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Ectopic expression of the homeobox gene Cdx2 is the key transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia; a novel mechanism in AML

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Die ektope Expression des Homeobox-Gens *Cdx2* ist der entscheidende transformierende Vorgang in einem Mausmodell einer t(12;13)(p13;q12) Akuten myeloischen Leukämie; ein neuer Mechanismus der AML-Entstehung

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1 INTRODUCTION

1.1 Hematopoiesis and hematopoietic stem cells

The term hematopoiesis refers to the formation and development of the cells of the blood. In humans, this process begins in the yolk sac in the first weeks of embryonic development. By the third month of gestation, stem cells migrate to the fetal liver and then to the spleen. Between 3 and 7 months of gestation these two organs play a major hematopoietic role whereas later on the bone marrow becomes the major hematopoietic organ and hematopoiesis ceases in the liver and spleen (Fig.1).

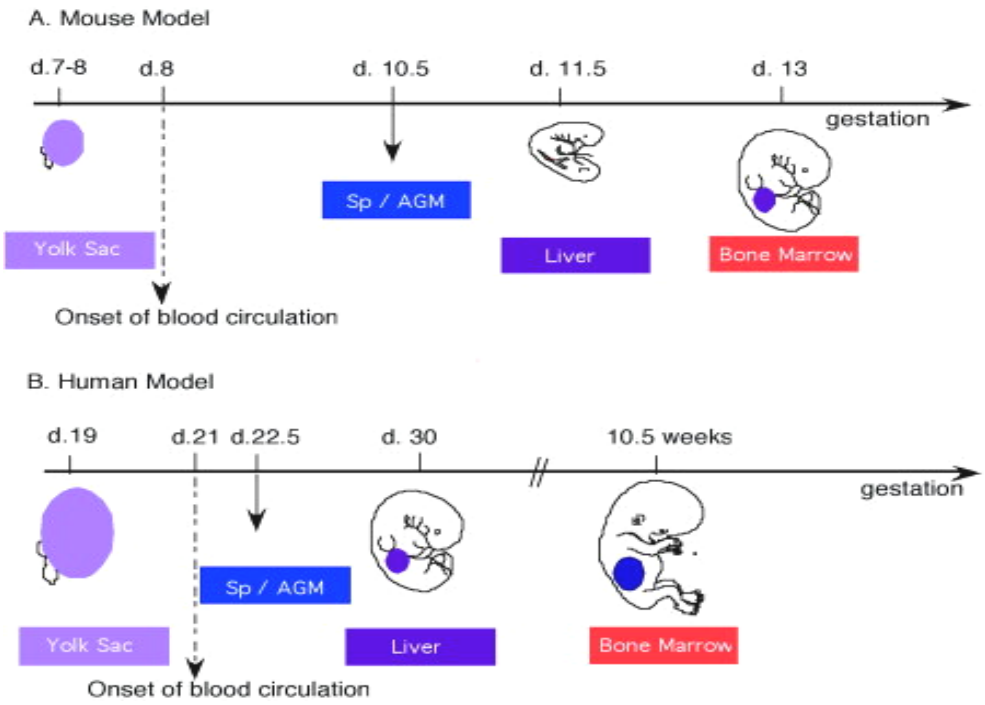


Figure 1. Ontogenic emergence of the hematopoietic system (figure adapted from Bonnet, 2003)

As the embryo develops, this primitive hematopoiesis is superseded by the definitive multilineage blood system (Bonnet, 2003). Every day, the body produces billions of new blood cells. All of these are derived from rare cells in the bone marrow called the hematopoietic stem cells (HSC). Adult stem cells are defined as cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation (Reya et. al., 2001). The first evidence for the existence of such a cell–type came from experiments by Ray Owen and co-workers in 1945, which showed that bovine fraternal twins, sharing a single placenta and blood circulation, retained production of blood cells genetically defined to be from both throughout their life. The hallmark properties of the hematopoietic stem cells (HSCs) were defined in 1963 by Till and McCulloch (Siminovitch et. al., 1964). In the murine model, the authors provided evidence for the existence of HSCs in bone marrow. These HSCs could reconstitute the hematopoietic system, and hence rescue lethally irradiated recipient animals. Using serial transplantations, they showed that HSCs possess the ability of self-renewal. Based on these experiments, HSCs were defined as "cells with the abilities of multilineage differentiation as well as unrestricted self-renewal." This discovery marked the beginning of modern-day stem-cell research (Bonnet, 2003). The first attempts at purifying HSCs came from experiments carried out by Till and McCullogh in the Netherlands (Van Bekkum et. al., 1979; Worton et. al., 1969). Based on their work, it has become possible to routinely identify and isolate highly purified murine and human HSCs based mainly on characteristic cell surface proteins that are either present (Sca1 and c-kit) or absent (using markers of lineage committed cells such as CD38, Mac-1 and CD8) (Weissman, 2002).

In the hematopoietic system, stem cells are heterogeneous with respect to their ability to self-renew and can be divided into three different populations: long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors without detectable self-renewal potential (Morrison and Weissman, 1994; Morrison et. al., 1997). These populations form a lineage in which the long-term HSCs give rise to short-term HSCs, which in turn give rise to multi-

potent progenitors (Morrison et. al., 1997). As HSCs mature from the long-term self-renewing pool to multipotent progenitors, they progressively lose their potential to self-renew but become more mitotically active. Whereas long-term HSCs give rise to mature hematopoietic cells for the lifetime of the mouse, short-term HSCs and multipotent progenitors reconstitute lethally irradiated mice for less than eight weeks (Fig.2).

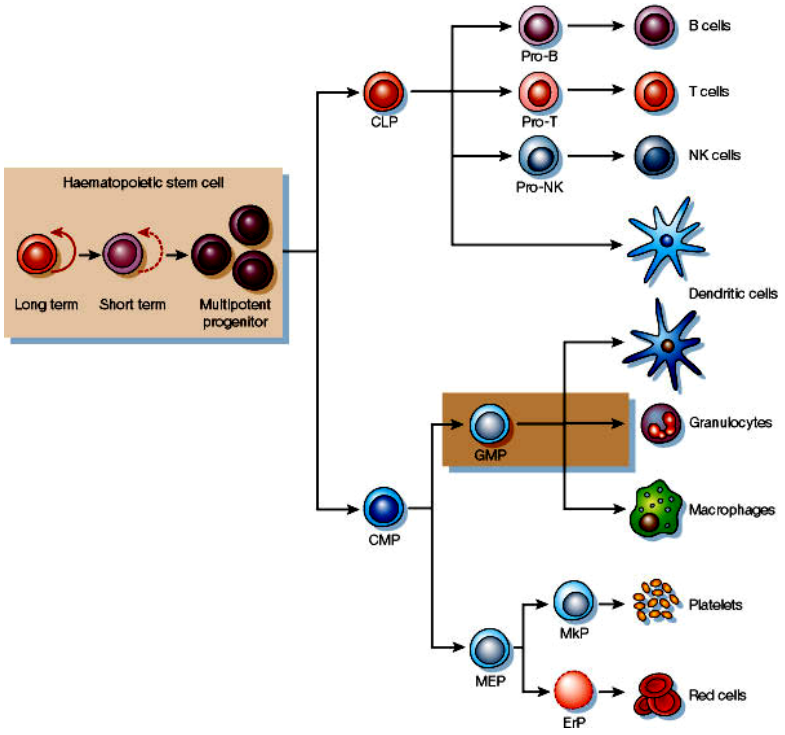


Figure 2. Development of hematopoietic stem cells. HSCs can be subdivided into long-term self-renewing HSCs, short-term self-renewing HSCs and multipotent progenitors (red arrows indicate self-renewal). They give rise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). Both CMPs/GMPs (granulocyte macrophage precursors) and CLPs can give rise to all known mouse hematopoietic cells (Figure adapted from Reya et. al., 2001)

1.2 Self-renewal and leukemogenesis

HSCs that enter cell division are faced with the fundamental options to self-renew or differentiate. Once multilineage cells are committed to differentiation, they either engage in myeloid differentiation to generate common myeloid pro-

genitors (CMP) or take the lymphoid path to generate common lymphoid progenitors (CLP) (Akashi et. al., 2000; BitMansour et. al., 2002; Hoang, 2004). The process of differentiation can be studied experimentally (spleen colony assay) through the capacity of these multipotent cells to give rise to other multipotent cells in their progenies and therefore to sustain multipotency. In contrast, the emergence of committed progenitors with restricted differentiation potential would lead to an extinction of multipotency (Hoang, 2004).

The characteristic of self-renewal is often described as a hallmark of normal stem cells and is perhaps the most important intrinsic cellular property that is subverted during stem cell tumorigenesis (Jordan and Guzman, 2004). Human cancer stem cells, identified in acute myelogenous leukemia (AML) (Bonnet and Dick, 1997), myeloid blast crisis of chronic myelogenous leukemia (Jamieson et. al., 2004), breast cancer and brain tumors (Al-Hajj et. al., 2003; Singh et. al., 2003) share functional properties with normal stem cells such as self-renewal, high proliferative potential, some differentiation capacity and ability to be serially transplanted (Jamieson et. al., 2004b; Passegue et. al., 2003). Huntly et. al. showed in a mouse model that leukemia-associated oncogenes have the capacity to confer self-renewal properties to hematopoietic progenitors that inherently lack the ability to self–renew (Huntly et. al., 2004). Constitutive expression of the *AML1-ETO* translocation product has been shown to increase the self-renewal frequency of stem cells (Mulloy et. al., 2002).

If dysregulation of signaling pathways that normally regulate stem cell self-renewal leads to tumorigenesis, then stem cells themselves might be the target of transformation in certain types of cancer. There are two reasons to think that this hypothesis may be correct: first, because stem cells have the machinery for self-renewal already activated, maintaining this activation may be simpler than turning it on de novo in a more differentiated cell; that is, fewer mutations may be required to maintain self-renewal than to activate it ectopically. Secondly, stem cells often persist for long periods of time, instead of dying like many mature cells in highly proliferative tissues. This means that there is a much greater

opportunity for mutations to accumulate in individual stem cells than in most mature cell types. Alternatively, self-renewal could also be acquired in certain leukemia's by the critical genetic alterations in downstream progenitors which convey stem cell characteristics to these cells. Though the molecular mechanisms of stem cell self-renewal are poorly understood, several genes and gene families have been implicated in the process including the Hox (Antonchuk et. al., 2001; Buske et. al., 2002) and Wnt gene families (Fig.3) (Reya et. al., 2003).

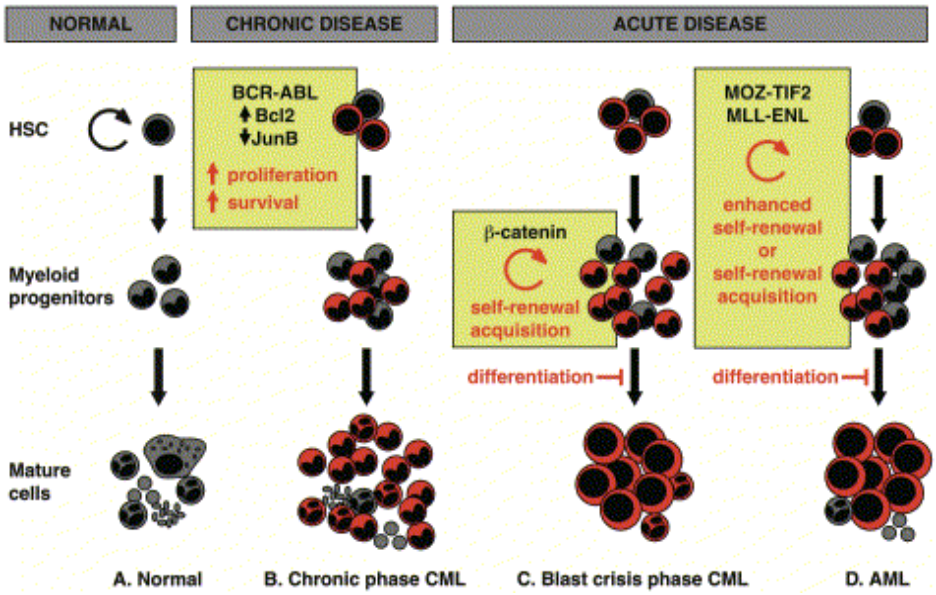


Figure 3. Oncogene hierarchy and role of self-renewal in the pathogenesis of leukemias. **A**. Normal myelopoiesis is distinguished by the orderly differentiation of HSC, which are the only cells with self-renewal capacity, into committed myeloid progenitors and their respective terminally differentiated progeny. **B.** Chronic diseases, such as chronic phase CML, are associated with preleukemic events that result in increased survival and proliferation within the stem and myeloid progenitor populations but continued production of terminally differentiated progeny. Acquisition of *BCR-ABL*, overexpression of *BCL2*, and inactivation of JunB expression are examples of such events that initially take place in HSC. **C and D:** Acute diseases such as blast crisis CML and AML are marked by acquisition of self-renewal capacity by progenitors that normally lack it or by enhanced self-renewal in HSC. β-catenin activation during the progression of CML to blast crisis is an example of such a leukemogenic event occurring in myeloid progenitors. MOZ-TIF2 and MLL-ENL are AML-associated translocation products that may enhance HSC self-renewal or endow myeloid progenitors with self-renewal potential. Together with a subsequent block in differentiation, these leukemogenic

events result in the accumulation of immature blast progeny and development of AML at either the HSC or myeloid progenitor stage (Figure adapted from Jamieson et. al., 2004b) .

1.3 Role of homeobox gene in normal and leukemic hematopoietic cells

The homeobox (HB) was first identified in the 1980s as a sequence motif that was shared among Drosophila homeotic genes (the HOM-C complex), where they play a key role in embryonic differentiation along the anterior–posterior (ap) axis, and is now known to be present in many genes in virtually all eukaryotic species (Stein et. al., 1996). It is estimated that the human genome contains at least 200 homeobox genes (Tupler et. al., 2001), of which only 39 are members of the HOX family. Unlike the HOM-C/HOX genes, which are organized in gene clusters, most homeobox genes are dispersed throughout the genome (Abate-Shen, 2002).

Homeobox-containing genes constitute a gene family characterized by a highly conserved 183-nucleotide sequence encoding a 61-amino acid domain, the homeodomain (HD). These homeodomains are structurally related to the helix-turn-helix motif of prokaryotic DNA-binding proteins and have sequence-specific DNA binding activity (Fig. 4) (Buske and Humphries, 2000). Sequences flanking the HD also influence specificity by coordinating interaction with cofactor proteins that influence DNA-binding properties. Homeobox genes are divided into two classes. Class I include clustered HB (HOX) genes and that comprises 39 members, while the Class II divergent HB (non-HOX) genes are dispersed through the genome, and include small families, such as the MSX, ParaHox (CDX), PAX, DLX and Engrailed (EN) groups, and are intermediate in size. Although individual members of homeobox families often share little sequence similarity other than the homeobox, some families have additional conserved sequence motifs that contribute to their distinct functional properties (Fig. 5) (Abate-Shen, 2002; Owens and Hawley, 2002)

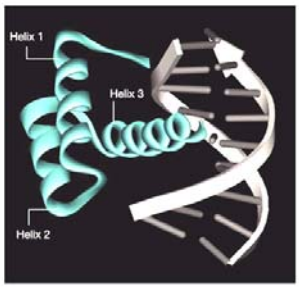


Figure 4. The structure of the homeodomain. Despite its considerable variation in primary sequence, the three-dimensional structure of the homeodomain has been well conserved, and corresponds to three α helices that have a relatively unstructured amino-terminal arm. The consensus amino acids, which are invariant among all homeodomains, are essential in maintaining structural integrity and making essential contacts with DNA. Amino acids that distinguish the various homeoprotein families contribute to differences in their DNA-binding specificities and other functional properties. The figure shows the Engrailed–DNA complex (Figure adapted from Abate-Shen, 2002)

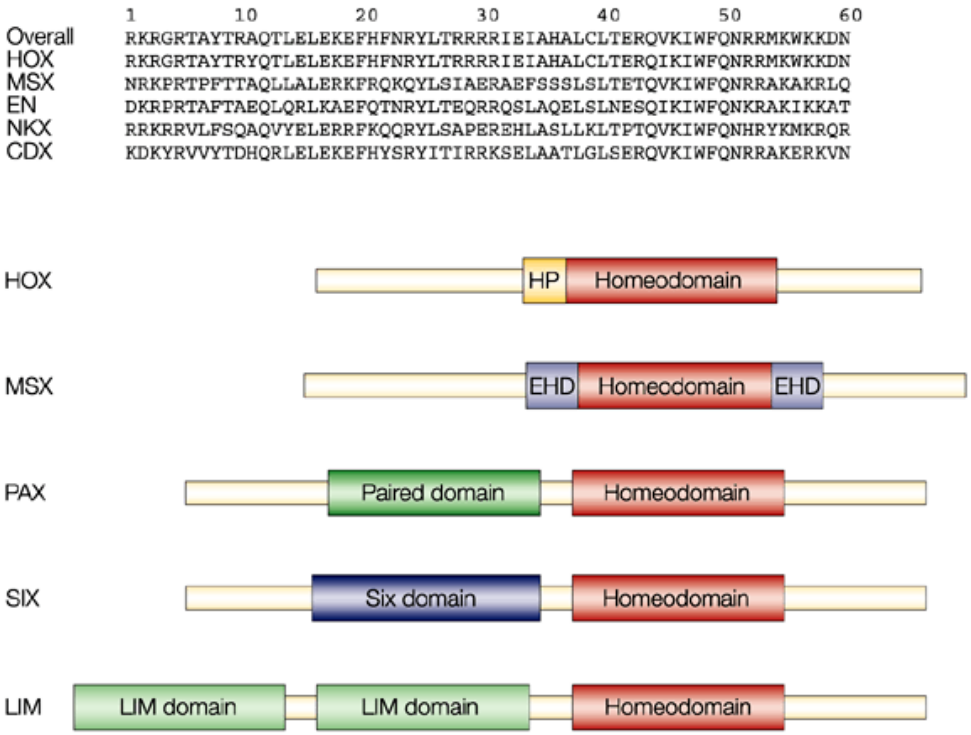


Figure 5. Generating diversity among homeoproteins. The distinct classes of homeoproteins show considerable sequence diversity within the homeodomain (top), and within the amino- and carboxy-terminal regions that flank this domain (bottom). The sequence diversity is believed to contribute to their distinct functional properties, by generating distinct DNA-binding specificities, promoting unique protein–protein interactions, and other mechanisms.

Functional diversity is also generated by additional conserved domains or motifs that occur in particular homeoprotein families. HOX proteins contain a hexapeptide (HP) motif—a short stretch of conserved residues that are responsible for mediating interactions with PBX homeoproteins. MSX proteins also have short stretches of conserved amino acids that flank the homeodomain, called the extended homeodomain (EHD), although their functions are unknown. PAX proteins contain an additional DNA-binding domain, known as the paired box. Members of the SIX family have a conserved amino-terminal domain called the Six domain, and LIM homeoproteins are named after a protein interaction motif, the LIM domain (Figure adapted from Abate-Shen, 2002).

1.3.1 HOX gene expression in normal and leukemic hematopoietic cells

Numerous studies have now shown that, in addition to embryonic tissue, HOX genes are expressed in mammalian adult tissues, both normal and neoplastic. These include normal and neoplastic skin (Care et al., 1996), kidney (Barba et al., 1993), human colonic mucosa (De Vita et al., 1993; Wang et al., 2001), testis (Watrin and Wolgemuth, 1993) and hematopoietic cells (Antonchuk et al., 2002; Buske et al., 2001; Giampaolo et al., 1995; Schnabel et al., 2000). These studies revealed that HOX gene expression in normal hematopoiesis is strictly regulated and differs strikingly between early progenitors and mature, differentiated hematopoietic cells. HOX genes of the A, B, and C cluster are expressed in early $CD34^+$ hematopoietic progenitors, whereas HoxD gene transcription is absent in this progenitor pool (Buske and Humphries, 2000). In sharp contrast, expression of certain Hox genes of the A, B, and C clusters is absent or substantially down regulated in the more mature $CD34^-$ compartment, (Giampaolo et. al., 1995; Lawrence et. al., 1996) indicating that HOX genes have a major role in primitive hematopoietic cells (Fig. 6).

The function of HOX genes in hematopoiesis has been investigated through knockout mouse models and overexpression from retroviral vectors in hematopoietic stem cells from murine fetal liver and bone marrow and in human cord blood progenitors. Overexpression of *Hoxb8*/*IL-3* in mice led to an aggressive transplantable leukemia in contrast to mice transplanted with *IL-3* only (Perkins

et. al., 1990). Leukemic blasts from many patients with acute myeloid leukemia (AML) show an aberrant pattern of *HOXA10* expression, further supporting the concept that dysregulated HOX gene expression may be a general feature of this malignancy (Kawagoe et. al., 1999; Lawrence et. al., 1995). Overexpression of HOXA10 in the progeny of human cord blood or fetal liver cells perturbs human lymphomyelopoiesis *in vitro* and *in vivo* (Buske et. al., 2001). Notably, the expression of *HOXA9* has been identified as one of the most consistent diagnostic markers of AML in humans and as the only single gene expression marker of more than 6800 cDNAs tested by DNA micro-array analysis that correlated with clinical outcome (Golub et. al., 1999). Overexpression of *Hoxa9* and *Hoxa7* caused AML in the BXH-2 mouse line (Nakamura et. al., 1996b). Aberrant expression of *HOXA9* has been reported to be an important component of leukemogenesis driven by MLL translocations (Armstrong et. al., 2002). Beside the aberrant expression of wild-type HOX genes in AML, recent molecular characterization of the two translocations t(7;11) and t(2;11) proved that HOX genes themselves such as *HOXA9* and *HOXD13* are direct targets of leukemia-associated genetic alterations (Iwasaki et. al., 2005; Kroon et. al., 2001; Nakamura et. al., 1996a).

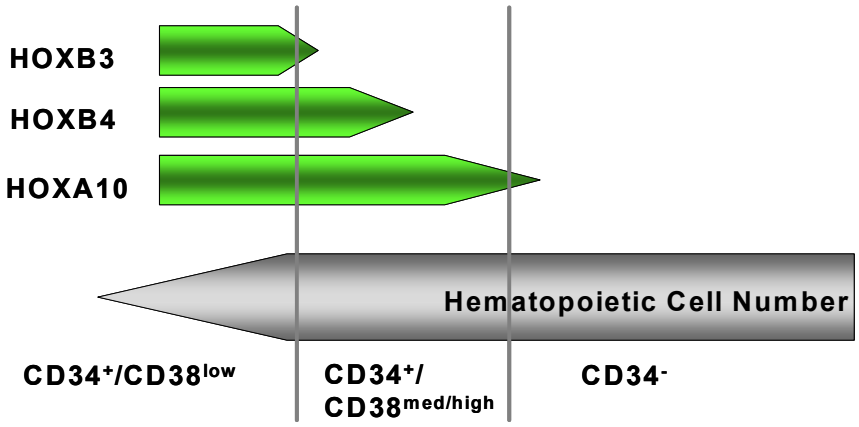


Figure 6. Expression of HOX genes in human BM cells. HOX genes are expressed in early progenitor cells. Their expression decreases during differentiation.

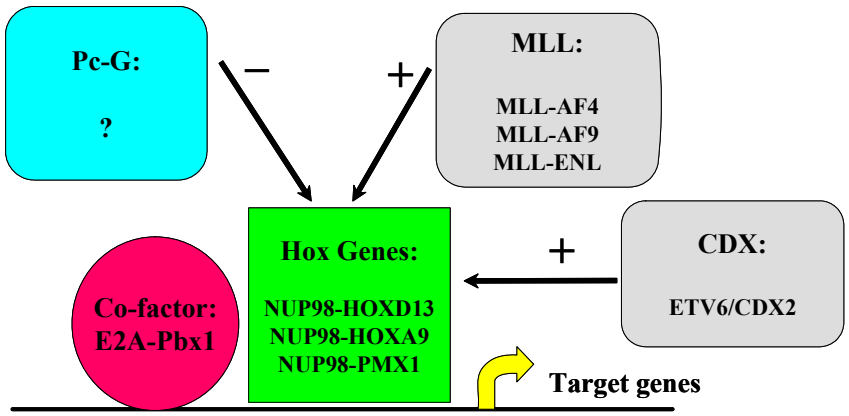


Figure 7. Homeobox genes are involved in leukemogenesis. The molecular characterization of chromosomal translocations has shown that Hox genes and their upstream regulators like members of the MLL and CDX family are directly involved as partner genes in leukemia-specific fusions as demonstrated for *HOXA9* and *HOXD13* in NUP98 fusions, for *PBX1* in the *E2A–PBX1* fusion and *CDX2*, a non HOX homeobox gene, with *ETV6*. Furthermore, the HOX upstream regulator *MLL* is one of the most frequently altered genes in human leukemia's.

1.3.2 Non-clustered homeobox genes in leukemogenesis

Several non-clustered HD proteins have been shown to function as cofactors for HOX proteins, interacting with HOX genes by a conserved sequence YPWMK and are co-expressed during embryonic development. These include the three-amino-acid-loop-extension (TALE) proteins, PBX, MEIS, and PREP1/KNOX1. PBX genes are widely expressed in fetal and adult tissues, although PBX1 transcripts are notably absent from lymphocytes (Monica et. al., 1991). The hematopoietically expressed (HEX) gene as another example of a non-clustered homeobox gene was initially identified in human hematopoietic cells, where it is expressed in multipotential progenitors, myeloid cells, and B cells, but not T or erythroid cells (Bedford et. al., 1993). HEX expression has also been detected in peripheral blood leukocytes from leukemia patients (Manfioletti et. al., 1995). Other non-HOX HB genes like *HOX11*, and members of the CDX, HLX, and PMX1 families are more restricted in their patterns of expression, and are involved in organogenesis or differentiation of specific cell types.

1.3.2.1 TALE homeobox genes

Members of the TALE family of non-HOX HD cofactor proteins, which include PBX, MEIS, and PREP1/KNOX1 proteins, are distinguished from other HD proteins by the inclusion of an additional three amino acids between α-helices 1 and 2 within the HD. Evidence from mouse models and human leukemic cells has indicated that inappropriate expression of TALE cofactors with certain HD proteins may contribute to leukemic transformation, while overexpression of TALE cofactors alone does not lead to disease development. One of these proteins, PBX1, has oncogenic potential in the form of a fusion protein with E2A. The *PBX1* gene was originally identified at the breakpoint of the t(1;19) translocation found in 10 to 20% of childhood pre-B-cell ALL (Shikano et. al., 1999). *MEIS1*, was capable of accelerating leukemogenesis when coexpressed with *HOXA9*, *HOXB3* and the fusion protein *NUP98-HOXA9* (Thorsteinsdottir et. al., 2001). Overexpression of *MEIS1* was reported in 50% of AML patients.

1.3.2.2 *CDX* genes are members of the ParaHox gene family

Cdx1, *Cdx2* and *Cdx4* are members of the caudal-related homeobox gene family based on their homology to the *caudal* gene of *Drosophila melanogaster* (James et. al., 1994; Suh et. al., 1994). The caudal homeobox gene in *Drosophila* is required for anterior-posterior regional identity (McGinnis and Krumlauf, 1992). Cdx members are upstream regulators of HOX genes. *Cdx1* and *Cdx2* loss of function models are associated with HOX gene inactivation. For example, a dominant negative *Xcad3* (the frog Cdx homolog) affects development of the posterior embryo in a manner that is reflected by loss of expression of a number of HOX genes, including *Hoxc6, Hoxa7, Hoxb7 and Hoxb9. Cdx4* mutant kgg embryos show deregulation of *hoxb4, hoxb5a, hoxb6b, hoxb7a, hoxb8a, hoxb8b and hoxa9a*. The hematopoietic phenotype of kgg embryos can be rescued by overexpressing *hoxb7a* or *hoxa9a* but not *hoxb8a* (Davidson et. al., 2003), indicating that the hematopoietic defect results

from perturbations in specific HOX genes (Davidson et. al., 2003). *CDX2* is expressed before most other HOX genes and it increases expression of *HOXA9* in cultured human colon cancer cells (Lorentz et al., 1997), indicating that Cdx family members are positive upstream regulators of HOX genes and play a role in embryogenesis. *Cdx1* and *Cdx2* which are expressed in the gut endoderm are involved in anterior-posterior patterning (Silberg et. al., 2000). *Cdx2* expression starts in the proximal intestine and decreases posteriorly, whereas *Cdx1* expression starts in the distal intestine with overlapping expression of *Cdx1* and *Cdx2* in the midgut (Fig.8).

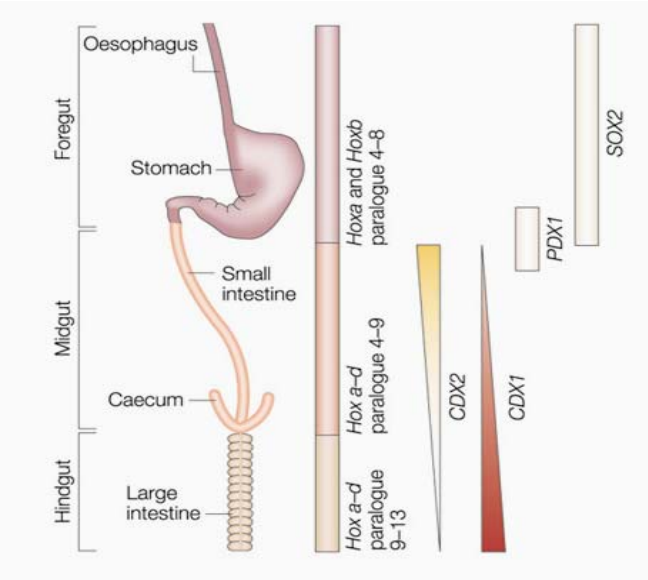


Figure 8. Regional expression of transcription factors in the developing gut. The transcription factors shown on the right have been mapped to specific regions of the endoderm, as shown on the left. There is a presumed rule of expression patterns of HOX genes in the developing gut. Each subdomain of the developing digestive tract (foregut, midgut and hindgut) seems to have a different HOX code: *Cdx2* expression starts in the proximal intestine and decreases (lightening bar) posteriorly, whereas *Cdx1* expression starts in the distal intestine and decreases anteriorly. Pdx1 expression has been observed in the antrum and duodenum. Sox2 is expressed from the pharynx to the stomach.

CDX2 plays an important role in development of the intestine and deregulated expression of *CDX2* is found in intestinal metaplasia and carcinomas (Bai et. al., 2002; da Costa et. al., 1999; Eda et. al., 2003; Eda et. al., 2002). *CDX2* is not normally expressed in adult hematopoiesis, but it has been identified as one

of the fusion partners in an AML-associated chromosomal translocation (Chase et. al., 1999).

1.4 CDX2

CDX2 (caudal type homeobox transcription factor 2) belongs to the caudal homeobox family and encodes a transcription factor. CDX2 is located on chromosome 13 and mouse Cdx2 on chromosome 5. The gene has three exons and two introns (Drummond et. al., 1997; James et. al., 1994). Human and mouse Cdx2 share overall 93% homology at the amino acid level. CDX2 is a 93kDa nuclear protein consisting of the N-terminal transactivation domain, a highly conserved pentapeptide domain, which allows binding of the TALE co-factor and the DNA binding homeodomain (Fig.9). Transcriptional activity of Cdx2 is regulated by the mitogen activated protein kinase (MAPK). The CDX2 activation domain is phosphorylated at serine 60 via the MAPK pathway. The S60-phosphorylated and S60-nonphosphorylated CDX2 have different transcriptional activity, as well as different spatial expression patterns in the intestinal epithelium (Rings et. al., 2001). p38 MAPK has been reported to regulate CDX2/3 function and intestinal cell differentiation (Houde et. al., 2001).

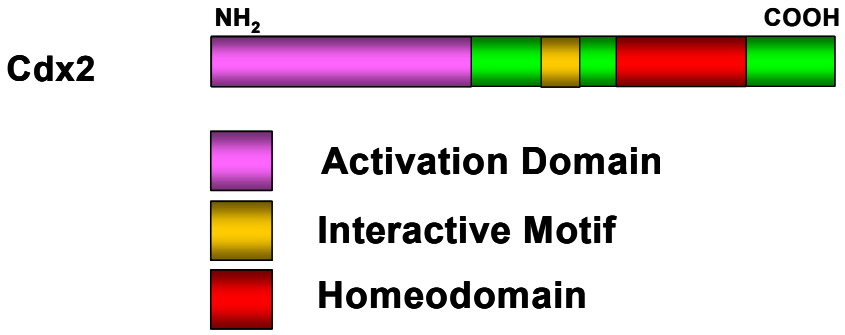


Figure 9. Cdx2 protein structure.

1.4.1 Role of *Cdx2* in embryogenesis and intestine development

Cdx genes have developmental roles and overlap in their expression patterns. *Cdx2* is expressed at 3.5 days postcoitum (dpc) in the trophectoderm but not in the inner cell mass (Beck et. al., 1995). At 7.5 dpc (Theiler stage 11), it is present in the chorion, ectoplacental cone, mesoderm of the developing allantoic bud, and posterior primitive streak. At 8.5 dpc (Theiler stage 13), expression is seen in all three germ layers at the posterior end of the embryo extending into the mesodermal root of the allantois, in the endodermal epithelium of the hindgut rudiment, and in the neural tube; the presegmented paraxial mesoderm expresses the gene, but the somites, lateral plate, and intermediate mesoderm do not. By 9.5 dpc (Theiler stage 15), the caudal pole of the embryo remains positive, as does the posterior neural tube and posterior gut endoderm. At 12.5 dpc (Theiler stage 20), expression is confined exclusively to the gut endoderm posterior to the foregut-midgut junction (Beck et. al., 1995; Chawengsaksophak et. al., 2004). *Cdx1* expression extends most anteriorly, followed by *Cdx2* and *Cdx4*, with all three genes expressed posteriorly.

Cdx2-null mutant embryos die between 3.5 and 5.5 dpc, and heterozygotes have tail abnormalities and exhibit anterior homeotic transformations involving the cervical and upper thoracic vertebrae, ribs, and midgut endoderm. 90% of Cdx2 heterozygote mutant mice develop multiple intestinal adenomatous polyps, particularly in the proximal colon, suggesting that Cdx2 mutations might be the primary event in the genesis of some intestinal tumours (Chawengsaksophak et. al., 2004; Chawengsaksophak et. al., 1997). Further studies by Traber et. al. have shown that *Cdx2* is strongly expressed at the crypt-villus junction and into the villus. *Cdx2* is important for transcription of a gene that is expressed in terminally differentiated intestinal epithelial cells, supporting that Cdx2 stimulates differentiation and expression of intestine–specific genes such as sucrase/isomaltase, carbonic anhydrase, and MUC2. In addition, *Cdx2* upregulates WAF1 (Silberg et. al., 2000; Suh et. al., 1994).

1.4.2 Silencing or aberrant overexpression of *CDX2* in metaplasia, carcinomas and myeloid leukemia

CDX2 is normally expressed in the gut during development and adulthood. However, its expression is lost in colorectal tumors and corresponding carcinoma cell lines (Ee et. al., 1995; Hinoi et. al., 2003; Hinoi et. al., 2001). A role for the loss of *Cdx2* expression in colorectal carcinoma development can be inferred from the frequent occurrence of adenomatous intestinal polyps in *Cdx2* heterozygous mutant mice. *CDX2* overexpression seems to inhibit growth and/or promote differentiation of colorectal cancer cells (Mallo et. al., 1998). *CDX2* inactivation in colon cancer results from defects in trans-acting pathways regulating *CDX2* transcription.

Beside the loss of function in colon cancer, aberrant and ectopic expression of *CDX2* is associated with intestinal metaplasia (Hinoi et. al., 2003; Ishikawa et. al., 2004; Satoh et. al., 2002), Barrett's epithelium (Eda et. al., 2003), gastric carcinoma (Silberg et. al., 2002) , but also with hematological neoplasias such as AML and chronic myeloid leukemia (CML) (Chase et. al., 1999; Rawat et. al., 2004). Normally *CDX2* is not expressed in adult hematopoietic cells. Ectopic expression of CDX2 in AML was reported by Chase et al. in a patient with AML M1 with a chromosomal translocation t(12;13)(p13;q12), creating a novel fusion of *ETV6* and *CDX2* (Chase et. al., 1999). In addition, aberrant expression of CDX2 was found in a case of blast crisis of chronic myeloid leukemia (CML-BC) with no detectable chromosomal abnormality (Chase et. al., 1999). The aim of the study presented here was to assess the oncogenic potential of both the *ETV6/CDX*2 fusion gene and the *CDX2* homeobox gene as will be discussed later in the thesis.

1.5 Chromosomal Translocations

Recurring chromosomal abnormalities have been identified in a variety of cancers, but are most frequently associated with leukemias, lymphomas and sarcomas (Rabbitts, 1994; Rowley, 1999). At present, more than 500 recurring

cytogenetic abnormalities have been reported in hematological malignancies. Three main cytogenetic changes have been detected in hematological malignancies: deletions, inversions and translocations. There are two principal consequences of chromosomal translocations: one consequence is that the breaks occur within a gene on each chromosome involved, creating a fusion gene which encodes a chimeric protein. Alternatively, a chromosomal translocation can induce aberrant or ectopic expression of a proto-oncogene by the juxtaposition of a coding region from one gene to the promoter of another gene (Rabbitts, 1994).

1.5.1 Generation of fusion genes with oncogenic properties

There are more than 300 recurring chromosomal translocations, of which more than 100 have been cloned. The Philadelphia (Ph) chromosome was the first chromosomal aberration that was found to be specifically associated with a human disease (Rowley, 1999), with more than 95% of CML cases bearing this fusion gene. This translocation leads to the formation of the fusion gene BCR-ABL which is a constitutively active tyrosine kinase. There are at least seven different chromosomal translocations associated with a myeloproliferative disease. These include the *BCR/ABL*, *ETV6/ABL*, *ETV6/PDGFRB*, *HIP1/PDGFRB*, *H4/PDGFRB, RAB5E/PDGFRB*, and *ETV6/JAK2* fusions (table 1) (Dash and Gilliland, 2001).

Table 1: Translocations involving tyrosine kinases

MPD = myeloproliferative disease

Translocation	Genes	Major structures	Disease
t(9;22)(q34;q11)	ABL1 BCR	TK GRB2/SOS binding	CML,ALL
t(8;13)(p11;q12)	FGFR1 ZNF198/FIM/RAMP	TK Cysteine rich MYM	MPD

t(6;8)(q27;p11)	FOP FGFR1	Leucine rich TK	MPD
t(8;9)(p12;q33)	FGFR1 CEP110	TK LZ	MPD
t(5;7)(q33;q11.2)	PDGFRB HIP1	TK Huntingtin inter-action	CMML
t(5;14)(q33;q32)	PDGFRB CEV14	TK LZ	AML
t(5;10)(q33;q21)	PDGFRB H4/D10S170	TK LZ	MPD

Besides tyrosine kinases, transcription factors are common targets of AML associated chromosomal translocations. In particular dysregulation of transcription factors, which play an important role in normal hematopoiesis, is a hallmark of AML. For example, there are more than a dozen chromosomal translocations that target subunits of the core binding factor (CBF) which is a heterodimeric transcription factor composed of CBFB and RUNX1 (AML1). CBF transactivates expression of a broad spectrum of genes that are critical for normal hematopoietic development (Speck et. al., 1999), as evidenced in part by a complete lack of definitive hematopoiesis in mice that are deficient in either AML1 (RUNX1) or CBFB (Castilla et. al., 1996; Okuda et. al., 1996). Thus, chromosomal translocations that resulted in loss of function of CBF would be predicted to impair hematopoietic development. The three most common translocations involving CBF are the t(8;21) (Erickson et. al., 1992), inv(16) (Liu et. al., 1993), and t(12;21) (Romana et. al., 1995) that result in expression of the *AML1/ETO*, *CBFB/MYH11* and *ETV6/AML1* (Barba et. al., 1993, Golub et. al. 1999) fusion proteins. AML associated chromosomal translocations result in the persistent expression of HOX family members which are downregulated during normal differentiation, resulting in a block in differentiation. Another gene frequently involved in leukemia–associated translocations is the *ETV6* (TEL) gene, which has been shown to be fused to over 17 different partner genes in hematological malignancies.

1.5.2 Aberrant expression of proto-oncogenes

In lymphoid leukemias and lymphomas, chromosomal translocations frequently lead to the transcriptional activation of proto-oncogenes by bringing their coding regions in the vicinity of immunoglobulin or T-cell receptor gene-regulatory elements, thus leading to the inappropriate expression of these proto-oncogenes (Kuppers and Dalla-Favera, 2001). Chromosomal translocations associated with Burkitt's lymphoma represent a typical example of this type of mechanism. Approximately 90% of Burkitt's lymphoma cases harbor a t(8;14)(q24;q32), which juxtaposes the *MYC* proto-oncogene to the immunoglobulin heavy-chain gene enhancer and promoter (IgH) (Kuppers and Dalla-Favera, 2001). In addition, the variant translocations t(2;8) and t(8;22) described in this disease also involve *MYC*, which translocates into the immunoglobulin κ and λ light-chain loci, respectively. In these translocations, the coding region of *MYC* is not altered, suggesting that its oncogenic activity is a result of its mis- or overexpression, although rare somatic mutations in the coding region of *MYC* have also been described in Burkitt's lymphoma (Showe et. al., 1985). MYC transgenic mice (TM) in which the MYC gene was expressed under the control of the IgH enhancer (Eμ-myc TM) developed clonal B lymphoid malignancies, after a long pre-lymphomatous phase characterized by the expansion of polyclonal pre-B cells (Langdon et. al., 1986). Aberrant expression of the BCL6 proto-oncogene caused by chromosomal translocations is also frequently reported in non-Hodgkin's lymphoma. BCL6 is normally expressed in B and CD4+ T cells in germinal centers (GC), and plays an essential role in GC formation (Ye et. al., 1997, Rego and Pandolfi, 2002). The homeobox gene HOX11 is deregulated in the translocation t(10;14) found in 7% of human T-cell acute lymphoblastic leukemia (T-ALL). HOX11 is not expressed in normal T-ALL (Hatano et. al., 1991).

Strikingly and in contrast to the situation in lymphomagenesis or in the development of lymphoblastic leukemias, aberrant and ectopic expression of a proto-oncogene induced by a chromosomal translocation has so far been not described as the key element for malignant transformation in myeloid leukemias.

The failure of certain fusion genes to induce leukemia on their own in animal models as well as the fact that more than 50% of acute myeloid leukemias do not have apparent cytogenetic abnormalities, point to the role of point mutations in or, alternatively, the aberrant expression of proto-oncogenes in the development of leukemia. These observations also suggest that the aberrant expression of proto-oncogenes might be more common than is generally believed. The sheer number of *ETV6* fusions in AML allows for the study of a diverse group of leukemias in which more than one of the aforementioned mechanisms might be operating. The t(12;13) translocation is particularly interesting in this regard as the expression of both the *ETV6/CDX2* fusion gene as well as the ectopic expression of the *CDX2* gene has been demonstrated.

1.6 ETV6

ETV6 (ETS variant gene 6) was originally called *TEL*, for translocation ets leukemia gene. ETV6 is a member of the ets (E-26 transforming specific) family of transcription factors. All ets family proteins share a conserved protein domain of about 88 amino acids in length, the so called *ets* domain (see Figure 10) (Bohlander SK 2005). The *ets* domain is a sequence specific DNA binding domain but it also mediates protein-protein interaction. The other evolutionarily conserved domain is the N terminally located *pointed* or SAM (sterile alpha motif) domain in the 652 amino acids of ETV6 (Bohlander SK 2005). This domain is also called HLH (helix loop helix) domain. It is found in yeast proteins and has been shown to be involved in homo and heterodimerization of transcription factors and in signal transducing proteins (e.g. of the MAPK pathway) (Fig.10). *ETV6* contains two alternative translational start codons (position 1 and position 43), leading to the expression of two isoforms of ETV6.

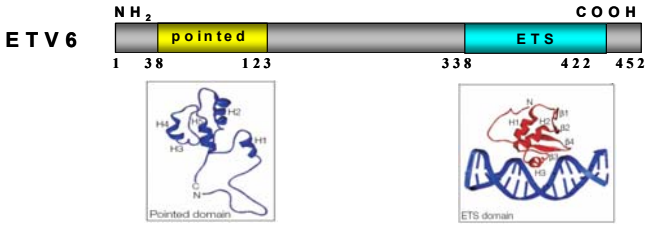


Figure 10. ETV6 protein structure.

1.6.1 Role of *ETV6* in hematopoiesis

ETV6 is widely expressed throughout embryonic development and in the adult. Embryos with a conventional knockout (KO) of the *Etv6* gene die by day 11 of embryonic development (E11) due to vascular abnormalities. Blood formation in the embryo is largely unperturbed (Wang et. al., 1997). Yet, studies using chimeric mice from Etv6-deficient embryonic stem (ES) cells suggested a requirement of *Etv6* in bone marrow hematopoiesis. Inducible and lineage-specific gene disruption of *Etv6* in adult hematopoiesis in mice suggests that it plays two important roles in hematopoietic differentiation. First, *Etv6* controls the survival of HSCs so that its disruption indirectly affects the majority of all hematopoietic cells which have limited clonal life spans and eventually will extinguish without constant regeneration from HSCs. Secondly, *Etv6* is required late in the development of the megakaryocyte lineage, where it presumably acts in concert with transcriptional regulators previously implicated in megakaryopoiesis (Hock et. al., 2004).

1.6.2 *ETV6* fusion partners

Translocations involving the *ETV6* gene contribute to leukemogenesis through at least 3 different mechanisms. One mechanism is the activation of kinases. The second mechanism is the loss of function of critical transcription factors and/or the formation of aberrant transcription factors and the third mechanism is the induction of ectopic and aberrant expression of the proto-oncogene by the

chromosomal translocation. Figure 11 shows the different breakpoints in the *ETV6* gene with regard to the different fusion partners.

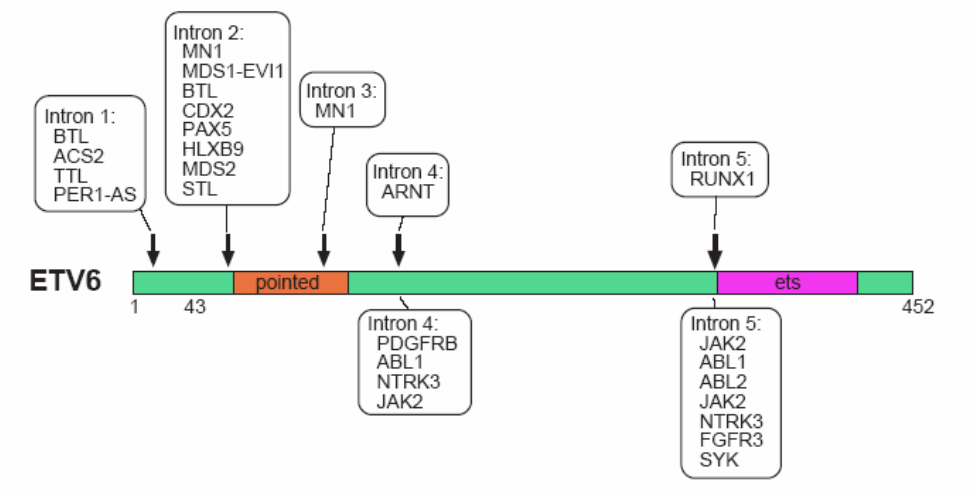


Figure 11. This is a schematic diagram representing the various breakpoints of *ETV6* (indicated by arrows) in the translocations involving different partner genes (indicated in closed boxes)

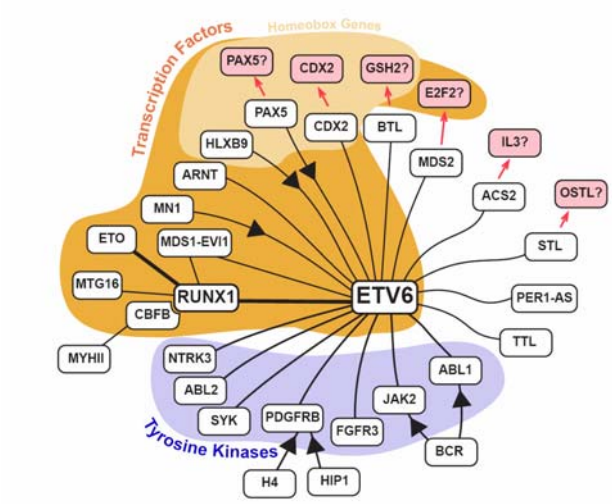


Figure 12. The wide variety of genes that fuse to *ETV6* can be sub-grouped into clusters with regards to their functional similarity. Two main clusters have been highlighted, the tyrosine kinases and transcription factors, which include the sub class of homeobox transcription factors (kindly provided by Prof. Stefan Bohlander)

1.6.3 Protein tyrosine kinase fusion partners of *ETV6*

The first identified fusion partner of *ETV6* was a protein tyrosine kinase (PTK), the platelet-derived growth factors receptor beta (*PDGFRB*) (Golub et. al., 1994). The fusion protein critical for the development of the chronic myelo-

monocytic leukemia is the *ETV6/PDGFRB* fusion and not the reciprocal *PDGFRB/ETV6* fusion. In the ETV6/PDGFRB fusion protein the N terminal portion of ETV6, which includes the pointed domain, is fused to the C-terminal two thirds of the PDGFRB protein, conserving the tyrosine kinase domain of PDGFRB. The fusion of the pointed domain of ETV6 in the N-terminal half with the tyrosine kinase domain in the C-terminal half of the fusion partner is characteristic of the class of *ETV6/PTK* fusions and is found in the fusions of *ETV6* with *ABL1, ABL2, JAK2, NTRK3, FGFR3* and *SYK* (Fig. 11*)* (Table 2) (Papadopoulos et. al., 1995; Cazzaniga et. al., 1999; Knezevich et. al., 1998; Kuno et. al., 2001; Peeters et. al., 1997)

Table2. Tyrosine kinase fusion partners of ETV6

Translocation	**Fusion partner**	**Breakpoint in ETV6**	**Disease**
t(5;12)(q31;p13)	PDGFRB	Intron 4	CMMoL
t(9;12)(q34;p13)	ABL1	Intron 4 and 5	AML, ALL
t(1;12)(q25;p13)	ABL2	Intron 5	AML-M3,-M4,T-ALL
t(9;12)(p24;p13)	JAK2	Intron 4 or 5	Pre B cell ALL, T-ALL
t(12;15)(p13;q25)	NTRK3	Intron 4 or 5	Congenital fibrosarcoma, mesoblastic nephroma, secretory breast carcinoma, AML
t(4;12)(p16;p13)	FGFR3	Intron 5	Peripheral T-cell lymphoma
t(9;12)(q22;p12)	SYK	Intron 5	MDS

1.6.4 Transcription factors and other fusion partners of *ETV6*

The *ETV6/RUNX1* (ETV6/AML1) fusion is the most common fusion gene in childhood acute B cell lymphoblastic leukemia (Shurtleff et. al., 1995). Re-

porter gene assays showed that the ETV6/RUNX1 fusion protein acts as a repressor by binding to the promoter and enhancer regions of RUNX1 target genes. This repression function is dependent on the pointed and on the central domain of ETV6, which are both part of the ETV6 portion of the ETV6/RUNX1 fusion protein (Fenrick et. al., 1999). ETV6/ARNT and HLXB9/ETV6 chimeric proteins are other examples of this class of ETV6 fusions found in AML and the HLXB9/ETV6 hybrid is detected in up to 20% of pediatric cases with AML (Beverloo et al., 2001) A potential mechanism of transformation for these fusions is that the ETV6/ARNT and HLXB9/ETV6 proteins interact with the wild-type ETV6 through the *pointed* domain, thereby interfering with normal ETV6 function (Beverloo et. al., 2001).

1.6.5 Ectopic and aberrant expression of a proto-oncogene gene

A number of *ETV6* translocations including the *ETV6-MDS1/EVI1* and the *ETV6-CDX2* fusion only contain the transcription/translation start of *ETV6* (Peeters et al., 1997, Chase et al., 1999). In these cases ectopic expression of the transcription factors *EVII* and *CDX2* was detected in addition to the expression of the fusion gene. The potential importance of the ectopic expression of a proto-oncogene in this class of ETV6 fusions was further underlined by observations that the ectopic expression of the proto-oncogene also occurred when the fusion gene itself was not translated into a protein product. For example patients with the t(4;12) positive leukemia show ectopic expression of the ParaHox gene *GSH2*. However the fusion gene *CHIC2-ETV6* generated by the chromosomal translocation was not translated into a protein product. Furthermore, expression of IL-3 was observed in a CML case with a t(5;12), lacking the *ETV6-ACS2* fusion protein. These results suggest that ectopic expression of *GSH2* and *IL-3* could be the key leukemogenic mechanism in these leukemias (Cools et. al., 2002).

1.7 The t(12;13)(p13;q12) translocation in myeloid leukemia.

The t(12;13)(p13;q12) translocation was discovered by Chase and coworkers in 1999 in a rare case of AML and was shown to result in the novel fusion between ETV6 and caudal related homeobox gene *CDX2* (Chase et. al., 1999). Interestingly, it was noted at that time that a non-rearranged *CDX2* mRNA was expressed at high levels in this patient. Normally, *CDX2* is not expressed in adult hematopoiesis. In addition, the aberrant expression of *CDX2* was reported in a case of CML-BC with no cytogenetically visible abnormality (Chase et al., 1999) (Fig. 12). This situation resembles other ETV6 translocations as described above (1.6.5). Thus, deregulated expression of CDX2 could be a key event in the pathogenesis of this AML subtype.

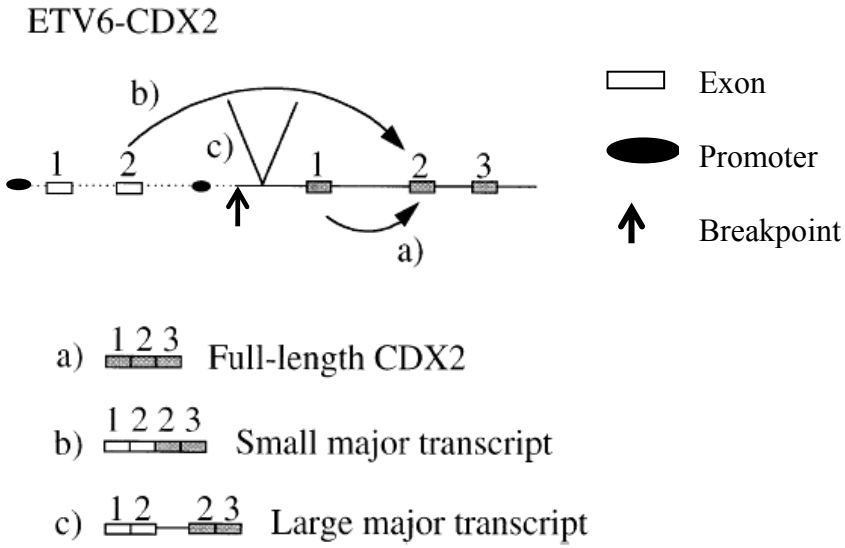


Figure 13. Schematic diagram to illustrate a possible mechanism for expression of an apparently normal full-length *CDX2* transcript (**a**) and *ETV6-CDX2* fusion transcripts **b**) and **c**). (Figure adopted and modified from Chase et. al., 1999)).

1.8 Aim of this research

The deregulation of proto-oncogenes by chromosomal translocations is a common and frequent observation in ALL and lymphomas. In contrast, generation of fusion genes with transforming properties is the pathogenetic hallmark of AML, and so far there is no functional proof that activation of a proto-oncogene by a balanced chromosomal translocation can be the key event for the development of AML. However, the observation that a significant number of ETV6-associated chromosomal translocation induces aberrant and ectopic expression of a proto-oncogene, indicates that this mechanism might play a key role also in myeloid leukemogenesis.

Aim of the project was to test this hypothesis in the case of the t(12;13) positive AML and analyze whether ectopic expression of *CDX2* is the key step in the development of AML. For this we established a mouse model of this subtype of leukemia aiming at providing the first functional proof of this mechanism for myeloid leukemogenesis.

2 MATERIAL

2.1 Mammalian cell lines

293T	(human embryonic kidney cell line)
GP+E86	(mouse fibroblast cell line)
32D	(mouse myeloid cell line)
NIH 3T3	(mouse fibroblast cell line)

2.2 Plasmids:

MIG/MIY:	murine stem cell virus derived vector. A bi-cistronic vector with an internal ribosomal entry site (IRES) and an EGFP or EYFP cassette 3' of the IRES) (kindly provided by Prof. Dr. R.K. Humphries, Vancouver, Canada)
pCDNA6/V5-His:	mammalian expression vector used for tagging genes (Invitrogen, Carlsbad, CA)
pEGFP-C1:	(Clontech)
Ecopac:	A packaging vector coding for the gag, pol, and env viral proteins.

2.3 Cell culture Medium:

DMEM high glucose	(GIBCO/BRL and PAN)
RPMI 1640	(GIBCO/BRL)

2.4 Chemicals and Reagents:

Agarose	(Sigma)
Acetic acid	(Sigma)
ATRA	(Sigma)
Calcium chloride	(Sigma)
Chloroform	(Sigma)
Dextran sulphate	(Sigma)
DNAzol	(GIBCO/BRL)
DNase I	(Invitrogen)
EDTA	(Sigma)
Ethidium bromide	(Sigma)
Formamide	(Sigma)
Fetal bovine serum	(PAN)
Glycine	(Sigma)
Giemsa	(Merck)
Hydrochloric acid	(Merck)
Isopropanol	(Merck)
Methocult M 3434	(Stemcell Technologies)
May-Grunwald's Eosin	(Merck)
Protamine sulfate	(Sigma-Aldrich)
Quikchange XL site-directed Mutagenesis Kit	(Stratagene)
Sodium chloride	(Sigma)
Sodium hydroxide	(Sigma)
Sodium dodecyl sulphate	(Sigma)
Skim milk	(Sigma)
Trizma base	(Sigma)
Trizol	(GIBCO/BRL)
5-flurouracil	(Medac)
ThermoScript RT-PCR	(Invitrogen)
Megaprime DNA labeling system	(Amersham)

GFX PCR DNA gel band elution (Amersham)

Microspin S-300 HR columns (Amersham)

ECL Western blotting analysis system (Amersham)

Denaturation solution: 1.5M NaCl, 0.5N NaOH.

20 X SSC: Dissolve 3M sodium chloride and 0.3Msodium citrate in 800 ml deionized water and adjust pH to 7.0 and make up the volume to one liter.

Pre-hybridization solution: Dissolve 0.2g skimmed Milk and 2.0g dextran sulphate in 17ml water and add 6ml 20 X SSC, 2ml formamide, 1ml 20% SDS and 80µl 500mM EDTA.

Telleyesnickzky's solution: 450ml EtOH + 25ml glacial acetic acid + 25ml formaldehyde

2.5 Antibodies:

Name	Clone	company	Label
Gr-1	RB6-8C5	BD Pharmingen	PE
Mac-1 (CD11b)	M1/70	BD Pharmingen	APC
Sca-1	D7	BD Pharmingen	PE
c-kit	2B8	BD Pharmingen	APC
B220	RA3-6B2	BD Pharmingen	APC
Ter119		BD Pharmingen	PE
CD4	GK1.5	BD Pharmingen	PE
CD8	53-6.7	BD Pharmingen	APC
Anti-histidine monoclonal antibody		Sigma	
Anti-CDX2 monoclonal antibody		DCS Innovative	

2.6 Cytokines*:*

mIL3	(Tebu-bio)
mIL6	(Tebu-bio)
M-SCF	(Tebu-bio)
G-CSF	(R&D systems)

2.7 Restriction Enzymes and DNA modifying Enzymes:

EcoRI, XhoI, HpaI, PmeI, EcoRV, T4 DNA Ligase, Calf intestine phosphatase all from New England Biolabs.

2.8 Instruments:

DNA Cross linker (GS Gene linker BIO-RAD)

Fluorescence Activated cell sorting (BD FACSVantage SE System)

Flow Cytometry (BD FACS Calibur System)

Cytospin (Cytospin 2 Shandon)

Microscope (Leitz Diavert Inverted Microscope, Ernst Leitz Wetzlar GmBH)

2.9 Mice

Parental strain mice were bred and maintained at the GSF animal facility. Donors of primary BM cells were > 12-week-old (C57Bl/6Ly-Peb3b x C3H/HeJ) F1 (PebC3) mice and recipients were > 8–12 week old (C57Bl/6J x C3H/HeJ) F1(B6C3) mice.

3 METHODS

3.1 cDNA constructs and retroviral vectors.

cDNAs of *ETV6/CDX2* (human) and murine *Cdx2* (with 93% overall identity and 98% identity in the DNA binding homeodomain to the human protein) were kindly provided by D.G. Gilliland (Division of Hematology/Oncology, Harvard Medical School, Boston, USA) and N. Cross (Department of Hematology, Hammersmith Hospital, London, UK). A poly-histidine tagged version of *ETV6/CDX2* was constructed by ligating a PCR product of the fusion gene in frame to the 3' end of the poly-histidine epitope of the pCDNA6/V5-His A plasmid (Invitrogen, Karlsruhe, Germany). The Cdx2 mutant lacking the first 179 N-terminal amino acids, which are deleted in the ETV6/CDX2 fusion, was generated by PCR and poly-histidine tagged following standard procedures (ΔN-Cdx2). For retroviral gene transfer into primary bone marrow (BM) cells, the different constructs were subcloned into the multiple cloning site of the modified MSCV 2.1 vector (Pineault et. al., 2003b) upstream of the internal ribosomal entry site (IRES) and the enhanced green or yellow fluorescent protein (GFP/YFP) gene. As a control the MSCV vector carrying only the IRES-EGFP cassette was used.

3.2 Site-Directed Mutagenesis:

Cdx2 mutants were created, to inactivate a putative PBX1 interacting motif (W167A-Cdx2) which is the docking site of Hox cofactors like *PBX1* and *Meis1* (Knoepfler and Kamps, 1995) or to inactivate the DNA binding ho-

meodomain (N51S-Cdx2) (Shanmugam et. al., 1999), using the QuikChange XL Site-Directed Mutagenesis Kit. PCR was done as recommended by the manufacturer (Stratagene, Amsterdam, Netherlands). PCR products were digested with *DpnI*, which digests only methylated DNA, to remove template DNA and transformed in competent cells supplied with the kit. Desired mutations were confirmed by sequencing.

Primer sequence for N51S-*Cdx2*:

Forward primer: 5'-TTTGGTTTCAGAGCCGCAGAGCCAAGGAGAG-3'

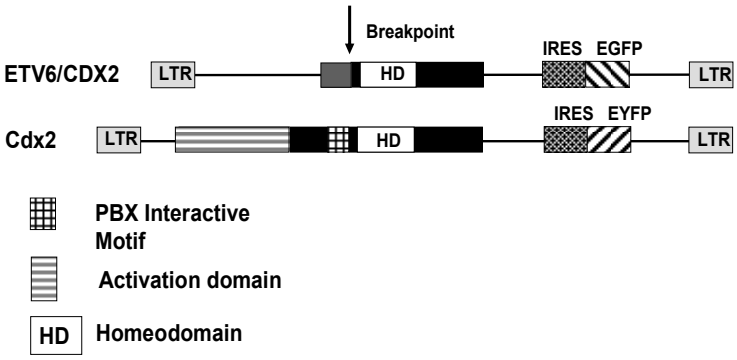
Reverse primer: 5'-CTCTCCTTGGCTCTGCGGCTCTGAAACCAAA-3'

Primer sequence for W167A-*Cdx2*:

Forward primer: 5'-GAAACCTGTGCGAGGCGATGCGGAAGCCC-3'

Reverse primer: 5'-GGGCTTCCGCATCGCCTCGCACAGGTTTC-3'

A



B

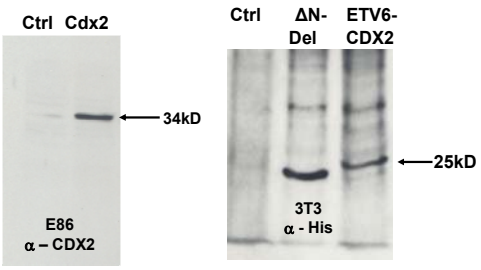


Figure 14. **Schematic diagram of constructs and proof of protein expression by Western blotting. a)** MSCV-based retroviral vectors were used to express ETV6/CDX2 and Cdx2 in murine bone marrow. LTR = long terminal repeats; IRES = internal ribosomal entry site. The figure 14**b**, shows the immunoblot analyses from the whole cell lysates of E86 and NIH 3T3

cells transfected with the different constructs and probed with the anti-human CDX2 antibody and anti-Histidine antibody.

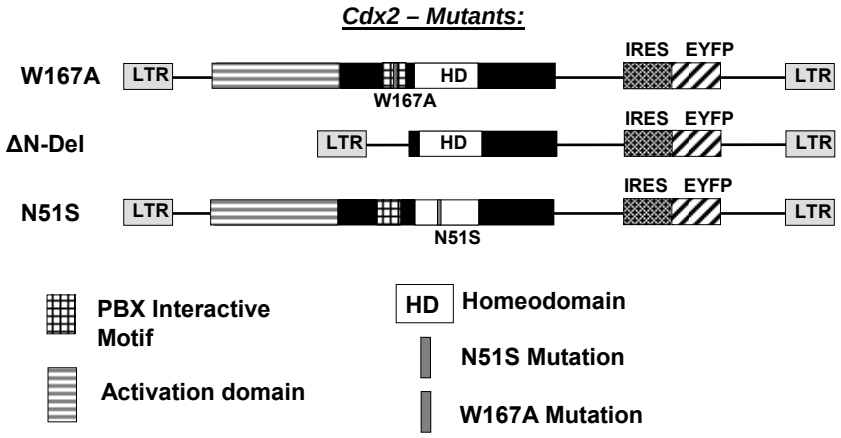


Figure 15 Schematic diagram of different mutants. MSCV based retroviral vector were used to express Cdx2 mutants in murine Bone marrow. LTR = long terminal repeats; IRES = internal ribosomal entry site.

3.3 Retrovirus Production.

Retrovirus was produced with the constructs mentioned before by individually co-transfecting 15 µg of the construct DNA with equal amounts of Ecopac DNA into 293T cells by using the calcium chloride precipitation method of transfection (Pineault et. al., 2003b). Virus conditioned medium (VCM) was collected 48h after transfection at time intervals of eight hours for forty-eight hours. Production of high-titer helper-free retrovirus was achieved by transducing the ecotropic packaging cell line GP+E86 (Pineault et al., 2003b) with virus conditioned medium (VCM) from 293T transfected cells. Successfully transduced GP+E86 cells were sorted out by FACS using GFP or YFP as a marker 4 days after transduction using standard procedures (Pineault et. al., 2003a).

3.4 Retroviral infection of primary BM cells.

Primary mouse bone marrow (BM) cells were transduced as previously described (Pineault et. al., 2003a). Briefly, BM cells were obtained by flushing both femurs and tibias of donor mice treated 4 days previously with 150 mg/kg 5-fluorouracil injected into the tail vein. 5-flourouracil eliminates cycling cells from the animal, thus enriching the bone marrow for primitive hematopoietic progenitors, which are non-cycling or quiescent in nature (Reya et. al., 2001). Cells were stimulated for 48 hrs in DMEM supplemented with 15% FBS, 10 ng/ml mIL-6, 6 ng/ml mIL-3 and 100 ng/ml murine stem cell factor (SCF). For transduction, cells were co-cultured for 48h with irradiated (40 Gy) $GP^{+}E86$ virus producing cells in the same medium with an addition of 5 µg/ml protamine sulfate with *ETV6/CDX2/GFP* or *Cdx2/YFP* $GP^{+}E86$ producers or with a mixture of 40 to 50% *CDX2/YFP* and 50 to 60% *ETV6/CDX2/GFP* producers in co-transduction experiments. Protamine sulfate prevents aggregation of viral particles, thus increasing efficiency of transduction. Loosely adherent and non-adherent BM cells were harvested from the co-culture 48h post transduction, BM cells were furtherer cultured in fresh medium with cytokine cocktail for 48h to allow expression of EGFP or EYFP. Transduced BM cells were sorted out by florescence activated cell sorting (FACS) using EGFP or EYFP as a marker.

In Vitro Assays

3.5 Proliferation Assay.

To study the proliferative potential *in vitro* of BM cells transduced with *ETV6/CDX2, Cdx2*, its mutants and *ETV6/CDX2* + *Cdx2*, we performed a proliferation assay by plating an equal number of successfully transduced bone marrow cells directly after sorting in DMEM supplemented with 15% FBS, 10 ng/ml mIL-6, 6 ng/ml mIL-3 and 100 ng/ml mSCF (standard medium) (Tebu-bio GmbH, Offenbach, Germany) at 37^{0}C in a humidified CO2 incubator. The

cells were subjected to half-media change every 7 days and their proliferation assessed on the same day by counting viable cells after trypan exclusion. To generate IL-3 dependent cell lines, successfully transduced bone marrow cells were cultured directly after sorting in DMEM, 15% FBS with mIL-3 alone (6ng/ml). A half-media change was done every 7 days.

3.6 Colony Forming Cells Assay (CFC-assay).

To check the differentiation and clonogenic potential of the BM cells expressing the various genes mentioned above, we performed a CFC assay by testing colony formation in methylcellulose. The CFC–assay was performed by culturing highly purified transduced cells (500 /dish) in 35mm-diameter Petri dishes directly after sorting in 1ml methylcellulose supplemented with cytokines (Methocult M3434), and incubated at 37^0C in humidified CO2 incubator. Colonies were counted microscopically on 7 to 9 days after plating according to standard criteria (Schwaller et. al., 1998). Re-plating capacity of clonogenic progenitors was assayed by re-plating the primary colonies on secondary methylcellulose dishes. Again colonies were counted day at 7 to 9 after the secondary re-plating.

3.7 Delta CFC Assay.

Normal bone marrow cells lose their clonogenic potential following in vitro culture. To analyse whether progenitors cells that expanded in the proliferation assay lose their clonogenic potential, we set up CFC-assays from proliferation assays by plating (3000 to 5000 BM cells/dish) cells in 35mm-diameter Petri dishes in 1 ml methylcellulose after culturing 7 days in standard medium (delta CFC-assay). Colonies were counted day 7 to 9 days post plating.

3.8 Cyto-Morphology.

Cyto-morphological analysis of bone marrow was done by transferring $5x10^4$ to $1x10^5$ bone marrow cells on glass slide by centrifugation at 500 rpm for 10 minute using a Shandon Cytospin2 centrifuge. Slides were stained with May-Grunwald's eosine-methylene blue and Giemsa solution using the standard protocol supplied by Merck.

3.9 Immunophenotyping:

Cell differentiation was determined and lineage distribution was checked by immunophenotyping. $5x10^4$ to $1x10^4$ cells were rinsed with PBS and stained for 30 minutes on ice with an appropriate concentration of phycoerythrin (PE) conjugated antibody to Gr-1, Sca1, Ter-119, CD4, and allophycocyanin-conjugated antibody for lineage markers Mac-1, cKit, B220 or CD8. The cells were then washed with PBS and stained with propidium iodide, and viable cells were analyzed with the FACS Calibur system.

3.10 Differentiation assay.

The differentiation capacity of proliferating cell populations was tested by culturing $1x10^5$ cells in DMEM, 15% FBS supplemented with G-CSF 100ng/ml or M-CSF 10ng/ml (R&D Systems, Wiesbaden, Germany) and all-trans retinoic acid (ATRA) at a final concentration of 1μM. After 5 days the morphology was determined by Wright-Giemsa stained cytospin preparations (Buske et. al., 2002; Pineault et. al., 2003a).

3.11 Delta Colony Forming Unit on Spleen (CFU-S) assay.

Day-4 5-FU mobilized primary BM cells from F1(PebC3) donor mice were transfected with the different viruses and retrovirally-transduced cells were purified based on the expression of GFP or YFP using a FACSVantage sorter

(Becton Dickinson, San Jose, USA). Transduced cells were cultured for 7 days in standard medium. The day 0 equivalent of 2.5– 3 x 10^4 cells was injected into the tail vein of lethally irradiated F1 (B6C3) recipient mice. The recovery of CFU-S cells was quantified by determining the number of macroscopic colonies on the spleen at day 12 post injection after fixation in Telleyesnickzky's solution.

3.12 BM transplantation and assessment of mice.

For bone marrow transplantation procedures, 8–10 week old recipient F1 (B6C3) mice were irradiated with 850 cGy from a 137Cs γ-radiation source. FACS purified transduced BM cells or defined ratios of transduced and untransduced cells were injected into the tail vein of irradiated recipient mice. PB or BM cell progeny of transduced cells were tracked using GFP or YFP fluorescence (Feuring-Buske et al., 2002). Lineage distribution was determined by flow cytometric analysis as previously described (Pineault et. al., 2003): Phycoerythrin-(PE) labeled Gr-1, Sca1, Ter-119, CD4 and allophycocyanin (APC) labeled Mac1, c-Kit, B220, CD8 antibodies were used for analysis (all Pharmingen Heidelberg, Germany). For histological analyses, sections of selected organs were prepared and H&E stained at the Academic Pathology Laboratory, GSF, Munich, using standard protocols. The mice were under observation for early signs of leukemia. Aspiration of the blood and bone marrow was performed at 8 weeks and subsequently at four week intervals and assessed for engraftment of the mouse with transduced cells and progression of disease. Early signs of leukemia included high engraftment efficiency and the presence of blast cells in the blood and bone marrow cytospin preparations and in blood smears. In the animal, the indications of initiation of disease were paleness of the feet, limited mobility or lethargy, short breaths or ruffled body hair. Moribund mice were sacrificed and analyzed as described.

3.13 Analysis of sacrificed/dead experimental mice.

Disease in the animals was determined by analyzing PB, spleen and BM of diseased sacrificed mice. PB was aspirated from heart with 1 ml insulin syringes; Heparin was used for anticoagulation. PB was used for blood smears on glass slides and RBC count. WBC count and immunophenotyping was performed after lysing the RBC with ammonium chloride. The size of the spleen in diseased mice was measured and the spleen was homogenized to single cell suspension in DMEM medium, for further analysis by FACS, cytospin or ex vivo assays. BM cells were obtained by flushing both femurs and tibias and subjected to WBC count, cytospin and immunophenotyping.

3.14 Preparation for histopathology.

The four limbs, tail and head of mice were pinned to a cork plate with needles after sacrificing the animals for histological analyses. Then the skin from the chin to the anus and also the abdominal muscles were opened in the median and also pinned (Fig.16). The mice were then bled by cutting one of the renal arteries, and the blood was sucked out with a tissue paper. Then the diaphragm was carefully cut away to allow the fixing liquid to get into the thorax. After this the whole mouse was placed into formalin over night, packed and sent to the histopathology, where the organs were cut and stained for analyses (Fig.16).

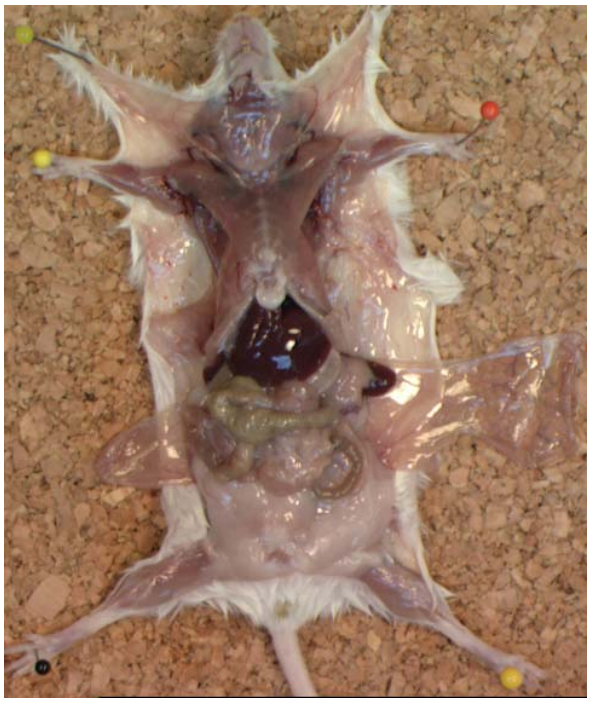


Figure 16. Dissected mouse before fixing in formalin

3.15 RT-PCR

Expression of *Hoxa9* and *Meis1* was assayed by RT-PCR in $Sca1^{-}/Lin^{+}$ cells sorted from a repopulated Cdx2 mouse or a control animal. Total RNA was isolated using Trizol reagent (GIBCO BRL) and treated with DNase I (amp grade) to remove contaminating genomic DNA. First strand cDNA was synthesized from 1 μg total RNA using the thermoScript RT-PCR system (all reagents from Invitrogen GmbH, Karlsruhe, Germany). Equal amounts of cDNA originating from 50 ng starting RNA were loaded to assess transcription levels. Intron spanning primer pairs were selected to avoid amplification of contaminating genomic DNA. The annealing temperature was 58°C and 60°C for *Meis1* and *Hoxa9*, respectively. The number of PCR cycles for each gene was chosen to stop the reaction in the linear phase of amplification (25 cycles for mβ-2 microglobulin, 35 cycles for *Meis1* and *Hoxa9*).

Primer sequence for *Hoxa9*:
Forward primer: 5'-GTTGCTCTGGTTGCTCTGTG-3'
Reverse primer. 5'-CTGTTGTGTTTGGTTGGTGAA-3'

Primer sequence for *Meis1*:
Forward primer: 5'-ATGTGACAATTTCTGCCACCG-3'
Reverse primer. 5'-CCTCAACGAGTGGATGCCGTG-3'

3.16 Southern blot analysis.

Southern blot analysis to assess proviral integration were performed by isolating DNA from bone marrow, spleen and peripheral blood of leukemic mice using DNAZOL reagent as recommended by the manufacturer. Southern blot was performed using standard protocols (Buske et al., 2001). DNA was digested with *EcoRI*, which cuts the proviral DNA once, to release a fragment specific to the proviral integration site. To check the full length integration, DNA was digested with *Nhe I*, which cuts in the long terminal repeats (LTRs) to release the proviral genome. After digestion DNA was loaded on a 0.7% agarose gel with 0.5 μg/ml ethidium bromide. After electrophoresis, the DNA was depurinated by incubating the gel in 0.2 N HCL for 8 minute and then in denaturing solution for 45 minutes. After denaturation, the DNA was transferred on a zeta-probe GT membrane by capillary action in 10X transfer buffer. Cross linking of the DNA with the membrane was done by incubating the membrane at 150 mJoule in a UV GS gene linker (BioRad). The probe used was a 700 bp GFP fragment, which was cut out from the pEGFP-C1 plasmid (Clontech) and labeled with α-^{32}P dCTP, using the Megaprime DNA labeling system (Amersham Biosciences). The probe was purified by using Microspin S-300 HR columms (Amersham Biosciences) Hybridization was done with α-^{32}P GFP overnight at 62^{0}C.

3.17 Western Blot.

Protein expression of the *ETV6/CDX2*, *Cdx2* and *Cdx2* mutants was documented by Western blotting using standard procedures (Reddy et. al., 2002). Total cellular protein was extracted using RIPA lysis buffer from NIH3T3 and E86 cells transduced with the different constructs. Whole cell lysates were

separated on 12% SDS-page gel and transferred to nitrocellulose membrane. Membranes were probed with an anti-histidine monoclonal antibody for ETV6/CDX2 and the ΔN-CDX2 mutant or with an anti-CDX2 human monoclonal antibody (kindly provided by DCS Innovative, Hamburg, Germany) for expression of the *Cdx2*, W167A-*Cdx2* and N51S-*Cdx2* mutants. Membranes were then incubated with horse radish peroxidase conjugated anti-rabbit immunoglobulin. Protein was visualized using an ECL plus kit, according to manufacturer's recommendations.

3.18 Statistical analysis

Data were evaluated using the t-test for dependent or independent samples (Microsoft Excel). Differences with p-values <0.05 were considered statistically significant.

4 RESULTS

In Vitro.

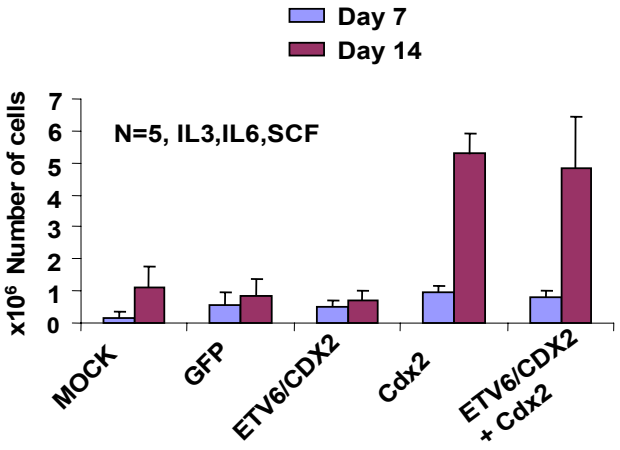
4.1 Ectopic expression of *Cdx2* induces proliferation and outgrowth of IL-3 dependent blast cell populations

To analyze whether the expression of the *ETV6/CDX2* fusion gene or the ectopic expression of the homeobox gene *Cdx2* is able to transform early murine hematopoietic progenitors we generated MSCV-based retroviral constructs. We checked the proliferative potential of bone marrow (BM) cells transduced with *ETV6/CDX2*, *Cdx2* and *ETV6/CDX2* & *Cdx2* in vitro by sorting for EGFP, EYFP or for cells co-expressing both fluorescent proteins, respectively. Expression of *Cdx2* induced a 26.81 ±2.60 (N=7) fold rise in cell number after 2 weeks in liquid expansion cultures (supplemented with IL-3, IL-6 and SCF) compared to BM cells transduced with GFP as a control (Fig. 17). Cells expressing *Cdx2* were able to grow in liquid culture supplemented only with IL-3 and showed blast cell morphology in both assay systems (Fig. 18, 17b). In contrast, cells expressing the *ETV6/CDX*2 fusion gene did not show any significant (0.70 ± 2.61, N=6) growth advantage compared to GFP positive cells in complete medium. In addition, cells expressing the fusion gene were not able to grow in medium supplemented only with IL-3 (Fig. 18). BM cells co-expressing *ETV6/CDX2* and the *Cdx2* showed a higher proliferation in complete medium compared to GFP control cells and were able to grow in medium supplemented only with IL-3, comparable to the cell populations transduced with Cdx2 alone (Fig. 17a, 18). Furthermore, we examined the leukemic prop-

erties of the cell population expressing *Cdx2* or *ETV6/CDX2* & *Cdx2* by injecting $1x10^6$ transduced and $1x10^6$ non-transduced cells per mice in lethally irradiated animals (N=4). All mice developed leukemia 8 weeks after transplantation.

Cytomorphological analysis of the cultured cells showed that BM cells expressing the fusion gene and GFP were able to differentiate to normal terminally differentiated macrophages and granulocytes. Of note, cells expressing *Cdx2* were blocked in differentiation at the blast cell stage (Fig. 17b).

a)



b)

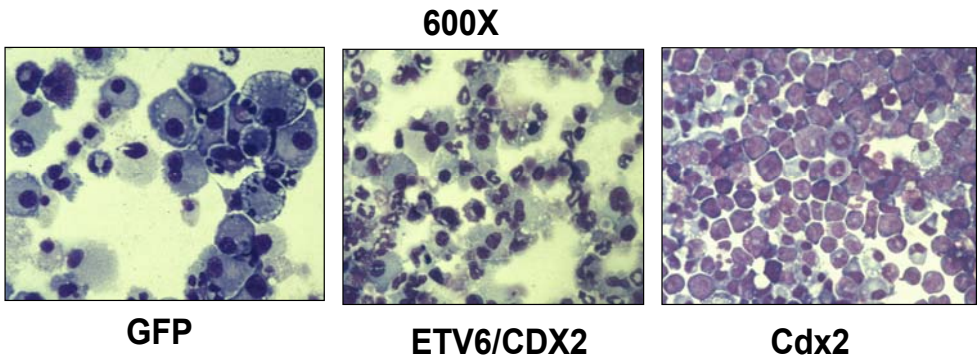


Figure 17. Ectopic expression of *Cdx2* increases the proliferation rate and blocks cell differentiation. a) BM cells expressing *Cdx2* or *ETV6/CDX2* + *Cdx2* showed a significant proliferative advantage compared to GFP transduced cells (N=5) in liquid expansion medium whereas expression of *ETV6/CDX2* did not show any growth advantage compared to GFP (N=5). **b)** Cytomorphological analysis of cultured cells infected with *Cdx2* shows blast mor-

phology indicating differentiation arrest whereas GFP and *ETV6/CDX*2 infected population were able to differentiate to normal terminally differentiated macrophages and granulocytes.

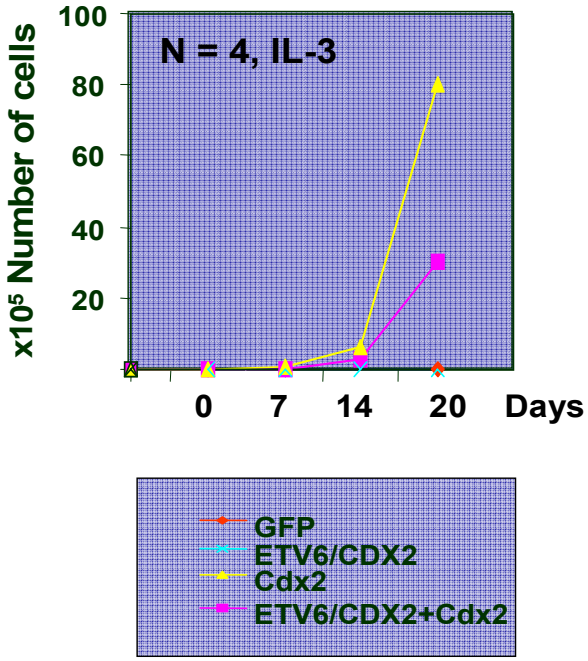


Figure 18. Expression of *Cdx2* and *ETV6/CDX2* + *Cdx2* induces outgrowth of IL-3 dependent blast cell populations. BM cells expressing *Cdx2* or *ETV6/CDX2* + *Cdx2* showed rapid growth in liquid expansion culture supplemented only with IL-3 (N=4), cells expressing the fusion gene did not grow under these conditions.

4.2 Expression of *Cdx2* upregulates the primitive marker Sca1

To confirm the myeloblastic nature of the cells, immunophenotyping was performed of the transduced BM cells propagated two week in complete medium or medium supplemented only with IL-3. Cells expressing *Cdx2* expressed the myeloid markers Mac-1 (72% ± 12, N=4), Gr-1 (48.15% ± 21, N=4)) and to a high percentage the primitive cell marker Sca1 (48% ± 8, N=4). In contrast, cells expressing *ETV6/CDX2* or GFP alone differentiated to mast cells under

these conditions and co-expressed Sca1 and c-kit (77% ± 2 and 88% ± 5, N=4) as previously described for the mast cell phenotype (Fig. 19).

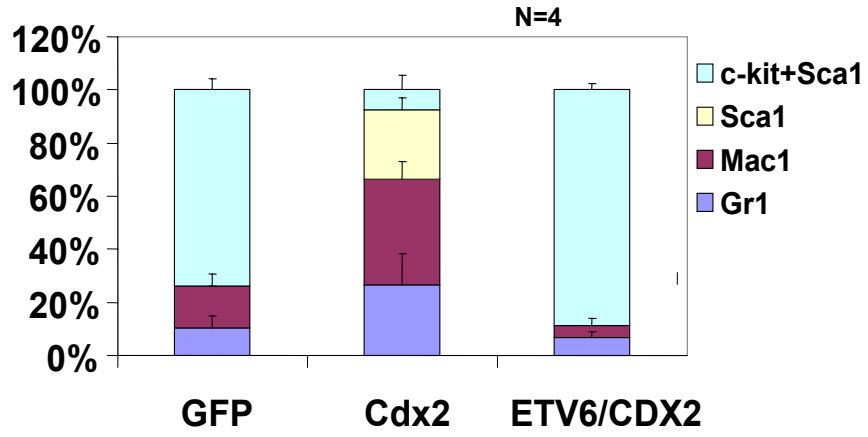


Figure 19. ***Cdx2* expressing cells express Sca1 and myeloid surface markers.** Immunophenotyping of the cells growing for 2 weeks in liquid culture supplemented with IL-3, IL-6 and SCF. *Cdx2* transduced cells expressed myeloid specific marker Gr-1, Mac-1 and were positive for primitive stem cell marker Sca1. Whereas >75% *ETV6/CDX2* and GFP cells were positive for the mast cell marker c-kit+Sca1.

4.3 Expression of *Cdx2* induces blast colony formation and increases the number of clonogenic progenitor cells

To test the differentiation potential of BM cells expressing *ETV6/CDX2*, *Cdx2* and *ETV6/CDX2* + *Cdx2* we performed the CFC assay by testing the colony formation on methylcellulose. *Cdx2* positive cells generated a higher number of primary CFC in methylcellulose compared to the GFP control (76 ± 22 vs. 41 ± 20 per 500 initially plated cells; n=5; $p<0.02$) (Fig. 20). Furthermore, Cdx2 positive colonies were significantly larger containing approximately 10 times more cells per colony than the controls (33 x 10^3 vs. 3.9 x 10^3/colony; n=5; $p<0.004$) (Fig. 21). Cells expressing the *ETV6/CDX2* fusion gene did not show any significant increase in colony number compared to GFP. We tested the proliferative potential of the primary colony by replating primary colonies in a

second methylcellulose dish: Cdx2 induced a 83 fold (N=5, p<0.0023) increase in the total CFU count and significantly a 3184 fold increase in CFU-blast count compared to the empty vector (N=5, p<0.007) (Fig. 22). Expression of *ETV6/CDX2 + Cdx2* showed a significant increase in the number of total primary CFCs and induced an increase in the total CFU-blast count in replating assay like Cdx2 alone (Fig. 20, 22).

In contrast, colonies expressing the *ETV6/CDX2* fusion gene did not show any significant increase in primary CFC. Replating primary colonies in a second methylcellulose dish, showed a 0.46 fold (N=5) decrease in the total CFU count compared to GFP (Fig. 20, 22). To check the surface marker of the colonies expressing different genes, we harvested the cells from 2°CFC. Immunophenotyping analysis showed that *Cdx2* and *ETV6/CDX2 + Cdx2* expressing cells were positive for the myeloid surface marker Gr-1 and Mac-1 (80 to 90% cells) and up-regulated the primitive stem cell marker Sca1 in 40 to 60% cells. Cells expressing *ETV6/CDX2* or GFP gene only, expressed both Sca1 and c-kit, which indicate a mast cell phenotype.

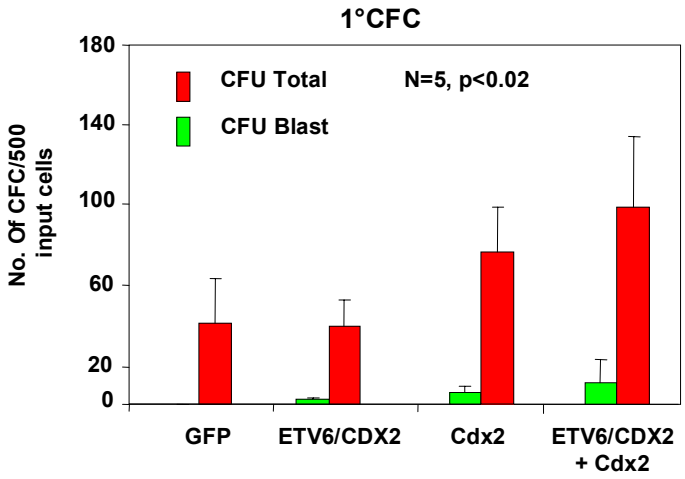


Figure 20. Expression of *Cdx2* or *ETV6/CDX2* & *Cdx2* increases the number of clonogenic progenitors. In the CFC-assay, BM cells expressing *Cdx2*, *ETV6/CDX2* & *Cdx2* showed a substantially higher number of colonies (N=5, p<0.02) compared to BM cells infected with the GFP control virus alone. *ETV6/CDX2* did not induce any increase in the total number of colonies compared to the control.

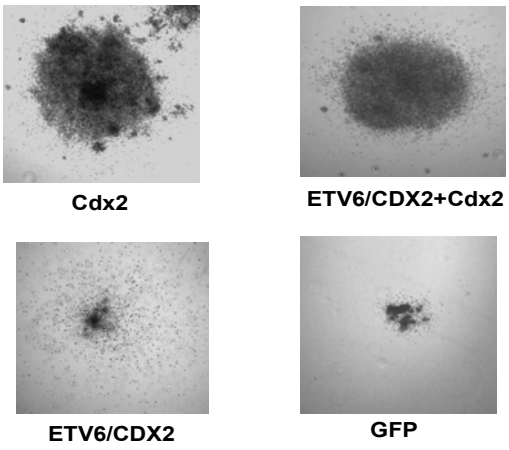


Figure 21. **Colonies expressing *Cdx2* or *ETV6/CDX2* & *Cdx2* were increased in size.** Microscopic picture of the colonies (50X) in the primary CFC-assay expressing *Cdx2* or *ETV6/CDX2* & *Cdx2*. The colonies consisted of more than 2000 cells. In comparison the average cell number per colony was 500 cells in the CFC-assay of BM cells expressing *ETV6/CDX2* alone or GFP.

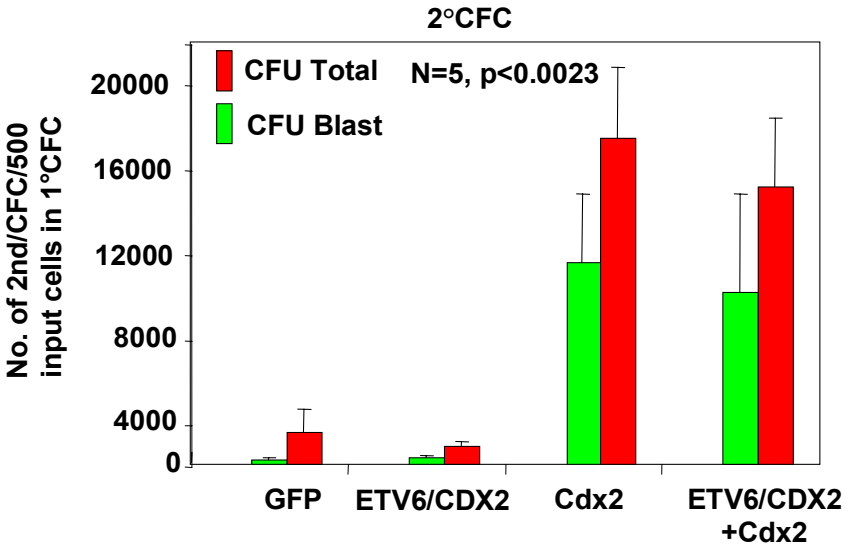


Figure 22. Re-plating was associated with increase in total CFU and formation of blast-like colonies in cell expressing *Cdx2*. Re-plating of primary colonies expressing *Cdx2* or *ETV6/CDX2* & *Cdx2* resulted in a significant increase in the total number of CFU and CFU-blasts (N=5, p<0.0023) compared to *ETV6/CDX2* and the GFP control.

4.4 *Cdx2* expression induces an increase in the yield of Δ CFU-S.

To investigate the effect of the expression of *ETV6/CDX2*, *Cdx2* and GFP on more primitive hematopoietic cells, progenitor enriched BM cells were transduced with the different constructs. Transduced cells were sorted out and injected into lethally irradiated mice after culturing them for 7 days in vitro. Spleen colonies of sacrificed mice were quantified macroscopically 12 days after injection (CFU-S assay). *Cdx2* expression induced a significant, over 10 fold increase in the yield of day12 CFU-S compared to the GFP control (n=8; p< 0.0001). In contrast, *ETV6/CDX2* (n=6) did not show any increase in the yield of day 12CFU-S compared to the GFP control. *Cdx2* did not only increase the colony numbers but also the size of the spleen colonies (Fig. 23 a,b).

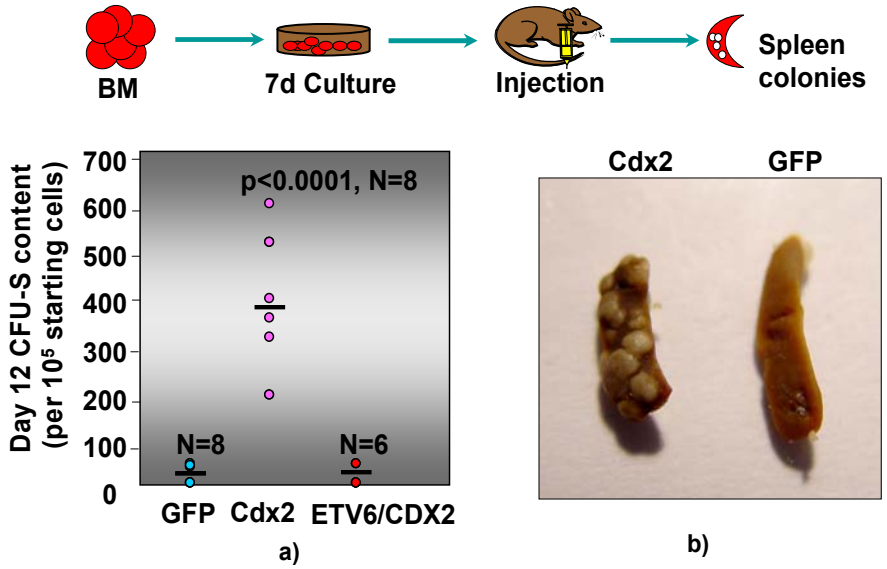


Figure 23. ***Cdx2* increases the yield of day 12 CFU-S. a).** Total number of day 12 CFU-S colonies derived per culture initiated with 1x 105 cells transduced with *Cdx2*, *ETV6/CDX2* and GFP as a control after 1week in liquid culture. The median is indicated. Cdx2 induced a significant, over 10 fold increase (N=8, p< 0.0001) in the yield of day 12 CFU-S compare to the GFP control. The fusion gene did not show any increase in CFU-S compared to the control. **b)** Spleens after fixing in Telleyesnickzky's solution showing large spleen colonies in the Cdx2 experimental arm compared to the control.

In Vivo

4.5 Ectopic expression of *Cdx2* causes AML in transplanted mice

To analyze whether the expression of the t(12;13) associated *ETV6/CDX2* fusion gene or the ectopic expression of the homeobox gene *Cdx2* is able to transform early murine hematopoietic progenitors *in vivo*, murine hematopoietic progenitors constitutively expressing *ETV6/CDX2* or *Cdx2* were FACS sorted to high purity by GFP+ or YFP+ expression respectively, and injected into lethally irradiated recipient mice directly after sorting (3–3.5 x 10^5 and 2–3.6 x 10^5 cells/mouse for *Cdx2* and *ETV6/CDX2*, respectively).

Mice transplanted with BM cells expressing *Cdx2* became moribund after a median of 90 days post transplantation (n=18) (Fig. 24). Diseased mice were characterized by cachexia, shortness of breath and lethargy, at which time they were sacrificed for further analysis. Diseased Cdx2 mice were characterized by elevated peripheral white blood count (WBC) (3.8-fold) with up to 48 x 10^6 circulating WBC/ml. Furthermore, moribund mice were anemic with a 5-fold decrease in peripheral erythrocyte count ($p<0.001$) (Table 3). All Cdx2 mice analyzed (n=7) suffered from spleenomegaly with an average spleen weight of 600 mg (range 0.4–0.9 g; $p<0.01$ compared to control animals) (Table 3). More detailed hematological analyses demonstrated that animals suffered from AML with a high percentage of blasts in the BM (42% ± 6), PB (14% ± 3) and spleen (35% ± 5) (n=8; $p<0.01$ compared to the control animals (Table 3).

Immunophenotypic characterization of PB, BM and spleen in diseased mice confirmed the predominance of myeloid Mac-1^+ and Gr-1^+ cells (84% ± 10 and 73% ± 15 in the PB, 65% ± 14 and 53% ± 14 in the spleen; n=4) compared to the GFP control mice (Mac-1^+ and Gr-1^+ cells 47% ± 5 and 25% ± 3 in the PB, 14% ± 9 and 10% ± 1 in the spleen; n=4) (Fig. 25 a, b, 26). Diseased mice showed expression of primitive stem cell marker c-kit (35% ±13 in PB and 38% ± 11.9 in spleen) compared to GFP (0.69% ± 0.2 in PB and 1.6% ± 0.6 in Spleen) (Fig. 25 a, b). Furthermore, the disease was characterized by a greatly

reduced normal $B220^+$ lymphoid population in the spleen and PB compared to controls (1.8% ± 1 versus 35% ± 8 and 1.3% ± 0.5 versus 46% ± 21 in the PB and in the spleen, respectively; n=4) (Fig. 25 a, b, 26).

Table 3. Hematological parameters of experimental mice

Mouse No.	Retroviral construct	survival days	RBC/ml $X10^9$	WBC/ml $X10^6$	Spleen Weight (mg)	BM % of blast	Spleen % of blast	PB % of blast	Lymphoid/ Myeloid Ratio in PB
1	GFP	90	6.0	4.5	150	0	0	0	5 : 1
2	GFP	90	4. 8	3.2	200	0	0	0	2 : 1
3	GFP	90	5.0	3.6	200	0	0	0	2 : 1
1*	CDX2	128	1.0	3.2	400	28	21	8	0.5 : 1
2*	CDX2	79	2.0	37	650	40	35	12	0.4 : 1
3*	CDX2	52	0.7	9	600	38	30	15	0.2 : 1
4*	CDX2	116	0.4	48	nd	ND	60	14	0.4 : 1
5*	CDX2	37	0.6	5	400	25	22	5	0.3 : 1
6*	CDX2	171	0.8	24	900	71	48	18	0.3 : 1
7*	CDX2	192	1. 1	10	800	60	41	30	0.5 : 1
8*	CDX2	84	0.4	28	400	32	24	8	0.8 : 1
1*	++	168	1.0	3.2	400	25	18	3	0.6 : 1
2*	++	230	1. 1	8	500	45	30	10	0.1 : 1
3*	++	151	0.2	8	600	58	37	16	0.4 : 1
4*	++	237	1. 5	2.4	300	25	18	5	0.6 : 1
5*	++	187	0.5	2.5	900	50	43	8	0.3 : 1
1	ETV6/CDX2	375	6. 5	2.4	160	10	8	0	0.3 : 1
2	ETV6/CDX2	375	5 .0	3.2	200	25	15	0	0.4 : 1
3	ETV6/CDX2	375	5 .2	6	180	15	9	0	2 : 1

* = diseased
++ = ETV6/CDX2 + CDX2, RBC = red blood cell count, WBC = white blood cell count, ND = not determined

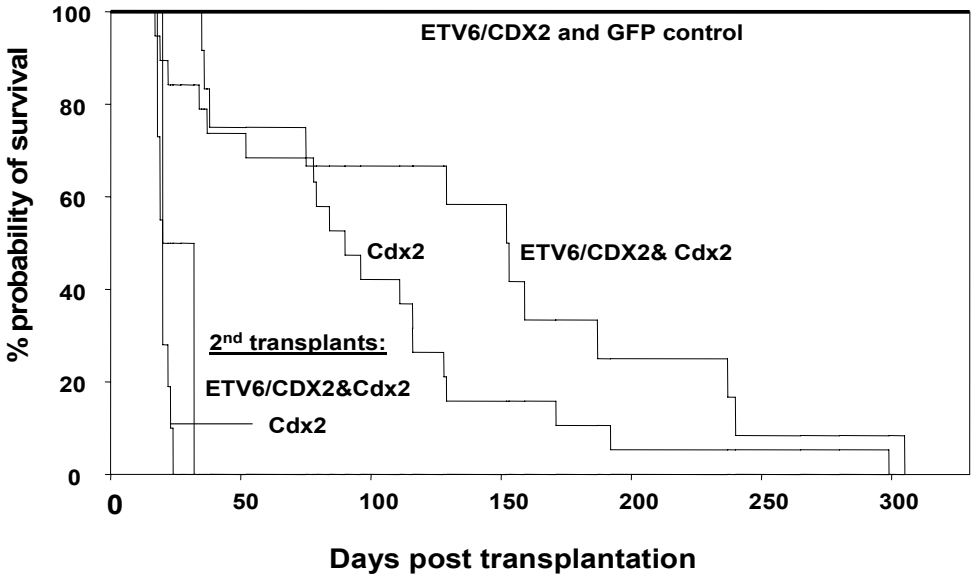
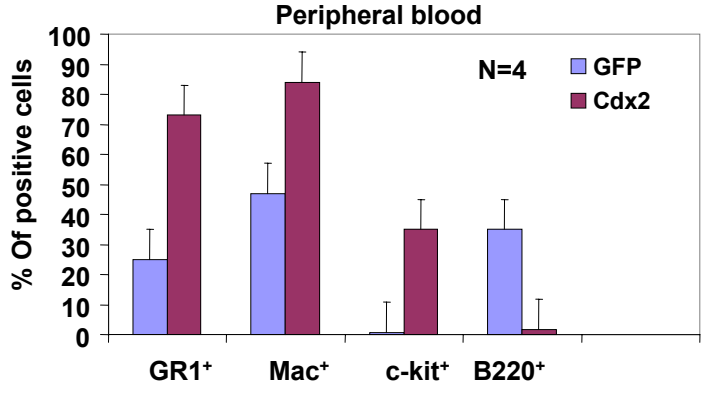


Figure 24. ***Cdx2* is highly leukemogenic in vivo, inducing an aggressive and transplantable AML in mice.** Survival curves of the mice transplanted with BM cells expressing *Cdx2* (N=18), *ETV6/CDX2* (n=9) or co-expressing *Cdx2* and the fusion gene (n=13). The control group was injected with BM infected with the GFP empty retrovirus (n = 7). The survival time of 2nd recipient mice transplanted with BM from diseased primary *Cdx2* or *Cdx2* & *ETV6-CDX2* recipients is indicated. Cdx2 mice died after a median of 90 days post transplantation. Mice transplanted with BM cells expressing *ETV6/CDX2* did not develop overt disease.

a)



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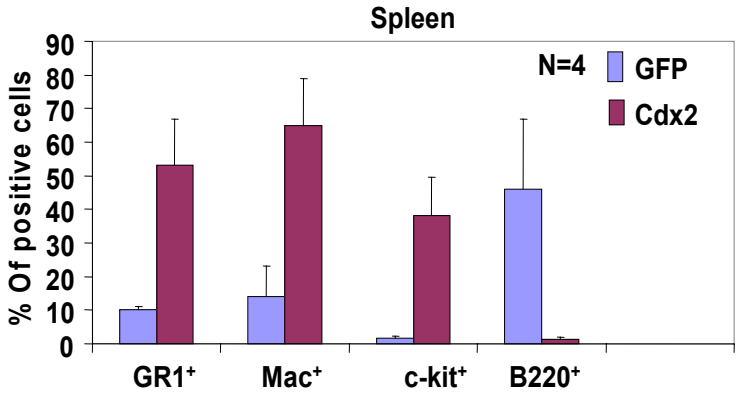


Figure 25. *Cdx2* expression increases the proportion of myeloid progenitors and reduces that of lymphoid cells. **a, b)** Immunophenotyping of the PB and spleen of *Cdx2* diseased mice showed a predominance of myeloid Gr-1$^+$, Mac-1$^+$ cells and >30% of this cells were positive for primitive stem cell surface marker c-kit$^+$ (N=4). **b)** The B220$^+$ lymphoid population in the spleen and PB was greatly reduced compared to GFP control mice.

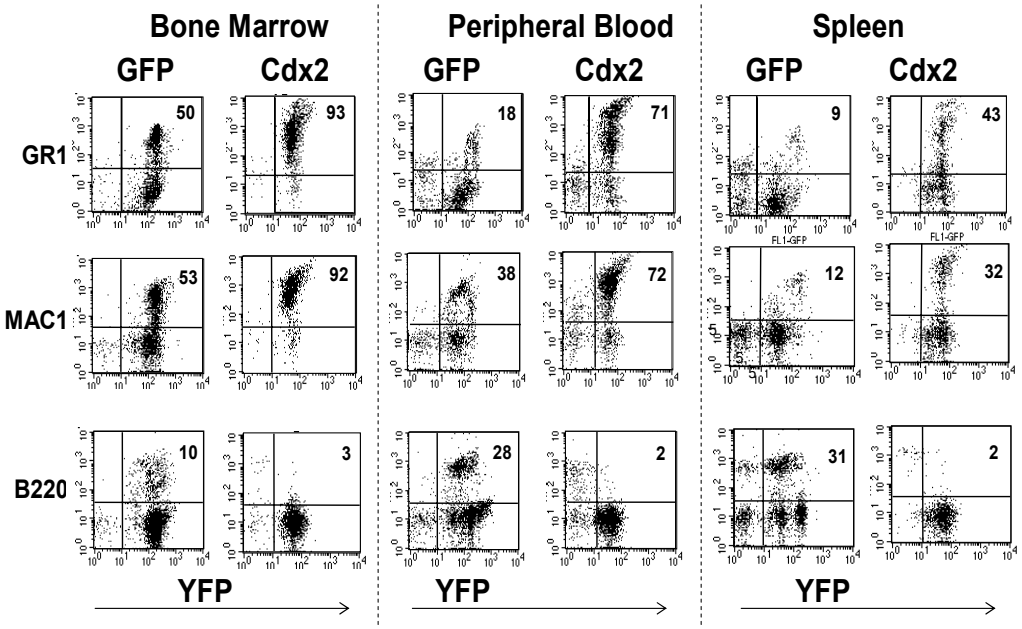


Figure 26. Flow cytometry from a representative leukemic *Cdx2* mouse. PB, BM and spleen from Cdx2 diseased mice in comparison to a GFP control. Cells were stained for the myeloid markers Gr-1 and Mac-1 and the lymphoid marker B220. The proportion of positive cells within the GFP$^+$ compartment is indicated.

4.6 Immunohistochemistry showed blast infiltration in multiple organs

Histopathology report of the diseased *Cdx2* mice showed a hypercellular BM. The normal hematopoiesis was almost completely effaced due to the presence of medium to large cells with a high nuclear/cytoplasmic ratio (Fig. 27 a). The nuclei were round with fine or blastic chromatin and one to three small nucleoli. The chloracetate esterase stain was negative (Fig. 27 b). The immunohistochemistry performed with anti-CD34 showed that approximately 30% of the cells showed a clear, strong membranous positivity suggesting for accumulation of primitive cells (Fig. 27 d). Cells were negative for N-acetyl-chloroacetate esterase, PAS and TdT, indicate that cells were positive for myeloid specific stain and negative for lymphoid specific (TdT) staining. Histologic analysis showing myeloid leukemic blasts ($CD34^+$,TDT^-) infiltrating in the spleen and liver are shown (Fig. 27 c, f). Histopathology of mice diagnosed acute myeloid leukemia. Wright-Giemsa-stained cytospins of PB, BM and spleen demonstated the meyloblastic nature of disease (Fig. 28).

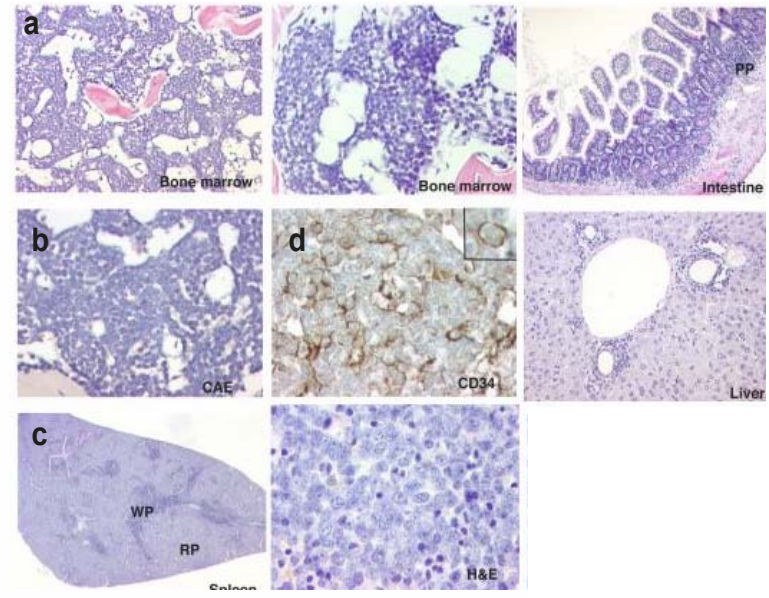


Figure 27. Histological analysis of diseased *Cdx2* mice showed multiple organ infiltration of blast cell population. Histological analysis of diseased Cdx2 mice a) BM (H&E). Immuno-histochemistry showed that the BM is hypercellular (200X) for **b)** N-acetyl-chloroacetate esterase stain was negative (400X) and **d)** 30% of the cells were $CD34^+$ (640X).

c) Histology of the spleen showed infiltration of the blast cells in the red pulp (H&E) (25X) and **e)** Giemsa staining (640X) and **f)** liver with perivascular infiltration (200X)

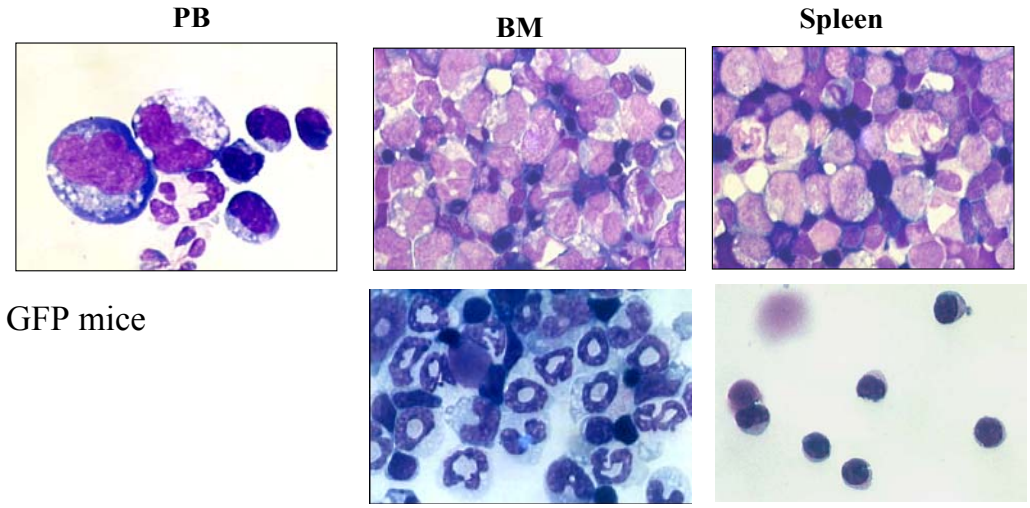


Figure 28. Cytospins of PB, BM and spleen of *Cdx2* diseased mice confirmed the myeloblastic phenotype compared to GFP mice. Cytospin preparations of PB, BM and spleen were analyzed after Giemsa staining and photographs were taken with an inverted microscope (Nikon) at 1000X and Spleen from GFP mice at 630X.

4.7 The *Cdx2*-Induced AML was transplantable

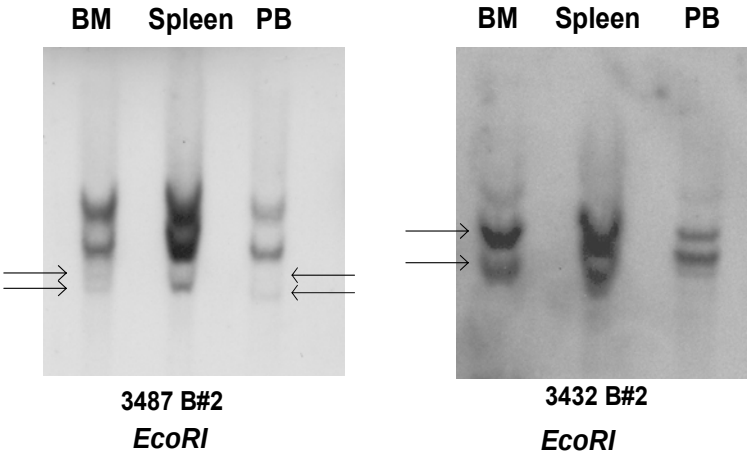
To test whether *Cdx2*-induced AML is transplantable, secondary mice were transplanted from the BM of primary diseased animals. The Cdx2–induced AML was transplantable and all lethally irradiated mice (n=11) died within 24 days after transplantation (Fig. 24). The morphology and immunophenotype of the disease in the secondary recipients was identical to the situation in the primary recipients.

4.8 *Cdx2*-induced diseased was oligoclonal

Analysis of the clonality of the *Cdx2*-induced disease was performed by Southern blot analysis. Genomic DNA from different tissues was digested with *EcoRI*, which cuts once in the proviral genome. GFP was used as a probe. Southern blot analysis demonstrated different intensities and patterns of provi-

ral signals in the different hematopoietic organs consistent with an oligoclonal nature of the disease (Fig. 28 a). Full-length integration of *Cdx2* was confirmed by digesting genomic DNA with *NheI*, which releases the proviral genome from LTR to LTR (Fig. 28 b).

a)



b)

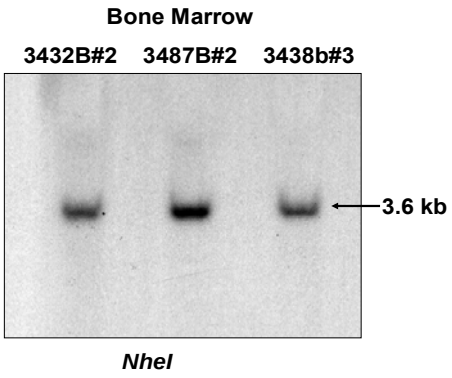


Figure 28. Southern blot showed oligoclonality of the disease: a) Southern blot analyses of genomic DNA from BM, PB and spleen of representative leukemic *Cdx2* mice. Genomic DNA was digested with *EcoR1*, which cuts once in the provirus, to determine the number of provirus integrants. Signals with lower intensity, indicating the presence of different leukemic clones, are indicated. **b)** Full-length provirus integration was documented by digestion with *NheI*, which cuts only in the LTRs of the provirus.

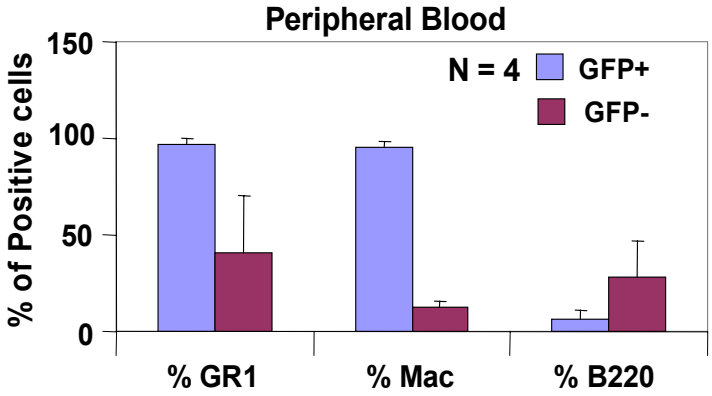
4.9 *Ex Vivo* analyses

Cdx2-transplanted mice were characterized by a 19-fold increase in frequency of clonogenic cells in the PB and an over 100-fold increase in the spleen compared to the control as quantified by ex vivo CFC assays (248 versus 13 clonogenic cells per $1x10^6$ cells/ml in the PB and 1400 clonogenic versus 13 cells per $1x10^6$ cells/ml in the spleen) (n=3). 28% (± 3) of these clonogenic progenitors were not able to terminally differentiate and formed blast colonies in methylcellulose with high serial replating capacity

4.10 *ETV6/CDX2* causes myeloproliferation but does not induce terminal disease

In striking contrast, mice transplanted with ETV6/CDX2 expressing cells did not succumb to terminal disease (n=9) (Fig. 29 a). However, when the PB of healthy *ETV6/CDX2* mice (n=4) was analyzed 40 weeks after transplantation, immunophenotyping analysis showed a high percentage of myeloid Gr-1^+/Mac-1^+ cells in the GFP compartment (Fig. 29a). In order to analyze whether the ETV6/CDX2 fusion caused subtle perturbations in hematopoietic development, healthy animals (n=3) were sacrificed 44 weeks after transplantation. Interestingly, two of the three animals showed an expansion of the mature neutrophil compartment in the PB with an inversion of the lymphoid/myeloid ratio (Table 3). In the GFP^+ positive compartment 87% and 68% were Mac-1^+/Gr-1^+ cells. Furthermore, the spleens from all mice were infiltrated with terminally differentiated myeloid cells (86% ± 0.9 Gr-1^+/Mac-1^+ cells) (Fig. 29b). However, none of the animals suffered from anemia, splenomegaly or the emergence of a blast population in the PB (Table 5). Thus, ETV6/CDX2 was able to induce a mild myeloproliferation without causing disease after a long latency time of 11 months after transplantation, but failed to induce leukemic transformation.

a)



b)

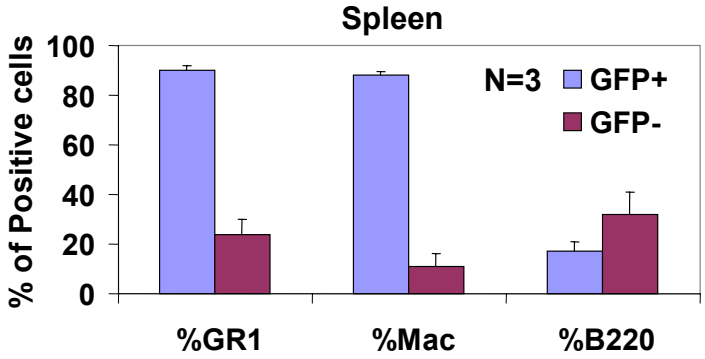


Figure 29. *ETV6/CDX2* induces myeloproliferation without causing disease in transplanted mice: a) *ETV6/CDX2* engrafted mice show an expansion of the myeloid Gr-1$^+$/Mac-1$^+$ cells in the GFP-positive compartment of PB 40 weeks after transplantation (n=4). **b)** Spleens of the engrafted *ETV6/CDX2* sacrificed mice were infiltrated with myeloid cells (86% ± 1 Gr-1$^+$/Mac-1$^+$ cells, n=3).

4.11 Co-expression of *ETV6/CDX2* and *Cdx2* does not accelerate or change the *Cdx2* induced phenotype

In addition, 13 mice were transplanted with a mixture of *ETV6/CDX2*, *Cdx2*, and *ETV6/CDX2* + *Cdx2* co–expressing cells, containing between 1.9 - 4.5 x 10^4 Cdx2 & *ETV6/CDX2* cells and less than 4000 Cdx2 cells per mouse. The addition of *ETV6/CDX2* + *Cdx2* co-expressing cells did not accelerate the course or change the phenotype of the disease compared to only *Cdx2* expressing cells (Fig. 24). All animals succumbed to AML and the leukemic

population consisted of *ETV6/CDX2* + *Cdx2* co-expressing or *Cdx2* expressing cells in all mice analyzed (n=4) (Fig. 30 a, b). These data indicate that aberrant expression of the wild-type *Cdx2* gene is crucial for malignant transformation in this model.

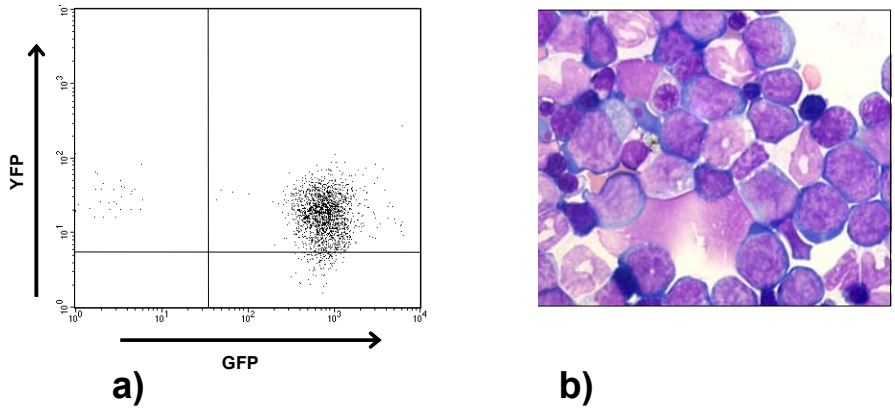


Figure 30. Co-expression of *ETV6/CDX2* + *Cdx2* causes AML in mice: **a)** Co-expression of *ETV6/CDX2* +*Cdx2* was confirmed by flow cytometry analysis of the BM cells of a leukemic mice using YFP/GFP as a marker. **b)** Giemsa staining of the cytospin of the BM cells of *ETV6/CDX2* +*Cdx2* mice showed the myeloblastic nature of the disease.

4.12 The transforming potential of *Cdx2* is dependent on the N-terminal transactivation domain and the intact homeodomain

In an effort to characterize the contribution of different motifs of *Cdx2* to the transforming capacity of the gene, three different mutants were designed: a homeodomain mutant of *Cdx2* (N51S-*Cdx2*), which results in loss of the DNA binding capacity, a Cdx2 mutant with an inactivating mutation in the putative PBX1 interacting motif (W167A-*Cdx2*) and a mutant lacking the N-terminal transactivation portion of Cdx2, which is also not present in the *ETV6/CDX2* fusion (ΔN-*Cdx2*). Protein expression of the mutants was confirmed by Western blot analysis (Fig. 31). Expression of wild-type *Cdx2* and W167A-*Cdx2* rapidly induced the outgrowth of IL-3 dependent cell lines in liquid cultures. The ΔN-*Cdx2* or the N51S-*Cdx2* mutants were not able to form cell lines i*n vitro*.

When the impact of the different constructs were tested on colony formation, W167A-*Cdx2* positive cells generated a higher number of CFU total and blast colonies in secondary CFC in comparison to the GFP control (10 fold and 40 fold per 500 initially plated cells, respectively; n=3; $p<0.02$) (Fig. 32 a, b). W167A-Cdx2 positive colonies were of the same size as Cdx2 colonies. In contrast, the expression of the N51S-Cdx2 and the ΔN-Cdx2 mutants did not show a significant increase in colony number or size of the colonies compared to GFP and did not induce growth of blast colonies (Fig. 31 a, b).

To investigate the effect of the different mutants on more primitive hematopoietic cells, progenitor enriched BM infected with the different viruses was injected into lethally irradiated mice after 7 days of in vitro culture and spleen colony formation was quantified 12 days after injection in sacrificed mice (CFU-S assay). The expression of *Cdx2* as well as the W167A-*Cdx2* mutant induced a significant, over 10 fold increase in the yield of d12 CFU-S compared to the GFP control (n=8; $p< 0.0001$) (Fig. 33). In contrast, deletion of the N-terminal portion of Cdx2 (n=5) or inactivation of the homeodomain (n=5) resulted in a complete loss of the Cdx2 activity (Fig. 33).

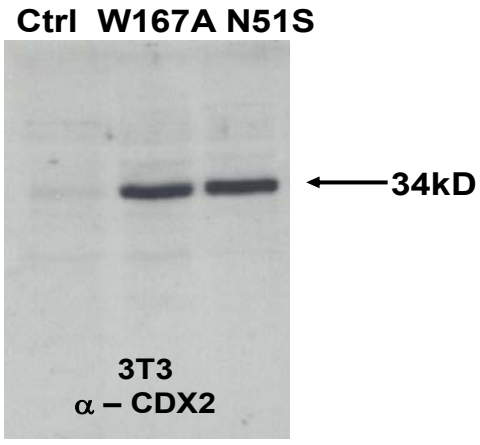
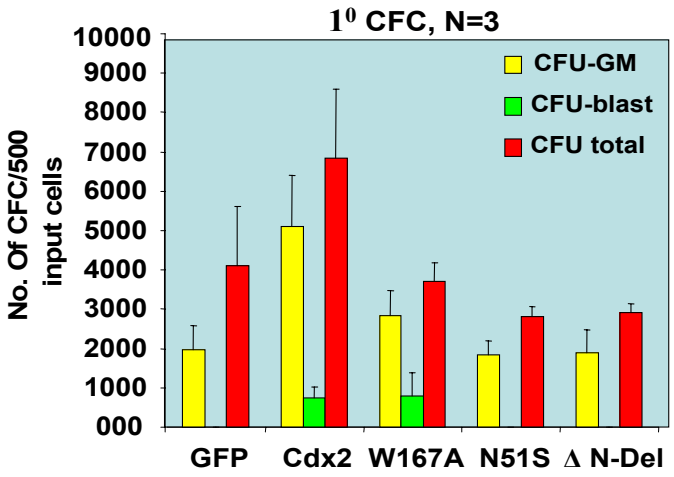


Figure 31. Protein expression of the *Cdx2* mutants: Immunoblot analysis from whole cell lysates NIH 3T3 cells transfected with the different mutants, probed with anti- human *CDX2* antibody.

a)



b)

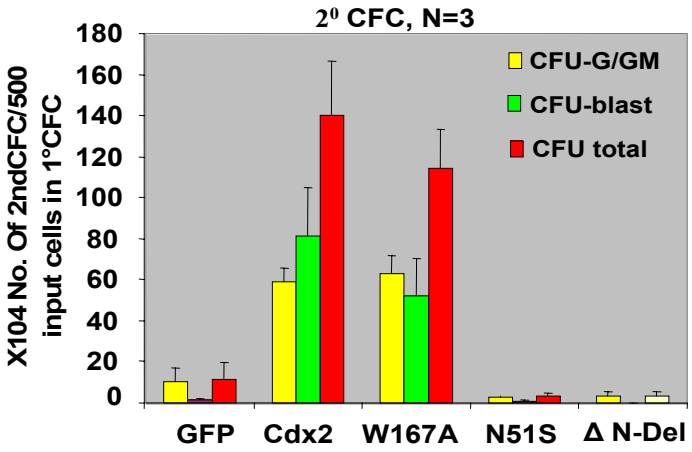


Figure 32. CFC assay showed that homeodomain dead mutant and mutant with a deletion of the transactivation domain lose their clonogenic potential: a) *Cdx2* and W167A-*Cdx2* showed a substantial increase in the number of CFU-blast in primary CFC (N=3) compared to the GFP control. **b)** In the re-plating assays N51S-*Cdx2* and ΔN-*Cdx2* failed to increase the number of secondary colonies compared to *Cdx2* wild-type.

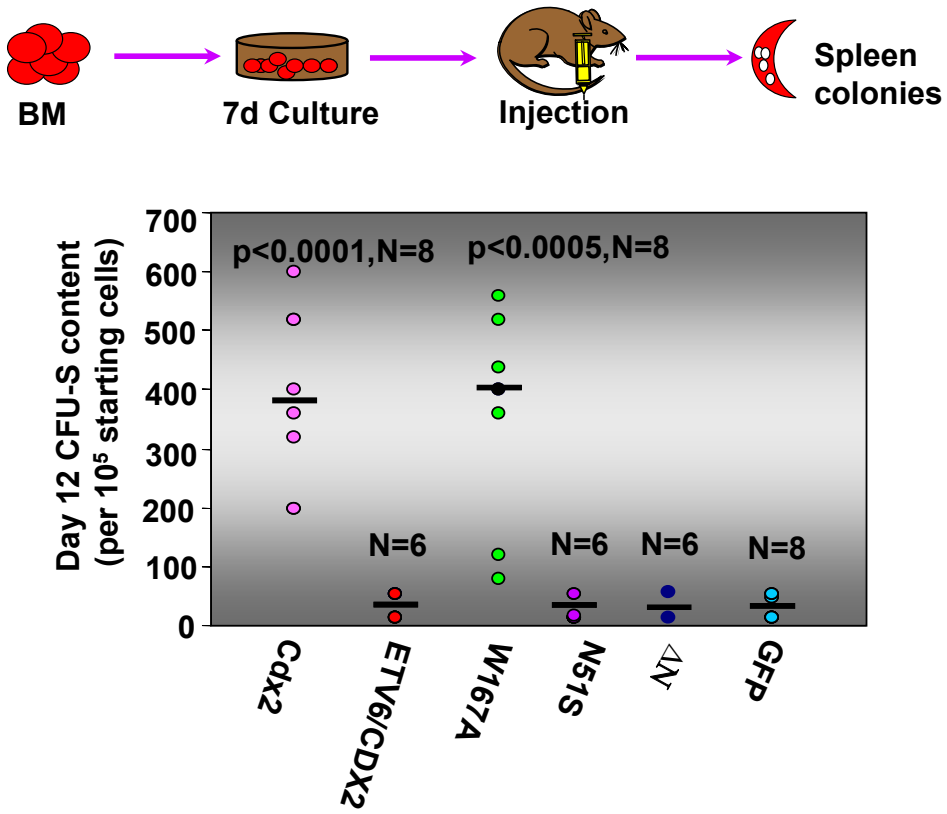


Figure 33. Deletion of the transactivation domain and inactivation of the homeodomain results in loss of activity in the CFU-S assay compared to wild-type *Cdx2*. Total number of day 12 CFU-S colonies derived per culture initiated with 1x 105 cells transduced with different vectors. The median is indicated. *Cdx2* and W167A-*Cdx2* induced a significant, over 10 fold increase (N=8, p< 0.0001) in the yield of day 12 CFU-S compared to the GFP control. N51S-Cdx2 and ΔN-Cdx2 did not show any increase in the yield of day 12 CFU-S compared to the GFP control.

4.13 The expression of *Hoxa9* and *Meis1* is not up-regulated by ectopic expression of *Cdx2*

As *Cdx2* is an upstream regulator of Hox genes, we asked whether *Cdx2* would perturb expression of leukemogenic homeobox genes such as Hoxa9 or Meis1. First, expression of and Meis1 determined in the 32D cell line, transduced with the, the *ETV6/CDX2* or the GFP-only virus, by RT–PCR. Compared to the control *Cdx2* did not upregulate expression of *Hoxa9* or *Meis1*. In addition, $Scal^{-}/Lin^{+}$ differentiated cells were recovered and highly purified from a *Cdx2* mouse and a control animal, a cell population with normally has no detectable expression of *Hoxa9* and *Meis1* (Pineault et. al., 2002): specific amplification

products were not detectable by RT-PCR after 25 cycles in both experimental arms. Amplification products could be detected after 35 cycles but without considerable differences in the intensity between Cdx2 and the control (Fig. 34). Thus, ectopic expression of *Cdx2* was not associated with upregulation of Meis1 or Hoxa9 in this model system.

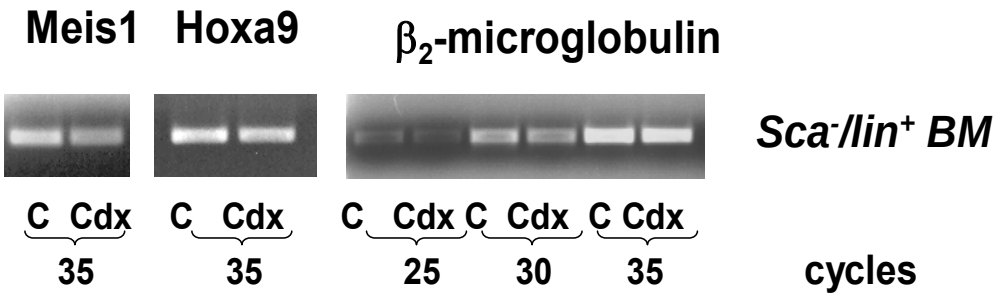


Figure 34. Ectopic expression of *Cdx2* in BM cells did not upregulate Hox genes: Expression of *Meis1* and *Hoxa9* analyzed by RT–PCR in $Scal^{-}/Lin^{+}$ BM cells isolated from a leukemic Cdx2 mouse or a control mouse. The number of PCR cycles for each gene was chosen to be in the linear phase of the amplification (25 cycles for mβ-2 microglobulin, 35 cycles for Meis1 and Hoxa9). C = control; Cdx = Cdx2

5 DISCUSSION

In general, chromosomal translocations can contribute to malignant transformation by two distinct mechanisms: first they can generate fusion genes with oncogenic potential. Secondly, they can induce aberrant or ectopic expressing of a proto-oncogene, thereby leading to malignant transformation. Both mechanisms are found in lymphoid neoplasia, with proto-oncogene activation being more frequently found in lymphomagenesis and in ALL.

The formation of fusion genes with oncogenic properties by balanced chromosomal rearrangements is considered to be the crucial step of leukemic transformation in patients with AML, and so far there is no proof that activation of a proto-oncogene by a balanced chromosomal translocation can be the key event for the development of AML (Cools et. al., 2002; Look, 1997). However, recent data have suggested that the activation of proto-oncogenes might be much more widespread in myeloid leukemogenesis than previously thought, because there are many AML cases, which do not express a functional fusion protein despite the occurrence of a balanced chromosomal translocation (Cools et al., 2002). Furthermore, many fusion proteins expressed in AML have clearly no leukemogenic potential in experimental in vivo models (Castilla et. al., 1996; Mulloy et. al., 2002; Pineault et. al., 2003), suggesting that additional or other functional alterations are the relevant pathogenetic events in these AML cases.

By using a murine BM transplantation model we now provide direct evidence that the induction of ectopic expression of the proto-oncogene *CDX2*, and not the creation of the fusion gene *ETV6/CDX2*, is the key transforming event in t(12;13)(p13;q12) positive AML. In addition, these data present the first direct evidence that the homeobox gene and Hox gene upstream regulator Cdx2, which so far has been linked to intestinal metaplasia and colon cancer (Silberg

et. al., 2002), is highly leukemogenic when ectopically expressed in hematopoietic progenitor cells.

Our data clearly show that Cdx2 transforms murine bone marrow cells *in vitro* and *in vivo*. As shown in the proliferation assays, *Cdx2*-transduced cells displayed a high rate of proliferation and were able to grow in medium supplemented only with IL-3 in vitro. Furthermore, these indefinitely growing cell populations displayed a myeloblastic phenotype and were able to cause leukemia when transplanted into lethally irradiated syngenic mice. This data indicated that ectopic expression of the *Cdx2* homeobox gene blocks differentiation of bone marrow cells. These data were supported by the observation that Cdx2 enhanced the in vitro clonogenic potential of BM cells and supported the outgrowth of blast colonies in the CFC-assay, indicating that Cdx2 increased the proliferation and blocked differentiation of these progenitor cells in methylcellulose. This effect was also confirmed when more primitive progenitor cells were analyzed in the CFU-S assay, in which Cdx2 expression significantly enhanced the number of spleen colony forming units.

The perturbation of the normal hematopoietic development by *Cdx2* might be linked to critical alterations in downstream Hox genes, which are central regulators of normal early hematopoietic development in the adult with a distinct expression profile in human and murine early progenitor cells (Buske and Humphries, 2000; Pineault et. al., 2002; Sauvageau et. al., 1994). Gene expression profiling of acute leukemias using DNA microarray technology linked aberrant expression of Hox genes such as *HOXA9*, *HOXA10* and of the non-clustered homeobox gene *MEIS1* to leukemogenesis (Debernardi et. al., 2003; Ferrando et. al., 2003; Golub. et al., 1999a; Yeoh et al., 2002). Retrovirally enforced expression of these genes induced severe perturbations of normal hematopoietic development in human and murine experimental models (Buske et. al., 2001; Thorsteinsdottir et. al., 2001). The perturbance of the expression of several Hox genes might be one of the reasons for the strong oncogenic potential of *Cdx2* (Lorentz et. al., 1997b; van den Akker et. al., 2002). However, RT–PCR analyses in the 32D cell line model and in $Sca1^{-}/lin^{+}$ BM population

of a mouse repopulated with Cdx2 expressing cells did not show up-regulation of Meis1 or Hoxa9 by Cdx2. However this does not exclude that perturbation of other Hox genes might play a role in the transformation process initiated by ectopic Cdx2 expression.

Of note, perturbed expression of HOX genes such as *HOXA9* or *HOXA10* in hematopoietic progenitor cells is not able to induce genuine AML in transplanted mice after a short latency time, but requires co-operation with the Hox co-factor MEIS1. In striking contrast, constitutive expression of *Cdx2* rapidly caused leukemia in recipient mice on its own. Moreover, mutating the PBX1 interacting motif (PIM) in Cdx2 was found to be insufficient to suppress the leukemogenic potential of the gene. This implies that Cdx2 differs from the other Hox genes in the requirement of the cofactors MEIS1 and PBX1 for its leukemogenic activity. The underlying cause for the difference in the leukemogenic activity between *Cdx2* and *HOXA9* or *HOXA10* is not known. But in contrast to HOXA9 and HOXA10, which are normally expressed at high levels in progenitor cells, *CDX2* is not expressed in hematopoietic cells (Chase et al., 1999). Thus, ectopic expression of *CDX2* might result in the activation of de novo downstream pathways, which are normally silent in early blood development.

Despite the differences in the oncogenic potential, many of the in vitro and in vivo hematopoietic effects induced by *Cdx2* are highly reminiscent of the effects of retrovirally overexpressed HOX genes as well as of leukemia–specific fusion genes such as *NUP98-HOXD13* with regard to the impact on short-term repopulating CFU-S or clonogenic progenitors (Pineault et. al., 2003a; Sauvageau et. al., 1997; Thorsteinsdottir et. al., 2001). The striking similarities of the phenotypes induced by the over-expression of homeobox genes of the HOX cluster and of *Cdx2*, as a member of the ParaHOX complex (Brooke et al., 1998), point to a high level of functional redundancy among homeobox proteins in hematopoiesis.

The hematopoietic activity of *Cdx2* was strictly dependent on an intact homeodomain, implicating that DNA binding of *Cdx2* is essential for its

transforming activity. Furthermore, deletion of the *Cdx2* N-terminal portion resulted in a complete loss of the activity in our assays. Of note, it was demonstrated that the N-terminal part of Cdx2 is necessary for transcriptional activation of HOX genes, supporting the concept that activation of downstream HOX genes is a potential key mechanism of Cdx2 induced transformation (Taylor et. al., 1997). Furthermore, it was demonstrated that the transcriptional activity of CDX proteins depends on the interaction of the p38 mitogen-activated protein kinase with the N-terminal transactivation domain of Cdx2 (Houde et. al., 2001). Serine-60 in the N-terminal domain has been shown to be phosphorylated by MAPK via ERK1/2 and the phosphorylated form of Cdx2 loses the transactivation capacity in intestinal epithelial cells. As a consequence, N-terminal deletion would diminish the transactivation capacity of *Cdx2*, thus abolishing its transforming capability. Importantly, the *ETV6/CDX2* fusion gene lacks the N-terminal portion of CDX2, presumably hampering its capability to transactivate target genes. This would explain the obvious discrepancy in the oncogenic potential between *Cdx2* and the *ETV6/CDX2* fusion gene. This is supported by the presented data, which demonstrate a complete loss of activity, when this N-terminal portion of *Cdx2*, which is not present in the *ETV6/CDX2* fusion gene, is deleted in the ΔN-*CDX2* mutant. Notably, mice transplanted with BM cells expressing the chimeric gene developed a mild myeloproliferation after a long latency time but without any clinical symptoms. This indicates that despite the loss of the N-terminal portion, the fusion gene is able to perturb hematopoietic development, although to a significantly lesser extent than full-length *Cdx2*. However, it cannot be excluded from our experiments that the first 54 amino acids of the ETV6 protein fused to CDX2 are responsible or at least contribute to the observed weak disturbances of hematopoiesis. However, to date there are no data that have shown a distinct function for these first 54 aa of ETV6. Taken together, our data propose a model, in which the chromosomal translocation t(12;13)(p13;q12) causes AML by activating the wild-type proto-oncogene *CDX2*. The mechanism of activation is not precisely known, but it was demonstrated that the chromosome 13 breakpoint

lies upstream of the *CDX2* gene. Therefore, one possible mechanism of *CDX2* activation could be that the full-length *CDX2* is translocated downstream to one of the two alternative ETV6 promoters, located between exon 2 and exon 3 of *ETV6* (Chase et. al., 1999). Intriguingly, recent data demonstrated the ectopic expression of the homeobox gene *GSH2* and of *IL3* in patients with AML and the translocations t(4;12)(q11-12;p13) and t(5;12)(q31;p13), respectively. Both translocations involve the *ETV6* gene, but do not create any functional fusion genes (Cools et. al., 2002). This suggests that activation of proto-oncogenes might be a common phenomenon in ETV6–associated leukemias. AML cases which do not express any functional fusion protein despite the occurrence of a balanced chromosomal translocation have also been reported. Furthermore, many fusion proteins expressed in AML fail to demonstrate leukemogenic potential in experimental *in vivo* models (Cools et al, 2002; Schwaller et. al. 1998; Mulloy et. al. 2002; Peeters et. al.1997b). Taking these findings into consideration it is tempting to speculate that activation of proto-oncogenes by chromosomal rearrangements might be a more widespread mechanism in myeloid leukemogenesis than previously thought. This is supported by observations in AML cases not affecting *ETV6*, in which expression of the putative proto-oncogene *EVI1* is upregulated by juxtaposition of the gene to the enhancer sequences of the ribophorin-I gene in patients with AML and 3q21 alterations (Nucifora, 1997). Our data now provide compelling evidence, that myeloid leukemogenesis can be initiated by this mechanism and stresses the relevance of proto-oncogene activation for the development of AML.

6 SUMMARY

In conclusion, the thesis demonstrates for the first time that activation of a proto-oncogene by a chromosomal translocation can be the key step in myeloid leukemogenesis, even if a fusion gene is generated and expressed in parallel. This mechanism of AML leukemogenesis was proven in a murine model of t(12;13)(p13;q12) AML, showing that myeloid leukemogenesis is induced by the ectopic expression of *Cdx2* and not by the *ETV6/CDX2* chimeric gene. Mice transplanted with bone marrow cells retrovirally engineered to express *Cdx2* rapidly succumbed to fatal and transplantable AML. In contrast, mice which were transplanted with BM cells expressing the fusion gene alone did not develop AML. The transforming activity of Cdx2 was dependent on an intact homeodomain and the N-terminal transactivation domain. Although mice transplanted with *ETV6/CDX2* expressing BM cells did not develop overt disease, these animals suffered from a mild myeloproliferation. Experiments testing the effect of simultaneous expression of the *Cdx2* and the fusion gene showed no acceleration or change in phenotype of the disease compared to expression of *Cdx2* alone, again demonstrating that the ectopic expression of *Cdx2* was the key event in this leukemia model. These findings link proto-oncogene activation to myeloid leukemogenesis, an oncogenic mechanism so far associated mainly with lymphoid leukemias and lymphomas. Our model constitutes the first functional proof that activation of a proto-oncogene by a chromosomal translocation is the key leukemogenic event in AML. As many fusion proteins expressed in AML have clearly no leukemogenic potential in experimental in vivo models, this mechanism might be more important for the development of AML than previously thought and should be included in the pathogenetic models of this disease.

7 ZUSAMMENFASSUNG

Schließlich zeigt diese Doktorarbeit zum ersten Mal, dass die Aktivierung eines Proto-Onkogens durch eine chromosomale Translokation der Schlüssel zur myeloischen Leukämogenese sein kann, selbst dann, wenn parallel dazu ein Fusionsgen generiert und exprimiert wird. Dieser Mechanismus für die Entstehung einer AML wurde in einem t(12;13)(p13;q12)AML-Mausmodell nachgewiesen, wobei gezeigt wurde, dass die myeloische Leukämogenese von der ektopischen *Cdx2*-Expression induziert wird, und nicht von dem *ETV6/CDX2*–Fusionsgen. Mäuse, die mit Knochenmarkzellen transplantiert wurden, welche durch retroviralen Gentranfer *Cdx2* überexprimierten, verstarben innerhalb kürzester Zeit an einer tödlichen und transplantierbaren AML. Im Gegensatz dazu entwickelten Mäuse, die mit Knochenmarkzellen transplantiert wurden, welche nur das Fusionsgen allein exprimierten, keine AML. Die Transformations-Aktivität von *Cdx2* war von einer intakten Homeodomäne und der N-terminalen Transaktivations-Domäne abhängig. Obwohl die Mäuse, die mit Knochenmarkzellen transplantiert wurden, die *ETV6/CDX2* exprimieren, nicht offensichtlich krank wurden, litten diese Tiere doch an einer leichten Myeloproliferation. Experimente, die den Effekt der gleichzeitigen Expression von Cdx2 und dem Fusionsgen untersuchten, zeigten keinerlei Beschleunigung oder Veränderung im Phänotyp der Erkrankung verglichen mit der Expression von Cdx2 alleine. Wieder wurde gezeigt, dass die ektopische Expression von Cdx2 eine Schlüsselrolle in diesem Leukämie-Modell untermauerte. Diese Ergebnisse zeigen die Bedeutung von Proto-Onkogen-Aktivierung in der myeloischer Leukämogenese. Dieser

Mechanismus wurde bisher vor allem mit lymphoiden Leukämien und Lymphomen in verbindung gebracht. Unser Modell stellt den ersten Beweis dafür dar, dass die Aktivierung eines Proto-Onkogens durch eine chromosomale Translokation der Hauptgrund für die Entstehung einer AML ist. Da manche Fusionsproteine, die bei einer AML exprimiert werden, keinerlei leukämieauslösendes Potential bei Experimenten mit in vivo-Modellen besitzen, dürfte dieser Mechanismus eine weitaus wichtigere Rolle bei der Entwicklung einer AML spielen als bisher angenommen. Deshalb sollte er auch in das pathogenetische Modell dieser Erkrankung aufgenommen werden.

8 REFERENCES

Abate-Shen, C. (2002).
Deregulated homeobox gene expression in cancer: cause or consequence? *Nat Rev Cancer 2, 777-785.*

Akashi, K., Reya, T., Dalma-Weiszhausz, D., and Weissman, I. L. (2000). Lymphoid precursors. *Curr Opin Immunol 12*, 144-150.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., and Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A 100, 3983-3988.*

Antonchuk, J., Sauvageau, G., and Humphries, R. K. (2001). HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. *Exp Hematol 29, 1125-1134.*

Antonchuk, J., Sauvageau, G., and Humphries, R. K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell 109, 39-45.*

Armstrong, S. A., Staunton, J. E., Silverman, L. B., Pieters, R., den Boer, M. L., Minden, M. D., Sallan, S. E., Lander, E. S., Golub, T. R., and Korsmeyer, S. J. (2002). MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat Genet 30, 41-47.*

Bai, Y. Q., Yamamoto, H., Akiyama, Y., Tanaka, H., Takizawa, T., Koike, M., Kenji Yagi, O., Saitoh, K., Takeshita, K., Iwai, T., and Yuasa, Y. (2002). Ectopic expression of homeodomain protein CDX2 in intestinal metaplasia and carcinomas of the stomach. *Cancer Lett 176, 47-55.*

Barba, P., Magli, M. C., Tiberio, C., and Cillo, C. (1993). HOX gene expression in human cancers. *Adv Exp Med Biol 348, 45-57.*

Beck, F., Erler, T., Russell, A., and James, R. (1995). Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. *Dev Dyn 204, 219-227.*

Bedford, F. K., Ashworth, A., Enver, T., and Wiedemann, L. M. (1993). HEX: a novel homeobox gene expressed during haematopoiesis and conserved between mouse and human. *Nucleic Acids Res 21, 1245-1249.*

Beverloo, H. B., Panagopoulos, I., Isaksson, M., van Wering, E., van Drunen, E., de Klein, A., Johansson, B., and Slater, R. (2001). Fusion of the homeobox gene HLXB9 and the ETV6 gene in infant acute myeloid leukemias with the t(7;12)(q36;p13). *Cancer Res 61, 5374-5377.*

BitMansour, A., Burns, S. M., Traver, D., Akashi, K., Contag, C. H., Weissman, I. L., and Brown, J. M. (2002). Myeloid progenitors protect against invasive aspergillosis and Pseudomonas aeruginosa infection following hematopoietic stem cell transplantation. *Blood 100, 4660-4667***.**

Bohlander S K (2005).
ETV6: a versatile player in leukemogenesis.*Semin Cancer Biol 15(3):162-74.*

Bonnet, D. (2003).
Hematopoietic stem cells**.** *Birth Defects Res Part C Embryo Today 69, 219-229.*

Bonnet, D., and Dick, J. E. (1997).
Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med 3, 730-737.*

Brooke, N. M., Garcia-Fernandez, J., and Holland, P. W. (1998).
The ParaHox gene cluster is an evolutionary sister of the Hox gene cluster. *Nature 392, 920-922.*

Buske, C., Feuring-Buske, M., Abramovich, C., Spiekermann, K., Eaves, C. J., Coulombel, L., Sauvageau, G., Hogge, D. E., and Humphries, R. K. (2002a).
Deregulated expression of HOXB4 enhances the primitive growth activity of human hematopoietic cells. *Blood 100, 862-868.*

.

Buske, C., Feuring-Buske, M., Antonchuk, J., Rosten, P., Hogge, D. E., Eaves, C. J., and Humphries, R. K. (2001).
Overexpression of HOXA10 perturbs human lymphomyelopoiesis in vitro and in vivo. *Blood 97, 2286-2292.*

Buske, C., and Humphries, R. K. (2000).
Homeobox genes in leukemogenesis. *Int J Hematol 71, 301-308.*

Care, A., Silvani, A., Meccia, E., Mattia, G., Stoppacciaro, A., Parmiani, G., Peschle, C., and Colombo, M. P. (1996).
HOXB7 constitutively activates basic fibroblast growth factor in melanomas. *Mol Cell Biol 16, 4842-4851.*

Castilla, L. H., Wijmenga, C., Wang, Q., Stacy, T., Speck, N. A., Eckhaus, M., Marin-Padilla, M., Collins, F. S., Wynshaw-Boris, A., and Liu, P. P. (1996).
Failure of embryonic hematopoiesis and lethal hemorrhages in mouse embryos heterozygous for a knocked-in leukemia gene CBFB-MYH11. *Cell 87, 687-696.*

Cazzaniga, G., Tosi, S., Aloisi, A., Giudici, G., Daniotti, M., Pioltelli, P., Kearney, L., and Biondi, A. (1999).
The tyrosine kinase abl-related gene ARG is fused to ETV6 in an AML-M4Eo patient with a t(1;12)(q25;p13): molecular cloning of both reciprocal transcripts. *Blood 94, 4370-4373.*

Chase, A., Reiter, A., Burci, L., Cazzaniga, G., Biondi, A., Pickard, J., Roberts, I. A., Goldman, J. M., and Cross, N. C. (1999).
Fusion of ETV6 to the caudal-related homeobox gene CDX2 in acute myeloid leukemia with the t(12;13)(p13;q12). *Blood 93, 1025-1031.*

Chawengsaksophak, K., de Graaff, W., Rossant, J., Deschamps, J., and Beck, F. (2004).
Cdx2 is essential for axial elongation in mouse development. *Proc Natl Acad Sci U S A 101, 7641-7645.*

Chawengsaksophak, K., James, R., Hammond, V. E., Kontgen, F., and Beck, F. (1997).
Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature 386, 84-87.*

Cools, J., Mentens, N., Odero, M. D., Peeters, P., Wlodarska, I., Delforge, M., Hagemeijer, A., and Marynen, P. (2002).
Evidence for position effects as a variant ETV6-mediated leukemogenic mechanism in myeloid leukemias with a t(4;12)(q11-q12;p13) or t(5;12)(q31;p13). *Blood 99, 1776-1784.*

da Costa, L. T., He, T. C., Yu, J., Sparks, A. B., Morin, P. J., Polyak, K., Laken, S., Vogelstein, B., and Kinzler, K. W. (1999).
CDX2 is mutated in a colorectal cancer with normal APC/beta-catenin signaling. *Oncogene 18, 5010-5014*.

Dash, A., Gilliland, D. G. (2001)
Molecular genetics of acute myeloid leukaemia. *Best Pract Res Clin Haematol 14, 49-64.*

Davidson, A. J., Ernst, P., Wang, Y., Dekens, M. P., Kingsley, P. D., Palis, J., Korsmeyer, S. J., Daley, G. Q., and Zon, L. I. (2003).
Cdx4 mutants fail to specify blood progenitors and can be rescued by multiple hox genes. *Nature 425, 300-306.*

De Vita, G., Barba, P., Odartchenko, N., Givel, J. C., Freschi, G., Bucciarelli, G., Magli, M. C., Boncinelli, E., and Cillo, C. (1993). Expression of homeobox-containing genes in primary and metastatic colorectal cancer. *Eur J Cancer 29A, 887-893.*

Debernardi, S., Lillington, D. M., Chaplin, T., Tomlinson, S., Amess, J., Rohatiner, A., Lister, T. A., and Young, B. D. (2003).
Genome-wide analysis of acute myeloid leukemia with normal karyotype reveals a unique pattern of homeobox gene expression distinct from those with translocation-mediated fusion events. *Genes Chromosomes Cancer 37, 149-158.*

Drummond, F., Putt, W., Fox, M., and Edwards, Y. H. (1997).
Cloning and chromosome assignment of the human CDX2 gene. *Ann Hum Genet 61 (Pt 5), 393-400.*

Eda, A., Osawa, H., Satoh, K., Yanaka, I., Kihira, K., Ishino, Y., Mutoh, H., and Sugano, K. (2003).
Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa. *J Gastroenterol 38, 14-22.*

Eda, A., Osawa, H., Yanaka, I., Satoh, K., Mutoh, H., Kihira, K., and Sugano, K. (2002).
Expression of homeobox gene CDX2 precedes that of CDX1 during the progression of intestinal metaplasia. *J Gastroenterol 37, 94-100.*

Ee, H. C., Erler, T., Bhathal, P. S., Young, G. P., and James, R. J. (1995). Cdx-2 homeodomain protein expression in human and rat colorectal adenoma and carcinoma. *Am J Pathol 147, 586-592.*

Erickson, P., Gao, J., Chang, K. S., Look, T., Whisenant, E., Raimondi, S., Lasher, R., Trujillo, J., Rowley, J., and Drabkin, H. (1992).
Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. *Blood 80, 1825-1831.*

Fenrick, R., Amann, J. M., Lutterbach, B., Wang, L., Westendorf, J. J., Downing, J. R., and Hiebert, S. W. (1999).
Both TEL and AML-1 contribute repression domains to the t(12;21) fusion protein. *Mol Cell Biol 19, 6566-6574.*

Ferrando, A. A., Armstrong, S. A., Neuberg, D. S., Sallan, S. E., Silverman, L. B., Korsmeyer, S. J., and Look, A. T. (2003).
Gene expression signatures in MLL-rearranged T-lineage and B-precursor acute leukemias: dominance of HOX dysregulation. *Blood 102, 262-268.*

Feuring-Buske, M., Frankel, A. E., Alexander, R. L., Gerhard, B., and Hogge, D. E. (2002).
A diphtheria toxin-interleukin 3 fusion protein is cytotoxic to primitive acute myeloid leukemia progenitors but spares normal progenitors. *Cancer Res 62, 1730-1736.*

Giampaolo, A., Pelosi, E., Valtieri, M., Montesoro, E., Sterpetti, P., Samoggia, P., Camagna, A., Mastroberardino, G., Gabbianelli, M., Testa, U., and et al. (1995).
HOXB gene expression and function in differentiating purified hematopoietic progenitors. *Stem Cells 13 Suppl 1*, *90-105.*

Golub, T. R., Barker, G. F., Lovett, M., and Gilliland, D. G. (1994).
Fusion of PDGF receptor beta to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. *Cell 77, 307-316.*

Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., *et al.* (1999a).
Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science 286, 531-537.*

Hatano, M., Roberts, C. W., Minden, M., Crist, W. M., and Korsmeyer, S. J. (1991).
Deregulation of a homeobox gene, HOX11, by the t(10;14) in T cell leukemia. *Science 253, 79-82.*

Hinoi, T., Loda, M., and Fearon, E. R. (2003).
Silencing of CDX2 expression in colon cancer via a dominant repression pathway. *J Biol Chem 278, 44608-44616.*

Hinoi, T., Tani, M., Lucas, P. C., Caca, K., Dunn, R. L., Macri, E., Loda, M., Appelman, H. D., Cho, K. R., and Fearon, E. R. (2001).

Loss of CDX2 expression and microsatellite instability are prominent features of large cell minimally differentiated carcinomas of the colon. *Am J Pathol 159, 2239-2248.*

Hoang, T. (2004).
The origin of hematopoietic cell type diversity. *Oncogene 23, 7188-7198.*

Hock, H., Meade, E., Medeiros, S., Schindler, J. W., Valk, P. J., Fujiwara, Y., and Orkin, S. H. (2004).
Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. *Genes Dev.*

Houde, M., Laprise, P., Jean, D., Blais, M., Asselin, C., and Rivard, N. (2001).
Intestinal epithelial cell differentiation involves activation of p38 mitogen-activated protein kinase that regulates the homeobox transcription factor CDX2. *J Biol Chem 276, 21885-21894.*

Huntly, B. J., Shigematsu, H., Deguchi, K., Lee, B. H., Mizuno, S., Duclos, N., Rowan, R., Amaral, S., Curley, D., Williams, I. R., *et al.* (2004).
MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell 6, 587-596.*

Ishikawa, A., Sasaki, M., Ohira, S., Ohta, T., Oda, K., Nimura, Y., Chen, M. F., Jan, Y. Y., Yeh, T. S., and Nakanuma, Y. (2004).
Aberrant expression of CDX2 is closely related to the intestinal metaplasia and MUC2 expression in intraductal papillary neoplasm of the liver in hepatolithiasis. *Lab Invest 84, 629-638.*

Iwasaki, M., Kuwata, T., Yamazaki, Y., Jenkins, N. A., Copeland, N. G., Osato, M., Ito, Y., Kroon, E., Sauvageau, G., and Nakamura, T. (2005). Identification of coopera-

tive genes for NUP98-HOXA9 in myeloid leukemogenesis using a mouse model. *Blood 105, 784-793.*

James, R., Erler, T., and Kazenwadel, J. (1994).
Structure of the murine homeobox gene cdx-2. Expression in embryonic and adult intestinal epithelium. *J Biol Chem 269, 15229-15237*.

Jamieson, C. H., Ailles, L. E., Dylla, S. J., Muijtjens, M., Jones, C., Zehnder, J. L., Gotlib, J., Li, K., Manz, M. G., Keating, A., *et al.* (2004a). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med 351, 657-667***.**

Jamieson, C. H., Weissman, I. L., and Passegue, E. (2004b).
Chronic versus acute myelogenous leukemia; A question of self-renewal. *Cancer Cell 6, 531-533.*

Jordan, C. T., and Guzman, M. L. (2004). Mechanisms controlling pathogenesis and survival of leukemic stem cells. Oncogene *23*, 7178-7187.

Kawagoe, H., Humphries, R. K., Blair, A., Sutherland, H. J., and Hogge, D. E. (1999).
Expression of HOX genes, HOX cofactors, and MLL in phenotypically and functionally defined subpopulations of leukemic and normal human hematopoietic cells. *Leukemia 13, 687-698*.

Knezevich, S. R., McFadden, D. E., Tao, W., Lim, J. F., and Sorensen, P. H. (1998).
A novel ETV6-NTRK3 gene fusion in congenital fibrosarcoma. *Nat Genet 18, 184-187.*

Knoepfler, P. S., and Kamps, M. P. (1995).

The pentapeptide motif of Hox proteins is required for cooperative DNA binding with Pbx1, physically contacts Pbx1, and enhances DNA binding by Pbx1. *Mol Cell Biol 15, 5811-5819*

.

Kroon, E., Thorsteinsdottir, U., Mayotte, N., Nakamura, T., and Sauvageau, G. (2001).

NUP98-HOXA9 expression in hemopoietic stem cells induces chronic and acute myeloid leukemias in mice. *Embo J 20, 350-361*.

Kuno, Y., Abe, A., Emi, N., Iida, M., Yokozawa, T., Towatari, M., Tanimoto, M., and Saito, H. (2001).

Constitutive kinase activation of the TEL-Syk fusion gene in myelodysplastic syndrome with t(9;12)(q22;p12). *Blood 97, 1050-1055.*

Kuppers, R., and Dalla-Favera, R. (2001).

Mechanisms of chromosomal translocations in B cell lymphomas. *Oncogene 20, 5580-5594.*

Langdon, W. Y., Harris, A. W., Cory, S., and Adams, J. M. (1986).

The c-myc oncogene perturbs B lymphocyte development in E-mu-myc transgenic mice. *Cell 47, 11-18.*

Lawrence, H. J., Sauvageau, G., Ahmadi, N., Lopez, A. R., LeBeau, M. M., Link, M., Humphries, K., and Largman, C. (1995).

Stage- and lineage-specific expression of the HOXA10 homeobox gene in normal and leukemic hematopoietic cells. *Exp Hematol 23, 1160-1166***.**

Lawrence, H. J., Sauvageau, G., Humphries, R. K., and Largman, C. (1996).

The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells 14, 281-291*.

Liu, P., Tarle, S. A., Hajra, A., Claxton, D. F., Marlton, P., Freedman, M., Siciliano, M. J., and Collins, F. S. (1993).
Fusion between transcription factor CBF beta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. *Science 261, 1041-1044.*

Look, A. T. (1997).
Oncogenic transcription factors in the human acute leukemias. *Science 278, 1059-1064.*

Lorentz, O., Duluc, I., Arcangelis, A. D., Simon-Assmann, P., Kedinger, M., and Freund, J. N. (1997).
Key role of the Cdx2 homeobox gene in extracellular matrix-mediated intestinal cell differentiation. *J Cell Biol 139, 1553-1565.*

Mallo, G. V., Soubeyran, P., Lissitzky, J. C., Andre, F., Farnarier, C., Marvaldi, J., Dagorn, J. C., and Iovanna, J. L. (1998).
Expression of the Cdx1 and Cdx2 homeotic genes leads to reduced malignancy in colon cancer-derived cells. *J Biol Chem 273, 14030-14036.*

Manfioletti, G., Gattei, V., Buratti, E., Rustighi, A., De Iuliis, A., Aldinucci, D., Goodwin, G. H., and Pinto, A. (1995).
Differential expression of a novel proline-rich homeobox gene (Prh) in human hematolymphopoietic cells. *Blood 85, 1237-1245.*

McGinnis, W., and Krumlauf, R. (1992).
Homeobox genes and axial patterning. *Cell 68, 283-302.*

Monica, K., Galili, N., Nourse, J., Saltman, D., and Cleary, M. L. (1991). PBX2 and PBX3 new homeobox genes with extensive homology to the human proto-oncogene PBX1. *Mol Cell Biol 11, 6149-6157.*

Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E., and Weissman, I. L. (1997).
Identification of a lineage of multipotent hematopoietic progenitors. D*evelopment 124, 1929-1939.*

Morrison, S. J., and Weissman, I. L. (1994).
The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity 1, 661-673.*

Mulloy, J. C., Cammenga, J., MacKenzie, K. L., Berguido, F. J., Moore, M. A., and Nimer, S. D. (2002).
The AML1-ETO fusion protein promotes the expansion of human hematopoietic stem cells. *Blood 99, 15-23*.

Nakamura, T., Largaespada, D. A., Lee, M. P., Johnson, L. A., Ohyashiki, K., Toyama, K., Chen, S. J., Willman, C. L., Chen, I. M., Feinberg, A. P., (1996a).
Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. *Nat Genet 12, 154-158*
.

Nakamura, T., Largaespada, D. A., Shaughnessy, J. D., Jr., Jenkins, N. A., and Copeland, N. G. (1996b).
Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. *Nat Genet 12, 149-153.*

Nucifora, G. (1997).
The EVI1 gene in myeloid leukemia. *Leukemia 11, 2022-2031.*

Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G., and Downing, J. R. (1996).
AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell 84, 321-330.*

Owens, B. M., and Hawley, R. G. (2002).
HOX and non-HOX homeobox genes in leukemic hematopoiesis. *Stem Cells 20, 364-379.*

Papadopoulos, P., Ridge, S. A., Boucher, C. A., Stocking, C., and Wiedemann, L. M. (1995).
The novel activation of ABL by fusion to an ets-related gene, TEL. *Cancer Res 55, 34-38.*

Passegue, E., Jamieson, C. H., Ailles, L. E., and Weissman, I. L. (2003). Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc Natl Acad Sci U S A 100 Suppl 1, 11842-11849.*

Peeters, P., Raynaud, S. D., Cools, J., Wlodarska, I., Grosgeorge, J., Philip, P., Monpoux, F., Van Rompaey, L., Baens, M., Van den Berghe, H., and Marynen, P. (1997a).
Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. *Blood 90, 2535-2540.*

Peeters, P., Wlodarska, I., Baens, M., Criel, A., Selleslag, D., Hagemeijer, A., Van den Berghe, H., and Marynen, P. (1997b).
Fusion of ETV6 to MDS1/EVI1 as a result of t(3;12)(q26;p13) in myeloproliferative disorders. *Cancer Res 57, 564-569.*

Perkins, A., Kongsuwan, K., Visvader, J., Adams, J. M., and Cory, S. (1990).
Homeobox gene expression plus autocrine growth factor production elicits myeloid leukemia. *Proc Natl Acad Sci U S A 87, 8398-8402.*

Pineault, N., Buske, C., Feuring-Buske, M., Abramovich, C., Rosten, P., Hogge, D. E., Aplan, P. D., and Humphries, R. K. (2003).

Induction of acute myeloid leukemia in mice by the human leukemia- specific fusion gene NUP98-HOXD13 in concert with Meis1. *Blood 101, 4529-4538*.

Pineault, N., Helgason, C. D., Lawrence, H. J., and Humphries, R. K. (2002).
Differential expression of Hox, Meis1, and Pbx1 genes in primitive cells throughout murine hematopoietic ontogeny. *Exp Hematol 30, 49-57.*

Rabbitts, T. H. (1994).
Chromosomal translocations in human cancer. *Nature 372, 143-149.*

Rawat, V. P., Cusan, M., Deshpande, A., Hiddemann, W., Quintanilla-Martinez, L., Humphries, R. K., Bohlander, S. K., Feuring-Buske, M., and Buske, C. (2004).
Ectopic expression of the homeobox gene Cdx2 is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia. *Proc Natl Acad Sci U S A 101, 817-822.*

Reddy, V. A., Iwama, A., Iotzova, G., Schulz, M., Elsasser, A., Vangala, R. K., Tenen, D. G., Hiddemann, W., and Behre, G. (2002).
Granulocyte inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. *Blood 100, 483-490*.

Rego, E. M., and Pandolfi, P. P. (2002).
Reciprocal products of chromosomal translocations in human cancer pathogenesis: key players or innocent bystanders? *Trends Mol Med 8, 396-405*.

Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R., and Weissman, I. L. (2003).
A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature 423, 409-414.*

Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001).
Stem cells, cancer, and cancer stem cells. *Nature 414, 105-111.*

Rings, E. H., Boudreau, F., Taylor, J. K., Moffett, J., Suh, E. R., and Traber, P. G. (2001).
Phosphorylation of the serine 60 residue within the Cdx2 activation domain mediates its transactivation capacity. *Gastroenterology 121, 1437-1450.*

Romana, S. P., Mauchauffe, M., Le Coniat, M., Chumakov, I., Le Paslier, D., Berger, R., and Bernard, O. A. (1995).
The t(12;21) of acute lymphoblastic leukemia results in a tel-AML1 gene fusion. *Blood 85, 3662-3670.*

Rowley, J. D. (1999).
The role of chromosome translocations in leukemogenesis. *Semin Hematol 36, 59-72.*

Satoh, K., Mutoh, H., Eda, A., Yanaka, I., Osawa, H., Honda, S., Kawata, H., Kihira, K., and Sugano, K. (2002).
Aberrant expression of CDX2 in the gastric mucosa with and without intestinal metaplasia: effect of eradication of Helicobacter pylori. *Helicobacter 7, 192-198.*

Sauvageau, G., Lansdorp, P. M., Eaves, C. J., Hogge, D. E., Dragowska, W. H., Reid, D. S., Largman, C., Lawrence, H. J., and Humphries, R. K. (1994).
Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. *Proc Natl Acad Sci U S A 91, 12223-12227.*

Sauvageau, G., Thorsteinsdottir, U., Hough, M. R., Hugo, P., Lawrence, H. J., Largman, C., and Humphries, R. K. (1997).
Overexpression of HOXB3 in hematopoietic cells causes defective lymphoid development and progressive myeloproliferation. *Immunity 6, 13-22.*

Schnabel, C. A., Jacobs, Y., and Cleary, M. L. (2000).
HoxA9-mediated immortalization of myeloid progenitors requires functional interactions with TALE cofactors Pbx and Meis. *Oncogene 19, 608-616.*

Schwaller, J., Frantsve, J., Aster, J., Williams, I. R., Tomasson, M. H., Ross, T. S., Peeters, P., Van Rompaey, L., Van Etten, R. A., Ilaria, R., Jr., (1998).
Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes. *Embo J 17, 5321-5333*.

Shanmugam, K., Green, N. C., Rambaldi, I., Saragovi, H. U., and Featherstone, M. S. (1999).
PBX and MEIS as non-DNA-binding partners in trimeric complexes with HOX proteins. *Mol Cell Biol 19, 7577-7588.*

Shikano, T., Kobayashi, R., and Ishikawa, Y. (1999).
Leukoencephalopathy in childhood acute lymphoblastic leukemia with t(1;19). *Leuk Lymphoma 33, 135-140.*

Showe, L. C., Ballantine, M., Nishikura, K., Erikson, J., Kaji, H., and Croce, C. M. (1985).
Cloning and sequencing of a c-myc oncogene in a Burkitt's lymphoma cell line that is translocated to a germ line alpha switch region. *Mol Cell Biol 5, 501-509.*

Shurtleff, S. A., Buijs, A., Behm, F. G., Rubnitz, J. E., Raimondi, S. C., Hancock, M. L., Chan, G. C., Pui, C. H., Grosveld, G., and Downing, J. R. (1995).
TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. *Leukemia 9, 1985-1989*.

Silberg, D. G., Sullivan, J., Kang, E., Swain, G. P., Moffett, J., Sund, N. J., Sackett, S. D., and Kaestner, K. H. (2002).
Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. *Gastroenterology 122, 689-696.*

Silberg, D. G., Swain, G. P., Suh, E. R., and Traber, P. G. (2000).
Cdx1 and cdx2 expression during intestinal development. *Gastroenterology 119, 961-971.*

Siminovitch, L., Till, J. E., and McCulloch, E. A. (1964).
Decline in Colony-Forming Ability of Marrow Cells Subjected to Serial Transplantation into Irradiated Mice. *J Cell Physiol 64, 23-31*.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., and Dirks, P. B. (2003)
Identification of a cancer stem cell in human brain tumors. *Cancer Res 63, 5821-5828.*

Speck, N. A., Stacy, T., Wang, Q., North, T., Gu, T. L., Miller, J., Binder, M., and Marin-Padilla, M. (1999).
Core-binding factor: a central player in hematopoiesis and leukemia. *Cancer Res 59, 1789s-1793s.*

Stein, S., Fritsch, R., Lemaire, L., and Kessel, M. (1996).
Checklist: vertebrate homeobox genes. *Mech Dev 55, 91-108*.

Suh, E., Chen, L., Taylor, J., and Traber, P. G. (1994).
A homeodomain protein related to caudal regulates intestine-specific gene transcription. *Mol Cell Biol 14, 7340-7351*.

Taylor, J. K., Levy, T., Suh, E. R., and Traber, P. G. (1997).

Activation of enhancer elements by the homeobox gene Cdx2 is cell line specific. *Nucleic Acids Res 25, 2293-2300.*

Thorsteinsdottir, U., Kroon, E., Jerome, L., Blasi, F., and Sauvageau, G. (2001).
Defining roles for HOX and MEIS1 genes in induction of acute myeloid leukemia. *Mol Cell Biol 21, 224-234.*

Tupler, R., Perini, G., and Green, M. R. (2001).
Expressing the human genome. *Nature 409, 832-833.*

Van Bekkum, D. W., van den Engh, G. J., Wagemaker, G., Bol, S. J., and Visser, J. W. (1979).
Structural identity of the pluripotential hemopoietic stem cell. *Blood Cells 5, 143-159.*

Van den Akker, E., Forlani, S., Chawengsaksophak, K., de Graaff, W., Beck, F., Meyer, B. I., and Deschamps, J. (2002).
Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development 129, 2181-2193.*

Wang, L. C., Kuo, F., Fujiwara, Y., Gilliland, D. G., Golub, T. R., and Orkin, S. H. (1997).
Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. *Embo J 16, 4374-4383*.

Wang, Y., Hung, C., Koh, D., Cheong, D., and Hooi, S. C. (2001). Differential expression of Hox A5 in human colon cancer cell differentiation: a quantitative study using real-time RT-PCR. *Int J Oncol 18, 617-622***.**

Watrin, F., and Wolgemuth, D. J. (1993).

Conservation and divergence of patterns of expression and lineage-specific transcripts in orthologues and paralogues of the mouse Hox-1.4 gene. *Dev Biol 156, 136-145.*

Weissman, I. L. (2002).
The road ended up at stem cells. *Immunol Rev 185, 159-174*.

Worton, R. G., McCulloch, E. A., and Till, J. E. (1969).
Physical separation of hemopoietic stem cells differing in their capacity for self-renewal. *J Exp Med 130, 91-103*.

Ye, B. H., Cattoretti, G., Shen, Q., Zhang, J., Hawe, N., de Waard, R., Leung, C., Nouri-Shirazi, M., Orazi, A., Chaganti, R. S., (1997).
The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nat Genet 16, 161-170*.

Yeoh, E. J., Ross, M. E., Shurtleff, S. A., Williams, W. K., Patel, D., Mahfouz, R., Behm, F. G., Raimondi, S. C., Relling, M. V., Patel, A.,. (2002).
Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell 1, 133-143*.

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Rawat VP. , Cusan M, Deshpande A, Hiddemann W, Quintanilla-Martinez L, Humphries RK, Bohlander SK, Feuring-Buske M, Buske C**.**

2. The *AML1-ETO* Fusion Gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice.***J. Clin Invest. (2005);115:2159-2168***

Schessl C, **Rawat VP**, Cusan M, Deshpande A, Schnittger S, Kern WG, Kohl TM, Spiekermann K, Rosten PM, Humphries RK, Hiddemann W., Quintanilla-Martinez L., Bohlander SK., Feuring-Buske M.& Buske C..

3. Characterization of the antiapoptotic (p35) gene homologue of Spodoptera litura nucleopolyhedrosis virus (SlNPV) ***Mol Biol Rep. 2001;28(3):167-73***

Kumar M, Shanker S, **Rawat VP**, Das RH.

4. Acute myeloid leukemia is propagated by a leukemic stem cell with lymphoid characteristics in a mouse model of CALM/AF10-positive leukemia. ***Cancer Cell. 2006 Nov;10(5):363-74***.

Deshpande AJ, Cusan M, **Rawat VP**, Reuter H, Krause A, Pott C, Quintanilla-Martinez L, Kakadia P, Kuchenbauer F, Ahmed F, Delabesse E, Hahn M, Lichter P, Kneba M, Hiddemann W, Macintyre E, Mecucci C, Ludwig WD, Humphries RK, Bohlander SK, Feuring-Buske M, Buske C.

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Ectopic expression of the homeobox gene Cdx2 is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia

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Ectopic expression of the homeobox gene *Cdx2* is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia

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Creation of fusion genes by balanced chromosomal translocations is one of the hallmarks of acute myeloid leukemia (AML) and is considered one of the key leukemogenic events in this disease. In t(12;13)(p13;q12) AML, ectopic expression of the homeobox gene *CDX2* was detected in addition to expression of the *ETV6-CDX2* fusion gene, generated by the chromosomal translocation. Here we show in a murine model of t(12;13)(p13;q12) AML that myeloid leukemogenesis is induced by the ectopic expression of *CDX2* and not by the *ETV6-CDX2* chimeric gene. Mice transplanted with bone marrow cells retrovirally engineered to express *Cdx2* rapidly succumbed to fatal and transplantable AML. The transforming capacity of *Cdx2* depended on an intact homeodomain and the N-terminal transactivation domain. Transplantation of bone marrow cells expressing *ETV6-CDX2* failed to induce leukemia. Furthermore, coexpression of *ETV6-CDX2* and *Cdx2* in bone marrow cells did not accelerate the course of disease in transplanted mice compared to *Cdx2* alone. These data demonstrate that activation of a protooncogene by a balanced chromosomal translocation can be the pivotal leukemogenic event in AML, characterized by the expression of a leukemia-specific fusion gene. Furthermore, these findings link protooncogene activation to myeloid leukemogenesis, an oncogenic mechanism so far associated mainly with lymphoid leukemias and lymphomas.

The molecular dissection of balanced chromosomal translocations in patients with acute leukemia has greatly advanced our knowledge of the pathogenesis of this disease, demonstrating that chromosomal translocations often affect genes that regulate hematopoiesis. Chromosomal translocations involve mainly two mechanisms that lead to malignant transformation: deregulation of the expression of a protooncogene by juxtaposition of a potent enhancer or promoter elements or creation of a fusion gene (1–3). Although both mechanisms are found in lymphoid leukemia or lymphoma, formation of a fusion gene predominates in acute myeloid leukemia (AML). In fact, to date, there are no experimentally confirmed instances in which the transcriptional deregulation of a protooncogene is the key leukemogenic event in a fusion gene-positive AML.

The oncogenic potential of fusion genes has been well documented experimentally. However, emerging data, mostly from murine *in vivo* models, have demonstrated that many of these fusion genes are not able to induce leukemia on their own. This observation suggests an important role for other genetic alterations that cooperate with fusion genes in patients with AML (4–6). The intriguing differences in the oncogenic potential of fusion genes are well documented for the large family of chimeric genes involving the *ets* transcription factor *ETV6*, located at 12p13. *ETV6* is one of the genes most frequently involved in chromosomal translocations. Chromosomal translocations affecting the *ETV6* locus have been reported with >40 different partners (7). Fusion partners of *ETV6* can be phosphotyrosine kinases (PTK) or transcription factors and genes of unknown function, dividing *ETV6* fusion genes into two distinct groups. Fusions of *ETV6* with PTKs such as *PDGFRB*, *JAK2*, *ABL1*, *ABL2*, or *NTRK3* create highly leukemogenic proteins in murine experimental models (8–12). In the group of *ETV6*-transcription factor fusions, the N-terminal portion of *ETV6* is fused to the partner gene in most cases, retaining (e.g., *ETV6-AML1*) or losing the *pointed* domain (e.g., *ETV6-CDX2*, *ETV6-MDS1/EVI1*) (13–15). Although data about the leukemogenic potential of this group of fusion genes are still limited, extensive analyses of the most frequent *ETV6* chimeric transcription factor, *ETV6-AML1*, failed to show any major transforming activity in a transgenic or bone marrow (BM) transplantation mouse model (16, 17). Based on these data, expression of an *ETV6*-transcription factor fusion might not be sufficient to induce disease. Indeed, recent evidence corroborates that *ETV6* acts as a tumor suppressor gene and that, in almost all cases of *ETV6/AML1*-positive acute lymphoblastic leukemias, there is a deletion or loss of expression of the nonrearranged *ETV6* allele (18, 19). Furthermore, several chromosomal translocations involving the *ETV6* locus associated with myeloid malignancies such as t(4;12), t(5;12), or t(12;17) do not form any functional fusion gene at all, pointing to a key variant oncogenic mechanism in these cases (20, 21). In this regard, the t(12;13)(p13;q12) associated with the *ETV6-CDX2* fusion gene in human AML is of notable interest. The translocation breakpoint leaves the *CDX2* gene intact, and expression of both the fusion gene and full-length *CDX2*, normally restricted to intestinal epithelial cells, was observed in leukemic cells, thus raising the possibility that ectopic expression of *CDX2* is the key pathogenic event (14).

To clarify this particular issue and to gain insight into alternative mechanisms of transformation in patients with AML and *ETV6* rearrangements, we established a mouse model for t(12;13)(p13;q12) human AML. We demonstrate that ectopic expression of *Cdx2* is the key transforming event that induces fatal AML in transplanted mice. In contrast, expression of the ETV6-CDX2 fusion protein is unable to induce leukemia. Furthermore, we show that the transforming potential of *Cdx2* depends on the integrity of its DNA-binding domain and the N-terminal domain of Cdx2. Our data point to a previously uncharacterized mechanism of leukemogenesis in patients with AML, in which a balanced chromosomal translocation contributes to malignant transformation by activating the expression of a protooncogene, a mechanism so far associated mainly with lymphoid leukemias or lymphomas (3).

Materials and Methods

cDNA Constructs and Retroviral Vectors. cDNAs of *ETV6-CDX2* and *Cdx2* (93% overall and 98% identity in the homeodomain between

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Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; YFP, yellow fluorescent protein; CFU-S, colony-forming unit–spleen; PB, peripheral blood.

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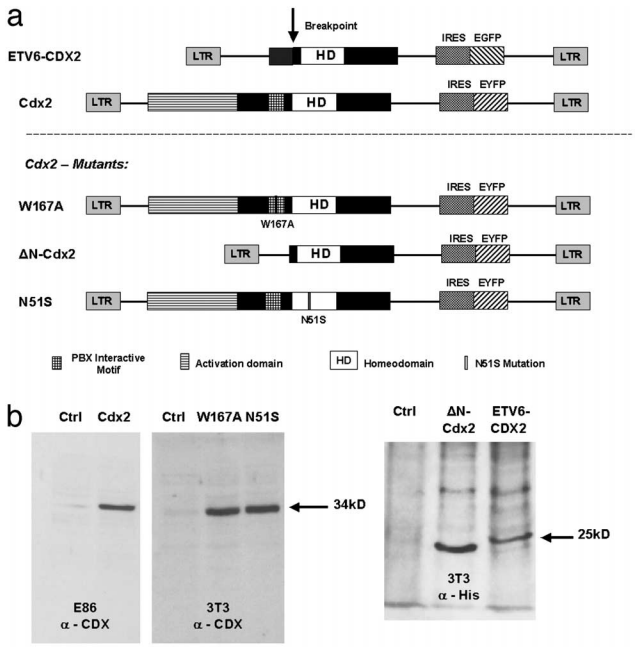

Fig. 1. (*a*) Retroviral vectors used to express *ETV6-CDX2*, *Cdx2*, and the different *Cdx2* mutants in murine BM. IRES, internal ribosomal entry site. (*b*) Western blot analysis of cellular extracts from NIH 3T3 or E86 cells transfected with the different constructs. The molecular mass is indicated.

the human and murine proteins) were kindly provided by D. G. Gilliland (Division of Hematology/Oncology, Harvard Medical School, Boston) and N. Cross (Department of Haematology, Hammersmith Hospital, London). A histidine-tagged version of *ETV6-CDX2* was constructed by ligating a PCR product of the fusion gene in frame to the 3′ end of the histidine epitope of the pCDNA6/V5-His A plasmid (Invitrogen), *Cdx2* mutants were created that were previously shown to inactivate a putative PBX1 interacting motif (W167A-Cdx2) (22) or to inactivate the DNA-binding homeodomain (N51S-Cdx2) (23) by using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). The *Cdx2* mutant lacking the first 179 N-terminal amino acids, which are deleted in the *ETV6-CDX2* fusion, was generated and histidine tagged by PCR following standard procedures (ΔN-Cdx2) (4). For retroviral gene transfer into primary BM cells, the different constructs were subcloned into the multiple cloning site of the modified murine stem cell virus (MSCV) 2.1 vector (4) upstream of the internal ribosomal entry site (IRES) and the enhanced GFP or yellow fluorescent protein (YFP) gene. As a control, the MSCV vector carrying only the IRES-enhanced GFP cassette was used.

Production of high-titer helper-free retrovirus was carried out following standard procedures by using the ecotropic packaging cell line GP^+E86 (4). The number of provirus integrants was determined by *Eco*RI digestion and full length integration by *Nhe*I digestion, followed by Southern blot analysis using standard techniques (24). Protein expression of the *ETV6-CDX2*, *Cdx2*, and *Cdx2* mutant plasmids was documented by Western blotting using standard procedures. Membranes were probed with an antihistidine monoclonal antibody (Sigma) for ETV6-CDX2 and the ΔN-Cdx2 mutant or with an anti-CDX2 monoclonal antibody (kindly provided by DCS Innovative, Hamburg, Germany) for expression of the Cdx2, W167A-Cdx2, and N51S-Cdx2 mutants (25) (Fig. 1).

***In Vitro* Assays.** Cell proliferation was assessed in DMEM supplemented with 15% FBS/10 ng/ml mIL-6/6 ng/ml mIL-3/100 ng/ml murine stem cell factor (standard medium) (Tebu-bio, Offenbach, Germany). Differentiation of clonogenic progenitors was analyzed by plating cells in methylcellulose supplemented with cytokines (Methocult M3434, StemCell Technologies, Vancouver). IL-3-dependent cell populations expressing Cdx2 or coexpressing ETV6-CDX2 and Cdx2 were established *in vitro* directly after sorting in DMEM/15% FBS with IL-3 alone (6 ng/ml). The differentiation capacity of cultured cells was tested in DMEM/15% FBS supplemented with granulocyte colony-stimulating factor 100 ng/ml or macrophage colony-stimulating factor 10 ng/ml (R & D Systems) and *all-trans* retinoic acid at 1 μM final concentration. After 5 days, the morphology was determined by Wright–Giemsa-stained cytospin preparations (4, 25).

Mice and Retroviral Infection of Primary BMC. Parental strain mice were bred and maintained at the GSF animal facility. Donors of primary BM cells were >12-wk-old (C57BL/6Ly-Pep3b × C3H/HeJ) F_1 (PepC3) mice, and recipients were >8- to 12-wk-old (C57BL/6J × C3H/HeJ) F_1(B6C3) mice. Primary mouse BM cells were transduced as described (4). For transduction, cells were cocultured with irradiated (40 Gy) ETV6-CDX2/GFP or Cdx2/YFP GP^+E86 producers or with a mixture of 40–50% Cdx2/YFP and 50–60% ETV6-CDX2/GFP producers in cotransduction experiments.

Colony-Forming Unit–Spleen (CFU-S) Assay. Primary BM cells from F_1(PepC3) donor mice treated 4 days previously with 5-fluorouracil were transfected with the different viruses, and retrovirally transduced cells were highly purified based on expression of GFP or YFP by using a FACSVantage (Becton Dickinson). Transduced cells were cultured 7 days in standard medium. The day 0 equivalent of $2.5–3 \times 10^4$ cells was injected into lethally irradiated F_1(B6C3) recipient mice. The recovery of CFU-S cells was quantified by determining the number of macroscopic colonies on the spleen at day 12 postinjection after fixation in Telleyesnickzky's solution.

BM Transplantation and Assessment of Mice. Recipient F_1(B6C3) mice (8–10 wk old) were irradiated with 850 cGy from a ^{137}Cs γ-radiation source. FACS-purified transduced BM cells, or a defined ratio of transduced and untransduced cells was injected into the tail vein of irradiated recipient mice. Peripheral blood (PB) or BM cell progeny of transduced cells were tracked by using the GFP or YFP fluorescence (26). The lineage distribution was determined by FACS analysis as described (4): phycoerythrin-labeled Gr-1, ScaI, Ter-119, CD4, and allophycocyanin-labeled Mac1, cKit, B220, or CD8 antibodies were used for analysis (all PharMingen). For histological analyses, sections of selected organs were prepared and hematoxylin/eosin-stained by using standard protocols.

RT-PCR. Expression of *Hoxa9* and *Meis1* was assayed by RT-PCR in $Sca\text{-}1^-Lin^+$ cells sorted from a mouse repopulated with Cdx2 expressing BM cells or a control animal. Total RNA was isolated by using Trizol reagent (GIBCO/BRL) and treated with DNase I (amp grade) to remove contaminating genomic DNA. First-strand cDNA was synthesized from 1 μg of total RNA by using the thermoScript RT-PCR system (all reagents from Invitrogen). Equal amounts of cDNA originating from 50 ng of starting RNA were loaded to assess transcription levels. Intron-spanning primer pairs were selected to avoid amplification of contaminating genomic DNA. The annealing temperatures were 58°C and 60°C for *Meis1* and *Hoxa9*, respectively. The number of PCR cycles for each gene was chosen to stop the reaction in the linear phase of amplification (25 cycles for mβ-2 microglobulin, 35 cycles for *Meis1* and *Hoxa9*).

Statistical Analysis. Data were evaluated by using the *t* test for dependent or independent samples (Microsoft EXCEL). Differences with P values < 0.05 were considered statistically significant.

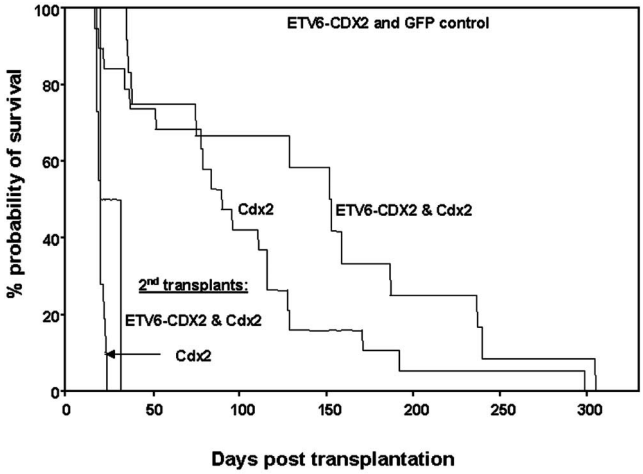

Fig. 2. Survival curve of mice transplanted with BM cells expressing *Cdx2* ($n = 18$), *ETV6-CDX2* ($n = 9$), or coexpressing *Cdx2* and the fusion gene ($n = 13$). The control group was injected with BM infected with the GFP empty retrovirus ($n = 7$). The survival time of secondary recipient mice, transplanted with BM from diseased primary *Cdx2* or *ETV6-CDX2* and *Cdx2* recipients, is indicated.

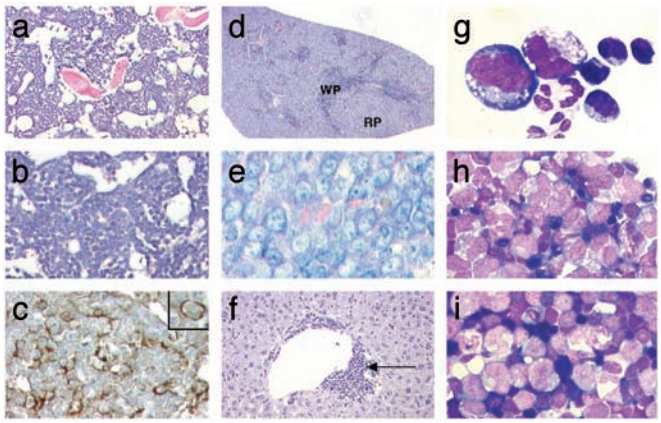

Fig. 3. Histological analysis of diseased *Cdx2* mice. (*a*) BM [hematoxylin/eosin (H&E)]. Immunohistochemistry of the BM (×200) for *N*-acetyl-chloroacetate esterase (×400) (*b*) and CD34 expression (×640) (*c*). Histology of the spleen H&E (×25) (*d*) and Giemsa staining (×640) (*e*) and liver with perivascular infiltration (×200) (*f*). Cytospin preparations from PB (*g*), BM (*h*), and spleen (*i*) (all ×1,000).

Results

Ectopic Expression of *Cdx2* Causes AML in Transplanted Mice. To analyze whether expression of the t(12;13)-associated *ETV6-CDX2* fusion gene and/or the ectopic expression of the homeobox gene *Cdx2* is able to transform early murine hematopoietic progenitors *in vivo*, we generated MSCV-based retroviral constructs and documented full-length protein expression by Western blotting (Fig. 1). Murine hematopoietic progenitors constitutively expressing *ETV6-CDX2* or *Cdx2* were highly purified by FACS based on GFP^+ or YFP^+ expression, respectively, and injected into lethally irradiated recipient mice directly after sorting (3–3.5 × 10^5 and 2–3.6 × 10^5 cells per mouse for *Cdx2* and *ETV6-CDX2*, respectively).

Mice transplanted with BM cells expressing *Cdx2* became moribund after a median of 90 days posttransplantation ($n = 18$) (Fig. 2). Diseased mice were characterized by cachexia, shortness of breath, and lethargy when they were killed for further analysis. In striking contrast, mice transplanted with *ETV6-CDX2*-expressing cells did not succumb to terminal disease ($n = 9$) (Fig. 2). Diseased *Cdx2* mice were characterized by elevated peripheral white blood count (WBC) (3.8-fold) with up to 48 × 10^6 circulating WBC per milliliter. Furthermore, moribund mice were anemic, with a 5-fold decrease in peripheral erythrocyte count ($P < 0.001$) (Table 1). All *Cdx2* mice analyzed ($n = 7$) suffered from splenomegaly, with an average spleen weight of 0.6 g (range 0.4–0.9; $P < 0.01$ compared to control animals) (Table 1). More detailed hematological analyses demonstrated that animals suffered from AML with a high percentage of blasts in the BM (42% ± 6), PB (14% ± 3), and spleen (35% ± 5) ($n = 8$; $P < 0.01$ compared to the control animal) (Table 1). Furthermore, leukemic mice showed multiple organ infiltration with blast cells. Thirty percent of the blasts expressed CD34 but were negative for *N*-acetyl-chloroacetate esterase, periodic acid/Schiff reagent, and terminal deoxynucleotidyltransferase, as shown by immunohistochemistry, consistent with an undifferentiated myeloblastic phenotype of the disease (Fig. 3). Immunophenotypic

Table 1. Hematological parameters of experimental mice

Mouse no.	Retroviral construct	Day of death	RBC per ml ×10^9	WBC per ml ×10^6	Spleen weight, mg	BM % blasts	Spleen % blasts	PB % blasts	Lymphoid/myeloid ratio in PB
1	GFP	90	6	4.5	150	0	0	0	5:1
2	GFP	90	4.8	3.2	200	0	0	0	2:1
3	GFP	90	5.0	3.6	200	0	0	0	2:1
1*	Cdx2	128	1.0	3.2	400	28	21	8	0.5:1
2*	Cdx2	79	2.0	37	650	40	35	12	0.4:1
3*	Cdx2	52	0.7	9	600	38	30	15	0.2:1
4*	Cdx2	116	0.4	48	nd	ND	60	14	0.4:1
5*	Cdx2	37	0.6	5	400	25	22	5	0.3:1
6*	Cdx2	171	0.8	24	900	71	48	18	0.3:1
7*	Cdx2	192	1.1	10	800	60	41	30	0.5:1
8*	Cdx2	84	0.4	28	400	32	24	8	0.8:1
1*	++	168	1.0	3.2	400	25	18	3	0.6:1
2*	++	230	1.1	8	500	45	30	10	0.1:1
3*	++	151	0.2	8	600	58	37	16	0.4:1
4*	++	237	1.5	24	300	25	18	5	0.6:1
5*	++	187	0.5	25	900	50	43	8	0.3:1
1	ETV6-CDX2	375	6.5	2.4	160	10	8	0	0.3:1
2	ETV6-CDX2	375	5	3.2	200	25	15	0	0.4:1
3	ETV6-CDX2	375	5.2	6	180	15	9	0	2:1

*, diseased; ++, ETV6-CDX2 and Cdx2; RBC, red blood cell count; WBC, white blood cell count; ND, not determined.

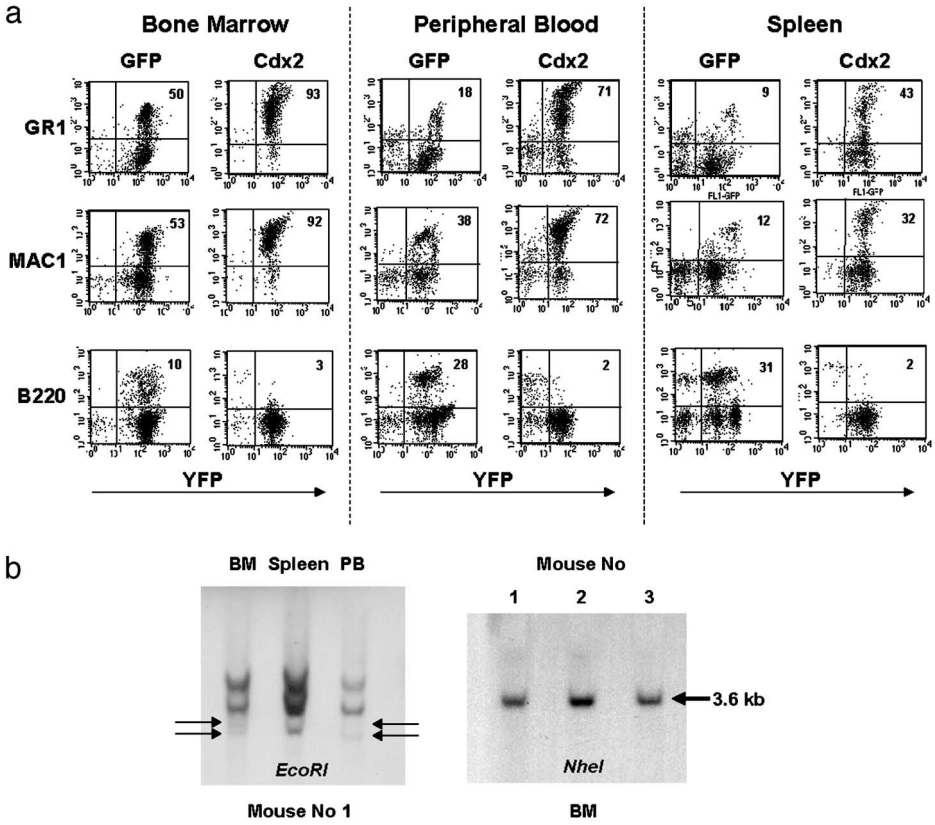

Fig. 4. (*a*) Flow cytometry from a representative leukemic *Cdx2* mouse from PB, BM, and spleen in comparison to a GFP control animal. Cells were stained for the myeloid markers Gr1 and Mac1 and the lymphoid marker B220. The proportion of positive cells within the GFP^+ compartment is indicated. (*b*) Southern blot analyses of genomic DNA from BM, PB, and spleens of representative leukemic *Cdx2* mice. Genomic DNA was digested with *Eco*RI, which cuts once in the provirus, to determine the number of provirus integrants. Signals with different intensity, indicating the presence of different leukemic clones, are indicated. Full-length provirus integration was documented by digestion with *Nhe*I, which cuts only in the LTRs of the provirus.

characterization of PB, BM, and spleen in diseased mice confirmed the predominance of myeloid $Mac1^+$ and $Gr\text{-}1^+$ cells (84% ± 10 and 73% ± 15 in the PB, 65% ± 14 and 53% ± 14 in the spleen, respectively; $n = 4$) compared to the GFP control mice ($Mac1^+$ and $Gr\text{-}1^+$ cells 47% ± 5 and 25% ± 3 in the PB, 14% ± 9 and 10% ± 1 in the spleen, respectively; $n = 4$). Furthermore, diseased mice were characterized by a greatly reduced normal $B220^+$ lymphoid population in the spleen and PB compared to controls (1.8% ± 1 vs. 35% ± 8 and 1.3% ± 0.5 vs. 46% ± 21 in the PB and in the spleen, respectively; $n = 4$) (Fig. 4*a*). Mice transplanted with *Cdx2*-expressing BM cells were characterized by a 19-fold increased frequency of clonogenic cells in the PB and a >100-fold increase in the spleen compared to the control as quantified by *ex vivo* CFC assays (248 vs. 13 clonogenic cells per 1×10^6 cells/ml in the PB and 1,400 clonogenic cells vs. 13 per 1×10^6 cells/ml in the spleen, respectively) ($n = 3$). Twenty-eight percent (±3) of these clonogenic progenitors were not able to terminally differentiate and formed blast colonies in methylcellulose with high serial replating capacity (data not shown).

The *Cdx2*-induced AML was transplantable and all lethally irradiated mice ($n = 11$) injected with BM cells of diseased *Cdx2* animals died within 24 days posttransplantation (Fig. 2). Analysis of the clonality of the disease by Southern blot analysis demonstrated different intensities and patterns of proviral signals in the different hematopoietic organs consistent with an oligoclonal nature of the disease (Fig. 4*b*).

To analyze whether the *ETV6-CDX2* fusion caused subtle perturbations in hematopoietic development, healthy animals ($n = 3$) were killed 44 wk after transplantation with *ETV6-CDX2*-expressing BM cells. Interestingly, two of three animals showed an expansion of the mature neutrophil compartment in the PB with an inversion of the lymphoid/myeloid ratio (Table 1) and 87% and 68% $Mac1^+/Gr1^+$ cells in the GFP-positive compartment. Furthermore, spleens from all mice were infiltrated with terminally differentiated myeloid cells (86% ± 0.9 $Gr1^+/Mac1^+$ cells). However, none of the animals suffered from anemia, splenomegaly, or the emergence of a blast population in the PB (Table 1). Thus, *ETV6-CDX2* was able to induce a myeloproliferation without causing disease but failed to induce leukemic transformation.

In addition, 13 mice were transplanted with a mixture of *ETV6-CDX2*, *Cdx2*, and *Cdx2* and *ETV6-CDX2* coexpressing cells, containing between $1.9–4.5 \times 10^4$ *Cdx2* and *ETV6-CDX2* cells and <4,000 *Cdx2* cells per mouse. The addition of *Cdx2* and *ETV6-CDX2* coexpressing cells did not accelerate the course or change the phenotype of the disease compared to only *Cdx2*-expressing cells. All animals succumbed to AML, and the leukemic population consisted of *Cdx2*- and *ETV6-CDX2*-coexpressing or *Cdx2*-expressing cells in all mice analyzed ($n = 4$) (Fig. 2). These data indicate that aberrant expression of the wild-type *Cdx2* gene is crucial for malignant transformation in this model.

The Transforming Potential of *Cdx2* Depends on the N-Terminal Transactivation Domain and the Intact Homeodomain. In an effort to characterize the contribution of different motifs of *Cdx2* to the transforming capacity of the gene, three different mutants were designed: a mutant inactivating the homeodomain (N51S-Cdx2), a Cdx2 mutant with an inactivating mutation in the putative PBX1-interacting motif (W167A-Cdx2), and a mutant lacking the N-terminal portion of Cdx2, which is not present in the *ETV6-CDX2* fusion (ΔN-Cdx2). Protein expression of the mutants was confirmed by Western blot analysis (Fig. 1*b*). Expression of wild-type Cdx2 and W167A-Cdx2 in primary bone marrow cells rapidly induced the outgrowth of IL-3-dependent cell populations in liquid cultures. The cells showed blast morphology, were $Gr^+/Mac1^+$-positive, and had lost their differentiation capacity when incubated with macrophage colony-stimulating factor, granulocyte colony-stimulating factor, or *all-trans* retinoic acid (data not shown). Furthermore, mice transplanted with 1×10^6 of these cells developed leukemia 8 wk posttransplant in contrast to mice injected with nontransduced or GFP-expressing control cells. Cells expressing

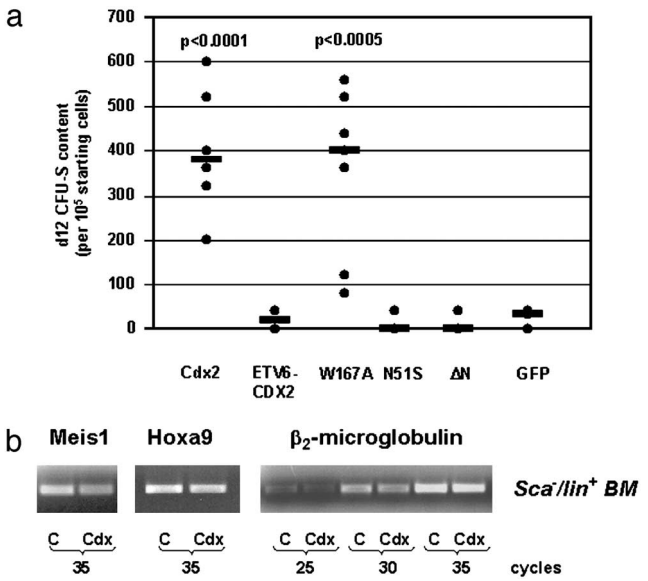

Fig. 5. (*a*) Total number of d12 CFU-S colonies derived per culture initiated with 1×10^5 cells transduced with the different viruses after 1 wk in liquid culture. The median is indicated. (*b*) Expression of *Meis1* and *Hoxa9* analyzed by RT-PCR in Sca^-lin^+ BM cells isolated from a *Cdx2* mouse or a control mouse. The number of PCR cycles for each gene was chosen to stop the reaction in the linear phase of the amplification (25 cycles for mβ-2 microglobulin, 35 cycles for *Meis1* and *Hoxa9*). C, control; Cdx, Cdx2.

ETV6-CDX2, the ΔN-Cdx2, or the N51S mutant as well as the control cells were not able to form blast cell populations *in vitro*. When colony formation was tested, Cdx2-positive cells generated a higher number of primary CFC in methylcellulose compared to GFP (76 ± 22 vs. 41 ± 20 per 500 initially plated cells, respectively; $n = 5$; $P < 0.02$). Furthermore, Cdx2-positive colonies contained ≈10 times more cells per colony than the controls (33×10^3 vs. 3.9×10^3 per colony, respectively; $n = 5$; $P < 0.004$). The expression of the other constructs did not change the size or number of colonies compared to the control. To investigate the effect of the different mutants on primitive hematopoietic cells, cells infected with the different viruses were injected into lethally irradiated mice after 7 days of *in vitro* culture, and spleen colony formation was quantified 12 days after injection in killed mice (CFU-S assay). Cdx2 expression as well as expression of the W167A-Cdx2 mutant induced a significant >10-fold increase in the yield of day 12 CFU-S compared to the GFP control ($n = 8$; $P < 0.0001$). In contrast, deletion of the N-terminal portion of Cdx2 ($n = 5$) or inactivation of the homeodomain ($n = 5$) resulted in complete loss of the Cdx2 activity in these assays. ETV6-CDX2 ($n = 6$) did not show any increase in CFU-S compared to the GFP control (Fig. 5*a*).

The Expression of *Hoxa9* and *Meis1* Is Not Increased by Ectopic Expression of *Cdx2*. Given the role of *Cdx2* as an upstream regulator of *Hox* gene expression, we asked whether *Cdx2* would perturb expression of leukemogenic homeobox genes such as *Hoxa9* or *Meis1*. First, expression of *Hoxa9* and *Meis1* was determined by RT–PCR in the 32D cell line transduced with the Cdx2, the ETV6-CDX2, or the GFP virus. Compared to the control, *Cdx2* did not increase expression of Hoxa9 or Meis1 (data not shown). In addition, Sca^-lin^+-differentiated cells were recovered and highly purified from a mouse transplanted with *Cdx2*-expressing BM cells and a control animal, a cell population with normally no detectable expression of *Hoxa9* and *Meis1* (27): specific amplification products were not detectable by RT-PCR after 25 cycles in both experimental arms. Amplification products could be detected after 35 cycle but without considerable differences in the intensity between *Cdx2*-transduced and control cells (Fig. 5*b*). Thus, ectopic expression of *Cdx2* was not associated with up-regulation of *Meis1* or *Hoxa9* in this model system.

Discussion

The formation of fusion genes with oncogenic properties by balanced chromosomal rearrangements is considered one of the crucial steps for leukemic transformation in patients with AML. By using the murine BM transplantation model, we now provide direct evidence that the ectopic expression of the protooncogene *Cdx2* and not the expression of the fusion gene *ETV6-CDX2* is the key transforming event in t(12;13)(p13;q12)-positive AML. Activation of protooncogenes by balanced chromosomal translocations is a well-known oncogenic mechanism in lymphoid leukemias or lymphomas but has, to our knowledge, not been functionally demonstrated for AML and translocations involving *ETV6* (3). In addition, these data present evidence that the homeobox gene and *Hox* gene upstream regulator *Cdx2*, which so far has been linked to intestinal metaplasia and colon cancer (28), is highly leukemogenic when aberrantly expressed in hematopoietic progenitor cells.

Cdx2 belongs to the large group of homeobox genes, which were originally described as master regulators of embryonic body development. The *Cdx* genes and their homologues *caudal* in *Drosophila* and *Xcad* in *Xenopus* belong to the ParaHox cluster, which is considered an ancient paralog of the *Hox* gene cluster (29). Although the *Cdx* genes show similarities to the 5′-located Abdominal-B like genes of the *Hox* gene cluster, they possess a Pbx recognition motif, a characteristic of 3′-located *Hox* genes (30). *Cdx* genes play a key role in the homeobox regulatory network, acting as upstream regulators of several *Hox* genes (30, 31). Thus, perturbation of *Cdx2* might be linked to critical alterations in downstream *Hox* genes that are central regulators of normal early hematopoietic development in the adult with a distinct expression profile in human and murine early progenitor cells (27, 32, 33). Gene expression profiling of acute leukemias using DNA microarray technology linked aberrant expression of *Hox* genes such as *HOXA9*, *HOXA10*, and of the nonclustered homeobox gene *MEIS1* to leukemogenesis (34–37). Retrovirally enforced expression of these genes induced severe perturbations of normal hematopoietic development in human and murine experimental models (24, 38). Altered expression of several *Hox* genes might be one of the reasons for the strong oncogenic potential of *Cdx2* (30, 39–42). However, RT–PCR analyses in the 32D cell line model and in Sca^-/lin^+ BM population of a *Cdx2* repopulated mouse did not indicate gross up-regulation of *Meis1* and *Hoxa9* by *Cdx2*. However, this does not exclude that perturbation of other *Hox* genes might play a role in the transformation process initiated by ectopic *Cdx2* expression.

Of note, perturbed expression of *Hox* genes such as *HOXA9* or *HOXA10* in hematopoietic progenitor cells is not able to induce frank AML in transplanted mice after a short latency time but requires collaboration with the *Hox* co-factor *MEIS1*. In striking contrast, constitutive expression of *Cdx2* rapidly caused leukemia in recipient mice. The underlying cause for the difference in the leukemogenic activity between *Cdx2* and *HOXA9* or *HOXA10* is not known. But, in contrast to *HOXA9* and *HOXA10*, which are normally expressed at high levels in progenitor cells, *CDX2* is not expressed in hematopoietic cells (14). Thus, ectopic expression of *CDX2* in leukemia patients might result in the activation of *de novo* downstream pathways, which are normally silent in early blood development.

Despite the differences in the oncogenic potential, many of the *in vitro* and *in vivo* hematopoietic effects induced by *Cdx2* are highly reminiscent of the effects of retrovirally overexpressed hematopoietic *HOX* genes as well as leukemia-specific fusion genes such as *NUP98-HOXD13* with regard to the impact on short-term repopulating CFU-S or clonogenic progenitors (4, 38, 43). The striking similarities of the phenotypes induced by the over-expression of

homeobox genes of the *Hox* cluster and of *Cdx2* as a member of the ParaHox complex (29) point to a high level of functional redundancy among homeobox proteins in hematopoiesis.

The hematopoietic activity of *Cdx2* strictly depended on its intact homeodomain, implicating that DNA binding of *Cdx2* is essential for its transforming activity. Furthermore, deletion of the Cdx2 N-terminal portion resulted in a complete loss of activity in our assays. Of note, it was demonstrated that the N-terminal part of Cdx2 is necessary for transcriptional activation of *Hox* genes, supporting the concept that activation of downstream *Hox* genes is a potential key mechanism of *Cdx2*-induced transformation (44). Furthermore, it was demonstrated that the transcriptional activity of CDX proteins depends on the interaction of the p38 mitogen-activated protein kinase and the N-terminal transactivation domain of Cdx2 (45). As a consequence, N-terminal deletion would diminish the transactivation capacity of CDX2. Importantly, the *ETV6-CDX2* fusion gene lacks the N-terminal portion of *CDX2*, presumably hampering its capability to transactivate target genes. This would explain the obvious discrepancy in the oncogenic potential between *Cdx2* and the *ETV6-CDX2* fusion gene; this is supported by our data, which demonstrate a complete loss of activity when this N-terminal portion of *Cdx2*, which is not present in the *ETV6-CDX2* fusion gene, is deleted in the ΔN-Cdx2 mutant. Notably, mice transplanted with BM cells expressing the chimeric gene developed myeloproliferation after a long latency time but without any clinical symptoms. These data indicate that, despite the loss of the N-terminal portion, the fusion gene is able to perturb hematopoietic development, although to a significantly lesser extent than full-length *Cdx2*. However, it cannot be excluded from our experiments that the first 54 amino acids of ETV6, which are fused to CDX2, are responsible for or at least contribute to the observed disturbances of hematopoiesis. Taken together, our data propose a model in which the chromosomal translocation t(12;13)(p13;q12) causes AML by inducing the ectopic expression of CDX2. The mechanism of transcriptional induction is not precisely known, but it was demonstrated that the chromosome 13 breakpoint lies upstream of the *CDX2* gene. Therefore, one possible explanation for the ectopic expression of CDX2 could be that the translocated protooncogene might now be under the control of one of the two alternative ETV6 enhancer/promoters, located between exons 2 and 3 of ETV6 (14). Intriguingly, it was recently shown that the homeobox gene GSH2 and IL-3 are ectopically expressed in patients with AML and the translocations t(4;12)(q11–12;p13) and t(5;12)(q31;p13), respectively. Both translocations involve *ETV6* but do not create any functional fusion genes (20). This observation suggests that activation of protooncogenes is a more common phenomenon in ETV6-associated leukemias than previously thought. Taking into consideration that several AML-associated fusion genes are not leukemogenic on their own, it is tempting to speculate that activation of protooncogenes by chromosomal rearrangements might be quite a widespread mechanism in myeloid leukemogenesis. This hypothesis is supported by observations in AML cases not affecting ETV6, in which expression of the putative protooncogene EVI1 is activated by juxtaposition to the enhancer sequences of the ribophorin-I gene in patients with AML and 3q21 alterations (46). Our data provide compelling evidence that myeloid leukemogenesis can be initiated by this mechanism and emphasize the relevance of protooncogene activation for the development of AML.

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1. Rowley, J. D. (1999) *Semin. Hematol.* **36,** 59–72.
2. Bohlander, S. K. (2000) *Cytogenet. Cell Genet.* **91,** 52–56.
3. Look, A. T. (1997) *Science* **278,** 1059–1064.
4. Pineault, N., Buske, C., Feuring-Buske, M., Abramovich, C., Rosten, P., Hogge, D. E., Aplan, P. D. & Humphries, R. K. (2003) *Blood* **101,** 4529–4538.
5. Kroon, E., Krosl, J., Thorsteinsdottir, U., Baban, S., Buchberg, A. M. & Sauvageau, G. (1998) *EMBO J.* **17,** 3714–3725.
6. Castilla, L. H., Garrett, L., Adya, N., Orlic, D., Dutra, A., Anderson, S., Owens, J., Eckhaus, M., Bodine, D. & Liu, P. P. (1999) *Nat. Genet.* **23,** 144–146.
7. Odero, M. D., Carlson, K., Calasanz, M. J., Lahortiga, I., Chinwalla, V. & Rowley, J. D. (2001) *Genes Chromosomes Cancer* **31,** 134–142.
8. Million, R. P., Aster, J., Gilliland, D. G. & Van Etten, R. A. (2002) *Blood* **99,** 4568–4577.
9. Schwaller, J., Frantsve, J., Aster, J., Williams, I. R., Tomasson, M. H., Ross, T. S., Peeters, P., Van Rompaey, L., Van Etten, R. A., Ilaria, R., Jr., *et al.* (1998) *EMBO J.* **17,** 5321–5333.
10. Tomasson, M. H., Sternberg, D. W., Williams, I. R., Carroll, M., Cain, D., Aster, J. C., Ilaria, R. L., Jr., Van Etten, R. A. & Gilliland, D. G. (2000) *J. Clin. Invest.* **105,** 423–432.
11. Iijima, Y., Okuda, K., Tojo, A., Tri, N. K., Setoyama, M., Sakaki, Y., Asano, S., Tokunaga, K., Kruh, G. D. & Sato, Y. (2002) *Oncogene* **21,** 4374–4383.
12. Wai, D. H., Knezevich, S. R., Lucas, T., Jansen, B., Kay, R. J. & Sorensen, P. H. (2000) *Oncogene* **19,** 906–915.
13. Golub, T. R., Barker, G. F., Bohlander, S. K., Hiebert, S. W., Ward, D. C., Bray-Ward, P., Morgan, E., Raimondi, S. C., Rowley, J. D. & Gilliland, D. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4917–4921.
14. Chase, A., Reiter, A., Burci, L., Cazzaniga, G., Biondi, A., Pickard, J., Roberts, I. A., Goldman, J. M. & Cross, N. C. (1999) *Blood* **93,** 1025–1031.
15. Peeters, P., Wlodarska, I., Baens, M., Criel, A., Selleslag, D., Hagemeijer, A., Van den Berghe, H. & Marynen, P. (1997) *Cancer Res.* **57,** 564–569.
16. Andreasson, P., Schwaller, J., Anastasiadou, E., Aster, J. & Gilliland, D. G. (2001) *Cancer Genet. Cytogenet.* **130,** 93–104.
17. Bernardin, F., Yang, Y., Cleaves, R., Zahurak, M., Cheng, L., Civin, C. I. & Friedman, A. D. (2002) *Cancer Res.* **62,** 3904–3908.
18. Wlodarska, I., Baens, M., Peeters, P., Aerssens, J., Mecucci, C., Brock, P., Marynen, P. & Van den Berghe, H. (1996) *Cancer Res.* **56,** 2655–2661.
19. Patel, N., Goff, L. K., Clark, T., Ford, A. M., Foot, N., Lillington, D., Hing, S., Pritchard-Jones, K., Jones, L. K. & Saha, V. (2003) *Br. J. Haematol.* **122,** 94–98.
20. Cools, J., Mentens, N., Odero, M. D., Peeters, P., Wlodarska, I., Delforge, M., Hagemeijer, A. & Marynen, P. (2002) *Blood* **99,** 1776–1784.
21. Penas, E. M., Cools, J., Algenstaedt, P., Hinz, K., Seeger, D., Schafhausen, P., Schilling, G., Marynen, P., Hossfeld, D. K. & Dierlamm, J. (2003) *Genes Chromosomes Cancer* **37,** 79–83.
22. Knoepfler, P. S. & Kamps, M. P. (1995) *Mol. Cell. Biol.* **15,** 5811–5819.
23. Shanmugam, K., Green, N. C., Rambaldi, I., Saragovi, H. U. & Featherstone, M. S. (1999) *Mol. Cell. Biol.* **19,** 7577–7588.
24. Buske, C., Feuring-Buske, M., Antonchuk, J., Rosten, P., Hogge, D. E., Eaves, C. J. & Humphries, R. K. (2001) *Blood* **97,** 2286–2292.
25. Buske, C., Feuring-Buske, M., Abramovich, C., Spiekermann, K., Eaves, C. J., Coulombel, L., Sauvageau, G., Hogge, D. E. & Humphries, R. K. (2002) *Blood* **100,** 862–868.
26. Feuring-Buske, M., Frankel, A. E., Alexander, R. L., Gerhard, B. & Hogge, D. E. (2002) *Cancer Res.* **62,** 1730–1736.
27. Pineault, N., Helgason, C. D., Lawrence, H. J. & Humphries, R. K. (2002) *Exp. Hematol.* **30,** 49–57.
28. Silberg, D. G., Sullivan, J., Kang, E., Swain, G. P., Moffett, J., Sund, N. J., Sackett, S. D. & Kaestner, K. H. (2002) *Gastroenterology* **122,** 689–696.
29. Brooke, N. M., Garcia-Fernandez, J. & Holland, P. W. (1998) *Nature* **392,** 920–922.
30. van den Akker, E., Forlani, S., Chawengsaksophak, K., de Graaff, W., Beck, F., Meyer, B. I. & Deschamps, J. (2002) *Development (Cambridge, U.K.)* **129,** 2181–2193.
31. Isaacs, H. V., Pownall, M. E. & Slack, J. M. (1998) *EMBO J.* **17,** 3413–3427.
32. Buske, C. & Humphries, R. K. (2000) *Int. J. Hematol.* **71,** 301–308.
33. Sauvageau, G., Lansdorp, P. M., Eaves, C. J., Hogge, D. E., Dragowska, W. H., Reid, D. S., Largman, C., Lawrence, H. J. & Humphries, R. K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12223–12227.
34. Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., *et al.* (1999) *Science* **286,** 531–537.
35. Debernardi, S., Lillington, D. M., Chaplin, T., Tomlinson, S., Amess, J., Rohatiner, A., Lister, T. A. & Young, B. D. (2003) *Genes Chromosomes Cancer* **37,** 149–158.
36. Ferrando, A. A., Armstrong, S. A., Neuberg, D. S., Sallan, S. E., Silverman, L. B., Korsmeyer, S. J. & Look, A. T. (2003) *Blood* **102,** 262–268.
37. Yeoh, E. J., Ross, M. E., Shurtleff, S. A., Williams, W. K., Patel, D., Mahfouz, R., Behm, F. G., Raimondi, S. C., Relling, M. V., Patel, A., *et al.* (2002) *Cancer Cell* **1,** 133–143.
38. Thorsteinsdottir, U., Kroon, E., Jerome, L., Blasi, F. & Sauvageau, G. (2001) *Mol. Cell. Biol.* **21,** 224–234.
39. Lorentz, O., Duluc, I., Arcangelis, A. D., Simon-Assmann, P., Kedinger, M. & Freund, J. N. (1997) *J. Cell Biol.* **139,** 1553–1565.
40. Charite, J., de Graaff, W., Consten, D., Reijnen, M. J., Korving, J. & Deschamps, J. (1998) *Development (Cambridge, U.K.)* **125,** 4349–4358.
41. Subramanian, V., Meyer, B. I. & Gruss, P. (1995) *Cell* **83,** 641–653.
42. Margalit, Y., Yarus, S., Shapira, E., Gruenbaum, Y. & Fainsod, A. (1993) *Nucleic Acids Res.* **21,** 4915–4922.
43. Sauvageau, G., Thorsteinsdottir, U., Hough, M. R., Hugo, P., Lawrence, H. J., Largman, C. & Humphries, R. K. (1997) *Immunity* **6,** 13–22.
44. Taylor, J. K., Levy, T., Suh, E. R. & Traber, P. G. (1997) *Nucleic Acids Res.* **25,** 2293–2300.
45. Houde, M., Laprise, P., Jean, D., Blais, M., Asselin, C. & Rivard, N. (2001) *J. Biol. Chem.* **276,** 21885–21894.
46. Nucifora, G. (1997) *Leukemia* **11,** 2022–2031.

The *AML1-ETO* fusion gene and the *FLT3* length mutation collaborate in inducing acute leukemia in mice

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The molecular characterization of leukemia has demonstrated that genetic alterations in the leukemic clone frequently fall into 2 classes, those affecting transcription factors (e.g., *AML1-ETO*) and mutations affecting genes involved in signal transduction (e.g., activating mutations of *FLT3* and *KIT*). This finding has favored a model of leukemogenesis in which the collaboration of these 2 classes of genetic alterations is necessary for the malignant transformation of hematopoietic progenitor cells. The model is supported by experimental data indicating that *AML1-ETO* and *FLT3* length mutation (*FLT3-LM*), 2 of the most frequent genetic alterations in AML, are both insufficient on their own to cause leukemia in animal models. Here we report that *AML1-ETO* collaborates with *FLT3-LM* in inducing acute leukemia in a murine BM transplantation model. Moreover, in a series of 135 patients with AML1-ETO–positive AML, the most frequently identified class of additional mutations affected genes involved in signal transduction pathways including *FLT3-LM* or mutations of *KIT* and *NRAS*. These data support the concept of oncogenic cooperation between *AML1-ETO* and a class of activating mutations, recurrently found in patients with t(8;21), and provide a rationale for therapies targeting signal transduction pathways in AML1-ETO–positive leukemias.

Introduction

The cloning of recurring chromosomal translocations and, increasingly, the molecular characterization of point mutations in patients with acute leukemia have substantially contributed to the understanding of the pathogenesis of this disease. In acute myeloid leukemia (AML), chromosomal translocations most frequently target transcription factors involved in the regulation of normal hematopoietic differentiation, whereas point mutations often affect genes involved in signal transduction pathways associated with cell proliferation (1–3). The systematic analyses of genetic alterations in patients with AML have demonstrated that genetic lesions of more than 1 transcriptional regulator, such as *AML1-ETO* (*RUNX1-MTG8*), *HOX* fusion genes, or *PML-RARA*, rarely occur in the leukemic clone. Similarly, patients with concurrent mutations of *FLT3*, *KIT*, or *NRAS* are rare. However, there are numerous examples in which fusion genes are identified together with activating mutations of receptor tyrosine kinases, exemplified by *PML-RARA* and the *FLT3* length mutation (*FLT3-LM*), which occur together in up to 35% of all patients with t(15;17)–positive AML (4).

These observations have favored a model of pathogenesis of acute leukemia in which the 2 groups of genetic alterations, 1 affecting transcriptional regulation and hematopoietic differentiation, the other altering signal transduction cascades associated with cell proliferation, collaborate in inducing acute leukemia (5). This concept is supported by experimental data demonstrating that *AML1-ETO*, one of the most frequent fusion genes in AML, is not able, on its own, to induce leukemia in experimental in vivo models but requires additional mutations in yet unknown genes for induction of hematological disease. In a conditional AML1-ETO murine model, for example, only mice treated additionally with *N*-ethylnitrosourea (ENU) developed AML as well as T cell lymphoblastic lymphoma, whereas untreated AML1-ETO mice showed only minimal hematopoietic abnormalities (6). Similar observations were reported from an hMRP8–AML1-ETO transgenic mouse model, which developed AML as well as T–acute lymphoblastic leukemia/lymphoma (T-ALL/lymphoma) only after ENU mutagenesis (7), and from a murine BM transplantation model inducing constitutive expression of AML1-ETO in hematopoietic progenitor cells by retroviral gene transfer (8). In a recent report, mice targeted to express AML1-ETO in the HSC compartment developed a nonlethal long-latency myeloproliferative syndrome but failed to develop acute leukemia (9).

To test the hypothesis of oncogenic cooperation between different classes of mutations, we analyzed a series of 135 patients with AML1-ETO–positive AML for the occurrence of activating mutations involving signal transduction pathways (*FLT3-LM*, *FLT3D835*, *KITD816*, *NRAS* codon 12/13/61). Because almost one-third of all AML1-ETO–positive patients had such activating mutations, we asked whether AML1-ETO would be able to collaborate with 1 of

Nonstandard abbreviations used: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; B6C3, C57BL/6J × C3H/HeJ (mice); CBF, core-binding factor; CFU-S, colony-forming spleen unit(s); cy, cytoplasmic; ENU, *N*-ethylnitrosourea; FLT3-LM, FLT3 length mutation; GSF, National Research Center for Environment and Health; IRES, internal ribosomal entry site; KD, kinase dead; LTR, long-terminal repeat; MSCV, murine stem cell virus; PepC3, C57BL/6Ly-Pep3b × C3H/HeJ (mice); VCM, virus-containing medium; YFP, yellow fluorescent protein.

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Table 1
Genetic alterations in patients with *AML1-ETO* rearrangement

	No. of patients analyzed	No. of patients with mutation detected
FLT3-LM	135	11 (8.1%)
FLT3D835	135	3 (2.2%)
KITD816	135	11 (8.1%)
NRAS codon 12/13/61	135	13 (9.6%)
FLT3, *KIT*, or *NRAS* mutation	135	38 (28.1%)
MLL-PTD	87	0

these alterations to induce leukemia. Here we demonstrated that retrovirally engineered coexpression of *AML1-ETO* and *FLT3-LM* potently synergizes to trigger the development of aggressive leukemia in a murine transplantation model.

This model will allow valuable insights into the pathogenesis of core-binding factor (CBF) leukemias and demonstrates, for the first time to our knowledge, the functional collaboration of AML1-ETO with a class of activating mutations frequently found in patients with t(8;21)–positive leukemia.

Results

AML1-ETO occurs frequently together with activating mutations involving signal transduction pathways in patients with AML. In order to characterize genetic alterations that occur together with the *AML1-ETO* fusion gene in AML, 135 patients with *AML1-ETO* (93 male, 42 female; median age 50.9, range 15.8–89.1) were screened for activating mutations in the receptor tyrosine kinases *FLT3* and *KIT* as well as in *NRAS* (*KITD816*, *NRAS* codon 12/13/61). Patients included 118 with newly diagnosed AML, 4 in first relapse, and 13 classified as having therapy-related AML. Activating mutations were detected in 38 patients (28.1%) and included mutations in the receptor tyrosine kinase *FLT3* or *KIT* (25 patients in total) or in *NRAS* (13 patients). In contrast, no *MLL-PTD* (partial tandem duplication) mutations were detected in 87 samples subjected to this analysis (Table 1). These data demonstrate that genetic alterations occurring with the *AML1-ETO* fusion gene frequently affect signal transduction pathways.

AML1-ETO cooperates with FLT3-LM in inducing acute leukemia in transplanted mice. To test the functional significance of the association of AML1-ETO with mutations involving critical signal transduction cascades, we used the murine BM transplantation model. Murine stem cell virus–based (MSCV-based) retroviral constructs carrying the AML1-ETO cDNA upstream of an internal ribosomal entry site–green fluorescent protein (IRES-GFP) cassette or the FLT3-LM cDNA upstream of an IRES–yellow fluorescent protein (IRES-YFP) cassette were generated to transduce and track hematopoietic cells expressing AML1-ETO (GFP^+), FLT3-LM (YFP^+), or both AML1-ETO and FLT3-LM (GFP^+/YFP^+) in vitro and in vivo (Figure 1). In order to investigate the impact of expression of AML1-ETO or FLT3-LM individually on primary primitive hematopoietic progenitor cells, we performed the colony-forming spleen assay (CFU-S). BM cells transduced with the *AML1-ETO*/GFP or *FLT3-LM*/YFP vector or both vectors were highly purified 96 hours after the start of infection by FACS. Their ability to form spleen colonies (day 0 equivalent) was measured by transplantation of transduced cells after purification into lethally irradiated recipient mice and quantification of spleen colony formation 12 days after injection. Constitutive expression of FLT3-LM did not increase the CFU-S content compared with the GFP control. In contrast, AML1-ETO increased the CFU-S content 3.1-fold compared with the control ($P < 0.002$). Strikingly, coexpression of FLT3-LM together with AML1-ETO increased CFU-S numbers a further 2.1-fold for a net increase of 6.5-fold CFU-S compared with the control ($P < 0.013$), thus demonstrating functional collaboration of these 2 genetic alterations in enhancing the CFU-S frequency (Figure 2A). In an effort to characterize the domains responsible for the collaboration of the 2 aberrations, an FLT3-LM and an AML1-ETO mutant were generated: the FLT3-LM mutant with loss of its kinase activity (kinase dead [KD]) (FLT3-LM-KD) and the AML1-ETO mutant with an L148D point mutation in the Runx1 domain of AML1-ETO (AML1-ETO-L148D), previously reported to lack DNA-binding activity. Expression of the constructs was tested by Western blot and FACS analysis, and FLT3-LM-KD was also tested for autophosphorylation as a surrogate marker for kinase activity and for its capacity to induce IL-3–independent growth in Ba/F3 cells (Figure 1, B, D, F, and G). Of note, AML1-ETO-L148D was not able to collaborate with FLT3-LM. Furthermore, the collaboration between AML1-ETO and FLT3-LM was dependent on the kinase activity of FLT3, as FLT3-LM-KD did not collaborate with the fusion gene (Figure 2A). Inhibition of the kinase activity of FLT3-LM by the protein tyrosine kinase (PTK) inhibitor PKC412 was tested in a ΔCFU-S assay after 48 hours of incubation with the inhibitor. The compound induced a 62% reduction of the day 0 equivalent of the CFU-S frequency (42 versus 16 per 1×10^5 initiating BM cells) of cells cotransfected with *FLT3-LM* and *AML1-ETO* compared with the untreated control, whereas the CFU-S frequency of cells infected with the GFP control vector was unchanged by the inhibitor (Figure 2B).

To further assess the potential collaboration of AML1-ETO with FLT3-LM, we carried out long-term BM transplantation studies using BM transduced with *AML1-ETO* or *FLT3*-LM alone or with both together. Over an observation period extending to 20.6 months, no disease developed in recipients of BM singly transduced with *AML1-ETO* (3×10^5 to 4×10^5 highly purified GFP^+ cells; $n = 9$) or *FLT3-LM* (7×10^4 to 2×10^5 highly purified YFP^+ cells together with 3×10^5 to 1×10^6 nontransduced helper cells; $n = 9$). To obtain mice engrafted with AML1-ETO/FLT3-LM–coexpressing BM cells, mice were injected with a mixture of GFP^+/YFP^+ cells (range 1×10^3 to 5.5×10^4 cells) and nontransduced normal BM cells (range 2.3×10^5 to 1.9×10^6). All recipients of doubly transduced BM ($n = 11$ from 5 independent experiments) succumbed to an aggressive acute leukemia after a median latency time of 233 days post-transplantation (Figure 3). These mice were engrafted with GFP/YFP–coexpressing cells that were positive for AML1-ETO and FLT3-LM transcripts in the RT-PCR analysis (Figure 1C). At diagnosis the mice were moribund, cachectic, and short of breath and suffered from splenomegaly (median spleen weight 441 mg) (Table 2). Peripheral blood and BM contained a high proportion of blasts, and peripheral blood wbc counts were highly elevated in 5 of 11 animals (range 2×10^6 to 430×10^6 cells/ml) compared with the GFP control (range 3.5×10^6 to 9×10^6) (Table 2), consistent with a diagnosis of acute leukemia. Additionally, mice were anemic, with a 45% reduction in erythrocyte counts compared with the mean count in the control.

Coexpression of AML1-ETO and FLT3-LM causes both acute myeloblastic and lymphoblastic leukemia. In 7 animals the morphology of the blasts was myeloblastic (Figure 4A), whereas 4 animals were characterized by a lymphoblastic cell population (Figure 4, B and C, and Table 2). In 2 of the 7 animals with AML (mice nos. 16 and 24) the blast population was accompanied by a dominant mast cell

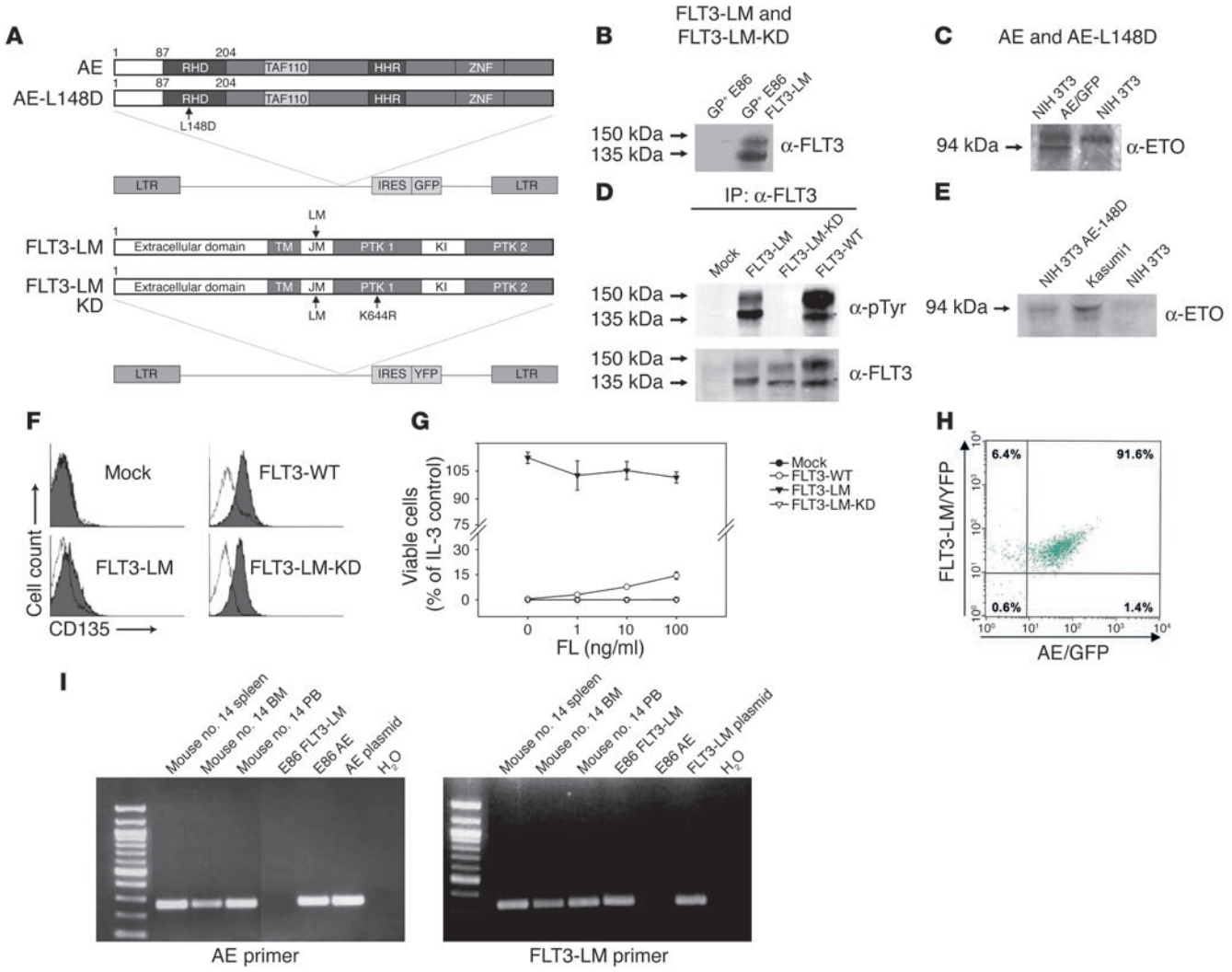

Figure 1

Schematic diagram and analysis of expression of different constructs. (**A**) Retroviral constructs for expression of AML1-ETO and of the AML1-ETO-L148D (31, 47), FLT3-LM, and FLT3-LM-KD mutant proteins. The GFP vector served as a control. AE, AML1-ETO; LTR, long-terminal repeat; RHD, runt homology domain; TAF110, TATA-binding protein–associated factor 110; HHR, hydrophobic heptad repeat; ZNF, zinkfinger; TM, transmembrane; JM, juxtamembrane; PTK, protein tyrosine kinase; KI, kinase insert. (**B**, **C**, and **E**) Western blot analysis of cellular extracts from GP+ E86 and NIH 3T3 cells transfected with the different constructs (the molecular mass is indicated). Kasumi cells served as a positive control. (**D**) α-pTyr plot demonstrating phosphorylation of FLT3-LM and FLT3-WT but not of FLT3-LM-KD. (**F**) FACS analysis of Ba/F3 cells transduced with the FLT3 constructs. (**G**) Growth of IL-3–dependent Ba/F3 cells infected with the different constructs. (**H** and **I**) Flow cytometry and RT-PCR analysis of cells coexpressing FLT3-LM/YFP and AML1-ETO/GFP, isolated from a representative leukemic mouse. FL, FLT3 ligand; PB, peripheral blood.

population with fine metachromatic granulation in the panoptic staining (Figure 4A). The BM and spleen were infiltrated with up to 80% and 80% blasts, respectively, in the mice with AML and up to 85% and 95%, respectively, in the mice suffering from ALL (Table 2 and Figures 4 and 5).

In order to determine more precisely the immunophenotype of the leukemic population, flow cytometric analyses from BM cells were performed. Seven animals suffered from AML with a Gr-1/Mac-1–positive cell population in the transduced compartment, which coexpressed Sca-1 (45.2%, range 19–73%). Of note, in 4 of the 7 animals with AML, coexpression of CD4 was detected in 21%, 27%, 31%, and 32% of BM cells (animals nos. 15, 16, 22, and 23, respectively; Table 2). Three mice suffered from B-lymphoblastic leukemia, with 90.4% of the transduced cells expressing B220 (range 85–97%) and lacking expression of myeloid antigens (Gr-1–positive 1.5%, range 0.4–2.3%; Mac-1–positive 1.7%, range 1.4–1.9%). One animal developed T cell leukemia, with coexpression of CD4 and CD8 (99% $CD8^+$, 86% $CD4^+$ in the transduced compartment) and expression of Sca-1 in 76% of all cells (Figure 4C). Histological tissue sections and immunohistochemistry were performed in 2 diseased mice with AML, including 1 of the animals with an increase in mast cells in the peripheral blood (mouse no. 16). Both animals showed multiple-organ infiltration into hematopoietic and nonhematopoietic organs with effacement of the normal follicular architecture of the spleen (Figure 5, D and E [right side]) and an infiltration with leukemic blasts in the liver and spleen (Figure 5, A–D and F). Immunohistochemistry

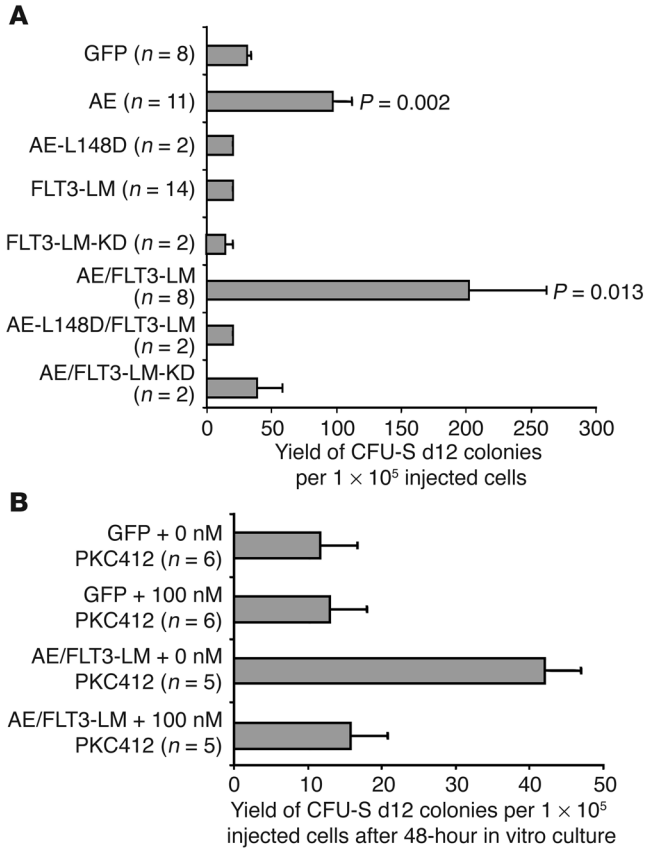

Figure 2
Analyses of CFU-S frequencies. (**A**) Primary BM cells retrovirally transduced with GFP, AML1-ETO, AML1-ETO-L148D, FLT3-LM, or FLT3-LM-KD vectors or with combinations of the different vectors were isolated by FACS 48 hours after infection and injected into lethally irradiated mice to assess initial (day 0) CFU-S numbers. CFU-S frequency per 1×10^5 initiating BM cells was determined in 3 independent experiments. The number of analyzed mice and the *P* value compared with the GFP control are indicated. (**B**) CFU-S frequency of primary BM cells infected with GFP or with both AML1-ETO and FLT3-LM and treated with the inhibitor PKC412 for 48 hours compared with untreated controls.

confirmed the diagnosis of AML: blasts were positive for myeloperoxidase but showed differentiation into more mature myeloid cells with positivity for chloracetatesterase (mouse no. 14; Table 2 and Figure 5, B, C, and F). In the second mouse with AML, infiltration of organs with cells expressing mast cell–specific tryptase and CD117 could be confirmed in the primarily and secondarily transplanted mouse, indicating the presence of a malignant infiltrating mast cell population in this animal (mouse no. 16).

The leukemias were readily transplantable and had the same histomorphology within 106 days after transplantation (median survival 68 days, range 57–106 days; $n = 5$) (Figure 3 and Figure 5, G–O). Southern blot analyses of BM from leukemic mice revealed modest numbers of proviral integrations, consistent with double infection and monoclonal or, at most, oligoclonal disease (Figure 6A). Monoclonal or oligoclonal disease is consistent with the relatively small transplant doses used but could also reflect a possible contribution of retroviral insertional mutagenesis to the transformation process. To further explore this latter possibility, 10 retroviral integration sites were subcloned and sequenced from 4 leukemic mice; all 10 sites were unique, and thus there was no indication of a common integration site associated with the leukemic transformation. Moreover, 5 sites were intergenic or not linked to known genes. The remaining sites were in introns in a 5′ to 3′ orientation most likely to lead to gene knock down rather than activation (Figure 6B and Table 3).

Since we observed coexpression of CD4 in leukemic cells of the majority of mice who developed AML in our model, we analyzed expression of CD4 and cytoplasmic (cy) CD3 in patients with AML1-ETO–positive AML; 17 of 52 patients analyzed (32.7%) and 39 of 50 patients analyzed (78%) were positive for CD4 or cyCD3, respectively. Furthermore, 31 of 52 patients (59.6%) expressed the B cell antigen CD19, 27 of 52 patients cyCD22 (51.9%), and 38 of 39 patients cyCD79a (97.4%) (Figure 7). There was no difference in the extent of coexpression of lymphoid antigens in AML1-ETO–positive AML with additional activating mutations of *FLT3*, *KIT*, and *NRAS* ($n = 14$) versus cases without this class of mutations ($n = 38$) (data not shown). This indicates that coexpression of lymphoid and myeloid antigens in myeloblastic leukemia, which is detected in the murine model, is a common characteristic in patients with AML1-ETO–positive AML.

Discussion

The translocation t(8;21)(q22;q22), which generates the AML1-ETO fusion gene, is one of the most frequent chromosomal translocations, detected in 12% of all AML patients and in up to 40% of FAB-M2 AML patients (10, 11). The translocation targets *AML1* (*RUNX1*), a member of the RUNX family characterized by a DNA-binding *Runt* domain at the amino terminus that is retained in the fusion gene (12). This domain is necessary for DNA binding and heterodimerization of AML1 with CBFB, the non–DNA-binding subunit of the complex. As predicted by the discovery that the *AML1* gene is rearranged in human hematopoietic disease, the AML1/CBFB complex was shown to be a key regulator of definitive hematopoiesis, and loss of either of these genes resulted in embryonic lethality with complete lack of definitive HSCs (13). In addition, it was recently reported that $AML1^{+/-}$ adult mice suffer from a 50% reduction of long-term repopulating stem cells (14). Although it is yet not fully understood how the

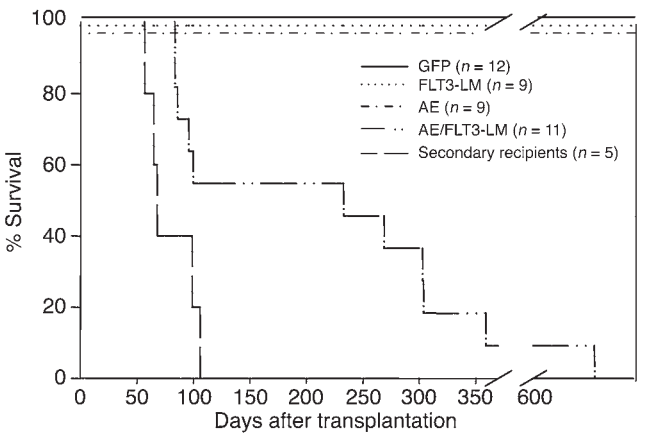

Figure 3
Survival of transplanted mice. Survival curve of mice transplanted with BM cells expressing AML1-ETO ($n = 9$), FLT3-LM ($n = 9$), or GFP ($n = 12$), of mice transplanted with marrow cells coexpressing AML1-ETO and FLT3-LM ($n = 11$), and of secondarily transplanted mice ($n = 5$).

Table 2
Hematological parameters of analyzed experimental mice

Mouse no.	Retroviral construct	Day of sacrifice	rbc/ml $\times 10^9$	wbc/ml $\times 10^6$	Spleen size (mm)	Spleen weight (mg)	BM % blasts	Spleen % blasts	PB % blasts
1	GFP	ND	4.8	7.6	ND	ND	1	0	0
2	GFP	ND	6.4	8.1	ND	ND	0	0	0
3	GFP	90	7	5	14 × 4	51	2	0	0
4	GFP	ND	5.4	9	ND	ND	0	0	0
5	GFP	689	5.6	4.5	15 × 4	78	0	0	0
6	GFP	721	7.25	3.5	ND	ND	3	0	0
7	AE	444	6	13	13 × 3.5	63	8	0	0
8	AE	479	3.1	15	14 × 4	60	11	0	0
9	AE	493	5.7	6.9	14 × 4	82	2	0	0
10	AE	615	5	7.6	16 × 5	117	14	0	0
11	FLT3-LM	88	4.5	10	15 × 4	95	4	ND	0
12	FLT3-LM	ND	4.5	13	ND	ND	2	ND	0
13	FLT3-LM	ND	5.6	8.3	ND	ND	1	ND	0
14[A]	AE/FLT3-LM	233	ND	23	27 × 7	600	80	52	20
15[A]	AE/FLT3-LM	100	0.85	26.5	19 × 6	166	48	80	75
16[A]	AE/FLT3-LM	612	1.7	12.5	14 × 4	118	20	50	25
17[B]	AE/FLT3-LM	84	ND	430	24 × 9	572	80	22	60
18[B]	AE/FLT3-LM	84	4.4	3.3	21 × 7	270	85	43	62
19[B]	AE/FLT3-LM	94	7.7	7.2	12 × 4	ND	85	20	95
20[B]	AE/FLT3-LM	96	3.8	60	21 × 6	310	40	95	60
21[A]	AE/FLT3-LM	269	4	2	28 × 9	650	38	62	30
22[A]	AE/FLT3-LM	303	2.6	10	15 × 3	200	24	76	38
23[A]	AE/FLT3-LM	304	2.6	2.5	29 × 10	1,400	27	55	78
24[A]	AE/FLT3-LM	359	2.5	35	28 × 9	760	39	77	64

[A]AML; [B]ALL. PB, peripheral blood; ND, not determined; AE, AML1-ETO.

AML1-ETO fusion gene contributes to leukemogenesis, it is thought that 1 key mechanism is the suppression of AML1- and C/EBPα-dependent activation of genes responsible for myeloid development (15, 16). Perturbation of hematopoiesis by expression of *AML1-ETO* results in an increase in the replating capacity of murine clonogenic progenitors and in the growth of primitive human progenitor cells in vitro (6, 17). Furthermore, in vivo and ex vivo analyses demonstrated alterations in the differentiation pattern and proliferative capacity of murine hematopoietic cells expressing the fusion gene (8, 9, 18, 19). However, numerous murine in vivo models documented that *AML1-ETO* on its own is not able to induce leukemia (6, 7, 9, 18, 19). The observation that *AML1-ETO* as a single factor is nonleukemogenic is further supported by findings that nonleukemic *AML1-ETO*–expressing progenitor cells can be isolated from healthy individuals as well as AML patients in remission, which suggests that additional mutations in these AML1-ETO–positive progenitors are necessary for the transformation into leukemia-initiating cells (20–22). The importance of collaborating genetic events in the pathogenesis of AML1-ETO–positive leukemias has indeed been shown in different murine models, such as a conditional *AML1-ETO* murine model as well as an hMRP8–*AML1-ETO* transgenic mouse model. Only mice treated additionally with ENU developed AML or T cell lymphoma (6, 7). Furthermore, retrovirally expressed *AML1-ETO* induced myeloblastic transformation in vivo only in a background deficient in the IFN-regulatory factor IFN consensus sequence-binding protein (18). These data strongly suggest that genetic alterations cooperating with *AML1-ETO* play a role in inducing leukemia.

In order to characterize genetic alterations that potentially collaborate with *AML1-ETO*, we screened 135 patients with AML for activating mutations of signal transduction pathways or mutations affecting the *MLL* gene. Whereas *MLL-PTD* mutations, which exemplify genetic alterations involved in transcriptional regulation, were not found at all, 28% of the patients were positive for activating mutations such as *FLT3-LM*, *FLT3D835*, *KITD816*, or *NRAS*. The frequent coexistence of such mutations with *AML1-ETO* fits well in the model of leukemogenesis in which the collaboration of 2 classes of genetic alterations, 1 affecting transcription factors associated with hematopoietic differentiation, the other affecting signal transduction pathways associated with cell proliferation, is necessary for the malignant transformation of hematopoietic progenitor cells (3). Using the murine BM transplantation model, we obtained direct evidence for a functional collaboration of *AML1-ETO* with *FLT3-LM* in inducing leukemia, supporting the aforementioned model of leukemogenesis. Furthermore, these data demonstrate the collaboration of the 2 most frequent genetic alterations in AML, providing an important model for the understanding of both the AML1-ETO–positive and the FLT3-LM–positive leukemias.

Of note, 4 of 7 AML mice reported here expressed the T cell antigen CD4. Although the mechanisms underlying the coexpression of myeloid and lymphoid antigens in AML1-ETO–positive myeloid leukemia are not clear, one possibility is that in this AML subtype an early progenitor cell with a lineage-overlapping mixed phenotype is the target of leukemogenic transformation, as recently proposed for hematological malignancies (23). Of note, it was recently demonstrated that, in AML1-ETO–positive leukemia, evidence of lineage overlap is not restricted to the expression of cytoplasmic or surface antigens but extends to the transcriptional apparatus, since *PAX5* is selectively expressed in one-third of patients with t(8;21) AML in

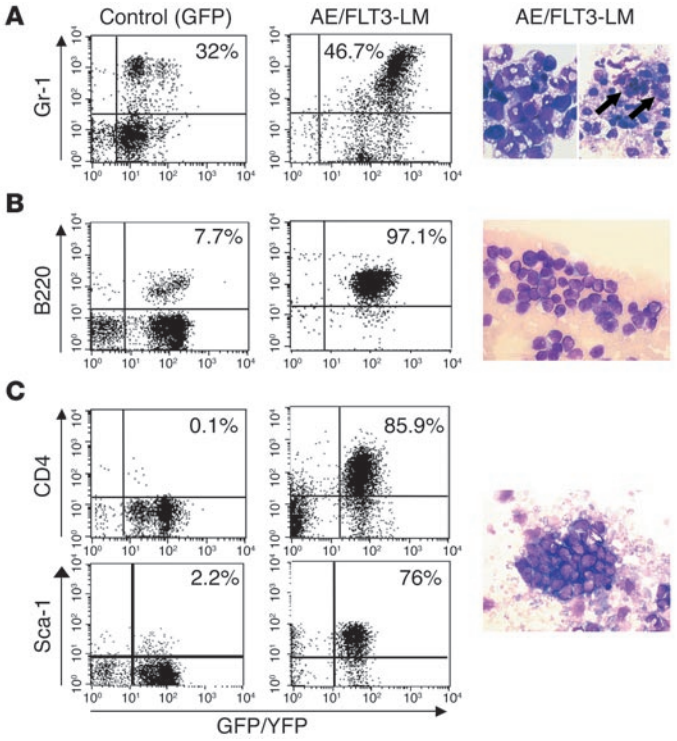

Figure 4
Immunophenotype and morphology of hematopoietic cells recovered from leukemic mice. The plots show representative FACS profiles from BM cells in comparison with cells from GFP control animals, with indication of the proportion of positive cells within the GFP^+/YFP^+ compartment. The photographs show cytospin preparations (H&E; magnification, ×630) from peripheral blood (**A**, right image; **B** and **C**) and from BM (**A**, left image). (**A**) AML with a dominant mast cell population (marked by arrows) (mouse no. 16). (**B**) B-ALL (mouse no. 17). (**C**) T-ALL (mouse no. 20).

contrast to all other cytogenetically defined AML subtypes (24). Furthermore, both myeloblastic and lymphoblastic leukemias of B and T cell type developed in this model of *AML1-ETO* and *FLT3-LM* cooperation. This observation was also reported in other murine models: in a conditional *AML1-ETO* murine model, mice treated additionally with ENU developed AML as well as T cell lymphoblastic lymphoma, although most of the T cell neoplasms did not express the fusion gene (6). Similar observations were reported from an hMRP8–*AML1-ETO* transgenic mouse model, which developed AML as well as T-ALL/lymphoma after ENU treatment (7). In addition, expression of *AML1-ETO* might contribute to the lymphoid phenotype of the leukemias in our model, as it was reported that *FLT3-LM* is able to induce a long-latency T cell lymphoma–like disease in the C57BL/C3H background (25). However, the association of *FLT3-LM* with a lymphoid disease seems to depend on the genetic background of the mouse strain, as *FLT3-LM* induced a myeloproliferative syndrome in BALB/c mice (26). In our model, also using the C57BL/C3H background, constitutive expression of *FLT3-LM* alone did not induce any perturbation of the hematopoietic development in vivo; this result was also recently reported in a mouse model of collaboration of *FLT3-LM* with *MLL-SEPT6* using the C57BL/6 strain (27).

Of note, overexpression of FLT3 and activating FLT3 mutations are associated with ALL in humans, in particular in cases of ALL with hyperdiploidy or *MLL* rearrangement, characterized by a primitive B cell or a mixed lymphoid-myeloid phenotype (28–30). The observations that ETV6-PDGFBR, which already by itself causes a lethal myeloproliferative syndrome in transplanted mice, induces exclusively a myeloblastic leukemia when coexpressed with *AML1-ETO* might point to the importance of the collaborating partner for the phenotype of the induced leukemia (31). Another possible explanation for the development of lymphoid malignancies in our model is that, in individual mice, lymphoid-committed stages of differentiation were hit by the retrovirus, resulting in lymphoblastic leukemia in these animals. This would potentially be a key difference from the human situation, in which both *AML1-ETO* and *FLT3-LM* are already present in the HSC pool (21, 32). An important question is whether the results were influenced by retroviral insertional mutagenesis. Most of the leukemic animals were transplanted with a low transplant dose and then suffered from monoclonal or oligoclonal disease. Although the number of retroviral integration events was low in the mice, insertional mutagenesis might have contributed to the leukemogenesis. However, analyses of the retroviral integration sites in the diseased animals showed integration into intergenic regions or introns of genes, more likely resulting in their knock down than in their activation. These data suggest that retroviral insertional mutagenesis might not play the key part in disease development, an issue that might be more accurately addressed in mouse models expressing *AML1-ETO* from an endogenous promoter. The long latency of the leukemias, even of secondary disease, however, strongly argues that additional secondary in vivo genetic events in the animals contributed to disease development.

To our knowledge, this is the first functional evidence of a leukemogenic collaboration of *AML1-ETO* with a complementary class of mutation, recurrently found in patients with t(8;21). It facilitates our understanding of acute leukemias associated with 2 of the most frequent genetic alterations in this disease. Furthermore, our experimental data support recent reports that show the functional relevance of activating mutations in patients with CBF leukemias [*AML1-ETO* or *CBFB-MYH11*] by demonstrating a significantly shortened overall and event-free survival for AML1-ETO–positive leukemias harboring activating mutations of *FLT3* or *KIT* compared with those without these mutations. In contrast, *RAS* mutations did not affect the treatment outcome (S. Schnittger, unpublished observations) (33, 34). In line with these findings, it was recently shown that in patients with AML1-ETO–positive leukemia, most leukemic cells at diagnosis additionally harbored mutations in *KIT*, whereas in 3 patients analyzed in complete remission, only the fusion gene, but not the *KIT* mutation, could be detected by PCR; this strongly supports the concept of a stepwise development of disease involving 2 collaborating genetic aberrations (35). These observations encourage the systematic screening of activating mutations in patients with CBF leukemias in prospective clinical trials to evaluate more precisely their prognostic impact, and they form a rationale to consider treatment strategies targeting the signal transduction apparatus in this AML subtype.

Methods

Patient samples. BM samples from 135 adult patients with newly diagnosed AML — de novo AML (n = 118), secondary AML after treatment of a previous malignancy (n = 13), and AML at relapse (n = 4) — were analyzed. The diagnosis of AML was performed according to the French-American-British criteria and the WHO classification (36, 37). Cytomorphology, cytochemistry, cytogenetics, and molecular genetics were applied in all cases

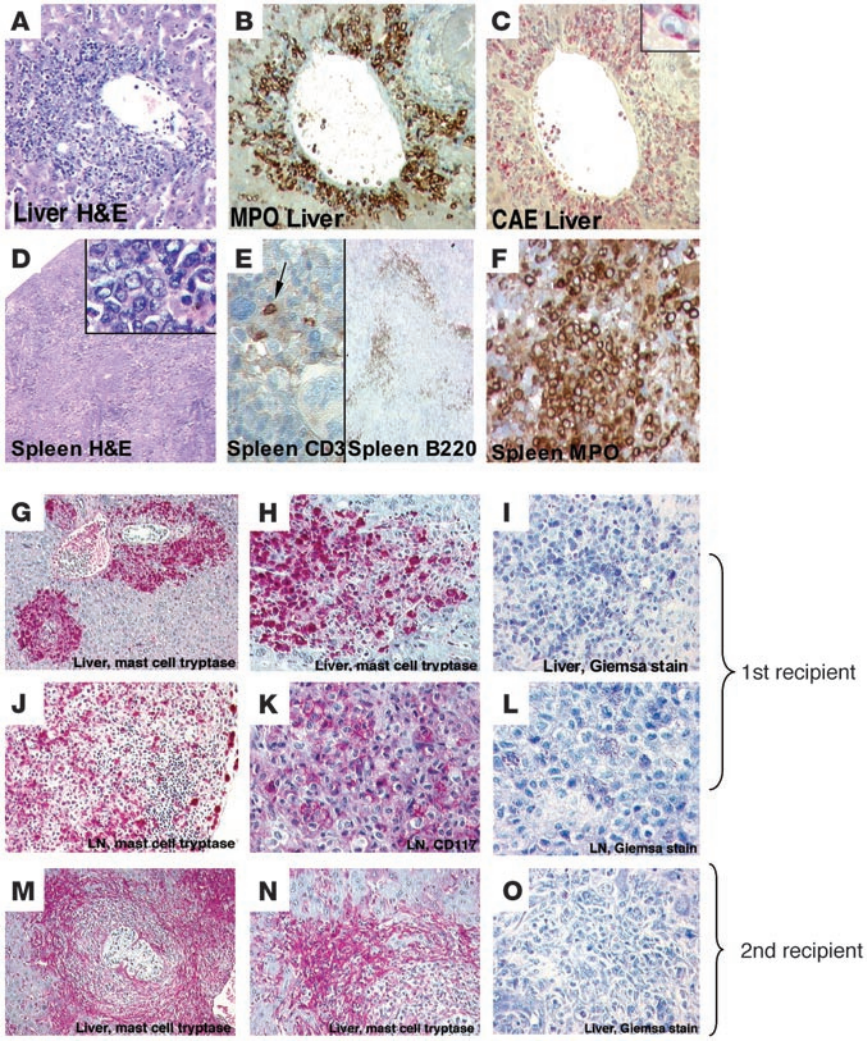

Figure 5
Histological analyses of leukemic mice. (**A**–**F**) Histological analyses of AML (mouse no. 14). Original magnifications: **A**–**C**, ×200; inset in **C**, ×1,000; **D** and right side of **E**, ×250; inset in **D**, ×650; left side of **E**, ×400; **F**, ×400. (**G**–**O**) Histological analyses of AML with a dominant mast cell population (mouse no. 16). (**G**–**L**) Primary recipient. (**M**–**O**) Secondary recipient. Original magnifications: **G** and **M**, ×100; **H**, **J**, and **N**, ×200; **I**, **K**, and **O**, ×400; **L**, ×600. Mast cells with metachromatic granulation in the Giemsa stain are indicated by an arrow. MPO, myeloperoxidase; CAE, *N*-acetyl-chloroacetate esterase.

as described below. Both animal and human studies were approved by the Ethics Committee of Ludwig Maximilians University and abided by the tenets of the revised World Medical Association Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm).

Cytogenetic and FISH analysis. Cytogenetic analyses were performed using standard techniques. For FISH, a commercially available AML1-ETO probe was used according to the manufacturer's instructions (Vysis Inc.) (38).

PCR. Molecular genetic analysis for *AML1-ETO* (38), *MLL-PTD* (39), *FLT3-LM* (4), *NRAS* mutations, *FLT3D835*, and *KITD816* (40) in patient samples was performed as has been described previously (41). In leukemic mice, expression of AML1-ETO and FLT3-LM was assessed by RT-PCR in animals transplanted with BM cells coexpressing AML1-ETO/GFP and FLT3-LM/YFP. Preparation of cDNA was performed as previously described (41). For AML1-ETO the primer forward 5′-ATGACCTCAGGTTTGTCGGTCG-3′ and the primer reverse 5′-TGAACTGGTTCTTGGAGCCTCCT-3′ (corresponding to positions nucleotide 395 and nucleotide 633 of GenBank accession number D13979, respectively) were used; for *FLT3-LM* the primer forward 5′-GCAATTTAGGTATGAAAGCCAGC-3′ and the primer reverse 5′-CTTTCAGCATTTT-GACGGCAACC-3′ (corresponding to positions nucleotide 1,704 and nucleotide 1,920 of GenBank accession number NM_004119, respectively) were used. The annealing temperature was 57°C. The number of PCR cycles for each gene was chosen to stop the reaction in the linear phase of amplification (35 cycles for AML-ETO and FLT3-LM). The integrity of the RNA in all samples was confirmed by mβ-2 microglobulin RT-PCR.

For the linker-mediated PCR (LM-PCR), integrated long-terminal repeats (LTRs) and flanking genomic sequences were amplified and then isolated using a modification of the bubble LM-PCR strategy (42, 43). Aliquots of the cell lysates from leukemic mice were digested with *Pst*I or *Ase* (New England Biolabs Inc.), and the fragments were ligated overnight at room temperature to a double-stranded bubble linker (5′-CTCTCCCTTCTCGAATCGTAACCGTTCG-TACGAGAATCGCTGTCCTCTCCTTG-3′ and 5′-ANTCAAGGAGAGGACGCTGTCTGTCGAAGG-TAAGGAACGGACGAGAGAAGGGAGAG-3′). Next, a first PCR (PCR-A) was performed on 10 μl (one-tenth) of the ligation product using a linker-specific Vectorette primer (5′-CGAATCGTAACC-GTTCGTACGAGAATCGCT-3′) (Invitrogen Corp.) and an LTR-specific primer (LTR-A: 5′-CAACACA-CACATTGAAGCACTCAAGGCAAG-3′) under the following conditions: 1 cycle of 94°C for 2 minutes, 20 cycles of 94°C for 30 seconds and 65°C for 1 minute, and 1 cycle of 72°C for 2 minutes. The bubble linker contains a 30-nucleotide nonhomologous sequence in the middle region that prevents binding of the linker primer in the absence of the minus strand generated by the LTR-specific primer. A 1-μl aliquot of the PCR-A reaction (one-fifteenth) was then used as a template for a second nested PCR (PCR-B) using an internal LTR-specific primer (LTR-B: 5′-GAGAGCTCCCAGGCTCA-GATCTGGTCTAAC-3′) and the same linker-specific Vectorette primer as was used in PCR-A, with the following conditions: 1 cycle of 94°C for 2 minutes, 30 cycles of 94°C for 60 seconds and 72°C for 1 minute, and 1 cycle of 72°C for 2 minutes. Ten microliters (one-half) of the final PCR-B product was electrophoresed using 2% agarose tris-acetate-EDTA gel. Individual bands were excised and purified using the QIAEX II Gel Extraction Kit (QIAGEN) and then cloned into PCR2.1 (Invitrogen Corp.) before sequencing of the integration site of the retrovirus.

Multiparameter flow cytometry. Immunophenotypic analyses were performed as previously described (44). The following combinations of antibodies were used: CD34/CD2/CD33, CD7/CD33/CD34, CD34/CD56/CD33, CD11b/CD117/CD34, CD64/CD4/CD45, CD34/CD13/CD19, CD65/CD87/CD34, CD15/CD34/CD33, HLA-DR/CD33/CD34, CD4/CD13/CD14, CD34/CD135/CD117, CD34/CD116/CD33, CD90/CD117/CD34, CD34/NG2(7.1)/CD33, CD38/CD133/CD34, CD61/CD14/CD45,

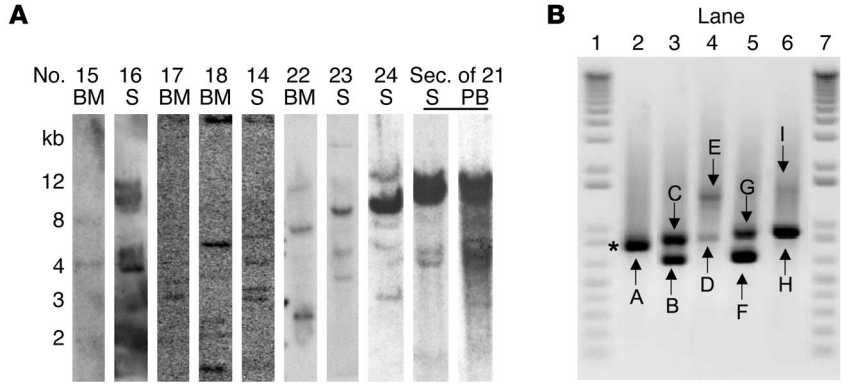

Figure 6
Analysis of proviral integrations. (**A**) Southern blot analysis of genomic DNA from different primary mice and a secondary recipient to detect clonal proviral integrations. DNA was digested with *Eco*RI, which cuts once in the proviral sequence, and blots were hybridized to a GFP/YFP probe. The mouse numbers are indicated (corresponding to those in Table 2). S, spleen; PB, peripheral blood; sec. of 21, second mouse of 1° mouse no. 21. (**B**) Bubble PCR analyses of retroviral integration sites in diseased mice. The bands (A–I) were isolated, subcloned, and sequenced. A description of the PCR products is given in Table 3. Asterisk indicates resolution as 2 unique bands after subcloning and sequencing of integration sites.

CD36/CD235a/CD45, CD15/CD13/CD33, CD9/CD34/CD33, CD38/CD34/CD90, CD34/CD79a/CD19, TdT/cyCD33/cyCD45, myeloperoxidase/lactoferrin/cyCD15, TdT/cyCD79a/cyCD3, and TdT/cyCD22/cyCD3. All antibodies were purchased from Beckman Coulter Inc., except for CD64 and CD15 (Medarex Inc.), CD133 (Miltenyi Biotec), and myeloperoxidase and lactoferrin (CALTAG Laboratories). For the analysis of cytoplasmic antigens, cells were fixed and permeabilized before staining with FIX & PERM (CALTAG Laboratories). Multiparameter flow cytometry analysis was performed with a FACSCalibur flow cytometer (BD).

The purity of all samples was 80–100%. Furthermore, immunophenotyping was performed with triple staining in all cases as indicated above, testing simultaneous expression of myeloid and lymphoid antigens to exclude contaminating normal lymphoid cells.

In mice, immunophenotypic analysis of single-cell suspensions from BM, spleen, and peripheral blood was performed by flow cytometry (FACSCalibur cytometer; BD) using PE-labeled Sca-1, Gr-1, Ter-119, and CD4 antibodies and allophycocyanin-labeled Mac-1, Kit, B220, and CD8 antibodies (all from BD Biosciences — Pharmingen), as previously described (45). The surface expression of the FLT3-LM construct and the FLT3-LM-KD mutant of Ba/F3 cells was confirmed by FACS analysis (Figure 2B) using anti–human CD135–PE mAb (BD) and an isotype-matched IgG1-PE control (Beckman Coulter Inc.).

cDNA constructs and retroviral vectors. For retroviral gene transfer into primary BM cells, AML1-ETO cDNA was subcloned into the multiple cloning site of the modified murine stem cell virus (MSCV) 2.1 vector (41) upstream of the enhanced GFP (EGFP) gene and the internal ribosomal entry site (IRES). The FLT3-LM cDNA was subcloned into the identical MSCV vector construct carrying the enhanced YFP (Figure 1A). The MSCV vector carrying only the IRES-EGFP cassette was used as a control. The cDNA of FLT3-LM was kindly provided by D.G. Gilliland (Harvard Medical School, Boston, Massachusetts, USA) and contained a 28–amino acid duplicated sequence (CSSDNEYFYVDFREYEYDLKWEFPRENL) inserted between amino acids 610 and 611. The AML1-ETO cDNA was provided by S.W. Hiebert (Vanderbilt University School of Medicine, Nashville, Tennessee, USA).

The FLT3-LM-KD mutation K672R (a point mutation of Lys644 to Arg that disrupts an ion pair with Glu661 that is critical for nucleotide binding in FLT3-WT; ref. 46), and the L148D AML1-ETO point mutation (31, 47) to prevent AML1-ETO DNA binding, were generated from the full-length human FLT3-LM cDNA and the AML1-ETO cDNA, respectively, using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The correct sequences of the constructs were confirmed by complete nucleotide sequencing, and expression was proved by Western blot and FACS analysis (Figure 1).

Cell culture. Gag-pol and envelope (GP^+ E86) packaging cells, NIH 3T3 cells, and 293T cells were grown in DMEM with 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2. Primary murine BM cells were plated in transplant medium consisting of DMEM supplemented with 15% FBS, 1% penicillin/streptomycin, 6 ng/ml IL-3, 10 ng/ml IL-6, and 100 ng/ml SCF (tebu-bio GmbH). IL-3–dependent Ba/F3 cells stably expressing the empty vector alone, FLT3-WT, FLT3-LM, and FLT3-LM-KD were seeded at a concentration of 0.05×10^6 per milliliter in the presence or absence of IL-3 and FLT3 ligand, as described previously (48). At 72 hours, viable cells were counted in a standard hemacytometer after staining with trypan blue.

Retrovirus production. High-titer helper-free retrovirus was produced with the constructs above by individual cotransfection of each construct with Ecopac (Cell Genesys Inc.) into 293T cells using calcium chloride precipitation. The retrovirus was subsequently collected in the conditioned medium.

Table 3
Identity of retroviral integration sites in diseased mice

Lane[A]	PCR product[A]	Gene	Protein family	Chromosome	Mouse no.
2	A 1	Intergenic		17qE3	15
	A 2	Intron 1 of Rnf8 alias AIP37 and 5 kbp 3′ of Pim1	Ring finger protein 8	17qA3	
3	B	Intergenic		15qE2	16
	C	Intron 1 of Ptp4a3	Protein tyrosine phosphatase 4a3	15qD3	
4	D	Hypothetical gene		10qC2	12
	E	Intron 3 of GATA	Transcription factor	6qD1	
5	F	Intergenic		18qD3	
	G	Intron 4 of SF3b	Splicing factor subunit b3	8qE1	
6	H	Intergenic in intron of hypothetical gene		3qF1	18
	I	Intergenic in intron of hypothetical gene		16qA1	

[A]Lanes and PCR products according to Figure 6B.

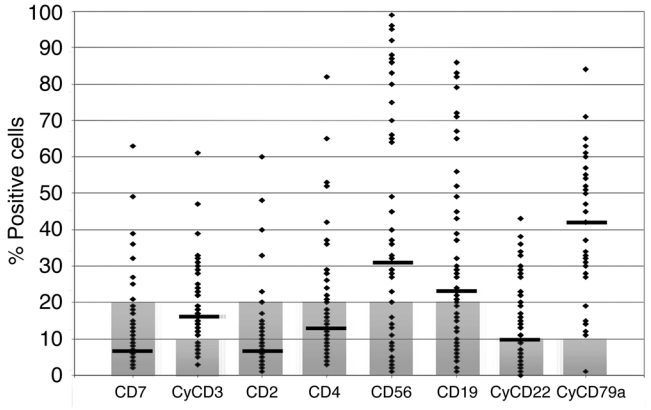

Figure 7
Expression of lymphoid antigens on 52 samples of patients with AML1-ETO–positive AML, determined by immunophenotyping. Samples were defined as negative for expression of cytoplasmic antigens when less than 10% of the cells stained with the antibody, and as negative for expression of surface antigens when less than 20% of the cells stained with the antibody (shaded areas).

To optimize transduction efficiency, the virus-containing medium (VCM) of different constructs was used to transfect GP$^+$ E86 cells to establish stable packaging cell lines, or to directly infect 5-FU–mobilized BM cells in the case of FLT3-LM. Ba/F3 cells were transfected with the different constructs as previously described (48, 49).

Mice and retroviral infection of primary BM cells. Parental-strain mice were bred and maintained at the GSF animal facility. Donors of primary BM cells [(C57BL/6Ly-Pep3b × C3H/HeJ) F_1 (PepC3) mice] and recipient mice [(C57BL/6J × C3H/HeJ) F_1 (B6C3)] were more than 8 weeks old. Primary mouse BM cells were transduced as previously described (41). For transduction of AML1-ETO, cells were cocultured in transplant medium with AML1-ETO/GFP producer cells irradiated with 40 Gy of ^{137}Cs γ-radiation. For infection with the FLT3-LM virus, BM cells were cultured in FLT3-LM/VCM, supplemented with cytokines (IL-3, IL-6, and SCF), to achieve optimal transduction efficacy. For coinfection with the FLT3-LM and AML1-ETO retroviruses, BM cells were cultured on a mixture of 30–50% AML1-ETO/GFP and 50–70% FLT3-LM/YFP producer cells in transplant medium or FLT3-LM/VCM supplemented with IL-3, IL-6, and SCF. Retroviral transfection of primary BM cells with the AML1-ETO mutant L148D and the FLT3-LM-KD mutant was performed as described for FLT3-LM, by cultivation of the BM in VCM supplemented with IL-3, IL-6, and SCF. All transductions were performed with the addition of 5 μg/ml protamine sulfate. Infected cells were highly purified (FACSVantage; BD) based on expression of GFP (for AML1-ETO alone), expression of YFP (for FLT3-LM alone), or coexpression of GFP and YFP (for AML1-ETO/FLT3-LM cotransduction) before transplantation.

BM transplantation and assessment of mice. FACS-purified transduced BM cells or ratios of transduced and nontransduced cells (if less than 3×10^5 transduced cells per recipient were available) were injected into the tail vein of 8- to 10-week-old irradiated recipient F_1 (B6C3) mice (800 cGy from a ^{137}Cs γ-radiation source). Peripheral blood or BM cell progeny of transduced cells were tracked using the GFP and/or YFP fluorescence in vivo (41). For transplantation of secondary mice, 1×10^6 to 2×10^6 cells of diseased primary animals were injected into the recipients after 800 cGy irradiation.

CFU-S and ΔCFU-S assay. 5-FU–mobilized primary BM cells from F_1 (PepC3) donor mice were retrovirally transduced with AML1-ETO; AML1-ETO-L148D; FLT3-LM; FLT3-LM-KD; both AML1-ETO and FLT3-LM; both AML1-ETO and FLT3-LM-KD; or both AML1-ETO-L148D and FLT3-LM. Cells transfected with the empty GFP vector served as control. Successfully transduced cells were isolated 48 hours after termination of infection by FACS (FACSVantage; BD). To assess initial (day 0) CFU-S numbers, purified cell populations were injected into lethally irradiated F_1 (B6C3) recipient mice 96 hours after the start of infection (45). To study the effect of the selective protein tyrosine kinase inhibitor PKC412 on double-positive cells, freshly sorted cells were cultured in transplant medium with 0 and 100 nM PKC412. After 48 hours, the cells were injected into lethally irradiated mice as described above, and the day 0 equivalent of the CFU-S frequency was calculated for both experimental groups. In this ΔCFU-S assay the base-line frequency of CFU-S is lower than in the CFU-S assay. The recovery of CFU-S cells was quantified by determination of the number of macroscopic colonies on the spleen at day 12 postinjection after fixation in Telleyesniczky's solution.

Southern blot. Genomic DNA was isolated from BM, spleen, and peripheral blood of diseased mice with DNAzol as recommended by the manufacturer (Invitrogen Corp.). Southern blot analysis was performed as previously described (45). DNA was digested with *Eco*RI and probed with a ^{32}P-labeled GFP/YFP DNA. Hybridizing bands were visualized by autoradiography.

Western blot. Protein expression of AML1-ETO, AML1-ETO-L148D, FLT3-LM, and FLT3-LM-KD was demonstrated by Western blotting using standard procedures (41). Membranes were probed with an anti-ETO polyclonal goat antibody and an anti-FLT3 polyclonal rabbit antibody (Santa Cruz Biotechnology Inc.). Protein expression of FLT3-LM showed 2 bands, as previously reported: in detail, the FLT3 receptor occurs in 2 different forms due to glycosylation that can be resolved in SDS-PAGE gradient gels — a 158- to 160-kDa membrane-bound protein that is glycosylated at N-linked glycosylation sites in the extracellular domain and an unglycosylated 130- to 143-kDa protein that is not membrane bound (50–52). Phosphorylation of FLT3 was tested by Western blot using 293T cells that were starved for 12 hours at 37°C, 5% CO_2. After cell harvesting and lysis, 300 μg of the lysates was immunoprecipitated with polyclonal rabbit anti-FLT3 antibody (s-18; Santa Cruz Biotechnology Inc.). Immunoprecipitates were analyzed by SDS-PAGE with mouse monoclonal anti-phosphotyrosine antibody (PY-99; Santa Cruz Biotechnology Inc.) and reprobed with anti-FLT3 antibody.

Histology. For histological analyses, sections of selected organs were prepared and stained at the Academic Pathology Laboratory, GSF (Munich, Germany), using standard protocols, as previously described (41). The mast cell–specific tryptase and the CD117 antibody were purchased from Dako-Cytomation. All the tumors were histopathologically classified according to the Bethesda proposals for classification on nonlymphoid and lymphoid hematopoietic neoplasms in mice (53, 54).

Statistical analysis. Data were evaluated using the 2-tailed Student's *t* test for dependent or independent samples (Microsoft Excel 2002, Microsoft Corp.). Differences with *P* values less than 0.05 were considered statistically significant.

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1. Look, A.T. 1997. Oncogenic transcription factors in the human acute leukemias. *Science.* **278**:1059–1064.
2. Rowley, J.D. 1999. The role of chromosome translocations in leukemogenesis. *Semin. Hematol.* **36**:59–72.
3. Gilliland, D.G., and Tallman, M.S. 2002. Focus on acute leukemias. *Cancer Cell.* **1**:417–420.
4. Schnittger, S., et al. 2002. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood.* **100**:59–66.
5. Gilliland, D.G. 2002. Molecular genetics of human leukemias: new insights into therapy [review]. *Semin. Hematol.* **39**:6–11.
6. Higuchi, M., et al. 2002. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. *Cancer Cell.* **1**:63–74.
7. Yuan, Y., et al. 2001. AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. *Proc. Natl. Acad. Sci. U. S. A.* **98**:10398–10403.
8. de Guzman, C.G., et al. 2002. Hematopoietic stem cell expansion and distinct myeloid developmental abnormalities in a murine model of the AML1-ETO translocation. *Mol. Cell. Biol.* **22**:5506–5517.
9. Fenske, T.S., et al. 2004. Stem cell expression of the AML1/ETO fusion protein induces a myeloproliferative disorder in mice. *Proc. Natl. Acad. Sci. U. S. A.* **101**:15184–15189.
10. Nucifora, G., et al. 1993. Detection of DNA rearrangements in the AML1 and ETO loci and of an AML1/ETO fusion mRNA in patients with t(8;21) acute myeloid leukemia. *Blood.* **81**:883–888.
11. Downing, J.R. 1999. The AML1-ETO chimaeric transcription factor in acute myeloid leukaemia: biology and clinical significance. *Br. J. Haematol.* **106**:296–308.
12. Peterson, L.F., and Zhang, D.E. 2004. The 8;21 translocation in leukemogenesis. *Oncogene.* **23**:4255–4262.
13. Wang, Q., et al. 1996. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc. Natl. Acad. Sci. U. S. A.* **93**:3444–3449.
14. Sun, W., and Downing, J.R. 2004. Haploinsufficiency of AML1 results in a decrease in the number of LTR-HSCs, while simultaneously inducing an increase in more mature progenitors. *Blood.* **104**:3565–3572.
15. Licht, J.D. 2001. AML1 and the AML1-ETO fusion protein in the pathogenesis of t(8;21) AML. *Oncogene.* **20**:5660–5679.
16. Pabst, T., et al. 2001. AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukemia. *Nat. Med.* **7**:444–451.
17. Mulloy, J.C., et al. 2002. The AML1-ETO fusion protein promotes the expansion of human hematopoietic stem cells. *Blood.* **99**:15–23.
18. Schwieger, M., et al. 2002. AML1-ETO inhibits maturation of multiple lymphohematopoietic lineages and induces myeloblast transformation in synergy with ICSBP deficiency. *J. Exp. Med.* **196**:1227–1240.
19. Rhoades, K.L., et al. 2000. Analysis of the role of AML1-ETO in leukemogenesis, using an inducible transgenic mouse model. *Blood.* **96**:2108–2115.
20. Nucifora, G., Larson, R.A., and Rowley, J.D. 1993. Persistence of the 8;21 translocation in patients with acute myeloid leukemia type M2 in long-term remission. *Blood.* **82**:712–715.
21. Miyamoto, T., Weissman, I.L., and Akashi, K. 2000. AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proc. Natl. Acad. Sci. U. S. A.* **97**:7521–7526.
22. Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. 2001. Stem cells, cancer, and cancer stem cells. *Nature.* **414**:105–111.
23. Miyamoto, T., et al. 2002. Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev. Cell.* **3**:137–147.
24. Tiacci, E., et al. 2004. PAX5 expression in acute leukemias: higher B-lineage specificity than CD79a and selective association with t(8;21)-acute myelogenous leukemia. *Cancer Res.* **64**:7399–7404.
25. Kelly, L.M., et al. 2002. PML/RARalpha and FLT3-ITD induce an APL-like disease in a mouse model. *Proc. Natl. Acad. Sci. U. S. A.* **99**:8283–8288.
26. Kelly, L.M., et al. 2002. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. *Blood.* **99**:310–318.
27. Ono, R., et al. 2005. Dimerization of MLL fusion proteins and FLT3 activation synergize to induce multiple-lineage leukemogenesis. *J. Clin. Invest.* **115**:919–929. doi:10.1172/JCI200522725.
28. Armstrong, S.A., et al. 2002. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat. Genet.* **30**:41–47.
29. Armstrong, S.A., et al. 2004. FLT3 mutations in childhood acute lymphoblastic leukemia. *Blood.* **103**:3544–3546.
30. Taketani, T., et al. 2004. FLT3 mutations in the activation loop of tyrosine kinase domain are frequently found in infant ALL with MLL rearrangements and pediatric ALL with hyperdiploidy. *Blood.* **103**:1085–1088.
31. Grisolano, J.L., O'Neal, J., Cain, J., and Tomasson, M.H. 2003. An activated receptor tyrosine kinase, TEL/PDGFbetaR, cooperates with AML1/ETO to induce acute myeloid leukemia in mice. *Proc. Natl. Acad. Sci. U. S. A.* **100**:9506–9511.
32. Levis, M., et al. 2005. Internal tandem duplications of the FLT3 gene are present in leukemia stem cells. *Blood.* doi:10.1182/blood-2004-05-1902.
33. Boissel, N., et al. 2004. Incidence and prognosis of RTKs and RAS mutations in CBF AML. A retrospective study of French adult ALFA and pediatric LAME trials [abstract]. *Blood.* **103**:2022.
34. Cairoli, R., et al. 2004. Prognostic impact of C-Kit mutations in core binding factor-leukemia [abstract]. *Blood.* **103**:2013.
35. Wang, Y.Y., et al. 2005. AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec. *Proc. Natl. Acad. Sci. U. S. A.* **102**:1104–1109.
36. Bennett, J.M., et al. 1976. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br. J. Haematol.* **33**:451–458.
37. Harris, N.L., et al. 1999. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting. Airlie House, Virginia, November 1997. *J. Clin. Oncol.* **17**:3835–3849.
38. Schnittger, S., et al. 2003. New score predicting for prognosis in PML-RARA+, AML1-ETO+, or CBFBMYH11+ acute myeloid leukemia based on quantification of fusion transcripts. *Blood.* **102**:2746–2755.
39. Schnittger, S., Wormann, B., Hiddemann, W., and Griesinger, F. 1998. Partial tandem duplications of the MLL gene are detectable in peripheral blood and bone marrow of nearly all healthy donors. *Blood.* **92**:1728–1734.
40. Spiekermann, K., et al. 2002. A new and recurrent activating length mutation in exon 20 of the FLT3 gene in acute myeloid leukemia. *Blood.* **100**:3423–3425.
41. Rawat, V.P., et al. 2004. Ectopic expression of the homeobox gene Cdx2 is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **101**:817–822.
42. Riley, J., et al. 1990. A novel, rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. *Nucleic Acids Res.* **18**:2887–2890.
43. Imren, S., et al. 2004. High-level β-globin expression and preferred intragenic integration after lentiviral transduction of human cord blood stem cells. *J. Clin. Invest.* **114**:953–962. doi:10.1172/JCI200421838.
44. Kern, W., et al. 2004. Determination of relapse risk based on assessment of minimal residual disease during complete remission by multiparameter flow cytometry in unselected patients with acute myeloid leukemia. *Blood.* **104**:3078–3085.
45. Pineault, N., et al. 2003. Induction of acute myeloid leukemia in mice by the human leukemia-specific fusion gene NUP98-HOXD13 in concert with Meis1. *Blood.* **101**:4529–4538.
46. Griffith, J., et al. 2004. The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. *Mol. Cell.* **13**:169–178.
47. Lenny, N., Meyers, S., and Hiebert, S.W. 1995. Functional domains of the t(8;21) fusion protein, AML-1/ETO. *Oncogene.* **11**:1761–1769.
48. Spiekermann, K., et al. 2003. The protein tyrosine kinase inhibitor SU5614 inhibits FLT3 and induces growth arrest and apoptosis in AML-derived cell lines expressing a constitutively activated FLT3. *Blood.* **101**:1494–1504.
49. Spiekermann, K., Faber, F., Voswinckel, R., and Hiddemann, W. 2002. The protein tyrosine kinase inhibitor SU5614 inhibits VEGF-induced endothelial cell sprouting and induces growth arrest and apoptosis by inhibition of c-kit in AML cells. *Exp. Hematol.* **30**:767–773.
50. Stirewalt, D.L., and Radich, J.P. 2003. The role of FLT3 in haematopoietic malignancies. *Nat. Rev. Cancer.* **3**:650–665.
51. Lyman, S.D., et al. 1993. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell.* **75**:1157–1167.
52. Carow, C.E., et al. 1996. Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. *Blood.* **87**:1089–1096.
53. Kogan, S.C., et al. 2002. Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. *Blood.* **100**:238–245.
54. Morse, H.C., 3rd, et al. 2002. Bethesda proposals for classification of lymphoid neoplasms in mice. *Blood.* **100**:246–258.

Acute myeloid leukemia is propagated by a leukemic stem cell with lymphoid characteristics in a mouse model of *CALM/AF10*-positive leukemia

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Summary

A challenge for the development of therapies selectively targeting leukemic stem cells in acute myeloid leukemia (AML) is their similarity to normal hematopoietic stem cells (HSCs). Here we demonstrate that the leukemia-propagating cell in murine *CALM/AF10*-positive AML differs from normal HSCs by B220 surface expression and immunoglobulin heavy chain rearrangement. Furthermore, depletion of $B220^+$ cells in leukemic transplants impaired development of leukemia in recipients. As in the murine model, human *CALM/AF10*-positive AML was characterized by CD45RA (B220)-positive, *IG* DH-JH rearranged leukemic cells. These data demonstrate in a murine leukemia model that AML can be propagated by a transformed progenitor with lymphoid characteristics, which can be targeted by antibodies that do not crossreact with normal HSCs.

Introduction

For most cancers, the cell that propagates tumor growth and is thought to play a key role in recurrence of disease is unknown. The study of normal and malignant hematopoiesis has formed a roadmap for the detailed analysis of the concept of the tumor-initiating cell and has demonstrated that, in leukemia, the malignant clone is organized in a hierarchy in which only a small subpopulation of cells—the leukemic stem cells (LSCs)—are able to initiate and propagate the disease (Hope et al., 2004). In AML most studies indicate that malignant transformation targets cells within the hematopoietic stem cell compartment. However, more recent data demonstrated that multipotential progenitors or more committed myeloid progenitors can also acquire leukemic stem cell properties when transformed by appropriate oncogenes (Cozzio et al., 2003; So et al., 2003). These reports clearly demonstrated that cells other than hematopoietic stem cells can be the target of fully transforming oncogenes, which are characterized by their ability to transfer self-renewal potential to committed stages of differentiation (Huntly et al., 2004; Jamieson et al., 2004). The identification of leukemic stem cell properties is a key step for an improved understanding of the biology of acute leukemias and might also have clinical implications: because of its essential role for the initiation and maintenance of the leukemic clone, a curative therapeutic strategy should be able to eradicate the LSC in the diseased patients.

SIGNIFICANCE

The identification of tumor stem cells is one of the major goals of cancer research. We report on a murine model demonstrating that myeloid malignancies such as acute myeloid leukemia can be propagated by a transformed progenitor with lymphoid characteristics. These data extend our previous knowledge about leukemia stem cell candidates in acute leukemia and indicate that leukemia-propagating cells in AML might differ from the myeloid blast population as well as from the normal hematopoietic stem cell in their surface antigen profile. These findings suggest that such leukemic stem cell candidates can be targeted with antibodies that spare the normal stem cell pool. This may pave the way for innovative antibody-based therapeutic strategies in this disease.

At the same time, such a strategy should spare normal hematopoietic stem cells (HSCs) to avoid intolerable therapy-associated stem cell toxicity. However, the major challenge for the development of such therapeutic approaches is the often striking similarity between leukemic and normal HSC, e.g., with regard to their cell surface markers (Reya et al., 2001; Warner et al., 2004). The limitations of the therapeutic tools available today are well known: although standard chemotherapy is highly effective in inducing remissions in patients with AML, the majority of patients finally relapse and succumb to this disease (Hiddemann et al., 2005). One of the reasons for treatment failure is that even dose-intensive conventional chemotherapy is not able to efficiently target the LSC, demonstrated, for example, by the observation that patients in morphological complete remission harbor $CD34^+/CD38^-$ progenitor cells with the leukemia-specific genetic alteration in their bone marrow (Feuring-Buske et al., 1999). A major step toward the development of stem cell-targeted AML therapies would be the identification of key differences between normal HSCs and the LSC. It has been reported that the LSC differs from its normal counterpart by the expression of CD90 or the interleukin-3α receptor, and in a preclinical NOD/SCID mouse model a diphtheria toxin (DT) human interleukin 3 fusion protein (DT388IL3) was able to selectively kill LSCs in patients with different subtypes of AML (Blair et al., 1997; Feuring-Buske et al., 2002; Jordan et al., 2000).

The recent reports demonstrating that acute leukemias can also arise from transformed committed progenitor cells might improve the chances to define biological differences between the LSC and the HSC (Huntly et al., 2004; So et al., 2003). However, leukemic stem cell candidates such as multipotent progenitor cells (MPPs) might be difficult to distinguish from normal HSCs, although first strategies were established in mice to discriminate MPPs from long-term repopulating stem cells by immunophenotype (Forsberg et al., 2005; Morrison et al., 1997). In this report we present experimental evidence that acute myeloid leukemia can be propagated by a transformed progenitor, which differs from normal hematopoietic stem cells by surface expression of B220 and immunoglobulin heavy chain (*IG* DH-JH) rearrangement. We established a murine model of *CALM/AF10* (C/A)-positive acute myeloid leukemia using the bone marrow transplantation model and retroviral gene transfer to constitutively express the fusion gene in hematopoietic progenitor cells. The established model well predicted our findings in patients with *C/A*-positive AML, who showed a cell population expressing CD45RA, the human homolog of B220. This human $CD45RA^+$ cell population displayed *IG* DH-JH rearrangements, was positive for the fusion gene as detected by fluorescence in situ hybridization (FISH), and was able to form CFU-blast colonies in vitro. In these patients the fusion gene is formed by the translocation t(10;11) (p13;q14), which involves the *CALM* gene (associated with clathrin-mediated endocytosis) and *AF10* (a putative transcription factor). This fusion gene is found in a variety of leukemias, which are all characterized by a very poor prognosis (Dreyling et al., 1998). Of note, the malignant phenotypes associated with C/A span AML, undifferentiated leukemia, acute lymphoblastic leukemia, and T cell lymphoma, thus making it appealing to determine the nature of the leukemic stem cell candidate(s) in *C/A*-positive disease. The results of this study demonstrate in a murine leukemia model that a transformed progenitor cell population with lymphoid characteristics can play a key role in propagating *C/A*-positive AML.

Results

CALM/AF10 induces acute myeloid leukemia in mice

To test the impact of constitutive expression of *C/A* on murine hematopoietic development, we employed the murine bone marrow (BM) transplantation model. The fusion gene was expressed in a murine stem cell virus (MSCV)-based retroviral construct carrying the *C/A* cDNA upstream of an internal ribosomal entry site (IRES)-green fluorescence protein (GFP) cassette that has been shown to efficiently transduce hematopoietic stem and progenitor cells (Figures S1A–S1C in the Supplemental Data available with this article online).

All mice (n = 13) transplanted with highly purified GFP^+ *C/A* transduced cells (median retroviral transduction efficiency of 5.7, range 1.2%–16.4%) with or without nontransduced helper cells developed a disease after a median of 110 days posttransplantation (range 46–366) (Figure 1A) and suffered from cachexia, lethargy, and shortness of breath. Detailed analyses of the hematopoietic compartments showed a more than 10-fold elevated white blood cell count in the peripheral blood (PB) of all animals with a median of 4.9×10^7 cells/ml (range 3.2–8.5×10^7) compared to the GFP control (median 0.48, range 0.32–0.8×10^7) ($p < 0.002$). Furthermore, mice were anemic, with a 7.3-fold reduced median red blood cell count of 0.75×10^9 cells (range 0.6–1.25×10^9) versus 5.5×10^9 (range 4.8–7×10^9) in the control mice ($p < 0.0001$), and suffered from splenomegaly (Table 1). Panoptic staining of PB smears and cytospin preparations of BM and spleen cells revealed an accumulation of blasts accounting for medians of 32%, 40%, and 39%, respectively, of all cells (Table 1; Figure 1B). The spleen showed a massive infiltration with myeloid cells (median of 86.9% $Mac1^+$ cells [range 72.8%–91.1%] versus 12% in the control [range 7%–26.5%] and 62.5% $Gr1^+$ cells [range 40%–76.2%] compared to 9.5% in the control [range 7.5%–11.3%]) (Figure 1B). Histological sections demonstrated infiltration of blasts in multiple nonhematopoietic organs, including the brain (Figure 1C). Immunohistochemistry showed positivity of the blasts for myeloperoxidase (*MPO*) and chloracetate esterase and negativity for B220 and CD3, indicating the myeloid nature of the population (Figure 1D). Taken together, the mice suffered from acute myeloid leukemia according to the Bethesda proposal for classification of nonlymyphoid and lymphoid hematopoietic neoplasms in mice (Kogan et al., 2002; Morse et al., 2002).

Transplantation of BM cells of leukemic mice into secondary recipients (n = 22) rapidly induced leukemia with the same characteristics after a median of 15 days (range 14–21 days) posttransplantation (Figure 1A). Bone marrow aspirations of anesthetized primary recipients 8 weeks posttransplantation did not show any myeloproliferative or lymphoproliferative syndrome before the onset of leukemia (n = 5). Analysis of proviral integration sites demonstrated oligoclonal disease in primary and secondary mice (Figure S2). Sequencing of the retroviral integration sites (n = 18) in the diseased mice showed 10 out of 18 sites in or near regions described as common integration sites (CIS) in the RTCGD database (http://rtcgd.ncifcrf.gov/) (Akagi et al., 2004). No recurrent integration sites or any association of the leukemic phenotype with individual proviral integrations were observed (Table S1). In summary, these data demonstrated that constitutive expression of *CALM/AF10* induced short-latency AML in transplanted animals.

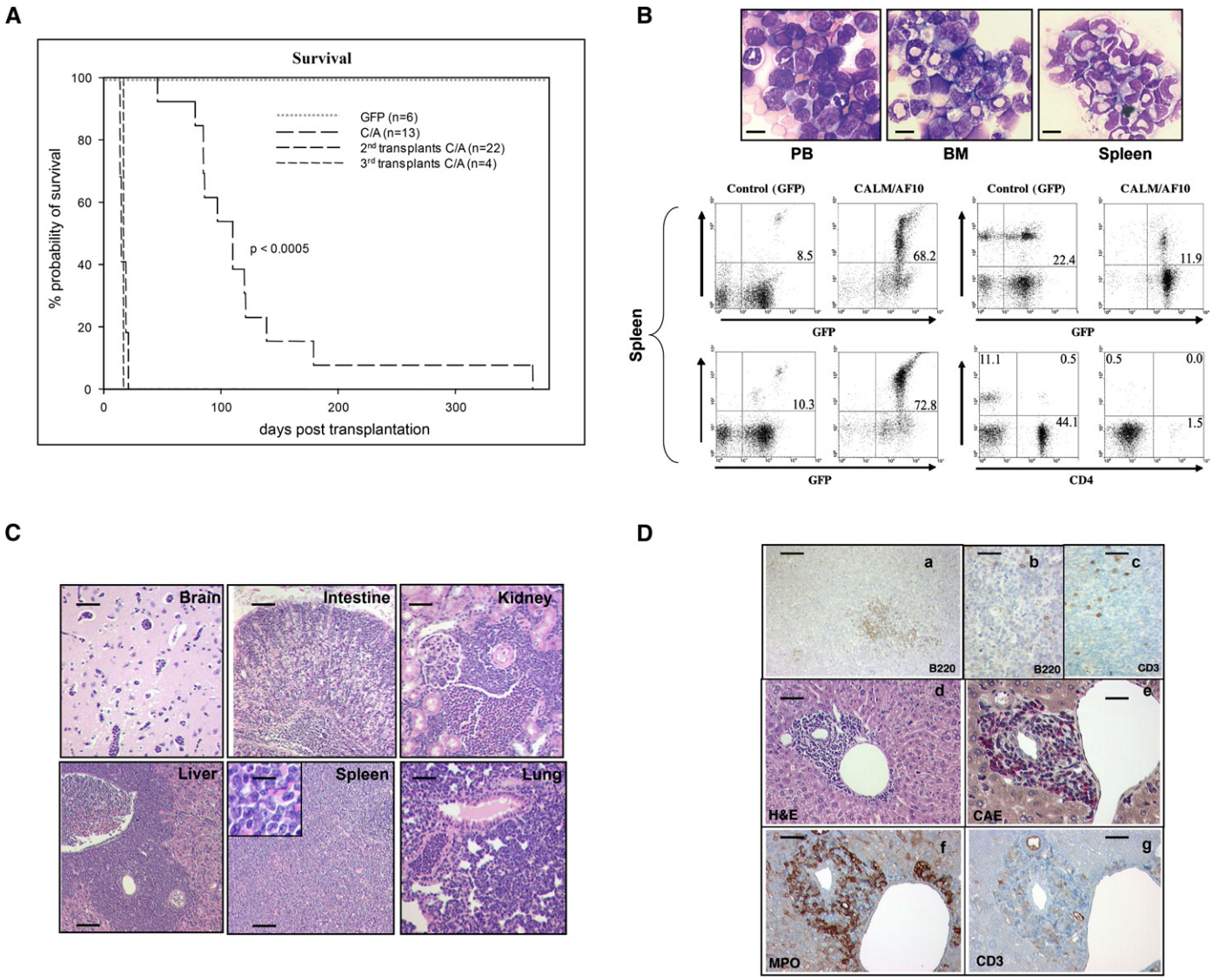

Figure 1. CALM/AF10 induces transplantable AML

A: Survival curve of mice transplanted with BM cells expressing *CALM/AF10* (n = 13, originating from five independent sets of transduction experiments). The control group was transplanted with BM infected with the GFP empty retrovirus (n = 6). The survival time of secondary recipient mice, transplanted with BM from diseased primary *CALM/AF10* mice or tertiary recipient mice injected with BM from leukemic secondary mice, is indicated. C/A, CALM/AF10.

B: Cytospin preparations from PB, BM, and spleen and immunophenotype of the spleen of a representative leukemic *CALM/AF10* mouse in comparison to a GFP control animal. Cells were stained for the myeloid markers Gr1 and Mac1 and the lymphoid markers B220, CD4, and CD8. The proportion of positive cells within the GFP^+ compartment is indicated; the CD4/CD8 costaining was gated for GFP^+ cells. BM, bone marrow; PB, peripheral blood. Scale bar, 10 μm.

C: Histological analysis of diseased *CALM/AF10* mice. Blast infiltration in the different organs is shown (brain, intestine, glomerular and tubular areas of the kidney, sinusoidal and portal areas of the liver, spleen, and lungs) Scale bar, 100 μm; scale bar in inset picture of spleen, 25 μm.

D: Histochemical and immunohistological analysis of the spleen and liver of a diseased *CALM/AF10* mouse. Anti-B220 (**Da** and **Db**) and anti-CD3 staining (**Dc**) of spleen sections and hematoxylin-eosin (**Dd**), CAE (**De**), MPO (**Df**), and anti-CD3 staining (**Dg**) of liver sections. MPO, myeloperoxidase; CAE, N-acetyl-chloroacetate esterase. Scale bars, 100 μm in **Db**–**Dg**, 25 μm in **Da**, 50 μm in **Dd**.

CALM/AF10-induced leukemia is propagated by a transformed $B220^+$, immunoglobulin DH-JH rearranged progenitor cell

More detailed analyses of the leukemic BM population (GFP^+ cells) revealed that, in addition to a majority of cells expressing myeloid markers (MM) ($Mac1^+$ cells, 82.9% [±8.6]; $Gr1^+$ cells, 86.4% [±3.7]), there was a small $B220^+/MM^-$ cell population in all animals obtained from five independent sets of experiments, which accounted on average for 6.7% (±2.1%) of the cells, comparable to the proportion of these cells in control mice (on average 9.4% ± 3%). An average of 26.0% (±8.6) and 32.5% (±13.2) of the BM cells coexpressed B220 with Mac1 or B220 with Gr1, respectively (n = 4) ($B220^+/MM^+$). Highly purified cells of the $B220^+/MM^-$ population had an immature, blast-like appearance, whereas the $B220^-/MM^+$ population included blasts as well as terminally differentiated myeloid cells (Figure 2A). Interestingly, all three subpopulations, including the cells expressing solely the MMs Mac1 or Gr1, showed DJ

Table 1. Hematological parameters in representative experimental mice

Mouse no.	Retroviral construct	Day of sacrifice	RBCs/ml × 10^9	WBCs/ml × 10^7	Spleen weight (mg)	Percent BM blasts	Percent spleen blasts	Percent PB blasts	IgH DJ rearrangement
1	C/A	10	0.75	3.5	700	30	32	27	ND
2	C/A	78	0.75	5.0	400	40	30	33	+
3	C/A	85	0.6	8.5	250	65	60	61	+
4	C/A	121	0.82	5.2	300	38	38	31	+
5	C/A	85	0.6	4.8	200	78	40	ND	+
6	C/A	86	1.25	3.2	400	ND	42	32	+
7[a]	C/A	67	4	2.5	240	32	29	33	+
8[a]	C/A	98	2.65	ND	350	36	ND	28	–
9	GFP	90	6	0.45	150	0	0	0	ND
10	GFP	157	4.8	0.32	200	0	0	0	ND
11	GFP	85	5	0.36	200	0	0	0	ND
12[b]	GFP	ND	4.8	0.76	ND	1	0	0	ND
13[b]	GFP	ND	6.4	0.8	ND	0	0	0	ND
14	GFP	90	7	0.5	51	2	0	0	ND

C/A, CALM/AF10; ND, not determined; RBC, red blood cell; WBC, white blood cell; BM, bone marrow; PB, peripheral blood.
[a]Mice were injected with highly purified B220-negative progenitor cells with IgH in germline configuration.
[b]Determined by bone marrow aspiration and bleeding of the mouse.

rearrangements of the heavy chain of the IgH locus, indicating that the myeloid population originated from DJ rearranged cells (Figure 2A). The bulk leukemic population isolated from diseased mice showed unlimited IL3-dependent growth in vitro and retained its characteristic phenotype with appearance of the $B220^+/MM^-$ as well as the $B220^+/MM^+$ and the $B220^-/MM^+$ population (n = 3).

To determine whether the growth potential differed between the three subpopulations, single cells of the $B220^+/MM^-$, $B220^+/MM^+$, and the $B220^-/MM^+$ population were sorted and tested for their proliferative capacity: strikingly, only the $B220^+/MM^-$ population displayed high clonal proliferative potential at the single-cell level, with a mean seeding efficiency of 29.3% compared to 1% in the other two populations (Figure 2B). When competitive transcriptional profiling of highly purified $B220^+/Mac1^-$ and $B220^-/Mac1^+$ cells was performed by cDNA microarray analysis, 944 genes out of a total of 3643 present genes were differentially expressed between the two populations. The gene categories, which were significantly overrepresented in the $B220^+/Mac1^-$ cells, comprised genes linked to mitosis and S phase, to response to DNA damage, to translation, and to DNA repair and replication. In contrast, the $B220^-/Mac1^+$ cells showed overrepresentation of genes belonging to the categories cell death, cell communication, and actin cytoskeleton organization, reflecting the profound differences in the transcriptome and the proliferative status between the two populations (http://www.ncbi.nlm.nih.gov/geo/; GEO accession number GSE5030) (Figure 2C). Importantly, a single $B220^+/MM^-$ cell isolated from leukemic mice could give rise to the $B220^+/MM^+$ and the $B220^-/MM^+$ populations in vitro, further indicating the hierarchical organization of the leukemic population (Figure 2D). These $B220^-/MM^+$ cells were functionally intact myeloid cells and were able to actively phagocytose yeast cells (Figure 2D). Furthermore, all the $B220^+/MM^+$ and the $B220^-/MM^+$ cells derived from a single $B220^+/Mac1^-$ cell in vitro displayed identical genomic DJ rearrangements like the original progenitor (Figure 2E). In conclusion, these data demonstrated that the $B220^+/MM^-$ cells isolated from leukemic mice are able to give rise to DJ rearranged myeloid cells and have the highest proliferative potential at the single-cell level compared to the MM^+ subpopulations.

Next it was determined whether highly purified $B220^+/MM^-$ cells generated from a single cell in vitro would be able to cause acute myeloid leukemia in vivo: all animals (n = 3) succumbed to acute myeloid leukemia after a short latency time of 35 days posttransplantation when 1×10^6 cells per mouse were injected. Thus, the data demonstrated that single $B220^+/MM^-$ cells isolated from a leukemic mouse can initiate the leukemia of the same phenotype as bone marrow cells freshly transduced with CALM/AF10 retrovirus. In order to test the hypothesis that the leukemia-propagating cell resides in the $B220^+/MM^-$ compartment, we purified the three distinct populations from leukemic primary recipients and performed limiting dilution transplantation assays that allowed us to determine the frequency of the leukemia-propagating cell in the three different subpopulations as previously described (Kroon et al., 1998). Importantly, the frequency of the leukemia-propagating cell was 1 in 36 cells in the $B220^+/MM^-$ population as opposed to 1 in 437 cells in the $B220^+/MM^+$ and 1 in 19,717 cells in the $B220^-/MM^+$ compartment. Thus, the frequency of the leukemia-propagating cell was over 548-fold higher in the $B220^+/MM^-$ population compared to the $B220^-/MM^+$ population (Figure 3A; Table S2). Strikingly, in our model removal of the $B220^+$ cells from the leukemic transplant prevented the development of leukemia in mice, in contrast to the animals, which were transplanted with leukemic $B220^+/MM^-$ cells and died after a median of 34 days posttransplantation (Figure 3B).

To test the possibility that the leukemic stem cells would also reside in the cell compartment lacking expression of B220 and the MMs Gr1 and Mac1 ($B220^-/Gr1^-/Mac1^-$), bone marrow cells of leukemic mice were costained with B220-APC, Gr1-PE, and Mac1-PE: the proportion of this cell population in the bone marrow was very small, with a median of 0.03% (range 0.01%–0.05%) in four mice tested. Furthermore, only very few cells expressed the stem cell-associated markers Sca1 and c-Kit (Figure S3A). To test this population functionally, we highly purified this subpopulation from leukemic mice and compared the clonogenic potential of these cells with the $B220^+/MM^-$ subpopulation: while the $B220^-/Gr1^-/Mac1^-$ population was not able to form any colonies in vitro, an equal number of $B220^+/MM^-$ cells generated replatable colonies with a frequency of 85 ± 13 blast colonies per 1000 cells (data from three mice

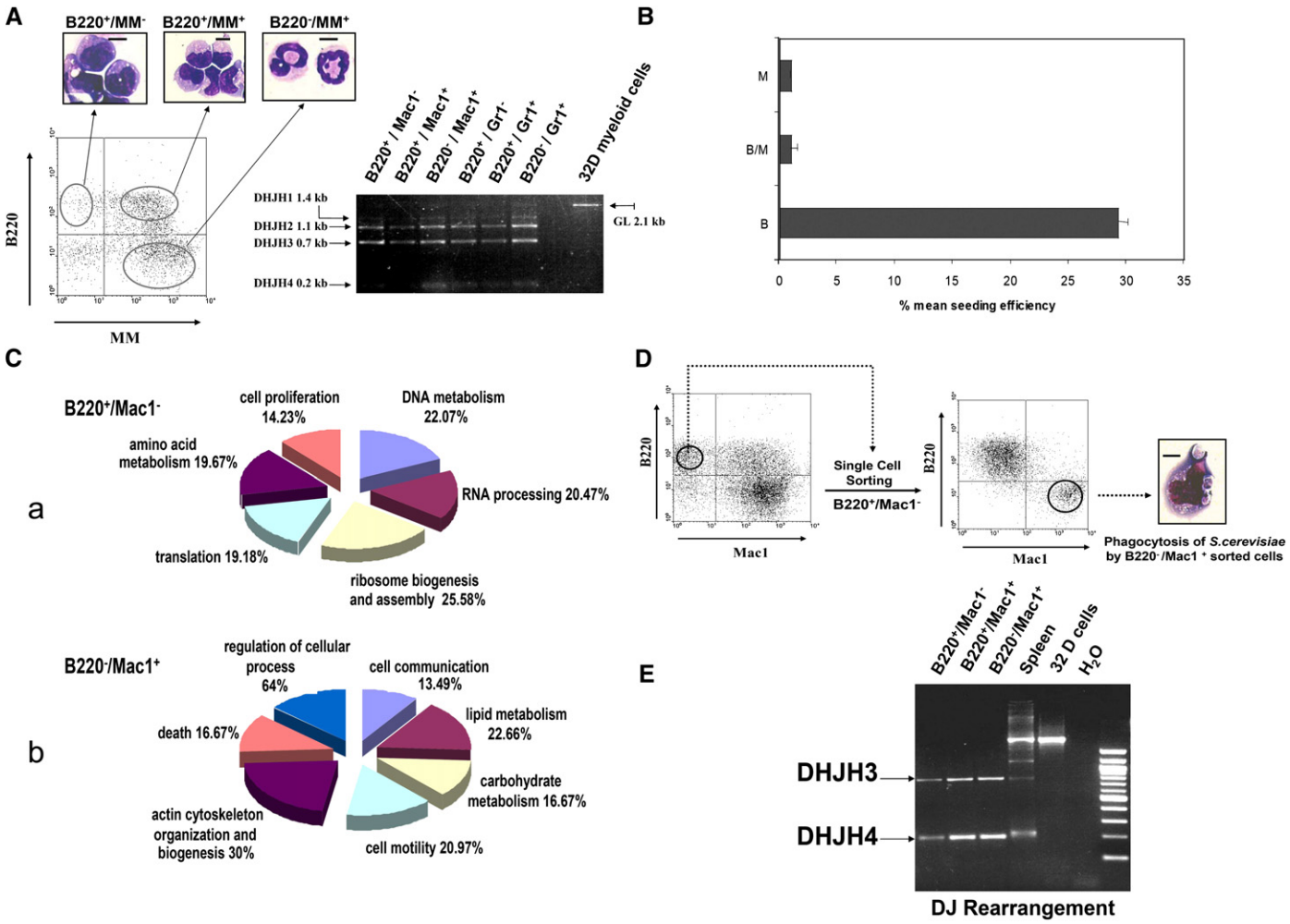

Figure 2. Transformed B220$^+$/MM$^-$ cells are IgH DH-JH positive and can generate IgH DH-JH rearranged myeloid cells at the single-cell level

A: Morphology and genomic DH-JH recombination of the immunoglobulin locus in different populations. Wright-Giemsa-stained cytospins of highly purified B220$^+$/MM$^-$, B220$^+$/MM$^+$, and B220$^-$/MM$^+$ cells and the genomic DH-JH status in different highly purified subpopulations from a representative leukemic *CALM/AF10* mouse. DH-JH status in B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, B220$^-$/Mac1$^+$, B220$^+$/Gr1$^-$, B220$^+$/Gr1$^+$, and B220$^-$/Gr1$^+$ cells is indicated; the myeloid cell line 32D was used as a DH-JH naive germline control. GL, germline; MM, myeloid marker. Scale bar, 10 μm.

B: Seeding efficiency of the different populations. Single-cell sorting of the three subpopulations, B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, and B220$^-$/Mac1$^+$, isolated from a leukemic bulk cell population was performed in 96-well plates, and single-cell growth was determined after culture for 4–5 weeks in IL3-supplemented medium. The mean seeding efficiency for each population is indicated (n = 3, plotted as mean values ± SD).

C: Distribution of gene categories significantly overrepresented in highly purified B220$^+$/Mac1$^-$ compared to B220$^-$/Mac1$^+$ cells (**Ca**) and in B220$^-$/Mac1$^+$ versus B220$^+$/Mac1$^-$ cells (**Cb**). Analysis was performed by DNA microarray-based expression profiling using a chip with 20,172 PCR-amplified, sequence-verified, gene-specific DNA fragments (LION Biosciences).

D: Phagocytosis by B220$^-$/Mac1$^+$ cells derived from a single-cell-sorted B220$^+$/Mac1$^-$ cell. Single-cell-sorted B220$^+$/Mac1$^-$ cells were expanded in vitro, and the B220$^-$/Mac1$^+$ cells generated from these cells were tested for phagocytosis of the yeast *S. cerevisiae*. A representative picture of a B220$^-$/Mac1$^+$ phagocytosing cell is shown. Scale bar, 5 μm.

E: Clonal DH-JH rearrangements in the three subpopulations. B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, and B220$^-$/Mac1$^+$ cells generated from a single B220$^+$/Mac1$^-$ sorted cell all showed the identical clonal DH-JH3 and DH-JH4 biallelic rearrangements. Genomic DNA from splenic cells from a nontransplanted mouse and the myeloid cell line 32D were used as positive and negative controls for recombination, respectively. B, B220$^+$/Mac1$^-$; B/M, B220$^+$/Mac1$^+$; M, B220$^-$/Mac1$^+$.

transplanted initially with independently transduced bone marrow; input number 100–1000 cells/dish in both arms). Importantly, the B220$^-$/Gr1$^-$/Mac1$^-$ cells did not show leukemic engraftment into secondary recipients or induced leukemia, whereas as few as 25 B220$^+$/MM$^-$ cells caused leukemic engraftment of the same phenotype as described before (input number between 25 and 113 cells for the B220$^-$/Gr1$^-$/Mac1$^-$ cells [n = 5] and an equal number of cells per mouse for the B220$^+$/MM$^-$ population [n = 5]) (Table S3). Taken together, the high frequency of the leukemia-propagating cell in the B220$^+$/MM$^-$ population, together with its ability to proliferate and to give rise to a myeloid DJ rearranged cell population, demonstrated that the leukemia-propagating cell resided in the B220$^+$/Mac1$^-$ cell compartment in this murine model.

More detailed analysis of the cell surface phenotype of the B220$^+$/MM$^-$ cells characterized them as CD43$^+$/AA4.1$^+$/CD24$^{low\text{-}pos}$/FLT3^{med}/IL-7R$^{low\text{-}neg}$, c-Kit$^{low\text{-}neg}$, CD19$^-$, and Sca1$^-$ (Figures 4A and 4B). The cells did not express CD21, CD23, sIgM, λ5, Bp1, or Igβ (Figures S4A and S4B), or CD4 or NK1.1 (data not shown). Transcriptional profiling by RT-PCR

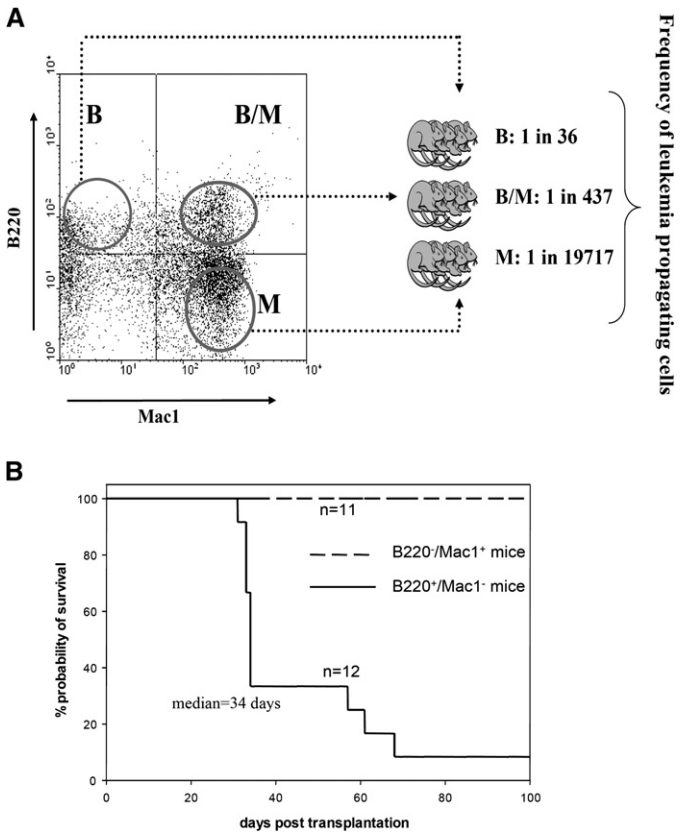

Figure 3. Frequency of leukemia-propagating cells

A: Determination of the frequency of leukemia-propagating cells. Cohorts of secondary mice were injected with highly purified B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, and B220$^-$/Mac1$^+$ populations from primary leukemic mice in a limiting dilution assay. The frequency of leukemia-propagating cells in each population is indicated. Details of cell numbers, number of mice per arm, and the median latency of disease are described in Table S2.

B: Survival of mice transplanted with leukemic BM depleted of B220$^+$ cells (B220$^-$/Mac1$^+$cells, n = 11; 50 cells [n = 4], 500 cells [n = 4], and 5000 cells [n = 3] isolated from the primary leukemic population). In comparison the survival of mice (n = 12) transplanted with B220$^+$/Mac1$^-$ cells with the same range of cell numbers (50 [n = 4], 500 [n = 4]), and 5000 cells [n = 4]) is shown.

showed lineage-promiscuous expression of the lymphoid factors *EBF*, *VpreB*, and *Rag2* and the MM *MPO* (Figure 4C; Figure S4C). Importantly, there was no transcription of *Pax5*, which is compatible with the incomplete rearrangement status at the IgH locus (data not shown) and the CD19 negativity of the cells (Figure 4B). In the B220$^+$/Mac1$^+$ and in the B220$^-$/Mac1$^+$ cells, transcripts for the M-CSF receptor became detectable, whereas transcription for *EBF* was downregulated. There was no detectable transcription of *Gata3*, *Zfpn1a3* (*Aiolos*) (Figure 4C), or *mb1* and only low expression of *λ5* transcripts (Figure S4C). In addition, we could not detect rearrangements of the TCRγ receptor, specifically Vγ 1.1-Jγ4, Vγ 2/4-Jγ1, or Vγ 5/7-Jγ1 by PCR (data not shown). The surface marker expression as well as the transcription factor expression profile collectively defined the B220$^+$/MM$^-$ population as a lymphoid progenitor (Figure S5).

In an effort to determine whether an earlier B220-negative cell could be transformed by the *CALM/AF10* fusion gene, we next analyzed whether transduction of *CALM/AF10* into highly purified B220-negative cells with IgH germline configuration resulted in the induction of AML of the same phenotype as described for the transduction of unfractionated bone marrow: two of two animals tested developed acute myeloid leukemia after transplantation of 1–2 × 10^5 B220-negative cells proven to have IgH germline configuration by PCR before injection, indicating that early B220-negative progenitors with IgH germline configuration can acquire leukemogenic potential after *CALM/AF10* transduction. However, whereas one of the AML cases showed B220$^+$ cells with IgH DJ rearrangement as described before, the other case did not display a B220$^+$ subpopulation. In addition, the leukemic bulk had a germline IgH configuration, a phenotype not observed in the transplantation assays using 5-FU-mobilized unfractioned bone marrow for viral infection (Figure S3B and Table 1). In contrast to early B220$^-$ progenitors, murine cell populations comprising pro-B cells (B220+/CD43+/CD25−; input number 2 × 10^6 cells per mouse; transduction efficiency, 10%) and pre-B cells (B220+/CD43+/CD25+ cells; input number 2 × 10^5 cells per mouse; transduction efficiency, 2%) did not acquire in vivo repopulating activity with no short- or long-term engraftment in lethally irradiated mice 4 or 8 weeks posttransplantation, respectively (data not shown).

To examine whether our findings in the murine CALM/AF10 leukemia model parallel characteristics of human *CALM/AF10*-positive AML, patient samples were analyzed for *IG* DH-JH rearrangement status and the presence of a CD45RA (the human homolog to B220) population. In addition, we further characterized the CD45RA$^+$ population by performing CFU-blast colony assays in vitro and FISH analysis for the presence of the *CALM/AF10* fusion gene. Table S4 summarizes the characteristics of patients diagnosed with AML or in one case with acute undifferentiated leukemia (AUL); two of the nine patients were children, six of nine patients had additional genetic alterations to the *CALM/AF10* t(10;11) translocation. Treatment consisted mainly of chemotherapy with high-dose Ara-C and anthracyclines, and outcome was dismal in all the patients with longer follow-up. Multiplex PCR and clonality analysis by GeneScanning techniques showed that seven out of nine AML samples demonstrated clonal *IG* DH-JH rearrangements (Table 2 and Figure 5A). We could also detect immature TCRδ (Dδ2,Vδ2) and TCRβ (TCRβ DJ) as well as TCRγ (Vγ10) rearrangements in six out of six patients tested (Table 2).

In all three patients tested, a substantial proportion of CD45RA$^+$ cells could be detected, ranging from 50.6% to 99.8% of bone marrow cells. In two of the three patients, the CD45RA-positive population coexpressed CD34. In all the patients tested, the CD34$^+$/CD45RA$^+$ cells harbored the *CALM/AF10* fusion gene as detected by FISH analysis (Figure 5Bb) (Table 3). Furthermore, CD34$^+$/CD45RA$^+$/CD10$^+$ cells, corresponding to the stage of early human lymphoid progenitors (Haddad et al., 2004), were also positive for the fusion gene (data not shown). The involvement of the CD45RA population in the malignant transformation process was further supported by the observation that the CD45RA population generated myeloid CFU-blasts (Table 3) in vitro, which showed the identical *IG* DH-JH rearrangement status at a single-colony level as detected in the myeloid bulk population (Figure 5C). To test whether early CD34$^+$/CD45RA$^-$ cells could be positive for the CALM/AF10 fusion gene, we highly purified CD34$^+$/CD45RA$^-$/lineage marker (lin)-negative (lin: CD38$^-$/CD19$^-$/CD10$^-$) cells of one patient with *C/A*-positive AML. Notably, the fusion gene could be detected in the majority of cells (92%) in this primitive

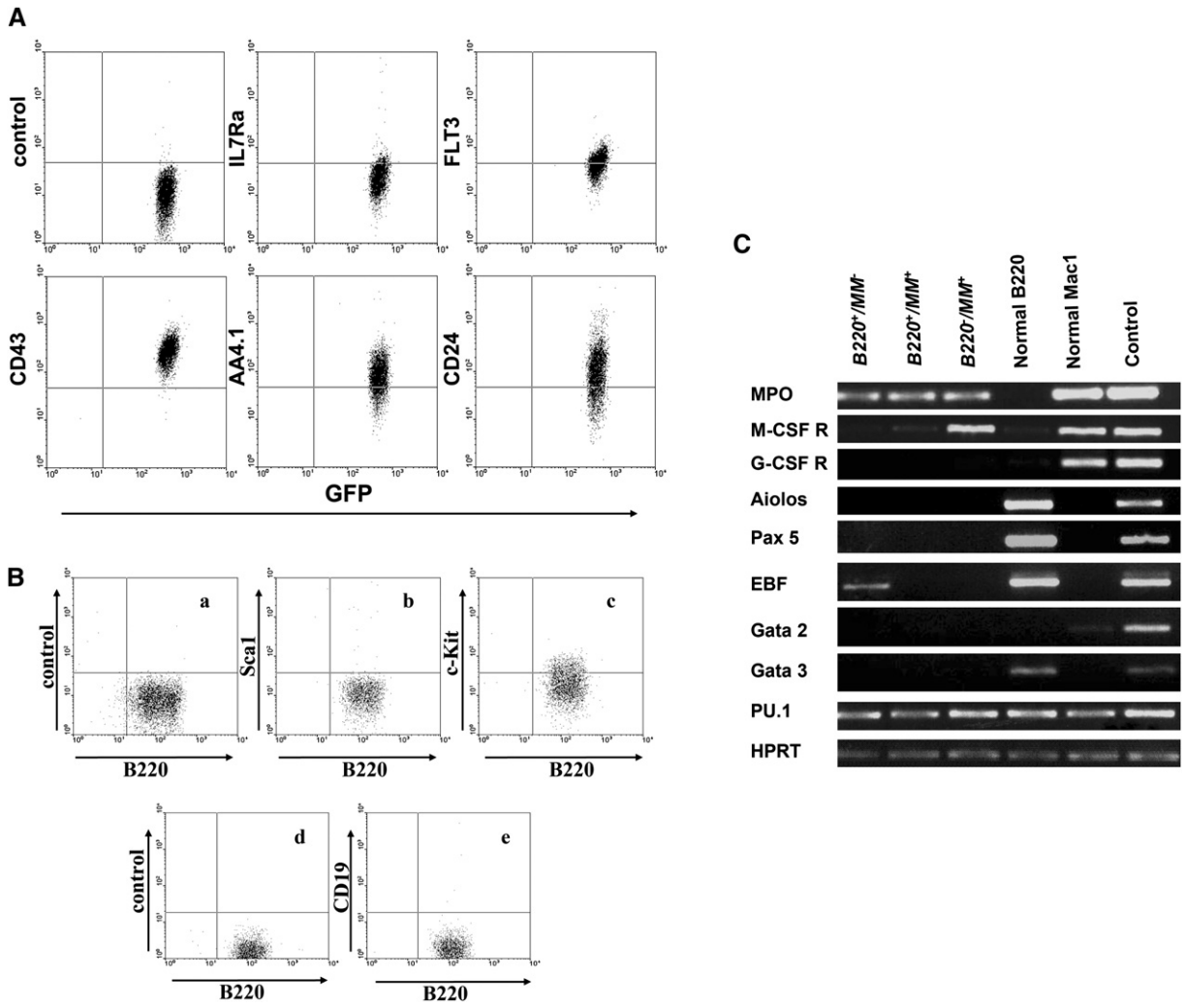

Figure 4. Leukemic B220$^+$/Mac1$^-$ cells express lymphoid markers

A: Expression of B cell-associated markers on leukemic B220$^+$/Mac1$^-$ cells: flow cytometric analysis after staining with various markers as indicated.
B: Expression of early and late markers on leukemic B220$^+$/Mac1$^-$ cells propagated in vitro for Sca1 (**Bb**) and c-Kit (**Bc**) or CD19 (**Be**); **Ba** and **Bd** are controls as indicated.
C: RT-PCR analysis of lineage associated genes. B220$^+$/MM$^-$, B220$^+$/MM$^+$, and B220$^-$/MM$^+$ cells clonally generated from a single B220$^+$/Mac1$^-$ cell were analyzed for the expression of genes associated with different hematopoietic lineages as indicated. B220$^+$ sorted cells from the spleen, Mac1$^+$ sorted cells from the bone marrow, and unsorted BM cells (all populations isolated from a nontransplanted control mouse) were used as controls for lymphoid and myeloid transcripts, respectively. MPO, myeloperoxidase; EBF, early B cell factor; HPRT, hypoxanthine phosphoribosyl transferase; *B220$^+$/MM$^-$*, B220$^+$/Mac1$^-$ cells; *B220$^+$/MM$^+$*, B220$^+$/Mac1$^+$ cells; and *B220$^-$/MM$^+$*, B220$^-$/Mac1$^+$ cells.

cell compartment (Figure 5Ba, patient number 9), indicating the involvement of early CD45RA-negative cells in the transformation process in this patient. In summary, these findings indicate that patients with *CALM/AF10*-positive AML are characterized by *IG* DH-JH rearrangements of their leukemic blasts and a CD45RA-positive population, which is positive for the leukemia-specific fusion gene and is able to generate myeloid blast colonies with the clonal *IG* DH-JH rearrangement ex vivo, reminiscent of several characteristics of murine CALM/AF10-induced AML in transplanted mice.

Discussion

Leukemias are considered to originate from a rare subset of transformed cells, which are able to initiate and maintain the disease. In particular, studies in patients with acute myeloid leukemia have shown that the transformation process involves early hematopoietic progenitor cells characterized by expression of CD34 and lack of CD38 and the different lineage markers (Blair et al., 1998; Dick, 2005). The concept that hematopoietic stem cells (HSCs) are a key target of leukemic transformation was further supported by murine models, which demonstrated that HSCs could acquire leukemic stem cell properties by forced expression of appropriate oncogenes, recurrently found in patients with AML (Huntly and Gilliland, 2005; Reya et al., 2001). Furthermore, inactivation of certain transcription factors such as JunB caused leukemia only when the targeted deletion took place in the HSC compartment and not in the more differentiated cell pool (Passegué et al., 2004). However, more recent data have provided in vivo evidence that also more

Table 2. IgH DJ and TCR rearrangements in CALM/AF10 AML patients

Patient no.	Type	IgH DJ rearrangements		TCR rearrangements		
		IgH DJ	IgH DJ sequences	TCRγ	TCRδ	TCRβ DJ
1	AML M0	monoclonal DH3	D39/ATTTTGACTGGTTATTATAAC*GT*CTGGTACTT CGATCTCTGGGGCCT/JH2	monoclonal Vγ10	Dδ2 biallelic	polyclonal
2	AML M2	monoclonal biallelic DH2 DH6	D2-15/TGGTAGCTGCTACTC*TT*TACGGTATGGAC GTCTGGGGCCA/JH6B D6-13/GGGTATAGCAG C*GGCTACAAGGGT*ACTACTGGGGCCA/JH4B	monoclonal Vγ10	Vδ2	monoclonal D1
3	AML M1	polyclonal DH1–7	—	polyclonal	Dδ2/Dδ3 biallelic	polyclonal
4	AUL	monoclonal DH1	DH1-26/TATAGTGGGAGCTACT*GTG*CTACTGG GGCCA/JH4B	monoclonal Vγ1-8 and Vγ10-11	Dδ2	monoclonal TCRβ DJ and TCRβ JA
5	AML M1	monoclonal DH7	ND	polyclonal	Dδ2 biallelic	polyclonal
6	AML M1	monoclonal DH7	ND	monoclonal Vγ10-11	Dδ2 biallelic	monoclonal TCRβ DJ
7	AML M1	monoclonal DH6	DH6-6/TATAGCAGCTCGT*TGT*ACTACTTTGACT ACTGGGGCCA/JH4B	ND	ND	ND
8	AML	monoclonal DH7	DH7-27/TAACCACTGGGGAC*CTCCCGGG* CTTTGACTACGGGGCCA/JH4B	ND	ND	ND
9	AML M5a	polyclonal DH1-7	—	ND	ND	ND

ND, not determined.

differentiated cells can acquire properties of leukemic stem cells: in a murine bone marrow transplantation model, retrovirally driven expression of the *MLL-GAS7* fusion gene induced acute leukemias with myeloid, lymphoid, or mixed phenotype. Immunophenotyping, transcriptional profiling, and the ability of the LSC to induce three distinct leukemia subtypes indicated

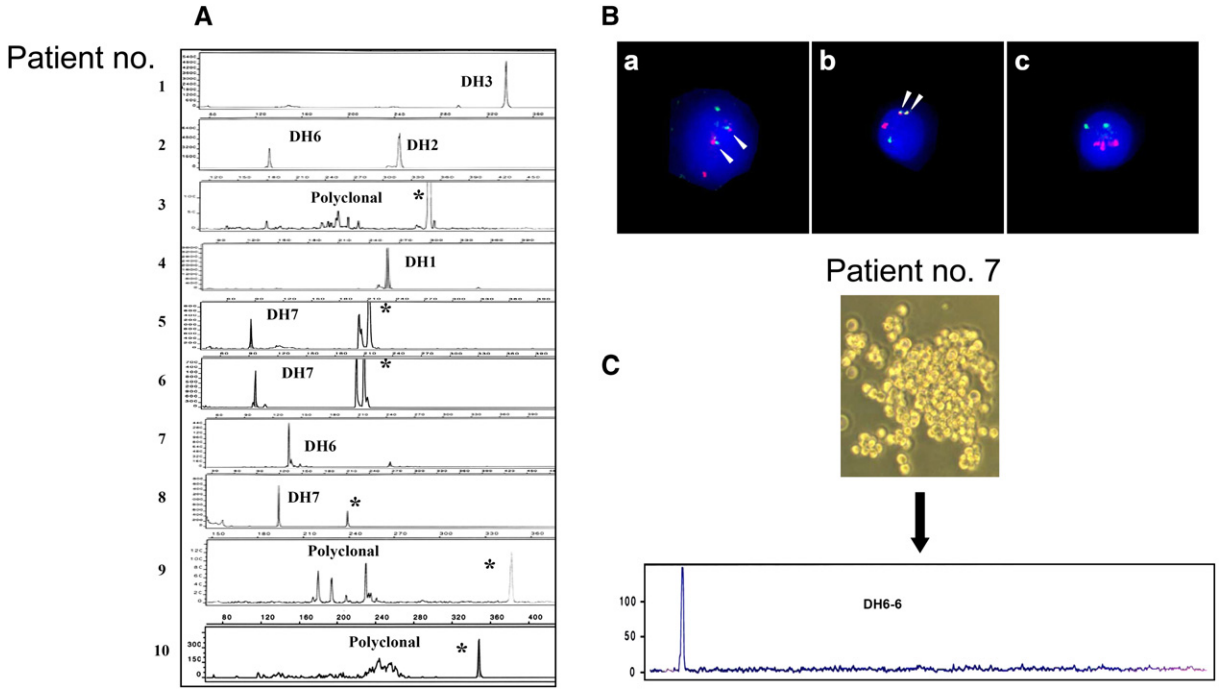

Figure 5. CALM/AF10-positive human AML samples show IgH DH-JH rearrangements and express CD45RA(B220)

A: GeneScanning analysis of IgH DH-JH rearrangements in nine cases of human CALM/AF10-positive AML using bone marrow of the leukemic patients with >80% myeloid blasts and polyclonal nonleukemic control cells. GeneScanning demonstrated a clonal IgH DH-JH rearrangement in seven out of nine cases. Unspecific background peaks resulting from annealing to nonrearranged alleles in the reactions are indicated with an asterisk.

B: Detection of the CALM/AF10 rearrangement in flow-sorted patient cells. **Ba:** A representative CD34+/CD45RA$^-$/lin$^-$ cell with the CALM/AF10 fusion, showing a normal AF10 (red) and a normal CALM (green) locus as well as two red/green signals, indicating the two reciprocal fusion genes (arrowheads) (92% of the cells were positive for the fusion gene; one representative CALM/AF10-negative cell of the same compartment is shown in **Bc**) (patient number 9). **Bb:** A representative CD34$^+$/CD45RA$^+$ cell carrying the CALM/AF10 fusions, showing the same signal pattern as in **Ba** (fusion signals are indicated by arrowheads) (patient number 7). **Bc:** A CD34$^+$/CD45RA$^-$/lin$^-$ cell without CALM/AF10 fusion showing two normal AF10 (red) and two normal CALM (green) signals (patient number 9).

C: Myeloid blast colony formation in methylcellulose of CD45RA$^+$ cells of a representative human AML patient (patient 7, magnification 100×). Lower panel shows the clonal IgH DJ rearrangement peak from a singly isolated myeloid blast colony analyzed by GeneScan. The rearrangement was identical to the rearrangements detected in the leukemic bulk of the patient (Figure 5A).

Table 3. Proportion of CD45RA-positive cells and CFU-blast frequency of C/A-positive AML patients

Patient no.	$CD34^+$/$CD45RA^+$	Overall $CD45RA^+$	CD34+/$CD45RA^-$/lin$^-$[b]	Positivity for CALM/AF10[c]		CFU-blast frequency[a]	IgH DJ of CFU-blast colonies
				$CD34^+/CD45RA^+$	$CD34^+/CD45RA^-$/lin$^-$		
3	94.52%	98.59%	0.13%	+	ND	92 ± 14	NA
7	96.21%	99.8%	0.01%	+	ND	12 ± 3	monoclonal DH6
9	1.68%	50.6%	33.07%	ND	+	ND	ND

ND, not determined; NA, not applicable.
[a]Per 10^6 $CD45RA^+$ bone marrow cells isolated from C/A AML patients (0.2–2 × 10^5 cells plated per dish).
[b]Lineage markers comprised CD38, CD19, and CD10.
[c]As determined by CALM- and AF10-specific FISH probes.

that the fusion gene targeted a multipotential progenitor in this murine model (So et al., 2003). In addition, retroviral expression of the *MLL-ENL* or *MOZ-TIF2* fusion gene in murine myeloid committed progenitor cells clearly demonstrated that committed progenitor cells can function as leukemic stem cells in these models and that transformation of HSC is not mandatory for the development and maintenance of acute leukemia (Cozzio et al., 2003; Huntly et al., 2004). The murine data presented here now provide evidence that a progenitor cell with lymphoid characteristics can propagate acute myeloid leukemia. Detailed characterization of the leukemic stem cell showed that the cells expressed B220, CD43, AA4.1, and CD24 but lacked expression of MMs as well as Sca1. Importantly, transcriptional profiling demonstrated a lineage-promiscuous expression of lymphoid factors such as *EBF*, *VpreB*, and *Rag2* (Busslinger et al., 2000) with progressive downregulation following differentiation into the $B220^+/Mac1^-$ and the $B220^-/Mac1^+$ cells together with the MM *MPO*. Of note, we did not detect transcription of *Pax5*, which is compatible with our finding that the $B220^+$ cells had an incomplete rearrangement status at the *IG* DH-JH locus and did not express CD19. Even though this progenitor showed characteristics of the early B lineage, the classification of this CALM/AF10 leukemia-propagating cell according to the different B cell classification systems remains difficult because of its leukemogenic characteristics and the potential impact of the fusion gene on its cell surface phenotype and transcriptional network. The phenotype we observed differed from the $B220^+/CD19^+$ cell stage, which is associated with *Pax5* positivity and the entire commitment to the B cell lineage (Hardy, 2003), but also from the profile of the earliest lymphoid progenitor, the common lymphoid progenitor (CLP), which lacks expression of B220 (Kondo et al., 1997).

In our model, a single transformed Pax5-negative progenitor with lymphoid characteristics was able to generate myeloid cells with IgH immunoglobulin rearrangements. Interestingly, $Pax5^{-/-}$ B cell progenitors show lineage-promiscuous transcription of B-lymphoid and myeloid genes, display a remarkable multilineage potential in vitro, and are able to be directed into the myeloid lineage (Heavey et al., 2003; Schaniel et al., 2002). In addition, the latent myeloid differentiation capacity of the CLP, which does not express *Pax5* yet, has become obvious due to the observation that it can be redirected to the myeloid lineage by retrovirally induced *IL-2Rβ* chain or *GM-CSF* receptor expression (Kondo et al., 2000). Thus, one intriguing possibility is that *CALM/AF10* instructed myeloid development of an early Pax5-negative lymphoid progenitor in our model. This commitment into the myeloid pathway was irreversible in the *C/A* model, as highly purified $B220^+/Mac1^-$ cells were not able to differentiate along the B lineage pathway in vitro on stromal OP9 feeders in lymphoid growth medium (data not shown).

The scenario that expression of *C/A* directs the lineage choice of *Pax5*-negative lymphoid progenitors is only one potential explanation for the myeloid phenotype of the leukemia. We cannot exclude that the fusion gene instructed myeloid lineage decision at the later stage of *Pax5*-positive cells, as it was recently shown that the myeloid transcription factors C/EBPα and C/EBPβ could convert *Pax5*-positive B cells into macrophages by downregulation of *Pax5* expression and upregulation of myeloid genes (Xie et al., 2004). However, we could not observe any significant downregulation of *Pax-5* expression following CALM/AF10 expression for 48 hr in the in vitro cultivated murine pro-B cell line Ba/F3 or primary murine fetal pre-B cell lines by real-time quantitative PCR and did not observe repopulating ability of murine pro-B or pre-B cells in lethally irradiated mice after transduction with CALM/AF10 (data not shown).

Another important scenario would be that the CALM/AF10 fusion gene targets a rare and early B220-negative progenitor with IgH germline configuration and activates lymphoid developmental programs in these cells, resulting in a $B220^+/MM^-$ IgH rearranged cell population with high leukemia-propagating potential as described in the murine model. Indeed, highly purified B220-negative progenitor cells with IgH germline configuration acquired leukemogenic properties in vivo in two independent experiments after infection with the CALM/AF10 retrovirus, indicating that an early progenitor cell can be transformed by the fusion gene. Interestingly, one of the mice developed an AML that lacked a $B220^+$ population and displayed an IgH germline configuration, which was never observed when unfractionated bone marrow was infected with the *CALM/AF10* retrovirus and transplanted into recipients. One explanation for this might be that C/A can target IgH rearranged $B220^+$ lymphoid progenitors, giving rise to AML with the consistent phenotype of a $B220^+$ transformed progenitor population, as well as early $B220^-$ progenitors with IgH germline configuration, resulting in AML with or without the $B220^+$ cell population.

Importantly, the experiments performed in this study do not rule out the other possibility, that the *CALM/AF10* fusion gene had an instructive effect on myeloid progenitors, initiating a lymphoid-specific program in these cells. In this scenario a myeloid cell would acquire lymphoid characteristics as a result of transformation by an appropriate oncogene. Another possibility is that *C/A* directly transformed a rare cell subset of B/macrophage progenitors, which was identified in fetal liver but also in adult bone marrow (Lacaud et al., 1998; Montecino-Rodriguez et al., 2001). However, this cell was described to be

CD19/*Pax5* positive and negative for CD45R/B220, a phenotype different than the phenotype of the LSC described here.

Of note, these different scenarios would result in the development of AML with a $B220^+$/MM-negative cell population with a very high frequency of leukemia repopulating cells, which would be accessible to an antibody-based B220 depletion. Indeed antibody-mediated depletion of the $B220^+/MM^-$ cells from leukemic transplants efficiently prevented leukemic development in mice, underlining the key role of this population for disease propagation. One implication of our findings is that in a subset of AML the leukemia-propagating cell might show a distinct cell surface and transcriptional phenotype, setting it apart from the leukemic myeloid bulk population, but also from the normal stem cell pool. In particular, the expression of lymphoid antigens would allow to discriminate this cell from the normal hematopoietic stem cells, which would facilitate the development of treatment strategies using the lymphoid surface antigens to target the leukemia-propagating cell but to spare the normal stem cell (Passegué et al., 2003). As illustrated in the murine CALM/AF10 leukemia model, such a strategy would have therapeutic potential. Depletion of the $B220^+$ transformed progenitor cells would fail to eradicate the malignant clone, if a rare leukemic B220-negative progenitor cell population existed, which gives rise to the $B220^+/MM^-$ population described. But even in this scenario, diminishing the B220-positive leukemia-propagating cells would promise clinical benefit, as mice transplanted with B220-depleted bone marrow failed to develop leukemia up to an observation time of 100 days, compared to rapid development of AML in mice transplanted with $B220^+$ transformed progenitors.

Recently, substantial progress has been made to characterize very early stages in human B cell development (Haddad et al., 2004; Hou et al., 2005; LeBien, 2000; Ryan et al., 1997). These reports have shown that similar to the murine system these early human progenitors express the B220 human homolog CD45RA, as well as CD34 and CD10, but lack CD19 and *Pax5* expression. In an effort to determine potential similarities between the murine CALM/AF10 AML and human disease, we analyzed nine patients with *CALM/AF10*-positive AML. Interestingly, the cases tested showed a CD45RA population with coexpression of CD34 in two of three patients. Furthermore, in seven out of nine patients tested, clonal IgH immunoglobulin rearrangements could be detected in the myeloid bulk. The leukemic nature of the CD45RA population in these cases was indicated by the presence of the fusion gene, its clonal IgH rearrangement, and its ability to form CFU-blast colonies ex vivo. Interestingly, in the $CD34^+/CD45RA^-/lin^-$ progenitor compartment of one patient we detected a mosaic of cells carrying the fusion gene or showing normal signal pattern by FISH, indicating that this cell compartment might be involved in the malignant transformation process.

In addition to IgH rearrangements, six of six patients were also positive for clonal rearrangements of the T cell receptor (TCR). These observations are in line with reports associating the *CALM/AF10* fusion gene with T-ALL, in particular with T-ALLs expressing the γ/δ TCR (Asnafi et al., 2003). In the murine model the constitutive expression of CALM/AF10 failed to induce T cell leukemia, and the observed myeloid leukemias did not show the TCRγ rearrangements that were analyzed (Vγ 1.1-Jγ4, Vγ 2/4-Jγ1, or Vγ 5/7-Jγ1). This could be attributable to various factors predisposing the murine model to leukemias of the myeloid lineage, such as the cell population initially used for retroviral infection; the in vitro culture conditions with IL3, IL6, and SCF stimulation before transplantation; or the level of expression of the fusion gene in the murine system compared to human disease. However, real-time PCR quantification of *CALM/AF10* transcripts showed low *CALM/AF10* expression in both the murine and human myeloid bulk (data not shown). Insertional mutagenesis was identified as an important factor in murine leukemogenesis (Akagi et al., 2004; Suzuki et al., 2002) and might explain the predisposition to AML development in the CALM/AF10 model: in an analysis of the proviral integration sites in leukemic mice, 10 of 18 detected sites were described before as common integration sites or transposon-tagged cancer genes according to the Retroviral Tagged Cancer Gene Database (RTCGD; http://RTCGD.ncifcrf.gov/) (Akagi et al., 2004). However, there were no recurrent insertion sites or a correlation of the integrations with the consistent phenotype of the leukemias in this model. As described for human *CALM/AF10*-positive T-ALLs (Dik et al., 2005; Soulier et al., 2005), the murine AML cases also displayed aberrant expression of *HoxA7* and *Meis1* (unpublished data); furthermore, we also observed deregulation of HOX genes in patients with *CALM/AF10*-positive AML (unpublished data). This indicates that deregulation of this transcription factor family is a common characteristic of CALM/AF10 leukemias independent of the phenotype.

In summary, this murine leukemia model demonstrates that a progenitor cell with lymphoid characteristics and a phenotype that differs from that of normal hematopoietic stem cells and the leukemic myeloid bulk, is able to propagate AML. These data provide a rationale for the development of therapeutic strategies targeting the leukemia-propagating stem cell without harming the normal stem cell pool and raise the hope that such innovative concepts may improve treatment outcome in a subgroup of patients with AML in the future.

Experimental procedures

Retroviral transduction of primary bone marrow cells and BM transplantation

Breeding and maintenance of the mice were conducted as described previously (Rawat et al., 2004). Production of high-titer helper-free retrovirus was carried out following standard procedures by using the ecotropic packaging cell line GP^+E86 (Schessl et al., 2005). Lethally irradiated (0.80 Gy) mice were transplanted with either highly purified $EGFP^+$ cells alone (4×10^5 cells per mouse) (FACSVantage, Becton Dickinson) or with a mixture of transduced and nontransduced cells (on average 1.76×10^5 transduced cells with 1.9×10^5 nontransduced cells per mouse). Lethally irradiated secondary and tertiary recipients were injected with 1×10^6 BM cells from a primary and secondary diseased mouse, respectively, with an equal number of nontransduced cells from a syngenic disease-free mouse bone marrow for radioprotection. Both animal and human studies were approved by the Ethics Committee of Ludwig Maximilians University and abided by the tenets of the revised World Medical Association Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm).

In order to determine the frequency of leukemia-propagating cells, $B220^+/Mac1^-$, $B220^+/Mac1^+$, and $B220^-/Mac1^+$ cells were isolated and highly purified from the BM of a leukemic primary mouse. The sort purity of these cells was analyzed with the FACSCalibur and determined to be over 98%. In each cohort 10-fold serial dilutions of these cells were injected intravenously (50 cells to 5×10^5 cells per mouse cohort) into lethally irradiated secondary recipient mice. Carrier cells (1×10^6) (nontransduced BM from a syngenic disease-free mouse) were added to each sample for radioprotection. Diseased mice were sacrificed and assessed for leukemia development. Mice that did not develop disease within 20 weeks posttransplantation were sacrificed and

tested for engraftment. The frequency of leukemia-propagating cells was calculated using Poisson statistics using the L-Calc Limiting dilution analysis software (Version 1.1 StemSoft Inc., Vancouver, Canada) (Buske et al., 2002).

Patient samples

BM samples from adult patients diagnosed with AML were analyzed. The diagnosis of AML was performed according to the French-American-British criteria and the World Health Organization classification (Bennett et al., 1985; Harris et al., 1995). Cytomorphology, cytochemistry, cytogenetics, and molecular genetics were applied in all cases as described below. The clinical features of patients are provided in Table S4. The study abided by the rules of the local internal review board and the tenets of the revised Helsinki protocol (http://www.wma.net/e/policy/b3.htm).

Sorting of cells from human AML patient samples

Frozen mononuclear peripheral blood or bone marrow cells from patients with AML were rapidly thawed and were washed twice in IMDM with 10% FBS, resuspended in PBS, and stained with various antibodies. For lineage depletion experiments, immunostaining was done with FITC-labeled anti-human CD34 (Beckman Coulter, Marseille, France) and PE-labeled anti-human CD38, PE-labeled anti-human CD19, PE-labeled anti-human CD10, and PE-labeled anti-human CD45RA (all from BD Pharmingen, Heidelberg, Germany). Cells were incubated with appropriate antibodies for 20 min on ice and washed with cold PBS prior to sorting. The trial was approved by the responsible local ethics committees, and all patients gave written informed consent according to the Helsinki Declaration.

FISH

Dual Colour Dual Fusion (DCDF) probes were developed with bacterial artificial chromosome (BAC) clones RP11-418C1 for the 5′-AF10 region (Texas Red; red in Figure 5B), RP11-249M6 for the 3′-AF10 region (red), RP11-12D16 (FITC, green) for the 5′-CALM region, and RP11-90K17 (green) for the 3′-CALM region (kindly provided by Dr. Mariano Rocchi, Bari). BAC DNA preparation, labeling, and FISH were performed as described (Crescenzi et al., 2004). Normal nuclei display a two-red and two-green (2R2G) hybridization pattern, whereas nuclei carrying the translocation display a one-red, one-green, twored/green fusion (1R1G2F) pattern. Other hybridization patterns, e.g., 1R1G1F (found in 0.5%–2% of nuclei) or 1R1G3F (found in 0.5%–1% of nuclei), were interpreted as technical artifacts.

The cells were directly sorted onto aminoalkylsilane-coated slides (SilanePrep Slides, Sigma, Germany) with or without preloading of 15 μl 3% bovine serum albumin solution in PBS and fixed in 1:3 acetic acid-methanol fixative.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, five supplemental figures, and five supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/5/363/DC1/.

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References

Akagi, K., Suzuki, T., Stephens, R.M., Jenkins, N.A., and Copeland, N.G. (2004). RTCGD: Retroviral tagged cancer gene database. Nucleic Acids Res. *32*, D523–D527.

Asnafi, V., Radford-Weiss, I., Dastugue, N., Bayle, C., Leboeuf, D., Charrin, C., Garand, R., Lafage-Pochitaloff, M., Delabesse, E., Buzyn, A., et al. (2003). CALM-AF10 is a common fusion transcript in T-ALL and is specific to the TCRγδ lineage. Blood *102*, 1000–1006.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A., Gralnick, H.R., and Sultan, C. (1985). Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann. Intern. Med. *103*, 620–625.

Blair, A., Hogge, D.E., Ailles, L.E., Lansdorp, P.M., and Sutherland, H.J. (1997). Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. Blood *89*, 3104–3112.

Blair, A., Hogge, D.E., and Sutherland, H.J. (1998). Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)/CD71(−)/HLA-DR. Blood *92*, 4325–4335.

Buske, C., Feuring-Buske, M., Abramovich, C., Spiekermann, K., Eaves, C.J., Coulombel, L., Sauvageau, G., Hogge, D.E., and Humphries, R.K. (2002). Deregulated expression of HOXB4 enhances the primitive growth activity of human hematopoietic cells. Blood *100*, 862–868.

Busslinger, M., Nutt, S.L., and Rolink, A.G. (2000). Lineage commitment in lymphopoiesis. Curr. Opin. Immunol. *12*, 151–158.

Cozzio, A., Passegue, E., Ayton, P.M., Karsunky, H., Cleary, M.L., and Weissman, I.L. (2003). Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. Genes Dev. *17*, 3029–3035.

Crescenzi, B., La Starza, R., Romoli, S., Beacci, D., Matteucci, C., Barba, G., Aventin, A., Marynen, P., Ciolli, S., Nozzoli, C., et al. (2004). Submicroscopic deletions in 5q-associated malignancies. Haematologica *89*, 281–285.

Dick, J.E. (2005). Complexity of the human acute myeloid leukemia stem cell compartment: Implications for therapy. Biol. Blood Marrow Transplant. *11*, 9–11.

Dik, W.A., Brahim, W., Braun, C., Asnafi, V., Dastugue, N., Bernard, O.A., van Dongen, J.J., Langerak, A.W., Macintyre, E.A., and Delabesse, E. (2005). CALM-AF10+ T-ALL expression profiles are characterized by overexpression of HOXA and BMI1 oncogenes. Leukemia *19*, 1948–1957.

Dreyling, M.H., Schrader, K., Fonatsch, C., Schlegelberger, B., Haase, D., Schoch, C., Ludwig, W., Loffler, H., Buchner, T., Wormann, B., et al. (1998). MLL and CALM are fused to AF10 in morphologically distinct subsets of acute leukemia with translocation t(10;11): Both rearrangements are associated with a poor prognosis. Blood *91*, 4662–4667.

Feuring-Buske, M., Haase, D., Buske, C., Hiddemann, W., and Wormann, B. (1999). Clonal chromosomal abnormalities in the stem cell compartment of patients with acute myeloid leukemia in morphological complete remission. Leukemia *13*, 386–392.

Feuring-Buske, M., Frankel, A.E., Alexander, R.L., Gerhard, B., and Hogge, D.E. (2002). A diphtheria toxin-interleukin 3 fusion protein is cytotoxic to primitive acute myeloid leukemia progenitors but spares normal progenitors. Cancer Res. *62*, 1730–1736.

Forsberg, E.C., Prohaska, S.S., Katzman, S., Heffner, G.C., Stuart, J.M., and Weissman, I.L. (2005). Differential expression of novel potential regulators in hematopoietic stem cells. PLoS Genet *1*, e28. 10.1371/journal.pgen.0010028.

Haddad, R., Guardiola, P., Izac, B., Thibault, C., Radich, J., Delezoide, A.L., Baillou, C., Lemoine, F.M., Gluckman, J.C., Pflumio, F., and Canque, B. (2004). Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. Blood *104*, 3918–3926.

Hardy, R.R. (2003). B-cell commitment: Deciding on the players. Curr. Opin. Immunol. *15*, 158–165.

Harris, N.L., Jaffe, E.S., Stein, H., Banks, P.M., Chan, J.K., Cleary, M.L., Delsol, G., De Wolf-Peeters, C., Falini, B., Gatter, K.C., et al. (1995). Lymphoma classification proposal: Clarification. Blood *85*, 857–860.

Heavey, B., Charalambous, C., Cobaleda, C., and Busslinger, M. (2003). Myeloid lineage switch of Pax5 mutant but not wild-type B cell progenitors by C/EBPα and GATA factors. EMBO J. *22*, 3887–3897.

Hiddemann, W., Spiekermann, K., Buske, C., Feuring-Buske, M., Braess, J., Haferlach, T., Schoch, C., Kern, W., Schnittger, S., Berdel, W., et al. (2005). Towards a pathogenesis-oriented therapy of acute myeloid leukemia. Crit. Rev. Oncol. Hematol. *56*, 235–245.

Hope, K.J., Jin, L., and Dick, J.E. (2004). Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. Nat. Immunol. *5*, 738–743.

Hou, Y.H., Srour, E.F., Ramsey, H., Dahl, R., Broxmeyer, H.E., and Hromas, R. (2005). Identification of a human B-cell/myeloid common progenitor by the absence of CXCR4. Blood *105*, 3488–3492.

Huntly, B.J., and Gilliland, D.G. (2005). Leukaemia stem cells and the evolution of cancer-stem-cell research. Nat. Rev. Cancer *5*, 311–321.

Huntly, B.J., Shigematsu, H., Deguchi, K., Lee, B.H., Mizuno, S., Duclos, N., Rowan, R., Amaral, S., Curley, D., Williams, I.R., et al. (2004). MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell *6*, 587–596.

Jamieson, C.H., Weissman, I.L., and Passegue, E. (2004). Chronic versus acute myelogenous leukemia: A question of self-renewal. Cancer Cell *6*, 531–533.

Jordan, C.T., Upchurch, D., Szilvassy, S.J., Guzman, M.L., Howard, D.S., Pettigrew, A.L., Meyerrose, T., Rossi, R., Grimes, B., Rizzieri, D.A., et al. (2000). The interleukin-3 receptor α chain is a unique marker for human acute myelogenous leukemia stem cells. Leukemia *14*, 1777–1784.

Kogan, S.C., Ward, J.M., Anver, M.R., Berman, J.J., Brayton, C., Cardiff, R.D., Carter, J.S., de Coronado, S., Downing, J.R., Fredrickson, T.N., et al. (2002). Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. Blood *100*, 238–245.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661–672.

Kondo, M., Scherer, D.C., Miyamoto, T., King, A.G., Akashi, K., Sugamura, K., and Weissman, I.L. (2000). Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. Nature *407*, 383–386.

Kroon, E., Krosl, J., Thorsteinsdottir, U., Baban, S., Buchberg, A.M., and Sauvageau, G. (1998). Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. EMBO J. *17*, 3714–3725.

Lacaud, G., Carlsson, L., and Keller, G. (1998). Identification of a fetal hematopoietic precursor with B cell, T cell, and macrophage potential. Immunity *9*, 827–838.

LeBien, T.W. (2000). Fates of human B-cell precursors. Blood *96*, 9–23.

Montecino-Rodriguez, E., Leathers, H., and Dorshkind, K. (2001). Bipotential B-macrophage progenitors are present in adult bone marrow. Nat. Immunol. *2*, 83–88.

Morrison, S.J., Wandycz, A.M., Hemmati, H.D., Wright, D.E., and Weissman, I.L. (1997). Identification of a lineage of multipotent hematopoietic progenitors. Development *124*, 1929–1939.

Morse, H.C., III, Anver, M.R., Fredrickson, T.N., Haines, D.C., Harris, A.W., Harris, N.L., Jaffe, E.S., Kogan, S.C., MacLennan, I.C., Pattengale, P.K., and Ward, J.M. (2002). Bethesda proposals for classification of lymphoid neoplasms in mice. Blood *100*, 246–258.

Passegué, E., Jamieson, C.H., Ailles, L.E., and Weissman, I.L. (2003). Normal and leukemic hematopoiesis: Are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc. Natl. Acad. Sci. USA *100* (*Suppl 1*), 11842–11849.

Passegué, E., Wagner, E.F., and Weissman, I.L. (2004). JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. Cell *119*, 431–443.

Rawat, V.P., Cusan, M., Deshpande, A., Hiddemann, W., Quintanilla-Martinez, L., Humphries, R.K., Bohlander, S.K., Feuring-Buske, M., and Buske, C. (2004). Ectopic expression of the homeobox gene Cdx2 is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia. Proc. Natl. Acad. Sci. USA *101*, 817–822.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105–111.

Ryan, D.H., Nuccie, B.L., Ritterman, I., Liesveld, J.L., Abboud, C.N., and Insel, R.A. (1997). Expression of interleukin-7 receptor by lineage-negative human bone marrow progenitors with enhanced lymphoid proliferative potential and B-lineage differentiation capacity. Blood *89*, 929–940.

Schaniel, C., Bruno, L., Melchers, F., and Rolink, A.G. (2002). Multiple hematopoietic cell lineages develop in vivo from transplanted Pax5-deficient pre-B I-cell clones. Blood *99*, 472–478.

Schessl, C., Rawat, V.P., Cusan, M., Deshpande, A., Kohl, T.M., Rosten, P.M., Spiekermann, K., Humphries, R.K., Schnittger, S., Kern, W., et al. (2005). The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. J. Clin. Invest. *115*, 2159–2168.

So, C.W., Karsunky, H., Passegue, E., Cozzio, A., Weissman, I.L., and Cleary, M.L. (2003). MLL-GAS7 transforms multipotent hematopoietic progenitors and induces mixed lineage leukemias in mice. Cancer Cell *3*, 161–171.

Soulier, J., Clappier, E., Cayuela, J.M., Regnault, A., Garcia-Peydro, M., Dombret, H., Baruchel, A., Toribio, M.L., and Sigaux, F. (2005). HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). Blood *106*, 274–286.

Suzuki, T., Shen, H., Akagi, K., Morse, H.C., Malley, J.D., Naiman, D.Q., Jenkins, N.A., and Copeland, N.G. (2002). New genes involved in cancer identified by retroviral tagging. Nat. Genet. *32*, 166–174.

Warner, J.K., Wang, J.C., Hope, K.J., Jin, L., and Dick, J.E. (2004). Concepts of human leukemic development. Oncogene *23*, 7164–7177.

Xie, H., Ye, M., Feng, R., and Graf, T. (2004). Stepwise reprogramming of B cells into macrophages. Cell *117*, 663–676.

Accession numbers

The microarray data in Figure 2C can be accessed from NCBI's Gene Expression Omnibus (GEO) with accession number GSE5030.