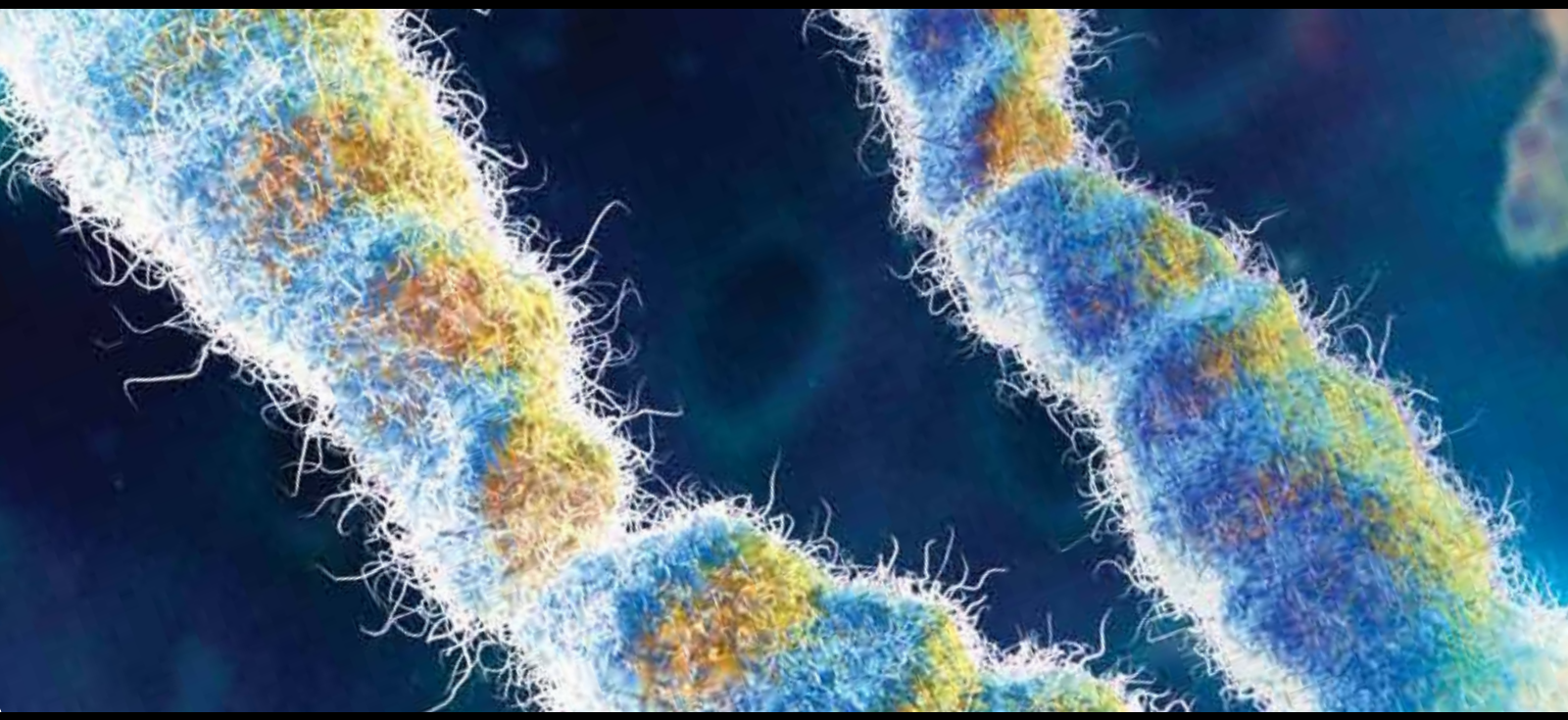
Journal of Aging Research

Mitochondria and Ageing

Guest Editors: Christiaan Leeuwenburgh, Reinald Pamplona, and Alberto Sanz



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Editorial Board

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Editorial

Mitochondria and Ageing

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During the last two decades, interest relating to mitochondria in the field of aging has grown exponentially. Nowadays, it is known that mitochondria are more than just the powerhouses of the cell. They participate in almost every aspect of metabolism. The more we learn about mitochondria, the more fascinating they become and the more they seem to be implicated in aging. Originally, the gerontological community became interested in mitochondria due to their role in the generation of reactive oxygen species (ROS). D. Harman proposed his renowned free radical theory of aging in 1956 [1], reformulating it 16 years later as the mitochondrial free radical theory of aging (MFRTA) [2] in order to emphasise the fact that mitochondria were simultaneously both the main generators and the main targets of free radicals. Since then, many laboratories have published an extensive volume of data both supporting and refuting the MFRTA. Presently, new theories have been put forward suggesting a role for mitochondria in aging which is independent of the generation of ROS. For example, it has been proposed that aging is caused by the accumulation of mutations and large-scale deletions in mitochondrial DNA (mtDNA), which may partly arise from the inherent error rate of mtDNA polymerase gamma rather than oxidative damage [3]. It has also been proposed that aging can be caused by an alteration of the redox homeostasis, since mitochondria regulate the relative levels of NADH/NAD+, NADPH/NADP+, and GSH/GSSG [4].

The considerable attention that mitochondria have attracted is well represented in this special issue. Different authors present new discoveries from their laboratories or review the latest advances in their fields of expertise. The role of mtDNA in aging and cancer is a popular and highly debatable topic. This is illustrated by three articles in the "Mitochondria and Aging" special issue. Specifically, R. Gredilla in "*DNA damage and base excision repair in mitochondria and their role in aging*" describes the substantial progress that has been achieved in understanding the repair mechanisms relevant to mtDNA. It is currently known that mtDNA has a sophisticated repair system adapted to the particular needs of an environment characterised by high levels of ROS. C. Desler et al. in "*The importance of mitochondrial DNA in aging and cancer*" and A. M. Czarnecka and E. Bartnik in "*The role of the mitochondrial genome in ageing and carcinogenesis*" analyse the contribution of mitochondria to both the origin and progression of cancer. Several interesting correlations are revealed; however, further research is needed before more definite conclusions can be reached. This will require the implementation of new techniques to measure every aspect of mitochondrial function in detail in combination with high-throughput screening. L. Staunton et al. in "*Proteomic profiling of mitochondrial enzymes during skeletal muscle aging*" describe how proteomic profiling can help to achieve this, whereas E. Barbieri et al. in "*Morpho-functional and biochemical approaches for studying mitochondrial changes during myoblasts differentiation*" demonstrate a practical application of the techniques in the study of cellular fate. Although the role of mitochondria in cell differentiation is not yet understood, available data indicate that they play a major role and that certain changes in mitochondrial function are essential to complete cellular differentiation.

Dietary restriction (DR) is the most widely used intervention to extend lifespan. Currently, numerous laboratories worldwide are searching for genetic targets that will allow an increased lifespan without reducing the number of calories. It is well established that mitochondria are major contributors to the cellular adaptations needed to prolong lifespan during DR. However, the mechanisms or pathways involved have yet to be completely elucidated. Organisms with a short lifespan, such as yeast, offer an excellent opportunity for screening. B. Li et al. in "*Identification of potential calorie restriction-mimicking yeast mutants with increased mitochondrial respiratory chain and nitric oxide levels*" use this approach to identify new genes implicated in lifespan extension by DR. For example, they show evidence that nitric oxide produced by complex IV triggers changes that elicit lifespan extension. Several mechanisms have been proposed to underlie the increase in lifespan by DR, an increase in the deacetylase activity of sirtuins and an augmentation of mitochondrial biogenesis being two of them. G. R. Wagner and R. M. Payne in "*Mitochondrial acetylation and diseases of aging*" discuss the role of mitochondrial deacetylases in aging. The importance of mitochondrial sirtuins has recently been documented by articles describing their essential role in the activation of protection against oxidative damage during DR [12, 13]. Mitochondrial biogenesis is another leading topic in aging research. One of the major regulators of this process is peroxisome-proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). T. Wenz in "*Mitochondria and PGC-1α in aging and age-associated diseases*" discusses the alteration of mitochondrial biogenesis in aging and how PGC-1α can be used as a novel therapy. New treatments are needed to protect against neurodegenerative diseases, one of the most devastating consequences of aging. Calcium is a key player in the intercommunication of different cellular organelles as reviewed by J. P. Decuypere et al. in "*IP_3 receptors, mitochondria, and Ca^{2+} signaling: implications for aging.*" Upon intercommunication damage, cellular homeostasis is lost, resulting in cellular death or transformation. R. Kumar and H. Atamna in "*Therapeutic approaches to delay the onset of Alzheimer's disease*" propose two complementary approaches to delay the onset of Alzheimer's disease. Boosting mitochondrial metabolism should decrease amyloid-β peptide accumulation. Coincidentally, exercise increases mitochondrial function and decreases the concentration of markers of Alzheimer's disease [16]. Deregulation of metal transition homeostasis is increasingly recognized as a major problem during aging [17].

If the past is described as good and the present as promising, then the future will likely be very bright. However, there are many challenges that must be overcome to understand completely the role of mitochondria in aging and enable the development of new therapeutic approaches. The development of new techniques to study mitochondrial function *in vivo* is of the utmost priority. Most of our knowledge about how mitochondria work is based on data from isolated organelles. As *in vitro* systems are prone to artifacts, an *in vivo* approach would prove much more reliable. Cell culture offers an alternative approach, but is restricted by the fact that oxygen pressure is a major determinant of mitochondrial function and levels used are often not representative of physiological concentrations. For success, we must aim for excellence. Therefore, we must strive for the situation which enables the quantification of measurements in model organisms and human patients *in vivo*. Difficult? Maybe. Possible? Yes. In summary, for all of us interested in understanding these fascinating organelles, we are living in exciting times. But if we work hard, the future will be even more promising.

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Review Article

DNA Damage and Base Excision Repair in Mitochondria and Their Role in Aging

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During the last decades, our knowledge about the processes involved in the aging process has exponentially increased. However, further investigation will be still required to globally understand the complexity of aging. Aging is a multifactorial phenomenon characterized by increased susceptibility to cellular loss and functional decline, where mitochondrial DNA mutations and mitochondrial DNA damage response are thought to play important roles. Due to the proximity of mitochondrial DNA to the main sites of mitochondrial-free radical generation, oxidative stress is a major source of mitochondrial DNA mutations. Mitochondrial DNA repair mechanisms, in particular the base excision repair pathway, constitute an important mechanism for maintenance of mitochondrial DNA integrity. The results reviewed here support that mitochondrial DNA damage plays an important role in aging.

1. Introduction

Our DNA, both nuclear and mitochondrial, is constantly exposed to endogenous and exogenous agents that induce DNA lesions and DNA instability, which includes blockage of DNA replication and transcription as well as chromosomal rearrangements [1]. In order to maintain genomic integrity, different DNA repair pathways have evolved in cells. Without efficient cellular DNA repair mechanisms, DNA stability and cellular survival are seriously compromised. DNA repair occurs both in prokaryotes and eukaryotes. In eukaryotes, DNA repair mechanisms have been described to take place not only in the nucleus, but in all cellular compartments containing DNA. Although these mechanisms have mostly been investigated in the nucleus, our knowledge regarding mitochondrial DNA repair pathways has significantly increased during the last decade. Because DNA repair pathways are important determinants of DNA stability, they are thought to play an important role in the aging process [2–4].

DNA repair mechanisms have been extensively investigated in the nucleus. Depending upon the type of DNA lesion that has been generated, a specific DNA repair pathway proceeds. Thus, bulky lesions, which are induced on DNA by UV light as well as by carcinogenic compounds, are removed by the nucleotide excision repair (NER) pathway, whereas DNA lesions such as oxidation products generated by ROS are repaired by the base excision repair (BER) pathway [5]; on the other hand, mismatches in DNA are removed by the mismatch repair pathway (MMR) and lesions such as interstrand cross-links are processed by recombinational DNA repair [1]. The relevance of the DNA repair pathways in the maintenance of the genome integrity and cellular survival is stressed by the critical consequences in the survival of the organisms when deficiencies in key enzymes of the DNA repair pathways occur [1, 2]. Moreover, several homologues between prokaryotes and eukaryotes have been reported among the different components of the DNA repair pathways [6], and proteins of the main pathways are highly conserved [3, 7–9].

Mitochondrial DNA (mtDNA) mutations and mitochondrial dysfunction are thought to play an important role in the aging process [10–13], and increased levels of mutations in DNA have been described to occur in brain and other tissues during normal aging leading to DNA instability [14–16]. According to the mitochondrial-free

radical theory of aging, the accumulation of reactive oxygen species-(ROS-)-induced mtDNA damage and mtDNA mutations over time are the main contributors to deleterious changes leading to cellular dysfunction and aging [17, 18].

In eukaryotic cells, the integrity of mtDNA is constantly challenged by the endogenous production of mitochondrial ROS, which are generated during mitochondrial respiration. ROS are particularly important genotoxic agents and can generate a large number of DNA lesions, including oxidized DNA bases, abasic sites, and single- and double-strand breaks. Because many of the ROS-induced DNA lesions show mutagenic or cytotoxic effects, mitochondrial-free radicals are thought to be an important source of DNA mutations and DNA instability, particularly in mitochondrial DNA. The proximity of mtDNA to the main sites of mitochondrial ROS generation, located within complexes I and III of the mitochondrial electron transport chain in the inner mitochondrial membrane, is the major reason for the higher steady-state levels of oxidative lesions and the higher instability observed in mtDNA when compared to nuclear DNA [19, 20]. For many years, such high level of oxidative lesions in mtDNA was considered to be a consequence, at least in part, of (i) the absence of efficient DNA repair mechanisms in mitochondria and (ii) the lack of protective histones in mtDNA. However, different studies have shown that mtDNA is organized into protein-DNA complexes called nucleoids, where DNA has been described to be associated with different proteins and anchored to the inner mitochondrial membrane [21–23]. These membrane-associated structures may provide more protection to the mtDNA against oxidative attack than was formerly thought. On the other hand, it is now well established that various DNA repair pathways actively take place in mitochondria. The initial studies on mitochondrial DNA repair capacity focused on UV light-induced DNA damage repair, and they provided the basis for the notion that mitochondria lacked functional DNA repair mechanisms [24]. Thirty years later, it is known that various DNA repair pathways do take place in mitochondria [25]. Most of the studies on mitochondrial DNA repair have focused on the BER mechanisms, which are the DNA repair pathway coping with oxidative lesions and it is the best-characterized DNA repair pathway in mitochondria [26, 27].

2. DNA Repair and Mitochondria

As mentioned above, DNA repair research has focused mainly on the nuclear compartment; however, the investigations performed in the last two decades have confirmed that mitochondria do possess effective DNA repair mechanisms, and the understanding of how these mechanisms function has significantly increased in the last few years. The first DNA repair pathway that was described to actively take place in mammalian mitochondria was the BER pathway. Today, other DNA repair mechanisms that were thought to occur exclusively in the nucleus have been described to take place in mammalian mitochondria such as MMR [28, 29] and the long-patch BER [30–32]. Moreover, classical nuclear DNA repair enzymes have been identified in mitochondria [28, 31, 33–35], with similar function than the one observed in the nucleus [31, 35], or with novel functions according to its mitochondrial location [33].

However, and despite the increased knowledge in mtDNA repair, important questions still remain opened, such as the precise mechanisms for the repair of some specific lesions in mtDNA such as double-strand breaks or DNA adducts derived from lipid peroxidation. The latter are likely generated in mtDNA, since mitochondrial phospholipids are main targets of mitochondrial-free radicals [36] and mtDNA is associated with the inner mitochondrial membrane; DNA adducts derived from lipid peroxidation are known to be processed by NER [37, 38], but whether mitochondria possess the capacity to repair DNA lesions others than UV light-induced lesions via the NER pathway remains to be elucidated.

3. Mitochondrial Base Excision Repair

BER is the primary pathway known for repair of small DNA modifications caused by alkylation, deamination, or oxidation. BER takes place both in the nucleus and in mitochondria, and although the mechanisms are similar, mitochondria possess an independent BER machinery. Both nuclear BER and mtBER are based on a cascade of reactions starting with the recognition of the damage followed by enzymatic processing steps that aim to remove the lesion and restore genomic integrity. All the components of the mtBER pathway are nuclear encoded and imported into mitochondria [39]. The BER pathway includes 4 distinct steps: (i) recognition and removal of the modified base, (ii) processing of the generated apurinic/apyrimidinic (AP) site, (iii) incorporation of the correct nucleotide(s), and (iv) strand ligation.

3.1. Mitochondrial BER: Early Steps, Recognition, and AP Site Processing. The first step of BER is catalyzed by DNA glycosylases, which recognize the modified base and cleave the N-glycosidic bond, creating an abasic site. In addition to the cleavage activity towards the N-glycosidic bond, some DNA glycosylases also have AP lyase activity, which allows the cleavage of the DNA backbone. These DNA glycosylases are called bi-functional glycosylases while those possessing only cleavage activity are named monofunctional. Oxidized bases are generally removed by bifunctional DNA glycosylases. Among all the different DNA glycosylases that have been described to be present in the nucleus, only some of them have been detected in mitochondria [26, 40]. As the rest of enzymes involved in mtBER, nuclear genes encode mitochondrial DNA glycosylases. Mitochondrial and nuclear isoforms of the same DNA glycosylases are encoded by the same gene and are generated by alternative transcription initiation sites and alternative splicing [41]. That is the case of two of the main mitochondrial DNA glycosylases, 8-oxoguanine DNA glycosylase-1 (Ogg1) and Uracil-DNA glycosylase (UNG).

UNG was the first DNA-glycosylase to be identified [42]. Uracil in DNA is generated by deamination of cytosine or by misincorporation of dUMP. The removal of uracil

from DNA is crucial due to its ability to pair with adenine, causing GC to AT transition mutations upon replication. Despite UNG was the first DNA-glycosylase investigated, it is Ogg1 the one that has been investigated most extensively. Ogg1 is a bifunctional DNA glycosylase that recognizes and cleaves 8-hydroxi-guanine (8-oxoG) from double-strand DNA. For many years, this DNA lesion has been the most broadly used as DNA damage marker, both in nuclear and mitochondrial DNA, mainly because 8-oxoG is relatively easy to detect and measure. However, it does not necessarily mean that 8-oxoG is the most frequent and biologically relevant DNA lesion. Actually, oxidative DNA lesions such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formamidopyrimidine (FapyA) have been detected in genomic DNA at similar or even higher levels than 8-oxoG. Recently a new group of DNA glycosylases, NEIL glycosylases, has been identified in mammalian tissues primarily excising FapyG and FapyA. These glycosylases are homologous of the *Escherichia coli* DNA glycosylases Fpg/Nei and three main isoforms, NEIL1, NEL2, and NEIL3, have been described [43, 44], being present both in nucleus and mitochondria [34, 44, 45]. Moreover, some of them have been involved in repair mechanisms of oxidative lesions in transcribed or replicating DNA [46]. The identification of this group of DNA glycosylases in mammals was of great interest, particularly when the knock-out (KO) mouse for NEIL1 was developed [47]. Due to the partial redundancy in the activity of DNA glycosylases, KO mice for most of them do not exhibit a significant phenotype. However, KO mice for NEIL1 accumulate mtDNA deletions to a higher extent than wild-type mice and develop symptoms associated with the metabolic syndrome [47].

After recognition and cleavage of the modified or erroneous base by the specific DNA glycosylase, an abasic site is generated. Repair of these lesions shares common steps with that of ROS-induced DNA single-strand breaks and spontaneously generated AP sites. These DNA lesions are continuously generated and various enzymatic processes have evolved to repair them. Among those processes, AP endonuclease (APE) is the most important and ubiquitous [48]. Although two isoforms of APE, APE1 and APE2, have been described in mammalian tissues, APE1 is the main AP endonuclease in mammalian cells taking part of the BER processes both in nucleus and mitochondria [49]. The absence of significant APE-specific activity of the recombinant APE2 has suggested that it functions in other cellular processes different than BER [50]. Together with its activity in DNA repair processes, APE1 also has a redox activity that is involved in signal transduction. APE1 participates in several important cellular mechanisms such as apoptosis, proliferation, and differentiation functioning as a transcriptional coactivator [51–53]. Unlike most of the known enzymes taking part in the BER process in mitochondria, mitochondrial APE nature remains unclear. It is still discussed whether the mitochondrial APE is an N-terminal truncated product of APE1 [54]. The deletion of the N-terminal residues containing the nuclear localization signal has been described to induce a 3-fold increase in the specific activity of APE1 [55], and APE activity is higher in mitochondrial fractions than in nuclear fractions. However, the presence of this truncated isoform of APE1 has only been reported in bovine liver mitochondria, while no truncated product has been observed in mitochondrial extracts of different human cell lines [55]. The relevance of APE1 in mammalian cells is stressed by the reports showing a significant increase in single-strand breaks as well as AP sites when APE1 is inactivated [56, 57]. Moreover, and in contrast to DNA glycosylase KO mice, cellular survival is critically compromised in the absence of APE1, and KO mice for APE1 show embryonic lethality [58].

3.2. Mitochondrial BER: Final Steps, Short- and Long-Patch BER. Once the AP site has been processed by APE1, the following step in the BER pathway is catalyzed by a DNA polymerase, which inserts the correct nucleotide(s) in the generated gap. There is only one known DNA polymerase in mammalian mitochondria, the DNA polymerase gamma (polγ), which is involved in all replication and repair of mtDNA. Two different BER subpathways exist depending on whether polγ inserts one nucleotide or more: short-patch (SP) or long-patch (LP) BER [26]. During the SP-BER, one single nucleotide is incorporated into the gap by polγ, while the LP-BER involves the incorporation of several nucleotides, typically 2 to 7. Long-patch BER processing of a DNA lesion is more complex than SP-BER, since the incorporation of various nucleotides results in the exposure of the original DNA strand as part of a single-strand overhang or flap structure. Additional enzymatic activities are required in order to process such structure [27]. In the nucleus, the flap structure is recognized and cleaved by the flap endonuclease 1 (FEN-1), which is an essential enzyme for LP-BER [59]. Why BER proceeds through the short- or long-patch is not completely understood. It seems to depend, at least in part, on the type of lesion and on the intermediates that are generated during the process. For instance, AP sites can be further oxidized, and this sort of DNA lesion requires to be processed by LP-BER [60].

Until recently it was believed that SP-BER occurred both in nucleus and mitochondria, whereas LP-BER only took place in the nucleus. However, considering the constant exposure of mtDNA to mitochondrial-free radicals, it was likely that certain types of lesions, such as oxidized AP sites, were generated at a significant rate in mtDNA [60]. Therefore, SP-BER would be insufficient to cope with all the collection of lesions inflicted in mtDNA. Various investigations have recently shown that, likewise nuclear BER, mitochondrial BER can also progress in a long-patch manner [30–32, 61]. Furthermore, and similarly to nuclear long-patch BER, different studies support that FEN-1 plays an important role in mitochondrial LP-BER [31, 61]. Besides, during the processing of the 5′ flap structure FEN-1 interacts with Dna2, a helicase/nuclease protein, in human HeLa cell mitochondria [35].

The final step of the mitochondrial BER pathway, the nick sealing, is catalyzed by a DNA ligase. While two DNA ligases are described in the nucleus (I and III), in mammalian mitochondria only DNA ligase III has been detected, acting both in replication and repair. DNA ligase

III is a splice variant from the LIG3 gene encoding for both the nuclear and mitochondrial enzymes. The mechanism of action as well as the interaction with other proteins of the mitochondrial variant of DNA ligase III is still unclear.

3.3. Mitochondrial BER Proteins, Organization, and Interactions. Some of the proteins involved in mtDNA repair have been described to be components of the nucleoids [62, 63]. One of the essential components of the nucleoids is the mitochondrial transcription factor A (TFAM) that plays a significant role in the organization and structure of the mitochondrial nucleoids [64, 65]. TFAM has recently been associated with mtBER activity, modulating DNA repair efficiency in mitochondria [66]. Likewise, essential proteins of the mtBER like polγ, in particular its accessory subunit polGβ, have also been described to be key factors in the organization of these nucleoprotein complexes [67]. Investigations on how the organization of mtDNA in mitochondria affects and modulates BER is increasing, and recent studies have suggested that mtDNA association to the inner membrane is critical for proper base excision repair [68]. Moreover, the cockayne syndrome group B (CSB) protein, which was thought to be present exclusively in the nucleus, has been recently localized to mitochondria [33]. Mutations in the CSB gene are responsible for the Cockayne syndrome, a segmental premature aging syndrome [69]. The Cockayne syndrome is associated with severe development deficiencies and neurodegeneration. The CSB protein plays a role in genomic maintenance and transcription regulation in the nucleus, and it had also been suggested to play a role in mtBER, although the mechanism was obscure [4]. Recent studies have indicated that CSB may play a role in general mitochondrial maintenance [70]. Interestingly, the investigation on CSB protein and its location in mitochondria have suggested that CSB protein could play a direct role in mtBER by interacting and stabilizing BER proteins in the protein-DNA complexes associated with the inner mitochondrial membrane when mtDNA repair takes place [33].

4. Mitochondrial DNA Damage, DNA Repair, and Aging

The increased susceptibility to cellular loss and the progressive functional decline observed during aging has been associated with accumulation of damage to macromolecules, particularly to mtDNA [10, 13]. According to the mitochondrial-free radical theory of aging free radicals generated by mitochondria are the main contributors to the accumulation of damage. Over the years, several investigations have linked mitochondrial ROS production and accumulation of mtDNA mutations to the aging process, and because mtDNA repair mechanisms are essential in order to avoid increased accumulation of mtDNA mutations, they are thought to play an important role in the aging process [2]. Although mitochondrial ROS and mtDNA mutations were supported as main contributors of aging mainly by descriptive data and correlative studies, the generation of a knock-in mouse expressing proof-reading deficient polγ, but conserving its replicative function, is considered by some authors as an important support of accelerated mtDNA mutation rate resulting in increased aging rate [12, 71]. This mouse model, the mutator mouse, appears as an interesting model in which to investigate the causative link between mtDNA mutation accumulation and aging, and the role that mtBER may play in the process [72, 73]. In various tissues of this mouse model, mtDNA point mutations as well as mtDNA deletions have been described to accumulate at a much higher rate than in the wild-type mice [12, 71, 74]. However, how the accumulation of mtDNA mutations lead to the loss of mitochondrial function and aging is still discussed [74]. Investigations on brain and heart samples have suggested that it is the accumulation of large mtDNA deletions and clonal expansion, instead of mtDNA point mutations, that drive the premature aging phenotype [16, 75]. However, after performing research on hepatic and cardiac tissues, Edgar et al., recently reported that circular mtDNA molecules with large deletions represent only a minor proportion of the total mtDNA in this mouse model and suggested that random point mutations occurring in mtDNA are the driving force behind the premature aging phenotype [76]. Discrepancies could be due, at least in part, to the different mechanisms that have been suggested to be responsible of the generation of mtDNA mutations in postmitotic and mitotic tissues [77]. It has been proposed that in postmitotic cells, such as neurons, mtDNA deletions occur primary during repair of damaged DNA, whereas in mitotic tissues mtDNA point mutations are more likely to be caused during replication [78]. This could lead to significant differences in the type of mtDNA mutations being detected in postmitotic and mitotic cells during normal aging. Thus, for substantia nigra neurons from aged individuals and Parkinson's disease patients, direct evidence exists of mitochondrial dysfunction due to accumulation of mtDNA deletions [79, 80], while mtDNA point mutations have been reported to occur rarely in these neurons during aging [77]. If mtDNA deletions are actually generated during repair of damage mtDNA, alteration in mtBER fidelity with age would play an important role in mtDNA deletion accumulation and aging. A recent study has reported the presence of large linear mtDNA fragments in liver mitochondria in the mutator mouse model [81]. The authors suggest that due to the increased errors inserted during DNA synthesis, replication arrest would occur as a result of an effort to execute DNA repair. In a scenario of a high mutant load, replication pausing would be extended and strand breakage would likely occur at pause sites, leading to formation of mtDNA linear fragments. Although the level of these mtDNA fragments does not increase with age in the mutator mice, Bailey et al. [81] suggest that the long-term impact of aberrant mitochondrial DNA replication might contribute to the premature aging phenotype of these mutator mice. Moreover, they suggest that as a result of aberrant mitochondrial replication, essential factors involved in DNA metabolism would be mobilized to mitochondria and eventually would become exhausted. Because nuclear and mitochondrial DNA metabolism share several of those essential factors, the collapse of DNA factors in mitochondria could probably have an impact on nuclear DNA metabolism, contributing to the premature aging phenotype.

The impact of mtDNA deletions on nuclear DNA has also been suggested as a potential determinant of aging in different organisms. Various studies have investigated whether the migration of mtDNA sequences from mitochondria to the nucleus could be relevant in the aging process. Gene transfer from organelles to the nucleus has occurred repeatedly during eukaryote evolution [82], but inappropriate *de novo* insertion of mtDNA fragments into human chromosomal DNA has been reported to result in rare severe genetic diseases [83].

In the filamentous ascomycete fungus *Podospora anserina*, mtDNA instability is thought to play an important role in the aging process [84] and rearrangements of the mtDNA are considered as a hallmark of senescence in wild-type strains. These rearrangements include the systematic amplification of the first intron of the cytochrome c oxidase subunit-I gene as circular DNA molecules [85]. Twenty years ago, integration of these mtDNA plasmids within the nuclear genome was reported to take place during senescence in *P. anserina* [86]. In the last few years the hypothesis of insertion of mitochondrial DNA fragments into nuclear DNA as aging factor [87] has been tested by different research groups. It has been reported that migration of mitochondrial DNA fragments into the nucleus affects the survival rates in *Saccharomyces cerevisiae* [88]. In *S. cerevisiae* mtDNA fragments are initially present as circular molecules in the nucleus, and the frequency of integration events into chromosomal DNA increases during lifespan [88]. Cheng and Ivessa have reported that in *S. cerevisiae* mutants with a higher translocation rate of mtDNA fragments into the nucleus than in wild-type strains, chronological lifespan is reduced, while in mutants showing a reduced translocation rate of mtDNA fragments in the nucleus, the chronological lifespan is extended. Moreover, Caro et al. [89] have reported that mitochondrial DNA sequences into nuclear DNA accumulate with aging in rat liver and brain. Furthermore, the integration of the mitochondrial sequences seems to occur at specific sites of the chromosomes instead of randomly.

Taken together, it appears likely that mtBER has an important role in the aging process due to its role avoiding mtDNA mutations. Caloric restriction (CR) is a well-known experimental manipulation that reduces the accumulation rate of mtDNA mutations [90, 91] and increases maximum life span in several species [92]. Various investigations have studied the role of DNA repair mechanisms on the beneficial effects of CR. Caloric restriction has been reported to promote genomic stability, at least in part, by a general enhancement of nuclear DNA repair mechanisms [93]. However, although total [94] and nuclear [95, 96] BER capacity in different rat brain regions and tissues have been shown to be increased in CR animals when compared to *ad libitum* fed animals [94], investigation of the BER activities in the mitochondrial compartments has shown that mtBER capacity did not change in liver and even decreased in the brain and kidney of caloric restricted rats. [96]. The lower mtBER capacity observed in restricted rats would be in agreement with the fact that mitochondria from caloric restricted rodents have been shown to generate ROS and accumulate oxidative damage to mtDNA at lower rates than *ad libitum* fed animals [92]. Hence, when the generation of mitochondrial ROS and the levels of mtDNA damage are significantly reduced, it may be possible for the organism to invest less energy in mtDNA repair capacity without negative consequences.

On the other hand, different studies have reported that deficiencies and changes in mitochondrial BER fidelity occur during aging, particularly in postmitotic tissues. In vertebrates, the most important postmitotic tissue is probably the brain, due to its main role in homeostasis of the organism. Mitochondrial BER capacity has been described to be organ-specific, with the brain being one of the tissues with the lowest capacity [97]. Various studies have reported an age-related decline in DNA glycosylase activities brain cortical mitochondria in rats [98] and mice [45, 99], suggesting that mtBER may play a critical role in the maintenance of the central nervous system during aging [100]. In addition, important differences in mtBER have been observed among various brain regions during aging [45]. The cortical region and the cerebellum have been described to accumulate less mtDNA lesions with aging and to be more resistant to oxidative stress conditions [101]. Interestingly, those regions showed higher BER capacity than hippocampus, which has been described to be a much more vulnerable region in the brain [101, 102].

A recent investigations has reported that the age-related decline in brain mtBER occurs specifically at the synapses [103], stressing the relevance of the central nervous system heterogeneity in these processes. Moreover, deficiencies in mtBER in skeletal muscles during aging have been suggested to contribute to age-related sarcopenia [104]. Finally, potential age-related changes in mtBER have been investigated in other aging models such as *P. anserina* as well; again, aging was associated with a decrease in mitochondrial BER [7], probably contributing to the observed mtDNA instability in the aged fungi [105].

In conclusion, several investigation support that age-related mtBER impairment and increased mtDNA instability contribute to aging. Further investigations are required to clarify the precise mechanisms and whether other DNA repair mechanisms mitochondrial and/or nuclear are also involved in the aging process. Moreover, better knowledge of how mitochondrial DNA repair mechanisms function will help to find strategies to potentially retard mtDNA mutation generation and hence cellular dysfunction observed during aging.

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Review Article

The Importance of Mitochondrial DNA in Aging and Cancer

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Mitochondrial dysfunction has been implicated in premature aging, age-related diseases, and tumor initiation and progression. Alterations of the mitochondrial genome accumulate both in aging tissue and tumors. This paper describes our contemporary view of mechanisms by which alterations of the mitochondrial genome contributes to the development of age- and tumor-related pathological conditions. The mechanisms described encompass altered production of mitochondrial ROS, altered regulation of the nuclear epigenome, affected initiation of apoptosis, and a limiting effect on the production of ribonucleotides and deoxyribonucleotides.

1. Introduction

Mitochondria are semiautonomous organelles present in almost all eukaryotic cells in quantities ranging from a single copy to several thousands per cell. Important mitochondrial functions include ATP production by oxidative phosphorylation, β-oxidation of fatty acids, and metabolism of amino acids and lipids. Furthermore, mitochondria have a prominent role in apoptosis initiation. The circular mitochondrial DNA (mtDNA) is more susceptible to DNA damages in comparison to nuclear DNA (nDNA). Importantly, mtDNA molecules are not protected by histones, they are supported with only rudimentary DNA repair and are localized in close proximity to the electron transport chain (ETC), which continuously generates oxidizing products known as reactive oxygen species (ROS). Thus, the mutation rate of mtDNA has been reported to be up to 15-fold higher than observed for nDNA in response to DNA damaging agents [1].

Mitochondrial dysfunction and especially dysfunctions caused by mutations of the mtDNA have been implicated with a wide range of age-related pathologies, including cancers, neurodegenerative diseases and, in general, processes that regulate cellular and organismal aging. The mitochondrial genome encodes peptides essential for the function of the ETC and production of ATP by oxidative phosphorylation. Electrons are primarily donated to the ETC from the Krebs cycle, but other sources also contribute. The human enzyme dihydroorotate dehydrogenase (DHODHase), an integral part of the *de novo* synthesis of pyrimidines, is coupled to the ETC [2, 3]. The activity of the enzyme is dependent on its ability to transfer electrons to the ETC. ATP is the primary product of oxidative phosphorylation, but certain molecules of ROS are also generated continuously [4, 5]. At subtoxic concentrations, ROS has been demonstrated to function as second messenger molecules proposed to report oxygen availability for oxidative phosphorylation and energetic yield, affecting epigenetic marking of nDNA and regulating nuclear transcription factors, kinases, and phosphatases as reviewed by Weinberg and Chandel [6]. However, at increased levels, ROS induces oxidative damage to lipids, proteins, RNA, and DNA.

Mutation of mtDNA has been correlated with aging and cancer. Mutations of mtDNA that results in an aberrant expression of mitochondrial encoded ETC subunits have been demonstrated to impair the activity of the ETC and be correlated with a decreased capacity for oxidative phosphorylation [7, 8]. In turn, as will be reviewed, ETC dysfunction has been demonstrated to affect the production of ATP and ROS, alter the expression of several nuclear genes, affect the regulation of proteins, and disturb the synthesis of cellular nucleotides. The focus of this paper is on the immediate effects of mtDNA alterations and their potential role in premature aging and tumor progression.

2. mtDNA Fidelity Is Correlated with Aging and Cancer

The lifespan of both mice and aging human cultured cells has been associated with decreases in the number of mitochondria and changes of mitochondrial morphology [9–11]. These alterations are accompanied by an accumulation of mutations in the mtDNA [12–16]. Accordingly, an age-related decline of mitochondrial capacity for oxidative phosphorylation has been demonstrated in both human skeletal muscle and rat hearts [7, 8, 17].

Increasing evidence suggests an important role of accumulating mtDNA mutations in the pathogenesis of many age-related neurodegenerative diseases as well as a number of age-related pathological alterations of heart, skeletal muscle, and the vascular system [18–21]. A strong correlation between the fitness of mtDNA and age-related pathologies have been demonstrated with the independent construction of two mouse lines expressing mutated versions of the mitochondrial polymerase gamma. Both mouse lines developed a mtDNA mutator phenotype, linking the increase of somatic mtDNA mutations with symptoms of premature aging and reduced lifespan [22, 23]. Furthermore, in a longevity study, human life length could be associated with the life length of the mother but not the father, suggesting an influence of the maternal inherited mitochondrial genome [24, 25].

Warburg formulated a relationship between mitochondria and cancer with the discovery that most tumors relied on ATP production by glycolysis rather than oxidative phosphorylation [26]. Alterations of mtDNA have been correlated with tumor progression and have been reported in a variety of cancers including ovarian, thyroid, salivary, kidney, liver, lung, colon, gastric, brain, bladder, head and neck, and breast cancers [27–29]. Reported alterations include point mutations, deletions, and depletions. Alterations of mtDNA might merely be a consequence of tumor progression, however, it has been demonstrated that the invasive phenotype of human cells depleted of mtDNA can be reversed by reintroducing exogenous wild-type mitochondria [30, 31]. Furthermore, construction of a cybrid cell line of prostate cancer cells harboring specific mtDNA mutations has in nude mice been demonstrated to have a growth advantage over cybrids of prostate cells with functional mtDNA [32, 33]. As a result, cybrid cancer cells with mtDNA mutations have the potential of forming a tumor 7-fold larger than cybrid cancer cells with functional mtDNA [32]. Together these indications suggest that mtDNA alteration is directly involved in tumor progression and not merely a consequence of it.

3. Accumulation of mtDNA Mutations

The circular human mtDNA are present in several copies in each mitochondrion. The mtDNA molecule consists of 16539 base pairs that constitute 37 genes encoding 22 tRNAs, 2 rRNAs, and 13 polypeptides. The encoded polypeptides comprise few but essential subunits of the ETC and ATP synthase [34, 35]. The ETC maintains an electrochemical potential gradient between the intermembrane space and the matrix of the mitochondria. This gradient is utilized by the ATP synthase to generate ATP by oxidative phosphorylation. The ETC consists of four membrane-bound enzyme complexes (complex I–IV), two electron carriers (ubiquinon and cytochrome c), and is located in the inner membrane of the mitochondria. Mutations in mtDNA affect the function of the ETC, the electrochemical gradient, and the generation of ATP by oxidative phosphorylation. Furthermore, mutations of mtDNA can result in an elevated production of ROS.

Somatic mutations in the mtDNA have been suggested to accumulate during the life span of humans. Accordingly, it has been demonstrated that brain, muscle, heart, and skeletal muscle of aging humans harbor an increased mutational load of the mitochondrial genome when compared to corresponding tissues of young [5, 8, 12, 36]. The age-correlated accumulation of mtDNA mutations has been proposed to be the cumulative result of a mitochondrial vicious circle and a preferential accumulation of specific mtDNA mutations [37]. According to the theory of a mitochondrial vicious circle, mutations in either nuclear or mitochondrial genes encoding subunits of the ETC will impair electron transfer leading to increased ROS production [38]. Consequent oxidative damage to mtDNA induces alterations of the mtDNA-encoded polypeptides of the ETC leading to further ROS production, thus establishing a vicious circle resulting in an accumulation of mtDNA mutations.

In a variety of diseased and aging tissue, cells of the tissue are a mosaic of cells containing non-mutated mtDNA molecules and cells containing mutated mtDNA molecules [16, 36, 39, 40]. Cells containing mutated mtDNA are often homoplasmic indicating that the mutations are not the result of random oxidative damages from mitochondrial ROS. Rather, these mutations are the consequence of a preferential accumulation of a specific mutated mtDNA molecule, which has become the predominant species within a single cell. The mechanism of this selection is unknown, however it has been hypothesized that mitochondria containing mutated mtDNA causing a reduced respiratory function will have a lower production of ROS and consequently have a lower risk of oxidative damage of the mitochondrial membrane. Mitochondria with a reduced respiratory function will therefore have a lower risk of suffering lysosomal degradation allowing the affected mitochondria to populate the cell [41]. In contrast, *in Silico* models have demonstrated the potential of a single mutated mtDNA molecule to clonally expand in postmitotic cells without the requirement of any types of selection but rather through random genetic drift in a pool of replicating mtDNA molecules [42]. Age-related accumulation of mtDNA mutations is therefore likely either a result of a mitochondrial vicious circle, preferential accumulation of specific mtDNA mutations, or the cumulative outcome of both factors.

4. Mitochondria-Produced ROS Can Damage Cellular Components

More than 90% of cellular oxygen uptake is utilized in the process of oxidative phosphorylation which continuously

generates ROS such as superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$), [5, 43]. ROS are generated to an extent of 1–4% of the oxygen consumed by the mitochondria [4, 5]. Complex I and especially complex III are the prime sites for electron leakage to molecular oxygen yielding $O_2^{\cdot -}$ [44, 45]. The production of ROS is inversely correlated with the rate of electron transport, increasing exponentially when complex I or III are impaired [46]. The main mediator of electron leakage is the reduced form of ubiquinone, ubiquinol, which is able to reduce molecular oxygen [47]. In order to neutralize the produced ROS, a number of antioxidant defenses are active within the mitochondria. $O_2^{\cdot -}$ is neutralized by intramitochondrial Mn superoxide dismutase (SOD2) catalyzing the formation of H_2O_2. The latter either diffuses out of the mitochondria or is inactivated by reaction with glutathione catalyzed by glutathione peroxidase [48–50]. If the amount of produced ROS exceeds the capacity of SOD2 and glutathione peroxidase, $O_2^{\cdot -}$ and H_2O_2 levels will rise. In the presence of transition metals, such as iron or copper, highly reactive $OH^{\cdot}$ can be produced by Haber-Weiss or Fenton reactions. $OH^{\cdot}$ can in turn give rise to a plethora of ROS, which can further damage proteins, lipids, and DNA [43, 51, 52].

The mitochondrial production of ROS is an essential component of the free radical theory of aging formulated by Harman [53–55]. According to the theory, mitochondria-produced ROS induces oxidative damage to lipids, proteins, and DNA in mitochondria and potentially in nucleus. Consequently, ROS production is considered one of the causes of aging and age-related pathologies [38, 56–58] and a contributing factor in the formation of cancers [59, 60]. In support of this theory it has been demonstrated that inactivation of the *Sod2* gene in mice lead to a 2-3-fold increase of oxidative damages of the nuclear DNA in heart and brain tissue when compared to mice expressing SOD2 [5]. Furthermore, it has been demonstrated that an overexpression of a human catalase targeted for the mitochondria, prolonged median and maximal lifespan of mice by approximately 20% and enhanced exercise performance when compared to wild-type littermates [61, 62]. Mice overexpressing the mitochondrial targeted catalase did not display any adverse side effects, but rather a decrease in a select group of age-related pathologies [63]. Induced mutations in genes encoding subunits for complex II have been demonstrated to result in increased production of $O_2^{\cdot -}$ and H_2O_2 in hamster fibroblasts. The increased production of ROS was cooccurring with an increase of aneuploidy that could be reversed by expression of complex II subunits without mutations [59]. This led the authors to augment that the increase of mitochondria-produced ROS resulted in genomic instability.

5. Mitochondrial ROS Functions as Second Messenger Molecules

Despite having been described mainly as a detrimental byproduct of oxidative phosphorylation for more than 50 years, it has become evident that certain types of ROS function as second messengers under subtoxic concentrations. As such, ROS has been demonstrated to regulate gene expression by controlling transcription factors and to affect protein activity by regulating kinases and phosphatases as reviewed by Weinberg and Chandel [6]. The regulative effects of ROS are exerted through their redox potential. As an example, several ROS sensitive molecules contain cysteine rich proteins, in which ROS-induced oxidation can result in formation of disulfide bonds in the same molecule or between two cysteine rich molecules. The formation of disulfide bonds can therefore lead to conformational changes of a molecule or result in the dimerizations of two or more molecules, thereby modulating activation and activity of the molecules [64].

Molecules that are regulated by ROS have been demonstrated to be involved in cell survival, cell cycle control, apoptosis, differentiation, and several stress responses. Abnormal signaling elicited by aberrant ROS production can therefore affect essential pathways of the cell, potentially initiating an incorrect cellular response to a given situation, increasing the risk of senescence or tumorigenesis [6, 65]. Mitochondria-produced ROS have been demonstrated to affect the transcription factor hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimer consisting of the constitutively expressed subunit HIFβ and the oxygen sensitive subunit HIF-1α. Upon expression HIF-1α is marked by ubiquitination and subsequent degraded by the proteasome [66]. However, an increased production of mitochondrial ROS has been demonstrated to stabilize HIF-1α, allowing the subunit to dimerize with HIFβ and forming the active nuclear transcription factor. Active HIF-1 is known to regulate the expression of glycolytic enzymes, the angiogenic factor VEGF and affect pathways promoting apoptosis [67–69] and aberrant activity of HIF-1 has been correlated with tumorigenesis as reviewed by Weinberg and Chandel [6]. Furthermore, loss of HIF-1α has been demonstrated to accelerate premature cellular senescence in mice [65].

Mitogen-activated protein kinases (MAPK) are a superfamily of serine/threonine protein kinases that function as signal transducers that propagate stimuli from growth factors and a wide variety of cellular and extracellular stresses [70]. The MAPKs are critical for correct regulation of gene expression, cell cycle progression, apoptosis, and other cellular activities in response to these stimuli. Mitochondria-produced ROS has been demonstrated to induce phosphorylation and thereby activation of the p38 MAPK in cardiomyocytes [71]. Furthermore, the ERK1/2 MAPK is activated by exogenously added H_2O_2 [72] and in eosinophils this activation is inhibited if the cells are treated with rotenone, an inhibitor of complex I of the ETC [73]. The authors interpreted this to the importance of mitochondrial respiration in activation of ERK1/2, however, rotenone is demonstrated to induce the production of mitochondrial ROS [74], and it is therefore possible that mitochondrial ROS rather than mitochondrial respiration is the effector of the ERK1/2 regulation. MAPKs are critical for correct cellular response to a variety of stimuli including mitogenic factors and stresses, and concurrently aberrant function and regulation of p38 MAPK and ERK1/2 has been correlated

with both senescence, misregulation of apoptosis, and tumor initiation [75–77].

HIF-1, p38 MAPK, and ERK1/2 constitute a nonexhaustive list of biological molecules regulated either directly or indirectly by mitochondria-produced ROS. The purpose of this regulation is most likely for the cell to react upon oxygen availability for oxidative phosphorylation, energetic yield, and to respond to increased oxidative load and mitochondrial stresses. As such, the targets of this regulation are therefore involved in critical cellular pathways, and it is no surprise that the majority of pathways that can be regulated by mitochondrial ROS have been correlated with cancer and senescence upon deregulation.

An abnormal production of mitochondrial ROS can therefore elicit detrimental effects on the cell, causing both oxidative damages on cellular components and most likely, an incorrectly regulated second messenger affecting critical cellular pathways.

6. Mitochondrial Regulation of the Nuclear Epigenome

Mitochondrial dysfunctions invoke mitochondria-to-nucleus retrograde responses in human cells [78]. Cells devoid of functional mitochondria (ρ^0) are useful in studies aiming to gain insight into the possible role of mitochondria in regulating or being associated with the epigenetic alterations of the nuclear genome, either gene specific or genome wide, particularly at the level of DNA methylation. It has been shown that depletion of the mitochondrial genome results in aberrant methylation of promoter CpG islands (high CG rich regions) normally unmethylated in the parental cell line. Conversely Smiraglia et al. [79] has shown that, at specific loci, the 51 UTR comprising a CpG island was partially hypomethylated in ρ^0 cells compared to the parental cell line in which this region was completely hypermethylated. It was suggested that the partial loss of genomic DNA methylation could be associated with the loss of mtDNA and mitochondrial function. Repletion of wild-type mitochondria in ρ^0 cells (mtDNA deficient) resulted in the partial re-establishment of some methylation profiles to the original parental state. Accordingly, this study provides an interesting depiction of the possible role of mitochondria in establishing or maintaining genomic (nuclear) DNA methylation. Interestingly, increasing evidence is emerging on an interdependent relationship between the mitochondria and the nuclear genome. It is well established that expression of nuclear genomic DNA is regulated by epigenetic factors, and consequently, an understanding of how mitochondria regulate nuclear epigenetics is of great importance.

7. mtDNA and Apoptosis

Mitochondria play a central role in initiation of the intrinsic pathway of apoptosis by releasing mitochondrial proteins, which normally reside in the intermembrane space, into the cytosol. This triggers assembly of the apoptosome and activation of procaspase-9, leading to a cascade of events which ultimately leads to cell death. Several studies have indicated that mtDNA mutations are associated with mitochondrial induced apoptosis in aging mouse models. It has been demonstrated that a genetically engineered mouse model expressing proofreading-deficient mitochondrial DNA polymerase gamma accumulate mtDNA mutations and display a premature aging phenotype [23]. It was further demonstrated that cleaved caspase-3 levels increased with aging in various organs of the mouse model, suggesting increased apoptotic activity [23]. Furthermore, Hiona et al. [80] demonstrated an increased level of mitochondria-elicited apoptosis in muscle tissue of the same mouse line by observing increased caspase-9 activity and a significant positive correlation between caspase-9 and caspase-3 activity. The increased level of apoptosis was accompanied by a reduction in the mitochondrial membrane potential [80]. Reduction in the mitochondrial membrane potential has been demonstrated to affect the mitochondrial matrix condensation *in vitro*, and thus the release of proapoptotic cytochrome c into the cytosol [81]. It has also been demonstrated that aging human colonic cells displaying respiratory chain deficiency have a significant higher apoptotic frequency compared to normal human colonic cells [82], indicating that respiratory deficiency induces apoptosis. However, Hiona et al. [80] demonstrated that mtDNA mutations in the mouse model of their study caused no change in activity of the ETC complexes or ROS production, however, they did find a marked decrease in ETC complexes and ATP production, suggesting that accumulation of mtDNA mutations associated with ETC dysfunction and altered membrane potential may lead to activation of the intrinsic apoptotic pathway. Thus these studies indicate a link between increased apoptosis and aging.

8. Mitochondrial Dysfunction and the Cytosolic Nucleotide Metabolism

Rate-limiting steps of the metabolism of cytosolic ribonucleotides and deoxyribonucleotides take place in the mitochondria and can be affected by the fitness of the organelle as reviewed by Desler et al. [83]. In accordance, several studies have demonstrated a correlation between mitochondrial dysfunctions affecting the ETC and aberrant synthesis of cytosolic ribonucleotides and deoxyribonucleotides [78, 84, 85]. Deoxyribonucleotides are exclusively destined for DNA synthesis in the form of deoxyribonucleotide triphosphates (dNTP), but ribonucleotides have a multitude of roles in RNA synthesis in the form of ribonucleotide triphosphates (rNTP), as chemical transporters in form of ATP and in the form of basic second messenger molecules. Disruption of the intracellular levels of deoxyribonucleotides or ribonucleotides is unfavorable as imbalance of the dNTP pools can induce a variety of genetic changes such as base substitutions, frameshift mutations, delay of replication fork progression, and DNA replication as well as increase in the frequency of fragile sites [86–91]. Decreased levels of rNTP pools inhibit RNA synthesis, likely by inhibiting the initiation frequency of RNA polymerase I, and thereby inhibiting the

synthesis of rRNA [92]. Furthermore, inhibition of purine and pyrimidine synthesis induces a p53-mediated cell cycle arrest and inhibits cell proliferation, ultimately leading to increased cytotoxicity [93–96].

The *de novo* synthesis of pyrimidines is directly linked to the ETC by the flavoenzyme dihydroorotae dehydrogenase (DHODHase). DHODHase catalyzes the conversion of dihydroorotate to orotate by oxidation. Subsequent catalytic steps convert orotate into uridine monophosphates that can be further converted to UTP and CTP, and ultimately, dTTP and dCTP, respectively. DHODHase is located in the inner membrane of the mitochondria with the active site facing the inner membrane [2]. DHODHase is functionally connected to the ETC by a flavin prosthetic group that couples dihydroorotate oxidation to respiratory ubiquinone reduction [3]. From ubiquinol, the flow of electrons continues through the ETC. Leflunomide and brequinar are inhibitors of DHODHase that bind to the quinone-binding site, thereby blocking interaction between ubiquinone and the flavin prostethic group of DHODHase [97]. Treatment of human lymphocytes with leflunomide or brequinar arrests the cells in G1 phase and inhibits both RNA and DNA synthesis [98–100]. The inhibitory effects are suppressed by addition of uridine which can be salvaged to UMP, where by the *de novo* synthesis of pyrimidines is bypassed. Treating the human leukemic cell line CCRF. CEM with leflunomide or brequinar cause a significant reduction in the levels of CTP and UTP, while the levels of purine nucleotides remain unaffected by leflunomide but increased by brequinar [101]. Furthermore, our unpublished data indicate that a leflunomide mediated inhibition of DHODHase in a human cervical cancer cell line results in decreased levels of dTTP and dCTP (Data not shown). Together these results demonstrate the importance of the activity of DHODHase for cytosolic levels of pyrimidine nucleotides. It has been suggested that any dysfunction of the ETC; lack of oxygen, presence of inhibitors, or mutations of complex III and IV, would entail impairments of the *de novo* UMP synthesis, and a subsequent decrease in the *de novo* synthesis of cytosolic nucleotides [78, 85]. In support of this argument it has been demonstrated that a chemical inhibition of the ETC causing a buildup of ubiquinol has an inhibitory effect on DHODHase [85]. Inhibition of the ETC caused by dysfunctional ETC subunits encoded by mutated mtDNA is therefore likely to have an effect on the activity of DHODHase. Treatment of cells with chloramphenicol inhibits mitochondrial protein synthesis, and mimics an mtDNA-induced mitochondrial dysfunction, impairing ETC activity. In chick embryo cells, treatment with chloramphenicol was demonstrated to inhibit DHODHase activity and cell growth [102]. Growth inhibition was reversed by addition of pyrimidines to the growth media. This indicates that mitochondrial dysfunction affecting the ETC has an inhibitory effect on DHODHase activity that is comparable to inhibition with leflunomide or brequinar. This conclusion is substantiated by the fact that cultured mammalian cells devoid of mtDNA are auxotrophic for pyrimidines, and must be routinely grown in the presence of a uridine supplement [103].

In summary, the DHODHase links cytosolic nucleotide metabolism with the ETC. Inhibition of DHODHase or the ETC has been demonstrated to decrease the levels of cytosolic pyrimidines. It is possible that an inhibition of the ETC induced by damaged ETC subunits encoded by mutated mtDNA can cause an imbalance of cytosolic nucleotides, which in turn can induce detrimental genetic changes that are also apparent in tumor formation and aging.

9. Conclusion

For the better half of a century, both aging and cancer formation have been associated with divergent mitochondrial function. Since first suggested, the correlation between mitochondrial dysfunctions and aging and cancer has been debated as merely symptoms of aging and cancer. However, with the generation of mouse lines containing a mtDNA mutator phenotype [22, 23] and with the usage of cybrid mechanisms in cell cultures [30–33], it has been demonstrated that mtDNA aberrations can be correlated with at least certain aspects of aging and cancer progression. In this paper we have described different immediate effects of mtDNA aberrations, and their potential role in inducing premature aging, or promote tumor progression. These effects encompass (1) altered mitochondrial production of ROS, (2) mitochondrial regulation of the nuclear epigenome, (3) mitochondrial initiation of apoptosis, and (4) mitochondria induced inhibition of de novo synthesis of rNTP and dNTP (see Figure 1). Where 1, 3, and 4 can be directly linked to the mtDNA aberrations and their effect on the ETC, the link between mitochondria and regulation of the nuclear epigenome is unknown and likely multifaceted. All of the effects have been correlated to either cellular senescence or organismal premature aging and cancer progression. Even though, all the described mechanisms can be related to aging and cancer, the effect exerted by these mechanisms in response to mtDNA aberrations is most likely dependent on the type of tissue and cells exposed. For example, a mitochondrial induced inhibition of *de novo* synthesis of rNTP and dNTP will not have an adverse effect on cells that are primarily reliant on rNTP and dNTP produced by salvage pathways. Similarly, a burst of mitochondrial ROS will have a smaller risk of inducing cellular damage if the cell or tissue type affected has sufficient antioxidant defenses.

The activity and efficiency of the ETC and thereby the capacity for oxidative phosphorylation is directly correlated with the fidelity of the mitochondrial genome [7, 8]. The fidelity of the mitochondrial genome is determined by a variety of factors including the specific genome region, the type of mutation, and the ratio between unaffected and affected mtDNA molecules in a mitochondrion and in the cell. Correspondingly, ROS production is low in mitochondria with a functional ETC and increased when especially the mtDNA encoded subunits of complex I and III are mutated [44, 45]. However, the fidelity of the mitochondrial genome can be compromised to a threshold where the mtDNA-encoded peptides become overloaded with mutations such that the ETC is unable to support an

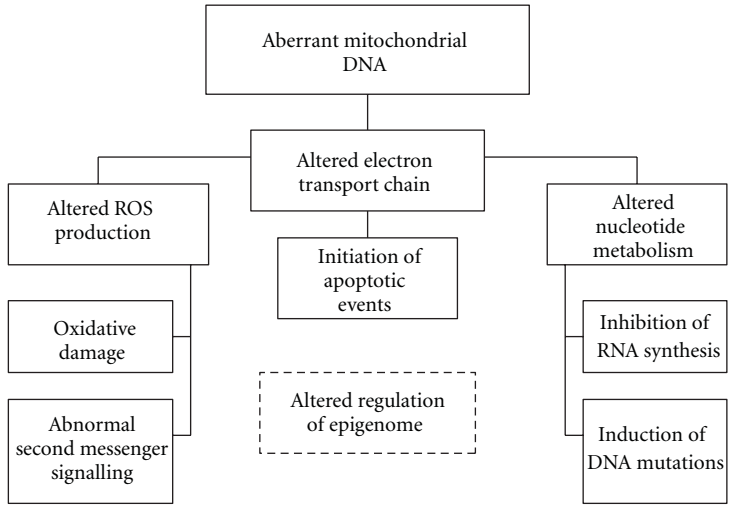

Figure 1: Overview of effects of mtDNA aberrations correlated aging and cancer progression. Aberrant mitochondrial DNA causes a reduced fidelity of the electron transport chain, which in turn can alter the production of reactive oxygen species (ROS), prematurely initiate apoptotic events and alter the nucleotide metabolism. An increased production of ROS can cause oxidative damage to cellular components including nuclear DNA, while both a decrease and increase of ROS can compromise its role as a second messenger molecule, affecting several cellular mechanisms. An altered nucleotide metabolism can result in a altered production of ribonucleotides and deoxyribonucleotides increasing the risk of inhibition of RNA synthesis and DNA mutations, respectively. Aberrant mitochondrial DNA can also alter the regulation of the nuclear epigenome even though the mechanism responsible has not been elucidated.

electron transport. In this situation, the ETC can neither perform oxidative phosphorylation nor produce ROS. As the capacity for oxidative phosphorylation and the production of mitochondrial ROS are correlated with the fidelity of the mitochondrial genome, the immediate effects of mtDNA aberrations most likely elicits their maximal destructive effects at different points in the course of the degradation of the mitochondrial genome. The ability of mitochondria-produced ROS to induce oxidative damage to cellular components are maximal in situations where the fidelity of the mitochondrial genome allows transport of electrons through the ETC, but when electron leak is so abundant that the cellular antioxidative defenses are saturated and an overload of harmful ROS molecules are allowed to form. In contrast, the role of ROS as signal molecules is affected in situations where the production is both increased and decreased. In cases where ROS as a signal molecule mediates a stress response, an increased production of ROS will potentially trigger an unnecessary range of responses, including stalling of cell cycle or initiation of apoptosis. In contrast decreased production of ROS mediate improper stress responses with affected cells more vulnerable to a given situation. Accordingly both an increased and decreased level of ROS can theoretically increase the risk of senescence or contribute to tumor initiation and progression, in light of the second messenger role of ROS. The activity of the DHODHase is inversely correlated with the activity of the ETC. Therefore, the risk of a mitochondrial induced imbalance of the cellular rNTP and dNTP pool is most pronounced when the ETC is unable to transport electrons.

It is an important realization that aberrations of mtDNA can induce the risk of cellular senescence, organismal premature aging, and cancer progression through a variety of different mechanisms, dependent on the type of mtDNA alteration and the cell or tissue type affected. As a consequence, the mechanism linking mitochondrial dysfunction to cancer and aging can no longer be perceived as a simple mechanism where oxidative damage to cellular components induced by mitochondria-produced ROS is the main mediator. Rather this pathway is complex and mediated by several critical cellular events affected by mitochondrial function.

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Review Article

The Role of the Mitochondrial Genome in Ageing and Carcinogenesis

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Mitochondrial DNA mutations and polymorphisms have been the focus of intensive investigations for well over a decade in an attempt to understand how they affect fundamental processes such as cancer and aging. Initial interest in mutations occurring in mitochondrial DNA of cancer cells diminished when most were found to be the same mutations which occurred during the evolution of human mitochondrial haplogroups. However, increasingly correlations are being found between various mitochondrial haplogroups and susceptibility to cancer or diseases in some cases and successful aging in others.

1. Introduction

Mitochondria play essential and diverse roles in the physiology of eukaryotic cells. These structures are not only indispensable for ATP production and participate in numerous intermediate metabolic reactions but also play a central role in calcium homeostasis, apoptosis, cell signaling, and differentiation. Impairment of mitochondrial functions has been implicated in a wide variety of human pathologies including cancer and age-related diseases such as type II diabetes mellitus (DM II), Alzheimer's disease, degenerative arthritis, ischemic heart disease, and age-related macular degeneration [1–4].

Until recently aerobic glycolysis of cancer cells received little attention in oncology despite being one of the first observations made in the field [5]. Recent progress in the understanding of cancer molecular genetics, biochemistry, and proteomics has renewed interest in "mitochondrial oncology" [2, 4]. It is now widely understood that, unlike most normal cells, cancer cells are dependent on glycolysis for energy production and that an energetic switch is an indispensible step in malignant transformation [6], since aerobic glycolysis confers a growth advantage to tumor cells [7] and disrupted OXPHOS provides a proliferative advantage [8]. New attempts to analyze the significance of mitochondria in oncology encompass the increasingly important debate whether mtDNA mutations or polymorphisms are actually the cause or an effect of tumor progression. A growing number of reports support the hypothesis that mitochondrial functions are profoundly altered in transformed cells and that mtDNA polymorphisms are important for the processes of cell transformation [3, 4, 9, 10].

Somatic mtDNA mutations are a general phenomenon occurring during cell transformation and ageing. In the last ten years mtDNA mutations have been found in solid tumors including carcinomas and sarcomas, and also leukemias and lymphomas [1, 3, 11–13]. At the same time reports of premature aging in mutant mice with greatly increased rates of mitochondrial DNA mutagenesis—so-called "mitochondrial mutator mice"—confirmed that accumulation of mtDNA mutations is a key mechanism of aging [14]. Comprehensive scanning of mtDNA in cancer cells detected functionally relevant point mutations [15–17], but also *specific mtDNA polymorphisms* in polypeptide-encoding genes and the D-loop were found in as many as 50% of cancer patients

[18–20]. MtDNA mutations and polymorphisms can cause alterations in the encoded proteins [21, 22] and in consequence may compromise the respiratory chain function and stimulate abundant ROS production [15, 23]. Proteins with altered sequences as the result of somatic mtDNA mutations have been shown to have disturbed assembly and stability and therefore defective structure of respiratory chain complexes leading to OXPHOS deficiency [21, 22, 24, 25]. Such mutations could also affect cells during aging.

An analogous correlation has been proposed for cells with specific rare mtDNA polymorphisms [26–30]. Thus, the different mtDNA sequences reported in a variety of human cancer cells are thought to contribute to oxidative respiratory malfunction. In particular a few reports confirm that mtDNA genome sequence heterogeneity is the cause of subsequent mitochondrial function alterations including OXPHOS deficiency, ROS overproduction, and finally upregulation of signaling pathways associated with cell maturation, proliferation, and cell death [12, 25, 31, 32]. The codependence of cancer growth rate, metastatic potential and mtDNA mutations has recently been established in a mouse model [15]. Moreover, the apparent conundrum of whether mtDNA mutations found in tumors are a cause or a consequence of the carcinogenic process has recently also been partially solved after the demonstration that pathogenic mtDNA mutations lead to excessive reactive oxygen species signaling [33, 34], diminish cellular apoptotic potential [35], initiate mitochondria-nucleus signaling that promotes a cellular invasive phenotype [36–38], regulate epigenetic modifications in the nucleus [10], and affect nucleotide pools and chromosomal stability [39]. Although numerous mtDNA polymorphisms and mutations have been identified in various tumors, the pathogenic implications (cell growth or transformation promotion? metastatic potential promotion?) of many of these mtDNA variants remain unclear and need critical evaluation in order to avoid pitfalls, misinterpretations, and erroneous conclusions [40, 41].

Comprehensive analysis by Bandelt et al. [41–43] shows that oncology, forensic science, population genetics, and clinical literature on mtDNA is often compromised by unsatisfactory results in many studies, including flawed sequence data, artificial recombinant sequences or interpretative shortcomings [43–45]. In particular phantom mutations and phantom mutation hotspots lead to misidentification of pathologic somatic mutations and to misinterpretations in all kinds of aspects of clinical mtDNA studies [46, 47]. Many listed mutations when reanalyzed were found to actually be polymorphisms, including polymorphisms known to define major haplogroups. It should be stressed that those artifacts have been inadvertently regarded as novel and pathogenic. Bandelt et al. proposed a paradigm of evaluation of sequence quality and detection of potential problems when inferring the pathogenic status of an mtDNA mutation to avoid false positive results [48]. Some authors have also called for stringent quality control of mtDNA data by haplogroup-directed database comparisons [47, 49]. Therefore, in this paper we mainly focus on mtDNA polymorphisms and mitochondrial haplogroups and their role in cancer and aging.

2. Mitochondrial DNA Polymorphisms in Cancer

The use of mitochondrial DNA polymorphisms as biomarkers is rapidly expanding in metabolic disease diagnostics, the analysis of human migration patterns, and human identification in forensic sciences [2, 11, 12]. Although somatic mtDNA mutations have been reported in many types of cancer, very few reports have documented the prevalence of inherited mitochondrial DNA polymorphisms in cancer patients and its comparison to healthy control populations.

The first interesting and widely investigated mtDNA polymorphism in the cancer field was A10398G, first described as causative factor in breast cancer development [50–52]. In particular we have shown the abundance of the 10398G polymorphism in a Polish breast cancer cohort. 23% of patients with biopsy-proven breast cancer carried the 10398G variant, while in the healthy group only 3% ($P = .0008$). This polymorphism is of great interest as 10398 *locus* in mtDNA is highly polymorphic. While the revised Cambridge reference sequence [53] lists the wild type base as A, the alternative base (G) is also prevalent in many populations [54]. Amongst 2704 complete coding region sequences reported in the mtDB database, the base is A in 54% (1461), G in 46% (1242), and *T* in 1 case [54, 55]. Moreover, analyses of this polymorphism in the clinical context yielded contradictory findings. The 10398A appears to increase the risk of Parkinson's disease [56], Alzheimer's disease [57], and amyotrophic lateral sclerosis [58], but in contrast it protects against cardiomyopathy in patients with Friedreich's ataxia [59]. At the same time Canter et al. found an increased frequency of the 10398A in African-American women with invasive breast cancer [60]. In contrast, in another study, the 10398A frequency was not increased in an African American breast cancer cohort when compared with controls [50]. The significant difference reported by us between 10398G distribution in breast cancer patients and breast cancer-free women with negative family history seems to prove the unique role of the A10398G polymorphism in breast cancer predisposition. We believe that A10398G might be considered as a contributing factor in carcinogenesis and as variant modifying the risk of developing breast cancer [19, 61]. In contrast, inheritance of 10398A has been postulated to increase incidence and severity of prostate cancer in African-American men as compared with other ethnic groups [52]. Concerning the pathophysiological role of A10398G it must be underlined that the amino acid with 10398A is threonine, and with 10398G alanine. Amongst 61 analyzed species (including humans), the amino acid at position 114 is threonine in 44%, and alanine in 30% and a different amino acid in 26% [62]. The substitution of threonine—a polar, neutral amino acid with hydropathy index −0.7 for alanine—a nonpolar, neutral amino acid with hydropathy index of 1.8 [63]—is significant and relatively nonconservative as reflected by the Grantham value of 58 [64]. The methyl group of alanine is nonreactive and is thus almost never directly involved in protein function and bonding [65, 66].

Concerning other mtDNA loci, multiple interesting mtDNA polymorphisms have been reported in prostate [67], thyroid [68], and oral cancer cases [69] with breast cancer as the most widely explored research topic [20, 27, 70–74]. It was shown that breast cancer patients with multiple *mtDNA D-loop* (CA)(n) polymorphisms have significantly poorer disease-free survival than those with one copy of the mtDNA D-loop (CA)(n) polymorphism [70]. mtDNA variation, particularly in the D310 segment, was indicated to be a causative factor of breast cancer development. In particular the C16189T polymorphism seems to be of potential pathophysiological impact as it has been associated with susceptibility to dilated cardiomyopathy (DCM) [75], insulin resistance, and development of DM II in adult life and vascular pathologies including lacunar cerebral infarction and cardiovascular diseases [76]. In a *breast cancer* cohort study we detected a significantly greater incidence of mtDNA polymorphisms T239C, A263G, and C16207T and a significant lower incidence of A73G, C150T, T16183C, T16189C, C16223T, and T16362C compared to controls [27]. In breast cancer cohort NADH-dehydrogenase mtDNA encoded genes were also rich in polymorphisms, in particular very rare polymorphisms such as A4727G, G9947A, A10044G, A10283G, T11233C, and C11503T [19].

At the same time we have also reported multiple mtDNA polymorphisms in *vulvar cancer* cases, in particular 19 specific uncommon polymorphisms and one polymorphism not reported previously (C498delC). In vulvar cancer cases overrepresented polymorphisms included C16192T, C16256T and C16270T, what is interesting is that, all are specific for haplogroup U, whereas the 16223T polymorphism characteristic for haplogroups I, W, and X was underrepresented [28, 54]. In all the studies, including breast cancer, endometrial adenocarcinoma, and vulvar carcinoma, polymorphisms were predominantly found in mtDNA hypervariable regions HV1 (16024–16383) and HV2 (57–333) [26–28].

In the case of *hepatocellular carcinoma* (HCC), polymorphisms including 16266T, 16293G, 16299G, 16303A, 242T, 368G, and 462T were associated with increased risk for alcohol-dependent HCC. At the same time the 523A/del was associated with increased risks of both alcohol-dependent, and independent HCC. Moreover, 489T/152T, 489T/523A, and 489T/525C haplotypes were significantly reduced in HBV-positive HCC patients in comparison to HBV-negative patients. Haplotypes 489C and 152T, 249A, 309C, 523Del, or 525Del were associated significantly with an increase of alcohol-HCC risk [77].

6221C and 7389C polymorphisms were significantly associated with *prostate cancer* ($P < .05$) and in strong linkage disequilibrium with each other ($r(2) > 0.6$) in African-American men [78], while no specific mtDNA polymorphisms appeared to have a significant effect on a predisposition to prostate cancer in the Korean population [79].

In summary we and others have hypothesized that patients bearing some of the common mitochondrial polymorphisms are at higher risk of cancer development, with a particularly interesting role of haplogroup-specific mtDNA polymorphisms. Moreover, it might be tempting to suggest that in cancer patients a similar situation is found as that reported by Lehtonen et al. [80] for patients with sensorineural hearing impairment—that is, in both cases, affected persons have more rare substitutions in their mitochondrial DNA than the healthy control population. Similar results have been obtained for other diseases related to mitochondria [81]. It is difficult to explain this at present, but possibly these rare mutations have an effect on mitochondrial function making persons in whom they are present more susceptible to certain diseases.

3. Mitochondrial Mutations and Polymorphisms in Aging

Numerous papers have analyzed the increased occurrence of mitochondrial mutations and deletions in aging cells in various organisms [82–84]. The fact that older cells in various tissues, especially in postmitotic ones, accumulate both point mutations and deletions in mitochondrial DNA is undisputed, but there have been a number of problems interpreting these results. First, relatively high percentages of mutations are known to be required to affect the respiratory chain, on the basis of data from patients with mitochondrial diseases. Second, the correlation of point mutations and deletions with aging is not proof of causality—only of coexistence. Another problem which has been controversial is what the effects of the mutations are—do they cause a vicious cycle of ROS release, as was postulated by Harman [85], with more and more new mutations leading inevitably to cell death?

In the last years two models have resolved some, but not all of the problems which have been raised in studies of aging and mitochondria. The first was mentioned above—the mutator mouse, obtained independently by two groups [86, 87] with a mutated mitochondrial DNA polymerase which led to accumulation of large numbers of mutations in the mitochondrial DNA. The mice showed symptoms of premature aging—and, as the mutations came before the aging this was proof of causality. There are still, however, some problems with the interpretation of the results obtained with these models. Why are heterozygous mice which accumulate a high number of mutations healthy? Are deleted mtDNA molecules the cause of aging? Do they occur? The different techniques used and different results obtained by the two groups make it difficult to analyze what exactly is happening during the aging process, but some things do appear to be clear—there are numerous point mutations, they lead to cell death and eventually aging, and all this takes place without any excessive production of ROS [88–90].

The second result was perhaps less spectacular than those obtained with the mutator mouse but is very important for solving the problem of whether and how low levels of mitochondrial DNA mutations can affect the functioning of cells. Dufour et al. [91] found that a mitochondrial respiratory chain deficiency in neurons which was caused by a nuclear mutations when present in only 20% of

the mitochondria caused degeneration of adjacent neurons. This points to a solution of the problem that the levels of mutations found in aging tissues are too low.

Thus, mitochondrial mutations are now generally believed to be involved in the aging process. The involvement of ROS will be discussed in a separate section as will the effects of mitochondrial polymorphisms—through their grouping in haplogroups.

4. Mitochondrial Haplogroups in Cancer and Aging

Human mtDNA is highly polymorphic. On the basis of certain SNPs present in mtDNA, the human population can be divided into haplogroups. Analysis of their distribution among the population enables reconstruction of ancient human migrations. Mutations creating certain haplogroups (that became polymorphisms over time) were suggested to affect coupling efficiency of the electron transport chain, enabling adaptation to life in different climatic conditions [2]. It is estimated that around 25% of mtDNA polypeptide sequence variation, 10–20% of tRNA variation, and at least some of rRNA variation contributes to this effect.

MtDNA haplogroup variation seems to confer genetic susceptibility for several conditions, as indicated by numerous studies. Mitochondrial genetic background was shown to affect the risk of visual failure caused by Leber's Hereditary Optic Neuropathy (LHON), as mutation occurrence in patients with haplogroup J or K correlated with an elevated risk of developing the condition, whereas haplogroup H had a protective effect [2, 11, 92, 93]. A similar effect was reported for haplogroups M8a and M7b that diminished and enhanced, respectively, clinical manifestation of LHON symptoms in a Chinese population [94]. Haplogroup H1 was shown to have protective effects on ischemic stroke risk among Portuguese patients [95]. Parkinson's disease (PD) was less frequent among K haplogroup individuals in the Italian population [96], whereas a study on Polish PD patients reported a protective effect of haplogroup J in males [97]. JTWIX supercluster background was associated with a higher number of nonsynonymous mutations in complex I genes and an increased risk of PD. At the same time haplogroups U and K seem to neutralize the harmful effect of the APOE4 variant in Alzheimer's disease [98] and be connected with psychiatric disorders [99]. Moreover, polymorphisms characteristic for haplogroup J and M were shown to be associated with longevity in European and Japanese populations, respectively [100]. On the other hand, haplogroup J was reported to confer susceptibility to type II diabetes [101].

In the cancer field, significant increased risk for *breast cancer* development was reported for haplogroup K [102]. Our analysis revealed that mitochondrial haplogroup distribution in patients with breast cancer greatly differs from cancer-free controls and the general Polish population. Haplogroup I was overrepresented in individuals with cancer (14% versus 3%; $P = .017$) in comparison to the general Polish population, and if the breast cancer cohort was compared to cancer-free centenarians, haplogroup H was underrepresented (38% versus 58%; $P = .019$) [27]. It was shown that haplogroup M individuals have an increased risk of breast cancer compared with haplogroup N in China [103]. In a specific Chinese population, patients with mtDNA haplogroup M exhibited an increased risk of breast cancer development ($P = .040$) and that this haplogroup-risk effect was even more pronounced in a subhaplogroup of M, subhaplogroup D5 ($P = .030$) [104].

Concerning other types of cancer not as much research was done as in the case of breast cancer; nevertheless, it was proven that haplogroup D4a is associated with an increased risk of *thyroid cancer* development ($P = .028$) [104]. The inheritance of mitochondrial haplogroup U was shown to be associated with an approximately 2-fold increased risk of prostate cancer and 2.5-fold increased risk of *renal cancer* in white North American individuals and it was indicated that individuals with this mitochondrial haplotype are in a high-risk group. Because mitochondrial haplogroup U is found in 9.35% of the white United States population, more than 20 million individuals were shown to be in a high risk group [105]. On the other hand, in a Middle European population mitochondrial haplogroup frequencies did not differ significantly between patients with prostate cancer and control population, implying no impact of inherited mitochondrial DNA variation on predisposition to PC [106]. *Esophageal cancer* (EC) predisposition was investigated in Taihang Mountain and Chaoshan areas in China. In this study D5 haplogroup was associated with EC at the general population level in the Taihang Mountain area and in women below 60 years of age in the Chaoshan area. It was also shown that D4a and D5 haplogroups might be risk markers of EC development, in particular D4a and D5 for the Taihang Mountain area and D and D5 for the Chaoshan area [107].

Mitochondrial haplogroups were also in the focus of gynecological oncology. In *vulvar cancer* patients, we again found significant differences with underrepresentation of haplogroup H in the cancer cohort. Moreover, in the comparison with the general Polish population a trend towards haplogroup U and K overrepresentation was also noticed (44 versus 19%, $P = .009$) [28]. The underrepresentation of haplogroup H ($P = .023$) in vulvar cancer patients was particularly interesting. Haplogroup H is marked by the T7028C polymorphism. Therefore, if haplogroup H is underrepresented, 7028T polymorphism is overrepresented. For vulvar cancer the C7028T RFLP test has Odds Ratio (OR) and Diagnostic Odds Ratio = 3.11 and indicates Relative Risk (RR) = 2.43 in comparison to the control (cancer-free centenarians) [28]. A similar trend was reported for *endometrial adenocarcinoma* cases. Analysis revealed that the endometrial carcinoma population haplogroup distribution differs from the Polish population with haplogroup H being strongly underrepresented ($P = .003$). In this group patients with endometrial adenocarcinoma had the T7028C 12% of the time while the general Polish population carried the T7028C polymorphism 42% of the time. As a result one had a 5.53 higher chance to be T7028C carrier and not develop cancer with specificity of prediction as high as 0.775 (0.755–0.815) [26]. In both

vulvar and endometrial cancer cases Diagnostic Odds Ratio of C7028T test was in the range of 3–6, and therefore may provide a strong clue in investigations of etiology [108]. In another study patients with *endometrial cancers* clustered in haplogroup D with a significantly higher frequency [109].

In summary we believe that, because the patterns of germline polymorphisms in cancer patients and controls are different, it is possible that the inheritance of specific mitochondrial genotypes predisposes individuals to cancer [110, 111]. Moreover, the co-occurrence of multiple inherited mtDNA SNPs may influence the disease phenotype as shown in the case of 12308G and 10398G in breast cancer [61] or G10680A and T14484C in LHON [112]. One polymorphism may have a modifying role for increasing the penetrance and expressivity of other mtDNA genome variants. In particular, the mitochondrial haplotypes may play a synergistic role in the development of cancer as is the case of vision loss in the families carrying the LHON-associated primary mtDNA mutations. In LHON patients the mitochondrial haplotype has been shown to influence the clinical expression [94].

The relationship between mitochondrial haplogroups and aging in humans is based on analysis of centenarians in various populations and appropriate control groups; in some populations certain haplogroups were found to be overrepresented in relation to controls [113]. A very attractive hypothesis has been proposed by Wallace [2] that successful aging is due to uncoupling of the electron chain. In brief, persons with more uncoupled haplogroups would produce more heat (an adaptation to colder climates), be more calorie restricted, and thus age better, but also be more susceptible to certain mitochondrial diseases. This has not been confirmed directly, as comparison of uncoupling in cell lines with identical genetic backgrounds repopulated with mitochondria from haplogroups H and T did not show any differences [114], but there are some results concerning human lifespan under conditions of good and poor supply which indicate that for instance persons with haplogroup H live longer than persons with haplogroup U under conditions when the food supply is limited [115]. Differences in transcription between haplogroups have also been observed [116], though a direct difference in uncoupling was not analyzed. There are also some data indicating that mice with more uncoupled mitochondria live longer than mice without the uncoupling [117]. However, there is a problem that few of these association studies would fulfill the criteria proposed by Samuels et al. [118] as the analyzed groups were often too small. Thus, the conclusion would be that there are interesting suggestions of correlations of mitochondrial haplogroups and aging, but the evidence is still too weak and may indeed remain that way. On the other hand, there are new papers indicating differences between some haplogroups for example, between haplogroups H and UK in respect to among others oxygen consumption and protein synthesis [119] and persons with haplogroup H have been shown to have higher mitochondrial oxidative damage than those with haplogroup J [120], and it is possible that this problem will be solved in the future.

5. Reactive Oxygen Species in Carcinogenesis and Ageing

Multiple papers have indicated that oxidative stress is central in the pathogenesis of a wide variety of diseases including cancer and a special role in mitochondria-related ageing has been attributed to reactive oxygen species (ROS) [1, 2, 121]. The mitochondrial respiratory chain is the major source of ROS, which in excess obviously destroys mtDNA, in turn contributing to the promotion of a vicious cycle of cancer development and possibly aging. The constant generation of ROS within the mitochondria and the increased oxidative stress in cancer cells may cause further damage to the electron transport chain, and amplify respiratory malfunction and cancer dependency on glycolysis. It is ROS production that increases when electron transport is reduced or inhibited, as in the case of low aerobic respiration rate resulting from mtDNA mutations and aberrant OXPHOS protein structure [122]. The main sources of mitochondrial ROS are complex I and complex III since mitochondrial generation of ATP through oxidative phosphorylation involves formation of O_2^- and subsequently derived ROS such as hydrogen peroxide and ·OH. This is the case of complex I flavin active site, complex I and complex III iron-sulfur centers and ubisemiquinone transferring electrons to oxygen and giving rise to superoxide anions. At the same time if the respiratory chain is inhibited downstream of complex III (as in the case of some mtDNA mutations), electrons coming from succinate oxidation also increase superoxide anion generation [123]. Subsequently, oxidative stress develops and ROS scavenging is overloaded [124, 125].

The scenario that emerges from ROS, mtDNA mutations and cancer studies is rather complex [124, 125], since different mtDNA mutations do not result in the same profile in terms of ROS production [23, 40]. For example, it has been proven that only mtDNA mutations that affect the function of complex I and III, but not COX mutations, are triggers of ROS production [33] and that only specific mutations in complex I genes with subsequent ROS overproduction may promote tumor growth [17] and cancer metastases [15]. In contrast Zielonka and Kalyanaraman [126] recently have proven that Ishikawa et al. [15] failed to show evidence for formation of superoxide and hydrogen peroxide, presumed to be generated from complex I deficiency associated with mtDNA mutations in metastatic cells.

The involvement of ROS and mitochondria in cancer appears to be generally accepted [127]. On the other hand, the role of ROS in aging appears to be less well established, in spite of the very long period in which it has been the fundamental hypothesis for much of aging research. During the last years a number of papers have suggested that aging does not require ROS formation [128] and the free radical theory of aging has been increasingly under attack and appears to be no longer the major theory explaining this phenomenon [129, 130]. However, as is often true for fields as complex as aging, the situation is not simple and discussions as to the role of ROS will continue. This paper deals essentially with mammals; however, recent work on Drosophila melanogaster has shown that mitochondrial

DNA controls longevity in females regardless of ROS production, and moreover a number of predictions of the mitochondrial free radical theory of aging were not verified [131].

Interpretation of many results is quite complex. For instance, Schriner et al. [132] have shown that overexpression of catalase in mitochondria in mice extends their lifespan. This could be construed as evidence that ROS removal affects longevity. The overexpression of this enzyme not only alleviates aging symptoms in the heart in normal mice [133] but also in the mutator mouse model [134]. Thus, the question is, if the mutator mouse ages without ROS production, what does the catalase do in the heart of these mice? Dubec et al. [135] on the basis of a mouse model with a proofreading-deficient mitochondrial polymerase targeted only to the heart proposed that cell death and cardiomyopathy are due to apoptosis caused by rare mutations in mitochondrial DNA leading to the release of an apoptosis-promoting peptide.

Where does this leave mitochondria? There are still numerous data indicating their involvement in the aging process—from model organisms [129, 136] to humans [1, 2]. Moreover, the exact mechanisms through which mitochondrial function affects aging is still unknown, what does appear to be clear is that neither are ROS required for aging nor does their production necessarily cause it.

6. Summary and Perspectives

Precise understanding of the molecular mechanisms whereby specific mtDNA polymorphisms predispose to cancer is currently lacking. Although the mechanisms of generation and functional impact of mtDNA polymorphisms are still not clear, there is a high incidence and broad distribution in human cancers making them a potential marker for cancer detection, but the role of mtDNA polymorphisms in the maintenance of the tumor cell phenotype or in tumorigenesis remains to be elucidated [2, 3, 137, 138]. Although it is generally believed that polymorphisms may have pathogenic potential, it remains to be established whether they contribute to neoplastic transformation by changing cellular energy capacities, increasing mitochondrial oxidative stress, or modulating apoptosis. The significance of each individual polymorphism for mitochondrial function and tumorigenesis is unknown and only functional analysis in relation to cell behavior, proliferation, and apoptosis can determine the significance of these polymorphisms and their association with tumorigenesis. We believe that polymorphisms in mtDNA, both in the D-loop and in the coding region (including haplogroup-specific positions), may cause subtle differences in the encoded protein structure and function [4, 12, 139]. Particular mtDNA genotypes may predispose an individual to an earlier onset of degenerative cellular processes, such as the accumulation of somatic mtDNA variation, decline in OXPHOS capacity or faster cancer progression, as shown in transmitochondrial cybrids [140]. The mtDNA haplotype may also influence its further mutagenesis, as has been shown for haplogroup J that predisposes the mitochondrial genome to mutate at locus 14484, possibly through near-neighbor effects as previously described to occur during mtDNA evolution [141]. All these phenomena may affect both cancer and aging.

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Review Article

Proteomic Profiling of Mitochondrial Enzymes during Skeletal Muscle Aging

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Mitochondria are of central importance for energy generation in skeletal muscles. Expression changes or functional alterations in mitochondrial enzymes play a key role during myogenesis, fibre maturation, and various neuromuscular pathologies, as well as natural fibre aging. Mass spectrometry-based proteomics suggests itself as a convenient large-scale and high-throughput approach to catalogue the mitochondrial protein complement and determine global changes during health and disease. This paper gives a brief overview of the relatively new field of mitochondrial proteomics and discusses the findings from recent proteomic surveys of mitochondrial elements in aged skeletal muscles. Changes in the abundance, biochemical activity, subcellular localization, and/or posttranslational modifications in key mitochondrial enzymes might be useful as novel biomarkers of aging. In the long term, this may advance diagnostic procedures, improve the monitoring of disease progression, help in the testing of side effects due to new drug regimes, and enhance our molecular understanding of age-related muscle degeneration.

1. Introduction

The neuromuscular system is severely affected during the natural aging process [1]. Pathophysiological cycles of denervation and impaired reinnervation, the loss of entire motor units, unloading due to prolonged periods of disuse, and excitation-contraction uncoupling may trigger a substantial loss in skeletal muscle mass and function [2]. Although considerable interindividual differences exist in the functional decline of the musculature during aging, most elderly people experience a general loss in skeletal muscle strength [3]. While regular physical activity and a protein-rich diet can partially counteract severe muscle wasting [4], a sedentary life style and certain medical conditions, such as diabetes, cancer, renal failure, chronic obstructive pulmonary disease, or congestive heart failure [5–7], clearly promote muscle degeneration [8]. Skeletal muscle wasting plays a crucial role in physical disability, frailty, and loss of independence in aged people [9]. Skeletal muscle wasting in the elderly has been termed sarcopenia of old age, whereby this muscular impairment is probably due to multiple factors [10], as outlined in Figure 1. On the cellular level, a variety of abnormal structural, physiological, and biochemical processes have been identified that are directly or indirectly associated with age-dependent muscle wasting. This includes a severe decline in contractile efficiency [11], increased apoptosis [12], denervation-associated atrophy [13], bioenergetic changes [14], impaired ion homeostasis [15], excitation-contraction uncoupling [16], decreased capacity for fibre regeneration [17], a partially diminished cellular stress response [18], and an altered equilibrium of hormones and growth factors crucial for the maintenance of contractile function [19], as well as oxidative stress and mitochondrial abnormalities [20–22]. The general issue of fibre type shifting during aging is still controversial. Although individual muscles in aged humans and animal models of sarcopenia exhibit alterations in the molecular composition of contractile fibres and changes in their glycolytic and aerobic capacity, findings on distinct shifts in fibre types with aging are highly variable [23–26]. However, since mitochondrial functions are clearly impaired in senescent muscle tissues, it was of interest to summarize the impact of recent mass spectrometry-based proteomic studies on the molecular fate of mitochondrial enzymes in senescent fibres. This paper briefly outlines

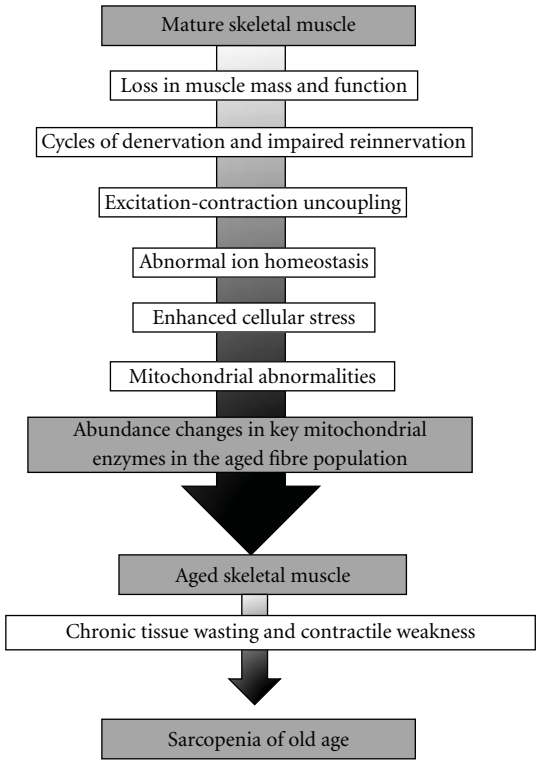

Figure 1: Overview of the multifactorial etiology of sarcopenia. Shown are the main physiological and biochemical events that trigger chronic tissue wasting and severe contractile weakness in senescent skeletal muscles. One of the most striking age-related changes is a drastic alteration in the abundance of mitochondrial enzymes.

the proposed role of mitochondria in cellular senescence and recent achievements of mitochondrial proteomics and then focuses on findings from proteomic profiling studies of aged skeletal muscle preparations and the identification of mitochondrial elements as potential markers of fibre aging.

2. Mitochondria and Cellular Senescence

Mitochondria are the primary site for energy generation via oxidative phosphorylation and play a key role in protein transport, intermediary metabolism, cell cycle progression, calcium signaling, and the regulation of apoptosis [27]. Proteomic cataloguing studies of this crucial organelle suggest the existence of approximately 1,500 mitochondrial proteins [28–30], whereby altered expression levels within the mitochondrial proteome are critical factors for normal development and numerous diseases [31–33]. Changes in mitochondria have long been associated with playing an integral role during natural aging [34–37], and the pharmacological application of antioxidants for counteracting mitochondria-specific symptoms of senescence is being extensively studied [38]. Interestingly, the mitochondrial theory of aging also encompasses the mechanisms that may lead to cellular senescence in contractile tissues [39–41]. Altered levels of mitochondrial activity in aged muscle tissues have been well established and extensively reviewed [42–44]. The detrimental accumulation of mitochondrial DNA deletions and mutations on the genetic level and deficiencies in the mitochondrial electron transport chain on the biochemical level are clearly associated with muscle aging. The pathological consequences of an age-related decline in mitochondrial function are the impairment of essential ATP-dependent cellular processes [45] and amplified oxidative stress in senescent tissues due to the increased release of reactive oxygen species from the mitochondrial respiratory chain [46, 47]. In general, senescent muscle tissues are exposed to an enhanced production of mitochondrial reactive oxygen species, increased mitochondrial apoptotic susceptibility, disturbed mitochondrial bioenergetic functions, and a reduced transcriptional drive for mitochondrial biogenesis [22, 48]. Although these functional impairments clearly occur in skeletal muscle mitochondria during aging, biochemical studies have also demonstrated considerable age-related changes in the abundance and posttranslational modifications of key mitochondrial enzymes.

3. Profiling of the Mitochondrial Proteome

Proteomics is concerned with the large-scale and high-throughput identification and characterization of the global protein constellation of a given biological entity, such as cells, tissues, or body fluids [49]. Protein complements are separated by standard methods, including gel electrophoresis and liquid chromatography [50–52], and individual protein species are usually identified by mass spectrometry [53–55]. The verification of proteomic data is routinely carried out by biochemical, immunological, cell biological, and physiological assays. Skeletal muscle proteomics, in particular, involves the comprehensive biochemical analysis of protein populations from defined muscle tissues, individual muscles, specific fibre populations, or distinct subcellular fractions [56–58]. Figure 2 outlines the standard workflow for the identification of novel aging-associated biomarkers using gel electrophoresis-based proteomics. Total crude tissue extracts, detergent phase-extracted proteins, or mitochondria-enriched fractions are routinely used as starting material for the determination of new mitochondrial markers. The main analytical steps involved in skeletal muscle proteomics are the extraction of a distinct protein population from crude extracts, subcellular fractions, or affinity-purified protein complexes, the efficient separation of proteins by one-dimensional gel electrophoresis, two-dimensional gel electrophoresis, or liquid chromatography, the densitometric mapping of altered protein concentration levels, the unequivocal identification of protein species by mass spectrometry of protease-generated peptide mixtures, and finally the independent validation of proteomic data by enzyme assays, immunoblot analysis, ligand binding assays, or immunofluorescence microscopy.

Since the concentration range of proteins is not a static entity, but highly dynamic, and because the density of proteins spans several orders of magnitude in complex cellular systems, proteomic studies of crude extracts result

mostly in the cataloguing of abundant and soluble protein species. Thus, conventional gel electrophoresis-based proteomics underestimates certain classes of proteins, such as high-molecular-mass proteins, integral membrane proteins, extremely basic or acid proteins, and low-abundance proteins [50–52]. Over the last few years, proteomic approaches have been refined in order to reduce sample complexity by subcellular fractionation protocols and affinity separation techniques [59–61]. Mass spectrometry-based proteomics suggests itself as an ideal analytical method to determine global changes in the mitochondrial protein complement [62, 63]. Mitochondrial proteomics is concerned with the establishment of the entire organelle-associated protein complement and the dynamic nature of posttranslational modifications in mitochondrial components, as well as differential expression patterns within mitochondrial protein populations due to physiological adaptations or pathological insults [64–66]. Considerable tissue-specific differences exist within the mitochondrial proteome and reflect the diversity of mitochondrial functions in individual organs [67–69]. As listed in Table 1, proteomic maps of mitochondria exist for numerous organs from several different species.

The first comprehensive survey of human mitochondria detected approximately 1,500 spots on a silver-stained reference map and identified 46 mitochondria-associated proteins in placental tissue [70]. Subsequent studies have discovered several hundred mitochondrial proteins by mass spectrometry, using differential centrifugation or density gradients consisting of percoll, metrizamide or nycodenz for prefractionation purposes [71–77]. Proteomic analyses yielded 615, 680 and 940 distinct mitochondrial proteins in human and mouse heart, respectively [71–73], 182 and 192 mitochondrial proteins in mouse and rat liver, respectively [74, 75], 823 mitochondrial proteins in human skeletal muscle [76], and 723 and 1,198 mitochondrial proteins in brown and white fat cell lines, respectively [77]. Several proteomic studies have investigated mitochondrial protein populations in several organ systems in parallel, including liver, muscle, heart, kidney, and brain [67–69]. The most comprehensive comparative report on the mitochondrial proteome has created a compendium of 1,098 genes and their protein expression across 14 mouse tissues [78]. Detailed listings of proteomic studies that have focused on mitochondria in health and disease can be found in recent extensive reviews of this specialized field of subproteomics [28, 30, 65]. These crucial cataloguing exercises form now the basis of detailed comparative investigations into disease-dependent alterations in mitochondria [64], including studying the effects of aging on the mitochondrial proteome [79, 80].

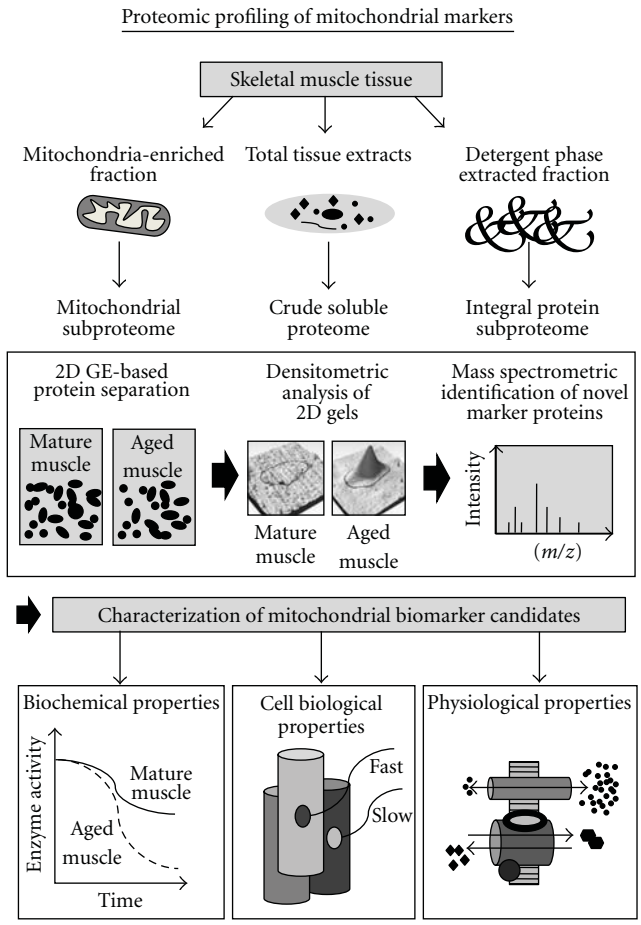

Figure 2: Flowchart of the proteomic workflow to identify and characterize novel mitochondrial markers involved in sarcopenia of old age. Shown are the various steps involved in the high-throughput proteomic screening of tissue specimens, including sample preparation, gel electrophoretic separation, densitometric analysis, and mass spectrometric identification of new candidate proteins.

4. Mitochondrial Markers of Skeletal Muscle Aging

Over the last decade, a large number of proteomic studies have identified potential biomarkers of muscle aging [81]. Studies of aged human muscle and the most widely employed animal model of sarcopenia-related abnormalities, the senescent rat [82], have revealed changes in proteins involved in the regulation of excitation-contraction coupling, ion homeostasis, muscle contraction, muscle relaxation, metabolite transportation, energy metabolism, and the cellular stress response [83–99]. Table 2 lists recent proteomic studies that have identified the potential involvement of mitochondrial elements in sarcopenia of old age. The proteomic analysis of total extracts from aged human vastus lateralis muscle has identified numerous aerobic markers with an increased density, including the mitochondrial enzymes ATP synthase, ubiquinol-cytochrome c reductase, and oxoglutarate dehydrogenase during muscle aging [86]. In analogy, elevated levels of mitochondrial enzymes, such as succinate dehydrogenase, isocitrate dehydrogenase, ATP synthase, malate dehydrogenase, ubiquinol-cytochrome c reductase, and pyruvate dehydrogenase, were also shown to occur during the aging of rat gastrocnemius muscle [93, 94]. These investigations were performed with the fluorescence difference in-gel electrophoretic technique, one of the most powerful biochemical methods to compare

Table 1: List of major profiling studies of the mitochondrial proteome.

Proteomic studies	Mitochondrial protein identification	Reference
Proteomic analysis of human placenta	First comprehensive cataloguing of the mitochondrial proteome, which resulted in the identification of 46 distinct proteins	Rabilloud et al. [70]
Analysis of human and mouse heart	Identification of 680 human mitochondrial proteins and 940 mouse mitochondrial proteins in heart muscle	Taylor et al. [71], Gaucher et al. [72], Zhang et al. [73]
Proteomic profiling of mouse and rat liver	Identification of 182 mouse proteins and 192 rat proteins that are associated with liver mitochondria	da Cruz et al. [74], Fountoulakis et al. [75]
Proteomic profiling of human skeletal muscle	Identification of 823 mitochondrial proteins in human vastus lateralis muscle	Lefort et al. [76]
Proteomic profiling of brown and white adipose cell lines	Identification of 723 mitochondrial proteins in brown adipose cell line and 1,198 mitochondrial proteins in white adipose cell line	Forner et al. [77]
Comparative studies for the establishment of the mammalian mitochondrial proteome from various tissues	Identification of tissue-specific expression patterns of mouse and rat mitochondrial proteins from liver, skeletal muscle, kidney, brain, heart, and various other organs. The most comprehensive comparative study established the mitochondrial protein complement in 14 different tissues	Mootha et al. [67], Forner et al. [68], Reifschneider et al. [69], Pagliarini et al. [78]

concentration changes of distinct protein species in soluble proteomes [100]. The recent proteomic profiling of the detergent phase-extracted protein complement from senescent rat gastrocnemius muscle confirmed a changed concentration of numerous mitochondrial enzymes during aging. The mitochondrial marker enzymes ATP synthase and isocitrate dehydrogenase were found to be significantly increased in aged muscle tissue [99]. In contrast to the highly discriminatory difference in-gel electrophoretic technique used for studying muscle aging [86, 93, 94, 97], proteomic approaches with conventional protein dyes or dyes that cover a limited dynamic range have identified considerably fewer changes in mitochondrial markers [85, 87].

Subproteomic studies of mitochondria-enriched fractions from aged skeletal muscles have shown differential effects on the abundance of mitochondrial enzymes [90, 91, 96–98]. Chang et al. [90] have studied the effect of aging and caloric restriction on the rat mitochondrial proteome. In skeletal muscles, isocitrate dehydrogenase and malate dehydrogenase were shown to be increased in 25-month-old Fisher 344 rats, as compared to 6-month-old rats. Caloric restriction appears to have only a minor effect on age-related changes in the mitochondrial protein complement [90]. Severe metabolic changes in aged skeletal muscle were confirmed by an extensive proteomic survey of mitochondrial preparations from 3-month-old versus 26-month-old rat gastrocnemius muscles [97]. These muscle specimens represent young adult versus senescent contractile tissues, respectively. The fluorescent difference in-gel electrophoretic analysis demonstrated an age-dependent elevation in numerous mitochondrial proteins, including NADH dehydrogenase, ATPase synthase, succinate dehydrogenase, the mitochondrial inner membrane protein mitofilin, peroxiredoxin isoform PRX-III, mitochondrial fission protein Fis1, succinate-coenzyme A ligase, acyl-coenzyme A dehydrogenase, ubiquinol-cytochrome c reductase core I protein, prohibitin, and porin isoform VDAC2 (Figure 3).

Proteomic studies of posttranslational changes in aged skeletal muscle have revealed increased nitration levels in succinate dehydrogenase [83], decreased phosphorylation levels in cytochrome c oxidase and aconitase [92], and altered carbonylation levels in ATP synthase, NADH dehydrogenase, pyruvate dehydrogenase, and isocitrate dehydrogenase [91] during muscle aging [101]. Abnormal posttranslational modifications may alter protein stability, subcellular targeting, intra- and intermolecular interactions, as well as coupling efficiency between substrates and active sites in affected mitochondrial enzymes. This might partially explain impaired mitochondrial functioning in senescent fibres. Thus, natural aging of skeletal muscles appears to be associated with distinct changes in posttranslational modifications of important mitochondrial enzymes. Recently, Ferreira et al. [102] compared the proteomes of subsarcolemmal versus intermyofibrillar mitochondria from rat skeletal muscle. A differential expression pattern was established for 38 mitochondrial proteins. In the future, refined proteomic studies might be able to determine whether intermyofibrillar mitochondria are differently affected by muscle aging as compared to subsarcolemmal mitochondria.

5. Conclusion

Since improved nutritional intake and exercise intervention can only partially alleviate the symptoms of sarcopenia, there is an urgent need to develop novel pharmacological strategies to prevent age-related muscle wasting [103]. Recent publications by working groups on the etiology, epidemiology, potential interventions, and the clinical assessment of sarcopenia show that a general definition of this common geriatric syndrome is still evolving [104–109]. In the future, it will be crucial to establish reliable sarcopenia-specific biomarkers to develop superior diagnostic tools for the correct classification of this age-dependent muscle pathology [110]. Mass spectrometry-based proteomics suggests itself

Table 2: Proteomic identification of mitochondrial proteins during skeletal muscle aging.

Proteomic study	Changes in mitochondrial marker proteins	Reference
Analysis of total extracts from aged human vastus lateralis muscle	General increase in aerobic markers, including mitochondrial enzymes such as ATP synthase, ubiquinol-cytochrome c reductase, and oxoglutarate dehydrogenase during muscle aging	Gelfi et al. [86]
Analysis of total extracts from rat gastrocnemius muscle	Increase in mitochondrial enzymes, such as succinate dehydrogenase, isocitrate dehydrogenase, ATP synthase, and malate dehydrogenase during muscle aging	Doran et al. [93]
Analysis of total extracts from rat gastrocnemius muscle	Differential effect on the abundance of mitochondrial isoforms of aconitase during muscle aging	O'Connell et al. [87]
Analysis of total extracts from aged rat gastrocnemius muscle	Moderate effect on cytochrome c oxidase and isocitrate dehydrogenase during muscle aging	Piec et al. [85]
Analysis of total extracts from rat gastrocnemius muscle	Increase in many enzymes involved in oxidative metabolism, such as ATP synthase, isocitrate dehydrogenase, ubiquinol-cytochrome c reductase, and pyruvate dehydrogenase during muscle aging	Capitanio et al. [94]
Subproteomic study of the effect of aging and caloric restriction on rat muscle mitochondria	Increased levels of isocitrate dehydrogenase and malate dehydrogenase in aged muscle mitochondria. Caloric restriction appears to have only a marginal effect on the mitochondrial proteome	Chang et al. [90]
Subproteomic analysis of mitochondria-enriched fraction from aged rat gastrocnemius muscle	Increased levels of mitochondrial creatine kinase, NADH dehydrogenase, ATP synthase, succinate dehydrogenase, and ubiquinol cytochrome c reductase during muscle aging	O'Connell and Ohlendieck [97]
Analysis of total extracts and mitochondria-enriched fraction from aged ratgastrocnemius muscle	Differential effect on mitochondrial enzymes, such as pyruvate dehyrdogenase, cytochrome c oxidase, isocitrate dehydrogenase, and ATP synthase during muscle aging	Lombardi et al. [96]
Subproteomic analysis of mitochondria-enriched fraction from aged mouse hind limb muscles	Differential effects on the abundance and carbonylation of various mitochondrial enzymes, including NADH dehydrogenase, cytochrome c oxidase, and ATP synthase during muscle aging	Alves et al. [98]
Analysis of detergent phase-extracted protein complement from aged rat gastrocnemius muscle	Increase in mitochondrial marker enzymes, such as ATP synthase and isocitrate dehydrogenase during muscle aging	Donoghue et al. [99]
Proteomic analysis of nitration in aged rat skeletal muscle	Increased nitration levels in succinate dehydrogenase	Kanski et al. [84]
Phosphoproteomic analysis of total extracts from aged rat gastrocnemius muscle	Decreased phosphorylation levels in cytochrome c oxidase and aconitase during muscle aging	Gannon et al. [92]
Proteomic analysis of carbonylation in aged rat skeletal muscle mitochondria	Altered carbonylation levels in numerous mitochondrial proteins, including ATP synthase, NADH dehydrogenase, pyruvate dehydrogenase, and isocitrate dehydrogenase during muscle aging	Feng et al. [91]

as an ideal analytical tool for the study of skeletal muscle aging. The biochemical establishment of a robust protein marker signature for sarcopenia of old age will be extremely useful for (i) formulating a coherent cellular theory of muscle aging, (ii) the development of proper diagnostic criteria that can differentiate between different degrees of age-related muscle weakness, (iii) the identification of novel therapeutic targets to counteract cellular stress and fibre degeneration during aging, and (iv) the evaluation of improved treatment regimes to slow down the aging process.

Recent proteomic studies of mitochondria-enriched fractions and total skeletal muscle extracts have demonstrated altered levels of key mitochondrial enzymes in senescent muscle tissues. Aged neuromuscular systems appear to contain a higher degree of certain mitochondrial enzymes. Thus, although mitochondrial dysfunction and oxidative stress are associated with sarcopenia, muscle aging is also clearly linked to metabolic alterations. This suggests that abundant mitochondrial enzymes may be useful for general muscle profiling and are excellent biomarker candidates for

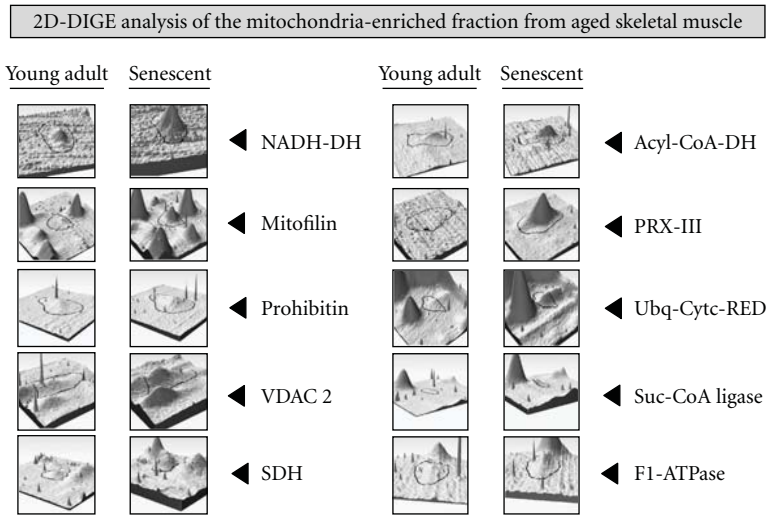

Figure 3: Proteomic profiling of the mitochondria-enriched fraction from aged skeletal muscle tissue. Shown is the comparative graphic representation of distinct two-dimensional protein spots with a changed expression in aged muscle as judged by fluorescence difference in-gel electrophoretic analysis [97]. Individual panels document alterations in the abundance of NADH dehydrogenase (NADH-DH), the inner mitochondrial membrane protein mitofilin, prohibitin, the porin isoform VDAC 2, succinate dehydrogenase (SDH), acyl-coenzyme A dehydrogenase (Acyl-CoA-DH), peroxiredoxin isoform PRX-III, ubiquinol-cytochrome c reductase core I protein (Ubq-Cytc-RED), succinate-coenzyme A (Suc-CoA) ligase, and mitochondrial F1-ATPase.

the biochemical classification of cellular changes during the natural aging process.

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Research Article

Morphofunctional and Biochemical Approaches for Studying Mitochondrial Changes during Myoblasts Differentiation

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This study describes mitochondrial behaviour during the C2C12 myoblast differentiation program and proposes a proteomic approach to mitochondria integrated with classical morphofunctional and biochemical analyses. Mitochondrial ultrastructure variations were determined by transmission electron microscopy; mitochondrial mass and membrane potential were analysed by Mitotracker Green and JC-1 stains and by epifluorescence microscope. Expression of *PGC1α*, *NRF1α*, and *Tfam* genes controlling mitochondrial biogenesis was studied by real-time PCR. The mitochondrial functionality was tested by cytochrome c oxidase activity and *COXII* expression. Mitochondrial proteomic profile was also performed. These assays showed that mitochondrial biogenesis and activity significantly increase in differentiating myotubes. The proteomic profile identifies 32 differentially expressed proteins, mostly involved in oxidative metabolism, typical of myotubes formation. Other notable proteins, such as superoxide dismutase (MnSOD), a cell protection molecule, and voltage-dependent anion-selective channel protein (VDAC1) involved in the mitochondria-mediated apoptosis, were found to be regulated by the myogenic process. The integration of these approaches represents a helpful tool for studying mitochondrial dynamics, biogenesis, and functionality in comparative surveys on mitochondrial pathogenic or senescent satellite cells.

1. Introduction

Skeletal muscle represents an important model for studying mitochondrial behaviour during cell growth and differentiation. Myoblasts cultured *in vitro,* if induced by cell confluence and serum deprivation, follow a myogenic program, which includes an active proliferation, withdrawal from the cell cycle, synthesis of muscle-specific proteins, and fusion into multinucleated myotubes [1, 2]. This event is accomplished by the activation of specific myogenic regulatory factors (MRFs) [3–5].

Recent studies suggest that mitochondria are involved in the regulation of the skeletal muscle physiology and play a critical role in cell growth, cell proliferation, cell death, and cell differentiation [6–13]. In particular, mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression, the activity of myogenic factors, and by control of *c-Myc* expression [8, 14, 15]. Furthermore, differentiation appears to be a program which is dependent on both mitochondrial function and mitochondrial biogenesis, as indicated by the rapid increase in mitochondrial mass/volume, mtDNA copy number, mitochondrial enzyme activities, and mRNA levels within the first 48 hrs of myoblast differentiation [6, 7]. Mitochondrial DNA transcription and replication are key events in cellular differentiation, which requires interaction between the nucleus and the mitochondrion [16].

Several aging theories are associated with mitochondrial damage or with a decline in mitochondrial energy production in which links between mitochondria genome expression and senescence symptoms are not always recognized [17–19]. Our interest is particularly focused on the role that mitochondria may play in the proliferative and differentiation capacity of satellite stem cells. It is well documented that with aging, satellite stem cells lose both mitogenic and myogenesis abilities and may decrease in numbers in both mice and humans [20–23]. The C2C12 cell line *satellite* myoblasts could offer a suitable model for studying mitochondrial behaviour during the differentiation program.

In this study, we combined a morphological and biomolecular approach to analyze changes in mitochondrial phenotype, ultrastructure, biogenesis, and functional activity during C2C12 myoblast differentiation. Although the contribution of the proteomic profile of mitochondria during the myogenesis program is significant, it has not been described in the literature. In this paper we aim to better define the involvement of mitochondria in the regulation of muscle cell differentiation and discover new proteins potentially involved in the crosstalk between nuclei and mitochondria.

2. Material and Methods

2.1. Cell Line. Mouse C2C12 myoblasts were grown in flasks in the presence of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine at 37°C, and 5% CO_2. To induce myogenic differentiation, when 80%–90% confluence was obtained, the medium was changed to DMEM supplemented with 1% FBS. Cells were analyzed at the undifferentiated stage and at the early-, middle-, and late-differentiation stage. In order to eliminate divergences in the differentiation time points analyzed, we assessed several differentiation markers. The cells, grown in the presence of 10% fetal calf serum until 80% cell confluence, were considered undifferentiated cells, corresponding to day 0 of the differentiation process (T0). To induce differentiation, cells at T0 were switched to differentiation medium. They were analyzed in the early-differentiation stage, 24 h after serum removal (T1), in mid-differentiation, 3–5 days after serum removal, when myotubes containing one of two nuclei appeared (T3–5), and in the late-differentiation stage, that is, 7–10 days after serum removal, in the presence of long multinucleated myotubes (T7-10).

2.2. Estimation of Myoblast Fusion. Myoblasts and myotubes were methanol fixed and air dried under different experimental conditions. They were then stained with water 1 : 10 May Grunwald-Giemsa solution, washed, and mounted to evaluate cell fusion. Cells were considered fused if they contained two nuclei within one cytoplasmic continuity as reported by Ferri et al. [5]. The fusion percentage was evaluated as the number of nuclei in myotubes divided by the total number of nuclei in myoblasts and myotubes magnified by 100 (×40 objective) using a TE 2000-S Nikon reverted microscope (RM) with a digital Nikon DN100 acquisition system. Twenty optical fields were randomly chosen. Data were expressed as means ± S.E.M.

2.3. Mitochondrial Ultrastructure. Undifferentiated and differentiated cell monolayers were washed and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 15 min, gently scraped, and centrifuged at 1200 rpm.

Cell pellets, as well as purified mitochondria, were further fixed by glutaraldehyde for 1 h. All specimens were OsO_4 postfixed, alcohol dehydrated, and embedded in araldite, as previously described [24]. Thin sections were stained with uranyl acetate and lead citrate and analysed with a Philips CM10 electron microscope. Mitochondrial density was calculated in 20 different areas of 10×15 cm at 28000 magnification. Mitochondrial sizes were evaluated at 28000 magnification using the Philips CM10 microscope and Megaview software system.

2.4. Mitochondrial Mass and Membrane Potential. The fluorescent dye Mito Tracker Green FM (Molecular Probes), which covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues regardless of membrane potential (DWm) and JC-1 (Molecular Probes), a mitochondrial membrane potential sensor, were used to monitor mitochondrial mass and membrane potential respectively [25, 26]. The medium was removed from the culture dish and replaced with prewarmed growth medium containing 100 nM Mito Tracker Green or 2 μg/mL JC-1. After incubation for 20 min at 37°C, cells were immediately washed twice in cold PBS and analyzed using a Zeiss LSM 510 metaconfocal microscope. The variation of JC-1 signals was also analyzed by Zeiss LSM Image Examiner software.

2.5. Nucleic Acid Extraction and cDNA Synthesis. At each differentiation step, plates ($n = 3$) were washed with PBS, and nucleic acids were isolated. Total DNA and total RNA were extracted using QIAmp DNA kit (Qiagen, Chatsworth, Calif, USA) and RNeasy Mini Kit (Qiagen, Chatsworth, Calif, USA), respectively, following the manufacturer's instructions. Nucleic acid concentrations were estimated spectrophotometrically (DU-640; Beckman Instruments, Milan, Italy) at 260 nm. One microgram of DNase-treated total RNA was reverse transcribed using Omniscript RT (Qiagen, Chatsworth, Calif, USA) and random hexamers in a final volume of 20 μL as suggested in the manufacturer's protocol.

2.6. Construction of the Reference Plasmid pDGC. To construct the reference plasmid pDGC, a 98 bp amplicon of the mouse GAPDH, Acc. no. NM_008084, and a 100 bp sequence of mouse mtDNA located within the *COXII*, Acc. no. NP_904331.1, were inserted into the TA cloning and HindIII restriction sites, respectively, of the polylinker region of pDrive (Qiagen, Chatsworth, Calif, USA). The resultant dual-insert plasmid of 4048 bp, renamed pDGC, was purified by using DNA plasmid purification Kit (Qiagen, Chatsworth, Calif, USA) and was verified as having only one copy

of each insert by restriction enzyme digestion as well as DNA sequencing. Plasmid concentration was estimated spectrophotometrically (DU-640; Beckman Instruments, Milan, Italy) at 260 nm and was adjusted to give a stock solution of 1×10^9 molecule/μL. Further 10-fold serial dilutions down to a concentration of 1×10^1 molecule/μL were prepared.

2.7. Determination of mtDNA Content and mRNA Expression Levels by Quantitative Real-Time PCR. All quantitative real-time PCR reaction were carried out in a Bio-Rad iCycler iQ Multi-Color Real-Time PCR Detection System using 2x Quantitect SYBR Green PCR kit (Qiagen). The PCR conditions were set up as follows: hot start at 95°C for 10 min then 40 cycles of the two steps at 95°C for 30 sec and at 60°C for 30 sec. Reaction mix (25 μL final volume) consisted of 12.5 μL Mix Hot-Start (Qiagen), total DNA (50 ng) or cDNA (1 μL) template, 2 μL SYBR Green, and 0.3 μM of each primer (Table 1). Threshold cycle (Ct) was determined on the linear phase of PCRs using the iCycler iQ Optical System software version 3 (BioRad, Milan, Italy). The specificity of the amplification products obtained was confirmed by examining thermal denaturation plots, by sample separation in a 3% DNA agarose gel and by sequencing. A precise determination of mitochondrial DNA (mtDNA) copy number was determined amplifying both *COXII* and GAPDH as mtDNA and nDNA targets, respectively. Quantification of mtDNA was performed by reference to a single recombinant plasmid (pDGC) containing a copy of each target DNA sequence (mitochondrial and nuclear). *COXII* and GAPDH gene copy number were determined by interpolating the threshold cycle (Ct) from standard curves that were obtained using serial dilution of the recombinant plasmid pDGC. The mtDNA/nDNA ratio was obtained, relating the mitochondrial and nuclear DNA quantities. The relative expression of *Tfam, PGC1-α* transcription factors, and *COXII* were quantified using 1 μL of cDNA template and the PCR condition already described above. The amount of each target transcript was related to that of the reference gene (the ribosomal protein S16) using the method described by Pfall [28]. In fact, previous experiments have shown that S16 mRNA is stable during the differentiation process [5]. All oligonucleotide primers were designed using Primer Express version 1.0 (Perkin-Elmer Applied Biosystem) from the GenBank database and are listed in Table 1.

2.8. Preparation of Mitochondria for Enzymatic and Proteomic Analyses. About 3×10^7 cells were harvested and washed with 1 × PBS buffer. The pellet was resuspended in 5 mL of an ice-cold solution containing 5 mM K^+-Hepes, pH 7.4, 210 mM mannitol, 1 mM EGTA, 70 mM sucrose, and 55 μg/mL digitonin and homogenized by 10 strokes in an ice-cold glass homogenizer. Nonlysed cells and nuclei were pelleted by centrifugation at 750 g for 20 min at 4°C, and the supernatant was centrifuged again at 8000 g for 15 min at 4°C. The resulting mitochondrial pellet was resuspended in 1 mL of 5 mM K^+-Hepes, pH 7.4, 210 mM mannitol, and 70 mM sucrose at 37°C and treated for cytochrome oxidase activity and proteomic analysis as described below.

2.9. Enzymatic Activity of Cytochrome c Oxidase. Cytochrome c oxidase activity was determined spectrophotometrically using the Cytochrome c Oxidase Assay Kit (Sigma, MO, USA). Reactions were started by the addition of ferrocytochrome c. The difference in extinction coefficients ($\Delta\varepsilon^{mM}$) between ferrocytochrome c and ferricytochrome c is 21.84 at 550 nm. One unit of enzyme will oxidize 1.0 μmole of ferrocytochrome c per minute at pH 7.0 at 25°C. The proteins were determined according to the method of Lowry et al. [29] using bovine serum albumin as the standard.

2.10. Proteomic Analysis. Mitochondria were resuspended in urea lysis buffer (8 M urea, 4% CHAPS, 65 mM DTE, and 40 mM Tris base) and sonicated for 5 s on ice. Following centrifugation at 21000 g, protein concentration was determined by Bradford assay [30]. Aliquots were then stored at −80°C until use. Two dimensional electrophoresis (2-DE) was carried out as previously described [31]. Briefly, isoelectric focusing was made on Immobiline strips providing a nonlinear pH 3–10 gradient (GE Healthcare Italy, Milan, Italy) using an IPGphore system (GE Healthcare) and applying an increasing voltage from 200 V to 3500 V during the first 3 h, then stabilized at 5000 V for 20 h. After IPG strip equilibration, the second dimension was carried out in a Laemmli system on 9%–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) at 40 mA/gel constant current, until the dye front reached gel bottom. Forty-five μg (analytical runs) or 500 μg (semipreparative runs) of proteins were used for each electrophoretic run.

Analytical gels were stained with silver nitrate [32], while semipreparative gels for mass spectrometry analysis were stained with Brilliant Blue G-Colloidal (Sigma- Aldrich, Saint Louis, USA) according to the manufacturer's procedure. Gel images were acquired by Fluor-S MAX multi-imaging system (BioRad Laboratories Italy, Segrate, Italy), and the data were analysed with ImageMaster 2D Platinum software. To test the significant differences in the relative protein levels for each spot, a paired Student's *t*-test statistic was applied at a significant level of $P < .05$.

The gel digestion procedure was adapted from Shevchenko et al. [33] as previously described [34].

LC-ESI-MS/MS analysis was performed using a Q-TOF microTM mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray nanoflow electrospray ion source and a CapLC system. The sample was analyzed using a Symmetry C18 nano column (Waters, Milford, Mass, USA) as an analytical column. For protein identification, MS/MS spectra were searched by MASCOT (Matrix science,www.matrixscience.com, UK) using the database of NCBI nr. For unmatched peptides, however, good quality MS/MS spectra were manually sequenced using de novo sequencing process (carried out by PepSeq of the Masslynx 4.0 software, Micromass), and the obtained sequence was subsequently used in Expasy TagIdent.

2.11. Statistical Analysis. Unless noted otherwise, the results were expressed as mean values ± S.E.M. for the indicated number of measurements. Results from PCR real-time

Table 1: List of primer pairs.

Genes	Primers (forward)	Primers (reverse)	References
Mouse COXII	5′-CATCTGAAGACGTCCTCCACTCAT-3′	5′-TCGGTTTGATGTTACTGTTGCTTGAT-3′	this study
Mouse TfamA	5′-GGGAGCTACCAGAAGCAGAA-3′	5′-CTTTGTATGCTTTCCACTCAGC-3′	this study
Mouse PGC1-α	5′-CGGAAATCATATCCAACCAG-3′	5′-TGAGGACCGCTAGCAAGTTTG-3′	[27]
Mouse S16	5′-TGAAGGGTGGTGGACATGTG-3′	5′-AATAAGCTACCAGGGCCTTTGA-3′	[5]
Mouse GAPDH	5′-TGACGTGCCGCCTGGAGAAA-3′	5′-AGTGTAGCCCAAGATGCCCTTCAG-3′	[27]

Table 2: Mitochondrial area and number variability during differentiation by means of ultrastructural observations of resin-embedded sections.

Differentiation day	Δ cell mitochondria area/10 × 15 cm total surface	Δ isolated mitochondria area/10 × 15 cm total surface	Number of mitochondria/10×15 cm total area
$T = 0$	3.30E−02 ± 0.005	6.90E−02 ± 0.009	6 ± 0.89
$T = 1$	9.30E−02 ± 0.008	9.80E−02 ± 0.008	10 ± 1.14
$T = 4$	8.20E−02 ± 0.004	8.10E−02 ± 0.004	13 ± 0.91
$T = 7$	3.40E−02 ± 0.008	5.40E−02 ± 0.005	15 ± 0.86

analysis were compared with the ANOVA test, followed by a post hoc test using Tukey's multiple comparison test. The threshold of significance for the ANOVA and the Tukey's test was fixed at $P \leq .05$.

3. Results

3.1. Cell Differentiation. The monolayer organization, as directly analysed at RM and by means of Giemsa staining, deeply changes from undifferentiated myoblasts to myotubes. In the undifferentiated condition (Figures 1(a), 1(b), and 1(c)), myoblasts appear as fusiform or star-shaped cells, mostly flattened and closely adherent to the substrate. At the initial differentiation stage (Figures 1(d), 1(e), and 1(f)), intercellular spaces disappear, cells progressively align, and, occasionally, elongate. Four days after differentiation induction (Figures 1(g), 1(h), and 1(i)), early myotubes, with 2 or more centrally located nuclei, appear ($T = 4$, fusion index = 38 ± 3.4%). The late differentiation condition (7 days) is characterized by the presence of highly structured myotubes (Figures 1(j), 1(k), and 1(l)). These are 100–600 μm syncytia and contain even more than 20 nuclei, mainly centrally located or, occasionally, aligned in parallel rows ($T = 7$, fusion index 84.6 ± 6%).

3.2. Morphofunctional Changes in Mitochondrial Content. Changes in mitochondrial ultrastructure were determined by transmission electron microscopy (TEM). Figure 1 shows the progression of C2C12 cell differentiation and the related mitochondrial behaviour. Their number per area significantly increases from the undifferentiated condition (c), through the initial (f) and the intermediate (i) differentiation stages, to the final phase, characterized by myotubes, which show the maximal mitochondrial content (l). Conversely, the size of single mitochondria, appears to change throughout differentiation. It increases in the undifferentiated stage (c) reaching maximal values at initial differentiation condition (f). It then steadily decreases (f, i), showing minimal values in the late differentiation stage (l). TEM of isolated mitochondria further highlights mitochondrial changes. Table 2 represents mitochondrial number and area variability during differentiation. They undergo a progressive rounding from 0 (Figure 1, inset c) to 7 day (Figure 1, inset l) after differentiation induction.

Analysis of mitochondria suggests a numerical increase of mitochondrial cristae from the undifferentiated to differentiated condition (Figure 1, insets: c, f, i, and l) probably correlated with the reported increase in enzymatic activities [6].

Figure 2 describes mitochondrial characteristics during differentiation, analysed by confocal microscopy, after Mito Tracker green (a–d) and JC-1 (e–h) staining, both specific mitochondrial dyes. The first covalently binds to mitochondrial proteins and is generally considered an available indicator of mitochondrial mass. The second undergoes characteristic fluorescence changes according to the mitochondrial membrane $\Delta\Psi$, thus revealing functional mitochondrial alterations. In myoblasts (a, b, c, and d), both fluorescent probes show a perinuclear mitochondrial distribution. Indeed, at initial differentiation stages, numerous mitochondria can be identified as clearly distinguishable single organelles. Moreover, after differentiation induction, mitochondrial mass increased appearing uniform in myotubes (e and f). Mitochondrial membrane potential also increased, highlighted by JC-1 main red staining (g), still more evident in late differentiation condition shown in (h). Graphs of lower panel show the increasing level of red fluorescence JC-1 intensity from myoblasts (i) to late myotubes (j).

3.3. mtDNA Content. To ensure accurate quantification of mtDNA, we applied a PCR-based assay using a dual-insert reference plasmid, containing both mtDNA and nuclear DNA targets [35]. In this work, pDrive plasmid was used to

Table 3: Identification of mitochondrial protein differentially expressed during myogenesis.

No.	Protein	Score	NCBI nr	Peptides	MW	PI	Localization
1	Malate dehydrogenase, mitochondrial (MDH2)	162	DEMSMM	IFGVTTLDIVR, VDFPQDQLATLTGR, IQEAGTEVVK	35589	8.93	Mitochondrial matrix
2	Malate dehydrogenase precursor (MDH2)	345	DEMSMM	VAVLGASGGIGQPLSLLLK, IFGVTTLDIVRANTFVAELK, VDFPQDQLATLTGRIQEAGTEVVK, MIAEAIPELK	35589	8.93	Mitochondrial matrix
3	Aldehyde dehydrogenase 2, mitochondrial (Aldh2); putative uncharacterized protein	143	Q3TVM2_MOUSE	TIPIDGDFFSYTR, VAEQTPLTALYVANLIK, EAGFPPGVVNIVPGFGPTAGAAIASHEGVDK	56560	7.03	Mitochondrial matrix
4	Aldehyde dehydrogenase precursor, mitochondrial	411	I48966	TIPIDGDFFSYTR, LGPALATGNVVVMK, TFVQENVYDEFVER, TEQGPQVDETQFK, GYFIQPTVFGDVK, TIEEVVGR, YGLAAAVFTK	56502	7.53	Mitochondrial matrix
5	Voltage-dependent anion channel 1 (VDAC1)	161	VDAC1_MOUSE	LTFDSSFSPNTGK, VTQSNFAVGYK, LTLSALLDGK	32331	8.55	Mitochondrial outer membrane
6	Voltage-dependent anion channel 1 (VDAC1)	344	VDAC1_MOUSE	GYGFGLIK, WTEYGLTFTEK, LTFDSSFSPNTGK, VTQSNFAVGYK, VNNSSLIGLGYTQTLKPGIK, LTLSALLDGK	32331	8.55	Mitochondrial outer membrane
7	Pyruvate dehydrogenase (lipoamide) beta. (Pdhb protein)	288	Q99LW9_MOUSE	TYYMSAGLQPVPIVFR, DFLIPIGK, IMEGPAFNFLDAPAVR, VTGADVPMPYAK, VLEDNSVPQVK	34814	5.63	Mitochondrial matrix

Table 3: Continued.

No.	Protein	Score	NCBI nr	Peptides	MW	PI	Localization
8	Prohibitin	396	A39682	DLQNVNITLR, ILFRPVASQLPR, IYTSIGEDYDER, VLPSITTEILK, FDAGELITQR, AAIISAEGDSK, AAELIANSLATAGDGLIELR, NITYLPAGQSVLLQLPQ	29802	5.57	Mitochondrial intermembrane space
9	ATP synthase D chain, mitochondrial (ATP5H)	131	ATP5H_MOUSE	ANVAKPGLVDDFEK, YTALVDQEEKEDVK	18607	5.52	Mitochondrial inner membrane
10	Ubiquinol-cytochrome *c* reductase core protein 1	135	Q3THM1_MOUSE	TDLTDYLNR, IQEVDAQMLR	52806	5.89	Mitochondrial inner membrane
11	Fumarate hydratase precursor, mitochondrial (FH)	170	UFRT	AAAEVNQEYGLDPK, AIEMLGGELGSK, VAALTGLPFVTAPNK	54429	9.06	Mitochondrial
12	Superoxide dismutase precursor	72	DSRTN	GDVTTQVALQPALK	24659	8.96	Mitochondrial matrix
13	Aconitase 2, mitochondrial (ACO2)	340	Q3UDK9_MOUSE	DINQEVYNFLATAGAK, SQFTITPGSEQIR, NTIVTSYNR, FNPETDFLTGK, NAVTQEFGPVPDTAR, WVVIGDENYGEGSSR	85376	8.08	Mitochondrial matrix
14	Dihydrolipoamide dehydrogenase (DLDH)	139	Q99LD3_MOUSE	ADGSTQVIDTK, EANLAAAFGHPINF	54238	7.99	Mitochondrial matrix

Table 3: Continued.

No.	Protein	Score	NCBI nr	Peptides	MW	PI	Localization
15	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit (ATP5B)	998	Q3TFD7_MOUSE	LVLEVAQHLGESTVR, TIAMDGTEGLVR, VLDSGAPIK, IPVGPETLGR, IMNVIGEPIDER, VVDLLAPYAK, IGLFGGAGVGK, TVLIMELINNVAK, EGNDLYHEMIESGVINLK, VALVYGQMNEPPGAR, VALTGLTVAEYFR, FTQAGSEVSALLGR, AIAELGIYPAVDPLDSTSR, IMDPNIVGNEHYDVAR, ILQDYK, FLSQPFQVAEVFTGHMGK	56207	5.25	Mitochondrial inner membrane
16	Protein disulfide isomerase A3 (Pdia3)	94	PDIA3_MOUSE	DASVVGFFR, GFPTIYFSPANK, ELNDFISYLQR	56472	5.88	Endoplasmic reticulum, also present in mitochondria (see discussion).

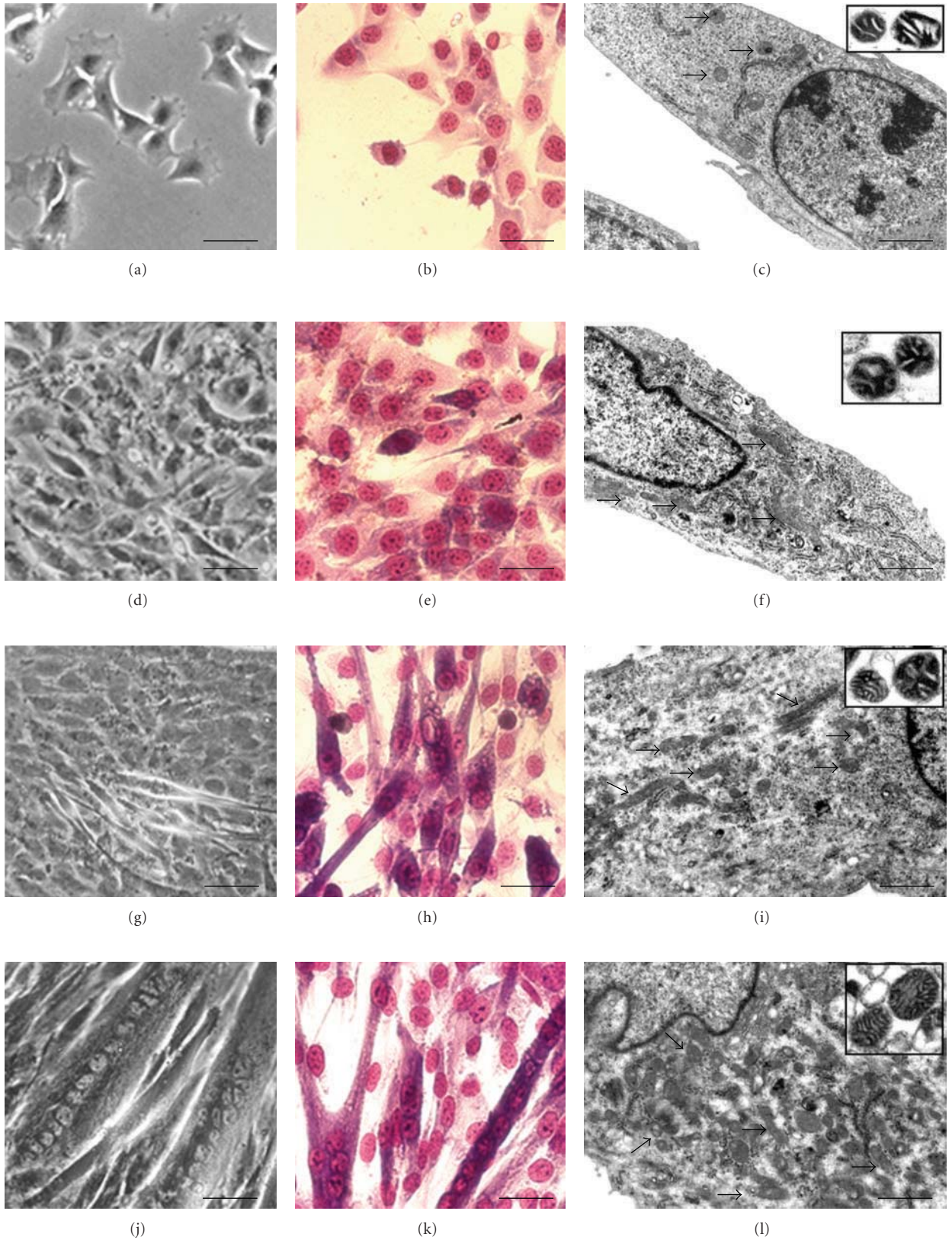

Figure 1: Undifferentiated (a, b, c), early differentiation (d, e, f), intermediate differentiation (g, h, i) and late differentiation stages (j, k, l), are indicated by RM (a, d, g, j), Giemsa staining (b, e, h, k), and TEM (c, f, i, l). Mitochondrial morphology is further detailed by the correspondent insets, showing TEM analysis of isolated mitochondria. C2C12 cell differentiation morphological progression is evident, as well as mitochondrial behaviour in the various stages. (a, b, d, e, g, h, j, k): Bar = 20 μm; (c, f, i, l): Bar = 0.5 μm; insets, Bar = 0.1 μm.

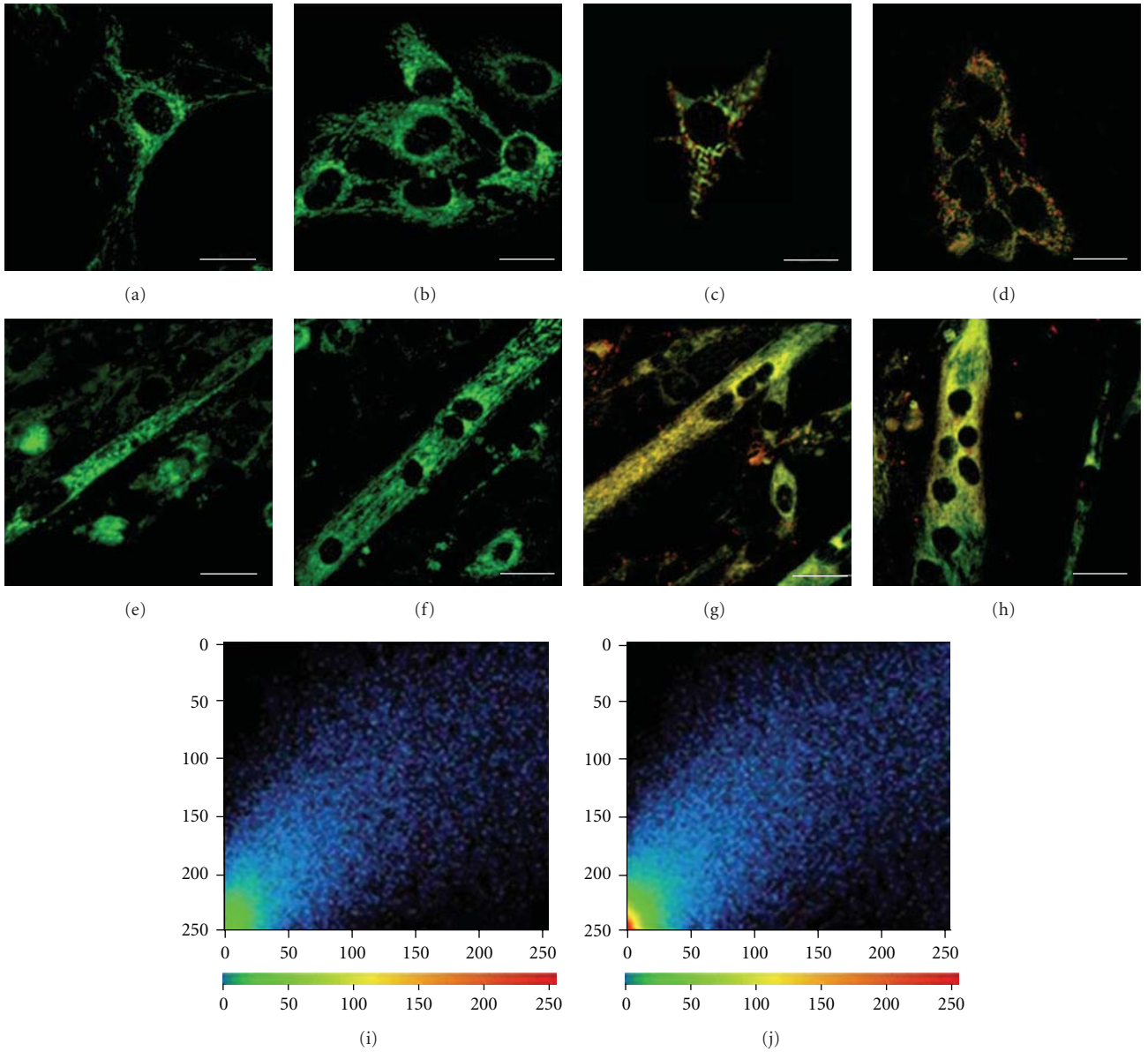

Figure 2: Confocal microscopy of C2C12 myoblasts (a–d) and late myotubes (e–h), after Mito Tracker (a, b, e, f) and JC-1 (c, d, g, h) staining. Graphs of lower panel show the different fluorescence JC-1 intensity in myoblasts (i) and late myotubes (j). (a–h): Bar = 20 μm.

construct the reference plasmid pDGC, containing a single copy of *COXII* and GAPDH segments, the mitochondrial and nuclear target genes, respectively.

As shown in Figure 3(a), twenty-four hours after differentiation induction, the relative amount of mtDNA undergoes a 2-fold increment at the intermediate period of differentiation ($T = 3$) reaching a plateau level at the final stage of maturation ($T = 7$).

3.4. mRNA Expression Level of Mitochondrial Biogenesis "Master" Genes. An increase in mitochondrial biogenesis reflects an enhanced expression of nuclear and mitochondrial genes [36–38]. Two master genes involved in the mitochondrial biogenesis, the nuclear transcriptional coactivator peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (*PGC1α*), and the mitochondrial transcription factor A (*Tfam*) were quantified using RT real-time PCR during differentiation. *PGC1α* induces mitochondrial biogenesis by interacting with several nuclear transcription factors [36–39], and *Tfam* is involved in the mitochondrial genome transcription [40, 41], replication [42], and it is also crucial for maintaining mitochondrial DNA [43].

As shown in Figure 3(b), *PGC-1α* expression does not change during the first 24 h from the induction of differentiation while progressively increasing up to 9.2-fold in differentiated myotubes on the 7th day compared to the myoblasts at time T0.

The *Tfam* expression level during the myoblasts differentiation is slightly shifted compared to the *PGC-1α*

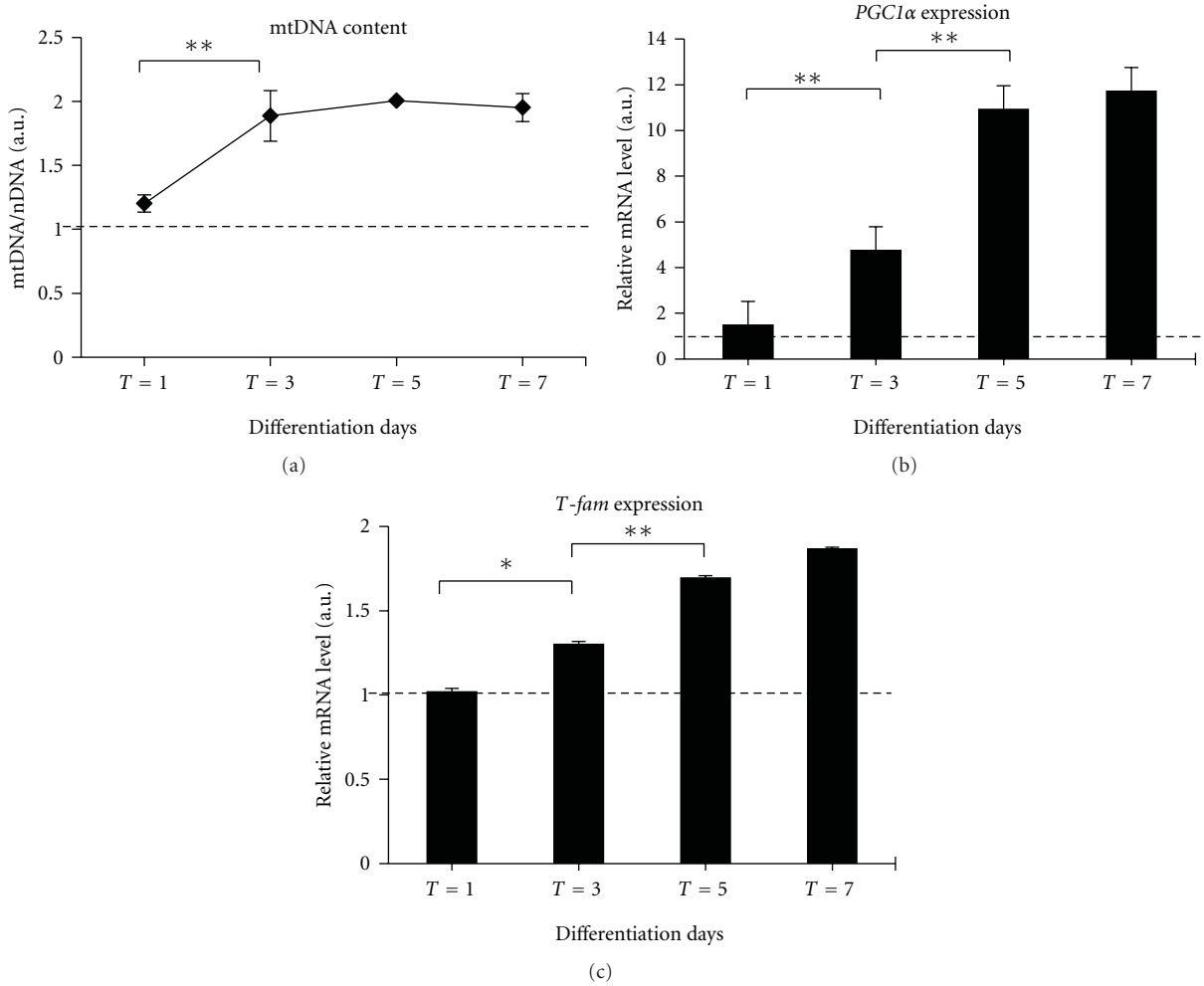

Figure 3: Evaluation of mitochondrial biogenesis during myoblast differentiation. In (a), determination by real-time PCR of mtDNA content expressed as mtDNA/nDNA ratio (*COXII*/GAPDH), as described in Section 2. In (b), quantitative analyses of *PGC-1α* and *T-fam* by real-time PCR. The amount of each target transcript was related to that of the reference gene (the ribosomal protein S16). Data are expressed as the mean ± SEM of three experiments; all samples were analyzed in triplicate. Results from PCR real-time analysis were compared with the ANOVA test, followed by a post hoc test using Tukey's multiple comparison test. The threshold of significance for the ANOVA and the Tukey's test was fixed at $^{*}P \leq .05$; $^{**}P \leq .01$.

expression; in fact, it increased significantly between days 3–7 (Figure 3(c)).

3.5. Cytochrome c Oxidase Activity and COXII Expression Level. The mitochondrial enzymatic activities of cytochrome oxidase reflecting the respiratory chain activities were significantly higher in myoblasts able to differentiate (Figure 4(a)).

In addition, we evaluated the expression level of the corresponding gene coding for the subunit II of mitochondrial cytochrome c oxidase *(COXII)*, which represents a target gene for mitochondrial transcriptional activity [27, 44, 45]. On days 3–7, the mitochondrial *COXII* transcript levels were significantly higher than in proliferating myoblasts (Figure 4(b)).

3.6. Changes in Mitochondrial Proteomic Profile. To highlight significant changes in mitochondrial proteome during differentiation, we performed a 2D page on mitochondria isolated from C2C12 myoblasts over a 7-day time span differentiation. A total of 994 spots (mean) could be resolved on a silver-stained large 2DE gel, where we loaded 45 μg of total protein. A larger amount of protein *per spot* was necessary for protein identification, thus we used preparative gels stained with Brilliant Blue G-Colloidal. To evaluate the possible presence of cellular contaminants, we compared the mitochondrial map with that of the whole cellular lysate in which we had previously identified several cytosolic and membrane proteins [46]. The comparison of 2D maps of mitochondria and whole cell lysate allowed us to state that the preparation of mitochondria contained little or no cellular contaminants.

The study of quantitative changes of individual proteins in a purified mitochondrial fraction showed that 32 mitochondrial proteins increased significantly in abundance

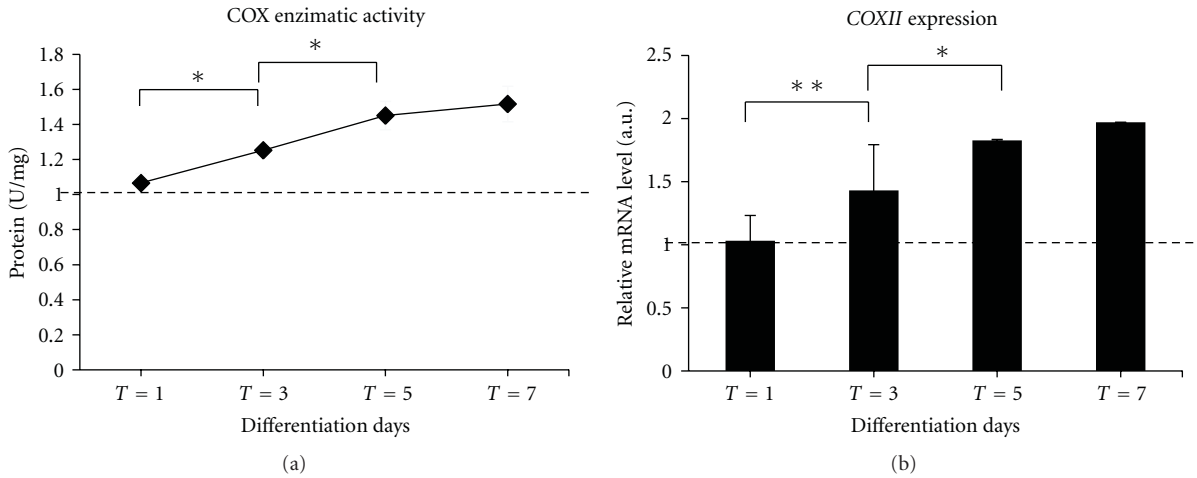

FIGURE 4: Time course change of cytochrome oxidase (COX) enzymatic activity and transcription level of *cytochrome oxidase* subunit II (*COXII*) gene at progressive differentiation stages. (a) Quantitative analysis enzymatic activity. (b) The expression level of *COXII* is related to S16 mRNA gene level. Results from PCR real-time analysis were compared with the ANOVA test, followed by a post hoc test using Tukey's multiple comparison test. The threshold of significance for the ANOVA and the Tukey's test was fixed at $^{*}P \leq .05$; $^{**}P \leq .01$.

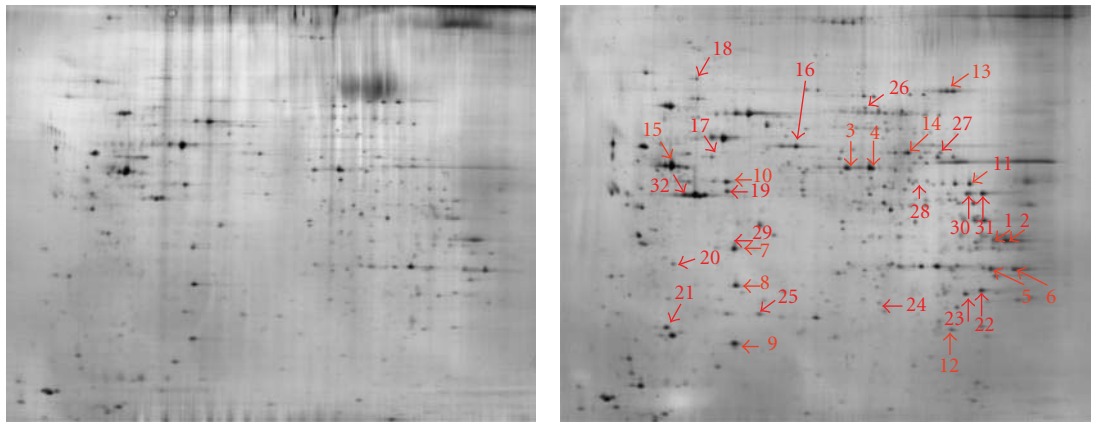

FIGURE 5: Image of a silver-stained 2-DE gel of 45 μg purified mitochondrial proteins from C2C12 myoblasts at 0 (a) and 7 (b) days of differentiation time. Differentially expressed spots are indicated by arrows and numbered according to Table 3.

(Figure 5). The proteins showing the greatest expression changes were also characterized by electrospray ionisation (ESI) tandem mass spectrometry. In particular, the major changes occurred between *T*1 and *T*4 time of differentiation, while fewer differences were shown between *T*0-*T*1 and *T*4–*T*7 (Table 3).

The main mitochondrial proteins which could be detected in fully differentiated syncytia were involved in the citric acid cycle (malate dehydrogenase: MDH2, fumarate hydratase: FH, and aconitase 2: ACO2) or belong to the pyruvate dehydrogenase complex (pyruvate dehydrogenase, lipoamide beta: PDHB, dihydrolipoamide dehydrogenase), complex III (ubiquinol-cytochrome c reductase core protein 1: UQCRC1) and complex V (ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit: ATP5B, and ATP synthase d chain: ATP5H) of the respiratory chain.

This was also interesting for the superoxide dismutase (MnSOD), a voltage-dependent anion-selective channel protein 1 (VDAC1), and the protein disulfide-isomerase A3 (Pdia3) that were differentially expressed during differentiation.

4. Discussion

In this study, we described temporal mitochondrial changes during the myogenic program of C2C12 myoblasts by analyzing complementary key parameters for mitochondrial dynamics, biogenesis, and functionality. Of particular interest is the contribution of the proteomic approach to better define the pattern of mitochondrial protein expression accompanying differentiation in myotubes and potentially involved in the crosstalk between nuclei and mitochondria.

Morphological analysis performed by fluorescence microscopy with markers of mitochondrial mass/volume and $\Delta\Psi$, as well as ultrastructural analysis, allowed us to acquire more information regarding the mitochondrial organization and dynamics in C2C12 myoblast differentiation.

Mitochondrial organization in myoblasts was perinuclear, and it was possible to discriminate individual mitochondrion by both MitoTracker Green and JC1 staining. This type of mitochondrial distribution is described in the literature for other cell types including fibroblasts [47], pancreatic acinar cells [48, 49], astrocytes, and neurons [50].

On the contrary, in myotubes, morphological observation by epifluorescence did not allow us to discriminate individual mitochondrion, showing homogeneous staining, representative of the mitochondrial network, well described in skeletal muscle tissue [51, 52]. TEM analysis showed a mitochondrial remodeling during differentiation and alignment of organelles along the myotubes.

At this level, we cannot show the formation of a network equal to that which is found in skeletal muscle fibers, where mitochondria are arranged in crystal structures closely related to the sarcoplasm [51, 52]. Indeed, the sarcomeres of myotubes are only sketched [53], but they may support the development of a mitochondrial network during myotube maturation.

The mitochondrial counting *per* area of cell surface, obtained by TEM, showed that the number of mitochondria increased from undifferentiated to differentiated conditions. Mature myotubes contained approximately 2-fold more mitochondria than myoblasts. However, in the first 24 hours after induction of differentiation, the mitochondria increased in size up to 3-fold gradually decreasing in size only after the intermediate phases to reach the same size observed in myoblasts at $T = 0$, in mature myotubes ($T = 7$). This observation suggests that mitochondria first undergo fusion and then fission, which allows their distribution during syncytia formation as previously reported [54]. Nevertheless, the mitochondria in myotubes showed a greater extension of mitochondrial cristae than mitochondria in myoblasts. Marked stimulation of the biosynthesis of the phospholipid cardiolipin during the differentiation phases has been observed in previous studies on L6E9 myoblasts and other cells [54, 55]. It is probably necessary to supply the proper amount of functional mitochondrial inner membrane for the respiratory chain proteins involved in oxidative metabolism [7].

All the parameters observed through morphological analysis confirm a linear increase in mitochondrial biogenesis during differentiation. The morphological analysis corroborates the progression of the myogenic process and the increase in biochemical markers such as the transcription factors of mitochondrial biogenesis *PGC-1 α* and *Tfam*.

Of particular interest was the timing of mtDNA replication compared to mitochondrial biogenesis. Although mitochondrial biogenesis increased linearly during differentiation, mtDNA content increased significantly from the early days of differentiation already reaching a plateau at the intermediate stage. Hence, our investigation shows a slight difference in timing between DNA replication and mitochondrial biogenesis. This shift could be explained by the biological cycle of mitochondria [56]. Mitochondrial fission is preceded by an extension of the organelles and the mtDNA replication phase. Although there is a slight shifting, the correlation between the number of copies of mtDNA and mitochondrial biogenesis is positive ($r^2 = 0.85$, data not shown).

In several studies, the measure of mtDNA copy number has been considered proportional to the number of mitochondria, a golden star for mitochondrial density [57–60]. However, changes in mitochondrial abundance regardless of the mtDNA copy number may occur, especially in peculiar conditions such as during alterations in the rates of intracellular ROS generation [61]. Franko et al., investigating C2F3 mouse myoblasts, showed that an increment in mtDNA does not always correlate with the proliferation of mitochondria or with their activity [62]. In this investigation, the mtDNA copy number of C2C12 myoblasts significantly increased during the early-intermediate differentiation phases ($T = 1$ and $T3$) up to 2-fold remaining constant during the myotube maturation. Likewise, over the course of myoblast differentiation in rat cell line L6, a small but significant increase in mitochondrial DNA copy number was observed by [27]. Furthermore, in a recent study on the regulation of mitochondrial biogenesis during myogenesis, mtDNA copy number was determined as a marker for mitochondrial density using QPCR, and the mtDNA copy number was 4-fold higher in fully differentiated myotubes than it was in myoblasts [60].

Interestingly, during differentiation, an increased mtDNA transcriptional activity and oxidative metabolism correspond to an enhanced mitochondrial biogenesis, as highlighted by the upregulation of *COXII* mRNA levels and cytochrome c oxidase activity ($r^2 = 0.83$ and $r^2 = 0.97$, resp., data not shown).

In our investigation, we integrated the mitochondrial changes observed by multiple key determinants with proteomic analysis.

In the literature, mitochondrial proteomic maps of differentiating myoblasts are not available; hence, this work presents the first proteomic profile of mitochondria during the myogenesis program. Previously, proteome-based investigations have been carried out to provide a description of the myogenic differentiation program [46, 63, 64]. We employed a proteomic approach using two dimensional electrophoresis, particularly helpful for investigating the subset of cellular proteins, such as organellar proteins, due to the reduced complexity of the protein sample [65].

In particular, analyzing the differentially expressed proteins in the mitochondrial proteome map during the myogenic process, we observed that also the enzymes involved in cellular respiration, such as pyruvate dehydrogenase, MDH2, FH, ACO2, and more markedly HB and 5B ATP synthase subunits, representative of oxidative phosphorylation, increase linearly with the mitochondrial biogenesis showing a positive correlation ($r^2 = 0.915$, data not shown). These findings are consistent with the differentiating cells' greater reliance on aerobic metabolism compared to the glycolytic

metabolism that characterizes the undifferentiated myoblasts [7].

Moreover, our observations are in agreement with Moyes and coworkers who demonstrated an mRNA increment for pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase, cytochrome c oxidase, and NADH dehydrogenase [6]. The increment of Krebs cycle and respiratory chain proteins supports the augmented mitochondrial functionality also confirmed by the COX enzymatic activity during myoblast differentiation [6, 7].

The data obtained using the proteomic approach are consistent with the increase in mitochondrial function and membrane depolarization highlighted by JC-1 and in agreement with the increase of mitochondrial cristae observed by TEM. These data support the evidence described by Sauvanet (2010) assuming that mitochondrial bioenergetics and dynamics are linked and that mitochondrial morphology reflects their functional status [66].

Proteomic analysis revealed other notable proteins involved in the crosstalk between nuclei and mitochondria, such as MnSOD, a recently described cell protection molecule whose role is the maintenance of myoblast mitochondrial function and the preservation of the potential of myoblast stem cell differentiation [67]. In our model, the increment of MnSOD expression is highlighted during myotube formation ($T = 4$–$T = 7$), as recently documented by Kislinger and collaborators [64].

Another interesting protein associated with the progressive myoblast differentiation is the protein transport voltage-dependent anion-selective channel protein 1. It has been reported that its gene expression changes during myoblast differentiation [68], and it has been recognized as a key protein in mitochondria-mediated apoptosis, since it is a target for the pro- and antiapoptotic Bcl2-family of proteins, and for its function in releasing apoptotic proteins located in the intermembrane space [69]. Apoptosis is considered to be essential for normal skeletal muscle development by eliminating cells with defects or undergoing damage during differentiation [70–73]. The detection of mitochondrial myogenesis-correlated proteins, known to play a role in apoptosis, supports the link between differentiation and this type of cell death [46, 74].

Another protein induced during differentiation belongs to the protein disulfide isomerase (PDI) family: disulfide-isomerase A3 (Pdia3). These molecules are best known for their role as chaperones in protein-folding reactions in the endoplasmic reticulum [75]. However, mitochondrial localization has been documented in the outer membrane of rat liver mitochondria [76–78], bovine liver mitochondria [79], in a mitochondrial fraction of *Arabidopsis* [80] and a chloroplast isoform in *Chlamydomonas* [81]. PDI may act in mitochondria in several ways: enhancing protein folding of newly synthesized proteins, reducing disulphides required to activate proteins, controlling mitochondrial membrane permeability, and playing a role in the assembly and function of some enzymatic systems [76, 77].

In conclusion, this investigation demonstrates that the proteomic approach, integrated with the classical morpho-functional and biochemical analyses, provides a complete *scenario* of mitochondrial dynamics, biogenesis and functionality useful in comparative surveys of mitochondrial pathogenic or senescent satellite cells.

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Research Article

Identification of Potential Calorie Restriction-Mimicking Yeast Mutants with Increased Mitochondrial Respiratory Chain and Nitric Oxide Levels

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Calorie restriction (CR) induces a metabolic shift towards mitochondrial respiration; however, molecular mechanisms underlying CR remain unclear. Recent studies suggest that CR-induced mitochondrial activity is associated with nitric oxide (NO) production. To understand the role of mitochondria in CR, we identify and study *Saccharomyces cerevisiae* mutants with increased NO levels as potential CR mimics. Analysis of the top 17 mutants demonstrates a correlation between increased NO, mitochondrial respiration, and longevity. Interestingly, treating yeast with NO donors such as GSNO (*S*-nitrosoglutathione) is sufficient to partially mimic CR to extend lifespan. CR-increased NO is largely dependent on mitochondrial electron transport and cytochrome c oxidase (COX). Although COX normally produces NO under hypoxic conditions, CR-treated yeast cells are able to produce NO under normoxic conditions. Our results suggest that CR may derepress some hypoxic genes for mitochondrial proteins that function to promote the production of NO and the extension of lifespan.

1. Introduction

Calorie restriction (CR) extends lifespan in a variety of organisms and has also been shown to ameliorate many age-associated diseases such as diabetes and cancers [1–3]. However, the molecular mechanisms underlying CR-induced beneficial effects are still not fully understood. Owing to a short lifespan and well-established molecular genetic techniques, the budding yeast *Saccharomyces cerevisiae* represents a powerful system to identify new components in CR signaling pathways and to study these factors at the molecular/genetic level. Yeast lifespan can be studied in two distinct ways: replicative lifespan (RLS) and chronological lifespan (CLS). RLS measures the number of cell divisions an individual yeast cell undergoes before senescence (division potential), whereas CLS measures the length of time cells remain viable at a nondividing state (postmitotic survival). RLS may serve as a model to understand the mechanisms of replicative senescence such as in stem cells, while CLS may be more relevant to postmitotic cell senescence in adult animals [4, 5].

Moderate CR can be imposed in yeast by reducing the glucose concentration from 2% to 0.5% in rich media [6–9], which extends both RLS and CLS. In yeast, CR is suggested to function through reducing the activities of conserved nutrient-sensing pathways. Decreasing the activities of the Ras-cAMP/PKA (cyclic AMP-activated protein kinase A) pathway, Sch9 (homolog of mammalian S6K kinases) and Tor1 kinases have been shown to mimic CR and extend lifespan [6, 10, 11]. The recent identification of additional CR-specific longevity genes provides further insight into the molecular mechanisms underlying CR and the resulting metabolic alterations [7, 12–17]. The Sir2 family proteins (sirtuins) are among identified CR downstream targets; they are conserved longevity factors that were originally discovered and studied in yeast [3]. Sirtuins are NAD^+-dependent

protein deacetylases that are responsive to metabolic changes and stress and have been shown to play important roles in several CR models [3, 18, 19].

Mitochondria have also been shown to play important roles in CR. In yeast, CR induces a shunting of carbon metabolism from fermentation to the mitochondrial TCA cycle [12]. This metabolic shift to respiration is necessary and sufficient for the activation of Sir2-mediated lifespan extension in certain yeast strains [12]. A link between CR and increased mitochondrial metabolism has also been reported in higher eukaryotes including mammals [13, 14, 20–22]. Notably, the age-dependent decline in expression of genes encoding components of the mitochondrial respiratory chain has been reported in several species [22–24]. Since mitochondria are the major sites of energy production in eukaryotic cells, these findings highlight the crucial role of energy metabolism in CR. Since CR regimens involve the reduction of nutrient input, it is believed that a global change in nutrient sensing and regulatory pathways as well as changes in the mitochondrial respiratory chain are translated to physiological responses to counteract age-induced effects [25–27].

The role of the mitochondrial respiratory chain in CR is still unclear. In yeast, it has been suggested that CR activates mitochondrial respiration to prevent the accumulation of toxic metabolites [28–30]. Although the mitochondrial electron transport chain is also the primary site for reactive oxygen species (ROS) generation in the eukaryotic cell, increased mitochondrial electron flow during CR would be expected to reduce ROS levels [3, 31–33]. Recently, cytochrome c oxidase (COX) of the mitochondrial respiratory chain has been shown to catalyze the reduction of nitrite to nitric oxide (NO) [34]. When considered with the findings that the respiratory chain is involved in CR and that NO has been implicated in CR, it is of interest to ask if the NO involved is produced by mitochondrial COX.

In order to begin to elucidate how CR modulates complex genetic and metabolic networks to alter stress resistance, genomic stability, and lifespan, it is essential to uncover additional factors in the CR pathway. Towards this end, we have explored the relationship between NO and CR and have identified new genes in CR.

2. Materials and Methods

2.1. Yeast Strains and Media. Yeast strains BY4742 *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* and the genome-wide gene deletion collections (nonessential genes) were acquired from Open Biosystems. Medium used for replicative lifespan (RLS) analysis was YEP (2% bacto peptone, 1% yeast extract, 1.5% agar) supplemented with filter-sterilized glucose at a final concentration of 2% or 0.5%. Medium used for chronological lifespan (CLS) analysis was minimal synthetic SD (6.7 g/L yeast nitrogen base) supplemented with 4x auxotrophic amino acids (leucine, histine, uracil, and lysine) and glucose to a final concentration of 2% or 0.5%. Gene deletions were made by replacing the wild-type genes with the reusable Kan^r marker as described in [16] and verified by Polymerase Chain Reaction (PCR) using oligonucleotides flanking the genes of interest.

2.2. GSNO Treatments. Different concentrations of GSNO were added to yeast cells during chronological lifespan assays at different time points. GSNO was made as follow: 200 μL of 0.5 M GSH in 0.5 M HCL was added to 200 μL of 0.5 M $NaNO_2$ and then incubated at 4°C in the dark. After 30 minutes, 400 μL of 0.25 M NaOH was added to neutralize the solution. This reaction generated GSNO at final concentration of ~125 mM. Inactivated GSNO was generated by exposing GSNO to UV illumination for 30 min (photolysis) [35].

2.3. Measurement of Relative NO Levels Using the DAF-FM DA Fluorescence Dye. Diaminofluorescein-FM diacetate (Kamiya Biomedical) was diluted to 1 μM in reaction buffer (0.2 M phosphate buffer, pH 7, 1 mM EDTA, 0.1% glucose) immediately prior to the assay. Cells at different growth stages were harvested, washed twice, and then resuspended into 120 μL of reaction buffer (0.2 M potassium phosphate buffer, pH 7, 1 mM EDTA, 0.1% glucose). 100 μL of cell suspension was added to 100 μL reaction buffer with 1 μM of DAF-FM DA in 96-well fluorescence assay plates (~ 5×10^6 cells were analyzed in each well). After 2 hours of incubation (30°C), fluorescence signals were detected using a plate reader with excitation at 485 nm and emission monitored at emission at 535 nm [36, 37].

2.4. Nitric Oxide (NO) Measurement by an NO-Specific Electrode in Intact Cells. Cells were grown in YPD media containing either 0.5% or 2% glucose on a shaker (200 rpm) at 28°C and harvested in mid-exponential phase. Cells were washed twice with cold distilled water, pelleted, and kept on ice until use. Nitrite-dependent NO synthesis was measured essentially as described in [34, 38]. Briefly, cells were resuspended in 2 mL PBS (pH 6.5) in an NO chamber and bubbled 5 min with nitrogen. Then the chamber was closed, and NO production was measured at 28°C after the addition of 1 mM sodium nitrite using an NO electrode (amiNO-700, Innovative Instrument) as described in [34, 38].

2.5. Polarographic Measurement of NO Production and O_2 Consumption by Mitochondria. Cells were grown as above and harvested in mid-exponential phase. Mitochondria were prepared as described in [39]. NO production was measured with an amino-700 nitric oxide sensor connected to an inNOII nitric oxide measuring system (Innovative Instruments, Inc). Simultaneous measurement of O_2 concentration in the chamber was performed by a 2 mm Clark-type electrode Oxy-2 (Innovative Instruments, Inc) connected to the inNOII. Except where noted, all solutions were nitrite-free. Measurements were performed at 28°C in a final reaction volume of 2 mL, in a thermostated chamber with a close-fitting lid containing ports for the electrodes and a Hamilton syringe. Assays for NO production by the mitochondrial respiratory chain were performed in NO Assay Medium (6 mM succinate, 650 mM mannitol, 10 mM

K_2HPO_4 [pH 6.5], 0.1 mM EDTA, and 10 mM KCl). Except where noted, the NO assay medium was prebubbled with air in order to be saturated with oxygen (200 μM) prior to addition of $NaNO_2$, which was added to a final concentration of 1 mM, and the measurement of NO production.

2.6. Screens for Genetic Mimics of CR Base on Relative Levels of NO. Each mutant strain from the deletion collection (Open Biosystems) was inoculated into SD in 96-well plates and allowed to grow for 2 days. 4 μL saturated culture was then added into 400 μL fresh SD and allowed to grow for 12 hr. To prepare for NO measurement, cells were spun down in 96-well plates at 3000 RCF, washed, and then resuspended into 120 μL reaction buffer (0.2 M phosphate buffer, pH 7, 1 mM EDTA, 0.1% glucose). 100 μL cell suspension was added to 100 μL reaction buffer with 1 μM of DAF-FM DA (Kamiya Biomedical) prealiquated in 96-well fluorescence assay plates. Multiple wild-type (WT) controls grown under both normal (2% glucose) and CR (0.5% glucose) conditions were included on each 96-well plate as internal controls. Average NO levels of WT controls grown under normal condition (after normalized to cell number) were set to 1. NO levels of the mutants were normalized to the WT controls on the same plate. We have determined the variations among different plates and different experiments to be ~20% (data not shown), which was used as the standard deviation for the initial screen.

2.7. Chronological Lifespan (CLS). Three to four single colonies derived from each strain were analyzed in each experiment as described in [4] with a few modifications. Cells were grown in 10 mL SD (at a starting OD_{600} of 0.1) in 50 mL tubes on a roller drum set at 250 rpm to ensure proper aerations. Cell viability (~2×10^8 cells were analyzed in each sample) was monitored every 2-3 days by plating a fraction of the culture onto fresh media to determine the colony-forming units (CFU). Cell survival rates were calculated by normalizing the CFU of each time point to the CFU of day 0 (48 hr after starting CLS, when cells just entered stationary phase). Numbers on the x-axes of CLS graphs denote the number of days after entering stationary phase (nonmitotic state). GSNO-treated cells were grown in the dark for the first 24 hr.

2.8. Replicative Lifespan (RLS). All RLS analyses were carried out on YEP agar plates supplemented with glucose at a final concentration of 2% or 0.5% with 50 cells per strain per experiment [16] using a micromanipulator under a microscope. All RLS assays were repeated at least twice by two different individuals.

2.9. Oxygen Consumption. Oxygen consumption was monitored using a Clark-type electrode as previously described in [16].

2.10. GSNO and NO Metabolizing Activities in Cell Lysates. About 300 OD_{600} units of yeast cells grown to different stages were harvested and homogenized in 1.5 mL lysis buffer containing 20 mM Tris-HCl, pH 8, 0.5 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma), and protease inhibitor tablet (Roche) using a beads beater. The GSNO reductase (GSNOR) activity of Sfa1 was determined by measuring GSNO-dependent NADH consumption using fluorescence (340/460 nm) as described in [40] in 200 μL reaction buffer (200 μM NADH, 20 mM Tris-HCl, pH 8, 0.5 mM EDTA) with 15 μg (total proteins) cell lysate and 0 or 200 μM GSNO at room temperature. The NO metabolizing activity of Yhb1 was determined by measuring NO-dependent NADH consumption using fluorescence (340/460 nm) as described in [41] in a 200 μL reaction mix containing 200 μM NADH, 20 mM Tris-HCl, pH 8, 0.5 mM EDTA, 15 μg lysate, and 0 or 300 μM MAHMA NONOate (Cayman Chemical) at room temperature. The NADPH-dependent NO-metabolizing activity was measured by MAHMA NONOate-dependent consumption of NADPH using fluorescence (340/460 nm) as described in [42] in a 200 μL reaction mix containing 250 μM NADPH, 20 mM Tris-HCl, pH 8, 0.5 mM EDTA, 15 μg lysate, and 0 or 300 μM MAHMA NONOate at room temperature.

2.11. CYC7 Reporter Assay. Approximately 1 kb of the *CYC7* promoter was cloned into the *URA3+*, pYES2.1 vector (Invitrogen) using the BamHI site immediately upstream of the β-galactosidase gene. For the liquid β-galactosidase assay, 5 OD_{600} units of yeast cells grown in SD w/o uracil for 18 hrs (starting $OD_{600} = 0.1$) under normoxic condition were washed, lysed by bead beating in breaking buffer (100 mM Tris-HCl pH 8, 20% glycerol, 1 mM DTT, 0.02% SDS, 8 mM PMSF), and separated from debris by centrifugation. 50 μL of lysate was then added to 450 μL of Z buffer (0.1 M sodium phosphate buffer pH 7, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), preincubated at 30°C for 5 min, and then 100 μL 4 mg/mL ONPG in Z buffer was added. After 1 hr incubation at 30°C, the reaction was stopped with 250 μL of 1 M $NaCO_3$ and read at 420 nm. This reading was then normalized to protein concentration of the lysate and finally normalized to the WT 2% glucose sample.

2.12. L-NAME Treatment for NO Quantitation. Cells were first inoculated in SD (staring $OD_{600} = 0.1$) and allowed to grow for 12 hrs. The arginine analogue and NOS inhibitor L-NAME (Sigma) was then added to these cells at 200 mM final concentration as previously described in [43]. After 2 hr incubation at 30°C, cells were washed and then incubated with the DAF-FM DA fluorescence dye to determine relative NO levels as described previously.

2.13. Hydrogen Peroxide and GSNO Toxicity Tests. For hydrogen peroxide treatments, cells were pregrown in SD with 2% or 0.5% glucose (CR) for 20 hrs (staring $OD_{600} = 0.1$) then were treated with 0.5% H_2O_2 for 1 hr. Following this treatment, cells were plated on YPD in 5-fold serial dilutions. For GSNO toxicity test, cells were pregrown in SD with 2% or 0.5% glucose (CR) or pretreated with 25 μM GSNO for 18 hr. GSNO was then added to cells at a final concentration of 1.5 mM. After 24 hr treatment, cells were removed from

the GSNO-containing SD, plated on YPD in 5-fold serial dilutions, and then allowed to grow for 2 days at 30°C.

2.14. Statistical Analyses. Statistical analysis of CLS was carried out using the AUC method (area under the curve) [44, 45]. *P* values were calculated for each pair of lifespans as shown in Supplemental Table 2 (see Supplementary Material available online at doi: 10.4061/2011/673185). Statistical analysis of RLS was carried out using the JMP statistics software (SAS), and the Wilcoxon rank sums tests *P* values were calculated for each pair of lifespans as shown in Supplemental Table 3. All other *P* values were calculated using Student's *t*-test (two-tailed).

3. Results

3.1. Calorie Restriction Induces a Significant Increase in NO Production. Moderate CR in yeast results in a metabolic shift from fermentation to mitochondrial respiration [12]. In mice, CR-induced mitochondrial biogenesis was attributed to increased expression of endothelial nitric oxide synthase (eNOS) [20]. To further understand the role of NO in CR, we determined whether CR increased NO levels in yeast using an NO-sensitive dye, diaminofluorescein-FM diacetate (DAF-FM DA). Although DAF-FM DA is fairly specific to NO and NO oxidation products [36, 37], we further confirmed that it did not cross-react with other reactive oxygen species (such as superoxide and hydrogen peroxide) *in vivo* under our assay conditions (Supplemental Figure 1). As shown in Figure 1(a), CR significantly increased NO-related signals during early growth stages, while cells were actively dividing. The long-lived *tor1*Δ and *hxk2*Δ mutants [6, 11] also showed increased NO signals (Figure 1(b)). In addition, CR, *tor1*Δ, and *hxk2*Δ also conferred resistance towards nitrosative stress (treatments of a physiological NO donor *S*-nitrosoglutathione, GSNO) (Figure 1(c)). We next determined whether the activities of major NO detoxification enzymes were increased by CR. Yeast flavohemoglobin (Yhb1), which catalyzes the conversion of NO to nitrite or nitrous oxide, and GSNO reductase (GSNOR, Sfa1) are the two major enzymes that protect yeast cells from nitrosative stress [40, 41, 46]. Interestingly, Yhb1 activity determined in the crude cell lysate [41] was increased by CR (Figure 1(d)), whereas Sfa1 activity [40] was not increased by CR, suggesting that Yhb1 may play an important role in CR. In addition, CR also slightly induced NADPH-dependent NO-metabolizing activity (Figure 1(d)), which has been reported in mammalian cells [42]. Together, these results demonstrate that CR-induced increases in NO are not due to decreased NO degradation.

3.2. A Genetic Screen for High NO Mutants as Potential CR Genetic Mimics. To identify additional components in the CR pathway, we screened the yeast deletion collection (~4500 strains) for mutants with elevated NO levels similar to CR. About 157 high-NO mutants were identified and ranked by NO levels, growth rate, and resistance to GSNO (Supplemental Table 1). CR increases both replicative and chronological lifespan (RLS and CLS). Therefore, we used these criteria to classify potential "CR genetic mimics." Among the top 15 hits, 5 mutants (*hxt17*Δ, *pkh2*Δ, *gup1*Δ, *hhf1*Δ, *and soy1*Δ) showed extended CLS and RLS (Table 1). Hxt17 is a high-affinity hexose transporter, which may mimic CR by decreasing the activity of the glucose sensing pathways [6]. Pkh2 is a Ser/Thr protein kinase that activates Sch9 [47], and *pkh2*Δ is likely to behave similar to *sch9*Δ, which has been shown to mimic CR [11]. Recently, *pkh2*Δ was identified as an RLS-extending mutant [48], providing further validation for our screen. Gup1 is an ER membrane-localized O-acyltransferase involved in remodeling the fatty acid moiety of the GPI anchor [49]. *HHF1* encodes one of two identical histone H4 proteins. Depleting histone H4 has been shown to alter the expression levels of specific yeast genes including many genes that regulate energy homeostasis [50, 51]. Soy1/Aim4 is suggested to participate in mitochondrial genome maintenance [52]; however, its molecular function remains unclear.

We next determined whether these long-lived mutants function in the same pathway as CR to extend lifespan. As expected, CR did not significantly alter CLS (Figures 2(a)–2(d)) or RLS (Figures 3(a)–3(d)) of most of these long-lived mutants, suggesting that these mutations might be in genes that function in the same (or overlapping) pathway to extend CLS and/or RLS (detailed statistical analysis of CLS and RLS is shown in Supplemental Tables 2 and 3, resp.). Two notable exceptions were observed in our studies: the *gup1*Δ (Figures 2(c) and 3(c)) and the *soy1*Δ (Figure 3(b)) mutants. CR further increased *gup1*Δ RLS (*gup1*Δ versus *gup1*Δ, CR; $P = .038$) and vice versa (CR versus *gup1*Δ, CR; $P = .004$) (Figure 3(c)), suggesting a positive interaction between *gup1*Δ and CR in RLS extension. In addition, although CR-induced CLS was not further increased by *gup1*Δ (CR versus *gup1*Δ, CR; $P = .073$), the *gup1*Δ mutant exhibited longer CLS under CR (*gup1*Δ versus *gup1*Δ, CR; $P = .035$). Interestingly, CR appeared to decrease *soy1*Δ-induced RLS (*soy1*Δ versus *soy1*Δ, CR; $P = .008$) (Figure 3(b)), suggesting a negative interaction between *soy1*Δ and CR. However, *soy1*Δ did not significantly change CR-induced RLS (CR versus *soy1*Δ, CR; $P = .49$). A potential positive interaction was also observed between *hhf1*Δ and CR. CR appeared to extend RLS more significantly when combined with *hhf1*Δ (CR versus *hhf1*Δ, CR; $P = .001$). However, CR did not significantly change *hhf1*Δ-induced RLS (Figure 3(d)) (*hhf1*Δ versus *hhf1*Δ, CR; $P = .19$).

3.3. CR-Induced Nitric Oxide Production Is Most Likely Generated by Enhanced Mitochondrial Respiration and the Cytochrome c Oxidase. We next determined whether these long-lived, high-NO mutants displayed an increase in mitochondrial respiration similar to CR cells. As shown in Figure 4(a), all of these mutants showed increased oxygen consumption, demonstrating a correlation between increased mitochondrial respiration and NO production. In addition, a functional electron transport chain was required for CR-induced lifespan. CR failed to extend RLS [7, 12] and CLS (Figure 4(b)) in the respiratory-deficient *cyt1*Δ mutant

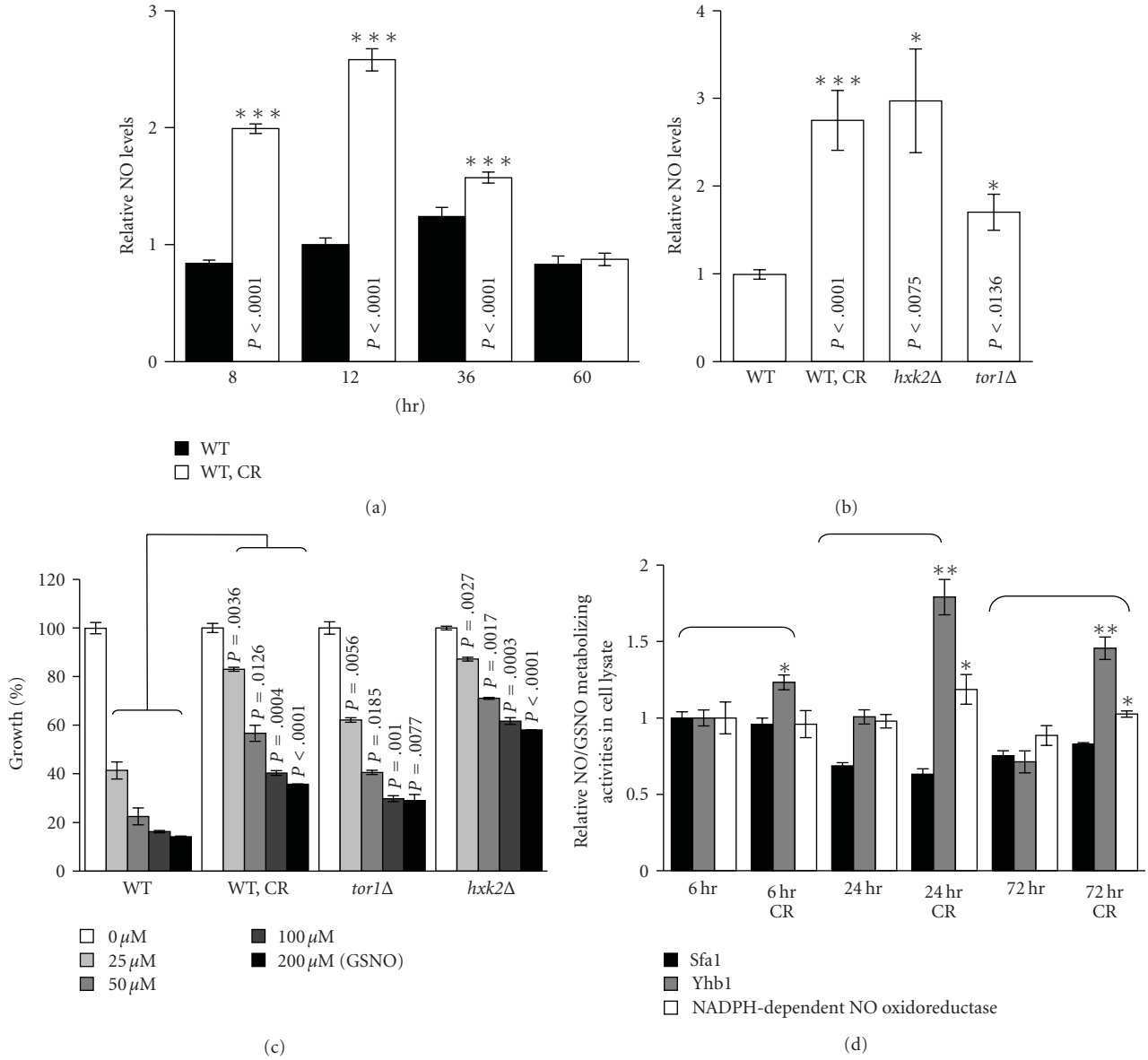

Figure 1: Calorie restriction (CR) and CR genetic mimics increase nitric oxide (NO) levels and confer resistance to nitrosative stress in yeast cell. (a) CR increases NO-related signals during early growth stage. (b) Long-lived *tor1*Δ and *hxk2*Δ mutants also show increased NO-related signals. (c) CR and CR mimics confer resistance to *S*-nitrosoglutathione- (GSNO-) induced toxicity. Results show percentages (%) of growth normalized to no treatment controls after treated with GSNO at indicated concentrations for 20 hr. (d) Yeast cells grown in CR show increased NO metabolizing activities determined in cell lysates. *P* values are calculated comparing CR cells and the *tor1*Δ or *hxk2*Δ mutants to WT control. WT: BY4742 wild-type control; CR: 0.5% glucose; Δ: gene deletion. One representative set of three independent experiments is shown. Error bars denote standard deviations.

lacking cytochrome c1. Deletion of *CYT1* also eliminated the long RLS (Figure 4(c)) and CLS (B. Li and S.-J. Lin, unpublished) of potential CR mimics. We then further examined how CR increased NO production. Although there is biochemical evidence that NOS homologs are present in yeast [43], the existence of nitric oxide synthases in yeast remains controversial since no NOS sequence homolog has been identified. In contrast, cytochrome c oxidase (COX) has been shown to produce NO in a nitrite- (NO_2^--) dependent manner under hypoxic conditions [34]. Therefore, the mitochondrial electron transport chain is a potential source of NO production in CR cells. Our results (Figure 4(d)) showed that CR- and CR mimics-induced NO increases were largely abolished in cells lacking *CYT1*. In addition, CR-induced NO production was not inhibited by the NOS inhibitor (arginine analog) L-NAME (Figure 4(e)).

Since the COX complex was reported to produce NO under hypoxic conditions [34], we examined whether calorie-restricted yeast cells could synthesize NO under normal (normoxic) conditions. Using an NO-specific electrode, we verified that calorie-restricted BY4742 WT (ρ^+) cells could produce NO when grown under normoxic

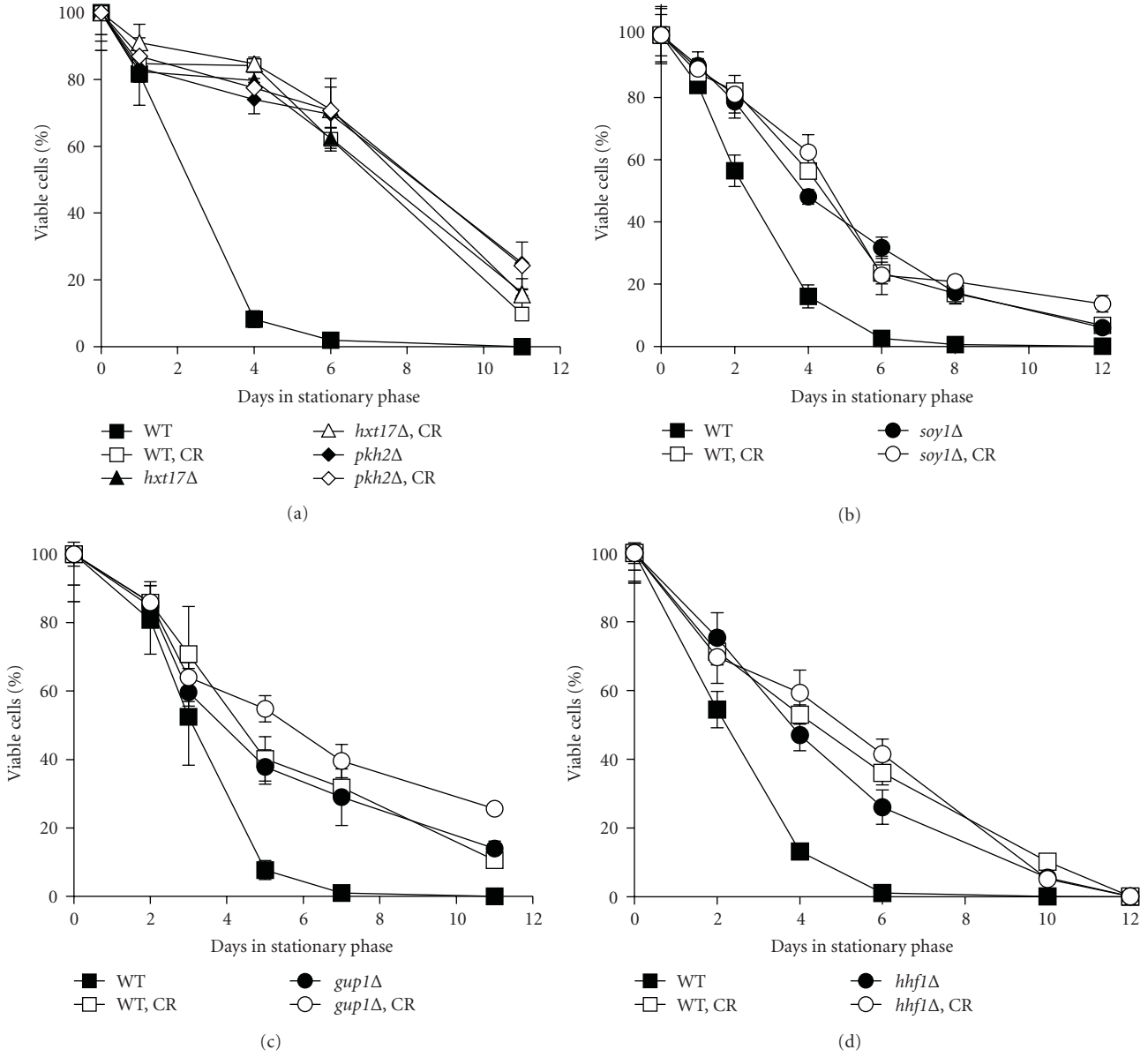

Figure 2: Chronological lifespan (CLS) analysis of potential CR genetic mimics. (a)–(d) Potential CR-mimicking high-NO mutants show extended CLS, and most do not significantly further increase the CLS of cells grown in CR. (c) Although CR further increases *gup1*Δ-induced CLS (*gup1*Δ versus *gup1*Δ, CR; $P = .035$), *gup1*Δ does not further increase CR-induced CLS (CR versus *gup1*Δ, CR; $P = .073$). (d) Although CR further increases *hhf1*Δ-induced CLS (*hhf1*Δ versus *hhf1*Δ, CR; $P = .02$), *hhf1*Δ does not further increase CR-induced CLS (CR versus *hhf1*Δ, CR; $P = .28$). One representative set of three independent experiments, each conducted in quadruplicate, is shown. Error bars denote standard deviations. WT: BY4742 wild-type control; CR: 0.5% glucose; Δ: gene deletion. Numbers on the *x*-axes denote the number of days after entering stationary phase (nonmitotic state). Pairwise statistical analysis of CLS is shown in Supplemental Table 2.

conditions (Figure 4(f)). In contrast, calorie restriction failed to induce NO production either in the BY4742 (ρ^0), a rho^0 strain lacking mitochondrial DNA, or in the respiratory-deficient *cyt1*Δ mutant under normoxic conditions. These findings further support the conclusion that the NO synthesis in calorie-restricted yeast cells requires a functional mitochondrial respiratory chain. To explore this further, we examined the oxygen sensitivity of nitrite-dependent NO synthesis by mitochondria isolated from WT cells. As shown in Figure 4(g), oxygen sensitivity reported previously for nitrite-dependent mitochondrial NO synthesis [34] was reduced in mitochondria isolated from WT cells grown under CR. These results demonstrated that yeast mitochondria are capable of NO synthesis at oxygen concentrations that are in the normoxic range experienced by yeast cells grown in colonies or in liquid culture.

Eukaryotic cells harbor both aerobic and hypoxic isoforms for several components of the electron transport chain to ensure proper electron transfer in response to different oxygen concentrations [32, 55–57]. It has been reported that,

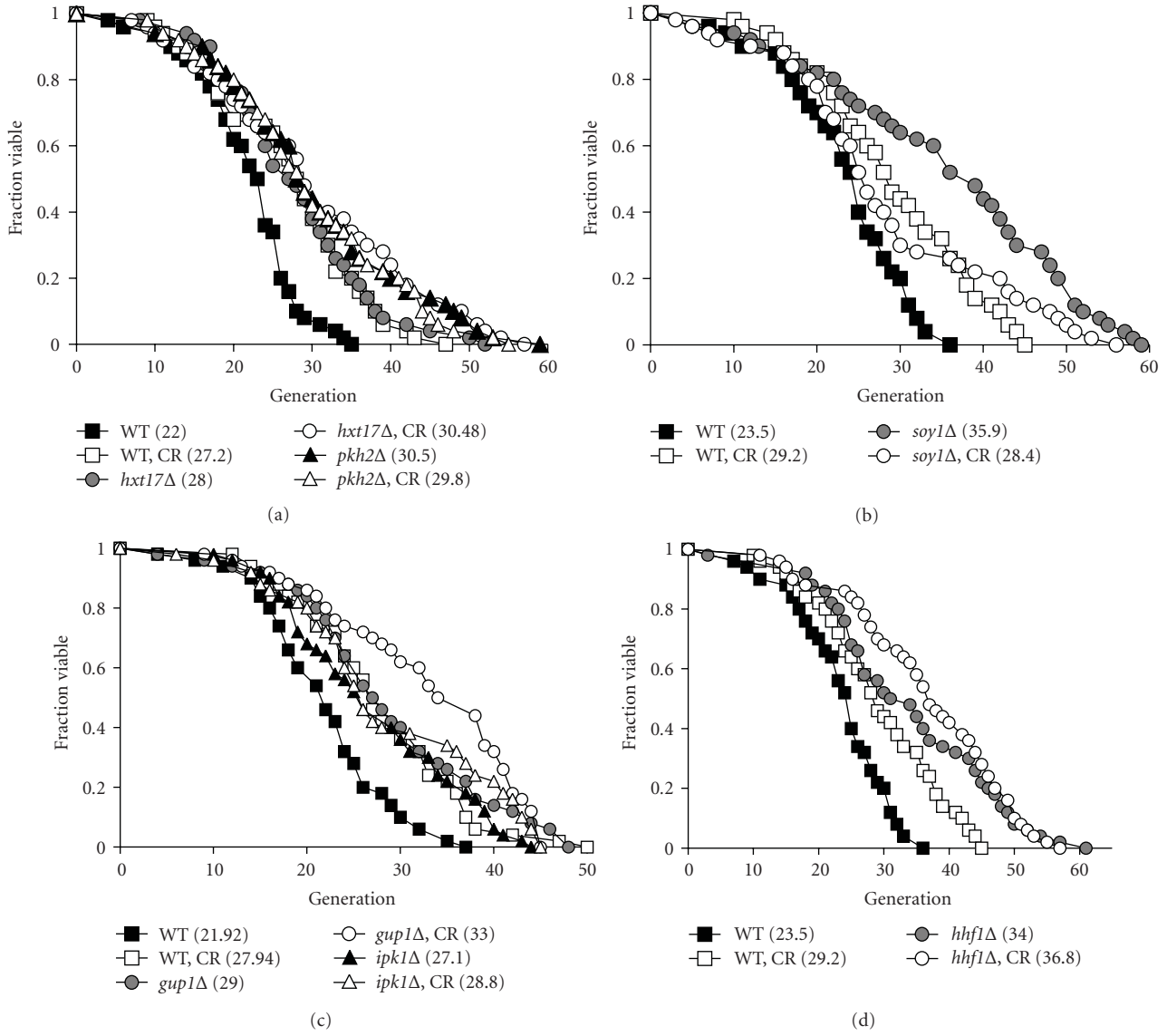

Figure 3: Replicative lifespan (RLS) analysis of potential CR genetic mimics. (a) Potential CR mimicking high-NO mutants show extended RLS and do not significantly further increase the RLS of cells grown in CR. (b) CR decreases *soy1*Δ-induced RLS (*soy1*Δ versus *soy1*Δ, CR; $P = .008$); however, *soy1*Δ does not significantly change CR-induced RLS (CR versus *soy1*Δ, CR; $P = .49$). (c) CR further increases *gup1*Δ-induced RLS (*gup1*Δ versus *gup1*Δ, CR; $P = .038$). *gup1*Δ also further increases CR-induced RLS (CR versus *gup1*Δ, CR; $P = .004$). (d) Although *hhf1*Δ further increases CR-induced RLS (CR versus *hhf1*Δ, CR; $P = .001$), CR does not further increase *hhf1*Δ-induced RLS (*hhf1*Δ versus *hhf1*Δ, CR; $P = .19$). One representative set of three independent experiments is shown. WT: BY4742 wild-type control; CR: 0.5% glucose; Δ: gene deletion. Pairwise statistical analysis of RLS is shown in Supplemental Table 3.

although COX-specific NO synthesis is strongly inhibited by oxygen when COX contains the aerobic isoform of subunit V (Va), the oxygen inhibition is relieved when COX contains subunit Vb, the hypoxic subunit V isoform [38]. Interestingly, CR increased the expression of the hypoxia inducible cytochrome c *CYC7* (Figure 4(h)) as well as *COX5b*, which encodes COX hypoxic subunit Vb (P. Castello and R. Poyton, unpublished). Therefore, CR might induce NO production in part by derepressing expression of the hypoxic isoforms of mitochondrial electron transport complexes and perhaps additional hypoxia responsive genes.

3.4. NO Detoxification Enzymes Are Partially Required for CR-Induced Lifespan Extension. Next, we examined the roles of NO detoxification enzymes Yhb1 and Sfa1 in CR. CR failed to extend RLS in the *sfa1*Δ*yhb1*Δ mutant (Figure 5(a)), indicating that Yhb1 and Sfa1 are required for optimal NO homeostasis during CR-induced RLS. Deleting *YHB1* partially abolished CR-induced RLS ($P = .03$, CR versus CR, *yhb1*Δ), whereas deleting *SFA1* alone had no significant effect. Therefore, it appeared that Yhb1 and Sfa1 play redundant roles under our CR/RLS assay condition. The lifespan of potential CR mimics *hxt17*Δ, *pkh2*Δ, *and*

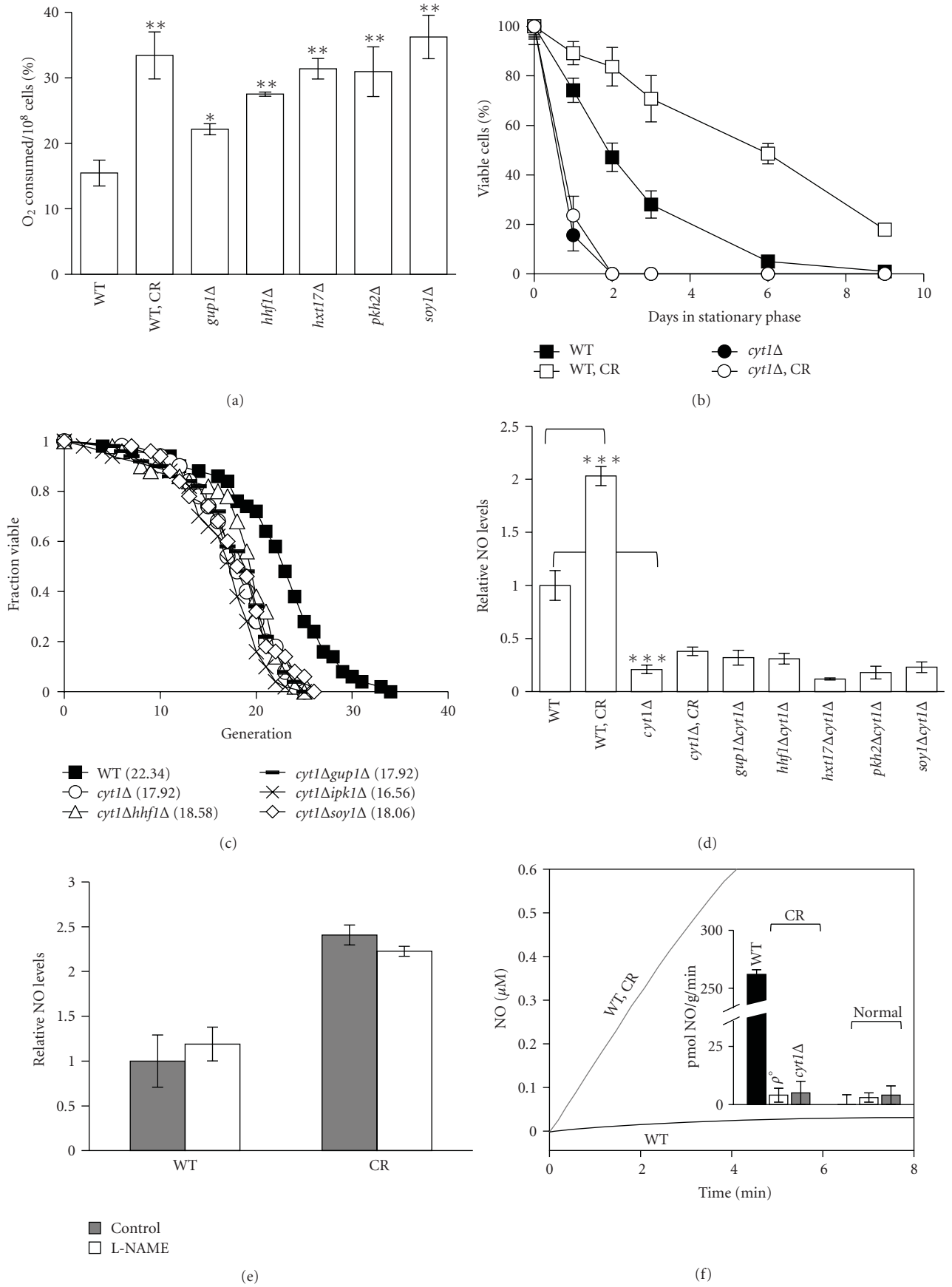

Figure 4: Continued.

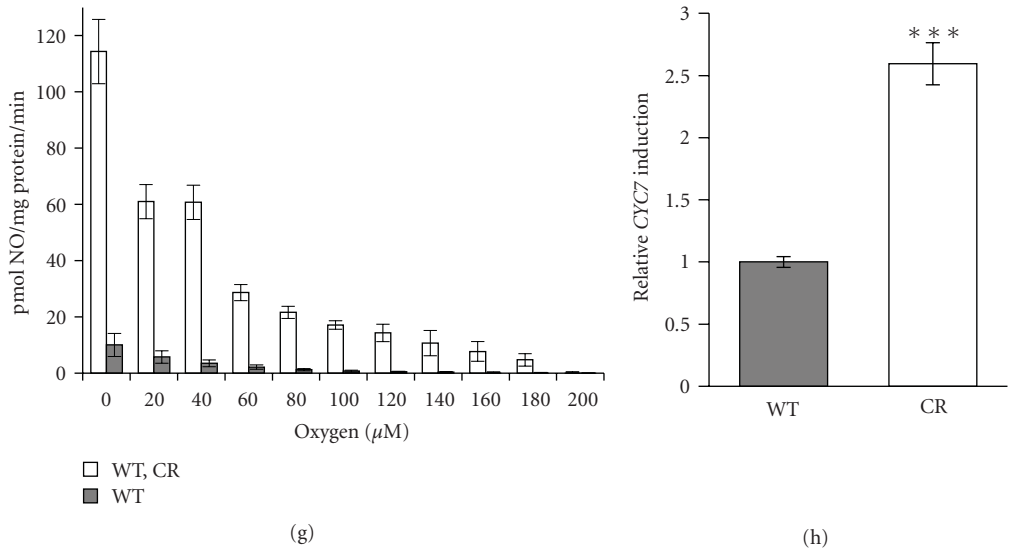

Figure 4: Mitochondrial respiration and NO production play important roles in CR-induced lifespan. (a) All potential CR mimic mutants show increased oxygen consumption. (b) CR-induced CLS is prevented in the *cyt1*Δ mutant. Numbers on *x*-axes denote the number of days after entering stationary phase (nonmitotic state). (c) CR mimic mutants-induced RLS is prevented in the *cyt1*Δ mutant. (d) Increases in NO by CR or CR mimic mutants are largely abolished in the *cyt1*Δ mutant. (e) CR-induced NO production is not inhibited by L-NAME, an NOS inhibitor. (f) Involvement of the respiratory chain in cellular NO production. Cells (160 mg) were suspended in 2 mL of PBS and prebubbled for 5 min with N_2 to create anoxic conditions. After 5 min of prebubbling, $NaNO_2$ was added to a final concentration of 1 mM, and NO production was measured with an NO polarographic electrode. Insert: NO production rates for BY4742 ρ^+ (black), BY4742 ρ^0 (white), and *cyt1*Δ (grey) for cells that were grown to log phase in 2% glucose (normal) or 0.5% glucose (CR). (g) Effects of oxygen concentrations on mitochondrial NO production. Isolated mitochondria from cells grown under normal and CR conditions were assayed for NO production in assay medium as described in the Materials and Methods Section. (h) CR increases *CYC7* gene expression using a β-galactosidase reporter-based promoter activity assay. Original values: 39 ± 2 nmoles ONPG converted/min/mg protein for WT; 105 ± 4 nmoles ONPG converted/min/mg protein for CR. WT: BY4742 and BY4742 (ρ^+) wild-type control; ρ^0: cells lacking mitochondrial DNA; CR: 0.5% glucose. One representative set of experiment is shown. Error bars denote standard deviations. *P* values are calculated using Student's *t*-test ($^*P < .05$; $^{**}P < .01$; $^{***}P < .005$).

*hhf1*Δ was also reduced to WT levels in the *yhb1*Δ*sfa1*Δ mutant (Figure 5(b)). Deleting *YHB1/SFA1* was not likely to prevent CR-induced RLS solely by reducing mitochondrial activity. As shown in Figure 5(c), CR-induced increases in oxygen consumption and NO production were only partially reduced in the *yhb1*Δ*sfa1*Δ mutant. Interestingly, *yhb1*Δ*sfa1*Δ did not abolish CR-induced CLS (Figure 5(d)), suggesting that additional NO-detoxification enzymes and stress response factors might compensate for the loss of Yhb1/Sfa1 for CR-induced CLS. In line with our results, it has been reported that the stress response factors such as Rim15 and superoxide dismutases play important roles in CR-induced CLS [9, 58].

3.5. Treatment with GSNO Extends Yeast CLS. We then examined whether treating cells with NO donors was sufficient to mimic CR to extend lifespan. Interestingly, GSNO appeared to be the most effective NO donor for lifespan extension among the different NO donors tested (B. Li and S.-J. Lin, unpublished). As shown in Figures 6(a) and 6(b), both a single treatment of 25 μM *S*-nitrosoglutathione (GSNO) (which caused mild growth inhibition, Figure 1(c)) and multiple treatments of 5 μM GSNO (no growth inhibition observed) extended CLS to a level similar to CR. GSNO did not further increase CR-induced (Figures 6(a) and 6(b)) or CR mimics-induced (Figure 6(c)) CLS suggesting that GSNO and CR may function in the same pathway to extend CLS. Interestingly, GSNO treatments partially rescued the short CLS of the respiratory-deficient *cyt1*Δ mutant (Figure 6(d)), suggesting that it may partially compensate for the loss of respiration or may also confer some respiration-independent beneficial effects. For example, GSNO may extend lifespan by promoting specific protein modifications [59]. However, GSNO treatment (25 μM) did not appear to have an impact upon RLS (B. Li and S.-J. Lin, unpublished); this could be an effect of GSNO instability in the plate-based RLS assay. It is also likely that GSNO only confers partial beneficial effects of CR. Since CR confers resistance to various stresses, we examined whether GSNO might mimic CR by increasing stress resistance. As shown in Figure 6(e), cells pretreated with a low dose of GSNO were more resistant to GSNO-induced toxicity. Pretreatment of GSNO also slightly increased the resistance to hydrogen peroxide compared to calorie-restricted cells (Figure 6(f)). Therefore, GSNO may extend CLS in part by increasing stress responses,

Table 1: List of potential genetic mimics of CR.

Deletion	NO[a]	CLS[b]	RLS[c]	O_2[d]	Biological function
*ypk1*Δ	4.1 ± 1.4	E (0.006)	N (21.5^{wt}; 23.2^{mt}; 0.15)	0.8 ± 0.03	Ser/Thr protein kinase; sphingolipid signaling
*tps1*Δ	3.9 ± 0.9	N (0.18)	N (22^{wt}; 21.4^{mt}; 0.65)	1.1 ± 0.04	Trehalose biosynthesis; response to stress
*reg1*Δ	2.9 ± 0.4	E[e]	S (21.5^{wt}; 17.6^{mt}; 0.0014)	1.2 ± 0.1	Negative regulation of glucose-repressible genes
*soy1*Δ	2.9 ± 0.4	E (0.0024)	E (23.5^{wt}; 35.9^{mt}; 0.0001)	2.4 ± 0.2	Function unknown; associates with nuclear pore complex
*hxk2*Δ	2.5 ± 0.8	N[f]	E[g]	∼3[h]	Hexokinase; glycolysis
*erg4*Δ	2.1 ± 0.3	E (0.005)	N (21.5^{wt}; 23.9^{mt}; 0.54)	1 ± 0.03	Ergosterol biosynthesis
*fyv5*Δ	1.8 ± 0.5	E (0.004)	N (22.3^{wt}; 21.9^{mt}; 0.25)	1.5 ± 0.02	Ion homeostasis
*ipk1*Δ	1.8 ± 0.7	N (0.07)	E (21.9^{wt}; 27.1^{mt}; 0.0065)	2.3 ± 0.2	Inositol phosphate biosynthesis
*gup1*Δ	1.7 ± 0.1	E (0.026)	E (21.9^{wt}; 29^{mt}; 0.0001)	1.4 ± 0.05	Remodeling the GPI anchors; glycerol transport
*lip5*Δ	1.7 ± 0.3	N (0.86)	E (22.5^{wt}; 28.4^{mt}; 0.01)	0.7 ± 0.05	Biosynthesis of lipoic acid
*hhf1*Δ	1.7 ± 0.4	E (0.005)	E (21.3^{wt}; 27.7^{mt}; 0.0001)	1.8 ± 0.02	Histone H4 proteins; chromatin assembly
*dse2*Δ	1.7 ± 0.1	E (0.0005)	N/A[i]	1.6 ± 0.02	Required for cell separation
*avl9*Δ	1.6 ± 0.1	N (0.091)	N (23.4^{wt}; 26.02^{mt}; 0.2)	0.9 ± 0.01	Conserved protein involved in exocytic transport
*utr1*Δ	1.6 ± 0.2	E (0.001)	N (22.5^{wt}; 24.48^{mt}; 0.52)	1 ± 0.07	Cytosolic NAD kinase
*zap1*Δ	1.5 ± 0.1	E (0.02)	E[j] (22.5^{wt}; 29.8^{mt}; 0.0124)	1.6 ± 0.1	Zinc-regulated transcription factor
*pkh2*Δ	1.4 ± 0.2	E (0.0035)	E (22^{wt}; 30.5^{mt}; 0.0001)	2 ± 0.25	Ser/Thr protein kinase; sphingolipid signaling
*hxt17*Δ	1.3 ± 0.1	E (0.0004)	E (22^{wt}; 28^{mt}; 0.0014)	2 ± 0.1	Hexose transporter

This table lists top 15 hits from our screen and the *pkh2*Δ and *hxt17*Δ mutants. Mutants listed here were first selected by their "growth" (at least 75% of WT; no severe growth defects) and by "resistance to GSNO" (at least 150% of WT) and then were ranked by NO levels.

[a]Numbers show average relative intracellular NO levels ± standard deviation (normalized to wild-type control) (for details, see Section 2). [b]CLS: chronological lifespan, E: extended; N: normal; *P* values are calculated using the AUC method (area under the curve) (see Section 2). Results of one set of representative experiment (each conducted in triplicate or quadruplicate, $n = 3$ or 4 independent colonies) are shown. About 2×10^8 cells were analyzed in each sample. [c]RLS: replicative lifespan; E: extended; N: normal; S: short; N/A: not determined. Numbers show (average wild-type RLS; average mutant RLS; *P* values). *P* values are calculated using the Wilcoxon rank sums tests. Results of one set of representative experiment are shown. 50 cells ($n = 50$) were analyzed for each strain in each experiment. [d]O_2: relative average oxygen consumption rate ± standard deviation (normalized to wild-type control). [e]Reported [53]. [f]Reported [54]. [g]Reported [6]. [h]Reported [12]. [i]N/A: not available. Cells are sticky, unable to separate. [j]Exhibits early cell death.

possibly via inducing hormesis effect. Interestingly, not all of the high-respiration activity CR genetic mimics examined showed increased resistance towards hydrogen peroxide (Figure 6(f)), although these mutants all showed increased resistance towards GSNO (Supplemental Table 1). It is possible that different mutants only partially mimic CR by activating a different subset of stress response pathway. It is also very likely that additional mechanisms play important roles in these mutants.

4. Discussion

Calorie restriction (CR) extends longevity and ameliorates age-related diseases in many organisms. Understanding how CR induces these beneficial effects may therefore provide insights into the molecular mechanisms underlying age-associated diseases. Considerable progress has been made in the discovery of genes/proteins and metabolites that affect longevity. However, many molecular aspects of CR-induced events remain unclear. Uncovering additional long-lived CR-mimicking mutants will likely contribute to the understanding of the molecular mechanisms of CR. To identify new components in the CR pathway, we developed a nitric oxide- (NO-) based screen for mutants that showed higher intracellular levels of NO as potential CR mimics.

Nine of the top 17 high-NO mutants summarized in Table 1 show a positive correlation between increased mitochondrial respiration and NO production. There is also a positive correlation between increased respiration and longevity. These nine mutants showed increased oxygen consumption (9 out of 17) and increased CLS and/or RLS. In addition, mutants that showed both extended RLS and CLS (5 out of 17) all showed increased oxygen consumption as well as NO levels (Table 1). Two mutants (*avl9*Δ and *tps1*Δ) that did not show either extended CLS or RLS also did not show increased oxygen consumption. However, it is noteworthy that 5 long-lived mutants (with extended RLS or CLS) did not possess increased oxygen consumption.

Although CR cells have increased NO levels, a higher intracellular NO concentration is not sufficient to extend lifespan under all growth conditions and/or genetic backgrounds. For example, the *avl9*Δ and *tps1*Δ mutants have higher NO levels but do not show increased lifespan (Table 1). It is possible that increases in the stress response

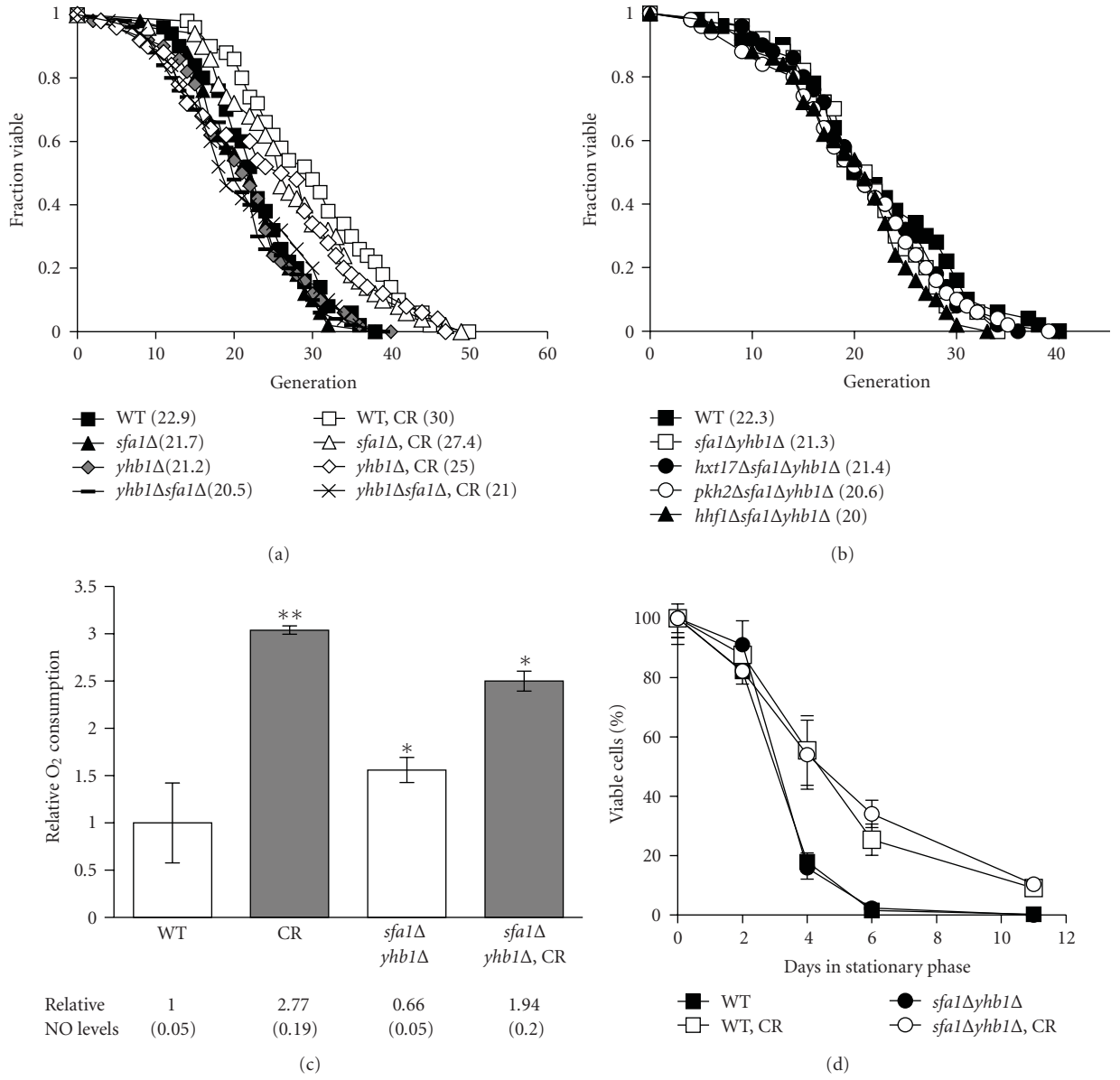

Figure 5: Balanced NO metabolism is required for CR-induced beneficial effects. (a) CR fails to extend RLS in cells lacking Sfa1 and Yhb1. (b) CR genetic mimics fail to extend RLS in cells lacking Sfa1 and Yhb1. (c) Deleting Sfa1 and Yhb1 partially affect CR-induced increase in oxygen consumption and NO levels. (d) Sfa1 and Yhb1 are not required for CR-induced CLS. One representative set of experiments is shown. Error bars denote standard deviations. P values are calculated using Student's t-test ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .005$).

as well as functional mitochondria are also required for NO-induced lifespan extension. Supporting this model, our results showed that treating the respiratory deficient *cyt1*Δ mutant with GSNO only partially restored its CLS (Figure 6(d)). In addition, it has been shown that several stress response factors play important roles in CLS and CR-induced CLS in *S. cerevisiae* [9, 33]. In *Schizosaccharomyces pombe*, the stress-responsive MAP kinase Sty1 was demonstrated to be essential for CR-mediated CLS extension [60].

NO detoxification enzymes Yhb1 and Sfa1 appear to play an important role in CR-induced RLS (Figure 5(a)) but not CLS (Figure 5(d)). One possible explanation is that perhaps actively dividing cells are more susceptible to CR-induced NO stress. In line with this model, CR increases NO more significantly in early growth stages during which most cells were still actively dividing (Figure 1(a)). Yhb1 and Sfa1 are dispensable for CR-induced CLS, which may be due to the fact that other NO metabolizing factors and/or stress response factors play more important roles in CR-induced CLS. Interestingly, the *sfa1*Δ*yhb1*Δ double mutant (under non-CR conditions) showed decreased NO levels (Figure 5(c)) suggesting that functional NO detoxification is required to support NO production in yeast cells. These results highlight the differences in sensitivity towards NO production and detoxification activities for CR-induced RLS and CLS. It is possible that yeast RLS and CLS have different sensitivities towards certain NO metabolites such as peroxynitrite. Peroxynitrite is formed by the reaction

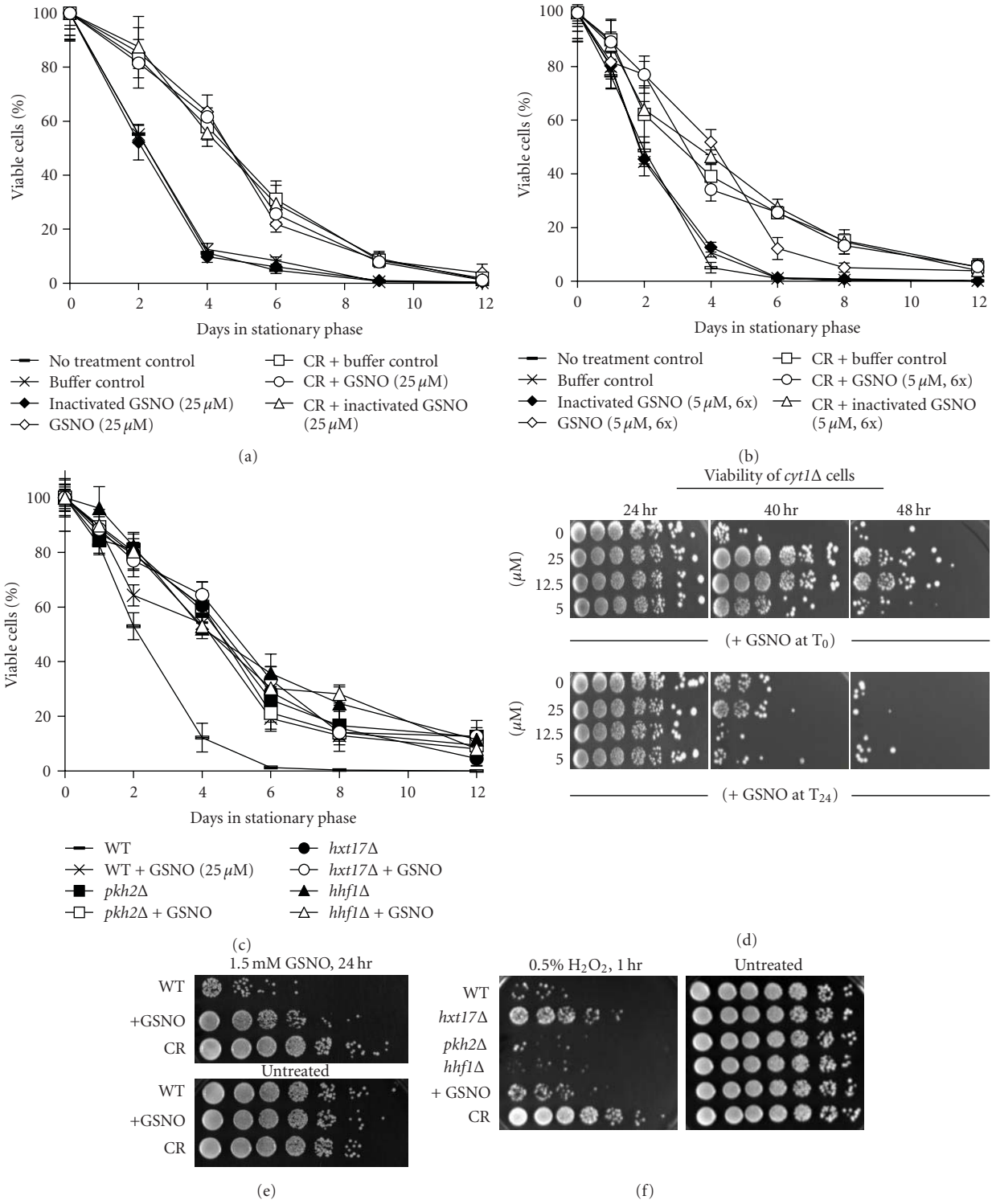

Figure 6: GSNO treatments extend chronological lifespan (CLS). (a and b) Treating cells with low doses of GSNO extends CLS. (a) Cells are treated with a single dose of GSNO at a final concentration of 25 μM upon starting the CLS assay (at 0 hr). (b) Cells are treated with GSNO at a final concentration of 5 μM at six different time points (6x): 0, 3, 6, 9, 18, and 24 hr. (c) GSNO treatment does not further increase the long CLS of representative CR mimic mutants. (d) GSNO treatments partially rescue the short CLS of the *cyt1*Δ mutant. Results show relative viability of cells taken from GSNO-treated CLS culture (in 5-fold serial dilutions). T_0: treating cells with GSNO upon starting CLS; T_{24}: treating cells with GSNO 24 hr after starting CLS. (e) Cells pretreated with 25 μM GSNO (+GSNO) show resistance towards GSNO-induced toxicity. (f) Cells pretreated with 25 μM GSNO (+GSNO) show mild resistance towards hydrogen peroxide-induced toxicity. WT: BY4742 wild-type control; CR: 0.5% glucose. One representative set of three independent experiments, each conducted in quadruplicate (for a, b, and c), is shown. Error bars denote standard deviations. For (a) to (c), numbers on *x*-axes denote the number of days after entering stationary phase (nonmitotic state).

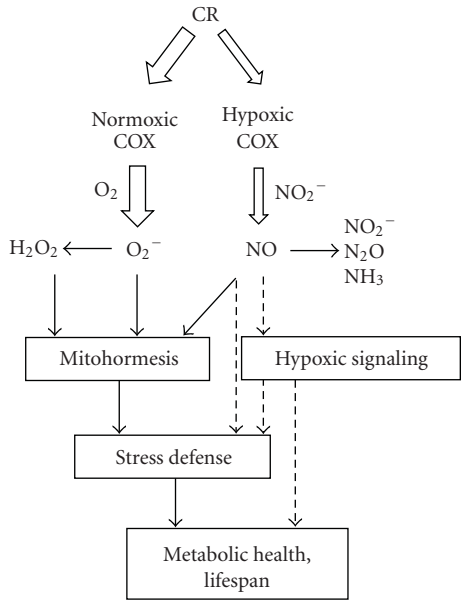

Figure 7: A proposed model for the roles of NO and mitochondrial respiration in CR. In yeast, the cytochrome c oxidase (COX) is likely to be responsible for CR-induced NO production. Under normoxic conditions, CR can activate both oxygen-dependent respiration and nitrite-dependent NO production, and both require the cytochrome c oxidase (COX). CR may increase NO production by derepressing expression of the hypoxic isoform subunits of COX and cytochrome c, which are normally repressed by oxygen. ROS detoxification enzymes protect cells from various ROS-associated damages and are required for certain CR-induced beneficial effects. NO and other mitochondrial ROS may induce adaptive gene expression changes as well as a metabolic shift, which optimize metabolism and improve cellular defense system against the oxidative stress that accumulates with age. For clarity and simplicity, many important longevity factors are not shown. Other pathways may work independently or in concert with mitochondrial respiration and stress response to mediate CR.

of NO with superoxide, both of which can be produced by the electron transport chain [32]. It is possible that during replicative aging, superoxide detoxification is not as efficient, making NO scavenging more important. During chronological aging, the high activity of superoxide dismutases can maintain consistently low levels of superoxide, compensating for the lack of NO scavenging. Supporting this possibility, several stress response factors including superoxide dismutase activities have been reported to be upregulated during CLS [9, 33, 58]. In addition, lower levels of superoxide have also been observed in calorie-restricted yeast cells during CLS [58]. Transcription factors associated with respiration (Hap4), stress response (Msn2), and a MAP kinase important in osmoregulation (Hog1) can drive the expression of Yhb1 [61, 62], Sfa1 [63, 64], or both and may regulate increased RLS and resistance to nitrosative stress during CR.

Our results indicate that COX is a major source of NO production under CR (Figure 7) because deleting components of the electron transport chain in the *rho*^{0} (Figure 4(f)), *cyt1*Δ (Figures 4(d) and 4(f)), and COX (B. Li and S.-J. Lin, unpublished) mutants largely abolishes CR-induced NO production and increased longevity (Figures 4(b) and 4(c)). Moreover, GSNO treatments could only partially compensate the loss of respiratory capacity (Figure 6(d)), indicating that both mitochondrial respiration and mitochondrial NO synthesis are required for optimal NO-induced longevity. Interestingly, the hypoxic isoforms of cytochrome c and COX subunit V, which are normally repressed by oxygen, are activated by CR (Figure 4(h)) (P. Castello and R. Poyton, unpublished). It is therefore possible that CR activates/de-represses factors that are normally induced by hypoxia to further augment NO production and respiration capacity. The upregulation of Cox5b in CR cells could explain both the increased rate of NO synthesis and the relaxation of oxygen sensitivity because a COX isozyme containing subunit Vb has been shown to support a higher rate of NO synthesis at high oxygen concentrations [38]. In addition, increased NO has also been suggested to function in the induction of hypoxic genes (hypoxic signaling) [32, 34, 65]. It would be interesting to determine the roles of CR-induced NO production and hypoxic signaling in mitochondrial hormesis (mitohormesis), which also plays an important role increasing stress responses and lifespan [66]. It remains unknown whether COX also plays important roles in CR-induced NO production in mammals, although rat liver mitochondrial COX and human endothelial cell COX also exhibit NO producing activity [34, 65]. In mammals, mitochondrial NO could also come from eNOS, which has been reported to physically interact with the outer mitochondrial membrane [67]. eNOS has been suggested as a mediator of CR in mice, and induction of eNOS caused both mitochondrial biogenesis and enhanced expression of the NAD-dependent protein deacetylase SIRT1 [20]. Together, these studies suggest that NO-mediated signaling and mitochondrial respiration work in concert to adapt cells to metabolic changes induced by CR leading to enhanced stress response and lifespan extension (Figure 7).

5. Conclusions

In summary, we have demonstrated that calorie-restricted yeast cells produce NO under normoxic conditions perhaps by derepressing the hypoxic subunit isoforms of COX and cytochrome c. CR-induced NO production may extend lifespan by increasing the stress response (mitohormesis). Our study may have uncovered potential novel components in the CR pathway and provided tools to analyze the interconnections between NO, mitochondrial respiration, CR, and longevity. Although the CR mimics identified in this study share similar NO levels, lifespan, and oxygen consumption phenotypes with CR, they may activate NO production and regulate mitochondrial respiration and lifespan via different mechanisms. It will be enlightening to examine these differences in future studies. Finally, we propose that CR likely confers its beneficial effects via a mitochondria-NO-mediated adaptive metabolic shift, which optimizes metabolism and at the same time improves cellular defense system against the oxidative stress that accumulates with age.

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Conflict of Interests

The authors have declared that no conflicts of interest exist.

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Review Article

Mitochondrial Acetylation and Diseases of Aging

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In recent years, protein lysine acetylation has emerged as a prominent and conserved regulatory posttranslational modification that is abundant on numerous enzymes involved in the processes of intermediary metabolism. Well-characterized mitochondrial processes of carbon utilization are enriched in acetyl-lysine modifications. Although seminal discoveries have been made in the basic biology of mitochondrial acetylation, an understanding of how acetylation states influence enzyme function and metabolic reprogramming during pathological states remains largely unknown. This paper will examine our current understanding of eukaryotic acetate metabolism and present recent findings in the field of mitochondrial acetylation biology. The implications of mitochondrial acetylation for the aging process will be discussed, as well as its potential implications for the unique and localized metabolic states that occur during the aging-associated conditions of heart failure and cancer growth.

1. Introduction

Over millions of years, eukaryotic organisms evolved fine-tuned metabolic mechanisms for orchestrating the conversion of diverse carbon substrates into cellular energy. These mechanisms include changes in gene transcription, allosteric regulation of metabolic enzymes by fluxing pools of intermediary metabolites, and direct regulation by posttranslational modifications (PTMs), such as phosphorylation. One PTM, lysine acetylation, has been traditionally studied in the context of nuclear histone modifications and is well known to influence changes in gene expression [1]. However, recent proteomics surveys have revealed that lysine acetylation is a widespread cellular modification that is particularly abundant on mitochondrial proteins, suggesting a new posttranslational mechanism for coordinating the metabolism of carbon sources [2, 3]. Accordingly, several studies have demonstrated that reversible lysine acetylation can, by targeting key enzymes, modulate the activity of mitochondria-localized fatty acid β-oxidation, the tricarboxylic acid cycle (TCA), urea cycle, and oxidative phosphorylation in a nutrient-responsive manner [4–7]. The reversible and nutrient-sensitive manner of many of these acetyl modifications has strongly implicated the mitochondrial-localized members of the NAD^+-dependent deacetylases (sirtuins) as regulatory mediators of these fundamental metabolic processes [8]. Sirtuins are also believed to be part of a genetic program influencing the development of age-related conditions such as heart disease, neurodegenerative disease, and cancer [9]. Although seminal discoveries have been made in the basic biology of mitochondrial acetylation, an understanding of how acetylation states influence enzyme function and metabolic reprogramming during age-associated pathological states remains largely unknown.

In this paper we will review mammalian acetate metabolism and provide a brief history of lysine acetylation as a means to discussing more recent advances in mitochondrial acetylation biology. We then suggest a role for alterations in mitochondrial acetylation states during age-related conditions of heart failure and cancer, as well as review its potential role in human longevity.

2. Acetyl-CoA and Metabolism

Acetate is an ancient energy precursor molecule of metazoan metabolism (Figure 1). In eukaryotic systems, the principal

source of cellular acetate is generated by the mitochondrial processes of glucose-derived pyruvate oxidation, amino acid catabolism, and the oxidation of even-numbered fatty acyl chains. These distinct metabolic pathways are all capable of yielding the activated form of cellular acetate, or acetyl-CoA. Thus, acetyl-CoA represents a common convergence point for carbohydrate, amino acid, and lipid catabolism. How mitochondrial acetyl-CoA is utilized throughout the cell is heavily dependent on the cell type and the metabolic state of the organism. However, the primary function of acetyl-CoA throughout all cell types is as a carbon donor in the tricarboxylic acid (TCA) cycle. Originally characterized by Hans Krebs and Albert Szent-Györgyi, the TCA cycle is a series of substrate oxidation and decarboxylation reactions that serve to liberate carbon dioxide and generate electron donors in the form of the reduced coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) [10]. The coenzymes NADH and $FADH_2$, in turn, serve as high-energy electron donors which are oxidized by the respiratory chain complexes to generate the proton-motive electrochemical gradient that is ultimately responsible the formation of energy-rich ATP [11]. Acetyl-CoA also serves as a vital component of anabolic cellular processes. In general, during a state of nutrient excess (fed state), a proportion of the excess acetyl-CoA generated within the mitochondria is exported into the cytosol in the form of citrate, where it is reconverted into acetyl-CoA by ATP-citrate lyase [12]. This cytosolic acetyl-CoA can then participate in *de novo* synthesis of fatty acids and sterols that are vitally important for the formation and maintenance of lipid membranes, as well as the synthesis of steroid hormones, triacylglycerols, cholesterol, and fat-soluble vitamins [13, 14]. Lipogenesis primarily occurs in hepatocytes and adipocytes, whereas cholesterol synthesis primarily occurs in hepatocytes. In contrast to the fed state, during a nutrient-depleted state, the liver uses TCA cycle intermediates for gluconeogenesis which depletes oxaloacetate and limits the entry of acetyl-CoA into the TCA cycle. As the liver continues to oxidize fatty acids for fuel, acetyl-CoA begins to accumulate and is converted into the transport forms of acetate known as ketone bodies. Ketone bodies are then used as an alternative fuel source in the brain, heart, and skeletal muscle during fasting or glucose-scarce conditions [15]. Free cytoplasmic and mitochondrial acetate can also be converted into acetyl-CoA via the ATP-dependent mechanisms of acetyl-CoA synthetase 1 and 2, respectively [16, 17]. Interestingly, acetyl-CoA synthetase 2 (AceCS2) was recently found to be an enzyme critically involved in thermogenesis during fasting conditions [18]. Through its well-established roles in the generation of cellular energy and as a macromolecular building block, acetyl-CoA stands as a critical chemical intermediate that is inextricably linked to cellular energy homeostasis. However, the fundamental and ancient link between acetyl-CoA, mitochondria, and metabolism is underappreciated in the context of acetyl-CoA's exciting and dynamic use as the acetyl group donor for acetyl-lysine posttranslational modifications. Interestingly, a recent study highlighted this often overlooked aspect of acetyl-CoA metabolism by demonstrating that the metabolic enzyme ATP-citrate lyase, in addition to its previously mentioned roles in lipid and steroid synthesis, also regulates global patterns of histone acetylation and corresponding changes in gene expression [19]. Therefore, it is nutrient availability and metabolism that largely determine the cellular pool of acetyl-CoA available for acetyl-lysine protein modifications.

3. N^{ε}-Acetylation

Acetylation occurring on the epsilon-amino group of lysine residues (N^{ε}-acetylation) was discovered on histone proteins purified from calf thymus over forty years ago [20]. The ensuing studies of N^{ε}-acetylation focused almost exclusively on histone substrates [21–23]. It was not until 1996 that Taunton et al. purified the first histone deacetylase enzyme [24]. This important discovery quickly expanded research interest in the field of histone acetylation and in the past 15 years has led to the discovery of numerous enzymes that catalyze the addition and removal of acetyl groups, termed histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. The dynamic and opposing functions of HATs and HDACs form the basis for the transcriptional modulation of chromatin and the histone code hypothesis [1, 25]. Due to their role in global cellular processes of transcription, HDACs have become attractive chemotherapeutic targets. Vorinostat (suberoylanilide hydroxamic acid) and Romidepsin (FK228) are FDA-approved HDAC inhibitors that are currently being used for the treatment of cutaneous T-cell lymphomas, and there are a number of related compounds in clinical trials for other neoplastic indications [26].

Owing to the highly conserved nature of HATs and HDACs across multiple eukaryotic species, we commonly classify them according to their homologs in yeast [27]. There are three primary families of HATs. These include the MYST family, the Gcn-5-related N-acetyltransferases (GNATs), and the E1A-associated protein of 300 kDa/CREB-binding protein (p300/CBP) family [28]. HDACs are broadly classified into the Rpd3/Hda1 family of deacetylases, and the silent information regulator-2-(Sir2-) like NAD^+-dependent deacetylases/mono-ADP-ribosyltransferases, or sirtuins. The Rpd3/Hda1 family are further grouped into classes I, II, and IV, while the sirtuins represent class III deacetylases [9, 29]. Due to the histone-centric nature of the founding studies on acetylation, these acetylase and deacetylase enzymes are traditionally referred to as HATs and HDACs. However, research in the past 10 years has established that the so-called HATs and HDACs have multiple nonhistone and extranuclear targets including, among many others, the tumor suppressor p53, the oncogene β-catenin, and the molecular chaperone HSP90 [30–33]. Accordingly, these enzymes are now more generally referred to as lysine acetyltransferases (KATs) and lysine deacetylases (KDACs).

Biochemically, the positively charged ε-amino group of lysine residues is important for protein stability via hydrogen bonding with nearby amino acid residues and can also serve as a base during enzyme catalysis [34] (Figure 2). The covalent enzymatic addition of an acetyl moiety to the ε-amino

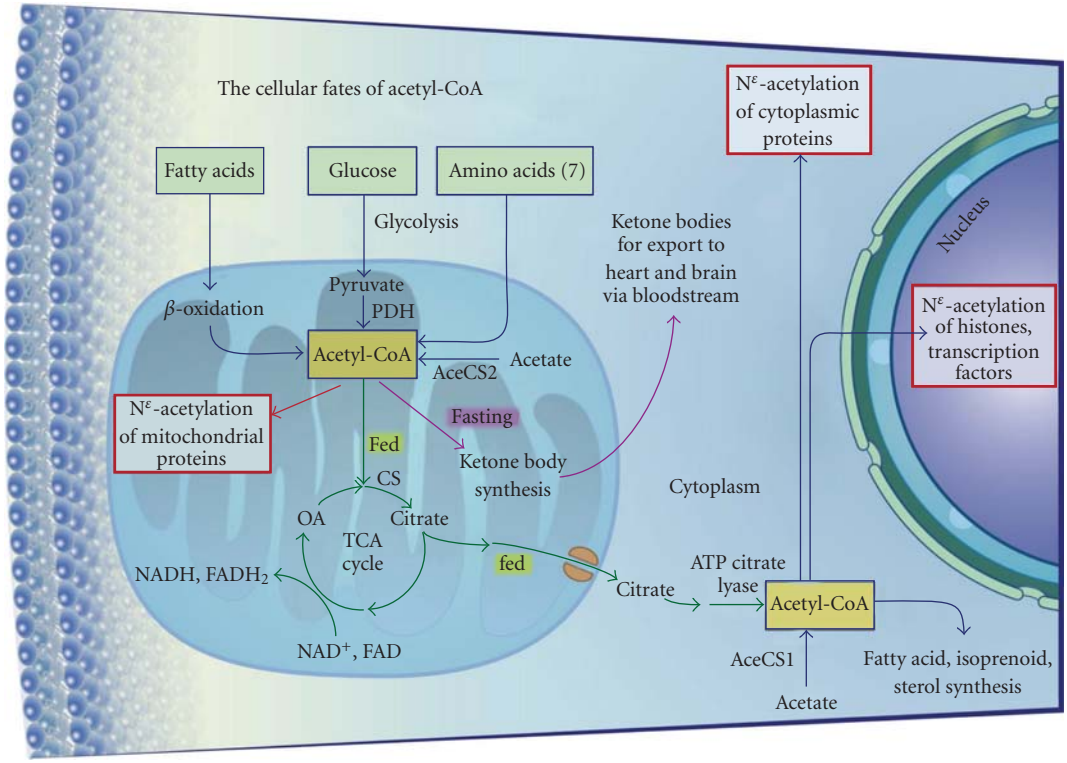

Figure 1: The cellular fates of acetyl-CoA. Please see section entitled "Acetyl-CoA and Metabolism" for a detailed description of the figure. PDH: pyruvate dehydrogenase complex, AceCS2: acetyl-CoA synthetase 2, CS: citrate synthase, OA: oxaloacetate, AceCS1: acetyl-CoA synthetase 1.

group on a lysine residue effectively neutralizes its native positive charge, which changes its propensity to interact with nearby residues, other proteins, or can alter the activity of an enzyme. In its well-established chromatin context, acetylation neutralizes positively charged lysine residues on histone tails, thereby reducing their charged interactions with chromatin and providing an epigenetic mark which is recognized and used by bromodomain-containing proteins to recruit transcriptional machinery and other chromatin-modifying elements [1]. In another context, acetylation of lysine 88 on the enzyme ornithine transcarbamylase (OTC) changes its affinity for its substrate, carbamoyl phosphate, and negatively regulates the detoxification of ammonia in the urea cycle [35]. Additionally, acetylation of lysine 685 on STAT3 promotes its stable dimerization and subsequent function as a transcriptional activator [36]. The ε-amino groups of lysine residues are also the targets of a number of other posttranslational modifications including methylation, ubiquitination, SUMOylation, and NEDDylation. These other modifications may confer their own unique properties to proteins and protein functions that could be coordinated with acetylation/deacetylation. Ubiquitination of lysine residues, in particular, is known to cooperate with acetylation to regulate the proteolytic degradation of cellular proteins [37]. It has also been proposed that acetylation may also act in concert with other distinct posttranslational modifications to achieve a broad spectrum of functional protein outcomes that has been termed the "protein modification code," analogous to the "histone code" for transcriptional regulation [38]. In general, it has become clear in recent years that N^{ε}-acetylation is a widespread and functionally diverse posttranslational modification.

4. NAD^+ Dependent Deacetylases (Sirtuins)

In their traditional nuclear context, KATs catalyze the acetyl-CoA-dependent addition of acetyl groups to disordered histone tails—a mark that generally induces a "loose" and transcriptionally active conformation of chromatin. In contrast, the traditional functional understanding of KDACs holds that they catalyze the H_2O-dependent removal of acetyl groups from histone tails to induce "tight" and transcriptionally repressed chromatin [25]. This well-established paradigm for transcriptional modulation was broadened with the discovery of the mechanistically distinct sirtuin class of deacetylases/mono-ADP ribosyltransferases.

There are 7 mammalian sirtuin enzymes. The nuclear-localized family members consist of SIRT1, SIRT6, and SIRT7, while SIRT2 is concentrated in the cytosol [9]. Interestingly, SIRT3, SIRT4, and SIRT5 exhibit mitochondrial localization [9, 39, 40]. A recent report has also suggested that SIRT1 could function within the mitochondria [41]. Together, the sirtuin family of KDACs are implicated in the regulation of a broad scope of biological processes including, but not limited to, transcriptional repression, development, apoptosis, DNA repair, cellular stress responses, and

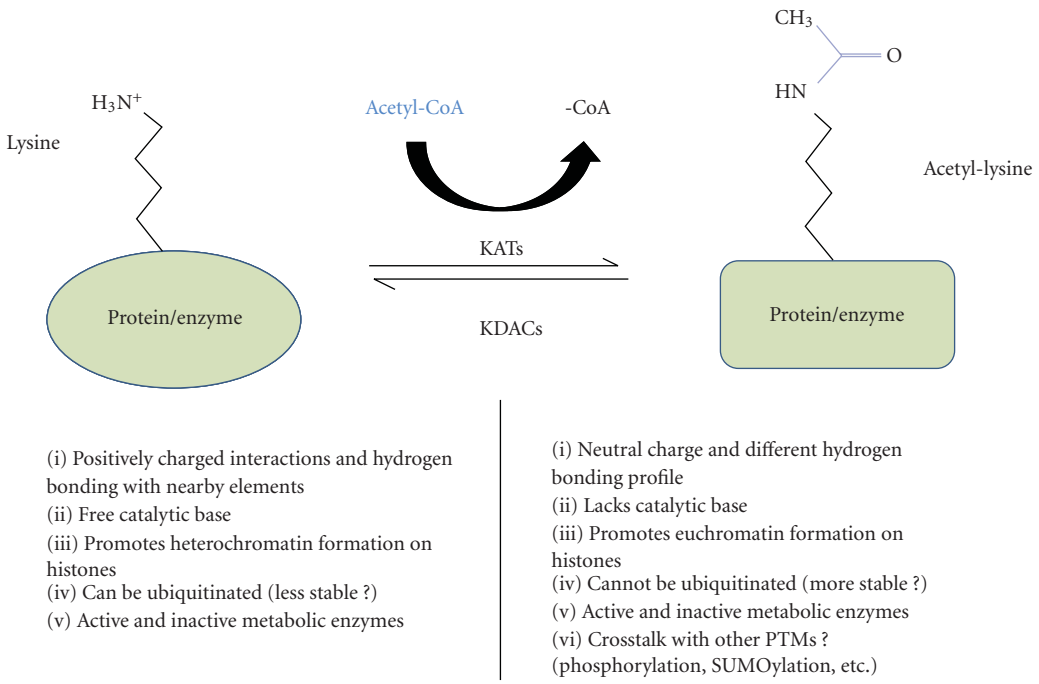

Figure 2: Changes in protein function and stability known to be associated with lysine acetylation. KATs: lysine acetyltransferases, KDACs: lysine deacetylases.

metabolism [42–46]. Sirtuins are unique among the four families of mammalian KDACs in that they catalyze the removal of acetyl groups in a fashion that is dependent on the cellular coenzyme NAD^+. The reduced form of NAD^+, NADH, is generally produced during cellular catabolic processes that serve to derive energy from nutrients, whereas the oxidized form, NAD^+, accumulates within cells during nutrient scarce conditions. Thus, the ratio of the reduced form to the oxidized form, or NAD^+/NADH, is indicative of the overall cellular energy state. Sirtuin deacetylase activity is responsive to this measure of cellular energetic balance and requires NAD^+ as the acceptor of an acetyl group from a target acetyl-lysine residue or for protein ADP ribosylation [47]. The NAD^+-dependent deacetylase reaction yields a deacetylated target protein, 2′-O-acetyl-ADP-ribose, and nicotinamide, which can then act to inhibit the sirtuin reaction mechanism [48]. In general, when the cellular NAD^+/NADH ratio is elevated during times of exercise, nutrient restriction, or outright fasting, sirtuins are presumed to be more actively deacetylating their target proteins, whereas during a fed or basal state, NADH predominates, and sirtuins are less active. It is via their nutrient and stress-sensing capacity to modify transcriptional landscapes and modulate enzyme function that sirtuins are believed to mediate the healthful benefits of caloric restriction—the only natural means of consistently extending lifespan and delaying the onset of age-related pathologies such as cancer in mammalian systems [8, 49–52]. It is the recently understood and growing influence of the sirtuin family on the biological mechanisms of aging that has excited the research community and has made the sirtuins highly attractive therapeutic targets for diseases of aging [53].

5. Mitochondrial Sirtuins and N^ε-Acetylation

Mitochondria are key organelles for intermediary metabolism as they coordinate the conversion of cellular carbon sources into useable energy in the form of adenosine triphosphate (ATP). The metabolic processes culminating in ATP synthesis are well characterized and are regulated by various mechanisms including nutrient availability, phosphorylation, allosteric mechanisms, reactive oxygen species, and divalent cations such as calcium and magnesium [54–57]. In light of the established understanding that mitochondria are the primary cellular generators of the acetyl-CoA necessary for enzymatic acetylation, it is surprising that N^ε-acetylation within the mitochondria remained virtually unstudied until the last decade. The discovery that the sirtuin family members SIRT3, SIRT4, and SIRT5 localize to the mitochondrial matrix has strongly suggested that acetylation could also play an important regulatory role within this organelle [39, 40]. An early study by Shi et al. supported this suggestion by demonstrating that SIRT3 regulates adaptive responses to cold exposure in brown adipose tissue by increasing the expression of mitochondrial uncoupling protein-1 (UCP1) and thereby increasing mitochondrial respiration [58]. However, the data in this study were generated with a SIRT3 construct lacking a mitochondrial targeting sequence, and, thus, it is not clear whether the results are due to SIRT3 acting within the mitochondria. Soon after this study was published, Schwer et al. and Hallows et al. independently confirmed that mammalian SIRT3 directly regulates the activity of the mitochondrial protein acetyl-CoA synthetase 2 (AceCS2) via NAD^+-dependent deacetylation [59, 60]. These findings demonstrated an

ancient metabolic regulatory mechanism conserved from the prokaryotic bacterium *Salmonella enterica* to mammals [61]. Taken together, these early studies not only confirmed reversible N^{ε}-acetylation as an important regulatory mark within mitochondria but also implicated the sirtuins as key nutrient-responsive modulators of the functional changes acetylation confers upon its target proteins.

The initial studies of mitochondrial acetylation gave way to global proteomics surveys. A 2006 proteomics survey conducted by Kim et al. revealed that over 20% of liver mitochondrial proteins and enzymes are acetylated and that many change acetylation states in response to acute fasting [2]. Furthermore, enzymes of the major mitochondrial carbon conversion pathways such as the TCA cycle and β-oxidation were found to be particularly enriched in acetyl modifications. These findings suggested that reversible acetylation could serve as a finely-tuned mechanism for globally regulating the use and conversion of carbon energy sources. The authors also demonstrated that a number of the mitochondrial enzymes they found to be acetylated have known implications in processes of mammalian aging and longevity, such as manganese superoxide dismutase (MnSOD) and NADH:ubiquinone oxidoreductase (respiratory complex I) [2]. Complementing the discovery that global acetylation profiles can change in response to nutrient availability, Schwer et al. showed that global mitochondrial acetylation profiles change in multiple organ systems in response to long-term caloric restriction (CR) [62]. Surprisingly, this study found that the vast majority of mitochondrial enzymes exhibited increases in acetylation during caloric restriction, which counters the belief that sirtuin-mediated deacetylation mediates the beneficial effects of caloric restriction. This finding could suggest that mitochondrial sirtuin-mediated deacetylation plays a relatively minor role in the metabolic changes that accompany caloric restriction and that there are more complex and undiscovered adaptive mechanisms at play. Additionally, Wang et al. and Zhao et al. further confirmed the regulatory importance of acetyl-modifications in metabolism and in the coordination of cellular carbon utilization [3, 63]. These proteomic surveys have exposed the prevalence of acetylation within mitochondria and reinforced its importance as a regulatory modification. Ongoing studies that aimed at understanding the mechanisms of acetylation dynamics are likely to reveal this as an essential component of mitochondrial biology.

Further focused investigations of acetylation within mitochondria have concentrated on the role of mitochondrial sirtuins. By generating SIRT3, SIRT4, and SIRT5 systemic knockout mouse models, Lombard et al. demonstrated that mice lacking SIRT3 exhibit global hyperacetylation of mitochondrial proteins in liver and brown adipose tissue, whereas the acetylation states in mice lacking SIRT4 and SIRT5 were relatively unchanged [64]. This evidence suggested that SIRT3 primarily functions as a deacetylase and potentially targets a wide range of mitochondrial proteins, whereas the deacetylase activities of SIRT4 and SIRT5 are much more restricted.

Although it is widely accepted that SIRT3's functional role is predominantly within mitochondria, several studies have explored potential nuclear and cytosolic roles for this sirtuin family member. The study mentioned earlier conducted by Shi et al. suggested that SIRT3 can regulate the expression of the nuclear-encoded genes coding for mitochondrial uncoupling protein 1 and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [58]. Similarly, SIRT3 has also been found to regulate the expression of catalase and manganese superoxide dismutase in the heart [65]. Yet another study indicated that SIRT3 can indirectly regulate the association between the glycolytic enzyme hexokinase II and the mitochondria [66]. These studies have formed the basis for future work investigating potential roles for SIRT3 outside of the mitochondria.

Early characterization of SIRT3 null mice indicated that they were phenotypically unremarkable, despite a significant reduction in their liver, heart, and kidney ATP content associated with hyperacetylation and reduced activity of mitochondrial respiratory complex I [7]. A more thorough investigation, however, revealed that $SIRT3^{-/-}$ mice display hepatic lipid accumulation during fasting resembling human disorders of fatty acid oxidation [4, 67]. This study found that SIRT3 directly deacetylates and activates long-chain acyl-CoA dehydrogenase (LCAD), illustrating a critical mechanism for this sirtuin in the metabolic adaptations to fasting and nutrient restriction [4]. Consistent with SIRT3's role in oxidative metabolism, Shulga et al. demonstrated that SIRT3 can stimulate oxidative phosphorylation and a shift away from glycolysis via inactivation of cyclophilin D, which consequently destabilizes the association of hexokinase II with the mitochondria [66]. Furthermore, mitochondrial isocitrate dehydrogenase (IDH2) is deacetylated and activated by SIRT3, thereby regulating a key step in the TCA cycle as well as the supply of NADPH necessary for mitochondrial antioxidant defense mechanisms [5, 68]. Additional studies have exposed SIRT3 as a regulator of protein translation via deacetylation of the mitochondrial ribosome and in the activity of respiratory complex II (succinate dehydrogenase) [69, 70]. It is now clear that SIRT3 can influence the activity of oxidative phosphorylation, fatty acid oxidation, and the TCA cycle, positioning this particular sirtuin as a major player in the regulation of intermediary metabolism.

Studies of mitochondrial SIRT4 and SIRT5 are less extensive than those for SIRT3. Earlier characterization of mammalian SIRT4 revealed that it chiefly functions as an NAD^{+}-dependent mono-ADP-ribosyltransferase that specifically ADP-ribosylates and inactivates mitochondrial glutamate dehydrogenase (GDH), thereby regulating amino acid-induced insulin secretion in pancreatic β cells [71, 72]. Later studies also identified GDH as a target of SIRT3-mediated activation via deacetylation, but potential cross-talk with SIRT4-mediated ADP ribosylation has not been explored [5, 64]. Recently, it was demonstrated *in vivo* that adenoviral-mediated shRNA knockdown of SIRT4 in mouse liver increases the expression of genes involved in fatty acid metabolism [73]. Together, these lines of evidence suggest that SIRT4 could play an important role in the disruption of lipid homeostasis associated with insulin resistance in type 2 diabetes. Further characterization of mitochondrial SIRT5 has shown that it regulates the first

step in the detoxification of ammonia during the urea cycle via deacetylation-mediated activation of carbamoyl-phosphate synthetase 1 [6]. Accordingly, SIRT5$^{-/-}$ mice develop hyperammonemia during metabolic states demanding greater protein catabolism such as fasting and caloric restriction [6, 74]. Another investigation has suggested that SIRT5 can deacetylate cytochrome *c*, a protein involved in mitochondrial respiration and apoptosis [5]. However, the functional importance of this relationship has not been determined and is also inconsistent with findings in the previously mentioned study indicating that SIRT5 is localized to the mitochondrial matrix. Future studies of mitochondrial sirtuins 4 and 5 are likely to elucidate their full regulatory capacity in response to various cellular stresses and stimuli.

Interestingly, other studies have implicated reversible mitochondrial acetylation in mechanisms of cell growth and survival. An early investigation of SIRT3's function in an epithelial cancer cell line indicated that it is required for JNK-regulated cell death via silencing the apoptotic regulator B-cell lymphoma 2 (bcl-2) [75]. In contrast to its role in epithelial cancer, SIRT3 was shown to serve a prosurvival function in cardiomyocytes via deacetylating Ku70 and promoting the sequestration of the proapoptotic protein Bax [43]. In addition to its apoptotic functions, SIRT3 was more recently demonstrated to be a bona fide tumor suppressor by Kim et al. [76]. This study showed that SIRT3$^{-/-}$ mouse embryonic fibroblasts display reduced antioxidant defenses, which result in a lower threshold for tumorigenesis—a finding that confirmed earlier work that established SIRT3's involvement in the transcriptional regulation of antioxidant genes [65, 77]. Furthermore, the transformation-permissive effect of SIRT3 ablation can be reversed upon the addition of exogenous MnSOD [76]. Consistent with these findings, two more recently published investigations independently confirmed that SIRT3 directly deacetylates and activates MnSOD, thereby linking nutrient availability to antioxidant defenses [78, 79]. A different study conducted by Yang et al. established that the levels of mitochondrial NAD^+ are a critical determinant to cell survival [80]. Mitochondrial NAD^+ levels were shown to be regulated by the rate-limiting enzyme in the mammalian NAD^+-salvage pathway, nicotinamide phosphoribosyltransferase (Nampt). Importantly, the authors demonstrated that NAD^+-responsive mitochondrial SIRT3 and SIRT4 are required for improved cell survival when mitochondrial NAD^+ levels rise under conditions of fasting and induced genotoxic stress [80]. Recently, two independent approaches also implicated SIRT3 in the regulation of p53-mediated growth arrest [81, 82]. Taken together, these studies indicate that mitochondrial SIRT3 is a critical mediator of cell growth and survival which may hold therapeutic implications in the treatment of various cancers.

There are also several interesting acetylated mitochondrial proteins involved in cellular stress responses and apoptosis on which the effect of reversible acetylation has not been studied. These proteins include apoptosis-inducing factor (AIF) responsible for DNA fragmentation during apoptosis, cytochrome *c* which forms an important component of the apoptosome as well as voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT), both of which are believed to form the mitochondrial permeability transition pore during apoptosis [2, 83]. Furthermore, all of the previously mentioned mitochondrial proteins are reversibly acetylated in a nutrient-sensitive manner, suggesting that the apoptotic functions of these proteins may be regulated by cellular energy status [2]. The study of mitochondrial acetylation states in processes of cell death and survival represents an entirely open field of study that is rife with intriguing biological questions that could provide a new layer of understanding to the mechanisms and regulation of apoptosis and cell growth.

The majority of the studies in the field of mitochondrial acetylation thus far have focused on the mitochondrial localized members of the sirtuin family of deacetylases, while the search for the counterbalancing mitochondrial acetyltransferase(s) has so far been unsuccessful. Although it is possible that certain nuclear-encoded, mitochondrial targeted proteins acquire acetylation marks prior to mitochondrial import, the confirmed acetylation of the mitochondrial confined ATP synthase subunit 8 protein makes the existence of a mitochondrial acetyltransferase(s) highly likely [2, 83]. The marked enrichment of acetyl-modifications occurring on enzymes involved in major processes such as β-oxidation and redox chemistry (64% and 44%, resp.) over the total pool of acetylated mitochondrial proteins (22%) also suggests finely tuned enzymatic mechanisms for regulating carbon flux within mitochondria [2]. Furthermore, the amino acid sequence motifs favoring acetylation within mitochondria differ from those of the nucleus and cytosol, suggesting a mitochondrial-specific acetyltransferase(s) possessing its own distinct set of substrates [2]. Despite the strong evidence for a mitochondrial acetyltransferase(s), it is also important to consider the possibility of nonenzymatic acetylation occurring in response to changes in the overall mitochondrial acetyl-CoA pool. Indeed, nonenzymatic acetylation is known to occur on histone substrates and may account for the unexpected increases in mitochondrial acetylation in response to caloric restriction [62, 84].

How do large changes in the acetylation state of a given mitochondrial protein pool affect protein function? Furthermore, how do large-scale changes in global mitochondrial acetylation states influence mitochondrial biology and overall cellular energy status? Although little work has been conducted to address these questions, some insights can be drawn from the literature. In the work of Hirschey et al., the authors performed semiquantitative mass spectrometry of the fatty acid oxidation protein LCAD in wild-type and SIRT3$^{-/-}$ livers in an effort to identify differentially acetylated lysine residues of interest [4]. The analysis discovered eight acetylation sites on LCAD; however, only one particular acetyl-lysine residue, K42, was found to be critically important for the SIRT3-dependent modulation of LCAD's enzymatic activity. Constitutively mimicking deacetylation at K42 induced a nearly 60% increase in LCAD activity. Furthermore, acetylation at only K42 accounted for over 70% of the total LCAD acetyl-lysine signal in SIRT3$^{-/-}$ liver mitochondria as quantified by Western blot

densitometry. Earlier work performed by this group found a similar pattern with K642 of the mitochondrial enzyme acetyl-CoA synthetase 2 [59]. These findings suggest that large changes in acetylation signal and protein function could be induced by relatively limited or restricted changes in the acetylation state of a given protein. Accordingly, global changes in mitochondrial acetylation, such as those observed in the SIRT3$^{-/-}$ mitochondria, might represent an unexpectedly limited number of acetylation state changes corresponding to large functional and metabolic consequences. Future work in this field will surely provide exciting insights into these questions, and the imminent discovery of the mitochondrial acetyltransferases(s) or alternative acetylation mechanisms will provide a critical step toward a more complete understanding of mitochondrial acetylation biology and its implications for disease states.

6. Mitochondrial Acetylation and Heart Failure

The recently understood prevalence of acetyl modifications within the mitochondria that can potentially modulate processes of intermediary metabolism suggests that altered acetylation could potentially contribute to age-associated disease states in which there are recognized alterations in mitochondrial carbon utilization, such as heart failure and cancer.

The unfortunate progression from increased cardiac workload to pathological cardiac hypertrophy and, ultimately, heart failure occurs when the long-term metabolic demands of the body exceed the ability of the pumping heart to meet them. Although there are a number of human conditions capable of leading to heart failure including renal insufficiency, high blood pressure, and cardiac arrhythmias, the leading risk factor is increasing age [85]. The role of mitochondrial dysfunction in heart failure and the aging process in general is well established. Mitochondrial disorders are often characterized by myopathies of the heart and skeletal muscle, as these highly energetic organs are heavily dependent on mitochondria-derived energy generation [86]. Furthermore, cardiomyopathy and heart failure in the general population are often regarded as diseases of impaired energy homeostasis associated with a depletion of the cardiomyocyte ATP and creatine pools [87, 88]. Interestingly, mice lacking the predominant mitochondrial NAD$^+$-dependent deacetylase SIRT3 also exhibit depleted cardiac ATP levels and further display global hyperacetylation of mitochondrial proteins in liver and brown adipose tissue [7, 64]. These data suggest a role for mitochondrial SIRT3 dysfunction in the pathophysiology of heart failure. Several additional studies also stand in support of this notion. As discussed earlier, Sundaresan et al. have shown that SIRT3 promotes cardiomyocyte cell survival during genotoxic and oxidative stress [43]. This group has also revealed that SIRT3 transgenic mice are resistant to agonist-induced cardiac hypertrophy via SIRT3-mediated upregulation of antioxidant genes [65]. Moreover, exogenous administration of NAD$^+$ into the hearts of mice was found to abrogate agonist-induced cardiac hypertrophy in a SIRT3-dependent manner [89]. Similarly, transgenic mice harboring cardiac-specific overexpression of the NAD$^+$-salvaging enzyme Nampt display reduced infarct size following ischemia-reperfusion injury, raising the possibility that the cardioprotective effect of increased NAD$^+$ is mediated by the mitochondrial sirtuins [90]. Pathophysiological cardiac remodeling and heart failure in humans are also known to develop in response to alterations in the renin-angiotensin system (RAS) [91]. One of the RAS signaling proteins, angiotensin II, is often used to induce hypertension and cardiac hypertrophy in experimental animals. Interestingly, ablation of the angiotensin II type I receptor in mice reduces cardiac injury, increases lifespan, and upregulates the expression of SIRT3 and its upstream regulator Nampt in the kidney [92]. Collectively, these studies implicate the mitochondrial deacetylase SIRT3 as a major cardioprotective enzyme, the modulation of which may hold therapeutic promise in the treatment of cardiac disease.

Mitochondrial oxidative phosphorylation is responsible for generating over 90% of the energy-rich ATP in the adult human heart [93, 94]. Under basal conditions, as much as 70% of this ATP is derived from fatty acid β-oxidation, and the remainder is generated from the oxidation of glucose, lactate, and ketone bodies [95]. The energetic substrate preferences of the heart can change in response to acute stimuli or stressors such as exercise, energy demand, hormonal regulation, ischemia, and substrate availability [95, 96]. Thus, the healthy heart is a versatile and adaptive consumer of energy substrates. The failing heart, however, experiences complex alterations in energy metabolism and substrate utilization that are incompletely understood [97]. Studies on rodent and canine models generally indicate that the failing myocardium suffers downregulation of mitochondrial respiratory chain complexes, decreased oxygen consumption and ATP generation, decreased flux through the creatine kinase system, and impaired fatty acid oxidation [95, 97–100]. Many have proposed targeting these metabolic rearrangements and altering substrate utilization as a therapeutic strategy to treat heart failure [101]. Interestingly, numerous mitochondrial enzymes of the respiratory chain and those involved in the oxidation of glucose and fat carry one or more acetylation marks that are reversible in a nutrient-sensitive manner. These include respiratory complexes I, II, and V, pyruvate dehydrogenase, several acyl-CoA dehydrogenases, and carnitine palmitoyltransferase 1 and 2, among many others [2, 62, 83]. It has also been demonstrated that the mitochondrial deacetylase SIRT3 can directly promote oxidative metabolism via deacetylation-mediated activation of respiratory complex 1, long-chain acyl-CoA dehydrogenase (LCAD), and cyclophilin D as discussed earlier [4, 7, 66]. The notion that acetylation within the mitochondria can modulate the activity of metabolic enzymes implies that there may be altered acetylation states within the mitochondria and that they could contribute to the pathophysiology of heart failure. More importantly, if this is true, devising strategies that directly target mitochondrial acetylation may provide therapeutic benefit in pathological rearrangements of cardiac energy metabolism.

7. Mitochondrial Acetylation and Cancer

In 1924, Warburg and Negelein made the seminal observation that many cancer cells prefer to metabolize glucose into lactate even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation [102]. This phenomenon, known as the "Warburg Effect" or aerobic glycolysis, was hypothesized by Warburg to be a defect in the mitochondrial metabolism of tumor cells [103]. In the roughly 70 years following its discovery, little progress was made in uncovering the biochemical mechanisms underlying the propensity of tumor cells to prefer aerobic glycolysis. The last 15 years of oncology research, however, have experienced a renewed interest in understanding the Warburg Effect, and a number of important discoveries have been made. Notably, it has been observed that tumor cell lines almost exclusively express the embryonic isoform of the glycolytic enzyme pyruvate kinase (PKM2), which contributes to the glycolytic preference and lactate production observed in cancer cells [104, 105]. Recent work conducted by Vander Heiden et al. has even provided early evidence of an alternative carbon utilization pathway in tumors expressing PKM2 [106]. Further work on tumor metabolism has revealed that malignant cells also consume large amounts of glutamine in an effort to replenish the TCA cycle intermediates necessary for a high rate of macromolecular synthesis that is characteristic of rapidly proliferating cells [107]. This "glutamine addition" can partially be explained by induction of a transcriptional program that favors glutaminolysis via the activation of the proto-oncogene Myc, which can occur in many forms of human cancers including neuroblastoma, lymphoma, and small cell lung cancer [108–112]. Together, these studies and the recent revitalized interest in cancer bioenergetics have unveiled some key mechanisms underlying the remarkable ability of transformed cells to proliferate. Despite these recent advances, there is still much that we do not understand about a tumor cell's aptitude for reprogramming metabolism to meet its rapid growth demands, and this represents an active area of investigation.

Orchestrating malignant changes in cellular behavior often occurs at the level of posttranslation regulation. Indeed, many cancers are characterized by mutations in, or altered activity of, certain kinases including tumor suppressor LKB1, Src, phosphatidylinositol-3-kinase (PI3K), and the mutant BCR-ABL fusion kinase, which often occurs in chronic myelogenous leukemia (CML) [113–116]. Additionally, there is an appreciated role for cancer-associated transcriptional changes mediated by nuclear KATs and KDACs [26, 117]. Interestingly, the predominant mitochondrial deacetylase SIRT3 has recently been reported to function as a tumor suppressor [76]. This work demonstrated that SIRT3$^{-/-}$ fibroblasts exogenously transformed with the oncogenes Myc and Ras display reduced activity of mitochondrial respiratory complexes I and III [76]. These results are consistent with earlier work demonstrating direct activation of mitochondrial complex I via SIRT3-mediated deacetylation [7]. Moreover, these findings suggest that there is impaired oxidative phosphorylation and a heavier reliance on glycolytic metabolism in order to satisfy the energetic requirements of transformed SIRT3$^{-/-}$ fibroblasts, representing the primary hallmarks of the Warburg Effect. Coupling these findings with the knowledge that SIRT3 could potentially be regulating numerous mitochondrial proteins suggests that SIRT3 dysfunction may represent an important factor contributing to the poorly understood metabolic reprogramming that occurs during tumorigenesis [64]. Consistent with this notion, SIRT3 is known to be downregulated in certain breast cancers [76]. Furthermore, if SIRT3 can function as a tumor suppressor, it is logical to conceive that the putative mitochondrial acetyltransferase(s) counterbalancing the tumor-suppressive effects of SIRT3 would be characterized as oncogene(s). A multitude of mitochondrial proteins are now known to be acetylated, many of which are modified in a reversible and nutrient-responsive manner. Accordingly, alterations in mitochondrial acetylation states, and, hence, alterations in carbon substrate utilization, may contribute to the unusual preference for aerobic glycolysis and glutaminolysis often observed in numerous forms of cancer.

In addition to understanding the emerging importance of mitochondrial acetylation in age-associated diseases, it is also important to briefly discuss evidence for its involvement in the aging process in general. Heart failure and cancer are two pathophysiological states that display an exponentially increasing incidence with advancing age, and a role for the mitochondrial deacetylase SIRT3 in these respective conditions has been discussed [85]. Complementing SIRT3's emerging role in diseases of aging is the interesting though controversial observation that SIRT3 is the only member of the sirtuin family reportedly linked to longevity in humans. An early study of the human SIRT3 gene identified a guanine to thymine (G477T) single nucleotide polymorphism (SNP) located in exon three to be associated with survivorship in elderly males [118]. Later, Bellizzi et al. discovered a variable number of tandem repeat (VNTR) polymorphisms possessing enhancer activity at the SIRT3 locus [119]. The authors of this study further demonstrated that the inactive form of the SIRT3 enhancer is underrepresented in a population exceeding 90 years of age, and thus concluded that the active VNTR enhancer is associated with longevity. Additional work has identified the activating elements that bind to this intronic VNTR enhancer sequence [120]. In contrast to these studies, a meta-analysis of SIRT3 SNP data coupled with a larger association study of the SIRT3 chromosomal region in centenarians indicated no significant genomic variation that could be linked to longevity, with the exception of one possible SNP, rs939915 [121]. Future analysis of genomic variation at the mammalian SIRT3 locus, transgenic animals, and functional studies of SIRT3 and its deacetylation targets will provide much-needed insight into the possible role that this sirtuin plays in longevity and the aging process.

8. Summary

Although the acetylation of lysine residues has been known as a posttranslational modification for over 40 years, it was not until the last 15 years that advances in technology

and molecular techniques have enabled the rigorous study of this highly interesting regulatory mechanism. We now understand that lysine acetylation rivals phosphorylation, in respect to both the sheer number of acetylated proteins in mammalian cells as well as in its regulatory capacity. Furthermore, global cellular acetylation states are largely dependent on mitochondrial-derived processes of acetyl-CoA formation. Many proteins within the mitochondria, itself, are now known to be reversibly acetylated and, in some cases, acetylation can regulate metabolism and the fate of carbon energy sources. Thus, reversible acetylation of mitochondrial proteins represents a new layer of protein regulation mediating acute and adaptive changes in mammalian metabolism. This new framework for understanding metabolism suggests that altered mitochondrial acetylation patterns can contribute to the pathological shifts in energy generation that are known to occur during heart failure, the rapid proliferation of malignant tumor cells, and, indeed, the aging process in general. It is also worth noting that mitochondrial acetylation in neurodegenerative pathologies is currently an unstudied area. Mitochondrial acetylation biology is an infant field of study that, in the future, will have the potential to bridge our understanding of cellular metabolism, cell death and survival, and the inevitable pathological consequences of aging.

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Review Article

Mitochondria and PGC-1α in Aging and Age-Associated Diseases

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Aging is the most significant risk factor for a range of degenerative disease such as cardiovascular, neurodegenerative and metabolic disorders. While the cause of aging and its associated diseases is multifactorial, mitochondrial dysfunction has been implicated in the aging process and the onset and progression of age-associated disorders. Recent studies indicate that maintenance of mitochondrial function is beneficial in the prevention or delay of age-associated diseases. A central molecule seems to be the peroxisome proliferator-activated receptor γ coactivator α (PGC-1α), which is the key regulator of mitochondrial biogenesis. Besides regulating mitochondrial function, PGC-1α targets several other cellular processes and thereby influences cell fate on multiple levels. This paper discusses how mitochondrial function and PGC-1α are affected in age-associated diseases and how modulation of PGC-1α might offer a therapeutic potential for age-related pathology.

1. Introduction

In the last 20 years, mitochondrial dysfunction has been recognized as an important contributor to an array of human pathologies [1–3]. Mitochondrial dysfunction is particularly associated with the onset and progression of many age-related disorders such as neurodegenerative and cardiovascular diseases as well as metabolic disorders and age-related muscle wasting. In most cases it is not clear if the mitochondrial dysfunction is causative of the disease or if it is a secondary effect of the disease. Also, it is not understood if mitochondrial dysfunction is an aggravating factor in disease progression. Recent work suggests that maintenance of mitochondrial function is beneficial in at least some age-related diseases [4]. The peroxisome proliferator-activated receptor (PPAR) γ coactivator α (PGC-1α) integrates regulation of mitochondrial function into the modulation of different, tissue-specific metabolic pathways and thereby links mitochondrial function to important cellular signaling pathways that ultimately control cell survival [5, 6]. The following review discusses how mitochondrial dysfunction is associated with age-related diseases and what impact PGC-1α and its targets have in these diseases and their prevention.

2. Mitochondrial Function, ROS, and Aging

2.1. Mitochondrial Function and OXPHOS. Mitochondria play a central role in the cell metabolism: besides being key player in apoptosis, mitochondria house major cellular metabolic pathways. The fatty acid oxidation and citric acid cycle convert nutrients absorbed from ingested food to electron donors to NADH and FADH. These redox equivalents are fed into the oxidative phosphorylation system (OXPHOS), which supplies the majority of the cellular ATP supply. Here electrons are transferred from the substrates NADH and FADH via OXPHOS complex I-IV to the terminal electron acceptor oxygen. During this process, protons are transferred from across the inner membrane generating a proton gradient. This gradient is the driving force for complex V, the ATP-Synthase, to synthesize ATP [7].

2.2. Mitochondrial ROS Production and Mitochondrial Theory of Aging. Since OXPHOS complexes I-IV transfer electrons and consume most of the cellular oxygen, it is assumed that OXPHOS is the main cellular producer of reactive oxygen species (ROS) [8]. Leakage of electrons from the electron transfer chain can reduce oxygen to form the superoxide

anion radical. Superoxide production precedes reactions that form more reactive and potentially more dangerous ROS such as hydroxyl radical and hydrogen peroxide [9]. The superoxide anion can also oxidize cellular sulphite and nitric oxide resulting in further ROS [9].

The cells and in particular mitochondria have an antioxidant program to remove ROS. Superoxide dismutases (SODs) convert superoxide into hydrogen peroxide, which in turn is transformed into water by catalase or by peroxidases such as glutathione peroxidase (GPX). Additionally, several small molecules have ROS scavenging activity such as flavonoids, glutathione, and ascorbate [10].

Under physiological conditions, ROS production is estimated to be ~0.2% to 5% of the consumed oxygen [11]. The mitochondrial theory of aging states that since mitochondria are the major site of ROS production in the cell, the organelle is the prime target for oxidative damage leading to oxidized damaged lipids, proteins and nucleic acids resulting in dysfunctional mitochondria [12]. A vicious cycle is thought to occur, as oxidative stress leads to mitochondrial (mt) DNA mutations, which in turn can result in enzymatic abnormalities and further oxidative stress. While links between aging and oxidative stress are not new and were proposed over 50 years ago, there is much debate over whether mitochondrial changes are causes of aging or merely characteristics of aging. The relationship between ROS-induced damage, mitochondrial function and aging remains still unclear and the contribution of ROS in the aging process is poorly understood.

Dysfunctional mitochondria do not necessarily produce more ROS. There are in fact many examples of mouse model with dysfunctional OXPHOS that only have minor or no oxidative stress [13–15]. One notable study in mice with depleted proofreading function of the mitochondrial DNA polymerase γ (POLG) demonstrated shortened lifespan but no increase in reactive oxygen species despite increasing mtDNA mutations, suggesting that mtDNA mutations can cause lifespan shortening by other mechanisms [14]. However, it should be noted that this particular mouse models acquires a mtDNA mutation load that is much higher than observed in aged individuals. Although the POLG mice develop age-like symptoms, the questions remain, how "normal" aging is driven and what role ROS plays in the "normal" aging process.

Humans and model organisms alike accumulate oxidative damage to lipids, proteins and nucleic acids during aging supporting the mitochondrial theory of aging [16]. However, animal models with decreased antioxidant defense have increased oxidative stress, but with a normal lifespan and reproduction rate [17, 18]. Data from mice overexpressing antioxidant enzymes are conflicting: mice overexpressing superoxide dismutases have decreased ROS production, but fail to get an extended lifespan [19, 20]. In contrast, mice with mitochondrially targeted catalase (mCAT) have extended lifespan and seem to have a decreased susceptibility towards age-associated pathologies such cancer and cardiomyopathy associated with decreased oxidative damage [21–24].

The effect of ROS on lifespan regulation might be tissue specific. ROS seems to play a role in stem cell aging. SOD2 deficient hematopoietic stem cells have impaired capacity to maintain red blood cell homeostasis and an increase in ROS levels has been associated with impaired stem cell function [25]. Mitochondrial dysfunction associated with oxidative damage is suggested to play a central role in the aging process of cochlear cells and thus play an important role in age-related hearing loss [26]. Several studies have shown that ROS are generated in cochlear exposed to high-intensity noise and that cochlear hair cell loss is enhanced in mice lacking SOD1 [27], whereas mCAT mice have reduced cochlear cell damage in mice suggesting that mitochondrial ROS may play a role in age-related hearing loss [28]. In the murine aging heart, over-expression of mCAT attenuated age-related changes including decline of diastolic function, myocardial performance as well as ventricular fibrosis [22, 23]. These findings suggest that mitochondrial ROS and/or the mitochondrial antioxidant defense together with the protein degradation and protein synthesis machinery to remove and replenish oxidized protein partially might be involved in the development of the phenotype.

While increased ROS and antioxidant defense aggravate phenotypes in mouse model of several degenerative diseases such as ALS and Alzheimer's [29–32], it is still under debate what happens during "normal" aging [33]. Short-term ROS production is apparently important in prevention of aging by induction of a process named mitohormesis and redox signaling [34]. This process seems to be particulary important for the insulin sensitizing effect of exercise [35]. Recent evidence suggests that suppression of ROS production fails to extend lifespan in worms and may even decrease lifespan in humans, presumably due to the reduction of the ROS signaling, which seems to be important for different cellular processes [36, 37]. ROS is also an important signal for induction of autophagy: starvation-induced autophagy can be suppressed by antioxidants suppressing the well-known prosurvival function of starvation-induced autophagy [38]. ROS is also involved in the regulation of the insulin/IGF-1 pathway [39].

Another factor that is discussed for playing a role in mitochondrial ROS production is the 66 kDA isoform of the growth factor adaptor shc ($p66^{shc}$). $p66^{shc}$ is activated by stress and generates ROS within mitochondria and seems to be also required for cytochrome *c* release and opening of the permeability transition pore, which is crucial for apoptosis [40]. It remains to be clarified, what exact role $p66^{shc}$ plays in the aging process and how it is connected to other aging-relevant pathways.

In conclusion, the exact relationship between mitochondria, oxidative stress, and aging has not yet been settled. An import aspect to consider is that oxidative damage is the sum of actual ROS production, capacity of the cellular antioxidant defense and last the clearance of damaged molecules by repair or protein degradation. Any of these factors might contribute to increased oxidative damage, so that, for example, under normal ROS production, defective clearance of damaged molecules results in increased oxidative damage. Hence oxidative damage has to be carefully assessed in the context of ROS production, antioxidant response and damage control.

3. The Peroxisome Proliferator-Activated Receptor (PPAR) γ Coactivator α (PGC-1α) and Mitochondrial Biogenesis

Mitochondria derive from dual genetic origin, the nuclear and mtDNA, so that biosynthesis from both genomes has to be coordinated. Mammalian mitochondria have been estimated to have up to ~1500 proteins. The vast majority of these proteins including structural genes and assembly factors for mitochondrial proteins are encoded in the nuclear DNA, are synthesized in the cytoplasm and are imported into mitochondria. mtDNA encodes only for 13 subunits of the OXPHOS enzymes CI, III, IV, and V as well as 2 rRNAs and 22 tRNAs. Expression of mtDNA-encoded proteins and RNA species is governed by the mitochondrial transcription and translation machinery, whose protein factors are encoded in the nuclear DNA [41].

It is now apparent that a relatively small number of nuclear factors serve to coordinate the transcriptional expression of nuclear and mitochondrial respiratory proteins. Among these are the nuclear respiratory factors NRF-1 and NRF-2 (GA binding protein, GABP), which are implicated in the expression of mitochondrial genes (Figure 1). In addition to the NRFs, stimulatory protein 1 (Sp1), estrogen related receptor α (ERRα), and yin yang 1 transcription factor (YY1) have also been linked to many genes required for respiratory chain expression and function. These factors are controlled by a common key component, namely, peroxisome proliferator-activated receptor (PPAR) γ coactivator α (PGC-1α) [42]. PGC-1α is a transcriptional coactivator and interacts with nuclear receptors and transcription factors to activate transcription of their target genes [43]. PGC-1α activity is responsive to multiple stimuli including but not limited to nutrient availability, calcium, ROS, insulin, thyroid and estrogen hormone, hypoxia, ATP demand, and cytokines [43].

Besides PGC-1α, other members of the PGC-1 family of coactivators, namely PGC-1β and PGC-related coactivator (PRC), are also implicated in modulating mitochondrial function, but their exact role is not understood [42]. Also, it is likely that other, yet unidentified factors, are involved in orchestrating mitochondrial biogenesis.

PGC-1α is the first responder to stimuli and interacts with transcription factors such as NRF1, which is an intermediate transcription factor which stimulates the synthesis of TFAM (Figure 1). TFAM is crucial for mtDNA transcription and in addition plays an important role in mtDNA maintenance and mtDNA nucleoid formation [44].

PGC-1α activity is regulated on both the expression and posttranslational level (Figure 1): expression is mainly regulated by the peroxisome proliferator-activated receptors (PPAR) and other tissue-specific factors such as cAMP responsive element (CREB) in skeletal muscle. PPARs respond to external stimuli and metabolic demands and by activating PGC-1α, they link this changes to mitochondrial biogenesis [41]. Activation of PPARs by pharmacological agonists used in treatment for metabolic syndrome successfully induced mitochondrial biogenesis [45–47]. Recently, it has been found that PGC-1α expression is decreased by methylation of the promoter by DNA methyltransferase 3b (DNMT3B) [48]. This kind of regulation leads to long-lasting changes in PGC-1α transcription and might be potentially relevant in several pathophysiologies. PGC-1α regulates its own transcription via YY1. YY1 is a common target of mammalian target of rapamycin (mTOR) and PGC-1α. mTOR directly modulates the physical interaction of PGC-1α with YY1 and thereby modulates mitochondrial activity. Decrease in mTOR activity likely inhibits YY1-PGC-1α function resulting in decreased expression of mitochondrial genes [42, 43].

Very little is known about negative regulators of PGC-1α. So far, only RIP140 and 160MYP have been identified. Both molecules suppress mitochondrial biogenesis [49, 50].

PGC-1α activity can also be modulated by posttranslational modifications. AMPK, Akt and p38 MAPK target PGC-1α phosphorylation sites. Important key players in this respect are the AMP-activated kinase (AMPK) and the sirtuin Sirt1 [51]. AMPK is involved in the adaptive response to energy deficit. Direct phosphorylation by AMPK not only activates PGC-1α, but also promotes PGC-1α-dependent induction at the PGC-1α promoter [51]. Activity of PGC-1α is also regulated through inhibitory acetylation by GCN5 and stimulatory deacetylation through Sirt1 [51]. Sirt1 is a member of the Sirtuin family that has been implicated in longevity in yeast, worms and flies [4]. Activation of Sirt1 through caloric restriction induces PGC-1α activity and enhances mitochondrial function [52, 53]. While it is observed that resveratrol indirectly activates PGC-1α and induces mitochondrial biogenesis [52, 53] it is under dispute whether this indirect mechanism involves Sirt1 or might function indirectly through AMPK [54, 55].

Since AMPK senses AMP/ATP ratios and Sirt1 is NAD+ dependent, both AMPK and Sirt1 modulate PGC-1α activity in response to cellular energy supply. Insulin reduces PGC-1α expression, but also induces phosphorylation of PGC-1α through Akt and thereby inhibits its activity [56, 57]. The p38 mitogen-activated protein kinase (p38 MAPK) phosphorylates and activates PGC-1α [58]. This phosphorylation enhances PGC-1α half-life, disrupts interaction with the corepressor p160MBP in myoblasts and thereby enhances PGC-1α cotranscriptional activity [49]. PGC-1α is also phosphorylated by glycogen synthase kinase 3β (GSK3β) and thereby inhibited under oxidative stress [59]. However, Sirt1 is activated under the same conditions and activates PGC-1α by deacetylation. It is so far unclear how Sirt1 and GSK3b act in concert to modulate PGC-1α activity.

Recent work also identifies SUMOylation, ubiquitination as well as *O*-linked β-N-acetylglucosamination and methylation suggesting that PGC-1α activity can be fine-tuned depending on cellular needs by various ways [60, 61].

4. Mitochondrial Function and PGC-1α in Age-Related Pathologies of Muscle, Heart, Liver, and Brain

Aging is most likely a multifactorial process. Recent findings suggest a causal role of mitochondrial dysfunction in the

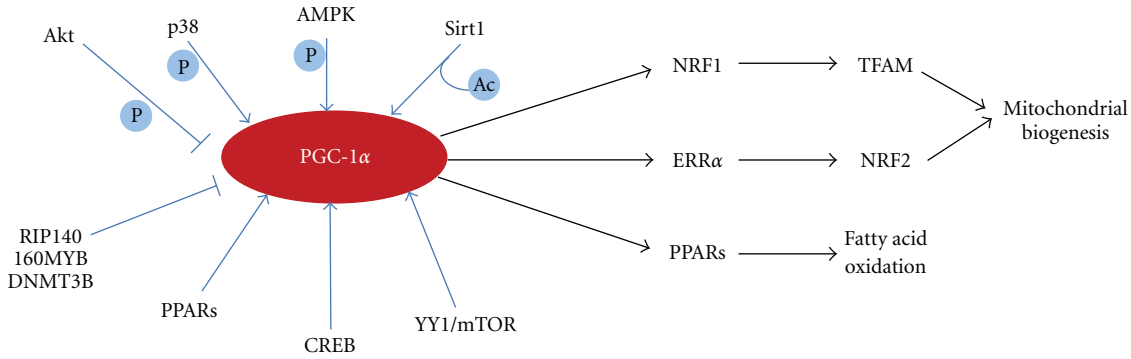

Figure 1: Modulation of PGC-1α and its targets. Regulation of PGC-1α activity on transcriptional and posttranscriptional levels as well as by interaction with inhibitory factors is summarized. The diagram shows the PGC-1α targets that are involved in metabolic regulation of mitochondria. The details are discussed in the text.

aging process and a central role for mitochondrial adaptation in the mechanism of aging retardation by caloric restriction and exercise. Mitochondrial disorders often present as neurological disorders, but can manifest as myopathy, diabetes, multiple endocrinopathy, or a variety of other systemic manifestations [62]. During aging, the decline of mitochondrial function often correlated with the onset and progression of similar pathologies [63, 64].

Many other factors have been discussed to play major roles in the aging process including mTOR, Sirt1 and insulin/IGF signaling as well as stem cell aging [65]. Sirt1 is clearly connected to PGC-1α function. Also, mTOR and PGC-1α pathways are linked through YY1 (see above). This regulation would allow the cell to connect nutrient pathways to activate mitochondrial function and ensure energy supply for cellular activities. Very recently, also a connection between telomere dysfunction, an additional player in causing cellular senescence and age-related pathology, and the PGC-family of coactivators was established [66].

Mitochondria are the most damaged organelle during aging. Hence removal and synthesis are necessary for proper energy homeostasis. An increase in mitochondrial turnover might be beneficial for cells resulting in better maintenance of the organelle. PGC-1α, poised centrally in multiple pathways affecting mitochondrial dysfunction and cellular function should play a key role in this prevention. Age-associated reductions in PGC-1α itself as well as in modulating proteins such as AMPK activity may be an important contributing factor in the reduced mitochondrial function associated with aging [67, 68].

The role of mitochondrial and PGC-1α likely affects different tissue. The following paragraph focuses on the effect in skeletal muscle, heart, liver, and brain, since those are the tissue, where PGC-1α function is best understood (Figure 2).

4.1. Skeletal Muscle and Sarcopenia. PGC-1α seems to be mediator of many known beneficial effects of exercise on muscle physiology. In skeletal muscle, PGC-1α expression is linked to muscle contraction [69]. A major mediator is the activation of Ca2+/calmodulin-dependent protein kinase IV (CamKIV) and calcineurin A, which are activated through the changes in calcium within the muscle in response to exercise. The heightened calcium signaling activates several important transcription factors such as CREB, which is a target of CamKIV, and myocyte enhancer factors 2 (MEF) [70]. Another factor that regulates PGC-1α expression upon exercise involves p38 MAPK, which activates MEF2 and transcription factor 2 (ATF2). p38 MAPK in conjunction with ATF2 results in increased expression of PGC-1α [71]. p38 MAPK also stimulates PGC-1α by phosphorylation in response to cytokine stimulation in muscle cells [72]. Finally, also AMPK as an ATP gauge is activated by exercise and enhancing PGC-1a transcription as well as activity (see above). The changed transcription program upon exercise induces changes in muscle plasticity such as a fiber-type switching towards more oxidative fibers and induces the mitochondrial antioxidant program [70, 73, 74].

PGC-1α is also involved in regulation of muscle function and integrity: PGC-1α regulates the neuromuscular junction program by being recruited to GABP-complex to stimulate a broad neuromuscular junction gene program [75]. In addition, PGC-1α inhibits FoxO3 activity on transcription of atrophy-specific genes and thereby decreases muscle atrophy [76]. Transgenic PGC-1α mice show smaller decrease in muscle fiber diameter and smaller induction of atrogenes in denervation-induced muscle atrophy and aging muscle by suppressing FoxO3 action [68, 76].

Additionally, increased muscular PGC-1α seems to be involved in the regulation of apoptosis and protein degradation during aging [68]. Loss of function studies in PGC-1α knockout animals additional suggest that PGC-1α modulates local or systemic inflammation and might regulate the expression of inflammatory cytokines and inflammatory markers such as TNFα and IL6 [77, 78], but the exact mechanism that links PGC-1α and the inflammatory response is not known. PGC-1α additionally controls angiogenesis in muscle by controlling VEGF expression and thus improves delivery of oxygen and substrates to muscle tissue [79].

In the aging muscle, zones of metabolically inactive tissue have been observed due to expansion of mitochondria that become damaged during aging [80]. This mitochondrial

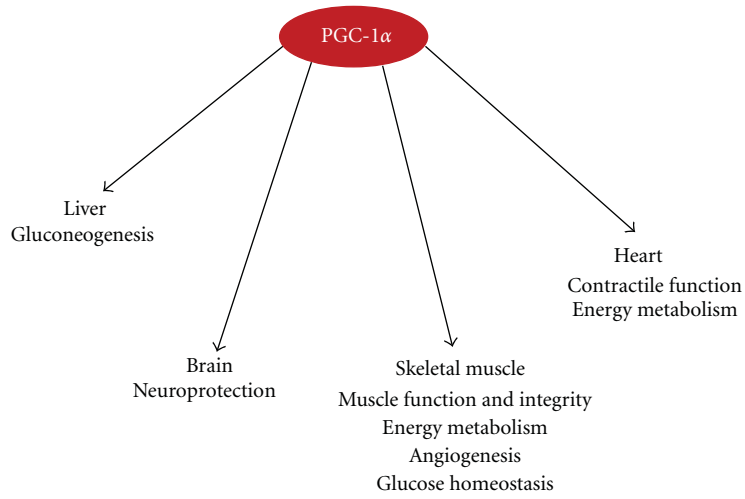

Figure 2: Tissue-specific function of PGC-1α relevant to age-related pathologies. Different functions of PGC-1α in heart, liver, brain, skeletal muscle and heart are depicted. These functions might be beneficial in age-associated pathologies as described in detail in the text.

dysfunction has been implicated in the development of sarcopenia, the age-related muscle loss [81].

Several studies have shown that elevated PGC-1α levels and maintenance of mitochondrial function in muscle prevent muscle wasting in muscular disorder such as mitochondrial myopathy [46], denervation-induced muscle atrophy [76] and Duchenne muscular dystrophy [75]. Elevated PGC-1α levels also have a therapeutic effect on the onset and progression of age-related loss of muscle mass (sarcopenia) [68]: here, transgenic muscle-specific expression of PGC-1α significantly reduced the loss of muscle mass and maintained exercise capacity during the aging process. The elevated PGC-1α levels in the aging muscle increased mitochondrial content and thereby maintained ATP supply. Additionally, transgenic PGC-1α mice showed decreased markers for apoptosis and proteolysis as well as a balanced autophagy, which most likely resulted in the decreased muscle atrophy. This maintenance of muscle mass in transgenic PGC-1α mice was associated with a "younger" neuromuscular junction phenotype and decreased fibrosis, which most likely also contributed to an improved muscle function. The prevention of sarcopenia in mice with elevated PGC-1α and maintenance of muscle as a metabolic tissue resulted in improved insulin sensitivity and prevented hypoglycemia during aging. Additionally, muscle-specific PGC-1α expression also ameliorated other pathological factors observed during aging on a systemic level: elevated muscle PGC-1α levels decreased gain of fat mass and osteoporosis in mice. Additionally, the level of circulating inflammatory markers usually observed during aging and in part be caused by the muscle atrophy were markedly reduced in transgenic PGC-1α animals [68].

While the precise mechanism of the observed protective effects is not entirely clear, the following possibilities could explain the effect of PGC-1α: the regulation of mitochondrial mass might help to prevent the energy crisis associated with many muscular diseases [46, 68]. PGC-1α also reduces the transcription of atrophy-specific genes by inhibiting FoxO3 [76]. Additionally, *de novo* protein synthesis is activated and the neuromuscular junction is stabilized [75]. Apoptosis and protein degradation, which are hallmarks of muscle wasting, are reduced [68]. These effects likely contribute to the antimuscle wasting properties of PGC-1α. Maintenance of the metabolic properties of the muscle tissue as well as prevention of the muscle atrophy most likely resulted in the observed systemic effects underlining the importance of muscle function and integrity for whole-body function.

4.2. Heart and Age-Related Cardiovascular Disorders. In the heart, PGC-1α strongly induces mitochondrial function and fatty acid oxidation [82]. Normal growth and response to exercise are controlled by PGC-1α similar to skeletal muscle [83, 84]. Absence of PGC-1α in the heart reduces the cardiac reserve under stress conditions and diminished the cardiac capacity under exercise conditions [85, 86]. In the failing heart, as what occurs in heart diseases and during aging, metabolism is switched from fatty acid to glucose utilization and expression of PGC-1α is reduced [87]. In contrast to skeletal muscle, muscle elevated PGC-1α seems to have an adverse effect in heart: elevated increased expression of PGC-1α in the heart causes cardiomyopathy and heart failure in mice [88]. Also, transient activation of PGC-1α diminishes cardiac contractile recovery after ischemia-reperfusion injury [89]. These findings suggest that PGC-1α levels in heart need to be tightly regulated to prevent pathology. The adverse effect of PGC-1α might be attributed to tissue-specific differences in the availability of transcription factor partners for PGC-1α, differences in cell signaling or other heart-specific metabolic requirements.

Despite these effects of elevated PGC-1α in the heart, PGC-1α may nevertheless affect cardiac function. Sirt1 and PPARα, two proteins that regulate PGC-1α expression and activity, are major players in protecting the heart from typical age-related pathologies such as hypertrophy, metabolic dysregulation and inflammation [53]. These effects could be also

observed by administration of resveratrol, which is also an indirect activator of PGC-1α implying that a PGC-1α might be involved in the cardioprotective effect.

A major factor contributing to the development of heart disorders during aging is the failing vasculature. PGC-1α seems to have an important role in the vasculature wall itself [90]. Endothelial dysfunction is an early feature of cardiovascular disease and is associated with increased levels of ROS. The antioxidant property of PGC-1α might hence be beneficial to maintain vasculature function and thus contribute to the prevention of cardiovascular diseases. Indeed, activation of PGC-1α in endothelial cells prevents oxidative damage and cellular apoptosis and prevents endothelial dysfunction in vivo [90]. It remains to be seen what effect endothelial PGC-1α has on angiogenesis and atherosclerosis, two major contributing factors of cardiovascular disease.

4.3. Brain and Age-Related Neurodegenerative Diseases. PGC-1α is expressed in all brain tissues and plays an important role in normal brain function and a major role in the oxidative stress response [91]. In mice, PGC-1α deficiency causes behavioral changes including anxiety and hyperactivity as well as hind limb clasping. These behavioral changes are associated with spongiform-like vacuolization primarily in the striatum associated with gliosis and leads to reduced expression of several brain-specific genes that are all associated with normal brain function. Substantia nigra and CA1 neurons are more susceptible to neurodegeneration in response to neurotoxins suggesting an important role of PGC-1α in neuronal maintenance [92]. PGC-1α also seems to be involved in the control of neurite growth and neuronal synaptic function [93].

While mitochondrial dysfunction affects the whole organisms during aging its effects might be especially deleterious at the level of the CNS [94]. PGC-1α might potentially relieve this defect and together with the above described brain-specific function influence age-associated neurodegeneration. In fact, PGC-1α has been implicated in the onset and progression of neurodegenerative diseases. Postmortem brain samples of patients with Huntington's disease (HD) had a decreased level of PGC-1α mRNA [95, 96]. Polymorphism is also associated with the onset of AD [97]. PGC-1α is repressed by a mutant form of the Huntington protein which leads to mitochondrial dysfunction and neurodegeneration. Over-expression of PGC-1α rescues cells from the deleterious effect of Huntington's, whereas loss of PGC-1α in HD mice aggravated neurodegeneration [95]. Moreover, PGC-1α KO mice show Huntington's like phenotype and neuronal lesions suggesting that PGC-1α is crucial for maintenance of striatal function. Additionally, PGC-1α SNPs are associated with the age of onset of HD. In a PD mouse model, PGC-1α deficiency caused an increased degeneration of dopaminergic neurons in the substantia nigra associated with oxidative damage [91].

Interestingly, activators of PGC-1α such as resveratrol have a neuroprotective effect in acute and chronic brain injury as well as in neurodegenerative diseases suggesting a role for PGC1-α in modulating the outcome of the disease [98].

4.4. Liver and Metabolic Disorders. In liver, PGC-1α is induced by fasting in response to glucagon and regulates most of the metabolic changes that occur during the transition from fed to fasted state [99]. The most relevant metabolic pathways in this regard are gluconeogenesis, fatty-acid-beta oxidation, ketogenesis, and heme biosynthesis [5]. Absence of PGC-1α results in a blunted hepatic fasting response as well as fasting hypoglycemia and hepatic steatosis [86]. PGC-1α associates in liver with several transcription factors such as HNF4-α and FoxO1 and thereby induces the expression of several gluconeogenic enzymes [100, 101]. Glucagon induces cAMP and CREB as well as p38 MAPK over cAMP and PKA [102]. p38 MAPK increases PGC-1α transcription as in muscle and seems to be also necessary for the expression of PGC-1α in response to free fatty acids to stimulate gluconeogenesis [103].

There has also been considerable interest in mitochondrial dysfunction as a contributing factor in the development of metabolic disorders. Although the involvement of mitochondrial dysfunction in insulin resistance is under dispute [104–106], several lines of evidence suggest that decreased mitochondrial function may be the underlying defect that causes insulin resistance during aging: the age-associated decline in mitochondrial function in elderly might contribute to the age-related insulin resistance [68, 107]. Increase in mitochondrial function during aging increases fuel handling, fatty acid oxidation and protects from insulin resistance [52, 68, 108, 109]. Interestingly, PGC-1α promoter methylation and hence decreased PGC-1α expression in skeletal muscle was found to be more prevalent in patients with diabetes compared to healthy subjects [48]. In addition, mitochondrial functional insufficiency and decreased PGC-1α levels have been found in the insulin-resistant offspring of patients with T2D. The fact that this occurs in healthy individuals that are not diabetic suggests that an inherent defect in oxidative phosphorylation may be a contributing factor [110]. Severeness of steatosis is associated with impaired PGC-1α expression and reduced mitochondrial gene expression [111]. Interestingly, rosiglitazone attenuates age-associated liver pathology in nonalcoholic steatohepatitis [112]. Rosiglitazone is indirectly activating PGC-1α via PPAR, implying that PGC-1α activating is beneficial in liver pathologies.

4.5. Role of PGC-1α Responsive Proteins in Age-Related Pathologies. Also downstream targets of PGC-1α may play a role in lifespan regulation and maintenance of tissue function. Over-expression of TFAM, for example, can reverse age-dependent memory impairment in mice, presumably through the prevention of mitochondrial dysfunction in microglia [113]. Over-expression of TFAM also protects against beta-amyloid-induced oxidative damage [114] and in addition seems to be also a target for therapeutic strategies in cardiac failure [115].

Both NRF1 and NRF2 have a broad spectrum of target genes besides mitochondrial genes. A screen for NRF1 binding sites revealed significant overlap to E2F, a transcription factor family which is involved in the regulation of cell growth. NRF1 is also involved in the regulation of the

expression fragile X mental retardation-1 gene and NRF1 promoter binding is increased in the mouse brain during aging which might be linked to the age-related deficiency in learning, memory, and cognition [116]. NRF2 is implicated in the regulation of the cell cycle, myeloid genes, T-cell development and other target genes [117, 118]. In muscle, NRF2 regulates the transcription of the nicotinic acetylcholine receptor and utrophin genes and plays a crucial role in formation and maintenance of the neuromuscular junction [119]. In conjunction with PGC-1α, NRF2 (GABP) regulates the NMJ program [75] (see above).

A novel identified target of PGC-1α is the mitochondrial-localized Sirtuin 3 (Sirt3) [120]. Sirt3 is a major player in mitochondrial biogenesis, regulation of metabolic enzymes and ROS suppression [121]. Recent findings show that Sirt3 also targets the mitochondrial permeability transition pore (mPTP) and prevents mPTP opening by modulating cyclophilin D [122]. mPTP opening plays a fundamental role in myocardial cell death and the development of heart disease [123].

5. Mitochondria and PGC-1α in Antiaging Strategies

There is an abundance of studies that provide indirect and direct evidence in support of a role for PGC-1α and regulation of mitochondrial function in antiaging strategies. As outline above, therapeutic modulation of PGC-1α has huge potential for treatment of patients with various mitochondrial dysfunction associated with disease and age-related disorders. In the following, antiaging strategies targeting mitochondria and PGC-1α in controlling age-related pathology are discussed.

5.1. Caloric Restriction. CR has been shown to induce longevity in many different organisms and is a common treatment for sarcopenia and insulin resistance [124–128]. Recent findings suggest a central role for mitochondrial adaptation in the mechanism of aging retardation by CR [4]. The decline in oxidative capacity in skeletal muscle during aging is prevented in CR animals [63, 129]. The slower decline of PGC-1α gene expression during aging in CR animals suggests a better maintenance of mitochondrial biogenesis during aging [63].

CR has been shown to exert a positive effect on mitochondria, boosting mitochondrial activity and hence providing some of the salutary effects of CR [3, 4]. The effects of CR are thought to be mediated by the regulatory family of sirtuins, mainly SIRT1, an NAD-dependent protein deacetylase [4]. Sirt1 has many proven targets involved in protein homeostasis and metabolisms [130]. Among them is PGC-1α [131]. Additionally, Sirt1 activates the endothelial nitric oxide synthase eNOS, which stimulates mitochondrial activity [132]. The resulting increase in mitochondrial activity is thought to be related to longevity effect of CR and Sirt1 upregulation. In caloric-restricted mice, Sirt1 activity is increased and protects form cancer, neurodegeneration, inflammatory disorders, metabolic, and cardiovascular disease [133]. Intriguingly, enforced Sirt1 activity seems to result in a CR-like physiology and protection from degenerative diseases [133, 134]. Recent evidence suggests that Sirt1 is also involved in mediating the response to dietary restriction and increasing health span in humans [64].

5.2. Exercise. Exercise is an excellent therapeutic intervention for conditions such as obesity, T2D, neurodegeneration, osteoporosis and sarcopenia [135, 136]. PGC-1α activation seems to be the mechanism that mediates those beneficial effects and increased PGC-1α levels promote an exercised phenotype [74, 137, 138]. Exercise also causes a reduction in the levels of systemic inflammation after exercise, presumably through the same mechanisms [139]. Exercise also activates the AMP-activated kinase (AMPK) [140]. AMPK is considered to be a key metabolic sensor that increases translocation of Glut4 to the plasma membrane [141]. AMPK also activates PGC-1α and thereby mitochondrial biogenesis in an attempt to compensate for the ATP depletion [137, 142]. This mechanism is thought to be the molecular basis for the therapeutic effect of exercise, which stimulates AMPK. Activation of PGC-1α in skeletal muscle has thereby the potential to compensate for mitochondrial dysfunction and affect other pathways and thus prevent insulin resistance as shown in an animal model [68, 74, 143].

5.3. Pharmacological Approaches. In addition to exercise, PGC-1α and hence mitochondrial biogenesis can be activated by a wide array of pharmacological substances. PGC-1α activity is controlled by the PPARs, AMPK and Sirt1 (Figure 2). Thus, drugs that activate PPARs, AMPK and Sirt1 could potentially result in PGC-1α activation. Such pharmaceutical activators include fibrates and rosiglitazone (PPAR), metformin and AICAR (AMPK) as well as resveratrol (Sirt1). Induction of mitochondrial biogenesis and/or PGC-1α activation has been demonstrated for most of these substances both *in vitro* and *in vivo* [45–47, 52, 141, 144]. Interestingly, bezafibrate administration to a mouse model of mitochondrial myopathy mimicked the effects of transgenic PGC-1α expression [46]. In mice, resveratrol prevents the decreased lifespan associated with obesity [52]. It remains to be seen what how pharmaceutical stimulated PGC-1α activation affects aging and lifespan.

6. Conclusion

Mitochondria have been implicated in the aging process and the onset and progression of age-associated diseases since decades. While the impact of mitochondrial ROS is in question, failing ATP supply due to increasing mitochondrial dysfunction seems to be a major contributing factor to the aging process. Modulating mitochondrial function and affecting several tissue-specific pathways by PGC-1α have been shown to have a beneficial effect. Notably, existing antiaging strategies as well as studies in mice suggest an important role of mitochondrial function and the PGC-1α cascade in the preventing of age-associated diseases. In the same

line, control and maintenance of mitochondrial function by PGC-1α activation have a huge therapeutic potential for age-related pathologies such as insulin resistance, sarcopenia and neurodegeneration. Findings on elevated PGC-1α levels in the heart caution against the systemic effects of elevated PGC-1α levels. Beneficial effects seem to be tissue specific, and remaining within a therapeutic window will be important.

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Review Article

IP_3 Receptors, Mitochondria, and Ca^{2+} Signaling: Implications for Aging

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The tight interplay between endoplasmic-reticulum-(ER-) and mitochondria-mediated Ca^{2+} signaling is a key determinant of cellular health and cellular fate through the control of apoptosis and autophagy. Proteins that prevent or promote apoptosis and autophagy can affect intracellular Ca^{2+} dynamics and homeostasis through binding and modulation of the intracellular Ca^{2+}-release and Ca^{2+}-uptake mechanisms. During aging, oxidative stress becomes an additional factor that affects ER and mitochondrial function and thus their role in Ca^{2+} signaling. Importantly, mitochondrial dysfunction and sustained mitochondrial damage are likely to underlie part of the aging process. In this paper, we will discuss the different mechanisms that control intracellular Ca^{2+} signaling with respect to apoptosis and autophagy and review how these processes are affected during aging through accumulation of reactive oxygen species.

1. Intracellular Ca^{2+} Signaling

Intracellular Ca^{2+} signaling is important in the regulation of multiple cellular processes, including development, proliferation, secretion, gene activation, and cell death. The formation of these Ca^{2+} signals is dependent on many cellular Ca^{2+}-binding and Ca^{2+}-transporting proteins, present in the various cell compartments of which the endoplasmic reticulum (ER) forms the main intracellular Ca^{2+} store [1]. The resting cytosolic $[Ca^{2+}]$ remains very low (~100 nM), through active extrusion of Ca^{2+} by pumps in the plasma membrane or in intracellular organelles, like the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump in the ER. Due to SERCA activity and intraluminal Ca^{2+}-binding proteins, the ER can accumulate Ca^{2+} in more than thousandfold excess compared to the cytosol [1, 2]. In the ER lumen, Ca^{2+} functions as an important cofactor for ER chaperones, thereby aiding in the proper folding of newly synthesized proteins [3]. Reciprocally, the Ca^{2+}-binding chaperones affect the Ca^{2+} capacity of the ER by buffering Ca^{2+} [2]. In addition, two tetrameric ER Ca^{2+}-release channels exist that, upon stimulation, release Ca^{2+} into the cytosol, thereby provoking Ca^{2+} signaling: the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) and the ryanodine receptor (RyR). They are similar in function and structure but differ in regulation, conductance, and expression profile [4, 5]. The rise in cytosolic $[Ca^{2+}]$ following its release from the ER results in various Ca^{2+}-dependent intracellular events. The exact cellular outcome depends on the spatiotemporal characteristics of the generated Ca^{2+} signal [6]. Since close contact sites between the ER and the mitochondria, involving direct molecular links with the IP_3R, exist (Figure 1), it is clear that ER-originating Ca^{2+} signals critically affect the mitochondrial function.

During aging, ER Ca^{2+} homeostasis alters and becomes dysregulated [7]. Most observations support a decline in ER $[Ca^{2+}]$ and in ER Ca^{2+} release (due to lower activity of SERCA, IP_3R, and RyR), but contradictory findings have been published, possibly related to the cell type under investigation (Figure 2). In addition, ER Ca^{2+} release and

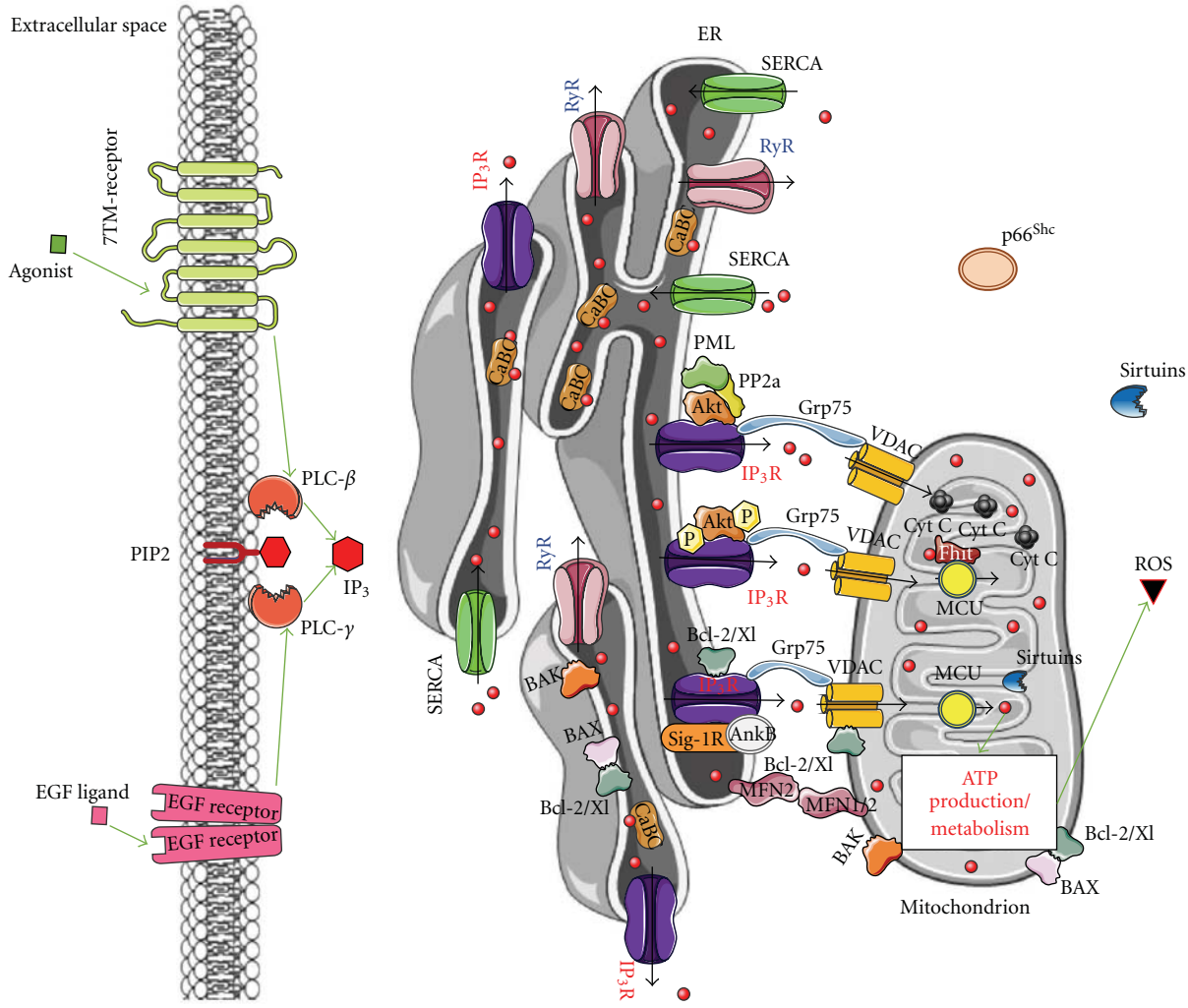

Figure 1: In a healthy cell, ER Ca^{2+}-handling components tightly regulate mitochondrial function and bioenergetics, representing the different key players involved in intracellular Ca^{2+} signalling with particular emphasis on the ER-mitochondria connections. The ER-Ca^{2+} content is regulated by channels and pumps (IP_3Rs, RyRs, SERCAs) and by Ca^{2+}-binding chaperones (CaBCs). IP_3 stimulates ER Ca^{2+} release and consequently the transfer of Ca^{2+} (red dots) from ER to mitochondria. Mitochondrial Ca^{2+}, transported via VDAC, is directly or indirectly involved in cellular energy metabolism and in the secondary production of reactive oxygen species (ROS). It is clear that IP_3R-mediated Ca^{2+} release ought to be tightly regulated to sustain mitochondrial activity and function. As a consequence, Ca^{2+}-flux properties of IP_3Rs are tightly and dynamically regulated by accessory proteins involved in cell death and survival, like Bcl-2, Bcl-Xl, PKB/Akt, Sigma-1 receptor (Sig-1R)/Ankyrin B (AnkB), and the recently identified PML. It is important to note that different regulatory mechanisms occur at the IP_3R, which may help cell survival (like Bcl-2, Bcl-Xl, PKB/Akt) or help to promote cell death (like PML). The latter is essential to prevent the survival of altered, damaged, or oncogenic cells. Thus, a tight balance between both outcomes is a requisite for cellular health and homeostasis, and a dynamic switch from prosurvival to prodeath is likely essential. In this paradigm, the production of ROS might contribute to the survival of cells by efficient detection of damaged/altered mitochondria and their removal by autophagy, while preventing excessive apoptosis. In addition, controlled apoptosis is likely to be important to eliminate cells, in which the removal of altered mitocondria by autophagy is not sufficient, thereby avoiding tumor genesis. In this process, the recently identified tumor suppressor PML may play a crucial role as it promotes IP_3R-mediated Ca^{2+} transfer from the ER into the mitochondria by dephosphorylating and suppressing PKB/Akt activity through PP2A. While PKB/Akt is known to suppress IP_3R-channel activity by phosphorylation of the IP_3R, the recruitment of PP2A via PML at the interorganellar ER/mitochondrial complex dephosphorylates and inactivates PKB/Akt. This suppresses PKB-dependent phosphorylation of IP_3R and thus promotes Ca^{2+} release through this channel and Ca^{2+} transfer into the mitochondria. At the mitochondrial level, the tumor suppressor Fhit has been shown to increase the affinity for the mitochondrial Ca^{2+} uniporter (MCU), thereby enhancing the uptake of mitochondrial Ca^{2+} at low and physiologically relevant levels of agonist-induced Ca^{2+} signals. Green arrows: stimulation; red lines: inhibition; black arrows: Ca^{2+} flux.

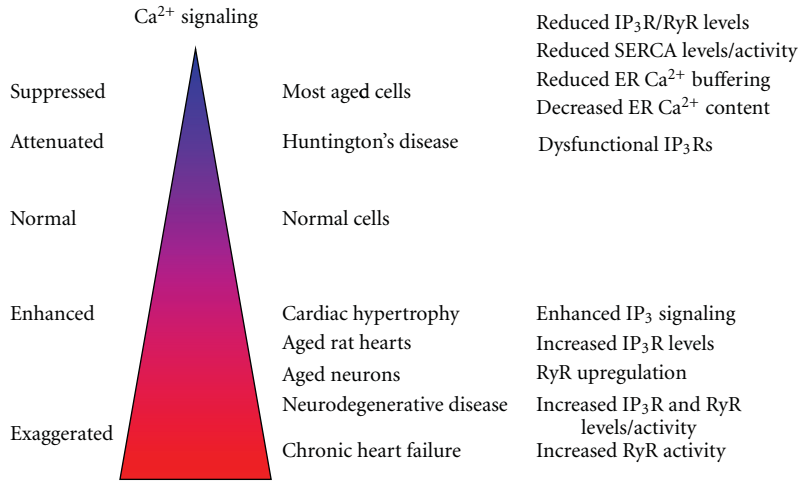

Figure 2: Altered Ca^{2+} signaling during aging and in age-related diseases. The Ca^{2+} dyshomeostasis during age is dependent on the cell type and the context. Most aged cells display decreased ER Ca^{2+} content and release, due to declined IP_3R or RyR levels, reduced SERCA activity, and decreased Ca^{2+} buffering by intraluminal Ca^{2+}-binding chaperones. However, in neurons and rat hearts, an enhanced Ca^{2+} signaling is found, caused by increasing IP_3R or RyR activity. Age-related diseases (neurodegeneration, cardiac hypertrophy, and chronic heart failure) are also characterized by enhanced Ca^{2+} signaling. However, this property may be disease dependent, since a mouse model for Huntington's disease displayed attenuated IP_3R1 activity due to impaired binding of Grp78 to IP_3R1. Hence, caution should be taken with general claims.

subsequent Ca^{2+} uptake by mitochondria regulate reactive oxygen species (ROS) production, autophagy, and cell death, processes implicated in aging.

In a previous review [8], we have focused on mechanisms regulating the Ca^{2+} content in the ER and its relevance for the development of physiological versus pathophysiological Ca^{2+} signalling. In the present review, we will focus on the subsequent step which is the mechanisms responsible for controlling Ca^{2+} transfer from the ER to the mitochondria. The Ca^{2+} level in the mitochondrial matrix plays an important role in the progression of apoptosis and autophagy [9, 10]. Here, we will especially analyze how the Ca^{2+} transfer to the mitochondria as well as apoptosis and autophagy are affected by the aging process in general and by reactive oxygen species in particular.

2. Mitochondrial Ca^{2+} Handling

In contrast with the role of the ER, the role of the mitochondria in physiological Ca^{2+} handling was underestimated or even ignored for a long time, but due to the seminal work of Rizzuto and his colleagues [11], this role is now generally accepted.

The electrochemical gradient ($\Delta\psi_m = -180\,\text{mV}$) between the inside and outside of energized mitochondria forms the driving force for the Ca^{2+} uptake in the mitochondrial matrix, which implies the transfer of Ca^{2+} ions over both the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM).

The Ca^{2+} ions taken up into the mitochondrial matrix stimulate the mitochondrial ATP production by regulating the activities of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and pyruvate dehydrogenase, three dehydrogenases of the Krebs cycle [12, 13]. Also other mitochondrial processes as fatty acid oxidation, amino acid catabolism, aspartate and glutamate carriers, the adenine-nucleotide translocase, Mn-superoxide dismutase, and F1-ATPase activity, are regulated by mitochondrial Ca^{2+} [12, 14, 15].

The ATP produced by the mitochondria is subsequently transferred to the cytoplasm; it will so especially regulate the activity of ATP-sensitive proteins localized in the close vicinity of the mitochondria. Two major proteins involved in Ca^{2+} transport, the SERCA, responsible for loading the ER, and the IP_3Rs, responsible for Ca^{2+} release from the ER, are stimulated by ATP. The bidirectional relation between Ca^{2+} release and ATP production allows for a positive feedback regulation between ER and mitochondria during increased energetic demand [16].

The uptake of Ca^{2+} in the mitochondria will also affect Ca^{2+} signaling. The local Ca^{2+} concentration near the mitochondria will depend on both the amount of Ca^{2+} released by the IP_3R and that taken up by the mitochondria. This will in turn depend on the efficiency of the coupling between both. Since both the SERCA pumps and the IP_3Rs are also regulated by Ca^{2+}, the local Ca^{2+} concentration in the vicinity of the mitochondria will determine the refilling of the ER and eventually the spatiotemporal characteristics of the subsequent Ca^{2+} signals. The way in which the Ca^{2+} signals are affected depends on the exact subcellular localization of the mitochondria, the production of ROS, the local Ca^{2+} concentration, the IP_3R isoform expressed, and may as well involve stimulation as inhibition of the signals [16–19]. Furthermore, the connection between mitochondria and the ER can be highly dynamic as the local Ca^{2+} concentration can also affect mitochondrial motility and ER-mitochondria associations in various ways [20].

3. Transport Proteins Involved in the Transfer of Ca^{2+} between ER and Mitochondria

3.1. IP_3Rs. The first key player is the IP_3R, the main Ca^{2+}-release channel in the ER of most cell types. The IP_3R consists of 4 subunits of about 310 kDa each (i.e., about 2700 a.a.). In mammals, three different IP_3R isoforms are expressed (IP_3R1, IP_3R2, and IP_3R3) while diversity is increased by splicing and the formation of both homo- and heteromeric channels [4, 21, 22]. All IP_3R isoforms are activated by IP_3, though with varying affinity [23]. Low Ca^{2+} concentrations stimulate but high Ca^{2+} concentrations inhibit the IP_3Rs [24–27]. Further modulation of the IP_3Rs is performed by ATP, phosphorylation, and protein-protein interactions [4, 28–30].

For efficient Ca^{2+} transfer between ER and mitochondria, it is important that IP_3Rs are localized very close to the mitochondrial Ca^{2+}-uptake sites. As different IP_3R isoforms exist, an important point is whether interaction with the mitochondria is isoform specific [31]. In CHO cells, IP_3R3 is the least expressed isoform, but it demonstrated the highest degree of colocalization with the mitochondria and consequently its silencing had the most profound effects on mitochondrial Ca^{2+} signals [32]. However, this does not represent a general rule as, for example, in astrocytes IP_3R2 was found to preferentially colocalize within the mitochondria [33]. These differences in intracellular localization of the IP_3R isoforms may be due to differences in relative expression levels of the various IP_3R isoforms and in subcellular localization among different cell types [34]. Moreover, the physiological setting [35] and the differentiation status [36] determine the subcellular localization of the various IP_3R isoforms in a given cell type.

3.2. Voltage-Dependent Anion Channels: The Main Ca^{2+}-Transport System across the OMM. The Ca^{2+} fluxes through the OMM are mainly determined by voltage-dependent anion channels (VDAC). Of the 3 existing VDAC isoforms, VDAC1 is the most abundant in most cell types [37]. It was demonstrated that the transient overexpression of VDAC in various cell types led to an increased Ca^{2+} concentration in the mitochondria, leading to a higher susceptibility for ceramide-induced cell death [38].

VDAC, however, allows also the transport of other ions and metabolites, including ATP. It has therefore multiple functions in the cell and is a central player in the crosstalk between the cytoplasm and mitochondria. In this manner, VDAC is also implicated in the induction of apoptosis by various stimuli [15].

The permeabilization of the OMM is a crucial step in apoptosis, but how this is exactly performed is not yet clear. Proteins belonging to the B-cell CLL/lymphoma-2 (Bcl-2)-protein family appear anyway to be necessary [39, 40]. Several Bcl-2-family members can affect the permeability of the OMM, for example, by binding to VDAC and regulating its properties or by forming multimeric channel complexes. Independently of the mechanism by which the increase in permeability of the OMM is achieved, it allows the release of the apoptogenic factors present in the intermembrane space to the cytoplasm and the progression of apoptosis [15, 40–42].

3.3. Ca^{2+}-Transport Systems across the IMM. In contrast to the Ca^{2+}-transport system across the OMM, that of the IMM is not yet well characterized. For a long time, the main IMM Ca^{2+}-transport system was named the mitochondrial Ca^{2+} uniporter. Additionally, a so-called rapid mode of mitochondrial Ca^{2+} uptake was described, but the nature of neither was known [43].

Three different highly Ca^{2+}-selective channels that may contribute to this process were meanwhile characterized, that is, MiCa [44], mCa1, and mCa2 [45]. Two of these channels, MiCa and mCa1, have properties compatible with the former uniporter and may represent species- and/or cell-type-dependent variability [43]. At the molecular level, the mitochondrial Ca^{2+}-uptake channels are not yet identified, but evidence for a role of a number of proteins has been presented [46, 47]. Recently, a Ca^{2+}-binding protein, named MICU1, which appears essential for mitochondrial Ca^{2+} uptake, was described [48]. It is, however, not known whether it actually forms (part of) a Ca^{2+} channel or functions as Ca^{2+} buffer or Ca^{2+} sensor. Interestingly, the tumor suppressor protein Fhit (fragile histidine triad) seems to promote mitochondrial Ca^{2+} uptake by increasing the affinity of the mitochondrial Ca^{2+} uniporter at the ER/mitochondrial microdomain [49].

Finally, the permeabilization transition pore (PTP) is another channel of still unknown nature [50]. It is voltage and Ca^{2+} dependent and is sensitive to cyclosporine A. It is not selective for Ca^{2+} as the open conformation of the PTP has a high conductance for all ions, including Ca^{2+}, and for molecules up to 1500 Da [51]. Its long-time activation leads to the demise of the cell, either by apoptosis or else by necrosis, depending on whether PTP opening occurs in only a small part of the mitochondria or in all of them, respectively [51, 52].

In addition, Ca^{2+}/Na^+ and Ca^{2+}/H^+ exchangers are also present in the IMM. Their main function is probably to export Ca^{2+} from the matrix, but they may also contribute to Ca^{2+} uptake under certain conditions [43].

4. Structural and Regulatory Proteins Involved in the Control of Ca^{2+} Transfer between ER and Mitochondria

Mitochondria-associated ER membranes (MAMs) were originally described as sites for lipid synthesis and lipid transfer between ER and mitochondria [53]. These MAMs are, however, also ideally suited for Ca^{2+} exchange [14]. Several proteins may participate in the stabilization of those MAMs and, through this stabilization, affect Ca^{2+} transfer between ER and mitochondria. Other proteins may be directly involved in regulating the Ca^{2+}-transport proteins described above.

4.1. Glucose-Regulated Protein 75. Glucose-regulated protein 75 (Grp75) belongs to the Hsp70 family of chaperones but is not inducible by heat shock [54, 55]. Importantly, it can couple the IP_3R to VDAC1 and allows for a better transfer of the Ca^{2+} ions from the ER to the mitochondrial matrix [56]. The increased Ca^{2+} signals in the mitochondria were not due to an increased ER-mitochondria contact area. These results indicate that Grp75 is probably not the main determinant for the ER-mitochondrial linkage but regulates the Ca^{2+} flux between ER and mitochondria by controlling the interaction between the IP_3R and VDAC1.

4.2. Sigma-1 Receptor. The ER chaperone proteins known as sigma receptors are targets for certain neurosteroids. Based on their biochemical and pharmacological properties, two subclasses, sigma-1 and sigma-2 receptors, are distinguished but only the sigma-1 receptor was cloned and properly characterized [57, 58]. The sigma-1 receptor is involved in many physiological functions as well as in several pathological conditions [58].

Sigma-1 receptors are especially enriched at the MAMs [59]. A specific interaction between the Ca^{2+}-binding chaperone BiP and the sigma-1 receptor was described [59]. This interaction depends on the ER Ca^{2+} concentration: a decrease in ER Ca^{2+} concentration leads to their dissociation, whereby both proteins become active chaperones.

The sigma-1 receptor regulates several ion channels, including the IP_3Rs [58]. Agonists of sigma-1 receptors could so potentiate agonist-induced Ca^{2+} release in NG108 cells [60]. Hereby, an interaction between the sigma-1 receptor, cytoskeletal ankyrin B, and IP_3R3 was demonstrated [61]. In CHO cells, the sigma-1 receptor also interacted with IP_3R3, but here ankyrin was not observed in the complex. Finally, a specific role was found for the sigma-1 receptor stabilizing the IP_3R3 present at the MAMs, and so regulating Ca^{2+} transfer between ER and mitochondria [59].

4.3. Mitofusins. Mitofusin 1 and 2 are two dynamin-related GTPases acting on mitochondria. Mitofusin 2 is enriched at MAMs. The absence of mitofusin 2 not only affected ER and mitochondrial morphology but also reduced the number of contact points between ER and mitochondria by about 40% [62]. Mitofusin 2 on the ER appeared necessary for connecting the two organelles by directly interacting with either mitofusin 1 or mitofusin 2 on the OMM. Moreover, the diminished interaction observed in the absence of mitofusin 2 affected Ca^{2+} transfer between the ER and the mitochondria. A too strong ER-mitochondria interaction may also be detrimental as overexpression of mitofusin 2 led to apoptosis [63].

4.4. Bcl-2-Family Members. Bcl-2 is the prototype of a large family containing both anti- and proapoptotic proteins. The antiapoptotic members of this family, including Bcl-2 itself, are characterized by the presence of 4 Bcl-2-homology (BH) domains (BH1 to 4). The proapoptotic members either have 3 BH domains (BH1, BH2, and BH3) as, for example, Bax and Bak, or only a single BH3 domain, as for example, Bim, Bid, and Bad (the so-called BH3-only proteins) [39].

The BH1, BH2, and BH3 domains of the antiapoptotic proteins, as Bcl-2 and Bcl-Xl, form together a hydrophobic cleft that can bind the amphipathic α-helical BH3 domain of proapoptotic proteins. In this manner, the antiapoptotic Bcl-2 family members antagonize apoptosis at the level of the mitochondria by binding and neutralizing proapoptotic Bax and Bak [39, 64]. In addition to this mitochondrial function, antiapoptotic Bcl-2 family members also act on the ER Ca^{2+} homeostasis [65, 66]. The exact mechanism is, however, not yet clarified, and effects on several Ca^{2+}-binding or Ca^{2+}-transporting proteins were described, including on the IP_3R [67–69].

Although there is an agreement that the antiapoptotic proteins as Bcl-2 bind to the IP_3R, there is among the various studies a discrepancy with respect to the exact binding site and to the functional consequences. The results obtained are summarized here below.

Firstly, cells lacking Bax/Bak displayed a decreased ER Ca^{2+}-store content, which was associated with an increased (i) amount of Bcl-2 bound to the IP_3R, (ii) protein-kinase-A-(PKA-) dependent phosphorylation of the IP_3R, and (iii) Ca^{2+} leak rate from the ER. Hence, increasing the ratio of antiapoptotic over proapoptotic Bcl-2-family members seemed to decrease the ER Ca^{2+}-store content by promoting the Ca^{2+} leak via hyperphosphorylation and hyperactivation of the IP_3R [70].

Secondly, IP_3Rs were described to be activated by Bcl-Xl. Bcl-Xl bound to all three IP_3R isoforms, thereby sensitizing them to low IP_3 concentrations [71, 72]. The interaction site was demonstrated to be the C-terminal part of IP_3R1 [71]. The binding of Bcl-Xl to the IP_3Rs is important for the protection of cells against apoptotic stimuli, since the overexpression of Bcl-Xl in IP_3R triple-knockout (TKO) cells did not provoke resistance against apoptotic stimuli. By ectopically overexpressing the different IP_3R isoforms in the TKO cells, it was found that all IP_3R isoforms were sensitized by Bcl-Xl and so conferred resistance against apoptotic stimuli. However, a decline in steady-state ER Ca^{2+} levels was only found in TKO cells ectopically expressing IP_3R3 [72], suggesting that decreased ER Ca^{2+} levels are not a requisite for cellular protection against apoptosis. The antiapoptotic action may therefore be due to the enhanced Ca^{2+}-spiking activity resulting from the sensitization of the IP_3Rs, and be mediated either by increased mitochondrial bioenergetics or by modulation of transcriptional activity and gene expression [71, 72]. A similar mechanism was recently proposed for Bcl-2 and Mcl-1 [73].

Thirdly, an inhibition of the IP_3-induced Ca^{2+} release by Bcl-2 was also demonstrated [74]. In contrast to the work discussed above, the interaction site was mapped to the regulatory domain of IP_3R1; moreover, the interaction was mediated through the BH4 domain of Bcl-2, a domain which is not involved in the interaction with the C-terminus of the IP_3R [73, 75]. A peptide corresponding to the Bcl-2-binding site on IP_3R1 specifically disrupted this interaction and in this way counteracted the functional effects of Bcl-2 on the IP_3R [75, 76].

4.5. PKB/Akt and Promyelocytic Leukemia Protein. Another regulatory mechanism of the Ca^{2+}-flux properties of the IP_3R is its phosphorylation via PKB/Akt [29, 77, 78]. Upon prosurvival stimulation of cells, the prosurvival kinase PKB/Akt binds and phosphorylates the IP_3R, thereby reducing its Ca^{2+}-release activity. This mechanism underpins the increased resistance of cells towards apoptotic stimuli by inhibiting the Ca^{2+} flux into the mitochondria and may be perused by tumor cells, yielding a survival advantage. The latter has been shown to occur in glioblastoma cells that display hyperactive PKB/Akt, leading to IP_3R hyperphosphorylation and suppression of IP_3R-channel activity [77].

Very recently, extranuclear promyelocytic leukemia protein (PML) has been shown to be present at the ER and mitochondrial-associated membranes, thereby promoting ER Ca^{2+} release. At these microdomains, PML controls the Ca^{2+}-flux properties of the IP_3R by recruiting PP2A, which dephosphorylates PKB/Akt. The latter suppresses its kinase activity and thus the PKB/Akt-mediated phosphorylation of the IP_3R, resulting in increased IP_3R-mediated Ca^{2+} transfer into the mitochondria and thus OMM permeabilization [79, 80]. This mechanism supplements the other known functions of PML in the nucleus of higher eukaryotes. PML nuclear bodies seem to contribute to its tumor suppressive action by inhibiting cell cycle progression and promoting cell death [81].

5. The Transfer of Ca^{2+} between the IP_3R and Mitochondria in Apoptosis and Autophagy

From the previous it is clear that Ca^{2+} transfer from the ER to the mitochondrial matrix is crucial for regulating mitochondrial functions, including bioenergetics. The mitochondrial Ca^{2+} signal can, however, also control the choice between cell survival and cell death, as it can participate in the induction and progression of apoptosis and autophagy [9, 10].

5.1. IP_3Rs and Mitochondrial Ca^{2+} in Apoptosis and Necrosis. Different studies have placed the IP_3R as central player in the transfer of Ca^{2+} into the mitochondria. Many cell types display the propagation of agonist-induced Ca^{2+} signals into the interior of the mitochondria [11, 82].

Ca^{2+} uptake in the mitochondria is crucial for multiple important cellular functions, but the risk of mitochondrial Ca^{2+} overload exists, which may result in the induction of cell death. At a high concentration, mitochondrial Ca^{2+} supports opening of the PTP in the IMM [51, 83]. This opening leads to the release of ions (including Ca^{2+}) and molecules (including ATP), mitochondrial depolarization, ROS production, cessation of oxidative phosphorylation followed by ATP hydrolysis, matrix swelling by osmotic forces, remodeling of the IMM, and eventually rupture of the OMM [52]. Subsequently various apoptogenic factors, including cytochrome C (CytC), apoptosis-inducing factor, Smac/Diablo, HtrA2/Omi, and endonuclease G, are released from the mitochondria [40]. These apoptogenic factors will activate effector caspases, as caspase-3 and caspase-7, and lead the cell into the execution phase of apoptosis. Permeabilization of the OMM is therefore considered as the decisive event in the development of cell death [84]. Given the proximity of IP_3Rs to the mitochondrial Ca^{2+}-entry sites, IP_3-induced Ca^{2+} spikes appear ideally suited for the stimulation of apoptosis [85], while the knockdown of the IP_3R by siRNA led to the suppression of the Ca^{2+} transfer to the mitochondria.

In addition to this canonical pathway, the group of Mikoshiba recently showed that not only excessive IP_3R-mediated Ca^{2+} release and the concomitant mitochondrial Ca^{2+} overload but also the loss of IP_3R function may lead to apoptosis by lowering the mitochondrial membrane potential [86]. In this study, it was shown that ER stress in neuronal cell leads to attenuation of IP_3R function by impairing the positive regulation of IP_3R1 by the ER chaperone Grp78, which acts as a major regulator of the unfolded protein response and thus prevents ER stress. The loss of Grp78 binding to the luminal domain of the IP_3R1 leads to impaired subunit assembly and thus dysfunctional channels. This property seems selective for IP_3R1, since Grp78 knockdown attenuated IP_3R1-mediated Ca^{2+} release but did not affect IP_3R2- or IP_3R3-mediated Ca^{2+} release. Hence, it is interesting to note that Ca^{2+} transfer from the ER to mitochondria requires a fine-tuned regulation, in which both suppressed and excessive Ca^{2+} transfer leads to apoptosis.

While a severe impairment of IP_3R1 function and attenuated Ca^{2+} release lead to mitochondrial apoptosis, low-level Ca^{2+} signaling from ER to mitochondria or enhancing ER-originating Ca^{2+} oscillations elicits a prosurvival action by stimulating the mitochondrial energy production or by inducing transcription of specific genes [9, 31, 67, 69, 87]. In this paradigm, Bcl-Xl has been proposed to promote cell survival through its direct action on the IP_3R by enhancing prosurvival Ca^{2+} signaling, increasing mitochondrial bioenergetics and activation of signaling via nuclear factor of activated T cells [71, 72].

Mitochondrial Ca^{2+} is a central factor in several neurodegenerative diseases as Alzheimer's disease, Parkinson's disease, and Huntington's disease [88]. The inhibition of cell death by preventing mitochondrial Ca^{2+} overload or by preventing the collapse of the mitochondrial membrane potential is likely therapeutically relevant for the treatment of these diseases. In contrast, enhancement of mitochondrial Ca^{2+} overload can lead to inhibition of tumor cell growth. Stimulation of the Ca^{2+} transfer between ER and mitochondria could lead to increased apoptosis and in this way inhibit uncontrolled cellular proliferation [89]. In this concept, it is not surprising that many tumor suppressor proteins emerge as regulators of the transfer of Ca^{2+} from the ER to the mitochondria, like Fhit and PML. Fhit acts at the mitochondrial level by increasing the affinity of the mitochondrial Ca^{2+} uniporter, thereby promoting mitochondrial Ca^{2+} elevations at low levels of agonist-induced Ca^{2+} signaling [49]. PML acts at the level of the ER, where it is recruited by the IP_3R via a phosphorylation-dependent process involving Akt and PP2A, thereby promoting Ca^{2+} transfer between the ER and the mitochondria and inducing cell death [79, 80]. Mutations or ablation of proteins, like Fhit

and PML, which may involve attenuated ER/mitochondrial Ca^{2+} transfers, has been associated with the development of tumors.

5.2. IP_3Rs and Mitochondrial Ca^{2+} in Autophagy. Autophagy is a delivery pathway used for the lysosomal degradation of long-lived proteins, protein aggregates, damaged organelles, and foreign pathogens. In stress situations (e.g., nutrient starvation), this process offers the cell a fresh pool of building blocks and has thus a prosurvival function [90]. Cells in those conditions have to make the decision between survival (autophagy) and death (apoptosis). Important crosstalks exist between these two pathways [91, 92]. Interestingly, Ca^{2+} and IP_3Rs have been implicated in both apoptosis and autophagy, although the role of Ca^{2+} in autophagy only recently emerged [9, 10, 93]. Nonetheless, Ca^{2+}/IP_3Rs may represent key players in the apoptosis-autophagy decision.

The first results on Ca^{2+} in autophagy even appeared contradictory. On the one hand, autophagy was activated by an increase of the cytosolic Ca^{2+} concentration [94–96]. On the other hand, autophagy was also activated by conditions that all would lead to a decrease of the IP_3R activity and/or cytosolic Ca^{2+} concentration and therefore potentially of the mitochondrial Ca^{2+} concentration [97–100]. In a recent report, it was shown that IP_3R activity is necessary to provide for a basal Ca^{2+} signal to the mitochondria, in order to control mitochondrial bioenergetics. IP_3R knockdown or inhibition will blunt these Ca^{2+} signals, thereby compromising mitochondrial ATP production. The resulting increase in AMP/ATP ratio will subsequently activate autophagy via AMP-activated protein kinase (AMPK) [87].

Other results indicate that IP_3Rs could inhibit autophagy through a scaffold function, via binding of both Bcl-2 and Beclin-1 (an essential autophagy protein), thereby promoting the anti-autophagic interaction between these two proteins. Treatment of HeLa cells with the IP_3R inhibitor xestospongin B promoted the release of Beclin-1 from the IP_3R-Bcl-2 complex, leading to autophagy activation [101].

So far, the data on Ca^{2+}-stimulated autophagy concern the Ca^{2+} in the cytosol [94–96] or ER [102, 103]. It is not yet clear whether the IP_3R is hereby involved, although treatment with an IP_3R inhibitor did blunt cadmium-induced autophagy stimulation [95]. The exact mechanism by which Ca^{2+} promotes autophagy is also still under debate. AMPK-dependent [94], AMPK-independent [96], or ERK-dependent pathways [95] are all possible.

Taken together, these data indicate that a specific, low-intensity Ca^{2+} transfer from ER to mitochondria is necessary to inhibit autophagy, while an increase of the cytosolic Ca^{2+} concentration would activate autophagy.

6. Implications of Ca^{2+} Signaling in Aging

6.1. Aging: A Process of Disorganization. All biological processes involved in the transformation of a fertilized egg into a mature individual capable of reproduction are driven by a purposeful genetic program. Through evolution, natural selection has favored individuals that are reproductively successful [104, 105]. Biological systems, like everything else in the universe, change as a result of entropic changes. Entropy is the tendency for concentrated energy to disperse when unhindered. Natural selection has resulted in sufficient relative strengths of the chemical bonds in our molecules to prevent entropic changes and also installed repair and replacement mechanisms. Evolution has therefore kept the biomolecules in a functional state until reproductive maturation.

After sexual maturation, there is no longer a species-survival benefit for indefinitely maintaining these energy states and, hence, the fidelity in most molecules. As we grow older, stochastic or random events not driven by a genetic program cause energy loss resulting in biologically inactive or malfunctioning molecules. Aging is therefore characterized by increasing entropy. The intrinsic thermodynamic instability of the molecules whose precise three-dimensional structures are no longer maintained leads to covalent modifications such as glycation, conformational changes, aggregation and precipitation, amyloid formation, altered protein degradation, synthesis rates, and nuclear and mitochondrial DNA damage and alterations. When the loss of structure and, hence, function ultimately exceeds repair and turnover capacity, vulnerability to pathology and age-associated diseases increases. Because of the randomness of the molecular disorder underlying aging, the loss of molecular fidelity varies within the body. The weakest links in this system will be the first that lead to disease, like in the vascular system and in cells with a high tendency for cancer development. The very heterogeneous aging process contrasts with the virtually identical stages of development until adulthood [106]. In this respect, we will here focus on the age-related disorganization in the Ca^{2+} signaling machinery, ROS production, and autophagy.

6.2. Mechanism Involved in Aging: ROS, Mitochondria, and Autophagy. The role of ROS accumulation and subsequent macromolecular damage in age-related degeneration has been supported by a plethora of cellular and biological data from various model systems and organisms [107]. Antioxidants act as ROS scavengers and protect against the detrimental effects of cellular ROS exposure. Genetically, genes that extend lifespan were clustered in the IGF-1/insulin-like signaling pathway in a variety of model systems [108]. Nongenetic mechanisms to extend lifespan in different organisms are achieved by caloric restriction and/or by physical activity [109–113]. The composition of the diet during caloric restriction is important; addition of antioxidants (like vitamins, flavonoids), minerals (like Zn and Se), and other compounds such as caffeine, omega 3, and fatty acids has been shown to enhance lifespan [114]. It should be noted, however, that most studies concerning these mechanisms were performed in yeast and animal models, but not yet in humans [115].

Here, we will discuss the molecular mechanisms of ROS underlying aging. First, we will discuss the remodeling of Ca^{2+} signaling during aging. This is important since

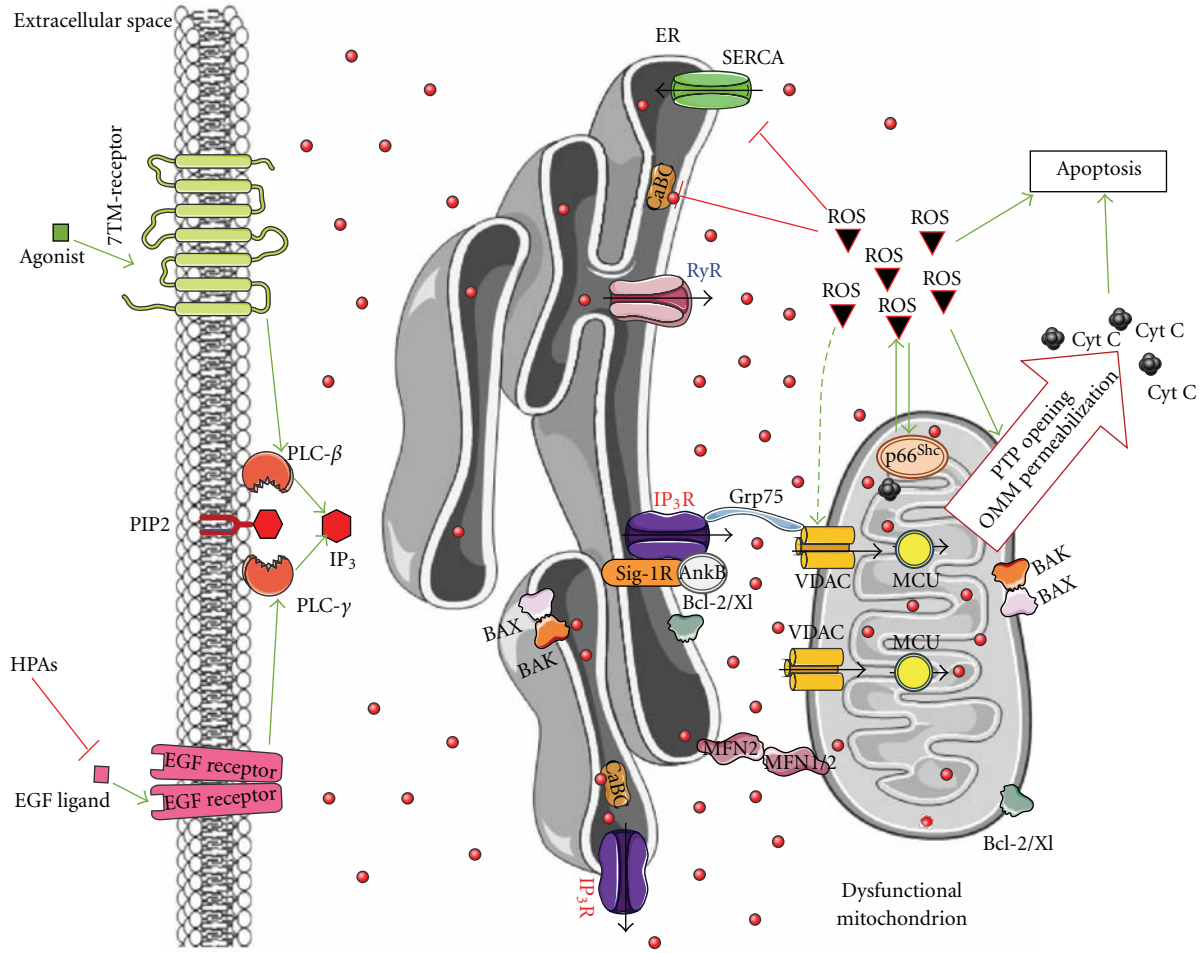

Figure 3: Ca^{2+} signalling and key events involved in aging. Aging cells display decreased function or expression of ER proteins (IP_3Rs, RyRs, SERCAs, Ca^{2+}-binding chaperones (CaBC)), increased cytosolic [Ca^{2+}], suppressed agonist-mediated signaling, and accumulation of damaged mitochondria due to declined autophagic activity. The simultaneous increase in disorganization and dysfunction of the Ca^{2+}-handling proteins and the decline in autophagy will result in the exaggerated production and excessive accumulation of ROS. These events may lead to both ER stress and mitochondrial dysfunction, like PTP opening and OMM permeabilization with the consequent release of apoptogenic factors and cell death. $p66^{Shc}$ and sirtuins take part in this scenario. $P66^{Shc}$ translocates to mitochondria upon oxidative-stress-induced PK$C\beta$ phosphorylation and peptidylprolyl isomerization by Pin1, thereby supporting ROS production. Sirtuins are downregulated and unable to exert its antiaging effect. It is important to note that while $p66^{Shc}$ ablation leads to lifespan extension, high levels of $p66^{Shc}$ have been observed in centenarians. While in normal cells, ROS help to detect and remove altered mitochondria through autophagy, thereby maintaining cellular health, the excessive release of ROS in combination with the decline in autophagy observed during aging may underpin the age-related cell-death processes. In this respect, the recently identified inhibitors of EGF-receptor signaling, the high-performance advanced age phenotype proteins (HPA-1 and HPA-2), whose knockdown promotes locomotory health span of *C. elegans*, may point towards an important role of proper agonist-induced Ca^{2+} signaling via the IP_3R axis. The relevance of these ligands or of attenuated agonist-induced signaling in humans needs to be established. However, recent evidence indicates that dysfunction of IP_3Rs during ER stress promotes cell death and underlies a neurodegenerative disease, like Huntington's disease. Given the central role of proper IP_3R function for mitochondrial bioenergetics and ATP production, the decline of IP_3R activity observed during ER stress or attenuated upstream signaling linked to IP_3 may be very relevant for age-related apoptosis but require further investigation. Green arrows: stimulation; red lines: inhibition; black arrows: Ca^{2+} flux; dashed-green arrow: stimulation/damage.

the OMM permeabilization is critically controlled by the elevation of the mitochondrial Ca^{2+} concentration, thereby serving as a coincidence detector with ROS [116]. Next, we will focus on the signaling cascade involving sirtuins, $p66^{Shc}$, and autophagy in the regulation of mitochondrial function. A schematic overview of the interaction between the different molecular key players in aging is provided in Figure 3.

6.2.1. Ca^{2+} Signaling in Aging. Altered intracellular Ca^{2+} signaling is a hallmark of neurodegeneration, like in Alzheimer's and Huntington's disease [117–120]. Different models have been proposed for familial Alzheimer's-disease-linked presenilin mutations, including the function of presenilins as Ca^{2+}-leak channels [121], an increase in the expression level of IP_3Rs [122], or the direct activation of IP_3Rs or

RyRs [123–125]. In any case, it is clear that exaggerated Ca^{2+} signaling is an upstream event in the pathophysiology of Alzheimer's disease and contributes to the ROS-mediated cell toxicity [126]. However, the changes in Ca^{2+} signaling that occur in neurodegenerative diseases may be dependent on the type of disease. For instance, a mouse model for Huntington's disease revealed dysfunctional IP_3R Ca^{2+}-release channel activity in the cerebrum and striatum, which was caused by a prominent decline in the association of Grp78, a positive regulator of the IP_3R1-channel formation, with the IP_3R1 [86].

Other age-related diseases also display altered Ca^{2+} signaling. Cardiac hypertrophy, for example, is characterized by enhanced IP_3 signaling, leading to spontaneous Ca^{2+}-release events that underlie arrhythmias [127]. Also chronic heart failure can be a consequence of excessive phosphorylation of RyR, leading to an increased Ca^{2+} leak [128] (Figure 2).

However, the role and mechanism of ER Ca^{2+} signaling in aging is less clear [129], although most studies suggest altered Ca^{2+} signaling during aging (Figure 2). In most cell types, ER Ca^{2+} dyshomeostasis was caused by a decreased ER Ca^{2+} content and a decreased Ca^{2+} release from the ER, while the cytosolic $[Ca^{2+}]$ was increased. These effects were the result of a decline in SERCA and/or IP_3R and/or RyR activity, caused by changes in mRNA or protein levels, phosphorylation events, or oxidative damage to SERCA [7]. In addition, intraluminal Ca^{2+}-buffering protein levels often decline during age, in part also through oxidative damage [130] (Figure 2). Also VDAC undergoes posttranslational modifications in aged cells, possibly through oxidative break-up of tryptophan residues, thereby increasing the susceptibility to apoptosis [131]. This is in line with evidence showing that superoxide can lead to mitochondrial permeabilization in a VDAC-dependent manner [132]. In yeast, this phenomenon can be protected by Cu/Zn-superoxide dismutase, a protein known for its protective role against aging [133].

Some cell types, however, display Ca^{2+} dyshomeostasis in a different way (Figure 2). Studies in aged rat hearts, for example, showed increased IP_3R levels [134]. Also aged neuronal cells displayed reduced sensitivity towards caffeine, which may be caused by a decline in the steady-state ER Ca^{2+} levels [135–137]. The latter may be due to a decreased SERCA Ca^{2+}-pump activity, a limited supply of ATP or an increased Ca^{2+} leak from the ER. Other studies pointed to a prolonged Ca^{2+}-induced Ca^{2+} release, resulting in an inhibition of synaptic strength and long-term potentiation [138, 139].

Interestingly, IP_3R characteristics also appear to be altered in aged brain tissues [140], as IP_3R density and IP_3 binding to the IP_3R were decreased in aged rat cerebellum. The same observation of decreased IP_3 binding was made in aged mice cerebellum [141]. However, the cellular IP_3 content increased with age [142]. These findings suggest a role for the phosphoinositide/Ca^{2+} signaling in the impaired neuronal responsiveness during aging. In this respect, more recent work revealed that stimulation of IP_3Rs in old astrocytes increased protection against ROS and subsequently neuroprotection [143].

Moreover, in aged MII-stage eggs, it was found that the IP_3R1 was proteolytically cleaved by caspase-3, resulting in a leaky 95-kDa C-terminal IP_3R1 fragment containing the channel pore [144, 145]. In contrast, when the C-terminal channel domain was recombinantly expressed in the mouse oocytes, the sperm-factor-induced Ca^{2+} oscillations were abolished and the eggs displayed an apoptotic and fragmented phenotype. Previously, we had shown that caspase-3-dependent cleavage of the IP_3R augmented the late phase of apoptosis by providing a prolonged ER Ca^{2+} leak [146]. However, in healthy cells, the Ca^{2+} leak through a recombinantly expressed C-terminal channel domain was very small. Hence, the caspase-3-dependent cleavage of the IP_3R may participate in cellular Ca^{2+} overload via a second-hit mechanism. In the case of aged oocytes, accumulated ROS may be the second hit. Currently, it is not clear whether IP_3R cleavage contributes to the aging process by overloading the mitochondria with Ca^{2+} and sensitizing them towards ROS accumulation. In addition, ROS may also directly regulate IP_3R activity, since it is known that oxidizing agents like thimerosal sensitize IP_3Rs by stimulating intramolecular interactions between the suppressor and ligand-binding domain [147]. Taken together, IP_3R/Ca^{2+} signaling appears to be affected in aged cells. Abnormal Ca^{2+} signals may then affect many processes (ROS production/protection, autophagy, apoptosis, synaptic transmission, etc.) that are altered during aging (summarized in Figure 5). Nevertheless, the overall changes in ER Ca^{2+} handling observed during aging seem relatively small compared to the changes found in Alzheimer's disease [129].

Recently, an elegant study on *Caenorhabditis elegans* re-enforced the paradigm that the activation of IP_3R pathways may be considered in therapeutic applications for treating age-related decline in skeletal muscle function (sarcopenia) [148]. Indeed, using an RNAi screen, the authors identified two critical factors that delayed the age-associated decline in locomotory health span of *C. elegans* in a high-performance advanced age phenotype (HPA-1 and HPA-2). The concept underpinning this study was that locomotory decline in humans contributes to frailty and loss of independence. Although the exact mechanism is not yet known, it is clear that HPA-1 and HPA-2 attenuate epidermal-growth-factor-(EGF-) dependent signaling via the EGF receptor [148]. When HPA-1 and HPA-2 are disrupted, EGF signaling via the EGF receptor will increase. The activation of the EGF-signaling pathway normally leads to cell proliferation, survival, integrity, and differentiation. Importantly, phospholipase C-γ (PLC-γ) and IP_3Rs were demonstrated to act downstream of EGF-receptor signaling, thereby contributing to prolonged health span in these animals. This is the very first report considering the role of EGF signaling in aging. Therefore, the exact mechanism of how these signaling pathways affect human aging remains to be further clarified, but restoring the attenuated IP_3R-mediated Ca^{2+} signaling and reestablishing normal mitochondrial function may be an attractive hypothesis in combination with chemical induction of autophagy (Figure 4). Nevertheless, a decline in G-protein-coupled receptor-dependent signaling has been observed in the skeletal muscle and intestine of aged

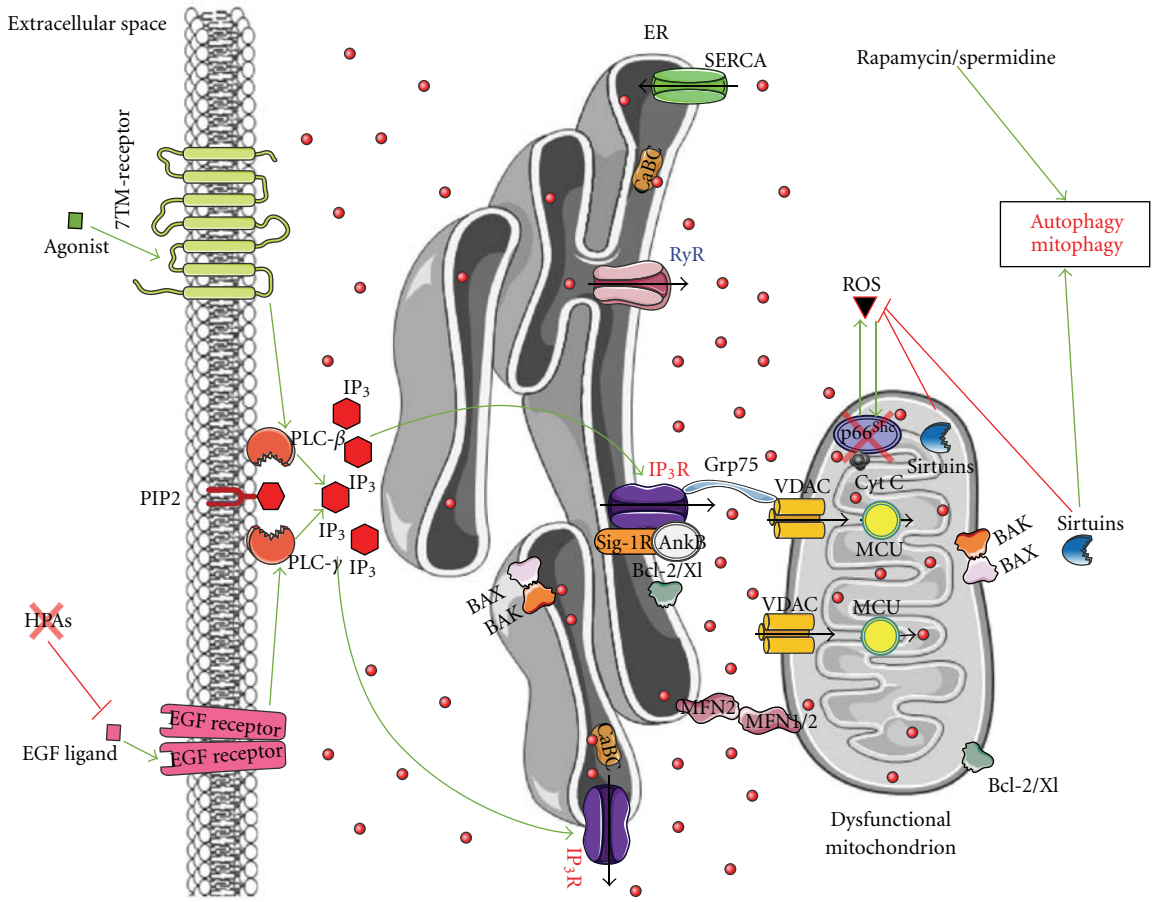

FIGURE 4: A speculative antiaging strategy based on restoring IP_3R-mediated Ca^{2+} signaling and chemical induction of autophagy. Provided the concept that aging cells are characterized by suppressed IP_3 signaling or attenuated IP_3R, Ca^{2+}-release activity is relevant in humans, and elevating IP_3 levels may compensate for the decline in the IP_3/IP_3R-signaling axis. This may contribute to a decline in the $p66^{Shc}$-mediated ROS production, an activation of sirtuin-dependent mitochondrial biogenesis, and the lowering of ROS production. The final step of this compensatory response consists in the autophagic removal of the damaged mitochondria. Hence, chemical induction of autophagy (e.g., by rapamycin or spermidine) is likely critical for successful and healthy aging in human beings. It is important to note that this concept is based on a recent report on *C. elegans*, in which ablations of inhibitors of EGF signaling enhance IP_3R signaling and promote healthy lifespan extension. Green arrows: stimulation; red lines: inhibition; black arrows: Ca^{2+} flux.

rats [149]. The underlying mechanism involved a prominent decrease in the levels of $G_{q/11}$ and G_i protein levels.

6.2.2. Sirtuins. Sirtuins are a conserved family of proteins that are linked to longevity and stress tolerance in *Saccharomyces cerevisiae* [150]. Sirtuins have been identified as antiaging genes, since increasing their activity prolonged lifespan not only in yeast, but also in *C. elegans* and *Drosophila melanogaster* and is thought to act similarly in mammals [151–153]. In this respect, age is often associated with reduced sirtuin levels. In aged mouse embryonic fibroblasts, progressive loss of the sirtuin-1 protein, but not mRNA, was observed [154]. However, other studies show that this is at least tissue specific; sirtuin-1 activity was reduced in rat hearts, but not in adipose tissue [155], and reduced sirtuin-1 expression was found only in distinctive parts of the mouse brain [156]. Sirtuins, which retard aging as a function of their gene dosage, display unique biochemical activities, that is, NAD-dependent protein deacetylase [157, 158]. The subsequent deacetylation of sirtuin substrates alters their activity (activation or inhibition). In mammals, sirtuin-1 deacetylates a variety of key transcription factors and cofactors, like p53 [159], FOXO proteins [160, 161], peroxisome proliferation activating receptor (PPAR)-γ coactivator-1α (PGC-1α) [162], and nuclear factor-κB [163]. The effects of sirtuin-1 on these factors elicit stress tolerance and metabolic changes reminiscent of caloric restriction, while caloric restriction upregulates sirtuin-1 levels, and mice lacking sirtuin-1 did not display phenotypic responses upon caloric restriction [160, 164–166]. Since sirtuins are regulated by NAD^+, their activity will be influenced by the NAD^+/NADH ratio and thus by the metabolic state of the cell [167]. Hence, sirtuins may be influenced not only by caloric restriction but also by physical activity, both associated with longevity and increased insulin sensitivity [168, 169].

Importantly, sirtuin-1 also regulates mitochondrial biology [150, 167], another key aspect in aging, since the number

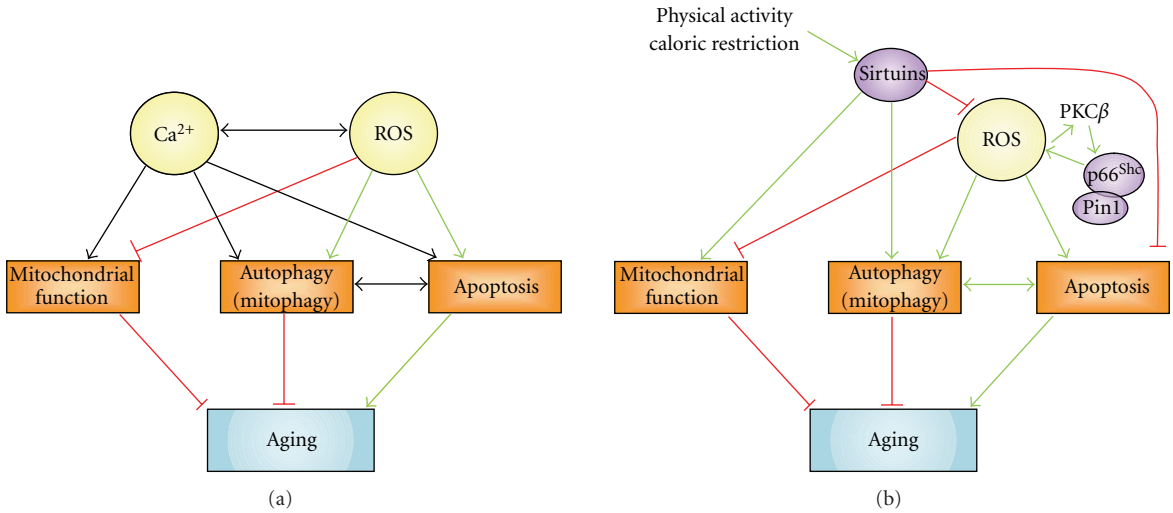

Figure 5: Network of interactions between sirtuins, $p66^{Shc}$, Ca^{2+}, and ROS, which affect mitochondrial function, autophagy, and apoptosis, thereby controlling aging-dependent processes. (a) Ca^{2+} signals may increase or prevent aging. Ca^{2+} signals are characterized by different spatiotemporal characteristics and subsequently different outcomes on mitochondrial function, autophagy, and apoptosis. For example, a constitutive Ca^{2+} transfer from ER to mitochondria would stimulate mitochondrial function and inhibit autophagy and apoptosis, while a mitochondrial Ca^{2+} overload would be proapoptotic. The interplay between mitochondrial Ca^{2+} elevations and ROS production is a critical determinant in the apoptotic outcome at the level of the mitochondria, which function as co-incidence detectors. Therefore, high mitochondrial Ca^{2+} concentrations and ROS act as a double-hit mechanism, triggering mitochondrial-dependent apoptosis. (b) Sirtuins are mainly antiaging genes via the promotion of mitochondrial function and autophagy and inhibition of apoptosis. They also act inhibitingly on ROS. Sirtuin function may be enhanced by restricting caloric intake or increasing physical activity, thereby extending lifespan. Increased ROS activate the Pin1- $p66^{Shc}$ complex, which, in turn, promotes the production of ROS and subsequently mitochondrial damage. Therefore, $p66^{Shc}$ may help to target damaged mitochondria and activate cellular processes that deal with dysfunctional mitochondria and oxidative stress. The outcome, however, can be dual: aging may be enhanced via a complete removal of the cell through apoptosis, while the selective removal of the damaged mitochondria through mitophagy, leaving the cell with predominantly healthy mitochondria, may slow down the aging process. Green arrows: stimulation; red lines: inhibition; black arrows: stimulation or inhibition.

of functional mitochondria is known to decline during aging. This has been proposed to underlie aging in diseases like type-2 diabetes [170, 171]. In contrast, increasing mitochondrial activity will increase the metabolic rate, enhance glucose metabolism, and improve insulin sensitivity. Even without an increase in the metabolic rate, caloric restriction might be beneficial by inducing mitochondrial biogenesis via sirtuin-1 [165, 172, 173]. Activation of sirtuin-1 has been shown to be involved in mitochondrial biogenesis and improved mitochondrial function by deacetylation of PGC-1α, thereby lowering ROS production [162].

Sirtuin-1 also suppressed stress-induced apoptosis, while the lack of sirtuin-1 inhibited autophagy *in vivo* [174]. In addition, the extension of lifespan upon caloric restriction was proposed to be dependent on the induction of autophagy by sirtuin-1 [175]. The underlying mechanism probably involves the deacetylation of certain autophagy proteins, such as Atg5, Atg7, and Atg8 [174, 175]. A schematic overview of the role of sirtuins in aging is depicted in Figure 5.

6.2.3. $p66^{Shc}$. Recent research revealed the role of $p66^{Shc}$, the 66 kDa isoform of the Shc (Src homolog and collagen homolog) family [176]. Although $p66^{Shc}$ forms stable complexes with Grb2, an adaptor protein for the Ras-exchange factor SOS, it has little effect on Ras-mediated signaling [177].

Nevertheless, $p66^{Shc}$ is activated by oxidative stress via phosphorylation on Ser36, and this mechanism is indispensable for $p66^{Shc}$'s lifespan regulation [178, 179]. Mice in which $p66^{Shc}$ has been deleted displayed a prolonged lifespan with a decreased mitochondrial metabolism and ROS production, while lacking pathophysiological characteristics or effects on body size. MEF cells from $p66^{Shc-/-}$ animals displayed resistance towards oxidative-stress-induced apoptosis in a p53-dependent manner [176].

ROS arise from the mitochondrial electron-transfer chain or from exogenous sources, like UV and ionizing radiations. $p66^{Shc}$ is involved in mitochondrial ROS production. In basal conditions, about one fifth of $p66^{Shc}$ is localized to the intermembrane space of the mitochondria, while oxidative stress dramatically increases the mitochondria-associated $p66^{Shc}$ due to its mitochondrial translocation from the cytosol [180]. In the mitochondria, $p66^{Shc}$ interacts with CytC, promoting the shuttling of electrons from CytC to molecular oxygen [181]. The latter may underlie the increased ROS production upon $p66^{Shc}$ overexpression and the decreased ROS production in $p66^{Shc}$ knockout cells. In addition, $p66^{Shc}$ knockout cells displayed decreased oxidative capacity, thereby redirecting metabolic energy conversion from oxidative toward glycolytic pathways. Therefore, $p66^{Shc}$ may provide a molecular switch to oxidative-stress-induced apoptosis by controlling mitochondrial ROS production.

It should be noted, however, that studies in yeast correlated higher respiration rates combined with decreased oxidative stress and increased lifespan [182]. This suggests that the respiration rate *per se* is not the important factor for ROS production, but more likely the electron transmit time and the availability of oxygen [183].

In normal cells, oxidative stress leads to compromised mitochondrial Ca^{2+} homeostasis, which is an early event of mitochondrial damage [107, 176]. This is observed as a decreased mitochondrial Ca^{2+} signal upon agonist stimulation in cells challenged with H_2O_2 despite a normal cytosolic Ca^{2+} signal. Importantly, cells lacking $p66^{Shc}$ seemed to be protected against oxidative challenge, since their mitochondrial Ca^{2+} signaling upon agonist stimulation was not impaired in the presence of H_2O_2 [176]. Similar results were found in MEF cells lacking Pin-1, a peptidylprolyl isomerase catalyzing *cis*/*trans* isomerization of phosphorylated Ser-Pro bonds, where the reduction of agonist-induced Ca^{2+} signals in mitochondria upon oxidative stress was significantly smaller. These findings suggest a phosphorylation-dependent conformational change in Pin-1 targets, like $p66^{Shc}$.

Recent work provided important mechanistic insights into the role of $p66^{Shc}$ in the early mitochondrial response to oxidative stress [178, 179]. ROS are known to activate a variety of kinases, including protein kinase C (PKC) β. The activation of PKCβ will cause the phosphorylation of $p66^{Shc}$ on Ser36, although other kinases may also participate in this process. Indeed, the mitochondrial fraction of $p66^{Shc}$ during oxidative challenge was severely reduced after treatment with PKCβ inhibitors. As a result, Ser36-phosphorylated $p66^{Shc}$ will interact with Pin-1. The catalytic activity of Pin-1 may result in *cis*/*trans* isomerization of Ser36-Pro37, thereby triggering the exposure of a mitochondrial targeting sequence or an interaction with mtHsp70, a mitochondrial heat-shock protein. This process may underlie selective targeting of $p66^{Shc}$ to mitochondria undergoing oxidative challenge. The mitochondrial targeting of $p66^{Shc}$ involves its protein-phosphatase-(PP-) 2A-mediated dephosphorylation and dissociation from mtHsp70, although the mechanism of their contribution is not fully elucidated. In the intermembrane space, $p66^{Shc}$ will interact with reduced CytC and enhance intramitochondrial H_2O_2 production. The latter and its more damaging reaction products, the hydroxyl radicals, have been shown to trigger the opening of the PTP [184]. This will perturb mitochondrial structure and function, resulting in mitochondrial permeabilization, CytC release, and apoptosis induction, and subsequently lead to a coordinated cell-death response and the removal of the cell containing damaged mitochondria. However, in addition to apoptosis, autophagy may be involved in removing the subpopulation of compromised mitochondria suffering from oxidative challenge. Interestingly, this autophagy-mediated removal of damaged mitochondria can be triggered through PTP opening [185]. This will result in the removal of the organelles that are damaged by the oxidative stress (a process termed mitophagy), while maintaining the healthy mitochondria. According to these findings, it is interesting to note that aging has been associated with declined autophagy activity [186], while autophagy activity is a requisite for lifespan extension in *C. elegans* [187]. In this way, $p66^{Shc}$ may be important for mitochondrial quality control through the autophagy-mediated removal of damaged mitochondria. However, during aging, the number of mitochondria suffering from oxidative stress may increase, while their cleanup by the autophagic system may become limiting, leading to the accumulation of unprocessed oxidation-damaged mitochondria. Importantly, in mouse models for aging, the levels of $p66^{Shc}$ seemed to decline, while its phosphorylation at Ser36 was enhanced [188]. This correlated with higher free-radical production and accumulation of damage caused by ROS.

Strikingly, fibroblasts obtained from centenarians displayed elevated levels of $p66^{Shc}$ [189], indicating that basal mitochondrial $p66^{Shc}$ plays an important role in normal cell-damage management of stress and in damage repair. Indeed, the selective removal of damaged mitochondria may contribute to lifespan extension. In addition, it is interesting to note that increased physical activity has been associated with lifespan extension and lower mortality, although this is associated with increased mitochondrial ROS production due to an increased metabolic rate. Therefore, it is conceivable that exercise may promote adaptation to ROS by upregulating ROS scavengers, causing a natural resistance against ROS or against cellular damage in general [167]. Hence, it may be worth investigating whether $p66^{Shc}$ levels are affected by exercise and whether this may contribute to increased cleanup of damaged mitochondria or resistance against ROS. A schematic overview of the role of $p66^{Shc}$ in aging is depicted in Figure 5.

6.2.4. Autophagy. It has become increasingly clear that autophagy plays a central role in the aging process, in which it is involved in the removal of damaged organelles or of protein aggregates by engulfment in autophagosomes followed by lysosomal degradation. First of all, autophagy was demonstrated to decrease with increasing life time [186]. Caloric restriction slowed down the age-related impairment of autophagy in skeletal muscle of rats [190]. In addition, chemical induction of autophagy by spermidine or by rapamycin prolonged lifespan [191, 192]. In contrast, animals with compromised capacity to perform autophagy were short living and displayed neurodegenerative phenotypes, probably due to the accumulation of deleterious accumulation of protein aggregates [193–195]. Moreover, it is clear that damaged mitochondria ought to be removed, while harboring the healthy mitochondria, which are needed for cell survival. In any case, the accumulation of damaged mitochondria and their impaired removal is a hallmark of aging and will contribute to decreased cell viability. Therefore, mitochondrial quality control is essential for proper cell survival.

The "selective" recognition of damaged mitochondria by autophagosomes without affecting healthy mitochondria remains very poorly understood. However, the first components essential for "selective" mitophagy have been identified in yeast: Uth1, an OMM protein, and Aup1, a mitochondrial phosphatase [196–198]. Additional components of

organelle-specific autophagy have been revealed in a systematic screen, including Atg11, Atg20, Atg24, Atg32, and Atg33 [199, 200]. Atg32 is proposed as the receptor for mitophagy via the local recruitment of Atg8, an essential component of the autophagosome formation. NIX/BNIP3L [201, 202], BNIP3 [203], PARKIN [204], and PINK-1 [205–210] were proposed to be involved in mitochondrial degradation in mammalian cells. PARKIN is selectively recruited by dysfunctional mitochondria, thereby mediating the engulfment of these mitochondria by the autophagosomes [204]. A recent study provided clear insights into the underlying mechanism, which required the accumulation of the kinase PINK-1 on damaged mitochondria. In healthy mitochondria, PINK-1 is maintained at a low level by voltage-dependent proteolysis [210]. In mitochondria with sustained damage, PINK-1 levels rapidly accumulated. The latter was required and sufficient to recruit PARKIN to the mitochondria providing a mechanism for the selective removal of damaged mitochondria by autophagy. Importantly, mutations in PINK-1 or PARKIN associated with Parkinson's disease abolished the recruitment of PARKIN by PINK-1 to the mitochondria, allowing the accumulation of damaged mitochondria. Another recent study revealed the mitochondrial protein NIX as the selective mitophagy receptor for the removal of damaged mitochondria by binding and recruiting LC3/GABARAP proteins [211]. The latter are ubiquitin-like modifiers required for the elongation of autophagosomal membranes.

Besides these mitophagy receptors, mitochondrial proteases and chaperones were needed to prevent the accumulation of misfolded and aggregated proteins within the mitochondria [167].

Finally, various studies point towards a role of ROS upstream of autophagy [212]. Accumulation of ROS directly affects different key players essential for the induction of autophagy, including the activation of the protein kinases AMPK and JNK, the inhibition of other kinases (Akt and TOR), and the inhibition of LC3 delipidation. These processes will stimulate autophagy, thereby alleviating the oxidative stress by removing the ROS-generating mitochondria.

7. Conclusions

Upstream Ca^{2+} and ROS signaling tightly control cellular homeostasis by regulating fundamental cell-death and cell-survival processes like apoptosis and autophagy. It is clear that many proteins that mediate apoptosis and autophagy directly affect Ca^{2+} signaling through interaction with the ER and mitochondrial Ca^{2+}-release and/or Ca^{2+}-uptake mechanisms. Furthermore, these Ca^{2+}-signaling proteins contribute to the functional and physical linking between ER and mitochondria. Importantly, the interplay between ER and mitochondrial Ca^{2+} signaling and ROS signaling mediates the detection, the efficient targeting, and removal of mitochondria with sustained damage. This is the key for cellular homeostasis as well as for homeostasis at the level of the whole organism. In this respect, the efficient and selective removal of damaged mitochondria by autophagy is a crucial element in the maintenance of cellular health, whereby the poisonous accumulation of ROS from dysfunctional mitochondria and eventual cell death via apoptosis are avoided. Recent studies point towards a central role for impaired autophagy and inadequate removal of damaged mitochondria during aging. At the level of the organism, apoptosis will be the ultimate resort to remove seriously damaged cells. This will particularly affect the lifespan of nondividing cells, like neurons, thereby affecting the lifespan of the whole organism.

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Review Article

Therapeutic Approaches to Delay the Onset of Alzheimer's Disease

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The key cytopathologies in the brains of Alzheimer's disease (AD) patients include mitochondrial dysfunction and energy hypometabolism, which are likely caused by the accumulation of small aggregates of amyloid-β (Aβ) peptides. Thus, targeting these two abnormalities of the AD brain may hold promising therapeutic value for delaying the onset of AD. In his paper, we discuss two potential approaches to delay the onset of AD. The first is the use of low dose of diaminophenothiazins (redox active agents) to prevent mitochondrial dysfunction and to attenuate energy hypometabolism. Diaminophenothiazines enhance mitochondrial metabolic activity and heme synthesis, both key factors in intermediary metabolism of the AD brain.The second is to use the naturally occurring osmolytes to prevent the formation of toxic forms of Aβ and prevent oxidative stress. Scientific evidence suggests that both approaches may change course of the basic mechanism of neurodegeneration in AD. Osmolytes are brain metabolites which accumulate in tissues at relatively high concentrations following stress conditions. Osmolytes enhance thermodynamic stability of proteins by stabilizing natively-folded protein conformation, thus preventing aggregation without perturbing other cellular processes. Osmolytes may inhibit the formation of Aβ oligomers *in vivo*, thus preventing the formation of soluble oligomers. The potential significance of combining diaminophenothiazins and osmolytes to treat AD is discussed.

1. Introduction

1.1. An Overview of Alzheimer's Disease. Alzheimer's disease (AD) is an irreversible brain disorder that slowly destroys memory and eventually a person's ability to perform the daily life tasks and activities. Memory problems are one of the first signs of AD, and as it progresses, decline in other cognitive abilities such as poor judgment and mood changes starts to surface. Eventually people with severe AD cannot communicate and become completely dependent on others for their care. Most people with AD have lateonset of disease, which usually develops after age 60. However, a silent preclinical phase which precedes the development of AD clinical symptoms may span 2–8 years.

AD is a disease with a complex etiology and no single therapeutic approach is likely to prevent or lead to a cure. All current treatments focus on several different aspects, including management of behavioral symptoms or temporarily slowing the progress of the disease. However, none of these treatments changes or alter the inevitable course of the disease. Thus, even after almost three decades of research, AD is still dementia with a progressive failure to form new memories, and thereby interfering with the basic mechanism of the disease.

In recent years, it has been well-accepted that one of the pathological mechanisms of AD relates to the accumulation amyloid-β (Aβ) peptide in certain brain regions [1]. Aβ is a product of Aβ precursor protein (AβPP) via natural proteolytic processing. AβPP is processed by three different proteases known as α-, β-, and γ-secretase. Each of these three proteases cleaves AβPP at different sites resulting in various Aβ species ranging from 39–43 amino acid residues [2]. It has been shown that long species of Aβ (A$\beta_{42,43}$) are strongly amyloidogenic and form aggregates readily compared to the short forms (A$\beta_{39,40}$). Aβ are the building blocks of insoluble extracellular Aβ deposits or senile plaques formation [3], a neuropathological hallmark of AD [3]. AD is also marked by neurofibrillary tangles, an intracellular filaments of highly

phosphorylated tau protein. Impairment of cellular function in AD is demonstrated by a set of cytopathologies (reviewed in [4]) such as decline in cytochrome *c* oxidase (complex IV), mitochondrial dysfunction, abnormal iron homeostasis, oxidative stress, dimerization of AβPP and synaptic dysfunction [5–9], and energy hypometabolism. Several lines of evidence point towrads strong connection between small aggregates of Aβ and mitochondrial dysfunction.

1.2. Role of Amyloid-β and Oligomers in Alzheimer's Disease. According to the oldest so-called "cholinergic hypothesis", AD is caused by reduced levels of the neurotransmitter acetylcholine, which is important for memory. However, medications intended to treat acetylcholine deficiency have not been very effective in modifying the course of the disease. In the early 90s, the "amyloid hypothesis" was postulated according to which Aβ deposits arising from AβPP cause AD. As a result, specific proteases that process AβPP were the focus of drug development as a possible mean to lower Aβ production. However, several complications stem from the significance of these proteases for other biological functions creating serious obstacles in this front.

It is now well-accepted fact that AD is a neurodegenerative disorder associated with protein aggregation and misfolding of AβPP, which may be triggered by genetic polymorphism, age-dependent alteration to Aβ metabolism, or environmental factors that may promote accumulation and aggregation of Aβ peptide [10–13]. Thus, therapies directed at reversing Aβ aggregation appeared promising. However, several limitations reduced the excitement in this approach as was the case for immunotherapy targeted using specific anti-Aβ antibodies. Additionally, the lack of chemical safety of the available drugs to prevent Aβ aggregation was also a major concern.

There are reports showing a correlation between an early cognitive impairment in AD and increased oligomerization of Aβ, which precede the appearance of senile plaques [14–16]. Oligomers of Aβ correlate with early cognitive impairment in AD [17, 18]. In recent years, studies using cells, mouse models, and human brain tissues strongly suggest that soluble Aβ oligomers could be a toxic forms of Aβ [19, 20]; however, experimental data for their direct in vivo toxicity is lacking [21]. Thus, preventive approaches may include also preventing the formation of Aβ aggregates. However, it is not entirely clear which cellular compartment is the primary target of Aβ toxicity. Interestingly, in addition to other extra- and intracellular compartments, Aβ is also found in the mitochondria [22, 23], suggesting that neurotoxic effects of Aβ may be widespread [24], unlike earlier views that the extracellular senile plaques are the only main neurotoxic factor in AD. Energy hypometabolism and synaptic dysfunctions are proposed to be the primary target of Aβ neurotoxicity [25, 26]. Thus, preventing mitochondrial dysfunction by identifying the primary metabolic pathway, specifically targeted by Aβ is a plausible approach to delay AD [27]. We propose that preventative approaches are more promising for lowering the prevalence of AD. These approaches could target the formation of Aβ oligomers and enhance mitochondrial activity to counter energy deficiency in AD.

2. Role of Mitochondrial Dysfunction in Alzheimer's Disease

Several lines of evidence suggest that impairment of mitochondrial function plays important role in the development of neurodegenerative diseases including AD. Mitochondrial dysfunction and impaired cellular energy is an early decline in metabolism seen in AD patients. In addition to the biochemical physiological changes, the brain mitochondria of AD patients exhibit substantial structural changes that included abnormal cristae, accumulation of osmophilic material, and smaller size compared to normal controls [23, 28]. It has been reported that mitochondrial fragmentation damages regions of nerve cell synapses. Excessive fragmentation of mitochondria causes synaptic injury leading to eventual nerve cell death. Since synapses are critical for learning and memory, their impairment leads to the dementia in AD patients. We have recently proposed that strong binding of Aβ with heme is a key factor associated with Aβ-mediated neurotoxicity [29, 30], which could be the primary metabolic pathway targeted by excess Aβ production [31–33], and thus interfering with mitochondrial structures and functions by increasing the production of nitric oxide (NO) leading to mitochondrial damage and impaired energy metabolism [34, 35]. One of the key cytopathologies of AD include decline in cytochrome c oxidase (complex IV) and αKGDH, which seems to contribute to mitochondrial dysfunction [36–38]. This, abnormal biology of the mitochondria may contribute to energy deficiency in AD [35, 39]. There are reports showing an abnormal interaction of Aβ with key brain metabolites such as zinc, copper, cholesterol, mitochondrial protein import machinery, HrtA2 protease, ABAD, and heme [22, 31, 40–43]; however, their relation to cytopathologies of AD is not clear.

The decline in complex IV, which occurs in heme deficient cells, leads to similar structural consequences on mitochondria (unpublished observations). Several lines of experimental evidence provided support that heme metabolism may be a specific metabolic pathway that is targeted by Aβ peptides [43–46]. We showed recently a specific heme-binding motif in human Aβ peptides [27]. Based on our recent work and other laboratories, we propose that depletion of regulatory heme and the formation of Aβ-heme peroxidase are key factors of mitochondrial dysfunction in the brains of AD patients. Due to phylogenic variation in the amino acid sequences of Aβ, differential heme-binding of Aβ could also explain why humans, but not rodents, develop AD-like neuropathology (Reviewed in [4]). Heme is responsible for the metabolic integrity of complex IV, which is a key factor in mitochondrial gene regulation systems; therefore, pathways that depend on heme may be properly regulated by lowering Aβ oligomeric forms, and enhanced heme synthesis may improve neuronal energy metabolism.

3. Therapeutic Approaches for Alzheimer's Disease and Challenges

AD has a devastating impact on both personal and community levels. AD is the most common age-related dementia manifested by widespread progressive cognitive deterioration and impaired behavioral skills. With the aging US populations, and widespread prevalence of AD in these populations warrants immediate need for the management of this deleterious disease. In spite of efforts from scientific community for several decades, the available drug therapies for AD are only remedial without proper understanding of the underlying mechanisms involved therein. Unless new treatments are developed to decrease the likelihood of developing AD, the number of individuals with this disease in the United States is expected to be more than 10 million in next three decades.

There is currently no specific cure for AD patients, but scientific research is unraveling the mysteries of AD, including the causes and the mechanisms of the disease progression, which might one day effectively solve the Alzheimer's puzzle. In recent years, some understandings have already provided critical information about how to prevent, delay, or slow the nerve cell damage that leads to AD, which may help maximize quality of life of these patients. However, drug treatments currently available are used to only manage the cognitive symptoms of AD by slowing the progression of symptoms for a while.

Despite the intensive research on AD, a therapeutic or preventive strategy for AD remains elusive or limited at the most [47], which has been a key obstacle for the development of effective therapies for AD. The efficacy of currently available drugs has further been hampered by the fact that the effectiveness of these drugs progressively declines with the progression of disease. Thus, there is an urgent need for a new generation of drugs to prevent or delay the onset of AD.

Except for imaging techniques, a precise and accurate diagnostic biomarker for AD is also lacking [48]. However, images results are excellent in following disease progress, but limited in revealing preclinical before protein deposits occur AD. The ultimate biomarker would preferably be a blood- or CSF-borne metabolite that indicates the risk for AD in advance of the clinical signs or protein deposits. Aβ-heme peroxidase could serve a unique biomarker if found in blood or CSF of AD patients. Aβ-heme has the advantage of being dependant on Aβ and it is tightly linked to a key brain metabolite (heme as mitochondrial metabolite), thus could indicate impairment in brain metabolism that depends on Aβ accumulation.

4. Mitochondria As Targets for Delaying the Onset of Alzheimer's Disease

Mitochondrial dysfunction in AD could serve as a therapeutic target an interest in developing mitochondrial drugs is emerging. Enhancing mitochondrial function and maintaining structural integrity of mitochondria could delay the onset of AD. Below, we discuss pharmacological approaches to enhance mitochondrial function and prevent the formation of Aβ toxic oligomers.

Figure 1: The chemical structure of methylene blue; a diaminophenothiazin.

Mitochondria are a major energy source, and it has been known that energy deficiency can result in synaptic dysfunction and neurodegeneration of the hippocampus and cortical regions of the brain [49]. The brain is particularly sensitive to mitochondrial dysfunction, the resulting oxidative stress, and impaired energy metabolism [50–52]. Thus, improved energy metabolism through enhanced mitochondrial activity in the brain might be an effective approach to delay the onset of AD.

Mitochondrial dysfunction in AD is associated with a decline in mitochondrial complex IV and energy deficiency. Due to involvement of mitochondria in cellular senescence and aging, it may contribute to neural dysfunction with age. Therefore, targeting mitochondria is an emerging field of research in finding therapeutic strategies to combat aging and neurodegenerative disorders. In fact, recent developments support this idea (reviewed in [53]), and potency of pharmacological agents to prevent or delay age-related neurodegeneration is under investigation [54]. Our recent results with methylene blue (3,7 Bis-dimethylaminophenazathionium; MB) in countering some mitochondrial dysfunctions including mitochondrial complex IV formation, enhanced cellular oxygen consumption and heme synthesis, and reversed premature senescence are aimed at enhancing mitochondrial function, which could contribute to the antisenescence activity of MB [55–57]. Due to the ability of MB to cross the Blood Brain Barrier [58], our studies may provide a potential future therapeutic tool for AD and other related diseases using MB.

MB is known as a redox indicator with a low redox potential, which allows it to cycle readily in mitochondria (Figure 1), and is easily soluble in both water and organic solvents, thus, MB and its derivative MBH_2 can enter the mitochondria and other intracellular compartments such as lysosomes [59]. MB is the first chemical to induce mitochondrial respiratory complex, and we propose a new medical use for MB by increasing brain's reserve of both complex IV and the capacity to synthesize heme [56]. Increasing the activity of complex IV is intriguing as a decrease in complex IV activity causes cytotoxicity leading to increased oxidants production and decreased energy charge of the mitochondria [60–62]. MB in turn may elevate the levels of complex IV and improve mitochondrial function. Complex IV consumes more than 95% of the O_2 that reaches cells, and thus, excess complex IV may play a key role in lowering the production of oxidants by decreasing the steady-state concentration of intracellular O_2 in the mitochondria. Complex IV activity correlates well with the metabolic activity of cells and thus

could improve cognitive performance. On the other hand, enhancing heme synthesis should help neural cells in delaying the onset of the consequences of sequestration of heme by huAβ. Together, these findings suggest that MB may delay the onset of Alzheimer's dementia. Further, MB at higher concentrations (μM range) are neurotoxic, and our findings show that MB is effective in improving mitochondrial function at nM range of concentrations, which is consistent with the intrabrain concentration that can be achieved upon chronic treatment with MB [56]. MB has a long-standing, extensive history of medical uses [63] with an extended medical and safety record in humans, and its FDA approval for clinical trials in connotation to aging and age-related disorders may not be difficult to obtain on safety grounds.

5. Mechanisms of Action of Methylene Blue and Its Clinical Applications

In spite of widespread clinical uses of MB for decades, the mechanism(s) of its diverse biological actions are not clear. MB is readily absorbed by various organs including brain [64], and has a long history of clinical uses including chronic treatments of congenital methemoglobinemia, methemoglobinemia, psychiatric disorders, and more recently in the prevention of the side effects of ifosfamide-induced encepholopathy chemotherapy [65], and hypotension in septic shock [66–68]. MB has also been shown to protect against cyclosporine injury to kidney [69], streptozotocin injury to pancreas [70], ischemic-reperfusion injury [71], radiation [72], and enhances β-oxidation of long chain fatty acids [73]. Clinical doses of MB ranges between 1-2 mg/kg/day for up to 6 times over 24 hrs [74]. Higher doses (>7.5 mg/Kg) of MB cause the formation of Heinz bodies in erythrocytes [75]. In addition, MB administration has been reported to improve the cognitive function in rats, and increase the activity of cytochrome c oxidase (complex IV) [76, 77] and decrease of monoamine oxidase activity in the brain, which may result in an increased dopamine concentration [78]. Some reports have proposed that MB may be acting by inhibiting the NO-activating soluble guanylate cyclase [79], nitric oxide synthase [80], and MAO activity [78]. There are also reports suggesting that MB may be acting as an antioxidant precursor [81, 82]. However, in recent years, it has become quite clear that the biological effects of MB are not consistent with these mechanisms [55, 56, 83]. This discrepancy may in part be due to different doses of MB used in these experimental conditions, which have ranged from >10 μM to <1 μM, suggesting that uses of MB at doses in the nM concentrations may follow a different mechanism [55, 56]. For example the effect of MB on complex IV depends on the dose used [55]. Thus, interpretation of the experimental findings should take into consideration MB's dose especially when high (non-therapeutic) dose is used [84]. Based on our data and others, for the first time, we proposed a molecular mechanism explaining how MB might be affecting mitochondrial function [55–57]. We propose that diaminophenothiazins cycling between the reduced and the oxidized forms may explain, in part, their mitochondria-protecting activity (Figure 1). This mechanism is proposed in Figure 3 and in [55]. It is now an open secret that adequate assembly and activity of complex IV depends upon heme-*a* [85]; thus, the increased rate of heme synthesis with MB treatment could provide cells with heme to support the assembly of complex IV that could result in delaying mitochondrial dysfunction, cellular senescence, aging, and AD.

6. Preventing Aggregation and Oligmerization of Amyloid-β with Osmolytes

Protein misfolding and/or instability leads to aggregate formation. Many neurodegenerative diseases exhibit deposits of aggregated proteins such as Aβ oligomers, senile plaques, phosphorylated tau (p-tau), α-synuclein, and polyglutamine; and all are key neuropathologies in many CNS disorders. Aβ oligomers in AD may progress to form large insoluble fibrils that form the plaques, a key hall mark of AD. Accumulation of these protein deposits exacerbate neurodegeneration due to the fact that the human brain has a limited capacity to prevent the formation or removal of these protein aggregates. It is known that limited protein degradation may contribute to the accumulation of these peptide deposits with age [86]. The aging brain also exhibits limited antioxidant activity and self-repair capacities (e.g., limited neurogenesis).

Our knowledge of the kinetics of the formation of protein aggregates *in vivo* in brain disorders as well as the limited understanding for the mechanism by which these aggregates interfere with neuronal metabolism function has impaired our capabilities for the development of preventative therapeutic strategies. In general, the intermediate species in the cascade of protein misfolding appear to be highly toxic and may interfere with basic metabolic activity of the brain. As for Aβ oligomers it is not clear how toxic they would be in vivo [21]. However, removal or preventing the formation of such intermediate species may prove to be of great clinical value. We demonstrated that the binding of Aβ monomers and oligomers with heme results in sequestration of regulatory heme leading to impaired cellular metabolism. Therefore, preventative approaches to treating AD could be targeted at blocking the formation of Aβ oligomers, enhancing the synthesis of heme and complex IV, in addition to the use of antioxidants.

6.1. Oligmers as Targets for Delaying Alzheimer's Disease. Protein functions depends on maintaining and stabilizing their active conformation(s) under physiological and stress conditions [87]. Thus, under severe physical and chemical stress conditions, biological systems created mechanisms to maintain their functional conformations. These mechanisms are directed at preventing structural perturbations in proteins due to thermodynamic or chemical stressful conditions [88–91]. Biological systems that fail to provide proteins structure stabilizing conditions also fail to adapt to such conditions. As a result, often protein misfolding and aggregation occurs leading to a partial or complete loss of function, in addition to the formation of protein aggregates [92, 93]. Both conditions have serious consequences on cell function and metabolism.

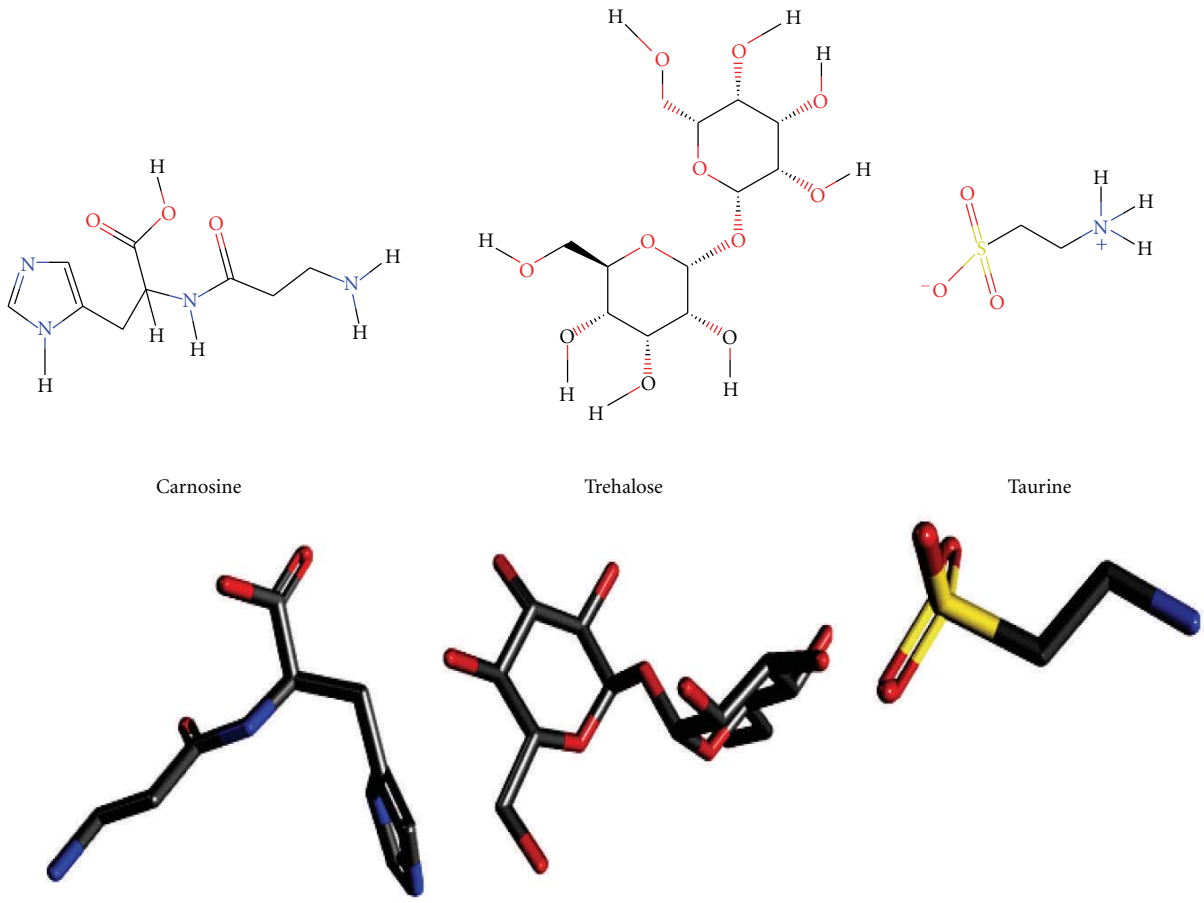

Figure 2: Chemical structure of selected osmolytes. Upper panel: structure in 2D; lower panel, structure in 3D configurations, respectively.

In order to adapt to stressful conditions, tissues created certain mechanisms such as degradation of misfolded proteins or accumulation of small organic solutes at high concentrations. These solutes can serve as antioxidants or play stabilizing role for intracellular structures of macromolecules. Osmolytes [88, 89, 94] are group of endogenous chemicals produced by cells and accumulate to concentration as high as millimolars (Figure 2). The exact function of these metabolites and significance for intermediary metabolism and organ function is still under investigation. We are interested in finding scientific reasoning that links to identify the role of osmolytes and the high tissue connections. Experimental evidence points that osmolytes maintain stability and folding of proteins without perturbing other cellular processes, an action that requires relatively high intracellular concentrations [90, 91, 93, 95–97]. It is well known that following protein synthesis, a highly disordered unfolded state of the polypeptide chain passes through well-defined partially structured transition states before the fully folded protein forms. Molecular chaperones that deter aggregation of incompletely folded species also play role in correctly folding newly synthesized proteins [98]. If under certain conditions, the cellular environment becomes less than optimal for proper protein folding, then newly synthesized proteins become prone to aggregation [99–101]. Similarly, under these conditions, intermediates in protein processing pathways may also be subject to accumulation, misfolding, and aggregation (e.g., Aβ). Protein aggregates, if not quickly removed, may transform to fibrils and other possible aggregates that accumulate in tissue and interfere with cell metabolism [102–107]. It is likely that small aggregates, as well as the highly organized fibrils and plaques, can give rise to pathological conditions, a common feature among many neurodegenerative diseases, including AD [97, 108].

The biological significance of naturally occurring osmolytes has intrigued scientists for many years. There are a number of well-known naturally occurring osmolytes, which fall into three chemical classes: methylamines (trimethylamine-N-oxide, Choline-O-sulphate, and sarcosine), polyols (sorbitol, glycerol, sucrose and trehalose), and certain amino acids and their derivatives (glycine, taurine, proline and betaine) (Table 1). The role of osmolytes in protein folding, cell senescence, cell homeostasis, and mitochondrial structure has been described in various studies. However, more investigations are still needed to evaluate the role of osmolytes in health and diseases and their therapeutic potential.

Osmolytes interact with the peptide backbone and amino acid side-chains [109]. The potency of an osmolyte to promote protein folding and solubility is determined by the balance of these interactions and the solvophobic effects of the osmolyte. There are several studies to support the view

Table 1: List of some known naturally occurring osmolytes in each class with their major presence in protecting the stability of specific proteins under harsh conditions.

Type	Name(s)	Used by
Polyols	mannitol, glycerol, sorbitol, inositol, pinitol	plants, algae, mammalian kidneys, insects, reptiles, fish
Amino Acid	glycine, alanine, and proline	mammalian cells
Amino Acids Derivatives	taurine, octopine, alanine	marine invertebrates, prokaryotes
Methylamines	trimethylamine-N-oxide, sarcosine, phosphorylcholine, glycine betaine	marine invertebrates, plants, mammalian kidneys

that the powerful solvophobic effects of osmolytes on the peptide backbone dominate, such that the relative Gibbs free energy (ΔG) of the unfolded state is less favorable than that of the folded state (ΔG of the peptide folding is more negative).

The presence of several osmolytes inside cells raises questions about their role in protecting intracellular macromolecules under stressful conditions. The antioxidative activity of the osmolytes has been also proposed. Since the protection provided by an osmolyte does not depend on specific chemical interactions with the macromolecules, in principle, any of the osmolytes should be capable of replacing each other, depending upon either endogenous or exogenous availability of particular osmolyte(s) [110]. Since the role of protein backbone is critical in determining thermodynamic stability and folding of proteins in osmolyte solutions [111–115], designing these small molecules (osmolytes) appears to be an excellent strategy and could be a critical step in preventing various critical proteins from misfolding or aggregation (6). This may have far-reaching consequences in understanding and preventing several deleterious diseases that are caused by protein misfolding/aggregation [116, 117]. Since organic osmolytes are naturally occurring molecules, they may have potential therapeutic applications without concerns of major toxic side effects [118].

Because of their capabilities to fold proteins into native-like functional species, osmolytes have been the focus of several studies related to neurodegenerative diseases in which the pathogenesis is associated with the misfolding of specific proteins [119, 120]. These diseases include AD, Huntington's disease (HD), and muscular dystrophy (MD). Trehalose, an osmoluyte, can significantly inhibit polyglutamine-mediated protein aggregation when orally administrated to the transgenic mouse model of Huntington's disease (a neurodegenerative disease) [114], and can increase the life span of Huntington's disease mouse model [114], indicating that trehalose is readily bioavailable. Osmolytes have similar effect of the folding of androgen receptor containing elongated polyglutamine chain length. Elongated polyglutamine chain in androgen receptor leads to formation of its aggregation and thus play role in causing neurodegeneration in Kennedy's disease [116].

Experimental finding suggests that AD toxicity could be linked to the formation of oligomeric forms of Aβ peptides and AD progression correlates with increasing aggregate formation of Aβ. At physiological concentration, Aβ_{40} peptide incubated in the presence of trehalose inhibits aggregation of this peptide in a dose-dependent manner [121], and this osmolyte-mediated inhibition of Aβ_{40} peptide aggregation correlates with its toxic effects in neuronal cell system [121].

In these pathological conditions, specific misfolded aggregate-prone proteins are resistant to the normal cellular processes of protein folding and turnover and we propose that osmolytes may interfere with the production and/or the removal of these toxic intermediate aggregates. Further, these osmolytes can be attractive molecules for delaying the onset of neurodegenerative diseases characterized by protein misfolding and toxic aggregation. In particular, osmolytes may have therapeutic potential for treating AD, because of their effect on Aβ oligomers. Osmolytes may also help stabilize the senile plaques, preventing the shedding of Aβ oligomers. Osmolytes are also known to function as antioxidants [122, 123], and their level seems to decline in AD patients [124].

The research on naturally occurring osmolytes suggests that they have a protective role in promoting brain health, including resistance to neurodegeneration. Thus, there is potential for both prevention and treatment of neurodegenerative diseases using osmolytes [125, 126]. The prospect of using natural osmolytes as a therapeutic tool for AD appears to be quite exciting and can have far reaching consequences in developing therapeutic tools for its prevention and/or management. However, while these proposals appear to be quite promising, more studies are needed to validate their effectiveness as a potential therapeutic target.

7. Summary

The mitochondrial role in health and disease has recently received immense scientific interest, particularly, because of the emerging field of mitochondria as a potential therapeutic target. Mitochondria play intricate role in energy, redox, and intermediary metabolism positioning them in cross road for health or disease. Genetic, environmental, and life style factors can lead to impairments in mitochondrial function. Impairment to mitochondrial function is found in numerous age-related degenerative diseases and disorders. Mitochondrial dysfunction leads to an increase in oxidative stress, energy hypometabolism, and impairs calcium homeostasis, ultimately leading to inadequate cellular function. Preventing mitochondrial dysfunction is presumed to have functional benefits, regardless of whether mitochondrial dysfunction is primary or secondary [57]. Thus, therapeutic strategies to improve mitochondrial function and delay the onset of age-related degenerative disorders are currently under investigation. We have shown that diaminophenothiazins (e.g., methylene blue, thionine, Figure 1) can delay cellular senescence by enhancing mitochondrial function, which are impaired in AD brains.

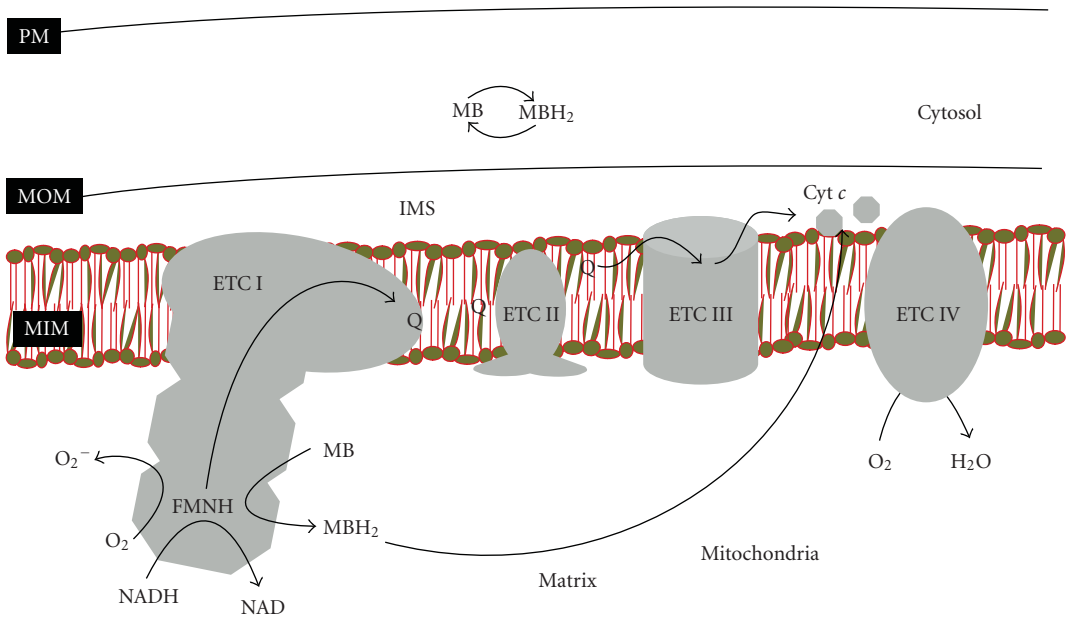

Figure 3: The proposed model for the interaction of methylene blue with specific mitochondrial and cellular components. ETC followed by roman number refers to the specific components of electron transport chain (ETC) of the mitochondria. MIM, MOM, and IMS refers to the mitochondrial inner membrane, mitochondrial outer membrane, and intermembrane space, respectively. MB and MBH_2 refer to oxidized and reduced forms of methylene blue, respectively. The four complexes are: complex I (ETC I), complex II (ETC II), complex III (ETC III), and complex IV (ETC IV) in addition to ATP synthase (i.e., complex V). The electron transfer through each one of the ETC starts at ETC I, which catalyzes two electrons oxidation of NADH and continues until water is formed on ETC IV. Coenzyme Q serves as low-molecular weight electron carrier from ETCs I and II to III. Cytochrome *c* (cyt *c*) serves as electron carrier from ETC III to ETC IV. Production of superoxide radical from complex I is proposed to be prevented by MB, which serves as electron carrier that competes with molecular oxygen on the electrons "leaking" from complex I. During this process MB is converted to MBH_2. Then MBH_2, a reduce MB, carries the electrons to cytochrome *c*, which is then oxidized by ETC IV [55].

Mounting evidence suggests a role of small aggregates of amyloid-β (Aβ) in the etiology of AD. Aβ aggregates impair mitochondrial function, synaptic function, Ca^{++} homeostasis and ultimately leading to cellular hypometabolism and neurdegeneration. Aβ peptide can also be localized within the mitochondria of AD patients. Experimental evidence show that osmolytes can stabilize and enhance cellular proteins to adopt physiologically compatible conformation. Osmolytes are efficient antioxidants that may also increase neural resistance to oxidative stress caused by Aβ. Thus, osmolytes may interfere with the aggregation of Aβ, enhance their proteolytic clearance, and counter oxidative stress [57].

We propose two different approaches to prevent or delay the onset of AD. The first is directed at enhancing mitochondrial activity using MB to enhance mitochondrial function (Figure 3). Energy deficiency in AD may be contributed by impaired insulin (glucose) metabolism and mitochondrial function. Thus, concentrating on single impairment at the time would not be enough to resolve the energy hypometabolism in AD. Glucose metabolism depends on adequately functioning mitochondria and vice versa. Since both glucose and mitochondrial metabolism are interconnected, it might be more beneficial for AD patients to develop a therapeutic approach that resolves (or delay) both impairments. Successful merger of treatment with MB and intranasal delivery of insulin to the brain may prove valuable for AD patients. MB exerts its effect at very low (nM) concentration, which in conjunction with its safety record in humans further minimizes any risk of side effects of chronic exposure to MB. The second approach is directed at preventing the aggregation of Aβ by using osmolytes, natural metabolites synthesized in the brain. Preventing the aggregation of Aβ may enhance their proteolytic removal and decrease the risk of their interference with heme and mitochondrial metabolism. MB can also induce heme synthesis, thus, when combined with osmolytes, may assist in preventing heme deficiency. We propose MB and osmolytes could help delay the onset of AD by preventing Aβ oligomers formation, enhancing mitochondrial function, and attenuating heme deficiency.

We propose that preventative approaches for AD could be targeted at blocking the formation of Aβ oligomers, enhancing the synthesis of heme and complex IV, in addition to the use of antioxidants.

Conflict of Interests

Dr. Atamna has applied for patent on MB to treat mitochondrial dysfunction.

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