Enhancing mitochondrial function in vivo rescues MDS-like anemia induced by pRb deficiency

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Erythropoiesis is intimately coupled to cell division, and deletion of the cell cycle regulator retinoblastoma protein (pRb) causes anemia in mice. Erythroid-specific deletion of pRb has been found to result in inefficient erythropoiesis because of deregulated coordination of cell cycle exit and mitochondrial biogenesis. However, the pathophysiology remains to be fully described, and further characterization of the link between cell cycle regulation and mitochondrial function is needed. To this end we further assessed conditional erythroid-specific deletion of pRb. This resulted in macrocytic anemia, despite elevated levels of erythropoietin (Epo), and an accumulation of erythroid progenitors in the bone marrow, a phenotype strongly resembling refractory anemia associated with myelodysplastic syndromes (MDS). Using high-fractionation fluorescence-activated cell sorting analysis for improved phenotypic characterization, we illustrate that erythroid differentiation was disrupted at the orthochromatic stage. Transcriptional profiling of sequential purified populations revealed failure to upregulate genes critical for mitochondrial function such as \textit{Pgc1b}, \textit{Alas2}, and \textit{Abcb7} specifically at the block, together with disturbed heme production and iron transport. Notably, deregulated \textit{ABCB7} causes ring sideroblastic anemia in MDS patients, and the mitochondrial co-activator \textit{PGC1b} is heterozygously lost in del5q MDS. Importantly, the anemia could be rescued through enhanced PPAR signaling in vivo via either overexpression of \textit{Pgc1b} or bezafibrate administration. In conclusion, lack of pRb results in MDS-like anemia with disrupted differentiation and impaired mitochondrial function at the orthochromatic erythroblast stage. Our findings reveal for the first time a role for pRb in heme and iron regulation, and indicate that pRb-induced anemia can be rescued in vivo through therapeutic enhancement of PPAR signaling. © 2020 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.

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hemoglobin, progressive decrease in cell size and nuclear condensation, and finally enucleation to become reticulocytes that mature in the bloodstream into biconcave erythrocytes [1]. Disruption at any stage of the process can result in anemia, a condition that results in reduced quality of life and impaired clinical outcome from a diverse set of diseases including chronic inflammation, cancer, and bone marrow disorders such as myeloid myelodysplastic syndrome (MDS) [2].

Erythropoiesis is intimately coupled to cell division; early erythroid progenitors are required to proliferate and self-renew to maintain the pool of more mature precursors, whereas late erythroid progenitors are dependent on cell cycle progression to initiate and progress through their terminal differentiation program [3]. Retinoblastoma protein (pRb) is a key cell cycle regulator controlling the G1-to-S phase transition [4], which is of particular importance for erythropoiesis. In a key commitment step from CFU-Es to terminal differentiation, erythroid progenitors are synchronized in S phase through downregulation of cyclin-dependent kinase (CDK) inhibitor p57Kip2 [5]. This leads to inhibition of PU.1 and activation of the erythroid master transcriptional regulator GATA-1 and the erythropoietin receptor (EpoR), which locks the cell cycle clock to the erythroid differentiation program. During the final steps of maturation, orthochromat erythroblasts undergo cell cycle exit and are arrested in G1 phase [5].

During the G1-to-S phase transition, pRb is hyperphosphorylated by CDKs, which in turn release E2F transcription factors necessary for cell cycle progression [6]. Deletion of pRb in hematopoietic stem cells (HSCs) using MxCre induces myeloproliferative disease. This is, however, not intrinsic to HSCs, but rather the consequence of an pRb-dependent interaction between myeloid-derived cells and the microenvironment caused by dual deletion in stem/progenitor cells and the bone marrow stroma [7]. Rb-deficient embryos have profound anemia, and cell-specific deletion has revealed an intrinsic requirement for pRb and several of its downstream E2F mediators for proper erythroid development [8]. Furthermore, erythroid-specific deletion of pRb was reported by Sankaran et al. [9] to result in inefficient terminal differentiation because of deregulated coordination of cell cycle exit and mitochondrial biogenesis. However, because of the methods available at the time, it remained unclear at what specific stage erythroid development was disrupted, and further characterization of the link between cell cycle regulation and mitochondrial function is needed.

To further decipher mechanisms downstream of pRb deficiency that cause anemia we took advantage of the erythroid-specific pRb conditional knockout mouse described by Sankaran et al. [9]. Herein we report that lack of pRb results in MDS-like macrocytic anemia despite elevated levels of Epo and accumulation of erythroid progenitors in the bone marrow, with a development block at the orthochromat erythroblast stage. Detailed transcriptional profiling of purified erythroid progenitor populations immediately around the differentiation block revealed that pRb-deficient erythroblasts in final maturation stages failed to upregulate large clusters of genes critical for mitochondrial function, heme synthesis, and iron metabolism. Importantly, we report that the in vivo overexpression of peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α), an essential co-activator of mitochondrial biogenesis [10], successfully rescued anemia induced by pRb deficiency. Treatment with bezafibrate [11], a small molecule activator of the same nuclear complex as PGC1α, normalized the blood parameters in a similar fashion, indicating that enhanced mitochondrial function rescues MDS-like anemia induced by pRb deficiency.

Methods

Mice, sampling, and transplantations
Epor-Cre Rosa26-eYFP<sup>lox/los</sup> Rb<sup>fl/fl</sup> conditional knockout mice (referred to as Epor-Cre pRb<sup>−/−</sup>) were bred in the clinical research center at Lund University and maintained at BMC Lund University, Lund, Sweden. All experiments were performed under ethical guidelines approved by the Animal Ethics Committee of Lund, Sweden.

Steady-state parameters were analyzed using littermate mice aged 8–9 months. Peripheral blood (PB) from tail vein punctures was collected in EDTA tubes and analyzed using Sysmex KX-21N (Sysmex Corp., Kobe, Japan). Bone marrow (BM) was flushed from femurs, spleens were weighed, and then crushed and single-cell suspensions were counted using Sysmex.

For transplantation studies, donor BM from femur, tibia, and iliac crest was harvested (pRb<sup>−/−</sup> or Cre-pRb<sup>−/−</sup>, CD45.2), counted, and depleted for lineage markers and Sca1. Cells were transduced with PGC1α overexpressing lentivirus or control for 12 hours and transplanted into lethally irradiated recipients (CD45.1, 2 × 500 cGy) together with 100,000 whole BM cells from congenic wild-type (WT) mice (CD45.1) for support. PB was analyzed 4 and 8 weeks post transplant and in BM and spleen on termination at 8 weeks.

Cohorts of Epor-Cre pRb<sup>−/−</sup> conditional knockout mice and Cre negative WT littermates were fed custom feed containing 0.5% bezafibrate (w/w), ssniff Spezialdiäten GmbH, Soest, Germany) or control feed without bezafibrate. PB samples were collected at days 0, 30, and 60 and analyzed on the Sysmex.

Flow cytometry
Four separate flow cytometry antibody stains were performed to analyze (i) early myelo-erythroid/erythroid progenitors in BM using Sca1, c-Kit, CD150, CD105, CD41, and lineage markers B220, CD3, Gr-1, and Mac-1; (ii) terminal erythroid differentiation in BM and spleen using Ter119, CD44, and FSC-A; (iii) lymphoid/myeloid lineages in BM using B220, CD3, Gr-1, and Mac-1; and (iv) donor-derived lineages in...
PB using CD45.2, Ter119, B220, CD3, and Gr1. DAPI; or 7-AAD was used to exclude dead cells, and MitoTracker deep red (Thermo Fisher Scientific, Waltham, MA, USA) was included to measure mitochondrial activity. Samples were sorted and analyzed on a FACS Aria III (Becton Dickinson).

### In vitro erythroid differentiation

C-kit–bone marrow cells from WT and pRb-deficient mice were enriched using CD117 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and transduced with PGC1β overexpressing lentivirus or control vector in SFEM (STEMSpan Stemcell Technologies, Vancouver, BC, Canada) erythroid culture medium with 3 U Epo/mL, 30% fetal bovine serum, 0.3 mg/mL holo-transferrin (Sigma Aldrich), and 0.4 μg/mL proteamine sulfate. Cells were cultured for 36 hours before analysis.

### Transcriptional analysis

For microarray analysis, 80,000 erythroid progenitors from the polychromatic (III), orthochromatic (IV), and reticulocyte (V) populations were sorted into RLT buffer (1% β-mercaptoethanol) and snap-frozen. RNAeasy Plus micro kit (Qiagen, Hilden, Germany) was used for RNA extraction followed by the NuGEN high-yield RNA amplification protocol. The microarray was performed by Kompetenzzentrum Fluoreszente Bioanalytik (KFB, Regensburg Germany) using a single-cartridge array (Affymetrix, mouse gene 2.0 ST).

For quantitative reverse transcription polymerase chain reaction (RT-qPCR), 20,000 erythroid progenitors from populations IV and V (from in vitro cultures 5,000–10,000 population IV cells) were sorted into RLT buffer. The RNAeasy Micro Kit (Qiagen) was used for RNA extraction. cDNA synthesis was performed according to the manufacturer’s protocol using the SuperScript III First-Strand Synthesis kit (Thermo Fisher). RT-qPCR assays were performed using the TaqMan Gene expression master mix protocol (Thermo Fisher) with pre-designed probe-based primers (Integrated DNA Technologies, Coralville, IA, USA) (Supplementary Table E1, online only, available at www.exphem.org).

### Apoptosis assay

BM cells were stained with antibodies against Ter119 (APC, BioLegend, San Diego, CA, USA) and CD44 (APC-Cy7, BD Bioscience), the PE Annexin V kit (BD Bioscience), and BioLegend, San Diego, CA, USA) and CD44 (APC-Cy7, BD Bioscience). Annexin V was added to each sample, gently vortexed and incubated at room temperature for 15 min in the dark, and analyzed on the LSR II (BD Bioscience).

### Cell cycle assay

Two hundred fifty thousand to four hundred thousand sorted erythroid progenitors (III, IV, V) were fixed with ice-cold 70% ethanol, incubated at −20°C overnight, washed with PBS, and stained individually with propidium iodide staining buffer (10 μg/mL, Sigma Aldrich). PI intensity in the sorted populations was analyzed on the LSR II.

### Heme assay

Five hundred thousand sorted erythroid progenitors (IV and V) were lysed in 500 μL of Cell Extraction Buffer (Thermo Fisher) and snap-frozen. Free heme was detected according to protocol using the Hemin Colorimetric Assay Kit (BioVision, Milpitas, CA, USA).

### Serum iron assay

Serum supernatant from PB was collected and analyzed according to protocol using the Iron Colorimetric Assay kit (Sigma Aldrich).

### Morphology and iron stain

Cytospin slides with 15,000 BM cells were stained with May–Grünwald (Merck) for 5 min and counterstained with 3% Giemsa (Merck) for 15 min. For analysis of iron accumulations, slides were stained with potassium ferrocyanide (20%) and hydrochloric acid (10%) at a 1:1 ratio for 20 min, and counterstained with eosin (0.2%) for 10 min.

### Statistical analysis

Statistical analysis was done using GraphPad Prism 7.00 (GraphPad Software, San Diego, CA, USA); significance was analyzed using Student’s unpaired parametric t-test when comparing WT with knockout groups, if not stated otherwise. Ordinary one-way analysis of variance (ANOVA) was used for analysis of the in vivo bezafibrate experiment and in vitro erythroid differentiation experiment. Pairwise comparisons were done where applicable.

A detailed description of all methods is available online in the Supplementary Data (online only, available at www.exphem.org).

### Results

**Erythroid-specific deletion of pRb results in MDS-like anemia with a developmental block at the orthochromic erythroblast stage**

To further understand the mechanism of impaired erythroid development caused by pRb deficiency, we used the mouse model previously described by Sankaran and colleagues, where lox-p flanked pRb is deleted specifically in the erythroid lineage using EpoR-driven Cre recombinase (Epor-Cre pRb<sup>fl/fl</sup>) [9]. To enable tracing of nucleated pRb-deficient cells, the mice were further crossed with Rosa26 yellow fluorescent protein (eYFP) mice with a lox-p flanked stop codon after the promoter, resulting in YFP labeling and pRb deletion in EpoR-expressing cells (Figure 1A) [12,13]. Erythroid specific pRb deletion was confirmed using qPCR in FACS-sorted erythroid, B, T, and myeloid cells (Supplementary Figure E1A,B, online only, available at www.exphem.org). Only erythroid cells became YFP+ consistent with our previously analysis of eYFP expression using Epor-Cre [12] (Supplementary Figure E1C). In line with Sankaran et al. [9], intrinsic deletion of pRb in the erythroid lineage resulted in anemia with
Figure 1. Erythroid specific knockout of pRb results in anemia despite the increased progenitors in bone marrow and spleen. (A) Schematic outline of erythroid-specific conditional YFP knockin and pRB knockout model driven by the Epo receptor (Epo-R) promotor. (B) Sysmex analysis on peripheral blood measuring red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit levels (HCT), mean corpuscular volume (MCV), and platelets (PLT) \((n=6)\). (C) Enzyme-linked immunosorbent assay measuring Epo serum concentrations \((n=8\) or \(9)\). (D) The gating strategy used to fractionate myelo-erythroid progenitors with indicated surface markers \((n=5)\). (E) Quantification of Pre granulocyte−macrophage (Pre GM), Pre megakaryocyte−erythroid (Pre Meg-E), Pre erythroid colony-forming units (Pre CFU-E), and erythroid colony-forming units (CFU-E cell) from freshly isolated bone marrow (BM), as determined by flow cytometry \((n=5)\) [14]. (F) Representative flow cytometry plots of terminal erythroid differentiation in BM, using cell size (FSC-A), CD44, and Ter119 to fractionate BM cells into pro-erythroblasts (I), basophilic erythroblasts (II), polychromatic erythroblasts (III), orthochromatic erythroblasts (IV), reticulocytes (V), and erythrocytes (VI) [15]. (G) Quantification of terminal erythroid populations in BM \((n=19\) or \(20)\). (H) Representative images (WT left, KO right) and (I) quantification of spleen (Spl) weight \((n=25−26)\). (J) Quantification of terminal erythroid populations in spleen \((n=10)\). (K) Representative flow cytometry plots for populations IV (left) and V (right) from WT mice, and (L) quantification of cell viability using Annexin V, DAPI, and flow cytometry \((n=4)\). Data are expressed as the mean ± SEM. \(*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.\)
significantly reduced red blood cell (RBC) counts, hemoglobin (HGB) concentration, and hematocrit (HCT) levels (Figure 1B). In addition, pRb-deficient erythrocytoid cells exhibited an increase in mean corpuscular volume (MCV) (Figure 1B). Deletion of pRb also resulted in reduced platelet numbers (Figure 1B) and an increased total number of bone marrow cells (Supplementary Figure E1D). Measurement of serum EPO concentration using enzyme-linked immunosorbent assay (ELISA) revealed a twofold increase in pRb-deficient mice compared with WT animals (Figure 1C), indicating a maintained physiological response to the anemia.

Previous analysis of Epor-Cre pRb<sup>fl/fl</sup> mice utilized only CD71 and Ter119 staining to provide an overview of erythroid development. More recently developed phenotyping strategies provide greatly improved fractionation of the erythroid populations and define distinct stages within the differentiation trajectory of murine erythropoiesis. To accurately determine at what stage erythroid differentiation was impaired, we applied a high-fractionation flow cytometry protocol efficiently distinguishing the different myelo-erythroid/erythroid progenitors in the bone marrow [14] including Pre-Meg-E, Pre CFU-E, and CFU-E (Figure 1D), revealing a 44% increase in the CFU-E population of pRb-deficient mice (Figure 1E; for absolute numbers see Supplementary Figure E1E). Despite an increased number of erythroid progenitors in the BM and elevated serum erythropoietin levels, pRb-deficient animals remained anemic. Analyses of terminal erythroid differentiation using the surface marker CD44 in conjunction with forward scatter (FSC-A) [15], separating Ter119+ BM erythroblasts into pro-erythroblasts (I), basophilic (II), polychromatic (III), orthochromatic (IV) erythroblast, reticulocytes (V), and mature RBCs (VI) (Figure 1F), revealed that pRb deficiency resulted in a 2.9-fold increase in orthochromatic (IV) cells, followed by a 51% decrease in reticulocytes (V) compared with wild type (Figure 1G).

Steady-state erythropoiesis occurs at a constant rate in the BM, while anemia induces an extramedullary stress response in the spleen to rapidly produce large numbers of new erythrocytes [16,17]. The spleen weight of pRb-deficient mice was increased 2-fold compared with wild type (Figure 1H,I). Similar to that observed in BM, orthochromatic erythroblasts accumulated in the spleen (5.3-fold increase), with compromised ability to differentiate into reticulocytes (Figure 1J). Even though absolute numbers of reticulocytes are compensated for by the vast increase in numbers of total white blood cells per femur and spleen (Supplementary Figure E1F,G), mice lacking pRb remain anemic.

Apoptosis analysis within each differentiation step using Annexin V and DAPI (Figure 1K), demonstrated a switch from increased to decreased cell survival adjacent to the cellular block (Figure 1L). Increased survival of early erythroblasts in the pRb knockout is potentially due to the increased circulating levels of Epo [18]. In summary, detailed phenotypic analysis revealed that erythroid-specific deletion of pRb resulted in macrocytic anemia despite enhanced levels of circulating erythropoietin and increased numbers of erythroid progenitors in the BM, with disturbed terminal differentiation specifically at the orthochromatic erythroblast stage. Such inefficient erythropoiesis is characteristic of anemia associated with MDS, a group of heterogeneous diseases characterized by abnormal and ineffective hematopoiesis [19].

Transcriptional analysis around the maturation block reveals multiple distinct patterns of gene deregulation compared with normal terminal erythroid differentiation

Transcriptional complexity is high during terminal erythroid development, with large changes in gene expression in each step of maturation accompanying the morphological transformation [20]. To elucidate mechanisms underlying the inability of pRb-deficient orthochromatic erythroblasts (IV) to mature into reticulocytes (V), microarray analysis was performed on FACS-sorted populations at and adjacent to the block (III, IV, and V) (Figure 2A; for morphology see Supplementary Figure E2, online only, available at www.exphem.org). Transcriptome analysis revealed that very few genes were significantly different compared with WT before the block (III), followed by a gradual increase in numbers of deregulated genes (Figure 2B). Analysis using Genesis [21] revealed that differentially expressed genes clustered into five distinct patterns of deregulation compared with the expected expression during the final stages of terminal erythroid differentiation in the WT: clusters of genes that (I) should be downregulated but failed to downregulate (Figure 2C); (II) should be downregulated but were upregulated (Figure 2D); (III) should have stable expression through differentiation but were downregulated (Figure 2E); (IV) should be upregulated but failed to upregulate either at the block (Figure 2F) (V) or in the final maturation to reticulocytes (Figure 2G; for gene lists per cluster, see Supplementary Table E2, online only, available at www.exphem.org). Having identified where the developmental block induced by pRb deficiency occurred allowed us to define sets of genes that were unable to adhere to normal expression patterns during terminal erythroid differentiation.

Cell cycle exit is perturbed in pRb-deficient orthochromatic erythroblasts

Analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [22] revealed
that the clusters of genes that failed to downregulate or were upregulated (Figure 2C, D) associated mainly with cell-cycle related functions (Figure 2H). A 2- to 12-fold increased expression of cell cycle-related genes compared with WT was confirmed in both populations IV and V using qPCR, including genes critical for cell cycle transitions (Ccne1, Cdk1, Cdk2, Tfdp1) and transcriptional repression (E2f7, E2f8) (Figure 2I). Notably, the expression of E2f2, a transcriptional activator normally increased during terminal differentiation [23], was decreased in pRb-deficient reticulocytes. In line with the transcriptional profiling, analysis using propidium iodide and FACS revealed that the cell cycle status of polychromatic erythroblasts (III) was...
unchanged, while orthochromic erythroblasts (IV) and reticulocytes (V) lacking pRb exhibited a 2-fold increase in cells remaining in S phase (Figure 2J,K). Taken together, our results confirm that pRb deletion results in an inability to properly exit cell cycle and extends previous findings to demonstrate that the defect is specific to the orthochromic erythroblasts.

**pRb deficiency results in deregulation of mitochondrial function including heme production and iron metabolism**

DAVID analysis of genes that should remain stably expressed during terminal erythroid differentiation, but were downregulated in Rb-deficient progenitors (Figure 2E), revealed that these clusters were highly related to mitochondrial function, with gene ontology terms including oxidative phosphorylation and NADH dehydrogenase activity (Figure 3A). Decreased expression of key genes involved in oxidative phosphorylation, such as *Ndufa1* (complex1, OXPHOS), *Atp5s* (ATP synthesis), and *Cox7b* (electron transfer), was confirmed using qPCR (Figure 3B). Furthermore, in line with reactive oxygen species (ROS) being a byproduct of mitochondrial respiration, the expression of mitochondrial antioxidant *Prdx3*, which is important for ROS homeostasis, was also decreased at the developmental block (Figure 3B). In accordance, flow cytometry analysis using Mitotracker DeepRed revealed that pRb deficiency resulted in reduced mitochondrial membrane potential in the populations around the maturation block (Figure 3C). Furthermore, analysis of mitochondrial ROS levels using MitoSox revealed a delay in the peak of MitoSox*high* cells (Figure 3D,E). Collectively, erythroid-specific deletion of pRb resulted in decreased expression of mitochondria-related genes, accompanied by a decreased mitochondrial membrane potential and a shift in mitochondria-produced ROS.

The clusters that failed to upregulate during terminal erythroid maturation (Figure 2F,G) included genes involved in heme synthesis and iron transport (Figure 3F), biological processes taking place in the mitochondria that are fundamental for proper RBC function. These included *Fech*, which catalyzes insertion of iron into heme [24], and *Alas2*, which encodes the rate-limiting enzyme in erythropoietic-specific heme synthesis [25]. Furthermore, the iron transporter *Abcb7* and protoporphyrinogen IX transporter *Tmem14c* exhibited decreased gene expression (Table 1), which was verified using qPCR (Figure 3G). In accordance with perturbed expression of iron transporters, Prussian blue staining of BM cells revealed increased iron accumulation in pRb-deficient erythroblast progenitors compared with wild type (Figure 3H), consistent with previous observations in the spleen [9]. Furthermore, total iron concentration in the serum of KO mice was significantly decreased (Figure 3I). Coordination of iron acquisition and heme synthesis is required for effective erythropoiesis, and iron availability is known to regulate heme production [26]. Measuring heme concentration in sorted IV and V progenitor populations revealed that wild-type cells increased their heme concentration from the orthochromic erythroblast (IV) to reticulocyte (V) stage (Figure 3J). In contrast, pRb-deficient cells had 1.9-fold higher heme concentrations in the orthochromic erythroblasts compared with WT, followed by decreased heme concentrations in reticulocytes (Figure 3J). Collectively, our data indicate that erythroid-specific deletion of pRb resulted in deregulation of heme production and iron metabolism, uncovering a previously unrecognized mechanism by which pRb regulates erythropoiesis.

**pRb-deficient erythroid cells exhibit aberrant expression of genes that are recurrently deregulated in human MDS**

Subtypes of MDS are characterized by mitochondrial iron accumulations resulting in refractory anemia with ring sideroblasts (RARS), which are caused by mutated *SF3B1* [27] and downstream aberrant splicing of genes including *Abcb7* [28]. Both *SF3B1* and *Abcb7* were deregulated in pRb-deficient erythroid progenitors together with other genes involved in MDS-related anemia such as *Tmem14c* and *Alas2* (Table 1). pRb-Deficient cells also displayed aberrant expression of several of the most recurrently mutated genes in MDS involved in other disease phenotypes [19], including *Srsf2* and *U2af1* affecting pre-mRNA splicing [29], *Mdm2* promoting enhanced cancer susceptibility [30], and *Kras* promoting survival and proliferation [31] (Table 1). Collectively, our data indicate that erythroid-specific deletion of pRb results in deregulation of a large number of genes frequently altered in human MDS.

**Improving mitochondrial function via an enhanced PPAR signaling pathway rescues MDS-like anemia in pRb-deficient animals**

Because of the impairment of mitochondria-related genes and function, we next asked if increased mitochondrial function could rescue the MDS-like anemia induced by intrinsic pRb deficiency. PGC1β is a transcriptional co-activator of mitochondrial biogenesis [10], which has previously been reported to be reduced in Ter119+ erythroblasts of pRb-deficient mice and when knockdown resulted in impaired erythroid maturation of G1E-ER cells [9]. Previous data, however, did not address whether there was a causative link, and if restoration of PGC1β expression could rescue the phenotype of pRb deficiency in vivo. Analyzing its expression in purified erythroid progenitor populations, we confirmed that PGC1β was downregulated
specifically in the orthochromatic erythroblasts where the developmental block occurs (Figure 4A). To determine if restoring expression of PGC1β could rescue the anemic phenotype in vivo, we created a lentiviral vector efficiently overexpressing PGC1β under the SFFV promotor (Figure 4B; Supplementary Figure E3A–D, online only, available at www.exphem.org). Transduced progenitor-enriched BM cells (initial transduction efficiency of 70%–80%, Supplementary Figure E3B) together with whole BM support cells were transplanted into lethally irradiated mice that were followed for recovery over time (Figure 4C). Strikingly, analyses of PB parameters after 8 weeks revealed complete normalization of the RBC count in mice transplanted with KO cells overexpressing PGC1β, whereas mice treated with the control vector remained anemic (Figure 4D). Equivalent improvements were seen for hemoglobin concentration and hematocrit volumes (Figure 4D). In addition, the increase in mean cellular volume resulting from intrinsic pRb deletion was close to normalized by overexpression of PGC1β (Figure 4D). Spleens remained enlarged (data not shown), indicating that extramedullary stress erythropoiesis is still required at 8 weeks to ameliorate the anemia. Further analysis of
Table 1. Genes commonly associated with MDS are deregulated in the pRb-deficient mouse model.

| Gene   | Function in MDS | Function of gene | Role in human MDS |
|--------|-----------------|------------------|-------------------|
| Sf3b1  | Deregulated pre-mRNA splicing, frequently mutated in MDS | Spliceosomal machinery | Malcovati et al. [27] |
| U2af1  | Deregulated pre-mRNA splicing, frequently mutated in MDS | Spliceosomal machinery | Shirai et al. [56] |
| Mdm2   | Deregulated DNA damage response | E3 ubiquitin − MDM2 leads to cancer susceptibility | McGraw et al. [30] |
| p53    | Tumor suppressor | SNPs within p53 | McGraw et al. [30] |
| Map3k7 | Negative regulation of apoptosis, deregulated in RARS | MAP kinase pathway | Nikpour et al. [43] |
| Kras   | Activating mutations in MDS, promotes survival and expansion | Tyrosine signaling pathway | Bejar et al. [31] |
| Cbl    | Negative regulator of receptor tyrosine kinase | Tyrosine signaling pathway | Bejar et al. [31] |
| Asxl1  | Frequently mutated in MDS | Epigenetic regulator | Chen et al. [57] |
| Ezh2   | Inactivating mutation in myeloid disorders | Epigenetic regulator | Ernst et al. [58] |
| Tmem14c| Altered splicing in RARS | Heme metabolism | Conte et al. [32] |
| Alas2  | Deregulated in RARS progenitors | Heme biosynthesis | Malcovati et al. [59] |
| Abcb7  | Iron transport from mitochondria | | |

FDR = false discovery rate; ns = not significant.

Gene expression analysis revealed that genes that are known to be frequently deregulated in human MDS [27, 30-32, 43-46, 61] were also deregulated in the pRb-deficient mouse model. Gene biological function and proposed role in MDS pathophysiology are indicated.

Although overexpression of PGC1β provides proof-of-principle that impaired mitochondrial function is causally linked to the erythroid differentiation defect, it is not a treatment relevant for clinical application. We therefore asked if we could ameliorate the anemia by pharmacological enhancement of mitochondrial function. To this end, we used bezafibrate, a pan-PPAR agonist acting on the same nuclear receptors that are activated by PGCs [11]. Strikingly, and consistent with the PGC1β overexpression, treating nontransplanted WT and KO mice with 0.5% bezafibrate in the food or control diet for 2 months resulted in amelioration of the refractory anemia with normalization of RBC count and HCT and MCV levels as compared with WT, as well as a trend toward normalization of HGB concentrations (Figure 4F; Supplementary Figure E4, online only, available at www.exphem.org).

To determine if enhanced mitochondrial function also affected the cell cycle, c-kit+ bone marrow cells from WT and pRb-knockout mice were transduced with PGC1β overexpressing lentivirus or a control vector and cultured in erythroid culture medium for 36 hours (Figure 5A). Pgc-1β was efficiently overexpressed (Figure 5B) and resulted in increased mitochondrial membrane potential in orthochromatic erythroblasts (IV), as measured with Mitotracker Deep Red and FACS (Figure 5C). Pgc-1β overexpression in vitro reversed the developmental block at the orthochromatic erythroblasts stage (IV) in the same way as seen in vivo (Figure 5D). Furthermore, transcriptional analysis within transduced population IV progenitors revealed that cell cycle genes were deregulated in pRb-deficient cells in a similar fashion in vitro and in vivo (Figure 5E). Interestingly, Pgc-1β overexpression normalized the expression of cell cycle genes critical for both cell cycle transitions and repression of transcriptional activity, including Cdk1, Cdk2, Ccne1, E2f7, and E2f8 (Figure 5E). In conclusion, improved 
mitochondrial function through an enhanced PPAR signaling pathway rescued MDS-like anemia, as well as normalized the enhanced expression of cell cycle genes induced by pRb deficiency.

Discussion
Deletion of pRb causes anemia in mice [9]. However, the precise stage of erythroid development when pRb is important and the underlying mechanisms have not been clearly defined. Using high-fractionation FACS protocols [14,15], we found that erythroid-specific deletion of pRb results in inefficient erythropoiesis reminiscent of MDS-related anemia, with a developmental block at the orthochromatic erythroblast stage similar to that reported in human MDS patients [32]. Chronic refractory anemia is present in more than 85% of MDS patients [33] and severely impairs the quality of life of patients [33,34], affects their clinical outcome [35], and correlates with increased risk of transformation into acute myeloid leukemia [36].

Consistent with pRb being a potent inhibitor of the G1-to-S phase transition [4] Rb-deficient erythroblasts failed to repress S-phase genes and to properly exit the cell cycle at the orthochromatic erythroblasts. Our results are in line with ex vivo studies indicating that pRb-null terminal erythroblasts from fetal liver continue to cycle in culture [37]. Previous analysis using the same mouse model did not detect a difference in proliferation [9]. This is likely due to the use of a heterogeneous phenotypic population of erythroblasts before the differentiation block (CD71high/Ter-119high), whereas we detected increased cycling in the populations after the maturation block. In agreement with the finding by Spike et al. [38] that pRb deficiency results in decreased enucleation in red cells from fetal liver [38], we detected propidium iodide-positive

Figure 4. Increased activation of the PPAR pathway through PGC-1β overexpression and bezafibrate treatment ameliorates pRb deficiency-induced anemia. (A) mRNA expression of PGC1β in erythroid populations IV and V using qPCR, revealing reduced expression in pRb-deficient cells (n = 2). (B) Schematic representation of PGC1β overexpressing lentiviral vector containing a spleen focus-forming virus (SFFV) promoter, internal ribosome entry site (IRES) element, and dTomato fluorescent reporter, and Western blot analysis of PGC1β protein expression in 3T3 cells transduced with spacer or PGC1b overexpression vector (n = 1). (C) Experimental layout of transplantation experiment in which lethally irradiated mice were transplanted with 100,000 transduced progenitor-enriched cells together with 100,000 whole bone marrow support cells. (D) Sysmex analysis at 8 weeks post transplant on peripheral blood from mice transplanted with WT or KO donor BM treated with spacer or PGC1β overexpression vector, measuring red blood cell (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT) levels, and mean corpuscular volume (MCV). (E) Quantification of flow cytometric analysis of dTomato+ and Ter119+ erythroid cells within the BM at 8 weeks. (F) Quantification of Sysmex analysis on peripheral blood at start, 1 month, and 2 months of 0.5% (w/w) bezafibrate chow treatment (n = 9 or 10). Data are expressed as the mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
reticulocytes in bone marrow from pRb-deficient mice, indicating less efficient enucleation. This effect was, however, transient as RBCs in PB from KO mice displayed normal enucleation.

Interestingly, our results indicate that activation of PPAR signaling through overexpression of PGC1\(\beta\) normalized the enhanced expression of cell cycle genes induced by pRb deficiency. In line with these results, activation of PPAR-\(\gamma\) in Rb-deficient mouse embryo fibroblasts has been reported to induce adipocyte terminal differentiation, which also requires cell cycle exit to occur [39]. Furthermore, activation of PPAR signaling has been found to inhibit cell growth in human colorectal cancer cell lines [40]. Further studies are required to conclusively establish the link between pRb-induced cell cycle arrest and PPAR signaling.

Apart from being a cell cycle regulator, pRb has recently been reported to also control various aspects of cell metabolism, including nucleotide synthesis, glutathione synthesis, glycolysis, and oxidative metabolism [41]. Coupling of cell cycle regulation to mitochondrial function has been observed in various cell types, including erythroid cells [9]. However, the specific stage of erythroid maturation at which pRb is important for metabolic control has not been previously defined. Here we report that pRb deficiency results in impaired oxidative phosphorylation and ROS regulation specifically at the final differentiation step from orthochromatic erythroblast to reticulocyte. This is the same stage at which erythroid differentiation becomes compromised, suggesting these are closely coupled features of the mechanism. This was accompanied by decreased mitochondrial membrane potential and deregulated mitochondrial production of ROS. Although a previous study using the same mouse model [9] did not detect a difference in ROS, our analysis of more highly purified phenotypic populations has now detected the shift in ROS production. Every division during terminal erythroid differentiation results in daughter cells that are significantly different from the mother cell. This emphasizes the importance of performing mechanistic studies in highly purified, stage-specific cell populations.

Mutations in mitochondrial genes have been reported in MDS patients [42], and somatic SF3B1 mutations, which are common in subtypes of MDS, are associated with downregulation of core mitochondrial pathways [32,43]. These include genes related to iron and heme homeostasis [27,32], suggesting that iron and heme homeostasis could play a role in the disease phenotype. Loss of the mitochondrial matrix chaperone Hspa9b in zebrafish recapitulates ineffective hematopoiesis including anemia, dysplasia, and increased cell apoptosis caused by compromised mitochondrial function and oxidative stress [44]. Furthermore, mice with accumulating mitochondrial mutations caused by defective Polg proofreading activity exhibit MDS-like macrocytic anemia [45], further suggesting a role for deregulated mitochondrial function in MDS pathogenesis.

Whereas pRb has previously been implicated in regulation of mitochondrial function [9,41], this is the first
time that pRb is described as important for functional heme synthesis and iron transport. We and others [46,47] have found that heme and iron metabolism genes are strongly upregulated during terminal erythroid differentiation. Furthermore, we found that pRb deficiency results in failure to enhance the expression of several genes required for heme production and iron transport. Interestingly, many of these genes are known to be involved in refractory anemia with ring sideroblasts (RARS) [28,48], a subtype of MDS characterized by iron accumulations in perinuclear mitochondria in the form of mitochondrial ferritin [27]. The formation of ring sideroblasts has proven very difficult to reproduce in mouse models of MDS [49]. Our results indicate that intracellular iron accumulations are significantly more frequent in BM cells from mice lacking pRb in the erythroid compartment, although at low frequency. The phenomenon was occasionally also observed, albeit at markedly lower frequency, in WT BM.

It is interesting to note that although RB itself is not commonly mutated in MDS, many of the genes that are deregulated in response to its deletion are known to be involved in MDS pathogenesis, as outlined in Table 1. In addition, PGC1β, which is downregulated specifically where the developmental block occurs, is located within the commonly deleted region of chromosome 5 of the del(5q) subtype of MDS [50]. Impaired erythropoiesis in del(5q) MDS has previously been linked to heterozygous deletion of RPS14 [51]. It is, however, unlikely that the full disease phenotype is attributed to loss of a single gene out of the entire commonly deleted region. Supporting that PGC1β deficiency potentially also contributes to the disease phenotype, patients with del(5q) MDS exhibit inefficient erythropoiesis, with increased levels of heme in the progenitors before the block and decreased levels of ALAS2 in later progenitor stages after the block [52], in a fashion very similar to that we report here. Furthermore, knockdown of PGC1β in G1E-ER cells resulted in inefficient erythroid differentiation [9]. Importantly, we report that in vivo overexpression of PGC1β ameliorates MDS-like anemia, which was reproduced using a small molecule therapy with the mitochondrial respiratory chain and can correct deficiencies of peroxisome proliferator-activated receptor pathway stimulating several of the hallmarks of MDS-related anemia, indicating that this model could be valuable for mechanistic studies. We further report that the anemia could be ameliorated by enhanced PPAR pathway signaling in vivo either genetically or therapeutically, suggesting that improved mitochondrial function could be beneficial—a potentially important step in finding new ways to treat MDS-related anemia. Further studies are needed to investigate if these findings can be translated to the human setting.

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