Molecular Cell, Vol. 7, 673–682, March, 2001, Copyright ©2001 by Cell Press

PUMA Induces the Rapid Apoptosis of Colorectal Cancer Cells

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Summary

Through global profiling of genes that were expressed soon after p53 expression, we identified a novel gene termed *PUMA* (*p*53 *u*pregulated *m*odulator of *a*poptosis). The protein encoded by *PUMA* was found to be exclusively mitochondrial and to bind to Bcl-2 and Bcl-X_L through a BH3 domain. Exogenous expression of *PUMA* resulted in an extremely rapid and profound apoptosis that occurred much earlier than that resulting from exogenous expression of *p53*. Based on its unique expression patterns, p53 dependence, and biochemical properties, PUMA may be a direct mediator of p53-associated apoptosis.

Introduction

Inactivation of the growth-controlling functions of p53 appears to be critical to the genesis of most human cancers (Hollstein et al., 1999; Hussain and Harris, 1999). The p53 protein controls tumor growth by inhibiting cell cycle progression and by stimulating apoptosis (Levine, 1997; Lane, 1999; Oren, 1999; Prives and Hall, 1999; Vogelstein et al., 2000). It has been shown that the inhibition of cell cycle progression by p53 is in large part due to its ability to transcriptionally activate genes that directly control cyclin-dependent kinase activity (reviewed in El-Deiry, 1998). For example, p53 induces $p21^{CIP1/WAF1}$, which binds to and inhibits several cyclin/cdk complexes (Harper et al., 1993; Xiong et al., 1993), and 14-3-3σ, which sequesters cyclin B/cdc2 complexes in the cytoplasm (Chan et al., 1999). In both cases, the induction results from p53 binding to cognate recognition elements in the promoters of these genes (El-Deiry et al., 1993; Hermeking et al., 1997).

Much less is known about the mechanisms through which p53 induces apoptosis, though this is also thought to be mediated by transcriptional activation of target genes (reviewed in Chao et al., 2000). The apoptotic function of p53 is highly conserved, as is evident from functional studies of the *Drosophila* p53 homolog (Brodsky et al., 2000; Jin et al., 2000; Ollmann et al., 2000). Moreover, the cell cycle inhibitory effects of p53 are inadequate to fully account for the tumor suppressor effects of p53, suggesting that apoptotic induction is a key component of p53's tumor suppression (Gottlieb and Oren, 1998; Symonds et al., 1994). Many studies have been performed to identify genes that are regulated by p53 and mediate apoptosis (El-Deiry, 1998). Among these candidates, those that encode mitochondrial proteins are particularly attractive because p53-initiated apoptosis appears to proceed through a mitochondrial pathway. In particular, the apoptosis stimulated by p53 involves disruption of mitochondrial membrane potential, accumulation of reactive oxygen species, stimulation of caspase 9 activity, and subsequent activation of a caspase cascade (Polyak et al., 1997; Li et al., 1999; Soengas et al., 1999; Schuler et al., 2000).

Three genes that are regulated by p53 and encode proteins that at least partly reside in the mitochondria have been identified. The first to be identified was *BAX*, the proapoptotic Bcl-2 family member that serves as the prototype for this class (Reed, 1999). More recently, *Noxa* and *p53AIP1* have been discovered and shown to encode proapoptotic mitochondrial proteins whose expression is controlled by p53 (Oda et al., 2000a, 2000b). To explore the role of these genes in colorectal cancers (CRC), we examined their expression patterns in detail. As described below, these three genes did not appear to be expressed at early enough times or at sufficiently robust levels to account for the dramatic apoptosis induced by p53 in CRC cells. This stimulated us to search for other mitochondrial proteins that might play a role in this process. Here we describe a novel gene encoding a mitochondrial protein that is tightly regulated by p53 and has several properties suggesting it mediates p53-associated apoptosis in CRC cells. In light of the rapid induction of this gene by p53 and its potent proapoptotic effect, it was named *PUMA* (*p*53 *u*pregulated *m*odulator of *a*poptosis).

Results

Discovery of *PUMA* and Its Regulation by p53

We have previously shown that the CRC cell line DLD1 undergoes apoptosis ~18 hr following expression of exogenous p53 under the control of a doxycycline-regulated promoter. Moreover, these cells are committed to apoptosis after only 9 hr of p53 exposure, as addition of doxycycline after this period does not diminish apoptosis (Yu et al., 1999). These observations, combined with the analysis of numerous p53-regulated genes in this system, led us to propose the following guidelines for candidates that might mediate apoptosis in CRC cells. First, their induction in DLD1 cells should be robust and rapid, with substantial expression by 9 hr. Second, they should be induced by p53 in other CRC lines, not just DLD1 cells. Third, they should be induced not only by high levels of exogenous p53, but also by elevated endogenous p53 following exposure to chemotherapeutic drugs. Fourth, their induction after such exposures should depend on an intact *p53* gene. Fifth, the candidate genes should exhibit biochemical and physiologic properties that suggest they can directly stimulate apoptosis through a mitochondrial pathway.

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We used the serial analysis of gene expression (SAGE) technique to study DLD1 cells inducibly expressing p53 (Velculescu et al., 1995; Yu et al., 1999). We identified only one gene, denoted *PUMA* and described here, which met the criteria described above. The *PUMA* gene was discovered through a SAGE tag that matched to ESTs (*e*xpressed *s*equence *t*ags) but to no known genes. The SAGE data indicated that *PUMA* was induced over 10-fold in DLD1 cells following p53 expression for 9 hr. Northern blotting showed that *PUMA* was induced as soon as 3 hr following doxycycline withdrawal, just as was $p21^{CIP1/WAF1}$ (Figure 1A). *PUMA* expression was maximal by 6 hr, well before the 9 hr "commitment point" for apoptosis determined previously (Yu et al., 1999). In each of four lines tested, there was significant induction of *PUMA* after infection with an adenovirus encoding wild-type (wt) p53, but none after expression of an analogous adenovirus encoding a R175H mutant p53 (Figure 1B). Furthermore, *PUMA* mRNA expression was found to be induced in HCT116 and SW48 cells following treatment with 5-FU (5-fluorouracil), the mainstay of treatment for CRC, as well as by the DNA-damaging agent adriamycin (Figure 1C). HCT116 and SW48 cells contain wt *p53* genes, and the results in Figure 1C demonstrate that endogenous levels of p53 were sufficient to induce *PUMA.* The apoptosis following 5-FU treatment is totally dependent on intact p53 (Bunz et al., 1999). Using HCT116 cells in which the p53 genes had been disrupted by targeted homologous recombination (Bunz et al., 1998), we found that the transcriptional induction of PUMA by 5-FU was also entirely dependent on p53 (Figure 1C).

It was informative to compare the transcriptional patterns noted above with those of the three other p53-induced genes encoding mitochondrial proteins (*BAX*, *Noxa*, and *p53AIP1*). SAGE revealed only a slight or insignificant induction of *BAX* and *Noxa* transcripts, as confirmed by Northern blotting (Figure 1A). *p53AIP1* transcripts were not detectable by either SAGE or Northern blotting in these experiments, consistent with previous results showing that this gene is activated only at late times following p53 induction (Oda et al., 2000b). Furthermore, only *PUMA* was induced in all four CRC lines tested after infection with p53 adenoviruses, and only *PUMA* was significantly induced by 5-FU in both HCT116 and SW48 cells (Figures 1B and 1C). In general, the transcriptional patterns of *PUMA* closely matched those of $p21^{CIP1/WAF1}$, while those of the other three genes were considerably different.

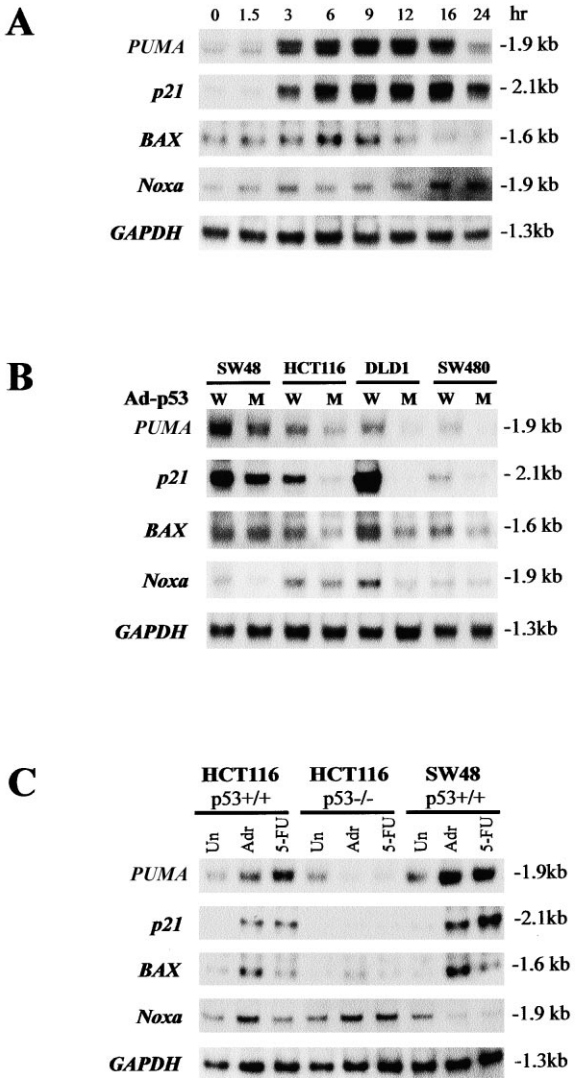

Figure 1. Induction of *PUMA* by p53 in CRC Cells

(A) Northern blot analyses of RNA samples prepared from p53-inducible DLD1 cells at the indicated time points are shown. The *PUMA* gene was induced as early as 3 hr after doxycycline removal, similar to that of *p21*, while the *BAX* and *Noxa* genes were not induced as robustly. *p53AIP1* transcripts were not detectable under these conditions. A *GAPDH* probe was used as a loading control.

(B) RNA from the indicated colorectal cancer cells lines infected with adenovirus expressing wt p53 (W) and mutant p53R75H (M) for 17 hr was analyzed by Northern blotting.

(C) RNA from the indicated colorectal cancer cells lines treated with adriamycin (Adr) or 5-Fluorouracil (5-FU) for 24 hr was analyzed by Northern blotting. RNA from untreated cells (Un) was used as a control.

Characterization of the Human *PUMA* Transcript and Gene

A combination of database searching, resequencing of EST clones, RT–PCR analyses, and 5′ RACE was used to obtain an apparently full-length cDNA for *PUMA* (Figure 2A). These efforts were complicated by an extremely GC-rich 5′ untranslated region. The final assembled cDNA was 1.9 kb in size, consistent with the size of the major induced transcript observed in Northern blots (Figure 1A). Comparison of the resultant sequences with that of genomic DNA revealed that the *PUMA* transcript was contained within four exons (1a, 2, 3, and 4), with the presumptive initiation codon in exon 2 (Figure 3A). *PUMA* was predicted to encode a 193-amino acid protein with no significant homologies to other known proteins except for the BH3 domain discussed below. RT–PCR analysis showed that *PUMA* was expressed at low but similar levels in each of eight different human tissues, and radiation hybrid mapping showed that the *PUMA* gene is located on chromosome 19q (data not shown).

An alternatively spliced form (AS) of *PUMA* devoid of exon 2 appeared in some RT–PCR experiments with

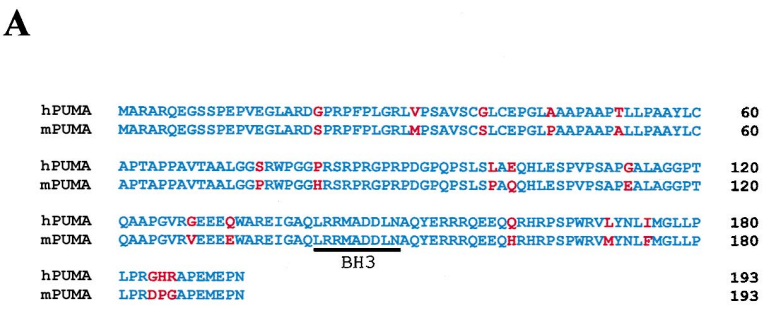

Figure 2. The PUMA Protein Contains a BH3 Domain

(A) Alignment of the predicted amino acids of human and mouse PUMA reveals 90% identity. The identical residues are colored blue, and nonconserved residues are colored red. The residues comprising AA128–165 were predicated to form an α helix by the Chou-Fasman method. The middle third of the α helix corresponding to the BH3 (AA141–149) domain is completely identical in both human and mouse PUMA.

(B) Alignment of BH3 domains of PUMA with other Bcl-2 family members. Conserved residues (contained in more than three members of the eleven shown) are colored blue, whereas the nonconserved residues are colored red.

B

PUMA	141	LRRMADDLN	149
EGL1	58	LAAMCDDFD	66
BAD	114	LRRMSDEFV	122
BID	90	LAQIGDEMD	98
BAK	78	LAIIGDDIN	86
BIM	152	LRRIGDEFN	160
BIK	61	LACIGDEMD	69
HRK	37	LKALGDELH	45
BAX	63	LKRIGDELD	71
Bcl-2	97	LRQAGDDFS	105
Bcl-xL	90	LREAGDEFE	98

human RNA templates and likely corresponded to the shorter mRNA species observed in Figure 1A. Sequencing of PCR products showed that the AS altered the open reading frame so that it no longer contained a BH3 domain, and we therefore did not evaluate this form further.

We searched for consensus p53 binding sites upstream of the *PUMA* gene and identified two such sites, BS1 and BS2, lying 230 and 144 bp upstream of the transcription start site, respectively (Figure 3A). To determine whether this region of the *PUMA* gene could mediate p53 responsiveness, we cloned a 493 bp fragment whose 5′ end was 336 bp upstream of the putative transcription start site and placed it in front of a luciferase reporter containing a minimal promoter. Inclusion of this region conferred a 60-fold activation when transfected into H1299 cells together with a p53 expression vector (Figure 3B). Deletion of the 5′ terminal 300 bp from this construct (a region that contained BS1 and BS2) led to loss of most of the p53 responsiveness (Figure 3B).

To determine which of the two binding sites was primarily responsible for the p53 responsiveness, we tested constructs containing four copies of either binding site, in wt or mutant form, inserted upstream of a luciferase reporter and minimal promoter (Figure 3C). In the mutant forms, two residues predicted to be critical for p53 binding were substituted with noncognate nucleotides. These experiments revealed that BS2 was likely to be the major p53-responsive element, as it was activated over 400-fold by exogenous p53 in H1299 cells, while BS1 was activated only 7-fold (Figure 3D). Cotransfection of the BS2 reporter with a mutant p53 R175H expression vector did not result in reporter activation (Figure 3D). Additionally, mutation of the BS2 sequence completely abrogated wt p53 responsiveness (Figure 3D). Finally, we transfected the BS2 reporter into HCT116 cells, which contain endogenous wt p53, in the absence of an exogenous p53 expression vector. Transfection of the BS2 reporter but not the BS1 or mutant BS2 reporters resulted in high levels of luciferase activity in HCT116 cells, suggesting that endogenous levels of p53 are sufficient for direct *PUMA* activation (Figure 3D).

The Murine *PUMA* Gene

The mouse homolog of *PUMA* was identified through searches of mouse EST and genomic databases. The predicted protein from the murine gene was 91% and 90% identical at the amino acid and nucleotide levels, respectively, to the human protein (Figure 2A). Moreover, the intron–exon structure of *PUMA* was remarkably conserved, consisting of four exons (1a, 2, 3, and 4) in both of the two species (Figure 3A). The sequence of the region upstream of the first exon in the murine gene revealed that the major p53 binding site in the human was present at a similar position in the mouse promoter (375 and 410 bp upstream of the 3′ end of exon 1a in mouse and human, respectively). As shown in Figure 3A, the sequence of the BS2 sites in the mouse and human genes were identical at 18 of 20 residues, with

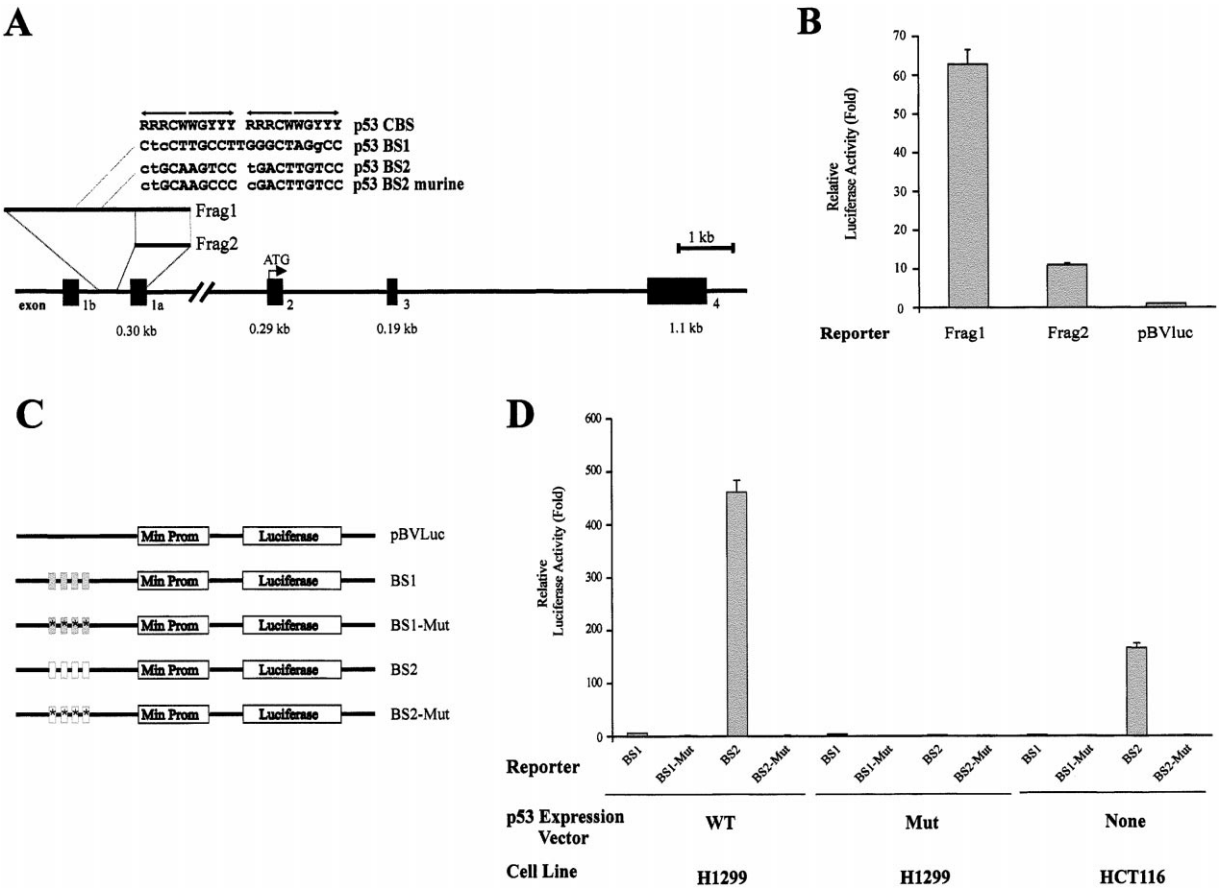

Figure 3. p53 Activates the *PUMA* Promoter

(A) The two potential p53 binding sites (BS1 and BS2) within 300 bp of the putative transcription start site are indicated. The major transcript we identified contained exons 1a, 2, 3, and 4, and the open reading frame (ORF) was predicted to begin at the indicated ATG. Several transcripts derived from the same gene are described by Nakano and Vousden in the accompanying article. The transcripts described by Nakano and Vousden contained an alternative first exon, labeled 1b in the figure, while the transcripts we detected all began with exon 1a. Fragments (Frag1 and Frag2) of the *PUMA* promoter containing the putative p53 binding sites were used to construct reporters. The previously characterized p53 consensus binding site (CBS) (El-Deiry et al., 1992) is shown above the BS1 sequence, with R denoting purine, Y denoting pyrimidine, and W denoting A or T. The sequences of exons 1a, 2, 3, and 4 and the BS2 site but not exon 1b or the BS1 site were conserved in mice. The sequence of the murine BS2 site is shown in the figure.

(B) The indicated fragments were cloned into pBVLuc and cotransfected into H1299 cells together with a wt or mutant (R175H) p53 expression construct (Baker et al., 1990). The ratio of luciferase activity in the presence of wt p53 compared to that in the presence of mutant p53 is plotted on the ordinate. All experiments were performed in triplicate with a β-galactosidase reporter included in the transfection mix for normalization, with means and one standard deviation indicated by the bars and brackets, respectively.

(C) Luciferase reporters containing either four copies of the potential p53 binding sites BS1 and BS2 or mutant versions of these sites were constructed as described in Experimental Procedures. "Min Prom" indicates the minimal promoter present in the vector (pBVLuc).

(D) Transfections were performed exactly as in (B) to test the reporters shown in (C). Results for each pair of reporters (wt and mutant) were normalized to the luciferase/β-galactosidase ratio of the mutant reporter. The "raw" luciferase/β-galactosidase ratios for these mutant reporters were 0.24 and 1.07 for BS1-Mut and BS2-Mut in H1299 cells transfected with p53 wt; 3.36 and 3.72 for BS1-Mut and BS2-Mut in H1299 cells transfected with p53R175H (mut); and 0.11 and 0.14 for BS1-Mut and BS2-Mut in HCT116 cells.

the two differences confined to C to T transitions predicted to have no effect on p53 binding (Cho et al., 1994).

PUMA Encodes a BH3 Domain-Containing Mitochondrial Protein that Interacts with Bcl-2 and Bcl-X_L

Two observations led us to test the hypothesis that *PUMA* encoded a mitochondrial protein. First, the PUMA protein was predicted to contain a BH3 domain (Figure 2B). BH3 domains are one of the four *B*cl-2 *h*omology domains present in the Bcl-2 family of proteins (Chittenden et al., 1995). Several of the proapoptotic members of this family contain the BH3 domain but not the BH1, 2, or 4 domains and reside at least partially in mitochondria (reviewed in Reed, 1997; Korsmeyer, 1999; Huang and Strasser, 2000). The BH3 domains are essential for their proapoptotic activities and for their ability to heterodimerize with other Bcl-2 family members (Wang et al., 1996; Zha et al., 1997; Wang et al., 1998). Second, a GenBank entry (accession number U82987) corresponding to a partial *PUMA* cDNA sequence carried the intriguing annotation of "human Bcl-2 binding component 3". The basis for this annotation was not specified, and the amino acid sequence included with this entry was out

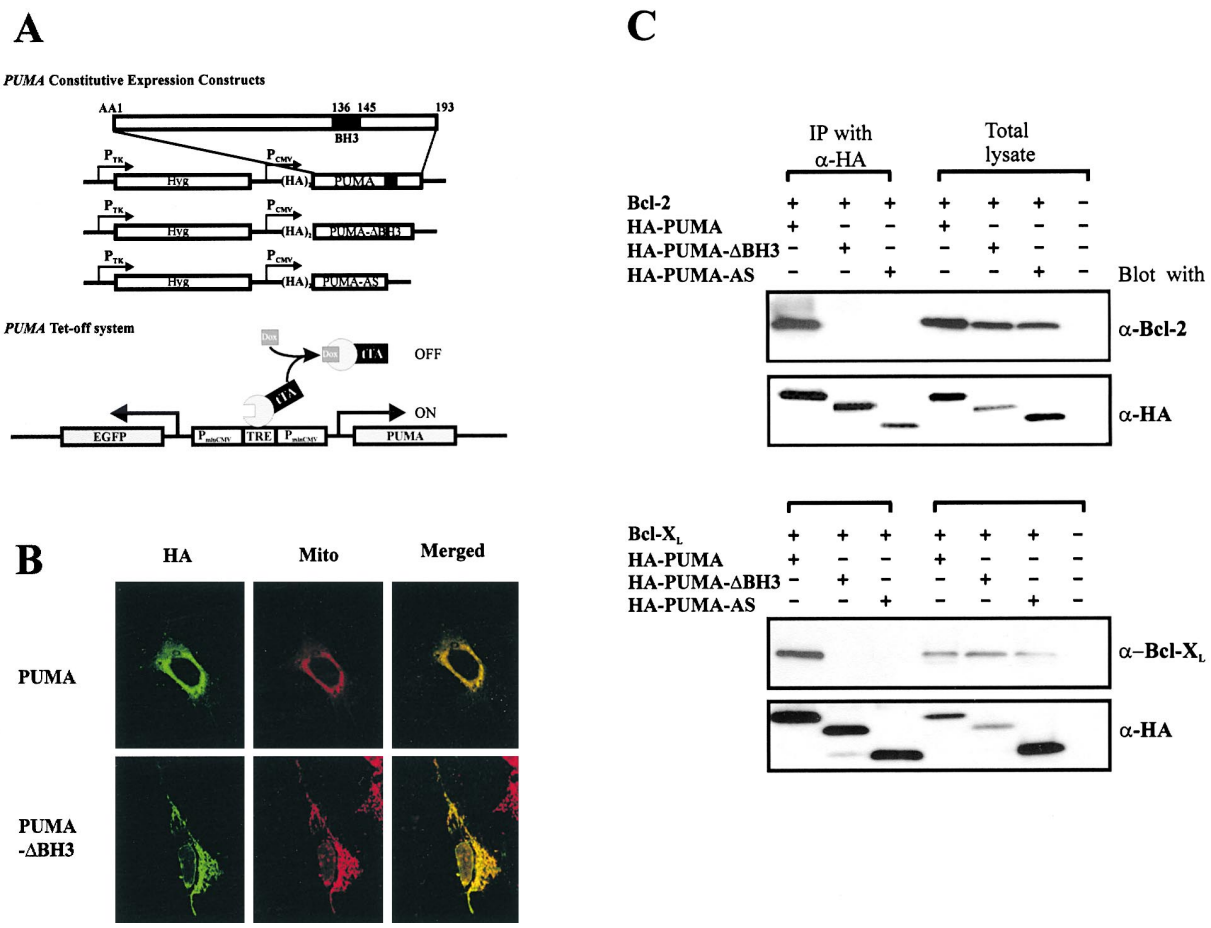

Figure 4. *PUMA* Encodes a Mitochondrial Protein that Interacts with Bcl-2 and Bcl-X_L

(A) Diagram of expression constructs. For constitutive expression, P_{TK} and P_{CMV} refer to the herpes virus thymidine kinase promoter and CMV promoter, respectively. Hyg stands for hygromycin B-phosphotransferase gene, conferring resistance to hygromycin B. For inducible expression, TRE, tetracycline-responsive elements; tTA, tet activator; and P_{minCMV}, minimal CMV promoter. This system was activated by removal of doxycycline (Dox). PUMA-AS denotes an alternatively spliced form of the gene.

(B) HA-tagged PUMA constructs were transfected into 911 cells and visualized by indirect immunofluorescence (green). MitoTracker Red dye was used to visualize mitochondria. PUMA-ΔBH3 encodes a tagged PUMA protein with a 15-amino acid deletion and is, therefore, missing the BH3 domain.

(C) Different pairs of expression constructs were transfected into 911 cells, and total lysates were immunoprecipitated with a rabbit anti-HA antibody, then analyzed by Western blotting with the indicated antibodies. The lanes labeled total lysate contain ~25% of the amount of lysate represented in the lanes containing immunoprecipitates.

of frame with respect to the major protein we predicted to be encoded by the *PUMA* gene.

To determine the subcellular localization of human PUMA, we constructed an expression vector encoding the full-length PUMA protein with an amino-terminal hemaglutanin (HA) tag (Figure 4A). This vector was expressed in 911 cells, which have a flat morphology that facilitates subcellular localization studies. Indirect immunofluorescence with an anti-HA antibody showed punctate perinuclear staining in all transfected cells (Figure 4B). Comparison of this localization with that of a dye that labeled mitochondrial membranes (MitoTracker Red) indicated complete colocalization (Figure 4B). Interestingly, the BH3 domain was not required for this localization, as the protein generated from another *PUMA* expression vector, PUMA-ΔBH3 (identical except for the deletion of the BH3 domain), was also found exclusively in mitochondria (Figure 4B). This lack of dependence on BH3 for mitochondrial localization is consistent with data on other BH3-containing proteins, though it distinguished PUMA from Noxa, in which the BH3 domain was required (Oda et al., 2000a).

We next tested whether PUMA interacted with Bcl-2. Using the *PUMA* expression vector described above, we expressed PUMA together with Bcl-2 in 911 cells. Immunoprecipitation experiments showed that a major fraction of Bcl-2 (50%) was bound to PUMA under these conditions (Figure 4C). The BH3 domain of PUMA was essential for this interaction, as deletion of the BH3 domain completely abrogated the binding (Figure 4C). A similar vector encoding the alternatively spliced form of PUMA provided an additional control in this experiment (Figure 4C).

Previous experiments have shown that Bcl-2 is not expressed in many CRCs, while Bcl-X_L is ubiquitously expressed (Zhang et al., 2000). To determine whether

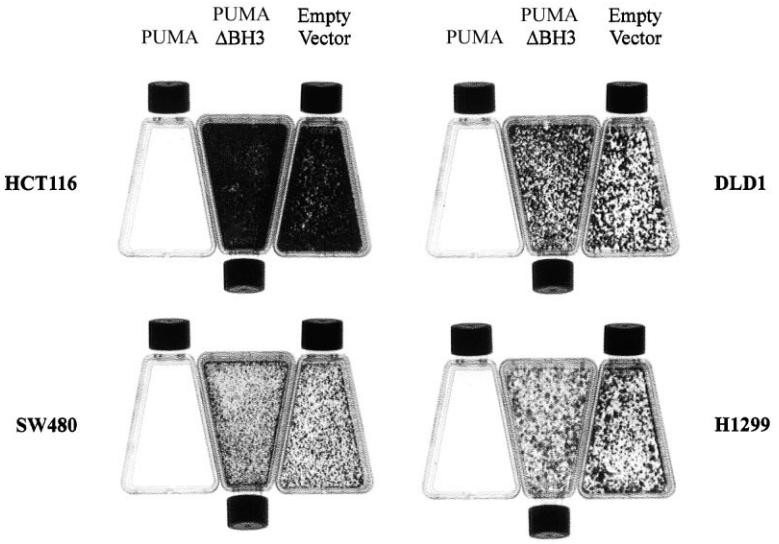

Figure 5. PUMA Potently Suppresses the Growth of Human Cancer Cells

The indicated cell lines were transfected with constructs encoding PUMA, PUMA-ΔBH3, or the empty vector. Cells were harvested 24 hr after transfection, and equal cell numbers were serially diluted in T25 flasks and grown under selection in hygromycin B for 17 days. Only the highest density flasks are shown. There was no observable difference in colony formation between transfection with PUMA-ΔBH3 and that with the empty vector, while the number of colonies obtained after transfection with the *PUMA* expression vector was reduced by more than 1000-fold.

PUMA also binds to Bcl-X_L, 911 cells were cotransfected with PUMA plus Bcl-X_L expression vectors, and analogous immunoprecipitation experiments were performed. As shown in Figure 4C, Bcl-X_L bound to intact PUMA, and the BH3 domain of PUMA was essential for this binding.

PUMA Expression Results in Complete and Rapid Cell Death

To determine the effect of PUMA expression on cell growth, we constructed an expression vector containing *PUMA* plus a hygromycin B resistance gene (Figure 4A) and transfected it into four different cancer cell lines. Following selection, there was a drastic reduction in colony formation after transfection with the *PUMA* expression vector compared to the empty vector or to an analogous vector encoding PUMA without its BH3 domain (Figure 5). This colony suppression was observed regardless of the *p53* genotype of the cells (wt in HCT116 cells, mutant in SW480 and DLD1, and null in H1299). Enumeration showed that *PUMA* expression reduced colony formation by over 1000-fold.

To determine the basis for this profound growth inhibition, we generated an inducible *PUMA* expression system in DLD1 cells, similar to the *p53* expression system used to obtain the SAGE data. The expression vector contained separate *GFP* and *PUMA* genes under the control of a doxycycline-regulated promoter (Figure 4A). Several clones of PUMA-expressing cells were obtained and tested, and each behaved identically. Upon withdrawal of doxycycline, virtually all cells underwent apoptosis, as evidenced by their condensed chromatin and fragmented nuclei (Figure 6A). Continued expression of PUMA completely eliminated the cells' ability to form colonies (Figure 6B). Staining with the JC-1 dye (Molecular Probes) showed that this apoptosis was preceded by a change in mitochondrial membrane potential (not shown). One remarkable feature of this apoptosis was its rapidity: nuclear condensation and fragmentation were significant within 6 hr of removal of doxycycline (Figure 6E).

We found during the course of these experiments that β-catenin was a particularly good marker of apoptosis, presumably due to its sensitivity to caspase activation. Degradation of β-catenin was already evident by 2 hr following PUMA expression, and a major fraction of β-catenin was degraded by 3 hr (Figure 6C). There was very little lag between the time at which PUMA was expressed and the time at which β-catenin began to be degraded (Figure 6C). Likewise, caspase 9 activation was detectable by 3 hr and was substantial at 6 hr. Interestingly, PUMA protein appeared to be unstable in these cells; perhaps a target of the caspases that were activated during the apoptotic process (Figure 6C).

For comparison, we analyzed the time course of caspase activation and apoptosis following p53 expression in DLD1 cells. Though expression of p53 and PUMA was induced immediately upon doxycycline withdrawal (Figures 6C, 6D, and data not shown), it took at least 9 hr longer for caspase 9 activation and β-catenin degradation to appear following p53 expression (note the different time scales in Figures 6C and 6D). Moreover, morphological signs of apoptosis, such as condensed chromatin and fragmented nuclei, appeared ~9 hr later in cells expressing p53 compared to cells expressing PUMA (Figure 6E).

Discussion

The results reported here suggest that p53-mediated cell death in colorectal cancer cells is in part mediated through the transcriptional activation of the *PUMA* gene. The results in Figure 3 show that this activation is likely the direct result of p53 binding to the BS2 sequences within the *PUMA* promoter. The time course of induction of *PUMA* (Figure 1A) and the ability of PUMA to cause a rapid and profound degree of apoptosis (Figures 5 and 6) support this model. It is also supported by a large body of literature showing that Bcl-2 family members, particularly those containing only BH3 domains, initiate apoptotic processes in organisms ranging from *C. elegans* to humans (Reed, 1997; Adams and Cory, 1998;

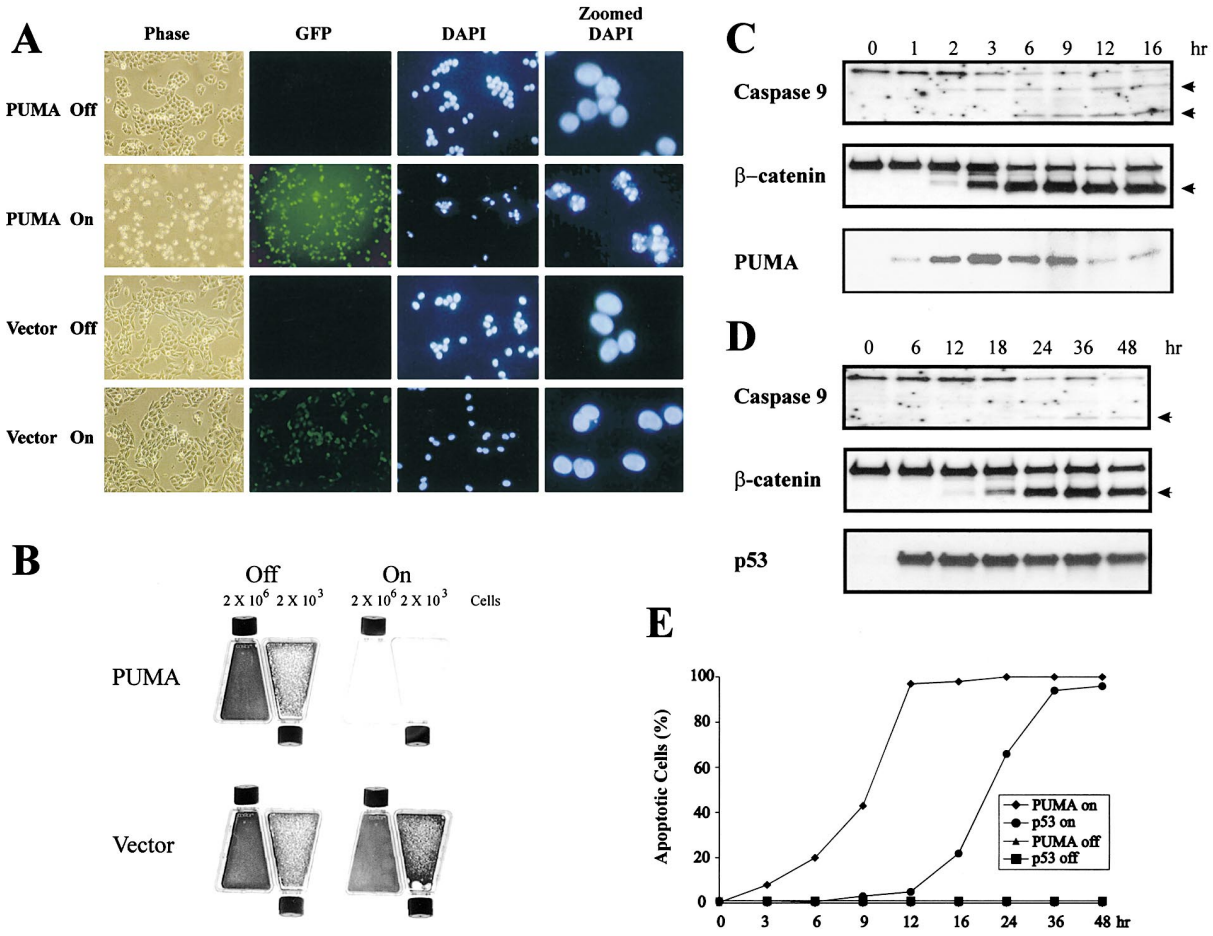

Figure 6. *PUMA* Induces Rapid Apoptosis in DLD1 Cells

(A) An expression vector containing separate cassettes for GFP and PUMA (Figure 4A) was used to establish inducible clones of DLD1 cells. Representative results are shown for cells that were maintained in the uninduced state (Off) or after induction by removal of doxycycline from the medium for 12 hr (On). The same fields are shown in the first two columns, as viewed under phase contrast (Phase) or fluorescence microscopy (GFP), for the clones that inducibly expresses both GFP and PUMA (PUMA) or GFP alone (Vector). The third and fourth columns (DAPI) shows nuclei of the same cell cultures harvested immediately after microscopy and stained with Hoechst 33528. Apoptotic cells stained with this dye have characteristic condensed chromatin and fragmented nuclei. Virtually all *PUMA*-induced cells were apoptotic by 12 hr.

(B) The indicated clones were grown in the presence (Off) or absence (On) of doxycycline for 10 days, then stained with crystal violet. Two different flasks, containing either 2,000,000 or 2,000 cells at the start of the experiment, are shown to illustrate the profound effect of PUMA induction.

(C) DLD1 cells inducibly expressing PUMA were harvested at the indicated times following doxycycline withdrawal. Whole-cell lysates were used in Western blots to assess activation of caspase 9 and cleavage of β-catenin. Cleavage products are indicated by arrows.

(D) Identical to (C), except that the DLD1 cells inducibly expressed p53 instead of PUMA. Note the different time scale.

(E) DLD1 cells induced to express either PUMA or p53 were assayed for apoptosis, as indicated by nuclear condensation and fragmentation, at the indicated time points. At least 300 cells were counted for each determination, and the experiment was repeated twice with identical results.

Korsmeyer, 1999; Vander Heiden and Thompson, 1999; Green, 2000). Finally, it is supported by previous studies showing that p53-mediated apoptosis proceeds through a mitochondrial pathway (Polyak et al., 1997; Li et al., 1999; Soengas et al., 1999; Schuler et al., 2000).

Following review of our paper, we were informed by the editors that another group had identified the same gene using a distinct experimental system. There were differences in the transcription products identified by the two groups. The major transcripts detected by Nakano and Vousden (2001 [this issue of *Molecular Cell*]) did not contain exon 1a and instead included exon 1b joined directly to exons 2 or 3. One of these transcripts (that joining exon 1b to exon 2) encodes the same protein (termed "α" by Nakano and Vousden) as that described here. The other transcript (joining exon 1b to exon 3) encodes a protein (termed "β" by Nakano and Vousden) that would be identical to α in the C-terminal 101 amino acids (corresponding to those encoded by exons 3 and 4), but would differ at the N terminus (92 amino acids predicted in α, and 30 completely different amino acids in β). It is notable that exons 1a, 2, 3, and 4, but not exon 1b, are highly conserved between human and mouse, so that only the α protein would be predicted to occur in mice. Further studies will be required to determine the relative abundance of the transcripts and proteins pro-

duced from this gene in various cell types in both humans and mice. Functionally, the discrepancies in the major proteins predicted by the two groups are likely to make little difference, as the apoptotic, mitochondrial localization, and BH3 domains of the protein are all located in exons 3 and 4. Moreover, the functional and biochemical properties of the proteins reported by the two groups were nearly identical despite their divergence at the N terminus. In an effort to simplify references to this gene in the future, the two groups have agreed to call it *PUMA* instead of the original names given it (*JFY-1* by our group and *SEPUKU* by Nakano and Vousden).

These results raise a number of questions that can now be addressed. The pore-forming abilities of Bcl-2 family members have been documented (Minn et al., 1997; Schendel et al., 1998), and it will be interesting to see whether *PUMA*, which is only related to the Bcl-2 family through its BH3 domain, affects pore formation when complexed with other Bcl-2 family members or with other mitochondrial proteins. Expression of high levels of *PUMA* is sufficient for apoptosis, but it is not known whether expression of this gene is necessary for apoptosis. Additionally, *PUMA* was expressed, albeit at very low levels, in all normal human tissues analyzed. This leads to the question of the normal function of this gene and its role in physiologic and disease-related apoptotic processes in vivo. Targeted deletions of *PUMA* in human somatic and mouse ES cells, facilitated by the sequence data provided in Figure 2, should provide answers to these questions in the future. Finally, the fact that *PUMA* expression led to a very rapid and profound apoptosis suggests that it should be considered as a substitute for p53 in cancer gene therapy.

Experimental Procedures

Cell Culture

The human colorectal cancer cell lines DLD-1, HCT116, SW48, SW480, and the human lung cancer cell line H1299 were obtained from ATCC. HCT116 cells with a targeted deletion of p53 have been previously described (Bunz et al., 1998). All lines were maintained in McCoy's 5A media (Life Technologies) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C. The retinal epithelial cell line 911 was kindly provided by A. J. Van der Eb of the University of Leiden and maintained as described (Fallaux et al., 1996). Chemotherapeutic agents were used at concentrations of 0.2 μg/ml (adriamycin) and 50 μg/ml (5-FU), and cells were treated for 24 hr. Transfections were performed with Fugene 6 (Boehringer Mannheim) according to the manufacturer's instructions.

Constructs

PUMA expression plasmids: the HA-tagged, full-length PUMA expression vector pHA-PUMA was constructed by cloning RT–PCR products into the pCEP4 vector (Invitrogen). Variants of this vector, containing PUMA with the BH3 domain deleted or the alternatively spliced form of PUMA, were constructed similarly. Sequences for the primers and details of vector construction are available from the authors upon request. In all cases, inserts of multiple individual clones were completely sequenced, and the ones that were free of mutation were subsequently used for experiments. The Bcl-2 expression vector was described previously (Pietenpol et al., 1994), and the V5-tagged Bcl-X_L expression vector was purchased from Invitrogen.

Reporter Constructs and Reporter Assay

Promoter-containing fragments were amplified from human genomic DNA of HCT116 cells and cloned into the pBVLuc luciferase reporter vector containing a minimal promoter (He et al., 1998). To test presumptive p53 binding sites, the following oligo pairs containing two copies of wild-type or mutant binding sites were used: 5′-CTAGGCTCCTTGCCTTGGGCTAGGCCACACTCTCCTTG CCTTGGGCTAGGCC-3′ and 5′-CTAGGGCCTAGCCCAAGGCAAG GAGAGTGTGGCCTAGCCCAAGGCAAGGAGC-3′ for BS1, 5′-CTAG GCTCATTACCTTGGGTTAAGCCACACTCTCATTACCTTGGGTTAA GCC-3′ and 5′-CTAGGGCTTAACCCAAGGTAATGAGAGTGTGGCT TAACCCAAGGTAATGAGC-3′ for BS1mut, 5′-CTAGGCTGCAAGTC CTGACTTGTCCACACTCTGCAAGTCCTGACTTGTCC-3′ and 5′-CTA GGGACAAGTCAGGACTTGCAGAGTGTGGACAAGTCAGGACTTGC AGC-3′ for BS2, and 5′-CTAGGCTGTAATTCCTGAATTATCCACAC TCTGTAATTCCTGAATTATCC-3′ and 5′-CTAGGGATAATTCAGGA ATTACAGAGTGTGGATAATTCAGGAATTACAGC-3′ for BS2mut. The annealed oligonucleotide pairs were concatamerized and cloned into the NheI site of pBVLuc. Transfections of 911 cells were performed in 12-well plates using 0.2 μg luciferase reporter plasmid, 0.2 μg pCMVβ, and 0.8 μg pCEP4 encoding either wt p53 or mutant p53R175H. The β-galactosidase reporter pCMVβ (Promega) was included to control for transfection efficiency. Luciferase and β-galactosidase activities were assessed 24–48 hr following transfection with reagents from Promega and ICN Pharmaceuticals, respectively. All reporter experiments were performed in triplicate and repeated on at least three independent occasions. Transfections with HCT116 cells were performed similarly, except that 0.4 μg luciferase reporter and 0.4 μg β-galactosidase reporter were used for each well, without p53 expression vectors.

Inducible Cell Lines

The method for generating inducible cell lines in DLD1 cells has been previously described (Yu et al., 1999). In brief, the HA-tagged full-length PUMA cDNA was cloned into pBi-MCS-GFP to create pBi-PUMA-GFP. Linearized pBi-PUMA-GFP and pTK-hyg (Clontech) was cotransfected into DLD1-TET cells at a molar ratio of 5 to 1. DLD1-TET cells are DLD1 derivatives containing a constitutively expressed tet activator (Gossen and Bujard, 1992; Yu et al., 1999). Single colonies were obtained by limiting dilution in the presence of 400 μg/ml G418, 250 μg/ml hygromycin B (Calbiochem), and 20 ng/ml doxycycline for 3–4 weeks. Clones that had low background GFP fluorescence and homogeneous GFP induction were selected and analyzed for the expression of PUMA by Western blot analysis.

Immunoprecipitation and Western Analysis

Immunoprecipitation was performed essentially as described (Chan et al., 1999), using two different immunoprecipitation buffers, one containing 0.1% Tween 20, and the other containing 0.5% NP-40; identical results were obtained with both. In brief, 911 cells were seeded in T75 flasks 18 hr prior to transfection with 5 μg of each of two expression constructs (10 μg total) and harvested 20 hr after transfection. The cell suspension was sonicated for 15 s in a total volume of 1 ml and incubated with 30 μl protein A:protein G beads (4:1; Boehringer Mannheim) for 1 hr at 4°C. The supernatants collected after centrifugation ("total lysates") were subsequently used for immunoprecipitation with rabbit antibody against HA (sc-805; Santa Cruz). Western blotting of total lysates and immunoprecipitates was performed as previously described (Chan et al., 1999). Other antibodies used in these experiments included a mouse monoclonal antibody against hemagglutinin (12CA5; Boehringer Mannheim), a rabbit antibody against caspase 9 (sc-7890; Santa Cruz), a mouse monoclonal antibody against Bcl-2 (OP60; Oncogene Sciences), a mouse monoclonal antibody against V5, (R960-25; Invitrogen), a mouse monoclonal antibody against β-catenin (C19220; Transduction Labs), and a mouse monoclonal antibody against p53 (DO1; a gift of D. Lane).

Immunofluorescence and Confocal Microscopy

911 cells were seeded on glass chamber slides (Nalge Nunc, Lab-Tek 177372) and transfected with PUMA expression constructs. Twenty hours later, MitoTracker Red (0.5 μM; Molecular Probes) was added to the medium, and the cells were incubated at 37°C for an additional 20 min. Cells were fixed with 4% paraformaldehyde in PBS, permeablized with cold acetone, and blocked with 100% goat serum for 1 hr at room temperature. After three washes in

PBST (PBS with 0.05% Tween 20), slides were incubated with anti-HA antibody (12CA5; Boehringer Mannheim) diluted 1:200 with 50% goat serum in PBST at 4°C overnight. After four washes in PBST for 5 min each, slides were incubated with Alexa^{488}-conjugated anti-mouse antibody (A-11001; Molecular Probes) diluted 1:250 in PBST for 30 min at room temperature. After four additional washes in PBST, slides were mounted and analyzed by confocal microscopy.

Cell Growth and Apoptosis Assays

Approximately 1×10^6 cells were plated in each T25 flask 18–24 hr prior to transfection. Twenty-four hours following transfection with constitutive PUMA expression constructs, cells were harvested by trypsinization and serial dilutions were plated in T25 flasks under hygromycin selection (0.1 mg/ml for HCT116, 0.25 mg/ml for DLD1, and 0.4 mg/ml for SW480 and H1299). Attached cells were stained with crystal violet 14–17 days later. For DLD1 lines containing inducible PUMA constructs, cells were grown in doxycycline and serially diluted in T25 flasks. Twenty-four hours after seeding, the medium was replaced with fresh growth media with or without doxycycline, and cells were allowed to grow for 10 days and then stained with crystal violet. To determine the fraction of apoptotic cells, all cells (attached and floating) were collected and stained with Hoechst 33258 as described (Waldman et al., 1996). Cells with characteristic condensed chromatin and fragmented nuclei were scored as apoptotic.

Northern Blot Analysis

Total RNA was prepared using RNAgents (Promega), and 10 μg of total RNA was separated by electrophoresis in 1.5% formaldehyde agarose gels. Probes for Northern blotting were generated by PCR, using cellular cDNA or ESTs as template, and labeled by random priming (Feinberg and Vogelstein, 1984). The sequences of the primers used to prepare all probes are available from the authors upon request. Northern blot analysis was performed and hybridized in QuickHyb (Stratagene) as described (Zhang et al., 1997).

Acknowledgments

We thank K. Nakano and K. H. Vousden for pleasant and productive interactions, L. Meszler for help with cell imaging and the members of the Kinzler/Vogelstein Laboratories for advice and discussion. This work was supported by the Clayton Fund and NIH grants CA 43460 and GM 07184.

Received November 1, 2000; revised January 23, 2001.

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GenBank Accession Numbers

The GenBank accession numbers for the human and murine *PUMA* cDNA and protein sequences are AF332558 and AF332559, respectively.

The GenBank accession numbers for the human and murine *PUMA* promoters are AF332560 and AF332561, respectively.