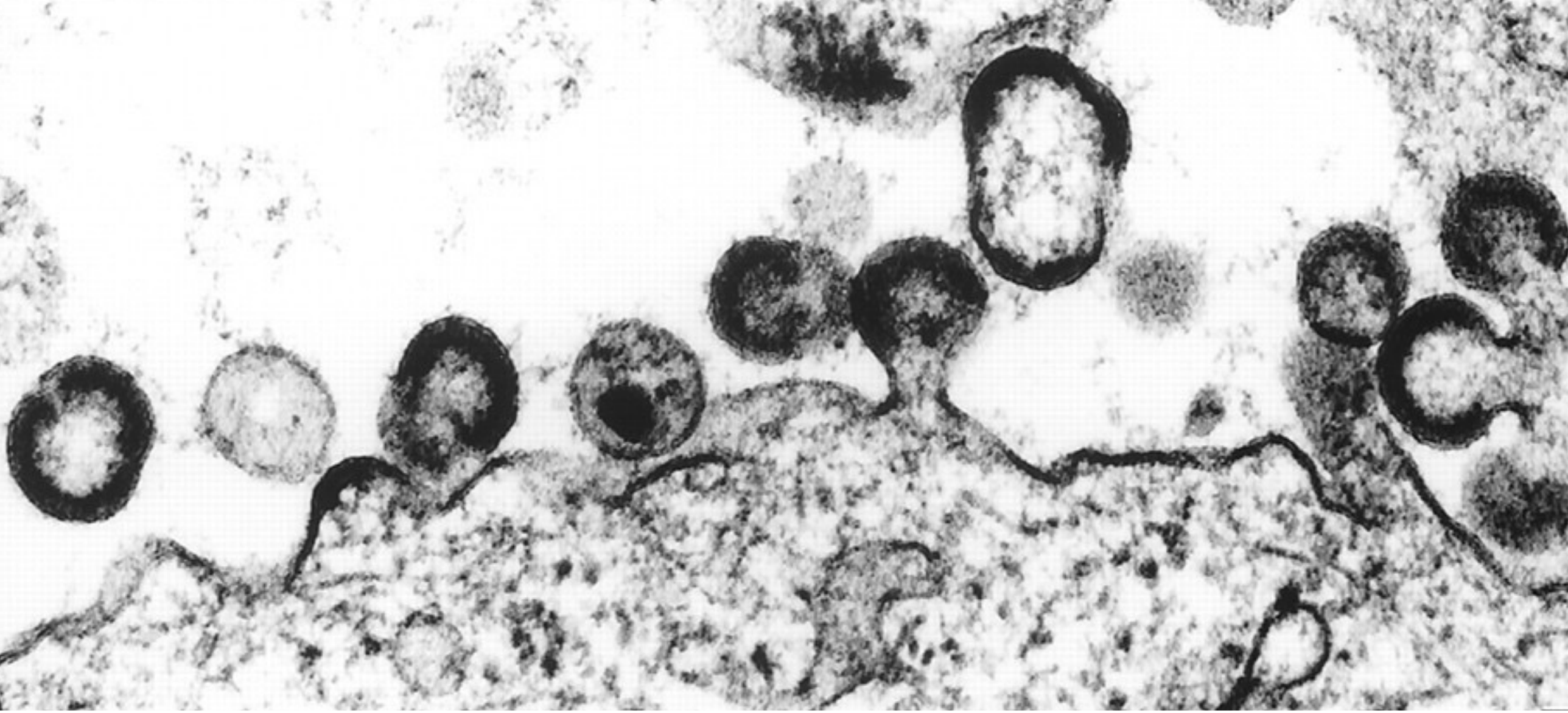

Lentiviral vector therapy for X-EDMD

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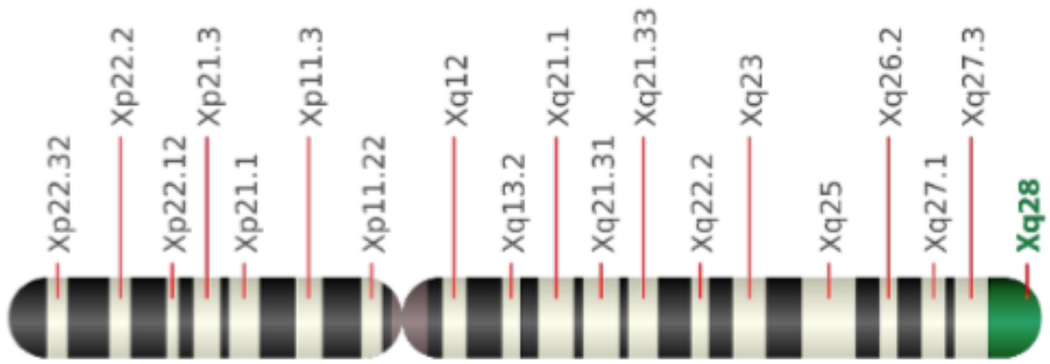
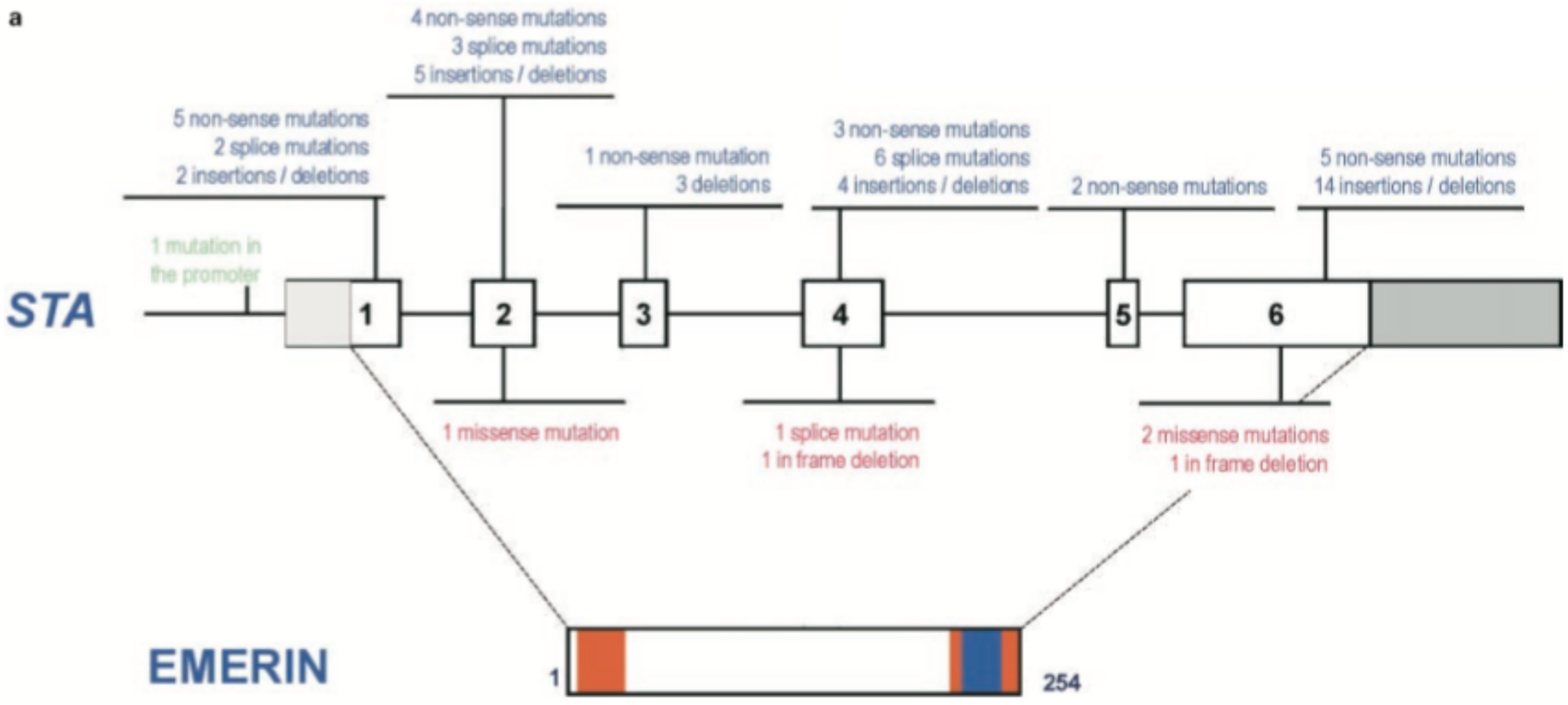
Prof. I Saggio
Gene therapy
17 Dic 2015

SAPIENZA
UNIVERSITÀ DI ROMA

X-linked Emery-Dreifuss Muscular Dystrophy

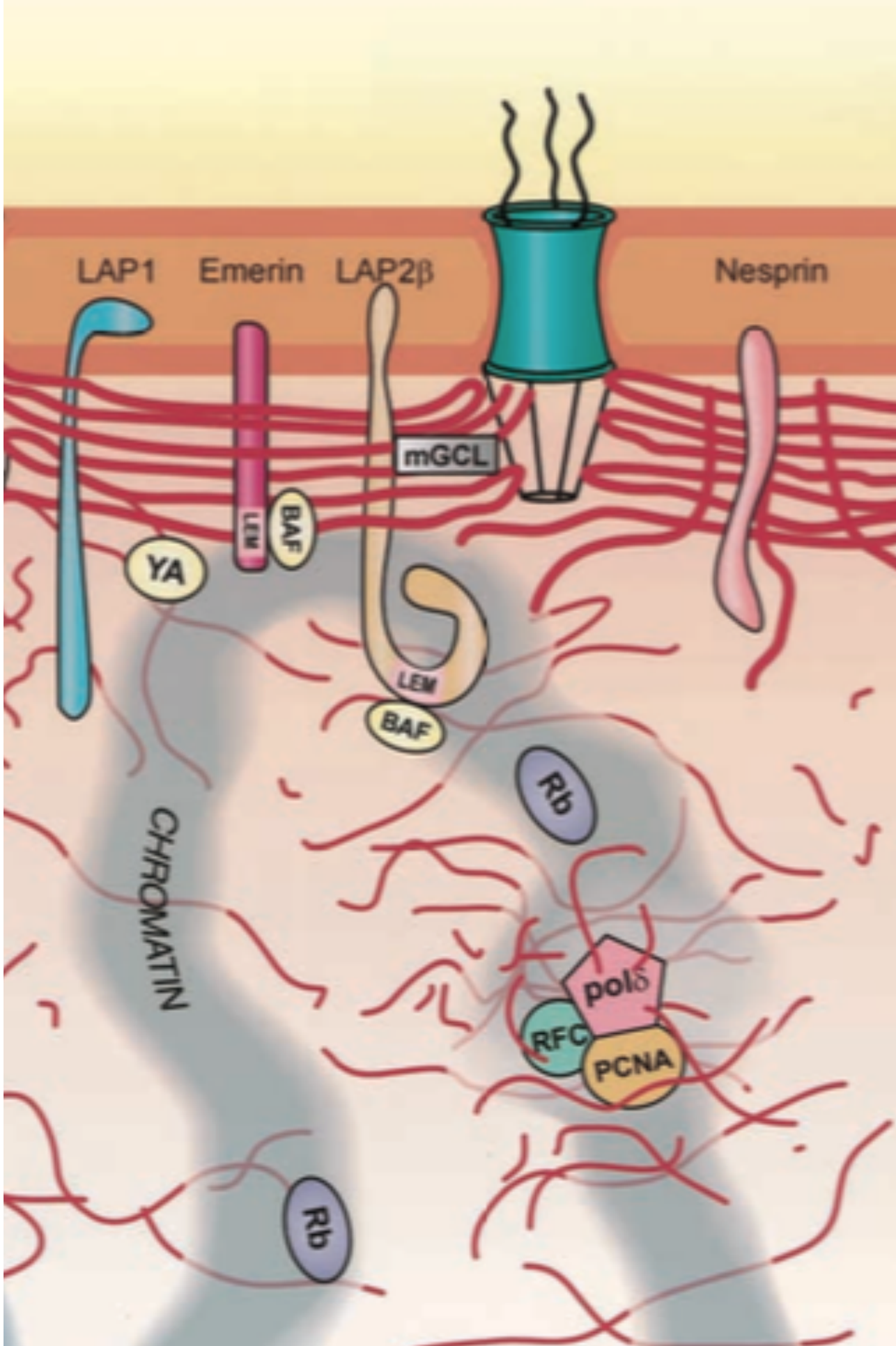
- prevalence about 1/100000 in the word
- inherited in an X-linked recessive pattern
- mutations occurring in STA gene on Xq28 coding for Emerin protein (LAP family).
- X-EDMD is a rare laminopathy





Molecular basis under the pathology

- Emerin is an inner nuclear membrane protein
- Its main role seems to be the interaction with different component of nuclear lamina, such as Lamin A and BAF, and DNA
- Nuclear lamins are involved in a number of critical processes including nuclear assembly, apoptosis, DNA synthesis, and possibly transcription



Genotype

mutation in STA gene
on X chromosome
-lack of emerin

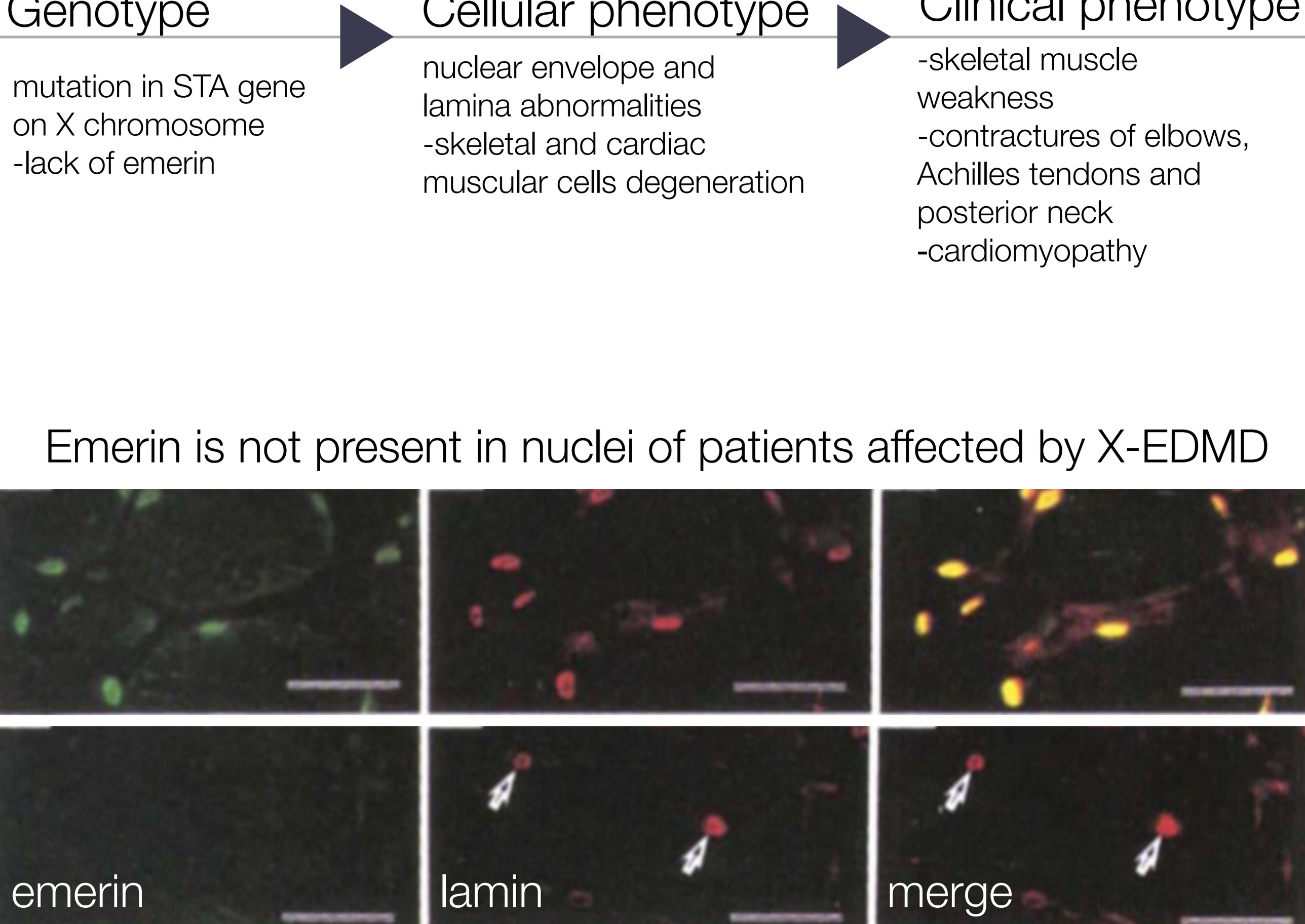
Cellular phenotype

nuclear envelope and
lamina abnormalities
-skeletal and cardiac
muscular cells degeneration

Clinical phenotype

-skeletal muscle
weakness
-contractures of elbows,
Achilles tendons and
posterior neck
-cardiomyopathy

Emerin is not present in nuclei of patients affected by X-EDMD



The Project

Curing the disease at every level using a lentivector

large transgene - infect non dividing cells - stable integration

Lentivector containing Emerin coding sequence

Injection targeting cardiac and skeletal muscles

Integration of genomic cassette in host genome

Emerin expression

Rescue of muscular phenotype of patients

Cure the disease

The Project

experimental plane

<u>In vitro</u> studies on human cells

- RG246, EMD-null myocytes and cardiomyocytes differentiated

<u>In vitro</u> studies on mouse cells

- cells derived from skeletal and cardiac muscles

<u>In vivo</u> studies on mouse

- Emd null-mice

WB ab α-emerin — lentivector transduction efficiency

qRT-PCR — mRNA presence and relative quantities

IHC α-emerin — protein localization

IHC laminin β1
functional assay

WB - Erk 1/2 activities
functional assay

Fluorescence microscopy
transduction relative efficiency

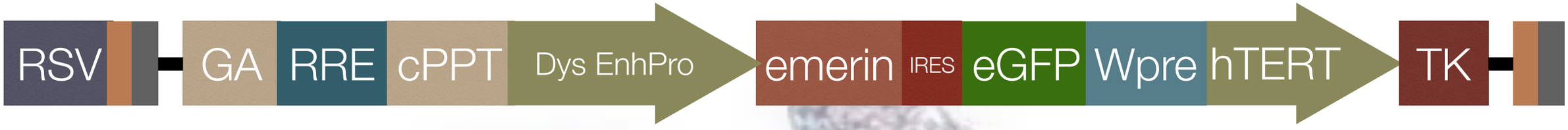
Rotarod test
functional assay

Electrocardiogram
functional assay

Vacuoles positioning
functional assay

The Vector

Lentiviral vector carrying emerin coding sequence and targeted towards skeletal muscle and heart



- dystrophin enh/promoter, promoter active just in skeletal muscle and heart - 3,8 Kb
- emerin - 762 bp
- IRES, internal ribosome entry site for bicistronic espression - 500 bp
- eGFP, reporter gene - 714 bp
- hTERT, in adult promoter active just in neoplastic cells, 1,7 Kb
- TK, Timidine Kinase, suicide gene - 179 bp

8Kb

The Vector

3rd generation viral particles production
via in trans complementation

- ELISA assay -> Titer determination

HEK 293T Cells

In vitro - human cell lines differentiation

undifferentiated ESCs

EB formation

migrating cells from EBs

+EGF

+5-AZA

+ITS

in vitro differentiation using myogenic induction media

+dexomathasone

myocytes

undifferentiated ESCs in MEF-CM

mechanical passage into Matrigel-coated plates

+bFGF for 6d

+BMP4

transfer in RPMI-B27 medium

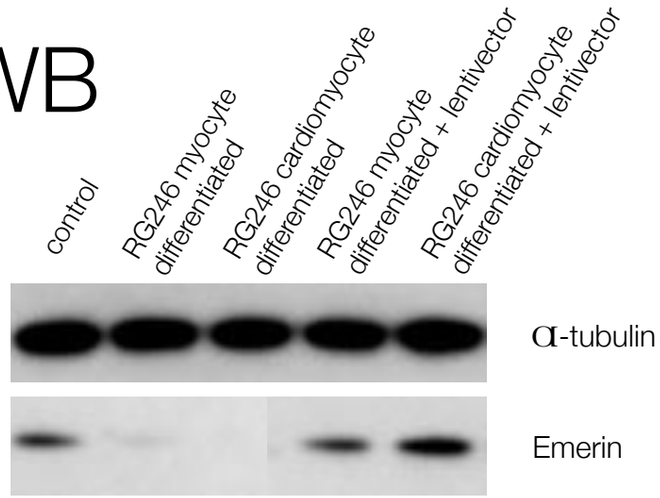
+Activin A

cardiomyocytes

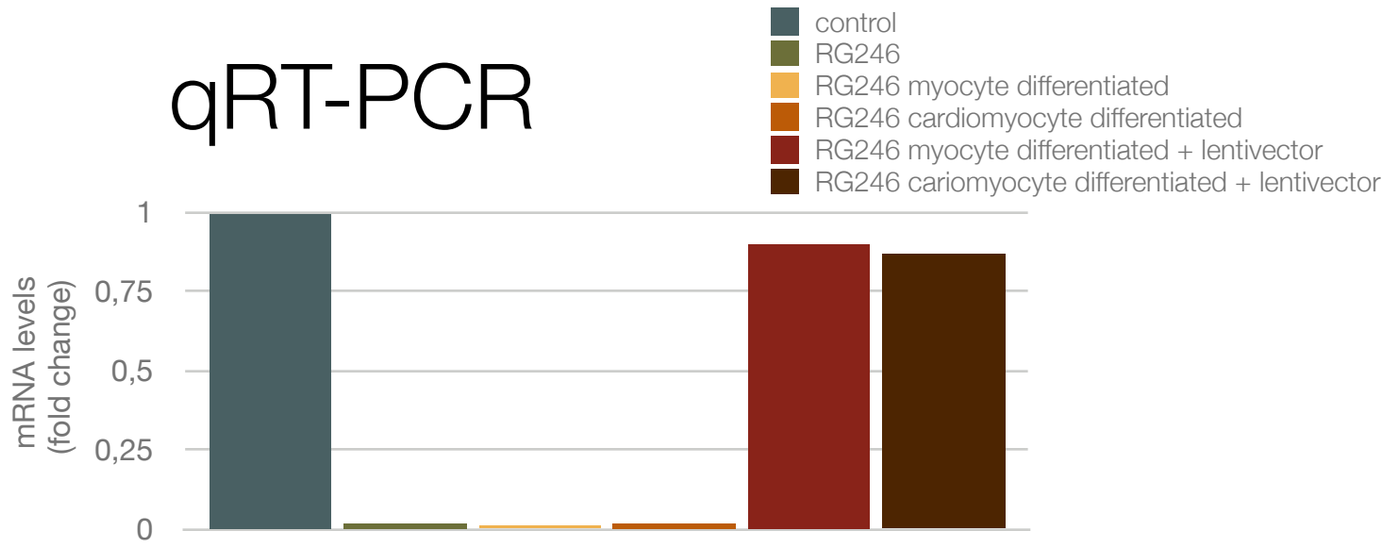
In vitro - human

- lentivector transduction efficiency
- mRNA presence and relative quantities
- protein localization
- functional assay

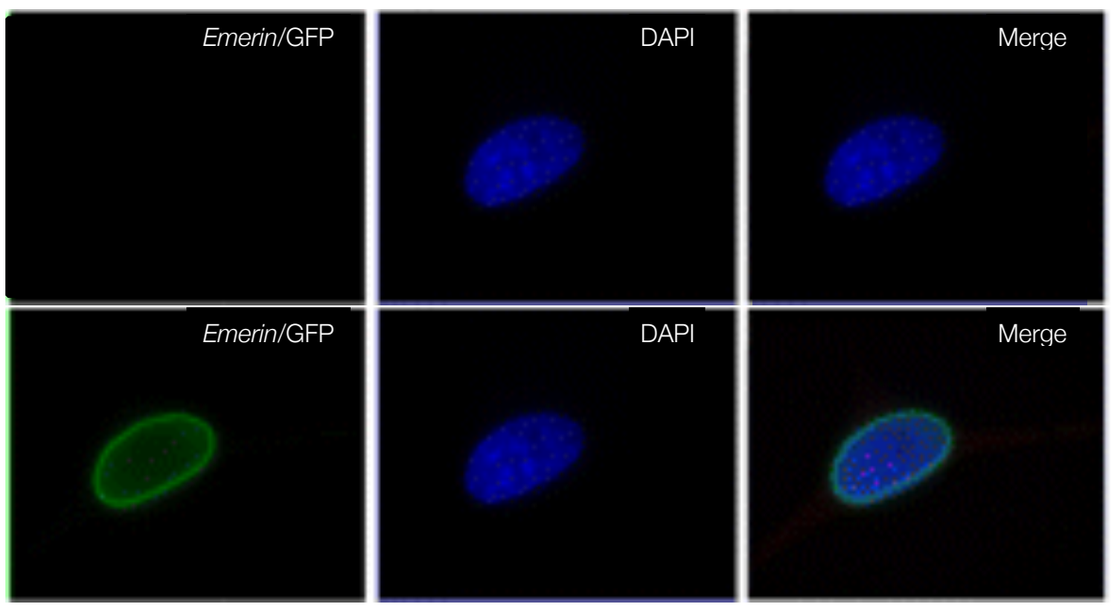
WB



qRT-PCR



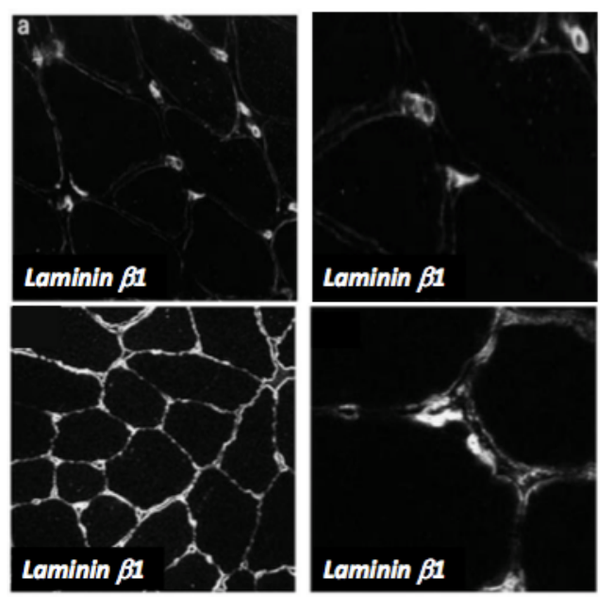
IHC



IHC

RG246

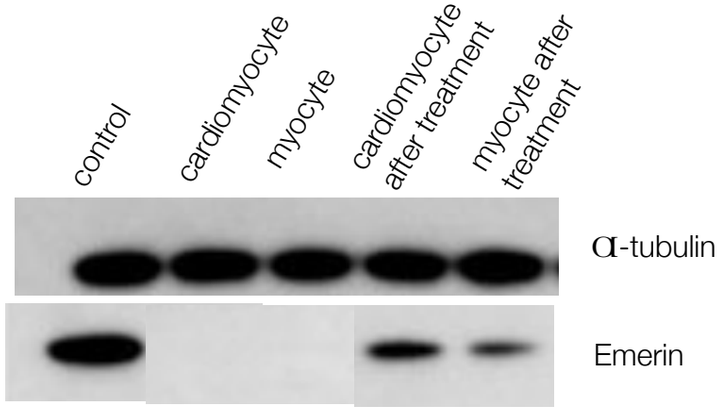
RG246 treated



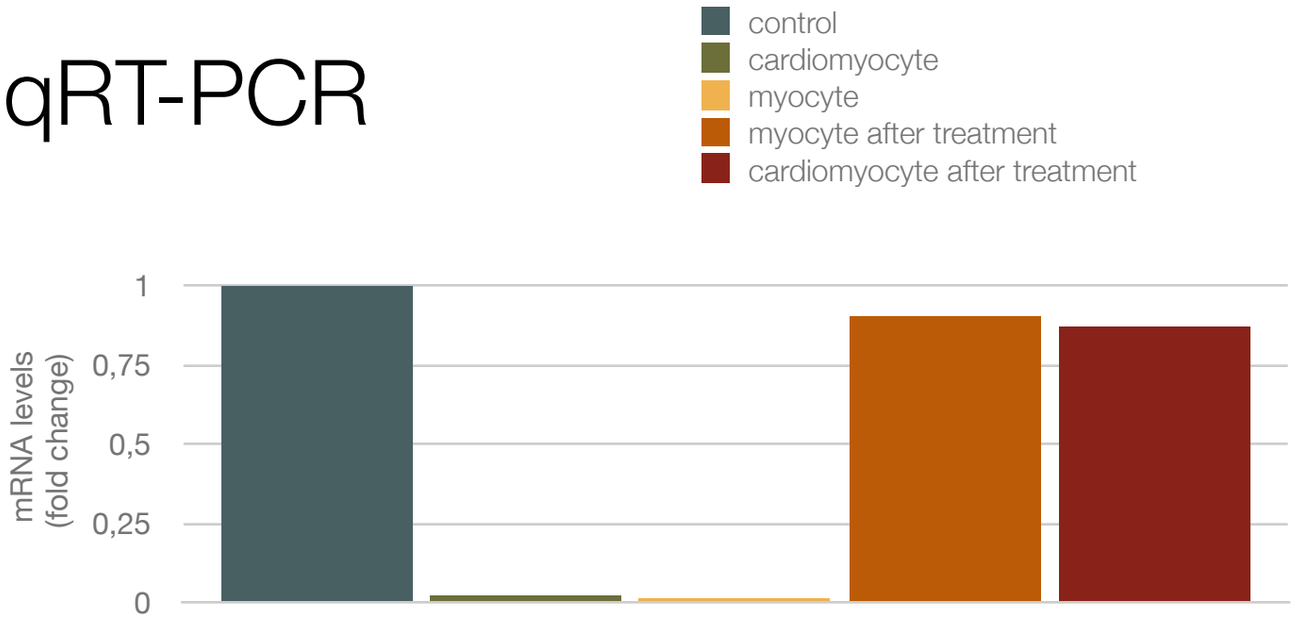
In vitro - mouse

- lentivector transduction efficiency
- mRNA presence and relative quantities
- functional assay
- targeted expression control

WB



qRT-PCR



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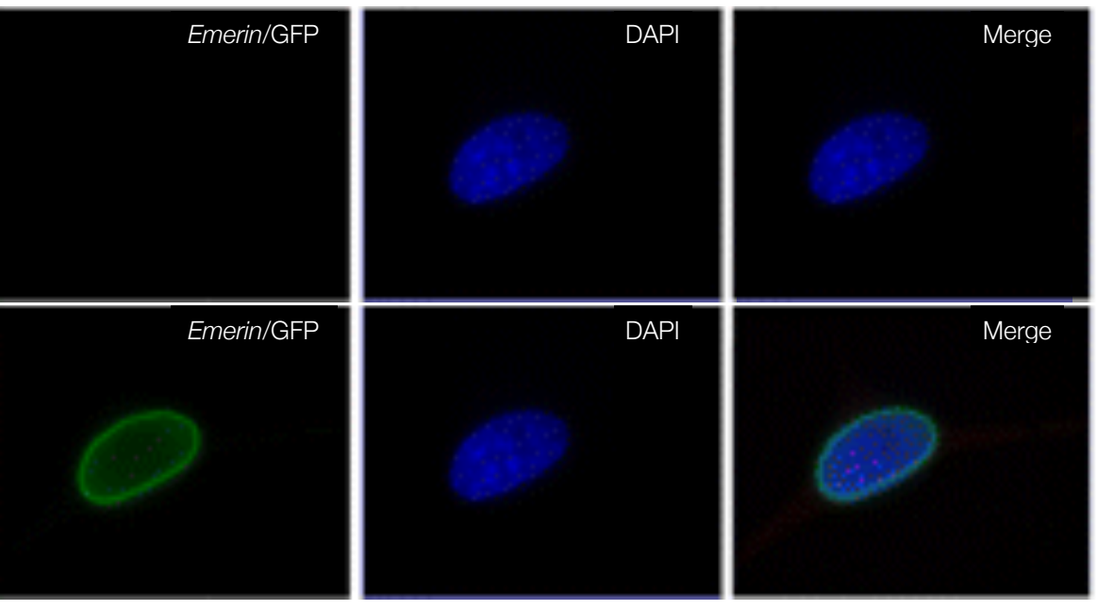
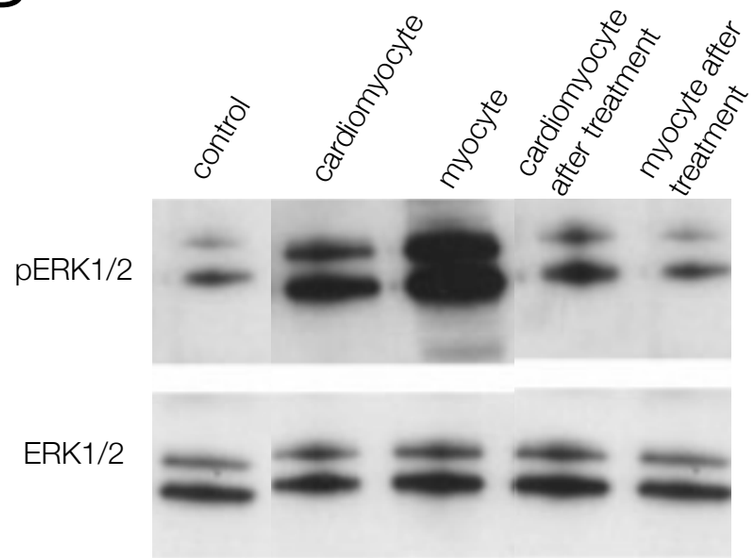


- cardiomyocyte
- myocyte
- control
- cardiomyocyte after treatment
- myocyte after treatment

WB



In vivo - mouse the model

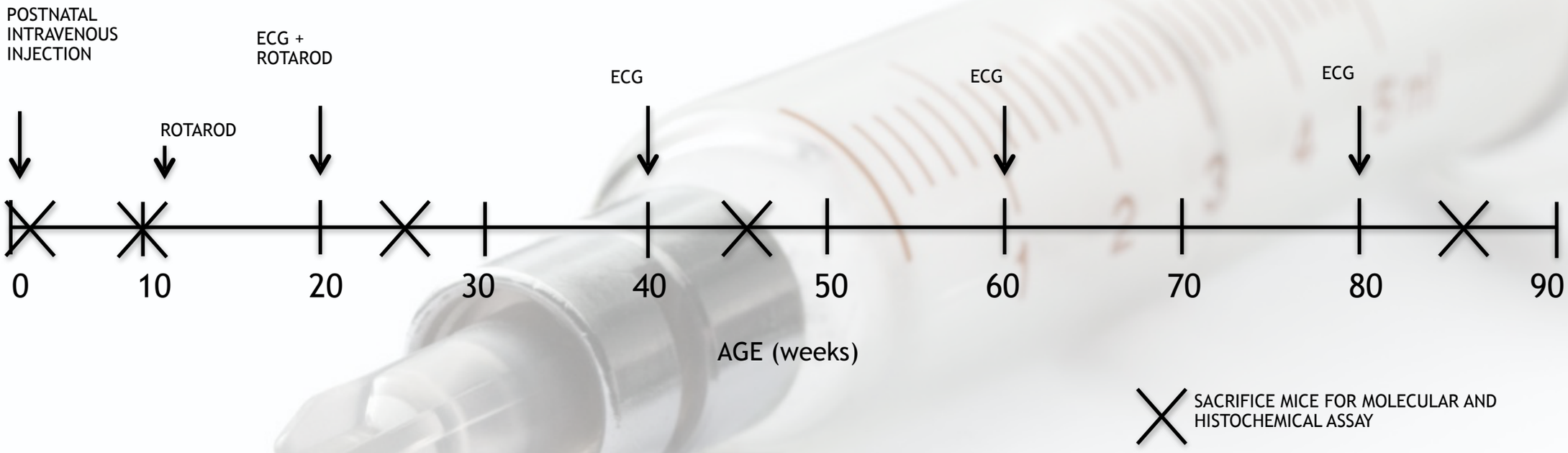
♂ *Emd y/-* ♀ *Emd -/-*

Emerin-Lacking Mice Show Minimal Motor and Cardiac Dysfunctions with Nuclear-Associated Vacuoles

- Rotarod test revealed altered motor coordination.
- Electrocardiography showed mild prolongation of atrioventricular conduction time in emerin-lacking male mice older than 40 weeks of age.
- Electron microscopic analysis of skeletal and cardiac muscles from emerin-lacking mice revealed small vacuoles , which mostly bordered the myonuclei.

In vivo - mouse

injection and timeline



Intravenous injection is used to deliver vector to heart and skeletal muscles

12 mice

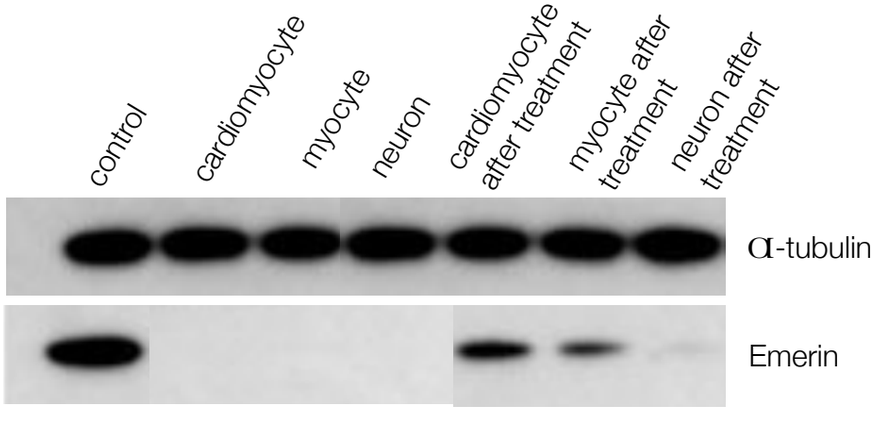
- 3 wt male
- 3 wt famale
- 3 emd -/y
- 3 emd -/-

During in vitro experiments two colonies are generated in order to obtain the whole number of mice needed for experiments

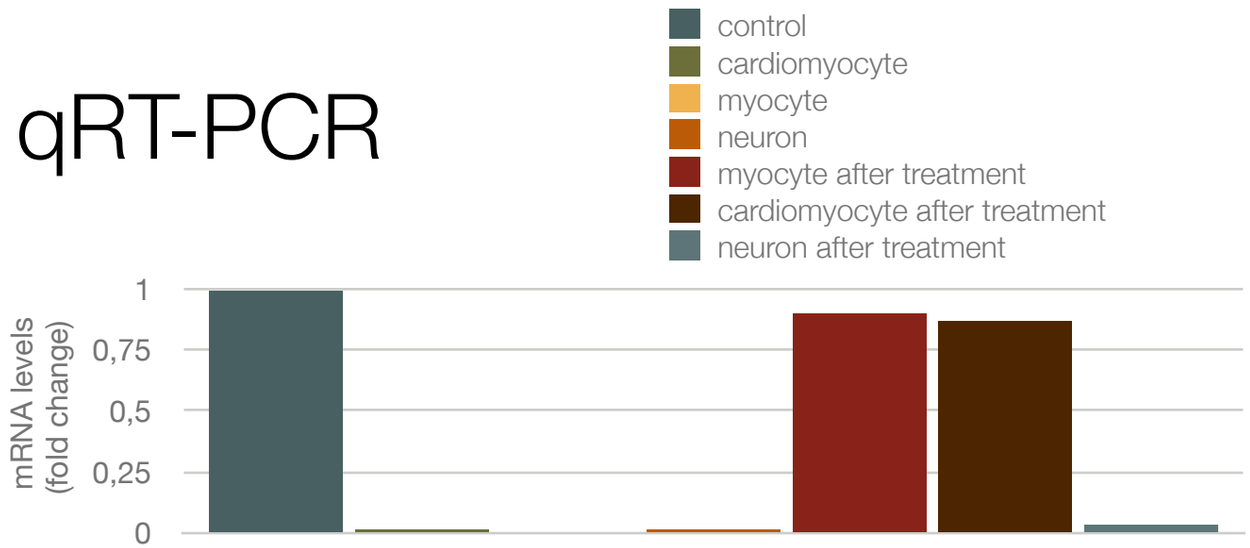
In vivo - mouse

- lentivector transduction efficiency
- mRNA presence and relative quantities
- protein localization

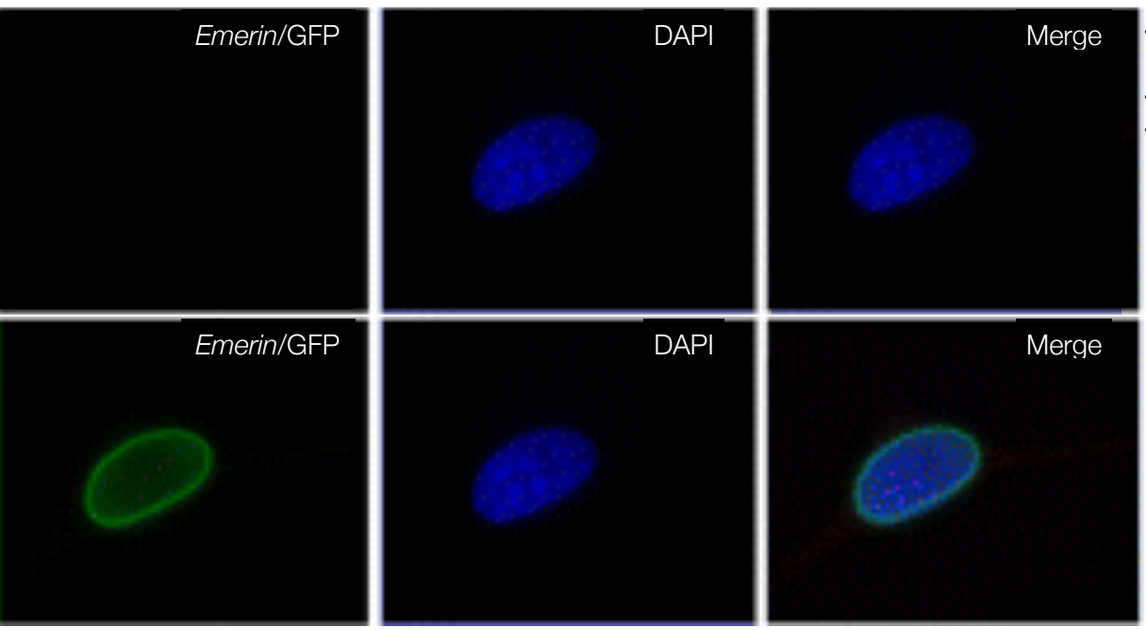
WB



qRT-PCR



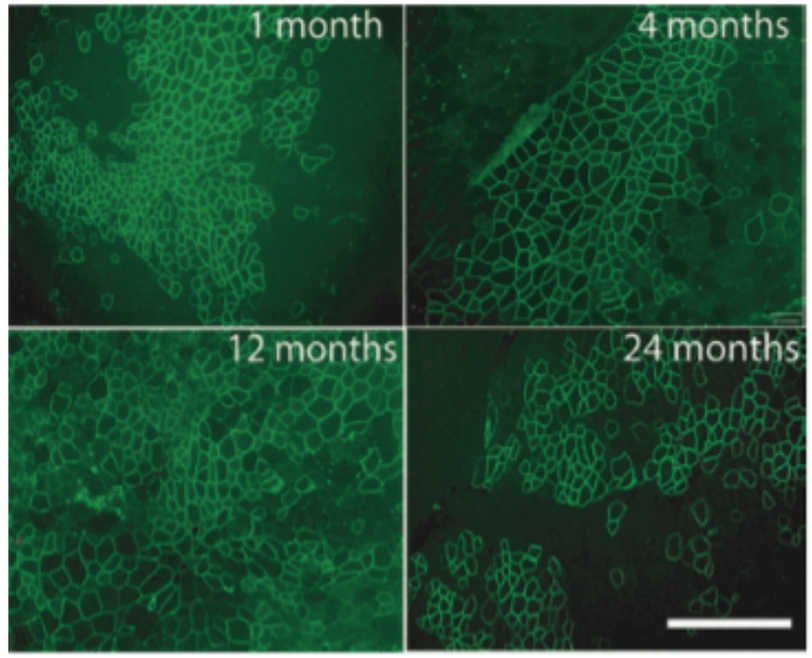
IHC



- cardiomyocyte
- myocytes
- neuron after treatment

- control
- cardiomyocite after treatment
- myocyte after treatment

fluorescence microscopy



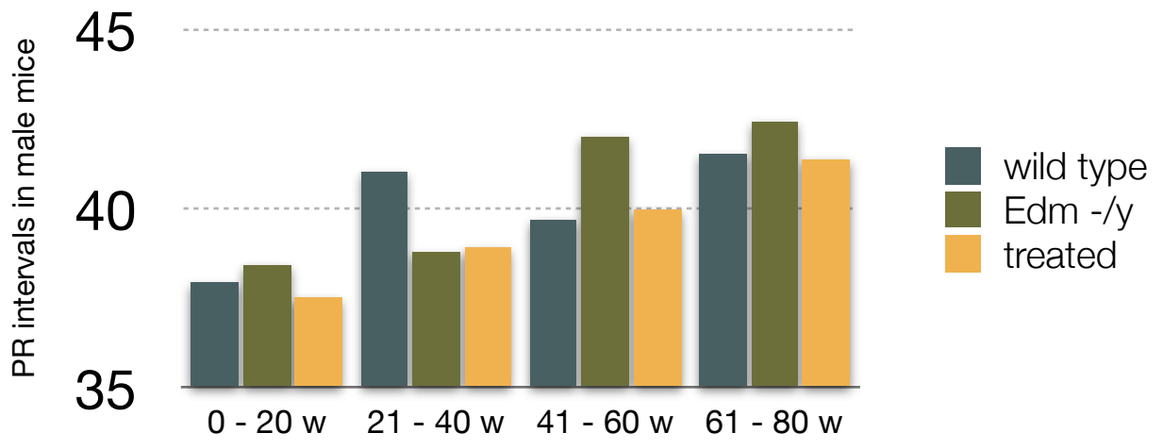
In vivo - mouse

Functional assays

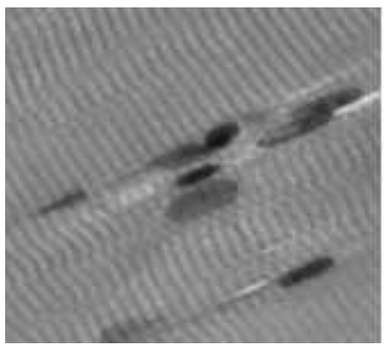
Rotarod test

	wt mice	Emd-null mice	Treated mice
4 - 10 W	100%	42%	98%
11 - 20 W	100%	30%	94%
Total	100%	36%	96%

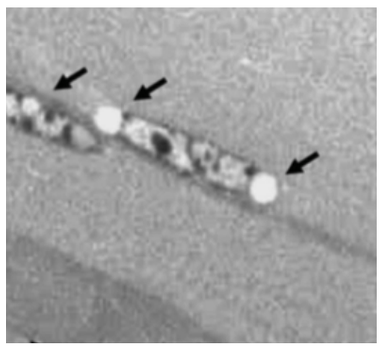
Electrocardiogram



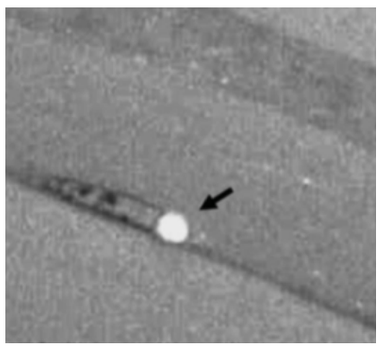
Electron microscopy



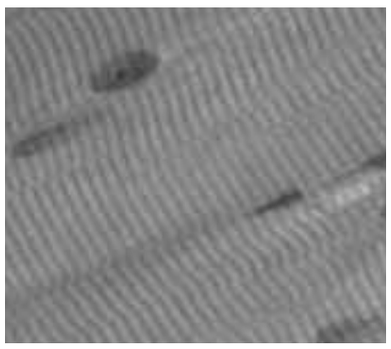
wt



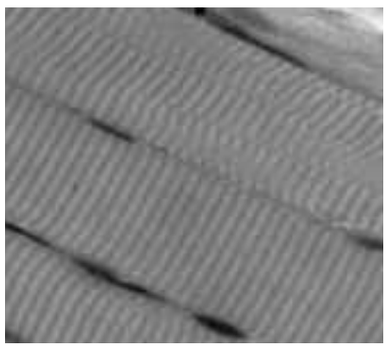
Emd -/y



Emd -/-



Emd -/y
+ lentivector



Emd -/-
+ lentivector

Pitfalls and future perspectives

- Exogenous Emerin protein could be non-functional because of a lack of post-transcriptional/translational modifications.
  This could be resolved using cDNA containing some important introns that are missing in our version.

- Transgene insertion can induce tumors formation because random integration of lentivirus could target an oncosuppressor.
  In vivo, we could perform a LAM-PCR to check the integration site of the virus.

- The TK suicide gene could not be efficient.
  In this case, we could change for another suicide gene or for another promoter.

- Lentivector delivery could be affected by difficulties.
  In this case, we could consider different injection protocols, or to combine two different.

- Observation of clear results can be complicated. Indeed the mouse model we chose shows minimal dysfunctions.
  We could use another mouse model that presents muscular phenotype closer to the one in humans or another mammalian model like dogs that are often used in muscular diseases projects.

Our studies born having as goal a therapy for a human disease, so hopeful results coming from these set of experiments could encourage us to project a clinical trial.

Trials made during neonatal phase are suggested, in order to avoid muscle degeneration.

During clinical trail great attention should give to control safety of treatment.

As far as tissue specific transgene expression is concerned, EboZ GP + DisEnh and promoter could be used in other muscular pathologies as a way to induce an efficient and cell-targeted gene expression.

Costs

40.000,00€ - 3 years

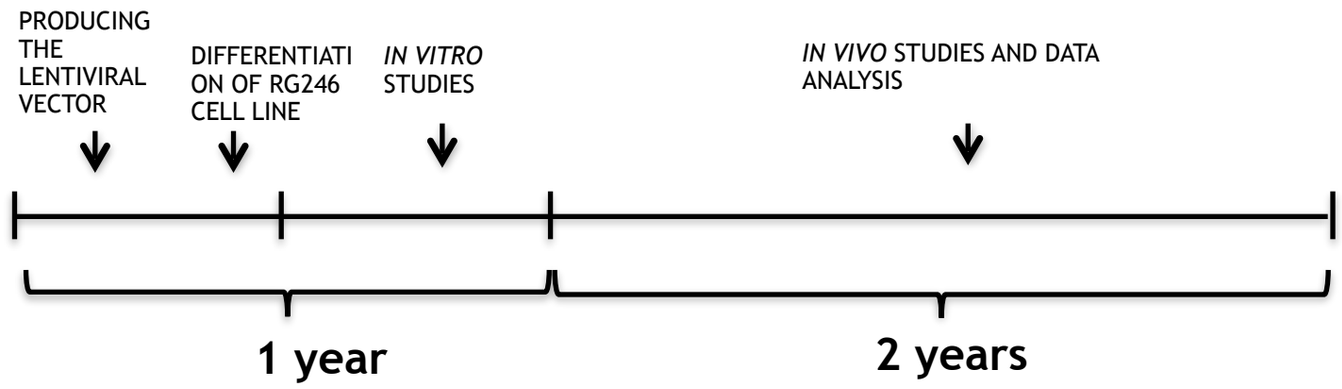
- Cell lines: - **RG246** contact Reproductive Genetic Institute
  - **293T** 575,00€
  - **Cell colture facilities and reagents for differentiation** 2500,00€

- Mice lines: - **C57/BL6 Mice (WT)** M: 20,15€ per mouse (3x 20,15=60,5€) F: 21,75€ per mouse (3x21,75€=65,2€)
  - ***Emd* -/- female Mice** 300,00€ per mouse (3x300,00=900,00€)
  - ***Emd* -/*y* male Mice** 300,00€ per mouse (3x300,00=900,00€)
  - **Stabulation** 1000,00€ per month 24 months of experiment TOT 24000,00€

- Lentiviral vector: 400,00-1200,00€
- ELISA kit for titer (ThermoFisher): 400,00€
- *In vitro* and *in vivo* experiment:

  Antibodies (good for both WB and Immunostaining)
  - **Emerin antibody Sigma** 310,00€
  - **Erk 1/2 antibody SantaCruz** 279,00€
  - **pErk 1/2 antibody SantaCruz** 279,00€
  - **Lamin β-1 antibody SantaCruz** 279,00€
  - **Secondary antibody (α-mouse)** 190,00€

  - **qRT-PCR kit** 100,00€



- Lab materials needed: - Immunofluorescence and Electron Microscopy
  (already present in the lab) - Real Time qPCR
  - Softron ECG Processor SP2000
  - Cages and other animals facilities (rotarod and other functional assay)
  - Chemical reagents, plastic tools, glassware and other basic instrument of molecular biology

The Jackson Laboratory

SANTA CRUZ BIOTECHNOLOGY

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