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Critical Review

Functional Organization of Mammalian Mitochondrial DNA in Nucleoids: History, Recent Developments, and Future Challenges

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Summary

Various proteins involved in replication, repair, and the structural organization of mitochondrial DNA (mtDNA) have been characterized in detail over the past 25 or so years. In addition, in recent years, many proteins were identified with a role in the dynamics of the mitochondrial network. Using advanced imaging and an increasing number of cytological techniques, we have begun to realize that an important aspect to mtDNA maintenance, in both health and disease, is its organization within the dynamic mitochondrial network in discrete protein–DNA complexes usually termed nucleoids. Here, I review recent developments in the study of nucleoid dynamics and proteins. I will discuss the implications of the organization of mtDNA in nucleoids in light of DNA replication, repair, gene expression, segregation, and inheritance.

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A BRIEF HISTORY

The existence of mitochondrial DNA (mtDNA) was first inferred in the 1940s in yeast as a heritable cytoplasmic element called the rho factor. It took many more years to visualize mtDNA (first in chick cells) (*1*), by electron microscopy, and to establish firmly that the presumed nucleic acid inside mitochondria of yeast did indeed correspond with the rho factor [for a comprehensive overview, see Williamson (*2*)]. From this point on things moved on rapidly and culminated in the 1980s in a series of important advances such as the identification of the unique mitochondrial genetic system with a typically variant genetic code, the identification of the various mtDNA encoded proteins (Fig. 1 shows the human mtDNA gene map), which are mostly involved in the essential process of oxidative phosphorylation (OXPHOS), the details of non-Mendelian inheritance, RNA splicing including self-splicing in yeast, and RNA editing in trypanosomes. Essential to these findings was the derivation of the complete sequences of human and mouse mtDNA in 1981 (*3–5*), followed by that of many other species, combined with the development of powerful genetic methods in yeast. Despite this, many important details remain unresolved, for example, the precise mechanism(s) of both yeast and mammalian mtDNA replication [see Holt (*6*), for a recent overview of the proposed mammalian mtDNA replication models and some of the outstanding issues].

Mitochondrial research has taken center stage via the discovery of the role of mitochondria in apoptosis and by the realization that a broad spectrum of human diseases involves mitochondrial biochemical and genetic pathways. A unique feature, first shown in the late 1980s, is that many mitochondrial disorders involve mutations in mtDNA itself. These disorders can be sporadic, or maternally inherited, or even autosomal, when caused indirectly by mutations in nuclear genes coding for proteins with a role in mtDNA maintenance. The genetics and phenotypic expression of these disorders can be complicated by the high mtDNA copy number within a cell, a genetic bottleneck during germline maturation, and often poorly understood tissue specificity and mtDNA segregation. The discovery of mtDNA involvement in human genetic disease highlighted our ignorance of many of the processes involved in mitochondrial genome maintenance.

Important issues for our understanding of human mitochondrial (disease) genetics include the organization of mtDNA in the mitochondrial network and the nature of the machinery of its replication, repair, distribution, mitotic segregation, and germline transmission. The first clues for our current knowledge of the organization of mtDNA came again from studies in yeast. It was shown in the mid-1970s that mtDNA in yeast could be specifically stained *in vivo* as submitochondrial structures later

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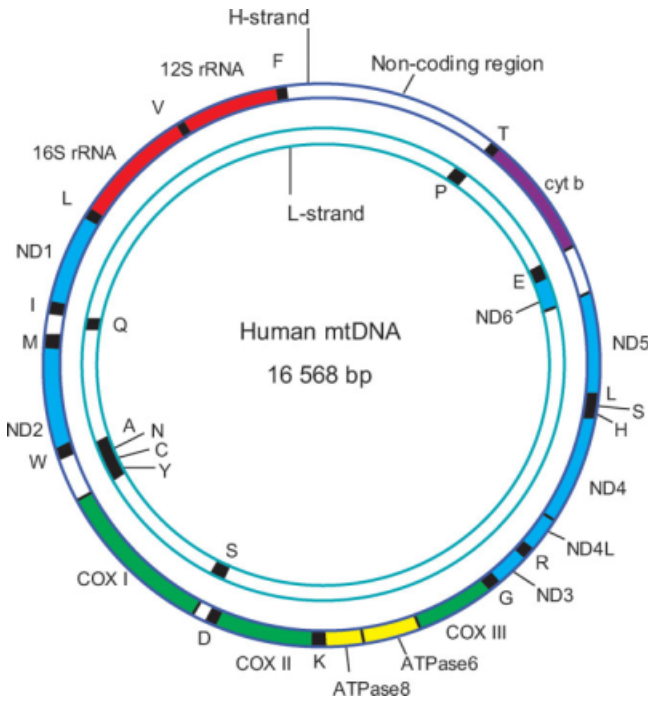

Figure 1. The human mitochondrial DNA gene map. Human mtDNA is a double-stranded, closed, circular molecule of 16.6 kb. Single letters indicate the positions of the corresponding tRNA genes. ND, NADH dehydrogenase genes; cyt b, cytochrome b gene; COX, cytochrome c oxidase genes; A6/8, ATP synthase genes 6 and 8; 12S/16S, ribosomal RNA genes.

called nucleoids. Additional genetic, biochemical, and cell biological approaches, as discussed in more detail later, have created the paradigm that (i) mtDNA molecules are usually clustered within the mitochondrial network in protein–DNA complexes called nucleoids; (ii) each cell type, depending on the species, growth conditions, developmental stage/differentiation, and so on, will typically contain tens (in *S. cerevisiae*) to hundreds (in mammals) of nucleoids, each containing several mtDNA copies; (iii) each nucleoid behaves as a relatively stable and inert genetic element. In yeast, there is good evidence that nucleoids are the units of genetic segregation.

Despite the long-established idea of mtDNA "compartmentalization" within yeast mitochondria, it has taken much longer for the concept to be widely accepted for mammalian cells perhaps mostly for the lack of a clear visual confirmation, whereas yeast nucleoids are readily visualized using DAPI (*7*). In fact, despite the fact that mtDNA–protein complexes were already identified and purified in the late 70s and early 80s and some researchers already at that time referred to mtDNA–protein complexes in vertebrates as nucleoids on the basis of the yeast paradigm [*e.g.*, (*8*)], even today mtDNA is often but erroneously referred to as "naked," especially in the context of susceptibility to oxidative DNA damage. However, already some of the earliest analyses of mammalian mtDNA *in situ*, using electron microscopy, had suggested mtDNA to be present in compact structures (*9*), that might represent the first visualization of mammalian mtDNA nucleoids.

Here, I will discuss recent advances in nucleoid organization, function, and dynamics with an emphasis on mammalian nucleoids and in the context of mtDNA maintenance and gene expression. It is not the intention of this review to give a full historical account of the research of nucleoids and mtDNA-binding proteins because this has already been expertly done relatively recently (*10, 11*). Although mostly concentrated on budding yeast and mammalian nucleoids, where appropriate, I will refer to other organisms and in the appropriate context will also discuss mitochondrial network dynamics but again refer to recent extensive reviews on this topic (*12–14*). The literature dealing with plastid nucleoids is mostly beyond the scope of this review. For a review on this subject, see (*15*).

S. CEREVISIAE OR *HOMO SAPIENS*

The discovery in the late 1980s of the association of mtDNA mutations with human disorders (*16, 17*) has given an enormous boost to mitochondrial genetic research. Nevertheless, in contrast to yeast, the study of human mitochondrial genetics is still seriously hampered by our inability to introduce directed mtDNA mutations. A second clear advantage in the use of *S. cerevisiae* is the ease with which to introduce nuclear-gene mutations and screen for complementation or synthetic lethality. So, why do we study human (mammalian) mitochondrial genetics if yeast provides such a versatile model system? The most obvious reason is that despite the many similarities, there are also many obvious differences. These include differences in mitochondrial genome size, organization and copy number, in mtDNA replication and repair, and in mitochondrial movement. More generally, there are vast differences in the mitochondrial proteomes, in cellular architecture, in reproduction, differentiation, and so on. In relation to the particular subject of mtDNA-nucleoids, it has become clear over the last 10 years that many of the proteins involved in mtDNA maintenance have opportunistically coevolved with mtDNA in the various branches of the eukaryotic evolutionary tree [see also (*18*)] to the extent that some proteins, such as the mtDNA helicase Twinkle, are found in metazoans but not in, for example, budding yeast (*19, 20*), whereas other proteins, such as aconitase, have adopted a clear second function in nucleoid maintenance in yeast (*21, 22*), but not as far as we know in mammals. Similarly, also in metazoan, or more specifically, mammalian evolution, various mitochondrial proteins with a conserved function in, for example, metabolism may have adopted an additional nucleoid function that will not be found in budding yeast. However, as I will also try to illustrate in this review, despite divergence in proteins there could be well considerable conservation of function deserving future studies. This illustrates, and I will try to further illustrate this throughout this manuscript, that despite a great divergence in nucleoid biology between yeast and mammals, the study of

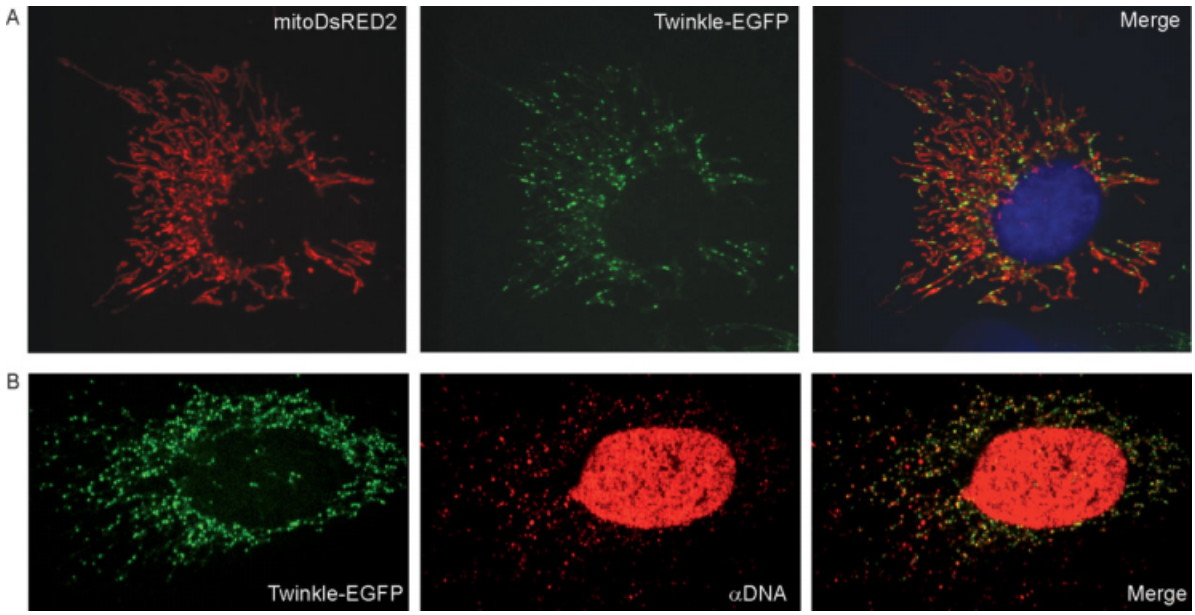

Figure 2. Twinkle is found in punctate foci within the mitochondrial network and colocalizes with mtDNA. (A) Human U2OS were cotransfected with a Twinkle-EGFP and mitochondrially targeted DsRED2 (Mito-DsRED2, Clontech) expression construct illustrating that Twinkle EGFP is found in punctate foci within the mitochondrial network (in the Merge panel). (B) Human U2OS were transfected with a Twinkle-EGFP expression construct while mtDNA (as well as nuclear DNA) was detected using an anti-DNA antibody and an anti-mouse Alexa-fluor 568 antibody, showing extensive overlap of signal with the Twinkle-EGFP protein (in the Merge panel) thus visualizing nucleoids. For both panels, cells were fixed and mounted using DAPI-containing mounting medium to visualize nuclei. Images were taken using confocal microscopy. For further experimental details on the general procedures, see (*25*).

yeast nucleoids has been extremely informative and useful for our understanding of mammalian nucleoids and without a doubt also is interesting in its own right.

Organization of Mammalian mtDNA in Nucleoids

As discussed earlier, a paradigm for the organization of mtDNA in nucleoids has existed in yeast for many years. A mammalian equivalent of nucleoids, however, has received relatively little attention despite the obvious relevance for our understanding of mtDNA maintenance, transcription, and genetics. A reappreciation of a mammalian equivalent of the yeast nucleoid paradigm has in part come from our need to better understand mtDNA genetics in the context of human disease (*23*). Stimulated perhaps by recent debates on the mechanisms of mtDNA replication and by the realization that a multitude of mtDNA maintenance proteins are defective in human disease, it has also been reappreciated that the existence of discrete mtDNA–protein complexes in mammals facilitates their isolation and can thus be used as a tool to identify new proteins of mtDNA maintenance. Nevertheless, even though as briefly alluded to above, mammalian mtDNA maintenance proteins have been identified by means of isolation of proteins tightly bound to highly purified mtDNA from the early 1980s onward, this has not told us much about the structural and spatial organization of mtDNA within the mitochondrial network *in vivo*, or of mtDNA dynamics.

The first evidence for a discrete localization of mtDNA within the mitochondrial network in mammals, similar to yeast mtDNA organization in nucleoids, has come from DAPI staining of HeLa cell mitochondria (*24*). Despite the advancement of imaging techniques, improvements in immunological and other staining protocols, and the routine use of fluorescent protein tags, clear *in vivo* evidence for specific association of protein(s) with mtDNA in nucleoid structures in mammalian cells was only shown 10 years later by the fortuitous strong and specific association with mtDNA of the newly identified mtDNA helicase Twinkle (*20*) (Fig. 2). The organization of mammalian mtDNA in discrete foci in cultured cells was however clearly demonstrated in a very much overlooked article in 1997 (*26*) where the use of Picogreen was also first introduced [see also (*27*)]. A second approach that confirmed a discrete spatial organization of mtDNA in mammalian cells used improved FISH techniques for the specific detection of mtDNA (*28*). These studies suggested that multiple mtDNA molecules can be present in each nucleoid, based either on tentative mtDNA copy number/nucleoid number comparison or on the high-resolution imaging of FISH samples. This was more carefully and quanti-

tatively determined for various cell lines (*29, 30*) showing between 2 and 10 copies of mtDNA/nucleoid. A further confirmation has come from time-lapse imaging, showing that larger sized nucleoid structures can divide to give smaller sized structures (*29, 31*). Again this was also observed previously using Picogreen staining (*26*). Finally, we and others could show by BrdU labeling experiments that most nucleoids specifically incorporated this label (*29–31*), suggesting at least a temporary existence of recently duplicated mtDNA molecules within single nucleoids.

Based on the simple notion that nucleic acid needs protection and usually is protected by DNA-binding proteins and based on the yeast mitochondrial and even the bacterial nucleoid paradigm, it seems obvious that not only Twinkle but also many other proteins are specifically associated with mtDNA in nucleoids. The most obvious candidates are the aforementioned DNA-binding proteins that were isolated in the 70s and 80s. Because of their abundance and resistance to medium to high salt washes, the two proteins that have been positively identified using this approach in various mammalian and nonmammalian species are the mitochondrial single-stranded DNA-binding protein (mtSSB, the mammalian Rim1p homolog) and TFAM (transcription factor A of mitochondria, also known as mtTFA; the mammalian homolog of Abf2p) (*8, 32–36*). Both yeast homologs have been positively identified as nucleoid components [see *e.g.*, (*21*)].

MtSSB was shown in *Drosophila* to be an essential protein of mtDNA maintenance (*37*). It has a proposed role in stabilization of replication intermediates and the D-loop structure in mammalian mtDNA. Using immunofluorescence (IF) for the endogenous protein, we showed its presence in nucleoids in human cell culture (*31*). Similarly, in more recent nucleoid isolation procedures, mtSSB was identified by mass spectrometry and found to consistently copurify with mtDNA in HeLa cells and *Xenopus* (*38–40*).

Although mtDNA packaging has been suggested as the main role for Abf2p, a packaging function for mammalian TFAM was originally suggested to occur mainly in the mitochondrial control region (*41*), while various estimations of human and mouse TFAM concentrations are too low for complete coverage of mtDNA (*42–44*). However, other studies suggest that each cell contains enough TFAM monomer to bind mtDNA at regular intervals of about 20 bp (*45–47*). In practice, TFAM functions as a homodimer (*48*) and both *in vitro* measurements as well as TFAM/mtDNA cosedimentation data suggest the possibility of TFAM-dimer binding at 35–40 bp intervals (*45, 46, 48*). Using IF, we and others have confirmed that mammalian TFAM at least partially colocalizes with mtDNA *in situ* (*30, 31, 46*). Of the mammalian nucleoid proteins, TFAM is one of the few factors that play a clear structural role in mtDNA organization in nucleoids, perhaps similar to the architectural function of nuclear histone proteins in nucleosome formation. As a protein that likely plays a role in the *in vivo* DNA topology (*48–50*), and whose binding to mtDNA is not entirely a sequence-specific (*41*), the importance of TFAM in mitochondrial transcription is not questioned. Certainly, all evidence points to the need for a tight regulation of TFAM levels in the regulation of mtDNA copy number, gene expression, and possibly nucleoid packaging (*50*).

Also, using biochemical isolation procedures similar to yeast nucleoid isolation procedures (*31, 46*), TFAM copurified with mtDNA, as already shown by many others (see earlier). By testing other proteins, also the mitochondrial DNA polymerase catalytic subunit, POLG1, copurified with mtDNA, although we could not unequivocally show its presence in nucleoids by IF (*31*). The POLG accessory subunit (POLG2) similarly copurified with mtDNA but only partially colocalized with nucleoids by IF.

In several articles, Bogenhagen et al. (*38–40*) identified either by immunoprecipitation and mass spectrometry or by formaldehyde crosslinking and sedimentation, proteins copurifying with *Xenopus laevis* oocyte mtDNA and HeLa cell mtDNA. In *Xenopus* this not only confirmed the presence of TFAM and mtSSB but also yielded the adenine nucleotide translocator and prohibitin 2, and more surprisingly, the E2 subunits of pyruvate dehydrogenase and branched-chain α-ketoacid dehydrogenase. Interestingly, also the E2 subunit of α-keto glutarate dehydrogenase was identified as a yeast nucleoid-associated protein (*21, 51*). Because the E2 subunits in these various enzymes perform similar enzymatic reactions and adopt a similar quaternary structure within larger enzyme complexes, the cross-species involvement of these subunits suggested a specific capability in mtDNA binding. They were suggested to provide a possible mtDNA inner membrane anchoring site (*38*). Nucleoids isolated from HeLa cells also showed the presence of the E2 subunit of branched-chain α-ketoacid dehydrogenase and many other proteins including most known replication factors that were not identified in *Xenopus*. It was argued that these differences could in part be explained by the fact that *Xenopus* nucleoids were purified from mature oocytes that show little or no mtDNA replication (*39*).

Using a somewhat different strategy from the ones used by Bogenhagen et al., a more restricted set of proteins was found associated with mtDNA (*52*). One of these proteins, ATAD3, was shown to be required for mtDNA maintenance by RNAi-induced depletion and to have specific D-loop binding properties. It was suggested that ATAD3 has a role in the organization of multiple copies of mtDNA and/or in segregation (*52, 53*) (see also later). More recently, however, it has been suggested that the putative D-loop binding domain of ATAD3 would be facing the mitochondrial intermembrane space (*40*) and that ATAD3 could therefore not perform its supposed nucleoid function as a D-loop binding protein, although other nucleoid functions were not rejected. However, these experiments also do not exclude that a subfraction of the protein would be in direct association with mtDNA via its D-loop binding domain. Indeed, IF of ATAD3 shows that certainly not all of the protein colocalizes with mtDNA at any given time. This illustrates an impor-

tant issue in future research directed at dissecting the roles of potential nucleoid proteins, which is that there could well be a core of nucleoid proteins that will always be found associated with most mtDNA, such as TFAM, but at the same time proteins with important functions in mtDNA maintenance will only temporarily associate with mtDNA and/or will associate with only a subset of nucleoids while performing a specific task. Such proteins could be mtDNA repair proteins (*54*) and proteins involved in, for example, nucleoid division such as perhaps ATAD3. A good case in point is also human Dna2. This protein in yeast has been implicated in various nuclear DNA maintenance processes but was recently shown to be also mitochondrial in humans (*55, 56*). We showed that the protein shows at best a partial colocalization with nucleoids but upon expression of various Twinkle mutants the nucleoid colocalization was close to 100%, suggesting the protein to be transiently nucleoid associated and unable to dissociate under some conditions of nucleoid dysfunction (*56*). Additional complications that can be anticipated in future mammalian nucleoid research are that a protein in question is not exclusively mitochondrial [see (*6*) for a review highlighting various recent examples] or that a protein has multiple functions within mitochondrial metabolism. Good examples for this have presented themselves in the past in yeast [see *e.g.*, (*10, 18, 22, 57, 58*) and references therein] and was also suggested recently for the human POLG accessory subunit (*25*). Because of the limited power of genetics in mammalian cell culture and time-consuming mouse genetics, it is anticipated that dissecting potential nucleoid protein function from other functions will take a long time.

Copy Number Control

Cell growth and proliferation require the continuous growth of cellular compartments, including the mitochondrial network. In postmitotic tissues/cells, in contrast to nuclear DNA replication, mtDNA continues to be replicated and turned over [see *e.g.*, (*59*)].

Because of the essential role of mtDNA in the synthesis of the OXPHOS enzyme complexes, and thus cellular energy production, mitochondrial and cellular growth and proliferation need to be balanced by mtDNA replication. Although not as strictly maintained as nuclear DNA, mitochondrial genome copy number (per cell) is maintained at a relatively constant level in a proliferating cell culture for a given cell type. However, depending on the tissue-/cell-type, steady-state mtDNA copy number has been shown to vary, with highest levels being present in the most energy demanding tissues and in the ovum with an estimated copy number of up to 200,000 [see *e.g.*, (*60, 61*)].

Although we are still far from fully understanding mtDNA copy number regulation in general terms in animal physiology, there are several clues suggesting important contributing factors and mechanisms that directly relate to mtDNA maintenance [see (*61–63*) for various reviews relating to this issue]. These stem in part from identification of nuclear gene mutations in human mtDNA depletion disorders and from the study of various mouse models. It is beyond the scope of this review to extensively discuss this topic but in brief, the factors identified so far that impact directly on mtDNA copy number fall in a few different categories that include proteins with a catalytic role in mtDNA replication, TFAM, most likely in its role as an mtDNA packaging factor, and proteins involved in nucleotide metabolism.

Despite the obvious involvement of various proteins, including nucleoid proteins, in mtDNA copy number control very little is known about the control of mtDNA copy number per nucleoid. To my knowledge, as yet no data are available concerning nucleoid-mtDNA copy number in physiologically relevant tissues, despite its obvious importance for our understanding of mitochondrial disease genetics. Nevertheless, nucleoid size and copy number per nucleoid have been shown to differ in different cultured cell lines/cell types [(*30, 53*) and unpublished observations]. Comparing A549 lung carcinoma cells with 143B osteosarcoma cells has suggested an inverse relationship between ATAD3 A/B expression levels and nucleoid size, whereas conversely nucleoid numbers related directly to ATAD3 expression levels (*53*). This suggested, also based on other properties of the protein such as its ability to bind mtDNA D-loops (*52*), that ATAD3 could have a role in nucleoid division, and it would therefore be one of the principle factors determining nucleoid mtDNA copy number. In apparent contrast, human POLG2 overexpression increased nucleoid size, reduced nucleoid number, but did not affect mtDNA copy number (*25*). Knockdown of POLG2 reduced mtDNA copy number, while at the same time increasing the number of nucleoids, thus reducing mtDNA copy number per nucleoid. By another measure (the presence of detergent-resistant mtDNA multimers analyzed by two-dimensional agarose gel electrophoresis), knockdown of both ATAD3 and POLG2 reduces mtDNA multimers, suggesting that ATAD3 also has a role in nucleoid formation. To bring together the various findings, it is suggested that both ATAD3 and POLG2 are involved in a cycle of mtDNA synthesis (POLG2), stabilization of multiple mtDNA copies in nucleoids (POLG2 and ATAD3), and ultimately nucleoid division (ATAD3 and other factors) (*25*). This model needs to be further substantiated, the involvement of other candidate nucleoid proteins tested, and the possible involvement of the mtDNA D-loop further investigated.

What Does Nucleoid Organization Mean for mtDNA Maintenance, mtDNA Distribution, Transcription, and Translation?

Organization of multiple copies of mtDNA in a protein-rich structure not only protects the DNA from various insults but is also likely to put constraints on any transactions involving the DNA, such as replication, repair, and transcription. On the other

hand, it could also provide the most appropriate microenvironment for the proteins involved in these processes and might facilitate, for example, a gene-conversion mode of mtDNA repair, which requires more than one copy of DNA.

Using BrdU labeling, both yeast and mammalian nucleoids have been shown to be sites of mtDNA replication (*29–31, 64*). Interestingly, not all nucleoids participate at any particular moment in mtDNA replication. In yeast this was further emphasized by the specific localization of a subset of proteins, Mgm101, Mmm1, and Mip1 (the yeast POLG1 homolog) near sites of BrdU incorporation, whereas the mtDNA packaging factor Abf2 was associated with all mtDNA foci on the basis of DAPI costaining (*64*). It was furthermore shown that Mgm101, Mip1, and Mmm1 are visible as discrete partially colocalized foci even in rho$^{\circ}$ cells, that these foci self-replicate, and are faithfully transmitted to buds. It was suggested that this assembly, termed TMS (for two-membrane spanning structure), functions as part of an mtDNA replication factory. It is of particular interest to note here that Mmm1p was previously suggested to be a mitochondrial outer membrane (MOM) protein. However, a new and very fascinating twist to this story is that Mmm1p appears to be a membrane protein of the endoplasmic reticulum (ER) and is part of a complex that forms ER-mitochondrial junctions (*65*). Other identified partners in this complex are Mdm10 and Mdm34 (previously known as Mmm2), which function as a MOM anchor, and Mdm12 as a subunit involved in connecting the MOM anchor with the ER-anchor, that is, Mmm1. The complex as a whole was coined ERMES for "ER-mitochondria encounter structure." Further work on the ERMES complex has shown that deletion of any of its constituents resulted in a reduction in cardiolipin levels (*65, 66*), which reflected a more general problem in phospholipid biosynthesis by limiting phospholipid exchange between the ER and mitochondria. Kornmann et al. (*65*) speculated that the vicinity of replicating nucleoids to these junctions could suggest a coupling of mtDNA genome maintenance with mitochondrial membrane upkeep. Although, as argued earlier, there is a considerable divergence between yeast and mammals in terms of nucleoid proteins and mtDNA maintenance, ER-mitochondrial junctions have been demonstrated in mammals and interestingly seem to involve proteins involved in mitochondrial network dynamics, in particular, mitofusin 2 (Mfn2), but possibly also mitofusin 1 (Mfn1) (*67*). Mfn2 was furthermore shown to be both a MOM protein and an ER-localized membrane protein. In mitochondrial membrane fusion reactions, Mfn1/2 are essential for membrane tethering by forming homotypic and heterotypic complexes between adjacent mitochondria [reviewed in (*68*)]. This suggests that ER-localized Mfn2 can mediate ER-mitochondrial tethering by forming a complex with mitochondrial Mfn1 or 2 (*67*).

Of the above yeast proteins involved in ER-mitochondrial junctions, Mdm10p and Mmm1p have also been implied in binding to cytoplasmic actin in budding yeast (*69*). The actin cytoskeleton has been shown to be important in budding yeast mitochondrial movement, whereas in mammals or, for example, *N. crassa* mitochondrial movement is largely dependent on the microtubular network (*14, 70*). Some roles for the actin cytoskeleton should also be considered but are as yet largely unexplored (*14*). Mdm10 and Mdm12 have also been shown to be required for the maintenance of normal mitochondrial morphology and distribution (*71, 72*). Mmm1 and Mdm34 were previously suggested to be required for maintenance of both normal mitochondrial and nucleoid structure as well as mtDNA stability (*73–75*).

However, the study of the ERMES proteins from a different perspective has shed yet another light on the role of the Mmm1, Mdm10, and Mdm12 by showing that these are also involved in the import and assembly pathway, in particular, the assembly of beta-barrel proteins in the outer mitochondrial membrane (*76*). Results in this article seem to suggest that at least the mitochondrial morphology phenotypes observed with Mmm1, Mdm10, and Mdm12 deletions could be secondary to defects in outer mitochondrial membrane assembly. Alternatively, the more recent findings that these proteins are involved in ER-mitochondrial junctions and phospholipids biosynthesis (see earlier) might well unify the beta-barrel protein assembly defect and the mitochondrial morphology phenotypes. The data suggesting that the ERMES protein complex also functions as an actin cytoskeleton attachment site and is at least found in the close vicinity of nucleoids still stands to my knowledge. This implies that the ERMES proteins still could have a primary role in mtDNA segregation. Two additional yeast proteins, the yeast Pumilio family members Puf1 and 3 provide part of the link between mitochondria via some of the ERMES proteins and the actin cytoskeleton (*13, 77, 78*). Puf3 preferentially binds cytoplasmic mRNAs for proteins targeted to mitochondria and presumably targets these mRNAs for cotranslational import and subsequent degradation.

The above findings provide an interesting parallel with the suggestion by Iborra et al. (*29*) that human nucleoid-derived transcripts preferentially locate in the vicinity of cytosolic translation and mitochondrial protein import sites, and it would be very interesting in the future to see if this can be further substantiated. Certainly, the recent identification by Bogenhagen et al. (*40*) of various mitochondrial and cytoplasmic ribosomal proteins as well as other translation factors in purified crosslinked nucleoids lends support to this idea, although at this point it is too early to exclude contamination perhaps caused by a crosslinking artefact. Affinity purification using a mitochondrial translation factor (mitochondrial ribosomal recycling factor, mtRRF) (*79*), apart from a long list of proteins involved in RNA-metabolism, mitochondrial translation, and protein quality control, also yielded a multitude of mitochondrial nucleoid proteins as identified in other studies, further confirming that mitochondrial transcription, translation, and OXPHOS assembly might be closely linked to nucleoids.

It is of interest that also mitofilin has been identified in human nucleoid isolations and was shown by IF to locate in the

vicinity of nucleoids (*39*). This inner membrane protein was proposed to be involved in cristae morphology (*80*) and part of a large supermolecular complex. Subsequent work has shown that various mitochondrial proteins consistently copurify using mitofilin-specific monoclonal antibodies, including several import/assembly factors of the outer mitochondrial membranes such as Sam50 (*81*). Sam50 is an outer membrane beta-barrel protein of the sorting and assembly machinery (SAM) complex, which is critically involved in beta-barrel protein assembly, thus drawing again an interesting parallel between yeast and mammalian proteins with a potential nucleoid function. Finally, overexpression of one of the components of the Tim complex, Tim17, was recently shown to rescue mtDNA-loss phenotypes both in yeast and a human disease cell culture model (*82*), although the mechanism is not likely to be directly nucleoid related.

To conclude we can perhaps propose the existence of a membrane scaffold structure, as suggested earlier (*83*), that is at least functionally conserved in all respiring eukaryotes and that would coordinate mtDNA maintenance with mitochondrial translation, cytoplasmic translation, protein import and assembly (Fig. 3), similar to the suggestion made by Iborra et al.

A high copy number of mtDNA in mammalian cells might perhaps have limited the need for a rigid control of nucleoid distribution during mitosis such as found in yeast, in which it is suggested that the actin-cytoskeletal attachment to the now recoined ERMES complex and which in turn is linked to nucleoids, ensures the transmission of nucleoid- and thus mtDNA-containing mitochondria to the bud (*69*). The most straightforward, yet speculative, scenario in mammalian cells assumes that only the nucleoid distribution over mitochondria is actively regulated, whereas nucleoid distribution to daughter cells is achieved indirectly by regulation of mitochondrial distribution. This means that as long as nucleoids are distributed more or less uniformly along the mitochondrial network, and each cell in a proliferating cell population receives its share of mitochondrial mass, mtDNA transmission to each daughter cell is ensured. So, how could nucleoids be distributed along the mitochondrial network? As active sites of mtDNA replication, it is first of all essential that as mitochondrial mass increases, nucleoids divide or release mtDNA molecules to generate daughter nucleoids and as discussed this has indeed been observed using time-lapse imaging (*26, 29, 31*). A second clue comes from the long-standing observation that mtDNA appears to be membrane associated (*8, 86*). In agreement with this, comparison of nucleoid with mitochondrial movement in COS7 cells showed similar direction and velocity (*31*). In contrast to our own previous suggestions that nucleoid movement might be highly restricted compared to mitochondrial movement (*23*), this would suggest that nucleoid dynamics are similar to mitochondrial dynamics and could be brought about by membrane growth and plasticity alone. In agreement with this, nucleoid and outer membrane-targeted GFP showed similar dynamics in cell fusion experiments (*30*). The above model would clearly be different than the model for yeast nucleoid dynamics in which nucleoid movement is restricted when compared with matrix and membrane protein movement (*87–90*).

Mitochondrial and Nucleoid Dynamics

Although still preliminary, the observations in mammalian COS7 cells suggested a distribution of nucleoids relative to sites of mitochondrial fission so that each daughter mitochondrion would have at least one nucleoid (*31*). This was further confirmed by Legros et al. (*30*) showing that most mitochondria have at least one nucleoid and furthermore by showing that cccp treatment resulted in a fragmented mitochondrial network with the majority of mitochondria still showing the presence of at least one nucleoid. However, more recent studies examining the effects of the absence of Mfn1/ 2 or Opa1 proteins in mouse cell lines show a different picture where mitochondrial network fragmentation results in many small mitochondria devoid of nucleoids (*91*). Interestingly, it appears from this publication that a double Mfn1+2 knockout resulted in increased nucleoid size, although no statistics were shown and only a single image was printed that might not have been representative for the whole cell culture. Also, no data were presented on mtDNA copy number. These finding are further corroborated by the recent finding that some Opa1 mutations in human disease result in mtDNA instability (*92–95*). Nevertheless, the results do suggest that there is an as yet largely unexplored interplay between mitochondrial network dynamics and nucleoid dynamics/segregation. The involvement of Mfn1 and 2 in ER-mitochondrial junction formation (see earlier) also warrants further investigation of these junctions and their potential relation to mtDNA nucleoid maintenance (see also Fig. 3). Interestingly, in one recent study, it was observed that inhibition of mitochondrial fission in a rhabdomyosarcoma cell line shifted the ratio of the A3243G mutation over wild-type mtDNA in favor of A3243G (*96*), suggesting a dynamic interplay between mtDNA segregation and mitochondrial network dynamics that warrants further studies.

Colocalization studies with Drp1 showed exclusion of nucleoids from putative fission sites (*29, 31*). A sensing mechanism that would position mitochondrial membrane growth and/or fission to occur between nucleoids remains speculative but is not unprecedented as evidenced by bacterial Z-ring formation. This process can involve active nucleoid occlusion and negative regulation of Z-ring assembly by the Min system [for reviews see (*97, 98*)]. Interestingly, *Arabidopsis* homologs of MinD and MinE have been implicated in placement of chloroplast fission sites (*99–101*). However, in contrast to mitochondrial fission, chloroplasts also use an FtsZ homolog, suggesting a system that overall is more closely related to the bacterial system than the mitochondrial system might be.

MtDNA Inheritance and Nucleoids: Heteroplasmy, Segregation, and Complementation

The association of a large spectrum of mtDNA mutations with human disease came as a surprise to many. The presence

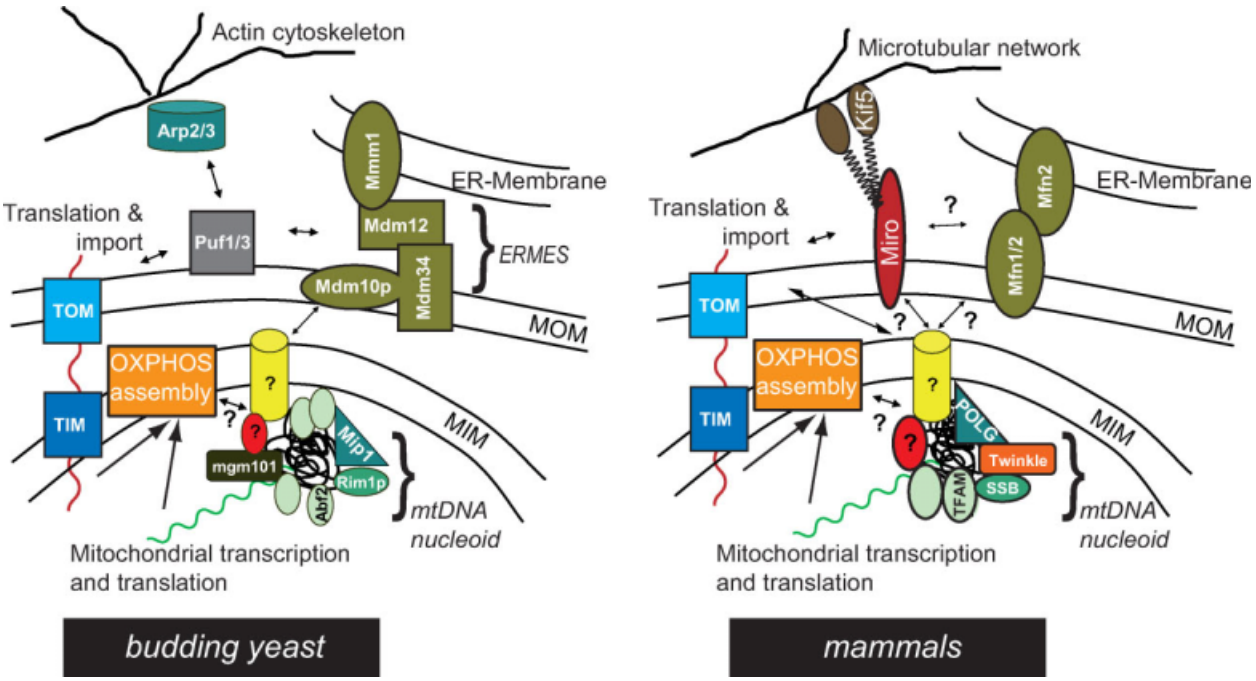

Figure 3. Is there conservation of function between budding yeast and mammalian nucleoid organization and dynamics? This figure illustrates recent advances in our understanding of nucleoid organization in budding yeast and mammals in the larger context of mitochondrial biology, in particular mitochondrial dynamics and biogenesis of OXPHOS complexes. In addition, the figure tries to illustrate that despite divergence of proteins there might be considerable conservation of function. Although this is still very speculative, it also points to directions nucleoid research may go in the near future and illustrates various outstanding questions. I will here briefly summarize both for yeast and mammals what is known to highlight the scientific grounds for this figure and refer to the main text for more details and most of the references. In yeast, a variety of nucleoid proteins have been well established some of which, mostly mtDNA replication and repair factors, are named here [for a more detailed overview see (*10*), on which part of this illustration is also based]. As in mammals, it is currently unknown which protein(s) and to what extent RNA and/or DNA are responsible for nucleoid membrane tethering. What is know in yeast is that replicating nucleoids are found in the close vicinity of the Mdm10/Mdm34/Mdm12/Mmm1 (ERMES) complex, that this complex is at least partly responsible for ER-mitochondrial tethering and that the actin cytoskeleton associates with this complex, involving the Arp2/3 and Puf 1/3 proteins. An important outstanding question for yeast is what makes the connection between the nucleoid proper and the ERMES complex? In mammals, ER-mitochondrial tethering can be mediated by MFN2 at the ER membrane and MFN1 and/or MFN2 at the mitochondrial membrane. There are no data to suggest that nucleoids are often found in the vicinity of these tethers, but MFN1or 2 and OPA1 knockout cells do show an apparent nucleoid defect. Nucleoids have been shown to often locate in the vicinity of KIF5B (*29*), while KIF5 proteins interact with Miro at the MOM and at least based on a recent publication this interaction is direct and does not need the mammalian Milton homolog Grif-1 (*84*). It is beyond the scope of this review to extensively review microtubule-mediated mitochondrial transport [for a recent review on this subject focussing on the Miro GTPases see (*85*)]. Finally, both in yeast and especially in mammals, biochemical isolation of nucleoids has suggested their possible vicinity to mitochondrial chaperones and proteins of mitochondrial transcription/translation and even cytoplamic translation, confirming IF data of Iborra et al. (*29*) and suggesting a coordination of mitochondrial transcription and translation, with cytosolic translation, import via the translocases of the outer and inner membrane (TOM and TIM, respectively) complexes and the coordinated assembly of the OXPHOS system, as also illustrated in Iborra et al. For clarity, many proteins, in particular nucleoid proteins that have been identified in recent years, are illustrated here only with a single red oval-shaped protein. The question mark illustrates the still tentative nucleoid association of many of these proteins and the fact that they have not, except for a few, been well characterized.

of hundreds to thousands of copies of mtDNA in the cytoplasm of a typical human cell not only accounts for many of the peculiarities of mitochondrial disorders but also explain why many of the more severe mtDNA mutations are tolerated, for example, during embryogenesis, while giving a disease phenotype at birth or, more usual, later in life.

Although beyond the scope of this review, it is good here to briefly describe the various aspects of mitochondrial genetics

that make mitochondrial disorders interesting from a genetic point of view but at the same time very complicated for genetic counseling and therapy. Many excellent reviews on this subject have been written over the years also in relation to mitochondrial network dynamics [see *e.g.*, (*102, 103*)]. MtDNA is inherited exclusively from the mother and a genetic bottleneck has been hypothesized to explain rapid selection of single mtDNA genotypes. Using mice with two easily distinguishable mtDNA genotypes, mtDNA genotypic variance in primordial germ cells was found to be significantly less than in primary or mature oocytes (*104*). It was concluded that this genetic bottleneck should therefore occur early in oocyte development. It was long thought that a severe reduction in mtDNA copy number during primordial germ cell differentiation was sufficient to explain this phenomenon (*105, 106*), but the work by Wai et al. (*106*) has shown that this is not the case. Instead, they observed that replication of mtDNA in a selected subset of nucleoids during folliculogenesis coincided with an increased genotypic mtDNA variance in primary and mature oocytes and could provide an explanation for the bottleneck. A very interesting question, raised from a nucleoid perspective, is what determines the selection of some nucleoids to initiate mtDNA copying while many others remain inert? And, does a similar mechanism perhaps play a role in the segregation of mtDNA mutations in postmitotic tissues of mitochondrial disease patients?

Because the paternal mtDNA contribution to the total mtDNA population of the egg is negligible as it is likely actively destroyed, and as a consequence of the mtDNA bottleneck, all cells of the developing fetus and later adult human body are usually of a single mitochondrial genotype (for the sake of simplicity, I will here largely disregard the occurrence and accumulation of somatic mtDNA mutations with age). This situation, be it at the organismal, cellular or mitochondrial level is referred to as homoplasmy. However, mtDNA mutations do occur, can coexist with wild-type mtDNA in the same cell or mitochondrion, and can be transmitted through the maternal germline despite the bottleneck: in fact, the bottleneck partly explains why mtDNA variants present in the mother at very low levels can become abundant in the offspring. The situation, where two or more mitochondrial genotypes coexist, is referred to as heteroplasmy. In case of two genotypes, one mutant and one wild-type, and because of the high mtDNA copy number in mammalian cells, heteroplasmy levels for one or the other genotype can vary along a continuous scale between 0.1 and 99.9%. Once two genotypes are present in an individual, heteroplasmy levels can change with increasing age by somatic segregation toward one or the other genotype. The tissue specificity of this segregation is still poorly understood, but elegant studies in mouse have shown that with age both random genetic drift and tissue-specific selectivity can occur within a single animal (*107, 108*). Genes for this selectivity are currently being mapped (*109*).

Known mtDNA disease mutations are to a large extent recessive as they only result in a phenotype when high percentages of mutant mtDNA are present, usually showing a rapidly increasing disease severity with increasing percentages. The recessive nature of most mutations shows that complementation of mutant mtDNA can be very effective under some circumstances even though mtDNA can be present throughout an extended mitochondrial network, for example, in a muscle syncytium. Complementation has thus been suggested to be the consequence of mitochondrial network dynamics allowing for rapid and extensive exchange of genetic material [also reviewed in (*102*) and more recently by (*110*)]. However, this concept has been intensely debated over, with on the one hand the proponents and on the other hand those who argue that complementation by "genetic" intermixing is a very rare event]for a discussion of those opposing views see (*111*) and reply]. Most experimental data for both views are based on similar approaches using cell or cytoplast fusion techniques. In the first study, $\rho+$ HeLa cytoplasts prestained for mtDNA with either DAPI or EtBr were fused with ρ° HeLa cells (*112*). Following a recovery of 4–6 h, the entire mitochondrial network of resulting cybrids, shown by R123 staining, was also uniformly stained with EtBr or DAPI. This was taken as evidence for rapid mitochondrial and mtDNA mixing. However, with our current day knowledge of mtDNA organization in human cells, the data of this article have to be interpreted with great caution because both EtBr and DAPI staining showed a uniform mitochondrial staining, contrasting the discrete foci now commonly observed for more specific nucleoid staining. Thus, by not taking this article into account, to date there is no direct visual evidence in favor of rapid mixing of mtDNA populations in mammalian cells. Nevertheless, based on recent cell fusion studies, there is clear evidence for rapid mixing of differentially labeled mitochondrial matrix proteins, mediated by fusion (*113, 114*). Although yeast studies have suggested that mtDNA mixing is slow compared with matrix or membrane protein mixing because of cytoskeletal "interference" with nucleoid movement, the lack of mammalian homologs for the proteins believed to be involved in active nucleoid distribution (see earlier) shows that we cannot at this point extrapolate the yeast findings even though it is tempting to speculate that nucleoid movement in mammals might be directed in part by the microtubular network (*29*) (see also Fig. 3).

The second approach again using cytoplast or whole cell fusion (creating cybrids and/or hybrids) directly examined the occurrence of complementation of various mtDNA mutations. Although the various studies showed that complementation does occur, the frequency of complementation ranged from being very low to almost 100%. The different outcomes of these experiments have been suggested to result from subtle differences in experimental procedures. For example, it was suggested that hybrids could have an increased rate of mitochondrial fusion because of imbalance in nuclear genes of mitochondrial dynamics (*111*). However, also tumor cell lines, commonly used to generate cybrids, have unstable nuclear genomes and are often aneuploid (*115*). Furthermore, the various cell lines in

the mentioned studies were all observed to have efficient mitochondrial fusion, either in cybrid or hybrid studies (*113, 114*). A further possible explanation for different results might be synergism of some of the mutations that were used in the different studies.

In the last approach, the first mouse model for a single mtDNA deletion/duplication has been studied for complementation at the tissue level. Surprisingly, and in contrast to human disorders, no tissue mosaicism for cytochrome c oxidase deficiency was observed with mtDNA deletion levels of up to 80% (*116*). A strong case was made for widespread exchange of genetic material, which would functionally complement the deleted mtDNA molecules. But, as pointed out by others (*111*), the same result can be obtained assuming a stable, multicopy heteroplasmic unit of inheritance be it a mitochondrion, nucleoid, or otherwise. We proposed such a principle, "the faithful nucleoid model," based on the yeast nucleoid model, suggesting that the organization of mtDNA in nucleoids might restrict frequent mixing of mtDNA molecules (*23*). This was recently put to the test by the creation of cell hybrids each carrying a distinct homoplasmic partial mtDNA deletion that could be distinguished using FISH. These experiments showed that, at least under the specific conditions of that test, stable mixing of nucleoids appears to be extremely rare or nonexistent even though functional complementation did occur (*110, 117*). This again shows, as earlier argued (*102*), that mitochondrial network dynamics likely is important in functional complementation even in the absence of direct mtDNA mixing.

CONCLUDING REMARKS AND FUTURE PROSPECTS

The Nucleoid Concept in mtDNA Genetics

Although the nucleoid concept in mammalian mitochondrial genetics will introduce new parameters that might complicate the modeling of mitochondrial gene segregation and complementation, some of the above discussed controversies might be better understood once we have answered a few very basic questions. These are as follows: (i) are nucleoids the units of mammalian somatic and/or germline mtDNA inheritance/segregation? (ii) to what extent and at what speed do mtDNA-nucleoids mix, if at all (*110, 117*), when mitochondria fuse. Although this has been now tested in a cell hybrid model, ideally this should be readdressed in a natural setting in an animal model that would also allow us to ask whether, for example, tissue-specific differences exist? (iii) are there tissue-specific and/or species-specific differences in the number of mtDNA molecules per nucleoid, and if so, what regulates nucleoid mtDNA copy number? Assuming nucleoids are the units of mtDNA inheritance and mtDNA copy number per nucleoid is low, segregation dynamics will likely be similar to the expected segregation assuming each mtDNA molecule behaves as independent [*e.g.*, (*118*)]. However, with high nucleoid mtDNA copy number, segregation dynamics can be very different from the dynamics that one would predict assuming each mtDNA molecule as a unit of inheritance. Assuming that there will be some degree of nucleoid intermixing, it will be an important determinant. Intuitively, a high degree of intermixing would push the model toward a segregation model that assumes that each mtDNA molecule behaves independently. Computer simulations will be needed to work out the predictions based on nucleoid models. As discussed earlier, a variety of complicating factors such as phenotypic selection and preferential replication of one mtDNA genotype over the other need to be addressed (*23, 119*).

Nucleoid Proteins and Molecular Mechanisms

As some of the recent biochemical isolations of human nucleoid proteins show relatively large sets of associated proteins (*39, 40*), an immediate question that arises is what defines a nucleoid protein? For example, the question comes to mind whether the various components involved in, for example, translation should be regarded as nucleoid proteins. Bogenhagen et al (*40*) have adopted a criterion that proteins that are present in native (non-crosslinked) nucleoids but not in crosslinked nucleoid samples should be regarded as peripheral and not as core nucleoid components (by using a formaldehyde crosslinking approach they could apply much more stringent detergent and salt conditions, such that only proteins that copurify with mtDNA under these conditions were considered as core nucleoid components). Nevertheless, they also admit that some abundant mitochondrial proteins or cytosolic ribosomal proteins present in crosslinked nucleoid samples inadvertently could have become crosslinked to nucleoid proteins and could thus be contaminants. In addition, currently applied procedures might not be particularly useful to identify transient nucleoid components, that is, components that are recruited to nucleoids in a temporal and/or spatial manner or under specific conditions. Certainly, such proteins should be considered *bona fide* nucleoid proteins. Principally, one could define a nucleoid protein as any protein that either temporarily or permanently associates directly with mtDNA or with other nucleoid proteins and plays a role in mtDNA maintenance. This should preferably be demonstrated both by biochemical fractionation and by immunofluorescent colocalization methods and further confirmed by functional studies.

Nucleoid purification strategies have so far always used moderate to high levels of detergents with isolated mitochondria, which might have resulted in the loss of specific membrane components, while one of the most urgent questions in mammalian nucleoid research is what determines nucleoid membrane association, and does a functionally similar two-membrane spanning structure as inferred in yeast exist in mammals?

Apart from the obvious need to determine the molecular function(s) of potential nucleoid proteins that have been identified in recent years, of particular interest will be their potential role in events at a more macroscopic level. For example, how do nucleoids divide, how are they distributed along the mito-

chondrial network, and what role does mitochondrial fusion and fission play in nucleoid dynamics? Also from the perspective of mtDNA maintenance and gene expression, many questions need answering. To name just a few: how does packaging of mtDNA in nucleoids affect the control of replication and transcription and are nucleoids organized as part of larger complexes for the import and assembly of the oxidative phosphorylation complexes? Certainly, mammalian mtDNA research is going through a small renaissance, and although the study of nucleoids is not the be-all and end-all of this research, it does provide a very useful conceptual framework to study many of the questions of mtDNA maintenance in health and disease we are currently struggling with.

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