A NOVEL STRATEGY TO RESTORE THE CORRECT FUNCTION OF TIN2 IN DYSKERATOSIS CONGENITA PATIENTS

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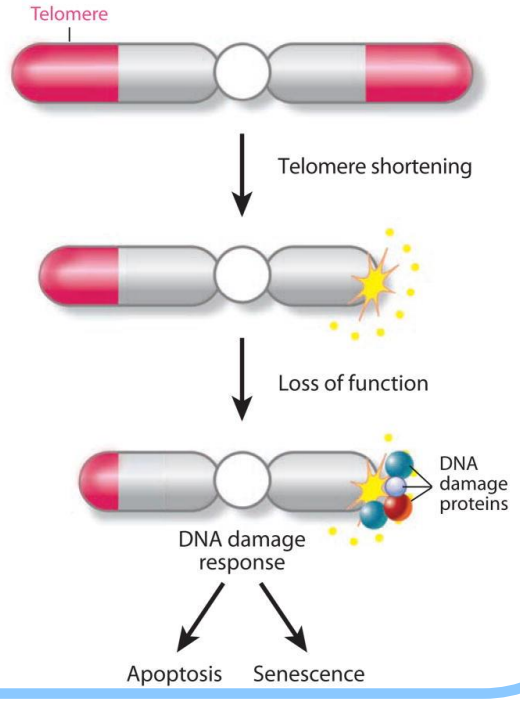
Background

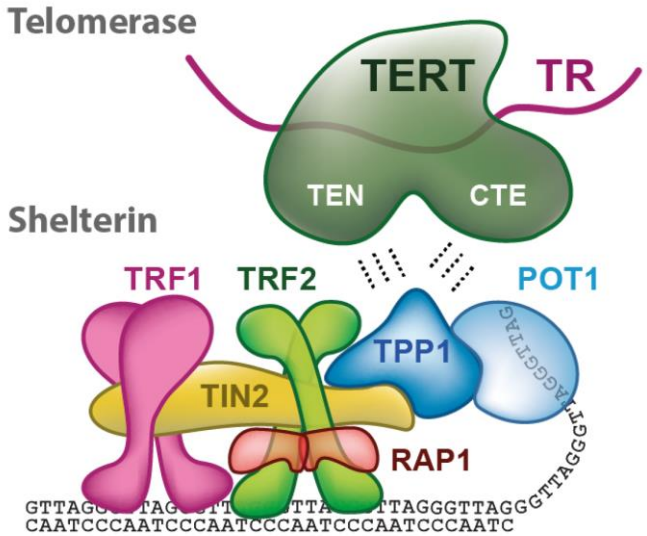
Dyskeratosis Congenita

Congenital Rare disease related to **telomere shortening** and bone marrow failuring



Shelterin complex

Protecting and maintaining the **telomeres** metabolism and their **length**



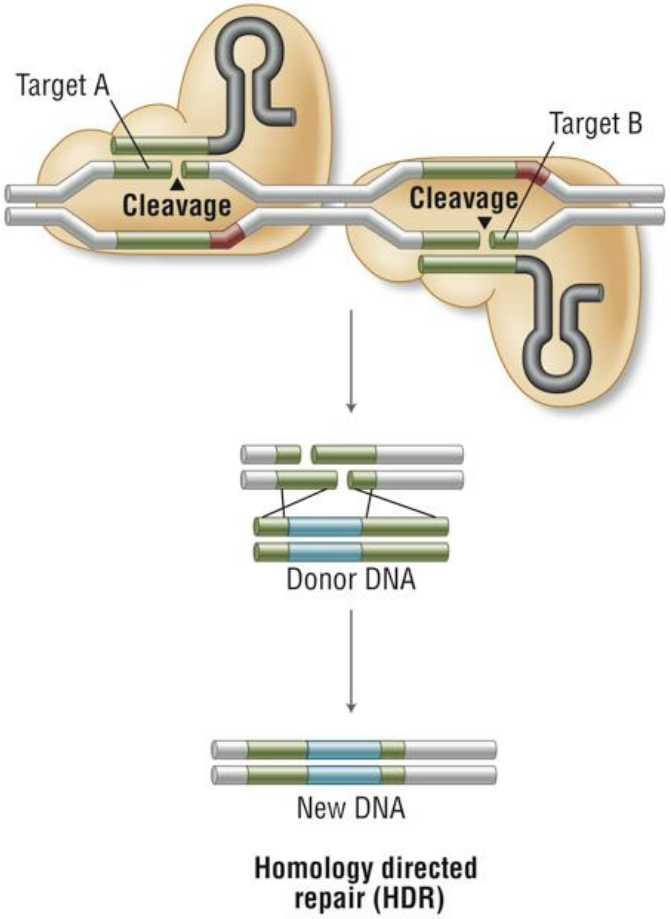
TIN2 protein

Involved in **Shelterin complex** function.

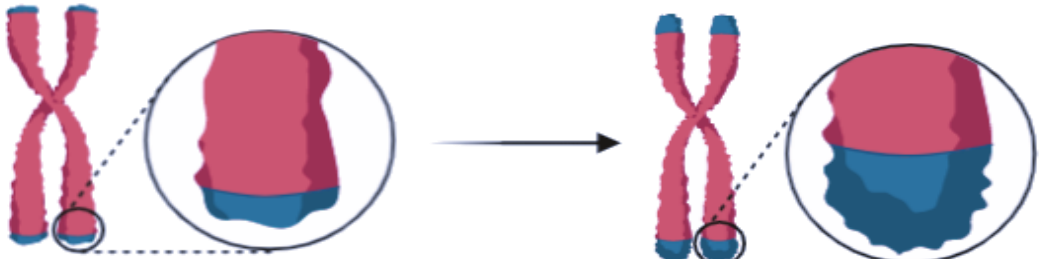
Encoded by ***tinf2***

Aim of the project

Recovering the ***tinf2*** WT phenotype thanks to **CRISPR/Cas9 Nickase** gene editing, which will lead to the restoration of **TIN2** ability to interact properly in the **Shelterin complex**, to correct telomerase recruitment and the telomeres metabolism.



TELOMERES ELONGATION



BONE MARROW REPOPULATION

Materials and methods

ANIMAL-MODEL

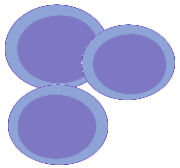
- **Mouse model TIN2$^{DC\text{-}cond}$** (K267E) Exon 6

CATAAAGAG >CATGAGGAG

CELL-LINES

- **MEF cells**

- **CD34+ HSPCs**

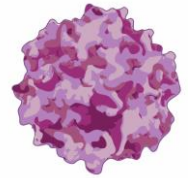


Why AAV6 and CRISPR/Cas9 Nickase?

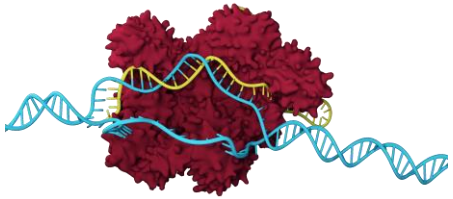
- Efficient delivery and editing tools
- No insertional mutagenesis
- Easier and cheaper

EDITING AND DELIVERING

- **AAV6**



- **CRISPR/Cas9 Nickase**



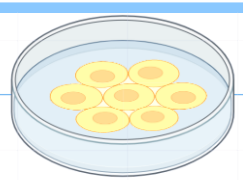
Experimental plan

1. In vitro

Design and develop of CRISPR/Cas9 Nickase and AAV6 vector

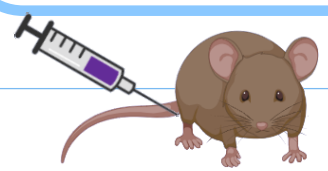
2. In vitro

Test the molecular efficiency of CRISPR/Cas9 Nickase in MEF-cell line



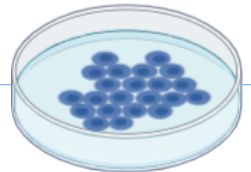
3. Ex vivo

Extract and transfect HSPCs from the engineered mouse



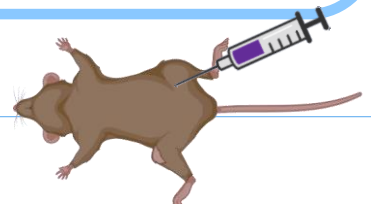
4. Ex vivo

Selection, monitoration and expansion of the transfected cells with CRISPR/Cas9 Nickase



5. Ex vivo

Transplant the transfected HSPCs into the mouse (intra-femoral)

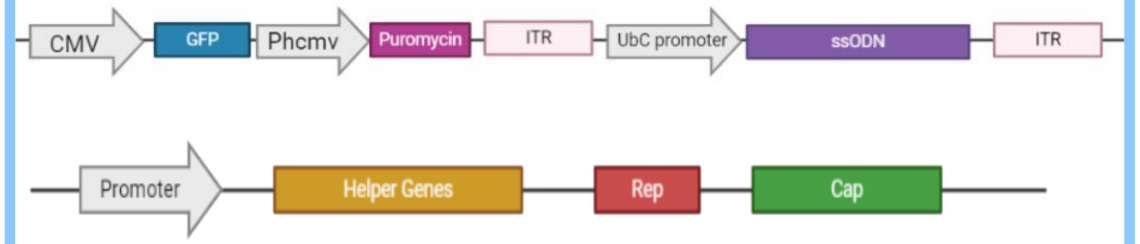


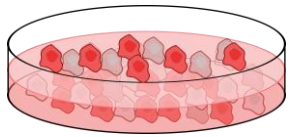
6. Final evaluation

-q-PCR
-Western Blot
-Whole genome NGS
-FISH
-FACS
-Proliferation assay
-Mouse health evaluation

In vitro

rAAV6





HEK293T

- **GFP**
- **Puromycin**
- **ssODN**
- **Serotype 6 capsid**

CRISPR/Cas9 Nickase

sgRNA candidates:

sgRNA1

5’ CGGGATTTTCGCTTTCCCAA AGG **3’**

sgRNA2

3’ CTACTCCCATTTAGGAACAT GGG **5’**

ssODN

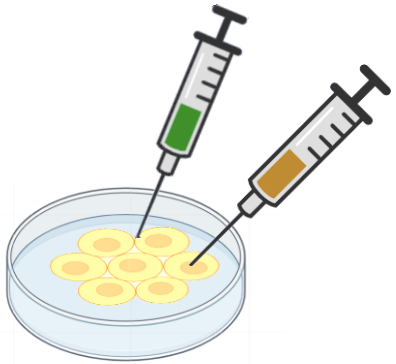
5’GCTTTAATCTGGCCCCTTTGGG
AAAGCGAAAATCCCGATCACATT
GGACATCGGCAAAGGCGTGCCA
TAAAGAG **3’**

eu.idtdna

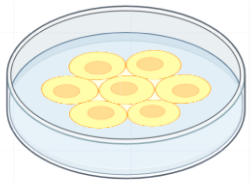
In vitro

TRANSFECTION

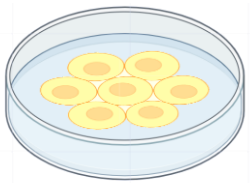
- RNP (Cas9 + sgRNA)
- AAV6 (ssDNA)
- PVA



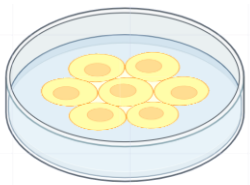
MEF RNP+ AAV6+ PVA+



MEF RNP+ AAV6+



MEF WT



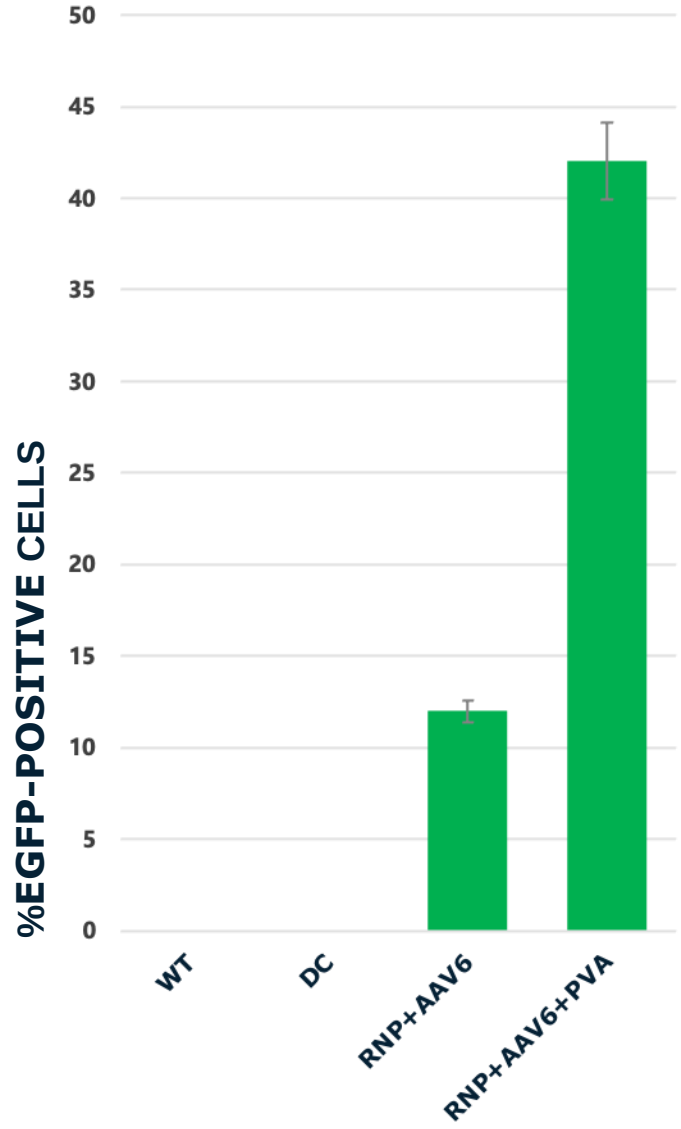
MEF DC

DOUBLE-SELECTION

PUROMYCIN RESISTANCE

GFP+ (FACS)

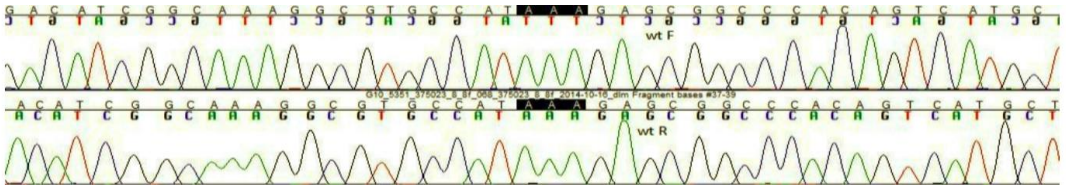
ANALYSIS



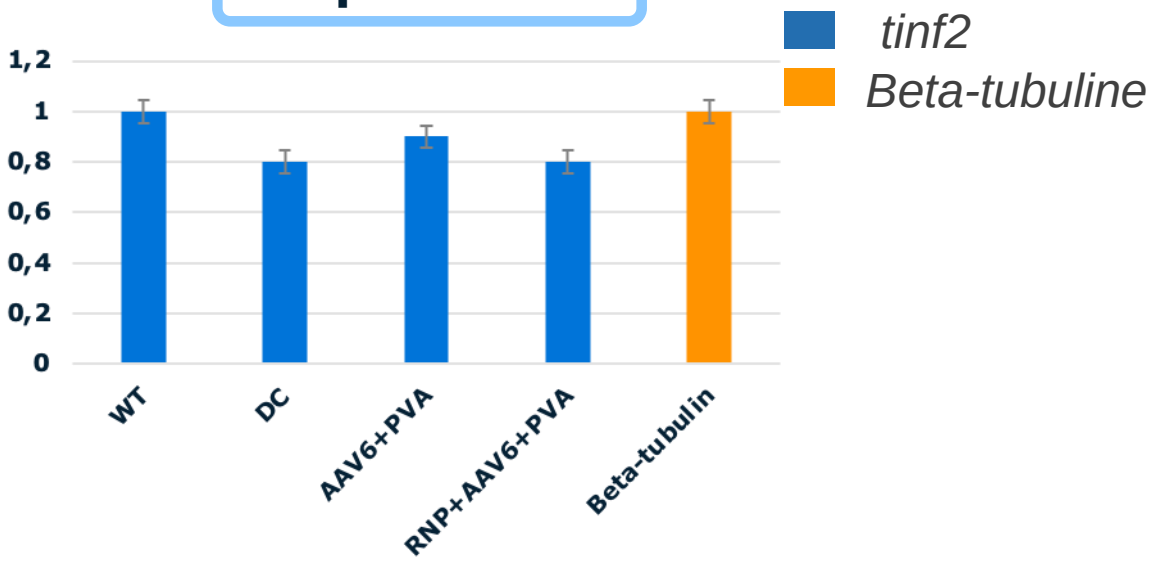
Is the *tinf2* sequence and its expression in MEF cells changed?

ON / OFF – TARGET DETECTION

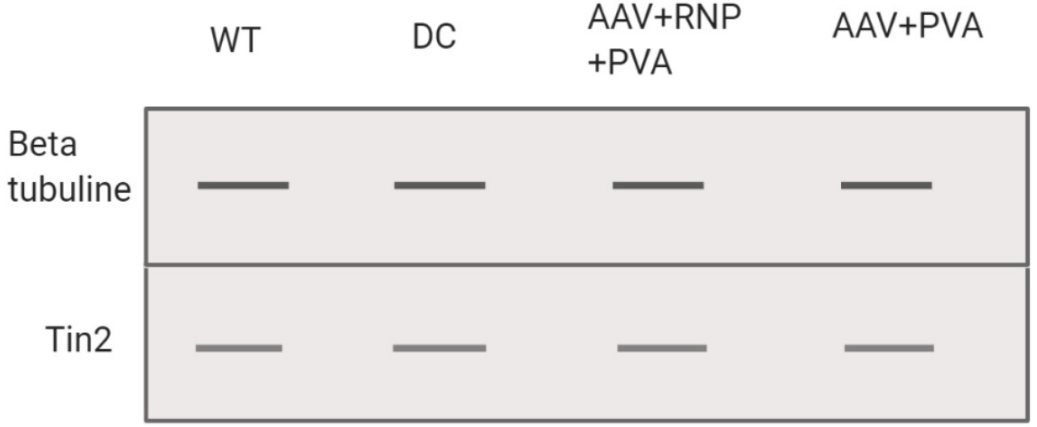
NGS(WHOLE GENOME SEQ)-TARGETED AMPLICON SEQ



q-PCR



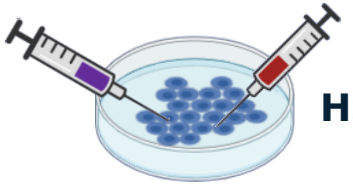
WESTERN BLOT



Ex vivo

EXTRACTION & TREATMENT

- 200’000 Bone marrow HSPCs



HSPCs

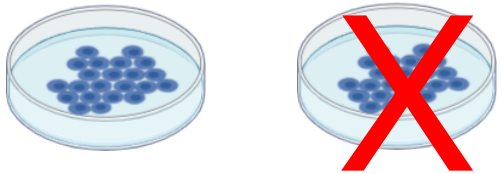
- Cell stimulation with cytokine

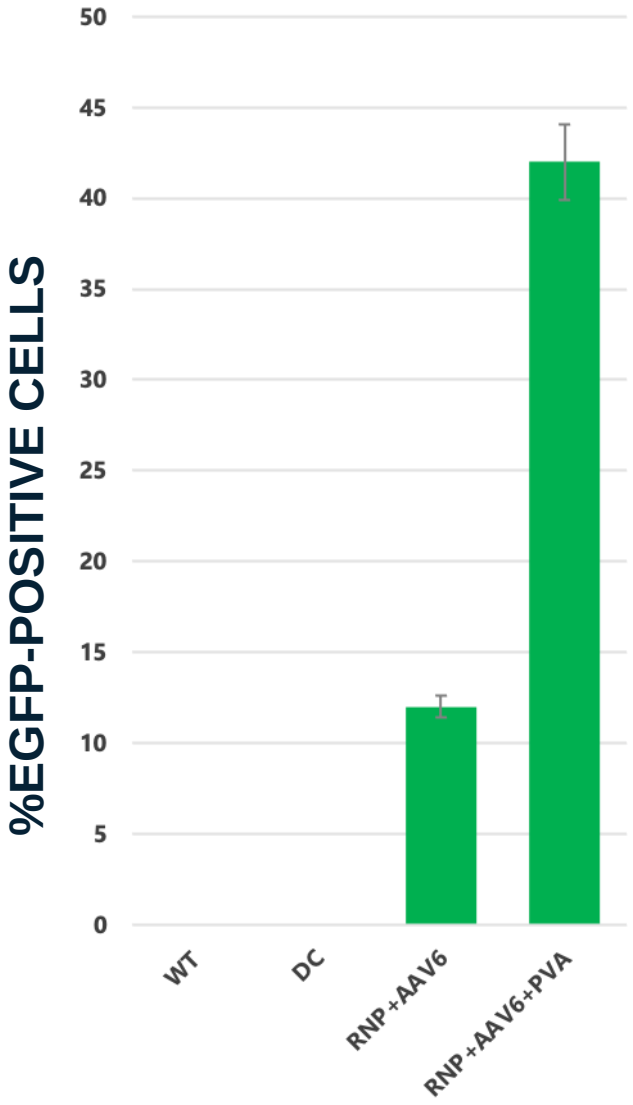
TRANSFECTION

DOUBLE-SELECTION

PUROMYCINE RESISTANCE

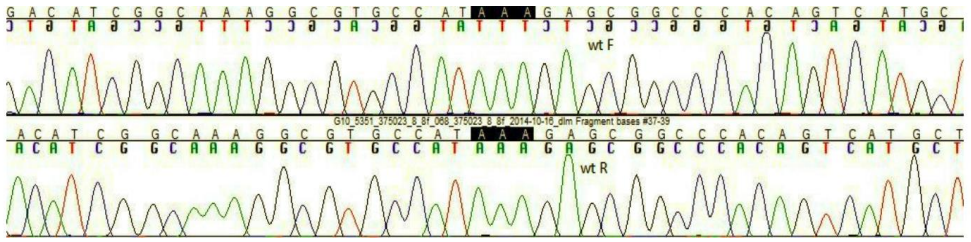
GFP+(FACS)



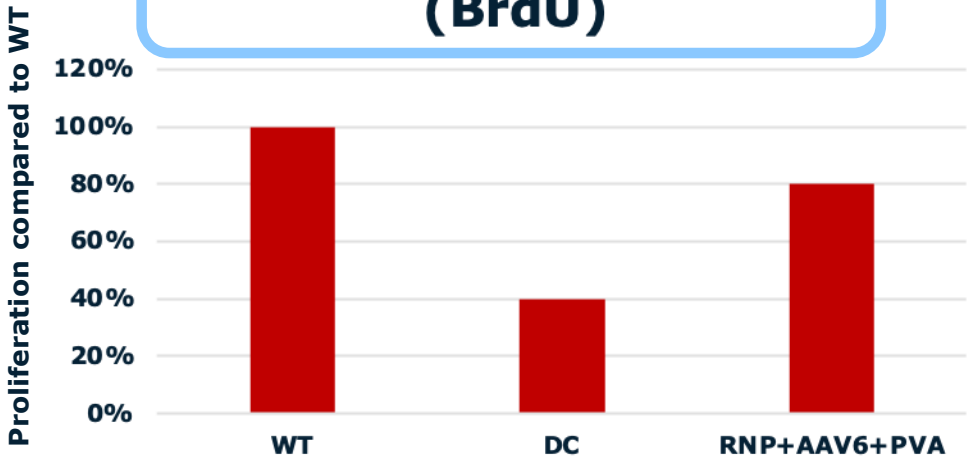


Is the *tinf2* sequence and its expression in HSPCs changed?

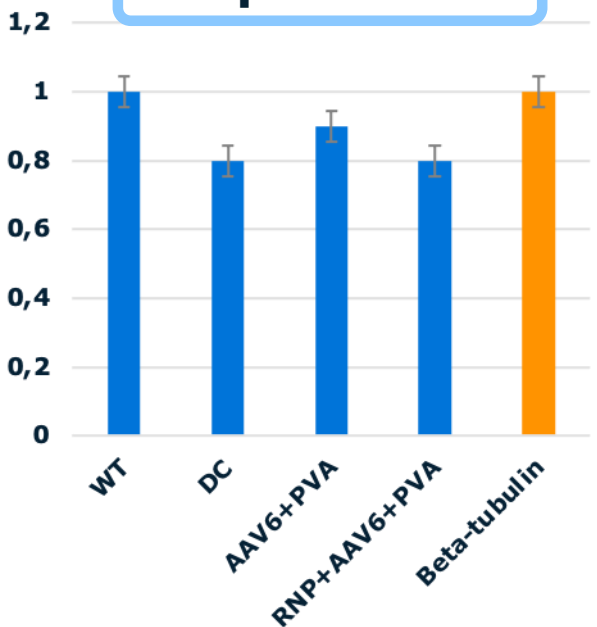
NGS(WHOLE GENOME SEQ) TARGETED AMPLICON SEQ



PROLIFERATION ASSAY (BrdU)



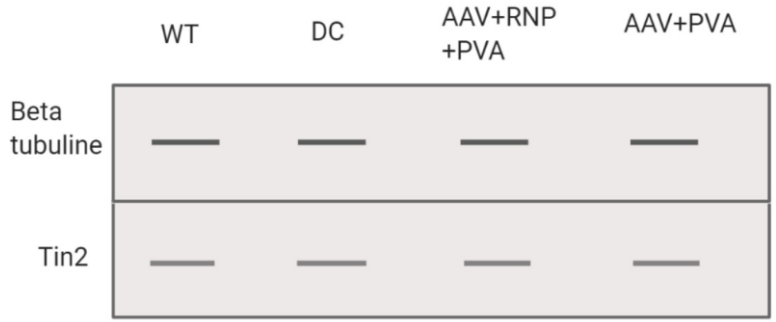
q-PCR



tinf2

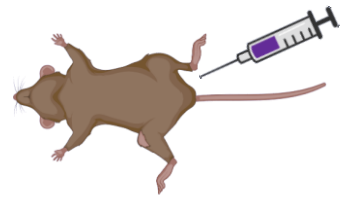
Beta-tubulin

WESTERN BLOT



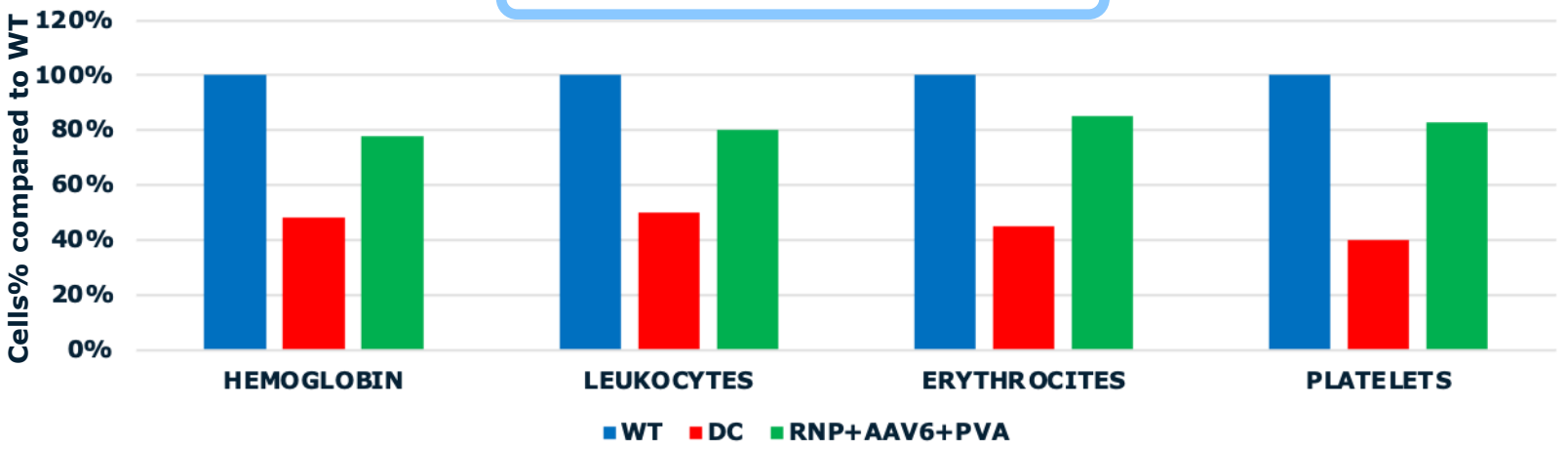
Cell expansion

Transplant



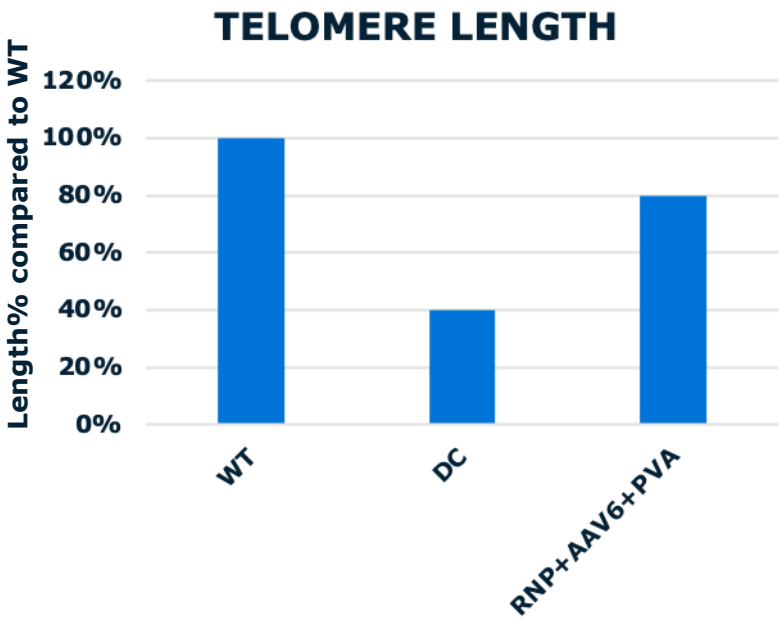
Is the Bone Marrow population growing?

BLOOD CELLS COUNT

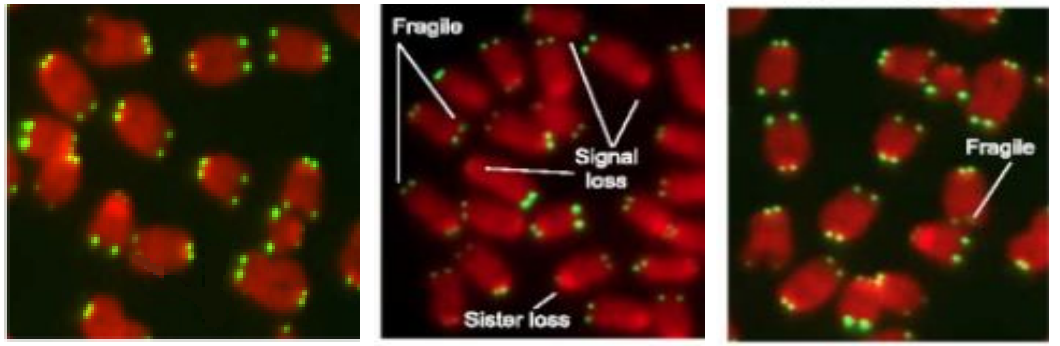


16 weeks after transplantation

WHAT ABOUT THE TELOMERE LENGTH?



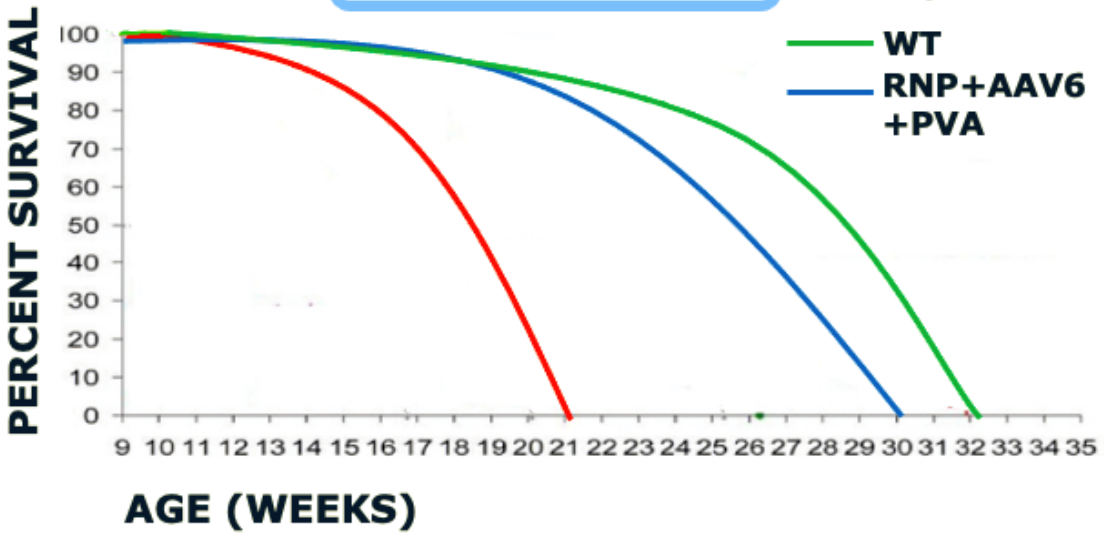
Q-FISH



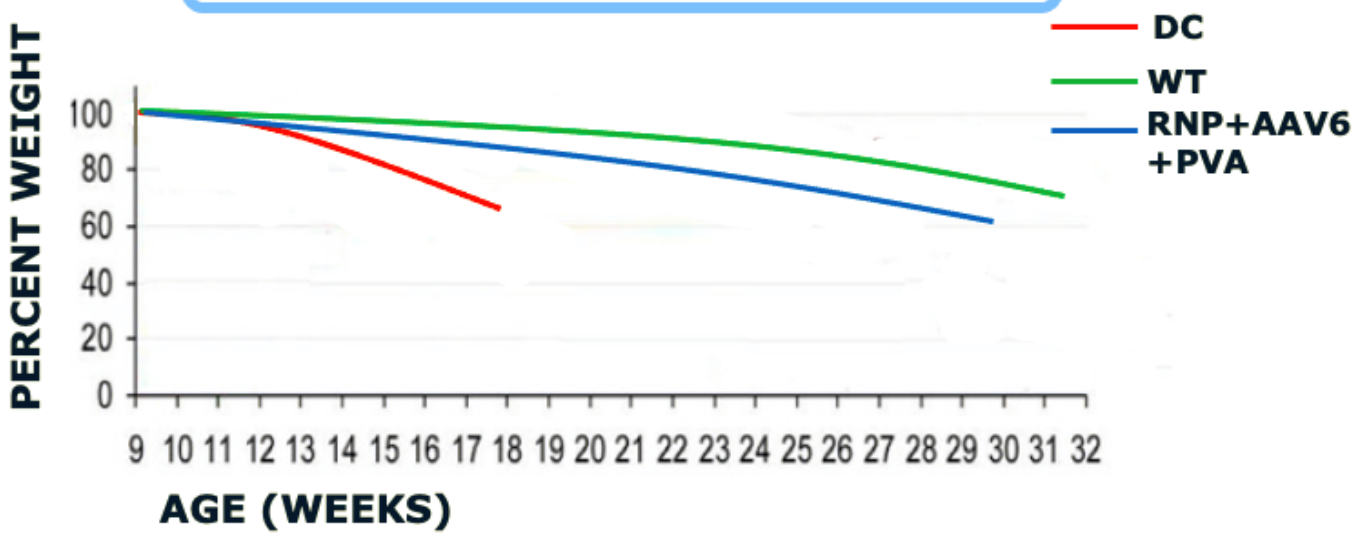
WT DC RNP+AAV6+PVA

Mouse health evaluation and results

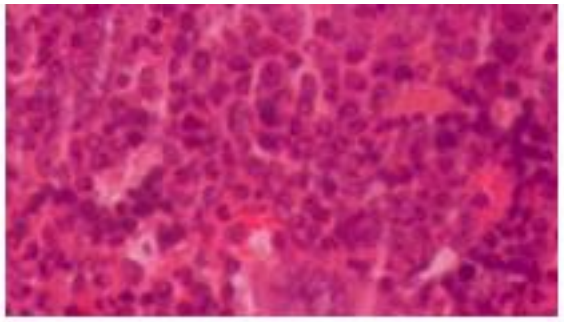
SURVIVAL



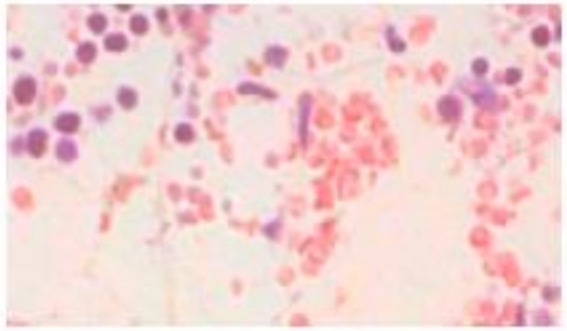
BODY WEIGHT PROGRESSION



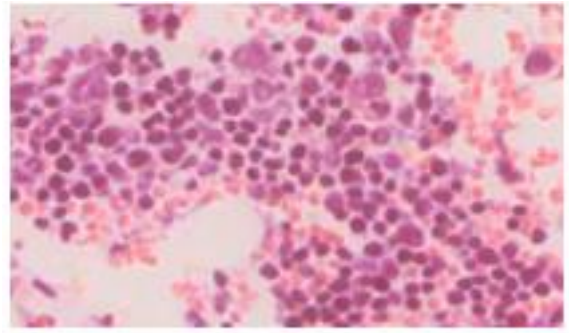
WT MOUSE BM



DC MOUSE BM



RNP+AAV6+PVA MOUSE BM



Conclusion

- **We expect a significant presence of TIN2 WT protein, which can allow the shelterin complex to carry out it's function to maintain the correct telomere length.**

- We also expect the bone marrow repopulation, proliferation and differentiation.

- **Ex vivo experiments**

1° year → **2° year**

- **Preparation of the vector**
- ***In vitro* experiments**
- **Checking results**

Future perspectives

Our strategy is just a starting point which will lead the DC research to a next level.
We can imagine the possibility to treat DC human patients in vivo and ex vivo, also in the other genes involved in the disease.
Our aim is to improve this approach, making it less invasive, using iPSCs, and developing new tools to make it more efficient.

Budget

Method	Price	Source
TINF2$^{DC\text{-}cond}$	€ 20000	Jax.org
MEF cell line	€200	Lgstandars-atcc.org
CRISPR/Cas9 Nickase +sgRNAs + ssODNs	€1500	Genescript.com
AAV6	€ 2000	Vigenbio.com
FISH, FACS, q-PCR, SEQS, WT-BLOT, Proliferation assay	€ 5000	
1 PI, 2PhD, 1 technician	€200’000	
Total	**Aproximately €230’000**	

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