High mitochondrial DNA copy number has detrimental effects in mice

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Mitochondrial DNA (mtDNA) is an essential multicopy genome, compacted into protein–DNA clusters called nucleoids. Maintaining an adequate mtDNA copy number is crucial for cellular viability. Loss of mtDNA results in severe human syndromes, whereas increased mtDNA copy number has been suggested to improve survival from myocardial infarction in mice and to be a promising therapeutic strategy for mitochondrial disease. The mechanisms that regulate mtDNA amount and organization are, however, not fully understood. Of the proteins required for mtDNA existence, only the mitochondrial helicase Twinkle and mitochondrial transcription factor A (TFAM) have been shown to increase mtDNA copy number in vivo, when expressed in physiological levels. Here we studied how Twinkle and TFAM affect mtDNA synthesis and nucleoid structure in mice. Using in vivo BrdU labeling, we show that Twinkle specifically regulates de novo mtDNA synthesis. Remarkably, high mtDNA copy number in mice is accompanied by nucleoid enlargement, which in turn correlates with defective transcription, age-related accumulation of mtDNA deletions and respiratory chain (RC) deficiency. Simultaneous overexpression of Twinkle and TFAM in bitransgenic mice has an additive effect on mtDNA copy number, increasing it up to 6-fold in skeletal muscle. Bitransgenic mice also exhibit further enlargement of nucleoids and aggravation of the RC defect. In conclusion, we show that Twinkle acts as a regulator of mtDNA replication initiation, and provide evidence that high mtDNA copy number and alteration of nucleoid architecture may be detrimental to mitochondrial function.

INTRODUCTION

Mitochondrial DNA (mtDNA) is a circular 16.5 kb molecule that encodes 13 essential proteins of the oxidative phosphorylation system, the main source of ATP for the eukaryotic cell. Primary and secondary defects of mtDNA are a common cause of human disease. Secondary mtDNA mutations are typically caused by defects in nuclear genes that encode mtDNA processing proteins or those affecting nucleotide pools, leading to progressive accumulation of mtDNA deletions or mtDNA depletion, and subsequently to mitochondrial respiratory chain (RC) deficiency (reviewed in 1). MtDNA maintenance defects have been shown to be a common cause of neurodegenerative disorders (2,3), and possibly have a role in mechanisms of aging (4,5).

MtDNA replicates continuously in post-mitotic cells, but the mechanisms that regulate mtDNA replication initiation and copy number are not well known. Likewise, the consequences of increased mtDNA amount in vivo are not well characterized. Two mtDNA maintenance proteins, mitochondrial transcription factor A (TFAM) and the mitochondrial helicase Twinkle, are known to correlate linearly with mtDNA copy number (6,7), but the mechanisms involved have not been previously established. Within the mitochondria, mtDNA exists as multimolecular clusters called nucleoids. A nucleoid contains ~2–8 mtDNA copies in primary or immortalized human cell lines (8), but knowledge of the nucleoid mtDNA content in vivo in different tissues is not available. Nucleoid organization is important for the maintenance, inheritance and segregation of mtDNA (9), and may
provide a layered scaffold for mtDNA replication, transcription and translation (10). However, the exact significance of nucleoid organization, and potential in vivo effects of alterations thereto, are presently not well defined.

TFAM, a high-mobility group-box protein, is an activator of mitochondrial transcription (11), and required for mtDNA replication, probably through providing RNA primers for replication (12). However, it also serves a histone-like function by packaging mtDNA (13,14) and as mtDNA levels follow TFAM levels (15), it is likely that naked mtDNA does not exist in mitochondria. TFAM knockout embryos were devoid of mtDNA (12) and the two strains of transgenic mice that ubiquitously overexpressed the human TFAM protein showed 1.5–2-fold increased mtDNA copy number (6,7). The advantage of using the human form of TFAM (hTFAM) is that it does not activate mtDNA transcription in mice, but preserves non-specific DNA-binding and packaging ability. Therefore, hTFAM overexpression is a means to study the consequences of increased mtDNA copy number while limiting potential other effects such as mitochondrial transcription initiation. Increased mtDNA copy number has not been associated with harmful effects in mice; on the contrary, the TFAM overexpressors exhibited improved survival after experimental myocardial infarction (16) and had reduced age-related memory impairment and microglial mtDNA damage (17).

Twinkle was discovered based on its similarity to the T7 phage helicase/primase (18) and is part of the minimal mtDNA replisome in vitro (19). Twinkle increased the mtDNA copy number when overexpressed in mice, and it was proposed to be a ‘licensing factor’ for mtDNA replication (7).

Increasing the amount of mtDNA has been suggested as a therapeutic approach in diseases with mitochondrial dysfunction. In this study, we address the molecular mechanisms by which Twinkle and TFAM regulate mtDNA copy number, and investigate the in vivo consequences of high mtDNA levels.

RESULTS

We utilized the formerly generated mouse models that ubiquitously overexpress mouse Twinkle [A-line (7), heterozygous, back-crossed to C57BL/6 background] or human TFAM (16), hence referred to as the ‘Twinkle-mice’ and ‘TFAM-mice’. To confirm that the mouse phenotypes were not caused by disruption of additional genes by transgenesis, we back-crossed to C57BL/6 background or human TFAM tously overexpress mouse Twinkle [A-line (7), heterozygous, back-crossed to C57BL/6 background]. We utilized the formerly generated mouse models that ubiquitously overexpress mouse Twinkle [A-line (7), heterozygous, back-crossed to C57BL/6 background] or human TFAM tously overexpress mouse Twinkle [A-line (7), heterozygous, back-crossed to C57BL/6 background].

Twinkle implements mtDNA replication in vivo

Increase in the steady-state mtDNA levels could potentially be achieved by increasing the initiation of mtDNA replication. We asked whether the amount of newly synthesized molecules increased upon overexpression of Twinkle or TFAM. We gave the mice i.p. injections of the thymidine analogue BrdU, which labels newly synthesized DNA, and compared the BrdU signal in mtDNA from the heart and the skeletal muscle to the total nuclear 18S rDNA amount by South-Western analysis after 24 h post-injection and bands were quantified from the same blot. The nuclear 18S rDNA gene was probed to control for loading. Representative bands are shown for clarity of comparison. (B) The number of replicating mtDNA molecules per muscle fibre nucleus was obtained as the ratio of BrdU signal against nuclear 18S rDNA signal for heart (n = 4) and muscle (n = 7 for WT and TFAM, n = 9 for Twinkle). (C) mtDNA was quantified by qPCR, from the skeletal muscle (n = 10–12), heart (n = 5–6) and brain (n = 3) tissue of 10-week-old mice and from the skeletal muscle tissue of 14-month-old mice (n = 1–2). The effects of Twinkle and TFAM were additive in both muscle and heart. WT, wild-type; Tw, Twinkle-mice; Tw+TFAM, bitransgenic mice. *P < 0.05, **P < 0.01, ***P < 0.001; Student’s t-test compared with wild-type. Error bars indicate SEM.

Figure 1. Twinkle implements de novo mtDNA synthesis. (A) South-Western analysis of mtDNA replication. BrdU incorporation into mtDNA was analyzed 24 h post-injection and bands were quantified from the same blot. The nuclear 18S rDNA gene was probed to control for loading. Representative bands are shown for clarity of comparison. (B) The number of replicating mtDNA molecules per muscle fibre nucleus was obtained as the ratio of BrdU signal against nuclear 18S rDNA signal for heart (n = 4) and muscle (n = 7 for WT and TFAM, n = 9 for Twinkle). (C) mtDNA was quantified by qPCR, from the skeletal muscle (n = 10–12), heart (n = 5–6) and brain (n = 3) tissue of 10-week-old mice and from the skeletal muscle tissue of 14-month-old mice (n = 1–2). The effects of Twinkle and TFAM were additive in both muscle and heart. WT, wild-type; Tw, Twinkle-mice; Tw+TFAM, bitransgenic mice. *P < 0.05, **P < 0.01, ***P < 0.001; Student’s t-test compared with wild-type. Error bars indicate SEM.
that lacks the D-loop, thus avoiding obscuring the result by inclusion of 7S DNA in the analysis.

**Twinkle and TFAM have an additive effect on mtDNA copy number in mice overexpressing both proteins**

Next we studied whether a further increase in mtDNA copy number could be achieved by crossing Twinkle mice with TFAM mice to generate bitransgenic Twinkle + TFAM overexpressors. The overall well-being and gross phenotype were similar to wild-type littermates in all the transgenic lines. In quantitative real-time PCR (qPCR) and in Southern blot analysis (Supplementary Material, Fig. S1A) of mtDNA from the skeletal muscle of 10-week-old mice, the mtDNA copy number of Twinkle mice was 1.9-fold, TFAM mice 3.2-fold and of the bitransgenic mice 5.8-fold higher than that of the wild-type mice. The copy number of the bitransgenic mice was significantly higher when compared with that of the wild-type mice ($P = 1.6 \times 10^{-7}$), the Twinkle mice ($P = 1.3 \times 10^{-5}$) and the TFAM mice ($P = 3.8 \times 10^{-4}$) (Fig. 1C). The findings were similar in the muscle of 14-month-old mice (Fig. 1C).

In the heart of 10-week-old mice, the effects of Twinkle and TFAM were also additive; fold-changes were 4.5 in the bitransgenic mice ($P = 6.2 \times 10^{-2}$), 2.2 in the Twinkle mice ($P = 0.012$) and 3.0 in the TFAM mice ($P = 9.6 \times 10^{-4}$; Fig. 1C). In the brain, mtDNA copy number was increased 1.7-fold in Twinkle mice ($P = 3.2 \times 10^{-4}$) and 1.9-fold in bitransgenic mice ($P = 3.8 \times 10^{-3}$), but was unchanged in TFAM mice (Fig. 1C). The TFAM transgene expression has been reported in the brain (17), but its level may be insufficient to increase the copy number of total brain mtDNA. No significant gender differences existed within any of the groups.

The observed increase in mtDNA copy number in bitransgenic mice was not related to changes in the abundance of human TFAM protein, since this was the same as in the TFAM mice (Supplementary Material, Fig. S1B). Likewise, endogenous mouse TFAM mRNA levels were unchanged in all of the transgenic mice (Supplementary Material, Fig. S1D). Twinkle protein was detectable only upon overexpression and its level was comparable in the bitransgenic mice and Twinkle mice (Supplementary Material, Fig. S1C).

These results show that Twinkle and TFAM have an additive effect on mtDNA copy number and demonstrate that mtDNA can be considerably upregulated in tissues.

**Overexpression of Twinkle or TFAM results in enlarged nucleoid size**

We investigated whether the mtDNA copy number increase in Twinkle and TFAM mice affected the number of mtDNA molecules per nucleoid. Nucleoids were visualized in the brain, muscle and heart by PicoGreen staining (Fig. 2A–L) and by immunohistochemistry with an anti-DNA antibody (Fig. 2M). The results from the two methods were in good agreement, supporting that the increase in PicoGreen signal was related to an increased nucleoid size rather than a change in DNA conformation. In wild-type mice, nucleoids could not be detected (Fig. 2A–C), suggesting that mtDNA content per nucleoid in wild-type tissues was lower than the detection sensitivity of PicoGreen or DNA antibody. In the muscle of TFAM mice and bitransgenic mice, enlarged mtDNA nucleoids were identifiable as large foci with a size range of 0.4–3 μm$^2$ (Fig. 2G and J, respectively). In Twinkle mice, muscle was comparable to wild-type (Fig. 2D), whereas the heart exhibited detectable nucleoids (Fig. 2E), similar to the TFAM mice and bitransgenic mice (size range 0.4–5 μm$^2$; Fig. 2H and K, respectively). In the brain, enlarged nucleoids were seen only in mice with increased mtDNA copy number, i.e. in Twinkle-mice and bitransgenic mice, but not in TFAM mice (Fig. 2 F, L and I, respectively).

Thus, Twinkle and TFAM increased nucleoid size *in vivo*, except in the skeletal muscle of Twinkle mice and the brain of TFAM mice.

**Nucleoid enlargement is associated with mtDNA deletions**

mtDNA deletions occur in normal aging and progressively accumulate in large amounts in some disorders that are caused by mtDNA maintenance defects (1). Reasoning that the altered nucleoid organization could lead to mtDNA instability, we searched for mtDNA deletions in the skeletal muscle, heart and brain of our mice by long-range PCR assay. Deletions were not present in any of the 10-week-old mice (Supplementary Material, Fig. S1E), which already showed enlarged nucleoids (Fig. 2). At 14 months of age, multiple deleted mtDNA molecules, represented by PCR products that were shorter than the full-length 16.5 kb band, were exclusively present in transgenic tissues that exhibited enlarged nucleoids: the heart and brain of Twinkle-mice; the muscle and heart of TFAM-mice; and the muscle, heart and brain of bitransgenic mice (Fig. 3). As a positive control, we used the muscle of the Deletor mouse that has late-onset mitochondrial myopathy and accumulation of multiple mtDNA deletions (20). Wild-type mice did not exhibit deleted molecules or enlarged nucleoids at any of the analyzed ages. Likewise, there were no deletions in the muscle of Twinkle mice or the brain of TFAM mice, which also did not have enlarged nucleoids.

These data show that, upon overexpression of Twinkle or TFAM, increased mtDNA amount per nucleoid is associated with formation of mtDNA deletions.

**Very large nucleoids are formed in TFAM and bitransgenic tissues but also when Twinkle expression is further increased**

Next we quantified the size and number of the visible mtDNA nucleoids. The bitransgenic mice exhibited the highest number of enlarged nucleoids in all size intervals (Fig. 4C). In the heart, the bitransgenic mice had a 2.3-fold higher total number of visible nucleoids than the TFAM mice ($P = 0.0019$), which in turn had a 4.3-fold higher total amount of visible nucleoids than the Twinkle mice (Fig. 4C). To find out whether the substantial difference in the number of large nucleoids between Twinkle mice and TFAM mice was caused by dominant-negative effects of the human TFAM protein or whether large-scale nucleoid enlargement could simply be achieved by increasing the amount of Twinkle,
we cross-bred Twinkle-mice to create Twinkle+/+ mice that were homozygous for the murine Twinkle transgene. The 10-week-old Twinkle+/+ mice had more Twinkle protein than heterozygous Twinkle mice (Supplementary Material, Fig. S1C). Accordingly, the Twinkle+/+ mice exhibited a marked further enlargement of nucleoids in the heart (Fig. 4B). In fact, the number of nucleoids per field was significantly higher than in TFAM mice (P = 0.040). Over the larger nucleoid size ranges (>2.2 μm²), the difference between Twinkle+/+ mice and TFAM mice was particularly pronounced, and the number of very large (>2.2 μm²) nucleoids in the heart of Twinkle+/+ mice approached that of the bitransgenic mice (Fig. 4C). It should be noted that the mtDNA copy number was similar in Twinkle−/− and Twinkle+/+ mice (Fig. 4A), which suggests that other factors become limiting for mtDNA replication when Twinkle is highly abundant.

In the skeletal muscle, the total amount of visible nucleoids per field was 2.2-fold higher in bitransgenic mice compared with TFAM-mice. Nucleoids remained undetectable in the muscle of homozygous Twinkle+/+ mice (Fig. 4B). Quantification of nucleoids from DNA–antibody-stained samples gave essentially the same result as the PicoGreen. Nucleoid enlargement was not associated with increased amounts of the nucleoid protein ATAD3 (21) in the skeletal muscle (Supplementary Material, Fig. S1F).

These results imply that Twinkle and TFAM have an additive effect on nucleoid size and that overexpression of Twinkle increases the average amount of mtDNA per nucleoid in a dose-dependent manner.

Figure 2. Twinkle and TFAM increase nucleoid size in tissues. DNA was detected by PicoGreen staining from frozen sections of the skeletal muscle, heart and occipital cerebral cortex of 10-week-old mice using confocal microscopy. The control mice showed only nuclear DNA signal (asterisks) in all tissues (A, B and C). The Twinkle-mice’ muscle was similar to wild-type (D), but they exhibited enlarged mtDNA nucleoids (arrows) in the heart (E) and brain (F). TFAM-mice showed enlarged nucleoids in the muscle (G) and heart (H), but not in the brain (I). The bitransgenic Twinkle + TFAM mice showed enlarged nucleoids in all the three tissues (J, K and L). (M) Immunohistochemistry of muscle and heart tissue using an anti-DNA antibody gave a similar result to PicoGreen staining. Enlarged mtDNA nucleoids were detected in Twinkle-, TFAM- and bitransgenic mice but not in wild-type mice. Scale bars 20 μm² (A–L) and 25 μm² (M).
Nucleoid enlargement inhibits mitochondrial transcription

We then examined whether nucleoid enlargement affected mitochondrial transcription. We used qPCR to measure the levels of mitochondrial RNA (mtRNA) transcripts of genes that are either proximal or distal to the promoters of the heavy strand (HS; Fig. 5A) and light strand (LS; Supplementary Material, Fig. S2A), in the muscle and heart of our transgenic mice. The relative positions of the assayed genes within the mitochondrial genome are schematically shown in Fig. 5B and given in Supplementary Material, Figure S2B. Mitochondrial transcript levels were normalized against nuclear genomic beta-actin expression.

In 10-week-old mice, we found that the tissues with a significant amount of very large (>2.2 μm²) nucleoids (Fig. 4C) had depletion of mtRNA transcripts: the muscle and heart of TFAM and bitransgenic mice, and the heart of homozygous Twinkle+/- mice (Fig. 5A). There was an inversely linear correlation between the number of very large nucleoids in the heart and the level of the promoter-proximal COXI mRNA transcript ($R^2 = 0.95$, $P = 0.023$; Fig. 5C). For the promoter-distal mRNA transcript CytB, a similar linear correlation was deduced from heterogeneous Twinkle mice, homozygous Twinkle+/- mice and bitransgenic mice ($R^2 > 0.99$, $P = 0.042$), but the TFAM mice did not fit well into the correlation (Fig. 4D). This suggested that the TFAM mice had lower levels of the promoter-distal transcripts than expected from nucleoid size alone. The LS-encoded promoter-distal tRNA transcripts were not significantly changed upon TFAM overexpression (Supplementary Material, Fig. S2A). We confirmed that the qPCR method was suited for detection of tRNA depletion by demonstrating lowered levels of the HS-encoded tRNA–His transcript (Supplementary Material, Fig. S2C).

These data suggest that high mtDNA content in a nucleoid has an inhibitory effect on mitochondrial transcription, and that human TFAM in mice has an additional deleterious effect on promoter-distal transcription that is independent from nucleoid enlargement. The differences in the levels of HS- and LS-encoded transcripts may be related to differing transcript half-lives, or to strand-specific differences in transcription regulation.

Curiously, in the skeletal muscle of Twinkle+/- mice, where no enlarged nucleoids were present, clear elevation of mtDNA copy number increase without nucleoid enlargement can lead to increased mitochondrial transcription or, alternatively, that Twinkle has a role in the regulation of transcription.

Partial deficiency of the RC complexes in TFAM mice, and aggravation of the phenotype in bitransgenic mice

The finding of mtRNA depletion in 10-week-old mice prompted us to measure the amount of RC complexes by blue native polyacrylamide gel electrophoresis (BN-PAGE; Supplementary Material, Fig. S3). The signals for complexes I, III and IV (CI–CIV, respectively) were normalized against that of complex II, which is entirely encoded by the nucleus. In the skeletal muscle of TFAM mice, CI was reduced to 43% ($P = 0.013$) and CIV to 56% ($P = 0.026$) compared with wild-type mice. The depletion was significantly more pronounced in bitransgenic mice, where CI was reduced to 10% ($P = 0.012$) and CIV to 41% ($P = 0.015$) compared with wild-type mice. Bitransgenic mouse muscle had significantly less of complexes I ($P = 0.041$), III ($P = 0.0017$) and IV ($P = 6.2 \times 10^{-4}$) than the TFAM mice (Supplementary Material,
Fig. S3A and C). In the heart, RC complex levels were more resistant to mtRNA depletion compared with the skeletal muscle, potentially due to increased protein half-life. We observed depletion of CIV to a level of 39% ($P = 0.023$) in the heart of bitransgenic mice, but the levels of the other complexes were not significantly altered (Fig. S3B and D).

**Progressive RC dysfunction in TFAM and Twinkle mice**

To understand the long-term effects of mtDNA deletions and RC complex depletion in our mice, we studied the activities of cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) by histochemical analysis of the skeletal muscle and heart of 14-month-old mice. COX-deficient fibers were observed in the skeletal muscle of TFAM mice and bitransgenic mice but not in wild-type or Twinkle mice (Fig. 6C, D, A and B, respectively). The proportion of COX-negative fibers was, on average, 0.8% in TFAM mice and 0.7% in bitransgenic mice. The COX-negative fibers were mostly white in TFAM mice (Fig. 6C) and mostly blue (Fig. 6D) in bitransgenic mice, meaning that mitochondrial hyperproliferation with increased SDH activity had occurred in the latter but not in the former. In the heart we observed COX-deficient cells in Twinkle mice, TFAM mice and bitransgenic mice.
In all mice and tissues, the presence of COX-deficient cells correlated with enlarged nucleoids and mtDNA deletions. Electron microscopic examination of aged bitransgenic mice revealed enlarged intermyofibrillar mitochondria with abnormal cristae (Fig. 6J) that contrasted with the dark, regular cristae in the subsarcolemmal mitochondria of the wild-type mouse (Fig. 6I). The mitochondria of bitransgenic mice also contained hypodense inclusions similar in size to the enlarged nucleoids that were seen in light microscopy (Fig. 6J, inset). The mosaic COX deficiency that was observed in 14-month-old mice with elevated mtDNA was similar, albeit less pronounced, to that found in aged Deletor mice (20), and was probably a consequence of a high mtDNA deletion load in individual cells. The RC complex depletion in 10-week-old mice (above), on the other hand, most likely reflected an early-onset transcriptional or translational defect.

**DISCUSSION**

A sufficient mtDNA copy number is critical for the upkeep of oxidative capacity and ultimately cell survival. Elucidating the mechanisms of mtDNA maintenance therefore has important implications for the understanding and treatment of mitochondrial diseases, as well as neurodegeneration and aging. Here we have provided evidence of the mechanisms by which Twinkle and TFAM regulate mtDNA levels

**Figure 6.** Mitochondrial function is impaired in Twinkle- and/or TFAM overexpression. Frozen skeletal muscle sections from 14-month-old mice were stained for COX (brown) and SDH (blue) activity. Only normal brown fibres were seen in skeletal muscle of wild-type (A) and Twinkle-mice (B). TFAM-mice (C) and bitransgenic mice (D) displayed COX-negative skeletal muscle fibres (arrows). In the heart, COX-deficient cells were observed in Twinkle-mice (F), TFAM-mice (G) and bitransgenic mice (H). The number of COX-deficient cardiac cells mounted to ~3–4 per section, and no COX-deficient cells were observed in wild-type heart. Scale bars 50 μm and 20 μm in inset (A–H). Electron micrographs of muscle from 14-month-old wild-type (I) and bitransgenic (J) mouse muscle showed intermyofibrillar accumulations of large mitochondria with abnormal cristae in the bitransgenic mouse. The bitransgenic mouse also harboured mitochondria with hypodense inclusions (J, inset, arrows). Scale bars 2 μm and 1 μm in inset (I and J).
mechanism. This mechanism may involve increased packaging and stability of mtDNA, as was suggested previously (6). Recent results using in vivo BrdU labeling and immunostaining for BrdU and TFAM showed that nucleoids form subpopulations with regard to replicative activity, and that nucleoids enriched in TFAM replicate less (23). Our results support a model wherein TFAM increases packaging and stability of mtDNA and elevates the proportion of non-replicating nucleoids. Wild-type TFAM could also affect mtDNA replication rate through providing RNA primers, although the robust effect of Twinkle on mtDNA synthesis suggests that Twinkle is the principal limiting factor for replication initiation under physiological conditions. It can be inferred then that a pathway for nuclear control of mtDNA replication must involve alterations in the level or activity of Twinkle.

The level of BrdU incorporation in Twinkle mice was more pronounced in the heart than in the muscle. Interestingly, the heart of Twinkle mice also exhibited nucleoid enlargement. This suggests that mtDNA synthesis rate must not be too high in order to allow proper mtDNA organization and nucleoid segregation. Evidently, other factors than Twinkle are also involved in nucleoid size regulation. We found that hTFAM markedly increased nucleoid size, which suggests that increased mtDNA packaging may be sufficient to increase nucleoid size in certain tissues. Another possible explanation for nucleoid enlargement in Twinkle and TFAM mice is the partial organization of mtDNA into covalently linked multienzyme clusters called catenanes. Catenane formation was previously found to occur in the hearts of mice overexpressing either Twinkle or TFAM (24), and was also prominent in normal human heart (24).

Compared with the heterozygous Twinkle+/− mice, homozygous Twinkle+/+ mice exhibited further nucleoid enlargement without a further increase in the total mtDNA level. Therefore, Twinkle+/+ mice arrange the same total amount of mtDNA into nucleoids that are fewer in number but larger in size. Analogously, an earlier study found that overexpression of the accessory subunit of polymerase γ in human cultured cells led to nucleoid enlargement without a corresponding increase in mtDNA copy number (25). Hence, it appears that mtDNA can organize itself into smaller or larger packages depending on the relative levels of regulatory proteins, and that mtDNA copy number per nucleoid does not necessarily directly reflect mtDNA copy number per cell. We show here that Twinkle is a gate-keeper molecule in initiating mtDNA synthesis, but when its level becomes as high as in Twinkle+/+ mice, other factors (e.g. endogenous TFAM or DNA polymerase γ) may become limiting. It is also possible that mtDNA replication becomes inefficient because the nucleoids are too large, and that mtDNA copy number expansion may require that nucleoid growth and segregation are balanced in order to maintain a nucleoid size that is ideal for replication.

The proteins required for nucleoid segregation are not known. One candidate is the AAA+ ATPase family member ATAD3 (21). ATAD3 binds mitochondrial D-loops and affects nucleoid structure (21), although it was recently questioned whether the protein binds mtDNA directly (10). We showed that the levels of ATAD3 protein did not correlate with nucleoid size in muscle. Therefore, ATAD3 is not stabilized against a large nucleoid and may not be required for nucleoid growth. Nevertheless, the tissue-specific nucleoid enlargement that occurred in our mice probably reflects different availability of factors that split the nucleoid, and ATAD3 may well be one of those factors.

We found age-related mtDNA deletion formation exclusively in tissues that harbored enlarged nucleoids already at the age of 10 weeks, which suggested a causal link between large nucleoid size and accelerated deletion formation. Abnormal mtDNA copy number per nucleoid could interfere with the progression of the mtDNA replisomes, causing polymerase stalling and induction of double-strand DNA breaks, which have been proposed to underlie multiple mtDNA deletions in humans (26–30). Previous studies have shown that, once formed, mtDNA deletions accumulate in individual cells through clonal expansion (31–33). This was attributed to a replicative advantage of small versus large mtDNA molecules (34). In Twinkle mice, the accumulation of deleted molecules may be enhanced due to the general increase in mtDNA synthesis. TFAM mice had a normal level of mtDNA synthesis, but accumulated deletions nevertheless. This suggests that Twinkle mice either had increased deletion formation at the large nucleoids, or that the turnover of the formed mtDNA deletions was decreased because of increased packaging of deleted mtDNA molecules by TFAM.

Nucleoid enlargement preceded the emergence of deletions by several months, meaning that the mtDNA deletions may be a consequence, but not a cause, of nucleoid enlargement. That said, it remains possible that nucleoid enlargement and deletion formation are the result of the same underlying problem rather than being causally linked. Specifically, the increased mtDNA replication upon Twinkle overexpression may cause a relative lack of polymerase γ, and insufficient polymerase or exonuclease activity could be the trigger of replication stalling and deletion formation.

The number of very large (>2.2 μm²) nucleoids correlated linearly with depletion of mtRNA. The depletion of promoter-distal mtRNA transcripts in TFAM mice was more severe than the expected based on the nucleoid size distribution alone and may partly be accounted for by a dominant-negative effect resulting from an increased occupancy of mtDNA by the transcription-defective hTFAM. Another possibility is that hTFAM interferes with the negative transcription regulator MTERF3, as mice that lack this factor develop premature transcription termination (35). Overexpression of wild-type TFAM in cultured cells led to a transcription defect (36), and it remains to be studied whether overexpression of mouse TFAM in mice would actually have the same effect. In either case, our results from homozygous Twinkle+/+ mice strongly suggest that nucleoid enlargement in itself inhibits mitochondrial transcription.

Increased mtDNA copy number upon TFAM overexpression has previously been reported to improve survival after experimental myocardial infarction (16) and decrease age-related memory impairment (17). Our results from two independent mouse models show that high mtDNA copy number can also be associated with nucleoid enlargement and defective replication and transcription. Further studies are needed to elucidate the physiological relevance of high mtDNA copy number and nucleoid enlargement in human
pathology. It is notable that an increased mtDNA copy number together with lowered mitochondrial transcript levels has been reported in human aging (37), and that nucleoid enlargement was found in cultured fibroblasts following administration of the anticancer drug doxorubicin, which can cause cardiac toxicity with mtDNA deletions and mitochondrial dysfunction in humans (38). Analysis of nucleoid morphology could thus provide clues to the mechanisms of mtDNA deletion formation in disorders where deletions occur due to yet-unknown reasons.

In conclusion, we have shown that the helicase Twinkle is a regulator of mtDNA synthesis and nucleoid structure in mice. The hTFAM protein similarly affects nucleoid structure, and high nucleoid mtDNA content appears to have detrimental effects. This suggests that treatment strategies aimed at increasing mtDNA levels must be undertaken with some caution.

MATERIALS AND METHODS

Transgenic animals

This study utilized two previously described mouse models: the Twinkle overexpressor (7) and TFAM overexpressor mice (16), both in C57BL/6 background and expressing the transgene under a ubiquitous beta-actin promoter. The Twinkle mice were back-crossed to C56BL/6 from FVB/N for more than 12 generations, and the congenicity was confirmed with the Mouse Medium Density SNP Panel (Illu-mina). Animals were interbred to generate bitransgenic mice, heterozygous for both transgenes. Genotypes were PCR-verified as described previously (7,16). All animals were housed and handled in accordance with regulations of the ethical boards of the Finnish National Public Health Institute and University of Helsinki (permit #STU575A/2004). Mice were sacrificed at 10–13 weeks of age, unless otherwise indicated.

Transgene insertion sites were identified using a Genome Walker Universal Kit (Clontech) according to the manufacturer’s instructions. The genomic insertion sites were analyzed in TFAM mice and homozygous Twinkle+/+ mice by expression array analysis using the Affymetrix GeneChip Mouse Genome 430 2.0 array (Affymetrix).

PicoGreen staining and confocal microscopy

Frozen sections from muscle and heart were covered with 1 μl PicoGreen (Molecular Probes) in 1 ml phosphate-buffered saline (PBS), and incubated 1 h at 37°C. Samples were mounted with SlowFade Gold reagent (Invitrogen) and imaged using a Zeiss LSM 510 Meta Confocal microscope. To count nucleoids, 8–10 fields (×63 Plan-Apochromat oil immersion objective) from heart of two Twinkle mice and three mice each for the other genotypes were photographed, and analyzed with the ImageJ 1.43f software (http://rsweb.nih.gov/ij/). Images were made binary, the smallest and largest visible nucleoids were identified as 0.4 and 5 μm², respectively, and particles were analyzed between these limits. The investigator was blinded to the genotype of the mice until data were ready.

in vivo BrdU labeling

Ten-week-old mice received a single i.p. injection of 1 mg/g BrdU (Sigma) in PBS. Mice were sacrificed 24 h later, and total DNA was isolated by routine phenol/chloroform extraction. Three microgram DNA was digested overnight using 10 U BsrXI restriction endonuclease (Fermentas) followed by agarose gel electrophoresis and blotting onto Hybond N+ membranes (Amersham). Membranes were blocked in 5% milk, and immunodetection was performed with polyclonal mouse anti-BrdU antibody (Becton Dickinson) at 1:1000 dilution in Tris-buffered saline (TBS) 0.1% Tween. Detection was performed with HRP-conjugated anti-mouse IgG (Molecular Probes) and ECL plus western blot detection system (Amersham). Nuclear 18S rDNA was detected by Southern hybridization as described previously (7).

Real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). One microgram total RNA was DNase digested using the Amplification Grade DNase I kit (Invitrogen). cDNA was synthesized with M-MLV reverse transcriptase (Promega) according to manufacturer’s instructions using random hexamers. The PCR amplification was done with 1:40 cDNA reaction with DyNAmo™ Flash SYBR® Green qPCR kit (Finnzymes) according to manufacturer’s instructions on an AbiPrism SDS 7000 machine (Applied Biosystems). Amplification conditions were as suggested by the supplier: 95°C for 7 min followed by 35 cycles of 95°C for 10 s and 60°C for 30 s. Dissociation curves ensured the existence of a single PCR product. Each sample was run in duplicate. qPCR data were analyzed using 7000 System Sequence Detection Software version 1.2.3 (Applied Biosystems). The amplification level of the assayed gene was normalized against the beta-actin gene. For mtDNA quantification, essentially the same protocol was used, with 25 ng total DNA used as template and normalizing the CytB gene amplification level against the nuclear beta-actin gene. Primer sequences are available on request.

Southern blotting

Southern blotting to detect mtDNA and the nuclear 18S rDNA gene was performed as described (7).

Immunoperoxidase staining

Formalin-fixed, paraffin-embedded samples of the skeletal muscle and heart were sectioned to 8 μm thicknesses, deparaf-finized and boiled in 10 mM citrate buffer, pH 6, for 10 min in a microwave oven followed by cooling for 20 min. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 min. Blocking was done with 1.5% normal horse serum in PBS 0.1% Tween for 45 min, and primary antibody incubation, using monoclonal mouse anti-DNA (Progen) at 10 μg/ml dilution in antibody diluent (Dako), overnight at +4°C. After washing, the samples were incubated 30 min in biotinylated anti-mouse IgM secondary antibody (Abcam) diluted at 1:500. After three additional washes in PBS, avidin/biotin complex was added from the Vectastain ABC
kit and incubated 30 min. Detection was done using 3,3′-diaminobenzidine (Sigma) for 3 min. Microscopy was performed with a Zeiss Axioplan 2 light microscope. Nucleoids were counted with the ImageJ 1.43f software as described under PicoGreen staining.

**Long PCR**

Long PCR to amplify the entire mitochondrial genome or selectively deleted mtDNA molecules was done using the Expand Long Template PCR System (Roche) with previously described primers (20). Twenty-five nanogram total DNA was used as template, with 200 μM dNTPs, 0.3 μM primers in 50 μl of enzyme buffer. Cycling conditions were: 92°C for 2 min followed by 30 cycles of 92°C for 10 s and 68°C for 12 min. PCR products were separated by electrophoresis on 1% agarose gels and visualized with a Typhoon 9400 scanner (Amersham).

**Blue native and SDS–polyacrylamide gel electrophoresis and immunoblot**

Mitochondrial protein enrichment, BN-PAGE and immunodetection of RC complexes was performed as described previously (39,40). A 2.5 μg enriched mitochondrial protein extract was loaded per well. The antibodies were mouse monoclonal antibodies against complex I (MS111), II (MS204), III (MS302) and IV (MS407) from Mitosciences diluted at 1:10 000 (CII) or 1:1000 (others) in TBS 0.1% (MS204), III (MS302) and IV (MS407) from Mitosciences diluted at 1:10 000 (CII) or 1:1000 (others) in TBS 0.1% Tween with 5% milk.

In SDS-PAGE, 10 μg enriched mitochondrial protein extract was separated on 10–20% gradient gels and transferred to Immobilon-FL transfer membrane (Millipore) using the Bio-Rad Western Transfer unit. The membranes were blocked in 5% milk in TBS 0.1% Tween. Antibody against ATAD3 (dilution 1:2000 in 5% milk TBS 0.1% Tween) was contributed by Dr Ian Holt. Polyclonal goat anti-TFAM and ATAD3 (dilution 1:2000 in 5% milk TBS 0.1% Tween) was blocked in 5% milk in TBS 0.1% Tween. Antibody against the Bio-Rad Western Transfer unit. The membranes were blocked in 5% milk in TBS 0.1% Tween. Antibody against ATAD3 (dilution 1:2000 in 5% milk TBS 0.1% Tween) was contributed by Dr Ian Holt. Polyclonal goat anti-TFAM and rabbit anti-Twinkle were purchased from Santa Cruz (sc-19050) and Aviva Systems Biology (ARP36483_P050), respectively, and diluted 1:1000. Corresponding secondary antibodies (Molecular Probes) were incubated with the membranes at a dilution of 1:10,000. ECL Plus Western Blotting Detection System (GE Healthcare) was used for the detection of signals with Typhoon 9400 (Amersham Biosciences) and quantified with the ImageQuant v5.0 software.

**COX/SDH activity stain and electron microscopy**

COX/SDH histochemical analysis was done as described previously (20). The numbers of COX-negative were counted from three independent mice of each genotype, counting at least 600 cells in each case. For electron microscopy, fixation, plastic embedding and sectioning were performed as described previously (20), and imaging was done with a JEOL 1400 Transmission Electron Microscope.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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