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

Vijay P. S. Rawat*†, Monica Cusan*†, Aniruddha Deshpande*†, Wolfgang Hiddemann*†, Leticia Quintanilla-Martinez‡, R. Keith Humphries§¶, Stefan K. Bohlander*†, Michaela Feuring-Buske*†, and Christian Buske*†‖

*GSF–Clinical Cooperative Group Leukemia and †Department of Medicine III, Grosshadern, Ludwig Maximilians University, 81377 Munich, Germany; ‡GSF–Department of Pathology, 85764 Neuherberg, Germany; and §Terry Fox Laboratory, BC Cancer Agency and ¶Department of Medicine, University of British Columbia, Vancouver, BC, Canada V5Z 4E6

Edited by Janet D. Rowley, University of Chicago Medical Center, Chicago, IL, and approved November 21, 2003 (received for review August 29, 2003)

**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/EVII*) (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

This paper was submitted directly (Track II) to the PNAS office.

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

‖To whom correspondence should be addressed. E-mail: buske@gsf.de.

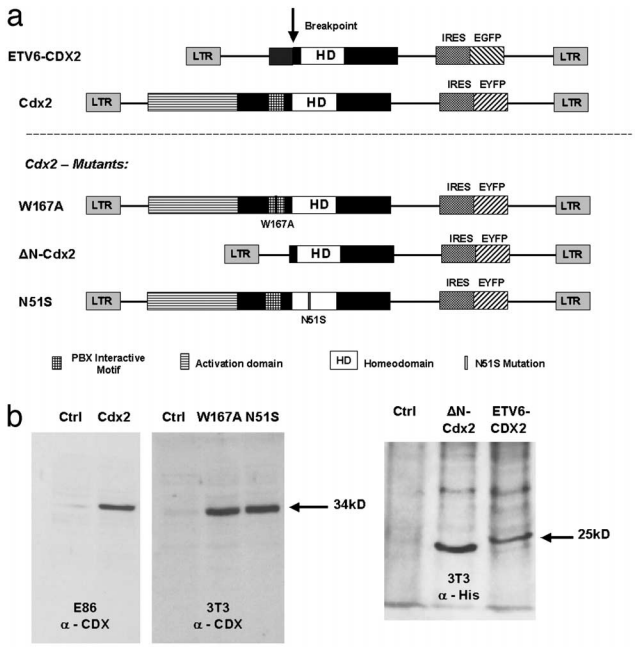


**Fig. 1.** (*a*) Retroviral vectors used to express *ETV6-CDX2*, *Cdx2*, and the different *Cdx2* mutants in murine BM. IRES, internal ribosomal entry site. (*b*) Western blot analysis of cellular extracts from NIH 3T3 or E86 cells transfected with the different constructs. The molecular mass is indicated.

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 $\mu$M 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 $\mu$g 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 m$\beta$-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.

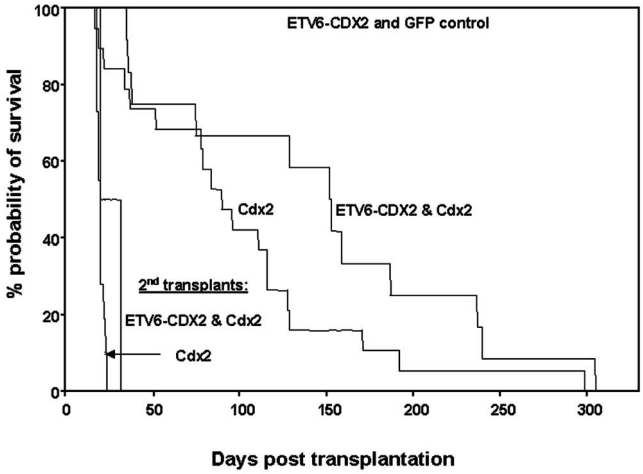


**Fig. 2.** Survival curve of mice transplanted with BM cells expressing *Cdx2* ($n = 18$), *ETV6-CDX2* ($n = 9$), or coexpressing *Cdx2* and the fusion gene ($n = 13$). The control group was injected with BM infected with the GFP empty retrovirus ($n = 7$). The survival time of secondary recipient mice,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

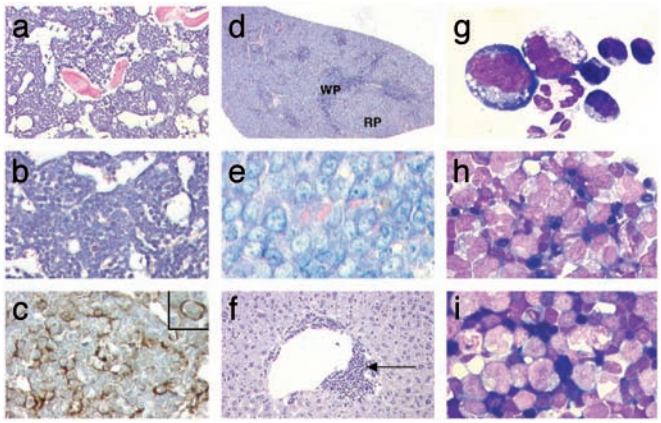


**Fig. 3.** Histological analysis of diseased *Cdx2* mice. (*a*) BM [hematoxylin/eosin (H&E)]. Immunohistochemistry of the BM (×200) for *N*-acetyl-chloroacetate esterase (×400) (*b*) and CD34 expression (×640) (*c*). Histology of the spleen H&E (×25) (*d*) and Giemsa staining (×640) (*e*) and liver 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.

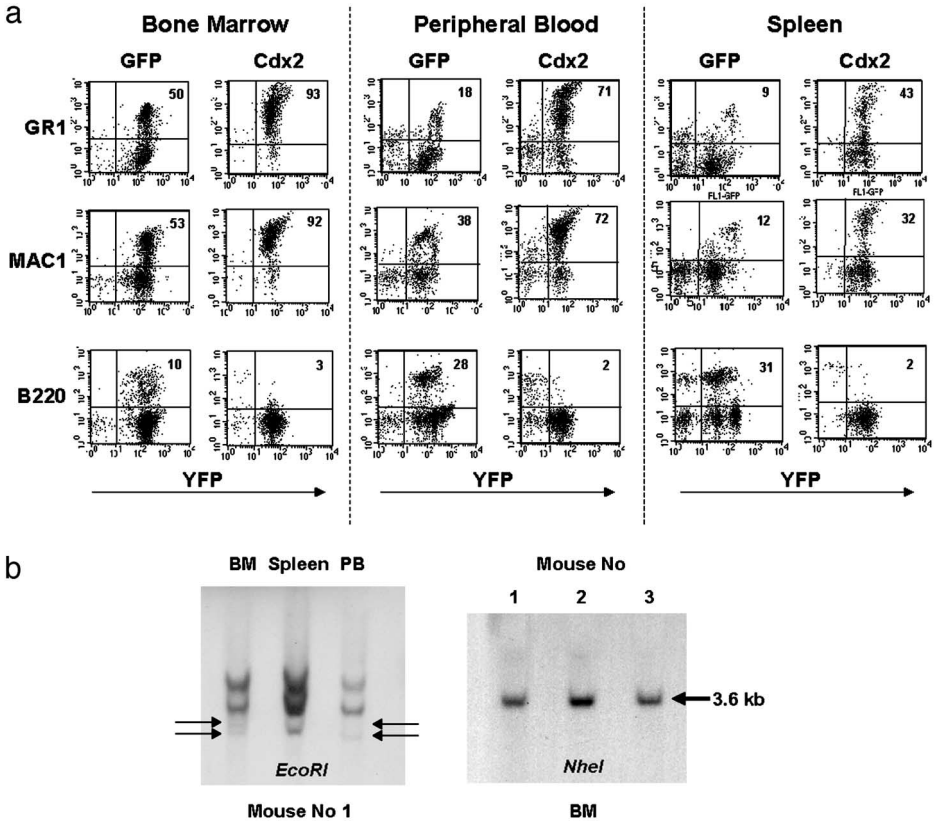


**Fig. 4.** (*a*) Flow cytometry from a representative leukemic *Cdx2* mouse from PB, BM, and spleen in comparison to a GFP control animal. Cells were stained for the myeloid markers Gr1 and Mac1 and the lymphoid marker B220. The proportion of positive cells within the $GFP^+$ compartment is indicated. (*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 $1 \times 10^6$ cells/ml in the PB and 1,400 clonogenic cells vs. 13 per $1 \times 10^6$ 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 $1 \times 10^6$ of these cells developed leukemia 8 wk posttransplant in contrast to mice injected with nontransduced or GFP-expressing control cells. Cells expressing

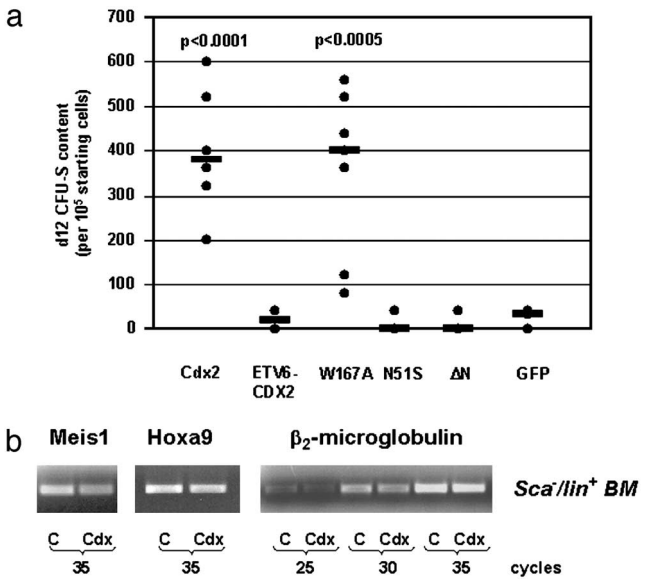


**Fig. 5.** (*a*) Total number of d12 CFU-S colonies derived per culture initiated with $1 \times 10^5$ cells transduced with the different viruses after 1 wk in liquid culture. The median is indicated. (*b*) Expression of *Meis1* and *Hoxa9* analyzed by RT-PCR in $Sca^-lin^+$ BM cells isolated from 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 m$\beta$-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 ($76 \pm 22$ vs. $41 \pm 20$ 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 ($33 \times 10^3$ vs. $3.9 \times 10^3$ 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.

We acknowledge D. G. Gilliland for generously providing the ETV6-CDX2 and Cdx2 cDNA, T. Haferlach and B. Ksienzyk for excellent technical assistance, and the members of the GSF animal facility for maintenance of the animals. This work was supported by Deutsche Forschungsgesellschaft Grant Bu 1177/2-1 (to C.B.)

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.