The mouse KLF1 Nan variant impairs nuclear condensation and erythroid maturation

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Abstract

Kruppel-like factor 1 (KLF1) is an essential transcription factor for erythroid development, as demonstrated by Klf1 knockout mice which die around E14 due to severe anemia. In humans, >140 KLF1 variants, causing different erythroid phenotypes, have been described. The KLF1 Nan variant, a single amino acid substitution (p.E339D) in the DNA binding domain, causes hemolytic anemia and is dominant over wildtype KLF1. Here we describe the effects of the KLF1 Nan variant during fetal development. We show that Nan embryos have defects in erythroid maturation. RNA-sequencing of the KLF1 Nan fetal liver cells revealed that Exportin 7 (Xpo7) was among the 782 deregulated genes. This nuclear exportin is implicated in terminal erythroid differentiation; in particular it is involved in nuclear condensation. Indeed, KLF1 Nan fetal liver cells had larger nuclei and reduced chromatin condensation. Knockdown of XPO7 in wildtype erythroid cells caused a similar phenotype. We propose that reduced expression of XPO7 is partially responsible for the erythroid defects observed in KLF1 Nan erythroid cells.

Introduction

Erythropoiesis is the process of red blood cell production; defects in this process lead to anemia and thus insufficient oxygen delivery to tissues and subsequent organ failure. Therefore, the formation of red blood cells has to be tightly controlled during embryonic development and homeostasis in the adult. KLF1 (previously known as EKLF) is a well-characterized, erythroid-specific transcription factor and one of the critical regulators of red blood cell maturation. KLF1 acts mainly as an activator and its target genes are involved in multiple processes of erythroid differentiation, including cell cycle regulation [1, 2], hemoglobin metabolism [3],
and expression of membrane skeleton proteins [4, 5]. The importance of KLF1 is illustrated by Klf1 knockout embryos which die around E14 due to the lack of functional erythrocytes [6, 7]. In contrast, heterozygous Klf1+/− mice survive into adulthood, showing that haploinsufficiency for KLF1 does not have a severe phenotype [8]. KLF1 has a N-terminal transactivation domain and a C-terminal DNA binding domain, composed of three zinc fingers. They mediate specific DNA binding to 5’-CACCC-3’ motifs [9]. Variants in human KLF1 are found across the entire gene. The majority are missense variants in the three zinc fingers, which presumably alter the DNA binding/sequence recognition properties of KLF1. Mutations in KLF1 are associated with different phenotypes in humans [10], such as In(Lu) blood group [11], hereditary persistence of fetal hemoglobin (HPFH) [12], zinc protoporphyria [13], increased HbA2 [14], and congenital dyserythropoietic anemia (CDA) type IV.

The Neonatal anemia (Nan) mouse is an ethylnitrosourea (ENU)-induced semi-dominant hemolytic anemia model first described in 1983 by Mary Lyon [15], who positioned the variant on chromosome 8 [16]. In 2010, Klf1 was identified as the gene responsible for this phenotype, due to a single point mutation in the second zinc finger (p.E339D) [17, 18]. While Klf1 Nan homozygous mice die around E10, KLF1 Nan heterozygous mice survive into adulthood displaying life-long hemolytic anemia. This indicates that the KLF1 Nan variant affects the function of wildtype KLF1 protein, as this phenotype does not occur in Klf1 haplo-insufficient mice [6–8, 17, 18]. Indeed, the DNA binding properties of KLF1 Nan may be altered due to steric clash between the carboxyl group of p.339D and the methyl group of thymidine, resulting in the deregulation of a subset of target genes [18], although alternative models have been proposed [17].

Until recently, research has focused on the effects of the KLF1 Nan variant in adult mice [17–19]. Given that KLF1 expression begins around E7.5 [20], it is of interest to investigate the impact of aberrant KLF1 activity during development. Here we investigated erythropoiesis during different stages of fetal development and observed impaired red blood cell maturation at E12.5, as assessed by flow cytometry analysis of the CD71 and Ter119 markers. In agreement with previously published RNA-seq analysis of Nan erythroid cells [21–23], expression profiling of E12.5 fetal liver cells revealed 782 deregulated genes in Nan samples including a host of known KLF1 targets such as Dematin and E2F2 [1, 4, 24]. Intriguingly, the nuclear exportin XPO7, which has recently been implicated in nuclear condensation and enucleation during erythroid maturation [25], was one of the deregulated genes. XPO7 expression was significantly downregulated in the presence of the KLF1 Nan variant erythroid progenitors, potentially contributing to increased nuclear size. We propose that this partially explains the erythroid defects observed in KLF1 Nan erythroid cells, providing a novel link between KLF1 and nuclear condensation.

Materials and methods

Mice

All animal studies were approved by the Erasmus MC Animal Ethics Committee. Well-being of the mice was monitored daily. The mouse strains used were Klf1Nan/wt (C3H101H-Klf1Nan/H, obtained from the MRC Harwell Institute, Harwell, UK [15]) and Klf1 knockout (Klf1tm1Nimr, stock maintained at Erasmus MC animal facility [6]). Animals used for the RNA-seq experiments also contained the single copy human HBB locus PAC8.1 transgene (Tg(HBB) 8.1Gvs) [26]. Mice were maintained by breeding Klf1wt/wt males with C57BL/6 females. Genotyping was performed by PCR using DNA isolated from toe biopsies. For Klf1 Nan genotyping, the PCR product was digested with DpnII. For timed pregnancies, Klf1Nan/wt males were mated with C57BL/6 females, and Klf1ko/wt males were mated with Klf1ko/wt females. The day of
vaginal plug discovery was considered E0.5. Mice were euthanized by cervical dislocation. Embryos were collected at E12.5, E13.5, E14.5 and E18.5; tail DNA was used for genotyping. Primer sequences are detailed in S1 File supplementary materials and methods.

**Blood analysis**

Peripheral blood (~50 μL) was collected from the mandibular vein of adult mice, and standard blood parameters were measured with an automated hematologic analyzer (Scil Vet ABC, Viernheim, Germany).

**Cell culture and transduction**

I/11 erythroid progenitors and primary mouse fetal liver cells were cultured as described [27]. To induce differentiation of I/11 cells we used StemPRO-34 SFM (10639–011, Life Technologies, Carlsbad, CA) supplemented with 500 μg/mL iron-saturated transferrin (Scipac, Crumlin, UK) and 10 U/mL Epo (Janssen-Cilag, Breda, NL). Lentiviral shRNAs targeting XPO7 were obtained from the MISSION shRNA library (Sigma-Aldrich, Saint Louis, MO). The clones used are detailed in S1 File supplementary materials and methods.

**RNA isolation and RT-qPCR analyses**

RNA was extracted using TRI reagent (Sigma-Aldrich). To synthesize cDNA, 2 μg of RNA were used together with oligo dT (Invitrogen, Carlsbad, CA), RNase OUT (Invitrogen), and SuperScript reverse transcriptase II (Invitrogen) in a total volume of 20 μL for 1 hour at 42˚C. 0.2 μL of cDNA was used for amplification by RT-qPCR. Other experimental details and primer sequences are detailed in S1 File supplementary materials and methods.

**Protein extraction and western blotting**

Total protein extracts from mouse fetal liver cells were prepared according to [28]. To visualize protein expression, cell lysates of ~3x10⁶ cells were loaded on 10% SDS-polyacrylamide gels for electrophoresis. The gels were transferred to nitrocellulose blotting membrane 0.45 μm (10600002, GE Healthcare, Chicago, IL) and probed with specific antibodies. Membranes were stained for Tubulin (T5168, Sigma-Aldrich) as loading control, and for XPO7 (sc390025, Santa Cruz Biotechnology, Dallas, TX).

**Flow cytometry, cell sorting, enucleation- and cell morphology analysis**

These procedures are described in detail in S1 File supplementary materials and methods.

**RNA-sequencing and analysis**

RNA-seq was performed according to manufacturer’s instructions (Illumina; San Diego, CA, USA), as described [29]. The sequenced reads were mapped against the mouse genome build mm10 using TopHat 2.0.6 [30] with the transcriptome gene annotation of Ensembl v73 [31]. Further details of the bioinformatics analyses are described in S1 File supplementary materials and methods. Files with the raw data have been deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena), accession number PRJEB30678.
Chromosome conformation capture combined with high-throughput sequencing (4C-seq) and data analysis

4C-seq experiments were carried out as described [32, 33]. Briefly, 4C-seq template was prepared from E13.5 fetal liver or fetal brain cells. In total, between 1 and 8 million cells were used for analysis. Further experimental details and of the bioinformatics analyses are described in S1 File supplementary materials and methods. Files with the raw data have been deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena), accession number PRJEB30678.

Statistical tests

Statistical analysis of blood parameters was performed by using analysis of variance with Bonferroni correction; flow cytometry data and gene expression results were analyzed by using Mann-Whitney tests. ImageStream data were analyzed by t tests. Excel 2010 (Microsoft, Redmond, WA) was used to draw the graphs. Values plus or minus standard deviations are displayed in all figures. Statistical analysis of RNA-seq and 4C-seq data is described in S1 File supplementary materials and methods.

Results

Characterization of KLF1 Nan fetal liver cells

The effect of the KLF1 Nan variant has been studied in adult mice [17–19], but data on its effect during gestation is limited. Hence, to study this variant during embryonic development, we used a Nan mouse model carrying one mutant allele (Klf1Nan/wt, from now on called Nan). At E12.5, E14.5, and E18.5, Nan embryos were paler than wildtype littermates, indicating anemia, but otherwise looked phenotypically normal. Flow cytometry analysis of E12.5, E14.5, and E18.5 fetal liver cells used the Kit, CD71, Ter119 and CD44 markers to trace red blood cell differentiation. A severe downregulation in expression of the Ter119 marker was observed at all three stages (Fig 1A and 1B). The CD71/Ter119 double-positive population was significantly decreased in the Nan samples, while the CD71 single-positive population showed an increase. No significant differences were observed for Kit and CD44 in the Nan samples (FigA in S1 File). In addition, similar results were obtained when assaying embryonic blood, with Ter119 being highly downregulated (Fig 1C and 1D). These results indicate that Nan embryos display delayed erythroid maturation compared to wildtype controls. This is in line with the observation that a higher percentage of cells is positive for CD71 in adult blood (Fig B in S1 File), indicative of higher percentage of circulating reticulocytes [18]. Consistent with this notion, analysis of standard blood parameters revealed a significant increase in red cell distribution width (RDW) in the Nan mice (Fig B in S1 File). Furthermore, in agreement with previously published data [18], we observed minor, yet significant, decreases in RBC (red blood cell), HGB (total hemoglobin), HCT (hematocrit), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration) values. Interestingly, when comparing Nan E14.5 fetal liver cytospins to wildtype controls, we observed a marked increase in the average size of the erythroid cells and their nuclei (Fig 1E). Taken together, these data show that erythroid maturation is impaired in Nan animals.

Identification of deregulated genes in E12.5 Nan fetal livers

In order to identify genes that are affected by the KLF1 Nan variant, a genome-wide RNA-seq was performed on samples derived from E12.5 Nan and wildtype fetal livers (N = 6 each), as at this stage the fetal liver is mainly composed of erythroid cells. 782 genes appeared to be
deregulated in the Nan mutants (false discovery rate [FDR] < 0.01, absolute fold change equal or greater than 1.5), of which 437 were upregulated and 345 downregulated (Fig 2A and 2B and S1 Table). Even though KLF1 has been mainly described as a transcriptional activator, the majority of the deregulated genes displayed increased activation in the Nan erythroid cells. This is consistent with previously reported RNA-seq data of the Nan model [21–23]. We
postulate that this might be due to secondary effects of KLF1 Nan on other transcriptional regulators and/or neomorphic effects of KLF1 Nan [21, 23]. To validate the data, we checked the expression of Epb4.9 and E2f2, genes known to be down-regulated in Nan erythroid cells (Fig 2C, left panel and FigC in S1 File). Indeed, a significant decreased expression of the transcripts of these two genes was detected in Nan fetal livers. Moreover a significant 2-fold down-regulation of BCL11A, a known target of KLF1 [12, 34], was observed indicating that the KLF1 Nan variant affects its expression (Fig 2C, left panel and FigC in S1 File). Given the role of BCL11A and KLF1 in globin switching, the expression levels of the β-like globin genes were checked; the embryonic Hbb-bh1 gene was upregulated and the KLF1 target gene Hbb-b1 was downregulated, consistent with previous reports [18]. In addition, the embryonic Hba-x gene was
upregulated in E12.5 Nan fetal livers (Fig 2C, right panel and FigC in S1 File). Collectively, these data are in accordance with the notion that intact KLF1 fulfils a crucial role in developmental regulation of globin gene expression [8] and deregulation of embryonic globin expression in adult Nan mice [18].

The nuclear exportin XPO7 is downregulated in Nan erythroid cells. The expression of the Xpo7 gene, encoding a nuclear exportin, was prominently downregulated in Nan E12.5 fetal livers (~4-fold decrease; Fig 2A; see also [21, 23]). This raised our interest since XPO7 was recently implicated in terminal erythroid differentiation, as a protein involved in enucleation [25]. To corroborate the RNA-seq data, XPO7 expression in Nan E14.5 fetal livers was reduced by approximately 90% in the Nan samples as determined by RT-qPCR (Fig 3A). Transcripts using the canonical first exon were barely detectable in all samples (not shown). Importantly, XPO7 protein levels were also reduced in Nan fetal liver cells (Fig 3B). To investigate whether XPO7 expression is dependent on KLF1, XPO7 mRNA and protein levels were measured in Klf1 null erythroid cells. In E13.5 Klf1 null fetal livers [6] expression of XPO7 mRNA and protein is significantly reduced (Fig 3C and 3D). Remarkably, downregulation of XPO7 was also observed in Klf1wt/ko fetal livers, although to a lesser extent than observed in Klf1 null fetal livers (Fig 3C and 3D). Thus, similar to BCL11A [12, 34], activation of XPO7 by KLF1 is dose-dependent. In agreement with the notion that KLF1 is a direct activator of the Xpo7 gene, KLF1 binds to the canonical promoter and first intron of the Xpo7 gene in mouse [21, 35–37] and human [35, 38] erythroid cells (Fig 3E).

The chromatin conformation of the Xpo7 locus is not affected in Nan erythroid cells

Since KLF1 is required to form an active chromatin hub in the β-globin locus [39], 4C-seq experiments were performed on the Xpo7 locus in E13.5 wildtype fetal livers and fetal brains and Nan fetal livers (Fig 4A). The canonical promoter of Xpo7 was used as viewpoint to investigate potential changes in chromatin conformation (Fig 4B). Interestingly, a loop was identified between the canonical promoter of Xpo7 (situated at the beginning of exon 1a) and the exon that produces the erythroid-specific form of XPO7 (exon 1b), indicating that these two regions are in spatial proximity in erythroid cells (Fig 4B), whereas this loop has lower contact frequencies in fetal brain. However, few local changes in the chromatin conformation were found between wildtype and Nan samples (Fig 4B). The experiment was repeated using the erythroid-specific promoter as viewpoint. This confirmed the results obtained with the canonical promoter as viewpoint. (Fig 4C). We suggest that this loop might recruit transcription factors binding to the area of the canonical promoter to the vicinity of the erythroid promoter, thereby facilitating expression of the erythroid-specific Xpo7 transcript. Of note, these results are consistent with the observation that KLF1 is responsible for inclusion of Xpo7 into transcription factories containing the Hbb and Hba loci [40].

Nan mouse fetal liver cells present defects in nuclear condensation

Since XPO7 has been implicated in enucleation of erythroid cells in vitro [25] enucleation in Nan fetal livers was analyzed. This was quantified in E14.5 fetal livers by flow cytometry using the erythroid marker Ter119 and Hoechst-33342 as a nuclear stain. Similar percentages of enucleated cells were observed between Nan and control fetal liver samples (Fig 5A and 5B). Similar results were obtained with E12.5 and E18.5 fetal liver cells (data not shown). To check whether the flow cytometry analysis could indeed discriminate nucleated from enucleated cells, we sorted the Hoechst-positive and Hoechst-negative populations and prepared cytopins. This showed that all the Hoechst-negative cells identified by flow cytometry had indeed
enucleated (Fig 5C). In addition, assessing enucleation levels of mouse fetal liver cells from embryos at E12.5 and E14.5 cultured in proliferative and in differentiation medium, similar ratios of nucleated versus enucleated cells were found in control and Nan samples (Fig D in S1 File). Nevertheless, a striking increase in the percentage of large cells in the fetal livers of the Nan embryos was observed. Quantification of cell size using flow cytometry revealed a significant increase in average cell size at E12.5, E14.5 and E18.5 in the Nan samples (Fig 5D). In line with this finding the nuclear area of the Nan fetal liver cells was significantly increased when

Fig 3. XPO7 expression in wildtype, Nan and Klf1 knockout fetal liver cells. (A) Xpo7 mRNA relative values in wildtype and Nan E14.5 fetal livers. * indicates p value <0.01. n indicates the number of embryos. (B) Western blot analysis of XPO7 protein in wildtype and Nan E14.5 fetal livers and quantification. β-tubulin was used as loading control. * indicates p value <0.01. n indicates the number of embryos. (C) Klf1 and Xpo7 mRNA relative expression values in wildtype, KLF1 heterozygotes and KLF1 knockout E13.5 fetal livers. * indicates p value <0.01. n indicates the number of embryos. (D) Western blot analysis of XPO7 protein in wildtype, Klf1 heterozygotes and Klf1 knockout E13.5 fetal livers and quantification. β-tubulin was used as loading control. * indicates p value <0.01. n indicates the number of embryos. (E) KLF1, GATA1, NF-E2, H3K4me2, H3K4me3, and p300 ChIP-seq data for the human XPO7 locus. KLF1 peaks in the region of the canonical XPO7 promoter and in the first intron are indicated by a red box. Data taken from [38].

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Fig 4. 4C-seq analysis of the Xpo7 locus. (A) Schematic representation of chromosome number 14. The green box indicates the zone where the Xpo7 gene resides. The RefSeq mm10 Xpo7 gene is indicated by rectangles (exons) and arrows (introns) that point to the direction of transcription. The location is indicated in Mega basepairs (Mb). The erythroid-specific first exon is indicated by a red box. (B-C) 4C-seq representation of the chromosome contact frequencies detected using the canonical promoter of Xpo7 (B) and the region of the erythroid specific Xpo7 exon (C) as viewpoints. The mean of a running windows of 21 restriction fragment-ends of the median value of the biological replicates with a maximum of 3000 are indicated by colored lines. Loci with a statistically significant (FDR < 0.05) higher contact frequencies and reads per million > 250 in wildtype fetal liver compared to the fetal brain are indicated by light grey boxes. Loci with a statistically significant (FDR < 0.05) higher contact frequencies and reads per million > 250 in fetal brain compared to the wildtype fetal liver are indicated by dark grey boxes. The red dotted line indicates the viewpoint and the red arrow the position of the loop. Purple, wildtype fetal liver; Orange, Nan fetal liver; Grey, wildtype fetal brain.

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compared to control fetal liver cells in cytospin slides stained with the nuclear dye Hoechst 33342 (Fig 5E). These data are consistent with the notion that XPO7 is involved in nuclear condensation, a process that precedes enucleation. However, despite the impaired nuclear condensation, the cells are still able to undergo enucleation as we observed similar ratios of nucleated versus enucleated cells in control and Nan fetal liver cells.

**XPO7 knock down in I/11 cells partially mimics the phenotype of Nan cells.** Due to the poor growth of cultured Nan fetal liver cells our attempts to rescue XPO7 deficiency by lentiviral overexpression consistently failed. Therefore, the role of XPO7 in erythroid differentiation was further analyzed by knocking down XPO7 in the factor-dependent immortalized...
mouse erythroid cell line I/11 [41]. Using three different shRNAs, an efficiency of ~70% knockdown was reached as shown by Western blot (Fig 6A). Before differentiation a minor difference in expression of the surface markers CD71 and Ter119 and no difference in cell size between the control and the knockdown cells was observed (data not shown). In contrast,
upon transfer to differentiation medium, the maturation of XPO7 knockdown cells was impaired, as shown by CD71 and Ter119 flow cytometry analysis (Fig 6B), and the average cell size was increased (Fig 6C). In addition, using an ImageStream flow cytometer showed the mean and median size of the nuclear area to be increased upon XPO7 knockdown when the cells were cultured under differentiation conditions (Fig 6D). This is consistent with the notion that XPO7 is required for nuclear condensation during terminal erythroid differentiation. Collectively, these findings suggest that XPO7 is partially responsible for the phenotype of Nan mice, establish that the Xpo7 gene is an erythroid target gene of KLF1, and that nuclear condensation is a process previously unrecognized to be regulated by KLF1.

Discussion

Erythropoiesis is a complex process that involves many players whose coordinated activity ensures the production of functional red blood cells. One of these players is KLF1, a transcription factor with multiple roles during terminal erythroid differentiation. Firstly, it is essential for globin regulation, in particular for direct activation of β-globin [6, 7]. In addition, it acts as a master regulator of genes activated during differentiation of red blood cells, such as membrane proteins, heme synthesis enzymes and cell cycle regulators [2, 4, 24]. Hence, it comes as no surprise that Klf1 knockout embryos die due to severe anemia, and that the phenotype is not rescued by exogenous expression of a β-like globin gene [42]. Accordingly, KLF1 variants can lead to diverse phenotypes in humans [10]. One example is a missense variant in the second zinc finger of human KLF1 (p.E325K) that causes CDA type IV [43–48]. This variant is believed to affect binding of KLF1 to its target genes thereby exerting a dominant-negative effect on wildtype KLF1 protein. Similar effects have been described for the Nan mouse model. These mice have a missense variant, p.E339D, in a position homologous to that of the human CDA type IV variant [17, 18]. Studies on the effect of the KLF1 Nan variant in adult mice have revealed that these animals display life-long anemia [17–19].

In this paper we present our findings on the effects of the KLF1 Nan variant on definitive fetal erythropoiesis and show that erythroid maturation is impaired in Nan fetal livers at E12.5, E14.5 and E18.5. We identified 782 differentially expressed genes in Nan versus control E12.5 fetal livers. In agreement with previous reports on erythropoiesis in Nan mice [18, 21–23], the expression of globin genes is altered in Nan fetal livers. In particular, the upregulation of embryonic βh1 globin might be explained by the significantly lower expression of BCL11A in Nan embryos, which normally suppresses βh1 expression [49]. Alternatively, the onset of definitive erythropoiesis may be delayed in Nan embryos, resulting in a higher contribution of primitive, yolk sac-derived, erythrocytes to the fetal liver. Primitive erythrocytes express βh1 globin at high levels [50]. A similar reasoning could be applied to Xpo7, one of the most downregulated genes in Nan E12.5 fetal liver, since it is expressed at lower levels in primitive erythroid cells [51]. However, Xpo7 was previously identified as a common deregulated gene between Nan fetal liver and adult spleen [22], supporting the notion that its expression is directly affected in Nan erythroid cells. Xpo7 caught our attention since a recent paper described that it is required for nuclear condensation and enucleation during terminal erythroid differentiation in vitro [25]. In addition, the observation that XPO7 expression was also reduced in Klf1 knockout fetal livers indicated that the Xpo7 gene might be a direct target of KLF1. Supporting this notion, data mining of ChIP-seq results revealed that KLF1 binds to the Xpo7 locus in mouse [21, 35–37] and human [35, 38] erythroid cells. Collectively, these data suggested that, similar to the β-globin locus [39], KLF1 might have a role in the spatial organization of the Xpo7 locus. 4C-seq analysis of the Xpo7 locus demonstrated that it adopts a different conformation in fetal liver cells compared to fetal brain cells. The
The presence of the KLF1 Nan variant doesn’t appear to mediate any major changes in the chromatin conformation of the Xpo7 locus. We note that the promoter of the Xpo7 gene contains so-called ‘category II’ KLF1 binding sites [18] which are recognized by wildtype KLF1 only. The presence of such ‘category II’ sites is a hallmark of downregulated genes in Nan erythroid cells. This suggests that in Nan cells wildtype KLF1 is still able to bind to the Xpo7 promoter and organize the erythroid-specific 3D conformation of the locus, but is unable to activate transcription efficiently. An interesting observation is the presence of a loop between the promoter of the canonical Xpo7 promoter (in front of exon 1a) and the erythroid-specific promoter (in front of exon 1b), which is absent in the fetal brain control. This loop is likely the consequence of recruitment of the two promoters to the same transcription factory [40, 52].

Previous work has shown that XPO7 knockdown in cultured mouse fetal liver cells impairs chromatin condensation and enucleation during terminal erythroid differentiation [25]. Although in Nan mice enucleation still occurs, the reduced XPO7 expression due to the KLF1 Nan variant may impair chromatin condensation during terminal erythroid differentiation. We propose that this contributes to the maturation defects of Nan erythrocytes in fetal and adult definitive erythropoiesis. Indeed, knockdown of XPO7 in immortalized mouse erythroblasts cells leads to impaired maturation of the cells, evident by dysregulation of the flow cytometry markers CD71 and Ter119 and the presence of larger cells with larger nuclei in the cultures. Our data are in reasonable agreement with the recent publication on the role of XPO7 in erythroid maturation [25]. It is important to keep in mind that we cannot compare the levels of XPO7 protein between our system and that of Hattangadi et al. [25]. An emerging question is how Nan cells manage to enucleate in the presence of reduced levels of XPO7. One possibility is that the level of XPO7 present in vivo in Nan mice might suffice for correct enucleation of the erythroblasts but still affects nuclear condensation. Alternatively, downregulation of XPO7 might just slow down nuclear condensation, but the cells eventually manage to shed their nucleus when condensation is completed. Lastly, a protein with a role similar to that of XPO7 may substitute for it, thus enabling enucleation. Upregulation of other Xpo genes might compensate for loss of Xpo7 expression. We note that our RNA-seq data do not indicate that this is the case. We favour a scenario in which chromatin condensation is crucial for enucleation [53–55], with XPO7 as an important effector. It is not clear whether enucleation can happen before nuclear condensation is completed. Our study suggests that impaired nuclear condensation contributes to the erythroid maturation defects observed in the Nan mice.

Understanding the role of KLF1 during erythroid maturation and the enucleation process has clinical significance for the production of red blood cells in vitro for transfusion purposes. In recent years, many efforts have been made to produce erythrocytes in vitro starting from hematopoietic stem cells, embryonic stem cells or induced pluripotent stem cells [56–58]. Efficient enucleation is one of several challenges that have to be overcome in order to produce sufficient numbers of fully functional erythrocytes in vitro. More in depth knowledge of this process might guide the development of improved strategies to achieve this goal.

**Supporting information**

S1 File. Supplementary materials and methods and FigsA–D.

(DOCX)

S1 Table. Differentially expressed genes E12.5 wildtype and Nan fetal liver.

(XLSX)
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