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Targeting autophagy and mitophagy for mitochondrial diseases treatment

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EXPERT OPINION

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Introduction: Mitochondrial diseases are a group of rare genetic diseases with complex and heterogeneous origins which manifest a great variety of phenotypes. Disruption of the oxidative phosphorylation system is the main cause of pathogenicity in mitochondrial diseases since it causes accumulation of reactive oxygen species (ROS) and ATP depletion.

Areas covered: Current evidences support the main protective role of autophagy and mitophagy in mitochondrial diseases and other diseases associated with mitochondrial dysfunction.

Expert Opinion: The use of autophagy and/or mitophagy inducers may allow a novel strategy for improving mitochondrial function for both mitochondrial diseases and other diseases with altered mitochondrial metabolism. However, a deeper investigation of the molecular mechanisms behind mitophagy and mitochondrial biogenesis is needed in order to safely modulate these processes. In the coming years, we will also see an increase in awareness of mitochondrial dynamics modulation that will allow the therapeutic use of new drugs for improving mitochondrial function in a great variety of mitochondrial disorders.

Keywords: AICAR, AMPK, autophagy, coenzyme Q₁₀, heteroplasmy, mitochondrial dynamics, mitophagy, mTOR, Parkin, rapamycin, ROS

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1. Introduction

1.1 Mitochondrial diseases

Mitochondria are vital organelles for every nucleated cell as they generate ATP by the oxidative phosphorylation (OXPHOS) system, performed by the mitochondrial respiratory chain (MRC). Mitochondria are also important for several aspects of cell function and metabolism, because they regulate apoptosis, calcium homeostasis, and the response against oxidative stress mainly caused by the mitochondrial production of reactive oxygen species (ROS).[1]

In general, the term mitochondrial disease refers to those inborn error disorders caused by total or partial dysfunction of the mitochondrial electron transport chain associated to the OXPHOS process.[2] The estimated prevalence of these diseases is at least one in 5000 people, although it could be higher.[3]

Mitochondrial proteins are encoded by either mitochondrial DNA (mtDNA) or nuclear DNA. Consequently, mitochondrial diseases can be caused by mutations in both genomes. This double genetic control of mitochondrial function gives rise to different patterns of inheritance of mitochondrial diseases and complicates their characterization and diagnosis. Mitochondrial diseases have a great variety of phenotypes which manifest mainly in organs with higher energy demand such as neuronal tissue, muscle, heart, and kidney.[4]



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6. Article highlights.

- Accumulation of dysfunctional mitochondria is one of the main causes of worsening of the pathophysiology in mitochondrial diseases.
- Autophagy and mitophagy seem to play a protective role in mitochondrial diseases.
- Disruption of autophagy and/or mitophagy is a characteristic of many diseases with mitochondrial dysfunction (e.g., neurological diseases).
- Promotion of nonselective autophagy and/or mitophagy by pharmacological approaches could be a promising therapeutic target for mitochondrial diseases because it permits the elimination of dysfunctional mitochondria.
- Mitophagy regulation could be a curative therapy for mitochondrial diseases caused by mtDNA mutations, because we could modulate the heteroplasmy load.
- Specific removal of dysfunctional mitochondria should be accompanied by an increase in mitochondrial biogenesis to prevent the energetic collapse of the cell. This box summarizes key points contained in the article.

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It is still difficult to establish the relationship between the molecular pathology of mitochondrial diseases and the variety of phenotypes associated with them, because the phenotype depends on the type of mutation, the patient genetic background, and the existence of other mutations, among other features.[5] In the case of mtDNA mutations, understanding the phenotypes is even more complicated due to the characteristic presence of heteroplasmy (coexistence of wild-type and mutant mtDNA in the same cell).

1.2 Molecular pathophysiology of mitochondrial diseases

Currently, the understanding of the pathogenic mechanisms of mitochondrial diseases is limited. ATP shortage and ROS excess are the main pathogenic factors that cause most of clinical manifestations of mitochondrial diseases.[6,7] In most of the cases, both pathological alterations are due to a dysfunction in the OXPHOS system, which leads to a chronic stage of energy insufficiency and increased oxidative stress.

Other molecular features of mitochondrial diseases are a decrease in the activity of the complexes of the OXPHOS system, an increase in the glycolytic pathway, an increase in lactate production, a lower respiratory capacity per mitochondria; a lower mitochondrial membrane potential ($\Delta\Psi$), an increase in cytosolic calcium levels, a decrease in coenzyme Q₁₀ (CoQ₁₀) levels, a reduction of insulin secretion, an increase of apoptosis levels, and premature aging .

In addition, autophagy and/or mitophagy have been recently demonstrated to be misregulated in some cellular and animal models of mitochondrial diseases.[8–11]

1.3 Current treatments for mitochondrial diseases

Nowadays, there is only supportive therapy for mitochondrial diseases, and its effectiveness is not complete for most of them.[12]

Among the challenges for finding a successful mitochondrial disorder treatment are the lack of large groups of similar patients and the heterogeneity of symptoms. The partial accessibility of bioactive molecules to mitochondria is also a difficulty that needs to be overcome.[13]

Currently, only a few compounds seem to be effective for mitochondrial diseases. The majority of therapies are a combination of creatine, CoQ₁₀, and lipoic acid.[14] Antioxidants such as riboflavin, thiamine, and vitamin E are also used to counteract the effects of oxidative stress caused by ROS.[15] CoQ₁₀ treatment has demonstrated an improvement in the pathophysiology of some cellular models of mitochondrial diseases,[16] although the results drawn from clinical trials are not conclusive.[17]

There are some potential treatments under study which target different molecular features of mitochondrial diseases (see Table 1). Most of them mainly promote mitochondrial biogenesis with the intention of increasing mitochondrial function by having a greater mitochondrial mass.[18] However, controversy exists regarding whether increasing biogenesis of dysfunctional, as well as normal, mitochondria will ultimately be beneficial or harmful.

Due to the intrinsic difficulties in the design of clinical trial for rare diseases, few randomized double-blinded placebo-controlled trials for mitochondrial disease have been completed.[18,19]

2. Autophagy and mitophagy in mitochondrial diseases**2.1 Biological importance of autophagy and mitophagy**

Autophagy is a process by which cytosol and organelles are sequestered by a *de novo*-formed membrane that elongates and seals on itself to form a double membrane vesicle termed autophagosome. The contents of the vesicle are then delivered to the lysosome/vacuole for hydrolytic degradation and

Table 1. Potential therapeutic targets of mitochondrial diseases under study.

Target	Strategy
Mitochondrial biogenesis promotion	Regulating AMPK, PGC1- α
Oxidative stress reduction	Antioxidant cocktails/overexpression antioxidant enzymes
Permeability Transition Pore (PTP)	Treatment with Cyclosporine A
Apoptosis inhibition	Caspases inhibitors

recycling of the resulting macromolecules. Autophagy is typically activated by fasting and nutrient deprivation to generate amino acids and metabolic intermediates to maintain ATP production. Furthermore, autophagy allows the removal of defective or useless organelles. In that context, some authors describe autophagy as a survival mechanism of the cell, because it represents a way to adapt to stressful situations, preventing death by apoptosis. Therefore, an appropriate regulation of autophagy is essential for cellular homeostasis, because only defective organelles have to be degraded.

Three main types of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In macroautophagy, cargo is sequestered from the rest of the cytosol by autophagosome and is degraded upon fusion of this vesicle with secondary lysosomes, which contain a broad range of enzymes required for hydrolysis of the cargo.[20] By contrast, during microautophagy, the lysosomal membrane invaginates to engulf the cytosolic components without any intermediaries.[21] In CMA, the cytosolic chaperone protein Hsc-70 (heat shock cognate protein of 70 kDa) recognizes targeted proteins through the exposure of a pentapeptide motif biochemically related to the pentapeptide KFERQ. The interaction between the chaperone and the substrate in the cytosol forms a complex, which is specifically translocated across the lysosomal membrane.[22]

This review specifically focuses on the role of macroautophagy in mitochondrial diseases (hereafter refer as autophagy). Autophagy can be a nonselective or selective process, because autophagy receptors and proteins of the growing autophagosome can interact specifically with the cytoplasmic component that needs to be eliminated.[23] The molecular mechanism underlying autophagy has been extensively researched in the past decade, and the genes involved in this process, denoted ATGs (Autophagy related), were found to be conserved from yeast to humans.[24] Among these ATGs, two major cytosolic kinase complexes, the beclin-1/phosphatidylinositol kinase type III (PI3K) complex and mammalian Target Of Rapamycin (mTOR) kinase and its accessory proteins regulate induction of autophagy, whereas autophagic-auxiliary proteins and lipids are involved in the formation of autophagosomes.[25]

Although autophagy usually refers to the nonselective elimination of any component of the cytoplasm, the term mitophagy was coined by J.J. Lemasters in 2005 to exclusively define the selective degradation of mitochondria by autophagy.[26] Damaged or useless mitochondria are selectively degraded by a regulated and complex process, which allows regulating the number of functional mitochondria in the cell, both for satisfying the metabolic demand and performing mitochondrial quality control.

Mitochondria produce ROS by cellular respiration, coupled to the electron transport chain. Defects in OXPHOS can increase ROS production, whereas ROS-mediated damage to biomolecules can have direct effects on

the components of the electron transport system.[27] Due to the production of these reactive species, mitochondria are highly exposed to oxidative stress, which causes the opening of mitochondrial permeability transition pores (MPTP) in the inner membrane that increases the permeability of mitochondrial membranes to molecules of less than 1.5 kDa, a process known as mitochondrial permeability transition or MPT. Induction of MPT can lead to mitochondrial swelling and cell death through apoptosis or necrosis depending on the particular biological situation.[28] Moreover, induction of MPT causes the loss of the proton electrochemical gradient, mitochondrial depolarization, and as a consequence, a defective production of ATP.

In addition, ROS are implicated in genotoxicity and may cause the accumulation of mutations in the mtDNA. The accumulation of oxidative damage in the mitochondria over time even can cause apoptosis due to release of proapoptotic proteins. Therefore, mitochondrial turnover must occur in the cell. This process is mainly performed by mitophagy.[29]

Understanding of mitophagy at the molecular level was pioneered in yeast.[30] In mammals, three different mechanisms of mitophagy are known. First, most mammalian erythrocytes lose their mitochondria during maturation by mitophagy mediated by NIX (NIP3-like protein X) and LC3 (Microtubule-associated protein light chain 3) proteins.[31] Second, it has been identified that intracellular iron chelation generates a strong mitophagy response by a mechanism that has not been well characterized.[32] Finally, the most important mitophagic mechanism in mammals is considered to be induced by depolarization of mitochondria due to damage accumulation and the harmful action of ROS.[33] This process is dependent on the interaction between Parkin and PTEN-induced putative kinase 1 (PINK1) kinase. In 2008, Parkin recruitment to depolarized mitochondria and the subsequent degradation of mitochondria by mitophagy was described.[34] Parkin, a protein which in humans is encoded by the PARK2 gene, is a cytosolic E3 ubiquitin ligase with an ubiquitin-like domain in its N-terminal end, which allows the transference of ubiquitin to lysine residues of target proteins, including Parkin itself.[35] Parkin catalyses a range of different ubiquitination events, from mono-ubiquitination (which has various cellular functions) until polyubiquitination of proteins that leads to proteasome degradation of the target protein.

On the other hand, PINK1 is a serine threonine kinase, which is found both in the cytosol and some mitochondrial compartments, most likely anchored to the outer mitochondrial membrane. Under steady-state conditions, PINK1 and Parkin regulate mitochondria morphology by interacting with the mitochondrial fusion/fission machinery. In healthy cells, PINK1 is imported into the inner mitochondrial membrane, cleaved by the protease Presenilins-associated rhomboid-like, and subsequently degraded.[36] However, dissipation of $\Delta\Psi$ hampers PINK1 import, causing it to accumulate at the outer mitochondrial membrane. As a

result, PINK1 levels are kept high on the outer membrane of depolarized mitochondria.[37] In these conditions, PINK1 recruits Parkin to mitochondria through direct phosphorylation. PINK1-mediated Parkin phosphorylation stabilizes the association of Parkin on mitochondria and activates its E3 ubiquitin ligase activities.[38] This process is especially accelerated when combined with phosphorylation of ubiquitin.[39]

Upon activation, Parkin ubiquitinates numerous proteins on the mitochondrial outer membrane and in the cytosol, including voltage-dependent anion channel 1, mitochondrial rho GTPase, translocase of outer mitochondrial membrane 20, and Mfn1 and Mfn2 (Mitofusins 1 and 2).[40]

Following Parkin-mediated ubiquitination of outer mitochondrial membrane proteins, the selective autophagy adapter protein p62/SQSTM1 (Sequestosome1), and NBR1 (Neighbor of BRCA1 gene 1 protein) are recruited to mitochondria and thought to play an essential role in mitophagy.[41,42] These proteins are able to interact with both ubiquitinated proteins of the outer membrane of mitochondria and autophagic membrane proteins such as LC3. The interaction between p62/SQSTM1 and NBR1 with LC3 allows the sequestration of depolarized mitochondria by the membrane of the autophagosome.[34,41]

In addition, ubiquitination and proteasomal degradation of Mfn1 and Mfn2 results in mitochondrial fission and fragmentation.[43] Mitochondrial fission has been shown to be important for mitophagy induction given that mitochondrial depolarization prevents fusion and leads to mitochondria isolation and subsequent degradation by mitophagy.[44] In contrast, increased mitochondria fusion has been shown to inhibit the mitophagy process.[45]

There are many studies about the importance of Parkin and PINK1 in cell viability. Knockdown of Parkin in differentiated human neuroblastoma cells (SH-SY5Y) reduced cell survival.[46] However, Parkin silencing had no effect in cell viability of HeLa cells, suggesting that Parkin silencing exerts effect on dopaminergic neurons, and the lack of Parkin is not necessarily incompatible with cell survival in another cell types.

2.2 Role of autophagy and mitophagy in mitochondrial diseases

2.2.1 Autophagy in mitochondrial diseases

Increased or imbalanced autophagy has been reported in several cellular and murine models of mitochondrial diseases. Autophagy could be induced as a compensatory mechanism to obtain energy or to eliminate damaged organelles whose accumulation could exacerbate mitochondrial dysfunction. Thus, in a mouse model of mitochondrial myopathy due to mutation in the mitochondrial helicase Twinkle, evidence of an increased recycling of mitochondria through autophagy in muscle fibers has been observed.[47] An increase of autophagy has also been reported in muscle biopsies of patients

harboring m.8344A>G mutation, the most common mutation that causes Myoclonic epilepsy with ragged-red fibers (MERRF) syndrome.[48]

Recently, the role of nonselective autophagy in a fibroblast model of mitochondrial dysfunction due to deficiencies in OXPHOS system has been described. An accumulation of autophagosomes and lysosomes has been observed in cells with mutations in genes encoding different components of the MRC, but a selective process of mitochondria degradation was not detected.[10] The authors suggest that the increase of autophagic markers in cellular models of mitochondrial diseases can be due to an impairment of the autophagic flux, which causes an accumulation of autophagosomes/autophagolysosomes.

Although there is no longer doubt about the importance of autophagy in mitochondrial diseases, there is not a common consensus about if autophagy has a protective or pathological role during the course of the diseases. The protective role of autophagy has been observed in multiple cellular types and pathological situations by a large number of studies. Among others, it has been observed that autophagy activation has a neuroprotective role during brain damage [49] and improves resistance under hyperoxia situations and oxidative stress in the lung endothelium.[50] However, autophagy has been demonstrated to be harmful in different situations such as establishment and progression of tumors [51] or progression of pathogenesis of different lung diseases.[52] Moreover, a recent study shows a protective effect of inhibition of autophagy on MRC disruption by an mTOR-independent mechanism.[53]

2.2.2 Mitophagy in mitochondrial diseases

Mitophagy can be considered a defense mechanism to face stress situations caused by mitochondria alterations (Figure 1). The protective role of mitophagy has been demonstrated in numerous cell models and pathological situations. Experiments with PINK1^{-/-} mice showed impaired mitochondrial respiration and an increase in large mitochondria, which can be related with the function of PINK1 to promote fission and mitophagy.[54] These effects were exacerbated with age, suggesting that mitophagy also has an important role in aging. Moreover, mitophagy has been reported to be an adaptive metabolism to prevent oxidative damage and hypoxia.[55]

In the particular case of mitochondrial diseases caused by mutations in the mtDNA, mitophagy may have an essential role in eliminating mitochondria harboring the highest percentage of mutated mtDNA, which will presumably be the most dysfunctional mitochondria. In this sense, a specific characteristic of some cell models of mitochondrial dysfunctions is the decreased $\Delta\Psi$ due to deficiencies in the electron flow through the MRC. This fact suggests that depolarized mitochondria can be selectively eliminated by mitophagy. Activation of mitophagy has been observed in dermal fibroblasts derived from patients with primary CoQ₁₀ deficiencies, Mitochondrial encephalomyopathy, lactic acidosis, and

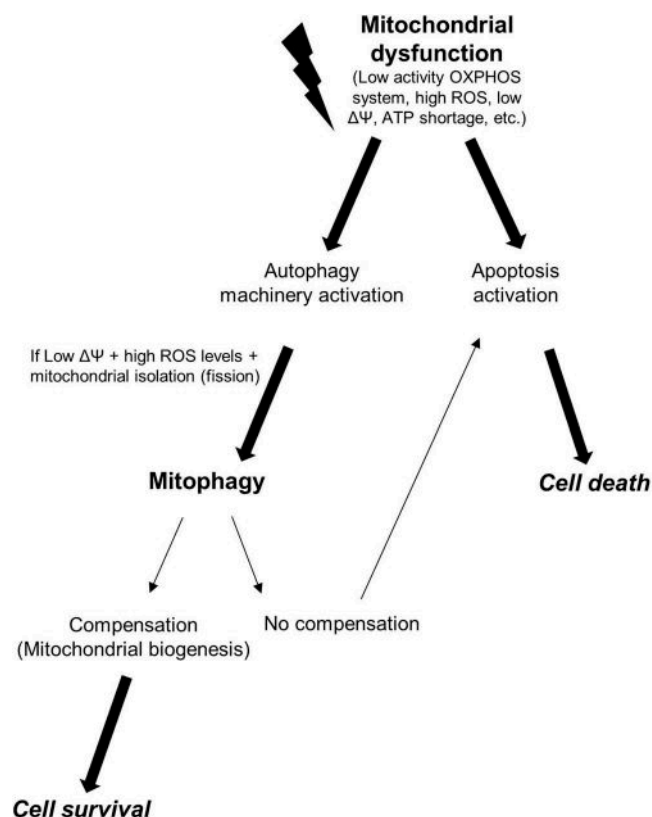


Figure 1. Mitophagy as a defense mechanism against mitochondrial dysfunction.

stroke-like episodes (MELAS) and MERRF mutations. In both cases, cells showed low $\Delta\Psi$, high level of ROS and CoQ₁₀ deficiency. In fact, CoQ₁₀ supplementation restored mitochondrial morphology, $\Delta\Psi$, ROS levels, and mitophagy rate to control values.[8,9,11,56] It is important to remark that in these cell models, extensive mitophagy was accompanied by an increase in autophagy markers, suggesting that activation of autophagic machinery is necessary to the specific elimination of mitochondria. Moreover, activation of mitophagy in mitochondrial diseases seems to be protective, because it has been demonstrated that disruption of mitophagy by autophagic sequestration using chemicals such as 3-methyl adenine or wortmannin or by genetic knockdown of ATG genes in MELAS or primary CoQ₁₀-deficient fibroblasts results in cell death by apoptosis.[8,11] However, it must be kept in mind that the use of PI3K inhibitors such as 3-methyl adenine or wortmannin has some limitations in terms of target specificity. Furthermore, some apparently specific ATG gene products may have autophagy-independent roles in apoptosis, endosomal function, and protein trafficking.[57] Therefore, the experimental conditions of inhibitor application and their side effects must be carefully considered.

Nevertheless, high activation of mitophagy in these cell models can suppose a loss of mitochondrial mass, resulting in

the energetic collapse of the cell. Therefore, if mitophagy activation is not compensated by increased mitochondrial biogenesis, degradation of dysfunctional mitochondria cannot be protective and becomes detrimental for cell survival.

However, in the majority of cases, mtDNA mutations are maintained over time. Most of patients with mitochondrial diseases show persistence and even an increase of mutational load with age, suggesting that the selective removal of dysfunctional mitochondria is not being effectively carried out. In light of these evidences, it is reasonable to think that mitochondrial dysfunction by itself is not sufficient to activate the mitophagic process. In this sense, some authors have suggested that mitochondrial diseases, like neurodegenerative diseases such as Parkinson's disease (PD) or Alzheimer's disease (AD), are associated with alterations of mitophagy regulation. In the case of neurodegenerative disorders, removal of aberrant mitochondria has been shown to play a protective role. Specifically, Parkin protein, whose loss of function causes PD, is involved in this process. Since Parkin is essential for mitophagy, PD may be at least in part associated with failure to eliminate dysfunctional mitochondria.[58] In the specific case of mitochondrial diseases, there is not a common agreement about the failure of mitophagy regulation. Since the expression level of PINK1 and/or Parkin in the cell is critical for mitophagy, some authors

propose that cells with mutated mtDNA have decreased levels of PINK1 and/or Parkin, possibly due to unknown transcriptional inhibition mechanisms. In fact, it has been observed that cybrids cells with mtDNA mutation (A3243G) have less Parkin expression levels than wild-type cybrids cells, and this fact may impair mitophagy of dysfunctional mitochondria.[59]

In support of this hypothesis, it has been reported significant differences between mitophagy rates of “Parkin-overexpressing” and “endogenous Parkin” cellular models. Overexpression of Parkin in cellular models treated with uncouplers of OXPHOS leads to a high Parkin translocation to depolarized mitochondria and mitophagy. However, in human primary fibroblasts with endogenous levels of Parkin, there is no significant activation of mitophagy after the loss of $\Delta\Psi$. [60] Low levels of Parkin in this model can be a consequence of Parkin self-ubiquitination, which leads to its own degradation and prevents its mitochondrial translocation attenuating further mitophagy. In fact, it has been demonstrated that inhibition of UPS (Ubiquitin Proteasome System) prevents Parkin ubiquitination and, as a consequence, the level of mitochondrially translocated Parkin increases.[60] However, the role of UPS in mitophagy must be further clarified because activation of UPS is crucial for Parkin-mediated mitophagy in several cell types.[61]

In light of these data, some authors have hypothesized that mitochondrial dysfunction *per se* is not sufficient to recruit mitophagic factors to damaged mitochondria and mitophagy activation. Although high levels of ROS and/or low $\Delta\Psi$ are necessary to promote mitophagy, Parkin expression levels may be critical to allow the degradation of dysfunctional mitochondria. Likewise, the presence of the nonselective

autophagy machinery would be necessary for the progression of mitophagy.

3. Modulating autophagy, mitophagy, and mitochondrial dynamics as a therapy for mitochondrial diseases

Autophagy and mitophagy modulation has been proposed as a possible therapy for numerous different diseases which present mitochondrial dysfunctions (see Table 2). Various autophagy inducers such as rapamycin, rapalogs (rapamycin derivatives), metformin, and trehalose and inhibitors (chloroquine, hidroxichloroquine) are already approved for the treatment of various diseases. Therefore, autophagy and mitophagy are very promising targets for the treatment of mitochondrial diseases, and the more studied strategies to pursue this approach are targeting two different autophagy regulation pathways: 5' adenosine monophosphate-activated protein kinase (AMPK) and mTOR pathways.

3.1 Targeting AMPK

Since autophagy and mitophagy could be some important targets in mitochondrial diseases treatment, one of the goals is modulating the function of the proteins responsible for the regulation of these processes. Among others, regulation of AMPK is gaining attention as a potential therapeutic target for mitochondrial diseases in the future, because it has many functions related to maintain the mitochondrial and cellular homeostasis.

Table 2. Autophagy and mitophagy as targets for different diseases.

Disease	Characteristics	Potential treatments
Neurodegenerative diseases (e.g., PD, AD, HD) [58]	Failure elimination of damaged mitochondria. Extensive accumulation of autophagosomes. Defects in mitochondrial dynamics.	Autophagy inducers [78]
Cancer	Autophagy is essential for tumor suppression in premalignant stages Autophagy promotes the pathogenesis of established malignancies	Autophagy inducers: Temsirrolimus, rapamycin [77] Autophagy inhibitors: chloroquine, hidroxichloroquine
Mitochondrial diseases	Autophagy/mitophagy imbalance	Autophagy/mitophagy inducers [12,97] Autophagy inducers
Chronic inflammation diseases (e.g., Chron disease) [98]	Defects of autophagy have been associated with higher production of proinflammatory cytokines implicated in pathogenesis of inflammation diseases.	Autophagy inducers
Lung diseases [52]	Protective or harmful role of autophagy depending on the type of lung disease	Autophagy inducers; Autophagy inhibitors
Diabetes type 2 [99]	Autophagy deficiency in β -cells could help to the progression from obesity to diabetes	Autophagy inducers (metformin)
Liver diseases [100]	Autophagy has a protective role (eliminating lipid droplets and protein aggregates), except for hepatitis B and C infections (virus promote their replication by autophagy)	Autophagy inducers; Autophagy inhibitors

AMPK is an important sensor of signals that control cellular energy balance in all eukaryotes and therefore has emerged as a metabolic master. AMPK functions as an initiator of autophagy via mTOR complex inhibition, and it also participates in the transcriptional control of lipogenesis and mitochondrial proliferation and regulates other metabolic processes such as glycolysis, lipolysis, or fatty acid oxidation.

AMPK is a heterotrimeric enzyme serine/threonine kinase composed of a catalytic subunit α and two regulatory subunits β and γ . [62] Activation of AMPK occurs in response to stress circumstances such as low glucose, hypoxia, ischemia, heat shock, increased ROS, and exercise. [63] Under these situations, the intracellular AMP +ADP/ATP ratio is increased, and then AMPK is converted from an inactive form to a catalytically competent form by phosphorylation of the activation loop within the kinase domain. AMPK can also be activated by Food and Drug Administration (FDA)-approved drugs, including the AMP analog 5-aminoimidazole-4-carboxamide-1- β -ribofuranoside (AICAR) or the anti-diabetic biguanide metformin and several natural products. [64]

AMPK is a key protein in autophagy regulation, because its activation can induce autophagy through different pathways. First, activated AMPK promotes autophagy by inhibiting mTORC1 activation. [65] Furthermore, under stress conditions

or starvation, AMPK is activated and directly phosphorylates ULK-1, an inducer of autophagy in mammals [66,67] (Figure 2). In addition, AMPK activation stimulates autophagy in skeletal muscle cells through its effects on the transcriptional function of FoxO3a and takes part in the initiation of autophagosome formation by interacting with ULK-1. [68]

Although autophagy is a general process in the cell, some authors suggest that activation of autophagy is necessary for mitophagy progression. [59] Therefore, induction of autophagy by activated AMPK, together with the recruitment of Parkin to defective mitochondria, contributes to induction of mitophagy in mammalian cells. In fact, it has been demonstrated that cells with AMPK deficiency exhibit defective mitophagy. [66] Therefore, increasing the activity of AMPK could ameliorate the symptoms of mitochondrial diseases that course with dysfunctional mitophagy. Thus, it has been recently proved that AICAR partially corrects the mitochondrial dysfunction in a mice model of Cytochrome C oxidase (COX) deficiency. [69]

Besides its importance in autophagy regulation, AMPK activation is also crucial for the metabolic switch from respiration to glycolysis caused by mitochondrial dysfunction. Thus, it has been observed that the AMPK activation induced an increase of the expression of several proteins involved in the glycolytic pathways in a fibroblast model of MERRF disease. [70]

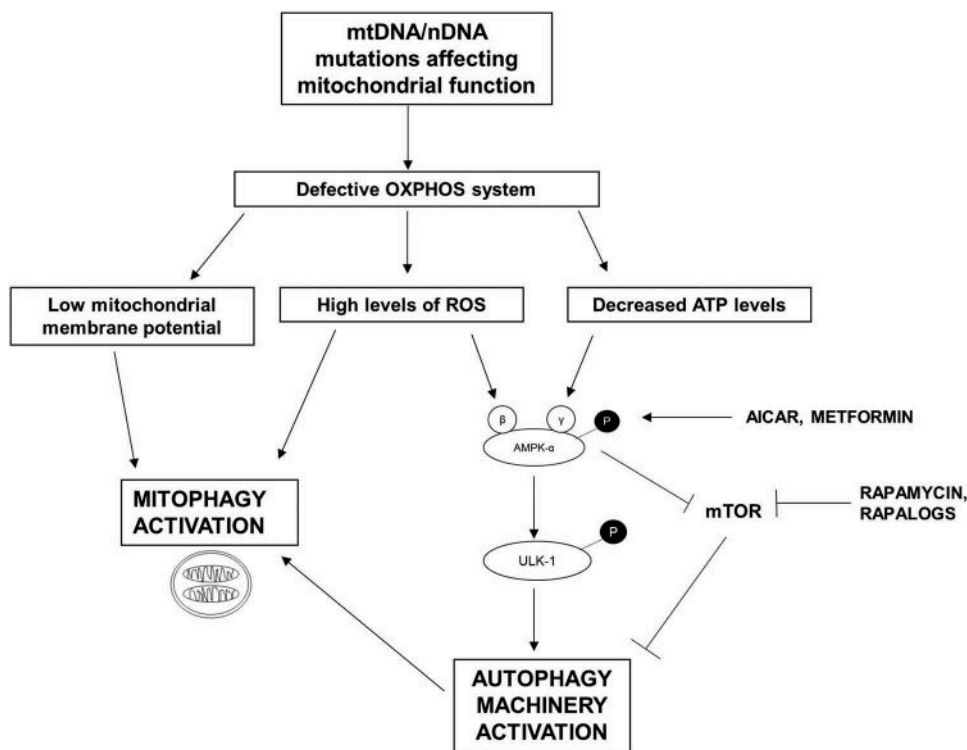


Figure 2. Mitochondrial dysfunction induces mitophagy and AMPK activation.

Likewise, AMPK has an important role in regulating mitochondrial biogenesis, which is closely related with mitophagy. The regulation of mitochondrial biogenesis in mitochondrial diseases depends on the activation of transcription factors which are downstream targets of AMPK, among others. The most important regulator of mitochondrial biogenesis is PGC1- α . [71] It has been observed that PGC1- α is directly phosphorylated and transactivated by AMPK. [72] Likewise, PGC1- α can be activated by Sirt-1-mediated deacetylation, which is also induced by AMPK activation. [73] PGC1- α regulates the expression of many mitochondrial proteins. In this way, it has been demonstrated that mitochondrial proliferation induced by PGC1- α activation enhanced OXPHOS capacity in a mitochondrial myopathy mouse model. [74] Moreover, overexpression of PGC1- α in cells from patients with mitochondrial diseases leads to an increase of respiration due to an improvement of MRC efficiency. [75] Therefore, modulation of AMPK could be a possible therapeutic strategy by increasing PGC1- α activation and mitochondrial biogenesis as a compensatory mechanism. Last but not the least, AMPK activation could protect cells with mitochondrial dysfunction against oxidative stress, because AMPK promotes the expression of antioxidant enzymes such as MnSOD and catalase by activating FoxO1 and FoxO3A transcription factors. [76] Therefore, AMPK has an essential role in mitochondrial adaptation to different dysfunctions and therefore could serve as a potential target for mitochondrial diseases treatment.

3.2 Targeting mTOR machinery

The use of pro-autophagic agents as a therapy for different diseases is increasing in recent years. Among these agents, rapamycin and its derivatives are the most studied as therapeutic agents for the treatment of tumors [77] and neurodegenerative diseases such as Huntington's disease (HD) or PD. [78]

Rapamycin exerts its pro-autophagic effect by competitive inhibition of mTOR complex (Figure 2). mTOR is a master regulator of different intracellular pathways that controls growth, proliferation, and survival. It is an effector in the PI3K/AKT pathway and performs its action by two different complexes, mTORC1 and mTORC2. mTORC1 promotes anabolism, protein synthesis, cell growth, and lipid biogenesis. At the same time, mTORC1 inhibits cellular catabolism by blocking autophagy. Instead, mTORC2 regulates cell survival, cell proliferation, metabolism, and the cytoskeleton. Due to the importance of autophagy in mitochondrial diseases is interesting to study the potential use of rapamycin for the treatment of these disorders.

Recently, the beneficial effect of rapamycin treatment in mitochondrial diseases and in other pathologies that present mitochondrial dysfunctions has been demonstrated. For example, it has been observed that treatment with rapamycin after cerebral ischemia promotes a reduced infarct volume, improves neurological outcomes, and inhibits mitochondrial

dysfunction in experimental animals. The beneficial effects of rapamycin are due to an increase in mitophagy. [79] In addition, promotion of mitophagy by rapamycin has demonstrated to be protective against the effects of rotenone, complex I inhibitor which causes mitochondrial dysfunction. [80] Using human chondrocytes treated with oligomycin to induce mitochondrial dysfunction, it has been proved that autophagy induction by rapamycin treatment protects chondrocytes from oxidative stress. [81] Therefore, regulation of autophagy could be a promising therapeutic target for aging-related musculoskeletal disorders too, because mitochondrial dysfunction has an important role in these diseases.

Rapamycin benefits have been also demonstrated in *in vivo* models of mitochondrial diseases. Rapamycin treatment has been demonstrated to improve survival and attenuate the progression of the disease in a mammalian model of Leigh syndrome, the *Ndufs4* knockout (*Ndufs4*^{-/-}) mouse. [82] Improved motor function was observed, as well as a decrease in brain inflammation and prevention of the brain lesions characteristic of Leigh syndrome.

3.3 Targeting heteroplasmy by promoting mitophagy

An important role of mitophagy in mitochondrial diseases caused by mtDNA mutations could be its capability to modulate the percentage of heteroplasmy. Since the percentage of heteroplasmy is directly proportional to the severity of mitochondrial diseases, [83] the modulation of heteroplasmy could be a promising therapeutic strategy. Different methods have been proposed to modulate and/or decrease the percentage of heteroplasmy in diverse models of mitochondrial diseases. [84,85] However, due to the possible side effects of the current methods, other strategies are needed for heteroplasmy modulation.

Mitophagy specifically eliminates damaged mitochondria with high level of ROS and/or low $\Delta\Psi$. Presumably, in mitochondrial diseases, mitochondria with these characteristics have higher percentage of heteroplasmy. Therefore, stimulating mitophagy seems to be a possible strategy to decrease the level of heteroplasmy, because only defective mitochondria, with high percentage of heteroplasmy, will be eliminated, and healthy mitochondria will proliferate.

Different strategies have been performed to stimulate mitophagy in diverse models of mitochondrial diseases. Overexpression of Parkin in cybrids with 80% of mutated mtDNA (cytochrome c oxidase I mutation) led to an increase in mitophagy, which allowed a reduction of the mutational load up to 26.7%, and most important, this reduction remained over time. [86] Moreover, COX activity was restored in cybrid cells enriched for wild-type mtDNA, indicating that decrease of heteroplasmy could improve the pathophysiology of the disease. Therefore, upregulation of Parkin expression may be beneficial for hereditary mitochondrial diseases.

These findings also indicate that endogenous Parkin levels may be a limiting factor for the negative selection of

dysfunctional mitochondria in some cell types. Thus, low levels of Parkin may prevent the physiological elimination of damaged mitochondria with high level of heteroplasmy, which are accumulated in the cells and cause the symptoms of the disease.

The importance of Parkin in heteroplasmy regulation has been recently examined in an *in vivo* model of mitochondrial dysfunction. Thus, in a strain of *Caenorhabditis elegans* carrying a heteroplasmic mtDNA truncation, it has been demonstrated that mutation in Pdr-1 (Parkin orthologue) caused an increase in the ratio mutated/wt mtDNA (up to 2.2-fold). [87] These results showed the first evidence of Parkin involvement in heteroplasmy modulation *in vivo*.

Other possible strategy to modulate the degree of heteroplasmy is to promote autophagy with the goal of secondarily activate mitophagy. Lengthy treatment with rapamycin of a culture of cybrids harboring the heteroplasmic mitochondrial mutation m.11778G>A, the most common in patients with Leber hereditary optic neuropathy, leads to decrease in the mutational load from 71% to 13%. [88].

3.4 Targeting mitochondrial dynamics

Mitochondrial shape in mammals is maintained in a dynamic equilibrium between proteins that promote organelle fusion such as Mfn1 and Mfn2 and optic atrophy protein 1 and those promoting fission such as mitochondrial fission protein 1 (Fis1) and dynamin-related protein 1 (Drp-1). [89,90] These opposing processes determine the organization and function of the entire mitochondrial network of the cell. [45]

Manipulating mitochondrial dynamics is potentially an attractive approach to treat mitochondrial diseases, because shifting the equilibrium of fission and fusion may allow damaged mitochondria to be rescued by mitochondrial fusion or else be selectively eliminated by mitophagy.

When mitochondria become dysfunctional, there is a disruption of the mitochondrial network and fragmented mitochondria appear in the cytoplasm. [44] Therefore, mitochondrial fission plays an important role in the removal of damaged mitochondria by mitophagy. As soon as individual parts of the mitochondrial network become dysfunctional, fission events induced by Fis1 and Drp-1 permit damaged mitochondria to become spatially isolated. Thus, dysfunctional mitochondria are distinguished on a morphological basis from the healthy mitochondria and can be presumably degraded by selective autophagy. [91]

Genetic approaches have shown that inhibiting mitochondrial fusion shifts the fusion/fission equilibrium towards fission, [92] and vice versa. [93] Thus, adding a pharmacological agent that inhibits fission to cells containing fragmented mitochondria should increase the proportion of tubular mitochondria. In fact, a compound termed mitochondrial division inhibitor 1 (mdivi-1), a Drp-1 inhibitor, selectively inhibits mitochondrial division. [94] More recently, mdivi-1 rescued mitochondrial morphological and functional defects induced by mutations in PINK1. [95]

Moreover, because a number of neurodegenerative diseases result from aberrant mitochondrial fusion, [90] promoting fusion *via* a fission-inhibiting compound could have therapeutic potential.

Furthermore, targeting mitochondrial dynamics can be another strategy to modulate heteroplasmy (Figure 2). Thus, it has been suggested that mitochondrial fission prevents the increase of heteroplasmy in cell models of mitochondrial diseases. In fact, it has been observed that the knockdown of Drp-1 causes an increase of percentage of heteroplasmy from 80% to 96% of m.3243A>G in a cell culture of rhabdomyosarcoma. [96] Therefore, the discovery of inhibitors of mitochondrial fusion and fission may be useful for the treatment of mitochondrial disorders, whether they are caused by dysfunctions on mitochondrial dynamics or not.

4. Conclusion

It is generally accepted that low levels of ATP and ROS accumulation are the main factors involved in the pathophysiology of mitochondrial dysfunctions. Because the accumulation of defective mitochondria seems to be the main cause of the worsening of mitochondrial diseases, autophagy, mitophagy, and mitochondrial dynamics are gaining importance as future therapeutic targets for these disorders.

5. Expert opinion

Among the molecular features of mitochondrial disorders, autophagy and mitophagy are gaining importance as potential therapeutic targets. Currently, there is no common agreement on the role that these processes play in cellular pathophysiology. Moreover, autophagy and mitophagy have to be comprehensively regulated in order to maintain the balance between cell survival and cell death (Figure 3).

Although the molecular pathways of nonselective autophagy are better known, the conditions under which the selective autophagy of mitochondria is promoted are still unclear. It seems that the presence of mtDNA mutations is not sufficient for the activation of mitophagy of dysfunctional mitochondria and may lead to the accumulation of defective mitochondria that can aggravate the disease. Under physiological or pathophysiological conditions, there is no clear agreement on the conditions that promote mitophagy, because depending on the mitochondrial disease model under study, activation or disruption of this process is observed. The only three common characteristics in mitophagy activation are decreased $\Delta\Psi$, increased ROS levels and mitochondrial isolation (fission), and the induction of the autophagy machinery. Therefore, treatment with autophagy inducers may allow improving mitochondrial function for both mitochondrial diseases and other diseases involving mitochondrial dysfunction. Moreover, the elimination or reduction of the percentage of mutated mtDNA *in vivo* could be an alternative therapy to gene therapy

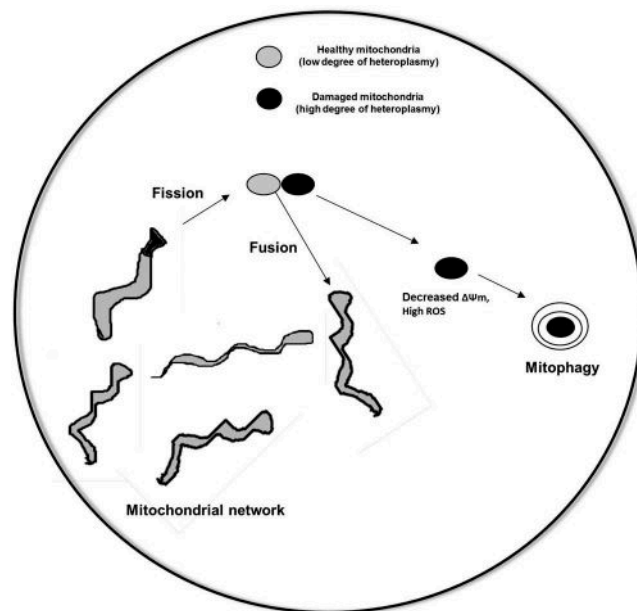


Figure 3. Interplay between mitochondrial dynamics and mitophagy.

by inducing mitophagy. However, a deeper investigation of molecular characteristics of Parkin-mediated mitophagy is needed in order to modulate this process.

The role of the proteasome machinery in mitophagy is also important and will be relevant in the near future. Some authors have suggested that the existence of a self-ubiquitination of Parkin leads to its own degradation. By studying and regulating the ubiquitination process, we could modulate the available Parkin levels and induce mitophagy in mitochondrial diseases. Indeed, preliminary studies with proteasome inhibitors such as MG132 show an increase in endogenous cytosolic Parkin by the prevention of its proteasome-mediated degradation. Based on these data, it has been suggested that the UPS is necessary to induce mitophagy in addition to autophagy activation.

Besides promoting autophagy/mitophagy, the activation of mitochondrial biogenesis is gaining importance as a potential treatment in mitochondrial diseases. In this sense, AMPK becomes decisive because it is capable of initiating a dual response which promotes both autophagy and mitochondrial biogenesis. Therefore, studying and modulating the activity of AMPK pathways and their possible role in mitochondrial disease are currently interesting investigation areas. However, a further knowledge about mitochondrial biogenesis regulation is needed because upregulation of mitochondrial biogenesis in the absence of balanced mitophagy may be maladaptive because of an increase in ROS generation, accumulation of damaged mitochondria, and cell death.

In the coming years, we will see an increase in awareness of mitochondrial dynamics that will allow the therapeutic application of already FDA-approved drugs (such as rapamycin,

resveratrol, or metformin) that can be used to improve mitochondrial function in different mitochondrial diseases.

A major advantage of these therapies is the variety of mitochondrial diseases which could be treated, because they look for the elimination of defective mitochondria with impaired OXPHOS system, in contrast with other more specific mitochondrial dysfunction therapies.

Preliminary results in cell and animal models of mitochondrial diseases are encouraging, because an improvement of mitochondrial pathophysiology in different mitochondrial diseases has been observed by treatments with autophagy/mitophagy modulators.

Declaration of interest

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