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PUMA, a potent killer with or without p53

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Abstract

PUMA (p53 upregulated modulator of apoptosis) is a Bcl-2 homology 3 (BH3)-only Bcl-2 family member and a critical mediator of p53-dependent and -independent apoptosis induced by a wide variety of stimuli, including genotoxic stress, deregulated oncogene expression, toxins, altered redox status, growth factor/cytokine withdrawal and infection. It serves as a proximal signaling molecule whose expression is regulated by transcription factors in response to these stimuli. PUMA transduces death signals primarily to the mitochondria, where it acts indirectly on the Bcl-2 family members Bax and/or Bak by relieving the inhibition imposed by antiapoptotic members. It directly binds and antagonizes all known antiapoptotic Bcl-2 family members to induce mitochondrial dysfunction and caspase activation. PUMA ablation or inhibition leads to apoptosis deficiency underlying increased risks for cancer development and therapeutic resistance. Although elevated PUMA expression elicits profound chemo- and radio-sensitization in cancer cells, inhibition of PUMA expression may be useful for curbing excessive cell death associated with tissue injury and degenerative diseases. Therefore, PUMA is a general sensor of cell death stimuli and a promising drug target for cancer therapy and tissue damage.

Keywords

PUMA; BH3 domain; Bcl-2 family; p53; apoptosis

Introduction

The Bcl-2 family proteins are evolutionarily conserved key regulators of apoptosis. PUMA (p53 upregulated modulator of apoptosis) is one of the most potent killers among the Bcl-2 homology 3 (BH3)-only subgroup of Bcl-2 family members. There are more than 300 articles published on PUMA, the majority of which describe its involvement in stress-induced apoptosis regulated by the tumor suppressor p53. Until now the activity of PUMA seems to be exclusively controlled by transcription, whereas other BH3-only proteins are often activated through multiple mechanisms including posttranslational modifications. In response to genotoxic stress such as DNA damage, *PUMA* is transactivated by p53. Along with another BH3-only protein, Noxa, which in most cases has a minor function, PUMA

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Conflict of interest

L Zhang acted as a consultant/an advisor for the Gerson Lehrman Group (New York, NY, USA) in April 2009. Together, J Yu and L Zhang hold two patents and have had several invention disclosures filed by the John Hopkins University (Baltimore, MD, USA) on the applications of SAGE technology and isogenic cell lines deficient in PUMA, Bax or SMAC. Both the authors are entitled to a share of the royalties for these inventions.

accounts for virtually all of the proapoptotic activity of p53. PUMA is also activated by other transcription factors to initiate p53-independent apoptotic responses to nongenotoxic stimuli, including growth factor/cytokine deprivation, endoplasmic reticulum (ER) stress and ischemia/reperfusion. In contrast to its prominent function in p53-dependent apoptosis, the function of PUMA in p53-independent apoptosis remains to be fully appreciated.

Similar to other BH3-only proteins, PUMA serves as a proximal signaling molecule that transduces death signals to the mitochondria where it acts through multidomain Bcl-2 family members to induce mitochondrial dysfunction and caspase activation. PUMA primarily acts to indirectly activate Bax and/or Bak by relieving the inhibition of these proteins by antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-X_L, Mcl-1, Bcl-w and A1. It has also been suggested that PUMA can trigger apoptosis by directly activating Bax, or through cytoplasmic p53 in some cells. With such versatile functions, it is perhaps not surprising that PUMA has been implicated in many pathological and physiological processes including cancer, tissue injury, neurodegenerative diseases, immune response and bacterial or viral infection. PUMA may well be an excellent therapeutic target, as activating PUMA inhibits tumor growth by restoring apoptosis in cancer cells, whereas inhibiting PUMA curbs excessive apoptosis associated with tissue injury and neurodegeneration.

Discovery

PUMA was independently cloned by three groups in 2001. Two groups identified *PUMA* as a transcriptional target of p53 through global gene expression profiling (Nakano and Vousden, 2001; Yu *et al.*, 2001), whereas another group identified PUMA as an interacting partner of Bcl-2 (hence named as Bcl-2-binding component 3 or BBC3) through yeast two-hybrid screening (Han *et al.*, 2001). *PUMA* is highly conserved between human and mouse, with over 90% sequence identity at both the DNA and protein levels (Yu *et al.*, 2001), and is found in six other vertebrate genomes. The genomic structure of *PUMA* is also very similar in human and mouse, with the human locus mapped to 19q31 (Yu *et al.*, 2001), and the mouse locus residing at 7A2. Five exons are identified in the *PUMA* locus, with the first two exons (1a or 1b) being noncoding (Figure 1). In addition to the two major transcripts, *PUMA*-α and *PUMA*-β, which both encode proteins containing the BH3 domain, extensive alternative splicing can result in multiple *PUMA* transcript species (Nakano and Vousden, 2001; Yu *et al.*, 2001). The functions of these splice variants, some of which lack the BH3 domain, are currently unclear. The genomic region including the *PUMA* promoter, exon 1a and intron 1 contains a high percentage of guanine and cytosine nucleotides, suggesting a propensity of forming secondary structures that inhibit transcription, which may account for low basal expression levels in unstressed cells (Yu *et al.*, 2001; Ming *et al.*, 2008). PUMA is conserved and fulfills similar functions in zebrafish (Sidi *et al.*, 2008). Although no PUMA homologue has been identified in lower eukaryotes, the BH3-only protein EGL-1 may be its functional counterpart in *Caenorhabditis elegans* (Horvitz, 1999).

Protein structure

Initial characterization of PUMA revealed that the protein belongs to the BH3-only subgroup of Bcl-2 family proteins, which share sequence similarity only within the BH3 domain (Figure 1). The BH3 domain of PUMA is required for its interactions with Bcl-2-like proteins, such as Bcl-2 and Bcl-X_L (Nakano and Vousden, 2001;Yu *et al.*, 2001). Structural analysis indicates that the BH3 domain of PUMA forms an amphipathic α-helical structure that directly binds to antiapoptotic Bcl-2 family proteins (Day *et al.*, 2008). The C-terminal portion of PUMA contains a hydrophobic domain that directs its mitochondrial localization (Figure 1;Yu *et al.*, 2003b). Both the BH3 domain and mitochondrial localization are essential for the ability of PUMA to induce apoptosis or suppress cell growth (Yu *et al.*, 2003b;Yee and Vousden, 2008). To date, no posttranslational modification on PUMA has been reported.

Regulation by transcription

PUMA is normally expressed at a very low level (Yu *et al.*, 2001), but is rapidly induced in response to a wide range of stresses in human and mouse cells of virtually every tissue origin that has been examined. To date, only transcriptional induction of *PUMA* has been reported. Bioinformatics analyses reveal potential binding sites for multiple transcription factors in the promoter, exon 1 and intron 1 regions of the *PUMA* gene. Several of these sites are conserved in the human and mouse genomes, such as those for p53, c-Myc and forkhead box O3A (FoxO3a). These transcription factors can activate reporters containing their respective sites in the *PUMA* gene, and are recruited to these sites following stresses *in vivo*.

Among the transcription factors that activate PUMA, the function of p53 is best understood. Within hours of DNA damage, p53 is recruited to the two p53-responsive elements in the *PUMA* promoter (Kaeser and Iggo, 2002; Wang *et al.*, 2007a). Gene targeting studies have indicated that both p53 and the p53 binding sites in the *PUMA* promoter are indispensable for *PUMA* induction by DNA damage (Yu *et al.*, 2001; Wang *et al.*, 2007a). The binding of p53 to the *PUMA* promoter facilitates modifications of the core histones such as acetylation of histones H3 and H4, which leads to opening of the chromatin structure and transcriptional activation (Kaeser *et al.*, 2004; Wang *et al.*, 2007a). In isolated cases, the p65 or p52 subunit of nuclear factor-κB can facilitate p53-dependent *PUMA* induction through p53-dependent recruitment to the *PUMA* promoter following some forms of DNA damage (Fujioka *et al.*, 2004; Schumm *et al.*, 2006).

In addition to p53, a number of other transcription factors are implicated in *PUMA* induction. The p53 homologue p73 can regulate *PUMA* expression independent of p53 by binding to the same p53-responsive elements in the *PUMA* promoter in response to a variety of stimuli (Melino *et al.*, 2004; Matallanas *et al.*, 2007; Ming *et al.*, 2008). The forkhead family member FoxO3a mediates *PUMA* induction by cytokine/growth factor withdrawal (You *et al.*, 2006a). C/EBP homologous protein (CHOP) and E2F1 are involved in *PUMA* induction following ER stress (Futami *et al.*, 2005; Li *et al.*, 2006; Zou *et al.*, 2009). The oncoproteins E2F1 and c-Myc can induce PUMA through their respective binding sites in the *PUMA* promoter (Fernandez *et al.*, 2003; Hershko and Ginsberg, 2004). Moreover, general transcription factors, including C/EBPβ, CREB, c-Jun and Sp1 have been implicated in *PUMA* induction, some of which may do so by cooperating with p53 or p73 (Qiao *et al.*, 2003; Hayakawa *et al.*, 2004; Koutsodontis *et al.*, 2005; Ming *et al.*, 2008). On the other hand, *PUMA* transcription is subject to negative regulation by transcriptional repressors, including Slug (Wu *et al.*, 2005), some alternative splice products of *p73* (ΔNp73 and p73α) (Melino *et al.*, 2004; Nyman *et al.*, 2005) or *p63* (ΔNp63) (Rocco *et al.*, 2006) and microRNA (Choy *et al.*, 2008).

Role in p53-dependent apoptosis induced by genotoxic stress

The tumor suppressor p53 eliminates damaged or stressed cells through induction of apoptosis (Vogelstein and Kinzler, 2004). PUMA is induced by DNA damage resulting from a variety of genotoxic agents, such as single- and double-strand breakers, inter- and intramolecular cross-linkers, nucleotide analogues and topoisomerase inhibitors, many of which are conventional chemotherapeutic drugs. The induction of PUMA by these agents is strictly p53 dependent, and is abolished in p53-deficient human cancer cells (Yu *et al.*, 2001, 2006). p53-dependent PUMA induction also occurs in response to other classes of agents,

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such as neurotoxins (Gomez-Lazaro *et al.*, 2005; Wong *et al.*, 2005), proteasome inhibitors (Yu *et al.*, 2003a), microtubule poisons (Giannakakou *et al.*, 2002) and transcription inhibitors (Kalousek *et al.*, 2007), likely owing to low levels of DNA damage elicited by these agents. PUMA is essential for p53-dependent apoptosis in a number of cell culture systems. *PUMA*-knockout HCT116 colon cancer cells are highly resistant to apoptosis induced by p53 overexpression or the DNA-damaging agents adriamycin, 5-fluorouracil (5-FU), cisplatin, oxaliplatin, etoposide, camptothecin, UV (ultraviolet) irradiation and γ-irradiation (Yu *et al.*, 2003b; Chipuk *et al.*, 2005; Ding *et al.*, 2007; Wang *et al.*, 2007a; Tsuruya *et al.*, 2008). Additional examples of p53-dependent and PUMA-mediated apoptosis include neuronal cell death induced by 6-hydroxydopamine (Biswas *et al.*, 2005), and cancer cell death induced by the proteasome inhibitors MG-132, bortezomib and epoxomicin (Concannon *et al.*, 2007; Ding *et al.*, 2007), and by the monoflavonoid wogonin (Lee *et al.*, 2008).

γ-Irradiation induces p53-dependent apoptosis *in vivo* in mouse thymocytes and intestinal crypts (Clarke *et al.*, 1993; Lowe *et al.*, 1993). PUMA is induced in a p53-dependent fashion by γ-irradiation in a variety of tissues and cell compartments, including but not limited to mouse embryonic fibroblasts (MEFs), neurons, thymocytes, hematopoietic system, spleen and intestinal crypts. *PUMA*-knockout mice are highly protected from apoptosis induced by γ-irradiation in all of these tissues except for primary MEFs, which enter cell-cycle arrest following γ-irradiation (Jeffers *et al.*, 2003; Villunger *et al.*, 2003; Akhtar *et al.*, 2006; Wyttenbach and Tolkovsky, 2006; Qiu *et al.*, 2008). However, transformed MEFs are also susceptible to DNA-damage-induced and PUMA-dependent apoptosis (Villunger *et al.*, 2003). These striking apoptotic deficiencies observed in *PUMA*-knockout mice resemble those found in *p53*-knockout mice. Importantly, recent studies demonstrated that p53-dependent PUMA induction is the major mechanism leading to γ-irradiation-induced apoptosis in the intestinal and hematopoietic stem/progenitor cells, and contributes significantly to the resulting tissue injury (Wu *et al.*, 2005; Qiu *et al.*, 2008). p53-dependent PUMA induction is also responsible for γ-irradiation-induced apoptosis in zebrafish embryos (Sidi *et al.*, 2008). PUMA is likely to be required for p53-dependent apoptosis induced by other types of DNA damage *in vivo*. Among over a dozen p53 downstream targets implicated in regulating apoptosis (Yu and Zhang, 2005), only deficiency in PUMA results in such striking phenotypes, leaving no doubt that it is an essential mediator of p53-dependent apoptosis *in vivo*.

Under most conditions, PUMA accounts for a majority, if not all, of the proapoptotic activity of p53 in response to DNA damage. However, depending on cell types, mouse strains, apoptotic stimuli and status of other genes, another BH3-only protein, Noxa, can complement the function of PUMA in p53-dependent apoptosis (Villunger *et al.*, 2003). Noxa has a minor function in apoptosis induced by genotoxic drugs or γ-irradiation in MEFs and thymocytes (Michalak *et al.*, 2008). The dominant function of PUMA might be explained by differential regulation of PUMA and Noxa by p53. The induction of PUMA by genotoxic stimuli is strictly p53 dependent, whereas that of Noxa is not (Yu *et al.*, 2001). The p53 binding sites in the *PUMA* promoter are among the few with the highest affinity for p53 following genotoxic stress (Kaeser and Iggo, 2002). Furthermore, PUMA was more potent than Noxa in apoptosis induction when overexpressed (Yu *et al.*, 2001; Cregan *et al.*, 2004). However, Noxa seems to have a prominent function in p53-dependent apoptosis induced by UV irradiation in oncogene-transformed MEFs and keratinocytes (Naik *et al.*, 2007), and in NIH3T3 cells (Shibue *et al.*, 2006).

In response to DNA damage, p53 also induces genes that regulate cell-cycle arrest such as the cyclindependent kinase (CDK) inhibitor p21, which is often antiapoptotic. Preferential activation of proapoptotic genes such as *PUMA* and/or suppression of *p21* is found in cells that are poised to die (Seoane *et al.*, 2002; Iyer *et al.*, 2004; Sykes *et al.*, 2006; Zhang *et al.*, 2006; Patel *et al.*, 2008). On the other hand, selective induction of cell-cycle regulators or suppression of *PUMA* occurs in cells that are resistant to DNA-damage-induced apoptosis (Wu *et al.*, 2005; Jackson and Pereira-Smith, 2006). Moreover, radiosensitivity of several mouse tissues is correlated with the expression of *PUMA*, but inversely with that of *p21* (Fei *et al.*, 2002). Therefore, the choice between cell-cycle arrest and apoptosis can determine cell fate through differential regulation of p53 targets.

Functions in apoptosis induced by other stimuli

Oncogenic stress

PUMA-mediated apoptosis functions as a safeguard mechanism against neoplastic transformation. The cellular proto-oncoprotein c-Myc is a potent transcription factor that can induce p53-dependent apoptosis (Hermeking and Eick, 1994). Large-scale chromatin immunoprecipitation (ChIP) analysis identified several high-affinity c-Myc-binding E boxes in the *PUMA* promoter (Fernandez *et al.*, 2003). c-Myc activates PUMA expression and facilitates PUMA-mediated apoptosis in colon cancer cells (Seoane *et al.*, 2002). Expression of Eμ-Myc activates PUMA and induces PUMA-dependent apoptosis in lymphoma (Jeffers *et al.*, 2003; Maclean *et al.*, 2003). *PUMA* was also identified as a potential transcriptional target of the proto-oncoprotein c-Myb in human mammary cells (Rushton *et al.*, 2003). Oncogenic E2F signaling, a common abnormality in cancer due to defects in the RB pathway, has been linked to p53 activation and apoptosis (Stanelle and Putzer, 2006). E2F1 expression activates *PUMA* and several other BH3-only proteins through the E2F sites in their promoters (Hershko and Ginsberg, 2004). Apoptosis induced by E2F1 is impaired in *PUMA*-knockout HCT116 cells (Hao *et al.*, 2007), and in *PUMA*-knockdown SAOS-2 osteosarcoma cells (Hershko and Ginsberg, 2004). On the other hand, oncoproteins such as ΔNp63 and ΔNp73 can inhibit apoptosis by suppressing *PUMA* (Simoes-Wust *et al.*, 2005; Rocco *et al.*, 2006; Klanrit *et al.*, 2008).

Several studies suggest that p53-dependent and PUMA-mediated apoptosis preferentially eliminates cells with chromosomal instability or compromised cell-cycle checkpoints, which are characteristics of transformed cells. For example, p53 overexpression or genotoxic agents normally induce cell-cycle arrest in HCT116 colon cancer cells, but trigger PUMA-dependent apoptosis in p21-deficient HCT116 cells (Yu *et al.*, 2003b). Apoptosis was detected mostly in the polyploid or M-phase p21-deficient cells, and could be inhibited by other CDK inhibitors including p16 and p27 (Le *et al.*, 2005). Interestingly, tetraploidy, which can lead to aneuploidy, triggered a higher rate of spontaneous apoptosis that is p53 and PUMA dependent (Castedo *et al.*, 2006).

Growth factor/cytokine withdrawal and kinase inhibition

The expression of PUMA is kept in check in healthy cells by survival signals, including growth factors and cytokines (Han *et al.*, 2001; Zou *et al.*, 2009). Blocking these signals through growth factor or cytokine withdrawal leads to p53-independent transcriptional activation of *PUMA* by FoxO3a or p73 (Han *et al.*, 2001; You *et al.*, 2006a; Ming *et al.*, 2008). The function of PUMA in p53-independent apoptosis under such conditions has been extensively studied in hematopoietic cells. Myeloid cells isolated from *PUMA*-knockout mice are resistant to apoptosis induced by withdrawal of interleukin (IL)-3 and IL-6 (Jeffers *et al.*, 2003; Ekert *et al.*, 2006). In mast cells, PUMA deficiency blocked apoptosis induced by cytokine withdrawal (Ekoff *et al.*, 2007). PUMA knockdown also suppressed serum starvation-induced apoptosis in leukemia cells (Ming *et al.*, 2008).

PUMA is induced by various kinase inhibitors targeting signaling pathways downstream of growth factors, conditions that can promote apoptosis in cancer cells. For example, the pankinase inhibitor staurosporine induces PUMA-dependent apoptosis in MEF and colon cancer cells (Villunger *et al.*, 2003; Wang *et al.*, 2007a). The mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 or small-interfering RNA (siRNA) against *MEK1* induces PUMA-dependent apoptosis in melanoma cells (Wang *et al.*, 2007b). Other kinase inhibitors shown to activate PUMA in cancer cells include phosphoinositide-3 kinase inhibitors wortmannin and LY294002 (Han *et al.*, 2001; Ming *et al.*, 2008), the human epidermal growth factor receptor/vascular endothelial growth factor receptor inhibitor BMS-690514 (de La Motte Rouge *et al.*, 2007) and the farnesyltransferase inhibitor BMS-214662 (Gomez-Benito *et al.*, 2005).

ER stress

Apoptosis can occur as a result of ER stress. *PUMA* was identified as an ER stress-responsive gene in global gene expression profiling studies (Reimertz *et al.*, 2003; Ward *et al.*, 2004), and in an siRNA library screening of genes that inhibit ER stress-induced apoptosis (Futami *et al.*, 2005). PUMA is induced by ER stress in multiple cell types (Luo *et al.*, 2005; Nickson *et al.*, 2007; Jiang *et al.*, 2008). The induction of PUMA in response to ER stress is p53 independent in most cases, and mediated, in part, by the transcription factors CHOP (Li *et al.*, 2006), E2F1 (Futami *et al.*, 2005) and TRB3 (Zou *et al.*, 2009). It is likely that other transcription factors also contribute to PUMA induction by ER stress.

PUMA mediates ER stress-induced apoptosis in a variety of cell types. Apoptosis induced by the ER poisons tunicamycin and thapsigargin is inhibited in PUMA-knockout HCT116 colon cancer cells (Reimertz *et al.*, 2003; Luo *et al.*, 2005), *PUMA*-knockdown cardiomyocytes (Nickson *et al.*, 2007) and neuronal cells (Zou *et al.*, 2009), and *PUMA*-knockout MEF cells (Li *et al.*, 2006). At the same time, other BH3-only proteins such as Bim and Noxa are also critical in ER stress-induced apoptosis in other cell types (Li *et al.*, 2006; Puthalakath *et al.*, 2007). Deficiency in PUMA or Bim protects motor neurons from ER stress-induced apoptosis and delays motor neuron loss in amyotrophic lateral sclerosis mice (Hetz *et al.*, 2007; Kieran *et al.*, 2007), suggesting their overlapping functions in neurodegeneration.

Altered redox status

Stimuli leading to altered redox status, including hypoxia, anoxia, oxidative stress and generation of reactive oxygen species (ROS), can upregulate *PUMA* expression *in vitro* and *in vivo*. PUMA and p53 are induced by hypoxia or anoxia in transformed baby mouse kidney cells (Nelson *et al.*, 2004), by ROS or anoxia in cardiomyocytes (Li *et al.*, 2008), by oxidative stress in neuronal cells (Steckley *et al.*, 2007) and by ROS in colon cancer cells (Macip *et al.*, 2003). Phenotypically, *PUMA*-knockout HCT116 cells are deficient in hypoxia-induced and p53-dependent apoptosis (Yu *et al.*, 2003b). PUMA-deficient neurons are remarkably resistant to apoptosis induced by multiple oxidative stress inducers (Steckley *et al.*, 2007). ROS induction impacts on p53-dependent and PUMA-mediated apoptosis in colon cancer cells (Macip *et al.*, 2003). Although ROS is generated during PUMA-mediated apoptosis as a result of mitochondrial damage (Liu *et al.*, 2005), induction of PUMA by ROS may provide a feed-forward mechanism for signal amplification in the execution of apoptosis. Multiple transcription factors including p53 may be involved in PUMA induction by ROS.

Ischemia/reperfusion

PUMA has been implicated in apoptosis induced by ischemia/reperfusion, a primary cause of tissue injury in neurological disorders, heart attack and gastrointestinal disorders (Webster, 2006). Ischemia/reperfusion induces p53-independent PUMA expression and apoptosis in mouse small intestine (Wu *et al.*, 2007). Similar findings have been described in cultured cardiomyocytes and an *ex vivo* ischemic heart model (Toth *et al.*, 2006), and in isolated neurons (Reimertz *et al.*, 2003; Ward *et al.*, 2004). At least two mechanisms might be responsible for triggering PUMA-dependent apoptosis in response to ischemia/ reperfusion, including ROS and ER stress (Nickson *et al.*, 2007; Wu *et al.*, 2007). Immune response may also significantly contribute to apoptosis induction in the later phase of tissue injury.

Immune modulation

PUMA is an important regulator of apoptosis in the immune system. It complements the function of Bim in controlling T-cell apoptosis to terminate an immune response (Bauer *et al.*, 2006; Fischer *et al.*, 2008). Combined losses of *PUMA* and *Bim* result in significant changes in cell numbers in various hematopoietic compartments (Erlacher *et al.*, 2006). Apoptosis induced by glucocorticoids, which has been implicated in the regulation of the immune response, is PUMA dependent in several cell types. PUMA is necessary for glucocorticoid-induced and p53-independent apoptosis in cultured thymocytes (Jeffers *et al.*, 2003; Villunger *et al.*, 2003), and in cerebellar neural progenitor cells (Noguchi *et al.*, 2008). Apoptosis induced *in vivo* by the glucocorticoid dexamethasone is delayed and reduced in *PUMA*-deficient thymocytes and mature lymphocytes (Erlacher *et al.*, 2005, 2006). Other immune modulators such as interferons can stimulate PUMA expression through p53 or Jak1 (Takaoka *et al.*, 2003; Gomez-Benito *et al.*, 2007). The *salmonella* flagellin protein induces a classical proinflammatory gene expression profile including *PUMA*, which is similarly activated by the cytokine tumor necrosis factor-α (Zeng *et al.*, 2003).

Infection

PUMA expression is also modulated in response to viral or bacterial infection. p53-dependent PUMA induction appears to be an important component of AIDS-associated pathology (Castedo *et al.*, 2005). Upregulation of p53 and PUMA was detected in peripheral blood mononuclear cells and lymph nodes (Castedo *et al.*, 2005), circulating $CD4^+$ lymphocytes, dying syncytia and neurons of HIV carriers (Nardacci *et al.*, 2005), and in HIV-1-infected primary lymphoblasts (Perfettini *et al.*, 2004). The HIV envelope (Env) protein appears to be responsible for activating PUMA through p53 to induce PUMA-dependent apoptosis (Perfettini *et al.*, 2004). *Helicobacter pylori* infection leads to p73-mediated PUMA induction in gastric epithelial cells (Wei *et al.*, 2008). Degradation or downregulation of PUMA has been reported to inhibit apoptosis in cells infected with *Chlamydia* (Fischer *et al.*, 2004; Dong *et al.*, 2005; Ying *et al.*, 2005) or Epstein–Barr virus (Choy *et al.*, 2008).

Other inducers

Table 1 summarizes the major stimuli that induce PUMA-dependent apoptosis, along with the related transcription factors and model systems. In addition to these stimuli, PUMA is necessary for apoptosis induced by kinase activators such as phorbol ester (Villunger *et al.*, 2003;Ming *et al.*, 2008), and during skeletal myoblast differentiation (Shaltouki *et al.*, 2007). A variety of other anticancer agents and toxins can induce PUMA expression and apoptosis in human cancer cells. Examples include chemopreventive agents celecoxib (Ishihara *et al.*, 2007;Liu *et al.*, 2008), genistein (Tategu *et al.*, 2008) and green tea polyphenol (Wang *et al.*, 2008b), arsenic trioxide (Morales *et al.*, 2008), the BH3 mimetic Gossypol (Meng *et al.*, 2008) and the synthetic retinoid analogue 4-hydroxybenzylretinone (Anding *et al.*, 2007). Induction of PUMA under these conditions is mostly p53 independent, but its function remains largely undetermined.

Mechanisms of PUMA-induced apoptosis

The proapoptotic activity of PUMA requires its interactions with other Bcl-2 family members and mitochondria localization (Nakano and Vousden, 2001; Yu *et al.*, 2001). Biochemical studies indicate that PUMA induces apoptosis by activating the multidomain proapoptotic protein Bax and/or Bak through its interaction with antiapoptotic Bcl-2 family members, thereby triggering mitochondrial dysfunction and caspase activation. Some reports suggest that PUMA can also directly activate Bax/Bak or cytoplasmic p53 to induce mitochondrial dysfunction (Figure 2).

Functions through multidomain Bcl-2 family members

Similar to apoptosis induced by other BH3-only proteins, PUMA-induced apoptosis requires Bax and/or Bak. *BAX*-knockout HCT116 colon cancer cells are completely resistant to apoptosis induced by PUMA overexpression, or several stimuli that induce PUMA-dependent apoptosis (Yu *et al.*, 2003b). PUMA over-expression leads to conformational change, multimerization and mitochondrial translocation of Bax (Yu *et al.*, 2003b). *In vivo* PUMA-dependent apoptosis induced by γ-irradiation or ischemia/reperfusion also involves Bax multimerization and mitochondrial translocation (Wu *et al.*, 2007; Qiu *et al.*, 2008). These changes in Bax appear to be critical for PUMA-induced apoptosis (Ming *et al.*, 2006). Although Bax is undoubtedly necessary, the function of Bak in PUMA-induced apoptosis cannot be ruled out (Wu *et al.*, 2007; Qiu *et al.*, 2008).

It is controversial whether direct interactions of BH3-only proteins with Bax/Bak are critical for their ability to induce apoptosis. In one model, a limited subgroup of BH3-only proteins is suggested to induce apoptosis by activating Bax or Bak through direct interactions (Letai *et al.*, 2002). A hierarchical regulation was thus proposed in which these proteins, including Bid, Bim and perhaps PUMA, are necessary for Bax activation and apoptosis induced by other BH3-only proteins (Kim *et al.*, 2006). Although an interaction between the BH3 domain of PUMA and the first α-helical region (Halpha1) of Bax has been detected in a cell-free system (Cartron *et al.*, 2004), many studies performed using intact cells or knockout animals argue against direct activation of Bax by PUMA. No interaction between PUMA and Bax could be detected following co-transfection into cancer or immortalized cells (Ming *et al.*, 2006). Inconsistent results were obtained from experiments where the BH3 peptides of PUMA were used to induce Bax multimerization or cytochrome *c* release from isolated mitochondria (Kuwana *et al.*, 2005; Kim *et al.*, 2006; Deng *et al.*, 2007). Even in cells where PUMA and Bax were found to interact, the C-terminal hydrophobic domain of PUMA that mediates this interaction was not required for apoptosis (Yee and Vousden, 2008). Furthermore, Bax and Bak mutants without a discernable interaction with BH3-only proteins are fully capable in apoptosis induction (Willis *et al.*, 2007). Apoptosis still occurs in *Bim*/*Bid* double knockout cells with knockdown of PUMA (Willis *et al.*, 2007). Collectively, these studies strongly suggest that BH3-only proteins do not rely on direct interactions with Bax/Bak to induce apoptosis.

On the other hand, PUMA is able to bind to all antiapoptotic Bcl-2 family members with high affinity. In 911 human embryonic kidney cells, PUMA co-precipitated with a large fraction of Bcl-2 or Bcl-X_L (Yu *et al.*, 2001). The interactions between PUMA and antiapoptotic proteins are solely dependent on the BH3 domain, as deletion or mutations in the BH3 domain completely abrogated these interactions (Yu *et al.*, 2001), and the BH3 peptide of PUMA is fully capable of binding to antiapoptotic proteins (Chen *et al.*, 2005; Kuwana *et al.*, 2005). Much of the current data support a model in which the interactions of PUMA with antiapoptotic proteins cause displacement of Bax and Bak, resulting in activation of the proapoptotic activities of these proteins (Figure 2). PUMA overexpression has been shown to dissociate Bax from Bcl-X_L, and PUMA deficiency blocked apoptosis and dissociation of Bax/Bcl-X_L complexes following DNA damage (Ming *et al.*, 2006). In cell-free binding assays, the BH3 peptide of PUMA suppressed the inhibitory effects of antiapoptotic proteins on Bax without directly binding to Bax (Kuwana *et al.*, 2005). Furthermore, the cell-killing activities of BH3 domains correlate remarkably well with their binding affinities for antiapoptotic proteins (van Delft *et al.*, 2006).

The notion that PUMA is extremely potent in inducing apoptosis in multiple cell types can be explained by its ability to promiscuously interact with different antiapoptotic Bcl-2 family proteins. The PUMA BH3 peptide can bind to all five antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-X_L, Mcl-1, Bcl-w and A1 (Chen *et al.*, 2005). In contrast, BH3 peptides derived from the BH3 domains of most other proapoptotic proteins, such as those from Bad and Noxa, exhibited selective binding to antiapoptotic proteins. The molecular basis of the specificities remains poorly understood. Structural studies provided some insight into how BH3 domains bind to the antiapoptotic proteins Mcl-1 and A1 (Day *et al.*, 2008; Smits *et al.*, 2008). These studies suggest that the conserved residues of the BH3 domains (Figure 1) are responsible for optimal binding to antiapoptotic proteins, which does not seem to explain the disparities in binding affinity among different BH3 domains. This important issue may be better addressed by combining structural information and studies using molecular approaches such as systematic mutagenesis and domain swapping.

Binding of PUMA with several other proteins besides the multidomain Bcl-2 family proteins was reported to modulate its proapoptotic activity. A p53 transcriptional target and antiapoptotic protein called apoptosis repressor with caspase recruitment domain (ARC) was recently shown to interact with PUMA, and displace PUMA from Bcl-2 (Li *et al.*, 2008). A small chaperone protein p23 binds to PUMA in healthy cells, and prolonged ER stress disrupts this interaction and promotes PUMA-dependent apoptosis (Rao *et al.*, 2006). The functions of these and other potential PUMA-interacting proteins remain to be investigated.

Induction of mitochondrial dysfunction

Upon binding to antiapoptotic proteins and activating Bax/Bak, PUMA-induced apoptosis proceeds through a typical mitochondrial pathway, which is characterized by mitochondrial membrane permeabilization and depolarization, release of mitochondrial apoptogenic proteins including cytochrome *c*, SMAC and apoptosis-inducing factor (AIF), and activation of caspases (Figure 2; Yu *et al.*, 2003b). Production of ROS, which is indicative of mitochondrial dysfunction, is associated with PUMA-mediated apoptosis (Macip *et al.*, 2003;Liu *et al.*, 2005). A SMAC-regulated mitochondrial feedback mechanism was recently identified in the execution of PUMA-induced apoptosis. SMAC deficiency suppressed PUMA-induced apoptosis and the release of cytochrome *c* and AIF (Yu *et al.*, 2007). It is possible that SMAC is involved in regulating caspase-mediated mitochondrial events as described in other studies (Lakhani *et al.*, 2006).

Several recent reports suggest that PUMA acts on cytoplasmic p53 to promote mitochondrial dysfunction (Figure 2). PUMA can displace cytoplasmic p53 from Bcl-X_L following UV irradiation in colon cancer cells, which leads to mitochondrial membrane permeabilization (Chipuk *et al.*, 2005). This intriguing model is supported by studies showing that pharmacological inhibitors of cytoplasmic p53 (Strom *et al.*, 2006), but not similar inhibitors that suppress p53-mediated PUMA induction (Steele *et al.*, 2008), can block DNA-damage-induced apoptosis. However, it is not understood why the structurally related inhibitors used in these studies exhibited such different activities. Furthermore, FoxO3a was shown to promote p53 cytoplasmic accumulation in addition to *PUMA* transcription, leading to apoptosis induction (You *et al.*, 2006b). Despite the evidence supporting a role for PUMA in disrupting p53/Bcl-X_L complexes, in most systems PUMA-mediated apoptosis is not dependent on p53. PUMA induces apoptosis in numerous cancer cell lines irrespective of their p53 status (Ito *et al.*, 2005;Yu *et al.*, 2006;Sun *et al.*, 2007), as well as in *p53*-knockout mouse lymphocytes (Callus *et al.*, 2008). Knockout of p53 binding sites in the *PUMA* promoter abolishes PUMA induction by p53 and genotoxic agents, and suppresses apoptosis induced by p53 and genotoxic agents to the same extent as *PUMA* targeting (Wang *et al.*, 2007a). Moreover, apoptotic events induced by cytoplasmic p53, such as Bax and Bak oligomerization and mitochondrial membrane disruption, are unaffected in *PUMA*-knockout cells (Wolff *et al.*, 2008), indicating that the apoptotic function of cytoplasmic p53 is PUMA independent in most cell types.

Function of PUMA in cancer

It is increasingly apparent that apoptosis acts as a barrier against oncogenesis. Deregulated apoptosis contributes to tumor formation, progression and impaired responsiveness to anticancer therapies (Johnstone *et al.*, 2002; Adams and Cory, 2007). Several lines of evidence suggest that the function of PUMA is compromised in cancer cells. First, more than half of human tumors contain *p53* mutations (Vogelstein and Kinzler, 2004), which abrogate the induction of PUMA by irradiation and many chemotherapeutic drugs (Yu and Zhang, 2005). Second, frequent overexpression of antiapoptotic Bcl-2 family proteins and other antiapoptotic oncoproteins in tumors antagonizes PUMA-induced apoptosis (Adams and Cory, 2007). Third, PUMA expression was found to be reduced in malignant cutaneous melanoma, and PUMA expression appears to be an independent predictor of poor prognosis in patients (Karst *et al.*, 2005). In addition, approximately 40% of primary human Burkitt's lymphomas do not express detectable levels of PUMA, which is attributable, in part, to DNA methylation (Garrison *et al.*, 2008). However, *PUMA* does not appear to be a direct target of genetic inactivation in human cancer (Hoque *et al.*, 2003; Kim *et al.*, 2007; Yoo *et al.*, 2007).

Several animal studies suggest a role for PUMA in tumor suppression, despite the fact that *PUMA*-deficient mice do not show increased risk for spontaneous malignancies (Jeffers *et al.*, 2003; Villunger *et al.*, 2003). Suppression of PUMA by short-hairpin RNAs enhanced Eμ-Myc-induced lymphoma by blocking p53-dependent apoptosis (Hemann *et al.*, 2004). Loss of *PUMA* in *Bim*-deficient mice exacerbated hyperplasia of lymphatic organs, and promoted spontaneous malignancies (Erlacher *et al.*, 2006). In a hypoxia-induced tumor model, loss of PUMA- and Bax/Bak-dependent apoptosis contributes to chromosomal instability and enhanced tumorigenesis (Nelson *et al.*, 2004). Whether PUMA deficiency facilitates tumorigenesis in other tumor models is currently under investigation. Nonetheless, existing data already indicate that PUMA suppresses tumorigenesis, perhaps through both p53-dependent and -independent mechanisms.

Roles and considerations in cancer therapy

Evidence of PUMA induction by therapeutic agents in patients has just begun to emerge. For example, analysis of tissue biopsies from breast cancer patients showed that *PUMA* mRNA was induced within 6 h of chemotherapy (Middelburg *et al.*, 2005). Increased expression of *PUMA* and *Bim* is associated with better prognosis in patients receiving 5-FU-based therapy in stage II and III colon cancer, and is an independent prognostic marker for overall and disease-free survival (Sinicrope *et al.*, 2008). PUMA was induced by dexamethasone in leukemia cells isolated from patients that were sensitive to the treatment, but not from those that were resistant (Xu *et al.*, 2006).

In a series of cell culture and xenograft studies, elevated PUMA expression, either alone or in combination with chemotherapy or irradiation, induced profound toxicity to cancer cells. A variety of cancer cells have been analysed, including those from the lung (Yu *et al.*, 2006), head and neck (Sun *et al.*, 2007), esophagus (Wang *et al.*, 2006), drug-resistant choriocarcinoma (Chen *et al.*, 2007), melanoma (Karst *et al.*, 2006), malignant glioma (Ito *et al.*, 2005), gastric glands (Dvory-Sobol *et al.*, 2007), breast (Wang *et al.*, 2008a) and prostate (Giladi *et al.*, 2007). Driving *PUMA* expression by promoters that are selectively active in cancer cells, such as those from *hTERT* (Ito *et al.*, 2005), β-*catenin/Tcf-4* (Dvory-Sobol *et al.*, 2007; Giladi *et al.*, 2007) and *survivin* (Wang *et al.*, 2008a), yields minimal toxicity to normal tissues. It is interesting to note that *PUMA* adenovirus appears to be more efficacious in apoptosis induction and chemosensitization than *p53* adenovirus (Wang *et al.*, 2006; Yu *et al.*, 2006; Sun *et al.*, 2007), suggesting that *PUMA*-based gene therapy may be particularly beneficial to patients whose tumors harbor cellular or viral proteins that inactivate p53. Furthermore, BH3 mimetics, including ABT-737 and several other small molecule inhibitors of Bcl-2 family proteins, have begun to show promise as novel anticancer agents in preclinical studies (Adams and Cory, 2007; Zhang *et al.*, 2007). A BH3 mimetic of PUMA or Bim that can bind to all antiapoptotic proteins might exhibit even better anticancer activity, especially when used in combination with standard chemotherapeutic agents, irradiation and newer generation targeted therapies such as selective kinase inhibitors.

Radiation and chemotherapy can cause severe side effects in rapidly proliferating tissues, such as bone marrow, hair follicles and the gastrointestinal tract, which limit the dose that can be used to treat patients. Preventing these therapy-induced side effects can be extremely beneficial to cancer patients. PUMA is an important mediator of γ-irradiation-induced injury in the hematopoietic and intestinal systems (Wu *et al.*, 2005; Qiu *et al.*, 2008). Therefore, delivery of PUMA inhibitors to these normal tissues would be expected to alleviate the side effects of chemo and radiation therapies. On the basis of data from mice, inhibiting PUMA expression should pose minimal risk for cancer development, and may be more desirable than inhibitors of p53 for manipulating adverse toxicities.

Conclusion

PUMA is induced by a wide range of apoptotic stimuli through both p53-dependent and -independent mechanisms. It is central in mitochondria-mediated cell death by interacting with all known antiapoptotic Bcl-2 family members. A better understanding of PUMA regulation and its interactions with other Bcl-2 family proteins will not only reveal missing mechanistic links in apoptotic signaling pathways that lead to mitochondrial dysfunction, but will also provide tools and insights to identify PUMA activators, mimetics or inhibitors for treating cancer, as well as diseases associated with excessive apoptosis. A major challenge in cancer therapy will be to determine the differences in the signaling pathways between cancer and normal tissues that will allow selective killing of cancer cells by manipulation of PUMA-mediated apoptosis.

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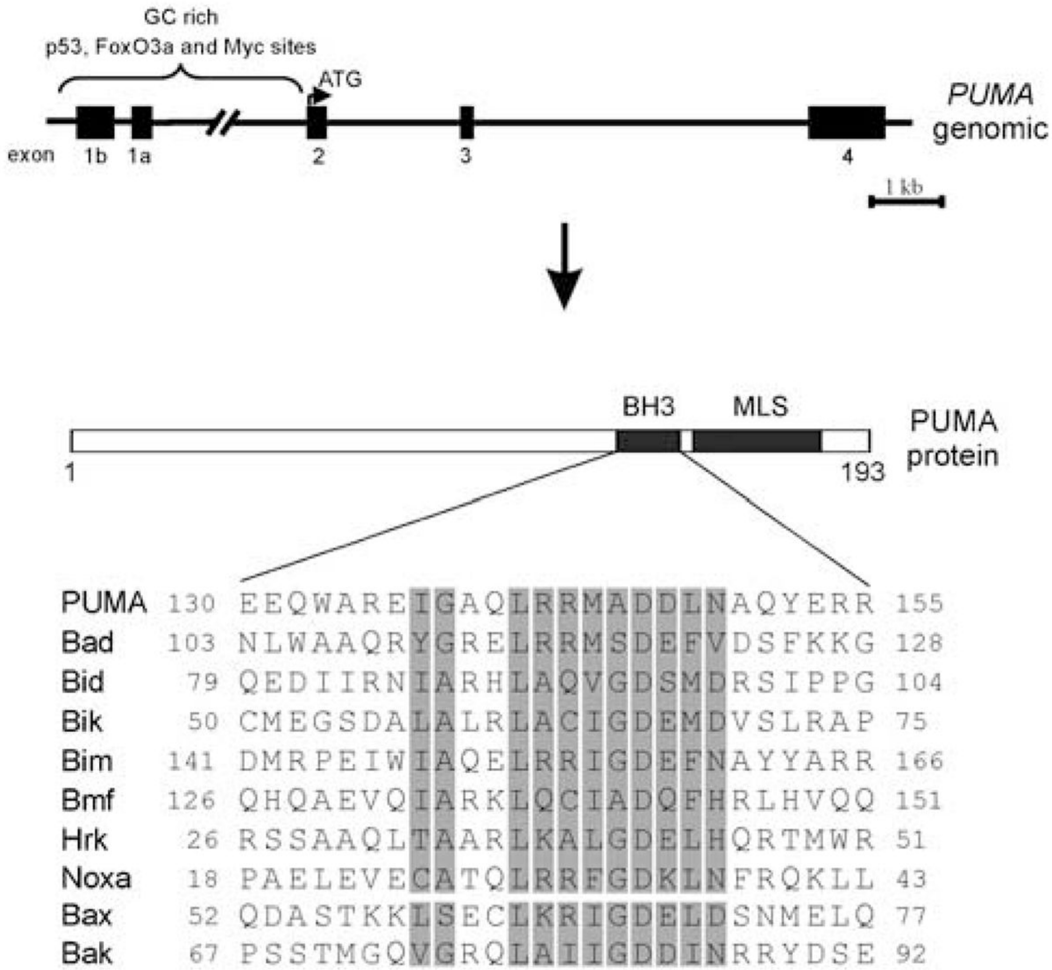

Figure 1.

PUMA gene and protein structure. The human *PUMA* gene contains three coding exons (exons 2–4) and two noncoding exons (exons 1a and b), all of which (except for exon 1b) are conserved in mouse. The binding sites of several transcription factors such as p53, c-Myc and FoxO3a are found in the regulatory regions of both human and mouse genes. The PUMA protein has two functional domains, the BH3 domain and a C-terminal mitochondria-localization signal (MLS), which is not well defined. Alignment of the BH3 domain of PUMA with those of other proapoptotic Bcl-2 family members is shown with the conserved residues shaded in gray.

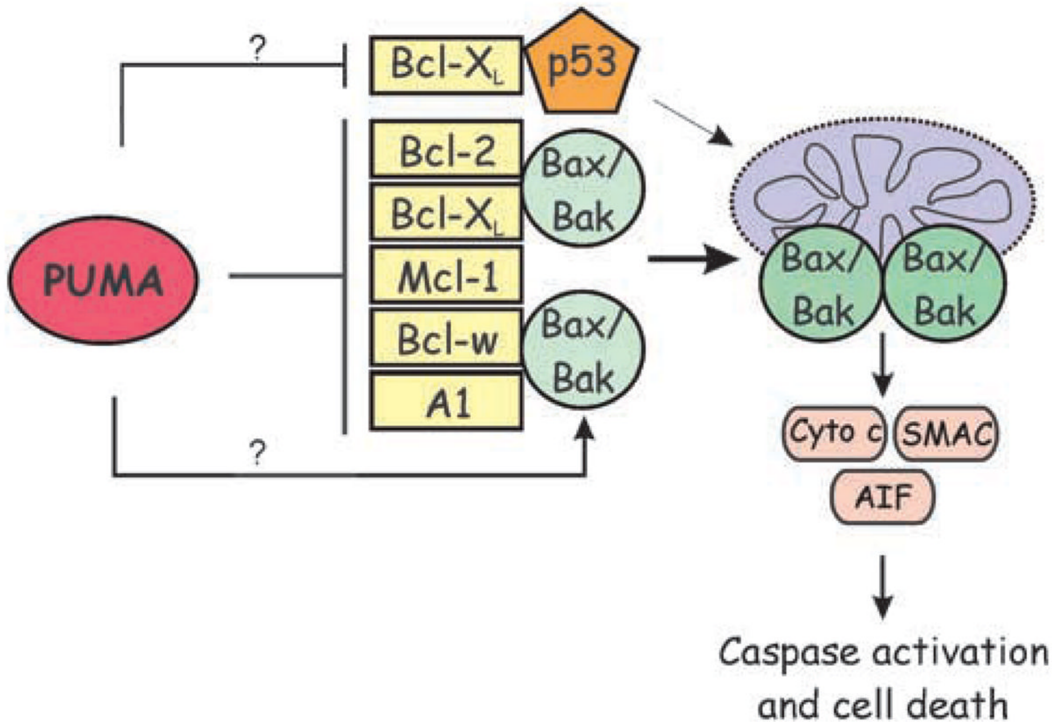

Figure 2.

A model for PUMA-mediated apoptosis. PUMA activates Bax/Bak indirectly by relieving the inhibition of all five antiapoptotic Bcl-2 family proteins to promote mitochondria dysfunction and release of the mitochondrial apoptogenic proteins cytochrome *c* (Cyto *c*), SMAC and apoptosis-inducing factor (AIF), which lead to caspase activation and cell death. Under some conditions, PUMA might directly activate Bax/Bak, or cytoplasmic p53 (by displacing it from Bcl-X_L) to promote cell death through the mitochondria.

Table 1

Stimuli and transcription factors that induce PUMA-dependent apoptosis

Stimuli	Transcription modulators	*In vitro* system	*In vivo* system	References
Genotoxic drugs	p53, p73, c-Myc, E2F1	Cancer, neuronal and renal cells	Neuron	Wong *et al.*, 2005; Akhtar *et al.*, 2006; Wyttenbach and Tolkovsky, 2006; Wang *et al.*, 2007a; Tsuruya *et al.*, 2008
Proteasome inhibitors	p53	Cancer cells		Concannon *et al.*, 2007; Ding *et al.*, 2007
γ- and UV irradiation	p53	Cancer, fibroblast and hematopoietic cells	Hematopoietic system, small intestine and neuron	Jeffers *et al.*, 2003; Villunger *et al.*, 2003; Chipuk *et al.*, 2005; Wu *et al.*, 2005; Wang *et al.*, 2007a, b; Qiu *et al.*, 2008; Sidi *et al.*, 2008
Oncogenes	c-Myc, Eμ-Myc, E2F1	Cancer and hematopoietic cells		Jeffers *et al.*, 2003; Hershko and Ginsberg, 2004; Hao *et al.*, 2007
Polyploidy	p53	Cancer cells		Le *et al.*, 2005; Castedo *et al.*, 2006
Viral infection	p53, miRNA	Cancer cells		Perfettini *et al.*, 2004; Choy *et al.*, 2008
Hypoxia and oxidative stress	p53	Cancer and neuronal cells		Yu *et al.*, 2003b; Steckley *et al.*, 2007
ER stress	CHOP, E2F1, p53	Cancer, fibroblast, cardiomyocyte and neuronal cells	Neuron	Reimertz *et al.*, 2003; Futami *et al.*, 2005; Luo *et al.*, 2005; Li *et al.*, 2006; Kieran *et al.*, 2007; Nickson *et al.*, 2007; Zou *et al.*, 2009
Kinase inhibition	p53-independent	Cancer cells		Wang *et al.*, 2007a, b
Cytokine, growth factor and nutrient deprivation	FoxO3a, p73	Hematopoietic and cancer cells		Jeffers *et al.*, 2003; Ekert *et al.*, 2006; Ekoff *et al.*, 2007; Ming *et al.*, 2008
Ischemia/reperfusion	p53-independent		Small intestine and heart	Toth *et al.*, 2006; Wu *et al.*, 2007
Termination of immune response	Unknown		T cells	Bauer *et al.*, 2006; Chang *et al.*, 2007; Cumont *et al.*, 2007; Fischer *et al.*, 2008
Glucocorticoids	p53-independent	Hematopoietic and neuronal cells	Hematopoietic system	Jeffers *et al.*, 2003; Villunger *et al.*, 2003; Erlacher *et al.*, 2005; Noguchi *et al.*, 2008
Homeostasis	Unknown		Hematopoietic system	Erlacher *et al.*, 2006

Abbreviations: CHOP, C/EBP homologous protein; FoxO3a, forkhead box O3A.