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Identification of novel hypomorphic and null mutations in *Klf1* derived from a genetic screen for modifiers of α-globin transgene variegation

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ABSTRACT

Position-effect variegation of transgene expression is sensitive to the chromatin state. We previously reported a forward genetic screen in mice carrying a variegated α-globin GFP transgene to find novel genes encoding epigenetic regulators. We named the phenovariant strains "*Mommes*" for *m*odifiers of *m*urine *m*etastable *e*pialleles. Here we report positional cloning of mutations in two *Momme* strains which result in suppression of variegation. Both strains harbour point mutations in the erythroid transcription factor, Klf1. One (*D11*) generates a stop codon in the zinc finger domain and a homozygous null phenotype. The other (*D45*) generates an amino acid transversion (H350R) within a conserved linker between zinc fingers two and three. Homozygous *MommeD45* mice have chronic microcytic anaemia which models the phenotype in a recently described family. This is the first genetic evidence that the linkers between the zinc fingers of transcription factors have a function beyond that of a simple spacer.

1. Introduction

Mutagenesis screens have been used in *Drosophila melanogaster* to identify modifiers of position effect variegation [1,2]. In these screens, researchers have used flies that displayed variegated expression of the *w* locus in the eye resulting in red and white patches. The establishment of the eye phenotype is due to stochastic gene silencing within a subset of cells within a field. Similarly, we have used a murine transgenic line that expresses GFP in red blood cells in a variegated manner to screen for modifiers of variegation [3,4]. The transgene includes the human *α1-globin* gene promoter (−570 to +40) and a 4.1-kb fragment containing the human α-globin locus enhancer region (HS-40) linked 3′ to the GFP expression cassette (*Line3* from [3]). This construct is specifically expressed in approximately 60% of circulating erythrocytes [5]. By using *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis we aimed to identify new genes involved in the establishment and maintenance of the epigenetic state in the mouse. We screened offspring of ENU treated males for changes in the expression of GFP in erythrocytes by flow cytometry as described [6]. Mutant mice are easily identified using a drop of blood. The screen can detect both suppressors and enhancers of variegation. We named the phenovariant strains "*Mommes*" (*m*odifiers of *m*urine *m*etastable *e*pialleles) [6]. Efficacy of the screen has been repeatedly demonstrated with the identification of known genes that play roles in epigenetic gene regulation [6,7]. Although the aim was to find mutations in epigenetic regulators of expression, one might also expect to identify genes that specifically bind and regulate the *α1-globin* gene promoter or HS-40 enhancer.

Krüppel-like factor 1 (*Klf1*, formerly known as *Eklf*) is specifically expressed in the erythroid cell lineage [8] and plays an essential role in erythroid development and differentiation [9,10]. Klf1 controls globin gene switching by directly activating *β-globin* gene expression and facilitating stage-specific interactions between the *β-globin* promoter and

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the locus control region (LCR) at the expense of the γ-*globin* gene promoters [11]. It also binds the two murine α-*globin* gene promoters and the murine HS-26 regulatory element which is equivalent to human HS-40 [12]. There is strong evidence that the human HS-40 regulatory element interacts with the α1- and α2-globin gene promoters by looping, and KLF1 is a likely participant [13]. Klf1 also regulates the human γ- to β-*globin* switch indirectly via direct regulation of transcriptional repressors such as *Klf3* and *Bcl11a* [14–16]. Mice deficient in *Klf1* have impaired haematopoiesis in the foetal liver and β-thalassemia [17,18]. Crosses to human β-globin locus YAC transgenic mouse strains confirmed incomplete γ- to β-*globin* gene switching. *Klf1*$^{-/-}$ mice have additional defects in the cell membrane, cytoskeleton, enucleation, and cell cycle which together lead to severe hemolytic anaemia [17,18].

Human mutations in *KLF1* have been described that result in different severities of blood disorders such as the In(Lu) blood group phenotype [19], hereditary persistence of foetal haemoglobin (HPFH) [20] and congenital dyserythropoietic anaemia (CDA) [21,22]. The latter is an inherited blood disorder characterised by ineffective erythropoiesis, abnormal morphology of erythrocytes and hemolysis. The causative point mutation is present within the second zinc-finger of KLF1 (E325K), an amino acid residue that interacts directly with DNA. There is an ENU-derived mouse mutation at the identical residue which leads to a similar dominant hemolytic anaemia [23].

Here, we report the identification and characterisation of two new ENU-generated mouse mutations in the *Klf1* gene detected in a screen for modifiers of expression of an α-*globin*-GFP variegated transgene [6]. We named the strains *MommeD11* and *MommeD45*. *MommeD11* carries a mutation in the second zinc finger of Klf1 which is predicted to disrupt DNA binding, whereas *MommeD45* carries a mutation in the linker-peptide between zinc-fingers two and three. This is a novel and interesting mutation which is similar in position to a recently described mutation in human KLF1 [24]. *MommeD45* homozygous mice are viable but have a partially compensated anaemia with splenomegaly. This new hypomorphic mutation in *Klf1* combined with similar human mutations demonstrates the heterogeneity of the severity of anaemia which is possible with different *Klf1* mutations. *KLF1* mutations should be considered in humans with mild congenital anaemia which is otherwise unexplained.

2. Results

2.1. Identification of two novel mutations in Klf1 that suppress transgene variegation

The α-*globin*-GFP transgene is expressed in a variegated manner such that mice homozygous for the transgene express GFP in ~55% of their erythrocytes [6]. The two mutant mice lines described, *MommeD11* and *MommeD45*, are enhancers of variegation (Figs. 1A and B) as determined by a shift of the percentage of red blood cells expressing the GFP transgene. Heterozygous mutant *MommeD45* and *D11* mice show a higher percentage of GFP expressing cells than the wild-type littermates. In order to identify the linked interval containing the mutation in *MommeD11* and *MommeD45*, *MommeD* lines were backcrossed twice to a C57BL/6J congenic mouse strain homozygous for the GFP transgene (*Line3C*), to generate a large cohort of G2 offspring (see Materials and methods). The recombined DNA regions of the offspring were determined using SNP markers by SNP chip. Both mutations in *MommeD11* and *MommeD45* mapped to the same interval in mouse Chr8 (Fig. 2A). For *MommeD11* a 37 Mb linked genetic interval was identified using SNP array analysis (Chr8: 72 Mb (rs1349830)–109 Mb (rs3662808)). Fine mapping, using ~200 recombinant G2 tails, reduced the interval to ~4 Mb (Chr8: 87–91 Mb; D8MIT78, D8MIT207). Whole exome sequencing was carried out to identify putative causative mutations. A single heterozygous variant unique to *MommeD11* was identified within the genomic interval identified by linkage. For *MommeD45* the genomic linked interval identified via SNP array analysis was ~17 Mb (Fig. 2A).

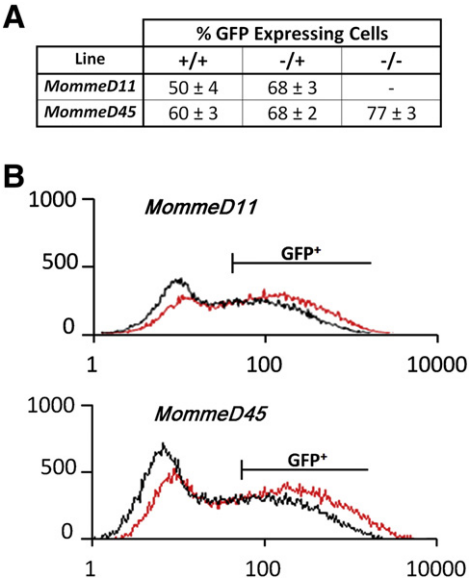

Fig. 1. *MommeD11* and *MommeD45* show enhanced and less variegated expression of an α-globin-GFP transgene. A. Table showing percentage of GFP positive circulating red blood cells at three weeks of age according to genotype (mean ± SD). No *MommeD11* homozygous mice were born alive. B. Representative FACS histograms showing intensity of GFP expression in circulating red bloods cells (x-axis) vs the number of cells detected at each fluorescence level (y-axis) from heterozygous *MommeD11* or *MommeD45* mice (red) versus wild-type litter mate controls (black). The GFP+ gate was set to exclude 99.9% of wild-type cells, i.e. cells with no GFP transgene.

Further fine mapping reduced the interval to 13.5 Mb. One of the candidate genes in this region was *Klf1*. Sanger sequencing of the three exons of the gene using wild-type and mutant mice based on the GFP profile validated mutations in *MommeD11* and *MommeD45* occurred within the third exon of the *Klf1* gene (Fig. 2B). There was a strict correlation between GFP variegation and heterozygous mutations.

The mutation in *MommeD11* is a T-to-A transversion leading to a new stop codon (C330X) in the second zinc-finger of Klf1. This cysteine is one of the critical Zn^{2+} ion co-ordinating residues which are critical for the structural integrity of the finger. We designated this allele as *Klf1*^{MommeD11}. The mutation in *MommeD45* is a missense mutation in the linker-peptide between fingers two and three that consists in a A-to-G transversion, where a histidine is substituted by an arginine (H350R). We have designated this allele as *Klf1*^{MommeD45} (Figs. 2B and C). We found that neither mutation decreased mRNA levels (Fig. 2C).

The mutation in *MommeD11* introduces a premature stop codon in the middle of the second Klf1 zinc-finger (Fig. 2A). This is a very similar mutation to that observed in many cases of In(Lu) whereby a truncation of a section of the zinc-finger is thought to result in a non-functional KLF1 protein with haploinsufficiency leading to the phenotype [25]. In the case of *MommeD45*, the mutation instead leads to an amino acid change (H350R). Interestingly, this particular amino acid resides within a conserved linker between zinc-fingers and is thus not likely to interfere with DNA-binding. H350 is conserved between the mouse and chicken but not between the mouse and human proteins (Fig. 2A). We note that mouse, human, chicken and zebrafish Klf1 proteins all have a conserved arginine at the next amino acid, R351, so the *MommeD45* mutation would lead to an additional misplaced arginine, R350. To ensure that the *MommeD45* mutations did not result in altered protein stability, we undertook Western blotting to compare levels of Klf1 expression between erythroid cells with different *MommeD45* genotypes. *MommeD45* homozygous and heterozygous erythroid cells expressed wild-type levels of Klf1 protein whereas *Klf1*$^{-/-}$ mice had no detectable protein (Fig. 2D). The intensity of non-specific cross-reactive bands was used to verify similar protein loadings.

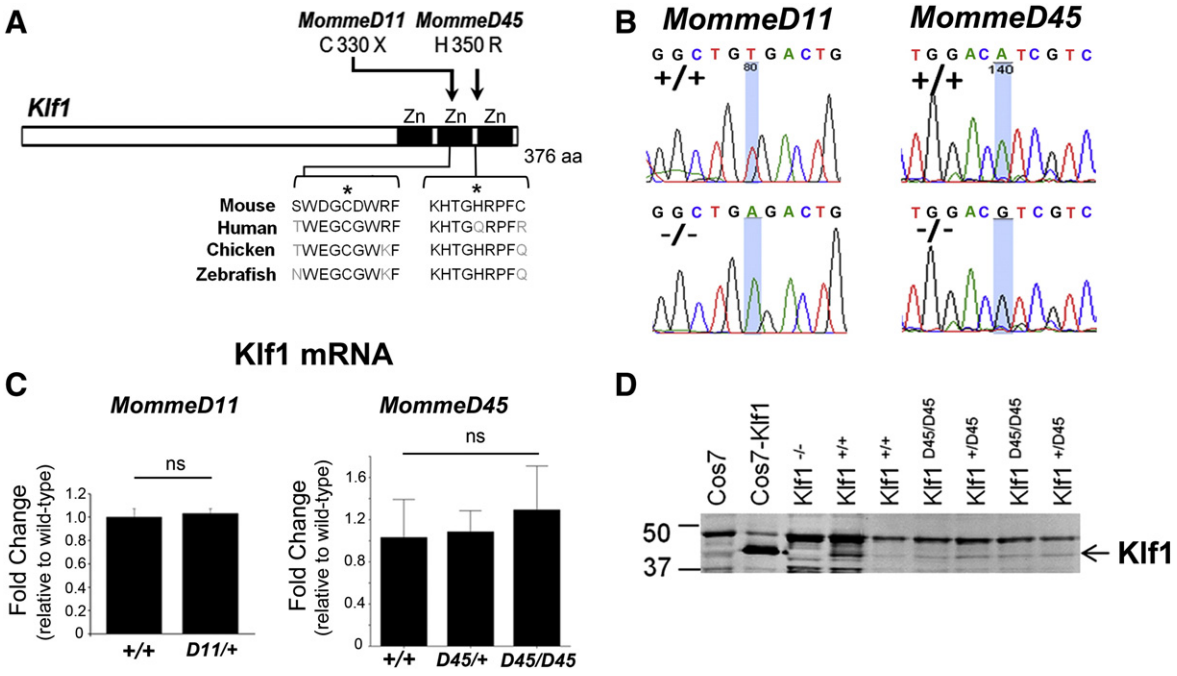

Fig. 2. *MommeD11* and *MommeD45* harbour point mutations in the coding region of Klf1. A. Schematic of murine Klf1 with the three C-terminal zinc fingers in black. The amino acid changes resulting from MommeD11 and MommeD45 mutations are shown by *. An amino acid sequence alignment between mouse, human, chicken and zebrafish Klf1 is shown. B. Representative Sanger sequencing traces of amplicons derived from $Klf1^{+/+}$, $Klf1^{MommeD11/MommeD11}$ and $Klf1^{MommeD45/MommeD45}$ mice. C. Real-time RT-PCR analysis of Klf1 mRNA levels in adult spleen from wild-type, $Klf1^{MommeD11/+}$, $Klf1^{MommeD45/+}$ and $Klf1^{MommeD45/MommeD45}$ mice. Fold change relative to wild-type controls is presented (mean ± SEM). At least four individuals were assayed for each genotype. Students *t*-test was used to evaluate differences between groups; ns, not significant. D. Western blot for Klf1 in COS7 cells (negative control), COS7 cells transiently transfected with pSG5-EKLF (positive control) [8], from $Klf1^{-/-}$ mice [18] (lane 3, negative control), $Klf1^{+/+}$ mice (lane 4) or a litter from $Klf1^{MommeD45/+}$ inter-cross with the genotypes as shown. Klf1 (arrow) is present at normal levels in $Klf1^{MommeD45/MommeD45}$ foetal liver extracts. A cross-reactive band was used to assess equivalent protein loading.

2.2. $Klf1^{MommeD11/MommeD11}$, but not $Klf1^{MommeD45/MommeD45}$ homozygotes show abnormal embryonic development

Heterozygous inter-crosses of *MommeD11* did not produce any viable homozygous mice at weaning. Consequently, we performed timed matings followed by embryonic dissections at different time points during embryonic development and the obtained embryos were genotyped. Homozygous *MommeD11* embryos were recovered between 13.5 and 14.5 dpc but at less than expected Mendelian ratios. This data suggests that embryonic lethality in *MommeD11* occurs between 12.5 and 14.5 dpc. Although $Klf1^{-/-}$ embryos were previously reported to be viable at 13.5 dpc, we think that this slightly earlier lethality reflects the genetic background (i.e. FVB/NJ for *MommeD11* versus BALB/c genetic backgrounds for the *Klf1* knockout mouse colony that we currently house). *MommeD11* homozygous embryos had pale and small foetal livers resembling that of $Klf1^{-/-}$ embryos which is consistent with *MommeD11* being a null allele of *Klf1*.

MommeD45 heterozygous inter-crosses generated homozygous pups at weaning at the expected Mendelian ratios (Fig. 3A). The homozygous mice obtained from these crosses were fertile (data not shown) and had no visible defects. Also, in contrast to $Klf1^{MommeD11/MommeD11}$ homozygotes, $Klf1^{MommeD45/MommeD45}$ embryos and foetal livers appeared grossly normal (Fig. 3B).

2.3. $Klf1^{MommeD45/MommeD45}$ mice have a mild compensated anaemia

In order to further investigate whether the mutation in *MommeD45* caused any defect in haematopoiesis, we analysed different blood parameters of adult mice using a haematological blood analyser (Fig. 4). In addition, we measured relative spleen weight and examined blood smears by microscopy. The levels of haemoglobin (g/L) were significantly decreased in homozygous *MommeD45* mice in comparison with heterozygous and wild-type littermates (129.7 ± 3.1 g/L, 125.5 ± 3.9 g/L and 110.5 ± 3.3 g/L for wild-type, *MommeD45* heterozygous and *MommeD45* homozygous animals respectively) (Fig. 4A). A similar trend was observed for red blood cell counts however these results were not statistically significant (Fig. 4B). Blood smears for $Klf1^{MommeD45/MommeD45}$ mutants showed a mild erythrocyte microcytosis

A **Heterozygous Intercrosses**

Line	Age	+/+	-/+	-/-	χ^2
MommeD11 $^{-/+}$	13.5-14.5dpc	13 (25)	33 (65)	5* (10)	<0.005
MommeD11 $^{-/+}$	3 weeks	13 (30)	31 (70)	0 (0)	<0.001
MommeD45 $^{-/+}$	3 weeks	30 (21)	83 (59)	28 (20)	ns

* 4/5 $Klf1^{MommeD11/MommeD11}$ mutants had pale livers

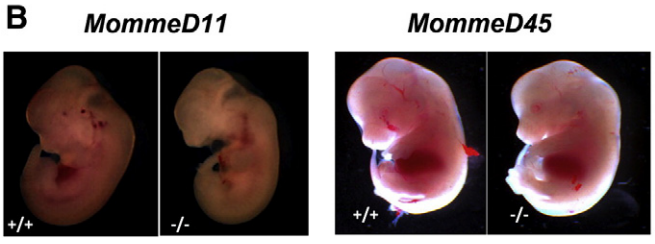

Fig. 3. $Klf1^{MommeD11/MommeD11}$ embryos die at mid-gestation from anaemia whereas $Klf1^{MommeD45/MommeD45}$ mice are born alive at normal Mendelian ratios. A. Genotypes of offspring observed from heterozygous inter-crosses of $Klf1^{MommeD11/+}$ mice, at 13.5–14.5 dpc or three weeks of age and $Klf1^{MommeD45/+}$ mice at three weeks of age. Data show the number of embryos/mice observed (and in brackets the percentage). The proportions of genotypes observed of normal appearance were compared to expected Mendelian ratios using the χ^2 test. B. Representative photomicrographs of $Klf1^{MommeD11/MommeD11}$ and $Klf1^{MommeD45/MommeD45}$ homozygotes and $Klf1^{+/+}$ litter mates at E12.5 (left) and E13.5 (right).

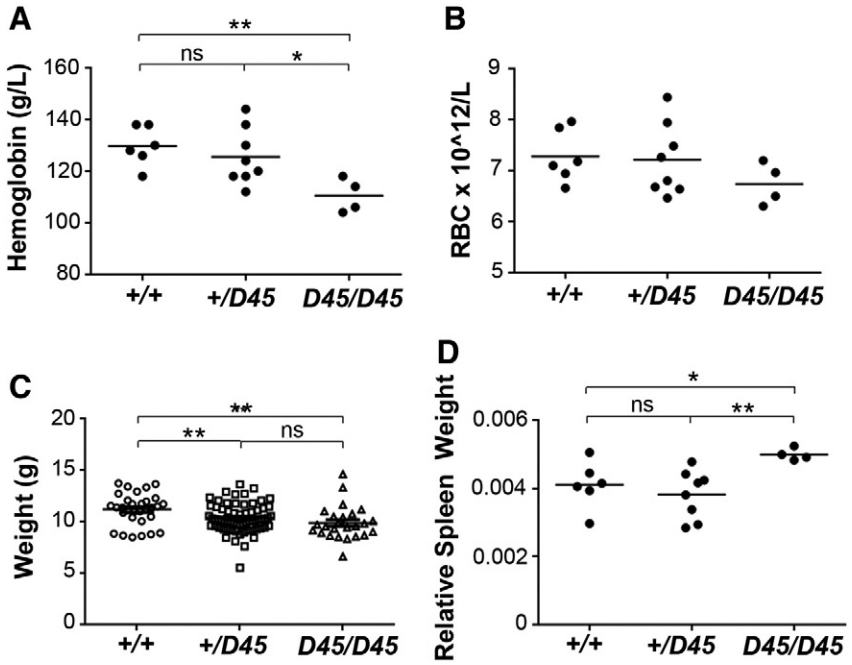

Fig. 4. Compensated anaemia in homozygous *Klf1*$^{MommeD45/MommeD45}$ adults. Measurement of haemoglobin (A), RBC count (B), body weight (C) and relative spleen weight (spleen/body weight) (D) arranged by genotype. Bars represent the mean value and dots are individual measurements. *P value < 0.05; **P value < 0.01; ns, no significant difference between groups based on Student's *t* test.

and a mild increase in reticulocyte numbers (data not shown). Expression of the endogenous *α-globin* and *β-globin* genes in spleen RNA from *Klf1*$^{MommeD45/MommeD45}$ mice was identical to that in *Klf1*$^{MommeD45/wt}$ and *Klf1*$^{wt/wt}$ mice by qRT-PCR (data not shown).

Heterozygous and homozygous *Klf1*^{MommeD45} mice weighed slightly less than wild-type litter mates, possibly due to mild intrauterine anaemia (Fig. 4C). However, homozygous mutant mice have significantly larger spleens as a function of total body weight than heterozygous and wild-type mice (0.41 ± 0.03%, 0.38 ± 0.03% and 0.50 ± 0.01% for wild-type, *Klf1*$^{MommeD45/wt}$ and *Klf1*$^{MommeD45/MommeD45}$ mice respectively) (Fig. 4D). Together, these results show that mild compensated microcytic anaemia is present in *Klf1*$^{MommeD45/MommeD45}$ mice.

2.4. Expression of endogenous α- and β-globin genes is normal in *Klf1*$^{MommeD45/MommeD45}$ mice

Given the published role for Klf1 in the direct regulation of the *α-* and *β-globin* genes, we were surprised to find increased GFP transgene expression in MommeD45 mice. We interrogated the expression of the endogenous *Hba-a1* and *Hbb-b1* globin genes in the spleen by RT-PCR (Fig. 5) and found no differences between wild-type, *Klf1*$^{MommeD45/wt}$ and *Klf1*$^{MommeD45/MommeD45}$ mice. Thus, the behaviour of the transgenic reporter with respect to Klf1 dependency is opposite to that of the endogenous α-globin gene locus (see Discussion). Also, the Klf1^{MommeD45} variant protein must function normally with respect to some target genes such as *α-* and *β-globin* but abnormally with respect to others, leading to compensated anaemia.

3. Discussion

In this study, we describe two novel mutations in the *Klf1* gene in two mouse lines identified by an ENU mutagenesis screen for epigenetic modifiers of gene regulation. Previously, this same screen has been employed successfully for the discovery of genes primarily involved in gene silencing [6]. Loss of function mutations in many genes previously led to enhanced expression levels and/or reduced variegation of the GFP transgene in erythrocytes. Examples of genes discovered in this way include the DNA methyltransferases (*Dnmt1* and *Dnmt3a*), the histone

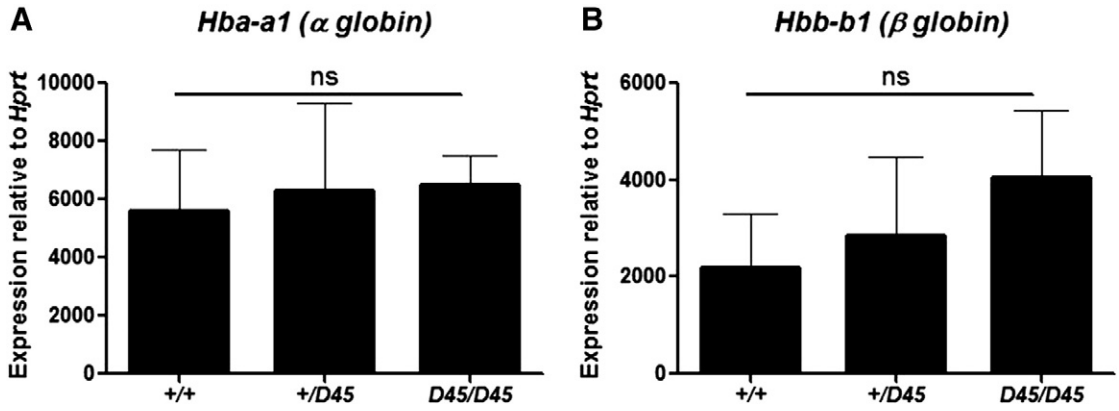

Fig. 5. Normal expression of endogenous *α-* and *β-globin* genes in homozygous *Klf1*$^{MommeD45/MommeD45}$ adults. Real time RT-PCR for *Hba-a1* and *Hbb-b1* in spleens from 10 week old wild type (+/+), heterozygous (+/D45) and homozygous (D45/D45) *Klf1*^{MommeD45} mice. Expression is presented as a function of HPRT expression levels. The bar graphs represent the mean of three biological replicates for each genotype and the error bars represent the standard errors of the means.

deacetylase (*Hdac1*) and *Smchd1*, a gene known to be involved in X chromosome inactivation [6].

ENU mutagenesis screens in mice have been useful for the study of a large number of physiological processes including haematopoiesis [26–28]. In most cases the mutations responsible for a phenotype have been loss of function mutations which read out as dominant (or gene dosage-dependent loss of function) mutations. In some cases the nature of the mutation can be very informative about functional domains such as those involved in DNA-binding specificity or key protein–protein interactions [23,29].

Klf1 is an essential erythroid-specific transcription factor that activates *β-globin* expression and other erythroid genes by binding to extended CACCC-box elements (CCMCRCC) in promoters and enhancers [8,9,12,17,18,30]. It is known to act primarily as a transcriptional activator in vivo although there may be some contexts in which it can act as a transcriptional repressor [31,32]. ENU mutagenesis screens have previously identified an interesting *Klf1* mutant with altered DNA-binding specificity and reduced expression of certain target genes [23]. The mutations in *MommeD11* and *MommeD45* are novel and they have different biological consequences. The mutation in *MommeD11* introduces a stop codon in the second zinc-finger of the protein and the homozygous embryos die in utero after 14.5 dpc. *Klf1*$^{-/-}$ mice also die at this time [17,18]. mRNA levels for *Klf1* in *MommeD11* and *MommeD45* did not differ between wild-type and heterozygous mice, suggesting that the respective nonsense and missense transcripts synthesised were stable. Furthermore, Klf1 was detected in nuclear extracts of erythroid cells from *MommeD45* homozygous mice at the same level as wild-type litter mates (Fig. 2D) suggesting that the mutant protein is stable and finds its way to the nucleus. It is known that some human mutations in the zinc fingers of KLF1 have disastrous consequences and lead to a wide range of blood-related disorders such as CDA [21,22]. However, *MommeD45* homozygous mutant mice only show a mild compensated microcytic anaemia and splenomegaly.

Surprisingly, we found that heterozygous mutations in *Klf1* led to an increase in the percentage of red blood cells expressing an *α-globin* GFP reporter gene. This would be consistent with a transcriptional repressor function for Klf1 at the *α-globin* gene promoter or the HS-26/HS-40 enhancer, but there are alternate possibilities. Klf1 could regulate the expression of genes involved in epigenetic silencing in erythroid cells, so that a heterozygous loss of function allele could lead to enhanced expression at many loci, particularly sensitive variegated loci such as the *α-globin* GFP transgene. Our searches of previously published RNA-seq and expression array datasets have not identified Klf1 target genes involved in epigenetic regulation but this remains a possibility [30,31,33,34].

Interestingly, *Klf3*$^{-/-}$ mice crossed to this same transgene display similar upregulation of mean GFP levels in erythrocytes and reduced variegation [35]. Like Klf1, the transcriptional repressor, Klf3, binds the *α-globin* transgene promoter in vivo. As the *Klf3* gene is a direct Klf1 target [14], it is possible that the reduced variegation in *MommeD11* and *MommeD45* is indirect and via loss of *Klf3* repression. Klf1 is known to bind the endogenous *α-globin* and *β-globin* gene promoters and distant regulatory elements (including murine HS-26 which is equivalent to human HS-40) and induce transcriptional activation in vivo [12] and in erythroid cell lines (Kevin Gillinder, personal communication). However, the mutation in *MommeD45* does not affect endogenous *α-* or *β-globin* gene expression. This strongly suggests that the apparent activation of the GFP transgene upon loss of function of Klf1 is dependent on the non-endogenous context of the transgene regulatory elements. In other words, Klf1 could normally be interfering with the function of this transgene by binding to the promoter and/or the HS-40 site but not facilitating transcription as it would do at the endogenous promoter because the context is wrong. For example, it may not be able to facilitate normal enhancer–promoter interactions because the normal architecture of the endogenous locus is disrupted in the transgene design. Klf1 may actually interfere with the interactions between the HS-40 enhancer and the basal *α-globin* promoter which directs GFP expression in this artificial context. Thus, loss of function mutations would lead to suppression of variegation. We are certain from the nature of the mutation and similarity to the *Klf1*$^{-/-}$ mouse phenotype that *MommeD11* is a null mutation in *Klf1*. Since the phenotype with respect to GFP transgene behaviour *in MommeD45* and *MommeD11 is similar*, we believe that the former is also a hypomorphic mutation.

In summary, we have identified two novel mutations in *Klf1* which result in different degrees of anaemia. One mutation is a loss of function mutation and which leads to a phenotype identical to the *Klf1*$^{-/-}$ phenotype [17,18]. The other mutation is novel and interesting. We believe that it is a hypomorphic allele. The highly conserved linkers between the zinc-fingers of all 17 KLFs [36] and related C_2H_2 type transcription factor families (such as SP1, Ikaros and Egr/Krox families) are believed by many to be inert flexible bridges which permit the three highly structured zinc-fingers to wrap around the major groove of DNA. According to our findings, this assumption might need to be revised. Indeed, we suggest that this conserved linker might play some functional role in either stabilising the overall structure of the DNA-binding domain, facilitating DNA-interactions, or even protein–protein interactions. Indeed, there is some evidence that the linker between the DNA-binding zinc fingers of Ikaros and SP1 is phosphorylated on threonine specifically in mitosis [37,38]. There is speculation that a kinase might recognise this linker and the H350R mutation might interfere with recognition. We note that H350R introduces a second arginine which interferes with the normal 3 amino acid spacing between T348 and R351. We speculate that this might perturb recognition of the peptide motif by a kinase. There is a recently described mutation in human *KLF1* very close to the murine H350R mutation [24]. These authors describe a family with a mutation (hG335R) which is just one amino acid N-terminal to the *MommeD45* mutation (Fig. 2A). The paternal carrier of this mutation has a normal erythroid phenotype but the child, who inherited this allele along with a loss of function paternal allele, has significant anaemia. This human family together with our description of the *MommeD45* phenotype strongly suggests that the linker region has an important function rather than just being an inert spacer between zinc-fingers. The *MommeD45* phenotype is mild so hypomorphic mutations in *KLF1* might be more common in humans than previously realized. Resequencing of *KLF1* in humans with inherited mild anaemias which are not typical of CDA should be considered.

4. Material and methods

4.1. Mouse strains

Procedures with wild-type and inbred C57BL/6J and FVB/NJ mice were approved by the Animal Ethics Committee of the QIMR Berghofer Medical Research Institute. The ENU screen was performed in the FVB/NJ transgenic line (*Line3*), which contains the GFP construct, as previously described [4]. The transgene contains the human *α1-globin* gene promoter (−570 to +40) linked to GFP with a 4-kb fragment encompassing human αHS-40 linked on the 3′ end, as described in detail for an identical LacZ containing transgenic construct [5,6]. Mutant founders and offspring were screened for changes in GFP expression at three weeks of age. Mutant mouse lines were backcrossed to non-mutagenized *Line3* mice for at least five generations, to remove additional mutations, before being used for additional experiments. C57BL/6J mice that contain the GFP construct are named as *Line3C* and they were produced by crossing the *Line3* to C57BL/6J mice for ten generations and selecting mice that carried the heterozygous transgene by flow cytometry. Wild-type inbred C57BL/6J and FVB/NJ mice were purchased from ARC Perth (Perth, WA, Australia). Sperm from *MommeD11* and *MommeD45* mutant mouse lines has been cryopreserved and is available via the Australian Phenomics Facility, Canberra.

4.2. Flow cytometry

GFP fluorescence was analysed by flow cytometry in mice at three weeks of age. For this, a drop of blood was collected in Osmosol buffer (Lab Aids Pty, Narrabeen, NSW, Australia) and analysed on a Guava easyCyte HT (Merck/Millipore), exciting at 488- and 550-nm. The 488-nm channel predominantly measures GFP fluorescence, and the 550-nm channel measures auto-fluorescence. The data obtained was analysed using Guava InCyte software, which is set up to exclude 99.9% of wild-type erythrocytes. Mean fluorescence was calculated using cells within the positive gate.

4.3. Linkage analysis

Linkage analysis was performed on tail-tip DNA samples. Heterozygous mutant *MommeD* mice, at least five generations from the founder, were backcrossed twice to *Line3C* and phenotyped for GFP expression by flow cytometry. The Illumina GoldenGate genotyping assay (Mouse Medium Density Linkage Panel) was used to perform linkage analysis on at least 15 wild-types and 15 heterozygous mice from *MommeD11* and *MommeD45*. The Mouse Medium Density Linkage Panel contains 766 measurable single nucleotide polymorphisms (SNPs) between C57BL/6J and FVB/NJ. Samples were genotyped as described previously [6]. Genotype calls were made using the Genotyping Module of GenomeStudio v1.1 software. Only samples with a call rate >95 were accepted. Linked intervals were identified based on LOD scores of 3 or higher.

4.4. Whole-exome sequencing

Whole-exome sequencing and variant calling were carried out as described in [39] using reagents supplied by Roche NimbleGen (SeqCap EZ Mouse Exome, version Beta 2, 110603_MM9_exome_rebal_2EZ_HX1, Madison, WI, USA). Those variants located within the *MommeD11* linked interval, and were also unique to *MommeD11*, were considered putative ENU mutations.

4.5. Genotyping assay

After the identification of point mutations in *MommeD11*, genotyping was carried out by gene sequencing. The forward and reverse primers used in *MommeD11* were 5′ TTGGCTCTACTTCATCAAGGG 3′ and 5′ GTGAGGTTTGTCCTGAAGGC 3′ respectively. For *MommeD45*, genotyping was carried out by PCR amplification followed by enzyme restriction and gel electrophoresis, as the point mutation in *MommeD45* created a novel restriction site. The forward and reverse primers used in *MommeD45* were 5′ TTGGCTCTACTTCATCAAGGG 3′ and 5′ GTGAGGTT TGTCCTGAAGGC 3′ respectively and the restriction enzyme used was ZraI. Genotypes were validated by Sanger sequencing.

4.6. Embryo dissections

Embryo dissections were performed at the indicated times (Fig. 3A) after timed matings. Heterozygous mutants were mated and 0.5 day post-coitum (dpc) was considered after the observation of a vaginal plug.

4.7. Fine mapping

Point mutations were mapped to relatively small regions of the genome using PCR followed by enzyme restriction or microsatellite markers that differ between FVB/N and C57BL/6J strains. DNA from ~100 wild-type and mutant offspring, phenotyped by the percentage of GFP positive erythrocytes, of the second backcross between FVB and C57 was used for the mapping. Mice wild-type for the mutation should only have C57 chromosomes at the linked interval, while heterozygous mice for the mutations should have both FVB and C57 chromosomes.

4.8. RNA isolation and quantitative real-time RT-PCR

RNA from *MommeD11* and *MommeD45* mice was isolated from the adult spleen using TRI reagent (Invitrogen). cDNA was synthesized from total RNA using SuperScriptIII reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR was performed with the Platinum SYBR Green qPCR Super-Mix-UDG (Invitrogen) with primers designed to span exon/intron boundaries (available on request). All reactions were performed in triplicate and normalized to the housekeeping gene *Hprt*. PCRs were run on a Viia7 (applied Biosystems). The forward and reverse RT-PCR primers used for *Klf1* were 5′ ATGAGGCAGAAGAGAG AGAGGA 3′ and 5′ AAATCCTGCGTCTCCTCAGA 3′ respectively and for *Hpr* were 5′ GGCCAGACTTTGTTGGATTT 3′ and 5′ ACTGGCAACATCAA CAGGACT 3′. RT-PCR primers for *Hba-a1* and *Hbb-b1* have previously been reported [40].

4.9. Measurement of blood parameters

The progeny of *MommeD45* heterozygous crosses were analysed at 5 weeks of age in order to determine the erythroid phenotype. Animals were anesthetised and blood was collected in heparinised tubes for analysis using a COULTER AcT.*diff* whole blood analyser (Beckman Coulter). Blood smears were also prepared, stained using May Grunwald–Giemsa stain and visualised by light microscopy. Mice were subsequently sacrificed and spleens removed by dissection and weighed to determine the proportion of total body weight. Greater than 3 mice for each genotype were analysed and an unpaired Student's *t* test was used to determine statistically significant differences between groups.

4.10. Western blotting

Nuclear extracts were made from 14.5 dpc foetal liver erythroid cells harvested from *MommeD45* heterozygous crosses. *Extracts from Klf1*$^{+/-}$ heterozygous crosses [18], and COS7 cells transfected with pSG5-EKLF [8] were used as controls. Equivalent amounts of extracted nuclear proteins as determined by BCA (Pierce) were separated on a NuPAGE® Novex® 4–12% Bis-Tris mini gel (Invitrogen), and then transferred to a PVDF membrane using an XCell II™ dry Blot Module (Invitrogen) according to the manufacturer's instructions. The membrane was blocked for 1 h in 3% BSA in PBST. A 1:2000 dilution of a rabbit polyclonal antibody which recognises the N-terminus of Klf1 [18] was bound to the membrane in 1% BSA in PBST overnight. The membrane was washed in PBST (4 × 10 min) and hybridised with IRDye® 800-conjugated affinity purified anti-Rabbit IgG antibody (Rockland Immunochemicals) (1:5000 dilution of a 1.0 mg/mL in 1% BSA in PBST). The membrane was washed in PBS–Tween20 and scanned on an Odyssey Infrared Imaging System (Li-COR® Biosciences).

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