

## Micromanagement of the mitochondrial apoptotic pathway by p53

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## 1. ABSTRACT

It is now well established that p53 is the primary arbiter of stress-response and the principal barrier to neoplastic processes at the cellular level. Perhaps the most potent weapon in p53's tumor suppressive arsenal is apoptosis, enacted as a last resort when all other remedies are exhausted. Initially, the mechanism was thought to be simply activation or repression of Bcl-2 family members by p53. More recently, evidence of a more rapid pathway emerged whereby p53 physically interacts with Bcl-2 family members to tip the balance toward apoptosis. This review details the multiple levels of regulation of mitochondrially-directed apoptosis by p53, including recent findings of how p53 translocation is regulated.

## 2. INTRODUCTION

p53 is the governor of cell fate. It rules a cellular bureaucracy that detects every possible stressor, weighs the gravity of the insult, and dictates the appropriate response. Although discovered nearly thirty years ago, the full reach of its powers is still being appreciated. The range of responses available to p53 is now known to include cell cycle arrest, repair of DNA and other cell division mechanisms, apoptosis, senescence, and autophagy, all of which are tumor suppression mechanisms (1, 2). This concentration of power makes p53 the principal barrier to neoplasia and hence one of the most frequently mutated genes in cancer (3).

**Table 1.** Classification of Bcl2 family of proteins (11, 46-48, 87) and their mechanisms of action (88)

<b>Pro-apoptotic members</b>	
Bax, Noxa, Puma, Bid, Bim, Bad, Bik	
1. Mitochondrial membrane potential disruption	Bax and Bak
2. Activators of Bax and Bak	
2a. Activators: Direct activators of Bax and Bak	Bim and Bid
2b. Enablers: Indirect activators of Bax and Bak act by releasing them after binding to anti-apoptotic members	Bad and Bik
2c. Both activator (40, 44) and enabler (35)	p53
<b>Anti-apoptotic members</b>	
Sequestering agents of pro-apoptotic proteins	Bcl2, BclxL, Mcl1
<b>Non-Bcl2 family</b>	
p53, AIP1 and mtCLIC/CLIC4 (74, 89)	

For many years, p53 was thought to govern cell fate exclusively by regulating transcription of nuclear genes whose products did the actual work. Indeed, in the context of apoptosis, p53 client genes form a veritable Who's Who list of effectors of the mitochondrial pathway. These include the anti-apoptotic genes Bcl-2 and Bcl-xL, and the pro-apoptotic Bax, Bid, Noxa, Puma, and many others (4, 5). However, this simple model was belied by results of experiments with transcriptional and translational inhibitors that showed that p53 could induce apoptosis rapidly in stressed cells without new gene expression (6, 7). Since those early studies, evidence for the direct participation of p53 in the mitochondrial pathway has continued to amass and amaze. Here we review the evidence for p53 interaction with Bcl-2 family members and consider some recent twists in the story.

## 3. MODULATION OF APOPTOSIS BY THE BCL-2 FAMILY

Before discussing the role of p53, we must first consider how the intrinsic pathway of apoptosis functions in its absence.

The Bcl-2 superfamily comprises a homologous group of proteins whose purpose is to regulate mitochondrial outer membrane permeabilization (MOMP) (Table 1). MOMP permits the escape of pro-apoptotic molecules such as cytochrome C into the cytosol where they trigger a macromolecular apoptotic cascade. The Bax subfamily, Bax and Bak, oligomerize to form the actual pore, while other family members either foil or abet this activity (8-12).

In non-stressed cells, Bax is predominantly cytosolic. However, in response to cytotoxic stimuli, Bax undergoes conformational changes and is directed towards the mitochondrial outer membrane (Figure 1) (13). Anti-apoptotic members of the Bcl-2 family, e.g., Bcl-2 and Bcl-xL, are also localized to the outer membrane where they inactivate Bax by forming complexes with it (14). Most other pro-apoptotic members such as Bim, Bad, and Bid (termed BH3-only proteins) activate Bax and stimulate oligomerization, translocation, or permeabilization by Bax and Bak (8) (Table 1).

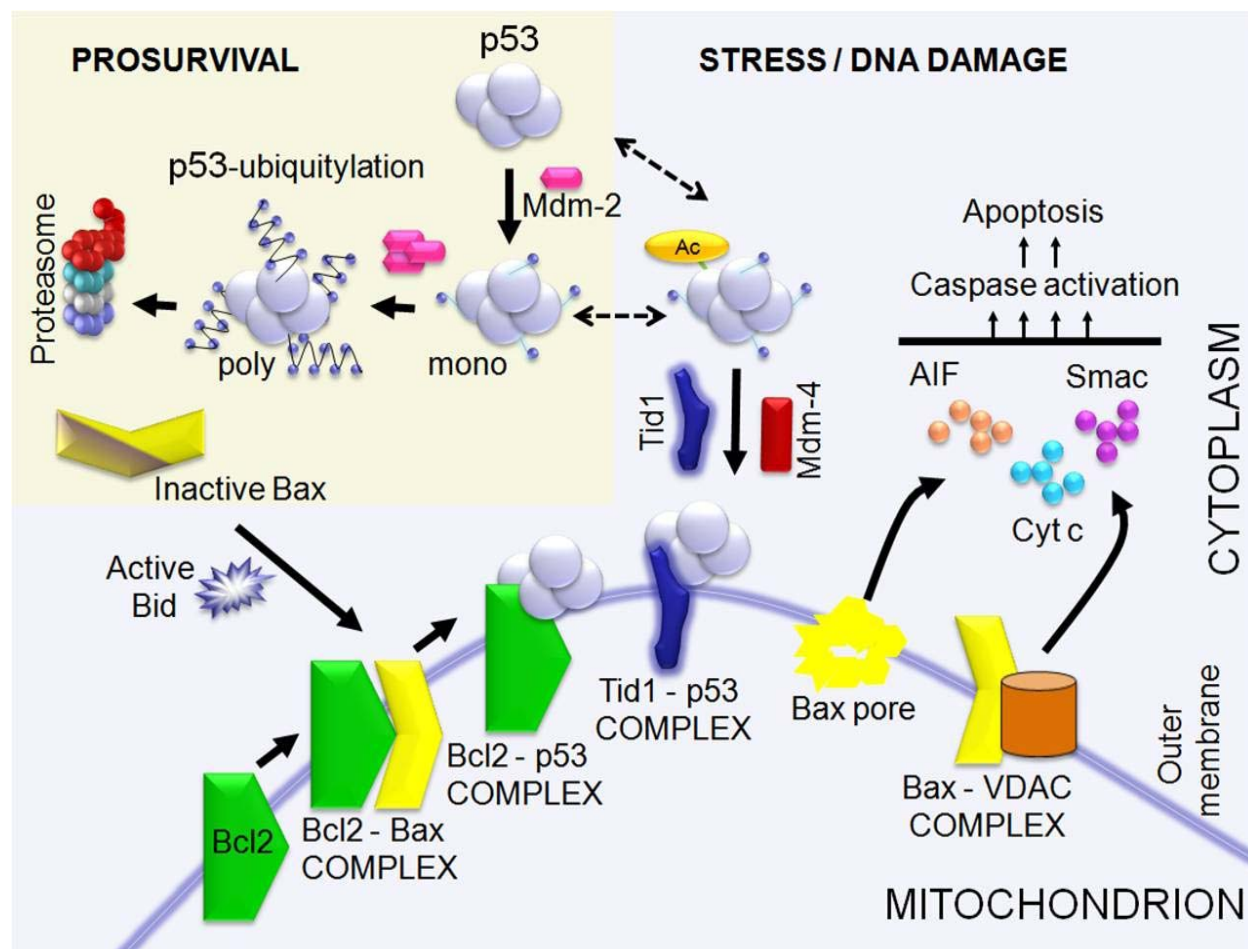
Nearly all family members are regulated by conformational shifts. Only the activated conformation of Bcl-2 can prevent oligomerization of Bax and release of cytochrome c and SMAC/Diablo (13, 15, 16) (Figure 1). On the other hand, interaction of Bax with Bim or Bad

shifts Bax to the active conformation, resulting in translocation and insertion of alpha helices 5, 6 and 9 into the bilayer (15). In the presence of a death signal, p53 induces Bax gene expression, and increased levels of Bax are now able to escape sequestration by Bcl-2 and oligomerize (16). The exact stoichiometry of the Bax-Bcl2 complex and the number of Bax subunits required to form a pore are not yet known.

These observations suggest two cellular phenomena by which pro-apoptotic proteins induce MOMP and cause apoptosis: first, the down-regulation of the anti-apoptotic members so that pro-apoptotic members are not sequestered and inactivated, and second the complexing of anti-apoptotic members thus displacing pro-apoptotic proteins from anti-apoptotic members. The latter would be possible only if some other proteins have higher affinity for the anti-apoptotic members and thereby shift the equilibrium in favor of apoptosis.

Consistent with its anti-apoptotic role, Bcl-2 was first discovered as an oncogene, and it was its mitochondrial localization that first brought the tumor-suppressive role of mitochondria to light (17). BclXL, about 45% identical to Bcl-2, is similarly anti-apoptotic but apparently not identical in function (18). While BclXL expression in breast cancer correlates with higher grade or rapidly growing tumors and an increase in nodal metastases (19), higher Bcl2 expression in breast cancers and small cell lung cancer is associated with low grade or slowly growing tumors and better prognosis (20, 21). Unexpectedly, some studies suggest that Bcl2 overexpression is responsible for cellular senescence and cell cycle arrest at G1 phase (22, 23) mediated by p27Kip1 (24). These findings suggest some functional divergence between Bcl2 and BclXL and a corresponding need to define the extent of their functional similarities.

Although the dance between Bax/Bak and Bcl-2/BclXL determines whether the cell lives or dies, BH3-only proteins also play an essential role. These proteins are specialized to respond to different forms of stress, sometimes in different cell types, to initiate an apoptotic cascade by tipping the balance in favor of Bax/Bad. While Bim and Bid can activate Bax/Bak directly, Bad and Bik enable Bax/Bak by freeing them from inactive complexes with anti-apoptotic proteins (Table 1). In turn, Bcl-2 and Bcl-xL sequester and bind to Bim and Bid and thus inhibit their pro-apoptotic action (10). Bad fights back by releasing Bim and Bid from their inactive complex with Bcl-2 and Bcl-xL (9, 25). In short, the levels of the Bcl-2



**Figure 1.** Formation of pores in the outer mitochondrial membrane is regulated by p53 translocation to the mitochondria. Under normal conditions, Mdm2 checks p53 level by regulating p53 ubiquitylation. Under genotoxic or cytotoxic stress, monoubiquitylated and acetylated p53 is translocated to mitochondria. p53 translocation to the mitochondria is mediated by Tid1 and Mdm4. p53 promotes translocation of Bax to the mitochondria (not shown) and displaces Bax from Bax-Bcl2 complex by itself complexing with Bcl2. Bax alone, or by complexing with VDAC, forms pores in the outer mitochondrial membrane. This releases cytochrome C, AIF and Smac, which form apoptosomes, activate caspases and initiate the intrinsic apoptotic pathway.

family of proteins regulated by p53 at the outer mitochondrial membrane are the determinants of cell fate. Increased levels of anti-apoptotic proteins favor cell survival while the reverse is true for the pro-apoptotic members.

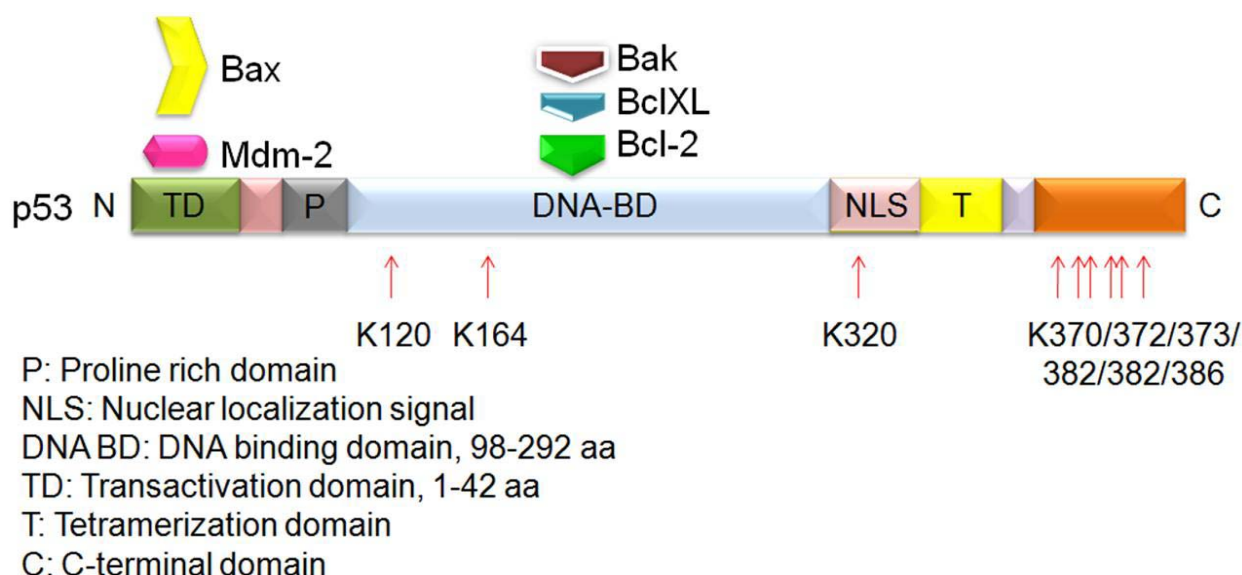
Noxa and Puma are two other p53-induced BH3-only proteins with potent pro-apoptotic effects. Both promote oligomerisation of Bax and Bak and cause MOMP (26, 27). Studies have shown that NOXA-deficient intestinal crypt cells and E1A-expressing MEFs (5, 27), but not thymocytes (5), are resistant to apoptosis induction in response to DNA damaging agents. On the other hand, PUMA-deficient thymocytes, neurons and E1A-expressing MEFs are deficient in p53-mediated apoptotic response after challenging with DNA damaging agents and serum withdrawal (5, 28). This cell type specificity could arise from PUMA and NOXA being expressed in different cell types or from the fact

that they target different anti-apoptotic proteins that are cell-type specific (29).

#### 4. INHIBITION OF BCL-2 AND ACTIVATION OF BAX BY TRANSLOCATION OF P53 TO MITOCHONDRIA

What is the evidence that p53 is physically involved in the mitochondrial pathway of apoptosis? The first indications of an extranuclear role for p53 came from studies by Karin's and Oren's laboratories showing that transcriptionally defective p53 mutants could still effect apoptosis, and this could occur in the presence of transcriptional and translational inhibitors (6, 7). These intriguing observations were widely reproduced but lacked mechanistic explanation.

That came from breakthrough studies by Marchenko and coworkers showing that a subpopulation of p53 translocated to mitochondria in response to various types of stress, including irradiation, DNA damaging



**Figure 2.** Schematic of p53 protein showing functional domains and sites of interaction with Bcl-2 family members. TD, Transactivation domain (amino acids 1-42) binds Mdm2 and Bax. DNA BD, DNA binding domain (amino acids 98-292) binds Bak, Bcl-2 and BclXL. p53 acetylation sites are shown by arrow. P, proline rich domain. NLS, nuclear localization domain. T, tetramerization domain. C, C-terminus, and N, N-terminus, of p53.

agents, hypoxia, and oncogene activation in cultured cells (30-37). This translocation occurred within 30-60 minutes, much earlier than transcriptionally induced apoptotic events and did not occur during p53-mediated cell cycle arrest (30, 34). The event was independent of transcriptional activation by p53; prolonged arrest of RNA polymerase II activity resulted in mitochondrial translocation of p53Ser15pSer46p in the absence of any increase in p21Waf1, an early p53 client gene (30).

The functional relevance of mitochondrial translocation was demonstrated by deliberate targeting of p53 to mitochondria by addition of a mitochondrial signal sequence (34, 35, 38). These authors found that the fusion protein provoked translocation and apoptosis in several cell lines. However, the situation appears to be more complex. Jaenicke's group found that while translocation occurred in response both to topoisomerase inhibitors and irradiation, apoptosis occurred only in response to topo inhibitors, while the irradiated cells senesced. Ectopic expression of wild-type p53 overcame senescence and induced apoptosis, while expression of mitochondrially-directed p53 did not (39). A comparison of molecular associations of mitochondrial p53 in cells subjected to either stress may prove illuminating.

A study by Chipuk and coworkers in 2004 provided additional support and linked mtp53 to Bcl-2 family members (40). The study used an inhibitor of nuclear import, wheat germ agglutinin (WGA), to subtract nuclear activities of p53 from the equation. WGA prevented p53 transcriptional responses in UV-treated MEFs but did not prevent apoptosis. However, Bax-/- MEFs failed to undergo apoptosis in the presence of WGA but not in its absence, implying that Bax is required for p53

action at the mitochondrion. In isolated mitochondria, both Bax and p53 were required for release of cytochrome C. Similar results were obtained in a liposomal model of mitochondria that measured release of fluorescent-tagged dextran. However, two subsequent reports conclude that Bak can be oligomerized by p53, leading to MOMP, and that Bax is not required (41, 42).

## 5. PHYSICAL INTERACTION OF P53 WITH BCL-2 FAMILY PROTEINS

The nature of p53 action at the outer membrane of the mitochondrion became clear when direct protein-protein interaction with Bcl-2 family members was investigated (30-36). A direct physical interaction of the p53 DNA binding domain with Bcl-2 and Bcl-XL has been demonstrated by mutational, nuclear magnetic resonance (NMR), and molecular modeling studies (35, 43, 44). The placement of these interaction sites in the DNA binding domain makes teleological sense because that domain should be unoccupied in cytosolic p53, while the N- and C-termini may engage in numerous other interactions (Figure 2). Recently it became clear that in a stressed cell, wild type p53 can free Bax from complexes with Bcl-2 or BclXL (45). On the other hand, a fifty-fold higher concentration of Bax is required to disrupt the p53 - Bcl-xL complex (40). This difference in affinity suggests that p53 sequesters Bcl-2 protein to inhibit its anti-apoptotic function and free Bax from the inactive Bax-Bcl-2 complex. At the same time, p53 works as a transcription factor to increase the concentration of not only Bax but also allied proteins such as Noxa and PUMA (46-48). On the other hand, anti-apoptotic genes such as Bcl-2 are repressed by p53 (49). Thus p53 emerges as a micromanager of Bax-mediated pore formation, initially by displacing it from

Bax/Bcl-2 complex and then by transcriptional upregulation of Bax. The relationship of p53 with Bak is similar. p53 can liberate Bak from its complex with another anti-apoptotic protein, Mcl1 (42). However, in this case, p53 can be shown by chemical cross-linking to complex with Bak oligomers via its DNA-binding domain. In contrast, p53 does not seem to form a stable complex with Bax, although a transient interaction with the p53 N-terminus has been reported (40). This led to the proposal of a “hit and run” model based on previous observations of transient interaction between Bax and tBid (40, 50).

It should be pointed out that most p53 mutations in cancer are in the DNA binding domain. Recent studies have shown, as expected, that only wild type p53 has the ability to bind Bcl-2 via its DNA binding domain; tumor-derived mutant p53 is incapable of complexing with Bcl-2 and inducing MOMP (51). Thus, a mutation in the DNA binding domain may disable both transcriptional and mitochondrial actions of p53, a jackpot for the nascent tumor cell (35).

### 6. MODULATORS OF P53 MITOCHONDRIAL HOMING

How is p53 translocated to mitochondria in stressed cells? Although monoubiquitination by MDM2 is required (52), MDM2 does not appear to serve as a shuttling protein. Instead, a mitochondrial chaperone protein known as Tid1, or mtHsp40, provides this function. Ahn and coworkers found that Tid1 forms a complex with p53 in hypoxic cells that directs p53 to mitochondria (Figure 1). These events required both the N-terminal mitochondrial signal sequence of Tid1 and its DnaJ domain, by which it interacts with Hsp70 proteins (53). Loss of Tid1 inhibited p53 translocation, and, remarkably, the translocation and apoptotic defects of p53 DNA-binding mutants could be suppressed by overexpression of Tid1 (53).

Other studies have found that p53 protein has an inherent affinity for the phospholipid cardiolipin that is unique to mitochondrial membranes. Using a series of deletion constructs, Li and co-workers showed these interactions to be mediated by C-terminal nuclear localization motifs (54). Knockdown of cardiolipin reduced p53 recruitment and apoptosis (54).

These C-terminal motifs were implicated in translocation in a second study that found that acetylation of lysines at positions 320, 373, and 382 was required for Bax activation and apoptosis (Figure 2) (55). Acetylation of these residues was found to empower p53 to free Bax from its inactive complex with Ku70, which p53 was found to bind with high affinity.

One well known inhibitor of p53 transactivation function, MDM4, has a surprisingly different function in mitochondria. In contrast to MDM2, which regulates p53 protein stability and antagonizes its apoptotic function, MDM4 has been thought to primarily antagonize the cell cycle arrest functions of p53. However, some more recent

evidence suggests that MDM4 may also promote p53 apoptotic functions under conditions of stress. Mancini and co-workers found that in stressed cells, MDM4 localizes to mitochondria, where it binds to Bcl-2 and helps to recruit p53-Ser46p to the complex, resulting in MOMP and apoptosis. Accordingly, MDM4 knockdown was found to decrease DNA-damage-induced apoptosis (56).

The association of p53 with mitochondria can be reinforced by downstream proapoptotic events. Translocation of p53 to mitochondria causes MOMP, followed by cytochrome c and SMAC/Diablo release (34). Cytochrome c interacts with Apaf-1 to form apoptosomes, which cleave procaspases into activated caspases. In addition, p53 enhances expression of cytosolic Apaf-1 and thereby promotes caspase activation (57). Caspase-mediated post-translational modification causes activation or inactivation of more than 280 proteins (58-61). p53 was recently found to be one of those activated. Sayan and coworkers demonstrated that cleavage of p53 by caspases generates four fragments, two of which lack nuclear localization signals and are translocated to the mitochondria. This translocation was sufficient to induce depolarization of the mitochondrial membrane and suggested positive feed forward regulation of p53 by caspases (62).

### 7. REGULATION OF OTHER PRO-APOPTOTIC MITOCHONDRIAL PROTEINS BY P53, VDAC AND CLIC4

In addition to the pores formed by Bax and Bak oligomers, there is an ion channel complex involved in loss of mitochondrial membrane integrity, the permeability transition pore complex (PTPC) that is present at the contact site of the outer and inner mitochondrial membranes. This complex consists of a voltage dependent anion channel (VDAC) on the outer mitochondrial membrane and adenine nucleotide translocase (ANT) on the inner mitochondrial membrane (63). VDAC and ANT form pores in the mitochondrial membrane either alone or in collaboration with Bax. Various cytotoxic and genotoxic stresses can activate these channels (63). However whether or not they are modulated by p53 is still an open question.

The CLIC (chloride intracellular channel) family of proteins are putative regulators of ionic homeostasis, pH and organellar volume in multiple organelles (64, 65). Among the several members of CLIC family CLIC4 (also known as mtCLIC, p64H1, RS43) is the most widely expressed and studied. Both its transcription and protein localization are regulated by p53. Initial studies showed that CLIC4 is localized to the mitochondria and cytosol of skin keratinocytes. Later, immunogold electron microscopy indicated their localization to the inner mitochondrial membrane (66, 67).

Functionally, CLIC proteins behave as chloride-selective channels (68-70), and are associated with differentiation of 3T3-L1 fibroblasts into adipocytes and mammary fibroblasts into myofibroblasts in the presence of TGFbeta (71). CLIC homologues are essential in organ

(excretory canal) development in *Caenorhabditis elegans* (72) and CLIC4 is among the very few genes that are upregulated in stem cells (73). In keratinocytes, DNA damage induces CLIC4 expression, and overexpression of mtCLIC causes cytochrome c leakage by loss of mitochondrial membrane potential and finally apoptosis (74).

Direct overexpression of CLIC4 activates intrinsic apoptosis, and its suppression prevents p53-mediated apoptotic response (74). Upregulation of mtCLIC mRNA and protein by Ad-p53 infection (66) induces p53-mediated apoptosis (74). CLIC4 is induced by TNF $\alpha$  at both transcriptional and translational levels and is subsequently translocated to the nucleus in a p53-independent manner (66, 75). Deletion of the Apaf-1 gene had no effect on apoptosis induced by nuclear targeted CLIC4, suggesting that the apoptosome is not required (71). These findings indicate that CLIC4 is induced by p53 and TNF $\alpha$ , and both nuclear and mitochondrial translocation of CLIC4 is associated with strong apoptotic response even if the mitochondrial apoptotic pathway is disabled.

Recent findings by the Yuspa lab showed that CLIC4 suppression via antisense enhanced TNF $\alpha$ -induced apoptosis. TNF $\alpha$  on the one hand can induce NF $\kappa$ B-mediated anti-apoptotic response and epithelial mesenchymal transition (EMT), while on the other hand it can induce apoptosis. They showed that antisense suppression of CLIC4 does not alter TNF $\alpha$ -induced NF $\kappa$ B anti-apoptotic activity but increases TNF $\alpha$ -mediated apoptosis in SaOS and U2OS cell line derivatives. Furthermore, suppression of CLIC4 antitumor effect was enhanced by TNF $\alpha$  administration in *in vivo* cancer models (76). They note that this is not unique; in cancer cell biology either overexpression or suppression of a protein can induce apoptosis. For example, apoptosis is induced by upregulation of c-myc in fibroblasts but by downregulation in hematopoietic cells (77, 78). How CLIC4 integrates into the Bcl-2-p53 regulatory network is a still open and fascinating question.

## 8. FUTURE DIRECTIONS

A fair question is whether the discovery that p53 acts directly at mitochondria has any therapeutic utility. Given that strategies to re-introduce p53 into p53-deficient tumors using viral vectors have met with mixed success (79), what new directions do these results provide? It should be noted that several factors limit the transcriptional success of ectopically expressed p53 in the tumor cell environment. The first is that p53 binds DNA as a tetramer, so that transduced tumor cells that express endogenous mutant p53 will develop predominantly mixed tetramers that may be transcriptionally inactive. Second, the pro-apoptotic targets of p53 may be epigenetically silenced, so that ectopic p53 is ineffectual (80). Third, many p53-client genes such as p21 are cytostatic and actually oppose the apoptotic program (81). In contrast, a mitochondrially targeted p53 would be purely apoptotic and immune to these limitations.

Considerable strides have already been made in developing such a tool by Ute Moll and coworkers. They directed p53 to the mitochondrion by fusing a mitochondrial signal sequence to its N-terminus and used viral vectors to transduce it into cultured cells or xenografted animals (38). Mitochondrially-restricted p53 was able to suppress growth of tumor cells even when an overexpressed mutant p53 was present (82, 83). This approach may find more clinical success than attempts to restore wild-type p53.

Beyond its orchestration of Bcl-2 family members, VDAC, and CLIC4 in apoptosis, p53 may play other roles in the mitochondrion. Several nuclear transcription factors, including AP-1, CREB, and NF $\kappa$ B have been detected in mitochondria (84), and p53 itself has been detected in the mitochondrial matrix (34). Moreover, p53 was reported to bind to a mitochondrial transcription factor in stressed cells (85). Potential p53 binding sites exist in the promoters of several mitochondrial genes, and genetic studies show lower expression in the absence of p53 (86). Clearly, the study of p53 localization, its client genes, and the molecules with which it associates continues to provide new insights into how the mammalian cell copes with stress and suppresses neoplasia.

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