Puma is an essential mediator of p53-dependent and -independent apoptotic pathways

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Summary

***Puma* encodes a BH3-only protein that is induced by the p53 tumor suppressor and other apoptotic stimuli. To assess its physiological role in apoptosis, we generated *Puma* knockout mice by gene targeting. Here we report that Puma is essential for hematopoietic cell death triggered by ionizing radiation (IR), deregulated c-Myc expression, and cytokine withdrawal. Puma is also required for IR-induced death throughout the developing nervous system and accounts for nearly all of the apoptotic activity attributed to p53 under these conditions. These findings establish Puma as a principal mediator of cell death in response to diverse apoptotic signals, implicating Puma as a likely tumor suppressor.**

Introduction

The p53 tumor suppressor maintains normal growth control and genomic stability by enforcing a G1 cell cycle arrest or inducing apoptosis in response to DNA damage and other forms of cell stress (Vogelstein et al., 2000). Disruption of this pathway promotes tumorigenesis, which frequently occurs in human cancers through the inactivation of p53 or its upstream regulators (e.g., $p19^{ARF}$ and ATM/ATR). The apoptotic function of p53 is essential for its tumor suppressor activity (Schmitt et al., 2002); however, the mechanism by which p53 physiologically controls cell death is ill defined. This response likely depends on p53's transcriptional regulation of target genes that now includes ~70 candidates, half of which have a purported activity in triggering apoptosis. It also has been proposed that no single gene may account for p53-dependent apoptosis and that combinations of targets may be required in a coordinated manner to elicit an apoptotic signal (Zhao et al., 2000).

Recent interest in how p53 triggers cell death has focused on Puma/Bbc3 (*p*53 *u*pregulated *m*odulator of *a*poptosis/*B*cl-2 *b*inding *c*omponent-*3*; referred to hereafter as Puma), a BH3-only proapoptotic Bcl-2 family member. *Puma* was identified as a p53-inducible gene by differential expression analyses and as a Bcl-2 binding protein in a yeast two-hybrid screen (Nakano and Vousden, 2001; Yu et al., 2001; Han et al., 2001). One feature that sets *Puma* apart from other p53 targets is that *Puma* expression is regulated not only by p53 through consensus p53-responsive elements located within its promoter, but also by glucocorticoids and serum deprivation (Han et al., 2001). Puma associates with the mitochondria and induces cell death when overexpressed in various cell lines and its apoptotic activity requires an intact BH3 domain. These in vitro studies, as well as findings derived from a somatic knockout tumor cell line (Yu et al., 2003), suggested that Puma may play an important role as an in vivo regulator of apoptosis.

Results and Discussion

To examine the physiological contribution of Puma to apoptosis, we generated *Puma*-deficient mice by deleting exons 1–3, which includes the transcription and translation start sites and the entire BH3 domain (Figure 1). $Puma^{+/-}$ animals were identified using PCR and Southern blot analysis (Figure 1B and data not shown) and bred to generate homozygous mutant mice. Viable $Puma^{-/-}$ mice were obtained and no obvious developmental defects were evident (Figure 1C). Western blot and real-time PCR analysis demonstrated that Puma is not expressed in hematopoietic cells derived from these knockout mice (Figure 1D and Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/4/4/321/DC1).

Induction of DNA damage in primary thymocytes by γ-irradiation (γ-IR) provokes rampant apoptosis (Lowe et al., 1993;

SIGNIFICANCE

The p53 tumor suppressor protects against cancer by eliminating cells that have suffered DNA damage or proliferate in an uncontrolled manner, by inducing apoptosis. We describe here the mechanism by which p53 elicits cell death and show that this occurs through the induction of Puma, a BH3-only proapoptotic protein. The establishment of Puma as an essential mediator of p53-dependent apoptosis reveals a novel target that could be exploited for either sensitizing or conferring resistance to chemo- and radiation-based therapies.

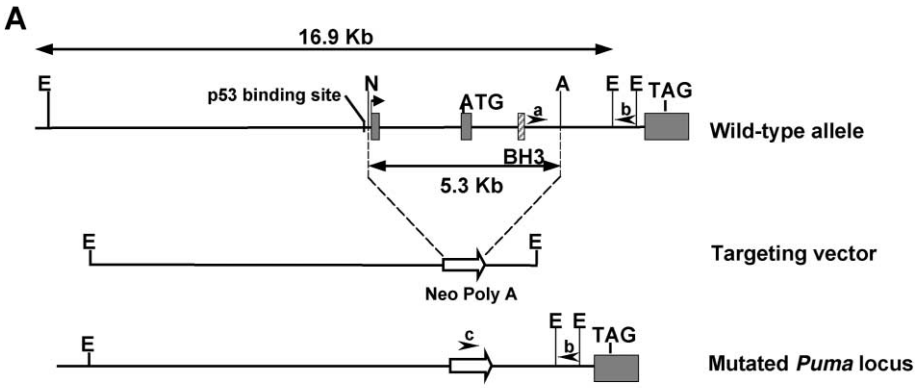


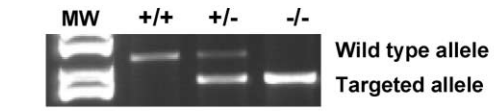
Figure 1. Generation of *Puma*-deficient mice

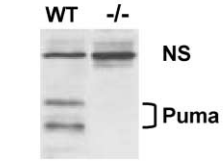
A: Genomic structure of the *Puma* locus and the targeting scheme for generating *Puma*$^{-/-}$ mice.

B: PCR-based genotyping of WT and *Puma*-deficient animals.

C: Genotyping results of litters derived from *Puma*$^{+/-}$ crosses at weaning.

D: Western blot analysis of Puma expression in primary myeloid progenitors derived from WT and *Puma*$^{-/-}$ mice (NS, nonspecific band).





C

Genotype of offspring (4 wks): *Puma*$^{+/-}$ x *Puma*$^{+/-}$

WT	*Puma*$^{+/-}$	*Puma*$^{-/-}$	
26	68	26	n = 123 Total

Clarke et al., 1993). Susceptibility to cell death following DNA damage specifically requires p53, as *p53*$^{-/-}$ thymocytes are resistant to γ-IR yet readily undergo apoptosis in response to other stimuli such as glucocorticoids. Thymocyte apoptosis can therefore occur through at least two distinct pathways: (1) a DNA damage-induced response, which requires an intact p53 pathway; and (2) a glucocorticoid-mediated process that is independent of p53 status. Since Puma expression is induced by p53 as well as glucocorticoids, the role of Puma in these processes was addressed by preparing thymocytes from wild-type (WT), *Puma*$^{+/-}$ and *Puma*$^{-/-}$ littermates at 6–7 weeks of age. The cells were placed in culture and divided into three groups: (1) control; (2) dexamethasone (1 μM, Dex); and (3) DNA damage (5 Gy, γ-IR). Samples were harvested at specific intervals following treatment and cell viability assessed by propidium iodide staining and FACS analysis (Figure 2). As expected, γ-IR efficiently induced p53 expression and the death of WT thymocytes. Strikingly, *Puma*$^{-/-}$ thymocytes maintained viability that equaled the survival of *p53*$^{-/-}$ cells following γ-IR despite the fact that p53 was efficiently induced in *Puma*$^{-/-}$ cells (Figure 2). *Puma*$^{+/-}$ thymocytes displayed an intermediate apoptotic response, suggesting that Puma is rate limiting in this process. *Puma*$^{-/-}$ thymocytes were also refractory to Dex-induced apoptosis when compared to WT and p53 null cells, but ultimately underwent cell death by 46 hr (Figure 2 and Supplemental Figure S1 on *Cancer Cell* website). The partial sensitivity of *Puma*$^{-/-}$ thymocytes to Dex suggests that additional factors participate in a redundant manner in triggering this cell death response. One such candidate is *Bim*, a BH3-only protein whose expression is also induced by dexamethasone (Wang et al., 2003). Similar to the *Puma* knockout, loss of *Bim* provides a partial protection against Dex at earlier times but not at later times (Supplemental Figure S1 online). Although Bim had also been reported to contribute to IR-induced thymocyte apoptosis (Bouillet et al., 1999), *Bim* deficiency had little effect on the survival of thymocytes following γ-IR, especially when compared to *Puma*$^{-/-}$ cells (Figure 2 and Supplemental Figure S1 on *Cancer Cell* website). As expected (Knudson et al., 1995), *Bax*-deficient thymocytes were

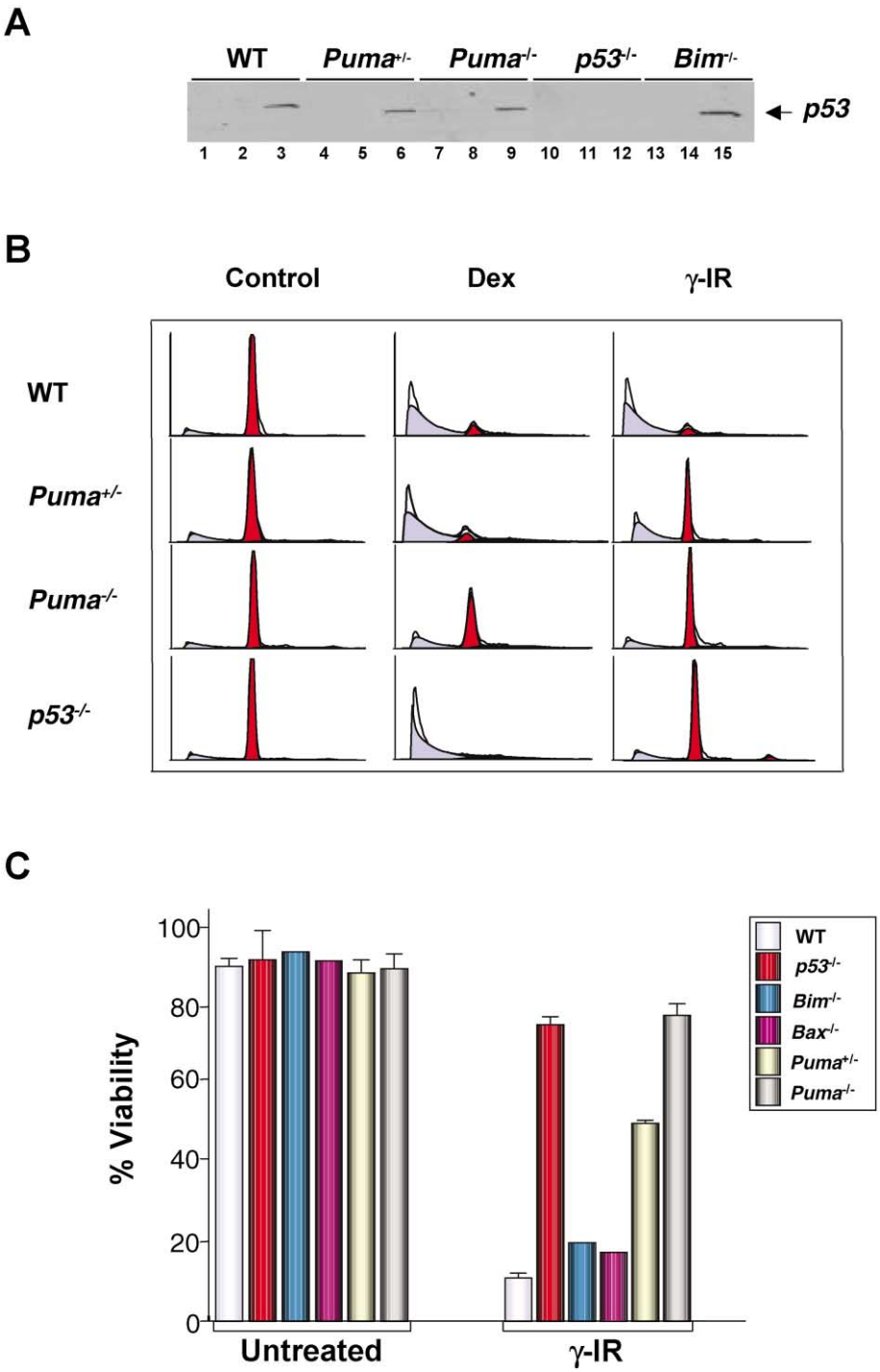


Figure 2. *Puma*-deficient thymocytes are resistant to DNA damage-induced apoptosis

Thymocytes from ~6-week-old mice were placed in culture and treated with either 1 μM dexamethasone (Dex), 5 Gy ionizing radiation (γ-IR), or vehicle-only (control) for the indicated times.

A: Two hours following treatment, cells were harvested and analyzed for p53 expression by Western blot analysis in Control (lanes 1, 4, 7, 10, and 13), Dex (lanes 2, 5, 8, 11, and 14), and γ-IR-treated (lanes 3, 6, 9, 12, and 15) samples.

B: Sixteen hours following treatment, cells were harvested and analyzed for viability by propidium iodide staining and FACS analysis. Apoptotic cells exhibited sub-G1 DNA (light blue) and viable cells displayed G1 and G2 DNA content (red).

C: Quantitation of cell death in response to γ-IR. Results are representative from two independent experiments.

also sensitive to γ-IR and underwent massive cell death (Figure 2C). The resistance to γ-IR-induced apoptosis was also evident in *Puma*-deficient myeloid progenitors and B cells (Supplemental Figures S3 and S4 online). Thus, Puma is a critical physiological mediator of p53-induced apoptosis of primary thymocytes in response to DNA damage and contributes to glucocorticoid-mediated cell death.

The role of Puma in DNA damage-induced death in the thymus, as well as throughout the developing nervous system, was also explored in the whole animal (Figure 3). Postnatal day 5 (P5) WT and *Puma*-deficient littermates were exposed to 18 Gy γ-IR and allowed to recover for 6 hr. Tissues were collected and analyzed for cell death by morphological and TUNEL-based assays. As observed in $p53^{-/-}$ mice, there was an almost complete abrogation of radiation-induced apoptosis in the thymus of *Puma* null animals after γ-IR (Figures 3e and 3f). Consistent with our ex vivo findings, apoptosis was compromised in the $Puma^{+/-}$ thymus compared to WT, although cell death was

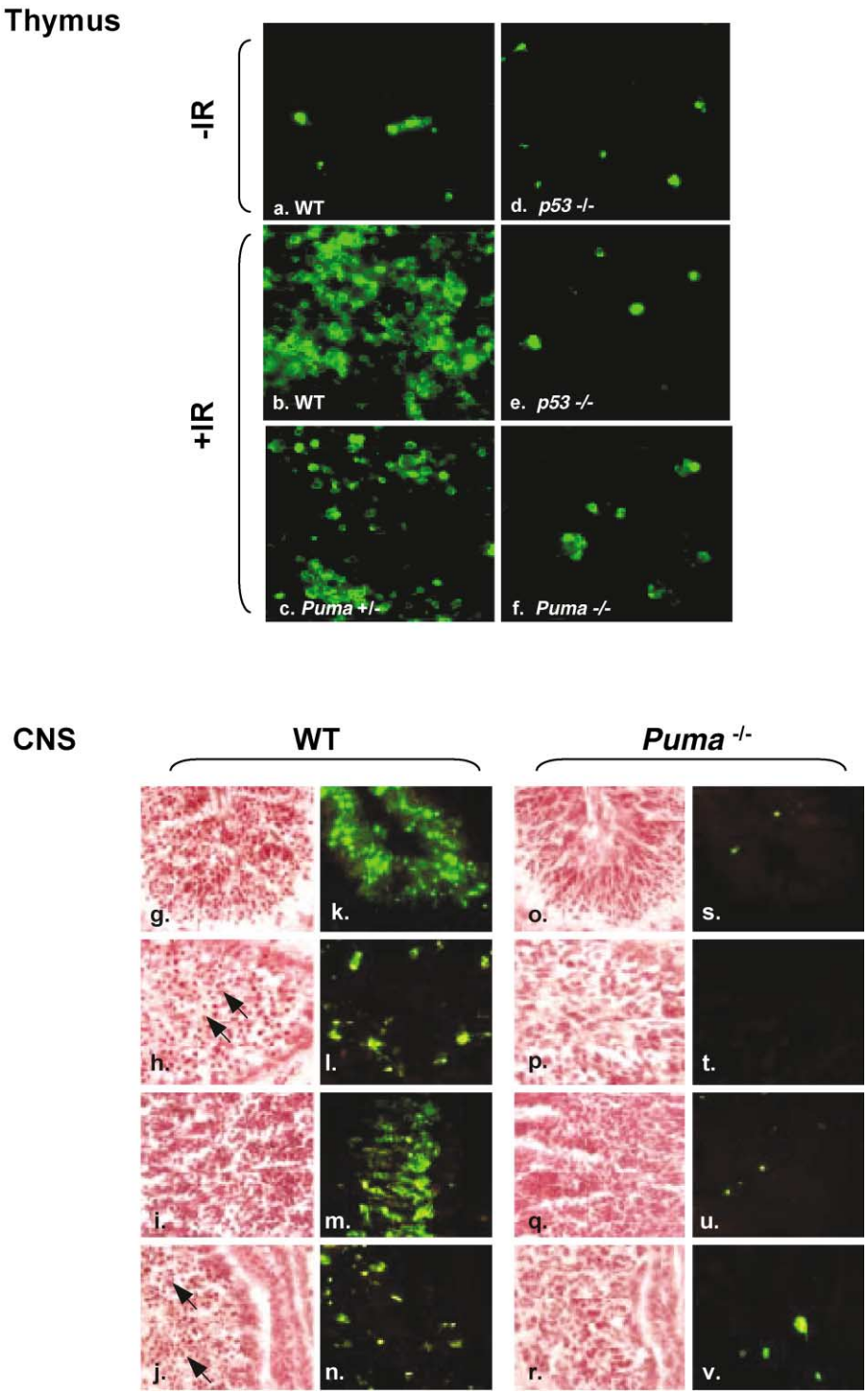
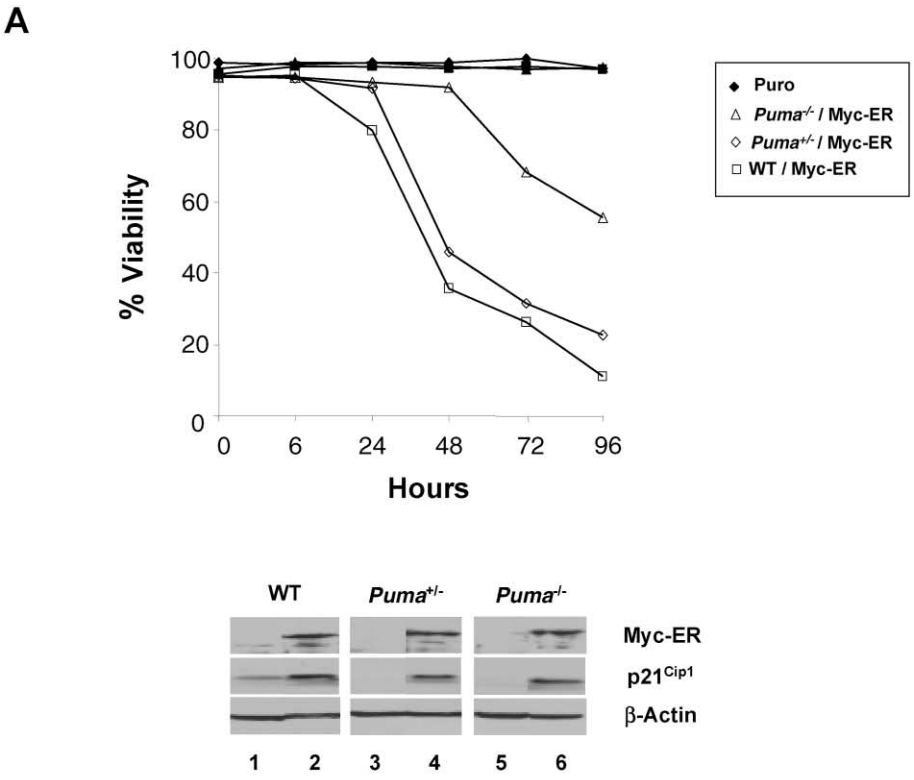


Figure 3. *Puma* is required for γ-IR-induced apoptosis in vivo in the thymus and nervous system

Top panels: Puma mediates p53-dependent death in the thymus following γ-IR. Abundant apoptosis in WT thymus (**b**), but markedly reduced apoptosis in the $Puma^{+/-}$ thymus (**c**) was identified using TUNEL staining 6 hr after 18 Gy of γ-IR. $Puma^{-/-}$ (**f**) or $p53^{-/-}$ (**e**) thymi display an almost complete resistance to γ-IR-induced apoptosis. Basal levels of apoptosis in unirradiated $p53^{-/-}$ (**d**) and WT (**a**) thymi are also shown. **Bottom panels:** γ-IR-induced apoptosis in the developing CNS is mediated by Puma. Widespread IR-induced apoptosis was, as expected, obvious in the cerebellar external granule layer (**g** and **k**), the dentate gyrus (**h** and **l**), the retina (**i** and **m**), and the subventricular zone of the lateral ventricle (**j** and **n**) of WT mice. By contrast, $Puma^{-/-}$ tissues were highly resistant to γ-IR-induced apoptosis (**o–v**). Apoptosis was detected by neutral red staining (**g–j** and **o–r**) or TUNEL (**k–n** and **s–v**). Arrows indicate pyknotic cells. Magnification is 400×.

substantially higher than in the $Puma^{-/-}$ thymus (Figures 3b, 3c, and 3f). Exposure of the developing nervous system to ionizing radiation also leads to p53-dependent apoptosis in proliferating and immature cell populations (Herzog et al., 1998). Remarkably, Puma deficiency completely blocked cell death throughout the developing nervous system following γ-IR treatment (Figure 3). Specifically, the cerebellar external granule layer (EGL), the dentate gyrus (DG) of the hippocampus, the retina, and the subventricular zone (SVZ) of the lateral ventricle of $Puma^{-/-}$ animals were highly resistant to apoptosis, as judged by the absence of pyknotic figures (Figures 3o–3r), whereas extensive cell death occurred in WT tissues (Figures 3g–3j). Histological identification of pyknotic cells as being apoptotic was confirmed by TUNEL analysis, which revealed pronounced and widespread apoptosis in all γ-IR-treated WT CNS regions examined (Figures 3k–3n), while there were virtually no TUNEL-positive cells in the γ-IR-



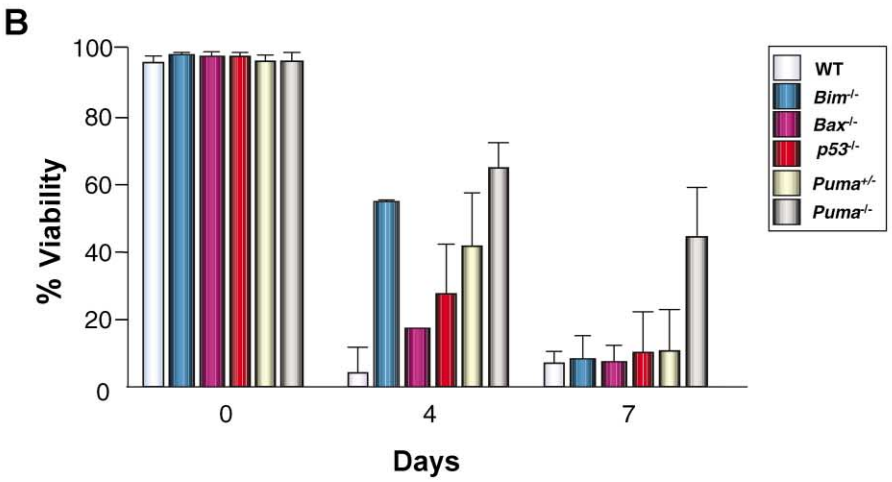


Figure 4. *Puma* is required for p53-dependent and -independent cell death signals

Primary bone marrow-derived myeloid stem cells were expanded in medium containing IL-3, IL-6, and SCF.

A: Puma mediates the p53 apoptotic checkpoint triggered by Myc. Primary myeloid progenitors were engineered to express a puromycin-resistance marker alone (Puro) or Puro and the conditional *Myc-ER* transgene. Levels of Myc expression and activity were equivalent between each genotype as demonstrated by Western blot analysis for Myc-ER and the induction of $p21^{Cip1}$ following 4-HT treatment (lower panel: lanes 1, 3, and 5, Puro-only; lanes 2, 4, and 6, Myc-ER). Viability was determined after activation of Myc-ER with 4-HT at the indicated times by trypan blue dye exclusion. Key: □, WT; ◇, $Puma^{+/-}$; Δ, $Puma^{-/-}$; closed symbols, Puro-only; open symbols, Myc-ER. Data shown represent three independent experiments.

B: The intrinsic apoptotic response to cytokine withdrawal is mediated by Puma. Cells were maintained in media containing 10% serum with and without cytokine for the indicated times and viability was determined by FACS and trypan blue dye exclusion. Data represent the average of five independent experiments using primary WT and *Puma*-deficient cell cultures.

treated $Puma^{-/-}$ CNS (Figures 3s–3v). Therefore, Puma is essential for apoptosis in the thymus and developing postnatal CNS after γ-IR and *Puma* loss recapitulates the radiation resistance observed in *p53* knockout mice.

Inappropriate cell proliferation stimulated by oncogenes triggers p53-dependent apoptosis of hematopoietic cells (Eischen et al., 1999; Schmitt et al., 1999), and the engagement of this cell death pathway provides a mechanism to eliminate potential cancer cells and suppress tumor development (Schmitt et al., 2002). The contribution of *Puma* to oncogene-induced apoptosis was examined by conditionally activating c-*Myc* fused to the 4-hydroxytamoxifen (4-HT)-responsive domain of the estrogen receptor (ER) in primary myeloid progenitors (Figure 4A). Western blot analysis demonstrated that the expression of Myc-ER was equivalent in WT, $Puma^{+/-}$ and $Puma^{-/-}$ cells (Figure 4A). Activation of Myc-ER in WT myeloid progenitors induced massive cell death (~10% survived at 96 hr), whereas $Puma^{-/-}$ cells were highly resistant and more than 50% of the population remained viable (Figure 4A). In fact, *Puma* null cells are comparable to $p53^{-/-}$ myeloid cells in their response to Myc-induced apoptosis (Eischen et al., 1999 and data not shown). Similarly, $Puma^{-/-}$ murine embryo fibroblasts, like $p53^{-/-}$ cells, were resistant to c-Myc-induced apoptosis (Supplemental Figure S5 on *Cancer Cell* website). Therefore, Puma also functions as an essential apoptotic regulator that protects against hyperproliferative signals emanating from oncogenes, such as c-*Myc*.

Primitive bone marrow-derived myeloid progenitors require interleukin-6 (IL- 6), IL-3, and stem cell factor (SCF) for growth and survival (Packham et al., 1998). Removal of these cytokines leads to G1 cell cycle arrest followed by protracted apoptosis, and this intrinsic cell death response is p53 independent (Packham et al., 1998; Eischen et al., 2001). Cytokine deprivation generally results in reduced expression of most genes; however, one exception is *Bim*, which is induced under these conditions (Shinjyo et al., 2001). Initial studies relying on 32D myeloid progenitor cells revealed that IL-3 suppresses *Puma* mRNA and protein levels (data not shown). To properly assess the role of Puma in cytokine withdrawal-induced death, primary bone marrow was obtained from the femurs of WT, $Puma^{+/-}$ and $Puma^{-/-}$ mice and cultured short term in medium containing IL-3, IL-6, and SCF to expand primary myeloid progenitor cells ($CD34^+$, c-Kit^+, $Sca1^+$, Lin^-, data not shown). Real-time PCR analysis demonstrated that *Puma* expression is induced 7- to 8-fold in primary myeloid cells following cytokine withdrawal (Supplemental Figure S2 online). Consequently, WT progenitors progressively lost viability when deprived of cytokines and by day 3 only 10%–15% of these cells were viable (Figure 4B and data not shown). Similarly, p53-deficient cells were only modestly resistant to cytokine deprivation. Consistent with previous findings (Bouillet et al., 1999), *Bim*-deficient cells exhibited extended survival in media lacking cytokines, but ultimately underwent cell death, and by day 7 less than 10% of these cells were viable (Figure 4B). By contrast, nearly 90% of the $Puma^{-/-}$ myeloid progenitors survived for 3 days in the absence of cytokines, and even after one week, approximately 50% of these cells remained viable (Figure 4B and data not shown). Nevertheless, $Puma^{-/-}$ progenitors remained dependent on cytokines for growth and arrested in G1 phase when deprived of hemopoietins (data not shown). Therefore, in contrast to p53, Puma plays an important role in the intrinsic cell death response suppressed by survival factors.

Although *Puma* null thymocytes, myeloid progenitors, and pro-B lymphocytes are remarkably resistant to apoptosis due to DNA damage, glucocorticoids, and/or limiting concentrations of cytokines, Puma does not appear to play a significant role in normal hematopoietic development. All lymphoid and myeloid subtypes, including those found in the bone marrow, thymus, spleen, and peripheral lymph nodes, are present and maintained at normal levels in *Puma* knockout mice (Supplemental Figure S6 on *Cancer Cell* website). A likely explanation for these findings is that similar to p53, Puma may contribute to cell death within these cell types only during pathological stress responses.

Our results demonstrate that Puma is a critical mediator of p53-induced cell death and actually accounts for nearly all of the apoptotic activity attributed to p53 in primary hematopoietic cells and the CNS in response to γ-IR or oncogenes (c-*Myc*). Recent studies have suggested a model whereby p53 promotes cell death by directly forming complexes with Bcl-2 and Bcl-X_L at the mitochondria (Mihara et al., 2003; Dumont et al., 2003). By contrast, our findings demonstrate that endogenous wild-type p53 is not sufficient in the absence of Puma to induce efficient cell death. Rather, Puma is required for p53-mediated cell death and this most likely occurs through the direct transcriptional induction of *Puma* expression by p53 and the subsequent association of Puma with Bcl-2 family members at the mitochondria. In light of the significant number of putative pro-apoptotic p53-regulated genes, the loss of just *Puma* having such a profound effect on apoptosis after DNA damage and other apoptotic stimuli is quite remarkable. Indeed, Puma is as essential to the induction of apoptosis during DNA damage as $p21^{Cip1}$ is to p53-dependent cell cycle arrest (Deng et al., 1995).

The contribution of PUMA to cell death was recently assessed in somatic knockout cancer cells (Yu et al., 2003). Homozygous deletion of *PUMA* in human colon carcinoma HCT116 cells that had been previously targeted to not express $p21^{CIP1}$ renders these double-knockout cancer cells resistant to p53-mediated apoptosis. It was also shown that *BAX* null HCT1166 cells are completely resistant to overexpression of *PUMA*, placing BAX downstream of PUMA and demonstrating a strict requirement for BAX in PUMA-mediated cell death. One limitation of this approach, however, is the reliance on tumor cells that have obviously undergone multiple genetic alterations. By specifically targeting *Puma* in mice, primary cells of different tissue types can be readily analyzed in vivo and ex vivo without the complication of additional genetic hits. Using this strategy, we demonstrated that (1) *Puma* is required for γ-IR-induced death of thymocytes and that *Bax* is dispensable for this response (Figure 2) and (2) *Puma* is essential for γ-IR-induced apoptosis throughout the developing CNS (Figure 3), including the retina, which does not require Bax (Chong et al., 2000). Thus, Puma is required for apoptosis in cell types and under stress conditions where Bax is dispensable.

Interestingly, recent studies demonstrate that tumors can tolerate wild-type p53 if appropriate survival measures are implemented, such as enforced Bcl-2 expression, indicating that p53′s apoptotic activity is its primary tumor suppressor function (Schmitt et al., 2002). Our results are not entirely consistent with this notion and suggest that the combined activities of p53 cell cycle arrest and cell death serve as checkpoints in cancer. Consistent with this hypothesis, *Puma* knockout mice do not appear to be inherently predisposed to developing spontaneous tumors. Presently, there are 14 $Puma^{-/-}$ mice over the age of 6 months (including animals that are over 1 year of age), which is well beyond the time frame for tumor development in *p53* knockout mice. Indeed, the only tumor-related death that we have observed within the entire Puma colony was a $Puma^{+/-}$ mouse at 8 months of age that developed medulloblastoma, which is not observed in *p53* knockout mice unless bred onto other genetically deficient backgrounds (e.g., $Ptc1^{+/-}$) (Wetmore et al., 2001). Whether tumor incidence increases in aged *Puma*-deficient mice, or in cooperation with other genetically predisposing alterations, is currently being examined.

Experimental procedures

Generation of Puma-deficient mice

A 16.9 Kb EcoRI fragment encompassing the entire *Puma* gene was isolated from an ES 129/SvJ BAC library (Incyte Genomics) and subcloned into pGEM-3Z. A portion of the 5′ promoter region, transcription start site, and 85% of the coding region were deleted by digestion with NotI and AscI and substituted with a 1.1 Kb XhoI–XbaI fragment of *Neo-Poly A*. E14 embryonic stem cells were electroporated with the linearized *Puma* targeting vector, selected in G418, and properly recombined clones were microinjected into C57BL/6 blastocysts. Germline transmission was achieved and hemizygous animals were identified by PCR and Southern blot analyses. The primer pair for amplification of the WT allele was Puma a (5′-TTATAGCCGGTGAGTGCA-3′) and Puma b (5′-AACAGCTTATTAAGAGCCAGCC-3′). The primer pair for amplification of the mutant allele was Puma c (5′-TTGACGAGTTCTTCT GAGGG-3′) and Puma b. Southern blot analysis was performed after XmnI

digestion using an outside probe (218 bp PCR-generated fragment located 3′ to the targeting vector).

Cell cultures

Thymocytes were prepared from ~6-week-old animals by passing the cells through a 100 μM sterile nylon mesh into DMEM containing 5% fetal bovine serum (FBS), 1% L-Glutamine, 1% penicillin/streptomycin, and 25 mM HEPES at 1×10^6 cells/ml. The cells were cultured at 37°C under 5% CO_2 and either treated with 1 μM dexamethasone or 5 Gy γ-irradiation. Myeloid progenitors were prepared from the bone marrow of 6- to 7-week-old mice by expanding the cells in RPMI 1640 medium containing 10% FBS and supplemented with IL-3 (20 U/ml), IL-6 (10 ng/ml; R&D Systems), and SCF (10 ng/ml; R&D Systems) (Packham et al., 1998). The immunophenotypes of these cells were determined by fluorescence-activated cell sorting (FACS) and were $CD34^+$, c-Kit^+, Sca-I^+, Lin^-. Primary B lymphocytes were derived from the bone marrow of 6- to 7-week-old mice cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 55 μM 2-mercaptoethanol, 2 mM glutamine, 10 μg/ml IL-7 (R&D Systems), 1% penicillin and streptomycin (GIBCO) on S17 stromal cells. The resulting cell cultures were immunophenotyped by FACS and shown to be 80% pro-B ($B220^+$, $CD43^+$, IgM^-, negative for T cell or myeloid/macrophage-specific markers) and 20% pre-B ($B220^+$, IgM^-, $CD43^-$) cells.

Viral transduction

Primary myeloid progenitors and mouse embryo fibroblasts (MEFs) were infected as described (Zindy et al., 1998) with the murine stem cell virus (MSCV) Myc-estrogen receptor (Myc-ER)-internal ribosome entry site (IRES)-green fluorescent protein (GFP) virus or with the MSCV-IRES-GFP control virus. Infected GFP-positive cells were sorted by FACS and expanded in culture under the appropriate growth conditions. Levels of Myc-ER fusion protein in all cultures were comparable as established by Western blot analysis. Addition of 1 μM 4-hydroxytamoxifen (4-HT) (Sigma) was used to activate Myc-ER by promoting its translocation from the cytoplasm to the nucleus, where it activates transcription (Littlewood et al., 1995).

Western blot analysis

Whole-cell protein extracts were prepared and quantified by the Bradford method (Bio-Rad) as previously described (Packham et al., 1998). Protein (100–150 μg/lane) was electrophoretically separated in 7.5% or 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The filters were blotted with antibodies specific for the Puma (AbCAM), p53 (Ab-7, Calbiochem), $p21^{Cip1}$ (C-19, Santa Cruz), and c-Myc (06-340, Upstate Biotechnology) and the proteins detected using the appropriate secondary antibodies and ECL reagents as recommended by the manufacturer (Amersham).

Cell cycle and viability

Cells were resuspended at 1×10^6 cells/ml in 0.1% sodium citrate containing 50 μg/ml propidium iodide and treated with 1 μg/ml RNase at room temperature for 30 min. DNA fluorescence was measured using a FACSCalibur Cell Sorter (Becton Dickinson) and quantitated using ModFit software (Verity Software House). Cell viability was also measured as a function of cell membrane integrity by trypan blue dye exclusion (Life Technologies).

Histology and immunohistochemistry

Mice were irradiated 5 days after birth with 18 Gy (^{137}Cs; 4.3 Gy/min) and allowed 6 hr of recovery. Tissues were collected after fixation by transcardial perfusion with 4% paraformaldehyde, cryoprotected in 20% sucrose/PBS, and cryosectioned (10 μm sagittal sections). Staining was performed with 1% Neutral Red (Aldrich Chemical) in 0.1 M acetic acid (pH 4.8) for 1 min followed by dehydration in ethanol. TUNEL staining was performed on cryosections using the ApopTag kit (Intergen) according to the manufacturer's directions.

Acknowledgments

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