Neurodegeneration in Accelerated Aging

Morten Scheibye-Knudsen

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Official opponents:
Alexander Bürkle, University of Konstanz
Lars Eide, University of Oslo

Correspondence: Center for Healthy Aging, Department of Cellular and Molecular Medicine, Faculty of Health and Medical Sciences, University of Copenhagen

E-mail: mscheibye@sund.ku.dk

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INTRODUCTION

The global elderly population has been progressively increasing throughout the 20th century and this growth is projected to persist into the late 21st century resulting in 20% of the total world population being aged 65 or more by the year 2100 (Figure 1). 80% of the total cost of health care is accrued after 40 years of age where chronic diseases become prevalent [1, 2]. With an exponential increase in health care costs, it follows that the chronic diseases that accumulate in an aging population poses a serious socioeconomic problem. Finding treatments to age related diseases, therefore becomes increasingly more pertinent as the population ages. Even more so since there appears to be a continuous increase in the prevalence of chronic diseases in the aging population [3]. In other words it seems that the aging population is not only growing larger it is also becoming increasingly unhealthy compared to the elderly population decades ago. Research into the mechanisms of aging and age related diseases is therefore imperative.

Recent decades have brought a wealth of knowledge about aging in various organisms. Genetic studies in yeast and worms have shown several conserved and overlapping molecular pathways

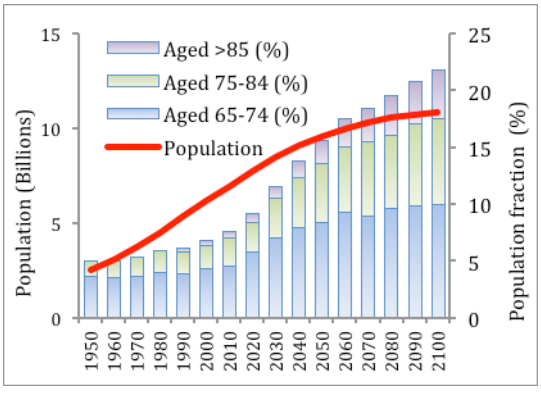

Figure 1. Recorded and projected population growth and age demographics (source: United Nations)

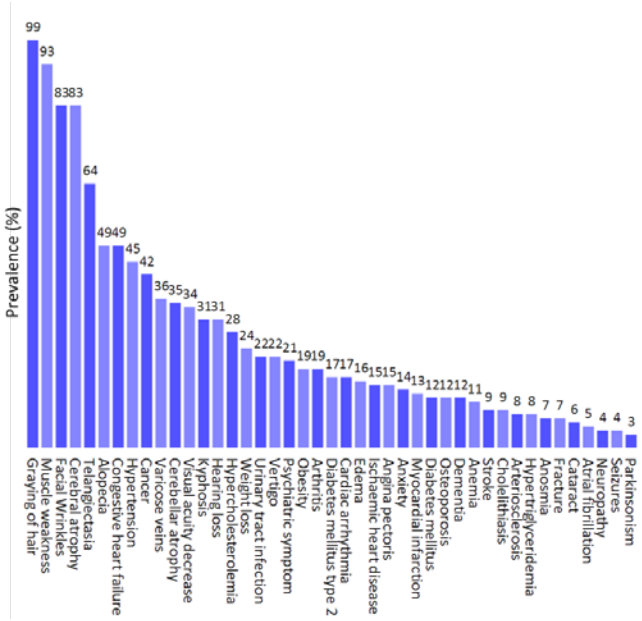

Figure 2. The phenotype of human aging.

that appear to regulate the aging process [4,5]. These include the insulin and IGF-1 signaling cascades [4], protein synthesis and quality control [6], regulation of cell proliferation through factors such as mTOR [7], stem cell maintenance 8 as well as mitochondrial preservation [9]. Most of these pathways are conserved through evolution and appear to regulate aging in many lower organisms. In humans, the discovery of a number of inherited disorders characterized by accelerated aging and defects in DNA repair has underscored the importance of genome maintenance in aging. Interestingly, each of these accelerated aging disorders shows only certain features of aging, hence these diseases are also termed segmental progerias. Although there is some overlap in the clinical picture of the disorders, there are also distinct features that distinguish one disease from another. This may indicate that the underlying cause of aging is multifactorial [10]. Indeed, normal human aging also show a distribution of signs and symptoms indicating that we do not all age in exactly the same way (Figure 2) [11-24]. For example only a subset of us will suffer from stroke, dementia, cancer etc. In that sense it is likely that multiple genetic, epigenetic and environmental factors regulate the aging process leading to variation in the aging phenotype among people. Interestingly, graying of hair, muscle weakness and facial wrinkles are almost universally present with age. These traits could thus represent outcome measures in a human aging intervention trial. From an interventional perspective studying normal human aging may, however, be problematic particularly because of the long lifespan of humans. In that regard, the accelerated aging disorders represent a significant opportunity to study the aging process in a setting where the genetic defect has been identified. In the following I will briefly go through the genetic background and clinical traits of these complex accelerated aging disorders.

Disease	Gene	Protein function	Tissues affected
Ataxia-telangiectasia	ATM	DNA repair	Brain, vasculature, immune cells, bone growth
Bloom syndrome	BLM	DNA repair and replication	Gonads, immune cells, vasculature, skin, endocrine pancreas, bone growth
Cockayne syndrome	ERCC6 (CSB), ERCC8 (CSA)	DNA repair and transcription	Brain, vasculature, bone growth
Dyskeratosis congenita	TERC, TERT, DKC1, NOLA3	Telomere maintenance	Skin, bone marrow, lung
Fanconi anemia	FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN, FANCP, RAD51, XPF	DNA repair	Skin, bone marrow, bone growth, gonads, brain
Hutchinson-Gilford progeria	LMNA	Nuclear architecture	Skin, bone, vasculature
Nestor-Guillermo progeria	BANF1	DNA replication (?)	Skin, bone
Rothmund-Thomson syndrome	RECQ4	DNA repair and replication	Skin, bone, vasculature
Werner syndrome	WRN	DNA repair and telomere maintenance	Skin, bone, vasculature, gonads, endocrine pancreas
Xeroderma pigmentosum	XPA, XPB, XPC, XPD, XPE, XPF, XPG, XPV	DNA repair and transcription	Skin, brain, bone growth

Table 1. A list of diseases that could be characterized as displaying some signs of accelerated aging including mutated gene(s), possible altered pathways and the main tissues affected.

ACCELERATED AGING

Since the early 1900s close to a dozen diseases, that show similarities to normal human aging have been characterized (Table 1). It should be noted that there is currently no consensus of which diseases should be included in a list of accelerated aging disorders and the list presented herein includes some diseases that may only bear slight similarities to normal aging. There is, however, substantial clinical overlap between many of these disorders, perhaps reflecting some commonality in the etiology of the diseases. Indeed, these disorders all have alterations in pathways involved in genome maintenance, highlighting the importance of preserving the integrity of DNA as we age. In the following sections I will briefly introduce these disorders.

Werner syndrome

Werner syndrome is an autosomal recessive disease caused by mutations in the DNA helicase Wrn [25]. Helicases are a group of enzymes able to unwind a double stranded DNA molecule into two single stranded DNA molecules. Wrn is a part of the RecQ family of helicases together with RecQL1, Blm, RecQL4 and RecQL5 [26, 27]. Werner syndrome is most commonly characterized by normal development until the teenage years, although the Wrn syndrome patients generally are of short stature [28-30]. Patients typically develop an aged appearance with gray hair, hair loss, hoarse voice and thin/scleroderma-like skin in their twenties. Later in life, patients develop cataracts, diabetes, osteoporosis and atherosclerosis, and they often die from cardiovascular disease in their fifties. Although, major neurodegeneration is not usually present in this disease global cerebral atrophy occurs at a high prevalence [29]. Based on the clinical progression, Werner syndrome therefore relatively closely phenocopies human aging. Even though the genetic cause of Werner syndrome has been determined, the exact pathogenesis is still unknown. The WRN helicase has been shown to participate in DNA repair and replication [26] as well as in more direct maintenance of telomeres, the specialized DNA structure that protects the end of the chromosomes [31]. These processes are all particularly important for maintaining the functionality of dividing cells and the clinical phenotype, where pathology in proliferating tissues predominate, could thus reflect the known biochemical activities of the Wrn helicase.

Hutchinson-Gilford progeria syndrome

Hutchinson-Gilford progeria syndrome (HGPS) is an autosomal dominant disease predominantly caused by a single mutation in the LMNA gene leading to alternative splicing of the transcript and the formation of a truncated protein termed progerin [32]. The accelerated aging features manifest early in the childhood of HGPS patients with growth delay, loss of adipose tissue, hair loss and progressive vascular changes [33]. Children usually die in their teens due to complications to their accelerated atherosclerosis [33]. Like the other accelerated aging disorders the exact pathogenesis is unknown. Progerin accumulates in HGPS cells and is believed to interfere with the normal function of the LMNA gene product, lamin A. Lamin A is involved in the organization of the nuclear matrix and morphological changes to the nucleus is a hallmark cytological feature of HGPS patients [34]. It has been proposed that progerin may interfere with transcription, DNA repair as well as alter mitochondrial function [35]. Interestingly, progerin may also accumulate with aging in cells from normal individuals although its role in the normal aging process is currently speculative [36].

Nestor-Guillermo progeria syndrome

Nestor-Guillermo progeria syndrome is a recently characterized disease caused by mutations in the gene BANF1 [37, 38]. Only two patients have been described suffering from this exceedingly rare disorder. The disease is, however, clinically quite interesting. The patients appear to be of short stature, develop skin atrophy and pigmentation changes, show loss of adipose tissue, hair loss and skeletal deformities. The overall clinical phenotype is thus rather similar to HGPS. Surprisingly, the patients do not develop cardiovascular changes or diabetes as is common in HGPS perhaps indicating that the mechanisms of vascular aging may be separate from for example skin aging. BANF1 encodes a gene possibly involved in maintaining the structure of the nuclear envelope through interactions with emerin and, notably, lamin A [39, 40].

Bloom syndrome

Bloom syndrome is an autosomal recessive disorder caused by mutations in the RecQ family DNA helicase BLM [41, 42]. The disease is characterized by severe short stature, skin pigmentation changes, telangiectasia and a predisposition to cancer [43]. The Blm helicase is believed to be particularly important for the specific pathway of DNA repair that deals with double stranded breaks (see below) and this deficiency may explain the greatly increased cancer risk these patients display [26, 27]. Notably, the spectrum of cancers is close to what is seen in the normal population albeit at an earlier age. Thus, one could argue that the carcinogenesis in Bloom syndrome may represent an aspect of accelerated aging. In addition to the role in double stranded DNA repair, this enzyme is believed to participate in DNA replication [44] perhaps explaining the severe growth deficiency that is seen in Bloom syndrome patients. Mental retardation has been reported in a few patients [45], however, neurodegeneration is not commonly described in Bloom syndrome patients.

Rothmund-Thomson syndrome

Rothmund-Thomson syndrome (RTS) is caused by mutations in the fourth member of the RecQ family of DNA helicases, RecQL4 [46]. The disease is characterized by characteristic skin changes occurring in early childhood [47]. The prodrome is typically an erythematous butterfly rash covering the cheeks while poikiloderma (skin atrophy, telangiectasia and pigmentary changes) become manifest at later stages. In addition, the patients display skeletal malformations, such as radial ray defects, short stature and hair loss [47]. Interestingly, these patients appear particularly prone to develop osteosarcoma, a type of cancer that is very rare in the general population [47, 48]. Neurodegeneration has not been reported in RTS patients. Mechanistically, RecQL4 is believed to participate in DNA replication as well as in telomere maintenance, however, the pathogenesis in RTS remain largely unknown [25, 26].

Dyskeratosis congenita

Dyskeratosis congenity (DC) is caused by recessive or dominant mutations in genes involved in the maintenance of telomeres. The most commonly mutated genes are TERT, TERC, NOLA2, NOLA3 and TINF2 and mutations in these enzymes cause telomere shortening and the development of DC [49]. DC is characterized by leukoplakia, nail dystrophy, abnormal skin pigmentation and later bone marrow failure [50]. In addition, hair loss, short stature, pulmonary fibrosis, developmental delay and cancer occur to minor degrees [50]. The pathogenesis is to some extent better explained in DC than in many other accelerated aging disorders. As mentioned, telomeres are the specialized DNA structures that form the ends of the chromosomes. Exposed ends of DNA, will under normal circumstances be interpreted by the cell as a broken DNA strand and activate a DNA damage response. It is therefore generally believed that the telomere structures are needed to avoid the activation of a DNA damage response [51]. In addition, a small portion of the very end of the telomere is lost with each replicative cycle and progressive telomere shortening has been shown to occur at least in some tissues [52]. When telomeres become sufficiently short they will no longer be able to suppress a DNA damage response and senescence, transformation or apoptosis will ensue. In DC accelerated telomere loss occur particularly in rapidly proliferating tissues such as skin and bone marrow. Nevertheless, some DC patients also develop neurological decline as well as cerebellar degeneration [50, 53]. These patients are, however, relatively rare and neurodegeneration is not a common feature of DC [50].

Fanconi anemia

Fanconi anemia is characterized by progressive bone marrow failure, growth retardation, skeletal deformities and a predisposition for malignancies [54-56]. Anemia is relatively prevalent in the elderly population and although Fanconi anemia is often not included in lists of accelerated aging diseases this disorder could potentially represent bone marrow aging. Interestingly, the patients also show alterations in endocrine regulation such as hyperinsulinemia, hypothyroidism and others [57]. The disease is caused by mutations in a growing list of genes that appear to partake in DNA replication and the repair of the rare but toxic DNA lesion, the inter-strand crosslink (see below) [58]. Cells from Fanconi anemia patients display chromosomal instability particularly in the bone marrow potentially explaining the defect in this rapidly replicating tissue. Although many patients show microcephaly, progressive neurodegeneration is not common [54-57].

Ataxia-Telangiectasia

Ataxia-telangiectasia (AT) is autosomal recessively inherited and caused by mutations in ATM. It is characterized by cerebellar degeneration leading to ataxia, telangiectasia, immune deficiency and a predisposition to cancer [59, 60]. In addition, AT patients show growth retardation and commonly display weight loss despite normal food intake [59]. The patients usually present in early childhood with progressive ataxia leading to loss of ambulation at around 10 years of age. Neuropathy and muscle weakness follow. Mechanistically, ATM is a kinase that is activated upon genotoxic stress and is thereby involved in the signaling cascade following DNA damage, particularly double stranded DNA breaks [61]. Although the role of ATM in this signaling cascade is rather well elucidated, it remains unknown why deficiency in this enzyme leads to a strong neurodegenerative phenotype. Indeed, other diseases with defective repair of double stranded DNA breaks such as Bloom syndrome, Werner syndrome, RTS, Nijmegen breakage syndrome and Warsaw breakage syndrome do not show the characteristic cerebellar degeneration observed in this disorder [28, 43, 47, 62, 63]. This potentially indicates that other pathways than the canonical double stranded DNA repair pathway may be altered in AT patients.

Cockayne syndrome

Cockayne syndrome is an autosomal recessively inherited early onset accelerated aging disorder caused by mutations in either ERCC6 (CSB) or ERCC8 (CSA). The disease is characterized by severe neurodegeneration, cachexia and dwarfism as well as hypersensitivity to sunlight but interestingly no predisposition to cancer [64]. Patients usually develop normally the first year of life before growth retardation and psychomotor regression becomes apparent. The neurodegeneration is characterized by severe cerebellar and cerebral degeneration as well as leukodystrophy and neuropathy [65]. In addition, sensorineural hearing loss, pigmentary retinopathy and seizures are common [66]. CSA and CSB were first characterized as being components of a DNA repair pathway called transcription coupled nucleotide excision repair [67-70]. This pathway is responsible for the removal of UV induced DNA damage explaining the hypersensitivity that patients show towards sunlight. UV irradiation does not penetrate to the

Clinical trait	XPA	CS	ATM
Ataxia	+	+	+
Cerebellar atrophy	+	+	+
Peripheral neuropathy	+	+	+
Short stature	+	+	+
Cancer	+		+
Chorea	+		+
Dysarthria	+		+
Cerebral atrophy	+	+	
Developmental delay	+	+	
Sensorineural hearing loss	+	+	
Sun sensitivity	+	+	
Mental retardation	+		
Microcephaly	+	+	
Areflexia		+	+
Demyelination		+	+
Nystagmus		+	+
Weight loss		+	+
Athetosis			+
Dystonia			+
Immune deficiency			+
Increased blood α-foeto protein			+
Oculomotor apraxia			+
Strabismus			+
Telangiectasia			+
Basal ganglia pathology	+	+	
Cataracts		+	
Contractures		+	
Dental caries		+	
Hyperactive reflexes		+	
Hypertension		+	
Kyphosis		+	
Lactic acidosis in the CNS		+	
Leukodystrophy		+	
Optic atrophy		+	
Pruritus		+	
Retinitis pigmentosa		+	
Seizures		+	
Tremor		+	
Vomiting		+	
Xerophthalmus		+	

Table 2. The clinical features of XPA, Cockayne syndrome and ataxia-telangiectasia show substantial overlap with mitochondrial diseases. Left column denotes clinical features. The features marked in red are commonly seen in mitochondrial disorders (see www.mitodb.com).

central nervous system and alternative roles for CSA and CSB have been proposed to explain the strong neurodegenerative phenotype. These include roles in basal transcription 71, base excision DNA repair 72, nucleosome remodeling 73 and others.

Xeroderma pigmentosum

Xeroderma pigmentosum is an autosomoal recessive disease caused by mutations in one of several genes (XPA, XPB, XPC, XPD, XPE, XPF, XPG, XPV) 74. Interestingly, XP was the first disease linked to defects in DNA repair and thus laid the foundation for an entire field of research and numerous later discoveries [75]. The disease is characterized by sun sensitivity leading to early onset skin cancer. In addition to the dermatological involvement several complementation groups show neurological features that are similar to what is seen in Cockayne syndrome and ataxia-telangiectasia [59, 64, 74, 76]. In particular patients belonging to the XPA complementation group appear to have a strong neurodegenerative phenotype with severe cerebral and cerebellar degeneration, ataxia, neuropathy, hearing loss, basal ganglia pathology and other [76]. Mechanistically, the XP group of proteins are involved in the DNA repair pathway nucleotide excision repair [77]. Further, several XP proteins are a part of the TFIIH complex thereby regulating transcription [77]. A few of the proteins have also been implicated in the DNA repair pathway that deals with interstrand DNA cross links [78]. The mechanistic pathogenesis for skin cancer and sun sensitivity is well explained by the role of XP proteins in the DNA repair of UV induced lesions, however, as in the case of Cockayne syndrome the pathogenesis leading to neurodegeneration has been speculative.

From a neuronal perspective Cockayne syndrome, ataxia-telangiectasia and xeroderma pigmentosum complementation group A are of particular interest because of the high prevalence of neurodegeneration in these diseases. Notably, cerebellar degeneration leading to ataxia, neuropathy and sensorineural hearing loss as well as a number of other neurological features are particularly prominent features in these disorders [64, 76, 79]. Further, in Cockayne syndrome lactic acid accumulates in the brain [65]. These neurodegenerative features are strikingly similar to what is observed in many bona fide mitochondrial disorders (Table 2) [80].

Since it has been difficult to explain the pathogenesis in these disorder based solely on the known functions in DNA repair the underlying pathogenesis of the neurodegeneration in Cockayne syndrome, ataxia-telangiectasia and xeroderma pigmentosum, group A, could thus be hypothesized to be due to mitochondrial dysfunction. I will in the following introduce this organelle and some pathways that are important for maintaining a healthy and functional pool of mitochondria.

MITOCHONDRIA

More than a billion years ago before complex organisms had evolved a roaming eukaryotic progenitor (pro-eukaryote) engulfed a small oxygen consuming prokaryote. For inexplicable reasons, the prokaryote escaped its grim fate and the two organisms started the arguably most successful collaboration in the history of life on this planet. The pro-eukaryote could shelter the prokaryote while the prokaryote could utilize the increasing atmospheric oxygen tension to produce a surplus of energy. A billion years later this collaboration led to the formation of multicellular organisms and to the very text you are reading at this moment. We have named our prokaryotic guest “the mitochondria” and have named the description of this event the endosymbiotic theory of the origin of the mitochondria [81]. Indeed, with a single exception [82], all metazoans contain mitochondria.

Because of the exogenous prokaryotic origin of this organelle it contains its own circular genome. Over the millennia genes have slowly been transferred to the nucleus with only 37 genes now remaining in the 16.6 kilobase genome. Of these only 13 are translated into proteins, while 22 are tRNAs and 2 are rRNAs. The vast majority of the more than 1500 proteins in the mitochondrial proteome are thus encoded in the nucleus [83]. Among these are all

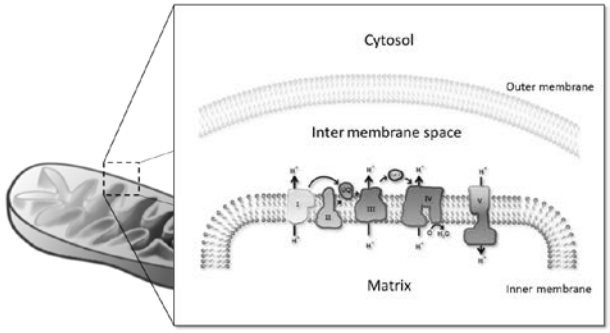

Figure 3. A simplified representation of the mitochondrial architecture. The mitochondrion is made up of an outer lipid bilayer (outer membrane), an inter membrane space, an inner membrane and a matrix space. Oxidative phosphorylation takes place in the matrix utilizing an electrochemical gradient generated by transfer of matrix protons to the inter membrane space by complex I,III and IV. Complex V uses the gradient to phosphorylate ADP to ATP.

the genes involved in mitochondrial DNA metabolism and maintenance. Nuclear encoded proteins are transported to the mitochondria via a transport mechanism that commonly, but not exclusively, recognizes a mitochondrial targeting sequence in the N-terminal amino acid code of the translated protein [84]. Protein transport is further complicated by the specialized architecture of the mitochondria that contain two lipid bilayers. After initial import of the protein further downstream regulation of the final destination is thought to occur in part through various sorting sequences in the N-terminal region of the protein [85]. Thus, the mitochondria are compartmentalized via an outer and an inner lipid bilayer into several partitions. These are from the outside in: the outer membrane, the inter membrane space, a highly folded cristae forming inner membrane and the matrix (Figure 3).
This interesting architecture allows for the formation of specialized milieus that facilitates efficient execution of mitochondrial function. Arguable the most important function of this organelle is ATP production. ATP is the energy currency of the cell and is required for numerous reactions that make up the living organism. The generation of ATP occurs through the formation of an electrochemical gradient across the inner membrane by the electron transport chain (complex I-IV) driven by the reduction of molecular oxygen to water as well as NADH or FADH2 to NAD+ or FAD respectively. The energy stored in the electrochemical gradient is then used to phosphorylate ADP to ATP in the matrix at complex V. In addition to ATP production through oxidative phosphorylation this organelle is involved in many other processes such as apoptosis, calcium regulation, heat production, heme- and hormone synthesis and others [86]. In the following sections I examine the role of mitochondria in aging and discuss a few pathways involved in the maintenance of a healthy pool of mitochondria.

Mitochondrial theory of aging

The mitochondria facilitate the metabolism of carbohydrates, fatty acids and under certain conditions amino acids to CO¬2. This allows a far higher energy output per carbon moiety compared with simple glycolysis. O2¬, NADH and FADH2 are, due to their redox potential, the vehicles that drive the turnover of carbohydrates through oxidative phosphorylation. Here, O2 is used in the electron transport chain to form an electrochemical membrane potential by pumping H+ from the matrix across the inner mitochondrial membrane. This membrane potential is then utilized by the ATP synthase (complex V) to synthesize ATP and in addition facilitates secondary transport of biomolecules across the inner membrane. However, the substantial reactivity of O2 represents a double edged sword. Notably, electrons may escape the electron transport chain and reduce O2 leading to the formation of a reactive oxygen species (ROS), the superoxide radical O2-. This molecule can be further converted into H2O2 (hydrogen peroxide) by the enzyme superoxide dismutase and/or to the highly reactive hydroxyl radical (OH•) via the Haber-Weiss reaction. ROS can readily react with macromolecules such as proteins, lipids and nucleic acids in their surroundings. It was proposed almost 60 years ago that the accumulation of oxidative damage to these molecules would lead to the decline of the organism and finally death [87]. Later this theory has been refined into a number of theories. For example, that damage may be particularly detrimental when affecting DNA and further, that an accumulation of mitochondrial DNA damage may be driving the aging process. In this model progressive mitochondrial dysfunction leads to increased ROS production that will facilitate further mitochondrial damage thereby forming a vicious cycle. At some hypothetical point the mitochondrial damage becomes too great for survival of the cells, tissue and organism.
A number of studies suggest that mitochondrial maintenance may be important in aging. It was early recognized that mitochondria accumulate deletions and oxidative damage with aging [88-92]. In support of the idea that oxidative mitochondrial damage may be important in aging, a transgenic mouse overexpressing the antioxidant enzyme catalase targeted to mitochondria was found to have increased lifespan [93]. Further, a mouse model that harbored a mutated version of the mitochondrial DNA polymerase (pol gamma) that introduces mutations in the mitochondrial genome during replication showed significantly shortened lifespan 9. In addition, HIV patients undergoing antiretroviral treatments show accumulation of mitochondrial DNA damage that may contribute to an HIV related accelerated aging phenotype [94]. Moreover, mitochondrial dysfunction has been shown in almost every age associated chronic disease such as Alzheimer's disease [95, 96], Parkinson's diseases [97], cardiovascular disease [98] and diabetes [99]. Despite these studies corroborating a mitochondrial

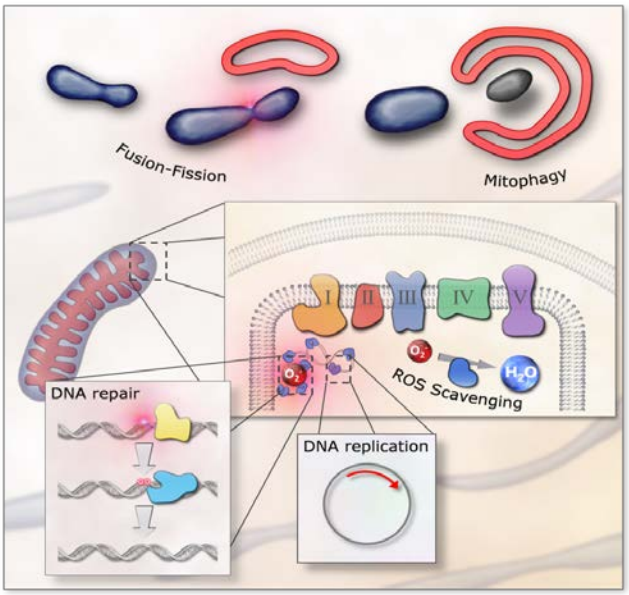

Figure 4. Mitochondrial maintenance pathways. Several pathways have evolved that maintain mitochondrial integrity. These include faithful DNA replication; efficient DNA repair; enzymes and molecules that remove reactive oxygen species (ROS); mitochondrial morphology regulation through fission and fusion; and whole organelle removal through mitophagy.

theory of aging, other studies have questioned this model. For example in yeast and nematodes slightly decreased mitochondrial function and increased ROS production leads to increased longevity [100-102]. In addition, although older literature has suggested an increased mutational load with aging, next generation sequencing has shown less clear results. For example there was no correlation with mutational load and aging in normal C57Bl6 mice [103] and in the brain of healthy humans point mutation frequencies were several orders of magnitude lower than previously reported 104. Since the mutational load has to reach as much as 60-90% of all mtDNA molecules to yield pathology [105, 106] it is difficult to understand how the reported low frequency of mutations/deletions can add to an aging phenotype. One proposed hypothesis that could explain the low frequency of mitochondrial DNA alterations in aging is the idea of a clonal expansion of mitochondria containing deletions in a subset of the cells in a tissue [107]. Although the role of mitochondrial DNA mutations in aging is still being elucidated, many pathways
associated with longevity such as the sirtuins [108], mTOR [109], IGF-1 [110], FOXO [111] etc. also regulate mitochondrial function . In summary, there appears to be a complex relationship between mitochondrial function and aging, however the mitochondrial theory of aging has not yet answered the central question: Why do we age?

MITOCHONDRIAL MAINTENANCE

A number of pathways appear to be necessary for proper mitochondrial DNA maintenance and segregation. These include mitochondrial DNA replication, repair, ROS scavenging as well as macromolecular dynamics such as fusion-fission processes and whole mitochondrial degradation termed mitophagy (Figure 4). Based on the volatile nature of ROS it is not surprising that pathways have evolved that attempt to minimize or repair the damage that may occur as a result of oxidative phosphorylation. I will in the following sections focus specifically on some processes that deal with ROS regulation and damage reversal.

Uncoupling proteins

The uncoupling proteins (UCPs) are believed to represent an early step in the limitation of ROS production. These proteins allow protons to fall back through the inner mitochondrial membrane thereby decreasing the membrane potential. Since mitochondrial ROS production is thought to be caused by the escape of electrons from the electron transport chain (ETC) a decrease in the electrochemical gradient attenuates the resistance in the ETC allowing more electrons to make it safely to complex IV. Five members of the UCP family are found in humans. The first, UCP1, was discovered in brown adipose tissue where it facilitates the production of heat by uncoupling respiration from ATP production [112]. While UCP1 is predominantly expressed in brown adipose tissue UCP2 is more ubiquitously expressed [113]. Interestingly, UCP2 is strongly expressed in the central nervous system and this may be important for the pathogenesis of neurodegeneration in DNA repair disorders as I shall discuss later. UCP2 is tightly regulated by a number of pathways and has an unusually short half-life (~30 minutes) several orders of magnitude shorter than the average mitochondrial protein [114, 115]. One important regulator of UCP2 is the central master transcription factor peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1α) [116]. Indeed, the PGC-1 family of transcription factors was first discovered to regulate UCP1 expression in brown adipose tissue as a part of the adaptive thermogenesis response [117]. A year later PGC-1α was found to regulate mitochondrial biogenesis and UCP2 possibly through nuclear respiratory factor 1 (NRF1) [118]. We now know that PGC-1α is regulated through a number of processes, including key longevity regulators such as the mechanistic Target of Rapamycin (mTOR), AMP activated protein kinase (AMPK) and SIRT1 [119-121]. In the brain PGC-1α may be particularly important since PGC- 1α knockout mice display neurodegeneration and PGC-1α deficient neurons show defective synaptic plasticity [122, 123]. The PGC-1α-UCP2 axis may be particularly pertinent since UCP2 confers neuroprotection to various stresses [124]. Indeed, as I will describe in greater detail below, we find that UCP2 may be central in the pathogenesis of neurodegenerative DNA repair disorders.

Reducing agent	Reaction
Superoxide dismutase	$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$
Peroxiredoxins	$2RSH + H_2O_2 \rightarrow RSSR + 2H_2O$
Glutathione peroxidase / Glutathione	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$
Vitamin E/C	$Vit_{red} + RO_2 \rightarrow Vit_{Ox} + RH$

Table 3. A list of common enzymatic and non-enzymatic antioxidants.

Antioxidants

In addition to UCPs the mammalian organism has evolved a number of ways to eliminate excess ROS formation. These are generally known as antioxidants (Table 3).
The organism utilizes both enzymatic reactions such as the catalase reaction as well as non-enzymatic reactions such as glutathione, vitamin E and vitamin C. Interestingly, some evidence supports the idea that ROS quenching may lead to lifespan extension. This is most strongly supported by transgenic mice overexpressing catalase targeted to mitochondria [93]. Based on the idea that ROS generation may attenuate age associated diseases a number of large scale population studies have investigated the effect of oral supplementation with antioxidants. Unfortunately, these trials have been largely unsuccessful and this therapeutic avenue has by-and-large been abandoned by the scientific community [125-127]. Although it is still rather speculative a possible reason for these disappointing results may be the idea that ROS molecules play important roles as signaling molecules as well as in the immune response. Indeed, as mentioned mild increased ROS production extends lifespan in roundworms probably through an adaptive increase in stress resistance pathways [128]. Attenuating these pathways could thereby represent an unfortunate side effect of antioxidant supplementation. For more in-depth description of the topic of oxidative stress and free radical scavenging I refer the reader to recent reviews [129, 130].

DNA repair

If antioxidants fail to scavenge the ROS these molecules can react with most macromolecules in the cell including proteins, lipids, RNA and DNA. The organism has evolved a number of sophisticated pathways to deal with this damage and, although a wealth of research has underlined the importance of lipid and protein oxidation, I will focus on DNA damage. Given the complexity of the DNA molecule it is not surprising that a myriad of different lesions occur in the genome. The continuous bombardment of the DNA constitutes a significant evolutionary pressure that early on led to the formation of a number of specific pathways that can repair these lesions. Thus most of the repair pathways that are ongoing

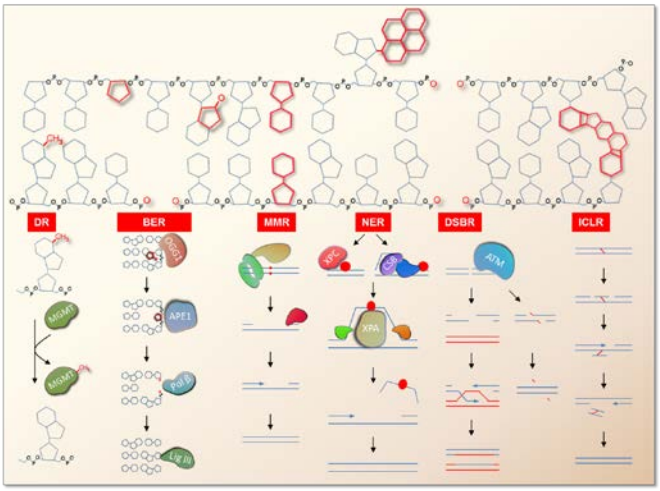

Figure 5. A simplified model of the DNA repair pathways present in the nucleus. The top represents the DNA helix with examples of lesions highlighted in red. DR: Direct reversal. BER: Base excision repair. MMR: Mismatch repair. NER: Nucleotide excision repair. DSBR: Double stranded break repair. ICLR: Interstrand cross-link repair.

in mammals also take place in yeast [131] and in bacteria [132-134]. DNA repair thus appears to be a prerequisite to life as we know it. Based on the type of lesion repaired, the pathways are: 1) Direct reversal; 2) Base excision repair; 3) Mismatch repair; 4) Nucleotide excision repair; 5) Double stranded break repair; 6) Interstrand cross-link repair (Figure 5). I will in the following sections briefly describe these pathways.

Direct reversal describes the process by which a chemically modified base in the DNA molecule is repaired without the removal of the base itself [135]. Several proteins are believed to be able to remove modification from DNA directly. Most notably, the protein O-6-methylguanine-DNA methyltransferase (MGMT) catalyzes the transfer of methyl groups from guanine to MGMT thereby inactivating itself in what can be termed a suicide reaction [136]. DNA methylation occurs both spontaneously and as a consequence of alkylating chemotherapeutic agents and direct reversal, and in conjunction with base excision and mismatch repair, acts to protect the genome from these insults [137, 138]. It should be noted that the DNA methylation that occurs as a consequence of alkylating agents is different than the methylation that occurs under physiological conditions as part of gene expression regulation [139].

Base excision repair deals with single base modifications including the common 8-hydroxyguanine and abasic sites. It is the main repair pathway that deals with oxidative lesions to the DNA [140, 141]. Two sub-pathways of base excision repair, long-patch and short-patch, have evolved that supplement each other in their ability to remove damage from the DNA [142]. Base excision repair is initiated by the recognition of the altered base typically by a glycosylase (such as OGG1) that removes the damaged base often leaving the ribose-backbone intact creating an abasic site (apurinic/apyrimidinic or AP- site). The abasic site is recognized by apurinic/apyrimidinic endonuclease 1 (APE1) that cleaves the ribose back-bone leaving a single stranded DNA gap. Subsequently the gap is filled by a DNA polymerase (typically DNA pol-β) either by single base (short-patch) or by several bases displacing a small oligonucleotide (long-patch) that is then removed by a flap-endonuclease (eg. FEN1). A ligase (such as ligase I or III) together with the scaffolding protein XRCC1 seals the DNA backbone concluding the predominantly error free repair of the damage. The importance of this pathway is evident from the fact that loss of essential base excision repair enzymes, such as APE1, XRCC1 and DNA polymerase β, are embryonic lethal in mice [143-145]. In addition, base excision repair has been involved in aging, cancer and neurodegeneration [146-148].

Mismatch repair is a postreplicative DNA repair pathway that deals with pairing of non-corresponding bases that occur during replication . The pathway entails three steps: a) detecting of the mispaired base; b) removal of a short oligo containing the mispaired base; c) resynthesis of DNA to fill the gap resulting in high fidelity repair [149]. The incorrect base, is believed to be recognized by the Msh2/Msh6 or Msh2/Msh3 complex, leading to the recruitment of the Mlh1 and the endonuclease Pms1. Pms1 recognizes the newly synthesized strand based on lack of methylation and cleaves the strand 5' or 3' prime of the lesion. An exonuclease, typically Exo1, is recruited and digests the single stranded DNA containing the mismatched nucleotide. The gap in the DNA, can then be resynthesized by a DNA polymerase to finalize the repair process. Loss of mismatch repair strongly predisposes individuals to cancer. This is most apparent from the finding that defects in mismatch repair leads to greatly increased risk of non-polyposis colorectal cancer [150, 151]. In addition, mismatch repair is believed to be involved in class switch recombination and somatic hypermutation in the immune system as well as in the pathogenesis of triplet repeat expansion diseases [151].

Nucleotide excision repair is a versatile repair pathway that deals with larger lesions encompassing several bases or with bulky adducts to the DNA [152]. Two pathways, global genomic and transcription coupled repair, have evolved that differ in the way the DNA damage is detected. In global genomic DNA repair the damage is thought to be detected by a protein complex containing XPE, DDB1, Cul4 and Rbx1 that presumably by random chance detects a lesion in the DNA and signals further repair. This complex stabilizes XPC and HR23b at the lesion leading to the recruitment of downstream factors. Transcription coupled repair is believed to be initiated by the stalling of an RNA polymerase possibly leading to the recruitment of CSA and CSB, the two proteins that are mutated in Cockayne syndrome. Both global genomic and transcription coupled repair converge on the recruitment of XPA, proteins mutated in xeroderma pigmentosum groups A, as well as helicases, endonucleases and single stranded binding proteins. The helicases, XPB and XPD, open the transcription bubble on each site of the lesion while XPA stabilizes the lesion and the single stranded DNA binding protein RPA coats the undamaged template. Next the endonucleases, XPG and XPF, cleave the oligonucleotide on each side of the lesion allowing the release of the oligonucleotide containing the lesion. The resulting single stranded DNA will serve as a template for re-synthesis of DNA and thus high fidelity DNA repair occurs with little chance of introduction of mutations. Interestingly, many of the components of nucleotide excision repair also function in normal transcription [153].

Double stranded DNA break repair is the pathway responsible for the repair of breaks to both DNA strands. Two major sub-pathways, homologues recombination and non-homologues end joining, have evolved to deal with these types of lesion. Homologues recombination uses the sister chromatid as a repair template, and will thus only be available during the late S to G2 phase of the cell cycle. The process entails the exonucleolytic 5'-resection of the DNA strand at the break by the MRN complex, Exo1 and other nucleases. The exposed single stranded DNA invades the DNA duplex (or displaces the DNA strand) of the sister chromatid and

uses the complementary strand as a template for high-fidelity re-synthesis of the DNA. Non-homologues end joining relies on microhomology between the broken ends. In this pathway only slight resection of the DNA happen and the re-ligation of the broken DNA ends relies on minor homologues sequences in the vicinity of the break. Annealing and ligation takes place resulting in the deletion of nucleotides surrounding the lesion. Since the non-homologues enjoining pathway does not use a sister chromatid as a template, this repair pathway can function in all stages of the cell cycle. Loss of nucleotides may, however, introduce detrimental mutations in the genome that could potentially lead to malignant transformation or cell death. It should be noted that two sub-pathways of non-homologues end joining have evolved, classical and alternative, that differ in the proteins involved, yet have similar outcome [154]. Although non homologues end joining may intuitively appear to be the less favorable due to the potential introduction of mutations, the pathway is essential for a proper working immune system. During lymphocyte development and later during the antibody maturation process, termed class-switching, cells purposefully utilized non homologues end joining to rearrange the V(D)J region of the T-cell receptor and immunoglobulin genes respectively to generate diversity in antigen binding [155]. I shall not go into any greater detail regarding DNA double stranded break repair, but point out that the ataxia-telangiectasia mutated (ATM) kinase is involved in the signaling cascade that leads to the recruitment and retention of DNA repair factors at the DNA break facilitating down-stream repair [156].

Arguably the most complex repair pathway is the process that deals with interstrand DNA cross-links. These highly toxic lesions are extraordinarily difficult to deal with in part because both strands are affected and thus no strand can act as a template for the other strand. The proteins involved in this pathway are still being elucidated but consists of proteins mutated in the disease Fanconi Anemia, where a growing number of genes are being discovered. The process may differ depending on the cell cycle but entails the recognition of the lesions and incision by an endonuclease (such as XPF) on both the 5' and 3' side of the lesion on one strand (unhooking). The modified base is flipped out of the back-bone and DNA is synthesized across the damaged base by a translesion DNA polymerase such as pol zeta. The modified base is then believed to be further processed through nucleotide excision repair following the steps elucidated above. It should, however, be noted that anemia and immune deficiency is uncommon in classic nucleotide excision repair diseases, perhaps indicating that a secondary pathway may act to remove the modified base in interstrand cross-link repair. Nevertheless, translesion synthesis is error prone so this repair pathway represents a way to introduce mutations in the DNA. In addition, defects in this pathway can, during DNA replication, lead to the formation of double stranded breaks potentially resulting in chromosomal rearrangements, a hallmark feature of cells from Fanconi anemia patients. All of these DNA repair pathways are present, active and necessary for the repair of nuclear DNA. However, many of these pathways are highly complex requiring a large number of proteins and only a few are believed to be present in the mitochondria (Figure 6). Direct reversal may occur within the mitochondria although the protein responsible for this reaction has yet to be described [157]. Base excision DNA repair represents a prominent repair pathway in mitochondria and all the enzymes necessary for this pathway are found in this organelle [158]. In contrast to nuclear base excision repair, where DNA polymerase beta is the main associated polymerase, mitochondria rely on DNA polymerase gamma for both replication and repair. In addition, to the standard polymerase activity this enzyme also contains an exonuclease activity that is able to remove single mismatched nucleotides from the DNA. This exonuclease domain gives the protein the capability to proof-read and excise misincorporated bases while polymerization is ongoing. Famously, removal of the proof-reading activity by mutating the exonuclease activity of pol gamma leads to accelerated aging in mice [9]. Although speculative, this proof reading capability may decrease the likelihood of DNA mismatches and thus classic mismatch DNA repair is not thought to occur in the mitochondria. Recently, a backup pathway utilizing YB-1 as a mismatch recognizing enzyme has been suggested within the mitochondria, although further work is needed for illuminating the downstream process [159]. Neither nucleotide excision repair nor DNA interstrand cross-link repair is believed to occur within the mitochondria. Simple ligation of double stranded breaks occur in the mitochondria [160] and a few proteins involved in nuclear double stranded break repair, such as Rad51 and Mre11 [161, 162], have been found in this organelle. Canonical homologues recombination or non-homologues end joining may, however, not occur given the complexity of these pathways. In addition, central proteins involved in this repair pathway have not been found in proteomics analysis of mitochondrial components (for a review of databases of mitochondrial proteins please see [163]). Base excision repair therefore appears to be the major pathway responsible for mitochondrial DNA repair. Since base excision repair mainly deals with oxidative damage to DNA, the preservation of this pathway in mitochondria may reflect the significant oxidative environment that exists within the mitochondrial matrix.

Oxidative DNA damage may be particularly important in the context of aging since this type of DNA damage appear to accumulate

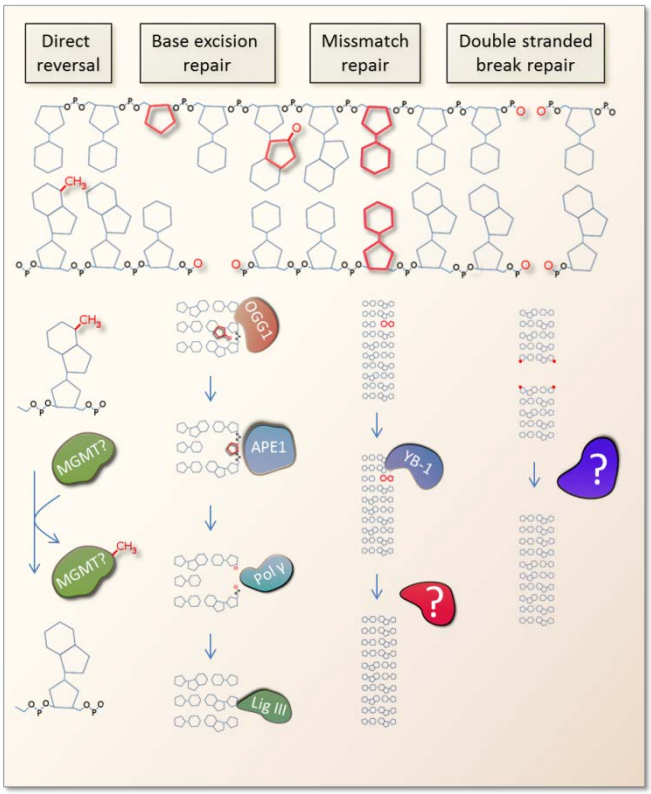

Figure 6. Putative DNA repair pathways in the mitochondria.

in the nucleus with aging particularly in non/slow-proliferating tissues [164]. This has been replicated in a number of studies, however, recently these findings have been questioned due to the significant technical difficulty in determining oxidative DNA damage in vivo [165]. Indeed, recent data using a sensitive PCR-based assay did not find any age associated increase in DNA damage neither in nuclear nor mitochondrial DNA [166]. It has therefore been proposed that accumulation of DNA mutations, and not DNA damage per se, may be the driving force in aging [167]. Nevertheless, increased activation of the DNA damage responsive enzyme poly-ADP-ribose polymerase 1 (PARP1) has been shown with age, perhaps indirectly reflecting an accumulation of nuclear DNA damage [168, 169]. Interestingly, higher capacity for PARP1 activation is a determinant of lifespan across species perhaps indicating that species with longer lifespan have a more active DNA damage response and thus more efficient DNA repair [170, 171]. PARP1 is activated by single stranded and double stranded DNA breaks and may thereby be particularly important in BER and DSBR. A number of other DNA lesions and structures, histone modifications etc. may, however, also activate PARP1 [172, 173]. Interestingly, it has been proposed that PARP1 activation by single stranded breaks is caused by the propensity of a nicked DNA to bend [174, 175]. Indeed, PARP1 is activated by bends in undamaged DNA to the same extend as a nick in the DNA [174]. This observation raises the possibility that PARP1 could be activated by helix distorting lesions, a class of lesions not usually considered to be repaired by BER or DSBR. Accordingly, it has been suggested that UV irradiation, that is known to induce helix distorting lesions, activates PARP1 [176, 177]. Lesions induced by UV irradiation are primarily repaired by NER thereby linking PARP1 to this pathway. UV lesions are, however, not repaired in the mitochondria and this organelle thus has to deal with the damage in an alternative fashion, potentially by whole organellar degradation in a process termed mitophagy.

Mitophagy

Another defense against damage is the process of mitophagy. This evolutionarily conserved pathway facilitates the removal of whole mitochondria through a mechanism entailing the delivery of the mitochondrion to the lysosome for digestion. Mitophagy is a sub-pathway of macro autophagy, a process by which the cell can degrade cellular components contributing to the maintenance of cellular homeostasis of macro- and micromolecular structures [178]. Macroautophagy describes the removal of larger protein aggregates and organelles such as endoplasmic reticulum, termed ER-phagy, and mitochondria, termed mitophagy. Interestingly, a growing number of diseases ranging from neurodegenerative disorders to autoimmune diseases have been suggested to have defects in autophagy [179]. The process is regulated by proteins, such as the Atg family, that for the most part was first characterized in yeast with later discovery of mammalian homologues. Mechanistically, autophagy involves the formation of a double lipid membrane, the phagophore, which engulfs part of the cytoplasm to form the autophagosome. Degradation and recycling of the content of the autophagosome is mediated through fusion with a lysosome resulting in a structure called the autolysosome (Figure 7).

Recently, defective autophagy has been shown in the rare disease Vici syndrome where the ability to fuse the autophagosome with the lysosome may be lost [180]. Interestingly, patients suffering from Vici syndrome display multisystem degeneration and structural developmental defects, highlighting the importance of autophagy in maintaining organismal health. Notably, increased autophagy has been speculated to underlie the life-extension observed in animal models treated with rapamycin [181]. This may entail the inhibition of mTOR (mechanistic/mammalian Target Of Rapamycin), a conserved kinase of the phosphatidylinositol 3-kinase-related kinase family [182]. Inhibition of mTOR may activate autophagy possible through AMPK leading to activation of the proautophagic enzymes Vps-34 and Ulk-1 [183, 184]. Quite strikingly other members of the phosphatidylinositol 3-kinase-related kinase family consist of the core DNA damage responsive kinases ATM, ATR and DNA-PKCS as well as the SMG1 and TRRAP kinases. Although, to my knowledge, no direct role of mTOR in the DNA damage response has been demonstrated, mTOR may be indirectly implicated in the DNA damage response through regulation of ATM [185].

Considerable interest in mitophagy came from the landmark findings that two proteins mutated in familial Parkinson's disease, PINK1 and Parkin, were involved in mitophagy [186, 187]. At least

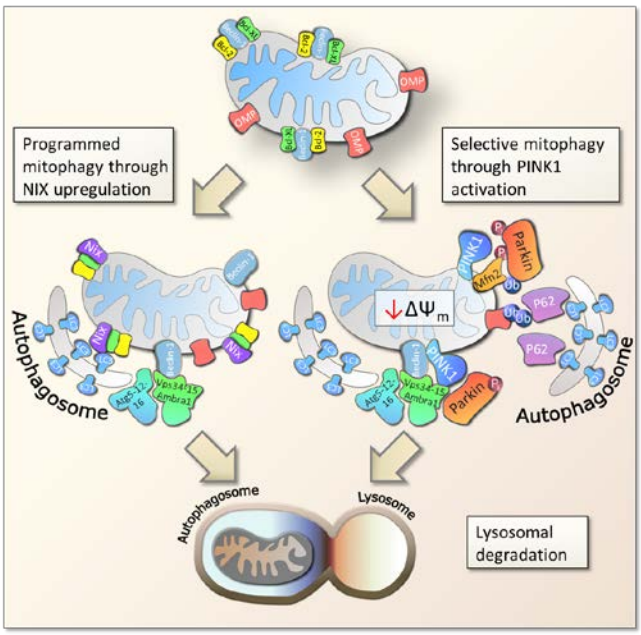

Figure 7. The mechanism of mitophagy. Mammalian mitophagy is believed to occur through at least two pathways, programmed mitophagy and selective mitophagy, although significant cross talk between these has been found. Programmed mitophagy was discovered in maturing red blood cells through upregulation of the protein NIX that may facilitate the dissociation of the anti-mitophagic proteins Bcl-2 and Bcl-XL from the pro-mitophagic protein Beclin-1. This potentially derepresses Beclin-1 allowing recruitment of the Vps34-15-AMBRA complex that in turn will associate with the autophagosome elongation machinery atg5-12/atg16 leading to the formation of an autophagosome. NIX coats the mitochondria and associates directly with LC3 which binds to the growing autophagosome. Mitophagy is completed by the fusion of the mitophagosome with a lysosome. In selective mitophagy the initiating event is believed to be mitochondrial inner membrane depolarization leading to PINK1 accumulation at the outer membrane. PINK1 phosphorylates Parkin, Mfn2 and other proteins leading to the activation of Parkin. Parkin is a E3-ubiquitin ligase that upon activation ubiquitinates outer mitochondrial membrane proteins, possibly VDAC, are ubiquitinated. Vps34-15-AMBRA complex and the Atg5-12/atg16 complex is recruited by activated PINK1/Parkin. Outer membrane ubiquitination leads to the recruitment of p62 which will associate with LC3 on the growing autophagosome. When the autophagosome is completed, fusion with a lysosome will lead to complete degradation of the mitochondria.

two distinct mitophagy pathways exist in mammals: programmed mitophagy and selective mitophagy. Programmed mitophagy was first demonstrated in erythroblasts where mitochondria are removed as part of the development to mature erythrocytes [188]. The apparent mechanism involves the upregulation of the pro-mitophagic protein NIX that may induce mitophagy by sequestering the anti-mitophagic factors Bcl-2 and Bcl-XL and de-repressing the pro-mitophagic protein Beclin-1 [189]. Alternatively, NIX upregulation may lead to mitochondrial membrane depolarization and the recruitment of the canonical mitophagic apparatus (see below) [190]. Further, NIX acts as a scaffolding molecule between the autophagosome associated protein LC3 and the mitochondria [191]. Interestingly, programmed mitophagy may also occur during fertilization selectively degrading paternally derived mitochondria while leaving the maternal mitochondria intact [192, 193]. More recently these findings have, however, been questioned and further research is warranted [194].

Selective mitophagy is believed to entail the specific degradation of depolarized mitochondria through the accumulation of PINK1 at the outer mitochondrial membrane [195]. Here, PINK1 phosphorylates and activates the E3-ubiquitin ligase Parkin [187]. Upon activation, Parkin ubiquinates a large number of proteins that presumably participate in the degradation of the mitochondria [196]. One of these proteins is the mitochondrial fusion protein Mfn2 that upon ubiquitination is degraded leading to fragmentation of the mitochondria, allowing easier digestion of the mitochondria through mitophagy [197-200]. Ubiquitination of the outer mitochondrial membrane also facilitates the association between the scaffolding protein p62 (aka SQSTM1), ubiquitin moieties and LC3 [201]. Recently, a PINK1-Parkin independent pathway has been suggested, indicating the likely scenario that redundancies exist in mitophagy as it is the case for other mitochondrial maintenance pathways [202]. Indeed, if PINK1 and Parkin were the only enzymes responsible for degradation of damaged mitochondria, mutations in these proteins should intuitively result in a much more severe clinical phenotype than the late onset neurodegeneration observed in Parkinson's disease.

What is a mitochondrial disease?

Mitochondrial diseases are inherently complex and recognition of a potential mitochondrial involvement in disorders of unknown pathogenesis poses a significant problem for clinician and basic scientists as well [203]. The combined prevalence of mitochondrial diseases has been proposed to be 1:5000 and thereby represent a significant patient group [203]. Only a few mitochondrial diseases are currently treatable and early identification of these patients are of great importance. Although mitochondrial diseases are clinically exceedingly diverse, some common clinical features do appear at a high frequency in these disorders 80. These include lactate accumulation, muscle weakness, hypotonia, developmental delay and ataxia (Figure 8, insert). Mitochondrial diseases can be caused by defects in a number of processes, including mtDNA replication, protein import, proteases, oxidative

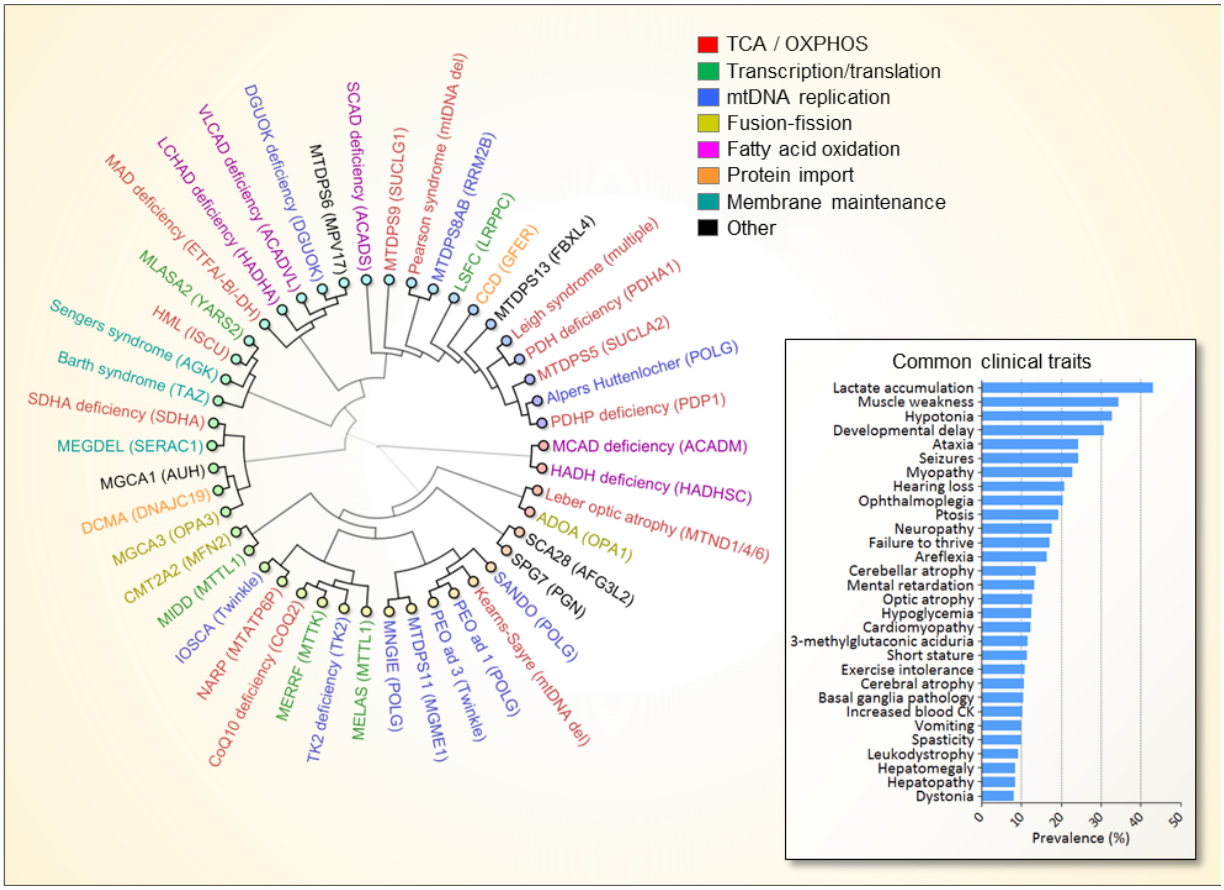

Figure 8. The complex clinical relationship between mitochondrial disorders. The dendrogram show the association between diseases based on the prevalence of clinical traits in the diseases. The closer two diseases are the more signs and symptoms are shared ei. the more similar the diseases are. Genes commonly mutated in these disorders are shown in the parentheses. The top dendrogram shows primary mitochondrial diseases and the colors represent the specific putative pathway that may be affected in that disease. The insert shows the average prevalence of commonly altered clinical traits in the mitochondrial disorders depicted in the dendrogram.

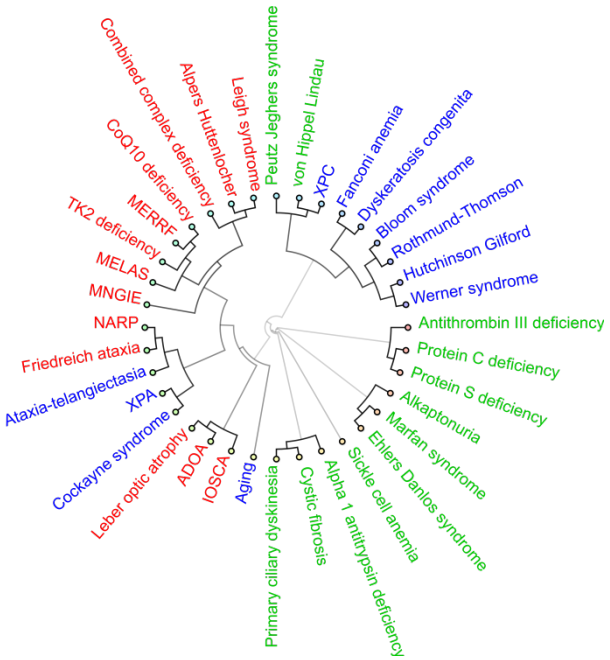

Figure 9. A condenced hierarchical clustering of diseases based on their phenotypical traits made using www.mitodb.com. Mitochondrial: red; Non-mitochondrial: green; Aging/progerias: blue.

phosphorylation, lipid membrane maintenance, fatty acid oxidation etc. To better understand the phenotype of bona fide mitochondrial disorders, we recently created a database of these diseases, describing the prevalence of signs and symptoms (see appendix, paper 1) [80]. Based on the prevalence of these clinical parameters we can perform hierarchical clustering and create dendrograms showing the clinical association between diseases (Figure 8). The shorter the distance between two diseases in the dendrogram the more similar the diseases are in their clinical presentation. A prominent message gathered from this approach is that the primary process that is defective in a disease can yield diverse outcomes. This is, for example, illustrated by the great variability in the phenotype between various syndromes caused by mutations in the genes associated with mitochondrial DNA replication (POLG, Twinkle, TYMP, MGME1, TK2, DGUOK, RRM2B). In addition to the variability in the clinical picture, there is substantial variation in the severity of the disease even within the same defined syndrome [204-206].

Besides the known mitochondrial diseases we added a number of diseases not believed to have any mitochondrial component as part of their pathogenesis as a control group. We further added a number of other diseases to the database including the accelerated aging disorders (Figure 9). Using this approach we found that the accelerated aging diseases with a significant neurodegenerative phenotype associated with mitochondrial diseases. To facilitate additional measurements of mitochondrial dysfunction in various diseases we designed a number of bioinformatics tools to yield further quantitative and qualitative measures of possible mitochondrial involvement in diseases of unknown pathogenesis. These included a support vector machine, a mito-score, a network algorithm and a mitochondrial barcode. To summarize the findings from this published work, we showed that a number of diseases, including Cockayne syndrome and ataxia-telangiectasia, had a significant mitochondrial phenotype [80]. We have since then expanded the database and found in silico mitochondrial involvement in a number of diseases laying the foundation for further research. I recommend the reader to visit www.mitodb.com to familiarize him/herself with the database. Interestingly, normal aging did appear to share some features of mitochondrial disorders, perhaps indicating that this organelle could contribute to age associated pathologies.

Metabolic and mitochondrial alterations in Cockayne syndrome

Based on the phenotypical similarities with mitochondrial diseases and the accelerated aging, we and others have investigated the mitochondrial function and oxidative stress in Cockayne syndrome. For a thorough discussion of this I recommend reading our review on possible pathogenic pathways that may be responsible for the mitochondrial dysfunction in Cockayne syndrome (See appendix, paper 2) [207]. In brief, it was early on speculated that oxidative lesions accumulate in the mitochondrial DNA in cells from Cockayne syndrome patients possible through the regulation of base excision repair enzymes [208, 209]. This view has, however, to some extend been questioned since more recent reports show no consistent accumulation of mitochondrial DNA damage in various tissues and cell lines in this disease [210-213]. Oxidative nuclear DNA damage has, however, been consistently found [211, 212]. A potentially important finding was that CSA and CSB may be found within the mitochondria [214]. This let us to investigate the metabolic and mitochondrial phenotype in greater detail leading to the publication of paper 3 (see appendix) [215]. We used the Cockayne syndrome mouse model, designated $Csb^{m/m}$ mice, that harbors a truncation mutation in murine Ercc6 [216]. We thoroughly investigated the brain using MRI and histology of the central and peripheral nervous system. Although we did find slightly smaller brain sizes as well as loss of cells in the spiral ganglion in the inner ear of old $Csb^{m/m}$ mice compared with controls, there was no genotype difference in the histology of most neuronal tissues (Figure 10, unpublished data). We did, however, find significant loss of adipose tissue, both subcutaneous and visceral, in old $Csb^{m/m}$ mice perhaps reflecting the cachexia phenotype observed in human Cockayne syndrome patients. This loss of fat was not caused by decreased food intake but increased metabolism as reflected by an increase in whole body oxygen consumption. These findings were reproduced in vitro, in cell lines where oxygen consumption rates appeared to be increased in CSB deficient cell lines. This is in accordance with some early literature [208] while a newer study found decreased respiration in CSB deficient cell lines [212]. The latter study cultured cells in galactose media to facilitate increased reliance of the cells on the energy production from the mitochondria. Interestingly, some of the cell lines in this publication did appear to be able to metabolize galactose and the results gathered from this study is therefore difficult to interpret. Nevertheless, in our study we further investigated the mitochondrial phenotype using flow cytometry and found increased mitochondria content and increased mitochondrial membrane potential in CSB deficient cells

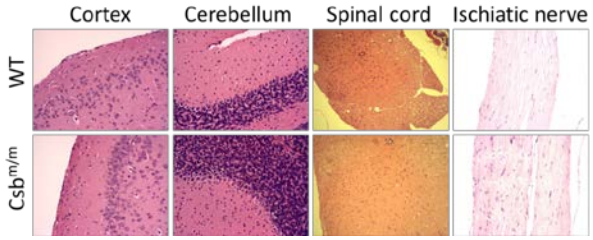

Figure 10. There is no significant neurodegeneration in 20 month old $Csb^{m/m}$ mice compared with WT.

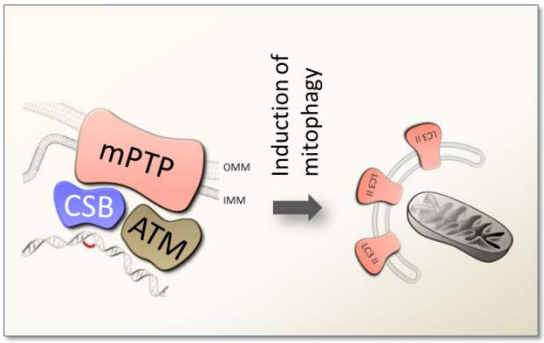

Figure 11. Proposed hypothetical model for how pro-mitophagic signaling could occur via CSB or ATM from within the mitochondrial matrix.

compared with isogenic WT controls. This was quite surprising since primary mitochondrial dysfunction is most commonly associated with loss of membrane potential and decreased oxygen consumption rates. We therefore speculated that these alterations may be caused by increased energy consumption. Indeed, glucose and ATP consumption were almost two fold higher in CSB deficient cells. Since mitochondrial content was increased while mitochondrial biogenesis was not, we went on to measure mitophagy in these cells. Basal autophagy seemed unchanged while the cells appeared to have a specific defect in the induction of mitophagy upon treatment with the mitochondrial toxin rotenone. Importantly, the mitochondrial phenotype in CSB deficient cells could be rescued using the autophagic stimulator rapamycin, an inhibitor of mTOR (see appendix, paper 4).

Based on the idea that CSA and CSB may be present in mitochondria we proposed that these enzymes may act within the mitochondrial matrix to sense mitochondrial DNA damage and induce mitophagy. This could occur through the induction of the mitochondrial permeability transition pore, a structure that had previously been shown to regulate mitophagy [217]. Loss of these proteins would therefore lead to defects in mitophagy through an unknown pathway (Figure 11). Notably, stalling the mitochondrial RNA polymerase using ethidium bromide [218] did not induce mitophagy in WT or CSB deficient cells indicating that a putative DNA damage sensing pathway in the mitochondria is different from the canonical transcription coupled nucleotide excision repair pathway seen in the nucleus. An additional issue with this hypothesis was that the mitochondria seemed to be highly functional with higher membrane potential as well as increased oxygen consumption rates. We therefore proposed an alternative theory that the mitochondrial dysfunction might be secondary to a nuclear DNA repair defect. Specifically, we proposed that activation of the DNA damage responsive enzyme PARP1, an enzyme previously reported to interact with CSB [219], might be involved in the increased ATP consumption. Among several questions arising from this work two may be particularly important: First, what is the molecular mechanism behind the defect in mitophagy? Second, can various interventions exacerbate or attenuate a possible neurodegenerative phenotype in $Csb^{m/m}$ mice?

Defective mitophagy in accelerated aging disorders

The observation that xeroderma pigmentosum group A show similar clinical features of neurodegeneration as Cockayne syndrome and ataxia-telangiectasia led us to investigate the mitochondrial phenotype in this disease in greater detail. XPA patients show significant neurodegeneration while XPC patients, on the other hand, do not display neuronal involvement [74]. Like XPA patients, individuals suffering from ataxia-telangiectasia (AT) also show cerebellar degeneration, ataxia and neuropathy. Based on these observations we investigated the mitochondrial phenotype in XPA, XPC and AT cells leading to a publication in Cell and a short review in Autophagy (see appendix, paper 5 and 6) [220, 221]. Strikingly, XPA and AT cells, but not XPC cells, showed increased mitochondrial membrane potential, increased mitochondrial content and increased ROS production, similar to what we had previously found in CS cells [215]. Both ATM, CSA and CSB have been shown to be present in mitochondria [214, 222, 223]. We therefore hypothesized that the mitochondrial dysfunction might stem from a process within the mitochondria. Surprisingly, XPA was not found in the mitochondria indicating that the defect in mitophagy might be secondary to a defect in nuclear DNA repair. The mitochondrial membrane potential is regulated through a number of mechanisms; one of them is the UCPs. We therefore investigated the levels of UCPs and found that UCP2 was decreased in XPA, CS and AT cells. Since UCP2 is regulated by PGC-1α and PGC-1α is regulated by the NAD+ dependent deacetylase SIRT1 [224] we investigated these proteins. Indeed, PGC-1α and SIRT1 levels were decreased in XPA, CS and AT cells. SIRT1 is in turn regulated by NAD+ levels and has been shown to be negatively regulated by the DNA damage enzyme PARP1 [168]. Because XPA, CS and AT are caused by defects in DNA repair we speculated that perhaps PARP1 was activated, leading to loss of NAD+, decreased activity of SIRT1 and loss of UCP2. Indeed, that is what we found, and importantly this pathway was conserved from nematodes, mice and rats to humans. Thus activation of PARP1 leads to loss of UCP2 and increased mitochondrial membrane potential. The increased membrane potential in DNA repair disorders with neurodegeneration leads to decreased accumulation of PINK1 at the outer mitochondrial membrane and deficient recruitment of Parkin to damage mitochondria. Interestingly, PARP inhibition or replenishment of NAD+ using NAD+-precursors rescue the lifespan defect in XPA deficient nematodes as well as the mitochondrial alterations in a mouse model of XPA. In summary, we were able to find a new nuclear-mitophagic cross talk important for the maintenance of mitochondrial health. Further these results may indicate that other diseases, such as Parkinson's disease, associated with defects in mitophagy might be treatable with NAD+ precursors. For a model see Figure 12.

Dietary interventions in Cockayne syndrome

To further investigate potential neurological alterations in the $Csb^{m/m}$ mice we fed the mice various diets under the hypothesis that the diets might act as metabolic stressor leading to an exacerbation or attenuation of a CS like phenotype. We chose a high fat diet (HFD), because this had been shown to exacerbate the neurodegenerative phenotype in models of Alzheimers disease [225], a 40% caloric restrictive diet (CR), since this intervention had shown neuroprotective effects in models of Alzheimers disease [225], and a diet supplemented with resveratrol, because this intervention had decreased the mutational load in $Csb^{m/m}$/OGG1-/- double transgenic mice [226]. This project resulted in a paper in Cell Metabolism (See appendix, paper 7).

In brief, the mice were put on the diets for 8 months after which various measurements were performed including metabolomic, transcriptomic, histological, behavioral and physiological investigations. Interestingly, the high fat diet appeared to rescue the phenotype of $Csb^{m/m}$ mice. Specifically, the increase in metabolism seen in $Csb^{m/m}$ mice was rescued by the high fat diet. Neurologically, alterations in the cerebellar transcriptome in $Csb^{m/m}$ mice were normalized by both resveratrol and the high fat diet and importantly the high fat diet appeared to reverse the hearing

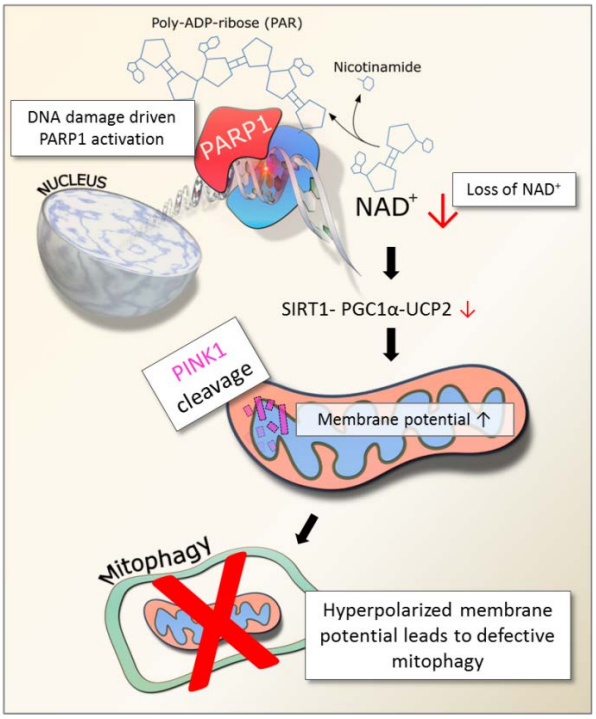

Figure 12. DNA repair deficiency leads to defective mitophagy through activation of PARP1 and depression of SIRT1. PARP1 is activated in DNA repair deficient disorders. This leads to loss of NAD^+ and a decreased activity of NAD^+ dependent enzymes. SIRT1 is depressed leading to decreased activation of PGC-1α and loss of UCP2. Consequently the mitochondrial membrane potential increases and PINK1 is cleaved. Parkin is no longer recruited to damaged mitochondria and mitophagy becomes defective.

loss in $Csb^{m/m}$ mice and attenuate the loss of cells in the spiral ganglion in the inner ear. Conversely, the CR treated $Csb^{m/m}$ were deaf and showed substantial loss of cells in the spiral ganglion. Notably, the $Csb^{m/m}$ mice, contrary to the WT, did not develop glucose intolerance, hyperinsulinemia or steatosis on the high fat diet indicating that the mice were able to compensate for the increased caloric intake.

At the biochemical level we found increased circulating lactate levels as well as increased lactate production in CSB deficient human cells as previously reported [65, 212, 215]. The increased lactate production appeared to be caused by a shift in the equilibrium of lactate/pyruvate towards lactate. The NAD/NADH ratio regulates the lactate/pyruvate equilibrium and loss of NAD+ could lead to increased lactate production. Indeed, PARP1 activation and NAD+ loss was found in CSB deficient cells and mouse tissue explaining the increased lactate production. Concomitantly to the decreased levels of NAD+, SIRT1 activity was decreased. In addition, SIRT1 protein levels were also decreased in CSB deficient cells, a phenomenon we also observed in XPA and ATM deficient cells as well as in normally aged nematodes. To explain why SIRT1 protein levels were decreased we speculated that acetyl-CoA levels might be decreased in CSB deficient cells leading to less histone acetylation and less expression of histone deacetylases, such as SIRT1. Indeed, in CSB deficient cells, the increased lactate production leads to less formation of acetyl-CoA and this either directly or indirectly resulted in decreased histone acetylation. Treatment with the high fat diet led to increased tissue and circulating levels of the ketone β-hydroxybutyrate (BOHB). BOHB is taken up and converted into acetyl-CoA by extrahepatic tissues, particularly the brain. PARP inhibition leads to increased shunting of glucose to acetyl-CoA likely due to a rescue of the NAD/NADH equilibrium. Thus, both PARP inhibition and BOHB treatment was able to normalize acetyl-CoA levels and increase histone acetylation in CSB deficient cells. BOHB or PARP inhibition also appeared to rescue the lifespan shortening in CSB deficient nematodes in a non-additive fashion indicating that these interventions act in the same pathway. Indeed, inhibition of the histone acetyl transferase PCAF in WT cells leads to a similar mitochondrial phenotype as CSB deficiency. In addition, PCAF inhibition or knockdown leads to loss of SIRT1. The loss of SIRT1 was not caused by alterations in transcription, but rather increased degradation of SIRT1 protein. It thus appeared that loss of acetyl-CoA in Cockayne syndrome leads to loss of SIRT1 protein expression through the histone acetyl transferase PCAF.

At the molecular level CSB is retained at laser induced damage sites by two PAR binding motifs in the N-terminal PARP1 binding domain of CSB. Indeed, mutations in these domains or PARP inhibition leads to decreased retention of CSB at laser induced damage. In vitro, recombinant CSB is able to displace activated PARP1 from damaged DNA thereby shutting down the PARylation activity of PARP1. In summary, under normal conditions CSB is recruited to sites of DNA damage through transcription [227]. At the damage site CSB is retained by PAR-polymers and displaces PARP1 to shut down its activity. Loss of CSB leads to persistent activation of PARP1, loss of NAD+ and downstream metabolic derangement. All these findings are summarized in a model in Figure 13.

LOOKING AHEAD

The overarching findings presented herein yields evidence towards a nuclear-mitochondrial crosstalk that may be important in the aging process. From the initial observations of increased metabolism in Cockayne syndrome to the later discoveries that these alterations appear universal across multiple neurodegenerative

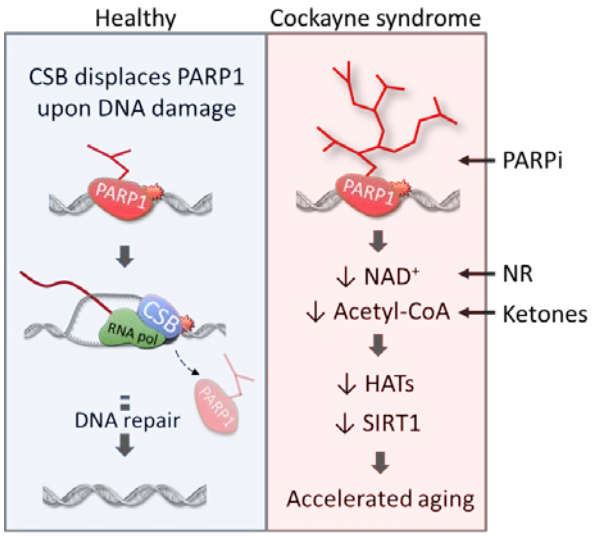

Figure 13. A proposed mechanism for the pathogenesis of Cockayne syndrome. Under normal conditions CSB will displace PARP1 from the DNA after PARP1 has been activated. In Cockayne syndrome, CSB is deficient and PARP1 gets hyperactivated. This leads to loss of NAD^+ and loss of acetyl-CoA. This results in decreased activity of histone acetyl transferases (HATs) and SIRT1 and subsequent mitochondrial dysfunction, metabolic derangement and accelerated aging.

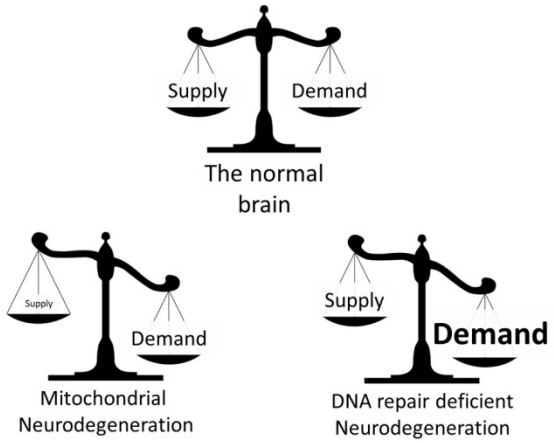

Figure 14. An energetic theory of DNA repair deficient neurodegeneration. Under normal healthy conditions energetic supply and demand is balanced. In mitochondrial diseases energetic output is decreased from the mitochondrial leading to energetic failure and neurodegeneration. In neurodegeneration associated with DNA repair disorders energetic output appears to be normal (or increased) while demand is increased leading to similar neurodegeneration as seen in mitochondrial disorders.

DNA repair disorders gives us new insight into the downstream effects of nuclear DNA damage. The possible signaling mechanism entails hyperactivation of the DNA responsive enzyme PARP1 and loss of NAD+. Indeed, NAD+ replenishment either through PARP inhibition or exogenous addition of NAD+ precursors such as NMN and NR appear to rescue age associated changes in these disorders. Notably, increased PARP activation and NAD+ depletion has been observed during nematode, mouse and human aging [111, 169, 228] and various age associated features in yeast, nematodes and mice can be rescued by treatment with NAD+ precursors or PARP inhibitors [168, 169, 229-231]. This PARP mediated aging pathway thus appears to be conserved across the eukaryotic phylogeny.

It is, however, still unknown what specific lesions that cause the age associated PARP activation. Persistent activation of a DNA damage response has been shown both in vivo and in vitro using confocal microscopy and quantification of γH2AX or 53BP1 foci [232, 233]. Further, the activation of a persistent DNA damage response may be driving senescence at least in cultured cells 233. Interestingly, the persistence of DNA damage foci may possibly not be caused by a global loss of DNA repair within the cell, because most foci are resolved while only a fraction appear to become permanent [233]. This could indicate that whatever is activating the DNA damage response contain structures that are irreparable or more slowly repaired. Notably, only a few persistent foci (more than 3) appear to be enough to drive the senescent phenotype. This means that the lesion frequency that leads to senescence is about 1 in a billion base pairs, which is several orders of magnitude lower than a number of other lesions such as single stranded breaks, depurination or 8-oxoguanine [234]. Because of the rarity of these persistent lesions, pathways to repair the damage may not have evolved because a) the lesions may not accumulate to a level that interferes with human physiology until significantly after reproduction and b) the energetic cost to maintain proteins involved in the repair of extremely rare lesions may exceed the survival benefit gained from such a repair pathway. From an aging perspective one could thus envision the random occurrence of exceedingly rare DNA modifications that are driving a persistent DNA damage response, including PARP1 activation, leading to senescence and aging. What the specific damage may be is currently unknown and a difficult question to answer experimentally. It is also possible that the persistent DNA damage response is not caused by DNA damage at all but secondary protein structures that for some reason constitutively activate a DNA damage response in the cell.

How would PARP1 activation lead to the specific neurological phenotype that is so close to what is observed in primary mitochondrial disorders? In mitochondrial disorders the current hypothesis for why the brain is predominantly affected is that this organ is highly energetically active. Loss of mitochondrial function leads to loss of energy production and thus energetic failure in tissues with high energy demand such as the brain and heart. PARP1 activation consumes energy in at least two ways: First, short term inhibition of energy production comes from shifts in the NAD/NADH ration that will lead to less conversion of pyruvate to acetyl-CoA [235] ; Second, NAD+ molecules that are metabolized by PARP1 have to be resynthesized. This happens through a recycling pathway initiated by the enzyme poly-ADP-ribose glycohydrolase (PARG) that degrades the PAR-polymer and involves several subsequent steps. The net reaction converts 2 ATP molecules to 2 AMP molecules for every molecule of NAD+ and is thus relatively energetically costly. Increased ATP consumption is seen in CSB and XPA deficient cells and the ATP drain could potentially come from PARP activation. Thus in mitochondrial neurodegeneration, defects in the supply of ATP fails to keep up with the energetic demand of the brain, while in neurodegeneration associated DNA repair defects, ATP output is not able to keep up with the increased ATP demand leading to energetic failure (Figure 14).

Although, I believe the proposed mechanism of PARP induced mitochondrial dysfunction in Cockayne syndrome could explain the close clinical correlation between this disease and mitochondrial disorders a number of questions remain. For example, if the role of CSB is to remove activated PARP1 from DNA what role does CSA play? CSA is a WD40 repeat protein that can act in complex with Cul4, DDB1 and Rbx to form a functional ubiquitin E3-ligase able to ubiquitinate RNA polymerase II and CSB [236]. Interestingly, PARP1 interacts with DDB2 (also known as XPE), an alternative partner to Cul4, DDB1 and Rbx [237]. DDB2 share sequence

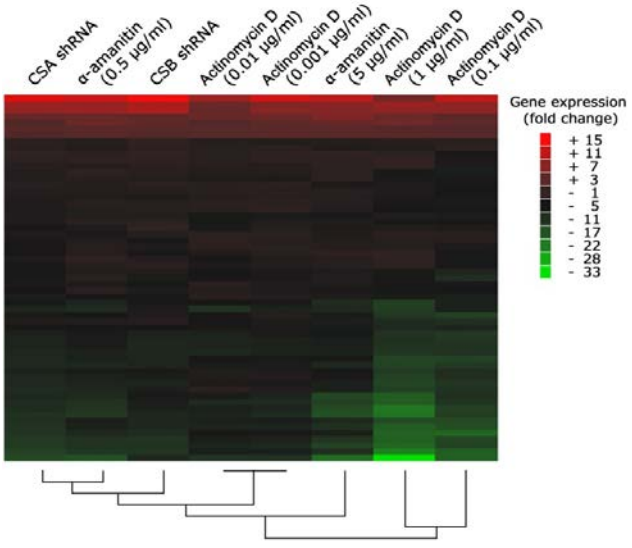

Figure 15. Heat map and hierarchical clustering of gene expression changes in SH-SY5Y treated with transcriptional inhibitors or subjected to stable knockdown of CSA or CSB (Unpublished)**.**

homology with CSA and, although completely speculative, perhaps PARP1 interacts with CSA as well. Indeed, CSB as well as XPA are known interaction partners of PARP1 [219, 238] and ubiquitination regulates PARP1 protein levels [239]. If CSA and PARP1 interact perhaps CSA is involved in the ubiquitination of activated PARP1 and subsequent degradation.

A second question that we have begun answering is how mutations in two very different proteins, CSA and CSB, can lead to the same clinical phenotype. Using the neuroblastoma cell line SH-SY5Y we made stable knockdowns of CSA and CSB and performed gene expression arrays. Interestingly, we find significant overlap in the altered genes indicating, perhaps not surprisingly, that these two proteins function in the same pathway. The pathways altered appear to a large extend to belong to three classes of proteins. Proteins involved in transcription appeared to be downregulated while translation and mitochondrial ATP production appeared to be upregulated. This could indicate a defect in transcription that led to a compensatory increase in translation. To further understand this we treated wild type SH-SY5Y cells with transcriptional inhibitors, α- amanitin and actinomycin D. Strikingly, treatment of normal cells with these inhibitors led to similar gene expression changes indicating that the defect in Cockayne syndrome may indeed be related to transcriptional defects (Figure 15). We then speculated that transcriptional inhibition may lead to PARP activation. Indeed, that appeared to be the case (data not shown). We confirmed these findings using a number of other inhibitors that target RNA polymerase I, II or III (CX-5461, ML-60218 and triptolide respectively; data not shown). These observations indicate that simple stalling of an RNA polymerase may lead to activation of PARP and downstream pleiotropic effects.

Another interesting topic to pursue is whether PARP activation could be driving the neurological phenotype in other DNA repair disorders. This would be particularly pertinent to investigate in the context of xeroderma pigmentosum where different complementation groups show various risks of neurological involvement [74]. Based on our hypothesis we would predict that XPB, XPD, XPG and XPF deficient cells lines also show the same mitochondrial phenotype.

CONCLUSION

In closing, the ideas presented herein could represent a unifying pathogenesis to the neurodegeneration seen in accelerated aging disorders. In addition, since normal aging has been associated with increased PARP activation, the pathogenesis involving NAD+ depletion and SIRT1 attenuation leading to mitochondrial dysfunction could be similar. Treatments that augment NAD+ or attenuate the consequences of PARP activation, such as ketone treatment, could thus represent new alternative treatments for age associated neurodegeneration.

Summary

The growing proportion of elderly people represents an increasing economic burden, not least because of age-associated diseases that pose a significant cost to the health service. Finding possible interventions to age-associated disorders therefore have wide ranging implications. A number of genetically defined accelerated aging diseases have been characterized that can aid in our understanding of aging. Interestingly, all these diseases are associated with defects in the maintenance of our genome. A subset of these disorders, Cockayne syndrome, Xeroderma pigmentosum group A and ataxia-telangiectasia, show neurological involvement reminiscent of what is seen in primary human mitochondrial diseases. Mitochondria are the power plants of the cells converting energy stored in oxygen, sugar, fat, and protein into ATP, the energetic currency of our body. Emerging evidence has linked this organelle to aging and finding mitochondrial dysfunction in accelerated aging disorders thereby strengthens the mitochondrial theory of aging. This theory states that an accumulation of damage to the mitochondria may underlie the process of aging. Indeed, it appears that some accelerated aging disorders that show neurodegeneration also have mitochondrial dysfunction. The mitochondrial alterations may be secondary to defects in nuclear DNA repair. Indeed, nuclear DNA damage may lead to increased energy consumption, alterations in mitochondrial ATP production and defects in mitochondrial recycling, a term called mitophagy. These changes may be caused by activation of poly-ADP-ribose-polymerase 1 (PARP1), an enzyme that responds to DNA damage. Upon activation PARP1 utilizes key metabolites that attenuate pathways that are normally protective for the cell. Notably, pharmacological inhibition of PARP1 or reconstitution of the metabolites rescues the changes caused by PARP1 hyperactivation and in many cases reverse the phenotypes associated with accelerated aging. This implies that modulation of PARP1 or the downstream metabolites may be a therapeutic strategy for treating accelerated aging disorders and potentially age-associated neurological decline seen in the normal population.

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