

GENOME EDITING IN A DILATED CARDIOMYOPATHY MOUSE MODEL *IN VIVO*



SAPIENZA
UNIVERSITÀ DI ROMA

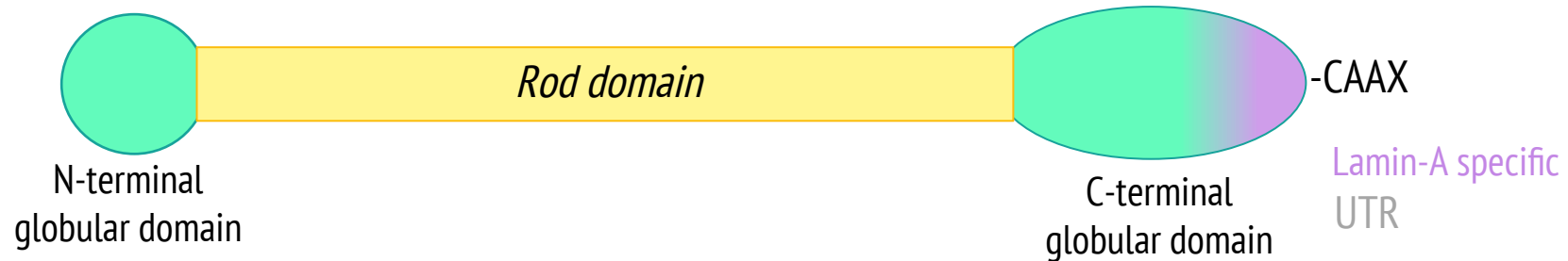
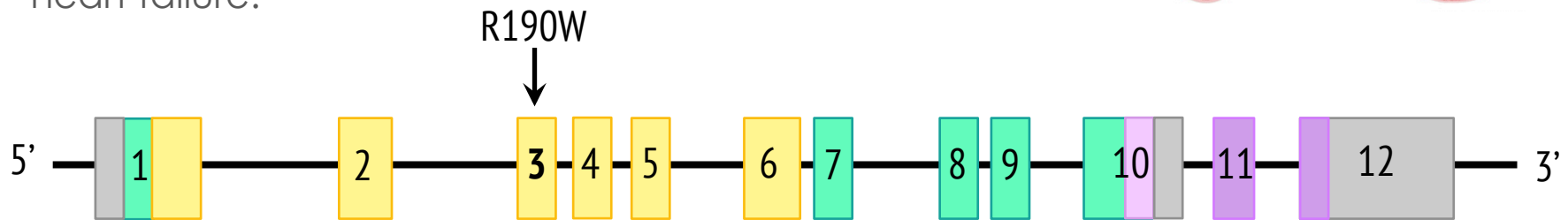
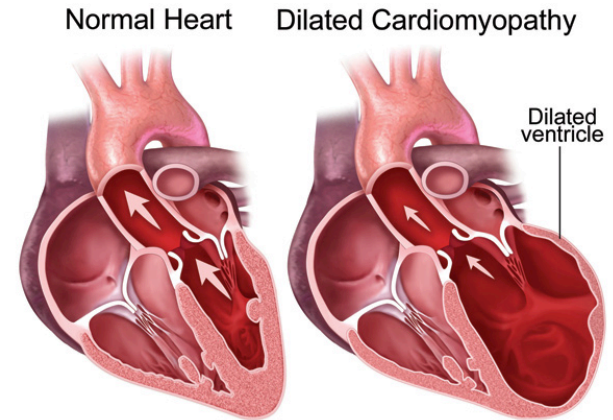
Professor Isabella Saggio
2017/2018

A GENE THERAPY PROJECT BY:

- LUCREZIA CAMICIA
- MARTA CARREÑO
- MARCO GUARNACCI
- VALENTINA SILENZI
- TOMMASO VICANOLO
- ERIKA VITIELLO

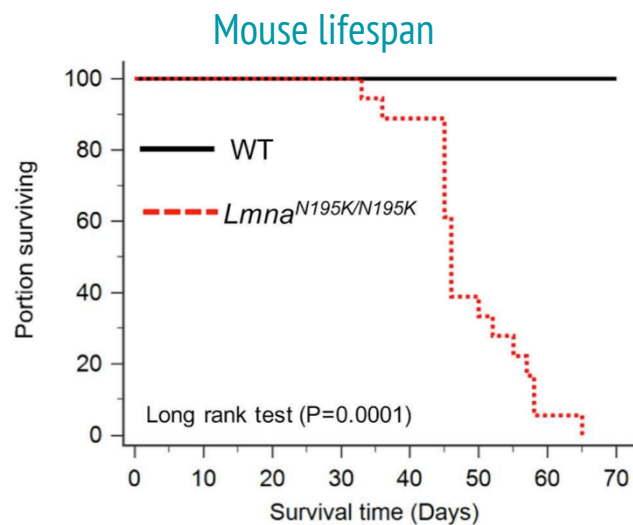
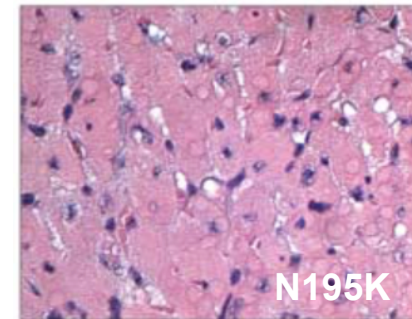
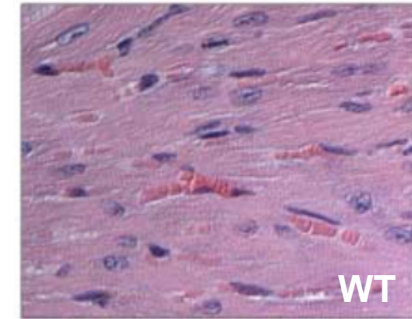
DILATED CARDIOMYOPATHY

- **WHAT?** Laminopathy: R190W mutation in exon 3 of the LMNA gene.
- **WHY?** Mutational hot spot.
- **SYMPTOMS?** Dilation of the left ventricle and heart failure.

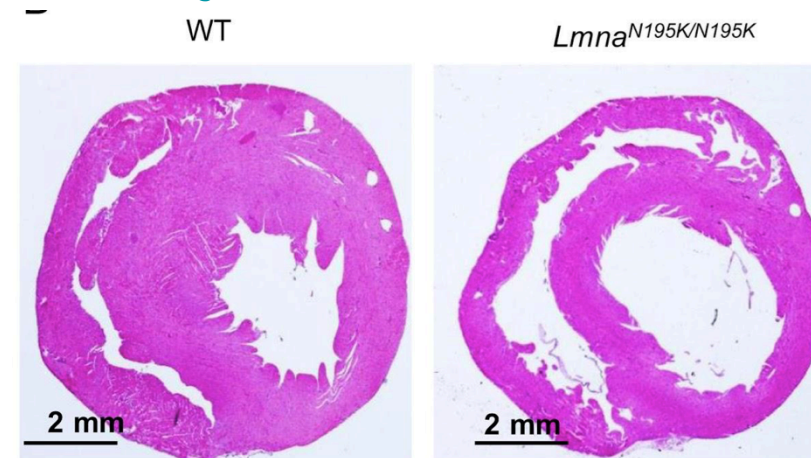


METHODS: Mouse Model

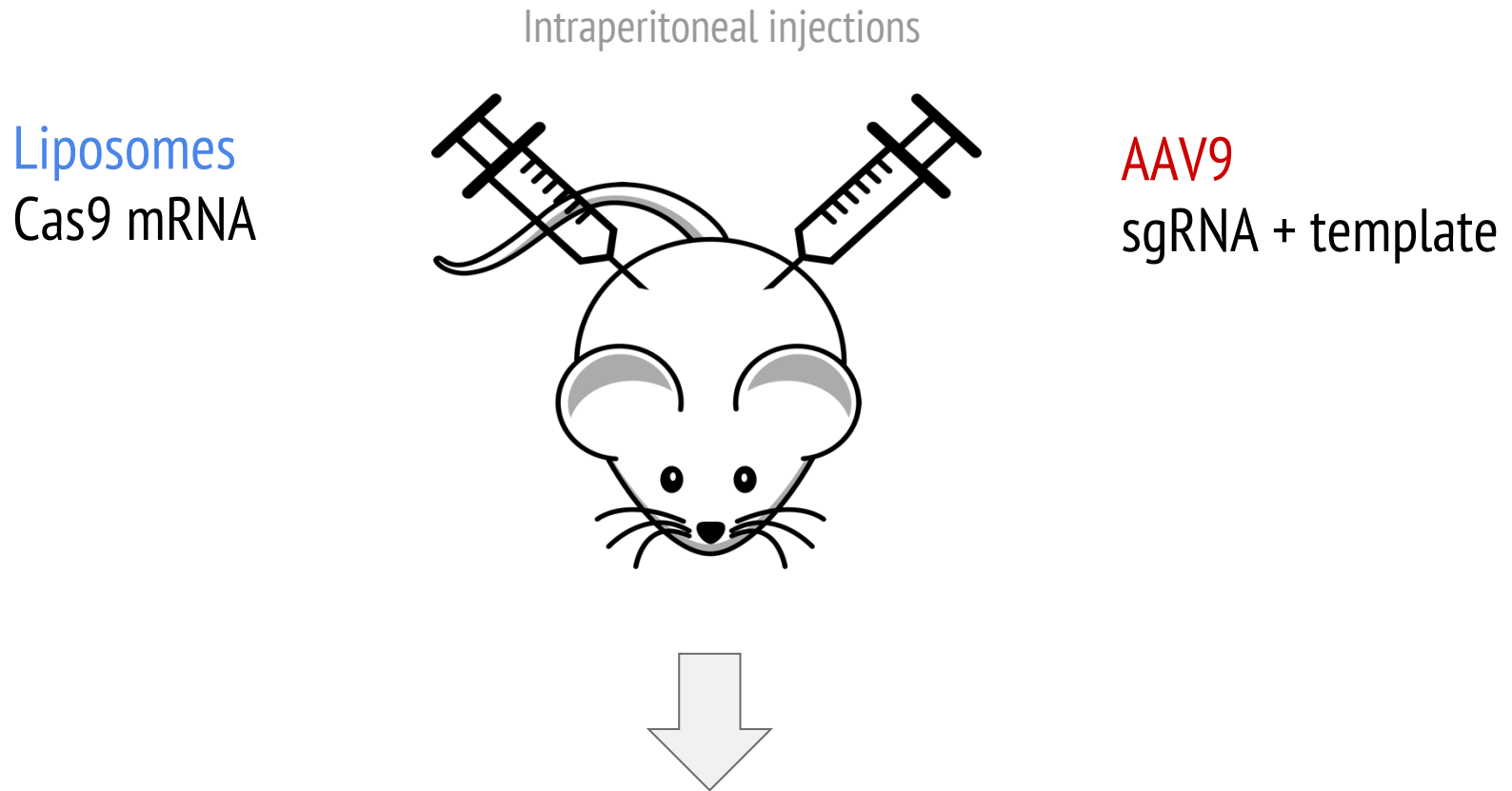
- N195K mice
- Homozygous for the mutation on exon 3 (LMNA^{-/-})
- Death when 2-3 months old due to arrhythmia.



H&E staining of ventricles

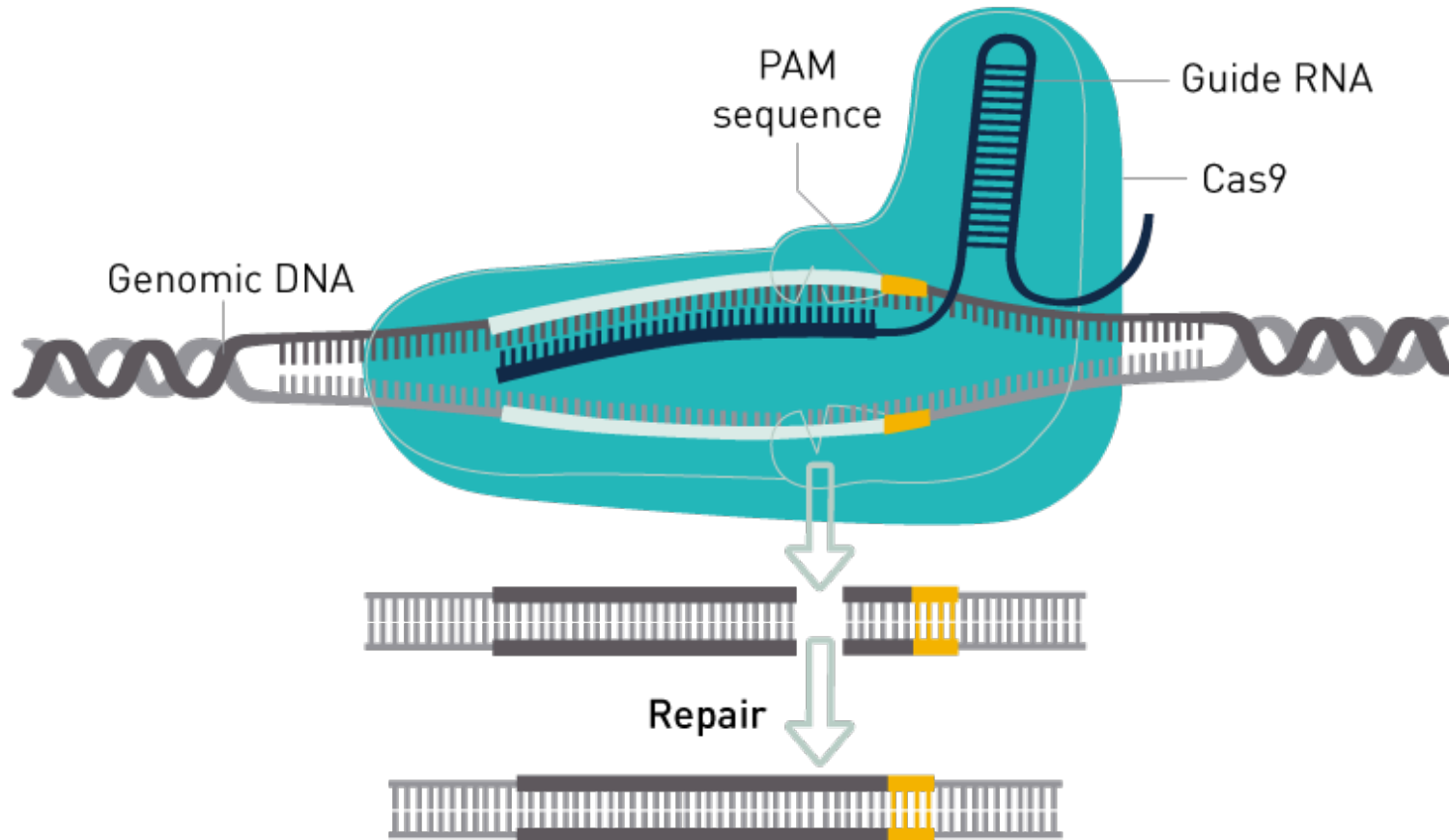


Our combined approach

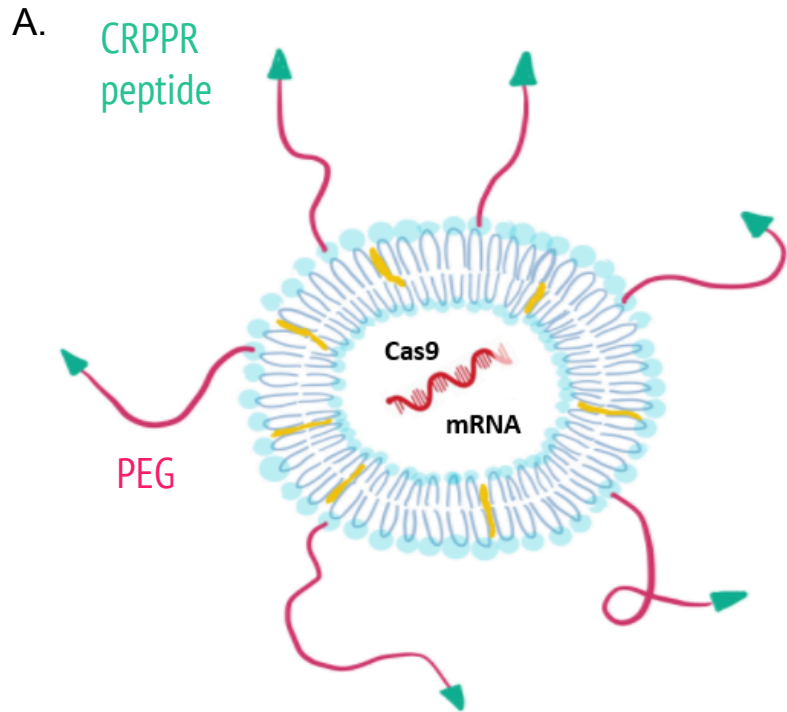


Chengzu Long et al., *Science*, 2015; Yin et al., *Nature Biotechnology*, 2016.

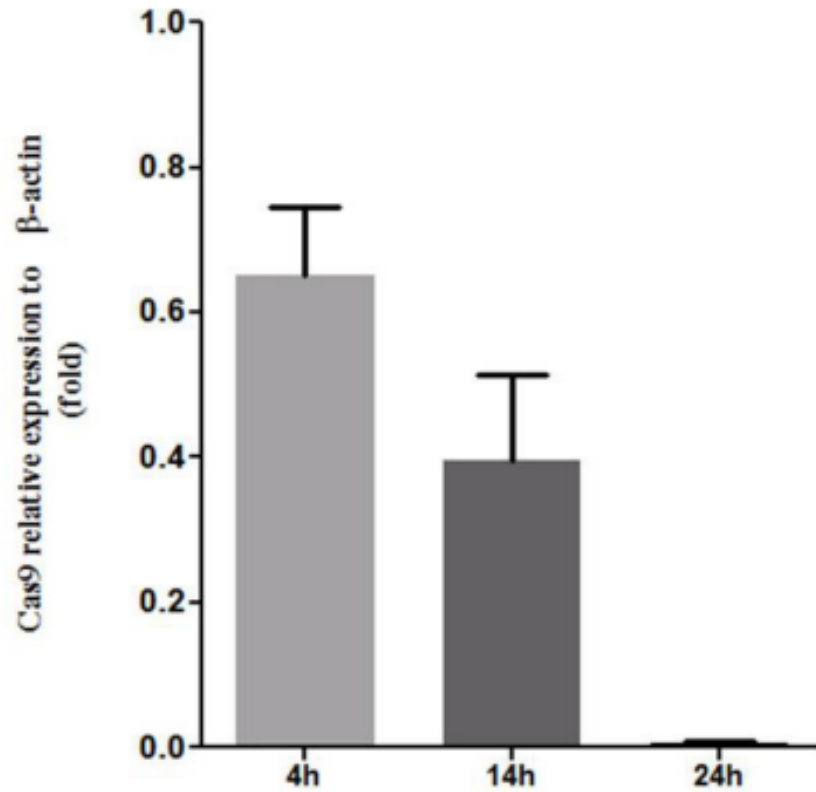
METHODS: CRISPR/Cas9



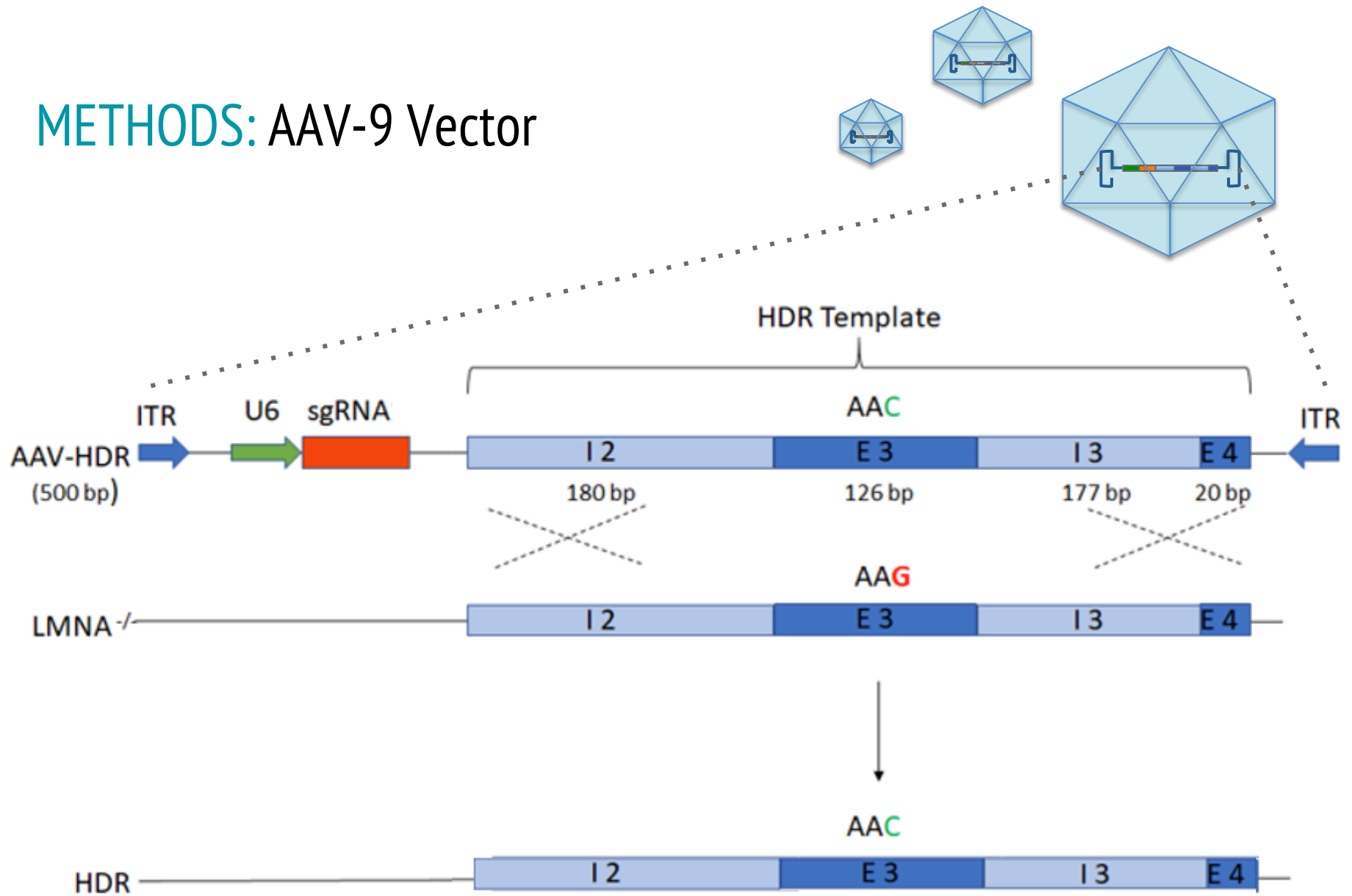
METHODS: Liposome Vector



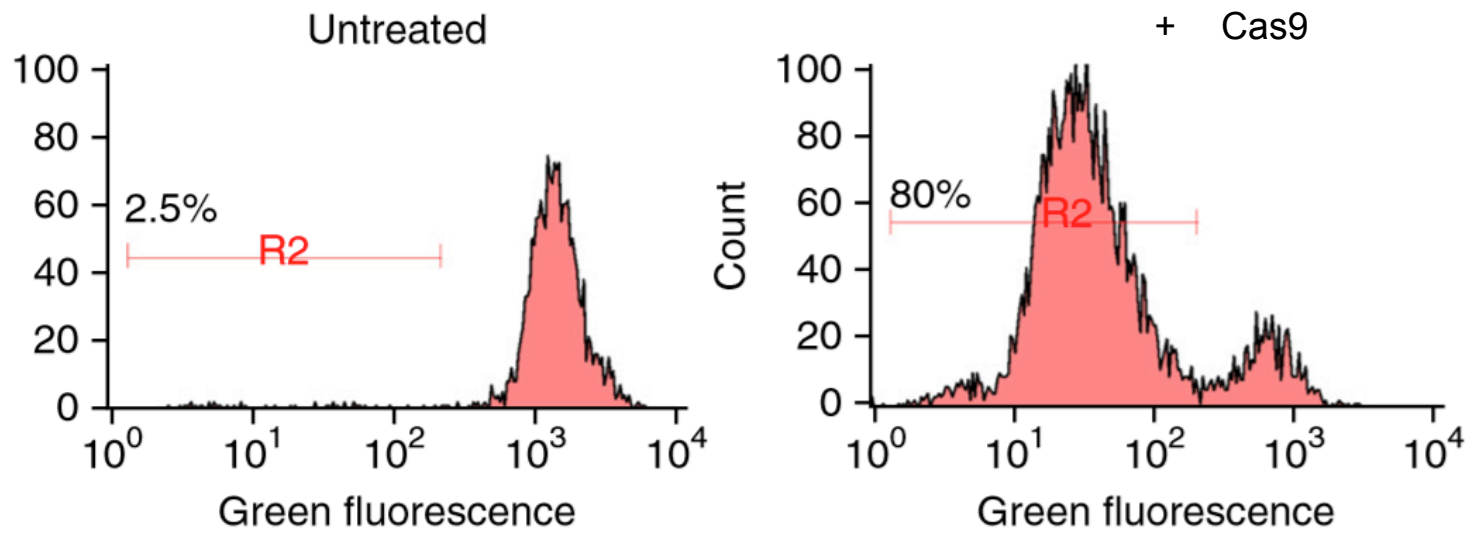
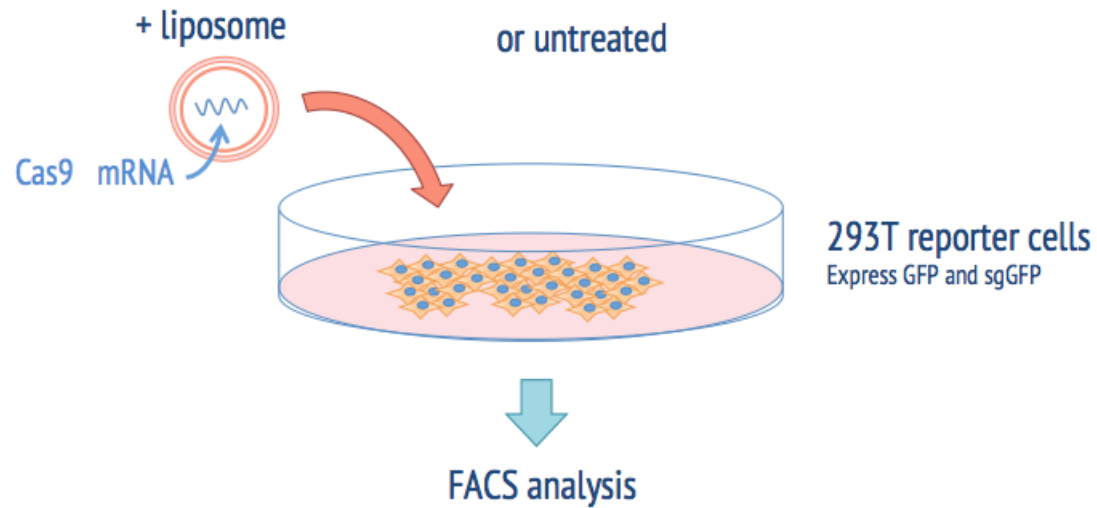
B. Cas9 persistence *in vivo*



METHODS: AAV-9 Vector



IN VITRO: Efficiency of targeting

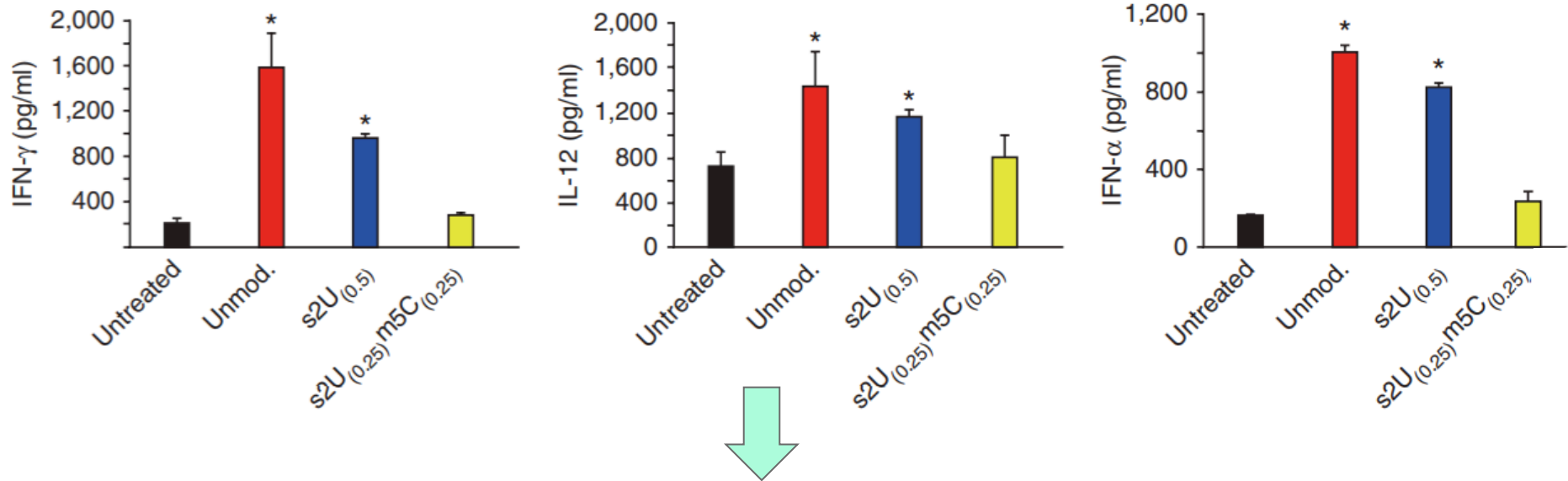


Is the Cas9 mRNA immunogenic?

mRNA Immunogenicity Assay

25% U → 2-thiouridine
25% C → 5-methyl-cytidine

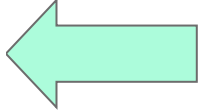
Incubation of Cas9 mRNA (modified or unmodified) in liposomes with N195K mouse whole blood.



Comparison of the levels of pro-inflammatory cytokines released after 0, 6 and 24 hours.

Selecting our sgRNAs

	score	sequence	PAM
Guide #1	75	GAACAGGCTACAGACGCTGA	AGG
Guide #2	62	GGATGAGATGCTGAGGCGAG	TGG
Guide #3	58	GGCGAGTGGATGCTGAGAAC	AGG

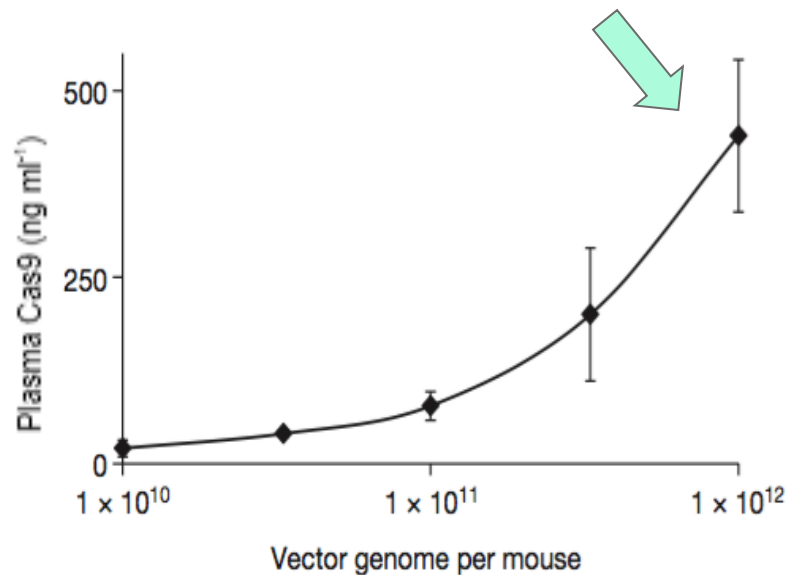


Top 3 ranked predicted off target sites

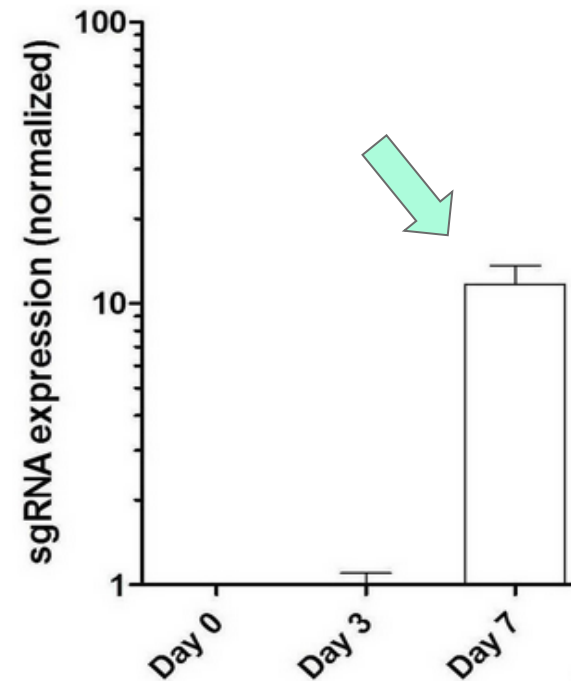
gene	sequence	score	mismatches	UCSC
	GCACAGCATCCAGACGCTGAAGG	0.9	4MMs [2:7:8:10]	NM_010546
	GAACGGCCTGGAGACGCTGAGAG	0.5	4MMs [5:7:10:11]	NM_183390
	GAGGAGGCTGCAGAGGCTGACAG	0.4	4MMs [3:4:10:15]	NM_011437

<http://crispr.mit.edu>

How much and when should AAV be administered?

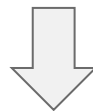
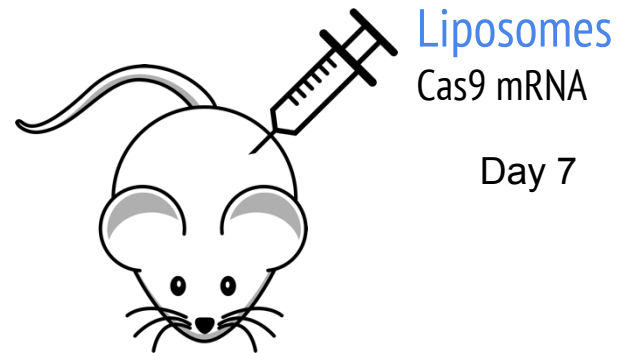
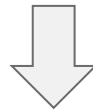
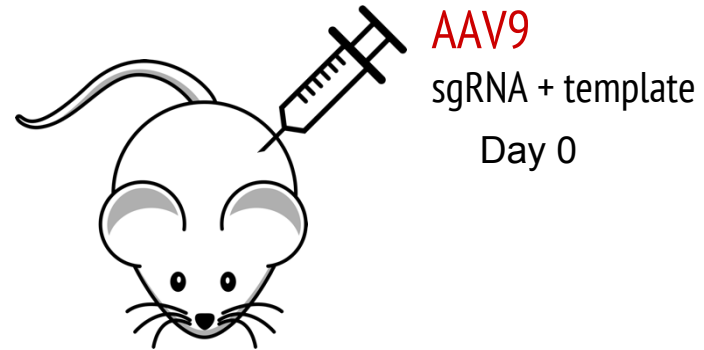


Intraperitoneal injection with 1×10^{12} genome copies of AAV-HDR.



Peak of expression of AAV at day 7. Injection of liposomes after a week to ensure maximal co-expression of all components.

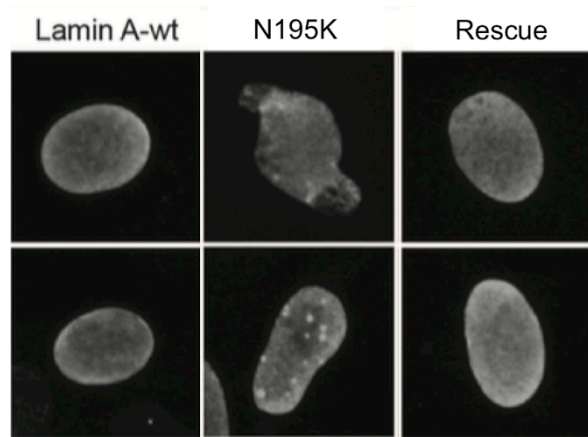
Our combined approach



Functional tests to see the rescue of the lamina

Has the wt phenotype been rescued?

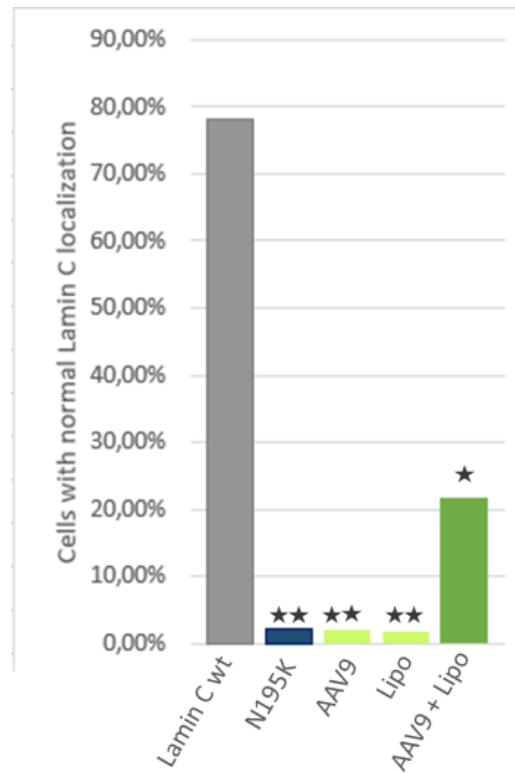
Immunostaining of lamin C
in mouse cardiomyocytes



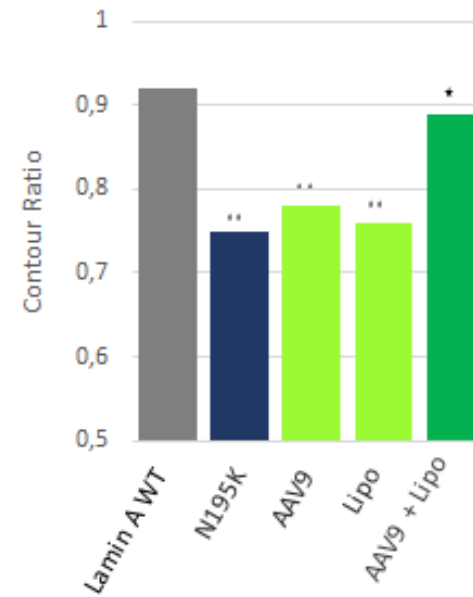
Structural Integrity of the nucleus



Lamin C localisation

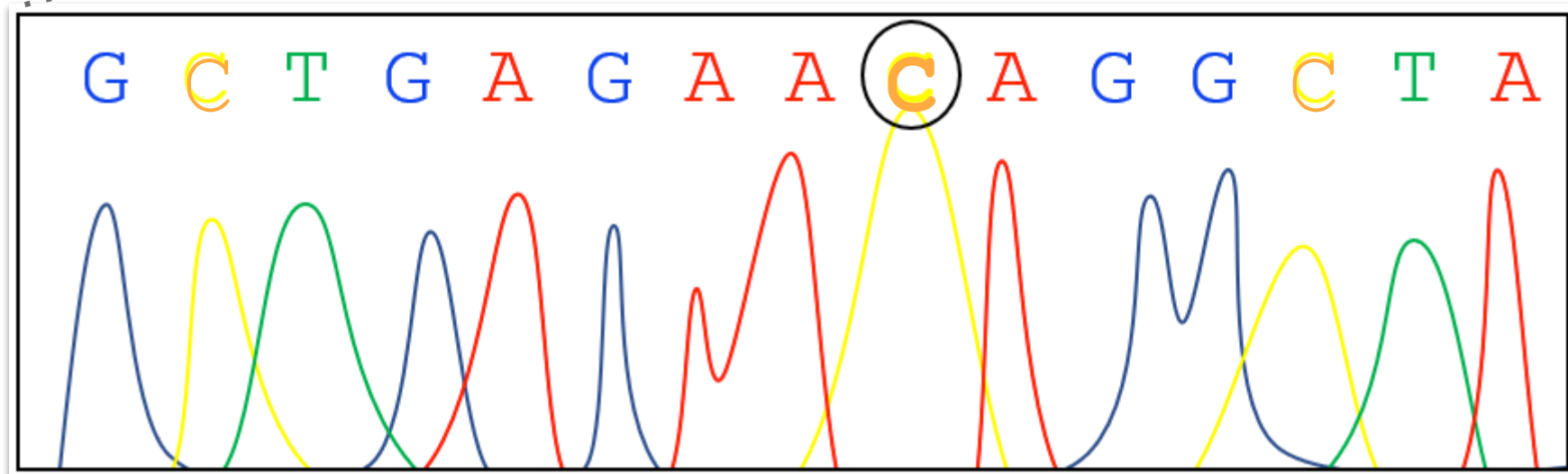


Shape of the nucleus

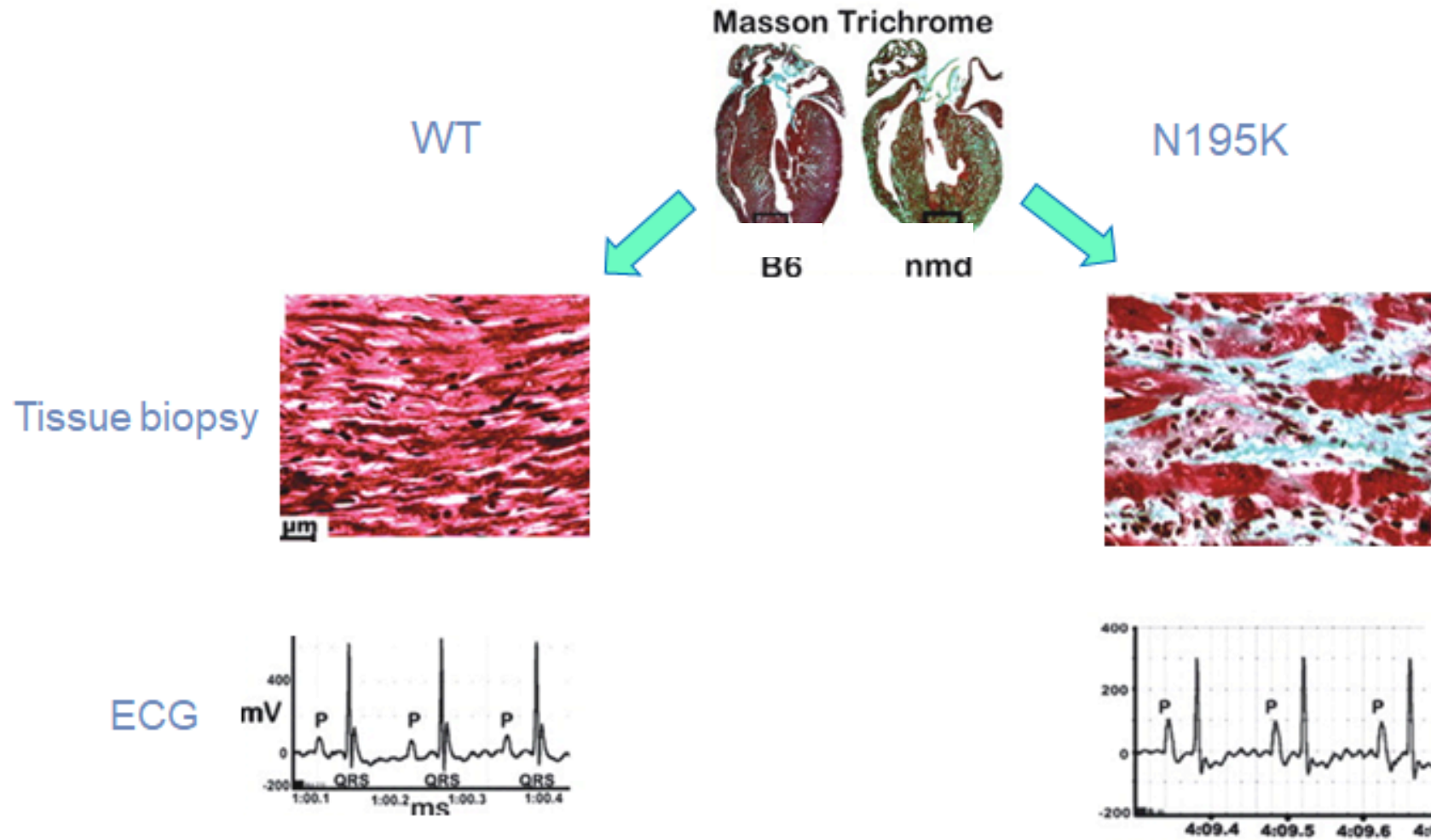


280 nuclei were analysed in total for each condition.

Is the wild-type LMNA sequence restored?

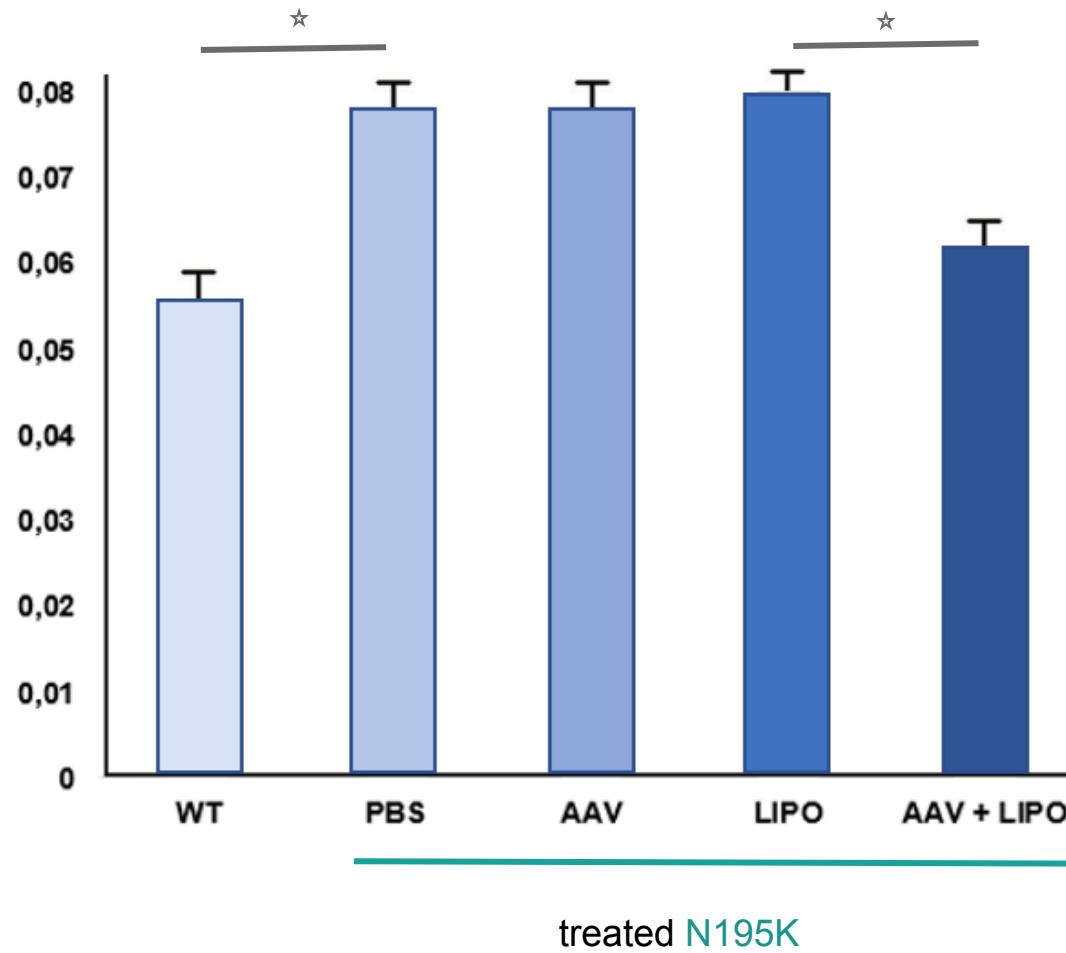


Functional tests



Adapted from Maddatu et al., *Human Molecular Genetics*, 2005.

Physical test



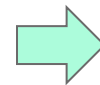
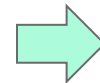
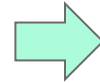
Adapted from Meifang Liu et al., *Elsevier*, 2014

CONCLUSIONS

- The combined approach described can correct the mutation causing dilated cardiomyopathy in *in vivo* mouse model;
- The phenotypic effects shown suggest an extension of lifespan and amelioration of the quality of life;
- The transient expression of Cas9 mRNA allows the treatment to be safer, compared to other expression methods described in literature so far;
- Numerous PAM sites have been identified on the LMNA gene → our approach could be tailored to address different mutations other than N195K.

PITFALLS

- The combined treatment can lead to a less efficient transfection of all CRISPR/Cas9 components in the same cell;
- Further studies are required to progress onto clinical trials.
- In humans, the mutation is heterozygous, as opposed to mice, meaning that the efficiency of the same combined approach, if used, would be halved.



SOLUTIONS

- Targeting methods can help both the viral and non viral vectors address cardiomyocytes only;
- We are waiting for a peptide to be discovered that can target liposomes to human cardiomyocytes;
- The use of sgRNA covering the mutated nucleotide in humans will result in addressing the mutated allele only.

COSTS - 65.000,00 €

- Cas9 mRNA 15 µg (Thermo fisher) → €275,00
- sgRNA with U6 promoter €55,00
- PEG-Liposome In Vivo Transfection Reagent 0.5 ml (10 injections) → € 394,00 / 1.5 ml (30 injections) → € 826,00
- 293T-GFP cell line (GenTarget) €297,00
- Chemically modified nucleosides, 10 µL each (2-Thio-UTP + 5-Methyl-CTP; Jena Bioscience) → € 163,82
- AAV9 (10¹² GC/ml) x 16ml → €10.800,00
- Lamin C Monoclonal Antibody 100 µL (EM-11; Invitrogen) → €184,00
- Lamin A/B Monoclonal Antibodies, 100 µL (Ab-392; Abcam) → €304,00
- Sequencing: € 750,00
- 4 x C57BL6 (WT) mice: € 500,00
- 16 x N195K mice: € 2.525,00
- Genomic DNA Purification Kit (Thermo Fisher) → €118, 00
- q-PCR kit (Qiagen) → €263,00
- Ecocardiography: € 1.300,00
- ELISA kit: Single-Analyte ELISArray Kits (Qiagen) → €296,00
- Molecular biology lab apparatus: €5.000,00

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