Trends in Cancer

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Review

Mitochondria, Bioenergetics and Apoptosis in Cancer

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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 ultrastructure 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.

Dual Role of Mitochondria in Energetics and Apoptosis

Mitochondria are double-membraned organelles believed to have been integrated into modern eukaryotes via symbiosis of proteobacteria into an anaerobic pre-eukaryotic (host) cell 1.5–2 billion years ago [1]. According to modern thinking (pioneered by Mitchell; [2]), an essential role of mitochondria is to produce 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)** (see Glossary) complexes. This electrochemical gradient acts as a store of energy. **ATP synthase** uses this stored energy to convert 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 hemeprotein cytochrome C (CytC), as well as Smac/Diablo, 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 that indicate that the mitochondrial ultrastructure [3] is intimately involved in the relationship between the bioenergetics [4] and apoptotic [5–8] (cell life/death decision) roles of mitochondria. The discussion summarizes recent advances in (i) new understanding of the role of ultrastructure and the location of membrane complexes on mitochondrial bioenergetics; (ii) new understanding of the role of ultrastructure in apoptosis; and (iii) an emerging, albeit incomplete, model for the relationship between (i) and (ii). 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 cancer [9], a better understanding of the

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The elements of the electron transport chain are not uniformly distributed along the inner membrane. ATP synthase (Complex V) forms dimers. The other four complexes form resparisomes.

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 affected 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 resparisomes.

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.

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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 points. (i) The mitochondria sustain a membrane potential that is not uniform along the inner membrane due to their ultrastructure and distribution of membrane proteins involved in the OXPHOS process. (ii) The change in the ultrastructure (remodeling) occurs during both metabolic changes and apoptosis. (iii) A growing body of recent evidence indicates that the bioenergetic and apoptotic functions are linked through this ultrastructure. (iv) A comprehensive model that explains this link is only in its early stages and incomplete. (v) 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 a 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). (vi) 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

Sources and Sinks of the Membrane Potential Are not Uniformly Distributed along the Inner Membrane

In 1961, Mitchell postulated [2] 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 that drives ATP synthesis. Now known as the chemiosmotic hypothesis (Box 1), this model is widely accepted. While the molecular constituents of the ETC complexes and ATP synthase are mostly identified, this 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 ultrastructure (Figure 2). (Anatomically/histologically the tubular type of mitochondria has different cristae

Box 1. Mitchell's Chemiosmotic Hypothesis

In 1961, Mitchell postulated [2] 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:

$$\Delta p = \Delta\Psi_m - 59\,\Delta\,pH \quad [I]$$

where $\Delta\Psi_m$ 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 four ETC complexes (I–IV) that pump protons across the inner membrane to create this gradient (see Figure 1 in main text). 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, and V is synonymous with $\Delta\Psi_m$. However, it also creates a pH gradient across the membrane as more protons end up outside the membrane. ATP synthase (Complex V) is a complex that 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 Complex III to IV

CytC is a reducible, soluble heme protein that plays the role of electron shuttle in the ETC. It is reduced by Complex III, and then subsequently reduces Complex IV. It also plays a key role in apoptosis, as will be discussed below. The location of CytC is believed to be 85% within the cristae [22] (see Figure 2 in main text).

Glossary

Apoptosis: systematic, ordered process of programmed cell death in response to external stimuli or internal stress.
ATP synthase (Complex V): complex that uses electrochemical energy stored in the membrane to convert ADP + P_i to ATP, hence coupling oxidation of nutrients to phosphorylation of ADP (OXPHOS).
BID: a proapoptotic member of the BH3-only subset of the BCL-2 family of proteins. **BID** is activated by cleavage by caspase 8 to form truncated **BID** (**tBID**). Other members of this family, which number over 10, include for example **BIM** (Bcl-2-interacting mediator of cell death).
Cytochrome C (CytC): heme protein that 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.
Electron transport chain (ETC): series of protein complexes on the mitochondrial inner membrane which pump protons across the membrane.
Mitochondrial outer membrane permeabilization (MOMP): believed to be caused by oligomerization of BAX/BAK proteins at the outer membrane, triggered by BID.
Tetramethylrhodamine, ethyl ester (TMRE): fluorescent dye used to track membrane potential.

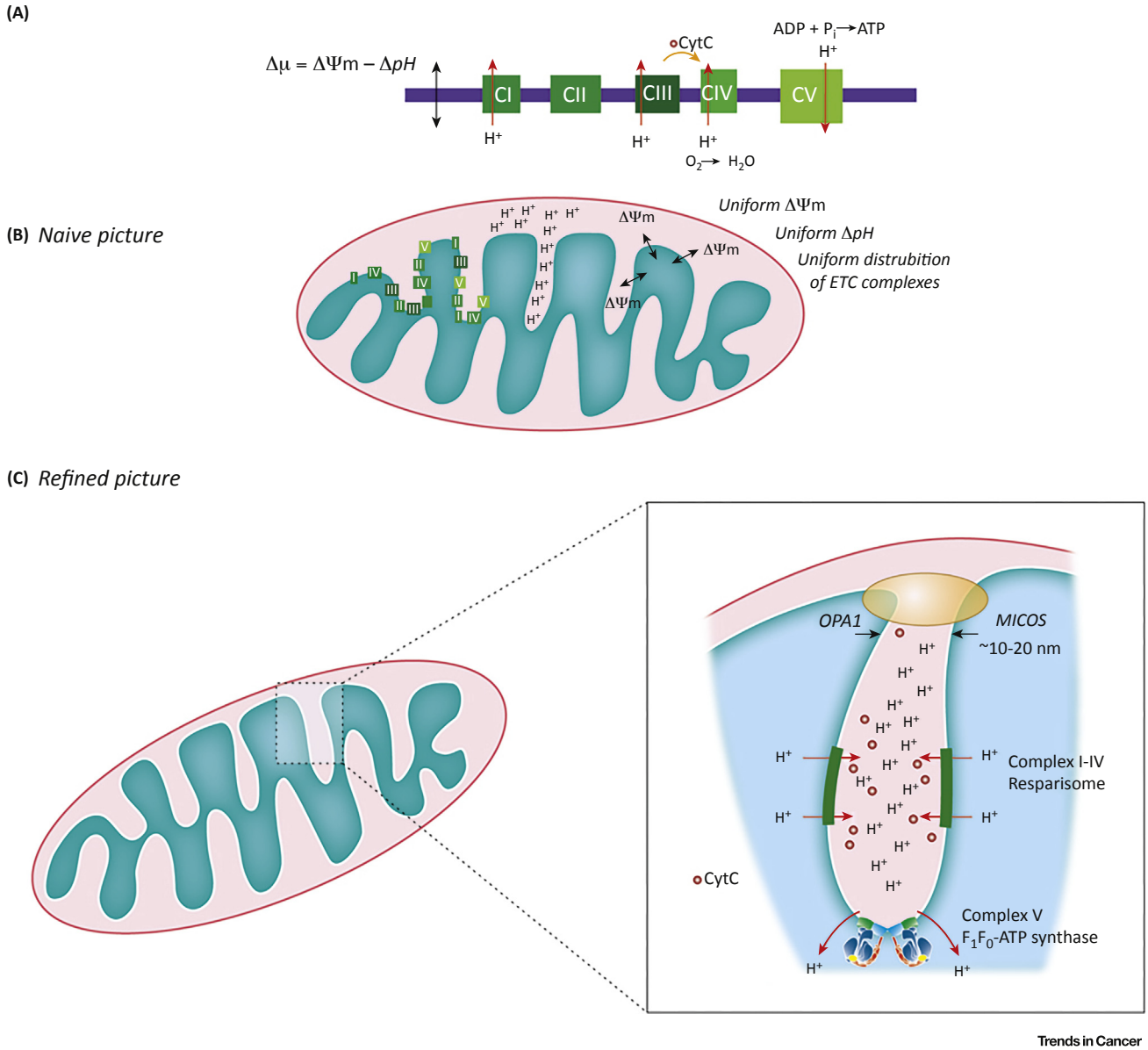

Figure 1. (A) ETC (top). (B) Naïve picture of distribution of the ETC components assumes they are uniformly distributed along the mitochondrial inner membrane, and that the electrochemical potential also is uniform. Because technology does not exist to directly measure these quantities accurately and with nanoscale spatial resolution, this naïve picture has persisted for many years. (C) 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. Abbreviations: CI–IV, Complex I–IV; ETC, electron transport chain; MICOS, mitochondrial contact site and cristae organizing system; OPA1, optic atrophy 1.

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) has shown that the ATP synthase complex *in vivo* is distributed as dimers along the inner folds of the cristae [10,11]

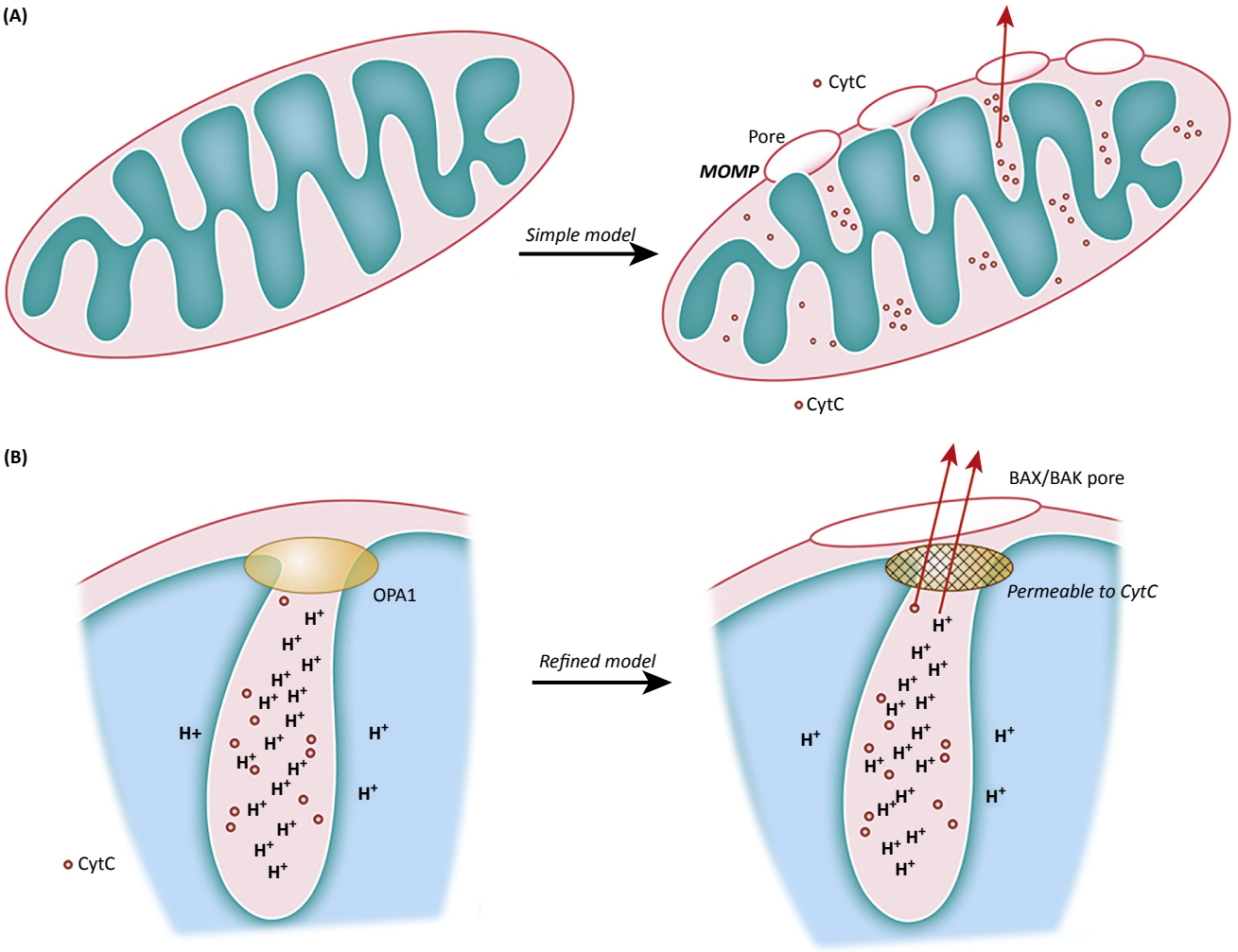

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Figure 2. (A) Process of MOMP gives rise to a BAX/BAK pore in the outer membrane, followed by CytC release, triggering caspases and irreversibly committing the cell to apoptosis. (B) A more refined model takes into account that the vast majority of CtyC 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. Abbreviations: CytC, cytochrome C; MICOS, mitochondrial contact site and cristae organizing system; MOMP, mitochondrial outer membrane permeabilization; OPA1, optic atrophy 1.

(Figure 1). Super-resolution optical microscopy (OM) supports this conclusion [12]. *In vitro* studies of vesicles with monomeric versus dimeric ATP synthase demonstrate that the dimerization induces a strong curvature to the membrane [11]. Furthermore, yeast and human knockout cell lines that disable dimerization of ATP synthase result in mitochondrial morphologies without cristae [13,14]. 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 a pH gradient [15]. 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 proton sink [10] and hence generates a pH gradient along the membrane. This would cause the regions within the cristae to have a higher pH gradient than the rest of the intermembrane space. Evidence for this hypothesis is presented in our recent work in more detail below.

ETC Complexes Form Supercomplexes along the Walls of the Inner Cristae

The ETC complexes (I–IV, Box 1) that participate in pumping protons from the matrix to the intermembrane space have also recently observed (via immuno-gold TEM [16]) to be localized inside the cristae, and not in the rest of the intermembrane 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 respar-isomes [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 2).

A Series of Protein Complexes Maintains the Mitochondrial Ultrastructure (Cristae Structure)

Mitochondrial Contact Site and Cristae Organizing System (MICOS)

A complex of proteins called MICOS [18,19] staples the cristae together at the junctions. This large (>1 MDa) hetero-oligomeric protein complex, conserved from yeast to humans, has at least seven identified units (Mic10, Mic12, Mic19, Mic25, Mic26, Mic27, and Mic60). Experiments (including both EM and super-resolution OM [20]) have shown MICOS to be localized at cristae junctions [21].

Optic Atrophy (OPA)1

Mitochondria constantly undergo fission and fusion. Mitochondrial fusion is mediated by two proteins on the outer membrane (mitofusins MFN1 and MFN2), and the inner membrane protein 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 that 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 crista, 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 rest of the intermembrane space (Figure 2). Experimental evidence for the CytC stores is in [22]. Direct experimental evidence for the high H^+ concentration is lacking because researchers have long focused on the membrane potential component of Δp, neglecting to study the ΔpH [23], but we recently demonstrated indirect evidence [24], discussed below.

Box 2. How Is Mitochondrial $\Delta p = \Delta\Psi_m - 59\ \Delta$ pH and Structure Measured?

$\Delta\Psi_m$ is usually measured via fluorescence [54]. Most charged species are hydrophilic, but by distributing the charge some moieties can be hydrophobic/lipophilic, for example, **tetramethylrhodamine, ethyl ester (TMRE)**, allowing it to cross the otherwise impermeable inner membrane. When such a species is fluorescent, its concentration can be qualitatively (and sometimes quantitatively) 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, $n_1/n_2 \approx e^{-\Delta\Psi_m/kT}$. In mitochondria with $\Delta\Psi_m \approx 100$ mV this gives a 100–1000× increased concentration inside, which is usually measured as a surrogate for the membrane potential. However, the limited number of photons and the sensitivity of mitochondria to bright light generating ROS limits the temporal resolution to ~100 ms [55]. 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 Δp, has long been neglected until recently [23]. GFP can be integrated into the genome and targeted to the mitochondrial compartment for *in vivo* real-time pH measurements [56]. Recently, we have applied nanotechnology using a sensitive graphene electrode to assay extra mitochondrial pH undergoing MOMP in real time (Figure I) [24]. This has the advantage that it can be scaled with microfabrication 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 structure [55]. 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 this [48].

Two methods are used to study mitochondrial structure: electron microscopy (EM) and super-resolution optical microscopy (OM). EM always require fixation and therefore only capture a snapshot of mitochondrial structure. Super-resolution OM 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 mitoplasts [57]. 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.

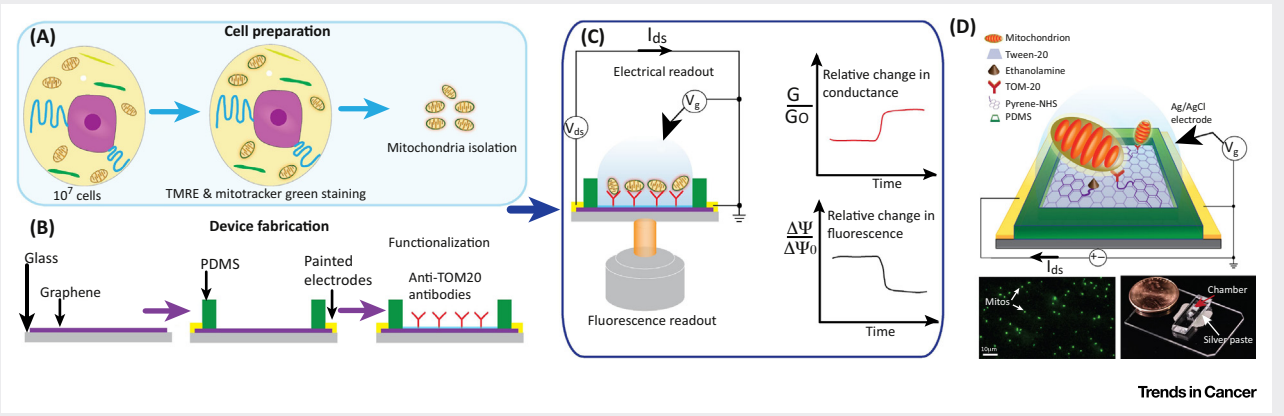

Figure I. (A) Mitochondria are isolated and loaded on (B) a prefunctionalized device with anti-TOM20 antibodies to immobilize the mitochondria. (TOM20 is a protein expressed on the mitochondrial outer membrane.) The PDMS chamber is for fluid handling. (C) After a brief incubation, mitochondrial external pH is measured with the graphene electrode, and membrane potential is measured via florescence with TMRE. (D) 3D schematic of the setup, optical micrograph showing mitochondria labeled with Mitotracker green dye, and optical micrograph of macroscopic device on cover slide. Abbreviations: PDMS, polydimethylsiloxane; TMRE, tetramethylrhodamine, ethyl ester. Adapted, with permission, from [24].

Humans have eight different isoforms of OPA1, including long and short forms (L-OPA1 and S-OPA1). These are present in nearly equimolar forms under basal conditions [25]. The L form can be cleaved to the S form by the mitochondrial zinc metalloprotease OMA1 when the mitochondrial membrane potential is low [26]. 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 $\Delta\Psi_m$, the mitochondrial membrane potential. This provides an important clue that relates a metabolic parameter to a protein involved in regulation of mitochondrial ultrastructure, but the detailed mechanism of this relationship is not clear yet.

The Cristae Structure Remodels in Response to the Metabolic Needs of the Cell

More than 50 years ago, Hackenbrock [27] observed condensation of rat liver mitochondria under different metabolic conditions. Nowadays, this topic is finding renewed interest because of new findings about its 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.

Spatial Distribution of the Electrochemical Gradient along the Mitochondrial Inner Membrane: Knowns, Unknowns, and Prospects

It is clear that the spatial distribution of the electrophysiologically active ETC components is organized in hierarchies (dimers and supercomplexes), and that these change in response to the metabolic needs of the cell. If the sources of the electrochemical potential Δp are non-uniformly distributed, how is the pH and $\Delta\Psi_m$ 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 regions inside the cristae and the rest of the intermembrane space? A recent GFP-based experiment demonstrated a pH difference of 0.3 between ATP synthase and CIV of the ETC [30] consistent with the expectation of a gradient of the pH along the membrane due to the spatial separation of the sink (ATP synthase) and source (ETC complex resparisomes) 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 approach [31]. Although the spatial distribution is not uniform for the ETC complexes, pH, and (presumably) $\Delta\Psi_m$, 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 nontrivial 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 3).

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 4). 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 cofactors 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.

Box 3. What Is the Role of the Mitochondrial Permeability Transition Pore (mtPTP) in Apoptosis?

The mtPTP is a name given to a complex of membrane proteins at the inner and outer membrane that are believed to form a large pore. The molecular identity of each of the components is controversial and not completely known [58–60], despite intense effort in the area for >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 inter-relationship 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 I and [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 I 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 best [55], and spatial resolution to 1 voxel ≈ one mitochondrion, 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, cyclophilin D, etc. A vast literature documents inducers of mPTP 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^{2+} overload, short openings of mtPTP might serve as an emergency release of accumulated Ca^{2+} ions and a mechanism allowing the partial dissipation of $\Delta\Psi_m$, reducing ROS generation. However, once the inner membrane is compromised via the mtPTP, the organelle ruptures via osmotic swelling and this releases CytC, triggering apoptosis. The original discovery studies of the mtPTP >10 years ago implicated it *in vivo* as a significant participant in apoptosis but since then researchers have questioned whether 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 flicker [63] 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 'flashes' of ROS, a bursting superoxide signal followed by a transient alkalization signal. These recent experiments are done using genetically engineered GFP technology. In fact the frequency of these flashes has been correlated with lifespan in some organisms [64].

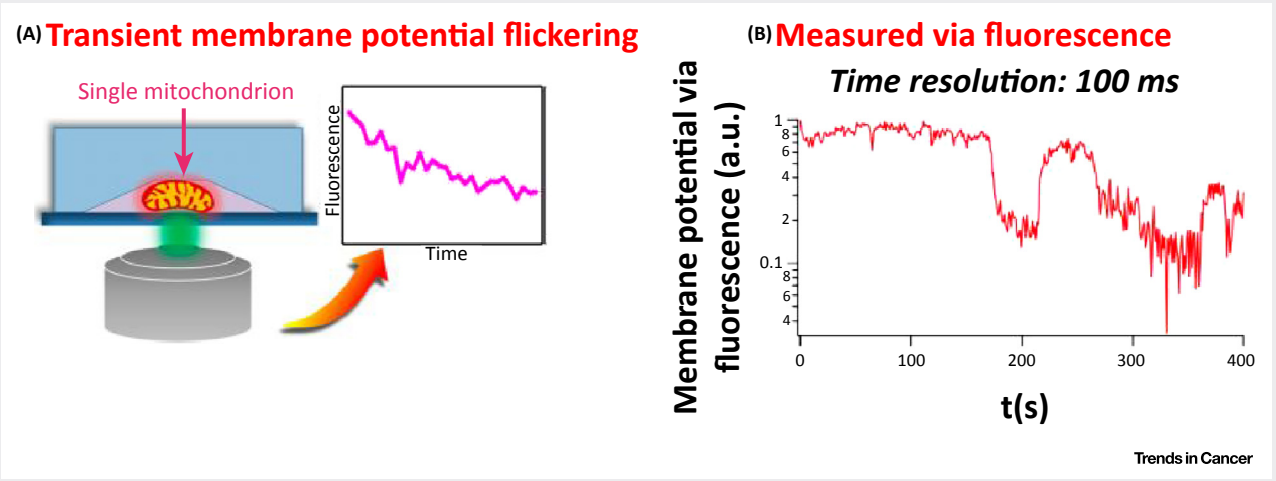

Figure I. (A) Single, vital, isolated mitochondria in a nanofluidic channel, labeled with TMRE (schematic). (B) TMRE fluorescence intensity versus time indicating flickering of the membrane potential prior to complete depolarization. Abbreviations: TMRE, tetramethylrhodamine, ethyl ester. Adapted, with permission, from [61].

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 >15 years ago to predominately reside in the regions inside the cristae, and not the rest of the intermembrane 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 precondition for apoptosis), one hypothesis is that cristae remodeling must occur to open the junctions.

Box 4. Apoptosis Pathway, MOMP, and Cancer Chemotherapy

In the mitochondrial pathway of cell death (apoptosis), various upstream effectors (DNA damage, ROS, stress, cell death signals, etc.) result in 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 CtyC into the cytosol. This release of CytC irreversibly activates the caspases that carry the apoptotic process to completion. Resistance to apoptosis is a hallmark of cancer [9]. In fact, eventually, every chemotherapy is believed to result in apoptosis as its final mechanism of action, regardless of the initial point of attack [49]. 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 CytC 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 CytC is contained in the cristae [22], the morphology of mitochondria is a key parameter to study.

Mitochondrial Stores of CytC Are Releases during Apoptosis

The release of the mitochondrial stores of CytC 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 understood [50] is the permeabilization of the mitochondrial outer membrane (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 >20 related proteins that share four homology domains (BH1–4). During MOMP, the outer membrane becomes permeable with pores, allowing high-molecular-weight proteins such as CytC to pass. Thus, it was initially believed that the MOMP process led to the release of CytC directly. Later, more refined thinking about cristae remodeling followed.

The MOMP process is the result of oligomerization of BAX or BAK 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 super-resolution OM, Jakobs *et al.* have imaged MOMP as large lipidic pores up to >100 nm in diameter [51]. These always occur with CytC release, making it impossible to dissect the order or cause/effect relationship. Their work is consistent with lipidic pores observed in liposomes with TEM [51] as well as AFM studies [52].

Furthermore, the regulation of BAX/BAK-mediated MOMP has been shown to be positively and negatively affected by the BCL-2 family of proteins. Although there are 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 clinically significant results. For example, venetoclax (ABT-199) has been developed as a chemical inhibitor of the antiapoptosis BCL-2 gene. It has received FDA approval for use in patients with chronic lymphocytic leukemia (CLL) with the 17p deletion. Additional clinical trials (Phase I–III) are underway for CLL, non-Hodgkin's lymphoma (including diffuse large B-cell lymphoma, mantle cell lymphoma, and follicular lymphoma), acute myeloid leukemia, multiple myeloma, systemic lupus erythematosus, and breast cancer [53].

Scorrano *et al.* found in 2002 that cristae remodeling occurs during apoptosis (opening the neck from 18 to 56 nm) [22], and found this was caused by the peptide **BID** (a proapoptotic member of the BH3-only subset of the BCL-2 family of proteins; Box 2) in a way that was independent of BAX/BAK oligomerization. BAX/BAK are proteins believed to oligomerize, forming pores which permeabilize the outer membrane (hence MOMP) (Box 4). 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 intermembrane space, and MOMP enables CytC to escape from the intermembrane space, cross the outer membrane (through the MOMP pores), and into the cytosol. This followed by work of Frezza in 2006 [32], who showed that OPA1 prevents CytC release and hence is the glue that holds the cristae together. Additional work supporting the importance of cristae junction opening to release CytC was also presented by Cipolat *et al.* [33]. Oligerimization of OPA1 was found to be the mechanism that regulates apoptosis by maintaining the tightness of the cristae junctions.

Sun *et al.* [34] 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 al.* [35] found that OPA1 oligomerization indeed blocks CytC release from the cristae, and that the disassembly of the OPA1 oligomers causes CytC release. This release of CytC is found even if the diameter of the cristae junctions is halved (from 18 to 9 nm) during apoptosis. Although both Scorrano *et al.* and Yamaguchi *et al.* found that BAX/BAK oligomerization is required for CytC release through the outer membrane, only Yamaguchi *et al.* found that BAX/BAK and BH3-containing BID are required for CytC release from the cristae junctions.

Regardless, both found that 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 that controls 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 imaging [36], 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 a Nobel prize in 2017 [37]. 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.

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, for example, exposure to staurosporine, actinomycin, etoposide, or UV. During this process, the cytosolic pH, mitochondrial membrane potential $\Delta\Psi_m$, 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 drops [38], and the cytosolic pH drops slightly [40]. The drop of the membrane potential and respiration rate is consistent with 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 is 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 constant [38], even though CytC is released from the mitochondria. Upon permeabilization of the cell plasma membrane by digitonin, the membrane potential and respiration rate drop [38]; these can be restored by the introduction of exogenous CytC into the buffer, which restores the

respiration rate [38] and $\Delta\Psi_m$ [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** (proapoptotic members of the BH3-only subset of the BCL-2 family of proteins; Box 2) to the suspension medium. In isolated mitochondria, upon induction of MOMP, metabolic assays show decreased respiration rate [39,41] and membrane potential [24,39,41], as well as a reduction in the buffer pH [24]. When exogenous CytC is introduced back into the buffer, these parameters are restored (when measured [24,39,41]). These data support a model where the loss of CytC from the mitochondria stalls the ETC. The pH change is discussed below. For permeabilized cells, the data are similar: upon BID-induced MOMP, $\Delta\Psi_m$ goes down [42], which is recovered upon addition of exogenous CytC into the buffer [38]. It should be noted that peptides have 100–1000-fold less activity than full-length proteins, suggesting caution is required when interpreting the results of experiments using peptides.

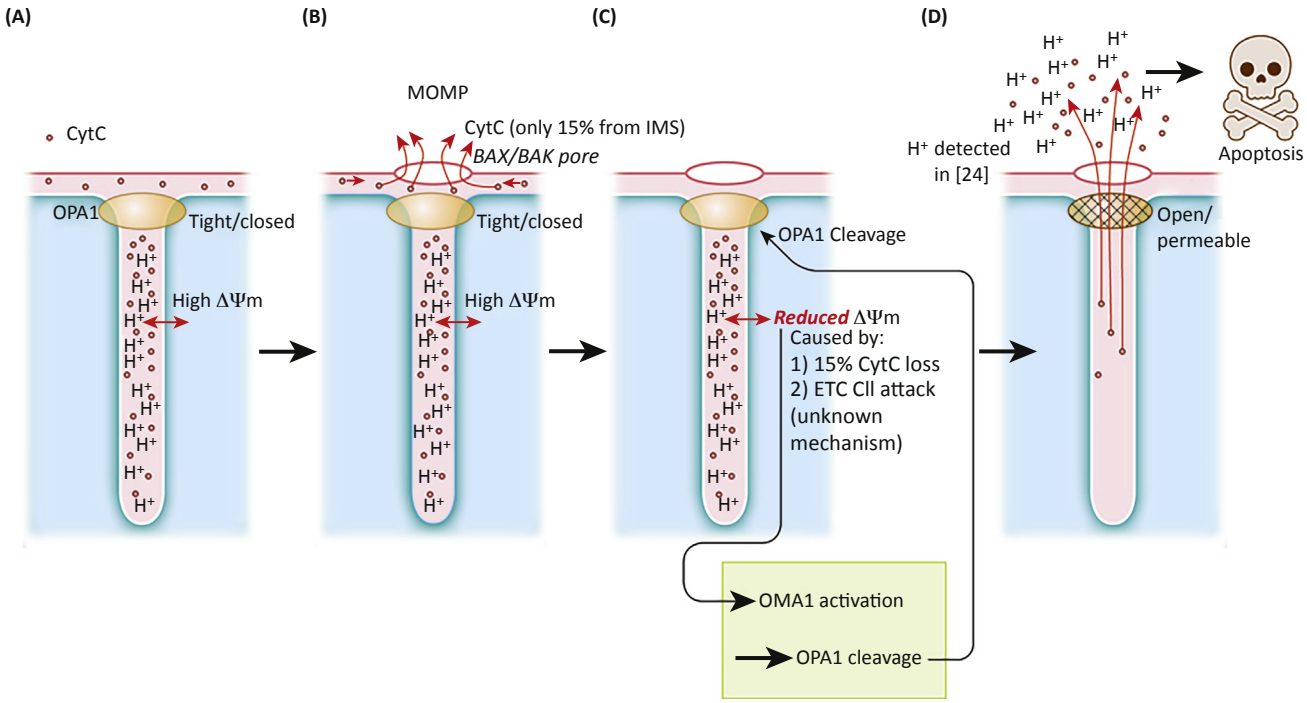

Figure 3. Emerging Model of How CytC Is Released. (A) Healthy mitochondria. Eighty-five percent of CytC is contained in the cristae and cannot escape. Because of the localization of the ETC resparisomes (not shown) in the cristae, the H^+ concentration is high there. (B) On MOMP, BAX/BAK pores form in the outer mitochondrial membrane (red). This releases a fraction of the CtyC into the cytosol; the remainder is still trapped by the tightly closed cristae junctions. (C) The mitochondrial membrane potential drops in response to either (i) loss of 15% of CtyC [24] or (ii) enzymatic attack on CII of the ETC by an unknown enzyme, in response to MOMP via an unknown mechanism [45]. This drop in $\Delta\Psi_m$ 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 cytosol [24], which may enhance the activity of the executioner caspases triggered by CytC. Abbreviations: CII, Complex II; CytC, cytochrome C; $\Delta\Psi_m$, membrane potential; ETC, electron transport chain; IMS, intermembrane space; MOMP, mitochondrial outer membrane permeabilization; OMA1, mitochondrial zinc metalloprotease; OPA1, optic atrophy 1.

Emerging Model

An early indication of the link between membrane potential and apoptosis was found in 2003 [43], 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 are dissembled is not known. The discovery of the dependence of OMA1 cleavage of OPA1 on $\Delta\Psi_m$ [26] has given a critical insight into the relationship between these parameters. In particular, OMA1 suppression prevents CytC release [44]. Therefore, cleavage of OPA1 must be required for CytC release. The relationship between $\Delta\Psi_m$, OPA, and cristae junction remodeling provides the missing link that gives 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 a small fraction of CtyC stored in the intermembrane space to be released into the cytosol, hence reducing the membrane potential $\Delta\Psi_m$ by reducing the activity of the ETC. Another, parallel pathway that reduces $\Delta\Psi_m$ after MOMP is degradation of Complex II of the ETC. Although the mechanism by which Complex II is degraded after MOMP is not known, evidence [45] confirms the model that the membrane potential degradation (actually ETC Complex II degradation) occurs upstream of OMA1 activation. Once OMA1 is activated due to reduction of $\Delta\Psi_m$, 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 ($\Delta\Psi_m$), mitochondrial ultrastructure (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 intermembrane space pH and membrane potential thus stabilizing OPA1 and blocking detectable changes in proton and CytC release.

Dynamin-Related Protein Plays a Significant Role in Cristae Remodeling

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. Super-resolution OM studies have shown BAX/BAK rings form on the outer membrane [46]. However, in DRP knockdown cells in the same work, these rings (which seemingly indicate large holes in the mitochondrial outer membrane) are not followed by CytC release. More recently, Otera *et al.* [47] have shown that DRP-1 knockout cells completely resist cristae remodeling and CytC release during apoptosis, and in these cell lines OPA1 oligomers are disassembled but the cristae are 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 ultrastructure, as the structures are smaller than the wavelength of light and only barely addressable by newly developed deep-sub wavelength microscopies (Box 2). 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, electrophysiological (electrical) information such as: membrane potentials, proton currents and fluxes, and pH gradients, with resolution smaller

Outstanding Questions

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 ultrastructure 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 noninvasive technologies be developed to peer inside the nanoscale structures of mitochondria and monitor their electrophysiological and structural activities in real time?

than the size of the structure, that is, at the 1-nm scale (see Outstanding Questions). Real-time nanoprobes such as atomic force microscopy (AFM)-based tools, quantum dots, or even nanoelectronic probes [24,48], 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.

Acknowledgments

This work was funded by the National Cancer Institute grant # IMAT R33CA183384. Extensive input based on an extremely productive long-term collaboration from Professor Douglas C. Wallace of the Center for Mitochondrial and Epigenomic Medicine, Children's Hospital of Philadelphia, Philadelphia, PA, USA and Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA regarding the models and mechanisms have been instrumental in formulating the concepts presented in this paper.

References

1. Lane, N. and Martin, W. (2010) The energetics of genome complexity. *Nature* 467, 929–934
2. Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* 191, 144–148
3. Jayashankar, V. *et al.* (2016) Shaping the multi-scale architecture of mitochondria. *Curr. Opin. Cell Biol.* 38, 45–51
4. Cogliati, S. *et al.* (2016) Mitochondrial cristae: where beauty meets functionality. *Trends Biochem. Sci.* 41, 261–273
5. Kasahara, A. and Scorrano, L. (2014) Mitochondria: from cell death executioners to regulators of cell differentiation. *Trends Cell Biol.* 24, 761–770
6. Perkins, G.A. and Ellisman, M.H. (2016) Remodeling of mitochondria in apoptosis. In *Mitochondria and Cell Death* (Hockenbery, D. M., ed.), pp. 85–110, Springer
7. Otera, H. and Mihara, K. (2012) Mitochondrial dynamics: functional link with apoptosis. *Int. J. Cell Biol.* 2012, 821676
8. Cleland, M.M. and Youle, R.J. (2011) Mitochondrial dynamics and apoptosis. In *Mitochondrial Dynamics and Neurodegeneration* (Lu, B., ed.), pp. 109–138, Springer
9. Hanahan, D. and Weinberg, R. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674
10. Davies, K.M. *et al.* (2011) Macromolecular organization of ATP synthase and complex I in whole mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* 108, 14121–14126
11. Strauss, M. *et al.* (2008) Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. *EMBO J.* 27, 1154–1160
12. Klotzsch, E. *et al.* (2015) Superresolution Microscopy reveals spatial separation of UCP4 and F0F1 -ATP synthase in neuronal mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* 112, 130–135
13. Paumard, P. *et al.* (2002) The ATP synthase is involved in generating mitochondrial cristae morphology. *EMBO J.* 21, 221–230
14. Habersetzer, J. *et al.* (2013) Human F1F0 ATP synthase, mitochondrial ultrastructure and OXPHOS impairment: a (super-)complex matter? *PLoS One* 8, e75429
15. Bitbol, A.-F. *et al.* (2012) Lipid membrane deformation in response to a local pH modification: theory and experiments. *Soft Matter* 8, 6073–6082
16. Wilkens, V. *et al.* (2013) Restricted diffusion of OXPHOS complexes in dynamic mitochondria delays their exchange between cristae and engenders a transitory mosaic distribution. *J. Cell Sci.* 126, 103–116
17. Acín-Pérez, R. *et al.* (2008) Respiratory active mitochondrial supercomplexes. *Mol. Cell* 32, 529–539
18. Pfanner, N. *et al.* (2014) Uniform nomenclature for the mitochondrial contact site and cristae organizing system. *J. Cell Biol.* 204, 1083–1086
19. Van der Laan, M. *et al.* (2012) Role of MINOS in mitochondrial membrane architecture and biogenesis. *Trends Cell Biol.* 22, 185–192
20. Jakobs, S. and Wurm, C.A. (2014) Super-resolution microscopy of mitochondria. *Curr. Opin. Chem. Biol.* 20, 9–15
21. Jans, D.C. *et al.* (2013) STED super-resolution microscopy reveals an array of MINOS clusters along human mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* 110, 8936–8941
22. Scorrano, L. *et al.* (2002) A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev. Cell* 2, 55–67
23. Santo-Domingo, J. and Demaurex, N. (2012) The renaissance of mitochondrial pH. *J. Gen. Physiol.* 139, 391–393
24. Pham, T.D. *et al.* (2016) Cristae remodeling causes acidification detected by integrated graphene sensor during mitochondrial outer membrane permeabilization. *Sci. Rep.* 6, 35907
25. MacVicar, T. and Langer, T. (2016) OPA1 processing in cell death and disease – the long and short of it. *J. Cell Sci.* 129, 2297–2306
26. Head, B. *et al.* (2009) Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells. *J. Cell Biol.* 187, 959–966
27. Hackenbrock, C.R. (1966) Ultrastructural bases for metabolically linked mechanical activity in mitochondria. *J. Cell Biol.* 30, 269–297
28. Cogliati, S. *et al.* (2013) Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. *Cell* 155, 160–171
29. Patten, D.A. *et al.* (2014) OPA1-dependent cristae modulation is essential for cellular adaptation to metabolic demand. *EMBO J.* 33, 2676–2691
30. Rieger, B. *et al.* (2014) Lateral pH gradient between OXPHOS complex IV and F0F1 ATP-synthase in folded mitochondrial membranes. *Nat. Commun.* 5, 3103
31. Song, D.H. *et al.* (2013) Biophysical significance of the inner mitochondrial membrane structure on the electrochemical potential of mitochondria. *Phys. Rev. E* 88, 62723
32. Frezza, C. *et al.* (2006) OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* 126, 177–189
33. Cipolat, S. *et al.* (2006) Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. *Cell* 126, 163–175

34. Sun, M.G. *et al.* (2007) Correlated three-dimensional light and electron microscopy reveals transformation of mitochondria during apoptosis. *Nat. Cell Biol.* 9, 1057–1072
35. Yamaguchi, R. *et al.* (2008) Opa1-mediated cristae opening is Bax/Bak and BH3 dependent, required for apoptosis, and independent of Bak Oligomerization. *Mol. Cell* 31, 557–569
36. Yamaguchi, R. and Perkins, G. (2009) Dynamics of mitochondrial structure during apoptosis and the enigma of Opa1. *Biochim. Biophys. Acta — Bioenerg.* 1787, 963–972
37. *Scientific Background on the Nobel Prize in Chemistry 2017 The Development of Cryo-Electron Microscopy*. https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2017/advanced-chemistryprize2017.pdf
38. Waterhouse, N.J. *et al.* (2001) Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. *J. Cell Biol.* 153, 319–328
39. Mootha, V.K. *et al.* (2001) A Reversible component of mitochondrial respiratory dysfunction in apoptosis can be rescued by exogenous cytochrome c. *EMBO J.* 20, 661–671
40. Matsuyama, S. *et al.* (2000) Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat. Cell Biol.* 2, 318–325
41. Giordano, A. *et al.* (2005) tBid induces alterations of mitochondrial fatty acid oxidation flux by malonyl-CoA-independent inhibition of carnitine palmitoyltransferase-1. *Cell Death Differ.* 12, 603–613
42. Ryan, J. and Letai, A. (2013) BH3 profiling in whole cells by fluorimeter or FACS. *Methods* 61, 156–164
43. Gottlieb, E. *et al.* (2003) Mitochondrial membrane potential regulates matrix configuration and cytochrome c release during apoptosis. *Cell Death Differ.* 10, 709–717
44. Jiang, X. *et al.* (2014) Activation of mitochondrial protease OMA1 by Bax and Bak promotes cytochrome c release during apoptosis. *Proc. Natl. Acad. Sci.* 111, 14782–14787
45. Jiang, X. *et al.* (2016) A small molecule that protects the integrity of the electron transfer chain blocks the mitochondrial apoptotic pathway. *Mol. Cell* 229–239
46. Große, L. *et al.* (2016) Bax assembles into large ring-like structures remodeling the mitochondrial outer membrane in apoptosis. *EMBO J.* 35, 402–413
47. Otera, H. *et al.* (2016) Drp1-Dependent mitochondrial fission via MiD49/51 is essential for apoptotic cristae remodeling. *J. Cell Biol.* 212, 531–544
48. Zhou, W. *et al.* (2015) Detection of single ion channel activity with carbon nanotubes. *Sci. Rep.* 5, 9208
49. Sarosiek, K.A. *et al.* (2013) Mitochondria: gatekeepers of response to chemotherapy. *Trends Cell Biol.* 23, 612–619
50. Shamas-Din, A. *et al.* (2013) Mechanisms of action of Bcl-2 family proteins. *Cold Spring Harb. Perspect. Biol.* 5, a008714
51. Schafer, B. *et al.* (2009) Mitochondrial outer membrane proteins assist Bid in Bax-mediated lipidic pore formation. *Mol. Biol. Cell* 20, 2276–2285
52. Salvador-Gallego, R. *et al.* (2016) Bax assembly into rings and arcs in apoptotic mitochondria is linked to membrane pores. *EMBO J.* 35, 389–401
53. Deeks, E.D. (2016) Venetoclax: first global approval. *Drugs* 76, 979–987
54. Brand, M.D. and Nicholls, D.G. (2011) Assessing mitochondrial dysfunction in cells. *Biochem. J.* 435, 297–312
55. Zand, K. *et al.* (2017) Resistive flow sensing of vital mitochondria with nanoelectrodes. *Mitochondrion* 37, 8–16
56. Santo-Domingo, J. *et al.* (2013) OPA1 Promotes pH flashes that spread between contiguous mitochondria without matrix protein exchange. *EMBO J.* 32, 1927–1940
57. Bertholet, A.M. *et al.* (2017) Mitochondrial patch clamp of beige adipocytes reveals UCP1-positive and UCP1-negative cells both exhibiting futile creatine cycling. *Cell Metab.* 25, 811–822.e4
58. Bernardi, P. (2013) The mitochondrial permeability transition pore: a mystery solved? *Front. Physiol.* 4, 95
59. He, J. *et al.* (2017) Persistence of the mitochondrial permeability transition in the absence of subunit c of human ATP synthase. *Proc. Natl. Acad. Sci.* 114, 201702357
60. Shanmughapriya, S. *et al.* (2015) SPG7 is an essential and conserved component of the mitochondrial permeability transition pore. *Mol. Cell* 60, 47–62
61. Zand, K. *et al.* (2013) Nanofluidic platform for single mitochondria analysis using fluorescence microscopy. *Anal. Chem.* 85, 6018–6025
62. Huser, J. and Blatter, L.A. (1999) Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. *Biochem. J.* 343, 311–317
63. Wang, W. *et al.* (2016) Mitochondrial flash: integrative reactive oxygen species and pH signals in cell and organelle biology. *Antioxid. Redox Signal.* 25, 534–549
64. Shen, E.-Z. *et al.* (2014) Mitoflash frequency in early adulthood predicts lifespan in *Caenorhabditis elegans*. *Nature* 508, 128–132