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The Multiple Levels of Mitonuclear Coregulation

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Abstract

Together, the nuclear and mitochondrial genomes encode the oxidative phosphorylation (OXPHOS) complexes that reside in the mitochondrial inner membrane and enable aerobic life. Mitochondria maintain their own genome that is expressed and regulated by factors distinct from their nuclear counterparts. For optimal function, the cell must ensure proper stoichiometric production of OXPHOS subunits by coordinating two physically separated and evolutionarily distinct gene expression systems. Here, we review our current understanding of mitonuclear coregulation primarily at the levels of transcription and translation. Additionally, we discuss other levels of coregulation that may exist but remain largely unexplored, including mRNA modification and stability and posttranslational protein degradation.

1. BACKGROUND

Mitochondria are double membrane–bound organelles that generate the majority of cellular energy through oxidative phosphorylation (OXPHOS). Four respiratory complexes that reside in the mitochondrial inner membrane create an electrochemical gradient across this membrane. The Complex V ATPase uses this gradient to generate ATP (**Figure 1*a***). In addition to their role in OXPHOS, mitochondria are integral to the regulation of metabolism, apoptosis, and cytosolic calcium concentration.

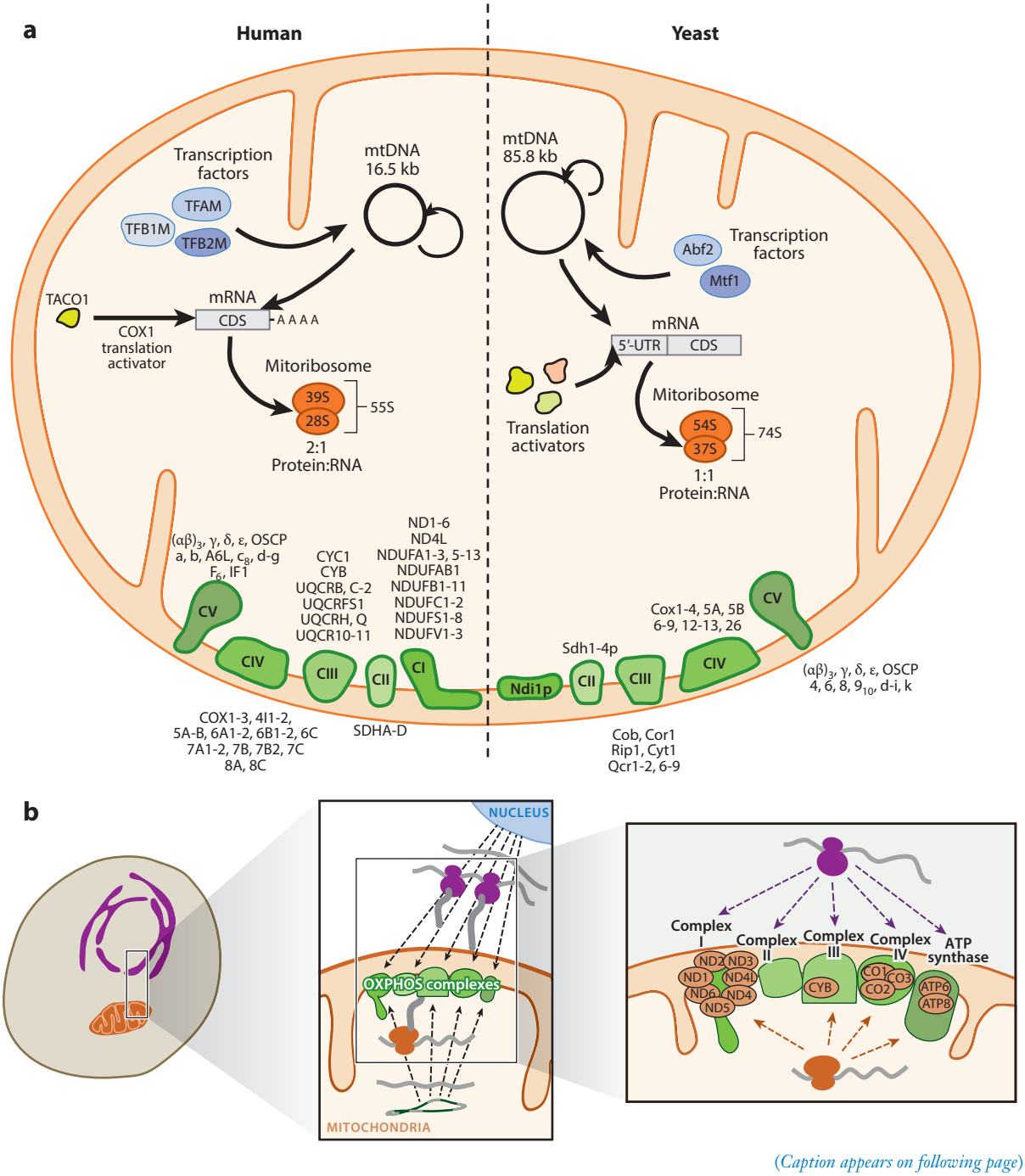
1.1. Regulation of Mitochondrial Gene Expression

Biogenesis of OXPHOS complexes presents a unique problem for human cells because their components are encoded by two different genomes. Collectively, these complexes contain approximately 80 subunits, of which just 13 are encoded by the mitochondrial genome; the remainder are encoded by the nuclear genome (**Figure 1*b***). Accordingly, to ensure efficient assembly and function, the biogenesis of these dual-origin complexes requires coordinated expression of the nuclear and mitochondrial genomes. Further complicating the matter, due to the evolutionary origin and size reduction of the mitochondrial genome (see the sidebar titled The Origins and Evolution of the Mitochondrial Genome), the mitochondrial expression machinery is distinct from the nuclear and cytosolic systems (**Figure 1*a***). The mitochondrial genome is transcribed by a single-subunit RNA polymerase most similar to enzymes from bacteriophage (76, 98), whereas translation is carried out by a dedicated ribosome within the mitochondria, the mitoribosome (42). Mitochondrial mRNAs lack both the 5′-caps of nuclear transcripts (59) and the Shine–Dalgarno sequences of prokaryotic mRNAs (105). Mitochondria also use slightly altered genetic codes that are read out by specific, mitochondrial-encoded tRNAs (8, 16). Therefore, the proper timing of subunit expression and correct assembly of OXPHOS complexes require the coordinated effort of both nuclear and mitochondrial regulatory pathways. Improper function of any of these pathways can lead to myriad human diseases with a wide range of symptoms and age of onset (reviewed in 147).

Although many of the principles of mitochondrial gene expression and regulation are conserved, species differ in several key regards, including the size and shape of the mitochondrial genome and the details of gene regulatory pathways. The human mitochondrial DNA (mtDNA) is a 16.5-kb circular plasmid (5), whereas the genome of *Saccharomyces cerevisiae* is 85.8 kb but appears to exist as linear arrays (45, 94). In both species, the mitochondrial genome is transcribed as polycistronic transcripts (34) that are posttranscriptionally processed and modified in a species-specific manner. In general, human mitochondrial mRNAs lack standard 5′-caps (59), whereas those of budding yeast have characteristically long 5′ untranslated regions (5′-UTRs). Yeast mitochondrial mRNAs also lack the polyA tails of their human counterparts (25, 110). The regulation of mitoribosome translation differs between species; budding yeast depend on transcript-specific translation factors that act through the long 5′-UTRs (reviewed in 63). By contrast, human mitochondrial mRNAs have short or no 5′-UTRs, and their translational regulation is less well understood (**Figure 1**).

1.2. Cross-Compartment Coregulation of Dual-Origin Complex Genes

All mitochondrial-encoded proteins contribute to dual-origin complexes. To ensure that such complexes are produced in a timely and efficient manner, some mechanism must balance the accumulation of mitochondrial- and nuclear-encoded proteins. OXPHOS complex assembly is particularly tricky in this regard because assembly intermediates can aberrantly produce reactive



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Mitochondrial gene regulation in humans and budding yeast. (*a*) The human mitochondrial genome is a 16.5-kb circular chromosome (5). By contrast, the *Saccharomyces cerevisiae* mitochondrial genome is much larger at 85.8 kb. Moreover, while it is typically depicted as circular, it is thought to exist as linear tandem arrays (94). Both the yeast and human mitochondrial genomes encode core protein components of the OXPHOS systems as well as tRNAs and rRNAs. The yeast genome also encodes a subunit of the mitoribosome, Var1. mRNAs are transcribed as polycistronic transcripts that are posttranscriptionally processed. Yeast mRNAs contain 5′- and 3′-UTRs of varying lengths, and human mitochondrial mRNAs also contain polyadenylated tails (110). Mitoribosomes are composed of large and small subunits that are themselves made up of rRNA and protein factors (42). Human mitoribosomes have a higher protein-to-RNA ratio than those of yeast and bacteria (137). Translation of yeast mRNAs requires specific translational activators (63). In humans, only one such factor has been described, translational activator of COX1 (TACO1) (158). The OXPHOS Complexes I–IV are responsible for generating a proton gradient that is used by the Complex V ATPase to produce ATP from ADP and inorganic phosphate. The OXPHOS system of both humans and yeast are very similar, although yeast have evolved a less complex, nuclear-encoded NADH reductase that does not contribute to the proton gradient. (*b*) Dual-origin OXPHOS complexes residing within the inner mitochondrial membrane consist of nuclear- and mitochondrial-encoded subunits. Mitoribosomes engage mRNAs transcribed from the mitochondrial genome and synthesize subunits that assemble with their nuclear counterparts, which are produced in the cytosol and imported into the mitochondria. The mitochondrial-encoded subunits are in the minority but serve as the core subunits of these inner membrane complexes. Abbreviations: CI, Complex I; CDS, coding sequence; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; UTR, untranslated region.

oxygen species (ROS), which damage DNA and proteins. Consistent with this, unbalanced OXPHOS biogenesis leads to disease and aging phenotypes (56, 66, 82). Thus, while cooperation between physically separated genomes expressed by distinct gene regulatory systems poses a formidable logistical challenge, achieving this coordination is clearly of physiological importance.

Subunits of nuclear-encoded complexes are often coregulated by synchronous transcription. The first genome-wide microarray experiments demonstrated that genes in the same pathways and complexes are induced with comparable dynamics (33), a principle so consistently observed in subsequent genome-wide analyses that it underlies unbiased bioinformatic approaches to place unannotated genes into biological processes (64, 67). Translation is also coregulated to promote

THE ORIGINS AND EVOLUTION OF THE MITOCHONDRIAL GENOME

Mitochondria originated from the endosymbiotic incorporation of a free-living α-proteobacterium that retained a version of its own genome (reviewed in 1, 57). Following endosymbiosis, the mitochondrial precursor experienced radical minimization. Consequently, mitochondrial genomes vary greatly in size and gene content, ranging from 3 protein-coding genes in the apicomplexan *Plasmodium falciparum* to 67 in the flagellate *Reclinomonas americana* (51, 142). The mitochondrial-retained genes are all involved in respiration or protein synthesis, although the set of retained genes varies between species (reviewed in 19). Why have mitochondria retained their genomes rather than completing the genetic transfer? The cell uses a great amount of energy to regulate two vastly different gene expression systems. One prevailing hypothesis is that highly hydrophobic proteins are difficult to import into mitochondria and sort into the correct membrane (reviewed in 119, 156). Consistently, the only protein-coding genes retained across mitochondrial genomes, *COB* and *COX1*, encode the most hydrophobic proteins in mitochondria, presumably presenting a challenge for cytoplasmic import (26). An alternative, but not mutually exclusive, explanation is the colocalization for redox regulation theory (reviewed in 2), which posits that mitochondrial-encoded genes allow local regulation of OXPHOS machinery independent of other mitochondria in the same cell. Other proposed explanations for mitochondrial genome retention include the risks that cytosolic translation of some mitochondrial-encoded proteins is toxic and that differences in the nuclear and mitochondrial genetic codes restrict gene transfer.

balanced synthesis of complex subunits. Analysis of ribosome profiling data in bacteria and yeast revealed that the translation rates of mRNAs encoding complex subunits corresponded closely to known complex stoichiometry (89), although this association is not as pronounced in mammalian cells (72, 86). The widespread coregulation of nuclear-encoded complexes suggests that cells benefit from balanced expression of subunits. The advantages of such coordination are clear. However, coregulation of dual-origin complexes poses a significantly greater logistical challenge to the cell. Furthermore, the stakes are high; imbalanced subunit production during OXPHOS biogenesis runs the risk of OXPHOS defects and ROS-induced toxicity.

In this article, we review our current understanding of the coregulation of mitochondrial and nuclear genomes. We describe current knowledge of coregulation at each stage of gene expression and discuss the control mechanisms that may be responsible. We close by discussing the necessity of coregulation and the role of protein turnover in balanced OXPHOS production.

2. COREGULATION OF NUCLEAR AND MITOCHONDRIAL TRANSCRIPTIONAL PROGRAMS

Nuclear-encoded OXPHOS genes often cluster in transcriptome-wide gene expression analyses, suggesting a shared transcriptional control mechanism (87, 106, 141). Indeed, RNA expression profiles of nuclear genes that encode mitochondrial proteins are so similar that coexpression analyses can be used to correctly predict mitochondrial localization of proteins of unknown function (11, 107). In addition, individual OXPHOS subunits exhibit even tighter coexpression with subunits of the same complex than with subunits of separate complexes (152). This level of regulation implies that in addition to a master regulator of all nuclear-encoded OXPHOS genes, the cell also has fine-tuning mechanisms specific to each complex. How is the carefully controlled transcription of nuclear-encoded OXPHOS genes coordinated with the transcription of their mitochondrial-encoded counterparts?

It is well established that the nuclear and mitochondrial OXPHOS transcription programs are sequentially coregulated through numerous transcription programs launched by mitochondrial–nuclear signaling pathways (recently reviewed in 44, 120, 130). For example, through various anterograde pathways, the nucleus can direct mitochondrial activity and biogenesis by activating nuclear-encoded genes responsible for mtDNA replication and transcription. Several master regulators, including NRF-1, PGC-1α, and PGC-1β, are activated in response to metabolic sensors and regulate the vast majority of nuclear-encoded mitochondrial proteins, including mitochondrial gene regulatory factors, to promote the balanced expression of OXPHOS subunits (12, 35, 54, 155). In the reverse direction, mitochondria generate retrograde signals that can alter nuclear gene expression in response to stimuli such as OXPHOS dysfunction, DNA damage, and loss of mitochondrial membrane potential (4, 6, 69, 90, 91–93, 133, 136, 143).

At steady state, the anterograde and retrograde signaling pathways tend to promote stoichiometric OXPHOS subunit production at the mRNA and protein levels. However, these pathways take hours or days to remodel the nuclear/cytosolic transcriptome, which then produces proteins that remodel the mitochondrial transcriptome. Are those changes fast enough to produce a balanced production of OXPHOS subunits? Recently, we investigated whether nuclear- and mitochondrial-encoded OXPHOS genes are simultaneously transcriptionally coregulated in *S. cerevisiae*. To this end, we analyzed the kinetics of transcript accumulation following a switch to a nonfermentable carbon source (31) (**Figure 2*a***). Both nuclear- and mitochondrial-encoded OXPHOS transcripts increased following the carbon source shift, but with markedly different kinetics. Nuclear OXPHOS transcripts rapidly increased in abundance, whereas mitochondrial

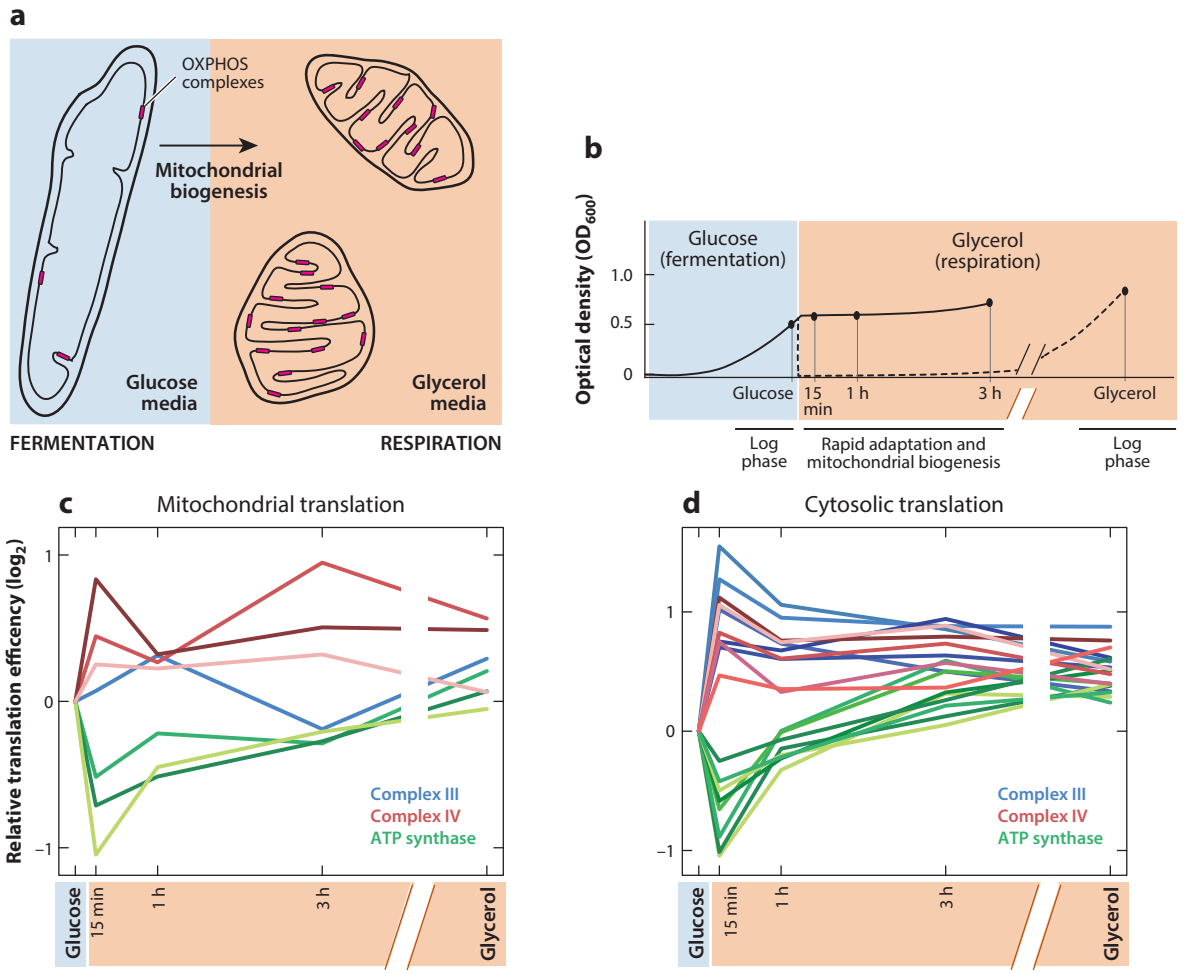


Figure 2

Coregulation of mitochondrial and nuclear gene expression during mitochondrial biogenesis in *Saccharomyces cerevisiae*. (*a*) In glucose-containing media, yeast obtain most of their energy from fermentation and therefore do not contain abundant OXPHOS complexes. When they are shifted into glycerol-containing media, yeast need to respire and consequently must undergo mitochondrial biogenesis to support the change in metabolism. During this adaptation, OXPHOS genes are upregulated across the mitochondrial and nuclear genomes. (*b*) To study gene expression during carbon source adaptation, a yeast culture was followed after a shift to glycerol-containing media. (*c*) Mitoribosome and (*d*) cytoribosome profiling following the transcripts that are engaged by ribosomes throughout the adaptation period (31). Translation efficiencies are displayed as fold change relative to efficiency in glucose media. Translation efficiency describes the translation regulation for each message and is calculated by normalizing the translation rate (ribosome footprint density) by the relative RNA abundance (RNA-seq density). The gene-specific translation programs during carbon source adaptation, color-coded by complex, are synchronized across cellular compartments. **Figure 2** is modified from Reference 31 with permission. Abbreviations: OXPHOS, oxidative phosphorylation; RNA-seq, RNA sequencing.

transcripts were upregulated at a much slower rate. These data suggest that transcription of OXPHOS components is not coordinated on short timescales. However, this has been investigated only in the context of mitochondrial biogenesis induced by a shift to nonfermentable media in *S. cerevisiae*, and it remains to be determined whether this will hold for other biogenesis pathways and in other species.

In our study, when cells were grown on glucose, the steady-state levels of mitochondrial OXPHOS mRNAs were higher than those of the nuclear OXPHOS mRNAs; the induction of nuclear transcripts upon the nutrient shift merely reduced the difference in these levels (31). It is possible that mitochondria maintain higher levels of their genomic transcripts, requiring that the cell upregulate nuclear transcription only when OXPHOS biogenesis is induced. Consistent with this notion, mitochondrial transcripts in several human cell lines are present at higher copy numbers than their nuclear counterparts (36, 84, 102, 132).

Given the lack of rapid coordination of OXPHOS transcriptional programs and observed differences between the abundances of nuclear- and mitochondrial-encoded mRNA, downstream regulatory mechanisms, such as translation regulation or protein turnover, are required to enforce stoichiometric assembly of dual-origin complexes.

3. COREGULATED MITOCHONDRIAL AND CYTOSOLIC TRANSLATION

Translation regulation has a major influence on the composition of the proteome (135). Recently, we showed that cytosolic and mitochondrial translation programs are synchronized during mitochondrial biogenesis in *S. cerevisiae* (31). To explore this issue, we analyzed the yeast translation programs by cytosolic and mitochondrial ribosome profiling during adaptation to respiratory growth (**Figure 2**). These data revealed that both cytosolic and mitochondrial ribosomes initially preferred mRNAs encoding OXPHOS Complex III and IV subunits over those encoding ATPase synthase subunits. Thus, despite being separated by two membranes and regulated by different mechanisms, these separate pools of ribosomes undergo the same regulatory program during carbon source adaptation. The mechanisms underlying the synchrony of the two translation programs remain unknown. Nevertheless, our initial analysis revealed that the translation programs are actively coregulated, in part through unidirectional communication from the cytosolic translation machinery to mitochondrial ribosomes (31).

The cytosolic and mitochondrial ribosome profiling methods differ substantially due to the differing stabilities of the two types of ribosome. Consequently, our analysis of mitochondrial and cytosolic translation could not determine whether the synchronized translation programs result in stoichiometric OXPHOS synthesis, and our results cannot be compared with those of the studies that demonstrated the stoichiometric cytosolic synthesis of complex subunits in yeast (89). Nonetheless, our findings clearly show that both compartments distribute their ribosomal resources similarly during mitochondrial biogenesis.

Mitochondrial translation is controlled by multiple mechanisms driven by nuclear-encoded proteins that presumably enable the synchronous cytosolic and mitochondrial translation programs. Yet, how regulation of mitochondrial translation is exercised in concert with cytosolic translation regulation is an area of ongoing research.

3.1. Translational Activators and Repressors

Pioneering genetic screens focusing on yeast respiratory control identified genes that control mitochondrial biogenesis, including mtDNA gene expression (38). Subsequent screens revealed that a subset of these factors are necessary for the synthesis of specific mitochondrial-encoded proteins (28, 30, 46). Many of these nuclear-encoded factors directly induce the translation of particular mitochondrial-encoded mRNAs and are therefore called translational activators. The biochemical mechanism by which they influence mitochondrial translation remains unclear, largely because mitochondrial translation has yet to be reconstituted biochemically. Nevertheless,

genetic evidence indicates that regulation by translational activators requires the 5′-UTRs of their mRNA targets and presumably involves binding of the activators to specific sequences within the UTRs (17, 29, 37). Translational repressors also exist; the first of these to be identified controls Atp6/8 synthesis (122).

Mitochondrial translational regulators are clear candidates for nuclear-encoded proteins that contribute to the synchronization of translational programs during yeast mitochondrial biogenesis. Indeed, cytosolic ribosome profiling data indicate that these regulators are themselves translationally upregulated when yeast are shifted from glucose- to glycerol-containing media (31). Because mitochondrial translational regulators are rate-limiting for production of their target proteins, their increased synthesis following a media shift has the potential to exert rapid translational control (58, 97, 144).

In humans, whether translation programs are synchronized during mitochondrial biogenesis remains unknown. Furthermore, most *S. cerevisiae* translational activators are not conserved in humans, and those that are appear to serve different functions (108). To date, only one translational activator has been identified in human cells. Analysis of a mutation associated with late-onset Leigh syndrome revealed a defect in the translation of *COX1* (158). Consequently, the responsible gene product, subsequently named TACO1, is considered a translational activator, although its mechanism of action needs to be determined before it will be clear whether it acts similarly to the way yeast translational activators act or by a distinct mechanism. Unlike yeast mitochondrial RNA, most mammalian mitochondrial messages either contain a minimal (<4-nt) 5′-UTR or lack one entirely (149). Therefore, any translational activators or repressors in mammals must use a mechanism distinct from that in yeast. Thus, it remains an open question whether synchronized translation programs exist in mammalian cells, and if so, how the two translation systems could be coregulated.

3.2. Nuclear-Encoded OXPHOS Subunits Control Mitochondrial Translation

Mitochondrial translation is stimulated in yeast by the arrival of nuclear-encoded subunits. For example, translation of mitochondrial-encoded Atp9 is stimulated by the expression of nuclear-encoded Atp9 during a shift from fermentation to respiration (47, 48). Here, the coordinated expression involves the variable stability of a cytosolic multisynthase complex, AME, which consists of a glutamyl- and a methionyl-tRNA synthetase (cERS and cMRS, respectively). AME dissociates during the metabolic shift from fermentation to respiration (47, 48). Free cERS localizes to mitochondria, where it stimulates mitochondrial translation. Simultaneously, free cMRS localizes to the nucleus and associates with RNA Pol II subunits, presumably controlling nuclear transcription. The nuclear localization of cMRS during the metabolic shift alters the expression of nuclear-encoded Atp1, which is responsible for an increase in mitochondrial-encoded Atp9 synthesis. In mammalian cells, the arrival of nuclear-encoded COX4 regulates the mitochondrial synthesis of COX1 (125). Without COX4, COX1 synthesis stalls as a nascent chain–ribosome complex. Such complexes may be primed for the arrival of COX4, presumably to ensure stoichiometric synthesis of Complex IV.

In yeast, translational activators provide the molecular mechanism connecting the arrival of nuclear-encoded subunits to the stimulation of mitochondrial translation. Many of these mitochondrial translational activators function as chaperones for the nascent polypeptide produced by their mRNA targets. Thus, unassembled mitochondrial-encoded subunits sequester translational activators, prohibiting further translation until the subunits are properly assembled with their nuclear-encoded counterparts. For example, cytochrome *c* oxidase (Complex IV) assembly requires the Cox1 translational activator Mss51, which also forms an assembly intermediate with

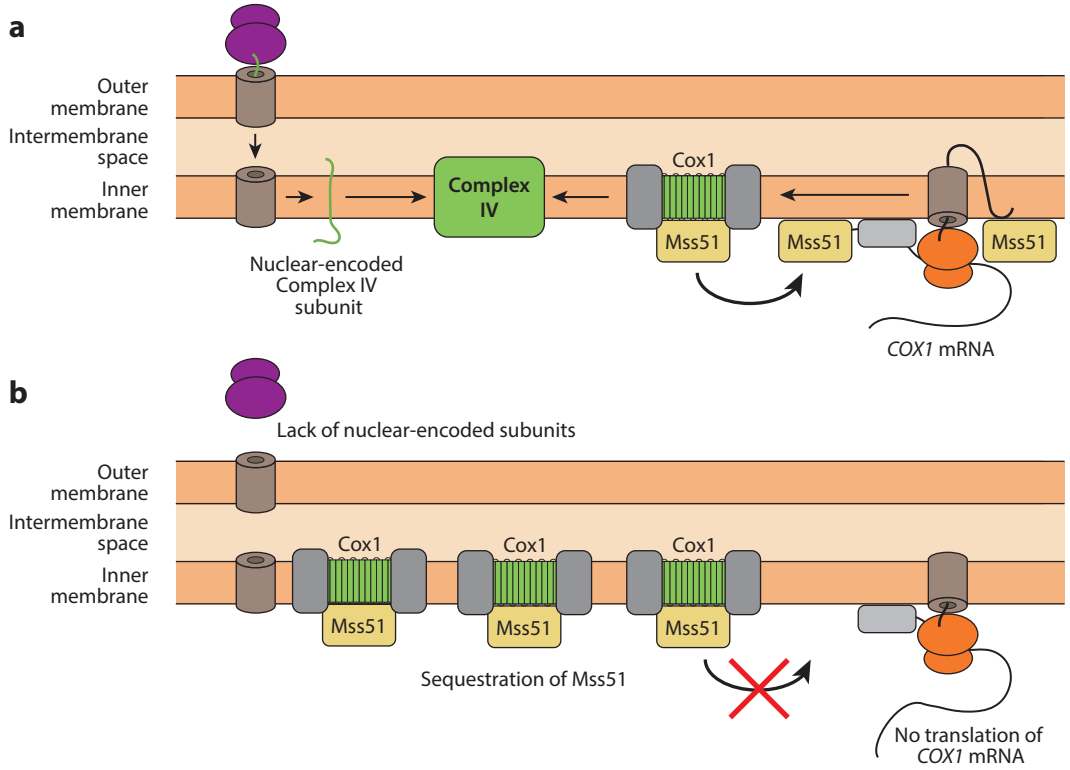


Figure 3

Coregulation of Complex IV assembly in *Saccharomyces cerevisiae*. OXPHOS subunits are encoded by the nuclear and mitochondrial genomes. Nuclear-encoded subunits are translated by cytosolic ribosomes and are actively transported across the mitochondrial membranes. In contrast, mtDNA-encoded subunits are translated by mitoribosomes near the mitochondrial inner membrane within which the nuclear- and mitochondrial-encoded OXPHOS subunits assemble into functional complexes. (*a*) In the case of Complex IV (cytochrome *c* oxidase), synthesis of mtDNA-encoded Cox1 requires the nuclear-encoded translational activator Pet309 Mss51 through an association with the *COX1* mRNA 5′-UTR. Importantly, Mss51 also binds the newly synthesized Cox1 protein, acting as an assembly chaperone (116). (*b*) If import of nuclear-encoded Complex IV subunits is decreased, unassembled Cox1 bound by Mss51 accumulates, thus sequestering Mss51 away from binding *COX1* mRNA and preventing further *COX1* translation (9, 10, 77, 103, 140). This prevents mitochondrial translation of *COX1* until enough nuclear-encoded subunits are imported, ensuring stoichiometric production of the nuclear and mitochondrial subunits. Abbreviations: OXPHOS, oxidative phosphorylation; mtDNA, mitochondrial DNA; UTR, untranslated region.

newly synthesized Cox1 (116) (**Figure 3**). Cox1, the core subunit of Complex IV, has 12 transmembrane domains and is thought to be cotranslationally inserted into the inner membrane (62). Following membrane insertion, Cox1 associates with Mss51 until it can assemble with the rest of the Complex IV subunits (9, 10, 77, 103, 140). Subsequently, Mss51 is presumed to be liberated to induce the next round of Cox1 synthesis. Because unassembled Cox1 sensitizes yeast cells to oxidative stress, sequestration of free Cox1 could serve a protective role (78). The role of Mss51 as both a translation activator and a chaperone suggests a feedback regulatory mechanism; when Cox1 is safely coassembled with other Complex IV subunits, then another round of Cox1 synthesis is initiated. Furthermore, this feedback mechanism positions Mss51 and other translation regulators as gatekeepers of mitochondrial translation, blocking synthesis until the arrival of nuclear-encoded OXPHOS subunits, which in turn ensures the correct stoichiometry of dual-origin complexes.

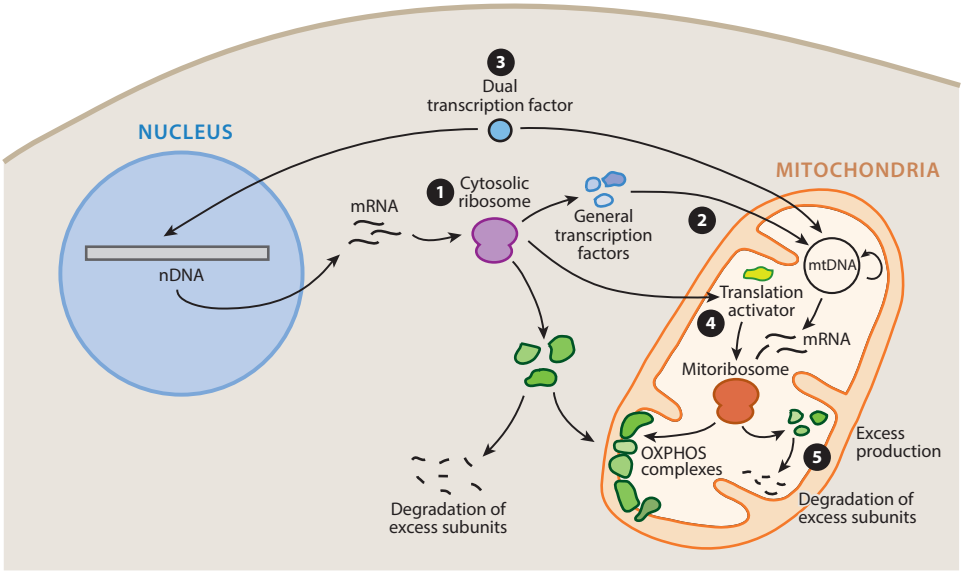


Figure 4

Levels of coregulation between the nucleus and mitochondria. ❶ Cytosolic ribosomes translate factors directly involved in mitochondrial control, including transcription factors, translational activators, and nuclear-encoded OXPHOS subunits. ❷ Transcription factors in mitochondria regulate both transcription and replication of the mitochondrial genome, which itself encodes the remaining subunits of the OXPHOS complexes. ❸ Some transcription factors localize to both the nucleus and mitochondria and could play direct roles in the regulation of both genomes. ❹ At least in *Saccharomyces cerevisiae*, translational activators are required for the efficient translation of each mitochondrial transcript. ❺ Subunits produced in superstoichiometric quantities in the cytosol and mitochondria are degraded through independent, compartment-specific pathways. Abbreviations: mtDNA, mitochondrial DNA; nDNA, nuclear DNA; OXPHOS, oxidative phosphorylation.

4. POSSIBLE MECHANISMS FOR COREGULATION ACROSS STAGES OF GENE EXPRESSION

At each stage of gene expression, the mitochondrial genome is regulated by nuclear-encoded factors that are imported into mitochondria. Many of these regulatory mechanisms likely contribute to the balanced accumulation of nuclear- and mitochondrial-encoded OXPHOS subunits (**Figure 4**).

4.1. Nucleoid Structure

The mitochondrial genome exists as a nucleoprotein complex, called a nucleoid, that can house multiple copies of the genome. Genome structure likely plays a critical role in the regulation of gene expression in mitochondria, as it does in both prokaryotes and eukaryotic nuclei. Recently, analysis of DNA-seq data from the Encyclopedia of DNA Elements (ENCODE) project revealed cell type–specific DNase hypersensitivity patterns across the mitochondrial genome that corresponded with differential gene expression, suggesting a link between nucleoid structure and transcriptional output (102). Mitochondrial transcription factor A (TFAM; Abf2 in yeast) is thought to be the main structural component of the nucleoid, as it can bend and compact DNA in vitro (75 , 83). Both overexpression and depletion of TFAM in vivo cause defects in genome replication, segregation, and expression, suggesting that either under- or overcompaction interferes with proper function

(39, 75). It is possible that other DNA-binding factors contribute to genome structure and thus to gene regulation. As the proteins involved in mitochondrial genome compaction are nuclear-encoded, the nuclear genome could directly dictate changes in the nucleoid structure, thereby influencing mitochondrial transcription.

4.2. mtDNA Copy Number

mtDNA exists in hundreds to thousands of copies per cell, depending on cell type (153). In humans, reduced mtDNA copy number is associated with a number of mitochondrial diseases (reviewed in 113). Several mitochondrial factors, including TFAM and the replicative helicase TWINKLE, have been implicated in the regulation of mtDNA copy number regulation (49, 68, 151). Mitochondrial mRNA levels could be controlled by mtDNA copy number (159). For example, a reduction in the amount of mtDNA could lead to an increased TFAM:mtDNA ratio, which has been shown to reduce mRNA levels, possibly due to overcompaction of mtDNA (100). Control of mtDNA copy number represents another potential mechanism of mitonuclear coregulation.

4.3. Noncanonical Roles for Nuclear Transcription Factors in Mitochondria

Research over the past two decades has revealed that nuclear transcription factors may have secondary functions within mitochondria. Many of these claims, however, have been met with skepticism due to the difficulty in experimentally demonstrating a separate mitochondrial role of a nuclear protein (32, 88, 118). To argue that a nuclear protein functions in mitochondria, it is first necessary to definitively show that the factor localizes to mitochondria, and this process can be confounded by protein contamination from other cellular compartments. Additionally, many of the nuclear transcription factors with putative mitochondrial roles lack mitochondrial translocation sequences, making it difficult to identify them bioinformatically. Furthermore, many of these factors regulate mitochondria through their nuclear functions, making characterization of a direct mitochondrial role even more challenging.

Evidence for the localization of many of these factors has been obtained using a variety of methods. Many of these proteins, including cyclic-AMP response element-binding (CREB) protein, thyroid hormone receptor p43, estrogen receptors ERα and ERβ, glucocorticoid receptor, MOF, Jun-D, c-Jun, MEF2D, and NFκB, localize to mitochondria in multiple cell types, as determined by subcellular fractionation, fluorescence microscopy, and immunogold electron microscopy (14, 20–24, 27, 32, 60, 71, 80, 95, 138). One concern with these types of experiments is that aggregating and unfolded proteins are imported into the mitochondrial matrix for degradation (128). Therefore, it is important to show that the folded versions of these proteins can be imported, such as through in vitro mitochondrial import assays. This has been achieved for CREB, p43, and MEF2D (21, 32, 138).

In addition to showing physical localization, it is important to demonstrate that these proteins have a mitochondrial-specific regulatory role. To this end, many studies have investigated whether mitochondrial-localized nuclear transcription factors bind mtDNA. Although in vitro DNA-binding assays are informative, it is also critical to show that they bind in vivo through assays such as chromatin immunoprecipitation (ChIP)-seq and ChIP-qPCR. Furthermore, bioinformatic analyses must be carefully performed to avoid mapping sequences to nuclear pseudogenes of mitochondrial origin, also known as *numts* (13, 61). Such analysis of both human and mouse genome-wide data sets has identified a handful of transcription factors with a putative role in mitochondria. Many of these, including CREB, ERα/β, and MafK, have been mapped by ChIP to the noncoding D-loop region or various mitochondrial gene bodies (14, 20, 21, 24, 32, 41, 43, 60, 71, 88, 95).

Do these proteins have a discernible function in mitochondria? Targeting CREB to mitochondria specifically increases the levels of *ND1*, *ND4*, and *ND5* transcripts (138). *ND6* mRNA and protein levels are decreased by MEF2D knockdown and increased by the targeting of active MEF2D to mitochondria (138). In another example, the lysine acetyltransferase MOF, as well as two of its accessory proteins, was recently shown to localize to mitochondria (22). Knockdown of MOF in HeLa cells decreases the levels of mitochondrial transcripts, with the exception of *ND2*, without inducing significant changes in key nuclear mitochondrial regulatory factors. In addition, MOF knockouts in mouse hearts result in mitochondrial degeneration and cardiac failure. In *Caenorhabditis elegans*, the transcription factor ATFS-1 is normally imported into mitochondria and degraded. However, during mitochondrial dysfunction, ATFS-1 traffics to the nucleus where it induces stress response genes. Further, a fraction of ATFS-1 remains in the mitochondria where it binds to mtDNA and prevents the accumulation of mtDNA-encoded transcripts (112). Although these observations support the roles of nuclear transcription factors in mitochondria, the mechanisms by which they act still need to be determined. In all cases, careful examination of compartment-specific activity is necessary to distinguish between mitochondrial and nuclear regulatory roles. Nevertheless, the potential activities of nuclear transcription factors is a topic that opens an interesting branch of mitonuclear communication and regulation.

4.4. Mitochondrial RNA Processing and Turnover

RNA processing provides another opportunity for coregulation between the mitochondrial and nuclear genomes. One example is the regulated degradation of MRPP3 during the mitochondrial unfolded protein response (mtUPR) (109). MRPP3 is one of the three protein subunits of mitochondrial RNAse P, which cleaves the 5′ ends of tRNA, simultaneously releasing the tRNA and adjoining mRNA from the polycistronic transcripts (114). In the absence of functional RNAse P during mtUPR, many mRNAs accumulate with unprocessed 3′-ends, potentially decreasing their efficient translation and thus preventing further aggregation of newly synthesized and unfolded proteins.

RNA degradation is also regulated (52). Considering the polycistronic nature of mitochondrial transcription, one would expect that mRNAs derived from the same transcript would be present in equimolar amounts. However, mRNA levels exhibit a striking degree of variability (81, 102), for which differential RNA degradation provides the most parsimonious explanation. Consistent with this, in HeLa cells, mitochondrial mRNA abundance and mRNA half-life are well correlated (25). RNA degradation in mitochondria is controlled by nuclear-encoded degradation factors such as the helicase SUV3 and the nuclease PNPase (Dss1 in yeast) (18, 148). In addition, nuclear-encoded mRNA-stabilizing factors such as LRPPRC and SLIRP play a significant role in shaping the mitochondrial transcriptome (11, 84). However, very little is known about how and to what extent these factors are themselves regulated. The importance of posttranscriptional RNA processing and degradation is highlighted by the fact that mutations in genes involved in these processes cause severe diseases. For instance, mutations in both PNPase and LRPPRC cause Leigh syndrome (reviewed in 147).

4.5. RNA Modifications

Translation could also be regulated by RNA modifications. It has long been known that many noncoding RNAs, such as rRNAs and tRNAs, are extensively modified, but recent high-throughput experiments using next-generation sequencing have revealed that mRNA modification is widespread (53). Some of these modifications are associated with translation

regulation. For example, the m1A modification, which is prevalent in mammalian mitochondrial-encoded mRNA, negatively correlates with translation efficiency and is coincident with sites of translational pausing (possibly by abrogating base pairing) (131), although the functional consequence of these pauses remains unexplored.

Another class of modification is noncanonical RNA capping. Mitochondrial RNA is typically uncapped, but an NAD^+ cap is present on some cytosolic and mitochondrial RNAs in yeast and mammals (70, 157). In yeast, only mitochondrial-encoded mRNAs with unprocessed 5′-ends are NAD-capped, suggesting that NAD^+ is incorporated as the initiating nucleotide during transcription (157). If this holds true in humans, then NAD caps are unlikely to have a major regulatory impact, because there are only three transcription start sites in the human mitochondrial genome. In any case, the function of noncanonical caps is not known.

4.6. Cytosolic RNA Import

Multiple types of nuclear-encoded RNAs, including tRNA and miRNA, are imported into mitochondria in a species-dependent manner (tRNA import has been reviewed in depth elsewhere, e.g., in 134). Regulation of mitochondrial tRNA abundance by controlling import could modulate mitochondrial translation elongation. Consistent with this idea, alterations in tRNA abundance influence cytosolic translation, and mutations in a mitochondrial tRNA gene alter mitoribosome pausing (127). tRNAs must be imported in some species, because their mitochondrial genome does not encode tRNAs that recognize all codons. However, import also occurs in species whose mitochondrial genome encodes a complete set of tRNAs. The *S. cerevisiae* mitochondrial genome encodes 24 tRNAs that are sufficient to support mitochondrial translation, but the nuclear-encoded Lys_{CUU}, Gln_{CUG}, and Gln_{UUG} tRNAs are still imported into the mitochondria (74, 79, 126). Of these three tRNAs, the functional consequences of only the Lys_{CUU} tRNA import have been investigated. Typically, the mitochondrial-encoded Lys_{UUU} tRNA is used to decode AAG lysine codons through a wobble uridine. Consequently, import of the Lys_{CUU} tRNA is redundant under normal conditions. However, under heat shock, import is essential because the wobble uridine becomes hypomodified and can no longer decode AAG (73). Notably, in this regard, mitoribosomes pause more frequently at AAG codons during carbon source adaptation than steady-state growth, indicating that the same mechanism may impact translating mitoribosomes after a carbon source shift (M. Couvillion & L.S. Churchman, unpublished observation). In mammalian cells, it is less clear whether tRNA import occurs, although there are anecdotal reports that describe the enrichment of the Gln_{UUG} tRNA in mitochondria (102, 129).

In addition to tRNAs, many RNAs of other types are localized within mitochondria (reviewed in depth in 154), but whether this localization serves a regulatory function remains unknown. Moreover, due to the intrinsic challenges of biochemical purification of mitochondria with sufficiently low background, it is difficult to definitively determine whether RNAs are specifically imported into mitochondria. One example of a nuclear-encoded RNA that influences mitochondrial gene regulation is the myogenesis-specific miRNA, miR-1. During muscle cell differentiation in mouse, miR-1 localization to the mitochondrial matrix increases the rate of mitochondrial translation (160). The effect is dependent on miRNA base pairing to mitochondrial-encoded transcripts and the mitochondrial localization of the Argonaute family protein AGO2. In contrast to cytoplasmic RNA interference, which normally represses translation, mitochondrial-localized miR-1 boosts translation. The role of mitochondrial import of nuclear-encoded RNAs in coregulation of the nuclear and mitochondrial genomes has not been characterized in detail but merits deeper investigation in future studies.

5. POSTTRANSLATIONAL REGULATION OF PROTEIN COMPLEX SUBUNIT ABUNDANCE

Although the multiple layers of transcriptional and translational regulation work together to ensure coordinated protein production (**Figure 4**), it remains unclear exactly how precise this coordination needs to be. While unicellular organisms synthesize complex subunits in nearly perfect stoichiometric proportions, as judged by ribosome profiling experiments (89), metazoan protein synthesis is less proportional, and substantial posttranslational fine-tuning of the proteome takes place (72). Nonstoichiometric production of complex subunits gives rise to excess unassembled orphan subunits, which are degraded primarily by the ubiquitin–proteasome system (55). For example, cytosolic ribosomal proteins are synthesized in the cytosol and transported to the nucleolus for assembly. In the nucleus, many unassembled subunits are degraded before they are incorporated into the holoenzyme, while the incorporated subunits are stabilized (86). This type of Interaction-based stabilization has been observed for many complexes, for example, the nuclear pore complex (150), T cell receptor (104), and conserved oligomeric Golgi complex (139). Mitochondrial proteins also exhibit interaction-dependent stabilities, and degradation of orphaned subunits is common.

5.1. Superstoichiometric Production and Degradation of Mitochondrial Proteins

Mitochondrial subunit overproduction happens both at steady state and during adaptation. In *S. cerevisiae* growing on glucose, we observed by ribosome profiling that mitochondrial proteins were being rapidly translated yet did not accumulate (31). However, this mismatch between synthesis rate and protein level was abolished by a shift to the nonfermentable carbon source glycerol. Because the ubiquitin–proteasome system, which is responsible for removal of unassembled complex subunits in the cytosol and nucleus, has limited access to mitochondrial contents, a set of dedicated mitochondrial proteases handle elimination of unassembled complex subunits (reviewed in 7).

For example, in *S. cerevisiae*, the membrane-bound *i*-AAA protease, which faces the intermembrane space, degrades unassembled mitochondrial-encoded subunits of the OXPHOS complex cytochrome *c* oxidase (COX) in deletion strains lacking the nuclear-encoded electron carrier, cytochrome *c*, or the COX complex subunit Cox4 (111, 115). Similarly, in humans, the inner membrane–bound *m*-AAA protease, which faces the mitochondrial matrix, is responsible for the degradation of mitochondrial-encoded proteins when the mitochondrial-encoded COX1 is mutated (65). In addition, shRNA-mediated depletion of *i*-AAA in HEK293 cells leads to accumulation of unassembled NDUFB6, ND1, and COX4, indicating that these are superstoichiometrically produced, with excess subunits being degraded, under normal conditions (145). Furthermore, proteome-wide pulse–chase experiments in human cell lines confirmed that multiple OXPHOS complex subunits are particularly unstable when newly synthesized (101), with many becoming stabilized in cell lines with nonfunctional *i*-AAA or *m*-AAA proteases (E. McShane & M. Selbach, unpublished data).

5.2. Complex Assembly and Protein Stabilization

Interaction-dependent stabilization of a protein can be demonstrated by its destabilization after depletion of an interaction partner. For example, many Cox subunits become destabilized after their nuclear interaction partners are eliminated. A recent study analyzed the effects of systematically deleting the 31 accessory subunits of Complex I in human cells on the levels of the remaining subunits (146). In the majority of cases, removal of a single subunit destabilized the interacting proteins within the same structural module. Similarly, after siRNA-mediated

elimination of specific assembly factors, ND1 cannot be incorporated into Complex I and is degraded by the *m*-AAA protease (161).

Another dual-origin mitochondrial complex is the mitoribosome, which consists of nuclear-encoded proteins and mitochondrial-encoded RNAs (in yeast, one protein subunit of the mitoribosome is also mitochondrial-encoded). Like the OXPHOS complexes, the mitoribosome is also affected by interaction-dependent stabilization. For example, one of the underlying genetic causes of Leigh syndrome is a mutation in the *mrps34* gene, which encodes a component of the small mitoribosome subunit (85). In both mice and humans, mutation in *mrps34* leads to a reduction in MRPS34 protein levels and destabilization of the other proteins of the small ribosomal subunit and the 12S ribosomal RNA (85, 123). Similarly, depletion of small mitoribosome subunit components MRPS16 and MRPS22 destabilizes MRPS11 but not the proteins of the large mitoribosome subunit (40). Interestingly, unstable orphaned mitoribosome subunits are produced in human cell lines without known mitoribosome-associated mutations (15, 101).

A final example of interaction-dependent protein stabilization is provided by the effects of deletion of the mitochondrial elongation factor mtEF4 (50), which increases the rate of mitochondrial translation. In somatic cells of mice, mtEF4 deletion activates mTOR, prompting cytosolic protein synthesis of OXPHOS components. The more abundant cytosolic subunits stabilize the overproduced mitochondrial subunits, thereby increasing the steady-state levels of the complexes. By contrast, in testes, mTOR is not activated upon mtEF4 deletion; the mitochondrial subunits remain superstoichiometric, and the excess proteins are degraded.

5.3. Possible Rationale for the Superstoichiometric Production of Mitochondrial Subunits

Several explanations have been proposed for the constitutive overproduction and degradation of complex subunits (101). Some of the hypotheses put forward are worth discussing in a mitochondrial context. First, protein interactions are the result of the interacting proteins' affinity and concentration. Overproduction of subunits with lower affinities could thereby facilitate complex assembly by assuring that sufficient interactions take place (96, 99). In other words, equimolar production of multiprotein complex subunits will not automatically allow sufficient complex formation if subunits harbor different interaction affinities. Second, during OXPHOS biogenesis, overproduction of interaction partners could be protective by preventing certain toxic subunits or intermediates from accumulating. For example, in the absence of Cox2, Cox11, or Sco1, mitochondria assemble a partial Complex IV that is prooxidant and toxic to the cell (78). In wild-type cells, production of excess subunits downstream of the partially assembled Complex IV could decrease oxidative stress by promoting full assembly of the complex. Similarly, overproduction of downstream subunits could prevent partially assembled ATP synthase from accruing, minimizing proton leakage from the intermembrane space to the matrix (121, 124). These protective mechanisms might be especially important during adaptation, when the synthesis of potentially harmful proteins must be quickly upregulated. Finally, upregulation of OXPHOS biogenesis might benefit from a rate-limiting subunit because only one genome (the one encoding the rate-limiting subunit) would need to rapidly respond to environmental changes, thus significantly decreasing mechanistic complexity.

It is interesting to consider whether posttranslational fine-tuning by intramitochondrial protein degradation could significantly facilitate the complicated task of coordinating protein production from two separate genomes. To fully assess the impact of protein degradation on the mitochondrial proteome, it will be necessary to perform systematic studies of the degradation rates of new and preexisting proteins during mitochondrial biogenesis.

6. OUTLOOK

A great deal remains to be discovered about the regulation of mitochondrial gene expression and its integration with nuclear gene regulation. How the nuclear and mitochondrial genomes coordinately respond throughout development, aging, stress, and disease processes represents an exciting and expansive area of analysis. Although our understanding remains incomplete, we have outlined here the aspects of these fundamental questions that have been explored to date, even when those reports have been anecdotal, to serve as a starting point for future research.

Most models of mitonuclear coregulation focus on nuclear control over mitochondrial gene regulation. Clearly, the mitochondrial genome requires nuclear genes for its expression, regulation, and replication. However, in many contexts, it remains unknown whether mitochondrial genes are activated or repressed independently of nuclear transcription. In one example in yeast, mitochondrial transcription is regulated in part through ATP sensing, which is enabled by the adenines at the start of most mitochondrial-encoded transcripts (3). Other instances in which the mitochondrial genome responds independently of its nuclear counterpart remain to be discovered.

Eukaryotic life arose at the cost of coexisting genomes, which were reduced via the minimization of mitochondrial genomes (see the sidebar titled The Origins and Evolution of the Mitochondrial Genome). Yet we retain two disparate genomes whose coexpression is responsible for metabolism and mitochondrial function. Accordingly, the coordination of mitochondrial and nuclear gene expression contributes broadly across cell biological, developmental, disease, and aging processes. Compared with nuclear gene regulation, our understanding of mitochondrial gene expression has lagged behind. In fact, the mitochondrial genome has been referred to as the neglected genome (117) due to the tendency of researchers to overlook its importance when analyzing large genomic data sets. We expect this trend to change. Through the application of sophisticated, modern genomic approaches to the compact mitochondrial genome, it is only a matter of time before we understand mitochondrial gene regulation as well as (or better than) nuclear gene regulation. Determining the mechanisms that coordinate these genomes will open many new areas of investigation, further our understanding of mitochondrial regulation, and clarify how a disruption to these pathways leads to disease and the effects of aging.

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