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Energizing Genetics and Epi-genetics: Role in the Regulation of Mitochondrial Function

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Abstract: Energy metabolism and mitochondrial function hold a core position in cellular homeostasis. Oxidative metabolism is regulated at multiple levels, ranging from gene transcription to allosteric modulation. To accomplish the fine tuning of these multiple regulatory circuits, the nuclear and mitochondrial compartments are tightly and reciprocally controlled. The fact that nuclear encoded factors, PPARγ coactivator 1α and mitochondrial transcription factor A, play pivotal roles in the regulation of oxidative metabolism and mitochondrial biogenesis is paradigmatic of this crosstalk. Here we provide an updated survey of the genetic and epigenetic mechanisms involved in the control of energy metabolism and mitochondrial function. Chromatin dynamics highly depends on post-translational modifications occurring at specific amino acids in histone proteins and other factors associated to nuclear DNA. In addition to the well characterized enzymes responsible for histone methylation/demethylation and acetylation/deacetylation, other factors have gone on the "metabolic stage". This is the case of the new class of α-ketoglutarate-regulated demethylases (Jumonji C domain containing demethylases) and of the NAD^+-dependent deacetylases, also known as sirtuins. Moreover, unexpected features of the machineries involved in mitochondrial DNA (mtDNA) replication and transcription, mitochondrial RNA processing and maturation have recently emerged. Mutations or defects of any component of these machineries profoundly affect mitochondrial activity and oxidative metabolism. Finally, recent evidences support the importance of mtDNA packaging in replication and transcription. These observations, along with the discovery that non-classical CpG islands present in mtDNA undergo methylation, indicate that epigenetics also plays a role in the regulation of the mitochondrial genome function.

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1. INTRODUCTION

Energy metabolism and mitochondrial function hold a core position in cellular homeostasis. The thorough analysis of several eukaryotic genomes and the experimental tools/techniques developed in the last twenty years have opened unprecedented opportunities in this field. Understanding the multifaceted aspects of energy metabolism is an obligate step to gain insights into the pathogenesis of metabolic disorders, i.e. diabetes, obesity, etc. Moreover, metabolic implications have recently emerged in other fields, such as tumoral proliferation, inflammation, development, stem cell biology and aging [1, 2].

The aim of the present review is to provide an updated survey of the genetic and epigenetic mechanisms involved in the control of energy metabolism and mitochondrial function.

1.1. Overview on Mitochondria

Since the first observation of their existence in the middle of the 19th century, mitochondria stimulated a great deal of interest in cell biologists. After the postulation of the chemiosmotic theory by Mitchell in 1961 [3], our understanding of mitochondrial physiology has evolved greatly. The current view of these fascinating organelles is of an intricate and dynamic network that controls a wide range of cellular functions. Mitochondria are deeply involved in numerous cellular processes, such as steroidogenesis, apoptosis to name a few, and, they also exert a tight control on energy metabolism. In fact, many metabolic pathways such as the tricarboxylic acid cycle (TCA), fatty acid β-oxidation, amino acid oxidation and oxidative phosphorylation (OXPHOS) are localized in mitochondria. The importance of these organelles is also underscored by the fact that mitochondrial dysfunction is associated with or causative of several disorders, such as type 2 diabetes [4], neurodegenerative diseases, Parkinson disease [5] and cancer [6].

Originating from a α-proto bacterion, mitochondria maintain almost all of their primordial bacterial shape: two membranes, the inner (IMM) and the outer (OMM) mitochondrial membranes, separated by an intermembrane space. The inner space is filled with the mitochondrial matrix. Text-book illustrations have depicted mitochondria as bean-shaped organelles freely fluctuating in the cytoplasm. However, advanced imaging techniques coupled to genetic manipulations

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have clearly demonstrated that they exist as a tangled network that interacts dynamically with the cytoskeleton to reach and sustain energy metabolism in every corner of the cell [7]. Such a dynamic network undergoes continuous rearrangement, due to fission and fusion events [4], deeply influencing the efficiency of the electron transport chain (ETC) and the overall mitochondria function [8].

Besides their ostensible simple structure, mitochondria also inherit a little genome from their bacterial ancestors, the mitochondrial DNA (mtDNA). mtDNA copy number can vary from 1.000 to 10.000 in each cell. The mitochondrial genome is a double strand DNA sequence with a length ranging from 14,000-17,000 base pairs. It encodes only 13 proteins, all belonging to the hydrophobic portions of some of ETC complexes, 22 tRNAs (mt-tRNAs) and only 2 rRNAs (mt-rRNAs) [4]. Unlike the nuclear DNA (nDNA), mtDNA is circular, and lacks telomeres and intronic /intergenic portions. In addition mtDNA displays different GC content, therefore the two strands of the mtDNA are termed heavy strand (HS, enriched in G) and light strand (LS, enriched in C); the mtDNA also contains a non-coding region containing the displacement loop (D-loop) and displaying control functions in replication and transcription. Replication and transcription of the mitochondrial genome are under the control of several nuclear-encoded proteins [4, 9]. The very well characterized mitochondrial transcription factor A (Tfam) is the most relevant regulator of mtDNA replication and transcription whose expression is, in turn, controlled by other nuclear-encoded transcription factors [4, 9, 10].

In contrast to the limited coding capacity of mitochondria, the set of mitochondrial proteins encoded by the nuclear genome, accounts for 1500 genes, as recently emerged from an extensive analysis of the mitochondrial proteome [11]. The great disparity between mitochondria-encoded proteins and the mitoproteome highlights the unquestionable relationship between mitochondria and nucleus. Nevertheless, at present, a complete view of the signaling pathways operating between the two organelles is still lacking.

1.2. Epigenetics: Basic Concepts

In eukaryotic cells DNA and histones form nucleosomes that build up chromatin. Chromatin is a dynamic structure that can be accessible and transcriptionally active (euchromatin) during gene transcription or condensed and transcriptionally less active (heterochromatin) when transcription is quenched [12].

Epigenetics is the study of short or long-term modifications of chromatin structure. Unlike genetics that is based on DNA sequence alterations, epigenetics consists of a variety of chemical reactions including post-traslational modifications on histones or the addition of methyl groups to DNA mostly on CpG sites.

DNA methylation and histone modifications are the main epigenetic modifications [13]. Post-translational acetylation, methylation, ADP-ribosylation and phosphorylation can occur on amino acid residues on the histone tails, modifying their chemical structure [14]. These histone modifications are part of the "histone code" that, by influencing chromatin condensation status [15], regulates gene transcription (Fig. **1**). Histone modifications can be activating or repressing: acetylation of histone H3 and H4 is associated with active transcription, whereas deacetylation compacts chromatin and makes it less accessible to transcriptional coactivators [16]. Histone methylation can play activating or silencing role depending on the target residues [17].

In the following sections, we will summarize current knowledge on the implications of epigenetics in the regulation of energy metabolism, with an emphasis on mitochondria. We will also address the role of sirtuins that, although not directly involved in the post-translational modifications of histones, profoundly affect gene transcription and/or the activity of mitochondrial proteins.

Finally, we will discuss the classical and emerging mechanisms governing mitochondrial functions, from nuclear regulation of mtDNA transcription and replication, to the regulation of mtRNA stability and mitoepigenetics.

2. EPIGENETIC REGULATION OF THE NUCLEAR GENOME AND ITS RELEVANCE TO ENERGY METABOLISM

2.1. Histone Methylation/demethylation

The enzymes responsible for the methylation of histones are termed methyltransferases; they modify one single lysine or arginine residue on a single histone protein and their output can be either activation or repression of transcription [17] (Fig. **1B**).

Lysine methylation sites implicated in activation of transcription are lysines at position 4, 36 and 79 of histone 3 (H3K4, H3K36, and H3K79), while lysine methylation sites linked to transcriptional repression are H3K9, H3K27, and H4K20 [17].

The histone methyltransferases (HMTs) can be grouped into three classes: SET domain lysine methyltransferases, non-SET domain lysine methyltransferases and arginine methyltransferases. All three classes use S-adenosylmethionine (SAM) as coenzyme to transfer methyl groups [18]. The SET-domain class is the best characterized group of methyltransferases and has been reported to correlate with metabolic disorders [19, 20].

Lysine residues methylation is a dynamic process since, in last decades, the existence of different histone demethylases (HDMs) has been demonstrated. Lysine-specific demetylase 1 (LSD1) was the first histone demethylase that was discovered [21]. This enzyme, demethylating H3K4, represses transcription [21]. On the other end, by forming a complex with the androgen receptor it can also demethylate H3K9 and activate transcription [22]. LSD1 is a FAD-dependent oxidase, composed of two domains: a FAD-binding and a substrate-binding domain [23]. Different multiprotein complexes recruits LSD1 to its target, where it can demethylate mono- and di-methylated H3K9 or K4, leading to transcriptional activation or repression [21, 22].

More recently, another demethylase domain, the *Jumonji* C (JmjC) motif, was described; JmjC-domain-containing proteins were identified as transcriptional coregulators involved in the demethylation of H3K9 [24]. There are five

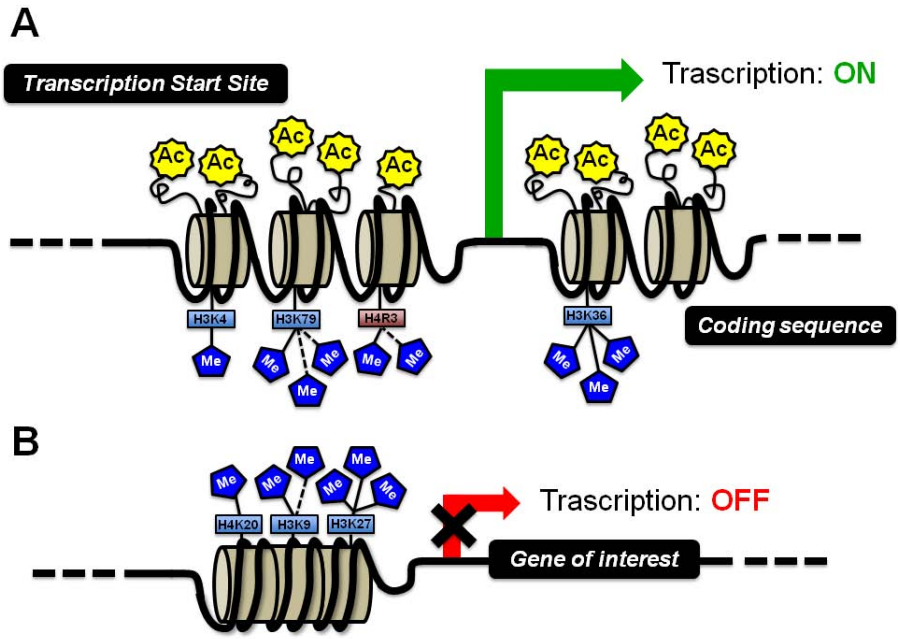

Fig. (1). Specific histone modifications that promote chromatin compaction or relaxation.

Histone acetylation and methylation at specific lysine or arginine residues (H3K4, H3K79, H3K36 and H4R3) opens chromatin and activates gene transcription (panel A). On the other hand, histone methylation at H4K20, H3K9 and H3K27 silences gene transcription (panel B).

subfamilies of JmjC lysine demethylases: JHDM1, JHDM2, JMJD2, JMJD3, and JARID1 [24]. Another class of HDM is JMJD6, which specifically demethylates methylated arginine residues such as H3R2me2 and H4R3me2. The identification of this HDM demonstrates that also arginine methylation is reversible [25]. JmjC-domain containing proteins display a α-ketoglutarate (α-KG) /Fe^{2+}-dependent dioxygenase activity: α-KG stabilizes the enzyme/substrate complex, binds the catalytic iron center and is oxidatively decarboxylated thus generating succinate [18].

2.2. Histone Methylation is Related to Cellular Energy Status

The activity of HMTs and HDMs depends on specific metabolic coenzymes [24] and their biosynthesis is related to intracellular ATP levels. SAM required for histone methylation is obtained by the reaction occurring between methionine and ATP catalyzed by S-adenosyl methionine transferase (MAT) [15]. Recently it has been shown that MAT is localized in the nucleus, where it controls the local SAM supply, and its presence correlates with H3K27 trimethylation [26]. Interestingly, Chiang and colleagues reported that high intracellular energy levels increase SAM concentration, with a consequent enhancement of DNA methyl transferase activity, leading to a higher global DNA methylation [27]. However, it has not been clarified yet whether HMT activity and histone methylation can be affected by SAM abundance in the cell.

HDMs (LSD1 or *Jumonji* domain classes) require flavin adenine dinucleotide (FAD) [23] and α-KG [24] to catalyze demethylation reaction. For this reason also histone demethylation is strictly dependent on energy status: ATP produced by intracellular metabolism of nutrients is the key molecule for biosynthesis of FAD and α-KG. In fact riboflavin kinase (RFK) catalyzes the conversion of riboflavin into flavin mononucleotide (FMN) that is then converted to FAD by FAD-synthase (FLAD). RFK and FLAD are ATP-dependent enzymes, which explain the close dependence of LSD1-mediated Lys-methylation on the energy homeostasis. Mitochondria play a central role in energy metabolism of cells since they usually provide most of the ATP by oxidative phosphorylation. The oxidative processes occurring in cells degrade fuel molecules yielding NADH and $FADH_2$, which are used as electron donors for the electron transport chain [28]. Also α-KG, a cofactor of the JmjC class of HDMs, is linked to oxidative metabolism since it is a key intermediate in the TCA cycle; therefore, fluctuation of its level may reflect mitochondrial function. α-KG is converted to succinyl-CoA by the α-ketoglutarate dehydrogenase in the presence of FAD and NAD, while $FADH_2$, NADH and succinyl-CoA inhibit the reaction. α-KG is indeed a powerful regulator of both DNA and histone methylation through the control of Ten eleven translocation methylcytosine dioxygenase (TET) and Jmj C domain containing demethylases, underlying that these epigenetic processes could be related to the oxidation of metabolic substrates in mitochondria.

Because aging is strictly related to metabolism [29], a role of HMTs/HDMs in these processes has been proposed recently [30]. At this regard, it was reported that histone H3 demethylase Ndy1/KDM2B protects cells from replicative senescence [31]. Senescence is related to abnormal production of reactive oxygen species (ROS) [32], strictly linked to mitochondrial metabolism [33]. ROS are produced in living cells by a variety of mechanisms: electrons escaping from the respiratory chain in mitochondria target oxygen to form superoxide anion [34]; NADPH oxidase and related enzymes produce superoxide anion upon activation, and nitric oxide synthase produces superoxide anion, $NO^{\cdot}$, and H_2O_2 [35].

Polytarchou and colleagues [31] showed that the JmjC domain histone demethylase Ndy1 protects from H_2O_2-induced apoptosis and inhibits ROS-mediated signaling and DNA damage. On the other hand, knockdown of Ndy1 yielded opposite effects, confirming a further relationship between histone methylation and mitochondrial function.

Despite these recent evidences a solid link between histone methylation and oxidative metabolism is currently missing therefore some key questions still remain open: i) how do physiological and developmental changes impact the levels of metabolic coenzymes?; ii) how can variations in the level of these molecules act as energy signals for transcriptional regulation mediated by HMTs and HDMs [15]? Metabolic intermediates, such as TCA cycle metabolites, can be considered ideal messenger molecules in the signal transduction from the mitochondria to the nuclei [30]. Nonetheless, the mechanism by which HMTs and HDMs, enzymes localized in the nucleus, capture molecules produced in mitochondria or in the cytoplasm is still unclear.

2.3. Histone Methylation/demethylation and Metabolic Disorders

In recent years, a number of studies demonstrated the role played by specific HMTs or HDMs in the onset of severe metabolic diseases. Lee and colleagues [36] reported that deletion of MLL3, a SET-domain HMT, leads to a lower accumulation of white adipose tissue, with a concomitant amelioration of metabolic phenotype. Villenueve and collaborators [37] investigated the role of LSD1 in the vascular complications of diabetes, describing higher inflammation and atherogenesis in vascular smooth muscle cells derived from *db/db* mice. The authors found an increase in the H3K4 dimethylation mark (activating transcription) on the promoters of the inflammatory genes monocyte chemoattractant protein 1 and interleuchin 6, concomitant with a reduced LSD1 recruitment to these regions. Moreover, they reported that treatment of cultured human vascular smooth muscle cells with high glucose recapitulated the same effect suggesting that glucose itself is able to increase expression of inflammatory genes. This evidence could strengthen the hypothesis of a direct correlation between energy intake and histone methylation.

In 2009, two different studies reported the key role of histone demethylase JHDM2a in metabolism, showing that JHDM2a null mice develop obesity and hyperlipidemia [38, 39]. At this regard, Tateishi and collaborators demonstrated that *Jhdm2a* expression was induced by β-adrenergic stimulation [39], a signal involved in cold-induced thermogenesis in brown adipose tissue (BAT) [40]. Jhdm2a was shown to directly regulate the expression of BAT markers, such as the Uncoupling protein 1 (*Ucp1*), and of the peroxisome proliferator-activated receptor α (*Ppara*). Therefore the authors concluded that obesity observed in JHDM2a knockout mice is linked to impaired BAT function. On the other hand Inagaki and colleagues did not detect impairment of brown fat functionality and demonstrated that obesity onset is related with reduced expression in white adipose tissue of a set of genes including the anti-adipogenic transcription factor *COUP-TFII*, the inhibitor of fat storage *ApoC1*, and the insulin-dependent glucose transporter *Glut4* [38].

However, further studies are needed to elucidate the molecular basis underlying the metabolic effect mediated by histone methylation/demethylation. The identification of new pathways regulating energy homeostasis may represent a new target for the development of future interventions in metabolic disorders.

2.4. Chromatin Acetylation and Deacetylation

Biological acetylation of histone tails is one of the most studied and common forms of chromatin modification [17]. It occurs at the ε-amino group of internal lysine residues and is a critical step in gene regulation processes because of the dramatic changes in chromatin structure. The acetylation state of chromatin is regulated by two families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs) that dynamically modulate chromatin packaging [41]. HATs transfer an acetyl group from acetyl-CoA to the ε-amino group of lysine residues, masking their positive charge. This results in chromatin relaxation and increases accessibility of transcriptional activators; on the contrary, HDACs promote the removal of the acetyl groups, therefore they compact chromatin and silence gene transcription [42]. Notably, HDACs also act on transcription factors and cofactors, thus regulating their activity [43]. In the last decades, thanks to the evolving proteomic techniques, a great number of proteins bearing acetylated lysines, including many mitochondrial proteins, have been discovered. In fact, in tissues rich in mitochondria, such as skeletal muscle, brown fat and heart, many proteins belonging to the TCA cycle and to ETC undergo acetylation [44].

Eukaryotic HDAC family consists of 18 members, grouped in two subfamilies of proteins exhibiting different deacetylating activity: HDACs and sirtuins [45].

Eleven zinc-dependent enzymes belong to the canonical mammalian HDACs subfamily and they carry out their deacetylating activity by means of a nucleophilic attack of water on the acetylylated lysines; histones bearing non acetylated lysines wrap DNA more tightly (Fig. **1A**). HDACs are classified into three classes based on their homology with yeast HDACs [46]. Class I, closely related to the yeast transcriptional regulator Rpd3, consists of HDAC1, 2, 3, and 8, which are ubiquitously expressed and mainly localized in the nucleus. Class II displays high homology with the yeast Hda1 [47] and includes HDAC4, 5, 6, 7, 9, and 10; these members are found in the cytoplasm but they can translocate into the nucleus in response to specific cellular signals and depending on their phosphorylation status [48]. HDAC11 is the only member of class IV and its role is still not clear [49]. Class III HDACs are related to the yeast orthologous silent information regulator 2 (Sir2), therefore, they are also known as sirtuins. In contrast to the other HDACs, sirtuins are NAD^+-dependent deacetylases which transfer an acetyl group from the lysine residues of a substrate to NAD^+ [50]. Indeed, they are considered metabolic intracellular sensors because sensitive to changes of the NAD^+/NADH ratio [51]. Sirtuins are involved in the regulation of several functions, such as metabolism, lifespan, cellular stress [52, 53] and are central players in mitochondrial homeostasis [54, 55]. In mammals there are seven sirtuins, grouped in four classes: Class I (SIRT1, SIRT2 and SIRT3), Class II (SIRT4); Class

III (SIRT5) and Class IV (SIRT6 and SIRT7) [56]. They show different localizations: SIRT1 and SIRT2 are able to shuttle from cytoplasm to nucleus [57, 58]; SIRT3, SIRT4 and SIRT5 localize in mitochondria and, among them, SIRT3 is the main mitochondrial deacetylase. The pivotal role of SIRT3 is demonstrated by the fact that SIRT3 knockout mice show higher level of protein acetylation, compared to mice lacking either SIRT4 or SIRT5 [59]. SIRT6 is mainly localized in the nucleus while SIRT7 is nucleolar.

As discussed above, the repressive effect mediated by HDAC activity is counteracted by the action of HATs. They consist of five families [60]: the GNAT (GCN5-related N-acetyltransferase) family is characterized by a carboxy-terminal bromo-domain and an acetylase activity even on non-histone proteins [61]. GCN5 (general control of amino acid synthesis 5) represents the most studied member of this class. The MYST family comprises five human HATs, Tip60, MOZ, MORF, HBO1 and MOF, containing a highly conserved MYST domain composed of an acetyl-CoA binding motif and a zinc finger [62]. The members of the MYST family participate in the formation of transcriptional co-activator complexes and, being involved in several nuclear pathways, their dysfunction or inactivation is linked to several diseases such as cancer [63]. The p300/CBP (CREB-binding protein) family is characterized by an acetyl-CoA binding domain, three zinc finger regions and a bromo-domain. P300/CBP can interact with several transcription factors [64] acting as co-activators. Another family of HATs is the basal transcription factor family, which is related to mammalian TAFII250, the largest subunit of the transcription factor complex TFIID2 [65]. The fifth HAT family is the nuclear receptor cofactors family, which includes nuclear receptor co-activators such as steroid receptor co-activators (SRC1) and clock circadian regulator (CLOCK) [66, 67]. In 2014 Karmodiya *et al.* [68] identified a novel family of HATs, namely Camello proteins, holding the HAT domain and showing specificity towards histone H4 acetylation.

2.5. HDACs Affect Mitochondrial Function and Energy Metabolism

The environment and the nutritional state can affect chromatin structure and cause abnormalities in epigenetic regulation associated to metabolic disorders, such as obesity, insulin resistance, diabetes, cancer, and altered apoptotic pathways [69]. Notably, these pathological conditions are often linked to defects in oxidative metabolism. In this respect, a growing number of studies provided further evidences on the involvement of HDACs in the regulation of energy metabolism.

In particular, a study published by Gao and coworkers [70] pinpointed the involvement of class I and class II HDACs in the control of energy expenditure. The authors reported that sodium butyrate, a dietary component that inhibits class I and II HDACs, improved the metabolic dysfunction in mice fed a high-fat diet, increasing energy expenditure and fatty acid oxidation. As a result, treatment with sodium butyrate prevented diet-induced obesity and insulin resistance. The beneficial effects of sodium butyrate occurred at the level of brown adipose tissue and skeletal muscle. In fact treated mice showed enhanced adaptive thermogenesis, reduced fat accumulation and brown adipocytes size and a greater number of oxidative fibers, coupled with enhanced fatty acid oxidation and mitochondrial function [70]. Since sodium butyrate *in vivo* activates PGC-1α, a master regulator of mitochondrial biogenesis, HDAC inhibition may induce its transcription. However other evidences showed that butyrate affected energy metabolism via HDAC-independent mechanism [71]. Recently, our laboratory contributed to unravel the role of HDACs in energy expenditure. Indeed we observed that a class I selective HDAC inhibitor induced PGC-1α expression, increased mitochondrial biogenesis and oxygen consumption in cultured myotubes and primary brown adipocytes [72]. More interestingly, treatment with the class I HDAC inhibitor in *db/db* mice promoted energy expenditure, enhancing oxidative metabolism in skeletal muscle and adipose tissue, consequently reducing body weight and improving insulin resistance. This phenotype is mediated by increased PGC-1α action in skeletal muscle and enhanced PPARγ/PGC-1α signaling in adipose tissue, due primarily to HDAC3 inhibition [72].

To corroborate the role of HDACs in energy metabolism, in 2011 Yamamoto *et al.* [73] pointed out that NCoR1 regulates muscle mass and its oxidative capacity. Nuclear Receptor Corepressor 1 (NCoR1) is a transcriptional corepressor that forms repressive complexes with HDAC3, HDAC4, 5, 7 and 9 [74]. Yamamoto *et al.* observed that muscle-specific NCoR1 knockout mice (NCoR1skm$^{-/-}$) exhibit increased muscle mass, locomotor activity and oxygen consumption, with a reduction in the respiratory exchange ratio, meaning that fat is their preferential energy source [73]. Consistent with these evidences, mice lacking NCoR1 showed increased mitochondrial content and activity in the gastrocnemius, reduced number of glycolytic MyHC2b-positive fibers and increased number of oxidative MyHC2x- and 2a- positive fibers. Interestingly, these authors showed that the absence of NCoR1 favored the acetylation and activation of mediated by p300/CBP (Fig. **2A**). These events ultimately result in induction of MEF2 target genes and increased muscle mass, coupled with induction of genes involved in mitochondrial respiration and fatty acid catabolism.

NCoR1 also plays a central role in adipose tissue [75]. In this tissue it acts as corepressor in multi-protein coregulatory complexes at the promoters of PPARγ target genes, regulating in turn PPARγ-dependent functions, such as adipocyte differentiation, insulin sensitivity and adipokine secretion [76-78]. In epididymal white adipose tissue of NCoR1 AKO (adipocyte-specific NCoR1 knockout) mice, the authors detected increased expression of typical adipogenic genes [75].

Another evidence of the importance of HDACs as metabolic regulators is that they also play a role in cardiac lipid metabolism. Postnatal deletion of HDAC3 in heart and skeletal muscle did not affect survival when mice were fed normal chow [79]. On the contrary, when fed a high fat diet, mice lacking HDAC3 in heart and skeletal muscles displayed signs of severe hypertrophic cardiomyopathy and heart failure. Decreased expression of myocardial mitochondrial bioenergetic genes, specifically those involved in fatty acid oxidation and lipid metabolism, led to cardiomyopathy and death. These data indicate that HDAC3 deletion impedes cardiac mitochondria response to nutritional changes [79].

2.6. Sirtuins and Energy Metabolism

A large number of studies highlighted the crucial role of sirtuins in the control of the cellular energy status in mammals. Under calorie restriction, the reduced availability of nutrients causes decrease of oxidative pathways and an increase of NAD^+/NADH ratio that activates sirtuins; therefore, their activity can be considered strictly linked to lipid and glucose metabolism. One of the main sensors of energy status is SIRT1: during fasting, in the liver, activated SIRT1 deacetylates PGC-1α that, consequently, transactivates the transcription of gluconeogenetic genes [80]. Consistent with these evidences, SIRT1 knockdown in fasted liver led to reduced glucose production and fatty acid oxidation. These effects were due to decreased expression of gluconeogenesis and fatty acid oxidation genes, secondary to 1α inhibition [81].

SIRT1 also enhances hepatic fatty acid oxidation and inhibits hepatic lipogenesis by the activation of the AMPK/LKB1 signaling [82]. Moreover, in the liver SIRT1 positively regulates PPARα, as in mice bearing liver-specific deletion of SIRT1, PPARα signaling was impaired leading to decreased fatty acid β-oxidation [83]. Moreover, during fasting, deficiency of SIRT1 causes reduced mobilization of free fatty acid from white adipocytes [84].

Another SIRT1 target is the transcription factor FOXO1 that, if deacetylated, regulates the expression of adipose triglyceride lipase gene (Atgl), the rate-limiting lipolytic enzyme [85]. It has been recently demonstrated that SIRT1, by deacetylating PPARγ, induces "browning" of subcutaneous white adipose tissue (WAT) due to the recruitment of the BAT program coactivator PR domain containing 16 (PRDM16) by PPARγ. As a consequence, BAT specific genes are induced while visceral WAT marker genes, typically associated with the onset of insulin resistance, are repressed [86].

In skeletal muscle, SIRT1-dependent deacetylation of PGC-1α leads to its activation and increases mitochondrial biogenesis and the expression of fatty acid oxidation genes [87] (Fig. **2B**). Notably, SIRT1-dependent activation of PGC-1α is required for induction and maintenance of fatty acid oxidation in response to low glucose concentrations leading to the switch from glucose to fatty acid oxidation. PGC-1α and FOXOs deacetylation leads to increased mitochondrial respiration and lipid oxidation through regulation of genes such as ERRα (mitochondria regulatory gene), IDH3α (TCA cycle), Cyt-c and COXVα (ETC), MCAD, CPT1b and PDK4 (fatty acid utilization) and PCG1α itself. To support these evidences, treatment of mice with resveratrol, a SIRT1 activator, improved their mitochondrial function, reducing the occurrence of diet-induced obesity and its associated insulin resistance [88].

An important feature of SIRT1 is its interaction with other sirtuins. For example SIRT6 negatively regulates the expression of the genes encoding enzymes involved in lipogenesis such as glycerol kinase (Gk), liver pyruvate kinase (Lpk), fatty acid synthase (Fas), acetyl-CoA carboxylase (Acc1), fatty acid elongase 6 (Elovl6) and stearoyl-CoA desaturase (Scd1) [89]. SIRT1 activates the expression of SIRT6, creating a complex together with FOXO3A and the transcription factor NRF1 at the SIRT6 promoter, consequently inducing fatty acid oxidation and inhibiting glycolysis and triglyceride synthesis via H3K9 deacetylation.

During fasting also the mitochondrial sirtuin, SIRT3, is activated and by deacetylating the long chain acyl-CoA dehydrogenase (Acadl) it induces fatty acid β-oxidation [90]. In BAT SIRT3 is activated by caloric restriction or cold exposure and increases the expression of PGC-1α and UCP1, leading to elevated thermogenesis and oxygen consumption [91]. SIRT3 is induced in the heart by exercise or pathological stimuli and protects against maladaptive cardiac remodeling, improving survival following pressure overload [92]. In neonatal rat ventricular myocytes, SIRT3 deacetylates the nuclear protein Ku70, prevents mitochondrial translocation of the proapoptotic protein Bax, and enhances tolerance against H_2O_2. Furthermore, SIRT3 deacetylates FOXO3A,

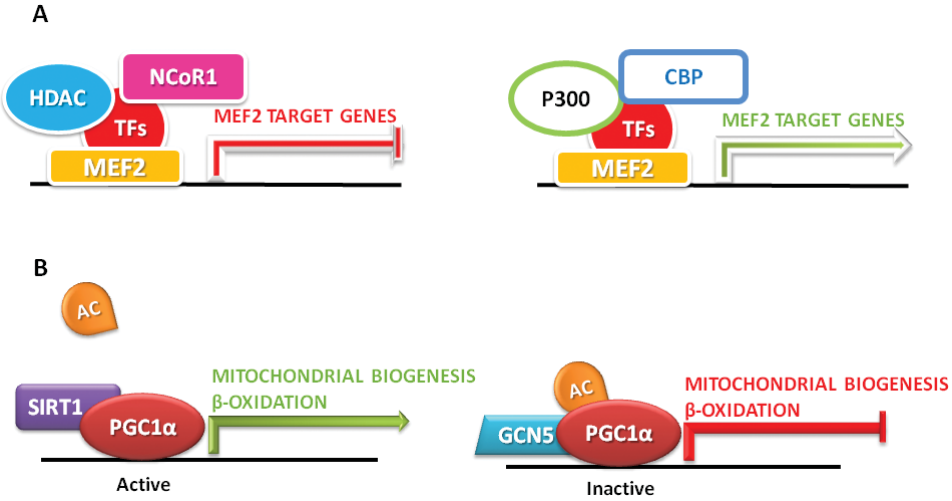

Fig. (2). Acetylation and deacetylation balance affects gene expression.

The nuclear repressor NcoR1 promotes switch of HDACs/HATs association with MEF2 repressing/activating gene transcription (panel A). Deacetylation/acetylation of PGC-1α by SIRT1 and GCN5, respectively, regulate mitochondrial biogenesis and β-oxidation (panel B).

thus increasing the expression of manganese-dependent superoxide dismutase (MnSOD also known as Sod2) that decreased the accumulation of superoxide in mitochondria [92].

SIRT3 also plays an important role in the maintenance of basal ATP levels and as a regulator of mitochondrial electron transport [93]. SIRT3 null mice showed 50% reduction of ATP basal levels in liver, heart and kidney accompanied by a markedly elevated acetylation of mitochondrial proteins and of several components of Complex I of ETC (e.g., NDUFA9, NADH dehydrogenase ubiquinone 1 alpha subcomplex subunit 9), thus resulting in the inhibition of Complex I activity [93].

Recently, interesting evidences shed new light on SIRT7, whose function was still poorly understood. In a recent paper, Ryu and coworkers [94] identified SIRT7 as a main actor in the regulation of mitochondrial homeostasis. By means of bioinformatic, molecular and *in vivo* studies the authors showed a SIRT7-dependent acetylation switch of GABPb1 (GA binding protein, also known as NRF2), a nuclear transcription factor involved in several adaptive processes in mitochondria. According to their results, SIRT7 deacetylates GABP allowing the formation of the transcriptionally active GABPa/GABPb heterotetramer, which activates mitochondrial gene expression. Through this pathway SIRT7 is able to prevent mitochondrial related diseases. SIRT7-knockout mice, in fact, exhibit lactate accumulation, hepatic microvescicular steatosis, reduced exercise performance, cardiac hypertrophy and dysfunction, hearing defects. The observed abnormalities suggest that Sirt7 null mice suffer from a multisystemic mitochondrial dysfunction since they are commonly found in patients with mitochondrial disorders [95, 96].

2.7. HATs and Energy Metabolism

As discussed above, lysine acetylation processes in mitochondria are closely linked to nutrient availability and represent a very important regulation of mitochondrial function. As previously discussed in relation to enzymes with deacetylating activity, such as HDACs and sirtuins, also HATs play a role in this scenario contributing to the control of mitochondrial functionality and energy metabolism. In fact, GCN5L1 (General Control of Amino Acid Synthesis 5-Like 1) was recently discovered as a putative mitochondrial acetyltransferase in HepG2 cells that counteracts acetylation and respiratory effects of SIRT3 [97]. Indeed GCN5L1 promoted acetylation of SIRT3 respiratory chain targets and reverted global SIRT3 effects on mitochondrial protein acetylation, respiration and bioenergetics acting on the ETC subunits NDUFA9 and ATP5A (ATP synthase subunit α) [97]. In addition, GCN5 is also in contrast with the action of another sirtuin, SIRT1. In fact it acetylates PGC-1α [98] and it reduces the expression of the gluconeogenic genes PEPCK (Phosphoenolpyruvate carboxy kinase) and G6Pase (Glucose 6-phosphatase), causing a phenotype similar to that of SIRT1 deficient hepatocytes. In myotubes, GCN5-mediated induction of PGC-1α acetylation decreases PGC-1α target genes expression [87]. Collectively these evidences suggest that GCN5 can induce metabolic changes depending on nutrient availability.

2.8. DNA Modifications

Recent findings demonstrate that DNA (hydroxyl)-methylation patterns stabilize DNA secondary structure elements [99], consequently altering binding of methyl CpG binding proteins, and subsequently quantitatively modulating gene expression [100] or alternative splicing of the target gene by loss and gain of those modifications [101]. The mechanism was reviewed in detail by Wu and colleagues [102]. 5-HmC, a biological intermediate between methylated and unmethylated cytosine with distinct biological functions, can be easily oxidized to demethylate substrates [103]. Passive demethylation is thought to occur through decreased activity of DNA methyltransferases [104]. Active processing requires enzymes catalyzing the oxidation of the methyl-residues on cytosines. Ten-eleven translocation methylcytosine dioxygenase (TET) involved in base excision repair (BER), are enzymes that convert 5-mC to 5-hydroxymethylcytosine [105] making them susceptible to further oxidation and cleavage. TET activity can be enhanced and mediated interacting with proteins like PPARγ or PRDM14 [106, 107].

Analysis of 42 healthy human cell types from various tissues resulted in the dynamic regulation of only 21.8% of autosomal CpGs overlapping mostly with enhancers and transcription-factor binding sites [108]. Naturally reconfiguration of methylome occurs in the human brain during development, accumulating highly conserved non-CG methylation in neurons. This process is highly dynamic and organized [109], and misregulation lowers synaptic plasticity, impairs memory in mice or leads to the loss of cell identity in olfactory neurons [110, 111]. 5-hmC additionally plays a role in asymmetric renewal of distributed stem cell [112]. It regulates their number, survival, homeostasis, and cell differentiation [113, 114]. Malnutrition and obesity can both lead to inheritable, sex specific methylation pattern changes in mice and humans, and consequently to metabolic adaptation, and reprogramming of the epigenetic machinery itself [115, 116].

Among other 44 mitochondrial function genes, PGC-1α and nuclear respiratory factor NRF promoters specifically are hypermethylated in type 2 diabetes (T2D). First, it is a non-heritable and individually acquired CpG modification in humans [117]. Contrasting the individuality in humans, PGC-1α promoter methylation and gene expression in murine muscle is influenced by maternal lifestyle [118]. NRF1 controls Tfam [119], whilst NRF2 determines the expression of translocator of outer mitochondrial membrane (TOMM 70), by a binding stabilized by two CpG island [120]. The acquisition of a metabolic memory linked due to long term high glucose exposure, results in hypermethylation and downregulation of DNA polymerase subunit gamma (POLG), with reduction of mitochondrial gene expression [120].

Two key enzymes involved in lipid oxidation as carnitine palmitoyltransferase 1a (CPT1a) and acetyl-CoA carboxylase (ACACA), are regulated by methylation of the promoter or by intronic CpGs as in the case of CPT1a [121, 122]. Uncoupling in adipose tissue converts stored energy into heat and is mediated by uncoupling proteins (UCP). UCP1 is regulated by DNA methylation and histone modifications. Their expression is triggered by hypomethylation and altera-

tion of histones methylation pattern in adipose tissue [123]. The differences of tissue functions of white adipose tissue as a storage of energy compared to brown adipose tissue as a consumer, and the controlled expression of UCP1 in a methylation driven manner altered by cold treatment demonstrates a tissue specific functional response to environmental changes and DNA methylation states in both mice and humans. The coalteration of histone modification and DNA methylation at UCP1 locus is another important aspect of epigenetic control. Notably, the binding of ligand bound NRFs [124] and ligand-dependent nuclear receptor corepressor (NCoR) [125] to receptor-interacting protein 140 (RIP140) assembles and recruits DNA methylases and histone methyltransferases (HMTs) to their target, indicating a precisely tuned mechanism of epigenetic regulation [126].

Methylation of mitochondrial DNA might be another control mechanism of mitochondrial function. mtDNMT1 expression is upregulated by NRF1 and PGC-1α in response to hypoxia, and by loss of p53, affecting the expression of mitochondrial transcripts [127]. DNMT3A and mtDNA methylation was also implicated in amyotrophic lateral sclerosis (ALS), revealing a decrease of mtDNMT3A in early disease stage and alteration of 16S RNA DNA methylation in nervous tissue and skeletal muscle of mice [128].

As discussed before, the functionality and number of mitochondria in diabetes and obesity are impaired, leading to cell stress and chronic inflammation. At this regard, there are several examples of alterations of mtDNA methylation. Interestingly, mitochondria are altered by CpG pattern change, which in turn may alter CpGs themselves. Diabetes and obesity significantly alter CpG methylation of hypoxia induced factor 3 A (HIF3A) locus in humans [129]. HIFs in turn are regulated by mitochondrial complex 3, reviewed in detail in [130]. Under hypoxic condition the cells and their environment are changed, influencing DNA (cytosine-5)-methyltransferase 3A's (DNMT3A) location and function in smooth muscle cells [131]. White adipocyte excreted hormone leptin controls metabolism and it also sensitizes heart muscle cell mitochondria to calcium signaling as well as cell death, a very prominent aspect of obesity related myocardial infarction [132]. In fact, leptin positively correlates with acute myocardial infarction in human [133] and is regulated by CpGs in an inheritable, nutrition dependent way, in mice, leading to higher leptin levels in offspring of mother mice subject to malnutrition [134].

Altogether, these examples clearly illustrate how alterations of DNA methylation are involved in several pathophysiological conditions relevant to human disease.

2.9. Epigenetic Inheritance and its Potential Role in the Regulation of Metabolism

As discussed above, metabolic adaptation in a given organism also occurs by means of epigenetic mechanisms. Lately, the concept that "metabolic signatures" inherited from parents or grand-parents might be also due to transmission of epigenetic marks has been proposed. This concept appears in contrast with the knowledge that germ cells undergo multiple rounds of epigenetic reprogramming. Indeed, some metabolic disorders, such as type 2 diabetes and obesity, can be transmitted to offspring independently of the genomic trait [135]. Of note, the investigation of transgenerational inheritance of epigenetic marks in mammals is hampered by the occurrence of *in utero* events that may affect the epigenome. For such reason, male mice represent a more suitable model to assess the heritability of epigenetic marks among generations.

In this context, Carone *et al.* [136] demonstrated that male mice subject to a low-protein diet generated mice with altered lipid metabolism associated to changes in cytosine methylation. Interestingly, the authors observed reproducible changes in methylation at a putative enhancer of PPARα in the livers of offspring. Although this epigenetic trait is undoubtedly linked to paternal diet, therefore transmitted through generations, the exact mechanism, e.g. chromatin modifications, small RNAs etc, whereby it occurred was not clearly deciphered.

More recently, LXRα, another nuclear receptor involved in lipid metabolism, was demonstrated to be the target of inherited epigenetic modifications, ultimately increasing age-related obesity and glucose intolerance in offspring [137]. More specifically, Martinez *et al.* found that germ cells of male mice that underwent *in utero* malnutrition were characterized by a hypomethylation pattern at the 5'UTR of *Lxra* that was also found in the livers of the next generation. These results suggest that environmentally induced epigenetic marks can be transmitted through the gametes. In line with these observations, Wei *et al.* [138] showed that paternal prediabetic condition affects glucose metabolism and insulin sensitivity in offspring. Specifically, the authors found an altered DNA methylation profile at the level of several genes involved in the insulin signaling pathway, both in sperm of the fathers and in the pancreatic islets of offspring. Based on their results, the authors propose that epigenetic information carrier in sperm responding to environmental conditions is largely heritable.

Despite the great interest in this field and the experimental evidences mentioned above, the mechanisms by which epigenetic traits can be transmitted among generations need further investigation to clearly establish their relevance to human health.

3. REGULATION OF THE MITOCHONDRIAL GENOME

3.1. mtDNA Transcription and Replication

Replication and transcription of the nuclear genome are two distinct processes, whereas they are mutually dependent in the case of mtDNA [9] (Fig. **3**). The activity of the mitochondrial transcription initiation complex is mostly regulated by three transcription factors: Tfam, transcription factor B1 mitochondrial (Tfb1m) and transcription factor B2 mitochondrial (Tfb2m). Their presence is necessary for the activity of the bacteriophage-related mtRNA polymerase (POLRMT) [139]. This complex starts its activity from the heavy strand promoters (HSP) 1 and 2 and light strand promoter (LSP) in a bidirectional way following the entire LS and HS [140]. The region containing these promoters is also known as D-loop and is the only untranscribed region of mtDNA.

The mtDNA replication machinery requires multiple proteins such as the helicase TWINKLE, the DNA polymerase

POLG and the single stranded DNA binding protein, mitochondrial (SSBP1) [140-143]. New findings showed that mutations of any of the members of the mtDNA maintenance machinery are associated with several mitochondrial dysfunctions. For example, the group of Suomalainen [144] demonstrated accumulation of mtDNA deletions coupled with mitochondrial dysfunction in a transgenic mouse model that expresses a mutated form of the helicase TWINKLE. Notably this mutation is associated with the mitochondrial disease chronic progressive external ophthalmoplegia. Similarly, depletion of Tfb1m in mouse Langerhans islets results in mitochondrial dysfunction associated with type 2 diabetes and insulin secretion impairment [145, 146].

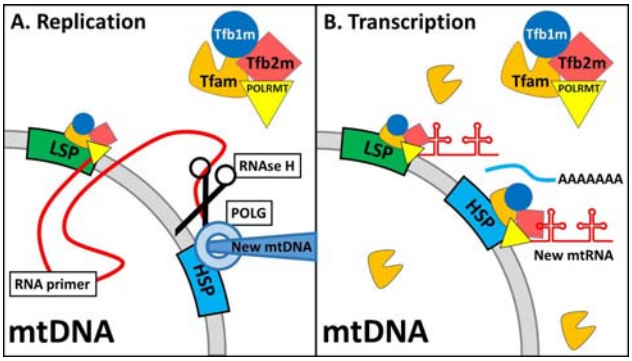

Fig. (3). The replication and transcription machineries acting at the mitochondrial promoters.

The LSP and HSP promoters play a key role in both replication and transcription of the mitochondrial genome. The replication machinery (left panel) includes the DNA polymerase POLG, the RNA polymerase POLRMT, the helicase TWINKLE (not shown), the mitochondrial SSBP1 (not shown), and the initiation factors Tfam, Tfb1m, Tfb2m. POLRMT together with the factors Tfam, Tfb1m, and Tfb2m constitute the transcription machinery (left panel).

Tfam is the main mediator of a great variety of molecular cues directed to mitochondria [147]. Tfam is a highly conserved protein with a molecular weight of about of 25 kDa and its ability to bind DNA is due to the presence of two distinct high mobility group (HMG) boxes [148]. The essential role of Tfam is demonstrated by the fact that its ubiquitous deletion brings to complete impairment of embryonic mitochondrial content and subsequent death at E10.5 [149]. Altered levels of Tfam in specific tissues or cell types bring to heavy mitochondrial dysfunction and decreased mitochondrial biogenesis. In fact, specific ablation of Tfam in pancreas results in mitochondrial loss, mitochondrial function impairment and insulin secretion deficiency [150]. In addition, WAT and BAT adipose tissue specific Tfam depletion results in a decreased expression of the ETC complexes, increased insulin resistance and BAT whitening, suggesting mitochondrial biogenesis loss in this tissue [151]. The murine model of genetic ablation of Tfam in Schwann cells shows severe mitochondrial dysfunction associated with metabolic impairment in the peripheral nervous system and peripheral neuropathy [152]. On the other hand, its deficiency at the level of dopamine neurons in the central nervous system leads to Parkinsonism [153].

Evidences gathered in the last few years demonstrated that the regulation of replication and transcription by Tfam goes beyond the mere binding to HSP and LSP (Fig. **4**). Indeed, Tfam is the main component of nucleoids, nucleoprotein structures anchored to IMM, where it displays a histone-like function in mtDNA packaging [154, 155]. The Tfam:mtDNA ratio is extremely important for mtDNA transcription and replication and the underlying mechanisms were recently reviewed [154, 156] The degree of Tfam dimerization and the number of Tfam molecules assembled at both HSP and LSP deeply and mutually influence replication and transcription. Specifically, in a monomeric form, Tfam coats LSP within the D-loop thus allowing transcription of a short sequence that acts as a primer for HS replication. However, when dimer/monomer ratio increases, HS transcription starts while its replication halts. mtDNA transcription is interrupted when the Tfam:mtDNA ratio is very high. Thus, mtDNA transcription and replication are finely tuned by the levels of Tfam, its degree of dimerization, and by the Tfam:mtDNA ratio. Furthermore, Tfam is able to bind mtDNA also at aspecific sites outside the D-loop. In this way, the random dimerization between Tfam molecules associated at distal position causes packaging of mtDNA and blocks the assembling of the transcription initiation complex [157]. These models were recently verified experimentally in *in vitro* reconstituted nucleoids [158].

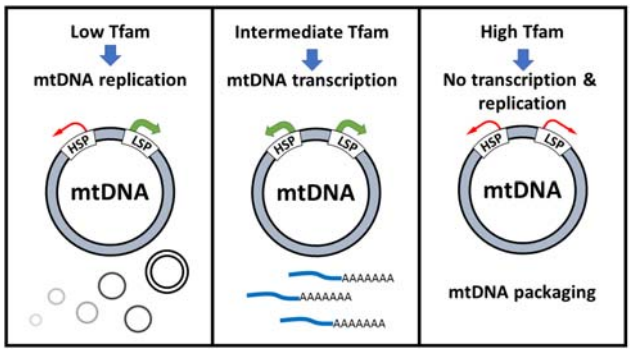

Fig. (4). Tfam levels modulate mtDNA replication and transcription.

When present at low levels, Tfam coats the LSP, predominantly in monomeric form, and promotes mtDNA replication (left panel). Increased expression and/or stability of Tfam protein leads to its dimerization, a condition favouring mtDNA transcription (middle panel). Very high levels of Tfam lead to its binding at many aspecific sites along the mtDNA; this event causes higher compaction of the mitochondrial genome and the turning off of its replication/transcription (right panel).

Given its essential role in governing mtDNA transcription and replication, it is conceivable that Tfam accessibility to mtDNA be tightly monitored. To this end, degradation of unnecessary and/or misfolded proteins is guaranteed by a quality control machinery containing typical proteases [159]. Specifically, mitochondrial levels of Tfam are regulated by the AAA^+ Lon protease (LonP) in a phosphorylation-dependent manner [160]. Specific knockdown and chemical inhibition of LonP results in increased Tfam abundance and mtDNA copy number [160]. On the contrary, its overexpression brings to Tfam selective degradation and mtDNA transcription impairment [161]. Additional data demonstrated that LonP silencing in colon cancer cells decreased mtDNA transcripts and oxygen consumption rate and increased ROS production [162].

The expression of Tfam, is mainly regulated by PGC-1α [140, 163, 164], which interacts with and co-activates the transcription factor nuclear respiratory factor 1 (NRF1). It is worthwhile to mention that PGC-1α controls the expression of many other mitochondrial genes through different transcription factors and nuclear receptors such as NRF2, PPARα, and the estrogen related receptor α (ERRα) [10].

PGC-1α is highly sensitive to environmental conditions, representing one of the most outstanding links between external cues and nuclear transcription. PGC-1α expression is deeply influenced by cold, physical exercise and cell energy status [165-167]. On the other hand its activity is deeply affected by post-transcriptional modifications such as phosphorylation by AMP-activated protein kinase [168], acetylation by SIRT1 [88], or direct interaction with other proteins such as the mammalian target of rapamycin (mTOR) or the transcriptional repressor YY1 (Yin Yang-1) [169, 170]. As mentioned above, mice lacking SIRT1 in the liver show fatty acid accumulation, PGC-1α and ETC subunits expression decrease [83]. Specific ablation of YY1 in skeletal muscle brings to severe mitochondrial myopathy associated with a decreased expression of Tfam and OXPHOS complexes [171]. Many factors are involved in mtDNA expression and maintenance, also resulting from the tight crosstalk between the nucleus and the mitochondria. Thanks to new experimental approaches and the increasing number of mouse models, the regulation of mtDNA replication and transcription is now largely described. Nevertheless, the precise molecular mechanisms underlying these processes are far to be fully deciphered, also because new regulators are constantly emerging, broadening the complexity of these processes. In fact, we are now starting to figure out how mtDNA transcription and replication are controlled not only at the genomic level but as we will see below, also at different stages of mRNA processing.

3.2. Mitochondria RNAs Processing

As mentioned above, mtDNA does not contain intronic sequences and this implies two important consequences. First, mitochondria transcribe all mtRNA sequences at the same time in a single polycistronic sequence, without wasting energy regulating the transcription of individual sequences. Second, mitochondria must have an efficient enzymatic repertoire to cut the polycistronic mRNA and get the individual open reading frames (ORF) [172]. Until the early '90, when the tRNA punctuation model was formulated by Ojala *et al.* [173], the existence of a sophisticated regulatory mechanism controlling mtRNAs processing, stability and decay was not experimentally evident. In more recent years, applications of innovative technologies and new experimental approaches have allowed to identify new regulators of mtRNA processing that influence mitochondrial function and physiology.

The first and limiting step in mtRNA maturation is the cleavage of tRNA 5'-end and 3'-end between open reading frames (ORF). The ribozyme ribonuclese P (RNase P) and the ribonuclease RNase Z (encoded by the ELAC2 gene) carry out the cleavage of the polycistronic transcript at the 5'-end and at 3'-end of tRNAs sequences, respectively [174, 175]. ELAC2 mutations in humans result in complex I deficiency and cardiomyopathy, in addition to mtRNA precursors accumulation in different tissues [176]. Once cleaved, the nascent mRNA undergoes maturation through the addition of a polyadenylated tail at the 3'-end. This crucial step is accomplished by mitochondrial poly(A) polymerase (mtPAP) and polynucleotide phosphorylase (PNPase) that synthesize polyA tails ex novo. Missense mutation in the human PNPase gene results in the substitution of the asparagines at position 478 to aspartic acid (N478D) in the encoded protein. This mutation impairs mtRNAs polyadenylation leading to the loss of ETC complexes I and IV transcripts and to a severe decrease in mitochondrial translation [177]. Altogether, these observations provide insights into the relevance of mtRNA maturation in mitochondrial physiology. On the other hand, PNPase and mtPAP have a double-edge role. In fact, recent results demonstrated that transient association of PNPase-mtPAP with the helicase SUV3 leads to the formation of the degradosome that also contains RNase P and RNase Z/ELAC2 [178, 179]. The degradosome, by shortening polyA sequences on mtRNAs modulates their function and, consequently, their translation. SUV3 deletion in mammalian cells results in mitochondria morphology defects, impairment in OXPHOS transcription, decreased levels of ATP and mtDNA and matrix accumulation of mtRNA [180]. The main steps and factors involved in the maturation and processing of the mitochondrial RNA precursor are represented in (Fig. **5**).

The stability of RNA sequences seems to be regulated also by the leucine-rich pentatricopeptide repeat-containing (LRPPRC) protein [181], which counteracts the negative effects of SUV3-PNPase-mtPAP on mtRNAs half-lives [178]. The SUV3-PNPase-mtPAP complex preferentially degrades double strand RNAs, therefore LRPPRC could prevent mtRNAs degradation by two possible mechanisms. LRPPRC could prevent the formation of a loop and, consequently, of a double strand structure in the RNA molecule. Additionally, LRPPRC could remove the polyA tail and prevent the formation of a double strand framework with the polyU sequences within the mt-RNA [182]. LRPPRC down-regulation has been demonstrated to alter ND5-cytochrome B precursor transcript [183]. Moreover, its specific depletion in mouse heart leads to a decreased ATP production, due to ATPase translation impairment and in abnormal production of ROS [184].

The rising of new technologies coupled with bioinformatics analyses has provided new possibilities to identify previously unknown proteins involved in mtRNA maturation. By applying accurate methods to quantify mtRNAs, coupled with information derived from the mitoproteome, Wolf and Mootha recently identified a new regulator of mtRNAs half-life [185]. By this means they were able to identify FASTKD4, a nuclear-encoded mitochondrial protein with predicted ability to interact with RNA. Its silencing was shown to decrease mtRNA stability and consequently to decrease the expression of some ETC complexes despite mtDNA increase compared to control.

In the nucleus, the stability of mRNAs is also affected by small non coding RNA sequences called microRNAs [186]. Mitochondrial small non-coding RNAs, produced by not yet identified ribonucleases, were recently detected, suggesting

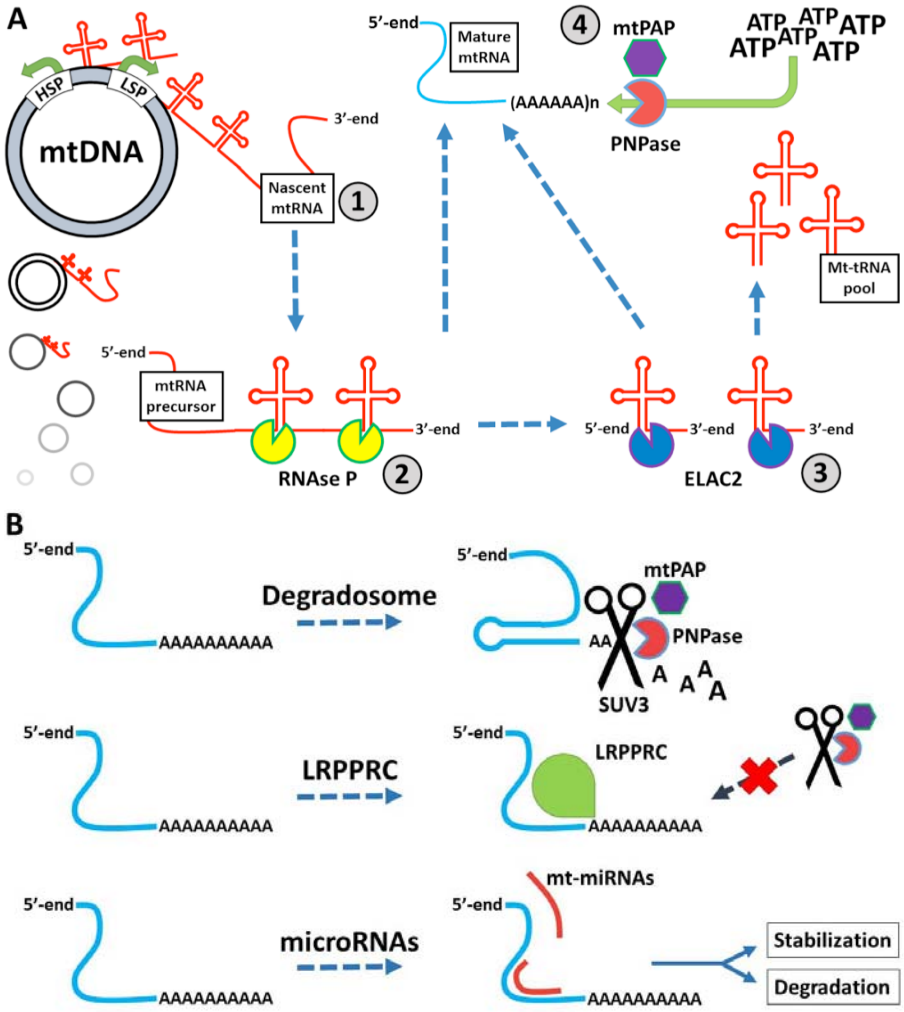

Fig. (5). Maturation and processing of the mitochondrial polycystronic RNA precursor.

Maturation of mtRNAs is depicted in panel A. mtDNA is transcribed to a single polycystronic mtRNA precursor (step 1). RNase P cleaves contiguous mRNA/rRNA and tRNA sequences, generating a free 3'end of the mRNA/rRNA molecule and a free 5'end of the tRNA molecule (step 2). Cleavage at the 3'end of tRNA is carried out by the endonuclease ELAC2 (step 3). Formation of the polyA tail of mt-mRNAs is accomplished by the combined action of mtPAP and PNPase (step 4).

Panel B summarizes the pathways affecting mt-mRNA stability. mtPAP, PNPase and SUV3 form a complex termed the degradosome that, by shortening the polyA tail, decreases mRNA stability. The activity of the degradosome is counteracted by LRPPRC, an RNA interacting protein. Physical interaction between mRNA and LRPPRC prevents the formation of double strand stretches within the mRNA molecule, thus preventing the action of the degradosome.Finally, microRNAs localized in mitochondria (mt-miRNAs) can promote either stabilization or degradation of target mt-mRNAs (see text for more details).

that mitochondrial mRNA stability is more complex than expected [187]. In addition, recent data demonstrated that stability and expression of target mt-mRNAs also depends on interactions between mitochondrial non-coding RNAs and RNA-binding proteins, such as GRSF1, which directly target long non-coding mtRNAs. Its loss entails serious consequences on mitochondrial homeostasis such as mitochondrial ribosome biogenesis impairment, mtRNAs destabilization and dysregulation of mitochondrial proteins synthesis [188].

As mentioned above, mitochondrial mRNAs are not the only RNA sequences encoded by mtDNA. Mitochondria-encoded tRNAs and rRNAs hold a fundamental role in mitochondrial proteins translation. mt-tRNAs originate from the cleavage of the polycistronic sequence, as previously discussed in the section on mt-mRNAs maturation. RNase P and RNase Z/ELAC2 5'-end and 3'-endcutting activity holds a crucial part in the formation of tRNAs. Likewise mt-mRNAs sequences, mt-tRNAs are subjected to an intricate post-transcriptional maturation process that includes base substitution and conjugation with taurine [189]. The clinical importance of mitochondrial tRNAs was evident as early as in 1990 when a mutation in a mt-tRNA was associated with encephalomyopathy [190]. Since then, an increasing amount of data has been collected demonstrating that many diseases and syndromes are associated with mt-tRNAs mutations and processing defects [191].

Furthermore, 12S and 16S mt-rRNA stability is also necessary for a proper mitochondrial translation even if, to our knowledge, there are few clinical manifestations linked to

mt-rRNAs mutations [192-194]. However, there are growing evidences of the complexity of mt-rRNA post-transcriptional modifications and of their role in mitochondrial physiology. For example, Metodiev *et al*. recently reported that Tfb1m is responsible for 12S mt-rRNA methylation and thus the proper mitochondrial ribosome stability in mammals [195]. Moreover, a recent study from Lee *et al*. demonstrated that specific silencing of the mitochondrial rRNA methyltransferase family member RNMTL1 decreased 16S subunit methylation resulting in impaired mitochondrial translation [196]. Summarizing, given the initial polycistronic structure of mtRNA, its proper cleavage and processing are key steps for a correct mitochondrial translation and function. Moreover, several factors involved in mtRNA transcription seem to participate also in other steps of mtRNA processing, suggesting that many of these proteins may have multiple roles. Together with the obscure role of non-coding mtRNAs, this is a promising area of future investigations.

3.3. Mitochondrial Translation

Besides 12S and 16S mt-rRNA subunits, many nuclear proteins constitute the mammalian mitochondrial ribosome (mitoribosome). The overall molecular weight of the mitoribosome is about 2.7 MDa; it consists of a small 28S (SSU) and a large 39S (LSU) subunit [197]. Mitoribosomes translate only mitochondrial membrane proteins, and consequently they lie anchored to the matrix side of the IMM [198]. Mitochondrial translation is a multistep process that comprises mitoribosome assembly, which, unlike cytoplasmic ribosomes, proceeds along with mt-rRNA and mt-mRNA maturation, initiation, elongation, and termination. For a detailed discussion of protein biosynthesis in mitochondria interested readers may refer to recent reviews [197, 198]. Briefly, two factors are involved in the initiation of protein synthesis in mitochondria. The mitochondrial initiation factor 3 (mtIF3) dissociates the 28S and the 39S subunits whereas mitochondrial initiation factor 2 (mtIF2) transfers mt-mRNAs into the mitoribosome and activates the first methionine-tRNA through the conversion of a GTP molecule to GDP. Once activated, translation is maintained active mainly by two proteins, the mitochondrial elongation factor Tu (mtEFTu) and the mitochondrial elongation factor G1 (mtEFG1) with the intervention of GTP molecules. mtEFTu escorts aminoacylated tRNAs to the ribosomal A site while mtEFG1, assists the translocation of the nascent peptidyl chain to the P site and of the discharged tRNA to the E site [199]. A mutated form of mtEFG1 is associated with a severe mitochondrial dysfunction where the translation of all mitochondrial encoded proteins is heavily affected [200]. Finally, translation termination is due to the presence of four possible different stop codons (UAA, UAG, AGA, and AGG) and of four proteins named mtRF1, mtRF1a, ICT1, and C12orf65 whose precise role is still obscure [201].

Overall, it is clear that molecular paradigms of nuclear gene translation do not apply to mitochondria. Even though mtDNA is smaller and shorter than nuclear chromosomal DNA, the mitochondrial translation machinery seems very complex and distinct. More work is needed for a deeper comprehension of how the mitoribosome assembles and how nuclear proteins can affect the efficiency of mitochondrial proteins translation.

3.4. Mitochondrial Import of Proteins and RNAs

Mitochondrial localization and import of large molecules (polypeptides and RNAs), mainly coming from the nucleus, is fundamental for the life and maintenance of the organelle. For example, nuclear-transcribed mRNAs encoding some ETC hydrophobic subunits are localized closer to the OMM than other mRNAs encoding for non hydrophobic peptides. This phenomenon could be explained by the presence of the RNA-binding protein Y-box binding protein-1 in the OMM [202]. This allows efficient and rapid import of nuclear-encoded mitochondrial proteins, immediately after translation.

Whereas protein transport in mitochondria is largely documented [203, 204], mitochondrial import of RNA sequences still needs to be more characterized. PNPase, which, as mentioned above, is involved in mtRNAs polyadenylation, is the most characterized mitochondrial RNAs carrier. Once imported inside the OMM, PNPase binds the translocase of the inner mitochondrial membrane 23 (TIM23) and, through physical interaction with the i-AAA protease Yme1, is shuttled to the intermembrane space [205, 206]. These protein-protein interactions allow proper RNAs import into the mitochondrial matrix (Fig. **6**). A recent study showed that PNPase is essential for import of the ribozyme RNase P, the mRNAs encoding mitochondrial ribosome proteins (MRP) and 5S RNA sequences in yeast mitochondria [207]. Moreover, ablation of PNPase in mice leads to decreased mitochondrial oxygen consumption [207]. A missense mutation at position 1424A>G of the human PNPase gene associated with deafness was demonstrated to be involved in impaired RNA import to mitochondria [208].

Import of nuclear RNAs into mitochondria, in addition to its biological significance, could represent a valid tool to deliver small RNA sequences (20 nucleotides) to mitochondria to bypass mtRNAs defects and counteract mitochondrial syndromes. Wang al collaborators developed such a system in 2012 using a fusion RNA oligonucleotide, bearing the non coding H1 RNA portion of RNase P that efficiently reached mitochondria [209]. Furthermore, they confirmed previous data according to which 3'-untranslated region (3'-UTR) of some nuclear mRNAs, such as the one encoding cytochrome c oxidase subunit II (COXII), carries a mitochondrial localization sequence [210].

The studies mentioned above clearly show that the characterization of new molecular mechanisms will provide important insights and tools to face mitochondrial dysfunctions in an innovative and specific manner.

3.5. Mitochondria Epigenetics and microRNAs

The apparent absence of classical CpG islands in mtDNA covered mtDNA epigenetics with general skepticism. Furthermore, mitochondria lack histones, and the associated histone modifying enzymes. During the last years increasing evidences demonstrated the presence of active DNA methyl transferases in the matrix of these organelles, such as DNMT3a, and methylated nucleotides in mtDNA, like 5-methylcytosine and 5-hydroxymethylcytosine [211]. Recent data showed that the D-loop is methylated at non-CpG islets, suggesting a possible epigenetic control of mtDNA tran-

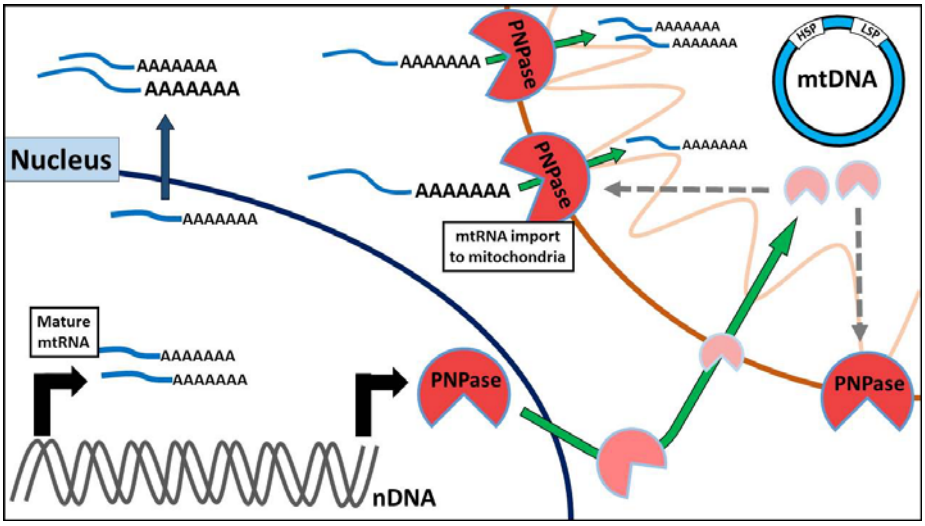

Fig. (6). Trafficking of mRNAs between nucleus and mitochondria.

The nuclear encoded PNPase also plays a role in the trafficking of nuclear mRNAs to mitochondria. PNPase localizes in the OMM where it captures nuclear mRNAs, promoting their translocation to mitochondria. Molecular recognition is thought to be mediated by a specific sequence at the 3'UTR of nuclear mRNAs.

scription and replication [212], even though mtDNA methylation can also occur in several ORFs. Indeed, the genes encoding NADH dehydrogenase subunits are hypermethylated during oxidative stress and aging whereas 12S rRNA methylation is decreased in aged males [211]. Furthermore, new evidences showed that air particles increase the methylation state of phenylalanine-tRNA, 12S ribosome subunit and the D-loop region, suggesting a direct influence of environment on mtDNA transcription [213].

Epigenetic mechanisms are not the only emerging regulators of mtDNA transcription. Non-coding sequences expressed in the nucleus are rising as a new and sophisticated control system of mtDNA transcription and replication. MicroRNAs (miRNAs) are 20-22 nucleotide non-coding sequences, which bind their target mRNA sequences in the 3'-UTR. miRNAs participate in many pathways and cellular processes, both physiological and pathological, such as the immune response, cell differentiation during development, and cancer [214-216]. On the basis of their target genes and of their cellular localization, miRNA can be classified in three different groups: miRNA encoded in the nucleus, which control the expression of different mitochondrial components and/or regulators; miRNA that are translocated into mitochondria where they affect the expression of mitochondria encoded genes; miRNA encoded by mtDNA.

In the last few years, miRNA have emerged as important both positive and negative regulators of mitochondrial function, broadening the long list of mitochondrial controlling pathways. Recent evidences showed that at least 80 pre-miRNAs and miRNAs generated from the nuclear genome localize in mitochondria, suggesting also the possible existence of a pre-miRNA processing system in the organelle [217]. Among the 80 putative mitochondrial miRNAs, miR-1 is the most characterized and its role in mitochondrial function is now well established. A very recent study demonstrated that miR-1 is imported inside the organelle where, along with argonaute-2 (Ago2), it coordinates translation of the mitochondrial genome during C2C12 cell differentiation to myotubes [218]. miR-1 expression increases during C2C12 differentiation and its downregulation decreases mitochondrial encoded ND1 and COX1 protein levels without affecting their gene expression [218].

On the other hand, new evidences also showed the fundamental role of miRNAs that regulate the expression of mitochondrial proteins in the nucleus. The hypoxia-induced miR-210 reduces the nuclear encoded iron-sulfur cluster scaffold homolog (ISCU) and COX10 expression shifting cancer cell lines from oxidative to glycolytic metabolism and is highly expressed in high-altitude placental cell cultures [219, 220]. A recent study demonstrated that hypoxia also increases the expression of miR-199a-2:miR-214 cluster in cardiomyocytes [221]. Overexpression of miR-199a-2:miR-214 cluster blunts the expression of PPARδ, whereas their silencing has positive effects on ETC complexes expression [221]. Diabetes, obesity and metabolic syndromes are also characterized by many compromised miRNA pathways. Mohamed *et al.* recently demonstrated that miR-149 expression decreases in the skeletal muscle of diet-induced obese mice [222]; in contrast its overexpression selectively inhibits poly(ADP-ribose) polymerase-2 (PARP-2), subsequently increasing the activity of the SIRT1-Pgc-1α complex, ultimately ameliorating the mitochondrial phenotype of skeletal muscles. On the other hand, miR-378 and miR-378* are upregulated in obese and insulin resistant mice liver interfering with PGC-1β mRNA [223]. Specific ablation of these oligonucleotides increases liver cells oxygen consumption rate and succinate dehydrogenase complex subunit D (Sdhd) and fumarate hydratase (Fh) expression [223]. miRNAs can also affect lipid biosynthesis the regulation of which is tightly linked to that of β-oxidation.miR-33a and miR-33b are transcribed along with sterol regulatory element binding factor 2 and 1 (SREBF2 and SREBP1) as they are found in the intron 16 of SREBF2 and intron 17 of SREBF1, respectively. Their main role is to contrast lipid catabolism to sup-

port lipid biosynthesis, in fact their overexpression down-regulates several β-oxidation key enzymes as CROT, CPT1a, HADHB and AMPKα [224].

miRNAs and mtDNA epigenetics represent supplementary and well established levels regulating the expression of nuclear genes. Latest results demonstrated that mtDNA epigenetics and specific miRNAs are more and more enlarging the list of mitochondrial regulators, and we are probably scraping the edge of a huge and fascinating iceberg.

4. CONCLUSION AND FUTURE PERSPECTIVES

The constantly growing understanding of the epigenetic mechanisms underlying gene regulation has refreshed our perception of genetics. Moreover, the discovery of regulatory pathways, by which non coding RNAs finely regulate gene transcription, has added higher levels of complexity. These novel concepts provided an extraordinary contribution to our understanding of how energy metabolism is regulated in response to environmental cues. This is especially true for mitochondria where the crosstalk between two genomes involves a highly regulated trafficking of proteins, mRNAs, miRNAs and much more.

Although the tremendous advances accomplished in the field, some gaps still need to be filled.

- The intensive research carried out by several groups in the last twenty years allowed us to gain a comprehensive appreciation of the multiple roles of sirtuins in metabolism. The same applies to some members of the HDAC superfamily whose metabolic effects have been fully characterized, by means of genetic and/or biochemical approaches. Less is known on members such as HDAC6, 8, and 11, whose functions and roles are still elusive. To this end, more studies are needed to characterize the structure and cellular localization of these proteins and their molecular functions (catalytic activity, scaffolding properties, etc).
- The number of enzymes involved in histone methylation/demethylation has grown rapidly in the last ten years, especially after the discovery of the JmjC domain as a demethylase signature motif. The requirement of α-KG, a key intermediate in the TCA cycle, as cofactor represents a peculiar feature linking epigenetics to metabolism, and vice versa. It is expected that future research on the Jumonji family members will add novel facets to the transcriptional and epigenetic regulation of energy metabolism.
- Mapping of the methylome in different models and cell systems suggests a strong association between this epigenetic mark and patho-physiology. Base methylation at non-classical CpG sites in the mitochondria genome is an emerging and still controversial aspect of epigenetics, potentially correlated with mitochondrial dysfunction. Technical limitations in the analysis of regions characterized by a low methylation state, such as mtDNA, might hamper the discovery of "hot spots" linked to energy metabolism. To this end, novel procedures designed to overcome limits, such as the immunoprecipitation step of methyl cytosine containing fragments, will be instrumental.
- Epigenetics mostly relies on metabolite-sensitive modifications. It follows that local levels of metabolites in the nucleus and mitochondria play a crucial role, tightly linking key metabolic pathways to epigenetics. In this respect compartimentalization of acetyl-CoA metabolism is paradigmatic: it provides conceptual support to the observations that protein acetylation is highly favored in mitochondria and that histone acetylation greatly depends on acetyl-CoA intracellular trafficking. Quantitative mapping of key intermediates in distinct cellular compartments is necessary for a deeper understanding of metabolite-driven epigenetics.
- Transgenerational inheritance of epigenetic marks is a fascinating and highly debated component of the "metabolic signature" induced by environmental conditions. How chromatin modifications and DNA methylation in parental germ cells can withstand epigenetic reprogramming, thus being transmittable to offspring is presently unclear. Several gaps concerning the epigenetic state in germ cells, zygote, and embryo at different pre- and post-implantation stages still need to be filled. Unfortunately, the experimental tools currently available appear insufficient for a complete understanding of the full range of factors influencing the epigenetic signature during embryonic development.
- mtDNA packaging is emerging as a mechanism to regulate transcription and replication of the mitochondrial genome, ultimately affecting mitochondrial function. Breakthroughs in the role played by Tfam in mtDNA compaction have been achieved in recent years. However, it is conceivable that other proteins might interact with mtDNA, contributing to its compaction. The possibility to reconstitute nucleoids *in vitro* and to investigate their structure by applying high content imaging techniques will provide valuable insights in mitoepigenetics.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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LIST OF ABBREVIATIONS

BAT	=	Brown Adipose Tissue
CBP	=	CREB-binding Protein
D-loop	=	Displacement Loop

CPT = Carnitine Palmitoyl Transferase
ERRα = Estrogen Related Receptor α
ETC = Electron Transport Chain
FLAD = FAD-synthase
GCN5 = General Control of Amino Acid Synthesis 5
H3 = Histone 3
H4 = Histone 4
HAT = Histone Acetyltransferase
HDAC = Histone Deacetylase
HDM = Histone Demethylase
HIF = Hypoxia Induced Factor
HMT = Histone Methyltransferase
HS = Heavy Strand
HSP = Heavy Strand Promoter
IMM = Inner Mitochondrial Membrane
JmjC = Jumonji C
LonP = AAA^+ Lon Protease
LRPPRC = Leucine-rich Pentatricopeptide Repeat-containing Protein
LS = Light Strand
LSD1 = Lysine-specific Demetylase 1
LSP = Light Strand Promoter
MAT = S-adenosyl Methionine Transferase
MEF2 = Myocyte enhancer factor 2
miRNA = microRNA
mtDNA = Mitochondrial DNA
mtEFG1 = Mitochondrial Elongation Factor G1
mtEFTu = Mitochondrial Elongation Factor Tu
mtIF2 = Mitochondrial Initiation Factor 2
mtIF3 = Mitochondrial Initiation Factor 3
mt-mRNA = Mitochondrial mRNA
mtPAP = Mitochondrial Poly(A) polymerase
mtRNA = Mitochondrial RNA
mt-rRNA = Mitochondrial rRNA
mt-tRNA = Mitochondrial tRNA
NCoR1 = Nuclear Receptor Corepressor 1
OMM = Outer Mitochondrial Membrane
ORF = Open Reading Frames
OXPHOS = Oxidative Phosphorylation
PGC-1α = PPARγ Coactivator 1α
PLRMT = mtRNA Polymerase
PNPase = Polynucleotide Phosphorylase
POLG = Mitochondrial DNA Polymerase
PPAR = Peroxisome Proliferator Activated Receptor
RFK = Riboflavin Kinase
RIP140 = Receptor-interacting Protein 140
RNase P = Ribozyme Ribonuclese P
RNase Z = Ribonuclease Z
ROS = Reactive Oxygen Species
SAM = S-adenosylmethionine
SIRT = Sirtuin
SRC1 = Steroid Receptor Co-activator
SSBP1 = Mitochondrial Single Stranded DNA Binding Protein
TCA = Tricarboxylic Acid
Tfam = Mitochondrial Transcription Factor A
Tfb1m = Transcription Factor B1 Mitochondrial
Tfb2m = Transcription Factor B2 Mitochondrial
Ucp1 = Uncoupling Protein 1
WAT = White Adipose Tissue
YY1 = Yin Yang-1
α-KG = α-ketoglutarate

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