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Mitochondrial morphology – Emerging role in bioenergetics

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Abstract

Dynamic change of mitochondrial shape is a cellular process mediated mainly by fission and fusion of mitochondria. Studies have shown that mitochondrial fission and fusion are directly and indirectly associated with mitochondrial maintenance, bioenergetic demand, and cell death. Changes in mitochondrial morphology are frequently observed in response to changes in the surrounding cellular milieu such as metabolic flux that influences cellular bioenergetics. Connections between morphological regulation and the bioenergetic status of mitochondria are emerging as reciprocally responsive processes, though the nature of the signaling remains to be defined. Given the pivotal role mitochondria play in cellular fate, tight regulation of fission and fusion is therefore critical to preserving normal cellular physiology. Here we describe recent advancements in understanding of the mechanisms governing mitochondrial morphology and their emerging role in mitochondrial bioenergetics.

Introduction

Living organisms take up foods and metabolize them to produce energy necessary for a myriad of cellular processes. In most eukaryotic organisms, this task is ascribed to specialized cellular organelles, mitochondria, which perform high-efficiency energy production. Mitochondria are considered to be bacterial in origin, taking up residence inside eukaryotic cells through an ancient endosymbiotic event. During evolution, the host might have taken away the autonomy from the new residents by removing the majority of the bacterial genome and incorporating them into the host's genome. In this manner, the host could control the energy-producing activity at the levels of not only gene expression but also protein import. As eukaryotic cells evolved into more complex and differentiated cells and organs with specialized functions, the specific subcellular distribution of mitochondria inside the cell became critical to meet energy demands in localized areas. To control their cellular distribution, the host might have put a leash on mitochondria, trapping them on the cytoskeleton. While these scenarios are conceivable tactics that the host cells might have employed to dictate mitochondria for efficient production and delivery of energy, morphological plasticity of mitochondria is a remaining cell biological enigma in terms of its functional significance. Mitochondria change their shape continuously through fission, fusion, and other undefined processes such as branching/de-branching and extension/

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retraction. If the morphological plasticity of mitochondria has evolved out of the same purpose by the host cells, one could assume that mitochondrial shape change has important biological roles associated with the energy-producing activity of mitochondria. While this article is not intended to discuss evolutionary aspects of mitochondrial shape change, we will briefly review mitochondrial fission and fusion mechanisms and pathophysiological signals that affect mitochondrial morphology, and venture to seek evidence for bioenergetic significance of mitochondrial morphology.

Fission and fusion of mitochondria

Mitochondrial morphology encompasses a vast spectrum from small spheres/ovals and short tubules to elongated tubules and reticular networks depending on cell types in varied tissues and organs. Among many types of mitochondrial shape changes, fission and fusion are the most defined and studied processes. Mitochondrial fission and fusion involve enzymatic reactions mediated by dynamin-related large GTPases, known for their membrane remodeling activities. These proteins are dynamin-like protein 1 (DLP1), two isoforms of mitofusin (Mfn1 and 2), and OPA1 (optic atrophy 1) in mammals. DLP1, frequently referred to as Drp1 for dynamin-related protein 1, is a soluble cytosolic protein mediating mitochondrial fission. Mfn and OPA1 are associated with outer and inner membranes, respectively, where they mediate fusion of their respective membranes. Mechanisms and mechanisms of mitochondrial fission and fusion are the subject of many prior reviews [1–4]. In this section, we will discuss recent developments in mechanisms governing mitochondrial fission and fusion.

Mitochondrial membrane remodeling by dynamin-related GTPases

DLP1 is the central component for mitochondrial fission. DLP1 contains a GTPase domain at the N-terminus followed by middle (MID) domain, unconserved (UC) domain, and coiled-coil (CC) domain at the C-terminus [5, 6] (Fig. 1). Two alternatively splicing regions were identified in rat DLP1, one within the GTPase domain and the other in the UC domain (Fig. 1), which possibly generates 12 different spliced variants, ranging 79–85 kDa in size [7]. The UC domain contains a brain-specific exon and thus often is referred to as B-insert in human DLP1 [5] (Fig. 1). The alternative exon in the GTPase domain was found to be also brain-specific in rat DLP1 [7]. The CC domain is also called GED for GTPase effector domain or assembly domain. The recently solved crystal structure of dynamin 1 has defined the functional importance of the MID and CC domains [8, 9], as they form a stalk structure that has a large binding interface for multimerization of dynamin and possibly DLP1. *In vitro* studies using recombinant DLP1 and liposomes reported the first evidence for a mechanical-pinching activity of this protein [10]. DLP1 was shown to assemble into ring-like structures that are stacked to form tubes in the presence of a non-hydrolyzable GTP analogue. The DLP1 assembly on the liposomes constricted them to form lipid tubules with ~30 nm diameter, suggesting the mechanical activity of DLP1 [10]. Studies using the yeast DLP1 homologue Dnm1 showed a similar activity, although the diameter of the Dnm1-induced lipid tubule was ~100 nm [11]. Detailed structural analyses of cryo-EM images demonstrated that, upon GTP hydrolysis, the outer diameter of Dnm1-decorated lipid tubules significantly decreases to ~60 nm from ~120 nm, and Dnm1 rapidly releases from the lipid tubules [12]. This contractile activity of Dnm1 likely mediates the final scission during mitochondrial fission. An interesting observation of the Dnm1-lipid tube was the presence of a 3–4 nm gap between Dnm1 and the lipid surface in averaged 3-dimensional reconstruction, indicative of an unstable interaction of Dnm1 with lipid. It is possible that this gap may accommodate additional cofactors during mitochondrial fission in cells to enhance the contractile activity of Dnm1 [12].

Mfn and OPA1, the mitochondrial fusion-promoting dynamin-related proteins, are less defined in terms of their mechanical activity mediating membrane fusion. Mfn contains the N-terminal GTPase domain as well as coiled coil motif-containing two heptad repeat regions (HR1 and HR2) at opposite sides of the two C-terminal transmembrane domains (Fig. 1). The two transmembrane domains are closely spaced and span the outer membrane twice, which orients most of the Mfn molecule (GTPase, HR1, and HR2) in the cytosol. Two Mfn isoforms in mammals, Mfn1 and Mfn2, show different tissue expression pattern. At the mRNA level, Mfn1 is more generally expressed throughout tissues whereas Mfn2 shows high abundance in heart and skeletal muscle [13]. At the protein level, however, Mfn2 is predominant in brain whereas Mfn1 is abundant in heart and testis [14]. In mitochondrial fusion, Mfn mediates tethering of apposing mitochondria prior to the membrane fusion. A study using differentially labeled mitochondria showed that tethering between Mfn1 molecules is more efficient than Mfn2-Mfn2 tethering [15]. In addition, Mfn1 has 8-fold higher GTPase activity compared to Mfn2 [15]. However, Mfn1 and Mfn2 can form heterotypic as well as homotypic complexes [16]. Recent studies indicated that the Mfn1-Mfn2 heterotypic trans complex is more efficient than the homotypic complex for fusion, suggesting that different levels of the two Mfn isoforms may control the fusion efficiency in different tissues [17]. Crystal structure of the C-terminal HR2 of the Mfn1 isoform revealed a dimeric anti-parallel coiled coil, suggesting that this interaction tethers apposing mitochondria during mitochondrial fusion [18]. This tethering link is approximately 10 nm and the gap between the opposing membrane was measured to be ~16 nm [18], which exceeds the 1–2 nm distance necessary for lipid bilayer mixing [19, 20]. It is presumed that the GTPase activity would further draw two lipid bilayers closer for fusion; however, no direct evidence for this mechanical activity has been shown for Mfn to date.

OPA1 is anchored at the inner mitochondrial membrane by the N-terminal transmembrane domain, which leaves the bulk of the molecule in the intermembrane space. The N-terminal region of OPA1 contains an alternative splicing region, which produces 8 different splice variants [21]. In addition, OPA1 undergoes proteolytic cleavage at the N-terminal sites (S1, S2) downstream of the transmembrane domain to generate short soluble forms of OPA1 (Fig. 1) [22], which still remain associated with the inner membrane. *In vitro* mixing of soluble OPA1 and cardiolipin-enriched liposomes induced protrusions on the liposome surface, forming lipid tubules of varying diameter ranging from 50 nm to > 100 nm [23]. These observations indicate that OPA1 has capacity to constrict membranes.

Because of the two distinct membrane systems of mitochondria, completing mitochondrial fusion in one step is topologically impossible. Mfn initiates the tethering as discussed and fuses the outer membranes of apposing mitochondria, which allows spatial contact between the inner membranes and subsequent fusion through the OPA1 function. The sequential fusion of outer and inner membranes indicates the highly coordinated actions of Mfn and OPA1 in completing mitochondrial fusion. This notion is supported by observations that the lack of OPA1 function generally causes mitochondrial fragmentation even in the presence of functional Mfn at the outer membrane [24–27]. While OPA1 has been shown to require the Mfn1 isoform for mitochondrial fusion [24], the mechanisms of the coordinated fusion of outer and inner membranes are not fully understood.

Additional factors in mitochondrial fission and fusion

Mitochondrial fission requires the action of DLP1 for the final membrane severing. As indicated above, DLP1 is cytosolic and requires translocation to mitochondria for fission. Conventional dynamin contains pleckstrin homology domain and proline-rich domain which facilitate membrane binding through an interaction with phosphatidylinositol-4,5-bisphosphate and SH3 domain-containing proteins, respectively. However, DLP1 lacks both

these domains, necessitating an alternative recognition of DLP1 at its target membranes. Multiple proteins have been identified in mammals as potential mitochondrial receptors for DLP1. Despite characteristics consistent with their roles as the receptor for DLP1, their specificities and mechanisms in the DLP1-dependent fission process are still open to question. A small mitochondrial outer membrane protein, Fis1, was initially regarded as a receptor for DLP1, given that increasing the number of Fis1 molecules in mitochondria through exogenous expression resulted in fragmented mitochondria [28–30]. In addition, Fis1 interacts with DLP1, and furthermore, inhibiting DLP1 decreased the Fis1-induced mitochondrial fragmentation, suggesting that Fis1 functions in DLP1-mediated fission as a limiting factor [28–30]. Mitochondrial fission factor (Mff) was also identified to function in mitochondrial fission [31]. Both Fis1 and Mff have C-terminal single transmembrane domains that anchor them at the outer mitochondrial membrane [28, 29, 31]. A more recent study by Otera et al reported that Mff induced mitochondrial fragmentation when overexpressed and the Mff-dependent fission does not require Fis1 function [32]. Mff overexpression fragmenting mitochondria is directly contradictory to the earlier study in which no morphological change of mitochondria was observed upon Mff overexpression [31]. One potential factor for the conflicting results is the use of different splice variants for their studies. Nine different Mff splice variants were identified in human cells [31]. The smaller 25 kDa variant was used by one group [31] whereas the other group used the ~35kDa one [32], which might have resulted in different experimental findings. Studies directly comparing the functions of different splice variants will be necessary. Regardless, both Mff and Fis1 weakly interact with DLP1 as co-immunoprecipitation requires chemical crosslinking. However, Mff forms distinct foci that co-localize with DLP1 in mitochondrial tubules unlike Fis1 that diffusely distributes throughout the mitochondrial surface. Furthermore, knockdown of Mff decreased mitochondrial localization of DLP1, and targeting Mff to the plasma membrane redistributed DLP1 to the new location, strongly suggesting that Mff recruits DLP1 [31]. Although it is clear that Mff and Fis1 participate in mitochondrial fission, further studies are necessary for defining relative contributions of Mff and Fis1 to DLP1-mediated mitochondrial fission.

Mitochondrial dynamics proteins of 49 kDa and 51 kDa (MiD49 and MiD51) and mitochondrial elongation factor 1 (MIEF1) were recently identified to interact with DLP1 [33, 34]. MiD51 and MIEF1, independently characterized, are the identical protein. MiD49 and MiD51/MIEF1 are 45% homologous and are anchored at the outer mitochondrial membrane through a N-terminal transmembrane domain [33, 34]. Overexpression of MiD49 or MiD51/MIEF1 induced an elongated mitochondrial phenotype. However, contradictory results were reported in changes of mitochondrial morphology upon genetic knockdown of these proteins. Zhao *et al.* showed that MIEF1 downregulation induced mitochondrial fragmentation [34] whereas Palmer *et al.* reported that knockdown of both MiD49 and 51 promoted the formation of long fused mitochondria [33]. Thus, two opposite roles for this protein were proposed: pro-fusion by Zhao *et al* and pro-fission by Palmer *et al.* Palmer *et al.* hypothesized that long fused mitochondria upon overexpression of MiD proteins, which appeared conflicting with their suggested pro-fission role, was the result of overexpressed MiD49/51 mis-targeting DLP1 away from the site of fission [33]. On the other hand, based on the observation that MIEF1 interacts with DLP1 and Fis1 separately, Zhao *et al.* speculated that MIEF1-DLP1 interaction inhibits DLP1 function, resulting in pro-fusion phenotype and that the MIEF1-Fis1 interaction also produces profusion phenotype by preventing the DLP1-Fis1 interaction [34]. Similar to this postulation, the fission protein DLP1 has been shown to interact with the fusion protein Mfn2 and can function as a pro-fusion factor [35]. It is possible that relative amounts and differential regulation of Fis1, Mff, MIEF1/MiD51, and Mfn2 on the mitochondrial surface may determine the role of DLP1 in either fission or fusion [35, 36].

Mitochondria are in close contact with the endoplasmic reticulum (ER), which facilitates transfer of lipids and calcium between the two organelles. This close physical contact has recently been shown to have additional function in mitochondrial fission. ER tubules were observed to wrap around the mitochondrial tubules and constrict them [37]. While DLP1 foci were seen at these contacts, this event occurs independently of Mff and DLP1, suggesting that the ER-mediated mitochondrial constriction is an early event and marks the future fission site [37]. ER-mitochondria association has been reported to involve a tethering role of the mitochondrial fusion protein Mfn2 [38]. A subpopulation of Mfn2 was observed to be associated with the ER and interacted with the mitochondrial Mfn proteins, which appeared to play a role in calcium transfer between these two organelles [38]. However, the ER-mediated mitochondrial constriction is independent of the Mfn2 protein [37], suggesting that these two ER-associated events are functionally and mechanistically distinct processes.

Mitochondrial bioenergetic parameters appear to play a role in mitochondrial morphology. While this aspect will be further discussed in the later section, the mitochondrial inner membrane potential, a critical element for mitochondrial function, is required for mitochondrial fusion [22, 39, 40]. The aforementioned proteolytic cleavage of OPA1 is an important process for mitochondrial fusion and appears to be regulated by the mitochondrial membrane potential [22, 41, 42]. Several inner membrane associated proteases have been shown to cleave OPA1. These include PARL (presenilin-associated rhomboid-like), i-AAA (Yme1L), m-AAA (paraplegin and AFG3L1 and 2), and the inner membrane metalloprotease OMA1 [22, 42–47]. Although it is unclear why mammals have evolved to employ multiple different proteases for OPA1 cleavage, the proteolytic regulation of OPA1 has an important function in the selective fusion event in which damaged mitochondria remain isolated and are prevented from fusion, which facilitates to maintain healthy mitochondrial population [48]. Both m-AAA and OMA1 were shown to mediate mitochondrial depolarization-induced OPA1 cleavage [22, 47]. OMA1 is normally sequestered in the inner membrane microdomain. A loss of the inner membrane potential triggers OMA1 release and OPA1 cleavage to block the undesired fusion events [47].

Signaling mechanisms regulating mitochondrial fission and fusion

Mitochondrial morphology appears to coordinate with changes in the surrounding cellular milieu such as alterations in metabolic flux and also with processes of mitochondrial maintenance and ultimately tissue and system homeostasis. In proliferative cells, mitochondrial fission/fusion is regulated during cell cycle for adequate supply of energy as well as proper mitochondrial inheritance to daughter cells. Tight regulation of the fission and fusion processes is therefore critical for preserving normal cellular and tissue physiology, and their dysregulation is associated with many pathological conditions.

Protein modifications regulating mitochondrial morphology

As discussed, it is likely that a mitochondrial receptor recruits cytosolic DLP1 during mitochondrial fission. Additionally, DLP1 has been reported to undergo modifications including phosphorylation [49–51], S-nitrosylation [52], SUMOylation [53, 54], ubiquitination [55–57], and O-GlcNAcylation [58], which affects its subcellular distribution, stability, and function.

Mitotic cell division requires adequate segregation of mitochondria between two daughter cells, which is facilitated by DLP1-mediated mitochondrial fission. Phosphorylation of DLP1 at a C-terminal serine residue (–IMPAS^PQKG–) by the mitosis-promoting kinase Cdk1/cyclin B promotes mitochondrial fission to fragment mitochondria at the onset of mitosis (Fig. 1) [59]. Recently, a kinase cascade leading to the mitotic phosphorylation of DLP1 was defined [60]. The mitotic kinase Aurora A phosphorylates/activates the small

Ras-like GTPase RALA. Activated RALA relocates from the plasma membrane to mitochondria along with the RALA effector RALBP1, which promotes DLP1 recruitment to mitochondria. RALBP1 in mitochondria acts as a scaffold to bring Cdk1/cyclin B to the DLP1 for mitotic phosphorylation of DLP1 [60]. Knockdown of RALA or RALBP1 resulted in a reduction in ATP levels and the number of metabolically active cells presumably through successive cell divisions in the absence of proper distribution of mitochondria to daughter cells [60], underscoring the importance of a regulated coupling of mitochondrial fission to cell division. While dephosphorylation of DLP1 is predicted to restore tubular mitochondria upon exit from mitosis, DLP1 was found to be degraded through a cell cycle E3 ubiquitin ligase, APC/C (anaphase-promoting complex/cyclosome), to facilitate the formation of tubular mitochondria [57]. Cell cycle control of mitochondrial morphology also involves the promotion of fusion, as mitochondria were found to become hyperfused transiently at the G1-S transition [61]. The hyperfused mitochondria were metabolically active, producing more ATP compared to those in other cell cycle stages, and capable of inducing cyclin E buildup necessary for the S phase entry [61]. However, direct regulation of mitochondrial fission or fusion to produce hyperfused mitochondria at the G1-S transition is not known. Unrelated to cell cycle control, the same serine residue of DLP1 was found to be phosphorylated by protein kinase C delta (PKC β) in a rat model of hypertensive neuroencephalopathy [62]. Similar to the Cdk1-mediated phosphorylation, DLP1 phosphorylation by PKC β increased mitochondrial fission and was associated with the fragmented mitochondrial morphology in oxidative stress and hypertensive rat brains [62]. Additionally, the MAP-kinase ERK1/2 was also implicated in DLP1 phosphorylation at the same serine residue in high glucose-induced mitochondrial fragmentation [63].

Another serine residue in the DLP1 C-terminal region is phosphorylated to alter the DLP1 activity and location. This serine residue locates at 20 amino acids downstream from the Cdk1 site and is within the protein kinase A (PKA) consensus motif (–VARKLSAR–) (Fig. 1). Phosphorylation of DLP1 by PKA decreases its GTPase activity and keeps DLP1 in the cytosol, inducing a fused mitochondrial phenotype [50, 51]. A kinase anchoring protein 1 (AKAP1) targets PKA to the mitochondrial outer membrane for DLP1 phosphorylation [64]. The Ca²⁺-dependent phosphatase calcineurin (protein phosphatase 2B) as well as protein phosphatase 2A/B β 2 dephosphorylate DLP1 at this site to promote mitochondrial fission [49, 51, 65]. Stress, exercise, or β -adrenergic activation have been shown to increase the PKA-mediated DLP1 phosphorylation in mouse heart muscle cells [51]. The same serine residue was reported to be phosphorylated by CaMKI α in neurons upon calcium influx through voltage-dependent calcium channels. In opposition to the phosphorylation by PKA, the CaMKI α -mediated phosphorylation increased the Fis1-DLP1 interaction, mitochondrial association of DLP1, and hence mitochondrial fission [66]. A recent study has shown that Rho-associated coiled coil-containing protein kinase 1 (ROCK1) phosphorylates DLP1 at the same serine residue and fragments mitochondria in diabetic kidney [67], consistent with the observation made with DLP1 phosphorylation by CaMKI β .

Additional modifications other than phosphorylation have been identified for fission/fusion proteins. An E3 ubiquitin ligase, MARCH5/MITOL, interacts with the fission proteins DLP1 and Fis1 and the fusion protein Mfn2 [55, 68]. MARCH5 ubiquitinated DLP1 and Fis1 for proteasomal degradation, and therefore MARCH5 downregulation caused mitochondrial fragmentation by stabilizing fission proteins and increasing fission [55, 68]. However, another report showed that inhibiting MARCH5 induced mitochondrial elongation and net formation [69]. Parkin is a Parkinson's disease-associated cytosolic E3 ligase. DLP1 was shown to be ubiquitinated by Parkin for degradation [56]. As described above, DLP1 is a novel substrate of the APC/C E3 ubiquitin ligase for cell cycle regulation of mitochondrial morphology [57]. Related to ubiquitination, small ubiquitin-related modifier (SUMO) ligation modifies DLP1. Mitochondrial-anchored protein ligase (MAPL) is a mitochondrial

SUMO E3 ligase that SUMOylates DLP1 and is shown to enhance DLP1 stability and increase fission [54]. DLP1 has been shown to be S-nitrosylated by nitric oxide. DLP1 S-nitrosylation is associated with brain damage in Alzheimer's disease patients. S-nitrosylated DLP1 was shown to have increased dimerization and GTPase activity to induce mitochondrial fragmentation [52].

More recently, DLP1 was shown to undergo glycosylation. O-linked-N-acetyl-glucosamine glycosylation (O-GlcNAcylation) was detected at two consecutive threonine residues (--QEPTTGNW--) in the UC domain (B-insert) of DLP1 (Fig. 1) [58]. DLP1 O-GlcNAcylation was enhanced in cardiomyocytes exposed to the elevated glucose level and in the hearts from diabetic mice [58]. Consequences of the O-GlcNAcylation of DLP1 included a decrease of the DLP1 phosphorylation at the serine residue phosphorylated by PKA, an enhanced affinity for GTP, and increased mitochondrial association of DLP1. These changes in DLP1 increased mitochondrial fission, consistent with the previously described fragmented mitochondrial morphology upon high glucose insult in cells and animal models [63, 70–74]. Interestingly, OPA1 was also O-GlcNAcylation modified, which was significantly increased in high glucose treatment [75]. The OPA1 O-GlcNAcylation contributes to the fragmented mitochondrial phenotype in high glucose conditions, as decreasing the extent of the modification increased mitochondrial elongation [75].

Stress signaling of mitochondrial fission and fusion

Mitochondrial morphology and the bioenergetic status of the cell are intimately linked, and as such, substrate availability affects mitochondrial morphology. Nutrient depletion *in vitro* or fasting animals *in vivo* is reported to induce mitochondrial elongation [76, 77]. The pro-fusion phenotype observed in starvation is the result of an inhibitory phosphorylation of DLP1 at the downstream serine residue by PKA [76, 77], and additionally through the dephosphorylation of the upstream serine, which decreases the DLP1 fission activity [77]. Unopposed fusion then results in elongated mitochondria, which were characterized to be resistant to autophagic removal while efficiently producing more ATP through enhanced cristae density and ATP synthase dimerization [76]. OPA1 null or Mfn null cells were unable to induce mitochondrial elongation under nutrient deprivation, resulting in an ATP depletion-driven cell death, suggesting a protective role of mitochondrial elongation under nutrient depletion [76]. During starvation, cells operate with a limited nutrient supply obtained from autophagic degradation of cellular constituents to preserve essential cellular processes. Autophagy also degrades mitochondria (mitophagy) and mitochondrial fission has been shown to be necessary for mitophagy [48]. However, bulk mitophagy under starvation would eliminate the most efficient energy-generating process, which would be detrimental to cell survival. It is likely that the formation of elongated mitochondria by decreasing fission is a mechanism to spare mitochondria under starvation stress. At the same time, expansion of cristae surface and ATP synthase dimerization induced in elongated mitochondria also serve as a mechanism to enhance the efficiency of ATP production.

Promoting pro-fusion phenotype of mitochondria in the starvation conditions is similar to the previously reported stress-induced mitochondrial hyperfusion (SIMH) [78]. SIMH was observed in stressed conditions including transcription/translation inhibition, UV irradiation, and serum and amino-acid deprivation. Fusion proteins Mfn1 and OPA1, as well as stomatin-like protein 2 (SLP-2) are required for SIMH [78]. SLP-2 participates in SIMH by protecting OPA1 from proteolytic cleavage, preserving the long form of OPA1. Similar to the just described mitochondrial elongation in starvation, SIMH also increased the oxidative phosphorylation and ATP production. Prolonged incubation with SIMH-inducers causes apoptosis. SIMH-incompetent Mfn1 null or SLP-2 deficient cells were found to be highly

sensitive to apoptosis in stress conditions compared to SIMH-competent cells. Interestingly, low dose treatments of stress stimuli were found to enhance resistance against apoptosis following a secondary insult, suggesting that SIMH serves as a pro-survival mechanism, possibly a type of preconditioning [78].

Alternative states of metabolic depletion, such as oxygen suppression or removal in hypoxia and anoxia, result in a strikingly different phenotype from starvation, enhancing mitochondrial fragmentation [79, 80]. The mitochondrial A-kinase anchoring protein 121 (AKAP121) was found to play a role in mitochondrial shape change in hypoxia by regulating the PKA-mediated DLP1 phosphorylation [80]. In normoxia, AKAP121 is associated with PKA at the mitochondrial surface, facilitating DLP1 phosphorylation. In hypoxia, AKAP121 was ubiquitinated by the ubiquitin ligase Siah2 and degraded, which decreased DLP1 phosphorylation by PKA thereby fragmenting mitochondria [80]. In an alternative mechanism, AKAP121 binds to Fis1 in normoxia, which blocks the DLP1-Fis1 interaction necessary for mitochondrial fission. The hypoxic degradation of AKAP121 releases Fis1 for DLP1 binding and induces mitochondrial fragmentation [80]. Accordingly, an *in vivo* cardiac ischemic injury model showed that *Siah2*^{-/-} mice maintained less impaired mitochondrial morphology and were less susceptible to ischemic injury with smaller infarct size compared to wild type mice [80]. This result is congruent with a previous study in which inhibition of DLP1 GTPase activity with the small molecule inhibitor mdivi-1 reduced infarct size in an ischemia/reperfusion model [79].

Recently, it has been reported that several microRNA species regulate mitochondrial fission to enhance mitochondrial fission in cardiac ischemia/reperfusion injury and apoptosis [81–83]. Under stress conditions, the p53 tumor suppressor can arrest cell growth or induce apoptosis. The microRNA miR-30 normally functions to suppress p53 levels. Upon exposure to apoptotic stimuli, the level of miR-30 decreased, releasing its suppressive effect and upregulating p53, which in turn transcriptionally induced DLP1 expression to increase mitochondrial fission, triggering apoptosis [81]. Another microRNA, miR-499 has been found to regulate DLP1 through targeted suppression of calcineurin [82]. In cardiac ischemia/reperfusion, the level of miR-499 was decreased. Decreased levels of miR-499 augmented calcineurin levels, which dephosphorylates DLP1 at the PKA site to induce mitochondrial translocation of DLP1 and mitochondrial fragmentation. Hence, increasing the miR-499 level was effective in decreasing cardiac infarct size and apoptosis as well as improving cardiac function in ischemia/reperfusion. Interestingly, the expression of miR-499 is under the control of p53, in which increased levels of p53 suppress the miR-499 expression [82], positioning miR-30 and miR-499 up- and down-stream of p53, respectively, for both transcriptional and post-translational regulation of DLP1. Additionally, the fission protein Fis1 is also regulated by microRNA [83]. miR-484 was found to target Fis1, and thereby increasing miR-484 levels attenuated cell injury. Expression of miR-484 was suppressed in anoxia, which in turn lead to enhanced mitochondrial fission and apoptosis. The expression of miR-484 was activated by Foxo3a [83]. Interestingly, the expression of antioxidant enzymes Mn superoxide dismutase [84] and catalase [85] are also regulated by this transcription factor. Given that reactive oxygen species (ROS) is a critical factor in cell injury in ischemia/reperfusion, regulating Foxo3a would be an attractive strategy to ameliorate the ischemia-reperfusion injury.

Stress has also been shown to change the fusion protein Mfn2 to promote mitochondrial fragmentation and apoptosis [86]. In stress conditions such as chemotherapeutic agent treatment, Mfn2 was found to be uniquely phosphorylated at serine27 (–NASPLK–) through the MAP kinase JNK that is known for activating apoptotic pathways. This phosphorylation promoted selective degradation of Mfn2 through the ubiquitin-proteasome system. While the known E3 ligase Parkin has been shown to target Mfn and other

mitochondrial proteins for mitophagy [56, 87, 88], stress-induced selective Mfn2 degradation was mediated by the HECT domain ubiquitin ligase Huwe1 [86]. Serine27-phosphorylated Mfn2 was suggested to recruit Huwe1 by binding to the Huwe1 BH3 domain, which degrades Mfn2 and in turn promotes mitochondrial fragmentation and apoptosis in stress conditions.

Mitochondrial Morphology and the Energetic State of Mitochondria

Inhibition of oxidative phosphorylation and mitochondrial morphology

Respiratory chain complexes I – IV generate a proton motive force across the mitochondrial inner membrane to drive ATP synthesis through complex V, ATP synthase, in oxidative phosphorylation (OXPHOS). Treatment with chemical inhibitors of these complexes inhibits ATP synthesis and also results in disrupted mitochondrial morphology, generally mitochondrial fragmentation [89–91]. Treatment with mitochondrial chemical uncouplers is also associated with fragmented mitochondrial morphology [39, 40, 92]. Inhibition of either complex I or III with rotenone or antimycin A, respectively, resulted in mitochondrial fragmentation in CV1-4A cells, MEFs, and neurons [90, 93]. Complex II is irreversibly inhibited by 3-nitropropionic acid (3-NP) resulting in both swollen and fragmented mitochondria [91]. In this treatment, ATP levels were suppressed within an hour and significant increases in mitochondrial fragmentation lagged behind, reaching significance in 3 hours, suggesting that morphological disruption was the consequence of the OXPHOS dysfunction. Enhanced fragmentation following 3-NP treatment was partially suppressed by treatment with N-acetylcysteine to reduce the increased ROS levels, indicating the causal role of ROS for mitochondrial fragmentation [91]. However, the absence of full recovery of mitochondrial morphology leaves open the possibility that suppressed membrane potential may also contribute.

A study quantifying the mitochondrial morphology changes using antimycin A or FCCP showed that a shifted balance toward fission was responsible for the mitochondrial morphology change [94]. In measuring the rates of change using time-lapse fluorescence videos, the study demonstrated that the first 5 minute treatment for FCCP and 30 minute treatment for antimycin A were sufficient for the changes in morphology with a new equilibrium established after the 30 minute. The rate of fission and fusion in the new equilibrium was much lower compared to the original rate observed prior to the treatment [94]. Consistent with these observations, the feedback control of fission/fusion rates based on the length of mitochondrial tubule and the number of mitochondrial tips has been postulated, which predicts a permanent shift to either fission or fusion inevitably lowering the rate of both events [4]. In many cases, OXPHOS inhibition leads to mitochondrial depolarization, resulting in mitochondrial fragmentation through OPA1 cleavage. Mitochondrial depolarization following fission events has been shown to be important for cellular energy maintenance through selective disposal of depolarized mitochondria by autophagy [48]. In this context, chemical inhibition of electron transport chain (ETC) components may reflect the cellular response to accumulated dysfunctional mitochondria that require disposal, eliciting mitochondrial fission.

An additional phenotype observed with chemical inhibition of ETC components is the formation of donut-shape mitochondria. These mitochondria were observed upon inhibition of complex I or III, and chemical uncoupling with FCCP [89, 94]. Enhanced donut formation was observed in hypoxic conditions and during reoxygenation, corresponding to loss of the membrane potential [95]. Formation of donuts followed mitochondrial swelling induced by opening of the mitochondrial permeability transition pore or K^+ channels. End-to-end or end-to-side fusion of a mitochondrial tubule results in donut shape, which requires full fusion of both outer and inner membranes through Mfn and OPA1. Partial detachment

of mitochondria from microtubules was observed prior to fusion, which appears to be a requirement for donut formation. Donuts become fragmented and opened following washout of FCCP. Interestingly, the recovery of membrane potential was more rapid in fragmented mitochondria originating from donut shaped mitochondria, compared to longer tubular mitochondria. Based on these observations, it was hypothesized that the donut formation may enhance mitochondrial volume tolerance and preservation of mitochondrial machinery, and thus is advantageous in adverse metabolic conditions [95]. OXPHOS inhibition inevitably leads to morphological alterations of mitochondria. These observations clearly indicate that functional states of mitochondria are reflected in mitochondrial morphology. Signals from mitochondrial bioenergetics activities likely regulate mitochondrial fission/fusion machineries to mediate the morphological change.

Disrupted fission/fusion and mitochondrial function

As discussed, OXPHOS inhibition and therefore altered bioenergetic status affects mitochondrial morphology. Disrupting mitochondrial fission/fusion has been found to affect mitochondrial and cell function, indicating that the reciprocal effect, morphology determining function, also holds true. The deletion of both Mfn1 and 2 resulted in mitochondrial fragmentation and a deleterious effect on mitochondrial oxygen consumption [96]. The suppression of ETC function was associated with reduced oxygen consumption with substrates for complexes I, III, and IV. A suppression of OPA1 expression mirrored these results suggesting a global effect in decreasing energy production in the inhibition of mitochondrial fusion. Fusion inhibition correlated with a widespread loss of the membrane potential, supportive of a role for morphology affecting ETC function [96]. Aforementioned studies support such a mechanism with fusion promoting ATP production in starvation, at a specific cell cycle stage, and under stress [61, 76–78]. Mitochondrial fusion appears to support an ideal organization of ETC components to support efficient OXPHOS and ATP synthesis.

Decreases in mitochondrial fission are also reported to result in impaired mitochondrial function. DLP1 knockdown in HeLa cells induced the formation of hyper-fused mitochondria, which was accompanied by decreases in inner membrane potential, oxygen consumption, ATP levels, and cell proliferation [97]. The disrupted mitochondrial function was found to lead to increases in ROS levels and oxidative damage, along with the loss of mitochondrial DNA and activation of autophagy [89, 97]. In contrast to these deleterious effects of decreased fission, complete elimination of DLP1 appeared to have little effect on mitochondrial bioenergetic parameters [98]. Mitochondrial membrane potential, respiration, cellular ATP contents, mitochondrial DNA, and autophagy were unaffected in DLP1-knockout mouse embryonic fibroblasts [98]. Although the reason for this discrepancy is unclear, it is possible that sustained loss of mitochondrial fission in DLP1 knockout cells might have triggered cellular protective or adaptive mechanisms that preserve mitochondrial parameters. More recent observations in primary hepatocytes with the dominant-negative fission mutant DLP1-K38A revealed that blocking mitochondrial fission induces an inner membrane proton leak (uncoupling) [74]. No defects in respiratory complex activities were found with fission inhibition along with no change in the maximal respiration capacity. Remarkably, the mechanism of the fission inhibition-induced uncoupling was identified to be the repeated cycles of large-scale loss and recovery of the inner membrane potential. Interestingly, the DLP1-K38A-induced fluctuation of the inner membrane potential was insensitive to inhibitors of established mitochondrial channels and pores, suggesting a novel uncoupling mechanism [74].

Mitochondrial fission and fusion are postulated to maintain bioenergetic health of mitochondria through selective fusion which maintains a healthy mitochondrial population, and fission which separates damaged mitochondria from healthy networks [48]. This process

is coupled to the autophagic removal of dysfunctional mitochondria and, therefore, disrupted fission results in accumulation of energetically impaired mitochondria [48, 99].

Human diseases associated with defects in mitochondrial fission/fusion

Mutations in two mitochondrial fusion GTPases, Mfn2 and OPA1, are associated with Charcot-Marie-Tooth disease type 2 and autosomal dominant optic atrophy, respectively [100–102]. Mutated DLP1 was also discovered to be lethal in one human case [103]. As discussed, changes in the abundance of these proteins can have deleterious effects on the bioenergetic function of the cell. In addition to direct mutations in fission/fusion genes, other human diseases including neurodegeneration and metabolic diseases have implicated the mitochondrial morphogenic proteins in their etiologies. It was found that a mutant huntingtin, the Huntington's disease protein, associates with DLP1 and stimulates DLP1 enzymatic activity, resulting in enhanced mitochondrial fragmentation [104]. Importantly, mitochondrial fragmentation preceded the onset of huntingtin aggregation and neurological symptoms, which was reversed by the expression of DLP1-K38A. This observation is in accordance with prior reports that overexpression of mutant huntingtin induced mitochondrial fragmentation [105] and that calcineurin-mediated dephosphorylation of DLP1 was increased in Huntington's disease patients [106]. In addition, an increased DLP1 level has been reported in conjunction with a suppression of Mfn1 expression in Huntington's disease patients [107]. The familial forms of Parkinson's disease also converge on mitochondrial morphology through PINK1 and Parkin [108], mitochondrial serine/threonine kinase and ubiquitin ligase, respectively, which lead to Parkinsonian symptoms when mutated. Loss of PINK1 expression was characterized by mitochondrial fragmentation and associated with loss of membrane potential [109]. PINK1 and Parkin have been found to coordinate ubiquitination of Mfn1 and 2, which would promote mitochondrial fragmentation and underscores the role of these proteins in mitophagy [87, 88]. It has also been reported that Parkin targets DLP1 for degradation through ubiquitination, which is possibly dysregulated to predispose neurons to the disease [56]. While familial Parkinson's disease only accounts for 5–10% of Parkinson's disease occurrence, which includes the aforementioned PINK1 and Parkin maladies, disrupted mitochondrial morphology is likely to occur in sporadic Parkinson's disease as well. Patient samples were documented to have deficiencies in respiratory chain complex I often showing systemic and oxidative damage in complex I subunits [110, 111]. Studies to model sporadic Parkinson's disease utilize complex I inhibitors such as MPP⁺ (1-methyl-4-phenylpyridinium), 6-hydroxydopamine, and rotenone which are known to induce mitochondrial fragmentation through DLP1 activity [56, 112]. Alzheimer's disease is characterized by extracellular deposit of amyloid β (A β) protein derived from amyloid precursor protein (APP). Overexpression of APP or A β treatment of neurons alters levels of mitochondrial fission/fusion proteins and causes abnormal distribution of fragmented mitochondria [113, 114]. Changes in the levels and expression of mitochondrial fission/fusion proteins were also detected in hippocampal tissues of Alzheimer's disease patients [115]. S-nitrosylation of DLP1 was shown to enhance dimerization and GTPase activity of the DLP1 molecule, and reported to be associated with neuronal damage in Alzheimer's disease patients [52, 115].

Metabolic diseases also show altered mitochondrial morphology through changes in mitochondrial fission and fusion. Studies in endothelial cells of diabetic patients and animals revealed fragmented mitochondrial morphology [73, 116]. Treating cultured human aortic endothelial cells with an elevated glucose concentration resulted in fragmented mitochondria with increased levels of Fis1 and DLP1. Mitochondrial fragmentation has been shown to be associated with increased ROS levels [71, 72, 116]. The enhanced ROS was found to blunt the activation of eNOS and cGMP production [116]. In mouse coronary endothelial cells of type I diabetic mice, a suppression of OPA1 expression was also observed along with

enhanced DLP1 [73]. The *in vivo* treatment of diabetic animals with the superoxide scavenger TEMPOL decreased the diabetic oxidative stress. Interestingly, the TEMPOL treatment also restored mitochondrial morphology without restoring the levels of OPA1 and DLP1. Similar findings were observed in cultured endothelial cells. Here, superoxide overproduction caused mitochondrial fragmentation without changing fission/fusion protein levels, suggesting that oxidative stress and superoxide were the cause of the fragmented mitochondrial morphology in these experimental systems [73]. On the other hand, studies have demonstrated that decreasing mitochondrial fission and thus restoring tubular mitochondrial morphology prevents the ROS increase in other cell types under diabetic milieu [48, 63, 67, 71, 72, 74, 116]. In type II diabetic patients regardless of the presence of an obese phenotype, Mfn2 expression was significantly decreased in skeletal muscle [117], which predicts short and fragmented mitochondrial morphology [96]. Skeletal muscle from non-diabetic obese patients also revealed reduced Mfn2 expression [117]. Although a positive correlation was found between Mfn2 expression and insulin sensitivity, insulin infusion did not affect the Mfn2 expression in obese subjects or diabetic patients. However, body weight loss in obese patients increased Mfn2 expression. Pro-inflammatory signals may contribute to the dysregulation of Mfn2 expression in obesity and type II diabetes [117]. Altered mitochondrial morphology has been observed in nonalcoholic steatohepatitis (NASH) [118, 119]. Mega-mitochondria were observed as large 'swollen' mitochondria with a loss of discernible cristae structure that contain crystalline inclusions. However, whether this morphological change requires mitochondrial fission/fusion proteins remains to be investigated.

Perspectives - Mitochondrial fission/fusion as therapeutic target

We have briefly discussed how mitochondrial fission and fusion are mediated and how they are associated with cellular signaling networks. Also described is how the morphological plasticity of mitochondria is linked to bioenergetics in physiological and stress conditions. Mitochondrial morphology has now become a new parameter for evaluating mitochondrial functionality, and as such the field has produced a great number of research articles dealing with mitochondrial morphology in physio-pathology in recent years. While we have attempted a focused approach, it has been a challenge to include all available information and we regret that many findings are inevitably left out. Notwithstanding, an emerging theme throughout this review is the close association of mitochondrial morphology and bioenergetic status and their reciprocal influence on one another. As discussed, mitochondrial toxins that decrease the bioenergetic function disrupt normal mitochondrial morphology, indicating that functional abnormality invokes morphological alterations, which is likely the case of terminally deformed mitochondria observed in many human diseases. Conversely, genetic defects in mitochondrial fusion/fission genes cause human diseases or lethal consequence, likely through impairing mitochondrial bioenergetic function.

Another emerging theme that we consider more important is that fused interconnected mitochondria are energetically active and are generally associated with survival/protective function whereas short and fragmented mitochondria are mostly found with a decreased activity in pathologic conditions. These observations are consistent with findings that maintaining or restoring tubular mitochondria largely decreases pathologic parameters and cell injury in models of diabetes, cardiac and renal ischemia-reperfusion, and neurodegeneration to list a few [52, 67, 74, 79, 120]. However, whether the improved outcome by enforcing mitochondrial elongation is mediated by increased mitochondrial bioenergetics is unclear. While bioenergetic activity of morphologically restored mitochondria in pathological conditions remain to be evaluated, a recent study identified the increased uncoupling as an underlying mechanism in relieving diabetic complications

through decreasing mitochondrial fission [74]. This study demonstrated that fission inhibition induces inner membrane proton leak, thereby decreasing mitochondrial ROS production and oxidative stress in an increased metabolic flux of diabetic conditions. Increased ROS production from mitochondria and subsequent oxidative stress play a large part in many pathology developments including organ ischemia-reperfusion, metabolic diseases, neurodegeneration, and aging. Although it is not discussed in this review, it has been shown that apoptosis is accompanied by mitochondrial fragmentation, and preventing mitochondrial fragmentation blocks or delays apoptotic progression. Apoptosis is an unavoidable course in most pathological developments. Provided that increasing mitochondrial connectivity can decrease both oxidative stress and apoptosis, manipulating mitochondrial fission/fusion is an attractive strategy for ameliorating many progressive human maladies (Fig. 2). Permanent alterations of mitochondrial fission or fusion are detrimental as manifested in the mitochondrial fission/fusion diseases. Defining the temporal correlation of the progression of pathological parameters and mitochondrial morphology change within specific pathologies will help identify temporal windows for effective targeting of morphological manipulation. As described here, studies have identified many factors necessary for mitochondrial fission and fusion. In addition to direct manipulation of fission/fusion GTPases, additional components including signaling processes, will provide increased opportunities for manipulating and targeting mitochondrial fission and fusion in many pathological conditions.

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- Mitochondrial morphology is responsive to surrounding cellular milieu
- Deformed mitochondrial morphology is coincident with many disease pathologies
- Mitochondrial morphology has a reciprocal relationship with bioenergetic status
- Manipulating mitochondrial fission and fusion can be used as a therapeutic strategy

Figure 1. Molecular structure of dynamin-related GTPases in fission and fusion

GTPase, Middle (MID), and GED are the dynamin core domain and are found in DLP1 and OPA1. Purple boxes in DLP1 are alternatively splicing regions (sp1 and sp2). The sp2 is also referred to as “B” for brain-insert. The serine residues undergoing phosphorylation in DLP1 and Mfn2 are in red whereas the two threonine residues that are glycosylated in DLP1 are in blue. Arrowheads in the OPA1 indicate proteolytic cleavage sites. Mitochondrial transit sequence (MTS) is removed by the mitochondrial processing peptidase (MPP) after import. S1 and S2 are in the two alternatively splicing exons (5, 5b) that are cleaved by paraplegin (S1) and Yme1L (S2), respectively, to produce short isoforms of OPA1. DLP1 (Drp1), dynamin-like (related) protein 1; Mfn1/Mfn2, mitofusin 1 and 2; OPA1, optic atrophy1; GED, GTPase effector domain; HR1, heptad-repeat 1; HR2, heptad-repeat 2; TM, transmembrane; CC, coiled coil domain

Figure 2. Controlling mitochondrial morphology as a therapeutic strategy

Metabolic or other insults induce mitochondrial deformation, which is accompanied by increases of ROS (reactive oxygen species) and oxidative stress and/or apoptosis. Maintaining or restoring normal mitochondrial morphology by manipulating fission and fusion (morphological intervention) prevents not only the ROS increase and oxidative stress (1) but also apoptotic progression (2), decreasing pathological outcome.