561

ARCUS Gene Editing to Eliminate MELAS-associated m.3243A>G Mutant Mitochondrial DNA

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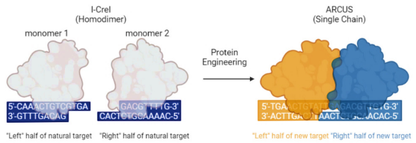
INTRODUCTION

- Mitochondria contain their own multi-copy, double-stranded circular genome that encodes 37 genes critical for oxidative phosphorylation.
- Pathogenic mitochondrial DNA (mtDNA) mutations are commonly heteroplasmic, where both wild-type (WT) and mutant genomes co-exist in the same cell.
- Clinical symptoms manifest once the percentage of mutant mtDNA exceeds a particular threshold.
- Diseases resulting from mtDNA mutations are multi-system, complex disorders with no available cures.
- Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like epidodes (MELAS) is a mitochondrial disease primarily caused by a point mutation in mt-tRNA$^{Leu(UUR)}$. This mutation (m.3243A>G) is the most common pathogenic mtDNA mutation and impairs the enzymatic activity of the mitochondrial respiratory chain.
- ARCUS is derived from I-CreI, a homodimeric protein that recognizes and cleaves a semi-palindromic 22 base pair target sequence in the chloroplast genome of *Chlamydomonas reinhardtii*.
- ARCUS is a small (~350 amino acids), monomeric protein that does not require a guide RNA to edit DNA and can be optimized to improve specificity.



OBJECTIVE

- Mitochondria lack an efficient double-strand break repair mechanism and instead rapidly degrade any linearized mtDNA molecules.
- There is a tightly controlled mechanism for maintaining mtDNA copy number that results in the replication of any genomes remaining following mtDNA degradation.
- **Therapeutic approach:** deliver mitochondrial-targeted ARCUS (mitoARCUS) to affected cells to selectively cleave and eliminate mutant mtDNA while leaving wild-type mtDNA to repopulate the cell, resulting in a shift in heteroplasmy and improvement in mitochondrial function.
- Nuclease specificity is paramount, as the WT and mutant mtDNA sequences only differ by a single nucleotide.

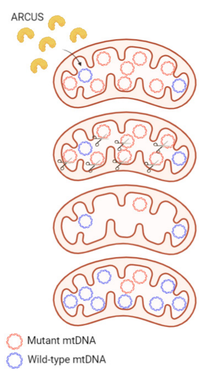
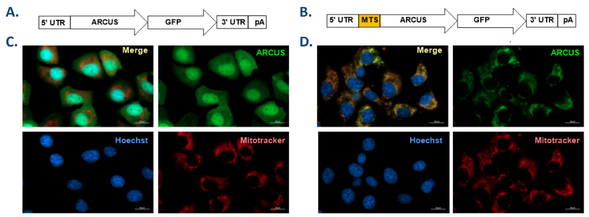


FIGURE 1. mitoARCUS 22 base pair binding site and corresponding WT sequence

Mutant mtDNA: C A G **G** C C C G G T A A T C G C A T A A A
Wild-type mtDNA: C A G **A** G C C C G G T A A T C G C A T A A A

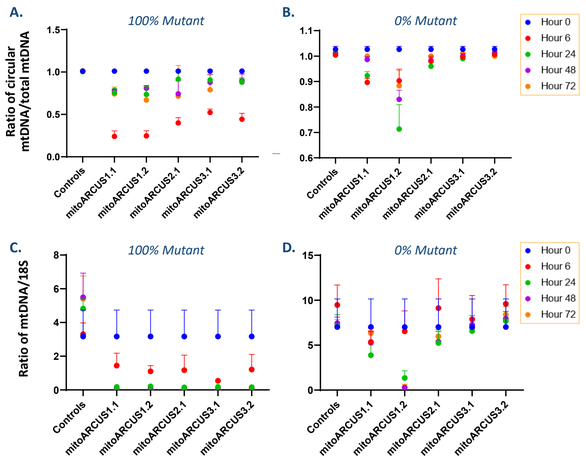
RESULTS

FIGURE 2. mitoARCUS is efficiently trafficked to the mitochondria using an N-terminal MTS



- Two ARCUS constructs were transfected into cells: ARCUS lacking a subcellular targeting sequence (**2A**) or ARCUS with a mitochondrial targeting sequence (MTS) fused at the N-terminus (**2B**). The cells were stained at 24 hours for Hoechst 33342 and Mitotracker.
- Without a targeting sequence, ARCUS shows diffuse, cytoplasmic/nuclear localization (**2C**). With an MTS, ARCUS co-localizes with MitoTracker showing efficient mitochondrial delivery (**2D**) without evidence of nuclear off-target editing (data not shown).

FIGURE 3. Protein engineering and optimization results in two distinct nucleases that efficiently eliminate mutant mtDNA without significant cleavage of WT mtDNA



- Two homoplasmic cell lines (100% or 0% mutant) were used to evaluate various mitoARCUS nucleases for on-target (mutant mtDNA) and off-target (WT mtDNA) activity, using a high dose of 1.5x10^5 mRNA copies/cell. Activity at each site was measured in terms of mtDNA cleavage (**3A**, **3B**) and mtDNA depletion (**3C**, **3D**).
- All nucleases exhibited high on-target activity, as evidenced by the robust cleavage (**3A**) and elimination (**3C**) of mutant m.3243G mtDNA. However, the nucleases varied in their ability to discriminate against WT m.3243A mtDNA (**3B**, **3D**). mitoARCUS3.1 and mitoARCUS3.2 were statistically insignificant from the controls in both linearization and depletion of WT mtDNA, indicating a high degree of specificity.

FIGURE 4. mitoARCUS shifts heteroplasmy and improves mitochondrial function in 96% mutant m.3243G cybrid cells without deleterious impacts to respiration during transient mtDNA depletion

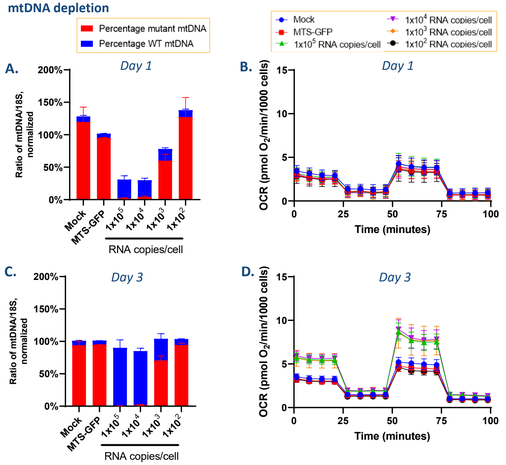


Table 1. Statistical significance relative to MTS-GFP control, day 1[a]

Condition	mtDNA copy number	% mutant mtDNA	% WT mtDNA	Basal respiration	Maximal respiration
Mock	ns	ns	ns	ns	ns
1x10^5 RNA copies/cell	**	****	****	ns	ns
1x10^4 RNA copies/cell	**	****	****	ns	ns
1x10^3 RNA copies/cell	ns	****	****	ns	ns
1x10^2 RNA copies/cell	ns	ns	ns	ns	ns

Table 2. Statistical significance relative to MTS-GFP control, day 3[a]

Condition	mtDNA copy number	% mutant mtDNA	% WT mtDNA	Basal respiration	Maximal respiration
Mock	ns	ns	ns	ns	ns
1x10^5 RNA copies/cell	ns	****	****	****	****
1x10^4 RNA copies/cell	ns	****	****	****	****
1x10^3 RNA copies/cell	ns	****	****	****	****
1x10^2 RNA copies/cell	ns	ns	ns	ns	ns

- 96% mutant m.3243G cells were transfected with mitoARCUS mRNA at 10-fold dilutions. At days 1 and 3 post-transfection gDNA was isolated from the cells and analyzed for heteroplasmy and mtDNA copy number (**4A, 4C**) and the live cells were evaluated for respiratory changes using the Seahorse Cell Mito Stress Test (**4B**, **4D**).
- At day 1, a dose-dependent mtDNA depletion was observed that was significant for the two highest mRNA doses. This depletion corresponded to the selective cleavage of mutant mtDNA. Despite the mtDNA depletion, respiration was unaffected (**Table 1**).
- At day 3, mtDNA copy number was restored to statistically insignificant levels. The cells treated with the three highest mRNA doses exhibited a significant decrease in percentage of mutant mtDNA and a significant increase in percentage of WT mtDNA. The dose-dependent shifts in heteroplasmy resulted in concomitant improvements in basal and maximal respiration (**Table 2**). Notably, small shifts in heteroplasmy (66.5% mutant ± 3.4%) confer a similar functional benefit to a complete shift.

FIGURE 5. mitoARCUS-induced mtDNA depletion does not impact respiration, even when cells are grown in galactose

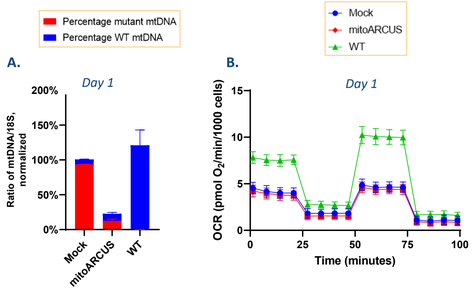
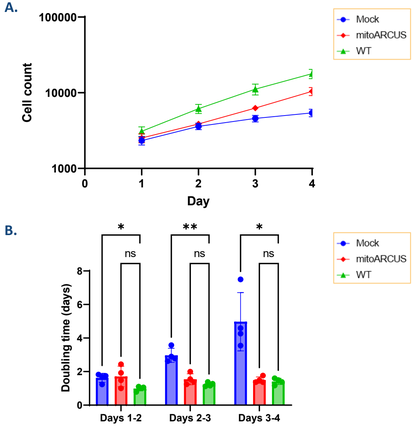


Table 3. Statistical significance relative to mock 96% m.3243G control, day 1[a]

Condition	mtDNA copy number	% mutant mtDNA	% WT mtDNA	Basal respiration	Maximal respiration
mitoARCUS	**	****	****	ns	ns
WT	ns	****	****	***	****

- 96% mutant m.3243G cells were transfected with 1x10^5 mitoARCUS mRNA copies/cell. A 0% mutant (WT) cell line was included as a control. All conditions were plated into media containing galactose, rather than glucose, immediately following the transfection. At day 1 post-transfection, gDNA was isolated from the cells and analyzed for heteroplasmy and mtDNA copy number (**5A**) and the live cells were evaluated for respiratory changes using the Seahorse Cell Mito Stress Test (**5B**).
- As expected, a significant mtDNA depletion was observed in the cells treated with mitoARCUS, corresponding to the selective cleavage of mutant mtDNA. However, respiration was not impacted, despite the galactose-induced reliance of these cells on oxidative phosphorylation to generate ATP (**Table 3**).

FIGURE 6. mitoARCUS-induced heteroplasmy shift rescues impaired proliferation of 96% mutant m.3243G cybrid cells grown in galactose



- Using the cells described in Figure 5, 2x10^3 cells/well were plated into replicate 96 well plates at day 0. On days 1-4, one plate was stained with Hoechst 33342 and counted using ImageXpress Pico (**6A**). The resulting cell count was used to calculate the doubling time of each condition (**6B**).[a]
- Untreated 96% mutant cells grown in galactose exhibit a growth impairment and significantly greater doubling time compared to WT cells, which is compounded over time.
- mitoARCUS-treatment rescues the growth impairment, resulting in a doubling time that is statistically insignificant from WT cells.

CONCLUSIONS

1. mitoARCUS can successfully be trafficked to the mitochondria and, once there, specifically cleave and eliminate m.3243G mutant mtDNA resulting in a shift in heteroplasmy and improvement in mitochondrial function.
2. Transient mtDNA depletion did not negatively impact oxygen consumption, even when cellular reliance for ATP production was shifted to oxidative phosphorylation.
3. Precision BioSciences' protein engineering and optimization platform allows for the generation of highly specific nucleases that can accurately discriminate a single nucleotide difference.
4. Together, these data support the development of mitoARCUS as an *in vivo* gene editing therapeutic for the treatment of disease-causing heteroplasmic mtDNA mutations.

*[a]Statistics were calculated using an ordinary one-way ANOVA, Dunnett's multiple comparisons test (ns: P>0.05, *: P≤0.05, **: P≤0.01, ***: P≤0.001, ****: P≤0.0001).*