Amgen Award Lecture

Expression and Maintenance of Mitochondrial DNA *New Insights into Human Disease Pathology*

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Mitochondria are central players in cellular energy metabolism and, consequently, defects in their function result in many characterized metabolic diseases. Critical for their function is mitochondrial DNA (mtDNA), which encodes subunits of the oxidative phosphorylation complexes essential for cellular respiration and ATP production. Expression, replication, and maintenance of mtDNA require factors encoded by nuclear genes. These include not only the primary machinery involved (eg, transcription and replication components) but also those in signaling pathways that mediate or sense alterations in mitochondrial function in accord with changing cellular needs or environmental conditions. Mutations in these contribute to human disease pathology by mechanisms that are being revealed at an unprecedented rate. As I will discuss herein, the basic protein machinery required for transcription initiation in human mitochondria has been elucidated after the discovery of two multifunctional mitochondrial transcription factors, h-mtTFB1 and h-mtTFB2, that are also rRNA methyltransferases. In addition, involvement of the ataxia-telangiectasia mutated (ATM) and target of rapamycin (TOR) signaling pathways in regulating mitochondrial homeostasis and gene expression has also recently been uncovered. These advancements embody the current mitochondrial research landscape, which can be described as exploding with discoveries of previously unanticipated roles for mitochondria in human disease and aging. (*Am J Pathol* 2008, 172:1445–1456; DOI: 10.2353/ajpath.2008.071163)

Stereotypical Roles for Mitochondria in Human Disease and Aging

Mitochondria are complex and dynamic organelles that are absolutely required for the development, function,

and longevity of virtually all eukaryotic cells and organisms. Mitochondria are central players in metabolism by virtue of harboring dozens of enzymes within their double-membrane structure, including, for example, the TCA cycle and those involved in the catabolism or biosynthesis of fatty acids, amino acids, heme, and steroids. Genetic defects that result in altered expression or activity of such enzymes cause many classic inborn errors of metabolism. An important subset of these involves the oxidative phosphorylation (OXPHOS) complexes I to V in the inner mitochondrial membrane that produce ATP, the most often touted primary function of mitochondria. In humans, these complexes comprise ~80 protein subunits, 13 of which are encoded by maternally inherited mtDNA.¹ Thus, unlike most other mitochondrial metabolic diseases, which are inherited in a Mendelian manner, OXPHOS disorders can also be strictly maternally inherited via mutations in mtDNA. Somatic mutations in mtDNA also accumulate in tissues with age and are thought to contribute to age-related disease pathology and the aging process itself.^{2–7}

Although defective energy metabolism resulting from loss of ATP production is certainly a common attribute of most OXPHOS diseases, it is not usually a stand-alone feature. In fact, there are a number of additional salient downstream consequences that stem from the nature of the

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electron transfer processes that underlie the OXPHOS mechanism, as well as the many additional functions of the organelles, which reach well beyond metabolism *per se*. These may include the enhanced production of damaging reactive oxygen species (ROS) by the electron transport chain (usually complex I or III),² aberrant apoptosis,⁸ and altered ion homeostasis.⁹ Finally, it has become evident that cellular signaling pathways sense and control mitochondrial function,¹⁰ providing yet another potential pathogenic consequence of OXPHOS disruption, defective signal transduction, that is only beginning to be understood.

Expression and Maintenance of Human mtDNA: Basic Principles

In humans, mtDNA is a double-stranded circle of 16,569 bp that encodes 37 genes: 13 mRNAs, 2 rRNAs, and 22 tRNAs.¹ The mRNAs specify essential integral membrane components of the OXPHOS complexes, the rRNAs are subunits of mitochondrial ribosomes, and the tRNAs mediate translation of the 13 mRNAs by these dedicated mitochondrial ribosomes. Genes are located on both mtDNA strands, which are called the heavy (H) strand and light (L) strand based on their relative buoyant densities in denaturing CsCl gradients. Transcription initiates at one of two promoters on the H-strand (called HSP1 and HSP2) and a single L-strand promoter (the LSP). The LSP and HSP1 are located in the major noncoding region of the molecule called the displacement loop (D-loop) regulatory region, whereas HSP2 is located downstream of HSP1 within the tRNA^{Phe} gene. Transcripts derived from HSP2 and LSP can be near genome-length polycistronic products, whereas those from HSP1 are terminated at a specific site (in the tRNA^{Leu} gene) downstream of the 16S rRNA and produce mainly the two rRNAs (12S and 16S). Interestingly, this termination event is linked physically to initiation at HSP1 mediated by simultaneous binding of the termination factor, mTERF, to the termination site and the promoter, thus forming a regulatory loop.¹¹ Because of the unique gene arrangement in mtDNA (ie, the rRNAs and most mRNAs are immediately flanked by tRNAs), tRNA processing is believed to be the major mechanism that liberates the majority of the 37 mature RNA molecules from the polycistronic primary transcripts. In mammals, the mature mRNAs are devoid of significant 5'-untranslated sequences, and thus the mechanism of ribosome binding and mitochondrial translation initiation remains obscure.

In mammalian cells, mtDNA is maintained at a high copy number. Generally, there are thousands of copies per cell with the precise number apparently regulated by tissue-specific factors. Within the mitochondrial matrix complexes of 2 to 10 mtDNA molecules are packaged into nucleoprotein complexes called nucleoids that are primarily inner-membrane associated.¹²⁻¹⁴ Thus, each nucleoid-containing mitochondrion by definition has multiple mtDNA copies. However, the notion of specifying the number of mtDNA molecules/mitochondrion is a vague one given that mitochondria usually form a dynamic,

branched network that can fuse, divide, and intermix components, including nucleoids.¹⁵

There are additional hallmark features of mammalian mtDNA that are worthy of discussion. First, in addition to the three promoters there are several other *cis*-acting sequences that are of regulatory significance. For example, in the D-loop regulatory region there are four sequence elements (conserved sequence blocks: CSB I, CSB II, CSB III; and origin of H-strand synthesis: O_H) that are postulated to be important for initiation of transcription-primed, leading-strand DNA synthesis according to the asymmetric model of mtDNA replication.¹⁶ These elements are downstream of the LSP and are involved in configuring and processing the LSP transcript to form RNA primers for initiation by the mtDNA polymerase, Pol γ .¹⁷ Therefore, the RNA primers for leading-strand mtDNA replication are generated by POLRMT (the mitochondrial RNA polymerase). Second, approximately two-thirds the distance around the mtDNA molecule from O_H is O_L, which is a primary site of initiation of lagging-strand mtDNA synthesis, according to the asymmetric replication model. This site lies in an unusual region of the genome where there is a clustering of five adjacent tRNA genes. Although this site is a major region of initiation of lagging-strand synthesis, it appears that other sites on the molecule may serve as alternative initiation sites.¹⁸ Third, one of the first features of mtDNA to be recognized is the D-loop itself. This is a stable three-stranded DNA structure of ~570 to 665 nucleotides in length (in humans) that begins at O_H and extends downstream where it ends at a few distinct sites.¹⁹ This structure has all of the features of a stalled (or terminated) leading-strand replication intermediate; however, whether this is actually the case has not been strictly determined. Since its discovery, the significance of the need to maintain this structure in a subset (a large subset in some cells) of mtDNA molecules has eluded the field, but it is logical to assume it is of regulatory importance with regard to expression, replication, and/or inheritance of mtDNA. Lastly, in addition to the asymmetric model of mammalian mtDNA replication (on which I have expounded above) other models have been proposed.²⁰⁻²² These remain controversial and have been debated in the literature.²³⁻²⁵ Clearly, additional molecular and mechanistic studies are merited to test all of these models critically and to address the possibility that multiple mechanisms may be involved that operate under different conditions or in a tissue-specific manner.

With regard to regulation of mtDNA expression and maintenance, a key point to re-emphasize is that, except for the mtDNA-encoded rRNAs and tRNAs, all of the factors required for transcription, RNA processing, translation, replication, and repair of mtDNA are encoded by nuclear genes, translated by cytoplasmic ribosomes, and imported into mitochondria to their sites of action. In other words, there is an important and relatively large subset of the ~1500 nucleus-encoded proteins in the mitochondrion that is devoted to mitochondrial gene expression and mtDNA maintenance. From this situation it follows that signaling pathways must exist to coordinate the activities of these distinct genetic compartments (the

nucleus and mitochondria) to maintain and modulate mitochondrial gene expression and OXPHOS activity, not to mention the many other functions of these amazing organelles. These mitochondrial regulatory factors and pathways are yet another potential underlying cause of human disease. In fact, pathogenic mutations in nuclear genes encoding proteins required for mitochondrial translation^{26,27} and mtDNA maintenance²⁸ are well documented. One goal of this review is to summarize published evidence that the human mitochondrial transcription machinery and signaling pathways that regulate mitochondrial gene expression and DNA maintenance contribute to human disease pathology and aging in unprecedented ways, as described below.

The Core Human Mitochondrial Transcription Machinery Is a Mixed Three-Component System

Transcription of human mtDNA is directed by a dedicated mitochondrial RNA polymerase, POLRMT, that is a member of the bacteriophage T3/T7 family of single-subunit RNA polymerases.²⁹ Although the prokaryotic origin of mitochondrial RNA polymerases was perhaps expected given the bacterial ancestry of the organelle, it was nonetheless surprising when the first mitochondrial RNA polymerase to be cloned and sequenced (that of budding yeast) was found to be homologous to these bacteriophage enzymes as opposed to the multisubunit bacteria RNA polymerases themselves. The carboxy-terminal portion of the ~140-kDa POLRMT contains the conserved bacteriophage-related sequence motifs that compose the catalytic domain based on structure-function studies of the phage enzymes.²⁹ Unlike the bacteriophage RNA polymerases, however, POLRMT has a large amino-terminal extension that contains two pentatricopeptide repeat (PPR) domains, which are conserved only in vertebrate mtRNA polymerases.³⁰ The function of the PPR domains and the rest of the amino terminal domain of POLRMT remains to be determined, but likely involves coupling other processes (eg, RNA processing or translation) to transcription as we have shown for the amino terminal domain of yeast mitochondrial RNA polymerase.^{30–33} Such a function is also logical to propose based on the fact that other PPR domain proteins have documented roles involved in RNA regulation.^{34–36}

Also unlike bacteriophage RNA polymerases, which do not require additional protein factors to proceed through the various stages of transcription, most, if not all, mitochondrial RNA polymerases require associated transcription factors for function. The first mammalian mitochondrial transcription factor to be identified was human mitochondrial transcription factor A (h-mtTFA or TFAM), which was identified by its ability to facilitate specific promoter-driven transcription by partially purified POLRMT that alone had only nonspecific RNA polymerase activity.^{37,38} This function of h-mtTFA is mediated

through its binding to DNA elements directly upstream of the LSP and HSP1 initiation sites.³⁹ As a member of the high-mobility-group (HMG) box family of proteins,⁴⁰ h-mtTFA binds DNA in a relatively sequence-nonspecific manner and bends and wraps DNA via the namesake HMG-box DNA binding domains of this class of proteins.^{41,42} In h-mtTFA there is a linker between the two HMG-boxes and a 25-amino acid C-terminal tail that is critical for specific DNA binding upstream of the promoters and its ability to stimulate specific transcription initiation.⁴³

Studies of the mitochondrial transcription machinery in the budding yeast, *Saccharomyces cerevisiae* (referred to from here on as yeast), revealed the involvement of a second type of mitochondrial transcription factor that is unrelated to h-mtTFA, called sc-mtTFB (or Mtf1p by standard yeast nomenclature), which led to speculation about the existence of orthologs in higher eukaryotes.⁴⁴ Although biochemical evidence for an mtTFB-like transcription factor activity in *Xenopus* was reported,⁴⁵ we provided the first direct proof of a vertebrate ortholog of yeast mtTFB with the isolation of a cDNA encoding this transcription factor, now called h-mtTFB1 or TFB1M.⁴⁶ Our subsequent studies revealed that h-mtTFB1 binds DNA nonspecifically and stimulates transcription initiation *in vitro* by binding to the C-terminal tail of h-mtTFA.^{46,47} Very soon after we identified h-mtTFB1, Falkenberg and colleagues⁴⁸ reported the isolation of the same protein as well as a second human paralog called h-mtTFB2 (or TFB2M), which also interacts with the C-terminal tail of h-mtTFA.⁴⁷ They also reconstituted specific mitochondrial transcription *in vitro* using h-mtTFA and a complex of POLRMT with either h-mtTFB1 or h-mtTFB2, with all proteins produced from recombinant sources.⁴⁸ Altogether, these seminal studies defined the core human mitochondrial transcription system as a mixed three-component system (consisting of POLRMT, h-mtTFA, and either h-mtTFB1 or h-mtTFB2), which is absolutely required to achieve specific initiation at HSP1 and LSP (Figure 1). The transcription factor requirements for initiation at HSP2 have not been determined.

Intriguingly, the cloning of h-mtTFB1⁴⁶ and h-mtTF2 revealed that these proteins are related to a family of site-specific rRNA adenine methyltransferases⁴⁸ that are homologous to the bacterial KsgA rRNA methyltransferase that was presumably present in the original endosymbiont.^{49,50} The presence of two h-mtTFB paralogs in mammals appears to be the result of an early gene duplication event that occurred before the split of fungi and metazoans in eukaryotic evolution.⁴⁹ Based on their ability to complement an *Escherichia coli* ksgA mutant, h-mtTFB1 and h-mtTFB2 have rRNA methyltransferase activity that modifies the conserved stem-loop in the mitochondrial 12S rRNA^{49,51} (Figure 1). In the case of h-mtTFB1, this enzymatic activity is not required for its transcription factor function *in vitro*,⁴⁷ indicating these functions are separable. The biological significance of the rRNA methyltransferase activity of these transcription factors will be discussed in a subsequent section.

Figure 1. The human mitochondrial transcription machinery and potential mechanism through which the human mitochondrial transcription factor/rRNA methyltransferase, h-mtTFB1, influences the A1555G deafness-associated mtDNA mutation. The minimal protein requirements for transcription initiation from human mtDNA promoters *in vitro* (bottom right) are shown associated with a segment of mtDNA (gray helix). POLRMT, the bacteriophage-related RNA polymerase (largest blue shape) is shown initiating RNA synthesis from an unwound promoter (RNA is represented by the black line exiting the bubble in the mtDNA strands). Formation of this open promoter complex requires promoter-bound h-mtTFA (dark blue L-shape), a dual high-mobility group-box protein, and either h-mtTFB1 or h-mtTFB2 (light blue shape) shown bridging an interaction between the C-terminal tail of h-mtTFA and POLRMT. The two mitochondrial ribosomal RNAs (12S and 16S) are mtDNA encoded and produced by processing of primary transcripts synthesized by POLRMT. Both h-mtTFB1 and h-mtTFB2 are also rRNA methyltransferases that can methylate a conserved stem-loop in the mitochondrial 12S rRNA, based on studies in bacteria. At the top left, h-mtTFB1 (blue shape) is shown methylating the 12S rRNA stem-loop before the eventual assembly of the RNA into mitochondrial ribosomes (green ovals). Each small red oval represents a methyl group added to one of two tandem adenines in the loop of the stem-loop (each of the adenines is dimethylated at ring position N6). The A1555G deafness-associated mtDNA point mutation (X) is in close proximity to the methylated stem-loop, and the structure of this region of the ribosome is affected by this mutation, as well as by the h-mtTFB1-mediated methylation events. It is through these structural perturbations (attributable to alterations in methylation status of the 12S rRNA) that h-mtTFB1 most likely influences the pathogenicity of the maternally inherited A1555G deafness mutation.

The Mechanism of Human Transcription Initiation

Because we¹ and others⁵² have recently described in detail the experimental results that form the basis for models describing the mechanism of transcription initiation at human mtDNA promoters, I will only summarize these here. The essence of the current model, for which there appears to be a general consensus in the field, is that promoter recognition and open complex formation is mechanistically similar to that of bacteriophage T7. However, instead of the polymerase itself being sufficient to recognize and melt the promoter, as is the case for T7, POLRMT requires the simultaneous presence and coordinated activities of h-mtTFA and either h-mtTFB1 or h-mtTFB2 (Figure 1). That is, consistent with its bacteriophage ancestry, POLRMT binds directly to critical promoter nucleotides and hence contributes significantly to the sequence specificity of initiation at mtDNA promoters.⁵³ However, its ability to do so is dependent of several additional conditions being met, including it being in a 1:1 complex with either h-mtTFB1 or h-mtTFB2, the presence of promoter-bound h-mtTFA, and physical distortion of the pro-

motor region into an initiation-competent configuration (eg, a particular bend or kink) (Figure 1). Currently, it is this last requirement that remains obscure. Specifically, whether the bending and unwinding of the promoter template occurs before or after promoter recognition by POLRMT is unknown. In the former case, the promoter could be presented to POLRMT in a unique configuration that allows it to be discerned, which could be provided by the unique DNA properties of the HMG boxes of h-mtTFA.⁴¹ In the latter case, POLRMT might be capable of binding the promoter unaided (like T7 RNA polymerase) whereas other factors assist subsequent initiation or stabilization of an open promoter complex. Finally, which of the three core components provides the promoter-melting function is unclear. Again, h-mtTFA could be the principle promoter modulator in this regard via its aforementioned unique DNA-distorting capabilities. However, a second possibility that is equally likely (and not mutually exclusive) is that h-mtTFB1 or h-mtTFB2 stabilizes the open complex formation by binding to the unwound template or non-template promoter DNA strands. Such a single-stranded DNA binding function could in principle be a functional manifestation of their rRNA methyltransferase ancestry, in that this activity likely requires the ability of h-mtTFB1 and h-mtTFB2 to bind single-stranded nucleic acid to a significant extent.⁴⁶ Based on this synopsis, one can ascertain that many important mechanistic details have been elucidated with regard to the mechanism of human mitochondrial transcription. However, many important questions remain that are worthy of future study. The relatively simple nature of this system provides a facile model system to learn not only more about fundamental aspects of transcription but also which of the core transcription components are regulatory with regard to mitochondrial gene expression and function.

The h-mtTFB1 and h-mtTFB2 Mitochondrial Transcription Factors Retain Ancestral rRNA Methyltransferase Activity and Coordinately Regulate Mitochondrial Gene Expression, Activity, and Biogenesis

That mammals have two paralogs in the mtTFB family of mitochondrial transcription factors that are homologous to KsgA-type rRNA methyltransferases led to several immediate questions surrounding whether they actually had rRNA methyltransferase activity and how their dual functions were parsed with regard to specific effects on mitochondrial gene expression. In our original cloning of h-mtTFB1, we demonstrated that it binds the methyl-donating co-factor S-adenosylmethionine,⁴⁶ providing the first clue that it might have enzymatic function. We subsequently showed that both h-mtTFB1 and h-mtTFB2 have this enzymatic activity as evidenced by their ability to methylate the homologous bacterial 16S rRNA substrate when expressed in a *ksgA*-null *E. coli* strain.^{49,51} Interestingly, h-mtTFB2 has significantly less activity on this heterologous substrate than h-mtTFB1,⁴⁹ which is opposite of their relative transcription factor activity pro-

files.⁴⁸ This poses the interesting possibility that both factors possess both functions but vary in their relative activities of each. Based on this premise, we proposed⁴⁹ that differential expression of the two factors in tissues could be regulatory by allowing differential modulation of transcription/replication (via their different transcription factor activity) and translation (via their different rRNA methyltransferase activity). In this regard it is interesting to note that the relative abundance of h-mtTFB1 and h-mtTFB2 protein in HeLa cell mitochondria is not equal, but that their expression is coordinately regulated to some degree.⁵⁴

To date only two studies have been published regarding the *in vivo* roles of the mtTFB1 and mtTFB2 proteins. In human HeLa cells or *Drosophila* Schneider cells, overexpression of mtTFB2 results in increased mtDNA copy number and steady-state levels of mitochondrial transcripts,^{54,55} whereas knockdown of *Drosophila* mtTFB2 by RNAi had the opposite effect on these parameters.⁵⁵ Similar experiments with human and *Drosophila* mtTFB1 point to a primary role in mitochondrial translation and not transcription.^{54,56} These results are consistent with the simple interpretation that mtTFB1 is the primary rRNA methyltransferase (Figure 1) and mtTFB2 is the primary transcription factor, as one might predict from their relative rRNA methyltransferase and transcription factor activity profiles. However, these studies do not formally eliminate the possibility of partially overlapping methyltransferase and transcription factor functions or that there is crosstalk between the two factors. With regard to the latter possibility, we found that overexpression of h-mtTFB1 in HeLa cells also resulted in an increase in mitochondrial mass, suggesting a novel role for h-mtTFB1 in inducing mitochondrial biogenesis in addition to its function in translation.⁵⁴ Furthermore, overexpression of h-mtTFB2 results in a concomitant increase in the steady-state level of h-mtTFB1 and an increase in mitochondrial membrane potential along with an increase in mitochondrial mass.⁵⁴ Altogether, these results hint at the exciting possibility that rRNA methylation (Figure 1) is a previously unrecognized signal for mitochondrial biogenesis and that a retrograde pathway operates to ensure coordinate regulation of h-mtTFB1 and h-mtTFB2 to properly modulate mitochondrial biogenesis and function. The presence of such an intricate pathway might begin to explain why maintenance of two mtTFB paralogs has been selected for during evolution in most metazoans, including humans. Finally, in addition to the linkage of h-mtTFB1 and h-mtTFB2 to rRNA methyltransferases, another connection between mitochondrial transcription and translation is the binding of mitochondrial ribosomal protein L12 (MRPL12) directly to POLRMT.⁵⁷ This interaction may couple transcription and translation directly or provide a means to coordinate transcription of mtDNA-encoded rRNAs with the import of nucleus-encoded ribosomal mitochondrial ribosomal proteins. In either case, this poses the interesting possibility that key aspects of mitochondrial ribosome assembly are monitored by cells, perhaps as a signal to determine the rate of overall mitochondrial biogenesis or as a metric of mitochondrial functional capacity.

The h-mtTFB1 Gene Is a Nuclear Modifier of Maternally Inherited Deafness

Several mutations in mtDNA cause maternally inherited deafness.^{58,59} The most extensively characterized of these is the relatively common A1555G mutation in the 12S rRNA gene associated primarily with nonsyndromic and/or aminoglycoside antibiotic-induced deafness.⁶⁰ Early pedigree analysis and cell culture studies with mutant patient-derived cells revealed that the associated deafness and mitochondrial phenotypes are influenced strongly by the nuclear genetic background.^{58,61–63} In fact, several nuclear modification loci of the A1555G mutation have been described.^{64–67} These nuclear genetic background influences likely explain some of the variability in individuals within and between A1555G deafness pedigrees in the age of onset of hearing loss and resistance to the ototoxicity of aminoglycosides.

After our isolation of h-mtTFB1 and demonstration that, in addition to acting as a transcription factor, it also methylates a conserved stem-loop in mitochondrial 12S rRNA^{46,51} (Figure 1), Bykhovskaya and colleagues⁶⁴ reported that a polymorphism near the h-mtTFB1 gene (*TFB1M*) provides a protective effect in individuals with the deafness-associated A1555G mutation. Although the mechanism through which h-mtTFB1 modifies the A1555G deafness phenotype remains unknown, it is likely that it is through its impact on mitochondrial translation either indirectly, via its transcription factor function, or directly, via its methylation of the 12S rRNA.⁶⁸ At present, the latter seems most likely. This is because h-mtTFB1 methylates two adjacent adenine residues in a highly conserved stem-loop structure near the 3'-end of the 12S rRNA that, perhaps not coincidentally, is immediately downstream of where the A1555G point mutation occurs (Figure 1). The potential relevance of this relationship and h-mtTFB1 in mitochondrial deafness syndromes was originally noted by us⁵¹ based on the fact that expression of h-mtTFB1 in *E. coli* restored methylation of the 16S stem-loop and sensitivity to the antibiotic kasugamycin in a strain lacking KsgA (the bacterial ortholog h-mtTFB1). Given that the A1555G mutation also predisposes individuals to aminoglycoside antibiotic-induced deafness,^{58,60} we hypothesized (based on the bacterial analogy) that the methylation status of the 12S rRNA in human mitochondria could modify the phenotype of the A1555G mutation. This idea was promoted further by Bykhovskaya and colleagues⁶⁴ who proposed that the chromosome 6 marker near the *TFB1M* gene provides its protective effect by altering h-mtTFB1 activity. For example, they suggest that decreased expression of h-mtTFB1 could cause a corresponding decrease in 12S rRNA methylation that suppresses the loss of ribosomal function resulting from the A1555G mutation. This seems plausible because both the A1555G mutation and the methylation status of this stem-loop alter the structure of the rRNA in the same domain of the ribosome.^{60,69} Thus, an altered and malfunctioning ribosome conformation imposed by the A1555G mutation could, in principle, be restored by lack (or alteration) of methylation of the nearby stem-loop. In

this manner, lower or altered h-mtTFB1-driven rRNA methylation would reduce the penetrance of the deafness phenotype. That the A1555G mutation increases or decreases rRNA methylation and hence plays a role in the deafness phenotype also remains a distinct possibility. Testing molecular models such as these is key to understanding how mtDNA mutations cause deafness in the first place and how nuclear modifiers such as h-mtTFB1 operate to modulate their pathogenic consequences.

Regulation of Mitochondrial Gene Expression and Respiration by the TOR Pathway Limits Life Span

Mitochondria have long been implicated in aging, and many reviews on the mitochondrial theory of aging have been published. The mitochondrial theory^{2,70} is often married to the so-called free radical theory of aging,⁷¹ because of the fact that ROS, an important source of biological free radicals, are produced by mitochondrial respiration and are postulated to damage cellular components and ultimately to lead to loss of normal cell and tissue function that underlies the aging process. In fact, a vicious cycle of mitochondria-driven oxidative stress, whereby mitochondrial ROS damage components of the resident respiratory chain, which, in turn, leads to even more mitochondrial ROS production, is often postulated as a key feature of aging and age-related pathology.^{3,7,72} Thus the acquisition of mitochondria might represent an example of antagonistic pleiotropy, where the obvious early growth benefits of harnessing the energy in nutrients via oxygen-mediated catabolism are ultimately offset by the deleterious consequences of oxygen-mediated damage throughout the course of an individual's life time. Although weighing the pros and cons of this theory is beyond the scope of this review, I will expound on recent results from aging studies in yeast that point to a key role for respiration in regulating life span and some of the first direct evidence for the operation of the vicious cycle of mitochondrial ROS production in its regulation. Interestingly, these studies point to oxidative damage of factors other than mtDNA (eg, the OXPHOS system itself) as a major contributor to aging in yeast, which is perhaps relevant to finding that increased ROS and oxidative damage are not observed in mice engineered to accumulate mtDNA mutations at a high rate.^{3,6,8}

Because of its conditional ability to survive in the absence of mitochondrial respiration (ie, if grown on a fermentable carbon source), yeast has remained an invaluable model organism for understanding mitochondrial function.^{73,74} Yeast has also come to the forefront of aging research, by providing investigators two experimental approaches to measure life span and characterize genes and pathways involved.⁷⁵ The first is replicative life span, which is defined as the number of times that a mother cell can divide (ie, bud) before senescence, and the second is chronological life span (CLS), which represents the amount of time that cells remain viable under nondividing conditions (eg, in stationary phase cultures).

There is now substantial evidence that oxidative stress and mitochondrial respiration are involved in both forms of yeast aging. For example, increased expression of the ROS-detoxifying enzymes superoxide dismutase (encoded by the genes *SOD1* and *SOD2*) results in extended CLS, whereas deletion of these genes has the opposite effect.^{76,77} In addition, increased mitochondrial respiration is required for the longevity-promoting effects of caloric restriction on replicative lifespan⁷⁸ and CLS,⁷⁹ whereas defective respiration can increase⁸⁰ or decrease⁸¹ CLS depending on the precise nature of the mitochondrial defect. Similar effects of respiration and oxidative-stress resistance on life span have been observed in other model eukaryotes.^{82–87} Despite these correlations, what remains unclear is the precise relationship between respiration rate, mitochondrial ROS production, and life span.³ That is, higher rates of respiration can result in higher or lower rates of mitochondrial ROS production, and depending on the model system and conditions studied, life span can be increased or decreased. This dispels the commonly stated view that higher rates of respiration necessarily increase ROS production and curtail life span (promote aging phenotypes). Our recent work on the role of the TOR pathway in regulating yeast CLS is a salient case in point.⁸⁸

It has been shown in multiple model systems that down-regulation of the pro-growth TOR signaling pathway extends life span.^{89–91} To examine the mechanism underlying this conserved response, we examined yeast strains in which TOR signaling was reduced via deletion of the *TOR1* gene (encoding one of the two TOR kinases in this organism) and showed that these strains have an approximately threefold increase in median CLS compared to their wild-type counterparts.⁸⁸ However, the more significant finding from this study is that the ability of reduced TOR signaling to increase CLS absolutely required mitochondrial respiration. Specifically, deletion of *TOR1* results in increased translation and steady-state levels of mtDNA-encoded respiratory chain enzyme subunits and elevated rates of mitochondrial respiration. Furthermore, the increased respiration and the extended lifespan of *tor1*-null cells are glucose- and oxygen-dependent. Our conclusion from these results is that TOR normally inhibits respiration and that ROS-mediated damage (or some other oxygen-dependent phenomenon) during glucose-dependent growth limits stationary phase survival (ie, CLS). We went on to speculate that the salient life span-limiting parameter was ROS-mediated damage to the mitochondrial components themselves, setting into motion a vicious cycle of oxidative stress.^{3,88} This, however, awaits further experimental confirmation. Finally, it was recently reported that the mammalian TOR (mTOR) pathway regulates respiration in cultured human Jurkat T cells;⁹² thus, that mTOR signaling limits life span or impacts aging phenotypes in mammals via its effects on respiration and ROS production remains an intriguing possibility. The fact that mTOR and one of its negative regulators is physically associated with the mitochondrial outer membrane is engaging in this regard.^{92,93}

Beyond Salvage: dNTPs Derived from the de Novo Ribonucleotide Reductase Pathway Contribute to mtDNA Maintenance

It is obvious that mitochondria require a constant source of deoxynucleoside triphosphates (dNTPs) to allow continuous replication and repair of their mtDNA. In contrast to nuclear DNA replication, which occurs exclusively in S phase, mtDNA replication is not strictly cell cycle-dependent. In fact, mtDNA replication occurs in all stages of the cell cycle and persists even in nondividing cells.^{19,94} For these reasons, it has been generally accepted that deoxynucleotide salvage pathways provide the dNTPs for mtDNA replication, a notion that is supported by the presence of salvage pathway enzymes (eg, deoxynucleoside and deoxynucleotide kinases) in mitochondria.^{95,96} Furthermore, that salvage pathways are critical for mtDNA maintenance is evidenced convincingly by inherited human mtDNA depletion syndromes, a subset of which is caused by mutations in genes encoding mitochondrial and cytoplasmic deoxynucleotide salvage enzymes.^{28,95,96} Nonetheless, early observations pointed to the potential involvement of the *de novo* pathway of dNTP synthesis in mtDNA replication and maintenance,⁹⁶ that is, dNTPs derived by ribonucleotide reduction by the enzyme ribonucleotide reductase (RNR). Although a report of a mitochondria-localized form of RNR surfaced,⁹⁷ it has not been verified or generally accepted in the field. In fact, the presence of transporter proteins that facilitate import of deoxynucleosides (for salvage phosphorylation) and presumably also deoxynucleotides (from cytoplasmic RNR reactions) would seem to obviate the need for bona fide mitochondrial ribonucleotide reduction.⁹⁵

Substantial evidence from budding yeast has amassed showing that alterations in the activity or abundance of large R1 subunit of RNR have corresponding effects on mtDNA copy number and stability. For example, overexpression of *RNR1* rescues mtDNA instability caused by point mutations in or haploinsufficiency of Mip1p, the yeast mtDNA polymerase.⁹⁸ In addition, we have shown that strains that overexpress or harbor an activating point mutation in *RNR1* or that recapitulate increased flux through the Mec1-Rad53 signaling pathway (which increases RNR expression and activity) have increased amounts and stability of mtDNA.^{99–101} Conversely, others have shown that defective Mec1-Rad53 pathway signaling decreases mtDNA stability (ie, increases petite mutant formation).^{102,103} Taken together these results clearly point to RNR pathway-driven alterations in the cytoplasmic deoxynucleotide pool as a significant parameter that can influence mtDNA replication and/or stability. Whether these effects are levied at the level of replication, repair, or stability of mtDNA remains an open question. At the time, the relevance of these results to mammalian cells was also questionable given that yeast does not have deoxynucleotide salvage pathways¹⁰⁴ and is forced to obtain dNTPs for mtDNA replication and repair from the *de novo* pathway. However, that yeast Mec1p and Rad53p have orthologous signaling kinases in mammalian cells, ATM/ATR and CHK1/CHK2,¹⁰⁵ respectively,

led us to propose that this pathway may represent a conserved mechanism through which mtDNA replication and stability are modulated.¹⁰¹

Recent reports have shed significant new insight into the role of the *de novo* RNR pathways in mammalian mtDNA maintenance. For example, there is good evidence for ribonucleotide reduction in resting cultured human cells,¹⁰⁶ conditions previously thought to be under the purview of the salvage pathways alone. In addition, even in noncycling cells, the R1 and p53R2 subunits of RNR make a small, but significant contribution to the dNTP pool that, in principle, could contribute to mtDNA replication and repair (as well as basal nuclear repair).¹⁰⁷ This hypothesis was unequivocally confirmed by the recent report by Bourdon and colleagues,¹⁰⁸ who found linkage of mutations in the p53R2 subunit of RNR in mitochondrial disease patients with severe mtDNA depletion in muscle. Finally, we recently reported that pharmacological inhibition of RNR in wild-type human fibroblasts or RNAi-mediated inhibition of the R1 or R2 subunits of RNR in HeLa cells results in mtDNA depletion.¹⁰⁹ Thus, it has become quite evident that both salvage and *de novo* pathways contribute significantly to mtDNA replication and maintenance in mammalian cells. Furthermore, it appears that RNR-derived dNTPs are used for mtDNA replication in both cycling and noncycling cells. This poses the exciting possibility that different cell and tissue types have evolved to depend differentially on these two pathways for replication and repair of mtDNA. Understanding the underlying dynamics involved may finally shed some light onto how tissues maintain characteristic amounts of mtDNA and why mtDNA depletion syndromes display such exquisite tissue-specific pathology. Finally, based on these new concepts, it is logical to predict that the genes encoding the R1 and R2 subunits of RNR, like p53R2,¹⁰⁸ will represent disease loci for mtDNA depletion syndromes or other mtDNA-based diseases.

A Newly Uncovered Role for ATM in RNR and Mitochondrial Homeostasis May Contribute to the Complex Pathology of Ataxia-Telangiectasia (A-T)

ATM is a serine/threonine protein kinase (related to the phosphoinositide 3-kinases) with a well documented role in sensing nuclear DNA damage.^{110,111} In particular, it responds to double-strand breaks in DNA and initiates a signaling cascade that leads to cell cycle arrest and DNA repair or, under some circumstances, apoptosis. However, ATM and the related kinase ATR are orthologs of yeast Mec1p, which, as discussed in the previous section, signals to RNR and modulates mtDNA copy number and stability.^{99,101} This led us to investigate whether such a function for ATM was conserved and likewise involved in mtDNA maintenance and dynamics in mammals. To this end we first analyzed A-T patient fibroblasts that have a null mutation in ATM for disruption in mtDNA metabolism. Here, we found that A-T cells exhibit conditional

mtDNA depletion even in the absence of DNA damage and failed to promote the RNR-dependent increase in mtDNA when DNA damage was induced by ionizing radiation.¹⁰⁹ These cells also have significant disruptions in the steady-state levels of all three RNR subunits in the presence or absence of DNA damage as well as other disruptions in mitochondrial homeostasis. Tissue-specific alterations in mtDNA copy number were also observed in ATM-null mouse tissues, as was a general reduction of the R1 subunit of RNR in all tissues examined.¹⁰⁹ Additional evidence for ATM's involvement in mitochondrial homeostasis was reported previously by Stern and colleagues¹¹² and also recently by Ambrose and colleagues.¹¹³ Thus, ATM clearly has a role in regulating RNR and mitochondrial function under normal growth conditions as well as in response to DNA damage (Figure 2). Some of the downstream consequences of ATM action are mediated by p53,^{114,115} including the induction of p53R2 expression in response to DNA damage.^{116,117} Given that p53 is also implicated in the regulation of mitochondrial respiration^{118–121} and mtDNA maintenance,^{122–124} it is likely that some of the effects of loss of ATM on mitochondria we and others have reported are attributable to disruptions of p53 function (Figure 2).

These newly delineated aspects of ATM function bring to the table the potential involvement of RNR and mitochondrial dysfunction in the pathogenesis of A-T (Figure 2), a disease with a wide range of clinical symptoms.¹²⁵ In addition to the hallmark ataxia, because of degeneration of cerebellar neurons, A-T patients are prone to lymphoma, sterility, diabetes, and premature aging phenotypes. With the exception of lymphoma, all of these symptoms are commonly observed in patients with mitochondrial diseases,^{125–128} supporting the notion that A-T may at some level be a mitochondrial disorder. Perhaps even more compelling, however, is that oxidative stress and aberrant apoptosis are key features of the disease,^{129,130} which are commonly invoked mechanisms of mitochondrial-driven pathology as already discussed. Thus, one model that is worthy of future investigation is that the underlying mitochondrial dysfunction in A-T patient cells and tissues leads to enhanced mitochondrial ROS production, setting into motion a vicious cycle of oxidative stress that causes tissue damage and dysfunction in A-T patients (Figure 2). Such a scenario would presumably be exacerbated by the loss of the normal nuclear DNA damage response in the absence of ATM. Finally, the reported disruptions in RNR in the absence of ATM¹⁰⁹ may contribute to the observed pathology as well, either by causing the mitochondrial dysfunction (attributable to mtDNA copy number and stability defects) or by negatively impacting the nuclear DNA damage response (through inappropriate dNTP pool management). Although, at this point, these ideas should be considered in the realm of speculation, they are certainly deserving of serious consideration given they hold the promise for novel therapeutic strategies for this devastating disease.

Figure 2. Speculative model of A-T pathology that incorporates recently uncovered roles for ATM in mitochondrial homeostasis. Three key proteins in the proposed pathogenic pathway (**blue rounded rectangles**) are ATM, p53, and ribonucleotide reductase (RNR). RNR comprises a large R1 subunit and one of two small subunits, R2 and p53R2. Solid blue lines represent known or well-accepted relationships between these factors and normal downstream cellular processes (**top**, white half of the figure) or pathogenic features of A-T (**bottom**, shaded half of the figure). For example, the role of ATM in nuclear DNA damage sensing is well established, as is the nuclear genomic instability that contributes to A-T pathology because of loss of this function. **Bold black arrows** indicate newly identified roles for ATM and RNR in the regulation of mitochondrial function described in the text. **Dashed arrows** represent speculative pathogenic consequences of disrupted mtDNA metabolism and mitochondrial respiration because of loss of ATM signaling in A-T patient cells. These include increased mitochondrial ROS production that would cause or contribute to the known oxidative stress associated with the disease and other consequences of loss of mitochondrial function that are also common to mitochondrial diseases (eg, neurodegeneration, ataxia, and diabetes). As depicted in the **top**, white half, these mitochondrial defects in A-T patient cells could be precipitated from the loss of ATM acting directly on mitochondria, through the alteration of p53 function (which can influence mitochondria directly or via RNR) or through direct (ie, non-p53-mediated) influence on RNR expression.

New Insights into Mitochondrial Disease Pathology and Aging

Although mitochondrial diseases resulting from mtDNA mutations are relatively rare, when nuclear mutations that cause mitochondrial dysfunction are also considered, the incidence of primary mitochondrial diseases is estimated to be at least 1:8000 births.¹³¹ However, undoubtedly, even this is a major underestimate of the role of mitochondria in human disease. Mitochondria are being implicated in a multitude of cellular processes at an unprecedented rate, which brings into view a corresponding increase in new mechanisms through which mitochondrial dysfunction can contribute to human disease pathology. Our studies directed toward deciphering the basic

mechanism of mitochondrial gene expression and mtDNA maintenance have almost invariably led us to novel disease connections and insights into aging and the regulation of life span. This indicates to me that the contribution of mitochondria to human health and aging is grossly underestimated and that the mechanisms involved extend far beyond the stereotypical metabolic maladies typically associated with these organelles. As we continue to learn their basic properties, diverse roles in cellular homeostasis, and complex pathogenic mechanisms in the coming years, mitochondria will likely represent a rational therapeutic target not only for primary mitochondrial diseases but also for more common health problems such as diabetes, heart disease, common neurodegenerative diseases, cancer, and age-related pathology.^{7,132}

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References

- Bonawitz ND, Clayton DA, Shadel GS: Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. *Mol Cell* 2006, 24:813–825
- Balaban RS, Nemoto S, Finkel T: Mitochondria, oxidants, and aging. *Cell* 2005, 120:483–495
- Bonawitz ND, Shadel GS: Rethinking the mitochondrial theory of aging: the role of mitochondrial gene expression in lifespan determination. *Cell Cycle* 2007, 6:1574–1578
- Kujoth GC, Bradshaw PC, Haroon S, Prolla TA: The role of mitochondrial DNA mutations in mammalian aging. *PLoS Genet* 2007, 3:e24
- Kujoth GC, Leeuwenburgh C, Prolla TA: Mitochondrial DNA mutations and apoptosis in mammalian aging. *Cancer Res* 2006, 66:7386–7389
- Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly YM, Gidlof S, Oldfors A, Wibom R, Tornell J, Jacobs HT, Larsson NG: Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 2004, 429:417–423
- Wallace DC: A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet* 2005, 39:359–407
- Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, Hofer T, Seo AY, Sullivan R, Jobling WA, Morrow JD, Van Remmen H, Sedivy JM, Yamasoba T, Tanokura M, Weindrich R, Leeuwenburgh C, Prolla TA: Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* 2005, 309:481–484
- Giacomello M, Drago I, Pizzo P, Pozzan T: Mitochondrial Ca^{2+} as a key regulator of cell life and death. *Cell Death Differ* 2007, 14:1267–1274
- McBride HM, Neuspiel M, Wasiak S: Mitochondria: more than just a powerhouse. *Curr Biol* 2006, 16:R551–R560
- Martin M, Cho J, Cesare AJ, Griffith JD, Attardi G: Termination factor-mediated DNA loop between termination and initiation sites drives mitochondrial rRNA synthesis. *Cell* 2005, 123:1227–1240
- Bogenhagen DF, Rousseau D, Burke S: The layered structure of human mitochondrial DNA nucleoids. *J Biol Chem* 2008, 283:3665–3675
- Holt IJ, He J, Mao CC, Boyd-Kirkup JD, Martinsson P, Sembongi H, Reyes A, Spelbrink JN: Mammalian mitochondrial nucleoids: organizing an independently minded genome. *Mitochondrion* 2007, 7:311–321
- Kaufman BA, Durisic N, Mativetsky JM, Costantino S, Hancock MA, Grutter P, Shoubridge EA: The mitochondrial transcription factor TFAM coordinates the assembly of multiple DNA molecules into nucleoid-like structures. *Mol Biol Cell* 2007, 18:3225–3236
- Detmer SA, Chan DC: Functions and dysfunctions of mitochondrial dynamics. *Nat Rev Mol Cell Biol* 2007, 8:870–879
- Shadel GS, Clayton DA: Mitochondrial DNA maintenance in vertebrates. *Annu Rev Biochem* 1997, 66:409–435
- Kaguni LS: DNA polymerase gamma, the mitochondrial replicase. *Annu Rev Biochem* 2004, 73:293–320
- Brown TA, Cecconi C, Tkachuk AN, Bustamante C, Clayton DA: Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism. *Genes Dev* 2005, 19:2466–2476
- Clayton DA: Replication of animal mitochondrial DNA. *Cell* 1982, 28:693–705
- Holt IJ, Lorimer HE, Jacobs HT: Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. *Cell* 2000, 100:515–524
- Yang MY, Bowmaker M, Reyes A, Vergani L, Angeli P, Gringeri E, Jacobs HT, Holt IJ: Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication. *Cell* 2002, 111:495–505
- Yasukawa T, Yang MY, Jacobs HT, Holt IJ: A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA. *Mol Cell* 2005, 18:651–662
- Bogenhagen DF, Clayton DA: Concluding remarks: the mitochondrial DNA replication bubble has not burst. *Trends Biochem Sci* 2003, 28:404–405
- Bogenhagen DF, Clayton DA: The mitochondrial DNA replication bubble has not burst. *Trends Biochem Sci* 2003, 28:357–360
- Holt IJ, Jacobs HT: Response: the mitochondrial DNA replication bubble has not burst. *Trends Biochem Sci* 2003, 28:355–356
- Jacobs HT, Turnbull DM: Nuclear genes and mitochondrial translation: a new class of genetic disease. *Trends Genet* 2005, 21:312–314
- Smeltink JA, Elpeleg O, Antonicka H, Diepstra H, Saada A, Smits P, Sasarman F, Vriend G, Jacob-Hirsch J, Shaag A, Rechavi G, Welling B, Horst J, Rodenburg RJ, van den Heuvel B, Shoubridge EA: Distinct clinical phenotypes associated with a mutation in the mitochondrial translation elongation factor EFTs. *Am J Hum Genet* 2006, 79:869–877
- Copeland WC: Inherited mitochondrial diseases of DNA replication. *Annu Rev Med* 2008, 59:131–146
- Masters BS, Stohl LL, Clayton DA: Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell* 1987, 51:89–99
- Rodeheffer MS, Boone BE, Bryan AC, Shadel GS: Nam1p, a protein involved in RNA processing and translation, is coupled to transcrip-

- tion through an interaction with yeast mitochondrial RNA polymerase. *J Biol Chem* 2001, 276:8616–8622
31. Bryan AC, Rodeheffer MS, Wearn CM, Shadel GS: Sls1p is a membrane-bound regulator of transcription-coupled processes involved in *Saccharomyces cerevisiae* mitochondrial gene expression. *Genetics* 2002, 160:75–82
32. Rodeheffer MS, Shadel GS: Multiple interactions involving the amino-terminal domain of yeast mtRNA polymerase determine the efficiency of mitochondrial protein synthesis. *J Biol Chem* 2003, 278:18695–18701
33. Wang Y, Shadel GS: Stability of the mitochondrial genome requires an amino-terminal domain of yeast mitochondrial RNA polymerase. *Proc Natl Acad Sci USA* 1999, 96:8046–8051
34. Mili S, Pinol-Roma S: LRP130, a pentatricopeptide motif protein with a noncanonical RNA-binding domain, is bound in vivo to mitochondrial and nuclear RNAs. *Mol Cell Biol* 2003, 23:4972–4982
35. Small ID, Peeters N: The PPR motif—a TPR-related motif prevalent in plant organellar proteins. *Trends Biochem Sci* 2000, 25:46–47
36. Tavares-Carreón F, Camacho-Villasana Y, Zamudio-Ochoa A, Shingu-Vazquez M, Torres-Larios A, Perez-Martinez X: The pentatricopeptide repeats present in Pet309 are necessary for translation but not for stability of the mitochondrial COX1 mRNA in yeast. *J Biol Chem* 2008, 283:1472–1479
37. Fisher RP, Clayton DA: A transcription factor required for promoter recognition by human mitochondrial RNA polymerase. Accurate initiation at the heavy- and light-strand promoters dissected and reconstituted in vitro. *J Biol Chem* 1985, 260:11330–11338
38. Fisher RP, Clayton DA: Purification and characterization of human mitochondrial transcription factor 1. *Mol Cell Biol* 1988, 8:3496–3509
39. Fisher RP, Topper JN, Clayton DA: Promoter selection in human mitochondria involves binding of a transcription factor to orientation-independent upstream regulatory elements. *Cell* 1987, 50:247–258
40. Parisi MA, Clayton DA: Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. *Science* 1991, 252:965–969
41. Fisher RP, Lisowsky T, Parisi MA, Clayton DA: DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein. *J Biol Chem* 1992, 267:3358–3367
42. Thomas JO, Travers AA: HMG1 and 2, and related 'architectural' DNA-binding proteins. *Trends Biochem Sci* 2001, 26:167–174
43. Dairaghi DJ, Shadel GS, Clayton DA: Addition of a 29 residue carboxyl-terminal tail converts a simple HMG box-containing protein into a transcriptional activator. *J Mol Biol* 1995, 249:11–28
44. Shadel GS, Clayton DA: Mitochondrial transcription initiation. Variation and conservation. *J Biol Chem* 1993, 268:16083–16086
45. Bogenhagen DF: Interaction of mtTFB and mtRNA polymerase at core promoters for transcription of *Xenopus laevis* mtDNA. *J Biol Chem* 1996, 271:12036–12041
46. McCulloch V, Seidel-Rogol BL, Shadel GS: A human mitochondrial transcription factor is related to RNA adenine methyltransferases and binds S-adenosylmethionine. *Mol Cell Biol* 2002, 22:1116–1125
47. McCulloch V, Shadel GS: Human mitochondrial transcription factor B1 interacts with the C-terminal activation region of h-mtTFA and stimulates transcription independently of its RNA methyltransferase activity. *Mol Cell Biol* 2003, 23:5816–5824
48. Falkenberg M, Gaspari M, Rantanen A, Trifunovic A, Larsson NG, Gustafsson CM: Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat Genet* 2002, 31:289–294
49. Cotney J, Shadel GS: Evidence for an early gene duplication event in the evolution of the mitochondrial transcription factor B family and maintenance of rRNA methyltransferase activity in human mtTFB1 and mtTFB2. *J Mol Evol* 2006, 63:707–717
50. Shutt TE, Gray MW: Homologs of mitochondrial transcription factor B, sparsely distributed within the eukaryotic radiation, are likely derived from the dimethyladenosine methyltransferase of the mitochondrial endosymbiont. *Mol Biol Evol* 2006, 23:1169–1179
51. Seidel-Rogol BL, McCulloch V, Shadel GS: Human mitochondrial transcription factor B1 methylates ribosomal RNA at a conserved stem-loop. *Nat Genet* 2003, 33:23–24
52. Falkenberg M, Larsson NG, Gustafsson CM: DNA replication and transcription in mammalian mitochondria. *Annu Rev Biochem* 2007, 76:679–699
53. Gaspari M, Falkenberg M, Larsson NG, Gustafsson CM: The mitochondrial RNA polymerase contributes critically to promoter specificity in mammalian cells. *EMBO J* 2004, 23:4606–4614
54. Cotney J, Wang Z, Shadel GS: Relative abundance of the human mitochondrial transcription system and distinct roles for h-mtTFB1 and h-mtTFB2 in mitochondrial biogenesis and gene expression. *Nucleic Acids Res* 2007, 35:4042–4054
55. Matsushima Y, Garesse R, Kaguni LS: Drosophila mitochondrial transcription factor B2 regulates mitochondrial DNA copy number and transcription in Schneider cells. *J Biol Chem* 2004, 279:26900–26905
56. Matsushima Y, Adan C, Garesse R, Kaguni LS: Drosophila mitochondrial transcription factor B1 modulates mitochondrial translation but not transcription or DNA copy number in Schneider cells. *J Biol Chem* 2005, 280:16815–16820
57. Wang Z, Cotney J, Shadel GS: Human mitochondrial ribosomal protein MRPL12 interacts directly with mitochondrial RNA polymerase to modulate mitochondrial gene expression. *J Biol Chem* 2007, 282:12610–12618
58. Fischel-Ghodsian N: Mitochondrial deafness. *Ear Hear* 2003, 24:303–313
59. Kokotas H, Petersen MB, Willems PJ: Mitochondrial deafness. *Clin Genet* 2007, 71:379–391
60. Prezant TR, Agopian JV, Bohlman MC, Bu X, Oztas S, Qiu WQ, Arnos KS, Cortopassi GA, Jaber L, Rotter JL, Shohat M, Fischel-Ghodsian N: Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nat Genet* 1993, 4:289–294
61. Guan MX, Fischel-Ghodsian N, Attardi G: Biochemical evidence for nuclear gene involvement in phenotype of non-syndromic deafness associated with mitochondrial 12S rRNA mutation. *Hum Mol Genet* 1996, 5:963–971
62. Guan MX, Fischel-Ghodsian N, Attardi G: Nuclear background determines biochemical phenotype in the deafness-associated mitochondrial 12S rRNA mutation. *Hum Mol Genet* 2001, 10:573–580
63. Jaber L, Shohat M, Bu X, Fischel-Ghodsian N, Yang HY, Wang SJ, Rotter JL: Sensorineural deafness inherited as a tissue specific mitochondrial disorder. *J Med Genet* 1992, 29:86–90
64. Bykhovskaya Y, Mengesha E, Wang D, Yang H, Estivill X, Shohat M, Fischel-Ghodsian N: Human mitochondrial transcription factor B1 as a modifier gene for hearing loss associated with the mitochondrial A1555G mutation. *Mol Genet Metab* 2004, 82:27–32
65. Guan MX, Yan Q, Li X, Bykhovskaya Y, Gallo-Teran J, Hajek P, Umeda N, Zhao H, Garrido G, Mengesha E, Suzuki T, del Castillo I, Peters JL, Li R, Qian Y, Wang X, Ballana E, Shohat M, Lu J, Estivill X, Watanabe K, Fischel-Ghodsian N: Mutation in TRMU related to transfer RNA modification modulates the phenotypic expression of the deafness-associated mitochondrial 12S ribosomal RNA mutations. *Am J Hum Genet* 2006, 79:291–302
66. Li X, Guan MX: A human mitochondrial GTP binding protein related to tRNA modification may modulate phenotypic expression of the deafness-associated mitochondrial 12S rRNA mutation. *Mol Cell Biol* 2002, 22:7701–7711
67. Li X, Li R, Lin X, Guan MX: Isolation and characterization of the putative nuclear modifier gene MTO1 involved in the pathogenesis of deafness-associated mitochondrial 12S rRNA A1555G mutation. *J Biol Chem* 2002, 277:27256–27264
68. Shadel GS: A dual-function mitochondrial transcription factor tunes out deafness. *Mol Genet Metab* 2004, 82:1–3
69. Ballana E, Morales E, Rabionet R, Montserrat B, Ventayol M, Bravo O, Gasparini P, Estivill X: Mitochondrial 12S rRNA gene mutations affect RNA secondary structure and lead to variable penetrance in hearing impairment. *Biochem Biophys Res Commun* 2006, 341:950–957
70. Miquel J: An update on the oxygen stress-mitochondrial mutation theory of aging: genetic and evolutionary implications. *Exp Gerontol* 1998, 33:113–126
71. Harman D: Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 1956, 11:298–300
72. Mandavilli BS, Santos JH, Van Houten B: Mitochondrial DNA repair and aging. *Mutat Res* 2002, 509:127–151
73. Liu Z, Butow RA: Mitochondrial retrograde signaling. *Annu Rev Genet* 2006, 40:159–185
74. Tzagoloff A, Myers AM: Genetics of mitochondrial biogenesis. *Annu Rev Biochem* 1986, 55:249–285

75. Kaeberlein M, Burtner CR, Kennedy BK: Recent developments in yeast aging. *PLoS Genet* 2007, 3:e84
76. Fabrizio P, Liou LL, Moy VN, Diaspro A, Valentine JS, Gralla EB, Longo VD: SOD2 functions downstream of Sch9 to extend longevity in yeast. *Genetics* 2003, 163:35–46
77. Longo VD, Gralla EB, Valentine JS: Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. Mitochondrial production of toxic oxygen species in vivo. *J Biol Chem* 1996, 271:12275–12280
78. Lin SJ, Kaeberlein M, Andalis AA, Sturtz LA, Defossez PA, Culotta VC, Fink GR, Guarente L: Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature* 2002, 418:344–348
79. Smith ED, Kennedy BK, Kaeberlein M: Genome-wide identification of conserved longevity genes in yeast and worms. *Mech Ageing Dev* 2007, 128:106–111
80. Barros MH, Bandy B, Tahara EB, Kowaltowski AJ: Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in *Saccharomyces cerevisiae*. *J Biol Chem* 2004, 279:49883–49888
81. Bonawitz ND, Rodeheffer MS, Shadel GS: Defective mitochondrial gene expression results in reactive oxygen species-mediated inhibition of respiration and reduction of yeast life span. *Mol Cell Biol* 2006, 26:4818–4829
82. Dillin A, Hsu AL, Arantes-Oliveira N, Lehrer-Graiwer J, Hsin H, Fraser AG, Kamath RS, Ahringer J, Kenyon C: Rates of behavior and aging specified by mitochondrial function during development. *Science* 2002, 298:2398–2401
83. Ishii N, Fujii M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, Yanase S, Ayusawa D, Suzuki K: A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. *Nature* 1998, 394:694–697
84. Larsen PL, Clarke CF: Extension of life-span in *Caenorhabditis elegans* by a diet lacking coenzyme Q. *Science* 2002, 295:120–123
85. Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, Ruvkun G: A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. *Nat Genet* 2003, 33:40–48
86. Schirner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS: Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* 2005, 308:1909–1911
87. Walker DW, Hajek P, Muffat J, Knoepfle D, Cornelison S, Attardi G, Benzer S: Hypersensitivity to oxygen and shortened lifespan in a *Drosophila* mitochondrial complex II mutant. *Proc Natl Acad Sci USA* 2006, 103:16382–16387
88. Bonawitz ND, Chatenay-Lapointe M, Pan Y, Shadel GS: Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. *Cell Metab* 2007, 5:265–277
89. Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S: Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway. *Curr Biol* 2004, 14:885–890
90. Powers RW III, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S: Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes Dev* 2006, 20:174–184
91. Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Muller F: Genetics: influence of TOR kinase on lifespan in *C. elegans*. *Nature* 2003, 426:620
92. Schieke SM, Phillips D, McCoy Jr JP, Aponte AM, Shen RF, Balaban RS, Finkel T: The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *J Biol Chem* 2006, 281:27643–27652
93. Bai X, Ma D, Liu A, Shen X, Wang QJ, Liu Y, Jiang Y: Rheb activates mTOR by antagonizing its endogenous inhibitor. FKBP38. *Science* 2007, 318:977–980
94. Bogenhagen D, Clayton DA: Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. *Cell* 1977, 11:719–727
95. Elpeleg O, Mandel H, Saada A: Depletion of the other genome-mitochondrial DNA depletion syndromes in humans. *J Mol Med* 2002, 80:389–396
96. Mathews CK, Song S: Maintaining precursor pools for mitochondrial DNA replication. *FASEB J* 2007, 21:2294–2303
97. Young P, Leeds JM, Slabaugh MB, Mathews CK: Ribonucleotide reductase: evidence for specific association with HeLa cell mitochondria. *Biochem Biophys Res Commun* 1994, 203:46–52
98. Lecrenier N, Foury F: Overexpression of the RNR1 gene rescues *Saccharomyces cerevisiae* mutants in the mitochondrial DNA polymerase-encoding MIP1 gene. *Mol Gen Genet* 1995, 249:1–7
99. Lebedeva MA, Shadel GS: Cell cycle- and ribonucleotide reductase-driven changes in mtDNA copy number influence mtDNA inheritance without compromising mitochondrial gene expression. *Cell Cycle* 2007, 6:2048–2057
100. O'Rourke TW, Doudican NA, Zhang H, Eaton JS, Doetsch PW, Shadel GS: Differential involvement of the related DNA helicases Pif1p and Rrm3p in mtDNA point mutagenesis and stability. *Gene* 2005, 354:86–92
101. Taylor SD, Zhang H, Eaton JS, Rodeheffer MS, Lebedeva MA, O'Rourke TW, Siede W, Shadel GS: The conserved Mec1/Rad53 nuclear checkpoint pathway regulates mitochondrial DNA copy number in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2005, 16:3010–3018
102. Zhao X, Chabes A, Domkin V, Thelander L, Rothstein R: The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. *EMBO J* 2001, 20:3544–3553
103. Zhao X, Rothstein R: The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1. *Proc Natl Acad Sci USA* 2002, 99:3746–3751
104. Vernis L, Piskur J, Diffley JF: Reconstitution of an efficient thymidine salvage pathway in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 2003, 31:e120
105. Mallory JC, Petes TD: Protein kinase activity of Tel1p and Mec1p, two *Saccharomyces cerevisiae* proteins related to the human ATM protein kinase. *Proc Natl Acad Sci USA* 2000, 97:13749–13754
106. Pontarin G, Ferraro P, Hakansson P, Thelander L, Reichard P, Bianchi V: p53R2-dependent ribonucleotide reduction provides deoxyribonucleotides in quiescent human fibroblasts in the absence of induced DNA damage. *J Biol Chem* 2007, 282:16820–16828
107. Håkansson P, Hofer A, Thelander L: Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem* 2006, 281:7834–7841
108. Bourdon A, Minai L, Serre V, Jais JP, Sarzi E, Aubert S, Chretien D, de Lonlay P, Paquis-Flucklinger V, Arakawa H, Nakamura Y, Munich A, Rotig A: Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. *Nat Genet* 2007, 39:776–780
109. Eaton JS, Lin ZP, Sartorelli AC, Bonawitz ND, Shadel GS: Ataxia-telangiectasia mutated kinase regulates ribonucleotide reductase and mitochondrial homeostasis. *J Clin Invest* 2007, 117:2723–2734
110. Kastan MB, Lim DS: The many substrates and functions of ATM. *Nat Rev Mol Cell Biol* 2000, 1:179–186
111. Shiloh Y: The ATM-mediated DNA-damage response: taking shape. *Trends Biochem Sci* 2006, 31:402–410
112. Stern N, Hochman A, Zemach N, Hammel I, Shiloh Y, Rotman G, Barzilai A: Accumulation of DNA damage and reduced levels of nicotine adenine dinucleotide in the brains of Atm-deficient mice. *J Biol Chem* 2002, 277:602–608
113. Ambrose M, Goldstine JV, Gatti RA: Intrinsic mitochondrial dysfunction in ATM-deficient lymphoblastoid cells. *Hum Mol Genet* 2007, 16:2154–2164
114. Saito S, Goodarzi AA, Higashimoto Y, Noda Y, Lees-Miller SP, Appella E, Anderson CW: ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. *J Biol Chem* 2002, 277:12491–12494
115. Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan MB: DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev* 1997, 11:3471–3481
116. Nakano K, Balint E, Ashcroft M, Vousden KH: A ribonucleotide reductase gene is a transcriptional target of p53 and p73. *Oncogene* 2000, 19:4283–4289
117. Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, Takei Y, Nakamura Y: A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* 2000, 404:42–49
118. Donahue RJ, Razmara M, Hoek JB, Knudsen TB: Direct influence of the p53 tumor suppressor on mitochondrial biogenesis and function. *FASEB J* 2001, 15:635–644

119. Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, Hurley PJ, Bunz F, Hwang PM: p53 regulates mitochondrial respiration. *Science* 2006, 312:1650–1653
120. Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P, Moll UM: p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* 2003, 11:577–590
121. Zhou S, Kachhap S, Singh KK: Mitochondrial impairment in p53-deficient human cancer cells. *Mutagenesis* 2003, 18:287–292
122. Achanta G, Sasaki R, Feng L, Carew JS, Lu W, Pelicano H, Keating MJ, Huang P: Novel role of p53 in maintaining mitochondrial genetic stability through interaction with DNA Pol gamma. *EMBO J* 2005, 24:3482–3492
123. Chen D, Yu Z, Zhu Z, Lopez CD: The p53 pathway promotes efficient mitochondrial DNA base excision repair in colorectal cancer cells. *Cancer Res* 2006, 66:3485–3494
124. de Souza-Pinto NC, Harris CC, Bohr VA: p53 functions in the incorporation step in DNA base excision repair in mouse liver mitochondria. *Oncogene* 2004, 23:6559–6568
125. McKinnon PJ: ATM and ataxia telangiectasia. *EMBO Rep* 2004, 5:772–776
126. DiMauro S, Hirano M, Schon EA: Approaches to the treatment of mitochondrial diseases. *Muscle Nerve* 2006, 34:265–283
127. Pieczenik SR, Neustadt J: Mitochondrial dysfunction and molecular pathways of disease. *Exp Mol Pathol* 2007, 83:84–92
128. Trushina E, McMurray CT: Oxidative stress and mitochondrial dysfunction in neurodegenerative diseases. *Neuroscience* 2007, 145:1233–1248
129. Barzilai A, Rotman G, Shiloh Y: ATM deficiency and oxidative stress: a new dimension of defective response to DNA damage. *DNA Repair (Amst)* 2002, 1:3–25
130. Kamsler A, Daily D, Hochman A, Stern N, Shiloh Y, Rotman G, Barzilai A: Increased oxidative stress in ataxia telangiectasia evidenced by alterations in redox state of brains from Atm-deficient mice. *Cancer Res* 2001, 61:1849–1854
131. Thorburn DR: Mitochondrial diseases: not so rare after all. *Intern Med J* 2004, 34:3–5
132. McFarland R, Taylor RW, Turnbull DM: Mitochondrial disease—its impact, etiology, and pathology. *Curr Top Dev Biol* 2007, 77:113–155