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ORIGINAL CONTRIBUTIONS

# Generation of *N*-ethyl-*N*-nitrosourea-induced mouse mutants with deviations in hematological parameters

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**Abstract** Research on hematological disorders relies on suitable animal models. We retrospectively evaluated the use of the hematological parameters hematocrit (HCT), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), red blood cell count (RBC), white blood cell count (WBC), and platelet count (PLT) in the phenotype-driven Munich *N*-ethyl-*N*-nitrosourea (ENU) mouse mutagenesis project as parameters for the generation of novel animal models for human diseases. The analysis was carried out on more than 16,000 G1 and G3 offspring of chemically mutagenized inbred C3H mice to detect dominant and recessive mutations leading to deviations in the levels of the chosen parameters. Identification of animals exhibiting altered values and transmission of the phenotypic deviations to the subsequent generations led to the successful establishment of mutant lines for the parameters MCV, RBC, and PLT. Analysis of the causative mutation was started in selected lines, thereby revealing a novel mutation in the transferrin receptor gene (*Tfrc*) in one line. Thus, novel phenotype-driven mouse models were established to analyze the genetic components of hematological disorders.

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## Introduction

Hematological parameters are used routinely in clinical analyses for the diagnosis of the involvement of various organs and for the evaluation of therapeutic strategies in multifactorial and polygenic human diseases. Recently, genome-wide association studies were carried out using hematological parameters, including erythrocyte parameters, white blood cell count, and platelet count, for the identification of novel alleles to determine blood cell traits in humans (Andrews 2009 and refs. therein).

Biomedical research with mice as animal models includes the search for and the analysis of alleles that predispose for or protect against specific diseases. A strategy for the search of novel disease-related alleles consists of the random chemical mutagenesis of a large number of animals followed by systematic screening for clinically relevant disease phenotypes. A widely used mutagen is *N*-ethyl-*N*-nitrosourea (ENU), which is mutagenic for premeiotic spermatogonial stem cells. This allows the production of a large number of randomly mutagenized offspring from treated males. ENU predominantly induces point mutations that result in allelic series for the functional analysis of genes (Hrabé de Angelis et al. 2007). In recent years, ENU mouse mutagenesis projects were established for the systematic, genome-wide, large-scale production and analysis of mouse mutants as model systems for inherited human diseases. They used appropriate routine procedures which allowed the screening of large numbers of mice for a broad spectrum of parameters

(Cordes 2005; Hrabé de Angelis et al. 2000; Nolan et al. 2000). Mutant lines were established for various phenotypic parameters. ENU-induced mutant lines with the causative mutation already identified are successfully used in different areas of biomedical research (Barbaric et al. 2007; Takahasi et al. 2007 and refs. therein). The application of high-throughput sequencing techniques now facilitates the identification of the causative mutations in ENU-induced mouse lines (Boles et al. 2009).

In the phenotype-driven Munich ENU mouse mutagenesis project, a standardized screening profile of hematological parameters was established for the analysis of offspring of mutagenized inbred C3H mice in order to detect phenotypic variants (Gailus-Durner et al. 2005; Rathkolb et al. 2000). The parameters hemoglobin (HGB), mean corpuscular volume (MCV), red blood cell count (RBC), white blood cell count (WBC), and platelet count (PLT) were measured directly. The parameters hematocrit (HCT), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated from the measured parameters. Here we retrospectively evaluate the generation of mutant lines exhibiting deviations from the normal levels of hematological parameters.

## Materials and methods

### Mutagenesis and breeding of mice

The experiments were carried out on the inbred C3HeB/FeJ (C3H) genetic background as described previously (Hrabé de Angelis et al. 2000; Mohr et al. 2004). Ten-week-old male mice (generation G0) were injected intraperitoneally with ENU (three doses of 90 mg/kg in weekly intervals).

The screen for dominant mutations was performed on G1 animals which were derived from the mating of the mutagenized G0 males to wild-type C3H females. Inheritance of the observed abnormal phenotype was tested on G2 mice which were derived from the mating of the affected G1 mouse exhibiting the altered phenotype with wild-type C3H mice.

The screen for recessive mutations was carried out on G3 mice produced in a two-step breeding scheme from G1 mice. G1 males, which were excluded to exhibit dominant mutations by phenotypic analysis, were mated to wild-type C3H females for the production of G2 animals. Subsequently, six to eight G2 females were backcrossed to the G1 male to produce the G3 mice of the pedigree. The analysis of the inheritance of an observed abnormal phenotype in G3 mice was done on G5 mice. Therefore, the affected G3 mouse presumably harboring a homozygous recessive mutation was mated to a wild-type mouse for the production of the presumably heterozygous mutant G4 mice with an inconspicuous phenotype. Subsequently, the G5 mice derived from the intercross of G4 mice were tested for the abnormal phenotype. Alternatively, G5 mice derived from the backcross of a G4 mouse to the affected G3 animal were examined.

After the identification of the causative mutation, the provisional names of the established lines will be replaced according to the official nomenclature. Mouse husbandry was done under a continuously controlled specific-pathogen-free (SPF) hygiene standard according to the FELASA recommendations (Nicklas et al. 2002) (http://www.felasa.eu). All animal experiments were carried out under the approval of the responsible animal welfare authority (Regierung von Oberbayern).

### Hematological analysis

Blood from 3-month-old G1 and G3 mice was analyzed. Blood samples were obtained with the mouse under short-term ether anesthesia by puncture of the retro-orbital sinus with a 0.8-mm microhematocrit capillary (Laborteam K&K, Munich, Germany). Fifty microliters of blood was collected in EDTA-coated tubes (KABE Labortechnik, Elsenroth, Germany), mixed, and analyzed using an Animal Blood Counter (Scil, Viernheim, Germany) validated by the manufacturer for the analysis of mouse blood. Calibration and quality control were performed according to the manufacturer's protocols.

The following hematological parameters were directly measured in EDTA-treated blood: HGB, MCV, RBC, WBC, and PLT. The parameters HCT, MCH, and MCHC were subsequently calculated using the directly measured parameters. HCT was calculated as MCV $\times$ RBC. MCH and MCHC were calculated as HGB/RBC and HGB/HCT, respectively (Klempt et al. 2006a). In the cases where altered values below or above the defined normal range appeared, the mice were retested after 3 weeks.

### Linkage analysis of the causative mutation

Two backcross generations were produced from the identified outliers by using C57BL/6 mice as the second inbred strain. In mutant lines harboring a dominant mutation, phenotypic mutant C3H males were mated to female C57BL/6 mice. The resulting G1 mice were analyzed for heterozygous phenotypic mutants and were again bred to C57BL/6 mice. In mutant lines harboring a recessive mutation, homozygous phenotypic mutant C3H males were mated to C57BL/6 mice and the resulting G1 hybrid mice were intercrossed. In both cases, the G2 offspring were phenotypically classified into mice exhibiting normal or altered levels of the respective blood parameter. After sacrifice, tissue samples were collected for subsequent DNA extraction.

Tail clip samples were incubated over night with a lysis buffer consisting of 10 mM Tris-HCl (pH 8.0), 1% (w/v) SDS, 50 mM EDTA, and 300 μg/ml Proteinase K (Sigma-Aldrich, Taufkirchen, Germany). Automated DNA extraction from the lysates was performed using the AGOWA Mag Maxi DNA Isolation Kit (AGOWA, Berlin, Germany). For linkage analysis, a genome-wide mapping panel consisting of microsatellite and/or single nucleotide polymorphism (SNP) markers was applied. The markers used are available upon request. Genotyping of this panel was performed using MassExtend, a MALDI-TOF high-throughput genotyping system supplied by Sequenom (San Diego, CA, USA). Chromosomal positions of markers and genes are according to the NCBI m37 mouse assembly, 2011 (http://www.ensembl.org).

### Statistical analysis

The statistical analysis of the data was carried out using Microsoft Excel 2000 (Microsoft Corp., Redmond, WA, USA). Values are presented as medians and 95 and 90% ranges unless stated otherwise.

## Results and discussion

### Normal range of hematological parameters in C3H mice

In the phenotype-driven Munich ENU mouse mutagenesis project, the hematological parameters HGB, MCV, RBC, HCT, MCH, MCHC, WBC, and PLT were used in the phenotypic analysis. The parameters were first determined in about 200 male and 200 female 3-month-old wild-type C3H mice (Klempt et al. 2006b), and the 95% range of the values was defined as the normal range (Loeb and Quimby 1999). According to the chosen normal range, abnormally low and high values were determined in the G1 and G3 offspring of ENU-mutagenized mice. On the one hand, the chosen normal range must allow for the phenotypic detection of animals with heritable defects leading to altered levels of hematological parameters. On the other hand, it must exclude smaller deviations from the mean level that are not suitably robust to drive breeding of a mutant line over several generations. Because the Munich ENU project was carried out in two different mouse facilities over many years, we re-evaluated the cutoff levels for the hematological parameters in retrospect for the present study. Blood values of more than 16,000 male and female G1 and G3 offspring of ENU-mutagenized mice in both mouse facilities were included (Table 1). The 95% range of the values determined in the large resource of animals should more reliably represent the normal range of the hematological values over the whole time period of the study. The numbers of G1 and G3 mice that were expected to be primarily altered by ENU-induced mutations for a given blood parameter are negligible in this context. Normal ranges for hematological parameters have been published from other projects, including ones on C3H mice, which grossly confirmed the mean values identified in this study for all parameters except WBC and PLT. Higher mean values usually are published for these two values (Champy et al. 2008; Kile et al. 2003b) (http://www.interphenome.org). This may be due to study-specific factors thereby indicating that internal controls are indispensable.

Subsequently, deviations in hematological values were defined in our analysis for mice showing values below or above the limits of the 95% range in two measurements of a 3-week interval leading to the identification of phenotypic variants. Separate calculation of the annual hematological normal ranges for both mouse facilities involved in the study revealed facility-specific and time-specific differences in the normal ranges, which indicate the inherent impossibility of carrying out complete standardization in a long-term large-scale phenotyping project (data not shown). The nature of the time-specific variations was not analyzed further in detail. These values were also taken into account for the assessment of the phenotypic mutant animals.

### Establishment of mutant lines showing altered values for hematological parameters

In the Munich ENU project, more than 16,000 three-month-old G1 and G3 offspring of ENU-treated mice were screened for dominant and recessive mutations leading to hematological abnormalities (Table 1). The examined offspring included 11,000 G1 mice and 5,500 G3 mice. G1 and G3 phenotypic variants were tested for the inheritance of the deviations in hematological values on G2 offspring from the mating of G1 phenotypic variants to wild-type mice in the screen for dominant mutations, and on G4 × G3 backcross offspring after breeding G3 phenotypic variants to wild-type mice in the screen for recessive mutations. Wherever it was possible, sperm was cryopreserved from male phenotypic variants that were not mated because of breeding capacity limitations. A heritable altered blood level was diagnosed when offspring of the phenotypic variants showed values below/above the defined normal range in two measurements of a 3-week interval. The lack of the appearance of mutant offspring from phenotypic variants indicated the absence of a genetic cause for the altered phenotype (Aigner et al. 2009 and refs. therein).

The phenotypic analysis of the offspring resulted in mutant lines showing abnormal values for the direct parameters MCV, RBC, and PLT. As the hematological

**Table 1** Retrospective analysis of the normal ranges of hematological parameters in 3-month-old C3H mice of the Munich ENU project

| Parameter | Unit | Measurement/calculation | Sex | $n$[a] | Median | 95% range | 90% range |
|---|---|---|---|---|---|---|---|
| HGB | g/dl | Direct | m | 10010 | 14.8 | 12.9–17.0 | 13.2–16.6 |
| | | | f | 6635 | 14.6 | 12.6–17.1 | 13.0–16.6 |
| MCV | fl | Direct | m | 10012 | 50 | 47–52 | 47–52 |
| | | | f | 6635 | 50 | 47–53 | 48–52 |
| RBC | $\times 10^6/\mu l$ | Direct | m | 10012 | 9.2 | 7.8–10.6 | 8.1–10.4 |
| | | | f | 6630 | 8.9 | 7.5–10.4 | 7.9–10.1 |
| HCT | % | MCV × RBC | m | 10009 | 45.6 | 38.4–52.9 | 40.2–51.6 |
| | | | f | 6632 | 44.6 | 37.5–52.2 | 39.5–51.0 |
| MCH | pg | HGB/RBC | m | 10015 | 15.9 | 14.7–18.4 | 14.9–17.8 |
| | | | f | 6637 | 16.3 | 14.9–18.9 | 15.1–18.1 |
| MCHC | g/dl | HGB/HCT | m | 10015 | 32.3 | 29.5–37.7 | 30.0–36.0 |
| | | | f | 6636 | 32.5 | 29.5–38.3 | 30.0–36.4 |
| WBC | $\times 10^3/\mu l$ | Direct | m | 9981 | 3.6 | 1.5–7.8 | 1.8–7.0 |
| | | | f | 6627 | 3.6 | 1.6–7.8 | 1.8–6.9 |
| PLT | $\times 10^3/\mu l$ | Direct | m | 10016 | 622 | 346–844 | 400–795 |
| | | | f | 6638 | 594 | 313–824 | 361–779 |

*HCT* hematocrit, *HGB* hemoglobin, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *MCV* mean corpuscular volume, *PLT* platelet count, *RBC* number of red blood cells, *WBC* number of white blood cells, *m* male, *f* female

[a] G1 and G3 offspring of ENU-treated mice for the dominant and recessive screen. About 60% of the analyzed mice were males

traits examined are partially correlated with each other, some mutant lines showed alterations in additional parameters (Table 2). The success rate of the parameters used for the establishment of mutant lines depends on many factors, including the genetic background and the experimental procedures used. Absence of the description of additional hematological alterations in a given mutant line does not indicate identical values for these parameters in phenotypic mutants and phenotypic wild-type mice of the line. In-depth analysis of the additional hematological parameters may be preferentially carried out after the causative mutation is identified.

Breeding of the mutant lines by phenotypic analysis over several generations with matings of homozygous mutant, heterozygous mutant, and/or wild-type animals confirmed the presence of a dominant or recessive mutation in the lines with viable and fertile mutant offspring. The lines were bred for two or more generations without losing the altered phenotype, which indicated the monogenetic cause of the abnormal phenotype. In only the two lines, MVD007 with dominant mutation and PLT005 with recessive mutation, did less than half of the expected number of phenotypic mutants appear. The phenotypic penetrance of the mutations is sufficient for the further analysis of the lines. Statistical analysis also confirmed that the numbers of animals that exhibit abnormal values below/above the cutoff level were significantly ($\chi^2$ test, $P < 0.001$) higher in all lines compared to a normal C3H mouse population where only 2.5% of the mice are expected to show values below/above the cutoff levels defined by the 95% normal range.

The ENU-induced mutant lines established in the clinical chemical screen of the Munich ENU project (plasma substrates and plasma enzyme activities) showed a tendency toward increased frequency of the altered phenotype with increasing age of the mice, which may be explained by the progression of the pathological process (data not shown). Thus, the frequency of the mutant phenotypes may be underestimated in the present study as the mice were investigated at the age of 3 months. The hematological screen may be improved by analyzing the animals more frequently and/or at an older age. In addition, further in-depth analysis of chosen ENU-induced lines within defined short-term small-scale projects may reveal higher levels of the phenotypic penetrance as observed in the long-term large-scale phenotyping project (Aigner et al. 2009 and refs. therein).

The blood analysis of the Munich ENU project included the clinical chemical screening of plasma samples with substrates, electrolytes, and enzyme activities (Klempt et al. 2006b). However, to date few data for these additional parameters have been generated for the mutant lines with hematological alterations. They will be examined in the in-depth analysis of the lines after the identification of the causative mutations. This will include plasma ferritin, transferrin, and iron.

In addition to the mutant lines described in Table 2, one line with a recessive mutation was established in the Munich ENU project and showed deviations of different

**Table 2** Mutant lines derived from the Munich ENU mouse mutagenesis project with altered values of hematological parameters as primary phenotype according to the cutoff levels which were defined in this retrospective study

| Phenotype | Line name | Mode of inheritance | Offspring[a] *n* (m/f) | Altered blood values | | | | Additional phenotype[c] |
|---|---|---|---|---|---|---|---|---|
| | | | | *n* (m/f) | %[b] | Range | Median (m/f) | |
| MCV low | MVD001 | Dominant | 121 (79/42) | 59 (40/19) | 49 | 40–43.5 | 41/42 | MCH low |
| MCV low | MVD003 | Dominant | 46 (26/20) | 20 (11/9) | 43 | 45–46.5 | 46/46 | – |
| MCV low | MVD005 | Dominant | 160 (105/55) | 69 (54/15) | 43 | 43–46 | 45/45 | MCH low |
| MCV low | MVD007 | Dominant | 39 (21/18) | 8 (7/1) | 21 | 44–46 | 45/46 | – |
| MCV low | MVD009 | Dominant | 32 (19/13) | 11 (7/4) | 34 | 45–46 | 46/46 | – |
| MCV low[d] | MVD013 | Dominant | 147 (92/55) | 70 (44/26) | 48 | 42–45 | 43/44 | HCT high, |
| RBC high[d] | | | | 73 (47/26) | 50 | 10.8–15.6 | 13.2/12.6 | MCH low |
| MCV low[d] | MVD016 | Dominant | 84 (43/41) | 36 (14/22) | 43 | 39–46 | 43/44 | MCH low |
| RBC high[d] | | | | 36 (18/18) | 43 | 10.5–12.6 | 12.0/11.6 | |
| MCV low | MVD017 | Dominant | 59 (36/23) | 27 (17/10) | 46 | 40–43 | 42/42 | MCH low |
| MCV low | MVD018 | Dominant | 87 (42/45) | 42 (22/20) | 48 | 40.5–45 | 42/42 | RBC high, MCH low |
| MCV low | MVD019 | Dominant | 199 (135/64) | 98 (67/31) | 49 | 40.7–46 | 44/45 | – |
| MCV low | MVD021 | Dominant | 30 (20/10) | 9 (7/2) | 30 | 44.5–46 | 46/45 | – |
| MCV low | MVD023 | Recessive | 23 (10/13) | 3 (3/0) | 13 | 43–46 | 46/– | HGB low, HCT low |
| MCV high | MVU008 | Recessive | 118 (69/49) | 17 (5/12) | 14 | 54–59 | 55/56 | MCH high |
| PLT high | PLT001 | Dominant | 264 (144/120) | 75 (44/31) | 28 | 835–1162 | 898/884 | – |
| PLT high | PLT005 | Recessive | 101 (51/50) | 12 (12/0) | 12 | 858–1075 | 933/– | – |
| PLT low | PLT008[e] | Recessive | 15 (10/5) | 9 (4/5) | 60[e] | 97–142 | 117/121 | – |
| PLT high | PLT009 | Recessive | 30 (14/16) | 8 (3/5) | 27 | 827–1037 | 936/885 | – |

*HCT* hematocrit, *MCH* mean corpuscular hemoglobin, *MCV* mean corpuscular volume (fl), *PLT* platelets ($\times 10^3$/µl), *RBC* number of red blood cells ($\times 10^6$/µl), *m* male, *f* female

[a] Offspring after mating heterozygous phenotypic mutants to wild-type mice (screen for dominant mutations) and after breeding heterozygous mutant mice (screen for recessive mutations)

[b] After mating heterozygous mutants to wild-type mice in the lines with dominant mutations, 50% phenotypic mutants were expected in the offspring. After mating heterozygous mutant mice in the lines with recessive mutations, 25% phenotypic mutants were expected in the offspring

[c] Additional phenotype which occurs in more than half of the phenotypic mutants detected with the main parameter. The additional phenotype may also appear in few phenotypic wild-type animals

[d] The lines MVD013 and MVD016 show both low MCV and high RBC values as primary phenotype

[e] Offspring after mating homozygous mutant mice to heterozygous mutants are depicted

plasma enzyme activities (high alanine aminotransferase, high aspartate aminotransferase, high alkaline phosphatase, low α-amylase). The homozygous mutants in this line were phenotypically defined if at least three of the plasma enzyme activities were altered (Aigner et al. 2009). The female G3 founder mouse of the line also showed decreased values of HGB, RBC, and HCT, and PLT levels near the low cutoff level. Reanalysis of the hematological parameters as secondary parameters in the phenotypic mutants revealed PLT values below the cutoff level in 84% of the animals ($n = 31$ of 37). The other six homozygous mutants showed a mean PLT level of 371 $\times 10^3$/µl, which is also much decreased compared to the mean of the normal range. In addition, 54% (20 of 37) of the phenotypic mutants showed decreased HGB values and 43% (16 of 37) of the phenotypic mutants showed decreased RBC and HCT values. The alterations of the hematological parameters seemed to progress with age. This may be due to a consumption coagulopathy in the mutants of this line.

In addition to the mutant lines described in Table 2, many additional lines were set up from phenotypic variants with hematological parameters, including line MVD015 with dominant mutation which was successfully bred for only one generation to date and showed low MCV levels of 44.5–45.5 fl in 9 of 20 offspring, and lines in which the phenotype was suggested to be abnormal and attributed to the presumably mutant animals is not covered by the stringent cutoff levels defined in this retrospective analysis. This may be due to the rather wide 95% normal range defined in a large number of animals that were analyzed in a long-term project over several years. Thus, further matings have to be carried out and offspring analyzed in these

additional lines to clearly confirm an abnormal phenotype (data not shown).

Our previously published data on the use of altered plasma parameter levels for the establishment of ENU-induced mutant lines included hypocholesterolemia or hypercholesterolemia, hyperglycemia, increased plasma urea levels, and decreased or increased plasma enzyme activities as abnormal phenotypes. Breeding of five or more phenotypic variants was necessary to establish one mutant line with increased plasma parameter levels. Transmission of decreased levels from the phenotypic variants to the offspring was observed in every second or third mating; therefore, the establishment of mutant lines in these cases was more efficient than for elevated plasma levels (Aigner et al. 2009 and refs. therein). In this retrospective study, the success rate of the confirmation crosses was not analyzed in detail. However, the number of mutant lines established with decreased levels of hematological parameters as primary phenotype is higher than the number of mutant lines established with increased levels (Table 2), and the number of matings of phenotypic variants resulting in the absence of phenotypic mutant offspring was higher for phenotypic variants with increased levels compared to those with decreased levels (data not shown). Thus, these data confirmed the previous results.

## Linkage analysis of the causative mutations

According to the availability of resources in the ongoing ENU project, breeding mice for the linkage analysis of the chromosomal site of the causative mutation was already carried out in the lines MVD001, MVD017, and MVD019, harboring a dominant mutation (Table 2). All three mutant lines were bred according to low MCV values as primary phenotype. Breeding data over several generations confirmed that the abnormal phenotype of each line is caused by a single genomic locus. In the Munich ENU project, C57BL/6 wild-type mice normally were used as the second inbred strain. Compared to C3H, C57BL/6 mice and B6C3F1 and C3B6F1 hybrids displayed a lower normal range for MCV (Klempt et al. 2006a). Despite this, the detection of phenotypic mutant F1 hybrid mice and the classification of the N2 mice in phenotypic mutants and wild-type littermates according to the expected Mendelian ratio were possible in the approaches for all three lines.

In the line MVD001, backcross mice were classified as phenotypic mutants with MCV levels of 38–43 fl, whereas phenotypic wild-type mice had MCV values of 46 fl or higher. The classification was confirmed by the appearance of low MCH and high RBC values in most phenotypic mutants. In total, 112 of 221 mice (51%) were classified as phenotypic mutants. Linkage analysis of 64 N2 phenotypic mutants with 49 polymorphic microsatellite markers (2-3 markers per chromosome) detected the linkage of the causative mutation to MMU16. Additional fine mapping defined the region between 29.0 Mb (*D16Mit3*) and 35.6 Mb (*D16Mit211*). Bioinformatics analysis (http://omicspace.riken.jp/PosMed/) of this determined chromosomal region identified 102 Ensembl genes. The transferrin receptor (*Tfrc*) gene (32.6 Mb) was chosen for sequence analysis of the exons and flanking intronic regions. It detected a T-to-C exchange in the 5′ splice site consensus sequence at the 3′ end of exon 4. This results in the appearance of mRNA products lacking exon 4 (Fig. 1).

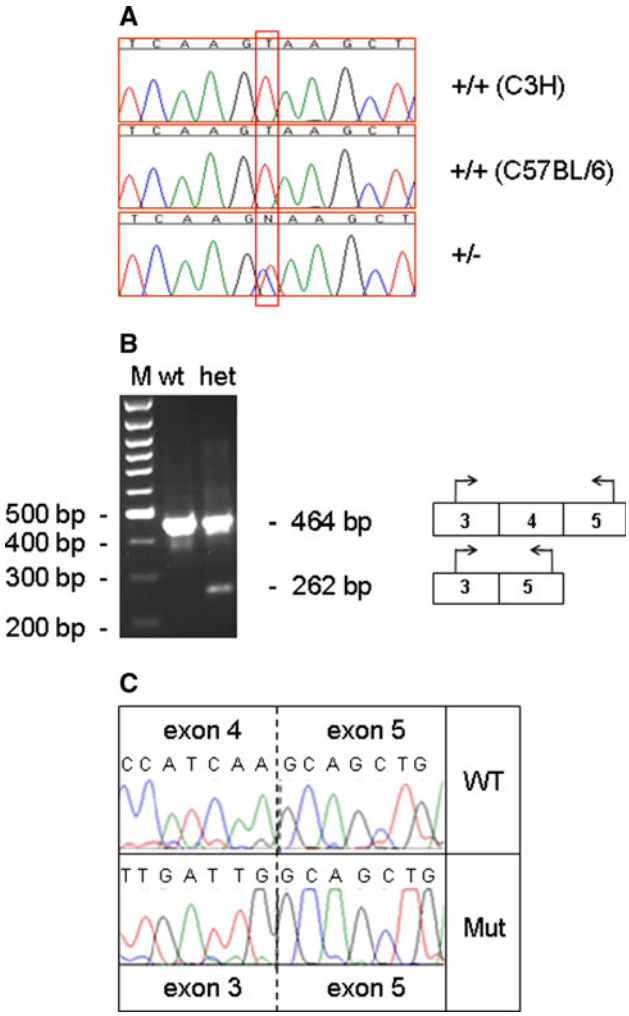


**Fig. 1** Identification of the causative mutation in the transferrin receptor gene (*Tfrc*) of line MVD001. **a** Sequence analysis of *Tfrc* exons and adjacent intronic regions detected a T-to-C exchange in the 5′ splice site consensus sequence at the 3′ end of exon 4 in heterozygous mutant (+/−) mice. **b** The point mutation results in the deletion of exon 4 during RNA processing. In the heterozygous mutants (het), RT-PCR analysis using oligonucleotides derived from exon 3 and 5 of *Tfrc* revealed 262-nt mutant fragments in addition to the 464-nt wild-type fragments. *M* 100-bp DNA ladder. **c** Sequence analysis of the RT-PCR fragments confirmed the deletion of exon 4 in the mutant *Tfrc* mRNA

Deletion of exon 4 results in a frame shift, and due to a premature stop codon, the mutant mRNA codes for an 81-aa Tfrc protein compared to the wild-type 763-aa protein. The probability of the existence of confounding nonsegregating mutations in the mapped chromosomal region is $P < 0.01$ (Keays et al. 2007).

Two *Tfrc* mutants have been described previously. Compared to the MVD001 line, heterozygous knockout mice ($Tfrc^{-/+}$) with the deletion of essential sequences from exons 3 to 5 showed identical hematological deviations such as low MCV, low MCH, a compensatory high RBC, and normal levels for HGB, HCT, WBC, and PLT (Levy et al. 1999). High RBC was also observed in line MVD001, albeit in less than half of the phenotypic mutants detected with the main parameter MCV. Homozygous mutant mice of the knockin line $Tfrc^{L622A}$ showed mild hypochromic microcytic anemia with low levels of MCV, MCH, HGB, and HCT. No data were published for heterozygous $Tfrc^{L622A}$ mutants (Schmidt et al. 2008). In addition, genome-wide association studies in humans identified new determinants of erythrocyte traits, including the *Tfrc* locus (Ganesh et al. 2009). Thus, an additional mutant *Tfrc* allele was established in line MVD001 for the functional analysis of *Tfrc*. Further work has to include the production and phenotypic analysis of homozygous mutants of line MVD001.

In line MVD017, the backcross animals of the linkage analysis showed lower MCV levels compared to those of wild-type C3H mice. Thus, phenotypic mutants were defined with MCV values of 43 fl or lower. Using this cutoff level, 186 of 401 mice (46%) were classified as phenotypic mutants. The classification was confirmed by the appearance of low MCH levels in most phenotypic mutants. The evaluation of the linkage analysis of the causative mutation in N2 phenotypic mutants using genome-wide polymorphic DNA markers is in progress.

In line MVD019, backcross mice were classified as phenotypic mutants with MCV levels of 39.5–45 fl, whereas phenotypic wild-type mice showed MCV values of 46 fl or higher. In total, 256 of 424 mice (60%) were classified as phenotypic mutants. Mapping analysis in 59 N2 phenotypic mutants and 32 N2 phenotypic wild-type mice detected the strongest linkage of the causative mutation to the marker *D9Mit140* (42.7 Mb) of MMU9. For this locus, 57 of the 59 N2 phenotypic mutants showed a heterozygous C3H/C57BL/6 genotype, whereas all 32 N2 phenotypic wild-type mice exhibited a homozygous C57BL/6 genotype.

Bioinformatics analysis (http://omicspace.riken.jp/PosMed/) of the mapped chromosomal region between 40.9 Mb (*D9Mit286*) and 47.4 Mb (*D9Mit130*) identified 111 Ensembl genes. The probability of the existence of confounding nonsegregating mutations in this determined chromosomal region is $P < 0.01$ (Keays et al. 2007).

A search for candidate genes in the mapped chromosomal fragment by using bioinformatics tools (http://omicspace.riken.jp/PosMed/) identified genes involved in erythropoiesis, including the genes for hydroxymethylbilane synthase (*Hmbs*, 44.1 Mb) and rho guanine nucleotide exchange factor 12 (*Arhgef12*, 42.8 Mb). The loci identified in genome-wide association studies for erythrocyte traits in humans (Ganesh et al. 2009; Soranzo et al. 2009) did not match the mapped chromosomal region.

### Mutant lines with deviations of hematological parameters derived from other phenotype-driven ENU projects

Major centers performing phenotype-driven ENU mutagenesis-based projects used hematology screens for the generation of novel mutant mouse models (Cordes 2005). Search for published chemically induced (ENU) mutants (as of 28 January 2011) in the "phenotypes and alleles" database of the Mouse Genome Informatics website (http://www.informatics.jax.org/searches/allele_form.shtml) revealed 2,266 alleles and 1,814 genes/markers. Among them, 245 alleles and 184 genes/markers were described with phenotypes influencing the "hematopoietic system."

Deviations in the same hematological parameters as in our study have been used as the primary phenotype for the establishment of mutant lines from phenotype-driven ENU mouse mutagenesis projects, including sensitized screens. In several lines, the causative mutation has already been identified (Table 3). In addition, more than 50 lines with the causative mutation not yet identified have been published (Kile et al. 2003a; Mason et al. 2007; Tian et al. 2008) (http://pga.jax.org, http://www.brc.riken.jp/lab/gsc/mouse). Compared to the successful use of the parameters MCV, RBC, and PLT for the establishment of mutant lines in our study, other studies also established mutant lines using other parameters used in our study (i.e., HGB, HCT, MCH, MCHC, and WBC). Due to the correlation of some hematological traits used, the primary phenotype in some mutant lines is defined by alterations of two or more hematological parameters. The ENU projects were carried out using various genetic backgrounds and different experimental procedures; therefore, the success rates of the parameters used for the establishment of mutant lines cannot be compared.

## Conclusions

In this study we retrospectively evaluated the use of hematological parameters in the phenotype-driven Munich ENU mouse mutagenesis project to detect dominant and recessive mutations leading to hematological disorders.

**Table 3** Published lines with the causative mutation already identified that were established by deviations in hematological parameters as primary phenotype derived from phenotype-driven ENU mouse mutagenesis projects (according to http://www.informatics.jax.org)

| Primary phenotype | Name/allele, gene | Inheritance | Chr | Reference |
|---|---|---|---|---|
| PLT↑ | 1190003J15Rik$^{plt2}$, RIKEN cDNA 1190003J15 gene | rec. | 7 | Stevenson et al. (2010) |
| MCV↓ | Ank1$^{RBC2}$, ankyrin 1, erythroid | dom. | 8 | Rank et al. (2009) |
| HCT↓ | Apc$^{Min}$, adenomatosis polyposis coli | dom. | 18 | Moser et al. (1990); Su et al. (1992) |
| PLT↓ | Bcl2l1$^{Plt16}$, BCL2-like 1 | dom. | 2 | Mason et al. (2007) |
| PLT↓ | Bcl2l1$^{Plt20}$, BCL2-like 1 | dom. | 2 | Mason et al. (2007) |
| PLT↓ | C1galt1$^{ptl1}$, core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1 | rec. | 6 | Alexander et al. (2006) |
| PLT↑ | Ep300$^{Plt6}$, E1A binding protein p300 | dom. | 15 | Kauppi et al. (2008) |
| RBC↓, WBC↓, PLT↓ | Erg$^{mld2}$, avian erythroblastosis virus E-26 (v-ets) oncogene related | dom. | 16 | Loughran et al. (2008) |
| PLT↓ | Gata1$^{Plt13}$, GATA binding protein 1 | dom. | X | Majewski et al. (2006) |
| PLT↓ | Gp1ba$^{Plt17}$, glycoprotein 1b, alpha polypeptide | dom. | 11 | Mason et al. (2007) |
| WBC↓ | Il7$^{hlb368}$, interleukin 7 | rec. | 3 | Feng et al. (2007) |
| WBC↓ | Il7$^{m1Jyen}$, interleukin 7 | rec. | 3 | Huang et al. (2007) |
| WBC↓ | Jak3$^{bfy}$, Janus kinase 3 | rec. | 8 | http://www.apf.edu.au |
| WBC↓ | Jak3$^{wil}$, Janus kinase 3 | rec. | 8 | http://www.apf.edu.au |
| PLT↓ | Mpl$^{hlb219}$, myeloproliferative leukemia virus oncogene | rec. | 4 | Chan et al. (2009) |
| PLT↑ | Myb$^{M303V}$, myeloblastosis oncogene | rec. | 10 | Sandberg et al. (2005) |
| PLT↑ | Myb$^{Plt3}$, myeloblastosis oncogene | dom. | 10 | Carpinelli et al. (2004) |
| PLT↑ | Myb$^{Plt4}$, myeloblastosis oncogene | dom. | 10 | Carpinelli et al. (2004) |
| WBC↓, RBC↓, PLT↓ | Pgm3$^{mld1}$, phosphoglucomutase 3 | rec. | 9 | Greig et al. (2007) |
| HGB↓, HCT↓, RBC↑, MCV↓ | Steap3$^{fred}$, STEAP family member 3 | rec. | 1 | Lambe et al. (2009) |
| PLT↓ | Stim1$^{Sax}$, stromal interaction molecule 1 | dom. | 7 | Grosse et al. (2007) |
| PLT↑ | Suz12$^{Plt8}$, suppressor of zeste 12 homolog (Drosophila) | dom. | 11 | Majewski et al. (2008) |
| HGB↓, MCV↓ | Tmprss6$^{msk}$, transmembrane serine protease 6 | rec. | 15 | Du et al. (2008) |
| PLT↓ | Wdr1$^{rede}$, WD repeat domain 1 | rec. | 5 | Kile et al. (2003a, 2007) |

*Dom.* dominant, *rec.* recessive

Some of the listed lines were established by using sensitized screens. The numbers of lines listed for a defined parameter may depend on multiple aspects and, therefore, do not reflect the success rate of the parameter for the establishment of mutant lines in ENU projects

Analysis of more than 16,000 G1 and G3 mice was carried out. To date, a high number of mutant lines showing abnormal values for the parameters MCV, RBC, and PLT were produced. Successful linkage analysis was already carried out for some of the mutant lines. The successful use of hematological parameters as primary diagnostic tools has also been described by other ENU mutagenesis projects. In summary, we established novel phenotype-driven mouse models for hematological disorders which are available on request for the further scientific examination. They complement the available models generated by targeted mutagenesis of candidate genes, altogether expanding the resource of mouse models that are available as tools for the functional analysis of the genome.

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