Gene therapy project

29/11/23

Project empowerment

Title

Background

Aim

Materials and methods

Results

Discussion

References

Pitfall and solutions

Budget

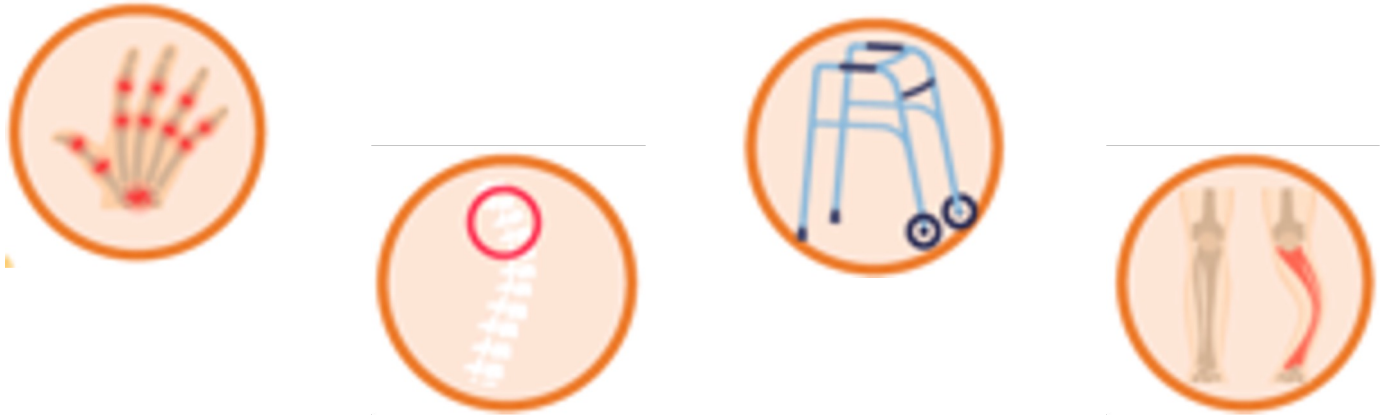
Gene therapy project

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 1A



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

Bastianelli, Bernardi, Colonnelli, Ilie

Background

Peripheral Nervous System Disease	Weakness in lower leg muscles, foot deformities ecc
Incidence of CMT	1/2500 → 80% of which are type 1A
Development	Ageing related
No resolutive treatment available	Only palliative

Molecular basis of the disease

Duplication of *PMP22*

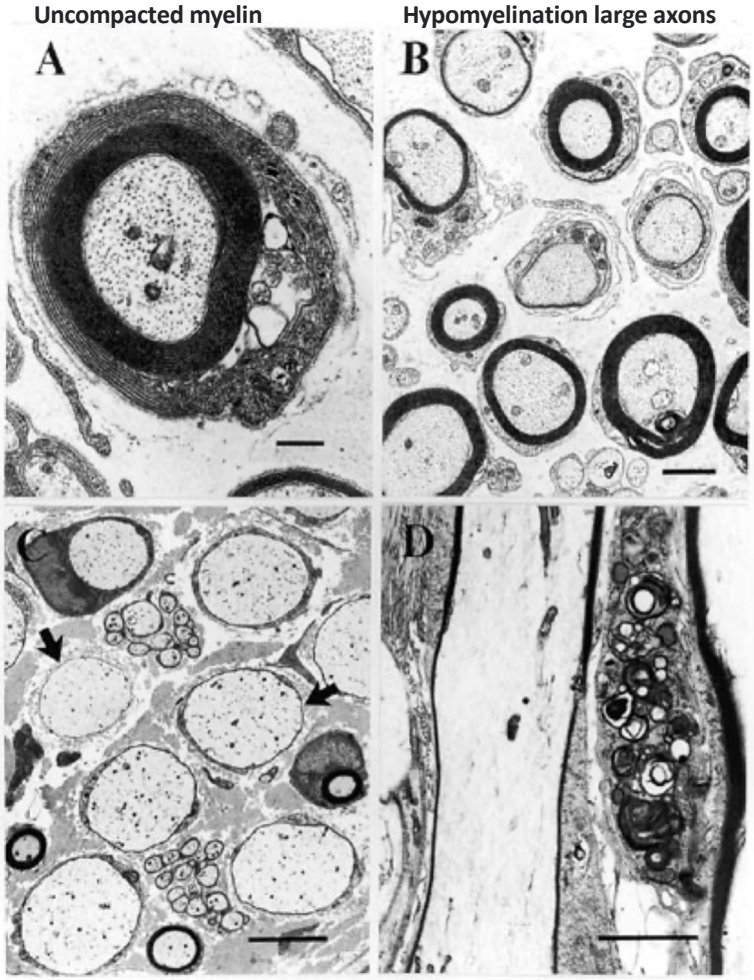
Overload of the Endoplasmic Reticulum (ER)

PMP22 aggregates

Dys and Demyelination

Onion Bulb formation

Secondary axonal degeneration



Huxley et al, Human Molecular Genetics, 1998.

Aim of the project

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

C61 heterozygotic mice

PMP22 Schwann cells*

→ Copy number of *PMP22:* 4

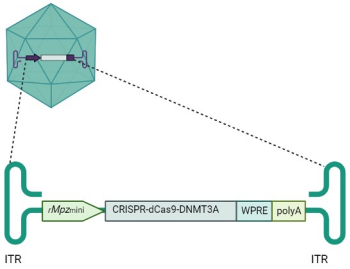
→ MCV: 25 m/s

→ Histology: mid demyelination

AAV 2/9

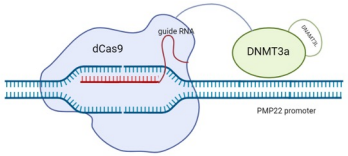
→ MPZ promoter-SC specific

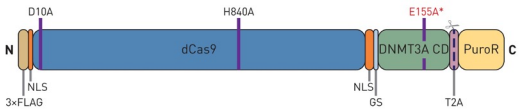
→ Intrathecal lumbar injection



CRISPR-dCas9-DNMT3A

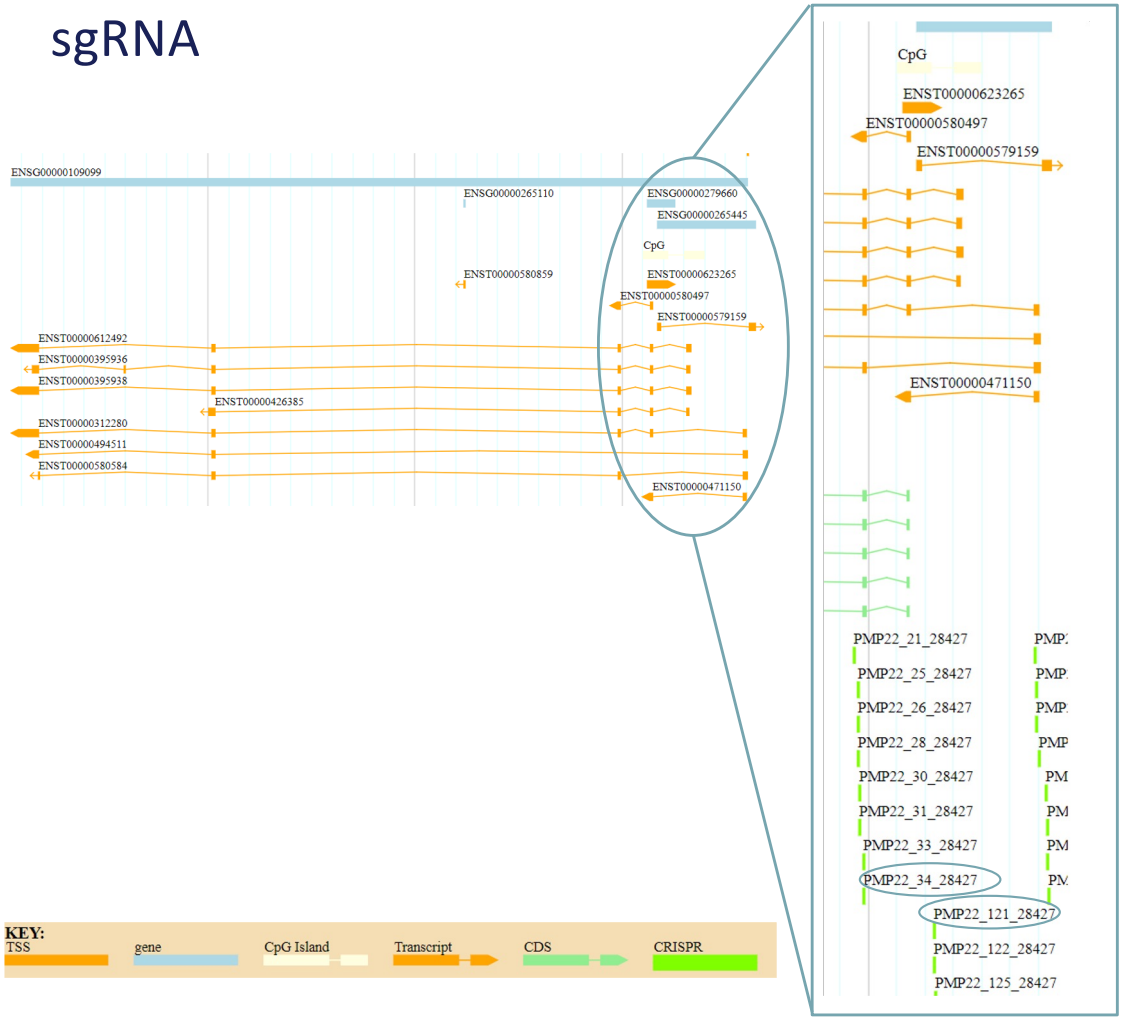
→ 2 sgRNA





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

sgRNA



sgRNA 34

PMP22_34_28427	GTGGACATTTCCTGA GGAAG NGG	S A E	ENSG00000109099::P MP22

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

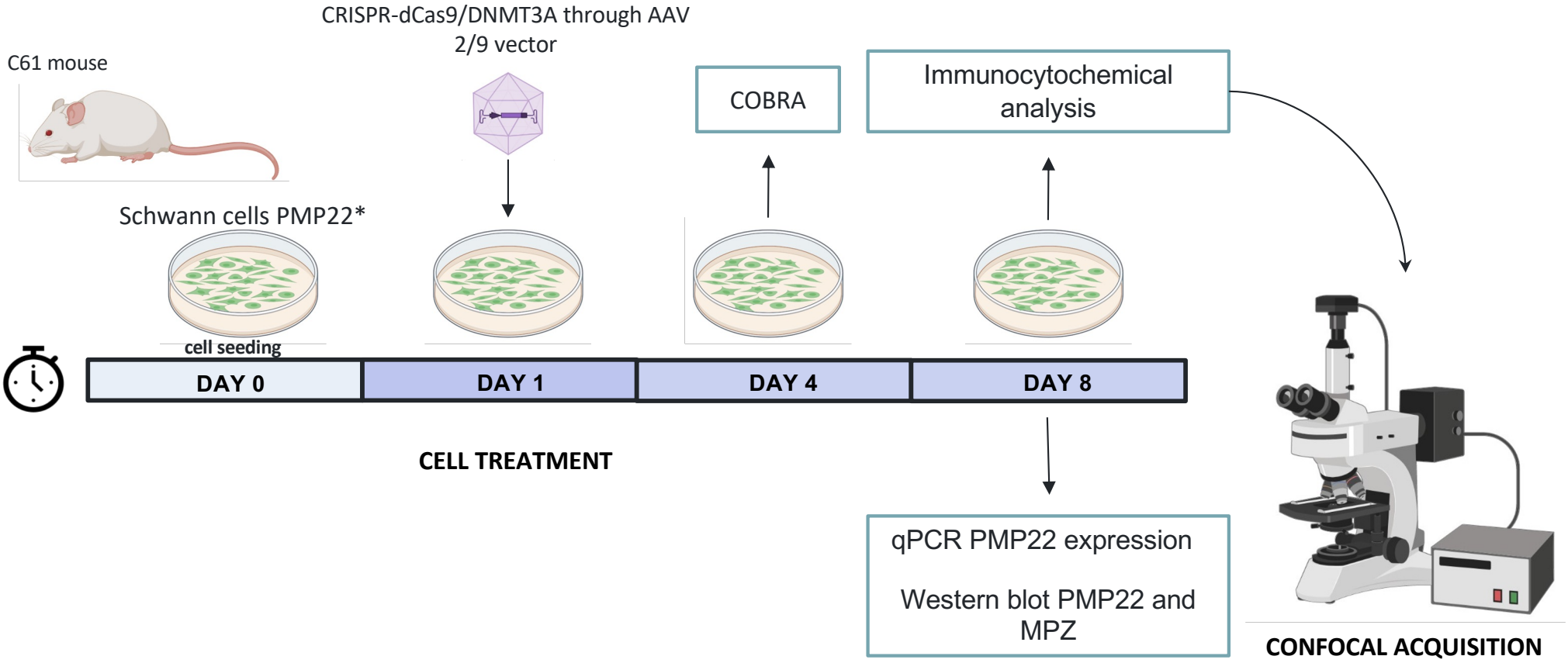
sgRNA 121

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::R P11-849N15.1

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

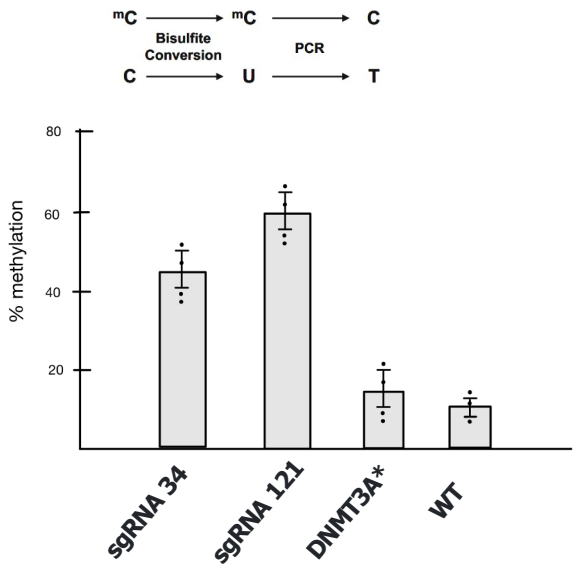
In vitro


CRISPR-dCas9/DNMT3A through AAV 2/9 vector
C61 mouse
COBRA
Immunocytochemical analysis
Schwann cells PMP22*
cell seeding
DAY 0
DAY 1
DAY 4
DAY 8
CELL TREATMENT
qPCR PMP22 expression
Western blot PMP22 and MPZ
CONFOCAL ACQUISITION

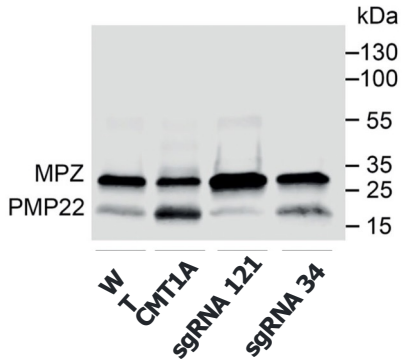
RESULTS: in vitro

Does Methylation downregulate expression?

COBRA

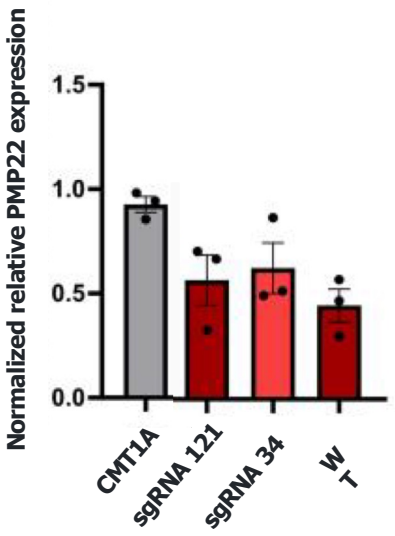


WESTERN BLOT



Adapted from Gautier et al. nature communications, 2021

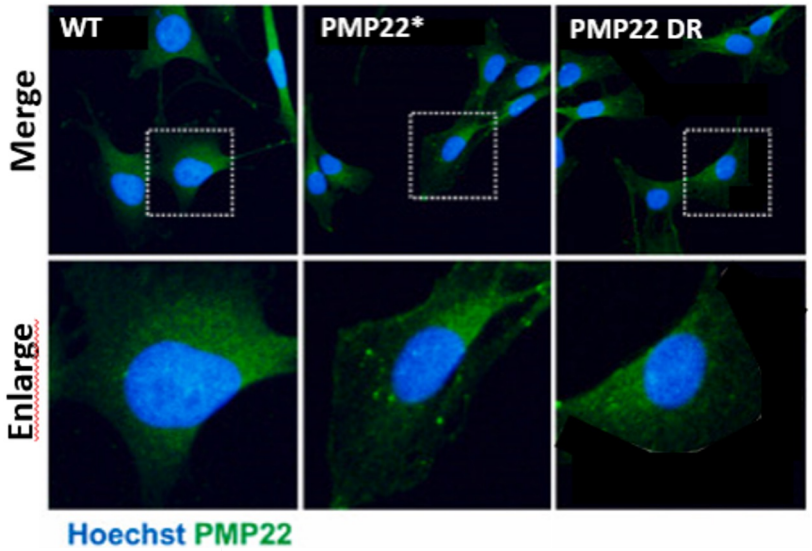
pPCR

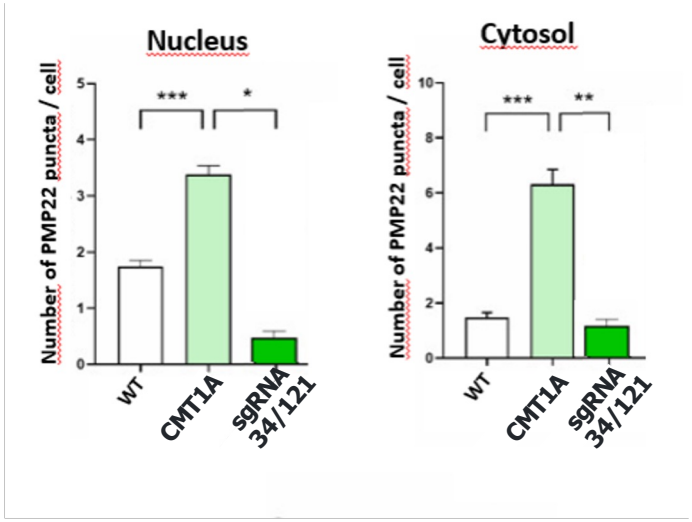


Adapted from Van Lent et al. Brain, 2023

Accumulation of *PMP22*

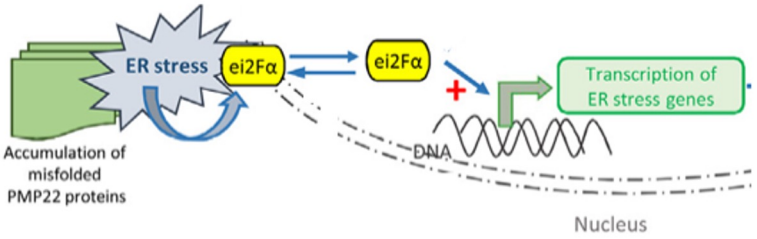
→ PMP22 aggregates

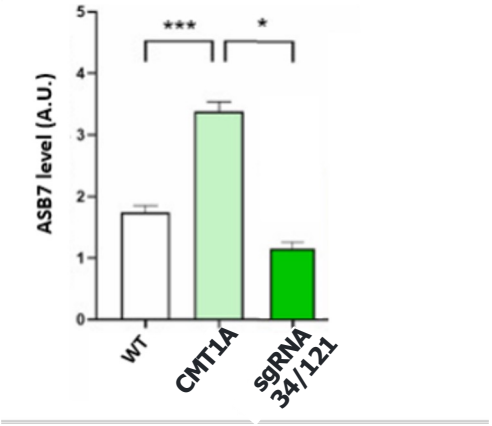




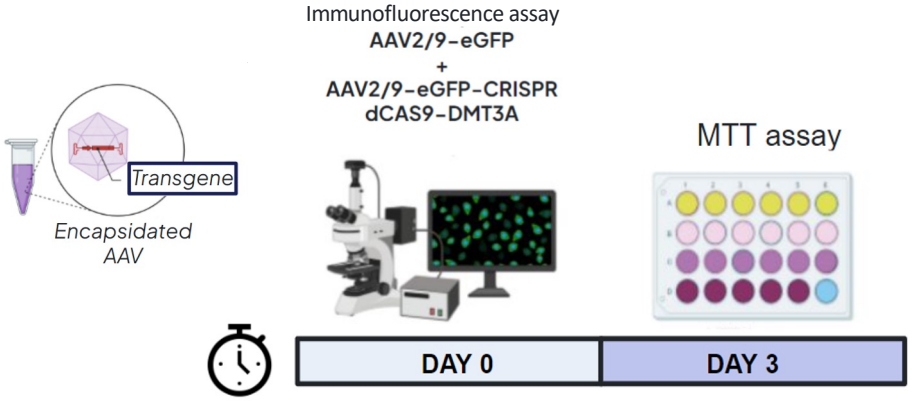
→ ER stress

→ increase ASB7 expression

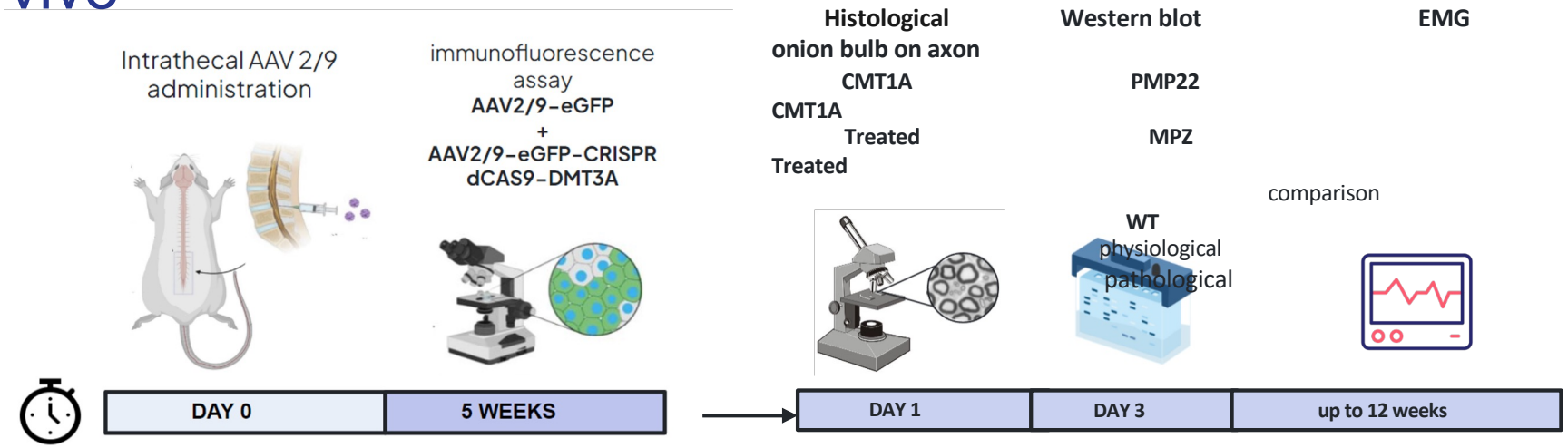




In vitro



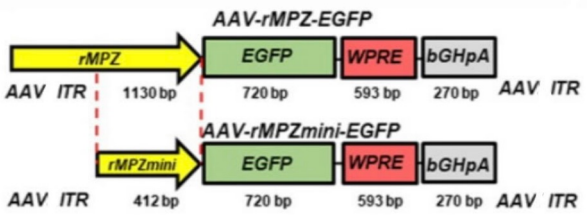
In vivo

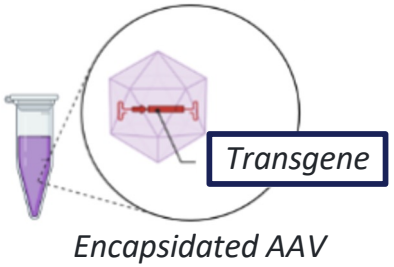


What is the system of delivery?

AAV2/9 → high tropism for Schwann cells

mock vector

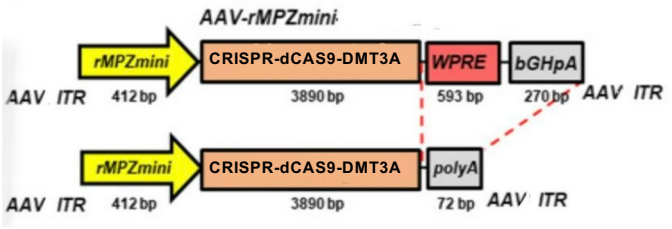




Encapsidated AAV

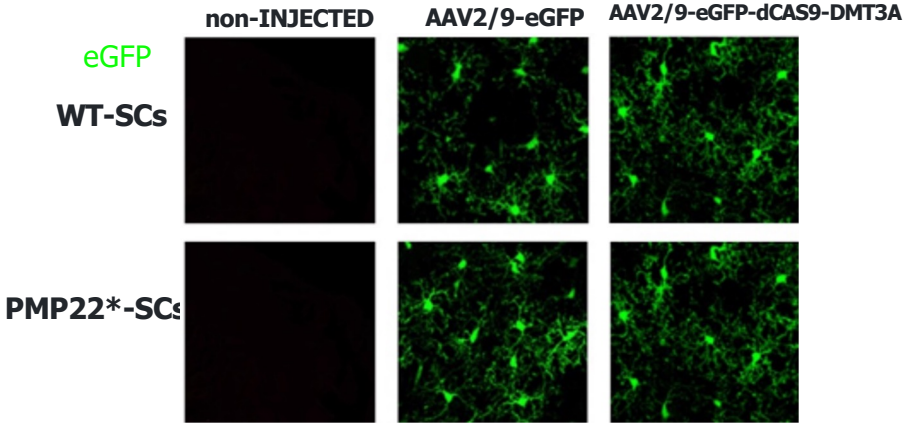
Therapeutic vector

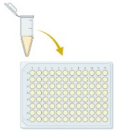
CRISPR-dCAS9-DMT3A



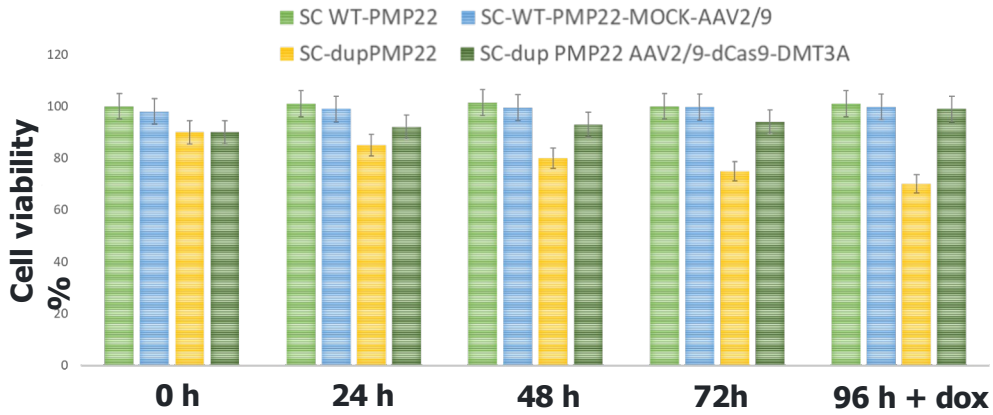
How to test AAV2/9 efficiency in vitro ?

Immunofluorescence assay



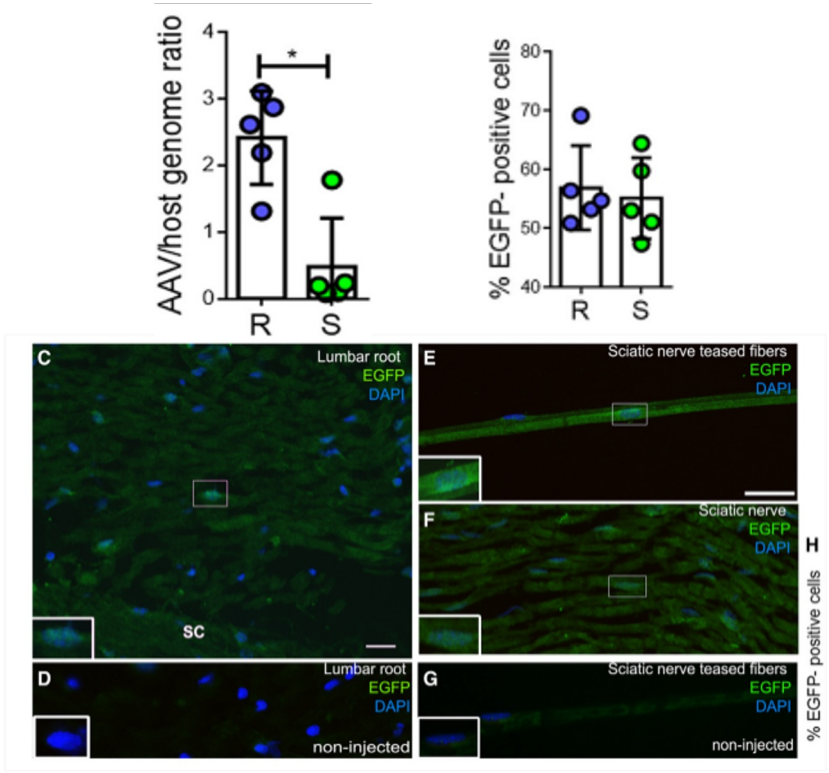


How to test the non-cytotoxicity of the treatment in vitro? *MTT assay*

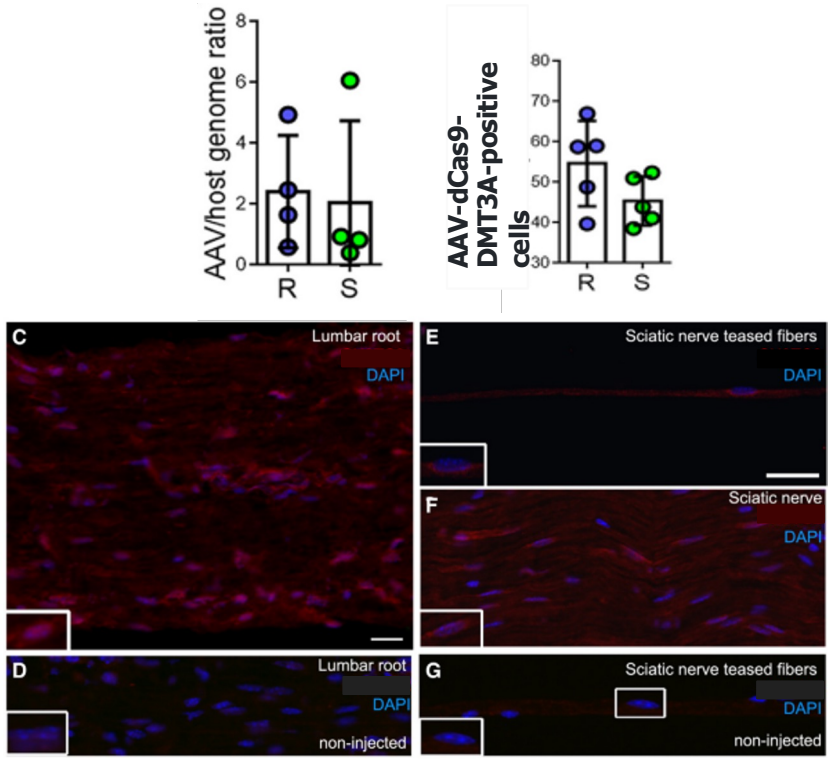


In vivo

Biodistribution of the mock **AAV2/9-EGFP** in PNS 5 weeks following lumbar intrathecal injection



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



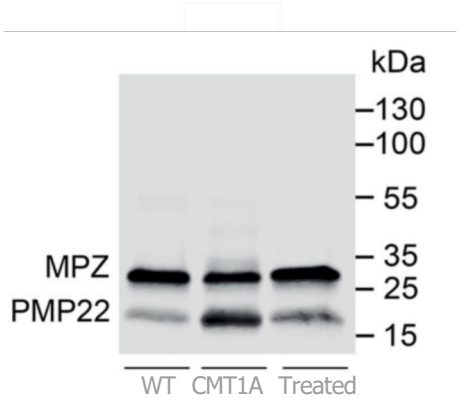
Adapted from Georgiou E. et al., 2023 Molecular Therapy

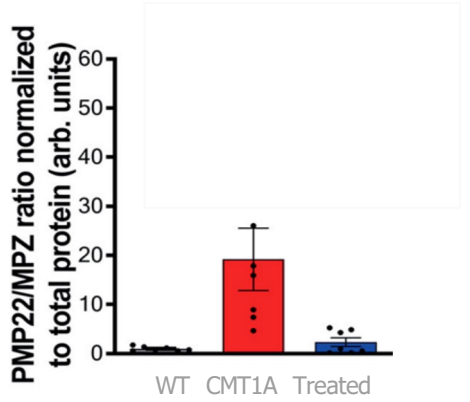
How to check the treatments efficiency ?

WESTERN BLOT

Myelin protein zero (MPZ):
→ expressed by Schwann cells
→ main structural component of the myelin sheath.

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





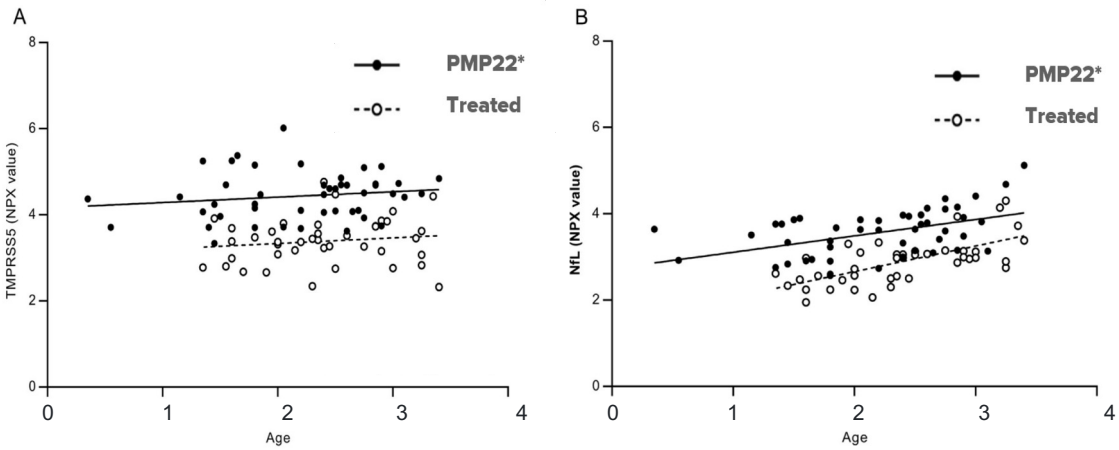
Adapted from Gautier et al. nature communications, 2021

Blood concentration values NPX (Normalized Protein eXpression)

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

- Nf-L (marker for axonal degeneration) ;
- TMPRSS5 (biomarker for myelinating).

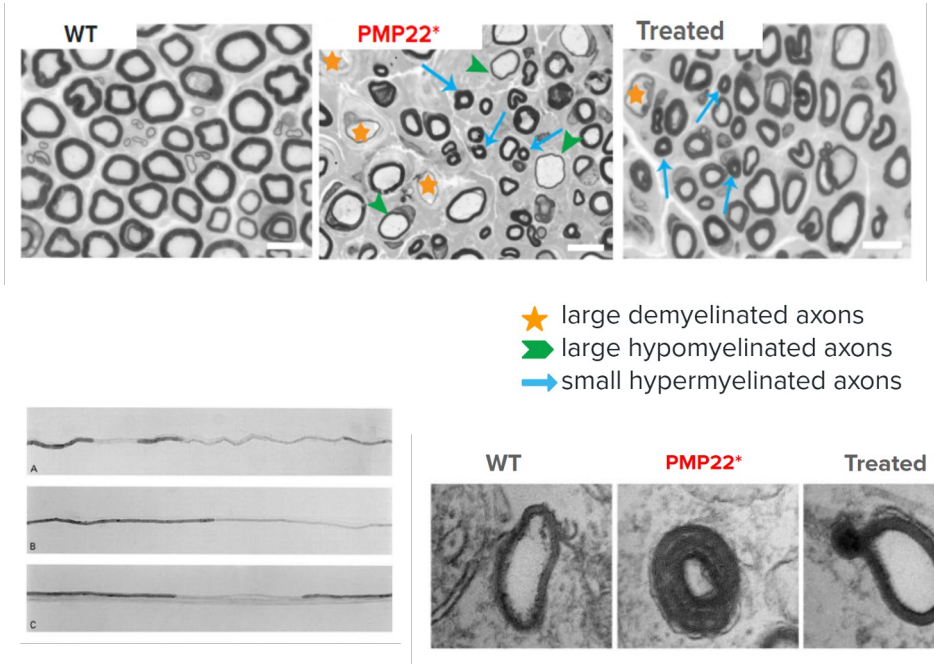
Adapted from Hongge Wang, et al. 2020



Histological

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

In

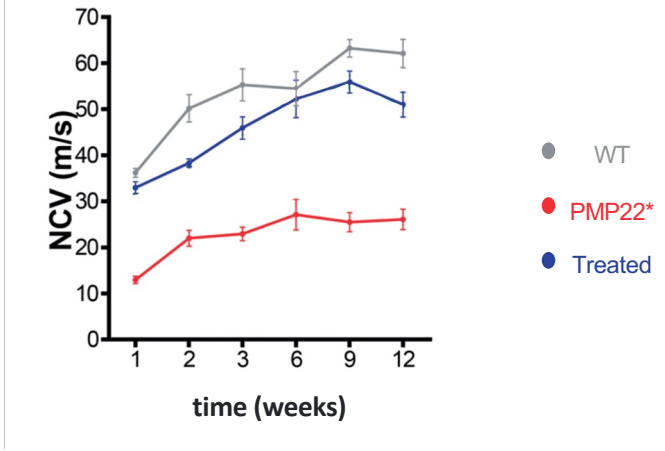


Adapted from Gautier et al. nature communications, 2021

EMG

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



Adapted from Gautier et al. nature communications, 2021

Budget

Thank you

& images by Freepik

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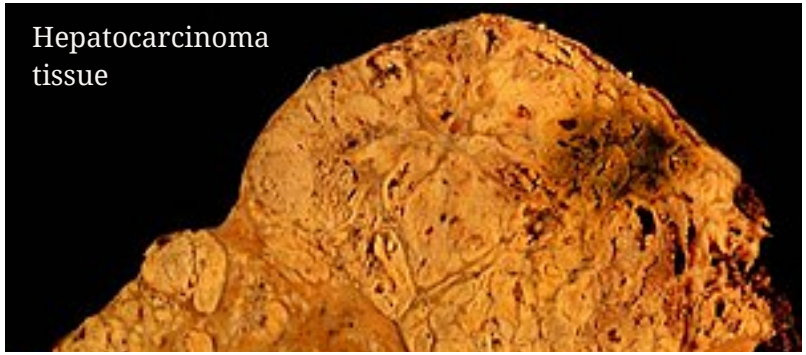
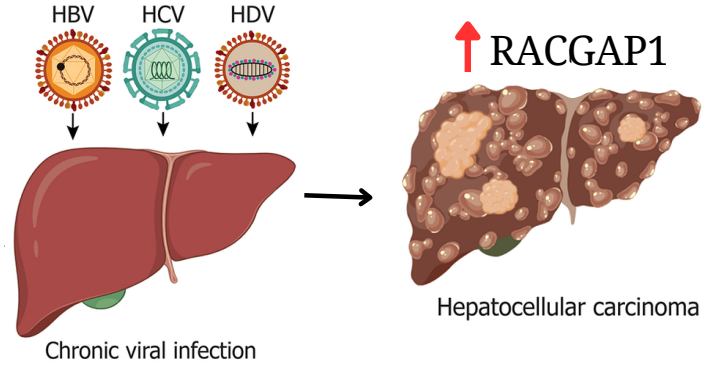
RACGAP1 competitive inhibition in hepatocellular carcinoma via vector based mRNA transfection

Lavinia Pace
Antonio Duarte
Ernest Serra
Miriana Santacroce
Luigi Fanelli

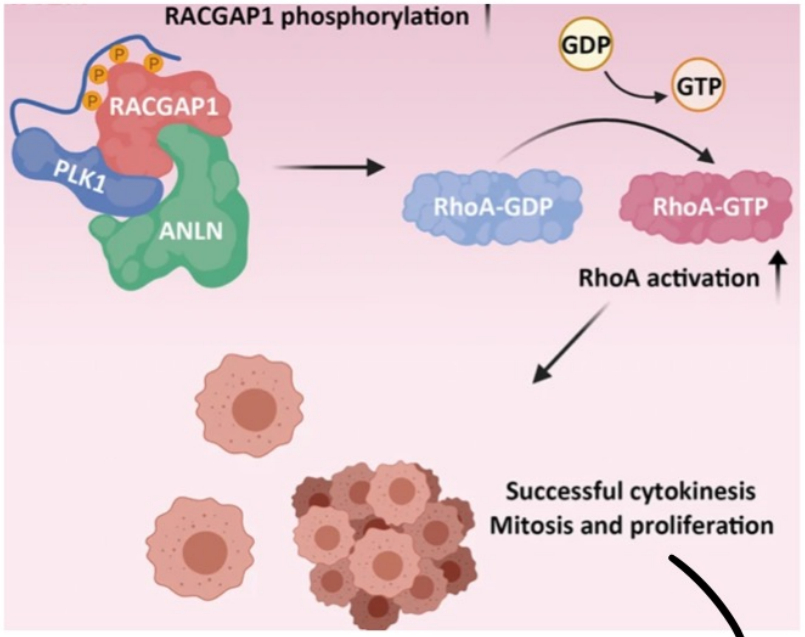
Background: panoramic on Hepatocellular Carcinoma (HCC)





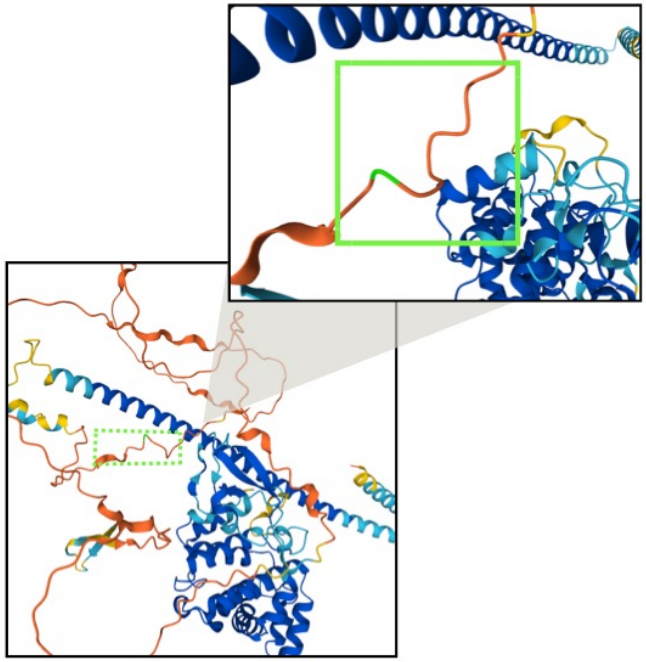
- Liver cancer is the **third most lethal cancer** globally. **Infection by hepatitis B\C** viruses is the main risk factor for HCC development
- The median age: > **60** years
- HCC **recurrence** is significantly associated with **RACGAP1 upregulation**: activation of RACGAP1/Rho/ERK signaling axis

Background: RACGAP1 pathway



RACGAP1 phosphorylation activation cascade

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
- Leading to a reduced activation of RhoA
- Inhibition of self proliferation

Experimental plan

In silico

- Sequence analysis
- Mutation of 4 serines into cysteins
- Sequence alignment
- Structure analysis

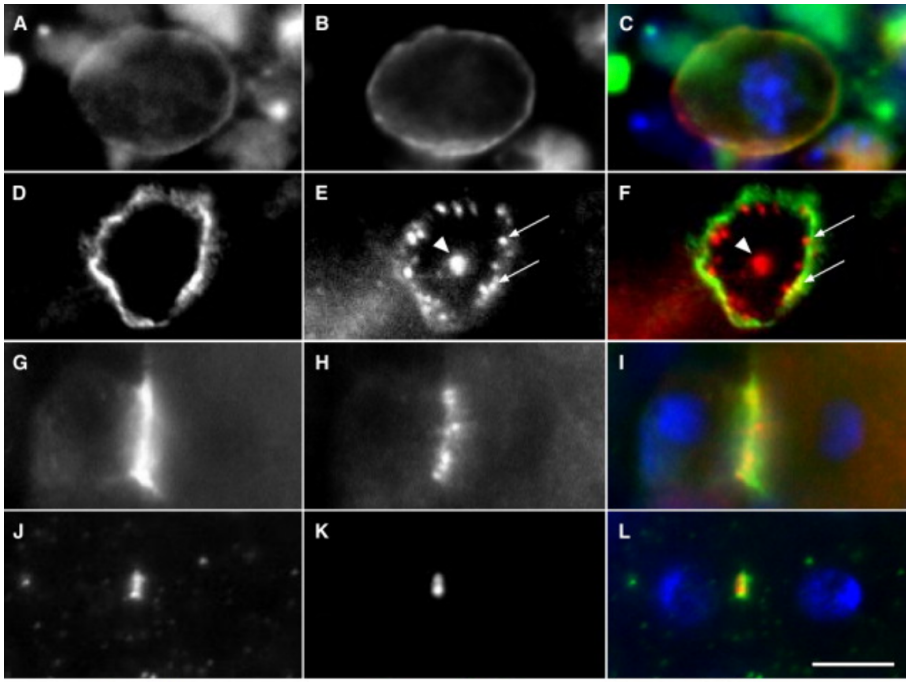
In vitro

- Cloning of mRNA seq
- Transfection into HCCLM3 cells
- mRNA expression
- Co-ChIP for complex formation
- Levels of phosphorylation
- RhoA activation
- Apoptosis and cell cycle analysis

In vivo

- Tumor mass analysis
- Tumorigenesis assay

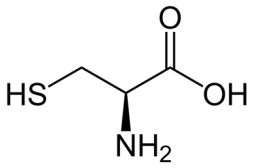
Where are RACGAP1 and ANLN located?



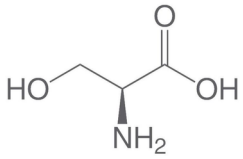
RACGAP and ANLN location on the cell during division:
https://www.sciencedirect.com/science/article/pii/S0960982207023354

Does the amino acidic change cause any effects on the protein?

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



Cysteine



Serine

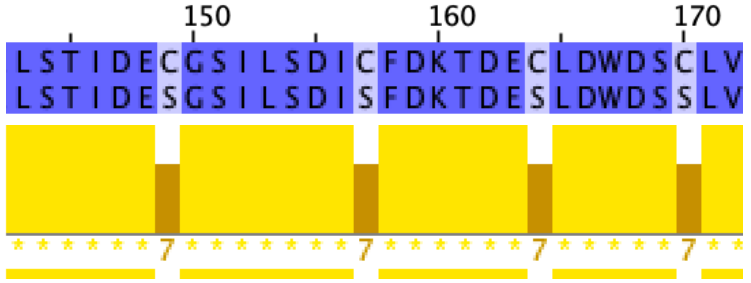
AMINOACID MODIFICATIONS - sequence

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

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

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

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



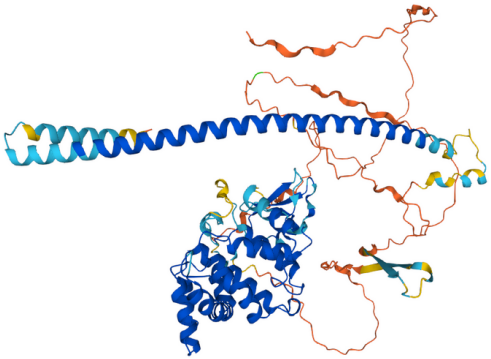
Number of conserved physico-chemical properties /10

https://www.jalview.org/help/html/calculations/conservation.html

MSA using T-coffe via Jalview

Aminoacid modifications - structural predictions

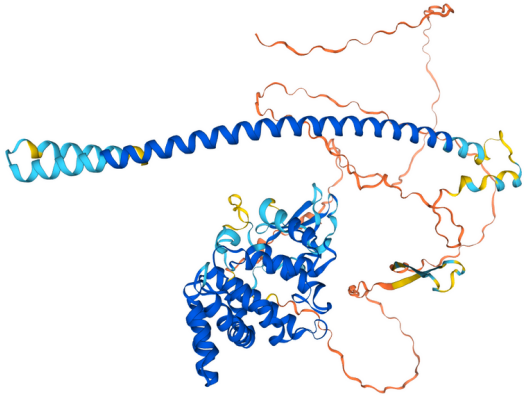
A **WT RACGAP1**



Structure prediction via alphaphold

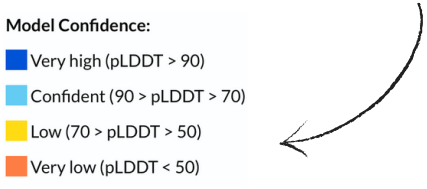
https://www.uniprot.org/uniprotkb/Q9H0H5/feature-viewer

B **RACGAP1(UGU)** Serra et al.



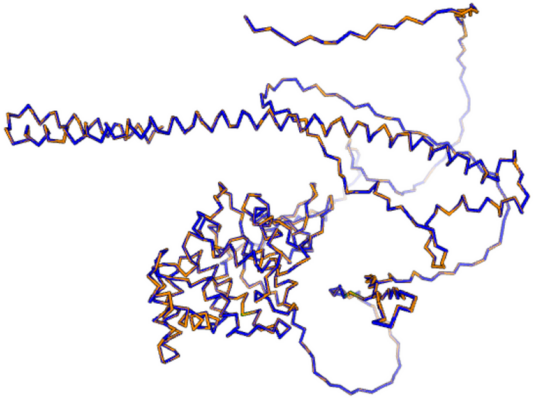
Structure prediction via Swissprot

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



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

C **WT RACGAP1/RACGAP1(UGU)**

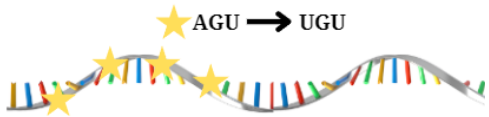


3D structure superposition via DALI

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/

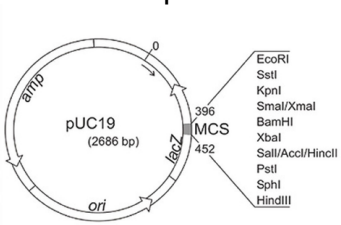
Cloning and transfection of RACGAP(UGU) mRNA



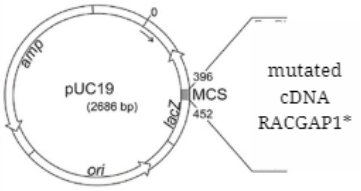
mutated mRNA

mutated cDNA

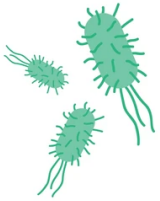
+



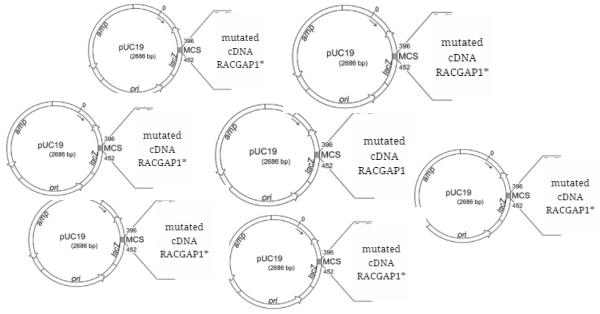
plasmid



plasmid with mutated RACGAP1(UGU) seq

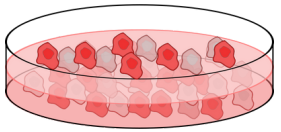


E. Coli DH5α



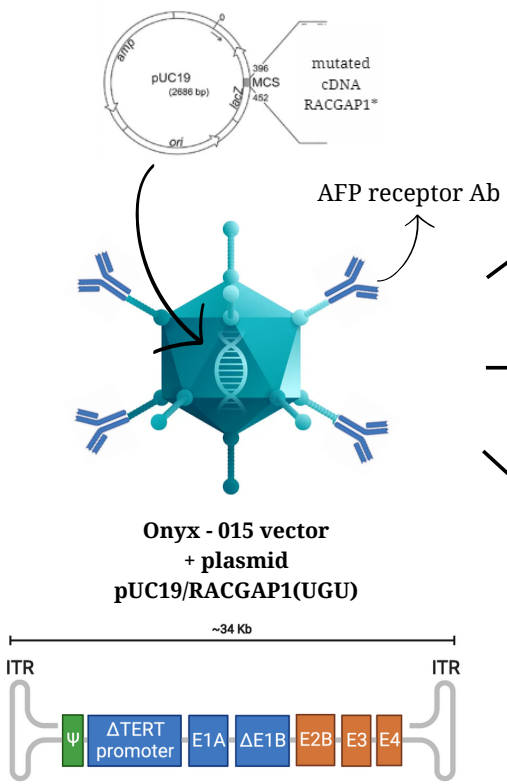
+

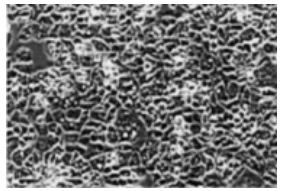
Onyx - 015 vector



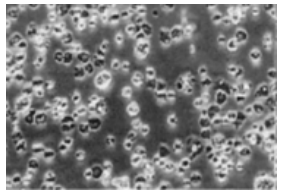
Transfection into HCCLM3 cells

What happens to the cells using ONYX-015?

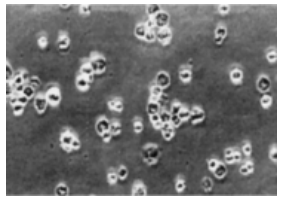




Healthy liver cells p53 wt and no alpha-feto protein receptor. The vector doesn't enter the cells and no healthy liver cells are killed

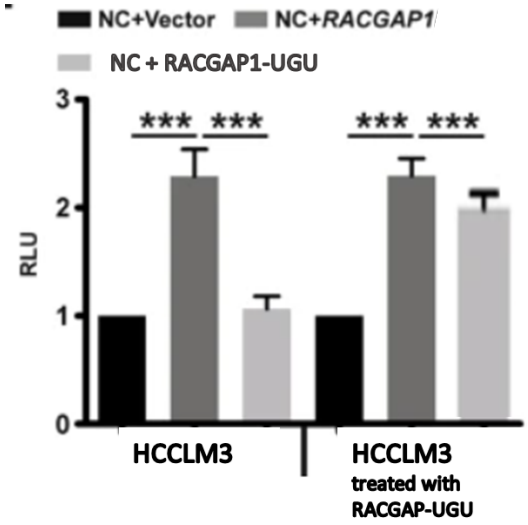


HCC cells p53 wt and with alpha-feto protein receptor on the cell surface. The vector enters the cells and delivers the RACGAP1(UGU) mRNA. The cells die by cytokinetic failure



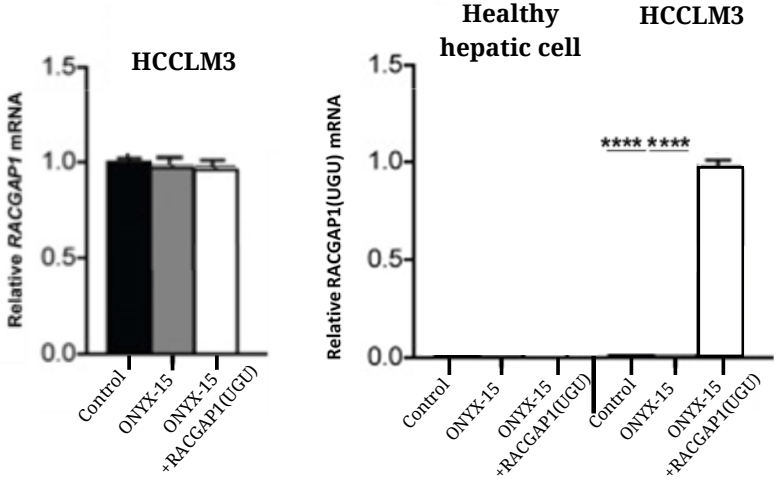
HCC cells Δp53 and with alpha-feto protein receptor on the cell surface. The vector enters the cells, delivers the RACGAP1(UGU) mRNA and activates the Δp53 onyx pathway. The cells die by both cytokinetic failure and cell lysis

In vitro: Is RACGAP1(UGU) expressed?



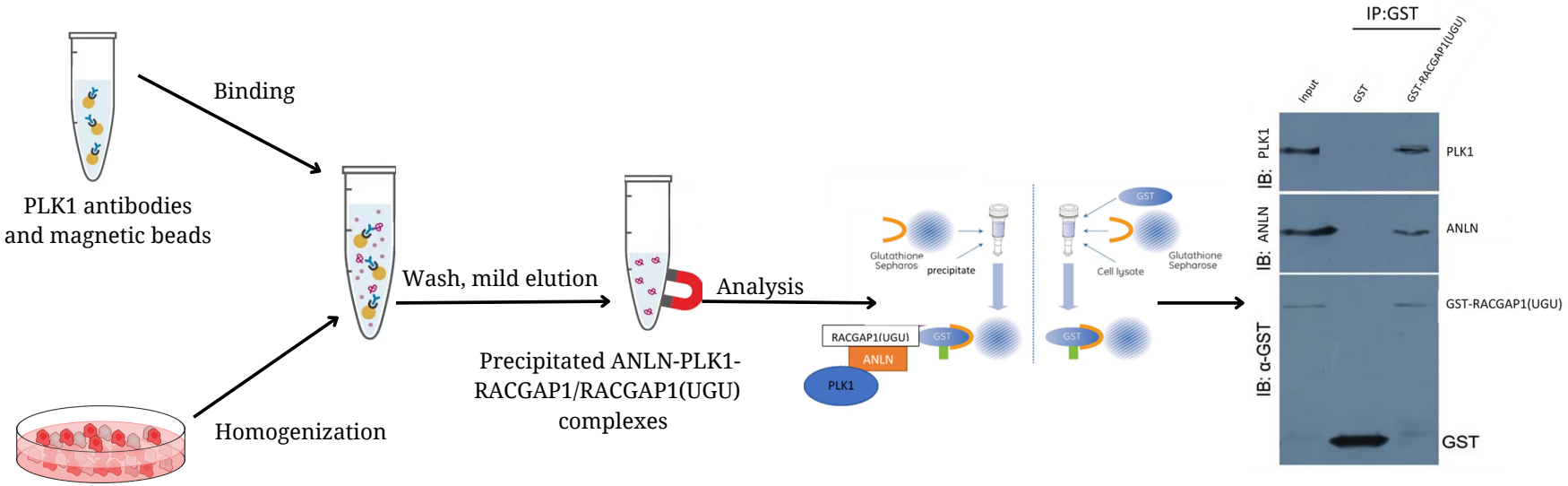
Luciferase essay- Expression of RACGAP1 and RACGAP1(UGU) in wt HCCLM3 cells and HCCLM3 cells after transfection with RACGAP1(UGU) mRNA

Adapted from https://www.nature.com/articles/s41419-019-1666-2



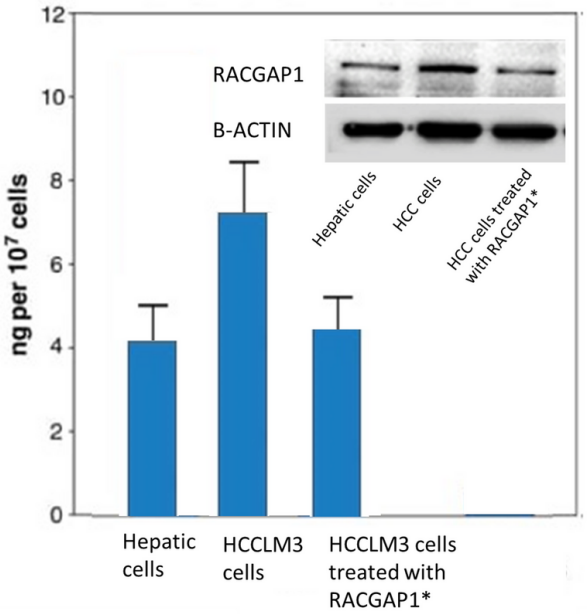
RT-PCR- Relative RACGAP1 and RACGAP1(UGU) mRNA level in HCCLM3. The expression of RACGAP1 and RACGAP1(UGU) is not influeced by the presence of the other

In vitro: Does the RACGAP1(UGU)/ANLN/PLK1 complex form?

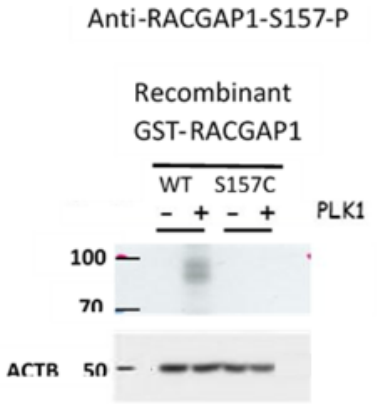


Pull down: RACGAP1(UGU)-ANLN-PLK1 complex formation

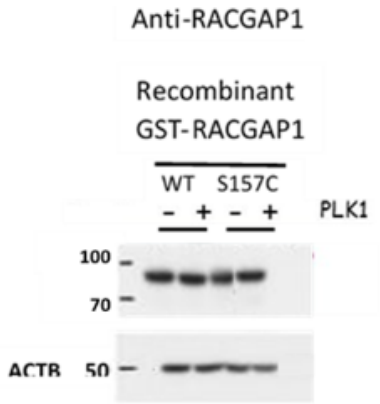
In vitro: Is RACGAP1(UGU) phosphorylated?



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

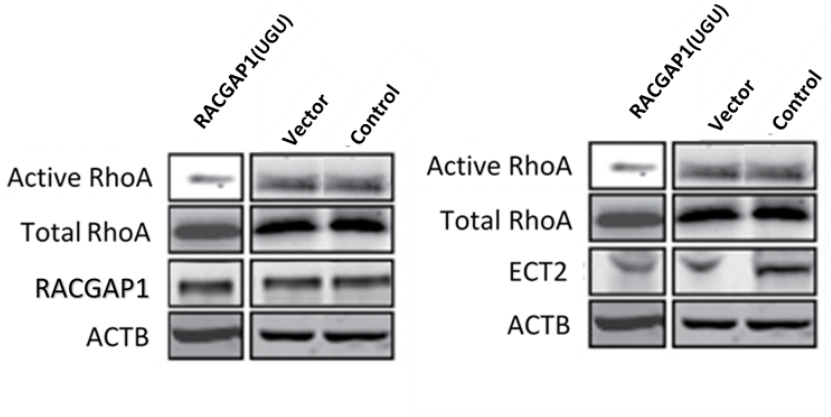


Anti-Phospho Ser157 of GST-RACGAP1 in presence of RACGAP1 (WT) or RACGAP1 (S157C)

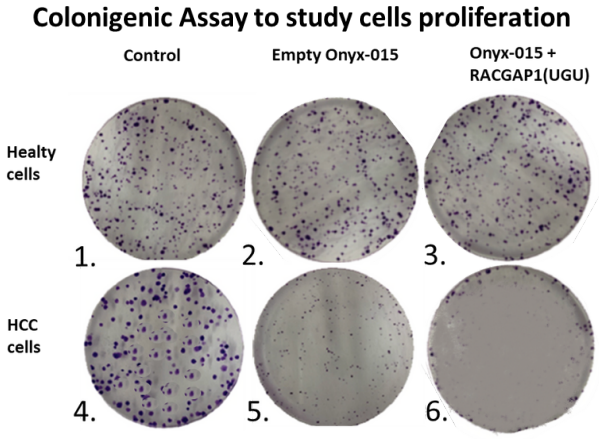


Anti-RACGAP1 of GST-RACGAP1 in presence of RACGAP1 (WT) or RACGAP1 (S157C)

In vitro: Is RohA activity decreased?

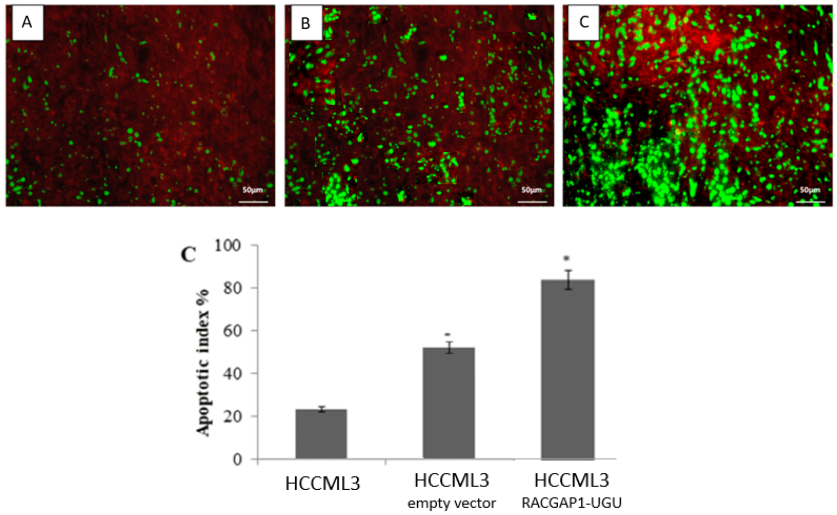


Western blot- Detection of RhoA activity and also ECT2 and RACGAP1 expression in HCCLM3 after trasfection of RACGAP1(UGU) Adapted from (Yang et al., 2018)



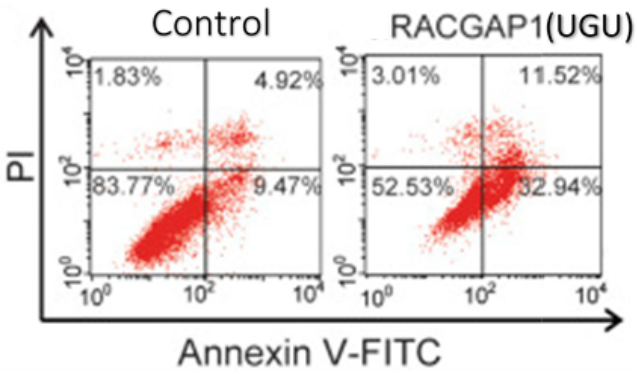
Clonogenic assay:
1. Healthy Hepatic cells,
2. Healthy Hepatic cells with transfection of empty Onyx-015,
3. Healthy Hepatic cells with transfection of RACGAP1(UGU) mutated protein,
4. Hepatocarcinoma HCCLM3 cells,
5. Hepatocarcinoma HCCLM3 with transfection of empty Onyx-015,
6. Hepatocarcinoma HCCLM3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

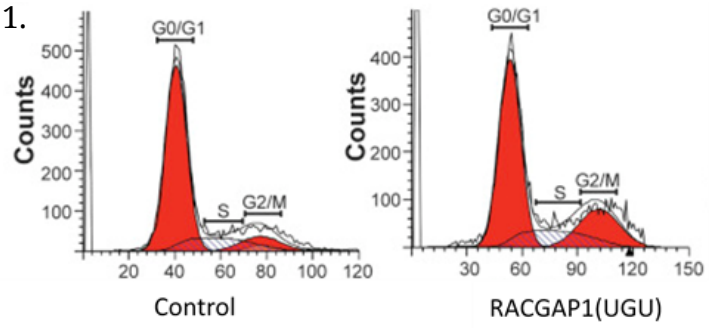


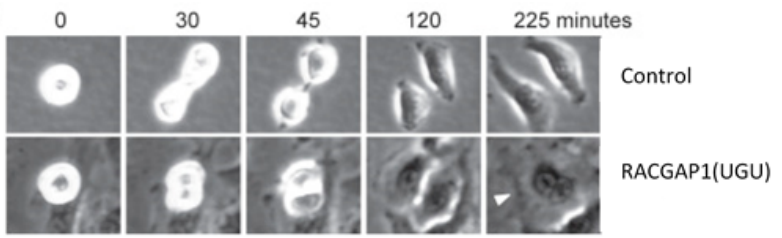
Flow cytometry analysis- Staining cells with the apoptosis marker Annexin V (FITC) and propidium iodide allows the discrimination of intact cells (FITC-PI-), early apoptotic (FITC+PI-) and late apoptotic or necrotic cells (FITC+PI+).

Adapted from (Yang et al., 2018)

In vitro: Does RACGAP1(UGU) cause cytokinesis failure?

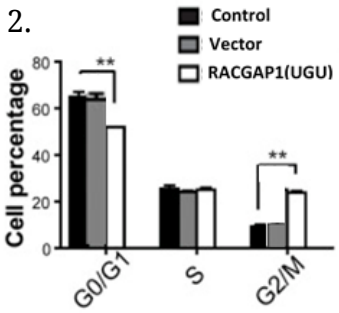
1.



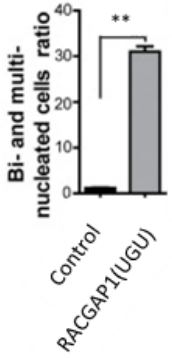


Cytokinesis analysis - Selected frames from time-lapse imaging of RACGAP1(UGU) and control HCCLM3 cells.

2.



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.

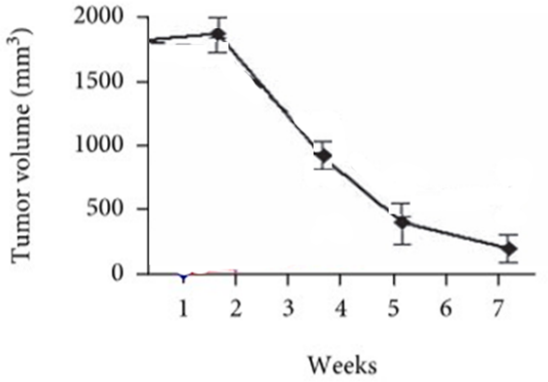


Statistics of bi- and multi-nucleated HCCLM3 cells after trasfection

Adaptedfrom (Yang et al., 2018)

In vivo: Is there a tumor mass decrease?

1.

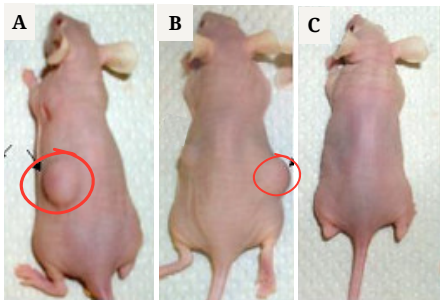


2.

1. Decrease of tumor mass during weeks with the treatment of RACGAP1(UGU) on BALB/c nude mice

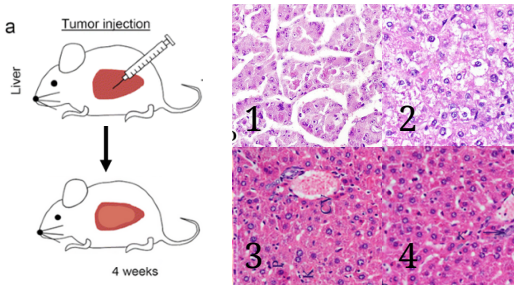
2. Representative images of tumors removed from mice

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



A. Nude mouse injected with HCCLM3 cells. Evident tumor mass.
B. Nude mouse injected with HCCLM3 cells transfected with empty vector, smaller tumor mass
C. Nude mouse injected with HCCLM3 cells transfected with RACGAP1(UGU) vector. No evidence of tumor growth.

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



HCC histological samples from BALB/c nude mouse

1. HCC tissue
2. Tissue sample injected with empty vecotr
3. Tissue sample injected with RACGAP1(UGU) vector
4. Healthy liver tissue

https://translational-medicine.biomedcentral.com/articles/10.1186/s12967-017-1247-z/figures/4

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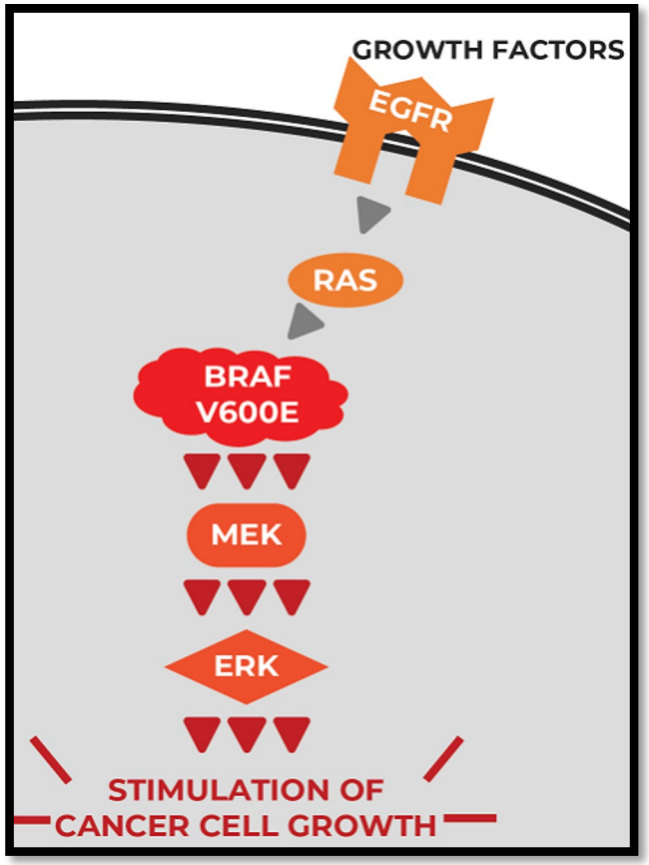
CRISPRi: A THERAPEUTIC APPROACH IN MANAGING ANAPLASTIC THYROID CANCER

Gabriele Virgilio, Alessandro Belvedere, Emanuela Unhe Jeong, Nashon Majaliwa

BACKGROUND

Anaplastic thyroid cancers (ATCs) are highly aggressive tumors and account for **30%** of US thyroid cancer deaths.

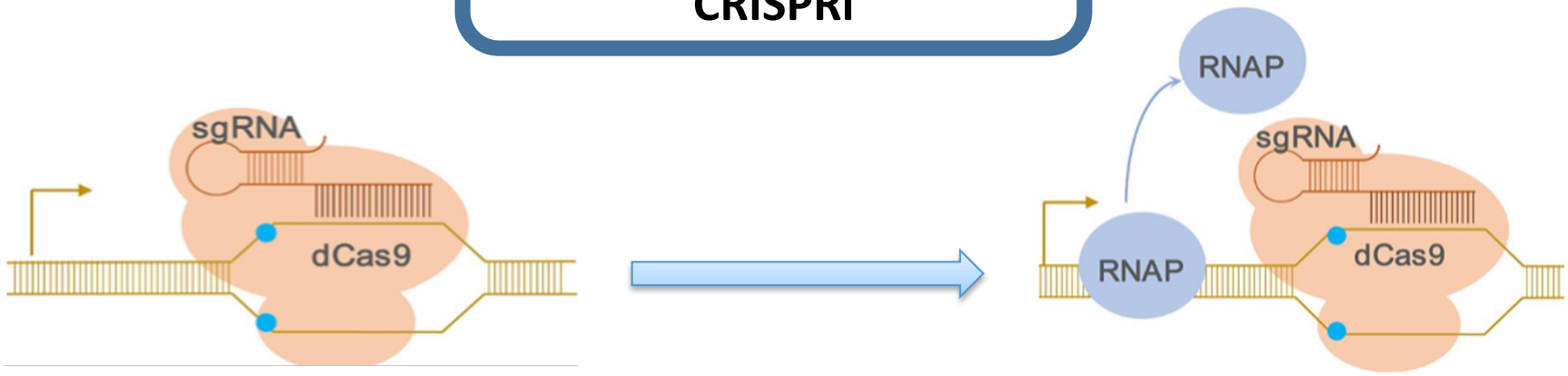
- ***BRAF*** is a proto-oncogene involved in the activation of the MAPK pathways;
- The ***BRAF^{V600E}*** **point mutation** is a common early molecular event and is detected in **45%** of ATC cases;
- Cancer cells expressing BRAF V600E are **less responsive** to **radioactive iodine therapy** due to **downregulation** of the sodium iodide symporter (**NIS**);
- BRAF chemical inhibitors can cause **paradoxical BRAF activation**, leading to the formation of secondary cancers.



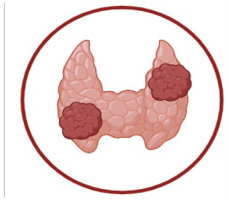
https://www.researchgate.net/publication/320341777_PIK3CAH1047R-induced_paradoxical_ERK_activation_results_in_resistance_to_BRAFV600E_specific_inhibitors_in_BRAFV600E_PIK3CAH1047R_double_mutant_thyroid_tumors

AIM OF THE PROJECT AND STRATEGY

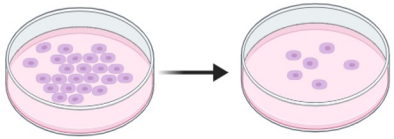
Suppress *braf* transcription through CRISPRi



Avoiding
secondary cancers



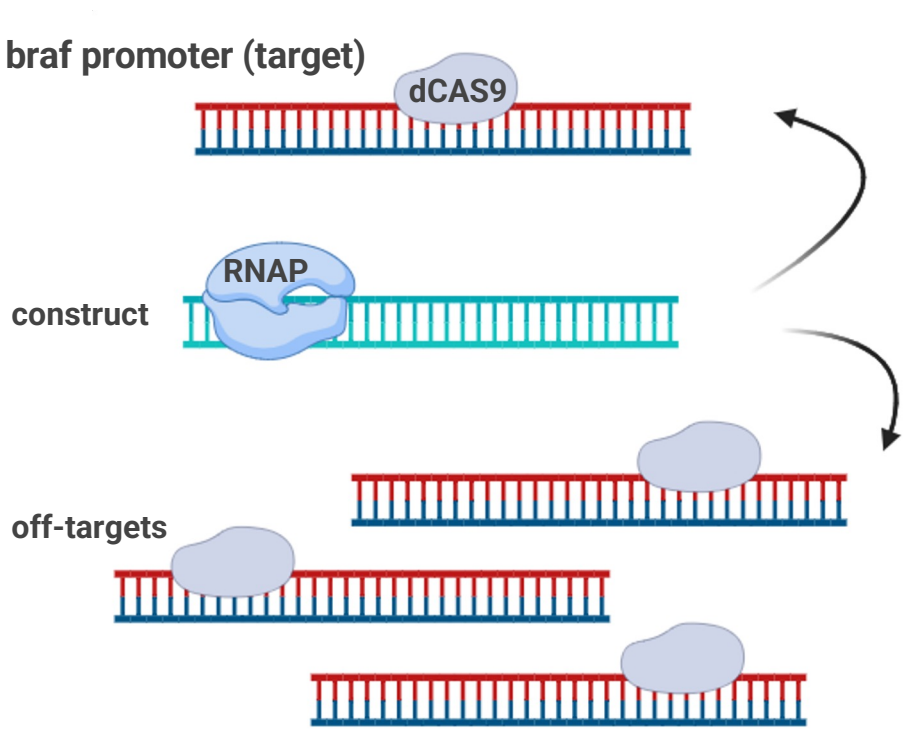
Slowing down
proliferation



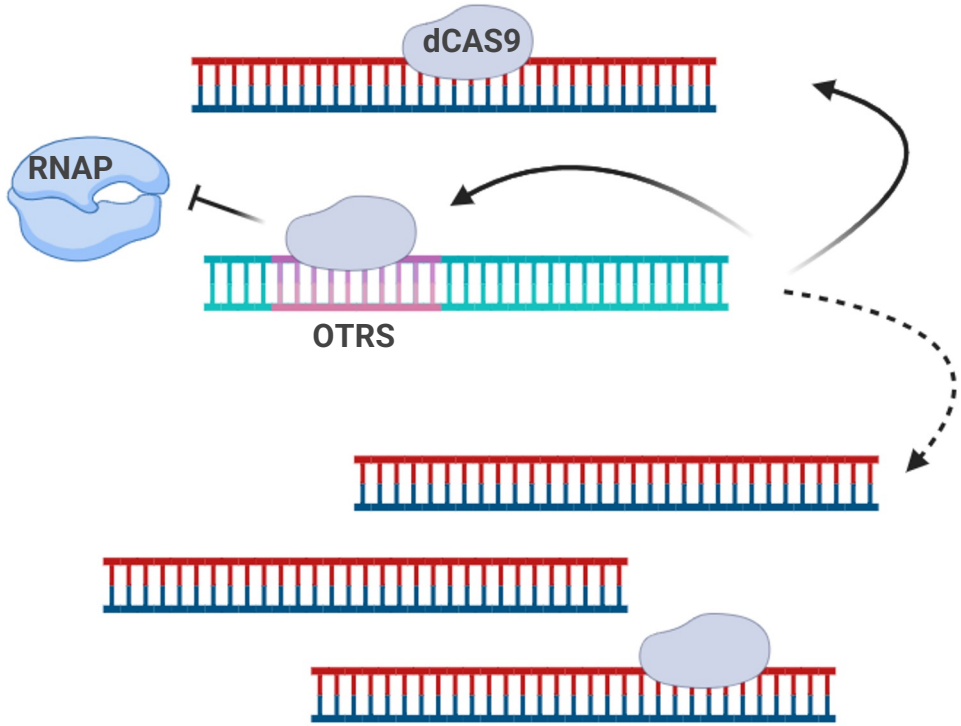
Restoring
sensitivity to radiotherapy

REDUCING SIDE EFFECTS

Off-Target Regulatory Sequence (OTRS)

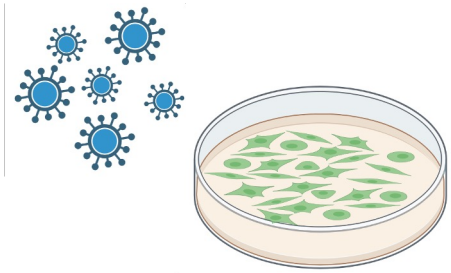


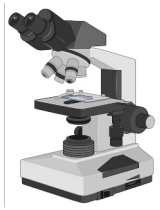
dCAS9 is produced and **binds** to the **target** and **off-target** sites

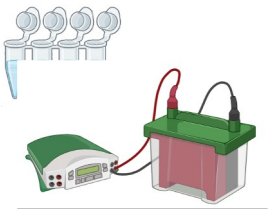


dCAS9 is produced and **binds** to the **target** and the **OTRS**, **preventing** further **dCas9 transcription** and **off target binding**

IN VITRO EXPERIMENTAL PLAN







Human thyroid cell lines carrying **BRAF^{V600E}** (**8505**c)

DAY 0

8505c transduction with adenovector, start selection

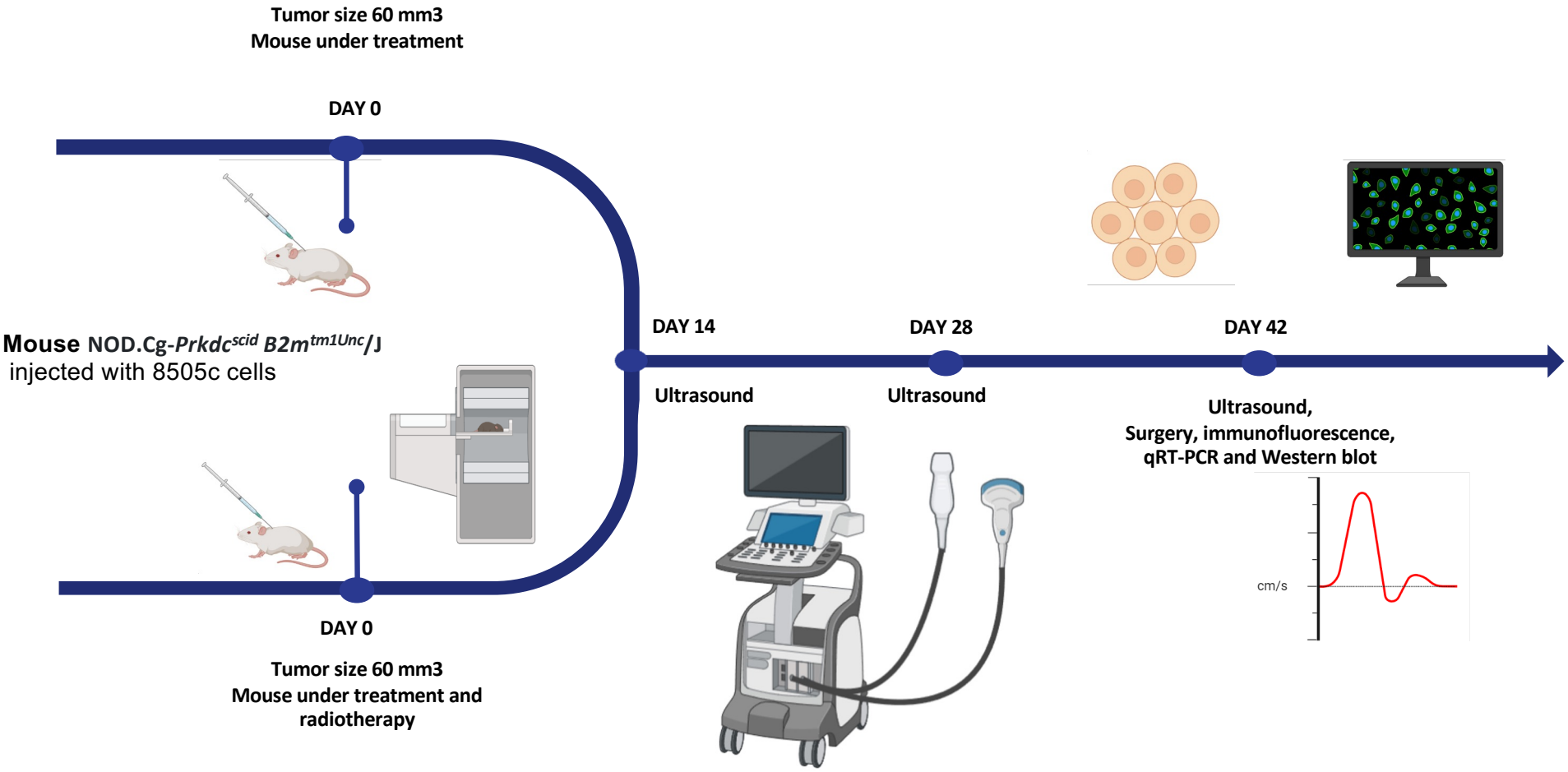
DAY 1, 2, 3

Cell count

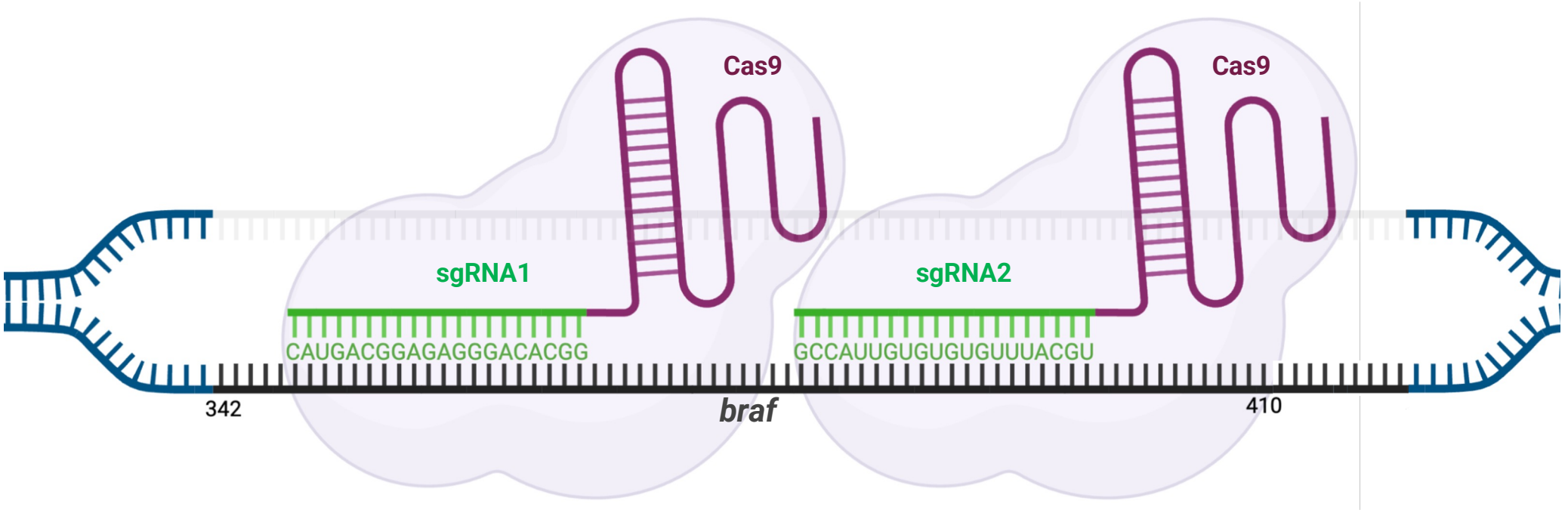
DAY 4, 5

Cell count, Western blot, qRT-PCR

IN VIVO EXPERIMENTAL PLAN



sgRNA DESIGN



OTRS

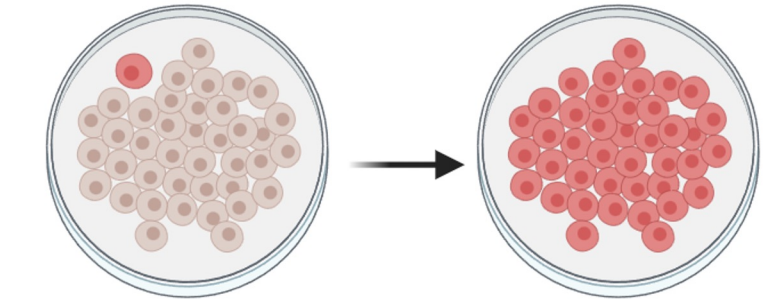
CATGGCGGCGCGGGACACGG

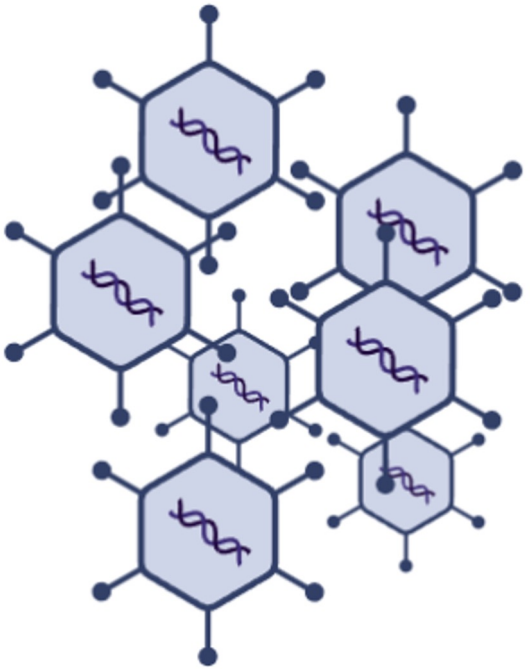
GCCATTTTGTGTGTTTGGGT

ADENOVECTOR DESIGN & PRODUCTION

Construct: dCas9 + sgRNA

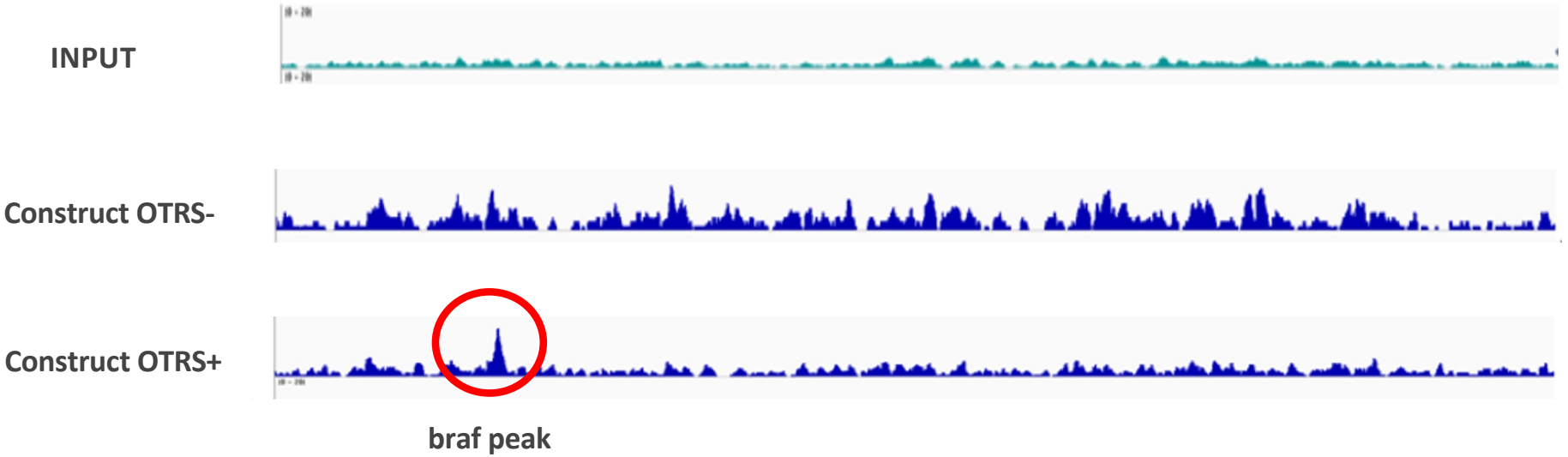
Transfection and selection of HEK293 cell with Ampicillin



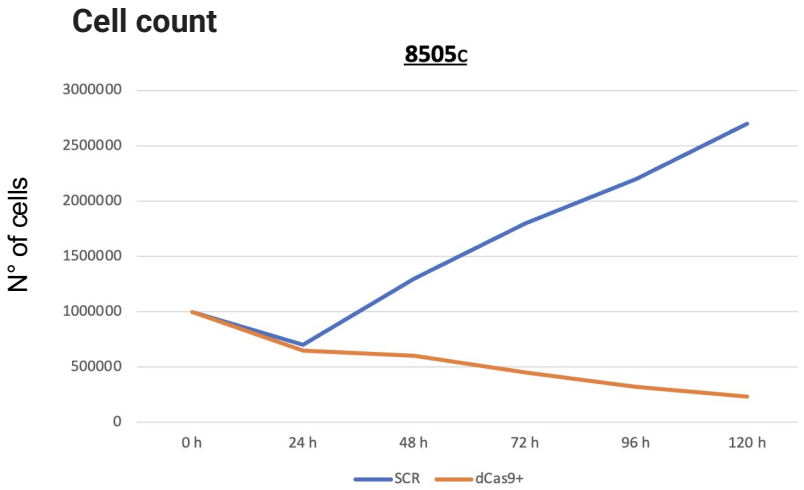


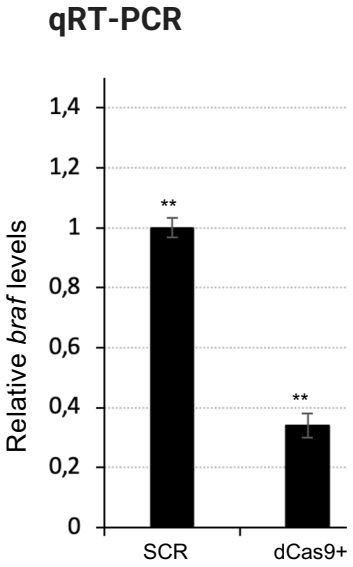
PROOF THAT THE SYSTEM WORKS

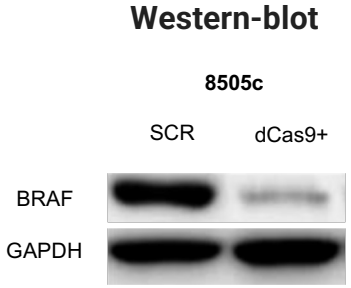
ChIP-seq: targeting dCAS9

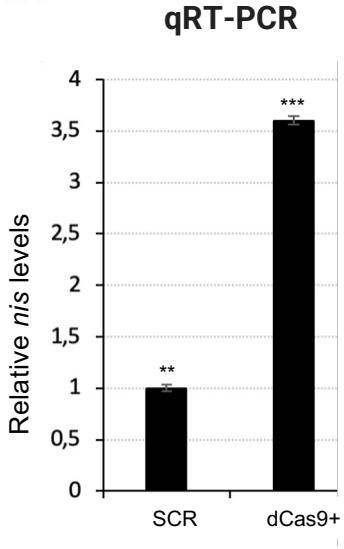


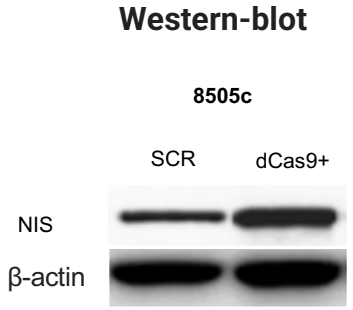
EXPERIMENT IN VITRO





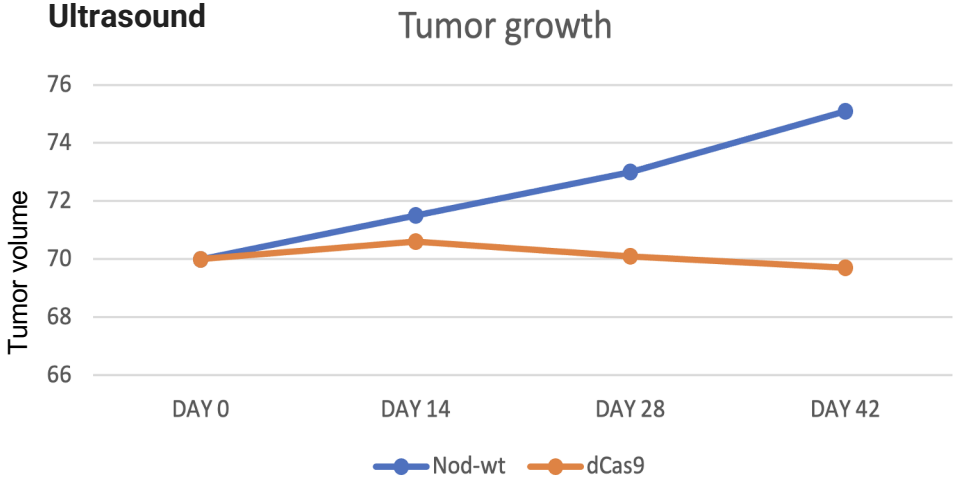




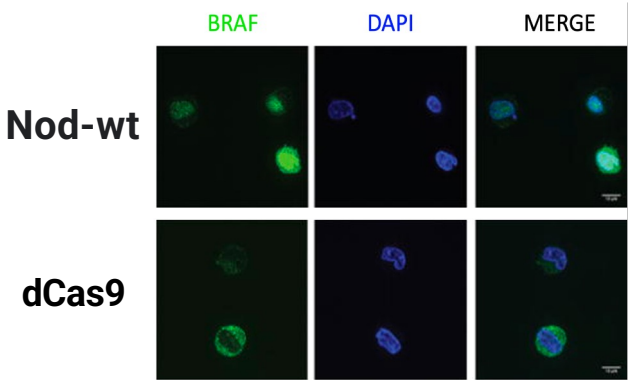


EXPERIMENT IN VIVO

Ultrasound

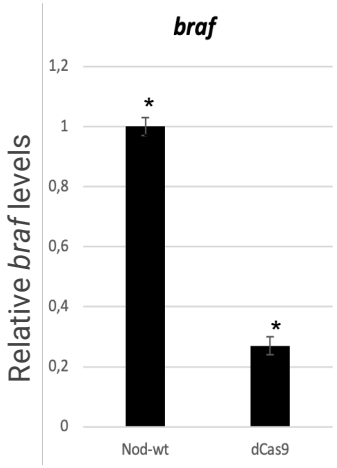


Immunofluorescence

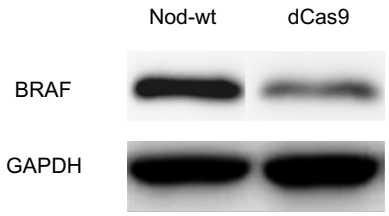


adapted from Hess G. T. et al. 2016

qRT-PCR

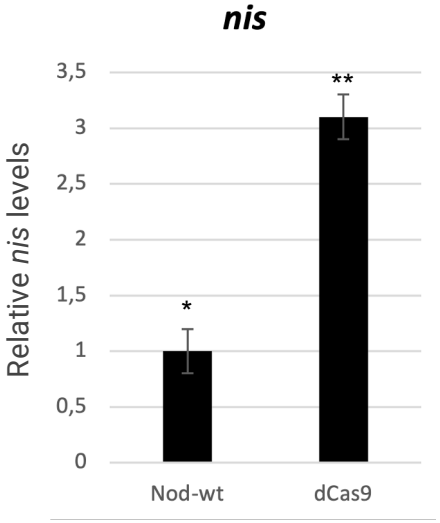


Western-blot

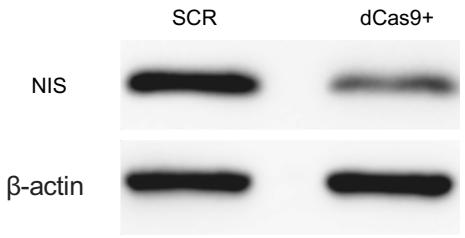


EXPERIMENT IN VIVO

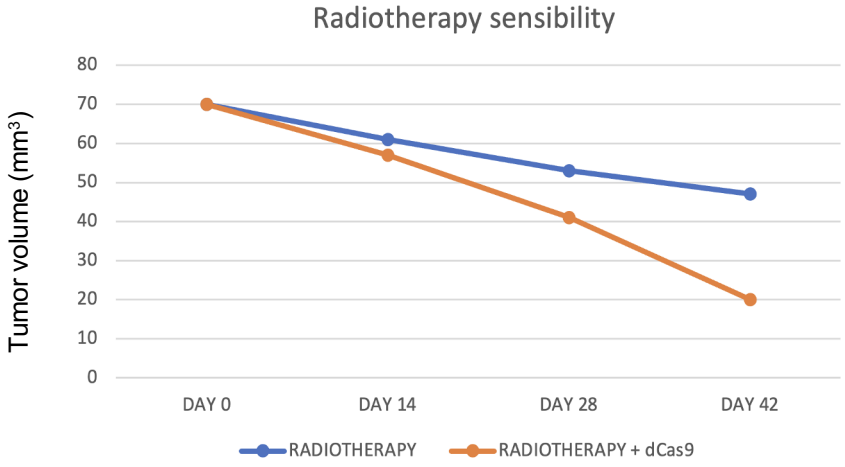
qRT-PCR



Western-blot



Ultrasound



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Materials

- HEK293T cell
- 8505c cell
- 4 mice
- Empty vector
- Vector with dCAS9 with the off-target
- Vector with dCAS9 without the off-target
- sgRNA1
- sgRNA2