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Review

Intersection of mitochondrial fission and fusion machinery with apoptotic pathways: Role of Mcl-1

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Mitochondria actively contribute to apoptotic cell death through mechanisms including the loss of integrity of the outer mitochondrial membrane, the release of intermembrane space proteins, such as cytochrome c, in the cytosol and the caspase cascade activation. This process is the result of careful cooperation not only among members of the Bcl-2 family but also dynamin-related proteins. These events are often accompanied by fission of the organelle, thus linking mitochondrial dynamics to apoptosis. Emerging evidences are suggesting a fine regulation of mitochondrial morphology by Bcl-2 family members and active participation of fission–fusion proteins in apoptosis. The debate whether in mitochondrial morphogenesis the role of Bcl-2 family members is functionally distinct from their role in apoptosis is still open and, above all, which morphological changes are associated with cell death sensitisation. This review will cover the findings on how the mitochondrial fission and fusion machinery may intersect apoptotic pathways focusing on recent advances on the key role played by Mcl-1.

Introduction

Mcl-1 protein is overexpressed in many types of human tumours [e.g., leukemia (Derenne et al., 2002), breast (Ding et al., 2007), central nervous system (Krajewski et al., 1997), prostate (Krajewska et al., 1996) and ovary cancers (Brotin et al., 2010)) and correlates with disease grade and survival predicting response to anti-cancer therapies (Backus et al., 2001; Cho-Vega et al., 2004; Saxena et al., 2004; Wuilleme-Toumi et al., 2005). Therefore, it has been thoroughly studied for the past 20 years. Mcl-1 is a member of the Bcl-2 family, an important class of proteins that finely regulates programmed cell death through its pro- and anti-apoptotic factors (Chipuk and Green, 2008; Youle and Strasser, 2008; Chipuk et al., 2010; Llambi and Green, 2011; Shamas-Din et al., 2013; Czabotar et al., 2014; Moldoveanu et al., 2014; Green and Llambi, 2015). Members of the Bcl-2 family share a common trait that is a highly conserved sequence named Bcl-2 homology (BH) 1–4 domains (Moldoveanu et al., 2014).

BH1, BH2 and BH3 motifs form a hydrophobic cleft involved in cell death and survival pathways; BH4 domain is critical for anti-apoptotic function, whereas BH3-only domain is critical for pro-apoptotic activities. Moreover, many Bcl-2 family members own a transmembrane (TM) domain with anti-apoptotic function at the C terminus. This TM domain plays a key role in anchoring intracellular membranes such as nuclear envelope, endoplasmic reticulum (ER) and the outer mitochondrial membrane (OMM) (Krajewski et al., 1993).

This review will focus on Mcl-1 isoforms generated by the alternative splicing (AS) of the gene. The full-length isoform of Mcl-1 (Mcl-1L) is a TM protein mostly localised at the OMM, whereas the short isoform (Mcl-1S) has a cytosolic localisation (Bingle

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Abbreviations: AS, alternative splicing; BH, Bcl-2 homology; Drp1, Dynamin-related protein 1; ER, endoplasmic reticulum; hFis1, Human fission protein 1; IMM, inner mitochondrial membrane; Mcl-1ES, myeloid cell leukemia 1 extra short form; Mcl-1L, myeloid cell leukemia 1 full-length form; Mcl-1S, myeloid cell leukemia 1 shot form; Mff, mitochondrial fission factor; Mfn1, mitofusin 1; Mfn2, mitofusin 2; MOMP, mitochondrial outer membrane permeabilisation; OMM, outer mitochondrial membrane; OPA1, optic atrophy 1; PEST, proline (P), glutamic acid (E), serine (S) and threonine (T); TM, transmembrane domain.

et al., 2000). A third extra short isoform (Mcl-1ES) has also been identified. It localises to the cytoplasm and mitochondrial membranes where it executes pro-apoptotic activities (Kim et al., 2009).

In healthy cells, mitochondria continuously divide and fuse together to form an interconnected and dynamic network (Rizzuto et al., 1996) sustained by a well-established molecular machinery participating in mitochondrial integrity. In the past, it has been discovered that changes in mitochondrial morphology influence pivotal cellular functions such as calcium signalling (Rizzuto et al., 1998; Rimessi et al., 2008; Giorgi et al., 2012), reactive oxygen species generation (Yu et al., 2006; Marchi et al., 2012), neuronal plasticity (Chang and Reynolds, 2006), muscular atrophy (Zuchner et al., 2004) and senescence (Lee et al., 2007). Moreover, mitochondrial morphology modifies the energetic state of the organelle and plays a key role in programmed cell death (Suen et al., 2008; Bonora et al., 2015). Growing evidence has shown that Bcl-2 family members are also involved in mitochondrial dynamics in healthy cells (Delivani et al., 2006, Frezza et al., 2006, Ryu et al., 2012, Li et al.,2013). Furthermore, connecting the processes of fission and fusion to apoptosis and vice versa, dynamin-related proteins are directly and indirectly implicated in programmed cell death, suggesting a finely regulated connection between these events (Lee et al., 2004).

Concerning the relationship between mitochondrial morphology and apoptosis, a debate is still open and the state of the art is being updated. For example, regarding dynamin-related-protein 1 (Drp1), several studies have revealed its involvement in apoptosis through interaction with Bax and Bak to increase OMM permeability (Cassidy-Stone et al., 2008). Furthermore, Frank et al., using a dominant-negative mutant to inhibit Drp1 (Drp1K38A), have prevented mitochondrial fission, loss of mitochondrial membrane potential and release of cytochrome c, thus blocking the on-going apoptotic pathway (Frank et al., 2001). On the other hand, available data suggest that inhibition of Drp1 only results in a delay in cytochrome c release without reducing cell death (Parone et al., 2006; Estaquier and Arnoult, 2007).

A question that still remains open about the connection between apoptosis and mitochondrial dynamics is which one occurs first in the case of a cause–effect relationship. It is certain that when cells undergo programmed cell death, a change occurs in mitochondrial morphology, but it is not clear whether these two events are simultaneous or not. In the past 5 years, interesting data obtained from the study of Mcl-1 in apoptosis have shed light on its involvement in mitochondrial dynamics (Perciavalle et al., 2012; Varadarajan et al., 2013, 2015; Morciano et al., 2016) suggesting that another Bcl-2 family member plays a key role in regulation of fission and fusion dynamics.

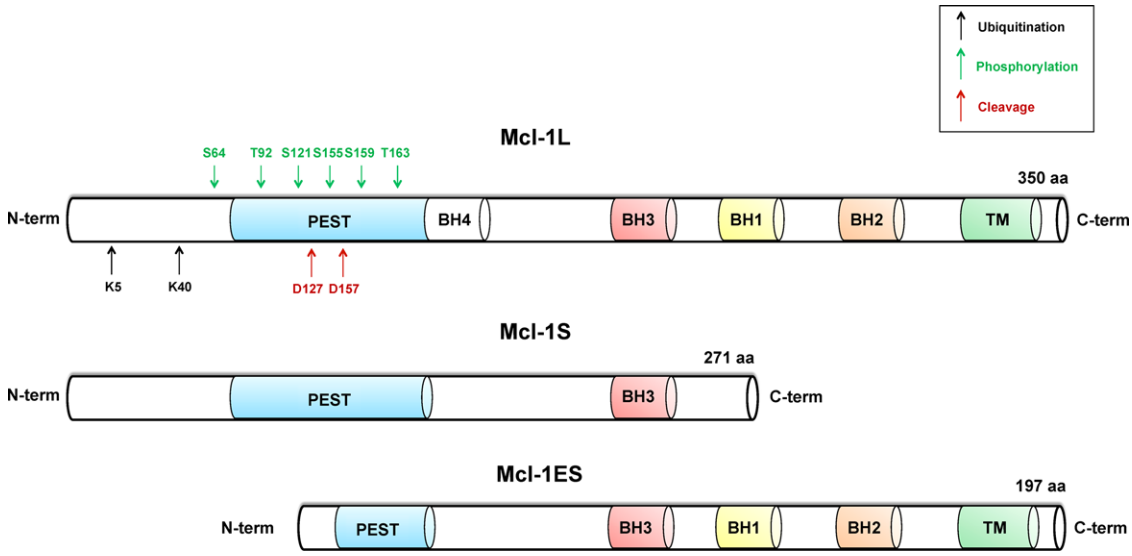
Mcl-1: Functional localisations

Although Mcl-1 has been object of several reviews (Warr and Shore, 2008; Akgul 2009), no one has discussed its AS-derived isoforms and functional localisations. Indeed, countless reports focused on its post-translational modifications, such as cleavage, phosphorylation and ubiquitination that tightly regulate Mcl-1 activity (see the following references).

A unique feature of Mcl-1 is a long N-terminus domain involved in the cleavage by caspases and granzyme B at two aspartate sites (Herrant et al., 2004; Han et al., 2005). This region contains PEST sequences (Kozopas et al., 1993; Akgul et al., 2000) enriched in proline (P), glutamate (E), serine (S) and threonine (T) residues and includes six different phosphorylation sites. Depending on which serine or threonine residue is phosphorylated, it will lead to an opposite consequence, that is protein stabilisation or degradation (Inoshita et al., 2002; Domina et al., 2004). Lysine residues of this region instead are poly-ubiquinated by different ubiquitin-ligases, leading to rapid proteasomal degradation of Mcl-1 protein (Zhong et al., 2005). Worth of notice, in this region the so long discussed and controversial BH4 domain resides. Mcl-1 like the other Bcl-2 family members shares highly conserved sequences in a maximum number of four (Moldoveanu et al. 2014). Unlike the other three BH domains, BH4 was initially found only in anti-apoptotic protein such as Bcl-2, Bcl-xL and Bcl-w where it is crucial for their anti-apoptotic activity, but later it was also observed in the effector proteins BAK and BAX (Chipuk et al., 2010) and finally detected in Mcl-1 (Kvansakul et al., 2008). This BH4 motif is characterised by $\varphi_1\ \varphi_2\ X\ X\ \varphi_3\ \varphi_4$ sequence, where X can be any amino acid, $\varphi_1\ \varphi_2$ and φ_4 are aliphatic residues and φ_3 is an aromatic residue (Figure 1). If that trait is clear in the sequences of Bcl-2, Bcl-x and Bcl-w, in Mcl-1 is more

Figure 1 | Molecular features of Mcl-1 protein isoforms

Functional domains and post-translational modification sites of ubiquitination, caspase cleavage and phosphorylation.
Mcl-1L: NP_068779, Mcl-1S: NP_877495, Mcl-1ES: NP_001184249
Abbreviations: BH: Bcl-2 homology domain; PEST: proline, glutamic acid, serine and threonine residues; TM: transmembrane domain.



controversial. Nevertheless, structure alignments illustrate the presence of the above motif in α1 helix of Mcl-1, Bak, Bax and viral Bcl-2-like proteins (Kvansakul et al., 2008). The other three BH domains form a hydrophobic cleft crucial for dimerisation with pro-apoptotic family members and for their activity inhibition, whereas BH3 domain is essential for pro-apoptotic function.

AS generates three distinct isoforms, which acquire a specific subcellular localisation (Table 1) and consequently different functions: (i) full-length form (Mcl-1L), a TM protein with anti-apoptotic activities mostly localised in the OMM, has a full size of 350 residues and a molecular weight of 40 kDa, as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It contains three putative BH domains, which are required to heterodimerise with other family members and a TM domain in the C-terminal portion; the N-terminal portion (as previously reported) is rich in PEST regions, characteristic of proteins with a short half-life (Akgul et al., 2000); (ii) the short form Mcl-1S (molecular weight: 32 kDa) possesses pro-apoptotic functions and cytosolic localisation (Bae et al., 2000) and derives from exon 2 skipping generating a shorter form of 271 amino acids lacking BH1, BH2 and TM domains due to a shift in the reading frame; (iii) the extra-short Mcl-1ES is composed of only 197 residues and localised both to cytoplasm and mitochondrial membranes (Kim et al., 2009) to execute its pro-apoptotic function. It migrates around 25 kDa when analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It lacks a portion of exon 1 that removes 53 amino acids corresponding to the PEST region maintaining all three BH and the TM domains (Figure 1).

In 1995, Yang and colleagues were the first to try to define the intracellular distribution of Mcl-1. In their preliminary experiments, the protein was largely localised to the cytoplasm and small dots appeared in the nucleus, but it was also associated with membranes through its carboxyl hydrophobic tail. Nevertheless, their further experiments showed a remarkable localization of Mcl-1 to mitochondria upon detection by various methods such as immunofluorescence microscopy, subcellular fractionation and affinity purification with anti-Mcl-1 antibody.

Mcl-1L at the OMM executes its anti-apoptotic peculiarity and cell survival by binding and sequestering pro-apoptotic protein such as Bak and Bax as well as BH3-only proteins preventing the beginning

Table 1 | Mcl-1 isoforms, localisation and main functions

Isoform type	Localisation	Function	Refs.
Mcl-1L	Intracellular membranes (mitochondria and ER)	Binds pro-apoptotic proteins of Bcl-2 family suppressing apoptosis	Thomas et al. (2010)
	Nucleus	Regulates cell-cycle progression by interaction with PCNA	Fujise et al. (2000)
		DNA damage response by means of regulation of Chk1	Jamil et al. (2008)
	Intracellular membranes and nucleus	Embryonic and neuronal development	Rinkenberger et al. (2000)Arbour et al. (2008)
		Promotes the survival of haematopoietic cells	
Mcl-1S	Cytosol and ER	Acts like a BH3-only protein promoting apoptosis	Bae et al. (2000)
Mcl-1ES	Cytosol and mitochondria	Suppresses Mcl-1L anti-apoptotic function promoting mitochondrial cell death by its own oligomerisation within the mitochondrial membrane	Kim et al. (2009)Kim and Bae (2013)

Abbreviations: ER: endoplasmic reticulum; Refs: references.

of the intrinsic pathway (Thomas et al., 2010). Moreover, Mcl-1L behaves like a development-specific protein since its deletion in murine embryos promotes a peri-implantation embryonic lethality (Rinkenberger et al., 2000). In 2008, it was demonstrated how this protein acts as key regulator of apoptosis during neuronal development in the survival of newly committed neurons and the regulation of injury-induced neuronal cell death (Arbour et al., 2008). Mcl-1 has an important role in promoting the survival of haematopoietic cells, maintaining the development of lymphocytes and early haematopoietic progenitors. Furthermore, Steimer et al., deleting anti-apoptotic Mcl-1 in the myeloid lineage, discovered that it is fundamental for neutrophil development whereas it is dispensable for the development of monocytes and macrophages (Steimer et al., 2009).

Mcl-1S, a splicing variant of Mcl-1 displaying opposite functions, was isolated from human placenta and human myeloid leukemia cells. This short splicing variant lacks 248 nucleotides, and this deletion causes a shift in the open reading frame leading to the complete loss of BH1, BH2 and TM domains and an altered C-terminal sequence. Thus, it cannot be associated with mitochondrial membranes and mainly localises to cytosol (Bae et al., 2000). This shorter protein does not interact with any other Bcl-2 family member except for Mcl-1L, generating heterodimers. This dimerisation can neutralise either the pro-apoptotic function of Mcl-1S enhancing survival or, vice versa, it induces apoptosis by binding and inhibiting Mcl-1L. Therefore, the balance of these splicing results can influence cell viability (Bae et al., 2000).

In 2009, Kim and co-workers identified another new splicing variant of human Mcl-1: Mcl-1ES, generating from the truncation of exon I at a non-canonical GC–AG donor–acceptor pair, this isoform lacks PEST motif but maintains the BH and TM domains (Kim et al. 2009). This variant localises to the cytoplasm and mitochondrial membranes (with the help of Mcl-1L) to execute apoptosis by interacting with Mcl-1L and repressing it. Later, the molecular mechanism underlying Mcl-1ES pro-apoptotic activity was elucidated. It consists of a Bax/Bak-independent pathway mediated by a unique interaction with Mcl-1L, facilitating its proper localisation to the mitochondria. Mcl-1ES forms mitochondrial oligomers followed by mitochondrial outer membrane permeabilisation (MOMP) and cytochrome c release in a Mcl-1L-dependent manner (Kim and Bae, 2013). Interestingly, the majority of cytochrome c resides in mitochondrial cristae, dynamic structures found in the inner mitochondrial membrane (IMM). This raises the possibility that cristae remodelling may also be involved in regulation of cytochrome c egress from mitochondria following MOMP. BH3-only molecules and activated Bax and Bak can also mediate cristae remodelling without

requiring MOMP [see Tait and Green (2013) for review] (Yamaguchi et al., 2008). For example, treatment of mitochondria with tBid, a BH3 protein, results in cristae remodelling and consequent release of cytochrome c from the cristae in a process that does not require the tBid BH3 domain (Scorrano et al. 2002).

Updating mitochondrial dynamics concept

The term mitochondrion, coined by Benda in 1898, comes from two Greek words that mean threads and grains. Although mitochondria have been thought to be static for many years, currently we know they are able to fuse and divide for a specific purpose and undergo redistribution in the intracellular space. In particular, their distribution as a network allows to join together at multiple contact sites to form specific domains with other organelles as mitochondria–ER associated membranes (Patergnani et al., 2011; Marchi et al., 2014; Giorgi et al., 2015) resulting in important consequences for cell fate (La Rovere et al., 2016).

In recent years, thanks to developments in the live-cell imaging and electron tomography, we have been able to observe the dynamic morphology and distribution of mitochondrial network (Dimmer and Scorrano, 2006). In healthy eukaryotic cells, the keeping of the overall mitochondrial shape depends on the balance between the opposite processes of mitochondrial fusion and fission. While an unbalanced fission leads to organelle fragmentation, an unbalanced fusion leads to its elongation. Each of these excesses reflects not only simple morphological changes but also signals governing cell lifespan. Indeed, mitochondrial dynamics govern a variety of functions, encompassing mitochondrial distribution and inheritance (Ishihara et al., 2009), remodelling of mitochondria during developmental processes (Hales and Fuller, 1997), migration of white blood cells (Campello et al., 2006), coordination of cell death programs (Szabadkai et al., 2006; Morciano et al., 2016), removal of damaged organelles by autophagy (Twig et al., 2008) and adaptation to the metabolic conditions of the cell (Tondera et al., 2009).

In healthy metabolically and respiratory active cells mitochondrial network appears interconnected, whereas small and fragmented mitochondria prevail in quiescent and respiratory inactive cells (Chen et al., 2005). Mitochondrial fusion enables efficient mixing of mitochondrial content which is advantageous under conditions of high-energy demand and is critical for maintenance of mitochondrial functions and to counteract cellular ageing (Balaban et al., 2005; Sato et al., 2006) (Figure 2).

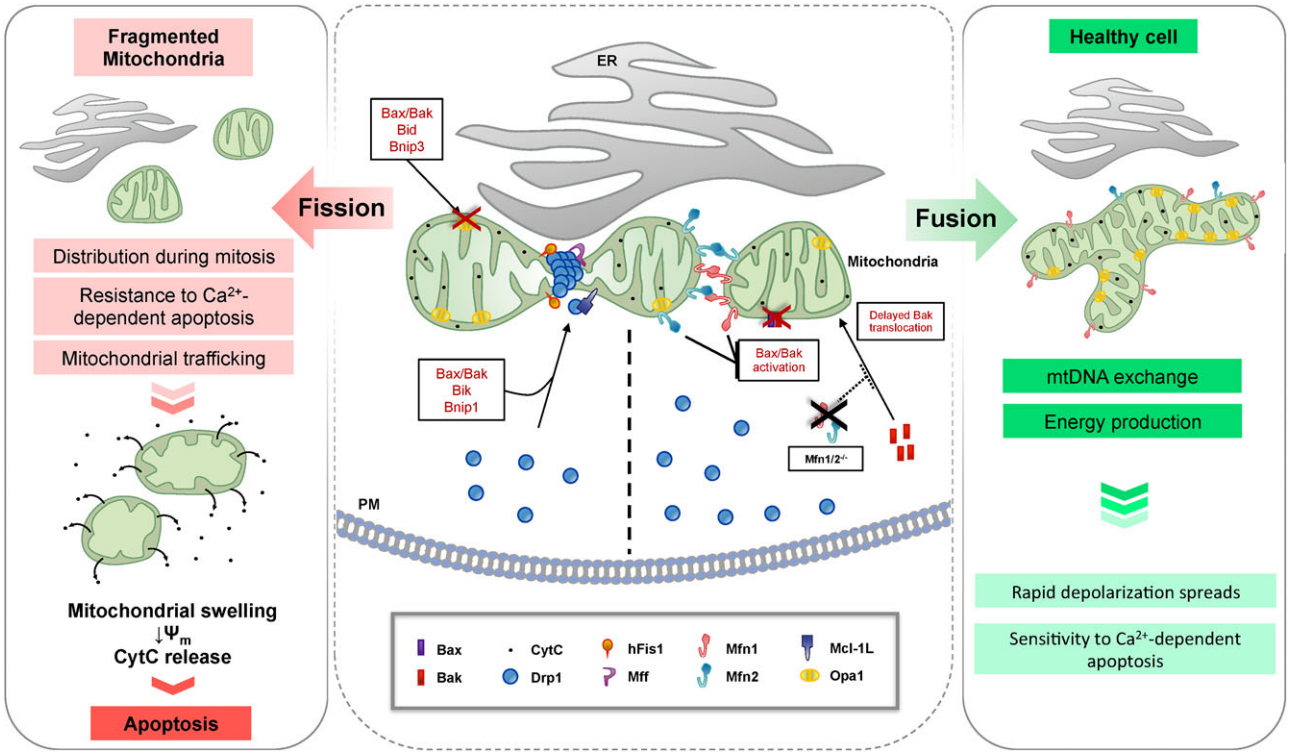
Mitochondrial division is crucial for cell division, release of cytochrome c and other intermembrane space proteins during apoptosis, generation of transportable mitochondrial units for movement along the cytoskeleton (Detmer and Chan, 2007) and it also eliminates damaged organelles from the mitochondrial network by autophagy (Twig et al., 2008). Alterations in the balance of the fusion/fission machinery have been observed in much pathology including neurodegenerative diseases (Chen et al., 2007; Costa et al., 2010), cancer (Wang et al., 2015; Morciano et al., 2016) and in ischemia-reperfusion injury to the heart (Cereghetti et al., 2008).

The core of this machinery consists of several GTPases, highly conserved during evolution with structural homologies to dynamin proteins. They possess several identifiable regions: a large highly conserved GTPase domain that participates in fusion, two helical regions named middle domains and a GTPase effector domain that can mediate protein–protein interactions, GTP hydrolysis and membrane remodelling even if most of these mitochondria-shaping proteins are integral membrane proteins (Liesa et al., 2009).

In mammalian cells, the major player of mitochondrial fission is the soluble Drp1. Drp1 and its function are conserved in all eukaryotes (Smirnova et al., 2001), including plants (Logan, 2010). It participates in the fragmentation of mitochondria, peroxisomes and ER (Schrader, 2006). Drp1 has a cytoplasmic localisation and reversibly associates with OMM at sites of mitochondrial division, where the fission occurs preferentially at ER-contact regions (Friedman et al., 2011). In particular, it shows the capacity to constrict membranes, upon assembly, into spirals and induce mitochondrial scission upon GTP hydrolysis (Frohlich et al., 2013) (Figure 2). The cytosolic expression makes Drp1 easily subject to several post-translational modifications that modulate recruitment, assembly, activity and stability to OMM in response to various cellular cues. In this scenario, the modulation of phosphorylation state of the

Figure 2 | Mitochondrial dynamics overview

Mitochondria are double membrane organelles with an intermembrane space (IMS) that separates the outer and the inner membranes. The main proteins involved in modulation of mitochondrial fusion (Mfn1, Mfn2, OPA1) and fission (hFis1, Mff) are principally TM proteins, except for Drp1 that requires some molecular anchors to bind to the OMM. When the balance hangs on the side of the fusion (right panel), the increased mitochondrial interconnection and elongation enhance oxidative phosphorylation and contact sites with the ER leading to rapid mitochondrial depolarisation and an increased sensitivity to Ca^{2+}-dependent apoptosis. Increased fission or reduced fusion events give a fragmented mitochondrial network (left panel) as observed during mitosis or upon severe stress (*i.e.*, hypoxia, apoptotic insults). Under apoptotic stimulation, the action of some Bcl-2 family members (Bax/Bak, Bik and Bnip) at the OMM induces Drp1 translocation and OPA1 oligomers disruption (via Bax/Bak Bid and Bnip3), increasing fragmentation and finally apoptosis. On the other hand, a fragmented network can protect cells from Ca^{2+}-dependent apoptosis and rapid depolarisation waves. Mfn1 and Mfn2 interfere with Bax/Bak activation (but not with the mitochondrial anchoring) and cytochrome c release.



conserved Ser637 of Drp1 represents the signal that controls protein translocation between mitochondria and the cytoplasm (Cribbs and Strack, 2007). Scorrano and co-workers have shown that, following mitochondrial depolarisation, a rise in cytosolic Ca^{2+} activates the cytosolic phosphatase calcineurin that, in turn, dephosphorylates Drp1 on Ser637, regulating its translocation to mitochondria and defragmentation (Cereghetti et al., 2008). This process of calcineurin-mediated Drp1 activation is crucial in ischemia reperfusion damage of the heart. Conversely, protein kinase A, after cyclic AMP activation, phosphorylates this residue, leading to the elongation of mitochondria and confers resistance to various pro-apoptotic insults (Cribbs and Strack 2007).

Drp1 translocation to mitochondria is a process that requires the binding to many adaptors on the OMM where the main component of this class of proteins is human fission protein 1 (hFis1).

hFis1 is anchored to the OMM via a single C-terminal TM domain. Drp1 and hFis1 interact transiently, and genetic modifications of (TPR)-like domain abolish mitochondrial fragmentation (Yu et al., 2005). Other important Drp1 "receptors" are

mitochondrial fission factor (Mff) (Loson et al., 2013), mitochondrial dynamics proteins 49 and 51 (MiD; MiD49 and MiD51) (Palmer et al., 2011) and Mcl-1L (Morciano et al., 2016) that has been recently discovered.

Mitochondrial fusion consists of reactions that involve both OMM and IMM rearrangements, revealing that mitofusins are required for outer membrane fusion whereas the dynamin-related protein optic atrophy 1 (OPA1) is essential for inner membrane processing (Meeusen et al., 2004). Mitofusins (Mfn1 and Mfn2) are two conserved highly homologous dynamin-related GTPases with a large N-terminal GTPase domain, two hydrophobic heptad repeats and two TM domains that insert them in the OMM (Rojo et al. 2002). Both the N- and C-terminal regions of these proteins protrude from the OMM into the cytosol, and this U-shaped TM domain allows to distinguish these proteins from other members of the dynamin family that are soluble and reversibly associated with membranes. As showed by Chen and co-workers, downregulation of both Mfn1 and Mfn2 leads to greatly reduced levels of mitochondrial fusion with a highly fragmented mitochondrial population in mutant cells caused by on-going mitochondrial fission (Chen et al., 2005). Mfn1 and Mfn2 appear to play slightly different roles in mitochondrial fusion, but both molecules are necessary to maintain a steady-state level of tubular network.

Since when the localisation of Mfn2 at ER membranes highlighted a possible role as a bridge between ER–mitochondria faces (de Brito and Scorrano, 2008), increasingly pathological roles have been suggested for Mfn2 impairment such as obesity, diabetes (Hernandez-Alvarez et al., 2010) and leptin resistance in the hypothalamus (Schneeberger et al., 2013). These disorders were associated with mitochondrial dysfunctions and ER stress following loss of mitochondria–ER contacts. Two independent and convincing recent studies (Cosson et al., 2012; Filadi et al., 2015) appear to challenge this view, showing how Mfn2 ablation or silencing increased the close contacts between the two organelles preventing an excessive, potentially toxic proximity between the two organelles.

Only Mfn1, but not Mfn2, is required in the interplay necessary for fusion of IMM mediated by the inner membrane OPA1. The mammalian OPA1, which is ubiquitously expressed, is coded by ORF comprising 30 alternatively spliced exons that lead to eight mRNAs (Delettre et al., 2001).

Various stress conditions, including mitochondrial membrane depolarisation and apoptosis, induce a raise of OPA1 processing into short forms (Ishihara et al., 2006; Griparic et al., 2007). The IMM fusion seems to require the presence of both long (uncleaved) and short (cleaved) forms of OPA1. This protein is also crucial for maintenance of the structure of IMM. As a matter of fact, its downregulation results in alteration of cristae morphology (Olichon et al., 2003) and the cristae junctions (Frezza et al., 2006). Instead, a mild OPA1 overexpression *in vivo* protects mice from denervation-induced muscular atrophy, ischemic heart and brain damage, as well as hepatocellular apoptosis, ameliorating mitochondrial cristae and blunting damage of highly metabolically active organs in response to apoptotic, necrotic and atrophic stimuli (Varanita et al., 2015).

Understanding mitochondrial structure–function relationships in apoptosis: Bcl-2 family, Mcl-1 and dynamin-related proteins

The eternal bond between structure and function is a recurring event in biology. In mitochondria, shape and function enhance this duet in controlling the route of a cell from its birth until death (Rimessi et al., 2015). Indeed, the structure of mitochondria has to sustain the whole energy state of a cell (Bonora et al., 2012) to organise and forward various stimuli from the cytosol, maintain the cross-talk with other organelles (Varadarajan et al. 2012) and facilitate the access to products of mtDNA expression. Not only shape influences crucial cell functions, but also vice versa, a behaviour totally wired to be responsive to cellular needs.

In the past years, studying both mitochondrial fission and fusion proteins, they appeared to modulate apoptosis through activities that were apparently distinct from their roles in mitochondrial morphology but which involved, most of the times, members of the Bcl-2 family (Table 2). Simultaneously, there was increasing evidence for other non-apoptotic roles of the Bcl-2 family, ranging from ionic homeostasis and autophagy to the regulation of fission–fusion dynamics in subcellular

Table 2 | Bcl-2 family members and dynamin-related proteins functional relationship in apoptosis pathways

Dynamin-related protein	Bcl-2 family partner	Function	Coupled to apoptosis	Refs.
Drp1	BNIP1	Increases Drp1 translocation rate from cytosol to mitochondria	Yes	Ryu et al. (2012)
	Bax/Bak	Drp1 stabilisation to mitochondria	Unknown	Wasiak et al. (2007)
	Bcl-XL	Forms a complex with Drp1, Mff and clathrin enhancing vesicle endocytosis during synaptic stimulation	No	Li et al. (2013)
	Bax	Translocates to OMM, causes the accumulation of mitochondrial Drp1 and Mfn2 inducing apoptotic fragmentation of mitochondria	Yes	Karbowski et al. (2002)
	Bik	Induces Drp1 recruitment, remodelling of mitochondrial cristae and cooperates to cytochrome c release	Yes	Germain et al. (2005)
	Mcl-1L	The reduction of Mcl-1L at the OMM prevents the interaction and consequently the normal physiologic functions of Drp1, producing a persistent hyperfused mitochondrial state	Yes	Morciano et al. (2016)
	Bid	$Drp1^{-/-}$ MEF are less susceptible to apoptosis by BID, showing a blunted cytochrome c release	Yes	Oettinghaus et al. (2016)
hFis1	Bax	hFis1 induces Bax translocation to mitochondrial where it creates foci with Drp1	Yes	Karbowski et al. (2002)
Mff	Bcl-XL	Forms a complex with Drp1, Mff and clathrin enhancing vesicle endocytosis during synaptic stimulation	No	Li et al. (2013)
Mfn1	Bax	Mfn1 inhibits the amino-terminal activation but not the mitochondrial translocation of Bax apoptosis in a GTPase- dependent manner	Yes	Ryu et al. (2012)
Mfn2	Bax/Bak	In healthy cells they induce mitochondrial fusion by activating assembly of the large GTPase Mfn2 and change Mfn2 submitochondrial distribution	No	Karbowski et al. (2006)
		Mfn2 interferes with Bax activation and cytochrome c release	Yes	Neuspiel et al. (2005)
	Bak/Bid	Double knock-out of Mfn1 and Mfn2 delayed Bak translocation to OMM that in turn slowly activates Bid-induced OMM permeabilisation and cytochrome c release	Yes	Weaver et al. (2014)
Opa1	Bid	Bid widens cristae junctions and disrupts OPA1 oligomer favoring remodelling of the cristae	Yes	Frezza et al. (2006)
	Bax/Bak	Bax/Bak and BH3-only proteins induce Opa1-dependent cristae remodelling	Yes	Yamaguchi et al. (2008)
	Bnip3	Bnip3 interacts with Opa1 triggering the complex disruption in a Bax- and/or Bak-dependent manner, leading to mitochondrial fragmentation and apoptosis.	Yes	Landes et al. (2010)

Abbreviations: BNIP1: BCL2/adenovirus E1B 19kDa interacting protein 1; Refs: references.

organelles, including the endoplasmic reticulum and mitochondria.

The first evidence that mitochondrial dynamics interact with cell death pathways, derived from Frank and co-workers who have shown that Drp1 regulates mitochondrial fission and swelling during apoptosis (Frank et al., 2001). The expression of the dominant-negative Drp1 inhibits the apoptosis-associated induction of OMM fragmentation and IMM depolarisation, thereby counteracting mitochondrial fission during cell death. The close relationship between morphology and sensitivity to cell death is further confirmed by depletion of hFis1 that produces greater resistance to apoptosis than Drp1 as inhibitory targets. This study has revealed an additional, major feature, that is the multi-step action of the dynamin-like proteins in the apoptotic pathway (Lee et al., 2004).

Two key studies have shown a role for Bax and Bak both in the regulation of mitochondrial fusion

in healthy cells (Karbowski et al., 2006) and the participation in mitochondrial fission during apoptosis (Brooks et al., 2007). Therefore, modulators of apoptosis have the capacity to interact and modulate the components of mitochondrial dynamics and vice versa blending the two mechanisms, even if it is not always accepted. Assuming that mitochondrial dynamics could be cell cycle-dependent and quite sensitive to culture condition and cell type leading to controversial results, an increased rate of mitochondrial fission does not always correlate with activation of apoptosis and the other way around. For instance, *Mfn1*$^{-/-}$ and *Mfn2*$^{-/-}$ cells show extensive mitochondrial fragmentation (Chen et al., 2003) but an unexpected slow response to tBid (Weaver et al., 2014). This appears to stem from the relative incapacity of some organelles to import Bak in *Mfn1*$^{-/-}$ and *Mfn2*$^{-/-}$ cells, which is correlated to a delayed release of cytochrome c and activation of apoptosis.

A recent study from Oettinghaus and co-workers (Oettinghaus et al., 2016) has shown that Drp1 participates in cytochrome c release by specific intrinsic death stimuli. Indeed, Drp1$^{-/-}$ mouse embryonic fibroblasts were protected from apoptosis induced by BH3 interacting-domain death agonist protein. However, they observe mitochondrial fragmentation after apoptotic stimulation in Drp1-deficient cells, correlating this phenomenon to diminished levels of profusion members. Therefore, they clarify that mitochondrial fission was caspase independent and Drp1 impinges on the cristae-remodelling pathway augmenting cytochrome c stash and release through OMM.

Some questions still remain unanswered: How does Mcl-1 protein take part in mitochondrial dynamics? Which shape and function depend on its expression? The role of Mcl-1 is still under intense study, and first results are partially controversial.

One of the first and recent reports that have focused on these intriguing mechanisms have been given by Perciavalle and co-workers who claimed that an amino-terminal truncated form of Mcl-1, localised to the IMM and exposed to the matrix, is required for mitochondrial fusion and supports mitochondrial bioenergetics. Moreover, it is implicated in the assembly of the F_oF_1-ATP synthase to support respiration (Perciavalle et al., 2012). The relevance of this work lies in the fact that Mcl-1 (by its matrix-localised form) could gain a separate (or indirectly related) function from its anti-apoptotic role.

More recently, Varadarajan et al. (2013) have examined the role of Mcl-1L in shaping mitochondria by using two putative inhibitors: BI97C1 and BI112D1.

BI97C1 is an apogossypol derivative and since its discovery it has been considered a pan-Bcl-2 inhibitor (Goff et al., 2013) (by targeting Bcl-2, Bcl-xL and Bcl-2A1 as well as Mcl-1). Instead, BI112D1 is its structural derivative, which was designed to display a higher selectivity for Mcl-1 over Bcl-2 or Bcl-xL (Wei et al., 2011). Both interact at the BH3 binding groove of Mcl-1 with different affinities (Placzek et al., 2011).

They have been able to induce apoptosis in a Mcl-1–dependent model (primarily through the mitochondrial pathway), and a dramatic time-dependent mitochondrial fragmentation was encountered (Varadarajan et al., 2013). This loss of connectivity was immediately evident and suggested a role for Mcl-1 in either promoting mitochondrial fusion or preventing fission. Inhibiting Mcl-1 action by pharmacological intervention enhanced fission in a Drp1 independent manner since neither its knockdown nor its dominant negative mutant affected the ability of drugs to induce mitochondrial fragmentation. The authors have ascribed the lack of tubular network to a time-dependent loss of the high molecular weight isoforms of OPA1. Moreover, fragmentation occurs upstream of Bax/Bak and independently from apoptosis (Varadarajan et al., 2013). Two years later, the same research group published new data by analysing other two Mcl-1 putative inhibitors, Maritoclax (marinopyrrole A) and Dinaciclib. Maritoclax was classified as a high selective inhibitor that antagonises Mcl-1 by binding to and targeting the protein for proteasome-mediated degradation (Doi et al., 2012), whereas Dinaciclib was mainly a cyclin-dependent kinase inhibitor that also reduced Mcl-1 expression (Gregory et al., 2015; Jane et al., 2016). Dinaciclib was more potent in inducing cell death and loss of Mcl-1 expression, but only Maritoclax resulted in extensive mitochondrial fragmentation, swelling and reactive oxygen species production (Varadarajan et al., 2015) in an analogous manner to other putative inhibitors (Varadarajan et al., 2013). Maritoclax-mediated mitochondrial perturbing was associated with a loss of Mfn1 and some OPA1 isoforms. Since it did not alter fission

proteins, it was more likely a loss in mitochondrial fusion.

Although the final effect of both inhibitors was the downregulation of Mcl-1L, their data strongly indicate the presence of simultaneous Mcl-1-independent pathways when analysing apoptosis and mitochondrial dynamics, making the understanding of the real connection between the two events difficult or impossible. Indeed, Maritoclax induced extensive mitochondrial fragmentation even in cells lacking Mcl-1, suggesting that Maritoclax-induced mitochondrial perturbation occurred irrespective of Mcl-1 status and it clearly can induce apoptosis even through other mechanisms. The use of pharmacological treatments with a wide background of side effects in analysing the interplay between Mcl-1 and mitochondrial dynamics can be misleading or of difficult analysis.

These studies summarise that the design of specific inhibitors is complicated by the complex regulation of Mcl-1, the composition of the BH3-binding groove and the existence of other isoforms of Mcl-1 that may participate in other cellular functions, including ER membrane reorganisation and mitochondrial fission–fusion dynamics.

It is feasible that inhibitors target different Mcl-1 isoforms in a different way depending on binding affinities and/or cellular distribution, and they may differentially affect the properties associated with these isoforms leading to a biological effect difficult to interpret.

In *in vivo* experiments, the complete loss of Mcl-1 in myocytes leads to extensive mitochondrial damage, as shown by using transmission electron microscopy (Thomas et al., 2013). Most mitochondria appear fragmented and exhibit extensive swelling and cristae remodelling. Analysis of mitochondrial fission and fusion proteins has revealed no changes in Mfn1/2, Opa1 and hFis1; instead Drp1 is completely undetectable in the mitochondrial fraction and in basal conditions (Thomas et al., 2013). Although an accumulation of these dysfunctional mitochondria occurs after loss of Mcl-1, autophagy is not active. Indeed, in the heart, Mcl-1 depletion decreases mitochondrial PINK1 expression leading to failed recruitment of PARK2 to the organelle, thus impairing mitophagy (Thomas and Gustafsson, 2013). The presence of Mcl-1 has been demonstrated to govern normal mitochondrial functions and cell survival upon infarction. Nevertheless, it is not yet known which Mcl-1 isoform is involved in autophagy and mitophagy.

Despite many experimental data recommend that increasing the rate of mitochondrial fission or decreasing the rate of mitochondrial fusion can stimulate apoptosis (when the two events are connected), numerous reports in the literature have confirmed that also in case of mitochondrial fusion cell death occurs by cell- and stress-dependent mechanisms (Szabadkai et al., 2004; Tondera et al., 2004; Westrate et al., 2014).

We have proposed an innovative approach to study Mcl-1 involvement in mitochondria remodelling and apoptosis. Our method, based on the antisense oligonucleotide technique, has targeted exonic splicing enhancer sequences in exon 2 of Mcl-1 primary transcript finely decreasing the Mcl-1L/S ratio. This has resulted in a hyperfused mitochondrial network and has increased sensitivity to apoptosis. Indeed, the shift in splicing from Mcl-1L to Mcl-1S by Mcl-1S3 was a priming stimulus for extensive cell death through the mitochondrial intrinsic apoptotic pathway. These findings do not involve any balancing mechanism activated by other anti-apoptotic proteins, such as Bcl-2 and Bcl-xL (Morciano et al., 2016).

Thus, if the inefficient mitochondrial fusion caused by total Mcl-1 inhibition/depletion is not related to altered susceptibility to cell death (Varadarajan et al. 2013), the hyperfused state induced by decreasing the Mcl-1L/S ratio might represent a crucial event for sensitising mitochondria to a wide range of apoptotic stimuli (Morciano et al., 2016). After excluding a possible role for Mfn1/2 and OPA1 proteins, it has been investigated whether the augmented mitochondrial fusion occurs in a Drp1-dependent manner. As previously mentioned, Drp1 is predominantly a cytosolic protein and moderately at the mitochondrial membranes, where it likely exerts physiological roles in maintaining a basal fission rate. Indeed its translocation from the cytosol to the mitochondrion is essential for its pro-fission functions. In Mcl-1L-depleted cells, Drp1 accumulation at mitochondria was less efficient and this coincides with the active fusion pathway. These findings suggest that Mcl-1L acts as a molecular anchor for mitochondrial Drp1 and further validate the pivotal role of Mcl-1L/S balance in the regulation of the fusion and fission machinery.

The clinical significance of understanding Mcl-1 features in cancer resides in the fact that none of its published inhibitors have demonstrated significant potency and/or specificity in a cellular experimental environment (Varadarajan et al., 2013). Moreover, validated and functional inhibitors of the Bcl-2 family, such as ABT-263 and ABT-199, are only effective against some proteins but do not target Mcl-1, which is commonly overexpressed in tumours and associated with chemoresistance and relapse of the pathology.

Concluding remarks

Key proteins involved in mitochondrial dynamics have been identified during the past years, and the role played by Bcl-2 family members in apoptosis is an old and ascertained issue born with the discovery of the Bcl-2 gene. Emerging evidence shows how the boundary between the two topics is not so marked as previously thought. In effect, it is generally agreed, but still debated, that mitochondrial fission/fusion dynamics are functionally related to apoptosis and cellular requirements. This fascinating point of view is also object of discussion, above all the multi-step action by which Bcl-2 family members regulate mitochondrial morphology and dynamin-related proteins promote cell death (Galluzzi et al., 2015). For translational and therapeutic purposes, we must establish (i) the identification of oncogenes as modulators of mitochondrial dynamics and functions such as metabolic efficiency and mitochondrial potential (Bittremieux et al., 2016; Vervliet et al., 2016); (ii) the related molecular mechanisms of action; and (iii) the exact timeline on which the two molecular events interface with each other. It is possible that different tumour types may be more or less sensitive to modulation of mitochondrial function depending on which oncogenic lesions drive that specific malignant condition. Further advancements in the development of small molecule Mcl-1 inhibitors with clear on target cellular effect will shed new light on the intersection of mechanisms of mitochondrial fusion and fission with apoptosis and may lead to new therapies for cancer.

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Conflict of interest statement

The authors have declared no conflict of interest.

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