VARIEGATION OF THE PHENOTYPE INDUCED BY THE GATA-1<sup>low</sup> MUTATION IN MICE OF DIFFERENT GENETIC BACKGROUNDS

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Running Title: GATA-1<sup>low</sup> mutation and genetic backgrounds

Key-words: GATA-1, myelofibrosis, aging, genetic background, gene modifiers

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Barbara Panetta: organized the data and performed statistical analysis.
Elena Alfani: performed all of the mating and kept a registry of the mouse colony.
Valentina Gatta: performed mice genotyping.
Alessandro Pancrazzi: performed histological analysis and quantitative RT-PCR determinations.
Costanza Bogani: prepared hemopoietic tissues for analysis.
Alessandro Maria Vannucchi: designed research, analyzed the data and wrote the paper.
Francesco Paoletti: designed research, analyzed the data and wrote the paper.
Giovanni Migliaccio: designed research, analyzed the data and wrote the paper.
Anna Rita Migliaccio: designed research, analyzed the data and wrote the paper.

All the authors have read the manuscript, concur with its content and state that its content has not been submitted elsewhere.
ABSTRACT

All the mice harboring the X-linked GATA-1<sup>low</sup> mutation in a predominantly CD1 background are born anemic and thrombocytopenic. They recover from anemia at 1-month of age but remain thrombocytopenic all their life and develop myelofibrosis, a syndrome similar to human idiopathic myelofibrosis, at 12-month. The effects of the genetic background on the myelofibrosis developed by GATA-1<sup>low</sup> mice was assessed by introducing the mutation, by standard genetic approaches, in the C57BL/6 and DBA/2 background and by analyzing the phenotype of the different mutants at 12-13- (by histology) and 16-20- (by cytofluorimetry) month of age. Although all the GATA-1<sup>low</sup> mice developed fibrosis at 12-13-month, variegations were observed in the severity of the phenotype expressed by mutants of different background. In C57BL/6 mice, the mutation was no longer inherited in a Mendelian fashion and fibrosis was associated with massive osteosclerosis. Instead, DBA/2 mutants, although severely anemic, expressed limited fibrosis and osteosclerosis and did not present tear-drop poikilocytes in blood or extramedullary hemopoiesis in liver up to 20-month of age. We propose that the variegation in myelofibrosis expressed by GATA-1<sup>low</sup> mutants of different strains might represent a model to study the variability of the clinical picture of the human disease.

INTRODUCTION

GATA-1 is the member of the GATA transcription factor family<sup>1</sup> essential for appropriate erythroid<sup>2</sup>, megakaryocytic<sup>3,4</sup>, eosinophilic<sup>5</sup> and mast<sup>6</sup> cell differentiation. Because of the highly conserved structure shared by the GATA proteins<sup>7</sup>, the functions of GATA-1 are highly dependent on appropriate lineage expression<sup>8,9</sup>. The importance of the region of the X chromosome upstream to GATA-1, that includes the DNA hypersensitive site I, for appropriate gene expression in erythroid<sup>10,11</sup> and megakaryocytic (Mk)<sup>12,13</sup> cells was demonstrated by its targeted deletion in 6-SV 295 embryonic stem cells that were used to generate GATA<sup>neo<sub>HS</sub></sup> (or GATA-1<sup>low</sup>) mutants, subsequently maintained by crossing with C57BL/6 mice<sup>10</sup>. GATA-1<sup>low</sup> mutants are anemic<sup>10,11</sup> and thrombocytopenic<sup>12,13</sup> and >95% of them die at birth. Anemia is due to the fact that the mutation reduces GATA-1 expression in erythroblasts increasing (up to 30%) their apoptotic rate<sup>10,11</sup> while thrombocytopenia is caused by lack of GATA-1 expression in Mks that remain immature and release abnormal platelets,
the megathrombocytes\textsuperscript{12,13}. These Mks alterations have been implicated in the massive bone formation observed in the mutants that survive up to 5-month of age\textsuperscript{14}.

When the mutation was transferred in the CD1 background, pups were still born thrombocytopenic and anemic but a higher number (>50\%) of them survived up to adulthood \textsuperscript{(15 and this manuscript)}. These mutants recovered from anemia at 1-month thanks to the homeostatic mechanism that recruits the spleen as hemopoietic site, and increases the erythroid output precisely as necessary to compensate the erythroblast apoptosis induced by the mutation\textsuperscript{15}. This mechanism, unique to mice, is also involved in the recovery from anemia induced by hemolytic agents\textsuperscript{16}, radiation\textsuperscript{17} and deletions of other erythroid genes, such as STAT-5\textsuperscript{18} and Lyn\textsuperscript{19}, but does not restore megakaryocytopoiesis. Therefore, GATA-1\textsuperscript{low} mutants remain thrombocytopenic all their life in spite of the numerous Mks present in their tissues\textsuperscript{20}. The abnormalities of GATA-1\textsuperscript{low} Mks include increased P-selectin localization on the demarcation membrane system (DMS) and pathological neutrophil emperipolesis\textsuperscript{21}, two defects also expressed by Mks from patients with idiopathic myelofibrosis (IM)\textsuperscript{22,23}, a clonal myeloproliferative disorder characterized by thrombocytopenia and/or anemia, marrow fibrosis, increased stem/progenitor cell trafficking and extramedullary hemopoiesis that may be associated with osteosclerosis and eventually evolve to leukemia\textsuperscript{24-26}. Indeed, GATA-1\textsuperscript{low} mice developed myelofibrosis, a syndrome similar to IM that manifested itself at 10-12-month of age with marrow and spleen fibrosis and, from 15-month on, with tear-drop poikilocytes in blood, increased stem/progenitor cell trafficking and extramedullary hemopoiesis in liver\textsuperscript{20}. In these mutants, marrow fibrosis was associated with osteosclerosis but bone formation never reached the levels described for the original C57BL/6/6-SV 295 mutants\textsuperscript{14,20}, suggesting that the genetic background might affect the complexity of the phenotype induced by the mutation.

Stem cells form C57BL/6 and DBA/2 mice are known for the differences in how their cycling properties change with age\textsuperscript{27,28}. These differences have been evidenced by many experimental models, the most clear of which is probably represented by chimeric DBA/2-C57BL/6 animals\textsuperscript{29}. In these mice, hemopoiesis is primarily from DBA/2 stem cells in young animals but derives mainly from C57BL/6 stem cells as the mice grow old\textsuperscript{29}. This is due to the fact that DBA/2 stem cells proliferate faster and initially overcome the C57BL/6 ones but, being also extinguished faster, are later on competed out by C57BL/6 cells\textsuperscript{27,28}. These stem cell differences are due to polymorphisms in loci termed quantitative trait loci (QTL)\textsuperscript{30,31}.
Since GATA-1\textsuperscript{low} mice developed myelofibrosis after 12-month of age, and the disease involved massive amplification of the progenitor cell compartments\textsuperscript{6,20}, QTL might cooperate with the mutation in determining the complexity of the phenotypic trait. To test this hypothesis, the mutation was introduced in the C57BL/6 and DBA/2 strains and the phenotype expressed by GATA-1\textsuperscript{low} mutants of different strains compared. Indeed, myelofibrosis was developed by all the GATA-1\textsuperscript{low} mutants but marked strain variegations were observed. In the C57BL/6 background, the mutation ceased to be inherited in Mendelian fashion and the mutants developed fibrosis quickly and with massive osteosclerosis. In contrast, DBA/2 mutants, although severely anemic, expressed limited fibrosis and osteosclerosis and did not present tear-drop poikilocytes in blood or extramedullary hemopoiesis in liver up to 20-month of age. This variegation suggests the existence of gene modifier(s), possibly represented by QTL, that might enhances/suppresses those traits pleiotropic to the GATA-1\textsuperscript{low} mutation itself, and might represent a model to study the variability of the clinical picture of IM in humans.

**MATERIALS AND METHODS**

**Mice.** The GATA-1\textsuperscript{low} colony was started by mating a genetically modified male\textsuperscript{10} (of mixed C57BL/6 and 6-SV 295 background, kindly provided by Dr. S. Orkin) with CD1 females (Charles River, Calco, Italy). Their F1 offspring was crossed again until a line of homozygous mutants, with a genotype 62.5% CD1 and 37.5% C57BL/6 plus 6-SV 295 in unknown proportion, was obtained\textsuperscript{15}. The colony was maintained thereafter at the homozygous stage. To transfer the mutation in different backgrounds, CD1, DBA/2 (DBA/2NCrIBR) and C57BL/6J (C57BL/6JCrIBR) females (Charles River, Calco, Italy) were mated with 3\textsuperscript{rd}-5\textsuperscript{th} generation GATA-1\textsuperscript{low/0} males as described in Table I. Littermates were genotyped at birth by PCR\textsuperscript{6,10} and housed for up to 2 years under good animal care practice conditions in the animal facilities of Istituto Superiore Sanità. Littermates negative for the GATA-1\textsuperscript{low} mutation, i.e. wild-type at the GATA-1 locus, were analyzed as normal controls. All the experiments were performed with sex- and age-matched mice under protocols approved by the institutional animal care committee.

**Hematological parameters.** Blood was collected from the retro-orbital plexus into ethylen-diamino-tetracetic acid-coated microcapillary tubes (20-40 µL/sampling). Hematocrit (Hct), white cells (WBC) and platelets (ptl) counts were determined manually.
**Histology.** Liver, spleen and femurs were fixed in 10% (v/v) phosphate-buffered formalin (Sigma, St. Louis, MO, USA), paraffin embedded and cut into 2.5-3 μM sections that were stained with Hematoxylin-eosin, Gomori-silver (MicroStain MicroKit, Diapath, Bologna, Italy) or the osteoclast-specific tartrate resistant acid phosphatase (387-A Kit, Sigma) staining. Microscopic evaluations were performed with a DM RB microscope (Leica LTD, Heidelberg, Germany) set in a transillumination mode and images acquired with the IM 50 system (Leica). The number of Mks was determined at 40x original magnification in randomly chosen multiple sections to cover a total area of 33,5 mm². Fibrosis was quantified on images of Gomori-stained sections according to Beham-Schmid et al. and modified by Vannucchi et al. Mean (± SD) numbers of fiber intersections were calculated from values obtained on at least three different sections/organ/mouse.

**Flow cytometry and cell sorting.** Mononuclear cells obtained from liver, spleen and marrow were suspended in Ca²⁺ Mg²⁺-free phosphate buffered saline supplemented with 1% (v/v) bovine serum albumin, 2 mM EDTA, 0.1 % NaN₃ and incubated for 30 min on ice with 1 μg/10⁶ cells of phycoerythrin (PE)-conjugated CD117 (anti-c-kit) and CD71, and fluorescein isothiocyanate (FITC)-conjugated anti-CD34, -Sca-1, and TER119 (all from PharMingen, San Diego, CA). Cell fluorescence was analyzed with a Beckman Coulter Epix Elite ESP (Beckman Coulter, Miami, FL). Frequencies of stem/progenitor cells in spleen were analyzed on light density (ρ<1.080) cells separated over Ficoll (Sigma). Blood mononuclear cells were enriched by hypotonic lysis (0.87 % NH₄Cl for 15 min. on ice). Dead cells and non-specific signals were excluded by propidium iodide staining (5 μg/mL, Sigma) and appropriate isotype controls (PharMingen). In selected experiments, cells in the prospectic stem/progenitor cell gates were sorted (80-90% pure upon reanalysis) with a cell sorter FACS ARIA (Becton Dickinson, Franklin Lakes, NJ).

**Progenitor cell counts.** The frequency of progenitor cells in purified marrow and spleen cells from representative normal and GATA-1low littermates was determined by plating 100 cells/mL in standard methylcellulose cultures (0.9% w/v) containing fetal bovine serum (30% v/v, Sigma) and recombinant growth factors [rat stem cell factor (SCF, 100 ng/mL), mouse interleukin-3 (IL-3, 10 ng/mL), granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) (50 ng/mL each) (all from Sigma) and human erythropoietin (EPO, 2 U/mL; Boehringer Mannheim, Mannheim,
The cultures were incubated at 37 °C in a humidified incubator containing 5% CO₂ in air and colonies derived from more or less mature progenitor cells scored at 8 and 15 days.

**RNA isolation and quantitative RT-PCR analysis.** RNA was prepared by lysing bone marrow with Trizol (Gibco BRL, Paisley, UK) and reverse transcribed with 2.5 µM random hexamers using the superscript kit (Invitrogen, Milan, Italy). TGF-β1 cDNA was quantified using a TaqMan RT-PCR assay (catalog no.: Mm00441724_m1, Applied Biosystem, Foster City, CA), while osteocalcin cDNA was quantified by SYBR-green technology (SYBR green PCR master mix, Applied Biosystem)34. GAPDH cDNA was concurrently amplified as control. Reactions were performed in a ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Data were analyzed and the cycle threshold (Ct) calculated with the SDS software. cDNA levels were expressed as ΔCt (ΔCt = target gene Ct - GAPDH Ct).

**Statistical analysis.** Statistical analysis was performed by analysis of variance (Anova test) using Origin 3.5 software for Windows (Microcal Software Inc., Northampton, MA).

**RESULTS**

Mendelian inheritance of the GATA-1 low mutation in DBA/2, but not in CD1 or C57BL/6 background.

To introduce the GATA-1 low mutation in different backgrounds, normal C57BL/6, DBA/2 and CD1 females were crossed with hemizygous GATA-1 low/0 males (from the same litter) and their F1 heterozygous female offspring backcrossed with normal males (either C57BL/6, DBA/2 or CD1, as appropriate) to obtain mutant F2 mice (75% C57BL/6 or DBA/2 and 90.6% CD1, respectively). The mating of normal females with the original GATA-1 low/0 males had low (14 to 25%) success rate that was not reflected by deviations in the expected Mendelian inheritance ratio in the F1 offspring (Table I). In fact, wild-type males and heterozygous females represented the expected 50:50 offsprings in both DBA/2 and C57BL/6 F1 litters. An exception was represented by CD1 F1 litters in which normal males prevailed over heterozygous females (77 vs. 23%). In contrast, in the F2 litters the expected 25% Mendelian rate was observed only among the DBA/2 offsprings. In CD1 F2 litters, GATA-1 low/0 males and GATA-1 low/+ females represented only 10% of the total offspring and GATA-
1low/0 males were never detected in C57BL/6 F2 litters. These results indicate that the GATA-1low mutation is lethal in C57BL/6 but not in DBA/2 mice.

**Changes with age in blood values and femur and spleen cellularity in GATA-1low mutants of different background.**

Blood values and marrow and spleen cellularity of 12-13- and 16-20-month old GATA-1low mice and normal littermates are presented in Table II. As expected, 12-13-month old wild-type mice of different strains expressed similar levels of Hct (44-49%), plt (0.9-1.3x10⁶/µL) and WBC (8-11x10³/µL) counts. These values did not change with age since the small Hct reduction observed in normal DBA/2 and CD1 mice at 16-20-month was not statistically significant (Table II).

All of the mutant mice expressed number of blood thrombocytes significantly lower than normal (Table II). Ptl counts were not affected by genetic background and age, but were sensitive to gene dose since plt counts of heterozygous F1+F2 females were 3-times higher than those of hemizygous F2 males (approximately 0.42x10⁶/µL vs. 0.17x10⁶/µL, respectively) (Table II). This is probably due to the fact that, because of X inactivation, in heterozygous females, hemopoiesis derives both from normal and GATA-1low stem cells. Clusters of normal platelets, likely derived from the normal stem cells, are presented in Figure 1C.

The observation that human females carrying missense mutations in the zinc finger domains of GATA-1 have normal platelet counts had suggested that stem cells expressing GATA-1 from the normal allele have a proliferation advantage over those expressing the mutant one ⁸⁵. In contrast, the platelets counts in the heterozygous mouse females did not increase with age (Table II). This difference might be due either to specie-specific GATA-1-function, or to the fact that alterations in levels of expression or in the structure of GATA-1 have different consequences at the stem/progenitor cell level.

At 12-13-month of age, all of the mutant mice expressed Hct similar to controls, with the exception of hemizygous DBA/2 males that were clearly anemic (Table II). At 16-20-month of age, the Hct became significantly lower than normal in DBA/2 mutants (both heterozygous females and hemizygous males) and in CD1 hemizygous males. Low levels of Hct were not necessarily associated with presence of tear-drop poikilocytes in blood. In fact,
Table I: Summary of the mating of GATA-1\textsuperscript{low} mutants with normal DBA/2, C57BL/6 and CD1 mice.

<table>
<thead>
<tr>
<th>GENOTYPE OF THE OFFSPRINGS (%)</th>
<th>PERCENTAGE OF MENDELIAN RATIO</th>
<th>BACKGROUND*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA-1\textsuperscript{low}</td>
<td>DBA/2 (100%)</td>
<td>C57BL/6 (100%)</td>
</tr>
<tr>
<td>♀</td>
<td>♂</td>
<td>♀</td>
</tr>
<tr>
<td>DBA/2 (100% DBA/2)</td>
<td>18.2</td>
<td>5.5</td>
</tr>
<tr>
<td>CD1 (100% CD1)</td>
<td>14.3</td>
<td>13</td>
</tr>
<tr>
<td>C57BL/6 (100% C57BL/6)</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>F1 GATA-1\textsuperscript{low/+} (50% DBA/2)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>F1 GATA-1\textsuperscript{low/+} (50% C57BL/6)</td>
<td>100</td>
<td>5.5</td>
</tr>
<tr>
<td>F1 GATA-1\textsuperscript{low/+} (81.25% CD1)</td>
<td>33.3</td>
<td>5</td>
</tr>
</tbody>
</table>

* For the purpose of this study, the contribution of each background to the offspring genotype was calculated under the assumption that each parent contributed with 50% of the alleles and that crossing-over events had negligible effects on allele transmission. Crossings which do not follow Mendelian inheritance are indicated in grey. The results represent the summary of at least 20-30 matings for each crossing.
Table II: Blood values and number of cells in femur and spleen from 12-13- and 16-20-month old mice harboring the GATA-1<sup>low</sup> mutation in the C57BL/6, DBA/2 and CD1 background, as indicated. The values obtained in age-matched normal littermates are also reported for comparison.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>12-13-month</th>
<th>16-20-month</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blood</td>
<td>Femur</td>
</tr>
<tr>
<td></td>
<td>Hct (%)</td>
<td>Ptl (x10&lt;sup&gt;6&lt;/sup&gt;/µL)</td>
<td>WBC (x10&lt;sup&gt;7&lt;/sup&gt;/µL)</td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+F2</td>
<td>Wild Type</td>
<td>44.8±5.0</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>F1+F2</td>
<td>GATA-1&lt;sup&gt;low/+&lt;/sup&gt;</td>
<td>48.6±7.0</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>F2</td>
<td>GATA-1&lt;sup&gt;low/0&lt;/sup&gt;</td>
<td>36.6±2.0</td>
<td>0.17±0.25</td>
</tr>
<tr>
<td>DBA/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+F2</td>
<td>Wild Type</td>
<td>44.0±2.3</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>F1+F2</td>
<td>GATA-1&lt;sup&gt;low/+&lt;/sup&gt;</td>
<td>48.8±3.2</td>
<td>0.42±0.27</td>
</tr>
<tr>
<td>F2</td>
<td>GATA-1&lt;sup&gt;low/0&lt;/sup&gt;</td>
<td>36.6±2.0</td>
<td>0.17±0.25</td>
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<td>CD1</td>
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<tr>
<td>F1+F2</td>
<td>Wild Type</td>
<td>48.5±3.4</td>
<td>0.9±0.2</td>
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<tr>
<td>F1+F2</td>
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<td>45.0±3.3</td>
<td>0.3±0.1</td>
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<tr>
<td>F2</td>
<td>GATA-1&lt;sup&gt;low/0&lt;/sup&gt;</td>
<td>45.0±3.3</td>
<td>0.08±0.03</td>
</tr>
</tbody>
</table>

Data represents the mean (±SD) of independent determinations on at least 4-10 mice per experimental group. Values statistically different (p<0.01) from those observed in the corresponding littermates wild-type at the GATA-1 locus are indicated in bold. n.d. = not done.
numerous tear-drop poikilocytes were already present on blood smears from C57BL/6 mutants that had normal Hct, at 12-month of age (Figure 1B) but were undetectable on smears from DBA/2 mutants, in spite of their anemia, up to 16-20-month (Figure 1C and results not shown). As expected\textsuperscript{20}, tear-drop poikilocytes appeared on blood smears from CD1 mutants at 16-20-month of age (not shown).

Although GATA-1 should not affect myeloid differentiation\textsuperscript{1}, most of the mutants had WBC counts significantly lower than normal (3.9-5.5x10\textsuperscript{3}/\mu L vs. 8-11x10\textsuperscript{3}/\mu L, respectively, Table II). This might be due to abnormalities in WBC mobilization due to splenomegaly and/or fibrosis. Unfortunately, this hypothesis cannot be supported by data on spleen weights that were not recorded.

As expected, femur and spleen cellularity of normal mice was greatly affected by genetic background. At 12-13-month, the average cell number of the femur from normal mice ranged from \(\sim25\times10^6\) (C67BL/6) to \(\sim13\times10^6\) (DBA/2), while that of the spleen ranged from 180x10\textsuperscript{6} (CD1) to 70x10\textsuperscript{6} (C57BL/6). In normal controls, the cell number per femur and spleen did not change with age, with the exception of a modest, not significant, decrement observed for the femur of DBA/2 mice and the spleen of CD1 animals (Table II).

The cell number in the femur from 12-13-month old mutants was 2-5-fold lower than normal in all of the backgrounds investigated (Table II). This number did not change within heterozygous C57BL/6 females, increased by 5-fold in heterozygous DBA/2 females and decreased by 2-3 fold in all of the hemizygous males. On the other hand, spleen cellularity strikingly increased in heterozygous females, independently from background, and in hemizygous males of CD1 background (Table II). Of interest, the total cell number in the spleen from hemizygous DBA/2 males, the most severely anemic, was not statistically higher than normal. The spleen cellularity and the Hct of the different mutant mice were directly correlated (p<0.05, Figure 2A).

Therefore, thrombocytopenia was homogenously expressed by all of the mutant mice analyzed while occurrence of anemia was greatly dependent on genetic background and inversely correlated with spleen cellularity.
Figure 1A-C. Histological analysis of the myelofibrotic trait in 12-month old heterozygous F1 GATA-1 low females carrying the mutation in a predominantly CD1 (A), C57BL/6 (B) or DBA/2 (C) background. Blood smears are shown on the top panels while Hematoxilin-eosin- and Gomori-staining of marrow, spleen and liver are shown on the left and right panels, as indicated. The genetic background of the mice analyzed is specified in Table I while the quantification of Mk frequency and of the fibrosis in each animal group is presented in Table III. The arrow in the liver section in 1A indicates an erythroid nest within the parenchyma already detectable at 12-month in CD1 mutants while the arrowheads on the blood smears in 1C indicate clusters of normal platelets, i.e. deriving from the stem cell population which had inactivated the X chromosome carrying the GATA-1 low allele. These clusters were detected at high frequency mainly on blood smears of heterozygous DBA/2 mutants. Similar results were obtained in at least 3-6 animals for experimental points. Original magnifications are indicated on the right of each panel.
Frequency of Mk and presence of osteosclerosis and fibrosis in hemopoietic tissues from 12-13-month GATA-1<sup>low</sup> mutants of different background.

It has been reported that the GATA-1<sup>low</sup> mutation increases the number of Mks present in the hemopoietic tissues<sup>12,20,21</sup>. Such increase was no longer detectable at 12-13-month in all the mutants. In F1+F2 heterozygous females, the frequency of Mks was similar to, and 2-fold higher than normal in marrow and spleen, respectively. In hemizygous males, the frequency of Mks was 3- and 10-fold higher than normal in marrow and spleen of CD1 mutants, but was similar to and 5-fold higher than normal, in marrow and spleen of DBA/2 mice (Figure 1, Table III). Interestingly, Mks, that were barely detectable in liver parenchyma from normal littermates and from F1-F2 heterozygous females of C57BL/6 and DBA/2 background, were consistently observed in liver from hemizygous DBA/2 males and from both heterozygous females and hemizygous males of CD1 background (Figure 1, Table III).

Limited tissue fibrosis is associated with the process of aging. Consistently with this, some fibrosis was detected in hemopoietic tissues from all 12-13-month old normal mice analyzed (Table III). Fibrosis, however, was significantly higher than normal in femur and spleen of all the mutants analyzed. The numbers of Mks and of fibers were not correlated in the marrow but were significantly (p<0.0001) and linearly correlated in the spleen (Figure 2B). Fibrosis was also detected in liver from DBA/2 and CD1 mutants (i.e. in all those cases in which significant numbers of Mks were detectable in this organ) (Figure 1, Table III).
Table III: Frequency of Mk and number of fibers in femur and spleen from 12-13-month old mice harbouring the GATA-1<sup>low</sup> mutation in the C57BL/6, DBA/2 and CD1 background, as indicated. The values obtained in age-matched littermates are also reported for comparison.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Femur</th>
<th>Spleen</th>
<th>Liver</th>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>20±18</td>
</tr>
<tr>
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<td>GATA-1&lt;sup&gt;low/+&lt;/sup&gt;</td>
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<td>2897±541</td>
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<td>DBA/2</td>
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<td>GATA-1&lt;sup&gt;low/0&lt;/sup&gt;</td>
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<td>340±125</td>
<td>3970±890</td>
<td>366±110</td>
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b.d. Below detection; Values statistically different (<0.01) from those observed in the corresponding wild-type littermates are indicated in bold.
The lack of correlation between number of Mk and extent of fibrosis in the marrow at 12-month of age might be due to the fact that the process of new fiber formation is no longer active in this organ. We had previously shown that, in GATA-1\textsuperscript{low} mutants, fibrosis is associated with high levels of TGF-β expression\textsuperscript{20}. Therefore, to prove whether fibrosis was still active in the marrow of old mutants, we compared, by quantitative RT-PCR, the levels of TGF-β expressed in this organ from GATA-1\textsuperscript{low} mice of different backgrounds. Comparable levels of TGF-β were expressed by the bone marrow of old wild type animals (Table IV). On the other hand, the bone marrow from the mutants expressed levels of TGF-β either similar (C57BL/6) or lower (DBA/2 and CD1) than normal (Table IV). These results confirm that active fibrosis was reduced in the marrow from old GATA-1\textsuperscript{low} animals.

Table IV: Quantitative RT-PCR analysis for the expression of TGF-β and of osteocalcin in the bone marrow from GATA-1\textsuperscript{low} mice harbouring the mutation either in the C57BL/6, DBA/2 or CD1 background, as indicated. The values obtained in age-matched littermates are also reported for comparison.

<table>
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<th>Osteocalcin</th>
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<tr>
<td></td>
<td></td>
<td>(\text{ΔCt})</td>
<td>(2^{\text{ΔΔCt}})</td>
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<td>GATA-1\textsuperscript{low/+}</td>
<td>6.7±0.9</td>
<td>0.5±0.3</td>
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<tr>
<td>DBA/2</td>
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<td>5.3±1.0</td>
<td>1.1±0.6</td>
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<td>GATA-1\textsuperscript{low/0}</td>
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<td>GATA-1\textsuperscript{low/0}</td>
<td>8.5±2.2</td>
<td>0.1±0.1</td>
</tr>
</tbody>
</table>

Results represent the mean (±SD) of the values determined for three individual mice per experimental group. Each mouse was assayed in triplicate. \(2^{\text{ΔΔCt}}\) values were calculated using the mean value of the corresponding control as calibrator. Values statistically different (<0.05-0.01) from those observed in the corresponding wild-type littermates are indicated in bold.

As shown in Figure 1, heterozygous females of different background differed greatly in the extent of osteosclerosis in femur. As expected\textsuperscript{14}, C57BL/6 mutants expressed massive bone formation while osteosclerosis was limited, or barely detectable, in CD1 or DBA/2
mutants, respectively (Figure 1). There was no association between frequency of Mks in marrow and extent of bone formation (Figure 1, Table II).

Bone formation is the result of the balance between osteoclast and osteoblast activity. To address whether differences in bone formation in different strains were due to differences in osteoclast/osteoblast activity, we determined osteoclast frequency (on the basis of tartrate-resistant acid phosphatase staining) and osteoblast proliferation activity (indirectly, as levels of osteocalcin expression) in the femur of normal and mutant mice of different strains. Although the complex structure of the bone precludes a precise quantification of the osteoclasts, no difference were appreciated in the frequency of these cells in bones from the mutant and the corresponding wild type animals. However, bones from CD1 mice (both GATA-1<sup>low</sup> and wild type) contained twice as many osteoclasts than those from mice of the other strains (Figure 3). On the other hand, similar low levels of osteocalcin were expressed by wild type mice of all backgrounds but mutant mice expressed at least 1-log more osteocalcin than the corresponding wild type animals (Table IV). Differences in osteocalcin levels expressed by mutants of different strains were observed but were not statistically significant by Anova analysis.

<table>
<thead>
<tr>
<th>C57BL/6</th>
<th>DBA/2</th>
<th>CD1</th>
</tr>
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<tbody>
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<tr>
<td>GATA-1&lt;sup&gt;low&lt;/sup&gt;</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 3**
Figure 3. Tartrate resistant acid phosphatase staining of representative bone marrow sections from 12-month old heterozygous F1 GATA-1<sup>Www</sup> females carrying the mutation in a predominantly C57BL/6 and in hemizygous F2 males of CD1 and DBA/2 background, as indicated. Staining of the corresponding sections from wild type littermates are also indicated. Results are representative of those obtained in three animals per experimental group. The presence of osteoblasts is indicated by arrows. Original magnification 20X.

Therefore, all the mutants analyzed, independently from background, developed fibrosis at 12-13-month of age. The number of Mks were not associated with either fibrosis or osteosclerosis in marrow but a direct linear relationship between number of mutant Mks and number of fibers was present in spleen.

Expression of stem (CD117<sup>pos</sup>/Sca-1<sup>pos</sup>) and progenitor (CD117<sup>pos</sup>/CD34<sup>pos</sup>) cell antigens, as well as of erythroblast (TER119<sup>pos</sup>/CD71<sup>pos</sup>) markers, on marrow cells from normal and mutants mice of different background.

It is known that in mice the expression of hemopoietic-specific antigens is profoundly affected by genotype<sup>36</sup> and age<sup>37</sup>. Therefore, in preliminary experiments, we compared the expression profile of antigens that define stem (CD117<sup>pos</sup>/Sca-1<sup>pos</sup>) and progenitor (CD117<sup>pos</sup>/CD34<sup>pos</sup>) cells, as well as of those that mark erythroblasts at different stages of maturation (TER119<sup>pos</sup>/CD71<sup>pos</sup>), in marrow cells from normal littermates of different background (Figure 4, Table V). The cells were also functionally characterized by clonogenic (progenitor assay) and morphological (May Grunwald-Giemsa staining) criteria.

Marrow cells from different strains expressed slightly different CD117 staining pattern but the differences did not involve the CD117<sup>pos</sup>/Sca-1<sup>pos</sup> population whose frequency was roughly equivalent in all the three strains analyzed (Figure 4, Table V). As control, CD117<sup>pos</sup>/Sca-1<sup>pos</sup> cells were sorted and their cloning efficiency determined in semisolid assay. In all the cases, 10% of CD117<sup>pos</sup>/Sca-1<sup>pos</sup> cells, but none of the CD117<sup>pos</sup>/Sca-1<sup>neg</sup> ones, formed colonies at day 15. Sca-1 recognizes Ly-6A/E<sup>38</sup>, an antigen that in Ly-6<sup>b</sup> mice, such as C57BL/6<sup>36</sup> and DBA/2<sup>39</sup>, marks 99% of the marrow repopulating activity of the CD117<sup>pos</sup> cells. Therefore, in the rest of the study, the frequency of CD117<sup>pos</sup>/Sca-1<sup>pos</sup> cells was taken as indication for the number of stem cells present in a tissue.

The expression pattern of CD34 on marrow cells from mice of different background was heterogeneous. In C57BL/6 mice, the majority (96%) of the CD117<sup>pos</sup> cells were CD34<sup>high</sup> and these cells contained all the colony-forming activity of the tissue (approximately 90 colonies/100 cells plated). In contrast, the CD117<sup>pos</sup> fraction of marrow from CD1 and
DBA/2 mice expressed a continuum pattern of CD34 staining (from dim to high) (Figure 4). Both CD117\textsuperscript{pos}CD34\textsuperscript{dim} and CD34\textsuperscript{high} cells, independently sorted and plated in standard methyl-cellulose culture, gave rise to >75 colonies (mostly day 15 or 8, respectively) per 100 plated cells. In mice, the CD34 antigen is expressed by all progenitor cells but only by fetal-neonatal stem cells\textsuperscript{37}. Adult stem cells, however, retain the ability to express CD34 when induced to cycle\textsuperscript{40}. The results on the cloning efficiency and these considerations, suggest that the CD34\textsuperscript{dim} fraction of CD117\textsuperscript{pos} cells represents an immature stem/progenitor cell population evident only in some strains. Therefore, for the purpose of this study, the frequency of all the CD117\textsuperscript{pos} cells (CD34\textsuperscript{high} in C57BL/6 mice and CD34\textsuperscript{dim-high} in CD1 and DBA/2 mice, all indicated as CD34\textsuperscript{pos} for clarity) was considered as a measure of the progenitor cells present in a tissue.

Figure 4. Dot plot analysis for the expression of the CD117/Sca-1, CD117/CD34 and CD71/TER119 antigens in marrow cells from 16-20-month F1-F2 normal littermates with predominant C57BL/6, DBA/2 or CD1 background, as indicated. The gates defining stem and progenitor cells as well as those corresponding to maturing erythroblasts, are CD117\textsuperscript{pos}/Sca-1\textsuperscript{pos}, CD117\textsuperscript{pos}/CD34\textsuperscript{dim} to high and CD71\textsuperscript{pos}/TER119\textsuperscript{pos}, respectively, as indicated. The number in each quadrant indicates the frequency of marrow cells in that gate. Similar results were obtained in at least 3 mice for each strain and mean (±SD) of results obtained in different mice is presented in Table V.

Bone marrow cells from normal mice of the three backgrounds analyzed, contained significantly different numbers of erythroid cells. In fact, TER119\textsuperscript{pos}CD71\textsuperscript{pos} cells represented 18 or 30\% of the total marrow cells from C57BL/6 and CD1 mice, respectively. Furthermore, the changes in TER119 and CD71 expression pattern supposedly associated with the process of erythroblast maturation were found to be extremely sensitive to genetic background. In DBA/2 mice, CD71 and TER119 expression divided the erythroid cells into the 4 classes (TER119\textsuperscript{med}CD71\textsuperscript{high} and TER119\textsuperscript{high}CD71\textsuperscript{high}, CD71\textsuperscript{med} and TER119\textsuperscript{high}CD71\textsuperscript{low}).
corresponding to pro-erythroblasts and basophilic, chromatophilic and orthocromatophilic erythroblasts, described by Socolovsky et al.\textsuperscript{18} (Figure 4 and not shown morphological analysis of sorted cells). Such distinction was conserved in the CD1 background (Figure 4) but not in C57BL/6 mice in which the levels of CD71 expression remained relatively constant with maturation, while that of TER119 became markedly increased (Figure 4), as described for this same mouse strain by Hall et al.\textsuperscript{41}. Because of these strain differences, no attempt was made in the rest of the study to quantify the proportion of different erythroid precursors and the frequency of the entire TER119\textsuperscript{pos}CD71\textsuperscript{pos} cell population was taken as an indication of active erythropoiesis in a tissue.

In the marrow, the frequency of CD117\textsuperscript{pos}Sca-1\textsuperscript{pos} and CD117\textsuperscript{pos}CD34\textsuperscript{pos} cells was significantly lower than normal only in hemizygous CD1 males, while that of TER119\textsuperscript{pos}CD71\textsuperscript{pos} cells was decreased only in hemizygous DBA/2 males (Figure 5 and Table V). However, since marrow cellularity of all the mutants (independently from their strain, Table II) was greatly lower than normal, the number of hemopoietic cells of all types (stem/progenitor cells and erythroblasts) was greatly reduced in marrow from all 16-20-month old mutants (Figure 6).

In contrast, in the spleen, the frequency of CD117\textsuperscript{pos}Sca-1\textsuperscript{pos} cells was significantly higher than normal in hemizygous DBA/2 males, while that of CD117\textsuperscript{pos}CD34\textsuperscript{pos} cells was significantly increased in all the DBA/2 and CD1 mutants (Table V). Over all, because of the great increase in cellularity detected in this organ in most of the mutants (Table II), the number of stem/progenitor cells resulted increased in spleens from all mutants (Figure 6). On the other hand, the numbers of maturing erythroblasts (both as frequency, Figure 5 and Table V, and as total number, Figure 6) was higher than normal only in spleens from hemizygous CD1 mutants.

Of note, stem/progenitor cells were detectable in blood mainly from CD1 mutants where the frequency of CD117\textsuperscript{pos}CD34\textsuperscript{pos} and Sca-1\textsuperscript{pos} cells, reached 2.5% and 7.5% of the total mononucleated cells, respectively (Figure 5A and data not shown). CD1 mutants were also the only one in which significant numbers of progenitor cells and maturing erythroblasts were detected in the liver at this age (Figure 5C, Table V).

Therefore, at 16-20-month of age the marrow was a poor hemopoietic site in all of the mutants analyzed while the spleen itself was still an active erythropoietic organ mainly in
CD1 mutants. These mutants were also the only one to express significant extramedullary hemopoiesis in liver.

**Figure 5A-C.** Dot plot analysis for the expression of the CD117/Sca-1 (A), CD117/CD34 (B) and CD71/Ter119 (C) antigens in cells from marrow, spleen, blood and liver from 16-20-month old GATA-1<sup>low</sup> mutants and normal littermates of CD1 background, as indicated. The number in each quadrant indicates the frequency of the cells in that gate. Similar results were obtained in at least 3 mice for each strain and mean (±SD) of results obtained in different mice is presented in Table V.

**Figure 6.** Total number of stem (a, CD117<sup>pos</sup>/Sca-1<sup>pos</sup>) and progenitor (b, CD117<sup>pos</sup>/CD34<sup>pos</sup>) cells and of maturing erythroblasts (c, TER119<sup>pos</sup>/CD71<sup>pos</sup>) in normal as well in hemizygous GATA-1<sup>low</sup> mutants of different background, as indicated. C57BL/6 mutants were not analyzed because never born. The total number of each cell population was calculated by multiplying its frequency (Table V) per the total number of cells per organ (Table II), taking into account that the cells in a femur correspond to 8% of the total marrow population of a mouse<sup>74</sup>. The relative contribution of the marrow, spleen and liver to each population is indicated in light blue, dark blue and yellow, respectively.
Table V. Frequency (in %) of CD117+/Sca1+, CD117+/CD34+ and TER-119+/CD71+ cells in the tissues from 16-20-month old mice harboring the GATA-1\textsuperscript{low} mutation in the C57BL/6, DBA/2 and CD1 background, as indicated. The values obtained in age-matched normal littermates are also reported for comparison.

<table>
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<th>Strain</th>
<th>Genotype</th>
<th>Bone Marrow</th>
<th>Spleen</th>
<th>Liver</th>
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<tbody>
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<td></td>
<td></td>
<td>CD117+/Sca1'</td>
<td>CD117+/CD34'</td>
<td>TER119'/CD71'</td>
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<td></td>
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<td>4.7±0.5</td>
<td>19.1±3</td>
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Data represents the mean (SD) of results obtained on at least 3-4 mice per experimental group. Values statistically different (p<0.05) from those obtained in the corresponding wild-type controls are indicated in bold. b.d. = below detection, n.d. = not done.
DISCUSSION

Thrombocytopenia, fibrosis and reduced hemopoiesis in the marrow were the only traits expressed by GATA-1<sup>low</sup> mutants independently from their genetic background (Figure 1, Table III). Strain variegations were instead observed in perinatal mortality, occurrence of anemia and complexity (tear-drop poikilocytes in blood, osteosclerosis, extramedullary hemopoiesis) of the myelofibrosis developed by these animals (see Table VI).

The strain-independent expression of thrombocytopenia and fibrosis is in agreement with the notion that Mks are direct targets of the GATA-1<sup>low</sup> mutation<sup>12,13</sup>. In fact, low GATA-1 in Mks hampers expression of Mk-specific genes, assembling of α-granules and formation of platelet territories<sup>12,13,21</sup>. On the other hand, in spleen, fibers and Mks numbers were directly correlated (Figure 2), in agreement with the assumption that fibrosis results from pathological P-selectin localization on the DMS of the Mks that triggers neutrophil emperipolesis, para-apoptosis of Mk and TGF-β release in the micro-environment<sup>21</sup>. High TGF-β levels in the microenvironment would, then, cause fibrosis by stimulating fibroblasts to produce reticulinic and collagen fibers<sup>42</sup>.

Mutant Mks might also be responsible for the osteosclerosis observed in GATA-1<sup>low</sup> mice by stimulating osteoblast proliferation in vivo, as they do in vitro under cell-cell contact conditions<sup>14</sup>. However, strain differences in bone formation were not directly related with the numbers of Mks present within the marrow. The observation that normal mice transplanted with stem cells infected with a TPO-expressing retrovirus develop both myelofibrosis and osteosclerosis while mice deficient for the osteoclast inhibitor osteo-protegerin transplanted with the same TPO-overproducing stem cells develop only myelofibrosis<sup>43</sup>, suggests that unbalanced osteoblast/osteoclast activity might contribute to strain differences in bone formation. In this regards, the high levels of osteocalcin in the bones indicate that all the mutants mice experience high osteoblast activity (<sup>14,20</sup> and this manuscript). Statistically significant (p<0.05 by t-test) differences in osteocalcin expression were observed in mice of different strains but it is still unclear whether these difference represent strain or individual variability. On the other hand, the number of osteoclasts in the bone from CD1 mutants, although normal for their strain, was higher than that of C57BL/6 and DBA/2 mutants (Figure 3). Therefore, strain-specific balances in osteoblast/osteoclast activity might be responsible, at least in part, for the differences in osteosclerosis of these mutants (Table VI).
Osteoblasts are key elements of the hemopoietic stem cell niche\textsuperscript{44}. Genetically engineered mutations that increase the number of osteoblasts increase also the number of stem cells in the marrow\textsuperscript{45,46} while genetic ablation of the osteoblast pool results in extramedullary hemopoiesis is spleen and liver\textsuperscript{47}. In contrast, in GATA-1\textsuperscript{low} mice, the stem cell frequency in the marrow appeared to be inversely related to osteoblast activity [lower in CD1 than in DBA/2 mice (Table V) with a bone mass lower in DBA/2 than in CD1 mutants (Figure 1)]. Since osteoblast proliferation is probably triggered by cell-cell interaction with mutated Mks, we suggest that establishment of this pathological interaction might dislodge stem cells from their niches. This dislodgment, however, is not sufficient to cause extramedullary hemopoiesis since hemopoietic cells were almost exclusively observed in livers from CD1 mutants (both at 12-13- and 16-20-month), i.e. in the strain in which stem cells remain functional with age. It is possible that establishment of extramedullary hemopoiesis is the result of both extrinsic (dislodgment from the niche) and intrinsic (retention of stem cell properties) mechanisms (Table VI).

Also increased erythroblast apoptosis is a direct consequence of the GATA-1\textsuperscript{low} mutation\textsuperscript{10,11}, but adult mutants express normal Hct by recruiting the spleen as hemopoietic site at 1-month\textsuperscript{15,48} and a direct correlation between total number of spleen cells and Hct was still present in mutants of 12-20-month of age (Figure 2, 6, Table II). Activation of spleen erythropoiesis in response to stress is controlled by the locus, Fv2, that encodes the Stk receptor and confers susceptibility to the Friend Leukemia Virus (FLV)\textsuperscript{49}. Strains susceptible to FLV (DBA/2 and CD1) express the Fv2\textsuperscript{s} allele, which encodes a truncated Stk, sf-Stk, transcribed from an alternative promoter containing a functional GATA binding site. This site is instead deleted in Fv2\textsuperscript{r}, the allele carried by strains resistant to FLV (C57BL/6)\textsuperscript{50}. When expressed, sf-Stk forms a complex with the EPO receptor required both for the first step in FLV leukemogenesis\textsuperscript{51} (i.e. constitutive activation of the receptor by the viral gp55\textsuperscript{52}) and to recover from erythroid stresses (i.e. phenyl-hydrazine-induced hemolytic anemia\textsuperscript{50}) that involve spleen erythropoiesis. It is possible that Fv2 might also favor compensation of the defective erythropoiesis induced by the GATA-1\textsuperscript{low} mutation (Table VI). In fact, the GATA-1\textsuperscript{low} mutation does not necessarily affect sf-Stk expression since CD1 mutants (carrying Fv2\textsuperscript{r}) retain the capacity to recover from phenyl-hydrazine-induced anemia\textsuperscript{15}. At birth, the mutation was progressively less lethal as the background increased from C57BL/6 (Fv2\textsuperscript{r}) to CD1 and DBA/2 (both Fv2\textsuperscript{s}) suggesting that Fv2\textsuperscript{s} protects from anemia at the end of fetal development.
On the other hand, the observation that 16-20-month old DBA/2 and CD1 mutants differed greatly in the number of TER119\textsuperscript{pos}/CD71\textsuperscript{pos} cells present in spleen and in occurrence of anemia (Table II, V, Figure 6) indicates that Fv2\textsuperscript{s} alone is not sufficient to sustain spleen erythropoiesis in old mice.

Table VI. Summary of the phenotypic trait expressed by GATA-1\textsuperscript{low} mutants in different backgrounds

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</tr>
<tr>
<td>Marrow fibrosis</td>
<td>Yes</td>
<td>Yes</td>
<td>Limited</td>
<td>None</td>
</tr>
<tr>
<td>Osteosclerosis</td>
<td>Yes</td>
<td>limited</td>
<td>No</td>
<td>? (osteoclast activity)</td>
</tr>
<tr>
<td>Stem/progenitor cell trafficking</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>bFGF signaling STAT-5a,b</td>
</tr>
<tr>
<td>Extramedullary hemopoiesis</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>bFGF signaling STAT-5a,b</td>
</tr>
</tbody>
</table>

* only data on heterozygous F1 + F2 females are available.

Discrepancies between genotype and function have been described for many inherited hematologic diseases\textsuperscript{53} and complicate the analysis of genetically modified mice\textsuperscript{54}. In an increasing number of cases, discrepancies are ascribable to polymorphisms in loci different from those determining the primary defect defined “gene modifiers”. The complex trait variegation expressed by GATA-1\textsuperscript{low} mutants of different background suggests that their phenotype might be affected by one (or more) gene modifier(s). Strain variegation was already manifested at the first backcrossing, when allelic heterozygosity was only 50-75%.
Therefore, eventual GATA-1 gene modifier(s), as all the QTL identified so far\textsuperscript{55}, must have low meiotic recombination rates. The infrequency of the chromosome regions with low recombination rates suggests that QTL and GATA-1 gene modifiers might be the same genes. The QTL identified up to now include a cluster on chromosome 7 and three clusters on chromosome 11\textsuperscript{55}. The chromosome 11 clusters have been identified on the basis of two experimental approaches: genetic segregation and differential expression profiling for genes highly expressed in DBA/2 stem cells\textsuperscript{55}. One of these clusters contains STAT-5a,b, and is only 30 cM distant from the region syntenic with the cytokine cluster on human chromosome 5q. Another one is syntenic with human 17q21 that contains BCRA1\textsuperscript{55}. The cluster on chromosome 7 and those on chromosome 11 are linked by the observation that chromosome 7 contains a STAT-5 gene modifier that restores the ability of STAT-5\textsuperscript{null} stem cells to engraft in competitive repopulation assays\textsuperscript{56}. It is possible that either STAT-5a,b, the chromosome 7 cluster, or any other QTL gene might affect the phenotype, including development of extramedullary hemopoiesis, of GATA-1\textsuperscript{low} mutants (Table VI).

Also human IM is an heterogeneous disease that manifests itself with extremely variable morbidity and mortality\textsuperscript{24-26}. Familiar predisposition toward development of this disease is only now starting to be appreciated\textsuperscript{57,58}. It is possible that, as perinatal mortality would have prevented the recognition of genetically transmitted myelofibrosis in C57BL/6 mutants, familiar predisposition in IM is masked by in utero mortality.

The primary molecular defect leading to IM, the IM locus, has not been identified as yet. Although it is unlikely that this locus is represented by GATA-1, Mks from these patients express the same morphological (i.e. pathological neutrophil emperipolesis)\textsuperscript{23} and biochemical (reduced GATA-1 content)\textsuperscript{59} abnormalities of GATA-1\textsuperscript{low} Mks. Many cytogenetic defects (mostly del(13q), del(20q) or partial trisomy 1q) have been associated with IM but none of them is “unique” to the disease\textsuperscript{60,61}. Other genes that have also been implicated in IM are classic tumor suppressor genes, such as the retinoblastoma, p53, p16 and the RAS family of proto-oncogenes\textsuperscript{62-64}. Since IM stem cells present either elevated expression\textsuperscript{65} or point mutations\textsuperscript{66,67} of c-kit, this gene has also been implicated with IM development. Furthermore, elements of the b-fibroblast growth factor pathway have been proposed as candidates for the IM locus\textsuperscript{68}. Interestingly, elements of this pathway represent QTL in mice\textsuperscript{59}. More recently, a V617F mutation in the Jak2 gene was discovered in 30-50% of IM patients\textsuperscript{59,70-73}. However, it is still unclear how mutations in any of these genes can
possibly induce the specific Mks abnormalities observed in IM. Our results suggest that although none of these genes might represent the IM locus, any of them, might represent a gene modifier(s) of GATA-1 and contribute to the overall phenotype of the GATA-1\textsuperscript{low} mice. Viceversa, GATA-1 gene modifiers represent likely candidates for the IM locus.

In conclusions, the variegation of the phenotype expressed by GATA-1\textsuperscript{low} mutants in different backgrounds suggests that this complex trait is the result of the interaction between the primary genetic defect, the GATA-1\textsuperscript{low} mutation, with one(more) gene modifier(s). Although most of the variegation observed remains unexplained, on the basis of the known the genetic differences among the strains investigated, possible candidates for GATA-1 gene modifiers were identified. It is possible that polymorphisms in these genes might underlay the variability of the clinical picture of human IM as well.

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Variegation of the phenotype induced by the GATA-1-low mutation in mice of different genetic background

Fabrizio Martelli, Barbara Ghinassi, Barbara Panetta, Elena Alfani, Valentina Gatta, Alessandro Pancrazzi, Costanza Bogani, Alessandro M Vannucchi, Francesco Paoletti, Giovanni Migliaccio and Anna Rita Migliaccio