PAR bZIP-bik is a novel transcriptional pathway that mediates oxidative stress-induced apoptosis in fibroblasts

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PAR bZIP (cells knockout for PAR bZIP transcription factors) proteins, thyrotroph embryonic factor (TEF), albumin D-site-binding protein (DBP), and hepatic leukemia factor (HLF), are a family of transcription factors that have been shown to contribute to the expression of genes involved in detoxification and drug metabolism. Recently, we showed that PAR bZIP proteins were able to regulate the BH3-only gene *bcl-gS* in tumor cells. Here, we have extended the role of these transcription factors in the control of apoptosis executors by analyzing the expression of BH3-only genes in PAR bZIP triple knockout mouse fibroblasts. We found that *bik* was the only BH3-only gene downregulated in knockout cells. Consistently, transfection of TEF or DBP induces the expression of endogenous *bik*, regardless of the presence of active p53. Moreover, both promoter-reporter and chromatin immunoprecipitation assays indicate that PAR bZIP proteins activate the *bik* promoter directly. Treatment with different stress stimuli reveals a higher survival of knockout fibroblasts compared with that of wild-type cells, especially after incubation with H_2O_2, which suggest that PAR bZIP proteins participate in oxidative stress-induced apoptosis. Furthermore, the apoptotic cell death promoted by treatment with H_2O_2 can be impaired by reducing the expression of Bik in wild-type fibroblasts or enhanced by the overexpression of Bik in knockout cells. These findings reveal a novel transcriptional pathway relevant in transducing the apoptotic response to oxidative stress.

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Mammalian homologs of the *Caenorhabditis elegans* protein CES-2 include thyrotroph embryonic factor (TEF), albumin D-site-binding protein (DBP), and hepatic leukemia factor (HLF),[1–3] which are members of the proline- and acid-rich (PAR) subfamily of basic region leucine-zipper (bZIP) transcription factors. Mice deficient for all three PAR bZIP proteins are highly susceptible to generalized spontaneous and audiogenic epilepsies that frequently are lethal.[4] These proteins have recently been shown to control the expression of many enzymes and regulators involved in detoxification and drug metabolism, such as cytochrome P450 enzymes, carboxylesterases, and constitutive androstane receptor.[5] In addition, we showed for the first time that PAR bZIP proteins regulate the expression of a gene, *bcl-gS*, involved in the execution of apoptosis. This transcriptional pathway can be activated by chemotherapeutic agents in cancer cells, thus contributing to the apoptotic response to chemotherapy.[6] Bcl-gS is a member of the pro-apoptotic subgroup of the Bcl-2 family of apoptosis regulators, which share only the short BH3 region with the rest of the family.[7] Genetic experiments have shown that these proteins are essential initiators of programmed cell death in species as distantly related as mice and *C. elegans*. BH3-only proteins include Bid, Bad, Bim, Puma, Noxa, Hrk, Bik, Bmf, Bnip3, Bnip3L, and Bcl-gS.[8–10] They are regulated transcriptionally and by post-translational modifications, such as phosphorylation, ubiquitination, and proteolytic cleavage.[11–14] The tumor suppressor p53 is one of the main transcription factors involved in the regulation of proapoptotic Bcl-2 family members. This protein plays a pivotal role in the decision of whether the outcome of DNA damage will be growth arrest or apoptosis, and this choice is based, at least in part, on its transcriptional capacity. Among the BH3-only genes, *puma*, *noxa* and *bik* have been shown to be induced by the p53 pathway.[15–17] Although these three genes can be transcriptionally regulated by p53, current data suggest that *puma* is the principal mediator of p53-induced apoptosis.[18] Other transcription factors, such as Foxo3a, the hypoxia inducible factor-1, and E2F1, have also been shown to promote the expression of BH3-only genes,[19–21] whereas the transcriptional repressor, DREAM, blocks the expression of *hrk*.[22] As of the key role of BH3-only proteins as molecular sensors of apoptotic signals, deciphering the mechanisms that control the expression or activity of these proteins is essential to understand the molecular scenario of apoptosis and contribute to identify novel therapeutic targets that serve to modulate this process in different pathologies, including cancer and neurodegenerative diseases.

We report here that mouse fibroblasts deficient for all three PAR bZIP proteins show a selective downregulation of *bik* among the pro-apoptotic BH3-only genes. These transcription factors, mainly TEF, bind to a consensus site in the promoter region of *bik* and induce its expression. PAR bZIP (−/−) fibroblasts are less susceptible than wild-type cells to apoptosis induced by oxidative stress, which may be explained, at least in part, by the capacity of PAR bZIP proteins to transactivate *bik*. This is the first time that PAR bZIP transcription factors are involved in the cellular response to oxidant agents.

Results

PAR bZIP proteins are necessary for the expression of *bik* in fibroblasts.

We have shown earlier that PAR bZIP proteins modulate the expression of the BH3-only gene *bcl-gS* in human tumor cells.[6] As this was the first described role for PAR bZIP proteins in the regulation of a gene involved in the execution of apoptosis, we tried to extend this functional association to other members of the BH3-only subfamily. For this purpose, we compared the expression of different BH3-only genes in fibroblasts obtained from the tail of wild-type and PAR bZIP knockout mice, which are defective of TEF, DBP, and HLF. As shown in Figure 1a, all four PAR bZIP (−/−) fibroblast cultures showed no or very low expression of *bik* mRNA as compared with wild type cells. However, no differences were observed when we analyzed other BH3-only genes, including *bnip3*, *bnip3L*, *puma*, *bad*, *bid*, *bim*, *bmf*, and *hrk*. In addition, *noxa* and *bcl-gS* were not detected in these cells. Consistent with the mRNA levels, the expression of Bik protein was significantly higher in wild-type fibroblasts, as can be seen in a western blot analysis of protein extracts from wild-type and PAR bZIP (−/−) cell cultures (Figure 1b). Wild-type number 2 and PAR bZIP (−/−)

number 3 cells were subsequently used for viability analyses. To confirm the association between PAR bZIP proteins and *bik* expression, we transfected wild-type and triple knockout fibroblasts with either TEF or DBP, and found that the overexpressed proteins were able to upregulate the mRNA levels of *bik* in both cell populations, although it was more evident in PAR bZIP (−/−) cells (Figure 1c). In all transfection experiments, TEF resulted to be the most effective PAR bZIP protein in promoting the expression of *bik*. We then studied the expression of PAR bZIP genes and *bik* in a number of human cell lines, and as can be seen in Figure 2a, at least 9 out of 12 cell lines show a good correlation between *tef* and *bik* mRNA levels. The relevance of PAR bZIP proteins in the regulation of *bik* in human cells was shown by overexpressing TEF. As p53 has been described to promote the expression of *bik*,[17] we transfected p53-deficient and wild-type HCT116 cells with pcDNA3-Flag-TEF and then analyzed the expression of *bik* by RT-PCR. Western blot analyses revealed the levels of the transfected protein (Figure 2b). As shown in Figure 2b, the mRNA levels of *bik* were significantly increased (about three-fold by real-time RT-PCR) in response to TEF regardless of the presence of p53. Thus, TEF was able to upregulate *bik* in a p53-independent manner.

Bik gene is transcriptionally activated by TEF.

To verify the transcriptional regulation of *bik* by PAR bZIP proteins, we searched for consensus sequences in the human *bik* gene promoter and found a putative PAR bZIP-binding site of 413 bases upstream from the transcription start site (Figure 3a), which closely matched the consensus sequence of these transcription factors.[4] Then, a 877-bp fragment from the promoter region of *bik*, encompassing the transcription start site, was cloned into a promoterless luciferase reporter vector (*bik*-Luc). We found that the levels of luciferase activity in HEK293T cells co-transfected with the reporter
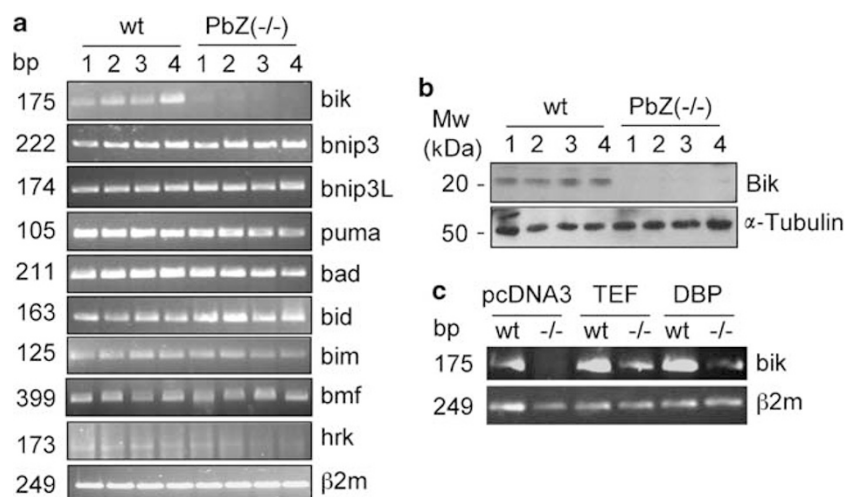

Figure 1 Expression of BH3-only genes in wild-type and PAR bZIP knockout fibroblasts. (**a**) Primary tail fibroblasts from four wild-type (wt) and four PAR bZIP knockout mice (PbZ(−/−)) were analyzed for the expression of BH3-only genes by semiquantitative RT-PCR. (**b**) The expression levels of Bik protein were determined in the same primary cultures by western blot. The levels of α-tubulin are also shown as a loading control. (**c**) Wild type and PAR bZIP (−/−) fibroblasts were transfected with pcDNA3-based constructs containing TEF or DBP cDNAs or with the empty vector and after 48 h, total RNA was extracted and analyzed for the expression of *bik* by RT-PCR. *β2-microglobulin* (β2m) mRNA was used as an amplification control
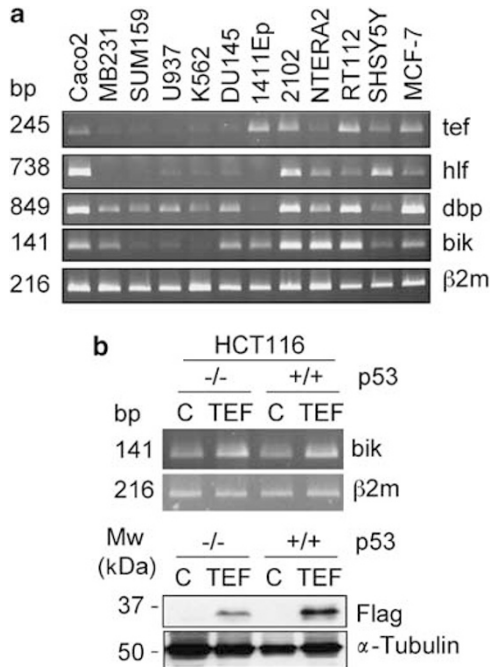
Figure 2 The expression of PAR bZIP genes correlates with the levels of *bik*. (**a**) The mRNA levels of *tef*, *hlf*, *dbp*, and *bik* were analyzed in a number of human cell lines from colon (Caco2), breast (MB231, SUM159, and MCF-7), testicular (1411Ep, 2102, and NTERA2), neural (SHSY5Y), prostate (DU145), bladder (RT112), and hematopoietic (U937, K562) origin. (**b**) HCT116 cells null for the p53 alleles ($-/-$) or containing wild-type p53 ($+/+$) were transfected with TEF cDNA fused to the Flag epitope sequence and then analyzed after an overall culture time of 48 h for *bik* expression by semiquantitative RT-PCR. Transfection efficiency was determined by western blot with anti-Flag antibodies. The levels of α-tubulin were determined to assure equal loading. β2-*microglobulin* (β2m) mRNA was used as an amplification control

construct and TEF were about four-fold higher than those in cells transfected with the vector alone (Figure 3b). Other PAR bZIP proteins were not as efficient as TEF in activating transcription, as we described earlier.[6] However, mutagenesis of the PAR bZIP site abrogated the transactivation capacity of the *bik* promoter in response to TEF. A consensus site for p53 was also present between the PAR bZIP sequence and the transcription start site of the *bik* promoter, but as shown in Figure 3b, the luciferase activity increased very modestly in response to p53. As expected, this activation was not modified when the PAR bZIP site was mutagenized, although the basal level of luciferase activity (cells transfected with the empty vector) was drastically reduced, suggesting that endogenous TEF but not p53 maintains a basal activity of the promoter. In addition, TEF-transfected HEK293T cells were also analyzed for the expression of Bik. As shown in Figure 3c, a significant increase in the protein levels of Bik was detected in these cells compared with empty vector-transfected cells. As expected by the luciferase results, the overexpression of p53 was less effective than TEF in upregulating Bik. The interaction of TEF with the endogenous *bik* promoter was shown by a chromatin immunoprecipitation (ChIP) assay. As no anti-TEF antibodies were available for this purpose, we transfected HEK293T cells with Flag-tagged TEF and

observed a significant enrichment of the promoter (amplified fragment spans the region from -363 to -538) when using an anti-Flag antibody (Figure 3d).

Similar data were obtained when we analyzed the mouse *bik* promoter (Figure 3e). A region of 1938 bp was cloned upstream from the luciferase gene. As shown earlier in its human counterpart, TEF transactivated the mouse promoter as determined by the level of luciferase activity in transfected cells (about eight-fold increase compared with the empty vector transfectants). However, no conserved PAR bZIP or p53-binding sites are present in this promoter. Thus, we constructed a series of deletion fragments (1367, 862, 525, and 197) from the 5′-end, and found that all of them retained the capacity to induce luciferase in response to TEF overexpression (data not shown). The remaining 197 nucleotides were analyzed by electrophoretic mobility shift assay (EMSA), using overlapping probes to delimit the region required for TEF binding. Only 1 of the 10 probes used (5′-GTTAAAGG GGCGGGACGGGGAGGGC), which shares 4 consecutive nucleotides with the TEF site in the human promoter, formed a complex that was disrupted in the presence of specific antibodies (Figure 3f).

The absence of PAR bZIP proteins promote resistance to stress stimuli. To identify the signals that promote apoptosis through the PAR bZIP-*bik* transcriptional pathway, we studied the response of wild-type and PAR bZIP ($-/-$) fibroblasts to different stress stimuli. Wild-type cells showed a higher decrease in viability after treatment with mitoxantrone or cyclophosphamide (<2-fold) or incubation in the absence of serum (a difference of about 40%) compared with the knockout fibroblasts (data not shown). A third stress stimulus was H_2O_2 and, interestingly, we detected the biggest difference in response between the two cell populations. Wild-type cells virtually died after 24 h of culture in the presence of H_2O_2, whereas cell viability was above 40% in knockout fibroblasts (Figure 4a). By 48 h of treatment, these differences were maintained or even increased. Oxidative stress promotes DNA strand breaks and, consistently, the number of cells with damaged DNA was clearly lower in PAR bZIP ($-/-$) fibroblasts treated with H_2O_2, as determined by a comet assay (Figure 4b). We later confirmed that cell death triggered by treatment with H_2O_2 was due to an apoptotic process, as wild-type but not PAR bZIP ($-/-$) cells underwent a characteristic nuclear fragmentation determined by nuclei staining with Hoechst (Figure 4c). To further show the relevance of TEF in signaling oxidative stress stimuli, we transiently transfected HEK293T cells with either a TEF cDNA-containing construct or the empty vector and then analyzed cell viability in response to H_2O_2. As shown in Figure 4d, the overexpression of TEF promoted $\sim30\%$ reduction in cell viability after treatment with 200 μM H_2O_2. Similar results have been obtained with other cell lines, including PAR bZIP knockout fibroblasts (data not shown).

Bik contributes to the PAR bZIP-mediated apoptotic response to oxidative stress. We then studied whether Bik was a relevant PAR bZIP target gene during the response to an oxidative stress stimulus. For this purpose, we stably
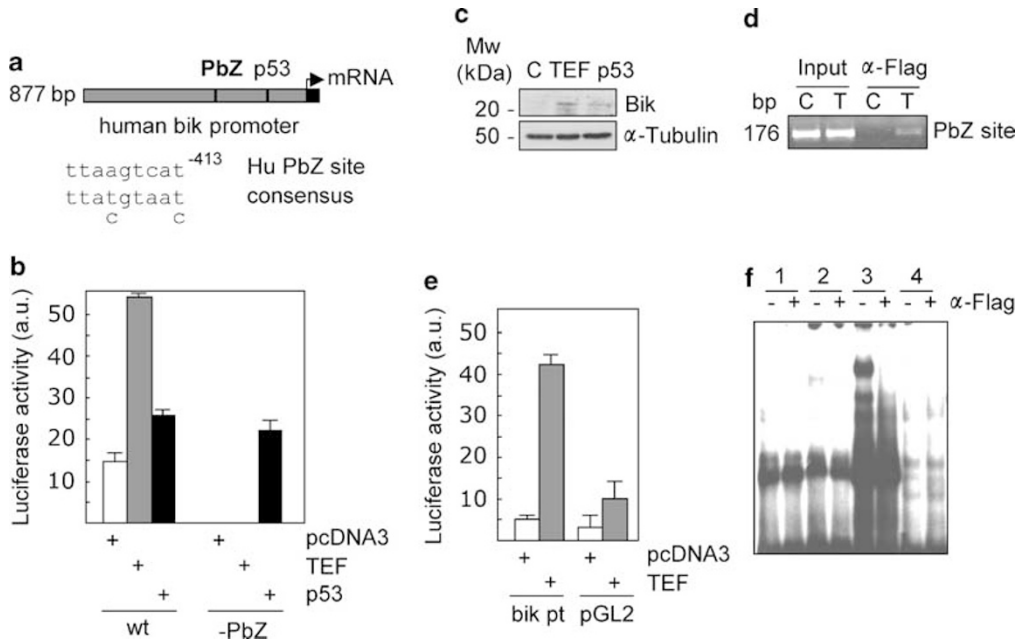
Figure 3 PAR bZIP proteins transactivate the *bik* gene. (**a**) Scheme of human *bik* promoter, fragment-cloned upstream of the luciferase gene showing the sequence of the PAR bZIP (PbZ) site. Numbers indicate the relative nucleotide position upstream of the transcription start site. (**b**) HEK293T cells were co-transfected with a human *bik* promoter-luciferase reporter vector containing wild-type (wt) or mutant (−PbZ) PAR bZIP site and with TEF or p53 cDNAs. After 24 h of transfection, cell extracts were prepared and analyzed for the relative luciferase activity. The mutant promoter lacks the sequence TTAAGT within the PbZ site (**c**) The same cell line was transfected with TEF or p53, and the protein levels of Bik were determined by western blotting after 24 h. (**d**) A ChIP assay was performed using HEK293T cells transfected with a control empty vector, C, or a Flag-tagged TEF cDNA, T. Cross-linked chromatin was incubated with an antibody against Flag, and immunoprecipitates from each sample were analyzed by PCR using primers flanking the PAR bZIP site of the *bik* promoter. As a positive control, a sample representing 0.1% of the total input chromatin was included. (**e**) HEK293T cells were co-transfected with a luciferase reporter vector containing the promoter region of mouse *bik* (*bik* pt) or the empty vector (pGL2), and with a pcDNA3-based construct containing TEF cDNA, and luciferase activity was determined after 24 h. Results were normalized for transfection efficiency with values obtained with pRSV-β-gal. All data points represent the means ± S.D. of three independent experiments. (**f**) HEK293T cells were transfected with FLAG-tagged TEF cDNA, and after 24 h, nuclear extracts were analyzed for the formation of protein–DNA complexes by EMSA using a series of overlapping probes. The figure shows a representative experiment with four probes that cover a region from −197 to −113 and includes the only sequence that binds TEF as determined by using anti-FLAG antibodies

transfected wild-type fibroblasts with a shRNA-containing vector specific for *bik* or with a non-target shRNA control vector. Figure 5a shows a significant reduction of Bik protein levels in cells transfected with the specific interference RNA, which correlated with a higher viability (57%) compared with the control cells (30%) in response to H_2O_2 (Figure 5b). A similar strategy aimed at blocking the expression of a PAR bZIP-independent BH3-only gene, *bnip3L*, did not modify the response to H_2O_2 (data not shown). As this oxidant promotes the generation of reactive oxygen species (ROS), we incubated H_2O_2-treated cells with dihydroethidine (DHE), a widely used fluorigenic indicator for ROS detection, and showed that the population of DHE-stained cells decreased from 64.6% in control transfectants to 25% in *bik*-defective cells (Figure 5c). The different response of these two cell populations was also determined by studying the morphology of fibroblasts in culture (Figure 5d). When treated with H_2O_2, control shRNA-transfected cells detached from the flask surface and acquired a round shape before cell death. This cell death phenotype was clearly attenuated in fibroblasts expressing lower levels of Bik. Consistent results were obtained when the loss of membrane potential, a feature of apoptosis, was determined in transfected cells after treatment with the oxidant agent. Apoptosis was reduced from 20% in control cells to about 5% in *bik* shRNA-transfected

cells (Figure 5e). To further confirm the relevance of Bik in the TEF-mediated apoptotic pathway, we stably over-expressed Bik in PAR bZIP (−/−) fibroblasts (Figure 6a). Consistently, ROS generation, measured as the percentage of DHE-stained cells, was higher in Bik transfectants (45.6%) than in control cells (24%) (Figure 6b). As shown in Figure 6c, fibroblasts overexpressing Bik but not empty-vector-transfected cells responded to H_2O_2 by detaching from the flask and dying, as determined by morphological changes. A similar result was obtained when cells were analyzed for the loss of membrane potential (Figure 6d). Apoptosis increased to more than 15% in *bik*-transfected cells even in the absence of H_2O_2, and it was clearly enhanced (more than 50%) after treatment with the oxidant agent. In contrast, under the same culture conditions, apoptosis was lower than 5% in control-transfected fibroblasts. No variation in the response to H_2O_2 was observed in knockout cells overexpressing Bnip3L (data not shown).

Discussion

Mice devoid of PAR bZIP transcription factors are epilepsy prone, age at an accelerated rate, and die prematurely.[4] In addition, gene expression analyses in different organs indicated that these transcription factors control the expres-
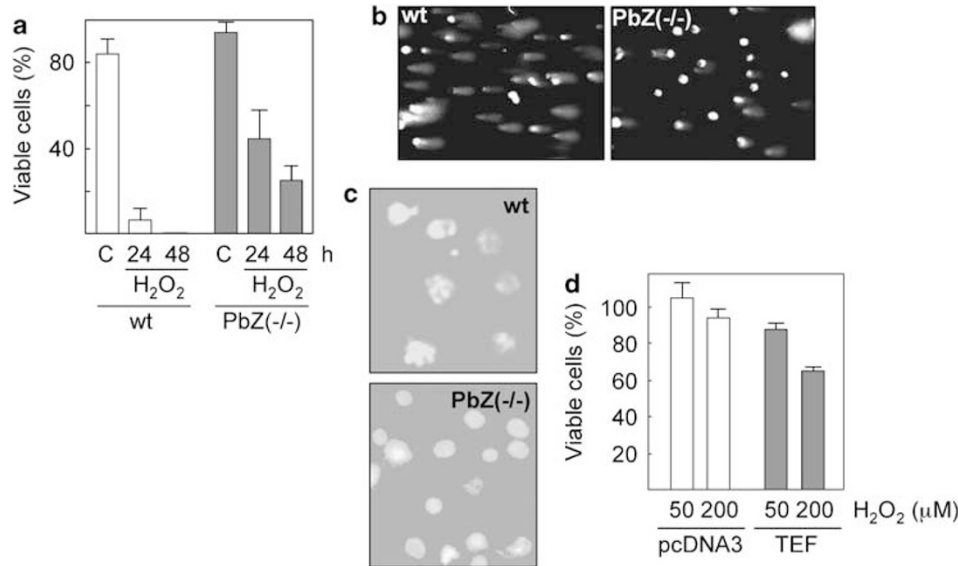
Figure 4 PAR bZIP $(-/-)$ fibroblasts are more resistant to oxidative stress. Wild-type (wt) and PAR bZIP knockout (PbZ$(-/-)$) fibroblasts were cultured in the presence of 300 μM H_2O_2 for 2 h and then maintained in the absence of the oxidant agent for an additional 22 h period. (**a**) Cells were treated as indicated or cultured in H_2O_2-free medium for 46 h after a 2 h-exposure to the oxidant, and cell viability was analyzed by WST-1 assay. C, untreated control cells. Histogram represents the mean + S.D. of independent fibroblast cultures from three wild-type (wt) and three PAR bZIP knockout (PbZ$(-/-)$) mice. (**b**) H_2O_2-treated fibroblasts were analyzed for DNA strand breaks by the comet assay. Ethidium bromide-stained tailed nuclei were visualized under a fluorescence microscope. Note the higher number of comets observed in wild-type cells. (**c**) Nuclear fragmentation of wild-type and PAR bZIP $(-/-)$ cells treated with H_2O_2 was detected after staining with the specific DNA dye Hoechst and analyzed by the fluorescence microscope. (**d**) HEK293T cells were transfected with a pcDNA3-based construct containing TEF cDNA or with the empty vector. After 24 h of transfection, cells were treated with two different concentrations of H_2O_2 for the time period described above, and then cell viability was determined by the WST-1 assay. Histogram represents the mean $\pm$ S.D. of three independent experiments

sion of many enzymes and regulators involved in detoxification and drug metabolism.[5] However, the relevance of PAR bZIP proteins at the cellular level has not been thoroughly investigated. By using mouse tail fibroblasts from PAR bZIP $(-/-)$ mice, we showed that PAR bZIP proteins, mainly TEF, were needed to maintain the basal mRNA levels of *bik*, a BH3-only proapoptotic member of the Bcl-2 family. Interestingly, we did not observe defective expression of other BH3-only genes, including *bnip3*, *bnip3L*, *puma*, *bad*, *bid*, *bim*, *bmf*, and *hrk*, whereas *noxa* and *bcl-gS* were not detected in these cells. Consistently, overexpression of TEF promoted the upregulation of *bik*. This proapoptotic gene has been shown to be induced by p53,[17] and not surprisingly the protein levels of Bik were increased in cells overexpressing p53. In line with these results, analysis of the promoter revealed the presence of functional PAR bZIP and p53-binding sites, although the contribution of p53 to the activation of *bik* promoter appeared to be modest. Furthermore, the overexpression of TEF induced *bik* regardless of the presence of functional p53, which helps confirm the independent transcriptional activity of TEF. Although TEF is expressed in the pituitary gland during embryonic development, it appears in several tissues in the mature organism, including brain, lung, liver, spleen, and kidney.[3] In line with this, we showed the expressions of *tef*, *dbp*, and *hlf* in a number of human cell lines, and also found a good correlation with the mRNA levels of *bik*. To test the functional relevance of this novel transcriptional pathway, we challenged wild-type and PAR bZIP knockout fibroblasts with different stress stimuli, including chemotherapeutic drugs, H_2O_2, and serum deprivation.

Interestingly, the biggest difference in viability between both cell populations was observed in response to H_2O_2. It is worth noting that treatment of neural cell lines with H_2O_2 upregulated the mRNA levels of *bik* as determined in microarray experiments,[23] suggesting a proapoptotic role of Bik in oxidative stress-induced neuronal apoptosis. In our fibroblast cell model, *bik* was constitutively expressed and did not show significant variation in response to H_2O_2. A result that we have confirmed in four different human cell lines (RT112, HCT116, HEK293T, and U937). However, we found that blockade of *bik* by RNA interference rendered wild-type fibroblasts more resistant to H_2O_2-induced apoptotic cell death. A likely explanation is that Bik may be held in check by anti-apoptotic proteins, such as Bcl-2, Bcl-x$_L$ or Mcl-1, and treatment with H_2O_2 may overcome this blockade by reducing the levels or activity of these proteins, allowing Bik to promote apoptosis. In line with this, Bik fails to relieve Mcl-1-mediated inhibition of Bak, but knockdown of Mcl-1 overcomes this inhibition and allows for the Bak activation by Bik.[24] Alternatively, down-regulation of anti-apoptotic proteins that block the activation of caspases, including IAP family members, may be necessary for Bik-mediated apoptosis in these cells. Consistent with this hypothesis, it has been recently shown that the loss of XIAP renders cells highly sensitive to oxidative stress.[25] In addition, suppression of Bik expression by using siRNA has been shown to block apoptosis induced by a stress stimulus (growth factor deprivation) in breast cancer cell lines.[26] We recently showed for the first time that PAR bZIP proteins are involved in the regulation of apoptosis through the transcriptional control of the BH3-only gene, *bcl-gS*.[6] Blockade of the *bcl-gS*
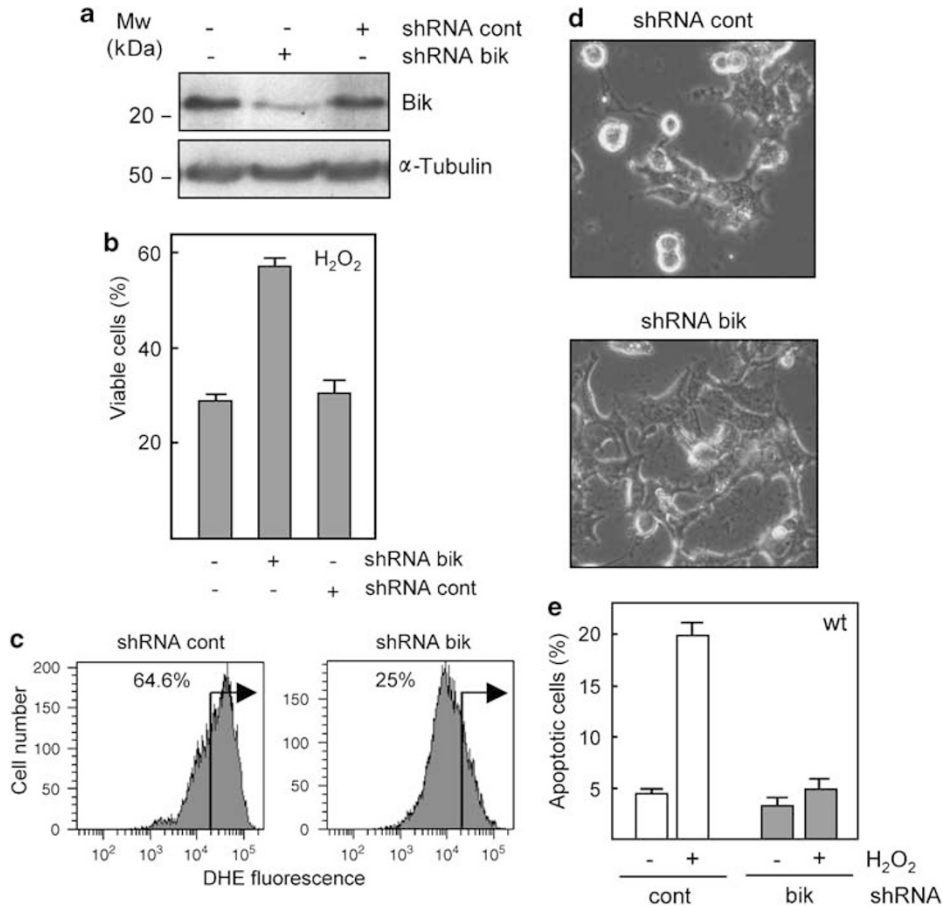
Figure 5 Blockade of Bik expression promotes resistance of wild-type fibroblasts to oxidative stress-induced apoptosis. (**a**) Wild-type fibroblasts were stably transfected with a non-target control or *bik*-specific interference RNA, and after the selection of transfectants, the reduced levels of Bik protein were assessed by western blot analysis. (**b**) After treatment with 200 μM H_2O_2, viability of transfected cells was analyzed by the WST-1 assay. Viability in untreated cells was referred to as 100%. (**c**) Transfected cells were treated with H_2O_2 and then ROS generation was quantitated by flow cytometry after dihydroethidine (DHE) staining. Arrows indicate markers used to determine the percentage of DHE-stained cells. The results of one representative experiment out of three are represented. (**d**) Alterations in the morphology of transfected fibroblasts after treatment with H_2O_2 recorded with phase-contrast microscopy. Note that shRNA *bik*-transfected cells remain attached and spread. (**e**) The percentage of apoptotic cells was determined by the loss of mitochondrial membrane potential after treatment with H_2O_2. All histograms represent the means $\pm$ S.D. of three independent experiments

expression in tumor cells by siRNAs give rise to reduced levels of apoptotic cell death after treatment with chemotherapeutic drugs. Thus, PAR bZIP proteins seem to transduce different stress stimuli through the regulation of different BH3-only genes.

A significant fraction of cellular Bik localizes to the endoplasmic reticulum membranes, where it can promote the release of Ca^{2+} that leads to the activation of effector caspases.[27] Oxidative stress triggers the endoplasmic reticulum Ca^{2+} release and therefore, the presence of Bik may be critical to set the apoptotic cascade in motion after exposure to oxidant agents. In addition, it has been recently shown that BH3 mimetics induce the production of superoxide in neurons, which is followed by depolarization and fragmentation of mitochondria.[28] Our model of *bik* shRNA-transfected wild-type fibroblasts is also consistent with these data, as cells produced lower amounts of ROS and showed morphological and biochemical evidences of decreased apoptosis in response to H_2O_2. Therefore, our data suggest that in fibroblasts, PAR bZIP proteins maintain a basal level of Bik, which is needed for the production of ROS, and a proper apoptotic response to oxidative stress; thus, downregulation

of this transcriptional pathway makes cells less sensitive to the stress stimulus. Of the BH3-only subfamily, *puma*, *noxa*, and *bim* have been reported to be upregulated in cortical neurons exposed to oxidative stress, although only the absence of *puma* makes neurons resistant to the induction of apoptosis by multiple oxidative stressors.[29] *Puma* is a known target gene of p53 and does not appear to be regulated by PAR bZIP proteins. Therefore, it is likely that both p53-*puma* and PAR bZIP-*bik* represent two transcriptional pathways needed for a proper apoptotic response to oxidative stress, and the relevance of each one may depend on the type or status of the cell. Oxidative stress has been implicated as a key trigger of cell death in several human disorders, including ischemia, neurodegenerative diseases, and diabetes.[30] Oxidant exposure has also been involved in the apoptotic cell death of repopulating fibroblasts during wound healing.[31] Furthermore, fibroblasts from old individuals have a reduced capacity to undergo apoptosis in response to oxidative stress compared with younger cells,[32] which may contribute to a number of age-dependent disorders. Thus, the results presented here provide a novel pathway that may help
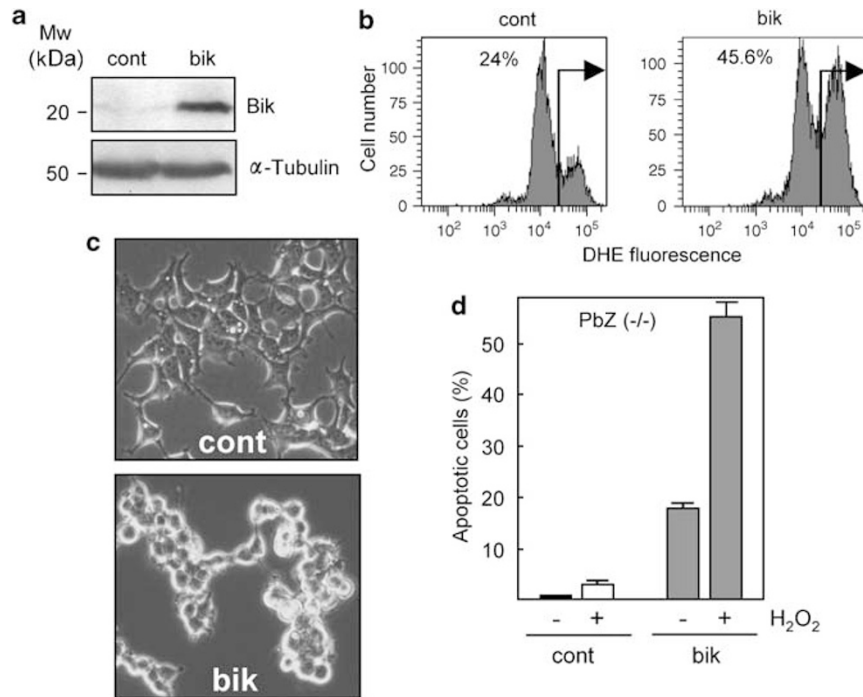
Figure 6 Overexpression of Bik sensitizes PAR bZIP knockout fibroblasts to oxidative stress-induced apoptosis. (**a**) PAR bZIP ($-/-$) cells were stably transfected with a *bik* cDNA-containing construct or an empty expression vector (cont), and after the selection of transfectants, the expression levels of Bik protein were determined by western blotting. (**b**) Transfected cells were treated with 200 μM H_2O_2, and then ROS generation was quantitated by flow cytometry after dihydroethidine (DHE) staining. Arrows indicate markers used to determine the percentage of DHE-stained cells. The results of one representative experiment out of three are represented. (**c**) Alterations in the morphology of transfected cells after treatment with H_2O_2 recorded with phase-contrast microscopy. Note that *bik*-transfected cells detach and acquire a round shape. (**d**) The percentage of apoptotic cells was determined by the loss of mitochondrial membrane potential after exposure to H_2O_2. All histograms represent the means $\pm$ S.D. of three independent experiments

unravel the molecular basis of the responses to oxidative stress in pathological or physiological conditions.

Together, this study shows a novel role for PAR bZIP proteins in the transcriptional regulation of *bik*, and supports that this pathway is necessary to activate the apoptotic cascade in response to oxidative stress. Further studies will be needed to deepen the understanding of the biological relevance of this transcriptional pathway in the stress response of different cell types, including neurons and cancer cells, and in the pathophysiology of degenerative diseases. It will also be of interest to decipher the molecular mechanism that activates PAR bZIP proteins in response to oxidative stress, and to find other targets of PAR bZIP proteins involved in apoptosis in different cell systems.

Materials and Methods

Cells. Fibroblasts were obtained from wild-type and PAR bZIP knockout mice,[4] as described.[33] In brief, mouse tails were minced and digested with 1 mg/ml type 1 collagenase in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 100 units/ml penicillin and 100 μg/ml streptomycin for 1 h at 37 °C. After letting the tissue fragments settle, the supernatant was collected and one-tenth volume of fetal calf serum (FCS) (Flow Laboratories, Irvine, CA, USA) was added to stop the collagenase action. The remaining tissue fragments were redigested for an additional 1 h, and the resulting cell suspension was centrifuged and re-suspended in DMEM with 10% FCS and antibiotics. Thus, each culture was derived from one tail, without any clonal selection. In all experiments, both wild-type and PAR bZIP ($-/-$) cells were used between passages 3 and 6.

HEK293T cells were maintained in DMEM supplemented with 10% FCS. Colorectal carcinoma cells HCT116 and their p53 ($-/-$) derivatives,[34] a gift from

B Vogelstein, were grown in McCoy's 5A medium (Invitrogen) supplemented with 10% FCS.

Cells were treated with 200 μM H_2O_2 unless otherwise indicated.

Analysis of cell death and ROS generation. Cell viability was analyzed by using the WST-1 assay (Clontech, Mountain View, CA, USA) following the manufacturer's recommendations. Hoechst 33258 staining (Sigma, St Louis, MO, USA) was used to visualize nuclear changes and apoptotic body formation, which are characteristic of apoptosis. Cells (0.5×10^6) were re-suspended in PBS and incubated with 1 μg/ml Hoechst at 37 °C for at least 30 min and then visualized in a fluorescence microscope (AxioVision, Carl Zeiss, Germany). To determine mitochondrial membrane potential, cells were incubated with 20 nM 3,3'-dihexyloxa-carbocyanine iodide and propidium iodide for 30 min at 37 °C and analyzed immediately by flow cytometry. In all experiments, viability of untreated wild-type and PAR bZIP ($-/-$) cells were above 85%.

For ROS generation analysis, cells were exposed to 200 μM H_2O_2 for the indicated time period and then incubated with 5 μM DHE (Molecular Probes, Eugene, OR, USA) at 37 °C for 15 min. In the presence of ROS, DHE is oxidized to red fluorescent ethidium. After incubation, cells were washed with cold PBS and ROS generation was determined by flow cytometry.

Analyses of RNA. Total RNA was prepared using TRIZOL reagent (Invitrogen). To assess mRNA expression, semiquantitative RT-PCR and quantitative real-time PCR were performed as described earlier.[35] The generated cDNA was amplified by using primers for mouse *bik* (5′-GGAGCCTGTGAGAGAC GTGG and 5′-GACACCTGTCCGGCTGTAGG), *bnip3* (5′-GCTCCCAGACACCAC AAGAT and 5′-TGAGAGTAGCTGTGCGCTTC), *bnip3L* (5′-GCAATGGCAATGAG AATGG and 5′-CATCTTCTTGTGGTGAAGG), *puma* (5′-TCCTGGAGCCCCAGAA ATGG and 5′-CTGGCCAGGGTGTCAGAAGG), *bad* (5′-GGGATGGAGGAGGAGC TTAG and 5′-CCCACCAGGACTGGATAATG), *bid* (5′-ACAGACCTGCTGGTGTTC GG and 5′-TTCCTTGGTTGAAGGAGCGG), *bim* (5′-CTCAACCCCAGACACACGG and 5′-GCATGGCTTGGTGCAGCGG), *hrk* (*dp5*) (5′-AGCCCGGACCGAGCAACA

GG and 5′-GTGGCTCTCTCTGTAGCTGG), *bmf* (5′-ATGTGTTCCAGTCAGAGG AT and 5′-GGAACTGGTCTGCAATACAC) and *β2-microglobulin* (5′-GGCCTGTAT GCTATCCAGAAA and 5′-GGCGTATGTATCAGTCTCAGTGG), and primers for human *bik* 19, *tef* (5′-TGGTCCTGAAGAAGCTGATGG and 5′-TCCAGGTCCATGT ACTCCAG), *hlf* (5′-CGTGTGGTGCTATCAGACAGG and 5′-CCCTTCCCTATGA CGGAGAT), *dbp* (5′-TCCTAGGATTTGCAGCGTTCTGGA and 5′-AGAAGCGATG TCTTCGAGGGTCAA), and *β2-microglobulin* (5′-GAGACATGTAAGCAGCATCA and 5′-AGCAACCTGCTCAGATACA).

Western blot analysis. Protein expression was determined by western blotting as described earlier.[36] Proteins (30 to 60 μg) were separated on a 12% polyacrylamide gel and transferred to nitrocellulose. Blots were blocked with 3% bovine serum albumin and incubated with rabbit anti-Bik (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse anti-Flag and anti-α-tubulin (both from Sigma) antibodies, and then incubated with goat anti-rabbit or anti-mouse antibody conjugated to alkaline phosphatase (Sigma). Bound antibody was detected by a chemiluminescence system (Applied Biosystems, Foster City, CA, USA).

EMSA. Cells transfected with a TEF cDNA inserted into a FLAG-containing vector were lysed and nuclear fractions were re-suspended in 20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 20% glycerol. Nuclear extracts (5–10 μg of total protein) were incubated with ^{32}P-labeled double-stranded DNA probes from the promoter region of the mouse *bik* gene. A total of 10 overlapping probes (9 of 25 bp and 1 of 17 bp) covering a region of 197 bp upstream the transciption start site were used. Samples were run on a 5% non-denaturing polyacrylamide gel in 200 mM Tris-borate, 2 mM EDTA. The gels were dried and visualized by autoradiography. Whenever indicated, nuclear extracts were preincubated with monoclonal anti-FLAG antibodies (Sigma).

ChIP assay. Purification of sonicated nuclear lysates and immunoprecipitation were performed as described elsewhere.[37] Precipitates were heated at 65 °C for at least 4 h to reverse the formaldehyde cross-linking. Proteins were digested in the presence of 20 μg/μl proteinase K, and then DNA fragments were purified with a QIAquick Spin Kit (Qiagen, Valencia, CA, USA). For PCR, 2 μl from each 40 μl DNA sample and 30 cycles of amplification were used.

Comet assay. DNA strand breaks were determined as described elsewhere.[38] Briefly, 50 000 cells re-suspended in PBS were added to 0.5% low-melting agarose and pipetted onto a precoated slide. An additional agarose layer without cells was added after solidification of the first layer. After solidification, the slides were placed in alkaline solution and the cells were lysed in the dark at 4 °C for 1 h. The slides were then subjected to electrophoresis, rinsed with neutralization buffer, and allowed to dry. The slides were stained with 0.5 μg/ml ethidium bromide just before examination under the microscope.

Gene silencing and overexpression of *bik*. Wild-type fibroblasts were transfected with 2 μg of non-target or *bik*-specific shRNAs (both from Sigma) by using lipofectamine 2000 (Invitrogen). After 24 h, transfected cells were selected in the presence of 1.2 μg/ml puromycin for 7 days and then analyzed for the expression levels of Bik protein. A pcDNA3-based construct containing human *bik* cDNA was used to transfect PAR bZIP (−/−) fibroblasts by lipofectamine 2000 and transfectants were then selected with neomycin and analyzed for cell viability in response to stress stimuli.

Gene reporter assays. A genomic PCR fragment from the promoter region of human *bik* (877 bp) was cloned into *Kpn*I and *Hind*III sites of the pGL2-basic luciferase reporter vector (Promega Corp., Madison, WI, USA). The authenticity of the construct was confirmed by sequencing. HEK293T cells were co-transfected with 1 μg of the promoter-containing pGL2 construct, 1 μg of TEF or p53 cDNAs cloned in the pcDNA3 expression vector, and 20 ng of pRSV-β-gal by lipofection using Superfect (Qiagen). After 24 h of transfection, cell extracts were prepared and analyzed for the relative luciferase activity by a dual-light reporter gene assay system (Applied Biosystems). Results were normalized for transfection efficiency with values obtained with pRSV-β-gal. Mutagenesis of the TEF consensus site in the *bik* promoter was carried out using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with primers 5′-GAACGCTGGCTTCACGCTG TCATCATTTCGTCCTCT-3′ and 5′-AGAGGACGAAATGATGACAGCGTGAAGC CAGCGTTC-3′. The *bik* promoter DNA insert was sequenced to verify the

mutations. A PCR fragment of 1938 bp from the promoter of mouse *bik* gene and a series of deletion fragments from the 5′-end were also cloned in the luciferase reporter vector. HEK293T cells were co-transfected with the mouse promoter constructs and TEF, and 24 h later, cell extracts were analyzed for the relative luciferase activity as described above.

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