SAPIENZA
UNIVERSITÀ DI ROMA

LM Genetica e Biologia molecolare nella Ricerca di Base e Biomedica aa 2013/2014

Corso di Terapia Genica Prof. Isabella Saggio

WERNER'S SYNDROME GENE THERAPY DESIGN

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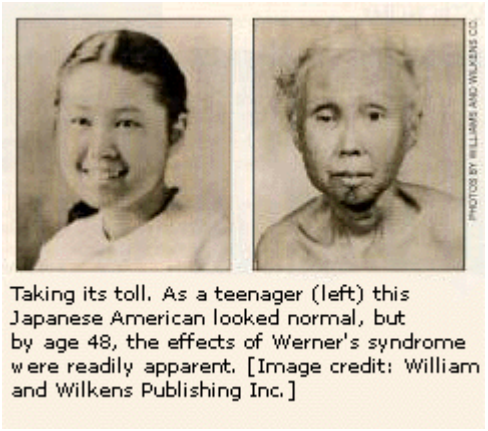
INTRODUCTION

Werner syndrome (WS) is a rare autosomal recessive progeroid disease which affects the adults.

From Chun and Yee, Cancer Biol. Ther. 2010



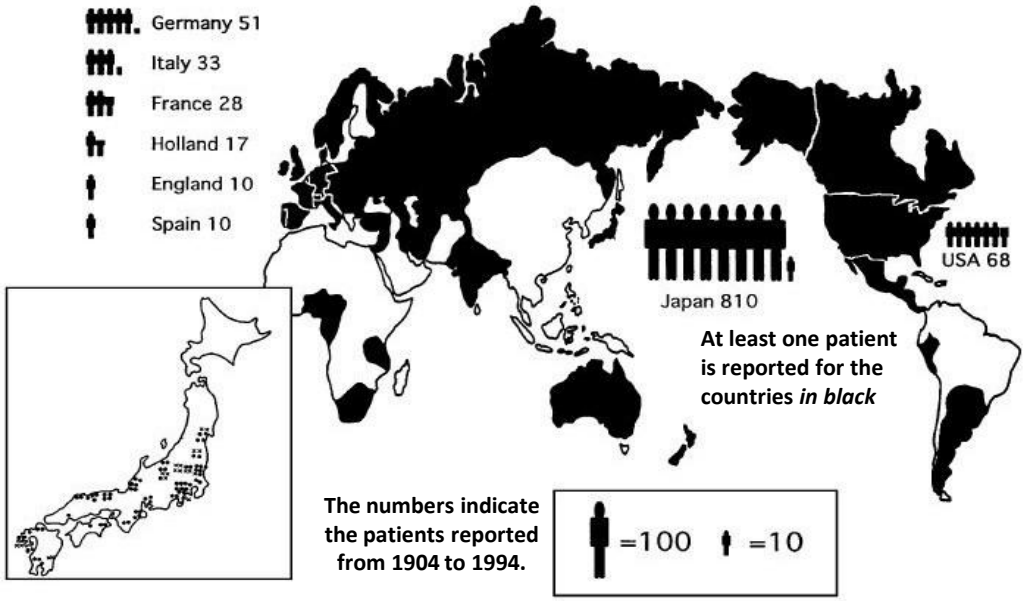
Taking its toll. As a teenager (left) this Japanese American looked normal, but by age 48, the effects of Werner's syndrome were readily apparent. [Image credit: William and Wilkens Publishing Inc.]

SYMPTOMS

- Dermatologic pathologies (atrophy, tight skin, ulceration, hyperkeratosis)
- Premature hair graying
- Bilateral cataracts
- Voice changes
- Osteoporosis
- Type II diabetes mellitus
- Cardiovascular disease
- Cancer (pancreas, skin, thyroid, colorectum)
- Soft tissue sarcomas

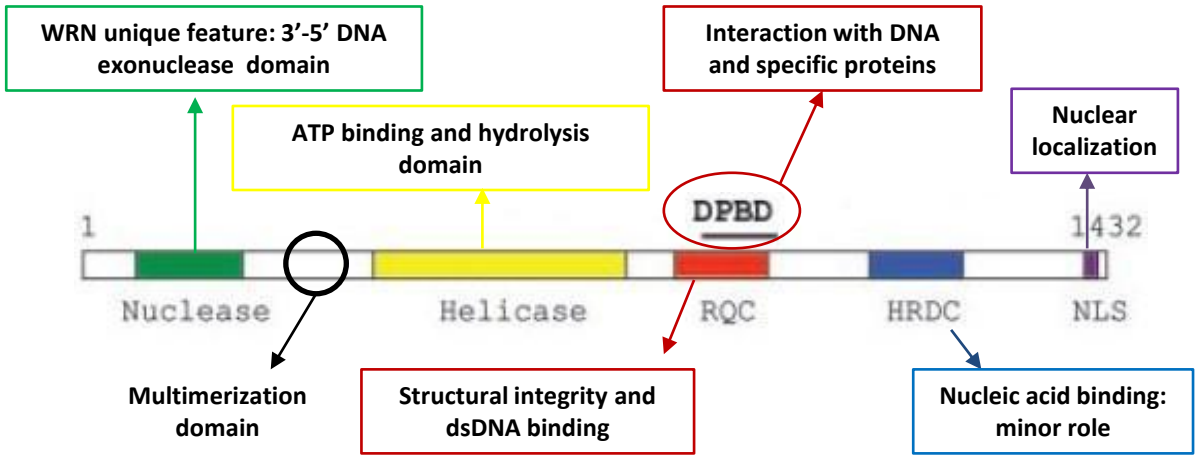
CAUSES OF DEATH

- Pancreatic cancer
- Myocardial infarctions
- Cerebrovascular accidents
- Other neoplasms



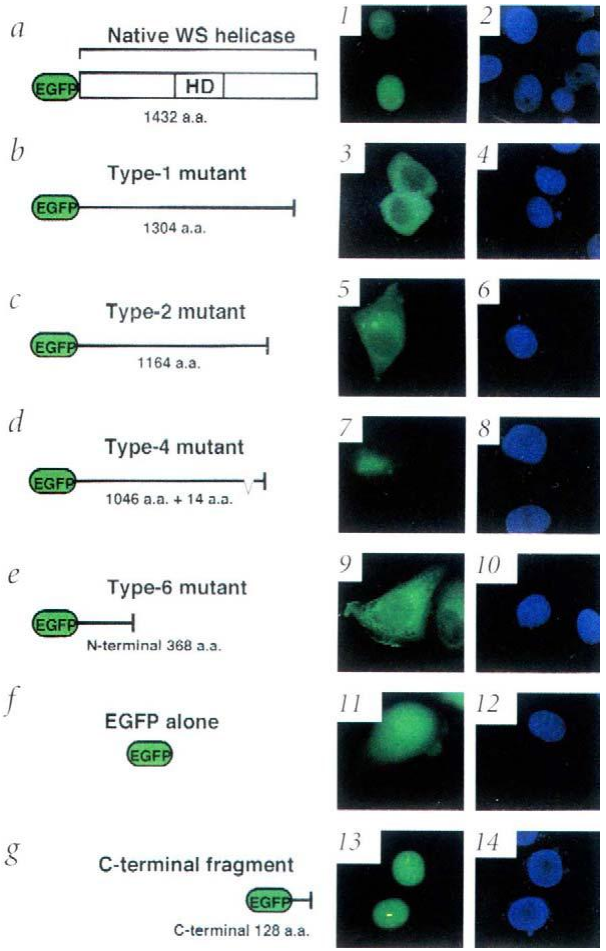
From Matsumoto et al.*, Hum Genet, 1997*

PANCREATIC DYSFUNCTIONS SEEMS TO BE THE PRIMARY CAUSE OF DEATH

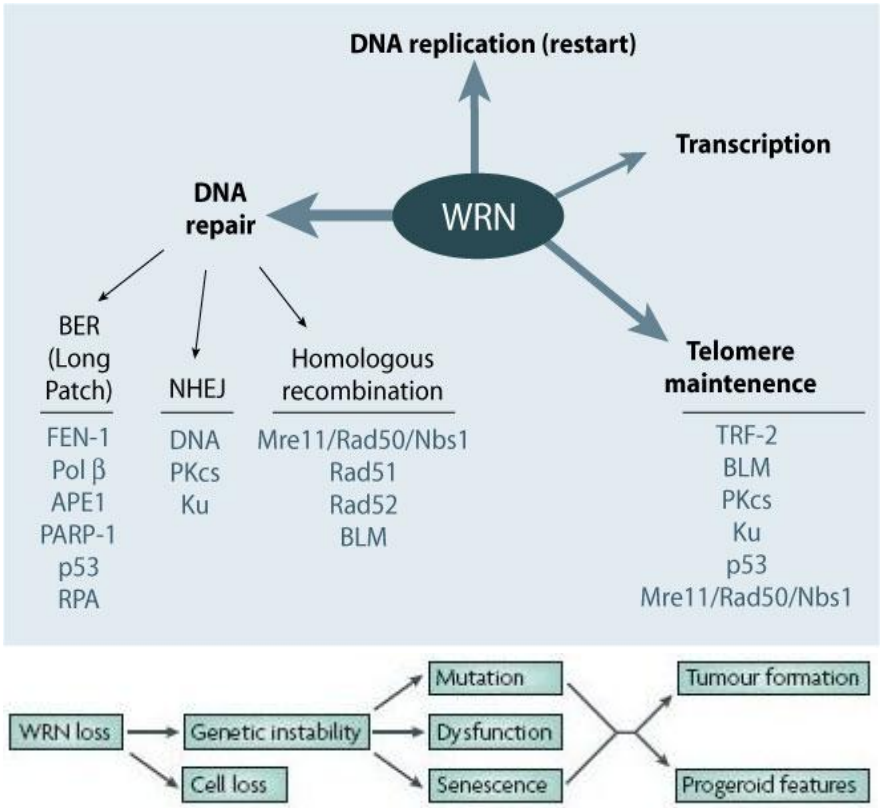


Modified from Hu et al., *PNAS 2005*

Mutations on *wrn* gene usually produce truncated proteins which are **unable to localize to the nucleus.**



B A C K

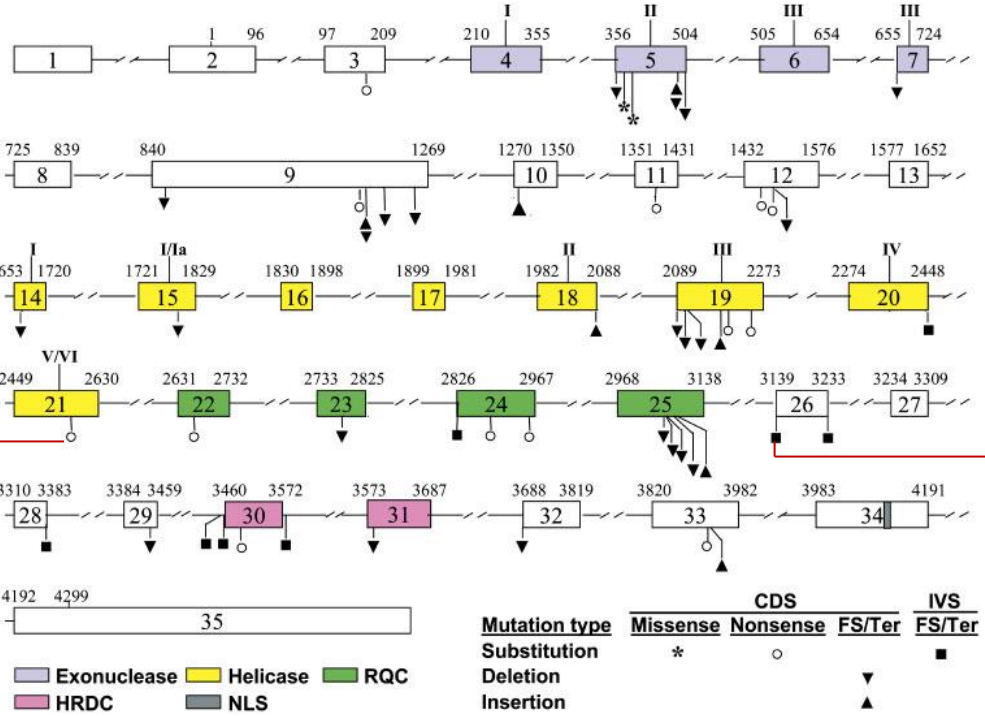


Modified from Kudlow et al., *Nature, 2007*

Matsumoto et al., *Nat. Genet. 1997*

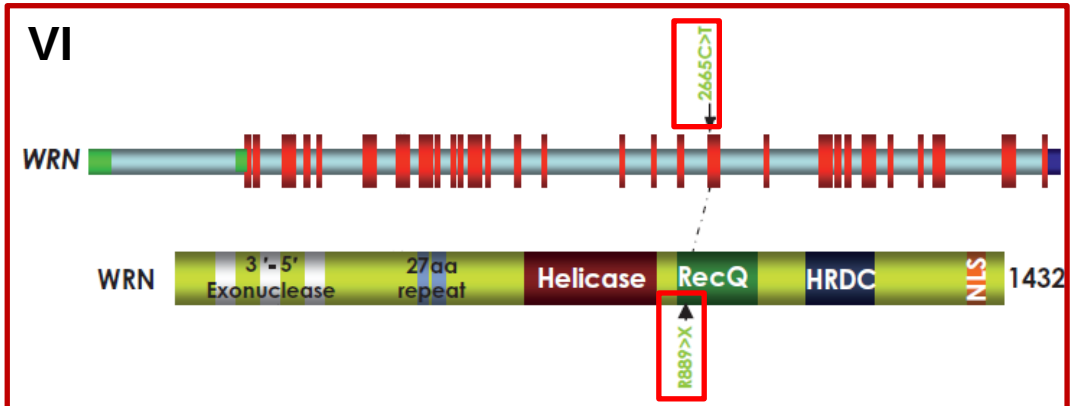
GROUND

Most frequent WS mutations



From Huang et al., *Hum. Mutat. 2006*

VI

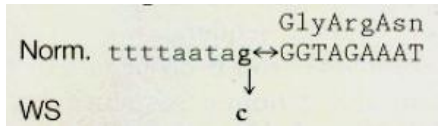


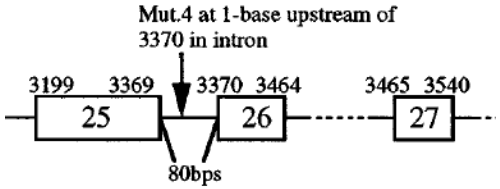
Modified from Ramirez et al.,*Cell. Mol. Life Sci. 2007*

C>T in the coding sequence

Stop codon in exon 21

IV





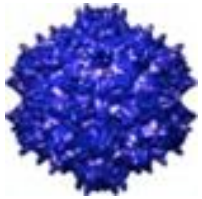
Modified from Yu et al., *Science 1996, Matsumoto* et al.,*Hum. Genet. 1997*

G>C on a site acceptor

Loss of exon 26 and frameshift

Stop codon in exon 27

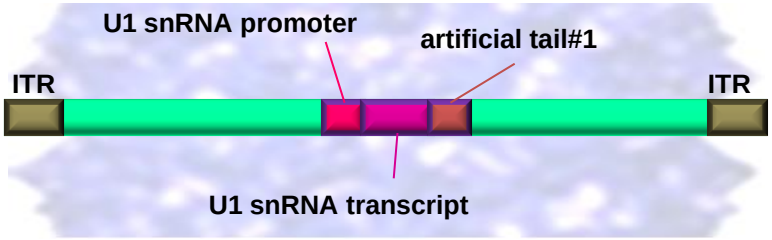
OBJECTIVES

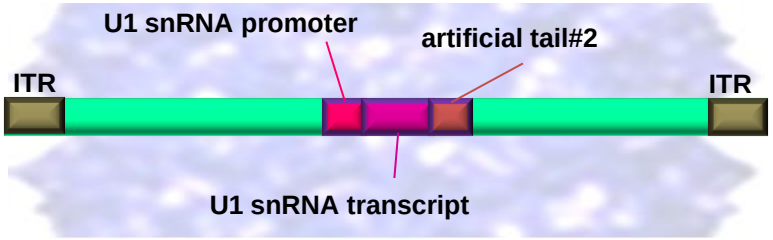


ADENO-ASSOCIATED VIRUS VECTORS

- Successfully transfered to the nucleus
- Persist as extrachromosomal elements
- Small size, small genome

- Expressed for a sustained period of time
- Lack of toxicity
- Weaker immunogenicity (see *Pitfall and solutions*)





EXON 21 SKIPPING

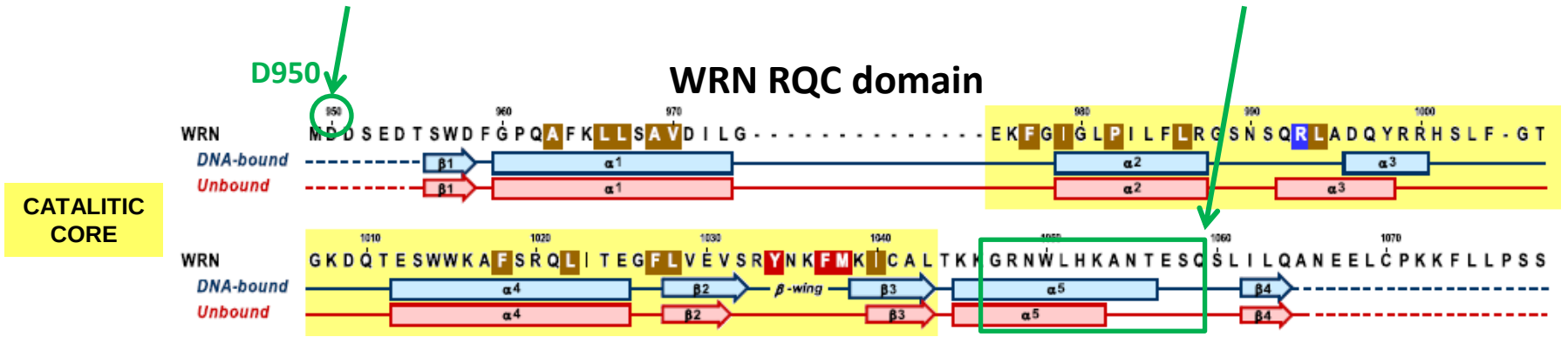
R877-R909 deletion

EXON 26 RESCUE

G1047-Q1059 deletion
i.e. part of α-helix5

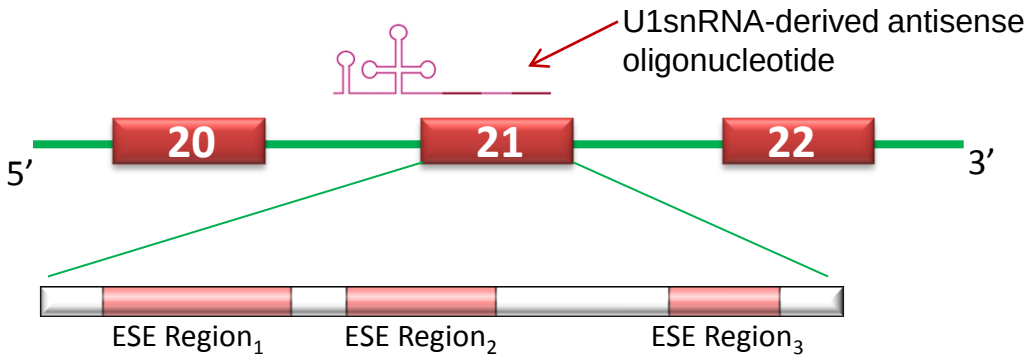
UPSTREAM the RCQ domain

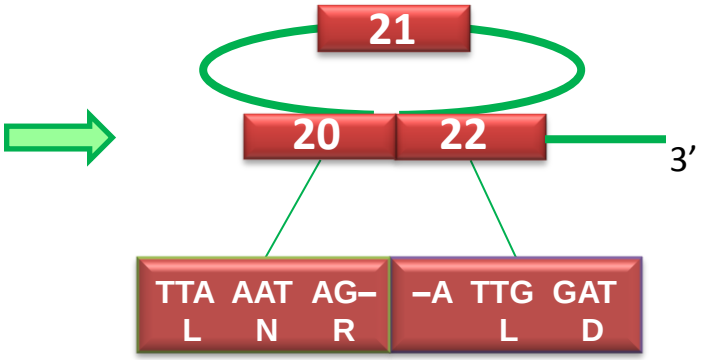
NO CATALITIC CORE LOSS



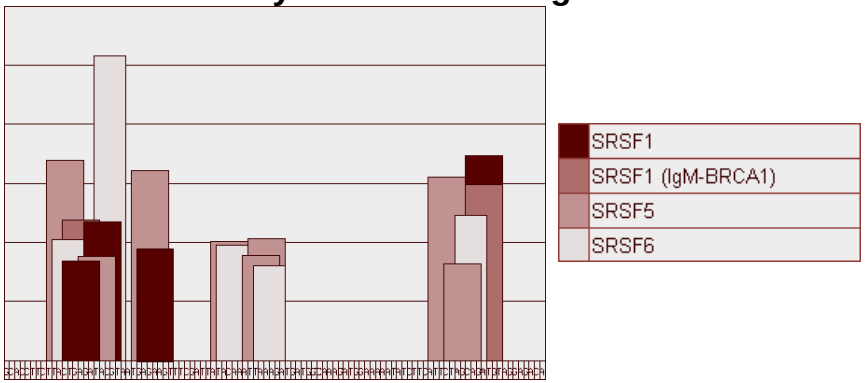
Modified from *Kitano* et al., *Structure 2010*

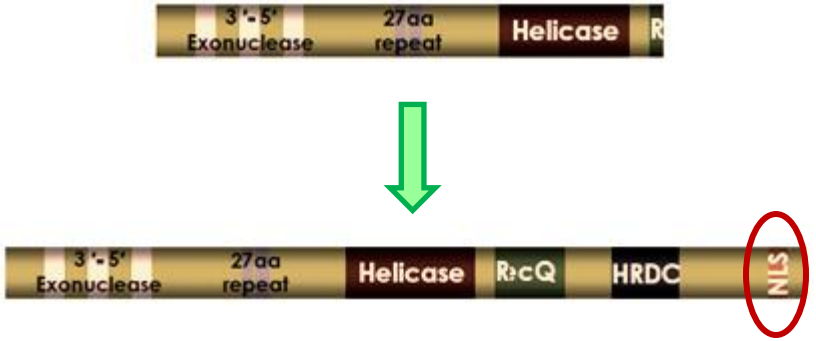
EXON SKIPPING





Probability of SRSF binding on Exon 21





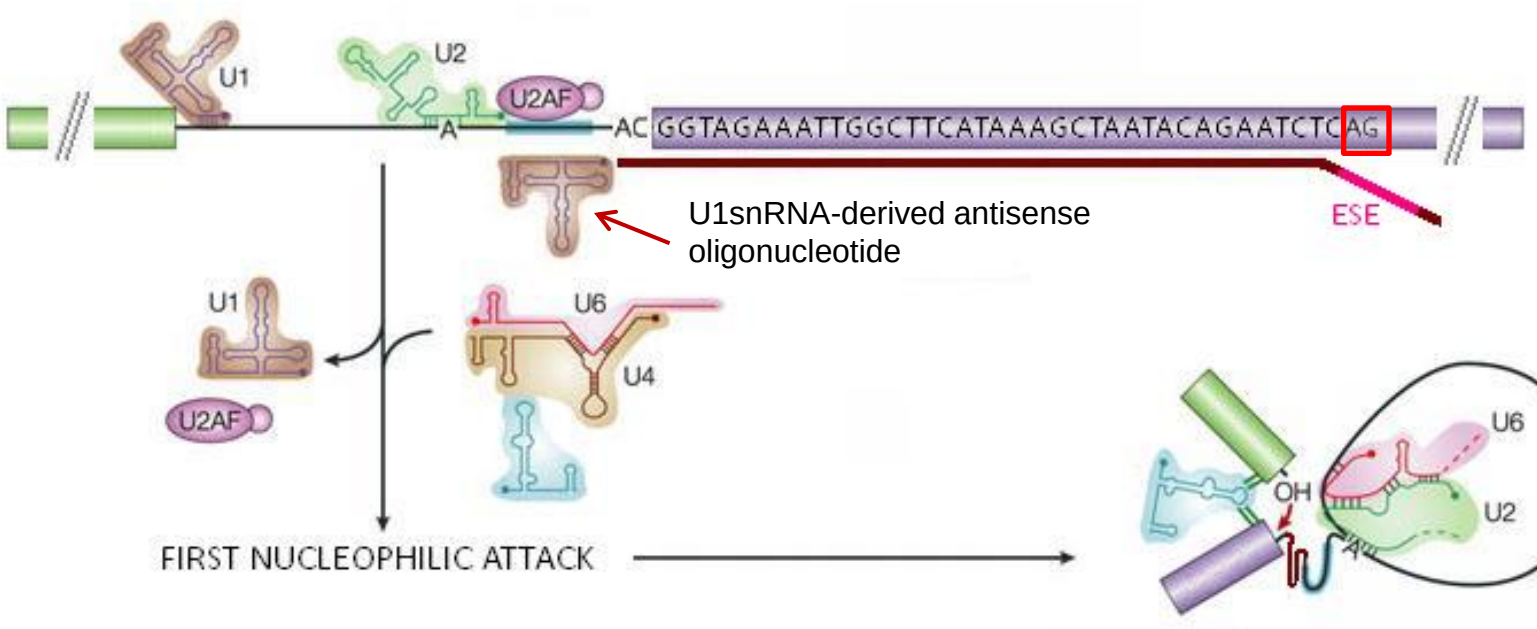
Three possible constructs:

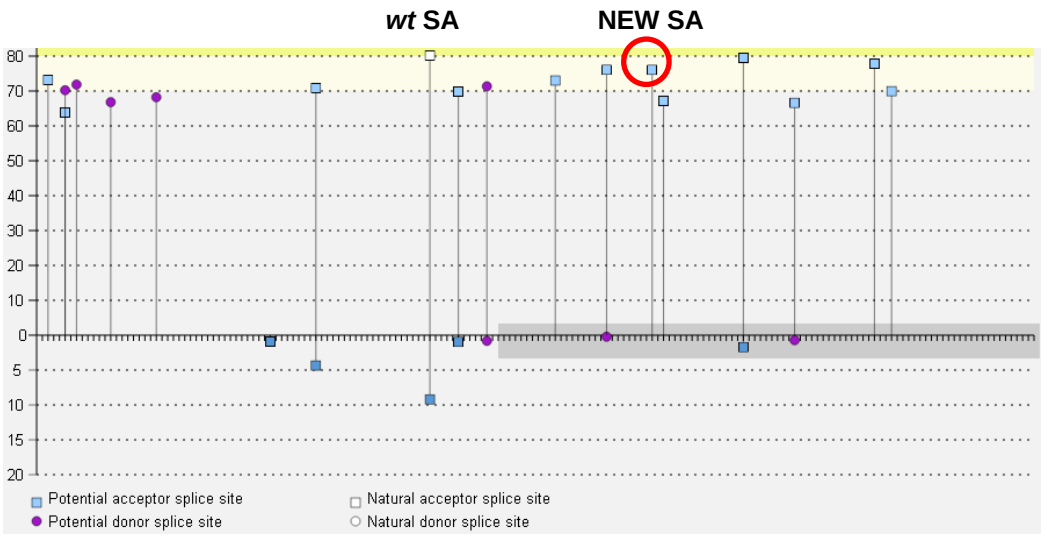
	αESE-R1	αESE-R2	αESE-R3
#1	+	+	
#2		+	+
#3	+		+

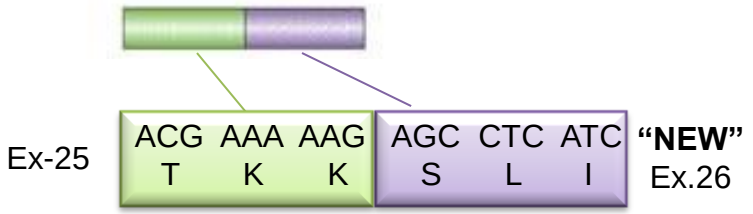
Loss of UGA stop codon allows the recovery of the full-length WRN protein

Prediction made with http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home

EXON RESCUE





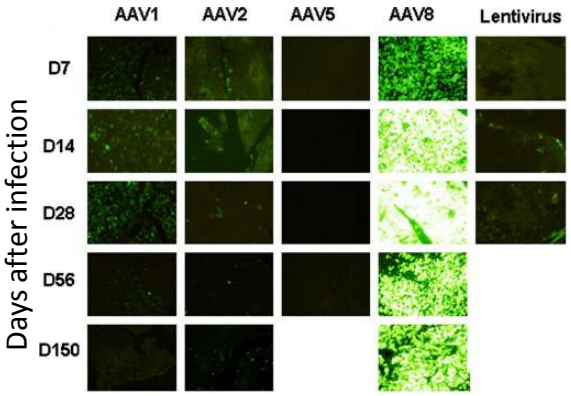


"New" exon 26 inclusion allows the recovery of the full-length WRN protein

Prediction made with http://www.umd.be/HSF/

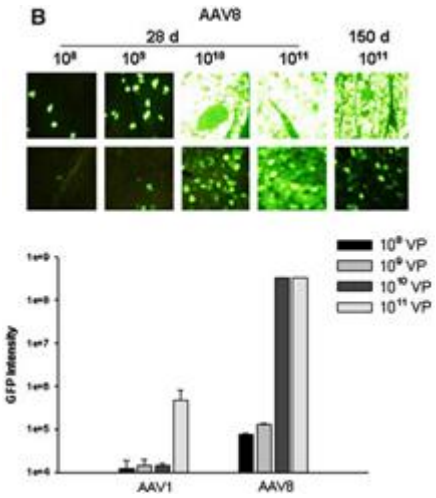
rAAV2/8 VECTOR

PERSISTENCE

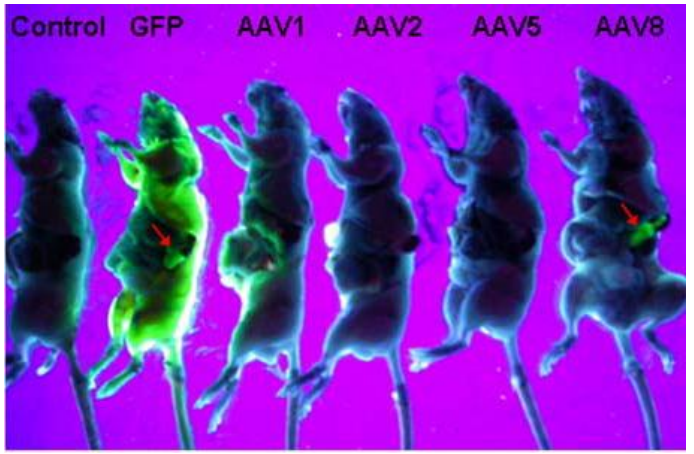


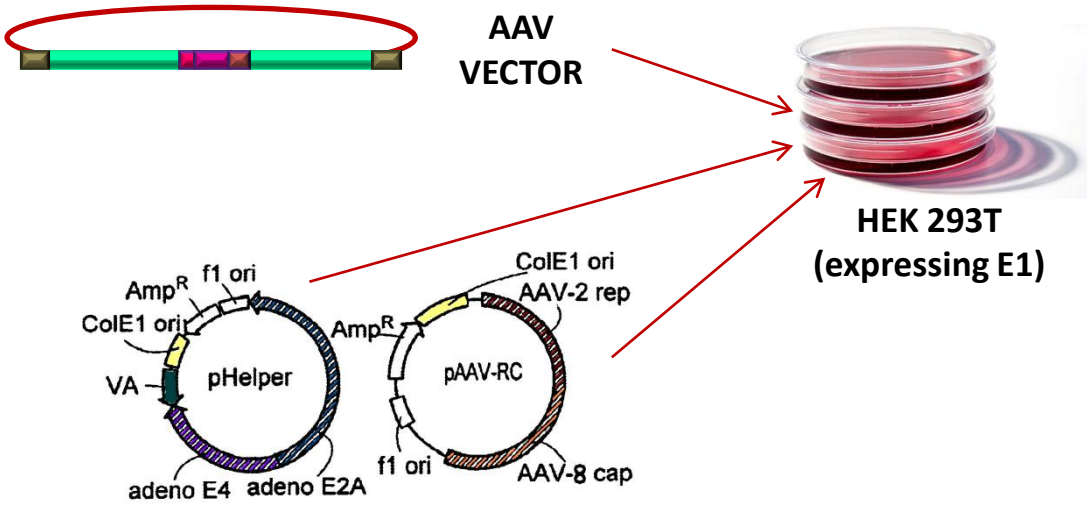
From Cheng et al.*, J. Biomed. Sci. 2007*

HIGH EXPRESSION



SPECIFITY





Purification:

- Cell lysis with benzonase-containing buffer
- Iodixanol density gradiend centrifugation and heparin-sepharose affinity chromatography

OR

CsCl gradients.

- Dialysis

Purity determined by silver-stained sds-page.

Titer determined with a dot-blot assay

PANCREATIC CELLS INFECTION

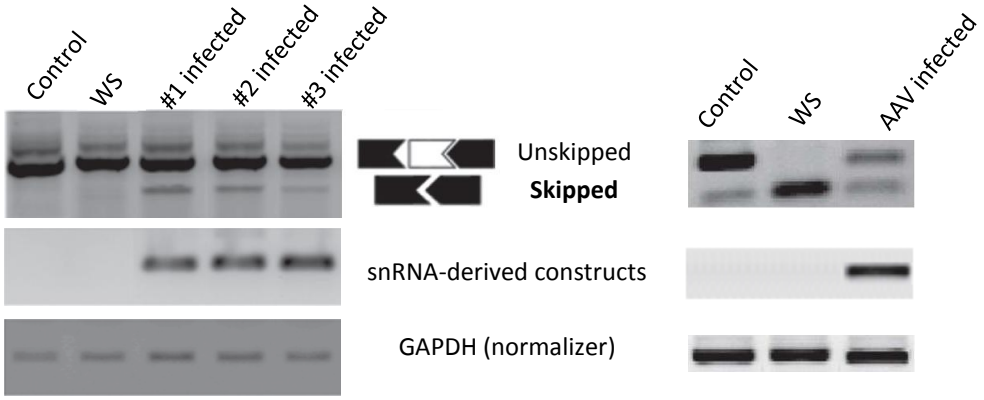
AAV2/8 transduction of **exocrine and endocrine pancreatic cells explanted from a WS patient** who shows one of the two mentioned mutations.

Control cell line: Isolated human pancreatic islet cells according to Ricordi *et al*., *Diabetes* 1988.

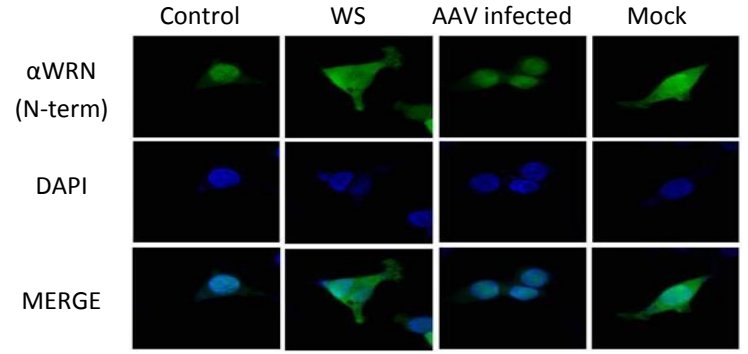
Mock samples *(shown just in IF example, but well considered for every method)*: WS pancreatic cells infected with an empty vector

Samples collected after 0-3-7-10 days after infection. STeLA, IF and proliferative analysis: also 2-3-4-5-6-7 weeks after infection.

RT-PCR: exon skipping and exon rescue



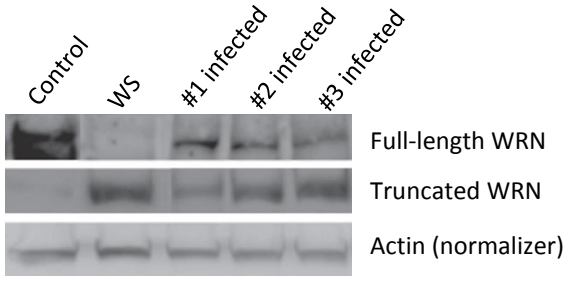
ImmunoFluorescence (IF)



Colocalization of TRF2, PARP1, Rad52 and p53 will be tested.

PCR products sequencing

Western blot (αWRN N-term)



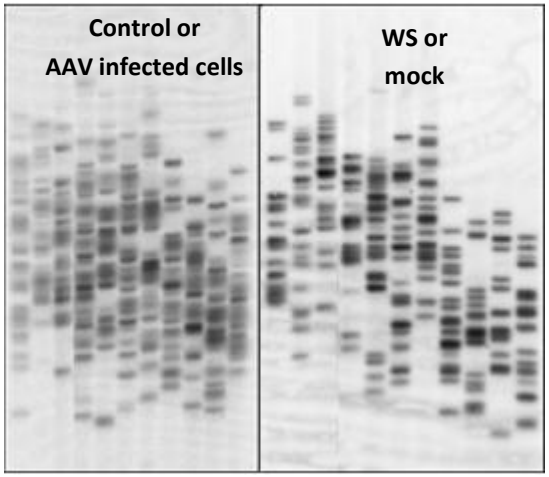
Biochemical assays

- Exonuclease activity
- Helicase activity
- Binding with known interacting proteins (e.g. TRF2, PARP1, Rad52, p53)

Proliferation analyses

Single Telomere Length Assay (STeLA)

1. An adapter (telorette) is ligated at the end of telomeres.
2. Selective amplification of X/Y telomeres.
3. Southern Blotting



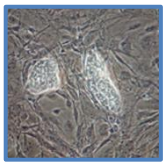
Adapted from
Cazzella et al., *Mol. Ther. 2012*
Duterte et al., *Nat.Struct.Mol.Biol. 2010*

Chen et al., *BJMedBiolRes 2013*
Azzalin et al., *PLoSOne 2012*

WS MOUSE MODEL CHARACTERIZATION

We will follow the protocol described in Chang *et al.*, Nat. Gen. 2004

C57/BL6N Inbred mouse (*wt*)



C57/BL6N **ESC**
Terc$^{Gt(IST14003C8)Tigm}$

Our **specific wrn $^{-/-}$** mouse model is not commercially available so we need it to be **created**.

Mouse and human WRN are very similar.

We plan to request a quote to buy two mice, one for each mutation we are studying.

Human Exon21 encoded aa in mouse WRN

```
NLNRHLLTEIRNEKFRLYKLKMMAKMEKYLH  904
N +R+LL EI +EKFRLYKLKMM KMEKYLH
NTSRNLLIEIHDEKFRLYKLKMMVKMEKYLH  869
```

Human Exon26 pre-mRNA homolog in mouse

```
TTTTAATAGGGTAGAAATTGGCTTCATAAAGCTAA
TTTTGATAGGGTAGAAAGTGGCTTGGAGAAGCCAG
```

Terc $^{-/-}$ x *wt*

↓

Terc$^{+/-}$

↻ Intercross and screening

→

G3 Terc $^{-/-}$
No evidence of telomere dysfunction

x

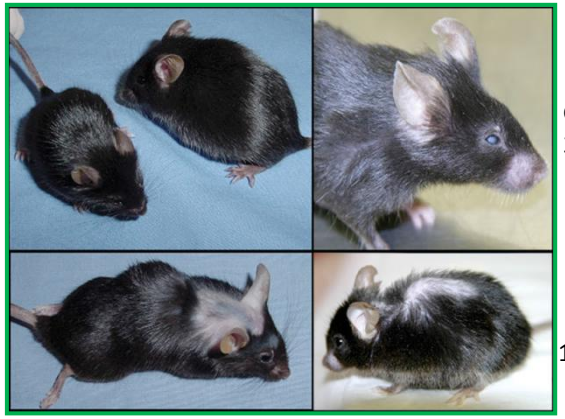
wrn $^{-/-}$

→ **Terc $^{+/-}$ wrn $^{+/-}$** → **Intercross in a cousin-mating scheme**

→ **Terc $^{-/-}$ wrn $^{+/-}$**

Terc $^{-/-}$ wrn $^{-/-}$

Intercross to produce G2 G3 G4 G5 G6
homozygous and heterozygous mice for wrn with a Terc $^{-/-}$ background



Terc $^{-/-}$ wrn $^{+/+}$ on the right G5 4-week-old

G5 3-months-old

G5 8-week-old

G6 12-week-old

From Chang et al.*, Nat. Gen. 2004*

WS MICE ANALYSIS

- **GROWTH AND AGING:** mice will be weighted until 1year of age and examined for ill health.

PROFOUNDLY ILL MICE WILL BE SACRIFIED AND THEIR ORGANS COLLECTED

- **Hair health** analysis
- **Bone density** determination
- **Cataracts**
- **Glucose tolerance** test and **serum insulin** analysis
- **TUMOR INCIDENCE** and **cardiovascular health**
- **Cytogenetic**, quantitative telomere FISH and spectral karyotyping analysis

WS MOUSE MODEL AAV INFECTION

AAV2/8 infection of C57BL6 Terc^{-}/Wrn^{-} **after anesthesia and a lateral incision** on the left side of the abdominal cavity.

Efficient *in vivo* transduction of a **control AAV2/8 vector expressing GFP** will be tested in *wt* mice as described in Cheng *et al.*, J Biomed Sci 2007

Control: *wt* C57BL6 mice

Mock: empty vector in C57BL6 Terc^{-}/Wrn^{-} mice

- **Biopsy after 0-7-14 -28-56-150 days and after infection.**

- **RT-PCR** for exon skipping and exon rescue analyses
- **Western blot** and **IF** with αWRN (N-terminal)
- **Biochemical assays**: catalitic analysis; IP and Western blot to assay the binding of some known interacting proteins (e.g. TRF2, p53, PARP1, Rad52)
- **Colocalization** with the same interacting protein assayed with the IP analysis
- **STeLA**
- **Proliferation and karyotype analyses**

- **Monitoring of mice well-being**

- Measurement of **blood glucose**
- **Insuline detection** from mice pancreas sections
- Measurement of **LDL** ("bad" cholesterol) and **HDL** ("good" cholesterol)
- **Noninvasive imaging** such as micro-TC (Fig.1) and PET (Fig.2) (also for tumor incidence)

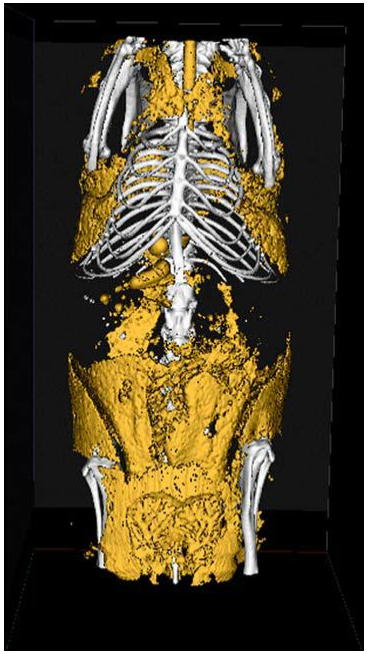


Fig. 1:Three-dimensional mouse rendering. Shown in *yellow is the adipose*
tissue revealed by segmentation based on computed tomographic value of
fat. Reconstruction with 93-µm voxel.
(Grassi et al.*, Radiol Med. 2009)*

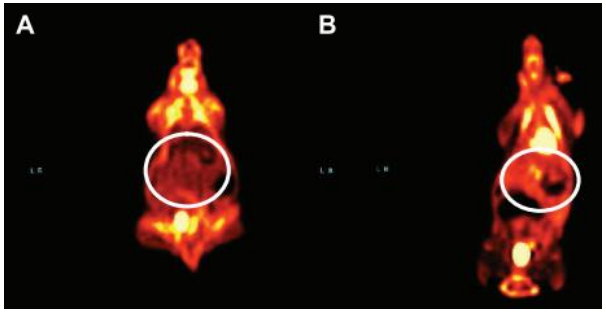


Fig. 2: PET scans. Circles in A and B draw attention to lack and presence of pancreatic tumour respectively.
(Grassi et al.*, Radiol Med. 2009)*

PITFALLS AND SOLUTIONS

- **A large amount of AAV2/8 vector could be needed** to have a sufficient effect on *wrn* mRNA splicing. This and the need of an incision to administer the vector can cause a **high immune response.**

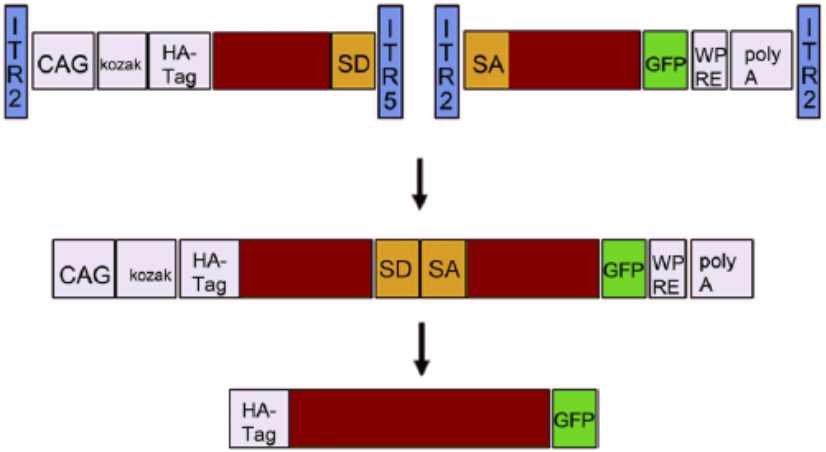
  We can introduce more U1snRNA-derived genes per AAV genome so that one single vector expresses more than one single snRNA-derived AON. It is necessary to keep in mind that one snRNA-derived gene is about 600bp long and AAV vectors have a packaging capacity of 4400bp.

- **Exon 26 rescue could be unsuccessful**. For example, the duplex between the 5' of exon 26 and the U1snRNA-based AON could represent a steric obstruction for the splicing reaction.

  We propose an exons 26/27 skipping which would preserve the correct frame and lead to a G1047-K1103 deletion i.e. α-helix 5 and β-sheet 4 loss which won't compromise the biochemical activity of the RQC domain. (Hu *et al.*, PNAS 2005)

  - **Exons 26/27 skipped protein could bind its interactors with a minor affinity.**

    We propose then to assay a **double transduction with trans-splicing vectors** following the DMD trial as a guide line (Koo *et al.*, Hum. Gene Ther. 2013) which would produce the whole WRN protein.



Modified from Koo et al.*, Hum. Gene Ther. 2013*

CONCLUSIONS

Once our tests on *wrn* mouse model will confirm the real efficacy of our therapeutic system, clinical trial on human patients will be able to start. It has to be reminded that immune response in humans represent the critical point of every gene therapy approach. Therefore the amount of AAV vector administrated to patients has to be chosen very carefully, also depending on the number of administrations needed. We finally suggest the use of both our treatment and classical therapeutic approaches to reduce also Werner's Syndrome effects which are not connected to pancreas dysfunction (e.g., therapies for cataracts, pioglitazon for diabetes mellitus type II, thiazolidinetione and rosiglitazione to reduce insuline resistance).

MATERIALS AND COSTS

- 293AAV Cell Line, Cell Biolabs, 350$ every 10^6 cells + delivery costs
- pAAV-MCS Promoterless Expression Vector, 455$ every 10µg + delivery costs
- pAAV rep2/cap8, quote to be requested
- pAd-helper, quote to be requested
- pAAV-GFP Control Vector, Cell Biolabs, 395$ every 10µg + delivery costs
- *(eventually)* AAV purification and quantification reagents
- *(eventually)* AAV purification standard kit, Cell Biolabs, 230$ every kit + delivery costs
- *(eventually)* AAV quantification kit, Cell Biolabs, 230$ every kit + delivery costs
- about 900-1000$ per mouse
- Stabulation costs
- Cell cultures reagents
- RT PCR, Western blot, IF, IP, biochemical assays, STeLA reagents (+ other assays ones) (e.g. Abcam Ab200 390€ every 100µl, Ab66601 380€ every 100µl)
- PCR purification + sequencing, Biofab research, 13,30€ per sample
- chemical reagents, plastics.

~12.000-14.000€/year (4-5 years predicted)

(we excluded instruments and materials that can be possibly collected thanks to collaboration with medical department e.g. *ws* or *wt* cells, imaging instruments)

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