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Review

PUMA, A CRITICAL MEDIATOR OF CELL DEATH – ONE DECADE ON FROM ITS DISCOVERY

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Abstract: PUMA (p53 upregulated modulator of apoptosis) is a pro-apoptotic member of the *BH3-only* subgroup of the Bcl-2 family. It is a key mediator of p53-dependent and p53-independent apoptosis and was identified 10 years ago. The *PUMA* gene is mapped to the long arm of chromosome 19, a region that is frequently deleted in a large number of human cancers. PUMA mediates apoptosis thanks to its ability to directly bind known anti-apoptotic members of the Bcl-2 family. It mainly localizes to the mitochondria. The binding of PUMA to the inhibitory members of the Bcl-2 family (Bcl-2-like proteins) via its BH3 domain seems to be a critical regulatory step in the induction of apoptosis. It results in the displacement of the proteins Bax and/or Bak. This is followed by their activation and the formation of pore-like structures on the mitochondrial

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Abbreviations used: ADI/II – activation domain I/II; Apaf-1 – apoptosis protease activating factor-1; Bad – Bcl-2 associated death promoter; Bak – Bcl-2 antagonist killer; Bax – Bcl-2 associated protein x; Bcl-2 – B-cell leukemia/lymphoma-2; BH1-4 – Bcl-2 homology domains 1-4; Bid – BH3 interacting domain death agonist; Bik – Bcl-2 interacting killer; Bim – Bcl-2 interacting mediator of cell death; Bmf – Bcl-2 modifying factor; Bod – Bcl-2-related ovarian death gene; Bok – Bcl-2 ovarian killer; BS1/2 – p53 binding site 1/2; CHOP – C/EBP homologous protein; DEN – diethylnitrosamine; GSK-3 – glycogen synthase kinase-3; HRK – harakiri, activator of apoptosis ; HSPCs – hematopoietic stem/progenitor cells; HSV-1 – human herpes virus; IKK – IκB kinase; IL-3 – interleukin 3; Lys – lysine; Mcl-1 – myeloid cell leukemia-1; MEFs – mouse embryo fibroblasts; miRNA/miR – microRNA; MLS – mitochondrial localization signal; MOMP – mitochondrial outer membrane permeabilization; NOXA (PMAP1) – phorbol-12-myristate-13-acetate-induced protein 1; OMM – outer mitochondrial membrane; PCD – programmed cell death; PI3K – phosphoinositide 3-kinase; PUMA – p53 upregulated modulator of apoptosis; ROS – reactive oxygen species; Ser – serine; Smac/DIABLO – second mitochondria-derived activator or caspases/direct IAP binding protein with low pI; TRB3 – tribbles 3 homolog; UV-γIR – ultraviolet-gamma irradiation

membrane, which permeabilizes the outer mitochondrial membrane, leading to mitochondrial dysfunction and caspase activation. PUMA is involved in a large number of physiological and pathological processes, including the immune response, cancer, neurodegenerative diseases and bacterial and viral infections.

Key words: Apoptosis, *BH3-only* proteins, Carcinogenesis, Inhibitory members of the Bcl-2 family, Intrinsic apoptosis pathway, p53, Pro-apoptotic members of Bcl-2 family, PUMA, Post-translational regulation, Transcription factors

INTRODUCTION

In animals, apoptosis (or programmed cell death, PCD) is the main mode of cell death during tissue development and homeostasis. It is accepted that diseases such as cancer, immunodeficiency syndromes and neurological disorders not only contribute to alterations in susceptibility to apoptosis, but can also enhance resistance to conventional therapy [1-4]. This is particularly true of neoplasms. During the progression of cancer, transformed cells are subjected to a large number of apoptotic stimuli that are the consequence of stress, such as oncogene stimulation, genotoxic damage or hypoxia [5].

Apoptosis can be induced by extrinsic factors, such as ligands for cell surface death receptors, or intrinsically through the response to damage and stress-inducing stimuli [6, 7]. Activation of the intrinsic apoptotic pathway, also termed the mitochondrial apoptotic pathway, plays a key role in mediating the response to stress stimuli.

In most cases, cells undergo apoptosis via the mitochondrial pathway. This pathway is regulated by proteins of the Bcl-2 (B-cell leukemia/lymphoma-2) family through complex interactions that dictate the integrity of the outer mitochondrial membrane (OMM) [8]. It is initiated by mitochondrial outer membrane permeabilization (MOMP), an important event that results in the release of soluble proteins (e.g. cytochrome c, Smac/DIABLO) into the cytosol [9, 10]. Cytochrome c can then engage the adapter protein Apaf-1 to oligomerize into the place of caspase-9 activation called the apoptosome [11]. Active caspase-9 cleaves executioner procaspases-3 and -7 [9, 12]. Active caspases-3 and -7 cleave a large number of cellular substrates, which results in cellular destruction [13].

The Bcl-2 family proteins are functionally classified as either anti-apoptotic or pro-apoptotic on the basis of their functions and the number of Bcl-2 homology (BH) domains [14-16]. These proteins may share 1 to 4 BH domains. The anti-apoptotic Bcl-2 proteins, including Bcl-2, Bcl-X_L, Mcl-1, Bcl-w and A1/Bfl1, usually contain 3 or 4 BH domains. The pro-apoptotic Bcl-2 family members are divided into multidomain effector proteins, such as Bax (Bcl-2 associated protein x), Bak (Bcl-2 antagonist killer) and Bok (Bcl-2 ovarian killer), and the *BH3-only* proteins, which possess only 1 of 4 BH domains. The *BH3-only* proteins form the largest subgroup. They share an amphipathic α helical BH3

domain. *BH3-only* proteins, such as PUMA/BBC3 (p53 up-regulated modulator of apoptosis/Bcl-2 binding component 3), Bid (Bcl-2 interacting domain death antagonist) and Bim/Bod (Bcl-2 interacting mediator of cell death/Bcl-2-related ovarian death gene), are apical sensors of diverse apoptotic signals that function to inhibit Bcl-2 family anti-apoptotic proteins (Bcl-2 like) and activate Bax/Bak [15, 17-24].

BH3-only proteins bind to anti-apoptotic proteins by inserting their BH3 domain into the hydrophobic pocket made by the folding of the BH1, BH2 and BH3 domains of inhibitory Bcl-2 family members [14]. These proteins are latent killers that need to be activated. Their activation mechanisms include a variety of transcriptional pathways and post-translational modifications [22]. Some of the proteins of this subgroup, i.e. PUMA, NOXA (phorbol-12-myristate-13-acetate-induced protein 1 or PMAP1) and Bik (Bcl-2 interacting killer) are transcriptionally up-regulated by p53-dependent or p53-independent transcription factors [23, 24]. This review focuses on the main achievements made regarding the structure and functions of PUMA during the decade since its discovery [25-27].

PUMA GENE AND PROTEIN STRUCTURE

PUMA was firstly cloned in 2001 by two independent laboratories as a transcriptional target of p53 through global gene expression profiling using microarrays or serial analysis of gene expression approaches [25, 26]. In the same year, Han *et al.* identified the *bbc3* gene (Bcl-2 binding component 3) using a yeast two-hybrid screening that corresponds to the *PUMA* cDNA [27]. Importantly, *bbc3* mRNA was induced by p53-dependent and p53-independent apoptotic stimuli, including dexamethasone treatment of murine thymocyte and deprivation of serum or growth factors in several cancer cell lines. These data support the idea that the regulation of *PUMA/bbc3* mRNA levels, and thus the pro-apoptotic activity of the encoded PUMA protein, represents a common target in different cell death pathways [27].

The *PUMA* gene (19q13.3) leads to the expression of pro-apoptotic *BH3-only* protein, which was originally named SEPUKU [26] or JFY-1 [28]. RT-PCR studies indicated that *PUMA* was expressed at low but similar levels in all of the examined tissues. Vogelstein's laboratory observed a significant induction of the new gene after infection with an adenovirus encoding wild-type p53 of four colorectal cancer cell lines [25]. A high level of *PUMA* activity was also confirmed in SAOS-2-p53 and H1299-p53 cells following their treatment with doxycycline [26]. Actinomycin D-induced activation of endogenous p53 in the tumor cell lines RKO, MCF-7 and U2OS also resulted in an elevation in *PUMA* expression. Importantly, this activation did not occur in RKO cells expressing human papillomavirus E6 protein, where p53 was inactivated by degradation. The length of the *PUMA* transcript is about 1.6-1.9 kb. *PUMA* has been reported to encode four different forms, α, β, γ and δ, but only PUMA α and PUMA β, which contain the BH3 domain, display pro-apoptotic activity. Both proteins

differ only in the N-terminal region of chain. They can interact with Bcl-2 family members in the mitochondrial membrane to drive cytochrome c relocation from the mitochondria to the cytoplasm and activate procaspases-9 and -3 [26]. Yu *et al.* described that the *PUMA* transcript (1.9 kb) contains four exons (1a, 2, 3 and 4) with a presumed initiation codon in exon 2 (Fig. 1A) [24, 25]. The predicted length of the main protein encoded by transcript 4 was 193 amino acids. PUMA revealed no significant homologies to other known proteins, except for those containing the BH3 domain. An alignment of the sequence of human and mouse PUMA indicated about 90% identity [25].

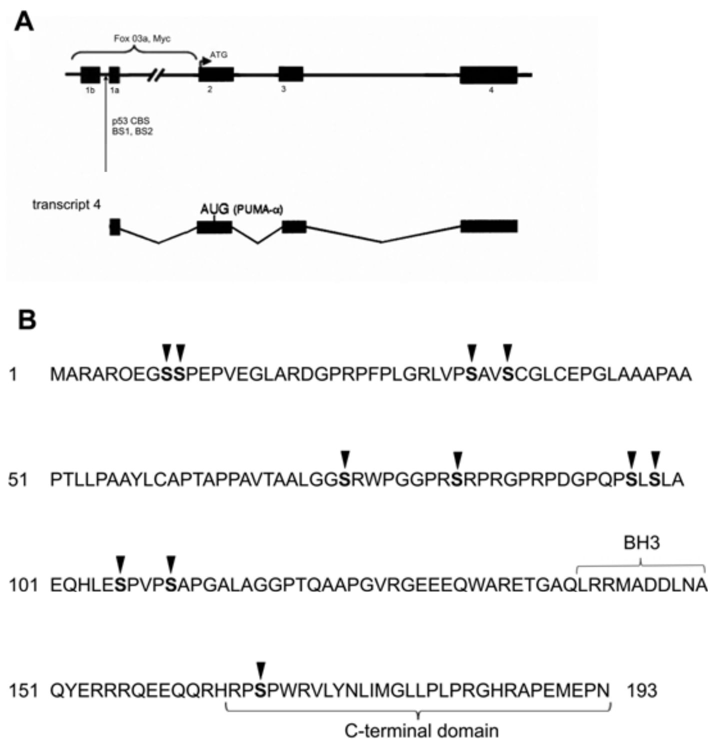

Fig. 1. Structural organization of the *PUMA* gene and main transcript, described as 4(A) and an alignment of the human PUMA sequence (B). The binding sites of the FoxO3a and Myc transcription factors are indicated. Conserved serines (S) in the amino acid sequence of PUMA are indicated by arrowheads and the BH3and C-terminal domains by brackets (according to: [22, 24, 26] modified).

PUMA possesses two functional domains: the BH3 domain; and the mitochondrial localization signal (MLS), which is localized in the C-terminal region of the molecule [28, 29]. The C-terminal structure has still not been precisely determined. A phylogenetic analysis of the BH3 domain-containing

members shows the presence of a seven-amino acid motif (the core LXXXGDE, where X is any amino acid, although neither Gly nor Glu are strictly conserved). Recently, based on structural and sequence studies, a 13-amino acid consensus sequence has been suggested, but without strict conservation (Fig. 1B) [16, 30]. It has been accepted that Leu and Asp, the most conserved elements of the "classic core", appear to be key residues in the interaction with the pro-survival Bcl-2 proteins [16, 18, 30]. Genetic experiments revealed consensus p53-binding sites (CBSs) within intron 1, namely BS1 and BS2, respectively located at 230 and 144 bp upstream of the transcription start [25]. Two distinct activation domains were described in the structure of p53, ADI and ADII. Both are needed for PUMA activity depending on the studied cellular systems. Using adenoviral-mediated gene delivery, reconstitution experiments and mice carrying a knock-in mutation in the *p53* gene, it was demonstrated that the ADI of p53 is crucial for the induction of several apoptotic genes, including *PUMA* in neuronal cells. The target *NOXA* and *Apaf-1* genes could be effectively induced by either of the two activation domains. Interestingly, ADI and ADII are required for the significant increase in *PUMA* gene activity and induction of the PUMA protein that can drive the apoptosis of neuronal cells [31].

It has been demonstrated in a localization study with MitoTracker dye that human PUMA colocalized with this dye in mitochondrial membrane, where it interacts with anti-apoptotic Bcl-2 family members to activate the pro-apoptotic proteins Bax/Bak and trigger mitochondrial dysfunction [28, 32, 33]. This is followed by the release of pro-apoptotic mitochondrial proteins, including cytochrome c and Smac/DIABLO, leading to caspase activation and cell death. PUMA was shown to be targeted at mitochondria by the C-terminal hydrophobic domain, where the MLS motif is present [28, 29]. The generation of several PUMA mutants after transient transfection in U2OS cells revealed that either mutation in the BH3 domain or deletion of the C-terminal 43-amino acid region resulted in the loss of pro-apoptotic capacity. Interestingly, deletion mutants without the last 43, 36 or 29 C-terminal amino acids retained apoptotic activity that was comparable with that of wild-type PUMA but abrogated their exclusive mitochondrial localization [29].

PUMA mediates apoptosis induced by both nuclear and cytoplasmic p53. It binds to the inhibitory proteins Bcl-2 and Bcl-X_L via its BH3 domain and reveals their inhibitory effect on the pro-apoptotic Bcl-2 family members Bax and/or Bak [33, 34]. This member of the *BH3-only* subgroup also drives apoptosis induced by p53-independent signals, such as growth factor deprivation or exposure to glucocorticoids or phorbol ester [23, 27].

Frequent deletion encompassing the *PUMA* locus in chromosome 19q13.3 was reported in a large number of neoplasms including B cell malignances and neural, colorectal and ovarian cancer cell lines [24, 25]. Very recently, RNA interference mechanisms have been shown to regulate apoptosis directly by targeting *PUMA* in glioblastoma, A549 lung and MCF-7 breast cancer cell lines via microRNA-221 and microRNA-222 [35, 36].

REGULATION OF PUMA ACTIVITY

PUMA is characterized by very highly efficient pro-apoptotic action. It is believed that it is one of the most powerful "killers" among the *BH3-only* proteins of the Bcl-2 family. The process of apoptosis conducted with the participation of PUMA is highly effective. Cancer cells are eliminated in the time span of a few hours [23, 24, 28]. PUMA functions are induced by various stimuli as a crucial mediator of p53-dependent and p53-independent apoptosis. Such complexity of PUMA functioning in the organism results from the fact that this protein is involved in a variety of disorders and pathological processes [23, 24]. Regulation of *PUMA* expression during PCD is orchestrated with the involvement of different transcription factors. One of the best known and most important regulators of *PUMA* is p53, which is described as tumor suppressor protein [23-28, 37]. Regulation of PUMA activity may also be determined by the activity of p73 [38, 39], Sp1 [38], FoxO3a [40, 41], E2F1 [39, 42], CHOP [43-48], TRB3 [24], AP-1 [48] and c-Myc [49, 50].

PUMA IN p53-DEPENDENT APOPTOSIS

PUMA represents one of the most potent pro-apoptotic *BH3-only* proteins. It is a key mediator of p53-dependent apoptosis (Fig. 2). The mutual cross-talk between p53 and PUMA is an excellent mechanism for preventing the growth and division of abnormal cells, thereby protecting them against the development of cancer [51]. p53-dependent activation of PUMA function occurs when cells receive a wide variety of stress signals. These include genotoxic agents such as UV, γ-IR, double- and single-stranded DNA breaks, purine analogues, and topoisomerase inhibitors, which are used as chemotherapeutic agents. The elevated expression of *PUMA* in cells through p53-dependent induction is also noticed in the response to oxidative stress, neurotoxins, changes in microtubule structure, deficiency of growth factors, hypoxia and viral infection [24, 25, 52-54]. Yu *et al.* [28] compared apoptosis induction in human colorectal cancer cells (HCT116 line) with targeted disruptions of both alleles of *PUMA* (*PUMA* $^{-/-}$) and *p21* (*p21* $^{-/-}$). The results revealed that these cells exhibited disorders of cell cycle control and did not undergo apoptosis, even in the presence of p53. Subsequent experiments carried out on the cell line HCT116 also showed that the absence of PUMA caused their high resistance to apoptosis induced by DNA-damaging agents, such as adriamycin, 5-fluorouracil, cisplatin, oxaliplatin, UV and γ-IR [24].

The effects of PUMA absence were also evaluated in mice exposed to γ-IR. Activation of *PUMA* expression in these conditions is strictly controlled by p53. Tests were performed in different cell types – fibroblasts, neurons, thymocytes and intestinal progenitor cells [23, 55, 56]. During their development, *PUMA*-depleted mice showed normal appearance and body weight and normal appearance of major internal organs. They lived for at least one year. These data

suggest that the loss of the gene and consequent lack of the PUMA protein did not result in an increased tendency to develop cancer. However, those mice that did not synthesize p53 (*p53* $^{-/-}$) lived for a significantly shorter period (about 6 months). The rodents were characterized by high susceptibility to spontaneous tumors, particularly thymic lymphomas [55]. The obtained results indicated that murine cells from various *PUMA*-depleted tissues had high resistance to apoptosis. In the absence of PUMA, there was no mobilization of the p53-dependent PCD, even if the mice were subjected to considerable DNA damages [23, 55, 56].

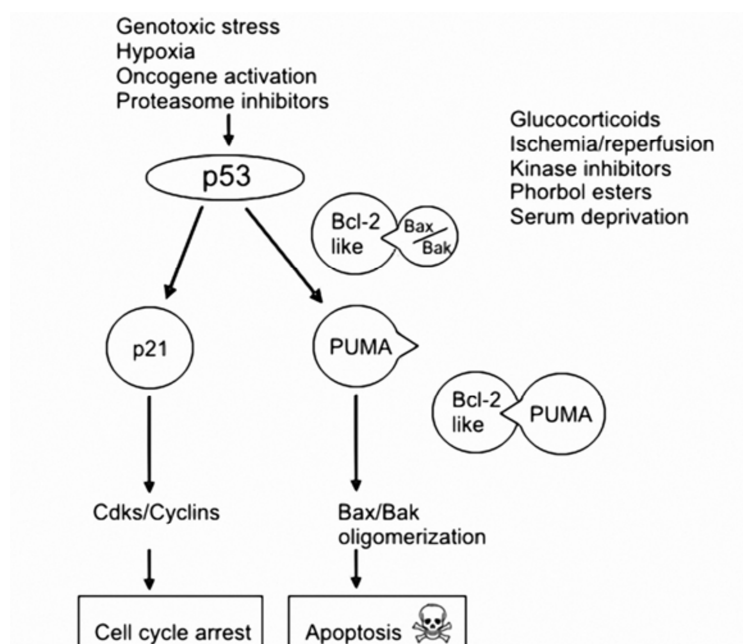

Fig. 2. PUMA is an essential mediator of p53-dependent and -independent apoptosis. p53 induces either cell cycle arrest or apoptotic death. PUMA is implicated in p53-dependent apoptosis induced by p53-independent stimuli such as glucocorticosteroids, ischemia/reperfusion, kinase inhibitors, phorbol esters and serum starvation. Binding of the Bcl-2 like proteins by PUMA via its BH3 domain (triangle) leads to activation and oligomerization of Bax/Bak followed by transmission of apoptotic signals via the mitochondrial pathway. p21 protein is identified as an essential mediator of p53-induced cell cycle arrest.

It has recently been demonstrated that the transcription factor p53 is required for transcriptional induction of *PUMA* in response to DNA damage [57]. HCT116 colorectal cancer cells with a targeted deletion of the p53-binding sites in the *PUMA* promoter did not undergo apoptosis induced by DNA damage, even in the presence of p53. These results revealed that p53-dependent transcriptional

activation of PUMA requires the presence of p53-binding sites in the *PUMA* promoter.

p53, described as a "guardian of the genome" [53, 54], is engaged in tumor growth inhibition. In the course of all genetic instabilities and DNA damage, p53 protein molecules operate in two ways: they can contribute to the inhibition of the cell cycle and DNA repair; or they can cause apoptosis by activating the transcription of several genes, including *PUMA*. Recent studies have reported that p53-dependent regulation of pro-apoptotic *PUMA* expression and subsequent apoptosis are dependent on the functioning of glycogen synthase kinase-3 (GSK-3) and the acetyltransferase Tip60. These enzymes are involved in the control of p53 function choice between cell cycle arrest and apoptosis [58-60]. GSK-3 is a serine/threonine kinase that exists in two isoforms, α and β [60, 61]. It is believed that it acts primarily as a promoter of PCD [61]. GSK3 activity is controlled via the PI3K pathway, through inhibitory phosphorylation by AKT. Inhibition of PI3K contributes to the increased activity of GSK-3. Very recently described results indicate that GSK-3 activity is required for the induction of p53-dependent *PUMA* [58]. The pharmacological inhibition of GSK-3, or kinase gene mutagenesis (*GSK-3* $^{-}$/$^{-}$), contributes to the elimination of *PUMA* expression and causes long-term survival of cells exposed to γ radiation. The active GSK-3-controlled, p53-dependent expression of PUMA in DNA damage also requires the participation of the above-mentioned acetyltransferase Tip60. Kinase assays demonstrated that GSK-3 phosphorylation of Tip60 on Ser86 induces its acetyltransferase activity. The activated form of Tip60 directly acetylates p53 on Lys120 and mediates the acetylation of histone H4 at the *PUMA* promoter. As revealed in previous studies [59, 60], phosphorylation of p53 on the Lys120 residue, which is located within the DNA-binding domain, is required for p53-dependent induction of *PUMA* expression during DNA damage induced by genotoxic agents. Inhibition of Lys120 acetylation by mutations in *p53* or *Tip60* reduced the level of p53-dependent transcription of *PUMA*.

To sum up, the fate of cells with genetic instability and the role played by p53 are affected by GSK-3 and Tip60. The enzymatic activity of these two proteins is required for p53-dependent induction of PUMA and apoptosis. It is interesting that GSK-3 affects the *PUMA* gene with high specificity. Importantly, no influence of GSK-3 has been observed on the expression of other pro-apoptotic p53 target genes, such as *NOXA* and *Bax* [58]. GSK-3 is also involved in hepatocyte lipoapoptosis caused by non-alcoholic fatty liver disease. During this pathological condition, which can lead to hepatocellular carcinoma, activation of JNK kinase is dependent on GSK-3. This kinase promotes hepatocyte PCD predominantly by inducing *PUMA* expression. It was described that inhibition of GSK-3 results in a reduction in JNK activity, a decrease in PUMA activity and a consequent attenuation of hepatocyte lipoapoptosis [61].

PUMA IN p53-INDEPENDENT APOPTOSIS

The published data indicate that PUMA plays a significant role in p53-independent apoptosis during cytokine/growth factor withdrawal, which is a strong signal for apoptosis (Fig. 2). The increase in the *PUMA* expression level under these conditions is due to the activity of transcription factors such as FoxO3a, Sp1 or p73 [27, 38, 40]. Cells with deletion of *PUMA* (*PUMA* $^{-/-}$) were resistant to apoptosis during the deficiency of cytokine/growth factors or following serum starvation [38, 40].

The pro-apoptotic activity of PUMA is also involved in the removal of damaged cells under conditions of ischemia/reperfusion. These conditions can lead to irreversible damage in cells and tissues, heart attack, and neurological diseases [43, 46, 47]. Alterations in *PUMA* p53-independent expression and apoptosis of damaged cells were described under such conditions. The elevated gene expression may be due to the generation of reactive oxygen species (ROS), endoplasmic reticulum stress (ER stress) induced by ATP deficiency, disorder in calcium levels and tissue acidosis [46]. It is not entirely clear how the activation of *PUMA* expression occurs during ischemia/reperfusion. The major transcription factors engaged in *PUMA* expression in these pathological conditions seem to be: E2F1, p73 and the ER stress-specific transcription factor C/EBP homologous protein (CHOP) [43, 46, 47]. As mentioned above, ER stress can also lead to apoptosis running with the involvement of PUMA [44, 45, 48, 62-64]. The increase in the *PUMA* mRNA level and activity of the encoding protein during endoplasmic reticulum dysfunctions were examined in various cell types, such as human hepatoma cells (Huh-7) [48], HCT116 cells [63], human melanoma cells [64], neonatal cardiac myocytes [45] and mouse embryo fibroblasts (MEFs) [44]. Regulation of *PUMA* transcription during ER stress occurs mainly without the participation of p53 [64]. The appropriate level of *PUMA* mRNA is regulated by the transcription factors, such as CHOP, E2F1, TRB3, and AP-1 [24, 44, 45, 48, 62]. It is likely that *PUMA* expression control depends on cell types in these pathological conditions [64].

Immune modulation, infections

PUMA is also involved in the complex mechanism of the immune response. PUMA-dependent induction of PCD is reported after bacterial and viral infections [65-67]. Recently, an increased expression of PUMA and NOXA has been described in gastric epithelial cells infected with *Helicobacter pylori* [65]. Cell response to bacterial infection increased with the activity of the p53 transcription factor family, particularly a robust up-regulation of p73. The appropriate balance between cell death and the development of immune cells is a prerequisite for its homeostasis. The immune response comes with strong T cell proliferation. The T cells subsequently develop into effector cells. After elimination of the pathogen, it is necessary to reduce the number of T cells through apoptosis. This process is a key step during the shutdown of an acute immune response. Two *BH3-only* proteins are primarily responsible for

T cell apoptosis: Bim and PUMA. Loss of *Bim* and/or *PUMA* causes significant changes in cell levels in various hematopoietic compartments [24, 52]. These proteins ensure the proper functioning of the immune system and prevent pathological conditions such as neoplasia or autoimmunity [68-71]. The results of experiments on mice infected with human herpes virus (HSV-1) revealed that T cells isolated from animals lacking *PUMA* were characterized by an abnormally long life span. These cells did not undergo apoptosis. The obtained data indicate a key role of PUMA in immune response regulation. Control of *PUMA* transcription in antigen-activated T cells that undergo PCD is driven mainly by p53 and FOXO3a transcription factors [68-70].

Changes in redox status

Interestingly, stimuli that alter redox status, including oxidative stress, anoxia, hypoxia and ROS generation, can up-regulate *PUMA* expression with the involvement of p53 and/or other transcription factors [24, 72]. The participation of PUMA in the rapid induction of cell apoptosis with oxidative damage is a strong defense mechanism and essential to maintain homeostasis. PUMA plays a major role in the removal of neuronal cells affected by neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease or amyotrophic lateral sclerosis [72].

POST-TRANSLATIONAL REGULATION OF PUMA

Little is known about the post-translational regulation of the pro-apoptotic activity of PUMA. The first results that provided undeniable evidence that this protein is subject to post-translational control by phosphorylation came from Ryan *et al.* [22]. Phosphorylation of the described *BH3-only* protein appears to be another very important point of control and decision of cell fate. As demonstrated, PUMA phosphorylation may occur in response to serum and interleukin-3 (IL-3) stimulation [73]. Thin-layer chromatography (TLC) of PUMA from transiently transfected HeLa cells with constructs encoding N-terminally HA- or Flag-tagged PUMA indicated that this protein is phosphorylated on Ser residues in multiple sites, i.e. 9, 10, 36, 96, 106 and 166, with the major site of modification being Ser10. None of the threonine or tyrosine residues are phosphorylated [22, 73]. Post-transcriptional modification by phosphorylation at Ser10 promotes protein turnover, represses PUMA-induced cell death, and promotes cell survival. Interestingly, it was described that mutation of Ser10 (to Ala) increased the half-life of PUMA, suggesting that phosphorylation at this residue destabilizes the protein, thereby keeping its apoptosis-inducing potential in check. This extremely important discovery contributes to a better understanding of the functioning of one of the strongest apoptotic killers. As demonstrated in a study of kinase assays, PUMA phosphorylation (Ser10) occurring at IL-3 signaling is responsible for IKK1 (IκB kinase 1) activity. It forms part of the anti-apoptotic signaling complex

IKK1/IKK2/Nemo. IKK1 belongs to a family of kinases engaged in promoting cell proliferation and survival. Through the phosphorylation of the inhibitor of NFκB, IκB, it indirectly regulates the transcriptional activation of this transcription factor's target genes.
The IKK1 activation pathway involves IL-3 receptor signaling, but this mechanism is still not precisely understood. It is likely that induction of IKK1 activity requires the presence and participation of a number of multiple components downstream of the IL-3 receptor. It remains unknown how other phosphorylation sites affect PUMA function.

PUMA AND APOPTOSIS

Ten years ago, scientists [25-27] discovered a PUMA protein that binds to the anti-apoptotic members of Bcl-2 family with high affinity. It is not surprising that the pro-apoptotic activity of PUMA also cooperates with that of the other members of this large family. Induction of apoptosis by PUMA is associated with the mitochondrial pathway and therefore also with two key multidomain pro-apoptotic proteins, Bax and Bak. It was observed that the total loss of Bax and Bak results in increased cell resistance to apoptosis [74, 75]. PUMA ensures their correct placement in the outer mitochondrial membranes, thus contributing to their activation [76-78]. In the active conformation, both proteins form ion channels in the mitochondrial membranes. These events ultimately lead to permeabilization of the outer membranes in the mitochondria and the release of apoptogenic factors such as cytochrome c, Smac/DIABLO and caspases from the intermembrane space of these organelles [20, 79-81].
Although a decade has passed since the discovery of PUMA, there are still some questions with no clear answers. One of them is how PUMA activates Bax and Bak thus leading to MOMP. The controversial question is whether PUMA serves as the direct or indirect activator (sensitizer/de-repressor) of the mitochondrial apoptosis pathway. At present, there are two main hypotheses concerning *BH3-only* proteins and their involvement in the activation of Bax and Bak: the direct activation model and the indirect activation model, which is also called the displacement model.

Direct activation model

In the direct activation model, *BH3-only* proteins are divided into distinct subgroups. The first group is called activators and includes Bim, tBid and PUMA. Activators can bind to Bax and/or Bak directly and lead to their activation.
The remaining *BH3-only* proteins form a second subgroup known as sensitizers or de-repressors. These include Bad, Noxa, Bik, BMF (Bcl-2 modifying factor), HRK (activator of apoptosis harakiri) and PUMA, which can probably act both as an activator and sensitizer/de-repressor. These proteins bind exclusively with the anti-apoptotic proteins of Bcl-2 family. Their function is to neutralize anti-apoptotic members of Bcl-2 family and to allow the subsequent release of the inhibited activators [15, 17, 20, 75, 81-85].

Recently published data [76, 77] revealed that PUMA can directly activate Bax and Bak in a similar manner to tBid and Bim. The presence of the BH3 domain enables direct interaction of PUMA with Bax and Bak, ultimately leading to their activation, insertion into the mitochondrial outer membrane and homo-oligomerization. In living cells, the pro-apoptotic proteins Bax and Bak are maintained in an inactive state. Bak is permanently embedded in the MOM via its C-terminal α9 helix and interacts with mitochondrial channel VDAC isoform (VDAC2), which maintains molecules of this protein as inactive monomers [76, 86]. Bax is located in the cytosol in an inactive, globular monomer form. Its C-terminal α9 helix, which is responsible for anchoring Bax in MOM, is associated with and blocked by the canonical BH3-binding groove [76]. Interestingly, a novel BH3-binding site sequence has been identified. It is composed of helix α1 and α6 of Bax/Bak [87]. Upon receipt of apoptotic signal(s) by the cell, PUMA as a *BH3-only* direct activator begins the process of activation of Bax/Bak. Its BH3 domain associates with the Bax/Bak interaction α1/α6 site, resulting in the conformational change of the multidomain pro-apoptotic proteins. PUMA attacks the α1 helix of Bax exposing its N-terminal region, followed by the release of the C-terminal α9 helix of Bax from the canonical BH3-binding groove and the anchoring of the protein in the MOM. In the case of Bak, the stage of C-terminal α9 helix release is omitted due to the mitochondrial localization of this protein. After a conformational change of Bax and Bak, PUMA is associated with their N-terminally exposed α1 helix, which leads to the homo-oligomerization of Bax and Bak. The final result is PUMA-dependent activation of Bax and Bak followed by the formation of ion channels in the MOM and by the management of the mitochondrial pathway of apoptosis [76, 77].

Indirect activation model

The indirect activation or displacement model postulates that Bax and Bak are constantly active, even in living cells. Therefore, they must be inhibited by anti-apoptotic proteins to prevent spontaneous apoptosis. The role of *BH3-only* proteins is to displace and release Bax and Bak from the anti-apoptotic protein heterodimers and further to promote PCD [15, 17, 20, 75, 81-85]. Thus, apoptosis occurs only when all the anti-apoptotic molecules are effectively neutralized by the *BH3-only* proteins. Each of the *BH3-only* proteins has a specific affinity for the anti-apoptotic members of Bcl-2 family [81]. Only PUMA, tBid and Bim have comparable high affinities to all anti-apoptotic proteins of Bcl-2 family [17, 18, 81, 85, 88]. In the indirect activation model, these three proteins constitute the main “killers” among all the members of the *BH3-only* subgroup. They can very quickly and efficiently run the apoptosis pathway in cells by direct neutralization of all anti-apoptotic proteins [17, 75].

The results of numerous experiments indicate the involvement of PUMA in the indirect activation model, or as a de-repressor/sensitizer in the direct activation model [76, 77, 89-91]. This protein binds to all the anti-apoptotic Bcl-2 family

members (Bcl-2, Mcl-1, Bcl-X_L, Bcl-w, and A1) with high affinity, causing their inactivation and Bax/Bak liberation, leading to mitochondrial dysfunction, manifested as MOMP and PCD [17, 18, 81, 85].

To start apoptosis, PUMA can also activate the cytosolic form of p53 [92-94]. PUMA couples the nuclear and cytoplasmic pro-apoptotic functions of p53 [94]. In cells growing properly and in the absence of cell stress, cytosolic p53 is kept inactive by sequestration with the cytosolic anti-apoptotic protein Bcl-X_L. After receiving apoptotic signals, such as DNA damage, UV/γ-IR or mutations of oncogenes, nuclear p53 is activated. It acts as a transcription factor, activating transcription of its target gene, *PUMA*. In the next phase, PUMA releases the cytosolic p53 from the Bcl-X_L inhibition complex by forming a new one, Bcl-X_L/PUMA. Free cytosolic p53 molecules are able to activate monomeric Bax in the cytosol and, consequently, induce the mitochondrial pathway of apoptosis [92-95]. Experiments performed in MEFs with mutant p53 and cytosolic Bcl-X_L that bound p53 but not PUMA showed that the cells were resistant to p53-induced apoptosis even in the presence of pro-apoptotic protein. Apoptosis was possible only in wild-type MEFs that lacked mutant Bcl-X_L. The results of this research indicate that the release of cytosolic p53 from Bcl-X_L complex inhibition by PUMA is necessary to start apoptosis [94].

PUMA IN CARCINOGENESIS

Apoptosis constitutes one of the main safeguards against carcinogenesis [96, 97]. The Bcl-2 family is one of the major regulators of the mitochondrial pathway of apoptosis. Mutual quantitative regulations and an appropriate balance between pro- and anti-apoptotic proteins provide tissue homeostasis. Overexpression of proteins that promote survival or loss of apoptogenic factors is associated with cancer development [15]. A large number of results have shown that PUMA dysfunction occurs in many cancer types. There is often a complete observable lack of *PUMA* expression associated with mutation or deletion of *p53*. Loss of p53, which acts as a transcription factor, is confirmed in more than 50% of human cancers. The result of such events is the total abolition of *PUMA* induction expression, and thus, the resistance of tumor cells to PCD induced by UV, γ-IR, DNA damage and a large number of chemotherapeutic agents [98]. The deficiency of *PUMA* expression in cells undergoing neoplastic transformation can also be caused by complete deletion of the long arm of chromosome 19 (19q13.3), where the gene encoding this protein is located. Such aberrations were reported in the case of gliomas, neuroblastomas, certain types of B-cell lymphomas, and head and neck cancers [52, 99]. During B-cell lymphomagenesis, the inactivation of *PUMA* expression inhibited by epigenetic mechanisms associated with an increase in its methylation was detected [50]. PUMA activity correlated with its participation in malignant cell apoptosis can also be abolished by overexpression of anti-apoptotic Bcl-2 family proteins. Imbalance within the complex interactions of the members of the Bcl-2 family,

which is manifested by the increased level of proteins that promote survival, results in a lack of PUMA-dependent apoptosis in cancer cells, even when it is present [15].

It is worth emphasizing that in all the human tumors examined so far, no mutations have been detected directly within the *PUMA* gene. Therefore, this gene itself is not a direct target for mutagenesis during carcinogenesis [52, 99-101]. Genetic studies of *PUMA* from head, neck and lung carcinoma cell lines revealed that this gene is not a direct target of inactivation in these cancers. Similarly, mutational analysis of *PUMA* also showed that mutation is not a key event in inactivation during hepatic or colorectal carcinogenesis. There have also been no somatic mutations detected in the BH3 domain that could contribute to the inactivation of pro-apoptotic activity of PUMA [100, 101]. Deregulation of apoptosis with the participation of PUMA is also revealed very frequently in lymphoproliferative diseases. Genetic studies have shown the rapid development of lymphomas induced by *myc* oncogene product in murine cells with *PUMA* deficiency (PUMA $^{-/-}$) [50, 102]. It is estimated that about 40% of patients with Burkitt's lymphoma (BL) are characterized by very low or even undetectable levels of *PUMA* expression. The same is true for some tumors associated with DNA methylation. Such pathology in the BL cells is caused by epigenetic mechanisms, where there is hypermethylation of four CpG sites arranged within the promoter and the exons of *PUMA*. *PUMA* harbors an unusually high CpG dinucleotide content (55-76%) within its promoter and coding regions. The result is uncontrolled and excessive attachment of methyl residues, which is followed by gene expression silence and resistance of malignant cells to the type of apoptosis dependent on this protein. The use of inhibitors of DNA methyltransferases restored the normal activity of its gene and resulted in a significantly increased level of *PUMA* transcripts [50].

Recently published reports have also provided relevant information on the functioning of microRNAs (miRNAs or miR) as regulators directly targeting *PUMA*. It appears that some of these small non-coding RNAs (~22 nt) in addition to negatively regulating p27 and p57, also affect *PUMA* mRNA translation in glioblastomas (several cell lines) [36], lung cancer (A549 line) and breast cancer (MCF-7 line), which are common forms of human epithelial cancers [35]. miR-221/222 molecules directly interact with putative binding sites in 3'UTR *PUMA* mRNA, leading to translational inhibition and repression of this pro-apoptotic protein, and induce cell survival and tumor progression. Silencing of miR-221/222 resulted in the restoration of pro-apoptotic activity of PUMA and induction of glioblastoma, MCF-7 and A549 cell apoptosis, and consequently in the inhibition of tumor growth. Additionally, it was demonstrated that miR-221/222 molecules are also important regulators of hepatocyte apoptosis in fulminant liver failure [103]. Overexpression of these molecules led to abrogation of mouse liver cell apoptosis through negative regulation of *PUMA* at the post-transcriptional level and to inhibition of protein translation. miR-221/222 may therefore be a potential therapeutic target in the

treatment of hepatitis and liver failure, glioblastoma and epithelial cancers [35, 36, 103].

PUMA operates as one of the strongest inducers of apoptosis and one of the most effective "security guards" against the development of cancer. However, very recent studies provide surprising new information on the role of PUMA in certain cancer types, emphasizing the "dark side" of the function of this pro-apoptotic molecule. Surprisingly, it turns out that genetic ablation of *PUMA* paradoxically leads to a protective effect for hematopoietic stem/progenitor cells (HSPCs) against γ radiation and the induction of mutations in these cells [104, 105]. This inhibits the development of lymphomas [106, 107] and hepatocellular carcinoma under certain conditions [108]. This phenomenon is referred as "the PUMA paradox" [109]. Murine HSPCs that were defective in p53-induced apoptosis due to the loss of its target gene *PUMA* (puma $^{-}/^{-}$) and that had DNA damage induced by γ-irradiation were resistant to γIR-induced lymphomagenesis and failed to form thymic lymphomas. Inhibition or absence of *PUMA* led to long-term survival of mice exposed to high-dose irradiation. Moreover, under such conditions, no increased risk of developing cancer was found. In wild-type cells, DNA damage caused by γIR induced p53-dependent activation of *PUMA*. PUMA protein induced apoptosis of HSPCs with damaged DNA thereby promoting the formation of thymic lymphomas [104-107]. Interesting results were obtained in a diethylnitrosamine-induced (DEN-induced) liver carcinogenesis model. DEN treatment induced p53-independent *PUMA* expression, but required the JNK1/c-Jun pathway, PUMA-dependent hepatocyte apoptosis and compensatory proliferation led to the development of hepatocellular carcinoma. In the case of inhibition or deletion of JNK1/PUMA, there occurred ablation of carcinogenesis and attenuated initiation of DEN-induced apoptosis in hepatocellular carcinoma cells [108]. The "PUMA paradox" is explained by the activity of HSPCs, which can create or give rise to the conditions favorable for cancer development during apoptosis. Apoptosis of cells containing the mutation(s) simultaneously induces compensatory proliferation of neighboring HSPCs in order to regenerate tissues and organs. The described event is referred as the "Phoenix Rising" pathway and proceeds with the participation of active caspase-3 and -7 and phospholipase A2 [110]. It thus contributes to the proliferation of HPSCs with cancer-causing mutations that are not subordinate to apoptosis. This may create favorable conditions for an accumulation of secondary mutations in cells and may allow the spread of genome instability and ultimately malignancy. Amplification of residual damage is limited, which results in preserved genome stability and tissue homeostasis as well as a lack of tumor growth [109, 111, 112].

CONCLUSIONS

Deregulation of apoptosis is implicated in numerous disease states, including cancer, degenerative disorders and autoimmunity. Importantly, alterations in

apoptotic signaling contribute to carcinogenesis and confer resistance not only to physiological apoptotic signals but therapeutic options as well. The overexpression of anti-apoptotic Bcl-2 family members is a common feature in numerous cancers. Cancers can be inhibited by pro-apoptotic Bcl-2 family members, such as PUMA, which is the only protein able to bind and antagonize all five inhibitory members of the Bcl-2 family. At present, very exciting experiments have started to obtain a precise picture of PUMA-inhibitory Bcl-2 protein interactions. A detailed understanding of these interactions and the mechanisms of regulating PUMA expression might be of paramount importance for the treatment of human diseases in which expression of this protein plays an essential role.

REFERENCES

1. Green, D.R. and Reed, J.C. Mitochondria and apoptosis. **Science** 281 (1998) 1309-1312.
2. Zhivotovsky, B. and Orrenius, S. Cell cycle and cell death in disease: past, present and future. **J. Intern. Med.** 268 (2010) 395-409.
3. Caroppi, P., Sinibaldi, F., Fiorucci, L. and Santucci, R. Apoptosis and human diseases: mitochondrion damage and lethal role of released cytochrome c as proapoptotic protein. **Curr. Med. Chem.** 16 (2009) 4058-4065.
4. Plati, J. and Khosravi-Far, R. Apoptotic cell signaling in cancer progression and therapy. **Integr. Biol.** 3 (2011) 279-296.
5. Evan, G. and Vousden, K.M. Proliferation, cell cycle and apoptosis in cancer. **Nature** 111 (2001) 342-348.
6. Green, D.R. Apoptotic pathway: paper wraps stone blunts scissors. **Cell** 102 (2000) 1-4.
7. Hengartner, M.O. The biochemistry of apoptosis. **Nature** 407 (2000) 770-776.
8. Green, D.R. and Evan, G.J. A matter of life and death. **Cancer Cell** 1 (2002) 19-30.
9. Mohamed, N., Gutierrez, A., Nunez, M., Cocca, C., Marit, G., Cricco, G., Medina, V., Rivera, E. and Bergoc, R. Mitochondrial apoptotic pathways. **Biocell** 29 (2005) 149-161.
10. van Gurp, M., Festjens, N., van Loo, G., Saelens, X. and Vandenabeele, P. Mitochondrial intermembrane proteins in cell death. **Biochem. Biophys. Res. Commun.** 304 (2003) 487-497.
11. Cain, K., Bratton, S.B., Langlais, C., Walker, G., Brown, D.G., Sun, X.M. and Cohen, G.M. Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4 MDa apoptosome complex. **J. Biol. Chem.** 275 (2000) 6067-6070.
12. Hill, M.M., Adrian, C. and Martin, S.J. Portrait of a killer: the mitochondrial apoptosome emerges from the shadows. **Mol. Interv.** 3 (2003) 19-26.
13. Riedl, S.J. and Salvesen, G.S. The apoptosome: signaling platform of cell death. **Nat. Rev. Mol. Cell. Biol.** 8 (2007) 405-413.

14. Borner, C. The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. **Mol. Immunol.** 39 (2003) 615-647.
15. Adams, J.M. and Cory, S. The Bcl-2 apoptotic switch in cancer development and therapy. **Oncogene** 26 (2007) 1324-1337.
16. Lanave, C., Santamaria, M. and Saccone, C. Comparative genomics: the evolutionary history of the Bcl-2 family. **Gene** 333 (2004) 71-79.
17. Willis, S.N. and Adams, J.M. Life in the balance: how BH3-only proteins induce apoptosis. **Curr. Opin. Cell Biol.** 17 (2005) 617-625.
18. Lomonosova, E. and Chinnadurai, G. BH3-only proteins in apoptosis and beyond: an overview. **Oncogene** 27 (2008) 2-19.
19. Letai, A., Bassik, M.C., Walensky, L.D., Sorcinelli, M.D., Weiler, S. and Korsmeyer, S.J. Distinct BH3 domains either sensitize or acrivate mitochondrial apoptosis, serving as prototype cancer therapeutics. **Cancer Cell** 2 (2002) 183-192.
20. Chipuk, J.E., Moldoveanu, T., Llambi, F., Parsons, M.J. and Green, D.R. The BCL-2 family reunion. **Mol. Cell** 37 (2010) 299-310.
21. Elkholi, R., Floros, K.V. and Chipuk, J.E. The role of BH3-only proteins in tumor cell development, signaling and treatment. **Genes Cancer** 2 (2011) 523-537.
22. Fricker, M., O'Prey, J., Tolkovsy, A.M and Ryan, K.M. Phosphorylation of Puma modulates its apoptotic function by regulating protein stability. **Cell Death Dis.** 1 (2010) DOI: e59; doc: 10.1038/cddis. 2010.38.
23. Jeffers, J.R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K.H., Han, J., Chittenden, T., Ihle, J.N., McKinnon, P.J., Cleveland, J.L. and Zambetti, G.P. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. **Cancer Cell** 4 (2003) 321-328.
24. Yu, J. and Zhang, L. PUMA, a potent killer with or without p53. **Oncogene** 27 (2008) S71-S83.
25. Yu, J., Zhang, L., Hwang, P.M., Kinzler, K.W. and Vogelstein, B. PUMA induces the rapid apoptosis of colorectal cancer cells. **Mol. Cell.** 7 (2001) 673-682.
26. Nakano, K. and Vousden, K.H. PUMA, a novel proapoptotic gene, is induced by p53. **Mol. Cell.** 7 (2001) 683-694.
27. Han, J., Flemington, C., Houghton, A.B., Gu, Z., Zambetti, G.P., Lutz, R.J., Zhu, L. and Chittenden, T. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. **Proc. Natl. Acad. Sci. USA** 98 (2001) 11318-11323.
28. Yu, J., Wang, Z., Kinzler, K.W., Vogelstein, B. and Zhang, L. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. **Proc. Natl. Acad. Sci. USA** 100 (2003) 1931-1936.
29. Yee, K.S. and Vousden, K.H. Contribution of membrane localization to the apoptotic activity of PUMA. **Apoptosis** 13 (2008) 87-95.

30. Day, C.L., Smits, C., Fan, C.F., Lee, E.F., Fairlie, W.D. and Hinds, M.G. Structure of the BH3 domains from the p53-inducible BH3-only proteins Noxa and Puma in complex with Mcl-1. **J. Mol. Biol.** 380 (2008) 958-971.
31. Cregan, S.P., Arbour, N.A., Maclaurin, J.G., Callaghan, S.M., Fortin, A., Cheung, E.C., Guberman, D.S., Park, D.S. and Slack, R.S. p53 activation domain 1 is essential for PUMA upregulation and p53-mediated neuronal cell death. **J. Neurosci.** 24 (2004) 10003-10012.
32. Wang, X., Wang, J., Lin, S., Geng, J., Wang, J. and Jiang., B. Sp1 is involved in H_2O_2-induced PUMA gene expression and apoptosis in colorectal cancer cells. **J. Exp. Clin. Cancer Res.** 24 (2008) 27-44.
33. Ming, L., Wang, P., Bank, A., Yu, J. and Zhang, L. PUMA dissociated Bax and Bcl-X_L to induce apoptosis in colon cancer cells. **J. Bioch. Chem.** 28 (2006) 16034-16042.
34. Chipuk, J.E, Bouchier-Hayes, L., Kuwana, T., Newmayer, D.D. and Green, D.R. PUMA couples the nucler and cytoplasmic proapoptotic function of p53. **Science** 309 (2005) 1732-1735.
35. Zhang, C., Junxia, Z., Zhang, A., Wang, Y., Han, L., You, Y., Pu, P. and Kang, C. PUMA is a novel target of miR-221/222 in human epithelial cancers. **Int. J. Oncol.** 37 (2010) 1621-1626.
36. Zhang, C., Zhang, J., Zhang, A., Shi, Z., Han, L., Jia, Z., Yang, W., Wang, G., Jiang, T., You, Y., Pu, P., Cheng, J. and Kang, C. MiR-221 and miR-222 target PUMA to induce cell survival in glioblastoma. **Mol. Cancer** 9 (2010) 1-9.
37. Jabbour, A.M., Daunt, C.P., Green, B.D., Vogel, S., Gordon, L., Lee, R.S., Silke, N., Pearson, R.B., Vandenberg, C.J., Kelly, P.N., Nutt, S.L., Strasser, A., Borner, C. and Ekert, P.G. Myeloid progenitor cells lacking p53 exhibit delayed up-regulation of Puma and prolonged survival after cytokine deprivation. **Blood** 115 (2010) 344-352.
38. Ming, L., Sakaida, T., Yue, W., Jha, A., Zhang L. and Yu J. Sp1 and p73 activate PUMA following serum starvation. **Carcinogenesis** 29 (2008) 1878-1884.
39. Ray, R.M., Bhattacharya, S. and Johnson, L.R. Mdm2 inhibition induces apoptosis in p53 deficient human colon cancer cells by activating p73- and E2F1-mediated expression of PUMA and Siva-1. **Apoptosis** 16 (2011) 35-44.
40. You, H., Pellegrini, M., Tsuchihara, K., Yamamoto, K., Häcker, G., Erlacher, M., Villunger, A. and Mak T.W. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. **J. Exp. Med.** 203 (2006) 1657-1663.
41. Dudgeon, C., Wang, P., Sun, X., Peng, R., Sun, Q., Yu, J. and Zhang, L. PUMA induction by FoxO3a mediates the anticancer activities of the broad-range kinase inhibitor UCN-01. **Mol. Cancer Ther.** 9 (2010) 2893-2902.
42. Hershko, T. and Ginsberg, D. Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. **J. Biol. Chem.** 279 (2004) 8627-8634.

43. Wu ,B., Qiu, W., Wang, P., Yu, H., Cheng, T., Zambetti, G.P., Zhang, L. and Yu J. p53 independent induction of PUMA mediates intestinal apoptosis in response to ischaemia-reperfusion. **Gut** 56 (2007) 645-654.
44. Li, J., Lee, B. and Lee A.S. Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. **J. Biol. Chem.** 281 (2006) 7260-7270.
45. Nickson, P., Toth, A. and Erhardt, P. PUMA is critical for neonatal cardiomyocyte apoptosis induced by endoplasmic reticulum stress. **Cardiovasc. Res.** 73 (2007) 48-56.
46. Webster, K.A. Puma joins the battery of BH3-only proteins that promote death and infarction during myocardial ischemia. **Am. J. of Physiol. Heart Circ. Physiol**. 291 (2006) 20-22.
47. Toth, A., Jeffers, J.R., Nickson, P., Min, J-Y., Morgan, J.P., Zambetti, G.P. and Erhardt, P. Targeted deletion of Puma attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion. **Am. J. Physiol. Heart Circ. Physiol.** 291 (2006) 52-60.
48. Cazanave, S.C., Elmi, N.A., Akazawa, Y., Bronk, S.F., Mott, J.L. and Gores, G.J. CHOP and AP-1 cooperatively mediate PUMA expression during lipoapoptosis. **Am. J. Physiol. Gastrointest. Liver Physiol.** 299 (2010) 236-243.
49. Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S, Greene, J., Cocito, A. and Amati B. Genomic targets of the human c-Myc protein. **Genes Dev.** 17 (2003) 1115-1129.
50. Garrison, S.P., Jeffers, J.R., Yang, C., Nilsson, J.A., Hall, M.A., Rehg, J.E., Yue, W., Yu, J., Zhang, L., Onciu, M., Sample, J.T., Cleveland, J.L. and Zambetti, G.P. Selection against PUMA gene expression in Myc-driven B-cell lymphomagenesis. **Mol. Cell. Biol.** 28 (2008) 5391-5402.
51. Happo, L., Strasser, A. and Scott, C.L. BH3-only Proteins. in: **Cell Death** (Melino, G. and Vaux, D., Ed.), 1th edition, John Wiley&Sons – Ltd, 2010, 75-90.
52. Erlacher, M., Michalak, E.M., Strasser, A. and Villunger, A. The BH3-only proteins Puma and Noxa: Two Brothers in Arms. in: **Apoptosis and Cancer Therapy: From Cutting-edge Science to Novel Therapeutic Concepts**, (Debatin, K.M. and Fulda, S., Ed.), Wiley-VCH Verlag GmbH, Weinheim, Germany. DOI: 10.1002/9783527619665.ch13, 2008, 379-402.
53. Lozano, G. and Zambetti, G.P. What have animals models taught us about the p53 pathway? **J. Pathol.** 205 (2005) 206-220.
54. Zapaśnik, M. and Cymerys, J.M. p53 protein – guardian of the genome in the viral infection. **Post. Biol. Kom.** 36 (2009) 565-582.
55. Michalak, E.M., Villunger, A., Adams, J.M. and Strasser, A. In several cell types tumour suppressor p53 induces apoptosis largely via Puma but Noxa can contribute. **Cell Death Differ.** 15 (2008) 1019-1029.

56. Qiu, W., Carson-Walter, E.B., Liu, H., Epperly, M., Greenberger, J.S., Zambetti, G.P., Zhang, L., Yu, J. PUMA regulates intestinal progenitor cell radiosensitivity and gastrointestinal syndrome. **Cell Stem Cell** 2 (2008) 576-583.
57. Wang, P., Yu, J. and Zhang, L. The nuclear function of p53 is required for PUMA-mediated apoptosis induced by DNA damage. **Proc. Natl. Acad. Sci. USA** 104 (2007) 4054-4059.
58. Charvet, C., Wissler, M., Brauns-Schubert, P., Wang, S-J., Tang, Y., Sigloch, F.C., Mellert, H., Brandenburg, M., Lindner, S.E., Breit, B., Green, D.R., McMahon, S.B., Borner, C., Gu, W. and Maurer U. Phosphporylation of Tip60 by GSK-3 determines the induction of PUMA and apoptosis by p53. **Mol. Cell.** 42 (2011) 584-596.
59. Tang, Y., Luo, J., Zhang, W. and Gu, W. Tip60-dependent acetylation of p53modulates the decision between cell-cycle arrest and apoptosis. **Mol. Cell** 24 (2006) 827-839.
60. Sykes, S.M., Mellert, H.S., Holbert, M.A., Li, K., Marmorstein, R., Lane, W.S. and McMahon, S.B. Acetylation of the p53 DNA binding domain regulates apoptosis induction. **Mol. Cell** 24 (2006) 841-851.
61. Ibrahim, S.H., Akazawa, Y., Cazanave, S.C., Bronk, S.F., Elmi, N.A., Werneburg, N.W., Billadeau, D.D. and Gores, G.J. Glycogen synthase kinase-3 (GSK-3) inhibition attenuates hepatocyte lipoapoptosis. **J. Hepatol.** 54 (2011) 765-772.
62. Hetz, C. and Glimcher, L. The daily job of night killers: alternative roles of the BCL-2 family in organelle physiology. **Trends Cell Biol.** 18 (2007) 38-44.
63. Luo, X., He, Q., Huang, Y. and Sheikh, M.S. Transcriptional upregulation of PUMA modulates endoplasmic reticulum calcium pool depletioninduced apoptosis via Bax activation. **Cell Death Differ.** 12 (2005) 1310-1318.
64. Jiang, C.C., Lucas, K., Avery-Kiejda, K.A., Wade, M., deBock, C.E., Thorne, R.F., Allen, J., Hersey, P. and Zhang, X.D. Up-regulation of Mcl-1 is critical for survival of human melanoma cells upon endoplasmic reticulum stress. **Cancer Res.** 68 (2008) 6708-6717.
65. Wei, J., O'Brien, D., Vilgelm, A., Piazuelo, M.B., Correa, P., Washinghton, M.K., El-Rifai, W., Peek, R.M. and Zaika A. Interaction of Helicobacter pylori with gastric epithelial cells is mediated by the p53 protein family. **Gastroenterology** 134 (2008) 1412-1423.
66. Perfettini, J-L., Roumier, T., Casted, M., Larochette, N., Boya, P., Raynal, B., Lazar, V., Ciccosanti, F., Nardacci, R., Penninger, J., Piacentini, M. and Kroemer, G. NF-κB and p53 qre the dominant apoptosis-inducing transcription factors elicited by the HIV-1 envelope. **J. Exp. Med.** 199 (2004) 629-640.
67. Rodrigues, R., Paranhos-Baccala, G., Vernet, G. and Peyrefitte, C.N. Crimean-congo hemorrhagic fever virus-infected hepatocytes induced ER-stress and apoptosis crosstalk. **PLoS** 7 (2012) 1-11.
68. Bauer, A., Villunger, A., Labi, V., Fischer, S.F., Strasser, A., Wagner, H., Schmid, R.M. and Häcker, G. The NF-κB regulator Bcl-3 and the BH3-only

proteins Bim and Puma control the death of activated T cells. **Proc. Natl. Acad. Sci. USA** 103 (2006) 10979-10984.
69. Fisher, S.F., Belz, G.T. and Strasser, A. BH3-only protein Puma contributes to death of antigen-specific T cells during shutdown of an immune response to acute viral infection. **Proc. Natl. Acad. Sci. USA** 105 (2008) 3035-3040.
70. Häcker, G., Bauer, A. and Villunger , A. Apoptosis in activated T cells: what are the triggers, and what the signal transducers? **Cell Cycle** 5 (2006) 2421-2424.
71. Hildeman, D., Jorgensen, T., Kappler, J. and Marrack P. Apoptosis and the homeostatic control of immune responses. **Curr. Opin. Immunol.** 19 (2007) 516-521.
72. Steckley, D., Karajgikar, M., Dale, L.B., Fuerth, B., Swan, P., Drummond-Main, C., Poulter, M.O., Ferguson, S.S., Strasser, A. and Cregan, S.P. Puma is a dominant regulator of oxidative stress induced Bax activation and neuronal apoptosis. **J. Neurosci.** 27 (2007) 12989 -12999.
73. Sandow, J.J. Regulation of the BH3-only protein PUMA by growth factor signalling. **Ph.D. Thesis of the University of Adelaide, School of Medicine**, 2011, 1-144.
74. Dewson, G. and Kluck, R.M. Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. **J. Cell Sci.** 122 (2009) 2801-2808.
75. Häcker, G. and Weber A. BH3-only proteins trigger cytochrome *c* release, but how? **Arch. Biochem. Biophys.** 462 (2007) 150-155.
76. Kim, H., Tu, H.C., Ren, D., Takeuchi, O., Jeffers, J.R. , Zambetti, G.P., Hsieh, J.J. and Cheng, E.H. Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. **Mol. Cell** 36 (2009) 487-499.
77. Gallenne, T., Gautier, F., Oliver, L., Hervouet, E., Noël, B., Hickman, J.A., Geneste, O., Cartron, P.F., Vallette, F.M., Manon, S. and Juin, P. Bax activation by the BH3-only protein Puma promotes cell dependence on antiapoptotic Bcl-2 family members. **J. Cell Biol.** 185 (2009) 279-290.
78. Kuwana, T., Bouchier-Hayes, L., Chipuk, J.E., Bonzon, C., Sullivan, B.A., Green, D.,R. and Newmeyer, D.D. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. **Mol. Cell** 17 (2005) 525-535.
79. Westphalm D., Dewson, G., Czabotar, P.E. and Kluck, R.M. Molecular biology of Bax and Bak activation and action. **Biochim. Biophys. Acta** 1813 (2011) 521-531.
80. Lindsay, J., Esposti, M.D. and Gilmore, A.P. Bcl-2 proteins and mitochondria-specificity in membrane targeting for death. **Biochim. Biophys. Acta** 1813 (2011) 532-539.
81. Ghiotto, F., Fais, F. and Bruno, S. BH3-Only Proteins: The death puppeteer's wires. **Cytometry A** 77 (2010) 11-21.

82. Giam, M., Huang, D.S.C. and Bouillet, P. BH3-only proteins and their roles in programmed cell death. **Oncogene** 27 (2009) 128-136.
83. Shamas-Din, A., Brahmbhatt, H., Leber, B. and Andrews, D.W. BH3-only proteins: orchestrators of apoptosis. **Biochim. Biophys. Acta** 1813 (2010) 508-520.
84. Leber, B., Lin, J. and Andrews, D. W. Embedded Together: the life and death consequences of interaction of the Bcl-2 family with membranes. **Apoptosis** 12 (2007) 897-911.
85. Chipuk, J.E. and Green, D.R. How do BCL-2 proteins induce mitochondria outer membrane permeabilization? **Trends Cell Biol.** 18 (2008) 157-164.
86. Shore, G.C. Apoptosis: it's BAK to VDAC. **EMBO Rep.** 10 (2009) 1311-1313.
87. Gavathiotis, E., Suzuki, M., Davis, M.L., Pitter, K., Bird, G.H., Katz, S.G., Tu, H.C., Kim, H., Cheng, E.H., Tjandra, N. and Walensky, L.D. BAX activation is initiated at a novel interaction site. **Nature** 455 (2008) 1076-81.
88. Willis, S.N., Fletcher, J.I., Kaumann, T., van Delft, M.F., Chen, L., Czabotar, P.E., Lerino, H., Lee, E.F., Fairlie, W.D., Bouillet, P., Strasser, A., Kluck, R.M., Adams, J.M. and Huang, D.C.S. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. **Science** 315 (2007) 856-859.
89. Jabbour, A.M., Heraud, J.E., Daunt, C.P., Kaufmann, T., Sandow, J., O'Reilly, L.A., Callus, B.A., Lopez, A., Strasser, A., Vaux, D.L. and Ekert, P.G. Puma indirectly activates Bax to cause apoptosis in the absence of Bid or Bim. **Cell Death Differ.** 16 (2009) 555-563.
90. Chipuk, J.E., Fisher, J.C., Dillon, C.P., Kriwacki, R.W., Kuwana, T. and Green, D.R. Mechanism of apoptosis induction by inhibition of the anti-apoptotic BCL-2 proteins. **Proc. Natl. Acad. Sci. USA.** 105 (2008) 20327-20332.
91. Chen, L., Willis, S.N., Wei, A., Smith, B.J., Fletcher, J.I., Hinds, M.G., Colman, P.M., Day, C.L., Adams, J.M. and Huang, D.C. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. **Mol. Cell** 17 (2005) 393-403.
92. Vaseva, A.V. and Moll, U.M. The mitochondrial p53 pathway. **Biochim. Biophys. Acta** 1787 (2009) 414-420.
93. Vousden, K.H. Apoptosis – p53 and PUMA: a deadly duo. **Science** 309 (2005) 1685-1686.
94. Chipuk, J.E., Bouchier-Haues, L., Kuwana, T., Newmeyer, D.D. and Green D.R. PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. **Science** 309 (2005) 1732-1735.
95. Wolff, S., Erster, S., Palacios, G. and Moll, U.M. p53's mitochondrial translocation and MOMP action is independent of Puma and Bax and severaly disrupts mitochondrial membrane integrity. **Cell Res.** 18 (2008) 733-744.

96. Yoo, N.J., Lee, J.W., Jeong, E.G. and Lee, S.H. Immunohistochemical analysis of pro-apoptotic PUMA protein and mutational analysis of PUMA gene in gastric carcinomas. **Dig. Liver Dis.** 39 (2007) 222-227.
97. Kuroda J. and Taniwaki, M. Involvement of BH3-only proteins in hematologic malignancies. **Crit. Rev. Oncol. Hematol.** 71 (2009) 89-101.
98. Pietsch, E.C., Sykes, S.M., McMahon, S.B. and Murphy, M.E. The p53 family and programmed cell death. **Oncogene** 27 (2008) 6507-6521.
99. Hoque, M.O., Begum, S., Sommer, M., Lee, T., Trink, B., Ratovitski, E. and Sidransky, D. PUMA in head and neck cancer. **Cancer Lett.** 199 (2003) 75-81.
100. Ahn, C.H., Jeong, E.G., Kim, S.S., Lee, J.W., Lee, S.H., Kim, S.H., Kim, M.S., Yoo, N.J. and Lee, S.H. Expressional and mutational analysis of pro-apoptotic Bcl-2 member PUMA in hepatocellular carcinomas. **Dig. Dis. Sci.** 53 (2008) 1395-1399.
101. Kim, M.R, Jeong, E.G., Chae, B., Lee, J.W., Soung, Y.H., Nam, S.W., Lee, J.Y., Yoo, N.J. and Sug H Lee. Pro-apoptotic PUMA and anti-apoptotic phospho-BAD are highly expressed in colorectal carcinomas. **Dig. Dis. Sci.** 52 (2007) 2751-2756.
102. Michalak, E.M., Jansen, E.S., Happo, L., Cragg, M.S., Tai, L., Smyth, G.K., Strasser, A., Adams, J.M. and Scott, C.L. Puma and to a lesser extent Noxa are suppressors of Myc-induced lymphomagenesis. **Cell Death Differ.** 16 (2009).
103. Sharma, A.D., Narain, N., Händel, E-M., Iken, M., Singhal, N., Cathomen, T., Manns, M.P., Schöler, H.R., Ott, M. and Cantz, T. MicroRNA-221 regulates FAS-induced fulminant liver failure. **Hepatology** 53 (2011) 1651-1661.
104. Shao, L., Sun, Y., Zhang, Z., Feng, W., Gao, Y., Cai, Z., Wang, Z.Z., Look, A.T. and Wu, W.S. Deletion of proapototic Puma selectively protects hematopoietic stem and progenitor cells against high dose radiation. **Blood** 115 (2010) 4707-4714.
105. Yu, H., Shen, H., Yuan, Y., Xu-Feng, R., Hu, X., Garrison, S.P., Zhang, L., Yu, J., Zambetti, G.P. and Cheng, T. Deletion of Puma protects hematopoietic stem cells and confers long term survival in response to high-dose radiation. **Blood** 115 (2010) 3472-3480.
106. Labi, V., Erlacher, M., Krumschnabel, G., Manzl, C., Tzankov, A., Pinon, J., Egle, A. and Villunger, A. Apoptosis of leukocytes triggered by acute DNA damage promotes lymphoma formation. **Genes Dev.** 25 (2010) 1602-1607.
107. Michalak, E.M., Vandenberg, C.J., Delbridge, A.R.D., Wu, L., Scott, C.L., Adams, J.M. and Strasser, A. Apoptosis-promoted tumorgenesis: γ-irradiation-induced thymic lymphpomagenesis requires Puma-driven leukocyte death. **Genes Dev.** 24 (2010) 1608-1613.
108. Qiu, W., Wang, X., Leibowitz, B., Yang, W., Zhang, L. and Yu, J. PUMA-mediated apoptosis drives chemical hepatocarcinogenesis in mice. **Hepatology** 54 (2011) 1249-1258.

109. Llambi, F. and Green, D.R. Apoptosis and oncogenesis: give and take in the BLC-2 family. **Curr. Opin. Genet. Dev.** 21 (2011) 12-20.
110. Li, F., Huang, Q., Chen, J., Peng, Y., Roop, D., Bedford, J.S. and Li, C-Y. Apoptotic cells activate the "phoenix rising" pathway to promote wound healing and tissue regeneration. **Sci. Signal.** 3 (2010) 10.1126/scisignal.2000634.
111. Baumgartner, F., Villunger, A. Apoptosis: a barrier against cancer no more? **Hepatology** 54 (2011) 1121-1124.
112. Labi, V. and Villunger, A. PUMA-mediated tumor suppression. **Cell cycle** 9 (2010) 4269-4275.