Apoptotic proteins with non-apoptotic activity: expression and function in cancer

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Abstract

Apoptosis is a process of programmed cell death in which a cell commits suicide while maintaining the integrity and architecture of the tissue as a whole. Apoptosis involves activation of one of two major pathways: the extrinsic pathway, where extracellular pro-apoptotic signals, transduced through plasma membrane death receptors, activate a caspase cascade leading to apoptosis. The second, the intrinsic apoptotic pathway, where damaged DNA, oxidative stress, or chemicals, induce the release of pro-apoptotic proteins from the mitochondria, leading to the activation of caspase-dependent and independent apoptosis. However, it has recently become apparent that proteins involved in apoptosis also exhibit non-cell death-related physiological functions that are related to the cell cycle, differentiation, metabolism, inflammation or immunity. Such non-conventional activities were predominantly reported in non-cancer cells although, recently, such a dual function for pro-apoptotic proteins has also been reported in cancers where they are overexpressed. Interestingly, some apoptotic proteins translocate to the nucleus in order to perform a non-apoptotic function. In this review, we summarize the unconventional roles of the apoptotic proteins from a functional perspective, while focusing on two mitochondrial proteins: VDAC1 and SMAC/Diablo. Despite having pro-apoptotic functions, these proteins are overexpressed in cancers and this apparent paradox and the associated pathophysiological implications will be discussed. We will also present possible mechanisms underlying the switch from apoptotic to non-apoptotic activities although a deeper investigation into the process awaits further study.

Keywords Apoptosis · Cancer · Metabolism · Mitochondria · SMAC/Diablo · VDAC1

Introduction

In recent years, it has become clear that many proteins have more than their main well-characterized functions, possessing additional functions. Among these are pro-apoptotic proteins which can also perform non-apoptotic functions Apoptosis is a tightly regulated process of programmed cell death that plays an important role in tissue regeneration, in the development of normal organs in the embryonic stage, and in many other cellular processes. A number of pro-apoptotic proteins reside in the mitochondrial intermembrane space (IMS) and are released into the cytosol in response to an apoptotic signal, leading to caspase activation, where caspase activity is considered a hallmark of apoptosis [1].

In recent years, it has become clear that in addition to their well-characterized activities, many pro-apoptotic proteins also possess additional non-apoptotic functions [2–4] and, apparently paradoxically, many are overexpressed in a variety of cancers.

Here, we describe about 15 apoptotic proteins that also exhibit non-apoptotic activity, while focusing on 2 mitochondrial proteins that are overexpressed in cancer. The first is VDAC1, which is a mitochondrial gatekeeper and multifunctional protein involved in energy production and metabolism, Ca^{2+} homeostasis, and intracellular communication between the endoplasmic reticulum (ER) and mitochondria, as well as mitochondrial fission, autophagy, inflammation, and apoptosis [5–10]. Depletion of VDAC1was found to re-program cell energy and alter the expression and functions of several pro-apoptotic proteins with no activation

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of apoptosis, suggesting a cell energy level-controlled shift from a pro-apoptotic to a non-apoptotic state [11].

The second protein is the second mitochondria-derived activator of caspase/inhibitor of apoptosis-binding protein with low pI (SMAC/Diablo), which is released from the mitochondria to the cytosol in response to diverse apoptotic stimuli and activates apoptosis. However, SMAC is also overexpressed in cancers where it participates in non-apoptotic processes including cell proliferation in the tumor microenvironment, inflammation, the immune system, and lipid/phospholipid synthesis [12–14].

The duality of pro-apoptotic proteins also being involved in multiple processes essential for tumor growth and progression, suggests a potential as druggable treatment targets.

Apoptosis pathways

There are two major apoptotic signaling pathways [1]. The extrinsic pathway is induced by external ligands that bind to cell surface death receptors, such as Fas/CD95, tumor necrosis factor (TNFα), tumor necrosis factor receptor 1 and 2 (TNFR1, TNFR2), the TNF-related apoptosis-inducing ligand (TRAIL) receptors, and death receptors 4 and 5 (DR4 and DR5). Activation of these receptors promotes the formation of a death signaling complex (DISC), leading to activation of pro-caspase-8, which activates caspases-3 and -7 for the final execution of cell death [15, 16]. Caspase-8 can also activate mitochondria-mediated apoptosis by cleaving Bid and the cleaved Bid (tBid) then activates Bax and/or Bak, to mediate the release of pro-apoptotic proteins from the mitochondria, thereby, activating apoptosis.

The second pathway, the intrinsic mitochondrial-dependent apoptotic pathway, can be activated by intracellular signals such as DNA damage, Ca^{2+} overload, increased reactive oxygen species (ROS) levels, chemotherapy drugs [1], and VDAC1 overexpression [17]. The apoptotic signals change the permeability of the outer mitochondrial membrane (OMM), leading to the release of the apoptotic factors residing in the IMS. These released apoptotic proteins include cytochrome c (Cyto c), SMAC/Diablo, high-temperature requirement protein A2 (HtrA2/Omi), endonuclease G (Endo-G), and apoptosis-inducing factor (AIF) [18]. The released factors trigger a downstream cascade of cell death protease (caspase) activity. The released Cyto c binds to the apoptotic protease activating factor 1 (Apaf-1), which in the presence dATP forms an oligomeric structure that recruits cytoplasmic inactive pro-caspase-9 to form the apoptosome, where pro-caspase-9 undergoes activation and then activates caspase-3, and -7, leading to apoptosis [19] (Fig. 1).

A schematic presentation of the two apoptosis signaling pathways: (a) extrinsic apoptosis—binding of death ligands to their receptors at the plasma membrane (PM)

induce the assembly of DISC, which consists of an adaptor protein and the relevant ligand, and recruits and activates pro-caspase-8. Active caspase-8 activates the executioner caspase-3/7, which in turn, cleaves cellular substrates and brings about the formation of apoptotic bodies. (b) Intrinsic apoptosis—mitochondria-mediated pathway in which multiple stimuli such as UV, DNA damage, oxidants, chemotherapies, ROS, and Ca^{2+} overload, trigger the formation of the protein-conducting channel at the OMM, allowing the release of apoptogenic factors (Cyto c, AIF, SMAC/Diablo, HtrA2/Omi, Endo-G) from the IMS into the cytosol. The released Cyto c, together with the cytosolic Apaf-1 and procaspase-9 form the apoptosome in the presence of dATP, leading to caspase-9 activation, which cleaves effector caspases, such as caspase-3/7. The activated effector caspases cleave cellular substrates and are responsible for destroying the cell from within, while apoptotic bodies are formed. AIF is cleaved by calpains and/or cathepsins and translocated to the nucleus, where it activates chromatin degradation and condensation. Endo-G also translocated to the nucleus where it cleaves chromatin DNA into nucleosomal fragments. The released SMAC in the cytosol interacts with the inhibitors of caspase IAPs (including survivin), thereby antagonizing caspase inhibition. The crosstalk of extrinsic apoptosis and mitochondrial intrinsic apoptosis is mediated by caspase-8, which cleaves Bid to form the truncated protein (tBid). This protein is then translocated to the mitochondria and activates the intrinsic apoptotic pathway.

There are several possible mechanisms that could explain how the apoptotic initiators cross the OMM. These include non-specific release of IMS proteins due to OMM rupture [20], opening of the permeability transition pore (PTP) in response to overproduction of ROS or Ca^{2+} overload [21], formation of a large channel composed of Bax and/or Bak oligomers [22, 23], and the possible formation of hetero-oligomers of VDAC1 and Bax or VDAC1 oligomers [7, 24–34] (Fig. 2). Notably, multiple pathways of Cyto c release may co-exist in the cell [17].

Mitochondria-mediated apoptosis and VDAC1

Mitochondria are a common site for multiple metabolic, biosynthetic, and bioenergetic reactions as well as apoptosis, mitophagy, NETosis, pyroptosis, necroptosis, parthanatos, ferroptosis, alkaliptosis, and oxeiptosis, and they also regulate innate immunity, and epigenetics, among others [35]. Moreover, mitochondrial dysfunction is associated with many diseases [36].

VDAC1 is one of the key controllers of mitochondrial function and is located in the OMM. VDAC1 is composed of 19 transmembrane β-strands that are connected through
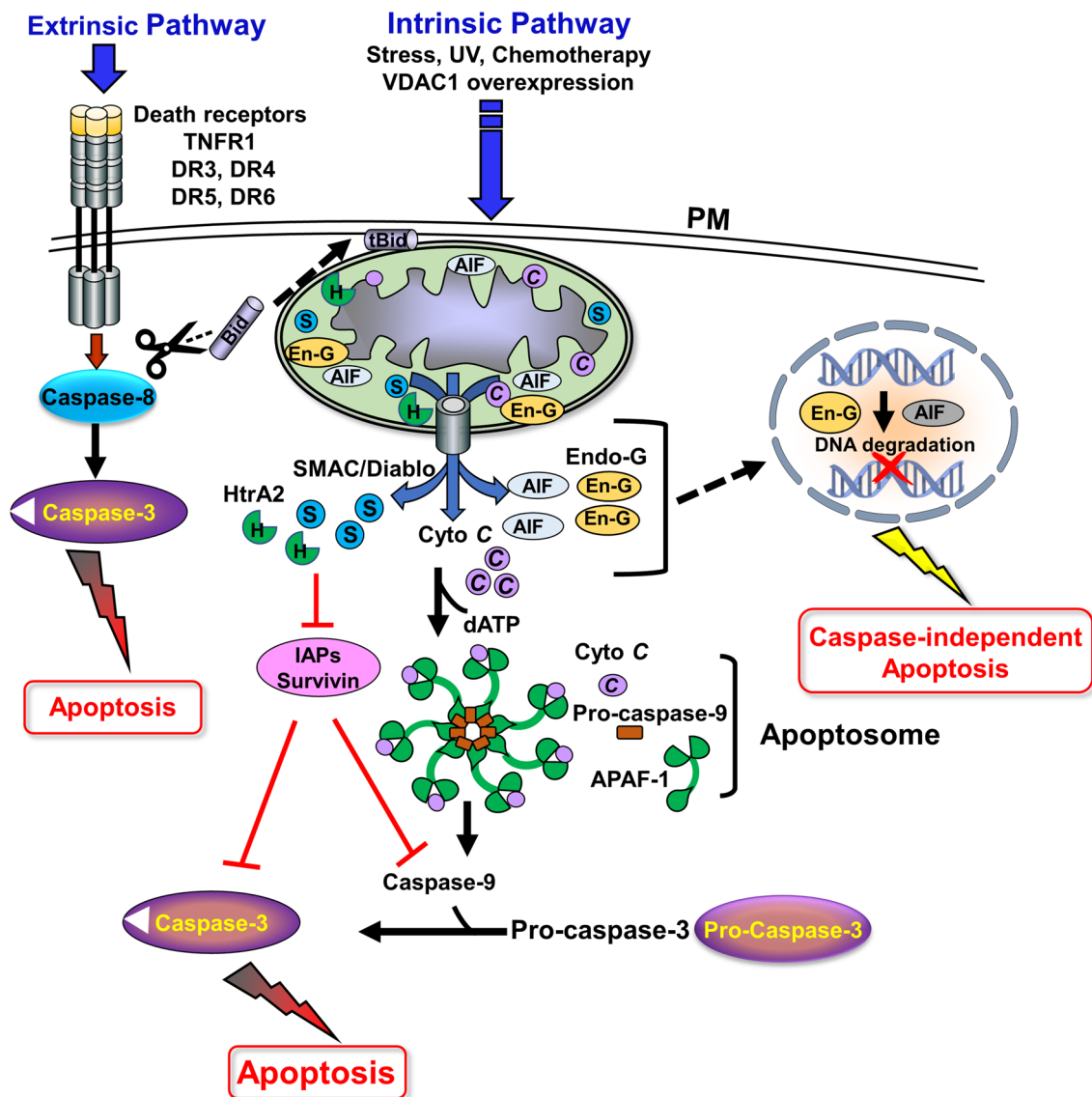
Fig. 1 Apoptosis pathways; Activation and regulation

flexible loops with strands β1 and β19 in parallel conformation. This forms a β-barrel [6] with a 25-residue-long N-terminal region that lies inside the pore and can translocate from the internal pore to the channel surface [37–40] (Fig. 3A). The mobility of the N-terminal region is important for channel gating, interaction with anti-apoptotic proteins, and for VDAC1 dimer formation [37]. In addition, the region serves as the site of interaction for apoptosis-regulating members of the Bcl-2 family (i.e., Bax, Bcl-2, and Bcl-xL) [26, 37, 41–43] and hexokinase (HK) [26, 44].

VDAC1 controls the metabolic and energetic crosstalk between the mitochondria and the rest of the cell by transporting metabolites such as pyruvate, malate, and succinate, nucleotides such as ATP/ADP and NADH/NAD+, and lipids, and ions including Ca^{2+} [5, 6, 17] (Fig. 3B).

The protein serves as a hub that interacts with over 100 proteins involved in key cellular functions including metabolism, signal transduction, anti-oxidation, regulation of DNA/RNA structure, and apoptosis [5, 45].

One important function of VDAC1 involves the release of mitochondrial pro-apoptotic proteins from the IMS to the cytosol where they interact with apoptosis regulatory proteins such as IAPs [5, 6, 17] (Fig. 2). It is now accepted that apoptosis induction leads to VDAC1 oligomerization, and results in the formation of a large channel within a VDAC1 homo-oligomer that serves as a release route for Cyto c and other pro-apoptotic proteins, leading to apoptosis activation [7, 24–34, 37, 46] (Fig. 2). Oligomeric VDAC1 also mediates mitochondrial DNA (mtDNA) release into the cytosol, thereby triggering the type-I
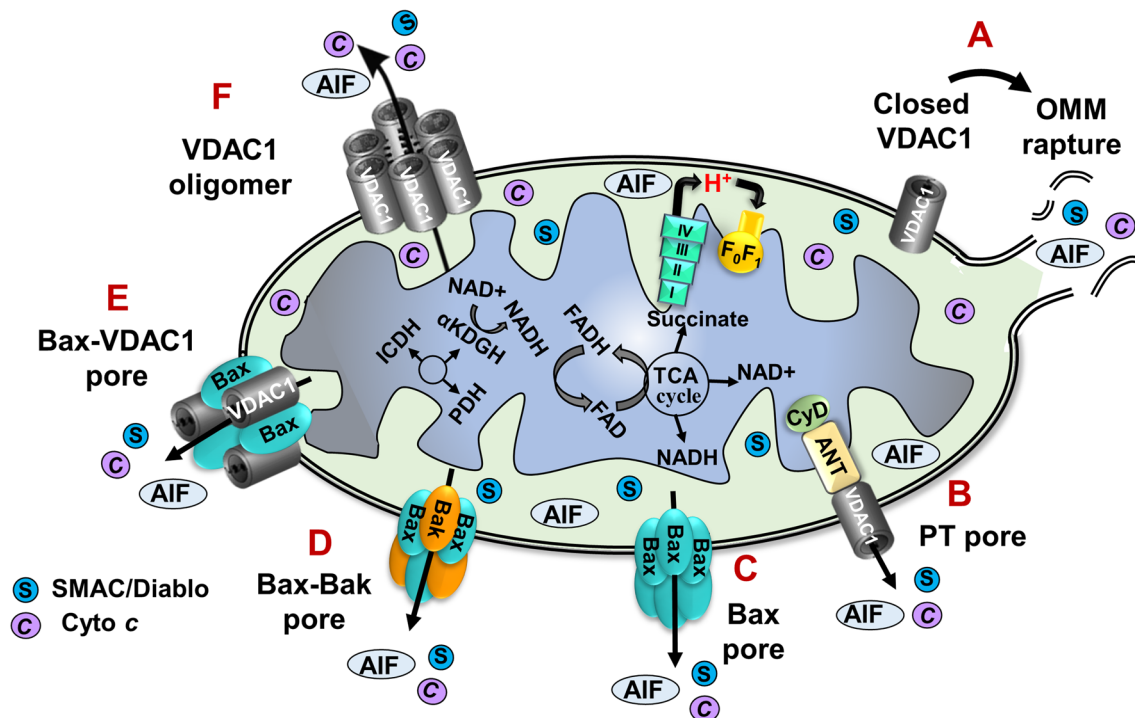
Fig. 2 Proposed mechanisms for apoptotic initiators crossing the OMM. A schematic representation of different models for the release of apoptogenic proteins. Apoptotic stimuli, stress conditions, or Ca^{2+} overload induce the assembly of a protein-conducting channel in the OMM, thereby allowing the release of Cyto c, AIF, HtrA2, Endo-G, and SMAC from the IMS to the cytosol where they trigger apoptotic cell death: (A) Prolonged VDAC1 closure leads to mitochondrial matrix swelling and OMM rupture, thereby providing a non-specific method of release of apoptogenic proteins; (B) Mitochondrial swelling leading to the assembly of a permeability transition pore (PTP); (C) Activation of the pro-apoptotic protein Bax leads to its association with the OMM and oligomerization to provide a protein-conducting channel; (D) Bax/Bak oligomerization and activation, provides a route for the release of pro-apoptotic proteins; (E) Bax and VDAC1 form hetero-oligomers that mediate the release; (F) VDAC1 oligomers serve as a protein-conducting channel that can mediate the release of apoptogenic proteins to the cytosol

interferon responses that cause systemic lupus erythematosus [24, 47].

Although VDAC1 overexpression is associated with apoptosis [17], it is somewhat unexpectedly also overexpressed in cancers [17, 36, 45, 48–50], as well as in other pathologies [36] such as Alzheimer's disease [51–54], in β-cells in type 2 diabetes [55], and in autoimmune diseases such as lupus [24], inflammatory bowel diseases [56], non-alcoholic steatohepatitis [57], acute liver injury [58], rheumatoid arthritis [59] cardiovascular diseases [60], and in the T cells of COVID-19 patients [61].

This apparent paradox may be explained by the finding that, while inhibition of apoptosis may lead to cancer and other severe disorders [62], activation of apoptosis may cause neurodegenerative and [54] autoimmune diseases [24, 56]. The latter can be targeted by inhibiting VDAC1 oligomerization, and thereby apoptosis [31, 52].

The involvement of VDAC1 in the control of pro- and non-apoptotic proteins is described below.

Apoptotic proteins with non-apoptotic function

The pro-apoptotic proteins caspases-3, -7, -8, and -9, AIF, APAF, survivin, SMAC/Diablo, Endo-G, HtrA2/Omi, TSPO, VDAC1, HK1, the Bcl2 family, and Cyto c [2, 3, 63] (Table 1) have all been shown to possess additional non-apoptosis related functions.

Caspases: Non-apoptotic functions of caspases

As already described, caspase activity is the hallmark of apoptosis. Caspases are a family of cysteinyl aspartate-specific proteases that usually exist in the cell as inactive proenzymes. However, in response to apoptosis initiation,

Fig. 3 VDAC1 as a multi-functional channel mediates the transport of metabolites, ions, lipids, and Ca²⁺ signaling, and regulates energy production, ER-mitochondria crosstalk, and apoptosis. **A** Side- and top views of the crystal structure of VDAC1 (PDB code: 3EMN). The β-barrel is formed by 19 β-strands, and the N-terminal domain is folded into the pore interior. **B**. (2) VDAC1 in the OMM controls transport of various metabolites and ions in and out of the IMS; (2) Transport of Ca²⁺ to the IMS, where it is transported to the matrix Ca²⁺ uniporter (MCU) complex; (3) MAVS (mitochondria anti-viral signaling), associated with VDAC1, enables anti-viral signaling Anti-viral activity via interaction with MAVS; (4) VDAC1 mediates the transfer of acyl-CoAs across the OMM to the IMS, where they are converted into acylcarnitine by CPT1a for further processing by β-oxidation. VDAC1 is involved in cholesterol transport as a constituent of the multi-protein complex termed the transduceosome, which contains Star/TSPO/VDAC1. (5) Mediation of cellular energy production by transportation of ATP/ADP and NAD⁺/NADH and

regulation of glycolysis via association with HK; The role of Ca²⁺ in regulating energy production is mediated via activation of the TCA cycle enzymes pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), and α-ketoglutarate dehydrogenase (α-KGDH) leading to enhanced activity of the TCA cycle. The electron transport chain (ETC) and the ATP synthase (F₀F₁) are also presented. (6) apoptotic signaling induced VDAC1 oligomerization leads to the formation of a large channel conducting apoptogenic proteins (e.g., Cyto c and AIF) transport from the mitochondrial IMS to the cytosol, leading to apoptosis; (7) VDAC1 oligomers from a large channel mediate the release of mtDNA, which is associated with inflammation; (8) VDAC1 structurally and functionally contributes to the mitochondria-associated membranes, where the inositol 3 phosphate receptor type 3 (IP₃R3), the sigma 1 receptor (Sig1R), binding immunoglobulin protein (BiP), the glucose-regulated protein 75 (GRP75/HSP70), and VDAC1 mediate Ca²⁺ transport from the ER to mitochondria

Table 1 Pro-apoptotic proteins with non-apoptotic functions

No	Protein	Apoptosis related function	Non-apoptosis function	References
1	Cytochrome *c* (Cyto *c*)	Upon release from the mitochondria, forms part of the apoptosome leading to caspase-9 activation and apoptosis induction	An electron transfer protein, function in the respiration chain. Influences cell differentiation and localizes to the nucleus under non-apoptotic conditions	[2, 11]
2	Caspase-3	Caspase-3 is the most important executioner caspase and is activated by both the intrinsic and extrinsic pathways. When activated, it cleaves multiple structural and regulatory proteins that are critical for cell survival and maintenance	Plays a role in cell proliferation and migration during neuronal differentiation, keratinocyte differentiation, in mammalian red blood cell nuclear extrusion, brain development, liver regeneration, and skin wound healing	[64, 74]
3	Caspase-7	Activated by initiator caspases and acts as an executioner caspase to cleave multiple proteins	Plays a pivotal role in bone formation and exhibits different functions depending on the developmental history of the bone	[82]
4	Caspase-8	Activated by the extrinsic apoptosis pathway, which itself is triggered by death receptors. When activated, it activates caspase-3 and Bid	Performs multiple non-apoptotic roles in cells, such as activation of NF-κB signaling, alteration of endosomal trafficking, and enhancement of cellular adhesion and migration	[93, 100]
5	Caspase-9	Activated by the mitochondrial apoptotic pathway in the apoptosome and activates other caspases such as -3 and -7	Induces differentiation of leukemic cancer cells, granulocytic cells, myoblasts, neuronal cells, and mouse embryonic stem cells	[103–106]
6	AIF	Is released from the IMS in response to apoptotic signals, then translocates to the nucleus, and activates DNA fragmentation and chromatin condensation to initiate a caspase-independent apoptosis	Transduces DNA-damage-induced signals, controlling both adipocyte differentiation and mitochondrial metabolism	[2]
7	APAF	A key factor in the apoptosome, that acts at the DNA damage checkpoint and is required for chromosomal stability	Protein functions in the DNA damage-induced cell cycle	[107–110]
8	Survivin	Inhibits caspase activation, thereby leading to negative regulation of apoptosis Direct and indirect binding of Survivin to initiator or effector caspases contributes to its inhibition of apoptosis [50]. Survivin binds to SMAC/DIABLO and prevents caspase activation [52]	Survivin in the cytosol and nucleus is associated with chromosome alignment and segregation during mitosis and cytokinesis. The protein is involved in the regulation of cell division and DNA-double-strand repair	[111–114]
9	SMAC	Is released from the mitochondria in response to apoptotic signals and interacts with IAPs in the cytosol to prevent their inhibition of caspases, thereby, initiating apoptosis	Regulates phospholipid synthesis by controlling PSD activity. It also modulates inflammation and immunity	[12, 14]
10	HtrA2/Omi	High-temperature requirement protein A2. This serine protease is localized to mitochondria and is released to the cytosol following apoptotic insult, where it interacts with XIAP, thereby allowing caspase activation	Regulates the cell cycle, inactivates premature aging in mammals, and promotes hepatic fibrogenesis. It is responsible for quality control in mitochondrial homeostasis	[115]
11	Endo-G	Endo-G is a mitochondrial protein, a DNA/RNA non-specific nuclease. Once released from mitochondria, it functions in the caspase-independent apoptotic pathway to induce nuclear DNA fragmentation	Regulates mitochondrial bioenergetics by cleaving DNA–RNA hybrids to yield the RNA primers necessary for mtDNA replication. It may play an important role in differentiated cardiomyocytes	[116, 117]
12	VDAC1	Participates in the release of mitochondrial pro-apoptotic proteins from the IMS to the cytosol and interacts with apoptosis regulatory proteins such as Bcl-2	Multifunctional protein involved in energy production and metabolism, Ca²⁺ homeostasis, intracellular communication, autophagy, and inflammation	[5–9, 17]
13	TSPO	Proposed to mediate ROS production and formation of PTP to mediate Cyto *c* release, thus, inducing apoptosis	Involved in cholesterol import, porphyrin transport, heme synthesis, regulation of cell proliferation, inflammation, quality control, and mitochondrial metabolism	[11, 118]
14	Bcl2 family	Pro- and anti-apoptotic proteins	BH3-only proteins inhibit autophagy, others induce autophagy, or modulate Ca²⁺ signaling through the ER	[119–129]
15	Hexokinase	Function as anti-apoptotic proteins when bound to VDAC1	Hexokinase catalyzes the phosphorylation of glucose	[44, 46, 130–137]

the initiator caspases are activated and activate the executioner caspases to cleave selected target proteins and execute cell death [64].

Recent studies have now reported caspase participation in multiple non-apoptotic cellular processes, including removal of protein aggregates, cell differentiation or de-differentiation, and rebuilding tissue [65–67]. Caspases are required in the early stages of differentiation [67] and for the terminal differentiation of cell types such as haematopoietic, epithelial, sperm, muscle, and trophoblast cells [2], as well as in the differentiation from human monocytes to macrophages [68]. Caspase activation is also required for terminal erythroid differentiation [69, 70]. The non-apoptotic functions of the pro-apoptotic proteins are summarized below and in Table 1.

Caspase-3 pro-apoptotic and non-apoptotic activities

Caspase-3 is an executioner caspase and a key mediator of both intrinsic and extrinsic pathway activated apoptosis (Fig. 1). Following activation by initiator caspases, casepase-3 cleaves proteins, leading to apoptotic cell death.

However, accumulating evidence now indicates that caspase-3 also possesses non-apoptotic functions, and is involved in regulating the growth and homeostasis of both normal and malignant cells and tissues in multicellular organisms [2, 3]. Caspase-3 activation has also been implicated in the differentiation of multiple cell types, including osteoblastic differentiation, and caspase-3-deficient mice accordingly exhibit delayed ossification and decreased bone mineral density [71–73]. The protein also affects the differentiation and maturation of human and mouse cell types [65], and is involved in cell proliferation and migration during neuronal, keratinocyte, and neuroendocrine cell differentiation, and the movement of neurons and neural and glial progenitor cells [2, 74]. Inhibition of caspase-3 prevents the differentiation of erythroid precursors [70] and affects brain development, liver regeneration, and skin wound healing [75]. The effects are seen in patient-derived glioma cells, where caspase-3 knockdown reduced the ability to form tumor spheres in culture [76].

Similarly, caspase-3-deficient mice are characterized by perinatal lethality with surviving newborns exhibiting various neural phenotypes [77], including specific aspects of cognition and behavior [78]. In contrast, caspase-3 overexpression has been reported in diverse types of malignant tumors [79] and high levels of caspase-3 mRNA expression have been found in neuroblastomas, Hodgkin's disease, and in hepatoma cell lines [80]. These findings support the theory that the non-apoptotic functions of caspase-3 may be beneficial for cancer.

Caspase-7 pro-apoptotic and non-apoptotic activities

Pro-caspase-7 is activated by caspase-9 and functions in apoptosis execution (Fig. 1). Non-apoptotic activities of caspase-7 have been described in hard tissue formation and in differentiating cells during odontogenesis [81], and the protein also plays a role during osteogenesis [82] and spermatogenesis [83]. Caspase-7 has been reported to regulate the cell cycle and differentiation of certain cell types, and it is essential for proliferation and cell growth [84], where caspase-7 deficiency has an adverse effect on cell cycle progression resulting in the reduction of cell proliferation [85].

Caspase-3/-7 double-deficient mice exhibit embryonic lethality, whereas mice singly deficient in either caspase are born at normal Mendelian ratios and display no gross abnormalities when maintained on a C57BL/6 genetic background [86].

Caspase-7 is downregulated in some cancers [87]. In breast cancer carcinoma, the protein is abnormally expressed and contributes to cell growth and proliferation, with the active forms localized in the nucleus apart from the cytosol [88]. Elevated levels of caspase-7 are associated with well-differentiated tumors in oral squamous cell carcinomas [89].

Caspase-8 pro-apoptotic and non-apoptotic activities

Caspase-8 is an initiator caspase in the extrinsic apoptosis pathway that activates caspases-3 and -7 for the final execution of cell death [90] (Fig. 1).

The protein was recently suggested to serve as molecular switch for apoptosis, necroptosis, and pyroptosis, and prevents tissue damage during embryonic development and adulthood [91], while caspase-8 auto-cleavage plays a role in regulating necroptosis and maintaining lymphocytes homeostasis [92].

The non-apoptotic functions of caspase-8 involve nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated pro-survival pathways, T- and B-cell proliferation, macrophage differentiation [68, 93, 94], and heart muscle differentiation [64]. Accordingly, loss-of-function mutations in the human caspase-8 gene cause defects in the activation of T, B and NK cells, culminating in immunodeficiency [95, 96].

In-vitro studies have indicated that caspase-8 induces the production of cytokines by acting as a scaffolding protein [97, 98]. In addition, caspase-8 enhances cleavage of focal adhesion substrates and cell migration, and its knockdown disrupts metastasis in neuroblastoma *in vivo* [99].

Caspase-8 deficiency has been reported in several cancer types, where it may contribute to tumorigenic transformation by interfering with cell differentiation [100]. Malignant neuroendocrine tumors, such as in neuroblastoma, primitive neuro-ectoderm tumors, medulloblastoma, glioblastoma, and small cell lung carcinoma frequently show a loss of caspase-8 [100].

Caspase 9 pro-apoptotic and non-apoptotic activities

Caspase-9 is an initiator of intrinsic apoptosis and when activated in the apoptosome, the protein stimulates downstream executioner caspases such as caspase-3 and caspase-7 (Fig. 1).

The non-apoptotic functions of caspase-9 include regulation of cellular differentiation/maturation, innate immunity, mitochondrial homeostasis, and autophagy, and activation of the NF-kB pro-survival pathway by interacting with cellular inhibitor of apoptosis protein-1 (cIAP1/BIRC2) [101]. Caspase-9 also promotes myocyte differentiation and proliferation, and its suppression may impede muscle [102] and neuronal cell differentiation [103].

The expression of caspase-9b is upregulated in several cancers, including non-small-cell lung cancer, thereby providing potential as a target for tumor treatment [101]. Caspase-9 is also involved in differentiating granulocytic and leukemic cancer cells [104].

Overall, the non-apoptotic functions of caspases are diverse, ranging from involvement in immune responses, cell proliferation, cellular remodeling, and cell fate determination, to cytoskeletal reorganization, with implications for cancer, neurodegeneration, wound repair, and regeneration.

Cytochrome c apoptotic and non-apoptotic functions

Cyto c is a key player in respiration, where it shuttles electrons between complex III and complex IV of the respiratory chain [138], but it also performs multiple other functions associated with cell life and death [139]. Cyto c release is a hallmark of the intrinsic apoptotic pathway in mitochondria-mediated apoptosis, and it forms the apoptosome in the presence of dATP and Apaf-1, thereby acting as an allosteric activator of caspase-9 [140, 141] (Fig. 1). The activity depends on the subcellular compartment in which the Cyto c resides, with electron transport activity associated with the IMS, and the pro-apoptotic functions seen when the protein is released into the cytosol.

Interestingly, in response to stress, such as DNA damage, Cyto c migrates to the nucleus before the cytoplasmic caspase are activated [142, 143].

Non-apoptotic functions of Cyto c include accumulation in the nucleus and induction of chromatin remodeling [144]. This localization of Cyto c to the cytoplasm or nucleus is associated with nitration, which thereby controls the cellular functions [145].

In non-cancerous systems, Cyto c appears to modulate differentiation across a wide spectrum of cell types [2], including differentiation associated with caspase-3 and -9 activation [4]. In cancers, elevated levels of Cyto c have been reported in patients with hepatocellular cancer [146], with downregulation in renal cell carcinoma tissues [147]. Cyto c has also been found in secretory granules in the pancreas and anterior pituitary [148].

Cyto c activities are tightly regulated by posttranslational modifications [149]. Specifically, phosphorylation can serve as the switch between the multiple activities of the protein [150].

AIF non-apoptotic functions

AIF is a pro-apoptotic protein that is released from the mitochondria upon apoptosis induction following its cleaved by calpains and/or cathepsins to yield the pro-apoptotic truncated AIF form, that translocates to the nucleus, where it participates in chromatin condensation and chromatinolysis [151–154].

Non-apoptotic pro-survival activities of AIF are related to oxidative phosphorylation. Murine *aif* knockout causes a defect in oxidative phosphorylation [155, 156].

AIF has been proposed to have an antioxidant function, which is important for optimal oxidative phosphorylation, as well as for the maintenance of mitochondrial morphology and cell-cycle regulation [154, 156]. An absence of AIF in murine cardiac tissue leads to the development of dilated cardiomyopathy and heart failure [157]. These mice also exhibit increased glucose tolerance, higher insulin sensitivity, decreased fat mass, and resistance to high-calorie-diet-induced obesity [158]. In this context AIF has been shown to control adipocyte differentiation [159].

AIF also possesses a pro-survival function as a component of the coiled-coil-helix-coiled-coil-helix domain containing 4 (CHCHD4)-dependent import pathway by which a set of nuclear-encoded cysteine-motif-carrying protein substrates are translocated to the IMS [160].

AIF levels are elevated in chronic lymphocytic leukemia (CLL) [161] and in prostate cancer, where the protein supports the growth and survival of aggressive prostate cancer cells [162]. The levels are also increased in colorectal cancer where AIF protect the cells from stress-induced

apoptosis [163]. Thus, elevated AIF protein levels benefit tumorigenesis.

SMAC as a non-apoptotic protein

SMAC is a pro-apoptotic protein that is released from the IMS upon apoptosis induction and interacts and antagonizes inhibitors of apoptosis proteins (IAPs) in the cytosol, thereby promoting the activation of caspases and apoptosis [164].

SMAC was found to be upregulated in a variety of tumors [12, 14, 161]. The non-apoptotic functions of overexpressed SMAC in cancer were recently identified [12, 14], and are presented below.

HtrA2/Omi as a non-apoptotic protein

The high temperature requirement protein A 2 (HtrA2) also known as stress-activated/regulated endoprotease (Omi), belongs to a family of serine proteases [165, 166]. HtrA2/Omi is released from the IMS to the cytosol in response to apoptosis signals, and once there, proteolytically removes IAPs and promotes both caspase-dependent and independent apoptosis [167, 168]. However, HtrA2/Omi has non-apoptotic activities include regulating the cell cycle by cleaving the large tumor suppressor kinase 1 (LATS1) protein and generating LATS1 fragments that inhibit the G1–S transition [169]. HtrA2/Omi inactivation has been reported to increase the accumulation of mtDNA deletions and deficiency of the protein induces mtDNA damage, DNA mutation, accumulation of unfolded proteins in the mitochondria, oxidative stress, and defective mitochondrial respiration, suggesting an important role in mitochondrial homeostasis [170]. An inactivating point mutation leads to premature aging in mammals [171].

HtrA2/Omi expression has been found to promote hepatic fibrogenesis and is suggested to be responsible for quality control in mitochondrial homeostasis [166].

Reduced Omi/HtrA2 activity has been connected to neurodegeneration. The protease activity of Omi/HtrA2 was shown to be required for the physiological processing of amyloid-β precursor protein and it is therefore suggested as a possible therapeutic target for Alzheimer's disease [172–174]. Omi/HtrA2 has also been linked to Parkinson's disease [175, 176].

Omi/HtrA2 has also been suggested to act as a negative regulator of cell cycle progression through the proteolytic processing of the mitotic kinase WTS/large tumor suppressor 1 (WARTS) [169].

Endo-G as an apoptotic and non-apoptotic protein

Endo-G is a mitochondrial endonuclease that is situated in the IMS and plays a role in mitochondrial DNA replication [177]. However, during apoptosis Endo-G is released from mitochondria and translocates to the nucleus, and induces nuclear DNA fragmentation cleaving chromatin DNA into nucleosomal fragments and induce cell death in caspase-independent pathways [116]. It was also proposed that Endo-G activates DNA damage response and triggers autophagy through its endonuclease activity [178].

Endo-G knockout mice are viable and grow to maturity with no evident defects, and fibroblasts derived from Endo-G-deficient animals showed no difference in sensitivity to a variety of intrinsic and extrinsic apoptotic stimuli, suggesting that Endo-G is not essential for early development or apoptosis [179].

The non-apoptotic functions of Endo-G include a role in recombination and repair during mitochondrial DNA replication [179]. The protein appears to be required for proliferation, as Endo-G-depleted cells accumulate at the G_2–M transition [180]. In addition, the protein plays an important role in differentiated cardiomyocytes [116]. Endo-G is a novel determinant of cardiac hypertrophy and mitochondrial function and is involved in regulating the estrogen-related receptor alpha (ERRα) and the peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) [181]. Endo-G knockdown causes the death of polyploid tumor cells [182].

Endo-G was detected in tumor than non-tumor parts of liver tissue of the patient. of the patient [183].

Survivin, BIRC5 as a non-apoptotic protein

Survivin, first identified in 1997, is a member of the IAP family of proteins with only one baculoviral IAP repeat (BIR) domain [111]. It has a dual function in preventing cell death by inhibiting apoptosis while playing an essential role in mitosis [111, 112]. Survivin, like other IAP family members, is thought to be a negative regulator of apoptosis [111, 112] and has been shown to interact with both caspase-3 and caspase-7 and to inhibit their activity [113]. In addition, the survivin suppression frequently reported in cancer cells is associated with caspase-3 activation and apoptosis enhancement [184–188]. Moreover, survivin has been shown to control the nuclear translocation of AIF, and hence negatively modulates the activation of caspase-independent apoptosis [20, 189–191].

Unlike the other IAP family members, survivin is not only involved in apoptosis regulation but also in promoting

mitosis by encouraging the formation of the chromosomal passenger complex [189, 192, 193]. Survivin in the nucleus is associated with chromosome alignment and segregation during mitosis and also controls cytokinesis, thereby regulating cell division, and the protein is also involved in DNA-double-strand repair [114, 194].

Survivin is highly expressed in most cancers and high levels are associated with chemotherapy resistance and increased tumor recurrence [190].

TSPO functions in apoptotic and non-apoptotic processes

TSPO, previously known as the peripheral-type benzodiazepine receptor (PBR), is located in the OMM [195]. The receptor is involved in the regulation of cellular proliferation, apoptosis, and mitochondrial functions [196].

Several studies suggest that TSPO functions in mitochondrial apoptosis [11, 118] by interacting with mitochondrial PTP, and with several other apoptosis-related proteins [197].

However, TSPO's main activities are unrelated to apoptosis and include cholesterol import, regulation of mitochondrial metabolism, cell proliferation, inflammation, porphyrin transport, heme synthesis [198], and mitochondrial quality control [118]. TSPO deregulates mitochondrial Ca^{2+} signaling, leading to activation of Ca^{2+}-dependent NADPH oxidase, and thereby, increasing ROS levels [199]. TSPO also regulates mitochondrial energy homeostasis through the modulation of fatty acid oxidation in steroidogenic cells [200].

TSPO is overexpressed in a wide variety of malignant human cells and in colon, brain, prostate, breast, ovarian, esophageal, gliomas, endometrial, and hepatic carcinomas [196], 201. Moreover, the TSPO expression level correlated positively with disease progression of some cancers suggesting that the multiple functions are advantageous to cancer growth and development [196].

Certain TSPO functions are thought to involve interaction with VDAC1 [202–204].

As presented below, the expression of most of these proteins is altered upon VDAC1 or SMAC depletion, suggesting that their expression is linked to cell metabolism.

Hexokinase (HK) dual functions in metabolism and apoptosis

Hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate, in an ATP dependent reaction that serves as the entry point for glucose to glycolysis. The four isozymes of HK present in mammalian tissues, termed HK-I to HK-IV, differ in their subcellular localization and metabolic function [205]. HK-I and HK-II are primarily mitochondrial and predominantly dock on to the cytosolic surface of the OMM by binding to VDAC [206]. Binding of HK to VDAC1 has been proposed to allow direct access to newly produced ATP in the mitochondria [207], thereby facilitating the maintenance of a high glycolytic flux rate in tumors with the accompanying increase in energy and metabolite production [130].

Interestingly, the results of both *in vitro* and *in vivo* studies suggest that HK-I and HK-II can function as anti-apoptotic proteins when bound to VDAC1, with their detachment enabling activation of apoptosis [44, 46, 130–137]. HK prevents Cyto *c* release and subsequent apoptosis [44, 46, 130–137] and also protects against Bax- or Bak-mediated apoptosis [131, 132, 208]. Thus, HK not only plays an important metabolic role, but also acts to inhibit apoptosis.

HK-I and HK-II are over expressed in many types of cancer, including colon, prostate, lymphoma, glioma, gastric adenomas, carcinomas, and breast cancers [209–213], as well as in hepatomas [214] and in aggressive tumors such as gliomas and colorectal cancer [215]. The observation of these elevated levels of HK-I and HK-II in cancer cells provide evidence that a high rate of glycolysis allows tumor cells to evade apoptosis, thereby allowing proliferation to continue [216].

In addition to promoting energy production and acting as an anti-apoptotic protein, HK binding to VDAC1 also regulates ROS production/efflux from the mitochondria [217] and increases the synthesis and uptake of cholesterol [131]. Thus, the multifunctional HK is beneficial for cancer cells and make HK dissociation from the mitochondria an attractive target for anti-cancer therapy [218, 219].

Non-apoptotic functions of other cell death regulatory proteins

Other proteins associated with apoptosis such as APAF1, Bax, XIAP, and the Bcl-2 family have also been shown to have non-apoptotic functions [220].

Bcl-2 family proteins: Bcl-2 family anti-apoptotic proteins such as Bcl-2, Bcl-XL, Bim, Bcl-w, and Mcl-1 [121, 126, 127], were found to inhibit autophagy via interaction with Beclin 1 [128, 129]. Conversely, pro-apoptotic BH3-only members of the Bcl-2 family such as BNIP3, Bad, Noxa, Puma, and others have been shown to induce autophagy by competing with Bcl-2 and Bcl-XL, and by interaction with Beclin 1 [121, 122]. Interestingly, both anti-apoptotic and pro-apoptotic proteins from the Bcl-2 family were shown to modulate Ca^{2+} signaling in the ER [123–125].

Bcl-2 and Bcl-XL were found to lower luminal Ca^{2+} levels by enhancing Ca^{2+} leakage from the ER, through

modulation of the gating of the inositol-1,4,5-trisphosphate receptor (IP3R) by its ligand [221, 222]. In contrast, Bcl-2, Bcl-xL, and Mcl-1 modulate Ca^{2+} homeostasis via interaction with IP$_3$Rs at the mitochondria-associated membranes (MAM) [223]. It has been suggested that Bcl-xL interacts with IP$_3$Rs to promote Ca^{2+} release, thereby regulating mitochondrial metabolism and neuronal function and development [224].

Conversely, Bax and Bak enhance the release of Ca^{2+} from ER stores, by involving the SERCA [119, 120].

Interestingly, Bcl-xL, but not Bcl2 can strongly suppress the mitophagy receptor FUN14 domain-containing 1 (FUNDC1)-mediated mitophagy by inhibiting the mitochondrial Ser/Thr phosphatase PGAM5, thereby preventing the dephosphorylation of FUNDC1 and blocking hypoxia-induced mitophagy [225]. When FUNDC1-mediated mitophagy is blocked by the microtubule inhibitor vinblastine, PGAM5 dephosphorylates FUNDC1 and mediates mitochondrial fission that aggravates vinblastine-induced cell death [226].

Bcl2 family members can affect the morphology of mitochondria. The activation of pro-apoptotic Bax and Bak promotes fragmentation of the mitochondrial network during apoptosis. This is not inhibited by the expression of Bcl-xL, MCL1, or other members of the Bcl2 subfamily [226].

Over expression of anti-apoptotic Bcl-2 members (e.g., Bcl-2, Bcl-XL, Mcl-1) is quite frequent in newly diagnosed cancer as well as after developing resistance to therapy [227]. Bcl2 and Bcl-xL are expressed at high levels in many types of cancer, such as colon, prostate, lymphoma, glioma, gastric adenomas, carcinomas, and breast cancer [155, 156]. When Bcl-2 is overexpressed in cancer cells, it may inhibit the pro-apoptotic signals, allowing the cancer cell to survive under stress [228].

Decreased Bak expression associated with increased expression of anti-apoptotic components has been shown in tumors of colon cancer patients [132]. It is now accepted that the ratio of expression of anti- and pro-apoptotic proteins in the cell determines the resistance to apoptosis induction.

APAF-1: Apaf-1, which is the main component of the apoptosome, a platform required for caspase 9 activation (Fig. 1), has been reported to function in the DNA damage checkpoint and is required for chromosomal stability [107–110]. The results of Apaf-1 knockdown in human cells and knockout in mice suggest that the protein functions in DNA damage-induced cell cycle arrest [107, 108, 110]. Apaf-1 mutants lacking the N-terminal CARD domain can replace endogenous Apaf-1 in the control of the DNA damage-induced cell-cycle blockade, suggesting that the cycle-arresting function of Apaf-1 is independent of caspase activation [107, 108, 110]. Apaf-1 depletion was also found to decrease the DNA damage induced, activating phosphorylation of the checkpoint kinase Chk1 [107, 108, 110], and

knockdown of Chk1 abrogates Apaf-1-mediated cell-cycle arrest. A study of biopsies from non-small cell lung cancer patients revealed a correlation between the nuclear translocation of Apaf-1 induced by DNA damaging agents, and the endogenous activation of Chk1.

In summary, the findings presented above suggest that proteins categorized as apoptotic proteins may also possess non-apoptotic functions ranging from regulating cell proliferation to affecting differentiation and modulating the immune response. Many of these apoptotic proteins are overexpressed in cancer, suggesting their importance for cancer development and survival.

The paradox of VDAC1 overexpression of in cancer

Exogenous overexpression of VDAC1 was found to induce apoptosis in the absence of any apoptosis stimuli and regardless of cell type [30, 46, 229]. Overexpressed VDAC1 undergoes oligomerization and forms a large channel that allows passage of IMS-located apoptotic proteins such as Cyto *c,* SMAC, and AIF, to cross the OMM and be released to the cytosol, where they induce apoptosis [7, 25–30, 230] (Fig. 3).

Moreover, VDAC1 expression could be increased by stimuli such as chemotherapy drugs, ROS, Ca^{2+}, and stress conditions, which activate mitochondria-mediated apoptosis via different mechanisms, but all lead to VDAC1 oligomerization [7, 29, 36].

In cancers, although VDAC1 is overexpressed [17, 36, 45, 48–50] (Fig. 4), there is no induction of apoptosis. This is due to the prevention of apoptosis by the concomitant over expression of anti-apoptotic proteins such as hexokinase and Bcl2 [17, 36, 45, 49].

Why do cancer cells overexpress VDAC1 despite its pro-apoptotic activity? Cancer cells reprogram their metabolism to support the increased biosynthetic and energetic demands required for their growth and motility. In these cells, VDAC1 is overexpressed to support cell energy and metabolism homeostasis [5, 6, 17]. Accordingly, downregulation of VDAC1 expression decreases metabolite exchange between the mitochondria and the cytosol and inhibits cell growth [49, 231]. Moreover, we demonstrated that depleting VDAC1 in tumor cells leads to metabolic reprogramming, with a resultant inhibition of cell proliferation and tumor growth, elimination of cancer stem cells, alteration of the inflammation in the tumor microenvironment, and induction of differentiation of the malignant cells to become normal-like cells [11, 49, 232–236].

Thus, VDAC1 can be considered as a pro-survival protein with pro-apoptotic activity that is controlled by the expression level and the interacting proteins.

Reducing VDAC1 expression in cancer alters the expression of pro-apoptotic proteins performing a non-apoptotic role

Silencing VDAC1 expression in tumors and cells in culture resulted in metabolic reprogramming [49, 231, 233] and alteration in the levels of expression of pro-apoptotic proteins [11]. Our results indicate that VDAC1 deletion leads to an upregulation of the pro-apoptotic proteins, caspases-3, -8, and -9, p53, and Cyto *c*, albeit with no induction of apoptosis, but rather, an association with cell differentiation [11, 232–236]. The increase in levels of Cyto *c* and the protein translocation to the nucleus in VDAC1-depleted tumors with no apoptotic cell death could be related to its role in cell differentiation [2]. The increase in caspase-3, -8 and -9 expression levels with no apoptosis seen when VDAC1 is depleted in a tumor [11, 235, 236] may be related to the proposed non-apoptotic functions of the caspases, for example in differentiation [2, 64].

Notably, the expression of other apoptosis-regulatory proteins such as SMAC, AIF, and TSPO are also decreased in VDAC1-silenced cells [11].

Thus, we can conclude that reprogramming cancer cell energy and metabolism shifts protein functions from pro-apoptotic to non-apoptotic, with effects on processes ranging from bioenergetics and metabolism to inflammation and differentiation.

Indeed, metabolic reprogramming via silencing VDAC1 expression encourages differentiation of cancer cells, both in culture and in mouse models [233–235, 237]. In glioblastoma (GBM), and in lung and breast cancers, cell metabolism rewired by VDAC1 depletion results in cell growth arrest and differentiation [49, 231–234, 238]. Similarly, in GBM tumors and in cells in culture, silencing VDAC1 expression results in differentiation of astroglia U-87MG cell into astrocyte- and neuron-like cells [233, 234]. In triple negative breast cancer, MDA-MB-231 cells, which correspond to a poorly differentiated tumor that does not express the estrogen, progesterone, or ERBB2/Her2 receptors [239], silencing VDAC1 expression in tumors and cells in culture increased the expression levels of all these receptors as well as that of prolactin [234, 237].

VDAC1 silencing also downregulated other pro-apoptotic proteins, including SMAC, AIF, and TSPO, which are associated with cell growth support [11].

AIF may offer advantages to cancer cells through non-apoptotic functions associated with pro-survival, oxidative phosphorylation, ROS detoxification, cell cycle regulation, and others [154]. Since VDAC1 depletion leads to a dramatic decrease in the levels of AIF [11], this should also reduce the AIF functions.

Similarly, a marked decrease in TSPO levels was observed in VDAC1-depleted tumors [11]. This decrease will affect the multiple functions of TSPO in cholesterol import, mitochondrial metabolism, cell proliferation, Ca^{2+} signaling, and oxidative stress regulation [240]. As TSPO binds to and acts via VDAC1 [202–204], the decrease in TSPO levels may result from VDAC1 depletion.

These findings reveal that metabolic reprogramming of tumors following silencing of VDAC1 expression both in cultured cells and in mouse models, results in changes in the levels of pro-apoptotic proteins, which then fulfil non-apoptotic functions, such as acting as regulators of cell growth and differentiation [233–235, 237] (Fig. 5).

To conclude, the results presented here suggest that players in apoptosis pathways may have a dual function in cancer and be both apoptotic and non-apoptotic, with the switch between these activities being controlled by the cell energy level on the metabolism-epigenetics axis. Accordingly, depletion of the mitochondria gatekeeper VDAC1 leads to a re-programming of energy and shifts the activity of these proteins from a pro-apoptotic to a non-apoptotic mode. This is accompanied by translocation of some of these proteins from the mitochondria/cytosol to the nucleus. Possible mechanisms that regulate the pro-apoptotic or non-apoptotic functions are discussed below.

The paradox of overexpression of the pro-apoptotic protein SMAC in cancer

SMAC is a 25-kDa protein requiring post-translational modification for maturation, activation, and translocation to IMS. Smac/DIABLO at 2.2 A resolution reveals that it homodimerizes through an extensive hydrophobic interface [241] (Fig. 7A). SMAC is a pro-apoptotic IMS-located protein, that, upon apoptosis induction, is released into the cytosol where it interacts with members of the IAP family (cIAP1, cIAP2, and XIAP) to neutralize their inhibition of caspases, thereby initiating apoptosis [164, 242, 243] (Fig. 1).

Unexpectedly, we and others have demonstrated overexpression of SMAC in many cancer types and cell lines, including cervical cancer, lung, ovarian, prostate, gastric and renal cell carcinomas, and different types of sarcomas (Fig. 6) [12, 14]. This may suggest that SMAC overexpression is beneficial to cancer cells, through the manifestation of non-apoptotic functions. Indeed, our results indicate that SMAC is required for the growth of cancerous, but not of non-cancerous cells in culture, and that silencing SMAC expression inhibits lung cancer-derived tumor growth, restores altered lipid metabolism, and modulates a number of oncogenic properties [12, 14] (Fig. 7).

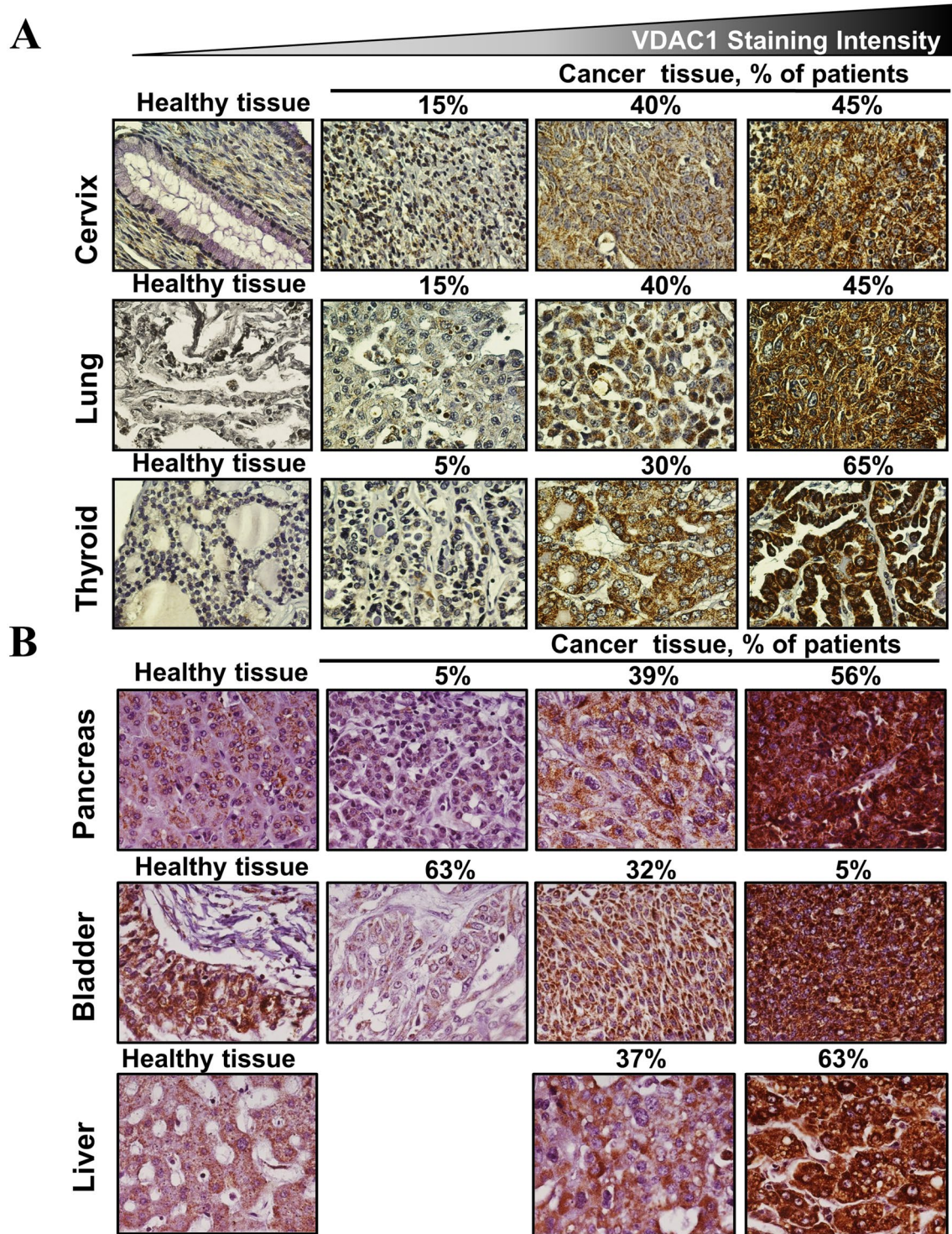This is particularly interesting, since mice lacking SMAC are viable, and grow, and mature normally, without

Fig. 4 VDAC1 overexpression in cancers. **A, B** Immunohistochemical staining of VDAC1 on tissue microarray slides obtained from Biomax: US slide (MC5003) comprising cancer tissues (n=18–20 patients for each cancer type) and normal tissue (n=5). Representative sections of cervix, lung, thyroid, pancreas, bladder, and liver are shown. The slides were incubated overnight at 4 °C with anti-VDAC1 antibodies (1:1000) in PBS containing 1% BSA and then with the appropriate secondary antibody (1:5000) diluted in PBS containing 1% BSA. The slides were subsequently treated with 3′3-diaminobenzidine tetra-hydrochloride (DAB) and counter-stained with hematoxylin. Negative controls lacked the incubation with the primary antibody. Sections of tissue were observed under an Olympus microscope and images were recorded at 200× magnification with the same light intensity and exposure time. The percentage of patients with positive staining at the intensity indicated by the scale at the top is shown

any histological abnormalities, and exhibit wild-type responses to all types of apoptotic stimuli [244]. This suggests that cancer cells require novel non-apoptosis-related functions.

SMAC possesses non-apoptotic functions required for cancer survival and development

The non-apoptotic functions of SMAC in cancer range from regulating cell proliferation to differentiation [12, 14].

The non-apoptotic function of SMAC was demonstrated by silencing its expression using specific siRNA in cells in culture or in sub-cutaneous lung cancer xenografts in mice [14, 245]. The non-apoptotic functions of SMAC in cancer range from inhibiting cell proliferation and tumor growth to differentiation [12, 14]. The si-hSMAC-treated residual tumor demonstrated morphological changes, including cell differentiation and reorganization into glandular/alveoli-like structures and elimination of surfactant-producing organs, the lamellar bodies [12, 14]. Similarly, CRISPER/Cas9 SMAC/Diablo-depleted A549 lung cancer cells displayed inhibited cell proliferation and cell migration [13]. Using the

proteomic approach of LC–MS/MS analysis, these SMAC/Diablo depleted cells, showed altered expression of proteins associated with lipid- and lipid signaling, vesicular transport and trafficking, metabolism, epigenetic, extracellular matrix, cell signaling and neutrophil-mediated immunity [13].

Moreover, tumors established from SMAC-KO A549 cells showed, altered expression of several components of the tumor microenvironment, reduced expression of inflammation-related proteins such as NF-kB and TNF-α and of the programmed death-ligand 1 PD-L1 associated with additive immune system suppression [13].

These results suggest that SMAC is involved in multiple processes that are essential for tumor growth and progression.

Next generation sequencing (NGS) analysis of lung cancer-derived tumors that were silenced for SMAC expression revealed altered expression of genes associated with transporters of metabolites, lipids, and ions, and of enzymes involved in the metabolism of cholesterol, lipids, and nucleotides [12, 14].

SMAC depletion altered expression of genes associated with cholesterol, lipid transport and synthesis, and lipid degradation and regulation [12, 14]. In addition, the expression of many genes associated with the formation of vesicles
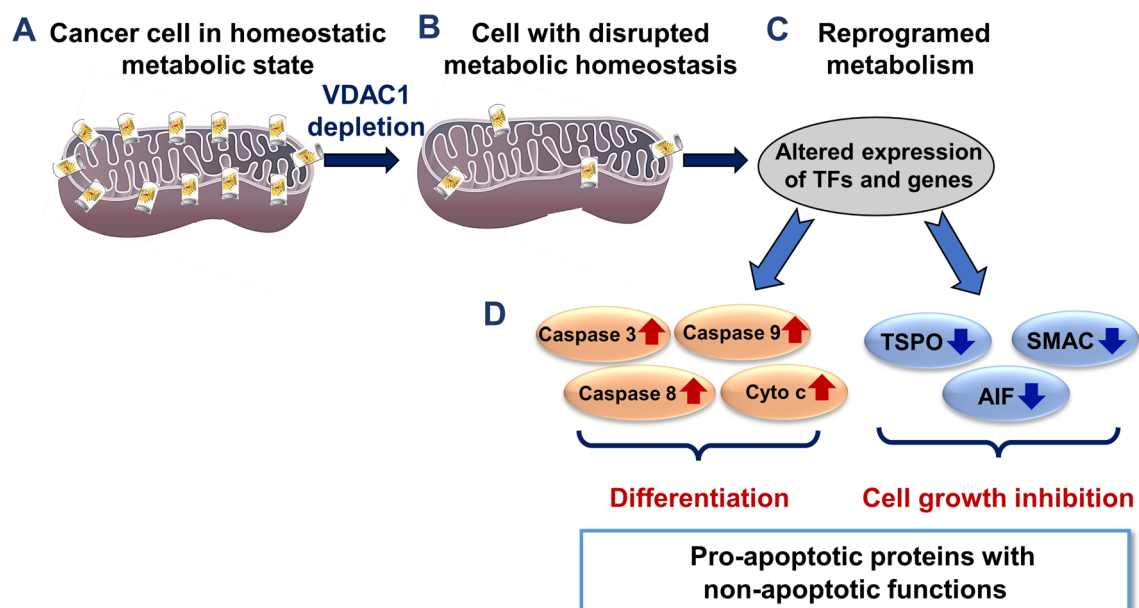

Fig. 5 Reprograming tumor metabolism via VDAC1 depletion alters the expression of pro-apoptotic proteins and shifts their activities towards arrested cell growth and induction of cell differentiation. A schematic presentation of cancer cell VDAC1-depletion leading to altered levels of apoptotic proteins and expression of their non-apoptotic functions associated with arrested cell growth and cell differentiation induction. Cells expressing VDAC1 maintain a homeostatic metabolic state supporting cell growth and survival (A). Following VDAC1 depletion, cells have impaired energy and metabolite generation (B). This results in altered expression of thousands of genes and transcription factors (TFs) such as metabolism regulators, increased p53 levels supporting cell differentiation, and decreased HIF1-α, c-Myc, and NF-kB/RelA levels, resulting in cell growth inhibition [11] (C). Metabolism reprogrammed tumors overexpress pro-apoptotic proteins that possess cell differentiation promotion functions, while pro-apoptotic proteins whose non-apoptotic function associated with cell growth promotion are downregulated (D)
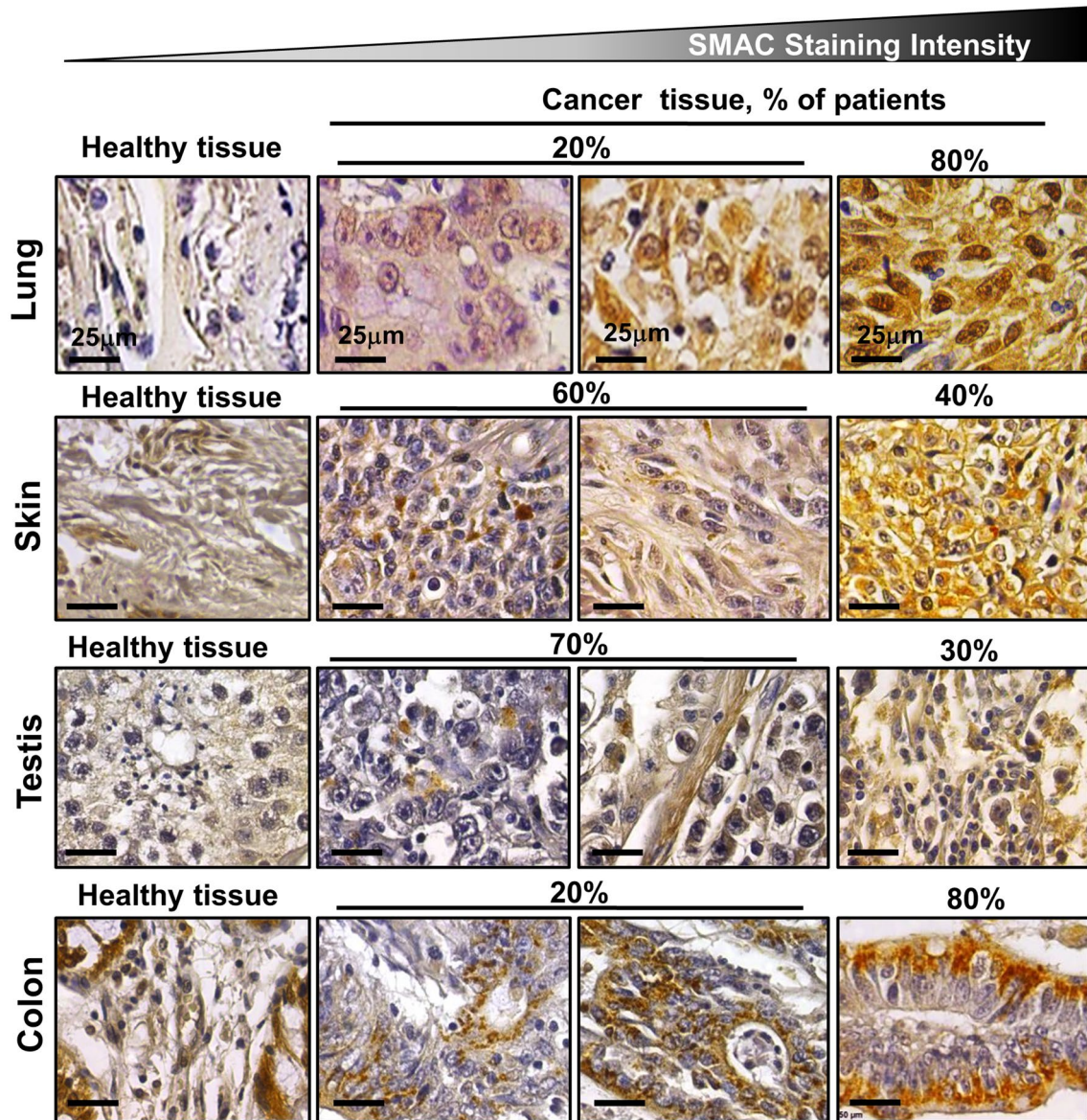
Fig. 6 SMAC overexpression in cancer. Immunohistochemical staining for SMAC was performed on tissue microarray slides obtained from Biomax US: MC5003 comprising cancer tissues (n = 10–20 patients for each cancer type) and normal tissues (n = 5). Representative lung, skin, testes, and colon are shown. The slides were incubated overnight at 4 °C with anti-SMAC antibodies (1:1000) in PBS containing 1% BSA and then with the appropriate secondary antibody (1:5000) diluted in PBS containing 1% BSA. The slides were subsequently treated with 3′3-diaminobenzidine tetra-hydrochloride (DAB) and counter-stained with hematoxylin. Negative controls lacked the incubation with the primary antibody. Sections of tissue were visualized under an Olympus microscope and images were recorded at 200× magnification with the same light intensity and exposure time. The percentage of patients with positive staining at the intensity indicated by the scale at the top is shown

that mediate intra- and extra-cellular transport and that are related to organelles, such as the ER, Golgi and exosomes, was modified [14]. In addition morphologically, the tumors exhibit differentiation and reorganization into glandular/alveoli-like structures. (Fig. 7B).

These non-apoptotic functions may explain the SMAC overexpression observed in multiple cancers.

SMAC regulates lipid metabolism in cancer via modulation of mitochondrial PSD activity

Metabolism reprogramming is a hallmark of cancer, with cancer cells demonstrating a high dependence on synthesis of the lipids [246], and phospholipids (PLs) that are essential
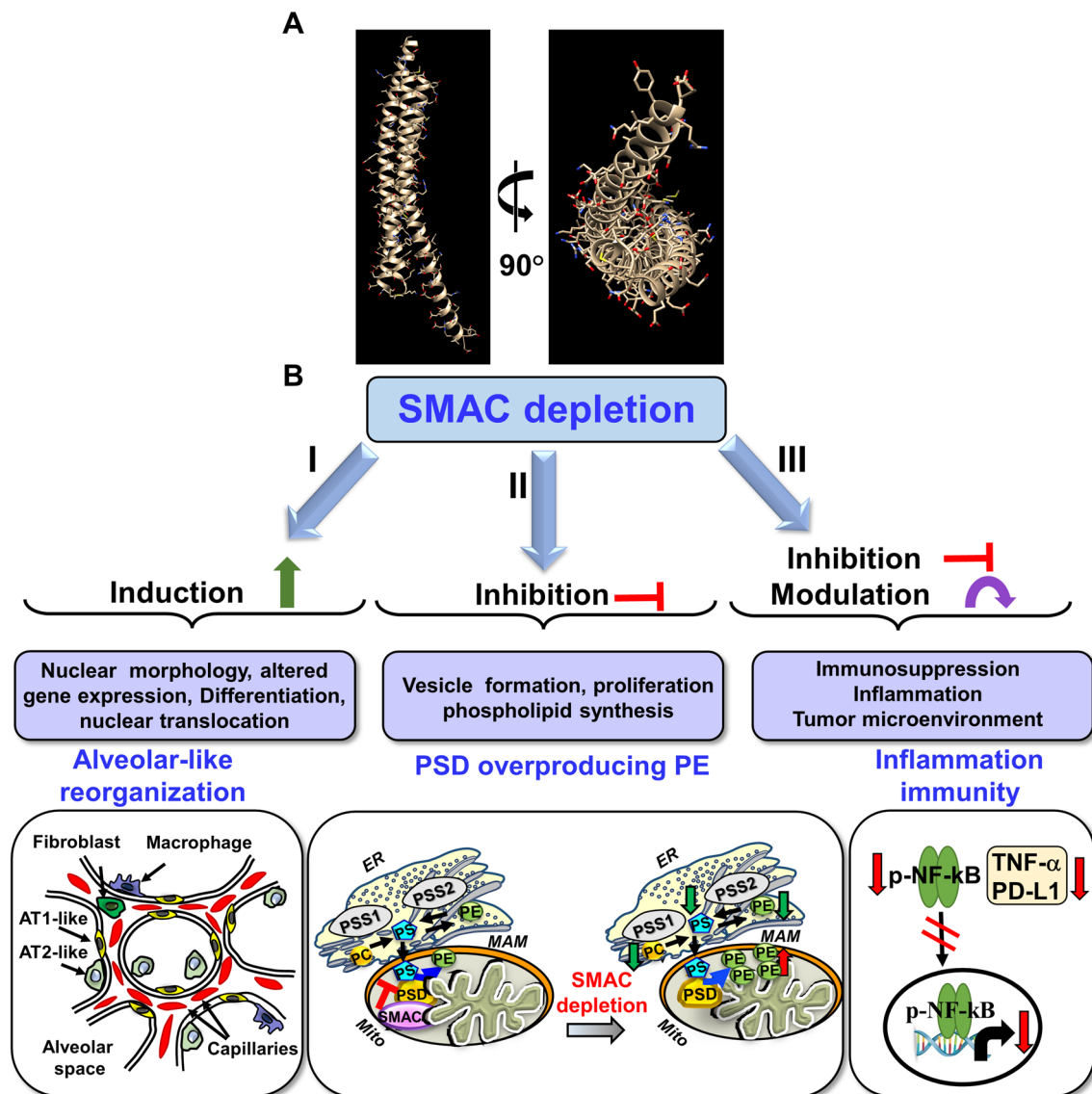
Fig. 7 SMAC depletion effects on tumor morphology and properties. **A** Crystal structure of SMAC, PDB https://doi.org/10.2210/pdb1F EW/pdb. **B** SMAC depletion leads to: (I) Induction of cell differentiation and reorganization into glandular/alveoli-like structures. Pulmonary alveoli cross-section showing the major cell types with the tumor implemented AT2-like cells were differentiation into AT1-like cells, and changes in blood capillary organization; (II) Inhibition of vesicle formation, cell proliferation in the tumor microenvironment, and phospholipid synthesis. (b) Induction of altered expression of genes associated with the cell membrane, exosomes, and ER- and Golgi-related proteins, nuclear translocation, and cell and tumor morphological changes. This is due to SMAC functions in the regula-

tion and maintenance of phospholipid synthesis [12]. In mammalian cells, distinct pools of PE are synthesized in either mitochondria or ER membranes [252]. The major site of phospholipid synthesis is in the ER [261], where PS is transferred from the ER to mitochondria [262]. In the ER, PS is produced from PC and PE by PSS1 and PSS2, respectively, and then transferred to the mitochondria, where it is converted into PE by PSD [250]. (III) Inhibition or modulation of inflammation and immunity in the small tumors developed from CRISPER/Cas9 SMAC/Diablo-depleted A549 lung cancer cells showed reduced expression of inflammation-related proteins such as NF-kB and TNF-α and of the programmed death-ligand 1 PD-L1 associated with additive immune system suppression [13]

for cancer growth, development, migration, invasion, and immune escape [247, 248]. PLs are essential building-block components of cellular membranes, but also function as regulators of various cellular functions such as cell adhesion and migration, signal transduction, vesicular trafficking, apoptosis, metabolism, and post-translational modifications

[249]. As presented above, lipid and phospholipid synthesis pathways are highly affected by depletion of SMAC in a tumor [12, 14].

We identified SMAC interaction with and modulation of the mitochondrial enzyme phosphatidylserine decarboxylase (PSD), which is also present in the IMS, as SMAC, and

converts phosphatidylserine (PS) to phosphatidyl-ethanol amine (PE) [12, 250]. Accordingly, SMAC deletion, resulted in about a 2-fold decrease in total PLs and phosphatidyl choline (PC) levels both in tumors and in CRISPR/Cas9 SMAC-KO cancer cells, while PE levels were increased 2-fold in the mitochondria [12]. Purified SMAC directly interacts with purified PSD and inhibited its activity, and SMAC downregulation resulted in enhanced PSD activity, thereby, overproducing mitochondrial PE, leading to cell depletion of PC and PS and inhibition of cancer cell proliferation [12] (Fig. 7D).

Such changes reflect significant differences in cellular physiology since PSD and PE are essential components. PSD-KO is embryonic lethal [251], and loss of PSD causes defects in mitochondrial morphology and function and disrupts electron transport chain complex formation [252]. PE is not only a membrane component, but also functions in the nucleus as a lipid chaperone signaling molecule where it modulates the structural organization of chromatin, nucleic acid synthesis, and DNA replication [253], and has a role in autophagy [254]. PE levels are elevated in several cancers, and it is a target of potent anticancer natural products [255].

SMAC-interacting network

SMAC has multiple effects on cancers which may be related to the interaction of SMAC with a variety of non-apoptotic proteins located in the IMS, cytosol, and nucleus [12, 14]. These include Bcl-2, mitogen-activated protein kinase [256], c-Jun N-terminal kinase [257], and protein kinase C (PKCδ) [258]. SMAC also interacts with NADE (p75NTR-associated cell death), thereby promoting TRAIL-induced apoptosis [259] SMAC interacts with two nuclear proteins, the aryl hydrocarbon receptor nuclear translocator, ARNT/HIF-1β, and the transcriptional co-activator the mastermind like transcriptional coactivator 2 truncated poly Q MAML2, [260].

We recently identified the binding sites of SMAC with ten SMAC-interacting proteins: baculoviral IAP repeat containing two (BIRC2), TNF receptor associated factor 2 (TRAF2), MAML2(ARENT/HIF-1β), the ubiquitin conjugating enzyme E2K (UBE2K), baculoviral IAP2 repeat containing five (BIRC5, survivin), HtrA2, and PSD [12, 14].

The diverse proteins that interact with SMAC, and their functions and subcellular localizations clearly explain the multiple non-apoptotic functions of SMAC that are essential for cancer cells.

In summary, the pro-apoptotic SMAC is overexpressed in many cancers, probably because of additional non-apoptotic functions that contribute to cancer cell survival. Reducing SMAC expression inhibits cancer cell proliferation, both in culture and in tumors. The hundreds of genes shown to be differentially expressed in SMAC-depleted tumors are associated with a wide variety of cellular and tissue morphological traits and functions, which probably reflects the non-apoptotic functions of SMAC that are essential for tumorigenesis. These results highlight the significance of SMAC for tumor growth and progression, and as a potential target for the development of new therapeutic approaches for cancer treatment.

What controls whether apoptotic proteins promote pro- or non-apoptotic functions?

A major question is how, when, and where is the transformation from a pro-apoptotic to a non-apoptotic protein initiated and regulated?

The signal or condition(s) that determine the pro- or non-apoptotic outcomes are unclear, although several mechanisms have been postulated [263]. One mechanism proposes that whether caspases fulfil apoptotic or non-apoptotic functions depends on the activation of substrates or cofactors that are unique to each event [264], or that are influenced by the timing and intensity of the signaling pathway [264]. Another proposed mechanism suggests that the caspases induce the expression of specific genes [265, 266]. Indeed, microinjection of active caspase-3 into cells was found to induce the expression of muscle-specific genes [266]. In caspase-8-deficient neuroblastoma cells, death effector domain expression attenuated tumor growth by a mechanism involving upregulation of p53 and differentiation markers [265].

Our results indicate that silencing VDAC1 expression, reduces cellular ATP levels and cell proliferation, induces metabolic reprogramming, inhibits tumor development and angiogenesis, and alters the tumor microenvironment and the expression of over 4000 genes, including epigenetic-related enzymes and transcription factors [49, 231–234, 238, 267]. These findings suggest that the rewiring of cancer cell metabolism induced by VDAC1 depletion, alters the expression of caspases, Cyto c, AIF, SMAC, and TSPO [11], most probably via an interplay between metabolism and epigenetics.

The result of pro-apoptotic versus non-apoptotic activity may also depend on the sub-cellular localization of the protein. Indeed, caspases, AIF, and Cyto c can be found either in the nucleus or the cytosol [144, 268], but in VDAC1-depleted tumors, Cyto c, AIF, caspase-8 and -9, and SMAC are present in the nucleus [11, 12, 14]. However, the molecular basis of protein sub-cellular localization remains unknown.

A further option is that other apoptotic regulatory proteins modulate the activity of the pro-apoptotic proteins. For example, ubiquitination-mediated XIAP degradation may be responsible for some caspase-3 non-apoptotic functions [269].

Finally, other suggested mechanisms for controlling protein pro- or non-apoptotic function include spatial restriction, as shown for caspase-9 activation during thrombopoiesis only in perinuclear granular structures, or temporal restriction as seen for caspase-3 activation only during early erythropoiesis, or by substrate specificity, as demonstrated in several models of cellular differentiation [2].

Perspectives

Numerous proteins have additional functions, over and their main and well-characterized activities.

Many pro-apoptotic proteins fulfil non-apoptotic, non-lethal physiological functions in the cell cycle, differentiation, metabolism, and inflammation.

Several of these apoptotic proteins are overexpressed in cancers where they perform non-apoptotic functions and thus, are potential targets for treating cancer.

Some of these pro-apoptotic proteins translocate to the nucleus to perform non-apoptotic functions that include regulating gene expression and modulating DNA and RNA structures.

The mechanisms orchestrating the transformation from a pro-apoptotic to a non-apoptotic protein are not clear. They may involve the timing and intensity of the signaling pathway, substrate or cofactor specificity, metabolism-epigenetics cross-talk, and sub-cellular localization, although the exact mechanisms await further study.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval Not applicable.

Data availability All data generated or analyzed during this study are included in this published articles and their supplementary information files.

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