SAPIENZA
UNIVERSITÀ DI ROMA

# TERC overexpression using AAV9-CRISPR in Dyskeratosis Congenita


PALCAU ALINA

PELLEGRINI FLAMINIA

PERCIBALLI ELISA

SCALZITTI SILVIA

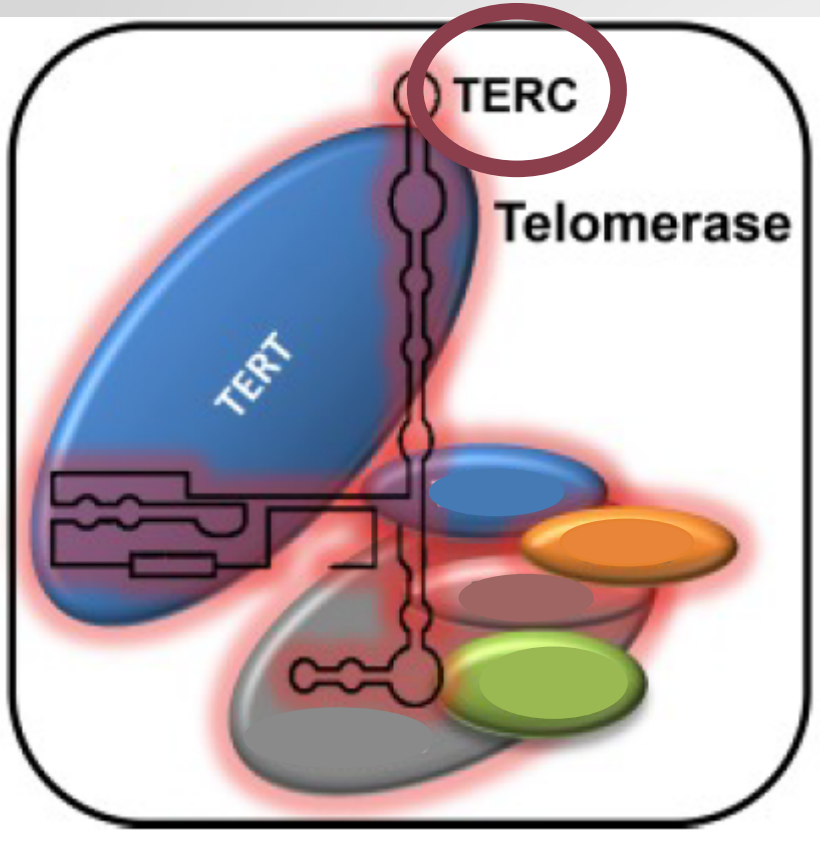

GENETICS AND MOLECULAR BIOLOGY

GENE THERAPY

PROF. ISABELLA SAGGIO

A.Y. 2018/2019

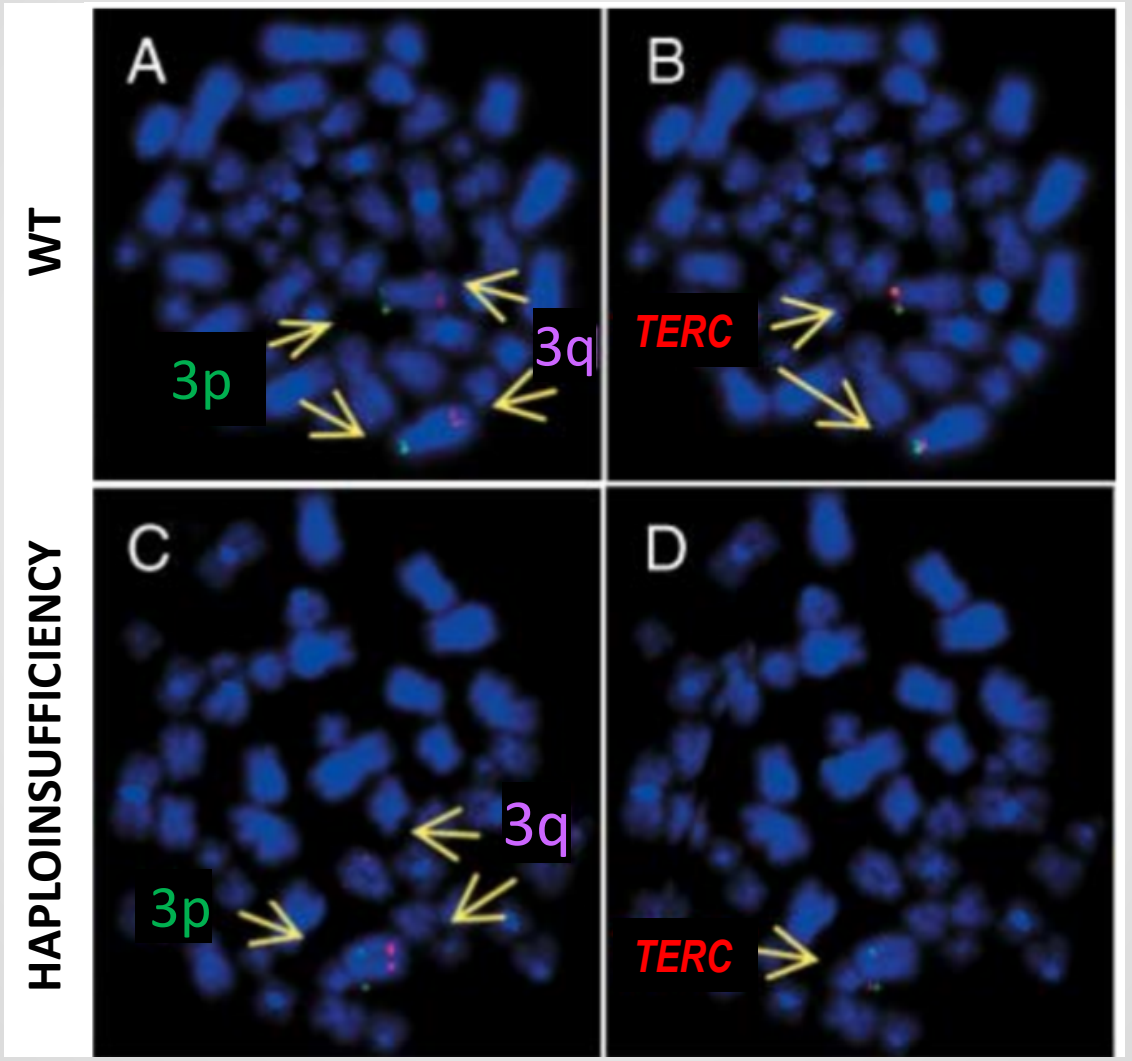
# TELOMERASE DEFECTS CAUSE TELOMEROPATHIES




Adapted from: Holohan B et al. J Cell Biol. (2014 )

- Telomerase maintains telomere length in proliferating cells.
- Telomerase is a ribonucleoprotein complex.
- Mutations in one of its components affect its activity.
- Overactivation of telomerase is an hallmark of cancer.

# TERC HAPLOINSUFFICIENCY IN DYSKERATOSIS CONGENITA

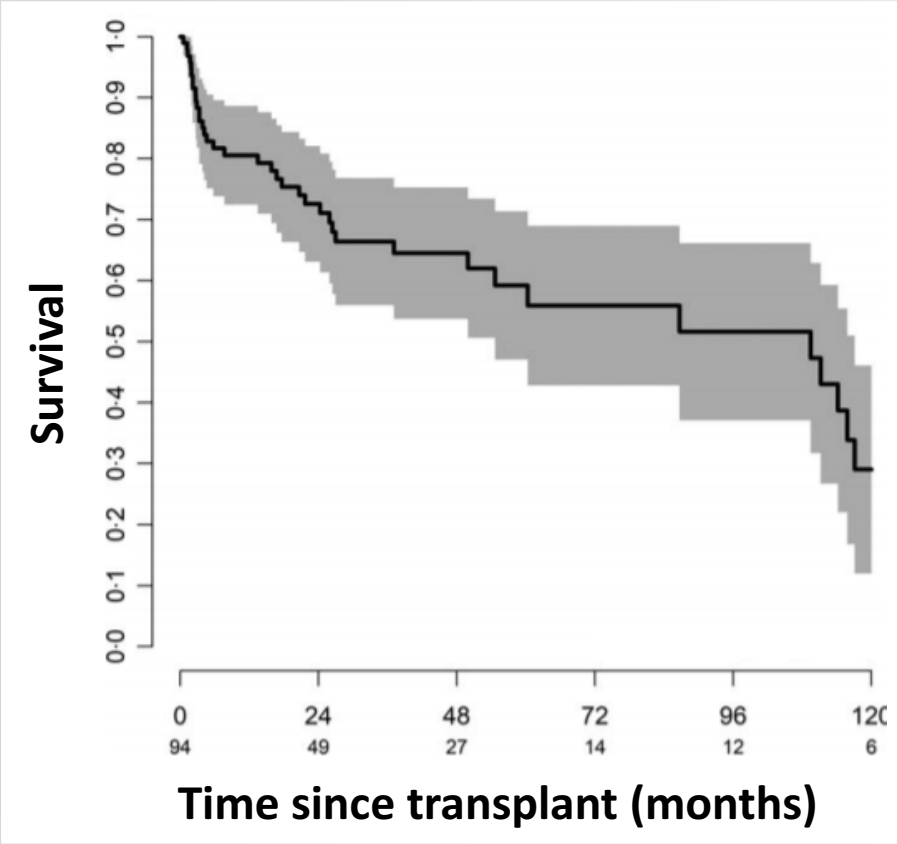



Adapted from: Du HY et al. Aging Cell (2007)

# CURRENT CLINICAL TRIALS

| STUDY TITLE | CONDITIONS | INTERVENTIONS | LOCATIONS | LAST UPDATE POSTED |
|---|---|---|---|---|
| **Investigation of the Genetics of Hematologic Diseases** | • Bone Marrow Failure Syndromes<br>• Erythrocyte Disorder<br>• Leukocyte Disorder | | St. Jude Children's Research Hospital<br>Memphis, Tennessee, United States | October 23, **2018** |
| **Treosulfan and Fludarabine Phosphate Before Donor Stem Cell Transplant in Treating Patients With Nonmalignant Inherited Disorders** | • Hematopoietic Cell<br>• Transplantation Recipient<br>• Non-Malignant | • **Procedure:** Allogeneic Bone Marrow Transplantation<br>• **Biological:** Anti-Thymocyte Globulin<br>• **Drug:** Cyclosporine | • Children's Hospital Colorado<br>Aurora, Colorado, United States<br>• Oregon Health and Science University<br>Portland, Oregon, United StateS<br>• Vanderbilt University | April 25, **2018** |
| **Hematopoietic Stem Cell Transplant for Dyskeratosis Congenita or Severe Aplastic Anemia** | • Dyskeratosis Congenita<br>• Aplastic Anemia | • **Drug:** Alemtuzumab<br>• **Drug:** Fludarabin<br>• **Drug:** Cyclophosphamide | University of Minnesota Medical Center, Fairview<br>Minneapolis, Minnesota, United States | September 25, **2018** |

# GOALS

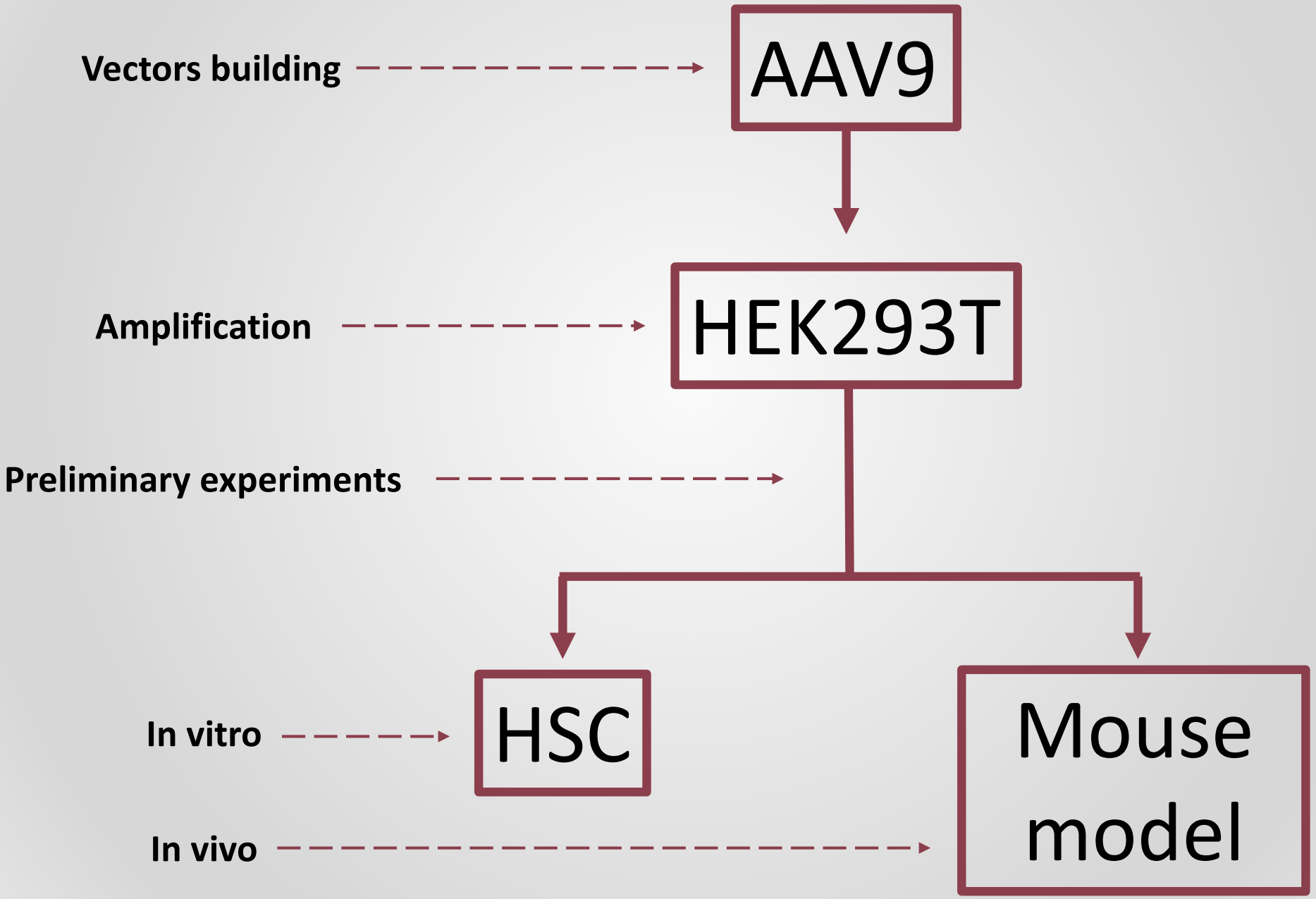



Francesca Fioredda et al. July (2018)

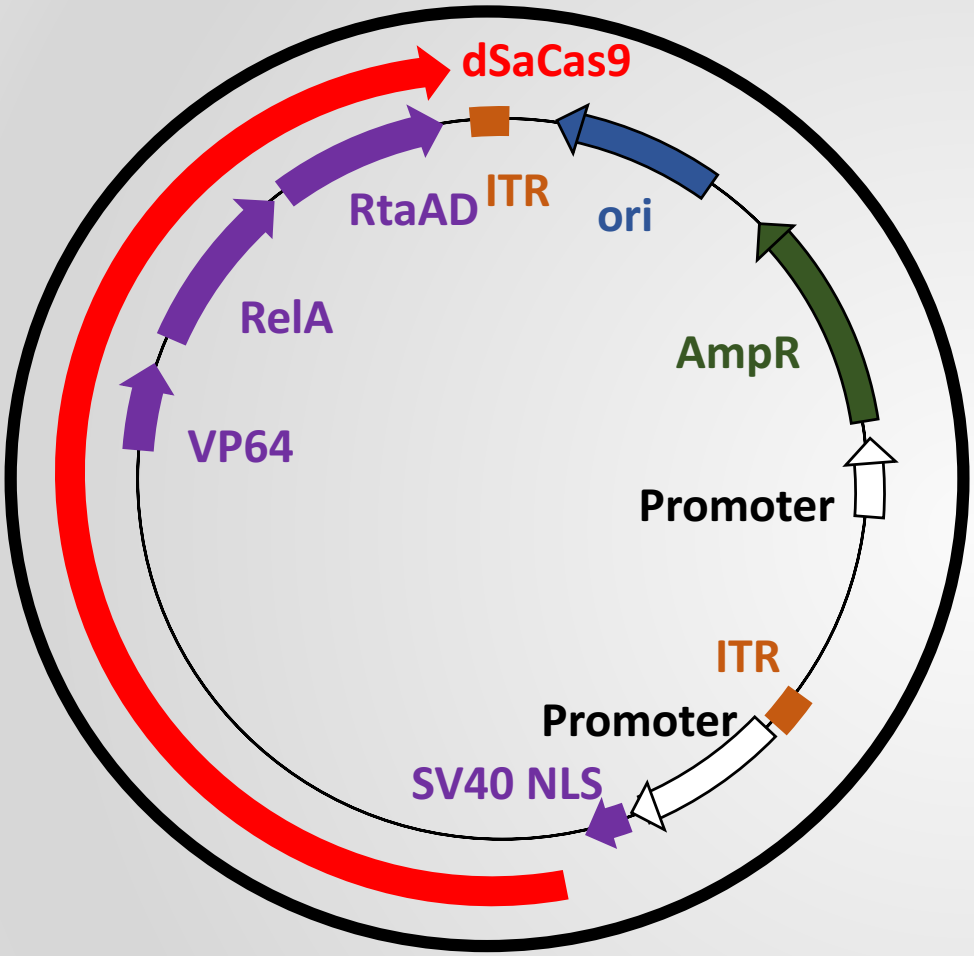
**Rescue of telomerase activity using a novel gene therapy approach: CRISPR/dSaCas9 mediated TERC overexpression.**

- Increase telomere length
- Increase telomerase activity
- Increase survival
- Avoid tumorigenesis

# EXPERIMENTAL PLAN

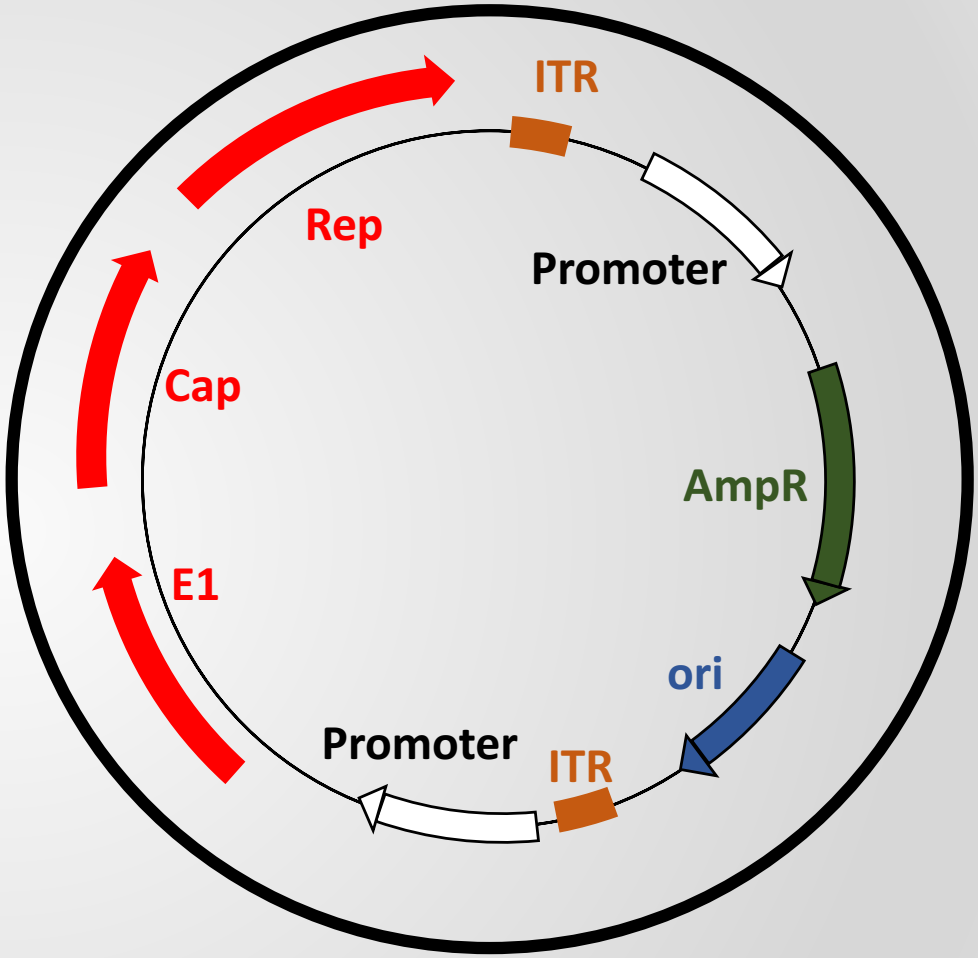


# VECTORS BUILDING




**AAV9-dSaCas9-VPR**

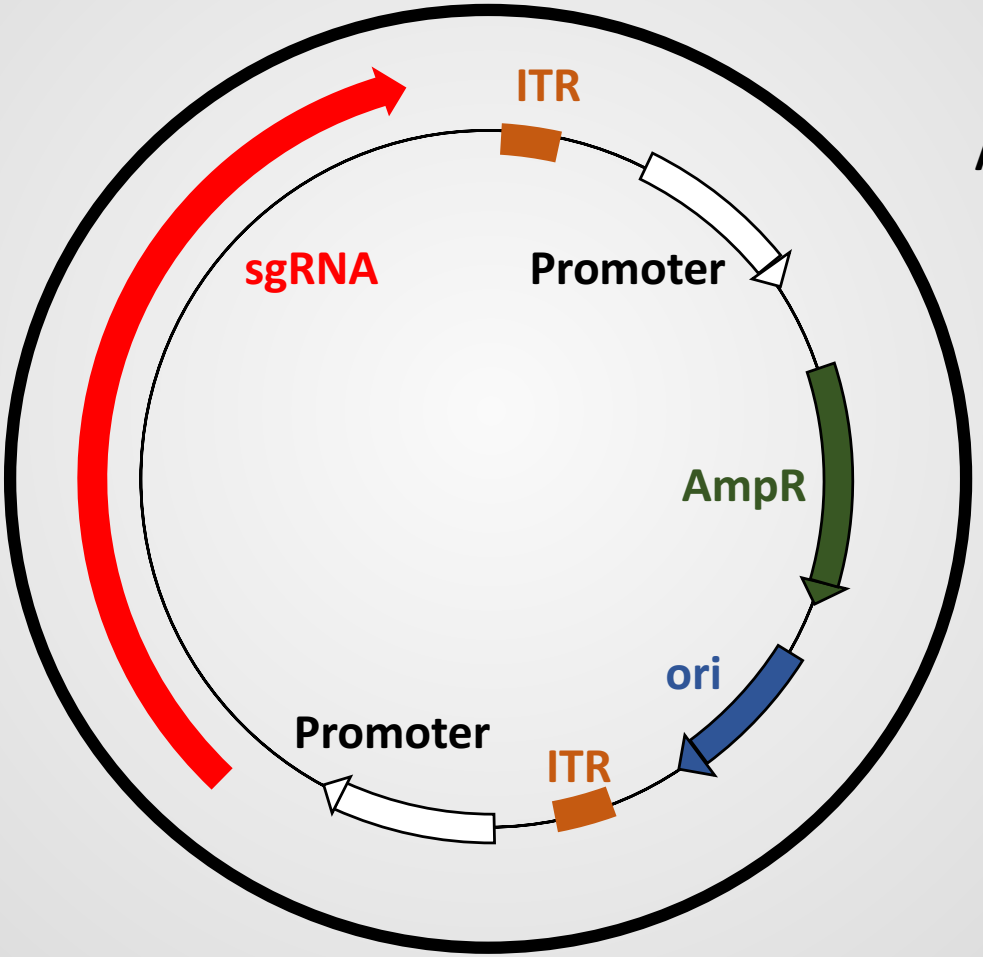
Modified from: https://www.addgene.org/68495/




**AAV9-helper**

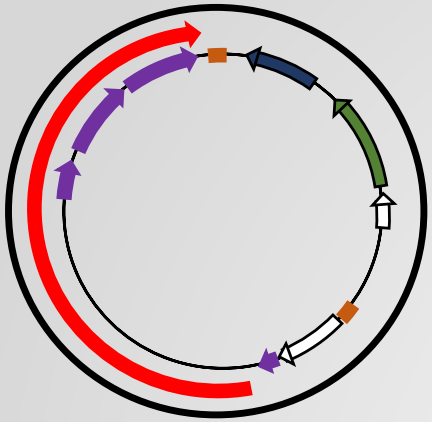
Modified from: https://www.addgene.org/81070/

# VECTORS BUILDING

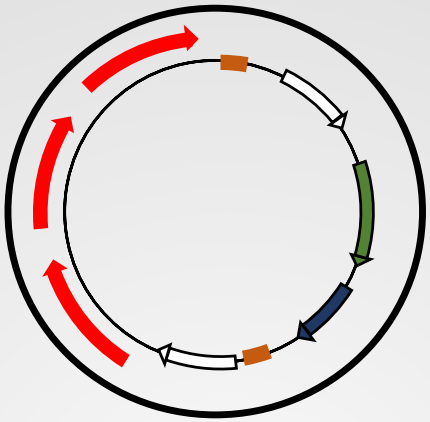



AAV9-sgRNA → TERC

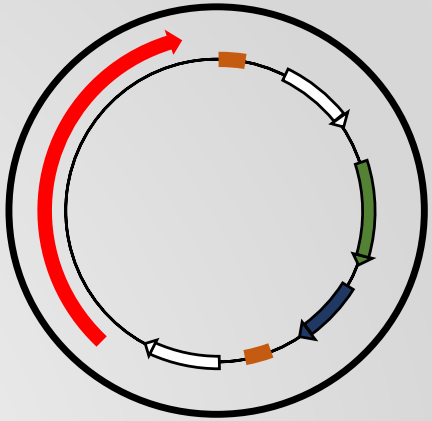
# AMPLIFICATION



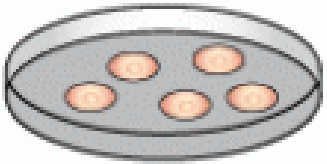
AAV9-dSaCas9-VPR



AAV9-helper



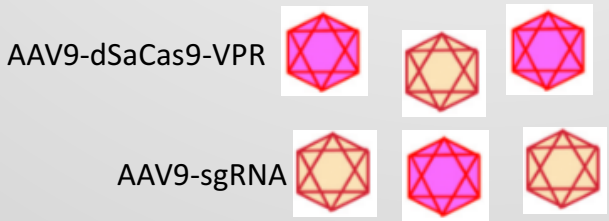
AAV9-sgRNA



Selection with Ampicillin

HEK293T

Purification in CsCl gradient



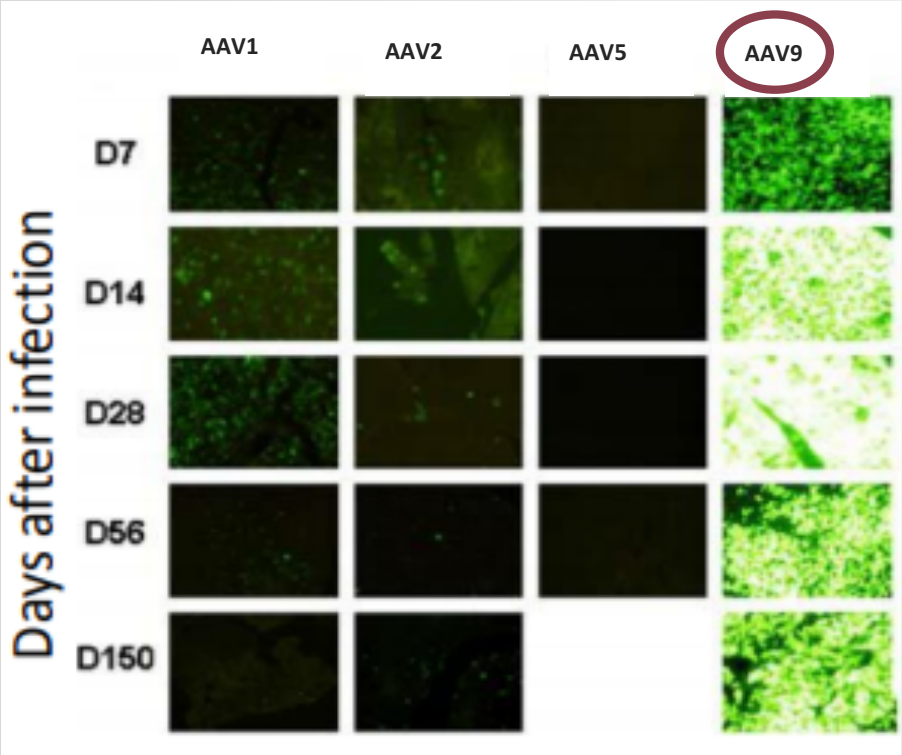
AAV9-dSaCas9-VPR

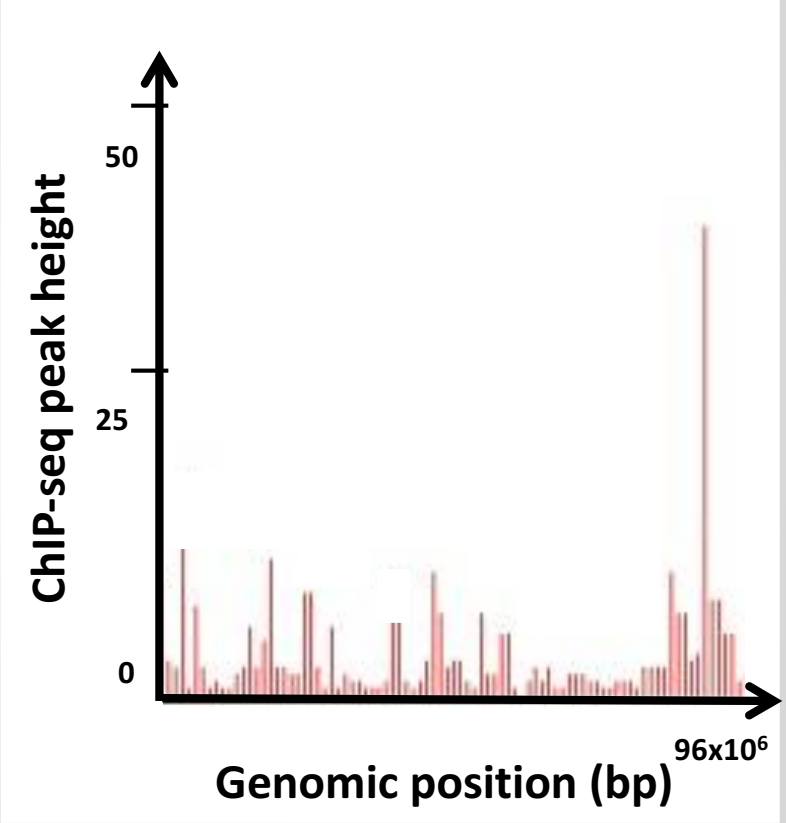
AAV9-sgRNA

Viral genome titer: qRT-PCR

# PRELIMINARY EXPERIMENTS: USE OF AAV9 SYEROTYPE AND SPECIFICITY OF dSaCas9

Adapted from: Kevin S. Myers et al. Methods (2015)

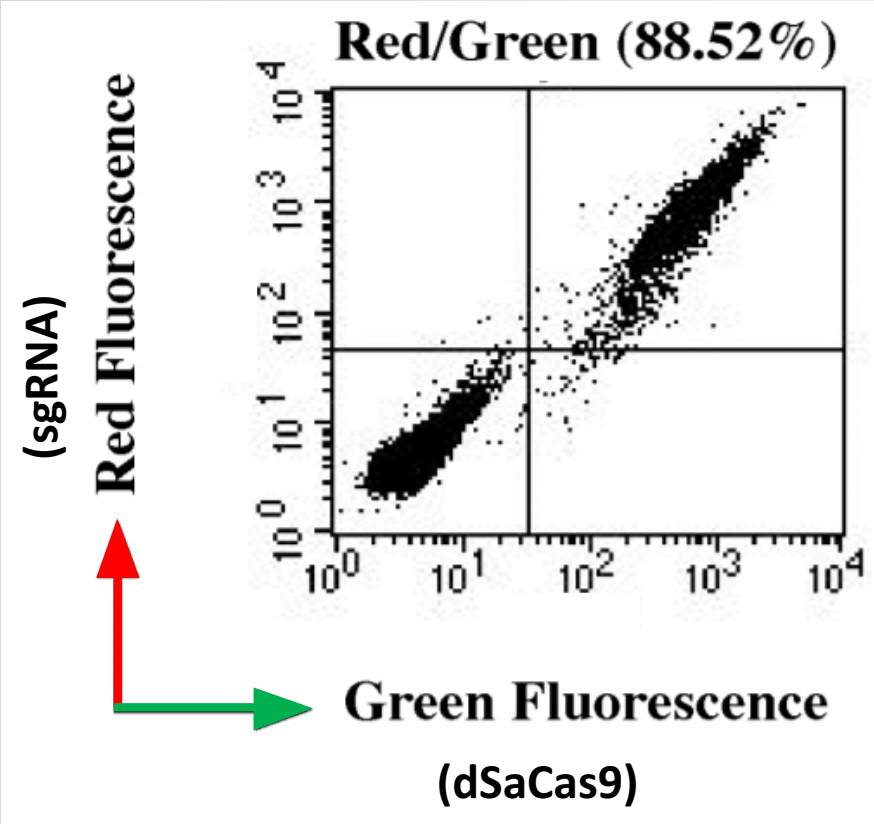


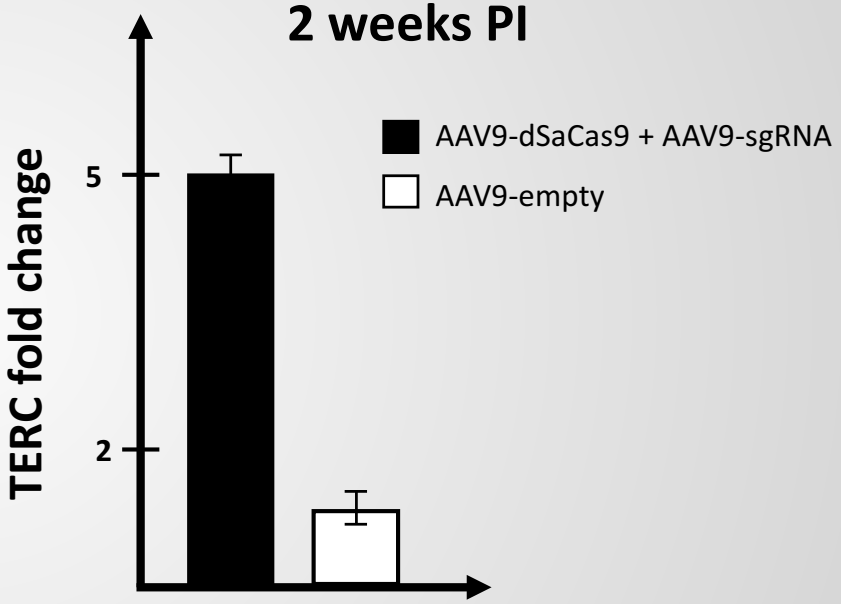




→ **Analysis of fluorescence:** AAV9 produces a high GFP expression, maintained overtime.

→ **ChIP-seq analysis:** the highest peak corresponds to the most frequent binding of dCas9, that is TERC sequence (chromosome 3).

# PRELIMINARY EXPERIMENTS: CO-TRANSFECTION AND VECTOR EXPRESSION ASSAYS

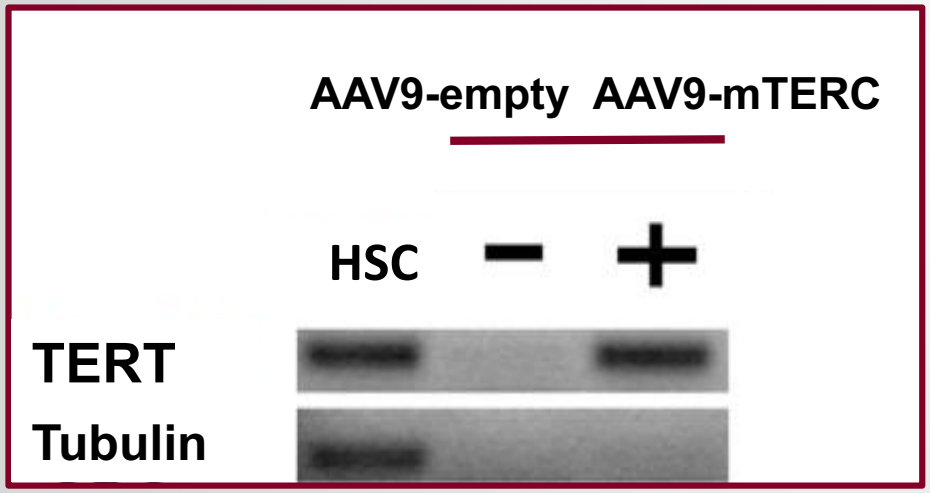


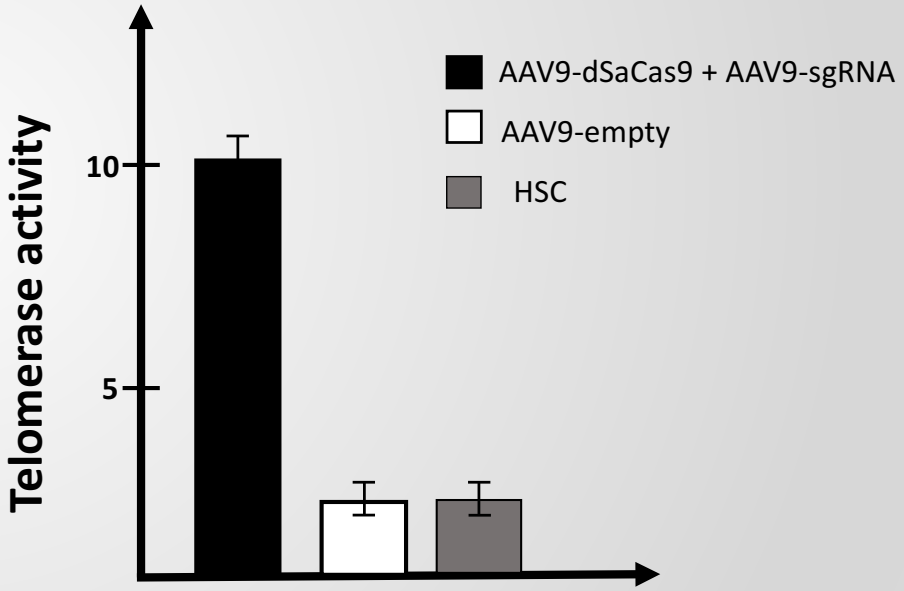

→ **FACS:** cotransfected cells are in the top right side of the graph and they are 88,52% of the total amount of HSC.

→ **qRT-PCR:** TERC expression in HSC is significantly increased after AAV9-dSaCas9 + AAV9-sgRNA

# IN VITRO: TERC-TERT INTERACTION

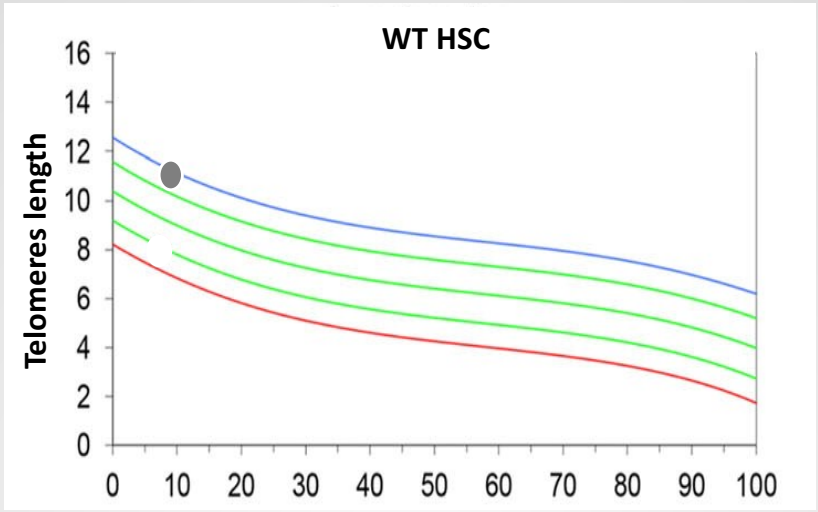

Adapted from: Christian B. et al. Bloodjournal (2018).

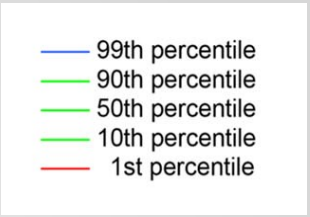


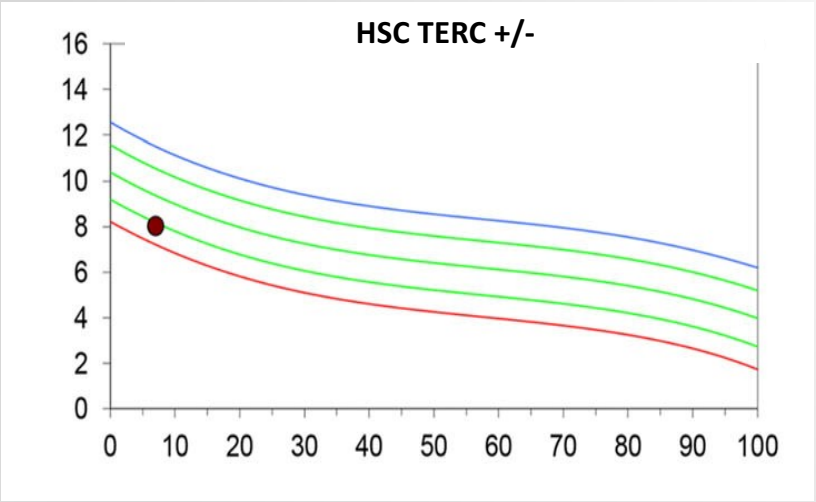

→ **Pull down assay:** exogenous TERC is able to interact with TERT

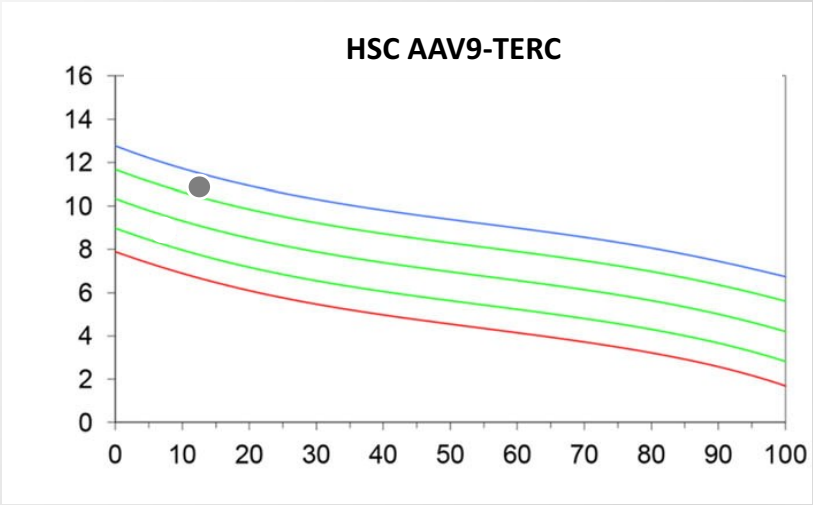
→ **TRAP assay:** telomerase activity is increased in treated cells

# IN VITRO: TELOMERE LENGTH ANALYSIS



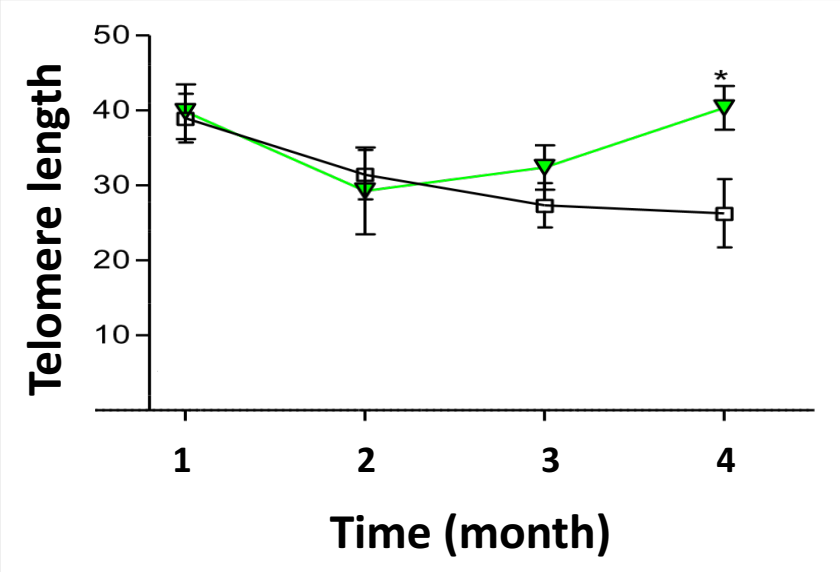




Brody H. et al. BMC Genomics (2016).

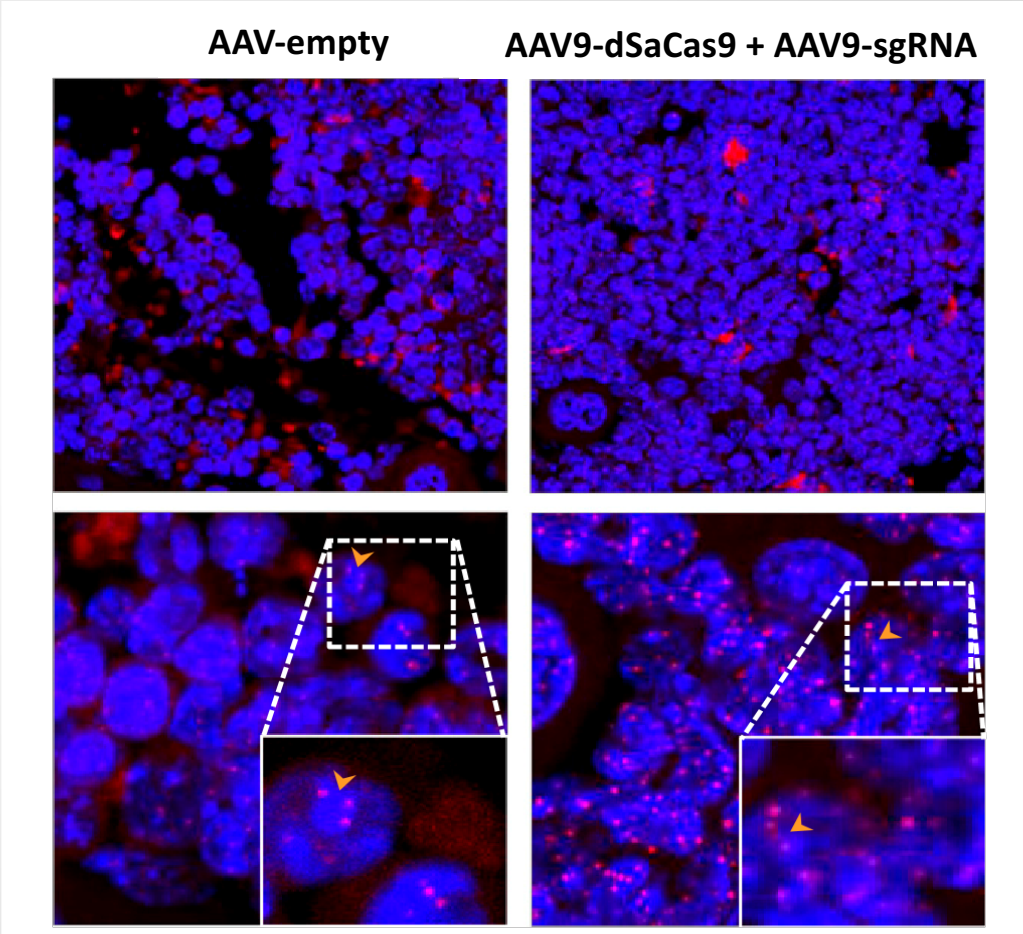
→ **FlowFish analysis:** TERC overexpression rescues telomere length.

# IN VITRO: TELOMERE LENGTH ANALYSIS




▼ AAV9-dSaCas9 + AAV9-sgRNA

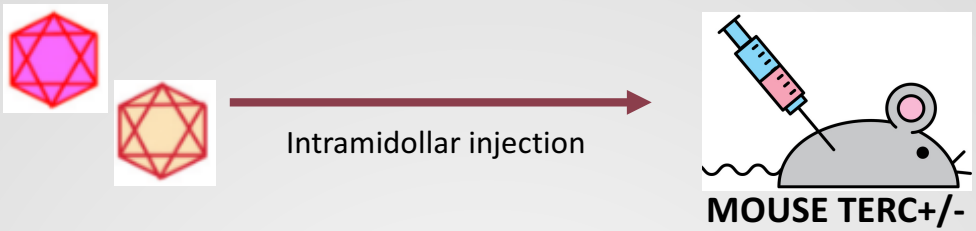
□ AAV9-empty

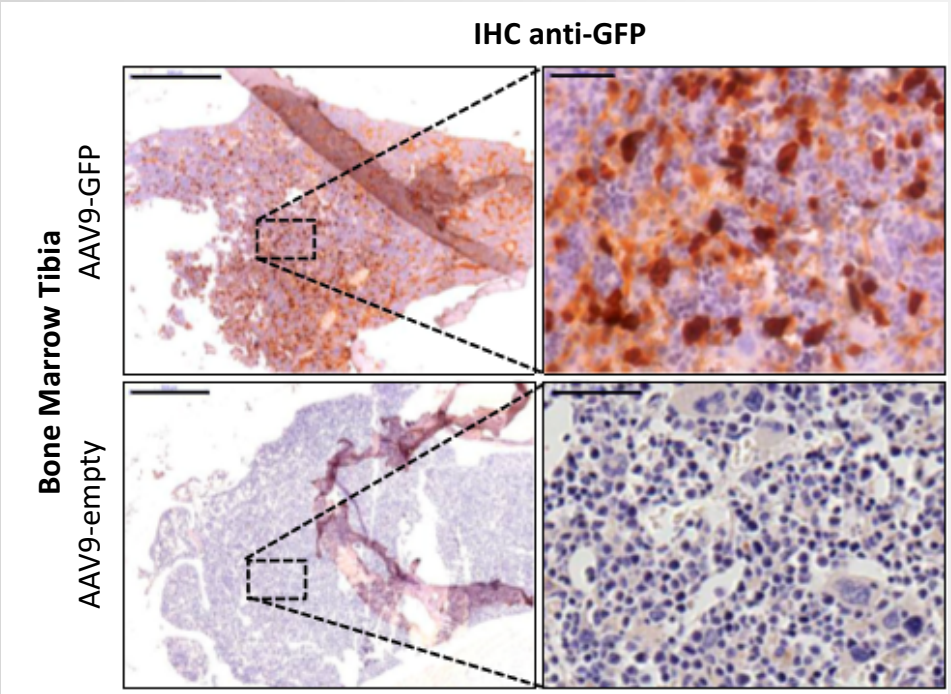


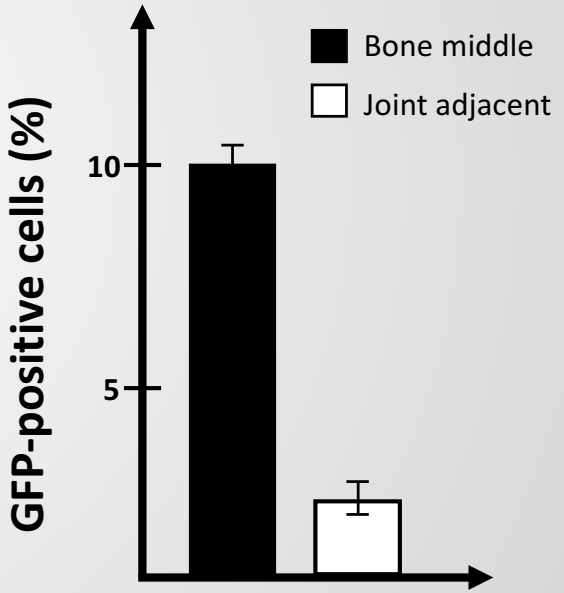

Adapted from: Christian B. et al. Bloodjournal (2018).

→ **qFISH:** AAV9-dSaCas9 + AAV9-sgRNA increase telomere length, compaired to the empty vector.

# IN VIVO: LOCALIZATION AND TRANSDUCTION



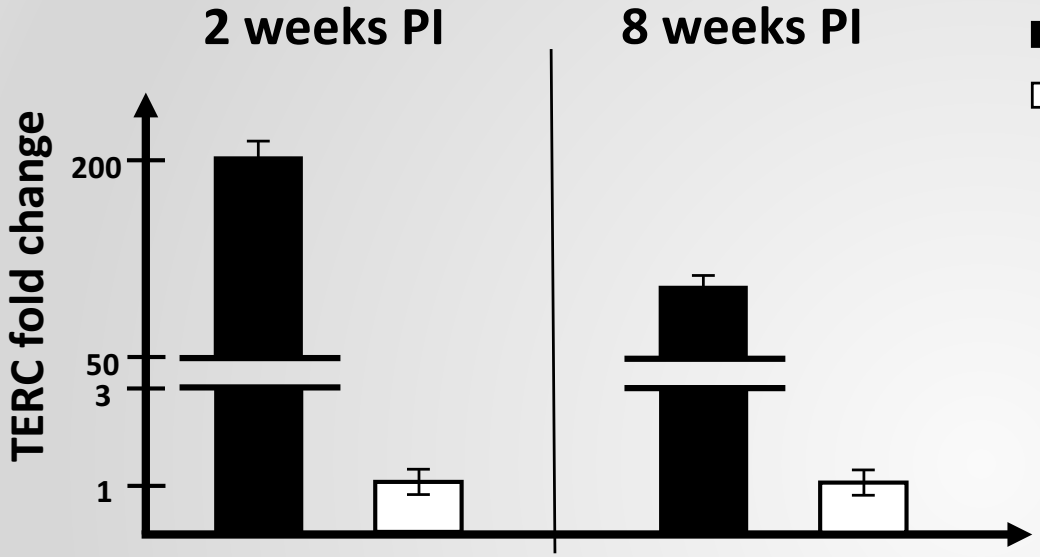



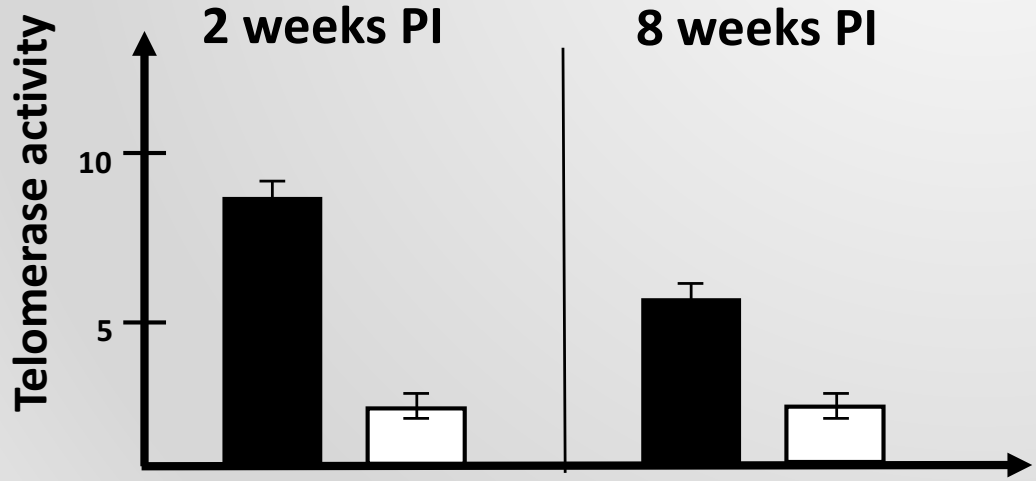

Adapted from: Blasco et al., Blood, 2016

→ **Immunoistochemistry:** the analisys with AAV9-GFP reporter showed the highest transduction level in bone middle regions.

# IN VIVO: TERC EXPRESSION AND TELOMERASE ACTIVITY

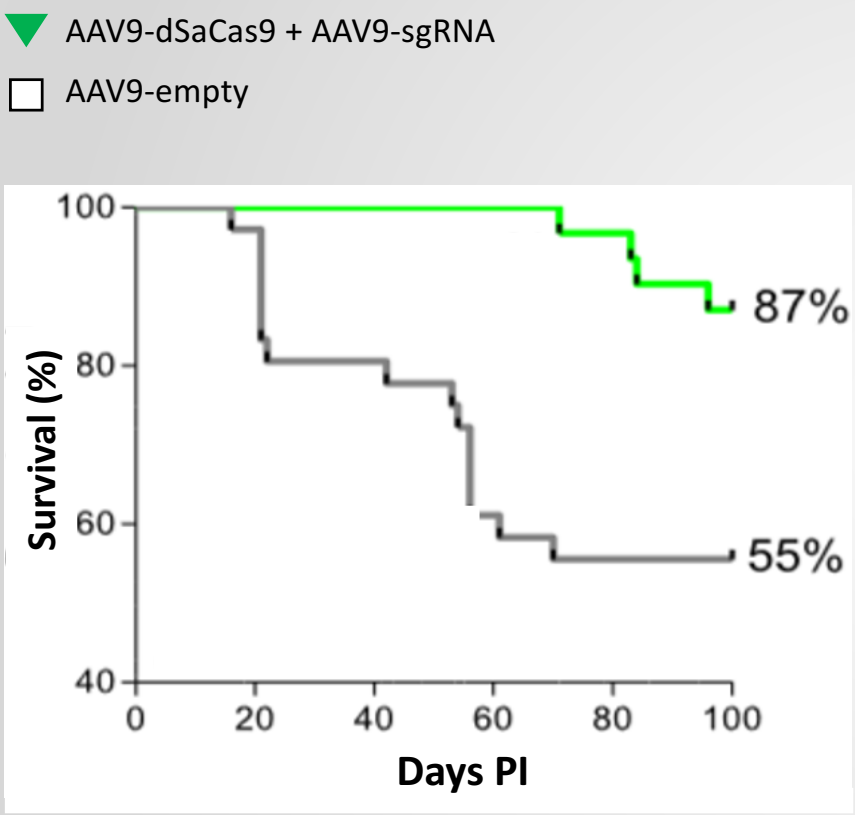



→ **qRT-PCR:** TERC expression is significantly increased in treated mice

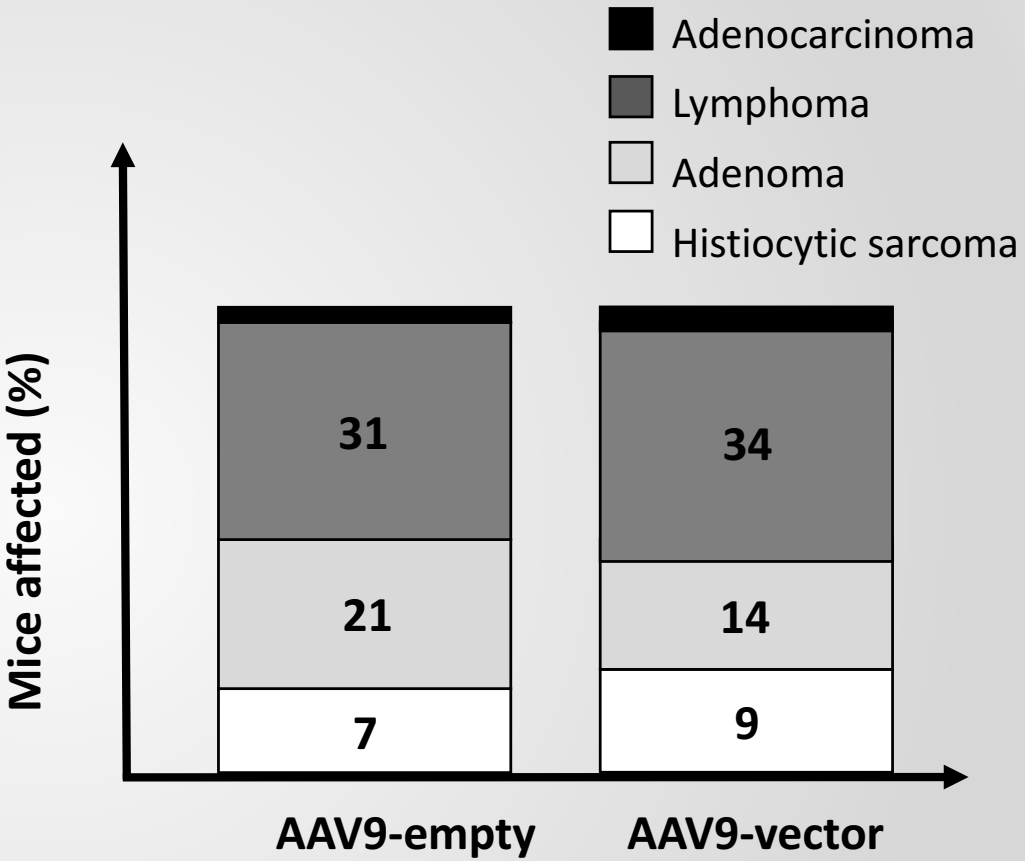



→ **TRAP assay:** telomerase activity is increased in treated mice

# IN VIVO: TERC OVEREXPRESSION INCREASES SURVIVAL WITHOUT INCREASING CANCER



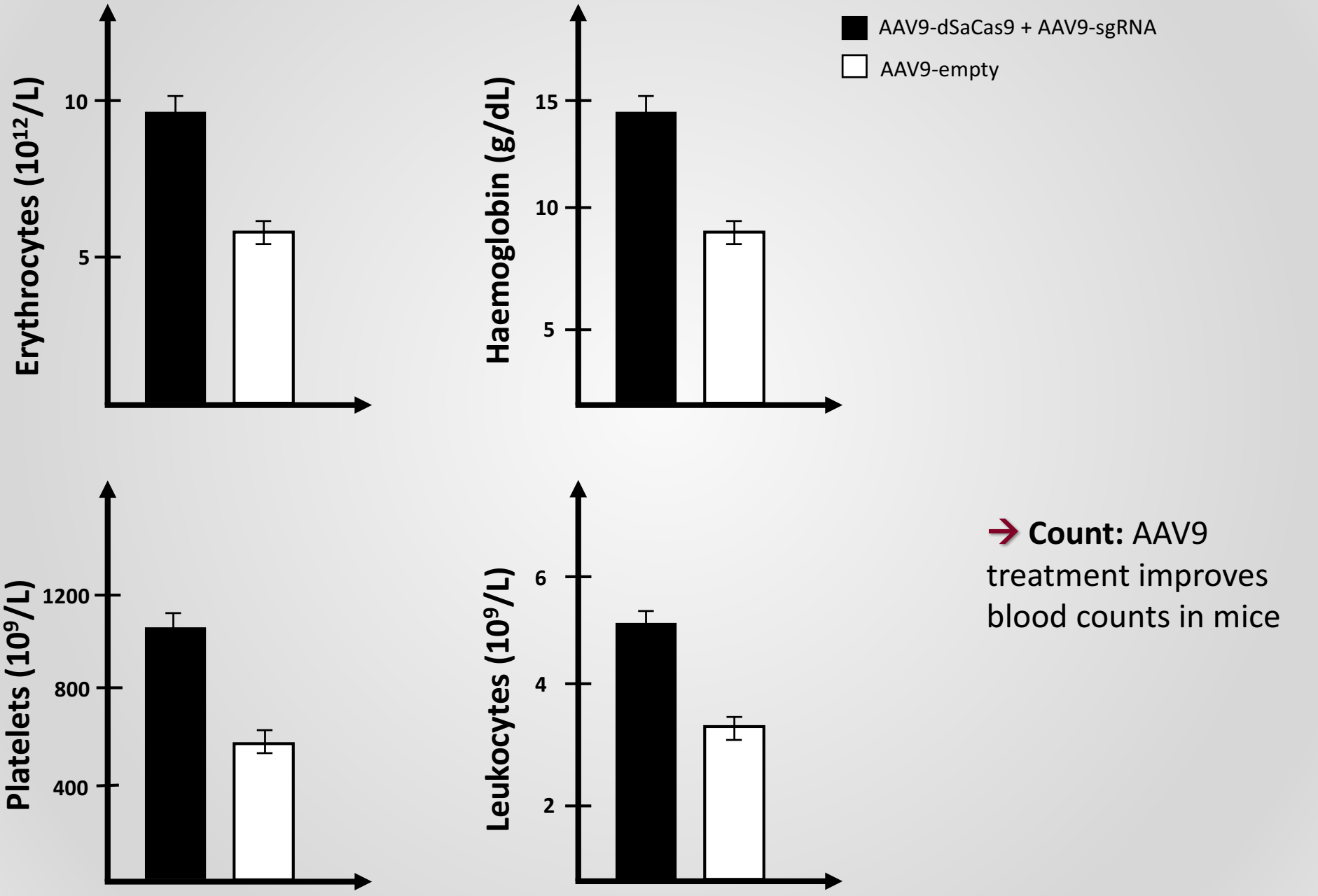

Adapted from "Blasco et al., Blood, 2016.




→ **Survival curves:** the treatment significantly rescues mouse survival.

→ **Pathological analysis:** of all mice under treatment at their time of death. Treated mice of both age groups did not show increased cancer incidence compared to the controls.

# IN VIVO: HUMAN ENDPOINT BLOOD COUNTS




→ **Count:** AAV9 treatment improves blood counts in mice

# PITFALLS AND SOLUTIONS

**OFF TARGETS EFFECTS**

- Guide RNA target with 2 G at 5’
- Codelivery of DNA decoy

**AAV-9 IMMUNOGENICITY**

- Empty capsid mutants can be used as decoys to overcome pre-existing humoral immunity by adsorbing antibodies in the bloodstream upon systemic delivery of both empty and functional AAVs1
- The AAV capsids can be genetically engineered or mutated to reduce the binding affinity and the neutralizing effects of AAV antibodies

**IMMUNE RESPONSE DUE TO PROLONGED EXPRESSION OF CAS9**

→ Conditional genome editing with light-inducible or chemical-inducible

# MATERIAL AND COSTS

| MATERIAL | COST |
|---|---|
| 293(E1) CELLS (*Cell Biolab*) | 350$ (every 10 alla 6 cells) |
| AAV-GFP CONTROL VECTOR (*Cell Biolabs*) | 395$ (every 10µg + delivery costs) |
| AAV trasduction kit 50 reactions (*Antiboies-online.com*) | 1005 $ |
| AAV-CMV-Null Titer : 1x1013 GC/ml- (*Vector Biolabs*) | 5662 $ |
| Stemline hematopoietic stem cell expansion medium (*Sigma Aldrich*) | 268 $ |
| FiSH Tag DNA multicolor kit, Alexa Fluor dye combination (*Thermo Fisher*) | 752 $ |
| RT-PCR, Western Blot, IF, IP, Biochemical assays | (e.g. Abcam Ab 200 390 $ every 100 µl, Ab 66601 380 $ every 100 µl) |
| PCR purification + sequencing (*Biofab research*) | 15.11 $ (per sample) |
| Plastic | 1500 $ (per year) |
| Stabulation | 800 $ (per month) |
| TRAP kit | 300 $ |
| (We excluded instruments and materials that can be possibly collected thanks to collaboration with medical department) | |

**TOTAL COST: 6821,11$**

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

QIAGEN®

abcam®
discover more

SAGE LABS
A Horizon Discovery Group Company

Bio Fab research
Servizi di Supporto alla Ricerca

SIGMA-ALDRICH

TGM
Texas A&M Institute for Genomic Medicine

ThermoFisher SCIENTIFIC

# References


- A Cayuela ML, Flores JM, Blasco M. The telomerase RNA component Terc is required for the tumour-promoting effects of Tert overexpression. EMBO Rep. (2005)
- Brian L Ellis, Matthew L Hirsch, Jenny C Barker, Jon P Connelly, Robert J Steininger III and Matthew H Porteus: "A survey of ex vivo/in vitro transduction efficiency of mammalian primary cells and cell lines with Nine natural adeno-associated virus (AAV1-9) and one engineered adeno-associated virus serotype" Virology Journal 2013, 10:74
- Brody Holohan , Wanil Kim , Tsung-Po Lai , Hirotoshi Hoshiyama , Ning Zhang, Anas M. Alazami , Woodring E. Wright , M. Stephen Meyn , Fowzan S. Alkuraya and Jerry W. Shay. Impaired telomere maintenance in Alazami syndrome patients with LARP7 deficiency. BMC Genomics (2016)
- Brody Holohan, Woodring E. Wright, and Jerry W. Shay. Telomeropathies: An emerging spectrum disorder. J Cell Biol. 2014 May 12; 205(3): 289–299.
- Bruno Bernardes de Jesus, Elsa Vera, Kerstin Schneeberger, Agueda M. Tejera, Eduard Ayuso, Fatima Bosch, Maria A. Blasco. Telomerase gene therapy in adult and old mice delays aging and increases longevity without increasing cancer. EMBO Mol Med. 2012 Aug;4(8):691-704.
- Capone, D'Alise, Ammendola *et al: "*Development of chimpanzee adenoviruses as vaccine vectors: challenges and successes emerging from clinical trials" *Expert Rev. Vaccines* 12(4), 379–393 (2013).
- Christian Bar, Juan Manuel Povedano, Rosa Serrano, Carlos Benitez-Buelga, Miriam Popkes, Ivan Formentini, Maria Bobadilla, Fatima Bosch, and Maria A. Blasco Telomerase gene therapy rescues telomere length, bone marrow aplasia, and survival in mice with aplastic anemia. (Blood. 2016;127(14):1770-1779)
- Cia-Hin Lau , Yousin Suh :"*In vivo* genome editing in animals using AAV-CRISPR system: applications to translational research of human disease" F1000Research 2017, 6(F1000 Faculty Rev):2153
- Du Hong-Yan et al., "Telomerase reverse transcriptase haploinsufficiency and telomere length in individuals with 5p– syndrome" *Aging Cell* (2007) Doi: 10.1111/j.1474-9726.2007.00324.
- Fred Goldman, Rachida Bouarich, Shashikant Kulkarni, Sara Freeman, Hong-Yan Du, Lea Harrington, Philip J. Mason, Arturo Londoño-Vallejo, and Monica Bessler. The effect of TERC haploinsufficiency on the inheritance of telomere length. Medical Sciences, (2005)
- Hao Li et al."Inhibition of HBV Expression in HBV Transgenic Mice Using AAV-Delivered CRISPR-SaCas9" Frontiers in Immunology, 2018. doi: 10.3389/fimmu.2018.02080
- Hong-Yan Du, Rachel Idol, Sara Robledo, Jennifer Ivanovich, Ping An, Arturo Londono-Vallejo, David B. Wilson, Philip J. Mason and Monica Bessler. Blackwell Publishing Ltd Telomerase reverse transcriptase haploinsufficiency and telomere length in individuals with 5p– syndrome. Aging Cell (2007) 6, pp689–697
- Ilgen Mender and Jerry W. Shay: "Telomerase Repeated Amplification Protocol (TRAP)" Bio Protoc., 2015. 5(22): e1657
- Kevin S. Myers, Dan M. Park, Nicole A. Beauchene, Patricia J. Kiley: "Defining Bacterial Regulons Using ChIP-seq Methods" Methods, 2015. doi: [10.1016/j.ymeth.2015.05.022]
- Lisa M. Kattenhorn, Christopher H. Tipper, Lorelei Stoica, Deborah S. Geraghty, Teresa L. Wright, K. Reed Clark, and Samuel C. Wadsworth :"Adeno-Associated Virus Gene Therapy for Liver Disease" Hum Gene Ther. 2016 doi: [10.1089/hum.2016.160]

# References


- Mason PJ, Wilson DB, Bessler M. Dyskeratosis congenita - a disease of dysfunctional telomere maintenance. Curr Mol Med. (2005)
- Michael F. Naso, Brian Tomkowicz, William L. Perry, William R. Strohl. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. BioDrugs (2017) 31:317–334
- Naoaki Mizuno, Eiji Mizutani, Hideyuki Sato, Mariko Kasai, Aki Ogawa, Fabian Suchy, Tomoyuki Yamaguchi and Hiromitsu Nakauchi. Intra-embryo Gene Cassette Knockin by CRISPR/Cas9-Mediated Genome Editing with Adeno-Associated Viral Vector. iScience. (2018)
- Pasqualina Colella, Giuseppe Ronzitti, and Federico Mingozz. Emerging Issues in AAV-Mediated *In Vivo* Gene Therapy. Mol Ther Methods Clin Dev. 2018 Mar 16; 8: 87–104.
- Rasmus O. Bak, Matthew H. Porteus: "CRISPR-mediated Integration of Large Gene Cassettes using AAV Donor Vectors" Cell Rep., 2017. 20(3): 750–756. doi:10.1016/j.celrep.2017.06.064.
- Ronald J. Mandel, Corinna Burger, and Richard O. Snyder*:"*Viral Vectors for In Vivo Gene Transfer in Parkinson's disease: Properties and Clinical Grade Production" Exp Neurol. 2008 doi: [10.1016/j.expneurol.2007.08.008]
- Shahinaz M. Gadalla, Carmem Sales-Bonfim, JeaneAe Carreras, Blanche P. Alter, Joseph H. Antin, Mouhab Ayas, Prasad Bodhi, Jeffrey Davis, Stella M. Davies, Eric Deconinck, H. Joachim Deeg, Reggie E. Duerst, Anders Fasth, Ardeshir Ghavamzadeh, Neelam Giri, Frederick D. Goldman, E. Anders Kolb, Robert Krance, Joanne Kurfberg, Wing H. Leung, Alok Srivastava, Reuven, Carol M. Richman, Philip S. Rosenberg, Jose Sanchez de Toledo Codina, Shalini Shenoy, Gerard Socié, Jakub Tolar, Kirsten M. Williams, Mary Eapen, Sharon A. Savage, Outcomes of Allogeneic Hematopoietic Cell Transplantation in Patients with Dyskeratosis Congenita, American Society for Blood and Marrow Transplantation. Published by Elsevier, 2013.
- Shyam Sushama Jose, Federico Tidu, Petra Burilova, Tomas Kepak, Kamila Bendickova, and Jan Fric: "The Telomerase Complex Directly Controls Hematopoietic Stem Cell Differentiation and Senescence in an Induced Pluripotent Stem Cell Model of Telomeropathy" Front Genet. 2018 doi: 10.3389/fgene.2018.00345
- Stefano Colloca*, et al.:"*Vaccine Vectors Derived from a Large Collection of Simian Adenoviruses Induce Potent Cellular Immunity Across Multiple Species" *Sci Transl Med* 4, 115ra2 (2012); DOI: 10.1126/scitranslmed.3002925