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ORIGINAL ARTICLE

p38α controls erythroblast enucleation and Rb signaling in stress erythropoiesis

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Enucleation of erythroblasts during terminal differentiation is unique to mammals. Although erythroid enucleation has been extensively studied, only a few genes, including retinoblastoma protein (Rb), have been identified to regulate nuclear extrusion. It remains largely undefined by which signaling molecules, the extrinsic stimuli, such as erythropoietin (Epo), are transduced to induce enucleation. Here, we show that p38α, a mitogen-activated protein kinase (MAPK), is required for erythroid enucleation. In an *ex vivo* differentiation system that contains high Epo levels and mimics stress erythropoiesis, p38α is activated during erythroid differentiation. Loss of p38α completely blocks enucleation of primary erythroblasts. Moreover, p38α regulates erythroblast enucleation in a cell-autonomous manner *in vivo* during fetal and anemic stress erythropoiesis. Markedly, loss of p38α leads to downregulation of p21, and decreased activation of the p21 target Rb, both of which are important regulators of erythroblast enucleation. This study demonstrates that p38α is a key signaling molecule for erythroblast enucleation during stress erythropoiesis.

***Keywords*:** p38α; enucleation; stress erythropoiesis; Rb

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Introduction

In mammalian erythropoiesis, erythroblasts at various differentiation stages associate with a central macrophage to form a functional erythroblastic island [1, 2]. Within the erythroblastic island, erythroblasts continue to expand by several rounds of division as they differentiate sequentially through basophilic, polychromatophilic and orthochromatic erythroblasts into fully mature erythrocytes [1, 2]. The production of mature erythrocytes is strictly regulated to maintain the homeostasis within the erythroid lineage, especially under stress conditions such as fetal erythropoiesis and hemolytic anemia [1-3]. Erythropoeitin (Epo) is one of the most important regulators of erythropoiesis [3]. It promotes erythroblast survival, proliferation and differentiation through several key signal transduction pathways, including the JAK-STAT, JNK and p38 pathways [4-6]. During stress erythropoiesis, the level of Epo can dramatically increase up to 1 000-fold [7]. Together with other stress-induced factors including SCF and glucocorticoids, Epo induces a rapid and massive expansion of erythrocytes to re-establish erythroid homeostasis [1-3, 7].

At the terminal differentiation stage of erythropoiesis, mammalian erythroblasts degrade intracellular organelles in autophagosomes and then extrude the pyknotic nucleus through a process that resembles cytokinesis [1, 2]. The underlying molecular mechanisms of erythroid enucleation remain largely undefined. A few genes that function specifically in DNA degradation and vesicle trafficking were recently shown to be involved in nuclear extrusion [8-12]. Importantly, the tumor suppressor protein retinoblastoma (Rb) was found to be required for erythroid enucleation under stress conditions [13]. Notably, Epo also plays important roles in regulating enucleation. Epo alone was shown to be sufficient to induce

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[4]Deceased. While this manuscript was in revision, Dr Hartmut Beug passed away after a heroic fight against cancer. We dedicate this work to his memory.

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differentiation of human CD34+ hematopoietic cells into enucleated erythrocytes [14]. Moreover, high Epo levels are essential in inducing irreversible enucleation of mouse erythroblasts [15, 16]. However, the molecular pathway through which Epo and other extrinsic signals trigger erythroid enucleation remains unknown.

p38α, as a member of mitogen-activated protein kinases (MAPKs), transduces extracellular signals of growth factors and stresses into various intracellular responses, including cell survival, proliferation and differentiation [17, 18]. We showed previously that erythroblasts deficient in p38α displayed increased proliferation [19]. The importance of p38α in differentiation has been revealed in several cell types. Deletion of *p38α* resulted in deregulated differentiation of lung stem and progenitor cells, both *in vivo* and *in vitro* [20]. Moreover, absence of p38α caused defective differentiation of myoblasts, coupled with delayed cell cycle exit and continuous proliferation [21]. In this study, we addressed the role of p38α in erythroid differentiation with a particular focus on enucleation.

Results

p38α activity is required for erythroblast enucleation ex vivo

We first analyzed the differentiation process of fetal liver-derived erythroid progenitors (FLEPs) *ex vivo*. These cells differentiate into enucleated and fully hemoglobinized mature erythrocytes in a differentiation culture with high Epo levels [15, 16]. Under these conditions, erythroid differentiation is highly synchronous and effectively completed within 3-4 days, involving minimal cell divisions. Epo was previously reported to increase phosphorylated p38α levels in erythroid cell lines [6, 22]. Western blot analysis showed that p38α phosphorylation levels were significantly elevated in FLEPs upon cultivation in differentiation medium (Figure 1A). As $p38\alpha^{-/-}$ embryos die around embryonic day 10.5 [23-26], we derived FLEPs from *Mx-cre*, $p38\alpha^{f/f}$ ($p38\alpha^{\Delta/\Delta}$) fetal livers, which showed complete deletion of *p38α* as previously reported [19]. FACS analysis showed that $p38\alpha^{\Delta/\Delta}$ FLEPs expressed attenuated levels of mature erythrocyte marker Ter119 (Figure 1B). Next, we analyzed expressions of globin genes, including embryonic globin genes *Hba-x*, *Hbb-y* and *Hbb-bh1* that are expressed in fetal livers till E14.5. mRNA levels of *Hbb-b1*, *Hba-x* and *Hbb-y* were decreased as determined by qRT-PCR (Figure 1C). The reduced expression of *Hba-x* and *Hbb-y* should reflect *p38α* deletion in primitive erythroid cells found in the fetal liver [15, 16, 27]. Importantly, total hemoglobin protein levels were reduced in these cells as measured by photometric assay (Figure 1D). However, $p38\alpha^{\Delta/\Delta}$ FLEPs differentiated to the stage of orthochromatic erythroblasts within 48 h (Figure 1E). Strikingly, almost all $p38\alpha^{\Delta/\Delta}$ FLEPs retained the nucleus at 48 h, while FLEPs from $p38\alpha^{f/+}$ control embryos differentiated into enucleated mature erythrocytes (Figure 1E). $p38\alpha^{\Delta/\Delta}$ FLEPs kept pyknotic nuclei even after a prolonged differentiation for 5 days (Supplementary information, Figure S1A).

Correlating with the retention of the nucleus, $p38\alpha^{\Delta/\Delta}$ erythroblasts reduced their cell size to that of orthochromatic erythroblasts at 7 μm, but not to the size of mature erythrocytes at 4 μm (Figure 2A). Electron microscopic analysis confirmed that enucleation of $p38\alpha^{\Delta/\Delta}$ erythroblasts was impaired (Figure 2B). In order to assess nuclear condensation, we quantified the heterochromatin area relative to the nuclear size. No difference in heterochromatin area was found in $p38\alpha^{\Delta/\Delta}$ compared to control erythroblasts (Supplementary information, Figure S1B). It was reported that histone deacetylation by histone acetyltransferases (HATs) and histone deacetylases (HDACs) is essential for nuclear condensation [28]. Among the HATs and HDACs that we have analyzed, no significant differences in expression levels were observed, implying that p38α deficiency may not affect histone acetylation (Supplementary information, Figure S1C). These results suggest that p38α-deficient erythroblasts appear to condense nucleus similar to that of controls. Moreover, p38α-deficient erythroblasts formed autophagosomes containing degraded mitochondria and other cellular organelles (Figure 2B). These data indicate that while erythroblasts lacking p38α undergo morphological changes characteristic for late stage of erythroid differentiation, p38α is specifically required for nuclear extrusion of erythroblasts.

In support of these findings, knockdown of p38α by shRNA led to reduced enucleation of erythroblasts (Figure 2C and 2D). To investigate whether the kinase activity of p38α is required, we infected erythroblasts with retroviruses carrying dominant kinase-dead p38α mutant (p38α-AF). p38α-AF overexpression significantly reduced the percentage of enucleated erythrocytes at 48 h of differentiation (Figure 2E). Moreover, treatment with SB202190, a compound that inhibits p38α kinase activity, induced similar enucleation defects in $p38\alpha^{f/+}$ erythroblasts (Figure 2F). These results indicate that kinase activity of p38α is essential for erythroblast enucleation.

Erythroid enucleation during fetal erythropoiesis depends on p38α

To investigate whether p38α could play similar roles

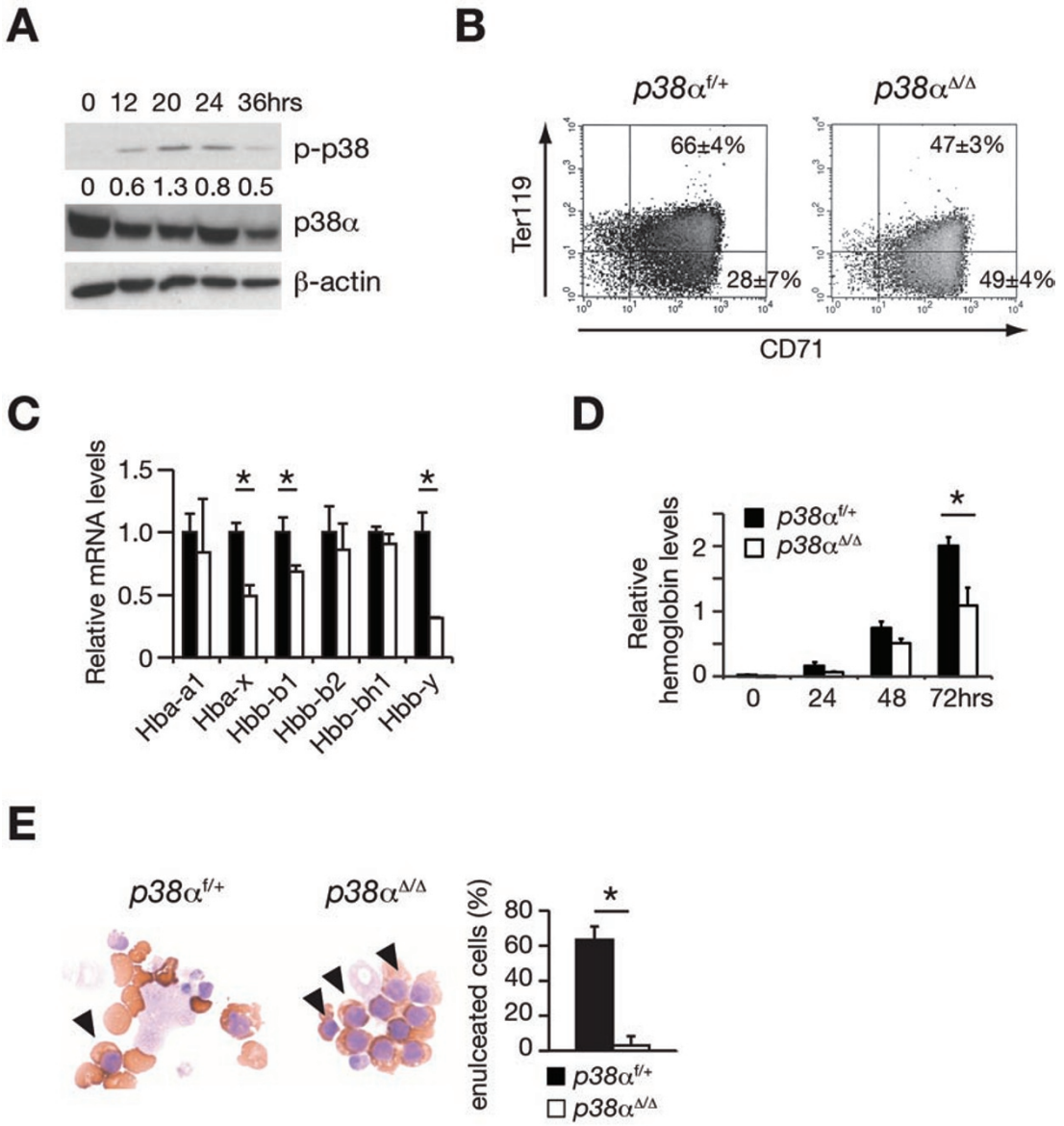

Figure 1 p38α kinase activity is essential in erythroblast enucleation *ex vivo*. **(A)** Levels of phosphorylated p38α and total p38α protein were analyzed by western blot in FLEPs during the course of differentiation. β-actin was used as a loading control. **(B)** FACS analysis of *p38*$\alpha^{f/+}$ and *p38*$\alpha^{\Delta/\Delta}$ erythroblasts in differentiating cultures using Ter119 and CD71 labeling. $P < 0.05$, *t*-test. **(C)** Relative mRNA levels of globin genes were determined by qRT-PCR in FLEPs after 48 h of differentiation. **(D)** Total hemoglobin levels in erythroblasts were measured by colorimetric assay. **(E)** *p38*$\alpha^{f/+}$ and *p38*$\alpha^{\Delta/\Delta}$ FLEPs were differentiated in culture for 48 h and stained by neutral benezidine and Giemsa staining. The percentage of enucleated cells to total cells in the culture was quantified. **P* < 0.05, *t*-test.

in vivo, we analyzed fetal erythroid erythropoiesis in *p38*$\alpha^{\Delta/\Delta}$ mice. Hematocrit analysis of E14.5 fetuses showed comparable hematocrit values in *p38*$\alpha^{\Delta/\Delta}$ and control embryos, implying that loss of p38α does not affect the overall erythroid homeostasis (Supplementary information, Figure S2A). Remarkably, nucleated erythrocytes were present in the peripheral blood of *p38*$\alpha^{\Delta/\Delta}$ mice after birth, but not in the peripheral blood of *p38*$\alpha^{f/+}$ control mice (Figure 3A and Supplementary information, Figure S2B). Nucleated erythrocytes contained highly pyknotic nuclei and expressed hemoglobin as shown by benzidine staining. Morphologically, these cells were similar to *p38*$\alpha^{\Delta/\Delta}$ FLEPs at late time points of differentiation. Moreover, FACS analysis showed that the $Ter119^{high}CD71^{low}$ population of mature erythrocytes was reduced in *p38*$\alpha^{\Delta/\Delta}$ fetal livers (Figure 3B). These data in-

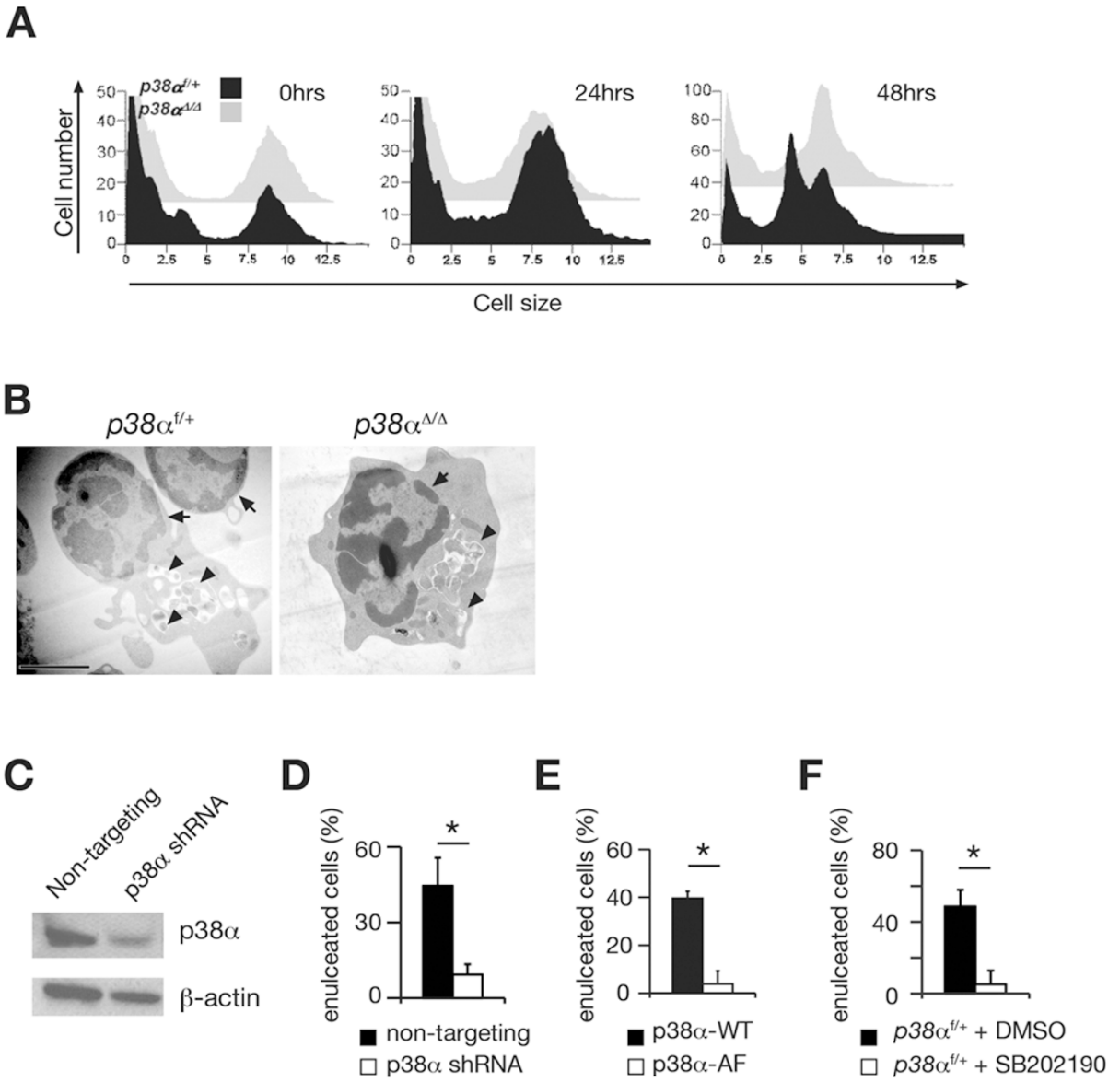

Figure 2 Deregulated differentiation in p38α-deficient erythroblasts. **(A)** Cell size profiles of *p38*$\alpha^{f/+}$ and *p38*$\alpha^{\Delta/\Delta}$ FLEPs after 0, 24 and 48 h of differentiation. Note that a large population of *p38*$\alpha^{f/+}$ FLEPs shifted to 4 μm after 48 h of differentiation, while *p38*$\alpha^{\Delta/\Delta}$ FLEPs were arrested at a size of 7 μm. **(B)** *p38*$\alpha^{f/+}$ and *p38*$\alpha^{\Delta/\Delta}$ erythroblasts were harvested at 48 h of differentiation and analyzed under transmission electron microscope. In both types of cells, nucleuses were condensed (arrows) and autophagosomes (arrowheads) were formed. Note that the nucleus is being extruded in the *p38*$\alpha^{f/+}$ erythroblasts, which is blocked in *p38*$\alpha^{\Delta/\Delta}$ erythroblasts. **(C)** The efficiency of lentivirus-mediated p38α knockdown was determined by western blot. **(D)** Lentiviral p38α knockdown caused a significant decrease of enucleated cell numbers during differentiation. **(E)** Erythroblasts were infected with retroviruses carrying wild-type p38α (p38α-WT) or dominant kinase-dead p38α mutant (p38α-AF). The percentage of enucleated cells was quantified in the culture. **(F)** Addition of the selective p38α inhibitor SB202190 (1 μM) to differentiating wild-type erythroblasts resulted in a decrease of enucleated cell numbers at 48 h. The percentage of enucleated cells was quantified. *$P < 0.05$, *t*-test.

dicate that fetal erythroblasts lacking p38α are defective in enucleation and terminal differentiation *in vivo*.

Since *p38*$\alpha^{\Delta/\Delta}$ mice die a few days after birth [19], we generated a mouse line with hematopoietic-specific deletion of *p38*α (*Vav-cre, p38*$\alpha^{f/f}$: *p38*$\alpha^{\Delta blood}$) to analyze the function of p38α in enucleation during adult erythropoiesis. *p38*$\alpha^{\Delta blood}$ mice were viable and born with Mendelian frequency. *p38α* was efficiently deleted in hematopoietic

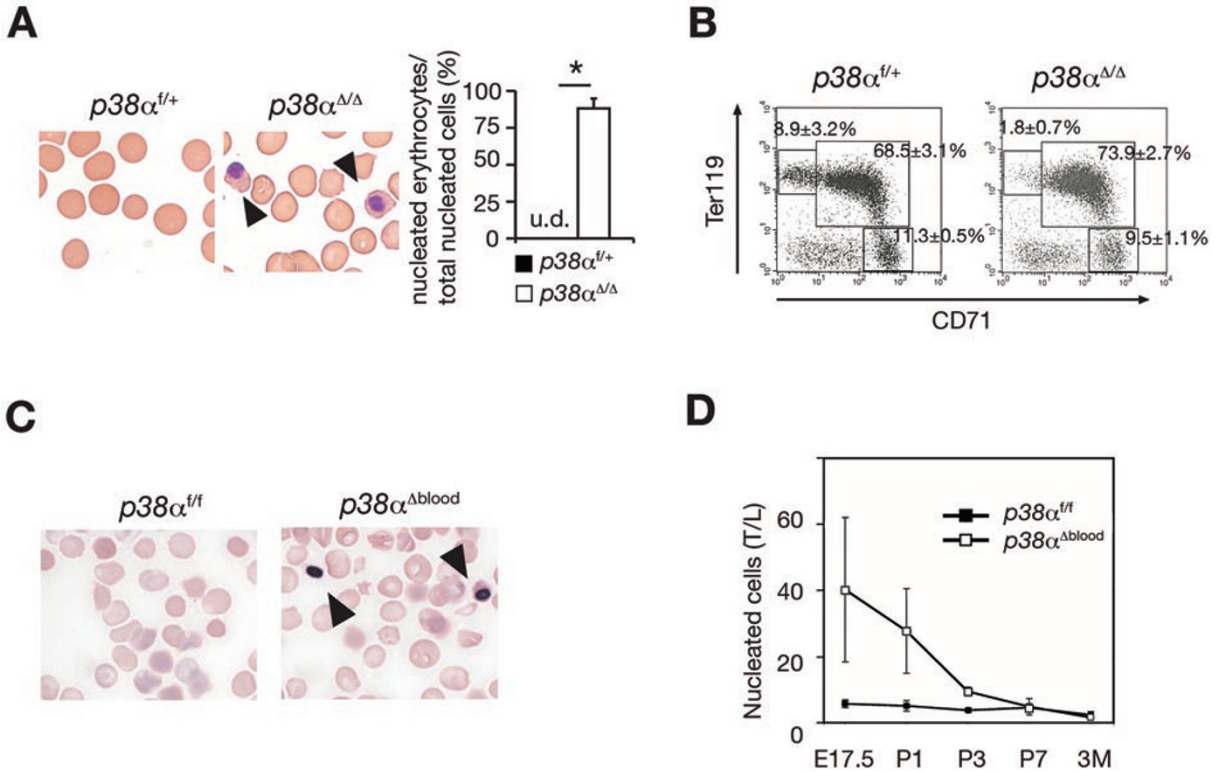

Figure 3 p38α regulates fetal erythroblast enucleation. **(A)** Neutral benzidine and Giemsa staining of peripheral blood smears from *p38*$\alpha^{f/+}$ and *p38*$\alpha^{\Delta/\Delta}$ newborns at postnatal day 0 (P0). Mature erythrocytes appear as yellow or brown cells without nucleus. Arrowheads indicate nucleated erythrocytes containing dark blue-stained nucleus. Erythroblasts were quantified as the percentage of total nucleated cells in peripheral blood. **(B)** Fetal liver cells from E17.5 fetuses were stained for Ter119 and CD71, and analyzed by FACS. $P < 0.05$, *t*-test. **(C)** Wright-Giemsa staining of peripheral blood smears of *p38*$\alpha^{f/f}$ and *p38*$\alpha^{\Delta blood}$ newborns at P0. Arrowheads indicate nucleated erythrocytes. **(D)** The numbers of nucleated cells in peripheral blood of *p38*$\alpha^{f/f}$ and *p38*$\alpha^{\Delta blood}$ fetuses at E17.5 and mice at P1, P3, P7 and 3 months (3M) of age was quantified by total blood count. *$P < 0.05$, *t*-test.

cells as determined by RT-PCR (Supplementary information, Figure S3A). Both *p38*$\alpha^{\Delta blood}$ fetuses and newborns displayed nucleated erythrocytes in peripheral blood and livers (Figure 3C and data not shown), indicating that impaired enucleation is due to *p38α* deletion in hematopoietic cells, but not in other cell types. Notably, the number of nucleated erythrocytes in peripheral blood of *p38*$\alpha^{\Delta blood}$ mice declined to levels of control mice a few days after birth (Figure 3D). These data show that p38α is required for erythroid enucleation in fetal definitive erythropoiesis, but not in adult definitive erythropoiesis under normal conditions.

p38α is required for enucleation during anemia-induced stress erythropoiesis

The *ex vivo* differentiation of FLEPs and the fetal definitive erythropoiesis are both characterized as stress erythropoiesis that is stimulated by high levels of Epo [2, 7, 15, 16]. We therefore analyzed whether p38α would be required for enucleation during stress erythropoiesis in adult mice. Phenylhydrazine (PHZ)-induced hemolytic anemia was employed as a model system for stress erythropoiesis, in which the spleen becomes the major erythropoietic organ to restore erythroid homeostasis. Importantly, phosphorylation of p38α was significantly increased in erythroblasts obtained from PHZ-treated spleens and fetal livers at E14.5 as shown by FACS analysis (Supplementary information, Figure S4). Strikingly, upon PHZ treatment, nucleated erythrocytes appeared in the peripheral blood of *p38*$\alpha^{\Delta blood}$ mice, but not in controls (Figure 4A and Supplementary information, Figure S2C). The numbers of nucleated erythrocytes were also found significantly increased in *p38*$\alpha^{\Delta blood}$ splenocyte cytospins (Figure 4B). FACS analysis further revealed that the number of mature Ter119^{high}CD71^{low} erythrocytes was decreased in *p38*$\alpha^{\Delta blood}$ spleens (Figure 4C). Interestingly, these nucleated erythrocytes disappeared from peripheral blood and spleen upon the resolution of anemia in these

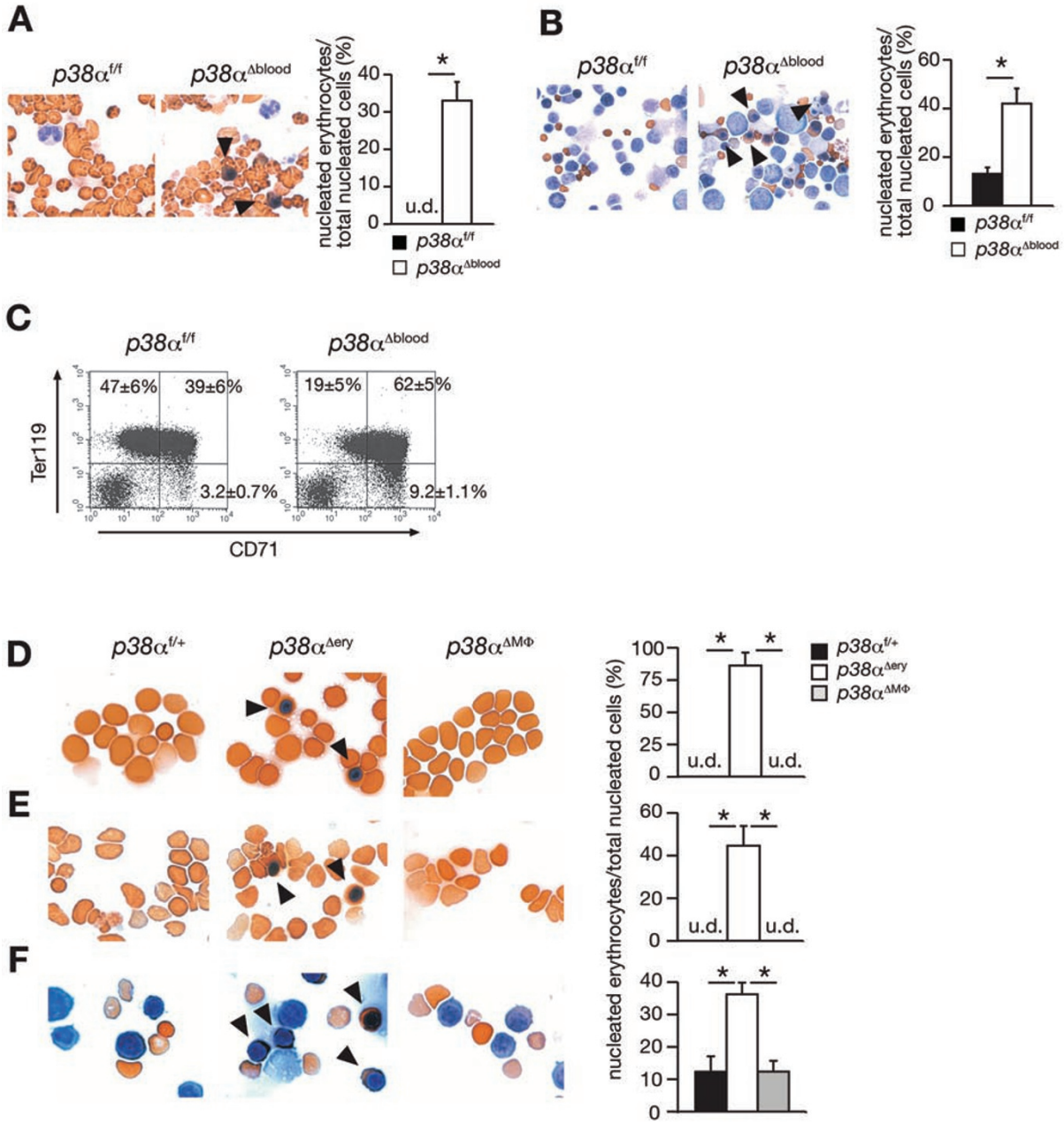

Figure 4 p38α regulates enucleation of stress erythropoiesis in a cell-autonomous manner. **(A**, **B)** Neutral benzidine and Giemsa-stained peripheral blood smears **(A)** and splenocyte cytospins **(B)** of *p38*$\alpha^{f/f}$ and *p38*$\alpha^{\Delta blood}$ adult mice at day 3 of PHZ-induced anemia. Arrowheads indicate nucleated erythrocytes. Nucleated erythrocytes were quantified as the percentage of total nucleated cells in peripheral blood or spleens. **(C)** Splenocytes from *p38*$\alpha^{f/f}$ and *p38*$\alpha^{\Delta blood}$ mice were analyzed by FACS after Ter119 and CD71 antibody staining at day 3 post PHZ treatment. $P < 0.05$, *t*-test. **(D)** Benzidine staining of peripheral blood smears of *p38*$\alpha^{f/f}$, *p38*$\alpha^{\Delta ery}$ and *p38*$\alpha^{\Delta m\Phi}$ newborns at P0. Peripheral blood smears **(E)** and splenocyte cytospins **(F)** from PHZ-treated *p38*$\alpha^{f/f}$, *p38*$\alpha^{\Delta ery}$ and *p38*$\alpha^{\Delta m\Phi}$ adult mice were stained with benzidine. Arrowheads indicate nucleated erythrocytes. Nucleated erythrocytes were quantified as the percentages of total nucleated cells in peripheral blood or spleens of these mice. *$P < 0.05$, *t*-test.

mice (data not shown). These data show that p38α is also required in adult mice for erythroblast enucleation during anemia-induced stress erythropoiesis.

p38α regulates enucleation in a cell-autonomous manner

Enucleation of erythroblasts is facilitated by several different types of cells, especially by macrophages in

the erythroblastic island [1]. In order to analyze whether impaired enucleation of erythroblasts is cell-autonomous or dependent on macrophages, we generated mouse lines with *p38α* deletion specifically in erythrocytes or macrophages, designated as *p38*$\alpha^{\Delta ery}$ (*ErGFPcre*, *p38*$\alpha^{f/f}$) and *p38*$\alpha^{\Delta mΦ}$ (*Lysm-cre*, *p38*$\alpha^{f/f}$), respectively. Both mouse lines were viable and born with Mendelian frequency. Efficient deletion of *p38α* in these two mouse lines was confirmed using purified erythroid cells and macrophages (Supplementary information, Figure S3B and S3C).

Notably, nucleated erythrocytes were only found in the peripheral blood of *p38*$\alpha^{\Delta ery}$ newborns, whereas *p38*$\alpha^{\Delta mΦ}$ newborns displayed normal enucleated erythrocytes (Figure 4D and Supplementary information, Figure S2D). Moreover, the clearance of nucleated erythrocytes in peripheral blood of *p38*$\alpha^{\Delta ery}$ mice showed similar dynamics as that in *p38*$\alpha^{\Delta blood}$ mice after birth (data not shown). We next treated adult *p38*$\alpha^{\Delta ery}$ and *p38*$\alpha^{\Delta mΦ}$ mice with PHZ to define the cell type responsible for the defective enucleation in anemia. Nucleated erythrocytes were observed in peripheral blood of *p38*$\alpha^{\Delta ery}$ adult anemic mice, but not in that of *p38*$\alpha^{\Delta mΦ}$ mice (Figure 4E and Supplementary information, Figure S2E). Moreover, splenocyte cytospins showed that the number of nucleated erythrocytes was increased in *p38*$\alpha^{\Delta ery}$ mice after PHZ treatment (Figure 4F). These data indicate that p38α regulates erythroblast enucleation in a cell-autonomous manner during fetal and anemic stress erythropoiesis.

p38α controls erythroid enucleation through activating the p21-Rb pathway

It was previously reported that p38α suppresses cell proliferation and differentiation via attenuating JNK activation [19, 21]. We found that JNK phosphorylation was also increased in differentiating *p38*$\alpha^{\Delta/\Delta}$ FLEPs (Supplementary information, Figure S5A). However, the JNK inhibitor SP600125 did not rescue impaired enucleation of *p38*$\alpha^{\Delta/\Delta}$ FLEPs (Supplementary information, Figure S5B).

To characterize the molecular mechanisms through which p38α regulates enucleation, we performed a gene expression profile analysis by comparing *p38*$\alpha^{f/+}$ and *p38*$\alpha^{\Delta/\Delta}$ FLEPs during differentiation. The Gene Set Enrichment Analysis (GSEA) software was applied to analyze whether expression profiles of gene sets with defined function were deregulated [29]. GSEA analysis showed that a set of genes enriched in hematopoietic progenitor cells was significantly upregulated in *p38*$\alpha^{\Delta/\Delta}$ FLEPs (Supplementary information, Figure S5C), whereas expression levels of genes enriched in mature hematopoietic cells were reduced (Supplementary information, Figure S5D). Moreover, we found that expression levels of the erythroid differentiation transcription factors Gata1 and C/EBPα (Supplementary information, Figure S5E and S5F) and downstream targets of these two genes (Supplementary information, Figure S5G and S5H) were substantially decreased in *p38*$\alpha^{\Delta/\Delta}$ FLEPs. These data further indicate that terminal differentiation of *p38*$\alpha^{\Delta/\Delta}$ FLEPs is impaired.

Markedly, GSEA analysis showed that genes repressed by Rb were significantly increased in *p38*$\alpha^{\Delta/\Delta}$ erythroblasts (Figure 5A). It has been reported that erythroblasts lacking Rb display attenuated hemoglobin accumulation, reduced expression of terminal differentiation markers and, importantly, impaired enucleation under stress conditions [13]. The remarkable similarities between p38α- and Rb-deficient erythroblasts led us to analyze whether the Rb pathway is deregulated in *p38*$\alpha^{\Delta/\Delta}$ erythrocytes. GSEA analysis revealed that target genes of E2F-1, a transcription factor suppressed by Rb, were significantly upregulated in *p38*$\alpha^{\Delta/\Delta}$ erythroblasts (Figure 5B). Furthermore, western blot analyses showed that Rb protein in *p38*$\alpha^{\Delta/\Delta}$ erythroblasts was hyperphosphorylated and thereby inactive (Figure 5C), with increased phosphorylation on various threonine and serine residues (Figure 5D). These results indicate that the activity of Rb is impaired in p38α-deficient erythroblasts.

It is well established that cyclin-dependent kinase inhibitors (CKIs), including p21 and p27, are key regulators of the Rb pathway. qRT-PCR and western blot assays showed that both mRNA and protein levels of p21 and p27 were significantly decreased in *p38*$\alpha^{\Delta/\Delta}$ FLEPs (Figure 6A and 6B). Consistent with decreased p21 and p27 levels and hyperphosphorylated Rb, cell cycle exit of *p38*$\alpha^{\Delta/\Delta}$ FLEPs also appeared to be delayed during differentiation (Figure 6C). To find out whether p21 and p27 themselves control erythroblast enucleation, we induced anemia in *p21*$^{-/-}$ and *p27*$^{-/-}$ mice by PHZ treatment. Markedly, numbers of nucleated erythrocytes were increased in *p21*$^{-/-}$ peripheral blood and spleens after PHZ treatment (Figure 6D), whereas *p27*$^{-/-}$ mice were unaffected (Supplementary information, Figure S6). Importantly, lentivirus-mediated p21 overexpression facilitated enucleation of *p38*$\alpha^{\Delta/\Delta}$ erythroblasts *ex vivo* (Figure 6E). These results suggest that p38α promotes erythroblast enucleation by activating p21 and Rb.

Discussion

Our data demonstrate for the first time that erythroid enucleation requires the activation of the stress-activated kinase p38α. We have previously reported that erythroblasts deficient in p38α display increased proliferation [19]. Using an *ex vivo* system specific for erythroblast

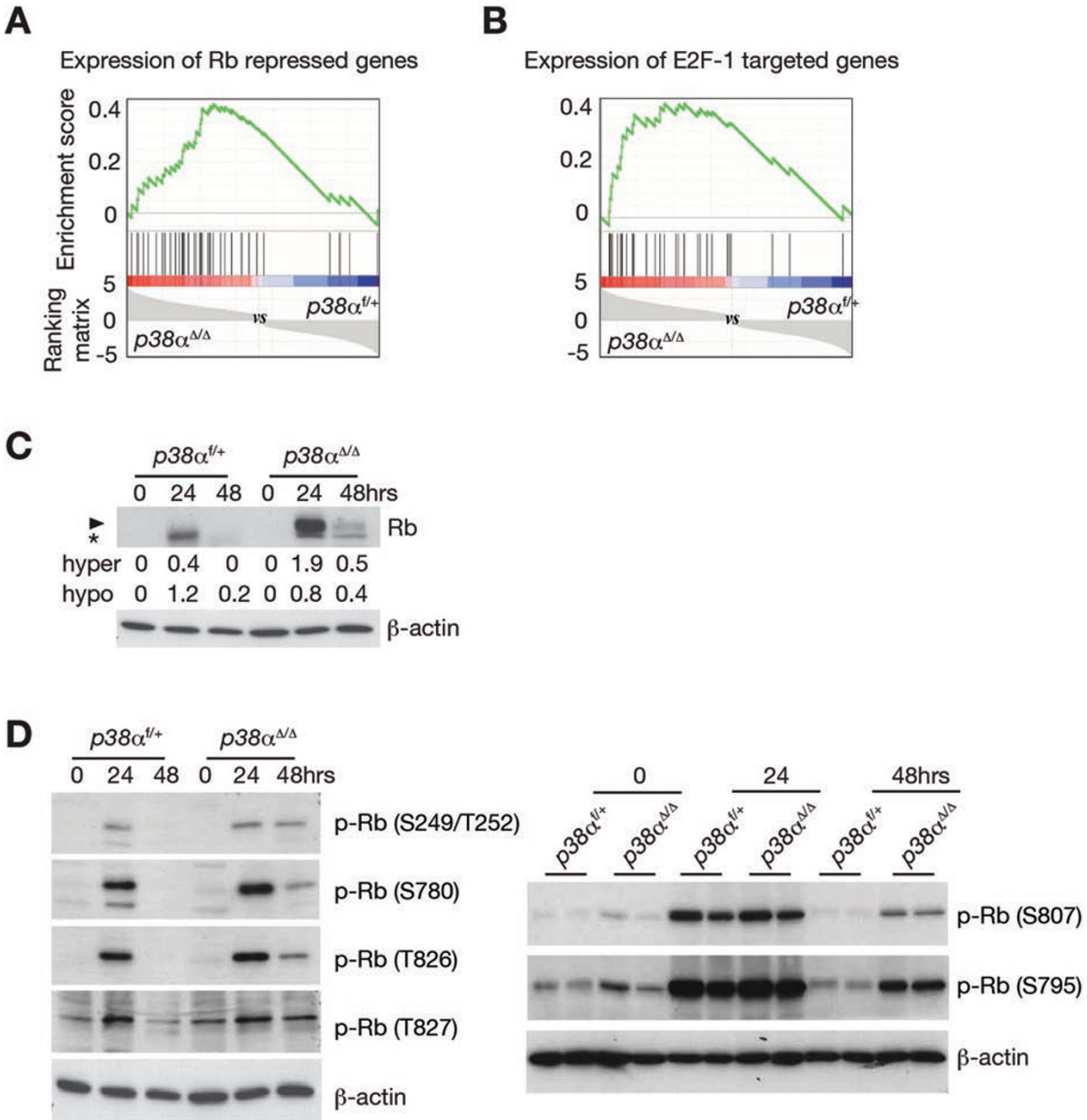

Figure 5 Erythroblasts lacking p38α show impaired Rb activation. **(A**, **B)** Gene expression profiles of *p38α*$^{f/+}$ and *p38α*$^{\Delta/\Delta}$ FLEPs at 36 h of differentiation were analyzed by GSEA. Expression profiles of genes repressed by Rb **(A)** and activated by E2F-1 **(B)** were analyzed by GSEA. **(C)** Total Rb levels were determined by western blot. The hyper- and hypo-phosphorylated Rb bands are indicated by the arrowhead and asterisk, respectively. Quantification of hyper- and hypo-phosphorylated Rb is shown. **(D)** Phosphorylation of Rb at various sites was determined by western blot during FLEP differentiation.

differentiation, we demonstrate that impaired enucleation is not due to enhanced proliferation, as p38α-deficient erythroblasts differentiate into orthochromatic erythroblasts, but fail in the terminal step of enucleation, even if differentiated for an extended time for 5 days (Supplementary information, Figure S1A). p38α deficiency delays erythroid differentiation in several aspects, as reflected by altered expression of transcription factors, erythroid markers and hemoglobin. Nevertheless, p38α-deficient erythroblasts enter the terminal differentiation stage, including cell size reduction and formation of autophagosomes. Strikingly, erythroblasts lacking p38α

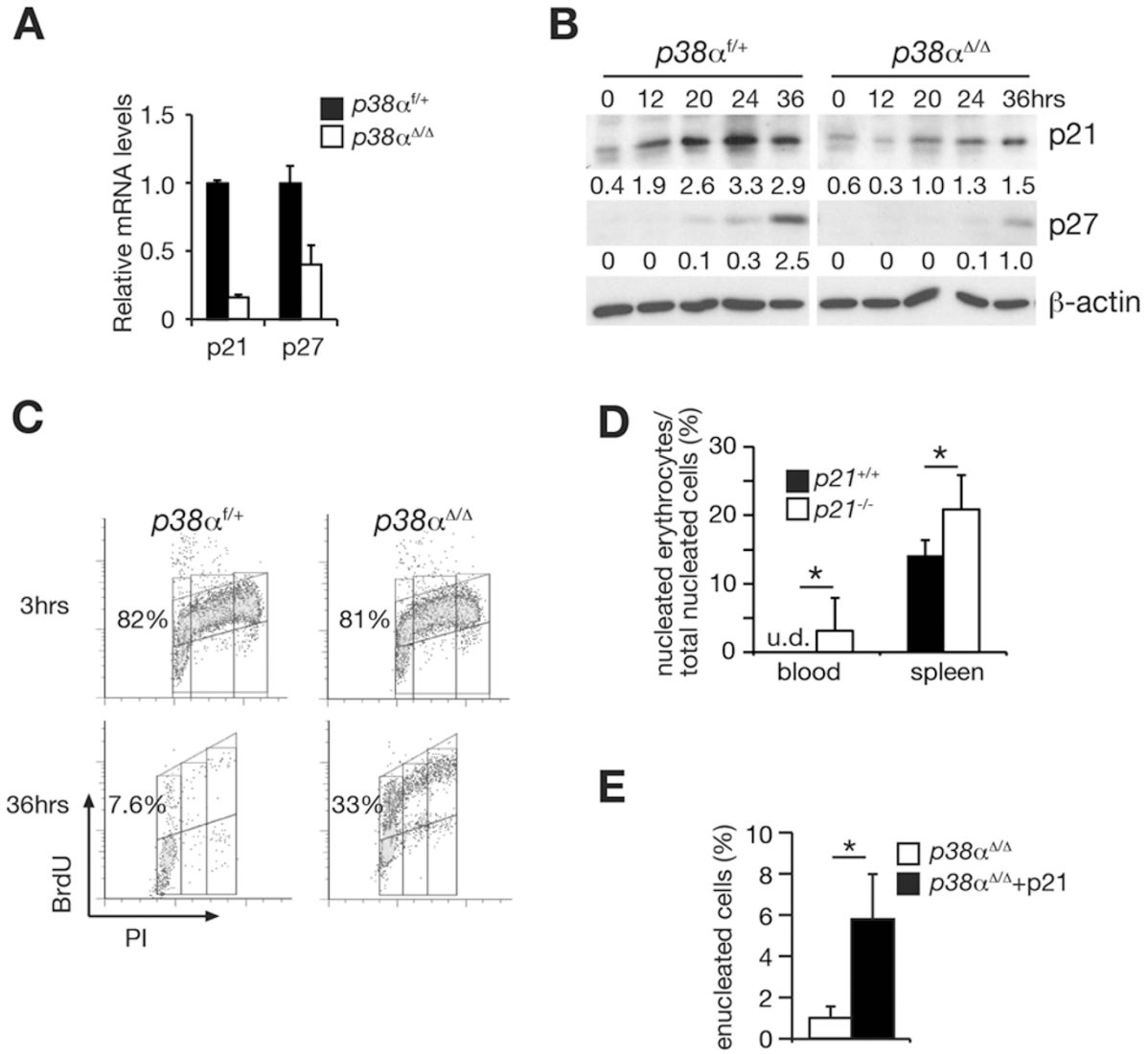

Figure 6 p38α regulates p21 during erythroblast enucleation. **(A)** p21 and p27 mRNA levels were measured by qRT-PCR in *p38*$\alpha^{\Delta/\Delta}$ FLEPs at 48 h of differentiation. **(B)** Protein levels of p21 and p27 were measured by western blot in FLEPs during differentiation. Quantification is shown in numbers. **(C)** Cell cycle profiles of one paired *p38*$\alpha^{f/+}$ and *p38*$\alpha^{\Delta/\Delta}$ FLEPs were analyzed by BrdU and PI double staining at 3 and 36 h of differentiation. Quantification of BrdU-positive cells is shown. **(D)** Nucleated erythrocytes were quantified in the peripheral blood and spleens of PHZ-treated *p21*$^{-/-}$ mice. **(E)** Overexpression of p21 by lentiviral infection increased the percentage of enucleated erythrocytes of *p38*$\alpha^{\Delta/\Delta}$ FLEPs. **P* < 0.05, *t*-test.

kinase showed almost a complete block in enucleation. Nucleated erythrocytes found in p38α-deficient mice are smaller than primitive erythrocytes, which eventually undergo enucleation [30]. Moreover, our previous studies showed that only definitive erythroblasts are generated in the *ex vivo* differentiation system [15, 16]. Therefore, nucleated erythrocytes found in p38α-deficient mice are unlikely to be primitive erythroid cells.

Engulfment of pyknotic nucleuses by macrophages is important in the completion of enucleation [1]. Using mouse lines with *p38α* deletion specific in the erythroid lineage or in the macrophage lineage, we unambiguously show a cell-autonomous function of p38α in enucleation. Chromosome condensation and autophagy-dependent organelle degradation are coupled with nucleus extrusion in erythoblasts during terminal differentiation [1-3, 11, 12]. Electron microscopy confirmed that p38α-deficient erythroblasts formed condensed chromatin and autophagosomes to a similar extent as controls, while nuclear extrusion was specifically blocked in these cells.

A recent study suggested that vesicle trafficking plays a role in erythroblast enucleation [10]. However, as p38α-deficient erythroblasts established autophagosomes, it is unlikely that p38α controls enucleation through promoting vacuole transportation and formation.

Furthermore, we found that p38α is required for enucleation only during fetal and anemic stress erythropoiesis, but not during erythropoiesis under normal conditions. In comparison to adult steady-state erythropoiesis, levels of Epo and other factors are dramatically increased under various stresses [7]. High levels of Epo were previously shown to enhance the activation of p38α [6, 22]. In agreement with these reports, p38α phosphorylation levels were increased in FLEPs, fetal livers, as well as anemic spleens (Figure 1A and Supplementary information, Figure S4). Epo stimulation induces substantial expression change of transcription factors necessary for erythroblast differentiation, including Gata1, C/EBPα, Gfi1, Gata2 and Fli1 [3]. It was also reported that p38α can regulate the activities of these key transcription factors, such as Gata1 and C/EBPα, through direct phosphorylation [31, 32]. Indeed, levels of these transcription factors and their target genes were found deregulated in p38α-deficient erythroblasts (Supplementary information, Figure S5 and data not shown), indicating that p38α is a key molecule converting extrinsic signals into intracellular responses during stress erythropoiesis.

The defective enucleation and differentiation of erythroblasts are highly similar between p38α- and Rb-deficient cells. Several studies described that Rb-deficient erythroblasts completely fail to extrude nuclei and display immature erythroid cell markers and impaired cell cycle exit *in vitro* [33, 34]. Moreover, Rb-deficient erythroblasts have impaired enucleation during fetal development and adult stress erythropoiesis. Importantly, we found that Rb phosphorylation levels and pathways downstream of Rb, e.g., E2F-1 and its target genes, were deregulated in erythroblasts lacking p38α. Previously, it was shown that knocking out *E2f-2* in Rb-deficient erythroblasts led to premature cell cycle exit and rescued the enucleation defect [35]. Apparently, the mechanism by which Rb signaling controls the process of enucleation remains to be further characterized. Our data indicate that the activation of the Rb pathway is dependent on p38α. However, it is unlikely that p38α directly regulates Rb phosphorylation, since Rb phosphorylation was increased in p38α-deficient cells. p21 and p27 levels were found to be reduced in differentiating erythroblasts lacking p38α. Strikingly, our data showed that p21 is also involved in enucleation during stress erythropoiesis. Therefore, these results suggest that p38α modulates Rb activity likely through increasing p21 expression, which is a well-characterized regulator of the Rb pathway [36, 37]. Taken together, the impaired enucleation of p38α-deficient erythroblasts could be explained by the deregulated p21 and Rb activities. Given that p38α regulates differentiation of other types of cells, such as skin epithelial cells, it would be of interests to characterize whether p21 and Rb are mediators of p38α signaling during differentiation in general.

Materials and Methods

Mice

Mice with *p38α*-floxed alleles ($p38\alpha^{f/f}$) and *MORE-cre*, $p38\alpha^{f/f}$ ($p38\alpha^{\Delta/\Delta}$), were described previously [19]. The Vav-cre, $p38\alpha^{f/f}$ ($p38\alpha^{\Delta blood}$); *LysM-cre*, $p38\alpha^{f/f}$ ($p38\alpha^{\Delta m\Phi}$); and *ErGFPcre*, $p38\alpha^{f/f}$ ($p38\alpha^{\Delta ery}$) mice, were generated by crossing $p38\alpha^{f/f}$ mice to *Vav-cre*, *LysM-cre* and *ErGFPcre* mice, respectively. The genetic background of the intercrosses was C57Bl6/J × 129sv. Deletion efficiency was confirmed by PCR on cDNA or genomic DNA.

PHZ-induced hemolytic anemia

PHZ was purchased from Sigma-Aldrich. Adult mice were injected intraperitoneally with 60 mg/kg PHZ or PBS at day 0 and day 1. On day 3 or day 6, blood samples from the tail vein were collected in EDTA-treated tubes. Blood smears were prepared using standard protocols. To obtain single cell suspension, spleens were squeezed through cell strainer and bone marrow cells were passed through a 27-gauge needle.

Erythroblasts expansion and differentiation culture

FLEPs were obtained from fetal livers isolated at E12.5 to 13.5 as described [15, 16]. Mouse embryonic stem cell-derived erythroid progenitors (ESEPs) were generated as described previously [15, 16]. FLEP and ESEP expanding cultures were daily adjusted to a concentration of 2×10^6 cells/ml in serum-free medium (StemPro34 plus nutrient supplement, Gibco/BRL), plus human recombinant Epo at 2 U/ml (Janssen-Cilag AG, Baar, Switzerland), murine recombinant Kit-ligand at 100 ng/ml (R&D Systems, Minneapolis, MN, USA), 10 μM dexamethasone (Sigma), and 40 ng/ml insulin-like growth factor 1 (Promega, Madison, WI, USA). For differentiation of FLEPs and ESEPs, dead and spontaneously differentiated cells were removed by Ficoll (Eurobio, France) purification and washed twice with PBS. Purified erythroblasts were cultivated at 2×10^6 cells/ml in StemPro34 plus nutrient supplement, supplemented with 10 U/ml Epo, 10 ng/ml insulin (Actrapid HM; Novo Nordisk), 30 μM glucocorticoid receptor antagonist ZK112.993 and 1 mg/ml iron-saturated human transferrin (Sigma). Cell number and size was determined using an electronic cell counter (CASY-1; Schärfe-System, Germany).

Lentiviral infection

p21 was cloned into pWPI lentiviral vector (Addgene). Lentiviral particles were produced as described before, using Lipofectamine (Invitrogen) and Plus Regent for transfection, following manufacturer's instructions. For infection, at day 0, erythroblasts were pelleted and resuspendend in viral supernatant containing 8 μg/ml polybrene at a concentration of 4×10^6 cells/ml. To enhance infection rate, erythroblasts with viral supernatant were spun for

30 min at 1 200 rpm in round bottom tubes. Erythroblasts were resuspended and adjusted to 2×10^6 cells/ml with erythroblasts proliferation medium. At day 1, erythroblasts were adjusted to 4×10^6 cells/ml using same volume of viral supernatant as used at day 0, and then diluted to 2×10^6 cells/ml with erythroblasts proliferation medium.

Histology

Blood smears and cytospins were benzidine-stained as described before, or stained with Wright-Giemsa staining according to the manufacturer's protocol (Sigma). Total hemoglobin contents were quantified by photometric assay using triplicate determinations, which were averaged and normalized to cell number and cell volume.

FACS analysis

Hematopoietic cells isolated from fetal livers and splenocytes were stained with mature erythrocyte marker Ter119 and erythroblast marker CD71 after treatment with ACK lysis buffer. Surface marker staining of erythroblasts was performed as described before [15, 16]. Ter119-PE and CD71-APC monoclonal antibodies were purchased from BD Biosciences. p-p38α antibody were purchased from Cell Signaling. FACS analysis of nuclear p-p38α was performed following the protocol provided by Cell Signaling. Bromodeoxyuridine (BrdU) and propidium iodide (PI) double staining was performed following manufacturer's instructions from BD Biosciences. 100 μM BrdU was added to differentiation culture 3 h before designated points of time. BrdU and anti-BrdU-FITC were purchased from BD Biosciences. PI was purchased from Sigma-Aldrich. FACS analysis was performed using BD Calibur Flow Cytometer (BD Biosciences) and analyzed with the CellQuest (BD Biosciences) or FlowJo (FlowJo) softwares.

Western blot analysis

Western blot analyses were performed using standard protocols (Amersham Biosciences). We used antibodies against the following proteins: p38α and p-p38α (Cell Signaling), p21 and Rb (BD Biosciences), p27 (Santa Cruz Biotechnology).

Gene chip hybridization and statistical analysis of data

The mouse microarray contained a set of 17 000 verified mouse cDNA clones printed onto polylysine-coated slides. Approximately 5 mg of total RNA from $p38\alpha^{f/+}$ and $p38\alpha^{\Delta/\Delta}$ FLEP cultures at 0, 24, 36 h of differentiation was primed in water with 1 μl of oligo Target Amp T7-oligo (dT) primer at 65 °C for 5 min. Reverse transcription was performed in a mixture containing first-strand reaction buffer provided with SuperScriptII (Invitrogen) for 30 min at 50 °C. Second-strand DNA synthesis was carried out using Taq polymerase (Fermentas) at 65 °C for 10 min. Amplification of RNA and amino allyl incorporation was performed using Amino Allyl MessageAmp aRNA Amplification kit (Ambion). cDNA was generated by incubation for 2 h with SuperscriptII at 42 °C. Remaining RNA was digested using RNase H. cDNA was purified (Qiaquick-kit) and dried in speedvac and probes were labeled with a reactive fluorescent dye from Molecular Probes (Alexa 555 and 647). The Cy3-dUTP and labeled probes were pooled and precipitated with blocking solution containing mouse Cot1 DNA at 80 °C for 20 min. The probe was then denatured at 94 °C for 1 min, followed by prehybridization at 50 °C for 1 h. It was then added to the microarray slides and incubated at 50 °C overnight. After the hybridization, the slides were scanned with a Gene Pix 4060 scanner. Analysis was performed using Gene Pix Pro 4.1. Raw expression data were normalized to $p38\alpha^{f/+}$ at 0 h of differentiation. Fold changes and *P*-values were calculated (ANOVA, $P < 0.05$). Technical repeats were averaged and further analyzed by GSEA software; *t*-test was applied for other statistics in the study.

Quantitative real-time PCR

Total RNA was isolated from FLEPs using TRIZOL (Invitrogen), following the manufacturer's instructions. cDNA synthesis was performed with the Ready-To-Go You-Prime-It First-Strand Beads (Amersham Biosciences). qRT-PCR reactions were performed using SYBR Green (Molecular Probes) on an Opticon2 Monitor Fluorescence Thermocycler (MJ Research).

Statistics

All data are presented as mean + sd. Data were subjected to Student's *t*-test for statistical significance ($P < 0.05$). At least three independent biological samples or repeated experiments were used for each analysis unless indicated.

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References

1 Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. *Blood* 2008; **112**:470-478.
2 Palis J. Ontogeny of erythropoiesis. *Curr Opin Hematol* 2008; **15**:155-161.
3 Richmond TD, Chohan M, Barber DL. Turning cells red: signal transduction mediated by erythropoietin. *Trends Cell Biol* 2005; **15**:146-155.
4 Witthuhn BA, Quelle FW, Silvennoinen O, *et al.* JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* 1993; **74**:227-236.
5 Nagata Y, Nishida E, Todokoro K. Activation of JNK signaling pathway by erythropoietin, thrombopoietin, and interleukin-3. *Blood* 1997; **89**:2664-2669.
6 Jacobs-Helber SM, Ryan JJ, Sawyer ST. JNK and p38 are activated by erythropoietin (EPO) but are not induced in apoptosis following EPO withdrawal in EPO-dependent HCD57 cells. *Blood* 2000; **96**:933-940.
7 Socolovsky M. Molecular insights into stress erythropoiesis.

Curr Opin Hematol 2007; **14**:215-224.
8 Kawane K, Fukuyama H, Kondoh G, *et al.* Requirement of DNase II for definitive erythropoiesis in the mouse fetal liver. *Science* 2001; **292**:1546-1549.
9 Ji P, Jayapal SR, Lodish HF. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. *Nat Cell Biol* 2008; **10**:314-321.
10 Keerthivasan G, Small S, Liu H, Wickrema A, Crispino JD. Vesicle trafficking plays a novel role in erythroblast enucleation. *Blood* 2010; **116**:3331-3340.
11 Sandoval H, Thiagarajan P, Dasgupta SK, *et al.* Essential role for Nix in autophagic maturation of erythroid cells. *Nature* 2008; **454**:232-235.
12 Schweers RL, Zhang J, Randall MS, *et al.* NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc Natl Acad Sci USA* 2007; **104**:19500-19505.
13 Walkley CR, Sankaran VG, Orkin SH. Rb and hematopoiesis: stem cells to anemia. *Cell Div* 2008; **3**:13.
14 Hebiguchi M, Hirokawa M, Guo YM, *et al.* Dynamics of human erythroblast enucleation. *Int J Hematol* 2008; **88**:498-507.
15 Dolznig H, Kolbus A, Leberbauer C, *et al.* Expansion and differentiation of immature mouse and human hematopoietic progenitors. *Methods Mol Med* 2005; **105**:323-344.
16 Carotta S, Pilat S, Mairhofer A, *et al.* Directed differentiation and mass cultivation of pure erythroid progenitors from mouse embryonic stem cells. *Blood* 2004; **104**:1873-1880.
17 Hui L, Bakiri L, Stepniak E, Wagner EF. p38alpha: a suppressor of cell proliferation and tumorigenesis. *Cell Cycle* 2007; **6**:2429-2433.
18 Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer* 2009; **9**:537-549.
19 Hui L, Bakiri L, Mairhorfer A, *et al.* p38alpha suppresses normal and cancer cell proliferation by antagonizing the JNK-c-Jun pathway. *Nat Genet* 2007; **39**:741-749.
20 Ventura JJ, Tenbaum S, Perdiguero E, *et al.* p38alpha MAP kinase is essential in lung stem and progenitor cell proliferation and differentiation. *Nat Genet* 2007; **39**:750-758.
21 Perdiguero E, Ruiz-Bonilla V, Gresh L, *et al.* Genetic analysis of p38 MAP kinases in myogenesis: fundamental role of p38alpha in abrogating myoblast proliferation. *EMBO J* 2007; **26**:1245-1256.
22 Nagata Y, Moriguchi T, Nishida E, Todokoro K. Activation of p38 MAP kinase pathway by erythropoietin and interleukin-3. *Blood* 1997; **90**:929-934.
23 Adams RH, Porras A, Alonso G, *et al.* Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Mol Cell* 2000; **6**:109-116.
24 Allen M, Svensson L, Roach M, *et al.* Deficiency of the stress kinase p38alpha results in embryonic lethality: characterization of the kinase dependence of stress responses of enzyme-deficient embryonic stem cells. *J Exp Med* 2000; **191**:859-870.
25 Mudgett JS, Ding J, Guh-Siesel L, *et al.* Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. *Proc Natl Acad Sci USA* 2000; **97**:10454-10459.
26 Tamura K, Sudo T, Senftleben U, *et al.* Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. *Cell* 2000; **102**:221-231.
27 England SJ, McGrath KE, Frame JM, Palis J. Immature erythroblasts with extensive *ex vivo* self-renewal capacity emerge from the early mammalian fetus. *Blood* 2011; **117**:2708-2717.
28 Jayapal SR, Lee KL, Ji P, *et al.* Down-regulation of Myc is essential for terminal erythroid maturation. *J Biol Chem* 2010; **285**:40252-40265.
29 Subramanian A, Tamayo P, Mootha VK, *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005; **102**:15545-15550.
30 Kingsley PD, Malik J, Fantauzzo KA, Palis J. Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. *Blood* 2004; **104**:19-25.
31 Stassen M, Klein M, Becker M, *et al.* p38 MAP kinase drives the expression of mast cell-derived IL-9 via activation of the transcription factor GATA-1. *Mol Immunol* 2007; **44**:926-933.
32 Qiao L, MacDougald OA, Shao J. CCAAT/enhancer-binding protein alpha mediates induction of hepatic phosphoenolpyruvate carboxykinase by p38 mitogen-activated protein kinase. *J Biol Chem* 2006; **281**:24390-24397.
33 Clark AJ, Doyle KM, Humbert PO. Cell-intrinsic requirement for pRb in erythropoiesis. *Blood* 2004; **104**:1324-1326.
34 Spike BT, Dirlam A, Dibling BC, *et al.* The Rb tumor suppressor is required for stress erythropoiesis. *EMBO J* 2004; **23**:4319-4329.
35 Dirlam A, Spike BT, Macleod KF. Deregulated E2f-2 underlies cell cycle and maturation defects in retinoblastoma null erythroblasts. *Mol Cell Biol* 2007; **27**:8713-8728.
36 Nabel EG. CDKs and CKIs: molecular targets for tissue remodelling. *Nat Rev Drug Discov* 2002; **1**:587-598.
37 Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta* 2002; **1602**:73-87.

(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)