Monocytic leukemia zinc finger protein is essential for the development of long-term reconstituting hematopoietic stem cells

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Monocytic leukemia zinc finger protein (MOZ), a transcriptional coactivator and member of the MYST family of histone acetyltransferases, is the target of recurrent translocations in acute myeloid leukemia. Since genes associated with translocations in leukemia are typically important regulators of blood formation, we investigated if Moz has a role in normal hematopoiesis. We generated mice carrying a mutation in the *Moz* gene. Homozygous *Moz* mutant mice died at birth. *Moz* mutant fetal liver hematopoietic cells were incapable of contributing to the hematopoietic system of recipients after transplantation. We observed profound defects in the stem cell compartment of *Moz*-deficient mice. Progenitors of all lineages were reduced in number. However, blood cell lineage commitment was unaffected. Together, these results show that Moz is essential for a fundamental property of hematopoietic stem cells, the ability to reconstitute the hematopoietic system of a recipient after transplantation and that Moz is specifically required in the stem cell compartment.

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Introduction

Moz is a MYST family coactivator with histone acetyltransferase activity (Yang 2004). Transcriptional coactivators form multiprotein complexes that are recruited to specific genomic loci by DNA-binding transcription factors. Coactivator complexes typically contain an enzyme subunit with chromatin-modifying activity, such as a histone acetyltransferase (Berger 2002). It has been suggested that the exchange of a corepressor complex with a coactivator complex, containing a histone acetyltransferase, may be a critical element in promoting gene expression (Glass and Rosenfeld 2000). Rather than forming a passive link between DNA-binding transcription factors and the basal transcriptional apparatus there is increasing evidence that coregulator complexes provide an additional level of regulation where gene expression patterns and consequently cellular phenotype are determined (Taatjes et al. 2004). Considerable progress has been made in identifying the DNA-binding transcription factors that direct the differentiation of specific blood

lineages (Zhu and Emerson 2002). Nevertheless, it is not clear how these transcription factors direct gene expression and hence the differentiation of diverse lineages. Paradoxically, a transcription factor may have several distinct functions in the differentiation of a single cell lineage (Zhu and Emerson 2002) as well as additional functions in completely different cell types. In addition, a DNA-binding transcription factor—for example, a nuclear receptor—may act under some conditions as a repressor, while under different conditions it might promote transcription (Glass and Rosenfeld 2000). Studies of the nuclear receptors suggest that the nature of the available coregulatory complexes plays an important role in determining the range of activity of DNA-binding transcription factors (Glass and Rosenfeld 2000). Since all developmental processes ultimately depend on precise regulation of transcription, identification of the coactivators involved is a necessary step in understanding the genetic regulation of development. We have previously shown that another member of the MYST family, Querkopf, is required for telencephalon and bone development (Thomas et al. 2000). Querkopf has an identical domain structure to Moz, and all functional domains are highly conserved (Thomas and Voss 2004).

The acetylation status of histones is determined by the opposing action of histone acetyltransferases and histone

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deacetylases (Strahl and Allis 2000). Deregulation of histone acetylation is involved in the pathogenesis of acute myeloid leukemia. The translocation t(8;21) causes the expression of a AML1-ETO fusion protein that leads to acute myeloid leukemia. It has been shown that this fusion protein associates with a histone deacetylase complex that represses AML1 target genes and blocks differentiation (Gelmetti et al. 1998; Lin et al. 1998; Lutterbach et al. 1998; Wang et al. 1998). The recruitment of histone deacetylases appears to be a significant factor in the pathology of at least some types of leukemia since it has been shown that inhibitors of histone deacetylase can promote differentiation of primary blast cells from acute myeloid leukemia patients (Ferrara et al. 2001). In addition, a histone deacetylase has been shown to associate with the Retinoic acid receptor α fusion proteins found in acute promyelocytic leukemia and block transcription (Grignani et al. 1998; Guidez et al. 1998; He et al. 1998) and histone deacetylase inhibitors in combination with other agents, have been shown to be effective in the treatment of acute promyelocytic leukemia (Redner et al. 1999). Together these reports show that disruption of the regulation of gene expression mediated by the deacetylation of histones is important in the pathogenesis of leukemia and suggests that proteins regulating chromatin structure, through acetylation of histones, have an essential role in normal hematopoiesis.

MOZ was originally identified in a recurrent chromosomal translocation t(8;16)(p11;p13) associated with acute myeloid leukemia (Borrow et al. 1996). This translocation defines a distinct subtype of M4/M5 acute myeloid leukemia. The prognosis of patients with this translocation is poor (Stark et al. 1995). The t(8;16)(p11;p13) translocation results in the in-frame fusion of the MOZ gene with the CBP gene. The resulting fusion protein consists of MOZ, without the C-terminal domains, fused to almost all of CBP, another coactivator of transcription. Other recurrent chromosomal rearrangements found in acute myeloid leukemia lead to the fusion of MOZ with the CBP-like coactivator, p300 (Chaffanet et al. 2000; Kitabayashi et al. 2001b). A case of myelodysplastic syndrome has been reported in which MOZ is fused to an uncharacterized gene (Imamura et al. 2003). In addition to translocations involving CBP or the closely related protein p300, other recurrent chromosomal rearrangements leading to leukemia result from the fusion of MOZ with the nuclear receptor coactivator, TIF2 (Carapeti et al. 1998; Liang et al. 1998). These fusion proteins also lack the C-terminal domains of MOZ. Studies of the MOZ-TIF2 fusion have shown that this protein is able to induce long-term self-renewal in committed myeloid progenitors and so initiate leukemic stem cell proliferation (Huntly et al. 2004).

Recently it has been reported that MOZ can interact with AML1 in vitro (Kitabayashi et al. 2001a). Chromosomal rearrangements of the AML1 (*RUNX1*) gene are the most common chromosomal rearrangements associated with leukemia (Downing et al. 2000). AML1 is required for the establishment of definitive hematopoiesis (Okuda et al. 1996; Wang et al. 1996), and has a role in

both megakaryocyte and T-cell differentiation (Ichikawa et al. 2004). AML1 has been shown to bind to MOZ in vitro suggesting that MOZ may act as a coactivator for AML1. An interaction with AML1 takes place through the C-terminal serine- and methionine-rich domains of MOZ. These are domains unique to MOZ and the very similar protein, Querkopf (Thomas et al. 2000). Binding of MOZ stimulates AML1-mediated transcription and the removal of these domains prevents MOZ activation of AML1 in vitro (Kitabayashi et al. 2001a).

Zebra fish (*Danio rerio*) carrying mutations in the *Moz* gene have been isolated in an ENU screen for fish exhibiting cartilage defects. The *Moz* mutant fish show pharyngeal segment identity defects (Miller et al. 2004). However the role of *Moz* in zebra fish hematopoiesis was not addressed.

To determine the physiological role of *Moz* during mammalian development we studied the effect of mutating the *Moz* gene. Since genes affected in recurrent chromosomal translocations leading to leukemia are likely to be critical regulators of blood formation (Orkin 2000), we concentrated this analysis of the *Moz* mutant mice on the development of the hematopoietic system.

Results

The Moz^Δ mutation causes perinatal lethality

We created a truncation of the mouse *Moz* gene (*Moz^Δ*) by the in frame insertion of the *neomycin phosphotransferase (neo)* coding sequence into exon 16. The insertion site is in an equivalent position to the t(8;16) breakpoint found in human leukemia and results in the deletion of the C-terminal domains of *Moz* (Fig. 1A–E). The truncation of the *Moz* coding sequence in these mice occurs in a similar position to the truncation resulting from a point mutation in the less severe of two ENU generated mutant zebra fish *Moz* alleles (Miller et al. 2004). We deleted the C-terminal domains of *Moz*, because a common feature of translocations involving the *MOZ* gene is that these domains are absent in the fusion protein as well as being shown, in vitro, to mediate protein–protein interactions. However, no protein product of the mutant locus could be detected in homozygous embryos by Western analysis even though mRNA was produced at normal levels (Fig. 1E,F; Supplementary Fig. 1). In addition, no mutant protein could be detected in the thymus, lung, kidney or bone marrow (tissues in which *Moz* is strongly expressed) of heterozygous adult animals. In these experiments the β -galactosidase–*neo* fusion protein produced by a gene trap allele of *Qkf* (Voss et al. 1998; Thomas et al. 2000; Thomas and Voss 2004) was used as a positive control. Since *lacZ*–*neo* fusion mRNA is expressed less strongly than the *Moz* mutant mRNA (Supplementary Fig. 1), and a corresponding β -galactosidase–*neo* protein could easily be detected, this suggests that the protein produced from the *Moz^Δ* allele may be unstable or inefficiently translated.

Homozygous *Moz^{Δ/Δ}* mice died at birth. Although not anemic, they failed to oxygenate their blood (Fig. 2E,F),

Figure 1. The *Moz*^Δ mutation. (A) Domain structure of Moz. (H15) H15 domain; (PHD) PHD zinc finger domain (C4HC3); (MYST) MYST histone acetyltransferase domain, the nucleosome-binding motif (C2HC) is indicated by a bar (arrowhead) within this domain; (Acidic) acidic domain; (Ser) serine-rich domain; (Met) methionine-rich domain. An arrow indicates where truncation of Moz protein was generated at amino acid 1538 by the in-frame insertion of the *neo* gene in the mutant allele. (B) Wild-type *Moz* allele showing 16 coding exons distributed over 80 kb. The C-terminal domains are encoded entirely by exon 16. (C) Mutant *Moz*^Δ allele after homologous recombination showing the insertion site of *neo*. (D) Southern analysis of BglII-digested genomic DNA showing the predicted fragment-length polymorphism after hybridization with a probe outside of the targeting construct, wild-type (+/+), heterozygous (Δ/+), and homozygous mutant (Δ/Δ) genomic DNA. (E) Northern analysis of *Moz*^{Δ/Δ}, *Moz*^{Δ/+}, and *Moz*^{+/+} littermate embryos at E13.5; note that the insertion of the *neo* coding sequences cause an increase mRNA of 1 kb. (F) Western analysis of *Moz*^{Δ/Δ}, *Moz*^{Δ/+}, and *Moz*^{+/+} littermate embryos using an antibody against *neo*. Note that no band corresponding to the Moz–neo fusion protein is detectable at the predicted size of 204 kD (indicated by arrow) in the Δ/Δ or Δ/+ lanes. In contrast, the two control lanes at the left show detection of the β-galactosidase–neo fusion protein (145 kD) produced in the cortex of Qk^{βgt} (gt/+) mice but not present in the cortex of a wild-type littermate (+/+). Filter was exposed for 1 h. Note that bands ≥50 kD are the result of nonspecific binding.

probably as a result of aortic arch defects, and exhibited facial abnormalities, including a cleft palate (data not shown). Craniofacial defects are also observed in zebra fish carrying mutations in the *Moz* gene and in both species similar structures are affected (Miller et al. 2004). At day 18.5 of gestation [embryonic day 18.5 [E18.5]] homozygous *Moz*^{Δ/Δ} fetuses had severe dysgenesis of both the thymus and spleen (Fig. 2G–N). While the *Moz*^{Δ/Δ} pups are of normal size, the thymic lobes of the mutant animals were approximately one-fourth the size of wild-type fetuses (Fig. 2C,D) and frequently only one lobe descended. Early in development the spleen anlage was indistinguishable between mutant and littermate control animals. However, there was a failure of the spleen to develop such that by E18.5 the site of spleen in the mutant animals could only be identified by the presence of a poorly developed blood vessel network within the mesentery (Fig. 2E,F). Histologically the mutant thymus appeared poorly organized, lacking a clear medulla. Ec-

topic cystic structures, probably of thymic origin, were observed (Fig. 2G,H). There were instances of minor bleeding associated with the thymus. The spleen in the mutant animals appeared as a slightly thickened region of mesentery in transverse sections (Fig. 2I,J). The E18.5 mutant animals had a normal hematocrit and all leukocyte lineages were present (Supplementary Fig. 2), although a slight, but statistically significant, elevation in the levels of nucleated red blood cells was observed in the mutants (Table 1). Maturation of erythrocytes was studied by flow cytometry and showed that differentiation from CD71^{high} Ter119^{low} through to Ter119^{high} CD71^{low} proceeded normally with the exception that the CD71^{high} Ter119^{high} population in the mutant fetal liver was larger than in the wild type (Supplementary Fig. 3). This shows that erythroid cell maturation oc-

Figure 2. The *Moz*^{Δ/Δ} phenotype. Wild-type littermate (A) and homozygous (B) *Moz*^{Δ/Δ} E18.5 pup of a *Moz*^{Δ/+} heterozygous intercross. Note that the homozygous *Moz*^{Δ/Δ} pup is cyanotic, indicating a lack of oxygenated blood (*n* = 32 E18.5 pups). Wild-type thymus (C) compared with *Moz*^{Δ/Δ} thymus (D) at E18.5. Wild-type spleen (E) compared with mutant spleen (arrow, F) at E18.5. Hematoxylin and eosin-stained transverse sections of E17.5 fetuses showing wild-type (G,I) and *Moz*^{Δ/Δ} littermate (H,J). Note dysgenic thymus (D,H) and dysgenic spleen (F,I) in mutant animals. (H) Minor bleeding and cystic remnants, probably of thymic origin (asterix). (J) The largest extent of the *Moz*^{Δ/Δ} spleen is shown (arrow). (A) Adrenal gland; (K) kidney; (Sp) spleen; (St) stomach; (T) thymus. Bar: G–J, 310 μm.

Table 1. Percent frequency blood cell count E18.5

Cell type	<i>Moz</i> ^{+/+}	<i>Moz</i> ^{Δ/+}	<i>Moz</i> ^{Δ/Δ}
Blast cells	2.2 ± 1.0	1.9 ± 0.9	0.3 ± 0.3
Meta/poly.	65.4 ± 7.5	48.0 ± 8.3	33.2 ± 7.9 ^a
Lymphocytes	15.2 ± 2.2	25.9 ± 6.3	11.0 ± 2.7
Monocytes	9.8 ± 1.4	12.7 ± 1.8	10.8 ± 3.1
Eosinophils	0.2 ± 0.2	0.1 ± 0.1	0.3 ± 0.3
Nucleated erythrocytes	7.2 ± 5.2	11.4 ± 5.8	44.3 ± 12.1 ^b

(Meta/poly.) Metamyelocyte/polymorphonuclear leukocyte. Means ± SEM; *n* = 8 *Moz*^{Δ/Δ}, 8 *Moz*^{Δ/+}, and 6 *Moz*^{+/+} E18.5 pups; *Moz*^{Δ/Δ} ≠ *Moz*^{+/+}; ^a*P* < 0.05; ^b*P* < 0.01.

curs. The slight increase in nucleated erythrocytes may be caused by the absence of a spleen in the mutant mice.

The Moz gene is widely expressed during development and in adult animals

We examined the expression pattern of the *Moz* gene by Northern blot, in situ hybridization and quantitative RT-PCR. During embryonic development *Moz* is widely expressed throughout the embryo, being present in all organs. However the level of expression varied substantially. The strongest expression domain at E13.5 was in the mesencephalon, with lower levels of expression in the spinal cord and telencephalon. Outside of the central nervous system, higher levels of expression were found in the thymus, the dorsal root ganglia, and the nasal epithelium at E13.5. Lower levels of expression were found in the lung, although expression was prominent in the epithelial layer, and in the fetal liver (Fig. 3A–C). At later stages of development strong expression was seen in the surface ectoderm, the incisors, and the whisker follicles (data not shown). *Moz* mRNA was detected in all adult organs examined by Northern blot with higher levels of expression seen in the thymus and lung. The mRNA levels in the thymus and lung were similar to the average level in the E12.5 embryo, whereas other adult organs contained lower levels of expression (Fig. 3D). *Moz* mRNA was detected in all hematopoietic progenitor populations as well as in the Sca-1⁺c-Kit⁺ lineage⁺ cell population enriched for hematopoietic stem cells. By quantitative RT-PCR the *Moz* mRNA levels in hematopoietic progenitor cell populations were similar to, or greater than, the mRNA levels found in whole midgestation embryos (Fig. 3E).

The Moz^Δ mutation causes a profound reduction in the number of hematopoietic progenitors; however, it does not affect lineage commitment

Since the hematopoietic system has a large capacity to compensate for defects in progenitor cell populations, we examined lineage-committed progenitors at E12.5 in the fetal liver and at E18.5 in both fetal liver and bone marrow using colony-forming cell assays. These assays showed that there was a profound reduction in the num-

ber of committed progenitors (Table 2). Significantly for the development of the fetus, erythroid progenitors were less severely affected than other lineages, although only ~30% of the normal number of erythroid colonies was present in the mutant fetal liver cells compared with wild-type cells. Bipotential granulocyte/macrophage progenitors and megakaryocyte/erythroid progenitors (Supplementary Fig. 2) were detected, suggesting that no significant difference in the hierarchy of blood formation occurs in the mutant animals as compared with wild-type animals. Although none of the rare eosinophilic colony-forming cells were found, the blood of *Moz*^{Δ/Δ} mutant fetuses contained eosinophils, indicating that progenitors for this lineage can be formed in homozygous animals. The *Moz*^{Δ/Δ} mutant cells were capable of

Figure 3. *Moz* is widely expressed both during embryogenesis and in the adult at low levels. (A–C) Radioactive in situ hybridization using a 2.8-kb probe to exon 16. Bright-field (A) and corresponding dark-field (B) images show that *Moz* is expressed in the thymus (Th, long arrow), the telencephalon (Tel), and nasal epithelium (NE, stippled arrow) at E13.5. In addition, *Moz* is expressed prominently in the dorsal root ganglia (Drg, short arrow), mesencephalon (Mes), and spinal cord (SC). (C) Sense control shows no hybridization above background. (D, top) Northern analysis, 10 μg total RNA per lane, shows *Moz* is expressed in most adult tissues, particularly in the thymus and lung where levels are similar to whole E12.5 embryo. Multipotent, proliferating cells isolated from adult brain and cultured as neurospheres also express *Moz*. (Bottom) *Hsp90β* mRNA as loading and transfer control. (E) Quantitative RT-PCR showing detection of *Moz* mRNA in cDNA prepared from hematopoietic stem cells (HSC), common lymphoid progenitors (CLP), common myeloid progenitors (CMP), megakaryocyte/erythroid progenitors (MEP), and granulocyte/macrophage progenitors (GMP) as compared with ES cells and E9.5 and E10.5 embryos.

Table 2. *Moz mutant E12.5 and E18.5 liver and bone marrow colony-forming cells in cultures stimulated with SCF + IL-3 + EPO per 20,000 cells plated*

Colony type	<i>Moz</i> ^{+/+}	<i>Moz</i> ^{Δ/+}	<i>Moz</i> ^{Δ/Δ}
Liver E12.5			
Blast cell	4.0 ± 2.7	4.5 ± 0.9	0.8 ± 0.5
Granulocyte	20.0 ± 6.8	7.2 ± 1.9 ^b	1.3 ± 0.5 ^{c,e}
Granulocytes/macrophage	8.5 ± 3.9	4.2 ± 1.4 ^b	3.3 ± 1.4 ^b
Macrophage	31.3 ± 14.3	13.2 ± 2.3 ^a	5.0 ± 1.9 ^{c,d}
Eosinophil	0.8 ± 0.5	0.1 ± 0.1 ^b	0 ± 0 ^b
Erythroid	93.5 ± 32.3	63.5 ± 11.3	28.5 ± 4.5 ^{a,d}
Megakaryocyte	7.8 ± 5.3	9.8 ± 2.6	1.0 ± 0.4 ^{a,d}
Megakaryocyte/erythrocyte	14.2 ± 5.3	9.8 ± 2.6	1.0 ± 0.4 ^{a,e}
Liver E18.5			
Blast cell	17.0 ± 2.6	6.0 ± 1.5 ^c	0 ± 0 ^{c,e}
Granulocyte	15.7 ± 3.1	10.3 ± 1.3 ^b	1.5 ± 0.7 ^{c,f}
Granulocyte/macrophage	16.0 ± 2.3	7.3 ± 2.7 ^b	2.8 ± 1.5 ^{c,d}
Macrophage	26.0 ± 3.5	20.5 ± 5.9 ^a	3.4 ± 2.0 ^{c,e}
Eosinophil	0.3 ± 0.3	0 ± 0 ^b	0 ± 0 ^b
Erythroid	8.3 ± 2.0	7.5 ± 1.5	2.8 ± 1.2 ^{a,d}
Megakaryocyte	5.0 ± 1.5	3.8 ± 1.9	0 ± 0 ^{b,d}
Megakaryocyte/erythrocyte	4.0 ± 1.2	5.0 ± 0.6	0 ± 0 ^{a,e}
Megakaryocyte/blast cell	1.7 ± 0.9	0 ± 0 ^b	0 ± 0 ^b
Bone marrow E18.5			
Blast cell	5.0 ± 2.0	6.0 ± 1.7	0 ± 0 ^o
Granulocyte	11.3 ± 3.0	12.0 ± 4.7	1.0 ± 1.0 ^{c,f}
Granulocyte/macrophage	11.7 ± 2.2	7.1 ± 2.2 ^b	0.7 ± 0.7 ^{c,d}
Macrophage	12.3 ± 1.8	8.7 ± 5.2	0.3 ± 0.3 ^{c,e}
Eosinophil	0.3 ± 0.3	0.7 ± 0.7	0 ± 0
Erythroid	12.0 ± 8.0	8.0 ± 4.6	0.7 ± 0.7 ^a
Megakaryocyte	3.3 ± 1.5	0.7 ± 0.7	0 ± 0 ^b
Megakaryocyte/erythrocyte	1.3 ± 0.7	0.7 ± 0.7	0 ± 0
Megakaryocyte/blast cell	0.7 ± 0.7	0 ± 0 ^b	0 ± 0 ^b

Means ± SEM; *n* = 3–5 *Moz*^{Δ/Δ}, 3–13 *Moz*^{Δ/+}, and 3–4 *Moz*^{+/+}; homozygotes and heterozygotes differ from wild types at significance levels indicated. Data were analyzed by two-factorial analysis of variance with genotype and developmental stage (E12.5 and E18.5 liver) or genotype and organ (E18.5 liver and bone marrow) as the two independent factors.

^a*P* < 0.05.

^b*P* < 0.01.

^c*P* < 0.001, homozygotes differ from heterozygotes at significance levels indicated.

^d*P* < 0.05.

^e*P* < 0.01.

^f*P* < 0.001.

differentiating into all cell lineages. However, we observed that the average size of the mutant colonies was reduced, particularly those formed from E18.5 fetal liver cells.

Overall fetal liver hematopoietic cellularity was reduced in the mutants at E12.5, E15.5, and E18.5 (77 ± 3% of wild type, *P* < 0.0001). The number of myeloid colony-forming cells was also significantly reduced in fetal liver of heterozygous animals at both E12.5 and E18.5 (Table 2). The mutant bone marrow at E18.5 was severely affected, having both low cellularity (32 ± 9% of wild type, *P* < 0.0001) and very low numbers of progenitors. Therefore, colonization of the bone marrow by progenitors takes place in the *Moz*^{Δ/Δ} animals, but hematopoiesis is not efficiently established. In addition, the relative number of colony-forming progenitor cells in the *Moz*^{Δ/Δ} fetus declined from 36.1 ± 9.8% of the wild-type littermate

value at E12.5 to 6.8 ± 2.5% of the wild-type value at E18.5 (*P* < 0.01), showing that there was a failure to maintain early progenitors numbers in the *Moz*^{Δ/Δ} mutant fetuses.

Early hematopoietic progenitors and stem cells in the fetal liver express a characteristic set of cell surface markers, including c-Kit (Morrison et al. 1995), Sca-1 (Morrison et al. 1995), AA4.1 (Jordan et al. 1995) and Flt-3 (Zeigler et al. 1994). The majority of lineage-specific cell surface markers are not detectable on these cells (Lin^{neg}). We used fluorescence-activated cell analysis to determine the size of the c-Kit⁺Sca-1⁺Lin^{neg}, c-Kit⁺Flt-3⁺Lin^{neg}, and c-Kit⁺AA4.1⁺Lin^{neg} populations at E14.5. The fraction of c-Kit⁺Lin^{neg} cells, representing predominantly committed progenitors with limited proliferative capacity, was normal in the mutant animals. However each of the early progenitor/stem cell

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Figure 4. Fluorescence-activated cell analysis of early progenitor/stem cell populations in *Moz*^{Δ/Δ}, *Moz*^{Δ/+}, and *Moz*^{+/+} littermate fetal livers at E14.5. Fetal liver cells were stained with a cocktail of FITC-conjugated antibodies against the lineage markers Ter119, Gr-1, CD4, CD8, B220, and CD19. Mac-1 was not included, since fetal liver stem cells have been reported to be mac-1-positive (Morrison et al. 1995). In addition, cells were labeled with APC-conjugated c-Kit antibody and with either PE-conjugated anti-Sca1 or anti-AA4.1 or biotinylated anti-Flt3 followed by PE-conjugated streptavidin. (A) Fluorescence-activated cell analysis profiles are shown for *Moz*^{+/+} fetal livers and *Moz*^{Δ/Δ} littermate fetal livers. The lineage-negative cell population was analyzed for c-Kit⁺, Sca1⁺, Flt3⁺, or AA4.1⁺ positive cells as indicated. (B) Quantification of fluorescence-activated cell analysis results: *n* = 6 *Moz*^{Δ/Δ}, 3 *Moz*^{Δ/+}, and 3 *Moz*^{+/+} littermate fetal livers; *Moz*^{Δ/Δ}; *Moz*^{Δ/+} ≠ *Moz*^{+/+}; (***) *P* < 0.001; (**) *P* < 0.01; *Moz*^{Δ/Δ} ≠ *Moz*^{Δ/+}; (ooo) *P* < 0.001. Note the reduction in early progenitor/stem cell numbers in the *Moz*^{Δ/Δ} mutant and the intermediate levels in the *Moz*^{Δ/+} heterozygotes. Data were analyzed by two-factorial analysis of variance with genotype and cell surface marker set as the independent factors, followed by Fisher's post-hoc test.

populations, c-Kit⁺Sca1⁺Lin^{neg}, c-Kit⁺Flt3⁺Lin^{neg}, and c-Kit⁺AA4.1⁺Lin^{neg} was reduced by >50% in mutant animals compared with controls (Fig. 4). Interestingly, all three of these early progenitor/stem cell populations showed an intermediate reduction in the heterozygous animals (Fig. 4).

The Moz^Δ mutation diminishes T-cell production in the thymus, but T cells mature normally

The thymus is a site of *Moz* gene expression (Fig. 3), and since the *Moz* mutant thymus was highly dysgenic, we

assessed lymphoid development in the thymus of *Moz*^{Δ/Δ} animals. We observed a substantial reduction in the number of lymphoid cells in the mutant thymus ($6.7 \pm 2\%$ of wild type, *P* < 0.0001) (Fig. 5A), similar to the reduction in myeloid and erythroid colony-forming cells in the fetal liver. Likewise, there was a significant reduction in the number of lymphoid cells in heterozygous animals. However, *Moz*^{Δ/Δ} mutant lymphoid progenitors were able to differentiate into CD8 and CD4 double-positive cells (Fig. 5B). The fraction of double-positive cells in the mutant and wild type was similar,

Figure 5. The thymus of *Moz*^{Δ/Δ} homozygous animals is able to support the differentiation of T cells. (A) Total cellularity of the *Moz*^{Δ/Δ} homozygous thymus is low at E18.5, with heterozygous animals having an intermediate cellularity: *n* = 5 *Moz*^{Δ/Δ}, 9 *Moz*^{Δ/+}, and 3 *Moz*^{+/+}; *Moz*^{Δ/Δ}; *Moz*^{Δ/+} ≠ *Moz*^{+/+}; (***) *P* < 0.0001; (**) *P* < 0.01; *Moz*^{Δ/Δ} ≠ *Moz*^{Δ/+}; (ooo) *P* < 0.0001. (B) Fluorescence-activated cell analysis shows the proportion of CD4/CD8 double-positive thymocytes is normal in *Moz*^{Δ/Δ} homozygous compared with littermate controls (*Moz*^{+/+}) at E18.5. There is a significant reduction in the number of double-negative cells in the *Moz*^{Δ/Δ} homozygotes (*P* < 0.05). (C,D) After 12 d of fetal thymic organ culture, thymocytes of both *Moz*^{Δ/Δ} homozygous and littermate controls (*Moz*^{+/+}) underwent further maturation to form both CD4 and CD8 single-positive cells (C) and recombined the T-cell receptor αβ subunits (TCRβ in D). Levels of TCRβ expression of single-positive cells gated in C are shown in D. Data were analyzed by one-factorial analysis of variance with genotype as the independent factor, followed by Fisher's post-hoc test.

~90%. However in the mutant animals there was a significant reduction in fraction of double-negative cells (Fig. 5B).

CD8/CD4 double-positive cells are intermediates in T-cell maturation (Anderson et al. 1999). Further maturation of T cells occurs after birth. To study subsequent steps in differentiation, fetal thymi were maintained in organ culture for 12 d. We found that *Moz*^{Δ/Δ} T-cell progenitors matured to form CD8 and CD4 single-positive cells (Fig. 5C) and underwent T-cell receptor recombination to become αβTCR-positive (Fig. 5D). In addition, γδTCR receptor cells were formed by *Moz*^{Δ/Δ} T-cell progenitors (data not shown). These results show that although the total number of lymphoid progenitors and total T-cell production was severely reduced in the *Moz*^{Δ/Δ} thymus, these cells were able to undergo normal T-cell maturation and the *Moz*^{Δ/Δ} thymic epithelium was able to support T-cell development.

Moz is required in hematopoietic stem cells

Together, the results presented above showed that the *Moz*^Δ mutation causes a severe reduction in the number of committed progenitors of myeloid, erythroid, and lymphoid lineages, but is compatible with differentiation of progenitors once they are formed. This implies that *Moz* is required in precursors of the lineage-committed progenitors, the hematopoietic stem cells (Fig. 6A). A characteristic feature of hematopoietic stem cells is their ability to reconstitute the hematopoietic system of a lethally irradiated mouse. To determine if *Moz* was required in stem cells we tested the ability of fetal liver cells from homozygous *Moz*^{Δ/Δ} mutant embryos at E15.5 to reconstitute the hematopoietic system of recipient mice. The irradiation conditions used were sufficient to ablate the recipients' hematopoietic system, making survival dependent on the ability of the donor cells to re-establish hematopoiesis (Kondo et al. 2003). A total of 17 recipients were injected with fetal liver cells from five different *Moz*^{Δ/Δ} embryos (three to five recipients per donor; 10×10^6 to 19×10^6 fetal cells per recipient). No recipients receiving donor cells from *Moz*^{Δ/Δ} embryos survived beyond 28 d (Fig. 6B). All recipients receiving fetal liver cells from either heterozygous or wild-type littermates survived, and remained healthy for at least 3 mo. The first few weeks of recovery from radiation require short-term repopulating cells to generate sufficient erythrocytes to avoid aplastic anemia. The rapid death of recipients receiving mutant cells suggests that *Moz*^{Δ/Δ} embryos have a severe lack of functional short-term repopulating hematopoietic cells.

The number of colonies formed on the spleens of irradiated recipients 12 d after reconstitution (CFU-S₁₂) is an assay of primitive, multipotent, hematopoietic cells (Till and McCulloch 1961; Jones et al. 1990) and primarily enumerates short-term repopulating cells (van der Loo et al. 1994). We performed CFU-S₁₂ assays using fetal liver cells isolated at E12.5, E15.5, and E18.5. At each stage, *Moz*^{Δ/Δ}-derived colonies of normal size were detected on spleens examined at day 12 after injection (Fig. 6C).

However, consistent with the reconstitution assay, these colonies were present in lower numbers than those produced from the fetal liver of wild-type littermate controls ($9.0 \pm 1.5\%$ of wild type; $P < 0.0001$) (Fig. 6D). In addition, at all stages examined, there was a reduction in the number CFU-S₁₂ in heterozygous animals (Fig. 6D).

To determine if *Moz*^{Δ/Δ} mutant embryos contain detectable numbers of long-term repopulating hematopoietic stem cells, we performed competitive reconstitution assays (Harrison et al. 1993). In these experiments, fetal liver cells isolated from E15.5 embryos derived from *Moz*^{Δ/+} heterozygous intercrosses were combined with wild-type fetal liver cells. The presence of wild-type cells in these experiments supplied sufficient short-term repopulating cells to prevent the recipients from developing severe anemia. Specific isoforms of the cell surface protein CD45 (Ly5) (Scheid and Triglia 1979) were used to distinguish wild-type "competitor" cells (CD45.1-positive) from "test" cells derived from *Moz*^{Δ/+} heterozygous intercrosses (CD45.1/45.2 double-positive) (Fig. 6E). Peripheral blood from recipients was examined at 6, 12–14, and 22 wk after reconstitution. While *Moz*^{+/+} fetal liver cells reconstituted the hosts' hematopoietic system to a larger extent than wild-type competitor cells, no contribution of littermate homozygous *Moz*^{Δ/Δ} cells could be detected above background in the peripheral blood at any stage (Fig. 6F,G). In addition, DNA was prepared from bone marrow of recipient mice at 22 wk and the presence of the mutant *Moz* allele was determined by Southern gel blot and by PCR. The mutant *Moz* allele was not detected in DNA purified from the bone marrow of recipients when the donor (test) cells were derived from a *Moz*^{Δ/Δ} fetal liver. Since the bone marrow contains large numbers of nucleated red blood cell progenitors, this indicates that the *Moz*^{Δ/Δ} fetal liver cells are unable to contribute to the erythroid lineage irrespective of the presence of wild-type fetal liver cells. A highly statistically significant reduction in the ability of fetal liver cells from heterozygous animals to compete with wild-type fetal liver cells was also observed. As in the CFU-S₁₂ assays (Fig. 6D) the heterozygous animals displayed an intermediate phenotype in competitive reconstitution assays (Fig. 6F).

In these experiments cells from individual *Moz*^{Δ/Δ} homozygous fetal livers were mixed with wild-type competitor cells in 1:1 and 4:1 ratios and injected into a total of six recipient mice. For each homozygous embryo ($n = 4$) a total of 15×10^6 fetal cells were transferred. Long-term repopulation units are more abundant in wild-type fetal liver cells than in bone marrow. A conservative estimate of one repopulating unit per 10^5 adult bone marrow cells (Harrison et al. 1993), and a minimum estimate of fourfold greater abundance of stem cells in fetal liver (Jordan et al. 1995; Harrison et al. 1997) implies that there are at least four repopulating units per 10^5 cells in a wild-type fetal liver. As we detected no long-term reconstituting cells in a total of 6×10^7 cells from the *Moz*^{Δ/Δ} fetal livers, the abundance of fully functional stem cells in the *Moz*^{Δ/Δ} mutants is reduced by >2000-fold when compared with the wild-type fetal liver.

In contrast, wild-type fetal liver “test” cells from littermate controls contributed more extensively to the hematopoietic system of recipient mice than the “competitor” cells (Fig. 6F,G).

Discussion

Hematopoiesis is organized in a hierarchy of differentiation steps, both in the fetal liver and in adult bone marrow, in which the progeny of stem cells become increasingly restricted to different lineages and in proliferative capacity (Dzierzak and Medvinsky 1995; Kondo et al. 2003). In the blood of *Moz*^{Δ/Δ} mice all mature cell types were present and, particularly important for fetal development, the hematocrit was normal. However, increasingly severe phenotypic effects were seen as we examined more immature cell types. A profound reduction in

the number of committed progenitors was seen. The number of colony-forming cells declined during fetal development of the *Moz*^{Δ/Δ} mice relative to wild-type controls and hematopoiesis was not effectively established in the bone marrow. Nevertheless, progenitors of all lineages were present. We examined the development of T cells in detail. There was a large decrease in the number of committed progenitors (thymocytes) and a corresponding decrease in total T-cell output, but in common with myeloid and erythroid progenitors, the few thymo-

Figure 6. Fetal liver hematopoietic cells from *Moz*^{Δ/Δ} homozygous animals are severely deficient in short-term and long-term repopulating stem cells. (A) Schematic diagram showing the relationship between long-term repopulating cells (HSC) detected by competitive repopulation unit (CRU) assays, short-term repopulating cells (ST-HSC) detected by spleen colony-forming unit assays (CFU-S₁₂), and lineage-committed progenitors identified in colony-forming cell assays. (B) Survival curve showing that lethally irradiated recipients receiving either heterozygous or wild-type E15.5 fetal liver cells from *Moz*^{+/+} intercrosses survive, whereas mice receiving homozygous littermate cells die within 28 d ($n = 17, 16$, and 8 mice receiving *Moz*^{Δ/Δ}, *Moz*^{+/+}, *Moz*^{+/+} cells, respectively). Mice were intravenously injected with 10×10^6 to 19×10^6 cells. There was no correlation between the survival times of mice receiving a small or large number of homozygous cells. (C) CFU-S₁₂ assays. Lethally irradiated recipients were injected with fetal liver cells from *Moz*^{Δ/Δ} intercrosses at E12.5, E15.5, or E18.5. (Top) A representative spleen of mice injected with E15.5 wild-type (*Moz*^{+/+}) fetal cells. (Bottom) A representative spleen of mice receiving cells from littermate *Moz*^{Δ/Δ} fetuses. (D) Absolute counts of spleen colonies larger than 0.5 mm: $n = 11$ *Moz*^{Δ/Δ} donors, 15 *Moz*^{Δ/+} donors, and 15 *Moz*^{+/+} donors; *Moz*^{Δ/Δ}, *Moz*^{Δ/+} ≠ *Moz*^{+/+}; (***) $P < 0.001$; (*) $P < 0.01$; *Moz*^{Δ/Δ} ≠ *Moz*^{Δ/+}; (ooo) $P < 0.0001$; (oo) $P < 0.01$. Note the paucity of CFU-S₁₂ cells derived from *Moz*^{Δ/Δ} heterozygotes (D). Data were analyzed by two-factorial analysis of variance with genotype and developmental stage as the independent factors, followed by Fisher's post-hoc test. (E) CRU assays. Lethally irradiated mice received a mixture of wild-type cells (1×10^6) and cells from *Moz*^{Δ/+} intercrosses at E15.5. Wild-type “competitor” cells were identified by the presence of the CD45.1 isoform, the “test” cells from *Moz*^{Δ/+} intercrosses expressed both CD45.1 and CD45.2 isoforms, and any residual cells from the irradiated recipient expressed CD45.2 only. In different experiments, competitor cells and test cells were mixed in ratios of 1:1 and 1:4. (F,G) Combined fluorescence-activated cell analysis of peripheral blood leukocytes results from all experiments performed at 12–14 wk (F), and at 22 wk (G) after reconstitution show extensive contribution of wild-type (*Moz*^{+/+}) fetal cells but no detectable contribution from homozygous littermates (*Moz*^{Δ/Δ}). (F) *Moz*^{Δ/+} heterozygotes show an intermediate ability to competitively reconstitute the hosts. (G) Contribution of each CD45-isoform-positive population is indicated for experiments using wild-type (*Moz*^{+/+}) and homozygous (*Moz*^{Δ/Δ}) fetal liver cells. Data are presented as combined percentage contribution after normalizing all input levels to a 1:1 ratio: $n = 4$ *Moz*^{Δ/Δ} donors, 9 *Moz*^{Δ/+} donors, and 7 *Moz*^{+/+} donors; *Moz*^{Δ/Δ}, *Moz*^{Δ/+} ≠ *Moz*^{+/+}; (***) $P < 0.0001$; *Moz*^{Δ/Δ} ≠ *Moz*^{Δ/+}; (ooo) $P < 0.0001$. Data were analyzed by one-factorial analysis of variance with genotype as the independent factor, followed by Fisher's post-hoc test.

cytes present were able to differentiate normally. These results show that the *Moz*^{Δ/Δ} mutation does not affect lineage commitment and differentiation of progenitors once they are formed, but that there is a lack of committed progenitors.

Interestingly, we observed a strong heterozygous phenotype in the number of colony-forming cells, the number of early progenitors and in reconstitution assays. This shows that there is a strong gene dosage requirement for *Moz*. Sensitivity to gene dosage has also been found in the coactivators CBP and p300 (Yao et al. 1998; Kung et al. 2000). These coactivators also contain a histone acetyltransferase domain (Yang 2004). The observation that the *Moz* mutation also shows strong gene dosage effects supports the notion proposed for CBP: that coactivators with histone acetyltransferase activity may be limiting in many cellular processes (Kamei et al. 1996).

Definitive hematopoiesis takes place in *Moz*^{Δ/Δ} mice, at reduced cellularity, and the full range of mature blood cell types can be produced. Aml1 is required for the development of definitive hematopoiesis (Okuda et al. 1996; Wang et al. 1996) and T-cell differentiation (Ichikawa et al. 2004). We have demonstrated that *Moz*, while able to stimulate activation of AML1 in vitro through its C-terminal domains (Kitabayashi et al. 2001a), is not essential for the Aml1 function in initiation of definitive hematopoiesis in vivo or for the lineage-specific function of Aml1 in T-cell development.

Our results show that mutating the *Moz* gene results in the absence of long-term repopulating stem cells and a substantial reduction in the number of multipotent cells able to form spleen colonies. In addition to these indirect assays, we have shown directly by flow cytometry that early progenitor/stem cell populations are greatly reduced in the *Moz*^{Δ/Δ} mice. These results show that *Moz* functions specifically in the hematopoietic stem cell compartment. In this context it is interesting that the MOZ–TIF2 fusion protein is able to induce leukemic stem cell self-renewal (Huntly et al. 2004). This activity requires the nucleosome-binding domain of MOZ and suggests that MOZ has the capacity to interact with the chromatin of genes controlling self-renewal (Deguchi et al. 2003; Huntly et al. 2004).

How do the *Moz*^{Δ/Δ} mice maintain relatively normal peripheral blood cellularity until birth with severe defects in the hematopoietic stem cell compartment? Our results are consistent with a requirement for *Moz* in maintaining stem cell numbers. We have shown that in *Moz* mutant mice hematopoietic cells differentiate, form multipotent progenitors with limited capacity for self-renewal, do not have a significant homing defect (as shown by the presence of cells able to form spleen colonies of normal size), and colonize the bone marrow of mutant fetuses. Multipotent cells are therefore present in sufficient numbers to produce lineage-committed progenitors early in gestation, and avoid phenotypic consequences of the loss of *Moz* function in the peripheral blood before birth, but cannot sustain long-term repopulation of an irradiated host after transplantation. A

gradual loss of hematopoietic capacity is, however, already apparent in the *Moz* mutant animals before birth, since the total number of colony-forming cells declines from E12.5 to E18.5.

In conclusion, we have shown that *Moz*, which is a target of translocations leading to leukemia, has an essential function in hematopoiesis. We have previously shown that the closely related protein Querkopf has an essential function in neural stem cells, both during fetal development (Thomas et al. 2000) and in the adult (Rietze et al. 2001). Together, these results show that these histone acetyltransferases specifically regulate diverse stem cell populations.

Materials and methods

Antibodies

Anti-Flt3 (A2F10.1), anti-CD19 (ID3), anti-B220 (RA3-6B2), anti-Gr.1 (RA6-8C5), anti-Ter119, anti-CD4 (GK1.5), anti-CD8 (YTS169.4), and anti-TCR (H57-597) were purified from hybridoma supernatants and biotinylated in the authors' laboratories. Anti-Sca1-PE, anti-c-Kit-APC, anti-AA4.1-PE, CD45.1-FITC, CD45.2-PE, and streptavidin-PE were purchased from BD Pharmingen. Anti-CD4-FITC and anti-CD8-PE were purchased from Caltag. Anti-Neomycin Phosphotransferase (polyclonal IgG raised against the full-length protein) was purchased from Upstate Biotechnology (#06-747).

Generation of the mutant allele

The homologous recombination construct was generated using a C57B/6 BAC, RP23-463A1, which contains all coding exons of the *Moz* gene. The 5' arm consists of 3.8 kb of *Moz* genomic sequences ending at a point equivalent to the break point in *Moz* in the t(8;16) translocation (Borrow et al. 1996) found in human leukemia. The 5' arm was partially generated by PCR and checked by sequencing. The 3' arm consisted of the 8.3-kb NcoI–BamHI fragment 3' of the break point. These fragments were cloned into a vector 5' and 3', respectively, of a promoterless *neomycin phosphotransferase* (*neo*) gene equipped with a *Pgk-1* polyadenylation sequence to produce the final construct pMoz17. The *neo* coding sequence was cloned in-frame with the remaining *Moz* coding sequences. This construct was linearized and electroporated into MPI-II cells (Voss et al. 1997). Transgenic embryonic stem (ES) cell clones were selected on the basis of G418 resistance. However, ES cell clones containing one homologously recombined *Moz* allele grew poorly, consistent with the *Moz*–*neo* fusion protein being present at low levels within the cells. Correctly targeted ES cell clones (11 from 104 G418-resistant clones) were identified by Southern gel blots, as shown in Figure 1, and chimeras were generated by morula aggregation (Voss et al. 1997). After homologous recombination, the last six amino acids encoded by the mutated *Moz* gene are His, Ser, Gln, Gln, Val, and Val. Germline chimeras were produced from two independent ES cell clones, Moz17–51 and Moz17–69. Due to very low fertility of heterozygous mice on inbred C57B/6 or 129Sv backgrounds, experiments were done using mice backcrossed to FVB/BalbC F1 hybrids. Since FVB mice carry the CD45.1 allele and BalbC mice carry the CD45.2 allele, this allows us to generate *Moz*^Δ intercrosses in which all progeny have the genotype CD45.1/CD45.2. The phenotype of homozygous mice derived from both Moz17–51 and Moz17–69 lines were identical; however, results are presented for line Moz17–51.

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Gross pathology and histology were performed as described previously (Thomas et al. 2000).

Colony-forming cell assays

Fetal liver colony-cell-forming assays were performed at E12.5 and E18.5. Suspensions of liver cells were cultured in 0.3% agar and stimulated to differentiate by the addition of purified murine growth factors, interleukin 3 (10^{-3} U/mL), stem cell factor (100 ng/mL), and erythropoietin (2 U/mL). Cultures were incubated for 7 d, then fixed, stained, and counted as described previously (Metcalf 1984).

Transplantation assays

CFU- S_{12} assays were performed as described previously (Till and McCulloch 1961). Briefly, a fetal liver cell suspension was prepared by triturating fetal livers in 1 mL of phosphate-buffered saline containing 3% heat-inactivated fetal calf serum. Cells were counted and viability assessed by trypan blue exclusion. Recipients were lethally irradiated with two doses of 5.5 Gy (550 rads) generated from a ^{60}Co γ -irradiation source, separated by 3 h. Two hours later, recipient mice were injected via the tail vein with a fetal liver cell suspension containing 10^6 cells. Three to five recipient mice were used per cell preparation. After 12 d, the spleens were dissected from recipient mice and fixed in Bouin's fixative. Colonies were counted under a dissection microscope. Spleens were sectioned and sections were stained with hematoxylin and eosin. For long-term reconstitution experiments, recipient mice were lethally irradiated as described above for the CFU- S_{12} assays and then injected with cell suspensions prepared from fetal livers. The recipient mice lacked a functional Rag-1 gene (Spanopoulou et al. 1994) and were backcrossed onto a C57B/6 background for >10 generations. The absence of T cells in these mice prevented host versus graft reaction. The donor cells could be distinguished from any residual cells derived from the recipient mice because these would have expressed only the CD45.2 isoform. Competitive reconstitution assays were performed as described previously (Harrison et al. 1993). Fetal liver cell suspensions were prepared as above. Competitor cells (10^6) from fetal livers dissected from mice carrying the CD45.1 allele were mixed with either 10^6 or 4×10^6 test cells from fetuses derived from *Moz* $^{\Delta/+}$ intercrosses (CD45.1/CD45.2 double-positive). After 6 wk, 12–14 wk, and up to 22 wk, the peripheral blood leukocytes of recipient mice were analyzed for the expression of the cell surface marker CD45 as shown (Fig. 3).

Fetal thymic organ cultures

The thymi from E18.5 fetuses were dissected and maintained in culture on a floating support as described previously (Purton et al. 2000). Thymi were maintained in culture for 12 d with a single medium change on day 6. On day 12, thymocytes were isolated from thymic lobes and the proportion of CD4 and CD8 single-positive cells in addition to TCR-positive cells was determined by flow cytometry.

Analysis of *Moz* gene expression

Northern blot analysis was performed using a 482-base-pair (bp) probe coding for part of the MYST domain (exons 10–12 and part of exon 9). RNA samples and procedure were identical to those described in Voss et al. (2003). Hematopoietic progenitor populations were isolated by FACS based on their cell surface marker profile as described previously (Nutt et al. 2005). These were

used to generate cDNA samples, and were then analyzed by quantitative PCR on an ABI prism 7900 (Applied Biosystems) using SYBR green (Qiagen QuantiTect) and protocols supplied by the manufacturer. *Moz* mRNA expression was normalized to *HPRT* mRNA.

Radioactive in situ hybridization, performed as described previously (Thomas et al. 2000) using a 2.8-kb probe to *Moz* exon 16. Briefly, the probe was labeled by incorporating [^{35}S]CTP (Amersham Biosciences) in an in vitro transcription reaction using either T3 or T7 RNA polymerase, hydrolyzed and incubated with deparaffinized tissue sections overnight at 55°C in a solution containing 50% formamide to increase stringency. Slides were washed at high stringency in SSC/50% formamide at 60°C followed by RNase A treatment to remove single-stranded RNA molecules. Slides were then dipped in liquid emulsion (LM-1, Amersham Biosciences) and stored for 2–3 wk at 4°C. Slides were then developed and lightly counter-stained with hematoxylin without differentiation.

Western blot analysis was performed using standard techniques. Briefly, embryos and adult organs were frozen on dry ice and stored at -70°C . Embryos and adult organs were lysed in ice cold KALB buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.5, 1% Triton X-100, 1 mM EDTA) supplemented with protease inhibitors (Roche). Lysates containing 60 μg of protein were subjected to electrophoresis on a 4%–20% PAGE gel and transferred to membranes (PVDF-Plus, Micron Separation, Inc.). Membranes were preincubated in 10% skim milk and 0.1% Tween 20. Membranes were then incubated in 1% BSA and 0.1% Tween 20 containing the anti-neo antibody diluted 1:750, washed, then incubated with goat anti-rabbit secondary antibody coupled to horseradish peroxidase (1:3000) before a final washing step. The antibody binding was visualized using chemiluminescence.

Statistical analysis

SAS StatView 5.0.1 software was used to perform one- or multifactorial analyses of variance followed by Fisher's post-hoc tests as indicated in the figure legends. The default α value of the StatView 5.0.1 software (5%) was used.

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