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Review

The relationship between mitochondrial shape and function and the cytoskeleton

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Abstract

Mitochondria are crucial organelles for life and death of the cell. They are prominent players in energy conversion and integrated signaling pathways including regulation of Ca^{2+} signals and apoptosis. Their functional versatility is matched by their morphological plasticity and by their high mobility, allowing their transport at specialized cellular sites. This transport occurs by interactions with a variety of cytoskeletal proteins that also have the ability to influence shape and function of the organelle. A growing body of evidence suggests that mitochondria use cytoskeletal proteins as tracks for their movement; in turn, mitochondrial morphology and function is regulated via mostly uncharacterized pathways, by the cytoskeleton.

Keywords: Mitochondria; Fusion; Fission; Cytoskeleton; Motility; Kinesin; Dynein; Trichoplein

1. Introduction

Mitochondria are essential organelles for life and death of the cell. They carry out a variety of important cellular functions including the production of the largest part of cellular ATP needed for endoergonic processes and the regulation of cytosolic Ca^{2+} transients [23,60,67]. Moreover, mitochondria play a crucial role in apoptosis [18,32]. Mitochondrial shape varies in living cells and can range from punctuate structures to tubular networks [5]. Mitochondrial distribution inside a cell can be strikingly heterogeneous: they are often enriched at cellular sites where energy demand is greater, or where their metabolic function is required, like at the level of the synaptic button. This implies that mitochondria are (i) mobile; (ii) use cytoskeletal proteins as tracks for their directional movement; (iii) are transiently or permanently stopped where their presence is required via interactions with specialized cellular structures.

The history of mitochondrial relationship with cytoskeletal proteins started with the identification of interactions with microtubules (MT) in different models, including insects, primary rat neurons and cultured mammalian cells [2,3,11,33,42,64,75]. Shortly after this, the interaction of our favorite organelle with intermediate filaments was uncovered [55,78]. Evidence that mitochondria associate with microfilaments has been unequivocally obtained in plants, while it remains uncertain whether and how this holds true also in mammalian cells.

Before we detail the liaison between cytoskeleton and mitochondria, we should briefly summarize the features of the different cytoskeletal components. Each type of filament is formed by a different protein component. Microfilaments are composed by a tight helix of oriented globular (G-) actin monomers [10]. α-actin is found in mammalian muscle, whereas β- and γ-actins constitute microfilaments of non-muscle cells. MT are cylindrical polymers of α/β-tubulin dimers. Nine to 16 linear polymers of tubulin heterodimers, assembled in such a way that β-tubulin in one dimer contacts α-tubulin in the next, constitute the walls of the microtubule [53]. MT are highly dynamic polar structures consisting of a plus end that is able to grow rapidly and a minus end that is located at the centrosome, next to the nucleus. The dynamic instability of MT requires an input of energy to shift the balance between polymerization and depolymerization. This energy comes from the hydrolysis of GTP. GTP binds to the β-tubulin subunit of

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the heterodimeric molecule and when a tubulin molecule adds to the end of the microtubule, this GTP molecule is hydrolysed to GDP [16]. MT are stabilized by binding of other proteins called microtubule-associated proteins (MAPs). There are two categories of MAPs, high molecular weight proteins (HMW) such as MAP-1 and MAP-2 and low molecular weight proteins such as Tau [15,29]. Intermediate filaments consist of a variety of tissue-specific proteins [35] and we will refer to them specifically when dealing with their interaction with mitochondria. Cytoskeletal components interact dynamically, a most striking example being the so-called "cortical platform" of a moving cell, where for example spektraplakins scaffold MT and filamentous actin and probably intermediate filaments (see [39] for a review).

A large number of molecular motors belonging to the superfamilies of kinesin, cytoplasmic dynein and myosin are crucial to bind organelles and vesicles to cytoskeletal elements. These motors provide a mean for the organelles to move along the cytoskeletal fibers. They consist of a motor domain connected by a stalk to a globular tail, which binds to the cargo. In proteins belonging to all three motor classes, ATP hydrolysis results in a small conformational change in the globular motor domain that is amplified and translated into movement [68,84].

Movement of the organelles must be coordinated with changes in their morphology. The shape of the mitochondrial network and of the individual mitochondria results from a net balance of fusion–fission processes regulated by a family of "mitochondria-shaping proteins" including specific mitochondrial dynamin-related proteins [70]. Dynamins are large, ubiquitous mechanoenzymatic GTPases that control the dynamics of membrane fusion, tubulation, budding and of vesicles formation [50]. The role of dynamins in controlling mitochondrial shape was first identified in yeast, where deletion of specific genes resulted in gross alterations of the mitochondrial network, and ultimately in functional abnormalities including loss of mitochondrial DNA, growth defects and petite strains [71]. In mammalian, known proteins regulating mitochondrial fission include DRP-1 [74], a cytosolic dynamin that translocate to fission sites where it interacts with its molecular adapter hFis1, an integral protein of the outer mitochondrial membrane (OMM) [38]. Mitofusin (MFN) 1 and -2 also reside in the OMM and regulate mitochondrial fusion [12]. The inner mitochondrial membrane protein OPA1 cooperates with MFN1 to fuse mitochondria [14].

Coordination between movement and changes in mitochondrial shape is particularly true when mitochondria are organized in a network of interconnected organelles: they must divide into mitochondrial "units" that can be readily transported. The coordination of these complex tasks suggests a crucial role for the cytoskeletal system to regulate not only mitochondrial movement, but also their morphology and perhaps their function. On the other hand, altered mitochondrial fusion–fission equilibrium could interfere with movement and localization of the organelle, and eventually with the integrated cellular function that requires a specific location of mitochondria.

In this review, we will provide a "mitochondrial" perspective on the liaison between mitochondria and cytoskeleton in yeast and mammalian cells, discussing how cytoskeleton can regulate mitochondrial shape and function.

2. Cytoskeleton and mitochondrial shape and distribution in yeast

2.1. Microfilaments

Genetic and biochemical studies have shown that mitochondria in *S. cerevisiae* bind to the actin cytoskeleton, which regulates both their positioning and transport. *S. cerevisiae* possesses a single, essential actin gene, making genetic dissection of actin–mitochondria interaction easier in this organism. Yeast strains containing temperature-sensitive lethal mutations in the actin gene grown at semi-permissive temperatures display abnormal mitochondrial distribution and lack mitochondrial movement [73]. Substitution of charged aminoacids with alanine residues uncovered a specific functional interaction between actin and mitochondria. The most severe effects on mitochondrial organization were caused by substitutions of aminoacids located under the myosin "footprint" on the actin monomer, suggesting that actin–myosin interactions might underlie mitochondrial organization. Mitochondrial fragmentation and defects in mitochondrial motility were also observed when cells were treated with actin-depolymerizing drugs such as latrunculin [22]. Isolated F-actin cables slide on immobilized mitochondria, demonstrating an actin-based motor activity on the surface of this organelle [73]. Deletion of the actin-stabilizing gene tropomyosin 1 affects mitochondrial morphology, distribution and long-distance movement [72], Tropomyosin overexpression can partially complement deletion of MDM20, which affects formation of the actin bundles and mitochondrial morphology. Further analysis showed that the movement of mitochondria is driven by actin polymerization via the Arp2/3 complex: Arp2p colocalizes with mitochondria probably by its interaction with the outer peripheral membrane Jsn1p [8,26]. The class V myosin Myo2p participates in mitochondrial movement regulating transmission or retention of the organelle to the bud, its activity being regulated by the rab-type small GTPase Ypt11p [7,37].

These studies suggest that actin cables are used as a scaffold for the attachment and movement of mitochondria in yeast cells. At the same time, several of the actin mutants whose mitochondrial movement is impaired also show defects in mitochondrial morphology, raising an important question: are these mitochondrial fragments the result of the accumulation of a required intermediate in the mitochondrial transport reaction? In other words, do we see these fragmented organelles because they were prepared by the fission machinery to be transported and this reaction went wrong? This would imply that there is a signal, like an intracellular "chemo attractant" that at the same time directs movement of mitochondria and recruits the fission machinery to prepare mitochondria for their transport. Actin

would then act as an active track to move the transportable organelle towards their destination.

2.2. Intermediate filaments

Less is known about the role of intermediate filaments in modulating mitochondrial morphology and movement in budding yeast. Most information resulted from the analysis of MDM1 mutants. MDM1 gene encodes a protein with small similarity to mammalian intermediate filament protein such as keratin and vimentin [48]. Purified Mdm1p self-assembles into 10-nm filaments in vitro, like other intermediate filament proteins, but it does not form a network of filaments in vivo [49]. Mdm1p form punctate structures throughout the cytoplasm which are not exclusively colocalized with mitochondria or other cytoplasmic organelles [48]. Mutations in the MDM1 gene result in abnormal mitochondrial morphology, growth defects and blocks both mitochondrial and nuclear migration. Yaffe and colleagues suggest that Mdm1p mediates mitochondrial motility by sequential binding and release of mitochondria to generate movement along Mdm1p filaments tracks [6].

2.3. Microtubules

MT do not seem to have a direct role in mitochondrial motility and distribution in *S. cerevisiae*. First of all *S. cerevisiae* does not have an extensive cytoplasmatic microtubule network [34]. Second, disruption of yeast MT using microtubule destabilizing drugs or mutations in β-tubulin does not affect the mitochondrial morphology. These observations are in accordance with the fact that budding yeast mitochondria do not colocalize with MT. In contrast, in the fission yeast *Schizosaccharomyces pombe* and the filamentous fungus *Neurospora crassa* MT are essential for mitochondrial distribution: mutations in α or β-tubulin or drugs that disrupt microtubule network result in unequally distributed mitochondria [86]. This strongly suggested that molecular motors such as cytoplasmic dynein or kinesin homologues might modulate mitochondrial movement, distribution and morphology in fission yeast. However, their depletion has no effect on mitochondrial positioning or morphology in S. pombe [86]. Mitochondrial motility and distribution in fission yeast are on the other hand largely controlled by polymerization of microtubule and elongation of the mitotic spindle [87], in analogy to the actin-polymerization driven movement of these organelles in the buddying yeast. The novel cytosolic EZ-HEAT protein Mmd1p, crucial to stabilize MT in S. pombe, was identified as the first component required for the alignment of mitochondria along MT in fission yeast [82].

3. Cytoskeleton and mitochondrial shape and distribution in higher eukaryotic cells

Mitochondrial movement in upper eukaryotic cells has been studied in particular in neurons, where it plays a crucial role to position mitochondria at the synaptic terminals. The picture that emerged from these studies and from what we know about movement of other organelles suggest that it the complex movement of mitochondria results from coordinated action of MT, microfilaments and intermediate filaments.

3.1. Microtubules

In upper eukaryotic cells, MT are essential cytoskeletal components that play a crucial role in events such diverse as cell motility and division, organelle transport and cell morphology and organization Mammals possess at least six forms of α- and β-tubulin, each of which is encoded by a different gene [63].

Interaction of mitochondria with MT and their movement along the MT tracks depends on microtubule-associated proteins (MAPs), a numerous set of proteins. MAPs can be divided into MT stabilizer and destabilizers, regulating MT polymerization and function, and molecular motors that are responsible for movement along MT of mitochondria and other membrane-bound organelles [59].

MT-associated motors include kinesins or dyneins. Dyneins transport their cargoes toward the minus end of MT and most kinesins transport cargos towards the plus end of MT, except for the few ones whose motor domain is positioned at the carboxyl-terminus of the protein, that direct transport to the minus of MT [79]. All kinesins display a high degree of homology in their motor domain, a region spanning for 340 amino acids and containing MT- and ATP-binding sites. The classification of kinesins into one of 14 families of the kinesin superfamily depends on their structural features. Human and mouse genomes encode 45 kinesins, whose functions classically are transport of cargo, including protein rafts, organelles and membrane-bound vesicles [51].

Members of six kinesin families (1, 2, 3, 4, 13 and 14) are implicated in transport of organelles. In particular, members of the kinesin-1 and -3 families are responsible for with mitochondrial movement [36]. Ablation of Kif5b, a member of the Kinesin-1 family, in the mouse results in mitochondrial clustering in the perinuclear region and in embryonic lethality. KIF5B is associated with the outer mitochondrial membrane, suggesting its crucial role as a motor for anterograde mitochondrial movement towards plus end of MT [77]. KLP67A is another motor that has been reported to have the unique role of positioning mitochondria near the mitotic spindle in *D. melanogaster* [61]. In *Drosophila*, the protein Milton serves as putative mitochondrial receptor for KIF5B, albeit it does not display any evident transmembrane domain. Milton has two homologues in human cells, hMilt1 and hMilt2, but none in lower eukaryotic cells [76]. In yeast, the tropomyosin 1 gene (Tpm1), displays 25% identity over 148 amino acids in the coiled-coil domain of Milton and distributes at mitochondria during cell division [72]. It is conceivable that Milton evolved from a Tpm1-like protein to take on a role in transport of mitochondria. This raises the question of whether the kinesin cargo encompasses proteins, like Milton, which might also bind actin and therefore represent the exchange capable of moving mitochondria from microtubule to actin tracks.

The Kinesin-3 family member KIF1Bα also colocalizes with mitochondria. Purified KIF1Bα is capable of driving anterograde

transport of mitochondria towards plus end of MT [56]. No KIF1Bα mitochondrial receptor is known: the protein KBP localizes to mitochondria and binds to KIF1Bα, but its probable function is to modulate the activity of KIF1Bα, as revealed by motility assays [85].

Fewer molecular details are known on the cytoplasmic dynein components involved in mitochondrial binding and transport. The cytoplasmic dynein light chain Tctex has been reported to associate with mitochondria, possibly via interaction with the outer membrane protein VDAC [69]. A tempting hypothesis is that BIM, a "BH3-only" member of the BCL-2 family of apoptosis regulators, which can bind to cytoplasmic dynein component LC8 [62] as well as to the mitochondrial proteins BCL-2 and BCL-XL [89] could serve as a scaffold for anchoring mitochondria to the carriers responsible of retrograde (minus end-directed) movement. Of note, during apoptosis induced by extrinsic stimuli mitochondria are known to redistribute and aggregate in the perinuclear region, suggesting a cross talk between the machinery that controls apoptosis and the one regulating movement of the organelle [19]. The interplay between cytoplasmic dynein and mitochondrial shape is further confirmed by a set of recent data from the group of G. Rutter. In a landmark paper, they showed that DRP-1, a dynamin-related protein required for fission of mitochondria [74], translocates from mitochondria to other intracellular membranes upon dynamitin-mediated disruption of cytoplasmic dynein function. DRP-1 normally resides in a complex with dynactin and dynamitin interferes with this interaction [80]. This work shows that the machineries controlling mitochondrial shape and movement interact and that integrity of the cytoplasmic dynein motor is required to shuttle pro-fission molecules on mitochondria. It furthermore provides an experimental basis for the concept that cargoing of mitochondria should be coupled to changes in shape that render elongate organelles amenable of easy transportation along MT tracks. It remains to be assessed whether a similar mechanism of shape-motion coordination exists for kinesin-mediated mitochondrial movement.

Movement of mitochondrial along MT tracks is influenced by second messengers generated during signaling events. For example, the direction of mitochondrial movement is influenced by levels of phosphatidyl inositol 4,5-bisphosphate (PtdIns(4,5)P2), since sequestration of (PtdIns(4,5)P2) by plekstrin-homology domains enhances plus-end-directed transport [20]. On the other hand, massive Ca^{2+} overload following activation of NMDA receptors results in early inhibition of mitochondrial motility [66]. Hajnockzy and coworkers clearly showed that indeed the mitochondrial movement stops following spatial and temporal characteristics of cytosolic Ca^{2+} spikes and oscillations during IP3- and ryanodine-receptor activation. This blockage of mitochondrial motility is associated with an increased mitochondrial Ca^{2+} uptake, suggesting that mitochondrial motility is diminished to enhance local Ca^{2+} buffering. Thus, mitochondrial movement is controlled in such a way that it meets local needs. The molecular determinants of this mitochondrial "brake" are still unknown: it will be of great interest to understand whether mitochondria stop because the motors are arrested in an inactive state, or because other cytoskeletal components serve as blocker that bind and halt the organelles.

Another level of regulation is accomplished by MAPs such as tau, whose expression is enhanced in pathologic conditions such as Alzheimer's disease, and MAP2 and 4. Levels of tau correlate with dynein-dependent fragmentation and clustering of mitochondria in the region, corresponding to the MTOC region [25], suggesting a role for DRP1 at least in the fission reaction involved in this. Microtubule affinity regulating kinase (MARK) and Par1 kinases are particularly efficient in detaching MAPs from microtubules. MARK2 can efficiently relieve the effect of tau on mitochondrial motion and clustering by reducing the level of microtubule-bound MAPs and thus removing obstacles for mitochondrial movement from the microtubule surface [47]. This would imply that fission follows blockage of mitochondrial movement, or that the same MARK2 kinase could actually regulate the mitochondrial fission machinery.

Besides motors, other MAPs bind to mitochondrial outer membrane. For example, specific sites of the outer membrane of purified mitochondria are involved in the MAP-mediated association of mitochondria to MT [43]. In the case of MT, MAP2 interacts with the outer mitochondrial membrane protein porin [45] and induces a change in the physicochemical properties of porin environment, suggestive of a modulation of porin function by MT binding [46].

3.2. Microfilaments

While actin cables represent the main tracks for mitochondrial movement in *S. cerevisiae*, in upper eukaryotic cells they are probably needed to assist MT in short-distance movement of mitochondria, in particular to microtubule-poor regions of the cell [36]. Actin-based motors responsible for cargoing mitochondria are not yet fully characterized. Indirect evidences suggest that members of the myosin I, II, V and VI families could act as molecular motors for mitochondrial movement along actin cables, but it should be pointed here that there is a large deal of uncertainty on their actual role. For example, Haynoczky and coworkers recently reported that myosin-Va, a Ca^{2+}-controlled motor protein, is present on mitochondria in situ in H9c2 cells and is also retained on isolated mitochondria [88]. On the other hand, in a pancreatic β-cell line myosin-Va only partially co-localizes with mitochondrial markers and a GFP-tagged C-terminal tail fragment of it does not colocalize with mitochondria nor does it affect movement of the organelle [81]. These differences could reflect the different cell types used, but still they rise an interesting question, whether binding of myosin-Va to mitochondria is mediated by a receptor protein on the surface of the organelle, whose expression is cell-type dependent.

The picture is even less clear for other myosins. The monomeric motor myosin I has been implicated in organelle transport, but its association with mitochondria is unclear. Part of the 130 kDa isoform of the protein is associated with the so-called "mitochondria associated membranes" (MAM), patches

of endoplasmic reticulum in close proximity with mitochondria [4]. Myosin II, a dimeric motor, has also been reported to associate with vesicular organelles in axoplasm, but its role in axonal transport is unclear [21]. Myosin VI drives movement toward the minus end of the actin filament, a quite attractive property that would explain the bidirectional movement of mitochondria on actin filaments in neurons [54], but it displays a punctate distribution typical of vesicular organelles rather than of mitochondria.

Irrespective of the proteins involved in the bridging of microfilaments with mitochondria, a growing body of evidence is starting to highlight that cytoskeletal changes are somehow transmitted to mitochondria, resulting in functional modification of the organelle. In yeast, decreased actin turnover, leading to the accumulation of large aggregates of F-actin, triggers an increase in the levels of reactive oxygen species (ROS) generated by mitochondria [31]. How this occurs is unclear, but the most likely candidate appears to be the Ras-cAMP pathway [9,30]. In mammalian cells some evidence implies a role for actin in ROS production, mitochondrial clustering and cell death following activation of death receptors [44]. Moreover, actin binding proteins appear to regulate the mitochondrial phase of apoptosis. Cofilin moves to mitochondria during the initiation phase of apoptosis and its levels correlate with cytochrome *c* release and apoptotic susceptibility [13]. Human Gelsolin, a Ca^{2+}-dependent actin regulatory protein prevents apoptotic mitochondrial changes [40,41]. Finally, the ER protein BAP31, which once cleaved by caspase-8 triggers mitochondrial fission during apoptosis [57], leaves its ã-actin binding sites following induction of cell death [24].

3.3. Intermediate filaments

Intermediate filaments (IF) are tough, ropelike structures that provide mechanical strength to cells and tissues. In humans, there are more than 50 differentially expressed different IF genes. They generally constitute 1% of total protein, although in some cells exposed to high loads of mechanical stress, such as epidermal keratinocytes and neurons, IFs are especially abundant, accounting for up to 85% of the total protein. Despite their diversity, members of the IF superfamily share a common structure: a dimer composed of two α-helical chains oriented in parallel and intertwined in a coiled-coil rod. The central rod domain is similar in all IF and regulates the assembly of the filaments.

Intermediate filaments in are grouped in three categories: keratins, vimentins and vimentin-related filaments and neurofilaments. Keratins represent the most diverse group, with more than 20 distinct members. Based on their amino acid composition, keratins can be divided in two groups: the type I (acidic) and the type II (neutral/basic) keratins, which can heteropolymerize. Vimentin is found in many cells of mesodermal origin including fibroblasts, endothelial cells and leukocytes, while the vimentin-related filament desmin is present mainly in the muscle cells. Vimentin can co-polymerize with desmin, but does not polymerize with keratins. Neurofilaments are the most abundant intermediate filaments in the nerve cells. Three groups of neurofilament proteins have been identified, called NF-L, NF-M and NF-H for low, middle and high molecular weight, respectively [28].

The role of IF in controlling mitochondrial movement, shape and function is unclear. It is reasonable that interaction of mitochondria with IF could participate in or at least modulate transport of the organelles. A conceivable hypothesis is that IF represent the “stop” signals that anchor mitochondria in the specific cellular locations where they are required.

The vimentin-like protein desmin is probably the IF for which physical and functional interactions with mitochondria are best characterized, thanks to the seminal work of Capetanaki and colleagues. Desmin forms a three-dimensional scaffold that seems to extend across the entire diameter of the mature striated myofiber. Thus, desmin could mechanically integrate the contractile actions of a muscle fiber. Ablation of Desmin in the mouse dramatically alters mitochondrial distribution, number, morphology, and function, showing for the first time the role and significance of the physical association of mitochondria with IFs. Extensive mitochondrial proliferation characterizes Desmin−/− cardiac muscle, in particular following work overload. Proliferation is often associated with mitochondrial swelling and loss of structured cristae. These morphological alterations are paralleled by a reduction in state 3 respiration measured in situ, but not in mitochondria isolated from Desmin-null fibers [52]. When antiapoptotic BCL-2 is expressed in the hearts of Desmin−/− mice, most mitochondrial defects, including changes in their ultrastructure, are blocked [83], showing a crosstalk between changes induced by ablation of this IF protein and activation of the proapoptotic pathway. A proteomic survey of the mitochondrial defects in Desmin−/− fibers showed a marked reduction in the levels of protein involved in acetate metabolism, amino-acid metabolism proteins, NADH shuttle and respiration, a result compatible with the diminished stimulated respiratory ability in situ [27].

Whether IF proteins interact directly with mitochondria or the interaction is mediated by an IF-associated protein that interacts with mitochondria is currently unknown. IF-linkers could play a role in this interaction. For example, plectin, an IF-linker protein that in muscle colocalizes with desmin, has been found in close proximity with the outer mitochondrial membrane [65]. Ablation of dystonin (BPAG1n), another linker protein that cross-links actin and desmin filaments, also affects mitochondrial distribution in mouse muscle [17]. On the other hand, these linkers all interact with desmin, raising the possibility that the mitochondrial alterations are secondary to a defect in desmin organization. Trichoplein, a novel keratin-binding protein with a low degree of homology with plectin, could represent an interesting candidate for such an IF-mitochondria interaction. Its intracellular distribution is indeed clearly punctuate, a pattern resembling that of mitochondria [58]. We explored the possibility that trichoplein is localized to mitochondria. Immunofluorescence as well as biochemical analysis on subcellular fractions identified it as a mitochondrial protein. Limited digestion with proteinase K confirmed that trichoplein is a associated with the outer membrane [1]. Of note,

expression of trichoplein resulted in a marked reduction in mitochondrial motility, while its ablation greatly enhanced the rate of directed mitochondrial movement. Binding of mitochondria to actin and/or MT was not affected by changes in the levels of trichoplein, suggesting that it modulates movement of mitochondria by linking them to IFs [1]. Thus, IFs appear to be crucial in positioning of mitochondria: they can directly or indirectly bind the organelles, detaching them from the actin or MT tracks.

4. Conclusions

A growing body of evidence is elucidating the relationship between cytoskeletal components and mitochondria. Most of the molecular components of this liaison are still uncharacterized, but we are starting to understand that mitochondria need cytoskeleton, as evidenced by the changes in organelle's function when cytoskeletal proteins are altered. The discovery of the linkers between mitochondria and cytoskeletal components, as well as of the signaling pathways regulating this interaction will shed new light on mitochondrial and cellular function.

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