Short title: Mitochondrial dynamics

Physiological functions of mitochondrial fusion

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Key words: mitochondrial fusion; mitofusin; OPA1; neurodegeneration

Abstract

In recent years, the dynamic nature of mitochondria has been discovered to be critical for their function. Here we discuss the molecular basis of mitochondrial fusion, its protective role in neurodegeneration, and its importance in cellular function. The mitofusins Mfn1 and Mfn2, GTPases localized to the outer membrane, mediate outer membrane fusion. OPA1, a GTPase associated with the inner membrane, mediates subsequent inner membrane fusion. Mutations in Mfn2 or OPA1 cause neurodegenerative diseases. Mouse models with defects in mitochondrial fusion genes have provided important avenues for understanding how fusion maintains mitochondrial physiology and neuronal function. Mitochondrial fusion enables content mixing within a mitochondrial population, thereby preventing permanent loss of essential components. Cells with reduced mitochondrial fusion, as a consequence, show a subpopulation of mitochondria that lack mtDNA nucleoids. Such mtDNA defects lead to respiration-deficient mitochondria, and their accumulation in neurons leads to impaired outgrowth of cellular processes and ultimately neurodegeneration.

Mitochondria are now recognized as highly dynamic organelles that move throughout a cell and constantly fuse and divide. Studies over the last several years indicate that these membrane-remodeling processes promote homogenization of the mitochondrial population by content mixing and thereby preserve mitochondrial function.[1,2] Mitochondrial fusion is clearly an important physiological activity; mutations in fusion genes in humans cause neurodegenerative diseases, and mice with reduced mitochondrial fusion show severe defects in multiple cell types. Current research in our lab focuses on the mechanics of mitochondrial fusion and the pathophysiological significance of this process.

Molecular mechanism

Fusion between two mitochondria entails the coordinated merging of four membranes–two outer membranes and two inner membranes. In mammals, three large GTPases are required for this function. The mitofusins, Mfn1 and Mfn2, are highly homologous, integral outer membrane proteins with the bulk of the polypeptide chain facing the cytoplasm. Both Mfn1 and Mfn2 can form protein complexes with themselves or each other.[3] Such physical interactions between Mfns on opposing mitochondria serve to tether the outer membranes together.[4] In the absence of Mfns, neither outer membrane fusion nor inner membrane fusion occurs.[4,5] It is currently unclear how Mfns promote outer membrane fusion beyond the initial tethering step. The presence of GTPase domains in Mfns suggests that they actively promote membrane merger via GTP hydrolysis.

OPA1, the third mitochondrial fusion protein, presents a more complicated story. Eight mRNA splice forms exist in mice and humans. In addition, post-translational cleavage of each mRNA product results in multiple protein isoforms. The "long" isoforms are integral inner membrane proteins, traversing the membrane once, whereas the "short" isoforms reside in the intermembrane space and are thought to be peripherally associated with the inner membrane. Under normal conditions, neither class of isoforms is sufficient to promote fusion. Rather, both a long and a short form of OPA1 must be present for mitochondrial fusion to occur.[6] By marking mitochondria with photoactivatable fluorescent markers, we have developed live imaging assays for outer membrane fusion versus full fusion. In mammalian cells, outer membrane fusion occurs in the absence of OPA1, but inner membrane fusion does not.[5] Therefore, although outer and inner mitochondrial membrane fusion are normally coordinated, the two processes can be functionally separated. These results indicate that mitofusins are essential for outer membrane fusion, whereas OPA1 functions subsequently in inner membrane fusion.

Pathophysiology

Human genetic studies show that mitochondrial fusion is particularly important to neurons.[1] Mutations in Mfn2 cause Charcot-Marie-Tooth disease type 2A (CMT2A), a peripheral neuropathy that primarily impairs motor and sensory neurons with the longest axons.[7] As a result, patients have the most severe and earliest symptoms in the distal extremities. Mutations in OPA1 cause autosomal dominant optic atrophy (DOA), a degenerative disease of the optic nerve.[8,9] Whereas some pathological OPA1 mutants

may be gain-of-function alleles, it is clear that at least some of the alleles result in haploinsufficiency, suggesting that loss of fusion is causal.

One question of great interest is why mutations in two mitochondrial fusion proteins cause such different diseases. Possible explanations include tissue specificity of expression, redundancy of Mfn1 and Mfn2, differences in function between outer and inner membrane fusion, and specific non-fusion functions of either Mfn2 or OPA1. Whereas all of these are possible, it is important to note that studies of more families have revealed a greater overlap in clinical manifestations of CMT2A and DOA patients than previously recognized.[1] DOA patients may present with peripheral neuropathies, whereas some CMT patients have optic atrophy. In addition, both OPA1 and Mfn2 mutations can cause cerebral and cerebellar abnormalities along with deafness, muscular dysfunction and psychiatric involvement. Therefore, mutations in both mitochondrial fusion genes can indeed affect a large range of partially overlapping tissues.

With both Mfn2 and OPA1 mutations, neuronal and muscular involvement is particularly widespread. This pattern is similar to that seen in disease caused by mitochondrial DNA (mtDNA) mutations. Interestingly, mtDNA deletions have been found in the muscle of a subset of DOA patients demonstrating myopathies.[10, 11] This observation suggests that a new avenue of exploration is how mitochondrial fusion affects mtDNA integrity.

Murine models

It is clear that much remains to be learned concerning the role of mitochondrial fusion *in vivo*, as well as the specific functions of Mfn1 versus Mfn2. To address these issues, we have established conditional knockout mice for *Mfn1* and *Mfn2*. With this approach, we can determine the relative importance of Mfn1 versus Mfn2 in any given tissue, eliminate mitochondrial fusion in one tissue at a time, and ascertain whether this affects the organism as a whole. Additionally, we can examine the cell biological consequences of mitochondrial fusion and determine how this pertains to the human diseases.

Deleting either *Mfn1* or *Mfn2* from all cells results in mid-gestational lethality.[3] When both Mfn1 and Mfn2 are eliminated, embryos die several days earlier. All of these embryonic lethalities are due to insufficient mitochondrial fusion in the placenta, because wildtype placentas will support the development of mutant embryos through birth.[12] Without Mfn2, the trophoblast giant cells, which provide the interface between embryonic and maternal cells, do not develop normally. However, mice that are *Mfn2*-/- but have a wildtype placenta survive for approximately 2 weeks after birth. *Mfn1*-/- placentas have normal giant cells but must harbor defects in other cell layers, because once again, a wildtype placenta will rescue the embryonic lethality of *Mfn1*-/- mice. In fact, these mice are not only born but survive into adulthood. When both Mfn1 and Mfn2 are missing in the embryo, though, the neonatal pups expire shortly after birth. Therefore, Mfn1 and Mfn2 serve both unique and redundant roles in development. It is important to note that their unique roles do not seem to reflect a functional difference between the proteins, but rather a difference in their expression patterns (discussed later).

Since CMT2A and DOA are both neurodegenerative diseases, we were particularly interested in whether attenuation of mitochondrial fusion affects neuronal cells. We found that Purkinje cells (PCs), the only efferent cells in the cerebellum, do not

develop properly in the absence of Mfn2.[12] In particular, their normally large dendritic arbors are only partially realized, thus limiting the formation of synapses. These defects eventually result in neuronal cell death, perhaps from lack of synaptic input. Thus *Mfn2*-/- mice have no ability to relay information from the cerebellum to the rest of the central nervous system. The subsequent lack of movement coordination prevents pups from feeding sufficiently and results in mortality at approximately two weeks of age. Interestingly, mitochondrial fusion is not only important for the development of PCs, it is also critical for their maintenance. Deletion of *Mfn2* from mature PCs causes massive degeneration of these neurons, resulting in severe ataxia in adult mice. Interestingly, the cerebellum can be affected in both CMT2A and DOA patients.

In contrast to *Mfn2*, deletion of *Mfn1* in either developing or mature PCs did not affect their mitochondria, and cerebellar function remained intact. Why is there this specificity if Mfn1 and Mfn2 both serve to mediate mitochondrial outer membrane fusion? RNA *in situ* analysis demonstrated a vastly greater level of *Mfn2* expression in PCs than *Mfn1*, suggesting that normally, mitochondrial fusion in PCs is largely dependent on Mfn2 rather than Mfn1. We therefore hypothesized that Mfn2 deletion would eliminate mitochondrial fusion whereas Mfn1 deletion would leave mitochondrial fusion activity largely intact, and it is this lack of fusion which causes the PC defects.[12] To test this idea, we established from mutant mice primary mixed cerebellar cultures, where we could exogenously express genes via lentiviral transduction. As expected, *Mfn2*-/- PCs showed mitochondrial abnormalities and poor viability in culture. However, transduction with either an Mfn2- or Mfn1-expressing lentivirus was equally effective at rescuing the PCs from cell death. A fusion-defective mutant, $Mfn2^{K109A}$, did not rescue the mutant phenotype. Therefore, it is not a unique function of Mfn2 that is required in PCs. It is simply that mitochondrial fusion in PCs *in vivo* depends largely on Mfn2 rather than Mfn1.

Our studies of *Mfn2* mutants identified in CMT2A further elaborate this concept of functional interplay between Mfn1 and Mfn2.[13] As an autosomal dominant disease, CMT2A generally involves mutation of only a single *Mfn2* allele, so that both wildtype Mfn1 and Mfn2 are also present in patients. Using Mfn1-null cells and Mfn2-null cells, we tested the ability of various $Mfn2^{CMT2A}$ alleles to form fusion-competent complexes with wildtype Mfns. Strikingly, whereas many $Mfn2^{CMT2A}$ alleles promote mitochondrial fusion in Mfn2-null cells, they are non-functional in Mfn1-null cells. This observation indicates that many $Mfn2^{CMT2A}$ alleles can promote fusion when coupled to Mfn1, but not with Mfn2. In most tissues of CMT2A patients, mitochondrial fusion proceeds normally, because Mfn1/Mfn1 and $Mfn2^{CMT2A}$/Mfn1 hetero-oligomeric complexes both support fusion. However, in tissues with low Mfn1 expression, mitochondrial fusion would be essentially abolished, because both these sources of mitochondrial fusion depend on Mfn1 function.

Cellular mechanisms

From a cell biological perspective, how does lack of mitochondrial fusion affect neuronal function and survival? Neurons are highly dependent on mitochondrial activity that is concentrated at dendritic and axonal terminals. Mitochondrial activity is required in dendritic spines for synapses to form and function properly. Neuronal survival, in turn, is dependent on sufficient synaptic activity. In both developing and adult PCs

lacking Mfn2, however, the mitochondria were found to be clustered in the cell body instead of spread throughout the neurites.[12] Furthermore, of those few mitochondria present, a large percentage was dysfunctional in their oxidative phosphorylation capabilities. Therefore, the combination of mitochondrial mislocalization and dysfunction impacts cellular function.

Mitochondria normally move rapidly along microtubule tracks, much like cars along a road. In fusion-deficient fibroblasts, though, time-lapse imaging shows that the fragmented mitochondria move with a more Brownian-like motion, without much net distance traveled.[3] In addition, analyses of branching junctions in dendrites by electron microscopy reveal pile-ups of mitochondria, which prevent transport to downstream sites.[12] The defect in directed movement (especially when long distances to synaptic termini must be traversed) and blockage of neurite junctions are likely to contribute to the congregation of mitochondria in the cell body of fusion-deficient neurons.

In both cultured cells and tissues from animals, it has been demonstrated that lack of fusion often leads to defects in oxidative phosphorylation. While the mechanism has not been fully elucidated, partial loss of mtDNA seems to contribute to these defects in cellular respiration. In wildtype cells, every mitochondrion typically contains at least one mtDNA nucleoid, as assessed by immunostaining with an anti-DNA antibody (Figure 1A). Fusion-deficient cells (Figure 1B, C), however, harbor a large percentage of mitochondria without any discernible DNA.[12] Since these mitochondria cannot fuse to other mitochondria and exchange contents, they have no ability to regain the requisite, mtDNA-encoded electron transport chain proteins, and are thus respiration-defective. Interestingly, defects in cerebellar function are also found in a large percentage of patients harboring pathological mtDNA mutations.[14, 15]

In summary, mitochondrial fusion protects mitochondrial function, most likely by enabling exchange of contents. In addition, fusion is important for mitochondrial motility, possibly through its effects on morphology. When fusion is attenuated, the combination of mitochondrial mislocalization (especially in highly polarized cells such as neurons) and dysfunction negatively impacts cellular and tissue function. In particular tissues at specific times, this can cause widespread consequences for the entire organism. For example, lack of fusion in adult Purkinje cells causes ataxia that the mice can largely tolerate. In developing pups, though, lack of fusion in the same cells invariably results in lethality for the mice. Because Mfn1 and Mfn2 play largely redundant roles in mitochondrial fusion, the expression patterns of the two proteins can dictate which tissues are selectively affected by the deletion of each gene. Tissue specificity in the human diseases CTM2A and DOA may also reflect expression pattern differences.

Figure legend

Figure 1: Mitochondrial fusion prevents loss of mtDNA nucleoids.
Mouse embryonic fibroblasts (MEFs) were stained with a monoclonal antibody against DNA. Mitochondria were visualized with by enhanced green fluorescent protein (EGFP) targeted to the mitochondrial outer membrane. In wildtype MEFs (A), each mitochondrial tubule contains one or more mtDNA nucleoids (small red spots). In contrast, MEFs lacking Mfns (B) or OPA1 (C) contain fragmented mitochondria, and a large subpopulation of these lack mtDNA nucleoids. Note that the anti-DNA staining also highlights nuclear DNA (large central oval). Scale bar indicates 10 microns.

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Figure 1, Chen and Chan