Pharmacological Research 160 (2020) 105196

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Review

SAM50, a side door to the mitochondria: The case of cytotoxic proteases

Stefania Lionello [a,b], Giovanni Marzaro [c], Denis Martinvalet [a,b,*]

[a] Department of Biomedical Sciences University of Padova, via U. Bassi 58/b, 35129, Padova, PD, Italy
[b] Veneto Institute of Molecular Medicine, Via G. Orus 2, 35129 Padova, PD, Italy
[c] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, via Marzolo 5, I-35131, Padova, PD, Italy

ARTICLE INFO

Keywords:
Mitochondria
Cytotoxic lymphocytes
Granzyme
SAM50
Mitochondrial protein import

ABSTRACT

SAM50, a 7–8 nm diameter β-barrel channel of the mitochondrial outer membrane, is the central channel of the sorting and assembly machinery (SAM) complex involved in the biogenesis of β-barrel proteins. Interestingly, SAM50 is not known to have channel translocase activity; however, we have recently found that this channel is necessary and sufficient for mitochondrial entry of cytotoxic proteases. Cytotoxic lymphocytes eliminate cells that pose potential hazards, such as virus- and bacteria-infected cells as well as cancer cells. They induce cell death following the delivery of granzyme cytotoxic proteases into the cytosol of the target cell. Although granzyme A and granzyme B (GA and GB), the best characterized of the five human granzymes, trigger very distinct apoptotic cascades, they share the ability to directly target the mitochondria. GA and GB do not have a mitochondrial targeting signal, yet they enter the target cell mitochondria to disrupt respiratory chain complex I and induce mitochondrial reactive oxygen species (ROS)-dependent cell death. We found that granzyme mitochondrial entry requires SAM50 and the translocase of the inner membrane 22 (TIM22). Preventing granzymes' mitochondrial entry compromises their cytotoxicity, indicating that this event is unexpectedly an important step for cell death. Although mitochondria are best known for their roles in cell metabolism and energy conversion, these double-membrane organelles are also involved in Ca^{2+} homeostasis, metabolite transport, cell cycle regulation, cell signaling, differentiation, stress response, redox homeostasis, aging, and cell death. This multiplicity of functions is matched with the complexity and plasticity of the mitochondrial proteome as well as the organelle's morphological and structural versatility. Indeed, mitochondria are extremely dynamic and undergo fusion and fission events in response to diverse cellular cues. In humans, there are 1500 different mitochondrial proteins, the vast majority of which are encoded in the nuclear genome and translated by cytosolic ribosomes, after which they must be imported and properly addressed to the right mitochondrial compartment. To this end, mitochondria are equipped with a very sophisticated and highly specific protein import machinery. The latter is centered on translocase complexes embedded in the outer and inner mitochondrial membranes working along five different import pathways. We will briefly describe these import pathways to put into perspective our finding regarding the ability of granzymes to enter the mitochondria.

1. Introduction

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells play a central role in the defense against pathogen-infected or transformed cells [1–6]. They kill their target cells mainly through the cytotoxic granule pathway, which relies on exocytosis of the granule content into the immune synapse. Once in the synaptic cleft, perforin facilitates the entry of granzyme serine proteases into the cytosol of the target cell [7–19]. There are five humans granzymes (A, B, H, K, and M) and 10 mouse orthologs (A, B, C, D, E, F, G, K, M and N) [20–22]. The granzyme A (GA) and granzyme B (GB) apoptotic cascades are the best characterized [20–22]. GA has a tryptase activity, meaning it cleaves after lysine or arginine residues. GA-induced cell death is independent of caspases and insensitive to B-cell CLL/lymphoma 2 (Bcl2), which are critical effectors and regulators of apoptotic cell death. Interestingly, GA does not trigger the mitochondrial outer membrane permeabilization (MOMP), another important hallmark of apoptotic cell death. However, although the GA pathway does not involve the caspases nor MOMP and is insensitive to the anti-apoptotic factor Bcl2, similar morphological features are observed after cell death via the GA pathway as in apoptosis

* Corresponding author at: Department of Biomedical Sciences University of Padova, via U. Bassi 58/b 35129, Padova, PD, Italy.
E-mail address: Denis.Martinvalet@unipd.it (D. Martinvalet).

https://doi.org/10.1016/j.phrs.2020.105196
Received 12 July 2020; Received in revised form 26 August 2020; Accepted 3 September 2020
Available online 9 September 2020

[23–27]. Like the effector caspases, GB displays Asp-ase activity, meaning it cleaves after aspartic acid residues. The GB cell death pathway is sensitive to Bcl2, although it can proceed both dependently on or independently of the caspases [22,28–32]. Human GB shares some common substrates with the effector caspases, such as ICAD (inhibitor of caspase-activated DNases), PARP1 (poly-(ADP-ribose) polymerase), lamin, NuMa (nuclear mitotic apparatus protein), DNA-PKc (DNA-dependent protein kinase catalytic subunit), and tubulin [26,29,33–37]. Like initiator caspases, GB proteolytically activates the pro-apoptotic Bcl-2-member BID to initiate the mitochondrial death pathway [35–38]. Consequently, Bax and Bak oligomerize to trigger MOMP, and the mitochondrial transmembrane potential dissipates. MOMP is required for release of cytochrome c (cyt c), HtrA2/Omi, endonuclease G (Endo G), Smac/Diablo, and apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space to the cytosol [26,39–43]. Strikingly, human GB attacks mitochondria in different ways. Indeed, even in the presence of caspase inhibitors, GB can induce loss of $\Delta\Psi m$ and cyt c release. GB can still induce cell death in a BID-, Bax-, and Bak-deficient background (proteins critical for MOMP), although at a lower rate and amplitude [39,41,44,45]. GA and GB also directly cleave subunits of the respiratory chain complex I to trigger reactive oxygen species (ROS)--dependent death [25,46–48]. Similarly, caspase 3 disrupts complex I subunits to trigger ROS-dependent death [49]. Direct cleavage of complex I subunits by GA, GB, and caspase 3 means that they must enter the mitochondria. None of the five human granzymes nor caspase 3 express mitochondrial targeting sequences, yet they enter this double membrane organelle, which breaks all the rules of mitochondrial protein import. The prevention of granzymes from entering mitochondria significantly alters their cytotoxicity. In this review, we provide a brief overview of mitochondrial protein import in order to discuss the significance of the granzymes' unorthodox mitochondrial entry for ROS-dependent cell death. We refer to [20,50,51] for comprehensive reviews on the granzymes.

2. The mitochondria

Mitochondria originated from endosymbiotic α-proteobacteria or from ancestors branching off before α-proteobacteria divergence [52–54]. Mitochondria have an outer membrane (OMM) enclosing a highly folded inner mitochondrial membrane (IMM), which protrudes into the matrix inner compartment. An intermembrane space (IMS) separates the OMM from the IMM. Both mitochondrial membranes stay connected at contact sites. These contact sites are necessary for the organization of the cristae, which are IMM invaginations [55–58]. The mitochondrial contact sites and cristae organizing system (MICOS) are crucial for the maintenance of IMM cristae [58–60]. Mitochondria are the critical site for cellular energy conversion and metabolism [61–63]. They are proficient in producing ATP via oxidative phosphorylation (OXPHOS) [64]. The OXPHOS system consists of the respiratory chain complexes I, II, III and IV plus the F_1F_O-ATP synthase, which are embedded in the IMM of the cristae and protrude into the matrix. NADH and $FADH_2$ provide reduced electrons to complexes I and II, respectively. These electrons transit via coenzyme Q10 to reach complex III. Then they reach complex IV via cyt c up to oxygen, the final acceptor, to produce water. The electron transfer provides energy stored as a proton gradient to fuel the F_1F_O -ATP synthase for ATP synthesis [65]. ROS production from complexes I and III is inevitable during this electron transport, even in physiological conditions [65]. Moreover, mitochondria are also crucial for Ca^{2+} homeostasis, the dysregulation of which can trigger mitochondrial calcium overload and cell death [66–68]. New evidence points to complex V as being the likely molecular determinant of the permeability transition pore involved in this Ca^{2+}-dependent cell death [69–71]. Mitochondria are also crucial for metabolite transport, cell cycle regulation, cell signaling, differentiation, stress response, redox homeostasis, aging, and cell death [66,72–82]. The pleiotropic function of mitochondria is matched by the complexity and plasticity of their proteome as well as their morphological and structural versatility [80,82–84]. In fact, mitochondria are continuously remodeled through events of fusion and fission. This mitochondrial remodeling is regulated by shaping proteins of the family of dynamin-related GTPases and their adaptors. OMM fusion is controlled by Mitofusin (MFN) 1 and 2, while optic atrophy 1 (OPA1) controls that of the IMM [74,85–90]. Mitochondrial fission requires dynamin-related protein (DRP) 1 redistribution from the cytosol to the OMM where it binds to its adaptor proteins hFis1 (human fission protein 1), MFF (mitochondrial fission factor), and MiD49/51 (mitochondrial dynamics 49-kDa and 51-kDa proteins) [74,85–94].

In humans, the mitochondrial proteome is estimated to include 1500 different proteins. These proteins are organized in complex protein machineries interacting in dynamic networks to ensure proper functioning of the mitochondria [80–82]. The vast majority of the mitochondrial proteome is encoded in the nuclear genome and translated on cytosolic ribosomes before import into the right mitochondrial compartment. To this end, the mitochondria are equipped with a very elaborate and highly specific protein import machinery hardwired on translocase complexes embedded in the OMM and IMM. These translocases work along five different protein import pathways [80,82,95]. Most mitochondrial precursor proteins have a targeting sequence necessary for their mitochondrial entry and correct addressing to their final mitochondrial compartments [80,82,95]. The TOM40 channel, the core subunit of the translocase of the outer mitochondrial membrane (TOM) complex, is the mitochondrial entry gate [96–98]. The interior of this channel is paved with hydrophilic and hydrophobic patches that act as paths of binding sites. These paths of binding sites are used by distinct import pathways for matrix hydrophilic precursors and hydrophobic precursors to the metabolite carriers [97]. Mitochondrial matrix proteins and proteins destined for the IMM have an N-terminal sequence that acts as a targeting signal. These proteins enter the organelle through TOM40 channels. They cross the IMM through TIM23 channels, the central subunit of the TIM23 complex, which are activated by the membrane potential [99–102]. Translocation across TIM23 channels to the final matrix destination is further assisted by the molecular motor presequence translocase-associated motor (PAM) [99–102]. On the way, mitochondrial processing peptidase (MPP) removes the presequence from the precursor proteins [103]. OMM proteins with N-terminal α-helical transmembrane segments (anchor sequence) and multi-spanning segments are inserted in the membrane by the mitochondrial import (MIM) complex [104]. Precursors of mitochondrial IMS proteins are taken by the mitochondrial import and assembly (MIA) machinery when they exit the TOM40 channel on the IMS side [105]. Precursors of metabolite carriers do not have a cleavable presequence. After exiting the TOM40 channel, they then reach the translocase of the inner membrane (TIM22) with the help of small TIM proteins acting as chaperones [106–108]. Precursors of OMM β-barrel proteins enter the organelle through TOM40 and from the IMS reach the sorting and assembly machinery (SAM) complex for their insertion into the OMM by SAM50, the core channel of this complex [109,110]. Mechanistically, β-barrel precursors enter the lumen of the SAM50 channel from the IMS side, to be released laterally into the OMM. This requires opening of the SAM50 lateral gate, which is mediated by displacement of the SAM50 endogenous carboxyl-terminal β-signal by the β-signal of the precursor protein being processed [111]. At the SAM50 lateral gate is a thinning of the lipid bilayer, where β-hairpin-like loops of the precursor accumulate and fold into de-novo β-barrel proteins before being released in the OMM [111]. Only a very small fraction of mitochondrial proteins (13 structural proteins) is encoded by mitochondrial DNA, transcribed by matrix nucleoids, translated on mitochondrial ribosomes, and imported into the IMM [52].

Recently, the absolute copy number of the translocases has been defined for yeast cells [80,82]. TOM40 and TIM23 are the most abundant translocases (19,500 and 11,500 copies per cell, respectively) in agreement with their central roles in mitochondrial protein import.

However, TOM40 and TIM23 copy numbers are relatively low compared to the very large numbers of their substrates [80,82]. This indicates the efficiency of the mitochondrial protein import machinery. The same conclusion is valid for both SAM50 and TIM22, which are least abundant, with 1500 and 1100 copies per cell, respectively [80,82]. However, when mitochondrial protein import is compromised, cytosolic accumulation of mitochondrial precursor proteins occurs and triggers the mitochondrial unfolded protein response (UPR^{mt}). In turn, the UPR^{mt} reduces cytosolic protein synthesis and increases proteasomal protein degradation, resulting in reduced accumulation of mistargeted proteins [112,113]. We refer the readers to [82,114] for recent reviews of mitochondrial protein import.

Mitochondria and the endoplasmic reticulum (ER) communicate at areas of contact called mitochondria-ER contacts (MERCs) [74,85–90,115,116]. MERCs are important for the regulation of mitochondrial Ca^{2+} homeostasis, the transfer of lipids, the initiation of autophagosome formation, and the determination of the mitochondrial fission sites [117–124]. At certain MERCs, the ER tubules wrap and constrict the mitochondria providing a platform for the recruitment of motor force-generating cytoskeletal proteins [117,125]. ER-bound inverted formin 2 (INF2) concentrates between the two organelles at these MERCs [121,125,126]. INF2 triggers the assembly of the actomyosin motor to provide the force required for the initial constriction of the mitochondria [117,121,125,126]. Then, DRP1 polymerizes and spirals around the mitochondria constricted by the associated ER to mediate their fission [117,121,125–129]. Interestingly, mitochondrial fragmentation and apoptosis seem directly connected. We have observed that GA induces mitochondrial fragmentation [48], but how GA triggers this fragmentation is still unclear. However, since mitochondrial morphology is also regulated by contact with the ER, granzyme-mediated mitochondrial fragmentation is likely to impact the mitochondrial–ER interoganellar contacts. Testing this possibility will require more investigations. During apoptosis, Bax, DRP1, and MFN2 localize at fission sites. BID/Bax/Bak complexes decrease mitochondrial fusion, probably due to the inhibition of MFN2, while increasing fragmentation in a caspase-dependent manner by stabilizing the docking of sumoylated DRP1 on the OMM [130–133]. During oxidative stress, DRP1 isoforms 1 and 3 are phosphorylated by protein kinase C (PKC) at residues S616 and S579, respectively, leading to mitochondrial fragmentation [134]. On the other hand, mitochondrial fragmentation is also observed during cell death due to the loss of the OPA1 long isoforms [135,136].

3. Granzymes induce mitochondrial ROS-dependent cell death

Although they activate very distinct death signaling pathways, GA and GB have the common property of triggering ROS-dependent cell death. We reported that both GA and GB attack the respiratory chain to cleave NDUFS3, NDUFV1, NDUFS1 and NDUFS2, four subunits of the same complex I (Fig. 1) [25,47,48,137]. Indeed, we found that granzymes cleave complex I subunits from isolated intact mitochondria or purified complex I. Wild-type complex I subunits are also cleaved from target cells during cytotoxic lymphocyte attack, while the granzyme uncleavable forms are not. These results convincingly showed that granzymes directly cleave the respiratory chain complex I [47,48]. Cleavage of complex I subunits dramatically increases electron leak from the respiratory chain, leading to a rapid and sustained mitocentric ROS production, loss of the complex I, II, and III activities, respiratory chain disorganization, mitochondrial respiration impairment, and loss of mitochondrial cristae junctions [25,46–48,137]. Interestingly, caspase 3 also attacks complex I by cleaving NDUFS1 to trigger ROS-dependent death [49]. Overall, three different death pathways (GA, GB, and caspase 3) converge at mitochondrial respiratory chain complex I to cause ROS-dependent death (Fig. 1). We showed the GB-mediated ROS are necessary for apoptogenic factor release, DNA oligonucleosomal DNA fragmentation, and lysosomal permeabilization (Figs. 1 and 2) [47]. Similarly, GA-mediated ROS are essential for the nuclear translocation of an ER-associated oxidative stress response complex called the SET complex (Fig. 2) [25,138–142]. The SET complex contains the base excision repair (BER) endonuclease Ape1, the endonuclease NM23-H1, the 5′-3′ exonuclease Trex1, the chromatin-modifying proteins SET and pp32, and a DNA-binding protein that recognizes distorted DNA, HMGB2. The SET complex is involved in the repair of abasic sites generated by oxidative DNA damage. By cleaving SET, GA transforms the DNA repairing SET complex into a DNA destroying complex, while interfering with BER by cleaving Ape1 and HMGB2 (Fig. 2) [138–142].

The core subunits of mammalian complex I are conserved in the bacterial complex I [143]. Therefore, it is not surprising that granzymes can also cleave bacterial complex I. Indeed, cytotoxic lymphocytes also kill intracellular bacteria after disruption of bacterial complex I. This is mediated by perforin-dependent delivery of granulysin and granzyme into the infected target cell cytosol. Then granulysin mediates granzyme entry across the cell wall into the bacteria. In the bacteria, GA and GB cleave subunits of the bacterial complex I. Granzymes also cleave oxidative stress response enzymes such as superoxide dismutase (SOD) and catalase [144]. Together, these results underline the importance of complex I disruption for ROS-dependent death as it is conserved across

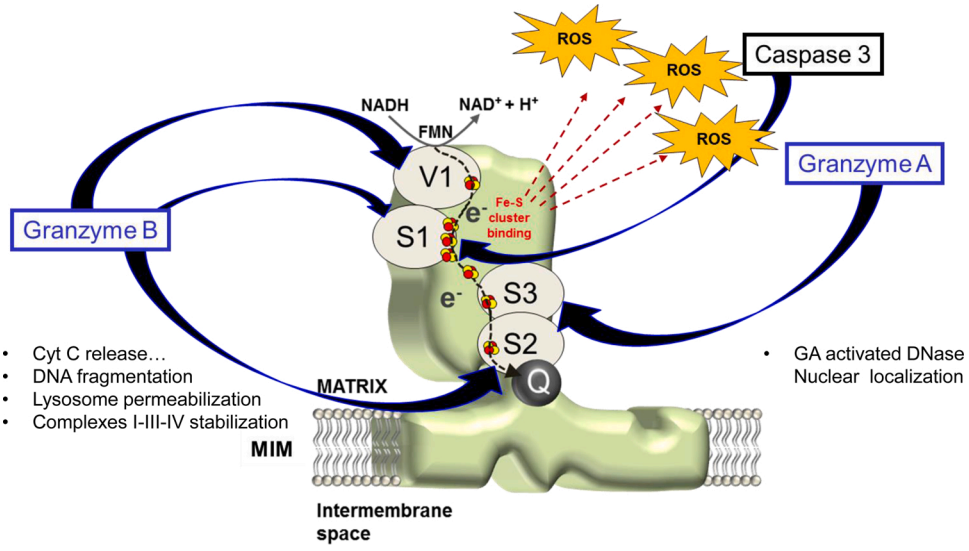

Fig. 1. Granzymes directly target respiratory complex I. GA cleaves NDUFS3. GB cleaves NDUFV1, NDUFS1, and NDUFS2. Caspase 3 cleaves NDUFS1. Cleavage of complex I subunits leads to ROS production. GA-mediated ROS are essential for GA-activated DNase nuclear translocation. GB-mediated ROS potentiate the release of apoptogenic factors cyt C, endo G, and SMAC; increase oligo nucleosomal DNA fragmentation; and increase lysosomal permeabilization and super complex stabilization.

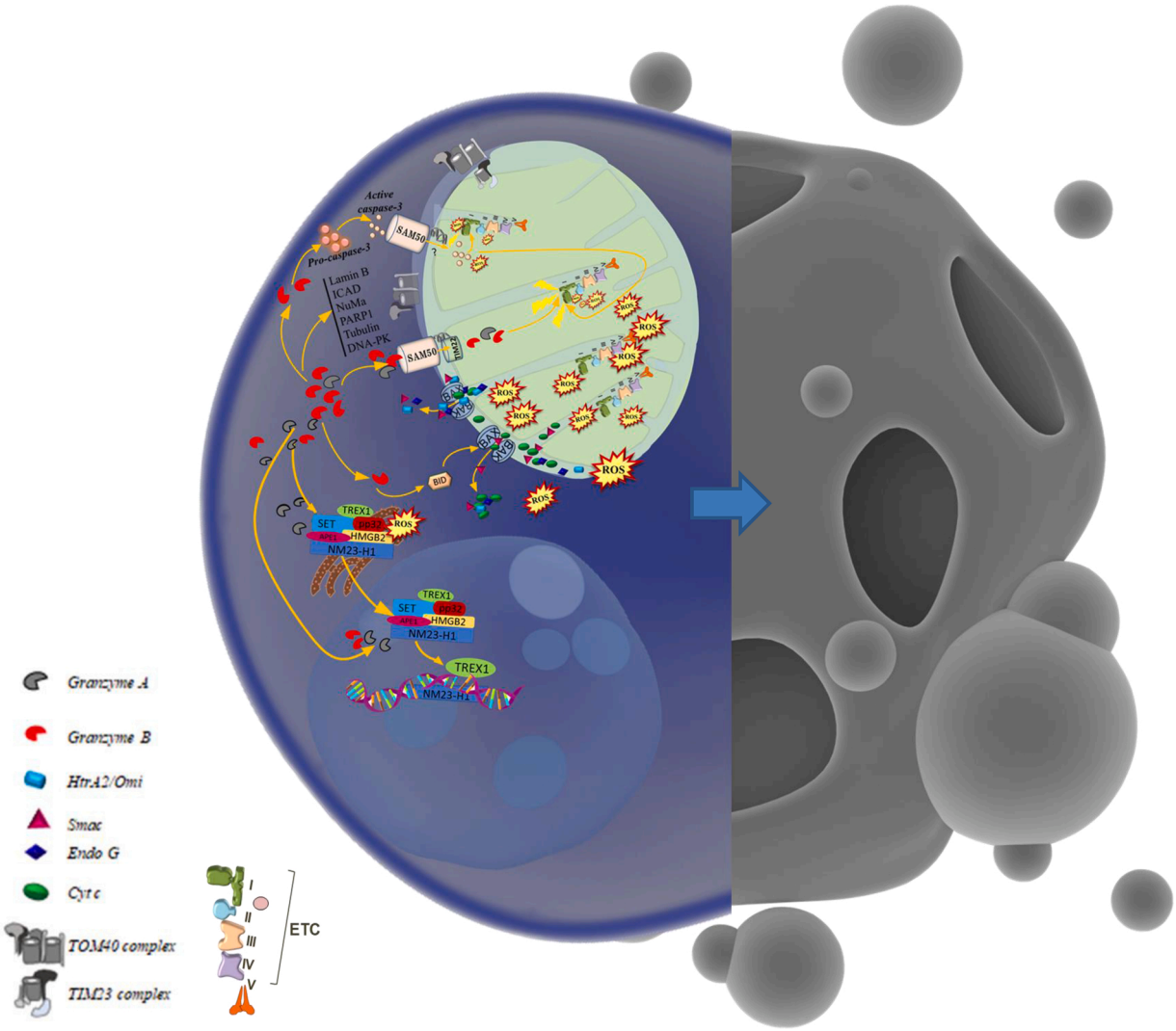

Fig. 2. Granzymes and caspase 3 enter mitochondria through the Sam50 channel. In the target cell cytosol, granzyme B initiates cell death by cleaving various substrates (ICAD, PARP1, lamin, NuMa, DNA-PKc, and tubulin). GB proteolytically activates BID to initiate the mitochondrial death pathway though the induction of Bax and Bak oligomerization, MOMP, and dissipation of the mitochondrial transmembrane potential (ΔΨm). MOMP is required for release of cytochrome c (cyt c), HtrA2/Omi, endonuclease G (Endo G), and Smac/Diablo. GA activates the ER bond SET complex containing the endonuclease Ape1 and NM23-H1, a 5′-3′ exonuclease Trex1, the chromatin-modifying proteins SET and pp32, and the DNA-binding protein HMGB2. Both granzymes and caspase 3 cross the outer and inner mitochondrial membranes through the Sam50 and Tim22 channels, respectively. Once in the matrix, granzyme and caspase 3 disrupt the electron transport chain (ETC) complex I and trigger a ROS increase. ROS potentiate apoptogenic factor release from the Bax/Bak pore (MOMP) and the nuclear translocation of the SET complex to trigger cell death.

phyla [47,48,144]. GB also induces the death of anaerobic bacteria by proteolyzing a set of highly conserved proteins involved in biosynthetic and metabolic pathways critical for the survival of bacterial under diverse environmental conditions [145]. Since mitochondria originated from endosymbiotic α-proteobacteria, it is reasonable to think that granzymes may disrupt similar mitochondrial biosynthetic and metabolic pathways, as they do in bacteria.

Although GA, GB, and caspase 3 do not express a mitochondrial targeting sequence, they still get inside the organelle independently of TOM40 and TIM23, the canonical mitochondrial import pathway to the matrix. Instead, GA and GB use SAM50 channels to cross the OMM and TIM22 channels to cross the IMM in a mtHSP70-dependent manner (Fig. 2) [46]. Both catalytically inactive and active granzymes enter purified intact mouse liver mitochondria in a manner that depends on the membrane potential. These results indicate that granzyme catalytic activity is dispensable in this pathway. Interestingly, granzymes also enter isolated yeast mitochondria, suggesting that the mechanism of granzyme mitochondrial entry is conserved from yeast to mammals. Taking advantage of the robust tools of yeast genetics, we found that isolated mitochondria from a yeast strain expressing thermosensitive alleles of TOM40 imported granzymes similar to the wild-type mitochondria at both permissive and non-permissive temperatures, indicating that TOM40 was not required in this pathway [46]. However, purified yeast mitochondria from strains expressing Tob55 (the yeast homolog of SAM50) under the inducible galactose promotor could not import granzymes when grown under glucose, although TOM40 expression was not affected [46]. This indicates that Tob55/SAM50 is required for granzyme mitochondrial entry. When mitochondria were used in import assays for granzymes and samples were resolved by Blue-Native gel electrophoresis, granzymes co-migrated with Tob55/-SAM50 but not with TOM40. Furthermore, only *Lactococcus lactis*, used as surrogate mitochondria, expressing Tob55 but neither TOM40 nor Por1 (the yeast homolog of VDAC [voltage-dependent anion-selective channel]) could import granzymes. These results indicate that Tob55/-SAM50 is necessary and sufficient for this translocation pathway and that the accessory subunits of the SAM complex are not needed (Fig. 2)

[46]. Using the same genetic approach, we showed that granzyme crossing of the IMM requires TIM22 and mtHSP70 but not TIM23 [46]. Lysine 243 (K243) and arginine 244 (R244), at the C-terminal extremity of GB, are necessary for its mitochondrial translocation. Although replacing these two residues with alanine did not alter GB catalytic activity, this was sufficient to prevent entry into target cell mitochondria during CTL attack. Most importantly, K243A/R244A substitution or silencing SAM50 to prevent GB entry into the mitochondria severely alters the cytotoxicity of GB [46]. SAM50 silencing also protects target cells from GA- and GM-mediated cell death [46]. In agreement with our finding, it was reported that GB enters the mitochondria to cleave Hax-1 (HS-1-associated protein X-1), a protein necessary to maintain the mitochondrial membrane potential [39]. Overexpression of wild-type or GB uncleavable Hax-1 protects the mitochondria against GB [39]. Taken together, these results clearly indicate that granzymes must enter the mitochondria in a process that we have dubbed Mitochondrial Entry of Cytotoxic Protease (MECP), an unexpected critical step for ROS-dependent cell death.

We found that caspase 3 enters isolated intact mitochondria in a SAM50- and membrane potential-dependent manner [46]. However, it was also reported that caspase 3 mitochondrial entry is downstream of MOMP [49]. It is likely that both mechanisms could take place to different extents depending of the cell type and the conditions. This would explain why silencing of SAM50 only partially inhibits active caspase 3-mediated cell death [46]. Understanding the relative contributions of MOMP and SAM50 in caspase 3 mitochondrial entry will require further investigation. GA mitochondrial entry is strictly dependent on SAM50, as GA does not trigger MOMP. In the case of GB, mitochondrial entry is independent of MOMP, since GB-mediated cleavage of complex I subunits still takes place in Bax and Bak double knockout cells [46,47,146]. Similarly, GB-mediated cleavage of complex I subunits takes place in isolated mitochondrial in the absence of S100 cytosolic factors, although addition of the latter increases the GB effect [46,47]. GB-mediated ROS increase apoptogenic factor release, suggesting that although important and required, MOMP could, in fact, be the tip of the iceberg [47]. MOMP provides the necessary disruption of the OMM. BID-dependent disruption of OPA1 oligomers triggers the opening of cristae junction [88]. Cytotoxic protease-dependent disruption of complex I generates the ROS necessary for the untethering of the apoptogenic factors from cardiolipin for efficient release [46,147,148]. SAM50-dependent GB entry into the mitochondrial requires an intact membrane potential, yet MOMP depolarizes the mitochondria. Together, these results indicate that SAM50-dependent GB mitochondrial entry must take place before MOMP or into mitochondria where MOMP has not occurred [46,47]. Determination of the hierarchy of events between MECP, ROS production, mitochondrial fragmentation, MOMP, and apoptogenic factor release will allow for further elucidation of the significance of MECP for cell death.

As discussed earlier, mitochondria are equipped with a very sophisticated and specific protein import machinery to maintain the vast diversity and plasticity of the organelle proteome, the hardware for the mitochondria pleiotropic functionality [96–102,149,150]. Granzymes break all the established rules of mitochondrial protein import. They do not have an N-terminal mitochondrial targeting sequence, and they use the SAM50 and TIM22 channels to cross the OMM and IMM, respectively, instead of the canonical TOM40-TIM23 axis. The TOM40-TIM23 translocation pathway is very selective. This selectivity may be viewed as a mechanism to protect mitochondrial integrity, which explains why they would not import 'mitotoxins' such as the granzymes. This agrees with the fact that TOM40-TIM23 are involved in the biogenesis and maintenance of the organelle. Therefore, cytotoxic molecules such as granzymes use SAM50-TIM22 as a side door to sneak into these organelles to disrupt the mitochondrial functions.

SAM50 is devoted to β-barrel protein insertion in the OMM and was not intended to function as a 'translocase' [111]. SAM50 is a member of the Omp85 family of β-barrel-channels [151,152]. This protein family is primarily known to function in the biogenesis of β-barrel proteins in bacteria or the outer membrane of mitochondria and chloroplasts. SAM50 is an approximately 7–8 nm diameter β-barrel channel inserted in the OMM (Fig. 3). It can accommodate 16–19 β-strands of the *de novo* β-barrel precursor protein being imported [110]. Together, this means that this channel is large enough to accommodate the granzymes [153]. Interestingly, new mechanistic insights into the operation of SAM50 revealed that the membrane insertion of β-barrel precursors requires opening of the SAM50 lateral gate at the thinning of the lipid bilayer where the precursor folds into de-novo β-barrel protein that is then released in the OMM [111]. Therefore, it is likely that during this process, conformational changes imposed by the opening of the SAM50 lateral gate by the precursor β-barrel protein facilitate the translocation of GB into the mitochondria. It was further proposed that this operating mode of SAM50, e.g. β-signal exchange, β-strand folding at the gate, and β-barrel lateral release into the membrane, could be extended to Omp85 family members of chloroplasts, and Gram-negative bacteria for the biogenesis of β-barrel protein [82,111]. Moreover, Toc75, the core channel of the translocase of the outer membrane of chloroplast functions as a pore translocase. The fact that Toc75 is a member of the Omp85 family implies that structurally similar proteins, like SAM50, can also function as transport pores. This is in agreement with our results showing that granzymes cross the OMM through the SAM50 channel [154].

Our results suggest that to enter and disrupt the function of mitochondria, granzymes may take advantage of SAM50 during its normal activity. However, granzyme mitochondrial entry appears to be very efficient, even if SAM50 and TIM22, the two channels hijacked during this pathway, are the least abundant of the mitochondrial protein import translocases [80,82]. Indeed, when mature catalytically inactive granzyme with Zs-Green fused at the N-terminus was over-expressed in HeLa cells, all the fusion proteins were localized within the mitochondria [46]. This indicates that this non-canonical mitochondrial entry was not saturated and fits well with the high efficiency of SAM50 and TIM22 in performing their normal functions [80,82]. It would be quite interesting to test the specificity of this alternative route to the mitochondria. We found that all five human granzymes enter the mitochondria, suggesting that this entry pathway could be shared by proteins possessing the granzyme chymotrypsin-like serine protease fold [46]. It will be very fascinating to characterize additional substrates of this alternative pathway, and to test whether it is dedicated only to proteins causing mitochondrial damage.

MECP of GA, GB, and likely caspase 3 requires SAM50 channel translocase activity, which seems to be sensitive to SAM50 phosphorylation, raising the question of how it is regulated. Interestingly, when performing homology modeling simulations of human SAM50 based on bacteria BamA structures (templates PDB-ID: 4K3C – BamA in open polypeptide-transport-associated [POTRA] domain conformation from *Haemophilus ducreyi*; 4K3B – BamA in closed POTRA domain conformation from *Neisseria gonorrhoeae*), we found that the protein could adopt an open conformation where the POTRA domain is extended and a closed conformation where this POTRA domain folds back on the IMS side of the barrel opening (Fig. 3A and 3B) [155–159]. Whether these conformational changes occur *in vivo*, how they are regulated, and how they could interfere with MECP are still open questions. A major difference between SAM50 and BamA proteins seems to reside in the electrostatic potential of the barrel inner surfaces. Indeed, while BamA protein shows a relatively neutral inner surface with a small negatively charged region, a more marked negative region facing a positively charged region was found in the homology model of SAM50 (Fig. 3C). Whether these differences in charge distribution are related to different channel selectivity is an interesting possibility that awaits investigation.

GB is also able to trigger the loss of cristae junctions from isolated mitochondria [47]. This interesting observation fits with the route of GB mitochondrial entry. SAM50 interacts with the MICOS complex to maintain the structure of the mitochondrial cristae [57–59,160,161]. In

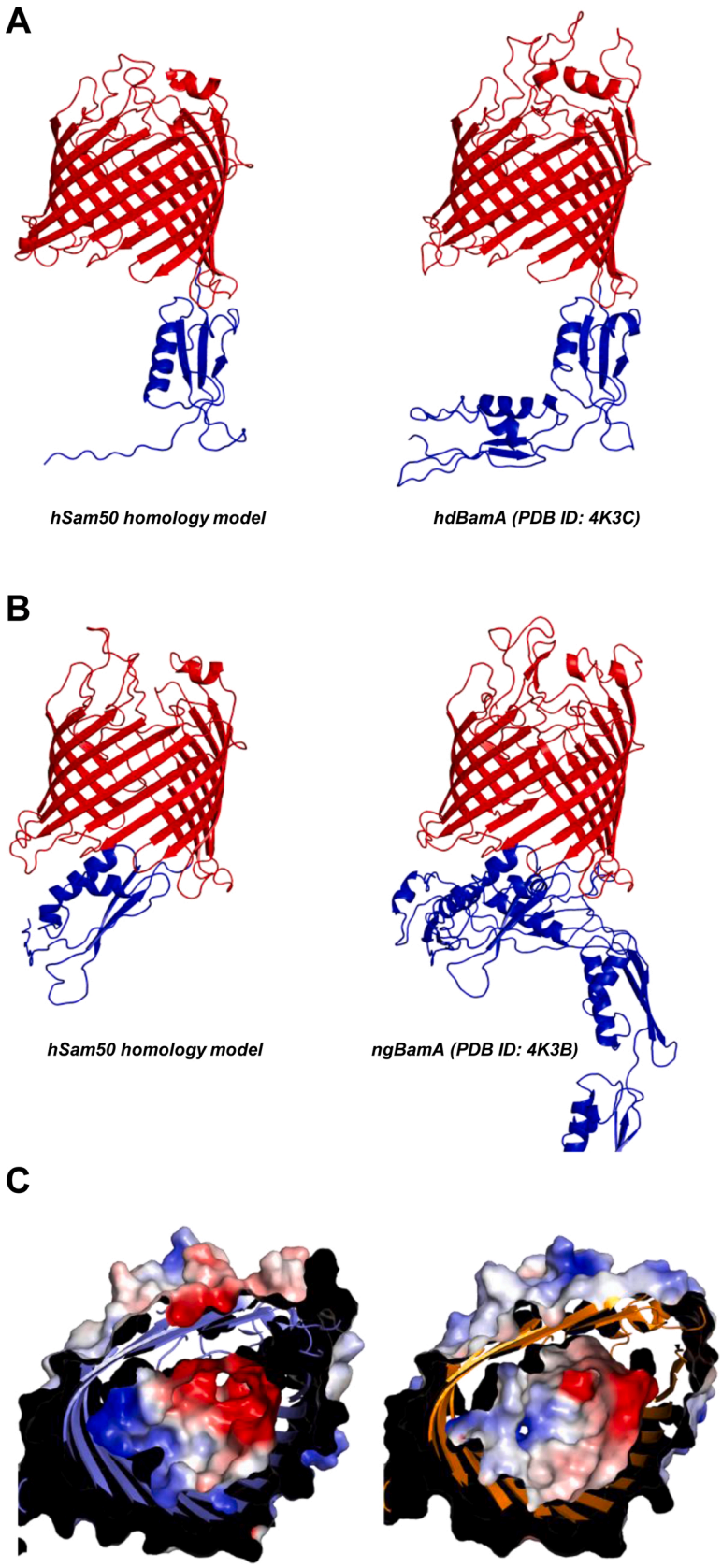

Fig. 3. Homology modeling simulations of human SAM50 based on bacteria BamA structure. Cartoon representation of membrane side views of the open conformation (A) and the closed conformation (B). Barrel domain: red; POTRA domain: blue. The electrostatic potential of the inner surface for SAM50 and BamA open conformation is also reported (C). Positively charged surfaces: blue; negatively charged surfaces: red; scale: -5/+5 KeV.

yeast, Mic60 or Mic10 deficiency leads to a complete loss of cristae junctions [57]. It is therefore possible that upon exiting the SAM50 channel, GB can alter some of the MICOS components to trigger the loss of cristae junctions.

4. Conclusion

Mitochondria are an integration and amplification hub for multiple death pathways, including those induced by granzymes, which must enter mitochondria to fully exert their cytotoxic action. Granzyme mitochondrial entry breaks all the rules of mitochondrial import pathways, as it uses the SAM50-TIM22 axis instead of the TOM40-TIM23 axis. MECP is conserved for all five human granzymes and most likely caspase 3. Preventing their mitochondrial entry compromises granzyme cytotoxicity, indicating that MECP is an important step for ROS-dependent cell death. In the future, it will be interesting to test whether other cytotoxic proteases similarly enter the mitochondria to determine to what extent MECP is conserved among cell death pathways. The characterization of additional substrates and further clarification of its regulation will be key to better understanding its physiological significance.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by an ERC starting grant ERC-2010-StG_20091118 and grants from UNIPD SID 2018, Foundation Boninchi, and Ambizione SNSFPZ00P3_126710/1. Stefania Lionello is supported by a Fellowship from the PhD school of the Department of Biomedical Sciences of the University of Padova, Italy.

References

[1] M.F. Bachmann, T.M. Kundig, G. Freer, Y. Li, C.Y. Kang, D.H. Bishop, H. Hengartner, R.M. Zinkernagel, Induction of protective cytotoxic T cells with viral proteins, Eur. J. Immunol. 24 (9) (1994) 2228–2236.
[2] D. Kagi, H. Hengartner, Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses, Curr. Opin. Immunol. 8 (4) (1996) 472–477.
[3] D. Kagi, B. Ledermann, K. Burki, H. Hengartner, R.M. Zinkernagel, CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity, Eur. J. Immunol. 24 (12) (1994) 3068–3072.
[4] M.E. van den Broek, D. Kagi, F. Ossendorp, R. Toes, S. Vamvakas, W.K. Lutz, C. J. Melief, R.M. Zinkernagel, H. Hengartner, Decreased tumor surveillance in perforin-deficient mice, J. Exp. Med. 184 (5) (1996) 1781–1790.
[5] L. Senovilla, I. Vitale, I. Martins, M. Tailler, C. Pailleret, M. Michaud, L. Galluzzi, S. Adjemian, O. Kepp, M. Niso-Santano, S. Shen, G. Marino, A. Criollo, A. Boileve, B. Job, S. Ladoire, F. Ghiringhelli, A. Sistigu, T. Yamazaki, S. Rello-Varona, C. Locher, V. Poirier-Colame, M. Talbot, A. Valent, F. Berardinelli, A. Antoccia, F. Ciccosanti, G.M. Fimia, M. Piacentini, A. Fueyo, N.L. Messina, M. Li, C.J. Chan, V. Sigl, G. Pourcher, C. Ruckenstuhl, D. Carmona-Gutierrez, V. Lazar, J. M. Penninger, F. Madeo, C. Lopez-Otin, M.J. Smyth, L. Zitvogel, M. Castedo, G. Kroemer, An immunosurveillance mechanism controls cancer cell ploidy, Science 337 (6102) (2012) 1678–1684.
[6] V. Shankaran, H. Ikeda, A.T. Bruce, J.M. White, P.E. Swanson, L.J. Old, R. D. Schreiber, IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity, Nature 410 (6832) (2001) 1107–1111.
[7] R.H. Law, N. Lukoyanova, I. Voskoboinik, T.T. Caradoc-Davies, K. Baran, M. A. Dunstone, M.E. D'Angelo, E.V. Orlova, F. Coulibaly, S. Verschoor, K. A. Browne, A. Ciccone, M.J. Kuiper, P.I. Bird, J.A. Trapani, H.R. Saibil, J. C. Whisstock, The structural basis for membrane binding and pore formation by lymphocyte perforin, Nature 468 (7322) (2010) 447–451.
[8] J. Thiery, D. Keefe, S. Boulant, E. Boucrot, M. Walch, D. Martinvalet, I.S. Goping, R.C. Bleackley, T. Kirchhausen, J. Lieberman, Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells, Nat. Immunol. 12 (8) (2011) 770–777.
[9] J. Thiery, D. Keefe, S. Saffarian, D. Martinvalet, M. Walch, E. Boucrot, T. Kirchhausen, J. Lieberman, Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis, Blood 115 (8) (2010) 1582–1593.
[10] G. Berke, T-cell-mediated cytotoxicity, Curr. Opin. Immunol. 3 (3) (1991) 320–325.
[11] G. Trenn, H. Takayama, M.V. Sitkovsky, Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes, Nature 330 (6143) (1987) 72–74.
[12] H.L. Ostergaard, K.P. Kane, M.F. Mescher, W.R. Clark, Cytotoxic T lymphocyte mediated lysis without release of serine esterase, Nature 330 (6143) (1987) 71–72.
[13] E. Rouvier, M.F. Luciani, P. Golstein, Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity, J. Exp. Med. 177 (1) (1993) 195–200.
[14] D. Kagi, B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel, H. Hengartner, Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice, Nature 369 (6475) (1994) 31–37.
[15] D. Kagi, F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, P. Golstein, Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity, Science 265 (5171) (1994) 528–530.
[16] P.J. Peters, J. Borst, V. Oorschot, M. Fukuda, O. Krahenbuhl, J. Tschopp, J. W. Slot, H.J. Geuze, Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes, J. Exp. Med. 173 (5) (1991) 1099–1109.
[17] J.H. Russell, T.J. Ley, Lymphocyte-mediated cytotoxicity, Annu. Rev. Immunol. 20 (2002) 323–370.
[18] G. Bossi, G.M. Griffiths, Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells, Nat. Med. 5 (1) (1999) 90–96.
[19] J. Lee, N.M.G. Dieckmann, J.R. Edgar, G.M. Griffiths, R.M. Siegel, Fas Ligand localizes to intraluminal vesicles within NK cell cytolytic granules and is enriched at the immune synapse, Immun. Inflamm. Dis. 6 (2) (2018) 312–321.
[20] D. Chowdhury, J. Lieberman, Death by a thousand cuts: granzyme pathways of programmed cell death, Annu. Rev. Immunol. 26 (2008) 389–420.
[21] R. Sattar, S.A. Ali, A. Abbasi, Bioinformatics of granzymes: sequence comparison and structural studies on granzyme family by homology modeling, Biochem. Biophys. Res. Commun. 308 (4) (2003) 726–735.
[22] D. Masson, J. Tschopp, A family of serine esterases in lytic granules of cytolytic T lymphocytes, Cell 49 (5) (1987) 679–685.
[23] P.J. Beresford, Z. Xia, A.H. Greenberg, J. Lieberman, Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation, Immunity 10 (5) (1999) 585–594.
[24] J. Lieberman, Granzyme A activates another way to die, Immunol. Rev. 235 (1) (2010) 93–104.
[25] D. Martinvalet, P. Zhu, J. Lieberman, Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis, Immunity 22 (3) (2005) 355–370.
[26] D. Zhang, P.J. Beresford, A.H. Greenberg, J. Lieberman, Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granule-mediated cytolysis, Proc. Natl. Acad. Sci. U.S.A. 98 (10) (2001) 5746–5751.
[27] P. Zhu, D. Zhang, D. Chowdhury, D. Martinvalet, D. Keefe, L. Shi, J. Lieberman, Granzyme A, which causes single-stranded DNA damage, targets the double-strand break repair protein Ku70, EMBO Rep. 7 (4) (2006) 431–437.
[28] S. Odake, C.M. Kam, L. Narasimhan, M. Poe, J.T. Blake, O. Krahenbuhl, J. Tschopp, J.C. Powers, Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolysis by isocoumarins, Biochemistry 30 (8) (1991) 2217–2227.
[29] V.R. Sutton, M.E. Wowk, M. Cancilla, J.A. Trapani, Caspase activation by granzyme B is indirect, and caspase autoprocessing requires the release of proapoptotic mitochondrial factors, Immunity 18 (3) (2003) 319–329.
[30] I.S. Goping, M. Barry, P. Liston, T. Sawchuk, G. Constantinescu, K.M. Michalak, I. Shostak, D.L. Roberts, A.M. Hunter, R. Korneluk, R.C. Bleackley, Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition, Immunity 18 (3) (2003) 355–365.
[31] D. Kaiserman, C.H. Bird, J. Sun, A. Matthews, K. Ung, J.C. Whisstock, P. E. Thompson, J.A. Trapani, P.I. Bird, The major human and mouse granzymes are structurally and functionally divergent, J. Cell Biol. 175 (4) (2006) 619–630.
[32] V.R. Sutton, D.L. Vaux, J.A. Trapani, Bcl-2 prevents apoptosis induced by perforin and granzyme B, but not that mediated by whole cytotoxic lymphocytes, J. Immunol. 158 (12) (1997) 5783–5790.
[33] F. Andrade, S. Roy, D. Nicholson, N. Thornberry, A. Rosen, L. Casciola-Rosen, Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis, Immunity 8 (4) (1998) 451–460.
[34] S.P. Cullen, C. Adrain, A.U. Luthi, P.J. Duriez, S.J. Martin, Human and murine granzyme B exhibit divergent substrate preferences, J. Cell Biol. 176 (4) (2007) 435–444.
[35] V.R. Sutton, J.E. Davis, M. Cancilla, R.W. Johnstone, A.A. Ruefli, K. Sedelies, K. A. Browne, J.A. Trapani, Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation, J. Exp. Med. 192 (10) (2000) 1403–1414.
[36] N.J. Waterhouse, K.A. Sedelies, K.A. Browne, M.E. Wowk, A. Newbold, V. R. Sutton, C.J. Clarke, J. Oliaro, R.K. Lindemann, P.I. Bird, R.W. Johnstone, J. A. Trapani, A central role for Bid in granzyme B-induced apoptosis, J. Biol. Chem. 280 (6) (2005) 4476–4482.
[37] J.B. Alimonti, L. Shi, P.K. Baijal, A.H. Greenberg, Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition, J. Biol. Chem. 276 (10) (2001) 6974–6982.

[38] L. Casciola-Rosen, M. Garcia-Calvo, H.G. Bull, J.W. Becker, T. Hines, N. A. Thornberry, A. Rosen, Mouse and human granzyme B have distinct tetrapeptide specificities and abilities to recruit the bid pathway, J. Biol. Chem. 282 (7) (2007) 4545–4552.
[39] J. Han, L.A. Goldstein, W. Hou, C.J. Froelich, S.C. Watkins, H. Rabinowich, Deregulation of mitochondrial membrane potential by mitochondrial insertion of granzyme B and direct Hax-1 cleavage, J. Biol. Chem. 285 (29) (2010) 22461–22472.
[40] G. Kroemer, L. Galluzzi, C. Brenner, Mitochondrial membrane permeabilization in cell death, Physiol. Rev. 87 (1) (2007) 99–163.
[41] G. MacDonald, L. Shi, C. Vande Velde, J. Lieberman, A.H. Greenberg, Mitochondria-dependent and -independent regulation of Granzyme B-induced apoptosis, J. Exp. Med. 189 (1) (1999) 131–144.
[42] J. Parrish, L. Li, K. Klotz, D. Ledwich, X. Wang, D. Xue, Mitochondrial endonuclease G is important for apoptosis in C. Elegans, Nature 412 (6842) (2001) 90–94.
[43] S.A. Susin, H.K. Lorenzo, N. Zamzami, I. Marzo, B.E. Snow, G.M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D.R. Goodlett, R. Aebersold, D.P. Siderovski, J.M. Penninger, G. Kroemer, Molecular characterization of mitochondrial apoptosis-inducing factor, Nature 397 (6718) (1999) 441–446.
[44] J.A. Heibein, M. Barry, B. Motyka, R.C. Bleackley, Granzyme B-induced loss of mitochondrial inner membrane potential (Delta psi m) and cytochrome c release are caspase independent, J. Immunol. 163 (9) (1999) 4683–4693.
[45] D.A. Thomas, L. Scorrano, G.V. Putcha, S.J. Korsmeyer, T.J. Ley, Granzyme B can cause mitochondrial depolarization and cell death in the absence of BID, BAX, and BAK, Proc. Natl. Acad. Sci. U.S.A. 98 (26) (2001) 14985–14990.
[46] V. Chiusolo, G. Jacquemin, E. Yonca Bassoy, L. Vinet, L. Liguori, M. Walch, V. Kozjak-Pavlovic, D. Martinvalet, Granzyme B enters the mitochondria in a Sam50-, Tim22- and mtHsp70-dependent manner to induce apoptosis, Cell Death Differ. 24 (4) (2017) 747–758.
[47] G. Jacquemin, D. Margiotta, A. Kasahara, E.Y. Bassoy, M. Walch, J. Thiery, J. Lieberman, D. Martinvalet, Granzyme B-induced mitochondrial ROS are required for apoptosis, Cell Death Differ. 22 (5) (2015) 862–874.
[48] D. Martinvalet, D.M. Dykxhoorn, R. Ferrini, J. Lieberman, Granzyme A cleaves a mitochondrial complex I protein to initiate caspase-independent cell death, Cell 133 (4) (2008) 681–692.
[49] J.E. Ricci, C. Munoz-Pinedo, P. Fitzgerald, B. Bailly-Maitre, G.A. Perkins, N. Yadava, I.E. Scheffler, M.H. Ellisman, D.R. Green, Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain, Cell 117 (6) (2004) 773–786.
[50] J.A. Trapani, M.J. Smyth, Functional significance of the perforin/granzyme cell death pathway, Nat. Rev. Immunol. 2 (10) (2002) 735–747.
[51] J.A. Trapani, Granzymes, cytotoxic granules and cell death: the early work of Dr, Jurg Tschopp, Cell death and differentiation 19 (1) (2012) 21–27.
[52] B.F. Lang, G. Burger, C.J. O'Kelly, R. Cedergren, G.B. Golding, C. Lemieux, D. Sankoff, M. Turmel, M.W. Gray, An ancestral mitochondrial DNA resembling a eubacterial genome in miniature, Nature 387 (6632) (1997) 493–497.
[53] R. Vendramin, J.C. Marine, E. Leucci, Non-coding RNAs: the dark side of nuclear-mitochondrial communication, EMBO J. 36 (9) (2017) 1123–1133.
[54] J. Martijn, J. Vosseberg, L. Guy, P. Offre, T.J.G. Ettema, Deep mitochondrial origin outside the sampled alphaproteobacteria, Nature 557 (7703) (2018) 101–105.
[55] C.A. Mannella, W.J. Lederer, M.S. Jafri, The connection between inner membrane topology and mitochondrial function, J. Mol. Cell. Cardiol. 62 (2013) 51–57.
[56] C.A. Mannella, M. Marko, P. Penczek, D. Barnard, J. Frank, The internal compartmentation of rat-liver mitochondria: tomographic study using the high-voltage transmission electron microscope, Microsc. Res. Tech. 27 (4) (1994) 278–283.
[57] M. van der Laan, M. Bohnert, N. Wiedemann, N. Pfanner, Role of MINOS in mitochondrial membrane architecture and biogenesis, Trends Cell Biol. 22 (4) (2012) 185–192.
[58] V. Kozjak-Pavlovic, The MICOS complex of human mitochondria, Cell Tissue Res. 367 (1) (2017) 83–93.
[59] H. Li, Y. Ruan, K. Zhang, F. Jian, C. Hu, L. Miao, L. Gong, L. Sun, X. Zhang, S. Chen, H. Chen, D. Liu, Z. Song, Mic60/Mitofilin determines MICOS assembly essential for mitochondrial dynamics and mtDNA nucleoid organization, Cell Death Differ. 23 (3) (2016) 380–392.
[60] C. Ott, E. Dorsch, M. Fraunholz, S. Straub, V. Kozjak-Pavlovic, Detailed analysis of the human mitochondrial contact site complex indicate a hierarchy of subunits, PLoS One 10 (3) (2015), e0120213.
[61] G. Attardi, G. Schatz, Biogenesis of mitochondria, Annu. Rev. Cell Biol. 4 (1988) 289–333.
[62] H.M. McBride, M. Neuspiel, S. Wasiak, Mitochondria: more than just a powerhouse, Curr. Biol. 16 (14) (2006) R551–60.
[63] M. Saraste, Oxidative phosphorylation at the fin de siecle, Science 283 (5407) (1999) 1488–1493.
[64] P. Mitchell, Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism, Nature 191 (1961) 144–148.
[65] M.P. Murphy, How mitochondria produce reactive oxygen species, Biochem. J. 417 (1) (2009) 1–13.
[66] J.M. Baughman, F. Perocchi, H.S. Girgis, M. Plovanich, C.A. Belcher-Timme, Y. Sancak, X.R. Bao, L. Strittmatter, O. Goldberger, R.L. Bogorad, V. Koteliansky, V.K. Mootha, Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter, Nature 476 (7360) (2011) 341–345.
[67] P. Bernardi, L. Scorrano, R. Colonna, V. Petronilli, F. Di Lisa, Mitochondria and cell death. Mechanistic aspects and methodological issues, Eur. J. Biochem. 264 (3) (1999) 687–701.
[68] G. Hajnoczky, G. Csordas, S. Das, C. Garcia-Perez, M. Saotome, S. Sinha Roy, M. Yi, Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis, Cell Calcium 40 (5–6) (2006) 553–560.
[69] P. Bernardi, A. Rasola, M. Forte, G. Lippe, The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology, Physiol. Rev. 95 (4) (2015) 1111–1155.
[70] A. Rasola, P. Bernardi, Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis, Cell Calcium 50 (3) (2011) 222–233.
[71] P. Bernardi, Why F-ATP synthase remains a strong candidate as the mitochondrial permeability transition pore, Front. Physiol. 9 (2018) 1543.
[72] D. De Stefani, A. Raffaello, E. Teardo, I. Szabo, R. Rizzuto, A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter, Nature 476 (7360) (2011) 336–340.
[73] M. Breitenbach, M. Rinnerthaler, J. Hartl, A. Stincone, J. Vowinckel, H. Breitenbach-Koller, M. Ralser, Mitochondria in ageing: there is metabolism beyond the ROS, FEMS Yeast Res. (2013).
[74] D.C. Chan, Mitochondria: dynamic organelles in disease, aging, and development, Cell 125 (7) (2006) 1241–1252.
[75] D.R. Green, G. Kroemer, The pathophysiology of mitochondrial cell death, Science 305 (5684) (2004) 626–629.
[76] S.W. Tait, D.R. Green, Mitochondria and cell death: outer membrane permeabilization and beyond, Nature reviews, Molecular cell biology 11 (9) (2010) 621–632.
[77] A. Kasahara, S. Cipolat, Y. Chen, G.W. Dorn 2nd., L. Scorrano, Mitochondrial fusion directs cardiomyocyte differentiation via calcineurin and Notch signaling, Science 342 (6159) (2013) 734–737.
[78] A. Kasahara, L. Scorrano, Mitochondria: from cell death executioners to regulators of cell differentiation, Trends Cell Biol. 24 (12) (2014) 761–770.
[79] R. Rizzuto, P. Bernardi, T. Pozzan, Mitochondria as all-round players of the calcium game, J. Physiol. (Paris) 529 (Pt 1) (2000) 37–47.
[80] M. Morgenstern, S.B. Stiller, P. Lubbert, C.D. Peikert, S. Dannenmaier, F. Drepper, U. Weill, P. Hoss, R. Feuerstein, M. Gebert, M. Bohnert, M. van der Laan, M. Schuldiner, C. Schutze, S. Oeljeklaus, N. Pfanner, N. Wiedemann, B. Warscheid, Definition of a high-confidence mitochondrial proteome at quantitative scale, Cell Rep. 19 (13) (2017) 2836–2852.
[81] S.E. Calvo, K.R. Clauser, V.K. Mootha, MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins, Nucleic Acids Res. 44 (D1) (2016) D1251–7.
[82] N. Pfanner, B. Warscheid, N. Wiedemann, Mitochondrial proteins: from biogenesis to functional networks, Nature reviews, Molecular cell biology 20 (5) (2019) 267–284.
[83] B. Westermann, Mitochondrial fusion and fission in cell life and death, Nature reviews, Molecular cell biology 11 (12) (2010) 872–884.
[84] D.C. Chan, Fusion and fission: interlinked processes critical for mitochondrial health, Annu. Rev. Genet. 46 (2012) 265–287.
[85] G.M. Cereghetti, V. Costa, L. Scorrano, Inhibition of Drp1-dependent mitochondrial fragmentation and apoptosis by a polypeptide antagonist of calcineurin, Cell Death Differ. 17 (11) (2010) 1785–1794.
[86] G.M. Cereghetti, A. Stangherlin, O. Martins de Brito, C.R. Chang, C. Blackstone, P. Bernardi, L. Scorrano, Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria, Proc. Natl. Acad. Sci. U.S.A. 105 (41) (2008) 15803–15808.
[87] K.S. Dimmer, L. Scorrano, (De)constructing mitochondria: what for? Physiology Bethesda (Bethesda) 21 (2006) 233–241.
[88] C. Frezza, S. Cipolat, O. Martins de Brito, M. Micaroni, G.V. Beznoussenko, T. Rudka, D. Bartoli, R.S. Polishuck, N.N. Danial, B. De Strooper, L. Scorrano, OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion, Cell 126 (1) (2006) 177–189.
[89] L.C. Gomes, G. Di Benedetto, L. Scorrano, During autophagy mitochondria elongate, are spared from degradation and sustain cell viability, Nat. Cell Biol. 13 (5) (2011) 589–598.
[90] L. Scorrano, Proteins that fuse and fragment mitochondria in apoptosis: con-fissing a deadly con-fusion? J. Bioenerg. Biomembr. 37 (3) (2005) 165–170.
[91] S. Gandre-Babbe, A.M. van der Bliek, The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells, Mol. Biol. Cell 19 (6) (2008) 2402–2412.
[92] H. Otera, C. Wang, M.M. Cleland, K. Setoguchi, S. Yokota, R.J. Youle, K. Mihara, Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells, J. Cell Biol. 191 (6) (2010) 1141–1158.
[93] C.S. Palmer, L.D. Osellame, D. Laine, O.S. Koutsopoulos, A.E. Frazier, M.T. Ryan, MiD49 and MiD51, new components of the mitochondrial fission machinery, EMBO Rep. 12 (6) (2011) 565–573.
[94] J. Zhao, T. Liu, S. Jin, X. Wang, M. Qu, P. Uhlen, N. Tomilin, O. Shupliakov, U. Lendahl, M. Nister, Human MIEF1 recruits Drp1 to mitochondrial outer membranes and promotes mitochondrial fusion rather than fission, EMBO J. 30 (14) (2011) 2762–2778.
[95] P. Rehling, K. Brandner, N. Pfanner, Mitochondrial import and the twin-pore translocase, Nature reviews, Molecular cell biology 5 (7) (2004) 519–530.
[96] K. Hill, K. Model, M.T. Ryan, K. Dietmeier, F. Martin, R. Wagner, N. Pfanner, Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins [see comment], Nature 395 (6701) (1998) 516–521.

[97] Y. Araiso, A. Tsutsumi, J. Qiu, K. Imai, T. Shiota, J. Song, C. Lindau, L.S. Wenz, H. Sakaue, K. Yunoki, S. Kawano, J. Suzuki, M. Wischnewski, C. Schutze, H. Ariyama, T. Ando, T. Becker, T. Lithgow, N. Wiedemann, N. Pfanner, M. Kikkawa, T. Endo, Structure of the mitochondrial import gate reveals distinct preprotein paths, Nature 575 (7782) (2019) 395–401.
[98] K. Tucker, E. Park, Cryo-EM structure of the mitochondrial protein-import channel TOM complex at near-atomic resolution, Nat. Struct. Mol. Biol. 26 (12) (2019) 1158–1166.
[99] M.F. Bauer, C. Sirrenberg, W. Neupert, M. Brunner, Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria, Cell 87 (1) (1996) 33–41.
[100] K. Malhotra, M. Sathappa, J.S. Landin, A.E. Johnson, N.N. Alder, Structural changes in the mitochondrial Tim23 channel are coupled to the proton-motive force, Nat. Struct. Mol. Biol. 20 (8) (2013) 965–972.
[101] P. D'Silva, Q. Liu, W. Walter, E.A. Craig, Regulated interactions of mtHsp70 with Tim44 at the translocon in the mitochondrial inner membrane, Nat. Struct. Mol. Biol. 11 (11) (2004) 1084–1091.
[102] H.C. Schneider, J. Berthold, M.F. Bauer, K. Dietmeier, B. Guiard, M. Brunner, W. Neupert, Mitochondrial Hsp70/MIM44 complex facilitates protein import, Nature 371 (6500) (1994) 768–774.
[103] G. Hawlitschek, H. Schneider, B. Schmidt, M. Tropschug, F.U. Hartl, W. Neupert, Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein, Cell 53 (5) (1988) 795–806.
[104] J. Popov-Celeketic, T. Waizenegger, D. Rapaport, Mim1 functions in an oligomeric form to facilitate the integration of Tom20 into the mitochondrial outer membrane, J. Mol. Biol. 376 (3) (2008) 671–680.
[105] A. Chacinska, S. Pfannschmidt, N. Wiedemann, V. Kozjak, L.K. Sanjuan Szklarz, A. Schulze-Specking, K.N. Truscott, B. Guiard, C. Meisinger, N. Pfanner, Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins, EMBO J. 23 (19) (2004) 3735–3746.
[106] N. Gebert, M. Gebert, S. Oeljeklaus, K. von der Malsburg, D.A. Stroud, B. Kulawiak, C. Wirth, R.P. Zahedi, P. Dolezal, S. Wiese, O. Simon, A. Schulze-Specking, K.N. Truscott, A. Sickmann, P. Rehling, B. Guiard, C. Hunte, B. Warscheid, M. van der Laan, N. Pfanner, N. Wiedemann, Dual function of Sdh3 in the respiratory chain and TIM22 protein translocase of the mitochondrial inner membrane, Mol. Cell 44 (5) (2011) 811–818.
[107] O. Kerscher, N.B. Sepuri, R.E. Jensen, Tim18p is a new component of the Tim54p-Tim22p translocon in the mitochondrial inner membrane, Mol. Biol. Cell 11 (1) (2000) 103–116.
[108] P. Kovermann, K.N. Truscott, B. Guiard, P. Rehling, N.B. Sepuri, H. Muller, R. E. Jensen, R. Wagner, N. Pfanner, Tim22, the essential core of the mitochondrial protein insertion complex, forms a voltage-activated and signal-gated channel, Mol. Cell 9 (2) (2002) 363–373.
[109] A. Klein, L. Israel, S.W. Lackey, F.E. Nargang, A. Imhof, W. Baumeister, W. Neupert, D.R. Thomas, Characterization of the insertase for beta-barrel proteins of the outer mitochondrial membrane, J. Cell Biol. 199 (4) (2012) 599–611.
[110] S.A. Paschen, T. Waizenegger, T. Stan, M. Preuss, M. Cyrklaff, K. Hell, D. Rapaport, W. Neupert, Evolutionary conservation of biogenesis of beta-barrel membrane proteins, Nature 426 (6968) (2003) 862–866.
[111] A.I.C. Hohr, C. Lindau, C. Wirth, J. Qiu, D.A. Stroud, S. Kutik, B. Guiard, C. Hunte, T. Becker, N. Pfanner, N. Wiedemann, Membrane protein insertion through a mitochondrial beta-barrel gate, Science 359 (6373) (2018).
[112] A.M. Nargund, M.W. Pellegrino, C.J. Fiorese, B.M. Baker, C.M. Haynes, Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation, Science 337 (6094) (2012) 587–590.
[113] X. Wang, X.J. Chen, A cytosolic network suppressing mitochondria-mediated proteostatic stress and cell death, Nature 524 (7566) (2015) 481–484.
[114] N. Wiedemann, N. Pfanner, Mitochondrial machineries for protein import and assembly, Annu. Rev. Biochem. 86 (2017) 685–714.
[115] D. Brough, M.J. Schell, R.F. Irvine, Agonist-induced regulation of mitochondrial and endoplasmic reticulum motility, Biochem. J. 392 (Pt 2) (2005) 291–297.
[116] M. Yi, D. Weaver, G. Hajnoczky, Control of mitochondrial motility and distribution by the calcium signal: a homeostatic circuit, J. Cell Biol. 167 (4) (2004) 661–672.
[117] J.R. Friedman, L.L. Lackner, M. West, J.R. DiBenedetto, J. Nunnari, G.K. Voeltz, ER tubules mark sites of mitochondrial division, Science 334 (6054) (2011) 358–362.
[118] R. Rizzuto, M. Brini, M. Murgia, T. Pozzan, Microdomains with high Ca2+ close to IP3-sensitive channels that are sensed by neighboring mitochondria, Science 262 (5134) (1993) 744–747.
[119] R. Rizzuto, P. Pinton, W. Carrington, F.S. Fay, K.E. Fogarty, L.M. Lifshitz, R. A. Tuft, T. Pozzan, Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses, Science 280 (5370) (1998) 1763–1766.
[120] A.A. Rowland, G.K. Voeltz, Endoplasmic reticulum-mitochondria contacts: function of the junction, Nature reviews, Molecular cell biology 13 (10) (2012) 607–625.
[121] F. Korobova, V. Ramabhadran, H.N. Higgs, An actin-dependent step in mitochondrial fission mediated by the ER-associated formin INF2, Science 339 (6118) (2013) 464–467.
[122] T. Hayashi, R. Rizzuto, G. Hajnoczky, T.P. Su, MAM: more than just a housekeeper, Trends Cell Biol. 19 (2) (2009) 81–88.
[123] M. Hamasaki, N. Furuta, A. Matsuda, A. Nezu, A. Yamamoto, N. Fujita, H. Oomori, T. Noda, T. Haraguchi, Y. Hiraoka, A. Amano, T. Yoshimori, Autophagosomes form at ER-mitochondria contact sites, Nature 495 (7441) (2013) 389–393.
[124] B. Kornmann, E. Currie, S.R. Collins, M. Schuldiner, J. Nunnari, J.S. Weissman, P. Walter, An ER-mitochondria tethering complex revealed by a synthetic biology screen, Science 325 (5939) (2009) 477–481.
[125] M.J. Phillips, G.K. Voeltz, Structure and function of ER membrane contact sites with other organelles, Nature reviews, Molecular cell biology 17 (2) (2016) 69–82.
[126] F. Korobova, T.J. Gauvin, H.N. Higgs, A role for myosin II in mammalian mitochondrial fission, Curr. Biol. 24 (4) (2014) 409–414.
[127] J.A. Mears, L.L. Lackner, S. Fang, E. Ingerman, J. Nunnari, J.E. Hinshaw, Conformational changes in Dnm1 support a contractile mechanism for mitochondrial fission, Nat. Struct. Mol. Biol. 18 (1) (2011) 20–26.
[128] E. Ingerman, E.M. Perkins, M. Marino, J.A. Mears, J.M. McCaffery, J.E. Hinshaw, J. Nunnari, Dnm1 forms spirals that are structurally tailored to fit mitochondria, J. Cell Biol. 170 (7) (2005) 1021–1027.
[129] Y. Yoon, K.R. Pitts, M.A. McNiven, Mammalian dynamin-like protein DLP1 tubulates membranes, Mol. Biol. Cell 12 (9) (2001) 2894–2905.
[130] C. Brooks, Q. Wei, L. Feng, G. Dong, Y. Tao, L. Mei, Z.J. Xie, Z. Dong, Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins, Proc. Natl. Acad. Sci. U.S.A. 104 (28) (2007) 11649–11654.
[131] M. Karbowski, D. Arnoult, H. Chen, D.C. Chan, C.L. Smith, R.J. Youle, Quantitation of mitochondrial dynamics by photolabeling of individual organelles shows that mitochondrial fusion is blocked during the Bax activation phase of apoptosis, J. Cell Biol. 164 (4) (2004) 493–499.
[132] M. Karbowski, Y.J. Lee, B. Gaume, S.Y. Jeong, S. Frank, A. Nechushtan, A. Santel, M. Fuller, C.L. Smith, R.J. Youle, Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis, J. Cell Biol. 159 (6) (2002) 931–938.
[133] S. Wasiak, R. Zunino, H.M. McBride, Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death, J. Cell Biol. 177 (3) (2007) 439–450.
[134] X. Qi, M.H. Disatnik, N. Shen, R.A. Sobel, D. Mochly-Rosen, Aberrant mitochondrial fission in neurons induced by protein kinase C{delta} under oxidative stress conditions in vivo, Mol. Biol. Cell 22 (2) (2011) 256–265.
[135] L. Griparic, T. Kanazawa, A.M. van der Bliek, Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage, J. Cell Biol. 178 (5) (2007) 757–764.
[136] N. Ishihara, Y. Fujita, T. Oka, K. Mihara, Regulation of mitochondrial morphology through proteolytic cleavage of OPA1, EMBO J. 25 (13) (2006) 2966–2977.
[137] D. Martinvalet, ROS signaling during granzyme B-mediated apoptosis, Mol. Cell. Oncol. 2 (3) (2015), e992639.
[138] P.J. Beresford, D. Zhang, D.Y. Oh, Z. Fan, E.L. Greer, M.L. Russo, M. Jaju, J. Lieberman, Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks, J. Biol. Chem. 276 (46) (2001) 43285–43293.
[139] D. Chowdhury, P.J. Beresford, P. Zhu, D. Zhang, J.S. Sung, B. Demple, F. W. Perrino, J. Lieberman, The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death, Mol. Cell 23 (1) (2006) 133–142.
[140] Z. Fan, P.J. Beresford, D.Y. Oh, D. Zhang, J. Lieberman, Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor, Cell 112 (5) (2003) 659–672.
[141] Z. Fan, P.J. Beresford, D. Zhang, J. Lieberman, HMG2 interacts with the nucleosome assembly protein SET and is a target of the cytotoxic T-lymphocyte protease granzyme A, Mol. Cell. Biol. 22 (8) (2002) 2810–2820.
[142] Z. Fan, P.J. Beresford, D. Zhang, Z. Xu, C.D. Novina, A. Yoshida, Y. Pommier, J. Lieberman, Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A, Nat. Immunol. 4 (2) (2003) 145–153.
[143] L.A. Sazanov, P. Hinchliffe, Structure of the hydrophilic domain of respiratory complex I from Thermus thermophilus, Science 311 (5766) (2006) 1430–1436.
[144] M. Walch, F. Dotiwala, S. Mulik, J. Thiery, T. Kirchhausen, C. Clayberger, A. M. Krensky, D. Martinvalet, J. Lieberman, Cytotoxic cells kill intracellular Bacteria through granulysin-mediated delivery of granzymes, Cell 157 (6) (2014) 1309–1323.
[145] F. Dotiwala, S. Sen Santara, A.A. Binker-Cosen, B. Li, S. Chandrasekaran, J. Lieberman, Granzyme B Disrupts Central Metabolism and Protein Synthesis in Bacteria to Promote an Immune Cell Death Program, Cell 171 (5) (2017) 1125–1137, e11.
[146] M.C. Wei, W.X. Zong, E.H. Cheng, T. Lindsten, V. Panoutsakopoulou, A.J. Ross, K. A. Roth, G.R. MacGregor, C.B. Thompson, S.J. Korsmeyer, Proapoptotic B.A.X. and BAK: a requisite gateway to mitochondrial dysfunction and death, Science 292 (5517) (2001) 727–730.
[147] G. Petrosillo, F.M. Ruggiero, G. Paradies, Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria, FASEB J. 17 (15) (2003) 2202–2208.
[148] G. Petrosillo, F.M. Ruggiero, M. Pistolese, G. Paradies, Ca2+-induced reactive oxygen species production promotes cytochrome c release from rat liver mitochondria via mitochondrial permeability transition (MPT)-dependent and MPT-independent mechanisms: role of cardiolipin, J. Biol. Chem. 279 (51) (2004) 53103–53108.
[149] A. Chacinska, C.M. Koehler, D. Milenkovic, T. Lithgow, N. Pfanner, Importing mitochondrial proteins: machineries and mechanisms, Cell 138 (4) (2009) 628–644.
[150] P. Rehling, K. Model, K. Brandner, P. Kovermann, A. Sickmann, H.E. Meyer, W. Kuhlbrandt, R. Wagner, K.N. Truscott, N. Pfanner, Protein insertion into the

mitochondrial inner membrane by a twin-pore translocase, Science 299 (5613) (2003) 1747–1751.
[151] I. Gentle, K. Gabriel, P. Beech, R. Waller, T. Lithgow, The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria, J. Cell Biol. 164 (1) (2004) 19–24.
[152] S. Moslavac, O. Mirus, R. Bredemeier, J. Soll, A. von Haeseler, E. Schleiff, Conserved pore-forming regions in polypeptide-transporting proteins, FEBS J. 272 (6) (2005) 1367–1378.
[153] E. Estebanez-Perpina, P. Fuentes-Prior, D. Belorgey, M. Braun, R. Kiefersauer, K. Maskos, R. Huber, H. Rubin, W. Bode, Crystal structure of the caspase activator human granzyme B, a proteinase highly specific for an Asp-P1 residue, Biol. Chem. 381 (12) (2000) 1203–1214.
[154] C. Andres, B. Agne, F. Kessler, The TOC complex: preprotein gateway to the chloroplast, Biochim. Biophys. Acta 1803 (6) (2010) 715–723.
[155] A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T. Heer, T.A.P. de Beer, C. Rempfer, L. Bordoli, R. Lepore, T. Schwede, SWISS-MODEL: homology modelling of protein structures and complexes, Nucleic Acids Res. 46 (W1) (2018) W296–W303.
[156] S. Bienert, A. Waterhouse, T.A. de Beer, G. Tauriello, G. Studer, L. Bordoli, T. Schwede, The SWISS-MODEL Repository-new features and functionality, Nucleic Acids Res. 45 (D1) (2017) D313–D319.
[157] N. Guex, M.C. Peitsch, T. Schwede, Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective, Electrophoresis 30 (Suppl 1) (2009) S162–73.
[158] P. Benkert, M. Biasini, T. Schwede, Toward the estimation of the absolute quality of individual protein structure models, Bioinformatics 27 (3) (2011) 343–350.
[159] M. Bertoni, F. Kiefer, M. Biasini, L. Bordoli, T. Schwede, Modeling protein quaternary structure of homo- and hetero-oligomers beyond binary interactions by homology, Sci. Rep. 7 (1) (2017) 10480.
[160] D.C. Jans, C.A. Wurm, D. Riedel, D. Wenzel, F. Stagge, M. Deckers, P. Rehling, S. Jakobs, STED super-resolution microscopy reveals an array of MINOS clusters along human mitochondria, Proc. Natl. Acad. Sci. U.S.A. 110 (22) (2013) 8936–8941.
[161] J. Xie, M.F. Marusich, P. Souda, J. Whitelegge, R.A. Capaldi, The mitochondrial inner membrane protein mitofilin exists as a complex with SAM50, metaxins 1 and 2, coiled-coil-helix coiled-coil-helix domain-containing protein 3 and 6 and DnaJC11, FEBS Lett. 581 (18) (2007) 3545–3549.