# A primer genetic toolkit for exploring mitochondrial biology and disease using zebrafish

Ankit Sabharwal[1], Jarryd M. Campbell[1,2], Zachary WareJoncas[1], Mark Wishman[1], Hirotaka Ata[1,2], Wiebin Liu[1.3], Noriko Ichino[1], Jake D. Bergren[1], Mark D. Urban[1], Rhianna Urban[1], Tanya L. Poshusta[1], Yonghe Ding[1,3], Xiaolei Xu[1,3], Karl J. Clark[1,2], Stephen C. Ekker[1,2*]

1. Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905, USA

2. Center for Clinical and Translational Sciences, Mayo Clinic, Rochester, MN 55905, USA

3. Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN 55905, USA

*Corresponding author: Ekker.Stephen@mayo.edu

## Keywords:

Mitochondria, Mitochondrial disorders, Zebrafish, gene editing,

TALEN, Gene Breaking Transposon

## Abstract

Mitochondria are a dynamic eukaryotic innovation that play diverse roles in biology and disease. The mitochondrial genome is remarkably conserved in all vertebrates, encoding the same 37 gene set and overall genomic structure ranging from 16,596 base pairs (bp) in the teleost zebrafish (*Danio rerio*) to 16,569 bp in humans. Mitochondrial disorders are amongst the most prevalent inherited diseases affecting roughly 1 in every 5000 individuals. Currently, few effective treatments exist for those with mitochondrial ailments, representing a major unmet patient need. Mitochondrial dysfunction is also implicated to be a common component of a wide variety of other human illnesses ranging from neurodegenerative disorders like Huntington's disease and Parkinson's disease to autoimmune illnesses such as multiple sclerosis and rheumatoid arthritis. The electron transport chain (ETC) component of mitochondria is critical for mitochondrial biology and defects can lead to many mitochondrial disease symptoms. Here we present a publicly available collection of genetic mutants created in highly conserved, nuclear-encoded mitochondrial genes in *Danio rerio*. The zebrafish system represents a potentially powerful new opportunity for the study of mitochondrial biology and disease due to the large number of orthologous genes shared with humans and the many advanced features of this model system from genetics to imaging. This collection includes 24 mutant lines in 18 different genes created by locus-specific gene editing to induce frameshift or splice acceptor mutations leading to predicted protein truncation during translation. Also included are 7 lines created by the random insertion of the gene-breaking transposon (GBT) protein trap cassette. All of these targeted mutant alleles truncate conserved domains of genes critical to the proper function of the ETC or genes that have been implicated in human mitochondrial disease. This collection is designed to accelerate the use of zebrafish to study of many different aspects of

mitochondrial function with the goal of widening our understanding of their role in biology and human disease.

## Introduction

Mitochondria are semi-autonomous organelles critical for eukaryotic cell function. The mitochondrial endosymbiotic genesis origin hypothesis proposes its evolution from an alpha proteobacterial ancestor, *Rickettsia prowazekii* [1,2] that were harnessed by a eukaryotic cell as the host billions of years ago [3]. The proteobacterium became a symbiote of the host cell, bringing with it a system for more efficient generation of cellular energy in the form of ATP.

During the course of evolution, the genetic material of the mitochondria underwent reductive expansion and was transferred in a retrograde fashion to the nuclear genome. The retrograde movement of genes from the mitochondria to its eukaryotic host paved the way for the mitochondria to specialize as energy production organelles rather than consuming energy repetitively replicating its own multi-copy genome. The mitochondrial-encoded genetic material at present is a vestige of the original proteobacterial genome [4–6] meaning despite having DNA of their own, mitochondria rely heavily on the nuclear genes for most of their functions.

Mitochondria have critical functions in metabolism, organ homeostasis, apoptosis and aging. They also play important but still largely mysterious roles in human pathology, as demonstrated by the enormous biological variation and diverse disorders in patients with mitochondrial disease [7–10]. Nearly every organ system can be compromised, but with highly variable and complex physiological and biochemical outcomes. Imaging and basic science of mitochondria showcase how this highly dynamic organelle responds differentially to extrinsic and intrinsic biological signals. However, understanding how mitochondria function in normal biology, and how human mitochondrial DNA variations contribute to health and disease, has been hampered by a lack of effective approaches to manipulate the powerhouse of the cell.

The vertebrate mitochondrial chromosome is circular and includes 37 genes, 13 encoding for protein subunits of the electron transport chain, 22 coding for transfer RNAs, and 2 encoding ribosomal RNAs **(Figure 1)** [11,12]. The mitochondrial gene order, strand specific nucleotide bias and codon usage is highly conserved [13]. However, mtDNA encoded genes lack introns and utilize a divergent genetic code than their nuclear counterparts [14,15]. For instance, AUA codon codes for methionine as per mitochondrial genetic code, whereas the same sequence codes for isoleucine in the nuclear genetic code. Similarly, nuclear stop codon UGA is read as the tryptophan amino acid by the mitochondrial codon cypher.

Mitochondria are unique cellular compartments with different DNA and RNA repair and editing rules, hampering attempts at directly manipulating these nucleic acid components. For example, DNA nucleases that introduce double stranded breaks and subsequent repair in nuclear DNA induce the degradation of mtDNA [16–18]. Indeed, none of the canonical DNA repair pathways found in the nucleus have been shown to be active in mitochondria [18–20]. Finally, no system has demonstrated the ability to deliver exogenous DNA or RNA to mitochondria, restricting the tools available for mtDNA editing [21,22]. All of these factors are distinct from the nuclear genome, making mtDNA a far less accessible genome for traditional gene editing methods and reagents.

The diverse functions that mitochondria are capable of, including oxidative phosphorylation, would not be possible with the small subset of 13 proteins encoded in mtDNA. Discoveries led by high throughput proteomic approaches have enhanced our knowledge of the mitochondrial proteome [23–27]. There are approximately 1158 nuclear-encoded proteins that localize to the mammalian mitochondria, exerting a dual genetic control via its nuclear counterpart. Nuclear-encoded mitochondrial proteins on the basis of their mitochondrial function can be broadly

classified into different categories including oxidative phosphorylation, energy production, membrane dynamics, genome maintenance, and ion/metabolite homeostasis.

Unlike the nuclear genome, where most cells have only two copies, each cell can harbor thousands of copies of mtDNA, depending on environmental needs, making mitochondrial genome engineering a population genetics challenge. When every mtDNA molecule is identical with non-mutagenic variants, the cell is in a genetic state, called homoplasmy, and the mitochondria functions normally. In people with mtDNA-based mitochondrial disease, mtDNA genomes harboring pathogenic mutations typically co-exist with the healthy genomes in a state called heteroplasmy. Homoplasmy may also arise when the cell harbors only mutant mtDNA molecules, resulting in severe clinical manifestations. [28]. The ratio of pathogenic-harboring to nonpathogenic-harboring mtDNA is critical to mitochondrial disease onset. A threshold exists for most mitochondrial disease, at which, the healthy mtDNA genomes can no longer compensate for the pathogenic genomes, resulting in disease onset leading to breakdown of oxidative phosphorylation and other mitochondrial function(s) [29].

Mitochondrial disorders are a heterogeneous group of clinical manifestations resulting from either inherited or spontaneous mutations in mtDNA or nDNA leading to altered structure or function of the proteins or RNA that reside in mitochondria [6,10,30,31]. The pathophysiology of mitochondrial disorders involves functions of both the nuclear and mitochondrial genomes, conferring additional complexity to the manifestation of the syndromes. The prevalence of mitochondrial disorders is about 1 in 5000 live births; however, the prevalence is often influenced by the presence of founder mutations and consanguinity in populations [31–33]. These conditions range in severity dependent on the specific mutation and its prevalence and

localization within the patient. In addition to diseases specifically progenerated by mitochondrial defects, mitochondrial dysfunction has also been implicated as a cofactor or by-product of many other conditions; including cancer, immune diseases, developmental delays and neurodegenerative condition such as Alzheimer's [34–38].

Mitochondrial dysfunction tends to affect primarily high energy systems and can therefore have devastating effects on a wide range of body systems including brain function, liver function, vision, hearing, immune function, and all muscle types [31,39]. The identification and characterization of novel mitochondrial genes in human genetic disorders have also enhanced our knowledge of mitochondrial function [40–44]. However, despite their known prevalence and severity, mitochondrial diseases remain understudied as compared to other genetic conditions, and the options for treatment are limited.

The conserved roles of mitochondria have been traditionally uncovered using accessible model systems such as the yeast *S.cerevisiae* [45–48] and invertebrates including *C.elegans* [49–52] and *D. melanogaster* [53–56]. Vertebrate mitochondria, however, are known to have additional innovations and encode unique subunits not present in invertebrate models [5,45,57–67]. Most exploration to date of vertebrate mitochondrial biology has been conducted either in cell culture or in mouse models. However, cells in a dish are missing important environmental cellular and organismal context, whereas mouse mitochondrial experimental work can be hindered by limited population size and invasive imaging techniques.

We provide here an initial genetic toolkit for modeling mitochondrial biology and disease in *Danio rerio* (zebrafish) in an effort to help catalyze the use of this invaluable model organism in mitochondrial research. The zebrafish is a tropical freshwater teleost that has proven to be an invaluable resource for the study of human disease and genetics [68–70]. Principal among the

many research amenable characteristics of zebrafish are their high genetic orthology to humans, high fecundity, their optical transparency (in the embryonic stage) that lends itself to facile imaging, and the ease of reagent delivery through microinjection into the single celled embryo, which is a full millimeter across before the first mitotic division. Zebrafish also serve as a potentially powerful vertebrate model organism to study human mitochondrial disorders because of conserved mitochondrial genome and mitochondrial genetic machinery. Zebrafish and human mitochondrial chromosomes display ~65% sequence identity at the nucleotide level, and share the same codon usage, strand specific nucleotide bias and gene order [12] **(Figure 1)**.

Over the recent years, zebrafish research has helped shed light on mitochondrial biology and further developed our understanding of the mechanisms of mitochondrial-associated pathology [71–76]. Zebrafish have also been successfully used as a model system to study mitochondrial targeting drugs with implications in development and cardiovascular function [71,77,78]. These drugs can simply be dissolved in the water housing the larvae and offer an advantage of large sample size with minimal volumes of drug administered. Drug-screening studies have aided in understanding the pathogenesis of various diseases and helped to identify targets for treatment [77].

To help engender an expansion of zebrafish deployment in mitochondrial research, we present a panel or “starter-pack” toolkit of genetic mutants made in nuclear-encoded mitochondrial genes in zebrafish. This mutant panel, which we have named the Marriot Mitochondrial Collection (MMC), consists of 30 zebrafish lines with mutations in 24 different mitochondrial genes. The mutant collection focusses primarily on genes that encode for components of the energy generating electron transport chain with at least one mutant in each complex of the ETC. Other mutants consist of assembly factors, protein chaperones that manage mitochondrial membrane

traffic, and genes related to mitochondrial replication. These mutants were made either by the use of targeted endonuclease [79], or curated from our research group's library of randomly generated insertional mutants [80]. We hope that this collection, in addition to being intrinsically useful, will also help serve as a primer to the modeling of mitochondrial biology and disease in zebrafish.

# Methods

## Zebrafish Handling

All animal work was conducted under Mayo Clinic's institutional animal welfare approvals (IACUC number: A34513-13-R16).

## Identification of zebrafish orthologs having putative mitochondrial function

A previous study combining discovery and subtractive proteomics with computational, microscopy identified 1098 mouse genes that could encode for proteins residing in mitochondria [24]. They further identified 1013 human orthologs for these genes, providing an initial inventory of the genes coding for proteins resident in mitochondria. Using literature assessment and HUGO database curation approaches, we identified 97 proteins that are involved in the biogenesis and assembly of electron transport chain in mitochondria. Using zebrafish orthologs of human genes from ZFIN (Zebrafish Information Network), 93 zebrafish mitochondrial orthologs were identified. These orthologs were systemically annotated with respect to clinical phenotype by extensive mining from PubMed based published case reports and OMIM database **(Supplementary Table 1)**.

## Mutant Generation

Mutant lines were created by one of two methods, either through the targeted use of Transcription Activator Like Effector Nucleases (TALENs) [79] or by screening Gene Breaking Transposon (GBT) lines for integrations into mitochondrial genes [80]. Reagents were delivered in both methods by the microinjection of either TALEN pairs or GBT transposon and Tol2 transposase into single cell zebrafish embryos.

## TALEN Design/Assembly/Delivery

The TALEN mutants in this collection were originally generated as part of a previously published study [79]. In brief, TALEN pairs were designed using the Mojohand software platform [81] (www.talendesign.org) to target highly conserved, and therefore likely functionally important, areas of nuclear-encoded mitochondrial genes. TALEN RVDs were then cloned into pT3Ts-GoldyTALEN (TALEN vector with a T3 transcriptional promoter for in vitro transcription) using the FusX rapid TALEN assembly system [79], which uses the RVD definitions: HD=C, NN=G, NI=A, NG=T. Following assembly, mRNA was synthesized in vitro using the mMessage Machine T3 kit (Ambion) and extracted by a phenol-chloroform extraction as prescribed in the mMessage Machine manual. The extracted mRNA was then delivered into single cell zebrafish embryos at 100pg doses (50pg per TALEN arm) by microinjection.

## TALEN Mutant Screening

Following microinjection, genomic DNA was extracted from F0 larvae three days post fertilization (dpf) by sodium hydroxide extraction. DNA for eight individual larvae was analyzed for NHEJ activity at the TALEN target site by Restriction Fragment Length Polymorphism (RFLP) analysis. Groups with high reported NHEJ activity by RFLP were raised to adulthood

and outcrossed to create an F1 generation. Suspected NHEJ mutants were further verified by Sanger sequencing. F1 larvae demonstrating NHEJ mutations confirmed by both RFLP and Sanger sequencing were raised to adulthood. Fin biopsies were performed on these adults and DNA was extracted by sodium hydroxide extraction.

**Gene Breaking Transposon System**

The Gene Breaking Transposon System of protein trap system and a complete repository of protocols for the creation and screening of GBT mutant lines has been described [80]. In short, protein trap transposons were delivered in combination with mRNA for Tol2 transposase (25pg each) into single cell zebrafish embryos by microinjection. Embryos were screened for GFP fluorescence at 3-4dpf and classified broadly into three classes [80]. Class three embryos, those with whole body GFP expression, were raised to adulthood and outcrossed to non-transgenic lines to create an F1 generation. mRFP-expressing F1 embryos were sorted by expression pattern, assigned a GBT number, and raised to adulthood. These adult fish were then outcrossed to non-transgenic lines to create an F2 generation upon which all subsequent propagation, testing and imaging was conducted.

To determine genes tagged by the protein trap system, rapid amplification of cDNA ends (RACE) was performed as described [82] with minor updates to primer sequences. cDNA was generated using a transposon-specific primer (5R-mRFP-P0) against 250ng of total mRNA in the reverse transcription reaction. PCR was then performed using the following gene-specific primers: 5R-mRFP-P1 and 5R-mRFP-P2. The resulting products were TA cloned for further amplification and then sequence verified for in-frame mRFP fusions by Sanger sequencing.

In some cases, inverse PCR was also conducted to RACE PCR as described [80]. Whole genomic DNA was extracted from individual F2 embryos using a sodium hydroxide extraction and 800ng was digested in a combination reaction using AvrII, NheI, SpeI, and XbaI restriction enzymes. Approximately 200ng of the product of this digestion was self-ligated and used as a template for PCR using the following nested and primary primers. 5' side: 5R-mRFP-P1 and 5R-mRFP-P2 paired with INV-OPT-P1 and INV-OPT-P2, respectively. 3' side: 5R-GFP-P1 and 5R-GFP-P2 with Tol2-ITR(L)-O1 and Tol2-ITR(L)-O3, respectively. The products of the final nested reaction were gel-extracted, cloned, and sequenced. Prospective in frame mRFP fusions were further verified by comparing suspected protein trap allele to GBT expression pattern and by PCR against DNA or cDNA from mRFP carrier siblings versus non-carrier siblings.

Protein trap lines as verified by these methods were catalogued for specific genomic fusions using the National Center for Biotechnology Information's (NCBI) Homologene Database. Human orthologs for tagged genes were further identified using blastX searches against the human genome. GBT lines with protein trap fusions to nuclear encoded mitochondrial genes with human orthologues have been included in this collection.

### *In silico* analysis to determine protein homology and alteration of DNA sequence by TALENs and GBT in zebrafish mutants

Human amino acid sequences were compared to both the zebrafish wild type sequence and each specific zebrafish mutant sequence associated with each allele. Amino acid sequence information for a particular gene was gathered for humans from https://www.uniprot.org/ along with the wild type amino acid sequence for the zebrafish. The first/most common isoform for each entry was used for this analysis. Any added information regarding functional domains or regions was also gathered. A protein-protein BLAST was conducted using https://blast.ncbi.nlm.nih.gov/Blast.cgi

to compare the sequences of the human and wild type zebrafish amino acid sequences. BLAST search settings used included the blastp algorithm. Regions of low and high homology were then mapped out for the sequences.

For zebrafish mutant to human comparisons, the cDNA or DNA sequences were gathered via sequencing and put through the translate tool at https://web.expasy.org/translate/. The standard genetic code was used for conversion. Stop codons arising from the frameshifted DNA sequence were found in all mutants. The BLAST analysis conducted on the mutants was taken from the starting methionine to the first encountered stop codon. Regions of low and high homology were then mapped for each sequence.

### Access to all reported reagents – zebrafish and sequences

All listed tools are immediately available through the Mayo Clinic Zebrafish Facility, and all fish lines will be available via ZIRC. Sequences needed for genotyping and related metadata are currently on zfishbook [83].

## RESULTS

### Generation of zebrafish mutant collection

We generated zebrafish mutants for a wide selection of nuclear-encoded mitochondrial genes by employing two genetic engineering approaches, gene breaking transposons and TALENs. The collection of zebrafish mutants is referred as the Marriot Mitochondrial Collection (MMC) and comprises of mutants for 24 nuclear-encoded mitochondrial proteins **(Figure 2)**. Out of these, 18 were created by TALEN indel mutagenesis and 6 were created by gene breaking trap mutagenesis. The mutants include proteins from all known functional pathways involved in mitochondrial homeostasis and ATP generation **(Figure 2)**.

Broadly, the pathways can be classified as subunits of oxidative phosphorylation complexes, chaperones for assembly of oxidative phosphorylation proteins, maintenance proteins for the mitochondrial genome (replication, mtRNA folding and translation), calcium homeostasis and mitochondrial protein import. All of these genes have human orthologs, nearly all with known mutations in which lead to severe clinical manifestations such as Leigh syndrome, cardiomyopathy, progressive external ophthalmoplegia, and oxidative phosphorylation deficiency **(Table 1)**.

**Table 1: List of nuclear-encoded genes for which zebrafish mutants were generated as part of MMC collection:** The current MMC resource list summarizes information on human gene, zebrafish ortholog, mouse ortholog, relevant clinical phenotypes and diseases, protein function, and OMIM ID. Green = Created by TALEN Indel Mutagenesis, Orange = Curated from GBT protein trap lines (Keywords: Chr- Chromosome; OMIM ID: Online Mendelian Inheritance in Man ID; NA-not available).

| Approved symbol | Approved name | Human chr | Zebrafish orthologs | Zebrafish chr | Allele designator | Mouse orthologs | Mouse chr | Clinical phenotype observed in humans | Disease | Biological function | OMIM ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *NDUFAB1* | NADH:ubiquinone oxidoreductase subunit AB1 | 16 | *ndufab1a*<br>*ndufab1b* | 1<br>3 | mn0135 | Ndufab1b | 7 | -na- | -na- | Oxidative phosphorylation enzymes - Complex I | 603836 |
| *NDUFAF6* | NADH:ubiquinone oxidoreductase complex assembly factor 6 | 8 | *ndufaf6* | 16 | mn0117 | Ndufaf6 | 4 | Focal right-hand seizures, ataxia, lactic acidosis, exercise intolerance, weakness and muscle tension | Leigh syndrome, mitochondrial complex I deficiency | Oxidative phosphorylation complex assembly - Complex I | 612392 |
| *NDUFS4* | NADH:ubiquinone oxidoreductase subunit S4 | 5 | *ndufs4* | 5 | mn0118 | Ndufs4 | 13 | Mitochondrial complex I deficiency | Mitochondrial complex-I deficiency | Oxidative phosphorylation enzymes - Complex I | 602694 |
| *SDHA* | Succinate dehydrogenase complex flavoprotein subunit A | 5 | *sdha* | 19 | mn0121 | Sdha | 13 | Dyspnea, cardiomegaly, cardiomyopathy, nystagmus, hypotonia, gastrointestinal stromal tumors, paragangliomas, pheochromocytoma, psychomotor regression, severe hyperandrogenism | Mitochondrial complex-II deficiency; Cardiomyopathy, Leigh syndrome; Paraganglioma | Oxidative phosphorylation enzymes - Complex II | 600857 |

| Approved symbol | Approved name | Human chr | Zebrafish orthologs | Zebrafish chr | Allele designator | Mouse orthologs | Mouse chr | Clinical phenotype observed in humans | Disease | Biological function | OMIM ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *BCS1L* | BCS1 homolog, ubiquinol-cytochrome C reductase complex chaperone | 2 | *bcs1l* | 9 | mn0102 | Bcs1l | 1 | Growth retardation, aminoaciduria, cholestasis,iron overload, lactic acidosis, early death, hypotonia, encephalopathy, microcephaly, psychomotor regression, hypoglycemia and hepatic failure | Björnstad syndrome: GRACILE syndrome; Leigh syndrome; Mitochondrial complex III deficiency, nuclear type 1 | Oxidative phosphorylation complex assembly - Complex III | 603647 |
| *UQCRQ* | ubiquinol-cytochrome c reductase complex III subunit VII | 5 | *uqcrq* | 14 | mn0128 mn0129 | Uqcrq | 11 | Severe psychomotor retardation and extrapyramidal signs, dystonia, athetosis and ataxia, mild axial hypotonia and marked global dementia | Mitochondrial complex III deficiency | Mitochondrial complex III: ubiquinol-cytochrome c reductase complex subunits | 612080 |
| *COX10* | Cytochrome c oxidase assembly factor heme A: farnesyltransferase COX10 | 17 | *cox10* | 12 | mn0107 mn0108 | Cox10 | 11 | Muscle weakness, hypotonia, ataxia, ptosis, pyramidal syndrome, status epilepticus, lactic acidosis, hypertrophic cardiomyopathy, hypoglycemia and metabolic acidosis | Leigh syndrome due to mitochondrial COX IV deficiency; Mitochondrial complex IV deficiency | Oxidative phosphorylation complex assembly - Complex IV | 602125 |
| *COX4I2* | Cytochrome C Oxidase Subunit 4I2 | 20 | *cox4i2b* | 23 | mn0104 | Cox4i2 | 2 | Exocrine pancreatic insufficiency, dyserythropoietic anemia, and calvarial hyperostosis | Exocrine pancreatic insufficiency, dyserythropoietic anemia, and calvarial hyperostosis | Oxidative phosphorylation complex enzymes - Complex IV | 607976 |
| *SURF1* | SURF1, Cytochrome C Oxidase Assembly Factor | 9 | *surf1* | 5 | mn0123 | Surf1 | 2 | Childhood onset neuropathy, lactic acidosis, mild intellectual disability ataxia, facial dysmorphism, encephalopathy, hypotonia, cerebellar ataxia, deafness, ophthalmoplegia, growth retardation and nystagmus | Charcot-Marie-Tooth disease, type 4K; Leigh syndrome, due to COX IV deficiency | Involved in biogenesis of cytochrome c oxidase complex | 185620 |
| *ATP5F1E* | ATP synthase F1 subunit epsilon | 20 | *atp5f1e* | 6 | mn0101 | Atp5f1e | 2 | Neonatal-onset lactic acidosis, 3-methylglutaconic aciduria, mild mental retardation, and peripheral neuropathy | Mitochondrial complex (ATP synthase) deficiency | Oxidative phosphorylation complex assembly - ATP synthase | 606153 |
| *TMEM70* | Transmembrane protein 70 | 8 | *tmem70* | 2 | mn0126 mn0127 | Tmem70 | 1 | Lactic acidosis, encephalopathy, histiocytoid cardiomyopathy, microcephaly, hypotonia, facial dysmorphism and 3-methylglutaconic aciduria psychomotor delay, hyperammonemia | Mitochondrial complex V (ATP synthase) deficiency | Biogenesis of mitochondrial ATP synthase | 612418 |

| Approved symbol | Approved name | Human chr | Zebrafish orthologs | Zebrafish chr | Allele designator | Mouse orthologs | Mouse chr | Clinical phenotype observed in humans | Disease | Biological function | OMIM ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *COQ2* | Coenzyme Q2, Polyprenyltransfe--rase | 4 | *coq2* | 5 | mn0106 | Coq2 | 5 | Multiple system atrophy, unsteadiness of gait, nystagmus, gait ataxia, dysarthria, speech difficulty, dysmetria, lactic acidosis, urinary dysfunction and nystagmus | Coenzyme Q10 deficiency, primary; Multiple system atrophy | Biosynthesis of CoQ, coenzyme in mitochondrial respiratory chain | 609825 |
| *PDSS2* | Decaprenyl diphosphate synthase subunit 2 | 6 | *pdss2* | 13 | mn0120 | Pdss2 | 10 | Hypotonia, seizures, cortical blindness, lactic acidosis, encephalopathy and nephrotic syndrome | Coenzyme Q10 deficiency | Involved in biosynthesis of coenzyme Q | 610564 |
| *MICU1* | Mitochondrial calcium uptake 1 | 10 | *micu1* | 13 | mn0132 | Micu1 | 10 | Proximal muscle weakness and learning disabilities | Myopathy with extrapyramidal signs | Regulator of mitochondrial calcium uptake | 605084 |
| *SMDT1* | Single-pass membrane protein with aspartate rich tail | 22 | *smdt1a*<br>*smdt1b* | 3<br>1 | mn0122 | Smdt1 | 15 | -na- | -na- | Core regulatory subunit of calcium channel in mitochondrial inner membrane | 615588 |
| *MCU* | Mitochondrial calcium uniporter | 10 | *mcu* | 13 | mn0111 | Mcu | 10 | -na- | -na- | Calcium transporter and mediates calcium uptake in mitochondria | 614197 |
| *TK2* | Thymidine kinase 2, mitochondrial | 16 | *tk2* | 7 | mn0125 | Tk2 | 8 | Infantile onset fetal encephalomyopathy, failure to thrive, muscle weakness, mild facial weakness, myopathy, axonal neuropathy, brain atrophy, hypertrophic cardiomyopathy, regression of motor development, psychomotor arrest, dysphonia, dysphagia, ptosis, impaired gait, bilateral ptosis, hypotonia, lactic acidosis and epilepsy | Progressive external ophthalmoplegia; Mitochondrial DNA depletion syndrome 2 (myopathic type) | Required for mitochondrial DNA synthesis | 188250 |
| *LRPPRC* | Leucine rich pentatricopeptide repeat containing | 2 | *lrpprc* | 13 | mn0235gt | Lrpprc | 17 | Delayed psychomotor development, mental retardation, mild dysmorphic facial features, hypotonia, ataxia, and the development of lesions in the brainstem and basal ganglia, seizures, dysphagia and hypertrophic cardiomyopathy | Leigh syndrome, French-Canadian type | Involved in translation of mitochondrial encoded cox subunits and mediation of folding of mitochondrial transcriptome | 607544 |
| *MTFMT* | Mitochondrial methionyl-tRNA formyltransferase | 15 | *mtfmt* | 7 | mn0114<br>mn0115 | Mtfmt | 9 | Psychomotor developmental delay, renal dysplasia, mild facial dysarthria and ataxia | Combined oxidative phosphorylation deficiency | Catalyzes the formylation of methionyl-tRNA | 611766 |
| *MRPS18B* | Mitochondrial ribosomal protein S18B | 6 | *mrps18b* | 19 | mn0425gt | Mrps18b | 17 | -na- | -na- | Part of small 28S subunit of mitoribosome | 611982 |

| Approved symbol | Approved name | Human chr | Zebrafish orthologs | Zebrafish chr | Allele designator | Mouse orthologs | Mouse chr | Clinical phenotype observed in humans | Disease | Biological function | OMIM ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *TIMM50* | Translocase of inner mitochondrial membrane 50 | 19 | *timm50* | 15 | mn0906gt | Timm50 | 7 | Severe intellectual disability, seizure and 3-methylglutaconic aciduria | 3-methylglutaconic aciduria, type IX; Mitochondrial complex V deficiency | Subunit of TIM23 inner mitochondrial membrane complex and recognizes mitochondrial targeting signal or pre-sequence | 607381 |
| *OGDH* | Oxoglutarate dehydrogenase | 7 | *ogdha*<br>*ogdhb* | 8<br>10 | mn0281gt | Ogdh | 11 | Colorectal cancer | Alpha-ketoglutarate dehydrogenase deficiency | Catalyzes the conversion of 2-oxoglutarate to succinyl-CoA and $CO_2$ | 613022 |
| *IDH2* | Isocitrate dehydrogenase (NADP (+)) 2, mitochondrial | 15 | *idh2* | 18 | mn0268gt | Idh2 | 7 | Acute myeloid leukemia and abnormal production of D-2-hydroxyglutaric acid | D-2-hydroxyglutaric aciduria 2 | Catalyzes the conversion of isocitrate to 2-oxoglutarate | 147650 |

## TALEN- and GBT-mediated targeting of nuclear-encoded mitochondrial genes

Amino acid analysis using protein-protein BLAST functions between the wild type human, wild type zebrafish, and mutant zebrafish sequences showed consistent results between the three conditions. The comparison of wild type human and wild type zebrafish sequences showed high areas of homology following the mitochondrial targeting domain in almost every gene, with 80-100% similarity in catalytic or active domains of the transcripts. Analysis of the mutant zebrafish and human wild type comparison showed a range of difference with predicted frameshift mutations leading to truncation of the protein very early in the transcript **(Figure 3A-X)**. Of the alleles created by TALEN mutagenesis in the MMC collection, all but one (*micu1)* showed a predicted frameshift mutation leading to a truncation event immediately following the NHEJ-mediated insertion or deletion. The GBT mutants showed high levels of homology prior to the transposon integration site followed by a nearly complete loss of normal transcript levels following splicing into the GBT cassette.

## Discussion

In a multicellular organism, each cell is able to carry out its functions due to well-orchestrated cross-talk between the nuclear and mitochondrial genomes. Many mitochondrial functions like energy production, genome maintenance, ion/metabolite homeostasis, membrane dynamics and transport of biomolecules can be attributed to approximately 1158 known nuclear proteins residing in the mitochondria [24,27]. Due to advancements in proteomic technologies in recent years, there has been a surge in documentation of many characterized and uncharacterized mitochondrial proteins. However, correlation between mitochondrial localization of these proteins and their physiological significance in disease progression remains largely unexplored. Out of the 1158 nuclear encoded proteins, only 245 have functional evidence in mitochondrial clinical manifestations [39]. For the remaining 913 proteins, mitochondrial involvement in disease progression has yet to be demonstrated. The obstacles in mitochondrial genetic research, and thus delays in finding effective treatments, are primarily due to the limited tools available to mitochondrial researchers, specifically the small number of available model systems and animals.

Most mitochondrial research thus far has made use of model systems that each present their own unique challenge to accurate study of human disease. Yeast, one of the most common laboratory eukaryotes, have been incredibly useful in mitochondrial research, but unlike humans and other animals they lack complex I [45]. Complex 1 deficiency is the most frequent among mitochondrial disorders caused by mutations in 28 out of 48 genes contributing to its assembly and biogenesis [84]. Also, complex 1 is responsible for the most ROS generation, which acts as a cue in various signaling pathways [85].

Human cell culture has been valuable in exploring mitochondrial disease models, principally through cytoplasmic hybrids that are made by replacing the mitochondria of an immortalized cell line with mutated patient mitochondria [86–89]. However, while these cells are a more faithful representation of human mitochondrial activity than yeast, they have different barriers to accuracy. First, immortalized cells, and cultured cells in general, often have modified metabolic pathways compared to cells *in vivo*. Second, as is common to all cell culture research, cells in a dish cannot accurately represent the complex interactions and systems biology inherent to a complete organism.

In this study, we aim to support the establishment of the zebrafish as a complementary animal model for understanding the role of nuclear-encoded mitochondrial proteins in biology and the pathophysiology of mitochondrial disorders. We propose that zebrafish can be an excellent model organism to study mitochondrial biology primarily because of their conserved genome, codon bias and synteny. Other advantages include their amenability to genetic manipulation and optical clarity, which facilitates direct observation. New gene editing techniques such as TALENs and CRISPRs have aided in the development of humanized disease models in organisms such as zebrafish, mice, and even pigs [90–97].

The MMC collection described here encompasses mutations in 24 different nuclear-encoded genes related to mitochondrial function enabling a diverse study of the important roles the mitochondria play in cellular biology. The collection includes mutants in all five complexes of the electron transport chain as well as many other crucial pathways related to protein transport, metabolite synthesis, mtDNA replication and expression, and calcium homeostasis. The first set of mutants were created using custom gene editing to exons coding for critical functional domains of the protein product. These domains were targeted because they shared high levels of

homology to their human ortholog. The second group of mutants consisted of fish that had been injected with a protein trap transposon system that truncates the expected protein products as well as sorting and imaging through fluorescent expression patterns *in vivo*. Included here, we have curated small group of mutants where the GBT integrated into a mitochondrial gene of interest.

In recent years zebrafish have been used to understand the pathophysiology of human mitochondrial disorders such as cardiovascular, multisystemic, neurological, and erythropoiesis, to name a few. Studies for loss of function have been carried out to model left ventricular non-compaction, a genetically heterogeneous cardiomyopathy caused by mutations in nicotinamide nucleotide transhydrogenase (*NNT*) gene [98,99]. Transiently suppressing the levels of *nnt* gene did mirror the symptoms, such as early ventricular malformation and contractility defects, observed in human patients [99]. Transient suppression of *ndufb11* protein in zebrafish results in cardiac anomalies, confirming the role of *NDUFB11* in histiocytoid cardiomyopathy pathogenesis [41].

Parkinson disease (PD) is another example where *parkin* and *pink* knockdown larvae display mitochondrial fragmentation accompanied by accumulation of oxidative species, mimicking the phenotype observed in PD patients. Loss of dendritic arborization and peripheral neurons were also observed in the knockdown animals [72,100–103]. The zebrafish model of Dravet syndrome shows altered metabolism associated with the pathophysiology [104]. Downregulation of the sodium channel, voltage-gated, type I like, alpha b (*scnl1ab*) in zebrafish leads to mitochondrial structural abnormalities accompanied by accumulation of oxidative species [104–106].

Sideroblastic anemia, caused by mutations in mitoferrin (*mfrn1*) gene, has been successfully modelled in zebrafish [107]. The elucidation of its role in zebrafish as iron importer does shed

some light on iron related defects in progression of anemia, accompanied by mitochondrial dysfunction. Zebrafish erythrocytes are nucleated and possess organelles such as mitochondria [108] in their cytoplasm, making them an ideal model organism to study ribosomopathies and hematological malignancies [109–114]. Mitochondria in erythrocytes may offer valuable insight into novel biological pathways resident in mitochondria.

Transient infantile liver failure and mitochondrial deafness, caused by mutations in tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (*TRMU*) gene [115] have been phenocopied in zebrafish [116]. Zebrafish *trmu* mutants exhibit abnormal mitochondrial morphology, deficient oxidative phosphorylation, defects in hair cells and decreases in the steady state levels of mt-tRNAs, resembling the observations made in patients suffering from this syndrome [116].

Taking cues from these studies, many of the genes selected here were prioritized on the basis of the mutations and clinical phenotype reported in patients. Detailed phenotypic analyses will be described in subsequent studies. The zebrafish mutants encode for genes that have been involved in myriad of clinical phenotypes such as encephalopathy, nephrotic syndrome, intellectual disability, psychomotor developmental delay, respiratory chain deficiency, lactic acidosis, iron overload, microcephaly, hypertrophic cardiomyopathy, etc. The superficial investigations of the tissue-specific phenotypes associated with, for example, housekeeping nuclear encoded proteins, have to be refined with organelle level investigations. The repository of mutants encoding for proteins involved in respiratory chain biogenesis and assembly offers the potential to understand the moonlighting role of these proteins. Mutations in these proteins are associated with organ-specific phenotypes such as neurological, paraganglioma, cardiovascular etc. [41,117]. Extensive energy demand by these tissues is one possible explanation for the progression of these

manifestations; however, the role of these proteins in organ-specific cellular niches or homeostasis is also a possibility. These models can help to decipher the role of these proteins in the mitochondrial interactome, when studied *in vivo*. This underpins the utility of this MMC collection in deciphering the role of mitochondrial proteins in tissue-specific biological pathways.

Zebrafish are also amenable to small molecule mediated pharmacological modelling of mitochondrial phenotypes. To test hypoxia as a potential protective therapy in mitochondrial disorders, Von Hippel-Lindau (*vhl*) null mutant zebrafish, when treated with antimycin mediated mitochondrial insult, exhibited improved survival. In addition, FG-4592 was found to improve survival in response to respiratory chain inhibition, possibly due to increase in hypoxia response [118]. A series of ETC complex specific pharmacological inhibitors such as rotenone (complex I), azide (complex IV), oligomycin (complex V) and chloramphenicol (mitochondrial protein translation) have been used to model respiratory chain dysfunction in zebrafish [78,119,120]. Zebrafish larvae display a series of phenotypes, such as developmental arrest, when treated with rotenone. Treatment with azide induced decreased heart rate, loss of motor function, inability to respond to tactical stimulation, neurological damage and mortality. Organ specific manifestations such as neurological and behavioral dysfunction have been reported in 6-7 days post fertilization (dpf) zebrafish larvae upon administration of a titred drug concentration [120]. These observations tilt the scales in favor of zebrafish for modeling mitochondrial clinical manifestations. Gaining insights from these studies, our compendium of mutants provides an excellent resource to test a large number of biological and chemical modulators as potential therapies for mitochondrial disorders for which treatment remains elusive.

Zebrafish offer many unique advantages for *in vivo* imaging experiments, as compared to their mammalian counterparts. A classical study corroborating this advantage is *in vivo* imaging of mitochondrial transport in a single axon, as demonstrated by Yang and his colleagues [121]. Seok and his colleagues generated a transgenic zebrafish line, expressing green fluorescent protein fused to mitochondrial localization sequence from cytochrome c oxidase [71]. Mitochondrial function is often measured by various parameters such as estimation of membrane potential, mitochondrial superoxide species and energy production. Superoxide activity and membrane potential in zebrafish has been measured by employing the use of cell permeable chemical probes such as MitoSox [122] and Dihydrorhodamine 123 (DH123) [73], respectively. Zebrafish transgenic lines expressing genetically encoded calcium and oxidation indicators, have also served as an excellent model to measure calcium homeostasis and oxidative status *in vivo* in models of mechanosensory hair cell damage and death [123]. Constructs such as Mitotimer allow the measurement of mitochondrial turnover, transport, and changes in the redox history of mitochondria during organogenesis events in zebrafish embryos [124]. This transgenic model enables a sweeping picture of the mitochondrial network, helping in studying cellular processes such as mitophagy and apoptosis.

Zebrafish have also provided novel mechanistic insights to interrogate the mitochondrial dynamics, to be called as "*in-vivo* life cycle of mitochondria" in healthy and diseased conditions [124]. One such example is that of Mitofish [74], that recapitulates mitochondrial network biogenesis, unraveling the role of the organelle in cell-type specific niches of different organ systems. Mitofish is a transgenic zebrafish line that fluorescently labels the mitochondria in the neurons, enabling non-invasive *in vivo* observation. Advancement in adaptive optics and lattice light sheet microscopy have empowered researchers to look at vibrant and colorful images of

organelles in zebrafish. These technologies have been applied in investigating the organellar dynamics in brain during early development and in the eye of an adult zebrafish [125]. These MMMC mutants provide an excellent platform to study mitochondrial remodeling and homeostasis in the progression of various clinical disorders by mapping the mitochondrial wiring of the cell.

These zebrafish mutants have been cryopreserved for the purpose of sharing the lines as a resource with the field for expanding the study of mitochondrial genetics and biology. In addition, our template for the creation of mitochondrial mutants in zebrafish should enable the creation of higher animal models of specific variants of interest. For the purpose of accelerating this research, all lines in this project have had their information deposited with ZIRC, and all of the lines have been made available via zfishbook (www.zfishbook.com) [83].

The goal of this study is to disseminate the use of zebrafish as a *noblesse oblige* in the field of mitochondrial biology and medicine, paving way for the development of novel insight for diagnostic and therapeutic strategies. Ultimately, using this collection of mutants we hope to unravel a small part of the mystery that shrouds one of the most crucial organelles in the cell. We hope that this MMMC mutant collection and the primer we provide for adding to it, will help to usher in a new mitochondrial research using the zebrafish.

## Acknowledgements

The authors thank Dr. Eric Schon and Dr. Vamsi Mootha for helping select the gene set targeted in this collection, and the following who contributed to the generation of these new zebrafish lines including Alexandra Cook, Roberto Lopez Cervera, and the Mayo Clinic Zebrafish Facility staff. Gabriel Martinez Galvez helped with paper illustration design. This work was sponsored

by the Mayo Foundation, a gift by the Marriott Foundation and by NIH grants DK84567, GM63904 and HG006431 to SCE.

## Author Contributions

The paper was written by AS, MW, ZWJ and SCE. Experiments were executed by JMC, MW, HA, NI, MDU, JDB, RMH, WL, YD and TLP with experimental guidance from XX, KJC and SCE. Data analysis was completed by JMC, NI, JDB, MDU, RMH, MW, WL, YD and AS.

## References

1. Andersson SG, Zomorodipour A, Andersson JO, Sicheritz-Ponten T, Alsmark UC, Podowski RM, et al. The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature. England; 1998;396: 133–140. doi:10.1038/24094

2. Muller M, Martin W. The genome of Rickettsia prowazekii and some thoughts on the origin of mitochondria and hydrogenosomes. Bioessays. United States; 1999;21: 377–381. doi:10.1002/(SICI)1521-1878(199905)21:5<377::AID-BIES4>3.0.CO;2-W

3. Gray MW, Burger G, Lang BF. Mitochondrial evolution. Science. United States; 1999;283: 1476–1481.

4. Thorsness PE, Weber ER. Escape and migration of nucleic acids between chloroplasts, mitochondria, and the nucleus. Int Rev Cytol. United States; 1996;165: 207–234.

5. Lynch M, Koskella B, Schaack S. Mutation pressure and the evolution of organelle genomic architecture. Science. United States; 2006;311: 1727–1730. doi:10.1126/science.1118884

6. Vafai SB, Mootha VK. Mitochondrial disorders as windows into an ancient organelle.

Nature. England; 2012;491: 374–383. doi:10.1038/nature11707

7. Elson JL, Samuels DC, Turnbull DM, Chinnery PF. Random intracellular drift explains the clonal expansion of mitochondrial DNA mutations with age. Am J Hum Genet. United States; 2001;68: 802–806. doi:10.1086/318801

8. DiMauro S, Hirano M. Mitochondrial DNA Deletion Syndromes. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, et al., editors. Seattle (WA); 1993.

9. Wallace DC. Mitochondrial DNA mutations in disease and aging. Environ Mol Mutagen. United States; 2010;51: 440–450. doi:10.1002/em.20586

10. Elson JL, Smith PM, Greaves LC, Lightowlers RN, Chrzanowska-Lightowlers ZMA, Taylor RW, et al. The presence of highly disruptive 16S rRNA mutations in clinical samples indicates a wider role for mutations of the mitochondrial ribosome in human disease. Mitochondrion. Netherlands; 2015;25: 17–27. doi:10.1016/j.mito.2015.08.004

11. Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nature genetics. United States; 1999. p. 147. doi:10.1038/13779

12. Broughton RE, Milam JE, Roe BA. The complete sequence of the zebrafish (Danio rerio) mitochondrial genome and evolutionary patterns in vertebrate mitochondrial DNA. Genome Res. United States; 2001;11: 1958–1967. doi:10.1101/gr.156801

13. Wolstenholme DR. Animal mitochondrial DNA: structure and evolution. Int Rev Cytol. United States; 1992;141: 173–216.

14. Osawa S, Jukes TH, Watanabe K, Muto A. Recent evidence for evolution of the genetic

code. Microbiol Rev. United States; 1992;56: 229–264.

15. Richter R, Pajak A, Dennerlein S, Rozanska A, Lightowlers RN, Chrzanowska-Lightowlers ZMA. Translation termination in human mitochondrial ribosomes. Biochem Soc Trans. England; 2010;38: 1523–1526. doi:10.1042/BST0381523

16. 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. United States; 2005;102: 14392–14397. doi:10.1073/pnas.0502896102

17. Srivastava S, Moraes CT. Double-strand breaks of mouse muscle mtDNA promote large deletions similar to multiple mtDNA deletions in humans. Hum Mol Genet. England; 2005;14: 893–902. doi:10.1093/hmg/ddi082

18. Nissanka N, Minczuk M, Moraes CT. Mechanisms of Mitochondrial DNA Deletion Formation. Trends Genet. England; 2019; doi:10.1016/j.tig.2019.01.001

19. Alexeyev M, Shokolenko I, Wilson G, LeDoux S. The maintenance of mitochondrial DNA integrity--critical analysis and update. Cold Spring Harb Perspect Biol. United States; 2013;5: a012641. doi:10.1101/cshperspect.a012641

20. Zinovkina LA. Mechanisms of Mitochondrial DNA Repair in Mammals. Biochemistry (Mosc). United States; 2018;83: 233–249. doi:10.1134/S0006297918030045

21. Patananan AN, Wu T-H, Chiou P-Y, Teitell MA. Modifying the Mitochondrial Genome. Cell Metab. United States; 2016;23: 785–796. doi:10.1016/j.cmet.2016.04.004

22. Gammage PA, Moraes CT, Minczuk M. Mitochondrial Genome Engineering: The

Revolution May Not Be CRISPR-Ized. Trends Genet. England; 2018;34: 101–110. doi:10.1016/j.tig.2017.11.001

23. Lopez MF, Kristal BS, Chernokalskaya E, Lazarev A, Shestopalov AI, Bogdanova A, et al. High-throughput profiling of the mitochondrial proteome using affinity fractionation and automation. Electrophoresis. Germany; 2000;21: 3427–3440. doi:10.1002/1522-2683(20001001)21:16<3427::AID-ELPS3427>3.0.CO;2-L

24. Pagliarini DJ, Calvo SE, Chang B, Sheth SA, Vafai SB, Ong S-E, et al. A mitochondrial protein compendium elucidates complex I disease biology. Cell. United States; 2008;134: 112–123. doi:10.1016/j.cell.2008.06.016

25. Elstner M, Andreoli C, Klopstock T, Meitinger T, Prokisch H. The mitochondrial proteome database: MitoP2. Methods Enzymol. United States; 2009;457: 3–20. doi:10.1016/S0076-6879(09)05001-0

26. Smith AC, Robinson AJ. MitoMiner, an integrated database for the storage and analysis of mitochondrial proteomics data. Mol Cell Proteomics. United States; 2009;8: 1324–1337. doi:10.1074/mcp.M800373-MCP200

27. Calvo SE, Clauser KR, Mootha VK. MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. Nucleic Acids Res. England; 2016;44: D1251-7. doi:10.1093/nar/gkv1003

28. Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet. England; 2005;6: 389–402. doi:10.1038/nrg1606

29. Rossignol R, Faustin B, Rocher C, Malgat M, Mazat J-P, Letellier T. Mitochondrial

threshold effects. Biochem J. England; 2003;370: 751–762. doi:10.1042/BJ20021594

30. Chinnery PF. Mitochondrial Disorders Overview. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, et al., editors. Seattle (WA); 1993.

31. Gorman GS, Chinnery PF, DiMauro S, Hirano M, Koga Y, McFarland R, et al. Mitochondrial diseases. Nature reviews. Disease primers. England; 2016. p. 16080. doi:10.1038/nrdp.2016.80

32. Skladal D, Halliday J, Thorburn DR. Minimum birth prevalence of mitochondrial respiratory chain disorders in children. Brain. England; 2003;126: 1905–1912. doi:10.1093/brain/awg170

33. Schaefer AM, McFarland R, Blakely EL, He L, Whittaker RG, Taylor RW, et al. Prevalence of mitochondrial DNA disease in adults. Ann Neurol. United States; 2008;63: 35–39. doi:10.1002/ana.21217

34. Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, et al. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science. United States; 1988;242: 1427–1430.

35. Swerdlow RH, Wooten GF. A novel deafness/dystonia peptide gene mutation that causes dystonia in female carriers of Mohr-Tranebjaerg syndrome. Ann Neurol. United States; 2001;50: 537–540.

36. Garone C, Tadesse S, Hirano M. Clinical and genetic spectrum of mitochondrial neurogastrointestinal encephalomyopathy. Brain. England; 2011;134: 3326–3332. doi:10.1093/brain/awr245

37. Urrutia PJ, Mena NP, Nunez MT. The interplay between iron accumulation, mitochondrial dysfunction, and inflammation during the execution step of neurodegenerative disorders. Front Pharmacol. Switzerland; 2014;5: 38. doi:10.3389/fphar.2014.00038

38. Singh RK, Saini S, Verma D, Kalaiarasan P, Bamezai RNK. Mitochondrial ND5 mutation mediated elevated ROS regulates apoptotic pathway epigenetically in a P53 dependent manner for generating pro-cancerous phenotypes. Mitochondrion. Netherlands; 2017;35: 35–43. doi:10.1016/j.mito.2017.05.001

39. Craven L, Alston CL, Taylor RW, Turnbull DM. Recent Advances in Mitochondrial Disease. Annu Rev Genomics Hum Genet. United States; 2017;18: 257–275. doi:10.1146/annurev-genom-091416-035426

40. Ghezzi D, Saada A, D'Adamo P, Fernandez-Vizarra E, Gasparini P, Tiranti V, et al. FASTKD2 nonsense mutation in an infantile mitochondrial encephalomyopathy associated with cytochrome c oxidase deficiency. Am J Hum Genet. United States; 2008;83: 415–423. doi:10.1016/j.ajhg.2008.08.009

41. Shehata BM, Cundiff CA, Lee K, Sabharwal A, Lalwani MK, Davis AK, et al. Exome sequencing of patients with histiocytoid cardiomyopathy reveals a de novo NDUFB11 mutation that plays a role in the pathogenesis of histiocytoid cardiomyopathy. Am J Med Genet A. United States; 2015;167A: 2114–2121. doi:10.1002/ajmg.a.37138

42. Wesolowska M, Gorman GS, Alston CL, Pajak A, Pyle A, He L, et al. Adult Onset Leigh Syndrome in the Intensive Care Setting: A Novel Presentation of a C12orf65 Related Mitochondrial Disease. J Neuromuscul Dis. Netherlands; 2015;2: 409–419. doi:10.3233/JND-150121

43. Floyd BJ, Wilkerson EM, Veling MT, Minogue CE, Xia C, Beebe ET, et al. Mitochondrial Protein Interaction Mapping Identifies Regulators of Respiratory Chain Function. Mol Cell. United States; 2016;63: 621–632. doi:10.1016/j.molcel.2016.06.033

44. Kohda M, Tokuzawa Y, Kishita Y, Nyuzuki H, Moriyama Y, Mizuno Y, et al. A Comprehensive Genomic Analysis Reveals the Genetic Landscape of Mitochondrial Respiratory Chain Complex Deficiencies. PLoS Genet. United States; 2016;12: e1005679. doi:10.1371/journal.pgen.1005679

45. Boumans H, Grivell LA, Berden JA. The respiratory chain in yeast behaves as a single functional unit. J Biol Chem. United States; 1998;273: 4872–4877.

46. Schwimmer C, Rak M, Lefebvre-Legendre L, Duvezin-Caubet S, Plane G, di Rago J-P. Yeast models of human mitochondrial diseases: from molecular mechanisms to drug screening. Biotechnol J. Germany; 2006;1: 270–281. doi:10.1002/biot.200500053

47. Altmann K, Durr M, Westermann B. Saccharomyces cerevisiae as a model organism to study mitochondrial biology: general considerations and basic procedures. Methods Mol Biol. United States; 2007;372: 81–90. doi:10.1007/978-1-59745-365-3_6

48. Denoth Lippuner A, Julou T, Barral Y. Budding yeast as a model organism to study the effects of age. FEMS Microbiol Rev. England; 2014;38: 300–325. doi:10.1111/1574-6976.12060

49. Addo MG, Cossard R, Pichard D, Obiri-Danso K, Rotig A, Delahodde A. Caenorhabditis elegans, a pluricellular model organism to screen new genes involved in mitochondrial genome maintenance. Biochim Biophys Acta. Netherlands; 2010;1802: 765–773. doi:10.1016/j.bbadis.2010.05.007

50. Harrington AJ, Hamamichi S, Caldwell GA, Caldwell KA. C. elegans as a model organism to investigate molecular pathways involved with Parkinson's disease. Dev Dyn. United States; 2010;239: 1282–1295. doi:10.1002/dvdy.22231

51. Moreno-Arriola E, Cardenas-Rodriguez N, Coballase-Urrutia E, Pedraza-Chaverri J, Carmona-Aparicio L, Ortega-Cuellar D. Caenorhabditis elegans: A useful model for studying metabolic disorders in which oxidative stress is a contributing factor. Oxid Med Cell Longev. United States; 2014;2014: 705253. doi:10.1155/2014/705253

52. Tissenbaum HA. Using C. elegans for aging research. Invertebr Reprod Dev. England; 2015;59: 59–63. doi:10.1080/07924259.2014.940470

53. Sanchez-Martinez A, Luo N, Clemente P, Adan C, Hernandez-Sierra R, Ochoa P, et al. Modeling human mitochondrial diseases in flies. Biochim Biophys Acta. Netherlands; 2006;1757: 1190–1198. doi:10.1016/j.bbabio.2006.05.008

54. Fernandez-Moreno MA, Farr CL, Kaguni LS, Garesse R. Drosophila melanogaster as a model system to study mitochondrial biology. Methods Mol Biol. United States; 2007;372: 33–49. doi:10.1007/978-1-59745-365-3_3

55. Guo M. Drosophila as a model to study mitochondrial dysfunction in Parkinson's disease. Cold Spring Harb Perspect Med. United States; 2012;2. doi:10.1101/cshperspect.a009944

56. Sen A, Cox RT. Fly Models of Human Diseases: Drosophila as a Model for Understanding Human Mitochondrial Mutations and Disease. Curr Top Dev Biol. United States; 2017;121: 1–27. doi:10.1016/bs.ctdb.2016.07.001

57. Lightowlers RN, Rozanska A, Chrzanowska-Lightowlers ZM. Mitochondrial protein

synthesis: figuring the fundamentals, complexities and complications, of mammalian mitochondrial translation. FEBS Lett. England; 2014;588: 2496–2503. doi:10.1016/j.febslet.2014.05.054

58. Pett W, Lavrov D V. Cytonuclear Interactions in the Evolution of Animal Mitochondrial tRNA Metabolism. Genome Biol Evol. England; 2015;7: 2089–2101. doi:10.1093/gbe/evv124

59. Lavrov D V, Pett W. Animal Mitochondrial DNA as We Do Not Know It: mt-Genome Organization and Evolution in Nonbilaterian Lineages. Genome Biol Evol. England; 2016;8: 2896–2913. doi:10.1093/gbe/evw195

60. Brown WM, George MJ, Wilson AC. Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci U S A. United States; 1979;76: 1967–1971.

61. Moyes CD, Suarez RK, Hochachka PW, Ballantyne JS. A comparison of fuel preferences of mitochondria from vertebrates and invertebrates. Can J Zool. NRC Research Press; 1990;68: 1337–1349. doi:10.1139/z90-201

62. Grossman LI, Wildman DE, Schmidt TR, Goodman M. Accelerated evolution of the electron transport chain in anthropoid primates. Trends Genet. England; 2004;20: 578–585. doi:10.1016/j.tig.2004.09.002

63. Gershoni M, Fuchs A, Shani N, Fridman Y, Corral-Debrinski M, Aharoni A, et al. Coevolution predicts direct interactions between mtDNA-encoded and nDNA-encoded subunits of oxidative phosphorylation complex i. J Mol Biol. England; 2010;404: 158–171. doi:10.1016/j.jmb.2010.09.029

64. Haen KM, Pett W, Lavrov D V. Parallel loss of nuclear-encoded mitochondrial aminoacyl-tRNA synthetases and mtDNA-encoded tRNAs in Cnidaria. Mol Biol Evol. United States; 2010;27: 2216–2219. doi:10.1093/molbev/msq112

65. Rea SL, Graham BH, Nakamaru-Ogiso E, Kar A, Falk MJ. Bacteria, yeast, worms, and flies: exploiting simple model organisms to investigate human mitochondrial diseases. Dev Disabil Res Rev. United States; 2010;16: 200–218. doi:10.1002/ddrr.114

66. Kuzmenko A V, Levitskii SA, Vinogradova EN, Atkinson GC, Hauryliuk V, Zenkin N, et al. Protein biosynthesis in mitochondria. Biochemistry (Mosc). United States; 2013;78: 855–866. doi:10.1134/S0006297913080014

67. Zhao J, Lendahl U, Nister M. Regulation of mitochondrial dynamics: convergences and divergences between yeast and vertebrates. Cell Mol Life Sci. Switzerland; 2013;70: 951–976. doi:10.1007/s00018-012-1066-6

68. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet. England; 2007;8: 353–367. doi:10.1038/nrg2091

69. Lin C-Y, Chiang C-Y, Tsai H-J. Zebrafish and Medaka: new model organisms for modern biomedical research. J Biomed Sci. England; 2016;23: 19. doi:10.1186/s12929-016-0236-5

70. Blanco-Sanchez B, Clement A, Phillips JB, Westerfield M. Zebrafish models of human eye and inner ear diseases. Methods Cell Biol. United States; 2017;138: 415–467. doi:10.1016/bs.mcb.2016.10.006

71. Kim MJ, Kang KH, Kim C-H, Choi S-Y. Real-time imaging of mitochondria in transgenic

zebrafish expressing mitochondrially targeted GFP. Biotechniques. England; 2008;45: 331–334. doi:10.2144/000112909

72. Flinn L, Mortiboys H, Volkmann K, Koster RW, Ingham PW, Bandmann O. Complex I deficiency and dopaminergic neuronal cell loss in parkin-deficient zebrafish (Danio rerio). Brain. England; 2009;132: 1613–1623. doi:10.1093/brain/awp108

73. Song Y, Selak MA, Watson CT, Coutts C, Scherer PC, Panzer JA, et al. Mechanisms underlying metabolic and neural defects in zebrafish and human multiple acyl-CoA dehydrogenase deficiency (MADD). PLoS One. United States; 2009;4: e8329. doi:10.1371/journal.pone.0008329

74. Plucinska G, Paquet D, Hruscha A, Godinho L, Haass C, Schmid B, et al. In vivo imaging of disease-related mitochondrial dynamics in a vertebrate model system. J Neurosci. United States; 2012;32: 16203–16212. doi:10.1523/JNEUROSCI.1327-12.2012

75. Steele SL, Prykhozhij S V, Berman JN. Zebrafish as a model system for mitochondrial biology and diseases. Transl Res. United States; 2014;163: 79–98. doi:10.1016/j.trsl.2013.08.008

76. Byrnes J, Ganetzky R, Lightfoot R, Tzeng M, Nakamaru-Ogiso E, Seiler C, et al. Pharmacologic modeling of primary mitochondrial respiratory chain dysfunction in zebrafish. Neurochem Int. England; 2017; doi:10.1016/j.neuint.2017.07.008

77. Pinho BR, Santos MM, Fonseca-Silva A, Valentao P, Andrade PB, Oliveira JMA. How mitochondrial dysfunction affects zebrafish development and cardiovascular function: an in vivo model for testing mitochondria-targeted drugs. Br J Pharmacol. England; 2013;169: 1072–1090. doi:10.1111/bph.12186

78. Polyak E, Ostrovsky J, Peng M, Dingley SD, Tsukikawa M, Kwon YJ, et al. N-acetylcysteine and vitamin E rescue animal longevity and cellular oxidative stress in pre-clinical models of mitochondrial complex I disease. Mol Genet Metab. United States; 2018;123: 449–462. doi:10.1016/j.ymgme.2018.02.013

79. Ma AC, McNulty MS, Poshusta TL, Campbell JM, Martinez-Galvez G, Argue DP, et al. FusX: A Rapid One-Step Transcription Activator-Like Effector Assembly System for Genome Science. Hum Gene Ther. United States; 2016;27: 451–463. doi:10.1089/hum.2015.172

80. Clark KJ, Balciunas D, Pogoda H-M, Ding Y, Westcot SE, Bedell VM, et al. In vivo protein trapping produces a functional expression codex of the vertebrate proteome. Nat Methods. United States; 2011;8: 506–515. doi:10.1038/nmeth.1606

81. Neff KL, Argue DP, Ma AC, Lee HB, Clark KJ, Ekker SC. Mojo Hand, a TALEN design tool for genome editing applications. BMC Bioinformatics. England; 2013;14: 1. doi:10.1186/1471-2105-14-1

82. Clark KJ, Geurts AM, Bell JB, Hackett PB. Transposon vectors for gene-trap insertional mutagenesis in vertebrates. Genesis. United States; 2004;39: 225–233. doi:10.1002/gene.20049

83. Clark KJ, Argue DP, Petzold AM, Ekker SC. zfishbook: connecting you to a world of zebrafish revertible mutants. Nucleic Acids Res. England; 2012;40: D907-11. doi:10.1093/nar/gkr957

84. Sharma LK, Lu J, Bai Y. Mitochondrial respiratory complex I: structure, function and implication in human diseases. Curr Med Chem. United Arab Emirates; 2009;16: 1266–

1277.

85. Liu Y, Fiskum G, Schubert D. Generation of reactive oxygen species by the mitochondrial electron transport chain. J Neurochem. England; 2002;80: 780–787.

86. King MP, Attardi G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science. United States; 1989;246: 500–503.

87. Koga Y, Davidson M, Schon EA, King MP. Analysis of cybrids harboring MELAS mutations in the mitochondrial tRNA(Leu(UUR)) gene. Muscle Nerve Suppl. United States; 1995;3: S119-23.

88. Vithayathil SA, Ma Y, Kaipparettu BA. Transmitochondrial cybrids: tools for functional studies of mutant mitochondria. Methods Mol Biol. United States; 2012;837: 219–230. doi:10.1007/978-1-61779-504-6_15

89. Wilkins HM, Carl SM, Swerdlow RH. Cytoplasmic hybrid (cybrid) cell lines as a practical model for mitochondriopathies. Redox Biol. Netherlands; 2014;2: 619–631. doi:10.1016/j.redox.2014.03.006

90. Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug RG 2nd, et al. In vivo genome editing using a high-efficiency TALEN system. Nature. England; 2012;491: 114–118. doi:10.1038/nature11537

91. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol. United States; 2013;31: 227–229. doi:10.1038/nbt.2501

92. Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP. Demonstration of

CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res. England; 2013;41: e188. doi:10.1093/nar/gkt780

93. Fujihara Y, Ikawa M. CRISPR/Cas9-based genome editing in mice by single plasmid injection. Methods Enzymol. United States; 2014;546: 319–336. doi:10.1016/B978-0-12-801185-0.00015-5

94. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. United States; 2014;157: 1262–1278. doi:10.1016/j.cell.2014.05.010

95. Zhang F, Wen Y, Guo X. CRISPR/Cas9 for genome editing: progress, implications and challenges. Hum Mol Genet. England; 2014;23: R40-6. doi:10.1093/hmg/ddu125

96. Hashimoto M, Bacman SR, Peralta S, Falk MJ, Chomyn A, Chan DC, et al. MitoTALEN: A General Approach to Reduce Mutant mtDNA Loads and Restore Oxidative Phosphorylation Function in Mitochondrial Diseases. Mol Ther. United States; 2015;23: 1592–1599. doi:10.1038/mt.2015.126

97. Niu D, Wei H-J, Lin L, George H, Wang T, Lee I-H, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science. United States; 2017;357: 1303–1307. doi:10.1126/science.aan4187

98. Meimaridou E, Kowalczyk J, Guasti L, Hughes CR, Wagner F, Frommolt P, et al. Mutations in NNT encoding nicotinamide nucleotide transhydrogenase cause familial glucocorticoid deficiency. Nat Genet. United States; 2012;44: 740–742. doi:10.1038/ng.2299

99. Bainbridge MN, Davis EE, Choi W-Y, Dickson A, Martinez HR, Wang M, et al. Loss of Function Mutations in NNT Are Associated With Left Ventricular Noncompaction. Circ Cardiovasc Genet. United States; 2015;8: 544–552. doi:10.1161/CIRCGENETICS.115.001026

100. Anichtchik O, Diekmann H, Fleming A, Roach A, Goldsmith P, Rubinsztein DC. Loss of PINK1 function affects development and results in neurodegeneration in zebrafish. J Neurosci. United States; 2008;28: 8199–8207. doi:10.1523/JNEUROSCI.0979-08.2008

101. Fett ME, Pilsl A, Paquet D, van Bebber F, Haass C, Tatzelt J, et al. Parkin is protective against proteotoxic stress in a transgenic zebrafish model. PLoS One. United States; 2010;5: e11783. doi:10.1371/journal.pone.0011783

102. Sallinen V, Kolehmainen J, Priyadarshini M, Toleikyte G, Chen Y-C, Panula P. Dopaminergic cell damage and vulnerability to MPTP in Pink1 knockdown zebrafish. Neurobiol Dis. United States; 2010;40: 93–101. doi:10.1016/j.nbd.2010.06.001

103. Xi Y, Ryan J, Noble S, Yu M, Yilbas AE, Ekker M. Impaired dopaminergic neuron development and locomotor function in zebrafish with loss of pink1 function. Eur J Neurosci. France; 2010;31: 623–633. doi:10.1111/j.1460-9568.2010.07091.x

104. Kumar MG, Rowley S, Fulton R, Dinday MT, Baraban SC, Patel M. Altered Glycolysis and Mitochondrial Respiration in a Zebrafish Model of Dravet Syndrome. eNeuro. United States; 2016;3. doi:10.1523/ENEURO.0008-16.2016

105. Baraban SC, Dinday MT, Hortopan GA. Drug screening in Scn1a zebrafish mutant identifies clemizole as a potential Dravet syndrome treatment. Nat Commun. England; 2013;4: 2410. doi:10.1038/ncomms3410

106. Grone BP, Qu T, Baraban SC. Behavioral Comorbidities and Drug Treatments in a Zebrafish scn1lab Model of Dravet Syndrome. eNeuro. United States; 2017;4. doi:10.1523/ENEURO.0066-17.2017

107. Guernsey DL, Jiang H, Campagna DR, Evans SC, Ferguson M, Kellogg MD, et al. Mutations in mitochondrial carrier family gene SLC25A38 cause nonsyndromic autosomal recessive congenital sideroblastic anemia. Nat Genet. United States; 2009;41: 651–653. doi:10.1038/ng.359

108. Bronnimann D, Annese T, Gorr TA, Djonov V. Splitting of circulating red blood cells as an in vivo mechanism of erythrocyte maturation in developing zebrafish, chick and mouse embryos. J Exp Biol. England; 2018;221. doi:10.1242/jeb.184564

109. Berman J, Hsu K, Look AT. Zebrafish as a model organism for blood diseases. Br J Haematol. England; 2003;123: 568–576.

110. Danilova N, Sakamoto KM, Lin S. Ribosomal protein S19 deficiency in zebrafish leads to developmental abnormalities and defective erythropoiesis through activation of p53 protein family. Blood. United States; 2008;112: 5228–5237. doi:10.1182/blood-2008-01-132290

111. Butler MG, Gore A V, Weinstein BM. Zebrafish as a model for hemorrhagic stroke. Methods Cell Biol. United States; 2011;105: 137–161. doi:10.1016/B978-0-12-381320-6.00006-0

112. Kulkeaw K, Sugiyama D. Zebrafish erythropoiesis and the utility of fish as models of anemia. Stem Cell Res Ther. England; 2012;3: 55. doi:10.1186/scrt146

113. Kretz CA, Weyand AC, Shavit JA. Modeling Disorders of Blood Coagulation in the Zebrafish. Curr Pathobiol Rep. United States; 2015;3: 155–161. doi:10.1007/s40139-015-0081-3

114. Avagyan S, Zon LI. Fish to Learn: Insights into Blood Development and Blood Disorders from Zebrafish Hematopoiesis. Hum Gene Ther. United States; 2016;27: 287–294. doi:10.1089/hum.2016.024

115. Prezant TR, Agapian J V, Bohlman MC, Bu X, Oztas S, Qiu WQ, et al. Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. Nat Genet. United States; 1993;4: 289–294. doi:10.1038/ng0793-289

116. Zhang Q, Zhang L, Chen D, He X, Yao S, Zhang Z, et al. Deletion of Mtu1 (Trmu) in zebrafish revealed the essential role of tRNA modification in mitochondrial biogenesis and hearing function. Nucleic Acids Res. England; 2018;46: 10930–10945. doi:10.1093/nar/gky758

117. DiMauro S, Schon EA. Mitochondrial respiratory-chain diseases. N Engl J Med. United States; 2003;348: 2656–2668. doi:10.1056/NEJMra022567

118. Jain IH, Zazzeron L, Goli R, Alexa K, Schatzman-Bone S, Dhillon H, et al. Hypoxia as a therapy for mitochondrial disease. Science. United States; 2016;352: 54–61. doi:10.1126/science.aad9642

119. Bretaud S, Lee S, Guo S. Sensitivity of zebrafish to environmental toxins implicated in Parkinson's disease. Neurotoxicol Teratol. United States; 2004;26: 857–864. doi:10.1016/j.ntt.2004.06.014

120. Byrnes J, Ganetzky R, Lightfoot R, Tzeng M, Nakamaru-Ogiso E, Seiler C, et al. Pharmacologic modeling of primary mitochondrial respiratory chain dysfunction in zebrafish. Neurochem Int. England; 2018;117: 23–34. doi:10.1016/j.neuint.2017.07.008

121. Xu Y, Chen M, Hu B, Huang R, Hu B. In vivo Imaging of Mitochondrial Transport in Single-Axon Regeneration of Zebrafish Mauthner Cells. Front Cell Neurosci. Switzerland; 2017;11: 4. doi:10.3389/fncel.2017.00004

122. Dowling JJ, Arbogast S, Hur J, Nelson DD, McEvoy A, Waugh T, et al. Oxidative stress and successful antioxidant treatment in models of RYR1-related myopathy. Brain. England; 2012;135: 1115–1127. doi:10.1093/brain/aws036

123. Pickett SB, Thomas ED, Sebe JY, Linbo T, Esterberg R, Hailey DW, et al. Cumulative mitochondrial activity correlates with ototoxin susceptibility in zebrafish mechanosensory hair cells. Elife. England; 2018;7. doi:10.7554/eLife.38062

124. Mandal A, Pinter K, Drerup CM. Analyzing Neuronal Mitochondria in vivo Using Fluorescent Reporters in Zebrafish. Front cell Dev Biol. Switzerland; 2018;6: 144. doi:10.3389/fcell.2018.00144

125. Liu T-L, Upadhyayula S, Milkie DE, Singh V, Wang K, Swinburne IA, et al. Observing the cell in its native state: Imaging subcellular dynamics in multicellular organisms. Science. United States; 2018;360. doi:10.1126/science.aaq1392

## Figures Legends:

**Figure 1: Circular representation of the zebrafish (NC_002333.2) and human mitochondrial genomes (NC_012920.1):** Both genomes share the same synteny, number of genes and are nearly identical in size.

**Figure 2: Zebrafish Marriot mitochondrial mutant collection:** Schematic representation of various mitochondrial resident pathways for which zebrafish mutants were generated. The nuclear-encoded mitochondrial proteins have been illustrated according to the function they are involved in mitochondrial maintenance and homeostasis. Mutants generated by gene editing are depicted as grey, whereas those generated by the GBT system are depicted in pink.

**Figure 3: A depiction of homology of the mutants created for proteins involved in different mitochondrial resident pathways: A-C: Complex 1 of mitochondrial respiratory chain;** A: NADH: ubiquinone oxidoreductase subunit AB1 (Ndufab1b); B: NADH dehydrogenase association factor 6 (Ndufaf6); C: NADH dehydrogenase iron sulfur protein 4 (Ndufs4); **D: Complex 2 of mitochondrial respiratory chain,** Succinate dehydrogenase subunit A (Sdha), subunit; **E-F: Complex 3 of mitochondrial respiratory chain;** E: Mitochondrial chaperone protein BCS1 (Bcs1l); F: Ubiquinol-cytochrome c reductase subunit VIII (Uqcrq); **G-I: Complex 4 of mitochondrial respiratory chain;** G: Protoheme IX farnesyltransferase (Cox10); H: Cytochrome c oxidase IV subunit 1 (Cox4i2b); I: Surfeit locus protein 1 (Surf1); **J-K: Complex 5 of mitochondrial respiratory chain;** J: ATP synthase subunit epsilon (Atp5e); K: Transmembrane protein 70 (Tmem70); **L-M: Biosynthesis of coenzyme Q;** L: 4-hydroxybenzoate polyprenyltransferase (Coq2) M: Decaprenyl-disphosphate synthase subunit 2 (Pdss2); **N-Q: Mitochondrial calcium homeostasis;** N: Mitochondrial calcium uptake protein 1

(Micu1); O: Calcium uniporter protein (Mcu) generated by TALEN and P; GBT; Q: Essential MCU regulator (Smdt1); **R-U: Mitochondrial genome maintenance;** R: Thymidine kinase 2 (Tk2); S: Leucine-rich pentatricopeptide repeat-containing (Lrpprc); T: Mitochondrial methionyl-tRNA formyltransferase (Mtfmt); U: 28s Ribosomal protein s18b, mitochondrial (Mrps18b); **V: Mitochondrial protein import;** Mitochondrial import inner membrane translocase subunit TIM50 (Timm50); **W-X: Mitochondrial metabolite synthesis;** W: 2-Oxoglutarate dehydrogenase (Ogdh); X: Isocitrate dehydrogenase (Idh2).

# Figures:

## Figure 1:

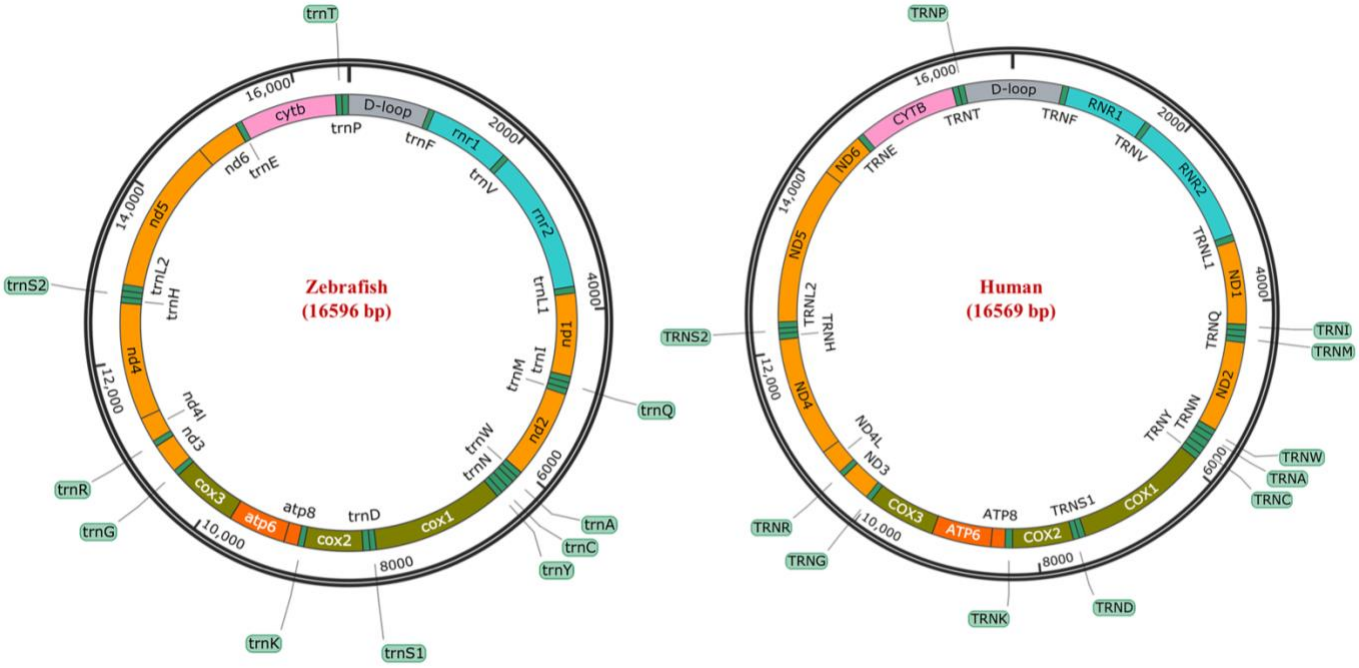


**Figure 2:**

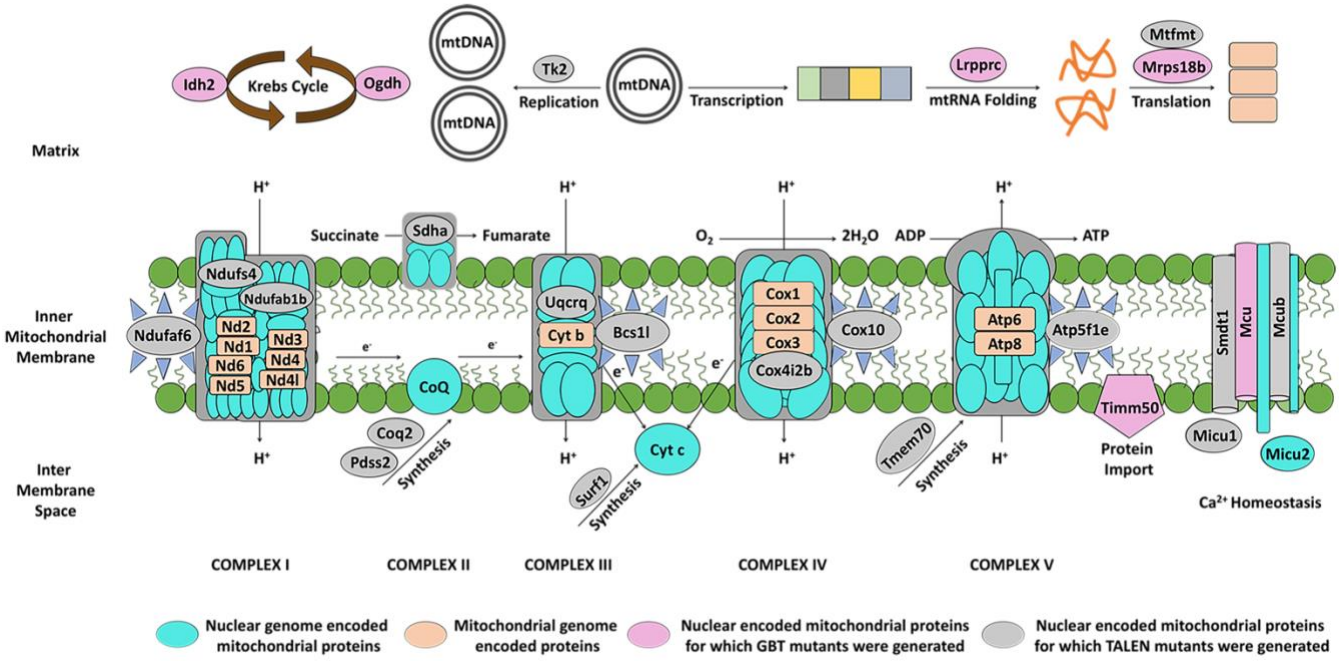
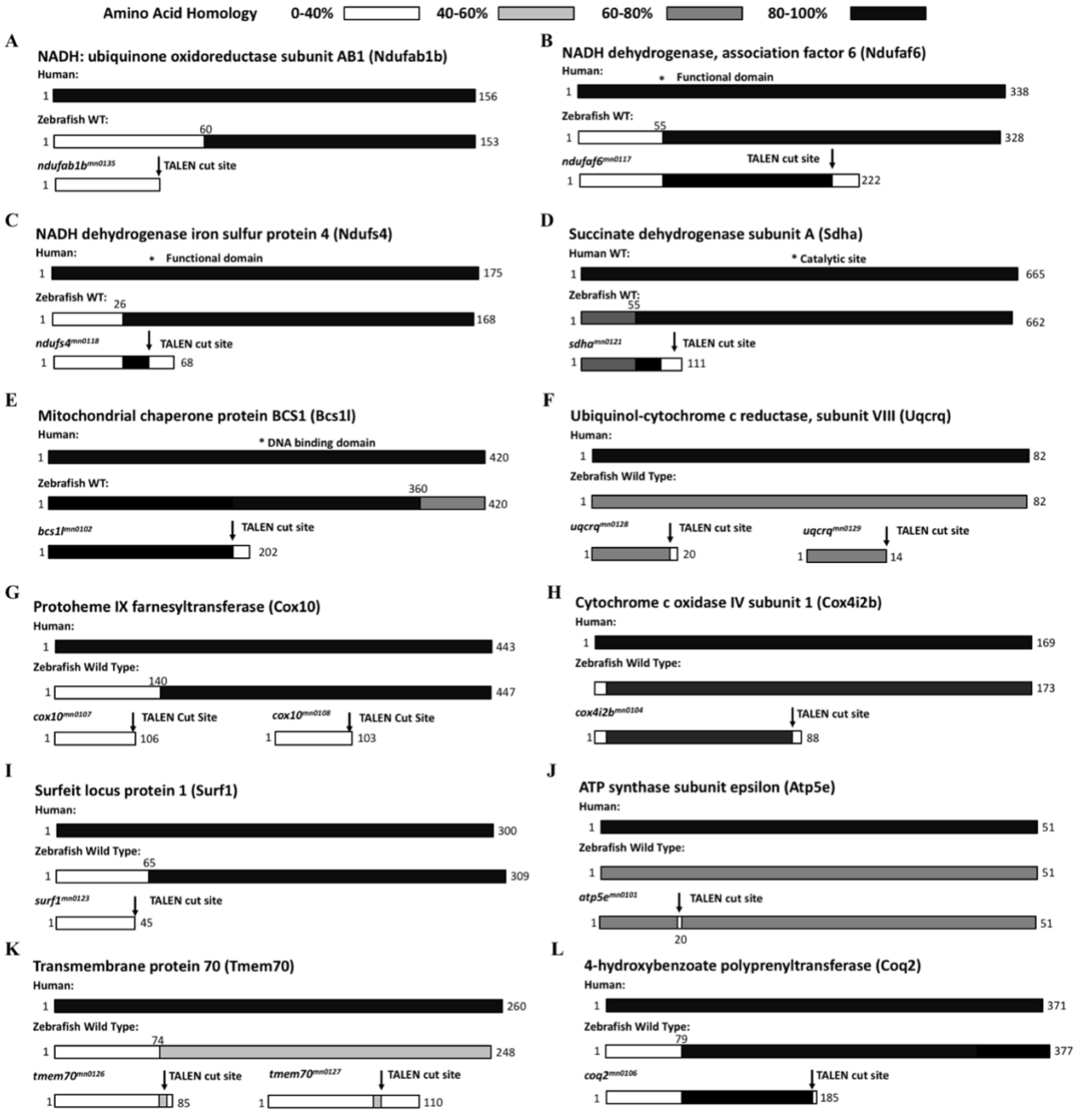
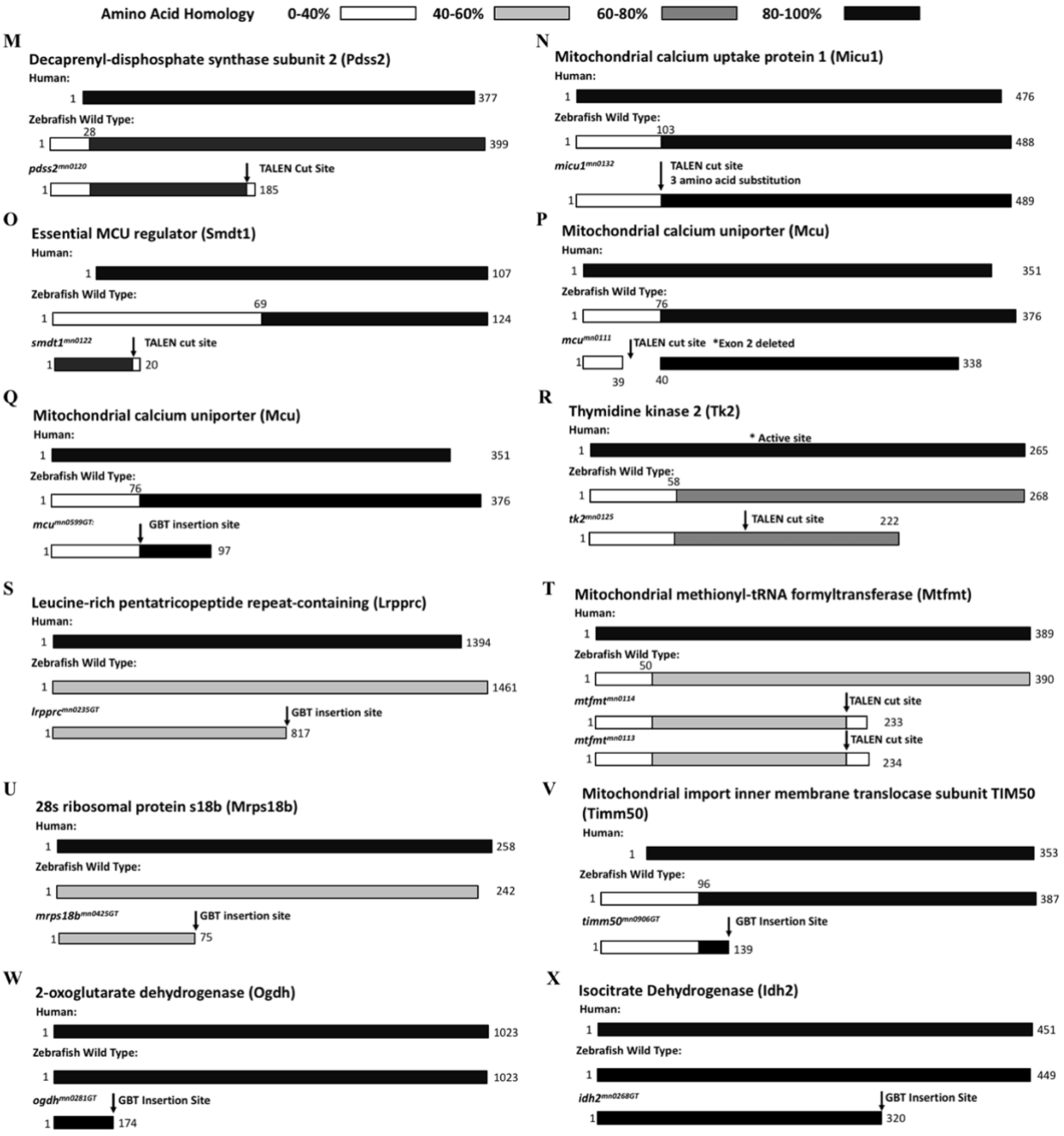

Matrix
Idh2
Krebs Cycle
Ogdh
mtDNA
mtDNA
Tk2
Replication
mtDNA
Transcription
Lrpprc
mtRNA Folding
Mtfmt
Mrps18b
Translation
Inner Mitochondrial Membrane
Inter Membrane Space
H+
Succinate
Sdha
Fumarate
O2
2H2O
ADP
ATP
Ndufs4
Ndufab1b
Ndufaf6
Nd2
Nd1
Nd3
Nd6
Nd4
Nd5
Nd4l
CoQ
Coq2
Pdss2
Synthesis
Uqcrq
Cyt b
Bcs1l
Cyt c
Surf1
Synthesis
Cox1
Cox2
Cox3
Cox4i2b
Cox10
Tmem70
Synthesis
Atp6
Atp8
Atp5f1e
Timm50
Protein Import
Smdt1
Mcu
Mcub
Micu1
Micu2
Ca2+ Homeostasis
COMPLEX I
COMPLEX II
COMPLEX III
COMPLEX IV
COMPLEX V
Nuclear genome encoded mitochondrial proteins
Mitochondrial genome encoded proteins
Nuclear encoded mitochondrial proteins for which GBT mutants were generated
Nuclear encoded mitochondrial proteins for which TALEN mutants were generated

**Figure 3:**


Amino Acid Homology
0-40%
40-60%
60-80%
80-100%
A
NADH: ubiquinone oxidoreductase subunit AB1 (Ndufab1b)
Human:
1
156
Zebrafish WT:
60
1
153
ndufab1b mn0135
TALEN cut site
1
B
NADH dehydrogenase, association factor 6 (Ndufaf6)
Human:
* Functional domain
1
338
Zebrafish WT:
55
1
328
ndufaf6 mn0117
TALEN cut site
1
222
C
NADH dehydrogenase iron sulfur protein 4 (Ndufs4)
Human:
* Functional domain
1
175
Zebrafish WT:
26
1
168
ndufs4 mn0118
TALEN cut site
1
68
D
Succinate dehydrogenase subunit A (Sdha)
Human WT:
* Catalytic site
1
665
Zebrafish WT:
55
1
662
sdha mn0121
TALEN cut site
1
111
E
Mitochondrial chaperone protein BCS1 (Bcs1l)
Human:
* DNA binding domain
1
420
Zebrafish WT:
360
1
420
bcs1l mn0102
TALEN cut site
1
202
F
Ubiquinol-cytochrome c reductase, subunit VIII (Uqcrq)
Human:
1
82
Zebrafish Wild Type:
1
82
uqcrq mn0128
TALEN cut site
1
20
uqcrq mn0129
TALEN cut site
1
14
G
Protoheme IX farnesyltransferase (Cox10)
Human:
1
443
Zebrafish Wild Type:
140
1
447
cox10 mn0107
TALEN Cut Site
1
106
cox10 mn0108
TALEN Cut Site
1
103
H
Cytochrome c oxidase IV subunit 1 (Cox4i2b)
Human:
1
169
Zebrafish Wild Type:
173
cox4i2b mn0104
TALEN cut site
1
88
I
Surfeit locus protein 1 (Surf1)
Human:
1
300
Zebrafish Wild Type:
65
1
309
surf1 mn0123
TALEN cut site
1
45
J
ATP synthase subunit epsilon (Atp5e)
Human:
1
51
Zebrafish Wild Type:
1
51
atp5e mn0101
TALEN cut site
1
20
51
K
Transmembrane protein 70 (Tmem70)
Human:
1
260
Zebrafish Wild Type:
74
1
248
tmem70 mn0126
TALEN cut site
1
85
tmem70 mn0127
TALEN cut site
1
110
L
4-hydroxybenzoate polyprenyltransferase (Coq2)
Human:
1
371
Zebrafish Wild Type:
79
1
377
coq2 mn0106
TALEN cut site
1
185


Amino Acid Homology
0-40%
40-60%
60-80%
80-100%
M
Decaprenyl-disphosphate synthase subunit 2 (Pdss2)
Human:
1
377
Zebrafish Wild Type:
28
1
399
pdss2mn0120
TALEN Cut Site
1
185
N
Mitochondrial calcium uptake protein 1 (Micu1)
Human:
1
476
Zebrafish Wild Type:
103
1
488
micu1mn0132
TALEN cut site
3 amino acid substitution
1
489
O
Essential MCU regulator (Smdt1)
Human:
1
107
Zebrafish Wild Type:
69
1
124
smdt1mn0122
TALEN cut site
1
20
P
Mitochondrial calcium uniporter (Mcu)
Human:
1
351
Zebrafish Wild Type:
76
1
376
mcumn0111
TALEN cut site
*Exon 2 deleted
1
39
40
338
Q
Mitochondrial calcium uniporter (Mcu)
Human:
1
351
Zebrafish Wild Type:
76
1
376
mcumn0599GT:
GBT insertion site
1
97
R
Thymidine kinase 2 (Tk2)
Human:
* Active site
1
265
Zebrafish Wild Type:
58
1
268
tk2mn0125
TALEN cut site
222
1
S
Leucine-rich pentatricopeptide repeat-containing (Lrpprc)
Human:
1
1394
Zebrafish Wild Type:
1
1461
lrpprcmn0235GT
GBT insertion site
1
817
T
Mitochondrial methionyl-tRNA formyltransferase (Mtfmt)
Human:
1
389
Zebrafish Wild Type:
50
1
390
mtfmtmn0114
TALEN cut site
1
233
mtfmtmn0113
TALEN cut site
1
234
U
28s ribosomal protein s18b (Mrps18b)
Human:
1
258
Zebrafish Wild Type:
1
242
mrps18bmn0425GT
GBT insertion site
1
75
V
Mitochondrial import inner membrane translocase subunit TIM50 (Timm50)
Human:
1
353
Zebrafish Wild Type:
96
1
387
timm50mn0906GT
GBT Insertion Site
1
139
W
2-oxoglutarate dehydrogenase (Ogdh)
Human:
1
1023
Zebrafish Wild Type:
1
1023
ogdhmn0281GT
GBT Insertion Site
1
174
X
Isocitrate Dehydrogenase (Idh2)
Human:
1
451
Zebrafish Wild Type:
1
449
idh2mn0268GT
GBT Insertion Site
1
320

866