Published in final edited form as:

Exp Hematol. 2009 February ; 37(2): 151–158. doi:10.1016/j.exphem.2008.10.008.

Erythropoietin-Induced Phosphorylation/Degradation of BIM Contributes to Survival of Erythroid Cells

Randolph M. Abutin^{a,*}, Jingchun Chen^{a,*}, Tina K. Lung^b, Joyce A. Lloyd^b, Stephen T. Sawyer^a, and Hisashi Harada^c

^a Department of Pharmacology/Toxicology, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA

^b Department of Human Genetics, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA

^c Department of Internal Medicine, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA

Abstract

Objective—A pro-apoptotic BH3-only protein BIM (BCL-2 interacting mediator of cell death) can link cytokine receptor signaling with the apoptotic machinery in hematopoietic cells. We investigated here the role of BIM in erythropoietin (Epo)-mediated survival in erythroid cells.

Methods—We down-regulated BIM in Epo-dependent HCD57 erythroid cells with shRNA, and used Real-time PCR, Western blots, and flow cytometry to characterize BIM expression and apoptosis. Hematologic analyses of BIM-deficient (*Bim*^{−/−}) mice were conducted.

Results—BIM expression increases in primary murine erythroid cells and HCD57 cells deprived of Epo. Whereas *Bim* mRNA increased less than 2-fold, BIM protein increased more than 10-fold after Epo withdrawal, suggesting post-transcriptional regulation of BIM. Epo treatment resulted in rapid phosphorylation of BIM at Serine 65 and phosphorylation correlated with degradation of BIM. Inhibition of ERK (extracellular signal-regulated kinase) by a MEK/ERK inhibitor, U0126, blocked both phosphorylation and degradation of BIM, resulting in apoptosis. Treatment with a proteasome inhibitor, MG-132, also blocked degradation of phosphorylated BIM. Down-regulation of BIM with the shRNA resulted in HCD57 cells more resistant to apoptosis induced by either Epo withdrawal or ERK inhibition. Although we observed no significant changes in the number of erythrocytes or reticulocytes in the circulation of *Bim*^{−/−} mice, erythroid progenitors from bone marrow in *Bim*^{−/−} mice were reduced in number and more resistant to apoptosis induced by U0126 MEK/ERK inhibitor.

Conclusion—Epo protects erythroid cells from apoptosis in part through ERK-mediated phosphorylation followed by proteasomal degradation of BIM.

Keywords

erythroid; erythropoietin; apoptosis; BIM; ERK

cTo whom all correspondence should be addressed: Hisashi Harada, Ph.D., Department of Internal Medicine, Massey Cancer Center, Virginia Commonwealth University, 401 College St., Richmond, VA 23298-0035, Phone: (804) 628-0064; Fax: (804) 827-1276; e-mail: hharada@vcu.edu.

*These authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Erythropoietin (Epo) is a hormone synthesized in the kidney in response to hypoxia that is required for the survival and proliferation of erythroid progenitors. Epo binds to its cognate receptor (EpoR) and activates pathways that include the phosphatidylinositol 3-kinase (PI3K), the RAS-extracellular signal-regulated kinase (ERK), and JAK/STAT pathways [1,2]. The BCL-2 family of proteins lies downstream of these proximal signals regulating survival/apoptosis. The BCL-2 family can be subdivided into three main groups based on regions of BCL-2 homology (BH) and function: multi-domain anti-apoptotic (e.g., BCL-2, MCL-1, BCL-X_L), multi-domain pro-apoptotic (e.g., BAX, BAK), and BH3-only pro-apoptotic (e.g., BAD, BID, BIM, PUMA) [3,4]. The BH3-only members identified to date function as upstream initiators of apoptosis. Among these members, BIM (BCL-2 interacting mediator of cell death) was identified as a BH3-only protein that induces apoptosis and can be antagonized by anti-apoptotic BCL-2 family members [5]. Studies of BIM-deficient mice/cells indicate important roles in lymphocyte homeostasis [6]. For example, BIM-deficient lymphocytes are resistant to certain apoptotic stimuli such as cytokine withdrawal [7]. BIM activity can be modulated at either transcriptional or post-translational level [6,8]. Transcriptional control of BIM involves contributions from Jun N-terminal kinase (JNK), PI3K, and Ras-ERK pathways. The presence of growth factors induces survival pathways including PI3K/AKT, which phosphorylates FOXO transcription factors and mediates their translocation from nucleus to cytosol, resulting in down-regulation of the *Bim* transcription [9]. AP1/c-JUN transcription factor is also involved in *Bim* expression in neuronal cells [10].

It has been reported that phosphorylation and ubiquitination of BIM can regulate its protein level [8]. We have shown that the phosphorylation status of BIM controls its pro-apoptotic activity [11]. Interleukin-3 (IL-3), a hematopoietic survival factor, induces ERK-mediated phosphorylation of BIM on three serine sites (Ser55/Ser65/Ser100). IL-3 withdrawal results in the dephosphorylation of BIM and only non-phosphorylated BIM interacts with the multi-domain pro-apoptotic effector BAX. Phosphorylation of BIM upon exposure of cells to IL-3 dramatically reduces the BIM/BAX interaction. A non-phosphorylatable BIM molecule (S55A, S65A, S100A) demonstrates enhanced interaction with BAX and enhanced pro-apoptotic activity. Thus, ERK-dependent phosphorylation of BIM in response to survival factor regulates BIM/BAX interaction and the pro-apoptotic activity of BIM. Another post-translational regulation of BIM activity has been proposed through sequestration to a microtubular dynein motor complex by interaction with dynein light chain/LC8 [12].

We have employed the Epo-dependent murine erythroleukemic cell line, HCD57 to study the Epo signal transduction pathways [13]. Using this system, we have shown that the coordinated activation of both PI3K/AKT and JAK2/STAT5 is necessary for Epo-mediated survival [14]. Furthermore, Epo-mediated ERK activation is associated with proliferation of erythroid cells [15]. It has also been demonstrated that ERK stimulation is involved in steady state erythropoiesis through JAK2 activation [16]. Downstream of these signaling pathways, BCL-X_L, an anti-apoptotic BCL-2 family protein, has been identified as a target molecule of JAK2/STAT5 for Epo-mediated survival [17]. Since BIM plays an important role in survival through ERK-dependent pathway in a variety of survival factors such as IL-2, IL-3, or nerve growth factor (NGF)[8], we investigated here the role of BIM in Epo-mediated survival in erythroid cells.

Materials and Methods

Reagents

U0126, LY294002, MG-132, and BIM antibody were purchased from Calbiochem (La Jolla, CA). Phospho-specific antibodies against ERK (Thr202/Tyr204), Akt (Ser473), GSK3 α/β (Ser21/9) were obtained from Cell Signaling Technologies (Beverly, MA). The antibodies for actin and ERK were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-phospho BIM Ser65 was prepared as described [11]. BAD antibody was obtained from BD Pharmingen (San Diego, CA).

Cell Culture

Early passage murine HCD57 and HCD57R cells were cultured in Iscove's modified Dulbecco's medium (IMDM)(Invitrogen, Carlsbad, CA) supplemented with 25% fetal calf serum (FCS; Hyclone, Logan, UT), 10 μ g/ml gentamicin (Invitrogen), and 1.0 U/ml Epo (Amgen, Thousand Oaks, CA) at 37°C in 5% CO₂. For Epo withdrawal studies, cells were washed with IMDM three times and incubated in IMDM supplemented with 25% FCS and 10 μ g/ml gentamicin in the absence of Epo for the indicated time. For Epo stimulation, cells were deprived of Epo and stimulated with 10 U/ml Epo for the indicated time. Primary erythroid progenitors were isolated from phenylhydrazine-treated mice and were cultured as previously described [18].

Western blot analysis

Fifty micrograms of protein were subjected to Western blot analysis as previously described [14]. Proteins were detected by enhanced chemiluminescence (GE Healthcare Bio-Sciences, Piscataway, NJ).

Transfections

The myristylated/constitutively active murine *Akt1* cDNA from pTRE-ca-Akt (kindly provided by Geoffrey Krystal, Virginia Commonwealth University) was cloned into the EcoRI site of pcDNA3.1 (Invitrogen, Carlsbad, CA). pSuper-BIM was described previously [11]. These plasmids were transfected into early passage HCD57 cells using the Gene Pulser electroporator (Bio-Rad, Hercules, CA) with 250 V, 950 μ F, and 50 Ω resistance in a 0.4 cm cuvette. Forty-eight hours after transfection, the cells were cultured with either 1200 μ g/ml of G418 (ca-Akt) or 0.5 μ g/ml puromycin (pSuper-BIM) for two weeks. Stable single ca-Akt clones were selected by limiting dilution for another two weeks. Pools of cells transfected with pSuper-BIM were selected for subsequent experiments.

Determination of apoptosis in erythroid cells

Apoptosis was determined by propidium iodide staining using flow cytometry analysis. Following cell treatment, cells were fixed in 70% ethanol overnight at 4°C. The cells were then washed twice in phosphate buffered saline and stained for 3 hours in 3 mM Sodium Citrate, 2 μ M propidium iodide, and 50 μ g/ml RNase A at 4°C in the dark. Cells were analyzed by the FACScan flow cytometer (BD Biosciences, Rutherford, NJ). Sub-G0/G1, indicative of apoptosis, was gated and a percentage of the total number of cells was determined. Samples were analyzed in triplicate and error bars represent standard deviation. Statistical analysis represents the results of a two-tailed, type 1 Student's t test with $p < 0.05$ considered as significant.

Real-time PCR

Each cDNA template was made from total RNA with Superscript II reverse transcriptase kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Quantitative analysis

of cDNA amplification was assessed by incorporation of SYBR Green into double-stranded DNA. For mouse *Bim* we used 5'-CGACAGTCTCAGGAGGAACC-3' as the forward primer and 5'-CCTTCTCCATACCAGACGGA-3' as the reverse primer [19]. For mouse *GAPDH* we used 5'-ATCACTGCCACCCAGAAGAC-3' as the forward primer and 5'-CACATTGGGGTAGGAACAC-3' as the reverse primer. PCR containing 50 ng of cDNA template, 0.5 μ M each of forward and reverse primers, and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was performed in a total volume of 25 μ l. Thermal cycling conditions were as follows: initial incubation of 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 1 min at 57 °C annealing temperature, and 30s at 72 °C. All cDNA samples were tested in duplicate using the ABI PRISM 7700 Sequence Detector and analyzed with ABI Prism Sequence Detection Software (Applied Biosystems).

CFU-e Progenitor Assays

Normal 8-week-old wild-type and *Bim*^{-/-} mice (kindly provided by Stanley Korsmeyer, Dana-Farber Cancer Institute, Boston, MA)[20] were prepared. Assessment of murine CFU-e colony formation was performed as previously described [18]. Briefly, bone marrow cells from wild-type or *Bim*^{-/-} mice were added to 3 ml methylcellulose media (Methocult M3334; Stem Cell Technologies, Vancouver, BC, Canada) containing 3 U Epo/ml in the presence of 0.1% DMSO (vehicle), 1 μ M, or 5 μ M U0126. A quantity of 1.1 ml of these cells was plated in duplicate onto 35-mm plates and cultured. CFU-e was counted 2 days after plating. Data are presented as the number of CFU-e per 35-mm plate, or as the percentage of control with or without U0126.

Results

Increased expression of BIM following Epo withdrawal

Primary erythroid progenitors were obtained from the spleens of mice treated with phenylhydrazine chloride to induce anemia [21]. The spleen cells we studied were >95% proerythroblasts as determined by a high level expression of both CD71 (transferrin receptor) and TER119 (glycophorin-A)[18]. These primary cells show apoptosis only 4 hours after Epo withdrawal. In contrast, the Epo-responsive erythroid cell line HCD57 undergoes extensive apoptosis following Epo withdrawal for 24 hours. The HCD57R cell line was subcloned from a single cell from HCD57 cells. This cell line is more resistant to Epo deprivation; apoptosis starts at 36–48 hours after Epo withdrawal [14]. We first examined the expression of BIM following Epo withdrawal in these three different kinds of cells. Three major BIM isoforms generated by alternative splicing [5] have been identified in both primary erythroid cells and HCD57 cells. The extra-long (BIM_{EL}) form was predominant in these cells, so we will refer to BIM_{EL} as “BIM”. Removal of Epo from primary erythroid cells and HCD57 cells resulted in more than 10-fold increase of BIM protein prior to the onset of apoptosis (Fig. 1A and 1B). We then quantified the relative expression of *Bim* mRNA in HCD57 cells by Real-time PCR. *Bim* mRNA increased to only 1.8-fold 2 hours after Epo withdrawal (Fig. 1C). *Bim* mRNA then decreased slightly as HCD57 cells became apoptotic in the 3 to 24 hours period. This small increase in *Bim* mRNA expression two hours following Epo withdrawal was statistically significant at p=0.05. These data suggest that the increase in BIM protein is mainly regulated by post-transcriptional mechanisms (see below).

ERK-dependent BIM phosphorylation/degradation induced by Epo

It has been well established that survival/growth factors such as IL-2, IL-3 and NGF induce ERK-dependent phosphorylation of BIM [8]. In this regard, we observed an Epo-dependent mobility shift on SDS-PAGE, indicative of phosphorylation of this protein, in primary erythroid (Fig. 2A), HCD57 (Fig. 2B), and HCD57R cells (Fig. 4B, see below). In primary erythroid and HCD57 cells, BIM protein was then decreased between 15–30 minutes following Epo stimulation. The time course of BIM phosphorylation was well correlated with the Epo-

induced ERK activation. To confirm whether Epo-activated ERK contributes BIM phosphorylation, we treated primary erythroid cells with a MEK/ERK inhibitor, U0126. The treatment inhibited the Epo-induced mobility shift of BIM on SDS/PAGE, resulting in increased BIM expression (Fig. 3A). We further confirmed BIM phosphorylation by Western blot analyses with a phosphorylation-specific antibody at Ser65 of BIM, which is known as a major ERK target site (Fig. 3B)[8,11]. ERK-induced BIM phosphorylation at Ser65 was completely inhibited by U0126 treatment. In accordance with increased BIM expression, treatment of HCD57 cells with U0126 enhanced apoptosis even in the presence of Epo (Fig. 3C). All of these data implicate that Epo-induced ERK phosphorylates BIM, resulting in suppression of apoptosis in erythroid cells.

We next assessed whether the decreased expression of BIM following Epo treatment was a result of BIM phosphorylation followed by degradation in proteasomes [8]. Pretreatment of HCD57 cells with a proteasome inhibitor, MG-132, resulted in the accumulation of BIM protein following Epo-mediated phosphorylation (Fig. 3D). The data suggest that Epo-induced phosphorylation of BIM results in proteasome-dependent degradation in erythroid cells.

Previous studies have implicated PI3K/AKT-mediated regulation of BIM expression [9]. Thus, we examined the effect of a PI3K inhibitor, LY294002, in the status of BIM expression/phosphorylation. HCD57R cells were deprived of Epo overnight, and then Epo was added back for 30 minutes. We observed a similar band-shift on SDS-PAGE after Epo re-addition, as shown in primary erythroid and HCD57 cells. However, treatment with LY294002 failed to inhibit phosphorylation of BIM following Epo stimulation in HCD57R cells (Fig. 4A). Extended culture of HCD57 cells with LY294002 in the presence of Epo did not reveal increased BIM protein expression (data not shown). The expression of constitutively-active AKT did not affect the expression and phosphorylation status of BIM in either HCD57 or HCD57R cells (Fig. 4B). These data suggest that the PI3K/AKT pathway is not involved in BIM expression/phosphorylation in erythroid cells.

ERK-regulated BIM is required for apoptosis following Epo deprivation in erythroid cells

To determine the significance of BIM in apoptosis induced by Epo deprivation, we established a pool of HCD57 cells that stably expressed shRNA for BIM [11]. Expression of BIM was strongly inhibited by BIM shRNA (HCD57-shBIM) compared to control cells (HCD57-CON) (Fig. 5A). Down-regulation of BIM in HCD57 cells resulted in a delay and reduction in apoptosis when Epo was withdrawn (Fig. 5B). These data strongly support the idea that BIM initiates apoptosis of erythroid cells after Epo deprivation, although the data also suggest the existence of BIM-independent pathways.

When Epo-activated MEK/ERK or PI3K is pharmacologically inhibited by U0126 or LY294002, respectively, HCD57 cells undergo apoptosis even in the presence of Epo. Thus, we next examined whether apoptosis triggered by U0126 is dependent on BIM (Fig. 5C). With U0126 treatment, HCD57-shBIM cells showed significantly less apoptosis compared to HCD57-CON cells. In contrast, the percentage of apoptosis triggered by LY294002, which did not affect BIM expression (Fig. 4), was not significantly different between HCD57-shBIM and HCD57-CON cells. Taken together, (i) BIM plays an important role in apoptosis induced by Epo deprivation, (ii) Epo protects erythroid cells from apoptosis in part through ERK-dependent phosphorylation of BIM followed by proteosomal degradation.

We then switched our attention to the role of BIM in the survival of erythroid progenitors *in vivo*. Blood from wild-type (*Bim*^{+/+}) and *Bim* gene knock-out (*Bim*^{-/-}) mice [20] was tested for hematocrit, number of red blood cells, white blood cells, and reticulocytes. Table 1 shows no significant differences in erythrocytes, reticulocytes, or any parameters of circulating blood, other than elevated numbers of white blood cells in the circulation of *Bim*^{-/-} mice as previously

reported [7]. We then examined erythroid cells from bone marrow and spleen for the expression of Ter119 and CD71 to determine the relative populations of immature to mature erythroid progenitors. The proportion of erythroid cells (Ter119⁺) in both bone marrow and spleen of *Bim*^{-/-} mice was significantly decreased, but the total number of Ter119⁺ erythroid cells from either the femur or spleen of *Bim*^{-/-} mice was not significantly different from wild-type (Table 2). This discrepancy is explained by the increased number of total nucleated cells in the femurs and spleens of *Bim*^{-/-} mice ($21.1 \pm 5.3 \times 10^6$ cells in *Bim*^{-/-} femurs versus $16.5 \pm 4.2 \times 10^6$ cells in wild-type femurs, $383 \pm 120 \times 10^6$ cells in *Bim*^{-/-} spleens versus $126 \pm 32 \times 10^6$ cells in wild-type spleens). We also did not find any significant difference in the ratios of immature and more mature erythroblasts between wild-type and *Bim*^{-/-} mice, when the percentages were normalized to the number of total Ter119⁺ cells (Table 3). We next tested whether the deletion of BIM affected the number of erythroid progenitors in the bone marrow by *in vitro* assays. Consistent with the reduction of the proportion of Ter119⁺ cells (Table 2), the number of CFU-e per total bone marrow cell cultured was significantly decreased to 30% of control in *Bim*^{-/-} mice compared to wild-type mice (Fig. 6A). A similar decrease in CFU-e was observed in the spleen of *Bim*^{-/-} mice as well as reduced BFU-e in both spleen and bone marrow (data not shown). We then tested whether a MEK/ERK inhibitor, U0126, would suppress CFU-e more in the bone marrow of wild-type mice compared to *Bim*^{-/-} mice, as was the case in the HCD57 cells following BIM down-regulation (Fig. 5). When normalized to the percent of control, the inhibition of CFU-e by U0126 was significantly less in *Bim*^{-/-} bone marrow than wild-type bone marrow (Fig. 6B). The *in vivo* experiments suggest that Epo-activated ERK triggers BIM degradation in murine bone marrow erythroid progenitors to promote survival; but ERK-BIM-independent pathway(s) also contribute EPO-regulated survival/apoptosis.

Discussion

We provide evidence here that BIM plays an important role in Epo-mediated survival in erythroid cells. BIM is induced by Epo deprivation, but re-addition of Epo induces ERK-dependent BIM phosphorylation followed by proteasome-dependent degradation in erythroid cells. Down-regulation of BIM through shRNA or deletion of the *Bim* gene enhanced survival in the presence of U0126 MEK inhibitor, implicating the significance of BIM regulated by ERK in survival/apoptosis in erythroid cells.

It is now clear that activation of BH3-only proteins by apoptotic stimuli initiates mitochondria-dependent cell death pathway. BH3-only proteins cause cytochrome c release by activating BAX and/or BAK, and the anti-apoptotic BCL-2 family of proteins prevents this process. Among BH3-only members, BIM and PUMA have been implicated to play a role in the context of apoptosis induced by survival factor withdrawal, since the gene knockout mice/cells of each are resistant for these stimuli [7,22,23]. PUMA, however, was undetectable in HCD57 cells (data not shown), and a recent report shows no effect in erythroid development in the PUMA null mice [24]. Our data suggest that BIM is a critical factor in the initial phase of apoptosis. Similar findings for *Bim* mRNA expression related to Epo exposure have been recently reported [25]. Down-regulation of BIM, however, does not prevent the eventual apoptosis in HCD57 cells. It has been reported that BCL-X_L, an anti-apoptotic protein, is required for survival at the late erythroblast phase of erythroid maturation but is not critical in the survival of proerythroblast dependent on Epo [26]. It has been recently demonstrated that NIX, another BH3-only protein, plays a critical role in reticulocytes in BAX/BAK-independent manner [27–29]. Thus other pro- and anti-apoptotic proteins seem to play a role in the mechanism by which Epo spares erythroid cells from apoptosis.

We did not find increased erythrocytes in the blood of *Bim*^{-/-} mice, but this result does not exclude a role of BIM in regulating apoptosis in primary erythroid cells. Possible reasons for failing to see elevated progenitors/precursors, erythrocytes and reticulocytes are: 1) normal

hematocrit count cannot increase significantly without inducing lethality [30–32]. 2) increased erythrocyte production would lead to increased oxygen in the circulation, which suppresses Epo production in the kidney and thereby returns the number of circulating erythrocytes to normal. Furthermore, the effect of deletion of *Bim* may not be manifested until erythropoiesis is stressed or suppressed in the *Bim*^{−/−} mice in future studies. This is true on murine hematopoiesis in *Stat5a*^{−/−}*Stat5b*^{−/−} or *Stat1*^{−/−} mice [33,34].

We show that control of BIM protein expression is independent of PI3K/AKT in HCD57 cells because exposure to the PI3K inhibitor, LY294002 failed to prevent BIM phosphorylation/ degradation and ectopic expression of constitutively-active AKT failed to suppress BIM expression with Epo deprivation (Fig. 4). We also did not observe alteration of BIM expression when a dominant-negative c-JUN (TAM-67) was expressed in HCD57 cells (data not shown) [35]. This is somewhat surprising in light of a number of studies that suggest a critical role of PI3K/AKT/FOXO and AP1/c-JUN transcription factors in inducing *Bim* expression. It is likely that the regulation of BIM expression depends on both survival factors and the cell system studied. Our analysis of HCD57 cells reveals at most 2-fold increase in *Bim* mRNA within 2 hours of Epo deprivation (Fig. 1C). Thus the large increase in total BIM protein expression when Epo is withdrawn and decrease of BIM following treatment with Epo are likely due to post-transcriptional control. Our finding that Epo-induced ERK phosphorylates BIM, resulting in rapid proteasome-dependent degradation is consistent with the post-transcriptional BIM regulation by other survival factors [8]. The study presented here recapitulates the importance of ERK activation and BIM expression in regulating survival/apoptosis in other cells.

Acknowledgements

The authors would like to thank Dr. Kimber White for measuring reticulocytes and hematological parameters and Katherine W. Falinski for statistical analysis. Portions of this work were presented in the abstract at the 45th Annual Meeting of the American Society of Hematology, and were included in a dissertation by RMA to satisfy the requirements of the Ph.D. degree of VCU. This work was supported by grants from the National Institutes of Health (R01DK39781 and R01HL65906 to STS) and Massey Cancer Center (to STS and HH).

References

1. Koury MJ, Sawyer ST, Brandt SJ. New insights into erythropoiesis. *Curr Opin Hematol* 2002;9:93–100. [PubMed: 11844990]
2. Richmond TD, Chohan M, Barber DL. Turning cells red: signal transduction mediated by erythropoietin. *Trends Cell Biol* 2005;15:146–155. [PubMed: 15752978]
3. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007;26:1324–1337. [PubMed: 17322918]
4. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004;116:205–219. [PubMed: 14744432]
5. O'Connor L, Strasser A, O'Reilly LA, et al. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* 1998;17:384–395. [PubMed: 9430630]
6. Strasser A. The role of BH3-only proteins in the immune system. *Nat Rev Immunol* 2005;5:189–200. [PubMed: 15719025]
7. Bouillet P, Metcalf D, Huang DC, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999;286:1735–1738. [PubMed: 10576740]
8. Ley R, Ewings KE, Hadfield K, Cook SJ. Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. *Cell Death Differ* 2005;12:1008–1014. [PubMed: 15947788]
9. Dijkers PF, Birkenkamp KU, Lam EW, et al. FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J Cell Biol* 2002;156:531–542. [PubMed: 11815629]

10. Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron* 2001;29:629–643. [PubMed: 11301023]
11. Harada H, Quearry B, Ruiz-Vela A, Korsmeyer SJ. Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. *Proc Natl Acad Sci USA* 2004;101:15313–15317. [PubMed: 15486085]
12. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999;3:287–296. [PubMed: 10198631]
13. Sawyer ST, Jacobs-Helber SM. Unraveling distinct intracellular signals that promote survival and proliferation: study of erythropoietin, stem cell factor, and constitutive signaling in leukemic cells. *J Hematother Stem Cell Res* 2000;9:21–29. [PubMed: 10738968]
14. Bao H, Jacobs-Helber SM, Lawson AE, et al. Protein kinase B (c-Akt), phosphatidylinositol 3-kinase, and STAT5 are activated by erythropoietin (EPO) in HCD57 erythroid cells but are constitutively active in an EPO-independent, apoptosis-resistant subclone (HCD57-SREI cells). *Blood* 1999;93:3757–3773. [PubMed: 10339482]
15. Jacobs-Helber SM, Penta K, Sun Z, Lawson A, Sawyer ST. Distinct signaling from stem cell factor and erythropoietin in HCD57 cells. *J Biol Chem* 1997;272:6850–6853. [PubMed: 9054369]
16. Menon MP, Fang J, Wojchowski DM. Core erythropoietin receptor signals for late erythroblast development. *Blood* 2006;107:2662–2672. [PubMed: 16332976]
17. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a^{-/-}5b^{-/-} mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* 1999;98:181–191. [PubMed: 10428030]
18. Jacobs-Helber SM, Sawyer ST. Jun N-terminal kinase promotes proliferation of immature erythroid cells and erythropoietin-dependent cell lines. *Blood* 2004;104:696–703. [PubMed: 15059850]
19. Zhang L, Insel PA. The pro-apoptotic protein Bim is a convergence point for cAMP/protein kinase A- and glucocorticoid-promoted apoptosis of lymphoid cells. *J Biol Chem* 2004;279:20858–20865. [PubMed: 14996839]
20. Takeuchi O, Fisher J, Suh H, et al. Essential role of BAX, BAK in B cell homeostasis and prevention of autoimmune disease. *Proc Natl Acad Sci U S A* 2005;102:11272–11277. [PubMed: 16055554]
21. Hara H, Ogawa M. Erythropoietic precursors in mice under erythropoietic stimulation and suppression. *Exp Hematol* 1977;5:141–148. [PubMed: 844518]
22. Jeffers JR, Parganas E, Lee Y, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* 2003;4:321–328. [PubMed: 14585359]
23. Villunger A, Michalak EM, Coultas L, et al. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003;302:1036–1038. [PubMed: 14500851]
24. Erlacher M, Labi V, Manzl C, et al. Puma cooperates with Bim, the rate-limiting BH3-only protein in cell death during lymphocyte development, in apoptosis induction. *J Exp Med* 2006;203:2939–2951. [PubMed: 17178918]
25. Sathyanarayana P, Dev A, Fang J, et al. EPO receptor circuits for primary erythroblast survival. *Blood* 2008;111:5390–5399. [PubMed: 18349318]
26. Rhodes MM, Kopsombut P, Bondurant MC, Price JO, Koury MJ. Bcl-x(L) prevents apoptosis of late-stage erythroblasts but does not mediate the antiapoptotic effect of erythropoietin. *Blood* 2005;106:1857–1863. [PubMed: 15899920]
27. Diwan A, Koesters AG, Odley AM, et al. Unrestrained erythroblast development in Nix^{-/-} mice reveals a mechanism for apoptotic modulation of erythropoiesis. *Proc Natl Acad Sci U S A* 2007;104:6794–6799. [PubMed: 17420462]
28. Schweers RL, Zhang J, Randall MS, et al. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc Natl Acad Sci U S A* 2007;104:19500–19505. [PubMed: 18048346]
29. Sandoval H, Thiagarajan P, Dasgupta SK, et al. Essential role for Nix in autophagic maturation of erythroid cells. *Nature* 2008;454:232–235. [PubMed: 18454133]
30. Kannel WB, Gordon T, Wolf PA, McNamara P. Hemoglobin and the risk of cerebral infarction: the Framingham Study. *Stroke* 1972;3:409–420. [PubMed: 4261794]

31. Harrison MJ, Pollock S, Kendall BE, Marshall J. Effect of haematocrit on carotid stenosis and cerebral infarction. *Lancet* 1981;2:114–115. [PubMed: 6113481]
32. Wannamethee G, Perry IJ, Shaper AG. Haematocrit, hypertension and risk of stroke. *J Intern Med* 1994;235:163–168. [PubMed: 8308479]
33. Socolovsky M, Nam H, Fleming MD, et al. Ineffective erythropoiesis in Stat5a(–/–)5b(–/–) mice due to decreased survival of early erythroblasts. *Blood* 2001;98:3261–3273. [PubMed: 11719363]
34. Halupa A, Bailey ML, Huang K, et al. A novel role for STAT1 in regulating murine erythropoiesis: deletion of STAT1 results in overall reduction of erythroid progenitors and alters their distribution. *Blood* 2005;105:552–561. [PubMed: 15213094]
35. Jacobs-Helber SM, Wickrema A, Birrer MJ, Sawyer ST. AP1 regulation of proliferation and initiation of apoptosis in erythropoietin-dependent erythroid cells. *Mol Cell Biol* 1998;18:3699–3707. [PubMed: 9632752]

Figure 1. The expression of BIM following Epo withdrawal in erythroid cells

Western blot analyses were performed with antibodies for BIM and actin. (A) Primary erythroid cells were isolated from the spleen in phenylhydrazine-treated mice. Cells were cultured in the absence of Epo for the indicated times. (B) Early passage HCD57 (lanes 1–3) or HCD57R (lanes 4–7) cells were deprived of Epo for the indicated times. (C) Quantification of *Bim* mRNA by RT-PCR. Total RNA from Epo-deprived HCD57 cells was subjected to RT-PCR. The results are the mean of duplicates. * $p=0.05$ when compared to time 0.

A

B

Figure 2. The expression of BIM following Epo stimulation in erythroid cells
 (A) Primary erythroid cells from phenylhydrazine-treated mice were cultured in the absence of Epo for up to three hours (lanes 1–4) and then stimulated with Epo (lanes 5–7) for the indicated times. (B) Early passage HCD57 cells were deprived of Epo (lanes 1–3) for up to four hours and then stimulated with Epo (lanes 4–9) for the indicated times. Western blot analyses were performed using the indicated antibodies.

A

B

Figure 3. Epo treatment results in BIM phosphorylation through an ERK-dependent pathway (A) Epo was withdrawn from splenic erythroid cells for 4 hours, followed by re-addition of Epo for the indicated times. Vehicle (DMSO) or a MEK inhibitor, U0126 (10 μ M) were added at the time of Epo deprivation. Western blot analyses were performed with the indicated antibodies. (B) Epo was withdrawn from HCD57cells for 4 hours, followed by re-addition of Epo for 30 minutes. Vehicle (DMSO) or U0126 (10 μ M) were added at the time of Epo starvation. Western blot analyses were performed with the indicated antibodies. (C) HCD57 cells were deprived of Epo (◆), deprived of Epo, but treated with U0126 (10 μ M) (▲), cultured with Epo (■), cultured with Epo + U0126 (×) for the indicated times. Cell death was determined by flow cytometry with propidium iodide-staining. Results are the mean $\pm$ SD of three independent experiments. (D) Epo stimulation results in proteasome-dependent degradation of BIM. Epo was withdrawn from HCD57cells for 4 hours, followed by re-addition of Epo for the indicated times. Vehicle (DMSO) or a proteasome inhibitor, MG-132 (100 μ M), were added at the time of Epo starvation. Western blot analyses were performed with the indicated antibodies.

A

B

Figure 4. The PI3 kinase/Akt pathway is not involved in BIM expression

(A) HCD57R cells were deprived of Epo overnight, pretreated with vehicle (lanes 1–2), 50 μ M LY294002 (lanes 3–4), 10 μ M U0126 (lanes 5–6), or LY294002 + U0126 (lanes 7–8) and stimulated with Epo for 30 min. Western blots were performed with antibodies for BIM and BAD. (B) HCD57 and HCD57R cells were stably transfected with a myc-tagged constitutive-active AKT (ca-AKT) construct. HCD57R (lanes 1–2), HCD57R-caAKT (lanes 3–4), early passage HCD57 (lanes 5–6), and HCD57-caAKT (lanes 7–8) cells were deprived of Epo and stimulated with Epo. Western blots were performed with the indicated antibodies.

A

B

Figure 5. Down-regulation of BIM slows the onset of apoptosis

(A) Western blot analyses were performed with HCD57 cells transfected with either pSuper-vector (HCD57-CON, lanes 1–4) or pSuper-BIM (HCD57-shBIM, lanes 5–8) in the absence of Epo for the indicated times. (B) Effect of BIM down-regulation in apoptosis. Cell death was determined by flow cytometry of propidium iodide-stained HCD57 (◆), HCD57-CON (■), and HCD57-shBIM (▲) cells following Epo withdrawal. (C) Effect of U0126 and LY294002 on apoptosis assessed by flow cytometry as in (B). HCD57-CON (□) and HCD57-shBIM (■) cells were deprived of Epo or cultured in Epo $\pm$ 10 μ M U0126 or 50 μ M LY294002 for 24 hours. Results are the mean $\pm$ SD of triplicate samples. * $p < 0.05$ when compared to the corresponding HCD57-CON cells.

Figure 6. Erythroid progenitors, CFU-e, are reduced in number in the bone marrow of *Bim*^{-/-} mice but are more resistant to MEK/ERK inhibition

(A) Bone marrow cells from wild-type or *Bim*^{-/-} mice were incubated in the presence of Epo with vehicle (DMSO) or U0126 in semisolid media. CFU-e were counted 2 days after the start of the experiment and are expressed as number of colonies per 35-mm plate. (B) Data in (A) were calculated as percentage of control with or without U0126. Error bars represent standard deviation from the mean. **p* < 0.01 and ***p* < 0.01 when compared to the control.

Table 1
Hematological parameters of whole blood in *Bim*^{-/-} mice

	<i>Bim</i> ^{-/-}	<i>Bim</i> ^{+/+}
White Blood Cells ($\times 10^3/\mu\text{L}$)	22.4 $\pm$ 8.6*	6.9 $\pm$ 1.9
Red Blood Cell ($\times 10^6/\mu\text{L}$)	8.7 $\pm$ 0.8	9.1 $\pm$ 0.4
Reticulocytes (%)	3.5 $\pm$ 0.8	4.2 $\pm$ 0.9
HGB (g/dL)	15.1 $\pm$ 1.1	16.3 $\pm$ 0.9
HCT (%)	48.1 $\pm$ 3.7	45.8 $\pm$ 4.8
MCV (fL)	51.5 $\pm$ 1.5	50.4 $\pm$ 0.6
MCH (pg)	17.9 $\pm$ 0.2	18.0 $\pm$ 0.1
MCHC (g/dL)	39.6 $\pm$ 0.3	39.3 $\pm$ 0.4
Platelets ($\times 10^3/\mu\text{L}$)	680 $\pm$ 90	792 $\pm$ 120

* Significantly different from *Bim*^{+/+} mice: p<0.05

HGB: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin content

The blood for 8–12 weeks *Bim*^{-/-} mice and their *Bim*^{+/+} littermates was collected in heparin coated tubes and applied to a K-1000 Hematology Analyzer (Roche Diagnostics).

Data are the result of three experiments $\pm$ SD.

Table 2
Number of erythroid progenitor/precursor cells in tissues

Tissue	<i>Bim</i> ^{-/-}	<i>Bim</i> ^{+/+}
Bone Marrow		
Percent of total	4.6 ± 0.2 % *	10.3 ± 0.5 %
Ter119 ⁺ cells/femur	1.0 ± 0.3 × 10 ⁶	1.7 ± 0.4 × 10 ⁶
Spleen		
Percent of Total	1.2 ± 0.2 % *	2.3 ± 0.3 %
Ter119 ⁺ cells/spleen	4.6 ± 1.4 × 10 ⁶	2.9 ± 0.7 × 10 ⁶

* Significantly different from *Bim*^{+/+} mice: p < 0.01

Total bone marrow or spleen cells were stained with Ter119 subjected to flow cytometry to assess erythroblast populations. The numbers indicate either the percentage of total cells that are Ter119⁺ or the total number of Ter119⁺ cells per femur or spleen. Data is the result of three experiments ± SD.

Table 3

Distribution of erythroid cells among stages of maturation

Erythroblast Maturation	Bone Marrow		Spleen	
	<i>Bim</i> ^{-/-}	<i>Bim</i> ^{+/+}	<i>Bim</i> ^{-/-}	<i>Bim</i> ^{+/+}
Region I	1.5 ± 0.3 %	3.3 ± 2.6 %	2.0 ± 0.6 %	3.3 ± 1.6 %
Region II	12.2 ± 3.2 %	14.3 ± 1.0 %	7.3 ± 2.5 %	11.0 ± 5.6 %
Region III	66.5 ± 5.2 %	67.1 ± 6.1 %	52.8 ± 4.8 %	57.5 ± 3.1 %
Region IV	20.3 ± 2.3 %	15.2 ± 4.4 %	36.2 ± 8.5 %	30.2 ± 6.1 %

Cells were obtained from spleen or bone marrow, subject to ammonium chloride treatment to remove erythrocytes, then stained with CD71 and Ter119, and subjected to flow cytometry to assess erythroblast maturation. The CD71 high, Ter119 low cells in region I are predominantly proerythroblasts, basophilic erythroblasts are in region II, late basophilic and chromatophilic erythroblasts are in region III, and Ter119 high, CD71 low cells in region IV are orthochromatophilic erythroblasts (as described by Socolovsky et al. [33]). Data were normalized to percentage of total Ter119⁺ cells (not total cells). Data are the result of three experiments ± SD. There were no significant differences in between *Bim*^{-/-} and *Bim*^{+/+} mice.