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Protein coding mitochondrial-targeted RNAs rescue mitochondrial disease *in vivo*

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Abstract

Mitochondrial encephalomyopathies (MEs) result from mutations in mitochondrial genes critical to oxidative phosphorylation. Severe and untreatable ME results from mutations affecting each endogenous mitochondrial encoded gene, including all 13 established protein coding genes. Effective techniques to manipulate mitochondrial genome are limited and targeted mitochondrial protein expression is currently unavailable. Here we report the development of a *mitochondrial-targeted RNA expression (mtTRES)* vector capable of protein expression within mitochondria (*mtTRES^{Pro}*). We demonstrate that *mtTRES^{Pro}* expressed RNAs are targeted to mitochondria and are capable of being translated using EGFP encoded constructs *in vivo*. We additionally test *mtTRES^{Pro}* constructs encoding wild type ATP6 for their ability to rescue an established *ATP6^L* *Drosophila* model of ME. Genetic rescue is examined including tests with co-expression of mitochondrial targeted translational inhibitors *TLI-NCL::ATP6* RNAs that function to reduce expression of the endogenous mutant protein. The data demonstrate allotopic RNA expression of mitochondrial targeted wild type *ATP6* coding RNAs are sufficient to partially rescue a severe and established animal model of ME but only when combined with a method to inhibit mutant protein expression, which likely competes for incorporation into complex V.

Keywords

Allotopic RNA expression; mitochondrial RNA import; *Drosophila*; Mitochondrial encephalomyopathy (ME)

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Declarations of Interest

MJP and AMC are co-inventors on a University of Pittsburgh patent describing a method to target RNAs to mitochondria as a possible gene therapy (patent 8883755 B2).

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Introduction

Numerous primary respiratory diseases known as mitochondrial encephalomyopathies (ME) are caused by mutations in mitochondrial DNA (mtDNA). Among the most severe MEs are those resulting from mutations directly affecting the mitochondrial DNA. There does not exist an efficacious treatment for mitochondrial disease and the development of a gene therapy approach would have a tremendous impact on patients. Gene therapies for ME patients are complicated by the fact that all established gene transfer methods introduce genes into the nucleus and there is no known method to introduce genes into mitochondria, although efforts to do so are actively being pursued (Niazi et al., 2012). Several groups have proposed mitochondrial-targeted protein-based gene therapies known as allotopic protein expression, however the available data remain somewhat controversial (Bietenhader et al., 2012; Bokori-Brown and Holt, 2006; Bonnet et al., 2008; Dunn and Pinkert, 2012; Ellouze et al., 2008; Figueroa-Martinez et al., 2010; Gray et al., 1996; Guy et al., 2009; Guy et al., 2002; Manfredi et al., 2002; Mukhopadhyay et al., 2006; Oca-Cossio et al., 2003). Expression of hydrophobic mitochondrial proteins within the cytosol could result in aggregation toxicity and it is not known whether such proteins would be efficiently imported or assembled into mature complexes. Current allotopic expression approaches do not address an inherent issue of competition by endogenous mutant proteins. Mitochondrial oxidative phosphorylation complexes are assembled from numerous protein subunits encoded by the mitochondrial as well as the nuclear genomes. Thus, allotopically expressed mitochondrial proteins will typically need to compete with mutant protein when assembling into a functional complex: a complication that has yet to be addressed.

Previously, we described a method to knock down mitochondrial-encoded proteins using mitochondrial targeted small chimeric RNAs that function as translational inhibitors (TLI) (Towheed et al., 2014). These small chimeric RNAs expressed from *mtTRES* vectors have a complementary region designed to interfere with mitochondrial translation initiation of a target gene and are able to specifically knock down protein levels in human cells and *in vivo*. Exploiting the endogenous RNA import pathway we sought to circumvent the technical hurdles associated with allotopic protein expression and asked whether we might be able to develop an allotopic RNA gene therapy. We have now developed vectors to express mitochondrial-targeted protein coding RNAs known as *mtTRES^{Pro}*. Here we demonstrate the effectiveness of *mtTRES^{Pro}* constructs to rescue ME phenotypes using our established *ATP6^I Drosophila* disease model, when combined with *mtTRES-TLIs*. Clinical therapeutic strategies may similarly be more effective by suppressing mutant protein expression and increasing relative wild-type or rescue-encoded protein levels.

Materials and Methods

Engineering *mtTRES^{Pro}* vector, constructs

The previously described *mtTRES* vector (Towheed et al., 2014) was utilized to create the *mtTRES^{Pro}* expression vector. Putative translational initiation elements (*TIEs*) were identified by alignment of sequences 5 prime to known mitochondrial translational start codons. *ATP6*, *EGFP*, and *rATP6* open reading frames (ORFs) were cloned as *TIE::ORF* fusion products using EagI-KpnI cloning sites to generate *mtTRES^{Pro}* vectors. All ORFs

were engineered for mitochondrial translation and stretches of poly T were recoded to prevent RNAPIII termination. Early cytosolic stop codons, which encode tryptophan in mitochondria, were introduced at codon position 58 in *EGFP* and codon position 20 in *ATP6/rATP6*. Genes were synthesized using Genewiz (South Plainfield NJ, USA). The *mtTRES^{Pro}-rATP6* was engineered to be resistant to translation inhibition, requiring numerous nucleotide changes. An EagI-AvrII DNA fragment bearing these changes was generated from synthesized oligonucleotides with complementing 3 prime ends using a primer dimer PCR-based strategy. Briefly, primers were synthesized by IDT (Coralville IA, USA) and utilized in a template-free PCR reaction with 15 cycles of amplification using a 50°C annealing temperature. The primer dimer DNA fragment was purified and directionally cloned into *mtTRES^{Pro}-ATP6* using a unique AvrII site within the *ATP6* coding region using standard techniques. Human myc tags (3 epitope repeats) were engineered at the N-terminus and C-terminus, and corresponding DNAs were synthesized by Genewiz (South Plainfield NJ, USA). All constructs were sequence verified using Genewiz (South Plainfield NJ, USA).

Fluorescence analysis of mtTRES^{Pro}-EGFP

Drosophila heads were removed, probosci dissected and fixed with Carnoy's fixative, as previously described (Palladino et al., 2002). Samples were paraffin processed, sectioned (5 µm) and imaged using confocal microscopy. Fluorescence quantification of the neuropil region was performed using *ImageJ* software (NIH, Bethesda MD, USA).

Drosophila transgenesis, locomotor function, and longevity assays

The PhiC31-mediated *attB* site in the *mtTRES^{Pro}* vector allowed for site-directed transgenesis. We utilized the *attP18* insertion site for the *ATP6*, *rATP6*, *EGFP*, and myc-tagged *rATP6* constructs on the first chromosome. *mtTRES-TLI::ATP6* constructs were previously described and utilized the *VK00027 attP* (*VK27*) insertion site. Control flies contained *attP18* and/or *VK27 attP* chromosomes, as appropriate. Standard methods were used to assay longevity (Celotto et al., 2011; Celotto et al., 2006; Palladino et al., 2000) and locomotor function (Celotto et al., 2011; Fogle et al., 2016; Roland et al., 2015).

Mitochondrial RNA import Assay

Cell Culture—HeLa cells were cultured at 37 C under humidified 95% air/5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher) containing glucose (4.5 gr/L), 1 mM pyruvate, 2 mM glutamine, Penstrep and 10 % fetal bovine serum (FBS).

Isolation of Mitochondria—Mitochondria and mitoplasts were isolated by the following protocol modified from Mercer et. al (Mercer et al., 2011). Mitochondria were prepared from $\sim 2 \times 10^7$ HeLa cells from three 10 cm² dishes transfected with either mtTRES-5s-HsCOX2TLI or mtTRESPro-5s-GFP 24 hours prior to collection using Attractene (Invitrogen). Cells were pelleted in PBS (200xg for 5 min at 4 °C) and resuspended in 800 uL of ice-cold Swell Buffer (10 mM NaCl, 1.5 mM MgCl₂ and 10 mM Tris-HCl, pH 7.5). Cells were allowed to swell for 5 min on ice and homogenized using 60 strokes in a 2 mL glass Dounce homogenizer. The sucrose concentration was then adjusted to 250 mM by adding 2 M sucrose in T₁₀E₂₀ buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.6). Nuclei

were pelleted (1,300 g for 3 min at 4 °C), and the centrifugation step was repeated on the supernatant until no visible pellet formed ~12x. Mitochondria were collected from the supernatant by centrifugation (15,000 g for 15 min at 4 °C). The supernatant was collected and centrifuged (15,000 g for 15 min at 4 °C) until no visible pellet formed. The mitochondrial pellet was resuspended in either 1 mL T₁₀E₂₀ buffer with 2M sucrose (continued with mitochondrial purification) or 50 uL STE/BSA Buffer (*100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.1% BSA*) (see Isolation of Mitoplasts below). To the mitochondrial suspension in T₁₀E₂₀ buffer with 2M sucrose, 1 uL of 100 ug/mL RNaseA (Thermo Fisher) was added and incubated on ice for 30 min. The RNaseA was quenched by adding 5 uL of RNaseOUT (Thermo Fisher). The pellet was washed 2x and resuspended in 100 uL T₁₀E₂₀ buffer with 2M sucrose.

Isolation of Mitoplasts—Mitoplasts were collected by first adding 500 uL of 6.25 mg/mL digitonin to the 50 uL mitochondrial suspensions in STE/BSA Buffer dropwise over two minutes with stirring on ice. Samples were then incubated for an additional 13 minutes with stirring on ice. The mitochondrial outer membrane was removed by adding 1 mL STE/BSA buffer and using 15 strokes on a 2 mL glass Dounce homogenizer. Mitoplasts were pelleted by centrifugation (10,000 g for 10 min at 4 °C) and resuspended in 100 uL STE/BSA buffer. RNase treatment was the same as isolated mitochondria.

Semi-quantitative RT-PCR—RNA was isolated from isolated mitochondria, mitoplasts, and 100 uL of the cytosolic fraction for each sample using the miRNeasy kit (Qiagen) according to manufacturer's instructions. RNA (1 ug) was incubated at 65 °C for 5 min. to remove secondary structure, then was put on ice for at least 1 min. RT was performed using Superscript RT (Invitrogen) and random hexamers for one hour, followed by an incubation at 70 °C for 15 min. to stop the reaction. PCR (95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 30 sec) was performed using GoTaq polymerase (Takara). Five uL from each reaction was taken out every five cycles beginning at cycle 10 and run on a 2% agarose gel.

Western blotting

ATP6 Western blots were performed with 4 adult thoraces homogenized in 60ul of sample buffer. Myc-Western blots were performed on purified mitochondria from 40 adult thoraces similar to a previously described protocol (Towheed et al., 2014). The mitochondrial pellet was suspended in sample buffer (60ul). All samples were heated at 95°C for 10 minutes and resolved using 12% SDS-PAGE. Anti-myc antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA) was used at 1:1000, and fly ATP6 antisera was used at 1:2000 (NeoBioSci, Cambridge MA, USA) (Towheed et al., 2014). Anti-COXII (Proteintech, Chicago IL, USA) was used as a loading control and was used at 1:2500. Anti-SOD2 (LifeSpan BioSciences, Inc., Seattle WA, USA) was used as a loading control at 1:2500. Secondary detection utilized anti-rabbit (1:4000) and anti-mouse (Biorad, USA) HRP conjugated antibodies. Standard methods were used for the analysis of the Western blots (Celotto et al., 2012).

Results

Development of *mtTRES*-protein coding vectors

We have previously developed a series of *mtTRES* (mitochondrial targeted RNA expression system) vectors that transcribe small chimeric mitochondrial-targeted RNAs in mammalian cells and *in vivo* (Towheed et al., 2014). *mtTRES* vectors express RNAs that function as translational inhibitors (TLIs) and decrease expression of endogenously encoded mitochondrial proteins (Towheed et al., 2014). We asked whether we could utilize the *mtTRES* system to express mitochondrial-targeted protein coding RNAs, and developed a series of *mtTRES*-protein coding (*mtTRES^{Pro}*) vectors to address this question. We engineered a translation initiation sequence into *mtTRES^{Pro}* utilizing the sequence 5' of ATP6 translation initiation (Figure 1A, 1B). To test the import and translatability of the RNAs encoded by *mtTRES^{Pro}* vectors *in vivo*, we generated a series of *mtTRES^{Pro}-EGFP* vectors using distinct non-coding leader sequences (NCLs) that target RNAs to mitochondria and bearing the coding region of the enhanced green fluorescent protein (EGFP; Figure 1B). Importantly, we engineered the EGFP for expression within mitochondria and utilized a UGA codon at position 58, which encodes a tryptophan within mitochondria but is a cytosolic translation termination codon. Thus, full-length fluorescent protein can be translated within mitochondria but not within the cytosol. Brain slices from transgenic *mtTRES^{Pro}-NCL::EGFP* flies exhibit fluorescence, indicating significant expression of EGFP *in vivo* (Figure 1C,D). Importantly, two independent transgenic strains utilizing either the *MRP* (fragment of *RMRP* or *RNase MRP*) or *RNP* (stem loop that directs the import of the RNA portion of RNase P) NCL resulted in significant and similar fluorescence expression, demonstrating translatability of *mtTRES^{Pro}-NCL::EGFP* RNAs *in vivo* (Figure 1C, 1D).

Rescue of ATP6¹ with *mtTRES^{Pro}-NCL::ATP6*?

ATP6¹ is a well-established animal model of ME owing to an endogenous mitochondrial loss-of-function mutation (Celotto et al., 2011; Celotto et al., 2006; Fogle et al., 2016; Ma and O'Farrell, 2015; Palladino, 2010). We asked whether *mtTRES^{Pro}-NCL::ATP6* could express wild type ATP6 and rescue *ATP6¹* mitochondrial disease in this animal model. Three independent *mtTRES^{Pro}-NCL::ATP6* transgenic animal strains utilizing three known NCLs, *MRP*, *RNP* and *5S^{mt}* were tested. As with the EGFP encoded constructs, the *ATP6* coding region was optimized for mitochondrial expression and contained a cytosolic stop codon at codon 20 in *ATP6*. This ensured the *mtTRES^{Pro}* protein was expressed from mitochondrial imported RNAs. As an initial test of *mtTRES^{Pro}-NCL::ATP6* function we generated transgenic animals and examined longevity, a well-characterized phenotype associated with the *ATP6¹* mutation. *mtTRES^{Pro}-5S^{mt}::ATP6* transgenic animal longevity was significantly different than *atp18* control flies, however, it did not significantly improve median age (Figure 2). *mtTRES^{Pro}-RNP::ATP6* and *mtTRES^{Pro}-MRP::ATP6* transgenes did not alter longevity and resulted in a small decrease in longevity (Figure 2). Overall experiments with the *mtTRES^{Pro}-NCL::ATP6* series of transgenes suggested these were ineffective at rescuing *ATP6¹*.

Mitochondrial import of *mtTRES* and *mtTRES^{Pro}* encoded RNAs

We and others have shown that many nuclear encoded RNAs are efficiently imported into mitochondria and some of these have been shown to function as mitochondrial targeting sequences or NCLs capable of targeting chimeric RNAs to mitochondria (Das et al., 2014; Entelis et al., 1998; Entelis et al., 2001a; Entelis et al., 2001b; Kolesnikova et al., 2004; Kolesnikova et al., 2000; Mercer et al., 2011; Noh et al., 2016; Sanchez et al., 2011; Smirnov et al., 2008; Sripada et al., 2012a; Sripada et al., 2012b; Tarassov and Entelis, 1992; Wang et al., 2010a; Wang et al., 2011; Wang et al., 2010b; Towheed et al., 2014). We have developed *mtTRES* vectors using NCLs that efficiently target chimeric RNAs to mitochondria that are both stable and functional, as in the case of mito-TLIs (Towheed et al., 2014). Using various import assays we have shown that *mtTRES* mitochondrial targeted RNAs are imported, however, these are relatively small RNAs (45–150 ribonucleotides in length) and not of sufficient length to encode useful proteins capable of rescuing an endogenous protein-coding gene mutation (Towheed et al., 2014). To determine whether the inability to rescue was due to ineffective expression or mitochondrial import, we examined the import of small *mtTRES* RNAs and larger *mtTRES^{Pro}* RNAs using a semi-quantitative RT-PCR assay and mitochondrial purified RNAs. We chose to examine *mtTRES^{Pro} 5S-eGFP* import as it was larger than *mtTRES^{Pro} 5S-ATP6* and to avoid possible complications by detecting the endogenous *ATP6* mRNAs. We generated mitoplasts, mitochondria with the outer mitochondrial membrane (OMM) removed, and used *Tim23* (an RNA known to bind to the OMM) as a rigorous control to ensure our mitoplasts were free of contaminating RNAs (Figure 3A). As previously reported small chimeric *mtTRES*-TLI RNAs were readily identified in the mitoplast fraction (Figure 3B). Interestingly the ~ 880 ribonucleotide *mtTRES^{Pro}* generated RNAs also demonstrated a strong enrichment in the mitoplast fraction (both easily detectable by cycle 31), suggesting the efficient import of small and large coding mitochondrial targeted RNAs (Figure 3).

Protein competition is a formidable hurdle to genetic rescue

Our data with *mtTRES^{exp}-NCL::ATP6* transgenic animals were unexpected, especially since *mtTRES^{Pro} RNAs* are efficiently imported and *mtTRES^{Pro}-NCL::EGFP* transgenic animals demonstrated translatability of mitochondrial imported RNAs by confocal microscopy. To better understand pathogenesis in *ATP6^I* and why this may be difficult to rescue, we performed Western blots with antisera to the ATP6 protein. These experiments revealed that steady state ATP6 protein levels were not changed in mutant *ATP6^I*, *sesB^I*, or *ATP6^I;sesB^I* double mutant flies from that of wild type control animals (Figure 4A and 4B). These data demonstrate that the missense mutation associated with *ATP6^I* does not significantly alter protein expression and suggests that competition with mutant protein for incorporation into the mature complex V represents a formidable obstacle to a genetic rescue.

Genetic rescue of *ATP6^I* with allotopic RNA expression

Previously we have shown that *mtTRES* expressed chimeric RNAs are capable of decreasing protein expression *in vivo* via translational inhibition (Towheed et al., 2014). To directly test the effect of mutant protein competition on efficacy of genetic rescue, we engineered a series

of ATP6 expressing vectors resistant to TLI (*mtTRES^{Pro}-NCL::rATP6*). To confer resistance, silent changes were made to the target site of *NCL-TLI::ATP6* RNA complementary element (Supplementary Figure 1). We generated *mtTRES^{Pro}-NCL::rATP6* transgenic animals utilizing the *5S^{mt}*, *MRP* and *RNP*NCLs, combined these each with *TLI-RNP::ATP6* and examined their ability to rescue the *ATP6^l* longevity phenotype. With co-expression of TLI RNAs, all *NCL::rATP6* RNAs were able to significantly rescue the longevity phenotype of *ATP6^l* control animals (Figure 5A). We examined locomotion in these same genotypes and all combinations of *mtTRES^{Pro}-NCL::rATP6*, and *TLI-RNP::ATP6* were able to provide significant improvement in locomotor function (Figure 5B). These data demonstrate a functional genetic rescue with three completely independent *mtTRES^{Pro}-NCL::rATP6* transgenes utilizing distinct NCL elements.

We have shown that *TLI-RNP::ATP6* and *TLI-MRP::ATP6* are each capable of knocking down ATP6 protein expression *in vivo* (Towheed et al., 2014). As an additional test of efficacy, we tested all three *mtTRES^{Pro}-NCL::rATP6* transgenes each in combination with *TLI-MRP::ATP6* expression to test their ability to rescue the *ATP6^l* longevity phenotype. Consistent with previous findings each of the *mtTRES^{exp}-NCL::rATP6* transgenes were able to significantly improve longevity over *ATP6^l* controls (Figure 6A). We examined locomotor phenotypes in all genotypes, as we had previously, and each showed a reduced mean recovery time. The *mtTRES^{Pro}-MRP::rATP6*; *MRP-TLI::ATP6* and *mtTRES^{Pro}-RNP::rATP6*; *MRP-TLI::ATP6* combinations were significantly improved over the *ATP6^l* control animals (Figure 6B). Together these data are consistent with the conclusion that allotopic RNA expression of mitochondrial-targeted coding RNAs using *mtTRES^{Pro}* are capable of genetic rescue with co-expression of mitochondrial-targeted TLI RNAs. These data additionally demonstrate the importance of mutant protein competition in the development of genetic rescue and gene therapy approaches for mitochondrial disease resulting from a protein coding missense mutations.

Genetic manipulation of ATP6 protein expression

Significant genetic rescue is achieved when we combine allotopic RNA-based expression of wild type ATP6 protein with a mechanism to specifically knock down endogenous mutant protein expression. Thus, endogenous expression of mutant ATP6 protein is a significant barrier to genetic rescue. As a biochemical test of our ability to genetically manipulate ATP6 protein levels, we performed Western blots with ATP6 antisera. Flies expressing *mtTRES^{Pro}-MRP::rATP6* do not significantly change ATP6 levels *in vivo* (Figure 7, lane 2), however, flies expressing *TLI-MRP::ATP6* RNAs knock down endogenous mutant ATP6 levels as predicted (Figure 7, lane 3). When we combined *mtTRES^{Pro}-MRP::rATP6* and *TLI-MRP::ATP6*, total ATP6 protein levels are significantly increased (Figure 7, compare lanes 1 and 4). These data demonstrate an ability to genetically manipulate ATP6 protein levels *in vivo* and support the conclusion that reducing mutant protein expression is necessary for an efficacious genetic rescue expressing wild type protein.

Demonstrating the import of allotopically expressed rATP6

As an independent confirmation that allotopic *mtTRES^{Pro}-MRP::rATP6* derived protein is being expressed, we sought to identify a suitable site to epitope tag the ATP6 protein. As

previous tagging information is not available for this protein, we engineered *mtTRES^{Pro}-MRP::rATP6^{MYC}* transgenic animals with tagged sites at both the N-term and C-term (Figure 8A). We asked whether myc-tagged ATP6 proteins could be detected by Western blot using anti-myc antibodies. Western blotting of *mtTRES^{Pro}-MRP::rATP6^{MYC}* transgenic animal proteins demonstrated significant expression of myc-tagged rATP6 proteins (Figure 8B). Importantly, the constructs expressed a protein of the expected ~ 29 kDa size and all were engineered to terminate prematurely producing a ~2.2 kDa or ~6.5 kDa protein if translation were to occur in the cytosol (which was not observed). Collectively, these data demonstrate expression of ATP6 protein expression from allotopic expressed mitochondrial-targeted coding RNAs utilizing *mtTRES^{Pro}*. We then asked whether the tagged constructs retained functionality and could still support a partial rescue of *ATP6^l*. *mtTRES^{Pro}-MRP::rATP6^{MYC}* with N-term tag completely abrogated functional rescue of *ATP6^l*, whereas, the C-term tag retained the ability to functionally rescue the *ATP6^l* mutant (Figure 8C).

Discussion

Type I mitochondrial encephalomyopathies (MEs) are severe untreatable diseases owing to mutations within the mitochondrial genome that impair an aspect of OXPHOS. Although gene therapies are being actively pursued, efficient methods of gene transfer into mitochondria do not currently exist in animals. Allotopic protein expression from the nucleus that targets proteins to the mitochondrion has been proposed by a number of laboratories with varying degrees of reported efficacy (Bietenhader et al., 2012; Bokori-Brown and Holt, 2006; Bonnet et al., 2008; Dunn and Pinkert, 2012; Ellouze et al., 2008; Gray et al., 1996; Guy et al., 2009; Guy et al., 2002; Manfredi et al., 2002). Noted throughout the literature are potential issues with the hydrophobicity of endogenously encoded mitochondrial proteins being expressed in the cytosol (Mukhopadhyay et al., 2006; Oca-Cossio et al., 2003). Import of proteins with multiple transmembrane spanning regions are likely to be exceptionally difficult (Figueroa-Martinez et al., 2011). In addition to their hydrophobicity, endogenously encoded mitochondrial proteins encode key OXPHOS subunits that are assembled into large multimeric mature complexes in order to function. ATP6 is no exception. The ATP6 protein is an ~ 25 kDa integral membrane hydrogen ion channel localized to the inner mitochondrial membrane that is highly hydrophobic and comprised of 5 transmembrane spanning regions with relatively small loops and termini. ATP6 is an essential component of the F₁F₀-ATP synthase (complex V) that is composed of dozens of individual subunits that make a mature functional holocomplex of ~ 600 kDa (Jonckheere et al., 2012).

We have proposed that targeting mRNAs to mitochondria, described here as allotopic RNA expression, could resolve the hydrophobicity problem and improve function as the proteins would be synthesized by endogenous mitoribosomes rather than cytosolic ribosomes. Many mechanistic details of mitochondrial RNA import pathways remain unclear and are still under investigation (Wang et al., 2015). However, a diverse group of RNAs have been shown to be imported into the mitochondria, including small hairpin RNAs (RNP and MRP), 5S rRNAs, tRNAs, and viral RNAs (Das et al., 2014; Entelis et al., 1998; Entelis et al., 2001a; Entelis et al., 2001b; Kolesnikova et al., 2004; Kolesnikova et al., 2000; Mercer et al., 2011;

Noh et al., 2016; Sanchez et al., 2011; Smirnov et al., 2008; Sripada et al., 2012a; Sripada et al., 2012b; Tarassov and Entelis, 1992; Towheed et al., 2014; Wang et al., 2010a; Wang et al., 2011; Wang et al., 2010b). We have previously shown the efficient import of small chimeric RNAs that are fusions between known imported RNAs with foreign sequences (Towheed et al., 2014). Here we report the development of *mtTRES^{Pro}* vectors and use this system to demonstrate the import of long RNAs capable of encoding proteins. We use a well-characterized ME *Drosophila* model to test the efficacy of *mtTRES^{Pro}* vectors *in vivo*. Our findings demonstrate for the first time the viability of the *mtTRES^{Pro}* vectors and allotopic RNA expression as gene therapy in an animal model of ME. Interestingly, we did not observe efficacy until we combined our allotopic RNA expression system with a method to knock down the endogenously expressed mutant protein. These results suggest there may be competition for inclusion into the mature complex V and that mutant proteins, such as ATP6, might function in a dominant-negative manner. Although it is difficult to formally demonstrate competition is the sole basis for the observed results, it is the most parsimonious explanation for the data. Nonetheless these findings demonstrate the importance of considering competition and other possible dominant-negative effects when developing rescue experiments and therapies. This is especially important since missense mutations are extraordinarily common causes of type I MEs.

We were able to utilize this system to test ATP6 tagging sites that would not disrupt the protein's function. Both termini of ATP6 were tested using a cassette of 3 myc epitopes. We demonstrate expression of the Myc-ATP6 (N-Term) and ATP6-Myc (C-Term) fusion proteins from mitochondrial targeted allotopic coding RNAs. The N-term tag appeared to disrupt the protein's function. Importantly, the tagging of the C-term did not disrupt function and supported a rescue of the *ATP6^I* longevity phenotype similar to the untagged protein.

mtTRES^{Pro} rescue of *ATP6^I* was only observed when combined with a method to knock down mutant protein expression. The rescue observed was significant and reproducible, and was observed using numerous different combinations of independently generated transgenic strains with distinct targeting elements. Although this is an important advancement, the rescues observed were not complete. We observed a maximum improvement of ~ 15–20% in longevity and ~ 25% in locomotor function. Collectively the data suggest the RNAs are efficiently generated and targeted to mitochondria but that their expression may not be optimal. It is possible that the robust targeting *mtTRES^{Pro}* RNAs to mitochondria results in toxicity that prevents a more complete rescue. It is also possible that the NCL targeting element is interfering with efficient mitoribosome translation initiation. Future studies are needed to resolve these possibilities and to optimize translation of the rescue constructs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *mtTRES^{Pro}* can target long coding allotopic RNAs to mitochondria
- Mitochondrial targeted RNAs encoding EGFP function as a reporter in *Drosophila*
- *mtTRES^{Pro}-ATP6* RNAs are tested for their ability to rescue ME *in vivo*
- Rescue, albeit incomplete, was observed when combined with a method to decrease mutant protein expression

Figure 1. Translation of mitochondrial imported *mtTRES^{Pro}-EGFP* RNAs in *Drosophila*
 (A & B) Design of *mtTRES^{Pro}-attB* vector showing location of RNAPIII initiation (orange), RNAPIII termination (brown), non-coding mitochondrial leader sequence (NCL) (blue), translation initiation sequence (red) and protein coding region (green). Full-length protein will only be translated within mitochondria (green Mito. protein), whereas, cytoplasmic translation will be truncated and non-functional (grey Cyto. protein). (C) Brain neuropil EGFP fluorescence detected by confocal imaging. (D) Quantification of the fluorescence intensity of brain slices (n > 85 for all genotypes).

Figure 2. Allotopic RNA expression of mitochondrial-targeted ATP6 coding RNAs
 Survival curves for *ATP6^l attP18/attP18* (black), *ATP6^l;MRP-ATP6/MRP-ATP6* (blue), *ATP6^l;RNP-ATP6/RNP-ATP6* (green) and *ATP6^l;5smt-ATP6/5smt-ATP6* (red) ($n > 150$ for each genotype). Bars represent $\pm$ SEM.

Figure 3. Mitochondrial import assays show strong enrichment of *mtTRES* and *mtTRES^{Pro}* encoded RNAs

(A) *TIM23* was used as a control as it is nuclear encoded but its mRNA is targeted to the OMM. As predicted, it is detectable in the cytosol, C, and in mitochondria, M, but is absent in purified mitoplasts, MP, whereas, the *5S-COXII-TLI* RNA is detectable in all fractions. (B) *mtTRES* RNAs are readily detectable in MPs. The *TIM23* control isn't detectable even at cycle 45. (C) *5S-eGFP* RNAs are also readily detectable in MPs in the absence of detectable *TIM23* demonstrating highly purified mitoplasts and efficient import of allotropic long coding RNAs. The *TIM23*, *5S-COXII-TLI*, and *5S-eGFP* amplicons were 111, 161 and 368 base pairs, respectively. In all cases (–) RT did not produce the observed amplicon. n=3.

Figure 4. Competition with endogenous ATP6 protein expression

(A) Immunoblotting of fly lysates to measure ATP6 protein levels. Mitochondrial localized SOD2 protein used as a loading control. (B) ATP6 protein levels expressed relative to wild-type control flies (black), *ATP6¹;sesB¹* (green), *ATP6¹* (red) and *sesB¹* (blue). *sesB¹* is a mutant allele encoding the *Drosophila* Adenine Nucleotide Translocator (ANT) and is the mutant strain in which the original *ATP6¹* was identified. n is greater or = to 3. Bars represent $\pm$ SEM. One-way ANOVA.

Figure 5. Allotopic RNA expression mitochondrial-targeted ATP6 coding RNAs with RNP::TLI
 (A) Survival curve for flies ATP6¹;attp18/attp18;;VK27/+ (**black, A**), ATP6¹;MRP-rATP6/MRP-rATP6;;RNP-TLI::ATP6 (**blue, B**), ATP6¹;RNP-rATP6/RNP-rATP6;;RNP-TLI::ATP6 (**green, C**) and ATP6¹;5smt-rATP6/5smt-rATP6;;RNP-TLI::ATP6 (**red, D**) Log-rank (Mantel-Cox) test was done for significance, bars represent $\pm$ SEM, $n > 150$ each genotype. Wild type control flies have a median age of ~ 70 days (not shown for clarity). (B) Mechanical stress sensitivity assay showing time to recovery for flies A, B, C and D ($n > 20$, bars denote $\pm$ SEM, One-way ANOVA). Wild type flies are not stress sensitive in this assay and all recover in 1-3 seconds (not shown). (A and B) * is $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.001$.

Figure 6. Allotopic RNA expression mitochondrial-targeted ATP6 coding RNAs with MRP::TLI (A) Survival curve for flies *ATP6¹;atp18/atp18;VK27/+* (**black, A**), *ATP6¹;MRP-rATP6/MRP-rATP6;;MRP-TLI::ATP6* (**blue, B**), *ATP6¹;RNP-rATP6/RNP-rATP6;;MRP-TLI::ATP6* (**green, C**) and *ATP6¹;5S^{mt}-rATP6/5S^{mt}-rATP6;;MRP-TLI::ATP6* (**red, D**) ($n > 150$ each genotype), Log-rank (Mantel-Cox) test for significance, bars represent $\pm$ SEM. (B) Mechanical stress sensitivity assay showing time to recovery for flies A, B, C and D ($n > 20$, bars denote $\pm$ SEM, One-way ANOVA). (A and B) ** is $p < 0.01$, *** is $p < 0.001$.

Figure 7. Genetic manipulation of ATP6 protein

(A) Western blot showing steady state level of ATP6 proteins in flies. *ATP6^l* (lane 1), *ATP6^l;MRP-*r*ATP6/MRP-*r*ATP6* (lane 2), *ATP6^l;TLI-MRP::ATP6* (lane 3) and *ATP6^l;MRP-*r*ATP6/MRP-*r*ATP6;TLI-MRP::ATP6* (lane 4). Mitochondrial protein COXII was used as a loading control. (B) Quantification of western blot data for ATP6 protein levels was normalized to the loading control. One-way ANOVA, n=3, $\pm$ S.E.M. * is p<0.05.

Figure 8. Myc-tagged allotopically expressed *rATP6* partially rescues mutant phenotype (A) Illustration of myc-tagged constructs. Untagged *mtTRES^{Pro}-MRP::rATP6* (no color). N-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (red), C-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (green). (B) myc-tagged ATP6 detected by western blot using anti-myc antibodies in mitochondrial preparations. Asc1 positive control yeast (lane 1), N-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (N-myc, lane 2), C-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (C-myc, lane 3) Mitochondrial protein COXII was used as a loading control. (C) Survival curves for external myc-tagged flies. Untagged mutant control *ATP6¹, atp18; VK27/+* (black), *mtTRES^{Pro}-MRP::rATP6* control (orange), N-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (red) and C-term *mtTRES^{Pro}-MRP::rATP6^{MYC}* (green). *** is $p < 0.001$.