

## The Intrinsic Pathway of Apoptosis and Carcinogenesis: An Update

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### ABSTRACT

A conserved, intact and tightly regulated apoptotic pathway is necessary for embryonic organogenesis, maintenance of tissue homeostasis and tumour suppression in healthy organisms. This pathway is also activated in response to hypoxia, oncogenic overexpression or DNA damage and serves to eliminate cells that have experienced lethal doses of genotoxic stress. Several studies suggest that resistance to apoptosis, also known as programmed cell death, results in the survival of mutant cells which can induce tumourigenesis. Therefore, inhibition of apoptosis is considered a requirement for tumourigenesis and thus an acquired resistance to apoptosis has become a “hallmark” for cancer. The intrinsic, or mitochondrial, pathway of apoptosis is mainly controlled by the interactions between mitochondrial and cytosolic pro- and anti-apoptotic proteins of the Bcl-2 family. Thus, resistance to apoptosis

can be acquired by mutations in pro- or anti-apoptotic genes of Bcl-2 family members or in the tumour suppressor gene p53 which is the DNA damage sensor in the intrinsic apoptotic signalling circuitry. Moreover, multiple epigenetic studies have illustrated the role DNA methylation plays in the aberrant apoptosis in many human cancers, and thus in the survival of tumours. Novel therapeutics that target the intrinsic pathway of apoptosis have also been the subject of considerable investigation.

**Key words:** Apoptosis; Carcinogenesis

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### INTRODUCTION

Apoptosis or programmed cell death is a crucial biological mechanism in most of biological systems for maintenance of tissue homeostasis and various cellular components. Failed or excessive apoptosis has been found to be involved in many human ailments particularly in autoimmune disorders and cancer. Apoptotic cell death pathways can be triggered at different entry sites including the mitochondria (intrinsic pathway) and the plasma membrane via death receptors (death receptor-initiated or extrinsic pathway). Although these two pathways can interconnect, they mainly differ in their induction conditions and regulation and thus play discrete roles in health and disease. Following various stress stimuli, the intrinsic pathway of apoptosis is mediated by intracellular signals that are transduced on the mitochondrial membrane leading to the activation of several proteins of Bcl-2 family including BAD, NOXA and PUMA which block anti-apoptotic Bcl-2 family members such as Bcl-x<sub>L</sub> and Bcl-2. This allows the release of “activator” proteins such as BID and BIM that activate the pro-apoptotic proteins BAK and BAX that in turn dimerise and translocate to the mitochondrial outer membrane leading to the permeabilisation of the mitochondrial outer membrane (MOMP). This enables several apoptogenic factors

such as the mitochondrial-AIF (apoptosis inducing factor), SMAC/DIABLO (second mitochondria-derived activator of caspases) and cytochrome *c* to be released concurrently to the cytosol. While AIF causes DNA condensation, SMAC/DIABLO can suppress the inhibitors of apoptosis proteins (IAPs) leading to the activation of several protease enzymes called “caspases”. Furthermore, since the caspases activator cytochrome *c* is also released from the inter-membrane space of the mitochondria into the cytosol, it binds to apoptotic protease activating factor-1 (Apaf-1) to then transform pro-caspase-9 into its active form of caspase-9. Caspase-9, an “initiator” caspase, in turn activates several “executioner” caspases, including caspase-3, caspase-6 and caspase-7 that mediate the cleavage of a specific set of substrates such as DNase inhibitors, leading eventually to DNA fragmentation and the disassembly and destruction of cellular proteins<sup>[1,2]</sup> (Figure 1). Data from several studies have indicated that the release of cytochrome *c* and caspase activation are sufficient for apoptotic cell death but not essential, indicating the presence of alternative caspase-independent apoptotic mechanisms such as AIF-mediated apoptosis and endonuclease-G<sup>[3,4]</sup>.

## THE BCL-2 FAMILY

More than 16 members of the Bcl-2 protein family have been characterised and recognised as the key regulators of the mitochondrial apoptotic circuitry. The Bcl-2 protein family is a large family that comprises proteins that either inhibit or promote apoptotic cell death. The permeabilisation of the mitochondrial outer membrane (MOMP) and therefore caspase-mediated proteolytic cascade are regulated by the great degree of functional overlap and interactions between various pro-apoptotic (such as BAX and BAK), anti-apoptotic (such as Bcl-x<sub>L</sub>, Bcl-2 and Mcl-1) and the BH3-only members of the Bcl-2 family<sup>[2,3,5,6]</sup>.

In non-stressed cells, various survival signals (e.g., growth factor) stimulate the production of anti-apoptotic Bcl-2 proteins including Bcl-2, Bcl-x<sub>L</sub> and Mcl-1 tend to bind and sequester “activator” BH3-only proteins such as BID and BIM that would otherwise interact with pro-apoptotic proteins such as BAX or BAK to form complexes that act as protein pores for apoptotic proteins and cytochrome *c* to be released and cause MOMP<sup>[7-10]</sup>. In response to stress, “sensitiser”

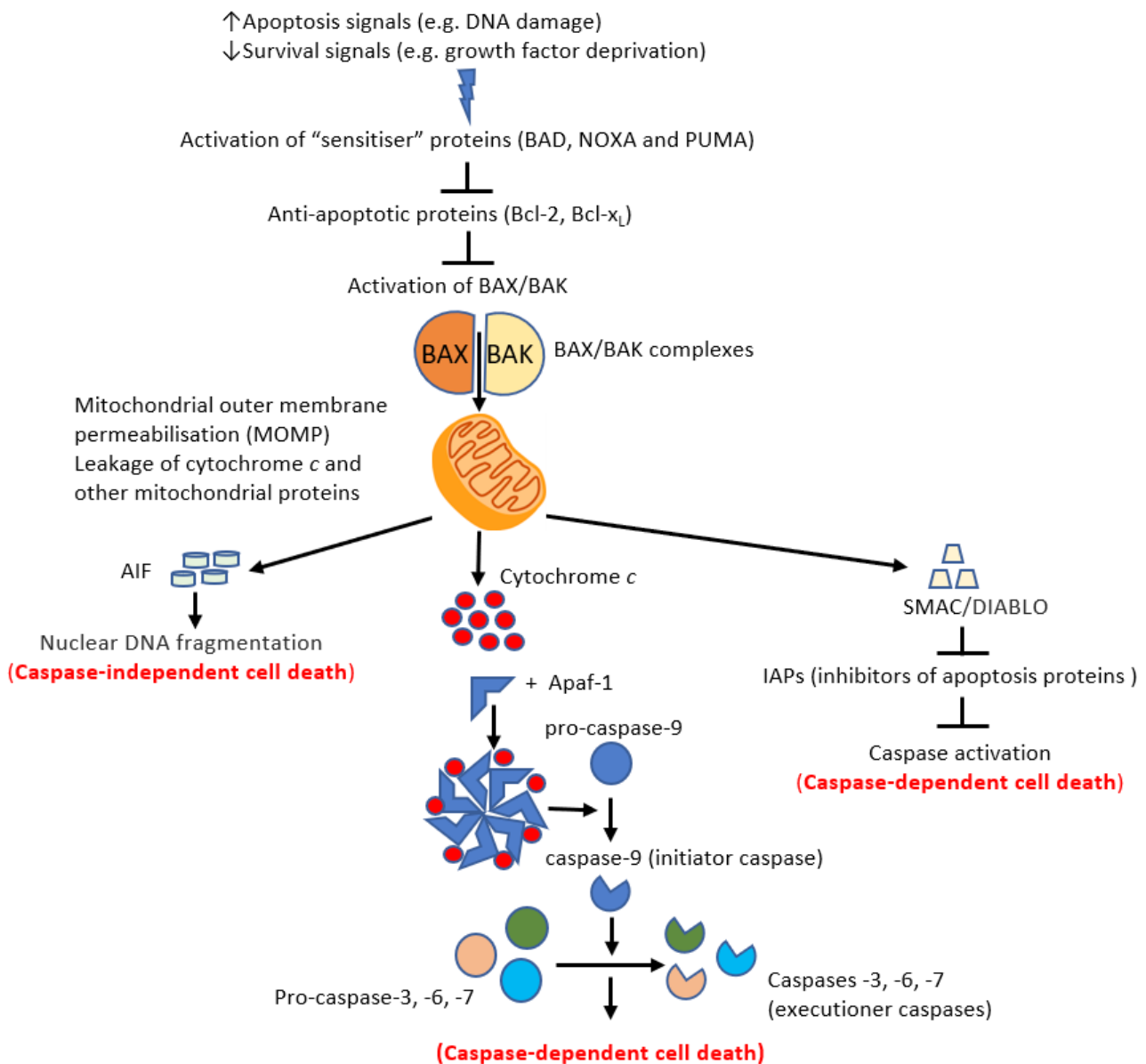


Figure 1 Schematic illustration of the molecular mechanisms of the intrinsic pathway of apoptosis.

BH3-only proteins such as BAD, NOXA and PUMA bind anti-apoptotic proteins to displace and release “activator” BH3-only proteins<sup>[9-11]</sup>. The “activator” BH3-only proteins then transiently activate BAK and BAX which undergo stepwise conformational changes and oligomerise to eventually trigger the release of cytochrome *c* and various apoptotic proteins<sup>[2,9,12]</sup>. In this context, it is important to note that although it is evident that BAK activation is sufficient to induce MOMP<sup>[13]</sup> and lack of BAX expression may elicit tumorigenesis in particular tissues<sup>[14]</sup>, BAK-deficient animal models do not necessarily manifest tumours<sup>[15]</sup>. It also remains to be established whether BAX and BAK are being constitutively activated by different mechanisms<sup>[16]</sup>. Moreover, whether alternatives to “activator” BH3-only proteins that can directly activate BAK and BAX exist remains questionable<sup>[5]</sup>. Another unresolved issue is whether “sensitiser” BH3-only proteins can directly activate BAX and BAK<sup>[5,17]</sup>. Other findings have also prompted inquiries into whether the “activator” BH3-only proteins are necessary to induce apoptosis<sup>[16]</sup>. These observations reveal the intricate yet delicate balance among these proteins which constitutes the cellular rheostat for controlling the mitochondrial pathway of programmed cell death.

## MCL-1

While Mcl-1 inhibits apoptosis by selectively sequestering BIM to prevent it from activating BAK or BAX in normal cells, apoptosis occurs when NOXA binds Mcl-1 to release “activator” BH3-only proteins. When NOXA binds Mcl-1, it also releases BAK since there is increasing evidence that Mcl-1 also binds directly to BAK to form stable complexes<sup>[18,19]</sup>. However, since BAK and BAX can be inhibited by different types of anti-apoptotic proteins, all pertinent proteins must be blocked for cell death to occur<sup>[6]</sup>. More importantly, unlike binding of Bcl-x<sub>L</sub> by “sensitiser” BH3-only proteins which does not induce Bcl-x<sub>L</sub> degradation, NOXA binds Mcl-1 not only to unleash BAK, but also to trigger E3-ligase-mediated degradation of Mcl-1 via the ubiquitin-dependent proteasomal system (UPS). This degradation appears to be necessary for downstream events, most importantly for translocation of BAX, cytochrome *c* release and caspase activation<sup>[6,20]</sup>. However, it must be borne in mind that a minor fraction of Mcl-1 can be eliminated independently of ubiquitination<sup>[21]</sup>.

Since anti-apoptotic Bcl-2 family proteins are over-expressed in most cancers, inhibition of anti-apoptotic proteins using “BH3 mimetics” such as ABT-737 has been effectively applied in cancer treatment. Nevertheless, as different cancer types tend to overexpress tumour-type dependent anti-apoptotic proteins, a comprehensive understanding of the selectivity of binding of various BH3-only proteins is crucial when designing highly selective anti-neoplastic agents able to target that tumour-specific anti-apoptotic proteins<sup>[1,7,9,10,16,22]</sup>. Further insight into protein regulation via the ubiquitin–proteasome system (UPS) suggests important implications for the development of proteasome inhibitors that lead to the accumulation of pro-apoptotic BH3-only proteins such as the proteasome inhibitors bortezomib (Velcade®) and MG-132 in myeloma<sup>[7,22,23]</sup>. Bortezomib’s ability to accumulate BIM, which is necessary for susceptibility of cancer to PTX (Taxol®), has supported the use of combination of PTX with bortezomib in melanoma<sup>[7]</sup>.

Even though the short half-life of Mcl-1 has been attributed to its unique (proline, glutamic acid, serine, threonine) PEST motifs<sup>[24]</sup>, deletions in the PEST-rich N-terminal region do not increase Mcl-1 half-life, suggesting that other regions may play a role in its stability<sup>[25]</sup>. Interestingly, mRNA of Mcl-1 is alternatively spliced

producing short pro-apoptotic and long anti-apoptotic isoforms. While the product of the full-length Mcl-1 gene induces anti-apoptotic activities, the naturally occurring spliced shorter isoform MCL-1S was found to trigger apoptosis in particular tissues<sup>[24,26,27]</sup>. This pro-apoptotic spliced Mcl-1 variant seems to lack C-terminal trans-membrane domains that are responsible for localisation of Mcl-1 to mitochondria<sup>[25]</sup>. These findings suggest that apoptosis could be regulated by the expression of altered Mcl-1 transcripts and that Mcl-1 may represent an evolutionary intermediate form between the anti- and pro-apoptotic Bcl-2 family members<sup>[26,27]</sup>. As the alternative splicing of Mcl-1 seems to be controlled by G2/M and/or mitotic spindle checkpoints, therapeutic approaches based on small interfering RNA (siRNA) that can target the splicing machinery or induce mitotic arrest are amenable to experimental pharmacological approaches. Remarkably, this disruption of cell cycle seems to alter the splicing of caspase-9 transcripts and trigger an extra pro-apoptotic activity<sup>[8]</sup>.

## P53

Once DNA damage occurs, it is sensed by ATM/ATR/DNA-PK kinases that activate CHEK2 which in turn phosphorylates p53 and subsequently blocks p53-MDM2 interaction, leading to inhibition of degradation of p53 and eventually release of p53. The p53 in turn either elicits transient or permanent cell cycle arrest or alternatively promotes irreversible p53-mediated apoptosis if the damage is excessive or beyond repair. If chronic genotoxic stresses or epigenetic silencing of genes responsible for cell cycle progression persist, p53 acts as a DNA-binding transcription factor and trans-activates a wide array of pro-apoptotic genes (such as BAX, BID, PUMA, NOXA) and the p53-induced protein with a death domain (PIDD) responsible for activation of caspase-2. Caspase-2 was found to be required for the release of cytochrome *c* and apoptotic cell death<sup>[5,22,28]</sup>. For apoptosis to occur, p53 affinity for the promoters in the pro-apoptotic genes should be higher than its affinity for binding sites in the DNA repair genes. To date, there is no solid evidence that a group of genes that are transcriptionally-regulated by p53 is essential to induce apoptotic cell death<sup>[29]</sup>. It has become evident that p53 translocates to the mitochondria and binds directly via its DNA-binding domain with Bcl-x<sub>L</sub> and Bcl-2 to form complexes which would ultimately lead to the release of cytochrome *c*<sup>[29,30]</sup>.

Recessive loss-of-function mutations in the sequence specific DNA-binding domain of p53 prevent the p53 protein from binding to several proteins such as Bcl-x<sub>L</sub> and Bcl-2 and destroy its transcriptional function and therefore prevent it from binding to its target genes. Similarly, p53 molecules with mutations in the homooligomerisation domain have been shown to oligomerise with wild-type p53 molecules and block their transcription function and thus exert a dominant negative effect over the wild-type p53 functions. Functional inactivation of p53 can disrupt the non-transcriptional mitochondrial or transcriptional cellular apoptotic functions and also impair the ability to repair non-lethal DNA damage in other genes during normal cell division and cell cycle arrest or apoptosis following DNA damage. This eventually leads to an accumulation of additional mutations and an increase the susceptibility to accumulate driver mutations in oncogenes, a state known as “mutator phenotype”, supporting the notion that certain mutations in p53 not only lose their tumour suppressor activity but gain oncogenic functions<sup>[22,28]</sup>. The p53 has been reported to be hypermethylated in many cancers and a resistance to various anti-neoplastic agents has been attributed to it. This has important implications for the development of novel

types of anti-tumour therapeutics such as DNA methyltransferase inhibitors which are attractive agents in the reversal of treatment resistance in such resistant cancers. Although initial findings are promising, further research is justified since histone deacetylation and chromatin remodelling are also key factors in the gene silencing process. Unlike tumours with wild type p53 alleles, that are relatively sensitive to chemo- and radiation-based therapies, tumours with bi-allelic mutated p53 alleles were found to be resistant to conventional treatment since underlying mechanisms of action for radiotherapy and chemotherapy entail killing cancerous cells mainly by creating DNA damage and thus inducing apoptotic cell death<sup>[28]</sup>.

Interestingly, certain gain-of-function mutations in p53 have been found to be associated with enhancement of tumorigenicity and resistance to various chemotherapeutic agents. The oncogenic effects of gain-of-function mutation in p53 are mediated by stimulating growth factors and/or growth factor receptors and transcriptional activity of topoisomerase I. These tissue-specific mutations can also contribute to tumourigenesis by both indirect and direct up-regulating various genes including the epidermal growth factor receptor (EGFR), proliferating cell nuclear antigen (PCNA), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF)<sup>[31]</sup>. Moreover, some gain-of-function p53 mutants compromise the efficacy of cancer chemotherapy and may lead to chemotherapy resistance via increasing the transcription of the multidrug resistance 1 (MDR-1) gene that codes for P-glycoproteins, which are membrane proteins of a superfamily of transporters capable of extruding chemotherapeutic agents under an ATP-dependent mechanism<sup>[31]</sup>. A number of studies have shown that p53 protein expression as a biomarker is an independent and robust prognostic tool for disease-free survival in certain subsets of cancers. Moreover, by adding Bcl-2 protein expression, immunohistochemical co-staining for Bcl-2 and p53 seems to have increased the accuracy of prediction and improved its clinical utility<sup>[32]</sup>. However, issues such as genetic heterogeneity of cancers might limit the use of these biomarkers in clinical practice.

On the other hand, and to avoid unwarranted or excessive apoptosis, p53 is negatively regulated by the proto-oncogene MDM2. The MDM2 protein transports p53 to the cytosol and reversibly ubiquitinates it leading to proteasome-mediated degradation. Efforts have been made to develop antisense oligonucleotides and small-molecules that inhibit MDM2 (e.g. Nutlins) or block the MDM2–p53 interaction. These drugs represent a promising avenue of novel anti-cancer therapeutics<sup>[23,33,34]</sup>. Intriguingly, anti-MDM2 antisense siRNAs have shown notable anti-tumour activity irrespective of p53 status, implying that MDM2 has p53-independent activity and might function as a ubiquitin ligase for other tumour suppressor proteins probably owing to alternative splicing that leads to distinctive MDM2 isoforms with potentially different functions<sup>[35]</sup>. Therefore, efficient cancer treatment might entail blocking the MDM2–p53 complex as well as inhibiting MDM2 expression to stop all the oncogenic effects of MDM2<sup>[23,35]</sup>.

## PUMA

PUMA, which is a target of p53 transcriptional activity and a critical mediator of p53-induced cell death, may bind to all anti-apoptotic Bcl-2 family proteins and therefore release p53 and “activator” BH3-only proteins which eventually lead to apoptotic cell death<sup>[5,9,12,36]</sup>. However, PUMA has not been proven capable of eliciting apoptosis in p53- or Bcl-x<sub>L</sub>-deficient cells<sup>[7]</sup>. There is increasing evidence that the cytoplasmic p53 is sequestered by Bcl-x<sub>L</sub> which subsequently blocks its transcription-dependent and -independent functions. This

can be reversed solely by PUMA which can bind Bcl-x<sub>L</sub> and release p53<sup>[22]</sup>. Although it has been suggested that PUMA is required for p53-mediated apoptosis<sup>[29,37]</sup>, loss of PUMA does not inherently elicit tumourigenesis. Instead, it delays it or even inhibits it in certain settings<sup>[29]</sup>. These observations are in contrast with loss of NOXA which accelerates tumour formation. A likely explanation for these findings is that PUMA-induced apoptosis can promote carcinogenesis by inducing apoptosis in hematopoietic progenitor/stem cells (HSCs), which are essential to prevent tumourigenesis. Studies have also highlighted that apoptosis in HSCs increases proliferation of neighbouring cells with mutations that may then acquire secondary mutations resulting in tumourigenesis<sup>[38,39]</sup>. These observations suggest that chronic administration of drugs that mimic BH3-only proteins such as PUMA that are able to target most of anti-apoptotic Bcl-2 family members can promote secondary tumours<sup>[38]</sup>. However, further studies are necessary for more definitive evidence.

## CONCLUSION

Since expansion of clonogenic cells in cancer demands that apoptosis remains low, dysregulation of certain apoptotic signalling pathways is characteristic for tumourigenesis. What remains highly controversial is that observations are not entirely consistent with the above-outlined patterns, suggesting the presence of a finely-tuned balance of combinatorial interactions that is as yet poorly defined. The multiplicity and complexity of the partially overlapping yet distinct roles of the various Bcl-2 network proteins in the apoptotic mitochondrial pathway represents a formidable challenge whose intricacies have as yet remained elusive. A deeper knowledge of the molecular mechanisms involved in the aberrant expression and regulation of apoptotic genes including p53 may prove fundamental in increasing our understanding. It is also essential to interrogate and accurately define the functions of pro- and anti-apoptotic genes in physiological and pathological contexts to achieve a quantitative rather than a phenomenological description of the cell death process and galvanise the development of novel anti-cancer therapeutics.

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