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Inventory of the Human Mitochondrial Gene Expression Machinery with Links to Disease

Timothy E. Shutt[1] and **Gerald S. Shadel**[1,2,*]

[1]Department of Pathology, Yale University School of Medicine, 310 Cedar Street, P.O. Box 208023, New haven, CT 06520-8023

[2]Department of Genetics, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208005, New haven, CT 06520-8005

Abstract

Mammalian mitochondrial DNA encodes thirty-seven essential genes required for ATP production via oxidative phosphorylation, instability or misregulation of which is associated with human diseases and aging. Other than the mtDNA-encoded RNA species (thirteen mRNAs, 12S and 16S rRNAs, and twenty-two tRNAs), the many remaining factors needed for mitochondrial gene expression (i.e. transcription, RNA processing/modification and translation), including a dedicated set of mitochondrial ribosomal proteins, are products of nuclear genes that are imported into the mitochondrial matrix. Herein, we inventory the human mitochondrial gene expression machinery, and while doing so highlight specific associations of these regulatory factors with human disease. Major new breakthroughs have been made recently in this burgeoning area that set the stage for exciting future studies on the key outstanding issue of how mitochondrial gene expression is regulated differentially in vivo. This should promote a greater understanding of why mtDNA mutations and dysfunction cause the complex and tissue-specific pathology characteristic of mitochondrial disease states and how mitochondrial dysfunction contributes to more common human pathology and aging.

1. Introduction

Mitochondrial dysfunction, including damage and mutagenesis of mitochondrial DNA (mtDNA) and deregulation of its expression are increasingly implicated in human disease, aging, and age-related pathology. Accordingly, unraveling the mechanism of mitochondrial gene expression is important to understand, and perhaps remedy, mitochondrial-based disease. Since the discovery of mtDNA over 40 years ago, much effort has been devoted to understanding the mode of transcription, replication and maintenance of this essential maternally inherited genome. Given the number of excellent and comprehensive reviews on many of these subjects in recent years [Falkenberg et al., 2007; Asin-Cayuela and Gustafsson 2007; Montoya et al., 2006; Shadel 2008], our goal here is to focus on aspects of mitochondrial gene expression that have been elucidated very recently or those subjects that have yet to be as extensively covered by others to date. Where appropriate we will highlight how these new findings are of pathological significance. Furthermore, we focus on those processes that occur within the mitochondrial compartment itself (*e.g.* transcription, RNA processing/modification, and translation) rather than reviewing the signaling pathways that regulate the expression and activity of the nuclear genes that control mitochondrial

*corresponding author: Department of Pathology, Yale University School of Medicine, P.O. Box 208023, New Haven, CT 06520-8023 phone: (203) 785-2475 FAX: (203) 785-2628 gerald.shadel@yale.edu.

biogenesis and function, which have also been covered well by others recently [Scarpulla 2008; Handschin and Spiegelman 2006].

2. General features of human mtDNA and its mode of expression

Human mtDNA is present at hundreds to thousands of copies per cell in most tissues and encodes thirteen protein subunits of four of the five large oxidative phosphorylation (OXPHOS) complexes in the inner mitochondrial membrane. Thus, mtDNA is essential for mitochondrial ATP production in virtually all cell types. In addition, mtDNA encodes the two ribosomal RNA subunits of mitochondrial ribosomes (12S and 16S rRNA), and twenty-two transfer RNAs needed for translation of the thirteen mRNAs. These thirty-seven genes are distributed on both strands of the circular, 16.5-kb mtDNA molecule, which are called the heavy (H) and light (L) strands based on their relative buoyant densities in denaturing CsCl gradients [Anderson et al., 1981; Clayton 1982]. In most, but not all cases, the mRNA and rRNA genes are flanked by tRNAs. Nearly full-length, polycistronic primary transcripts are generated from each strand and it is generally accepted that the next step in gene expression involves extensive RNA processing to excise the tRNAs from these in order to liberate the mature mRNAs and rRNAs (*i.e.* the so-called tRNA punctuation model [Ojala et al., 1981]). Furthermore, all of the genes are very closely spaced on the genome and hence little or no 5′ or 3′ flanking sequences exist on the mature mRNAs.

Due to the high-density gene arrangement, human mtDNA contains very little non-coding sequence. The major exception to this is the D-loop regulatory region, which contains three promoters required for transcription initiation, one L-strand promoter (LSP) and two H-strand promoters (HSP1 and HSP2), as well as evolutionarily conserved regulatory sequences involved in DNA replication and D-loop formation [Shadel 2008]. Transcription initiated from the LSP and HSP2 promoters results in long polycistronic transcripts from each strand, while that initiated from HSP1 is preferentially terminated at a specific site downstream of the two rRNA genes, producing a shorter H-strand transcript containing only $tRNA^{Phe}$ and the two rRNA species [Martinez-Azorin 2005; Montoya et al., 1983].

3. Mitochondrial Transcription

3.1 The core components required for efficient promoter-specific mitochondrial transcription initiation

The core machinery required for human mitochondrial transcription has been reviewed recently by us and others [Falkenberg et al., 2007; Bonawitz et al., 2006], thus it is only summarized here. It is now more or less generally accepted that the core machinery needed for the majority of basal mitochondrial transcription initiation is a three-component system consisting of POLRMT, h-mtTFB2, and h-mtTFA (a.k.a. Tfam), which are all needed together to obtain efficient promoter-specific initiation *in vitro* [Falkenberg et al., 2002; Gaspari et al., 2004]. POLRMT is a single-subunit RNA polymerase (POLRMT) of the T7 bacteriophage RNA polymerase family [Masters et al., 1987; Tiranti et al., 1997]. However, unlike T7 and other related phage polymerases, which do not require interactions with transcription factors to initiate transcription, mammalian POLRMT requires one of two orthologous rRNA methyltransferase-related transcription factors, h-mtTFB1 or h-mtTFB2, to initiate promoter-specific transcription from LSP and HSP1 *in vitro*. Efficient promoter-specific initiation also requires the high-mobility group (HMG) box DNA-binding protein, h-mtTFA (or Tfam), which was the first mitochondrial transcription factor identified [Fisher and Clayton 1985]. Human mtTFA binds upstream of the LSP and HSP1 promoters, most likely as a dimer [Gangelhoff et al., 2009; Kaufman et al., 2007], and facilitates initiation *in vitro* by binding to h-mtTFB1 or h-mtTFB2 in a manner that requires its C-terminal tail [McCulloch and Shadel 2003] and possibly interactions with HMG box B [Gangelhoff et al.,

2009]. The abundance of h-mtTFA in cells and tissues is a subject of some debate (see [Cotney et al., 2007]), resolution of which is relevant to ascertaining the degree to which this protein dynamically regulates transcription *in vivo* versus simply serves a mtDNA-packaging function like its yeast ortholog Abf2p [Diffley and Stillman 1992; Kucej et al., 2008].

While either h-mtTFB1 and h-mtTFB2 can sponsor transcription initiation in collaboration with the other two components *in vitro* [Falkenberg et al., 2002; McCulloch et al., 2002], recombinant h-mtTFB2 is significantly more active in this regard *in vitro*. Furthermore, several recent studies [Cotney et al., 2007; Cotney et al., 2009; Matsushima et al., 2005; Matsushima et al., 2004; Metodiev et al., 2009] are consistent with the notion that the functions of mtTFB1 and mtTFB2 have diverged such that mtTFB1 is primarily the 12S rRNA methyltransferase, important for ribosome biogenesis and mitochondrial translation, while mtTFB2 is the primary transcription factor. It is important to note, however, that h-mtTFB1 and h-mtTFB2 have each retained transcription factor activity *in vitro* [Falkenberg et al., 2002] and rRNA methyltransferase activity as ascertained in a heterologous *E. coli* system [Seidel-Rogol et al., 2003; Cotney and Shadel 2006]. Thus, the possibility remains that each contributes to rRNA methylation and transcription *in vivo*, albeit likely to very different relative degrees [Bonawitz et al., 2006]. While the recent knock-out of mtTFB1 in mouse heart shows clearly that this protein is needed for proper mitochondrial 12S rRNA methylation and ribosome biogenesis [Metodiev et al., 2009], as predicted from earlier studies in *E. coli* [Xu et al., 2008; Connolly et al., 2008] and cultured cells [Cotney et al., 2009], the fact that transcription is largely unaffected cannot be taken as definitive proof that h-mtTFB1 has no transcription factor function *in vivo*. Furthermore, the inability of Metovidev *et al.* to repeat the previously documented ability of h-mtTFB1 to stimulate transcription in mitochondrial lysates, a reaction that requires careful preparation of lysates that are sensitive to h-mtTFA addition as reported by others [McCulloch et al., 2002; Dairaghi et al., 1995], is not convincing evidence against a transcription function for h-mtTFB1. Nonetheless, it seems appropriate at this point to consider h-mtTFB2 as the primary transcription factor in mammalian mitochondria, while leaving open the possibility that h-mtTFB1 may play an ancillary role in transcription under certain circumstances or perhaps even a primary role in specific tissues other than heart.

Over-expression of h-mtTFB2 in HeLa cells leads to simultaneous up-regulation of h-mtTFB1 and induction of a mitochondrial biogenesis response (*i.e.* an increase in mitochondrial mass and housekeeping protein components/cell). In fact, this coordinate expression is needed to promote normal mitochondrial gene expression and maintain membrane potential [Cotney et al., 2009]. Thus, h-mtTFB1 and h-mtTFB2 are likely key downstream targets of mitochondrial biogenesis signaling pathways that do not act alone, but rather synergistically to promote different aspects of mitochondrial gene expression, biogenesis and function. A point mutation that eliminates the rRNA methyltransferase activity of h-mtTFB1 prevents the increase in mitochondrial mass associated with its over-expression in HeLa cells, pointing to a novel role for ribosome 12S methylation and assembly in controlling overall mitochondrial biogenesis [Cotney et al., 2009]. This same mutation does not affect the transcription factor activity of h-mtTFB1 *in vitro* [McCulloch and Shadel 2003] or the transcription factor and biogenesis phenotypes promoted by over-expression of h-mtTFB2 in HeLa cells [Cotney et al., 2009]. Over-expression of h-mtTFB1 alone causes hypermethylaton of the 12S rRNA and an aberrant mitochondrial biogenesis response, which is very likely relevant to its identification as a nuclear genetic modifier of the A1555G mtDNA mutation that causes maternally inherited and aminoglycoside-induced deafness (discussed in greater detail in the “RNA processing” section).

The metazoan mtTFB1 and mtTFB2 transcription factors evolved from the bacterial KsgA methyltransferase of the ancestral mitochondrial endosymbiont [McCulloch et al., 2002; Cotney and Shadel 2006; Shutt and Gray 2006]. Members of this class of enzymes are site-specific rRNA adenine N6-dimethyltransferases that dimethylate two adjacent adenine residues in a conserved stem loop of the small subunit rRNA (*e.g.* the 12S rRNA of human mitochondria). Both h-mtTFB1 and h-mtTFB2 can functionally replace the KsgA methyltransferase activity in *E. coli* [Seidel-Rogol et al., 2003; Cotney and Shadel 2006]. However, it is not unusual for methlytransferases of this class to perform additional functions. For example, the yeast homologue Dim1p is involved in nuclear rRNA processing [Lafontaine et al., 1995] and *E. coli* KsgA possesses DNA glycosylase/AP lyase activity involved in DNA repair [Zhang-Akiyama et al., 2009]. Whether h-mtTFB1 or h-mtTFB2 have additional functions such as these remains an intriguing open question.

3.2 Additional mitochondrial transcription components

While the core human mitochondrial transcription components required for initiation from HSP1 and LSP *in vivo* are POLRMT, h-mtTFA and h-mtTFB2 (or perhaps also h-mtTFB1 under certain circumstances), additional proteins that regulate mitochondrial transcription have also been identified recently. These include the mitochondrial ribosomal protein L12 (MRPL12) [Wang et al., 2007] and members of the MTERF family of transcription termination factors [Wenz et al., 2009; Kruse et al., 1989; Roberti et al., 2009]. Furthermore, there are now numerous reports of nuclear transcription factors that are also localized to mitochondria, where direct roles in mitochondrial transcription have been proposed [Psarra and Sekeris 2008a].

3.2.1 MRPL12, a link between transcription and translation in human mitochondria?—Several proteins have been identified in yeast mitochondria that interact with the mtRNA polymerase (Rpo41p) to couple transcription and translation [Rodeheffer and Shadel 2003]; however, no obvious homologs of these in higher eukaryotes have been indentified based on sequence information. In addition, transcription and translation occur concomitantly and are coordinately regulated in bacteria. In searching for proteins that interact with human POLRMT using an affinity-capture strategy, MRPL12 was identified as a direct binding partner that enhances mitochondrial transcription in h-mtTFA-dependent extracts and when over-expressed in HeLa cells [Wang et al., 2007]. While the significance of the POLRMT/MRPL12 interaction has not yet been elucidated mechanistically, we speculate that human MRPL12 may either directly couple transcription and translation in humans by binding simultaneously to POLRMT and ribosomes, or alone (*i.e.* outside of the ribosome) bind to POLRMT to activate its transcriptional activity in some way [Wang et al., 2007]. For example, the latter could be a mechanism to control mitochondrial ribosome biogenesis by coordinating the rate of synthesis of mtDNA-encoded rRNAs with import and assembly with the nucleus-encoded mitochondrial ribosomal proteins. In bacterial ribosomes, MRPL12 acts as a tetramer, but it is unknown whether the transcriptional stimulatory activity in human mitochondria is dependent on a monomeric or multimeric form of MRPL12.

That L12 family members can acquire separate functions outside of ribosomes appears to be a conserved feature of this class of proteins. For example, bacterial L12 is involved in autoregulation of its own mRNA [Johnsen et al., 1982] and yeast MRPL12 is associated with mtDNA nucleoids [Sato and Miyakawa 2004]. In *Drosophila*, MRPL12 is uniquely involved in cell cycle signaling [Frei et al., 2005] and human MRPL12 is a delayed-early gene in the response to growth signals [Marty and Fort 1996]. Most recently, MRPL12 was identified in an *in silico* screen for factors playing a role in regulating life span extension through calorie restriction [Goertzel et al., 2008]. Altogether, these observations suggest that

MRPL12 may be a unique mitochondrial target for controlling aging and cell growth that could also be of pathological or therapeutic significance.

3.2.2 The mTERF family of proteins—Transcription initiated at the HSP1 promoter is preferentially terminated within the $tRNA^{LEU(UUR)}$ gene immediately downstream of the 16S rRNA gene. This site-specific termination event is mediated by a DNA-binding protein called mTERF (now mTERF1) identified by Attardi and colleagues [Kruse et al., 1989], which is the founding member of a family of related proteins comprising mTERF1-4. This family is defined by the presence of several copies of the mTERF motif, a putative leucine zipper domain thought to confer DNA-binding capacity [Roberti et al., 2009; Fernandez-Silva et al., 1997].

Currently, there is a wealth of information on mTERF1, the most studied of the mTERF family to date. Purified mTERF1 from HeLa cell extracts is found in both a monomeric form capable of promoting transcription termination, as well as a trimeric form that does not exhibit this activity [Asin-Cayuela et al., 2004]. Termination by mTERF1 is bidirectional *in vitro* (*i.e.* can occur when its binding site is reversed in orientation) [Shang and Clayton 1994; Asin-Cayuela et al., 2005]. Recently, mTERF1 binding at the $tRNA^{LEU(UUR)}$ site has been demonstrated using an elegant "*in vivo* footprinting" approach that involves targeting of a DNA methyltransferase to mitochondria [Rebelo et al., 2009].

In addition to binding the termination site in $tRNA^{LEU(UUR)}$, mTERF1 also binds mtDNA in the HSP1 promoter region and stimulates transcription [Martin et al., 2005]. It has been proposed that simultaneous binding of mTERF1 to HSP1 and the termination site forms a loop that allows recycling of the transcription components for initiation after termination occurs. This so-called 'ribomotor' model [Martinez-Azorin 2005] is one way that the ~50 fold greater abundance of rRNAs compared to the downstream H-strand mRNAs can be explained [Gelfand and Attardi 1981], although differential stability of rRNA and mRNA species has been reported and likely also contributes to the higher levels of rRNA [Gelfand and Attardi 1981]. Another important aspect of the study by Martin *et al.*, is that transcription initiation from the HSP2 promoter was observed for the first time *in vitro*, which also required the presence of mTERF1 and its cognate termination site. The mechanistic basis for this remains unknown as does the precise protein components required for regulation at HSP2, the least studied of the three known human mtDNA promoters.

Despite a wealth of information on the action of mTERF1 *in vitro*, much less is known regarding its regulatory role *in vivo*. Recently, Jacobs and colleagues showed that mTERF1 binds several sites in mtDNA *in vivo* and that altering mTERF protein levels affects mtDNA replication pausing at these sites [Hyvarinen et al., 2007], leading to a model in which mTERF1 may mediate transcription and replication passage on the same mtDNA molecule. In addition, these authors also suggest that alterations in mTERF protein levels have little effect on steady-state levels of mitochondrial RNAs, however these data have yet to be published. In contrast, a *Drosophila* mTERF homolog, DmTFF, has been shown to regulate transcription *in vivo* [Roberti et al., 2006]. Clearly more work in this area is warranted.

Of potential pathological significance, the A3243G mtDNA mutation that causes the mitochondrial disease MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) occurs in the mTERF1 binding site in the $tRNA^{LEU(UUR)}$ gene. While this mutation decreases the ability of mTERF1 from mitochondrial extracts to terminate transcription *in vitro* [Shang and Clayton 1994; Hess et al., 1991], the relative steady-state levels of mRNA and rRNA are not altered in cybrid cell lines harboring this mutation [Chomyn et al., 1992]. Furthermore, *in vivo* footprinting in cells containing the A3243G mutation is similar to that from wild-type cells [Chomyn et al., 2000], suggesting that

mTERF1 binding at this site *in vivo* is not dramatically influenced by the A3243G mutation. While, at first glance, these results would appear to discount a role for altered mTERF1 binding in the MELAS pathology, it is important to keep in mind that termination may be regulated in a tissue-specific manner *in vivo* such that this interaction is pathogenically relevant. In addition, given that mTERF1 is needed for initiation at HSP2 and recycling of transcription at HSP1, potential effects on promoter regulation (in addition to termination) need to be addressed in this regard.

In 2005, with the availability of the complete human genome, Linder *et al.* identified three additional human mTERF paralogs (mTERF2-4) that were all predicted to localize to mitochondria [Linder et al., 2005]. Characterization of these paralogs has been the focus of several groups in recent years.

The function of mTERF3 (mTERF.D1, CGI-12) has been addressed through gene knock-out studies in mice. While a global knockout is embryonic lethal [Park et al., 2007], a heart-specific knock-out has been analyzed in detail. Heart tissue from these mice exhibits an aberrant mitochondrial transcription leading to decreased respiration and ultimately death. Specifically, transcripts (both mRNA and tRNA) proximal to the promoters are increased, while those more distal to the promoters are decreased. In addition, mTERF3 is able to bind mtDNA in the promoter region and its immunodepletion from mitochondrial extracts leads to increased transcription. These findings led Park *et al.* to posit that increased transcription initiation in the absence of mTERF3 leads to collision of transcription complexes on opposite mtDNA strands and hence incomplete transcription of each strand. These authors also provide indirect evidence for an additional role for mTERF3 in RNA processing.

The mTERF2 (mTERFL or mTERF.D3) homolog has been studied by several groups. This protein is reciprocally regulated with mTERF1 in serum starvation/stimulation experiments and its overexpression inhibits cell growth [Chen et al., 2005]. It is also able to bind non-specifically to DNA and is present in nucleoids at a level estimated to be ~5% that of h-mtTFA [Pellegrini et al., 2009]. Knock-out of the mTERF2 gene in mice is not lethal, but the mice exhibit respiratory defects when metabolically challenged with a high fat/low carbohydrate diet [Wenz et al., 2009]. On the high-fat diet, these mice exhibit decreased steady-state levels of most mRNA and tRNA transcripts and reduced translation of several proteins. However, observed decreases in translation do not always correlate with the decreased mRNA, a reminder that mitochondrial gene expression is regulated at several levels. Somewhat paradoxically, mTERF2 knockout mice fed a standard diet have normal levels of most mitochondrial RNA transcripts, with the exception of promoter-proximal tRNA species that are increased and promoter-distal tRNAs that are decreased. The imbalanced steady-state level of promoter-proximal and -distal tRNA species is reminiscent of the situation in the heart of the mTERF3 knockout mice [Park et al., 2007]. In contrast to the previous binding studies [Pellegrini et al., 2009], in this study mTERF2 exhibited specific DNA binding in the HSP promoter region that was confirmed *in vivo* by ChIP analysis [Wenz et al., 2009]. Most intriguingly, mTERF2 interacts with both mTERF1 and mTERF3 in coimmunoprecipitation experiments, but only in the presence of mtDNA. Altogether, these results suggest a role of mTERF2 as a positive modulator of mitochondrial transcription.

Given their unusual and important roles in various aspects of mitochondrial transcription, more study of this interesting class of proteins is needed. This could include studies guided toward understanding how these protein interact with the known transcription machinery, how they interact with each other, and how they are utilized differentially to control mtDNA expression *in vivo*. Finally, uncovering the role of the last of the known mammalian mTERF homolog, mTERF4 (mTERF.D2), remains a priority.

3.2.3 Nuclear transcription factors in mitochondria—Thyroid hormone has a well-documented effect on mitochondrial function and was the first nuclear transcription factor shown to potentially localize and function in mitochondria [Sterling and Milch 1975], where it apparently has direct effects on mtDNA transcription [Casas et al., 1999]. To date, numerous other nuclear transcription factors are reported in mitochondria (Table I). This subject has been have been reviewed recently [Psarra and Sekeris 2008a] and thus will only be summarized here.

Given the relatively simple transcription apparatus that operates in mitochondria, it is difficult to predict the effects of nuclear transcription factors on mitochondrial gene expression, especially given that these proteins have evolved to operate with the more complex multi-subunit RNA polymerases in the nucleus that have a separate evolutionary history. Thus, a direct role in mitochondrial transcriptional activation mediated through site-specific DNA binding should not be presumed *a priori* for nuclear transcription factors in mitochondria, even if predicted binding sites are found in mtDNA by sequence analysis. For example, STAT3 was recently shown to localize to mitochondria where it modulates respiration through direct interaction with OXPHOS subunits [Wegrzyn et al., 2009], as opposed to functioning in mitochondrial transcriptional regulation. Furthermore, mitochondrial localization of glucocorticoid receptor, Nur77 and NF-κB is involved in apoptosis [Adzic et al., 2009; Talaber et al., 2009; Wang et al., 2009a; Zamora et al., 2004]. It is thus tempting to speculate that the mitochondrial localization of certain nuclear transcription factors is more relevant for apoptosis regulation [Psarra and Sekeris 2008a; Psarra and Sekeris 2008b; Lee et al., 2008], perhaps through mtDNA binding and inhibition of mitochondrial transcription or interactions with apoptosis factors in the organelle.

Despite the above-mentioned caveats, it does appear that certain nuclear transcription factors may directly affect mitochondrial gene expression (*e.g.* ERalpha, ERbeta, GR, TR, CREB; Table I). The activities of these nuclear proteins is likely tissue-specific, as exemplified by thyroid hormone receptor stimulation of transcription in mitochondria purified from liver, but not heart [Fernandez-Vizarra et al., 2008]. Furthermore, because these proteins are known to affect nuclear transcription, which in turn can effect mitochondrial gene expression, it is difficult to parse out which effects are due to the direct actions of these proteins in mitochondria. The use of purified mitochondria to examine transcription effects *in organello* is a logical approach, but even this does not necessarily distinguish between effects on transcription and transcript stability. This is a fascinating new area of mitochondrial biology and the availability of totally recombinant mitochondrial transcription systems should allow researchers to directly test if and how these nuclear transcription factors interface with the core mitochondrial transcription components. Furthermore, the potential for novel functions of these factors in mitochondria that do not involve transcription per se is clearly high and needs to be considered seriously going forward.

4. Mitochondrial RNA Processing

4.1 A new twist on mitochondrial RNase P

According to the tRNA punctuation model, the long polycistronic H-strand and L-strand transcripts are processed into mature rRNA and mRNA indirectly through the cleavage and liberation of intervening tRNA species [Ojala et al., 1981]. This processing of tRNAs requires at least two endonuclease activities, RNase P and tRNase Z, which cleave the 5′ and 3′ termini, respectively. Mutations in mitochondrial tRNAs are a major cause of maternally inherited diseases in humans, and pathogenic consequences of mitochondrial tRNA mutations that specifically affect 3′-end processing represents a subset of these [Levinger et al., 2004]. Just as one example, several tRNA mutations that result in impaired tRNA

processing (*e.g.* the T7445C mutation in $tRNA^{SER(UCN)}$) cause non-syndromic deafness [Levinger et al., 2001].

Most RNase P enzymes are ribonucleoproteins that have an essential RNA component, as well as protein components (the simplest having one RNA and one protein component). While two distinct RNase P activities had been purified from mitochondria, [Rossmanith et al., 1995; Puranam and Attardi 2001], there remained some debate as to whether the activity identified by Puranam *et al*., which was reported to use the same RNA component as the nuclear enzyme, was an artifact of contamination by the nuclear enzyme [Rossmanith and Potuschak 2001]. While this still has not been resolved fully, Holzmann and colleagues identified a novel mitochondrial RNase P activity recently that is distinct from and evolutionary unrelated to nuclear RNase P [Holzmann et al., 2008]. Perhaps most surprising, this mitochondrial RNase P comprises three proteins (MRPP1-3) that form the active enzyme complex, apparently obviating the requirement for an RNA subunit altogether. The MRPP1 protein is homologous to m^1G_9 tRNA methyltransferases and is predicted to also perform this posttranscriptional modification to mammalian mitochondrial tRNAs. The MRPP2 protein, a member of the ubiquitous short-chain dehydrogenase/reductase (SDR) family, is another interesting bifunctional protein, mutations in which cause 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (MHBD) [Ofman et al., 2003]. Furthermore, this protein has been implicated in Alzheimer's disease [Lustbader et al., 2004] and X-linked mental retardation [Lenski et al., 2007]. It remains to be determined if any of these diseases involve disruption of this newly identified mitochondrial RNase P activity, but interesting possibilities along these lines have been discussed [Holzmann and Rossmanith 2009]. The third member of the mitochondrial RNase P complex, MRPP3, does not have any previous functional annotation, but contains a PPR domain often found in mitochondrial RNA binding proteins (the PPR domain will be discussed later). It will be interesting to learn mechanistically how this new RNase P complex performs this essential tRNA processing reaction, whether there are in fact two types of RNase P enzymes in human mitochondrial or not (*i.e.* is there a canonical RNase P ribonucleoproteion in human mitochondria as well), and the precise relationship of these proteins/activities to human disease.

4.2 tRNase Z

Mammals have two orthologous proteins, ELAC1 and ELAC2, which exhibit tRNase Z activity [Takaku et al., 2004; Takaku et al., 2003]. Because ELAC2 is predicted to localize to mitochondria [Levinger et al., 2004], it is the leading candidate for the mitochondrial tRNAse Z activity. Prior to the elucidation of its tRNase Z activity, ELAC2 was characterized as a susceptibility gene in prostate cancer [Tavtigian et al., 2001]. However, when several of the point mutations linked to prostate cancer susceptibility were characterized for tRNAse Z activity and no changes were observed, it was concluded that ELAC2 was not a prostate cancer susceptibility gene [Minagawa et al., 2005]. However, the activity assay employed used only the cytoplasmic $tRNA^{ARG}$ as a substrate for processing. Thus the possibility of mitochondrial (or other nuclear) tRNA processing defects being involved in prostate cancer susceptibility remains. Alternatively, impaired mitochondrial import or even a different function of the enzyme, separate from its tRNAse Z activity, could, in principle, be responsible for the prostate cancer susceptibility. The latter is worth consideration given that many mitochondrial proteins exhibit multiple functions and/or dual-targeting to other cellular locations.

4.3 CCA addition and polyadenylation

Following 5′ and 3′ cleavage of tRNAs, the addition of nucleotides to the 3′ end of mitochondrial RNA species is another critical step for proper function [Bobrowicz et al.,

2008]. Like all tRNAs, mitochondrial tRNAs require the addition of CCA to the 3′ end. This is performed by the mitochondrial tRNA-nucleotidyl transferase [Nagaike et al., 2001].

Another critical RNA processing event in mitochondria is polyadenylation of the mRNA and rRNA transcripts. In fact, in the case of some of the mRNAs, poly(A) tail addition is necessary to form a stop codon at the end of the open reading frame; underscoring how compact and densely packed the genes are in mammalian mtDNA [Ojala et al., 1981]. Two proteins have recently been implicated in mitochondrial polyadenylation: a mitochondrial poly(A) polymerase (MTPAP) [Tomecki et al., 2004; Nagaike et al., 2005] and a mitochondrial polynucleotide phosphorylase (PNPT1, which also contains 3′–5′ exoribonuclease activity) [Piwowarski et al., 2003], despite the latter being localized to the inner membrane space [Chen et al., 2006]. Knockdown of both MTPAP and PNPT1 by shRNA negatively affects polyadenylation, however, the initial oligoadenylation step still occurs, suggesting the existence of an additional enzyme involved in polyadenylation in mitochondria [Slomovic and Schuster 2008]. Intriguingly, MTPAP is a candidate gene implicated in extreme obesity [Xiao et al., 2006], while PNPT1 is linked to cellular senescence and ageing [Leszczyniecka et al., 2002].

The role of mitochondrial mRNA polyadenylation in RNA degradation is unclear as it may be important in both RNA stability and degradation [Bobrowicz et al., 2008]. The length of 3′ poly(A) tails has been implicated in stabilization of full-length mRNA species (reviewed in [Nagaike et al., 2008]), though conflicting reports of the effects of shortening poly(A) tails on mRNA stabilization have been published [Tomecki et al., 2004; Nagaike et al., 2005]. In bacteria, polyadenylation of RNAs plays a central role of their degradation [Dreyfus and Regnier 2002] and it appears as though mitochondria can utilize a similar mechanism as evidenced by the presence of transient or internal polyadenylation products in mitochondria [Slomovic et al., 2005]. These internal polyadenylation products are believed to result from the polyadenylation following the cleavage of RNA species. In this regard the RNA helicase SUV3 has been implicated in RNA degradation, in concert with PNPT1 with which it forms an RNA degradosome complex [Wang et al., 2009b]. The mechanism by which this complex distinguishes between RNA targets destined for degradation and stable mRNAs containing stable 3′ poly(A) tails remains another open question.

4.4 The PPR domain is found in many mitochondrial regulatory proteins

The pentatricopeptide repeat (PPR) family of proteins contain a degenerate 35-amino-acid motif most often in tandem repeats [Small and Peeters 2000]. This motif is commonly found in proteins involved in RNA metabolism and can bind RNA directly, but likely also mediates protein-protein interactions [Schmitz-Linneweber and Small 2008]. To date, seven PPR proteins have been identified in humans and they are all localized to mitochondria (Table II). The characterization of these PPR proteins to date suggests roles in mitochondrial RNA processing and translation.

The LRPPRC protein (also known as LRP130) is perhaps the best-characterized PPR protein in humans with several documented cellular functions [Mili and Pinol-Roma 2003; Cooper et al., 2006]. Mutations in LRPPRC cause a French Canadian variant of Leigh Syndrome characterized by cytochrome C oxidase deficiency [Mootha et al., 2003]. While LRPPRC affects the stability of mitochondrial COX1 and COXIII mRNAs and ultimately cytochrome oxidase assembly [Xu et al., 2004], these changes in mRNA could be due to direct binding of mitochondria mRNAs [Mili and Pinol-Roma 2003] or to downstream effects of a reported interaction between LRPPRC and PGC1alpha, a key nuclear transcriptional coactivator involved in mitochondrial biogenesis [Cooper et al., 2006], or both. Notably, the yeast Pet309 protein, a purported distant homolog of LRPPRC, is directly involved in translation

of mitochondrial COXI mRNA, but not stability of its mRNA [Tavares-Carreon et al., 2008].

The mitochondrial RNA polymerase, POLRMT, was the first PPR protein identified in humans and contains two PPR domains in the N-terminal region of the protein that is not part of the catalytic domain of the enzyme and shares no homology with the T7 family of RNA polymerases or even Rpo41p, the yeast mtRNA polymerase [Rodeheffer et al., 2001]. While the function of the PPR domains in POLRMT remains unknown, it has been suggested that they might coordinate transcription and translation, perhaps in collaboration with LRPPRC [Shadel 2004]. The mitochondrial ribosomal protein MRPS27 is a PPR protein that is a relatively recently acquired component of the small subunit (*i.e.* lacks homology to *E. coli* or yeast mitochondrial ribosomal proteins) [Koc et al., 2001a]. The function of this protein remains unknown. The most recently identified PPR-containing protein, MRPP3, contains two degenerate PPR domains and is a protein component of the novel mitochondrial RNase P complex already discussed [Holzmann et al., 2008]. Finally, three previously uncharacterized genes, annotated based solely on having PPR domains (PTCD1-3), have recently been studied [Lightowlers and Chrzanowska-Lightowlers 2008]. PTCD1, associates with incompletely processed mitochondrial RNA species and acts as a regulator of mitochondrial leucine tRNA abundance [Rackham et al., 2009]. PTCD2 is implicated in processing of cytochrome b mRNA [Xu et al., 2008]. As an interesting side note, PTCD2 and MRPS27 are located adjacent to one another in the nuclear genome (though transcribed in opposite directions) and also share significant sequence similarity in addition to their PPR domains [Xu et al., 2008]. Last, but not least, PTCD3 binds mitochondrial 12S rRNA, associates with the small subunit of the ribosome, and thus appears to have a general role in mitochondrial translation [Davies et al., 2009].

The PPR motif, along with tetratricopeptide repeats (TPR) and Sel-1 like repeats, form a larger structural family known as solenoid-repeat proteins [Karpenahalli et al., 2007]. Solenoid repeats, including PPR domains, are difficult to detect due to their degenerate nature. As a case in point, when the human genome was initially searched, only six proteins containing PPR domains were identified [Lurin et al., 2004], with MRPP3 initially being overlooked. To this end, we analyzed the seven annotated PPR domain containing proteins from the human genome with TPRpred, a novel program designed to search for more degenerate PPR motifs [Karpenahalli et al., 2007], and we were able to identify several additional PPR motifs within these proteins that were previously unrecognized (Table II). This exercise is proof-of-principle that other proteins likely exist in the human genome with degenerate, yet bona-fide PPR motifs. This is important to consider since such proteins would be excellent candidates for those involved directly in mitochondrial RNA metabolism and translation.

4.5 Post-transcriptional RNA modifications

Post-transcriptional modification of rRNAs and tRNAs is important for the proper function of these molecules and numerous types of modifications occur throughout the spectrum of life [Decatur and Fournier 2002; Gustilo et al., 2008]. Certainly, mitochondrial rRNAs and tRNAs are no exception to this rule [Dubin and Montenecourt 1970; Attardi and Attardi 1971]. However, because of the technical issues required to both identify modification sites/types and the enzymes required for their site-specific placement, our understanding of the RNA modification systems in mitochondria (and in general) is far from complete. Here, we shall review modification sites in mitochondrial tRNA and rRNA for which the respective modifying enzymes have been identified in humans and highlight how their impairment can lead to disease.

Ribosomal RNA methylation and pseudouridylation modifications have been mapped for mammalian mitochondrial ribosomes [Ofengand and Bakin 1997; Curgy 1985] and, while the number of modifications is less than that found in bacteria, the sites of modification are highly conserved, suggesting they serve important functions [Sirum-Connolly et al., 1995]. While genetic approaches in yeast have facilitated the unequivocal identification of rRNA modifying enzymes in mitochondria [Datta et al., 2005; Pintard et al., 2002; Ansmant et al., 2000], similar confirmation of the mammalian counterparts is lacking. One exception is the highly conserved N6-dimethylation of two adjacent adenine residues located near the end of the 12S rRNA, which is performed by h-mtTFB1 in human cells [Cotney et al., 2009; Cotney et al., 2009; Seidel-Rogol et al., 2003] and, as already discussed, confirmed by knock-out studies in mice [Metodiev et al., 2009]. Of pathological significance, h-mtTFB1 has been identified as a nuclear modifier of the A1555G mtDNA mutation that causes maternally inherited non-syndromic and antibiotic-induced deafness [Bykhovskaya et al., 2004b]. This mutation is located in the 12S rRNA gene in mtDNA, in close proximity to the stem-loop methylated by h-mtTFB1. We recently demonstrated that cybrids containing the A1555G mutation exhibit hypermethylation of the 12S rRNA, presumably due to a structural perturbation of the RNA that makes it a better substrate for h-mtTFB1 [Cotney et al., 2009]. These cells exhibit increased, but aberrant mitochondrial biogenesis and are more susceptible to stress-induced cell death, phenotypes shared by HeLa cells that are hypermethylated due to overexpression of h-mtTFB1. These results suggest that hypermethylation per se may be causing a mitochondrial retrograde signal that makes the cells more susceptible to apoptosis. This scenario may be relevant to the hair cell pathology and irreversible nature of the deafness in A1555G disease patients, which involves loss of these cells by apoptosis or other types of cell death. The homologous methyltransferase in *E. coli*, KsgA, methylates the small subunit rRNA during ribosome biogenesis, apparently providing an important checkpoint function for proper ribosome assembly [Xu et al., 2008; Connolly et al., 2008]. Thus, it is tempting to speculate that the rate of ribosome assembly is a signal for overall mitochondrial biogenesis and that this signal is enacted out of context due to hypermethylation abrogating a key ribosome assembly checkpoint in mitochondria. Furthermore, lowering the activity of h-mtTFB1 to reduce 12S hypermethylation is one potential explanation for how polymorphisms in the h-mtTFB1 gene can provide a protective effect in individuals with the A1555G mutation as reported [Bykhovskaya et al., 2004b]. Given that no mutations associated with the coding sequence of mtTFB1 were found in this study, it follows that the nuclear modifier activity is mediated through regulation of h-mtTFB1 expression, presumably its down-regulation. Thus, modulation of h-mtTFB1 expression and in turn 12S methylation is a potential avenue for prevention of deafness in A1555G carriers.

Modifications of tRNA molecules are critical for proper folding, recognition and base-pairing. The role of these modifications in folding may be even more important for mammalian mitochondrial tRNAs [Helm and Attardi 2004], which have evolved unusual structural features [Helm and Attardi 2004]. Several mitochondrial tRNA modifications have been identified in humans [Helm and Attardi 2004; Messmer et al., 2009; Helm et al., 1998; Juhling et al., 2009] and tRNA modification activities have been purified from HeLa cell mitochondria [Helm and Attardi 2004]. However, a comprehensive survey of all mitochondrial tRNA modifications has not yet been performed, nor have the specific proteins responsible for many of these modifications been identified.

Modifications of the anticodon stem to generate a so-called ‘wobble-base’ endows tRNAs with the ability to recognize multiple codons [Agris et al., 2007]. It has been observed that several pathogenic tRNA point mutations result in a lack of wobble-base modifications and this has been implicated in the pathogenesis of these mutations [Yasukawa et al., 2005]. The hypermodified nucleoside 5-methylaminomethyl-2-thiouridylate (mnm^5s^2U34) is a highly

conserved wobble-base modification also present in certain human mitochondrial tRNAs [Suzuki et al., 2002; Suzuki et al., 2001]. This modification requires a series of steps by multiple enzymes, of which three mitochondrial homologs (TRMU, MTO1 and GTPBP3) have been identified to date [Yan et al., 2005; Umeda et al., 2005]. Several mutations in the best characterized of these enzymes, TRMU, are linked to acute infantile liver disorder [Zeharia et al., 2009] and result in decreased 2-thiouridylation of mitochondrial tRNAs and impaired mitochondrial translation. Another mutation in TRMU also acts as a negative nuclear modifier of the A1555G and A1491G deafness-associated mtDNA mutations [Guan et al., 2006], while MTO1 and GTPBP3 are nuclear modifiers of the A1555G mutation [Bykhovskaya et al., 2004c]. The A1555G mutation exhibits impaired mitochondrial translation [Guan et al., 1996]. Thus, defects in wobble-base modification, which also globally decreases mitochondrial translation (via improper tRNA maturation), presumably act in concert to lower overall mitochondrial translation below the threshold required for normal cellular function [Guan et al., 2006].

Another example of a wobble-base modification that is specific to metazoan mitochondria is the 5-formylcytidine modification of $tRNA^{MET}$, which allows the decoding of non-canonical AUA start codons for translation initiation [Lusic et al., 2008; Takemoto et al., 2009]. While the enzyme required for this unusual modification remains unknown, mutations affecting this process might be expected to specifically affect expression of the proteins which utilize an AUA initiation codon (*i.e.* ND2, ND3 and ND5).

Pseudouridylation is another common RNA modification known to occur in mitochondria. Pseudouridylate synthase 1 (PUS1), which is predicted to localize both to mitochondria and the nucleus, is a candidate enzyme for the highly conserved pseudouridylation in the T-loop of tRNAs [Bykhovskaya et al., 2004a]. Recently, mutations in PUS1 have been implicated in myopathy, lactic acidosis and sideroblastic anemia (MLASA), in which decreased mitochondrial translation is presumably due to impaired mitochondrial tRNA pseudouridylation [Bykhovskaya et al., 2004a; Fernandez-Vizarra et al., 2007]. While several other pseudouridylate synthase paralogs are present in the human genome, their potential roles in mitochondrial RNA metabolism remain untested.

The MRPP1 component of the mitochondrial RNase P is one of three human homologs of the yeast tRNA m^1G_9 methyltransferase TRM10. In yeast, TRM10 methylates position $N^1$of the guanine residue located at position nine in several tRNAs. Unpublished reports also suggest MRPP1 modifies human mitochondrial tRNAs [Holzmann et al., 2008], however, the function of other known m^1G_9 methyltransferase homologs in humans remains unknown. Methylation at this location in the D-loop of human mitochondrial tRNAs is a recurring theme as a similar methylation of the adenine in position 9 of mitochondrial $tRNA^{LYS}$ (m^1A_9) is required for proper folding of this tRNA [Helm and Attardi 2004; Helm et al., 1998], while an m^2G_{10} methylation event in mitochondrial $tRNA^{LEU(UUR)}$ is known to be impaired in mitochondria harboring the pathogenic A3243G mutation in this tRNA gene that causes MELAS [Helm et al., 1999].

The identification of additional RNA modification enzymes in mitochondria remains an important goal as it very likely would illuminate novel genes involved in human disease. In this regard, it is important to remember that site-specificity of these modification enzymes is not necessarily conserved across orthologs from different species. The h-mtTFB2 protein, although primarily characterized as a mitochondrial transcription factor, maintains methyltransferase activity [Cotney and Shadel 2006]. However, it cannot compensate for the site-specific 12S rRNA methylation activity of mtTFB1 in mice [Metodiev et al., 2009]. One possibility is that h-mtTFB2 performs a different methylation of mitochondrial RNA. And, by this same logic, it remains possible that h-mtTFB1 (or other RNA modifying enzymes)

may also perform modifications in mitochondria in addition to those so far characterized. Clearly, deciphering the precise roles of specific post-transcriptional RNA modifications in mitochondrial RNA is an important and disease-relevant frontier.

5. Mitochondrial Translation

Recognition of mRNAs and initiation of translation by ribosomes is an important step in mammalian mitochondrial gene expression that is poorly understood. Unlike bacterial mRNAs, which contain a Shine-Dalgarno sequence, or yeast mitochondrial mRNAs, which have sequence elements in their 5′ UTRs that bind message-specific translational activators that facilitate ribosome binding and initiation of translation [Naithani et al., 2003], mammalian mitochondrial mRNAs generally have no sequences prior to the translation initiation codon that could be used as a ribosome-binding site [Montoya et al., 1981]. Until recently, our understanding of the signals and factors required for mitochondrial mRNA recognition and translation initiation has been completely deficient. Notably, a lack of predicted secondary structure at the 5′ end of mitochondrial mRNAs has been noted and suggested to be relevant with regard to facilitating initiation of translation in mitochondria [Jones et al., 2008]. It is also possible that internal ribosome entry sites are at play.

One breakthrough on the mitochondrial translation front is the identification of TACO1, a specific translational activator of COXI in human mitochondria [Weraarpachai et al., 2009]. Mutations in the TACO1 gene were identified as causative in a patient with cytochrome c oxidase deficiency and late-onset Leigh syndrome that had normal COXI mRNA levels. This potentially provides a new foothold for understanding how mitochondrial mRNAs are recognized for translation if the mechanism of action of TACO1 can be determined. This may also indicate that other gene-specific translation factors exist in human mitochondria to aid in translation of the remaining 12 mRNA species encoded in the mitochondrial genome, a situation reminiscent to that occurring in yeast mitochondria [Costanzo and Fox 1990]. One such candidate factor is SLIRP, an RNA-binding protein recently identified through computational methods used to search for previously uncharacterized regulators of OXPHOS [Baughman et al., 2009].

5.1 Ribosomes

Along with the OXPHOS complexes, mitochondrial ribosomes are the only cellular entities comprised of products encoded by both the nuclear and mitochondrial genomes. That is, the ~80 mitochondrial ribosomal proteins (MRPs) are encoded by nuclear genes that are imported into mitochondria, where they assemble with the two mtDNA-encoded rRNAs. This creates a unique situation for mitochondrial ribosome biogenesis compared to bacterial or even cytoplasmic ribosomes, which are synthesized and assembled in the same compartment. In addition, this requires coordination of nuclear and mitochondrial gene expression. As a result, mitochondrial ribosome assembly may be an important gauge of overall mitochondrial homeostasis as this process could serve to readout not only of how well both nuclear and mitochondrial gene expression is occurring and is coordinated, but also of the efficiency of mitochondrial import. The process of ribosome biogenesis is also energetically very expensive, providing yet another reason that mitochondrial ribosome assembly may be a process that is monitored carefully by cellular signaling pathways to estimate of the need for overall mitochondrial biogenesis.

Mitochondrial ribosomes are unusual in that they have smaller rRNA species in comparison to the ribosomes of their bacterial ancestors. Several groups have utilized proteomics approaches to identify a large complement of MRPs [Koc et al., 2001a; Koc et al., 2001b; Cavdar Koc et al., 2001a]. These studies, in combination with the determination of the three-dimensional structure of the bovine mitochondrial ribosome [Sharma et al., 2003], reveal an

increased protein content of ribosomes (69%) relative to bacteria (33%), which is thought to compensate for the smaller rRNAs [O'Brien 2003; O'Brien 2002]. This additional protein content, which is variable across eukaryotic species [Smits et al., 2007], is provided by the acquisition of new domains on canonical ribosomal proteins, as well as the addition of proteins having acquired a novel function in the ribosome. As such, it is perhaps not surprising that many mammalian MRPs have been demonstrated to exhibit extra-ribosomal functions [Cavdar Koc et al., 2001b] (Table III). Given the recurring theme of cell cycle regulation and cell death in the list of alternate MRP functions (Table III), it is tempting to speculate that mitochondrial ribosomes and translation play a previously underappreciated role in regulating these important cellular processes.

Given the essential role of mitochondrial translation in generating functioning OXPHOS complexes, it is not surprising that several MRP genes have recently been identified as candidate disease genes [O'Brien 2002; O'Brien et al., 2005; Sylvester et al., 2004; Jacobs and Turnbull 2005; Miller et al., 2004]. Proper import and assembly of MRPs into ribosomes is a key step required for efficient translation. One clinically relevant example of this is MRPL32, which requires processing via the mitochondrial m-AAA protease prior to assembly into ribosomes [Nolden et al., 2005], and is affected by loss of function of paraplegin, an essential component of the m-AAA protease, mutations in which cause hereditary spastic paraplegia and OXPHOS defects [Casari et al., 1998]. While the m-AAA protease also plays an important role in protein quality control, conceivably, the tissue-specific phenotypes associated with this disease could in part be due to impaired translation due to lack of proper processing of MRPL32 [Nolden et al., 2005].

5.2 Mitochondrial translation factors

In addition to the core components of the small and large ribosomal subunits, many other factors are also required for mitochondrial translation initiation, elongation and termination. Recent studies have identified several of these proteins, examined their roles in translation, and revealed important links to human disease (Table IV). Despite the differences in rRNA and protein content of bacterial and mitochondrial ribosomes, they are still more similar to one another than to eukaryotic cytoplasmic ribosomes. In fact, the conservation is such that hybrid ribosomes can be generated in *E. coli* by replacing one or more bacterial proteins with their mitochondrial homologues [Gaur et al., 2008;Soleimanpour-Lichaei et al., 2007]. Given that the bacterial ribosome is so well characterized, the generation of such hybrid ribosomes has been invaluable in studying the function of mitochondrial translation factors.

Translation initiation factors are conserved proteins required for assembly of ribosomes and initiation of translation. Unlike bacteria, which require three translation initiation factors (IF1-3), mammalian mitochondria utilize only two such factors [Koc and Spremulli 2002; Ma et al., 1995]. This discrepancy is likely due to the fact that mitochondrial initiation factor 2 (IF2mt, MTIF2) is able to functionally replace both IF1 and IF2 in *E. coli*, suggesting it also performs the functions of both in mitochondria [Gaur et al., 2008]. Mitochondrial initiation factor 3 (IF3mt, MTIF3) is involved in translation initiation through a direct binding with fMet tRNA and plays a role in subunit dissociation [Christian and Spremulli 2009]. Notably, mutations in the IF2mt gene have been implicated in susceptibility to Parkinson's disease [Abahuni et al., 2007].

The elongation factor Tu (EF-Tu) recognizes properly aminoacylated tRNA molecules and delivers them to the ribosome in a complex with the GTP required for translocation. The mitochondrial EF-Tu (EF-Tumt, TUFM) interacts with aminoacylated tRNAs [Hunter and Spremulli 2004] and is important for translational fidelity [Nagao et al., 2007]. Additionally, EF-Tumt has been implicated as a chaperone involved in protein folding in mitochondria [Suzuki et al., 2007]. To date a single mutation in EF-Tumt has been shown to cause

infantile encephalopathy due to decreased mitochondrial translation [Valente et al., 2007] as the result of decreased binding of EF-Tumt to aminoacylated tRNAs [Valente et al., 2009]. The guanine nucleotide exchange factor for EF-Tumt is the mitochondrial elongation factor Ts (EF-Tsmt, TSFM), which is required to regenerate GTP charged EF-Tumt. A mutation in EF-Tsmt was discovered in a patient exhibiting protein synthesis defects and OXPHOS deficiency [Smeitink et al., 2006].

In bacteria, the translation elongation factor G (EF-G) hydrolyses GTP to provide the energy required for the translocation step of protein elongation and also plays a vital role in ribosome disassembly through interactions with the ribosome recycling factor (RRF) [Savelsbergh et al., 2009]. Mitochondria, on the other hand, utilize two EF-G homologs, EF-G1mt (GFM1) and EF-G2mt (GFM2) [Hammarsund et al., 2001], which handle the distinct roles in translocation and ribosome recycling, respectively [Tsuboi et al., 2009]. Pathogenic mutations in EF-G1mt result in severely decreased mitochondrial translation [Valente et al., 2007; Coenen et al., 2004]. Consistent with the notion that EF-G1mt and EF-G2mt perform separate functions, overexpression of EF-G2mt cannot rescue translation in cells with mutant EF-G1mt [Coenen et al., 2004]. Furthermore, tissue-specific changes in the relative protein levels of EF-G1mt directly correlate with deficient mitochondrial translation [Antonicka et al., 2006].

Over 10 years ago, Zhang *et al.* identified (via homology to bacterial counterparts) two putative factors required for mitochondrial translation termination, ribosome release factor (mtRRF) and translation release factor (mtRF1) [Zhang and Spremulli 1998]. Recently, characterization of mtRRF (MRRF) revealed that it is localized to mitochondria, associates with ribosomes, and causes decreased mitochondrial translation when its expression is knocked down [Rorbach et al., 2008]. Furthermore, mtRRF specifically interacts with EF-G2mt to promote proper translation and ribosome disassembly [Tsuboi et al., 2009]. Meanwhile, two recent studies were unable to confirm the function of mtRF1 through *in vitro* characterization of the protein [Soleimanpour-Lichaei et al., 2007; Nozaki et al., 2008]. However, both studies identified another homolog, mtRF1a (MTRF1L), and demonstrated the capability of this novel release factor to terminate translation at UAA and UAG codons. Notably, only 11 of the 13 mitochondrial mRNAs utilize a UAA or UAG stop codon, with the remaining two utilizing the non-canonical stop codons AGA and AGG. It is has been speculated that the originally identified mtRF1, which is localized to mitochondria, may have specifically evolved to perform translation termination at these two non-canonical stop codons [Soleimanpour-Lichaei et al., 2007]. Thus, without specifically assaying for termination at these non-canonical sites the function of mtRF1 may have been missed. Alternatively, another yet-to-be-characterized protein may function at these non-canonical stop codons.

Clearly, there are many ways in which mitochondrial translation can be impaired leading to OXPHOS defects and ultimately pathology. Thus a more detailed understanding of mitochondrial translation is still needed before this complexity can be unraveled. It is notable that overexpression of EF-tumt and EF-G2mt can partially rescue the translation defects in the A3243G MELAS mutation, indicating that alterations in mitochondrial translation can be beneficial in some cases [Sasarman et al., 2008]. However, we must remain cautious as our understanding of this system is still premature in this regard. For example, overexpression of EF-tumt and EF-Tsmt in fibroblasts has dominant negative effects [Antonicka et al., 2006]. Additionally, the unexpected tissue-specific pathology associated with the nuclear-encoded EF-G1mt suggests that the mitochondrial translation apparatus and/or its regulation may be variable in different tissues [Antonicka et al., 2006] and thus much more complex than previously anticipated.

5.3 Regulation of mitochondrial translation

Post-translational modifications of ribosomal proteins are another means by which mitochondrial translation can be regulated. Phosphorylation is a common regulatory modification and has inhibitory effects on *E. coli* ribosomes [Soung et al., 2009]. Previous reports identified two components of mitochondrial translation apparatus that are phosphorylated. Phosphorylation of EF-Tumt has been observed in response to ischemia [He et al., 2001] and is predicted to inhibit translation (as is the case in *E. coli*). Meanwhile, phosphorylation of MRPS29 (also known as DAP3) may be relevant to either translation or its alternative role in apoptosis [Miller et al., 2008]. A more comprehensive study looking at bovine mitochondrial ribosomes identified phosphorylation of nearly one third (24 of ~80) of the total complement of MRPs and also showed that phosophorylation of mitorobosomes *in vitro* leads to inhibited translation activity [Miller et al., 2009]. Given that phosphorylation both of the *E. coli* and mitochondrial ribosome leads to reduced translation, this may be a highly conserved mechanism for modulating ribosome activity and translation output. Identification of the kinases that phosphorylate the mitochondrial ribosome and the physiological signals that regulate their activities will be an important advancement in our understanding of this process. Additionally, other ribosomal protein modifications, such as acetylation or methylation, are also likely to be important in the regulation mitochondrial translation. For example, methylation of the translation release factor mtRF1a is required for proper function of this protein [Ishizawa et al., 2008], while methylation of L3 and L12 is known to occur in *E. coli* [Polevoda and Sherman 2007]. The role of post-translational modification of mitochondrial regulatory proteins is yet another major area in mitochondrial biology that has not yet been studied in great enough depth.

6. Concluding Remarks

As we have endeavored to articulate, there have been many recent breakthroughs in the realm of identifying factors required for mitochondrial gene expression in humans. What is also quite evident is that, as a whole, this process it a hot bed of activity with regard to new human disease connections. In fact, if one considers the large number of nuclear genes that are required to simply maintain and express the thirteen mtDNA-encoded proteins at a steady-state in a cell, it becomes clear that this is one of the most susceptible cellular processes to nuclear genome mutagenesis and instability. This, coupled to the inherent susceptibility of mtDNA itself to oxidative damage and mutagenesis, makes the entire mitochondrial gene expression system a major axis for human genetic disease. Likewise, this is also why mitochondrial dysfunction is a key player in aging and age-related pathology, including cancer, neurodegeneration, diabetes and heart disease [Wallace 2005]. This argument becomes stronger yet, if genes for the OXPHOS complexes themselves, the many factors that are required for assembly of that system, and the nuclear and cytoplasmic factors that are needed for nuclear-mitochondrial signaling are included [Fernandez-Vizarra et al., 2009], which we did not have space to cover in this review.

With the inventory of factors required for mitochondria gene expression now large, and growing at an unprecedented rate, now is the time to delve more carefully into the regulation of the system. For example, we now have a good handle on the core machinery required for basal transcription of mtDNA in humans, but what is missing are detailed mechanistic studies on how these factors and the three mtDNA promoters are differentially regulated in vivo and in different tissues. The same can be said for understanding the regulation of the downstream processes of mitochondrial RNA processing and translation, as well as the relevance of post-transcriptional and post-translational modifications. In many ways, these areas can still be considered very much in their infancy and arguably in need of the most attention given the multiple connections to disease they already represent, despite the fact that only a subset of the requisite factors has been unequivocally identified to date.

Clearly, these are exciting times for the field of mitochondrial biology and disease and future investigations will no doubt yield new links to human pathology, insight into the aging process, and the promise of novel therapeutic strategies for a large number of important human health issues.

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7. References

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Table I

Nuclear receptors and transcription factors reported to localize to mitochondria

Protein	Nuclear Function*	Proposed Mt Function(s)	References
Estrogen receptor alpha	NR	Transcription	[Chen et al., 2004; Demonacos et al., 1996]
Estrogen receptor beta	NR	Transcription	[Chen et al., 2004]
Thyroid hormone receptor	NR	Transcription	[Casas et al., 1999; Fernandez-Vizarra et al., 2008; Wrutniak et al., 1995]
Glucocorticoid receptor	NR	Transcription Apoptosis	[Demonacos et al., 1993; Psarra and Sekeris 2009] [Adzic et al., 2009; Talaber et al., 2009; Sionov et al., 2006]
Nur 77	NR	Apoptosis	[Wang et al., 2009a; Li et al., 2000]
Retinoid X Receptor	NR	Transcription	[Casas et al., 2003]
Retinoic Acid Receptor	NR	Unknown	[Berdanier et al., 2001]
Androgen Receptor	NR	Unknown	[Solakidi et al., 2005]
PPARγ2	Coactivator	Interacts with TR	[Casas et al., 2000]
SLIRP	NR repressor	Mitochondrial mRNA stability	[Baughman et al., 2009]
CREB	TF	Transcription	[Lee et al., 2005; Ryu et al., 2005; De Rasmo et al., 2009]
AP-1	TF	Transcription	[Ogita et al., 2003]
NF-κB	TF	Transcription Apoptosis	[Cogswell et al., 2003] [Zamora et al., 2004]
I-κB	Inhibitor of NF-κB	Unknown	[Cogswell et al., 2003]
p53	TF	Apoptosis mtDNA replication/repair	[Marchenko et al., 2000] [Yoshida et al., 2003; Bakhanashvili et al., 2009; Bakhanashvili et al., 2008]
STAT3	TF	Translation	[Wegrzyn et al., 2009]
HMGA1	TF	DNA binding	[Dement et al., 2005]
Wnt13	TF	Unknown	[Struewing et al., 2006]

* NR and TF denotes Nuclear Receptor or Transcription Factor, respectively.

Table II

PPR domain containing proteins identified in the human genome

Protein	PPR Domains Reported/TPRpred*	Function(s)	Cellular Localization	Reference
POLRMT	2/1	Transcription	Mitochondria	[Rodeheffer et al., 2001]
LRPPRC	11/19	mRNA stability	Mitochondria	[Mootha et al., 2003]
		Nuclear transcription	Nucleus	[Cooper et al., 2006]
MRPS27	1/3	Translation	Mitochondria	[Xu et al., 2008]
MRPP3	2/2	RNA processing	Mitochondria	[Holzmann et al., 2008]
PTCD1	8/7	RNA processing	Mitochondria	[Rackham et al., 2009]
PTCD2	1/3	RNA processing	Mitochondria	[Xu et al., 2008]
PTCD3	9/9	Translation	Mitochondria	[Davies et al., 2009]

*TPRpred is a recently developed bioinformatic program that searches for PPR motifs http://toolkit.tuebingen.mpg.de/tprpred [Karpenahalli et al., 2007]

Table III

Mitochondrial ribosomal proteins implicated in additional functions other than translation.

MRP	Alternate function(s)	Other localization?	References
MRPS29 (DAP3)	Apoptosis	Nucleus	[Cavdar Koc et al., 2001b; Hulkko and Zilliacus 2002]
MRPS30 (PDCD9)	Apoptosis -Homologous to chicken pro-apoptotic protein p52		[Cavdar Koc et al., 2001b]
MRPL37	Apoptosis? -Homologous to MRPS30		[Levshenkova et al., 2004]
MRPL41 (BMRP)	Apoptosis / Cell cycle progression -Interacts with Bcl-2 and p53		[Chintharlapalli et al., 2005; Yoo et al., 2005; Kim et al., 2005]
MRPS36	Cell cycle progression		[Chen et al., 2007]
MRPL12	Mitochondrial transcription	Nucleolus	[Wang et al., 2007] [Andersen et al., 2005]
MRPS34	Unknown -Interacts with hDLG scaffold protein	Cytosol	[Ogawa et al., 2003]
MRPS18B	Viral infection and cell cycle regulation -Interacts with Rb protein and promotes entry to S-phase	Nucleus	[Kashuba et al., 2008]

Table IV

Mitochondrial Translation Factors.

Name	Official symbol	Function(s) in Translation	Associated Disease Relation
IF2mt	MTIF2	Initiation	Parkinson's Disease
IF3mt	MTIF3	Initiation Subunit disassociation	
EF-Tumt	TUFM	Delivers aminoacylated tRNAs Translation fidelity Protein folding chaperone	Infantile encephalopathy
EF-Tsmt	TSFM	Guanine nucleotide exchange factor for EF-Tumt	Encephalomyopathy Hypertrophic cardiomyopathy
EF-G1mt	GFM1	Translocation	Infantile encephalopathy Early-onset Leigh syndrome Progressive hepatoencephalopathy
EF-G2mt	GFM2	Ribosome recycling	
mtRRF	MRRF	Ribosome disassembly	
mtRF1	MTRF1	Translation termination?	
mtRF1a	MTRF1L	Translation termination	