

# Raf-1 Antagonizes Erythroid Differentiation by Restraining Caspase Activation

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## Abstract

The Raf kinases are key signal transducers activated by mitogens or oncogenes. The best studied Raf isoform, Raf-1, was identified as an inhibitor of apoptosis by conventional and conditional gene ablation in mice. *c-raf-1*<sup>-/-</sup> embryos are growth retarded and anemic, and die at midgestation with anomalies in the placenta and fetal liver. Here, we show that Raf-1-deficient primary erythroblasts cannot be expanded in culture due to their accelerated differentiation into mature erythrocytes. In addition, Raf-1 expression is down-regulated in differentiating wild-type cells, whereas overexpression of activated Raf-1 delays differentiation. As recently described for human erythroid precursors, we find that caspase activation is necessary for the differentiation of murine fetal liver erythroblasts. Differentiation-associated caspase activation is accelerated in erythroid progenitors lacking Raf-1 and delayed by overexpression of the activated kinase. These results reveal an essential function of Raf-1 in erythropoiesis and demonstrate that the ability of Raf-1 to restrict caspase activation is biologically relevant in a context distinct from apoptosis.

Key words: kinase • gene inactivation • erythropoiesis • fetal liver • apoptosis

## Introduction

Hematopoiesis is a complex process regulated by multiple growth factors supporting the renewal, differentiation, and survival of hematopoietic progenitors at different stages of maturation. Pluripotent, multipotent, and committed precursors can undergo a number of “renewal divisions” (1). During terminal differentiation, committed progenitors obey a fixed program that directs both the residual number of cell divisions and the specific timing of differentiation (2, 3). The correct balance between renewal and terminal differentiation is essential for homeostasis of the hematopoietic system, maintaining adequate numbers of precursors as well as mature cells, and is lost under pathological conditions such as leukemia and anemia.

Apoptosis is a finely regulated process equally essential for tissue development and homeostasis. During apoptosis, the cell is dismantled from within by cysteine/aspartic acid

proteases (caspases) that cleave proteins involved in the maintenance of cell shape, the integrity of the nucleus, as well as of DNA itself (4, 5). Cleavage of these so-called death substrates causes dramatic alterations in the shape of the cells, some of which are reminiscent of the changes seen during the differentiation of erythroblasts to mature erythrocytes. Indeed, caspase activation has recently been shown to be required for the differentiation of human erythroblasts in culture (6).

The Raf-1 kinase has been implicated in the transduction of signals directing cell proliferation, activation, and survival. However, conventional (7, 8) and conditional (9) ablation of Raf-1 have revealed that the essential role of this kinase is to prevent apoptosis rather than promote proliferation. Specifically, Raf-1 seems to act by preventing caspase activation (9). Raf-1-deficient embryos are anemic, exhibit various grades of growth retardation, and die at E12.5. The most prominent defects observed are placental insufficiency and liver apoptosis. Surprisingly in view of the anemia observed, apoptosis is restricted to the hepatoblast compartment and does not seem to affect the erythroid progenitors (7). Here, we address the cause of the anemic phenotype of the Raf-1-deficient embryos. We demonstrate that Raf-1 is degraded during erythroid maturation of

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primary mouse erythroblasts cultivated in physiologically relevant cytokines (10) and that this kinase delays erythroblast differentiation by restraining the caspase activation associated with it. Thus, the anemic phenotype of Raf-1-deficient embryos is likely due to premature erythroblast differentiation at the expense of renewal, which depletes the fetal liver of erythroid precursors. Our results indicate a novel role for Raf-1 in erythropoiesis and demonstrate that its essential function as a regulator of caspase activity is not restricted to the control of apoptosis.

## Materials and Methods

**Cell Isolation, Culture, Differentiation, and Infection.** Primary fetal liver cells were isolated from E11.5 or E12.0 mouse embryos from heterozygous Raf-1 intercrosses. Bone marrow cells were isolated from *Mx-Cre;c-raf-1<sup>flox/flox</sup>* and *c-raf-1<sup>flox/flox</sup>* mice after the induction of Cre expression by poly inosinic/cytidylic acid (poly I/C)\* treatment in vivo (400  $\mu$ g intraperitoneally, every other day, three injections total). Cells were collected 1 wk after the last injection and the conversion of the *flox* to the  $\Delta$  allele was examined by PCR analysis as previously described (9). Fetal liver- and bone marrow-derived cells and immortal mouse erythroblasts (I/11, p53-deficient; references 10 and 11) were expanded and maintained in serum-free erythroid medium (StemPro34™ plus nutrient supplement; Life Technologies) supplemented with 2 units/ml human recombinant erythropoietin (Epo; Janssen-Cilag), 100 ng/ml murine recombinant stem cell factor (R&D Systems), and  $10^{-6}$  M dexamethasone (Sigma-Aldrich). 40 ng/ml insulin-like growth factor 1 (Promega) was present in all media described (10). Cultures were maintained at densities between 2 and  $4 \times 10^6$  cells/ml and analyzed daily for cell numbers and cell size distribution in an electronic cell counter (CASY-1; Schärfe-System) to determine proliferation kinetics. Cumulative cell numbers were calculated as previously described (10).

For the determination of erythroid CFUs (CFUe), fetal livers were isolated at day E11.5 and resuspended in IMDM.  $10^5$  cells were seeded in duplicate in Methocult M3234® (StemCell Technologies Inc.) in the presence of 0.3 U/ml Epo. Colonies were counted by independent investigators after 2 and 3 d in culture.

For the experiments presented in Fig. 1 B through Fig. 4, fetal liver or bone marrow cells were cultured for 6 d in proliferation medium to enrich for erythroid progenitors. Immediately before the experiment, the cells were density purified by Ficoll centrifugation in 1.078 g/cm<sup>3</sup> lymphocyte separation medium (Eurobio) to remove dead and differentiated cells and obtain homogenous populations of proliferating erythroid progenitors (12). Cells were washed twice in PBS and reseeded at  $2-3 \times 10^6$  cells/ml in S-13 differentiation medium containing 12% FCS (Life Technologies) and supplemented with 10 units/ml Epo, insulin ( $4 \times 10^{-4}$  IE = 10 ng/ml, Actrapid® HM; Novo Nordisk),  $3 \times 10^{-6}$  M of the dexamethasone antagonist ZK112,993 (10), and 1 mg/ml iron-saturated human transferrin (Sigma-Aldrich). Differentiating erythroblasts were maintained at densities between 2 and  $4 \times 10^6$  cells/ml.

In selected experiments, the cells were preincubated for 1 h with the indicated concentrations of the broad range caspase inhibitors Z-VAD-FMK (R&D Systems) or Caspase Inhibitor III

(Boc-D-FMK; Calbiochem-Novabiochem). Fresh inhibitors were supplied with each medium change throughout differentiation.

I/11 erythroblasts were infected with a bicistronic retroviral vector in which the expression of mutant forms of Raf-1 coupled to enhanced green fluorescent protein (EGFP) via an internal ribosome entry site sequence is driven by the murine stem cell virus long-terminal repeats (13). 8 d after infection, I/11 cells were sorted on a FACSCalibur® (Becton Dickinson) for EGFP expression and single cells were expanded in 96-well plates.

**Cell Morphology, Histological Staining, FACS® Analysis, and Determination of Hemoglobin Content.** Erythroblasts at various stages of differentiation were cytocentrifuged onto glass slides and stained with histological dyes and neutral benzidine for hemoglobin (14). Nucleated and enucleated erythrocytes (brown- or orange-stained small cells), partially mature or immature cells (larger cells with gray or blue cytoplasm), and apoptotic/dead cells (fragmented/condensed nuclei, disintegrated cells) were counted after visual inspection in the microscope as previously described (14), evaluating >500 cells per sample on multiple, randomly selected fields. Bone marrow-derived cells cultured for 1 wk in erythroid medium were stained with antibodies against c-kit-APC, Ter119-PE, B220-APC, and Mac-1-FITC (all from BD Biosciences) before FACS® analysis. Hemoglobin content was analyzed by removing 50  $\mu$ l aliquots from the cultures and photometric determination was performed as previously described (10). Values obtained from triplicate determinations were averaged and normalized to cell number and cell volume. The highest value measured in a given experiment was taken as 100.

**Western Blot Analysis.** Erythroblasts were harvested after the determination of cell numbers, washed with ice cold PBS, and resuspended in  $2 \times$  SDS loading or lysis buffer (10 mM Tris-HCl, pH 7.0, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 1% Triton X-100). Insoluble material was removed by centrifugation (14,000 rpm for 5 min). Because hemoglobin, present in different amounts in the cells at various stages, interferes with any standard method of protein determination, samples were normalized with respect to both cell number and size and subjected to PAGE followed by electrophoretic transfer to nylon membranes. After blocking in TTBS (10 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.1% Tween 20) with 5% milk powder or 2% BSA (fraction V; Sigma-Aldrich), the membranes were probed with the appropriate primary antibodies (rabbit polyclonal antiserum against a COOH-terminal peptide of v-raf [SP63, CTLTTSPLRPVF], hsp90, caspase-1, caspase-3, caspase-9, and Lamin B; Santa Cruz Biotechnology, Inc.) in 1–2% BSA in TTBS before incubation with peroxidase-conjugated secondary antibodies and detection by enhanced chemiluminescence (Pierce Chemical Co.).

## Results and Discussion

We have previously demonstrated that Raf-1-deficient embryos are anemic and have hypocellular livers showing pronounced apoptosis of the hepatoblast compartment. Hepatoblast failure and the resulting lack of a proper microenvironment could be responsible for the impaired hematopoiesis and the anemic phenotype. Indeed, Raf-1-deficient fetal livers contained much less erythroid progenitors than WT livers as assessed in CFUe assays (Fig. 1 A). To determine whether this was due to a defect in the fetal liver environment or to a cell-autonomous defect of the erythroid progenitors, we investigated the ability of Raf-

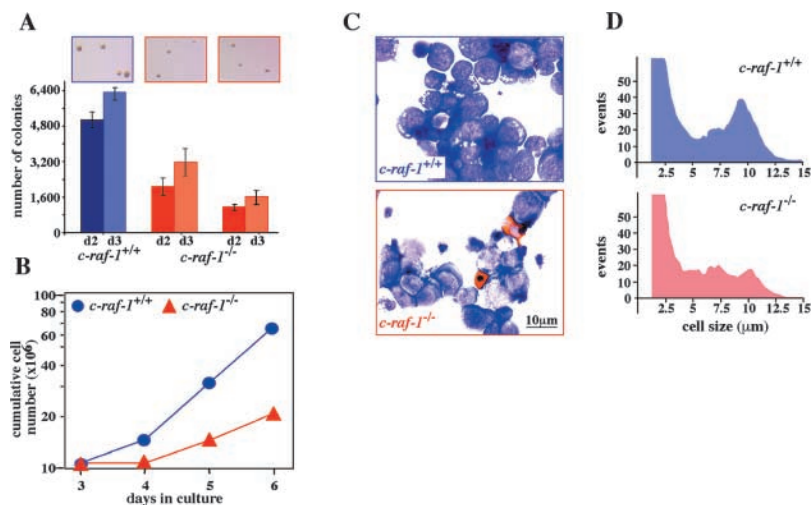
\*Abbreviations used in this paper: CFUe, erythroid CFU(s); EGFP, enhanced green fluorescent protein; Epo, erythropoietin; Poly I/C, poly inosinic/cytidylic acid.

1-deficient erythroblasts to proliferate and differentiate in vitro. Purified erythroblasts from E11.5 livers were cultivated in erythroid medium to favor proliferation but not differentiation of erythroblasts (10). Under these conditions, WT cells proliferated exponentially (eightfold total cell number increase from day 3 to 6), whereas *c-raf-1*<sup>-/-</sup> cells barely doubled (Fig. 1 B). This is in line with the previous observation that antisense Raf-1 oligonucleotides reduce Epo and insulin-like growth factor-induced proliferation of human erythroid progenitors (15). Analysis of cytopsin preparations after 6 d in culture showed that WT cultures contained mainly large, undifferentiated erythroid progenitors. In contrast, the Raf-1-deficient cultures were far more heterogeneous and contained immature cells, differentiating blasts, and mature hemoglobinized cells (Fig. 1 C). This heterogeneity and the presence of mature cells in the Raf-1-deficient cultures could be confirmed by cell size profile analysis. The WT cultures consisted mainly of large, blast-size cells (~10 μm Ø) and the *c-raf-1*<sup>-/-</sup> cultures contained a high proportion of smaller cells, including erythrocytes (~5 μm Ø, Fig. 1 D). Thus, Raf-1-deficient fetal liver cells fail to accumulate in culture, apparently due to accelerated differentiation at the expense of renewal.

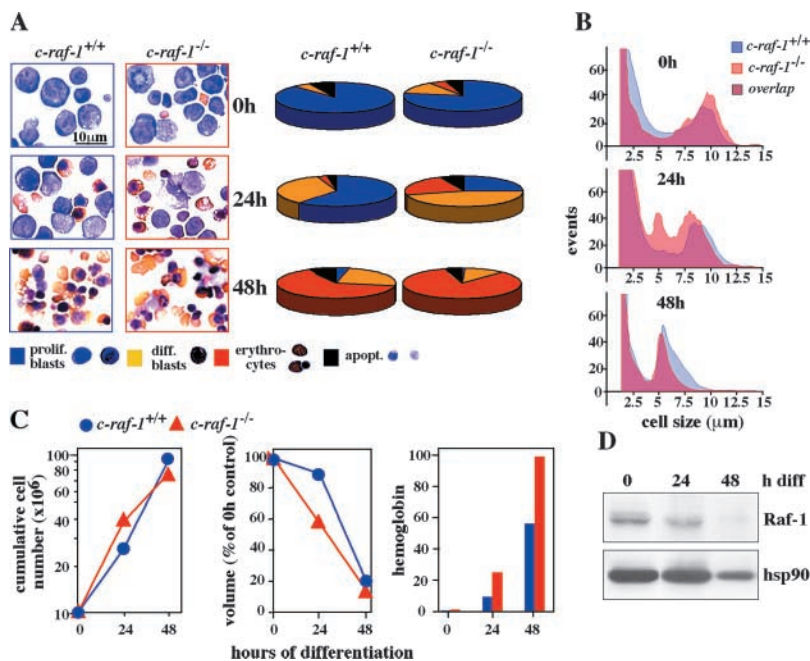
To further investigate the impact of the lack of Raf-1 on erythroid differentiation, density-purified erythroblasts from fetal liver cultures (refer to Materials and Methods) were induced to terminally differentiate by exposure to Epo plus insulin. Neither WT nor *c-raf-1*<sup>-/-</sup> cells underwent a significant degree of cell death during differentiation (2–7% dead cells/time point), and in both cultures differentiation was essentially complete after 48 h, although a larger residual proportion of partially mature cells was observed in the WT cultures (Fig. 2 A). At 24 h, however, *c-raf-1*<sup>-/-</sup> cultures showed a dramatic reduction of the number of erythroblasts (from 82 to 27%) compensated by a similarly dramatic increase in the numbers of nucleated (13–47%) and enucleated (3–22%) erythrocytes, as assessed by analysis of cytopsin and cell size profiles (Fig. 2, A and B). By

comparison, WT cultures after 24 h still contained 62% erythroblasts and only ~1.4% enucleated erythrocytes. Accelerated differentiation of Raf-1-deficient cells was also evident from quantitative analysis of other differentiation parameters, i.e., faster maturation-associated proliferation, cell size decrease, and hemoglobin accumulation (Fig. 2 C). To confirm that the defect observed in the Raf-1-deficient erythroblasts was independent from the fetal liver environment, we used erythroblasts derived from the bone marrow of *Mx-Cre*<sup>+</sup>; *c-raf-1*<sup>fllox/fllox</sup> mice treated with poly I/C to fully convert the flox allele to a null allele (Fig. 3 A, *c-raf-1*<sup>Δ/Δ</sup>; reference 9). Cells derived from poly I/C-treated *Mx-Cre*<sup>-</sup>; *c-raf-1*<sup>fllox/fllox</sup> animals were used as controls (*c-raf-1*<sup>fl/fl</sup>). After 6 d in culture in erythroid medium, both the *c-raf-1*<sup>fl/fl</sup> and *c-raf-1*<sup>Δ/Δ</sup> populations contained comparable amounts of erythroid cells, as determined by FACS<sup>®</sup> analysis (Fig. 3 B). These populations were density purified to remove dead and differentiating cells before the induction of terminal erythroid differentiation. Similar to the situation observed in fetal liver-derived erythroblast, a remarkable increase in nucleated (27%) and enucleated (22%) erythrocytes was apparent in the *c-raf-1*<sup>Δ/Δ</sup> culture 24 h after differentiation induction, whereas *c-raf-1*<sup>fl/fl</sup> cultures contained only 18% nucleated and 12% enucleated erythrocytes. Thus, the differentiation defect observed is intrinsic to the Raf-1-deficient erythroblasts and does not depend on organ environment.

These results suggest that delaying erythroid differentiation is an essential function of Raf-1. If so, differentiating WT cells should have a means of neutralizing this effect of Raf-1. Indeed, immunoblot analysis showed that Raf-1 expression declines rapidly during erythroid differentiation (Fig. 2 D). These data clearly define Raf-1 as a modulator of erythroid maturation, which ensures homeostasis by preventing the premature differentiation (and thereby the depletion) of actively proliferating erythroid precursors. This essential function of Raf-1 likely contributes to the ability of its viral counterpart, *v-raf*, to efficiently transform erythroid cells in vitro (16).



**Figure 1.** Fetal livers lacking Raf-1 contain lower amounts of CFUe and Raf-1-deficient erythroblasts show impaired expansion in culture. (A) Single cell suspensions ( $10^5$ , duplicates) from fetal livers of *c-raf-1*<sup>-/-</sup> or WT mice were plated in Methocult/Epo. Colonies were scored after 2 and 3 d. Mean values of quadruplicates  $\pm$  SD are shown. (B) Density-purified fetal liver erythroblasts from *c-raf-1*<sup>-/-</sup> or WT mice were cultured in erythroid medium. Cumulative cell numbers were determined daily. (C) The morphology of *c-raf-1*<sup>-/-</sup> and WT cells was analyzed on day 6. (D) Cell size profiles of erythroblasts were obtained by CASY measurement on day 6. For B–D, one representative experiment out of seven is shown.

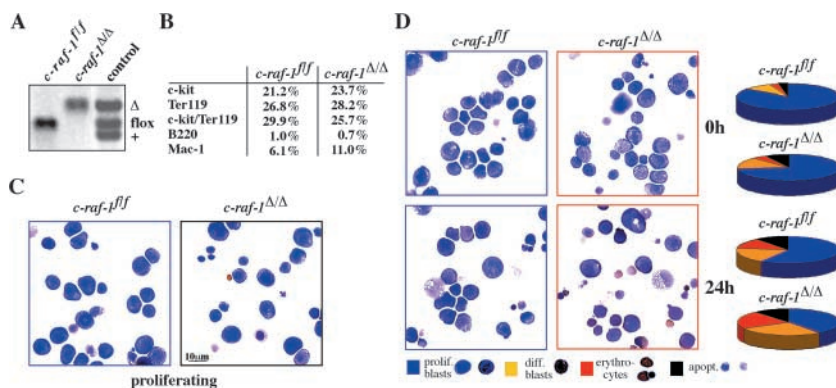


**Figure 2.** *c-raf-1*<sup>-/-</sup> erythroid progenitors differentiate faster than their WT counterparts. (A) Morphology of WT and *c-raf-1*<sup>-/-</sup> cells during differentiation. Cytopins stained with neutral benzidine plus histological dyes (left) were examined at each of the time points indicated (right). (B) Cell volume profiles measured daily show the characteristic decrease from a diameter of 10  $\mu\text{m}$  to 5  $\mu\text{m}$ . (C) Proliferation rate during differentiation (left), size decrease (middle), and hemoglobin content (right) of WT and *c-raf-1*<sup>-/-</sup> cells were determined daily during terminal differentiation. (D) The level of Raf-1 protein decreases during erythroblast differentiation. Whole cell lysates from WT cultures (15  $\mu\text{g}$ ) were subjected to immunoblot analysis with an anti-Raf-1 serum. hsp90, loading control. For A–C, one representative experiment out of seven is shown.

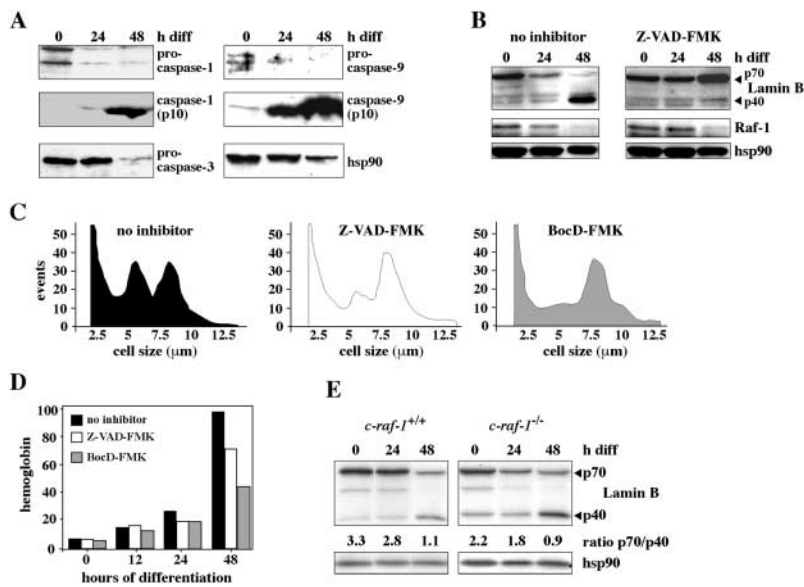
By what mechanism does Raf-1 control erythroblast differentiation? We have previously demonstrated that one essential function of this kinase is to restrain caspase activation during apoptosis (9). Recently, caspase activation has been found to be associated with and required for the differentiation of human erythroblasts (6). Therefore, we tested whether caspase activation was associated with the differentiation of murine erythroblasts in our *in vitro* system and whether Raf-1 delayed erythroid differentiation by restraining caspase activation. Caspases are synthesized as zymogens and converted into one large and one small subunit by limited proteolysis. Active caspases are tetramers of two large and two small subunits. We have monitored limited caspase proteolysis by immunoblotting, indicated by the disappearance of the zymogen (caspase-1, -3, and -9) and/or the appearance of the small subunit (caspase-1 and -9). Indeed, cleavage of the caspase-1, -3, and -9 zymogens was associated with erythroid differentiation (Fig. 4 A). Active caspases can, however, be efficiently counteracted by a group of proteins called IAPs, which bind to and inhibit the active caspase tetramer (17). To de-

termine whether the limited proteolysis of the caspase zymogens corresponded to caspase activation, we tested whether the cleavage of the death substrate Lamin B was associated with erythroid differentiation. In this context Lamin B is a relevant substrate because its cleavage is needed to dissolve the nuclear membrane and is therefore likely connected with enucleation (6).

Indeed, we observed cleavage of the 70-kD Lamin B protein to a 40-kD diagnostic proteolytic fragment during erythroblast differentiation of WT cells (Fig. 4 B). Treatment with the cell-permeable caspase inhibitor Z-VAD-FMK abolished the maturation-associated Lamin B cleavage (Fig. 4 B, top). In addition, both Z-VAD-FMK and a second broad-range caspase inhibitor, BocD-FMK, delayed erythroblast differentiation as measured by cell size decrease. After 36 h of differentiation, two distinct peaks were well discernible in the untreated culture: large-sized erythroblasts and small-sized mature cells. However, in inhibitor-treated cultures, the main peak at this time was represented by proliferating precursors (Fig. 4 C). Similarly, the inhibitors significantly delayed and reduced the accu-



**Figure 3.** *c-raf-1*<sup>Δ/Δ</sup> bone marrow-derived erythroid progenitors differentiate faster than their WT counterparts. (A) Total conversion of the floxed *c-raf-1* allele to a null allele shown by PCR analysis of bone marrow cells. (B) FACS<sup>®</sup> analysis and (C) morphology of *c-raf-1*<sup>fl/fl</sup> and *c-raf-1*<sup>Δ/Δ</sup> bone marrow-derived cells after 6 d of culture in proliferation medium and (D) after 0 and 24 h of culture in differentiation medium. The experiment was repeated twice with comparable results.

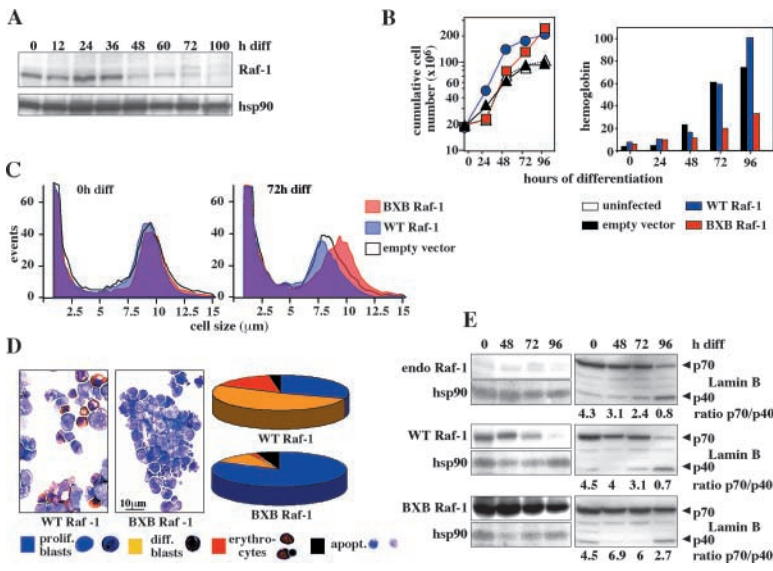


**Figure 4.** Caspase activation is necessary for the differentiation of erythroid progenitors. (A) Caspase activation accompanies the differentiation of erythroblasts. At the indicated times after the induction of differentiation, WT erythroblasts were lysed and the activation state of caspase-1, -3, and -9 was analyzed by immunoblotting. hsp90 was used as a loading control. (B) Caspase inhibitors block differentiation-associated caspase activation. WT erythroblasts were pretreated for 1 h with 150  $\mu$ M broad-range caspase inhibitor Z-VAD-FMK or left untreated (no inhibitor) before being switched to differentiation medium (with or without Z-VAD-FMK). Levels of p70 Lamin B and its cleavage product p40 in whole cell lysates (15  $\mu$ g) were detected by Western Blot analysis. (C and D) Caspase inhibitors delay erythroid differentiation. (C) Cell size profiles and (D) hemoglobin content of cultures treated with Z-VAD-FMK as in B or with 150  $\mu$ M BocD-FMK were analyzed daily. (E) *c-raf-1*<sup>-/-</sup> erythroblasts show increased Lamin B cleavage. WT or *c-raf-1*<sup>-/-</sup> erythroblasts were induced to terminally differentiate. At the time points indicated, the protein levels of p70 Lamin B and its cleavage product p40 were determined by Western Blot analysis (15  $\mu$ g). p70/p40 ratios were determined by densitometry using the program ImageQuant. hsp90, loading control.

mulation of hemoglobin in the differentiating cultures (Fig. 4 D). The inhibition of caspase activation, however, did not prevent differentiation-associated down-regulation of Raf-1 (Fig. 4 B, middle). To assess whether, in turn, the absence of Raf-1 had an effect on differentiation-associated caspase activation, we determined the extent of Lamin B cleavage in differentiating WT and *c-raf-1*<sup>-/-</sup> erythroblasts. Lamin B cleavage was comparable in the terminally differentiated cultures (Fig. 4 E, 48 h). However, more Lamin B cleavage product (p40), indicative of elevated caspase activity, was present in *c-raf-1*<sup>-/-</sup> cultures at the start as well after 24 h of differentiation (Fig. 4 E). This was not associated with increased apoptosis (Fig. 2 A and unpublished data) and correlated well with the differences observed in the amount of differentiated cells between WT and Raf-1-deficient erythroblast culture (Fig. 2). Thus, as observed in Raf-1-deficient macrophages (9) and fibroblasts treated by apoptotic stimuli (unpublished data), lack of Raf-1 is associated with an increase in caspase activation in differentiating erythroblasts.

If Raf-1 is indeed responsible for regulating erythroblast differentiation via caspase-mediated cleavage of important proteins, constitutive activation of Raf-1 should delay both cleavage of caspase substrates and erythroid differentiation itself. To confirm and extend these results, we have used the factor-dependent differentiation-competent erythroblast cell line I/11 (10, 11). As in primary fetal liver-derived erythroblasts, Raf-1 degradation accompanies the somewhat slower differentiation of these cells (Fig. 5, A and B). To assess whether Raf-1 overexpression would delay death substrate cleavage and differentiation and whether this required Raf-1 kinase activity, we infected I/11 cells with retroviral constructs directing the expression of full-length Raf-1 (WT Raf-1) and of a truncated, constitutively active form of Raf-1 (BXB Raf-1), and selected clones expressing these proteins. Bulk populations of infected cells

and clones expressing varying amounts of the exogenous proteins proliferated with similar kinetics in erythroid medium, indicating that neither WT nor constitutively active Raf-1 confer a selective proliferation advantage under these conditions (unpublished data). We consistently observed that the truncated, constitutively active (BXB) Raf-1 was expressed at much higher levels than WT Raf-1. The reasons for this are unknown. One might speculate that the overexpression of the WT protein, containing the regulatory domain that binds to upstream effectors (e.g., Ras), might compete with and block the activation of other downstream effectors necessary for survival. This would result in the counter selection of cells expressing high amounts of WT Raf-1, but not of BXB. Except slightly increasing proliferation during differentiation (Fig. 5 B, left), expression of WT Raf-1 did not have a major impact on erythroid differentiation as measured by hemoglobin accumulation (right), size decrease, and cytospin analysis (C and D). Expression of WT Raf-1 reduced Lamin B cleavage, albeit slightly, at all time points (Fig. 5 E, right), implying that a minimum threshold of caspase activity is necessary, above which differentiation starts and proceed normally. In contrast, constitutively active (BXB) Raf-1 delayed differentiation, as indicated by ongoing proliferation after 72–96 h, reduced hemoglobin accumulation (Fig. 5 B), increased cell size (C), and persistence of partially mature and immature cells that could be switched back to proliferation conditions (D and unpublished data). In parallel, BXB Raf-1 strongly delayed the cleavage of Lamin B (Fig. 5 E, right). In proliferating cells, WT Raf-1 was expressed at high levels compared with the endogenous protein but was down-regulated in the same manner during the course of differentiation. As mentioned above, BXB Raf-1 was expressed at higher levels and because of this persisted throughout differentiation although its amount decreased at the same rate as that of WT Raf-1 (Fig. 5 E, left).



**Figure 5.** Overexpression of active Raf-1 delays erythroid differentiation and differentiation-associated caspase activation in I/11 erythroblasts. (A) Raf-1 is down-regulated during differentiation of I/11 erythroblasts. I/11 erythroblasts were induced to differentiate. Levels of Raf-1 were detected at the indicated time points by immunoblot analysis of 50  $\mu$ g whole cell lysate. (B–D) Active Raf delays erythroid differentiation. The differentiation of uninfected I/11 was compared with that of cells expressing murine stem cell virus-EGFP (empty vector) with full-length Raf-1 (WT Raf-1) or a constitutively active Raf (BXB). Differentiation parameters analyzed were proliferation during differentiation (B, left), hemoglobin accumulation (B, right), and size decrease (C; overlays of cell size profiles obtained after 0 and 72 h of differentiation). (D) After 96 h of differentiation, the morphology and hemoglobin content of cells expressing exogenous WT Raf-1 and constitutively active Raf (BXB Raf) were compared. (E) Exogenous expression of Raf-1 mutants delays differentiation-associated caspase activation. Cell lysates were prepared from I/11 erythroblasts stably expressing Raf-1 mutants at different time points after the induction of differentiation. The amount of Raf-1 protein (left) as well as of Lamin B (right) were analyzed by immunoblotting whole cell lysates (15  $\mu$ g). Lamin B ratios were calculated as in Fig. 4 E. endoRaf-1, endogenous Raf-1; hsp90, loading control.

Several conclusions can be drawn from this experiment. First, by showing that overexpression of Raf-1 restrains caspase activation and delays erythroid differentiation, we confirm the role of Raf-1 as a negative regulator of both processes. Due to the differences in the expression of WT and BXB Raf-1, however, it is not possible to deduce whether BXB delays differentiation more efficiently than WT Raf-1 because of its constitutive kinase activity or simply because of its higher expression. Be that as it may, the experiment shows that a certain level of Raf-1 expression is needed at later maturation stages for restraining differentiation-induced caspase activation, whereas high level expression at the onset of differentiation has little effect on the progress of maturation. Second, the fact that the full-length protein expressed under the control of the retroviral promoter is down-regulated with the same kinetics and efficiency as endogenous Raf-1 indicates that the regulation of Raf-1 expression occurs at the posttranscriptional level, as also confirmed by Northern blot analysis (unpublished data). Interestingly, we have observed posttranscriptional Raf-1 down-regulation during pathogen-induced macrophage apoptosis (9). In this case, Raf-1 was degraded by the proteasome in a caspase-dependent manner. Thus, in macrophages, the relationship between Raf-1 and caspase activation was reciprocal, with the protease directing the degradation of the kinase that restrains its activation (9). In contrast, Raf-1 down-regulation in differentiating erythroblasts is either upstream of caspase activation or independently regulated. Defining the signal directing Raf-1 down-regulation and, if possible, interfering with it will be important in further elucidating the exact position of this kinase in the signal transduction cascade leading to erythroid differentiation. An additional unsolved question is how exactly Raf-1 controls caspase activation. It is possible that Raf-1, or a downstream effector, directly modifies the initiator protease(s), similar to how protein kinase B phos-

phorylates human caspase-9 and inhibits its protease activity (18). Raf-1 might also modulate the expression or activity of caspase inhibitors (17). In this context, in *Drosophila* activated D-Ras and D-Raf inhibit apoptosis by antagonizing *Hid* (19, 20), whose human orthologs have been shown to antagonize IAPs (21, 22). D-Raf, however, shares a greater degree of homology with B-Raf than Raf-1. Furthermore, at least in neurons, the regulation of IAP is an essential function of B-Raf and cannot be substituted for by Raf-1 (23). The exact modalities of Raf-1 action are unclear and will require additional study.

Raf-1 is activated by the erythroleukemia-inducing strains of Friend spleen focus-forming virus, and its inhibition by antisense oligonucleotide partially reduces proliferation of virus-infected cells (24). In addition, oncogenic *v-raf* versions cooperate with *myc* to transform erythroid cells (16). *p53*<sup>-/-</sup> cells can be likewise transformed by *v-raf*, giving rise to cell lines that can be propagated continuously and are highly tumorigenic (25). We now report a previously unrecognized function of Raf-1 as a modulator of erythroid homeostasis, which, by restraining caspase activation, prevents the premature differentiation of erythroblasts and safeguards the maintenance of the appropriate amount of proliferating precursors. A delay in precursor differentiation, as the one caused by mutation/overexpression of Raf-1 (BXB Raf-1 or *v-raf*), should tip the balance between proliferation and terminal maturation of erythroid cells toward precursor renewal and might represent the main contribution of Raf to the establishment of leukemia.

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