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Mitochondria, Bioenergetics & Apoptosis in Cancer

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Abstract

Until recently, the dual roles of mitochondria in ATP production (bioenergetics) and apoptosis (cell life/death decision) were thought to be separate. New evidence points to a more intimate link between these two functions, mediated by the remodeling of the mitochondrial ultra-structure during apoptosis. While most of the key molecular players that regulate this process have been identified (primarily membrane proteins), the exact mechanisms by which they function are not yet understood. Because resistance to apoptosis is a hallmark of cancer, and because ultimately all chemotherapies are believed to result directly or indirectly in induction of apoptosis, a better understanding of the biophysical processes involved may lead to new avenues for therapy.

Keywords

Mitochondria; apoptosis; cytochrome C; cristae

The dual role of mitochondria in energetics and apoptosis

The mitochondria is a double membraned organelle believed to have been integrated into modern eukaryotes via symbiosis of a proteobactirum into an anaerobic pre-eukaryotic (host) cell 1.5-2 billion years ago¹. According to modern thinking (pioneered by Mitchell²), an essential role of mitochondria is to produce adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS). In this process, the chemical energy stored in nutrients (carbohydrates, fats, etc.) is converted to an electrochemical gradient across the inner mitochondrial membrane via the electron transport chain (ETC) complexes. This electrochemical gradient acts as a “storage” of energy. ATP synthase uses this stored energy to convert adenosine diphosphate (ADP) to ATP. This bioenergetic picture of the role of mitochondria is now widely accepted. A second role of mitochondria is in the so-called “intrinsic” apoptosis pathway. This pathway converges (figuratively and literally) at the membrane of the mitochondria. Upon certain cell death signals (such as reactive oxygen species (ROS), DNA damage, etc), the outer membrane of mitochondria becomes permeable enough to release the soluble heme protein cytochrome C (cytc), as well as Smac/Diablo,

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endonuclease G, and other intermembrane space proteins, which irreversibly activate downstream caspases to carry out the apoptosis process.

This article summarizes several recent experimental results which indicate that the mitochondrial ultrastructure³ is intimately involved in the relationship between the bioenergetic⁴ and apoptotic^{5,6,7,8} (cell life/death decision) roles of mitochondria. The discussion summarizes recent advances in 1) New understanding of the role of ultrastructure and the location of membrane complexes on mitochondrial bioenergetics; 2) New understanding of the role of ultrastructure in apoptosis; 3) An emerging, albeit incomplete, model for the relationship between 1 and 2. The article is written from a biophysical perspective. The main message of this article is that one *must* approach mitochondria and cancer from this perspective in order to completely understand apoptosis and, hence, cancer; a classical DNA sequencing/biochemical pathway analysis approach is insufficient for a complete and useful understanding of the system. Since resistance to apoptosis is a hallmark of cancer⁹, a better understanding of the biophysical processes involved in apoptosis may lead to new avenues for therapy. Existing therapeutic trials targeting this mechanism are also summarized.

This message is substantiated with the following key (“tentpole”) points 1) The mitochondria sustain a membrane potential which is not uniform along the inner membrane due to its ultra-structure and distribution of membrane proteins involved in the OXPHOS process; 2) the change in the ultrastructure (“remodeling”) occurs during both metabolic changes and apoptosis; 3) A growing body of recent evidence indicates that the bioenergetic and apoptotic functions are linked through this ultrastructure, and 4) A comprehensive model that explains this link is only in its early stages and incomplete, 5) since this is a dynamic biophysical, active electrophysiological system, with localized pH and voltage gradients mediated by and affected by a hierarchical organization of active components (ionic, protein, membrane, organelle ultrastructure, and organelle-organelle interactions), there is significant technological challenge in pinpointing the location, function, and mechanism of all the components (membrane proteins, inner and outer membrane, inner membrane structure, and membrane pore formation during apoptosis), and 6) in spite of these challenges, pharmacological manipulation of apoptosis based on current understanding is a successful route for targeted therapies and indirectly is the target of all chemotherapies, making studies of this complicated system a priority for the field of cancer.

Bioenergetics: Electron Transport Chain and Cristae

The sources and sinks of the membrane potential are not uniformly distributed along the inner membrane

In 1961, Mitchell postulated² that energy is converted from chemical energy in carbohydrates and fats to an electrochemical gradient at the mitochondrial inner membrane, and that this electrochemical gradient is the energy source with drives ATP synthesis. Now known as the chemi-osmotic hypothesis (Box 1), this model is widely accepted. While the molecular constituents of the ETC complexes and ATP synthase are mostly identified, his model says nothing about their spatial distribution within the organelle, a key missing component (Figure 1). The mitochondrial inner membrane is invaginated into a dynamically

changing cristae ultra-structure (Figure 2). (Anatomically/histologically the tubular type of mitochondria has different cristae types.) It seems reasonable to ask: where are the ETC complexes and ATP synthases within this organelle, and why?

ATP synthase forms a proton “sink” at the folds of the cristae—Recently, high resolution transmission electron microscopy (TEM) experiments have observed that the ATP synthase complex in vivo is distributed as dimers along the inner folds of the cristae¹⁰¹¹ (Figure 1). Super resolution optical microscopy supports this conclusion¹². In vitro studies of vesicles with monomeric vs. dimeric ATP synthase demonstrate that the dimerization induces a strong curvature to the membrane¹¹. Furthermore, yeast and human knock-out cell lines which disable dimerization of ATP synthase result in mitochondrial morphologies without cristae structures¹³¹⁴. Therefore, the dimerization of ATP synthase clearly plays a causative role in the folding of cristae.

What is the biophysical mechanism for the relationship between the membrane curvature and the dimerization of ATP synthases? One hypothesis is that the pH gradient generated by the ATP synthase causes membrane curvature. Indeed, membrane curvature of isolated liposomes is observed in the presence of pH gradient¹⁵. However, how the dimerization of ATP synthase causes folding remains to be elucidated in detail.

What is the physiological role of the dimerization and localization at the tips of the cristae? One hypothesis is that the ATP synthase acts as a “sink” of protons¹⁰ and hence generates a gradient of pH along the membrane. This would cause the cristae to have a higher pH gradient than the rest of the inter membrane space. Evidence for this hypothesis is presented in our recent work in more detail below.

The ETC complexes form supercomplexes along the walls of the inner cristae—The ETC complexes (CI-CIV, Box 1) which participate in pumping protons from the matrix to the inner membrane space have also recently observed (via immuno-gold TEM microscopy¹⁶) to be localized inside the cristae, and not in the rest of the inter membrane space. Furthermore, rather than being randomly distributed along the inner membrane inside the cristae, there is increasing evidence that complexes are localized near each other into supercomplexes called “resparisomes”¹⁷. Therefore, it seems all the “action” is inside the cristae, in terms of both pumping protons (ETC complexes) and ATP synthesis (ATP synthase). One hypothesis is that the complexes are located close to the ATP synthase dimers to maximize the transfer of protons. However, it should be noted that the evidence for these findings is somewhat indirect. To date there are no demonstrated technologies to measure either the local membrane potential, pH gradient, nor the local distribution of the complexes in live cells with the accuracy needed for determination of their location within a single mitochondria or even along a single cristae (Box 3).

A series of protein complexes maintains the mitochondrial ultra-structure (cristae structure)

MICOS—A complex of proteins called MICOS¹⁸¹⁹ (mitochondrial contact site and cristae organizing system) staples the cristae together at the junctions. This large (> 1 Mda) heterooligomeric protein complex, conserved from yeast to humans, has at least 7 identified

units (Mic10, Mic12, Mic19, Mic25, Mic26, Mic27, Mic60). Experiments (including both electron microscopy and super resolution²⁰ optical microscopy) have shown MICOS to be localized at cristae junctions²¹.

OPA1—Mitochondria constantly undergo fission and fusion. Mitochondrial fusion is mediated by two proteins on the outer membrane (the mitofusins MFN1 And MFN2), and the inner membrane protein optic atrophy 1 (OPA1), named after its pathological consequences (degeneration of retinal ganglion cells sometimes follow by blindness) when mutated in the human inherited disease dominant optic atrophy. It is believed that OPA1 oligomerizes at the cristae junctions to form a diffusion barrier which traps cytc stores inside the cristae (see below). However, the exact mechanism of this is not known. (Do they form a circle at the entry to an individual cristae, or do they form long ridges along the length of extended cristae?) In addition, this may also trap protons, as they are generated by the ETC inside the cristae at high concentration. This will be discussed below in the context of apoptosis as well. For now the most consistent model has the cytc and H⁺ concentration higher inside cristae than the the rest of the inter membrane space, as shown in Figure 2. Experimental evidence for the cytc stores is in ref ²². Direct experimental evidence for the high H⁺ concentration is lacking because researchers have long focused on the membrane potential component of ψ , neglecting to study the pH ²³, but we recently demonstrated indirect evidence ²⁴, discussed below.

Humans have eight different isoforms of OPA1, including long form and short forms (L-OPA1, S-OPA1). These are present in nearly equimolar forms under basal conditions²⁵. The L form can be cleaved to the S form by OMA1 when the mitochondrial membrane potential is low²⁶. OMA1 normally is degraded by proteolysis, but in low membrane potential situations, OMA1 is stabilized and survives long enough to cleave L-OPA1 to S-OPA1. Thus, OPA pools are determined by the bioenergetic parameter Ψ_m . This provides an important clue that relates a metabolic parameter to a protein involved in regulation of mitochondrial ultra-structure, but the detailed mechanism of this relationship is not clear yet.

The cristae structure “remodels” in response to the metabolic needs of the cell

Already over 50 years ago, Hackenbrock²⁷ observed “condensation” of rat liver mitochondria under different metabolic conditions. New, roughly 40 years later, this topic is finding renewed interest because of new findings about the relevance to apoptosis and cancer. In 2013 Cogliati et al ²⁸ showed that changes in cristae structure affect metabolic efficiency of the ETC components and respirasomes. In 2014, Patten et al ²⁹ showed that OPA1-dependent cristae modulation is essential for adaption to metabolic conditions. Thus, OPA and cristae structure, separate from their role in apoptosis below, play a crucial role in response to metabolic needs of a cell.

The spatial distribution of the electrochemical gradient along the mitochondrial inner membrane: Knowns, unknowns, and prospects

It is clear that the spatial distribution of the electro physiologically active ETC components is organized in hierarchies (dimers, supercomplexes), and that these change in respond to the metabolic needs of the cell. If the “sources” of the electrochemical potential ψ are non-

uniformly distributed, how is the pH and Ψ_m gradient distributed and how is this related to (via cause or effect) the sources? One hypothesis is that the pH inside the pockets is low to maximize “efficiency”. If this is true, how do the junctions hold in the pH since protons are small and highly mobile? Next, if the pH sink is at the folds (ATP synthase dimers), what is the pH gradient along the inner membrane and what is it between the cristae and the rest of the inter membrane space? A recent green fluorescence protein (GFP) based experiment demonstrated a pH difference of 0.3 between ATP synthase and CIV of the ETC³⁰, consistent with the expectation of a gradient of the pH due to the spatial separation of the sink (ATP synthase) and source (ETC complex respirisomes) of protons. However, because of the technological difficulty for high spatial resolution studies of electrophysiology, that is the extent of our experimental knowledge at this time.

One can create biophysical models, but the size of the Debye length is comparable to the ultrastructure, making traditional electrochemical concepts challenging to apply in a straightforward manner, and requiring a more detailed mathematical approach³¹ Although the spatial distribution is non-uniform for the ETC complexes, pH, and (presumably) Ψ_m , a detailed model, biophysical / physiological understanding, and method to measure these quantities is still lacking. Without experimental methods to validate these models, their utility at the moment is limited. Because the spatial and temporal dynamics may be coupled to metabolism, and apoptosis, it is worth further investigations of models and tests. Just as for propagation of the action potential along neurons (the Hodgkins-Huxley model), the electrically active ETC complexes may be governed by a non-trivial equivalent circuit model governing the propagation of a voltage wave. It is entirely possible that a model similar to the Hodgkins-Huxley model, albeit undiscovered, can account for the temporal and spatial dynamics of the mitochondrial electrophysiology. Tantalizing evidence for this already exists (Box 4).

Apoptosis

Mitochondrial stores of cytc are released during apoptosis

The release of the mitochondrial stores of cytc into the cytosol is one of the key events in apoptosis (Box 2). During mitochondrial outer membrane permeabilization (MOMP), the outer membrane becomes permeable with pores allowing high molecular weight proteins such as cytc to spill into the cytosol. While cytc is the key protein, other co-factors are released, such as Smac/Diablo and endonuclease G. However, because cytc is the only one also involved in the ETC, it is a candidate to explain the link between bioenergetics and apoptosis. The extent to which these secondary proteins are involved in bioenergetics is unknown. It was initially believed that the MOMP process led to the release of cytc directly.

cytc must be released from the cristae: Is cristae remodeling required?

On closer inspection, the release of cytc following MOMP poses a conceptual problem, as, the stores of cytc were shown over 15 years ago to predominately reside in the cristae, and not the inner luminal space. Therefore, if MOMP allows the release of cytc, it must be free to pass through the cristae junctions. However, if this were true, the cytc would not be

concentrated in the cristae. Therefore, in order for the cytc to be released (a pre-condition for apoptosis), one hypothesis is that cristae remodeling must occur to open the junctions.

Scorrano found in 2002 that cristae remodeling occurs during apoptosis (opening the neck from 18 nm to 56 nm)²², and found this was caused by the peptide BID (a pro-apoptotic member of the 'BH3-only' subset of the BCL-2 family of proteins, Box 3) in a way that was independent of BAX/BAK oligomerization. BAX/BAK are proteins believed to oligomerize, forming pores with permeabilize the outer membrane (hence MOMP), Box 3. Thus, MOMP was claimed to be a different process than cristae remodeling. Both MOMP and cristae remodeling were found to be necessary for cytc release, as cristae remodeling allows cytc to escape from the cristae into the rest of the inner membrane space, and MOMP enables cytc to escape from the inner membrane space, cross the outer membrane (through the MOMP pores), and into the cytosol. This followed by work of Frezza in 2006³² who showed that OPA1 prevented cytc release and hence is the glue which held the cristae together. (Additional work supporting this the importance of cristae junction opening to release cytc was also presented in Cipolat³³.) Oligomerization of OPA1 was found to be the mechanism that regulates apoptosis by maintaining the tightness of the cristae junctions.

Sun³⁴ found that cytc release can occur prior to cristae remodeling, and that mitochondrial swelling occurs only late in apoptosis after the release of cytc and the loss of mitochondrial membrane potential. Follow on studies by Yamaguchi et al³⁵ found that OPA1 oligomerization indeed blocks cytc release from the cristae, and that the disassembly of the OPA1 oligomers caused cytc release. This release of cytc was found even though the diameter of the cristae junctions was halved (from 18 nm to 9 nm) during apoptosis. Although both Scorrano and Yamaguchi found BAX/BAK oligomerization was required for cytc release through the outer membrane, only Yamaguchi found BAX/BAK and BH3 containing BID were required for cytc release from the cristae junctions.

Regardless, both find the cytc permeability in the cristae junctions must change to enable release of cytc, so the physical size of the junction seems to be unrelated to the cytc permeability. One possibility is that OPA1 oligomers form a mesh impermeable to cytc across the cristae junction which is controlling cytc diffusion across the mesh, independent of the size of the cristae junction. These more refined hypothesis are shown in Figure 2.

Why is there such discrepancy between results? One of the most important factors is that TEM studies provide only a snapshot of a dynamic process. A second factor is that the processes may occur during preparation of the sample for TEM imaging³⁶, thus masking the effect to be imaged or significantly perturbing the "frozen" state from the in vivo, live state that is desired to be studied. Static structure studies have advanced significantly, even garnering the Nobel prize in 2017³⁷. However, this discrepancy illustrates that, in order to better understand the dynamic and functional properties of mitochondria, it is crucial to develop dynamic, real-time imaging technologies that can probe ultrastructure and electrophysiology at the nanoscale in vivo.

What is the mechanism that allows cytc to be released from the cristae and cause apoptosis? To answer this, one must look towards the link between bioenergetics and apoptosis in more

detail. Although extensive literature exists on both, the combination has not been reviewed until now.

The link between apoptosis and bioenergetics

Experimental evidence from key biophysical parameters

Whole cells—Whole cells in culture can be triggered to cause apoptosis using a variety of means, e.g. exposure to staurosporine, actinomycin, etoposide, UV, etc. During this process, the cytosolic pH, mitochondrial membrane potential Ψ_m , and respiration rate can be continuously monitored, giving insight to the role of mitochondria and bioenergetics in the process of apoptosis.

Upon induction of apoptosis in cultured cells, cytc is released into the cytosol, and the bioenergetic processes slow down or halt: The respiration rate is suppressed^{38,39}, membrane potential drops³⁸, and the cytosolic pH drops slightly⁴⁰. The drop of the membrane potential and respiration rate is consistent a model where the loss of cytc causes the ETC to stall (i.e. OXPHOS to stop) due to the lack of cytc in the mitochondria to act as an electron shuttle in the ETC. The pH change will be discussed below.

If the apoptosis process is initiated but caspase inhibitors are present, then release of cytc into the cytosol does not activate caspases and the apoptosis process is thus blocked. In this situation, the measurement of the respiration rate and membrane potential remains constant³⁸, even though cytc is released from the mitochondria. Upon permeabilization of the cell plasma membrane by digitonin, the membrane potential and respiration rate drop³⁸; these can be restored by the introduction of exogenous cytc into the buffer, which restores the respiration rate³⁸ and Ψ_m ³⁹. These data suggest that the loss of cytc from the cytosol is what causes OXPHOS to stop, as it can be “rescued” by exogenous cytc. (pH was not measured in these experiments). They also suggest that the caspases play an important (as yet undetermined) role in halting the OXPHOS process.

Isolated mitochondria—MOMP can be directly triggered in isolated mitochondria, or in mitochondria inside digitonin permeabilized cells, by the addition of BIM or tBid to the suspension medium. In isolated mitochondria, upon induction of MOMP, metabolic assays show decreased respiration rate^{39,41} and membrane potential^{39,41,24}, as well as a reduction in the buffer pH²⁴. When exogenous cytc is introduced back into the buffer, these parameters are restored (when measured^{39,41,24}). These data support a model where the loss of cytc from the mitochondria stall the ETC. The pH change will be discussed below. For permeabilized cells, the data are similar: upon Bid induced MOMP, Ψ_m goes down⁴², which is recovered upon addition of exogenous cytc into the buffer³⁸. It should be noted that peptides have 100-1000 fold less activity than full length proteins, suggesting great caution is required when interpreting the results of experiments using peptides.

Emerging Model

An early indication of the link between membrane potential and apoptosis was found in 2003⁴³, but that was before OPA and MICOS were well understood, and so it was purely phenomenological at the time. The role of OPA1 in cytc release is by now well established,

but the mechanism by which OPA1 oligomers was disassembled was not known. The discovery of the dependence of OMA1 cleavage of OPA1 on Ψ_m ²⁶ has given a critical insight into the relationship between these parameters. In particular, OMA1 suppression prevents cytc release⁴⁴. Therefore, cleavage of OPA1 must be required for cytc release. The relationship between Ψ_m , OPA, and cristae junction remodeling provided the missing link which gave rise to the following model (Figure 3):

The initial step in apoptosis of MOMP (BAX/BAK oligomerization) causes lipidic and/or proteinaceous pores in the mitochondrial outer membrane. This allows the small fraction of CyC stored in the inner membrane space to be released into the cytosol, hence reducing the membrane potential Ψ_m slightly by reducing the activity of the ETC. Another, parallel pathway which reduces Ψ_m after MOMP is degradation of CII of the ETC. Although the mechanism by which CII is degraded after MOMP is not known, evidence⁴⁵ confirms the model that the membrane potential degradation (actually ETC CII degradation) occurs upstream of OMA1 activation. Once OMA1 is activated due to reduction of Ψ_m , it can cleave OPA1. Cleavage of OPA1 (the glue that holds the cristae junctions together) results in disassembly of the cristae junctions. Disassembly of the cristae junctions allows complete release of cytc, as well as release of the high proton concentration inside the cristae, into the cytosol (whole cells) or buffer (isolated mitochondria). This model provides the critical link between a bioenergetic parameter (Ψ_m), mitochondrial ultra-structure (cristae remodeling), and apoptosis.

This model also explains why, in several different experiments reviewed above, addition of exogenous cytc to the medium surrounding the mitochondria would sustain the respiration required for maintaining the inter membrane space pH and membrane potential thus stabilizing OPA1 and blocking detectable changes in proton and cytc release.

DRP plays a significant role in cristae remodeling—Dynamin-related protein 1 (DRP1) is a cytosolic protein recruited to the mitochondrial outer membrane during mitochondrial fission. Its role in fission has been well studied. Although its activity is believed to be localized at the mitochondria outer membrane only, recent evidence suggests that DRP1 also has a role in cristae remodeling. Superresolution optical microscopy studies have shown BAX/BAK rings form on the outer membrane⁴⁶. However, in DRP knockdown cells in the same work, these rings (which seemingly indicate large holes in the OMM) are not followed by cytc release. More recently, Otera⁴⁷ has shown that DRP-1 knockout cells completely resist CR and cytc release during apoptosis, and in these cell lines OPA1 oligomers were disassembled but the cristae were not remodeled. How DRP, which mediates mitochondrial fission, causes cytc release or assists remains to be elucidated.

Concluding Remarks

The morphology of mitochondria is clearly critically important in apoptosis. There are serious technical challenges to dissect this ultra-structure, as the structures are much smaller than the wavelength of light and just barely addressable by newly developed deep-sub wavelength microscopies (Box 3). Most of these high resolution tools (including TEM) provide static, structural information. What is needed is information about the real-time,

dynamic processes, and in addition to structural information, also electrophysiological (electrical) such as: membrane potentials, proton currents and fluxes, and pH gradients, with resolution much smaller than the size of the structure, i.e. at the 1 nm length scale (see outstanding questions). Real time nano-probes such as AFM based tools, quantum dots, or even nanoelectronic probes⁴⁸²⁴, may enable future dissection of this electrophysiological active organelle. Just as the electrophysiological active neurons in the central nervous system give rise to information processing and human thought, the electrophysiological active mitochondria give rise to cellular energy as well as life/death decisions. A clear path forward is to develop new technology to assay the electrophysiological function of this organelle at the nanoscale in real time.

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Glossary

Apoptosis	A systematic, ordered process of programmed cell death in response to external stimuli or internal stress
ETC	Electron transport chain. A series of protein complexes on the mitochondrial inner membrane which pump protons across the membrane
IM Inner membrane	Inner membrane of the mitochondria, a double-membraned organelle, impermeable to small molecules
OM Outer membrane	Outer membrane of the mitochondria, a double-membraned organelle, permeable to small molecules

IBM	Inner boundary membrane
CM	Cristae membrane
TMRE	Tetramethylrhodamine, ethyl ester, a fluorescent dye used to track membrane potential
CJ, Cristae junction	Narrowing of invaginations of the inner membrane
GFP	Green fluorescent protein
ROS	Reactive Oxygen Species
cytc Cytochrome C	A heme-protein which has the dual role of shuttling electrons in the ETC and also activating caspases in the cytosol once released from the mitochondria to carry out the process of apoptosis
ATP synthase (CV)	a complex which uses this electrochemical energy stored in the membrane to convert $ADP + P_i$ to ATP hence coupling oxidation of nutrients to phosphorylation of ADP (OXPHOS)
MOMP	Mitochondrial outer membrane permeabilization, believed to be caused by oligomerization of BAX/BAK proteins at the outer membrane, triggered by BID.
BID	a pro-apoptotic member of the 'BH3-only' subset of the BCL-2 family of proteins

Box 1**Mitchell's Chemiosmotic Hypothesis**

In 1961, Mitchell postulated² that energy is converted from chemical energy in carbohydrates and fats to an electrochemical gradient at the mitochondrial inner membrane (proton motive force Δp) given by:

$$\Delta p = \Delta\Psi_m - 59\Delta pH, \quad \text{Eq. 1}$$

where Ψ_m is the membrane potential and ΔpH is the pH difference across the membrane. Throughout the years, this model became verified and the current model has 4 electron transport chain (ETC) complexes (CI-CIV) that pump protons across the inner membrane to create this gradient (Figure 1). The ETC components energetically couple pumping of protons across the inner membrane to oxidation of the chemical components of the Krebs cycle. This proton pumping creates a charge differential due to the positive charge of the proton, and hence a membrane potential across the inner membrane capacitance according to the well known relationship between the charge on a capacitor Q and the voltage drop across the capacitor V : $Q=CV$, where C is the capacitance of the membrane. (Here V is synonymous with Ψ_m .) However, it also creates a pH gradient across the membrane as more protons end up outside the membrane. ATP synthase (CV) is a complex which uses this electrochemical energy stored in the membrane to convert $ADP + P_i$ to ATP, hence coupling oxidation of nutrients to phosphorylation of ADP (OXPHOS).

cytc is a soluble protein that plays a key role shuttling electrons from CIII to CIV

cytc is a reducible, soluble heme-protein that plays the role of electron shuttle in the ETC. It is reduced by CIII, and then subsequently reduces CIV. It also plays a key role in apoptosis, as will be discussed below. The location of cytc is believed to be 85% within the cristae²², shown in Figure 2 schematically.

Box 2**Apoptosis pathway, MOMP, and cancer chemotherapies**

In the mitochondrial pathway of cell death (apoptosis), various upstream effectors (DNA damage, ROS, stress, cell death signals, etc.) result in cell apoptosis. The point of no return is an abrupt event that occurs at the membranes of the mitochondria: The organelle is eventually ruptured (or changed in some other way) and releases the stores of Cytochrome C (CytC) into the cytosol. This release of cytochrome C irreversibly activates the caspases which carry out the apoptotic process to completion. Resistance to apoptosis is a hallmark of cancer⁹. In fact, eventually, every chemotherapy is believed to result in apoptosis as its final mechanism of action, regardless of the initial point of attack⁴⁹. Because the mitochondrial pathway is highly regulated, it is a clear target for pharmacological manipulation. Because of its potential clinical significance, extensive efforts have been made to understand the mechanisms of cytochrome C release and its regulation. However, because of the small size of the organelle, the details of the release process are still far from understood. Since 85% of the cytochrome C is contained in the cristae²², the morphology of mitochondria is a key parameter to study.

Mitochondrial stores of cytochrome C are released during apoptosis

The release of the mitochondrial stores of cytochrome C into the cytoplasm is one of the key events in apoptosis. One of the processes that has been well studied but is still not completely understood⁵⁰ is the permeabilization of the mitochondrial outer membrane (Mitochondrial Outer Membrane Permeabilization (MOMP)). Historically, the finding of the BCL-2 gene that is implicated as an anti-apoptosis gene in B cell lymphoma, led to the study of a family of over 20 related proteins which share 4 homology domains (BH1-4). During MOMP, the outer membrane becomes permeable with pores allowing high molecular weight proteins such as cytochrome C to pass. Thus it was initially believed that the MOMP process led to the release of cytochrome C directly. Later, more refined thinking about CR remodeling followed.

The MOMP process is the result of oligomerization of BAX (BCL-2 antagonist killer 1) or BAK (BCL-2 associated X protein) proteins on the mitochondrial outer membrane. The details of this process are still being worked out. For example, structural studies of BAX/BAK oligomerization with AFM have demonstrated that, at least in some circumstances, BAX/BAK proteins oligomerize to form pores. With superresolution optical microscopy, Jakobs et al have imaged MOMP as large lipidic pores up to >100 nm in diameter⁵¹. These always occurred with cytochrome C release, making it impossible to dissect the order or cause/effect relationship. Their work is consistent with lipidic pores observed in liposomes with TEM⁵¹ as well as AFM studies⁵².

Furthermore, the regulation of BAX/BAK mediated MOMP has been shown to be positive and negatively affected by the BCL-2 family of proteins. Although several competing models for the details of how this regulation occur, it is clear both in vitro and in vivo that MOMP (hence apoptosis) can be regulated by the BCL-2 family of proteins. Furthermore, the development of several chemotherapies that manipulate this pathway by creating so-called BH3 mimetics has resulted in some clinical significant results. For

example, venetoclax (ABT-199) has been developed as a chemical inhibitor of the anti-apoptosis BCL-2 gene. It has received FDA approval for use in patients with chronic lymphocytic leukaemia (CLL) with the 17p deletion. Additional clinical trials (Phase I-III) are underway for CLL, non-Hodgkin lymphomas (including diffuse large B-cell lymphoma, mantle cell lymphoma, follicular lymphoma), acute myeloid leukaemia, multiple myeloma, systemic lupus erythematosus, and breast cancer⁵³.

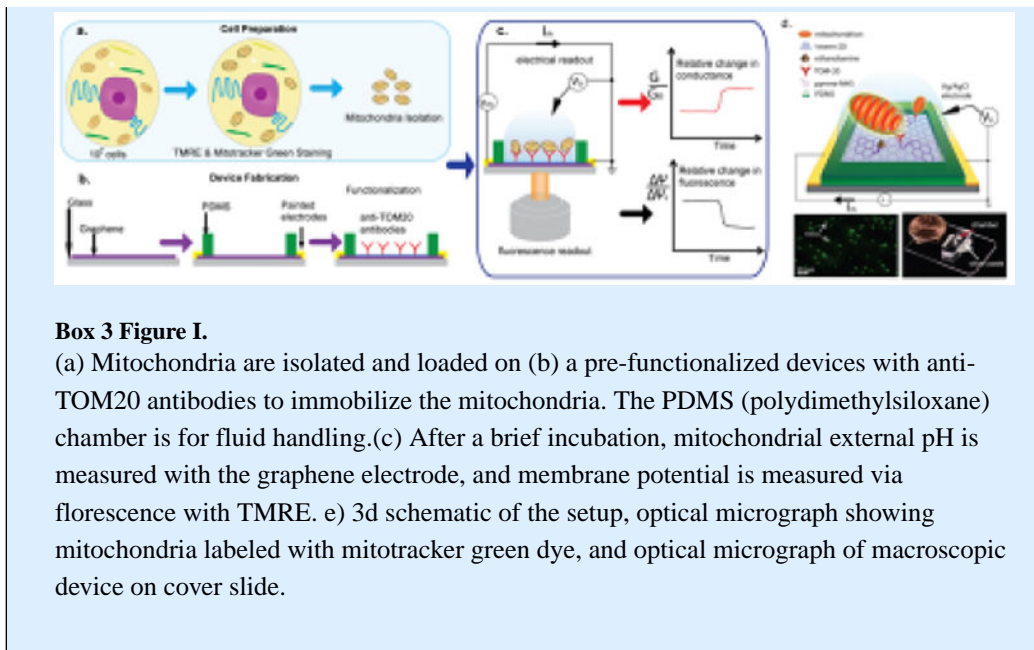
Box 3**How is mitochondrial $p = \Psi_m - 59$ pH and structure measured?**

Mitochondrial membrane potential Ψ_m is usually measured via fluorescence⁵⁴. Most charged species are hydrophilic, but by distributing a charge some moieties become hydrophobic/lipophilic, e.g. Tetramethylrhodamine, ethyl ester (TMRE), allowing it to cross the otherwise impermeable inner membrane. When such a species is fluorescent, its concentration can be qualitatively determined through a measurement of either fluorescence intensity or spectrum. When there is a membrane potential, the charged species will be distributed across the membrane via the Nernst relation, $n_1/n_2 \sim e^{\{-\Psi_m/kT\}}$. In mitochondria with $\Psi_m \sim 100$ mV this gives a 100-1000 \times increased concentration inside, which is usually measured as a surrogate for the membrane potential. However, the limited # of photons and the sensitivity of mitochondria to bright light generated ROS limits the temporal resolution to ~ 100 ms⁵⁵. The spatial resolution is also limited by the wavelength of light, of order the size of the mitochondria itself. Mitochondria may be only a single pixel in diameter but they can be many pixels long.

The pH, while an important component of p , has long been neglected until recently²³. GFP proteins can be integrated into the genome and targeted to the mitochondrial compartment for in vivo real time pH measurements⁵⁶. Very recently²⁴, we have applied nanotechnology using a sensitive graphene electrode to assay extra mitochondrial pH undergoing MOMP in real time (Figure I, from ref²⁴). This has the advantage that it can be scaled with micro fabrication for massively parallel assays. These new electronic techniques may enable high temporal resolution assays of mitochondrial electrophysiology with single mitochondria probed with single nanowire/nanotube structure⁵⁵. In addition, while none of the optical methods above have high enough spatial resolution for individual ion channel activity, new nanoelectronic technologies in the future may enable this⁴⁸.

Two methods are used to study mitochondrial structure: electron microscopy and super resolution optical microscopy. Electron microscopy always require fixation and therefore only capture a snapshot of mitochondrial structure. Super resolution optical microscopy also may require fixations. This, in addition to the necessary sample preparation and low throughput, has severely limited progress in the field.

For electrophysiology, patch clamp approaches have been successful only with mitoplasts⁵⁷. (A mitoplast is a mitochondrion that has been stripped of its outer membrane leaving the inner membrane intact.) Patching the intact organelle has proved challenging, because the inner membrane structure is smaller than pipette diameters.



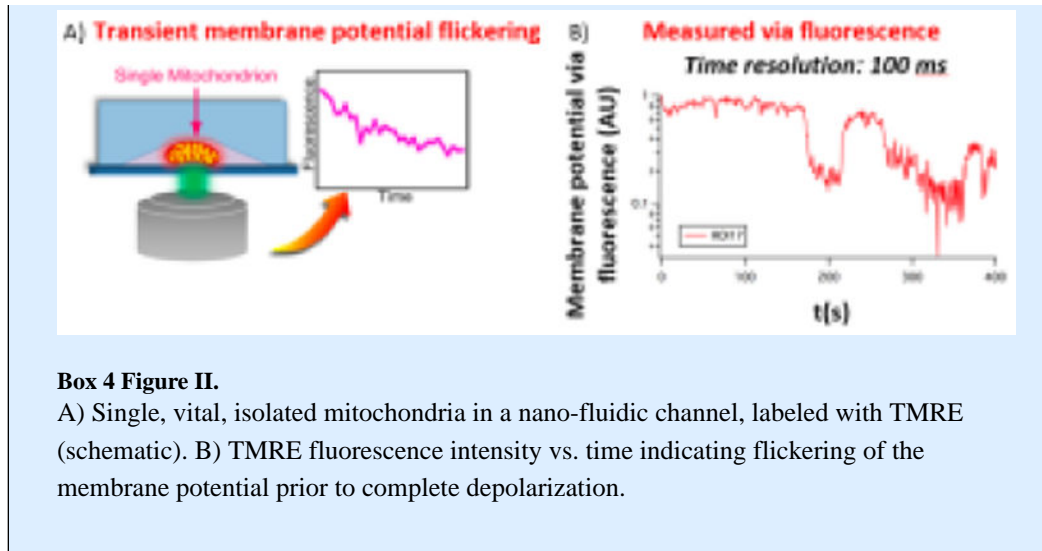
Box 4**What is the role of the mitochondrial permeability transition pore in apoptosis?**

The mitochondrial permeability transition pore (mtPTP) is a name given to a complex of membrane proteins at the inner and outer membrane which are believed to form a large pore. The molecular identity of each of the components is controversial and not completely known^{58,59,60} despite intense effort in the area for over 10 years. Although there are many ion channels, pumps, transporters, etc. in mitochondria (many of which can be assayed individually in suspended lipid bilayers), the interrelationship among these and their contribution to the mtPTP is still largely unresolved. A lot of this uncertainty stems from a lack of tools to measure electrophysiology at the nanoscale with high temporal resolution (Figure II from ⁶¹). What is known is that when mitochondria depolarize in response to ROS (e.g. light induced free radicals) and calcium overload, the membrane potential of the entire organelle (as measured by TMRE fluorescence intensity) “flickers” on and off before dropping to zero (Figure II and ⁶²). This flicker is believed to be the transient opening and closing of the mtPTP. However, because TMRE measurements are limited in time resolution to ~ 100 ms at best⁵⁵, and spatial resolution to one voxel ~ one mitochondria, little more is known about the electrophysiology of the mtPTP in vivo.

What regulates mtPTP? In the absence of direct patch clamp experiments in vivo, many additional clues can be gleaned by in vitro patch clamp of a variety of potential components of the mtPTP. This includes ATP synthase, BAX/BAK, Adenine nucleotide translocator (ANT), cyclophilin D, etc. A vast literature documents inducers of mtPTP opening.

What is the physiological role of the mtPTP? It is known to be heavily involved in ischemia/reperfusion injury in the brain and heart, but calcium overload is not a known mechanism of apoptosis. One possibility is that under conditions of prolonged oxidative stress or cellular Ca⁺ overload, short openings of mtPTP might serve as an emergency release of accumulated Ca⁺ ions and a mechanism allowing the partial dissipation of Ψ_m , reducing ROS generation. However, once the inner membrane is compromised via the mtPTP, the organelle ruptures via osmotic swelling and this does release cytochrome c triggering apoptosis. The original discovery studies of the mtPTP over 10 years ago implicated it in vivo as a significant participant in apoptosis but since then researchers have questioned if it is necessary for the mtPTP to open in order for apoptosis to occur. The most recent detailed studies of BH3 proteins, BAX/BAK oligomerization to form MOMP, and cristae remodeling do not seem to indicate a role for the mtPTP in apoptosis.

Are pH and ROS flicker⁶³ related to membrane potential flicker? Recent work has shown that, in addition to membrane potential flicker (measured via TMRE), pH may also flicker and the mitochondria release “puffs” or ROS. These recent experiments are done using genetically engineered GFP technology. In fact the frequency of these flashes has been correlated with lifespan in some organisms⁶⁴.



Trends box

- The elements of the electron transport chain are not uniformly distributed along the inner membrane. ATP synthase (Complex V) forms dimers. The other 4 complexes form respirisomes.
- The mitochondrial inner membrane is folded into cristae, which remodel in response to metabolic demand as well as cell death pathway signals (apoptosis). The cristae both effect and are effected by the electron transport chain.
- The membrane potential and pH gradient are not uniform along the inner membrane. The cristae create pockets of acidic regions, and the pH changes up to 0.3 between the ATP synthase (which consumes the energy stored in the membrane) and the respirisomes.
- The spatial and temporal dependence of these quantities is not possible to assay with current technology.
- Changes in the mitochondrial cristae (cristae remodeling) are an important step in apoptosis, and a potential target for future pharmacological manipulation.

Outstanding questions box

- Electrophysiology: How does the mitochondrial membrane potential and pH gradient change in space and time, and is there a physiological significance to this?
- Ultrastructure: In what ways does the mitochondrial ultra-structure change during apoptosis, and what is the mechanism for protein families that regulate and control this process?
- Cancer: Can an increased understanding of the link between bioenergetics and apoptosis be exploited for pharmacological manipulation of apoptosis?

Future directions: How can non-invasive technologies be developed to peer inside the nanoscale structures of mitochondria and monitor their electrophysiological and structural activities in real time

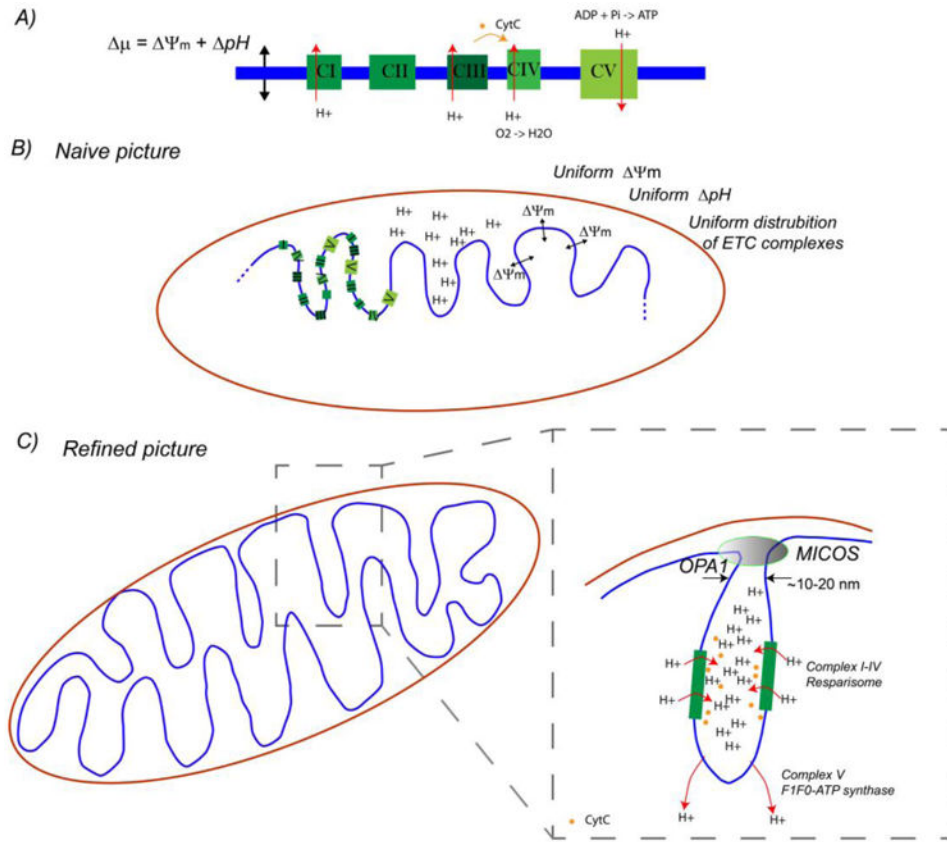


Figure 1.

A) Electron transport chain (ETC, top). B) Naive picture of distribution of the ETC components assumes they are uniformly distributed along the mitochondrial inner membrane (blue), and that the electrochemical potential also is uniform. Because technology does not exist to directly measure these quantities accurately and with nanoscale spatial resolution, this naive picture has persisted for many years. In the refined picture, put together through indirect observations over that last few years, the spatial distribution of the electrophysiology seems to be highly ordered and hierarchically organized. The coupled electrophysiology and biochemistry, although directly related to metabolism and apoptosis (hence cancer), remain to be explored and explained in detail.

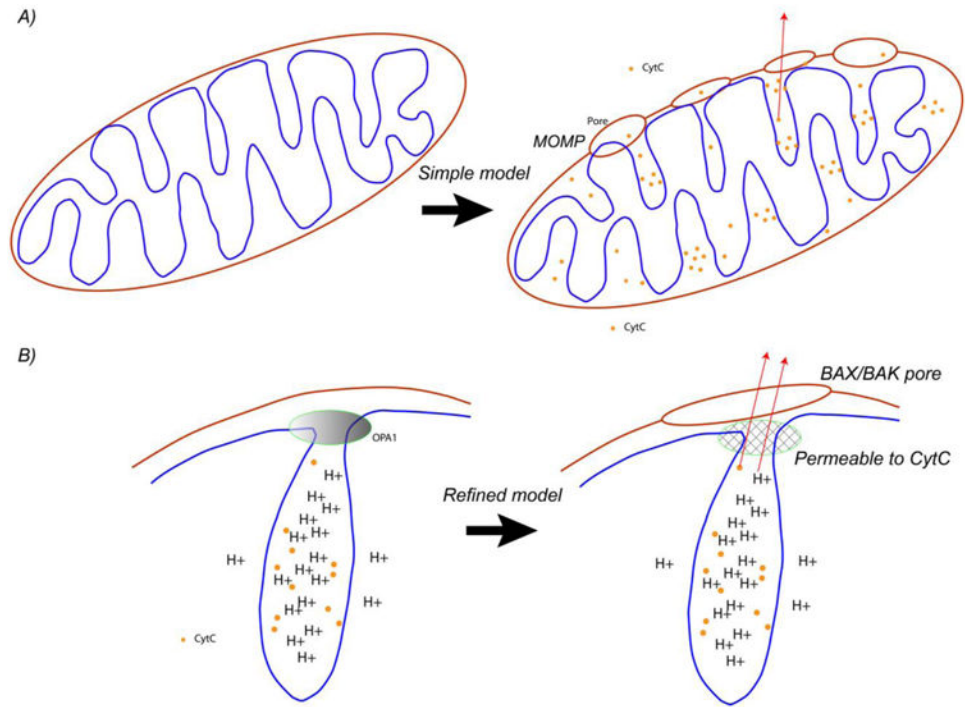


Figure 2.

A) Process of mitochondrial outer membrane permeabilization gives rise to a BAX/BAK pore in the outer membrane, followed by cytc release, triggering caspases and irreversibly committing the cell to apoptosis. B) A more refined model takes into account that the vast majority of CytC is stored in the cristae, and that the cristae junctions contain protein complexes which normally block cytc release, including MICOS and OPA1 oligomers. The exact mechanism by which the cristae junctions become permeable to cytc is unknown, hence the figure indicates a generic, permeable mesh.

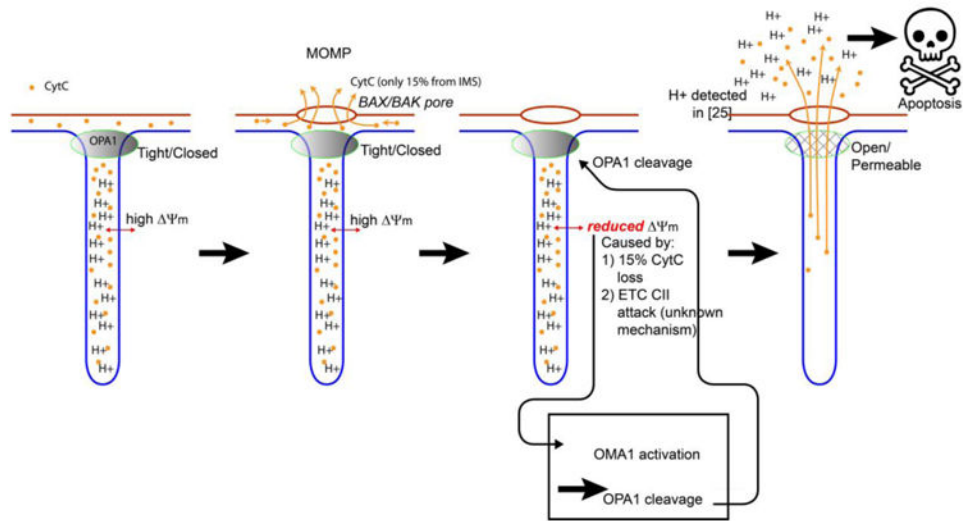


Figure 3.

Emerging model of how cytc is released. A) Healthy mitochondria. 85% of the cytc is contained in the cristae and cannot escape. Because of the localization of the ETC resparisomes (not shown) in the cristae, the H^+ concentration is high there. B) On MOMP, BAX/BAK pores form in the outer mitochondrial membrane (OMM, red). This releases a fraction of the CytC into the cytosol; the remainder is still trapped by the tightly closed cristae junctions. C) The mitochondrial membrane potential drops in response to either 1) loss of 15% of CytC²⁴ or 2) Enzymatic attack on complex II (CII) of the ETC by an unknown enzyme, in response to MOMP via an unknown mechanism⁴⁵. This drop in Ψ_m causes OMA1 activation, which cleaves the long form of OPA1. D) The cleaving of OPA1 opens the cristae junctions to allow complete release of cytc, triggering apoptosis. H^+ is also released causing slight acidification of the cytosol²⁴, which may enhance the activity of the executioner caspases triggered by cytc.