HHS Public Access

Author manuscript

Ann Hematol. Author manuscript; available in PMC 2020 September 01.

Published in final edited form as:

Ann Hematol. 2019 September ; 98(9): 2089–2096. doi:10.1007/s00277-019-03726-7.

BOK Promotes Erythropoiesis in a Mouse Model of Myelodysplastic Syndrome

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Abstract

Myelodysplastic syndromes are clonal hematopoietic stem cell disorders characterized by cytopenia and intramedullary apoptosis. BCL-2 Ovarian Killer (BOK) is a pro-apoptotic member of the BCL-2 family of proteins which, when stabilized from Endoplasmic Reticulum-associated degradation (ERAD), induces apoptosis in response to ER stress. Although ER stress appropriately activates the unfolded protein response (UPR) in BOK-disrupted cells, the downstream effector signaling that includes ATF4 is defective. We used Nup98-HoxD13 (NHD13) transgenic mice to evaluate the consequences of BOK loss on hematopoiesis and leukemogenesis. Acute Myeloid Leukemia developed in 36.7% of NHD13 mice with a *Bok* gene knockout between the age of 8 and 13 months and presented a similar overall survival to the NHD13 mice. The loss of BOK exacerbated anemia in NHD13 mice, and NHD13/BOK-deficient mice exhibited significantly lower hemoglobin, lower mean cell hemoglobin concentration, and higher mean cell volume than NHD13 mice. Hematopoietic progenitor cell assays revealed a decreased amount of erythroid progenitor stem cells (BFU-E) in the bone marrow of NHD13-transgenic/BOK-deficient mice. RT-QPCR analysis demonstrated decreased mean value of ATF4 in the erythroid progenitors of NHD13 and NHD13/BOK-deficient mice. Our results suggest that in addition to induction of apoptosis in response to ER stress, BOK may regulate erythropoiesis when certain erythroid progenitors experience cell stress.

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Conflict of interest

S-H. K., O.P. and M.M. declare no conflict of interest. S.G.K. serves as a SAB member for Dansar IT and Gene-in-Cell.

Compliance with ethical standards

All human and animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Keywords

BCL-2; BOK; myelodysplasia; erythropoiesis; apoptosis

Introduction

Myelodysplastic Syndromes (MDS) are clonal hematopoietic stem cell disorders defined by cytopenias, ineffective hematopoiesis and dysplasia eventually resulting in Acute Myeloid Leukemia (AML). Hematopoiesis is ineffective insofar as the bone marrow is often hypercellular with hematopoietic progenitors under stress to correct the cytopenias, but instead exhibiting prominent apoptosis. BCL-2, the BCL-2 family member PUMA, and the PUMA transcriptional regulator p53, which is mutated in 814% of MDS [1–4], all contribute to the antecedent apoptosis and subsequent development of AML. Like PUMA, BCL-2 Ovarian Killer (BOK) has been implicated as a tumor suppressor and in at least one context is upregulated by p53 [5].

BOK is a pro-apoptotic BCL-2 family member as transient overexpression leads to apoptosis [6–8]. Recent studies suggest that BOK is best characterized for its putative ability to induce apoptosis in response to Endoplasmic Reticulum (ER) stress, when stabilized from ER-associated degradation (ERAD) [8,9]. ER stress leads to the accumulation of unfolded proteins, which stimulate the unfolded protein response (UPR) via three signaling pathways mediated by IRE1α, ATF6, and PERK [10]. Although ER stress appropriately activates the UPR in BOK-disrupted cells, as measured by PERK and eIF2alpha phosphorylation, downstream effector signaling, including ATF4 and CHOP, is defective [9]. In agreement, the induction of CHOP during diethylnitrosamine-induced hepatocarcinogenesis was also greatly attenuated by the loss of BOK [11].

BOK's function as a tumor suppressor has been suggested due to its genetic location in one of the 20 most frequent, focally deleted chromosomal regions across all human cancers [12]. For instance, BOK was reported to act as a tumor suppressor by inhibiting epithelial-to-mesenchymal transition in non-small cell lung cancer (NSCLC) [13]. However, in the case of AML, BOK was identified in an RNAi screen as one of the 30 top-ranking genes whose depletion reduced the growth of OCI-AML2 cells suggesting leukemogenic potential of BOK [14]. Thus, a clear pathological role for BOK in leukemogenesis remains unknown. When investigating hematopoiesis, lymphoid organs and bone marrow of adult *Bok*$^{-/-}$ mice had a normal hematopoietic cell subset compartment [9], and Bok-deficient lymphoid and myeloid cells responded normally to apoptotic stimuli [15].

To evaluate the consequences of BOK loss on hematopoiesis and the development of AML, we used the *Nup98-HoxD13* (*NHD13*) transgenic mouse model of MDS/AML in which the t(2;11)(q31;p15) fusion protein is expressed in hematopoietic stem and progenitor cells under the control of the hematopoietic specific promoter, *Vav* [16]. *NHD13*-transgenic mice develop a highly penetrant MDS, showing macrocytic anemia and dysplasia starting at 3 months of age and increased apoptosis of bone marrow stem and progenitor cells. Approximately onethird of *NHD13* mice progress to AML within 10–14 months with high frequency acquisition of *NRas, Kras, or Cbl* mutations [17]. In this model, both

overexpression of anti-apoptotic BCL-2 and deletion of pro-apoptotic PUMA rescue cytopenias, but surprisingly delay progression to AML [18,19].

We hypothesized that loss of BOK may delay progression to AML in *NHD13* mice similar to the aforementioned overexpression of BCL-2 and deletion of PUMA. Notwithstanding, we found that loss of BOK does not appear to affect the development of AML in the *NHD13* mice. However, we did find that BOK contributes to erythropoiesis under stress conditions imparted by the *NHD13* translocation. Interestingly, the ATF4 pathway, which is regulated by BOK, has also been shown to coordinate stress erythropoiesis [20].

Materials and methods

Mice

NHD13 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Bok*$^{-/-}$ mice were generated as previously described [9]. *NHD13* mice were crossed with *Bok*$^{-/-}$ to generate *NHD13/Bok*$^{-/-}$ mice. All mice were maintained on a C57BL/6 background, such that WT mice were C57BL/6 mice and, whenever possible, litter mate controls. All procedures performed in studies involving animals were in accordance with the ethical standards of Yale University (2018–11514). For genotyping, loss of *Bok* was routinely confirmed by PCR as previously described [9] and *NHD13* transgene was also routinely confirmed with PCR procedure following the Jackson Laboratory's instruction. Genotyping was performed both shortly after birth and directly before an experiment with the mouse. To assess the development of leukemia and detect differences in hematological parameters, peripheral blood was obtained by retro-orbital bleeding from WT (n=11), *NHD13* (n=15), *NHD13/Bok*$^{-/-}$ (n=30), and *Bok*$^{-/-}$(n=12) mice from 3 months of age and complete blood counts (CBC) were performed using a Hemavet950 hematology analyzer (Drew Scientific, Oxford, CT, USA). Peripheral blood smears were made and stained with MayGrunwald and Giemsa solution to view the morphology of blood cells. The development of AML was defined by more than 20% blasts in peripheral blood smears. The overall survival of *NHD13* (n=30) and *NHD13/Bok*$^{-/-}$ (n=15) mice were compared in order to study the leukemogenic effects of BOK loss in the NHD13 model.

Hematopoietic progenitor cell assay

To detect differences in erythropoiesis and granulopoiesis, hematopoietic progenitor cell assays using Methocult M3434 (Stem cell technologies, Cambridge, MA, USA) were performed in 3 month-old mice (n=3, from each group). Mice femurs were grinded using pestle and mortar with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2mM EDTA until cell suspension was obtained. Red blood cells were lysed using BD Pharm Lyse (BD Biosciences) and bone marrow (BM) nucleated cells were obtained. The cells were seeded at a density of 1×10^5 cells per 35-mm dish in semisolid agar for colony forming unit granulocyte macrophage (CFU-GM) colony growth and blast-forming units-erythroid (BFU-E) growth. Cultures were incubated in 5% CO_2 for 8 days and CFU-GM and BFU-E colonies were counted manually. All progenitor assays were performed in triplicates.

Fluorescence-activated cell sorting (FACS)

To isolate the erythroid progenitors from our four groups of mice, FACS was performed as described previously [21]. Bone marrow samples were flushed from femurs of mice between 6 and 12 months of age using PBS/0.5% BSA. Flushed bone marrow cells were blocked with rat anti-mouse CD16/CD32 (BD Biosciences, Franklin Lakes, NJ, USA) and subsequently stained with FITC rat anti-mouse TER119 (BD Biosciences) and APC rat anti-mouse CD44 (BD Biosciences). Four groups of erythroblasts in different stages of maturation were isolated using FACS Aria (BD Biosciences). First, proerythroblasts were differentiated from other erythroid progenitors as $TER119^{-}$ and $CD44^{bright}$. Then, forward scatter (FSC) and CD44 were used to distinguish basophilic and polychromatophilic erythroblasts ($FSC^{high}CD44^{+}$); orthochromatophilic erythroblasts and reticulocytes ($FSC^{low}CD44^{mid/+}$); and red blood cells ($FSC^{low}CD44^{low}$).

Quantitative reverse transcription PCR

Total mRNA from the four isolated erythroblasts progenitor populations was extracted with the RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. The cDNA was amplified using iQ SYBR Green Supermix (Bio-Rad), and expression levels of ATF4, CHOP, β-globin major and GAPDH mRNA were determined using these primers: ATF4 forward, 5'-TCGATGCTCTGTTTCGAATG-3' and reverse, 5'GCAACCTGGTCGACTTTTA-3'; CHOP forward, 5'-GAGGTCACACGCACATCCC-3' and reverse, 5'-GGCACTGACCACTCTGTTTCC-3'; β-globin major forward, 5'-GAAGGCCCATGGCAAGAAA-3' and reverse, 5'-GCCCTTGAGGCTATCCAA-3'; GAPDH forward, 5′-ACCACAGTCCATGCCATCAC-3′ and reverse, 5′-CACCACCCTGTTGCTGTAGCC-3′. Cycle conditions were as follows: initial denaturation at 95°C for 20 s followed by 40 cycles of 95 °C for 10 s, 55 °C for 10 s, and 70 °C for 20 s, and an additional melt curve program at the end on a Bio-Rad CFX96 Dx Real-Time PCR System. PCRs for each sample were done in triplicate for all of the target genes and GAPDH.

Statistical analysis

Variables were compared between groups using non-parametric Mann Whitney Test. Overall survival was compared using Kaplan-Meier Method. A P-value of <0.05 was considered statistically significant. Statistical analyses were performed using GRAPHPAD PRISM 6 (GraphPad Software, San Diego, CA, USA). Significance is denoted as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Results

NHD13/Bok$^{-/-}$ mice develop a progressive anemia

Serial complete blood counts (CBCs) showed no significant difference between three to 6 months of age (data not shown). CBCs showed decreased hemoglobin as *NHD13/Bok*$^{-/-}$ mice age. Anemia was progressive from 7 months of age. The hemoglobin of 7 months old mice was significantly lower than those of 6 months old mice (Fig. 1). Serial CBCs showed

no differences for white blood counts (WBC) or platelets (PLT) as *NHD13/Bok*$^{-/-}$ mice age suggesting no age related detrimental effect on granulopoiesis and thromobopoiesis (Fig. 2).

NHD13/Bok$^{-/-}$ mice have a similar survival as the *NHD13* mice

To evaluate the leukemogenic effect of BOK loss added to *NHD13* translocation, CBC and blood smears were serially followed. AML developed in 36.7% (11/30) of *NHD13/Bok*$^{-/-}$ mice between the ages of 8 and 13 months (Fig. 3a, b). The number of *NHD13* mice with confirmed development of AML by blood smears and CBC was 11.1% (1/9). The percentage of AML development between the two groups was not statistically significant by Fisher's exact test. Median survival of *NHD13/Bok*$^{-/-}$ and *NHD13* mice were 355 and 340 days, respectively, which was not statistically significant by the Log-rank or Gehan-Breslow-Wilcoxon tests. Overall survival of *NHD13/Bok*$^{-/-}$ mice was similar to that of *NHD13* mice suggesting that the loss of BOK does not affect the leukemogenesis in *NHD13* mice (Fig. 3c).

NHD13/Bok$^{-/-}$ mice are significantly more anemic than *NHD13* mice by 7 months To find the implication of progressive anemia in *NHD13/Bok*$^{-/-}$ mice, red blood cell parameters were compared in the four groups of mice. Comparison of hemoglobin (Hb) at 3 months showed that the Hb of *NHD13* mice is significantly lower than that of wild type (WT) mice (Fig. 4a). Comparison of mean corpuscular volume (MCV) and red cell distribution width (RDW) at 3 months showed that red blood cells of *NHD13* mice are more macrocytic and vary in size more than those of WT mice indicating that dyserythropoiesis in *NHD13* mice is evident at 3 months (Fig. 4b, d). Hb of *NHD13/Bok*$^{-/-}$ mice at 3 months was not significantly different from that of *NHD13* mice but follow-up CBC revealed a significant decrease in hemoglobin in *NHD13/Bok* mice at 7 months compared with *NHD13* mice (Fig. 4a). Red blood cells of *NHD13/Bok*$^{-/-}$ mice showed significant increase of mean corpuscular volume (MCV) at 3 months and significant decrease of mean corpuscular hemoglobin concentration (MCHC) at 7 month compared with those of *NHD13* mice (Fig. 4b, c). These indicate that red blood cells of *NHD13/Bok*$^{-/-}$ mice are more macrocytic and hypochromic suggesting that red blood cells of *NHD13/Bok*$^{-/-}$ mice are more dyserythropoietic than red blood cells of *NHD13* mice. In other words, the loss of BOK exacerbated the anemia of the *NHD13* mice, which raised a potential connection between BOK and the regulation of erythropoiesis in cells experiencing stress from *NHD13* translocation.

NHD13/Bok$^{-/-}$ mice produce decreased BFU-E colonies at 3 months

To find if the abnormality in erythropoiesis is at the stem cell stage, methylcellulose hematopoietic progenitor cell assay was performed. The assay revealed that the numbers of BFU-E of *NHD13/Bok*$^{-/-}$ mice were significantly lower than those of *NHD13* (Fig. 5a) and that the numbers of CFU-GM of *NHD13/Bok*$^{-/-}$ were similar to those of *NHD13* (Fig. 5b). This finding indicates that *NHD13/Bok*$^{-/-}$ stem cells have less erythropoietic potential than *NHD13* hematopoietic stem cells, yet the granulopoietic potential of *NHD13/Bok*$^{-/-}$ stem cells remained similar to that of *NHD13* stem cells.

An increase of proerythroblasts in *NHD13* and *NHD13/Bok*$^{-/-}$ mice

Isolation of various stages of erythroid progenitors in the bone marrow by Fluorescence Activated Cell Sorting (FACS), as described previously [21], revealed that both *NHD13* and *NHD13/Bok*$^{-/-}$ mice have an increase in proerythroblasts relative to more mature red blood cells. This was verified by comparing the percentage of proerythroblasts to total cells isolated from femur bone marrow between *NHD13* and *NHD13/Bok*$^{-/-}$ mice (Fig. 6).

NHD13/Bok$^{-/-}$erythroid progenitors have decreased mean value of ATF4 and CHOP As described above, under ER stress the UPR is activated in *Bok*$^{-/-}$ cells but the downstream effectors, ATF4 and CHOP, are not upregulated [9]. It is also known that heme-regulated eIF2α kinase (HRI)-eIF2α-ATF4 signaling is necessary to promote erythroid differentiation [22]. Expression levels of β-globin, ATF4 and CHOP were measured and compared in the four groups of mice. We found that the expression of β-globin increases as cells mature, which agrees with a previous report [23] and that it is lower in basophilic, polychromatophilic and orthochromatophilic erythroblasts, reticulolcytes and red blood cells of *NHD13/Bok*$^{-/-}$ mice than that in those cells of WT, *NHD13*, and *Bok*$^{-/-}$ mice (Fig. 7a). We also found that the mean values of expression of ATF4 is lower in all erythroid forms of *NHD13* and *NHD13/Bok*$^{-/-}$mice than that in those cells of WT mice (Fig. 7b). We found that expression levels of ATF4 in *NHD13,* NHD13 /*Bok*$^{-/-}$ and *Bok*$^{-/-}$ are significantly lower than those of WT in the RBC population, as well (Fig. 7b). Finally, we found that the expression levels of CHOP in *NHD13, NHD13* /*Bok*$^{-/-}$ and *Bok*$^{-/-}$ are significantly lower than those of WT in RBC population, (Fig. 7c). These findings imply that mechanism of decreased erythropoiesis in *NHD13* and *NHD13/Bok*$^{-/-}$mice may involve the Activating Transcription Factor 4 (ATF4) pathway.

Discussion

The present study investigated the role of BOK in leukemogenesis and hematopoiesis using *NHD13* MDS/AML mouse model. A previous study using *BCL2* transgenic mouse with *NHD13* mice demonstrated that preventing apoptosis of premalignant cells delayed the development of AML [19]. Similarly, the loss of PUMA reduced apoptosis and significantly delayed the development of AML [18]. The loss of pro-apoptotic BOK was expected to delay the progression to AML in *NHD13* mice, but overall survival of *NHD13/Bok*$^{-/-}$ mice was not significantly different from that of *NHD13* mice. The percentages of AML development in *NHD13/Bok*$^{-/-}$ and NHD13 mice were 36.7% and 11.1%, respectively, which were not significantly different. This suggests that loss of BOK does not appear to affect the development of AML. These rates of AML development are similar to previous reports about the development of AML in the *NHD13* mice [16].

As mentioned above, role of BOK in cancer was demonstrated in NSCLC [13]. BOK protein levels significantly downregulated in NSCLC tumors compared with lung tissue and BOK downregulation was associated with tumor grade and lymph node metastasis suggesting a role as a tumor suppressor. BOK protein levels were also significantly decreased in colorectal cancer compared with normal tissue [24]. In terms of hepatocarcinoma, BOK promoted diethylnitrosamine (DEN)-induced hepatocarcinogenesis in mice through

induction of CHOP, BIM, and PUMA [11]. Here, a connection between BOK and CHOP was clearly demonstrated as previously published by Carpio, *et al.* [9].

The present study demonstrated that *NHD13* and *NHD13/Bok*$^{-/-}$ erythroid progenitors have decreased mean value of ATF4 mRNA compared with WT erythroid progenitors. Previously, knock-out of ATF4 in mice resulted in severe fetal anemia caused by proliferation defect of BFU-E and erythrocyte colony-forming unit (CFU-E) [25]. Knockdown of ATF4 with siRNA in mouse erythroid leukemic cells also resulted in lower mRNA level of β-globin major compared with the control siRNA and reduced number of benzidine positive cells [22]. It is also known that ATF4 was diminished in BOK disrupted cells under ER stress [9]. Thus, decreased ATF4 expression in *NHD13/Bok*$^{-/-}$ mice may contribute to their progressive anemia, but does not completely explain the added effects seen beyond the *NHD13* mice. Furthermore, this study concluded that the *in vivo* function of BOK for hematopoiesis is related to the erythropoiesis under cell stress condition such as is present with the *NHD13* translocation.

Acknowledgements

This work was supported by the NHLBI Grant 1R01HL131793, the NIDDK Grant U54DK106857, a Gabrielle's Angel Foundation award and the March of Dimes Basil O'Connor Starter Scholar research award. This work was also supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF- 2018R1D1A1B07040319).

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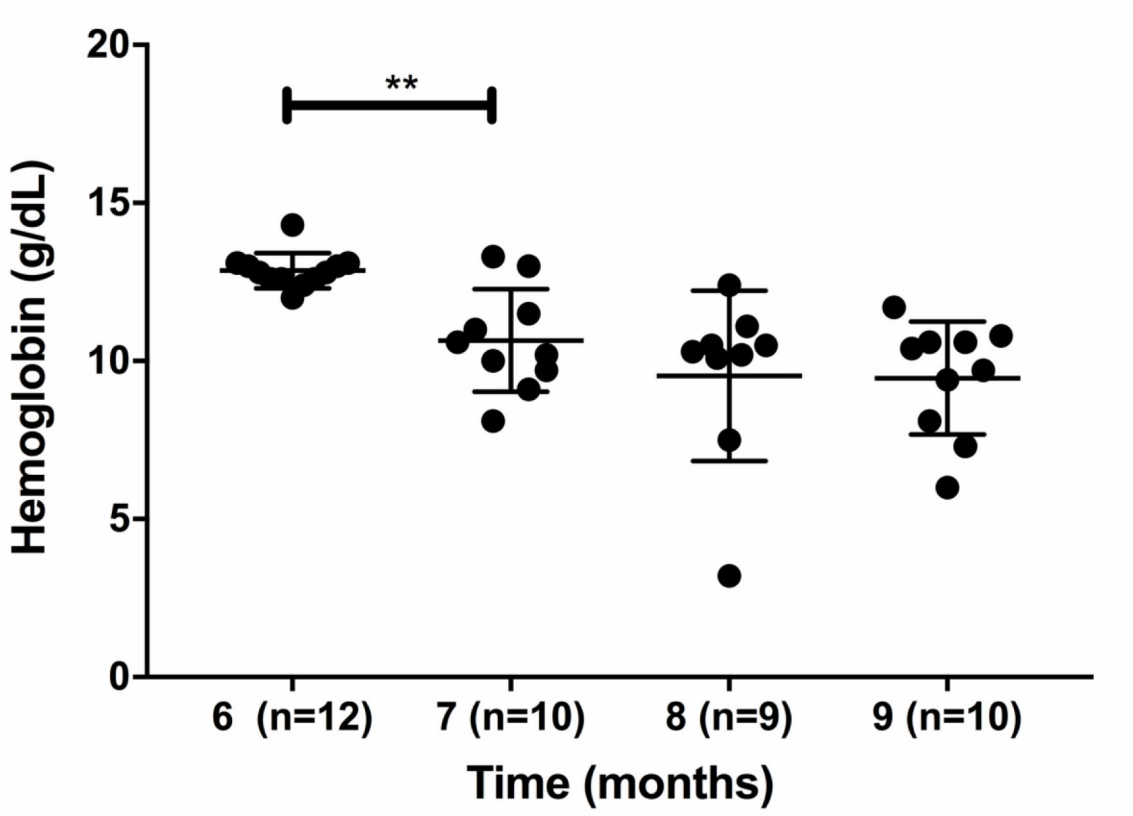

Fig. 1.
Serial hemoglobin in *NHD13/Bok*$^{-/-}$ mice (n=12). ** P <0.01.

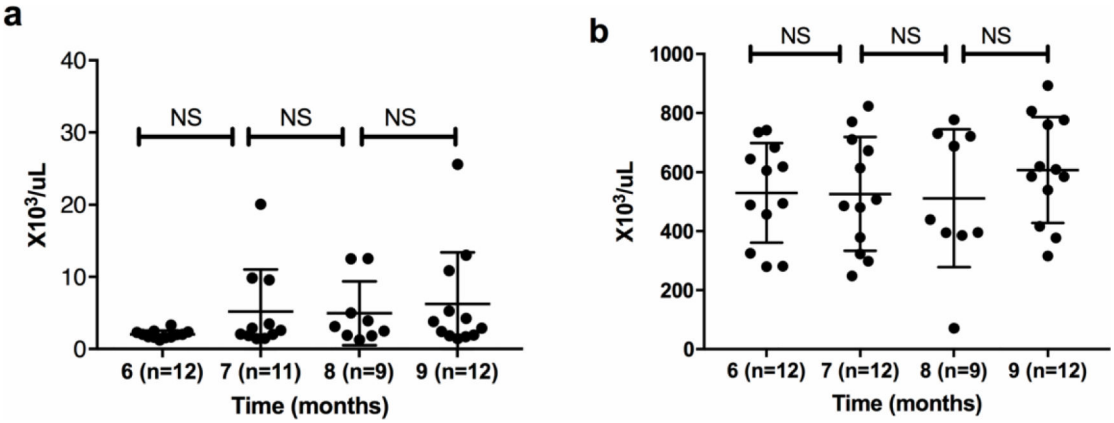

Fig. 2.

Serial complete blood counts in *NHD13/Bok*$^{-/-}$ mice (n =12). **a** White blood cell counts. **b** Platelet counts. NS, not significant.

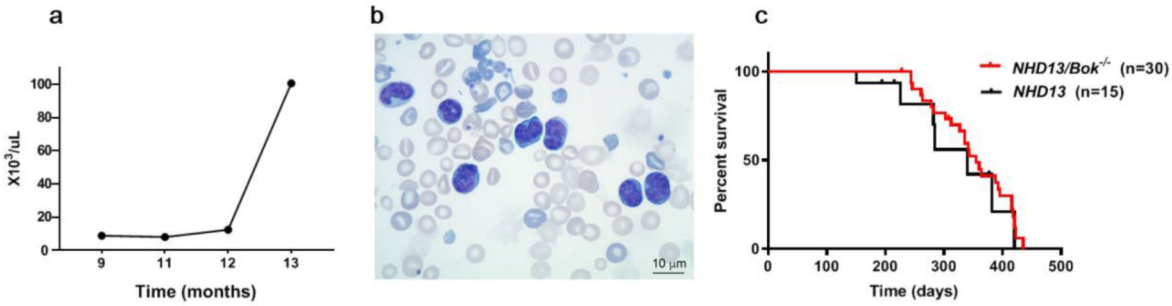

Fig. 3.

AML development in *NHD13/Bok*$^{-/-}$ mice. **a** An example of an increase of white blood cell count in a *NHD13/Bok*$^{-/-}$ mouse (n=1). **b** Morphology of leukemic cells (×1000). **c** Comparison of overall survival between *NHD13* (n=15) and *NHD13/Bok*$^{-/-}$ mice (n=30).

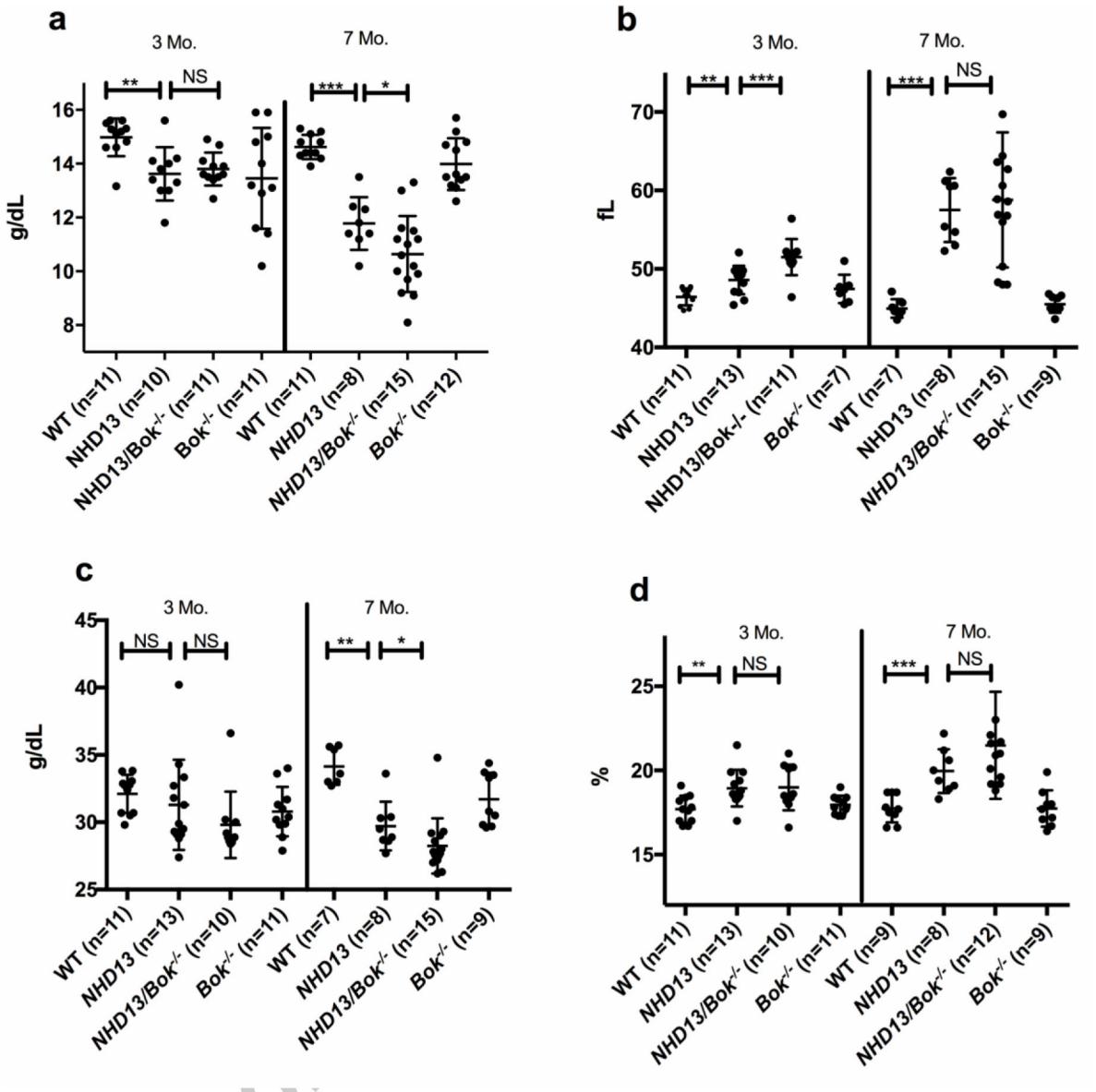

Fig. 4.

Comparison of red blood cell parameters in 3 months (Mo.) and 7 months (Mo.) old wild type (WT) (n=11), *NHD13* (n=12), *NHD13/Bok*$^{-/-}$ (n=15) and *Bok*$^{-/-}$ (n=12) mice. **a** Hemoglobin. **b** Mean cell volume. **c** Mean corpuscular hemoglobin concentration. **d** Red cell distribution width. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

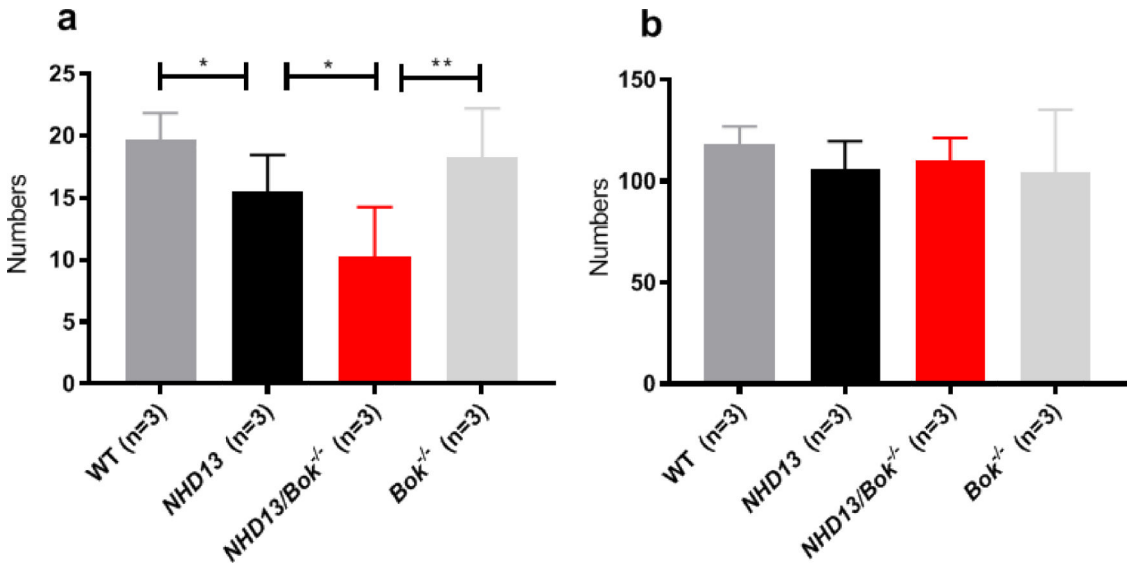

Fig. 5.

Hematopoietic progenitor cell assay in wild type (WT) (n=3), *NHD13* (n=3), *NHD13/Bok* $^{-/-}$ (n=3) and *Bok*$^{-/-}$(n=3) mice. **a** Blast-forming units-erythroid (BFU-E). **b** Colony forming unit granulocyte macrophage (CFU-GM).

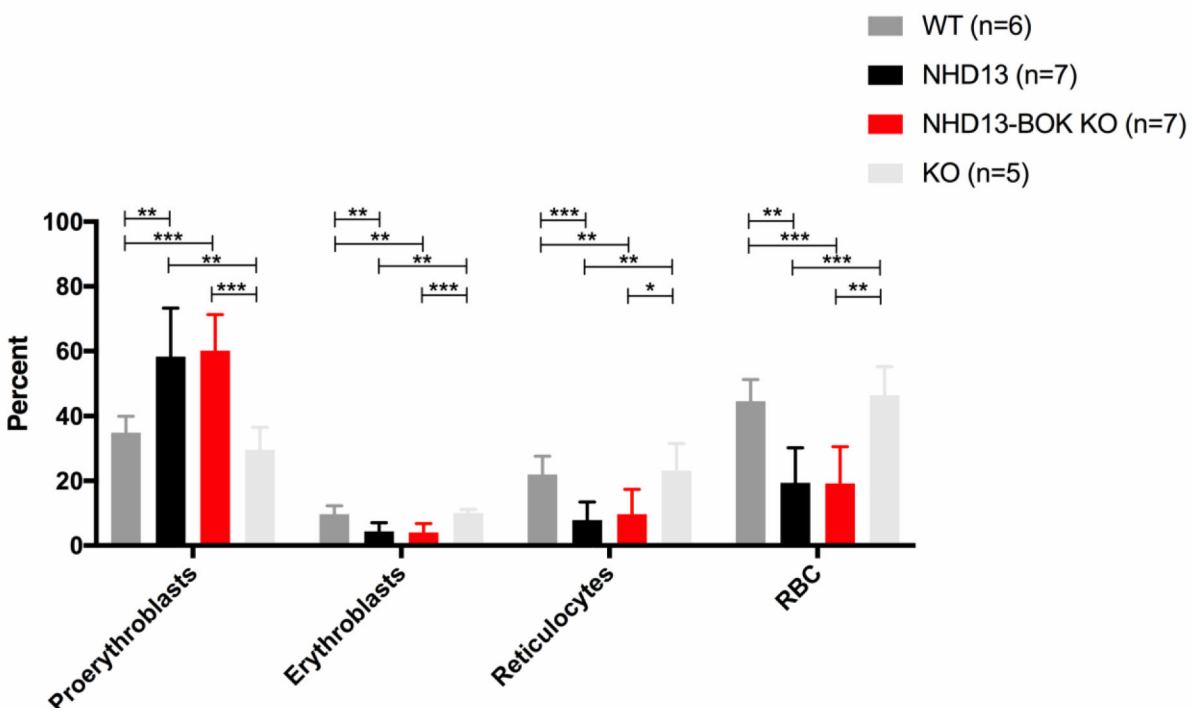

Fig. 6.

Percentages of maturing erythroid forms in wild type (WT), *NHD13, NHD13/Bok*$^{-/-}$ and *Bok*$^{-/-}$ mice. Proerythroblasts, basophilic and polychromatophilic erythroblasts (erythroblasts), orthochromatophilic erythroblasts and reticulocytes (reticulocytes) and red blood cells (RBC) were separated by FACS. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

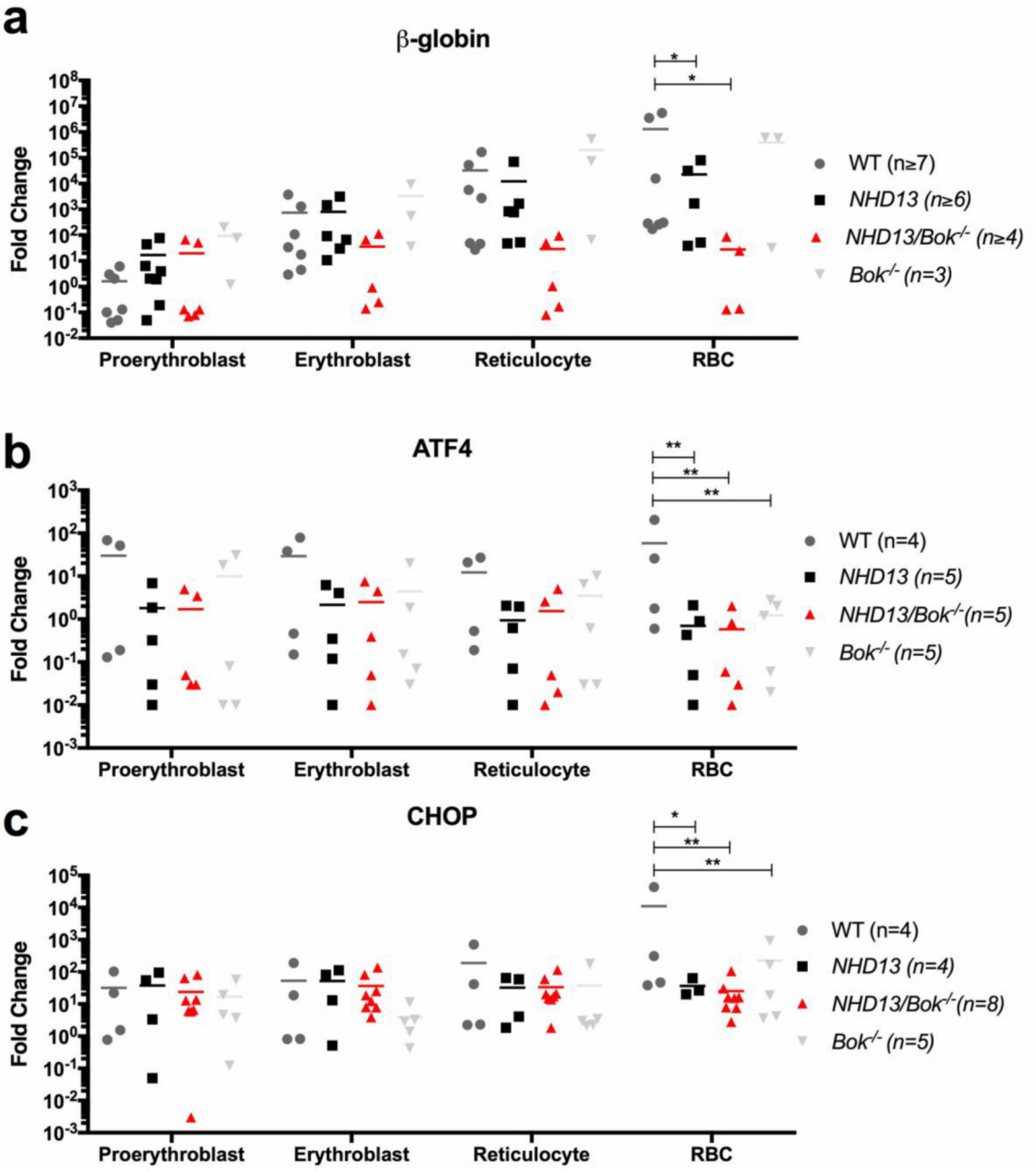

Fig. 7.

Expression levels of β-globin (**a**) ATF4 (**b**) and CHOP (**c**) in proerythroblasts, basophilic and polychromatophilic erythroblasts (erythroblasts), orthochromatophilic erythroblasts and reticulocytes (reticulocytes) and red blood cells (RBC). **P* < 0.05 and ***P* <0.01.