Mzf1 controls cell proliferation and tumorigenesis

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MZF1 is a transcription factor belonging to the Krüppel family of zinc finger proteins, expressed in totipotent hemopoietic cells as well as in myeloid progenitors. Here we have inactivated Mzf1 by gene targeting. Mzf1−/− mice develop lethal neoplasias characterized by the infiltration and complete disruption of the liver architecture by a monomorphic population of cells of myeloid origin reminiscent of human chloromas. Mzf1 inactivation results in a striking increase of the autonomous cell proliferation and of the ability of Mzf1−/− hemopoietic progenitors to sustain long-term hemopoiesis. These findings demonstrate that Mzf1 can act as a tumor/growth suppressor in the hemopoietic compartment.

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Zinc finger proteins play a crucial role in regulating normal hemopoiesis [Shviddasani and Orkin 1996]. Furthermore, several genes encoding zinc finger transcription factors are involved in chromosomal translocations associated with hemopoietic malignancies [Look 1997], and their functional deregulation or inactivation is an essential step in leukemogenesis [He et al. 2000 and references therein]. Myeloid zinc finger 1 [MZF-1] is a transcription factor of the Krüppel family of zinc finger proteins originally cloned from a cDNA library from a patient with chronic myeloid leukemia [Hromas et al. 1991]. Within the hemopoietic compartment MZF-1 expression is restricted to totipotent bone marrow [BM] progenitor cells and early myeloid progenitors and precursors, and is not detectable in fully differentiated blood cells [Bavisotto et al. 1991]. MZF-1 contains 13 zinc finger domains divided into two groups, which can bind DNA independently of each other [Morris et al. 1994; Hromas et al. 1996]. In vitro, in transient transfection experiments, MZF-1 can activate transcription in cells of hemopoietic origin, and it has also been found to repress transcription in nonhemopoietic cells [Morris et al. 1995; Hromas et al. 1996]. MZF1 antisense oligonucleotides inhibit granulocyte colony-stimulating factor (G-CSF)-driven granulocyte colony formation [Bavisotto et al. 1991]. However, MZF1 overexpression in embryonic stem [ES] cells also appears to interfere with the ability of these cells to undergo hemopoietic commitment as well as erythromyeloid colony formation [Perrotti et al. 1995]. These data may suggest a role for MZF1 in the control of myeloid differentiation. However, the biological function of MZF1 is presently unknown, also in view of the seemingly contradictory nature of these observations.

Here we show, in vivo, in knockout [KO] mice, that Mzf1 controls the proliferative potential of hemopoietic cells acting as a growth and tumor suppressor.

Results and Discussion

Generation of Mzf1+/− mice

We characterized and ablated the murine Mzf1 gene by homologous recombination. Sequencing of the Mzf1 coding region, which is retained in a single exon, revealed an 87.5% identity and a 97.2% similarity between the human and mouse proteins. The murine Mzf1 gene was found to be expressed in BM cells, but also in adult brain, testis, keratinocytes, and thymus [not shown]. Using a targeting vector for positive/negative selection in mouse ES cells, we replaced the Mzf1 coding region with the positive selectable marker (neomycin cassette), completely ablating the region encoding the Mzf1 DNA-binding domain, and obtained several Mzf1−/− ES cell clones. Mzf1−/− mutants in a pure 129Sv background were generated from 4 of 27 independently targeted ES cell clones [Fig. 1A–C; Materials and Methods]. Mzf1−/− mutants were born at Mendelian frequencies and were developmentally normal [not shown]. The disruption of Mzf1−/− was confirmed by RT–PCR analysis on RNA from BM cells [Fig. 1D; Materials and Methods].

Progressive accumulation of myeloid cells in the bone marrow from Mzf−/− mice

We characterized myeloid and lymphoid hemopoiesis in Mzf1−/− mice and control littermates. Analysis of peripheral blood [PB] cell populations with the use of an automated counter [Technicon H2], and by differential counts performed on Wright-Giemsa-stained smears, did not reveal significant differences between Mzf1−/− mice and wild-type sex-matched syngeneic littermates [not shown; Materials and Methods]. Spleen, BM, lymph nodes, and thymus from Mzf1−/− and wild-type mice were then analyzed by differential counts and by flow cytometry with antibodies specific for hemopoietic stem cells/progenitors, myeloid cells, T- and B-lymphocytes. No significant differences were found in the lymphoid...
compartment (not shown; Materials and Methods). However, we observed a progressive and consistent accumulation of the mature Mac-1+ (Sca-1 and Gr-1 negative) myeloid population in the BM of Mzf1−/− mice compared to syngeneic sex, age matched wild-type controls (increase ranging between 5%, at 1 mo of age, to 10% in 10-month-old mutants; 10 mutants and 10 wild-type controls were analyzed; mean difference 8.7%; P < .001) (Fig. 2A). Thus, unexpectedly, at the steady state, Mzf1 inactivation does not impair the ability of myeloid and lymphoid cells to terminally differentiate, but affects the size of the Mac-1+ myeloid compartment in the BM.

Mzf1−/− mice develop lethal myeloid neoplasias characterized by the infiltration and complete disruption of the liver architecture

To determine whether the inactivation of Mzf1 would result in overt disease, mice were followed throughout their life (during a 30-mo follow-up period). One group of mice was bled on a monthly basis, together with age-matched littermate controls, and sacrificed when developing manifest signs of disease (e.g., ascites; see below), or when hemoglobin levels dropped to <8 g/dL. Automated and differential counts, as well as morphological analysis of PB cells, were performed on each sample. In a second group, Mzf1−/− and wild-type controls were aged,

Figure 1. Targeted disruption of Mzf1 in the mouse germ line. (A) Maps of the wild-type Mzf1 locus (WT, top), the targeting vector (VEC, middle), and the predicted targeted gene (REC, bottom). The Mzf1 genomic sequence is depicted as a thick line with a gray box representing the Mzf1 coding sequence. Sequences from the PNT plasmid (Tybulewicz et al. 1991) are shown as thin lines, with an open box representing the neomycin resistance cassette (Neo), a dark gray box representing the herpes simplex virus thymidine kinase cassette (HSV-TK), and the filled black boxes representing the PGK promoters driving the expression of the HSV-TK and Neo genes. The Neo and Mzf1 genes are transcribed in opposite directions. The Mzf1 genomic fragments used as probes for Southern blot analysis are indicated [5' external probe and 3' external probe]. [B] BamHI, [H] HindIII, [X] XbaI restriction sites are shown. [B] Southern blot analysis of C7 untransfected cells and recombined clones after digestion with BamHI and hybridization with the three indicated probes. [C] Southern blot of mouse tail DNAs from one litter obtained by crossing Mzf1 heterozygous mice after digestion with BamHI. [D] RT–PCR analysis using oligonucleotides MZF-13 and MZF-7 confirming the complete inactivation of the Mzf1 locus in BM cells. Amplification with a Gapdh primer pair was used to normalize for the amount of cDNA.

Figure 2. Accumulation of myeloid cells and hepatic neoplasias in Mzf1−/− mice. (A) Flow cytometry analysis of BM cells in 10-month-old mice, stained with Mac-1 and Sca-1 antibodies. The genotype of the mice is indicated. At this age, Mzf1−/− mice do not show signs of overt disease. [B–D] Hepatic neoplasias in Mzf1−/− mice: wild-type [left] and diseased Mzf1−/− mice [right]. (B) Macroscopic illustration of liver. The liver of Mzf1−/− mouse is markedly enlarged in view of the infiltration of the neoplastic population. (C) Histopathological analysis of liver sections stained with hematoxylin and eosin (original magnification, 400×). The liver architecture is effaced by the dramatic infiltration of a monomorphic blast population. The black arrow indicates a residual hepatocyte. (D) Flow cytometric analysis of liver cells stained with Mac-1 and Gr-1 antibody. Mac-1 positive cells (upper left quadrant) infiltrate the diseased liver from a Mzf1−/− mutant.
and four from each genotype sacrificed every month for gross and microscopic examination of all organs, in particular PB, BM, spleen, liver, lymph nodes, and thymus. Cell populations in these organs were analyzed morphologically on smears, touch preparations, and cytospin preparations stained with Wright-Giemsa. By year 2, we observed extramedullary hemopoiesis in the liver or spleen in all Mzf1<sup>−/−</sup> mice analyzed (28 of 28 older than 2 yr). Extramedullary hemopoiesis affecting the spleen, but never the liver, was observed only in 5 of the 36 wild-type mice older than 2 yr [not shown]. Of the Mzf1<sup>−/−</sup> mice, 30% [n = 9] developed a peculiar neoplastic disease affecting the liver and the spleen of which they succumbed between 24 to 32 mo. In these mice a monomorphic blastic cell population completely effaced the liver architecture [Fig. 2B,C]. The morphological features of these cells were characteristic of hemopoietic blast with prominent nuclei. In some cases the neoplastic lesion was infiltrating the adjacent organs (i.e., the intestine). These neoplasias were highly reminiscent of solid tumor composed of myeloid cells. Surprisingly, the BM was never infiltrated by the neoplastic population [data not shown]. However, the BM of Mzf1<sup>−/−</sup> mice was almost invariably hypercellular. In one case the PB was affected, but even in this case the BM was normal [blasts <10%]. Four (14%) Mzf1<sup>−/−</sup> mice developed high-grade B-cell lymphomas. Of the 36 wild-type mice analyzed, four developed lymphomas [11%]. None of the wild-type mice developed the liver/spleen neoplasia.

To define the histological origin and the proliferative properties of the neoplastic cell population, we performed flow cytometry and immunohistochemistry analysis. The infiltrating cells were: [1] Mac-1 positive. Mac-1 (CD11b) is expressed either in macrophages/monocytes or in a very immature myeloid precursor [Fig. 2D]; [2] positive for GM-colony-stimulating factor receptor alpha [GM-CSF<sub>α</sub>], a marker also expressed on early myeloid progenitors [Fig. 3A–C]; [3] S100 negative. S100 is a marker normally expressed on mature histiocytes [not shown]; [4] strongly positive for the Ki67 proliferation marker [Fig. 3D–F]. Leukemic cells from Mzf1<sup>−/−</sup> mice were transplatable in 129/Sv syngeneic mice sublethally irradiated (500 Gy) [Material and Methods].

**Increased number of progenitor cell-derived colonies from the bone marrow and spleen of Mzf1<sup>−/−</sup> mice**

To unravel the mechanisms by which Mzf1 acts as tumor suppressor, we then assessed the cell autonomous hemopoietic potential of Mzf1<sup>−/−</sup> BM progenitors before the occurrence of the neoplasias in mice of 12–16 wk of age. To this end, we performed in vitro cultures of BM cells in standard cytokine and growth factor concentrations, from Mzf1<sup>−/−</sup> and control mice [Materials and Methods]. Mzf1 inactivation resulted in a marked increase of both myeloid and erythroid colonies: colony-forming unit-granulocyte, macrophage [CFU-GM], burst-forming unit-erythroid [BFU-E], and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte [CFU-GEMM], from both the BM and the spleen [Fig. 4]. Thus, under these concentrations of cytokines, Mzf1<sup>−/−</sup> BM and spleen progenitors possess an increased clonogenic capacity.

**Increased proliferation of Mzf1<sup>−/−</sup> hemopoietic progenitor cells**

To determine whether the greater numbers of BM- and spleen-derived colonies obtained from Mzf1<sup>−/−</sup> mutants are the result of an increased proliferative potential of hemopoietic progenitor cells, we performed <sup>3H</sup>thymidine suicide assays. This established procedure allows the estimation of the percent of progenitors in S-phase: incorporation of <sup>3H</sup>thymidine into DNA takes place in actively proliferating cells resulting in cell death [Material and Methods; Maze et al. 1992]. Thus, in this assay an increased proliferative potential of progenitor cells results in a reduced number of hemopoietic colonies. The BM and spleen cells from Mzf1<sup>−/−</sup> and wild-type mice were cultured in the absence or presence of high-specific activity <sup>3H</sup>thymidine as a pulse exposure. After removal of <sup>3H</sup>thymidine, cells were plated in methylcellulose, in the presence of growth factors, to trigger the formation of hemopoietic colonies. Mzf1<sup>−/−</sup> inactivation dramatically enhanced the proliferative capacity of hemopoietic progenitors and, in turn, their responsiveness to the hemopoietic growth factors present in the medium [Fig. 5A,B]. As previously mentioned, in vitro, Mzf1 is known to regulate c-Myb expression at the transcription level. However, the effects on cell proliferation observed in Mzf1<sup>−/−</sup> hemopoietic cells were not caused by an aberrant c-Myb expression. In fact, the levels of c-Myb were never found increased in Mzf1<sup>−/−</sup> mutants in both Western blot and TaqMan analyses performed on total BM cells or sorted early hemopoietic progenitors [not shown; Materials and Methods].

**Increased long-term proliferative potential of Mzf1<sup>−/−</sup> hemopoietic progenitors**

We then investigated whether the proliferative advantage of Mzf1<sup>−/−</sup> hemopoietic pro-
Genitors would result in an increased ability to support long-term hemopoiesis by performing long-term BM liquid cultures from $Mzf1^{-/-}$ mice and control littermates (Material and Methods). From these cultures, clonogenic assays in standard cytokines conditions were carried out on a weekly basis. A much greater number of CFU-GM was produced from $Mzf1^{-/-}$ BM cells throughout the 4-wk culture (Fig. 5C). Therefore, $Mzf1$ inactivation also augments the ability of progenitor cells to support long-term hemopoiesis.

In summary, the present study leads to three major conclusions:

1. $Mzf1$ is dispensable for myeloid/hemopoietic differentiation at the steady state. Although in vitro antisense experiments have shown that MZF1 might control myeloid hemopoietic differentiation (Bavasotto et al. 1991; Perrotti et al. 1995), the inactivation of $Mzf1$ in vivo in the mouse did not affect this process, at least at the steady state, but resulted instead in an accumulation of BM Mac-1$^+$ myeloid cells. In this respect, it is also worth noting that with gene targeting, the $Mzf1$ region coding for the DNA-binding domain was entirely deleted, thus resulting in the complete inactivation of $Mzf1$, including the $Mzf1$ isoform termed MZF1B or Mzf2 (Murai et al. 1997, 1998; Sander et al. 2000).

2. $Mzf1$ plays a crucial role in the negative regulation of the proliferative capacity of hemopoietic progenitors. Cells from the BM and spleen of $Mzf1^{-/-}$ mice yielded a greater number of colonies of all hemopoietic lineages in in vitro cultures. This may be explained either by an increased proliferation or by an increased commitment of progenitor cells to differentiate in response to the cytokines and growth factors present in the cultures. However, [$^{3}$H]thymidine suicide assays show that $Mzf1$ inactivation results in a marked increase in the proportion of hemopoietic progenitors that are actively cycling at a given time, both in the bone marrow and in the spleen. Furthermore, this increased proliferative rate allows self-renewal of progenitors, as demonstrated by the fact that $Mzf1^{-/-}$ BM cells gave rise to a greater number of colonies even after long-term BM culture. In contrast, analysis of apoptosis in early hemopoietic progenitor cells as evaluated by terminal deoxynucleotide end-labeling (TUNEL) and Annexin V assays did not reveal any significant difference between $Mzf1^{-/-}$ and wild-type mice (data not shown; Materials and Methods).

3. $Mzf1$ acts as growth and tumor suppressor in the myeloid hemopoietic compartment suggesting that $Mzf1$ inactivation or deregulated function could participate in tumorigenesis by lending a proliferative advantage to the neoplastic cells. From this point of view, it is intriguing that the human MZF1 gene is one of the most subtelomeric genes described so far located only a few kilobases from the subtelomeric repeat region of 19q, which may lead to MZF1 loss as a consequence of telomeric erosion (Hofman et al. 1996). The incidence of myeloid neoplasia in $Mzf1^{-/-}$ mice is higher.

Figure 5. Increased number of progenitor-derived colonies in $Mzf1^{-/-}$ mice. The clonogenic potential of $Mzf1^{-/-}$ hemopoietic progenitors was evaluated in a methylcellulose colony-forming assay by scoring CFU-GM, BFU-E, and CFU-GEMM obtained from (A) BM cells and (B) spleen cells and expressing these results per organ. The values are averages ± SE calculated from triplicates of one representative experiment carried out with five $Mzf1^{-/-}$ (black bars) and five syngeneic 129Sv control littermates (gray bars), plated as independent cultures.

Figure 4. Increased numbers of progenitor-derived colonies in $Mzf1^{-/-}$ mice. The clonogenic potential of $Mzf1^{-/-}$ hemopoietic progenitors was evaluated in a methylcellulose colony-forming assay by scoring CFU-GM, BFU-E, and CFU-GEMM obtained from (A) BM cells and (B) spleen cells and expressing these results per organ. The values are averages ± SE calculated from triplicates of one representative experiment carried out with five $Mzf1^{-/-}$ (black bars) and five syngeneic 129Sv control littermates (gray bars), plated as independent cultures.
than the incidence of myeloid leukemia in transgenic mice harboring potent oncogenes such as the PML-RARα fusion gene of acute promyelocytic leukemia (only 20% of these transgenic mice develop leukemia after a long latency; He et al. 1997). However, the fact that Mzf1−/− mice are not born with cancer and that, on the contrary neoplasias in Mzf1−/− mice occur after a latency period strongly suggests that additional mutations are needed for full-blown transformation. The progressive expansion of the hemopoietic compartment within the BM, liver, and spleen as a result of Mzf1 inactivation would then favor the occurrence of additional transforming events. Mzf1 inactivation could dictate the peculiar hepatic and splenic localization of these neoplasias by modulating the expression of adhesion molecule that would retain the blasts within these organs. These findings prompt investigating whether MZF1 mutations or loss of heterozygosity occur in human hemopoietic tumors: myeloproliferative syndromes and myeloid leukemias are possible candidates. However, it is also worth noticing that malignant myeloid tumors localized to both soft tissues such as the liver and the bones [also known as chloromas; Harrison 1987] have been described and are, in this respect, straightforward candidate for this analysis.

Materials and methods

**Generation of Mzf1−/− mice**

To characterize the mouse Mzf1 genomic locus, we screened a 129Sv mouse λ-phrase genomic library (Stratagene) with a human MZF1 cDNA probe encoding the acidic non-zinc finger N-terminal domain of the protein. Two overlapping clones were obtained. The coding sequence was determined by restriction enzyme mapping, DNA sequencing, and PCR. The targeting vector contains PGK-HSV-TK and PKG-NEO cassettes, the latter of which replaces, in the opposite orientation, the 1100-bp BamHI–MflI fragment of Mzf1 coding region [which codes for almost the entire DNA-binding domain of Mzf1 and its Mzf2 isoform: the first 11 zinc finger domains] and is flanked by Mzf1 genomic DNA [5′ arm, 7.0-kb HindIII–BamHI fragment; 3′ arm, 2.0-kb MflI–XbaI fragment]. In addition, the presence of the PGK-NEO cassette within Mzf1 coding region introduces stop codons in all three frames, thereby interrupting the translation of the remaining two C-terminal zinc finger domains of the Mzf1 protein. The NotI linearized vector was electroporated into CJ7 ES cells and transfecants were selected in G418 (500 μg/mL) and Gancyclovir [2 μM]. Resistant clones were screened by Southern blotting with the 3′ external probe using a BamHI digestion. Recombined ES cell clones were further characterized by Southern blotting with 5′ external and internal NEO probes, using a BamHI digestion. Twenty-six mutant CJ7 ES clones heterozygous for the Mzf1 deletion were obtained. Mzf1−/− mutants were generated from four independently targeted ES cell clones according to standard procedures [Barna et al. 2000]. Inactivation of the gene was confirmed by RT-PCR analysis. To this end, total BM RNA from 6– to 8-week-old mice was prepared using TRizol (GIBCO BRL). RT–PCR was performed with 2 μg of DNase I-treated RNA, by using cDNA Cycle Kit for RT–PCR [Invitrogen], according to the manufacturer’s protocol. Two microliters of the synthesized cDNA was used for PCR with the following primers: sense MZF-13, 5′-TGCGCTCACCAAGAGCGTCCA-3′; anti-sense MZF-7, 5′-TGGGCTGAGGGCCTGCTGCTTCC-3′; the quality of cDNA was assessed by amplification with Gapdh control primers: sense 5′-GTGAGATCTCTCAGTGAACCG-3′, antisense 5′-TATTATGGGATGCATTGGA-3′. The specificity of amplification was evaluated by hybridizing the PCR products, upon transfer on a nylon membrane, with internal primers (not shown).

**Analysis of hemopoiesis in Mzf1−/− mice**

Animals were analyzed throughout life along with littermate controls at bimonthly intervals [at least four mice from each genotype per time point]. Mice were bled by retroorbital venipuncture. Leukocyte, platelet, and red cell counts were performed with an automated counter [Technicon H2]. Differential counts of myeloid and lymphoid subpopulations were carried out on PB smears stained with Wright-Giemsa. Differential counts of three or four smears per animal were scored for a total of at least 300 cells. Single-cell suspensions from BM, spleen, lymph nodes, liver, and thymus were analyzed on a five-color FACStar Plus flow cytometer [Beckton Dickinson, Mountain View, CA]. Fluorochrome-conjugated antibodies for flow cytometry were CD45R/B220, CD34, CD43, TER119, Sca-1, c-Kit, Mac-1, CD3, CD4, CD8, CD24, GR-1 (Pharmingen).

**Autopsies, histopathology, and immunohistochemistry**

Animals were autopsied as needed and all tissues were examined regardless of their pathological status. Normal and tumor tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Sections (4–5 μm) were stained with hematoxylin and eosin according to standard protocols. Representative samples were selected for immunohistochemical analysis of GM-CSFRα and Ki-67 expression. For GM-CSFRα immunohistochemistry, liver sections from wild-type and Mzf1−/− mice were dewaxed in xylene, rehydrated through a graded series of alcohols, treated with 1% H2O2 in PBS, pretreated with 0.01 M citric acid, microwaved for 15 min, and incubated with anti-mouse GM-CSFRα antibody (Santa Cruz), overnight at 4°C. Positive signals were developed with diaminobenzidine [DAB] substrate using the avidin-biotin-peroxidase system and slides were counterstained with hematoxylin. Immunohistochemical analysis of Ki-67 (Novocastra) expression was performed on paraffin sections after deparaffining and rehydration through a graded series of alcohols, treatment with 1% H2O2, and incubated overnight at 4°C with the anti-Ki67. Positive signals were developed using DAB as mentioned above for GM-CSFRα staining. Immunohistochemical analysis was performed also for the following primary antibodies B220, CD45, TER119, CD34, CD8, and S100 (Dako), as described previously (Cordon-Cardo and Richon 1994).

**In vitro BM culture**

To score for BFU-E, CFU-GM, and CFU-GEMM, BM cultures from Mzf1−/− and wild-type mice were carried out by plating 5 × 104 cells in 1 mL of 1% methylcellulose Iscove’s modified Dulbecco medium (IMDM) with 30% FBS, 1 U/mL recombinant human erythropoietin (EPO), 5% [v/v] pokeweed mitogen mouse spleen cell-conditioned medium (PWMSCM), 50 ng/mL recombinant murine steel factor, and 0.1 mM hemin at 5% CO2 and lowered 5% O2. Colonies were counted after 7 d of incubation. A total of 10 Mzf1−/− and 10 control wild-type syngeneic littermates, sex and age [12-week-old] matched were analyzed in two independent experiments.

**Cycling status of hemopoietic progenitor cells**

The proportion of each progenitor cell type in DNA synthesis [S phase of the cell cycle] was estimated by means of the high-specific-activity (20 Ci/mM) [3H]thymidine kill technique, which is based on calculation of the reduction in the number of colonies formed in vitro after pulse exposure of cells for 20 min to [3H]thymidine, as compared with control nonradioactive thymidine [Maze et al. 1992]. Mzf1−/− and control wild-type syngeneic littermates sex and age [12-week-old] matched were used for this analysis.

**Long-term BM culture**

Cells were plated at low density, 103 per 1.5 mL of IMDM containing 20% FBS, 20 μM β-mercaptoethanol, 100 ng/mL murine Steel factor, 100 ng/mL Flt3-ligand, 50 ng/mL IL-6, and antibiotics [Pen-Strep-Ampo, BRL]. Independent cultures were set up for each mouse and 1 mL of medium was removed weekly. Progenitor assays were carried out as described above, on a weekly base for 4 wk, with 104 cells taken from these cultures. Mzf1−/− and control wild-type syngeneic littermates sex and age [12–16-week-old] matched were used for this analysis.

**Western blot and Real-time PCR (TaqMan)**

Analysis of Myb expression was performed by Western blot on whole-cell protein extracts prepared at different time points [0, 2, 4, and 6 d] from BM cultures treated with IL3 + SC-F + GM-CSF using with the 5E anti-c-Myb specific antibody [Sleeman 1993; courtesy of Joe Lipstick, Stanford University School of Medicine, CA]. TaqMan analysis of c-Myb expres-
tion was performed on total RNA extracted from sorted c-kit/FcγRII/II Receptor double positive myeloid progenitors using an ABI Prism 7700 Sequence Detection System (PE Biosystems). Sequences of primers and probe for mouse c-Myc are as follows: sense 5'-CGCGGGAACGACA AAAACATC-3'; antisense 5'-GGGACGCTTAGTACTATTACATGGCA-AAG-3'; 6FAM-CCAGTCACGTCCCTACCTGTCGGA-TAMRA. Early myeloid progenitors from a total of four MZF1−/− and four control wild-type syngeneic littermates, sex and age matched were analyzed in four independent experiments.

Apoptosis analysis by TUNEL and Annexin V Detection analysis of apoptosis was performed by Annexin V staining and fluorescein isothiocyanate (FITC) labeling of CD45-expressing myeloid cells from the liver or the spleen. Blasts and neoplasia in the liver or the spleen were also collected and spun on glass slides, fixed in 4% buffered formalin and stained by in situ TUNEL assay according to published protocols (Di Cristofano et al. 2001).

Transplantation Leukemic cells were transplanted essentially as described previously (Rego et al. 2000). Briefly, cells were collected from Mzf1−/− mice with liver neoplasia and leukemia as well as wild-type controls either by passing liver cells through a strainer to remove hepatocytes, or by flow-sorting CD45+ cells in the liver or spleen. Blasts (2 × 10⁵) were injected intraperitoneally into 129/Syngeneic sublethally irradiated mice (500 Gy), in triplicate, immediately after collection. The recipient mice were followed up for tumor growth on the site of injection and for leukemia development.

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