Perinatal synthetic lethality and hematopoietic defects in compound *mafG::mafK* mutant mice

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Prior studies exploring the mechanisms controlling erythroid gene regulation implicated MARE (Maf recognition element) cis-elements as crucial to the transcriptional activity of many erythroid genes. Numerous transcription factors can elicit responses through MAREs, including not only the AP-1 family proteins, but also a growing list of factors composed of Cap-N-Collar (CNC)–small Maf heterodimers. While these factors can activate transcription from MAREs in co-transfection assays, mouse germline mutations in *cnc* genes tested to date have failed to reveal primary erythroid phenotypes. Here we report that after combining the *mafK* and *mafG* targeted null alleles, mutant animals display several synthetic phenotypes, including erythroid deficiencies. First, compound homozygous small *maf* gene mutants survive embryogenesis, but die postnatally. Secondly, compound mutant animals develop severe neurological disorders. Thirdly, they exhibit an exacerbated *mafG* deficiency in megakaryopoiesis, specifically in proplatelet formation, resulting in profound thrombocytopenia. Finally, the compound mutant animals develop severe anemia accompanied by abnormal erythrocyte morphology and membrane protein composition. These data provide direct evidence that the small Maf transcription factors play an important regulatory role in erythropoiesis.

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spherocytosis

Introduction

The chicken ϵ/β -globin gene enhancer was the first distant transcriptional control element identified that was proven to be crucial for tissue-specific control over erythroid gene regulation (Choi and Engel, 1986; Hesse *et al.*, 1986). Analysis of enhancer activity by transfection of clustered array mutants into erythroid cells showed that one particular DNA sequence motif, specifying an AP-1 binding site, conferred the greatest contribution to enhancer activity (Reitman and Felsenfeld, 1988). The protein that bound

to this element, referred to as nuclear factor-erythroid 2 (NF-E2), was shown to be distinct from AP-1 through analysis of the erythroid-specific porphobilinogen deaminase gene promoter (Mignotte *et al.*, 1989). Over the past decade, this same sequence motif has been identified within *cis* regulatory elements of numerous erythroid genes. Despite the accumulated wealth of information demonstrating the importance of this binding site in erythroid gene regulation, there has been no formal demonstration of which transcription factor actually elicits responses from this *cis*-element in erythroid cells.

After its purification and cloning by reverse genetics, NF-E2 was found to be a heterodimeric basic region plus leucine zipper transcription factor. The larger subunit of this complex, called p45 (Andrews *et al.*, 1993a), displayed all the hallmarks of a hematopoietic cell-restricted transcription factor, while the smaller subunit, called p18 or MafK (Andrews *et al.*, 1993b; Igarashi *et al.*, 1994), consisted of little more than an amphipathic dimerization motif and a positively charged domain. MafK shared greatest sequence homology with the family of proteins related to the chicken v-Maf oncoprotein (Nishizawa *et al.*, 1989), while the p45 subunit was most closely related to the *Drosophila* nuclear regulatory Cap-N-Collar (CNC) protein (Mohler *et al.*, 1991).

Since those early observations, the number of proteins has expanded considerably that can form (either productive or unproductive) homo- or heterodimers, or that can specifically bind to the extended AP-1 sequence motif (called a MARE, for Maf recognition element; Kataoka *et al.*, 1994b; Motohashi *et al.*, 1997) present in the regulatory sequences of most erythroid as well as many non-erythroid genes. This group of MARE binding factors now includes four large Maf proteins (Swaroop *et al.*, 1992; Kataoka *et al.*, 1993, 1994a; Ogino and Yasuda, 1998), six CNC family members (Chan *et al.*, 1993, 1996; Itoh *et al.*, 1995; Johnsen *et al.*, 1996; Oyake *et al.*, 1996; Kobayashi *et al.*, 1999) and three small Maf proteins (Fujiwara *et al.*, 1993; Kataoka *et al.*, 1995), in addition to all of the previously identified members of the AP-1 (Jun/Fos) transcription factor families. Thus, the complexity of regulatory responses that can be controlled through the MARE element is vast: the factors capable of binding to these sites are pervasive, and the consequences of their binding have been shown to elicit transcriptional responses ranging from activation to repression (Engel, 1994; Kataoka *et al.*, 1995; Motohashi *et al.*, 1997).

Three of the six currently known *cnc* family members have been examined by germline mutagenesis. *p45* mutation conferred permanently impaired platelet formation as well as transient neonatal erythrocyte abnormalities, which were proposed to be an indirect consequence of thrombocytopenia since adult mutant animals had normal erythropoiesis (Shivdasani and Orkin, 1995; Shivdasani

The results of the compound mutant loss-of-function analyses shed significant new insight into the cellular processes controlled by small Maf–CNC heterodimer activity. First, compound homozygous *maf* mutant animals survive gestation. Animals missing both the *maff* and *mafK* genes suffer virtually no abnormalities, while compound mutants missing both *maff* and *mafG* differ only slightly from those missing *mafG* alone (our unpublished observations). However, unlike any of the individual small *maf* mutant or the *p45* mutant animals, *mafK::mafG* compound homozygous mutants succumb immediately after birth to postnatal lethality, which appears to be a consequence of differential contributions from three separate pathological deficiencies. First, compound homozygous mutants exhibit profound thrombocytopenia, thus exaggerating the homozygous *mafG*^{−/−} mutant phenotype to phenocopy precisely the *p45* mutation in its effects on megakaryopoiesis. Secondly, compound mutant animals bearing only one active *mafK* allele (e.g. *mafG*^{−/−}::*mafK*^{+/−}) display chronic posterior ataxia, exacerbating the previously documented *mafG*^{−/−} homozygous deficiency. Finally, homozygous compound mutants suffer from anemia, red cell fragility and erythroid cytoskeletal defects that resemble hereditary spherocytosis. Unlike the *p45* mutants, the *mafK::mafG* compound homozygous mutants display red cell morphological abnormalities at all developmental stages, including in definitive erythroid cells prior to birth. Furthermore, the small *maf* compound mutants

Fig. 1. Small *maf* mutant detection strategy. PCR screening of the *mafG* and *mafK* loci. Owing to the high similarity between the *mafG* and *mafK* loci, both are represented on a single diagram. (A) A common 5' primer was used with two distinct 3' primers, one corresponding to sequences within the second intron of the wild-type allele (top) and a 3' *lacZ* primer for the mutant allele (bottom). (B) Two percent agarose gel electrophoresis showing a typical distribution of wild-type, heterozygous and homozygous mutant animals from a compound heterozygous mutant intercross.

never survive postnatally. These experiments show for the first time an *in vivo* requirement for the small Maf family proteins in erythropoiesis, and they thus provide a key link in identifying *trans*-acting factor(s) that acts at this demonstrably crucial *cis* regulatory transcriptional control element.

Results

Synthetic perinatal lethality as a consequence of combining homozygous mafK and mafG mutant alleles

We first intercrossed *mafG*^{+/-} with *mafK*^{-/-} mutant mice (129/CD1 mixed hybrid background) to generate *mafG*^{+/-} :: *mafK*^{+/-} compound mutants; the compound heterozygotes were recovered at the expected Mendelian frequencies. Since those animals exhibited no apparent dysfunction, we intercrossed them and genotyped progeny between postnatal day 7 (P7) and P14. Genomic tail DNA was analyzed by PCR using common 5' *mafG*- and *mafK*-specific as well as separate 3' primers to distinguish between the wild-type and mutant alleles (Figure 1). Of 279 pups, all but one of the anticipated intercross genotypes were recovered at the expected Mendelian frequency (Table IA). Only four compound homozygous mutant (CM) animals were recovered, of 17 expected statistically. Although we increased the number of CM pups by initiating intercrosses with (phenotypically normal) *mafG*^{+/-} :: *mafK*^{-/-} breeding pairs, we still recovered only four CM pups out of 122 progeny from the latter intercrosses (of 30.5 expected; Table IB). Both results were statistically significant (Table IA, *p* < 0.05; Table IB, *p* < 0.01), demonstrating minimally that CM animals fail to thrive after birth. These CM pups were quite small compared with their littermates (Figure 2A) and did not survive to weaning. Therefore, we decided to kill CM pups at or before P14 to investigate further the consequences of combining these two null mutant alleles.

The identification of surviving postnatal CM animals suggested that the lethality of this genotype was probably

Fig. 2. Physiological characteristics of *mafG::mafK* CM pups. (A) The appearance of newborn CM pups. The CM pup (on the right) is both smaller and more pale than its control littermate (left); pups of this genotype most often suffer from hemorrhage in the lower abdomen [arrowhead in (B)] and head (not shown). (C) Small *maf* multiple allele mutant pups exhibit fully penetrant dyskinesia. The two pups shown are a control compound heterozygote (*mafG*^{+/-}::*mafK*^{+/-}; right) and a three-allele mutant (*mafG*^{-/-}::*mafK*^{+/-}; left) animal. The three-allele mutant adult was unable to right itself or control involuntary convulsions in its spastic hindquarters, and was therefore extremely ataxic.

Table I. Genotypes recovered from compound heterozygous mutant intercrosses

(A) *mafG*^{+/-}::*mafK*^{+/-} × *mafG*^{+/-}::*mafK*^{+/-}

Genotype	No. of 18.5 d.p.c. embryos (observed/expected)	No. of P14 pups (observed/expected)
<i>mafG</i> ^{+/+} :: <i>mafK</i> ^{+/+}	6/6.4	12/17.4
<i>mafG</i> ^{+/+} :: <i>mafK</i> ^{+/-}	10/12.9	47/34.8
<i>mafG</i> ^{+/+} :: <i>mafK</i> ^{-/-}	9/6.4	16/17.4
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{+/+}	15/12.9	40/34.8
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{+/-}	21/25.8	69/69.7
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{-/-}	18/12.9	40/34.8
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{+/+}	4/6.4	21/17.4
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{+/-}	13/12.9	30/34.8
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{-/-}	7/6.4	4/17.4*
Total	<i>n</i> = 103	<i>n</i> = 279

(B) *mafG*^{+/-}::*mafK*^{-/-} × *mafG*^{+/-}::*mafK*^{-/-}

Genotype	No. of P14 pups (observed/expected)
<i>mafG</i> ^{+/+} :: <i>mafK</i> ^{-/-}	38/30.5
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{-/-}	80/61.0
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{-/-}	4/30.5**
Total	<i>n</i> = 122

Interbreeding was performed between animals of mixed 129/CD1 background. Pups were genotyped between P7 and P14. The number of progeny of a specific genotype (observed) is compared with the frequency at which they would be expected given a normal Mendelian distribution (expected). The observed/expected difference is statistically significant: **p* < 0.05; ***p* < 0.01.

not embryonic in origin, but rather occurred at around the time of birth. We thus analyzed intercross progeny at 18.5 days post-coitus (d.p.c.), 1 day before birth, to determine whether or not CM embryos normally complete gestation. We recovered an expected number of CM pups (Table IA), which appeared grossly normal. Therefore, the loss of *mafG* and *mafK* together does not significantly affect embryonic development, and validates the hypothesis that the death of CM animals is a postnatal phenotype. Since none of the single gene small *maf* homozygous mutants had a shortened lifespan (Shavit *et al.*, 1998; Onodera *et al.*, 1999), the consequences of combining two of the mutant alleles resulted in this profoundly altered property, demonstrating that the origin of this perinatal

Fig. 3. Megakaryocyte morphology and *in vitro* platelet formation potential in *mafG*^{+/-}::*mafK*^{+/-} and *mafG*^{-/-}::*mafK*^{+/-} mice. Megakaryocytes were isolated from the bone marrow of *mafG*^{-/-}::*mafK*^{+/-} mice (A and B) or *mafG*^{+/-}::*mafK*^{+/-} mice (C and D). The morphology of both was examined by Giemsa staining (A and C). After plating into culture dishes, control megakaryocytes formed proplatelet projections after 24 h in culture (D), while megakaryocytes recovered from *mafG*^{-/-}::*mafK*^{+/-} mutants showed no propensity for megakaryocyte fragmentation or proplatelet formation (B).

CM attribute was due to the combination of the mutant *mafG* and *mafK* alleles, by definition a synthetic lethal phenotype.

Compound small *maf* mutants display dosage-dependent phenotypes and reveal *in vivo* functions for *mafK*

We previously reported platelet reduction and behavioral defects in *mafG*^{-/-} homozygous mutant animals (Shavit *et al.*, 1998). We therefore wondered whether there would be greater severity in these phenotypes if the animals were missing additional *mafK* alleles.

CM pups were bruised and had an ashen appearance (Figure 2A and B), possibly indicating that they might suffer from platelet deficiency and anemia. We therefore drew peripheral blood from 18.5 d.p.c., P14 and adult animals of various genotypes and performed complete blood analysis. As shown in Table II, all of the animals bearing at least one active *mafG* allele had similar hematological parameters. The blood data for *mafG*^{-/-}::*mafK*^{+/-}

Table II. Blood parameters in small *maf* compound mutant mice

(A) 18.5 d.p.c.							
Genotype	No. of RBC ($\times 10^6/\text{mm}^3$)	Hemoglobin (g/dl)	Hematocrit (%)	MCV (fl)	MCH (pg)	MCHC (%)	No. of platelets ($\times 10^3/\text{mm}^3$)
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{+/-}	3.35 $\pm$ 0.27	12.1 $\pm$ 1.3	29.5 $\pm$ 3.2	88.0 $\pm$ 3.6	36.1 $\pm$ 1.1	41.0 $\pm$ 0.9	334 $\pm$ 45
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{+/-}	3.32 $\pm$ 0.20	11.8 $\pm$ 0.5	28.8 $\pm$ 1.5	87.0 $\pm$ 10.1	35.7 $\pm$ 3.8	41.0 $\pm$ 1.0	78 $\pm$ 12
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{-/-}	3.03 $\pm$ 0.57	10.9 $\pm$ 0.9	25.8 $\pm$ 2.1	86.6 $\pm$ 10.7	36.6 $\pm$ 4.6	42.2 $\pm$ 0.2	98 $\pm$ 16
(B) P14							
Genotype	No. of RBC ($\times 10^6/\text{mm}^3$)	Hemoglobin (g/dl)	Hematocrit (%)	MCV (fl)	MCH (pg)	MCHC (%)	No. of platelets ($\times 10^3/\text{mm}^3$)
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{-/-}	6.18 $\pm$ 0.09	12.9 $\pm$ 0.2	37.0 $\pm$ 1.1	59.8 $\pm$ 0.8	20.9 $\pm$ 0.0	35.0 $\pm$ 0.4	654 $\pm$ 51
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{-/-}	6.03 $\pm$ 0.20	11.9 $\pm$ 0.7	33.6 $\pm$ 1.6	55.2 $\pm$ 1.0	19.8 $\pm$ 0.6	35.8 $\pm$ 1.0	635 $\pm$ 64
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{+/-}	6.34 $\pm$ 0.11	12.8 $\pm$ 0.4	34.4 $\pm$ 0.6	54.3 $\pm$ 0.0	20.2 $\pm$ 0.3	37.3 $\pm$ 0.6	164 $\pm$ 7
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{-/-}	3.50 $\pm$ 0.47	7.1 $\pm$ 1.6	16.7 $\pm$ 2.0	49.5 $\pm$ 2.0	17.9 $\pm$ 2.0	36.1 $\pm$ 2.6	20 $\pm$ 6
(C) Adult							
Genotype	No. of RBC ($\times 10^6/\text{mm}^3$)	Hemoglobin (g/dl)	No. of platelets ($\times 10^3/\text{mm}^3$)				
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{+/-}	8.34 $\pm$ 0.60	15.7 $\pm$ 0.5	1191 $\pm$ 80				
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{-/-}	8.91 $\pm$ 0.19	14.4 $\pm$ 0.3	1095 $\pm$ 48				
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{-/-}	9.29 $\pm$ 0.98	15.1 $\pm$ 0.8	1087 $\pm$ 79				
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{+/-}	8.99 $\pm$ 0.56	15.1 $\pm$ 0.7	529 $\pm$ 40				
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{+/-}	8.79 $\pm$ 0.63	14.5 $\pm$ 0.5	164 $\pm$ 25				
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{-/-}	ND	ND	ND				

Each value represents the mean $\pm$ SD of measurements of blood samples from at least three different animals. ND, not determined.

animals were also normal except for a markedly reduced platelet count, which was more severe than in *mafG*^{-/-} homozygous mutant animals (Table IIC). Since deletion of the second active *mafK* allele in the *mafG*^{-/-} mutant background reduces the platelet count essentially to zero (Table IIB), we conclude that consecutive deletion of *mafK* alleles in the *mafG* homozygous mutant background leads to a small Maf dosage-dependent loss of platelets.

We next examined the neurological phenotype of the mutants. Hind leg claspings was a late-onset symptom in *mafG*^{-/-} mutant mice, observed only after 5 or 6 months of age, and was not fully penetrant (Shavit *et al.*, 1998). In stark contrast, the onset of the same behavior in *mafG*^{-/-}::*mafK*^{+/-} animals occurred 3–4 weeks after birth, and was fully penetrant ($n = 14/14$). In addition, these same mutants displayed severe motor ataxia after 2 months of age, with intermittently spastic hind legs (Figure 2C), which appeared to be an exacerbated neurological defect previously documented in the *mafG*^{-/-} animals. Importantly, these findings showed that the loss of MafK in the *mafG*-null mutant background results in MafK-dependent neuromuscular phenotypes.

Perhaps most surprisingly, *mafG*^{+/-}::*mafK*^{-/-} mutants exhibited hematological parameters that were indistinguishable from those of wild-type mice and the animals displayed no neurological symptoms. After prolonged observation, we determined that no defects were observed in *mafG*^{+/-}::*mafK*^{-/-} mice. Analysis of the *mafG*^{+/-}::*mafK*^{-/-} mutants indicated that one active allele of *mafG* in a homozygous *mafK*-null mutant background was sufficient for the development, survival and fertility of these laboratory mice.

Table III. No proplatelet formation in small *maf* compound mutant mice

Genotype	No. of megakaryocytes with PPF/total megakaryocytes			Average PPF (%)
	Experiment No.			
	1	2	3	
<i>mafG</i> ^{+/+} :: <i>mafK</i> ^{+/+}	59/634	122/380	130/311	27.7 ± 16.7
<i>mafG</i> ^{+/+} :: <i>mafK</i> ^{-/-}	53/344	161/440	70/226	27.6 ± 11.0
<i>mafG</i> ^{+/-} :: <i>mafK</i> ^{-/-}	73/535	76/264	126/392	24.8 ± 9.8
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{+/+}	20/1333	4/420	2/413	0.98 ± 0.51
<i>mafG</i> ^{-/-} :: <i>mafK</i> ^{+/-}	0/268	0/828	0/866	0

Small *maf* mutant megakaryocytes are defective in proplatelet formation

Since thrombocytopenia was markedly exacerbated in both the *mafG*^{-/-}::*mafK*^{+/-} and the CM mice, we set out to clarify the nature of the small *maf* mutant-induced developmental block in platelet formation.

Megakaryocytes were isolated from the bone marrow of 6- to 8-week-old mice (Nagahisa *et al.*, 1996; Osada *et al.*, 1999). Most of the megakaryocytes purified from the *mafG*^{-/-}::*mafK*^{+/-} mutant mice were large and appeared to be mature (Figure 3A). In contrast, megakaryocytes from control mice represented different stages of maturation, ranging from small immature cells to large mature cells (Figure 3C). Thus, relatively mature megakaryocytes seemed to dominate in the bone marrow of *mafG*^{-/-}::*mafK*^{+/-} mice, which could reflect changes in their proliferation and/or differentiation in the *mafG*^{-/-}::*mafK*^{+/-} genetic background.

Fig. 4. Persistent fetal liver hematopoiesis in CM pups. Livers from P14 pups were examined. Hematopoietic foci [white arrowheads in (A)] are observed in P14 CM pup liver, while the liver of a littermate *mafG*^{+/-}:*mafK*^{+/-} pup harbors few hematopoietic cells (C). (B) Higher magnification of persistent hematopoietic cells of (A). The scale bar corresponds to 33 μ m (A and C) and 83 μ m (B).

When cultured *in vitro*, ~20% of normal megakaryocytes develop proplatelets, arrays of filamentous cell projections. We quantified the number of purified megakaryocytes with proplatelets after 1 day of *in vitro* culture, and calculated the incidence of proplatelet formation (PPF). Importantly, *mafG*^{-/-}:*mafK*^{+/-} megakaryocytes did not form proplatelets, in contrast to megakaryocytes of other genotypes having normal PPF incidence (Figure 3B and D; Table III). Thus, the results of inactivating one *mafK* allele and the consequent reduction in small Maf activity result in the complete loss of PPF in *mafG*^{-/-}:*mafK*^{+/-} animals. We concluded that one of the primary defects in megakaryocytes isolated from *mafG*^{-/-}:*mafK*^{+/-} mice was the loss of PPF activity, suggesting that two of the small Maf proteins, MafG and MafK, might act as direct regulators of the PPF process in megakaryocytes.

Small *maf* CM animals have irregular RBC morphology and increased RBC heterogeneity

In addition to bruising, the ashen appearance of the P0–P3 CM pups (Figure 2A) indicated that they might also be anemic. As shown in Table IIB, P14 CM pups had fewer red blood cells (RBCs) as well as lowered hemoglobin concentration accompanied by an extremely low platelet count, indicating that the CM animals indeed suffered from severe anemia. CM pup RBCs displayed reduced mean corpuscular volume (MCV) and mean cell hemoglobin (MCH), but mean corpuscular hemoglobin concentration (MCHC) was normal, which differs from the parameters observed in typical iron-deficient anemia caused by frequent hemorrhage. Curiously, CM mice at 18.5 d.p.c. exhibited virtually normal erythroid parameters (Table IIA). We noticed that the P14 CM pups had slight splenomegaly and unusual, persistent fetal liver hematopoiesis (Figure 4A and B; the control is shown in Figure 4C). Histological examination did not distinguish these hematopoietic cells from those in 18.5 d.p.c. CM fetal liver (data not shown), and both appeared normal except for megakaryocyte accumulation. We thus speculated that P14 CM pups displayed reactive extramedullary hematopoiesis due to anemia and that morphological changes of erythroid cells would be more apparent in peripheral blood.

We therefore prepared peripheral blood smears from 18.5 d.p.c. (Figure 5A and B), P10 (Figure 5C and D) and P14 (Figure 5E and F) littermates of compound

heterozygous mutant intercrosses. The RBCs in CM pups displayed aberrant morphology at all stages of definitive erythropoiesis (Figure 5B, D and F). As anticipated from MCV values, many of the CM RBCs were small and round with irregular shapes. By scanning electron microscopic (SEM) analysis, the aberrant RBCs were found to be spherocytes as well as other irregularly shaped cells (Figure 5G–J). It is clear that the erythroid abnormality is not caused by acute blood loss alone, since a majority of the RBCs were misshapen even in 18.5 d.p.c. embryos that displayed no anemia (Figure 5A and B; Table IIA). When we stained reticulocytes taken from the peripheral blood of CM pups and compared it with that of various other genotype littermates, not only had the reticulocyte frequency increased, but there were also many that were small and round, or irregularly shaped (Figure 5C and D). These abnormal reticulocytes strongly supported the hypothesis that an RBC deformity was a primary effect of compound disruption of the *mafG* and *mafK* genes, while leaving open the two possibilities that erythropoiesis is defective and generates misshapen RBCs or that fragility of CM RBCs ends in fragmentation during circulation. Thus, this phenotype differs from that of the *p45* mutants, which appear to suffer no permanent erythroid deficits (Shivdasani and Orkin, 1995).

The microscopic results indicated that CM mice might have disorganized erythroid membranes that are unable to maintain normal erythroid cell cytoskeletal morphology, thereby leading to hemolysis and anemia. In accord with this hypothesis, osmotic fragility tests showed that half of the RBCs taken from P14 CM blood were less stable than those from normal littermates (Figure 6). Interestingly, the other half of the CM RBCs were more stable. This divergent CM RBC stability may originate from alteration in RBC membrane composition, also reflected in an increased number of reticulocytes, which are more resistant to osmolarity change. This result revealed that CM RBCs displayed increased heterogeneity and that blood from CM pups contains RBCs that are more sensitive to osmolarity changes. To clarify the biochemical origins of the hypothetical erythroid membrane defect(s), SDS-PAGE and Western blot analyses were performed to examine the membrane protein constituents of mutant and control erythroid cell ghosts (gently lysed RBCs) (Figure 7). When compared with ghosts prepared from the blood of normal littermates, CM cytoskeletons had

Fig. 5. Irregular RBCs in CM mice. Smears of peripheral blood from *mafG*^{+/-}:*mafK*^{+/-} (A, C and E) or *mafG*^{-/-}:*mafK*^{-/-} (CM; B, D and F) 18.5 d.p.c. embryos (A and B) or P10 (C and D) or P14 (E and F) pups. In the CM blood smears, numerous spherocytes and poikilocytes (irregularly shaped RBCs) are observed. The P10 CM pups have an increased number of reticulocytes (C and D). Small and deformed reticulocytes are also observed [arrowheads in (D)], which are clearly distinguishable from densely stained platelets [arrows in (C)]. Note the absence of platelets in CM blood. Scanning electron micrographs of *mafG*^{+/-} (G) or CM (H, I and J) circulating erythroid cells from P14 pups are shown. The CM erythroid cells display spherocyte (H) or poikilocyte (I and J) morphologies.

relatively elevated levels of two proteins migrating with a mobility of apparent molecular sizes of 95 and 48 kDa (Figure 7A). The former (95 kDa) was similar to the electrophoretic mobility of kidney band 3 (Brosius *et al.*, 1989), which is an alternatively spliced kidney-specific isoform of the anion transport protein. The latter (48 kDa) was similar in size to band 4.9, dematin (Rana *et al.*, 1993). However, these proteins that had accumulated to abnormal abundance in the P14 CM RBC ghosts did not correspond to either the kidney-specific splice variant of band 3 or to band 4.9, as demonstrated on Western blots (Figure 7B). Other major membrane proteins, such as α - and β -spectrin (band 1 and band 2; 210–220 kDa) as well as erythroid band 3 (100 kDa) and actin (band 5; 42 kDa)

Fig. 6. CM blood contains erythrocytes more sensitive to alterations in osmolarity. Control and CM red blood cells were incubated at varying salt concentrations (abscissa) for 5 min. The hemoglobin concentration in 0% NaCl solution was taken as 100% lysis, and each percentage lysis value was determined. Half of the RBCs from CM pups were more sensitive to osmolarity changes than control RBCs. Another half were more resistant. Each value represents an average from two different animals, and the SD for each experiment is shown.

Fig. 7. Cytoskeletal defects in CM erythrocyte ghosts. (A) SDS-PAGE analysis of erythroid membrane protein recovered from P14 control (C; *mafG*^{+/-}:*mafK*^{+/-}) and CM (*mafG*^{-/-}:*mafK*^{-/-}) peripheral blood. The same number of cells were lysed and loaded on a 5–15% gradient polyacrylamide gel. Proteins were visualized by staining with Coomassie Blue. In the CM erythroid membranes, two proteins appeared to be overexpressed, and migrated with apparent *M_r* of 95 and 48 kDa. (B) Western blot analysis with anti-spectrin, anti-band 3 or anti-dematin antibodies. No difference was observed in the abundance of these three proteins in CM versus control RBC ghosts.

were expressed at approximately normal levels. We also analyzed the abundance of a number of other erythroid tissue-specific mRNAs that might be affected by the mutations using semi-quantitative RT-PCR (α - and β -globin, porphobilinogen deaminase, heme oxygenase, erythroid 5-aminolevulinatase synthase and ferrochelatase), but no differences were detected in the abundance of these

Fig. 8. Maf-independent transcription of the small *maf* genes. (A) Semi-quantitative RT-PCR analysis of RNAs recovered from 14.5 d.p.c. fetal liver and whole embryo of control and CM mice. The number of PCR cycles was 16, 18, 20, 22 or 24 for β -actin, and 24, 26, 28, 30 or 32 for MafF, shown by increasing triangle size. (B) MafK, MafG and MafF mRNA expression profiles were examined in *mafK*-null mutant, *mafG*-null mutant and wild-type adult mice by real-time quantitative RT-PCR. Expression levels of small *maf* genes were normalized for rRNA expression level in the same cDNA preparation. The expression levels were normalized for mRNA levels in the wild-type intestine (set as 1). Each value represents the mean $\pm$ SD of the relative levels from four independent experiments. Open bars, shaded bars and closed bars indicate wild-type, *mafK*-null mutant and *mafG*-null mutant mice, respectively. B.M. is bone marrow. (C) MafK, MafG and MafF mRNA levels were also analyzed by RNase protection assays. Antisense probes for MafK (lane 1), MafG (lane 2) or MafF (lane 3) were hybridized to 50 μ g each of total RNA from the intestine of a wild-type adult mouse. The three probes were mixed and hybridized to RNA samples from wild-type intestine (lane 4), *mafK*^{+/-} intestine (lane 5), *mafK*^{-/-} intestine (lane 6), wild-type liver (lanes 8 and 9), *mafG*^{+/-} liver (lanes 10 and 11), *mafG*^{-/-} liver (lanes 12–14) or tRNA (lanes 7 and 15). The migration positions of undigested probes are indicated by arrowheads, while the positions of protected fragments are indicated by arrows.

mRNAs recovered from CM versus normal animals (data not shown).

We conclude that the anemia observed in CM mice is a small *maf* mutant synthetic phenotype, and thus detectable only after combining two null *mafG* and *mafK* alleles. The erythroid cell spherocytosis and anemia were not observed if even one small *mafG* or *mafK* allele was active. Therefore, small *maf* CM pups appear to display severe anemia in part as a consequence of increased fragility of a significant fraction of their RBCs. We assume that the aberrant accumulation of 95 and/or 48 kDa erythrocyte membrane-associated proteins may cause the defect in RBC membrane integrity, which in turn leads to increased heterogeneity in RBC morphology and in osmotic fragility.

No cross-regulation between the three small *maf* genes

The observation that CM embryos can complete normal prenatal development led to the hypothesis that expression of the remaining small Maf protein, MafF, might be upregulated in CM mice to compensate for loss of the other two gene products (e.g. Foley *et al.*, 1998). To test this hypothesis, we determined the abundance of MafF mRNA in 14.5 d.p.c. fetal livers and embryos using semi-quantitative RT-PCR. As shown in Figure 8A, no increase in MafF accumulation was observed in CM fetal liver or whole embryos, suggesting that there is no compensating upregulation of the *mafF* gene even when MafG and

MafK are absent. Similarly, we found no evidence for upregulation of either of the remaining two small *maf* mRNAs in any single *maf* mutant when analyzed by real-time quantitative RT-PCR analysis (Figure 8B). These results were further supported by RNase protection assays also showing that there was no increase in the remaining small *maf* mRNAs in either *mafG* or *mafK* mutant mice (Figure 8C). These results therefore demonstrate that the transcription of the three small *maf* genes is not influenced by the loss of others, and thus that the abundance of small Maf transcript is determined by small Maf-autonomous mechanisms.

Discussion

This study vividly demonstrates that the small Maf proteins function in overlapping fashion in several developmental pathways *in vivo*. CM mice die perinatally from loss of both MafG and MafK, presumably due to absolute thrombocytopenia (exacerbation of a *mafG*-specific phenotype) and anemia (a unique *mafG::mafK* synthetic compound phenotype). *mafG*^{-/-}::*mafK*^{+/-} mutants display more severe manifestations of *mafG*-specific phenotypes, in a dosage-dependent manner, and thereby demonstrate that MafK functions in both hematopoietic and neuronal lineages, as we anticipated from their expression profiles (Motohashi *et al.*, 1996; Shavit *et al.*, 1998). Finally, analysis of *mafG*^{+/-}::*mafK*^{-/-} compound mutant animals demonstrates that *mafG* is the more critical gene for

survival, and that only one *mafG* allele is both necessary and sufficient for a semblance of normal reproduction and existence.

The observation that small *maf* CM mice exhibited severe thrombocytopenia and deformed erythrocytes was in part similar to the phenotypes exhibited by *p45* homozygous mutant mice (Shivdasani *et al.*, 1995) confirms the fact that the small Maf proteins and p45 are bona fide heterodimeric partners, certainly in megakaryocytes. However, the CM phenotype was clearly more severe than the *p45* mutation since no CM newborn ever survived to weaning. In contrast, once *p45* mutant animals survive beyond a specific critical point in neonatal development, they recover from neonatal injuries and develop normally (Shivdasani *et al.*, 1995). We suspect, but have no data to support the hypothesis, that the difference in transcription factor requirements between fetal liver and adult (bone marrow or spleen) hematopoiesis might account for the difference in the survivability of *p45* versus *maf* CM mice, and that the loss of small Maf proteins causes a broader and more severe defect in bone marrow hematopoiesis than does the *p45* mutation.

Embryonic analysis showed that *mafG*^{-/-}::*mafK*^{-/-} mutants could survive through gestation, and were recovered at normal Mendelian frequencies at 18.5 d.p.c. Therefore, the compound mutant lethality observed is peri- or postnatal. This was unexpected considering the dynamic expression patterns displayed by both *mafG* and *mafK*, with complementary expression patterns as early as 6.5 d.p.c., including only few apparent sites of overlapping expression from 8.5 d.p.c. onwards (Shavit *et al.*, 1998). Nonetheless, prominent sites of co-expression included pre-gastrulation mesenchyme, the ectoplacental cone, the yolk sac endoderm and the fetal liver. This expression pattern led us to hypothesize that compound mutant lethality, if encountered, would occur during one of these early critical stages during which those organs or tissue functions become important to the progress of embryogenesis (Shavit *et al.*, 1998). The experimental results that we described here suggest several alternative possibilities. One possible explanation is that MafF is able to compensate for the loss of both MafG and MafK in these tissues. The expression of the *mafF* gene in the developing embryo is relatively limited in comparison to *mafG* (Onodera *et al.*, 1999) and is not subject to up-regulation (Figure 7A), and therefore MafF seems unlikely to be a candidate for a factor that is able to compensate for the loss of *mafG* and *mafK*. It is nonetheless possible that minuscule levels of MafF, levels that are undetectable by β -galactosidase staining, could compensate for the loss of MafG and MafK. To test this hypothesis, we are currently attempting to generate animals in which all three small *maf* genes are ablated. An alternative explanation for normal embryonic development in the CM embryos is that other MARE and/or TPA-responsive element (TRE) binding factors, including large Mafs and Fos or Jun family members, may also compensate for MafG and MafK loss. Finally, it is also conceivable that other small *maf* genes are yet to be cloned, and that these overlap with *mafG* and *mafK* in their expression patterns or are induced in the mutants. However, since there is no evidence from numerous direct homology screens, two-hybrid screens or any other

functional assay that a fourth small Maf protein exists, we feel that this final possibility is unlikely.

The biochemical origin of poikilocytes in the CM mice is not clear. It is well known that deficiencies in major erythroid membrane skeletal proteins can elicit multiple disease phenotypes (spherocytosis, elliptocytosis, poikilocytosis or erythroid cell fragmentation; Clark and Wagner, 1989). It was reported earlier that disruption of the band 3 gene caused spherocytosis and hemolytic anemia (Southgate *et al.*, 1996). Based on the size of the proteins, we originally suspected that misregulation of the anion transporter (band 3) or dematin (band 4.9) proteins could be the cause of the strange RBC morphology. However, this hypothesis was definitively rejected by Western blot analysis, which showed that the band 3 and band 4.9 proteins were unchanged in abundance or size. Nonetheless, it is clear from SDS-PAGE that at least two proteins are markedly overexpressed in CM erythroid ghosts, suggesting that inappropriate excess of one or both of these proteins leads to pathophysiological disruption of the erythroid cytoskeleton.

These observations on mutant RBC ghosts are most consistent with the hypothesis that MafG and MafK homo- or heterodimers normally repress transcription of specific RBC target genes by virtue of small Maf binding to target MARE sites, and that in the absence of these small Maf proteins (in CM animals) these target genes are transcriptionally de-repressed and thereby inappropriately expressed. The consequences of this hypothetical overly abundant expression would then lead to the observed defective membrane integrity, suggesting that erythroid cytoskeletal proteins are among those Maf-repressed target genes. Small Maf proteins were shown previously to be effective repressors *in vitro* (Igarashi *et al.*, 1995a,b) and *in vivo* (H.Motohashi and M.Yamamoto, unpublished observations), thereby indirectly supporting this hypothesis, while the present data show that RBC ghosts in CM animals abnormally accumulate proteins associated with RBC ghosts, also supporting a 'Maf repressor' hypothesis.

Analyses of the *mafG*^{-/-}::*mafK*^{+/-} mutant animals were particularly informative in that they revealed the dosage-dependent phenotypes and demonstrated an *in vivo* role for MafK that was undetectable in the single small *maf* mutant animals (Kotkow and Orkin, 1996; Shavit *et al.*, 1998). The dosage-dependent decrease in platelet number with successive loss of MafK in the *mafG*-null background is particularly suggestive for the mechanism by which these proteins function *in vivo*: namely, that a single transcriptional event leading to platelet maturation requires, in a dosage-dependent fashion, small Maf activity. These multiple allele small *maf* mutant mice also exhibited exaggerated manifestations of the behavioral defects observed in *mafG*^{-/-} animals. The hind leg clasping phenotype was particularly obvious and was 100% penetrant in *mafG*^{-/-}::*mafK*^{+/-} animals. Finally, *mafG*^{-/-}::*mafK*^{+/-} mutants were 10–20% smaller than *mafG*^{-/-} littermates, which were themselves almost half the size of control animals. The dosage-dependent nature of all these behavioral and physiological deficits was further supported by analysis of the sole CM pup that survived until P19. It clasped its hind legs even before weaning and was half the size again of its *mafG*^{-/-} littermates. While CM animals

might have overcome the profound thrombocytopenia (as do 10% of the *p45* mutants; Shivdasani *et al.*, 1995), the additional stress from anemia due to the small *maf* erythroid deficit would certainly prevent them from surviving beyond adolescence.

Finally, we specifically note that the *mafG*^{+/-}::*mafK*^{-/-} mice were normal. This was quite surprising, since the data therefore imply that only one allele of *mafG* is necessary for all functions normally fulfilled by two wild-type alleles of *mafK* and *mafG*. This result suggests that MafK is less important to survival and reproductive capacity than is MafG in laboratory mice. However, the expression patterns of these two genes imply that MafK has unique roles unrelated to MafG, and that these roles may be compensated for by MafF or some other MARE binding protein(s).

Materials and methods

Genotyping of pups and embryos from compound heterozygous intercrosses

The individual *mafK* and *mafG* mutants (Shavit *et al.*, 1998) were intercrossed to generate the compound heterozygous mutants; these were then interbred for compound mutant analysis. Tails for genotyping were digested overnight in lysis buffer (100 mM NaCl, 1.0% SDS, 50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 0.35 mg/ml fresh proteinase K) at 55°C, phenol-chloroform extracted and precipitated.

Genotyping was performed by PCR. The sequences of the primers used for this analysis were: 5' *mafG* primer, MafG36: GCATGACTCGCCAGGAACAG; 3' *mafG* primer, MafG433: CCCAAGCCCA-GCCTCTCTAC; 5' *mafK* primer, MafKEx2.1: CCTACCGTTT-CTGTCTTTCCAG; 3' *mafK* primer, MafKIn346: AATTCCTGAGG-ACAAAGCTGAC; 3' mut. primer, LacZ4: CCGTAGCCAGCTTT-CATCAAC.

Preparation of peripheral blood smears

Litters of newborn pups were decapitated and a drop of blood was put onto a glass slide. The blood drop was spread with the edge of another glass slide, and then air-dried. The slides were subsequently fixed in methanol and processed with a Diff-quick™ (American Scientific Products) kit, which produces results similar to Wright-Giemsa staining. Peripheral blood from P10 pups (Figure 5C and D) was stained with Brecher's new methylene blue solution (Muto, Tokyo) for the detection of reticulocytes.

Hematological analysis

Blood was drawn into a heparinized capillary from newly isolated and freshly decapitated 18.5 d.p.c. embryos for hematological analysis; yolk sacs were genotyped later. Live mice were killed with CO₂ followed by cervical dislocation and 100–200 µl (2-week-old) or 500 µl (adult) of blood were drawn from the inferior vena cava into a syringe containing 2 µl (2-week-old) or 5 µl (adult) of 0.5 M EDTA. The blood samples from adults were analyzed by the Biological Research Laboratory of the University of Illinois at Chicago or by Anaylitics, Inc. (Gaithersburg, MD).

For 2-week old mice, it was difficult to determine precisely the platelet number in the compound mutant animals, since it appeared that small, fragmented RBCs might erroneously contribute to the automated platelet determination by cell size (see Figure 5). Therefore, the platelet:erythrocyte ratio was determined microscopically first from blood, then the actual platelet number was determined by counting the number of erythrocytes. The control platelet number quantified in this manner was ~75% of the value obtained by automated counting.

For histological analysis of persistent fetal liver hematopoiesis, the livers of P14 pups and neonates were fixed with 3.7% formaldehyde, embedded in paraffin, and then stained with hematoxylin and eosin.

Proplatelet formation assay

Bone marrow cells were collected from femurs and tibias of 6- to 8-week-old mice. Bone marrows were flushed with 2 ml of CATCH medium (Hanks' balanced salt solution, 1 mM adenosine, 2 mM theophylline, 0.38% sodium citrate pH 7.2) into a plastic dish. To enrich

the megakaryocytes, marrow cells were centrifuged in 50% Percoll/CATCH solution (density 1.065 g/ml) at 1100 r.p.m. at 20°C for 30 min. The intermediate layer was recovered, put on top of a bovine serum albumin (BSA) density gradient (16% BSA/CATCH at the bottom, 4% BSA/CATCH in the middle and 2% BSA/CATCH at the top) and allowed to stand at room temperature for 1 h. Cells were collected from the bottom and resuspended in Iscove's modified Dulbecco's medium (Gibco) supplemented with 1× Nutridoma-SP (Boehringer Mannheim). These enriched megakaryocytes were cultured in 5% CO₂ at 37°C for 24–36 h. An aliquot of the cells was mounted on slides by cytospin (Shandon) and stained with Giemsa.

Acetylcholinesterase activity was detected before counting megakaryocytes for PPF. Cells were fixed with 0.05% paraformaldehyde/phosphate-buffered saline (PBS) at room temperature for 30 min. After washing with PBS, 0.05% acetylthiocholine iodide in 75 mM sodium phosphate buffer pH 6.0, 5 mM sodium citrate, 3 mM CuSO₄, 0.5 mM KCNFe³⁺ was applied to the cells and incubated at 37°C for 30 min. The number of acetylcholinesterase-positive cells displaying obvious filamentous cell projections (e.g. Figure 3D) was counted and the PPF ratio was calculated (Table III).

Scanning electron microscopy

Washed RBCs were attached to a coverslip coated with poly-L-lysine by gravity and then fixed with 1% glutaraldehyde, 150 mM NaCl, 20 mM Tris-HCl pH 7.4 for 1 h. After dehydration with graded ethanol solutions and surface coating with Au/Pd, samples were imaged using a JSM-35CF JEOL SEM. The SEM images shown were pseudocolored in red using Adobe Photoshop.

Osmotic fragility tests

RBCs were recovered from 2-week-old CM or littermate control pups (*mafG*^{+/-}::*mafK*^{+/-} or *mafG*^{+/-}::*mafK*^{-/-}) and placed for 5 min in iso-osmotic (0.9%) to extreme hypo-osmotic (0.2%) NaCl solution. After removing unlysed RBCs by centrifugation, the hemoglobin concentration of the supernatant solution was determined by color reaction and the percentage lysis was determined (Briegleb *et al.*, 1993).

SDS-PAGE and Western blot analysis

Blood was drawn from P14 animals and RBC numbers were determined using a hemocytometer to normalize for cell number. A 20–30 µl volume (depending on cell number) of drawn blood was placed in an Eppendorf tube and after washing three times with 0.15 M NaCl, 5 mM sodium phosphate pH 8.0, 1 mM EDTA, cells were lysed by the addition of 0.5 ml of 0°C lysing buffer (10 mM sodium phosphate pH 8.0, 1 mM EDTA, 0.15 mM phenylmethylsulfonyl fluoride, 0.04 mM diisopropylfluorophosphate). After 5 min on ice, erythroid membrane proteins were isolated by centrifugation and subsequent washing. These erythroid membranes were electrophoresed on a 5–15% gradient polyacrylamide gel, and proteins were visualized by standard Coomassie Blue staining.

For Western blot analysis, anti-human band 3 polyclonal antibody, anti-mouse dematin polyclonal antibody and anti-human spectrin polyclonal antibody (Sigma) were used. Anti-human band 3 polyclonal antibody is reactive to both erythroid and kidney band 3 proteins (Southgate *et al.*, 1996). The dilution was 1:1000 for primary antibodies and 1:2000 for the appropriate secondary antibodies. Antibody reactivity was visualized by ECL color development (Amersham).

RT-PCR and RNase protection assay

The expression profiles of the *maff*, *mafG* and *mafK* genes were detected by real-time quantitative PCR (ABI PRISM 7700 Sequence Detection System). RNAs were extracted using ISOGEN (Nippon Gene) from tissues of 8-week-old mice obtained from the same mating colony. cDNAs were synthesized from these RNAs, and real-time PCR was performed as described previously (Onodera *et al.*, 1999). Radiolabeled Maff PCR was performed using conditions described previously (Onodera *et al.*, 1999).

For the RNase protection assays, RNA samples were isolated from 10-week-old mice by ultracentrifugation with guanidine isothiocyanate-cesium chloride. To prepare RNA probes for MafK, MafG and Maff mRNAs, pmMafK5, pmMafG2 and pmMaff2 were constructed. pmMafK5 was prepared by inserting a 0.3 kbp *NcoI* fragment of mouse MafK cDNA into the *NcoI* site of pGEM-5Zf(+) (Promega). pmMafG2 was made by inserting a 0.2 kbp *PvuII*–*ApaI* fragment of mouse MafG cDNA into *EcoRV*–*ApaI* sites of pBluescript SK(+) (Stratagene). pmMaff2 was made by inserting a 0.4 kbp *ApaI* fragment of mouse Maff cDNA into the *ApaI* site of pBluescript SK(+). Each of these

plasmids was digested with a restriction enzyme recognizing a unique site and then transcribed with Sp6 polymerase (pmMafK5) or with T7 polymerase (pmMafG2 and pmMafF2) in the presence of [α - 32 P]CTP. The probes were hybridized to 50 μ g of total RNA, digested with RNase A and T1, and electrophoresed as described previously (Motohashi *et al.*, 1996).

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