_008714
Flt3	1.5 - 2 kb upstream of gene	E	2	III	5G2	13q12.3	RTCGD		NM_010229
Tie-1	6-15 kb downstream of gene	C, E	2	III, V	4D1	1p34.2	x		NM_011587
c-Mpl	0.25-10kb upstream of gene	C, E	2	III, V	4D1	1p34	x		NM_010823
Edg3 (G-protein coupled)	18 kb upstream of gene	C, E	7/12§	II, III, IV	13B1	9q22.1	x	Edg1(RTCGD)	NM_010101
Lck-1	intron 1 (upstream of ATG)	C, E	1	III	4D2.3	1p34.3	RTCGD		XM_110515
Pim-1	9 kb downstream of gene	C, E	1	VI	17B1	6p21.2	RTCGD,[50]	Pim-2, Pim-3	NM_008842
Akt-2	11-14 kb upstream of gene	C, E	4/13	II, IV	7A3	19q13.1	RTCGD	Akt-1	NM_007434
PTPN5	exon 1 (upstream of ATG)	C, E, N	1/24	III	7B3	11p15.1	x	PTPN18, PTPNS1(RTCGD)	NM_013643
PTPN1, (PTP1B)	intron 1(downstream of ATG)	C, E, N	9/13	II, III, IV	2H3	20q13.13	RTCGD	PTPN18, PTPNS1	NM_011201
DUSP10 (MKP-5)	1 kb upstream of gene	C, E	1	III	1H4	1q41	RTCGD	DUSP2	NM_022019
Inpp5b	0.3 kb upstream of gene	C, E	6/13	I, III, V	4D1	1p34.3	x		NM_008385
Inpp4a	intron 14	C, E, N	6/12	III, IV, V	1B	2q11.2	RTCGD		NM_030266
Dok-1(p62)	26-28 kb from gene	C, E, N	10/10	I, II, III	6D1	2p13	x	dok1-pending (RTCGD)	NM_001381
Phospholipase D3 (Pld3)	25 kb upstream of gene	E	5/13	II, III, IV	7A3	19q13.1	RTCGD	Plaa, 2310004B05Rik	NM_011116
Dapp-1	0.8 kb upstream of gene - intron 1 (downstream of ATG)	C, E	11/13	II, III, IV	3H2	4q24	RTCGD		NM_011932
Edaradd	17 kb downstream of gene	C, E	1	III	13A2	1q43	RTCGD		NM_133643
Semaphorin 4b (Sema4b)	3 kb upstream of gene	C, E	1	V	7D2	15q26.1	RTCGD	Sema7a	XM_133534
Insuline receptor substrate 2	2.5 kb downstream of gene	C, E	2	III, IV	8A2	13q34	RTCGD		XM_146235
NF1 (Evi-2)	intron 35	C, E	2	III	11B5	17q11.2	RTCGD,[5]		NM_010161
PKI-gamma	66 kb upstream of gene	C, E	1	IV	2H3	20q13.12	RTCGD		U97170
Tao-2	3-4 kb upstream of gene	C, E	5/13	II, III, IV	7F4	16p13.11	RTCGD		NM_004783
Shc3	41 kb downstream of gene	C, E	712	II, III, IV	13B1	9q22.1	x		NM_009167
Calcyphosine	intron 4 (downstream of ATG)	C, E	2/13	II	15A2	5p13.3	x		NM_029341

TNF -related protein 6	2.5 kb downstream of gene	C, E	1	III	15E2	22q13.1	RTCGD	many	AF329842
lymphotoxin beta (TNF-C)	8.3 kb upstream of gene	C, E	1	III	17B2	6p21.3	RTCGD	many	NM_008518
phosphodiesterase 1	intron 3, intron 4 (downstream of ATG)	C, E	6/13	I, II, III, IV	10A3	6q22.31	x	phosphodiesterase4 b (RTCGD)	NM_006208
Socs-2	0.75 kb upstream of gene - intron 1 (downstream of ATG)	C	8/39	II, III, IV, V	10C3	12q22	x	Socs-1, Socs-7, Asb-2 (RTCGD)	NM_003877
ARF related protein	1.5 kb upstream of gene, intron 1 (upstream of ATG)	C, E	8/13	II, III, IV	2H4	20q13.13	x	ARF6, ARF2 (RTCGD)	AF217796
RAP-1	18 kb upstream of gene	C, E	1	III	15F2	12q12	x	RAP1a (RTCGD)	AF103905

a IL-2Rα, interleukin 2 receptor alpha; PKI-γ, phosphokinase gamma; TNF, tumor necrosis factor; ARF, ADP-ribosylation factor.
b Sequences from inverse PCR were analyzed with Celera (C), Ensembl (E), and National Center for Biotechnology Information (N) BLAST search programs, and the exact integration site was localized.
c Number of tumors found positive for integration by inverse PCR only (out of 69 analyzed) # or by inverse PCR followed by directed PCR§ (positive out of total analyzed).
d As defined in Table 1 and Fig. 2.
e Source (reference) for other studies in which the Gr-1.4 virus integration site was previously found.

Table 3. MuLV Gr-1.4 integration sites in transcriptional regulators

Gene [a]	VIS localization	Database[b]	Tumors [c]	Type of leukemia [d]	Chromosome		Graffi unique CIS (x) or source [e]	Family member identified in other studies	unique database identifier
					Mouse	Human			
JunB	10 kb upstream of gene	C	14/14	I, II, III, IV	8C3	19p13.2	RTCGD		NM_008416
Fli-1	between exon 1a -1 (upstream of ATG)	C, E, N	7	III, V	9A5.2	11q24	RTCGD, [8]		NM_008026
p53	intron 1, 5, 6 exon 5, 6, 7, 1.4 kb upstream of gene	C, E, N	7	III, V	11B4	17p13.1	RTCGD, [39]		NM_011640
Evi-1	2-14 kb upstream of gene	C, E	2	III, IV	3A3	3q26	RTCGD, [38]		NM_007963
N-Myc	intron 1(upstream of ATG)	C, E, N	1	II	12A2	2p25.1	RTCGD, [51]	Pvt1, C-Myc RTCGD, [51]	NM_008709
COE (OLF-1/EBF)	14 kb upstream of gene	C, E	4/48	II, III, IV	11B1.1	5q34	RTCGD		AF208502
Elf-4 (MEF)	5.5 - 0.25 kb upstream of gene, intron 1 (downstream of ATG)	C, E	11/12	II, III, IV	XA3.2	Xq26.1	RTCGD		NM_019680
YY1	0.5-1.5 kb upstream of gene	E, N	14/20	I, III	12F2	14q32.2	RTCGD, [9]		NM_009537
Sax-1 (NKX-1.1)	0.3 kb upstream of gene, intron 1, exon 2 (upstream of and downstream of ATG)	C, E, N	1	III	7F4	10q26.12	x	NKX-1.2 (RTCGD)	NM_009123
NK-2.3 homeobox	6 kb upstream of gene	C, E	2	III, IV	19D1	10q24.32	x	NKX-1.2 (RTCGD)	AF155583
Evi-6 (Hoxa9)	2 kb upstream of gene	C, E	1	IV	6B3	7p15.2	RTCGD, [43]	Hoxa7, 11, Hoxb4 (RTCGD)	NM_010456
mPLZF	1.5 kb upstream of gene - intron 1 (upstream of ATG)	C, E	3/59	III	9B	11q23	x		NM_010823
ELL	exon 1, intron 1 (downstream of ATG)	C, E	11/15	II, III, IV	8C1	19p13.11	x		NM_007924
Cesanne	16 kb, 0.8 kb upstream of gene	C, E	2/13	III, IV	3F2	1q23.1	x		NM_020205
nuclear hormone receptor NR1D1	8-10 kb upstream of gene	C, E	11/12	II, III, IV	11D	17q21.1	x		BC008989
Retinoid X receptor alpha	84 kb upstream of exon 1, 2 kb upstream of gene, intron 1 (upstream of ATG)	C	3/12	II, III, IV	2A3	9q34.2	x		NM_011305
N-COR-1	intron 1(downstream of ATG)	E	3/13	III	11B2	17p11.2	x	N-cor-2 (RTCGD)	NM_011308
HDAC1	14 kb upstream of gene	C, E	1/13	III	4D2.3	1p34.3	x	HDAC5, HDAC9 (RTCGD)	NM_008228
HDAC7A	8 kb - 100 bp upstream of gene, intron 3, 4 (downstream of ATG)	C, E	3/13	II, III	15F2	12q12	x	HDAC5, HDAC9 (RTCGD)	NM_019572
Smarcc2	0.55 kb upstream of gene	C, E	1	III	10D3	12q13.3	x	Smarcf1 (RTCGD)	NM_003075

HMG-17	28 kb upstream of gene, 1-2 kb downstream of gene	C, E	3/13	II, III	8C1	19p13.11	x	HMG-a1, a2, b1, cr (RTCGD)	AL590390

a COE, Collier/OLF-1/EBF; EBF, early B-cell factor; MEF, myeloid elf1-like factor; mPLZF, mouse PLZF.
b See Table 2, footnote b
c See Table 2, footnote c
d See Table 2, footnote d
e See Table 2, footnote e

Table 4. Other MuLV Gr-1.4 integration sites

Gene	VIS localization	Database[a]	tumors[b]	Type of leukemia	mouse chr.	human chr.	Graffi unique CIS (x) Reference [d]	identified family member [e]	unique database identifier
protein-arginine deiminase type- 1 (Pdi-1, PADI1)	0.5 kb upstream of gene, intron 1 (upstream of and downstream of ATG)	C, E	5/13	II, III, IV	4E1	1p36.1	RTCGD	Pdi-2 (RTCGD)	NM_011059
Myosin-IC	intron 1 (upstream of ATG)	C, E	12/39	II, III, IV	9D	15q22.2	RTCGD	Myosin-IB, IIIA (RTCGD)	XM_207764
synaptic vesicle glycoprotein 2A (Sv2A)	27 kb upstream of gene	E	1	III	3F2	1q23.1	RTCGD		NM_022030
Swap70	intron 3 (downstream of ATG)	C, E	1	IV	8C3	19p13.2	RTCGD		NM_009302
PAFAH1B2	11 kb downstream of gene	E, N	1	III	9B	11q23.3	RTCGD		NM_008775
netrin-1	intron 1 (downstream of ATG)	C, E	1	N.D.	11B3	17p11.2	x		XM_193630
multispanning membrane protein (TM9SF2)	1 kb upstream of gene	C, E	1	III	14E5	13q32.3	RTCGD		NM_080556
Histone H3.3A (H3F3A)	exon 1 (downstream of ATG)	C, N	1	IV	1H4	1q41	x	H1b, H1b, H1c, H2 (RTCGD)	Z85979
gene trap locus-13 (GTL1-13)	17 kb upstream of gene	C, E	2	III	2E1	11q12.1	x		NM_021512
tumor differentially expressed Tde1 (AF181684)	15 kb upstream of gene	C, E	1	IV	2H3	20q13.12	RTCGD		NM_012032
O-methyl transferase	4.5-25 kb upstream of gene	E	2	III	14B	10q22.2	x		AK007659
CHD2	17.3 kb - 18.8 kb upstream of gene	C, E	11/13	I, II, III, IV	7D1	15q26.1	x		NM_001271
Blm	3 kb- 0.8 kb upstream of gene	C, E	4/30	II	7D2	15q26.1	x		NM_007550
VDUP-1	1.6 kb upstream of gene - intron 1 (downstream of ATG) and exon 8 (downstream of stop) - 3 kb downstream of gene	C, E, N	14/14	I, III, IV	3F2	1q21	x		AF282826
PrdxII	2.4 kb upstream of gene - intron 1 (upstream of ATG)	C, E	14/14	I, II, III, IV	8C3	19p13.2	x		NM_011563
lymphocyte antigen (Y-6M, LY-6A.2/LY-6E.1, TAP)	6 kb downstream of gene	C, E	8/39	II, III, IV, V	10C3	12q22	x	lymphocyte antigen 108, 74, 6E (RTCGD)	NM_010738
Periaxin	57 kb upstream of gene	E	5/13	II, III, IV	7A3	19q13.1	x		AJ222968
ribonuclease H1 subunit	3 kb upstream of gene	C, E	14/14	I, II, III, IV	8C3	19p13.2	x		AK010292
Apoptosis inhibitory 5 (Api-5, fibroblast growth factor 2-interacting factor 2)	6 kb downstream of gene, intron 1 (downstream of ATG)	C, E	5/25	I, III	2E1	11p11.2	x		NM_007466

hematopoietic progenitor (CDCA4)	intron 1 (upstream of ATG)	C, E	2/13	IV	14E5	13q23.3	x		AK010535
U1 SNRNP binding protein	1 kb upstream of gene	C, E	2	II, III	5F	?	x		AK018232
unnamed	intron 1 (downstream of ATG)	C, E	3/13	II, III	15F2	12q12	x		AK002729
unnamed	1.5 kb downstream of gene	C, E	5/13	II, III, IV	7F4	16p13.11	x		AK004043
unnamed	0.1 - 1.5 kb upstream of exon 1	C, E	9/12	II, III, IV	8C1	19p13.11	x		AK013135
unnamed	intron 1(downstream of ATG)	E	5/25	I, III	2E1	11p11.2	x		AK001752
Lim domain protein Ril	intron 1 (downstream of ATG)	C, E	2/13	I, II	11B1.3	5q31.1	x		NM_019417
D6MM5e	intron 4 (downstream of ATG)	C, E, N	10/10	I, II, III	6D1	2p13	x		AF084364
Psmb-1	1 kb upstream of gene	C, E	1	N.D.	17A2	6q27	x	Psmb-8 (RTCGD)	NM_011185
ubiquitin	13 kb upstream of gene	C, E	9/12	II, III, IV	8C1	19p13.11	x	many (RTCGD)	NM_019639
Psme-1	0.34 kb upstream of gene	C, E	1	IV	14C1	14q11.2	x	Psmb-8 (RTCGD)	AB007136
Ddx21	2 kb downstream of gene	C, E	1	IV	10B4	10q22.1	x	Ddx-3, 6, 26 (RTCGD)	NM_019553
Actin-like	0.2 kb upstream of gene	C, E	1	III	10C2	12q23.1	x	actin-beta (RTCGD)	AK008409
Alpha-1 catenin	83 kb upstream of gene	C, E	1	III	18B3	5q31.2			NM_009818
VDAC-2	7.5 kb downstream of gene	C	1	III	14B	10q22.2	x	VDAC-1 (RTCGD)	NM_011695
Plau	5-7 kb upstream of gene	C, E, N	2	II, IV	14B	10q22.2	x		NM_008873
Proteoglycan, secretory granule (PRG1)	1.1 kb upstream of gene	C, E	1	I	10B4	10q22.1	x	PRG4 (RTCGD)	NM_011157
potasium channel Kcnk5	intron 1 (downstream of ATG)	C, E	1	II	14A3-B	6p21.2	RTCGD		NM_021542
FK506BP8	6.5 kb -0.2 kb upstream of gene	C, E	9/12	II, III, IV	8C1	19p13.11	x	FK506BP3, 10 (RTCGD)	NM_010223
MCT-related	10 kb upstream of gene	C, E	1	III	11B4	17p13.2	x	MCT4 (RTCGD)	AB041591
RNA binding motif 5	2.2 kb - 3 kb upstream of gene	E, N	3/11	1, III	9F2	3p21.31	x		AJ309168
RNA binding motif 6	3 kb - 4.8 kb downstream of gene	E, N	3/11	I, III	9F2	3p21.31	x		NM_011251

a TAP, T-cell-activating protein; MCT, monocarboxylate transporter.
b See Table 2, footnote b
c See Table 2, footnote c
d See Table 2, footnote d
e See Table 2, footnote e

Approximately 15% of the virus integrations identified by this inverse PCR-based screen were determined to be common (more than two integrations in a particular genomic locus) by the initial screen. To determine the frequency of common integrations more sensitively, we performed integration-specific PCRs with 49 Gr-1.4-targeted genes in multiple tumors (Tables 2 to 4); most appeared to be common. We sequenced the integrations in 19 genes derived from multiple (2 to 10) tumors; these included VDUP1, PrdxII, 11- to 19-lysine-rich leukemia (ELL), promyelocytic leukemia zinc finger (PLZF), and Edg3. In all instances, the integrations were located in the expected gene but at different positions, confirming the specificity of the Southern analysis and ruling out the possibility of cross-contamination by previously amplified PCR products. The genes flanking the virus integration sites were compared with data from the National Cancer Institute retrovirus-tagged cancer gene database (RTCGD) at http://genome2.ncifcrf.gov and other sources (Tables 2 to 4). The Gr-1.4 integrations that are included in this database or that were reported in other studies are indicated by the database name or references in Tables 2 to 4. Family members of Gr-1.4-targeted genes found in other studies are also indicated in those tables. Most of the virus integrations occurred in or near the 5' or 3' ends of the genes, suggesting that the levels of expression of these genes are deregulated as a consequence of virus integration. Eighteen CISs were located exclusively within the gene, conceivably causing gene disruption. This group comprises the previously reported virus targets Notch-1, NF1, PTPN1, Inpp4a, p53, SWAP70, and Kcnk5 and 11 new virus targets: calcyphosine, phosphodiesterase-1, ELL, NCOR-1, HDAC-7A, histone H3.3A, Api-5, Ril, D6Mm5e, and two unknown genes.

Discussion

In this study, we have used in vivo retroviral mutagenesis with the Gr-1.4 virus complex to identify novel disease genes specifically involved in the pathogenesis of AML. In comparable studies with other virus strains, e.g., Moloney, AKXD, or Cas-Br-M, the most prominently appearing tumor types are T and B-cell lymphomas; in the case of BXH2, myelomonocytic tumors are prominent [21, 30, 32, 37, 54]. In contrast, more than 80% of the leukemias induced by Gr-1.4 unequivocally exhibited immunophenotypic characteristics of myeloid cells, with immature morphological features (mainly myeloblasts), emphasizing the unique features of the Gr-1.4 virus complex as a tool for characterizing pathogenetic mechanisms in myeloid disease. Indeed, the majority of the CISs described here have thus far not been reported in extensive screens in lymphoma models, although some overlap was observed. The latter observation is not surprising in view of the fact that cell type specific events usually impinge on downstream common regulatory pathways that can be affected in multiple tumor types.

Although most of the Gr-1.4 CISs have been linked to candidate disease genes based on their proximity to these genes, proviral integrations have been reported to influence gene expression over distances of more than 100 kb. Thus, we cannot exclude the possibilities that genes located more distantly from the CISs also are deregulated and that multiple genes are affected by a single CIS [44]. In addition, three

other aspects of retroviral screens as they are currently being performed must be emphasized. First, malignancies induced by replication competent retroviruses are usually oligo- or polyclonal rather than monoclonal [9, 32]. Although this characteristic gives rise to high frequencies of CISs in relatively small cohorts of mice, it complicates the search for cooperating events within one leukemic clone. Second, the sensitive PCR-based techniques used to identify virus integrations do not allow distinction between CISs present in a majority of the leukemic cells, initiating an early pathogenic event, and CISs present in only a minor population of the cells, probably affecting leukemia progression genes. Finally, a recent study emphasized that MuLV strains have a preference for integration near transcriptional start sites [62]. Our data obtained with Gr-1.4 corroborate these conclusions and indicate that retroviral mutagenesis with MuLV preferentially, although not exclusively, identifies gene deregulation rather than gene disruption. Another important conclusion drawn by Wu and colleagues is that there appear to be no integration hot spots (preferred integration sites in certain loci) for MuLV [62]. Therefore, CISs found in viral screens are likely to play a role in leukemia.

Many of the Gr-1.4-affected genes appeared to be related to signaling; some have been linked to human leukemia already. For instance, the Tie-1 gene, which encodes a tyrosine kinase receptor that is normally expressed in vascular endothelial cells and hematopoietic stem cells, is overexpressed in chronic myeloid leukemia [59]. Importantly, high Tie-1 levels inversely correlate with the survival of chronic myeloid leukemia patients in the early chronic phase [59]. Markedly increased levels of Tie-1 also were detected in bone marrow samples from myelodysplastic syndrome and AML patients [58]. Notch-1 is another example of a CIS associated with human disease. The human homologue of the Notch (Tan-1) gene is involved in chromosomal translocation t(7;9)(q34;34.3), which results in a truncated receptor in human T-lymphoblastic neoplasms. Notch-1 is a proviral integration site in mouse lymphoid leukemias [12, 31]. Our data suggest that aberrant Notch signaling also may be involved in myeloid leukemia. Notably, the integrations in the Notch gene result in the constitutive formation of the truncated active form of Notch [31], which has been demonstrated to interfere with granulocyte colony-stimulating factor-induced myeloid differentiation in the 32D cell model [55]. Edg3 (endothelial differentiation gene 3) is a G-protein-coupled receptor (GPCR) involved in cell proliferation and survival [2]. The expression of Edg3 is downregulated during the differentiation of HL60 human leukemia cells and is assumed to mediate sphingosine 1-phosphate-induced Ca^{2+} responses [49]. Although the role of Edg receptors in normal hematopoiesis and malignancies is not yet clear, a closely related GPCR, cannabinoid receptor type 2, was found in a CIS in Cas-Br-M-induced leukemia [57]. Notably, perturbed expression of cannabinoid receptor type 2 recently was found to interfere with myeloid differentiation, supporting the notion that this type of GPCR may contribute to the pathogenesis of AML by inducing a maturation block [27].

Both protein and lipid phosphatases are crucial, mostly negative, regulators in growth factor signaling pathways [7, 64]. Two members of the protein tyrosine phosphatase nonreceptor (PTPN) family affected by Gr-1.4 are PTPN1 and PTPN5. The virus integrations in the PTPN1 gene are localized in intron 1 and conceivably disrupt its function. The PTPN1 gene was found in a CIS in BXH2-

induced leukemia [32]. Because TYK2, JAK2, and STAT5-JAK/STAT signaling intermediates involved in growth control by hematopoietic growth factors are all inactivated by PTPN1 (also known as PTP1B) [3, 16, 42], the disruption of PTPN1 is predicted to contribute to uncontrolled proliferation and survival of leukemic cells. Integrations in the PTPN5 gene occur in exon 1, upstream of the ATG translation start site. At present, little is known regarding the signaling function of PTPN5, which is alternatively termed STEP. Interestingly, recent data suggest that PTPN5/STEP shows a preferential affinity for p38 mitogen-activated protein kinase in a redox-sensitive manner, leaving the Erk kinases largely unaffected [40]. Inhibition of p38 mitogen-activated protein kinase activity by pharmacological inhibitors has been shown to interfere with terminal neutrophilic differentiation (G. J. van de Geijn et al., unpublished data) and to prevent apoptosis in neutrophils [10]. Conceivably, dephosphorylation of p38 by PTPN5/STEP may have similar effects and may promote the survival of immature myeloid cells. Other phosphatases that are targeted by Gr-1.4 are the inositol polyphosphate phosphatases Inpp4a and Inpp5b, which catalyze the hydrolysis of phosphatidylinositol polyphosphates at positions 4 and 5, respectively. Intriguingly, Inpp4a is a major target for transcription factor GATA-1 and inhibits the growth of megakaryocytes and NIH 3T3 fibroblasts [60]. Inpp5b is required for normal sperm development and function [17], but how this enzyme affects normal or leukemic blood cell development is not known. Reactive oxidant species (ROS) are generated by multiple cellular mechanisms, including metabolic processes, phagocytic reponses to pathogens (oxidative burst), and membrane receptor-mediated signaling. The tight regulation of ROS concentrations in the cell is controlled by both catabolic and redox mechanisms and is important for signal transduction, as it influences protein kinase and phosphatase reactions as well as the activities of transcription factors [46]. When ROS levels in a cell are too high, the resulting oxidative stress will affect normal protein function and genomic stability [46]. Genes involved in the redox-controlled regulation of ROS levels that are commonly targeted by Gr-1.4 are those for vitamin D3-upregulated protein 1 (VDUP1) and peroxiredoxin 2 (PrdxII). VDUP1 interacts with thioredoxin (TRX) and thereby counteracts the ameliorating effects of TRX on stress-induced apoptosis via apoptosis signal-regulating kinase 1 (ASK-1) and the function of TRX as an antioxidant [28]. The consequence of upregulation of PrdxII is as yet unclear, but it may interfere with the function of peroxiredoxin complexes and therefore inhibit the antistress activities of TRX [6]. Importantly, PrdxII is a TRX reductase and has been found to be overexpressed in human breast cancer [45].

The ELL and PLZF genes are two examples of genes that are part of fusion genes in human AML as a result of chromosomal translocations. In translocation t(11;19)(q23;p13.1), the ELL gene is fused to the trithorax-like mixed lineage leukemia (MLL) gene [56]. The ELL protein is an RNA polymerase II elongation factor [53]. Recently, it was demonstrated that both MLL-ELL and ELL inhibit p53 activity via binding of the C-terminal part of ELL to the transactivating domain of p53 [61]. The PLZF gene, involved in translocation t(11;17)(q23;q21), is fused in frame with RARα [22, 36]. PLZF is expressed in hematopoietic stem cells and normally is downregulated during differentiation. The overexpression of PLZF plays a role in the maintenance of the immature state of hematopoietic progenitor cells and promotes cell survival [52, 63]. The fact that PLZF is found as a common Gr-1.4-

targeted gene indicates that the deregulation of PLZF expression can contribute to leukemic development independent of its fusion to RARα. One of the major features of PLZF is its tight binding to transcriptional corepressors. Interestingly, several genes involved in transcriptional repression by chromatin modification, such as those for N-Cor-1 and histone deacetylases HDAC1 and HDAC7, are affected by Gr-1.4. Notably, family members of these proteins (N-Cor-2 and HDAC5) also were found in previous screens [54].

In conclusion, using high-throughput retroviral screens with the Gr-1.4 virus complex, we have identified novel pathways involved in myeloid leukemia. Currently, we are studying the consequences of aberrant gene expression or function by using gene transfer methodology with 32D cells and primary bone marrow cultures [9]. In addition, we are analyzing the significance of these pathways for human AML by screening expression levels in a panel of clinical samples with a gene expression array and quantitative reverse transcription-PCR technology. By following this combinatorial approach, we hope to develop new prognostic indicators that will discriminate further between low and high responses to therapy in different risk groups. Furthermore, the results obtained may open new avenues for developing specific and sensitive therapies based on the heterogeneous pathogenetic features of human AML.

Acknowledgments

We thank E. Rassart for providing the cell line that produced MuLV Gr-1.4. This work was supported by grants from the Dutch Cancer Society Koningin Wilhelminafonds.

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CHAPTER 3

Pathogenetic signatures of human acute leukemia unveiled by retrovirus-tagged cancer genes

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Submitted

Abstract

Acute myeloid leukemia (AML) is a heterogeneous disease with a variable response to treatment. Recurrent cytogenetic defects, detectable in approximately 50% of patients, are associated with AML subtype and prognosis [20]. Acquired mutations in regulatory genes, of which the majority remains to be disclosed, also largely determine the course of the disease. Gene expression profiling has been applied to further risk-stratify AML [3], but these studies did not reveal which genes are critical for the disease process [24, 28]. Here we show that mouse leukemia genes identified by provirus tagging are differentially expressed in prognostic subgroups of adult and pediatric AML. Genes directly flanking a virus integration site (VIS) but not more distantly located genes correlated significantly with gene sets responsible for classification, suggesting an involvement of these genes in the pathogenesis of human AML. VIS-flanking genes could be positioned in five regulatory networks by web-delivered pathway analysis. These results demonstrate the power of combining retroviral tagging of mouse cancer genes with gene expression profiling of patient samples to identify disease genes and to discover novel pathogenetic signatures of human AML.

Introduction

Human AML can be classified into distinct categories of variable prognostic risk on the basis of cytogenetic and molecular abnormalities. For instance, the chromosome translocations t(8;21), t(15;17) and inv(16), resulting in fusion proteins with altered transcription regulatory activities, are associated with a relatively favorable prognosis, whereas 3q26, 5q, 7q and 11q23 abnormalities correlate with unfavorable treatment outcomes, i.e., enhanced relapse rates and decreased overall survival [20]. Mutations in the genes encoding the transcription factor CCAAT/enhancer binding protein alpha (CEBPA) and the FMS-like tyrosine kinase3 (FLT3) have also been associated with favorable and adverse prognosis, respectively [1, 8]. AML cases with known (cyto-) genetic abnormalities make up for approximately half of the patients. Leukemogenesis in mouse models is a multistep process, requiring multiple genetic defects. Accordingly, mice harboring fusion genes derived from t(15;17), t(8;21) and inv(16) develop leukemia only after a long latency and with a low frequency, because additional events are required to fully transform hematopoietic progenitors into AML cells [9, 12, 15, 17, 30]. This likely also applies to AML patients, which in addition to chromosomal fusions, frequently carry mutations in, e.g., KIT or FLT3 [5]. A now widely held view is that perturbations leading to AML affect combinations of genes that control hematopoietic stem cell proliferation/renewal and myeloid differentiation [5]. Gene expression profiling has further highlighted the heterogeneous nature of human AML and has led to the identification of leukemia subsets based on distinctive gene expression signatures [3, 24, 28]. However, although the majority of genes involved in the pathogenesis and pathobiology of AML (disease genes) will be present in these signatures, there will be no distinction from genes merely associated with the variable maturation status of the leukemic cells (marker genes).

Retroviral insertion mutagenesis with murine leukemia virus (MuLV) is a powerful strategy to discover genes involved in leukemogenesis in the mouse. We have recently used this approach with Graffi-1.4 MuLV (Gr-1.4) to identify disease genes involved in myeloid leukemia [6]. Retroviral insertion can deregulate or disrupt gene expression [13]. Only when certain combinations of genes are affected, an infected cell will convert into a tumor cell and adopt a selective growth advantage [13]. Genes located near virus integration sites (VIS genes) can be rapidly identified using inverse PCR or splinkerette-based methods, combined with high through-put sequencing [7, 14, 19, 22, 26] and genome-wide BLAST searches [10, 16]. This results in still increasing lists of potential cancer genes (http://genome2.ncifcrf.gov/RTCGD) [7, 14, 19, 21, 22, 26], but only few of these genes have thus far been linked to human cancer. VIS genes found in multiple independent tumors, known as common VIS or CIS genes, are very likely disease genes. However, VIS genes not yet found common often also belong to gene classes associated with cancer [14, 19, 23, 26]. Conceivably, genes located more distantly from a virus integration may also be deregulated and qualify as proto-oncogenes [23].

Methods

Expression array analysis

Data were normalized by the global scaling method provided in the Affymetrix Microarray Suite, version 5.0 (MAS5.0) using a target average intensity value of 100 for the adult AML dataset [28] and a target average intensity value of 500 for the pediatric AML dataset [24]. Because these methods reliably identify signals with average intensity values of 30 and 100 and higher, minimum thresholds were set at those values for the adult and pediatric AML data sets, respectively. The level of expression of each probe set in every sample was calculated relative to the geometric mean and logarithmically transformed (base 2) to ascribe equal weight to gene-expression levels with equal relative distance to the geometric mean. Testing for differential expression between classes of patients was performed for all probe sets on the Affymetrix HGU133A GeneChip (n=22283) using the SAM method [27]. Patients from a specific class are tested compared to all remaining samples using a modified t-test statistic using sample-class permutations to assess statistical significance. Probe sets were considered to be differentially expressed when Fold Change was over 1.5 or under 0.67, score was over 4 or less than 4 and a q-value less than 5%, where False Discovery Rate was less than 5%.

Chi-square Pearson correlation analysis

Of all selected probe sets, a fraction is differentially expressed while remaining probe sets are not. A ratio was calculated for these two numbers. Similarly, a ratio was calculated for unselected probe sets. Chi-square Pearson correlation analysis was performed to assess the statistical significance for the difference between these ratios.

Pathway analysis

Ingenuity Pathway Analysis is a web-delivered application that enables discovery,

visualization and exploration of networks significant to experimental results, such as gene expression array data sets (http://www.ingenuity.com). Each gene identifier on the list was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB). The mapped genes, called Focus Genes, were then used as the starting point for generation of biological networks. To start building networks, the application queries the IPKB for interactions between Focus Genes and all other gene objects stored in the database and generates networks with a network size of 35 genes/proteins. A significance score, derived from a p-value indicating the likelihood of a network being found together due to random chance, was computed for each network according to the fit of the set of genes. A score exceeding 2 indicates that the network is not generated by random chance with at least 99% confidence. Biological functions, assigned by using the findings extracted from scientific literature and stored in the IPKB, are then calculated and assigned to each network. A Fisher's exact test is used to calculate a p-value determining the probability that the biological function assigned to that network is explained by chance alone.

Results and discussion

To assess the relevance of Gr-1.4 VIS and CIS genes for human AML, we determined their expression in different classes of adult and pediatric AML patients [24, 28]. Based on gene expression profiling, 285 adult AML cases were grouped into 16 distinct clusters of patients by unsupervised cluster analysis [28] (data available at http://www.ncbi.nlm.nih.gov/geo under accession number GSE1159). Using significance analysis of microarrays (SAM), which tests a class of AML patient samples for significantly differentially expressed genes compared to a second class of AML patient samples, we could link specific gene sets to these patient groups, by comparing each class to the remaining samples. In total, 5193 probe sets, representing 3644 genes, contributed to the signature of the 16 classes of adult AML (Suppl. Table 1a). The probability that a randomly selected gene is differentially expressed in one or more subclasses of the adult AML dataset is 0.28 (Table 1).

We performed Chi-square Pearson correlation analysis to test whether VIS and CIS genes have a higher probability to be differentially expressed in one of the AML subclasses. Four gene lists derived from the Gr-1.4-induced leukemia model and represented on the Affymetrix HGU133A GeneChip were analyzed: (I) VIS + CIS genes (n=115, represented by 234 probe sets), (II) CIS genes (n=51, 116 probe sets), (III) direct neighbors of CIS genes (n=53, 81 probe sets) (IV) genes that are located within a region of 1 mega base pairs of the CIS genes, with a maximum of 5 genes up- or downstream (n=279, 468 probe sets) (Figure 1; Suppl. Table 2). The VIS and CIS genes have a significantly increased probability to be differentially expressed in subclasses of adult AML compared to unselected genes (Table 1, genes are listed in supplementary Table 2). In contrast, such a correlation was not found for 2 or 10 neighboring genes of the CIS genes.

To determine the validity of these results for an independent AML profiling data set, we performed a correlation analysis using data from 130 childhood AML

Table 1. Virus integration sites projected on 285 adult AML and 130 pediatric AML samples

	Number of unique genes (probe sets)	Number of unique SAM genes in adult AML (probe sets)	Probability	P-value *	Number of unique SAM genes in pediatric AML (probe sets)	Probability	P-value *
All	12848 (22283)	3644 (5193$)	0.28	--	2093 (2736$)	0.16	--
Gr-1.4 CIS	51 (116)	22 (32)	0.43	0.002	16 (21)	0.31	0.0127
Gr-1.4 VIS	115 (234)	53 (74)	0.46	<0.0001	29 (42)	0.25	0.0050
2 adjacent genes	53 (81)	15 (18)	0.28	0.49 (n.s.)	7 (9)	0.13	0.9361 (n.s.)
10 adjacent genes	279 (468)	91 (123)	0.33	0.19 (n.s.)	50 (66)	0.18	0.2071 (n.s.)
BXH2 CIS/VIS	53 (111)	33 (51)	0.62	<0.0001	21 (25)	0.40	0.0001
AKxD CIS/VIS	119 (232)	72(104)	0.61	<0.0001	43 (60)	0.36	<0.0001
All CIS/VIS	237 (470)	122 (178)	0.51	<0.0001	69 (97)	0.29	<0.0001

$ Because some probe sets contributed to multiple classes, the total number of sets used in Chi-square analysis was 8739 for adult AML and 2955 for the pediatric AML cases. For details see supplementary Tables 1a and 1b.

* P-value determined by a two-tailed chi-square test with 95% confidence intervals.
n.s: not significant

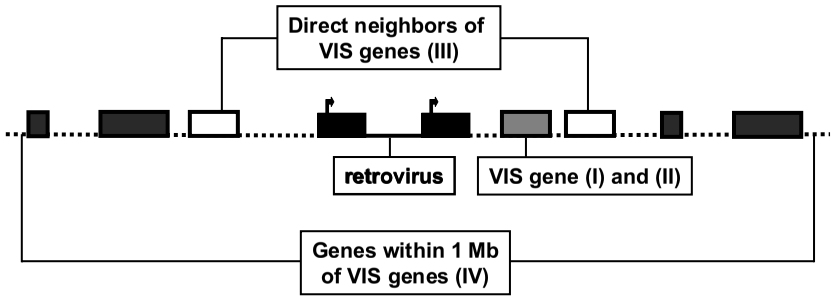

Figure 1. Genomic region of virus integration sites (VIS)
Four gene lists were derived from the Gr-1.4 -induced leukemia model; (I) genes directly flanking virus integration sites; (II) genes commonly targeted by virus integrations (CIS genes), (III) 2 direct neighbors of CIS genes and (IV) genes located within a region of one mega base pairs of CIS genes, with a maximum of 10. Virus integrations can be located up- or downstream or within the target gene.

samples (available at website http://www.stjuderesearch.org/data/AML1, also generated with Affymetrix HGU133A GeneChips) [24]. In this study, patients were grouped in 5 classes, i.e., cases with inv(16), t(15;17), t(8;21), translocations involving MLL and cases with megakaryoblastic leukemia (Supp. Table 1b). In total, 2736 probe sets, representing 2093 genes, contributed to the signature of the 5 patient classes of pediatric AML (Supp. Table 1b). The probability that a randomly selected gene is differentially expressed in one or more subclasses of the childhood AML dataset is 0.16 (Table 1). Similar to adult AML, Gr-1.4 CIS and VIS genes had a significantly increased probability to be differentially expressed in the distinct pediatric AML patient clusters (Suppl. Table 3), while again no such correlation was seen with the genes located more distantly from the virus integrations.

Analysis with candidate cancer genes identified in two other mouse leukemia models, BXH2 myeloid leukemia and AKxD B-lymphoid leukemia [19] (Suppl. Table 2) showed that these results are not unique for Gr-1.4 integrations. Both BXH2 and AKxD-derived VIS genes (http://genome2.ncifcrf.gov/RTCGD) were also significantly over represented in gene sets differentially expressed in the distinct adult and pediatric AML clusters (Table 1; Suppl. Table 3). The fact that this extends to genes involved in lymphoid AKxD-derived tumors suggests that disease mechanisms in B-lymphoid and myeloid leukemia do considerably overlap. Although this overlap may be related to the viral etiology of mouse leukemia, multiple examples of genes involved in both human B-lymphoid and myeloid malignancies exist as well. Taken together, the data from the three mouse leukemia models suggest that genes directly flanking virus integrations and that are differentially expressed in AML subtypes have a high probability to be involved in the pathogenesis of human AML.

To put the retrovirus-tagged genes relevant to human AML into regulatory networks, we imported all VIS/CIS genes from Gr-1.4, BXH2 and AKxD MuLV models that were differentially expressed in the adult AML panel (n=125, of which 110 genes, called focus genes were eligible for generation of networks) into the Ingenuity Pathway Analysis application (http://www.ingenuity.com). The focus genes could be linked to 5 highly significant networks that are prominently associated with cell growth and proliferation, hematopoietic cell development, cell cycle, and gene expression (Table 2, Figure 2, Suppl. Figures 1-5). Network 1 existed exclusively of focus genes (n=35), suggesting that different players within this network are commonly affected in AML. None of the AML subgroups with more than 15 focus genes showed a unique representation or exclusion of one or more of the 5 networks. (Suppl. Figure 6). We therefore hypothesize that the pathogenetic networks underlying the distinct AML subclasses are not completely separated, but rather are differentially affected. For instance, this applies to genes of network 1 in adult AML patient clusters 4 and 5 (Suppl. Figure 7). Genes involved in cytokine signaling, i.e., IL2RG, STAT5A and STAT5B, IL4R, HCK and IRS2 (encoding insulin receptor substrate, the transcriptional regulator SOX4, implicated in the pathogenesis of neuronal tumors [18] and lymphoma [25] and ZNF145, a transcriptional repressor also known as promyelocytic leukemia zinc finger (PLZF) involved in chromosome translocation (11;17) in acute promyelocytic leukemia (APL) are differentially perturbed within these clusters. None of the cases of cluster 4, in which ZNF145 is significantly upregulated, harbors a t(11;17), suggesting an

Table 2. Position of VIS/SAM genes in regulatory networks

	Focus genes in network*	Major global functions of network (number of Focus Genes >= 8) assigned by Ingenuity
Network 1	BTG2, CEBPB, DOK1, DSIPI, DUSP10, E2F2, ELF4, EVI1, FOS, FOSL1, HCK, HMGA1, HRAS, IL2RA, IL2RB, IL2RG, IL4R, IRS2, JUNB, LCK, LEF1, LTB, MADH3, MPL, NFKB2, NFKBIA, PLAU, RUNX1, SOX4, STAT5A, STAT5B, TP53, TRA1, ZFHX1B, ZNF145	Tissue Morphology (n=25) Cellular Growth and Proliferation (n=28) Cellular Development(n=27)
Network 2	CALD1, CCND2, CCND3, CTNNA1, ETS1, FLI1, HES1, MYB, MYC, MYCN, NFATC1, NOTCH1, NOTCH2, PAX5, PIM1, PRDM1, PRDX2	Cellular Development (n=13) Hematological System Development and Function (n=10) Cancer (n=12)
Network 3	BCL11A, CAPG,CCL4, CCL5, IFNGR2, IL6ST, INPP5A, KIT, MAP4K2, PTP4A3, PTPRE, PXN, SOCS2, SWAP70	Hematological System Development and Function (n=22) Cell Death (n=23) Immune Response (n=21)
Network 4	C3AR1,EPS15, HHEX, IFI30, MEF2C, MEF2D, NCOR1, NP, RXRA, ST13, TIE, ZFP36	Gene Expression (n=17) Cellular Development (n=12) Cancer (n=13)
Network 5	BCOR, CCND3, E2F2, GFI1, HOXA9, LMO2, MEIS1, TWIST1	Cancer (n=21) Gene Expression (n=22) Cellular Growth and Proliferation (n=22)

*: Complete networks are shown in Figure 2 and in supplementary Figures 1 - 5.

alternative mechanism of deregulation. Importantly, the PLZF protein has recently been implicated as a regulator of stem cell renewal [2, 4].

In conclusion, we have shown that genes located in or directly flanking virus integration sites in mouse leukemia correlate significantly with gene sets differentially expressed in distinct AML patient clusters identified by gene expression profiling. The combination of the two approaches thus facilitates recognition of novel disease genes and networks involved in the pathogenesis of human AML. It will be of interest to determine whether this strategy to discriminate between disease genes and "marker" genes extends to other types of cancer with a retroviral etiology in mice, e.g., lymphoma, breast cancer and brain tumors [11, 22, 26, 29]. With the VIS gene lists in the various mouse leukemia models not yet saturated and the possibilities of gene expression profiling of AML still growing, the power of this strategy will further increase. This will allow further refinement of the currently identified and presumably disclose additional pathogenetic networks. Ultimately this knowledge may be of use to further refine diagnosis and to identify target molecules for the development of directed therapeutic intervention.

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CHAPTER 4

The gene encoding the transcriptional regulator Yin Yang 1 (YY1) is a myeloid transforming gene interfering with neutrophilic differentiation

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Blood, 2003; 101:1111-1117

Abstract

The genetic defects underlying the pathogenesis of acute myeloid leukemia (AML) are still largely unknown. Retroviral insertion mutagenesis in mice has become a powerful tool to identify candidate genes involved in the development of leukemia and lymphoma. We have used this strategy with the 1.4 strain of Graffi murine leukemia virus (MuLV), which predominantly causes myeloid leukemias. Here, we report that Graffi-1.4-induced AML frequently harbors virus integrations in the gene encoding the transcription factor Yin Yang 1 (YY1). These integrations occurred in both orientations, and all were located in the 5' promoter region of the gene, 0.5 to 1.5 kb upstream of the major transcriptional start site. Luciferase reporter assays showed that virus integration in this region increases promoter activity and renders it independent of a functional binding site for Sp1, a major transcriptional regulator of YY1. We used the murine 32D model to study the consequence of perturbed YY1 expression for myelopoiesis. YY1 protein levels were high in 32D parental cells maintained in interleukin-3-containing medium, but they dropped when the cells were induced to differentiate by granulocyte-colony-stimulating factor (G-CSF). Strikingly, G-CSF-induced neutrophilic differentiation was reduced in 32D cell transfectants ectopically expressing YY1. In similar experiments on primary bone marrow cells, enforced YY1 expression blocked the outgrowth of CFU-GM colonies. Increased YY1 expression was seen in some cases of human AML. Collectively, these data imply a possible role of perturbed expression of YY1 in the development of AML through interference with the myeloid differentiation program in the leukemic progenitor cells.

Introduction

Human acute myeloid leukemia (AML) is a heterogeneous group of diseases with a variable treatment outcome. It is now well established that responses to different forms of therapy depend to a significant extent on the presence of certain genetic defects in the leukemic cells. For instance, chromosomal abnormalities t(8;21), inv(16), and t(15;17) are now being used as important prognostic indicators in AML [22]. However, it has also become clear from in vitro and in vivo models that, when isolated, these defects are insufficient to cause leukemia but that additional "hits" in as yet largely unidentified regulatory genes are required for full leukemic transformation of hematopoietic cells [17, 19]. In addition, in the significant proportion of AML patients who do not have cytogenetic abnormalities, the genes involved in the pathogenesis of the disease remain elusive. Retroviral insertional mutagenesis has become a powerful tool to identify genes implicated in leukemogenesis and lymphomagenesis [17, 19, 20]. In the past, this method has been tedious, but recently developed strategies using inverse polymerase chain reaction (PCR), direct nucleotide sequencing, and database screening now allow for a rapid and large-scale identification of potential disease genes [20]. Importantly, a number of genes identified by this strategy have also been shown to play a role in human hematopoietic malignancies [4, 12, 20, 21, 27, 37].

Graffi murine leukemia virus (Graffi-MuLV) is an ecotropic retroviral complex

causing leukemias in mice [29]. This viral complex does not contain oncogenic sequences, but it deregulates genes because of proviral integrations. A subclone of this complex, the Graffi-1.4 strain, predominantly induces myeloid leukemias [29]. In these leukemias, known proto-oncogenes such as c-myc, Pim-1, Fli-1, and Spi-1 are rarely affected [10], indicating that other genes involved in Graffi-1.4 MuLV-induced leukemias remain to be discovered.

YinYang 1 (YY1, NF-E1, delta, UCRBP, CF1) is a transcription factor of the GLI-Krüppel zinc finger protein family that controls many cellular processes [2, 32, 36]. Targeted disruption of the YY1 gene leads to embryonic lethality in mice from severe defects in the development of the embryonic and extraembryonic tissues [11]. The YY1 gene spans 23 kb comprising 5 exons and encodes a protein of 44 kDa. The gene is located on human chromosome 14q32 and on mouse chromosome 12 and is structurally highly conserved among these species (95% homology at mRNA level) [43]. Transcription of the YY1 gene is tightly controlled. Besides a major transcriptional start site, the existence of 10 alternative start sites in a (C+G)-rich region lacking a TATAbox have been reported [31]. YY1 expression is controlled by the transcription factor Sp1, which is expressed in many cell types, including hematopoietic cells [31, 30].

YY1 has been reported to activate or repress transcription of a large variety of cellular and viral genes [32, 33, 36]. Additionally, YY1 regulates gene expression in a cell-cycle-dependent fashion. This may, at least in part, be due to a control mechanism involving the retinoblastoma protein (Rb), which releases YY1 in the S-phase of the cell cycle [28]. The transcriptional activity of YY1 is positively regulated through acetylation of the protein by p300 and PCAF and is negatively regulated by deacetylation by histone deacetylases HDAC1, HDAC2, and HDAC3 [44].

In this paper, we report that the YY1 promoter region is a frequent target for integration of Graffi-1.4 MuLV and show that the regulation of YY1 expression is perturbed and becomes independent of Sp1 regulation as a result of these integrations. We further show that ectopic expression of YY1 has a negative effect on myeloid differentiation in a cell line model and prevents the outgrowth of myeloid progenitors from primary bone marrow cells. Finally, we present data indicating that in certain cases of human AML, YY1 expression is significantly increased compared with healthy bone marrow cells. Based on these findings, we suggest that the deregulation of YY1 expression can contribute to myeloid leukemia by interfering with the normal myeloid differentiation program.

Material and methods

Graffi-1.4 MuLV-induced leukemias

Newborn FVB/N mice (younger than 2 days) were injected subcutaneously with 100 μL of a cell culture supernatant of Graffi-1.4 MuLV-producing NIH3T3 cells (a gift from Dr E. Rassart, Departement des Sciences Biologiques, Universite du Quebec a Montreal, Canada). Mice were checked daily for symptoms of illness: apathy, white ears and tail, impaired interaction with cage mates, weight loss, and dull fur. Typically, leukemic mice had enlarged spleens, livers, thymuses, and lymph nodes. From these primary tumors, chromosomal DNA was isolated for PCR-based

screening. Blood samples were taken from the heart. For morphologic analysis, blood smears and cytospins were fixed in methanol, stained with May-Grünwald-Giemsa, and analyzed on a Zeiss Axioscope microscope (Carl Zeiss BV, Weesp, The Netherlands).

Immunophenotyping of the leukemic cells

Single-cell suspensions of different organs were analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Cells were labeled as described previously [18] with the following rat monoclonal antibodies: ER-MP54, ER-MP58, M1/70 (Mac-1), F4/80, RB68C5 (GR-1), ER-MP21 (transferrin receptor), TER119 (Glycophorin A), 59-AD2.2 (Thy-1), KT3 (CD3), RA3 6B2 (B220), and E13 161-7(Sca1). Immunodetection was performed using a goat anti-rat antibody coupled to fluorescein isothiocyanate (GαRa-FITC) (Nordic, Tilburg, The Netherlands).

Production of retroviral vectors

The plasmid pCMV-HA-YY1, containing hemagglutinin (HA)-tagged full-length human YY1 cDNA (a gift from Dr Y. Shi, Department of Pathology, Harvard Medical School, Boston, MA) was digested with XbaI and ApaI, and the HA-YY1 fragment was blunted and ligated into the HpaI site of pLNCX and pBabe retroviral vectors [24, 25]. PhoenixA packaging cells (from G. Nolan, Stanford, CA) were transfected with pLNCX-HA-YY1 or pBABE-HA-YY1 (Protection Mammalian Transfection Systems E-1200; Promega, Madison, WI). Supernatants containing high-titer, helper-free recombinant virus were harvested from 80% confluent producer cells cultured for 16 to 20 hours in Dulbecco modified Eagle medium, (DMEM; Gibco-BRL, Breda, The Netherlands) supplemented with 5% fetal calf serum (FCS), penicillin (100 IU/mL), and streptomycin (100 ng/mL). To determine titers of BABE-HA-YY1 and BABE control virus, the virus particles were pelleted by ultracentrifugation at 41.000 rpm (XL-90; Beckman, Mijdrecht, The Netherlands), and RNA was extracted with phenol (pH = 4.0) and spot-blotted on nitrocellulose filters. This blot was hybridized with a BABE-specific probe (SV40 fragment, BamHI-HindIII digest).

Cell culture and retroviral gene transfer

32D cells The interleukin-3 (IL-3)-dependent murine myeloid cell line 32D [15] containing the human wild-type G-CSF receptor (32D-WT1) were expanded and differentiated as described [8]. 32D-WT1 cells were infected with pLNCX-HA-YY1 virus and selected with G418 (Gibco-BRL). Several independent clones were expanded for further analysis.

Bone marrow cells

Hematopoietic cells were harvested from the femurs and tibiae of 8- to 12-week-old FVB mice as described [16]. After depletion of adherent cells, the remaining cells were fractionated on a Percoll gradient (AB 17-0891-01; Amersham Pharmacia Biotech, Uppsala, Sweden). Fraction 1 (density 1.058/1.0645) containing the earliest hematopoietic progenitor cells [6] was collected. Cells were washed twice in Hanks buffered salt solution (HBSS)/5% FCS/0.5% bovine serum albumin (BSA) and

then were prestimulated for 2 days at a final concentration of 5 x 10^5 cells/mL in Cell Gro (SCGM BE SP047; Boehringer Ingelheim Bioproducts Partnership Heidelberg, Germany) supplemented with a cytokine cocktail composed of murine (m) IL-3 (10 ng/mL), human (h) Flt3-ligand, human thrombopoietin (hTPO), murine stem cell factor (mSCF; 100 ng/mL) and granulocyte macrophage-colony-stimulating factor (GM-CSF; 2 U/mL). Retroviral infection was performed in culture dishes (Falcon 1008; Becton Dickinson) coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo T100A/B; Otsu, Japan)[5] at a concentration of 12 μg/mL. Before adding the bone marrow cells, the dishes were preincubated with virus supernatant (BABE-HA-YY1 or empty BABE) for 30 minutes at 37°C. subsequently, bone marrow cells were resuspended and mixed with fresh virus supernatant in a 1:1 ratio, and a fresh cytokine cocktail was added. Cells (5 x 10^5) were cultured overnight at 37°C and 5% CO_2. Virus supernatant and cytokine cocktail were refreshed again the next day, and cells were cultured for another 24 hours.

Colony assay

Bone marrow cells were plated at densities of 1 to 5 x 10^4 cells/mL per dish in triplicate in methylcellulose medium supplemented with 30% fetal bovine serum (FBS), 1% BSA, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, and GM-CSF (20 U/mL), with or without 2.5 μg/mL puromycin (Sigma, Zwijndrecht, The Netherlands) to evaluate the infection efficiency of the different retroviruses. Colonies consisting of more than 50 cells were counted on day 7 of culture.

Promoter activity assay

The YY1 promoter-containing subclone pδSS4.5 of λ24 [31] was digested with restriction enzymes MLuI and BglII. This YY1 promoter fragment was cloned in the luciferase reporter plasmid pGL3 (Promega). The Graffi-1.4 MuLV long terminal repeat (LTR) sequence was cloned in both orientations in the HpaI site. The Sp1 site was mutated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) by using primer SPIMUTF (5' GGACGGTTCGGGGC-GAGAGC 3') and primer SPIMUTR (5' GCTCTCGCCCCGAACCGTCC 3'). As a positive control, pRSVLuc [9] was used. pRSV-β-galactosidase (a gift from C. Berrevoets, Erasmus University Rotterdam, The Netherlands) was used for reference. Empty pGL3 vector served as a negative control. Luciferase assays were performed in HEK 293 cells. Cells were transfected by $CaPO_4$ precipitation [39] with a mixture of 5 μg derived pGL3 plasmid containing different promoter sequences and 2.5 μg pRSV-β-galactosidase, supplemented with empty vector, to a total amount of 20 μg total plasmid per milliliter. Luciferase assays were performed as described [8] and activities were calculated in arbitrary units, relative to β-galactosidase expression.

Twenty-five microliters cell lysate was mixed with 75 μL ONPG solution (0.028 g 2-nitro-phenyl-galacto-pyranosid; Boehringer-Mannheim, Almere, The Netherlands), 50 mL P-buffer (100 mM phosphate buffer, pH 7.0), 10 mM $MgSO_4$, 2.7 mM). Samples were incubated at 37°C for 1 hour. The extinction was measured at 450 nm in a Bio-Rad 450 microplate reader (Bio-Rad, Richmond, CA).

Inverse PCR on Graffi-1.4 MuLV-induced leukemias
Genomic DNA from the primary tumors was digested with HhaI. After ligation (Rapid ligation kit; Roche Diagnostics, Mannheim, Germany), a first PCR was performed using Graffi-1.4 MuLV (LTR) specific primers L1 (5' TGCAAGATGG-CGTTACTGTAGCTAG 3') and L2 (5' CCAGGTTGCCCCAAAGACCTG 3') (cycling conditions were 1 minute at 94°C, 1 minute at 65°C, and 3 minutes at 72°C; 30 cycles). For the second nested PCR, the primers L1N (5' AGCCTTATGGTGG-GGTCTTTC 3') and L2N (5' AAAGACCTGAAACGACCTTGC 3') (15 cycles) were used. The PCR reaction mixture contained 10mMTris(tris[hydroxymethyl]-aminomethane)-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 10 pmol each primer, and 2.5 U Taq polymerase (Pharmacia, Uppsala, Sweden). PCR fragments were analyzed on a 1% agarose gel and cloned in the TA cloning vector (Invitrogen BV, Carlsbad, CA) according to standard procedures.

Detection of virus integration in the YY1 gene by specific nested PCR
To determine the orientation and localization of the Graffi-1.4 provirus in the YY1 gene in an extended panel of leukemias, nested PCR was performed on 1 μg genomic DNA from primary tumors (Figure 1). For the first PCR, YY1 promoter region-specific primers Y1 (5' AGGAATCAGGAGCAGAAGAAAGTTTTGGGA 3') and Y2 (5' CAATAAAGTCTGCTCTGACGAGAAACGCC 3'), in combination with Graffi-1.4 MuLV LTR-specific primers L1 and L2 were used. Cycling conditions were 1 minute at 94°C, 1 minute at 65°C, and 3 minutes at 72°C, for 30 cycles. For the second PCR, the nested primers Y1N (5' AAACTCTCTGACTTAC-CTCCCTCTCCAAAGA 3') and Y2N (5' GTTCGTTTTGCCTTTACTCGTTAC-TCGGG 3'), in combination with the nested primers L1N and L2N (30 cycles), were used. The obtained PCR products were analyzed by Southern blotting. To determine the orientation of the Graffi-1.4 provirus, the blots were hybridized with radiolabeled Y1P (5' AAAACCTGCACAAGGACACCTTGCTAAGTATGTTT 3') and Y2P (5' AGCACACGGTCGGCTACGCTCCGTCCGCTACCGCA 3') at 45°C in Church buffer (0.5 M phosphate buffer, pH 7.2, 7% [wt/vol] sodium dodecyl sulfate [SDS], 10 mM EDTA [ethylenediaminetetra-acetic acid]) overnight. Signals were visualized by autoradiography according to standard procedures.

Nucleotide sequencing
PCR products were cloned in TA cloning vector and sequenced with the M13 forward primer (5' GACCGGCAGCAAAATG 3') and M13 reverse primer (5' CA-GGAAACAGCTATGAC 3') using an ABI 3100 sequencer (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands). Virus-flanking genomic sequences were identified using the National Center for Biotechnology Information (NCBI) database.

Western blotting
Lysates of 32D cells were prepared and subjected to Western blotting as described previously [3]. Antibodies used to visualize YY1 were goat anti-YY1 (Santa Cruz Biotechnology, CA) or rabbit anti-HA (Y-11, sc-805) for HA-tagged YY1. Rabbit anti-Sp1 (a gift from Dr G. Suske, Philipps University, Marburg, Germany) was used for the detection of Sp1. Goat anti-actin (I-19, sc-1616; Santa Cruz Biotechnology)

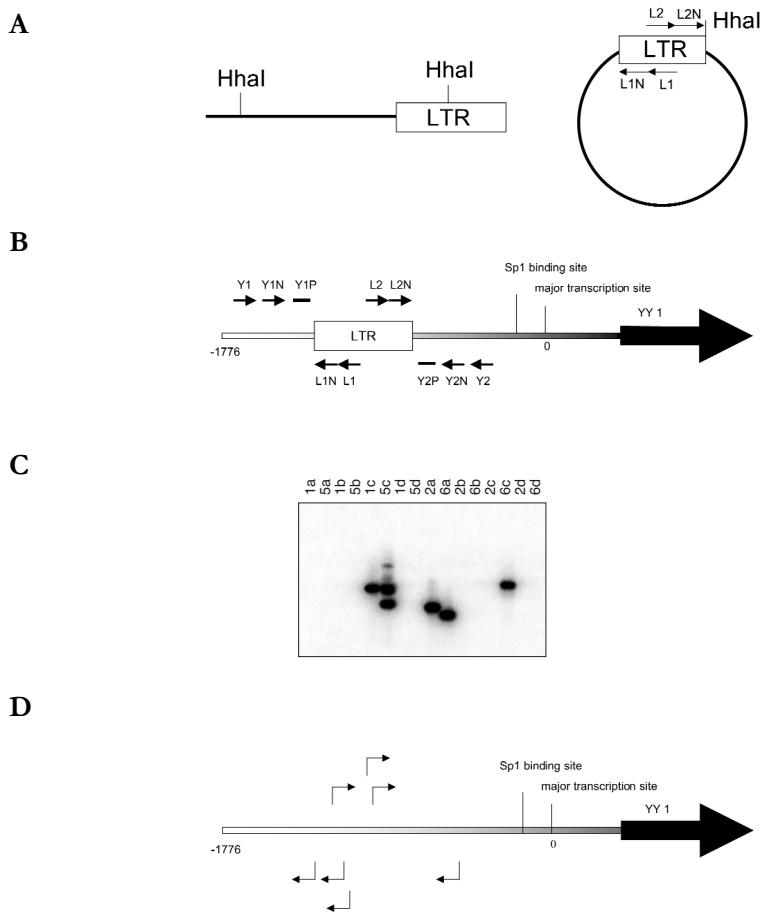


Figure 1. Identification of common virus integrations in the YY1 locus

A) Inverse PCR. Genomic DNA from leukemia cells was digested with HhaI. After ligation PCR was performed with primers L1 and L2 followed by a nested PCR with primers L1N and L2N to amplify LTR-flanking fragments from circularized DNA.

B) Nested PCR with LTR and YY1 primers to detect position and orientation of Graffi-1.4 MuLV integration in the YY1 promoter region. The lowercase letters indicate the position at the promoter in base pairs (bp). The first PCR was performed with the primer sets L1, Y1 (a), L1, Y2 (b), L2, Y1 (c), and L2, Y2 (d), followed by a nested PCR with primers L1N, Y1N (a) and L1N, Y2N (b), L2N, Y1N (c), and L2N, Y2N (d). Probes Y1P and Y2P were used to analyze the specificity of the PCR band by Southern blot.

C) Example of Southern blot analysis to determine virus integration and orientation in the 5 region of the YY1 gene. Results depicted are from DNA samples of leukemias 1, 2, 5, and 6. PCR products from the 4 different primer combinations (a, b, c, and d) were analyzed. In this example, the blot was hybridized with probe Y1P. The presence of the band in lane 1c indicates that tumor 1 has a virus integration in the reverse orientation. Tumor 5 has 2 YY1 virus integrations in the reverse orientation (lane 5c). Tumor 2 has an integration in the forward orientation (lane 2a). Tumor 6 harbors 2 integrations in both orientations (lanes 6a and 6c). All bands were sequenced to determine the exact location of virus integration.

D) Examples of virus integrations and orientations found in the YY1 promoter.

was used to control for equal loading of lysates.

Real-time quantitative PCR on human AML samples

Human AML cells were obtained following informed consent and were purified as previously described [23]. These purified blast fractions consisted of more than 95% myeloblasts. Total RNA was extracted with guanidinium thiocyanate and was purified by cesium chloride gradient centrifugation. RNA was transcribed into cDNA under standard conditions using Superscript (Life Technologies, Merelbeke, Belgium) and random hexamers. Real-time PCR amplification (ABI PRISM 7700 Sequence Detector; PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) was performed in a mix of 50 µL containing 2 µL cDNA, 250 µM dNTP (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 3 mM $MgCl_2$, 15 pmol primers, 200 nM Taqman probe, labeled 5' with reporter dye FAM (6-carboxy-fluorescein) and 3' with quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine; Eurogentec, Maastricht, The Netherlands), 1.25 U AmpliTaq Gold (PE Applied Biosystems), 5 µL 10 x bufferA (PE Applied Biosystems), and 30 µL H_2O. Cycling conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles of denaturation (15 seconds at 95°C), and annealing/extension (1 minute at 60°C). For YY1, forward primer (5' ATACCTGGCATTGACCT 3') and reverse primer (5' TGAGG-GCAAGCTATTGT 3') were used. The YY1 Taqman probe was 5' GAATGAAGC-CAAGAAAAATTAAAGAAGATGT 3'. The housekeeping gene porphobilinogen deaminase (PBGD) was taken as endogenous reference (PBGD forward primer 5' GGCAATGCGGCTGCAG 3'; PBGD reverse primer 5' GGGTACCCACGCGA-ATCAC 3'; PBGD Taqman probe 5' CATCTTTGGGCTGTTTTCTTCCGCC 3'). All samples were tested in duplicate, and average values were used for quantification. The expression of YY1 in patient samples relative to YY1 expression in healthy bone marrow (n = 6) was calculated according to manufacturer's instructions (user bulletin 2; ABI PRISM 7700 Sequence Detector; PE Biosystems).

Results

Graffi-1.4 MuLV-induced leukemias

Leukemias developed 4 to 6 months after subcutaneous injection of newborn FVB/N mice with Graffi-1.4 MuLV. Forty-eight of 59 (81%) leukemias analyzed exhibited morphologic characteristics of myeloid cells. Blast cell percentages in the bone marrow ranged from 24% to 90%, with an average of 48%. Leukemia cells expressed immunophenotypic marker profiles consistent with their myeloid appearance for example, ER-MP54$^+$, ER-MP58$^+$, CD3$^-$, GR-1$^+$. Six leukemias with blastlike morphology showed no immunophenotypic differentiation markers, suggesting that these tumors represented immature leukemias. Only 3 leukemias were of T-lymphoid origin (CD3$^+$/MP58$^-$/Thy1$^+$), and 2 showed mixed myeloid and erythroid features (Ter119$^+$/ ER-MP58$^+$/ F4/80$^+$). These results demonstrate that Graffi-1.4 MuLV infection predominantly induces myeloid leukemia in FVB/N mice.

Virus integrations in the YY1 gene

Inverse PCR was used to identify genomic sequences flanking Graffi1.4 MuLV integrations (Figure 1A). Integrations were found in several genes previously demonstrated to be involved in MuLV-induced leukemias (eg, Notch-1, Nf1, p53, Fli-1, Evi-1) and in several novel loci (Erkeland SJ, et al, manuscript in preparation). One of these newly identified integrations occurred in the YY1 locus, approximately 0.8 kb upstream of the transcriptional initiation site. In a subsequent analysis on independent cell samples using nested PCR with LTR and YY1 primers (Figure 1B-C), we found 23 integrations in the same region, 0.5 to 1.5 kb upstream of the transcriptional start site, in 14 of 20 leukemias tested. Integrations occurred in both orientations at an approximately equal ratio. Representative examples of these integrations are shown in Figure 1D. Searches for virus integrations in other parts of the gene using appropriate primer sets were negative (data not shown). It is of note that in some leukemia samples multiple integrations were detected, indicating that the Graffi-1.4 MuLV-induced leukemias are oligoclonal.

Integration of Graffi-1.4 MuLV LTR deregulates YY1 transcription

Because integration of the virus occurred in both orientations, we considered it most likely that viral enhancer sequences in the LTR cause increased YY1 transcription in these types of leukemia. Integration in the promoter region of a gene, however, does not always result in alterations in expression. For instance, the common ecotropic virus integration 12 (Evi12) is located approximately 1 kb upstream of the transcriptional start site of the Tra1/Grp94 gene but does not modulate its expression [38]. We first compared YY1 expression in the murine leukemias with and without virus integration by Northern and Western blot analyses but found no major differences between the groups (data not shown). However,

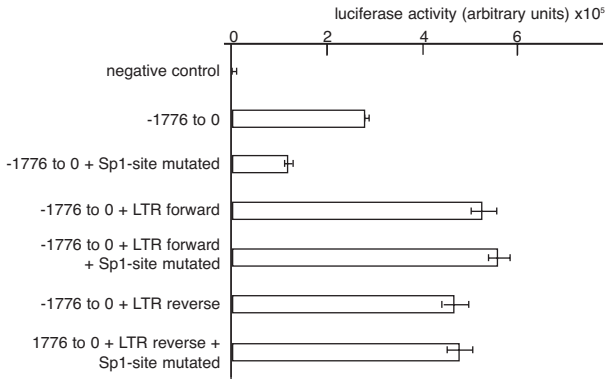


Figure 2. Effects of Graffi-1.4 LTR integration in the YY1 promoter region on gene transcription

Luciferase assays were performed in HEK 293 cells. One representative experiment of 3 is shown. Error bars represent the SD of the mean values of 3 independent experiments. Negative control: empty pGL3 vector. Reporter gene expression was calculated in arbitrary units, relative to β-galactosidase expression.

because virally induced leukemias are oligoclonal [17, 20] (and Erkeland SJ, et al, unpublished data), abnormalities in expression of YY1 in a subset of leukemia cells might go undetected by these techniques. As an alternative approach, we introduced the Graffi-1.4 LTR in the Hpa1 site at position 1417 in the YY1 promoter and quantified transcriptional activity using a transient reporter assay (Figure 2). Twofold higher YY1 promoter activity was measured following insertion of the Graffi-1.4 MuLV LTR, irrespective of the orientation of the LTR (Figure 2). Because an Sp1-binding site at position 48 to 39 has previously been shown to be important for the induction of YY1 transcription [31], we also studied the effects of insertion of the Graffi-1.4 MuLV LTR after disruption of this site. Although mutation of the Sp1-binding site resulted in a 50% reduction of normal promoter activity, the enhanced promoter activity caused by the integration of the LTR sequence was not affected (Figure 2). This result demonstrates that virus integration causes enhanced and Sp1-independent transcription of YY1.

Ectopic expression of YY1 blocks G-CSF-induced neutrophilic differentiation of 32D cells

The observation that integration of Graffi MuLV LTR alters the expression of YY1 in murine acute myeloid leukemia raised the question whether perturbed YY1 expression might affect myeloid cell differentiation. We have previously developed 32D cell clones expressing human G-CSF receptors (32D-WT1 cells) as a model to study neutrophilic differentiation [8]. Endogenous YY1 levels are high in parental 32D-WT1 cells cultured in IL-3-containing medium, under which conditions the cells remain immature (Figure 3). However, on transfer of the cells to a G-CSF-containing medium, in which cells undergo neutrophilic differentiation, YY1 protein levels declined from day 2 onward and remained low for the entire culture period (Figure 3). In view of the regulatory role of Sp1 in YY1 expression, we also looked at Sp1 levels and found that expression of Sp1 was down-regulated in the same time frame after switching the cells to G-CSF. These results raise the possibility that YY1 levels were reduced as a consequence of lowered Sp1-controlled transcription. To determine the consequences of perturbed YY1 expression for myeloid cell development, we ectopically expressed HA-tagged YY1 in these cells. As expected, YY1 expression in 32D-HA-YY1 cells remained high

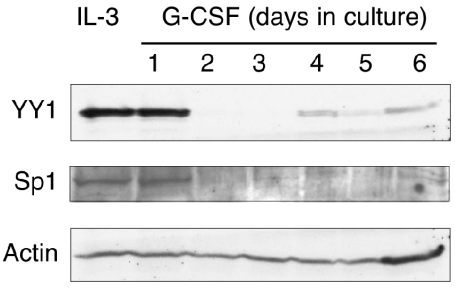
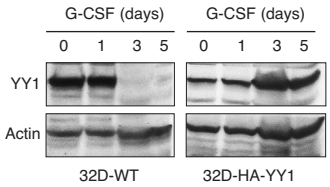


Figure 3. Western blot analysis of YY1 expression in 32D-WT1 cells

G-CSF responsive 32D-WT1 cells were cultured in IL-3–containing medium and then switched to G-CSF. Samples were taken daily and processed as described in "Materials and methods." The blot was hybridized with anti-YY1 or anti-Sp1, stripped, and rehybridized with anti-Actin to check for equal loading of cell lysates.

A



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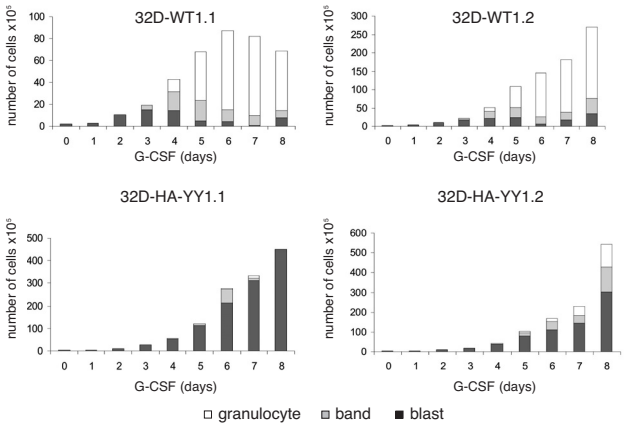

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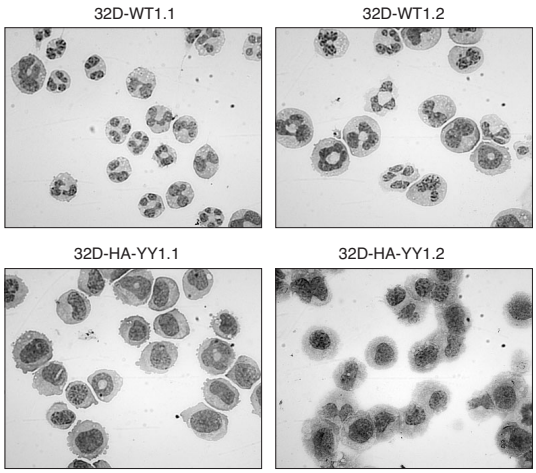

Figure 4. Ectopic expression of HA-YY1 in 32D cells inhibits neutrophilic differentiation
A)Western blot analysis with anti-YY1 in 32D-WT1 and with anti-HA antibodies in 32D-HA-YY1 cells after switching the cells from IL-3– to G-CSF–containing medium on t= 0 days. The blot was reprobed with anti-Actin antibodies for loading control.
B) Differential cell count (blasts, band form, and segmented nuclei) of 2 representative 32D-WT1 and 2 representative 32D-HA-YY1 clones.
C) Micrographs showing morphology of 32D-WT1 and 32D-HA-YY1 clones on day 5 (original magnification, 1000).

after the cells were switched to G-CSF (Figure 4A). Growth rate and morphology of 32D-HA-YY1 cells cultured in IL-3-containing medium were similar to nontransduced control cells (data not shown). Strikingly, when 32D-HA-YY1 cells were switched to G-CSF-containing medium, neutrophilic differentiation was markedly reduced (Figure 4B-C). Instead, cells with a blastlike morphology expanded exponentially for 8 days in these cultures (Figure 4B) and rapidly died thereafter as a result of apoptosis (data not shown). Thus, sustained YY1 expression prevented G-CSF-induced myeloid differentiation of 32D cells without affecting the apoptotic response that normally accompanies G-CSF-induced differentiation.

Ectopic expression of YY1 blocks CFU-GM colony formation

The above-mentioned experiments indicated that constitutive expression of YY1 interferes with G-CSF-induced myeloid differentiation in 32D cells. We next wanted to investigate how perturbed expression of YY1 affects the outgrowth of primary myelomonocytic progenitor cells (granulocyte macrophage-colony-forming unit [CFU-GM]). To this end, we retrovirally transduced mouse bone marrow cells with BABE-HA-YY1 or empty BABE virus and plated these in GM-CSF-containing colony assays. Notably, control bone marrow cells were subjected to equivalent titers of control virus (Figure 5A) to exclude that differences in colony outgrowth were

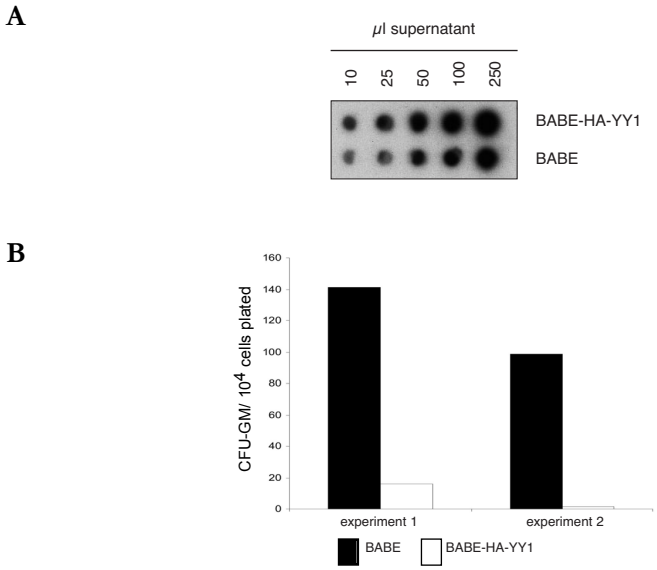

Figure 5. GM-CSF–induced colony formation by primary bone marrow cells after retroviral transduction of YY1

A) RNA spot blot analysis of supernatants containing BABE/HA-YY1 or BABE vector control virus, showing that titers used for infection were comparable. The filter was hybridized with a BABE-specific cDNA probe.

B) CFU-GM assay of primary bone marrow progenitor cells following infection with BABE-HA-YY1 or BABE control virus. Bone marrow cells were plated in triplicate at densities of 10^4 cells per dish in 1 mL methylcellulose medium containing GM-CSF (20 U/mL) and puromycin (2.5 μg/mL). Two independent experiments are shown.

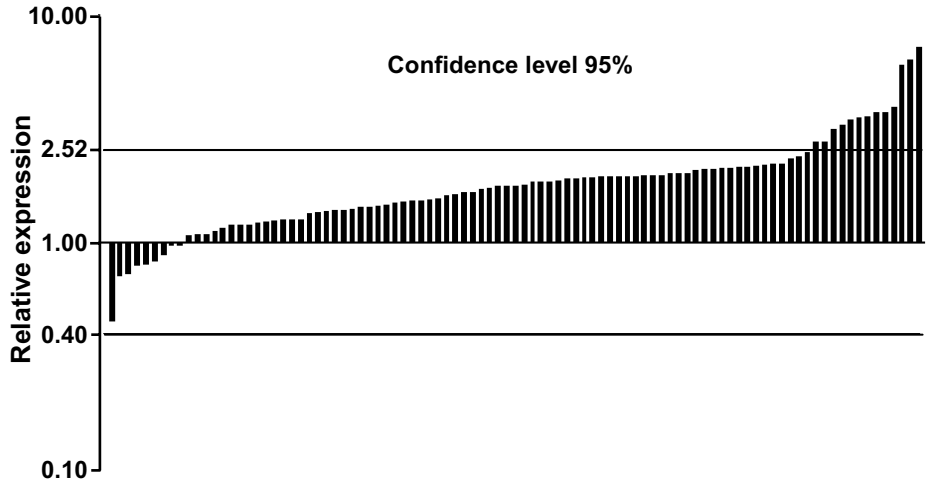

Figure 6 Real-time quantitative PCR analysis of YY1 transcripts in 94 patients with AML
Data are relative to the mean expression in healthy bone marrow samples (n=6), with 95% confidence limits indicated by the horizontal lines. AML data represent the mean of 2 independent experiments. The 95% confidence interval was calculated as: $Xmean_{(6\ NBM\ samples)} \pm (1.96\ SD)$.

caused by variations in transduction efficiencies. Strikingly, GM-CSF-induced colony formation by HA-YY1-transduced bone marrow cells was almost completely blocked (Figure 5B). This result suggests that perturbed expression of YY1, instead of interfering with differentiation, causes a growth arrest or results in premature apoptosis of CFU-GMs or their direct progeny.

YY1 expression in human AML

An important question is whether increased levels of YY1 expression can also be found in human AML cells, which would be suggestive of a possible involvement of abnormal YY1 expression in human disease. We performed real-time quantitative PCR on cDNAs from 94 patients with AML and 6 healthy volunteers. As shown in Figure 6, YY1 transcript levels in most patients did not significantly differ from healthy bone marrow cells. However, in 13 patients, mRNA levels significantly exceeded those of the healthy bone marrow controls.

Discussion

In this study, we demonstrated that the gene encoding the transcriptional regulator YY1 is located in a new common virus integration site in Graffi-1.4 MuLV-induced myeloid leukemia. The integrations occurred exclusively in the 5' promoter region of the gene, 0.5 to 1.5 kb upstream of the major transcriptional start site. Data from luciferase reporter assays strongly suggested that these integrations result in the dysregulation of YY1 expression. The pattern of virus integrations indicated that Graffi-1.4 MuLV-induced leukemias are oligoclonal, which is consistent with observations on leukemias induced by other MuLVs [17, 20] (Erkeland SJ, et al, unpublished data). The YY1 gene has already been identified once by inverse PCR

as a site of retroviral integration in BXH2 leukemia/lymphoma [20]. Although the exact site of integration and the type of leukemia/lymphoma were not specified in this study, the finding that the YY1 locus is affected by distinct MuLV types in different mouse strains indicates that the deregulation of YY1 may be more common in the development of various hematologic malignancies in mice.

Perturbed expression of YY1 inhibited G-CSF-induced myeloid differentiation in 32D cells and prevented the clonal outgrowth of CFU-GM progenitor cells. Importantly, we observed that integration of Graffi-virus LTR sequences enhanced YY1 transcription and made it entirely independent of the recognition site for the transcription factor Sp1, which has been shown to play a pivotal role in YY1 expression. Although Sp1 expression has been reported to be high in hematopoietic cells, it is down-regulated during differentiation in many cell types [30]. Indeed, we found that Sp1 declined in 32D cells after 2 days of G-CSF-induced differentiation (Figure 3). Therefore, we suggest that the bypass of Sp1 transcriptional control might be one of the mechanisms by which viral integration deregulates YY1 protein levels in leukemic cells.

To establish whether down-regulation of YY1 resulted from G-CSF-induced differentiation rather than from simply switching the cells from IL-3- to G-CSF-containing medium, we also analyzed YY1 expression in IL-3-dependent Ba/F3 transfectants expressing the G-CSF-R. These cells proliferate, but they do not undergo differentiation in response to G-CSF. Because YY1 levels remained high in Ba/F3-G-CSF-R cells (data not shown) cultured in G-CSF-containing medium, it appears most likely that YY1 expression is reduced during G-CSF-induced differentiation. This notion is further supported by observations in 32D cells expressing a truncated G-CSF receptor [40], which fails to differentiate in response to G-CSF and retains YY1 expression at a level comparable to that of cells cultured in IL-3 (data not shown).

A major question that remains to be addressed is how deregulation of YY1 expression leads to the observed defects in myeloid cell development. In view of the myriad proposed functions of YY1, multiple possibilities can be envisaged that are not mutually exclusive. First, it is conceivable that constitutive YY1 expression directly influences the transcriptional regulation of genes controlling myeloid cell development. This might be due either to the action of YY1 as a positive regulator of transcription or to its repressor activity. With regard to the latter, it is of interest that YY1 interacts with the EED-EZH (enhancer of Zeste) polycomb complex (PcG) involved in transcriptional repression. In this context, YY1 plays a role in targeting the PcG complex to specific DNA sequences and recruiting HDACs, which associate with both YY1 and EED [13, 42]. It is conceivable that the deregulation of YY1 could cause sustained repression of multiple target genes critical for granulocytic differentiation. In fact, altered recruitment of HDACs has already been implicated in the deregulation of myeloid differentiation by a number of transcription factor fusion proteins generated by specific translocations-PLZF-RARα t(11;17) [7], PMLRARα t(15;17) [41], and AML1-ETO t(8;21) [1, 14].

An alternative mechanism by which YY1 can influence cell fate is by forming complexes with other protein regulators, including the c-Myc and retinoblastoma (Rb) proteins [28, 34, 35]. A previous study has demonstrated that YY1 protein binds to Rb in a cell-cycle-dependent manner [28]. In the G0/G1 phase of the cell cycle, YY1

is in a complex with Rb and cannot bind DNA but is released from this complex when cells enter into S-phase, thereby regaining its DNA-binding activity [28]. The Rb protein family comprises 3 members, Rb, p107, and p130. Rb and p107, but not p130, are abundantly present in 32D cells proliferating in IL-3-containing medium. Conversely, on G-CSF-induced differentiation, Rb and p107 levels declined as p130 protein levels dramatically increased, suggesting that p130 plays a role in the induction of myeloid differentiation [26]. Based on these findings, we hypothesized that the overexpression of YY1 might result in sustained binding to p130 and thereby might interfere with differentiation. Although we confirmed that YY1 can form a complex with Rb in coimmunoprecipitation assays, we have been unable to detect such an interaction of YY1 with the p130 or p107 proteins (data not shown). An important extension of this work is to determine whether abnormalities in YY1 expression may also contribute to the pathogenesis of human hematopoietic malignancies. In a first attempt to address this question, we performed real-time quantitative PCR on cDNAs from purified human AML blasts and observed that in 13 patients, the expression of YY1 was significantly elevated compared with that in healthy bone marrow controls. Although this would argue in favor of the possibility that YY1 transcript levels are abnormal in these patients, accurate comparisons are difficult because healthy bone marrow contains a mixture of cell types of different lineages in various stages of maturation. Identification of the genes controlled by YY1 during myelopoiesis and a more detailed understanding of the signaling pathways influenced by YY1 in hematopoietic cells will be required to shed light on how perturbations in these processes contribute to the development of human AML.

Acknowledgments

We thank Dr E. Rassart for the Graffi-1.4 MuLV. We also thank Drs Joanna Prasher, Bart Aarts, and Marieke von Lindern for their comments on this manuscript and Karola van Rooyen for assistance with the preparation of figures.

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CHAPTER 5

WD40-repeat domain and SOCS box-containing proteins control granulocyte colony-stimulating factor signaling

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Abstract

In a murine retroviral screen for novel leukemia genes, we identified Wsb-2, encoding WD40-repeat domain and SOCS box-containing protein 2 as a common target. Virus integrations disrupted the Wsb-2 gene structure and WSB-2 expression was significantly decreased in a subclass of acute myeloid leukemia (AML) patients. Mammalian protein-protein interaction trap assays showed that Wsb proteins, via their WD40 domains, physically interact with the distal C-terminal region of the granulocyte colony-stimulating factor receptor (G-CSF-R), the major cytokine receptor involved in myeloid differentiation. Significantly, mutations truncating this region are common in severe congenital neutropenia patients who develop AML. Wsb binding reduced steady-state cell surface expression of the G-CSF-R and inhibited ligand-induced proliferation signaling. These effects were lost upon removal of the Wsb SOCS box, suggesting an involvement of ubiquitin ligase activity. These data suggest that Wsb-2 acts as a tumor suppressor in myeloid cells by controlling G-CSF-R routing to the plasma membrane, thus inhibiting G-CSF-induced expansion of myeloid progenitors.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by a block of differentiation and uncontrolled accumulation of leukemic blasts in the bone marrow (BM) and peripheral blood. In about half of AML cases, cytogenetic abnormalities and/or mutations in regulatory genes are found [37], which either interfere with the normal differentiation program of the leukemic cells or give rise to abnormal responses to growth stimuli. For instance, the chromosomal abnormalities t(8;21), t(15;17) and inv(16), and mutations in the gene encoding CCAAT/enhancer binding protein (CEBPA), result in altered transcriptional activities of proteins important for differentiation. On the other hand, mutations in the fetal liver tyrosine kinase receptor 3 gene (FLT3) result in the aberrant signaling function of this intrinsic tyrosine kinase receptor. Studies in mouse models have shown that neither of these abnormalities alone are sufficient to cause leukemia and that multiple (in)activating mutations in deregulating both the differentiation program as well as proliferation and survival signaling are required for full malignant transformation of myeloid progenitor cells [15, 35, 37]. In addition, no molecular defects have as yet been identified in 40% of AML patients. Hence, many underlying disease mechanisms in AML are still unknown [36].

We have recently performed a retroviral screen in mice to search for novel genes involved in myeloid leukemia [11]. One of the genes identified in this screen encodes the WD40-repeat and suppressor of cytokine signaling (SOCS) box-containing protein 2 (Wsb-2). Wsb-1 and Wsb-2 share 46% homology and belong to a protein family characterized by the SOCS box and a variety of distinct protein-protein interaction domains, such as SH2 domains in SOCS 1-7, ankyrin repeats in ASB 1-18, SPRY motifs in SSB 1-2, and WD40 repeats in WSB 1 and 2 proteins [22]. These proteins interact with Elongin B/C and cullin 2 or 5, thus forming so-called Elongin-Cullin-SOCS protein (ECS) complexes. ECS complexes function as E3

ubiquitin ligases, in which the SOCS-box proteins are thought to recruit the substrates that are target for ubiquitylation and subsequent proteasomal degradation [28, 29]. For instance, SOCS1 targets the signaling proteins JAK2, Vav, and insulin receptor substrate (IRS)-1 and IRS-2 for degradation via this mechanism [6, 46, 50]. However, substrate specificities of most of the SOCS protein family members are still unknown.

The granulocyte colony-stimulating factor receptor (G-CSF-R) is the major hematopoietic growth factor receptor involved in neutrophil development [5, 7]. G-CSF-R activates multiple signaling pathways involved in control of proliferation, survival and neutrophilic differentiation [5, 14, 59]. Among the different cytokine receptors involved in blood cell formation, G-CSF-R has the unique ability to initially induce cell proliferation, which is then followed by growth arrest and terminal differentiation towards neutrophilic granulocytes. The C-terminal membrane distal domain of G-CSF-R plays a major role in the control of differentiation [14]. In ~25% of patients with severe congenital neutropenia (SCN), this domain is truncated due to acquired nonsense mutations. Importantly, patients harboring such mutations are at high risk of developing AML [19, 63]. These truncated G-CSF-R mutants are severely defective in their signaling behavior. They confer a hyperproliferative response to G-CSF in knock-in mouse models and are unable to transduce differentiation signals in 32D and L-GM myeloid cell line models [1, 8, 18, 40, 60]. These phenotypic features can be partly explained by the loss of negative regulation by phosphatases and suppressors of cytokine signaling (SOCS). For instance, recruitment of the SH2 domain-containing protein tyrosine phosphatase (SHP-1), the SH2-containing inositol phosphatase (SHIP), and SOCS3, all implicated in the inhibition of G-CSF signaling, is disrupted by the truncation. In addition, both ligand-induced and constitutive internalization of the truncated G-CSF-R are severely affected, due to the loss of a critical di-leucine containing internalization motif (a.a. 749-769) [1, 52, 53]. However, neither of these mechanisms is exclusive for G-CSF-R. Moreover, a receptor mutant lacking the most distal residues 769-813 (G-CSF-R-Δ769), which remains fully sensitive to the negative effects of SOCS3, SHP-1 and SHIP and internalizes normally, also confers hyperproliferative signals in murine myeloid progenitors and is severely hampered in its ability to induce differentiation in 32D cells (Aarts LH, et al., in preparation). This result indicated that a novel mechanism, coupled to the distal portion of the G-CSF-R, plays a major role in controlling the proliferation/differentiation balance in myeloid progenitor cells.

Here, we show that Wsb-2 and the homologous Wsb-1 protein physically interact with the C-terminal membrane-distal domain of G-CSF-R and that this interaction affects cellular distribution and signaling function of G-CSF-R in a SOCS box dependent manner. Based on these activities and the affected expression in leukemia, we suggest that Wsb-2 acts as a tumor suppressor protein that dampens G-CSF-induced proliferation signals and thereby contributes to the induction of terminal myeloid differentiation, a process bypassed in AML.

Materials and methods

Detection of virus integrations in Wsb-2

To determine orientation and localization of Gr-1.4 integrations in Wsb-2 in an extended panel of leukemia samples, a nested PCR was performed on genomic DNA from primary tumors as described [11] (Fig. 1A). For the first PCR, Wsb-2 specific primers (a) 5'-GGGACCTGAATAAGCACG-3', (b) 5'-CTCCTGTGATTTCTCTCCTG-3', (c) 5'-AGACAACACTGCTTTGGTG-3', and (d) 5'-CTGTCATCTGCCACCGTAG -3' were used in combination with Gr-1.4 LTR specific primers L1: 5'-TGCAAGATGGCGTTACTGTAGCTAG-3' and L2: 5'-CCAGGTTGCCCCAAAGACCTG-3'. For the nested PCR, Wsb-2 specific primers (e) 5'-AGATCCAGGTGTTATCCG-3', (f) 5'-ACCAGTGTGATTATGTGGGA-3', (g) 5'-GGATTAGTGTGTAGGACCG-3' and (h) 5'-AGATACAAGCCTTCAGGTGAG-3' were used in combination with Gr-1.4 LTR specific primers L1N: 5'-AGCCTTATGGTGGGGTCTTTC-3' and L2N: 5'-AAAGACCTGAAACGACCTTGC-3', PCR fragments were analyzed on a 1% agarose gel and sequenced as described [11].

Yeast-two-hybrid (Y2H) analysis

Y2H experiments with yeast strain PJ69-4A [24] (obtained from Dr. Philip James, University of Wisconsin, Madison) [24] were performed as described [57]. A mouse 17-day embryo cDNA library (Matchmaker, Clontech laboraties, Palo Alto, CA) was used as prey library. For a second round screen, cDNA library plasmids were co-transformed together with the pGBT9-GCSFR762-813 or pGBT9-GCSFR762-790 bait. Prey inserts from this second selection were nucleotide sequenced (ABI 3100, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Y2H bait constructs

The G-CSF-R C-terminal 51 a.a. (G-CSF-R762-813) was obtained with primers Y2HGCSFRF1 (5'-CCGAATTCTCCTATGAGAACCTCTGGTTCCAG-3') and Y2HGCSFRR1 (5'-AACTGCAGTTTTAGTCATGGGCTTATGGACC-3'). G-CSF-R762-790 fragment was amplified with primers Y2HGCSFRF1 and Y2HGCSFRR2 (5'-AACTGCAGCTACAGGAGGGGGAAGTTGAG-3') using the pBabe-G-CSF-R [20] as template. PCR products were cloned in TA cloning vector (Invitrogen BV, Merelbeke, Belgium) and sequenced. EcoRI and PstI fragments were cloned in-frame with the DNA binding domain (BD) in pGBT9 vector (Clontech), resulting in pACT-BD-GCSFR762-813 and pACT-BD-GCSFR762-790.

Y2H prey constructs

Wsb-1 wild type and Wsb-1 without SOCS box (Wsb-1-ΔSB) sequences were amplified with primers WSB1fw (5'-AAGAATTCAAGCCAGCTTTCCCCCGAG-3') and WSB1r1: (5'-AACTCGAGTTAGCCCCTGTAGGAGAGAAACGCCAATA-3') using pMG2-WSB-1 and pMG2-WSB-1-ΔSB (see also Mappit constructs) as templates. Similarly, Wsb-2 and Wsb-2-ΔSB were amplified with primers WSB2fw (5'-AAGAATTCAAGAGGCCGGAGAGGAGCC-3') and WSB2r1 (5'-AACTCGAGCTAGAAAGTCCTGTATGTGAGGAACTC-3') using pMG2-WSB-2 and pMG2-WSB-2-ΔSB as templates. The PCR fragments were cloned in TA cloning vector, checked for correct sequence, EcoRI-XhoI digested and ligated into the

EcoRI and XhoI sites of the transcription activation domain (AD)-containing pACT prey vector (Clontech, Palo Alto, CA).

Immunoprecipitation
COS cells were transfected with pSG5-HAHA-G-CSF-R together with pMG2-WSB-1, pMG2-WSB-2, pMG2-FzR-WD40, or pMG2-SVT [12]. After 48 h, cell lysates were incubated with anti-FLAG (Sigma, Zwijndrecht, The Netherlands) or rabbit anti-HA antibodies (Santa Cruz Biotechnology, CA), immune-complexes gel fractionated and visualized by Western blotting as described [9].

Generation of PSG5-HAHA-G-CSFR
A G-CSF-R fragment was amplified using primers GCSFRHA (5'-GGAAGATCTCTACGCGTAATCTGGAACATCGTATGGGTACGCGTAAT-CTGGAACATCGTATGGGTAGAAGCTCCCCAGCGCCTC-3') and Fw7 creating a double HA-tag at the G-CSF-R C-terminus. The HpaI-BglII digested PCR fragment replaces the HpaI-BglII fragment of pBabe-G-CSF-R. The EcoRI-SalI fragment of pBabe-HA-G-CSF-R was ligated into pSG5 expression vector resulting in pSG5-HAHA-G-CSF-R.

Ligation of pACT-prey insert into pMG2
The pMG2 vector was mutated with QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA), using primers pMGEcoMutF (5'-GGCGGTTCGAGA-ATTCGAAGCA-3') and pMGEcoMutR (5'-TGCTTCGAATTCTCGAACCGCC-3') resulting in pMG2-mut vector. The EcoRI-XhoI fragment from pGBT9-Fzr-WD was cloned in the EcoRI and XhoI sites of the pMG2-mut vector resulting in pMG2-Fzr-WD.

Confocal laser scanning microscopy (CLSM)
CSLM was performed as described 1 using a Zeiss LSM510 confocal laser scanning microscope. HEK293 cells were transfected with pBabe-EGFP-fused G-CSF-R WT, mutants G-CSF-RΔ715, G-CSF-RΔ769 or G-CSF-RΔ749-769 [1] in combination with pMT2-MYC-WSB2 and fixed and stained as described using mouse α-MYC monoclonal antibodies (9E10, Santa Cruz Biotechnology, CA) and TRITC-conjugated Goat-α-Mouse secondary antibodies [1].

Generation of pMT2-MYC-WSB-1
WSB was amplified with WSBMYCf (5'-AAGAATTCAAGCCAGCTTTCCC-3') and WSB1r1 using pMG2-WSB as template and cloned in TA cloning vector. The EcoRI fragment was cloned in the EcoRI site of pMT2SM-MYC vector [1, 55].

MAPPIT
For MAPPIT experiments, G-CSF-R fragments were cloned in-frame with a MAPPIT bait receptor as described below. The bait receptor is a chimera of the extracellular domain of erythropoietin (Epo) receptor (Epo-R) and the transmembrane and intracellular portions of leptin receptor, which lacks the tyrosines that serve as STAT3 docking sites. Consequently, Epo-induced activation results in activation of Jak kinase but not STAT3 [13]. Prey sequences (Wsb-1, Wsb-2,

Wsb-1-ΔSB, Wsb-2-ΔSB, Shc, Grb2, FzR) were fused to a gp130-derived fragment that contains 4 functional STAT3 binding motifs. Upon interaction between prey and the G-CSF-R sequence linked to the leptin receptor bait backbone, Epo-induced Jak activation results in phosphorylation of the STAT3 recruitment sites in the gp130 moiety of the prey fusion and subsequent activation of the STAT3 complex, which is quantified by luciferase reporter assay. MAPPIT experiments were performed as described [12,13]. 293HEK-T cells ($2x10^5$) were transfected with bait and prey constructs in presence of luciferase reporter gene (pXP2d2-rPAP-Luci) and 48h after transfection, bait receptors were activated with Epo (0,5 U/ml) for 24 h. Cultures without Epo stimulation were run in parallel. Luciferase activity from a STAT3 luciferase reporter was determined in triplicate using the Steady-Glo luciferase assay system (Promega, Leiden, The Netherlands). Activities are expressed as fold induction, by dividing luciferase activity of the Epo-stimulated samples over non-stimulated values. Western blotting using anti-FLAG M2 monoclonal antibodies (1:6000) (Sigma-Aldrich Chemie, Steinheim, Germany) to determine expression levels of the FLAG-tagged prey proteins was performed as described [59].

Mappit bait constructs

The entire intracellular domains of G-CSF-R (G-CSF-R630-813) and G-CSF-R630-813-Y764F (intracellular domain lacking all tyrosines) were amplified with primers Mappit5 (5'-GGCGAGCTCAAGGAAGAATCCCCTCTG-G-3') and 1250RV using pLNCX-G-CSF-R and pLNCX-mutant "null", which lacks the tyrosines that serve as STAT3 docking sites [59] as templates. The C-terminal 65 amino acids fragment of human G-CSF-R was generated with primers Mappit11_749-769F (5'-GGCGAGCTCATCCACCTAGCCCCTCTTG-3') and 1250RV (5'-ATCTAGATT-AGTCATGGGCTTATGGA-3') with pLNCX-G-CSF-R and "null" [59] as template.The fragments were cloned in TA-vector, sequence checked, SacI and XbaI digested and ligated into pCEL(2L) vector, resulting in pCEL(2L)-C65 and -C65-Y764F, -G-CSF-R630-813 and -G-CSF-R630-813-Y764F, fused to the chimeric EPO receptor-leptin receptor mutant LepF3+2L [12,13,48]. C65 deletion mutants were made with QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA).

Mappit prey constructs

Human SHC was amplified using primers pMG2-SHCf (5'-AAGAATTCAACAA-GCTGAGTGGAGGCGGC-3') and pMG2-SHCr (5'-AACTCGAGTCACAGTT-TCCGCTCCACAGGTT-3') using pHMT-SHC as template [16]. Murine Wsb-1 and Wsb-2 were amplified from 32D cDNA with primers WSB1f1 (5'-AAGAATTCG-CCAGCTTTCCCCCGAGG-3') and WSB1r1 (Y2H constructs), WSB2f1 (5'-AAG-AATTCGAGGCCGGAGAGGAGCCGCT-3') and WSB2r1 (Y2H constructs). PCR products were cloned in TA vector, sequence checked and EcoRI-XhoI digested allowing ligation into pMG2 prey vector [13], thus resulting in SHC-, WSB-1- and WSB-2- Flag-tagged-gp130 fusions. The WSB-1-ΔSB and WSB-2-ΔSB mutants were made by site-directed mutagenesis (QuickChange) using primers WSB1stopf (5'-TGGGCCACTCCAAGGTAAGTCCCTAGCCTT-3'), WSB1stopr (5'AAGGC-TAGGGACTTACCTTGGAGTGGCCCA-3') and primers WSB2stopf (5'-TCCCCGGGTCTAGTCCTCAC-3'), WSB2stopr (5'-GTGAGGACTAGACCCG-

GGGA-3'), respectively.

Retroviral gene transfer in 32D cells

Production of retroviral vectors containing G-CSF-R-Wsb fusions. The G-CSF-R part of the G-CSFRΔ769-Wsb-1 fusion was amplified using primers Fw7 (5'-GTCCTCACCCTGATGACC-3') and 769WSB1fusionR (5'-AAAGCTGGCTGA-TCCTCCGAACCAGAGGTTCTCATA-3'), in which italics denote G-CSF-R sequence, underlined a flexible linker sequence [12] and regular fond Wsb sequence using pBabe-G-CSF-R [1] as template. The Wsb part was amplified using primers 769WSB1fusionF (5'-CTCTGGTTCGGAGGATCAGCCAGCTTTCCCCCGAG-G-3') and WSB1r1 (Y2H construct), and pMG2-WSB-1 and pMG2-WSB-1-SBD as template. Both amplification products were used as template and the annealed fragments could be amplified using primers Fw7 and WSB1r1. The G-CSF-R part of G-CSF-R 769-Wsb2 fusion was amplified using primer 769WSB2fusionR (5'-TCCGGCCTCTGATCCTCCGAACCAGAGGTTCTCATA-3') and Fw7. The Wsb-2 part was amplified using primers 769WSB2fusionF (5'-CTCTGGTTCGGA-GGATCAGAGGCCGGAGAGGAGCCGCT-3') and WSB2r1 (Y2H construct), with pMG2-WSB-2 and pMG2-WSB-2-SBD as input. These fragments were used as PCR template and amplified with primers Fw7 and WSB2r1. The fusion products were cloned in TA cloning vector and sequenced. pBabe-G-CSF-R was digested with HpaI and BglII, blunted (DNA polymerase I large fragment, Invitrogen) and dephosphorylated (shrimp alkaline phosphatase, Roche, Mannheim, Germany). Fusion products were digested with HpaI and XhoI, blunted, ligated in pBabe-G-CSF-R and checked by restriction analysis and nucleotide sequencing. Recombinant BABE virus was produced as described [10]. Interleukin-3 (IL-3)- dependent murine myeloid cell line 32D [17] were infected with the different BABE-based retroviral vectors and selected on 1 μg/ml puromycin (Sigma, Zwijndrecht, The Netherlands). For each construct multiple independent clones were expanded for further analysis.

Flow cytometric analysis of G-CSF-R expression on transduced 32D cells

To determine G-CSF-R expression, transduced 32D cells were labeled with biotinylated antihuman antibody recognizing an N-terminal extracellular epitope of the G-CSF-R (LMM741; Pharmingen, San Diego, CA), subsequently incubated with phycoerythrin-conjugated streptavidin (SA-PE; DAKO Diagnostics, Glostrup, Denmark) and analysed by flow cytometry using a FACScan (Becton Dickinson, Sunnyvale, CA) as described [1].

STAT3 and STAT5 electrophoretic mobility shift assay (EMSA)

EMSAs to determine activation of STAT3 and STAT5 in nuclear extracts of 32D cell transfectants were performed as described [1].

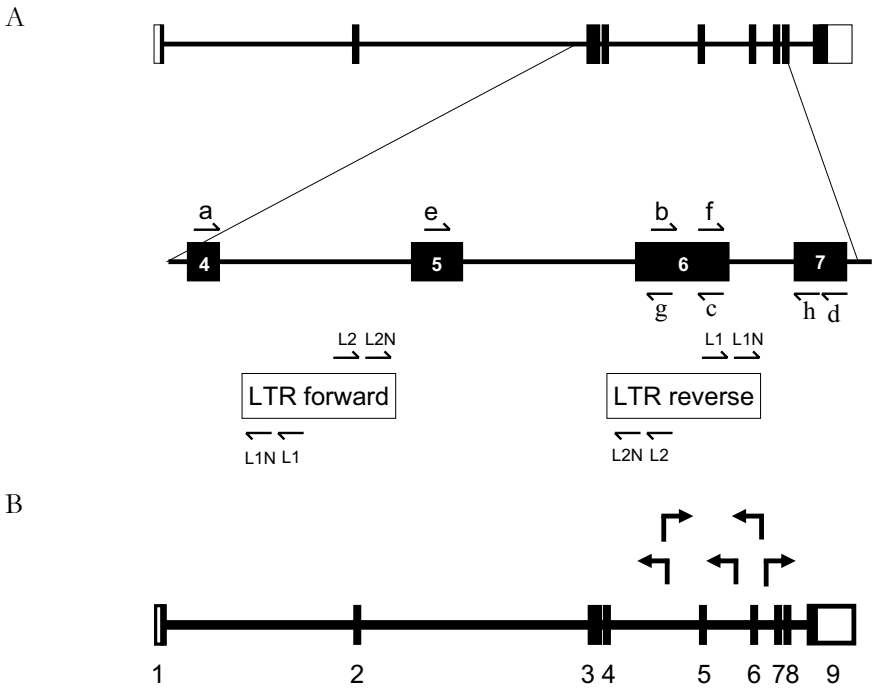

Figure 1. Wsb-2 is a common target for Gr-1.4 MuLV
A) Schematic overview of nested PCRs to detect position and orientation of Gr-1.4 integrations in Wsb-2. The 9 translated exons of Wsb-2 are indicated by black boxes; the white boxes represent untranslated regions of exons 1 and 9. For experimental details see Material and Methods.
B) Examples of virus integration sites in Wsb-2. Arrows indicate positions and orientation of the integrations.

Results

Wsb-2 gene disruption in Gr-1.4 MuLV-induced mouse leukemia

To determine integration frequency and position of the provirus in the Wsb-2 locus, we used a locus specific PCR strategy (Fig. 1A). Among 79 leukemia samples tested, 5 had integrations in Wsb-2. PCR products were sequenced to find the exact location and orientation of the virus integrations. All integrations were located in a region between exon 4 and exon 7 and occurred in both orientations (Fig. 1B). Strikingly, no integrations were found in the 5' region of the gene, which is usually the most common site of MuLV integrations [62] and is generally associated with enhanced and deregulated expression of the affected gene [27, 61]. The fact that integrations were found within the gene suggests that disruption of at least one Wsb-2 allele contributed to leukemia in the 5 affected samples. However, because of the non-clonal nature of MuLV-induced tumors [10, 11], we could not verify the status of the other allele nor determine to what extent Wsb-2 expression was affected in single leukemic clones.

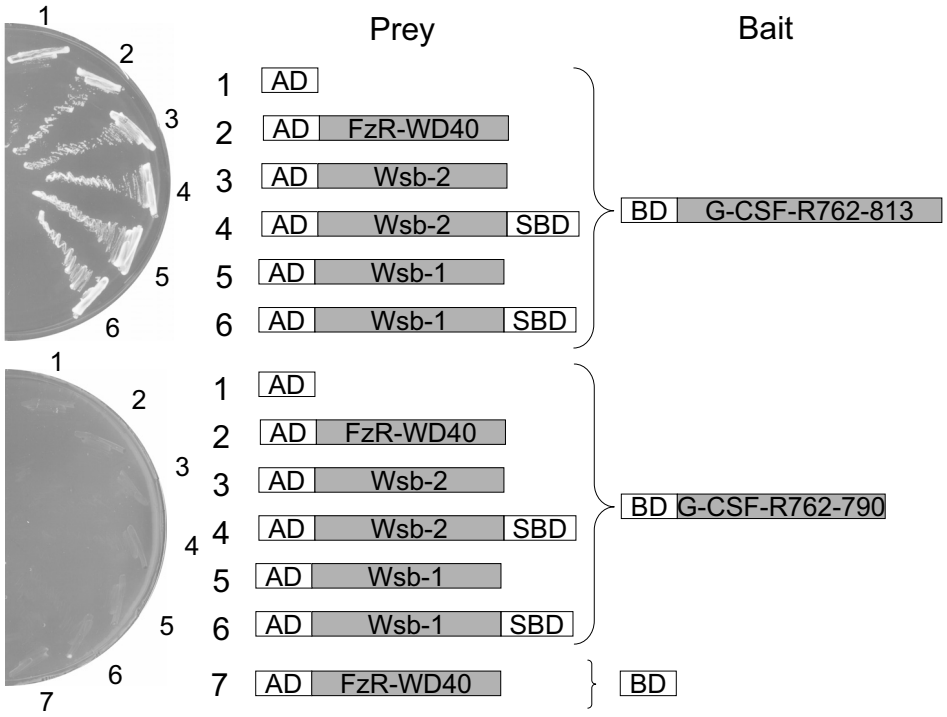

Figure 2. Yeast-two-hybrid analysis showing Wsb binding to the distal 23 amino acids (790-813) of G-CSFR

Yeast strain PJ69-4A was transformed according to the LiAc/SS-DNA/PEG procedure with either pGBT9-GCSFR762-813 (upper section) or pGBT9-G-CSF-R762-790 (lower section) in combination with Wsb-1, Wsb-1 without SOCS Box Domain (ΔSD), Wsb-2, Wsb-2ΔSD, Fzr-WD40 or empty prey vector expressing the AD-domain only.

Decreased WSB-2 expression in a subgroup of AML patients

Because human leukemia is virtually always a clonal expansion of malignant cells, we studied whether WSB-2 expression is deregulated in human AML samples. Gene expression profiling has led to the identification of leukemia subsets based on distinctive gene expression signatures [3, 45, 51]. Adult AML cases (n=285) have been grouped in 16 clusters based on unsupervised cluster analysis [51]. Significance analysis of microarrays (SAM) [49] revealed that WSB-2 expression is significantly decreased (Fold change = 0.45152, score = -6.33656, q-value = 0.109866108) in a subgroup of patients (cluster 4)[51] characterized by a high frequency of mutations in the gene encoding CCAAT enhancer binding protein (CEBPA). This suggests that reduced WSB-2 levels may contribute to the pathogenetic signature of this human AML subclass.

Wsb binding to G-CSF-R in Y2H and co-immunoprecipition assays

We concurrently performed Y2H analysis to detect proteins that associate with the C-terminal part (amino acids 762-813) of the G-CSF-R (suppl. Table 1), a region frequently truncated in patients with severe congenital neutropenia with disease

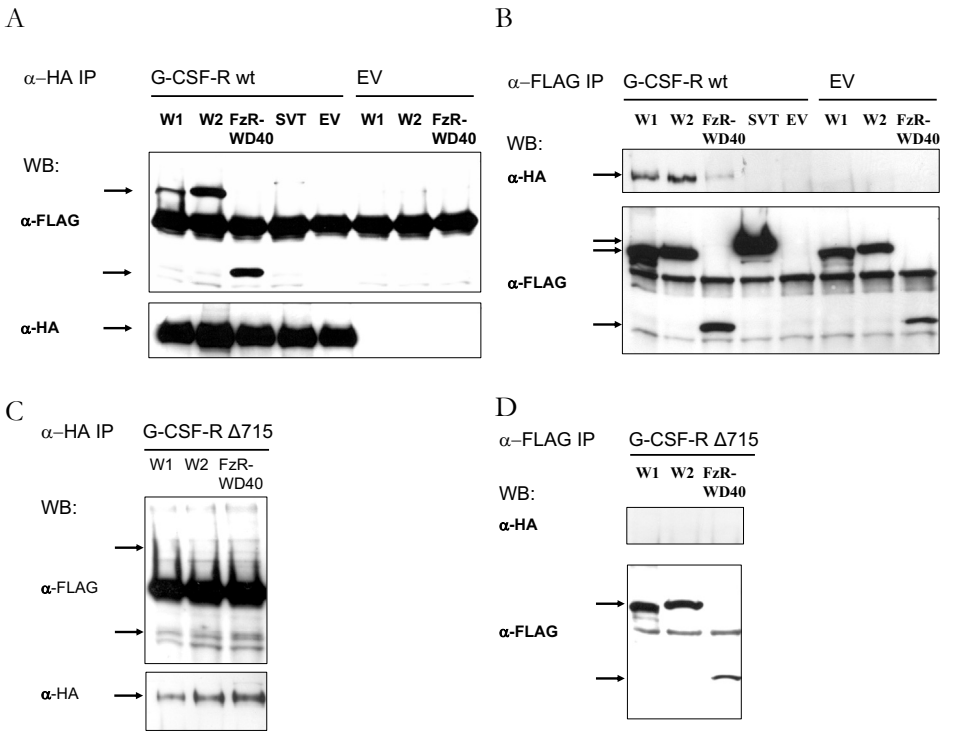

Figure 3 Wsb-1 and Wsb-2 co-immunoprecipitate with G-CSF-R in whole cell lysates
Cos-cells were transfected with pSG5-HAHA-G-CSF-R, pSG5-HAHA-Δ715 or pSG5-empty vector in combination with pMT2-FLAG-Wsb1, pMT2-FLAG-Wsb-2, pMT2-FLAG-FzRWD40 (positive control) or pMT2-FLAG-SVT (negative control). After 48 hours, cells were lysed and proteins immunoprecipitated with antibodies against HA-tagged G-CSF-R (A, C) or FLAG-tagged Wsb and control proteins (B, D). Blots were re-stained with anti-FLAG and anti-HA as indicated. Arrows mark positions of co-precipitated products (upper panels) and input of precipitates (lower panels).

progression to AML [52]. Using G-CSF-R-762-813 as bait, we found that the G-CSF-R C-terminus is a binding site for WD40-repeat domains (Fig. 2). From the mouse 17-day embryo cDNA prey library, we picked multiple clones containing the WD40 portion of Fizzy-related protein 1 (Fzr-1). Because the 7 WD40 repeat domain of Fzr-1 has a high structural homology with that of Wsb proteins, we asked whether Wsb-1/2 also bind to the G-CSF-R C-terminus. Although some background growth of G-CSF-R762-813 bait-containing yeast transformed with empty control vector was noted, growth rates of yeast expressing the Wsb-1/2 preys increased to levels comparable to the positive control (Fzr-WD40) (Fig. 2 upper panel). Deletion of the SOCS box domain (ΔSB) did not affect Wsb-1/2 binding to the G-CSF-R bait (Fig. 2, upper panel). The interaction depended on the most distal 23 amino acids of G-CSF-R; a bait construct lacking this stretch (G-CSF-R762-790) did not show detectable binding to any of the WD40-containing prey constructs (Fig. 2, lower panel). To investigate whether the interactions observed in yeast also take place in mammalian cells, we performed co-immunoprecipitation experiments in COS cells using HA-tagged G-CSF-R and Flag-tagged-Wsb proteins (Fig. 3). Wsb-1 and Wsb-2 co-precipitated with G-CSF-R in HA-IP's as efficiently as FzR-WD40, whereas no

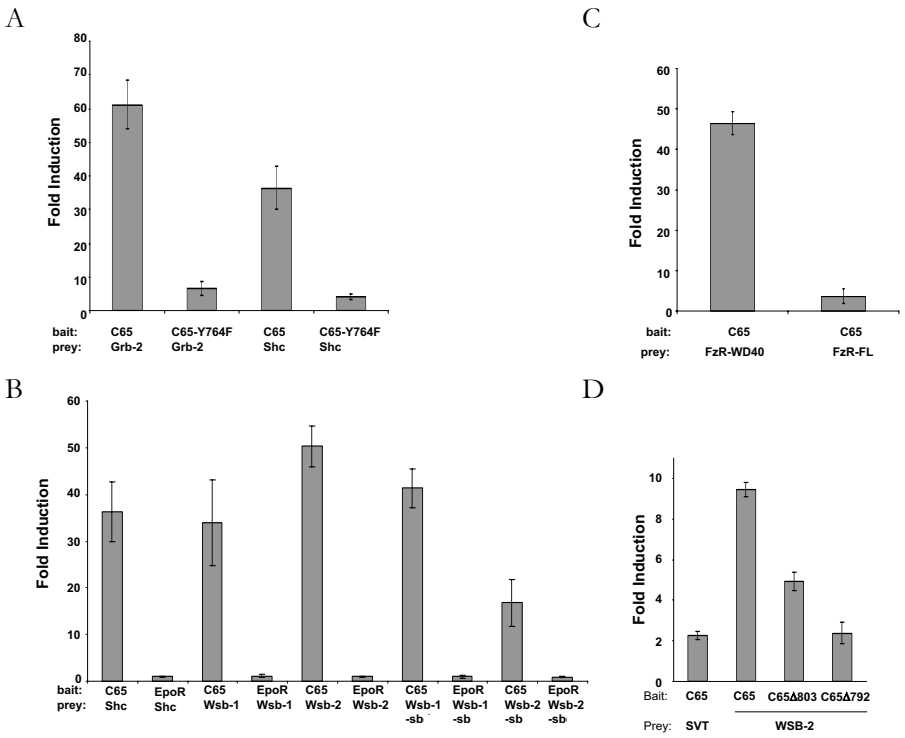

Figure 4. Specific interactions of Wsb, Shc and Grb2 to G-CSF-R determined by MAPPIT
HEK-T cells were transfected with Epo/LeptinR-G-CSF-R bait or Epo/LeptinR-EpoR (control) bait constructs and different gp130-based prey constructs as indicated withpresence of a STAT3-driven luciferase reporter (pXP2d2-rPAPluci). Transfected cells were either stimulated with Epo or not stimulated. Luciferase activity was measured 24 hours after stimulation. Fold induction was calculated by dividing luciferase values of Epo-stimulated cells by values of the non-stimulated cells. Empty prey vector was used as negative contol.
A) G-CSF-R748-813 bait with Y764 (C65) and with Y764 mutated to phenylalanine (C65-Y764F) and full-length Shc and Grb2 preys showing Y764 dependent Shc and Grb2 binding.
B) Interaction of C65 with Wsb-1, Wsb-2 and Wsb1/2 lacking the SOCS box (-ΔSB). C65/Shc and EpoR/Shc were taken along as positive and negative controls, respectively.
C) C65 bait interacts with WD40 domain of FzR but not with full length (FL) FzR.
D) Wsb-2 binding to C65 (748-813) and c-terminal deletion mutants.

interactions were seen with an irrelevant control protein (SVT) or with empty vector controls (Fig. 3A). A similar result was obtained in the reverse FLAG IP/HA Western blot experiment (Fig. 3B). As expected, in neither setting were interactions observed between G-CSF-R 715 and Wsb-1, Wsb-2, or FzR-WD40 (Fig. 3C and D).

G-CSF-R/Wsb interactions in mammalian protein-protein interaction trap (MAPPIT) assay

Preparation of lysates for immunoprecipitation analysis disrupts cell compartmentalization, which may cause artificial protein-protein interactions. To show that Wsb proteins physically interact with G-CSF-R in living cells, we performed MAPPIT, a cytokine receptor based two-hybrid assay [12, 13]. In these

experiments, intracellular parts of G-CSF-R were coupled to the C-terminus of the Epo/Leptin receptor hybrid bait protein [48]. First, we assessed the specificity of this system for G-CSF-R-containing bait constructs using preys containing full length Shc or Grb2, signaling proteins that bind directly to phosphorylated Y764 of G-CSF-R [59]. Shc and Grb2 interact efficiently with G-CSF-R a.a. 748-813 (C65), leading to approximately 40 to 60-fold induction of STAT3-driven reporter activity (Fig. 4A). Upon mutation of Y764 into phenylalanine (C65-Y764F), these activities were reduced to basal levels, supporting the specificity of the interactions. Importantly, Wsb-1 and Wsb-2 also interacted with the C65 bait, resulting in 30 to 50-fold induction of STAT3 activities (Fig. 4B). In contrast, full length Fzr-1 did not interact with the C65 bait, indicating that Wsb1/2, rather than Fzr-1 is a bona fide interaction partner of G-CSF-R (Fig. 4C). In addition, no Wsb-binding activity was observed with a control bait in which the cytoplasmic domain of Epo-R (amino acids 345-429) was fused to the bait backbone [13] (Fig. 4B). This result excludes that Wsb proteins bind to the Epo-R C-terminus and also rule out possible interactions of Wsb with the leptin receptor-based bait backbone. In agreement with the Y2H experiments, removal of the SOCS box did not abrogate binding of Wsb proteins to C65, although for Wsb-2, some reduction of MAPPIT activity was seen. This could indicate that the SOCS box may contribute to the stability of the Wsb-2 protein, as has been demonstrated for SOCS1 [42]. Wsb binding was strongly reduced after removal of the 10 distal amino acids (C65Δ803) and no longer detectable after removal of 21 distal amino acids (C65Δ792) (Fig. 4D). These results confirm and extend the data from Y2H experiments and the IP's and establish that Wsb proteins physically interact with the G-CSF-R C-tail, but not with other cytoplasmic subdomains of G-CSF-R.

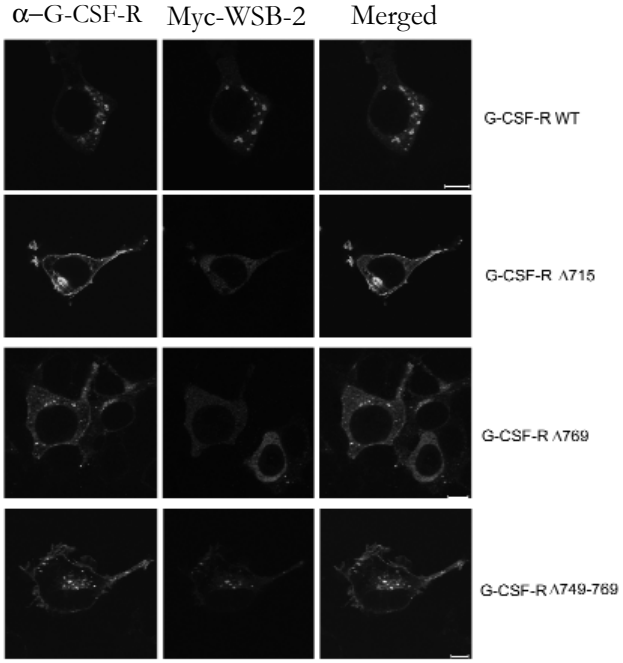

Figure 5. Co-localization of Wsb and G-CSF-R
(see also: suppl. Fig. 5)
Hek-T cells were plated on glass slides and cotransfected with G-CSF-R wt , d715, d749-769 or d769 together with myc-tagged WSB-2. Cells were fixed 2 days after transfection and immuno-stained for G-CSF-R (left panels; green) or myc-WSB (middle panels; red). Colocalization appears as a yellow after merging left and middle panels (right panels). Bar indicates 10 μM.

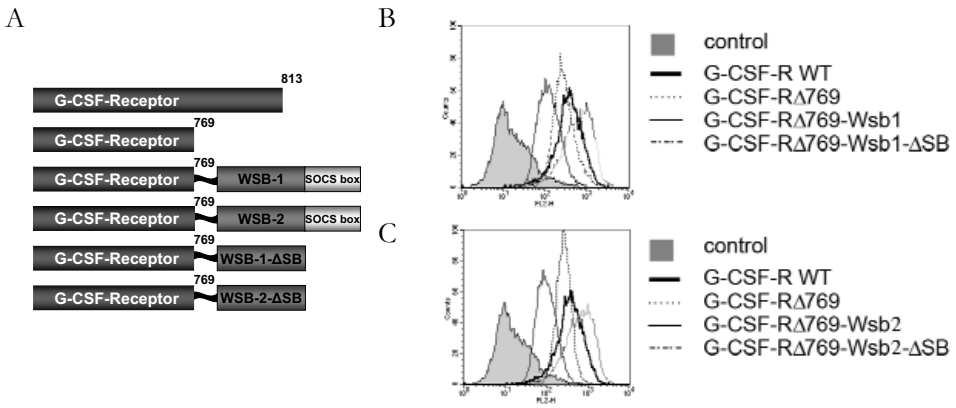

Figure 6. Expression of G-CSF-R-Wsb fusion proteins

A) Schematic overview of G-CSF-R constructs. Wsb-1 and Wsb-2 with or without SOCS box were fused to G-CSF-R 769 via a flexible linker.

B) Membrane expression of G-CSF-R, G-CSF-R 769 and G-CSF-R 769-Wsb-1 fusions on 32D cells analyzed by flow cytometry. Cells were stained as described in materials and methods.

C) Membrane expression of G-CSF-R, G-CSF-R 769 and G-CSF-R 769-Wsb-2 fusions on 32D cells analyzed by flow cytometry. Cells were stained as described in materials and methods.

Co-localization of Wsb with G-CSF-R in situ

To determine whether Wsb and G-CSF-R co-localize in intact cells, we performed confocal laser scanning microscopy. Upon co-expression of Myc-Wsb and EGFP-tagged wild type G-CSF-R proteins in HEK293 cells, both proteins accumulated and predominantly co-localized in intracellular vesicles (Fig. 5, Suppl. Fig. 5). In contrast, co-localization of Wsb was not observed with G-CSF-R-Δ715 and G-CSF-R-Δ769, mutants that lack the WD40-interacting distal 23 amino acids. Instead, Wsb distribution was largely diffuse in the cytoplasm of these cells. G-CSF-R-Δ749-769, a mutant defective in internalization but retaining its distal region again co-localized with Wsb, both on the plasma membrane as well as on intracellular vesicles, confirming the importance of the distal C-terminus of G-CSF-R.

Wsb reduces G-CSF-R membrane expression

WD40 repeat-containing proteins have the potential to interact with a wide variety of target substrates [43, 47]. To specifically assess the role of this interaction on the cellular distribution and signaling function of G-CSF-R we generated expression constructs in which Wsb-1 or Wsb-2 moieties were fused via a flexible linker to the G-CSF-R truncation mutant Δ769, which lacks the Wsb-binding region. In this way, activities of the Wsb proteins are confined to G-CSF-R (Fig. 6A). To determine the role of the SOCS box, we also generated G-CSF-RΔ769-Wsb fusions lacking this domain. Retroviral transduction in 32Dcl10 cells, which lack endogenous G-CSF-R, resulted in membrane expression of all G-CSF-R constructs (Fig. 6B and C). While the mean expression level of G-CSF-RΔ769 was consistently higher than wild type G-CSF-R, fusion of Wsb-1 or Wsb-2 to G-CSF-RΔ769 significantly reduced its expression on the plasma membrane. This effect of Wsb-1 or Wsb-2 was largely decreased upon removal of the SOCS box (Fig. 6B and C). These results suggest that Wsb proteins are important for the control of G-CSF-R cell surface expression.

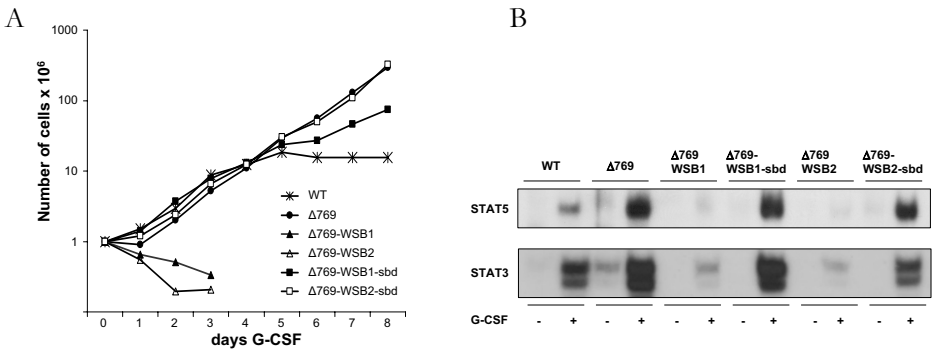

Figure 7 Wsb inhibits G-CSF induced STAT activation and proliferation
A) Proliferation of transduced 32D clones in G-CSF-containing suspension culture.
B) STAT3 and STAT5 EMSA. 32D clones transduced with indicated G-CSF-R constructs were serum deprived for 4 hours and then incubated with (+) or without (-) 100 ng/ml G-CSF for 10 min at 37 °C. Nuclear extracts were prepared and assayed by EMSA using STAT5 and STAT3 specific radiolabeled probes as described in material and methods. Results of one out of three representative clones per construct are shown.

Wsb inhibits G-CSF-induced STAT activation and proliferative signaling

G-CSF-RΔ769 exhibits prolonged signaling activities compared to the G-CSF-R, e.g., resulting in higher levels of STAT3 and STAT5 activation (Aarts LH et al, in preparation). To analyze whether the association of Wsb1/2 with the G-CSF-R could affect the kinetics of G-CSF-induced signal transduction, the 32D cells expressing the various G-CSF-R-Wsb fusion proteins were factor deprived, restimulated with G-CSF and activation of Stat3 and Stat5 was examined by EMSA (Fig. 7A). Compared to the G-CSF-RΔ769, the G-CSF-RΔ769-Wsb1/2 fusion proteins are strongly hampered in their ability to activate STAT3 and STAT5 in a SOCS box dependent manner (Fig. 7A). In long-term suspension culture, 32D/WT-G-CSF-R cells proliferated in response to G-CSF until day 5 of culture and then underwent growth arrest followed by terminal neutrophilic differentiation (Fig. 7B). In contrast, 32D/G-CSF-RΔ769 cells continued to expand, implicating that the distal C-terminus of G-CSF-R inhibits proliferation signaling. Fusion of full length Wsb-1 or Wsb-2 to G-CSF-RΔ769 strongly suppressed G-CSF-induced proliferation, again depending on the presence of the SOCS box.

Discussion

WD40 repeat and SOCS box-containing proteins and malignant transformation

In a retroviral mutagenesis screen, we have identified Wsb-2 as a novel candidate disease gene in myeloid leukemia. Virus integrations were located within the gene, conceivably disrupting Wsb-2 expression. Reduced WSB-2 transcript levels were found in a subgroup of AML patients, suggesting that loss of WSB function may contribute to human disease. The WD40 protein-protein interaction domain and the SOCS box are characteristic of the WSB proteins. Of the more than 40 proteins

that contain a SOCS box, a relationship with malignancy has currently best been characterized for the Von Hippel Lindau (VHL) protein. The VHL gene is a tumor suppressor gene mutated in the Von Hippel Lindau syndrome, a cancer prone condition predisposing to tumors of the kidney, central nervous system, and retina. The VHL protein targets hypoxia-inducible transcription factors 1 and 2 (HIF) for proteasomal degradation. Mutations in VHL patients result in disruption of the SOCS box and thus in the accumulation of HIF , leading to increased transcription of genes involved in angiogenesis and growth factor production (reviewed in [31]).
WD40 proteins are involved in a wide variety of processes, such as cell cycle control, chromatin assembly, DNA repair, signal transduction, apoptosis and vesicular trafficking [34, 58]. Despite their crucial involvement in these cellular processes, to date only few of the 136 human WD40 proteins have been associated with malignant disease [34]. For instance, Abelson helper integration site-1 (AHI-1), a novel gene encoding a WD40-repeat and SH3 domains, was recently identified as a frequent target for proviral integration in mouse pre B-cell lymphomas [25]. Moreover, AHI-1 deregulation was seen in Philadelphia chromosome-positive leukemia, suggesting a role for this protein in human leukemia [26].

WD40-repeat proteins in G-CSF signaling

We became specifically interested in determining the action of Wsb proteins on G-CSF signaling after having established in Y2H screens that the G-CSF-R C-terminus has affinity for WD40-repeats. Importantly, truncated G-CSF-R found in SCN patients who are prone to develop AML have lost this WD40-binding domain [52], which contributes significantly to the hyperproliferative signaling function of these mutant receptors (Fig. 7B). Although the experiments reported in this paper were specifically aimed at the role of Wsb proteins, it is likely that other WD40 proteins may also bind to this region. For instance, we found that the scaffolding protein Receptor for Activated C Kinase 1 (RACK1) also interacted with the G-CSF-R C-terminus in MAPPIT assays (data not shown). RACK1 may act as a scaffold for a variety of signaling proteins, e.g., activated protein kinase C and the SH2-containing protein tyrosine phosphatase Shp2 [38] and thereby modulate G-CSF signaling. Although this needs further investigation, it raises the possibility that the G-CSF-R C-terminus interacts with multiple WD40-containing regulatory proteins controlling the magnitude of G-CSF-induced proliferation. Importantly, MAPPIT appeared a valuable tool to discriminate between bona fide and irrelevant interactions of WD40 proteins with the G-CSF-R C-terminus. For instance, full length Fizzy-related or TEP1 [56], WD40 proteins that co-precipitated with G-CSF-R in IP's, did not interact with G-CSF-R in the MAPPIT system, possibly due to the fact that Fzr-1 and TEP1 are directed to different cellular compartments.

Mechanism of Wsb-induced downmodulation of G-CSF responses

To address how Wsb proteins affect the signaling function of the G-CSF-R, we generated chimeric proteins of G-CSF-R lacking the WD40-binding region (Δ769) and Wsb-1 or Wsb-2. In this way we could specifically examine the effects of Wsb binding to the G-CSF-R and circumvent phenotypes caused by overexpression of Wsb proteins resulting from (1) out-competition or increase of binding of alternate WD40 proteins (such as RACK1) to the G-CSF-R or (2) altered interactions with

other proteins with affinity for Wsb. Essentially, fusion of Wsb to G-CSF-RΔ769 recapitulated the role of the C-terminal amino acids 770-813 in a negative feedback loop, although the inhibitory effects of Wsb on both plasma membrane expression and signaling function were more pronounced than those of the G-CSF-R distal C-terminus (Figs. 6 and 7). We have currently no explanation for this difference. It is possible that Wsb expression levels under physiological conditions are rate limiting, leading to incomplete saturation of binding. Alternatively, other proteins that bind to amino acids 770-813 of G-CSF-R, for instance RACK1, may counteract the activities of Wsb. We also cannot exclude that the G-CSF-R-Wsb fusion proteins are more sensitive to ER-associated degradation (ERAD), a component of the protein quality control system, than wild type G-CSF-R, which would lead to decreased routing to the plasma membrane [39].

Role of SOCS box-containing proteins in G-CSF-R routing

Because deletion of the SOCS box almost completely abolished the effects of Wsb on G-CSF-R function, recruitment of E3 ligase activity through the ECS complex seems key to the observed decrease in G-CSF-R cell surface expression levels and to the reduced proliferative signaling. Whereas poly-ubiquitylation serves as a signal for proteasome-mediated protein degradation, mono-ubiquitylation may act as a sorting signal for delivery of proteins in the biosynthetic or endocytic pathway to multi vesicular bodies (MVB) and lysosomes [2, 21]. Direct or indirect association with E3 ligases regulates cell surface expression of a number of receptors. For instance, plasma membrane expression of AMPA-type glutamate receptors was shown to depend on mdm-2 E3 ligase-mediated degradation of its scaffold protein PSD-95 [4]. Moreover, cell surface expression levels of the transmembrane adhesion molecule SHPS-1 was shown to depend on the activity of the E3-ligase SCFNFB42 [41]. Kumar and colleagues have recently proposed a mechanism for degradation of the interferon receptor 1 (IFNAR1), involving the Homolog of Slimb (HOS) F-Box protein, which interacts with Skp1 and Cullin-1 to form the SCFHOS E3 complex [32, 33]. Similar to Wsb, HOS (alternatively termed β-TrCP2 or Fbw1b) binds to substrates via its WD40 domain. Three lysine residues in the cytoplasmic tail that are ubiquitylated by SCFHOS are essential for IFNAR1 degradation [32]. It is conceivable that Wsb-mediated association of ECS to G-CSF-R has a similar role in the down modulation of signaling via ubiquitylation of receptor lysines, although this needs further investigation. Indeed, preliminary data show that arginine substitution of the 5 lysine residues in the cytoplasmic domain of G-CSF-R results in prolonged signaling and altered intracellular routing (Aarts et al, manuscript in preparation). However, a major difference with SCFHOS-controlled lysosomal targeting of IFNAR1 is that binding of Wsb to G-CSF-R C-terminus does not require receptor activation, nor does serine phosphorylation seems critical for the interaction, given the absence of a conserved serine residue in the Wsb-binding region of G-CSF-R. This suggests that Wsb proteins are dispensable for ligand-induced internalization and routing and mainly affect the steady state distribution of G-CSF-R. A comparable role has recently been demonstrated for the suppressor of cytokine signaling SOCS5 in controlling homeostasis of the epidermal growth factor receptor [30]. Previous studies have shown that SOCS3 and to a lesser extent SOCS1 inhibit G-CSF responses. SOCS3 acts in a classical negative feedback loop,

involving G-CSF-induced activation of STAT3, STAT3-induced transcription of SOCS3 and subsequent binding of SOCS3 protein to phosphorylated tyrosine residue Y729 of G-CSF-R [20,23]. The inhibitory activity of SOCS3 on G-CSF-induced proliferation of myeloid precursors largely depends on the presence of the SOCS box [54]. In combination with our present results, these observations fit into a model in which both ligand-induced (via SOCS3) and constitutive (via Wsb proteins) recruitment of E3 ligase activities control signaling from the G-CSF-R. G-CSF is unique among different hematopoietic cytokines for its ability to induce transient proliferation followed by growth arrest and terminal myeloid differentiation, a bimodal response that depends on multiple mechanisms involving the membrane distal C-terminal region of G-CSF-R [52]. An attractive hypothesis is that Wsb contributes to this tightly balanced control of G-CSF-induced proliferation and differentiation by controlling distribution of the G-CSF-R to the plasma membrane. Whether this involves choices in specific routing mechanisms and/or accumulation in specific compartments or micro domains remains to be addressed.

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CHAPTER 6

The stress response gene encoding thioredoxin-interacting protein (Txnip) is a frequent target for deregulation by proviral integration in murine acute myeloid leukemia

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Abstract

Graffi-1.4 (Gr-1.4) and CasBrM murine leukemia viruses induce myeloid leukemias due to deregulation of genes by proviral integration. We recently identified the gene encoding thioredoxin-interacting protein (Txnip), alternatively known as 1,25-dihydroxyvitamin D-3 up-regulated protein-1 as a target for Gr-1.4 insertion. Txnip is involved in the regulation of reactive oxygen species (ROS)-induced stress responses. ROS influence signal transduction by modulating the activity of for instance protein and lipid phosphatases in cell differentiation processes at relatively low levels, whereas high ROS levels induce apoptosis. Perturbations of the cellular redox state have a major impact on these processes and are implicated in oncogenic transformation. Txnip regulates the cellular redox state by suppressing thioredoxin (Trx) activity, thereby controlling cell survival. Integrations in the gene encoding Txnip occurred at the 5' and 3' region with a frequency of 100% of the Gr-1.4 and 62% of the CasBrM induced leukemias. A hotspot of virus insertions was found in a region located 900 base pairs upstream of the ATG, near a newly identified heat shock element (HSE). The presence of Gr-1.4 LTR sequences in the Txnip 5' promoter region or 3' UTR results in a 2-2.5 times enhanced signal when compared to the wild-type promoter in luciferase assays. Normally, Txnip is expressed at moderate levels in myeloid cells and is significantly down modulated during granulocytic differentiation. Treatment of hematopoietic Ba/F3 and 32D cells with 200 μM H_2O_2 for 4 hours resulted in approximately 2 to 3 fold Txnip mRNA induction. Txnip mRNA is significantly increased in a panel of human adult and pediatric AML patients characterized by t(8;21). In addition, a subset of AML samples show enhanced Txnip protein expression compared to normal bone marrow and CD34 positive control cells. Ectopic expression of Txnip resulted in accelerated apoptosis and inhibited proliferation, indicating that deregulation as a single event does not confer a growth advantage and implying that additional events are needed for full leukemic transformation of myeloid precursors.

Introduction

Acute myeloid leukemia (AML) is characterized by the uncontrolled outgrowth of malignant immature myeloid cells in the bone marrow (BM) and peripheral blood [25-27, 33]. It is now generally accepted that leukemogenesis is a multistep process, requiring defects in multiple regulatory genes that may be caused by chromosomal translocations and deletions or by more subtle mutations (point mutations, small deletions or insertions). In approximately 20% of AML cases the genetic defects are unknown [25].

Thioredoxin (Trx) is a multifunctional protein present in the cytoplasm and nucleus. Trx also functions outside the cell and has been reported to stimulate proliferation of lymphocytes and tumor cells, and to induce chemotaxis of neutrophils, monocytes and T-cells [5, 6]. By retroviral mutagenesis using the Graffi-1.4 (Gr-1.4) and Cas-Br-M murine leukemia virus (MuLV), we recently identified the gene encoding thioredoxin-interacting protein (Txnip) as a common target for viral integration in myeloid leukemias [12]. The Txnip protein was originally described as

1,25-dihydroxyvitamin D-3 up-regulated protein-1 and first identified in the promyelocytic leukemia cell line HL-60 [9]. Txnip has been implicated in the control of the cellular redox status through binding to the redox-active site of Trx, thereby inhibiting its reducing activity [19, 40]. The Txnip gene is a stress responsive gene, induced by cellular stresses such as heat shock and high concentrations of intracellular reactive oxygen species (ROS) [19, 20, 28, 40, 47]. Txnip has been shown to prevent translocation of Trx to the nucleus, thereby inhibiting the redox-mediated transcription modulatory activity of Trx on NFkB and Ref-1 target genes [40]. Furthermore, Txnip binding to Trx results in the release of Ask-1, a mitogen-activated kinase kinase kinase involved in oxidative stress-induced apoptosis, from the Trx/Ask-1 complex [19, 24, 36].
In this study, we have mapped the provirus integrations in the Txnip gene in Gr-1.4 and CasBrM MuLV-induced leukemias and studied the consequences of these integrations for the regulation of Txnip transcription. In addition, we have studied the effects of enforced expression of Txnip in myeloid 32D cells and primary BM progenitors. Finally, we have investigated Txnip protein levels in human AML samples.

Materials and methods

Locus specific nested PCR

A directed PCR strategy to identify provirus integrations in the specific loci in mouse leukemia cells was recently described in detail [11, 12]. To determine the localization of Gr-1.4 and CasBrM provirus in the Txnip locus in an extended panel of leukemias, a nested PCR was performed on isolated chromosomal DNA from the primary tumors (Fig. 1). For the first PCRs, locus specific primers a (5'-CTCTAGTCAGCTCCTGAGGCATCTCTCAGC-3'), b (5'-TTTGTTTTCGAGT-TCCTGTCATTCTTTTCC-3'), c (5'-GCTCAGGTTTTCATAGTTTGTGTGTG-TG-3') and d (5'-AACAGGGCAGAGAACTGGGCTCAGAGATGG-3') were used in combination with Gr-1.4 MuLV LTR specific primers L1 (5'-TGCAAGAT-GGCGTTACTGTAGCTAG-3') and L2 (5'-CCAGGTTGCCCCAAAGACCTG-3') or with CasBrM LTR specific primers CL1 (5'-CGAACTTCCCTATTCTCAGT-TCTGTATTT-3') and CL2 (5'-CCCTGTGCCTTATTTGAACTAACCAAT-3'). Cycling conditions were 30 seconds at 94 ºC, 30 seconds at 60ºC and 4 min at 72ºC for 30 cycles. For the second, nested PCRs, locus specific primers e (5'-AAGGTTT-GCCAGATAACTAAGTGAAACTAA-3'), f (5'-CCCTCTGTCCTCTCTCCAAA-TTCACTAAA-3'), g (5'-TGCAGGTGGGAATGTGGTGCTTAGAAC-3') and h (5'-GTAAAAGACGCTTTCTGCGAAGCCTGAG-3') were used in combination with Gr-1.4 MuLV LTR specific nested primers L1N (5'-AGCCTTATGGTGGG-GTCTTTC-3') and L2N (5'-AAAGACCTGAAACGACCTTGC-3') or with CasBr LTR specific nested primers CL1N (5'-GCTCGCTTCTGGCTTCTGTTTG-3') and CL2N (5'-TGCTAAACCTGATGGTGGGTCTTTC-3'). Cycling conditions were 30 seconds at 94 ºC, 30 seconds at 60 ºC and 4 min at 72ºC for 30 cycles. To verify the specificity of the obtained PCR bands, we performed Southern- blotting using standard protocols. The blots were hybridized with radio labeled TXNIP locus specific probes P1: (5'-GCCGGTGAAATTGAAACAGGTTAGGGACAT-3') and

P2: (5'-GTCAGCTCCACTTTGTGGGACCCTGAGACC-3'). Signals were visualized by autoradiography according to standard procedures. Positive bands were cloned in TA-cloning vector (Invitrogen-N.V. Life Technologies S.A., Merelbeke, Belgium) and sequenced by using an ABI3100 sequencer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Southern-blotting

Chromosomal DNA samples (10 μg) isolated from primary leukemias [12] were digested with 50 units of restriction enzymes BamHI, EcoRI, or SacI overnight at 37 ºC. DNA fragments were separated at a 0.8 % agarose gel and analyzed by Southern blotting using radio labeled TXNIP locus specific probe.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Cells were incubated at 42 ºC and at 37 ºC respectively for 60 minutes. Nuclear extracts were prepared and assayed by EMSA as described previously [1, 48], using Santa Cruz buffer: 50 mM Tris pH=7.8, 250 mM NaCl, 5 mM DTT, 25% glycerol for probe binding buffer. The double strand Txnip probe 5'-GCATTCACCAGAA-AGGACTGA-3' and mutant Txnip probe 5'-GCATTCACCATTAAGGAATGAA-A-3' (negative control) were generated and labeled as described [1, 48].

Production of retroviral vectors, expression and promoter constructs

pLNCX2-Flag-Dusp10 vector

Primers FlagtagF1 (5'-CCCATGGATTACAAGGATGACGACGATAAGAGG-3') and FlagtagR1 (5'-CCTCTTATCGTCGTCATCCTTGTAATCCATGGG-3') were diluted in H_2O (1 μg/ml), heated at 94 ºC for 5 minutes and than slowly cooled down to 20 ºC (ramp = 0,1 ºC/sec. A kinase reaction was performed on 0,5 μg double strength DNA fragments using T4 kinase (Invitrogen-N.V. Life Technologies S.A., Merelbeke, Belgium) according to standard protocol and cloned in the StuI-site of pLNCX2 cloning vector (Clontech Laboratories, Palo Alto, CA). Total RNA was extracted from 32D-WT cells using TRIZOL reagent (Invitrogen-N.V. Life Technologies S.A., Merelbeke, Belgium) according to standard protocol. RNA was transcribed into cDNA using Superscript (Invitrogen-N.V. Life Technologies S.A., Merelbeke, Belgium) and random hexamers. Dusp10 was amplified with primers Dusp10f1: (5'-AACCCGGGATGCCTCCATCTCCTTTA-GACGAC-3') and Dusp10r1: (5'-AACCCGGGCGTTGTCACACAACTGTCTCC-AT-3') analyzed on 1 % agarose gel and cloned in TA cloning vector (Invitrogen-N.V. Life Technologies S.A., Merelbeke, Belgium). The SmaI fragment of TA-Dusp10 was cloned in the StuI site of pLNCX2-Flag vector.

pBABE-Dusp10 vector

Dusp10 was amplified with primers Dusp10_SmaIF (5'-AACCCGGGATGCCTC-CATCTCCTTTAGACGAC-3') and Dusp10_SmaIR (5'-AACCCGGGGTCACAC-AACTGTCTCCAT-3') using 32D derived cDNA (for details: pLNCX2-Flag-Dusp10 vector) as template. Amplified fragments were cloned in TA cloning vector and nucleotide sequenced. The SmaI fragment of TA-Dusp10 was cloned into the HpaI site of pBABE-Flag vector.

R780-Txnip vector
Txnip cDNA derived from 32D cells was amplified with primers TXNIP_SmaIF (5'-AACCCGGGATGGTGATGTTCAAGAAGATCAAGTC-3') and TXNIP-SmaIR (5'-AACCCGGGTCACTGCACGTTGTTGTTGTTGTT-3') as template. Amplified fragments were cloned in TA cloning vector and nucleotide sequenced. The SmaI fragment of TA-TXNIP was cloned into the HpaI site of pBABE-Flag vector. The EcoRI-SalI fragment of pBABE-Flag-TXNIP was cloned in the Not-1 site of R780 vector resulting in R780-Flag-TXNIP.

Promoter constructs (Fig. 4A)
To generate pGL3-V5, a 1448 nucleotide region upstream of the major ATG of the TXNIP open reading frame was amplified by PCR using primers VDUP-F1 (5'-AACGCGTCTCTAGTCAGCTCCTGAGGC-3') and VDUP-R2 (5'-CAGATCT-GATTGAGCCGAGTGGGTTCAAG-3'), and inserted into the MluI and BglII sites directly upstream of the luciferase gene in the vector pGL3-basic (Promega). A 1 kb fragment of the 3' UTR of the Txnip open reading frame was amplified with primers TXNIP-F3 (5'-CGTCTAGAGCCTGCAGGAAATGAAGCATC-3') and TXNIP-R5 (5'-CGTCTAGAAATGTAGAATGTCACCCAGG-3'), and cloned into the XbaI site downstream of the luciferase gene in pGL3-V5 to create pGL3-V5K3. The Gr-1.4 LTR was PCR amplified with primers Graffi-LTR1 (5'-GAAAGACCC-CACCATAAGGCT-3') and Graffi-LTR2 (5'-AATGAAGACCCCCGAGGTGG-3'). The LTR was inserted into the DraI site in the Txnip promoter region of pGL3-V5 and pGL3-V5K3, resulting in the plasmids pGL3-V5LF, and pGL3-V5LFK3, respectively. The Gr-1.4 LTR was also inserted in the NcoI site of pGL3, pGL3-V5K3LF, resulting in pGL3-V5K3LR.

Cell culture and retroviral transduction
32D and Ba/F3 cells
The (IL-3)-dependent murine hematopoietic cell-lines Ba/F3 and 32D containing the wild-type G-CSF receptor (32D-WT1) [14] were expanded and differentiated as described [10]. 32D-WT1 cells were infected with pLNCX-Flag-Dusp10 virus and selected with G418 (Gibco-BRL). Several independent clones were expanded for further analysis.

Primary bone marrow cells
Hematopoietic cells were harvested from the femurs and tibiae of 8 to 12 week old FVB mice, cultured and co-infected with equal amount of BABE virus together with recombinant R780-TXNIP or empty R780 virus as previously described [16]. Transduction efficiencies were measured by flow cytometry. Equivalent number of GFP positive cells were plated in triplicate in methocult (M3231) (Stemcell Technologies inc., Vancouver, Canada), either with human (h) granulocyte colony stimulating factor (G-CSF) (10 ng/ml), granulocyte macrophage (GM)-CSF (20 U/mL), or with a cocktail of murine (m) interleukin-3 (mIl-3) (10 ng/mL), hIL-6 (100 ng/mL), and stem cell factor (mSCF) (100 ng/mL), and puromycin (1.5 μg/mL). Colonies of more than 50 cells were counted on day 7 of culture.

Luciferase reporter assay

COS, HEK-T, and HEK-293 cells were seeded in 24-well plates at $0.5x10^6$ cells/well and transfected by $CaPO_4$ [46] with a 7:3 ratio of reporter plasmid and pcDNA3.1/mycHis/LacZ (Invitrogen), encoding LacZ as an internal control. Cells (32D, Ba/F3) were electroporated (280 V, 960 μFD capacitance) with 19 μg of reporter plasmid and 1 μg of LacZ plasmid per $10x10^6$ cells. One day after transfection, cells were lysed and assayed for luciferase and β-galactosidase activity as described [11].

Real-time reverse transcriptase PCR

RNA isolation and cDNA synthesis were performed as previously described [21]. The primers used for amplifications were: Txnip-forward 5'-TCCTTGCTGATCTACG-TCAG-3' and Txnip-reverse 5'-TGTCTTGAGAGTCGTCCACA-3', and ribonuclease inhibitor (RI)-forward 5'-TCCAGTGTGAGCAGCTGAG-3' and RI-reverse 5'-TGCAGGCACTGAAGCACCA-3'. The real-time PCR was performed, using TaqMan technology (PE Applied Biosystems model 7900 sequence detector; Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Reactions were carried out in a volume of 25 μL of a mixture containing 2 μL cDNA dilution, primers at 5 μM, and 12.5 μL 2 x SYBR Green PCR Master mix (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The amplification program used is previously described [21]. To confirm amplification specificity, the PCR products were subjected to melting curve analysis.

Results

Identification of virus integration sites in Txnip

The first two Gr-1.4 integration sites in the Txnip locus identified by inverse PCR (iPCR) were located 1.1 kb upstream of the transcriptional start site and 0.35 kb

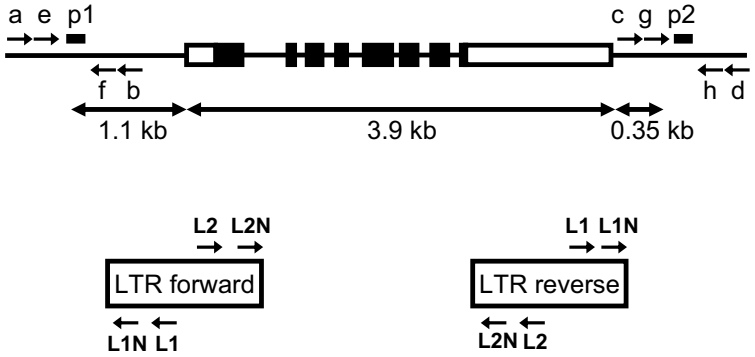

Figure 1. Nested PCR strategy to identify Gr-1.4 virus integration sites and orientation of the provirus

The first eight PCRs were performed with primers a, b, c and d in combination with LTR specific primers L1 and L2, using chromosomal DNA isolated from a panel of primary leukemias as template. To amplify the PCR products, nested PCRs were performed using primers e, f, g and h combined with the LTR specific nested primers L1N and L2N. The specificity of the PCR products was checked by Southern blot analysis with probes P1 and P2.

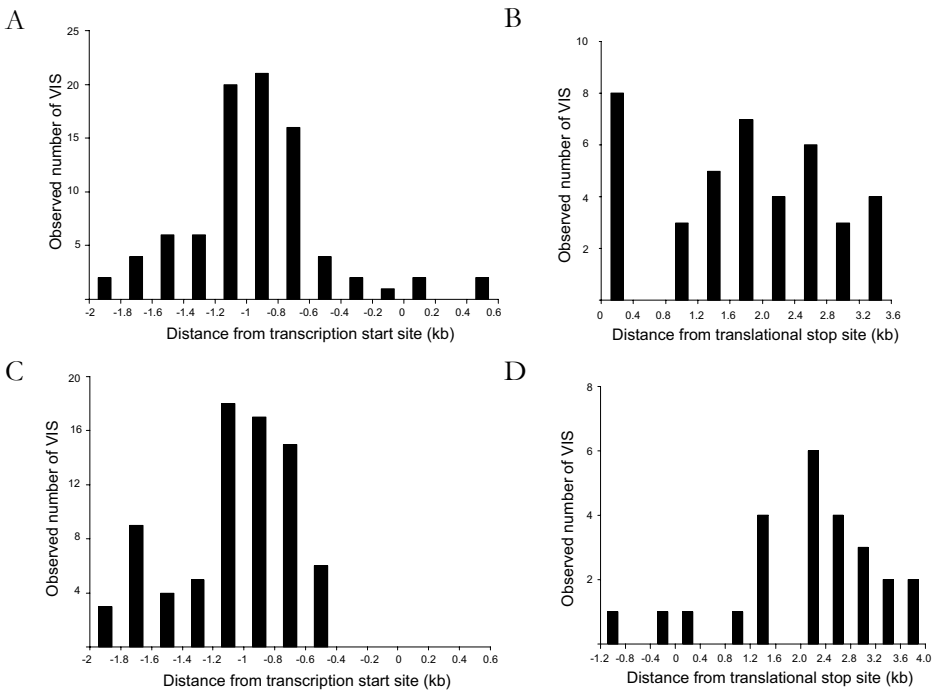

Figure 2. Viral integrations in Txnip
A,B) The 5' and 3' UTR of Txnip were divided in regions of 0.2 kb or 0.4 kb, respectively. From 14 leukemias, the amount of Gr-1.4 virus integration sites per defined region is depicted.
C,D) Same as A, B. From 24 leukemias the amount of CasBr-M virus integration sites per defined region is depicted.

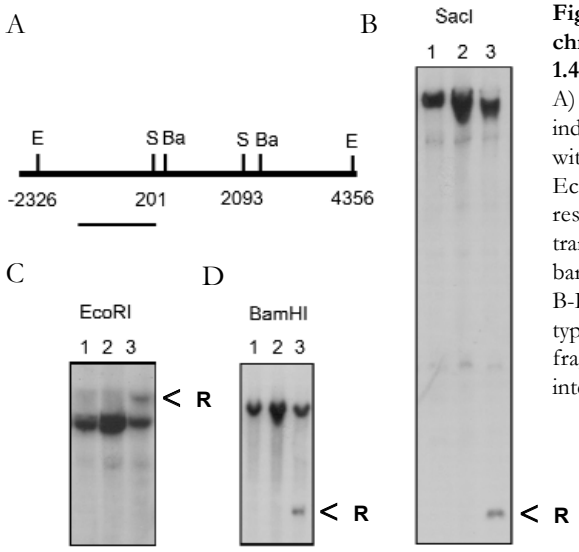

Figure 3. Southern blot analysis of chromosomal DNA isolated from Gr-1.4-induced tumors
A) Chromosomal DNA of three Gr-1.4-induced myeloid leukemias was digested with restriction enzymes BamHI (Ba), EcoRI (E), or SacI (S). Positions of restriction sites in Txnip relative to transcriptional start site are depicted. The bar marks the position of the probe.
B-D) Southern blot showing Txnip wild-type (WT) fragments (leukemia 1-3) and fragments rearranged due to proviral integration (R) (leukemia 3).

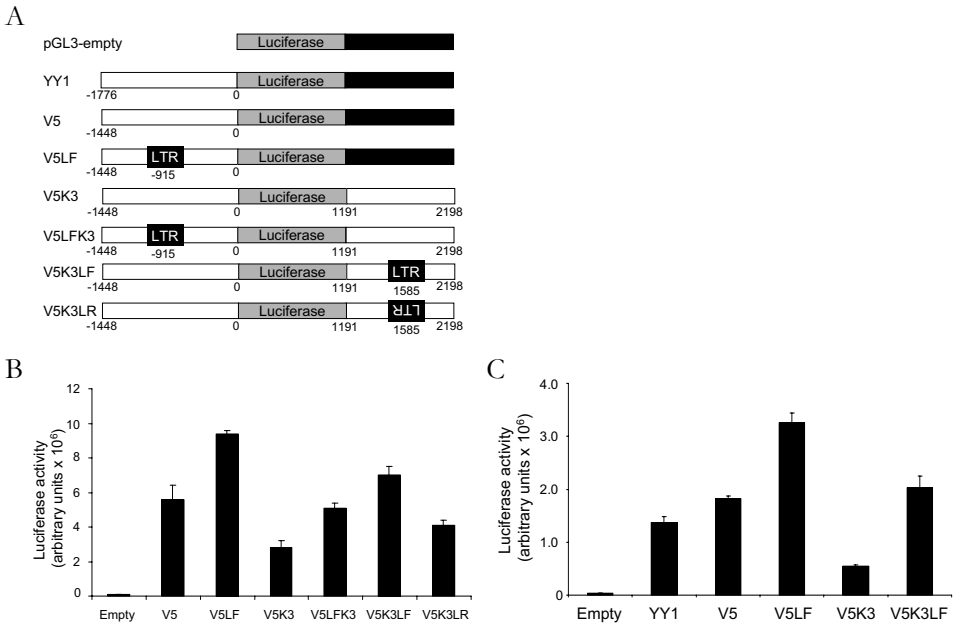

Figure 4. Transient TXNIP promoter assay

A) Reporter plasmids containing different promoter and 3' UTR sequences were generated as depicted (pGL3-empty, YY1= Yin-Yang-1 5' region (-1776 bp - 0) [11], V5='Txnip 5' region (-1448 bp - 0), V5LF=V5 + Gr-1.4 LTR forward orientation, V5K3= V5 + 3' UTR, V5LFK3= V5K3 + Gr-1.4 LTR in V5 region in forward orientation, V5K3LF/R= V5K3 + Gr-1.4 LTR in K3 region in forward or reverse orientation).

B, C) Cos cells (B) and myeloid 32D cells (C) were transfected with the different promoter plasmids by electroporation. One day after transfection, cells were lysed and assayed for luciferase activity. Luciferase activity values were normalized against β-galactosidase activity measured by spectrophotometry.

downstream of Txnip [12]. To further investigate the frequency of integrations in this locus, we performed directed PCR on chromosomal DNA isolated from 14 independent leukemias induced by Gr-1.4 (Fig. 1). Strikingly, all leukemia samples analyzed contained virus integrations in the 5' promoter as well as in the 3' UTR of Txnip. Proviral integrations occurred in both orientations with equal frequencies. Most virus integrations were found in a region -1.2 kb to -0.6 kb of the transcriptional start site (Fig. 2A). The Gr-1.4 integration in the 5' promoter region was confirmed by Southern blot analysis in one out of these leukemia samples (Fig. 3). Integrations 3' of the translational stop were found less frequently and were more equally distributed over a region of 4 kb (Fig. 2B). Directed PCR on chromosomal DNA isolated from a panel of CasBrM-induced leukemias gave comparable results (Fig. 2C and 2D), although the frequency of integration (14 out of 24, 58%) was somewhat less than in Gr-1.4-induced tumors. Notably, all leukemia samples with 3' UTR integrations also had integrations in the promoter region, suggesting that these insertions cooperate in deregulation of Txnip expression. These findings indicate that Txnip is a common target for provirus integration in mouse myeloid leukemia.

Integration of Graffi-1.4 MuLV LTR deregulates Txnip expression

To investigate the consequences of virus insertions in the Txnip locus, we performed luciferase reporter assays in myeloid 32D and in COS cells. Because insertions were found in the 5'- and 3' UTR of Txnip, we introduced Gr-1.4 LTR sequences in both of these regions (Fig. 4A). Approximately 2-2.5 fold higher luciferase activity was measured in COS cells and myeloid 32D cells following Gr-1.4 LTR insertion in the Txnip promoter (Fig. 4B and 4C). Notably, inclusion of the Txnip 3' UTR in the reporter construct significantly reduced luciferase activity (Fig. 4B, 4C), suggesting a possible role for the 3' UTR in mRNA stability or in translational regulation. Insertion of LTR sequences in the 3' UTR resulted in a 2 to 2.5-fold higher luciferase activity, indicating that virus insertions in this region

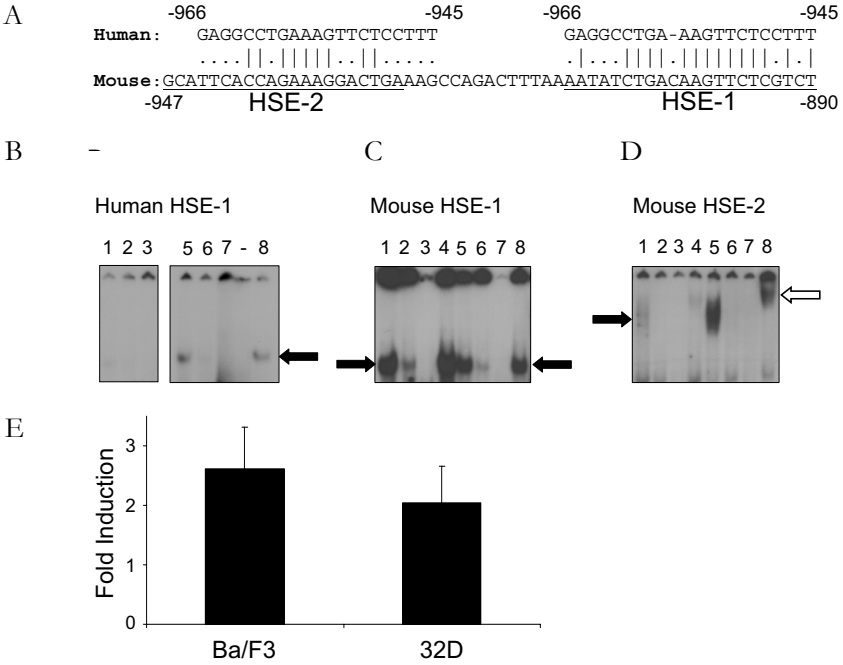

Figure 5. Activity of Stress-induced binding of HSF-1 to HSE-2 in the mouse Txnip 5'-promoter region

A) Alignment of human and mouse 5' Txnip regions encompassing HSE-1 (human), pseudo HSE-1 and HSE-2 (mouse).

B) Electrophoretic mobility shift assay (EMSA) using human TXNIP promoter fragment probe HSE-1. Nuclear extracts were isolated from Hek293 cells cultured at 37 °C (lanes 1-4) and after 1 h incubation at 42 °C (lanes 5-8). Lanes 1, 4, 5, and 8) contained radio labeled probe; lanes 2 and 6 probe mixed with 100 times excess of cold probe, lanes 3 and 7 radioactive mutant probe with mouse TXNIP pseudo HSE-1 probe. Closed arrows mark specific shifts.

C) Same as B. EMSA with mouse Txnip probe HSE-1.

D) Same as B. EMSA with mouse Txnip probe HSE-2. Open arrow marks specific super shift with monoclonal antibodies against HSF-1.

E) Ba/F3 and 32D cells were either stimulated with 200 μM H_2O_2 for 4 h or not treated. Cells were lysed for RNA extraction and cDNA synthesis. Real-time PCR was performed to determine Txnip mRNA levels relative to ribonuclease inhibitor expression (n=4). The fold induction was calculated by dividing the values of the stimulated samples by the non-stimulated.

enhance mRNA stability and/or promoter activity. These results demonstrate that virus LTR integrations in both the 5' promoter and 3' UTR enhance Txnip expression.

The 5' region of human TXNIP contains a heat shock factor (HSF)-binding element (HSE) [28], which is not completely conserved in the mouse promoter sequence (Fig. 5A). We found that the human HSE is indeed responsive to heat shock. However, super-shift analysis with anti HSF1 antibodies indicated that the factor involved is the heat shock factor-1 (HSF-1) (Fig. 5B). EMSA showed that the mouse partial homologue of the human HSE, which we called HSE1, is not responsive to heat shock (Fig. 5C). On the other hand, a HSE in the mouse Txnip promoter was identified proximal to the HSE1 sequence, which we designated HSE2 (Fig. 5A). Heat-shock treatment of 293Hek cells and super-shift analysis with monoclonal antibodies in EMSA showed that this element is a functional binding site for HSF-1 (Fig. 5D). Real-time RT-PCR showed that treatment of the hematopoietic cell lines Ba/F3 and 32D with 200 μM H_2O_2 for 4 h resulted in a 2 to 3 fold increase in Txnip transcript levels (Fig. 5E). Collectively, these data demonstrate that Txnip expression in hematopoietic cells is induced by cellular stress. We hypothesize that the virus LTR sequences integrated in the Txnip locus in mouse leukemia cells evoke Txnip transcription independent of stress-induced HSF activity. To test this possibility, we disrupted the HSF-binding element in reporter constructs containing or lacking LTR integration in the 5'-region (Fig. 5F).

Ectopic expression of Txnip inhibits 32D cell growth

Txnip is expressed at moderate levels in parental 32D-WT1 cells cultured in IL-3 containing medium, under which conditions the cells remain immature (Fig. 6A). However, upon transfer of the cells to G-CSF-containing medium, in which cells undergo neutrophilic differentiation, Txnip protein levels declined from day 2 onward and remained low for the entire culture period (Fig. 6A).

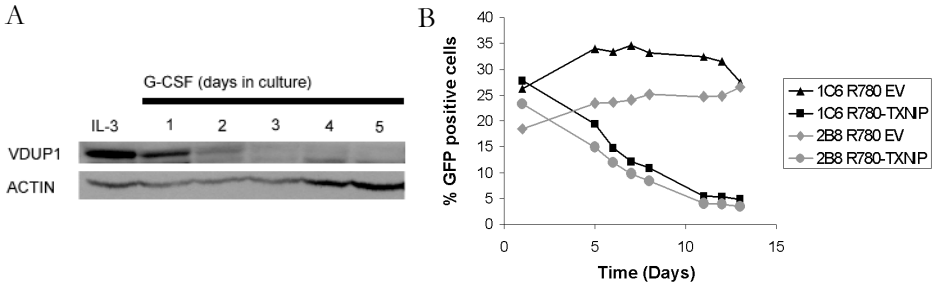

Figure 6. Ectopic expression of DUSP10 in 32D cells

A) Western blot analysis of endogenous TXNIP expression in 32D-WT1 cells. G-CSF responsive 32D-WT1 cells were cultured in IL-3-containing medium and then switched to G-CSF. Samples were taken daily and processed as described in Materials and Methods. The blot was first hybridized with monoclonal anti-Txnip antibodies, stripped, and then rehybridized with anti-Actin antibodies to check for equal loading.

B) 32D clones (2B8, 1C6) were infected with R780-TXNIP virus and cultured in IL-3 containing medium. The percentage of GFP positive cells was measured by flow cytometry at indicated time points.

To investigate the consequences of increased and sustained Txnip protein expression in myeloid cells, we infected 32D cells with R780-Txnip retroviral vector that also confers expression of GFP under the control of an IRES [42]. In the presence of IL-3, the percentage of Txnip-GFP transduced 32D cells gradually declined within 10 days of culture from 25% to approximately 3%, while the presence GFP-expressing control cells remained constant over this culture period (Fig. 6B). These results indicate that increased Txnip levels are not tolerated and supposedly induce apoptosis or growth inhibition in myeloid 32D cells as has been demonstrated in other cell types such as e.g., NIH3T3, vascular smooth muscle cells, 293 cells, HL60 cells, and the human stomach cancer cells SNU-16 and SNU-620 [15, 19, 40].

Txnip inhibits outgrowth of primary myeloid progenitors

We next investigated how increased TXNIP expression affects the outgrowth of primary colony forming units (CFU). To this end, we retrovirally transduced mouse bone marrow cells with R780-FLAG-Txnip or empty R780 virus and plated these cells in colony assays supplemented with GM-CSF, G-CSF, or a cocktail of IL-3, IL-6 and SCF. G-CSF, GM-CSF- and IL-3, IL-6, SCF-induced colony formation was significantly reduced in TXNIP-transduced bone marrow cells (Fig. 7). Cytospin analysis did not show differences in differentiation capacities of colony cells (data not shown). These results suggest that perturbed expression of Txnip causes growth arrest or results in premature apoptosis of primary progenitor cells when cultured in the presence of G-CSF, GM-CSF, or IL-3, IL-6, and SCF.

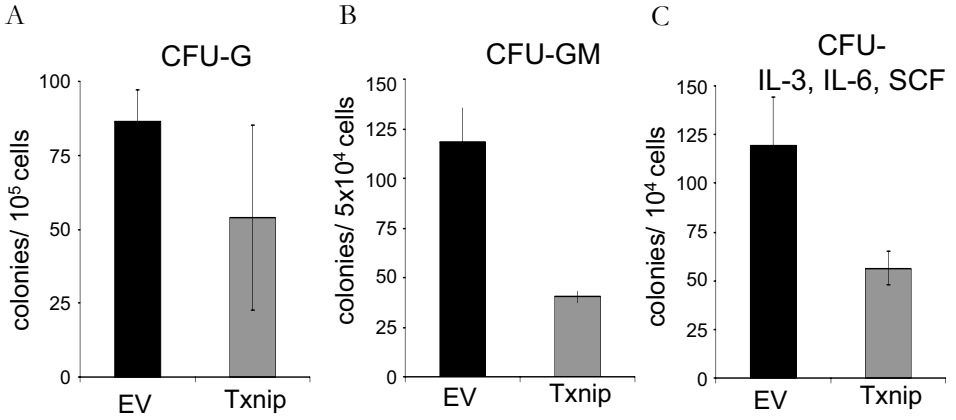

Figure 7. GM-CSF and G-CSF induced colony formation by primary bone marrow cells after retroviral transduction

A-C) CFU assay of primary bone marrow progenitor cells following infection with R780-Txnip and R780 control virus. Equivalent number of positive bone marrow cells was plated in triplicate at densities of 10^5 cells (G-CFU), $5x10^4$ cells (GM-CFU), or 10^4 cells (IL-3, IL-6, SCF) per dish in 1 ml methylcellulose medium containing G-CSF, GM-CSF, or a cocktail of Il-3, Il-6 and SCF.

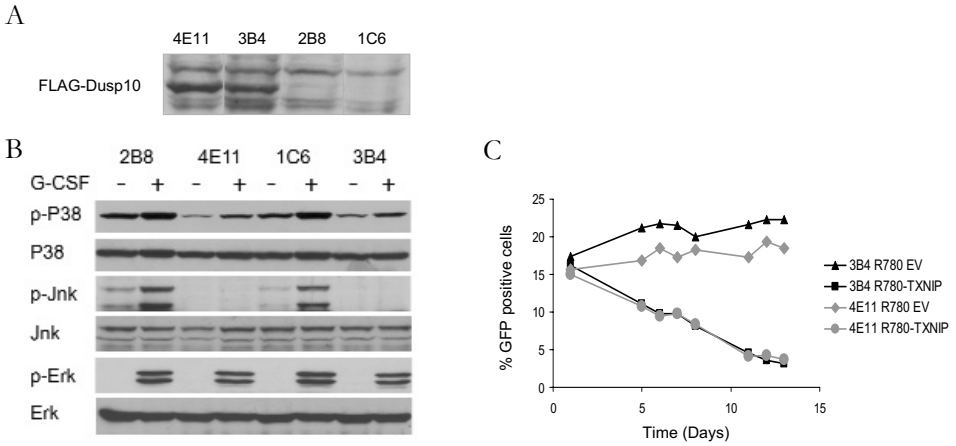

Figure 8. Ectopic expression of DUSP10 in 32D cells inhibits p38 and Jnk phosphorylation
A) Western blot analysis of lysates taken from pLNCX-FLAG-DUSP10 (4E11, 3B4) and pLNCX empty vector control (2B8, 1C6) transduced 32D clones with monoclonal anti-FLAG antibodies.
B) 32D clones (2B8, 4E11, 1C6, 3B4) were serum deprived for 4 hours followed by either G-CSF (100 ng/ml) or no ligand stimulation for 10 minutes. Cell lysates were analyzed by Western blotting using total- and phosphor- specific antibodies against p38, Jnk and Erk.
C) 32D clones (3B4, 4E11) were infected with R780-TXNIP virus and cultured in IL-3 containing medium. The percentage of GFP positive cells was measured by flow cytometry at indicated time points.

Txnip-induced growth inhibition occurs independent of p38MAPK and Jnk and is not counteracted by dual specificity phosphatase 10 (Dusp10)

A conceivable explanation for the growth inhibitory effects of Txnip is that it prevents Trx function, resulting in Ask-1 activation and apoptosis via phosphorylation and activation of p38 and Jnk [19, 36]. Genes encoding dual specificity phosphatases (Dusp's) are regularly found as targets for virus integration [12, 23, 29]. Because we identified the gene encoding Dusp10, a specific phosphatase for P-p38 and P-Jnk, but not for P-Erk, in a Gr-1.4 virus integration site in a leukemia that also harbor integrations in Txnip [12], we investigated whether ectopic expression of Dusp10 counteracts Txnip-induced growth inhibition. Stable Dusp10 overexpressing 32D clones (Fig. 8A) proliferated normally under cell expansion conditions and differentiated into granulocytic cells when treated with G-CSF, similar to the empty vector control cells (data not shown). As expected, ectopic Dusp10 expression strongly decreased cytokine induced P-p38 and P-Jnk levels and had no effect on P-Erk (Fig. 8B). However, Dusp10 expression only slightly inhibited ROS-induced apoptosis (data not shown) and despite the inhibitory activities on p38 and Jnk-controlled stress responses, Dusp10 did not revert the growth inhibitory effects of enforced Txnip expression (Fig. 8C).

TXNIP expression in human AML

Finally, we asked whether abnormal TXNIP levels could also be found in human AML cells, which would be suggestive of a role for TXNIP in human leukemia. To address this question, we determined TXNIP expression in different classes of adult and pediatric AML patients [35, 45]. Based on gene expression profiling, 285 adult AML

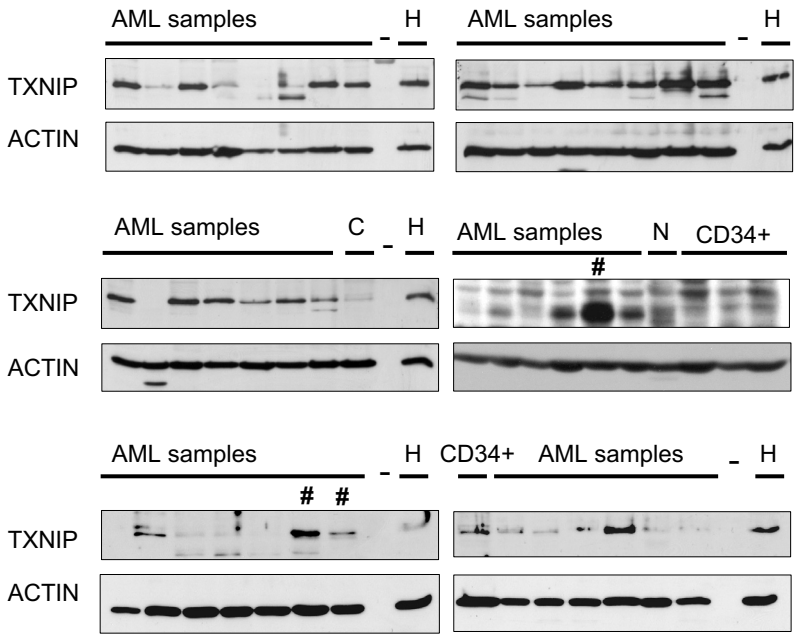

Figure 9. TXNIP protein levels in human AML samples
Western blot analysis of human AML samples, normal bone marrow (N), HL60 cells (H) and CD34+ cells (CD34+, C) protein samples with anti-Txnip antibodies. Actin was stained for loading control. Patients characterized by t(8;21) are marked (#).

cases were recently grouped into 16 distinct clusters of patients by unsupervised cluster analysis [45]. In a similar manner, a correlation analysis was performed using data from 130 childhood AML samples (available at website http://www.stjuderesearch.org/data/AML1). In the latter study, patients were grouped in 5 classes, i.e., inv(16), t(15;17), t(8;21), chromosomal aberrations involving MLL and cases with megakaryoblastic leukemia. Differential expression between classes of patients was performed for all probe sets on the Affymetrix HGU133A GeneChip using significance analysis of microarrays (SAM) [44]. This method tests a class of AML patients for significantly differentially expressed genes compared to a second class of AML patients, in this case the remaining samples. Probe sets of TXNIP were considered to be differentially expressed when Fold Change was over 1.5 or under 0.67, score was over 4 or less than 4, and a q-value less then 5%, where False Discovery Rate was less than 5%. Interestingly, TXNIP mRNA expression was significantly higher expressed with a Fold Chance value of 1.53 in cluster 13 of human adult AML [45] and with a Fold Change value of 1.83 in a cluster of pediatric AML. Both classes of patients are characterized by t(8;21) [45].
To extend this analysis to TXNIP protein expression, we performed Western blot analysis on 60 human AML samples and normal BM control samples, including purified CD34 cells from 4 healthy donors. Increased TXNIP protein level was measured in 60% of the cases. The three cases of AML t(8;21) which are included in figure 9, all demonstrate an increased TXNIP protein expression.

Discussion

In this study, we demonstrated that the gene encoding Txnip is frequently located in a CIS in both Gr-1.4 and CasBrMuLV-induced myeloid leukemia, suggesting that deregulation of Txnip is common in the development of myeloid leukemia in mice. The integrations occurred in the 5' promoter region, within a "hotspot" near a newly identified HSE that is located approximately 1 kb upstream of the transcriptional start site, and in the 3' region downstream of the translational stop. Insertion of Gr-1.4 LTR sequences in these regions enhanced transcriptional activity in a luciferase promoter assay. The number of virus integrations found per tumor indicates that Gr-1.4 MuLV and CasBrM-induced leukemias are oligo- or polyclonal, which is consistent with previous observations [11, 12, 23]. Inclusion of 3' UTR sequences in the promoter construct reduces luciferase activity, indicating that these sequences are involved in post-transcriptional regulation of Txnip. Post-transcriptional regulation of Txnip was already suggested, but no consensus AUUUA AU-rich elements (AREs), which are involved in down regulation of mRNAs, were noted within this 3' UTR sequences [28]. However, Txnip is predicted as a target for the microRNAs Mir-20, and Mir-106 [22]. These small RNA molecules are known to bind 3' UTR sequences and regulate translation of partially homologues mRNAs. Both MicroRNAs show homology with region 2188-2212 of the mouse mRNA sequence, suggesting a role for these molecules in regulation of Txnip.

We found that the stress activated transcription factor HSF-1 binds to the mouse Txnip promoter after heat shock treatment and that H_2O_2 treatment resulted in enhanced Txnip transcript levels in hematopoietic cells. These findings are in agreement with the studies showing that Txnip expression is regulated by several cellular stresses [19]. For example, Txnip was strongly induced in T-cell hybridomas (KMls-8) in response to ROS, ultraviolet (UV) light, and γ-ray exposure and in NIH3T3-, human embryonic kidney cell line 293-derived Bosc- and myeloma Sp2/0-AG14-cells after high density culture stress and heat shock treatment [19, 20, 28].

Our findings raise the possibility that TXNIP regulated pathways are also perturbed in human AML. The key question that remains to be addressed is how deregulation of TXNIP expression is involved in transformation of myeloid cells. TXNIP might influence transcription factor action via modulation of TRX activity on e.g., activator protein-1 (AP-1) via REF-1, p53 and Nuclear Factor-κB (NFκB) [7, 17]. In addition, binding of TXNIP to TRX competes with at least two other TRX-interacting proteins, thereby functionally modulating the activities of these molecules. First, TRX inhibits ASK1 by promoting ubiquitination and degradation [24, 36]. Enhanced TXNIP expression prevents TRX function, resulting in ASK1 activation [19, 36]. ASK1 is a mitogen-activated protein kinase kinase kinase and required cytokine- and oxidative stress-induced stress responses by activation of JNK and p38 pathways [18]. Second, TRX is an electron donor for multiple peroxiredoxins (PRDX), a family of thiol-specific antioxidants that reduces hydrogen peroxide (H_2O_2), and is functionally dependent of TRX [19]. Inhibition of thioredoxin system, thereby blocking its reducing function, will presumably result in increased levels of cellular ROS [30]. In agreement, recent studies demonstrated that enhanced inhibition of TRX by elevated TXNIP protein levels resulted in enhanced H_2O_2 concentration in SK-MEL-8 melanoma cells and human aortic smooth muscle cells (HSMCs), resulting in cellular oxidative stress [41, 49].

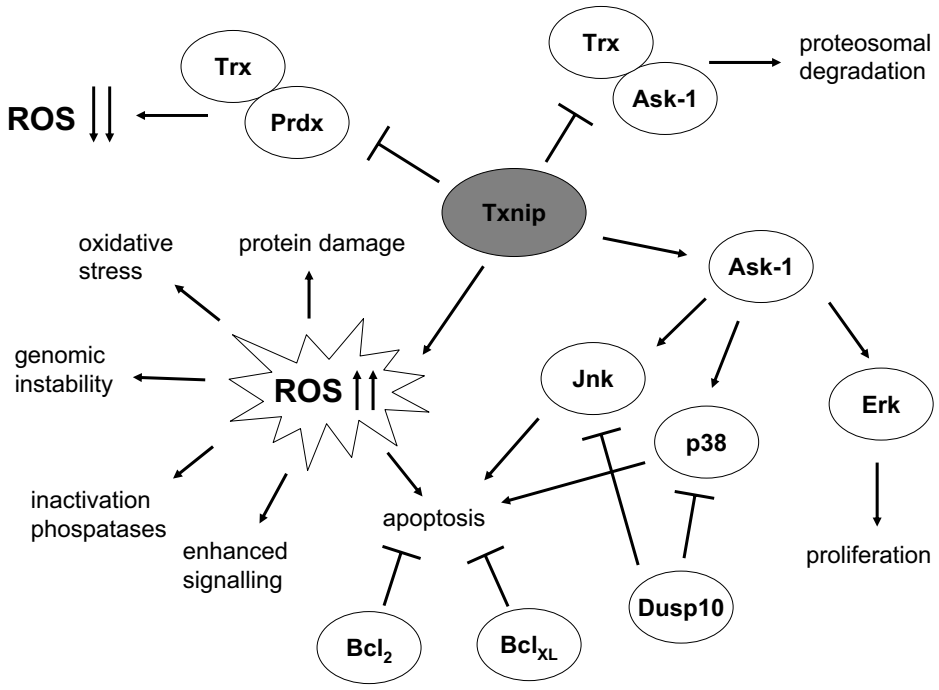

Figure 10. Schematic model of Txnip induced transformation
Enhanced Txnip expression leads to release of Ask-1 from the Trx-Ask-1 complex, thereby activating p38, Jnk and Erk stress kinases. In addition, Prdx reducing function is blocked by Txnip and results in increased cellular ROS levels, oxidative stress, protein damage, inactivation of phosphatases, enhanced signaling, genomic instability and apoptosis. Txnip induced cell death can be blocked by the apoptosis inhibitors Bcl2, Bclxl and Dusp10.

Cells generate low concentrations of ROS when stimulated by cytokines such as, e.g. interleukin-3 (IL-3), tumor necrosis factor-α (TNF-α), steel factor (SF), thrombopoietin (TPO), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte (G)-CSF [8, 38, 39, 49], upon for instance activation of a membrane bound NADPH oxidase mechanism stimulated by PI-3-kinase and RAC-1 [39]. It has now become clear that local intracellular ROS levels are critical regulators in signal transduction cascades [30, 39]. For example, ROS can either reversibly activate protein kinases such as, the STAT kinases JAK2 and TYK-2 [43] and tyrosine kinases in the ERK1/2 MAPK pathway [4, 32, 39] or inactivate protein tyrosine phosphatases (PTPs) such as PTP1B and SHP-2 and the lipid phosphatase Pten [2, 13, 34]. Moreover, abnormal cellular ROS levels are implicated in oncogenic transformation by influencing processes involved in proliferation, apoptosis, senescence and genomic stability [2-4, 13]. Interestingly, it was recently shown that the oncogene BCR/ABL elevates the level of ROS [37], resulting in numerous double strand breaks during genome duplication and division [31]. We are currently investigating if enhanced TXNIP expression contributes to this type of genomic instability and malignant progression.

The TRX-system (TRX, TRX-reductase and NADPH) reduces reactive oxygen species (ROS) via the redox-active sites of TRX. TXNIP inhibits thioredoxin

function by binding to its catalytic domain, thereby blocking the reducing activities of the TRX system [19]. We hypothesize that increased TXNIP levels may block reduction of cytokine induced cellular H_2O_2 levels, thereby inhibiting protein tyrosine phosphatase (PTP) activity that will result in prolonged activity of signaling molecules. This signal promoting effect of TXNIP will be effective only at TXNIP levels that do not induce apoptosis.

Collectively, enhanced Txnip expression causes block of Trx, thereby inhibiting down-modulation of ROS levels via peroxiredoxins (Fig. 10). In addition, Txnip releases Ask-1 from the Trx-Ask-1 complex, resulting in proliferation signals via activated Erk and induction of apoptosis signals via phosphorylation of Jnk and p38 (Fig. 10). High levels of ROS result in protein damage, oxidative stress, and genomic instability, inactivation of phosphatases, enhanced signaling and induction of apoptosis. Inhibition of Txnip induced cell death primarily by Dusps is unlikely in view of data shown in Figure 9. Consequently, for transformation of myeloid progenitor cells via Txnip induced ROS levels, additional hits, e.g. in Bcl-Xl/Bcl-2, and FAS, are needed for block of apoptosis.

In conclusion, we have identified Txnip as a common target of virus integration in mouse leukemia and thereby identified redox-controlled intracellular ROS genes as a mechanism involved in leukemogenesis in mice. Further understanding of pathways controlled by TXNIP during myelopoiesis and a more detailed understanding of the signaling pathways influenced by TXNIP in hematopoietic cells in normal state and during cellular stresses will be required to understand whether and how increased TXNIP levels contribute to the development of human AML.

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CHAPTER 7

General discussion & Summary

7.1 Retroviral insertion mutagenesis

The availability of the mouse genome sequence has made retroviral mutagenesis a powerful strategy for identification of novel disease genes involved in various types of cancer including different types of leukemia, mammary cancer, and brain cancer [24, 25, 27, 34, 36, 47, 50, 51]. To identify genes that are particularly involved in AML, we used the Graffi-1.4 (Gr-1.4) murine leukemia virus (MLV) for retroviral insertional mutagenesis in mice [15, 38]. To this end, a panel of new-born FVB/N mice was injected subcutaneously with Gr-1.4 virus. All mice developed leukemias after 4-6 months, which were mainly (up to 90%) of myeloid origin as characterized by immunophenotyping. An advantage of the retroviral mutagenesis strategy is that the virus provides a tag that simplifies identification of the affected genes. Inverse PCR (iPCR) technology combined with automated large-scale sequencing protocols was used for identification of these unknown virus-flanking sequences. Protocols often used for amplification of virus flanking sequences are for instance the inverse PCR (iPCR), vectorette- and splinkerette-PCR strategies [21]. The inverse PCR (iPCR) is a fast and reliable strategy. However, its efficiency is somewhat limited compared to the other methods due to the circularization by ligation step [21]. Nonetheless, the ample availability of tumor material allowed us to identify more than 100 candidate disease genes involved in multiple regulatory pathways, such as e.g., DNA-repair (Rad54/SNF2, Rad3-related), pathways involving reactive oxygen species (ROS)(VDUP1, PrdxII), and apoptosis (Api-5) (chapter 2 and chapter 3). Most of the virus-flanking genes are involved in signal transduction pathways and transcriptional regulation, indicating that deregulation of these processes is of major importance in myeloid leukemia. These results are partly overlapping with those from other MLV insertional mutagenesis studies [31, 47]. For instance, as, p53, Notch-1, Fli-1 and Evi-1 were already found by other investigators [1, 9, 10], and are likely to be involved in multiple types of mouse leukemia. In addition, we identified genes maybe more specifically involved in Gr-1.4-induced AML (Chapter 2).

When the virus is identified in a given locus multiple times in different tumors, the site of integration is called common integration site (CIS). Virus target genes located near CIS are believed to play an essential role in oncogenic transformation. The majority of the published CIS are defined by the minimal criterion of only two integrations within a genomic region of approximately 30 kb [35] and http://RTCGD.ncifcrf.gov. [1]. Moreover, even single insertions were scored as disease genes when known oncogenes were involved or their homologues counterparts [1, 31, 47]. We tried to extend the iPCR screen with locus specific directed PCR (Chapter 2). This strategy is much more sensitive than the iPCR, but can only identify virus flanking sequences in a short window up to approximately 10 kilo bases (kb) dependent on the polymerase and PCR protocol used. The directed PCR gives information about the frequency and distribution of the virus insertions in a specified locus. A drawback of iPCR and directed PCR strategies is that no information about the role of the potential target gene in the early onset of leukemia can be derived, since virus sequences in minor population of cells that arise in the clonal evolution of the tumors will also be detected. A Southern blot approach can be helpful to obtain this information.

A question that is frequently asked is whether CISs should always be considered as

disease genes, or represent in some cases preferred genomic sites for integration. Analysis of 500 HIV integrations in a human T-cell line shows that HIV viruses favor active genes and local hotspots [42]. However, analysis of 903 murine leukemia virus (MLV) intergration sites shows a different integration pattern and demonstrates that MLV prefers insertion in more active genes but near transcriptional start sites, instead of anywhere in the transcriptional unit. Importantly, no obvious local hotspots of integration were detected [53]. The bias of MLV insertion into active loci is not necessarily negative for the use of retroviruses in disease gene screens, because oncogenic events are mainly characterized by their involvement in normal proliferation, differentiation and survival of precursor cells [33]. Leukemias induced by retroviruses are characterized by a preleukemic phase in which millions infection events occur. Some cells, hit by virus insertions may gain an increased potential to grow out, but multiple additional events are required for full oncogenic transformation. Because these retroviruses replicate, precursor cells will be infected multiple times, resulting in additional mutations and tumor progression. Only cells that contain the right combination of oncogenic events will have enough malignant potential to grow out and cause disease.

An important question is how many retroviral integrations are required to transform a normal myeloid progenitor cell into a leukemic clone. Most investigators report approximately 3.5 to 6 virus integration sites per leukemia, but the amount of oncogenic "hits" may differ per tumor and will be dependent on the type of genes that are deregulated by the virus [27, 34, 47]. However, MLV-induced leukemias are often oligoclonal or even polyclonal [31] (and own observations), which makes it impossible to estimate the number of integrations in each separate tumor clone [35]. Investigation of virus integration sites in single cells using in situ hybridisation technology with leukemia virus specific fluorescent probes will probably give an answer to this question. Extension of this approach using gene specific probes in addition might give insight in the cooperation of virus insertion sites in cell transformation.

An often-discussed limitation of the retroviral insertional mutagenesis strategy is the low probability to identify tumor suppressor genes. There are two explanations for this disadvantage. First, MLV prefers integration near the start of transcriptional units of actively transcribed genes [53], presumably causing enhanced gene expression. Second, according to the Alfred Knudson's two-hit model [26], tumor suppressor genes requires retroviral integrations in both alleles, which reduces the probability to identify them. Intragenic viral insertions can produce dominant negative proteins [25, 51], but these are rarely found in retrovirus-based screens. However, there is growing evidence that haplo-insufficiency (inactivation of a single gene) results in lowering of tumor suppressor levels that is sufficient for oncogenesis [20, 41]. Examples of haplo-insufficient genes are acute myeloid leukemia 1 (AML1, core binding factor alpha 1, CBFA1), p53, inhibitor of cyclin dependent kinases p27 Kip1, and phosphatase and tensin homologue deleted from chromosome 10 (PTEN) [7, 20, 41]. Nevertheless, the fact that tumor suppressor genes are rarely identified suggests that in most cases inactivation of both alleles is required. Irradiation and N-ethyl-N-nitrosourea (ENU) treatment of mice to inactivate one allele before retroviral treatment, or utilizing mouse strains with high frequency of mitotic recombinations could potentially enhance the chance for identification of tumor suppressor genes [16]. To confirm that proviral insertion in a potential tumor suppressor gene is

functional in the disease, mice could be created that are heterozygous knockout for this particular gene [27]. Treatment of these mice with retrovirus will then result in a faster tumor development compared to the wild type littermates. These mice will either show loss of the wild-type gene or proviral insertion in the gene of interest, as has been shown for the Nf1 gene [29]. Retroviral insertional mutagenesis can also be used to search for tumor suppressor genes that are located on chromosome regions that are often lost in AML such as chromosome 7 and 5q deletions. Mice could be created that are hemizygous for these chromosomal regions that cover the not yet found tumor suppressor genes [27]. Such regions can be identified by homology analysis between the mouse and human sequences. These mice could then be treated with retrovirus and the tumors screened for insertions in the regions covered by the deletion on the wild type homologues chromosome.

We are currently investigating another approach to identify tumor suppressor genes. The retroviral DNA becomes integrated in the genome of the cell as a provirus and is highly influenced by the host cell gene regulation machinery. The long terminal repeat (LTR) sequences of the retrovirus are prone for methylation in mammalian cells. It is well known that epigenetic modification processes often silence retroviruses [4, 37]. We are investigating if retroviruses influence the expression of virus flanking genes by epigenetic silencing due to histone and DNA methylation "spreading" and as a result shut off promoters of target genes. In this case, the virus LTR sequences do not enhance expression of the target gene but instead inactivate gene expression. From most of the identified retrovirus-tagged genes, the effects of viral LTR sequences on expression of the target gene have not been investigated yet. A conceivable possibility is that multiple virus flanking genes are tumor suppressors. We are currently developing chromatin immuno-precipitation (ChIP) with methylated histones specific antibodies in combination with inverse PCR (iPCR) technology to identify tumor suppressor genes involved in retroviral mutagenesis induced mouse leukemias.

Tumor suppressor genes can also be identified by insertional mutagenesis using transposons. The Sleeping Beauty transposon is a synthetic Tcl/mariner family transposon that is derived from defective elements cloned from various Salmonid fish genomes [12, 39]. Sleeping Beauty is active in all vertebrate cell lines and primary cells tested. Moreover, Sleeping Beauty transposons are active in the mouse male germ line and at a rate that makes it suitable for large-scale mutagenesis screens [39]. Investigation of the Sleeping Beauty-mediated transposon insertion sites in HELA cells suggested that the sequence ANNTANNT is a favored site for integration [12]. Importantly, unlike mouse leukemia viruses (MLV), Sleeping Beauty transposons do not have a preference for promoter sequences. Moreover, it was recently shown that Sleeping Beauty transposons insert into genes (25-30% of the time) in regions all over the genome [22]. About one third of the genome is estimated to described which suggests that Sleeping Beauty transposons randomly integrates into the genome [22]. Combining the Sleeping Beauty model with mouse models that are prone for AML, for instance transgenic mice expressing a myeloid leukemia derived translocation, would provide an interesting approach for identification of tumor suppressor genes in mice. Alternatively, Sleeping Beauty transposons containing leukemia oncogenes could be generated [22, 28] and used for identification of cooperating tumor suppressor genes.

How many disease genes present in common virus integration sites still have to be identified? This question cannot be addressed at present. However, what became clear from the various virus-tagging screens is that a saturation point of disease gene identification has not been reached yet. In each screen sets of CISs were identified that were not found in other studies. Moreover, many insertion sites that are not yet found to be common may turn out to be disease genes as well [27] (Chapter 3). In fact, some of these single hits occur in known oncogenes. Moreover, the pathogenesis of human AML may be diverse and complex, as exemplified by the fact that more than 300 different chromosomal abnormalities have been observed in human AML [27] (http://cgap.nci.nih.gov/Chromosomes/Mitelman). It is likely that amongst the early events in malignant transformation are mutations in genes that are involved in maintenance of genomic stability [23]. Genomic instability will induce many additional mutations and chromosomal abnormalities that finally result in a full malignant cell that expands and forms a tumor. It has been estimated that there may be indeed thousands of mutations in the cancer cell genome [23].

7.2 Significance of retrovirus-tagged genes for human disease

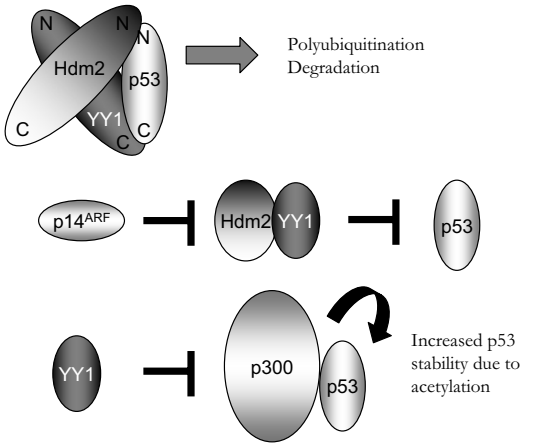
Genes identified in multiple screens using different virus strains and mouse models have an increased probability of representing true disease genes [1]. However, most data generated by retroviral insertional mutagenesis must be regarded as preliminary, as the oncogenic significance has yet to be confirmed for most of the identified virus flanking genes. Moreover, retroviruses can affect target gene expression over several hundreds of kilobases [35]. This makes an obvious definition of a potential virus integration target gene on basis of integration site impossible. We tried to tackle these issues by comparison of different groups of potential target genes, located near or more distantly from the insertion site, against genes differentially expressed in different subtypes of human leukemia (Chapter 3). We found that genes directly flanked by the virus integration site have the highest probability to be deregulated in subsets of human leukemia. Approximately 50% of the direct virus flanking-genes were found differentially expressed in subsets of human AML. However, not all the virus-flanking genes were differentially expressed in AML. There are multiple possible explanations for this. First, the subsets of AML were generated by unsupervised clustering analysis based on gene expression relative to the mean of all samples. Some disease genes might not be recognized because they are deregulated in samples that are not clustered with this approach. Extension of the analysis with supervised clustering may identify an additional set of mouse leukemia genes that are involved in human AML. Secondly, it is possible that a subset of virus flanking genes is involved in mouse leukemia but not in human disease. However, it is still possible that family members of identified genes are involved in human leukemia and it is of interest to further investigate this possibility. Thirdly, the deregulating activity of provirus integration is largely dependent on the long terminal repeat (LTR) sequences. These sequences consist of promoter and enhancer elements that are binding sites for cell type specific transcription factors and are indicators of the tissue specificity of the virus [5,6]. Some frequently identified CIS target genes might be oncogenic in the mouse model because of the fact that

their gene products activate the viral promoter [35]. Finally, some genes identified in mice might not be deregulated by expression levels but due to mutations that influence mRNA translation or protein function. To investigate this possibility, we are currently investigating a panel of human homologues of virus-tagged genes in human AML samples using high throughput mutation analysis screens.

7.3 The role of Yin Yang-1 (YY1) in AML

In chapter 4, it was shown that Gr-1.4 integrations in the YY1 promoter region deregulate YY1 expression, making it independent of Sp1 regulation. Moreover, ectopic expression of YY1 had a negative effect on differentiation of myeloid 32D cell line and prevented outgrowth of myeloid progenitors from primary bone marrow cells (Chapter 4). Furthermore, we found that in certain cases of human AML that YY1 expression is significantly increased compared to normal bone marrow cells. Interestingly, the YY1 gene was already identified previously by iPCR as a retroviral integration site in the BXH2 leukemia model [31]. In addition, increased YY1 expression was found in adenocarcinoma tumors [46], further supporting a role for YY1 in oncogenesis. Multiple possibilities of how YY1 contribute to leukemic progression have already been discussed in chapter 4, but recently some new interesting findings concerning the involvement of deregulated YY1 expression in malignant transformation have been reported [18, 46, 54]. These studies established that YY1 is a major regulator of the tumor suppressor p53 (Fig. 1).
Transcriptional activation via p53 is suggested to be controlled by YY1 in case of a subset of p53 targets such as p21 and Gadd45, proteins that regulate growth arrest and DNA repair respectively [54]. In agreement, enhanced expression of YY1 resulted in decreased p21, mouse double minute 2 protein (Mdm2) and p53 expression after etoposide and ultraviolet (UV) irradiation treatment, whereas inactivation of YY1 sensitized cells to DNA damage-induced apoptosis [18]. These results indicate that enhanced YY1 expression inhibits the activation of p53 in response to DNA damage, resulting in a strongly increased risk for genomic instability. YY1 is found to be a negative regulator of p53 function by stimulating human (H)dm2-mediated p53 poly-ubiquitination and proteasomal degradation. Loss of YY1 results in increased p53 levels, and conversely, overexpression of YY1 significantly decreases p53 levels. Expression of YY1 inhibits p53 binding to the coactivator p300, thereby preventing p300-mediated acetylation and stabilization of p53 [18]. YY1 interacts with Mdm2 and promotes Hdm2-p53 complex formation, resulting in enhanced Hdm2-mediated p53 ubiquitination and degradation [46]. The tumor suppressor p14ARF (p19ARF in mouse) interacts with Mdm2 and abrogates Mdm2 regulation of p53 [44, 55]. In addition, p14ARF interacts with YY1 and disrupts YY1 regulated Mdm2-mediated p53 ubiquitination, thereby inhibiting p53 degradation [46]. Conversely, enhanced YY1 expression can antagonize p14ARF dependent stabilization of p53 [46]. Interestingly, the p53 gene is frequently targeted by Gr-1.4 insertion, showing that p53 regulated pathways are commonly deregulated in mouse myeloid leukemia. Taken together, these data suggest that aberrant YY1 expression may be an important event in oncogenesis, but further studies are necessary to determine YY1 target genes and YY1 activity on important regulatory pathways.

A



B

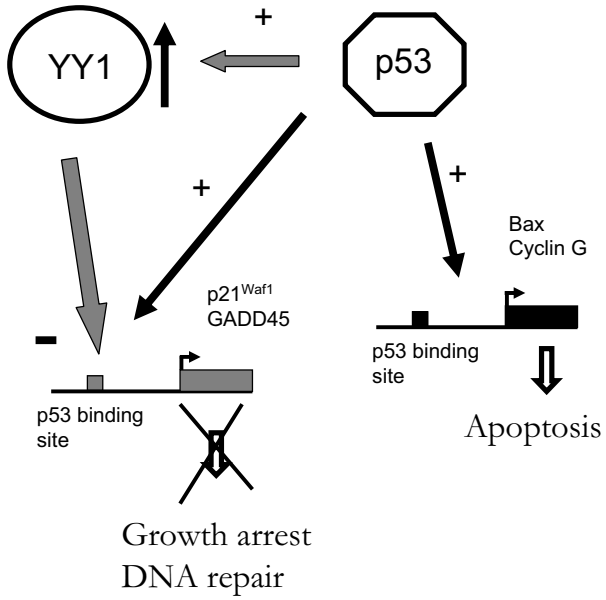


Figure 1: YY1 is a regulator of the tumor suppressor p53
A) Schematic diagram of the YY1-Hdm2-p53 ternary complex [46]. Overexpression of YY1 antagonizes the ability of p14ARF to stabilize p53. In addition p14ARF can disrupt YY1-Hdm2 interaction [46]. YY1 prevents p300-mediated acetylation of p53, thereby making p53 available for Hdm2-mediated ubiquitination and degradation [18].
B) Model for regulation of p53 dependent gene transcription by YY1. YY1 bind to the promoters of p21Waf1 and GADD45, regulators of growth arrest and DNA repair respectively, but not to Bax and Cyclin G promoters, both genes that are involved in apoptosis. YY1 interaction to the DNA binding site is enhanced by p53 [54]. Levels of YY1 may be critical for regulating p53-activated transcription of a panel of p53 targets.

7.4 The role of Wsb in leukemia

In Chapter 5 we described the findings concerning a new common virus integration site located in the gene encoding WD-repeat domain and a SOCS box-2 (Wsb-2). All virus insertions occurred between exon 4 and exon 7, strongly suggesting inactivation of WSB-2 gene expression. Wsb-2 mRNA level is significantly decreased in a subset of AML patients, suggesting a role in human disease. We found that the G-CSF-R C-terminus, a domain implicated in myelodysplastic syndrome (MDS) [52], severe congenital neutropenia (SCN) and AML [49], is an interacting domain for WD40 repeat containing proteins. Wsb-1, Wsb-2 and Rack-1, all proteins that contain domains involved in signal transduction, bind to the G-CSF-R distal domain. Wsb proteins reduce G-CSF-R membrane expression and inhibit G-CSF-induced STAT activation and proliferation signaling.
Our results described in chapter 5 suggest that Wsb protein is involved in down-modulation of the G-CSF-R signaling and intracellular routing. In a first attempt to investigate this function in more detail, we are currently using an RNAi based strategy to down modulate Wsb proteins levels. Wsb proteins have common characteristics and are most likely functionally redundant. Therefore both proteins Wsb-1 and Wsb-2, need to be sufficiently knocked down all at once. Our first Wsb RNAi experiments show approximately 75% Wsb-2 and 50% Wsb-1 mRNA reduction, which is probably not sufficient to modulate Wsb protein levels. This work is also hampered by the fact that RNAi effects on Wsb protein levels cannot be detected, as appropriate antibodies against Wsb proteins are not available yet. Generation of inducible Wsb knock-out models will probably be the best solution for further identification of the roles of Wsb proteins in leukemia.

7.5 The role of virus insertions in the Txnip gene in transformation of myeloid progenitors

We identified the gene encoding thioredoxin interacting protein (Txnip) as a frequent target for Gr-1.4 insertion (Chapter 6). Integrations in the Txnip gene occurred at the 5' and 3' region with a frequency of 100% of the Gr-1.4 and 58% of the CasBrM induced leukemias as detected by the directed PCR method. There are multiple reasons conceivable why so many virus insertions were found in this locus. First, knowing that Txnip is a stress responsive gene, deregulation of Txnip controlled stress pathways might be supportive for the retrovirus life cycle. In a condition in which cells are less responsive to virus induced stress, cells may produce more retroviruses and consequently generate genomic instability, resulting in a growth advantage for the targeted clone. Second, although the exact function of Txnip in the nucleus is not clear yet [19], Txnip might modulate transcriptional activity of factors that act on the virus enhancer and/or promoter regions, resulting in a stronger activation of oncogenes and their transforming potential. Third, virus integration in the Txnip locus may affect expression of multiple other genes in addition such as e.g., Junb and Prdx, which are located very close to Txnip (within approximately 10 kb). Cooperation of these genes in the process of transformation

will strongly enhance the probability to find virus insertions in this locus. The fourth possibility is that the Txnip locus is just a non-relevant hotspot for Gr-1.4 and CasBrM integration. However, analysis of 903 murine leukemia viruses (MLV) integration sites in the human genome showed that MLV preferred integration near transcriptional start sites (up- and downstream) of active genes but no regional hotspots for MLV integration were found in this study [53]. In addition, integrations in the Txnip locus were located up- and downstream of coding sequences, arguing against this possibility. On the other hand, different types of MLVs might have distinct integration features. In addition, MLV integration characteristics in the mouse genome might differ in human cells. Finally, it is possible that deregulation of Txnip expression is an exceptionally important oncogenic event in FVB/N mouse myeloid leukemia.

In chapter 6, it is shown that the presence of retroviral sequences in the Txnip 5' region or 3' UTR results in an enhanced signal when compared to the wild-type Txnip promoter. In addition, we found that TXNIP mRNA levels were increased in a subset of AML patients characterized by t(8;21). TXNIP is an inhibiter of thioredoxin (TRX). TRX is the major cellular disulfide reductase and is critical for redox regulation of protein functions [3]. Many functions of TRX have been described, e.g., reduction of H_2O_2, protein repair by methionine sulfoxide reduction, redox regulation of transcription factors and regulation of apoptosis [3]. Alternatively spliced forms of human TRX have been identified in multiple human tumor cell lines including three leukemia cell lines (CCRF-CEM, K562 and SR-23) [11]. This splice variant lacks exons 2 and 3, generating a catalytic inactive mutant protein that is unable to form a homodimer. Moreover, this variant is expressed at very low levels, presumably due to instability of the mRNA [11]. This suggests that decreased TRX function is implicated in tumorgenesis. In contrast, enhanced TRX expression is found in multiple primary tumors including lung, colon, cervix, liver, and pancreas and is associated with enhance proliferation and survival. TRX has been demonstrated to inhibit apoptosis and promote cell growth, indicating that TRX can act as an oncogene under certain circumstances. Enhanced TXNIP expression inhibits thioredoxin (Trx) function and in consequence increases ROS levels and oxidative stress in SK-MEL-8 melanoma cells [14] and human aortic smooth muscle cells (HSMCs) [43]. Oxidative stress is caused by reactive oxygen species (ROS) such as e.g., O^{2-} (superoxide radical), OH^- (hydroxyl radical) and H_2O_2 (hydrogen peroxide) [8, 45]. High amount of ROS induces base damage and double strand breaks in DNA and oxidizes RNA, lipids, proteins, and nucleotides, which results in cell impairment [45]. Multiple systems in the cell operate in the defence against oxidative damage to prevent genomic instability, including enzymatic removal of ROS and most known DNA repair mechanisms [45]. Damaged DNA that escapes repair is prone for additional mutations during DNA replication that may contribute to the initiation or progression of cancer [23]. High levels of ROS are important mediators of apoptosis. For instance, ROS induce apoptosis by regulating the phosphorylation and ubiquitination status of BCl-2 family members [30]. This results in increased levels pro-apoptotic Bad-Bax dimers due to decreased Bad phosphorylation and ubiquitination status and decreased anti-apoptotic Bcl-2-Bcl-Xl dimers due to decreased phosphorylation and increased ubiquitination of Bcl-2 [30]. Overexpression of the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL have been shown to

antagonize ROS-induced apoptosis and might cooperate in ROS-induced oncogenesis [13, 17, 32]. An increasing number of genes and cellular pathways are shown to be regulated by cellular redox status including several proto-oncogenes such as e.g., jun-B, c-myc and STAT5 [2]. In striking contrast, TXNIP expression was lost in human T-cell leukemia virus type I-dependent T-cell transformation, resulting in an IL-2 independent growth phenotype. Moreover, TXNIP expression was found down-regulated in several other tumors including a panel of human leukemias [40, 48], indicating that TXNIP might act as a tumor suppressor as well. While it is clear that TXNIP is involved in a variety of regulatory functions, more research is needed to understand the exact roles of this interesting protein in the transformation of normal progenitor cells into myeloid leukemia cells.

7.6 Conclusions

Retroviral insertional mutagenesis in mice with Gr-1.4 leukemia virus is a powerful strategy to identify novel genes involved in murine acute myeloid leukemia. Our investigation of newly identified Gr-1.4 targets such as YY1, Txnip and Wsb-2, suggest a role for these proteins in leukemic transformation of normal murine and human myeloid progenitors cells. We showed that the genes directly flanked by the virus integration site have the highest probability to be significantly deregulated in subsets of human AML. The combinatorial approach of gene expression profiling of human AML samples and gene tagging screens in mice allows identification of new networks and genes involved in the pathogenesis of human leukemia. Further functional in vivo analysis of the identified genes is needed to understand their roles in human leukemia development.

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Nederlandse samenvatting & Conclusies

Samenvatting

Acute myeloïde leukemie (AML) wordt geclassificeerd in verschillende risico categorieën op basis van cytogenetische en moleculaire afwijkingen. De chromosomale translocaties t(8;21), t(15;17) en inv(16) bijvoorbeeld, resulteren in fusie-eiwitten met afwijkende transcriptioneel regulerende activiteiten en worden geassocieerd met een relatief gunstige prognose, terwijl 3q26, 5q, 7q en 11q23 afwijkingen correleren met een verminderde overlevingskans na behandeling. Mutaties in het gen dat codeert voor de transcriptiefactor CCAAT bindend eiwit alpha (CEBPA) en de goeifactor receptor FMS-like tyrosine kinase3 (FLT3) zijn tevens geassocieerd met respectievelijk een goede en slechte prognose. Ongeveer de helft van de AML patiënten heeft bekende (cyto-) genetische afwijkingen. De ontwikkeling van AML is een meerstapsproces waarbij defecten in meerdere genen betrokken zijn. Dit is vooral duidelijk geworden in studies in muizenmodellen. Zo ontwikkelen muizen die fusiegenen herbergen afkomstig van t(15;17), t(8;21) en inv(16) pas na langere tijd en met een lage frequentie leukemie, hetgeen aangeeft dat additionele afwijkingen nodig zijn voor volledige transformatie van hematopoietische voorlopercellen in AML cellen. Algemeen wordt aangenomen dat AML ontstaat door afwijkingen van combinaties van genen die hematopoietische stamcel proliferatie, vernieuwing en myeloïde differentiatie controleren.

De beschikbaarheid van de sequentie van het muizengenoom heeft van retrovirale mutagenese een krachtige strategie gemaakt voor de identificatie van nieuwe ziektegenen die betrokken zijn bij diverse typen kanker, waaronder met name leukemie, lymfoom en borstkanker. Voor de identificatie van genen die voornamelijk een rol spelen in acute myeloïde leukemie (AML) hebben we Graffi-1.4 (Gr-1.4) muizen leukemievirus (MLV) gebruikt. Hiervoor zijn pasgeboren muizen subcutaan geïnjecteerd met Gr-1.4 virus. Al deze muizen ontwikkelden na 4-6 maanden een leukemie, voornamelijk van myeloïde origine (Hoofdstuk 2 en 4). Voor de identificatie van de virus-flankerende genen hebben we inverse PCR technologie gebruikt en gecombineerd met geautomatiseerde sequentie analyse. Met deze methode zijn meer dan 100 kandidaat ziektegenen geïdentificeerd die betrokken zijn in meerdere mechanismen, zoals bijvoorbeeld DNA herstel (Rad54/SNF2, Rad3-related), regulering van het niveau van reactieve zuurstof deeltjes (ROS)(Txnip, PrdxII) en geprogrammeerde celdood (Api-5) (Hoofdstuk 2). De meeste virus-flankerende genen maken deel uit van signaal transductie en transcriptionele netwerken (Hoofdstuk 2). Deze resultaten overlappen deels met die van andere MLV mutagenese studies. Zo werden bijvoorbeeld p53, Notch-1, Fli-1 en Evi-1 ook in eerdere screens gevonden. Deze genen zijn dus waarschijnlijk betrokken bij meerdere typen muizen leukemie. Daarnaast hebben we genen ontdekt die waarschijnlijk meer specifiek betrokken zijn bij myeloïde leukemie (Hoofdstuk 2).

Genen die in meerdere onafhankelijke mutagenese screens zijn gevonden zijn zeer waarschijnlijk belangrijk voor het ontstaan van leukemie in de muis. Het belang van deze genen voor humane leukemie staat daarmee echter niet vast. Bovendien kan een retrovirus de expressie van een targetgen over een afstand van enkele honderden kilobasen beïnvloeden. Dit bemoeilijkt een strikte definitie van een virus targetgen op basis van de plek van integratie. In hoofdstuk 3 hebben we geprobeerd dit probleem te benaderen door verschillende groepen potentiële targetgenen, dichtbij of verder weg gelokaliseerd van de virusintegratieplek, te vergelijken met genen die

differentieel tot expressie komen in subgroepen van AML. We vonden dat muizen leukemiegenen geïdentificeerd met retrovirale insertionele mutagenese differentieel tot expressie komen in subgroepen van AML bij zowel volwassenen als kinderen. Genen die direct grenzen aan de plek van virusintegratie correleerden significant met groepen van genen die verantwoordelijk waren voor classificatie van humane leukemie. Dit in tegenstelling tot genen die verderop liggen. Dit resultaat laat zien dat genen die het dichtst in de buurt liggen van virus integraties hoogst waarschijnlijk ook ziektegenen zijn met een rol in AML bij de mens. Deze genen konden geplaatst worden in vijf regulatoire netwerken. In dit proefschrift is de functionele rol van drie van dergelijke genen nader onderzocht; te weten de genen die coderen voor de transcriptiefactor Yin Yang 1 (Yy1), WD40-repeat domein en een SOCS box-2 (Wsb-2) en Thioredoxin bindend eiwit (Txnip).

In hoofdstuk 4 wordt beschreven dat Gr-1.4 integraties in de Yin Yang 1 (YY1) promoter regio YY1 expressie dereguleren en het onafhankelijk maken van regulatie door Sp1. Bovendien had kunstmatig verhoogde YY1 expressie een negatief effect op de differentiatie van myeloïde 32D cellen en voorkwam de uitgroei van myeloïde voorlopercellen vanuit primaire beenmerg cellen. Verder hebben we gevonden dat in een bepaalde groep van AML patiënten de YY1 expressie significant verhoogd is ten opzichte van normale beenmergcellen. Deze resultaten maken aannemelijk dat verstoorde regulatie van YY1 expressie interfereren met normale differentiatie programma van myeloïde voorlopercellen en daardoor kan bijdragen aan de ontwikkeling van leukemie.

Hoofdstuk 5 handelt over het gen dat voor een WD40-repeat domein en een SOCS box-2 (Wsb-2) codeert. Virusintegraties in Wsb-2 werden uitsluitend gevonden tussen exon 4 en exon 7, wat aangeeft dat het Wsb-2 gen waarschijnlijk geïnactiveerd wordt door de integraties. Ook bij een subgroep in humane AML werd gevonden dat het Wsb-2 mRNA niveau sterk verlaagd is. Wsb-2 blijkt te binden aan het distale domein van de G-CSF-R, een domein betrokken bij ernstige aangeboren neutropenie, myelodysplastisch syndroom (MDS) en AML. Verder vermindert Wsb-2 G-CSF-R membraan expressie en G-CSF-geïnduceerde STAT activatie en proliferatie signalen. Dit duidt erop dat Wsb-2 een tumoronderdrukkende werking uitoefent door remming van G-CSF geïnduceerde proliferatie van myeloïde voorlopercellen.

In hoofdstuk 6 worden de gegevens van integraties in het Txnip gen beschreven. Integraties in Txnip vonden plaats in de 5' en 3' regio in 100% van de Gr-1.4 en 58% van de CasBrM geïnduceerde leukemie gevallen. Integraties in deze regio's resulteren in een verhoogd signaal in luciferase assays, vergeleken met een normale Txnip promoter. TXNIP mRNA niveaus blijken te zijn verhoogd in een groep van AML patiënten ten opzichte van CD34+ normale beenmergcellen. Kunstmatige verhoogde expressie van TXNIP resulteerde in een versnelde geprogrammeerde celdood en vertraagde celdeling.

Conclusies

Retrovirale integratie mutagenese in muizen met Gr-1.4 leukemie virus is een goede methode om nieuwe genen te identificeren die betrokken zijn bij muizen AML. Muizen leukemiegenen die met deze methode zijn gevonden komen differentieel tot expressie in subgroepen van AML bij de mens. De resultaten beschreven in dit proefschrift omtrent de nieuw geïdentificeerde Gr-1.4 targets Yy1, Txnip en Wsb-2 suggereren een rol voor deze genen in transformatie van normale muizen en mensen hematopoietische voorlopercellen. Het combineren van gen expressie analyse in humane AML met screens van potentiële muizen kankergenen maakt het mogelijk nieuwe genen en netwerken van genen te identificeren die betrokken zijn bij de oorzaak van humane AML. Verder functioneel onderzoek in de muis is nodig om de rol van deze genen en netwerken in het ontstaan van leukemie te kunnen begrijpen.

Abbreviations

a.a.	amino acid
bp	base pair
BSA	bovine serum albumin
ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
BCR	breakpoint cluster region
BM	bone marrow
cDNA	complementary DNA, copy DNA
CFU	colony-forming unit
ChIP	chromatin immuno-precipitation
CIS	common insertion site
CLSM	Confocal laser scanning microscopy
C-terminal	carboxyl terminal
CSF	colony stimulating factor
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
ECS	Elongin-Cullin-SOCS
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EMSA	electrophoretic mobility assay
ENU	N-ethyl-N-nitrosourea
Epo	erythropoietin
Erad	ER-associated degradation
ES	embryonic stem
Evi	ecotropic virus integration site
FAB	French-American-British
FAM	6-carboxy-fluorescein
FCS	fetal calf serum
FISH	fluorescence in situ hybridisation
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
Gr-1.4	Graffi-1.4 MuLV
G-CSF	granulocyte colony-stimulating factor
G-CSF-R	granulocyte colony-stimulating factor receptor
GM-CSF	granulocyte macrophage colony-stimulating factor
h	human
HA	hemagglutinin
HSMC	human aortic smooth muscle cell
H2O2	hydrogen peroxide
IL-3	interleukin-3
inv(16)	inversion of chromosome 16
IP	immunoprecipitation
iPCR	inverse PCR
IPKB	Ingenuity Pathways Knowledge Base

IRES	internal ribosomal entry site
K	thousand
kb	kilo base
kd	kilo dalton
Luci	luciferase
LTR	long terminal repeat
m	mouse
MAPPIT	mammalian protein-protein interaction trap
MDS	myelodysplastic syndrome
mRNA	messenger RNA
MuLV(MLV)	murine leukemia viruses
Mo-MuLV	Moloney-MuLV
MVB	multi vesicular bodies
NCBI	National Center for Biotechnology Information
nt	nucleotide
N-terminal	amino-terminal
O2.-	superoxide radical
OH-	hydroxyl radical
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RARα	retinoic acid receptor α
RNA	ribonucleic acid
rPAP	rat pancreatitis-associated protein
ROS	reactive oxigen species
RPM	rounds per minute
RPMI	Rosswell park memorial institute medium
RT-PCR	real-time PCR
SAM	significance analysis of microarrays
SAP	shrimp alkaline phosphatase
SCF	stem cell factor
SCN	severe congenital neutropenia
SDS	sodium dodecyl sulfate
SOCS	suppressor of cytokine signaling
t(15;17)	translocation between chromosomes 15 and 17
TAMRA	6-carboxy-tetramethyl-rhodamine
TPO	thrombopoietin
TRITC	tetramethyl rhodamine isothiocyanate
Txnip	thioredoxin interacting protein
UTR	untranslated region
UV	ultraviolet
VIS	virus integration site
WBD	WD-40-binding domain
WD(40)-repeat	tryptophan-aspartic acid (WD)
WSB	WD-repeat domain and SOCS box-containing protein
wt	wild-type
Y2H	Yeast-two-hybrid
YY1	Yin Yang 1

Dankwoord

Het is dan eindelijk zover. Het boekje is af. Promoveren doe je echter niet alleen en voordat we gaan feesten wil ik dan ook een aantal mensen hartelijk bedanken.

Beste Ivo, ik wil je graag als eerste bedanken voor alle mogelijkheden, steun en vertrouwen die je mij de laatste jaren hebt gegeven. Al vanaf het sollicitatiegesprek was mij al duidelijk dat je een enthousiaste en gedreven onderzoeker bent die open staat voor nieuwe ideeën en ontwikkelingen. Jij hebt mij in de afgelopen jaren alle mogelijkheden gegeven om die dingen te onderzoeken die ik interessant vond en daar ben ik je zeer dankbaar voor. De soms eindeloze discussies over onze resultaten en onderzoeksrichtingen waren nuttig, motiverend en leerzaam en uiteindelijk waren we het altijd wel weer eens. Je bent eerlijk, behulpzaam, recht door zee en iemand die de puntjes op de i's wil zetten, en dat zijn waardevolle karakter-eigenschappen. Onze samenwerking heb ik dan ook als zeer prettig ervaren en ik hoop dat die nog lange tijd mag duren.

Tevens wil ik Bob hartelijk bedanken voor de mogelijkheid om op de afdeling Hematologie onderzoek te kunnen doen. Ik heb onze discussies op de vrijdagochtendbesprekingen en daarbuiten altijd enorm gewaardeerd.
Dr. Delwel, Prof.dr. Kanaar en Prof.dr. van Lohuizen wil ik bedanken voor hun bereidheid plaats te nemen in de kleine promotiecommissie. Ruud, ook erg bedankt voor de leuke samenwerking. Ik zal je moppen en grappen missen. Wat anderen ook zeggen, ik vind ze wel leuk (zelfs na tien keer) en misschien kan je zo af en toe wat grappen via de e-mail sturen.

Marijke (de allerbeste analiste) wil ik bedanken voor de zeer prettige samenwerking, vriendschap en gezelligheid. We hebben vanaf het begin samengewerkt en dat is altijd buitengewoon goed bevallen. Natuurlijk wil ik ook de andere twee topanalisten Onno en Alex hartelijk bedanken voor de enorme inzet, doorzettingsvermogen en gezelligheid. We hebben als een hecht team gewerkt. Ik zal jullie erg missen.

Ik wil Mirjam (altijd kritisch en precies) en Claudia (zeer vakkundig) bedanken voor de samenwerking betreffende het muizenwerk. Ik heb ontzettend veel van jullie geleerd. Jullie vertrek van de afdeling heb ik als een groot verlies ervaren. Bart wil ik bedanken voor de vele discussies betreffende het onderzoek aan de G-CSF-R. Van jou heb ik geleerd hoe met frustraties om te gaan (zoemmmmm). Lunchen zal nooit meer zo gezellig zijn. Gert-Jan, ook jij bedankt en veel succes met je nieuwe baan. Astrid, ik ga alweer voor de tweede keer afscheid van jou nemen. Heel veel succes met de voortzetting van het muizenproject. Charisma (de beste kok van de afdeling), hartelijk bedankt en veel succes met jouw promotie. Verder wil ik de studenten Antoinette, Pim, Claudia en Joost bedanken voor de korte maar leuke samenwerking en veel succes wensen met hun loopbaan. Roel, ook jij bedankt voor de goede samenwerking. Bio-informatica blijft toch een vreemd vak want wat betekenen die gegoochelde getallen nou eigenlijk voor de biologie? Ik heb veel van je geleerd. Albert Wölfler, Jan Tavernier en collega's wil ik bedanken voor de prettige internationale samenwerkingen.

Renee, het was kort maar erg leuk om met je gewerkt te hebben. Het is moeilijk, maar het gaat zeker lukken....., denk ik! We houden contact.

Marieke wil ik graag bedanken voor de talloze gesprekken tijdens het reizen naar Rotterdam en terug naar Leiden. Door jouw positieve karakter en gezelligheid zijn zelfs uren reisvertraging dragelijk. Ook de mensen in jouw groep zoals Martine, Thamar, Walbert, Lamberto, Emile en Montse wil ik hartelijk bedanken voor de prettige werksfeer en inbreng tijdens de werkbesprekingen. Peter, bedankt voor de goede samenwerking en adviezen betreffende het KWF-fellowship en Boston. Eric en Godfrey wil ik bedanken voor de steun, gezelligheid en het ontbijd met koffie in San Diego.

Verder wil ik (oud) kamergenoten Fokke, Basje, Sahar, Meri en Marieke bedanken voor de steun, koffie en gesprekken die altijd zorgden voor een leuke werksfeer.

Judith, Joanna, Mahban, en niet te vergeten de oudcollega's Daphne, Sigrid en Alister, bedankt voor jullie hulp en gezelligheid op de 13e. Eveline, Ans, Jan, Elwin, Hans, Karole en Ineke wil ik hartelijk bedanken voor de goede zorgen, adviezen en behulpzaamheid. Verder wil ik al mijn (oud)collega's die ik niet heb genoemd, bedanken voor de prettige werksfeer, hulp en collegialiteit op de werkvloer. Het waren 6 fantastische jaren!

Tot slot wil ik Sabine en Lars bedanken voor alle begrip, steun, liefde en geluk. Jullie zijn ook weer onmisbaar in het volgende avontuur in Boston.

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Curriculum Vitae

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2)doctoral degree (April 27, 2005)
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Erasmus Medical Center, Rotterdam, The Netherlands
Department of Hematology

Title thesis: **Identication, function and clinical Relevance of mouse leukemia Genes**

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-Identification of novel disease genes involved in Graffi-1.4 MuLV-induced acute myeloid leukemia in mouse models
-Investigation of the mechanisms of disease genes involved in leukemia using mammalian (myeloid) cell lines and murine myeloid progenitor culture systems
-Generation of promoter-specific, inducible, Cre-expressing mouse models for investigation of disrupted or activated disease genes at a defined time of myeloid development
-Identification of disrupted signaling pathways caused by a truncated Granulocyte-Colony stimulated Factor Receptor (GCSFR) and found in Severe Congenital Neutropenia patients by using in-vivo protein-protein interaction and Yeast Two Hybrid
-Identification of novel disease pathways cooperating with the truncated G-CSFR oncogene in murine AML
-Identification of mutations in the G-CSFR gene and novel disease genes in human AML
-Comparison of murine derived research data with human AML RNA expression Array data in cooperation with Bio-Informatics

Publications: # 1, 2, 4, and 5

-College of Advanced Technology:
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-Higher General Secondary School (1986 - 1989)
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Previous functions:

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-Identification of disease genes involved in acute myeloid leukemia in mouse models and murine leukemia cell lines
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-Generation of H2Kb-Apoptin transgenic mice
-Functional analysis of Apoptin protein domains using various immortalized and "normal" (non-transformed) cell lines
-Yeast Two Hybrid screen to identify Apoptin-binding proteins
-Generation of a BCL2 knock-out cell line model utilizing anti-sense constructs
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Publications: # 3, 6, and 7

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-Investigation of the localization of bullous pemphigoid antigen 180 (BP180) in hemidesmosomes
-Analysis of BP180 domains in complex formation with α6 and β4 integrins using molecular, biochemical and immuno fluorescence technologies
Supervisor: Dr. L. Borradori and Dr. A. Sonnenberg
Publication: #8

List of Publications:

1. Wolfler A, **Erkeland SJ**, Bodner C, Valkhof M, Renner W, Leitner C, Olipitz W, Pfeilstocker M, Tinchon C, Emberger W, Linkesch W, Touw IP, Sill H. A functional single nucleotide polymorphism of the G-CSF receptor gene predisposes to high-risk myelodysplastic syndrome. Blood. 2005 Jan 11.

2. **Stefan J. Erkeland**, Marijke Valkhof, Claudia Heijmans-Antonissen, Antoinette van Hoven-Beijen, Ruud Delwel, Mirjam H. A. Hermans and Ivo P. Touw. Large-scale identification of disease genes involved in acute myeloid leukemia. Journal of Virology. 2004 Feb;78(4):1971-80.

3. Danen-Van Oorschot AA, Zhang YH, Leliveld SR, Rohn JL, Seelen MC, Bolk MW, Van Zon A, **Erkeland SJ**, Abrahams JP, Mumberg D, Noteborn MH. Importance of nuclear localization of apoptin for tumor-specific induction of apoptosis. J Biol Chem. 2003 Jul 25; 278(30): 27729-36.

4. Van De Geijn GJ, Aarts LH, **Erkeland SJ**, Prasher JM, Touw IP. Granulocyte colony-stimulating factor and its receptor in normal hematopoietic cell development and myeloid disease. Rev Physiol Biochem Pharmacol. 2003; 149: 53-71.

5. **Erkeland SJ**, Valkhof M, Heijmans-Antonissen C, Delwel R, Valk PJ, Hermans MH, Touw IP. The gene encoding the transcriptional regulator Yin Yang 1 (YY1) is a myeloid transforming gene interfering with neutrophilic differentiation. Blood. 2003 Feb 1; 101(3): 1111-7.

6. Hoetelmans R, van Slooten HJ, Keijzer R, **Erkeland S**, van de Velde CJ, Dierendonck JH. Bcl-2 and Bax proteins are present in interphase nuclei of mammalian cells. Cell Death Differ. 2000 Apr; 7(4): 384-92.

7. Danen-Van Oorschot AA, Zhang Y, **Erkeland SJ**, Fischer DF, van der Eb AJ, Noteborn MH. The effect of Bcl-2 on Apoptin in 'normal' vs transformed human cells. Leukemia. 1999 Apr; 13 Suppl 1: S75-7.

8. Borradori L, Koch PJ, Niessen CM, **Erkeland S**, van Leusden MR, Sonnenberg A. The localization of bullous pemphigoid antigen 180 (BP180) in hemidesmosomes is mediated by its cytoplasmic domain and seems to be regulated by the beta4 integrin subunit. J Cell Biol. 1997 Mar 24; 136(6): 1333-47.