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Research Paper

Codon optimization is an essential parameter for the efficient allotopic expression of mtDNA genes☆

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ABSTRACT

Mutations in mitochondrial DNA can be inherited or occur *de novo* leading to several debilitating myopathies with no curative option and few or no effective treatments. Allotopic expression of recoded mitochondrial genes from the nucleus has potential as a gene therapy strategy for such conditions, however progress in this field has been hampered by technical challenges. Here we employed codon optimization as a tool to re-engineer the protein-coding genes of the human mitochondrial genome for robust, efficient expression from the nucleus. All 13 codon-optimized constructs exhibited substantially higher protein expression than minimally-recoded genes when expressed transiently, and steady-state mRNA levels for optimized gene constructs were 5–180 fold enriched over recoded versions in stably-selected wildtype cells. Eight of thirteen mitochondria-encoded oxidative phosphorylation (OxPhos) proteins maintained protein expression following stable selection, with mitochondrial localization of expression products. We also assessed the utility of this strategy in rescuing mitochondrial disease cell models and found the rescue capacity of allotopic expression constructs to be gene specific. Allotopic expression of codon optimized ATP8 in disease models could restore protein levels and respiratory function, however, rescue of the pathogenic phenotype for another gene, ND1 was only partially successful. These results imply that though codon-optimization alone is not sufficient for functional allotopic expression of most mitochondrial genes, it is an essential consideration in their design.

1. Introduction

Approximately 1 in 5000 individuals is affected by mitochondrial disease, a heterogeneous group of conditions caused by mutations in any of the > 1400 genes encoding components of the organelle responsible for aerobic respiration [1–5]. While the vast majority of mitochondrial proteins are encoded by the nuclear genome, translated in the cytosol, and imported into the mitochondrion, 13 core subunits of respiratory complexes are encoded by the reduced mitochondrial genome and synthesized within the mitochondrial matrix. Mutations in these 13 genes (or their associated non-protein-coding genes) tend to be especially severe, as all 13 proteins are core subunits of the OxPhos chain, and any disruption to subunit structure, stability, or function may have grave biochemical and physiological consequences [6]. Phenotypes, onset, and severity are variable [7,8], and range from mild symptoms such as exercise fatigue [9] to neonatal lethality [10].

The prospect of targeted mitochondrial therapies is therefore of great clinical interest, and a number of potential therapeutic approaches have been studied, including the use of small molecule drugs to combat symptoms [11,12], selective mitophagy to remove damaged

Abbreviations: ARMS qPCR, Amplification Refractory Mutation System-quantitative PCR; mtDNA, mitochondrial DNA; OxPhos, oxidative phosphorylation; MTS, mitochondrial targeting sequence; MELAS, mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes; LHON, Leber's Hereditary Optic Neuropathy; NARP, Neuropathy, Ataxia and Retinitis Pigmentosa; o, optimized; r, minimally-recoded; HEK293, human embryonic kidney cells; CI, Complex I; CIII, Complex III; CIV, Complex IV; CV, Complex V; BN-PAGE, Blue Native-PAGE; MPP, matrix processing peptidase; TIM, translocase of the inner membrane; IMS, intermembrane space; PMSF, phenylmethylsulfonyl fluoride; PIM, protease inhibitor cocktail; MIB, mitochondria isolation buffer; RSCU, relative synonymous codon use

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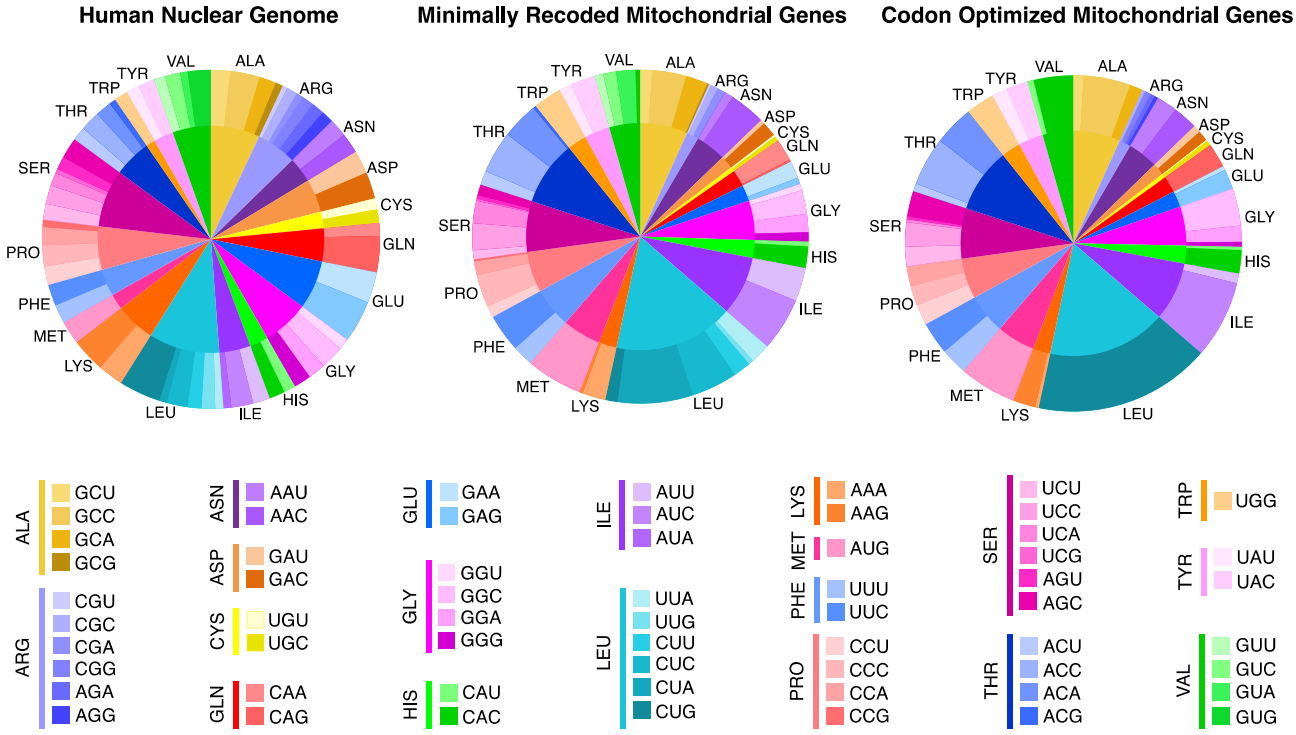

Fig. 1. Amino acid composition and codon use. Overall amino acid composition and codon frequencies for genes in the human nucleus and for minimally-recoded and codon-optimized mitochondrial genes. Represented as the fraction of global amino acid (inner circle) and codon (outer circle) use in CDSs of the nuclear genome and in each set of recoded or codon-optimized mitochondrial genes.

mitochondria [13–15] mitochondrial replacement therapy (MRT) for fertility treatments [16–18], and correction of mitochondrial mutations with gene editing tools such as mitoTALENS [19–23].

Gene therapy to target affected mitochondrial subunits is a promising alternative strategy which circumvents some of the technical challenges faced by the above approaches. One issue that remains, however, relates to the prokaryotic origin of the organelle. Translation within the mitochondrion deviates from the universal genetic code, utilizing machinery and codon frequencies more similar to its α-proteobacterial ancestry than to the mammalian nuclear genome [24–26]. Studies exploring the ability to recode mitochondrial genes to the universal genetic code originated in the 1980s with the work of Phillip Nagley and Rodney Devenish, who chemically synthesized versions of the yeast homologs of *ATP8* [27] and *ATP6* [28,29] for cytosolic expression. It was following these initial studies that the concept of allotopic expression was introduced, first as a research tool to probe the import and assembly of mitochondrial proteins and later for functional complementation studies.

Subsequently, allotopic expression has been suggested as a therapeutic tool to genetically remedy deleterious mtDNA mutations through nuclear complementation of the affected genes [30]. Since this insight, multiple attempts have been made to express mitochondrial genes allotopically, however results have been inconsistent and, at times, contradictory [28,31–38]. Because of this inconsistency in successful expression, modified approaches have been used to evaluate the influence of specific parameters, such as lowering mean hydrophobicity to improve protein import [39–42], utilization of specific upstream and downstream noncoding sequences [43–48], systematic testing of targeting sequence efficiencies [49,50], transkingdom expression [51–59] or piecewise import of proteins [38,60]. A critical, but often-overlooked consideration in these nuclear relocation studies, however, is the influence of the primary coding sequence on protein production.

The vast majority of these previous studies have utilized what may be considered “minimally-recoded” mitochondrial genes, wherein the only bases changed are those which differ from the universal genetic code (Trp, Met, Stop), often achieved through site-directed mutagenesis after cloning from mtDNA. While making these codon changes is essential to maintain amino acid sequence integrity during cytosolic translation, this minimal approach fails to account for other elements of primary sequence which can critically influence both gene and protein expression.

Extensive research has been conducted to determine optimal conditions for efficient heterologous protein production in various organisms, particularly for manufacturing industrial materials and biologics such as therapeutic proteins, monoclonal antibodies and enzymes. Because production at an industrial scale is often done in a transkingdom host, the target genes must not only be optimized for high levels of expression, but also adapted for efficient translation using non-native host machinery. In recombinant protein production, for example, it is well-known that the primary sequence can dramatically influence expression of a target, although the precise determinants of this effect remain poorly understood. It is accepted, however, that elements including the relative frequencies of codon use for each amino acid, local and global GC composition, mRNA secondary structure stability, and the presence of cryptic termination signals or splice sites are significant factors impacting the degree of protein expression and thus in the observed effect of synonymous codon changes [61–66]. Many commercial algorithms have therefore been developed to determine the optimal sequence and conditions for expression of a gene from a particular host. Though there are concerns regarding the use of codon optimization to increase homologous expression of a nuclear gene, such as the generation of novel or immunogenic peptides or structural perturbations in the encoded protein (reviewed by [67,68]), clinical gene therapy using a codon-optimized exogenous construct to compensate for mutations in a nuclear gene (e.g. hemophilia, [69]) is ongoing, and codon optimization continues to be widely utilized for the production of biotherapeutics.

Applying this principle to allotopic expression, we hypothesize that, given the bacterial origin of the mitochondrial genome, the coding sequences of minimally-recoded mitochondrial genes are dissimilar from

nuclear genes and are inefficiently translated by nuclear machinery, therefore resulting in poor allotopic expression (Fig. 1, Table S1). Viewing the human nucleus as a heterologous host for mitochondrial genes, we believe that codon optimizing these sequences may reduce the translational barrier caused by evolutionary divergence and facilitate functional protein production. Ironically, it was Nagley's original work engineering a yeast homolog of ATP8 in which his group first "optimized" for species codon use in a small gene cassette [27], hand-selecting codons for each amino acid based on their respective nuclear frequencies. Although they did not perform an extensive comparison of codon optimization vs minimal recoding, subsequent work in the field seems to have overlooked this important consideration almost entirely. One exception is the allotopic expression of the ND6 gene in a mouse model functionally null for ND6 ($ND6d^{KO}$), wherein the authors used Backtranslation-Tool v2 to generate optimal mouse nuclear expression constructs [47].

Here, we tested the allotopic expression of minimally-recoded and codon-optimized gene constructs for all 13 subunits of the respiratory chain encoded by the human mtDNA. We find that while several minimally-recoded genes will express transiently at low levels, all genes which have been codon-optimized for nuclear expression demonstrate robust transient expression, with proteins targeted to the mitochondria. Several of the optimized gene constructs express stably when integrated into nuclear DNA, and are targeted to the mitochondria. Furthermore, we tested this function in cell lines *null* for human ND1 and ATP8 subunits and a point mutant of mouse *mt-Atp8* and found that protein products from codon-optimized genes are capable of assembling into OxPhos complexes. From these data, we conclude that nuclear codon optimization is a necessary element for the successful allotopic expression of mitochondrial genes.

2. Results

2.1. Codon-optimization of mitochondrial genes enhances transient expression in mammalian cells

In this study, we are the first to demonstrate the application of codon-optimization, typically employed in heterologous expression systems, for the allotopic expression of all 13 human mitochondrial genes. We hypothesized that optimizing the codon usage of mtDNA genes for host nuclear expression is an important design consideration for successful allotopic expression. To test the influence of primary sequence optimization on cytosolic expression, we synthesized minimally nuclear-recoded (r) and codon-optimized (o) versions for the 13 human mitochondrial-encoded protein subunits, appended with an N-terminal MTS from nuclear-encoded Complex V subunit ATP5G1 to facilitate transgene localization [36] and a C-terminal FLAG tag for immuno-detection (Fig. 2A). For Complex I subunit ND6, only expression of the codon-optimized version (oND6) was tested, as the minimally-recoded version repeatedly failed synthesis for unknown reasons. We found that while only 3 of the 13 minimally-recoded mitochondrial genes (*rND3*, *rCOX1*, and *rATP8*) produced proteins detectable in mitochondrial fractions of transiently transfected HEK293 cells, all 13 codon-optimized genes resulted in exogenous mitochondrially-enriched proteins as judged by SDS-PAGE (Fig. 2B). Furthermore, paired mitochondrial lysates from cells transfected with minimally-recoded and codon-optimized constructs demonstrate that optimized genes result in substantially higher production of the desired protein product, implying that codon optimization of transgenes greatly enhances the transient expression of allotopically expressed proteins.

2.2. Codon-optimization is required for stable protein expression from allotopic genes in mammalian cells

It is well known that although many genes express transiently in cell culture, with plasmid copy numbers contributing to robust levels of protein expression, this often does not equate to similarly robust expression after stable nuclear integration. Additionally, while these data demonstrate that allotopically expressed genes are effectively expressed, translated, and targeted *in vitro* under transient expression conditions, the goal of a gene-therapy approach is the sustained benefit achieved by integration of the recombinant gene into nuclear DNA. Therefore, stable cell lines were generated to stably express the minimally-recoded or codon-optimized transgene from the nuclear genome.

We next sought to determine if the observed differences in transient protein expression between minimally-recoded and codon-optimized constructs are mirrored in their relative mRNA transcript abundance in stably expressing cells. Using whole cell mRNA from stably-selected HEK293 cells expressing either the nuclear-recoded or codon-optimized versions of each mitochondrial gene, we quantified the expression level of each gene by qPCR, normalized to the mRNA level of nuclear-encoded Complex IV subunit *COX10* (Fig. 3A). We found that in every case, transcripts for the codon-optimized gene was substantially higher than its minimally-recoded counterpart, with differences ranging from 5-180 fold between pairs. Both minimally-recoded and codon-optimized versions of each gene express at a level at least equivalent to endogenous *COX10*, indicating that any absence of detectable protein under stable conditions is not due to failed construct integration or silencing of the ORF promoter region. Additionally, despite dramatic differences in transcription between optimized and recoded versions, the majority of mRNAs are equal or one to two orders of magnitude lower in expression than the mRNA of housekeeping gene *GAPDH* (Fig. 3B), and the most robustly expressed optimized genes are present at no more than 1.38 times *GAPDH* (for oND3). From this we conclude that the observed differences in allotopically expressed protein levels are not due to gross overexpression of optimized transgenes, but likely due to increased translational efficiency and/or stability of the mRNA.

We next sought to determine whether the increased mRNA transcription of optimized constructs is also reflected in stable protein expression. We found that despite robust expression in transiently transfected HEK293 cells, codon-optimized constructs for oND5, oCYB, oCOX1, oCOX3, and oATP6 failed to maintain strong protein expression in mitochondria-enriched fractions from stably selected HEK293 cells. However, codon-optimized versions of the other 8 mitochondrially-encoded subunits maintained protein production stably, including 6 of the 7 Complex I (CI) subunits (oND1, oND2, oND3, oND4, oND4L, and oND6), Complex IV (CIV) subunit oCOX2, and oATP8 of Complex V (CV) (Fig. 3C). We found that of the minimally-recoded versions for each gene, only rND4 had any appreciable protein expression, though with detectable levels still well below its codon-optimized counterpart. Our present work acknowledges that while select minimally-recoded genes express protein transiently, no minimally-recoded gene produced a reliably detectable protein product following stable selection in mitochondrial fractions (Fig. 3C) aside from rND4.

Additionally, upon further examination of Western blot signals, we noted that more than one product was detected for many of the allotopically expressed proteins. Each construct tested was appended with an N-terminal mitochondrial targeting sequence (MTS) (from the ATP5G1 sequence), which is expected to be cleaved upon import to the mitochondrial matrix [36]. We therefore hypothesized that the additional protein signal may indicate a failed or inefficient MTS cleavage step following import. For oND1, oND3, and oATP8, the predominant band appeared to be the processed form, while oND2, oND4, and oND6 contained substantial amounts of both processed and unprocessed allotopically expressed proteins. Further, oND4L and oCOX2 appear to be overwhelmingly unprocessed (Fig. S1). In the case of oND4L and oCOX2, the majority of the protein in mitochondrial fractions appeared to be in the unprocessed form (Fig. S1; shown for oCOX2), while protein products of the remaining six stably expressed genes appeared to be predominantly cleaved (ND1, ND2, ND3, ND4, ND6, and ATP8) (Fig. 3C).

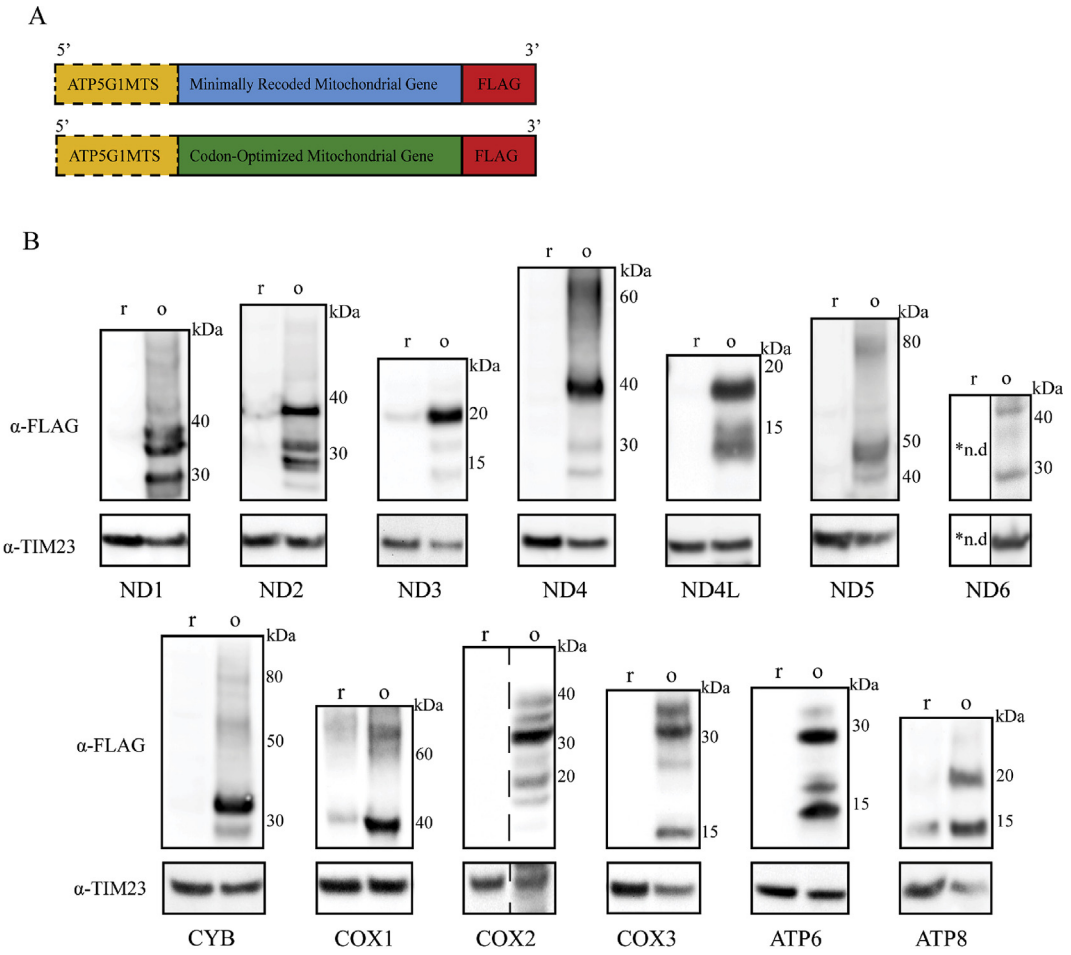

Fig. 2. Transient expression of mitochondrial gene constructs in HEK293 cells.
(A) Schematic of minimally-recoded (r) and codon-optimized (o) mtDNA gene constructs. All constructs were appended at the 5' end with the coding sequence for the ATP5G1 MTS plus the first five residues of the mature protein and have a FLAG epitope tag at the 3' end.
(B) Denaturing PAGE western blots of HEK293 cells expressing the minimally-recoded (r) or codon-optimized (o) gene construct for each of the 13 human mitochondrially-encoded proteins. Optimized and recoded constructs for each gene were transfected in parallel, and mitochondrial fractions from paired transfections were collected after 72 h. Each pair was prepared and run as a set, ~20 μg protein run per lane. Samples run in non-adjacent lanes are separated by a dashed line. The C-terminal FLAG epitope was immunodetected using mouse anti-FLAG antibody. Tim23 was used as the internal control. A representative blot for each gene transfection is shown (n = 3 biological replicates).

2.3. Stable expression in disease models

2.3.1. Rescue of complex I dysfunction: $ND1^{m}$ (m.3751insC) cell line

Complex I is the largest and most complex enzyme of electron transport, comprised of 45 individual subunits, including 7 of the 13 mitochondrially encoded proteins [70]. Mutations affecting any of these CI subunits can lead to mitochondrial disease, with isolated CI deficiency accounting for more than one-third of all biochemically characterized mitochondrial disorders [71,72]. Mitochondrially-encoded ND1 is among the core subunits of this complex and plays a critical role in its assembly and function [73,74]. Pathogenic mutations in the ND1 gene are known to cause congenital mitochondriopathies including mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), Leigh's Syndrome and Leber's Hereditary Optic Neuropathy (LHON) [75], and are also implicated in Alzheimer and Parkinson's diseases [76] and in tumor progression for some cancers [77,78].

Fibroblasts mutant for the ND1 protein ($ND1^{m}$) were obtained from the lab of Ian Trounce (University of Melbourne, Australia). The mutation m.3571insC was originally isolated from a patient glioma, and a homoplasmic cybrid cell line was generated using patient-derived fibroblasts and Rho0143BTK^{-} osteosarcoma cells [73,79]. This mutation introduces an additional C into a homopolymeric cytosine stretch in the first third of *MT-ND1* (m.3751insC), resulting in a premature stop codon (p.Leu89Profs*13) and truncation of the 318 amino acid protein product to 100 residues (Fig. 4A). Cells with this mutation are functionally *null* for the ND1 protein. In the absence of wild-type ND1, Complex I fails to assemble, resulting in impaired oxidative metabolism and failure to grow in galactose media [34,80].

Using this cell model, we tested the ability of exogenous ND1 to functionally express in a *null* background. Stably selected $ND1^{m}$ cells coding for oND1 expressed the FLAG epitope on denaturing western blots of mitochondrial protein isolates (Fig. 4C), with a major product detected at ~ 37 kDa and a minor product near 60 kDa, corresponding to the processed and unprocessed ND1 products, respectively. Neither the processed nor unprocessed form of ND1 was detected when cells were transfected with the minimally-recoded gene. As above in HEK293 cells (Fig. 3A and B), mRNA expression for the codon-optimized ND1 constructs was far in excess (~210 fold) of the minimally-recoded version (Fig. 4B).

To determine whether oND1 could incorporate into CI and restore complex assembly, we analyzed mitochondrial fractions using Blue Native (BN)-PAGE. Although the majority of CI is reported to exist in supramolecular assemblies in human cell lines [81], very high molecular weight complexes (greater than 1 MDa) are technically challenging to resolve in BN-PAGE gels. Therefore, mitochondrial lysates were

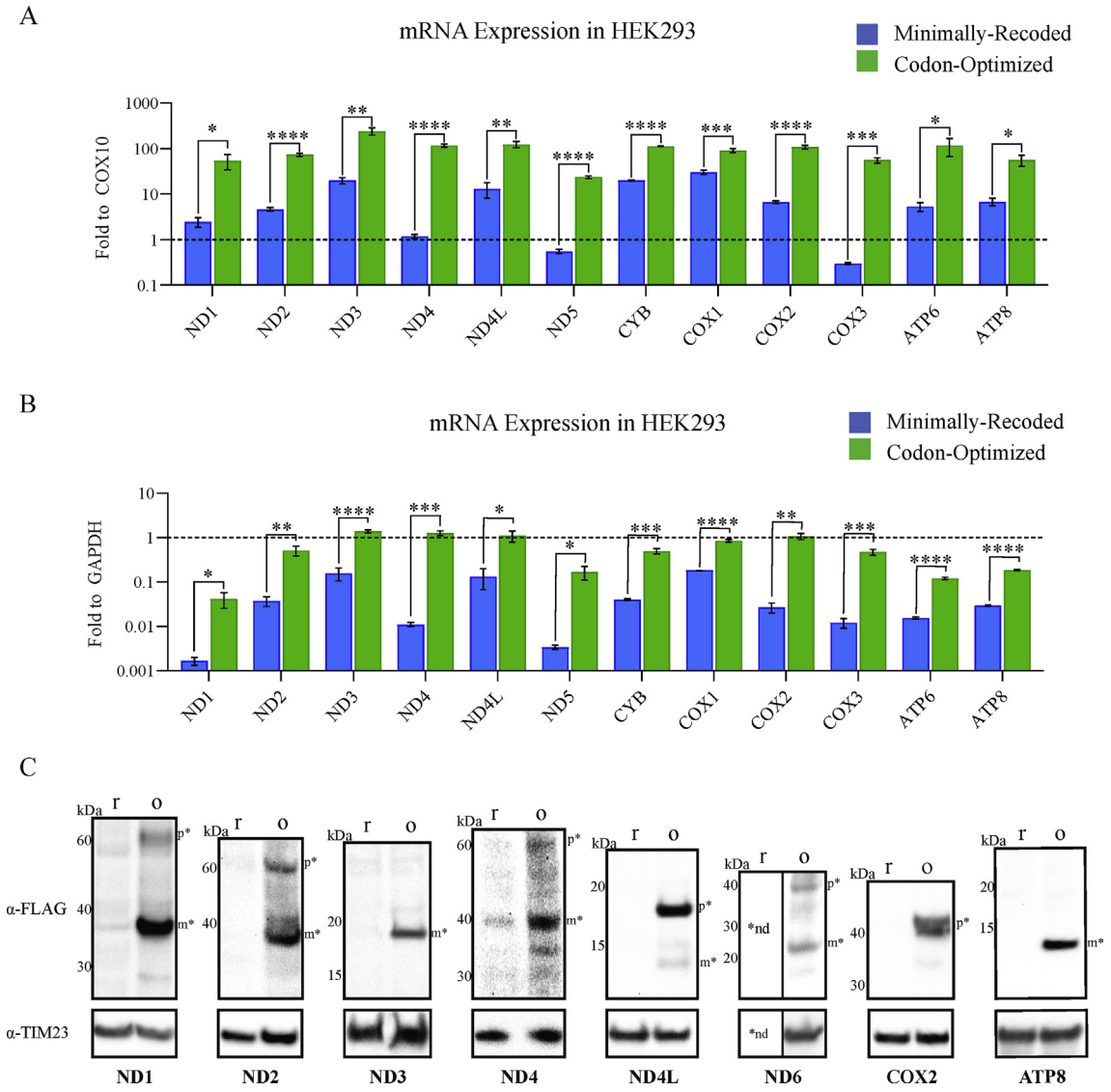

Fig. 3. Stable allotopic expression of mtDNA gene constructs in HEK293 cells.

(A) Quantitative RT-PCR detection of steady state mRNA levels for recoded (r) and optimized (o) mtDNA gene constructs after 4 weeks of selection in puromycin. The nuclear-encoded mitochondrial gene, *COX10*, was used as the reference gene. n = 3 biological replicates, performed in triplicate.

(B) As in (A), but with housekeeping gene *GAPDH* as the reference gene. n = 3 biological replicates, performed in triplicate.

(C) Denaturing PAGE western blots from purified mitochondrial fractions of stably-selected HEK293 cells expressing the recoded (r) or optimized (o) mitochondrial gene. For ND6, protein from only the optimized construct is shown, as rND6 was not available for comparison. ~20 μg protein/lane. The C-terminal FLAG tag was immunodetected using mouse anti-FLAG antibody. Tim23 was used as the loading control. A representative blot for each stable transfection is shown. n ≥ 3 biological replicates.

Data information: In (A–B), data are presented as mean expression level relative to control gene ± SEM. (A) $*P < 0.05$ [ND1 $P = 0.011$; ATP6 $P = 0.017$; ATP8 $P = 0.0051$] $**P < 0.005$ [ND3 $P = 0.00096$; ND4L $P = 0.00066$] $***P < 0.0005$ [COX1 $P = 0.00042$; COX3 $P = 0.00019$] $****P < 0.0001$ [ND2 $P < 0.0001$; ND4 $P < 0.0001$; ND5 $P < 0.0001$; CYB $P < 0.0001$; COX2 $P < 0.0001$] (unpaired Student's *t*-test). (B) $*P < 0.05$ [ND1 $P = 0.013$; ND4L $P = 0.0058$; ND5 $P = 0.0078$] $**P < 0.005$ [ND2 $P = 0.0032$; COX2 $P = 0.00055$] $***P < 0.0005$ [ND4 $P = 0.00018$; CYB $P = 0.00035$; COX3 $P = 0.00037$] $****P < 0.0001$ [ND3 $P < 0.0001$; COX1 $P < 0.0001$; ATP6 $P < 0.0001$; ATP8 $P < 0.0001$] (Student's unpaired *t*-test). In (C), p* marks predicted precursor (uncleaved) protein. m* marks predicted mature (cleaved) protein. Lanes with no data are separated by a solid line. nd* no data.

prepared using the detergent dodecyl-beta-D-maltoside (DDM), which is demonstrated to preserve CI while partially disrupting supercomplex structures. The FLAG-tagged oND1 protein could be detected in a high molecular weight complex on Native PAGE, (Fig. 4D, panel i) which comigrates with endogenous CI subunits GRIM19 (Fig. 4D, panel ii), and NDUFA9 (Fig. 4D, panel iii). Under these conditions, monomeric CI is observed exclusively in wild-type 143B mitochondria and in ND1^{m} mitochondria with allotopically-expressed oND1-FLAG, and is entirely absent in the ND1 *null* line. Additionally, in patients with primary Complex III (CIII) dysfunction, CI is also absent in ~50% of cases (combined Complex III/I dysfunction) due to the necessity of inter-complex associations for CI stability [81,83]. Thus, mitochondria lacking CI may also be expected to lack the CI/III unit. Indeed, when samples were probed for CIII subunit UQCRC2 (anti-CORE 2), both monomeric and dimerized CIII were unaffected in all cell lines tested. Meanwhile, Complex I-associated Complex III was only present in WT mitochondria and those expressing oND1, as evidenced by the comigration of CIII with the GRIM-19 and NDUFA9 subunits of CI, thus confirming failed CI/III association in the ND1 *null* line and supporting some restored complex assembly upon allotopic expression of the codon-optimized ND1 subunit (Fig. 4D, panel iv).

Because FLAG-tagged oND1 appeared to integrate into CI with

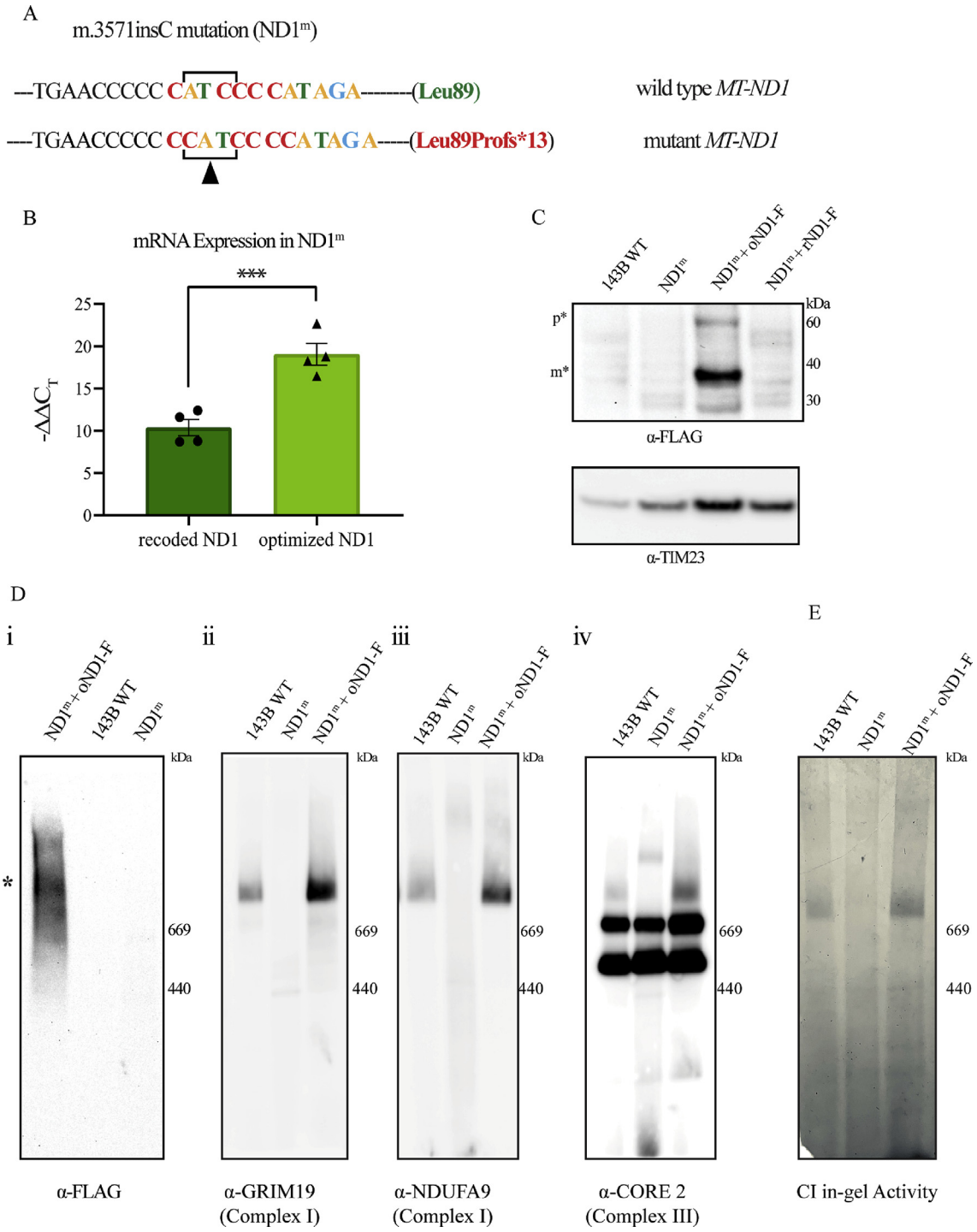

Fig. 4. Allotopic expression of ND1 in ND1^m (m.3751insC) cybrid cells.

(A) Schematic of the *MT-ND1* null mutation in the ND1^m cell line.

(B) Quantitative RT-PCR detection of steady state mRNA levels for *rND1* and *oND1* in stably selected ND1^m cells. The mRNA level of endogenous nuclear-encoded mitochondrial gene *COX10* was used as a reference. n = 4 biological replicates, performed in triplicate.

(C) Denaturing PAGE western blots of purified mitochondrial fractions from stably selected ND1^m cells expressing the recoded (r) or codon-optimized (o) mitochondrial *ND1* gene. ~20 μg protein/lane; the C-terminal FLAG tag was immunodetected using mouse anti-FLAG antibody. TIM23 was used as a loading control. Representative blot of n = 6 biological replicates.

(D) Blue Native PAGE western blots using 75 μg protein from purified mitochondrial fractions of wild type 143B cells (WT), homoplasmic ND1 mutant cells (ND1^m), and ND1^m cells stably expressing oND1 with the FLAG epitope (oND1-F). Exogenous protein was detected by immunoprobing for the FLAG epitope (i), and Complex I assembly was evaluated by probing for CI subunits GRIM19 (ii) and NDUFA9 (iii). Complex III disruption in the ND1^m line and the restored CIII profile in ND1^m expressing oND1 were detected using anti-CORE 2 antibody. Representative blots of n = 5 biological replicates.

(E) Complex I in-gel activity of DDM solubilized mitochondrial lysates. Samples were subjected to BN-PAGE followed by incubation of the gel in IGA assay buffer. Photographed at t = 2 h.

Data information: In (B), data are presented as mean -C_T normalized to reference gene *COX10* ± SEM. ***P = 0.0009 (Student's unpaired one-tailed *t*-test). In (C), p* marks predicted precursor (uncleaved) protein. m* marks predicted mature (cleaved) protein. In (D), signal of interest is denoted with *.

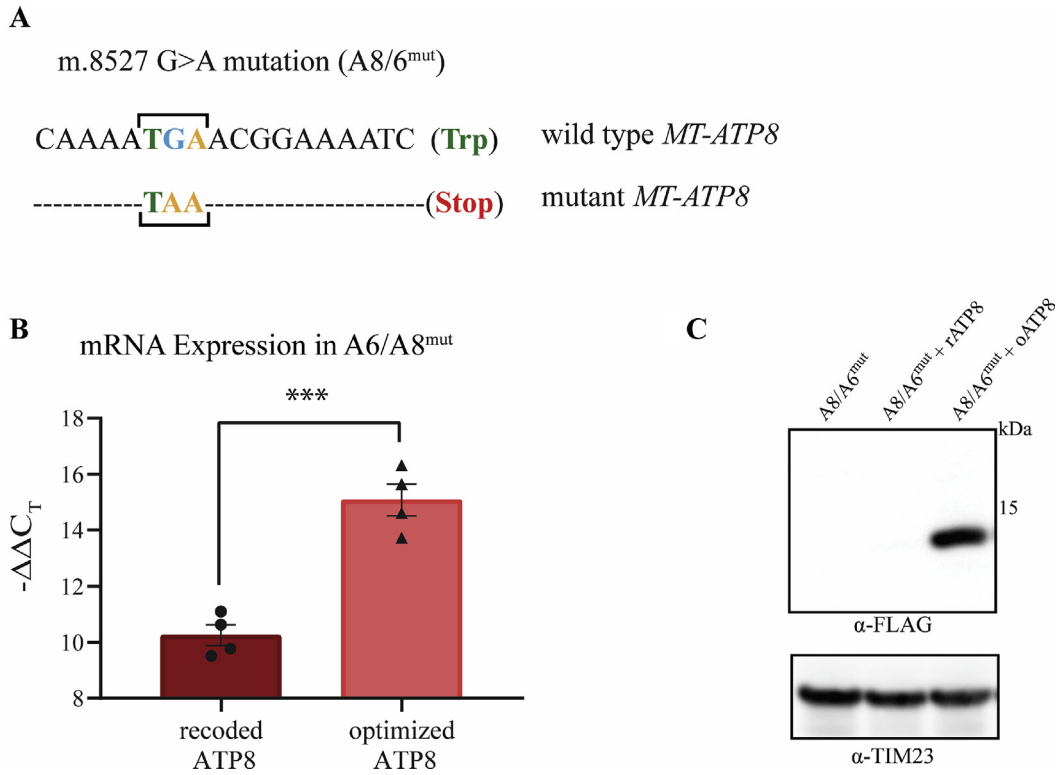

Fig. 5. Stable allotopic expression of codon-optimized human ATP8 in cells *null* for ATP8 protein. (A) Schematic of the *MT-ATP8* null mutation in the A8/A6^{mut} cell line.

(B) Quantitative RT-PCR detection of steady state mRNA levels for *rATP8* and *oATP8* in stably selected A8/A6^{mut} cells. The mRNA level of endogenous nuclear-encoded mitochondrial gene *COX10* was used as a reference. n = 3 biological replicates, performed in triplicate.

(C) Denaturing PAGE western blots for the stable allotopic expression of minimally-recoded (rATP8) or codon-optimized (oATP8) human ATP8 in A8/A6^{mut} cells. ~20 μg protein per lane. The C-terminal FLAG tag was immunodetected using mouse anti-FLAG antibody. Tim23 was used as a loading control. Representative blot of n = 3 biological replicates.

Data information: In (B), data are presented as mean -C_T, normalized to reference gene *COX10,* ± SEM. ***P = 0.0002 (Student's unpaired one-tailed *t*-test). In (C), m* marks predicted mature (cleaved) protein.

suboptimal efficiency, we created several cell lines with the same oND1 coding sequence in different expression plasmids, including a line with a C-terminal HA epitope tag. When lysed with the mild detergent digitonin, which preserves supercomplex structures, the HA-tagged oND1 protein could be detected in high molecular weight complexes near 950 and 830 kDa, consistent with the reported migration of monomeric CI and its late-stage assembly intermediate in human cells (Fig. S2A, panel i) [80,82]. GRIM19, however, migrates only with supercomplex-associated CI in wild-type 143B cells under these conditions, (Fig. S2A, panel ii), and the full molecular weight complex was not restored upon expression of the HA-tagged version. As expected, CIII/I associations were also absent upon incorporation of HA-oND1 (Fig. S2A, panel iii). In this case, the presence of HA-oND1 in a non-functional monomeric CI suggests that properties of the C-terminal tag may influence CI and supercomplex assembly profiles.

To determine if CI protein assemblies which incorporated tagged oND1 possessed enzyme function, samples were tested for CI-linked activity in-gel. Activity in wild-type and oND1-expressing mitochondria was present in CI monomers when lysed with DDM, with no detectable CI function in the ND1 mutant cell line (Fig. 4E), suggesting that the incorporated FLAG-tagged ND1 protein can indeed support functional Complex I activity. However, while some of the FLAG-tagged construct does incorporate into a functional complex, integration efficiency is not sufficient to restore activity to wild-type levels in a homoplasmic ND1 *null* mtDNA background.

The cybrid model is an artificial system, and under prolonged culture conditions we have found that the ND1 *null* mutation is prone to reverting to a heteroplasmic state. While we did observe partial CI assembly with some recovery of CI-linked activity upon expression of oND1 in homoplasmic ND1^{m} cells, we also found protein import and complex incorporation to be notably more robust when expressed in ND1 cybrids with partial reversion of the mutation (ND1$^{m/+}$), that is, some wild-type ND1 present [34]. In partially reverted ND1$^{m/+}$ lines, the FLAG epitope clearly incorporates into both Complex I monomers and supramolecular structures, comigrating with the endogenous ND1 subunit (Fig. S2B). While this presents a technical challenge for demonstrating functional rescue of OxPhos activity due to exogenous gene expression, a heteroplasmic model remains more representative of dysfunctional mitochondrial conditions in affected patients, and therefore the incorporation of allotopically expressed subunits in this context remains a clinically relevant result.

Although exogenous ND1 is inefficiently incorporated in the homoplasmic mutant line, we nonetheless sought to determine if CI function was improved in this model. We therefore conducted competitive growth assays in galactose media to assess respiratory status. It is well-documented that cell lines with severe CI mutations demonstrate restricted growth under normal conditions, with grossly impaired viability in the absence of glucose. Indeed, when cultured in glucose-containing media, ND1^{m} cells appeared to proliferate at approximately 25% the rate of wild-type 143B cells, and growth rate of ND1^{m} cells expressing a tagged oND1 was not significantly increased from the mutant. Meanwhile, the viability of wild type 143B cells was reduced to ~55% its viability when cultured in OxPhos restrictive media, and ND1^{m} cells were only ~11% viable. Mutant cells expressing the HA-tagged oND1 showed no significant difference in viability in either glucose or galactose media relative to ND1 *null* controls, consistent with

the absence of supercomplex assembly in native gels. Mutants expressing FLAG-tagged oND1, however, did demonstrate a modest, but statistically significant improvement in galactose survival, with an 18% average total viability, corresponding to 32% of wild-type viability in galactose (Fig. S2C). This suggests that although oND1 cannot fully complement the m.3751insC mutation in a homoplasmic null model, the modest improvement in OxPhos function may be sufficient to overcome the mutation threshold for some disease phenotypes.

2.3.2. Rescue of complex V dysfunction: mutations in ATP8

The F_1F_o ATP synthase is the terminal complex in the oxidative phosphorylation relay and is responsible for converting the proton motive force generated by Complexes I, III and IV into ATP. Two of the subunits of this multimeric complex are encoded by mtDNA, namely *MT-ATP8* and *MT-ATP6*, whereas, the genes for the remaining subunits reside in the nucleus [84]. Several pathological mutations in these two mtDNA subunits have been reported and manifest as NARP (Neuropathy, Ataxia, and Retinitis Pigmentosa), MILS (Maternally Inherited Leigh Syndrome) (www.mitomap.org), and cardiac hypertrophy [85,86] in patients. We previously reported the successful allotopic expression of the *MT-ATP8* gene and to a lesser extent the *MT-ATP6* gene, with functional rescue in a cybrid cell line A8/A6^{mut} (m.8529G-A) *null* (Fig. 5A) for the ATP8 protein [87]. We observed that expression levels of the codon-optimized o*ATP8* gene were many-fold higher than for minimally-recoded *rATP6*. Here, we compared the relative efficiencies of stable expression between recoded and codon-optimized human *ATP8* in this patient-derived cybrid cell line. Transient ATP8 expression indicated that protein products of both recoded and codon-optimized *ATP8* genes were detected in mitochondrial fractions of HEK293 cells (Fig. 2B), while stable expression was detectable only with the optimized version (Fig. 3C). Similarly, only the codon-optimized ATP8 protein is produced under stable expression in the A8/A6^{mut} *null* cell line (Fig. 5C). This distinction was also reflected in the steady-state mRNA levels for the two constructs (Fig. 5B), with a notable ~28 fold increase in oATP8 mRNA over rATP8 mRNA.

Considering the robust expression of oATP8 in *null* disease models, this gene can be an immediate candidate to evaluate *in vivo* for potential gene therapy applications. Prior to transgenic studies, however, we sought to verify that allotopically expressed oATP8 can similarly rescue mitochondrial defects in mouse cells, as well as effectively compete with an endogenous mutant protein, the likely scenario in many human mitochondrial diseases. The FVB mouse has a severe CV defect caused by several mutations, including a mitochondrial transversion mutation (m.7778G-T) resulting in an Asp-Tyr substitution within the conserved N-terminus of the ATP8 protein. To delineate the mtDNA contribution of this mutation to the overall defect, the model has been backcrossed onto the C57/BL6 background [88,89], resulting in the C57/BL6$^{(mtFVB)}$ mouse model. This is not a *null* model - the mutant ATP8 protein is synthesized and incorporated into Complex V (CV); however, C57/BL6$^{(mtFVB)}$ mice demonstrate measurable phenotypes and cells *in vitro* have subtle deficits indicative of disrupted CV function [90,91], making it an excellent model to test allotopically-expressed oATP8 function in a realistic disease cellular context.

Here, we tested for expression of mouse codon-optimized *oAtp8* in C57/BL6$^{(mtFVB)}$ fibroblasts bearing the (m.7778G-T) mutation in *mt-Atp8* (Fig. 6A). We created both minimally-recoded (*rAtp8*) and codon-optimized (*oAtp8*) versions of the mouse *mt-Atp8* gene and placed them into an inducible lentiviral expression vector (pCW57.1) to allow us control of exogenous gene expression. We then transduced allotopic expression constructs for the mouse mitochondrial *Atp8* gene into wild type and mutant (FVB) fibroblasts (obtained from the Ibrahim lab, University of Lübeck, Lübeck, Germany) and selected for stable transductants. Following induction with doxycycline, allotopically expressed ATP8 protein levels were observably greater with expression of *oAtp8* than with *rAtp8* (Fig. 6B), as in the *null* model system. The expressed protein was also able to integrate into CV on BN PAGE gels (Fig. 6C). We further assessed respiratory function in mutant cell lines through viability studies in galactose. The C57/BL6$^{(mtFVB)}$ mutation has been shown to cause various defects in inbred mice including anxiety and inflammation-related arthritis [91,92]. While the phenotype of this relatively mild polymorphism is difficult to measure *in vitro*, we did find that the ATP8 mutant mouse cells suffered a relative growth defect when grown on either glucose or galactose. Mutant cells expressing optimized msoATP8, however, grew significantly better than non-complemented cells under either growth condition (Fig. 6D). Although the C57/BL6$^{(mtFVB)}$ mouse line is not a *null* model, incorporation of allotopically expressed protein into pre-existing respiratory complexes is an important result as it remains unclear how efficiently exogenous proteins can compete with existing mutant proteins.

2.4. Codon optimization of mitochondrial genes reflects adaptation to nuclear code preferences

Finding that the levels of allotopic gene expression and protein levels differed considerably between minimally-recoded and codon-optimized versions of mitochondrial genes, we sought to examine the extent of similarity or dissimilarity between codon use in these genes in reference to the human nuclear genome by looking at codon bias. Because minimally-recoded gene sequences have only the changes necessary to preserve the amino acid sequence of a protein, they largely reflect codon preferences of the mitochondrial genome, while genes codon-optimized for nuclear expression should demonstrate coding patterns more similar to those of nuclear genes. To examine these patterns, we first looked at relative synonymous codon usage (RSCU) in the nuclear genome and in both recoded and codon-optimized mitochondrial genes. Mitochondrial genes encode membrane proteins which are highly hydrophobic, therefore RSCU values were calculated to address inherent bias due to the codon composition of hydrophobic amino acids (Table S2). When RSCU values were plotted as a heatmap, some notable differences in codon use patterns emerged (Fig. 7A). Several amino acids in recoded genes are encoded predominantly by synonymous codons which are overwhelmingly underrepresented in the nuclear genome. For example, of the two codons for Glutamine, the nuclear genome demonstrates strong bias for CAG over rarely used CAA, the predominant codon used for Glutamine in recoded genes. Similar usage patterns are also observed for the charged amino acids Lysine, Arginine, and Glutamic Acid, and for highly abundant Leucine, which alone constitutes 17% of the residues in mitochondrial protein-coding genes. Charged amino acids, though not abundant in mitochondrial proteins, are known to directly influence the import and topology of imported mitochondrial proteins. Leucine, meanwhile, is recognized by six synonymous codons: two strongly underrepresented, two mildly underrepresented, one mildly overrepresented, and one very strongly favored (CUG). While some recoded genes utilize the slightly favored CUC codon, none use the dominant CUG codon, and all recoded genes instead demonstrate strongly biased use of underrepresented codons UUA or CUA. As synonymous codon use of a genome can be loosely associated with translation, the high bias toward underrepresented nuclear codons for these amino acids reflects a preference for these codons in mitochondrial translation, and meanwhile suggests they may be poorly translated in the cytosolic context. Similarly, codon-optimized genes are comprised overwhelmingly of codons which show use bias in nuclear genes and avoid the use of underrepresented, or rare codons, likely contributing to their enhanced nuclear expression over minimally recoded versions. We therefore conclude that while properties of target proteins undoubtedly influence their ease of incorporation into the respiratory chain, the codon use profiles of mitochondrial genes are significant in facilitating heterologous nuclear expression and promoting effective translation.

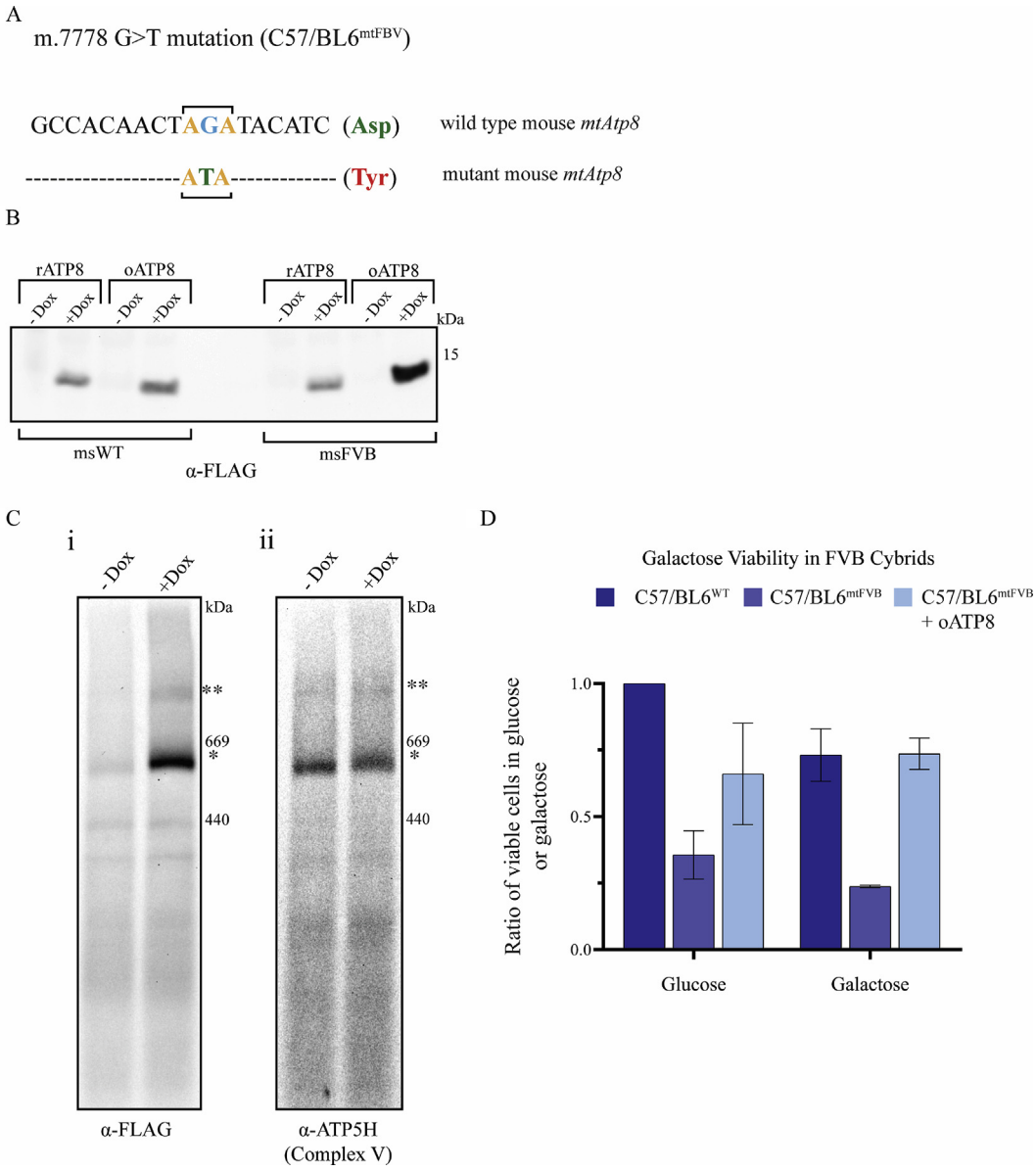

Fig. 6. Stable allotopic expression of codon-optimized mouse ATP8 in cells harboring a point mutantion for ATP8 protein.(A) Schematic of the *mt-Atp8* polymorphism in C57/BL6$^{(mtFVB)}$ mouse cells.

(B) Denaturing PAGE western blots for the stable allotopic expression of mouse ATP8 in C57/BL6$^{(mtFVB)}$ cells. Expression from recoded (rATP8) or codon-optimized (oATP8) constructs is shown before (-Dox) or after (+Dox) induction with Doxycycline. Each lane contains ~20 μg protein from the mitochondria-enriched cellular fraction. The C-terminal FLAG tag was immunodetected using mouse anti-FLAG antibody. Representative blot of n = 3 biological replicates.

(C) Blue Native PAGE western blots of purified mitochondrial fractions from C57/BL6$^{(mtFVB)}$ cells stably expressing mouse oATP8 in a doxycycline inducible system. Samples were immunoblotted for the FLAG epitope (i) and for endogenous ATP5h (ii) as a marker of Complex V and loading control. Representative blot of n = 3 biological replicates.

(D) Viability on galactose media. Wild type (C57/BL6^{WT}), mutant (C57/BL6$^{(mtFVB)}$) and mutant mouse cells stably expressing oATP8 (C57/BL6$^{(mtFVB)}$ + oATP8) grown in either glucose or galactose media were assessed for viability after 72 h. Shown as the mean ratio of viable cells in each growth condition. A ratio of 1.0 means viability is equivalent to viability of wild type (C57/BL6^{WT}) in glucose. n = 2 biological replicates.

Data information: In (C), * marks the Complex V monomer. ** marks the Complex V dimer. In (D), data is presented as the mean ratio with range bars.

3. Discussion

In this study, we are the first to demonstrate the application of codon-optimization, typically employed in heterologous expression systems, for the allotopic expression of all 13 human mitochondrial genes. Both inherited and acquired mutations in mtDNA cause severe diseases, including LHON, Leigh's syndrome, MELAS, MERRF, NARP and MILs, Kern-Sayer's syndrome, and reversible respiratory chain syndrome (reviewed in 2). Replicative errors, inefficient repair and close proximity to sites of reactive oxygen species production make mtDNA susceptible to damage with time. mtDNA mutations also accumulate with age and accompany a progressive decline in organelle function [4].

In principle, expressing the wild-type mitochondrial genes from the nucleus (allotopic expression) is a viable strategy to cure mtDNA diseases; however, this approach is difficult and limited by problems of expression and functional incorporation of the gene product. Here, we have demonstrated that merely optimizing the primary sequence of mitochondrial genes facilitates their nuclear expression in human cells, and in some cases, their assembly into respiratory complexes,

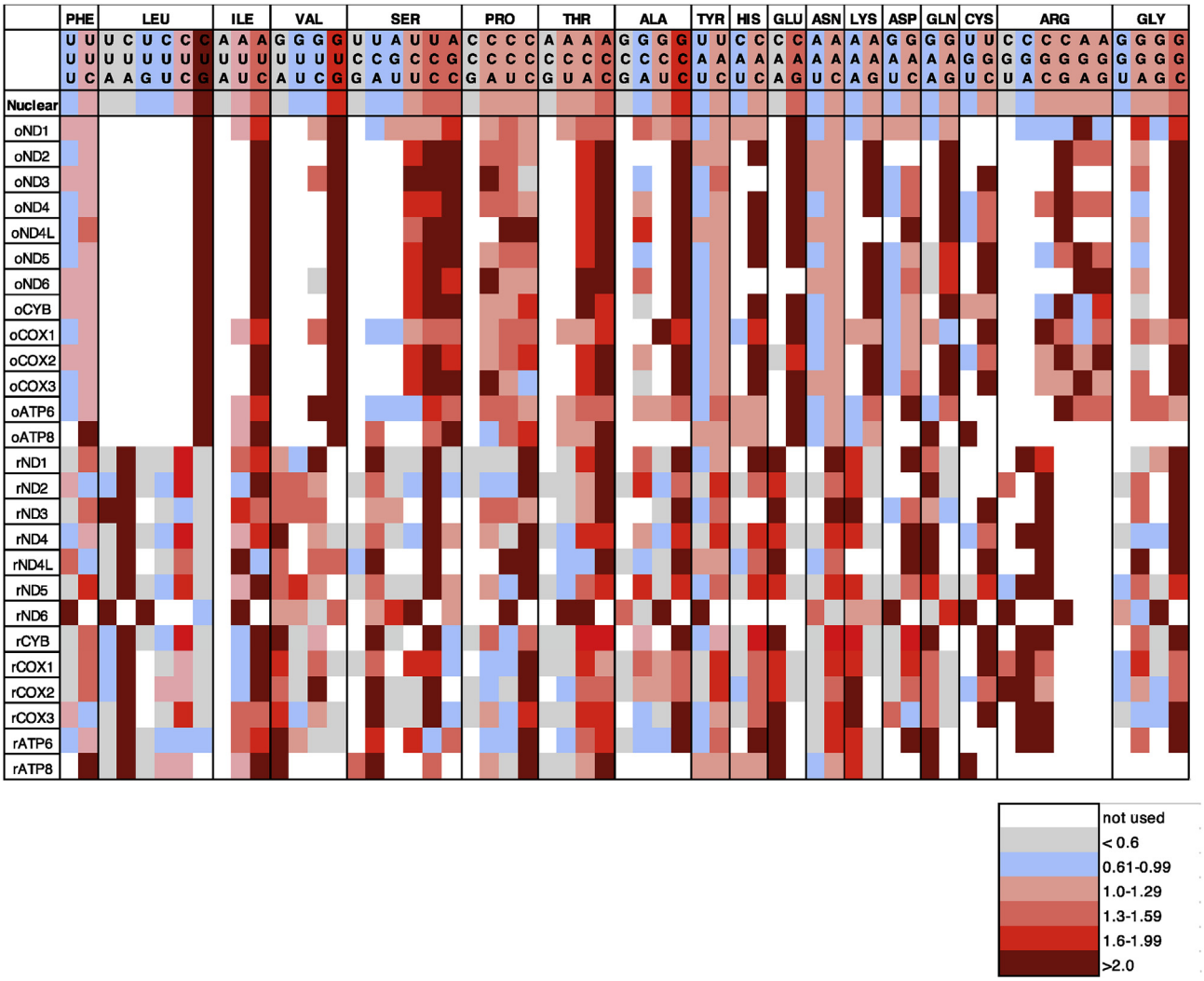

Fig. 7. Heatmap depicting per-codon synonymous codon use in each recoded and codon-optimized mitochondrial gene. Synonymous codons are listed beneath their corresponding amino acid and are shaded to reflect the RSCU value for that codon in nuclear genes, ordered from most under-represented to most overrepresented. A value of 1.0 indicates that there is no biased use of that codon, while values less than 0.6 and greater than 1.6 reflect under and over-representation, respectively. Codons which do not appear in a given gene are shown as white space.

surmounting one of the barriers to successful allotopic expression therapy for mitochondrial diseases. In total, eight of the codon-optimized genes were expressed stably at the protein level, significant considering that many reported studies have demonstrated expression only transiently. Of all thirteen minimally recoded genes, only rND4 demonstrated any protein expression stably; however, this was only a fraction of that detected for oND4 under similar conditions. Nonetheless, ND4 remains the only mitochondrial protein in production as a gene therapy and is currently in Phase 3 clinical trials for LHON-associated blindness. This, and various in-vitro and animal studies imply that the recoded ND4 gene may express sufficiently to partially rescue this disease phenotype [44,93].

Analysis of codon use patterns in minimally-recoded and codon-optimized gene constructs revealed that mitochondrial genes indeed utilize synonymous codons differently than nuclear genes, featuring a high percentage of codons considered to be rare or underrepresented in the nuclear genome, which may account for the poor or absent allotopic expression of minimally-recoded genes. Our results indicate that optimizing mitochondrial coding sequences to adapt to nuclear codon patterns is a critical step for the successful expression of these genes from the nucleus.

It is notable that while codon-optimization facilitates the allotopic expression of mitochondrial proteins, our results suggest that the appended MTS of many target genes, both recoded and optimized, may not be appropriately cleaved. Codon optimization of the target gene would not be expected to promote localization or processing of its encoded product, both of which are properties that are typically conferred by the protein sequence. Under stable expression conditions, the N-terminal MTS from *ATP5G1* directs the target protein to the mitochondrial matrix and is then cleaved by the matrix processing peptidase (MPP) upon entry through the translocase of the inner membrane (TIM). The protease cleavage site of this MTS has been determined and, for this study, we also included the first five amino acids of the mature ATP5G1 protein to facilitate processing of the precursor [36]. It is possible that in these fusion proteins the cleavage signal sequence is not efficiently recognized, or that secondary structures inhibit access to the protease site. Furthermore, the endogenous ATP5G1 MTS sequence is recognized by the processing peptidase of the mitochondrial matrix, and therefore would not be cleaved if the N-terminus of an allotopically expressed protein first adopts an orientation facing the intermembrane space (IMS). Despite incomplete processing of some allotopically expressed genes, the stable expression, mitochondrial localization, and import of the majority fraction are a promising result, suggesting that codon optimization technology may make allotopic gene therapy a

possibility for many, if not all mitochondrially-encoded genes.

In disease models, we demonstrate that expressing the codon optimized ATP8 gene from the nucleus can 1) rescue function in a patient cell line *null* for the ATP8 protein and 2) compete with pre-existing mutant proteins in mouse cell lines defective for ATP8 protein function. We also assessed respiratory function in mutant cell lines through viability studies in galactose. The C57/BL6$^{(mtFVB)}$ mutation has been shown to cause various defects in inbred mice including anxiety and inflammation-related arthritis [91,92]. While the phenotype of this relatively mild polymorphism is difficult to measure *in vitro*, we did find that the ATP8 mutant mouse cells suffered a relative growth defect when grown on either glucose or galactose. Mutant cells expressing optimized msATP8, however, grew significantly better than non-complemented cells under either growth condition (Fig. 5D). Although the C57/BL6$^{(mtFVB)}$ mouse line is not a *null* model, incorporation of allotopically expressed protein into pre-existing respiratory complexes is an important result as it remains unclear how efficiently exogenous proteins can compete with existing mutant proteins.

We also show that codon optimized oND1 can incorporate and partially restore OxPhos function in an ND1 *null* background, with greatly enhanced incorporation under heteroplasmic ND1$^{m/+}$ conditions. Severe mutations in mitochondrial subunits, such that of the ND1^{m} mutant line, disrupt OXPHOS to a degree that proliferation rates are markedly reduced even under normal growth conditions, with dramatically impaired viability in glucose-restricted media demanding oxidative metabolism. When cultured in the presence of glucose, ND1^{m} cells grew at less than 25% the rate of wild type 143B. Expression of oND1 did not significantly increase the rate of glycolyic growth in this cell line; however, when cultured in galactose media, viability was modestly, yet significantly increased to nearly half wild-type levels when the FLAG-tagged oND1 was expressed. It is notable that in this cell line, ND1 has previously been allotopically expressed to demonstrate the necessity of functional CI in the hypoxia-induced shift to a Warburg profile during tumorigenesis, however this was also only achieved under conditions of heteroplasmy [30]. Further, mitochondrial-nuclear crosstalk is well documented, and evidence suggests that mutations in the mitochondrial genome also influence nuclear gene expression profiles, even when phenotypes are mild [94]. Combined with our results, these data suggest that the efficiency of transgene import and complex integration may be influenced by mutation severity and associated secondary characteristics, such as altered mitochondrial membrane potential or the absence/presence of preexisting complex structures to act as scaffolds, and is a result which warrants further investigation.

Certainly other parameters such as the relative hydrophobicity of each subunit, complex stoichiometry, inner membrane topology, and inter-subunit contacts also play an important role in the functional incorporation of any subunit, and the relocation of mitochondrial genes to the nucleus may require systematic testing for each of these facets to achieve functional complementation. Nonetheless, the expression, import, and incorporation of allotopically expressed mitochondrial proteins into respiratory complexes described herein is noteworthy, particularly in disease models where mitochondrial function is perturbed. Furthermore, we note that while in most cases only a fraction of allotopically-expressed mitochondrial protein functionally integrates, and acknowledge the need for further engineering to increase processing and incorporation efficiency, we find it apparent that codon optimization facilitates the production of protein in an amount sufficient to observe an effect. Through this, we conclude that although making changes to coding sequence alone may not be sufficient for complete complementation of all mtDNA genes, host (nuclear) codon optimization is, for most, sufficient for stable expression, and is a necessary consideration for further allotopic expression studies and in gene therapy design.

4. Experimental procedures

4.1. Cell lines

Human Embryonic Kidney 293 (HEK293) cells were obtained from the ATCC. 143B osteosarcoma cells were obtained from Dr. Carlos Moraes, University of Miami, Miami, FL, USA. Cell lines with mtDNA mutations m.8529G-A (A8/A6^{mut}) [85,87] and m.3571dupC (ND1^{m}) [73] were obtained from other labs. The m.3571dupC cells were gifted to us by the Trounce lab, University of Melbourne, Melbourne, Australia. The m.8529G-A trans-mitochondrial cybrids were provided by the Rodenburg lab, Radboud University Medical Center, The Netherlands. Wild type and mutant mouse fibroblasts for the conplastic C57/BL6$^{(mtFVB)}$ strain were obtained from Dr. Saleh Ibrahim, University of Lübeck, Lübeck, Germany [88,91].

4.2. Homoplasmy assessment

We used Amplification Refractory Mutation System-quantitative PCR (ARMS qPCR) [95] to ascertain mutation load in cybrids during the course of this study. Primers used to distinguish wild type versus mutant mtDNA sequences for A8/A6^{mut} and ND1^{m} are listed in Supplementary Table S3. The mitochondrial genes *MT-CYB* or *MT-COX3* were used as reference genes. The A8/A6^{mut} cells were selected for homoplasmy as described previously [87]. The ND1^{m} cells were previously shown to be homoplasmic [79] and within our experimental limitations we were able to confirm that these cells were > 99.9% homoplasmic by ARMS qPCR (Supplementary Table S4) and Sanger sequencing (Supplementary Fig. S3). Homoplasmy in the mouse cell lines C57/BL6$^{(WT)}$, C57/BL6$^{(mtFVB)}$ and C57/BL6$^{(mtFVB)}$ stably expressing doxycycline inducible allotopic MsoATP8 was ascertained by Sanger sequencing using the primer indicated in Fig. S3B.

Briefly, genomic or mitochondrial DNA was isolated from 10^6 cells using the DNeasy Blood and Tissue kit from Qiagen (Hilden, Germany). The samples were not treated with RNase. DNA derived from 143B osteosarcoma cells (WT) and the parental mutant line (ND1^{m}) were used as controls. The *MT-ND1* insertion mutation is within a homopolymeric cytosine stretch, and can be difficult to specifically amplify, therefore the maximum detectable C_T difference between populations known to be homoplasmic for the wild-type (WT) or mutant (ND1^{m}) sequence was first evaluated. Gene fragments were synthesized for WT or mutant mtDNA region m.3441–3740, which spans the insertion mutation site in the ND1^{m} line. Samples comprised of 100% WT and 100% mutant *MT-ND1* gene fragments were then amplified with ND1 ARMS qPCR primers (Supplementary Table S3) using input copy numbers ranging from 10^6-10^9, and the amplification difference was used as a benchmark for homoplasmy in downstream testing of cell line samples (Supplementary Table S4). All ARMS qPCR reactions were performed in triplicate in a total volume of 20 μl in Power SYBR green master mix using 100 ng of genomic DNA or 5–25 ng of mitochondrial DNA as template for each reaction point. Primers and gene fragments (gBlocks) were synthesized by Integrated DNA Technologies (Coralville, IA) without any modifications and purified with standard desalting. Following qPCR, cycle threshold (C_T) values were obtained using the StepOne Software v2.3 and results expressed as ΔC_T ($C_{T\ Avg(mutant\ primers)}$ - $C_{T\ Avg(WT\ primers)}$). A no-template control was included in each reaction set to rule out non-specific priming. Further, in order to confirm that homoplasmy was preserved throughout the duration of lengthy experiments, we tested the mutation load in cybrid cell lines at various time points by ARMS qPCR. mtDNA was extracted using the DNeasy Genomic DNA Isolation Kit (Qiagen, Hilden, Germany) at various time points (for example before and after 4 weeks of puromycin treatment for stable selection). 12.5 ng of such purified DNA was used as template per reaction point (20 μl) in qPCR reactions. For evaluation of *MT-ND1* homoplasmy, copy number-controlled input was also used corresponding to gBlock copy numbers. The difference in C_T values

($\Delta C_{T\ avg(mutant\text{-}WT)}$) was evaluated and results expressed as the mean of three biological replicates each performed in triplicate.

4.3. Design and synthesis of expression constructs

Sequences for the 13 human mtDNA genes (*MT-ND1*, *MT-ND2*, *MT-ND3*, *MT-ND4*, *MT-ND4L*, *MT-ND5*, *MT-ND6*, *MT-CYB*, *MT-COX1*, *MT-COX2*, *MT-COX3*, *MT-ATP6,* and *MT-ATP8*) were downloaded from www.Mitomap.org. The mouse *mt-Atp8* gene sequence was obtained from the Mouse Genome Database.[2] The genetic code for each of these genes was edited to the standard code to generate minimally codon-corrected (r) sequences for productive translation in the nuclear-cytosol compartment. A second version, namely, a codon-optimized (o) sequence for each gene was generated using the OptimumGene algorithm by Genscript, Piscataway, NJ, USA. Both recoded and codon-optimized gene versions were synthesized by Genscript (Supplementary Data S1). Each gene construct was sub-cloned as an MluI-*Xho*I fragment into the pCMV6A (puromycin) expression vector from Origene, Inc (Cat# PS100025). A mitochondrial targeting sequence (MTS) was appended at the 5' end. We used the human/mouse ATP5G1 MTS including 5 amino acids from the mature protein in all our constructs [36]. Cloning using the XhoI site at the 3'end in the pCMV vector also introduced the Myc-FLAG epitopes at the C-terminus for ease of protein detection (Supplementary Fig. S4). The Myc tag was not utilized in this study and thus, for simplicity, tagged constructs will be referred to and labeled as only FLAG. We also generated versions of the optimized *ND1* allotopic construct with either a C-terminal 3x HA epitope tag or under the control of a CAG promoter. The minimally-recoded and codon-optimized mouse *Atp8-FLAG* gene fragments, including the mouse ATP5G1 MTS +5 amino acids at the 5' end, were cloned into a doxycycline inducible vector, namely, pCW57.1. This plasmid was a gift from David Root (Addgene plasmid # 41393; http://n2t.net/addgene:41393; RRID:Addgene_41393).

4.4. Expression of constructs in cultured mammalian cells

HEK293 cells were transfected (Xfect™; Clontech Laboratories, Inc. Mountain View, CA) with the pCMV6 expression plasmids (4 μg/well in a 6 well cell culture plate) containing recoded or codon-optimized gene constructs for all the 13 human mtDNA genes. For *ND6*, only the optimized version was transfected, as we were unable to successfully synthesize the minimally-recoded version of the gene. Mutant cybrids ($A8/A6^{mut}$ and $ND1^{m}$) were transfected with their corresponding minimally-recoded or codon-optimized gene constructs (pCMV-ATP5G1mts-r/oATP8-FLAG or pCMV-ATP5G1mts-r/oND1-FLAG respectively). We introduced mouse *Atp8* expression constructs (pCW57.1-ATP5G1 MTS-r/o MsAtp8-FLAG) into wild type and FVB mouse cell lines using lentiviral transduction. Transgene expression under the pCW57.1 promoter was induced by addition of 1μg/ml doxycycline to culture media. Protein expression under transient conditions was followed at 72 h post transfection or transfected cells were maintained in culture under selection pressure (puromycin at 1μg/ml) for 4 weeks to generate stable cell lines. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 mM L-glutamine, 1 mM Na-pyruvate, 50 μg/mL uridine and 1x antimycotic-antibiotic. Growth on galactose was performed by supplementing glucose-free DMEM medium with 10% (v/v) FBS, 1 mM L-glutamine, 1 mM Na-pyruvate, 50 μg/mL uridine, 1x antimycotic-antibiotic and 5 mM galactose.

[2] The nucleotide sequence for the mouse mtAtp8 gene can be accessed from the Mouse Genome Informatics (MGI) Database under the accession MGI:99926.

4.5. Competitive growth assays

10^4 cells were plated on either 5 mM glucose or 5 mM galactose-containing media as above and allowed to grow for 72 h at 37 °C. Cells were trypsinized and viable cells quantified by Trypan blue exclusion using a Countess Automated Cell Counter device (Thermo Fisher Scientific).

4.6. mRNA levels for recoded and codon-optimized expression constructs

Steady state mRNA levels for stably transfected optimized and recoded constructs were measured by RT-qPCR using the StepOnePlus system (Applied Biosystems, Foster City, CA). RNA was isolated from 10^6 cells for each cell line using the E.Z.N.A.® Total RNA Kit I (OMEGA Bio-Tek, Norcross, GA, USA). 500 ng of template mRNA was reverse transcribed to synthesize 10 μL of cDNA using the PrimeScript RT Reagent Kit (TaKaRa/Clonetech, Mountain View, CA, USA). For each qPCR reaction, 1 μl of cDNA was used as template. Primers were designed to amplify a specific region between the *ATP5G1* MTS and each respective mitochondrial gene (Supplementary Table S5). Amplifications were normalized to nuclear-encoded and mitochondria-targeted gene *COX10* and to housekeeping gene *GAPDH* as references in order to estimate both relative and absolute levels of exogenous gene expression.

4.7. Isolation of mitochondria

Two different methods were used to purify mitochondria in this study. When cell numbers were limited ($< 2 \times 10^7$) the detergent lysis method was used. For assays requiring greater amounts of concentrated mitochondrial protein, the mechanical lysis (Dounce) homogenization method was utilized.

4.8. Detergent lysis isolation of mitochondria

We have developed a protocol to rapidly and economically enrich mitochondria from limited numbers of mammalian cells. This method is suitable to process multiple samples in parallel and is comparable to commercially available kits. A total of 2×10^7 cells per sample were harvested by trypsinization. Cells were washed once with PBS and pelleted at $215 \times g$ for 5 min at 4 °C. Pelleted cells were then re-suspended in 500 μl of hypotonic buffer [50 mM HEPES KOH (pH 7.0), 1 mM EDTA, 1 mM DTT, 1 x Protease inhibitor cocktail (PIM) (Sigma Cat# 8340), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mg/ml Bovine Serum Albumin (BSA)] and incubated on ice for 5 min. Cells were lysed by adding 50 μL of 0.2% high purity Digitonin (CAS 11024-24-1) to achieve a final concentration of 0.02%, then vortexed vigorously (5–10 s) every minute for 5 min. A 1:1 vol (500 μL) of 2x isotonic buffer (100 mM HEPES KOH (pH 7.0), 1.2 M sorbitol, 2 mM EDTA, 2 mM DTT, 1 x PIM, 2 mM PMSF and 2 mg/ml BSA was added to the samples, followed by immediate centrifugation in a benchtop microfuge (Fisher Scientific, Hampton, NH) at $700 \times g$ for 10 min at 4 °C to remove unbroken cells and cell debris. The supernatant was carefully transferred to new tubes and centrifuged at $12{,}000 \times g$ for 15 min at 4 °C. The supernatant (cytoplasmic fraction) was removed, and the mitochondria-containing pellet stored at -80 °C until use.

4.9. Mechanical lysis isolation of mitochondria

Purification of mitochondria without detergent was performed as described previously [87]. Briefly, 2 × 15 cm fully confluent plates for each stable cell line or the parental cell lines were harvested by trypsinization and washed once with PBS) at $215 \times g$ in a swinging bucket table top centrifuge (Sorvall legend RT + centrifuge, ThermoScientific) for 5 min at 4 °C. Cells were gently resuspended in 7.5 ml of mitochondria isolation buffer (MIB; 20 mM Hepes/KOH, pH 7.5, 0.25 M

sucrose, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 1 x PIM, 1 mM PMSF) and passed once through a 1.5 inch, 25 gauge needle directly into a 15 ml Dounce homogenizer with 0.0010–0.0030 inch clearance. They were homogenized with 25 strokes, diluted with 7.5 ml of MIB and centrifuged at 2000 × *g*, 5 min, 4 °C. The supernatant was saved separately, and the pellet subjected to homogenization once more. The collected supernatants were centrifuged at 10,000 × *g*, 12 min, 4 °C. The pellet from the high-speed spin is the crude mitochondrial fraction. It was resuspended in MIB with 10% DMSO, flash frozen in liquid nitrogen and stored at 80 °C until use.

4.10. Immunoblotting and in-gel activity

Denaturing PAGE and Blue Native PAGE were essentially performed as described in Ref. [87]. SDS-PAGE was performed on NuPage™ 4–12% Bis-Tris gels using Novex™ Sharp Pre-Stained Protein Standard (Invitrogen, Carlsbad, CA). Native samples were run on NativePage™ 3–12% Bis-Tris gels, using the High Molecular Weight Calibration Kit (Amersham, GE Healthcare, Chicago IL.) for molecular weight standards. In-gel activity for Complex I was performed essentially according to Ref. [96]. Briefly, 75 μg of Dounce-isolated mitochondrial protein was resuspended in native lysis buffer [87] and solubilized with 20 μl 10% (w/v) n-Dodecyl-beta-D-maltoside (CAS 69227-93-6) for Blue Native PAGE. Following electrophoresis, the gel was washed twice with deionized water and then equilibrated in cold assay buffer (0.1 M Tris-HCl, pH 7.4) for 15 min with shaking. Buffer was then replaced with new assay buffer containing 0.1 mg/ml NADH (Sigma, St. Louis MO) and 2.5 mg/ml Nitrotetrazolium Blue Chloride (Alfa Aesar, Haverhill, MA) (IGA buffer). Color development was monitored at 10-min intervals for 2 h and gel was imaged over a transilluminator. The following antibodies were used in this study: Anti-FLAG antibody (1:1000; Cat# F1804) from Sigma, St. Louis, MO; anti-TIM 23 antibody (1:1000; Cat# sc-514463) from Santa Cruz Biotechnology Inc., Santa Cruz, CA; anti-TOMM 20 antibody (1:1000; Cat# A6774) from Abnova, Taipei City, Taiwan; anti-MT-ATP8 antibody (1:1000, 87); anti-ATP5G1MTS antibody (aa 1–61) (1:1000; Cat# ab96655) and anti-NDUFA9 antibody (1:1000; Cat# ab14713) from Abcam, Cambridge, UK; anti-UQCRC2:-Complex III Core2 protein antibody (1:1000; Cat# MS304) and anti-ATP5h antibody (1:1000; Cat# MS504) from MitoSciences, Eugene, OR. The antibody to human MT-ND1 was gifted by Dr. Anne Lombes, Institut Cochin, INSERM, Paris, France. Secondary antibodies to mouse (1:3000; Cat# G21040) and rabbit (1:3000; Cat# A16023) were obtained from Life Technologies, Carlsbad, CA, USA.

4.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.1 software. All qPCR calculations use the mean of each triplicate reaction for one data point. Normality of qPCR data sets was assessed using the Shapiro-Wilk test. mRNA expression in HEK293 cells was analyzed using an unpaired Student's *t*-test for each recoded and optimized gene pair. mRNA expression levels in mutant cell lines ($ND1^{m}$ and $A8/A6^{mut}$) were normalized to expression of *COX10* calculated as $-C_T \pm$ SEM. Viability of mutant lines ($ND1^{m}$ and $CL/BL6^{FVB}$) in galactose was determined from the mean number of live cells after 72h grown under normal or glucose-free conditions. Because the $ND1^{m}$ line demonstrates markedly slower growth under glucose conditions relative to wild-type, data for these cell lines were calculated as the ratio of viable cells in galactose to viable cells in glucose for each experiment. Data are represented graphically with wild-type (143B) viability in glucose set as the reference point (ratio of 1.0). Changes in viability were analyzed using Dunnett's multiple comparison test after one-way ANOVA, vs $ND1^{m}$ (mutant cells). Mouse cell lines ($CL/BL6^{WT}$, $CL/BL6^{FVB}$, and $CL/BL6^{FVB}$ + oATP8) are expressed as the ratio of total viable cells in each condition relative to viable wild-type cells ($C57/BL6^{WT}$) in glucose (ratio of 1.0). For all statistics, differences were considered significant when $P < 0.05$. *P*-values for main figures and supplementary figures can be found in figure legends.

4.12. Analysis of codon composition

Values for relative codon use in the human nuclear genome were obtained from the Codon Usage Database (kazusa.or.jp/codon/) and overall contribution to codon use from GtRNAdb (http://gtrnadb.ucsc.edu/genomes/eukaryota/Hsapi19/Hsapi19-summary-codon.html). Amino acid frequencies were derived from the sum of frequencies of synonymous codons. Codon and amino acid composition of minimally recoded and codon-optimized mitochondrial genes were computed using the CAIcal webserver (genomes.urv.es/CAIcal). Relative synonymous codon use (RSCU) values were calculated as the ratio of observed codon frequency for a synonymous codon to its expected frequency given equal use of all synonymous codons for a given amino acid, based on the standard genetic code. A value of 1.0 indicates no codon bias, while a value less than 0.6 or greater than 1.6 is given to indicate under- or overrepresentation, respectively. Single codon amino acids (Met, Trp) and stop codons were excluded from analysis.

Declarations of interest

None.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101429.

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