Molecular mechanisms of cell death: central implication of ATP synthase in mitochondrial permeability transition

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Abbreviations: CsA, cyclosporin A; MOMP, mitochondrial outer membrane permeabilization; MPT, mitochondrial permeability transition; PTPC, permeability transition pore complex.

Abstract

The term mitochondrial permeability transition (MPT) is commonly employed to indicate an abrupt increase in the permeability of the inner mitochondrial membrane to low molecular weight solutes. Widespread MPT has catastrophic consequences for the cell, *de facto* marking the boundary between cellular life and death. MPT results indeed in the structural and functional collapse of mitochondria, an event that commits cells to suicide via regulated necrosis or apoptosis. As MPT plays a central role in the etiology of both acute and chronic diseases characterized by the loss of post-mitotic cells, intense efforts have been dedicated not only at the understanding of MPT in mechanistic terms, but also at the development of pharmacological inhibitors. In this setting, multiple mitochondrial and extramitochondrial proteins have been suspected to critically regulate the MPT. Until recently, however, only peptidylprolyl isomerase F (PPIF, best known as cyclophilin D) appeared to constitute a core component of the so-called permeability transition pore complex (PTPC), the supramolecular entity that is believed to mediate the MPT. Here, after reviewing the structural and functional features of the PTPC, we summarize recent findings suggesting that one of its core components is represented by the c subunit of mitochondrial ATP synthase.

Mitochondrial permeability transition and cell death

The expression “mitochondrial permeability transition” (MPT) is commonly employed to indicate a brisk increase in the permeability of the inner mitochondrial membrane to low molecular weight (MW) solutes. This results in the osmotic influx of water into the mitochondrial matrix, followed by the structural and functional collapse of affected mitochondria.[1, 2] According to current models, the MPT would be mediated by the so-called permeability transition pore complex (PTPC), a supramolecular entity assembled at the interface between the inner and the outer mitochondrial membranes.[1, 3] The first description of MPT dates back to 1979, when this phenomenon was shown to stem from the accumulation of Ca2+ ions in the mitochondrial matrix and to be responsive to Mg2+ ions and ADP.[4] However, the interest in MPT dropped until the mid-1990s, when it became evident that mitochondria play a central role in the regulation of cell death elicited by several stimuli.[5, 6] Indeed, while a few mitochondria that have undergone the MPT do not cause major problems as they can be efficiently removed by the autophagic machinery,[7] widespread MPT commits the cells to death via regulated necrosis or apoptosis (Figure 1).[2] MPT-driven regulated necrosis mainly (but not only) reflects the bioenergetic consequence of MPT, i.e., the immediate dissipation of the mitochondrial transmembrane potential ($\Delta\psi m$) and arrest in all $\Delta\psi m$-dependent mitochondrial activities, including ATP synthesis [8, 9]. Conversely, MPT-driven apoptosis is mainly executed by mitochondrial intermembrane proteins that are released in the cytoplasm upon MPT, including (but not limited to) holocytochrome c, apoptosis-inducing factor, mitochondrion-associated, 1 (AIFM1, best known as AIF), and diablo, IAP-binding mitochondrial protein (DIABLO, also known as Smac) [10, 11]. As the apoptotic phenotype requires the activation of caspases,[12] cysteine proteases that operate in an ATP-dependent fashion,[13] whether MPT drives apoptosis or regulated necrosis may depend on the intracellular availability of ATP [14]. However, other parameters may determine, at least in part, the catabolic pathways activated by MTP, including the nitrosylation state of caspases [14]and the expression levels of endogenous caspase modulators.([15], [16])

Throughout the last two decades, robust genetic evidence has incriminated MPT as a major etiological determinant in a wide panel of acute and chronic disorders characterized by the unwarranted loss of post-mitotic cells. These conditions include, but are not limited to, ischemia/reperfusion injury of the brain,[17] heart, ([18], [19], [20]) and kidney; [21] neurodegenerative disorders [22] toxic syndromes, [23], [24]) diabetes [25] and myopathic/dystrophic disordersù [26, 27]. Along with the recognition that the MPT plays a critical role in multiple pathophysiological scenarios characterized by the excessive demise of post-mitotic cells, great interest gathered around the possibility that pharmacological inhibitors of MPT or mitochondrial outer membrane permeabilization (MOMP),[11] the major mechanism underlying intrinsic apoptosis, would mediate therapeutically relevant cytoprotective effects [28]. This translated into an intense wave of investigation that unveiled multiple mechanistic details about MPT and allowed for the characterization of various pharmacological and endogenous MPT modulators [29]. Thus, besides the accumulation of mitochondrial Ca^{2+}, major MPT stimulators include reactive oxygen species, inorganic phosphate, intracellular acidification, as well as atractyloside and carboxyatractyloside, which inhibit several members of the adenine nucleotide translocase (ANT) protein family by locking them in cytoplasmic side open conformation [3]. Conversely, among various molecules, the MPT is inhibited by ATP and ADP, NADH and NAD^+, glutamate, as well as by bongkrekic acid, which locks ANT family members in a matrix side open conformation, 5-isothiocyanato-2-[2-(4-isothiocyanato-2-sulfophenyl)ethenyl]benzene-1-sulfonic acid (DIDS), an inhibitor of voltage-dependent anion channel (VDACs), and cyclosporine A (CsA), which targets peptidylprolyl isomerase F (PPIF, best known as cyclophilin D, CYPD)[29].

The MPT-inhibitory potential of CsA has been documented so extensively, *in vitro* and *in vivo*, that this molecule is currently considered as the gold standard means for the confirmation of presumed instances of MPT.[30] Nonetheless, caution should be employed to interpret the effects of CsA, especially those observed *in vivo*, as this chemical is endowed with potent immunosuppressive

properties (reflecting its ability to indirectly inhibit calcineurin) [31].Thus, to ascribe with relative certainty a murine phenotype to the MPT, it is imperative to confirm the *in vivo* cytoprotective effects of CsA in *Ppif*$^{-/-}$ animals (see below), and to demonstrate that these two experimental interventions show a null epistatic interaction.

In spite of the intense experimental interest generated by the MPT throughout the last two decades, the precise molecular composition of the PTPC remains elusive. After summarizing the main structural and functional features of the PTPC discovered so far, here we discuss recent findings suggesting that one of its core components is represented by the c subunit of mitochondrial ATP synthase.

Architecture of the permeability transition pore complex

Core components. In the early 1990s, electrophysiological studies based on purified mitoplasts (i.e., mitochondria stripped of the outer membrane) demonstrated that the MPT corresponds to an significant increase in the conductance of the inner mitochondrial membrane, [32] pointing to the existence of a pore that would be responsible for this transition. Such a "mitochondrial megachannel" was rapidly found to share several features with the MPT, including its sensitivity to Ca^{2+} ions (which operate as activators) as well as to CsA and various divalent cations, including Mg^{2+} (all of which operate as inhibitors) [32, 33]. Shortly thereafter, the mitochondrial megachannel turned out to exhibit a voltage-dependent behavior, in thus far resembling VDAC [34]. In support of a critical role for VDAC in MPT, purified VDAC molecules reconstituted in planar bilayers or proteoliposomes were shown to form a dimeric channel that exhibited electrophysiological properties compatible with those of the mitochondrial megachannel [35]. Such an unexpected link between a protein of the outer mitochondrial membrane, VDAC, and a phenomenon that involves the inner mitochondrial membrane, the MPT, casted suspects on the actual composition of the mitochondrial megachannel, raising the possibility that it would be constituted by several proteins, not just one. Further supporting this hypothesis, a ligand of the peripheral benzodiazepine receptor (which was already known to involve VDAC, ANT and a third component) [36] was found to elicit currents from otherwise electrically silent mitoplasts [37].

Brdiczka and colleagues confirmed the supramolecular nature of the PTPC in 1996, when they documented a complex comprising VDAC, ANT, hexokinase 1 (HK1) and creatine kinase, mitochondrial 1 (CKMT1) exhibiting MPT-like electrical activity upon reconstitution in liposomes [38, 39]. Based on its interacting partners (including VDAC and ANT) [40] as well as on its pharmacological profile,[41, 42] CYPD was soon suspected to play a central role in the MPT. In the late 1990s, purified ANT molecules reconstituted in proteoliposomes were found to form an oligomeric channel exhibiting PTPC-like functional properties [43]. Cumulatively, these findings

inspired a first PTPC model according to which the MPT would be mediated by a supramolecular entity assembled at the interface between the inner and outer mitochondrial membrane by the physical and functional interactions of VDAC, ANT, HK1 and CKMT1. In line with its suborganellar localization (the mitochondrial matrix), CYPD was considered by this model as a regulator of the PTPC, but not as one of its pore-forming subunits.

Robust genetic data generated in the mid-2000s significantly challenged most components of its model. Thus, the simultaneous knockout of the genes coding for two distinct ANT isoforms, i.e., *Slc25a4* (encoding Ant1) and *Slc25a5* (encoding Ant2) failed to abolish the ability of murine hepatocytes to succumb to several MPT inducers, including the Ca^{2+} ionophore Br-A23187, in a CsA-inhibitable manner [44]. In line with this notion, mitochondria isolated from *Slc25a4*$^{-/-}$*Slc25a5*$^{-/-}$ hepatocytes retained the ability to undergo MPT *in vitro* upon exposure to a depolarizing agent, yet become irresponsive to atractyloside and ADP [44]. Similarly, the simultaneous genetic inactivation of three distinct VDAC isoforms, namely, Vdac1, Vdac2 and Vdac3, neither altered the propensity of murine fibroblasts to die when challenged with hydrogen peroxide (an MPT inducer), nor it influenced the ability of their mitochondria to undergo MPT in response to Ca^{2+} [45, 46]. At odds with these relatively minor effects, the standalone deletion of *Ppif* turned out to mediate major MPT-inhibitory and cytoprotective effects, *in vitro* as well as *in vivo*, in several models of acute ischemic injury [17-19, 47].In particular, the absence of CYPD was shown to dramatically increase the amount of Ca^{2+} ions required to trigger the MPT and to render this process completely insensitive to CsA [46, 47].

Taken together, these data apparently demonstrate that (1) ANT and VDAC are dispensable for both the execution and the regulation of the MPT; while (2) CYPD plays a crucial role in the process. This said, a central function for ANT in MPT cannot be formally excluded yet, as at least 2 additional ANT isoforms turned out to be encoded by the mammalian genome, namely, SLC25A6 (ANT3) and SLC25A31 (ANT4) [48, 49]). So far, no VDAC isoforms others than VDAC1, VDAC2 and VDAC3

have been identified (source http://www.ncbi.nlm.nih.gov/gene/). Nonetheless, the results of Baines and colleagues were obtained with *Vdac1*$^{-/-}$*Vdac3*$^{-/-}$ cells subjected to the temporary depletion of Vdac2 by small-interfering RNAs (siRNAs),[45, 46] an experimental system that appears somehow less robust than the simultaneous deletion of all Vdac-coding genes (which cannot be achieved as the knockout of *Vdac2* is lethal) [50]. Finally, it seems unlikely that CYPD, which is mainly localized within the mitochondrial matrix, would constitute the actual pore-forming component of the PTPC. In line with this notion, CYPD is currently viewed as the major gatekeeper of MPT, regulating the opening of the PTPC but not lining up the pore that physically allows for the entry of low MW solutes into the mitochondrial matrix. This said, the possibility that CYPD may change conformation and become able to form pores in the inner mitochondrial membrane during MPT, similar to what BAX does in the course of MOMP, [51] has not yet been formally excluded.

Inorganic phosphate has been identified very early as an MPT-promoting metabolite, [52]suggesting that the PTPC would possess a specific binding site. In physiological conditions, inorganic phosphate is transported across the inner mitochondrial membrane by members of the SLC protein family, including SLC25A3 (best known as PHC or PiC) and SLC25A24 (also known as APC1) [53]. While PiC imports inorganic phosphate into mitochondrial matrix coupled to either the co-import of H^+ ions or the export of OH^- ions, APC1 mediates this process along with the export of ATP and Mg^{2+} ions [54]. In 2003, APC1 was suggested to be responsible for the MPT-promoting activity of inorganic phosphate via an indirect effect on the mitochondrial pool of ATP and ADP, [55] a notion that has not been confirmed. Rather, it seems that SLC25A24 responds to increases in cytosolic Ca^{2+} levels by operating in reverse mode, thus favoring the mitochondrial uptake of ATP and ADP and inhibiting MPT [56]. In 2008, PiC was shown to bind CYPD and ANT1 *in cellula*, an interaction that was potentiated by MPT-inducing conditions and inhibited by CsA [57]. Along similar lines, a high-throughput genetic screen unveiled that PiC overexpression promotes mitochondrial dysfunction coupled to apoptotic cell death [58]. Also in this study PiC was found to interact with ANT1 (as well

as with VDAC1), especially in the presence of MPT inducers [58]. Moreover, the siRNA-mediate depletion of PiC exerted cytoprotective effects [58]. Together with previous data indicating that the reconstitution of liposomes with purified PiC molecules results in the formation of relatively unspecific pores[59]. These findings pointed to PiC as to the possible pore-forming unit of the PTPC. This hypothesis is incompatible with recent results indicating that a consistent reduction in PiC levels does not alter the ability of isolated mitochondria to undergo MPT in response to Ca^{2+} ions [60]. Thus, either PiC does not participate into the PTPC in a significant manner, either very small amounts of PiC are sufficient to mediate the MPT. As a corollary, this suggests that the cytoprotective effects of PiC depletion[58] may not stem from the modulation of MPT. The exact molecular mechanisms by which PiC promotes cell death under some circumstances remain to be elucidated.

Regulatory components. Several proteins have been shown to regulate the activity of core PTPC units (i.e., VDAC, ANT and CYPD). These regulatory components, which encompass cytosolic as well as mitochondrial proteins, appear to interact with the PTPC backbone in a highly dynamic manner [61].

The translocator protein (18kDa) (TSPO), a protein of the outer mitochondrial membrane, constitutes the benzodiazepine-binding component of the so-called peripheral benzodiazepine receptor, an oligomeric complex involving VDAC and ANT (see above)[36]. The physiological role of TSPO in steroid biosynthesis was described as early as in [62] and only a few years later circumstantial evidence implicating TSPO in the MPT began to accumulate. For the most part, these studies reported the ability of a series of endogenous (e.g., protoporphyrin IX) [63] and exogenous (e.g., PK11195, Ro5-4864, diazepam [64, 65] TSPO agonists to drive isolated mitochondria into MPT. In line with this notion, the incubation of purified mitochondria with a TSPO-blocking antibody reportedly inhibits several manifestations of MPT [66]. This said, the effects of TSPO ligands on cell death exhibit a great degree of variability, ranging from cytoprotective, [67, 68] to overtly lethal [69, 70].

Such a context dependency may stem from several causes, including (but presumably not limited to) model-intrinsic variables (including the expression levels of TSPO and other benzodiazepine receptors) and the concentration of TSPO-modulatory agents employed [71].

Various kinases have been shown to physically and/or functionally interact with core PTPC units, including CKMT1 (which is localized in the mitochondrial matrix), hexokinase 1 (HK1), HK2 as [61]. Some of these kinases, including CKMT1, HK1 and HK are unable to phosphorylate protein substrates, implying that their MPT-modulatory activity originates either from their physical interaction with core PTPC components or from their ability to catalyze metabolic reactions. CKMT1 not only binds VDAC1 and ANT1 [38, 39] but also phosphorylates creatine to generate phosphocreatine, a reaction that is tightly coupled to oxidative phosphorylation (and hence to the availability of ATP and ADP) [72, 73]. HKs catalyze the rate-limiting step of glycolysis, converting glucose into glucose-6-phosphate in an ATP-dependent manner [74]. Both HK1 and HK2 interact with multiple VDAC isoforms, hence gaining a preferential access to the export of mitochondrial ATP [75]. This configuration (i.e., the binding of HKs to VDAC) is associated with an optimal flux through glycolysis as well as with major cytoprotective effects [76]. In line with this notion, the administration of cell-permeant peptides or chemicals that competitively displace HK2 from VDAC1 has been shown to kill several types of cells upon MPT [77-80]. However, it remains unclear to which extent such a cytotoxic response reflects a direct modulation of the PTPC by HK2 over an indirect effect on the availability of antioxidants (cancer cells exploits glycolysis to boost the pentose phosphate pathway, which is critical for the regeneration of NAD(P)H and hence reduced glutathione) [81, 82]. The fact that the MPT-inducing activity of peptides disrupting the HK2/VDAC1 interaction is inhibited by CsA and bongkrekic acid, as well as by the ablation of *Ppif*, but not by that of *Vdac1* and *Vdac3*, [83] suggests that the PTPC-regulatory function of HKs mainly stem from a metabolic effect. Further supporting this notion, HK1 has recently been found to exert major cytoprotective effects in MPT-unrelated paradigms of death [84].

Contrarily to CKMT1 and HKs, GSK3β and PKCε exert MPT-modulatory functions that have been linked (at least partially) to their ability to phosphorylate core PTPC components [85-87]. For instance, active GSK3β has been reported to phosphorylate VDAC1, resulting in the MPT-stimulatory displacement of HKII, [85] and VDAC2, promoting the consumption of ATP by ischemic mitochondria (a process that is also expected to promote MPT), [88] while GSK3β phosphorylated on S9 (i.e., inactive) appears to inhibit the PTPC by physically disrupting the ANT1/CYPD interaction [89]. Recently, the activation of GSK3β has also been linked to the MPT-triggering phosphorylation of CYPD [90, 91]. However, formal evidence supporting the notion that GSK3β directly phosphorylates CYPD is lacking, specially in front of the fact that these proteins display different mitochondrial sub-compartimentalization (mitochondrial matrix for CYPD and external surface of outer mitochondrial membrane for GSK3β) [90]. PKCε has also been reported to phosphorylate VDAC1, yet this post-translational modification appears to promote, rather than destabilize, HK2 binding [86]. However, as the activation of PKCε by a synthetic peptide has been associated with the inactivating dephosphorylation of GSK3β[92]. it is not clear whether the effect of PKCε on the VDAC1/HK2 interaction *in cellula* actually reflect a direct phosphorylation event or a GSK3β-dependent signaling circuitry. As a matter of fact, the activation of several upstream signal transducers, including AKT1, mechanistic target of rapamycin (MTOR), protein kinase A (PKA) and protein kinase, cGMP-dependent, type I (PRKG1, best known as PKG) reportedly converge on the inactivation of GSK3β, hence mediating MPT-inhibitory effects [87, 93, 94]. A detailed description of these signaling pathways, which play a significant role in ischemic conditioning and cardioprotection, goes largely beyond the scope of this review [95].

Of note, the core units of the PTPC have been shown to interact with several components of the machinery that control MOMP, including both pro- and anti-apoptotic members of the Bcl-2 protein family [50, 96-103] as well as p53 [104]. BCL-2 and BCL2-like 1 (BCL2L1, best known as BCL-X_L) have been proposed to inhibit MPT by regulating the opening state of VDAC1 [99], [100]. This

said, whether the MPT-modulatory activity of anti-apoptotic BCL-2 family members originates from an increase or a decrease in VDAC1 conductance remains a matter of debate. Irrespective of this conundrum, BAX, BAK1 and BCL-2-like 11 (BCL2L11, a BH3-only protein best known as BID) reportedly promote MPT-driven apoptosis by interacting with ANT1 and/or VDAC1 [96, 98, 105]. Along similar lines, BCL2-associated agonist of cell death (BAD, another BH3-only protein) has been shown to trigger a VDAC1-dependent, BCL-X_L-responsive mechanism of MPT [101]. In this context, however, MPT appears to result from the BAD-dependent displacement of BCL-X_L from VDAC1 rather than from a physical BAD/VDAC1 interaction [101]. Finally, by sequestering the BAX-like protein BAK1, VDAC2 reportedly exerts MOMP-inhibitory functions [50]. Thus, the molecular machineries for MOMP and MPT engage in complex, mutually regulatory cross-talk.

Recent data indicate that a pool of p53 localized to the mitochondrial matrix participate in MPT-driven regulated necrosis by interacting with CYPD[104]. These findings add to an increasing amount of data arguing against the classical apoptosis/necrosis dichotomy. BAX and BAK1 are indeed being implicated in several paradigms of necrotic, as opposed to apoptotic, cell death, [20, 106] perhaps reflecting their ability to regulate mitochondrial dynamics,[20] or Ca2+ homeostasis [107-112]. Further studies are required to obtain precise insights into this issue.

In summary, in spite of a significant experimental effort, the precise molecular composition of the PTPC remains elusive (**Figure 2**). Accumulating evidence indicate that the mitochondrial ATP synthase, the multiprotein complex that catalyzes the synthesis of ATP while dissipating the chemiosmotic gradient generated by the respiratory chain across the inner mitochondrial membrane, constitutes a central PTPC component, as discussed below.

Mitochondrial ATP synthase: structure, function and implication in mitochondrial permeability transition

Molecular composition of mitochondrial ATP synthase. The mitochondrial ATP synthase is a large multiprotein complex consisting of a globular domain that protrudes into the mitochondrial matrix (F_1 domain, also known as soluble component) and an inner mitochondrial membrane-embedded domain (F_O domain), which are interconnected by a central and a lateral stalk. Owing to this molecular arrangement, the ATP synthase is also known as F_1F_O-ATPase, [113]. Mammalian ATP synthases contain 15 different subunits: α, β, γ, δ, ε, a, b, c, d, e, f, g, A6L, F6 and O (also known as oligomycin sensitivity-conferring protein, OSCP) forming a fully functional holoenzyme with a total MW of ~ 600 kDa. The α, β, γ, a, and c subunits exhibit a high degree of homology to their chloroplast and bacterial counterparts. Moreover, the overall topology of the mammalian ATP synthase as well as that of its F_1 and F_O components taken individually are highly conserved across evolution [113-115]. The mammalian F_1 domain is composed of three α/β dimers and interacts with one copy of the γ, δ and ε subunits (central stalk) as well as with the b, d, F6 and O subunits (peripheral stalk), providing a physical bridge between the soluble and proton-translocating (F_O) components of the holoenzyme [115-117]. The F_O domain contains a ring-shaped oligomer of c subunits stabilized by binding of cardiolipin, an lipid that is highly enriched in (if not confined to) the inner mitochondrial membrane, [115, 118]. Of note, the number of c subunits composing the so-called c-ring varies to a significant extent across species (10 in humans) [115]. These components of the F_O domain are highly hydrophobic and contain a critical carboxyl group (most often as part of a Glu or Asp residue) that is directly involved in the translocation of H+ ions across the inner mitochondrial membrane (see below)[119]. The remaining constituents of ATP synthase, i.e., the a, e, f, g, and A6L subunits, are also part of the F_O domain and interact with the c-ring. In particular, the a subunit provides a physical dock for the b subunit, while A6L appears to bridge F_O to other components of the peripheral stalk (**Figure 3**) [115-117, 120].

The roles of individual F_1F_O-ATPase subunits in ATP synthesis. Mitchell's chemiosmotic model, which is still largely accepted, postulated that the F_1F_O-ATPase is able to dissipate in a controlled fashion the electrochemical gradient generated across the inner mitochondrial membrane by respiratory chain complexes to condense ADP and inorganic phosphate into ATP [121]. Several decades of investigation, focusing for a large part on bacterial and bovine systems, have generated profound insights into the molecular mechanisms whereby the mitochondrial ATP synthase operate[115].

According to current models, the electrochemical gradient built up by the respiratory chain is dissipated as H^+ ions flow between the a subunit and the c-ring, imparting to the latter a relative rotation that is passed to the γ and ε subunits [122]. The rotation of the central stalk (approximate radius = 1 nm) inside a cylindrical lodge formed by the α3β3 hexamer (approximate radius = 5 nm) has been shown to cause conformational changes in the F_1 that drive ATP synthesis [122]. Each β subunit contains a nucleotide-binding site (which is localized at the interface with one of the adjacent α subunits) and can assume three discrete conformations: (1) the so-called βDP conformation, which is characterized by an elevated affinity for ADP; (2) the so-called βTP conformation, exhibiting a high affinity for ATP; and (3) the so-called βE conformation, displaying reduced affinity for ATP, [123]. Importantly, these three states invariably coexist on an individual F_1 domain, implying that the transition between conformations at distinct α/β interfaces is coordinately regulated [123].

The central stalk of ATP synthase can rotate up to 700 times/sec (depending on temperature, substrate availability and other factors), and each 360° turn results in the synthesis of three ATP molecules [123]. Detailed studies revealed that the γ subunit of the central stalk rotates in discrete 120° steps and that its interaction with a β subunit in the βTP conformation causes the release of ATP from the nucleotide-binding site (*i.e.*, the transition to the βTE state) [124]. Interestingly, it has been suggested that such discrete 120° steps might consist of 30-40° and 80-90° substeps, at least when "slow" ATPase variants (which release ATP at reduced rates) are concerned [125]. Of note, similar properties

could be ascribed neither to hybrid F_1 subunits containing only 1 or 2 slow β subunits, [126] nor to so-called V_1V_O-ATPases, [127, 128] variants of F_1F_O-ATPases that generally operate in reverse mode to catalyze the acidification of specific subcellular compartments [129]. Thus, whether the rotation of normal ATPases occurs in discrete substeps <120° remains to be formally demonstrated.

Irrespective of this unresolved mechanistic issue, ATP synthases appear to catalyze the condensation between ADP and inorganic phosphate by virtue of a functional cooperation between a "rotor" (formed by the c-ring coupled to the γ, δ and ε subunits) and a "stator" (consisting of the α3β3 hexamer plus the a, b, d, e, f, g, F6, A6L and O subunits) [114]. In this context, special attention should be devoted to the peripheral stalk (composed of the b, d, F6 and O subunits), which links the external surface of F_1 to the a subunit of F_O [130]. This separate substructure appears to play two important roles for ATP synthesis: (1) to counteract the tendency of the α3β3 hexamer to rotate along with the central stalk and the c-ring, and (2) to anchor the a subunit [114]. Interestingly, a and A6L are the only subunits of the F_1F_O-ATPase to be encoded by the mitochondrial genome, [131]and are the last ones to be incorporated into the assembling holoenzyme [132].

At the "top" of the F_1 domain, the N-terminal regions of α subunits interact with an OSCP monomer. Electron microscopy-based structural studies of the ATP synthase of *Saccharomyces cerevisiae* demonstrated that the C-terminus of the OSCP is located approximately 90 Å away from the F_1 domain [133]. Of note, the assembly of the latter appears to critically rely on the presence of the ε subunit of the central stalk, which may also be involved in the incorporation of c subunits into the c-ring [134]. These findings indicate that specific subunits of the ATPase synthase orchestrate the assembly of the catalytically active holoenzyme.

Supramolecular organization of the ATP synthase. Native blue electrophoresis-based experiments coupled to in-gel activity assays have been employed to demonstrate that the F_1F_O-ATPase exist not only as a monomer, but also as a dimer and higher-order oligomers (mainly tetramers and hexamers [135, 136]. Such oligomers are detectable when mitochondrial proteins are solubilized with mild

detergents, such as solutions that contain limited amounts of digitonin [137]. Conversely, when n-dodecyl β-D-maltoside is used for solubilization, most ATP synthase complexes are expected to appear in their monomeric form on native blue gels. Electron cryotomography-based studies demonstrated that the mammalian ATP synthase is arranged in 1 μm-long rows of dimeric supercomplexes that are located at the apex of mitochondrial cristae, a spatial configuration that favors effective ATP synthesis under proton-limited conditions [138]. Electron cryotomography followed by subtomogram averaging revealed that ATP synthase monomers from *S. cerevisiae* form symmetrical V-shaped dimers with an angle of 86° [139]. Specific components of the yeast F_1F_O-ATPase (i.e., the e and g subunits as well as the first transmembrane helix of subunit 4) appears to be required for the formation of ATP synthase dimers [139-141]. The critical involvement of the e and g subunits in the dimerization of the F_1F_O-ATPase has also been documented in the mammalian system [142, 143]. Moreover, the dimerization of the mammalian F_1F_O-ATPase appears to require the a and A6L subunits [132].

Of note, it appears that ATP synthase dimers contribute to the maintenance of the mitochondrial morphology as they the promote the formation of highly curved cristae ridges [139]. In line with this notion, as *Podospora anserina* (a filamentous fungus) ages, ATP synthase dimers dissociate into monomers, a degenerative process that is associated with the loss of mitochondrial cristae [144]. The ATPase inhibitory factor 1 (ATPIF1), a heat-stable protein that inhibits ATP synthesis as it stimulates F_1F_O-ATPase to operate in reverse mode[145, 146] has also been implicated in the dimerization of the ATP synthase [147]. Crystallographic and electron microscopy-based studies suggest indeed that dimeric ATPIF1 may stabilize ATPase dimers at the level of the F_1 domains [143, 147].

Importantly, the F_1F_O-ATPase synthesizes ATP from ADP and inorganic phosphate only in the presence an adequate proton-motive force (*pmf*). In mitochondria, such a *pmf* is generated by respiratory chain complexes, establishing a proton concentration gradient (ΔpH) across the inner mitochondrial membrane that underlies the $\Delta\psi_m$. Conversely, in the absence of an adequate *pmf*, the

F_1 hydrolyzes ATP avidly [148]. However, this mechanism accounts to a very limited extent for the lethal effects of MOMP and MMP [1, 11]. Indeed, in response to declines in the mitochondrial *pmf* (such as those induced by hypoxia), ATPIF1 inhibits the hydrolytic activity of F_1, hence avoiding a potentially lethal drop in intracellular ATP levels [149, 150]. In this context, it should be emphasized that the F_1F_O-ATPase would consume ATP of cytosolic origin only (1) if the $\Delta\psi_m$ exceeds the so-called "reversal potential" of ANT, i.e., the value of $\Delta\psi_m$ at which there is no net exchange of ADP and ATP across the inner mitochondrial membrane; and (2) ATP in the mitochondrial matrix cannot be provided by substrate-level phosphorylation [151-156]. ATPIF1 has recently been shown to limit the translocation of BAX to the outer mitochondrial membrane under pro-apoptotic conditions, presumably as it prevents mitochondrial remodeling [157]. These findings lend further support to the notion that the molecular machineries that regulate mitochondrial dynamics, MOMP and MPT engage in an intimate, mutually regulatory crosstalk [158-160].

The mitochondrial ATP synthase gives the "wedding ring" to the PTPC. Several parameters that alter the threshold for the induction of MPT have also been shown to regulate the catalytic activity of the ATP synthase,[161]. First, the hydrolytic activity of the F_1F_O-ATPase is strongly inhibited by the concurrent binding of ADP and Mg^{2+}, two potent MPT inhibitors, to its catalytic site, a situation known as Mg-ADP block [148]. ADP and Mg^{2+} ions are required for ATP synthesis and limit the catabolic activity of ATP synthase in a non-competitive manner that differs from simple product inhibition [162-166]. Of note, the Mg-ADP block can be resolved by an increase in *pmf*, expelling Mg^{2+} ions and ADP from the inhibitory site, [148, 167]. Inorganic phosphate, a prominent inducer of MPT, has also been proposed to relieve the Mg-ADP block, [148, 168, 169]. Thus, inorganic phosphate concentrations > 5mM robustly activate the hydrolytic activity of the F_1F_O-ATPase, [165, 170, 171]. Second, similar to ANT, [172] the ATP synthase is sensitive to the oxidation of specific cysteine residues (i.e., C294 and C103 in in the α and γ subunit, respectively), resulting in the formation of an inter-subunit, inhibitory disulfide bridge [173]. Moreover, the catalytic activity of the

F_1F_O-ATPase is influenced by $\Delta\psi_m$ and pH [148] which also affect the sensitivity of the PTPC to MPT inducers [174-176].

Similar to the PTPC, the ATP synthase also engages in physical and functional interactions with a large panel of mitochondrial proteins [177]. In particular, the F_1F_O-ATPase has been show to form supercomplexes with ANT family members and PiC (both of which have been involved in MPT and both of which contain oxidative stress-sensitive thiol residues), [178, 179] the so-called ATP synthasomes [180-182]. According to current models, the topological arrangement of ATP synthasomes would maximize the efficiency of ATP production and export, [177, 180-182]. Moreover, the F_1F_O-ATPase reportedly binds CYPD via the peripheral stalk, in particular though OSCP and subunit d [183]. This CsA-sensitive interaction reduces both the synthetic and hydrolytic activity of the ATP synthase [183]. However, the F_1F_O-ATPase-modulatory functions of CYPD only affect the intramitochondrial pool of adenine nucleotide, leaving its cytoplasmic counterpart unaffected, [184]. Finally, several members of the Bcl-2 protein family appear to interact, physically or functionally, with the ATP synthase [185-187]. In particular, BCL-X_L, which is known to inhibit the MPT upon binding to VDAC1 [99, 100] reportedly binds the F_1F_O-ATPase, hence enhancing its synthetic activity [185, 186]. Along similar lines, an amino-terminally truncated version of MCL-1 that localizes to the mitochondrial matrix (as opposed to the full-length MCL-1, which inserts into the outer mitochondrial membrane) not only promotes the activity of the mitochondrial respiratory chain, hence increasing the $\Delta\psi_m$ and stimulating ATP production, but also favors the oligomeric state of ATP synthase and thus preserves mitochondrial ultrastructure [187]. This said, whether MCL-1 physically interacts with one or more F_1F_O-ATPase subunits or whether its effects on the oligomerization of ATP synthase are indirect has not yet been clarified.

Pharmacological data also suggest a link between the F_1F_O-ATPase and MPT. For instance, oligomycin, which inhibits the catalytic activity of the ATP synthase upon binding to the F_O subunit, [188] has been shown to block MPT as induced by erucylphosphohomocholine (an antineoplastic

agent also known as erufosine) as well as by BAX- and tumor necrosis factor receptor 1 (TNFR1)-activating conditions [105, 189-191]. Of note, similar MPT-inhibitory effects could not be ascribed to piceatannol, which inhibits the F_1 domain of ATP synthase [191]. Taken together, these findings suggest that the ATP synthase (in particular the F_O domain) may play a central role in MPT.

In 2013, the suspects about the central implication of the F_1F_O-ATPase in MPT crystallized as Paolo Bernardi's group proposed that the pore-forming unit of the PTPC would consist in ATP synthase dimers [192, 193]. However, the demonstration that ρ^0 cells, which lack mitochondrial DNA, retain a functional PTPC argues against this model [194]. Indeed, in line with the fact that the dimerization of the F_1F_O-ATPase requires the a and A6L subunits (which are encoded by the mitochondrial genome), ρ^0 cells contains (highly unstable) ATP synthase dimers at extremely low levels [132]. Moreover, the dimerization of ATP synthase, which is promoted by ATPIF1 [147] has been associated with MPT-inhibitory and cytoprotective effects in several experimental paradigms [145].Conversely, the relative proportion of F_1F_O-ATPase dimers over monomers decreases in aged cells, correlating with increasing rates of cell death [144]. Of note, such a transition between the dimeric and monomeric form of the ATP synthase appears to stimulated by CYPD,[144] reinforcing the notion that F_1F_O-ATPase oligomers mediate cytoprotective, rather than cytotoxic, effects.

Among the components of the F_O domain, the highly conserved a, b and c subunits are sufficient to allow for the translocation of protons across lipid bilayers [195]. The c subunit has actually been ascribed with pore-forming properties [196, 197]. Moreover, a peptide displaying a high degree of similarity to the c subunit has been proposed to operate as a PTPC regulator, 8 [198, 199]. Driven by these observations and by the fact that the a subunit appears to be dispensable for MPT,[194]. we recently set out to determine the contribution of the c subunit to the PTPC [200].We found that the transient depletion of the c subunit (by means of ATP5G-targeting small-interfering RNAs) prevents MPT as induced by Ca^{2+} and oxidants, while its overexpression dramatically promotes MPT (and hence results in some extent of cell death *per se*) [200]. Of note, the MPT-regulatory effects of

depleting the c subunit were not influenced by the metabolic profile (glycolytic or respiratory) of the cells, nor were they mimicked by the transient depletion of the α subunit (ATP5A1). Moreover, the temporary depletion of the c subunit did not affect the mitochondrial ATP levels,[200] indicating that the effects on MPT that we observed did not reflect changes in the availability of adenine nucleotides. Subsequent work by another group demonstrated not only that the addition of purified c subunits to isolated mitochondria provokes MPT depending on its own phosphorylation state, but also that the c subunit binds Ca^{2+} ions [201]. However, the possibility that c-rings may exist in physiological conditions independently of the other components of ATP synthase has not yet been addressed.

Conclusions and perspectives

In spite of an intense wave of investigation, the precise molecular composition of the PTPC remains to be unveiled. As the MPT is triggered by conditions that promote protein unfolding, it has also been proposed that the PTPC would just assemble by the unspecific interaction of denatured proteins, (virtually) irrespective of their identity [1, 3, 202]. The evidence in support of this theory, however, is rather circumstantial. The study of the PTPC is actually problematic, for at least two reasons. First, several (presumed) core PTPC components exist in multiple isoforms, which significantly complicates the generation of adequate knockout models [44, 45]. Second, many proteins that have been involved in MPT exert key vital functions, a situation that is incompatible not only with the generation of murine knockout models, but also with strategies of stable cellular depletion [203, 204]. This latter issue could be circumvented by knock-in strategies aimed at replacing the wild-type protein with a mutant that is selectively impaired in its capacity to modulate cell death, an approach that was successful for the central MOMP regulator cytochrome *c*. [205].

Here, we propose that the ATP synthase plays a central role in MPT, based on the following observations: (1) the F_1F_O-ATPase and the PTPC share several pharmacological and endogenous modulators; (2) the F_1F_O-ATPase interacts with several MPT regulators, including ANT, PiC and CYPD; (3) the genetic modulation of the levels of the c subunit (the sole ATP synthase component with confirmed conducive capacity) influences the propensity of mitochondria to undergo MPT, *in vitro* and *in cellula*. As it stands, it seems premature to identify in the c subunit of the F_1F_O-ATPase the mysterious pore-forming component of PTPC. Perhaps, the ATP synthasome simply operates as a regulatory dock for an hitherto uncharacterized protein that disrupt the physical integrity of the inner mitochondrial membrane. Further studies based on robust genetic models will have to formally address these possibilities.

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Legends to Figures

Figure 1. **Lethal effects of the mitochondrial permeability transition.** When the inner mitochondrial membrane (IMM) becomes permeable to low molecular weight (MW) solutes, positively charged ions massively flow into the mitochondrial matrix driven by its electronegative nature. This phenomenon, which is commonly referred to as mitochondrial permeability transition (MPT) has two major consequences. First, it coincides with the dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$), virtually abolishing mitochondrial ATP synthesis and several other $\Delta\psi_m$-dependent mitochondrial functions. Second, it drives the massive entry of water into the mitochondrial matrix, causing an osmotic imbalance that results in the breakdown of both

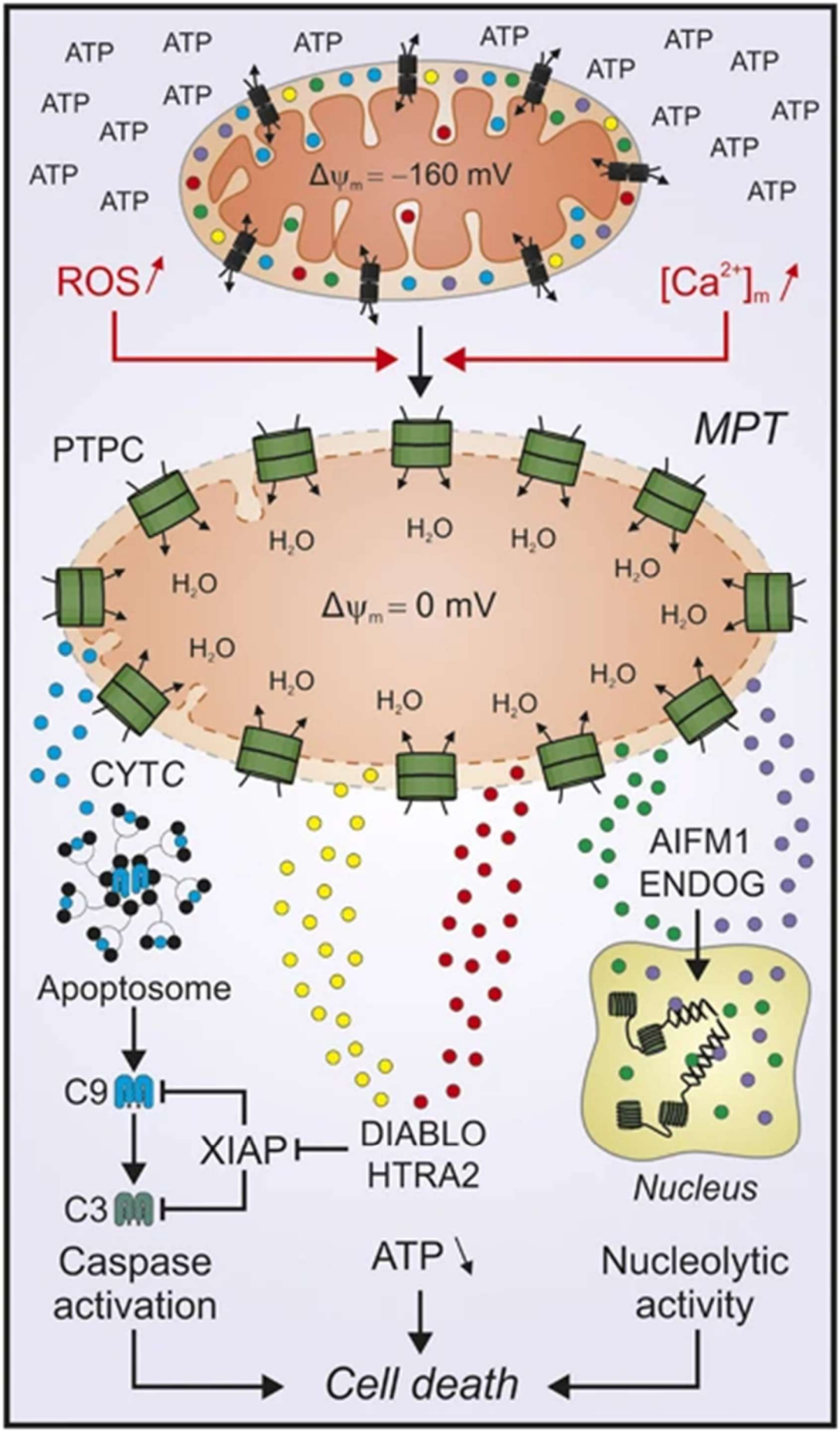
mitochondrial membranes. In turn, this provokes the release into the cytosol of several factors that are normally confined within the intermembrane space (IMS), including (but not limited to) holocytochrome c (CYT*C*), apoptosis-inducing factor, mitochondrion-associated, 1 (AIFM1), endonuclease G (ENDOG), diablo, IAP-binding mitochondrial protein (DIABLO) and HtrA serine peptidase 2 (HTRA2). Thus, depending on multiple parameters, including the global availability of ATP and perhaps the expression levels of caspase inhibitors such as X-linked inhibitor of apoptosis (XIAP), widespread MPT can induce necrotic as well as apoptotic instances of cell death. The latter are dominated by the CYT*C*-dependent activation of the caspase-9 → caspase-3 cascade, which is indirectly favored by both DIABLO and HTRA2. Conversely, the former originate in large part from the bioenergetic crisis that is provoked by MPT coupled to the caspase-independent endonucleolytic activity of AIFM1 and ENDOG. APAF1, apoptotic peptidase activating factor 1; OMM, outer mitochondrial membrane.

Figure 2. **Possible configuration of the permeability transition pore complex.** According to current models, the mitochondrial permeability transition (MPT) is mediated by the opening of a supramolecular entity assembled at the juxtaposition between mitochondrial membranes. Such a large multiprotein complex is commonly known as permeability transition pore complex (PTPC). Structural and functional studies performed throughout the past two decades suggest that multiple mitochondrial and cytosolic proteins intervene in the formation or regulation of the PTPC, yet the actual pore-forming unit of the complex remains elusive. These proteins include (but are not limited to): various isoforms of the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT) and hexokinase (HK); cyclophilin D (CYPD); the inorganic phosphate carrier (PiC), translocator protein (18kDa) (TSPO); creatine kinase, mitochondrial 1 (CKMT1); glycogen synthase kinase 3β (GSK3β); p53; as well as several members of the Bcl-2 protein family. The precise composition of the PTPC however, remains elusive. Recent data indicate that the mitochondrial ATP synthase, in particular the c subunit of the F_O domain, plays a critical role in MPT. Whether the c

subunit truly constitutes the pore-forming unit of the PTPC, however, has not yet been formally demonstrated. IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane.

Figure 3. **Molecular and supramolecular organization of the mammalian ATP synthase.** The mitochondrial ATP synthase consists of a globular domain that protrudes into the mitochondrial matrix (F_1 domain) and an inner mitochondrial membrane (IMM)-embedded domain (F_O domain), which are interconnected by a central and a peripheral stalk. Mammalian ATP synthases contain 15 different subunits: α, β, γ, δ, ε, a, b, c, d, e, f, g, A6L, F6 and O (also known as oligomycin sensitivity-conferring protein, OSCP). The F_1 domain consists of three α/β dimers and interacts with both the central stalk (an γ, δ and ε heterotrimer) and the peripheral stalk (which is composed by b, d, F6 and O subunits). The F_O domain involves a ring-shaped oligomer of c subunits stabilized by cardiolipin as well as the a, e, f, g, and A6L subunits. While the a subunit provides a physical dock for the b subunit, A6L appears to bridge F_O to other components of the peripheral stalk. Notably, the ATP synthase form dimers and higher order oligomer *in cellula*, a process that requires the a, e, g and A6L subunits. The formation of F_1F_O-ATPase dimers is significantly stimulated by ATPase inhibitory factor 1 (ATPIF1), perhaps as this small protein also form dimers that bridge adjacent F_1 domains. In yeast, ATP synthase monomers engaged in dimeric structures adopt a V-shaped conformation that forms an angle of 86°.

Figure 1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ROS
[Ca2+]m
MPT
PTPC
H2O
Δψm = 0 mV
CYTC
AIFM1
ENDOG
Apoptosome
C9
XIAP
DIABLO
HTRA2
C3
Nucleus
Caspase
activation
ATP
Nucleolytic
activity
Cell death

Figure 2

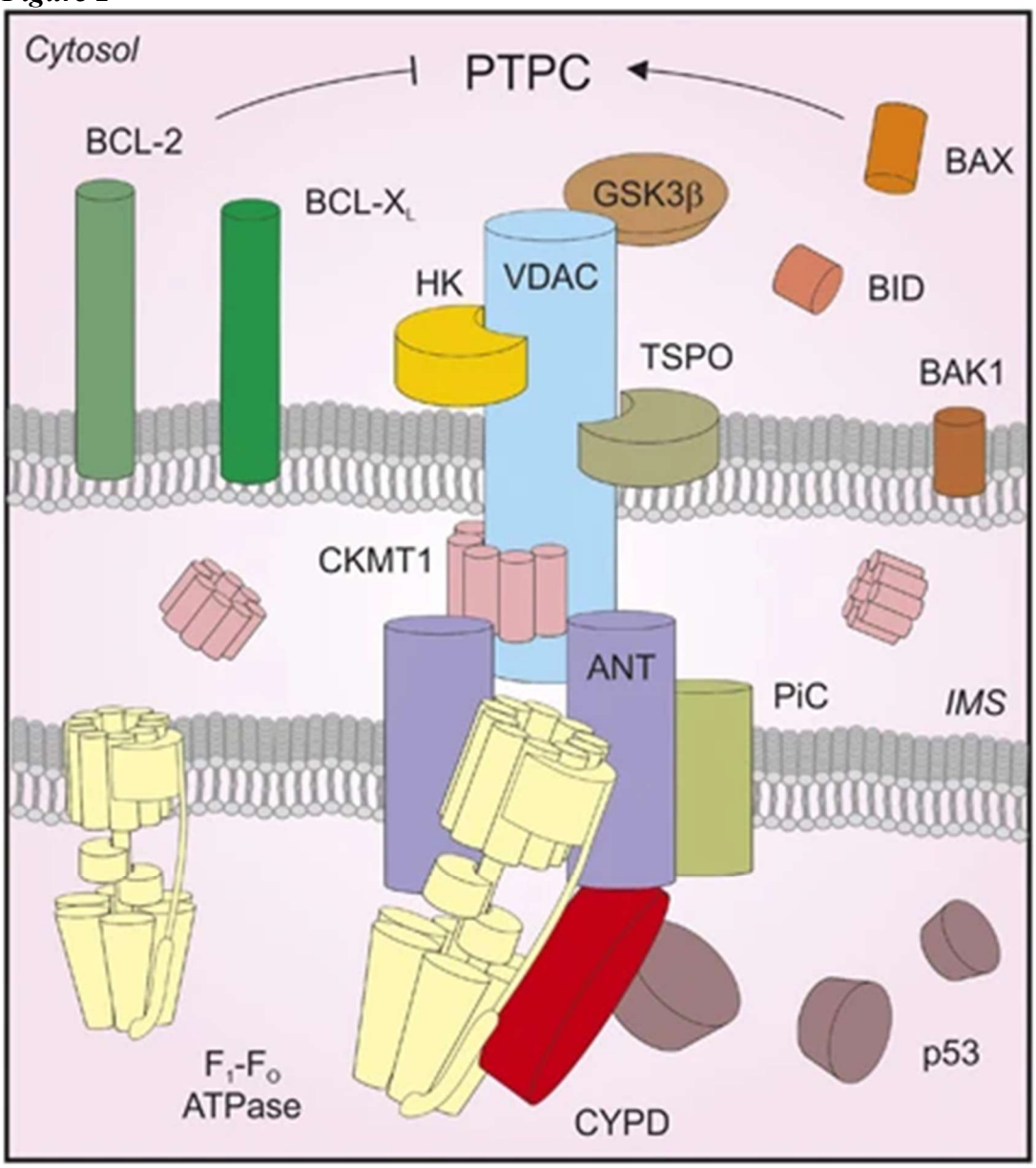
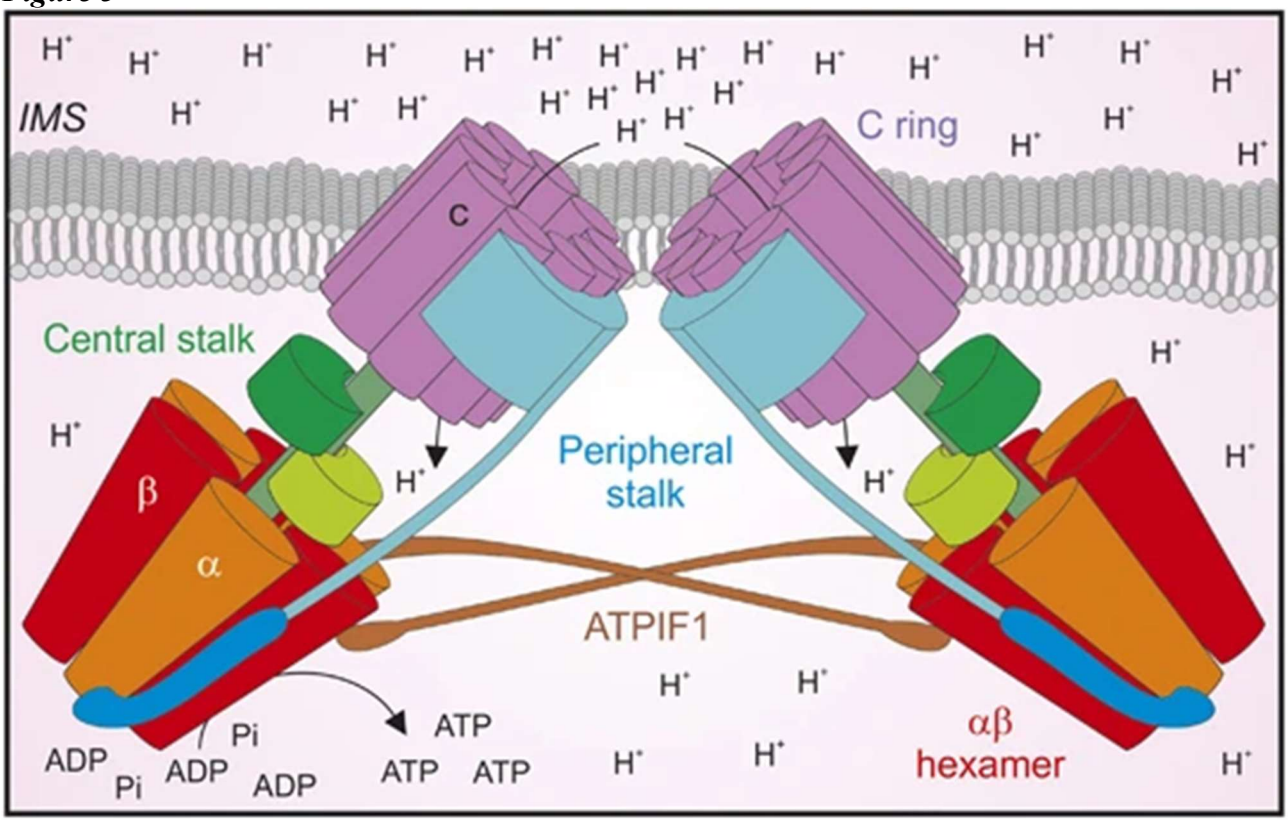

Cytosol
PTPC
BCL-2
BCL-X$_L$
BAX
GSK3β
HK
VDAC
BID
TSPO
BAK1
CKMT1
ANT
PiC
IMS
F$_1$-F$_o$
ATPase
CYPD
p53

Figure 3


IMS
C ring
c
Central stalk
Peripheral stalk
β
α
ATPIF1
ADP
Pi
ADP
Pi
ADP
ATP
ATP
ATP
αβ
hexamer