



HHS Public Access

Author manuscript

Contact (Thousand Oaks). Author manuscript; available in PMC 2022 November 02.

Published in final edited form as:

Contact (Thousand Oaks). 2022 ; 5: . doi:10.1177/25152564221133267.

Mitochondria from the Outside in: The Relationship Between Inter-Organellar Crosstalk and Mitochondrial Internal Organization

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Abstract

A fundamental role of membrane-bound organelles is the compartmentalization and organization of cellular processes. Mitochondria perform an immense number of metabolic chemical reactions and to efficiently regulate these, the organelle organizes its inner membrane into distinct morphological domains, including its characteristic cristae membranes. In recent years, a structural feature of increasing apparent importance is the inter-connection between the mitochondrial exterior and other organelles at membrane contact sites (MCSs). Mitochondria form MCSs with almost every other organelle in the cell, including the endoplasmic reticulum, lipid droplets, and lysosomes, to coordinate global cellular metabolism with mitochondrial metabolism. However, these MCSs not only facilitate the transport of metabolites between organelles, but also directly impinge on the physical shape and functional organization inside mitochondria. In this review, we highlight recent advances in our understanding of how physical connections between other organelles and mitochondria both directly and indirectly influence the internal architecture of mitochondria.

Keywords

mitochondrion (mitochondria); interorganellar (inter-organellar); cristae; Ca²⁺; endoplasmic reticulum; inner mitochondrial membrane; lipid droplet; phospholipid; lysosome

Introduction

Mitochondria perform myriad cellular functions and play a central role in cellular metabolism. The organelles are home to the TCA cycle, ATP synthesis via oxidative phosphorylation, phospholipid, amino acid and nucleotide biosynthesis, and many other processes, not to mention their essential role in iron-sulfur cluster biogenesis. The

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

coordination of these metabolic processes necessitates the complicated sub-organization of the double membraned organelle into distinct compartments, and the organelle is notorious for its elaborate cristae invaginations that give its distinct appearance in electron micrographs. The organization of the inner membrane into cristae is highly related to mitochondrial respiratory function and altered cristae morphology is associated with a large number of human diseases (Zick et al., 2009; Colina-Tenorio et al., 2020). The inner workings of the organelle must also be coordinated with global cellular organization. An area of intense focus in recent years has been understanding how inter-organelle contacts interplay with mitochondrial function. Mitochondria interact with nearly every other membrane-bound organelle in the cell and while traditionally thought of as hubs for lipid transport, and in the case of ER contact with mitochondria, Ca^{2+} homeostasis, new functions of MCSs are being continuously ascribed (Lackner, 2019). As our functional and mechanistic understanding of MCSs increases, we also have achieved a greater understanding of how mitochondrial organization and function are coupled to these MCSs. The goal of this review is to highlight our current understanding of how and why the behaviors and functions of inter-organelle MCSs influence the internal organization of mitochondria.

Spatial Organization of the Mitochondria interior

Before delving into the interplay between inter-organelle communication and the mitochondrial interior, we will first briefly cover what is known about what gives mitochondria their unique architecture. Mitochondria are a double membrane-bound organelle that organizes its multitude of functions at a sub-organelle level (Figure 1). While the outer mitochondrial membrane (OMM) serves as the interface with the cytosol and other membrane-bound cellular compartments, the inner mitochondrial membrane (IMM) undergoes more extensive physical organization. Cristae invaginations are connected to the boundary membrane, areas of apposition between the IMM and OMM, via cristae junctions (CJs). CJs are narrow tubular membrane necks that are thought to limit the free diffusion of ions and proteins between each subdomain (Mannella et al., 1994).

This distinct IMM architecture is established and maintained by several interdependent factors, the best-characterized of which we will focus on in this review. The IMM has a unique phospholipid composition from other cellular bilayers and is highly enriched for two phospholipids, cardiolipin and phosphatidylethanolamine (PE), which are each synthesized on the IMM (Tatsuta and Langer, 2017). Cardiolipin and PE are both non-bilayer lipids which induce negative curvature in membranes, allowing them to directly influence organelle shape. In addition to phospholipids, respiratory complexes also contribute to the distinctive interior shape of mitochondria. The terminal chain of the oxidative phosphorylation machinery, the ATP synthase, forms dimeric assemblies at a fixed angle that induce and stabilize membrane curvature (Strauss et al., 2008; Davies et al., 2012; Blum et al., 2019). These ATP synthase dimers oligomerize in a ribbon structure that lines and shapes the highly curved edges of cristae.

A third major determinant of IMM organization are structural protein complexes that influence membrane shape and organization. The Mitochondrial Contact Site and Cristae

Organizing System (MICOS) is a conserved multi-subunit complex that is enriched on the IMM at CJs, contributes to CJ stabilization and is required for normal cristae morphogenesis (for a more detailed review, see (Mukherjee et al., 2021)). While the exact mechanism of MICOS function has not been determined, multiple subunits have membrane bending capabilities, suggesting its subunits directly shape and stabilize CJs (Barbot et al., 2015; Hessenberger et al., 2017; Tarasenko et al., 2017). However, an additional feature of the complex is its ability to make protein-protein interactions with OMM proteins and complexes. These interactions are particularly stable in mammalian cells, where MICOS assembles into a supercomplex with the β -barrel sorting and assembly machinery (SAM) complex (Ott et al., 2012; Huynen et al., 2016). This supercomplex of IMM and OMM complexes, termed the mitochondrial bridging complex (MIB), spans the intermembrane space (IMS) and positions MICOS to sense and respond to cues from outside the organelle and directly influence cristae architecture.

In addition to MICOS, the IMM dynamin-related protein OPA1 (Mgm1 in yeast), which works as an IMM fusion machine (discussed below), also has been implicated as a key regulator of cristae. Depletion of Mgm1/OPA1 leads to defects in cristae organization and its oligomeric state is thought to maintain the integrity of cristae and regulate the release of cytochrome *c* that occurs during apoptosis (Olichon et al., 2003; Frezza et al., 2006; Meeusen et al., 2006). It has been complicated to disentangle the role of Mgm1/OPA1 in mitochondrial dynamics from its influence on cristae organization, though recent structural work indicates purified Mgm1 can form assemblies on the negative-curved inside of liposomes, supporting its involvement in cristae morphogenesis (Faelber et al., 2019).

While cristae are the most structurally distinctive feature of mitochondria, there are other organizing elements of mitochondria found on the interior of the IMM. Mitochondria have their own genome (mtDNA) which encodes for respiratory complex subunits as well as tRNAs and rRNAs. mtDNA exists in numerous copies per mitochondria and each is packaged in a proteinaceous nucleoid structure that resides in the mitochondrial matrix (Farge and Falkenberg, 2019). The mtDNA is transcribed and translated in the matrix as well, and both RNA granules and the mitoribosome can be found in close proximity to the nucleoid (Rey et al., 2020). Correlative superresolution microscopy and EM studies have revealed how these structures are spatially linked to cristae membranes and do not freely diffuse through the matrix (Kukat et al., 2011; Kopek et al., 2012; Stephan et al., 2019).

One of the principal reasons for the complicated ultra-structure of mitochondria is the sub-compartmentalization of functional processes within the organelle. Perhaps best recognized is that the respiratory complexes are localized to the cristae membranes (Vogel et al., 2006; Wurm and Jakobs, 2006; Wilkens et al., 2012). Interestingly, recent work has revealed that assembly intermediates of some of these complexes localize to the IMM boundary region, indicating that spatial control exists over the assembly process (Stoldt et al., 2018). Spatial control also exists over protein import into mitochondria as the two major mitochondrial translocases, the TOM and TIM complexes, reside on the OMM and IMM, respectively, and form supercomplexes that span the IMS at the boundary region and are enriched near CJs (Gold et al., 2017). Recent advances in microscopy have revealed additional functional elements of mitochondrial suborganization. As we will discuss, the placement and

organization of these processes does not occur in a vacuum, but instead is tightly linked and influenced by inter-organelle contacts and their functions.

How Metabolic Functions of Inter-Organelle Contacts are Coordinated with the Internal Spatial Organization of Mitochondria

While mitochondria-interorganelle contacts were first appreciated in EM micrographs in the mid-twentieth century, it was not until the biochemical identification of mitochondria-associated-membranes (MAMs) in 1990 by Jean Vance (Vance, 1990) that the functional behavior of mitochondria was suggested to be coupled to other organelles. With our increased understanding of the extent of contact between mitochondria and other organelles and the more recent identification of molecular tethers, we have gained a deeper understanding of the myriad functions of these pervasive MCSs and how they influence the inner workings of mitochondria (Figure 2).

Phospholipid Transport at ER-Mitochondria MCSs Influences Mitochondrial Organization

A fundamental role of ER-mitochondria MCSs is the transport of phospholipids between the organelles. In yeast, where the phospholipid trafficking and MCS tethers are best understood, the predominant tether is the ER-Mitochondria Encounter Structure (ERMES) complex. ERMES is a multi-subunit complex that spans the OMM and the ER membrane (Kornmann et al., 2009). Three ERMES subunits have cytosolic-facing phospholipid-binding SMP domains. *In vitro* work demonstrates the ability of such SMP proteins to bind and transport lipids, and consistent with this, cells deficient in the ERMES complex have altered mitochondria phospholipid composition (AhYoung et al., 2017; Jeong et al., 2017; Kawano et al., 2018). New work from the Kornmann group also provides long awaited *in vivo* evidence for the transport of phospholipids by ERMES (John Peter et al., 2022). While additional lipid transport proteins also have been linked to the regulation of mitochondrial membrane composition (for example the sterol transporter Lam6/Ltc1 (Elbaz-Alon et al., 2015; Murley et al., 2015), the ERMES complex in yeast has been demonstrated to be critical for mitochondrial organization.

Lipids provided to the mitochondria from inter-organelle contacts are utilized by phospholipid synthesis enzymes in the IMM to make cardiolipin and PE. Phosphatidic acid, which is presumably transported from the ER by ERMES, is subsequently transported across the IMS by the conserved Ups1/PRELID1 protein, before being converted into cardiolipin by IMM enzymes (Connerth et al., 2012). PE, meanwhile, is synthesized from PS that is also thought to be transported from the ER via ERMES, then across the IMS by the Ups1 paralog, Ups2/PRELID2 (Aaltonen et al., 2016).

Once generated, both cardiolipin and PE have direct and indirect roles in contributing to mitochondrial architecture. As discussed above, PE and cardiolipin can directly shape the IMM. Consistent with this direct role, the presence of either cardiolipin or PE is essential for life, as the simultaneous loss Crd1 and Psd1, which are required for the synthesis of cardiolipin and PE, respectively, is synthetically lethal in yeast (Gohil et al., 2005). However, phospholipids also work indirectly to influence architecture by contributing to protein

complex stability and function. ATP synthase dimerization, which stabilizes the highly curved edges of cristae, requires cardiolipin (Acehan et al., 2011). As another example, cardiolipin is required for assembly of one of two MICOS subcomplexes and influences the oligomeric state of the MICOS membrane shaping component Mic10 (Friedman et al., 2015; Rampelt et al., 2018). PE also plays a role in influencing mitochondrial organization. Both Psd1 and the IMS PS transporter Ups2 and its partner Mdm35 genetically interact with MICOS and influence mitochondrial ultrastructure and respiration (Aaltonen et al., 2016; Kojima et al., 2019). Thus, ER-mitochondria MCSs facilitate mitochondrial ultrastructure and organization indirectly through their lipid trafficking function.

However, ERMES may also play a more direct role in influencing cristae organization. In line with this, our lab recently determined that a subset of MICOS complexes form stable assemblies in wild type cells that appear to associate in proximity to ERMES-marked MCSs (Tirrell et al., 2020). In cells where the holo-MICOS complex was unable to assemble, the two MICOS subcomplexes each concentrated in assemblies that localized in proximity to ER-mitochondria MCSs. Interestingly, one of these subcomplexes disassembled in the absence of ERMES and cardiolipin, consistent with the model that a role of ER contacts may be to provide a lipid microdomain on the IMM that is favorable to promote MICOS, and perhaps cristae, assembly. The other MICOS subcomplex (consisting of Mic60 and Mic19) appears to stably associate in proximity to ER contacts independently of ERMES, suggesting the ER may influence MICOS assembly and cristae morphology by more than one mechanism.

Loss of ERMES in yeast leads to defects in both mitochondrial morphology and cellular respiratory capacity (Kornmann et al., 2009). Systematic identification of compensatory suppressor mutations of these defects has enhanced our understanding of how lysosome (yeast vacuole)-mitochondria MCSs can also serve as lipid transport hubs to mitochondria. Loss of ERMES can be suppressed by a dominant allele of Vps13, an N-chorein domain containing protein which has demonstrated to be involved in phospholipid binding and transport at multiple MCSs, including lysosome-mitochondria contacts (Lang et al., 2015; Park et al., 2016; John Peter et al., 2017; Bean et al., 2018; Kumar et al., 2018; Yeshaw et al., 2019; Li et al., 2020).

While Vps13 in yeast can target to the OMM via its receptor Mcp1, a phospholipid scramblase, and Vps13 is thought to mediate lipid transport between the lysosome and mitochondria, the two proteins are not thought to directly tether the organelles (John Peter et al., 2017; Bean et al., 2018; Adlakha et al., 2022). Instead, this occurs by the vCLAMP, a molecular tether between the vacuole-associated protein Vps39 and the OMM translocase subunit Tom40 (Gonzalez Montoro et al., 2018). Work from the Ungermann and Schuldiner labs determined that overexpression of Vps39 is sufficient to force extended vCLAMPs (Elbaz-Alon et al., 2014; Honscher et al., 2014). While the precise functional role of vCLAMPs is not clear, they may form a platform by which Vps13 can transport lipids. Interestingly, EM images from cells in which vCLAMPs are formed revealed that cristae are largely absent from these forced lysosome-mitochondria MCSs (Honscher et al., 2014). Thus, it is tempting to speculate that inappropriate phospholipid distribution caused by the formation of vCLAMPs has a major influence on cristae organization inside mitochondria.

Work from our lab found that one of the principal MICOS components, Mic60, specifically redistributed along the IMM when vCLAMPs are forced, potentially providing a molecular explanation for such cristae reorganization (Tirrell et al., 2020).

While mammalian cells lack sequence homologs of core ERMES complex subunits, recent work has identified that the human Vps13 homolog VPS13D targets to ER-mitochondria MCSs via an interaction with the Miro GTPase, a homolog of the ERMES-auxiliary subunit Gem1 (Guillen-Samander et al., 2021). Miro also physically interacts with MICOS and is required for normal cristae morphology, which we will discuss in more detail below, though it remains to be determined if these defects are caused by lack of VPS13D recruitment. Meanwhile, there is evidence that other lipid transport proteins, including ORP5 and ORP8, may work at the ER-mitochondria interface (Galmes et al., 2016). Interestingly, in a recent preprint, the Giordano group found that ORP5 and ORP8 physically interact with the MIB complex and that depletion of ORP5/ORP8 alters cristae morphology (Rochin et al., 2021). Altogether, these findings further support that a conserved role of inter-organelle contacts is as lipid transport hubs that shape the mitochondrial phospholipidome, and by extension, internal mitochondrial organization.

Ca²⁺ Regulation at ER-Mitochondria MCSs and its Influence on Mitochondrial Architecture

One of the primary functions of ER-mitochondria contacts is the regulation of cellular Ca²⁺ stores to mediate cellular signaling, oxidative metabolism, and cell death. Local release of Ca²⁺ from the ER via the inositol triphosphate receptor (IP3R) at sites of close apposition between the organelles allows for its uptake via the OMM voltage dependent anion channel (VDAC) and the IMM mitochondrial Ca²⁺ uniporter (MCU) (Pallafacchina et al., 2021). The local uptake of Ca²⁺ is thought to increase TCA cycle metabolism and promote electron transport complex activity.

Interestingly, recent works suggest that mitochondrial protein behavior and cristae shape at the ER-mitochondrial interface are dynamically modulated by Ca²⁺. The MCU is enriched on the IMM at the boundary region in cardiomyocytes (De La Fuente et al., 2016) and can dynamically reposition from cristae to the boundary upon induced Ca²⁺ spikes in other cell types via its interaction with its regulatory factor MICU1 (Gottschalk et al., 2019), suggesting mitochondria utilize compartmentalization of the IMM to spatially regulate Ca²⁺ dynamics. However, cristae themselves are also morphologically responsive to Ca²⁺. Cristae are enriched at ER-mitochondria MCSs more so than would be predicted due to chance (Booth et al., 2016) and those cristae in proximity to the ER appear to be less motile upon agonist-stimulated Ca²⁺ release from the ER (Gottschalk et al., 2018). Work from the Hajnoczky lab suggests that a consequence of agonist stimulated release of Ca²⁺ from the ER is the narrowing of cristae, causing compression of the intra-cristal space and the release of H₂O₂ across the OMM to the ER-mitochondrial junction, positively stimulating ER Ca²⁺ channels (Booth et al., 2016). Thus, a specialized role of ER-proximal cristae may be specific for regulating cellular Ca²⁺ homeostasis.

ER-proximal cristae may also serve as a cellular quality control determinant. Overload of Ca²⁺ into mitochondria can cause mitochondrial dysfunction and lead to apoptosis. Recent *in vitro* work suggests that cristae ultrastructure changes in response to elevated

levels of free Ca^{2+} . These changes include wider CJs and increased matrix-localized calcium-containing granules, and corresponds to reduced respiration (Strubbe-Rivera et al., 2021a). Cases of extreme Ca^{2+} overload are thought to lead to prolonged opening of the mitochondrial permeability transition pore (mPTP), an IMM channel of controversial identity but thought to be the ATP synthase and/or the adenine nucleotide transporter (Bonora et al., 2022). Opening of the mPTP leads to loss of membrane potential, mitochondrial swelling, cytochrome *c* release, and ultimately cell death. *In vitro*, Ca^{2+} overload stimulates opening of the mPTP and successive rupture of the OMM and IMM (Strubbe-Rivera et al., 2021b). While Ca^{2+} overload ultimately causes cell death *in vivo*, it remains to be determined whether apoptosis-associated de-oligomerization of OPA1 and CJ widening are directly caused by Ca^{2+} . Interestingly, depletion of MICU1 causes CJ widening to a similar extent as OPA1 depletion (Gottschalk et al., 2019), potentially indicating a related mechanism. It remains to be determined the molecular basis of CJ remodeling during milder Ca^{2+} stimulation, and whether the MICOS complex is involved in Ca^{2+} -mediated cristae dynamics. However, the MICOS/MIB complex physically interacts with the EF-hand containing protein Miro, depletion of which delays Ca^{2+} uptake into mitochondria (Modi et al., 2019), suggesting a potentially regulatory input.

Spatial Coupling of Mitochondrial Metabolism with Inter-Organelle MCSs

Localized Metabolism at ER-Mitochondrial Contact Sites.—An emerging area of focus in mitochondrial biology is the concept of spatially-compartmentalized metabolism at specific sub-organelle sites within the mitochondrial matrix, and in recent years, it has become clear that these metabolic hubs are spatially linked to inter-organelle MCSs. The discovery of the ERMES complex in yeast was instrumental in such discoveries, as visualization of the ERMES complex by fluorescence microscopy serves as a focal landmark to precisely map other spatially-linked processes relative to ER-mitochondria MCSs.

Through the use of high throughput screening in yeast, the Schuldiner lab made the important discovery that the mitochondrial pyruvate dehydrogenase complex (PDH), labeled with the enzymes Pda1 and Lpd1, localized to discrete foci in the matrix that co-localized with the ERMES complex (Cohen et al., 2014). Simultaneously, the authors found that peroxisomes, a second source of acetyl-coA via fatty acid β -oxidation in yeast, also exhibit significant co-localization with ERMES, interactions that are potentially mediated between ERMES and Pex11 (Mattiuzzi Ušaj et al., 2015) as well as by Pex34 and Fzo1 (Shai et al., 2018). The Schuldiner lab speculated that these two sources of acetyl-CoA may be spatially positioned in a manner to fuel the TCA cycle in the matrix (Cohen et al., 2014). Indeed, a recent systematic screen of metabolic enzymes revealed that many such enzymes also are spatially restricted within mitochondria in yeast. In addition to Pda1, several enzymes of the TCA cycle (including Kgd1, Mdh1, and Fum1) as well as other metabolic enzymes localize to discrete foci within mitochondria when GFP tagged (Noree et al., 2019). However, it remains to be determined how the compartmentalization of these enzymes inside the organelle influences metabolic flux and function, whether the focal assemblies of PDH and TCA machinery in the matrix are spatially linked to each other and to inter-organelle MCSs, and the precise functional relationship between MCSs and metabolic activity in the matrix.

CoQ metabolism has also been demonstrated to be physically linked to and dependent on ER MCSs. Recent work from the Nunnari and Schuldiner labs revealed that members of the CoQ biosynthetic pathway were spatially linked to ERMES complexes (Eisenberg-Bord et al., 2019; Subramanian et al., 2019). The assemblies of CoQ enzymes were revealed to form in a substrate-dependent fashion, indicating that enzymatic assemblies within mitochondria are sites of substrate channeling (Subramanian et al., 2019). Further, the studies revealed that disruption of the ER-mitochondria tethers impeded the formation of these assemblies as well as CoQ metabolism.

Together, these important findings reveal that inter-organelle contacts have a direct influence on the internal metabolic activity of mitochondria. However, it is unclear whether the ER contact site itself is important or whether the localized metabolism is key. An intriguing possibility is that Vps13-mediated bypass of ERMES loss will induce spatial re-localization of metabolic enzymes to vacuole-mitochondria contact sites. However, whether this occurs, and whether CoQ metabolism is spatially re-localized in a Vps13-remodeled background, remains to be determined.

Lipid Droplet MCSs Influence cristae Organization.—In mammals, where mitochondria perform fatty acid β -oxidation, fatty acid storage and mobilization at lipid droplets (LDs) and mitochondrial metabolism are intimately connected. An elegant study by the Shirihai lab performed careful *in vivo* characterization combined with differential biochemical fractionation to determine the character of mitochondria associated with LDs as opposed to non-LD-associated mitochondria in brown fat cells (Benador et al., 2018). Interestingly, the authors found that LD-associated mitochondria had functional differences from their counterparts, including higher respiratory capacity and, somewhat paradoxically, lower β -oxidation capacity. Corresponding to these functional differences, the authors found slight differences in cristae length and width of LD-associated mitochondria. The altered cristae appearance in LD-associated mitochondria in brown adipose tissue (BAT) is consistent with observations made in mouse cardiac tissue upon feeding with different diets (Varghese et al., 2019). As more insights are gained into the mechanisms that fine-tune cristae architecture, it will be fascinating to determine new ways in which mitochondrial structure and function are adaptively and dynamically tuned specifically at the LD interface.

While not examining mitochondrial morphology at LD MCSs directly, the Bickel group recently reported drastic alterations in overall mitochondrial morphology in BAT, where mitochondria make extensive LD interactions, upon cold stimulus (Gallardo-Montejano et al., 2021). The authors forced adaptive thermogenesis in mice and found that cristae density dramatically increased. In this work, they found that perilipin 5 (PLIN5), a LD-associated protein that promotes LD-mitochondria MCSs (Wang et al., 2011), increased in expression during cold exposure in BAT. By forcing PLIN5 overexpression in BAT in room temperature mice, cristae density increased to match cold-exposed mice in a manner dependent on triacylglycerol (TAG) lipolysis at LDs. New work by the Puigserver group has identified that this cristae density increase during adaptive thermogenesis in BAT may be driven by PERK-dependent ER-stress, which leads to increased import of the MICOS component MIC19 into mitochondria (Latorre-Muro et al., 2021). Altogether, these works suggest that

cristae organization may be influenced both directly and indirectly at the LD-mitochondrial interface and can be altered based on the metabolic demand of cells.

Iron Transport and Heme Metabolism at MCSs.—Another recently ascribed function of the mitochondrial interface with other organelles is the trafficking of iron and heme metabolism. Iron is a co-factor in heme assembly, and accordingly, the uptake of iron from transferrin receptors was found to be spatially coupled to endosome-mitochondria MCSs (Sheftel et al., 2007; Das et al., 2016). Interestingly, blocking transferrin release from endosomes prolonged the interaction between the organelles (Das et al., 2016).

The ER-mitochondria interface has been also implicated in iron transport as well as heme transport and metabolism. In yeast, disruption of the ERMES complex was found to lead to a cellular transcriptional response suggestive of iron deficiency, and a concomitant accumulation of iron in cells was observed (Xue et al., 2017). While it is not clear if ERMES directly transports iron, the phenotype was interestingly mimicked by deletion of the SAM/MIB complex component Sam37 (Xue et al., 2017). Mitochondrial iron is utilized by ferrochelatase, the last enzyme in the heme synthesis pathway, residing in the matrix. Interestingly, in yeast, ferrochelatase physically interacts with the MICOS subunit Mic60 and Mic60 is required for efficient activity of the enzyme, potentially linking cristae organization and heme synthesis (Dietz et al., 2021). Besides acting as a respiratory co-factor, heme is trafficked throughout the cell and the ERMES auxiliary subunit Gem1 was found to negatively regulate heme trafficking to the nucleus (Martinez-Guzman et al., 2020). While it is not yet clear if there is spatial coordination between iron uptake, heme synthesis, and transport, the idea of a heme metabolon has been proposed based on physical interactions between the synthesis enzymes (Medlock et al., 2015). Given the spatial control that exists over PDH and CoQ synthesis enzymes, it will be interesting to explore the possibility enzymes involved in heme synthesis are spatially linked to inter-organelle contacts.

Amino Acid Sensing Drives Mitochondria Remodeling at ER MCSs.—In addition to examples of sub-localization of mitochondrial enzymes within the matrix, the structural organization of the organelle is also tied into global cellular metabolism. In response to elevated cytoplasmic levels of branched-chain amino acids, mitochondria in yeast and mammalian cells undergo physical remodeling to form mitochondrial derived compartments (MDCs) (Schuler et al., 2020, 2021). While the mechanism is poorly understood, a number of proteins on the IMM and OMM, including carrier proteins required for amino acid transport across the IMM, are selectively recruited into vesicular structures that bud off from mitochondria and are ultimately targeted to autophagosomes for degradation (Hughes et al., 2016). These structures form at ER MCSs, and importantly, require Gem1/Miro for their formation (English et al., 2020; Schuler et al., 2020). However, the functional significance of spatially positioning MDCs near the ER is not yet clear. Interestingly, bypass of ERMES loss by Vps13 did not suppress MDC formation, leading the authors to speculate that ERMES interactions with the cytoskeleton, rather than a lipid transport role, may help promote MDC biogenesis (English et al., 2020). Indeed, mitochondrial remodeling also occurs to form mitochondrial derived vesicles (MDVs), another stress-responsive structure

that selectively removes IMM and OMM cargoes from mitochondria and forms in a Miro- and microtubule-dependent manner (Konig et al., 2021). The spatial positioning of MDV formation relative to ER MCSs has yet to be examined, and it would be interesting to determine if the ER MCS plays a functional role in the formation of each of these compartments beyond organizing cytoskeletal scaffolds.

The Interplay Between Mitochondrial Dynamics and Mitochondrial Internal Architecture

Not only do mitochondria have a highly complicated internal structure, the organelle is also highly spatially dynamic. Mitochondria form an extensive network of tubules in cells that dynamically change position by trafficking along the cytoskeleton and control their distribution and shape through the processes of division and fusion. As such, the internal structure of mitochondria must be coordinated with changes in the overall shape and movements of the organelle.

Mitochondrial Fission

Mitochondria divide via the recruitment of the large dynamin-related GTPase Drp1 (Dnm1 in yeast), which oligomerizes into a structure that circumscribes mitochondria and utilizes GTP hydrolysis to mediate constriction and division of the two mitochondrial membrane bilayers. While OMM receptors recruit Drp1 to the mitochondrial surface, the site of division is pre-marked in most cases by MCSs with the ER (Friedman et al., 2011; Guo et al., 2018; Kleele et al., 2021). Cytoskeletal components including actin and even other organelles are recruited to these ER contacts helping to facilitate division (Wong et al., 2018; Nagashima et al., 2020; Kraus et al., 2021).

While it is clear that mitochondrial division is spatially coordinated with ER contacts on the outer surface of the organelle, the internal structure of mitochondria must also be organized at the division site. Prior to the discovery of the connection between ER MCSs and mitochondrial division, mtDNA nucleoids were spatially linked to members of the ERMES complex in yeast (Hobbs et al., 2001; Meeusen and Nunnari, 2003). Nucleoids were later found to be associated with mitochondrial division at these ERMES-marked MCSs and frequently distributed to both tips of daughter mitochondria (Murley et al., 2013), suggesting mitochondrial division serves to distribute the mitochondrial genome. In mammalian cells, it was more recently discovered that ER-marked mitochondrial division sites are spatially linked to active sites of mtDNA replication marked by the DNA polymerase POLG2 (Lewis et al., 2016). Lewis et al. also found that manipulating the shape of the ER membrane altered mtDNA distribution within the organelle. Thus, MCSs are suggested to play a direct role in coordinating the internal distribution of mitochondrial nucleoids.

mtDNA nucleoids are two membrane bilayers away from the ER, and it is unclear precisely how the ER may be coupled to the workings of the inside of mitochondria. While it is clear that external force alone is sufficient to allow mitochondrial division to precede (Helle et al., 2017), multiple lines of evidence suggest that proteins on the IMM help facilitate or coordinate mitochondrial division. The IMM can constrict independently of functional

Drp1 (Labrousse et al., 1999; Lee and Yoon, 2014). Further, constrictions of the IMM can be transiently observed when Ca^{2+} homeostasis is pharmacologically altered (Cho et al., 2017; Chakrabarti et al., 2018). In these cases, IMM constriction and division can be observed to precede that of the OMM in a manner dependent on the activity of the Ca^{2+} uniporter MCU (Chakrabarti et al., 2018). Higgs and colleagues suggest this Ca^{2+} uptake and IMM constriction is promoted by ER-localized INF2 mediating actin polymerization after Ca^{2+} release by the ER, causing increased proximity of the ER and mitochondria. However, a recent preprint using cryo-EM analysis to characterize mitochondrial division sites in unprecedented detail has suggested that IMM constriction is simultaneous with OMM constriction, begging the question of whether an IMM machinery is necessary to coordinate the process (Mageswaran et al., 2021).

In yeast cells, Mdm33, has been proposed to help facilitate mitochondrial division, perhaps by regulating phospholipid metabolism (Messerschmitt et al., 2003; Klecker et al., 2015). Mdm33 assembles into higher order self-interacting structures, presumably via its matrix-facing coiled-coil domain. Loss of the protein causes mitochondrial morphology defects that protect against induced mitochondrial division and its overexpression causes mitochondrial fragmentation. However, Mdm33 is not absolutely required for mitochondrial division and is not conserved in mammalian cells. Thus, it remains unclear what mechanism may coordinate mtDNA replication with division, and if such a factor exists, whether it contributes directly or is an absolute requirement.

Elegant work from Suliana Manley's lab showed that while the majority of mitochondrial division events are ER-linked, a subset of division events at the tip of the organelle were mechanistically and functionally distinct (Kleele et al., 2021). These "peripheral" divisions occurred more frequently under cell stresses, and such events frequently resulted in daughter mitochondria that failed to contain mtDNA and had lower membrane potential and elevated ROS. Peripheral daughter mitochondria were enriched for the OMM protein FIS1 and these divisions were observed to be largely FIS1-dependent (Kleele et al., 2021). Kleele et al. suggest, based on the interaction between FIS1 and the Rab7 (late endosome/lysosome) GTPase activating protein TBC1D15 (Onoue et al., 2013; Yamano et al., 2014; Wong et al., 2018), that such peripheral divisions would occur at lysosome-mitochondria MCSs and be destined for degradation. It remains to be determined in what way, if any, the internal structure of mitochondria is linked to peripheral, degradative, division events, though the recent observation that individual cristae can independently maintain their own membrane potential (Wolf et al., 2019) led the authors to speculate a potential signaling mechanism may exist.

Mitochondrial Fusion

While mitochondrial fission was first established to be spatially linked to ER-mitochondria contacts, it has become clear that mitochondrial fusion is spatially linked to the ER as well (Guo et al., 2018; Abrisch et al., 2020). Mitochondrial fusion is also regulated by members of the dynamin superfamily, and two conserved machineries operate in the process. The mitofusins MFN1 and MFN2 (Fzo1 in yeast) mediate heterotypic and homotypic tethering and GTP hydrolysis-dependent fusion between apposing OMMs. IMM fusion is

subsequently mediated by Mgm1/OPA1. Consistent with fusion being spatially linked to the ER membranes, immunofluorescence of MFNs and OPA1 revealed that these proteins localize to discrete foci along mitochondria that co-localize with ER tubules and mark mitochondrial fusion sites (Anand et al., 2014; Abrisch et al., 2020). Interestingly, these MCSs represent hotspots where both mitochondrial division and fusion machineries reside and contribute to mitochondrial dynamics. Because of its spatial positioning, IMM fusion and cristae organization are necessarily linked to inter-organelle contacts. Mgm1/OPA1 and MICOS have been demonstrated to physically and functionally interact (Barrera et al., 2016; Glytsou et al., 2016) and it is implicit that there is a relationship between fusion, cristae organization, and inter-organelle contacts, though mechanistic details still need to be worked out.

In yeast cells, IMM fusion and OMM fusion are coordinated by the modified carrier protein Ugo1 (Coonrod et al., 2007; Hoppins et al., 2009). While a direct functional ortholog in mammalian cells is lacking, three OMM-localized carrier proteins exist: MTCH1, MTCH2, and SLC25A46. Recently, MTCH2 was shown to coordinate cellular metabolic status with mitochondrial fusion and is proposed to be a sensor of lyso-phosphatidic acid (Labbe et al., 2021). Labbé et al. suggest that MTCH1 and SLC25A46 could work in a related way and be modulators of mitochondrial fusion under other specific circumstances. Interestingly, SLC25A46 depletion was shown to cause mitochondrial hyperfusion as well as defects in MICOS stability and cristae organization (Janer et al., 2016). Thus, one possibility is that cellular metabolic status may be keyed into both mitochondrial network and cristae organization by these carrier-related proteins. As in the case of mitochondrial division, how the IMM must be coordinated during mitochondrial fusion and what the specific contribution of the ER is to influencing IMM architecture at these sites, whether through phospholipid transport, Ca²⁺ regulation, or other functional roles both direct and indirect, remains to be determined.

Mitochondrial Trafficking

Mitochondria rely on the cytoskeleton for their motility and spatial positioning throughout cells. While in yeast mitochondria rely primarily on the actin cytoskeleton, mammalian mitochondria utilize microtubules for longer-range movements, though in part rely on the actin cytoskeleton as well. Microtubule-based trafficking occurs via interactions between integral OMM Miro proteins (Miro1/RHOT1 and Miro2/RHOT2) and the kinesin adaptor proteins TRAK1/TRAK2 (aka Milton) (Devine et al., 2016). Miro1 and Miro2 are large GTPases homologous to the ERMES-auxiliary subunit Gem1 and, similarly to Gem1, Miro proteins have been suggested to concentrate on the OMM at ER-mitochondria MCSs (Kornmann et al., 2011; Qin et al., 2020).

Recent work in both *Drosophila* and human cells have implicated Miro in influencing not only overall mitochondrial morphology via transport, but also cristae organization within the organelle. In both flies and humans, a physical interaction between Miro and the MICOS component Mic60 have been observed in a manner influenced by the oxidation status of Mic60 (Tsai et al., 2017; Modi et al., 2019; Qin et al., 2020; Li et al., 2021). In *Drosophila*, MICOS components are required for Miro stability and, accordingly, loss

of Mic60 leads to defects in mitochondrial trafficking (Tsai et al., 2018). Conversely, like MICOS depletion, loss of Miro also has been observed to lead to aberrant cristae morphology (Modi et al., 2019). Superresolution microscopy revealed that Miro localizes in micro-clusters on the OMM that are in close proximity to IMM MICOS complexes (Modi et al., 2019). Interestingly, Modi et al. determined that MIC60 and the SAM complexes interact with TRAK in a Miro-dependent manner, potentially linking cristae organization with trafficking. As described above, the stable linkage between a subset of MICOS complexes and ERMES has been observed in yeast (Tirrell et al., 2020), supporting the idea of a conserved role. Another recent paper has identified thin Miro1-dependent mitochondrial tubule projections that occur at ER MCSs, contain mtDNA, and are proposed to be mediated by the Miro1-MIC60 interaction (Qin et al., 2020). Altogether these observations suggest the physical interaction between Miro and MICOS may couple internal spatial organization and mitochondrial motility. Despite these exciting advances, due to the multiple roles of Miro and the severe defects associated with its loss, careful structure-function analysis will be necessary to delineate its multiple functions and understand specifically how Miro and ER MCSs influences cristae organization.

Cristae Dynamics

With the improvement of high-speed super-resolution imaging technologies, it is now possible to image cristae dynamics in real time, leading to several important discoveries in recent years. Using Airyscan microscopy and membrane-potential dependent dyes, Orion Shirihai's group was able to visualize the sub-mitochondrial behavior of individual cristae in live cells (Wolf et al., 2019). This allowed for the important discovery that each cristae maintains its own membrane potential, acting as an individual unit. Other labs have utilized high speed super-resolution microscopy to make fundamental discoveries about cristae behavior. For example, live cell STED imaging was used to observe how mtDNA nucleoids behave relative to cristae as well as to observe cristae movements during mitochondrial division (Stephan et al., 2019). Others have examined the dynamic behavior of individual cristae in live cells, finding that they have the capacity to fuse, divide, and rearrange, and overall are much more dynamic than previously thought (Hu et al., 2020; Kondadi et al., 2020). These new technologies open the possibilities to explore previously unanswerable questions. For example, how does cellular metabolic status affect cristae dynamics and organization in real time, how do MCSs influence local cristae behavior, and how is the biogenesis of cristae during increased respiratory demand spatially regulated? As three-dimensional resolution of cristae in live cells improves, we can ask how the shape of individual cristae corresponds to dynamic behavior. Furthermore, as emerging technologies are applied in distinct cell types and even in living tissues, what distinctive behaviors can be observed? New phospholipid probes, organelle contact site markers, minimally invasive fluorescent labels, and improved imaging capabilities will converge to allow us to ask these and other questions, making it an exciting time to work at the intersection of mitochondrial ultrastructure and contact site biology.

Concluding Thoughts

Our understanding of two parallel areas of research—inter-organelle communication and mitochondrial ultrastructure and organization—have undergone transformative discoveries in the last 15 years. While many proteins and functions have been ascribed to inter-organelle MCSs, the list of molecular players is ever-expanding, and likewise, new roles are being continuously discovered. Likewise, advances in microscopy and high-throughput screening, both in yeast and now in human cells, continue to change our view of the complexity of mitochondria. We can now visualize the dynamics of individual cristae in real time, preserve mitochondrial ultrastructure with improved quality, and precisely pinpoint the localization of protein complexes inside the organelle with techniques like expansion microscopy and correlative light and cryo-EM tomography. Going forward, the ability to tease apart the molecular mechanisms at the intersection of these fields and visualize inner workings of mitochondria at unprecedented resolution will no doubt expand our understanding of the intimate connection between mitochondria and the world of organelles around them.

Acknowledgements

While doing our best to be comprehensive, there are several relevant studies that could not be covered due to space limitation, and we apologize to the authors of those works. We thank Laura Lackner and Mike Henne for critical review of this manuscript. JRF is supported by R35GM137894 and the UT Southwestern Endowed Scholars program.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Institutes of Health, (grant number R35GM137894).

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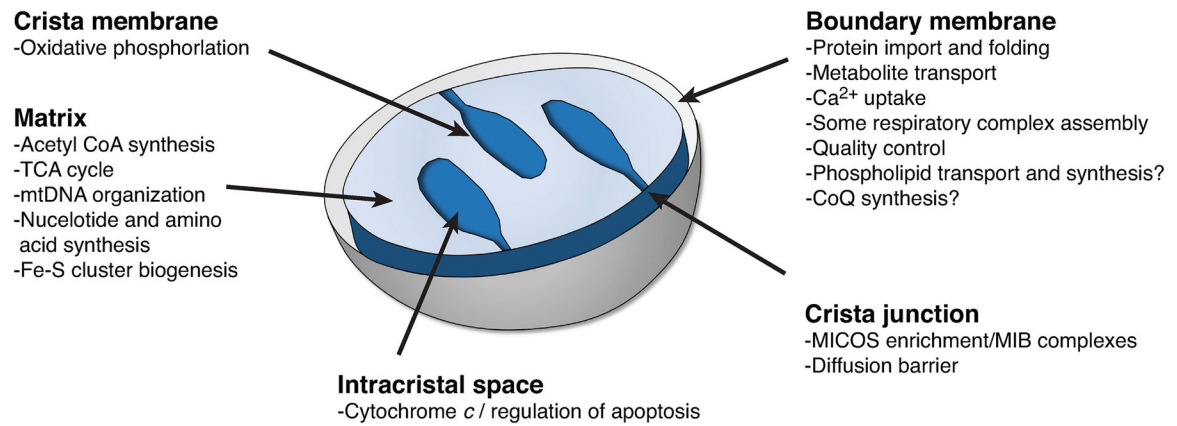
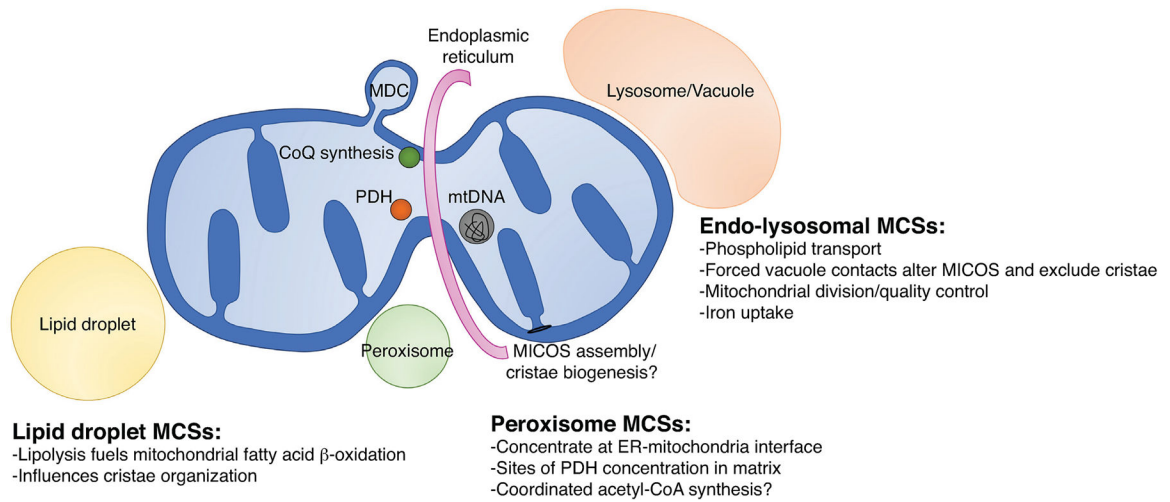


Figure 1. Spatial and Functional Organization of Mitochondria.

The mitochondria interior is spatially organized into distinct functional and morphological domains. Listed are examples of the functional processes that have been demonstrated to be spatially organized within each compartment. Note that this list is not exhaustive, and for many inner membrane localized processes, it is not yet clear whether these are restricted to the boundary or cristae membranes.

Endoplasmic reticulum MCSs:

- Phospholipid transport and synthesis on the IMM
- Ca²⁺ uptake and cristae enrichment
- Assembly sites of the MICOS complex and cristae biogenesis sites?
- mtDNA nucleoid coordination with division
- Mitochondrial fusion and cristae organization
- Miro-dependent trafficking and cristae organization
- CoQ synthesis enzymes
- MDC biogenesis site
- Iron transport and heme metabolism?

**Figure 2. Mitochondrial internal organization and function is spatially coordinated with inter-organelle MCSs.**

A current summary of the functional processes that occur inside mitochondria that have been shown to be coupled to MCSs between mitochondria and other organelles.