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Specific elimination of m.3243A>G mutant mitochondrial DNA using mitoARCUS in cultured cells and a novel xenograft mouse model

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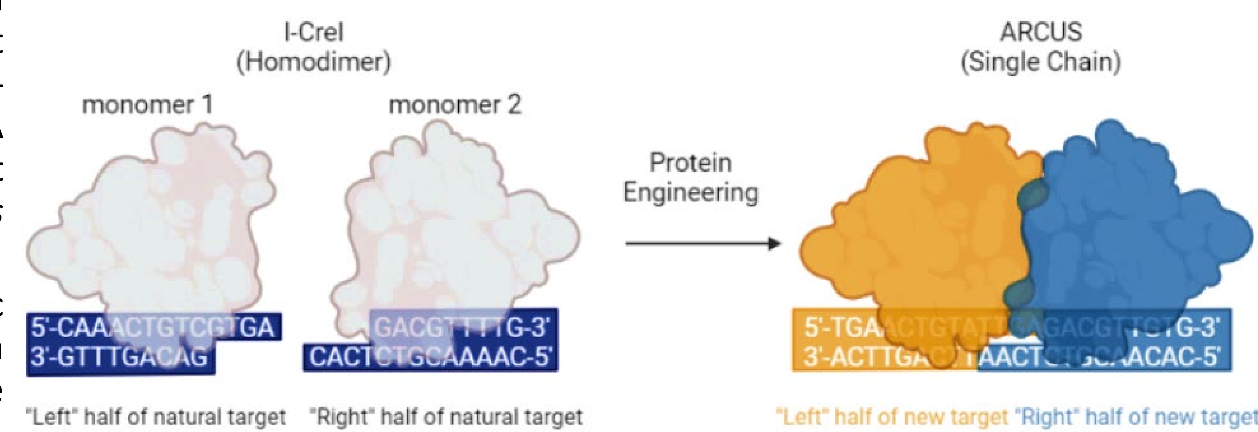
INTRODUCTION

- 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 episodes (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-*Cre*I, a homing endonuclease that recognizes and cleaves a semi-palindromic 22 base pair DNA target sequence in the chloroplast genome of *Chlamydomonas reinhardtii*.
- ARCUS is a small, monomeric protein that does not require a guide RNA to edit DNA and can be optimized to improve specificity.

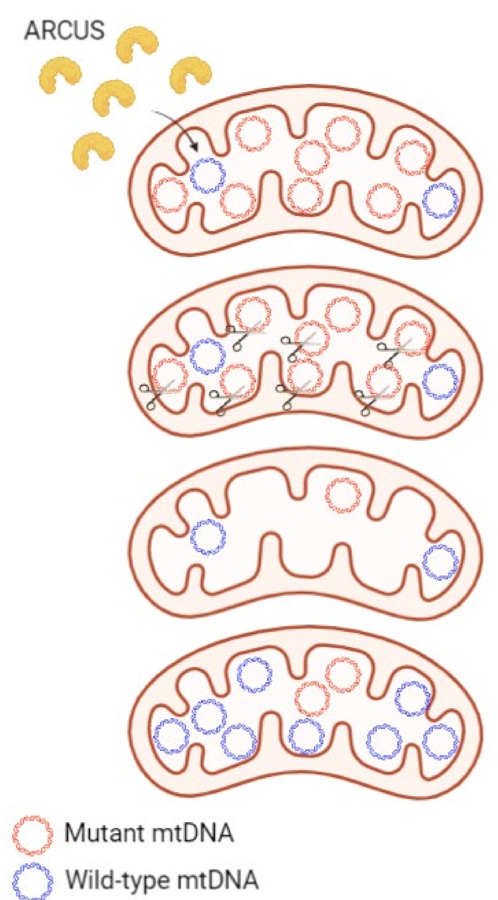


OBJECTIVE

- 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 WT 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.

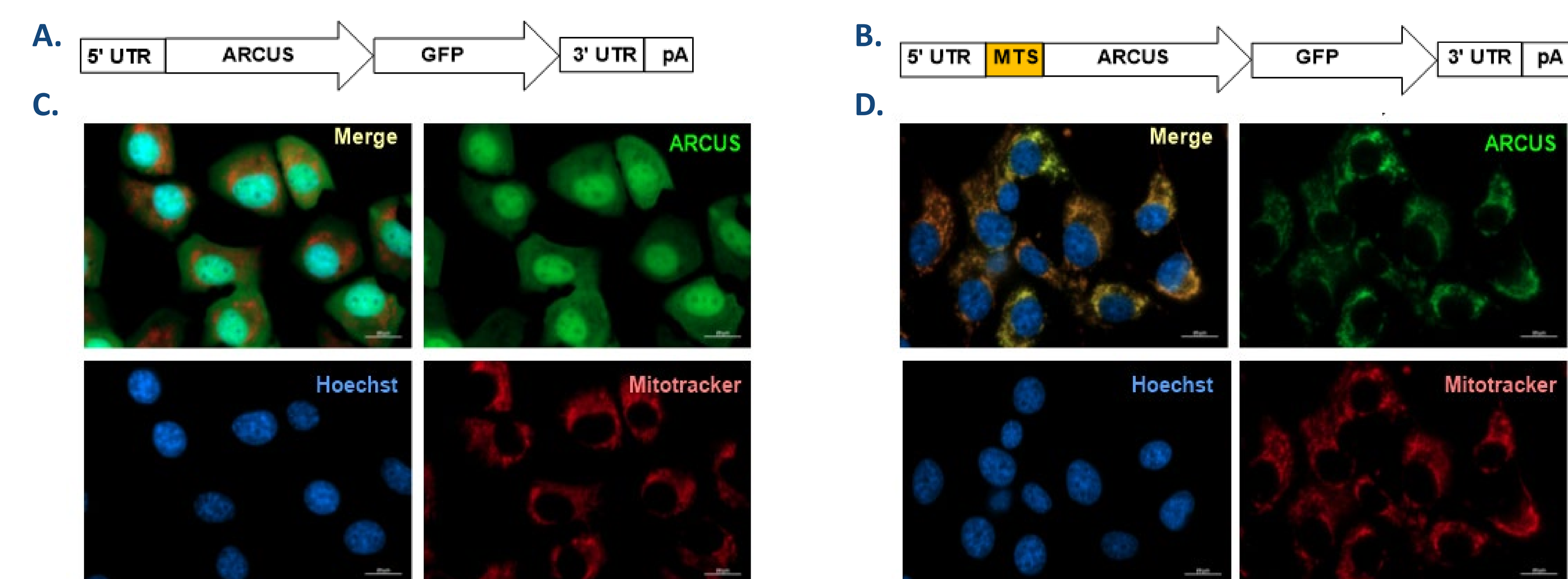
FIGURE 1. mitoARCUS 22 base pair binding site and corresponding WT sequence

Mutant mtDNA: C A G **G** 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



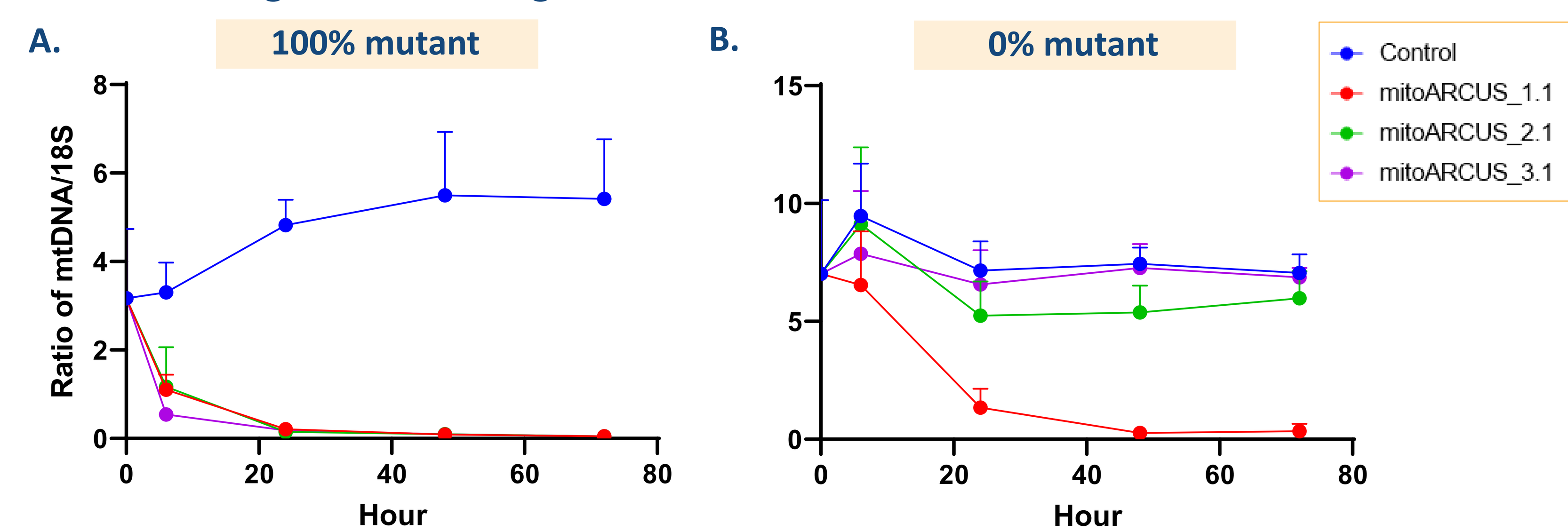
RESULTS

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 exhibits 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 optimization generates a nuclease that efficiently eliminates mutant mtDNA without significant cleavage of WT mtDNA



- Two homoplasmic cell lines (100% or 0% mutant) were used to evaluate three generations of mitoARCUS nucleases for on-target (mutant mtDNA) and off-target (WT mtDNA) activity. Activity at each site was measured in terms of mtDNA depletion.
- All three generations of nucleases exhibited high on-target activity, as evidenced by the robust elimination of mutant m.3243G mtDNA (**3A**). However, the nucleases varied in their ability to discriminate against WT m.3243A mtDNA and exhibited improved specificity with each subsequent generation (**3B**). mitoARCUS3.1 was statistically insignificant from the control at all measured timepoints, 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

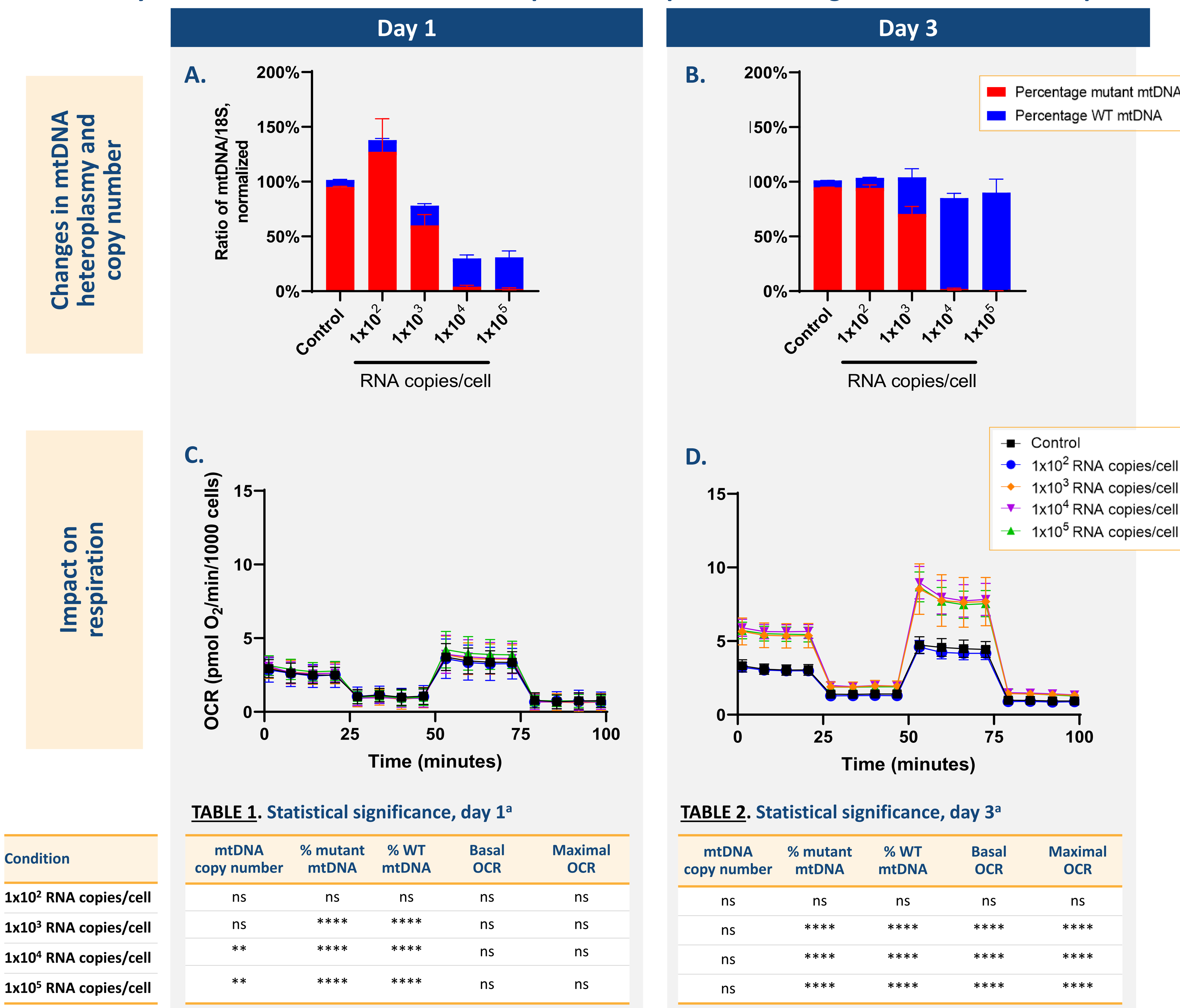


TABLE 1. Statistical significance, day 1[a]

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

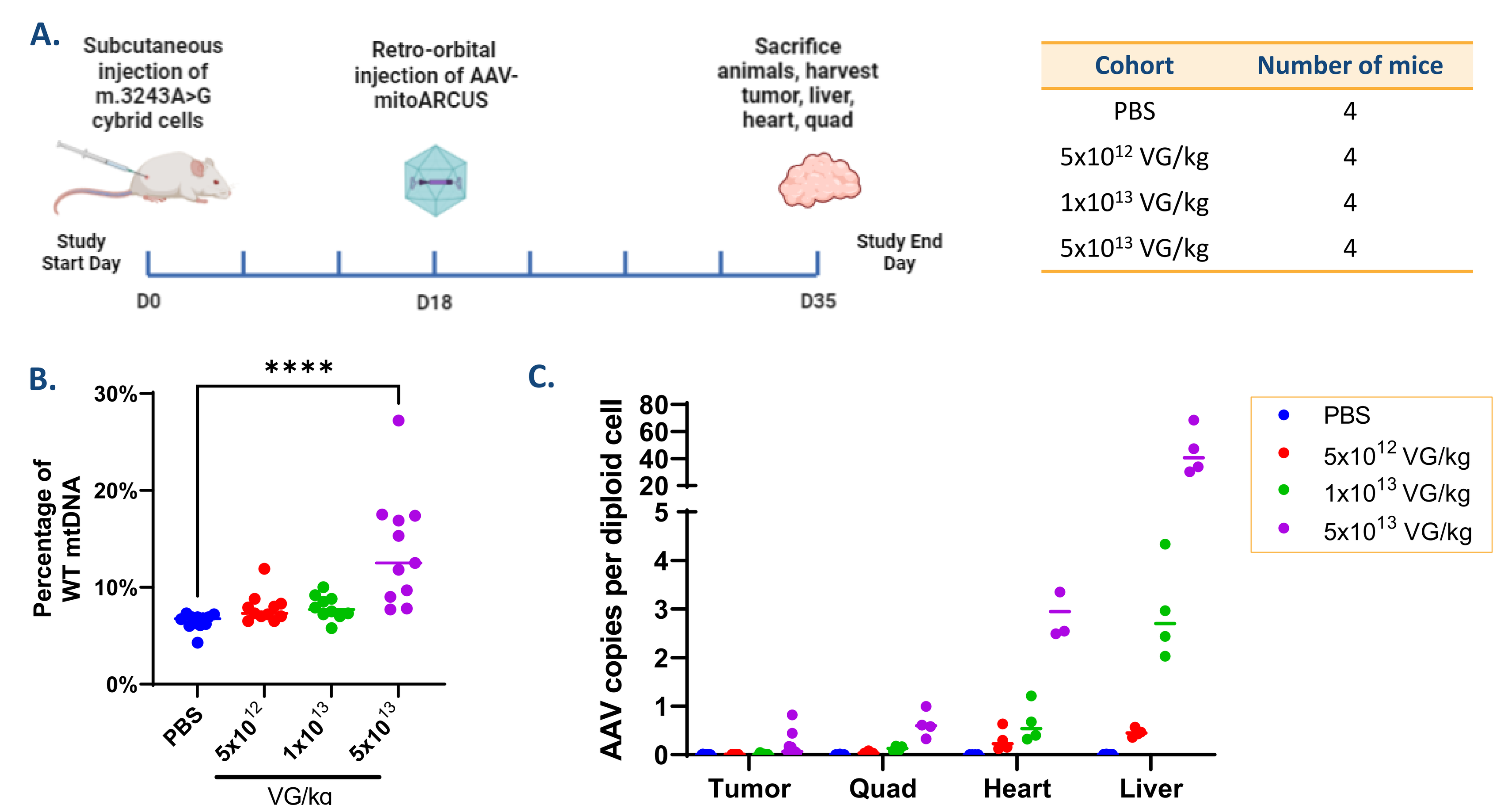
TABLE 2. Statistical significance, day 3[a]

Condition	mtDNA copy number	% mutant mtDNA	% WT mtDNA	Basal OCR	Maximal OCR
$1x10^2$ RNA copies/cell	ns	ns	ns	ns	ns
$1x10^3$ RNA copies/cell	ns	****	****	****	****
$1x10^4$ RNA copies/cell	ns	****	****	****	****
$1x10^5$ RNA copies/cell	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 and analyzed for heteroplasmy and mtDNA copy number (**4A**, **4B**) and the live cells were evaluated for respiratory changes using the Seahorse Cell Mito Stress Test (**4C**, **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 mutant mtDNA and a significant increase in 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.

[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).*

FIGURE 5. mitoARCUS shifts heteroplasmy *in vivo* in a novel xenograft mouse model



Cohort	Number of mice
PBS	4
$5x10^{12}$ VG/kg	4
$1x10^{13}$ VG/kg	4
$5x10^{13}$ VG/kg	4

- There are currently no available animal models for the m.3243A>G mutation. Therefore, we chose to generate a novel xenograft mouse model to test our therapeutic approach *in vivo*.
- 96% mutant m.3243G cells were injected subcutaneously into nude mice to generate the xenograft. At day 18, mice were systemically injected with either AAV-mitoARCUS or PBS. At day 35, all animals were humanely euthanized and tissues were collected for cellular DNA isolation (**5A**).
- A dose-dependent shift in heteroplasmy was observed in the AAV-mitoARCUS treated cohorts, which reached statistical significance at the highest dose (**5B**)[a]. There were no significant changes in mtDNA copy number in any of the cohorts (data not shown).
- There was a dose-dependent increase in AAV copies per cell across the isolated tissues, with the tumor having the lowest level of transduction (**5C**). Therefore, we hypothesize that greater shifts in heteroplasmy could be attained in disease-relevant tissues when the target DNA sequence is present.

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. Precision BioSciences' protein engineering and optimization platform allows for the generation of highly specific nucleases that can accurately discriminate a single nucleotide difference.
3. Using an AAV systemic delivery, mitoARCUS can be trafficked to therapeutically relevant tissues and induce shifts in heteroplasmy when the target DNA is present.
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.