Gene therapy project – poster and deadlines

- total 12 slides
- poster 90cm x 84cm

poster deadline: December 15 email to
isabella.saggio@uniroma1.it
mattia.latorre@uniroma1.it
romina.burla@uniroma1.it

email subject: genetherapy poster
file name: surnames_gt_date

Gene therapy project

Theme I: Aging

Group A: Bernardi, Ilie, Colonnelli, Bastianelli

Charcot marie tooth – pmp22

Group B: Hazrati, Bartolini, Glaudo, Montrone, Pourali

Werner syndrome

Theme II: Cancer

Group C: Belvedere, Jeong, Majaliwa, Virgilio

dCAS9 as a treatment for thyroid cancer

Group D: Santacroce, Pace, Serra, Fanelli, Duarte

Hepatic cancer – RACGAP1

Charcot Marie Tooth type 1A (CMT1A)

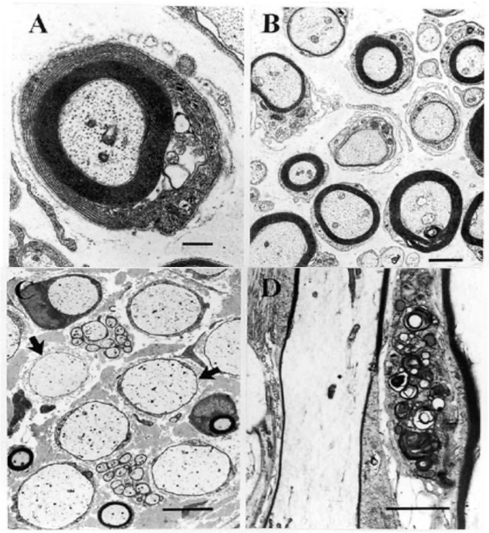
Silencing of PMP22 promoter 2 using a CRISPR/dCas9 combined with methyltransferase (DNMT3A)

Bastianelli, Bernardi, Colonnelli, Ilie

Background

Molecular basis of the disease:

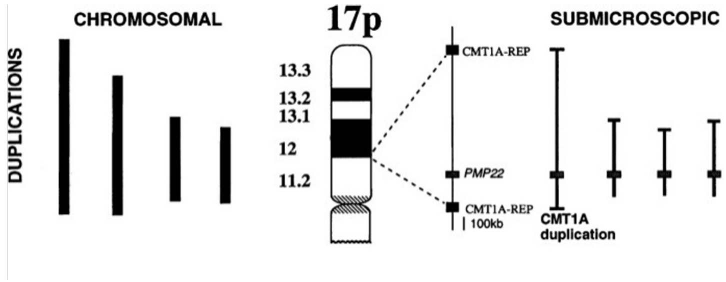
- PMP22 aggregates
- Dys Demyelination
- Onion Bulb formation
- Secondary axonal degradation



Electron micrographs from the sciatic nerve of C3 mouse

Duplication of *PMP22*

Peripheral Myelin Protein 22 (PMP22) gene on the 17p11.2-12.
Overload of the Endoplasmic Reticulum (ER)



Aim of the project

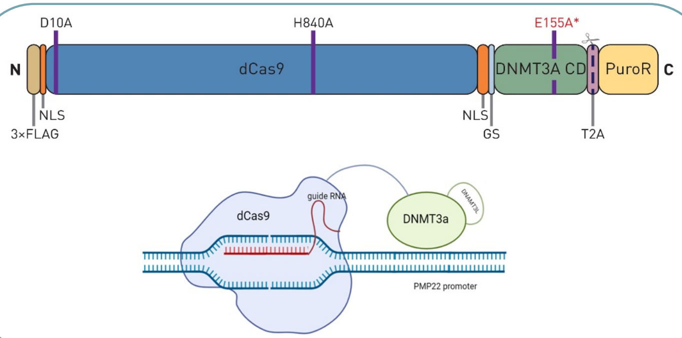
Use of CRISPR-dCas9 associated with DNMT3A to perform an epigenetic silencing of the Promoter 2 of PMP22

C3 mice
*PMP22**
Schwann cells

→ C … -4
→ Phenotype: mild demyelination

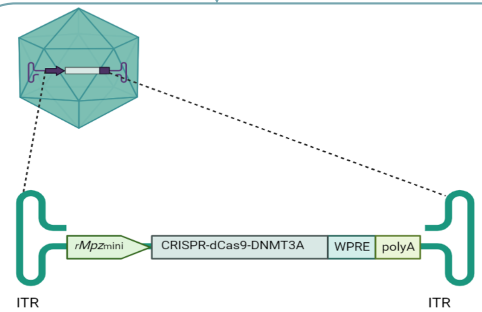
CRISPR-dCas9-DNMT3A

→ 2 sgRNA



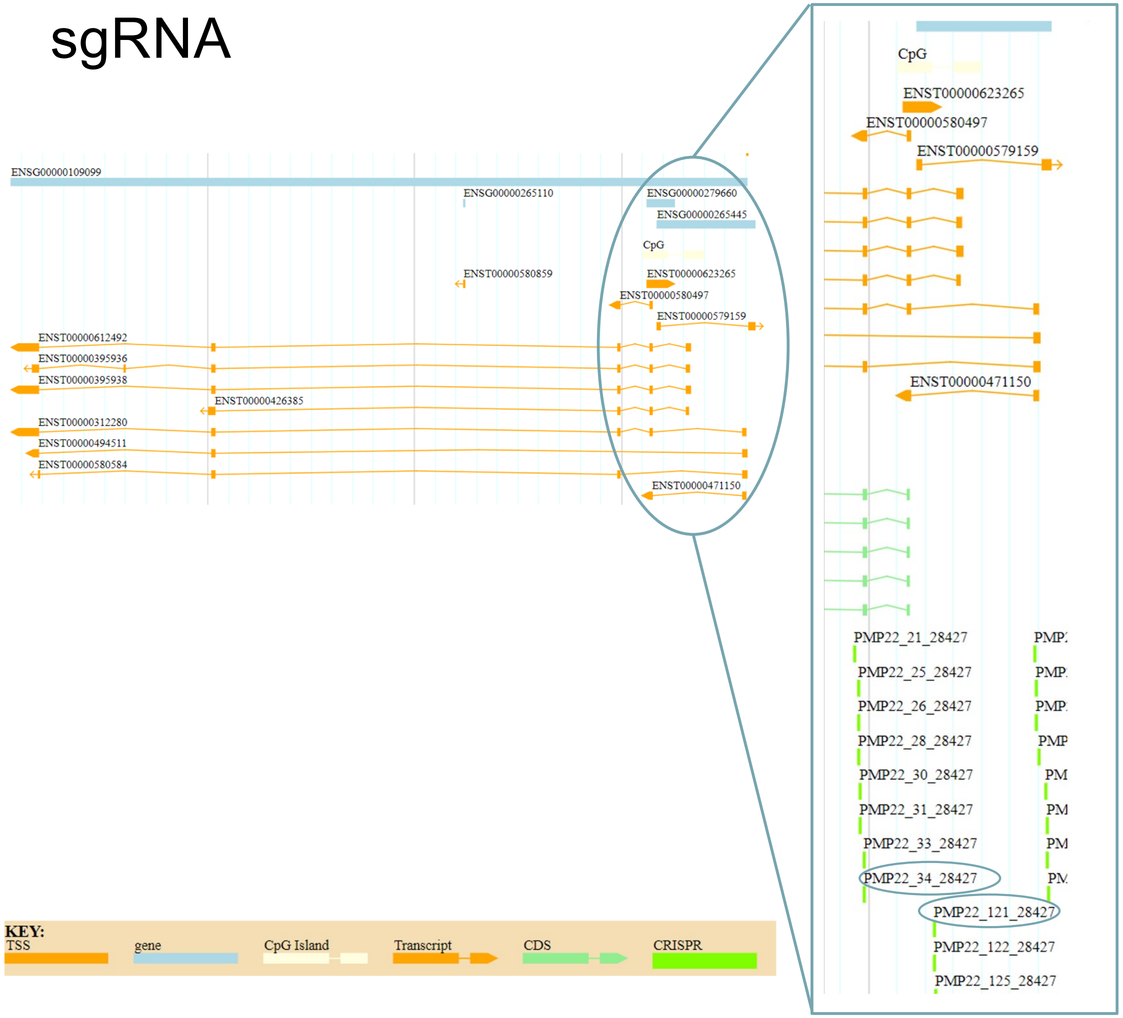
AAV 2/9

→ MPZ promoter-SC specific
→ Intrathecal lumbar injection



Adapted from Vojta et al. Nucleic acids research, 2016.

sgRNA



sgRNA 34

PMP22_34_28427	GTGGACATTTCCTGA GGAAG NGG	S A E	ENSG00000109099::PMP22

Target: |29832*| A G A A A C A G T G G T G G A C A T T T C C T G A G G A A G A G G T G C T A C A G T |29875*|
Matchstring: M M M M M M M M M M M M M M M M M M M M M M M
Query: G T G G A C A T T T C C T G A G G A A G N G G

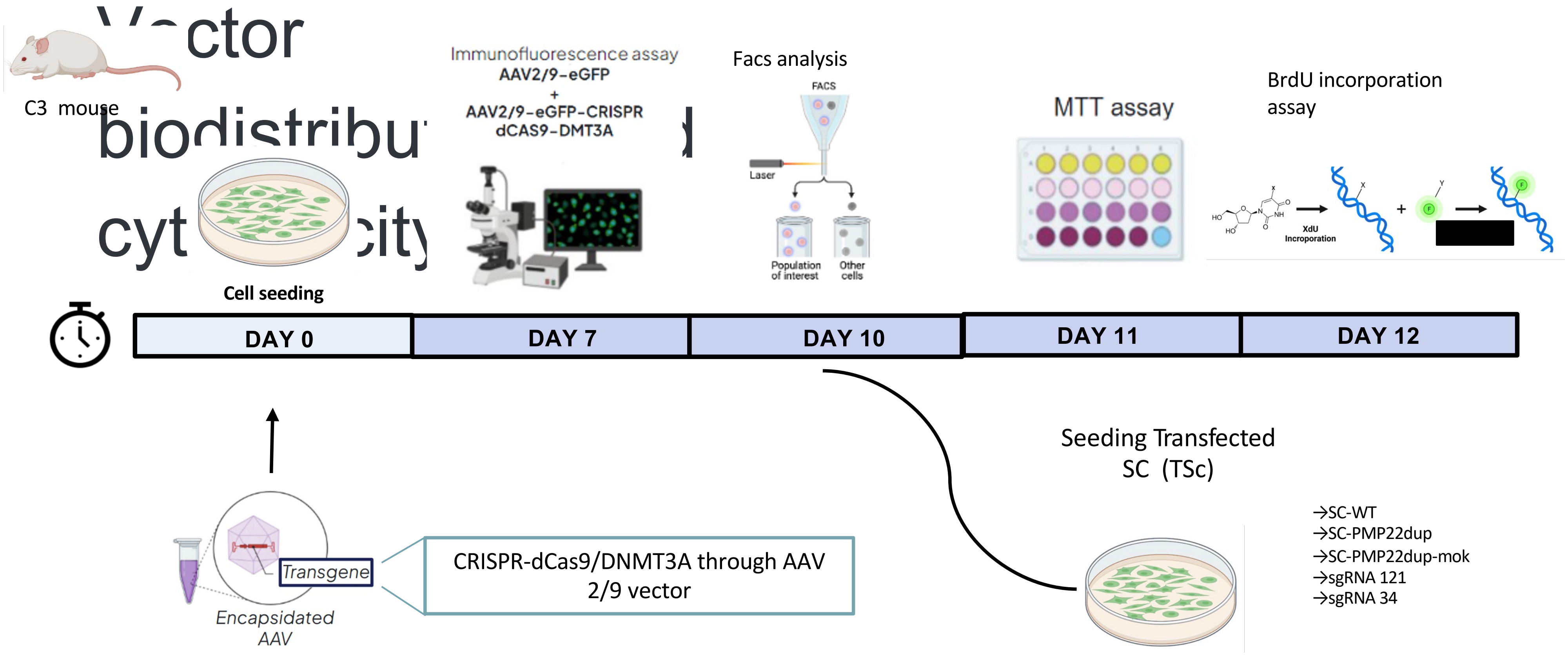
sgRNA 121

Target: |1052*| A G T C T G C A C C C C A G G C A C T C A A A G C C A G G A C A C G A A C C C C A A |1095*|
Matchstring: M M M M M M M M M M M M M M M M M M M M M M M
Query: C C N G G C A C T C A A A G C C A G G A C A C

PMP22_121_28427	GTGTCCTGGCTTTGA GTGCC NGG	S A E	ENSG00000265445::RP11-849N15.1

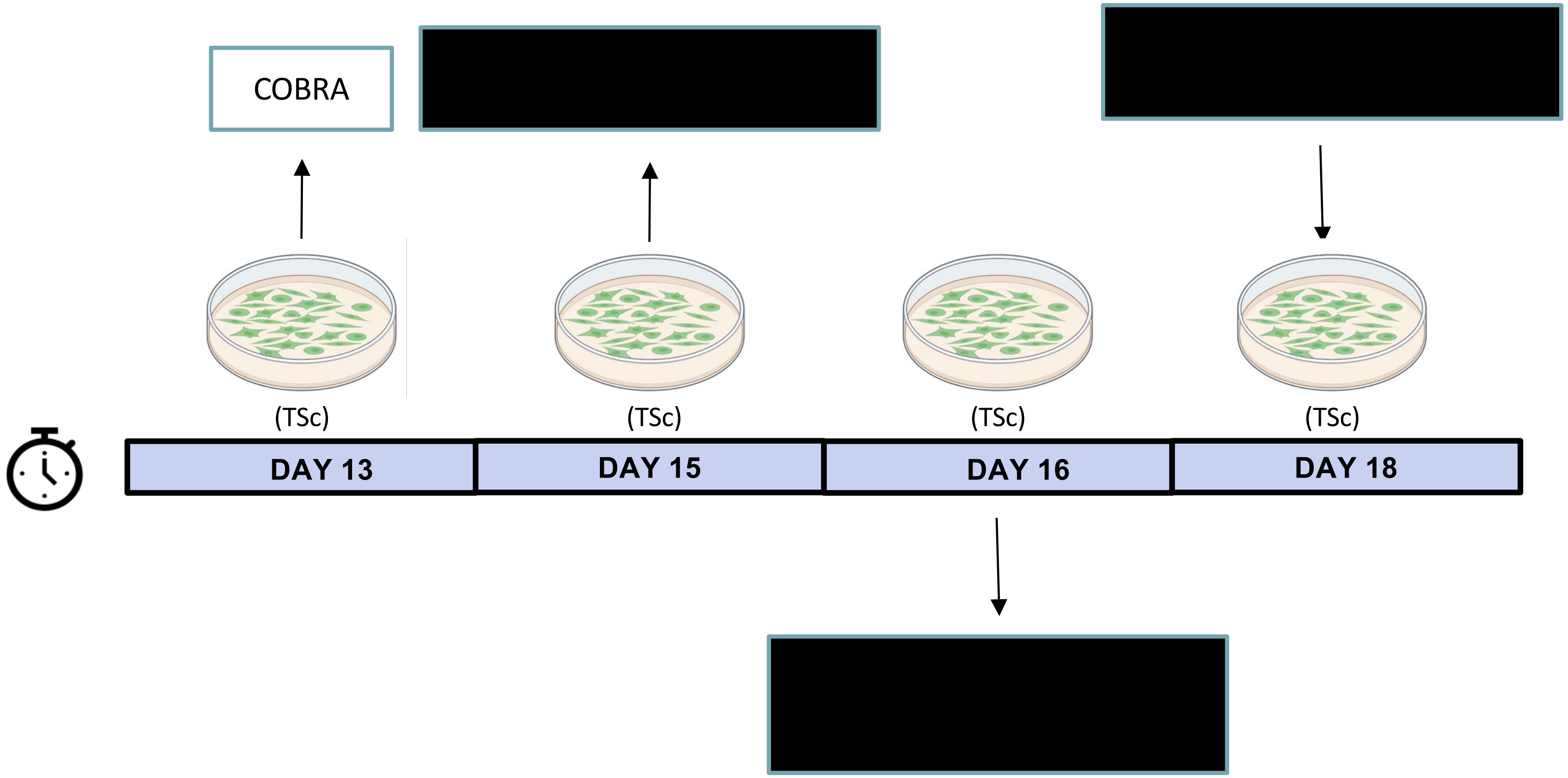
S: Specificity score
A: Annotation score
E: Efficiency score

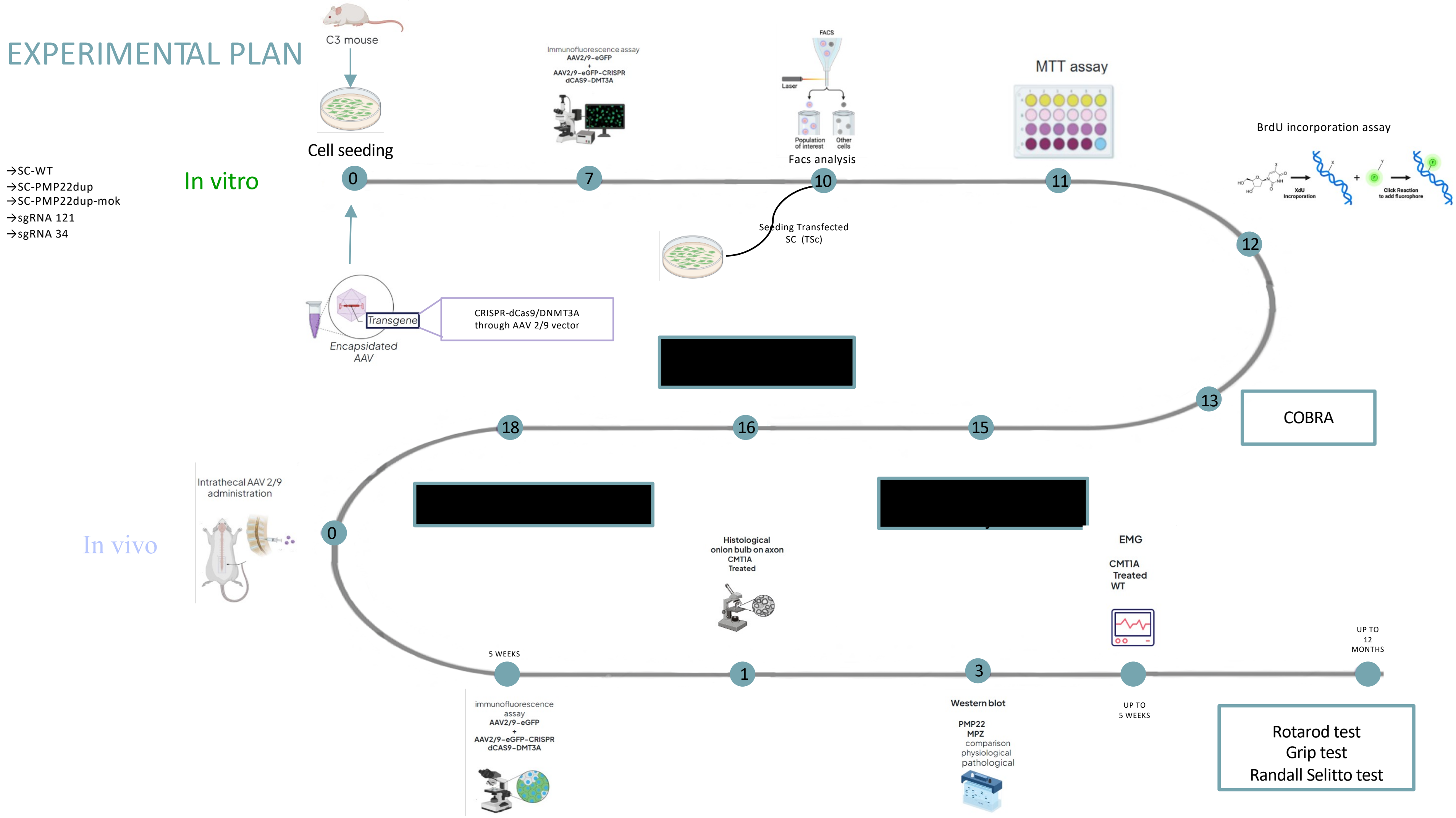
In vitro



C3 mouse
Cell seeding
Immunofluorescence assay
AAV2/9-eGFP
+
AAV2/9-eGFP-CRISPR dCAS9-DMT3A
Facs analysis
FACS
Laser
Population of interest
Other cells
MTT assay
BrdU incorporation assay
XdU Incorporation
DAY 0
DAY 7
DAY 10
DAY 11
DAY 12
Seeding Transfected SC (TSc)
→SC-WT
→SC-PMP22dup
→SC-PMP22dup-mok
→sgRNA 121
→sgRNA 34
Transgene
Encapsidated AAV
CRISPR-dCas9/DNMT3A through AAV 2/9 vector

In vitro


COBRA
[redacted]
[redacted]
(TSc)
(TSc)
(TSc)
(TSc)
DAY 13
DAY 15
DAY 16
DAY 18
[redacted]



EXPERIMENTAL PLAN
C3 mouse
Cell seeding
Immunofluorescence assay
AAV2/9-eGFP
+
AAV2/9-eGFP-CRISPR
dCAS9-DMT3A
FACS
Laser
Population of interest
Other cells
Facs analysis
MTT assay
BrdU incorporation assay
XdU incorporation
Click Reaction to add fluorophore
→SC-WT
→SC-PMP22dup
→SC-PMP22dup-mok
→sgRNA 121
→sgRNA 34
In vitro
0
7
10
11
12
13
15
16
18
Seeding Transfected SC (TSc)
Transgene
Encapsidated AAV
CRISPR-dCas9/DNMT3A through AAV 2/9 vector
[redacted]
COBRA
[redacted]
[redacted]
Intrathecal AAV 2/9 administration
In vivo
0
Histological
onion bulb on axon
CMT1A
Treated
EMG
CMT1A
Treated
WT
5 WEEKS
1
3
UP TO 12 MONTHS
immunofluorescence assay
AAV2/9-eGFP
+
AAV2/9-eGFP-CRISPR
dCAS9-DMT3A
Western blot
PMP22
MPZ
comparison
physiological
pathological
UP TO 5 WEEKS
Rotarod test
Grip test
Randall Selitto test

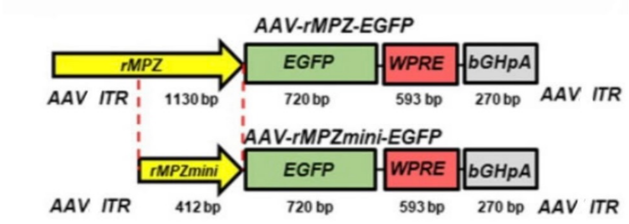
What is the system of delivery?

AAV2/9 → high tropism for Schwann cells

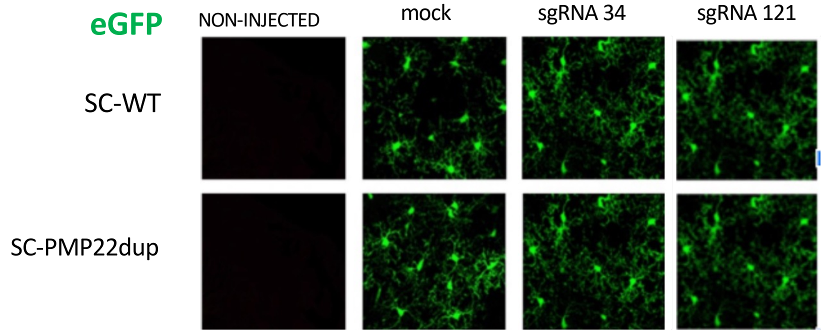
How to test AAV2/9 efficiency in vitro ?

Immunofluorescence assay

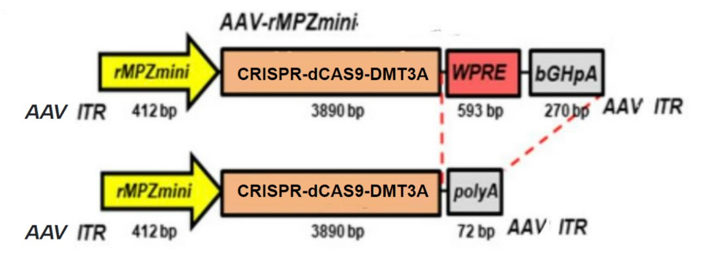
***AAV2/9* mock vector → high tropism for Schwann cells**

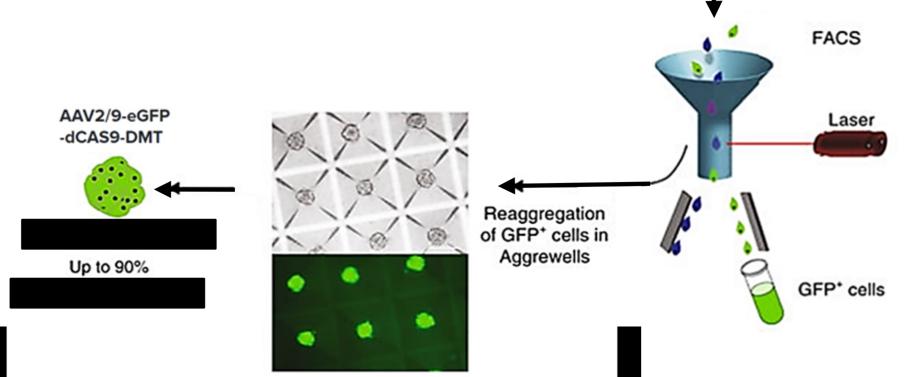


[redacted]



Therapeutic vector → CRISPR-dCAS9-DMT3A



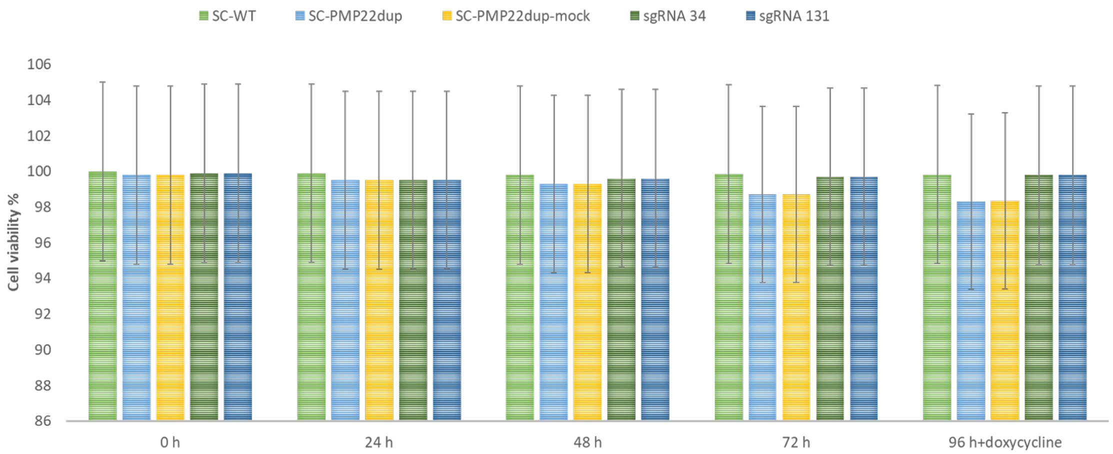


Adapted from Georgiou E. et al., 2023

Adapted from Gopika G. Nair et al. Nat Cell Biol. 2019

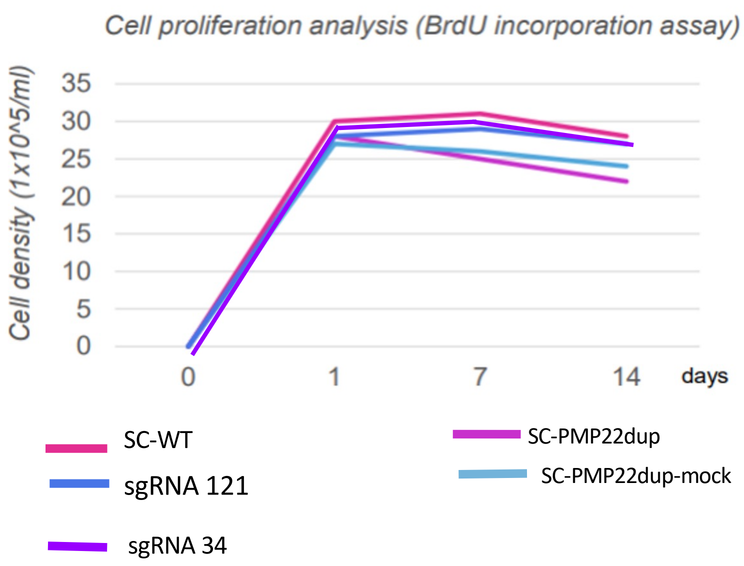
How to test the non-cytotoxicity of the treatment in vitro?

MTT assay



Adapted from Gao, F. et al. 2023

BrdU incorporation assay

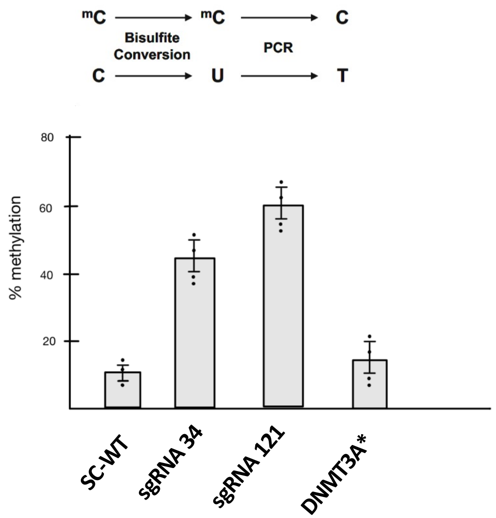


Adapted from Crane AM et al., Methods Mol Biol. 2013

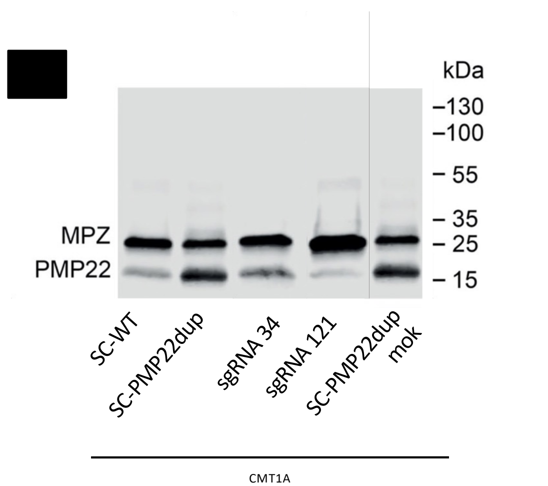
EXPECTED RESULTS: in vitro

Does Methylation downregulate expression?

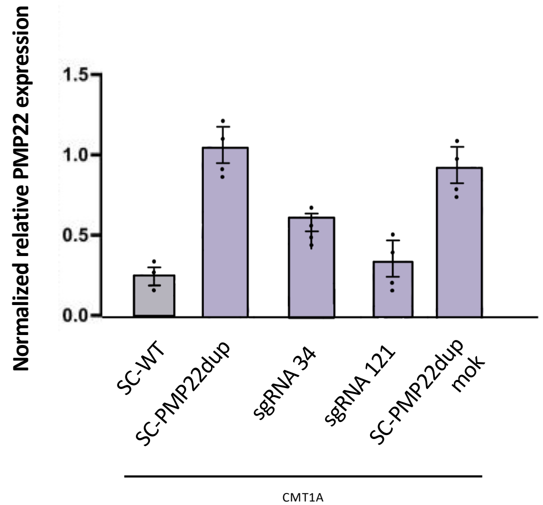
COBRA



WESTERN BLOT



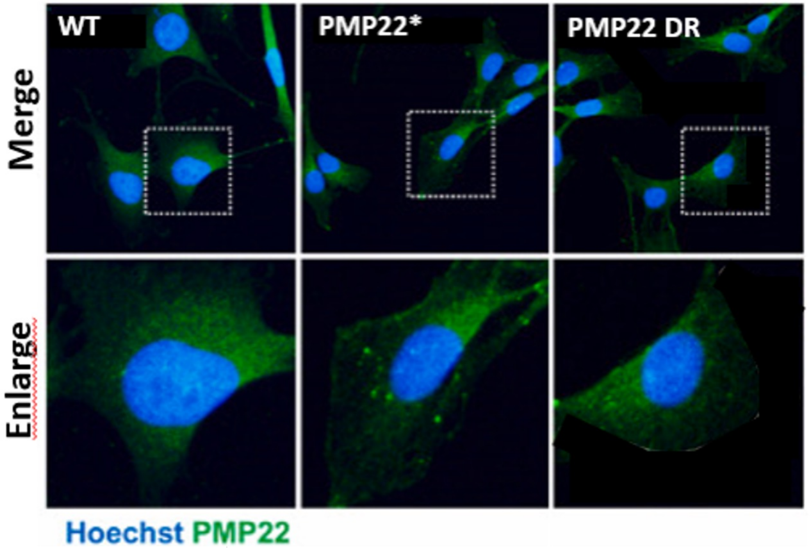
qPCR

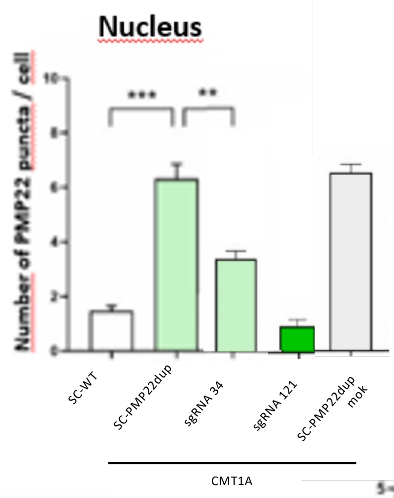


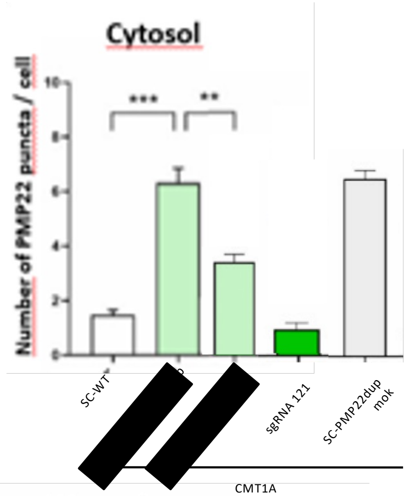
Adapted from Gautier et al. nature communications, 2021

Accumulation of *PMF*

→ PMP22 aggregates

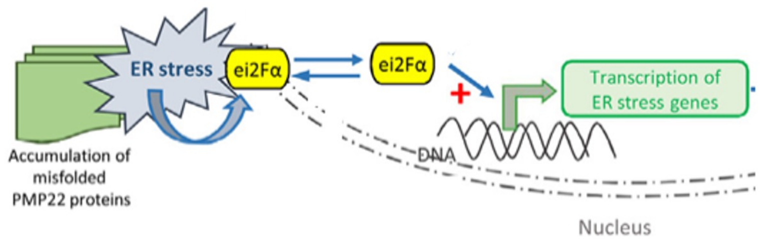


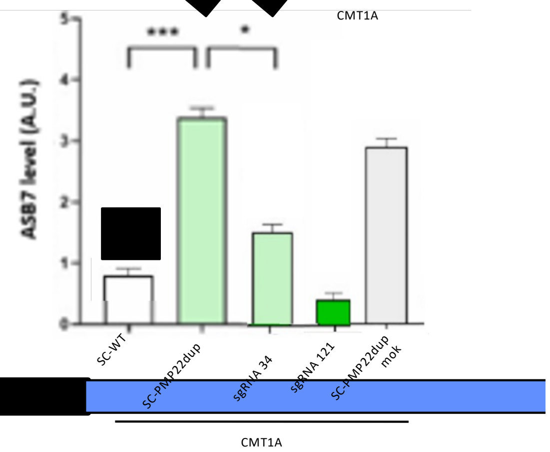




→ ER stress

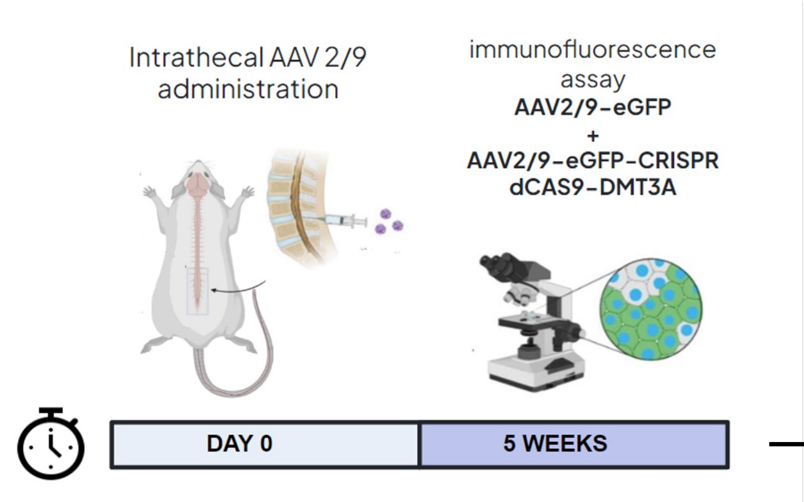
→ increase ASB7 expression



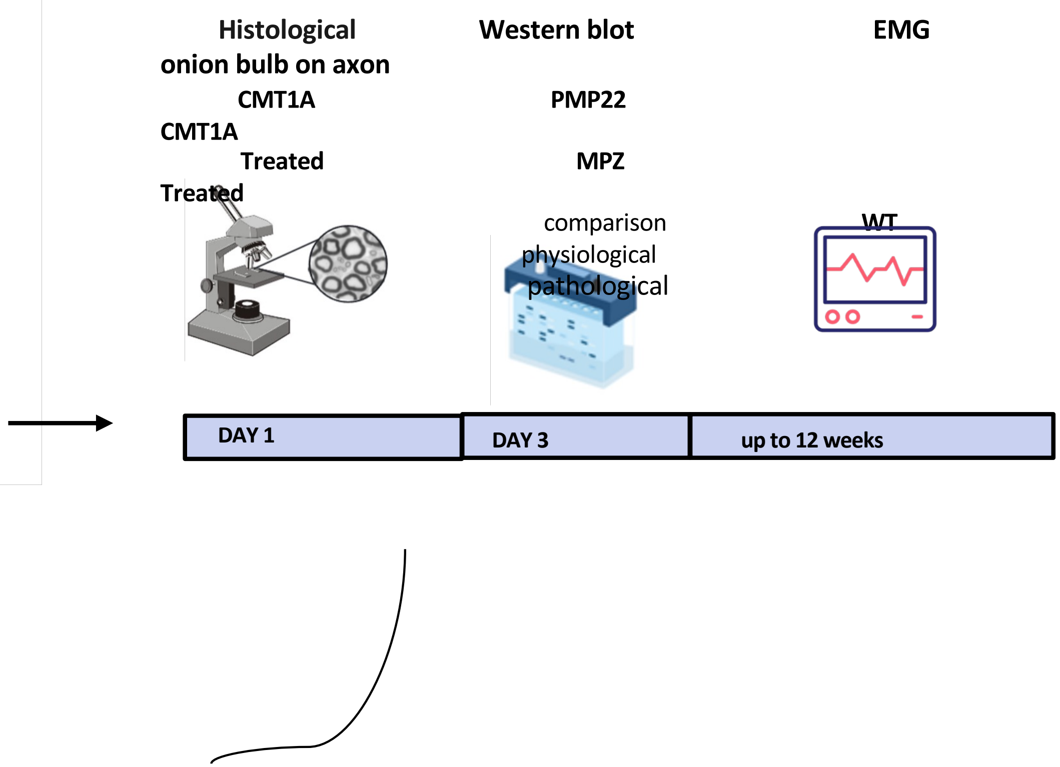


[redacted]

In vivo



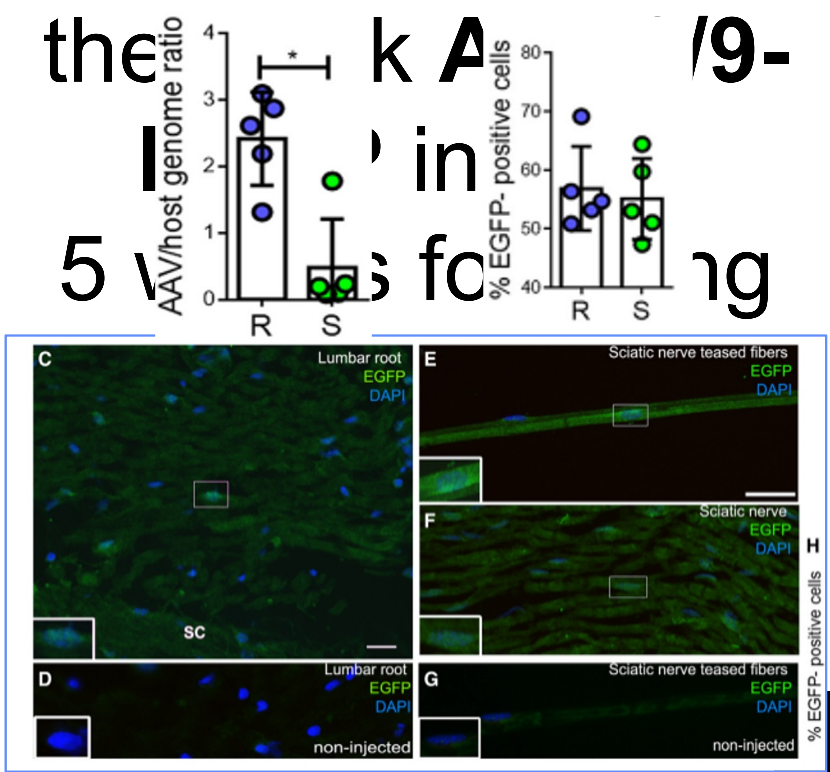
Intrathecal AAV 2/9 administration
immunofluorescence assay
AAV2/9-eGFP
+
AAV2/9-eGFP-CRISPR dCAS9-DMT3A
DAY 0
5 WEEKS



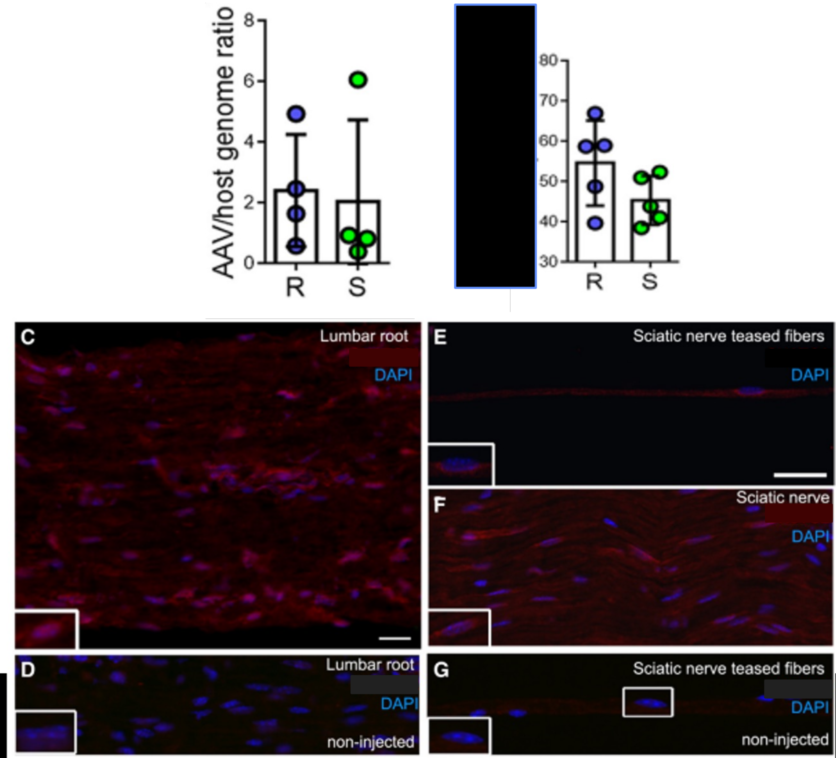
Histological
onion bulb on axon
CMT1A
CMT1A
Treated
Treated
Western blot
PMP22
MPZ
comparison
physiological
pathological
EMG
WT
DAY 1
DAY 3
up to 12 weeks

In vivo

Biodistribution of the k A /9- I in 5 w fo ng



Biodistribution of the therapeutic vector **AAV2/9-CRISPR-dCas9-DMT3A** in PNS 5 weeks following lumbar intrathecal injection



Adapted from Georgiou E. et al., 2023 Molecular Therapy

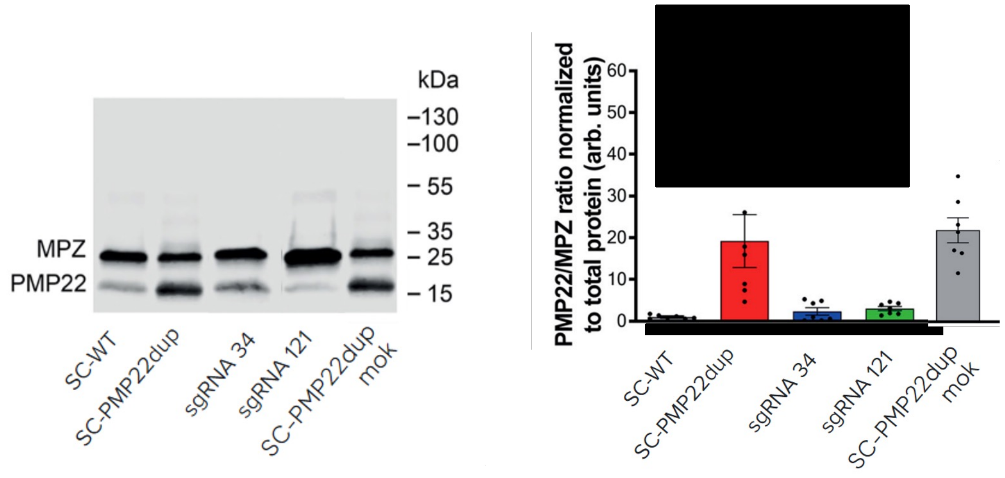
[redacted]

WESTERN BLOT

Myelin protein zero (MPZ):

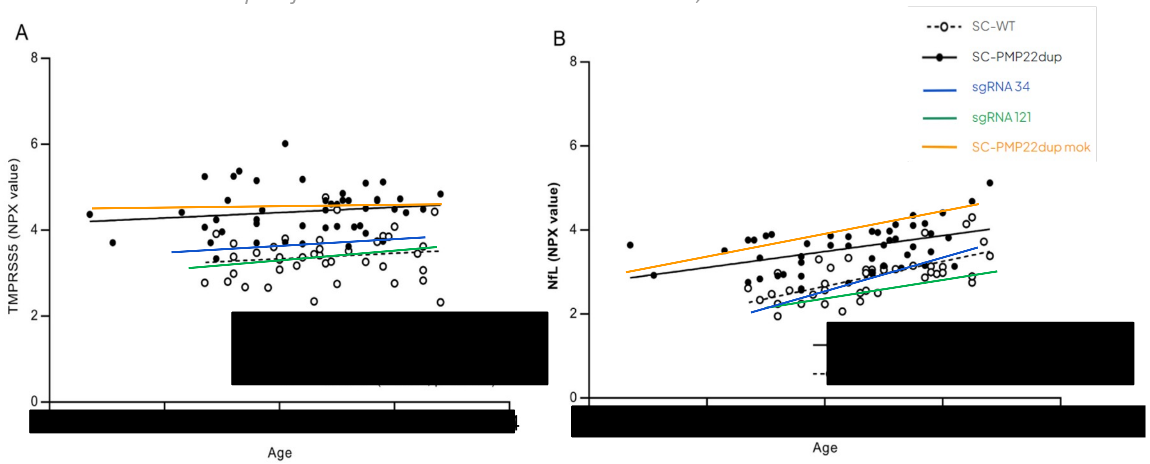
→ expressed by Schwann cells
→ main structural component of the myelin.

Pmp22 was upregulated relative to the myelin marker Mpz in CMT1A, resulting in higher expression of Pmp22



Adapted from Gautier et al. Nature communications, 2021

Blood concentration values
NPX (Normalized Protein eXpression)



High NPX value equals a high protein concentration. Circulating Biomarker:

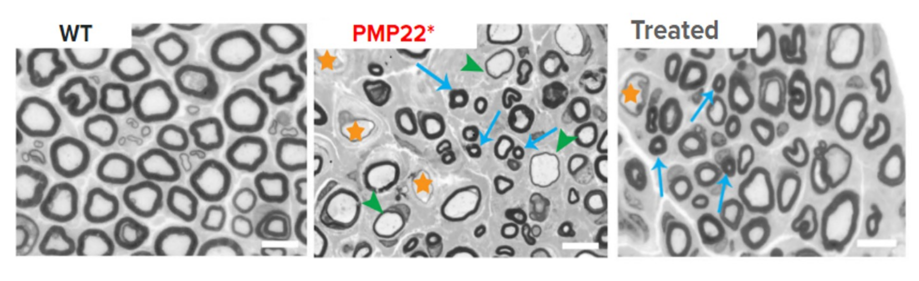
- Nf-L (marker for [redacted]
- TMPRSS5 (biom[redacted]

Adapted from Hongge Wang, et al. 2020

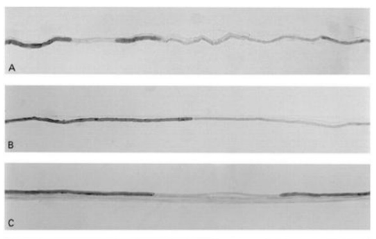
Histological

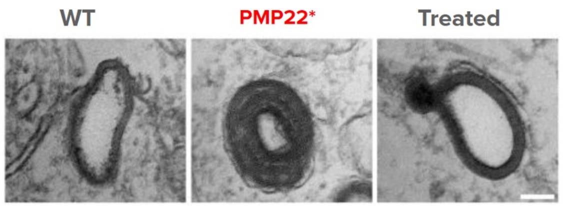
In CMT1A the PMP22 overexpression causes decreased myelination, recovery of axon myelination after treatment

In



★ large demyelinated axons
large hypomyelinated axons
small hypermyelinated axons



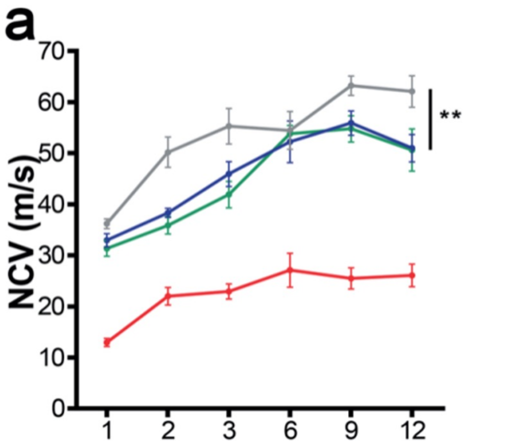


Adapted from Gautier et al. nature communications, 2021

Electromyography

The loss of myelin in CMT1A causes a delay in impulse transmission

After the treatment we can see an axonal recovery of the impulse, due to the correct reformation of the myelin



[redacted]

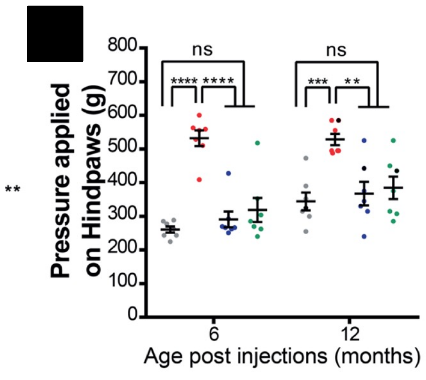
[redacted]

Adapted from Gautier et al. nature communications, 2021

Does the treatment restore motor and sensitive defects?

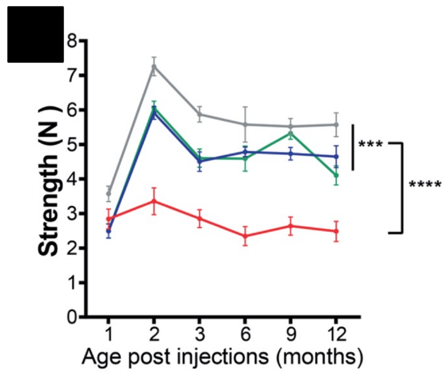
Randall Selitto test

(gram)



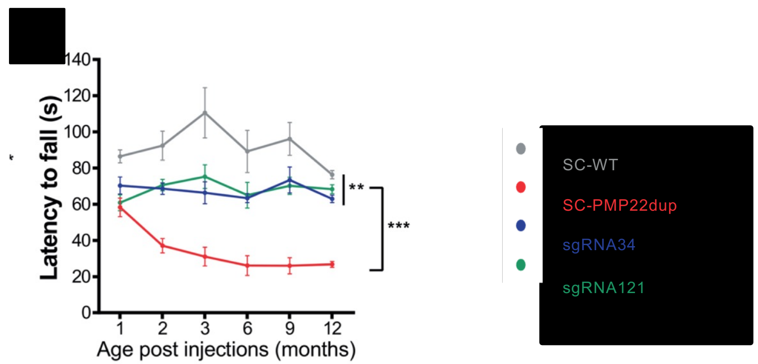
Grip test

(Newton)



Rotarod test

(second)



Adapted from Gautier et al. Nature communications, 2021

Budget and Materials

Materials	Costs
C3 mice PMP22dup Schwann cells + control animals + mice stabulation	595€ (x10) + 110 (x3) + 10.000€
Culture medium supplements (DMEM, FBS, GlutaMAX, Penicillin + Strepromicine, ViraDuctin AAV Transduction Kit)	600€
Packaging plasmid AAV 2/9	600€
MTT Assay Kit (Cell proliferation)	499€
eBioscience™ BrdU Staining Kit for Flow Cytometry FITC	724€
COBRA (validated Methylated Analysis Primer Set, 300 reactions)	745,6€
WB Analysis kit + Antibody (Anti-PMP22, MPZ)	200€
RT-qPCR kit and equipments (Thermo Fisher Scientific)	1200€
Monoclonal Antibody (NF-L; TMPRSS5)	500€
AssayLite Multi-color Conjugated Antibodies Flow Cytometry (FACS Analysis kit)	595€
Immunofluorescence assay (anti-PMP22; GFP)	330€
CRISPR-dCAS9-DNMT3A + 2sgRN + Cas9 protein	3500€
Research team	150.000€/year

TOTAL COST OF 325.773,6€

FOR A TOTAL OF **2 YEARS** OF RESEARCH

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Sapienza
Università di Roma

A Leap Back in Time: Lentiviral Vector-Mediated Expression of WRN Gene in Werner Syndrome

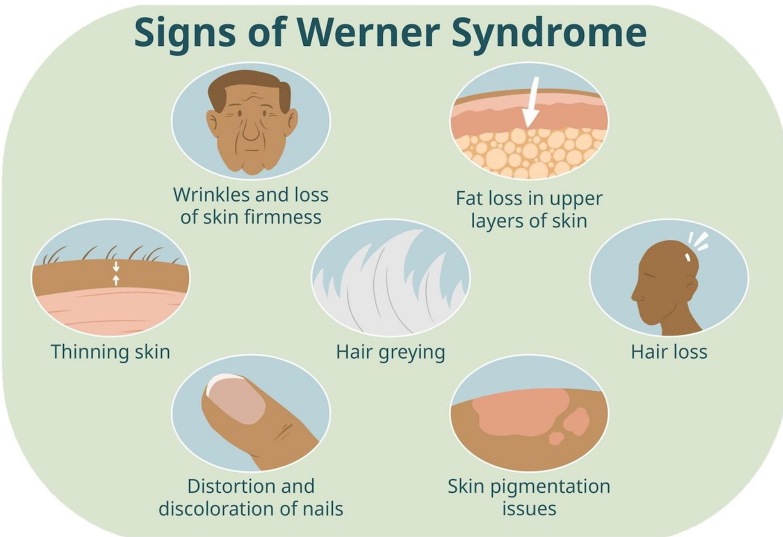
Sara Bartolini, Nicolò Glaudo, Mahsa Hazrati, Chiara Montrone, Raha Pourali

Winter School 2023-24

Background of Werner Syndrome

Rare autosomal recessive genetic disorder.

Loss of function in the WRN gene.



WS patient age 15 yrs | WS patient age 48 yrs

RecQ3: DNA helicase with a 3'→5' exonuclease activity.

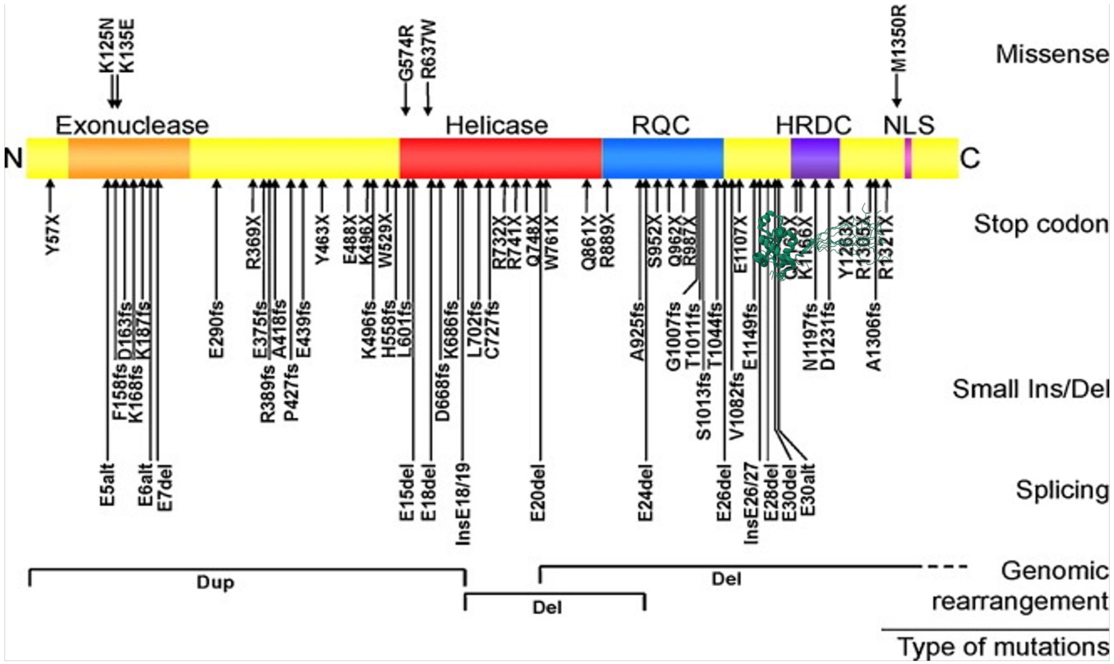
I. genome instability
II. DNA repair, replication, transcription and telomere maintenance
III. age-related diseases

Aim

Providing a functional WRN gene with a lenvi-viral vector as a potential treatment for Werner Syndrome

Why can't we correct the mutation?

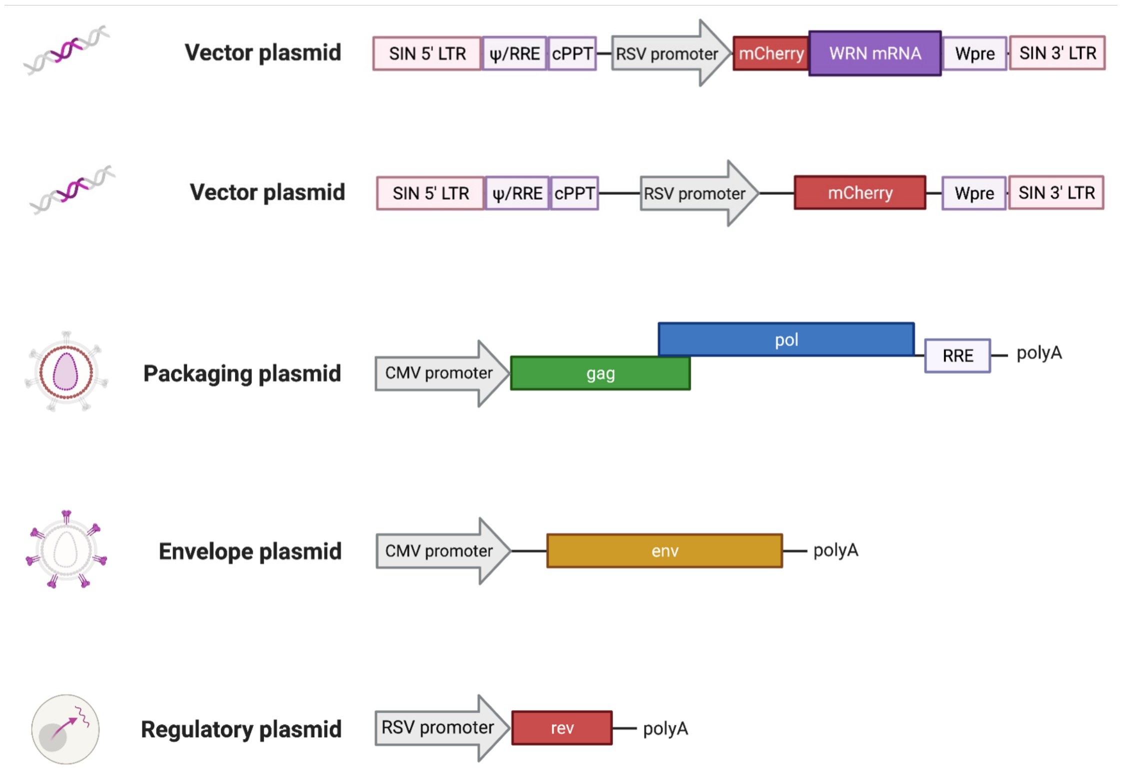
More than 80 different homozygous or compound heterozygous mutations in WRN gene



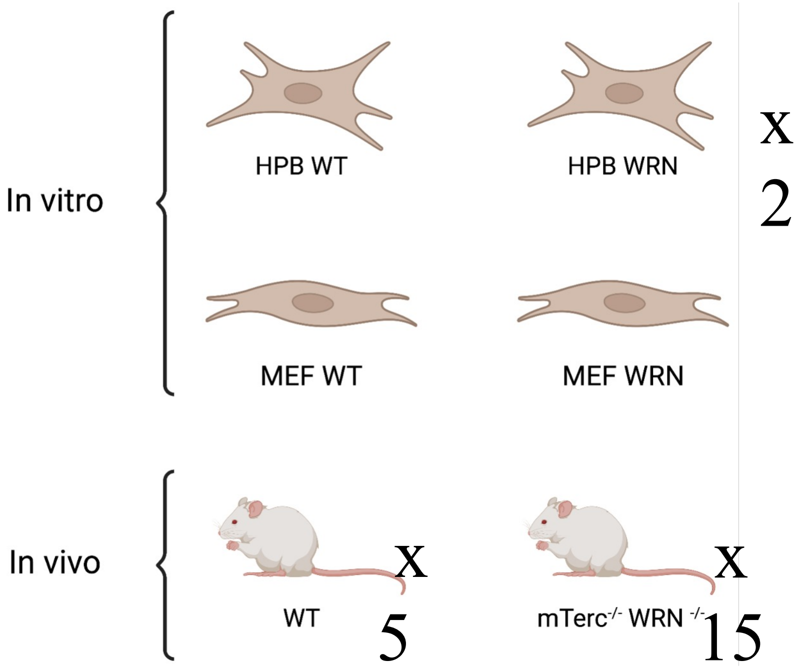
Junko Oshima, Julia M. Sidorova, Raymond J. Monnat, Werner syndrome: Clinical features, pathogenesis and potential therapeutic interventions, Ageing Research Reviews, 2017,

Materials

Third-Generation Lentiviral Vector System



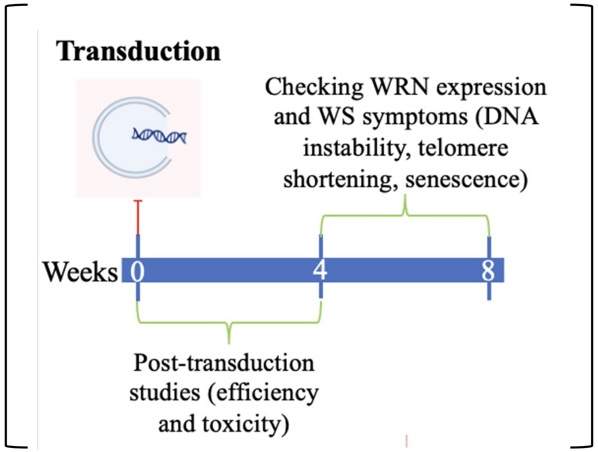
Model Systems



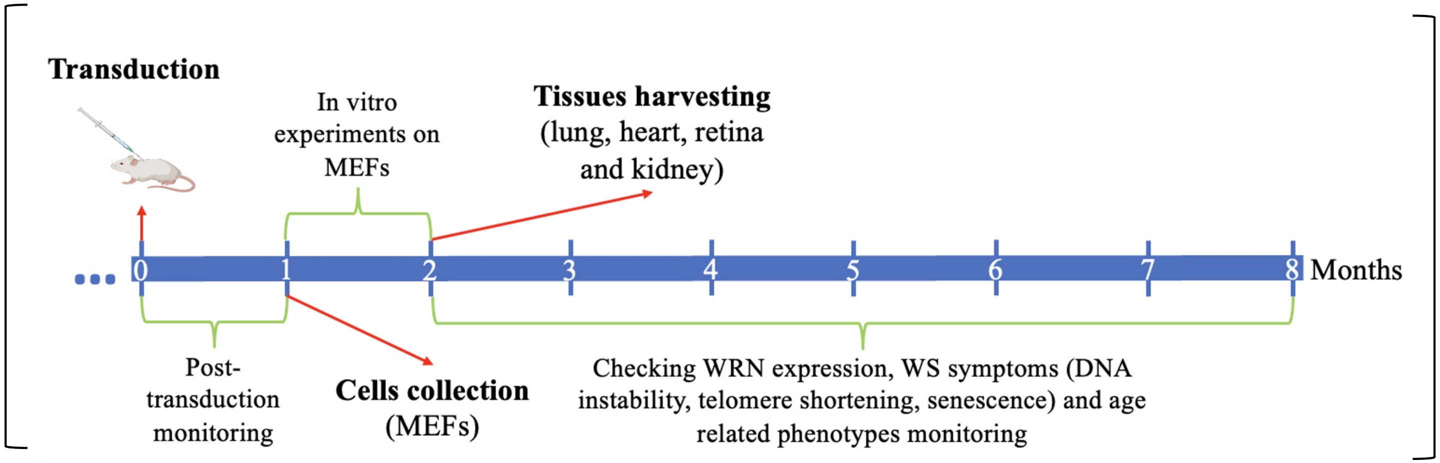
WRN-/- mice do not show WS phenotypes, due to their longer telomeres compared to humans. Instead, mTerc-/-WRN-/- mice shows premature aging phenotypes and heart failure.

Timeline

in vitro



in vivo



mice

- 5 WT
- 15 WRN → 5 WRN / 10 LV-WRN → 5 sacrificed at 2 months / 5 sacrificed at 8 months

In vitro

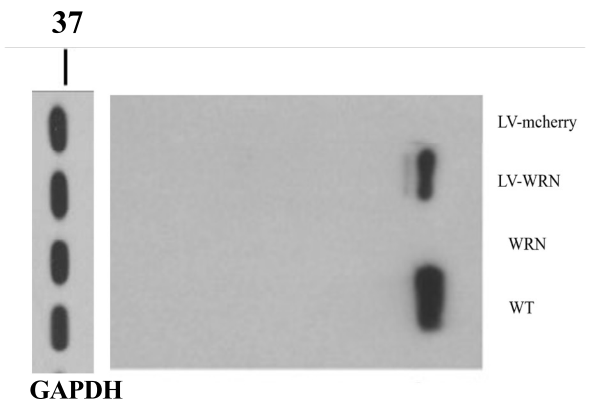
A. Is the transduction successful?

1) WB
2) IF
3) rt PCR

A. Is the treatment cytotoxic?

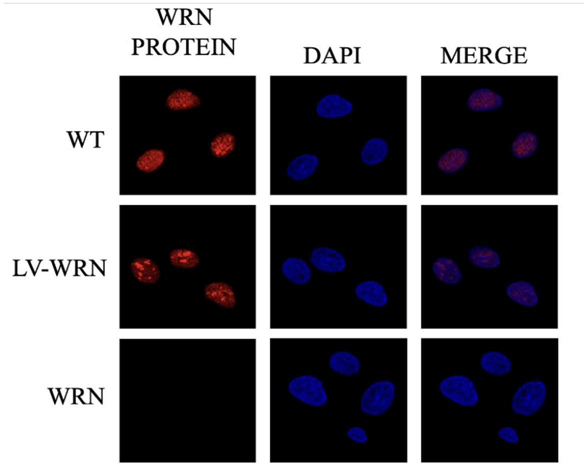
MTT assay

A.1)



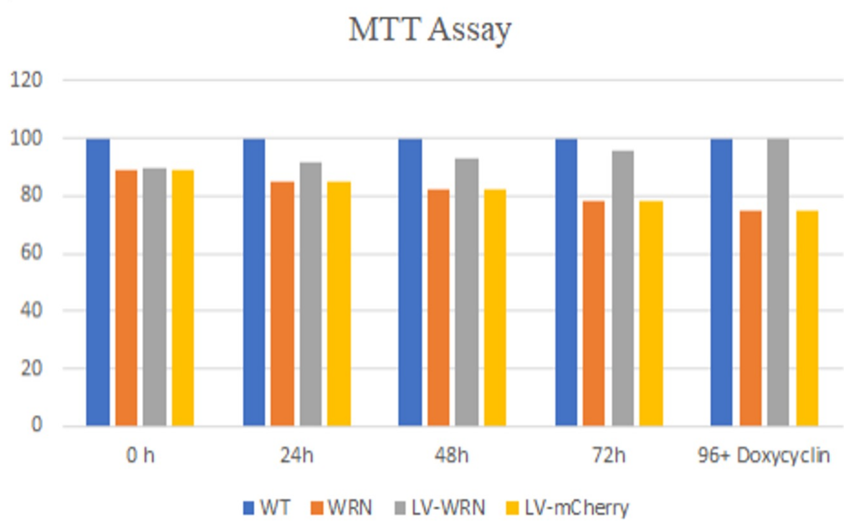
Adapted from Opresko et al, 2002.

A.2)

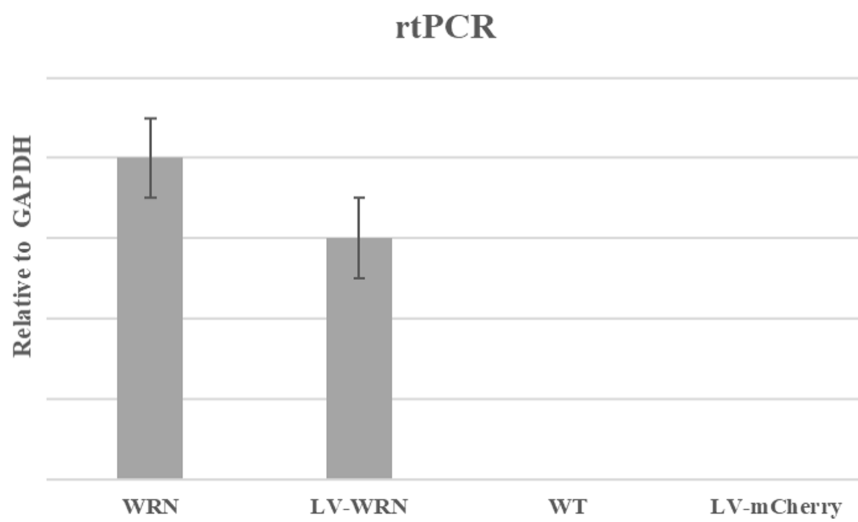


Adapted from Sadahira et al, 2014.

B)



A.3)

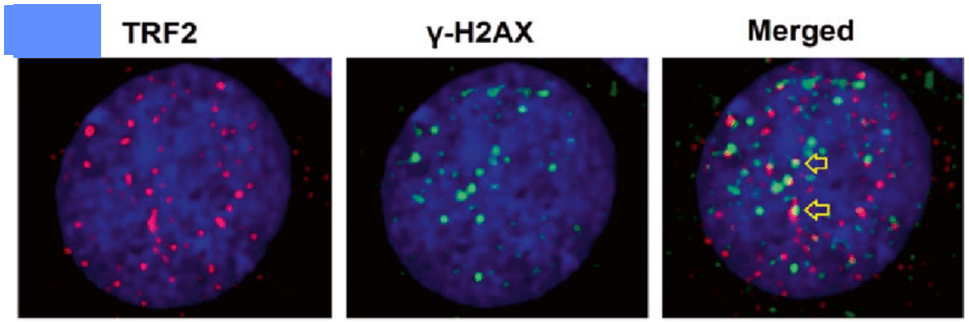


In vitro

C. Telomere length:

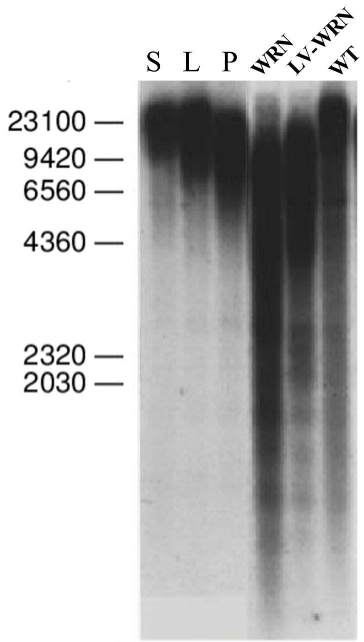
1) FISH
2) TRF
3) Pulse Field Electrophoresis

C.1)



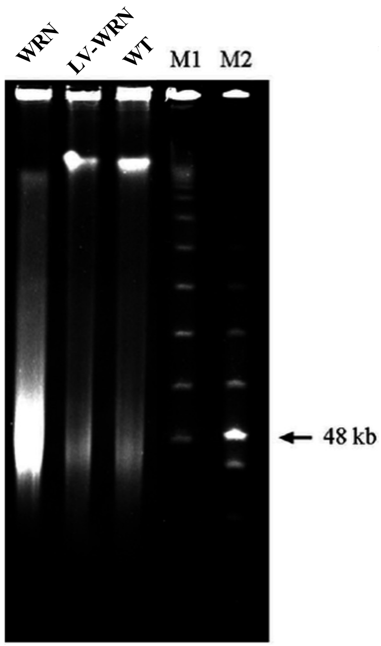
Adapted from Kroustallaki,2015

C.2)



Adapted from Tahara et al, 2017.

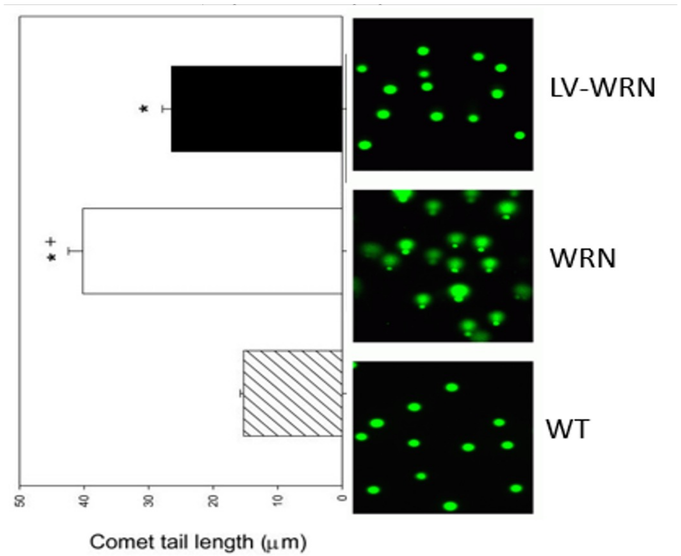
C.3)



Adapted from Ribas-Maynou et al, 2012.

D.

D)



Adapted from Guo et al, 2017.

In vitro

E. Monitoring the gene expression:

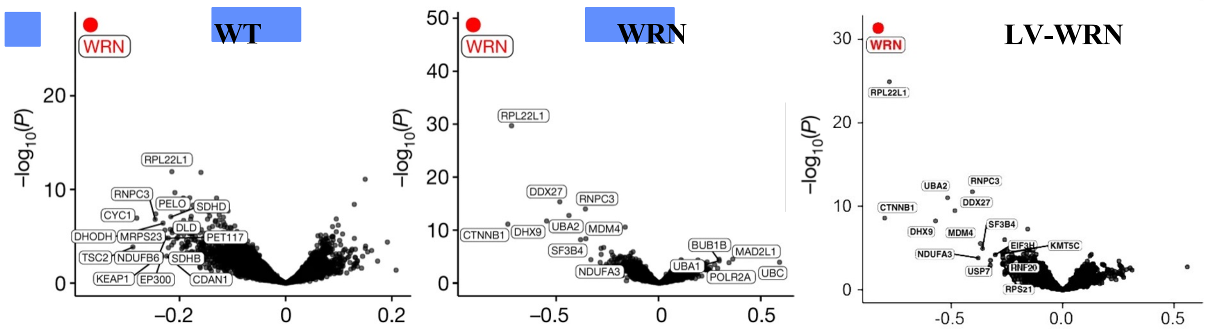
Verify through RNA-seq:

1) Volcano plot
2) Mutation Rate

F. Senescence:

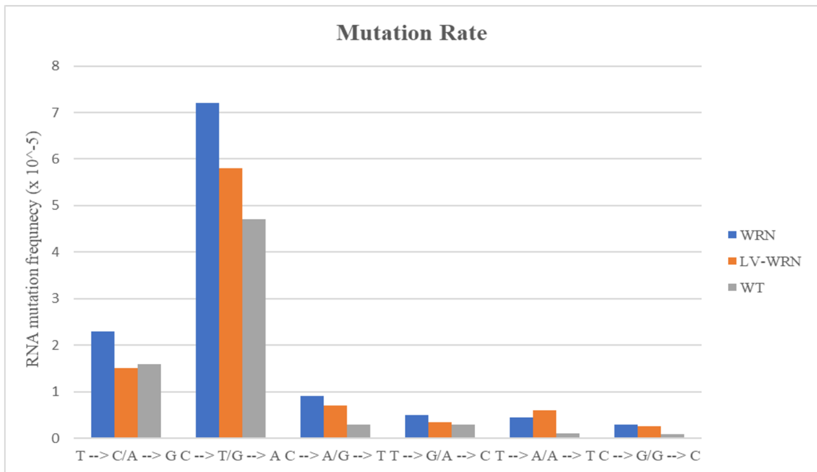
Senescence-associated β-galactosidase (SA-β-gal) Staining

E.1)

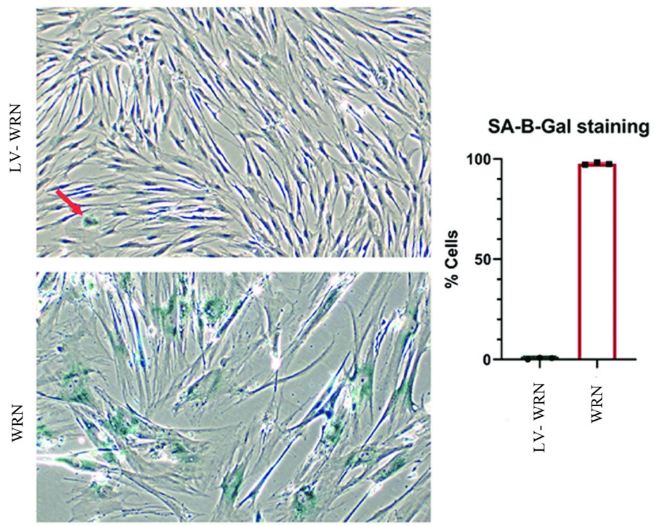


Adapted from Chan et al, 2019.

E.2)



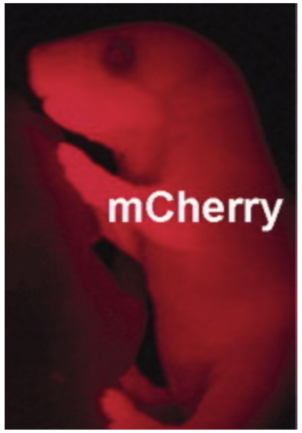
F)



Adapted from Neri et al, 2021.

In vivo

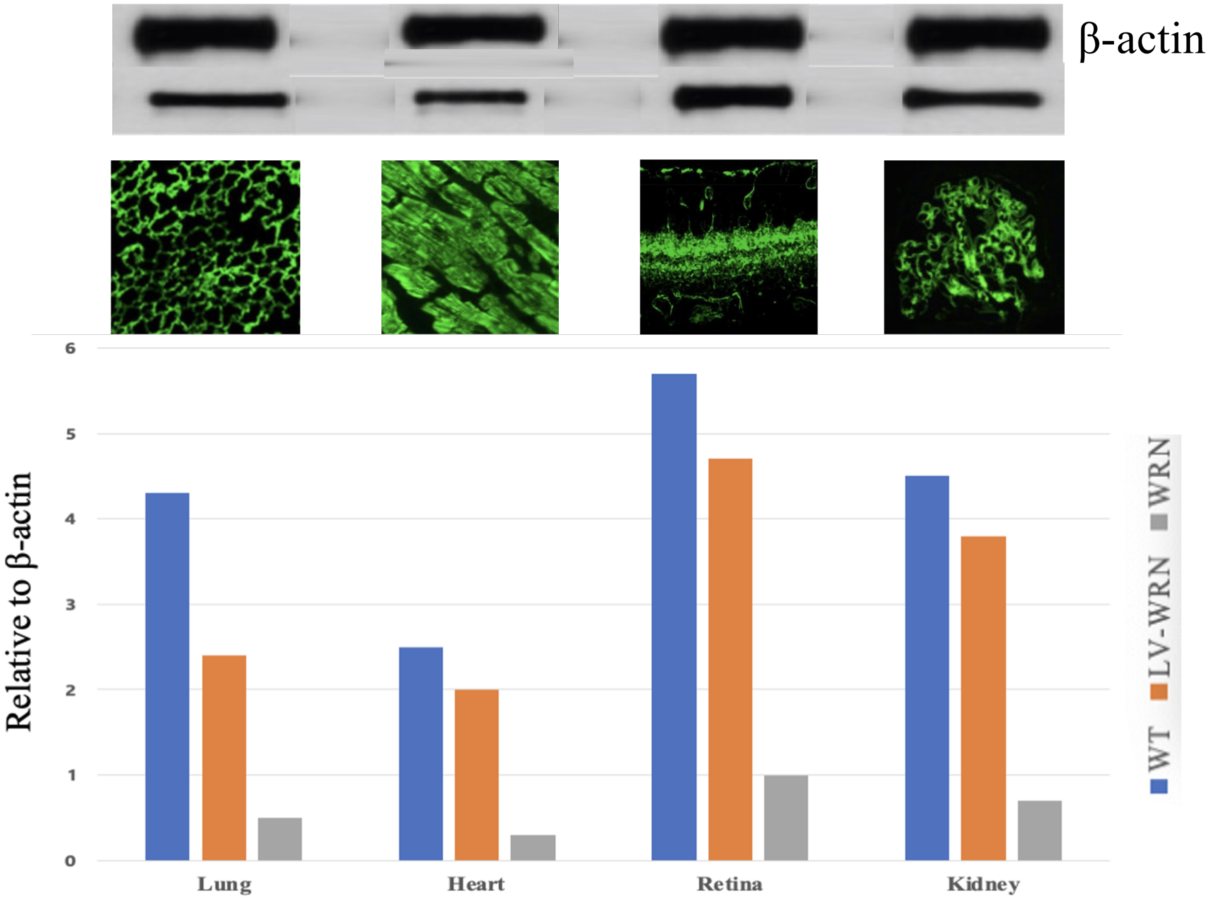
Does the vector effect all the organs?



Adapted from Fink et al, 2010.

Why do we choose the heart as a representative tissue?

Comparing WRN expression
in Lung, Heart, Retina and Kidney
by WB, IF and rt PCR

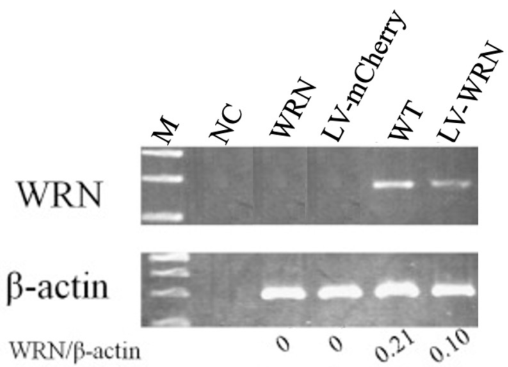


Adapted from Liu et al, 2014.

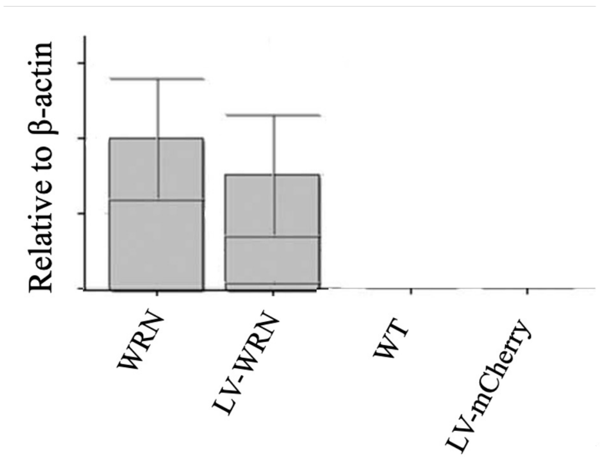
In vivo on Heart

A. Is the transduction successful?

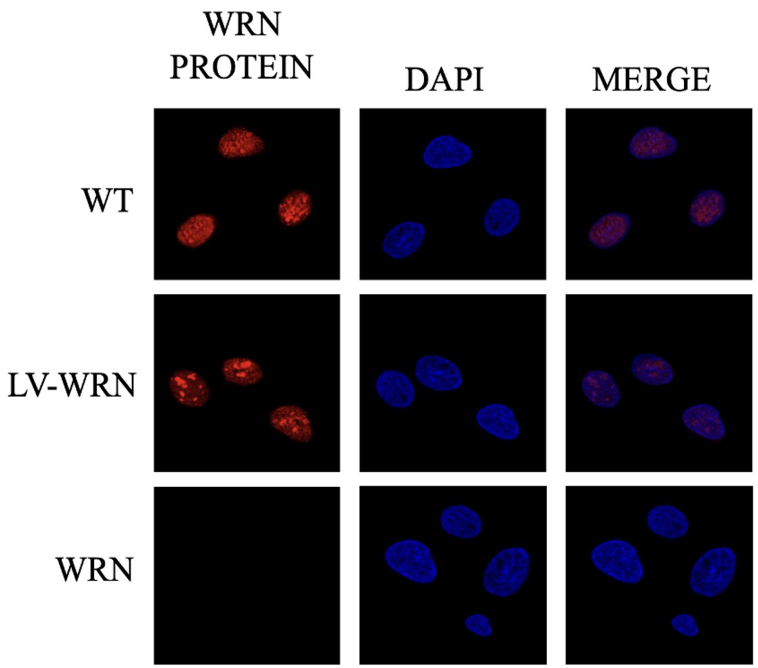
1) WB
2) rt PCR
3) IF



Adapted from Masuda, 2012.



Adapted from Liu, 2014.



Adapted from Sadahira et al, 2014.

A. 1)

A. 2)

A. 3)

In vivo
on Heart

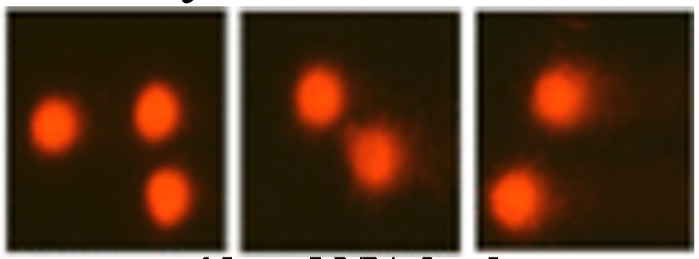
B. Do we see changes in the telomere length?

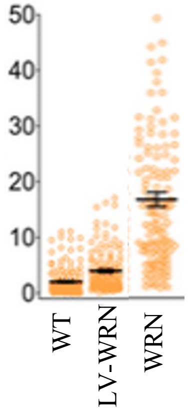
1) pulsed field electrophoresis
2) fluorescence in situ hybridization - FISH
3)

C
)

C. decrease the DNA damage level?

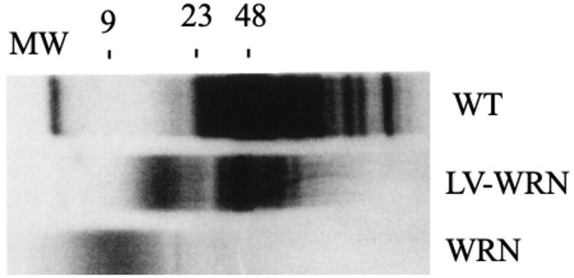
Comet assay





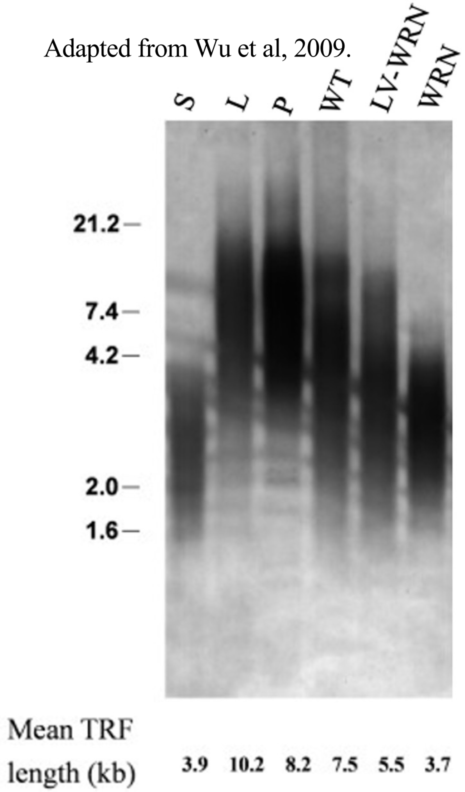
Adapted from Marabitti et al, 2020.

B.
1)



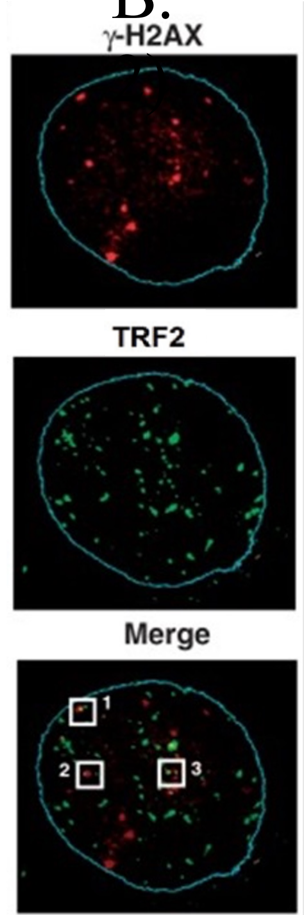
Adapted from Wu et al, 2009.

B.
3)



Adapted from Zhu et al, 2018.

B.



Adapted from Jullien et al, 2012.

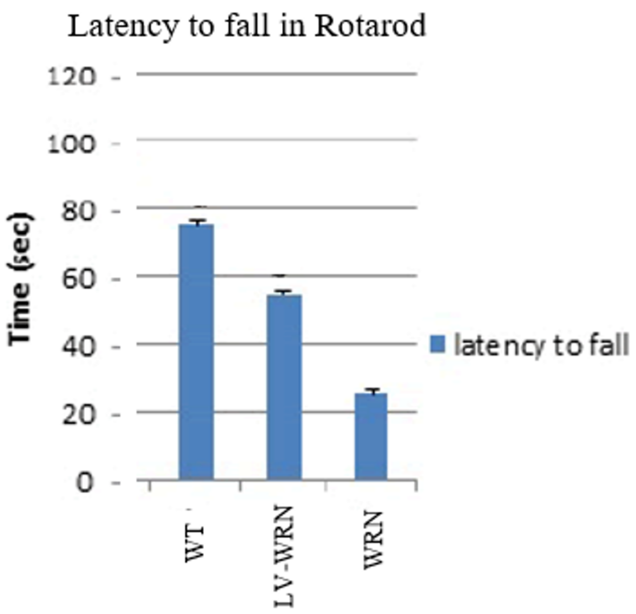
In vivo
on Heart

D. Do we see phenotypic changes?

E. Do we see any changes in behaviour?

Rotarod assay

E)



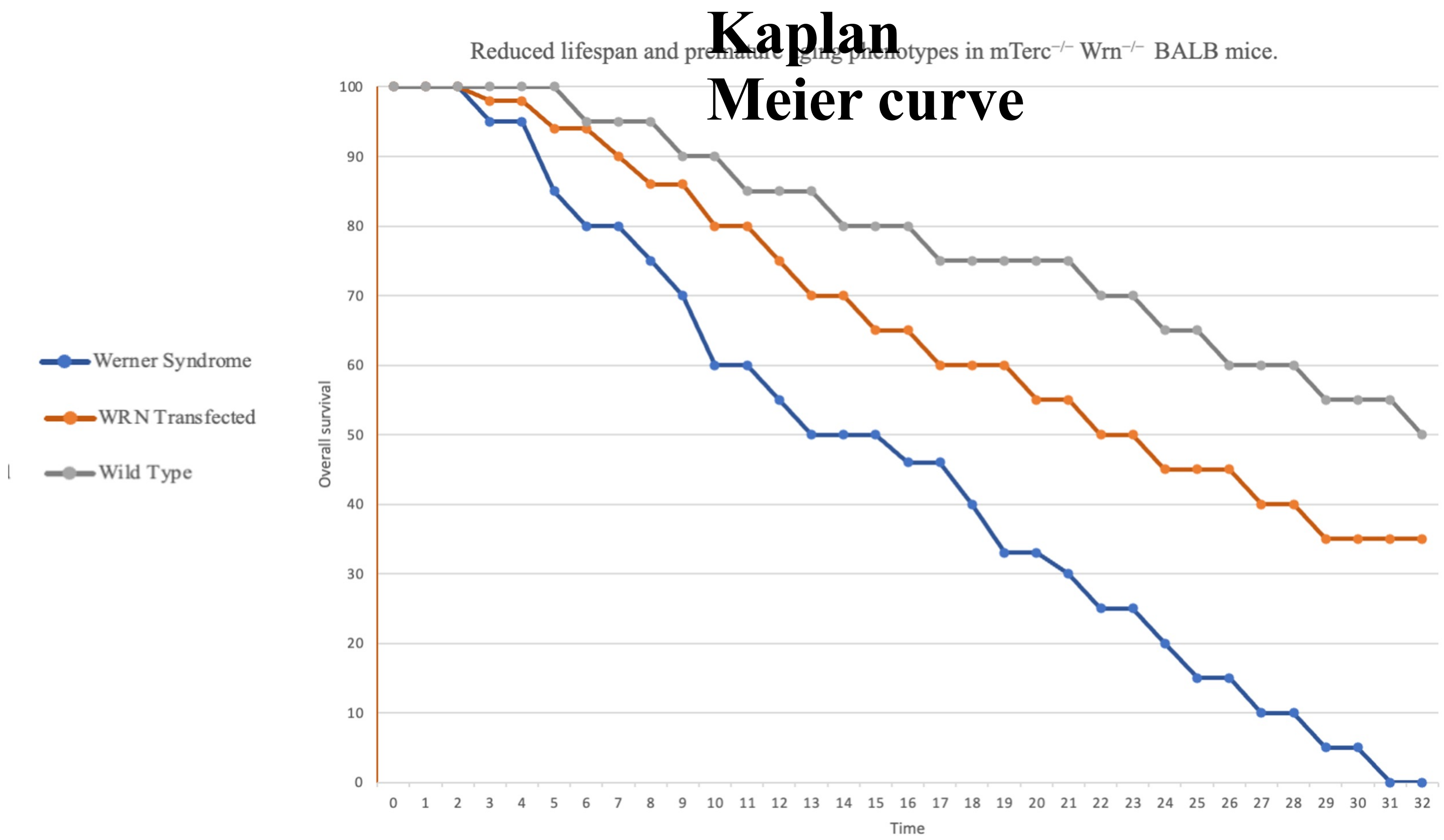
Adapted from Halder et al, 2017.

D)

	WT	LV-WRN	WRN
Glucose tolerance	-	-	++ at 4 months
Insulin resistance	-	-	+ at 4 months
Hair regrowth	-	-	+ at 4 months
Subcutaneous adipose	-	-	++ at 4 months
Gonad mass	-	-	+ at 4 months
Ophthalmic examinations	-	-	+ at 4 months
Voiding spot assay	-	-	+ at 4 months
Urinary Albumin Creatinine Ratio	-	+	+++ from 4 to 8 months
Bodyweight	-	-	++ at 8 months
Lordokyphosis	-	-	+ at 8 months
Osteoporosis	-	-	+ at 8 months
Cataract	-	-	++ at 8 months
Hair graying	-	-	++ at 8 months
Alopecia	-	-	+ at 8 months
Muscle mass	-	-	+ at 8 months
Spleen mass	-	+	+++ at 8 months

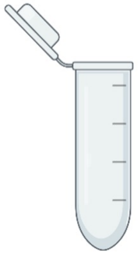
Adapted from Chang et al, 2005.

In vivo

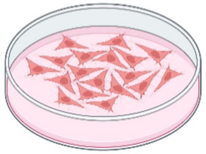

Kaplan Meier curve
Reduced lifespan and premature aging phenotypes in mTerc−/− Wrn−/− BALB mice.
Werner Syndrome
WRN Transfected
Wild Type
Overall survival
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
Time

Budget

Workers: 75K
PostDoc (x1): 30K each
PhD (x3): 15K each



Materials and Methods: 35K



Cell lines: 5K

Mice: 100K

Total: **215K / year**

for 2 years

Pitfalls and Solutions

Budget

Werner patients develop multiple, rare cancers which is the most common cause of death, and has no treatment at the moment.

Translatability between human patients and animal models.

Additional diagnosis tests for cancer such as Cytogenetic analysis.

Performing the therapy on humans for clinical trial.

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- Tsuge, K., & Shimamoto, A. (2022). Research on Werner Syndrome: Trends from Past to Present and Future Prospects. In *Genes* (Vol. 13, Issue 10). MDPI. https://doi.org/10.3390/genes13101802

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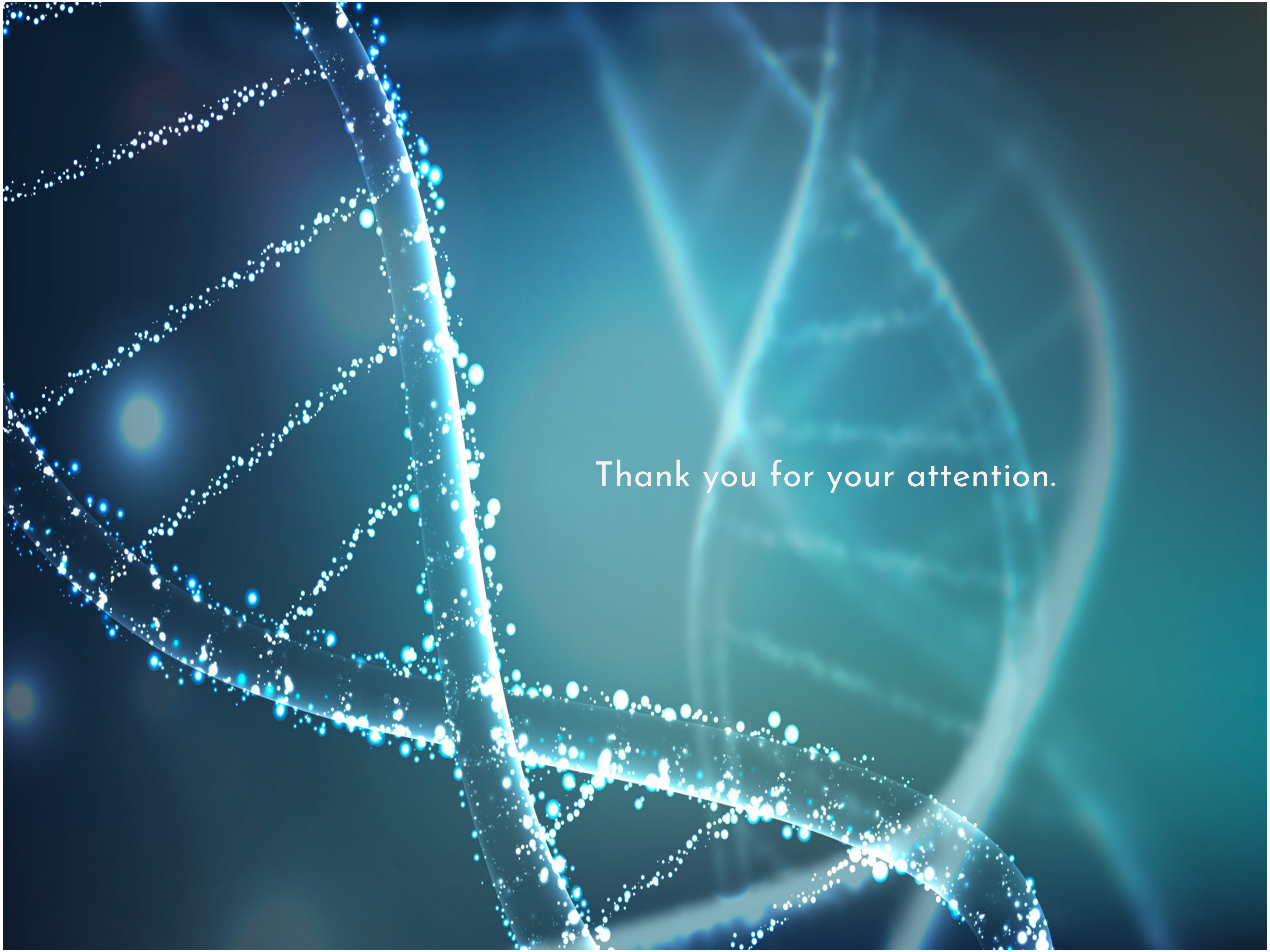
- Yamashita, M., & Emerman, M. (2006). Retroviral infection of non-dividing cells: Old and new perspectives. In *Virology* (Vol. 344, Issue 1, pp. 88–93). https://doi.org/10.1016/j.virol.2005.09.012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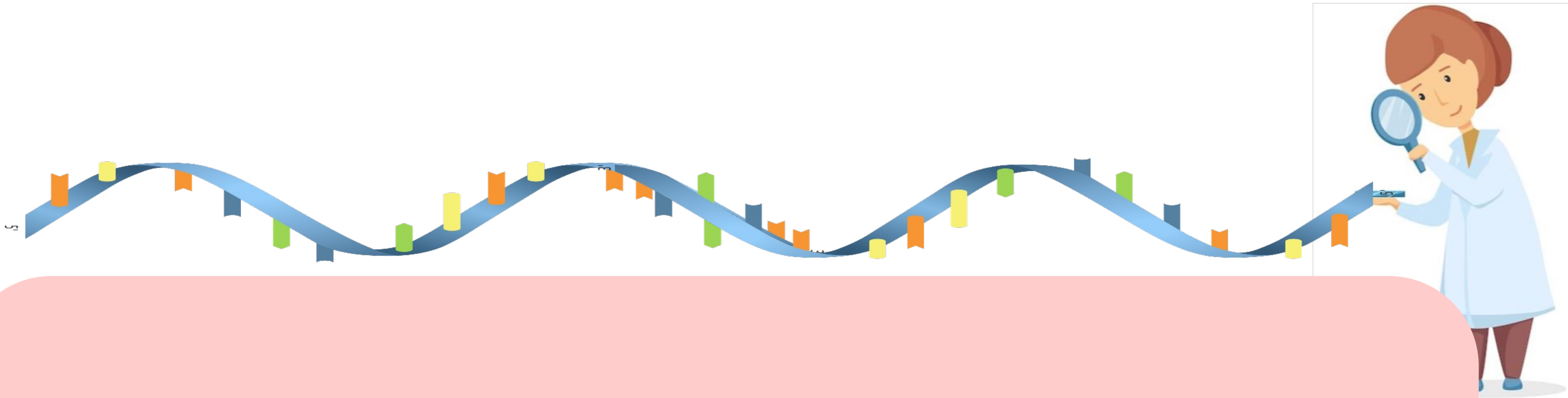
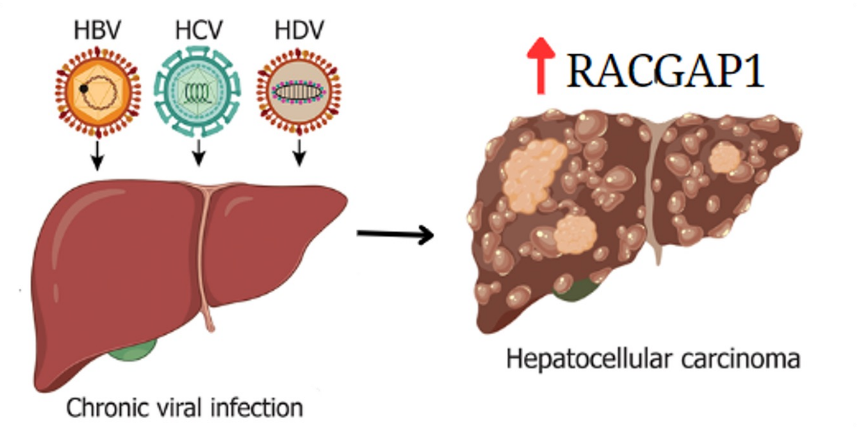


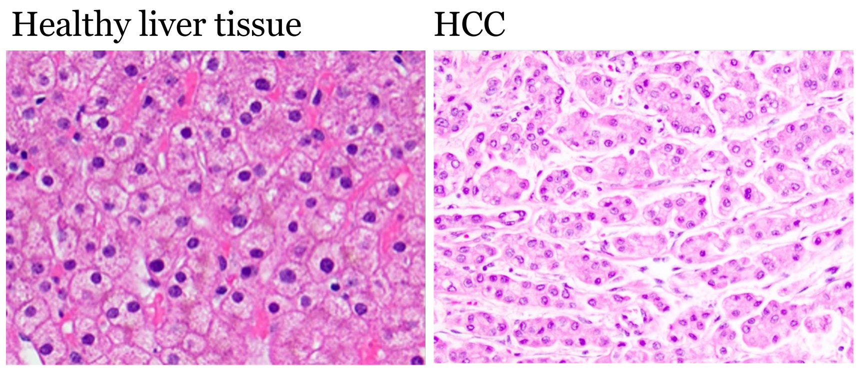
RACGAP1 competitive inhibition in hepatocellular carcinoma via vector-based mRNA transfection

Lavinia Pace
Miriana Santacroce
Ernest Serra
Antonio Duarte
Luigi Fanelli

SAPIENZA
UNIVERSITÀ DI ROMA

Background: Hepatocellular Carcinoma (HCC)



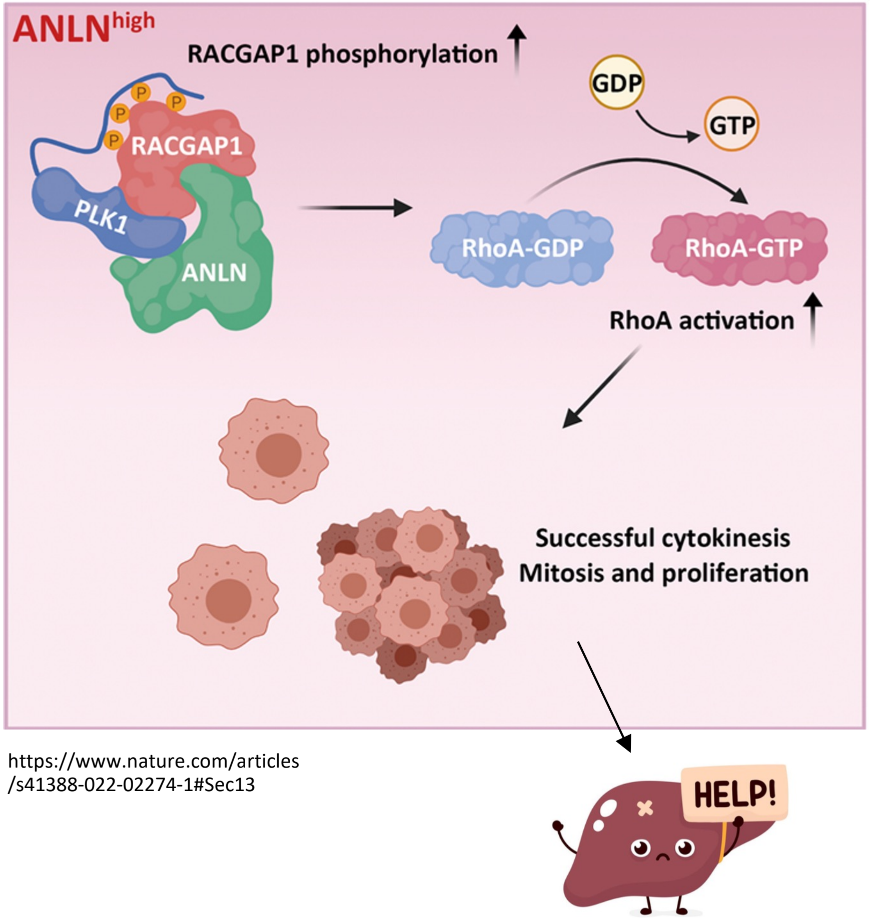


- Liver cancer is the **third most lethal cancer** globally. **Infection by hepatitis B\C** viruses is the main risk factor for HCC development

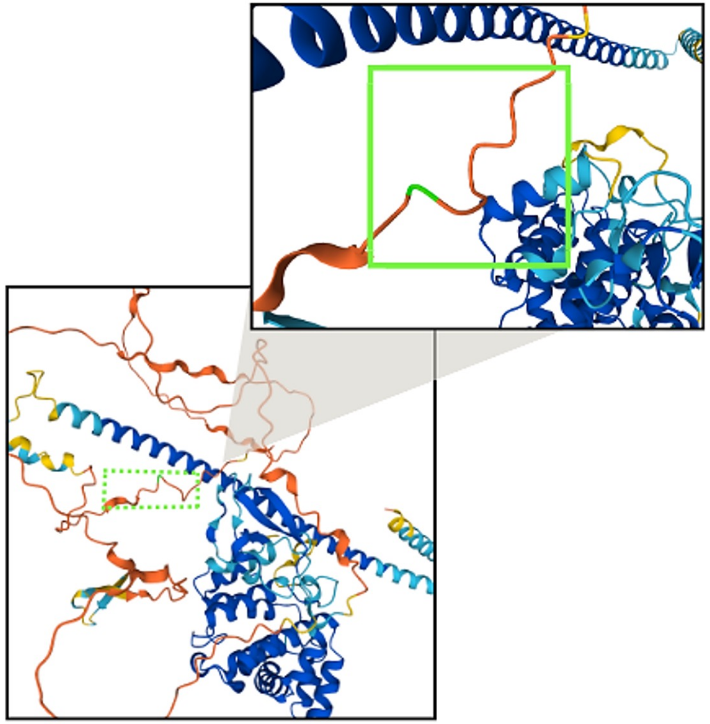
- The median age: > **60** years

- HCC **recurrence** is significantly associated with **RACGAP1 upregulation**: activation of RACGAP1/Rho/ERK signaling axis

Background: RACGAP1 pathway



https://www.nature.com/articles/s41388-022-02274-1#Sec13



RACGAP1 3D structure via Uniprot

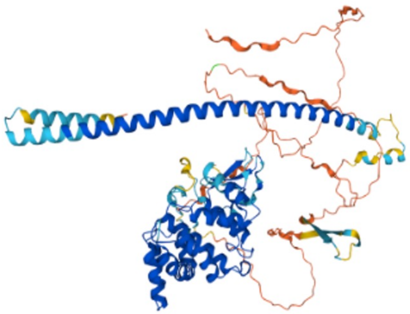
serine 149, 157, 164, 170 phosphorylated by PLK1

Aim of the project

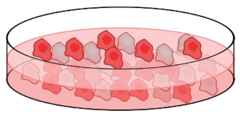
- Induce a **competitive inhibition of RACGAP1** by mutating its phosphorylation sites
- Reduced activation of Rho-A
- Inhibition of self proliferation

How do we do it?

In silico



In vitro



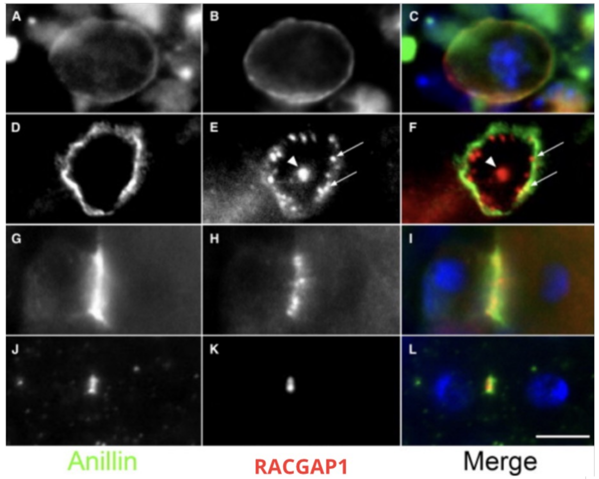
HCCLM3

BALB/c

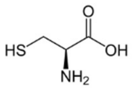
In vivo

In silico

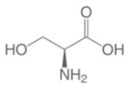
Amino acid modifications: does the aminoacidic change cause any effects on the protein?



RACGAP1 and ANLN location during cell division
https://www.sciencedirect.com/science/article/pii/S0960982207023354



Cysteine



Serine

```
WT          AETERSALDVKLKHARNQVDVEIKRRQRAEADCEKLERQIQLIREMLMCDTSGSIQLSEE   120
modified    AETERSALDVKLKHARNQVDVEIKRRQRAEADCEKLERQIQLIREMLMCDTSGSIQLSEE   119
            ************************************************************

WT          QKSALAFLNRGQPSSSNAGNKRLSTIDESGSILSDISFDKTDESLDWDSSLVKTFKLKKR   180
modified    QKSALAFLNRGQPSSSNAGNKRLSTIDECGSILSDICFDKTDECLDWDSCLVKTFKLKKR   179
            ****************************.*******.******.*****.**********

WT          EKRRSTSRQFVDGPPGPVKKTRSIGSAVDQGNESIVAKTTVTVPNDGGPIEAVSTIETVP   240
modified    EKRRSTSRQFVDGPPGPVKKTRSIGSAVDQGNESIVAKTTVTVPNDGGPIEAVSTIETVP   239
            ************************************************************
```

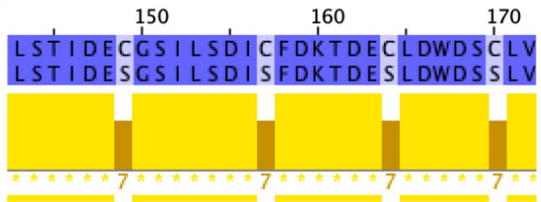
MSA, ClustalW from ebi
https://www.ebi.ac.uk

Intracellular proteins: Aminoacid effect changes on serine

Neutral → Cys (0) Asp (0) Glu (0) Lys (0) Gly (0) His (0) Asn (0) Pro (0)
Gln (0) Arg (0) Ala (0) Thr (0)

http://www.russelllab.org/aas/Ser.html

RACGAP1_HUMAN_MODIFIED_SEQUENCE
sp|Q9H0H5|RGAP1_HUMAN



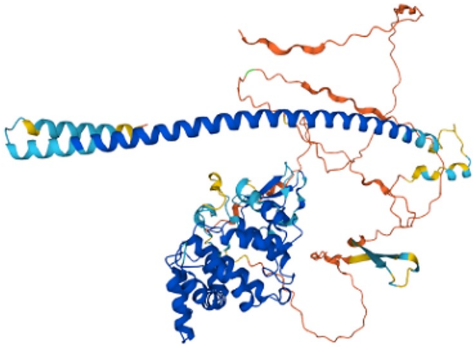
Conservation

Number of conserved physico-chemical properties /10
https://www.jalview.org/help/html/calculations/conservation.html

In silico

Structural predictions

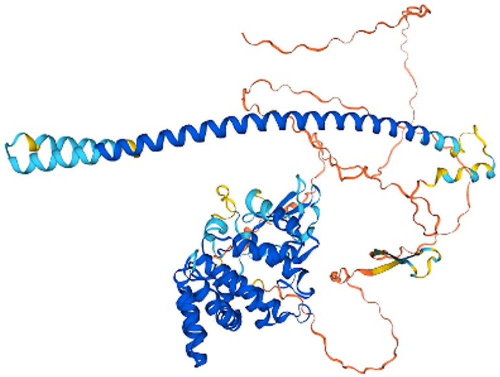
A WT RACGAP1



Structure prediction via alphaphold

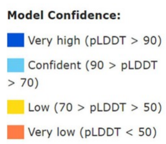
https://www.uniprot.org/uniprotkb/Q9H0H5/feature-viewer

B RACGAP1(UGU)



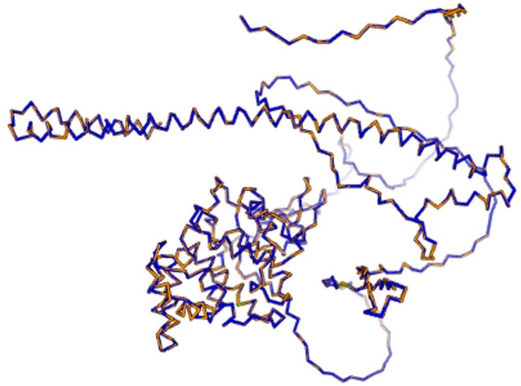
Structure prediction via Swissprot

https://swissmodel.expasy.org/interactive/Xk9YBQ/models/



https://www.uniprot.org/uniprotkb/Q9H0H5/feature-viewer

C WT RACGAP1/RACGAP1(UGU)



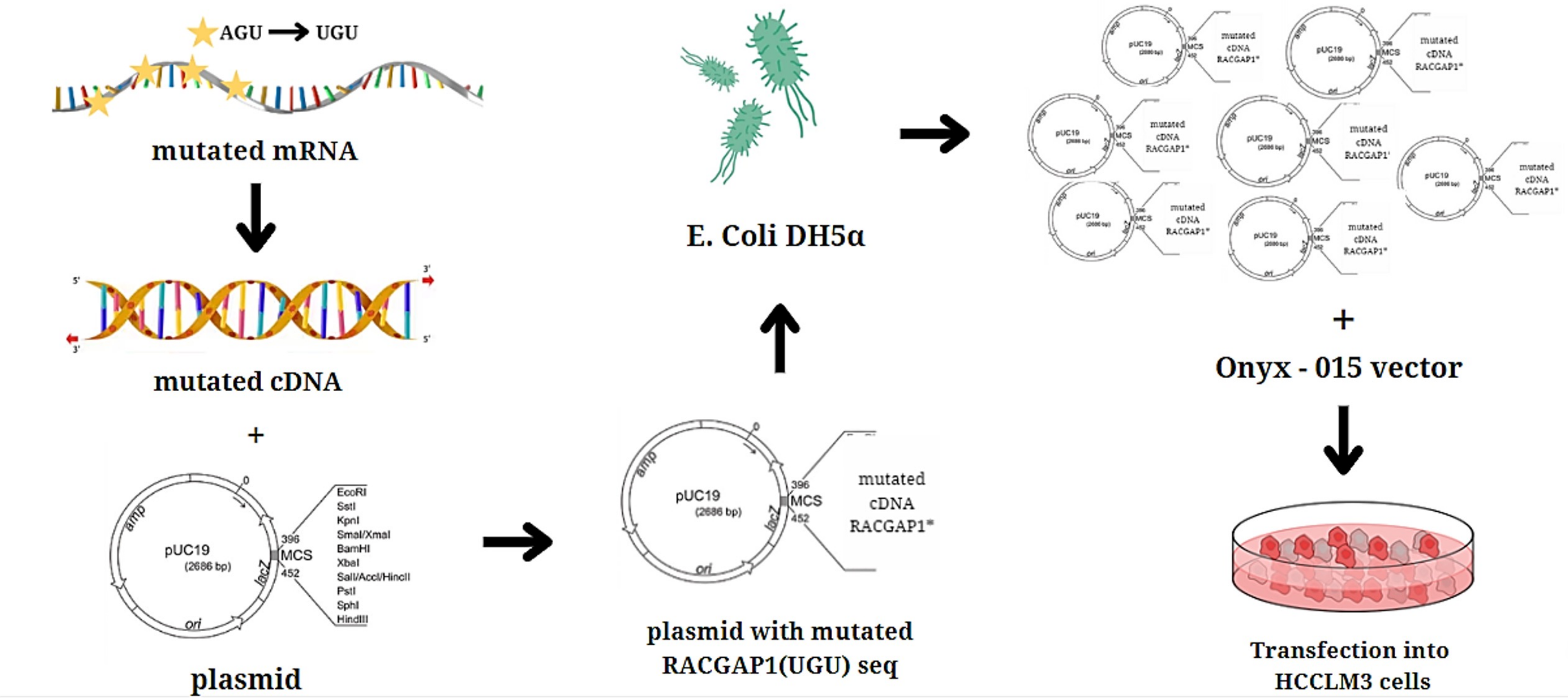
3D structure superposition via DALI

Legend: **Structure conservation**
Dark blue regions are structurally aligned
http://ekhidna2.biocenter.helsinki.fi/dali/DaliTutorial.pdf

Z-score=46.8
Significant similarities' have a Z-score above 2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2639270/

In vitro

Cloning and transfection of RACGAP1(UGU) mRNA

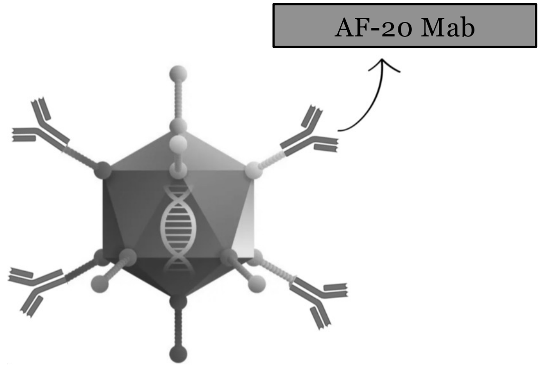


In vitro

How is the vector designed?

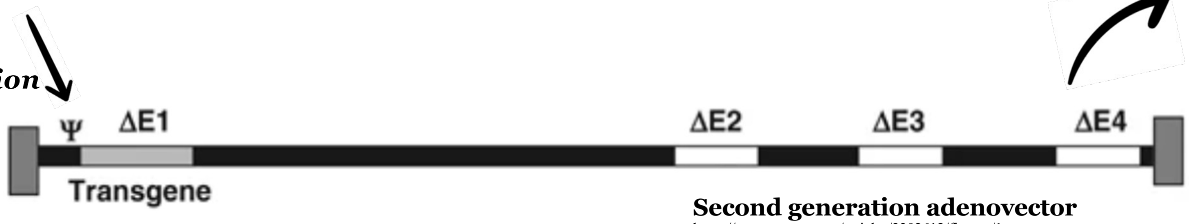
AF-20 Mab specific targeting for HCC
https://www.gastrojournal.org/article/S0016-5085(04)01616-6/fulltext?referrer=https%3A%2F%2Fpubmed.ncbi.nlm.nih.gov%2F

Also specific for HHCLM3 cell line
https://onlinelibrary.wiley.com/doi/10.1002/smll.201702037



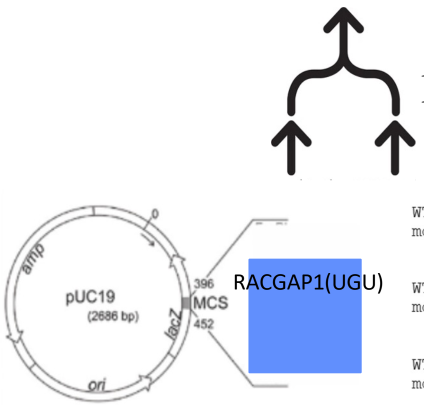
ONYX-015 +

Sequence introduction



Second generation adenovector
https://www.nature.com/articles/3302612/figures/1

Introduction of RACGAP1(UGU)

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WT          AETERSALDVKLKHARNQVDVEIKRRQRAEADCEKLERQIQLIREMLMCDTSGSIQLSEE   120
modified    AETERSALDVKLKHARNQVDVEIKRRQRAEADCEKLERQIQLIREMLMCDTSGSIQLSEE   119
            ************************************************************

WT          QKSALAFLNRGQPSSSNAGNKRLSTIDESGSILSDISFDKTDESLDWDSSLVKTFKLKKR   180
modified    QKSALAFLNRGQPSSSNAGNKRLSTIDECGSILSDICFDKTDECLDWDSCLVKTFKLKKR   179
            ****************************.*******.******.*****.**********

WT          EKRRSTSRQFVDGPPGPVKKTRSIGSAVDQGNESIVAKTTVTVPNDGGPIEAVSTIETVP   240
modified    EKRRSTSRQFVDGPPGPVKKTRSIGSAVDQGNESIVAKTTVTVPNDGGPIEAVSTIETVP   239
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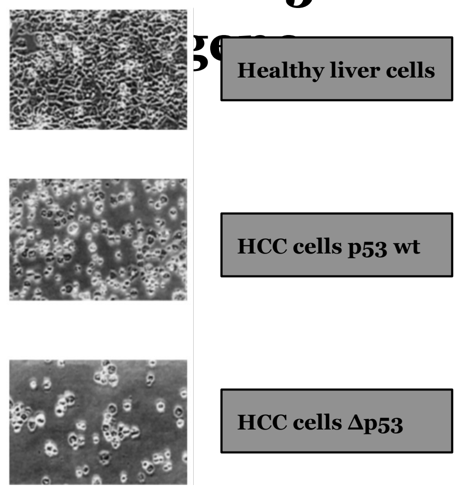
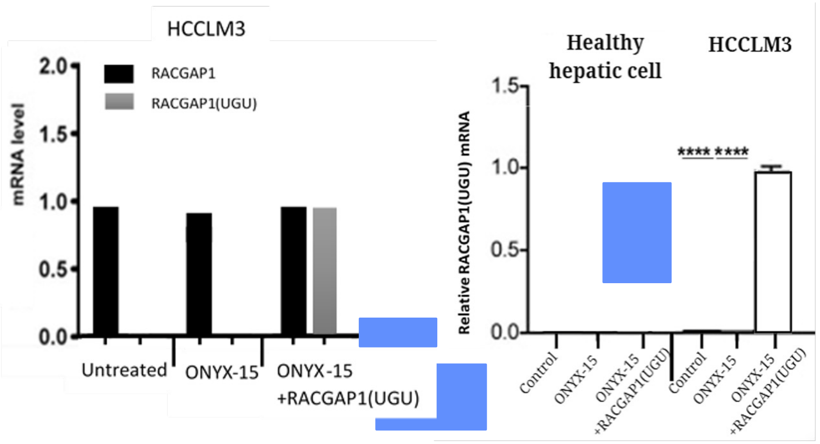


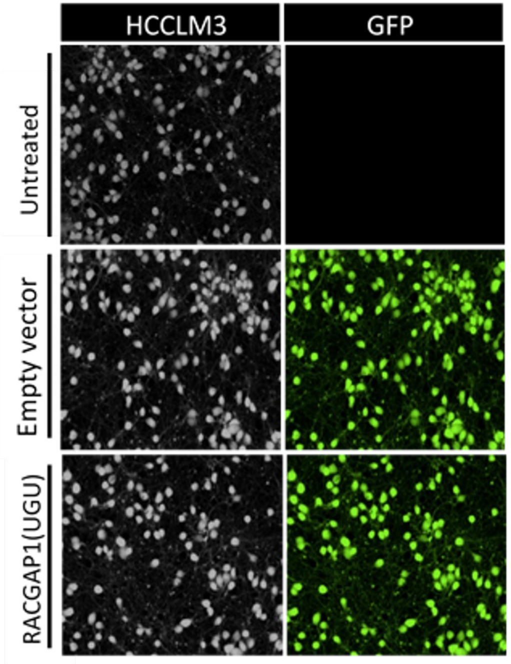
ONYX-015 + RACGAP1(UGU) effect on different cells
https://images.app.goo.gl/86RRRzDvhZAAyWtd6

In vitro

Is RACGAP1(UGU) expressed?

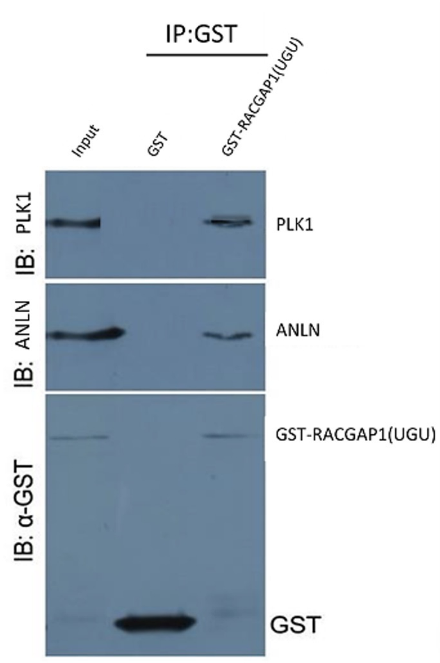


RT-PCR - Expression of RACGAP1 and RACGAP1(UGU) in HCCLM3 cells. Expression of RACGAP1(UGU) in healthy and tumural cell



Immunofluorescence assay-GFP expression in HCCLM3 treated with ONYX-15-GFP and ONYX-15-RACGAP1(UGU)-GFP

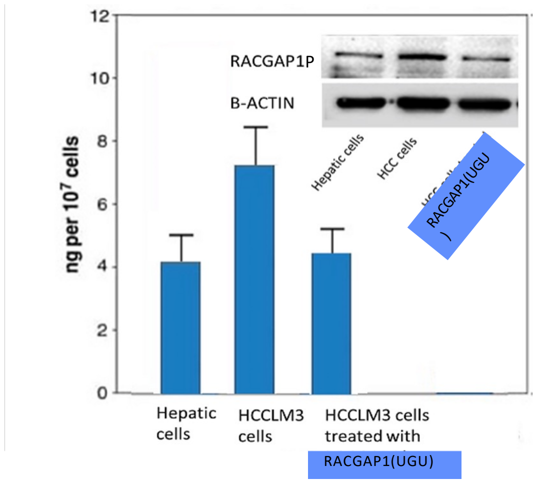
Does the RACGAP1(UGU)-ANLN-PLK1 complex form?



Pull down - RACGAP1(UGU)-ANLN-PLK1 complex formation

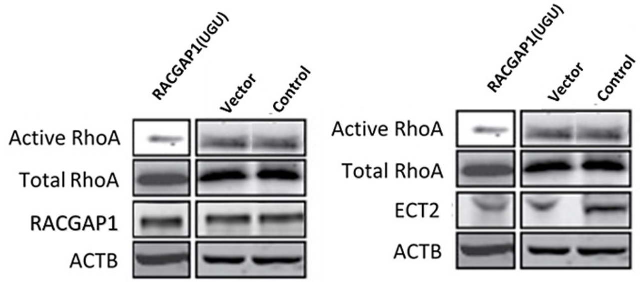
In vitro

Is RACGAP1(UGU) phosphorylated?



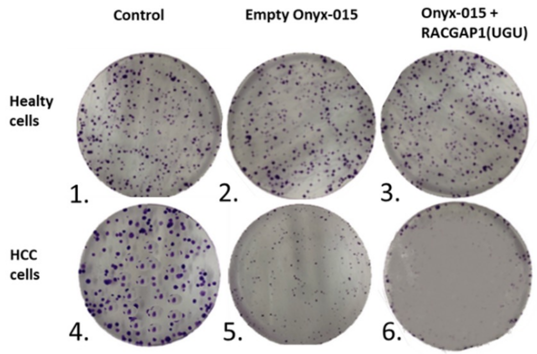
Phosphorylation assay - ELISA and Western Blot - Normal levels of RACGAP1P in healthy cells, elevetated levels of RACGAP1P in HCCLM3, reduced levels of RACGAP1P(UGU) in HCCLM3

Is RohA activity decreased?



Western blot - Detection of RhoA activity and also ECT2 and RACGAP1 expression in HCCLM3 after trasfection of RACGAP1(UGU) Adapted from (Yang et al., 2018)

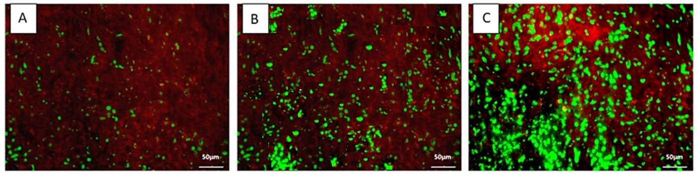
What happens to the cells?

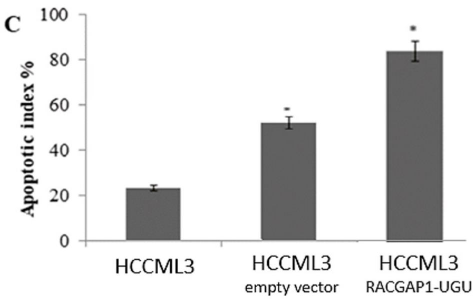


Clonogenic assay:
1. Healthy Hepatic cells,
2. Healthy Hepatic cells with transfection of empty Onyx-015,
3. Healthy Hepatic cells with transfection of RACGAP1(UGU) mutated protein,
4. Hepatocarcinoma HCCLM3 cells,
5. Hepatocarcinoma HCCLM3 with transfection of empty Onyx-015,
6. Hepatocarcinoma HCCLM3 with transfection of RACGAP1(UGU) mutated protein,

In vitro

Does RACGAP1(UGU) cause apoptosis?

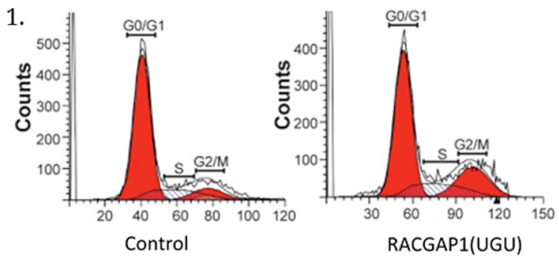


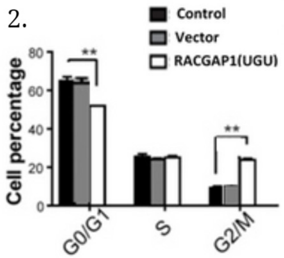


TUNEL assay - **A.** HCCLM3 non treated and no apoptotic cells are detected. **B.** HCCLM3 treated with emptyvector, no apoptotic cells are detected **C.** HCCLM3 treated with the mutated RACGAP1(UGU), incresead levels of apoptotic cells

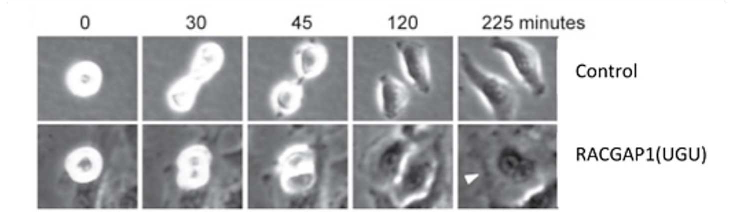
Adapted from
https://www.researchgate.net/publication/335679404_In_Vivo_Anti-Tumor_Effects_of_Citral_on_4T1_Breast_Cancer_Cells_via_Induction_of_Apoptosis_and_Downregulation_of_Aldehyde_Dehydrogenase_Activity

Does RACGAP1(UGU) cause cytokinesis failure?





Cell cycle analysis - **1.** Cell count in different cell cycle phases, RACGAP1(UGU) vs control HCCLM3 cells.
2. Cell percentage in different cell cycle pahses, RACGAP1(UGU) vs control HCCLM3 cells.



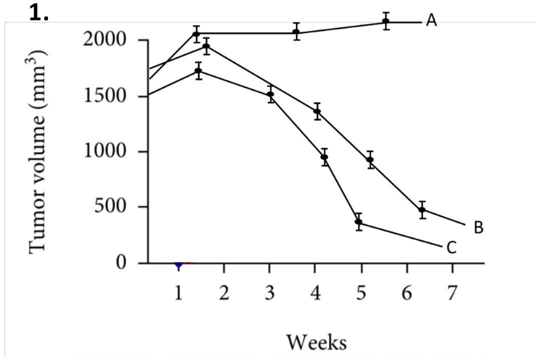
Cytokinesis analysis - Selected frames from time-lapse imaging of RACGAP1(UGU) and control HCCLM3 cells

Adapted from Adapted from (Yang et al., 2018)

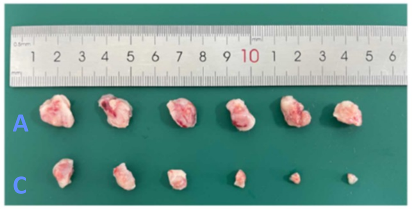
In vivo

Is there a change in the tumor mass?

1.

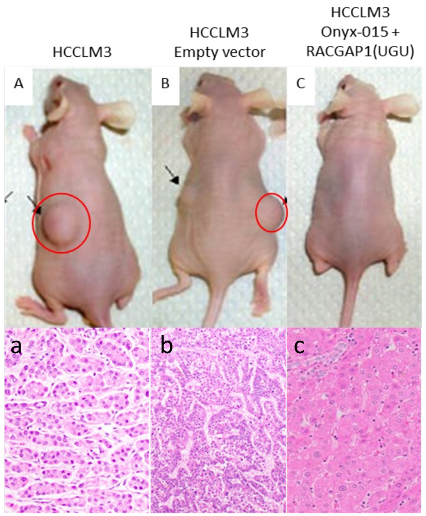


2.



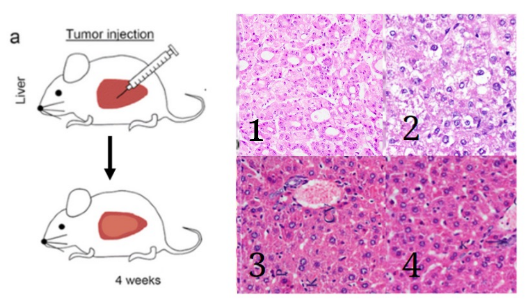
1.Analysis of tumor size on BALB/c mice injected with
A. HCCLM3
B. HCCLM3 treated with empty vector
C. HCCLM3 treated with onyx-015 + RACGAP(UGU)
2.Representative images of tumors removed from BALB/c mice from samples A and C

https://www.hindawi.com/journals/omcl/2022/3034150/



Tumor detection in BALB/c mouse injected with **A.** HCCLM3 cells; **B.** HCCLM3 cells + empty vector; and **C.** HCCLM3 cells + onyx-015 + RACGAP(UGU) and **relative histological samples.**

Adapted from https://bmccancer.biomedcentral.com/articles/10.1186/1471-2407-11-425/figures/7



HCC histological samples from C57BL/6 mouse
1. HCC tissue
2. Tissue sample injected with empty vector
3. Tissue sample injected with RACGAP1(UGU) vector
4. Healthy liver tissue

Adapted from https://pubmed.ncbi.nlm.nih.gov/15649325/

Project budget

Cloning and transfection

mRNA → $5 640 ($10/RNA base)
plasmid → $94
vector for in vitro and vivo → $2670
sequencing → $75
Total → $8479

In vitro

- reverse transcription → $490
- cell line→ $700
- plasmids→ $800
- qPCR → $800
- co-ChIP → $700
- Elisa → $700
- Western blot → $700
- clonogenic assay→ $200
- TUNEL→ $600
- cell cycle analysis→ $350
- **Total→ $6040**

In vivo

4 BALB/c nude mouse per group x3 (36 tot) → $2160
4 C57BL/6 mouse x3 (36 tot)→ $720
Mice maintance → $10 000
Total → $2880

Salaries

3 PhD → $60 000/year
2 post DOC → $ 50 000/year
Total → $110 000/year

Total for three years of work
$357 399

Pitfalls and solutions

What is the problem?		How do we solve it?
Low specificity of infection	→	Enhancement of cancer cell targeting
Overcoming already existing siRNA therapies	→	siRNAs can have off-targets and higher immunogenicity, whilst our protein endogenously exist in human
Possibility of introducing the RACGAP1(UGU) like a drug	→	More specificity towards the target with our therapy, more efficiency with the ONYX-015 coupled therapy