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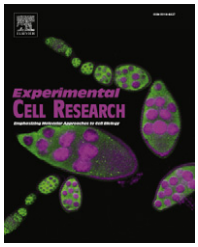

Review Article

Non-apoptotic roles for death-related molecules: When mitochondria chose cell fate

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ABSTRACT

The decision between death and survival is a difficult phase of a cell life. It may depend on the intensity of a stress stimulus, on the presence of invasive pathogens, or on specific signals from neighbouring cells. Death-related molecules are being shown to possess different, and sometimes opposite roles, which they play also according to a number of environmental clues. In this review, we will analyse some of these molecules and their roles, with particular regard to mitochondria-related factors, such as BCL2 family members, the apoptosome components, the autophagy/death cross-talkers and molecules regulating mitochondrial structure and functions. Turning the double-edged swords of death molecules into plougshares may turn out to be strategically crucial in molecular oncology.

Contents

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Introduction

Besides their important role in regulating or executing apoptosis, several key molecules in this process are being identified as key modulators of a number of alternative functions. After the seminal discovery of the mitochondrial pathway of cell death [1], it became clear that the definition 'death molecule' would have been quite limitative, if not inappropriate in most cases. The best example of this mysnomer is cytochrome-*c* (Cyt-*c*), the small and charged activator that, once released from mitochondria, enables Apaf1 conformational changes and apoptosome activation [1,2]. Cyt-*c* is an essential component of the electron transport chain in the oxidative phosphorylation process. Thus, it acts also as a key pro-survival factor in a cell life (and, in fact, its downregulation *in vivo* leads to early lethality, due to lack of energy production). But the list of dual-function or pleiotropic proteins in the context of apoptosis is growing fast. As we will review here, several proteins, which contribute to the maintenance or the change of mitochondrial structure, and rearrange their network in response to a number of stimuli, may also have a pro-death role [3]. *Vice versa*, the apoptosome key component Apaf1 [4] or the best known death-executors, the members of the caspase family of proteases [5], can sustain cell survival, by orchestrating several processes during cell differentiation and development [6]. Finally, on the verge between cell death and survival, other molecules can regulate both apoptosis and autophagy, a degradation pathway essential for recycling cell components both in basal conditions and upon a number of stressors [7,8].

All these new evidence are of the highest importance in cancer biology. Inducing apoptosis in cancer cells is, at present, the major goal of chemo- or radio-therapy [9] and the finding that molecules can play alternative roles within the cell, in some cases paradoxically opposite, can induce the research field to switch towards a more comprehensive analysis of the single molecular functions that can or cannot be modulated when treating tumors.

Cytochrome c, 'good' and 'bad' at the same time

Cyt-*c* is widely known as an efficient biological electron-transporter that, through the transition between the ferrous and ferric states of its heme group, plays its major role in cellular respiration by transporting electrons from Cyt-*c* reductase (Complex III) to cytochrome oxidase (Complex IV). This shuttling takes place at the level of the mitochondrial intermembrane space. Cyt-*c* null mice die very early in embryogenesis, due to a severe energy imbalance [10]. Besides this role as an essential pro-survival protein, a key role for Cyt-*c* has been established in mitochondria-mediated apoptosis: its release from the mitochondrial *cristae*, and from its cardiolipin-mediated anchoring to the mitochondrial membranes, is regulated by a number of BH3-containg pro- and anti-apoptotic protein and is permitted by *cristae* opening (see below) [11]. Once in the cytosol, Cyt-*c* binds the apoptosome core molecule Apaf1 and permits caspase-9 recruitment and its lethal activation [1,4]: A clear pro-death role, for such a generally *good* molecule. Of note, this function in apoptosis is absent in some lower eukaryotes (*Caenorhabditis elegans*) and controversial or very limited in others (*Drosophila melanogaster*) [4]. By elegant *in vivo* experiments, the double-side capacities of Cyt-*c* in vertebrates were finely dissected at the molecular level [12]. How can we explain these contradictory roles? A startling hypothesis is that Cyt-*c* pro-apoptotic activity is a secondary function, acquired from vertebrate cells during evolution to quickly respond with a death program to any mitochondrial damages above a certain threshold. A sensitivity that may have a crucial importance during embryogenesis, far more complicated in higher eukaryotes.

Caspase-3 and friends: problem solvers, not only killers

Cyt-*c* regulation of caspase-9 (Casp-9), through Apaf1 and the apoptosome assembly, allows the mitochondria to signal caspase-3 (Casp-3) towards a lethal program of destruction. Tens of targets for destruction, by this efficient cysteine aspartate protease, have been identified and, with a snap, a cell can be reduced in the goofy mass of an apoptotic body, ready to be orderly phagocytosed by professional or uncoventional phagocytes.

But this is not the only role for deadly Casp-3. Terminal differentiation of vertebrate lens fiber cells [13] and erythrocytes [14], as well as the transition from spermatid to spermatozoa in *Drosophila* [15], all involve proteolytic degradation of major cellular compartments by Casp-3. In these cases, Casp-3 helps removing huge portions of cytosol or controls an ordered DNA fragmentation mediated by caspase-activated DNases, in cells undergoing a terminal differentiation process. Of note, in the case of sperm maturation [15], a specific form of Cyt-*c* contributes to mitochondria-induced activation of Casp-3, indicating a possible secondary acquisition of this role for Cyt-*c* during evolution; and, more important, the fact that an ubiquitin-conjugating enzyme (dBruce), protects *Drosophila* sperm nucleus from degeneration, indicates the possibility that other regulators of Casp-3 (e.g., the inhibitor of apoptosis proteins, IAPs) may protect cells from a more 'general' destruction [16].

Obviously, all these processes are reminiscent of apoptosis by a number of viewpoints: from conceptual to molecular and morphological. The only difference with a death program is that cell destruction is partial, not complete, and functional to a specific morphological sculpture of the cytosol or nucleus.

But other roles, completely unrelated to apoptosis, can be played by caspases. A particular caspase, caspase-1, plays a key role in the inflammation process [17]; another of these cysteine protease, caspase-14, is a major regulator of keratinocyte terminal differentiation, which is important for the formation of the skin barrier [18].

Casp-3 does not make ecception. Disruption is its standard job, but at least another non-apoptotic function can be achieved by this enzyme. Indeed, caspase-3 may act in synaptic plasticity by specifically destroying dendritic spines upon physiological or pathological (such as excessive mitochondria depolarization in Alzheimer's Disease) signaling [19,20]. In the latter case, the accumulation of toxic Aβ (in its monomeric or oligomeric forms) drives the release of Cyt-*c* from post-synaptic mitochondria, thus activating Casp-3 (Fig. 1, bottom-right corner). This leads to Casp-3 targeting of the phosphatase calcineurin, whose cleavage product is constitutively active, being the main cause of dephosphorylation of the AMPA receptor subunit GluR1; internalization of de-phosphorylated GluR1 within the spine is the main trigger for the consequent spine degeneration [19].

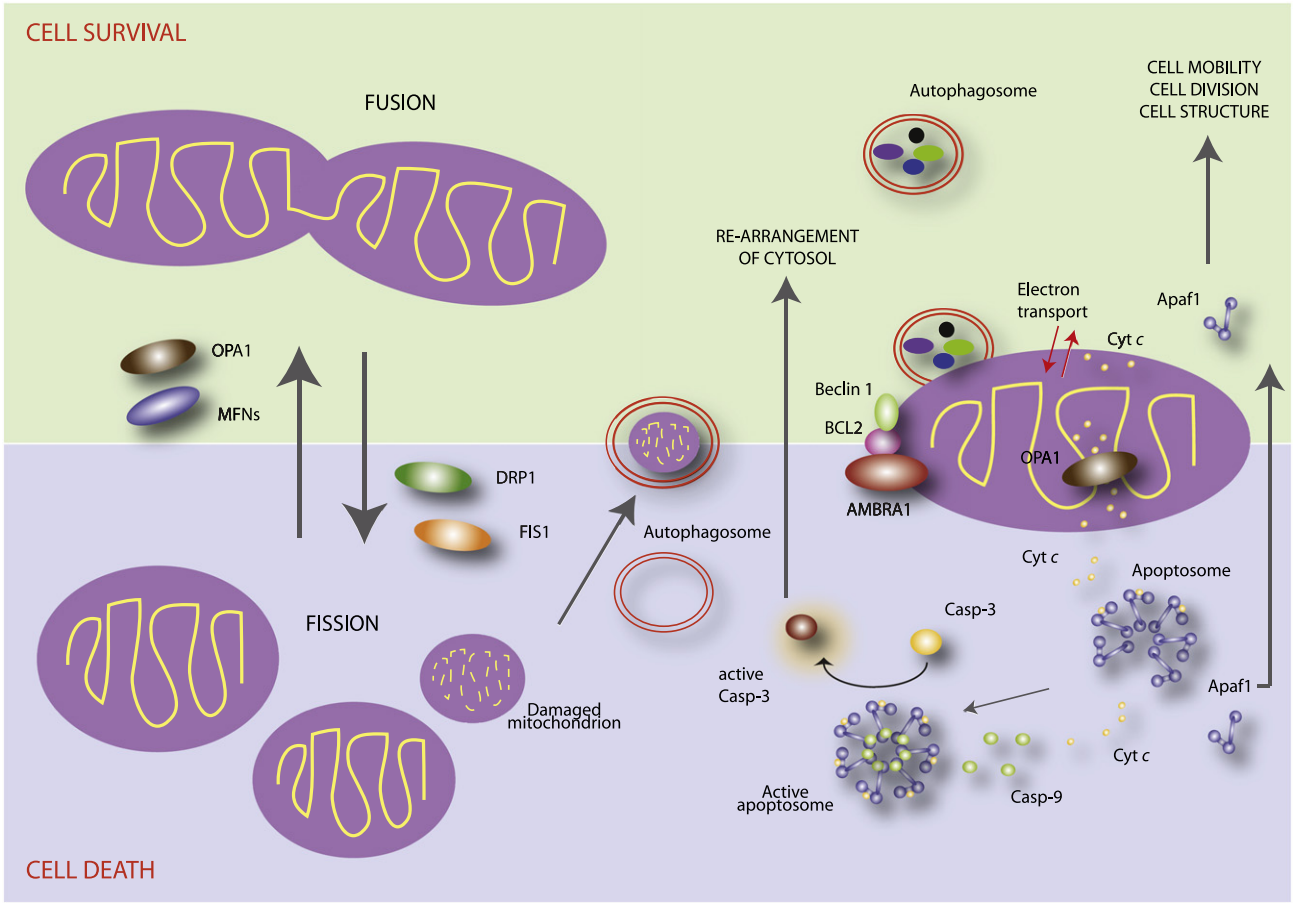

Fig. 1 – Multiple functions for death-molecules in a cell fate. Most of the pathways described in the text are represented. Mitochondria can regulate apoptosis, autophagy and a number of other functions, and their dynamics are strictly related to these regulations.

Is this again a sort of abrupted and local apoptosis, which turned out to be useful only during vertebrate evolution? Is this the consequence of a fine tuning of the enzyme, whose activity remains confined at a synaptic level? Is the evidence that the neuronal spine undergoes a fast decay, solid enough to explain the impossibility for Casp-3 activity to spread to other subcellular compartments? We still do not have answers to all these questions but, given the importance for this finding in the field of human neurodegeneration and CNS development, further investigation will certainly follow. One thing is clear: This activity still depends on mitochondria stimulation and Apaf1 function [19]. Therefore, also in this case, mitochondria are taking the leading decision.

Apaf1, the death adapter, can also ensure a number of regular cell functions

However, the best example of paradoxical effects for apoptotic molecules is given by the Apoptosis protease activating factor (Apaf1), an adapter factor activated (and conformationally regulated) by mitochondria-mediated Cyt-*c* release, enabling Casp-3 activity. Indeed, Apaf1 has an ancient evolutionary-conserved double role: In *C. elegans*, its homolog CED-4 can play pro- and anti-apoptotic functions by means of at least two differentially spliced isoforms [21]. In vertebrates, Apaf1 can translocate to the nucleus when a sublethal stress induces DNA damage. This shift of subcellular localization, by still unknown mechanisms, induces cell cycle arrest [22], and represents a relevant phenomenon in lung tumor prognosis. In this case, Apaf1 acts anyway in a stress response, even though its activity leads cells to less destructive fates.

More surprisingly, Apaf1 was shown to also be a clear *pro-survival* protein (Fig. 1): In a number of cell systems (mouse embryonic proneural cells and fibroblasts, both primary and immortalized), it modulates centrosome formation and cytoskeleton activities, i.e. cell migration, mitotic spindle formation and nuclear morphology maintenance [23]. HCA66, an Apaf1 interactor whose shuttling from the perinuclear envelope to the centrosome is also required for centrosome formation, is the mediator of this stunning pro-survival role.

Of note, both caspase and Apaf1 activity are targets for anti-cancer therapy. Apoptosis induction is widely believed as their only role, and their manipulation is often at the basis of a number of drugs used in clinical oncology [9]. Given their roles in survival, two aspects need now to be evaluated in this context: 1) the possibility that impairing caspase or Apaf1 activity (e.g. by using molecules interfering with their molecular domains, such as IAP analogs, or by disrupting their upstream activation pathway); and 2) the chance that when a tumor, independent of specific

death mutations, is established (e.g. a solid tumor dependent on adhesion or growth molecule dysregulation), their activating-triggers may, indeed, disturb differentiation or, paradoxically, augment the tumor survival rate.

Since, in most cases, death molecules with this double-edge-sword activity are dependent on mitochondrial signalling, and that mitochondria can also be targeted when their activities are disturbed, mitochondrial specific molecules can play important roles in this context. How do mitochondria structure and function deal with the death/survival decision? Which are the classes of molecules that can regulate this switch?

Mitochondrial dynamics at the verge between death and survival

Mitochondria are certainly at the cell crossroads between life and death. They can release molecules such as Cyt-*c* (and other factors, i.e. Endonuclease G, AIF, Smac/Diablo, Htr2/Omi, not discussed in this review) to push a cell towards a deadly end; and we have seen that the crucial molecules in this pathway can play other functions, when mitochondria are not undergoing specific stress or signal response.

Now, how mitochondria do react to stress and consent the release of such factors into the cytosol? Do the molecules, responsible of this release, play alternative roles within the mitochondria?

The morphology of the mitochondrial *cristae* (see above), for example, plays a relevant role on controlling Cyt-*c* and other pro-apoptotic effectors release, as it regulates their compartmentalization and therefore their release within the cytosol [24]. Mitochondria are highly dynamic organelles that continuously move, divide and fuse in a highly regulated fashion. The fusion/fission equilibrium determining the mitochondrial architecture is shifted towards a highly elongated or completely fragmented phenotype by stimuli related to the cell condition (e.g. stress), compartmentalization (e.g. neuronal dendrites), as well as the functional state of the organelles. Growing evidence indicates that regulation of mitochondria shape is a key process for many basic cellular functions, since changes in mitochondrial shape have been related to many key cellular processes such as neurodegeneration, calcium signaling, morphogenesis of specialized cells, regulation of migration of leukocytes, aging and, interestingly, cell cycle and apoptotic cell death (all reviewed in [3]). The balance between the opposing processes of mitochondrial fusion and fission is controlled by a growing family of 'mitochondria-shaping' proteins (Fig. 1). Members of this family either share structural homology with the large GTPases dynamins [25], or “non-conventional” members whose molecular function is less characterized. As reviewed in [3], MFN1 and 2 (mitofusin 1 and 2) are the principal regulators of outer membrane (OM) fusion in mammals; OPA1 (optic atrophy 1) is another key pro-fusion player, anchored to the inner membrane (IM), and mitochondrial division is mostly regulated by the cytosolic DRP1 (dynamin-related protein 1, that needs to be activated by dephosphorylation and recruited to mitochondria), and by another OM protein, FIS1 (mitochondrial fission 1). Complex post-transcriptional mechanisms tightly regulate the activity of OPA1, DRP1 and the other mitochondrial-shaping proteins.

Going back to apoptosis, a set of ultrastructural changes called '*cristae* remodeling' have been identified in the early stages of apoptosis, which are characterized by the widening of the tubular *cristae* junctions, and the fusion of *cristae*. This process is controlled by OPA1, independently from its effect on mitochondrial fusion: in healthy cells, an OPA1 complex in the IM functions as a staple that participates in keeping narrow the tubular junction of the *cristae* [24]. Upon apoptotic stimulus, OPA1 oligomers are disrupted during *cristae* remodeling allowing the release of the Cyt-*c* from the *cristae* stores. DRP1 is also involved in the *cristae* remodeling process, through the BH3-only protein BIK and an undefined mechanism probably independent on its pro-fission activity [26]. Thus, the dynamics and remodeling of mitochondrial *cristae* is a potential control key-point in the mitochondrial apoptotic pathway, and the proteins involved in this regulation play different roles in mitochondrial homeostasis.

Another important and highly regulated mitochondria structural alteration during apoptosis is the massive and reversible fragmentation of the network. This occurs immediately before or around the same time of Cyt-*c* release and before caspase activation. The mechanism by which the apoptotic fragmentation occurs is still unclear and it remains to be established whether it is concomitant to apoptosis or it causes it. DRP1, besides its role in *cristae* modeling, is certainly involved in the process of fragmentation, as a dominant negative mutant of DRP1 protects from fission, release of Cyt-*c*, and death [27]. At present, the concept that the pro-apoptotic function of DRP1 might be independent from its role in mitochondria fission is emerging and debated. It has been recently shown how the role of this protein in fission and apoptosis can be separated by using the small DRP1 inhibitor mdivi-1 [28].

However, the duality between mitochondria shaping, as a function of general mitochondrial homeostasis, and the role of OPA1, MFNs, FIS1 or DRP1, in the death process is not the only duality existing in mitochondria. Several evidence support the view that some ‘mitochondria-shaping’ proteins exert a regulatory role also on the cell cycle, having thus a potentially crucial role on the modulation of tumorigenesis. The depletion of the pro-fission FIS1 in cell lines, for example, not only enhances the elongation of the mitochondria network, but also reduces their proliferation [29]. Furthermore, cell proliferation can be partially restored when a non-cleavable form of OPA1 is over-expressed in Prohibitin 2-deficient cells, known to be defective in cell growth [30]. Finally, it has been demonstrated an anti-proliferative action of MFN2: Its expression is downregulated in vascular smooth muscle cells (VSMCs) under proliferative state conditions, such as atherosclerosis or restenosis; Also, MFN2 over-expression in VSMCs induces growth arrest and inhibits VSMC proliferation [31].

We have discussed how proteins related to mitochondrial dynamics can drive cell death or survival and trigger a relevant control of cell growth by modulating mitochondria structure and function. However, other classes of factors reside at the mitochondria membrane to *dialogue* about the decision to be taken. The BCL2-family members are well known as the main signallers of cell fate decision at the level of mitochondria; They also emerged as regulators of many alternative processes, from DNA damage [32,33], to Ca^{2+} signalling [34–36], to mitochondrial physiology and morphology [37,38]. In recent years, other actors were also added to this scenario: they are autophagy-regulating factors that continuously cross-talk with the BCL2 network to help mitochondria take the right decision.

The cross-talkers: autophagic and apoptotic proteins dialoguing at the mitochondria

Autophagy is a process in which cells self-digest organelles and long-living proteins. This process is controlled by a set of evolutionarily conserved gene (originally discovered in yeast), which are called autophagy-related genes (ATG), and a number of additional regulators, being in a few cases vertebrate-specific (reviewed in [7,8]). The process, by means of specific vesicles termed *autophagosomes* (Fig. 1), targets proteins and organelles to the lysosome in order to maintain cellular homeostasis, thus also regulating both natural turnover of organelles and defective organelles. For example, damaged mitochondria can be specifically removed by autophagosomes in a cell clearance sub-process termed *mitophagy*. The kinases AMPK, mTOR and ULK1, the adapter proteins Beclin 1 (BECN1) and AMBRA1 (activating molecule in beclin 1-regulated autophagy), the ubiquitin-like conjugation factors ATG5 and ATG7, the autophagosome cargo adapter LC3, are among the most relevant players in regulating the upstream signals for autophagosome formation and elongation [7,8]. Noteworthy, in recent years, autophagy has emerged as a subject of great interest for cancer therapy (see below).

A complex relationship between autophagy and cell death exists. Currently, the well-known anti-apototic factor BCL2 is a major player in this context [39]. The pool of BCL2 molecules resident at the endoplasmic reticulum (ER-BCL2) is able, together with NAF-1 (nutrient-deprivation autophagy factor-1), to negatively regulate BECN1-dependent autophagy. By contrast, the mitochondrial pool of BCL2 (mito-BCL2) has been initially described to exert only an anti-apoptotic function. BCL2-regulated apoptotic cell death has been extensively studied. Overexpression of BCL2 promotes a protective effect against a wide range of apoptosis inducers. This anti-apoptotic action derives from the fact that BCL2 neutralizes pro-apoptotic BCL2 family members, preventing mitochondrial membrane permeabilization and consequent cell death [40]. Recently, it has been shown that interference of ATG5 or BECN1 inhibited both autophagy and cell death induced by BCL2 siRNA, indicating that BCL2 knockdown induces autophagic cell death [41]; furthermore, previous studies have demonstrated that BECN1-mediated autophagy is negatively regulated through a direct interaction between BECN1 and ER-BCL2 [39]. The autophagy adapter molecule AMBRA1 is also a partner of BCL2 in mammalian cells and their binding is independent of BECN1 [42]. These findings indicate that AMBRA1 and mito-BCL2 bind BECN1 on the same site, AMBRA1 thus competing with mito-BCL2 to bind BECN1. As a consequence, AMBRA1/BECN1 interaction increases in the mitochondrial fraction whereas AMBRA1/BCL2 binding is disrupted. Altogether, these results lead to a model in which, under normal conditions, a pool of AMBRA1 is docked by BCL2 at the mitochondria, inhibiting its autophagic function; after autophagy induction, this mitochondrial pool of AMBRA1 separates from mito-BCL2 and increases its binding to BECN1 in order to favour the autophagic program. A possible understanding of this unexpected coupling of AMBRA1 with mito-BCL2 comes from recent work reporting that mitochondrial-derived membranes are utilized during autophagy, and that autophagosome formation is dependent on the ER-mitochondria connection [43], a process modulated by MFN2 [44]. Until now, it was established that autophagosome formation occurs at the rough ER on omegasome structure (reviewed in [45]). Taking into consideration this new 'mitochondrial model', it can be hypothesised that the mitochondrial pool of AMBRA1 can also be involved in autophagosome formation at the mitochondria. In addition, following autophagy induction, mitochondria fuse, becoming elongated tubules that are protected from autophagosomal degradation. This mechanism helps mitochondria to maximize energy production and to participate to autophagosomal membrane formation [46,47]. Since BCL2 family members (i.e., BAX and BAK) are also involved in the control of mitochondrial morphology [38], one can speculate that also the surprising AMBRA1/mito-BCL2 *couple* might play a role in autophagy-related mitochondrial dynamics. Other factors contribute to this open dialogue: the BCL2 like protein $BCLX_L$ can also interact with BECN1 at the ER; pro-autophagic ATG5 is cleaved by calpains upon enduring cell stress, this cleavage converting it in a mitochondrial pro-death factor; p53, one of the main regulator of the pro-apoptotic stress response at a transcriptional and cytosolic level, regulates autophagy by means of its nuclear and cytosolic isoforms (reviewed in [7]).

In sum, the choice of a cell between death and survival is based on a very complex network of molecular activities, often centered on the mitochondria. Autophagy and apoptosis are key processes at the endpoint of this dialogue and double-side molecules are continuously switched from one role to another, by their interactors or by specific post-translational modifications. The identification of these modifications and their manipulation may represent a new frontier in biomedicine.

Coda

Within the cell, pro-death and pro-life pathways can be modulated by several inputs, often taking advantage by the dual or multiple roles of proteins which have been considered only as death-regulators for a long while. The interface with the subcellular components, in this context, is often the mitochondrion. There the action takes place. Also, permeabilised mitochondria can be removed by mitophagy to prevent cell death, in a sort of continuous loop. In other subcellular domains, proteins can be sequestered and kept dormant, when the cell is undergoing a relatively stress-free phase. One of these domains is the cytoskeleton. Interestingly, protein belonging to the network of apoptosis (BIM), autophagy (AMBRA1) and mitochondrial dynamics (DRP1) regulation can be tethered by the dynein motor complex subunit DLC1 (dynein light chain 1) at the cytoskeleton docking site, and brought into action by phosphorylation events (mediated by key-kinases such as JNK or ULK1 or key-phosphatases, such as calcineurin) [48]. This regulation can be exerted in an one-by-one fashion or by mobilizing more proteins together, adding another level of synergic control to the system.

As emphasized before, this network of factors is of the highest importance in biomedicine. The dual role of Casp-3 has already been shown as a central switch in neurodegeneration or neurodevelopment in mammals. Apaf1, the major component of the apoptosome, is clearly playing other functions when not activated by the mitochondria; so many cell functions depend on its activity, that a broad spectrum of human diseases can result from its malfunctioning. However, due to the importance of the stress response in cancer, the choice between death and survival, operated on the mitochondria by most of the proteins we have discussed above, is clearly crucial in human tumors and in their sensitivity

to treatment. Human cancer cells are growing by evading cell death. Common strategies based on the modulation of BCL2-family members to treat cancer, should take now into account the importance of these proteins in regulating autophagy.

Also, mitochondrial dynamics and their modulation might be a crucial instrument for cancer cells in favor of a major functionality, an increased and efficient proliferation, as well as a manner to efficiently escape the programmed cell death machinery, the main defensive mechanism of the organism against tumorigenesis.

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