Review Article

Current progress with mammalian models of mitochondrial DNA disease

James Bruce Stewart1,2,3

1Max Planck Institute for Biology of Ageing, Cologne, Germany
2Wellcome Centre for Mitochondrial Research, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, UK
3Biosciences Institute, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, UK

Correspondence
James Bruce Stewart, Wellcome Centre for Mitochondrial Research, Faculty of Medical Sciences, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK. Email: jim.stewart@newcastle.ac.uk

Funding information
Marie-Sklodowska-Curie ITN European Training Network, Grant/Award Number: EU 721757; Max-Planck-Gesellschaft; United Mitochondrial Disease Foundation, Grant/Award Number: 13-053R

Communicating Editor: Avihu Boneh

Abstract
Mitochondrial disorders make up a large class of heritable diseases that cause a broad array of different human pathologies. They can affect many different organ systems, or display very specific tissue presentation, and can lead to illness either in childhood or later in life. While the over 1200 genes encoded in the nuclear DNA play an important role in human mitochondrial disease, it has been known for over 30 years that mutations of the mitochondria’s own small, multicopy DNA chromosome (mtDNA) can lead to heritable human diseases. Unfortunately, animal mtDNA has resisted transgenic and directed genome editing technologies until quite recently. As such, animal models to aid in our understanding of these diseases, and to explore preclinical therapeutic research have been quite rare. This review will discuss the unusual properties of animal mitochondria that have hindered the generation of animal models. It will also discuss the existing mammalian models of human mtDNA disease, describe the methods employed in their generation, and will discuss recent advances in the targeting of DNA-manipulating enzymes to the mitochondria and how these may be employed to generate new models.

KEYWORDS
animal models, heteroplasmy, homoplasmy, mitochondrial disease, mitochondrial DNA

1 | INTRODUCTION

Mitochondrial diseases constitute a range of heritable disorders that, through various mechanisms, end up leading to mitochondrial dysfunction. Despite all of these diseases leading to a similar cellular disorder, a staggering array of variable disease syndromes, tissue specificities, age of onset, and prognostic outcomes for the patient are known.1,2 Further complicating the diagnosis and understanding of these disorders, the causative mutations can be due to mutations of both the maternally inherited mitochondrial DNA (mtDNA) itself, or by genes whose products function within the mitochondria but are encoded by the nuclear DNA. Current estimates are that 1100 to 1500 nuclear encoded genes3 are translated by cytosolic ribosomes, and the protein products are then targeted to the various subcompartments of the mitochondria.

As a consequence of their proteobacterial origin, mitochondria maintain their own small genome (the mtDNA), which in animals, exists in multiple copies, which vary by different cell types.4 Animal mtDNA typically encodes subunits of mitochondrial respiratory complexes I, III, and IV (EC 7.1.1.2, 7.1.1.8, 7.1.1.9), plus the mitochondrial ATP synthase (complex V, EC 7.1.2.2).
To express these genes, the mtDNA also encodes two of the rRNA subunits of their own ribosomes⁵ and a mini-
ized array of 22 tRNAs to carry out the translation⁶ within the mitochondrial matrix. The mitochondrial
genome shows evidence of the loss of these mtDNA
encoded RNAs that are present in more ancestral-like
mtDNA genomes. For instance, animal mitochondrial
ribosomes have evolved to utilize a tRNA in place of the
5S RNA,⁸ they have also evolved a protein-only, multi-
subunit RNaseP enzyme⁹ (EC 3.1.26.5), and they gener-
al maintain only the minimal set of tRNAs to decode
the modified mitochondrial genetic code.⁴

For over 30 years, we have known that mutations of
the small, 16.5 kb mitochondrial genome are also impor-
tant causes of diseases.¹⁰,¹¹ Mutations by base changes,
small indels or by large deletions or partial duplication of
the mtDNA sequence are also known to lead to mito-
ochondrial disorders.² The high copy number and lack of
a coordinated mtDNA replication cycle leads to another
unusual feature of mtDNA, where two or more variants
of the mtDNA can coexist within the same organism, cell,
or even mitochondrion in a condition known as heter-
oplasmy.¹² In most of the mtDNA derived disorders, a
high relative proportion of the mutation is necessary to
lead to the disease state (referred to as the mitochondrial
threshold effect¹³). In some cases, the disease alleles can
be the only allele in the cell, tissue, or organ in a state
referred to as homoplasmy, such as seen in some mito-
ochondrial disease alleles leading to Leber Optic Atrophy
(LHON, OMIM #535000).

While each specific disease is quite rare in the popula-
tion, when summed together, frequency estimates for
childhood mitochondrial diseases can be as high as
1:6700,² and up to 1:4300 in adults.¹⁴ As with all rare dis-
ases, the infrequency of patients leads to many difficul-
ties in uncovering the molecular basis of disease and
hampers recruitment in clinical trials.¹⁵ The rarity of
these disorders unfortunately also provides economic dis-
incentives for corporate investment in preclinical and
clinical trials. In addition, the striking tissue specificity
in mitochondrial diseases leads to many instances where
hampers recruitment in clinical trials. In addition, the striking tissue specificity
in mitochondrial diseases leads to many instances where
cell lines, through genetic manipulation or when derived
from patient fibroblasts, can be aphenotypic, or not
reveal relevant biochemistry that underlies the pathology
of specific organ systems. Thus, the field has had a long
interest in animal models to move forward our under-
standing of these disorders, and for preclinical research
into interventions and cures.

Despite a long history of nuclear transgenesis, and
recent advances in genome editing, animal mtDNA
remains resistant to transgenic manipulation, and as such,
there are only a handful of models with relevant disease
mutations available. Fortunately, the mitochondrial

### Synopsis

To date, animal mitochondrial DNA has remained resistant to genome engineering
 technologies for the generation of animal models of these rare diseases; this review
will discuss the currently available mammalian models for mtDNA disease, how
they were generated, and future prospects for enhancing these resources.

#### 1.1 | ANIMAL MODELS IN MITOCHONDRIAL DISEASE RESEARCH

The selection of the animal model always remains a topic
of much discussion. Each model organism must be
weighed by the research question at hand. There has
been a long history of biomedical research using mouse
models, and for the nuclear-encoded mitochondrial
genes, these have been the models of choice in many pre-
vious studies (for reviews of models with nuclear genes
involved in mitochondrial disease, see References 16-20),
a trend that will continue with the ease of CRISPR-based
nuclear genome editing techniques.²¹ As a mammalian
model, mice are short lived, breed prolifically, have a
long history of transgenic manipulation of the nuclear
DNA and, from a purely phylogenetic perspective, share
with rats the closest genetic relationship of a model
organism to humans and other primates (Figure 1). Yet
this genetic closeness does not always result in the best
model of a human disease.

For instance, a very clinically relevant mouse model
of Leigh’s syndrome generated by disrupting the Complex
I NADH dehydrogenase-ubiquinone-FeS 4 (NDUFS4)
gene leads to a mouse model displaying ataxia,
encephalomyopathy, and other features that model the
human disorder.²² This model is now heavily used in pre-
clinical research.²⁴-²⁷ In contrast, mutations in the Surfeit
Locus Protein 1 (SURF1) gene, a cytochrome c oxidase
assembly factor, in patients also leads to a Leigh syn-
drome presentation with associated complex IV defi-
ciency.²⁶,²⁸ Yet a SURF1 knockout mouse line utilizing
the loxP-cre recombination system showed little patho-
logical phenotype and in one study, with knockout mice
that actually out-lived their wild-type littermates.³⁰ This
was despite a ~30% to 50% reduction in complex IV enzyme activity and decreased complex IV activity as assessed by histochemistry. More recent work with these SURF1 knockout mice has shown that the mild complex IV deficiency activates various mitochondrial stress response pathways which appears to fully compensate for the genetic deficiency under normal laboratory conditions. A recent experiment in pigs, where TALENs and CRISPR/Cas9 nucleases were used to generate SURF1−/− cells then were used to generate pigs by Somatic Cell Nuclear Transfer technologies. In contrast to the mice, the pigs suffered from failure to thrive, muscle weakness and highly reduced life span, with elevated perinatal mortality, and neuropathological disruptions consistent with a delay in central nervous system development. In this instance, the genetically more distantly related, yet larger and more anatomically similar pig better modeled the human pathology.

While this review will focus on mammals, and mostly mice, I wish to emphasize the important contributions of the classic invertebrate models, Drosophila melanogaster and Caenorhabditis elegans and Caenorhabditis briggsae. These powerful genetic models have begun to be commonly used to check for mitochondrial pathology of potential new disease alleles, and have strong potential in early high throughput screens for pathways of molecules in models of mitochondrial disease. As for mtDNA mutants, C. elegans and C. briggsae have a long history in the study of mtDNA deletions mutants (reviewed in), and recent work with targeted restriction endonucleases has allowed the generation of Drosophila models with mtDNA mutations, including very versatile temperature sensitive mutations. Another potential vertebrate model of mtDNA diseases is the zebrafish, as a number of studies of nuclear mitochondrial disease genes are underway. Recent work on mtDNA mutations and the germline, and the manipulation of the mitochondrial replication machinery portends the appearance of zebrafish mtDNA mutant models.

1.2 | TRANSGENIC MANIPULATION OF ANIMAL mtDNA—PROGRESS AND PROBLEMS

Unlike the mitochondria of plants, yeast, and Chlamydomonas, the mtDNA of animals has remained resistant to transfection. While we have long been able to move intact mitochondria between animal cells, or into the germ cells of Drosophila and mice, transfection of animal mitochondria remains elusive. In plants, yeast and Chlamydomonas, the methods to introduce DNA into the mitochondria are membrane disrupting, such as the induction of temporary pores through electroporation, or the use of bio-ballistic “gene guns” to punch DNA coated projectiles into the mitochondria to deliver the transgene. In animals, these treatments are likely to release cytochrome c from the mitochondria, which...
would be expected to induce the mitochondria-dependent apoptosis pathways which ultimately lead to the destruction of the cell bearing the treated mitochondria. In cell culture, the reported successful microinjection techniques of King and Attardi, viral-mediated membrane fusion of isolated mitoplasts, and even bacterial conjugation methods may have been able to limit cytochrome c release. Investigations into novel techniques, such as the photothermal nanoblade or liposome-based methods such as the MITO-porter system, give hope that high-efficiency ways to introduce exogenous DNA into mitochondria are forthcoming.

However, these methods would still require the introduction of an entire replacement mtDNA, as a second obstacle remains to the integration of smaller transgenic oligonucleotides into the mtDNA itself. Plant, Chlamydomonas, and yeast systems have clearly defined mechanisms of mtDNA recombination which allow for the integration of the transgene. However, recombination appears to be a rare phenomenon in animal mtDNA. The use of nuclease-driven genome-editing technologies currently in use to modify nuclear DNA would face specific problems. It is clear we can target functional nucleases into animal mitochondria, such as restriction enzymes zinc finger nucleases (ZFN), or transcription activator-like effector nucleases (TALEs). One recent article also reveals that the CRISPR nucleases CAS9, but more efficiently CAS12a, can be imported into mammalian mitochondria in cells, and that the gRNAs colocalize with the mitochondria. However, these authors concede that this gRNA localization may not represent mitochondrial matrix import, but only association with the mitochondrial membranes or the mitochondrial intermembrane space. History speaks to a number of cases where true mitochondrial matrix import was not actually achieved in attempts to import nucleic acids. An earlier report of mitochondrial CRISPR manipulation does not define the mitochondrial subcompartment localization of the gRNA. This leaves the possibility that the negative cell phenotypes observed may not have been due to the specific cutting of mtDNA but were overexpression artifacts, or may even represent reported RNA-independent DNA cleavage by the matrix-targeted CAS9. Indeed, Antón et al report mtDNA damage in their CAS12a import experiments and dramatic mitochondrial dysfunction when importing CAS9 to the mitochondria.

Regardless of the efficacy of importing protein-only or protein-RNA nucleases, mammalian mitochondria appear to lack the nonhomologous end joining, microhomology-mediated end joining, or homology-directed repair DNA repair pathways that lead to genome editing in the nucleus. Double strand breaks in mammalian mtDNA have been shown to be rapidly degraded, with only rare recircularization of the mtDNA to form deletion-bearing mtDNAs. These results were in agreement with earlier work on the mouse, which found that germline transmitted heteroplasmic mice rarely, if ever, recombine, implying a similar lack of recombination in the mouse germline, limiting the utility of these pathways in transgenesis. In contrast, transient expression of the mitochondrially targeted PstI to adult mouse neurons did demonstrate that these double-strand breaks were rejoined to form canonical mtDNA deletions. Also in Drosophila, the targeting of restriction enzymes has been successfully used to induce recombination in heteroplasmic flies, albeit at a very low rate and under a strong selective regime to recover the target recombinant mtDNA. Also, human cell lines heteroplasmic for the pathogenic mt-TL1 A3243G and mt-TI A4269G mutation, did display recombinant mtDNAs after 9 months of culture. These tissue-specific differences in these results warrant further study and characterization, as the ability to induce neuronal-like recombination within the germline may help in the generation of models bearing mtDNA deletions, and would be valuable for inducing yeast-like recombination for targeted manipulation of the mtDNA.

A major conceptual breakthrough has recently emerged, inspired by the CRISPR “base editor” strategies. In a classic case of unrelated research leading to a breakthrough for another field, researchers characterized a bacterial toxin deaminase (DddA) from the Gram-negative bacterium Burkholderia cenocepacia, which deaminated cytidines to uracil, leading to a C:G > T:A mutations at TC or TCC sites. Unlike other cytidine deaminases such as apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) or activation-induced cytidine deaminase proteins, DddA has specificity to double-stranded DNA and undetectable activity on RNA. Fortunately, mtDNA continues to replicate in postmitotic tissues outside of the cell cycle, allowing replication-associated mutations from C > U > T before the U is repaired in the mtDNA. This was demonstrated earlier when APOBEC1 had been targeted to the mitochondria of living flies, leading to robust C:G > T:A mutagenesis throughout the mtDNA without the need to induce strand breaks or limit base-excision repair systems.

To lead to specific editing, the team split the DddA protein into two parts, combining them with two TALE sequences, which would bring the domains together and limit the deamination to the space between the two TALE recognition sequences (dubbing the constructs DdCBEs). A similar strategy had been employed to induce ZFN-directed methylation of mtDNA, and to direct the paired FokI domains in mitochondrially
targeted TALE or ZFN nucleases to limit nuclease activity to specific mtDNA target sequences. Uracil glycosylase inhibitor sequences can also be included to inhibit repair of the uracil bases after targeting, and was shown to increase the editing activity with some constructs. When expressed in human cell lines, the more optimal DdCBE constructs induced up to 40% to 49% editing efficiency for the targeted base. Though all TC or TCC sequences between the TALE recognition sequences could be edited, outside of this region, off-target mutation screening found that other mutations were undetectable above background heteroplasmy levels for most of the TALE recognition sequences, demonstrating that good TALE specificity is the critical aspect of the specific editing in this system. This impressive work finally allows for targeting mutations to the 13 to 14% of the sites represented by the modifiable TC or TCC motifs with the mouse and human mtDNAs, respectively. This is particularly exciting for those using another developing model for the study of mitochondrial disease, the induced pluripotent stem cells (iPSCs) or organoid systems. The ability to engineer mtDNA mutations into the cells would remove the necessity of obtaining the cells from patients bearing these rare diseases, and would allow for mutant and control cells or organoids to be studied under identical nuclear genetic backgrounds. For iPSCs from nucleus-encoded disease mutations, such an editing strategy may also supply a means to remove unwanted mtDNA mutations that can arise during the development of iPSCs.

This technology will undoubtedly open the doors to the generation of targeted animal models, or in the reversion of pathogenic mutations back to wild-type alleles. However, of the 93 pathogenic human mtDNA mutations which are classified as “confirmed” disease causing in the MitoMap server (accessed July 2020) only 11 involve TC, TCC, GA, or GGA sites. There may be further limitations possible in the ability to generate appropriate TALE (or ZFN) nucleases for the construct, or the presence of other mutable bases in the vicinity. Due to variations in the mtDNAs, 13 of these human mtDNA mutations could be generated in analogous positions in the mouse mtDNA (Table S1). An expanded mutagenic toolkit to target different sequence contexts and different bases will be required to generate analogues of the common MELAS (OMIM #540000) and MERRF (OMIM #54500) alleles (mt-L1 3243A > G and mt-TK 8344A > G) or the Maternally Inherited Leigh Syndrome (#256000)/NARP (#551500) allele (mt-ATP6 8993 T > G). It would also caution against limiting the animal models to only direct sequence analogues of human mtDNA disease mutations, as species-specific differences are to be expected as to whether a specific substitution is pathogenic, or the relative severity of a mutation in another species. A striking example is the lack of sequence conservation in the TψC loop of the mt-TK which harbors the 8344A > G MERRF allele, making the design of the direct analog of the MERRF mutation impractical (see alignment at Reference 83).

As introduced previously, the overwhelming majority of mitochondrial proteins are encoded by the nuclear DNA, cytosolicly translated, and imported into the various mitochondrial subcompartments. Thus, a longstanding question has remained as to why the mitochondria maintain an independent chromosome, and why could we not just express recoded variants of the mtDNA-encoded proteins targeted to the mitochondria to produce animal models or to use as therapies? Such allotopic expression experiments have been reported, and remain quite controversial. There is one report of nuclear expression of mouse wild-type mt-ATP6 and a variant with an analogous Leigh syndrome/NARP 8993 T > G (L156R) mutation. Biochemically, the mice were indistinguishable from non-transgenic controls, but the mice expressing the allotopic ATP6 with the 8993 T > G mutation displayed poorer performance on a number of behavioral and physiological tests. More unusually, two independent rat models were reported where the human proteins derived from the LHON mt-ND4 G11778A (R340H) mutation were transfected into the eyes, where they were reported to lead to symptoms consistent with LHON pathology. The authors also reported being able to reverse the damage by a second round of transfection with the wild-type, human allotropic ND4. These results have led to clinical trials in human patients.

However, the claims have met with much skepticism. In the case of the rat work, is seems difficult to reconcile the apparent uptake of the human ND4 protein into the rat complex I, as cross-species mito-nuclear incompatibilities have often been reported, and the within species rapid coevolution of the nuclear and mitochondrial components of the respiratory complexes should provide a significant obstacle to cross-species complementation. Other work in cell culture has repeatedly failed to demonstrate that the allotopically expressed proteins are properly imported into the mitochondria, or that they are assembled into the respiratory chain complexes. The mitochondrially encoded proteins are very hydrophobic, and the mitochondrial ribosome has been demonstrated to interact directly with the inner membrane to cotranslationally insert the nascent polypeptides. It is unclear how the matrix import machinery would import these hydrophobic polypeptides and insert them in the correct orientation in the inner membrane. These concerns are supported by reports of polypeptide aggregates and failed incorporation of allotopically expressed proteins.
1.3  Indirect mutagenesis of mtDNA

To date, mutagenesis of the mtDNA in animals has been accomplished through indirect methods. The earliest examples were derived from the spontaneous appearance of deleterious mtDNA mutations in cell culture or from aged mouse tissues. Early manipulation of mtDNA in cell culture employed a combination of 4,5′,8-trimethylpsoralen and UV light exposure. Such cells have been used to transfer the mutants to embryonic stem (ES) cells for injection into oocytes to generate mouse models (see Section 2.2).

However, mammalian mtDNA has been shown to be remarkably resistant to chemical mutagenesis in the living mouse. One recent study treating live mice with the chemical mutagens benzo[a]pyrene and N-ethyl-N-nitrosourea showed the extensive mutagenesis of the nuclear DNA, and the presence of the expected chemical adducts on the mtDNA. Despite this, the mtDNA mutation rate in these mice remained indistinguishable from the untreated mtDNA mutation rate. In vivo mtDNA mutagenesis can be achieved through genetic means, through the use of manipulated components of the mitochondrial replication machinery. The first such mouse was the mtDNA mutator mouse, which bears an exonuclease activity-disrupting D257A mutation in the endogenous locus of the catalytic subunit of the mitochondrial DNA polymerase, polymerase gamma (PolG). Reports in the literature show this mouse having been independently generated in at least three labs. Phenotypically, all three display very similar strong progeria in human patients or through disrupted 5′-3′exonuclease MGME1 in human patients or the Mgme1-knockout mouse model. The persistence of these linear molecules can be explained by the compromised mtDNA degradation activity in the absence of MGME1 or functional PolG exonuclease activity.

Excess replication stalling by the polymerase is also reported in mtDNA mutator mice. Similar to exo-deficient polymerases used in cycle sequencing reactions, POLG acquires a strand-displacement activity, suggesting an alternative, replication-mediated model for the formation of the linear mtDNA fragments and control region multimers. There are also reports of classic mtDNA deletions in the mtDNA mutator mouse, and an increase in deletions when a mitochondrially targeted restriction endonuclease and the POLGD257A are coexpressed. mtDNA deletions in human patients can often be caused by other mutations in human PolG, or by mutations in the mitochondria’s replication-associated helicase, TWINKLE. Yet the levels remain much lower than the mtDNA deletion inducing models, such as the TWINKLE mutant “deleter” mice or even the MGME1 knockout mouse. These rearranged mtDNAs, including the canonical mtDNA deletions, have not been demonstrated to be transmitted through the mouse germline, limiting the available mouse models bearing mtDNA deletions.

These replication machinery mutants may be employed in cell culture to isolate mutations of interest. One group has been able to manipulate the expression levels of the exonuclease deficient POLG in order to limit mutagenesis to only one to two mutations per mtDNA to isolate pathogenic mtDNA point mutations within cell culture, resulting in 812 mtDNA mutations being identified in the study. Similar manipulations with TWINKLE or MGME1 cell lines may produce cell lines bearing mtDNA deletions that are of interest. A similar concept used the platelets of mtDNA mutator mice to create cybrids to capture and concentrate mtDNA mutations. Using heterozygous mtDNA mutator mice would reduce the mutation rate, and may generate cybrids with only a few mtDNA mutations, which would ease the correlation of phenotype to specific mutations in the downstream studies. These mtDNA mutation-bearing cell lines could be transferred into ES cells for implantation in mouse embryos (see Section 2.2).

2  GENERATING MOUSE MODELS—MITOCHONDRIAL TRANSFER METHODS

2.1  Introducing mtDNA into embryos

The first attempts at generating heteroplasmic mice utilized mitochondria bearing mtDNA mutations leading to chloramphenicol resistance (CAPR) in teratocarcinoma
and melanoma cell lines. Cybrids were derived and injected into blastocysts, where they generated mosaic animals. However, transmission of these mtDNAs to further generations was not documented and subsequent attempts to generate heteroplasmic CAP mice also did not result in a stable mouse line for analysis. The first models of heteroplasmic mtDNA transmission in transmitochondrial mice were generated by isolating cytoplasts from one mtDNA haplogroup, injecting and electrofusing these to the cells of an embryo carrying another mtDNA haplogroup, and then screening the progeny for heteroplasmic pups to produce heteroplasmic lines stably transmitting the mt-NZB and mt-BalbC mtDNA haplogroups. Shortly thereafter, karyoplast transfers from one mouse strain’s oocyte into another led to the generation of C57Bl/6 and NZB heteroplasmic mice (Figure 2A). Direct injection of mouse mitochondria isolated from nonsperm sources, into the oocyte also appears to be a potential way to introduce intact mitochondria bearing mtDNA into mice, but has not yet resulted in a disease model.

2.1.1 ΔmtDNA4696 “Mito-mice”

This cytoplast electrofusion method was employed by the Hayashi group to generate the ΔmtDNA4696 “mito-mice,” the first heteroplasmic mouse model with a pathogenic

---

**FIGURE 2** Three methods to generate mouse models with mtDNA mutations. A, Cytoplasts containing the desired mtDNA are generated to transfer mitochondria, but not the nucleus, to the embryo. Electrofusion merges some of the cytoplasm with cells of an early embryo, introducing the mtDNA, which can give rise to a chimeric founder (see Section 2.1). B, Cybrid embryonic stem (ES) cells are generated in cell culture to introduce the desired mtDNA through cytoplasmic fusion. The resulting ES cells can be homoplasmic or heteroplasmic, depending on whether ES cells are pretreated to remove their own mtDNA. ES cells are introduced into an early embryo to create chimeras (see Section 2.2). A + B, Female offspring are screened to determine if they are transmitting the desired mtDNA variants, and the correct founders are bred to create maternal lines of the mice. C, Breeding/screening methodology using female PolG<sup>D257A/WT</sup> females to transmit mtDNA to their offspring. After breeding N2 females to secure the female line, the mothers are sacrificed and their colons are screened for the presence of mitochondrial deficiency and the cosegregating mtDNAs are identified by sequencing of laser-captured colonic crypts (cartoon sequence traces showing a heteroplasmic C > T mtDNA mutation are illustrated below the histology section containing representative COX-deficient colonic crypts). Selected females are then bred to establish the heteroplasmic lines transmitting the desired mtDNA mutations.
mtDNA mutation. Synaptosomal fragments from mouse brains bearing mitochondria and mtDNA were isolated and then fused into a ρ⁰ mouse cell line. One cell line bearing a 4696 bp deletion breakpoint (positions mt-TK 7759 to mt-ND5 12 454) was selected to generate cytoplasts to fuse into mouse embryos (Figure 2A). While the first three generations of mice showed evidence of the mtDNA deletion, a 27.9 kb partially duplicated species in addition to the WT mtDNA sequence, the 27.9 kb molecule was reported to be lost during the early generations. Mice displayed decreased complex IV activity, and had a pronounced pathology of the kidneys. Surprisingly, the mice faithfully transmit this mtDNA deletion, despite this being an exceedingly rare phenomenon in humans.135

2.2 Manipulated via embryonic stem cells

In a refinement of the aforementioned method, mtDNA from a target cell line has been transferred into chromosomally normal ES cells, which were then injected into mouse oocytes. These experiments lead to chimeric founder animals, and the pups are screened in order to identify germline transmission of the ES cell, and the desired mtDNA variants in a manner similar to established mouse nuclear transgenesis techniques.136 This technique has been used to generate a number of relevant mouse models with mtDNA mutations (Figure 2B).

2.2.1 mt-COI T6589C (V421A) mice

The first mouse line was homoplasmic for a T6589C mutation in mt-COI, leading to a V421A amino acid substitution.137 This mtDNA mutation was identified in a B82COIM cell culture, where the cells were found to exhibit half of the expected mitochondrial complex IV activity. ES cells depleted of their own mtDNA through rhodamine-6G treatment were fused to enucleated B82COIM cells to introduce the mtDNA mutation of interest. These ES cells were microinjected into eight-cell-stage mouse embryos to generate the chimeric founder mice. The pups of the female chimeras were bred and screened for pups who had inherited the T6589C-bearing B82COIM mitochondria, to found this mouse line (Figure 2B). The authors reported that the mouse hearts showed reduced complex IV activity through COX histochemistry, increased blood lactate levels after glucose loading, and a slightly reduced body mass over 18 weeks. In a later study, the mice showed no lifespan differences compared to wild-type mice.138 Overall, the phenotype of the mouse was quite mild when compared to the ΔmtDNA4696 mito-mice.

An mtDNA chromosome from LA9 cells, homoplasmic for both a 13885insC frame-shift mutation in mt-ND6 and the same mt-COI T6589C mutation described above was utilized to independently generate the same animal model in a second laboratory. One ES cell line in which 4% of the mtDNA contained a spontaneous deletion (13885insCdelT) which repaired the mt-ND6 frameshift mutation led to the successful generation of ES cell cybrids and eventually a single female chimeric mouse after introduction into blastocysts.101 Intriguingly, the mice showed a profound selection against the 13885insC + T6589C mtDNA, in favor of the 13885insCdelT + T6589C. In the homoplasmic state, the T6589C mutation (in concert with 0-14% of the 13885insC) also led to decreases in complex IV activity, but showed evidence of ragged red fibers in the skeletal muscle, cardiomyopathy, and abnormal mitochondrial morphology in the heart and muscle at 1 year of age despite outwardly appearing no different from age-matched control mice.101 The apparent differences between the two lines of mice bearing the same mutation could be simply a matter of the differences in ages used, or by the exact housing condition of the mice used by the two groups in the respective papers. However, differences in the exact mouse strain used,111 the precise mtDNA haplogroup on which the mutation is carried,139 or the presence of the low levels of the 13885insC mt-ND6 frameshift mutation101 may all play a role in the divergent phenotypes reported.

2.2.2 mt-ND6 G13997A (P25L) mice

The next model was generated from a complex I deficient metastatic lung carcinoma cells bearing a G13997A mutation in mt-ND6.140 This substitution mirrored the human disease allele G14600A (P25L) which leads to Leigh syndrome with optic atrophy141 or Leigh Syndrome with sensorineural deafness.142 By 3 months of age, the mice showed evidence of impaired complex I + III activity, but normal complex II + III and IV activities, in line with an isolated complex I deficiency. Blood lactate was again elevated after glucose loading, and there were elevated H₂O₂ levels detected specifically in the bone marrow. Histology was carried out on the optic nerves and retina and no evidence of optic atrophy was observed.140 A later study on these mice at more advanced age found no significant differences in lifespans and no stark advancement of mitochondrial pathology, but the mice developed a glucose intolerance that was reported to be mitigated with treatment by the antioxidant N-acetylcysteine.138 The mice were seen
to develop hematopoietic tumors, especially B-cell lymphomas in the spleen, liver, and lungs at frequencies ×7.5 higher than the age matched controls, and these tumors appeared to have high propensity for malignancy.143

A second strain was generated by the Wallace lab, by psoralen/UV treatment of LMTK− cells. In their studies, they were able to detect swelling and then degeneration of the smaller retinal ganglion cells over the window of 14 to 24 months.106 More mitochondria, and those with abnormal morphology, were observed in the axons of the optic nerves and the mice showed signs of vision problems. The lab was also able to confirm an isolated complex I deficiency, and elevated ROS levels in the mice, but did not report the tumorigenesis phenotype in their mice.

The nuclear-encoded mitochondrial Adenine Nucleotide Translocator 1 gene (ANT1 or Slc25a4) is important to mitochondrial function by exchanging matrix ATP derived from mitochondrial complex V with ADP in the cytosol. A study of two ANTI1ko/ko strains, bearing either the mt-ND6(P25L), or the mt-CO1 (V421A) mtDNA showed that the ANTI1ko/ko, mt-ND6(P25L) combined mutations lead to an enhanced cardiomyopathy phenotype and shortened the lifespans of the mice. In contrast, the ANTI1KO/CO1KO mt-CO1(V421A) did not lead to a similar additive effect on the phenotype of the mice.144 Intriguingly, a similar observation was made in a line of Bcs1lS78G knock-in mice for the mitochondrial inner membrane AAA-family translocase BCS1L, a factor important for the Rieske iron-sulfur protein, UQCRFS1 subunit’s assembly into complex III. A homoplasmic, normally neutral haplogroup variant in mt-Cytb G14904A (D245N), when introduced into the Bcs1lS78G colony, led to a strong increase in the severity of the phenotype in this model.145 These studies again point to the importance of mito-nuclear epistasis effects, and the importance of understanding the exact strain of mice used,111 and additionally, knowledge of the precise mitochondrial haplogroup involved in your mouse models of mitochondrial disorders.

### 2.2.3 mt-TK G7731A mice

A mt-TK G7731A mutation was found and concentrated from P29 cell lines before introduction into ES cell cybrids. This mutation would disrupt base pairing in the anticodon stem of the tRNA^{Met}, and aligned to the known mt-TK G8328A mutation in humans.146,147 This early study reported that 4-month-old mice with >76% relative levels of the mutation were slightly smaller than their low-level counterparts or wild-type controls, that there was a general reduction of mitochondrial respiratory chain activity in the kidney and skeletal muscle, and the grip strength of the mice was reduced.102 Mice aged to 26 months retained these phenotypes, but began to show markers of dysfunction in the blood, including reduced hematocrit, hyperglycemia, and elevated blood urea nitrogen levels.143 Muscle atrophy, but the absence of ragged-red fibers, and renal failure were also discovered in postmortem analyses.

### 2.3 Breeding and selecting for mutations in POLG^{D257A/WT} lineages

Our method differs from other successful methods in producing mice with mtDNA mutations in that there is no embryo manipulation. Earlier, we had attempted to use mtDNA mutator mouse females to transmit mtDNA point mutations and small indel mutations through female lineages.112 We found that these mice contained far too many mtDNA mutations to lead to a clear genotype-phenotype association between any specific mutations. For instance, a mouse bearing a heteroplasmic mt-TM 3875delC and homoplasmic mt-TC T5425C mutations also bore 7 rRNA mutations, 3 additional tRNA mutations, a mutation in the OrfL region, and 23 protein coding gene mutations (10 synonymous and 13 non-synonymous).148 Despite the deletion-bearing tRNA^{Met} mutation, reduced tRNA^{Met} levels, and a germline selection limiting mice to <87%, no evidence of respiratory chain dysfunction was observed.

Further work on the mtDNA mutator mice revealed that the heterozygous females were also mutagenic, and that successive breeding of heterozygous females led to mutation accumulation, the eventual loss of fecundity, impairments in brain development,149 and decreased lifespan of POLG^{WT} animals that had accumulated these mtDNA mutations.150 Based on the observed mutation rate in WT siblings of mtDNA mutator mice, we estimated that heterozygous mtDNA females with no prior maternal exposure to the POLG^{D257A} allele would induce on average two to three mtDNA mutations per molecule that transmitted through the female germline. A later collaboration on heterozygous mtDNA mutator mice and the accumulation of different clonally expanded point mutations within each of their colonic crypts led us to an in vivo method to prescreen mouse lineages of interest.151 Using COX/SDH histochemistry to screen for individual colonic crypts displaying mitochondrial dysfunction, and using laser-capture microdissection to sequence the individual crypts, we were able to identify female lineages that were transmitting pathogenic mtDNA mutations, and identify the specific mutations cosegregating with the dysfunction while the mice still harbored subthreshold levels of the mtDNA mutation.
Thus, the mtDNA mutations from the POLG\textsuperscript{D257A/WT} female are transmitted via the oocytes to the first-generation backcross females (N1 animals), who are selected if they lack the POLG\textsuperscript{D257A} allele. These females are again backcrossed to produce an N2 generation of females bearing and segregating the mtDNA mutations from their mother. Once the N3 litters have produced females to stabilize the female line, the N2 mothers are sacrificed, and the colonic crypts screened for the presence of crypts bearing complex IV deficiency, as indicated by COX/SDH dual staining histochomistry, indicating the lines of interest for further breeding an characterization.\textsuperscript{152} COX deficient crypts are then laser-capture micro-dissected and the mtDNA amplified and sequenced to determine which mtDNA mutations are cosegregating with the mitochondrial deficiency (Figure 2C).

2.3.1 | \textit{mt-TA} C5024T mice

The first line derived by this method was found to contain two linked mtDNA mutations; \textit{mt-TA} C5024T mutation, and \textit{mt-ND6} C13715T (G119D). While no strong complex I phenotype was observed, and the G119D mutation sits in a poorly conserved region of \textit{mt-ND6}, it is impossible to rule out a contributing role of this mutation to the phenotype of the mice.\textsuperscript{152} The C5024T mutation disrupts the same aminocyl-stem basepairing as the human \textit{mt-TA} G5650A mutation found in a patient suffering from a strict mitochondrial myopathy phenotype,\textsuperscript{153} and in a complex case of CADASIL (OMIM #125310) where the patient had both a \textit{NOTCH3} mutation commonly associated with CADASIL and also the \textit{mt-TA} G5650A mutation.\textsuperscript{154}

In the mice, steady-state levels of the tRNA\textsubscript{Ala} were reduced in animals bearing higher relative levels of the mutation, eventually leading to decreased capacity in \textit{in organello} translation assays performed on mitochondria isolated from animal tissues.\textsuperscript{152} Mice retained less body fat than age-matched controls, and the heart mass was elevated, and showed elevated levels of the cardiomyopathy markers ANF and ATF5.\textsuperscript{155} Beyond the colonic crypts, mosaic complex IV deficiency was originally observed in the heart and colonic smooth muscle and very rarely in the skeletal muscle.\textsuperscript{152} However, our continued work has revealed that in mice above 75% relative levels of the mutation, the liver, kidneys, small intestine, choroid plexus of the brain ventricles, and the ocular muscles show signs of complex IV defects using NBTx histochemistry.\textsuperscript{31} The phenotype differs substantially from the strict myopathy in the young \textit{mt-TA} G5650A patient, but is surprisingly reminiscent of the mother in that study, who had high levels of the mutation and strikingly high levels of muscle complex IV deficiency, but had not succumbed to a disease phenotype at the time of the report.\textsuperscript{153} The scheme has also produced another, currently unpublished mouse line that we are currently characterizing.

2.4 | Naturally occurring mtDNA mutations

While this method has not led to the isolation of models of pathogenic mtDNA mutations in mice, spontaneous mutations have been studied in rats\textsuperscript{156,157} and veterinary practice has identified dogs with mitochondrial disease phenotypes.\textsuperscript{158-160} A molecular diagnosis of a \textit{mt-CYTB} G14474A (V98M) mutation was made in Australian Cattle dogs and Shetland Sheepdogs, which lead to a spongiform leukoencephalomyelopathy in the more severely afflicted dogs.\textsuperscript{161} More recently, Golden Retrievers from a maternal lineage were identified with sensory ataxic neuropathy,\textsuperscript{162,163} The animals were found to bear the small deletion \textit{mt-TY} 5304delA, which would disrupt the first basepairing after the variable loop, T\textsubscript{ψ}C stem of the tRNA, leading to an unstable tRNA with reduced steady-state levels. All afflicted dogs had >88% of this mutation, while animals with lower relative levels remained aphenotypic. ATP production rates and respiratory chain enzyme activities were reduced in the animals, and COX/SDH histochchemistry in the skeletal muscle showed a generalized reduction of COX staining, and bluish color due to the complex II counterstaining.\textsuperscript{162} Generally, these mutations have led to severe disability in animals bearing high relative levels of the mutation. Ongoing contact with veterinary medicine practitioners that observe classic mitochondrial phenotypes in pets and domestic animals may allow for unique collaborations, and expedite preclinical research. Such collaborations could be of great interest to pet owners while simultaneously being of help to human patients in expediting research and safety assessments into experimental treatments.

3 | PRECLINICAL WORK WITH MTDNA ANIMAL MODELS

These mtDNA mouse models generated to date (Table 1) have raised considerable interest, not only in allowing deeper investigations into the molecular mechanisms underlying the pathophysiology of mtDNA disease, but they have also allowed for preclinical investigations into potential therapies to treat human mtDNA disease patients. In 2005, the Hayashi group used the ΔmtDNA4696 “Mito-mice” to demonstrate that nuclear transplantation from these mice to an enucleated oocyte lacking the mtDNA deletion was possible, and protected the resulting pups from
the pathology of these “Mito-mice.”166 These early results appear to have inspired the human mitochondrial replacement therapies, which are now being refined for families transmitting mtDNA disease.167-170

One potential method to help patients with heteroplasmic mtDNA mutations would be to employ methods that would favor an increase in the relative amount of wild-type mtDNA while decreasing that of mutant mtDNAs within the cells. As the amount of mutant mtDNA is reduced below the biochemical threshold for the given mutation, one would expect the mitochondrial network within the cell to regain proper function and rescue the cellular phenotype. Early work on mtDNA NZB/BalbC heteroplasmic mice (see Section 2.1) showed that the targeted degradation of one of the mtDNA haplogroups with restriction endonucleases allowed for the selective removal of one mtDNA type. The cells were able to re-populate their mtDNA with the untargeted mtDNA haplotype, thereby protecting the cell from mtDNA depletion.63,171,172 Further studies using mitochondrially targeted TALE nucleases also successfully reduced the amount of the targeted mtNZB in mouse oocytes or early embryos.173

In back-to-back studies, the heteroplasmic mt-TA C5024T; mt-ND6 C13715T (G119D) strategy has been to alter mtDNA copy number within the cells. In an illustrative example, a pathogenic mitochondrial threshold of 81% in a cell with 100 mtDNA would predict that the 20 wild-type mtDNAs would be sufficient to maintain normal cellular function. By doubling the mtDNA content of the cell, levels as high as 90% would still provide the required 20 wild-type copies and maintain healthy mitochondrial function within the cell.176 It has been long known that manipulations of the

| Mutation          | Method of generation | Mouse genome informatics database ID and mutation | mtDNA sequence |
|-------------------|----------------------|--------------------------------------------------|----------------|
| CAP<sup>6</sup> mice | Embryo/cytoplast fusion | 3783752 (mt-Rnr2<sub>m1Dwa</sub>) | Inferred LA9 mtDNA<sup>139,164</sup> |
| ΔmtDNA<sup>4696</sup> Mito-mice | Embryo/cytoplast fusion | 3776665 (Del(MTmt-Tk-mt-Nd5)1Jiha) | C57Bl/6J<sup>165</sup> (GenBank NC_005089) |
| mt-COI T6589C (V421A) | ES cell/embryo fusion | 3777237 (mt-Co1<sub>m1Jiha</sub>) | Inferred C3H/An |
| mt-ND6 G13997A (P25L) | ES cell/embryo fusion | 4834201 (mt-Nd6<sub>m2Jiha</sub>) | Inferred C3H/An |
| mt-TK G7731A | ES cell/embryo fusion | 5469319 (mt-Nd6<sub>m3Dwa</sub>) | Inferred C3H/An |
| mt-TA C5024T; mt-ND6 C13715T (G119D) | PolgA<sup>D257A</sup> mutagenesis | 3777242 9mt-Nd6<sub>m2Dwa</sub> | Inferred LA9 mtDNA<sup>139,164</sup> |
| nt-TA C5024T; mt-ND6 C13715T (G119D) | PolgA<sup>D257A</sup> mutagenesis | 5902095 (mt-Ta<sub>M1Jbst</sub>) | Genbank MT937073 |
| nt-TA C5024T; mt-ND6 C13715T (G119D) | PolgA<sup>D257A</sup> mutagenesis | 5902096 (mt-Nd6<sub>M1Jbst</sub>) | Genbank MT937073 |

| Table 1 | Summary of the current mouse models of mtDNA disease |
|---------|------------------------------------------------------|

The Miniczuk group used AAV9.45 with their mitoZFN constructs to also reduce the levels of the mtDNA mutation, rescue the tRNA<sub>Ala</sub> steady-state levels, and found metabolomics signatures consistent with increased oxidative respiration with decreased dependence on glycolysis.65 At present, these methods rely on AAV-mediated gene therapy and studies into peptide delivery are still lacking. While gene therapy approaches are often complicated to extend to human treatments, work on delivering the mitoZNF to oocytes is underway174 which may potentially decrease the amount of the pathogenic mtDNA transmitted. Such a protocol could be coupled with in vitro fertilization techniques, or be combined with mitochondrial replacement therapies to limit the carryover of mutant mtDNA during these treatments. Aside from nucleases, other methods to selectively shift heteroplasmy are being explored. For instance, the use of small molecules that bind to mutations that produce G-quadruplex sequences have been shown in cell culture to selectively reduce the amount of the mutant mtDNA by inhibiting its replication.175 Unfortunately, no animal models with appropriate mutations are available to test this method in an in vivo study.

Instead of shifting mtDNA heteroplasm, another strategy has been to alter mtDNA copy number within the cells. In an illustrative example, a pathogenic mitochondrial threshold of 81% in a cell with 100 mtDNA would predict that the 20 wild-type mtDNAs would be sufficient to maintain normal cellular function. By doubling the mtDNA content of the cell, levels as high as 90% would still provide the required 20 wild-type copies and maintain healthy mitochondrial function within the cell.176

It has been long known that manipulations of the
TFAM gene, which serves a dual role as a component of the mitochondrial transcription initiation complex, and acts as a histone-like protein which binds to and compacts mtDNA, alters the cellular mtDNA copy number. TFAM heterozygous knockout mice are viable, with approximately half the normal mtDNA copy number, and overexpression of TFAM in the mouse leads to a 150% - 250% increase of the mtDNA copy number. Comparing the phenotypes of the standard mt-TA C5024T mouse and the mt-TA C5024T mice with both TFAM overexpression, and TFAM heterozygous knockout nuclear backgrounds supports this hypothesis. Mice with similar relative levels of the C5024T mutation showed improvements under TFAM overexpression, such as a recovery of body mass, suppression of the cardiac enlargement and cardiomyopathy markers, reduction of COX-deficient fibers, and a rescue of enzyme activity defects. While genetic overexpression of TFAM is most likely not feasible in patients, the authors point out that mitochondrial biogenesis is a process that has responded to small molecules, and may offer an avenue for the development of drug therapies for heteroplasmic mitochondrial diseases. Indeed, a few candidates such as bezafibrate, resveratrol and retinoic acid are already in use in experimental settings, but with contradictory results (reviewed in Reference 184).

4 | CONCLUSIONS

Progress is being made in our ability to generate mammalian models with pathogenic mtDNA mutations. To date, the mouse models of mtDNA disease have either been generated by introducing pathogenic mtDNA mutations within mitochondria into mouse embryos, introducing them into ES cells which are implanted into embryos, or by the generation and selection of mtDNA mutations produced by mtDNA mutator mice. To date, one striking difference between the mouse models and patients with mtDNA mutations has been a consistent trend towards a less severe phenotype in the mice. As mentioned earlier, some of the nuclear gene mouse models have also shown a rather mild phenotype in comparison to the human patients they were meant to model, such as the SURF1 knockout or the TWINKLE deleter mice. The disease phenotypes observed in the NDUFS4 knockout model of Leigh Syndrome does show that mice can, under the correct conditions, recapitulate human disease to some degree.

While some of this is likely due to differences in tissue specific metabolic demands between the species, another source of this variation may be the mechanism of mitochondrial biogenesis. The relaxed replication model of mtDNA, with its clonal expansion and vegetative segregation, is thought to lead to strong cell-by-cell mosaicism with respect to mtDNA mutations. While the human and mouse embryos do not start out with vastly different numbers of mtDNA inherited in the oocyte, humans are regarded to have substantially more cells. For instance, a human brain is estimated to contain ~1200 times more neuronal cells than that of a mouse. This drastically expanded amount of cell division and mitochondrial replication in human development could lead to much greater variation in the relative levels of the mtDNA mutation in a largely stochastic system. With more cells sampling these effects, a human will be far more likely to have cells that cross the mitochondrial threshold effect. This in turn is hypothesized to induce compensatory biogenesis mechanisms to maintain the cell’s mitochondrial function. However, this corrective function will eventually become self-defeating as the wild-type mtDNA molecules to copy become more rare, and thus eventually fail to compensate for the disrupted mitochondrial function. This phenomenon has been observed in patients with mtDNA disease. Thus, the smaller size and cell content of a mouse may inherently protect the mouse from some of the severe consequences of mtDNA disease.

A similar trend has been noted in aging studies with mouse models of mitochondrial dysfunction, as mice normally show little COX deficiency in their colons at the end of their life, a phenomenon quite common in aged humans. Yet heterozygous mtDNA mutator mice, with their ×10 increase in the mtDNA mutation load, do recapitulate the aged human levels of colonic COX deficiency in advanced age. Perhaps mouse models of human mtDNA diseases will require the use of compounded mutations within a mitochondrial pathway, such as the combined ANT1ko/ko, mt-ND6(P25L) or the mt-Cytb (D245N); Bcs1ko models, if a strong pathological phenotype is required.

Besides these current methods to generate the mouse models, we hope to see an expansion of other methods to generate these models. The use of mtDNA mutator cell lines, coupled to existing embryo manipulation techniques holds promise. More recently, targeted genome editing, such as that promised by the DdCBEs, will undoubtedly garner interest and hopefully lead to the generation of more models, again by manipulation of cells in culture, movement to ES cells, and then implantation into embryos. Direct delivery into the mouse may be more efficient, but germline transfection by AAVs are still not widely available. Finally, work into other base editor domains with other mutational signatures will greatly help in our ability to specifically mutate the mtDNA to both generate and potentially heal our animal
models, with the eventual goal of finding curative therapies to help the human patients suffering from these diseases.

ACKNOWLEDGMENTS
The author would like to acknowledge the past and ongoing collaborations with the Chinnery, Greaves, Larsson, Minczuk, and Moraes groups. The author would like to thank Nina Bonekamp and Laila Singh for their helpful comments on this manuscript. The author has received Research Group support from the Max Planck Society, funding from the United Mitochondrial Disease Foundation (13-053R), and support by a Marie-Sklodowska-Curie ITN European Training Network, “REMIX” (EU 721757). The author confirms independence from the funding sources and from Max Planck Innovation; the content of the article has not been influenced by the sponsors. Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST
The author is a named inventor for corporate licensing agreements offered by Max Planck Innovation, for commercial use of the mt-TA C5024T mouse mentioned in the review.

AUTHOR CONTRIBUTION
James Bruce Stewart is the sole author of the manuscript.

ETHICS STATEMENT
All reviewed animal work carried out in the author’s laboratory was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen in accordance with German and European Union regulations (Permits 84-02.042015.A103; 84-02.05.50.15.004).

ORCID
James Bruce Stewart  https://orcid.org/0000-0002-2902-4968

REFERENCES
1. Chinnery PF. Mitochondrial disorders overview. In: Adam MP, Ardinger HH, Pagon RA, et al., eds. GeneReviews ((R)). Seattle, WA: University of Washington; 1993.
2. Gorman GS, Chinnery PF, DiMauro S, et al. Mitochondrial diseases. Nat Rev Dis Primers. 2016;2(1):16080.
3. Calvo SE, Clauser KR, Mootha VK. MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. Nucleic Acids Res. 2016;44(D1):D1251-D1257.
4. Gray MW, Mootha VK. Evolutionary mitochondrial biology in titisee. IUBMB Life. 2018;70(12):1184-1187.
5. Greber BJ, Ban N. Structure and function of the mitochondrial ribosome. Annu Rev Biochem. 2016;85(1):103-132.
6. Watanabe K. Unique features of animal mitochondrial translation systems. The non-universal genetic code, unusual features of the translational apparatus and their relevance to human mitochondrial diseases. Proc Jpn Acad Ser B: Phys Biol Sci. 2010;86(1):11-39.
7. Burger G, Gray MW, Forget L, Lang BF. Strikingly bacterial-like and gene-rich mitochondrial genomes throughout jakobid protists. Genome Biol Evol. 2013;5(2):418-438.
8. Greber BJ, Bieri P, Leibundgut M, et al. Ribosome. The complete structure of the 55S mammalian mitochondrial ribosome. Science. 2015;348(6232):303-308.
9. Holzmann J, Frank F, Loffler E, Bennett KL, Gerner C, Rossmanith W. RNA Korean Russian: identification and functional reconstitution of the human mitochondrial tRNA processing enzyme. Cell. 2008;135(3):462-474.
10. Holt IJ, Harding AE, Morgan-Hughes JA. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature. 1988;331(6158):717-719.
11. Wallace DC, Singh G, Lott MT, et al. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science. 1988;242(4884):1427-1430.
12. Stewart JB, Chinnery PF. The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. Nat Rev Genet. 2015;16(9):530-542.
13. Rossignol R, Faustin B, Rocher C, Malgat M, Mazat JP, Letellier T. Mitochondrial threshold effects. Biochem J. 2003;370(Pt 3):751-762.
14. Gorman GS, Schaefer AM, Ng Y, et al. Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. Ann Neurol. 2015;77(5):753-759.
15. Vaquer G, Riviere F, Mavris M, et al. Animal models for metabolic, neuromuscular and ophthalmological rare diseases. Nat Rev Drug Discov. 2013;12(4):287-305.
16. Ylikallio E, Tyynismaa H. Generating mouse models of mitochondrial disease. In: LeDouxf MS, ed. Movement Disorders. Boston, MA: Academic Press; 2015:689-701.
17. Ruzzenente B, Rotig A, Metodiev MD. Mouse models for mitochondrial diseases. Hum Mol Genet. 2016;25(R2):R115-R122.
18. Wallace DC, Fan W. The pathophysiology of mitochondrial disease as modeled in the mouse. Genes Dev. 2009;23(15):1714-1736.
19. Torrance A, Diaz F, Vempati UD, Moraes CT. Mouse models of oxidative phosphorylation defects: powerful tools to study the pathobiology of mitochondrial diseases. Biochim Biophys Acta. 2009;1793(1):171-180.
20. Vempati UD, Torrance A, Moraes CT. Mouse models of oxidative phosphorylation dysfunction and disease. Methods. 2008; 46(4):241-247.
21. Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. Nat Biotechnol. 2020;38(7):824-844.
22. Hedges SB, Marin J, Suleski M, Paymer M, Kumar S. Tree of life reveals clock-like speciation and diversification. Mol Biol Evol. 2015;32(4):835-845.
23. Kruse SE, Watt WC, Marcinek DJ, Kapur RP, Schenkman KA, Palmeter RD. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. Cell Metab. 2008;7(4):312-320.
24. Ferrari M, Jain IH, Goldberger O, et al. Hypoxia treatment reverses neurodegenerative disease in a mouse model of Leigh syndrome. Proc Natl Acad Sci U S A. 2017;114(21):E4241-E4250.
25. McElroy GS, Recek CR, Reyfman PA, Mithal DS, Horbinski CM, Chandel NS. NAD+ regeneration rescues lifespan, but not ataxia, in a mouse model of brain mitochondri
dysfunction. Cell Metab. 2020;32(2):301-308.e6.
26. Silva-Pinheiro P, Cerutti R, Luna-Sanchez M, Zeviani M, Visconi C. A single intravenous injection of AAV-PHP.B-hNDUFS4 ameliorates the phenotype of Ndufs4 (−/−) mice. Mol Ther Methods Clin Dev. 2020;17:1071-1078.
27. Reynaud-Dulaurier R, Benegiamo G, Marrocco E, et al. Gene replacement therapy provides benefit in an adult mouse model of Leigh syndrome. Brain. 2020;143(6):1686-1696.
28. van Riesen AK, Antonicka H, Ohlenbusch A, Shoubridge EA, Wilichowski EK. Maternal segmental disomy in Leigh syn
drome with cytochrome c oxidase deficiency caused by homozygous SURF1 mutation. Neuropediatrics. 2006;37(2):88-94.
29. Verma IC, Bijarnia S, Saxena R, et al. Leber’s hereditary optic neuropathy with molecular characterization in two Indian families. Indian J Ophthalmol. 2005;53(3):167-171.
30. Dell’agnello C, Leo S, Agostino A, et al. Increased longevity and refractoriness to Ca(2+)-dependent neurodegeneration in Surf1 knockout mice. Hum Mol Genet. 2007;16(4):431-444.
31. Simard ML, Mourier A, Greaves LC, Taylor RW, Stewart JB. A novel histochemistry assay to assess and quantify focal cytochrome c oxidase deficiency. J Pathol. 2018;245(3):311-323.
32. Pulliam DA, Deepa SS, Liu Y, et al. Complex IV-deficient Surf1(−/−) mice initiate mitochondrial stress responses. Biochim J. 2014;462(2):359-371.
33. Quadrali C, Brunetti D, Lagutina I, et al. SURF1 knockout cloned pigs: early onset of a severe lethal phenotype. Biochim Biophys Acta Mol Basis Dis. 2018;1864(6 Pt A):2131-2142.
34. Chen Z, Zhang F, Xu H. Human mitochondrial DNA diseases and Drosophila models. J Genet Genomics. 2019;46(4):201-212.
35. Sen A, Cox RT. Fly models of human diseases: drosophila as a model for understanding human mitochondrial mutations and disease. Curr Top Dev Biol. 2017;121(1):1-27.
36. van der Blik AM, Sedensky MM, Morgan PG. Cell biology of the mitochondrion. Genetics. 2017;207(3):843-871.
37. Chiang AC, McCartney E, O’Farrell PH, Ma H. A genome-wide screen reveals that reducing mitochondrial DNA poly
merase can promote elimination of deleterious mitochondrial mutations. Curr Biol. 2019;29(24):4330-4336.e4333.
38. Kwon YJ, Guha S, Tuluč F, Falk MJ. High-throughput BioSorter quantification of relative mitochondrial content and membrane potential in living Caenorhabditis elegans. Mitochon
drion. 2018;40:42-50.
39. Foriel S, Renkema GH, Lasarzewski Y, et al. A drosophila mitochondrial complex I deficiency phenotype Array. Front Genet. 2019;10:245.
40. Ma H, O’Farrell PH. Selections that isolate recombinant mitochon
drial genomes in animals. eLife. 2015;4:e07247.
41. Xu H, DeLuca SZ, O’Farrell PH. Manipulating the metazoan mitochondrial genome with targeted restriction enzymes. Sci
cence. 2008;321(5888):575-577.
42. Otten ABC, Kamps R, Lindsey P, et al. Tfam knockdown results in reduction of mtDNA copy number, OXPHOS defici
cy and abnormalities in Zebrafish embryos. Front Cell Dev Biol. 2020;8:381.
43. Otten AB, Theunissen TE, Derhaag JG, et al. Differences in strength and timing of the mtDNA bottleneck between zebrafish germline and non-germline cells. Cell Reports. 2016;16(3):622-630.
44. Steele SL, Prykhozhij SV, Berman JN. Zebrafish as a model system for mitochondrial biology and diseases. Transl Res. 2014;163(2):79-98.
45. Otten AB, Stassen AP, Adriaens M, et al. Replication errors made during oogenesis lead to detectable de novo mtDNA mutations in zebrafish oocytes with a low mtDNA copy num
ber. Genetics. 2016;204(4):1423-1431.
46. Staudinger M, Kempfen F. Electroporation of isolated higher
plant mitochondria: transcripts of an introduced cox2 gene, but not an atp6 gene, are edited in organello. Mol Genet Genomics. 2003;269(4):553-561.
47. Mileshina D, Koulintchenko M, Konstantinov Y, Dietrich A. Transfection of plant mitochondria and in organello gene integration. Nucleic Acids Res. 2011;39(17):e115.
48. Bonnefoy N, Remacle C, Fox TD. Genetic transformation of Saccharomyces cerevisiae and Chlamydomonas reinhardtii mitochon
dria. Methods Cell Biol. 2007;80:525-548.
49. Hayashi J, Ohta S, Kikuchi A, Takemitsu M, Goto Y, Nonaka I. Introduction of disease-related mitochondrial DNA de
letions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. Proc Natl Acad Sci U S A. 1991;88(23):10614-10618.
50. Hayashi JI, Murakami J. Identification of cytoplasmically transferred mitochondrial-DNA in female germlines of drosophila and its propagation in the progeny. Mol Gen Genet. 1988;211(3):381-385.
51. Jenuth JP, Peterson AC, Fu K, Shoubridge EA. Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. Nat Genet. 1996;14(2):146-151.
52. Meirelles FV, Smith LC. Mitochondrial genotype segregation in a mouse heteroplasmic lineage produced by embryonic karyoplast transplantation. Genetics. 1997;145(2):445-451.
53. Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G. Mechanisms of cytochrome c release from mitochon
dria. Cell Death Differ. 2006;13(9):1423-1433.
54. King MP, Attardi G. Human cells lacking mtDNA: therapy for mutant mitochondria by delivering mitochondrial
mammalian cells. Transfection of plant mitochondria and in organello gene integration. Nucleic Acids Res. 2011;39(17):e115.
55. Bonnefoy N, Remacle C, Fox TD. Genetic transformation of Saccharomyces cerevisiae and Chlamydomonas reinhardtii mitochon
dria. Methods Cell Biol. 2007;80:525-548.
56. King MP, Attardi G. Human cells lacking mtDNA: therapy for mutant mitochondria by delivering mitochondrial
mammalian cells. Transfection of plant mitochondria and in organello gene integration. Nucleic Acids Res. 2011;39(17):e115.
57. Yoon YG, Koob MD. Transferring isolated mitochondria into tissue culture cells. Nucleic Acids Res. 2012;40(19):e148.
58. Wu TH, Sagullo E, Case D, et al. Mitochondrial transfer by photothermal nanoblade restores metabolite profile in mammalian cells. Cell Metab. 2016;23(5):921-929.
59. Kawamura E, Maruyama M, Abe J, et al. Validation of gene therapy for mutant mitochondria by delivering mitochondrial
RNA using a MITO-porter. Mol Ther Nucleic Acids. 2020;20:687-698.
60. Hagstrom E, Freyer C, Battersby BJ, Stewart JB, Larsson NG. No recombination of mtDNA after heteroplasmy for 50 gener
ations in the mouse maternal germline. Nucleic Acids Res. 2014;42(2):1111-1116.
61. Sato A, Nakada K, Akimoto M, et al. Rare creation of recombinant mtDNA haplotypes in mammalian tissues. *Proc Natl Acad Sci U S A.* 2005;102(17):6057-6062.

62. Srivastava S, Moraes CT. Double-strand breaks of mouse muscle mtDNA promote large deletions similar to multiple mtDNA deletions in humans. *Hum Mol Genet.* 2005;14(7):893-902.

63. Bayona-Bafaluy MP, Blits B, Battersby BJ, Shoubridge EA, Moraes CT. Rapid directional shift of mitochondrial DNA heteroplasmy in animal tissues by a mitochondrially targeted restriction endonuclease. *Proc Natl Acad Sci U S A.* 2005;102(40):14392-14397.

64. Alexeyev MF, Venediktova N, Pastukh V, Shokolenko I, Bonilla G, Wilson GL. Selective elimination of mutant mitochondrial genomes as therapeutic strategy for the treatment of NARP and MILS syndromes. *Gene Ther.* 2008;15(7):516-523.

65. Gammage PA, Viscomi C, Simard ML, et al. Genome editing in mitochondria corrects a pathogenic mtDNA mutation in vivo. *Nat Med.* 2018;24(11):1691-1695.

66. Bacman SR, Kauppila JHK, Pereira CV, et al. MitoTALEN reduces mutant mtDNA load and restores tRNA(Ala) levels in a mouse model of heteroplasmatic mtDNA mutation. *Nat Med.* 2018;24(11):1696-1700.

67. Pereira CV, Bacman SR, Arguello T, et al. mitoTev-TALE: a monomeric DNA editing enzyme to reduce mutant mitochondrial DNA levels. *EMBO Mol Med.* 2018;10(9):e8084.

68. Antón Z, Mullally G, Ford HC, van der Kamp MW, Szczelkun MD, Lane JD. Mitochondrial import, health and mtDNA copy number variability seen when using type II and type V CRISPR effectors. *J Cell Sci.* 2020;133(18):jcsc248468.

69. Hoogewijs K, James AM, Murphy MP, Lightowlers RN. Signed-for delivery in the mitochondrial matrix: confirming uptake into mitochondria. *Small Methods.* 2018;2(3):1700297.

70. Jo A, Ham S, Lee GH, et al. Efficient mitochondrial genome editing by CRISPR/Cas9. *BioMed Res Int.* 2015;2015:305716.

71. Sundaresan R, Parmeshwaran HP, Yosgha SD, Keilbarth MW, Rajan R. RNA-independent DNA cleavage activities of Cas9 and Cas12a. *Cell Rep.* 2017;21(13):3728-3739.

72. Yeh CD, Richardson CD, Corn JE. Advances in genome editing through control of DNA repair pathways. *Nat Cell Biol.* 2019;21(12):1468-1478.

73. Moreton A, Morel F, Macao B, et al. Selective mitochondrial DNA degradation following double-strand breaks. *PLoS One.* 2017;12(4):e0176795.

74. Fukui H, Moraes CT. Mechanisms of formation and accumulation of mitochondrial DNA deletions in aging neurons. *Hum Mol Genet.* 2009;18(6):1028-1036.

75. Mok BY, de Moraes MH, Zeng J, et al. A bacterial cytidine deaminase toxin enables CRISPR-free mitochondrial base editing. *Nature.* 2020;583(7817):631-637.

76. Andreazza S, Samstag CL, Sanchez-Martinez A, et al. Mitochondrially-targeted APOBEC1 is a potent mtDNA mutator affecting mitochondrial function and organisal fitness in drosophila. *Nat Commun.* 2019;10(1):3280.

77. Minczuk M, Papworth MA, Kolasinska P, Murphy MP, Klug A. Sequence-specific modification of mitochondrial DNA using a chimeric zinc finger methylase. *Proc Natl Acad Sci U S A.* 2006;103(52):19689-19694.

78. Hatakeyama H, Goto Y-I. Concise review: heteroplasmic mitochondrial DNA mutations and mitochondrial diseases: toward iPSC-based disease modeling, drug discovery, and regenerative therapeutics. *Stem Cells.* 2016;34(4):801-808.

79. Sison SL, Vermilyea SC, Emborg ME, Ebert AD. Using patient-derived induced pluripotent stem cells to identify Parkinson’s disease-relevant phenotypes. *Curr Neurol Neurosci Rep.* 2018;18(12):84.

80. Kang E, Wang X, Tippner-Hedges R, et al. Age-related accumulation of somatic mitochondrial DNA mutations in adult-derived human iPSCs. *Cell Stem Cell.* 2016;18(5):625-636.

81. Perales-Clemente E, Cook AN, Evans JM, et al. Natural underlying mtDNA heteroplasy as a potential source of intra-person hiPSC variability. *EMBO J.* 2016;35(18):1979-1990.

82. Lott MT, Leipzig JN, Derbeneva O, et al. mtDNA variation and analysis using mitomap and mitomaster. *Curr Protoc Bioinform.* 2013;44(1):1.23.1-1.23.26.

83. Putz J, Dupuis B, Sissler M, Florentz C. Mamit-tRNA, a database of mammalian mitochondrial tRNA primary and secondary structures. *RNA.* 2007;13(8):1184-1190.

84. Johnston IG, Williams BP. Evolutionary inference across eukaryotes identifies specific pressures favoring mitochondrial gene retention. *Cell Syst.* 2016;2(2):101-111.

85. Allen JF. The CoRR hypothesis for genes in organelles. *J Theor Biol.* 2017;434:50-57.

86. Dunn DA, Pinkert CA. Nuclear expression of a mitochondrial DNA gene: mitochondrial targeting of allotopically expressed mutant ATP6 in transgenic mice. *J Biomed Biotechnol.* 2012;2012:541245.

87. Qi X, Sun L, Lewin AS, Hauswirth WW, Guy J. The mutant human ND4 subunit of complex I induces optic neuropathy in the mouse. *Invest Ophthalmol Vis Sci.* 2007;48(1):1-10.

88. Ellouze S, Augustin S, Bouaita A, et al. Optimized allotopic expression of the human mitochondrial ND4 prevents blindness in a rat model of mitochondrial dysfunction. *Am J Hum Genet.* 2008;83(3):373-387.

89. Cwerman-Thibault H, Augustin S, Lechaue C, et al. Nuclear expression of mitochondrial ND4 leads to the protein assembling in complex I and prevents optic atrophy and visual loss. *Mol Ther Methods Clin Dev.* 2015;2:15003.

90. Lam BL, Feuer WJ, Abukhalil F, Porciatti V, Hauswirth WW, Guy J. Leber hereditary optic neuropathy gene therapy clinical trial recruitment: year 1. *Arch Ophthalmol.* 1998;273(23):14210-14217.

91. Barrientos A, Kenyon L, Moraes CT. Human xenomitochondrial fusion between mouse and human mitochondria is rapid and efficient. *Mitochondrion.* 2007;7(3):223-229.

92. Bayona-Bafaluy MP, Muller S, Moraes CT. Fast adaptive coevolution of nuclear and mitochondrial subunits of ATP synthetase in orangutan. *Mol Biol Evol.* 2005;22(3):716-724.

93. Werren JH, Richards S, Desjardins CA, et al. Functional and evolutionary insights from the genomes of three parasitoid Nasonia species. *Science.* 2010;327(5963):343-348.

94. Hill GE. Mitonuclear compensatory coevolution. *Trends Genet.* 2020;36(6):403-414.
96. Perales-Clemente E, Fernandez-Silva P, Acin-Perez R, Perez-Martos A, Enríquez JA. Allotopic expression of mitochondrial-encoded genes in mammals: achieved goal, undemonstrated mechanism or impossible task? *Nucleic Acids Res.* 2011;39(1):225-234.

97. Figueroa-Martínez F, Vázquez-Acevedo M, Cortés-Hernández P, et al. What limits the allotopic expression of nucleus-encoded mitochondrial genes? The case of the chimeric Cox3 and Atp6 genes. *Mitochondrion.* 2011;11(1):147-154.

98. Oca-Cossío J, Kenyon L, Hao H, Moraes CT. Limitations of allotopic expression of mitochondrial genes in mammalian cells. *Genetics.* 2003;165(2):707-720.

99. Brown A, Amunts A, Bai XC, et al. Structure of the large ribosomal subunit from human mitochondria. *Science.* 2014;346(6210):718-722.

100. Ott M, Herrmann JM. Co-translational membrane insertion of mitochondrially encoded proteins. *Biochim Biophys Acta.* 2010;1803(6):767-775.

101. Fan W, Waymire KG, Narula N, et al. A mouse model of mitochondrial disease reveals germline selection against severe mtDNA mutations. *Science.* 2008;319(5865):958-962.

102. Shimizu A, Mito T, Hayashi C, et al. Transmitochondrial mice as models for primary prevention of diseases caused by mutation in the tRNA(Lys) gene. *Proc Natl Acad Sci U S A.* 2014;111(8):3104-3109.

103. Inoue K, Nakada K, Ogura N, et al. Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. *Nat Genet.* 2000;26(2):176-181.

104. Moreno-Loshuertos R, Ferrin G, Acin-Perez R, et al. Evolution meets disease: penetrance and functional epistasis of mitochondrial RNA mutations. *PLoS Genet.* 2011;7(4):e1001379.

105. Bayona-Bafaluy MP, Movilla N, Perez-Martos A, Fernandez-Silva P, Enríquez JA. Functional genetic analysis of the mammalian mitochondrial DNA encoded peptides: a mutagenesis approach. *Methods Mol Biol.* 2008;457:379-390.

106. Lin CS, Sharpley MS, Fan W, et al. Mouse mtDNA mutant model of Leber hereditary optic neuropathy. *Proc Natl Acad Sci U S A.* 2012;109(49):20065-20070.

107. Valente WJ, Ericson NG, Long AS, White PA, Marchetti F, Bielas JH. Mitochondrial DNA exhibits resistance to induced point and deletion mutations. *Nucleic Acids Res.* 2016;44(18):8513-8524.

108. Trifunovic A, Wredenberg A, Falkenberg M, et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature.* 2004;429(6990):417-423.

109. Kijóth GC, Hiona A, Pugh TD, et al. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science.* 2005;309(5733):481-484.

110. Mito T, Kikkawa Y, Shimizu A, et al. Mitochondrial DNA mutations in mutator mice confer respiration deficits and B-cell lymphoma development. *PLoS One.* 2013;8(2):e55789.

111. Enríquez JA. Mind your mouse strain. *Nat Metab.* 2019;1(1):5-7.

112. Stewart JB, Freyer C, Elson JL, et al. Strong purifying selection in transmission of mammalian mitochondrial DNA. *PLoS Biol.* 2008;6(1):e10.

113. Bailey LJ, Cluett TJ, Reyes A, et al. Mice expressing an error-prone DNA polymerase in mitochondria display elevated replication pausing and chromosomal breakage at fragile sites of mitochondrial DNA. *Nucleic Acids Res.* 2009;37(7):2327-2335.

114. Pevea V, Blei D, Trombly G, et al. Linear mitochondrial DNA is rapidly degraded by components of the replication machinery. *Nat Commun.* 2018;9(1):1727.

115. Nissanka N, Bacman SR, Plastini MJ, Moraes CT. The mitochondrial DNA polymerase gamma degrades linear DNA fragments precluding the formation of deletions. *Nat Commun.* 2018;9(1):2491.

116. Bratic A, Kauppila TE, Macao B, et al. Complementation between polymerase- and exonuclease-deficient mitochondrial DNA polymerase mutants in genomically engineered flies. *Nat Commun.* 2015;6:8808.

117. Nicholls TJ, Zsurka G, Pevea V, et al. Linear mtDNA fragments and unusual mtDNA rearrangements associated with pathological deficiency of MGME1 exonuclease. *Hum Mol Genet.* 2014;23(23):6147-6162.

118. Matic S, Jiang M, Nicholls TJ, et al. Mice lacking the mitochondrial exonuclease MGME1 accumulate mtDNA deletions without developing progeria. *Nat Commun.* 2018;9(1):1202.

119. Medeiros TC, Thomas RL, Ghillebert R, Graef M. Autophagy balances mtDNA synthesis and degradation by DNA polymerase POLG during starvation. *J Cell Biol.* 2018;217(5):1601-1611.

120. Williams SL, Huang J, Edwards YJ, et al. The mtDNA mutation spectrum of the progeroid Polg mutator mouse includes abundant control region multimers. *Cell Metab.* 2010;12(6):675-682.

121. Macao B, Uhler JP, Siibak T, et al. The exonuclease activity of DNA polymerase gamma is required for ligation during mitochondrial DNA replication. *Nat Commun.* 2015;6:7303.

122. Vermulst M, Wanagat J, Kujoth GC, et al. DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice. *Nat Genet.* 2008;40(4):392-394.

123. Tynimsmaa H, Mjosund KP, Wanrooij S, et al. Mutant mitochondrial helicase Twinkle causes multiple mtDNA deletions and a late-onset mitochondrial disease in mice. *Proc Natl Acad Sci U S A.* 2005;102(49):17687-17692.

124. Baris OR, Enderer S, Neuhaus JF, et al. Mosaic deficiency in mitochondrial oxidative metabolism promotes cardiac arrhythmia during aging. *Cell Metab.* 2015;21(5):667-677.

125. Fayzulin RZ, Perez M, Kozhukhar N, Spadafora D, Wilson GL, Alexeyev MF. A method for mutagenesis of mouse mtDNA and a resource of mouse mtDNA mutations for modeling human pathological conditions. *Nucleic Acids Res.* 2015;43(9):e62.

126. Ishikawa K, Kobayashi K, Yamada A, Umehara M, Oka T, Nakada K. Concentration of mitochondrial DNA mutations precluding the formation of deletions. *Cell Metab.* 2018;9(1):2491.

127. Watanabe T, Dewey MJ, Mintz B. Teratocarcinoma cells as vehicles for introducing specific mutant mitochondrial genes into mice. *Proc Natl Acad Sci U S A.* 1978;75(10):5113-5117.

128. Sligh JE, Levy SE, Waymire KG, et al. Maternal germ-line transmission of mutant mtDNAs from embryonic stem cell-derived chimeric mice. *Proc Natl Acad Sci U S A.* 2000;97(26):14461-14466.
129. Levy SE, Waymire KG, Kim YL, MacGregor GR, Wallace DC. Transfer of chloramphenicol-resistant mitochondrial DNA into the chimeric mouse. Transgenic Res. 1999;8(2):137-145.

130. Marchington DR, Barlow D, Poulton J. Transmitochondrial mice carrying resistance to chloramphenicol on mitochondrial DNA: developing the first mouse model of mitochondrial DNA disease. Nat Med. 1999;5(8):957-960.

131. Meirelles FV, Smith LC. Mitochondrial genotype segregation during preimplantation development in mouse heteroplasmic embryos. Genetics. 1998;148(2):877-883.

132. Nishiyama H, Kanda H, Sato A, et al. Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. Genetics. 2000;156(3):1277-1284.

133. Irwin MH, Johnson LW, Pinkert CA. Isolation and microinjection of somatic cell-derived mitochondria and germline heteroplasmy in transmitochondrial mice. Transgenic Res. 1999;8(2):119-123.

134. Pinkert CA, Irwin MH, Johnson LW, Moffatt RJ. Mitochondria transfer into mouse ova by microinjection. Transgenic Res. 1997;6(6):379-383.

135. Wallace DC. Mitochondrial diseases in man and mouse. Science. 1999;283(5407):1482-1488.

136. Capecchi MR. The new mouse genetics: altering the genome by gene targeting. Trends Genet. 1989;5(3):70-76.

137. Kasahara A, Ishikawa K, Yamaoka M, et al. Generation of multiple mtDNA mutants and intermolecular recombination during clonal expansion of somatic cell-derived mitochondria. Hum Mol Genet. 2000;9(16):871-881.

138. Hashizume O, Shimizu A, Yokota M, et al. Specific mitochondrial DNA mutation in mice regulates diabetes and lymphoma development. Proc Natl Acad Sci U S A. 2012;109(26):10528-10533.

139. Fan W, Lin CS, Potluri P, Procaccio V, Wallace DC. mtDNA lineage analysis of mouse L-cell lines reveals the accumulation of multiple mtDNA mutants and intermolecular recombination. Genes Dev. 2012;26(4):384-394.

140. Yokota M, Shitara H, Hashizume O, et al. Generation of trans-mitochondrial Mito-mice by the introduction of a pathogenic G13997A mtDNA from highly metastatic lung carcinoma cells. FEBS Lett. 2010;584(18):3943-3948.

141. Malfatti E, Bugiani M, Invernizzi F, et al. Novel mutations of ND genes in complex I deficiency associated with mitochondrial encephalopathy. Brain. 2007;130(Pt 7):1894-1904.

142. Valente L, Piga D, Lamantea E, et al. Identification of novel mutations in five patients with mitochondrial encephalomyopathy. Biochim Biophys Acta. 2009;1787(5):491-501.

143. Shimizu A, Mito T, Hashizume O, et al. G7731A mutation in mouse mitochondrial tRNALys regulates late-onset disorders in transmitochondrial mice. Biochim Biophys Res Commun. 2015;459(1):66-70.

144. McManus MJ, Picard M, Chen HW, et al. Mitochondrial DNA variation dictates expressivity and progression of nuclear DNA mutations causing cardiomyopathy. Cell Metab. 2019;29(1):78-90 e75.

145. Purhonen J, Grigorjev V, Eikert R, et al. A spontaneous mitonuclear epistasis converging on Rieske Fe-S protein exacerbates complex III deficiency in mice. Nat Commun. 2020;11(1):322.

146. Blakely EL, Swalwell H, Petty RK, McFarland R, Turnbull DM, Taylor RW. Sporadic myopathy and exercise intolerance associated with the mitochondrial 8328G>A tRNALys mutation. J Neurol. 2007;254(9):1283-1285.

147. Houshmand M, Lindberg C, Moslemi A-R, Oldfors A, Holme E. A novel heteroplasmic point mutation in the mitochondrial tRNALys gene in a sporadic case of mitochondrial encephalomyopathy: de novo mutation and no transmission to the offspring. Human Mutation. 1999;13(3):203-209.

148. Freyer C, Cree LM, Mourier A, et al. Variation in germline mtDNA heteroplasmy is determined prenatally but modified during subsequent transmission. Nat Genet. 2012;44(11):1282-1285.

149. Ross JM, Stewart JB, Hagstrom E, et al. Germline mitochondrial DNA mutations aggravate ageing and can impair brain development. Nature. 2013;501(7467):412-415.

150. Ross JM, Coppotelli G, Hoffer BJ, Olson L. Maternally transmitted mitochondrial DNA mutations can reduce lifespan. Sci Rep. 2014;4:6569.

151. Baines HL, Stewart JB, Stamp C, et al. Similar patterns of clonally expanded somatic mtDNA mutations in the colon of heterozygous mtDNA mutator mice and ageing humans. Mech Ageing Dev. 2014;139:22-30.

152. Kauppila JHK, Baines HL, Bratic A, et al. A phenotype-driven approach to generate mouse models with pathogenic mtDNA mutations causing mitochondrial disease. Cell Rep. 2016;16(11):2980-2990.

153. McFarland R, Swalwell H, Blakely EL, et al. The m.5650G>A mitochondrial tRNAlys mutation is pathogenic and causes a phenotype of pure myopathy. Neuromuscul Disord. 2008;18(1):63-67.

154. Horvath R, Lochmuller H, Scharfe C, et al. A tRNA(Ala) mutation causing mitochondrial myopathy clinically resembling myotonic dystrophy. J Med Genet. 2003;40(10):752-757.

155. Filograna R, Koolmeister C, Upadhyay M, et al. Modulation of mtDNA copy number ameliorates the pathological consequences of a heteroplasmic mtDNA mutation in the mouse. Sci Adv. 2019;5(4):eaav9824.

156. Kim SB, Berdanier CD. Oligomycin sensitivity of mitochondrial tRNAAla mutation is pathogenic and causes a phenotype of pure myopathy. Neurouromuscul Disord. 2008;18(1):63-67.

157. Mathews CE, McGraw RA, Berdanier CD. A point mutation in the mitochondrial DNA of diabetes-prone BHE/Cdb rats. Am J Physiol. 1999;277(4):E702-E707.

158. Breitschwerdt EB, Kornegay JN, Wheeler SJ, Stevens JB, Baty CJ. Episodic weakness associated with exertional lactic acidosis and myopathy in old English sheepdog littermates. J Am Vet Med Assoc. 1992;201(5):731-736.

159. Vijayasarathy C, Giger U, Prociuk U, Patterson DF, Breitschwerdt EB, Avadhani NG. Canine mitochondrial myopathy associated with reduced mitochondrial mRNA and altered cytochrome c oxidase activities in fibroblasts and skeletal muscle. Comp Biochem Physiol A Physiol. 1994;109(4):887-894.

160. Paciello O, Maiolino P, Fatone G, Papparella S. Mitochondrial myopathy in a german shepherd dog. Vet Pathol. 2003;40(5):507-511.
161. Li FY, Cuddon PA, Song J, et al. Canine spongiform leukoencephalomyelopathy is associated with a missense mutation in cytochrome b. *Neuropathol Dis*. 2006;21(1):35-42.

162. Baranowska I, Jaderlund KH, Nennesmo I, et al. Sensory ataxic neuropathy in golden retriever dogs is caused by a deletion in the mitochondrial tRNATyr gene. *PLoS Genet*. 2009;5(5):e1000499.

163. Jäderlund KH, Örvind E, Johnsson E, et al. A neurologic syndrome in golden retrievers presenting as a sensory ataxic neuropathy. *J Vet Intern Med*. 2007;21(6):1307-1315.

164. Bibb MJ, van Etten RA, Wright CT, Walberg MW, Clayton DA. Sequence and gene organization of mouse mitochondrial DNA. *Cell*. 1981;26(2 Pt 2):167-180.

165. Inoue K, Ito S, Takai D, et al. Isolation of mitochondrial DNA-less mouse cell lines and their application for trapping mouse synaptosomal mitochondrial DNA with deletion mutations. *J Biol Chem*. 1997;272(24):15510-15515.

166. Sato A, Kono T, Nakada K, et al. Gene therapy for progeny of Mito-mice carrying pathogenic mtDNA by nuclear transplantation. *Proc Natl Acad Sci U S A*. 2005;102(46):16765-16770.

167. Craven L, Tuppen HA, Greggains GD, et al. Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. *Nature*. 2010;465(7294):82-85.

168. Herbert M, Turnbull D. Progress in mitochondrial replacement therapies. *Nat Rev Mol Cell Biol*. 2018;19(2):71-72.

169. Zhang J, Liu H, Luo S, et al. Live birth derived from oocyte spindle transfer to prevent mitochondrial disease. *Reprod Biomed Online*. 2017;34(4):361-368.

170. Tachibana M, Sparman M, Sritanaudomchai H, et al. Mitochondrial gene replacement in primate offspring and embryonic stem cells. *Nature*. 2009;461(7262):367-372.

171. Bacman SR, Williams SL, Duan D, Moraes CT. Manipulation of mtDNA heteroplasmy in all striated muscles of newborn mice by AAV9-mediated delivery of a mitochondrial-targeted restriction endonuclease. *Gene Ther*. 2012;19(12):1101-1106.

172. Bacman SR, Williams SL, Garcia S, Moraes CT. Organ-specific shifts in mtDNA heteroplasmy following systemic delivery of a mitochondria-targeted restriction endonuclease. *Gene Ther*. 2010;17(6):713-720.

173. Reddy P, Ocampo A, Suzuki K, et al. Selective elimination of mitochondrial mutations in the germline by genome editing. *Cell*. 2015;161(3):459-469.

174. McCann BJ, Cox A, Gammage PA, Stewart JB, Zernicka-Goetz M, Minzuk M. Delivery of mtZFNs into early mouse embryos. In: Liu J, ed. *Zinc Finger Proteins: Methods and Protocols*. New York, NY: Springer New York; 2018:215-228.

175. Naeem MM, Maheshan R, Costford SR, et al. G-quadruplex-mediated reduction of a pathogenic mitochondrial heteroplasmy. *Hum Mol Genet*. 2019;28(19):3163-3174.

176. Chinnery PF, Samuels DC. Relaxed replication of mtDNA: a model with implications for the expression of disease. *Am J Hum Genet*. 1999;64(4):1158-1165.

177. Shi Y, Dierckx A, Wanrooij PH, et al. Mammalian transcription factor A is a core component of the mitochondrial transcription machinery. *Proc Natl Acad Sci U S A*. 2012;109(41):16510-16515.

178. Kaufman BA, Durisic N, Mativetsky JM, et al. The mitochondrial transcription factor TFAM coordinates the assembly of multiple DNA molecules into nucleoid-like structures. *Mol Biol Cell*. 2007;18(9):3225-3236.

179. Kukat C, Wurm CA, Spahr H, Falkenberg M, Larsson NG, Jakobs S. Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA. *Proc Natl Acad Sci U S A*. 2011;108(33):13534-13539.

180. Ekstrand MI, Falkenberg M, Rantanen A, et al. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum Mol Genet*. 2004;13(9):935-944.

181. Larsson NG, Wang J, Wilhelmsson H, et al. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet*. 1998;18(3):231-236.

182. Jiang M, Kauppila TES, Motori E, et al. Increased Total mtDNA copy number cures male infertility despite unaltered mtDNA mutation load. *Cell Metab*. 2017;26(2):429-436 e424.

183. Nishiyama S, Shitara H, Nakada K, et al. Over-expression of Tfam improves the mitochondrial disease phenotypes in a mouse model system. *Biochem Biophys Res Commun*. 2010;401(1):26-31.

184. Viscomi C, Zeviani M. Strategies for fighting mitochondrial diseases. *J Intern Med*. 2020;287(6):665-684.

185. Herculano-Houzel S, Mota B, Lent R. Cellular scaling rules for rodent brains. *Proc Natl Acad Sci U S A*. 2006;103(32):12138-12143.

186. Durham SE, Samuels DC, Cree LM, Chinnery PF. Normal levels of wild-type mitochondrial DNA maintain cytochrome c oxidase activity for two pathogenic mitochondrial DNA mutations but not for m.3243A>G. *Am J Hum Genet*. 2007;81(1):189-195.

187. Greaves LC, Barron MJ, Campbell-Shiel G, Kirkwood TB, Turnbull DM. Differences in the accumulation of mitochondrial defects with age in mice and humans. *Mech Ageing Dev*. 2011;132(11-12):588-591.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Stewart JB. Current progress with mammalian models of mitochondrial DNA disease. *J Inherit Metab Dis*. 2020;1–18. [https://doi.org/10.1002/jimd.12324](https://doi.org/10.1002/jimd.12324)