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Small mitochondrial-targeted RNAs modulate endogenous mitochondrial protein expression *in vivo*

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Abstract

Endogenous mitochondrial genes encode critical oxidative phosphorylation components and their mutation results in a set of disorders known collectively as mitochondrial encephalomyopathies. There is intensive interest in modulating mitochondrial function as organelle dysfunction has been associated with numerous disease states. Proteins encoded by the mitochondrial genome cannot be genetically manipulated by current techniques. Here we report the development of a *mitochondrial-targeted RNA expression system* (*mtTRES*) utilizing distinct non-coding leader sequences (NCLs) and enabling *in vivo* expression of small mitochondrial-targeted RNAs. *mtTRES* expressing small chimeric antisense RNAs were used as translational inhibitors (TLIs) to target endogenous mitochondrial protein expression *in vivo*. By utilizing chimeric antisense RNA we successfully modulate expression of two mitochondrially-encoded proteins, ATP6 and COXII, and demonstrate the utility of this system *in vivo* and in human cells. This technique has important and obvious research and clinical implications.

Keywords

Mitochondrial disorders; translation inhibitors; non-coding small chimeric RNA; mitochondrial encoded proteins

Introduction

Mutations in the mitochondrial genome cause a set of devastating disease conditions categorized as primary respiratory chain diseases, also known as mitochondrial encephalomyopathies (MEs) (DiMauro and Schon, 2003). Mitochondrial gene therapy has

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been proposed as a treatment for ME, however, this approach remains controversial as there are limited preclinical data demonstrating efficacy and evidence suggesting this approach may have significant limitations (Manfredi et al., 2002, Bokori-Brown and Holt, 2006, Perales-Clemente et al., 2011).

Endogenously encoded mitochondrial proteins function within large well-characterized respiratory complexes that perform oxidative phosphorylation (OXPHOS). The mitochondrial genome is known to harbor hundreds of pathogenic mutations, including ones affecting all of the tRNA genes and over 260 distinct coding mutations. The vast majority of protein-coding gene mutations associated with human mitochondrial disease are missense mutations, accounting for ~225 of the pathogenic mitochondrial mutations (www.mitomap.org), implying that mutant protein is usually capable of being expressed in the disease state. We previously discovered and characterized a *Drosophila* model of ME with an endogenous missense mutation in the *ATP6* gene affecting the F_1F_o-ATPsynthase (complex V) (Celotto et al., 2006a, Palladino, 2010). Twenty-one distinct human missense mutations exist within the *ATP6* gene, fourteen of which have been shown to cause human MEs including Familial Bilateral Striatal Necrosis (FBSN), Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP), or Maternally Inherited Leigh's Syndrome (MILS) (Noji et al., 1997, Stock et al., 1999, D'Aurelio et al., 2010). *ATP6*^{1} mutant flies contain a missense mutation with high mutant heteroplasmy and exhibit phenotypes analogous to human symptoms including locomotor and progressive neural dysfunction, seizures, myodegeneration, and reduced longevity (Palladino, 2010).

Competition with mutant protein for incorporation into mature respiratory complexes is likely a major obstacle to a viable mitochondrial gene therapy: a fact that has largely been ignored. This competition may explain the controversial allotopic expression results and remains a formidable obstacle to the treatment of MEs resulting from any endogenous mitochondrial missense mutation. A method to specifically reduce expression of mitochondrial-encoded genes is not known.

Several RNAs are naturally imported into the mitochondria from the cytoplasm and detailed studies have provided critical insight into the import process and import substrates (Schneider and Marechal-Drouard, 2000, Tarassov et al., 2007, Lithgow and Schneider, 2010). Although the exact mechanism of RNA import into mitochondria is unknown, several pathways have been suggested to mediate mitochondrial RNA import (Mahapatra and Adhya, 1996, Wang et al., 2010, Schneider, 2011). We have identified a nuclear encoded mitochondrial *5S rRNA* isoform and engineered a novel vector to express small RNAs *in vivo*. We developed a mitochondrial-targeted translational inhibition (TLI) approach using small chimeric RNAs to regulate endogenous mitochondrial protein expression. Here we demonstrate the efficacy of mitochondrial-targeted TLIs by targeting two distinct loci encoding essential proteins of two different OXPHOS complexes, one *in vivo* and the other *in vitro*. The ability to selectively modulate mitochondrial protein expression in animals represents an important technological advance with obvious research and clinical applications.

Material and methods

Engineering *mtTRES* and *mtTRES-TLI* constructs

The *mtTRES* vector was created using the available *pUAST-attB* vector as a backbone (Bischof et al., 2007). A StuI site was added by site directed mutagenesis 5' to the *attP* integration site using Quick Change Lightning (Invitrogen, USA). The *5S rRNA RNAPIII* promoter (AE013599.4) and termination (AE013599.4) sequences were PCR amplified from wild type *Drosophila* genomic DNA and directionally inserted using standard cloning methods and the HindIII-EcoRI and StuIKpnI cloning sites, respectively. For the mammalian *mtTRES* vector the human *U6* promoter (NT_010194.17) was PCR amplified from p*Silencer* 2.1 (Invitrogen, USA), purified and inserted in place of the fly *RNAP III* promoter. The EcoRI-EagI cloning sites were used to insert NCLs. The *5S rRNA*^{mt} variant was identified as the most abundant mitochondrial isoform by clonal analyses (88%) from three independent cloning events and sequence analysis of 135 clones. The *5S rRNA*^{mt} was the major mitochondrial isoform in all three independent clonal populations (Supplementary material: Figure S1 and **GenBank: CR33451**). The *5S rRNA*^{mt} sequence was synthesized with flanking EcoRI-EagI cloning sites (GeneWiz, South Plainfield NJ, USA). The *MRP* and *RNAseP (RNP)* oligonucleotides were annealed and directionally cloned into EcoRIEagI cloning sites using published sequences (Wang et al., 2012b). TLI complementary sequences were synthesized as oligonucleotides, annealed and directionally cloned into EagIKpnI cloning sites. *TLI-5S*^{mt}*::ATP6(a)* is 25 bases long, whereas *TLI-5S*^{mt}*::ATP6(b)* is 26 nucleotides in length and the complementary region is shifted 3 nucleotides 5'. All oligonucleotides were commercially synthesized by IDT (Coralville IA, USA). The final constructs were sequence verified (GeneWiz, South Plainfield NJ, USA).

Drosophila transgenesis, longevity and locomotor assays

mtTRES vectors allow site-directed PhiC31-mediated *attP/B* transgenesis. We used the *VK00027 attP* insertion site and flies bearing the *VK27 attP* chromosome are the control for all transgenic experiments. DNA injections were performed by Genetic Services (Cambridge MA, USA) and successful transgenesis events were identified using *white*$^{mc+}$. Homozygous transgenic strains were tested. Previously established methods were used to test longevity (Palladino et al., 2003) and locomotor assays (Fergestad et al., 2006, Fergestad et al., 2008).

Western blotting and antisera production

Standard methods were used for western blot analyses (Celotto et al., 2012). Briefly, flies were carbon dioxide anesthetized and snap frozen in liquid nitrogen. Thoraces from 8 flies were dissected and homogenized in sample buffer (125 µl), heated at 95 °C for 5 min, loaded into the wells of an SDS-PAGE gel. Antisera was generated to fly ATP6 protein using purified HKEFKTLLGPSGHNGS peptide (hc17), immunized New Zealand rabbits and antigen affinity purification (NeoBioSci, Cambridge MA, USA). Anti-ATP6 antibody recognition specificity of hc17 peptide was confirmed by Southern Blot and ELISA (by NeoBioSci, Cambridge MA, USA). Western blotting identifies a single ~25kDa protein that enriches with mitochondria. Competitive ELISA (kit by Cell Biolabs Inc., USA) using fly lysates and increasing concentrations of hc17 peptide was used to further validate the specificity of the anti-ATP6 antibody (Supplementary material, Figure S2). ATP6 antisera is

used 1:2000. Anti-COXII antibodies (Proteintech, Chicago IL, USA) and anti-SOD2 antibodies (LSBio, Seattle WA, USA) were used at 1:2500 and 1:2000, respectively. Anti-ATP-alpha (a5-c antibody, Developmental Studies Hybridoma Bank, University of Iowa, USA) was used as a loading control. ATPalpha is a nuclear encoded plasma membrane protein (the catalytic subunit of the Na^+/K^+ ATPase). For HeLa cells, 1×10^6 cells were electroporated and harvested after ~48–72 hr for western blot. GAPDH (1:3000) (Abcam, USA) was used as a loading control. Secondary detection was performed using anti-rabbit (1:4000) (Biorad, USA) and anti-mouse (1:10000) (Biorad, USA) HRP conjugated antibodies. For all Western blots sub-saturation images have been quantified. In some cases, darker exposures of the quantified images are used in the figures.

RNA isolation and Quantitative RT–PCR

RNA was extracted from 12 whole flies, using 250 μl Trizol (Invitrogen, San Diego, USA) and the RNeasy mini kit (Qiagen, Valencia, USA). RNA was eluted in 100 μl dH2O and quantified. 5 μg RNA was used to perform a reverse transcription reaction (Superscript RT, Invitrogen). Quantitative Real-Time PCR [Mx3000P QPCR System, Stratagene] was performed using standard techniques with normalization to *RP49* expression (Celotto et al., 2006b). Only DNA-free cDNA samples were used. In a total reaction of 25 μl, 12.5 μl 2X-SYBR Green Supermix (Qiagen, Valencia, USA), 2 μl of cDNA and 400 nM each of forward and reverse primers (*ATP6*, *COXII*) were used. Fold change (FC) was determined using the equation, $FC=2^{-\Delta(\Delta Ct)}$. All QPCR experiments were performed with four biological replicates and the data were normalized to mRNA expression levels of *RP49*.

Isolation of mitochondria from HeLa cells

Mitochondria were isolated using standard differential centrifugation procedure. In short, 24 million cells were trypsinized and homogenized by Dounce homogenizer. Nuclear fraction was pelleted at 1000*g* for 15 min. The supernatant was then centrifuged at 10000*g* for 15min. The pellet contained the enriched mitochondria.

In vitro transcription and radiolabeling

Primers were designed to amplify $5S^{mt}$ and $TLI\text{-}5S^{mt}::COXII$ sequences from previously engineered $mtTRES\text{-}5S^{mt}$ and $mtTRES\text{-}5S^{mt}\text{-}TLI::COXII$ plasmids. *T7+5s_For* (TAATACGACTCACTATAGGGGCCAACGACCATACCACGCTGAATAC) and *5s_Rev* (AGGCCAACAACACGCGGT GTTC) primers were used for 5S DNA amplification. For $TLI\text{-}5S^{mt}::COXII$, *T7+5s_For* and *COX2_Rev* (TCCAAAAAATCTTAATGGCACATGCAGC) primers were used.

Using Thermo Scientific TranscriptAid T7 High Yield Transcription Kit and [α-32P] adenosine 5’-triphosphate (MP Biomedicals) *in vitro* transcription was performed, as per the manufacturer’s instructions. Unincorporated [α-32P] ATP was removed using NucAway™ Spin Columns (Ambion Inc. Austin, Texas). Specific activities of radiolabeled RNA products were quantified by LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter) and equal amounts used in the mitochondrial import assay.

Mitochondrial RNA import assay

Mitochondrial RNA import assay was modified from (Magalhaes et al., 1998, Bhattacharyya et al., 2002, Wang et al., 2012a). In short, mitochondrial pellets were suspended with RNA probes in the import buffer (200 μl final volume) containing 0.25M sucrose, 2 mM KH_2PO_4, 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 5 mM L-methionine, 1 mg/ml BSA, 5 mM ATP, 2 mM DTT, 20 mM succinate, 50 mM HEPES, [pH 7.1]. The mixture was incubated for 20–30 minutes at room temperature. Mitochondria were spun at 11000*g* for 5 min and washed once with wash buffer (0.6 M sorbitol, 20 mM Tris, [pH 8.0]). To remove RNA that was not imported in the mitochondria, the pellet was spun again and resuspended in 200 μl nuclease buffer containing 25 μg/ml of micrococcal nuclease (New England Biolabs Inc.) and incubated for 30 min at 27°C. Mitochondria were collected, solubilized in SDS buffer at 65°C for 5 min, RNA was purified using TRIzol® reagent (Life Technologies) and resolved by denaturing polyacrylamide urea gel (National Diagnostics). Autoradiography was performed using phosphor imager and gel was scanned using Image Quant software.

Results

Generation of *mtTRES* for TLI *in vivo*

The metazoan mitochondrial genome contains 37 genes that are critical for electron transport chain function. These include 13 protein-coding genes and RNAs to facilitate their expression: 22 tRNAs and 2 rRNAs (12S and 16S) (Anderson et al., 1981, Bibb et al., 1981). All other proteins and RNAs functioning within the mitochondrion are imported from the cytoplasm. It has previously been described that 5S rRNAs are expressed from large nuclear gene arrays (~100–200 genes) and that *5S* rRNAs can readily be found within mitochondria from flies to humans (Benhamou and Jordan, 1976, Artavanis-Tsakonas et al., 1977, Yoshionari et al., 1994, Magalhaes et al., 1998, Entelis et al., 2001, Smirnov et al., 2008, Smirnov et al., 2010). Although these gene arrays encode many *5S rRNA* isoforms, we identified a single common mitochondrial *5S rRNA* variant (*5S rRNA*^{mt}) representing the majority of *5S rRNAs* within *Drosophila* mitochondria. To enable studies *in vivo*, we developed the *mtTRES* (mitochondrial Targeted RNA Expression System) vector using *5S rRNA*^{mt} as a non-coding leader sequence (NCL) and employing *RNAP III* promoter and termination elements (Figure 1). The RNAPIII promoter was selected due to its ability to direct transcription of rRNAs, tRNAs and other small non-coding RNAs (Dieci et al., 2007).

We asked whether we might utilize this mitochondrial RNA targeting system to modulate expression of endogenous mitochondrial genes *in vivo*. To test this we generated transgenic *mtTRES* animals capable of expressing chimeric RNAs consisting of an NCL and a sequence complementary to a mitochondrial mRNA, specifically targeting the known translational start site (Figure 1B, C). Translational inhibition/repression has been demonstrated to be functional within the cytosol by antagonizing small ribosomal subunit docking and lowering translational efficiency (Doyle et al., 2001) but has never been demonstrated in mitochondria.

mtTRES ATP6 TLIs phenocopy *ATP6*^{1} longevity and locomotor dysfunction

Numerous *ATP6* missense mutations are associated with human disease and our detailed understanding of *ATP6*^{1} mutant phenotypes prompted us to initially ask whether we could functionally knock down ATP6 expression *in vivo*. We utilized *mtTRES* to generate two independent transgenic *ATP6* TLIs designated as *TLI-5S*^{mt}*::ATP6(a)* and *TLI-5S*^{mt}*::ATP6(b)*. Lifespan assays were performed to test whether *TLI-5S*^{mt}*::ATP6* TLIs affect the longevity of flies. We observed a significant decrease in the longevity of flies expressing *TLI-5S*^{mt}*::ATP6(a)* or *TLI-5S*^{mt}*::ATP6(b)* compared to wild type control flies (Figure 2A). These data demonstrate that *mtTRES* TLIs targeting *ATP6* reduce longevity consistent with a loss of ATP6 function *in vivo*.

TLI-5S^{mt}*::ATP6(a)* and *TLI-5S*^{mt}*::ATP6(b)* flies were tested for conditional locomotor function in response to sensory hyperstimulation (bang sensitivity), a progressive seizure-related phenotype resulting from loss of ATP6 function *in vivo* (Celotto et al., 2006a). Young *TLI-5S*^{mt}*::ATP6(a)* and *TLI-5S*^{mt}*::ATP6(b)* animals (day 5) were aphenotypic; however, aged animals (day 50) exhibited conditional locomotor impairment compared to wild type control animals (Figure 2B). Strikingly, both *TLI-5S*^{mt}*::ATP6* transgenic strains phenocopy the conditional locomotor dysfunction observed in *ATP6*^{1}, including the progressive nature of this mitochondrial seizure-related phenotype. Importantly, *ATP6*^{1} is of extremely high mutant heteroplasmy (98%) and results in severe locomotor and longevity phenotypes, whereas, *TLI-5S*^{mt}*::ATP6* results in an ~50% knockdown and the observed phenotypes are qualitatively similar but less severe, as would be expected for the hypomorphic condition.

Mitochondrial TLIs modulate protein levels

To more directly test the ability of *mtTRES* TLI's to modulate protein expression we performed western blotting with *TLI-5S*^{mt}*::ATP6(a)* transgenic fly lysates. Western blotting demonstrated a 34% reduction in ATP6 levels compared to lysates from wild type control animal (Figure 2C, D). These data demonstrate the *mtTRES* TLI approach is capable of endogenous mitochondrial protein modulation *in vivo*.

mtTRES TLI using distinct NCLs *in vivo*

Previously two small RNAs, *MRP* and *RNP*, were shown to be actively imported into mammalian mitochondria *in vitro* (Wang et al., 2010), suggesting their utility as NCLs. We generated two additional transgenesis vectors for *in vivo* animal studies, *mtTRES*^{MRP} and *mtTRES*^{RNP}. As an additional test of the functionality of the *mtTRES* system we generated *TLI-MRP:: ATP6* and *TLI-RNP::ATP6* that express chimeric RNAs targeting *ATP6* mRNAs for TLI using *mtTRES*^{MRP} and *mtTRES*^{RNP}, respectively. ATP6 protein levels were examined in *TLI-MRP::ATP6* and *TLI-RNP::ATP6* animal extracts and were shown to be significantly reduced similar to *TLI-5S*^{mt}*::ATP6* (Figure 2C, D). Together these data demonstrate the ability to reduce steady state ATP6 protein levels using several independent constructs *in vivo*. Importantly these experiments utilize three distinct NCL sequences, including two discovered in mammals (Chang and Clayton, 1987, 1989, Puranam and Attardi, 2001).

mtTRES TLIs modulate expression independent of RNA stability

mtTRES TLIs are designed to function by antagonizing translation and reducing steady state protein levels by an RNA-stability independent mechanism. To test whether these chimeric mitochondrial targeted RNAs are modulating protein levels by regulating RNA stability, we performed qRT-PCR analyses on total RNA from *TLI-5S^{mt}::ATP6(a)*, *TLI-5S^{mt}::ATP6(b)*, and wild type control animals to determine whether RNA levels of the targeted gene were altered. No changes in *ATP6* RNA levels were observed (Figure 3A, B). We also examined whether *TLI-5S^{mt}::ATP6(a)* or *TLI-5S^{mt}::ATP6(b)* altered the RNA levels of another mitochondrial expressed gene, *COXII*, and found no significant changes in *COXII* transcript levels with either of the TLIs (Figure 3C, D). Together these data are consistent with a translational inhibition/repression mechanism of action that is independent of alterations in RNA stability.

Mitochondrial TLIs specifically knockdown target proteins

The chimeric RNAs in the present study are predicted to knockdown mitochondrial protein expression levels by specifically inhibiting the docking of the small subunit of mitoribosomes on target mRNA akin to the cytosolic mechanism of action (Doyle et al., 2001). To test the specificity of *TLI-NCL::ATP6* chimeric RNAs, we examined COXII protein levels by western blot (Figure 4). *TLI-NCL::ATP6* chimeric RNAs were able to modulate ATP6 protein levels, however, COXII protein was not altered (Figure 4A, B). These data suggest that *TLINCL:: ATP6* chimeric RNAs do not globally alter translation and modulate target mitochondrial gene expression specifically.

Mitochondrial TLIs *in vitro*, import assay and scrambled control

We developed a mammalian version of the *mtTRES* vector to examine efficacy of import and *mtTRES-TLI* constructs in human cells. We created a series of *TLI-NCL::COXII* constructs designed to target human *COXII* mRNAs. For chimeric TLI RNAs to regulate expression of endogenous mitochondrial proteins via translational repression they must be efficiently imported into mitochondria. We directly examined mitochondrial import of *5S^{mt} rRNA* and chimeric *TLI-5S^{mt}::COXII* RNAs using an established import assay (Figure 5). RNA import was dependent on mitochondrial membrane potential, as evidenced by a lack of import when mitochondria were treated with FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) prior to adding the RNA probes. These data demonstrate robust import of the *5S^{mt} rRNA* used as an NCL and the chimeric *TLI-5S^{mt}::COXII* RNAs and suggest our constructs might function well as TLIs in mammalian systems.

We functionally tested our mammalian *TLI-NCL::COXII* constructs using each of the three NCLs previously tested *in vivo*. All three *TLI-NCL::COXII* constructs significantly decreased COXII protein levels (Figure 6). These data demonstrate the ability of *mtTRES* TLI constructs to modulate protein levels in human cells using multiple NCL targeting signals, although the *TLI-5S^{mt}::COXII* reliably gave the most significant knockdown.

To further test the specificity of the mitochondrial TLI we generated a vector with a scrambled *COXII* complementary sequence (*TLI-5S^{mt}::scCOXII*) and repeated our analysis

with this additional control. Western blot data shows that *TLI-5S^{mt}::COXII* reduced COXII protein, as it had previously, however, *TLI-5S^{mt}::sc-COXII* (scrambled) does not alter the target protein (Figure 7). Together these data demonstrate the utility of *mtTRES-TLI* constructs in human cells.

Conclusions/discussion

Mitochondrial dysfunction has been associated with the pathogenesis of numerous significant disease conditions (Wallace et al., 1988, Wallace, 2005, DiMauro and Schon, 2008, Wallace, 2010, 2012). The study of ME has been severely hampered by a limited number of animal models, especially those affecting endogenous mitochondrial genes (Palladino, 2010). Researchers have developed innovative methods to alter heteroplasmy using mitochondrial-targeted restriction enzymes (Xu et al., 2008) and reported manipulating mitochondrial DNA or general effects on mitochondrial translation via RNA import strategies (Kolesnikova et al., 2004, Mukherjee et al., 2008, Karicheva et al., 2011, Wang et al., 2012a, Bacman et al., 2013, Comte et al., 2013), however, the ability to directly manipulate the expression of mitochondrial-encoded proteins has remained elusive and has obvious basic and clinical applications. The identification of NCL sequences that direct RNAs to mitochondria enables a TLI approach using chimeric RNAs with a complementary element. The development of the *mtTRES* expression system enables expression of these chimeric RNAs *in vivo*. *mtTRES* utilizes RNAPIII promoter and termination elements such that NCLs and chimeric RNAs containing NCLs resemble natural substrates for RNA import. The demonstration that the *mtTRES* system is functional *in vivo* now enables the manipulation of mitochondrial genome expression, which opens up numerous avenues of investigation and is of immense value to the mitochondrial research and clinical communities.

These data demonstrate the general applicability of the approach by targeting two loci encoding proteins for which antibodies were available to verify functionality. *TLI-NCL::ATP6* RNAs achieved ~ 40–50% reduction in steady state protein levels *in vivo*. In human cells *TLI-NCL:: COXII* achieved 20–50 % reduction in protein levels, dependent upon the NCL employed. Differences in reduction between the various NCLs and TLIs could be due to sequence selective effects or differences in the stability of chimeric RNA secondary structures affecting import or availability of the complementary RNA sequence for targeting.

Although these data demonstrate general applicability, there is notable constraint in the design of TLIs that has the potential to restrict its application. There are data suggesting antisense targeting for translational repression, at least in the cytosol, must be directed to the start codon to be effective, restricting construct design options. RNAP III is known to terminate within stretches of poly T, potentially restricting the use for some genes in some organisms where a poly A exists proximal to the start codon. Lastly, our data demonstrate differing levels of functionality within the NCL. Although we cannot fully explain these differences at this time, such sequence selectivity could reflect the fact that within the chimeric RNA the NCL must be recognized and the complementary sequence must still be accessible in its native structure. Certain NCL- complementary sequence combinations will

form stable secondary structures that abrogate one of these functions in a manner that may not be fully predictable. Since the *5S*^{mt} NCL is larger and more highly structured with a lower delta G, it is predicted that this NCL will be more reliable but additional studies will be needed to fully test this prediction.

Mitochondrial-targeted TLIs were designed to function by antagonizing ribosome docking and lowering translational efficiency. Antisense RNAs are commonly employed to reduce expression of nuclear genes through a well-understood RNA interference mechanism that leads to target RNA degradation. We investigated this possibility by examining RNA levels of *ATP6* and *COXII* and the data demonstrate normal RNA levels *in vivo*, arguing against an RNA-destabilizing mechanism of action. To demonstrate that the 5S^{mt} RNA and *TLI-5S*^{mt}*::COXII* chimeric RNA are being imported into the mitochondria with similar efficiency we performed a direct *in vitro* RNA import assay. Earlier studies have suggested that RNA import is dependent on mitochondrial membrane potential. We observed that the RNA import into the mitochondria was almost negligible in presence of FCCP, which uncouples and depolarizes mitochondria.

The data presented here demonstrate the utility and general applicability of the *mtTRES* system as well as the ability to engineer TLI chimeric RNAs to modulate endogenous mitochondrial gene expression. The capability to modulate mitochondrial gene expression will enable detailed studies of mitochondrial function and organelle dysfunction *in vivo*. Efforts to develop a mitochondrial gene therapy face a formidable challenge of competing with endogenous mutant protein expression. The *mtTRES* TLI system described here has the potential to accelerate the realization of an effective gene therapy for mitochondrial diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We developed a novel series of *mtTRES* vectors that express small RNAs *in vivo*.
- Chimeric mitochondrial-targeted RNAs function as translational inhibitors (TLIs).
- We developed *mtTRES* TLI constructs that modulate expression of ATP6 *in vivo*.
- Expression of human COXII is modulated by chimeric TLI RNAs in HELA cells.

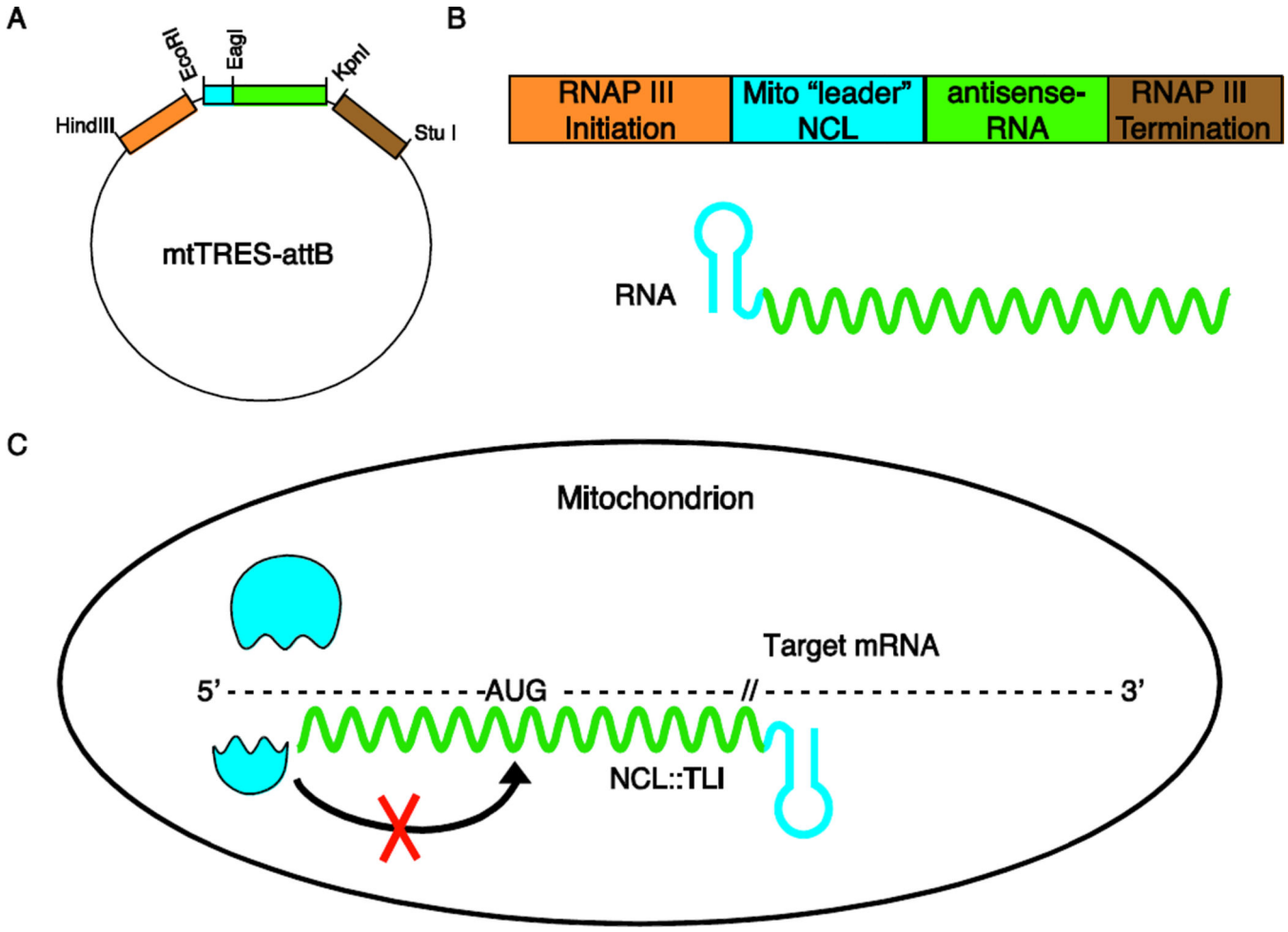

Figure 1. Design of the *mtTRES attB* transgenesis vector

(**A**) Restriction enzyme map of the *mtTRES-attB* vector. (**B**) Cartoon describing the linear 5' to 3' order of required components for allotopic RNA expression: RNAP III specific initiation and termination (**orange** and **brown**, respectively), non-coding leader sequence (NCL) RNA (**blue**) and the antisense RNA (TLI) (**green**). The subsequent RNA transcribed will be a chimeric NCL-TLI RNA. (**C**) Cartoon demonstrating the proposed mechanism of translational inhibition. The complementary sequence competes with the small subunit of the ribosome for binding thus inhibiting docking to the target RNA at the start codon (*AUG*).

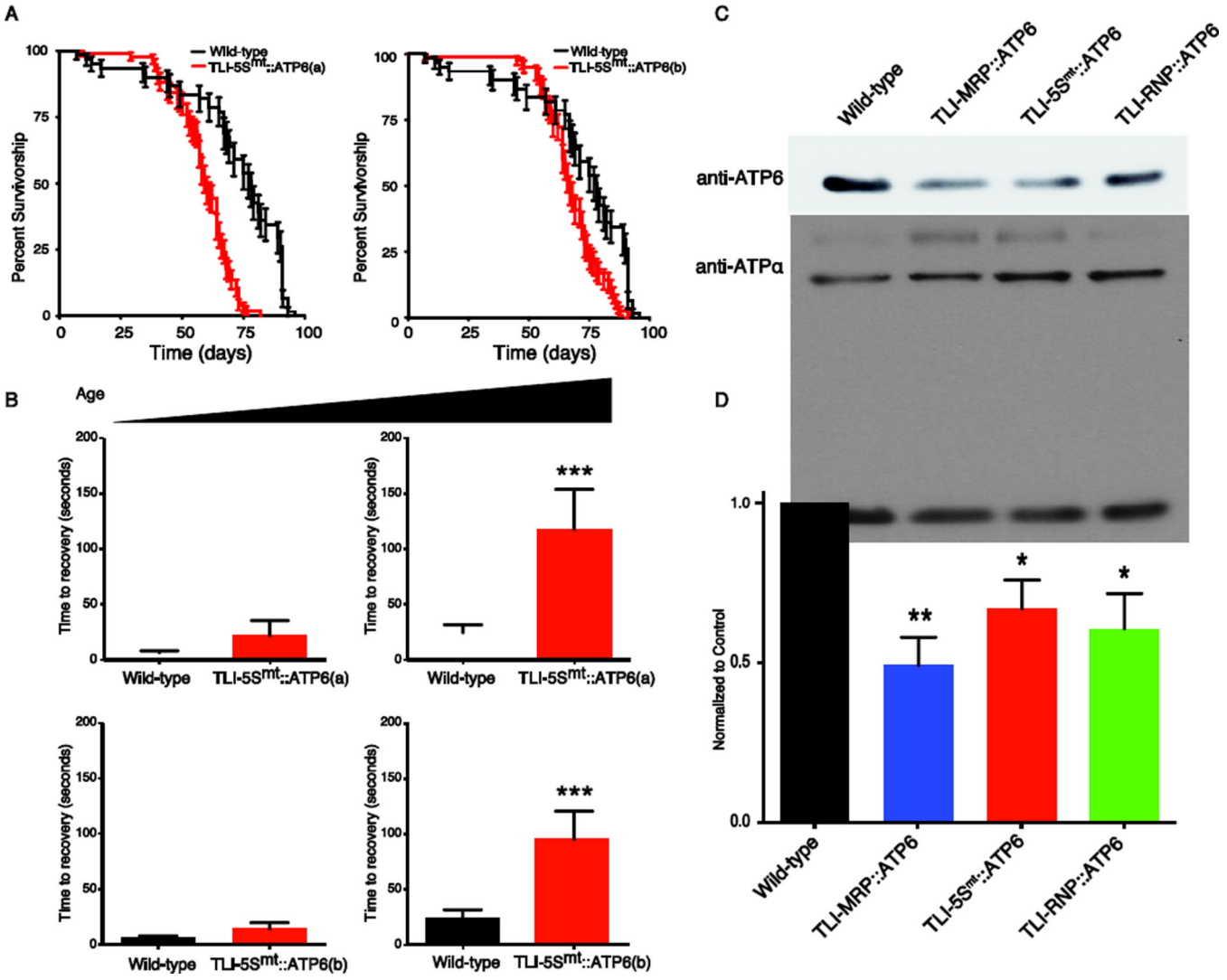

Figure 2. Translational inhibitors exhibit reduced longevity, mechanical stress sensitivity and lower steady state protein expression *in vivo*

(**A**) *TLI-5S^{mt}::ATP6(a)* (red) has a 24% reduction in survival as compared to the wild-type animals (**black**) (n=95, p<0.0001). *TLI- 5S^{mt}::ATP6(b)* (**red**) displays a 15% reduction in survival as compared to wild-type animals (in black) (n=83, p<0.0001). (**B**) *TLI-5S^{mt}::ATP6(a)* animals exhibit a progressive increase in mechanical stress sensitivity (day 5 and day 50 shown). (**C**) *TLI-NCL::ATP6(a)* fly extracts were probed with anti-ATP6 antibody to examine steady state protein levels. The expression levels were normalized to ATPalpha, the plasma membrane Na/K ATPase catalytic subunit (upper panel). (**D**) Quantitation of western blots show 50% decrease in *TLI-MRP::ATP6(a)*, 34% decrease in *TLI-5S^{mt}::ATP6(a)* and 40% decrease in *TLI-RNP::ATP6(a)*. Unpaired t-test was used as statistical test; *p<0.01, **p<0.001, ***=p< 0.0001; mean ± SEM, n=3–4.

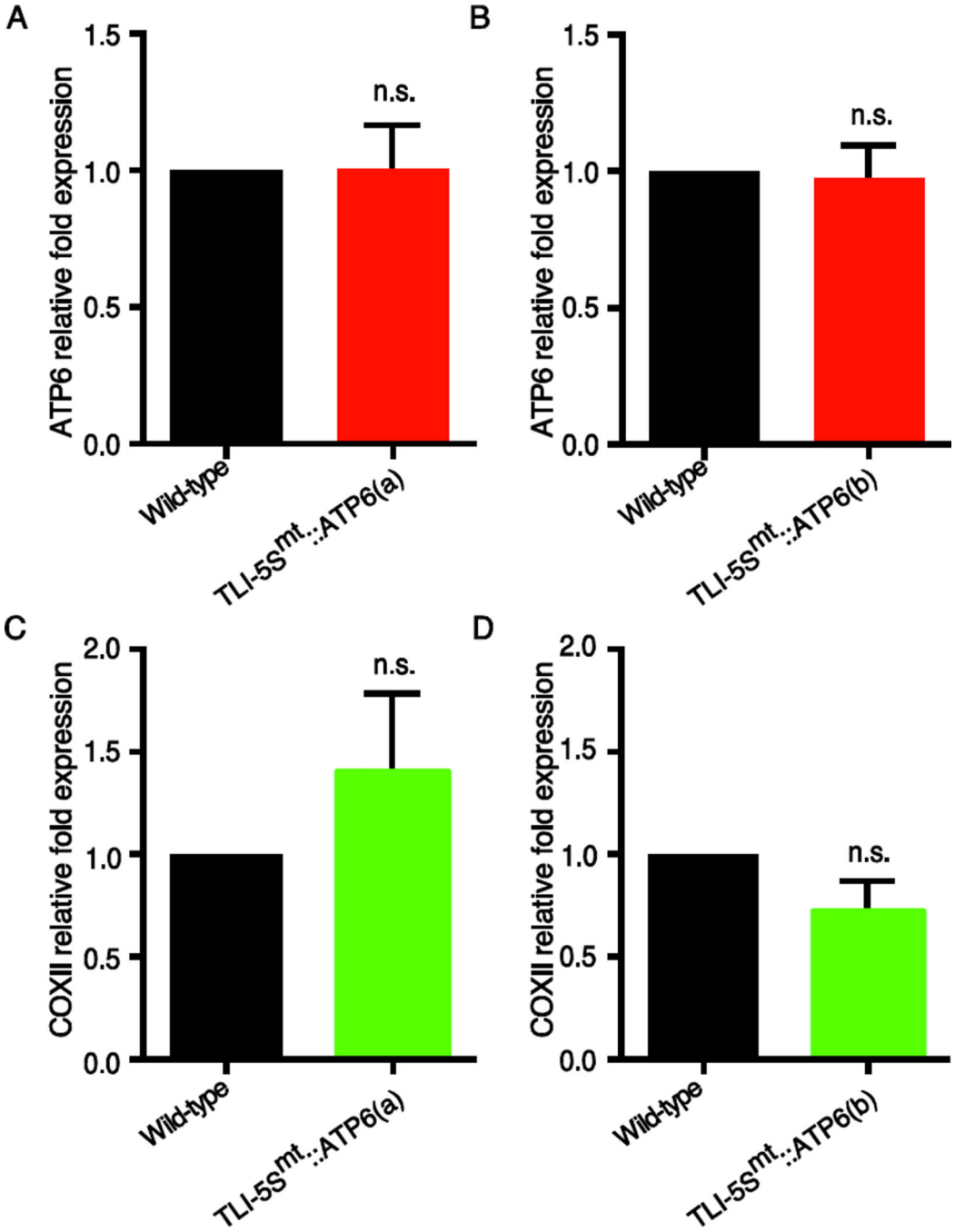

Figure 3. TLIs function by an RNA stability independent mechanism

Fold change in transcript levels determined by qRT-PCR. Fold change mRNA expression of *ATP6* (red) and *COXII* (green) is shown relative to wild type controls (black). (**A and C**) *TLI-5S^{mt}::ATP6(a)*. (**B and D**) *TLI-5S^{mt}::ATP6(b)*. All transcript levels were normalized to *RP49* expression. One-way ANOVA was performed to test significance; n.s. is $p > 0.05$; mean ± SEM, n=9 (3 biological and 3 technical repeats of each sample).

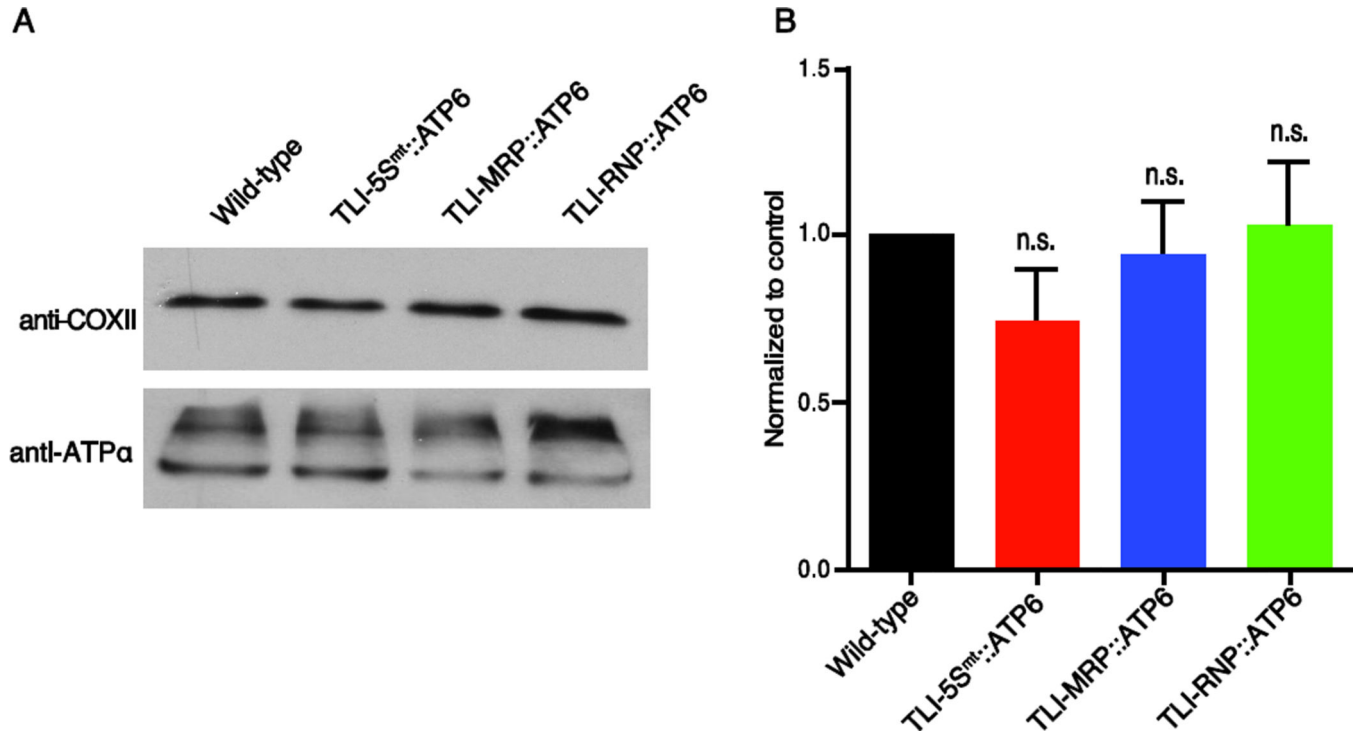

Figure 4. Translational inhibitors knockdown target protein specifically

(A) *TLI-NCL:: ATP6(a)* fly extracts were probed with anti-COXII antibody to examine steady state protein levels. The expression levels were normalized to ATPα (upper panel). (**B**) Quantitation of western blots showing no significant change in the COXII expression levels in *NCL::ATP6(a)*. One-way ANOVA with multiple comparisons was used to test significance; n.s. is $p > 0.05$; mean ± SEM, n=3.

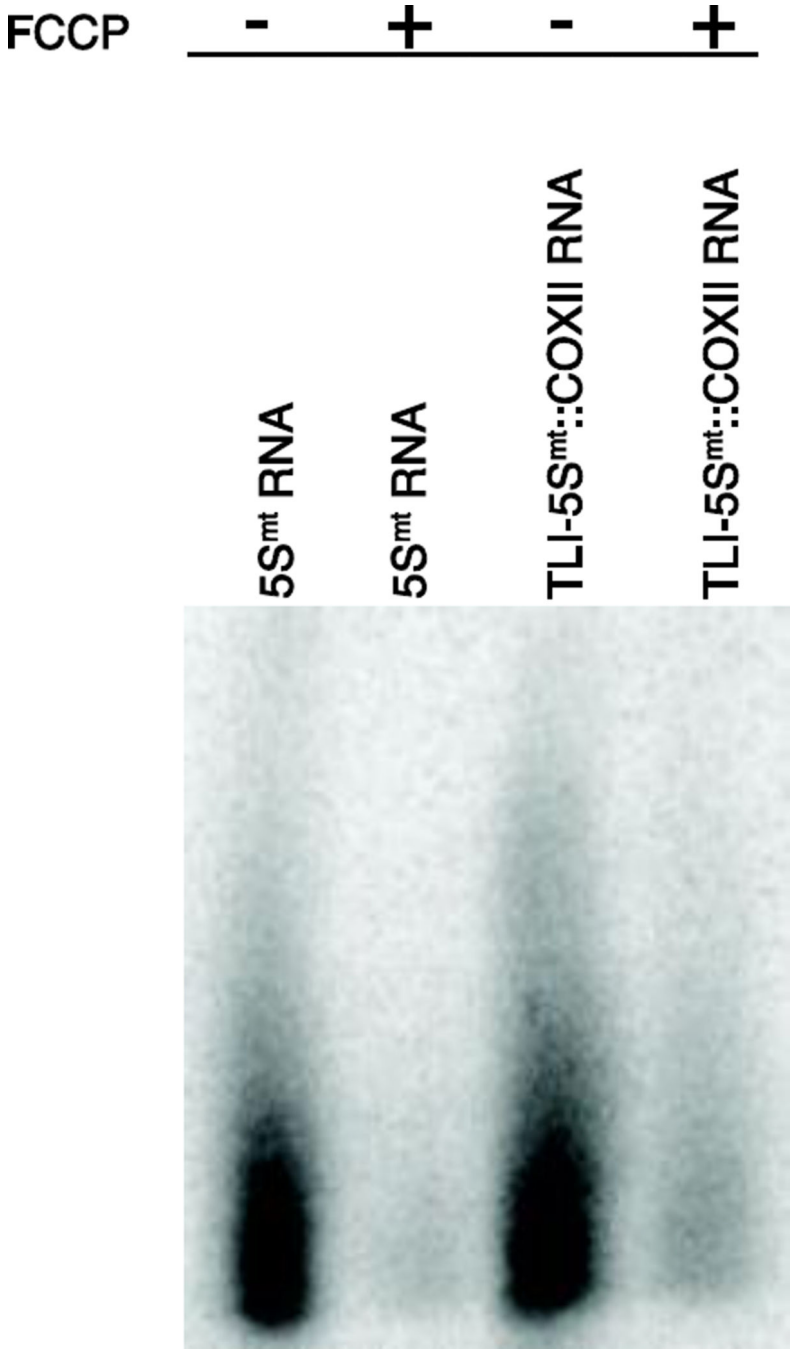

Figure 5. *In vitro* import of radiolabeled RNA into mitochondria

[α-32P] labeled *5S*^{mt} RNA and *TLI-5S*^{mt}*::COXII* RNA were transcribed *in vitro* and incubated with equal amounts of mitochondria isolated from HeLa cells in presence or absence of FCCP (+ or −). The pellet was treated with nuclease to digest non-imported RNAs and SDS treatment was performed to produce mitoplasts. Extracted RNAs were resolved using urea polyacrylamide gels and analyzed using Image quant (Storm 860 Molecular Imager). The experiment was repeated 3 times with a representative image shown.

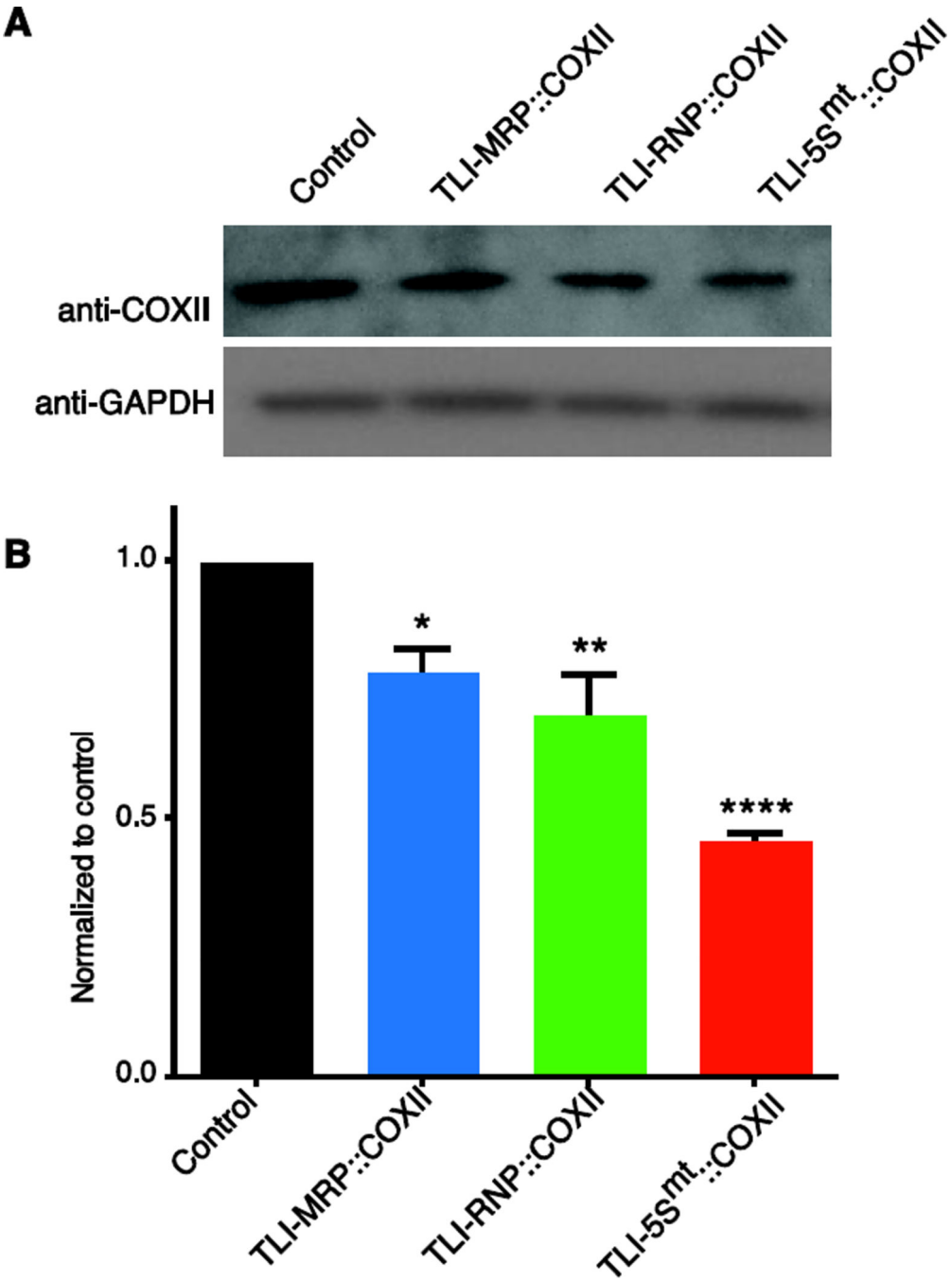

Figure 6. Translational inhibitors decrease steady state protein levels in HeLa cells

(**A**) HeLa cells were transfected with *mtTRES* plasmids expressing TLIs directed to human mitochondrial *COXII* RNAs. The cells were harvested at ~48–72 hrs and analyzed by western blot. (**B**) Quantification of steady-state COXII shown relative to the control plasmid (black) in cells transfected with mammalian *mtTRES* plasmids *TLI-MRP::COXII, TLI-RNP::COXII* and *TLI-5S^{mt}::COXII* revealed reduced expression of 22%, 30% and 55%, respectively. GAPDH was the loading control. One-way ANOVA with multiple

comparisons was performed to test significance; * is $p < 0.03$, ** is $p < 0.0005$; **** is $p < 0.0001$; mean ± SEM, n=3.

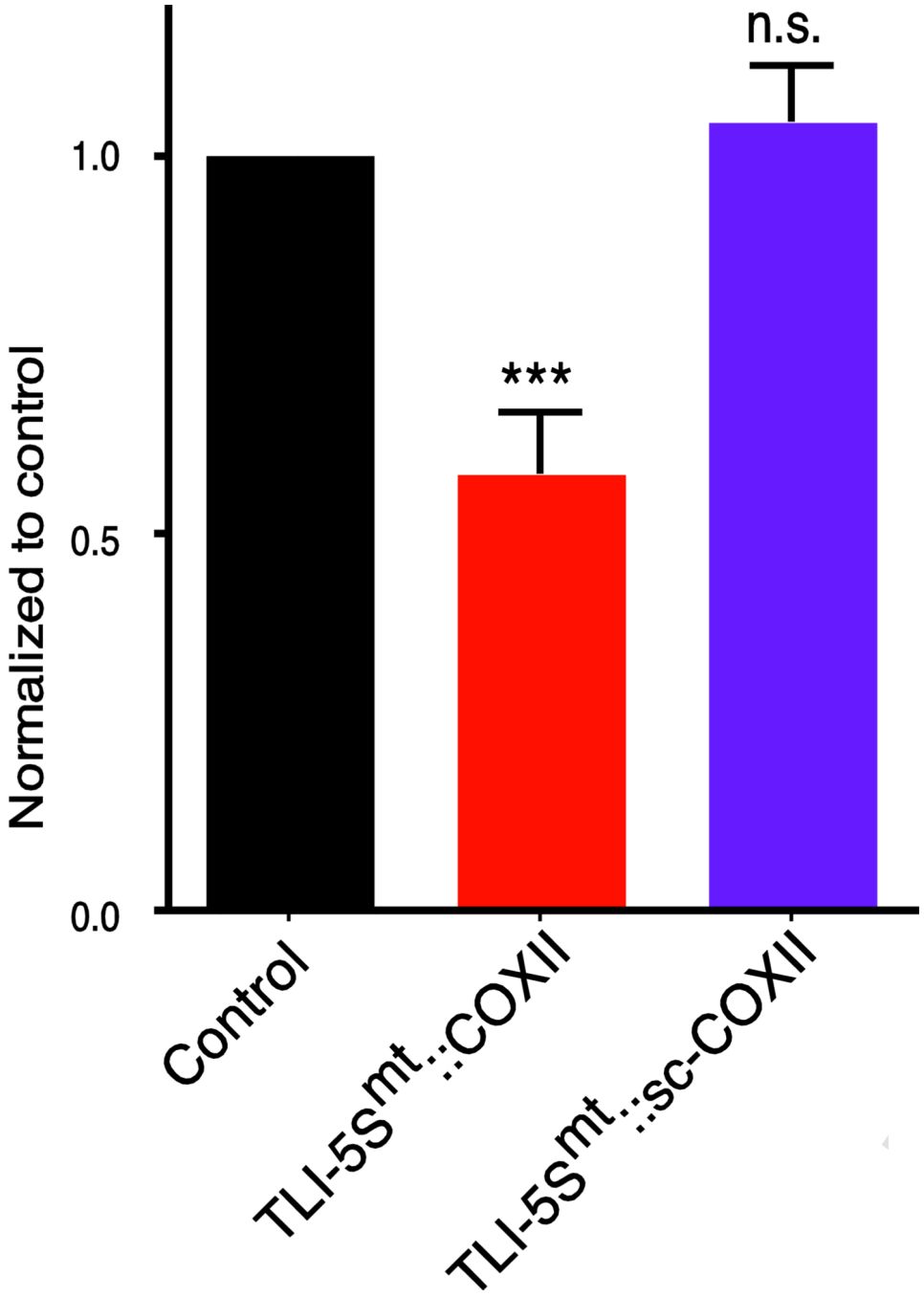

Figure 7. TLI with scrambled complementary sequence do not alter COXII protein level

TLI-5S^{mt}::COXII reduced COXII protein expression in transfected HeLa cells, as previously. *TLI-5S^{mt}::scCOXII* with an intact 5S NCL but a scrambled complementary sequence did not alter COXII protein levels. Quantitation of western blots showing no significant change in the expression levels of COXII in HeLa cells. One-way ANOVA with multiple comparisons was used to test significance; n.s. is $p > 0.05$, *** is $p < 0.0004$; mean ± SEM, n=3–6.