Mice lacking the mitochondrial exonuclease MGME1 accumulate mtDNA deletions without developing progeria

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Replication of mammalian mitochondrial DNA (mtDNA) is an essential process that requires high fidelity and control at multiple levels to ensure proper mitochondrial function. Mutations in the mitochondrial genome maintenance exonuclease 1 (MGME1) gene were recently reported in mitochondrial disease patients. Here, to study disease pathophysiology, we generated Mgme1 knockout mice and report that homozygous knockouts develop depletion and multiple deletions of mtDNA. The mtDNA replication stalling phenotypes vary dramatically in different tissues of Mgme1 knockout mice. Mice with MGME1 deficiency accumulate a long linear subgenomic mtDNA species, similar to the one found in mtDNA mutator mice, but do not develop progeria. This finding resolves a long-standing debate by showing that point mutations of mtDNA are the main cause of progeria in mtDNA mutator mice. We also propose a role for MGME1 in the regulation of replication and transcription termination at the end of the control region of mtDNA.
Mitochondrial diseases represent the most common group of inherited metabolic diseases in humans with a prevalence of about 1 in 5000. These genetically heterogeneous disorders can be caused by mutations in mitochondrial DNA (mtDNA) or in nuclear genes that encode proteins with mitochondrial function. Mutations in nuclear genes cause mtDNA instability resulting in mtDNA depletion or accumulation of deletions and/or point mutations, ultimately leading to impaired oxidative phosphorylation (OXPHOS). The vast majority of mutations causing human mtDNA instability map to genes encoding proteins involved in mtDNA replication, e.g., the catalytic subunit of mtDNA polymerase (POLGA), the accessory subunit of mtDNA polymerase (POLGB), the replicative helicase (TWNK), DNA replication helicase/nuclease 2 (DNA2), mitochondrial genome maintenance exonuclease 1 (MGME1) and ribonuclease H1 (RNASEH1), or nucleotide pool regulation, e.g., thymidine phosphorylase (TP), mitochondrial thymidine kinase (TK2), deoxyguanosine kinase (DGUOK), ATP-dependent succinate-CoA ligase (SUCLA2), and GTP-dependent succinate-CoA ligase (SUCLG1). Mitochondrial disorders are highly variable in disease severity and clinical presentation, and display tissue-specific manifestations. The basis for the clinical heterogeneity and tissue specificity of mutations in these ubiquitously expressed genes is unknown. The fact that mtDNA maintenance defects primarily involve high energy demanding tissues, such as brain and muscle, can only partially explain the tissue specificity seen in mitochondrial disorders. Other organs are also frequently affected, e.g., cardiomyopathy, diabetes mellitus, liver dysfunction, and optic neuropathy. Extensive in vitro work has led to significant progress in our understanding of the biochemical processes underlying mtDNA maintenance disorders, but animal models are nevertheless essential to understand the wide range of phenotypes and secondary metabolic consequences of mtDNA instability in different tissues.

**Fig. 1** Conditional knockout of Mgme1. a Targeting strategy for disruption of the Mgme1 gene. Magenta arrows loxP sequences; turquoise arrows FRT sites. b Schematic representation of Mgme1 cDNA. RT-PCR analysis of Mgme1 transcripts from control (+/+ ) and Mgme1 knockout mice (−/− ). c Western blot analysis of MGME1 levels in heart, liver, kidney, and spleen mitochondrial extracts of Mgme1 knockout (−/− ) and wild-type (+/+ ) mice and heart mitochondrial extracts of control (L/L) and tissue-specific knockout mice (L/L, cre). Mitochondrial HSP60 was used as a loading control.
Various animal models have increased our understanding of progression and disease mechanisms in mtDNA maintenance disorders and this will eventually open new avenues for therapeutic intervention\(^3\),\(^{10}\),\(^{18}\)–\(^{21}\).

To gain further insight into diseases of defective mtDNA replication, we created a knockout mouse model for the recently described disease gene encoding MGME1 (also known as Ddk1)\(^7\),\(^{22}\). Loss-of-function mutations in MGME1 were reported to cause a severe multisystem mitochondrial disorder in humans with depletion and rearrangements of mtDNA\(^7\),\(^{23}\). Patients with loss-of-function MGME1 mutations have progressive external ophthalmoplegia, skeletal muscle wasting/weakness, emaciation, respiratory distress, severe dilated cardiomyopathy, microcephalus, mental retardation, and severe gastrointestinal symptoms. Biochemically, MGME1 is a single-stranded DNA nuclease involved in processing of 5' mtDNA ends generated during replication\(^7\),\(^{22}\),\(^{24}\). Loss of MGME1 expression, either in siRNA treated cells or in patient fibroblasts, leads to an accumulation of 7S DNA\(^7\),\(^{25}\), which is the single-stranded DNA species formed by premature replication termination at the end of the control region of mtDNA\(^7\),\(^{25}\), thus suggesting a role for MGME1 in repressing formation or increasing turnover of these molecules.

We have studied the in vivo mtDNA replication phenotypes associated with MGME1 deficiency in various mouse tissues of knockout mice. Although MGME1 is not essential for embryonic development, its loss leads to accumulation of multiple deletions and depletion of mtDNA in a range of different mouse tissues. Furthermore, our data show that MGME1 is involved in regulation of heavy-strand replication and transcription termination. Remarkably, we report that MGME1 knockout mice display tissue-specific replication stalling patterns, with different tissues accumulating distinct replication intermediates, showing that this mouse model is a valuable tool to investigate tissue-specific pathology caused by the absence of MGME1.

**Results**

**Generation of Mgme1 knockout mice.** To study the in vivo function of the Mgme1 gene, we generated a conditional knockout allele (Fig. 1a). The mutated locus (Mgme1\(^+/\)loxp\(\text{-pur}\)) was transmitted through the mouse germline and the puromycin selection cassette was removed by mating with transgenic mice ubiquitously expressing the Flp-recombinase. Mice heterozygous for the loxP-flanked Mgme1 allele (Mgme1\(^+\)\(+\)/loxP\) were mated to mice ubiquitously expressing cre-recombinase (\(\beta\)-actin-cre) to obtain heterozygous Mgme1 knockout (Mgme1\(^+/\)\(-\)) mice. An intercross of heterozygous Mgme1\(^+/\)\(+\) animals gave viable homozygous knockout Mgme1\(^+/\)\(-\) pups at the approximate expected Mendelian ratios (genotyped pups \(n = 204\); Mgme1\(^+/\)\(+\) \(n = 36\); Mgme1\(^+/\)\(-\) \(n = 119\); Mgme1\(^+/\)\(+\) \(n = 49\)). Mgme1\(^+/\)\(-\) mice had a normal gross appearance and were followed until the age of 70 weeks (Supplementary Fig. 1). Reverse transcription (RT)-PCR analyses of the Mgme1 mRNA confirmed the absence of sequences corresponding to exon 3 (Fig. 1b) and the MGME1 protein was absent on western blots (Fig. 1c) in all investigated tissues of Mgme1\(^+/\)\(-\) mice. We also generated heart- and skeletal-muscle-
specific **Mgme1** knockout mice (**Mgme1**\(^{loxP/loxP}\); +/Ckmm-cre) that had no obvious phenotype when followed until 12 months of age. The MGME1 protein was absent in heart of the **Mgme1**\(^{loxP/loxP}\); +/Ckmm-cre animals (Fig. 1c). These results show that mice with both germline and tissue-specific knockout of **Mgme1** are viable and appear healthy.

**Widespread distribution of deleted mtDNA in Mgme1\(^{-/-}\) mice.** We performed long-extension PCR on total heart DNA to assess mtDNA integrity in **Mgme1**\(^{-/-}\) mice (Fig. 2a). When we used a primer pair (Supplementary Fig. 2a) designed to amplify the major arc of mtDNA, which is the region most commonly affected by deletions, we found multiple shorter molecules on long-extension PCR, consistent with the presence of mtDNA rearrangements in **Mgme1**\(^{-/-}\) mice (Fig. 2a). Moreover, Southern blot analyses of heart DNA from **Mgme1**\(^{loxP/loxP}\); +/Ckmm-cre mice and various tissues of **Mgme1**\(^{-/-}\) mice showed both mtDNA depletion and a prominent deletion of mtDNA (Fig. 2b–d). With restriction enzyme mapping, we established that the deleted species was a linear mtDNA molecule of ~11 kb that lacks sequences corresponding to the minor arc of mtDNA, which is the shorter region extending between the origin of replication of the leading (heavy, **O**\(_{1}\)) and lagging (light, **O**\(_{2}\)) strand of mtDNA. Consistent with the location of the deletion, both SacI (Fig. 2b, d and Supplementary Fig. 2d) and XhoI (Supplementary Fig. 2b) digested the linear mtDNA species into two fragments. In contrast, EagI, which only digests mtDNA in the minor arc region, did not cut the linear mtDNA molecule (Supplementary Fig. 2c). Similar to the findings in **Mgme1**\(^{-/-}\) mice, patients with pathogenic **MGME1** mutations display multiple mtDNA deletions in skeletal muscle, blood and urine on long-extension PCR analyses\(^7,23\). We have previously reported that the prematurely ageing mtDNA mutator (**PolgA**\(^{mut/mut}\)) mouse has high levels of a linear subgenomic mtDNA molecule on Southern blot analyses of fibroblast mtDNA\(^7,23\). We have previously reported that the prematurely ageing mtDNA mutator (**PolgA**\(^{mut/mut}\)) mouse has high levels of a linear subgenomic mtDNA molecule in addition to high levels of point mutations in mtDNA\(^25,26\). The linear subgenomic mtDNA molecules are very similar in size (Fig. 2b, Supplementary Fig. 2b, d) in **Mgme1**\(^{-/-}\) and **PolgA**\(^{mut/mut}\) mice. However, **Mgme1**\(^{-/-}\) mice accumulate more than double the amount of the 11 kb subgenomic fragment in comparison with **PolgA**\(^{mut/mut}\) mice (Supplementary Fig. 2d, e). The finding of linear subgenomic mtDNA molecules of a similar size and extension in patients and mice lacking functional MGME1 and in mtDNA mutator mice expressing mutant POLGA suggests that both enzymes function in the same pathway and that there is a common mechanism for the formation of those subgenomic fragments\(^27\).

**Mgme1 knockout mice do not age prematurely.** We assessed the mtDNA point mutation load in **Mgme1**\(^{-/-}\) mice by using a high-fidelity polymerase to amplify mtDNA fragments followed by cloning and sequencing of individual clones. We detected wild-type levels of point mutations in liver of young (11 weeks of age)
and old \(\text{Mgme}^{-/-}\) mice (70 weeks of age) (Supplementary Fig. 3a). Furthermore, we could not detect any significant changes in the load of mtDNA point mutations in spleen and skeletal muscle of 70 weeks old \(\text{Mgme}^{-/-}\) mice (Supplementary Fig. 3b). The increase in the point mutation load in mtDNA mutator mice is associated with premature onset of ageing-related phenotypes, such as decreased life span, male infertility and anemia\(^{25,28}\). In contrast, \(\text{Mgme}^{-/-}\) male mice are fertile with normal testis weight, normal sperm count, normal sperm motility and unaltered testis morphology (Fig. 3a–e).

At 20 weeks of age, \(\text{Mgme}^{-/-}\) mice have normal blood hemoglobin concentration, whereas older animals show a mild decrease in hemoglobin at the age of 70 weeks (Supplementary Fig. 4a, b). Importantly, the anemia of \(\text{Mgme}^{-/-}\) mice is much milder than the anemia in mtDNA mutator mice despite the observation that \(\text{Mgme}^{-/-}\) mice have much higher levels of subgenomic deleted mtDNA than mtDNA mutator mice\(^{30}\). At the age of 70 weeks \(\text{Mgme}^{-/-}\) mice have moderate anemia without reticulocytosis accompanied by moderate splenomegaly (Supplementary Fig. 4b–d), whereas mtDNA mutator mice at 40 weeks of age have profound anemia, marked reticulocytosis and massive splenomegaly with extramedullary hematopoiesis\(^{25,28}\).

Finally, we performed combined cytochrome c oxidase/succinate dehydrogenase (COX/SDH) enzyme histochemistry of heart, colon and skeletal muscle of \(\text{Mgme}^{-/-}\) mice and controls at the ages of 45 and 70 weeks. We found COX deficient cells in heart and colon from the age of 45 weeks in \(\text{Mgme}^{-/-}\) mice, whereas there were no changes in skeletal muscle (Fig. 4).

**Increased stability of 7S DNA in the absence of MGME1.** Given the observed effects on mtDNA quantity and integrity in both patients and mice lacking MGME1, we investigated regulation of replication in the control region. Typically, most mtDNA replication events initiated at the \(O_1\) region are abortive due to premature termination at the end of the control region, which results in the formation of a \(\sim 650\) nt long nascent DNA species (7S DNA) that creates a characteristic triple-stranded DNA structure, the displacement loop (D loop)\(^{31}\). Although the smear around 7S DNA in \(\text{Mgme}^{-/-}\) mice samples prevented us from accurate quantification of the 7S DNA band, Southern blot analyses suggested an increase of steady-state levels of 7S DNA in \(\text{Mgme}^{-/-}\) mice (Fig. 5a, b). To further investigate the mechanism behind the altered levels of 7S DNA levels, we performed in organello mtDNA replication experiments (Fig. 5c)\(^{32,33}\). Freshly isolated mitochondria from heart tissue were pulse labeled for 2 h with \(^{32}\)P-dATP followed by a one-hour chase to follow the synthesis and stability of de novo synthesized mtDNA (Fig. 5c). Surprisingly, no de novo synthesis of a distinct 7S DNA species was found in heart mitochondria from \(\text{Mgme}^{-/-}\) mice (Fig. 5c, lanes 2 and 6). However, we observed robust ongoing mtDNA synthesis as there was labeling of full-length mtDNA and a smear of shorter mtDNA species in \(\text{Mgme}^{-/-}\) mitochondria, similar to the findings in wild-type mitochondria (Fig. 5c). The replicative DNA helicase TWINKLE is essential for mtDNA replication\(^{34}\), and we, therefore, also analyzed \(\text{Twnk}^{-/-}\) knockout (\(\text{Twnk}^{loxP/loxP}^{-/-}/\text{Ckmm-cre}\)) heart mitochondria to ensure that the observed labeling is due to de novo mtDNA synthesis. As expected, no de novo mtDNA replication products were detected in \(\text{Twnk}^{-/-}\) knockout mitochondria (Fig. 5c, lanes 10 and 11).

Moreover, we performed chase experiments and found that 7S DNA is less stable than full-length mtDNA in wild-type mitochondria (Fig. 5c, lanes 1, 5, and 9 vs lanes 4, 8, and 12), consistent with previous observations in cultured cells\(^{35}\). To investigate the puzzling discrepancy between the low de novo synthesis and the high steady-state levels of 7S DNA in \(\text{Mgme}^{-/-}\) mice, we followed the stability of mtDNA with Southern blot analyses after inhibiting mtDNA replication with the chain-terminating nucleotide analog 2′,3′-dideoxycytidine (ddC) in mouse embryonic fibroblasts (MEFs). In the presence of ddC no 7S DNA is observed after three days of treatment. In contrast, 7S DNA is stabilized in the absence of MGME1 and can still be observed after 3 days of ddC treatment.
To elucidate whether the increased stability of 7S DNA was associated with structural changes, we defined the 3′ and 5′ ends of 7S DNA in Mgme1−/− mice (Supplementary Fig. 5a, b). Ligation-mediated (LM)-PCR analysis showed a tendency of shift towards longer 5′ DNA ends in the absence of MGME1 (Supplementary Fig. 5a, b). In contrast, LM-PCR analyses as well as 3′ polyadenylation-mediated PCR amplification revealed only modest changes in the 3′ ends of 7S DNA of Mgme1−/− mice (Supplementary Fig. 5a). Extended 5′ ends of 7S DNA have also been reported in fibroblasts from patients with MGME1 mutations and it has been speculated that this may explain the increased stability of 7S DNA.

**Fig. 5** Increased 7S DNA stability and diminished 7S de novo synthesis in Mgme1 knockout mice. a Southern blot analysis of SphI-digested mtDNA in heart from wild-type (+/+) and Mgme1 knockout mice (−/−). b Southern blot quantification: ratio of 7S DNA to mtDNA signal. Error bars represent the SEM. *P < 0.05; Student’s t-test. n = 4 biological replicates per genotype. c De novo DNA synthesis in heart mitochondria isolated from 8-week-old control (Mgme1+/+) and Mgme1 knockout (Mgme1−/−) mice and Twnk control (Twnk+/+) and tissue-specific knockout (Twnk+/−, cre) heart mitochondria. Mitochondria were pulse labeled for 2 h and the chase was performed for 1 h. d Southern blot analysis of SphI-digested mtDNA and 7SDNA from 3 days ddC treated (+) or untreated (−) mouse embryonic fibroblasts

(Fig. 5d, lane 4 vs lane 2). To elucidate whether the increased stability of 7S DNA was associated with structural changes, we defined the 3′ and 5′ ends of 7S DNA in Mgme1−/− mice (Supplementary Fig. 5a, b). Ligation-mediated (LM)-PCR analysis showed a tendency of shift towards longer 5′ DNA ends in the absence of MGME1 (Supplementary Fig. 5a, b). In contrast, LM-PCR analyses as well as 3′ polyadenylation-mediated PCR amplification revealed only modest changes in the 3′ ends of 7S DNA of Mgme1−/− mice (Supplementary Fig. 5a). Extended 5′ ends of 7S DNA have also been reported in fibroblasts from patients with MGME1 mutations and it has been speculated that this may explain the increased stability of 7S DNA.

**MGME1 interacts with mtDNA replication factors.** To further establish a role for MGME1 in mtDNA replication we performed a search for interacting partners by using a proximity-biotinylation assay (BioID) followed by affinity purification and mass spectrometry. The expression of human MGME1-BirA* in cultured human cells leads to an increase of protein biotinylation (Supplementary Fig. 6a) and the BirA tag does not interfere with the mitochondrial localization of the fusion protein (Supplementary Fig. 6b). Our results confirm that MGME1 interacts with the catalytic subunit of the mtDNA polymerase (POLGA) (Fig. 6). Furthermore, we identified a number of other mitochondrial replication-related proteins that could be interactors of MGME1, e.g., mitochondrial single-stranded DNA-binding protein (SSBP1), mitochondrial RNA polymerase (POLRMT) and TWINKLE (Fig. 6, Supplementary Data 1). As expected, several naturally biotinylated mitochondrial proteins, such as pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC), and methylcrotonyl-CoA carboxylase (MCCC1) were also identified in the affinity purification experiments. In addition, we identified very abundant mitochondrial proteins, such as proteases and respiratory chain subunits, which likely represent contaminants as we often find these proteins in various types of protein interaction studies.

**Lack of MGME1 influences mitochondrial transcription.** Next, we investigated the steady-state levels of mitochondrial rRNAs, tRNAs, and mRNAs in heart tissue from Mgme1−/− mice. Unexpectedly, there was a decrease in the abundance of
mitochondrial transcripts from HSP and an increase of promoter–proximal transcripts from LSP in Mgme1+/− mice (Fig. 7a, b, Supplementary Fig. 7a). Promoter-distal LSP transcripts, such as tRNAAsn and tRNACys, did not change significantly in Mgme1−/− mice. Normally, there is frequent termination of LSP transcription at CSB1 to generate the abundant polyadenylated 7S RNA (Fig. 7c) with a poorly understood function. The steady-state levels of 7S RNA were markedly decreased in Mgme1−/− mice (Fig. 7c, Supplementary Fig. 7b). In contrast, we found markedly increased levels of an antisense H-strand transcript that spans over the control region, the so called anti control region transcript (ACR) (Fig. 7d). This transcript, which has an opposite sense to LSP transcripts, has previously been reported to increase in abundance in response to decreased de novo formation of 7S DNA) the levels of the ACR transcript are increased.

Loss of Mgme1 does not affect mitochondrial protein levels. As loss of Mgme1 leads to decreased levels of full-length mtDNA and the formation of high levels of a linear subgenomic mtDNA molecule (Fig. 2), we decided to investigate the steady-state levels of mitochondrial proteins encoded by mtDNA and nuclear genes (Fig. 8). The steady-state levels of (OXPHOS) subunits were normal in mitochondrial protein extracts (Fig. 8a, b) and the organization of the respiratory complexes was unaltered on blue native polyacrylamide gel electrophoresis (BN-PAGE) of mitochondrial extracts from Mgme1−/− mice (Fig. 8c). We also found normal levels of proteins involved in mtDNA replication, transcription, and RNA maturation, such as mitochondrial transcription termination factor 1 (MTERT1), leucine-rich pentatricopeptide repeat-containing (LRPPRC), mitochondrial transcription factor A (TFAM), mitochondrial transcription factor A (POLRMT).

Tissue-specific replication stalling in Mgme1 knockout mice. To gain a more detailed insight into the integrity of mtDNA we performed next generation sequencing of mtDNA from liver (Fig. 9a), heart (Fig. 9d) and brain (Supplementary Fig. 8) of Mgme1−/− mice. The relative sequence coverage pattern of mtDNA from the liver of Mgme1−/− mice (Fig. 9a) was very similar to the previously published pattern in liver from Polga<sup>mut/mut</sup> mice and is consistent with the presence of a linear subgenomic mtDNA fragment extending from O<sub>H</sub> to O<sub>L</sub> in both Mgme1−/− (Fig. 2d, Supplementary Fig. 2d) and Polga<sup>mut/mut</sup> mice. Further analysis of mtDNA by neutral-neutral two-dimensional agarose gel electrophoresis (2DNAGE) showed a prominent and site-specific stalling of mtDNA replication in the region of O<sub>L</sub> (Fig. 9c) consistent with the sharp decline in sequence coverage after this point. In striking contrast, the sequence coverage pattern of mtDNA from heart (Fig. 9d) and brain (Supplementary Fig. 8a) of Mgme1−/− mice showed more of a gradual decrease with the most abundant sequences being present close to O<sub>H</sub>. 2DNAGE analysis of heart and brain mtDNA from Mgme1−/− mice showed a generalized increase in the abundance of replication intermediates at all points along the replication fork arc (Fig. 9e, f; Supplementary Fig. 8b, c; Supplementary Fig. 9). This is consistent with a non-specific replication stalling phenotype as documented by the decreased sequence coverage as the distance from the origin of replication increases.
**Discussion**

In this study, we report that the loss of MGME1 has marked effects on mtDNA replication in vivo. The \( \text{Mgme1}^{−/−} \) mice are viable but exhibit an mtDNA replication stalling phenotype that surprisingly shows a tissue-specific pattern and is associated with decreased mtDNA copy number and an accumulation of mtDNA deletions. Furthermore, we report an unexpected role for MGME1 in the regulation of mtDNA replication and transcription termination at the end of the D-loop region. Proximity labeling experiments with the Bio-ID method showed that MGME1 interacts with a number of components of the replication machinery. MGME1, thus, has an important role in the regulation of mtDNA replication.

Loss of MGME1 in mice causes an increase in the steady-state levels of 7S DNA, consistent with the previously suggested role of MGME1 in degrading this mtDNA species. Importantly, de novo replication of mtDNA occurs in the absence of MGME1, but the formation of 7S DNA is severely decreased. This striking discrepancy between impaired de novo synthesis and increased steady-state levels of 7S DNA is likely explained by increased stability of these molecules in the absence of MGME1 activity. It has been estimated that the 7S DNA in patient fibroblasts has a four-fold longer half-life than in control cells, and here we report increased stability of the 7S DNA species in \( \text{Mgme1}^{−/−} \) MEFs after mtDNA replication inhibition with the chain-terminating nucleotide analog ddC. Interestingly, the severely decreased de novo formation of 7S DNA argues that MGME1 may also modulate replication termination at the end of the D-loop region. Support for a regulatory role for MGME1 at the end of the D-loop region comes from the observation that transcription from HSP, which is normally terminated at this region, is aberrantly regulated in the absence of MGME1. Lack of HSP transcription termination results in accumulation of an ACR transcript over the D-loop region. This ACR transcript has also been described to form in response to thiapenecolin or ddC treatment of tissue culture cells. We have previously reported in vivo evidence for ACR transcript accumulation in transgenic mice with impaired mtDNA transcription initiation. In this mouse strain, knockout of \( \text{Tfam} \) in heart, which typically results in severe cardiomyopathy, was rescued by expression of the human \( \text{Tfam} \) gene. Interestingly, in addition to ACR transcript accumulation in those rescue mice the levels of 7S DNA were significantly reduced. We propose that MGME1 might be part of a regulatory switch acting at the end of the D-loop region that controls mtDNA replication and H-strand transcription termination. Similarly, we have recently shown that the mitochondrial transcription termination factor 1 (MTERF1) blocks transcription initiated from LSP, just downstream of the ribosomal transcription unit, preventing formation of an antisense transcript over the D-loop region and interference with the activity of the LSP promoter in the mouse. Moreover, MTERF1 was recently suggested to display contrahelicase activity and would therefore be able to counteract DNA unwinding by the TWINKLE helicase, which is a termination mechanism that may prevent the

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**Fig. 7** Steady-state levels of mitochondrial transcripts in \( \text{Mgme1} \) knockout mice. **a** Schematic representation of mtDNA with focus on NCR (non-coding region). Green line heavy-strand transcripts, pink line light-strand transcripts. Red line heavy-strand replication, HSP heavy-strand transcription promoter, LSP light-strand transcription promoter, \( O_h \) origin of heavy-strand replication, \( O_l \) origin of light-strand replication **b** Steady-state levels of mitochondrial mRNAs, rRNAs, and tRNAs; Nuclear 18S rRNA is used as a loading control. COX1 (Cytochrome oxidase subunit 1); COX2 (Cytochrome oxidase subunit 2); NDI (NADH-ubiquinone oxidoreductase chain 5); ND1 (NADH-ubiquinone oxidoreductase chain 1); ND6 (NADH-ubiquinone oxidoreductase chain 6); CytB (Cytochrome reductase subunit b). **c** Northern blot analysis of 7S RNA levels in heart from control (\( L/L \)) and \( \text{Mgme1} \) tissue-specific knockout mice (\( L/\), cre) and \( \text{Mgme1} \) knockout mice (\( −/− \)) and corresponding control (\( +/+ \)); loading control is nuclear 18S rRNA. Figures b and c are derived from the same northern blot membrane. **d** The ACR transcript mapped by northern blot analysis, using single-stranded riboprobe complementary to the mtDNA light-strand control region. ND1 and COX1 transcripts were used as size indicators and 18S nuclear rRNA was used as a loading control. ACR transcript is indicated by dotted box.
replication and transcription machineries from colliding. Interestingly, ChIP sequencing experiments recently demonstrated that TWINKLE may also be important for the regulation of replication at the 3′ end of 7S DNA in the control region. Importantly, the Bio-ID experiments we present here show an interaction between MGME1 and TWINKLE. The prevention of interference between the mitochondrial replication and transcription machineries, as well as prevention of promoter interference seems to require complex regulation on multiple levels.

In line with the above-discussed results, Mgme1−/− mice display reduced steady-state levels of the transcript originating from the HSP promoter, whereas promoter–proximal transcripts from LSP are increased and promoter–distal transcripts unaltered. It is likely that the ACR transcript, if abundant, interferes with the HSP promoter leading to a decrease of HSP transcription initiation. As a consequence, the transcription apparatus would shift to the LSP promoter thus resulting in an increased transcription initiation from LSP and higher levels of LSP promoter–proximal transcripts. Interestingly, despite boosted proximal transcription from LSP, the 7S RNA levels were decreased in Mgme1−/− mice. The function of this promoter proximal transcript is unknown, but it is often discussed in the context of replication primer formation. We propose that most LSP initiation events are used for DNA synthesis in our

Fig. 8 Protein and respiratory complexes steady-state levels in Mgme1 knockout mice. a Western blot analysis of steady-state levels of respiratory chain complex subunits and diverse mitochondrial proteins in heart mitochondrial extracts from 12 and 25-week-old control (+/+) and Mgme1 knockout mice (−/−) mice and heart from control (L/L) and Mgme1 tissue-specific knockout mice (L/L, cre). b Analysis performed as in (a) using liver mitochondria isolated from control (+/+), Mgme1 heterozygous (+/−), and Mgme1 knockout mice (−/−). c BN-PAGE analysis of Mgme1 knockout (−/−) and wild-type (+/+) heart mitochondria. Left panel, coomassie staining. Middle panels, in-gel enzyme activities of complexes I and IV. Right panel, western blot analysis of steady-state levels of respiratory chain complex II visualized by immunostaining against SDH2 subunit. InIIIn and InIIInIVn: respiratory chain supercomplexes.

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ATP5a (C V) - UQCR2 (C III) - COX1 (C IV) - SDHA (C II) - NDUFB8 (C I) -
LRPPRC - POLRMT - TWINKLE - MTERF1 - TFAM - VDAC -
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In agreement with this model, we report increased sequence coverage of the mouse mtDNA samples from liver of Mgme1−/− and controls. Mitochondrial genome position (x-axis) versus sequence coverage divided by maximum coverage for each sample. For each genotype two samples derived from different mice were analyzed. The approximate locations of the origins of light-strand (Ol) and heavy-strand (Oh) replication are indicated by dotted lines with arrows. Relative coverage—heart Relative coverage—liver

Lack of MGME1 causes mtDNA depletion and accumulation of mtDNA deletions in a range of different mouse tissues. A hallmark of MGME1 deficiency in patient fibroblasts and mice is an 11 kb linear mtDNA fragment spanning the entire major arc of the mtDNA, which has been previously described in mtDNA mutator mice and Mgme1−/− mice. Consistent with this hypothesis, Mgme1−/− mice do not accumulate point mutations and do not display a progeroid phenotype. In line with this finding, mtDNA subgenomic fragments have not been detected in tissues from aging mammals further indicating that this lesion on its own does not induce aging.

Both 2DNAGE and sequence analysis of mtDNA isolated from various Mgme1−/− tissues revealed remarkable tissue-specific molecular phenotypes. The 2DNAGE analysis of Mgme1−/− heart and brain showed a general and unspecific accumulation of replication intermediates along the replication arc, suggesting that replication is stalling along the entire restriction fragment. Similarly, downregulation of MGME1 in tissue culture cells induces a comparable non-specific stalling effect. This result was different to what was seen in liver tissue of the Mgme1−/− mice, where a very prominent stalling site was observed in the vicinity of Ol. Furthermore, deep sequencing of liver mtDNA from Mgme1−/− mice showed a sequence coverage plot that reflects the presence of linear fragments with a large deletion. A similar sequence coverage has been previously reported from liver samples of mtDNA mutator mice. The coverage profiles of Mgme1−/− samples from brain and heart displayed a different pattern including a pronounced peak of 7S DNA accumulation of replication intermediates along the replication arc, suggesting that replication is stalling along the entire restriction fragment. These differences between the liver on the one hand and heart tissue on the other hand are in agreement with the distinct replication stalling profiles revealed by 2DNAGE data from these tissues. The increased reads along the major arc in the liver indicate that replication has trouble passing Ol, predominantly replicating the major arc, explaining the specific stalling in the 2DNAGE. The unusual slope in sequence coverage from the heart suggests that replication is initiated faster than it can be completed, resulting in more reads closer to the origin and generalized stalling as seen on the 2D gel experiments.
The observed tissue specificity of our Mgme1−/− mouse model is in accord with the well-known tissue heterogeneity of mitochondrial disorders. The tissue-specific phenotypes may, at least partly, be explained by the observation that diverse tissues have different energy demands and biosynthetic capacities. The synthesis of mtDNA relies tightly on a balanced dNTP pool, which depends on the mitochondrial dNTP salvage pathways and assisted transport of cytosolic dNTPs to mitochondrial matrix. These processes are likely differentially regulated in various tissues, thereby contributing to the tissue-specific manifestations of mitochondrial mtDNA maintenance diseases as exemplified by patients carrying mutations in enzymes important for the mitochondrial nucleotide salvage pathways, e.g., TK210 and MPV1757.

In conclusion, we show here that Mgme1 is a replication-related nuclease, necessary for 5′-7S DNA processing and faithful mtDNA replication in mice. Absence of Mgme1 results in tissue-specific replication stalling and accumulation of deleted mtDNA molecules but does not create a progeroid phenotype in affected animals. We propose that Mgme1 is part of a termination complex acting at the end of the D-loop region where it modulates mtDNA replication and H-strand transcription termination. The tissue-specific molecular phenotypes associated with Mgme1 deficiency suggest that the Mgme1−/− mice will be a valuable model for investigation of the pathophysiology of mtDNA maintenance disorders.

Methods

Animals and housing. Knockout transgenic mice on a C57BL/6J background were housed in standard individually ventilated cages (45 × 29 × 12 cm) under a 12 h light/dark schedule in controlled environmental conditions of 22 ± 2 °C and 50 ± 10% relative humidity and fed a normal chow diet and water ad libitum. The study was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (reference numbers 84-02.04.2015-A103 and 84-02.30.15.004) and performed in accordance with the recommendations and guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

Generation of Mgme1 knockout mice. The targeting vector for disruption of Mgme1 in embryonic stem cells was generated using BAC clones from the C57BL/6J RPCI-731 BAC library and has been transfected into the Taconic Artemis C57BL/6N Tac ES cell line. To generate conditional knockout Mgme1 mice, exon III was flanked by loxP sites. The puromycin resistance cassette was introduced as a selectable marker and removed by tampering of Mgme1+loxPloxP mice with transgenic mice ubiquitously expressing Flp-recombinase. Mgme1+loxPloxP mice were mated with mice ubiquitously expressing cre-recombinase (β-actin-cre) to generate heterozygous knockout Mgme1+loxPloxP mice. Mgme1+loxPloxP mice were further intercrossed to generate homozygous knockout Mgme1−/− mice. To obtain tissue-specific (heart and skeletal muscle) knockout mice, Mgme1+loxPloxP mice were crossed with transgenic mice expressing cre-recombinase under the control of the muscle creatinin kinase promoter (CmMK-cre).

Isolation of mitochondria from mouse tissue. Mitochondria were isolated from mouse tissues using differential centrifugation as previously described. Briefly, fresh tissues were cut, washed with ice cold PBS and homogenized in mitochondrial isolation buffer (MIB) containing 310 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA by using a Potter S pestle (Sartorius). The homogenate was centrifuged at 1000 g for 10 min at 4 °C and the supernatant was subsequently spun at 10,000 g for 15 min at 4 °C to isolate mitochondria. Crude mitochondrial pellets were suspended in MIB supplemented with 1× Complete protease inhibitor cocktail (Roche). Protein concentration was determined by the Bradford method using BCA as a standard. Mitochondria from brain tissue were isolated following protocol from Milltenyi BioTec mitochondrial extraction tissue kit using TOM22 MicroBeads.

DNA extraction and Southern blot analysis. Genomic DNA was isolated by Gentra PureGene Tissue Kit (Qiagen) according to kit instructions. DNA quantification was performed with the Qubit 1.0 fluorometer (Thermofisher). A volume of 2 μg of DNA was digested with SacI, XhoI, SpeI, or EagI and DNA fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose membranes (Hybond™) and hybridized with a13C32P-dATP-labeled probes. For DNA extraction Southern samples were heated for 3 min at 93 °C prior to loading. List of probes can be found in Supplementary Data 2.

Uncrept scans of important Southern blots with marker lanes are presented in Supplementary Fig. 10.

In organello replication. A volume of 1 mg of freshly isolated heart mitochondria was resuspended in 0.5 μl of incubation buffer (25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K2HPO4, 0.05 mM EDTA, 5 mM MgCl2, 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 10 mM Tris–HCl, pH 7.4) supplemented with 1 mM Mg2+ for 1 h on a rotator wheel. Chase experiments were followed by incubation with non-radioabeled dATP (5 mM) for additional hour. After incubation, mitochondria were pelleted at 9000 rpm for 4 min and washed twice with 10% glycerol, 10 mM Tris–HCl, pH 6.8, 0.13 mM MgCl2. In the following step DNA isolation and Southern blot analysis were performed as described above.

Long-extension PCR. Mouse mtDNA was amplified from 2 ng of total DNA with the following primers (P1:488-510, P2:4021-4040) using LA Taq polymerase (TAKARA, Japan) and following PCR conditions: 98 °C for 10 s, 95 °C for 30 s, and 72 °C for 10 min, 35 cycles.

Northern blot analysis. Northern blot transcript analysis was performed as previously described. Briefly, RNA, 2 μg, was isolated using the TRIzol extraction kit (GIBCO-BRL) and transferred to Hybond-N membranes, GE Healthcare) by northern blotting. DNA probes, α-32P-dCTP-labeled, were used for visualization of mtRNA and mtDNA molecules. RNAs and mtDNA were detected using specific oligonucleotides labeled with γ-32P-ATP. For the detection of the ACR transcript riboprobe was synthesized using Riboprobe System (Promega). List of probes can be found in Supplementary Data 2.

Western blot analysis and BN-PAGE. A volume of 20 μg of isolated mitochondria was resuspended in 4× Lämmli BUFFER (4% SDS, 20% Glycerol, 120 mM Tris, 0.02% Bromophenol Blue), proteins were separated on 4–12% NuPage gels (Invitrogen) and transferred to Hybond–N+ membranes (GE Healthcare) by northern blotting. Antibodies used for western blotting were as follows: MitoProfile total OXPHOS antibody cocktail (MitoSciences), HS960 (Cell signalning, 1:1000), COXI (Invi- trogen, 1:1000), MTERF1 (Proteintech, 1:1000), TFAM (Abonva, 1:1000), SDH2 (MitoScience). Rabbit polyclonal antisera against MGME1, TINWINKLE, POLRMT, COX2, LRRPRC proteins were generated using recombinant mouse proteins.

For BN-PAGE 75 μg mitochondria were solubilized in solubilization buffer: 1% (w/v) digitonin (Calbiochem), 20 mM Tris, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (v/v) glycerol. Following 15 min of incubation on ice, non-solubilized material was removed by centrifugation and the supernatant was mixed with loading dye (5% (w/v) Coomassie Brilliant Blue G-250 (Serva), 100 mM Tris, pH 7, 500 mM Na2-aconamic acid). Samples were resolved on 3–13% (w/v) acrylamide gradient BN-PAGE gels. BN gels were further subjected to Coomassie Brilliant Blue R staining, in-gel activity assay or western blot analysis, as indicated.

Library preparation and Illumina pair-end DNA sequencing. Library preparation protocol/kit was the NEBNext Ultra DNA Library Prep Kit for Illumina. A volume of 150 ng gDNA with the Covaris sonicator to the requested insert size of >500 bp. The sequencing run conditions were 2 × 250 bp on the Illumina HiSeq2500, using HiSeq Rapid v2 Kits from Illumina. Each library was indexed individually with the provided barcodes and sequenced to 6,000,000 reads (tolerance range –30%). The reads were aligned to the C57Bl/6 J mouse mtDNA reference sequence (NC_005089.1), using the corona lite mapping algorithm (Applied Biosystems) with default settings. The first 49 bases of the mitochondrial genome. This alignment procedure attempts to map each read at full-length to the reference sequence, allowing for at most 6 mismatches for each 50 bp read.

2DNAGE. For two-dimensional gels, mtDNA was isolated from fresh sucrose gradient purified mitochondria from liver and heart tissues and by the sequential phenol-chloroform extraction. The resulting DNA (3 μg per panel) was digested with RCl, precipitated and loaded onto 0.4% agarose without ethidium bromide. The DNA fragments were followed by ethidium bromide stained and molten 1% agarose containing 500 μg/ml ethidium bromide was cast around the gel slices. Second-dimension gels were run at a constant 260 mA for 6 h at 4 °C. Gels were Southern blotted onto nylon membranes and hybridized with probes detecting either the OX1-containing fragment or the OX2-containing fragment. Primer sequences used for probe synthesis (5′–3′) were: OX1 forward, AATCAATGGTTCAGGTCATAAAATAATCATCAAC; OH reverse, AATCAATGGTTCAGGTCATAAAATAATCATCAAC; OH reverse.
Sperm motility analysis requested from the authors. Cells were then appropriately diluted with fresh medium to permit sperm motility. The cauda epididymis was then unclamped and pierced with the point of a scalpel blade to allow sperm to be collected. The cauda epididymis was collected in 2.4 ml lysis buffer (50 mM Tris-Cl, pH 7.4, 500 mM NaCl, 0.2% SDS, 1 × protease inhibitor (Edl Protease Inhibitor Cocktail, EDTA-free, Thermo Fisher Scientific), 1 mM EDTA. Subsequently, 240 μl of 20% Triton-X 100 (final concentration2% was added. After sonication samples were centrifuged at 16,500×g for 4 min. Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific) were incubated with the lysates on a rotator at 4 °C overnight. Following washing in 1.5 ml wash buffer 2 (0.1% (w/v) deoxycholic acid, 1% (w/v) TritonX-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES, pH 7.5) five successive washing steps with 1.5 ml 50 mM Tris-CL, pH 7.4, were applied. Samples were eluted in elution buffer (2 M Urea, 5 mg/ml Trypsin, 1 mM TCEP, 50 mM Tris-CL, pH 7.5) at room temperature. A volume of 5 mM CAAX was added to the samples and reaction was incubated at 37 °C overnight. The samples were then further analyzed by LC MS/MS mass spectrometry. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009138.

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Author contributions

S.M. performed experimental work, data analysis, and was involved in project planning. M.J., T.I.N., J.P.U., C.D.-S., M.-L.S., O.R., A.F., P.L.P., J.B.S., and M.D.M. helped with experimental work and were involved in project planning and data analysis. X.L. and I.A. analyzed MS data. M.F., A.F., O.R., and J.B.S. analyzed the data and discussed the results. D.M. and N.-G.L. conceived the project and wrote the manuscript.

Additional information

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