

## Chapter 2

# Relationships Between Mitochondrial Dynamics and Bioenergetics

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**Abstract** In this chapter we describe the fundamental mechanisms by which mammalian cells regulate energy production, and we put emphasis on the importance of mitochondrial dynamics for the regulation of bioenergetics. We discuss both the impact of shape changes of the mitochondrion on organellar energy production, and the existence of reverse mechanisms of regulation of mitochondrial fusion and fission by the cellular energy state. Hence, in complement to pioneering concepts of metabolic control which only considered the key controlling steps of energy fluxes at the level of the respiratory chain, the recent study of mitochondrial dynamics highlights new possibilities for OXPHOS control. The implications of such a regulatory loop between mitochondrial dynamics and bioenergetics impacts several fields of human biology, as diverse as embryonic development, energy storage, cell motility, lipid and membrane biogenesis, intracellular trafficking and cell death. In addition, most neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and Hereditary Spastic Paraplegia are associated with defects in mitochondrial dynamics and bioenergetics. Therefore, to unravel the fundamental mechanisms by which mitochondrial form interacts with mitochondrial function could permit to increase our basic knowledge on the regulation of energy metabolism and to decipher the pathophysiology of a group of rare neuronal diseases.

**Keywords** Mitochondria • Bioenergetics • Oxidative Phosphorylation • Dynamics • Neurodegenerative diseases

### Abbreviations

ADP    adenosine diphosphate  
ANT    adenine nucleotide translocator  
ATP    adenosine triphosphate

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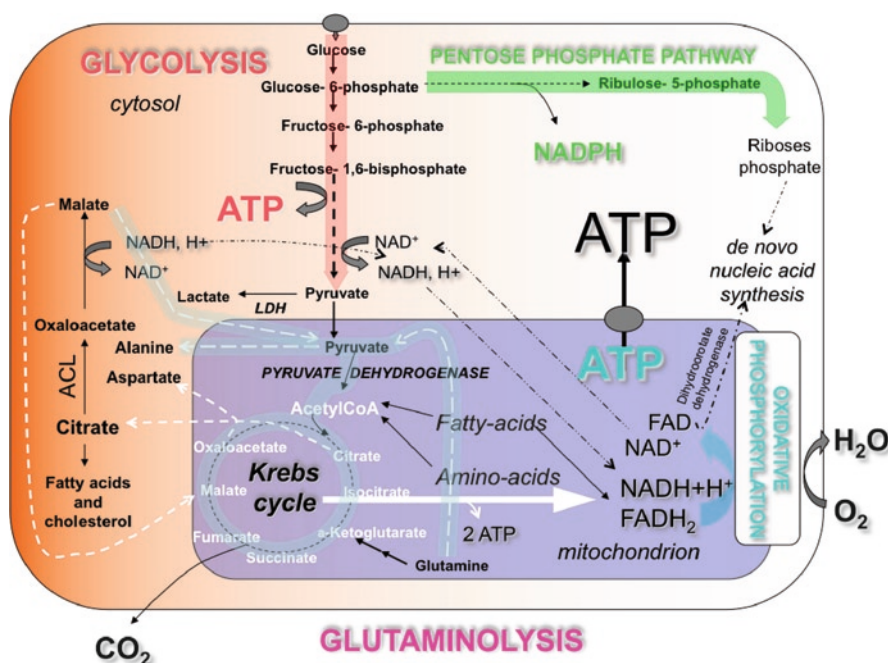
|                               |                                                                                                 |
|-------------------------------|-------------------------------------------------------------------------------------------------|
| CCCP                          | carbonyl cyanide m-chlorophenylhydrazone                                                        |
| COX                           | cytochrome c oxidase                                                                            |
| CM                            | cristae membrane                                                                                |
| CoQ                           | coenzyme Q                                                                                      |
| Cyt c                         | cytochrome c                                                                                    |
| DAPI                          | diamidino-4',6-phénylindol-2 dichlorhydrate                                                     |
| $\Delta\psi$                  | mitochondrial membrane electric potential                                                       |
| DNP                           | 2,4-Dinitrophenol                                                                               |
| DRP1                          | dynammin-related protein 1                                                                      |
| EGFP                          | enhanced GFP                                                                                    |
| EM                            | electron microscopy                                                                             |
| ETC                           | electron transfer chain                                                                         |
| FADH2                         | flavin adenine dinucleotide reduced form                                                        |
| FCCP                          | carbonylcyanide-p-trifluoromethoxyphenylhydrazone                                               |
| FISH                          | fluorescent in situ hybridization                                                               |
| FMN                           | flavin mononucleotide                                                                           |
| 4Pi MICROSCOPE                | confocal microscope with two opposing lenses used for high resolution imaging of fluorescence   |
| FRET                          | fluorescence resonance energy transfer                                                          |
| GFP                           | green fluorescent protein                                                                       |
| GTP                           | guanidin triphosphate                                                                           |
| H <sub>2</sub> O <sub>2</sub> | hydrogen peroxyde                                                                               |
| IBM                           | inner boundary membrane                                                                         |
| ICS                           | intra cristae space                                                                             |
| IM                            | inner membrane                                                                                  |
| IMS                           | inter-membrane space                                                                            |
| JO <sub>2</sub>               | respiratory rate                                                                                |
| MCA                           | metabolic control analysis                                                                      |
| mPTP                          | mitochondrial permeability transition pore                                                      |
| mt-NETWORK                    | mitochondrial network                                                                           |
| NADH                          | nicotinamide adenine dinucleotide reduced form                                                  |
| OM                            | outer membrane                                                                                  |
| OPA1                          | gene encoding a dynammin-related mitochondrial protein causing autosomal dominant optic atrophy |
| OXPHOS                        | oxidative phosphorylation                                                                       |
| PDH                           | pyruvate dehydrogenase complex                                                                  |
| PLD                           | phospholipase D                                                                                 |
| RCR                           | respiratory control ratio                                                                       |
| RFP                           | red fluorescent protein                                                                         |
| ROS                           | reactive oxygen species                                                                         |
| SDH                           | succinate dehydrogenase                                                                         |
| STED MICROSCOPY               | stimulated emission depletion microscopy                                                        |
| TMRM                          | tetramethyl rhodamine methyl ester                                                              |

## 2.1 Introduction

Rapid progress in mitochondrial research recently demonstrated a critical role for mitochondrial fusion and fission in the modulation of mitochondrial functions as diverse as apoptosis, energetics, calcium signaling, and ROS generation (Rossignol and Karbowski 2009). Alternative observations also reported changes in mitochondrial form upon activation of apoptosis, cell division, or adaptation to low energy states. Hence, the link between mitochondrial dynamics and energy production can be investigated in two directions. In this chapter we present first the observations in favor of a regulation of mitochondrial dynamics by the mitochondrial energy state, and secondly we summarize the experimental results indicating a possible control of mitochondrial energy production by organellar, shape changes. Prior to develop these two hypotheses we describe briefly the mechanisms of cellular and mitochondrial energy production, as well as their regulation, and we give a basic introduction on mitochondrial dynamics. At the end of this chapter we highlight the importance of the link between mitochondrial dynamics and energetics in human pathology.

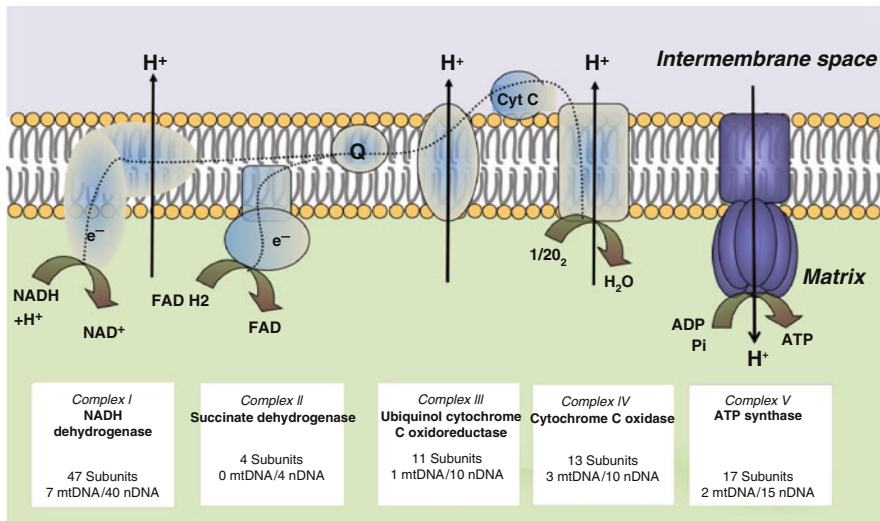
## 2.2 The Biochemistry of Energy Production

In most human tissues, mitochondria provide the energy necessary for cell growth, and biological activities. It has been estimated that about 90% of mammalian oxygen consumption is mitochondrial, which primarily serves to synthesize ATP, although in variable levels according to the tissue considered and the organism's activity status (Benard et al. 2006). Mitochondria intervene in the ultimate phase of cellular catabolism, following the enzymatic reactions of intermediate metabolism that degrade carbohydrates, fats and proteins into smaller molecules such as pyruvate, fatty acids and amino acids, respectively (Fig. 2.1). Mitochondria further transform these energetic elements into NADH and/or FADH<sub>2</sub>, through  $\beta$ -oxidation and the Krebs cycle. Those reduced equivalents are then degraded by the mitochondrial respiratory chain in a global energy converting process called oxidative phosphorylation (OXPHOS) where the electrons liberated by the oxidation of NADH and FADH<sub>2</sub> are passed along a series of carriers regrouped under the name of "respiratory chain" or "electron transport chain" (ETC), and ultimately transferred to molecular oxygen (Fig. 2.2). ETC is located in mitochondrial inner membrane, with an enrichment in the cristae. ETC consists of four enzyme complexes (complexes I – IV), and two mobile electron carriers (coenzyme Q and cytochrome c). These complexes are composed of numerous subunits encoded by both nuclear genes and mitochondrial DNA at the exception of complex II (nuclear only). It was demonstrated that these complexes can assemble into supramolecular assemblies called "supercomplexes" or respirasomes.



**Fig. 2.1** Main pathways of cellular and mitochondrial energy metabolism. The two main metabolic pathways, *i.e.* glycolysis and oxidative phosphorylation are linked by the enzyme complex pyruvate dehydrogenase. Briefly, glucose is transported inside the cell and oxidized to pyruvate. Under aerobic conditions, the complete oxidation of pyruvate occurs through the TCA cycle to produce NADH, H<sup>+</sup> and/or FADH<sub>2</sub>. These reduced equivalents are oxidized further by the mitochondrial respiratory chain

In presence of energy substrate (NADH or FADH<sub>2</sub>), the transfer of electrons from complex I (and/or II) to complex IV mediates the extrusion of protons from the matrix to the inter-membrane space, thus generating an electrochemical gradient of protons ( $\Delta\mu_{\text{H}^+}$ ) which is finally used by the F<sub>1</sub>-F<sub>0</sub> ATP synthase (*i.e.* complex V) to produce adenosine triphosphate (ATP) the energetic currency of the cell. This gradient has two components: an electric potential ( $\Delta\Psi$ ) and a chemical potential ( $\Delta\mu_{\text{H}^+}$ ) that can also be expressed as a pH gradient ( $\Delta\text{pH}$ ). According to the chemiosmotic theory (Mitchell 1961),  $\Delta\mu_{\text{H}^+} = \Delta\Psi - Z\Delta\text{pH}$ , with  $Z = -2.303 \text{ RT/F}$ . Under physiological conditions, mitochondrial energy production can alternate between two energy steady-states: basically, at state 4, respiration is slow and ATP is not produced ( $\Delta\Psi$  is high), while during state 3, respiration is faster and ATP is largely produced ( $\Delta\Psi$  is lower). In particular conditions, such as mitochondrial inner membrane permeabilization or the use of a chemical uncoupler,  $\Delta\Psi$  can be totally dispersed. As a consequence, respiration is accelerated and ATP production annihilated. The inhibition of respiratory chain complexes also generally decreases  $\Delta\Psi$ . Under physiological conditions, it is considered that mitochondria produce ATP in an intermediate state lying between state 3 and state 4. ATP is the only form



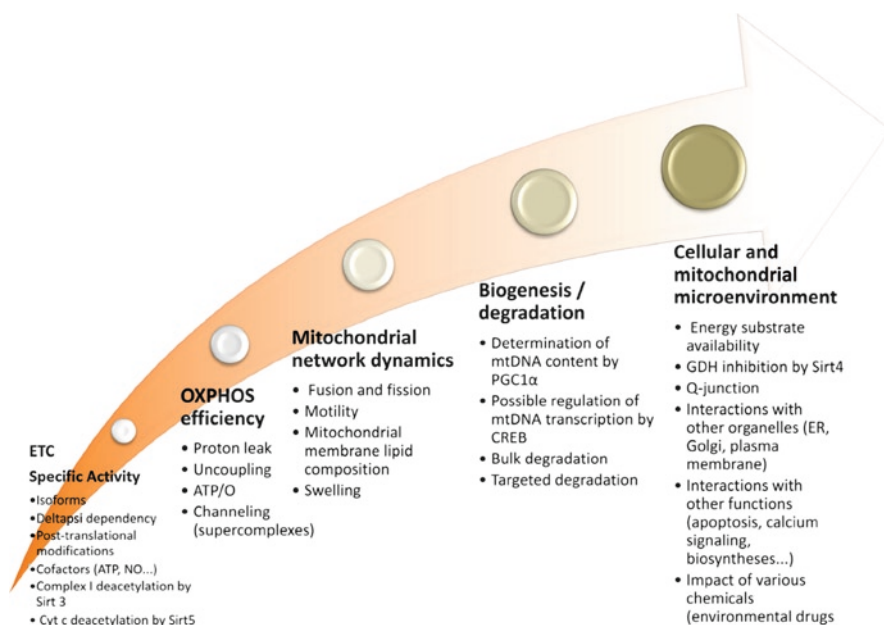
**Fig. 2.2** Mitochondrial respiratory chain. For mammals, the respiratory chain consists of four enzyme complexes (complexes I – IV) and two intermediary substrates (coenzyme Q and cytochrome c). The  $\text{NADH} + \text{H}^+$  and  $\text{FADH}_2$  produced by the intermediate metabolism are oxidized further by the mitochondrial respiratory chain to establish an electrochemical gradient of protons, which is finally used by the F1F0-ATP synthase (complex V) to produce ATP, the only form of energy used by the cell. In this simple representation of the respiratory chain, the supra-molecular organization (supercomplexes, dimers) is not shown

of energy used by the cell, and when produced in the mitochondrion it is exported to the cytosol by the adenine nucleotide translocator (ANT) in exchange for cytosolic ADP. Generally, the transport of energy metabolites, nucleotides and cofactors into and out of the mitochondrial matrix is performed by transporters located in the inner membrane (Palmieri et al. 1996). Mitochondria also contain “shuttle” systems that permit the transport of NADH.

## 2.3 The Regulation of Cellular and Mitochondrial Energy Production

In mammalian cells, energy homeostasis requires a constant coordination between cell activity, nutrient availability and the regulation of energy transformation processes. This is obtained via a complex system of signaling linking energy sensing and nutrient sensing to cellular effectors that include kinases and transcription factors. The AMP-activated protein kinases are activated upon alterations in the cellular AMP/ATP ratio, which is dictated by the balance between energy supply (ATP production) and energy demand (ATP consumption). When activated by AMP,

the AMPK initiate a cascade of phosphorylation to switch on the catabolic pathways that produce ATP (glycolysis, oxidative phosphorylation via the stimulation of mitochondrial biogenesis), and to switch-off the anabolic pathways that consume ATP (protein synthesis, fatty acid synthesis, cholesterol synthesis) (for review see (Hardie 2007)). More recently, it was discovered that AMPK further regulates energy metabolism through the activation of the sirtuin SIRT1 (Canto and Auwerx 2009; Canto et al. 2009) and the downstream modulation of SIRT1 targets that include the peroxisome proliferator-activated receptor- $\gamma$  coactivator 1 alpha (PGC1 $\alpha$ ), and the forkhead box O1 (FOXO1) and O3 (FOXO3a) transcription factors. In the present chapter, we will discuss how mitochondrial dynamics interacts with the regulatory pathway of energy metabolism governed by AMPK and sirtuins, and reciprocally. The regulation of mitochondrial energy production is concerted and multi-site (Benard et al. 2010). The different levels of OXPHOS regulation include (1) the direct modulation of respiratory chain kinetic parameters, (2) modulation of OXPHOS intrinsic efficiency by changes in the basal proton conductance or the induced proton conductance, (3) possible changes in the morphological state of the mitochondrial compartment (as discussed here), (4) modulation of mitochondrial biogenesis and degradation, and (5) *in situ* regulation of mitochondrial heterogeneity by the cellular and the mitochondrial microenvironment. Most of these regulatory mechanisms (Fig. 2.3) of mitochondrial energy production were discovered at the

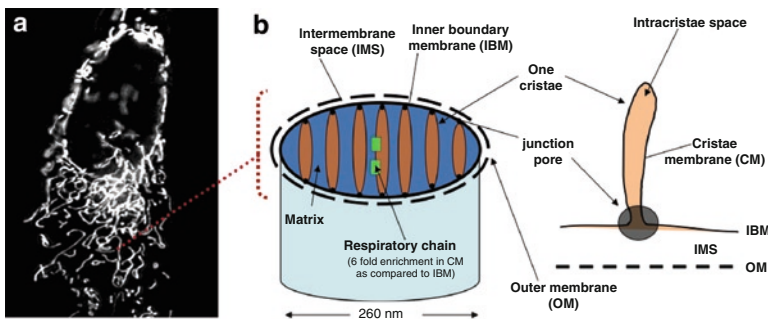


**Fig. 2.3** Multi-site regulation of mitochondrial oxidative phosphorylation. The modulation of OXPHOS capacity and activity occurs at different levels to adapt mitochondrial energy production to the cellular needs and environmental bioenergetic constraints

level of the respiratory chain and its surrounding lipid environment. Below, we discuss whether and how changes in mitochondrial fusion and fission impact these conserved mechanisms of bioenergetic regulation.

## 2.4 Mitochondrial Structure and Dynamics

In the past decade, the development of fluorescence microscopy has allowed the gathering of a three dimensional view of the mitochondrion in living human cells (Bereiter-Hahn and Voth 1994; Griparic and van der Bliek 2001; Yaffe 1999). In these studies, the mitochondria looked as an organelle that appeared more like a wide network of long tubules rather than a collection of small individual vesicles. In Fig. 2.4, we see the arborescence of the mitochondrial network in living human cells. Mitochondrial dynamics is performed by fusion proteins and fission proteins, which could reveal new possibilities for the control of mitochondrial energy production and cell viability (Benard and Karbowski 2009). In mammals, the proteins involved in mitochondrial fusion include the mitofusins MFN1 and MFN2, and OPA1 (different isoforms of OPA1 are generated by alternative splicing). SLP2 (Stoml2), MarchV, Bax and Bak interact with MFN2 to regulate fusion. In addition, different ATP-dependent or ATP independent proteases regulate fusion via OPA1 processing (Ehse et al. 2009). Low mitochondrial ATP levels, or the dissipation of the mitochondrial electric membrane potential across the inner membrane induce OPA1 cleavage by PARL and the matrix AAA (*m*-AAA) protease (Duvezin-Caubet et al. 2006). OMA1 mediates OPA1 processing if *m*-AAA proteases are absent or mitochondrial activities are impaired (Ehse et al. 2009). PISD and mito-PLD are proteins involved in the metabolism of phospholipid and could regulate mitochondrial



**Fig. 2.4** Mitochondrial structure. **(a)** Overview of the mitochondrial network in living human HeLa cells. The mitochondrial network was imaged by fluorescence microscopy (bi-photonic), using a matrix-targeted GFP. **(b)** Internal organization of the mitochondrial network; section of a tubule. This scheme illustrates the “cristae junction model” of mitochondrial interior. In this view, the pores could serve to regulate the release of cytochrome c during apoptosis

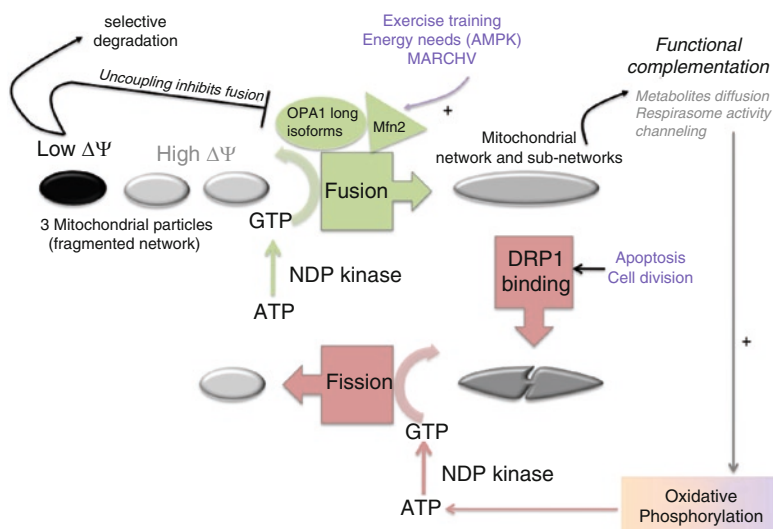
fusion via changes in membrane composition (Furt and Moreau 2009). The role of MIB is unclear, and might also participate to mitochondrial fusion via MFN2 binding. The proteins involved in mitochondrial fission include FIS1 and DRP1. Mitochondrial Fission Factor (MFF) also plays a role in mitochondrial fission. The regulators of fission include MARCHV, Mff, Bcl-w and different kinases required for the phosphorylation of DRP1. DRP1 can also be nitrosylated (see below). So far, the interaction between mitochondrial dynamics and mitochondrial energetics remain unclear.

To investigate the link between mitochondrial energy production and organellar shape changes, appropriate methods are needed to quantify changes both in mitochondrial form and function. Firstly, one can observe the shape of the mitochondrial network by epifluorescence or confocal microscopy in living cells using various fluorescent probes targeted to the mitochondrial matrix (GFPs, mitotrackers, TMRM...). For instance, inhibited fusion leads to a fragmented mitochondrial network while inhibited fission generates long tubules with signs of hyperfusion. The morphometric analysis of the mitochondrial compartment can be performed on microscopy images by using an automated computerized method to assess the length and branching degree of the mitochondrial particles (Koopman et al. 2005b). This method gives a quantitative evaluation based on the measurement of a form factor (combined measure of length and degree of branching), an aspect ratio (measure of length), and the overall content of mitochondria in the cell. In situations of altered fusion and fission (as occurs in neurological diseases caused by mutations in OPA1 and Mfn2, or DRP1, respectively) the viscosity of the mitochondrial matrix can also change (Benard and Rossignol 2008a; Koopman et al. 2008). Likewise, a test of mitochondrial fusion is available on cells containing different fluorescent proteins targeted to the mitochondrial matrix (matrix red fluorescent protein; mtRFP) or matrix green fluorescent protein (mtGFP). These two types of cells are fused with polyethylene glycol (PEG) to allow cell membrane fusion and the resulting polykaryons are analysed by confocal microscopy on the basis of their level of mtRFP and mtGFP colocalization. The mixing of green and red mitochondrial matrix-targeted proteins due to mitochondrial fusion leads to cells containing “yellow mitochondria” (Legros et al. 2002). Yet, the polyethylene glycol used for cell membrane fusion might also interfere with mitochondrial membrane fusion and perturbate the assay. Another possibility to measure mitochondrial fusion is to use mitochondrially targeted photoconvertible GFP (mito-Dendra) which changes color from green to red once activated by a blue laser. The mixing of the activated GFP with mitochondrial tubules generates yellow regions which can be counted as function of time to evaluate fusion, fission and transport (Koutsopoulos et al. 2010). Similar assays were also developed to investigate automatically the activity of mitochondrial fusion and fission (Jourdain and Martinou 2010). To further assess the activity of mitochondrial fission, it is also possible to use the so-called “CCCP-assay” which inhibits fusion and allows fission to proceed. The time required, or the CCCP amount needed to visualize fission gives a measure of its activity. This test is indirect and considers that OPA1 processivity is unchanged in the different conditions. This test was used in Ehses et al. (2009) and Ishihara et al. (2003, 2006).

## 2.5 Control of Mitochondrial Dynamics by Energy Metabolism (Hypothesis 1)

The possible control of mitochondrial fusion by energy state was first suggested by studies (Legros et al. 2002; Ishihara et al. 2003) showing that mitochondrial fusion is altered by the collapse of mitochondrial membrane potential ( $\Delta\Psi$ ). This was later partly explained by a  $\Delta\Psi$ -dependent cleavage of OPA1 (Duvezin-Caubet et al. 2006) and the subsequent inhibition of mitochondrial fusion. Fission can also be controlled by energy dependent processes since the phosphorylation of DRP1 by a cAMP-dependent kinase activates fission and promotes cell division. Hypothesis 1 (Fig. 2.5) was also inspired by a wide range of studies showing the alteration of cellular energy productions by the perturbation of mitochondrial fusion and fission (notably in different pathologies), as we reviewed previously in (Benard and Rossignol 2008b) and (Benard et al. 2010). Yet, the signals that mediate changes in energy state to the fusion-fission machinery remain poorly understood, and different possibilities can be proposed.

A conceivable hypothesis of a bioenergetic control of mitochondrial fusion and fission by the mitochondrial and/or the cellular energy states arise from the evidence that OPA1, MFN1, MFN2 and DRP1 require GTP to perform their mechanical activity. This idea was initially proposed by Petr Jezek in 2009 (Jezek and Plecita-Hlavata 2009; Jezek et al. 2009). The GTP formation by the Krebs cycle followed by GTP extrusion by the ANT could directly modulate the extent of mitochondrial fusion (Jezek and Plecita-Hlavata 2009; Jezek et al. 2009). The conversion of



**Fig. 2.5** Mutual link between mitochondrial bioenergetics and dynamics (schematic representation of hypotheses 1 and 2 described in the text)

ATP to GTP by nucleoside diphosphate kinases (NDP kinases) located either on the surface of the mitochondrion, or in the intermembrane space (Chen and Douglas 1987) might also play an important role in the bioenergetic control of these dynamins. The NDP kinase D (NM23-H4) was shown to be associated with the outer and inner mitochondrial membranes (Lacombe et al. 2000; Milon et al. 2000) and was not found in the soluble fractions. NDP kinase D and porin distribute similarly among the fractions, thus strongly suggesting that NDP kinase D is associated, like porin, with contact sites between the outer and the inner membrane. In addition to NDP-kinase D, intra-mitochondrial NDP kinases were found in pigeon. The Nm23-H6 partially colocalizes with mitochondria (Tsuiki et al. 1999).

Besides this possibility, the analysis of the different proteins involved in mitochondrial fusion or fission has evidenced post-translational modifications for DRP1, OPA1, and MFN2, which might also intervene in the modulation of mitochondrial dynamics and the subsequent control of OXPHOS capacity in response to cellular energy state and nutrients availability. First, DRP1 can be phosphorylated, ubiquitylated, nitrosylated and sumoylated. So far, the impact of these changes on mitochondrial energy production have not been investigated, and solely the effects on DRP1 recruitment to mitochondrial membrane, DRP1 turnover, fission activity or the sensitivity to apoptosis were analysed (Karbowski et al. 2007; Ishihara et al. 2003; Figueroa-Romero et al. 2009; Nakamura et al. 2006). The lowered expression of DRP1 by RNA interference induces the impairment of OXPHOS with a strong reduction of ATP synthesis capacity (Benard et al. 2007). Regarding OPA1, it is well documented that a loss of mitochondrial membrane potential (*i.e.* uncoupling by CCCP (Duvezin-Caubet et al. 2006)) triggers the cleavage of long isoforms of OPA1 to shorter forms, and reduces the ability for mitochondrial fusion (Legros et al. 2002). This cleavage can be performed by different m-AAA *proteases* which may or may not require ATP and can be controlled by prohibitins; the interaction of both the proteases and their regulators by the cellular energy states also remains to be clarified. How the energy state modifies their expression level and self-proteolytic state remains elusive. Likewise, 8 OPA1 isoforms result from alternate splicing of three exons (Ex4, Ex4b and Ex5b) and the impact of variable energy states on this pattern of OPA splicing is unknown. Alternative splicing is an important mechanism to create protein diversity and to regulate gene expression in a tissue- or developmental-specific manner. Interestingly, the activity of the spliceosome depends on the levels of ATP, since the PRP16 RNA-dependent ATPase is required for the second catalytic step of pre-mRNA splicing (Schwer and Guthrie 1992). The downregulation of OPA1 expression in human cells, or the occurrence of pathogenic mutations in this protein are associated with an alteration of OXPHOS functioning (Chen et al. 2005; Chevrollier et al. 2008; Olichon et al. 2003).

Similar results were obtained with Mfn2 downregulation in human cells or transgenic mice (Chen and Chan 2009). Again, it is still unknown how these changes in mitochondrial fusion and fission alter the mechanisms of energy production. Mfn2 can also be conjugated with MARCHV (Nakamura et al. 2006; Karbowski et al. 2007) to stimulate its fusion activity. Recent observations also describe a role for

MFN2 in controlling ATP/ADP exchanges, possibly through protein-protein interactions, which might offer an additional mechanism by which mitochondrial fusion could control mitochondrial energy production (Guillet et al. 2009). Lastly, Mfn2 was recently identified at the interface between the endoplasmic reticulum and the mitochondrion, with a possible role in the regulation of mitochondrial  $\text{Ca}_2^+$  uptake (de Brito and Scorrano 2008). Since matricial calcium can stimulate different mitochondrial dehydrogenases and transporters, which participate in energy production, Mfn2 might also be engaged in the regulation of mitochondrial energetics via calcium signalling. The hypothesis of a role for OPA1, DRP1 and MFN2 in the regulation of mitochondrial and cellular energy production is emphasized by the discovery of diseases caused by mutations in these proteins (see below). As discussed above, energy metabolism is regulated by different pathways involved in nutrient sensing, oxygen sensing or energy needs sensing. How these master regulators of energy metabolism interact with the mitochondrial fusion and fission proteins also remain to be investigated. The activation of Sirt1 by resveratrol stimulates AMPK activity and induces the upregulation of Mfn2 in neurons (Dasgupta and Milbrandt 2007). Likewise, in skeletal muscle, the expression of an active form of AMPK (transgenic mice Tg-AMPK $\gamma$ 3<sup>225Q</sup>) triggers the upregulation of MFN-2 (mitofusin 2), OPA-1 (dynamin-like GTPase-optic atrophy 1) and DRP-1 (dynamin-related protein 1) (Garcia-Roves et al. 2008).

## 2.6 Control of Mitochondrial Energetics by Organellar Dynamics (Hypothesis 2)

Theoretical studies on the regulation of oxidative phosphorylation in skeletal muscle mitochondria suggest that only a direct activation by “some cytosolic factors” of all oxidative phosphorylation enzymes is able to account for the large increase in  $\text{VO}_2$  and ATP turnover accompanied by only a very moderate increase in ADP concentration during the rest to work transition (Korzeniewski 2000; Korzeniewski and Mazat 1996). Yet, recent findings in mitochondrial physiology might allow to propose a hypothetical role for mitochondrial dynamics in the rapid regulation of OXPHOS output. Indeed, a recent study by the group of JC. Martinou evidenced a rapid stress-induced mitochondrial hyperfusion (SIMH), which is accompanied by an increase in mitochondrial ATP production (Tondera et al. 2009). This process involved the mitochondrial inner membrane protein SLP-2 and was dependent on mitochondrial oxidative phosphorylation since it was blocked in  $\text{Rho}^0$  cells that exhibited a normal mitochondrial membrane potential, but were unable to synthesize ATP. The SIMH was observed in situations of stress, as induced by actinomycin D, cycloheximide or UVs, which also induced a large increase in the cellular ATP content (220%, 160% and 120% of the untreated control, respectively). The increased ATP levels associated with SIMH could be attributed to a combined stimulation of OXPHOS and glycolysis, as well as a possible inhibition of the ATP consuming activities. Still, the mechanisms by which this

(hyper)fusion event could initiate an increase in mitochondrial and glycolytic ATP synthesis remain unexplained. It may be postulated that mitochondrial fusion could increase the speed of diffusion of energy metabolites (ADP, NADH) within the mitochondrial tubules, or that such conformational changes impact the respirasome organization and activate signalling cascades which remain to be deciphered. Likewise, the overexpression of mitofusin 2 improves the mitochondrial capacity to produce ATP and stimulates glucose oxidation (Bach et al. 2005). This could be explained by a higher fusion of the mitochondrial network, but also a stimulation of mitochondrial biogenesis, as Mfn2 plays a role in both processes (Zorzano et al. 2009a, b). Furthermore, MFN2 interacts with the proto-oncogene Ras, and Ras activity was associated with the modulation of mitochondrial respiration through the regulation of complex I (Baracca et al. 2010).

Hence, the interaction between Mfn2 and Ras could participate to the regulation of mitochondrial ATP production, but this point remains to be evaluated. Lastly, new insights into the critical relationships between mitochondrial dynamics and programmed cell death have demonstrated an interaction between Bax and Bak with Mfn2 (Karbowski et al. 2006) which might suggest that Bcl-2 family members may also regulate apoptosis through organelle morphogenesis machineries. Interestingly, Bcl2 can also regulate directly mitochondrial respiration, through the modulation of cytochrome c oxidase (COX) activity (Chen and Pervaiz 2009, 2007). A closer link between mitochondrial energetics and apoptosis could emerge in the coming years. In addition to Mfn2, the fusion protein OPA1 could also participate in the control of mitochondrial respiration since the removal of the *m*-AAA protease (which cleaves OPA1 in a  $\Delta\psi$ -dependent manner) limits the capacity of the oxidative phosphorylation system to supply ATP under conditions of high energy demand (Ehnes et al. 2009).

Hypothesis 2 (Fig. 2.5) also includes a control of mitochondrial dynamics by the cellular energy state via changes in mitochondrial membrane composition. Mitochondrial function relies fundamentally on organellar membranes properties, since chemiosmosis cannot exist without membrane impermeability to protons (Mitchell 1961), respiratory chain supercomplexes organization is conditioned by the lipid surrounding (Pfeiffer et al. 2003) and changes in mitochondrial membrane fluidity can alter bioenergetics (Benard et al. 2007; Benard and Rossignol 2008b, a; Aleardi et al. 2005). Thus, modifications of the phospholipid composition of mitochondrial membranes could participate to the control of energy production or apoptosis. For instance, it was shown that changes in organellar membranes fluidity alter both the functioning of OXPHOS and the overall dynamics of the mitochondrial network, in a mutual way (Benard et al. 2007). The control of the mitochondrial apoptotic pathway is also conditioned by changes in organellar membrane integrity (MOMP) and composition (Sandra et al. 2005; Newmeyer and Ferguson-Miller 2003; Wei et al. 2001; Kuwana et al. 2002). It remains to be shown whether changes in mitochondrial membrane composition induced by difference in energy state could contribute to the observed changes in mitochondrial network morphology. A study revealed that an ancestral member of the phospholipase D (PLD) superfamily of lipid-modifying enzymes is required for mitochondrial fusion by hydrolysing cardiolipin to generate phosphatidic acid (Choi et al. 2006). In this

study, the silencing of *MitoPLD* significantly reduced mitochondrial membrane potential and presumably associated mitochondrial energy production.

To conclude on hypothesis 2, only few observations are available regarding the stimulation of OXPHOS by an increase in mitochondrial fusion, while numerous reports indicate an impairment of mitochondrial respiration in situations of altered fusion or fission (Benard and Rossignol 2008b). Therefore, more evidences are needed to prove that mitochondrial fusion and fission proteins actively participate in the regulation of mitochondrial respiration.

## 2.7 Mitochondrial Fusion and Functional Complementation (Consequence of Hypothesis 2)

When two mitochondrial particles fuse, they can exchange materials that could permit compensation for a defective mitochondrial energy production at the cellular level. The study of Nakada et al. (2001) was the first to show the existence of mitochondrial genetic and functional complementation *in vivo* by analyzing the skeletal muscle of different transgenic mice containing various degrees of heteroplasmy (percentage of mutant mitochondrial DNA per cell). These authors never observed the coexistence of respiratory chain complex IV active and inactive mitochondria by histochemical staining within single muscle fibers despite the presence of 89% heteroplasmy. This might indicate that genetic complementation was attained in the muscle fibers, possibly via the inter-mitochondrial particles fusion and mixing of their content. During aging, the different proteins located in the mitochondrial network (as well as mitochondrial DNA molecules) can be progressively damaged by the high local concentration of reactive oxygen species (ROS), thus creating a heterogeneous situation where active DNAs and protein complexes could coexist with damaged ones along the tubules (heteroplasmy). In this case, the fusion of mitochondrial particles could allow functional complementation (Benard and Rossignol 2008b). Depending on the morphological state of the mt-network, the internal complementation can be variable. In the fragmented state, no exchange can occur between the different particles, creating an heterogeneous situation inside the cells. Therefore, reactions of fusion and fission could modulate the network organization and impact the capacity for compensating OXPHOS defects. Furthermore, we showed that such compensation exists until a threshold is reached (Rossignol et al. 1999, 2000, 2003) (upon strong inhibition of mitochondrial respiratory chain), and we also showed that mitochondrial fragmentation occurs once this threshold is reached (Benard et al. 2007). Accordingly, forced fusion of the mitochondrial network might allow a higher resistance (higher threshold) to a pathogenic respiratory chain impairment induced by a specific inhibitor of complex I. The mitochondrial threshold effect shows that large defects in the activity of respiratory chain complexes (induced by aging or pathogenic mutations) can occur in muscle or brain without triggering a reduction of the mitochondrial ATP production. We postulate that mitochondrial fusion and the exchange of materials such as energy metabolites and mitochondrial subunits could permit functional complementation.

The role of mitochondrial fusion in promoting intramitochondrial diffusion of metabolites could be important since studies indicate the existence of segmentation along the tubules that sequester the matrix into successive domains within the same tubule. This segmentation was observed in different analyses of mitochondrial interior physical continuity, using either JC<sub>1</sub> or photoactivable GFP, as well as FRAP experiments (Collins et al. 2002; Partikian et al. 1998; Twig et al. 2006, 2008a). The use of rosGFP1, or GFPpH also reveals zones of variable intensity in the mt-network of a same cell. In this case, the lipid nature of the intratubular barriers remains to be clarified, as well as their protein composition. The existence of internal barriers inside the tubule has important repercussions for the modalities of energy production, as they could abrogate the diffusion of substrates and metabolites required for oxidative phosphorylation. Different pre-fragmented domains could exist next to each other along the tubules, with differences in energy states, determined by the composition of OXPHOS complexes, the local concentration of substrates and activators of the energetic machinery, the variable volume of the different compartments (ICS and IMS), and the surrounding cytoplasm ATP needs. Thus, the idea of mitochondrial tubules operating as long uninterrupted electric cables might need to be revisited. Furthermore, it is also possible that the internal heterogeneity of mitochondrial tubule, in regard to OXPHOS activity, could be dictated by the local needs for ATP and the demand for oxygen (Mironov 2007; Mironov and Symonchuk 2006), but more studies are needed to validate this view. Mitochondrial networking could also play a role in genetic inter-mitochondrial particles complementation (Nakada et al. 2001), but this requires complete fusion and physical continuity. An alternative could be the existence of partial and transient sub-networks that will define larger functional domain. Twig et al. validated this view by using a matrix targeted photoactivable GFP (Twig et al. 2006). They showed that different subnetworks are coexisting and can undergo internal fission or fusion, without loss of their global architecture. Each network is defined by luminal continuity, equipotentiality, and boundaries which cannot be predicted without consideration of these parameters. This idea of subnetwork was previously proposed by the group of Dimitri Zorov that described the existence of *Clusters Formed by Chains of Mitochondria that were called Streptio mitochondriale* (Bakeeva et al. 1978). Taken together, these different observations permit the definition of four hierarchical levels of organization of the mitochondrion: the segment, the tubule, the sub-network and the global network. Each of these structures could play different roles in the synthesis and delivery of the vital ATP to various cellular areas, as well as to coordinate the different functions of the mitochondrion.

## 2.8 Mitochondrial Dynamics, Cell Cycle, and Organogenesis

The overall networked and ramified architecture of the mitochondrion could be conserved throughout species. In some eukaryotic microorganisms such as *Trypanosoma* the mitochondrion is typically observed as a single highly ramified organelle.

Likewise, the protozoan *Toxoplasma* present only one ramified mitochondrion. Yet, the comparison of the mitochondrial network in different cell types (Kuznetsov et al. 2009) as well as the study of its internal organization in muscle, heart, liver, kidney and brain taken from rats shows a large diversity in mitochondrial shape and organization which could be partly explained by differences in energy supply and demand, as well as a tissue-specific regulation of fusion and fission.

A link between the cell cycle and mitochondrial dynamics was evidenced in Hela cells, as the mitochondrial network becomes fragmented in early mitotic phase, and fused again in the daughter cells (Margineantu et al. 2002; Chang and Blackstone 2007). This process depends on Drp1 phosphorylation by Cdk1/cyclin. Mitochondrial dynamics might also play a determinant role during cell development as Campello S et al. showed that mitochondria specifically concentrate at the uropod during lymphocyte migration, by a process involving rearrangements of their shape (Campello et al. 2006). In this study, mitochondrial fission facilitated relocation of the organelles and promoted lymphocyte chemotaxis, whereas mitochondrial fusion inhibited both processes. In *Drosophila*, the polar granules are tightly associated with mitochondria in early embryos, suggesting that mitochondria could contribute to pole cell formation (Amikura et al. 2001a, b). It was even reported that mitochondrial large and small rRNAs are transported from mitochondria to polar granules prior to pole cell formation. Fujiwara S. and Satoh N. showed that during ooplasmic segregation, mitochondria are mainly distributed to the myoplasmic region in embryos of the ascidian *HaloCynthia roretzi* (Fujiwara et al. 1993). Recently, a transgenic mice model showed that brain-specific Drp1 ablation caused developmental defects of the cerebellum (Wakabayashi et al. 2009). Likewise, a primary culture of NS-Drp1(−/−) mouse forebrain showed a decreased number of neurites and defective synapse formation (Ishihara et al. 2009). Lastly, the removal of Mfn2 from the cerebellum in a transgenic mice model (Chen et al. 2007), triggered neurodegeneration caused by loss of mitochondrial fusion. In this model, during development and after maturity, Purkinje cells required Mfn2 (but not Mfn1) for dendritic outgrowth, spine formation, and cell survival.

## 2.9 Mitochondrial Dynamics, Energetics and Diseases

The recent discoveries of pathogenic mutations in genes essentials for fusion and fission of the mitochondrial network have complicated the physiopathological mechanisms of these diseases (Detmer and Chan 2007), and led to questions about the similarities between enzymatic defects in respiratory chain complexes versus the perturbation of mt-network dynamics. Why do they lead to similar clinical consequences, how do they interact, and what are the molecular and signaling bases that connect each other? Recent hypotheses in the field of mitochondrial physiopathology even consider that defects of the mitochondrial network could be the final cause of the disease, consequently to mutations affecting primarily the respiratory chain complexes. For instance, the work of Duvezin et al. (2007) analyzed the

fusion protein OPA1 in different cells models with a primary genetic defect in the respiratory chain complexes. These included cybrids created from a patient with myoclonus epilepsy and ragged-red fibers (MERRF) syndrome, mouse embryonic fibroblasts harboring an error-prone mitochondrial mtDNA polymerase gamma, heart tissue derived from heart-specific TFAM knock-out mice suffering from mitochondrial cardiomyopathy, and skeletal muscles from patients suffering from mitochondrial myopathies such as myopathy encephalopathy lactic acidosis and stroke-like episodes. They observed that dissipation of the mitochondrial membrane potential led to fast induction of proteolytic processing of OPA1 and concomitant fragmentation of mitochondria. Moreover, recovery of mitochondrial fusion depended on protein synthesis and was accompanied by resynthesis of large isoforms of OPA1. This could designate a novel mechanism by which the proteolytic processing of OPA1 could induce the fragmentation of energetically compromised mitochondrial segments. This could prevent the fusion of dysfunctional mitochondrial particle with the functional mitochondrial network. As discussed above, the relationships between mitochondrial energy production and mt-network organization are mutual (Benard et al. 2007). Hence, pathological mutations affecting either the respiratory chain, or the dynamic machinery, can both lead to OXPHOS deficiency and abnormal shaping of the mitochondrion. The observations of mt-network architecture in cells from patients with a genetic defect in respiratory chain show inconsistent abnormalities of the mt-network. The patterns of these changes can vary from one patient to another, as the clinical signs typically do. Most studies looking at these aspects concerned the complex I deficiencies, in human skin fibroblasts, since it is the most common cause of mitochondrial diseases. The group of Robinson B. analyzed the mitochondrial structure and motion dynamics in living cells with energy metabolism defects by real time microscope imaging (Pham et al. 2004). They concluded that skin fibroblasts from patients with mitochondrial complex I deficiency and normal fibroblasts treated with rotenone, or antimycin A, contained higher proportions of mitochondria in the swollen filamentous forms, nodal filaments, and ovoid forms rather than the slender filamentous forms found in normal cells. They also reported a decreased motility with more ovoid mitochondrial forms compared to the filamentous forms. Likewise, it was shown that when complex I activity was chronically reduced by 80% in human skin fibroblasts, using rotenone treatment, the percentage of moving mitochondria and their velocity decreased by 30% (Koopman et al. 2007). It was proposed that ROS generated by the ETC in pathological situations could be responsible for such observed changes in mt-network organization. To test this hypothesis, Werner Koopman and Sjoerd Verkaart developed a fine and reliable method for the simultaneous quantification of oxidant levels and cell spreading (Koopman et al. 2005a, b, 2006a, b), based the monitoring of CM-H<sub>2</sub>DCF conversion into DCF upon intracellular oxidation, by video-rate confocal microscopy. After an extensive validation of their protocol, these authors looked at the ROS steady-state levels in fibroblasts taken from patients with a complex I deficiency and demonstrated a 2.5-fold higher oxidative stress in these cells. In another study, the same group demonstrated that superoxide production is increased in complex I deficient cell lines, in proportion

to the enzymatic activity decrease. Interestingly, the redox state of these cells remained unaffected, even though oxidative stress was higher (Koopman et al. 2005b). Lastly, they analyzed the consequence of complex I deficiency on the mitochondrial network organization and observed a higher branching and elongation of the tubules, qualified as “mitochondrial outgrowth” (Koopman et al. 2005a).

The questions arise about the importance of mt-network adaptations for the diagnosis of mitochondrial diseases. This could allow a first check as an easy test on blood cells, with a rapid mitochondrial staining and direct visualization on a fluorescent microscope. All OXPHOS defects will not be identified this way (Guillery et al. 2008; Benard et al. 2007), but large deficiencies could be observed at the level of the mt-network, owing that the cell type under consideration expresses sufficiently the metabolic defect. It was reported that an heterozygous, dominant-negative mutation in the dynamin-like protein 1 gene (DLP1) could cause a pathological conditions associated with microcephaly, abnormal brain development, optic atrophy and hypoplasia, persistent lactic acidemia, and a mildly elevated plasma concentration of very-long-chain fatty acids. The DLP1 protein is involved in the fission of mt-network tubules, and we showed that its absence can impair the synthesis of ATP by the mitochondrion (Benard et al. 2007). Thus, the mitochondrial network organization is a new parameter that must be taken into account for physiopathological analyses of mitochondrial diseases.

The possible interaction between mitochondrial dynamics and energy production could offer an opportunity for the discovery of drugs that target mitochondrial fusion or fission with a stimulatory effect on energy metabolism, to the benefit of patients suffering from obesity, diabetes and mitochondrial/neuromuscular diseases (Zorzano et al. 2009a, b). Defective fusion or fission are the cause of rare human neurological disorders such as Charcot-Marie Tooth type 2a, Autosomal Dominant Optic Atrophy, Lethal Defect of Mitochondrial and Peroxisomal Fission, Spastic Paraplegia or a dominant form of spino-cerebellar ataxia, SCA28 (defective proteins MFN2, OPA1/3, DRP1, Paraplegin or AFG3L2 respectively), the pathophysiological molecular mechanisms of which still remain unknown. Recent evidence also suggest that mitochondrial dynamics is impaired in Parkinson’s disease, and that Parkin could be involved in the selective degradation of altered mitochondrial particles (Narendra et al. 2008, 2009). Twig and colleagues proposed a life cycle of mitochondrial particles where regions of the mitochondrial network with low membrane potential could be selectively targeted and brought to degradation (Twig et al. 2008a, b), presumably through a Parkin-mediated mechanism. In Alzheimer’s disease, elevated levels of S-nitrosylated Drp1 were found in brain samples from AD patients and AD mouse models, along with extensive mitochondrial fragmentation (Cho et al. 2009). For all of the above listed diseases, the impairment of mitochondrial energy production as observed in fibroblasts taken from patients with OPA1 or MFN2 mutations still remain unexplained at the molecular level.

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