CRISPR/Cas9-based Modelling of Mitochondrial Disorders

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1. Introduction

- Mitochondria are complex cellular organelles which provide an energy source for the body.
- A fault in any of the 1300 gene products required for healthy mitochondrial assembly and function could result in mitochondrial dysfunction, leading to mitochondrial diseases, which are associated with a wide range of symptoms, severities, and age of onset.

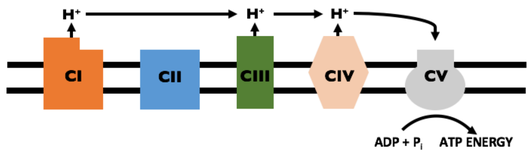


Figure 1: Simplified mitochondrial electron transport chain showing each of the five complexes embedded in the mitochondrial membrane.

- The CRISPR/Cas9 technique enables the generation of model cell lines carrying specific mitochondrial disease-causing mutations.
- This gene-editing system targets 'molecular scissors' to a specific locus within the genome, which cut the DNA and can introduce mutations.

Mitochondrial Disease Patient

- Mitochondrial disease symptoms (hypotonia, lactic acidosis, brain abnormalities) present from birth.
- Whole-exome sequencing identified a compound heterozygous mutation in the ***RTN4IP1*** gene.

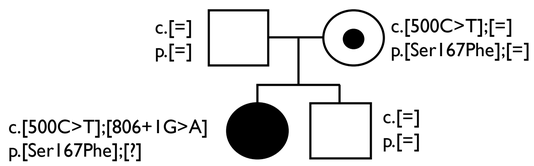


Figure 2: Family pedigree showing inheritance pattern of *RTN4IP1* mutations in patient's family.

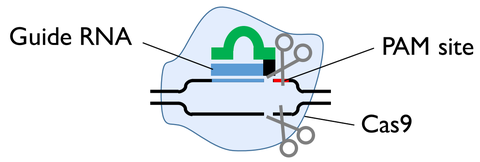
- Patient samples show a complete loss of RTN4IP1 protein and a tissue-specific mitochondrial Complex I defect in skin fibroblasts but not muscle tissue (unpublished data).

2. Aim

- Generate a human cell line harbouring a null mutation in the mitochondrial *RTN4IP1* gene using the genome editing CRISPR/Cas9 technique.
- Characterise the effect of the *RTN4IP1* null mutant on mitochondrial function.

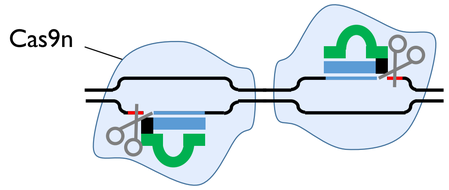
3. Methods

CRISPR/Cas9 Nuclease system



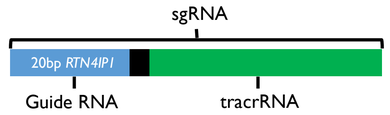
- The CRISPR/Cas9 nuclease system consists of a 20bp target DNA sequence (guide RNA) and a pair of molecular scissors (Cas9), which induce a double strand break in the DNA.
- Cas9 is guided via the 20bp guide RNA sequence to the gene of interest (*RTN4IP1*) and it requires a PAM site (NGG) in order to cut DNA.
- Cellular DNA repair machinery promotes gene editing via non-homologous end joining.

CRISPR/Cas9n Nickase system



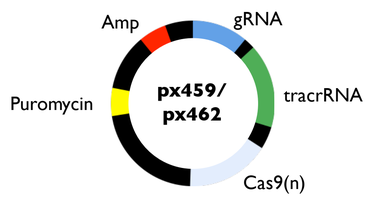
- The nickase system (Cas9n) induces single stranded breaks on the DNA .
- Two unique guide RNA constructs are required in order to produce the breaks.
- The nickase system is more precise than the nuclease system as there are fewer off-target effects.
- Gene editing is promoted via homology-directed repair.

1 CRISPR/Cas9 construct design



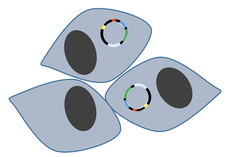
- Online CRISPR design tools (ZiFit, CRISPR Design) were used to identify a 20bp sequence (gRNA) that is complementary to target sequence in the *RTN4IP1*.
- To minimize potential off-target effects, gRNAs for the nickase system were designed.

2 Molecular cloning



- Molecular cloning of the 20 bp *RTN4IP1* target sgRNAs into CRISPR/Cas9 expression vectors (px459 nuclease or px462 nickase system)
- Transformation of vectors into competent DH5α *E. Coli* cells followed by plasmid extraction.

3 Nucleofection and isolation of clones



- U2OS human cells were transfected with px459*RTN4IP1* CRISPR/Cas9 vector using electroporation, followed by enrichment of CRISPR/Cas9-edited cells by antibiotic selection and clonal expansion.

4 Analysis of gene edited clones by PCR and Sanger sequencing

4. Results

A. Sanger sequencing of CRISPR/Cas9 clones identified two heterozygous and a compound heterozygous mutation in the *RTN4IP1* gene. Target sgRNA highlighted in grey.

Heterozygous clones 1 and 2 (+/-)

Alelle 1 - Wild type

ATGGAATTTCTGAAGACTTGTGTACTTAGAAGAAATGCATGCACTGCGGTTTGCTTCTGGAGAAGCAAAGTTG
TCCAAAAGCCTTCAGTTAGAAGGATTAGTACTACCTCTCCTAGGAGCACTGTCATGCCTGCTT

Allele 2 - c.112 C>T missense mutation

ATGGAATTTCTGAAGACTTGTGTACTTAGAAGAAATGCATGCACTGCGGTTTGCTTCTGGAGAAGCAAAGTTG
TCCAAAAGCCTTCAGTTAGAAGGATTAGTACTACCTCTTCTAGGAGCACTGTCATGCCTGCTT

Compound heterozygous clone 3 (-/-)

Allele 1 - 16bp deletion CCTAGGAGCACTGTCA

ATGGAATTTCTGAAGACTTGTGTACTTAGAAGAAATGCATGCACTGCGGTTTGCTTCTGGAGAAGCAAAGTTG

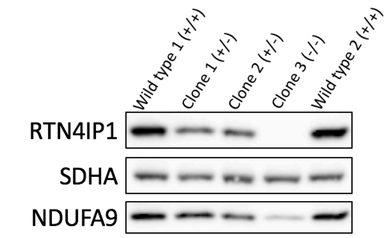
TCCAAAAGCCTTCAGTTAGAAGGATTAGTACTACCTCT----------TGCCTGCTT
CCTAGGAGCACTGTCA

Allele 2 - 5bp deletion CCTAG > TCAA insertion

ATGGAATTTCTGAAGACTTGTGTACTTAGAAGAAATGCATGCACTGCGGTTTGCTTCTGGAGAAGCAAAGTTG

TCCAAAAGCCTTCAGTTAGAAGGATTAGTACTACCTCT-TCAAGAGCACTGTCATGCCTGCTT
CCTAG>TCAA

B. Western blot analysis of *RTN4IP1* clones showed a partial loss of RTN4IP1 in clones 1 and 2 (+/-) and complete loss of RTN4IP1 protein in clone 3 (-/-) when compared to wild-type controls (+/+). The steady-state levels of the mitochondrial complex I subunit NDUFA9 were markedly reduced in clone 3. SDHA (complex II) was used as a loading control.



5. Summary and Future Work

- CRISPR/Cas9 generated human cell line carrying a null mutation in the *RTN4IP1* gene (clone 3) mimics the mitochondrial Complex I defect found in *RTN4IP1* patient fibroblasts.
- In order to reveal whether RTN4IP1 is a new Complex I assembly factor, the *RTN4IP1* CRISPR/Cas9 cell line will undergo a complexosome analysis.

6. Acknowledgements

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Sander, J. and Joung, J. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol*, 32(4), pp.347-355.