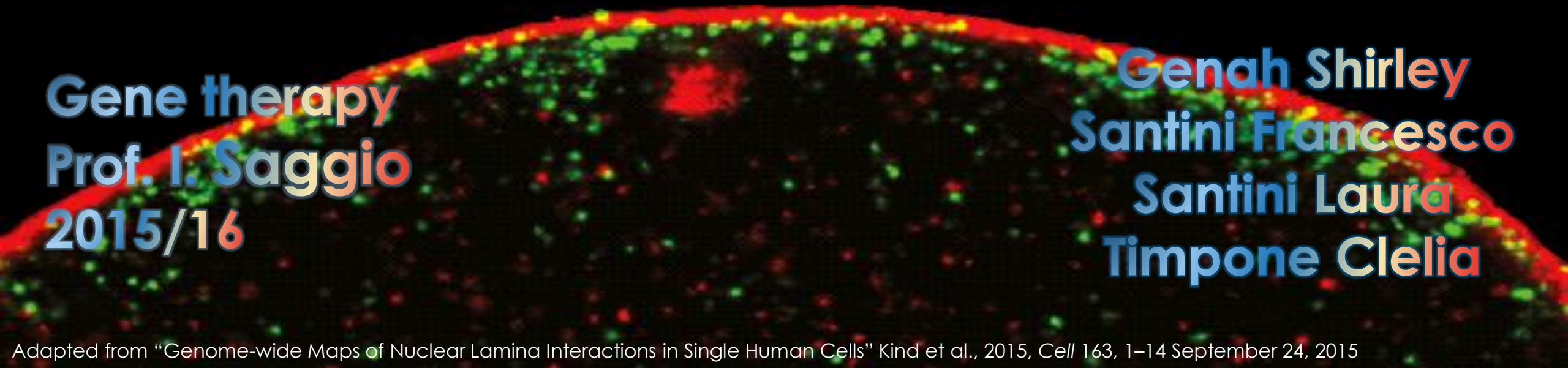
Laminopathies&non viral vectors

Novel therapeutical approach based on gene-therapy



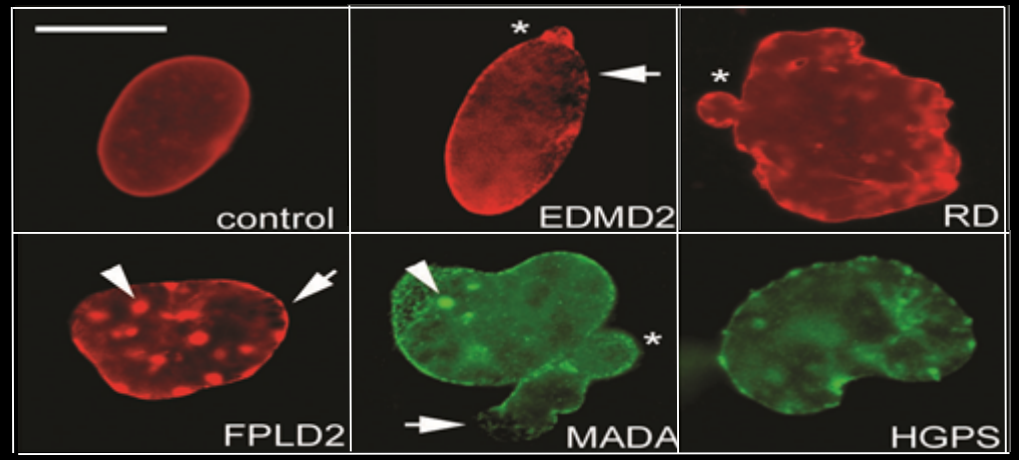
Gene therapy
Prof. I. Saggio
2015/16

Genah Shirley
Santini Francesco
Santini Laura
Timpone Clelia

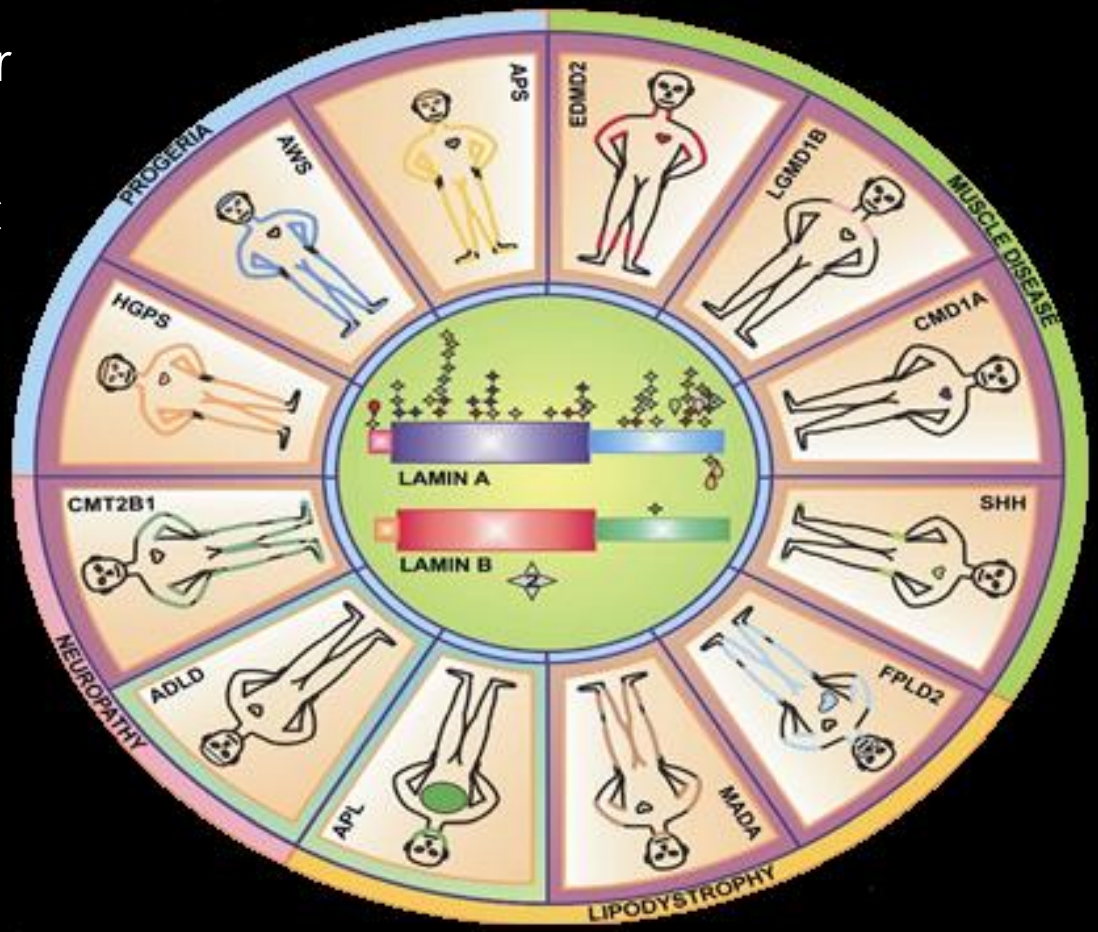
Adapted from "Genome-wide Maps of Nuclear Lamina Interactions in Single Human Cells" Kind et al., 2015, *Cell* 163, 1–14 September 24, 2015

Laminopathies are a group of genetic diseases caused by mutations in genes encoding *lamins*, the proteic components of nuclear lamina, or in proteins related to their post-trascriptional modification or interacting with them

Nuclear lamina has roles in maintenance of nuclear shape and structure, transcriptional regulation, nuclear pore positioning and function and heterochromatin. Lamins also interact with the LINC complex to coordinate chromatin dynamics with cytoskeleton and extra-cellular signals, influencing thereby citoplasmatic situation [2]



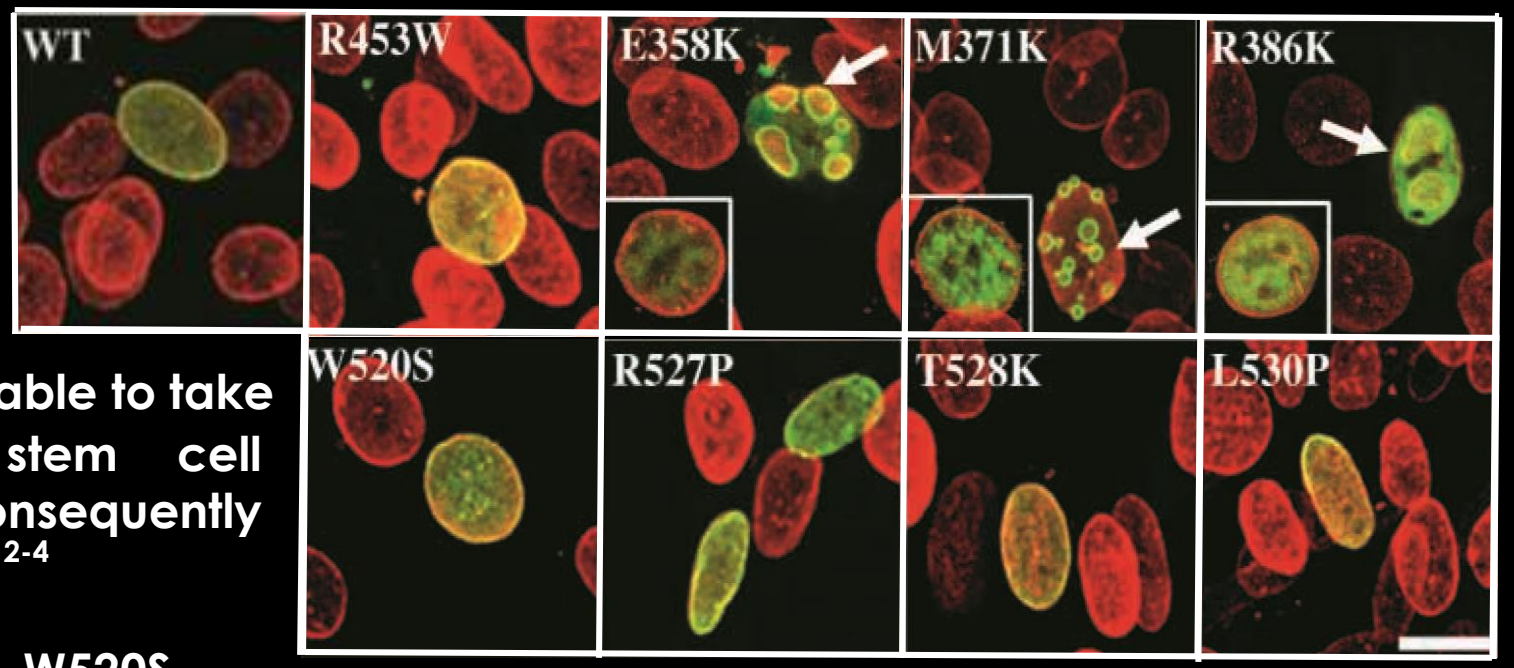
Adapted from Lattanzi et al *Nucleus* September/October 2014



Adapted from R. Rzepecki et al. *Cellular and molecular biology* 2011

AD-EMD2: Autosomal Dominant Emery-Dreifuss disease type 2

AD-EMD2 is a laminopathy caused by mutation with loss of function in *Lmna gene* (reported similarly in *Lmna* −/− muscle fiber nuclei). Main feature is muscolar distrophy : contractures of the elbows, Achilles' tendons and posterior neck and progressive muscle wasting encome early. Nuclei are enlarged, not able to take the right position during muscle stem cell differentiation and regeneration, with consequently wrong regulation of myonuclear domains [2-4]



Adapted from Ostlund et al Journal of Cell Science 2001

The most frequent mutations are R453W, W520S, R527P, T528K, L530P, E358K, M371K AND R386K: last three show the worse affection, and are all located in exon 6.[4]

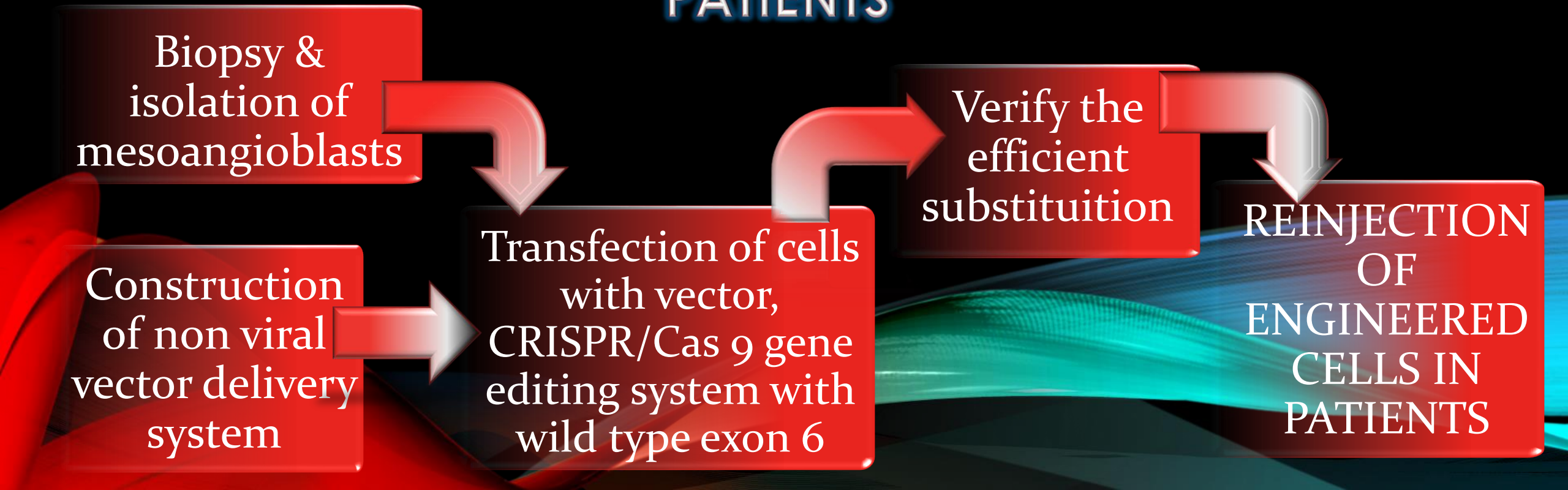
Adapted from Capell and Collins *Nature Reviews Genetics,* December 2006)

OUR APPROACH

A NOVEL GENE THERAPY STRATEGY TO REPLACE EXON 6 IN MABs TOOK FROM PATIENTS VIA GENE-EDITING

&

AUTOTRANSPLANTATION OF CELLS WITH SUBSTITUTION IN PATIENTS



Genome editing strategy

WHAT	WHY
CRISPR/Cas 9 system to direct the integration of our donor template in specific site in the genome. It's a powerfull tool, but it's not perfect: it can stand 5 nucleotides overhang [15]	So we chose an improved Cas9 nickase mutant form instead of the WT endonuclease: this improvement allows a very more specific cleavage and decreases the number of potential off-targets [11, 13, 15]
A NON VIRAL delivery system: a chimeric protein to target cells and bring our transgene into them	Safer than viral vectors, less citotoxyc than other chemical delivery system and less stressful than physical one [6]
A tiny sized gene vector, stable but with transient expression	The little size of our gene vector improves the expression of our transgene, but do not integrates itself in the host : we'll have safe expression but only for the time we need it! [7-10]

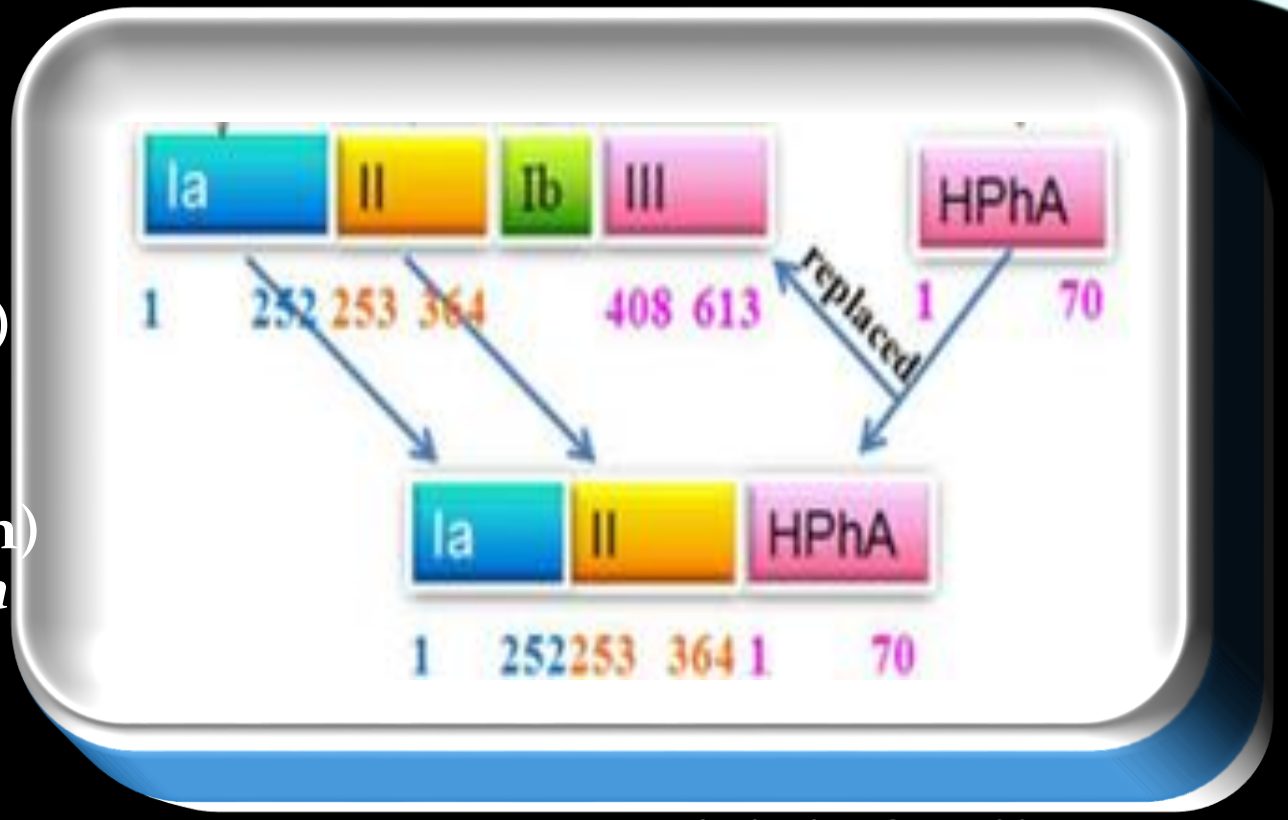
Chosen vector

The vector is the fusion protein rPE-HPhA composed by 2 parts:

1)Pseudomonas aerouginosa Exotoxin A(PE)

- Domain Ia (resposible of the recognition of LDL/α2-macro-globulin receptor)
- Domain II (responsible of the translocation)

2)Pyrococcus horikoshii Histon-like protein HPhA. Our vector can bind efficiently and with high affinity either receptor on cell surface and DNA



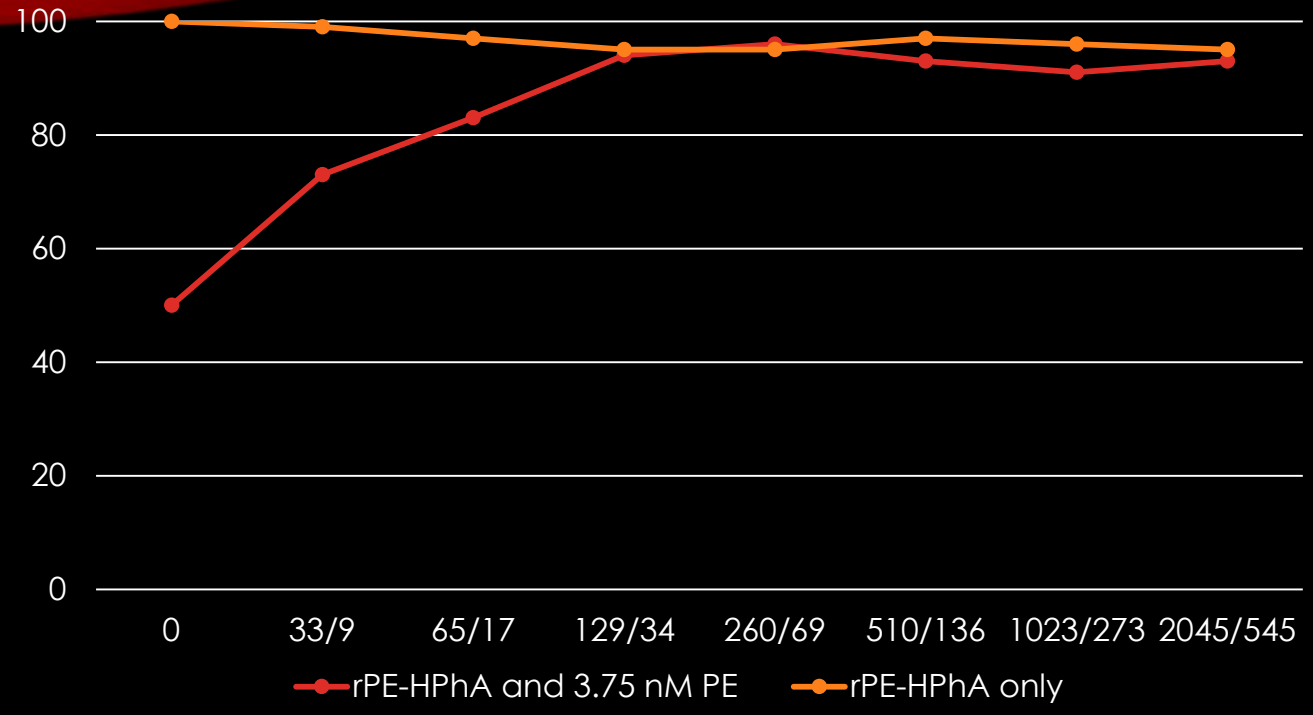
Deng et al, *Plos One*, 2015

?Why a proteic vector?

- NON VIRAL NO immunogenic response or inability to inject again the patient, NO restrictions about the size of the transgene, LESS costs and NO insertional mutagenesis
- LESS CYTOTOXIC AND MORE EFFICIENT THAN LIPOPLEXES the most used tools among nvv
- LESS STRESSFUL THAN PHISICAL DELIVERY
- CHEAP AND EASY PRODUCTION

All ref 6

The delivery sistem was tested for SAFENESS

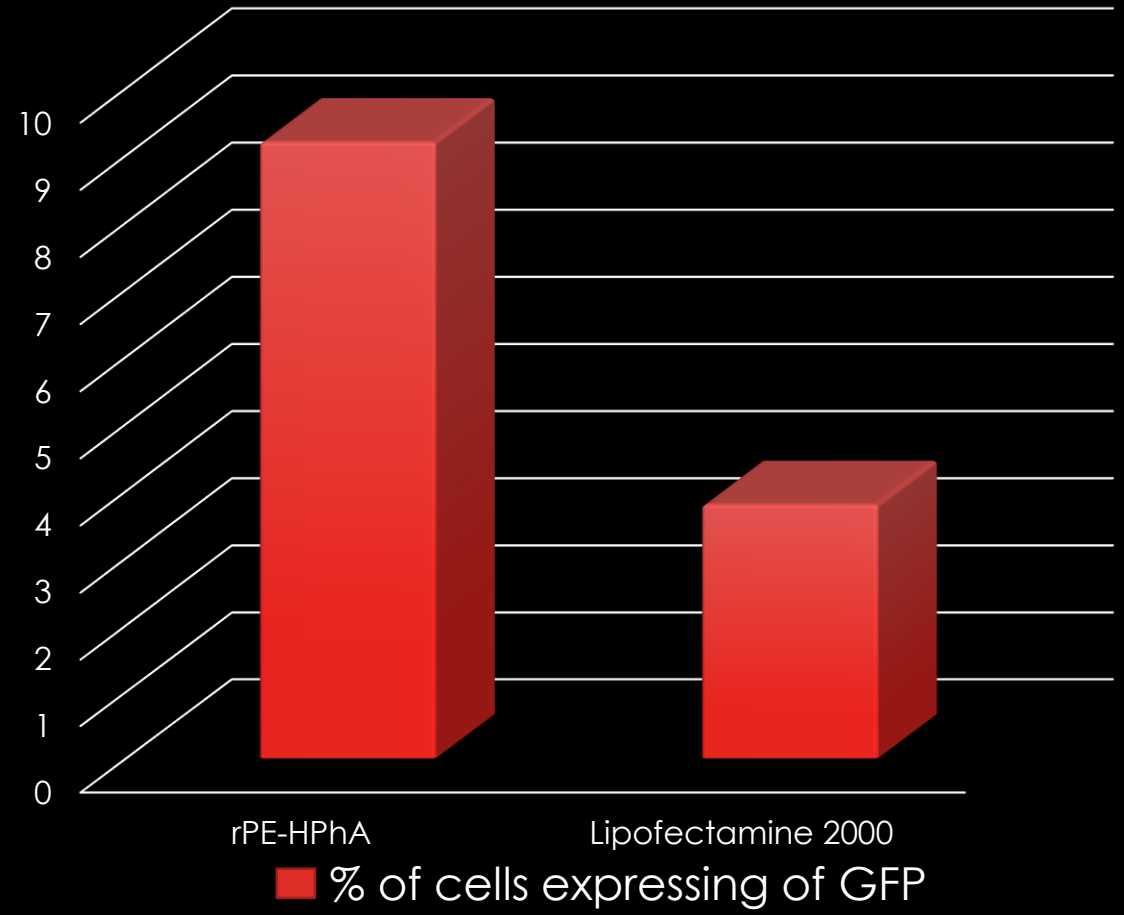


On x axys: the first number is the concentration of rPE-HPhA in nM and the second the ratio of rPE-HPhA and *Pseudomonas Exotoxin* 3.75 nM
On y axys: percentage of alive HeLa cells

The graph shows that pretty all cells are alive after treatment with even very high doses of rPE-HPhA and the effective competion between our vector and non engineered *Pseudomonas Exotoxin A* (PE) [6]

Adapted from Deng et al, *Plos One*, 2015

EFFICIENCY



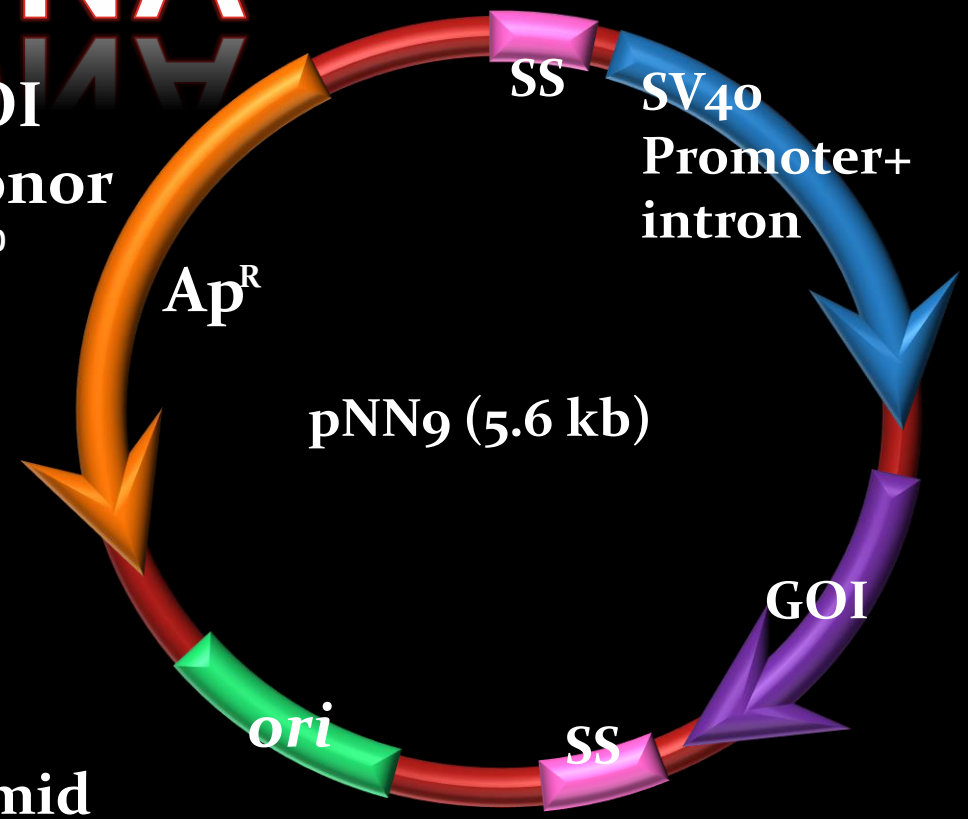
Compared to cationic liposomes (the non viral vector most used) our system shows higher efficiency of transduction, measured by GFP expression [6]

Adapted from Deng et al, *Plos One*, 2015

DNA vector:LCC DNA

Linear Covalently Closed DNA are minimized GOI expression cassettes produced starting from a donor plasmid by the cleavage of *TelN protelomerase* [7-10]

- **Tiny size:** 2-3 kb long
- **No-prokaryotic backbone:** immune response by the host cell is avoided
- **Integration in the genome occurs with a very low frequency**, and if it happens it results in **chromosome disruption and apoptosis**
- **Great efficiency of expression: >17 time respect plasmid**
- **Stability**: the flanking hairpins protect from degradation by endogenous nucleases, **AND ALSO TRANSIENT EXPRESSION TOO**
- **SS sequences** used for production already encode **DTS sequences**



LCC DNA (2.4 kb)

Adapted from Nafissi and Slavcev, *Microbial Cell Factories*, 2012

Our transgene

EXON 6 (221 bp) flanked by other sequences:

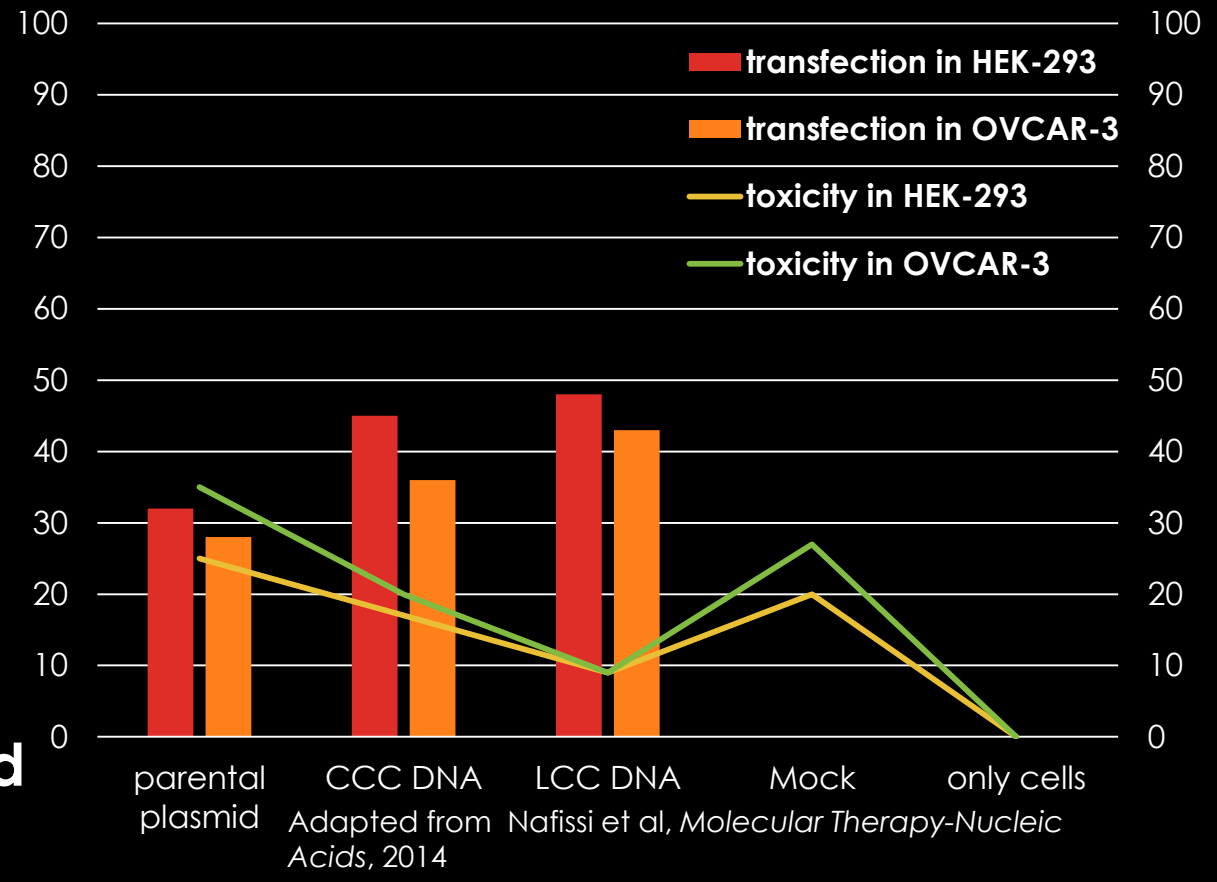
- upstream INTRON 5 (588 bp)
- downstream INTRON 6 (92 bp)

EXON 7 (223 bp)

PART OF INTRON 7 (300 bp)

To achieve Homologous Recombination (which request >500 bp us and ds the sequence) instead of Non Homologous End Joining (NEHJ) system [11,12]

Toxicity and efficience of LCC DNA in comparison with other vectors

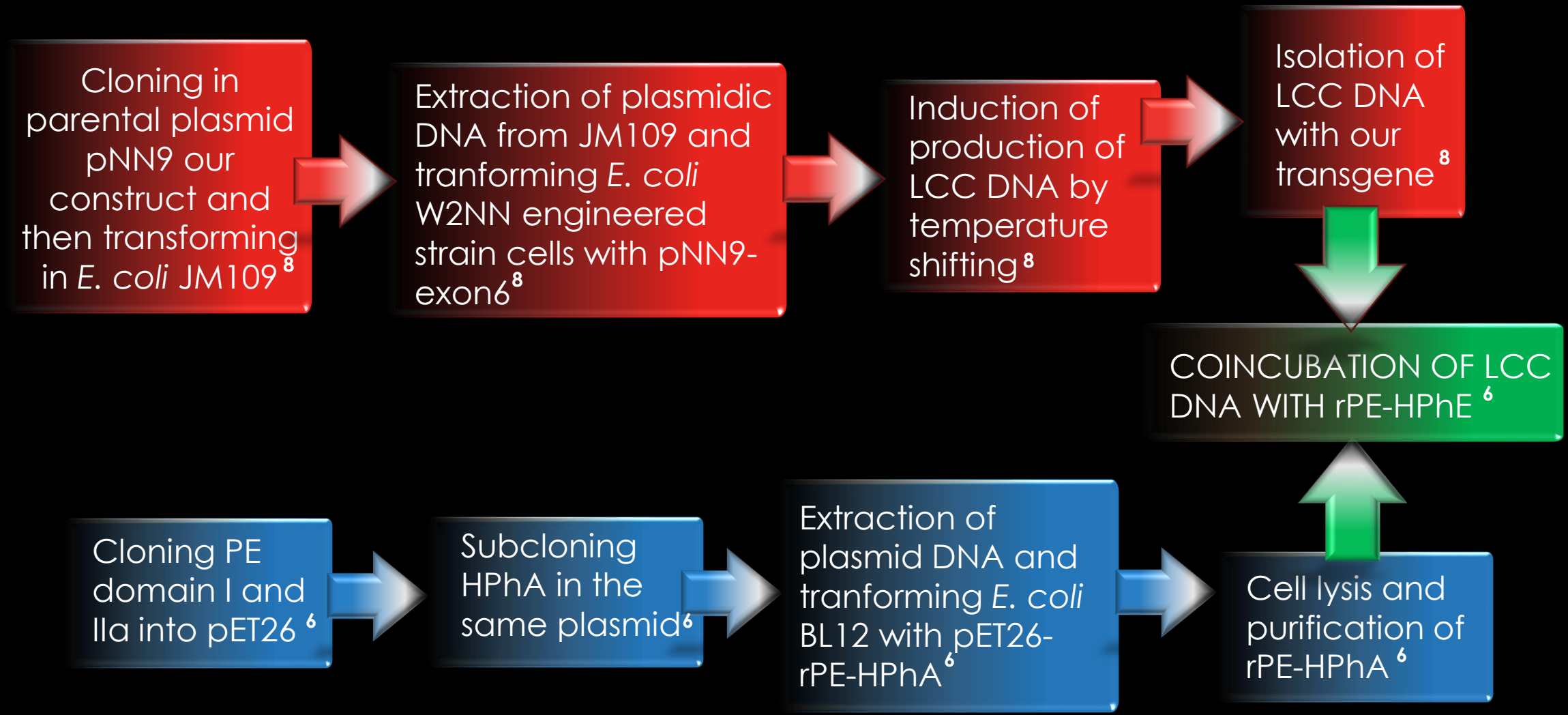


Adapted from Nafissi et al, *Molecular Therapy-Nucleic Acids*, 2014

In one of the introns we put a **unique** **5'-GTCGAC-3' SITE FOR *SalI*** [12, NCBI BLASTA, Restrictionmapper.org]

Instead of SV40promoter we put a REPORTER GENE: in this instance PUROMYCIN RESISTANCE

CONSTRUCTION OF rPE-HPhA-LCC DNA VECTOR

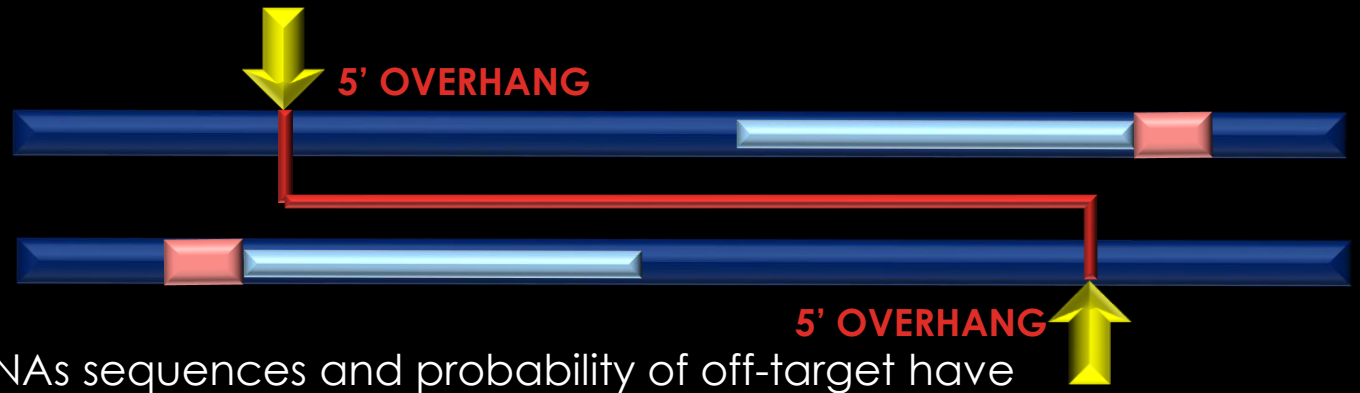


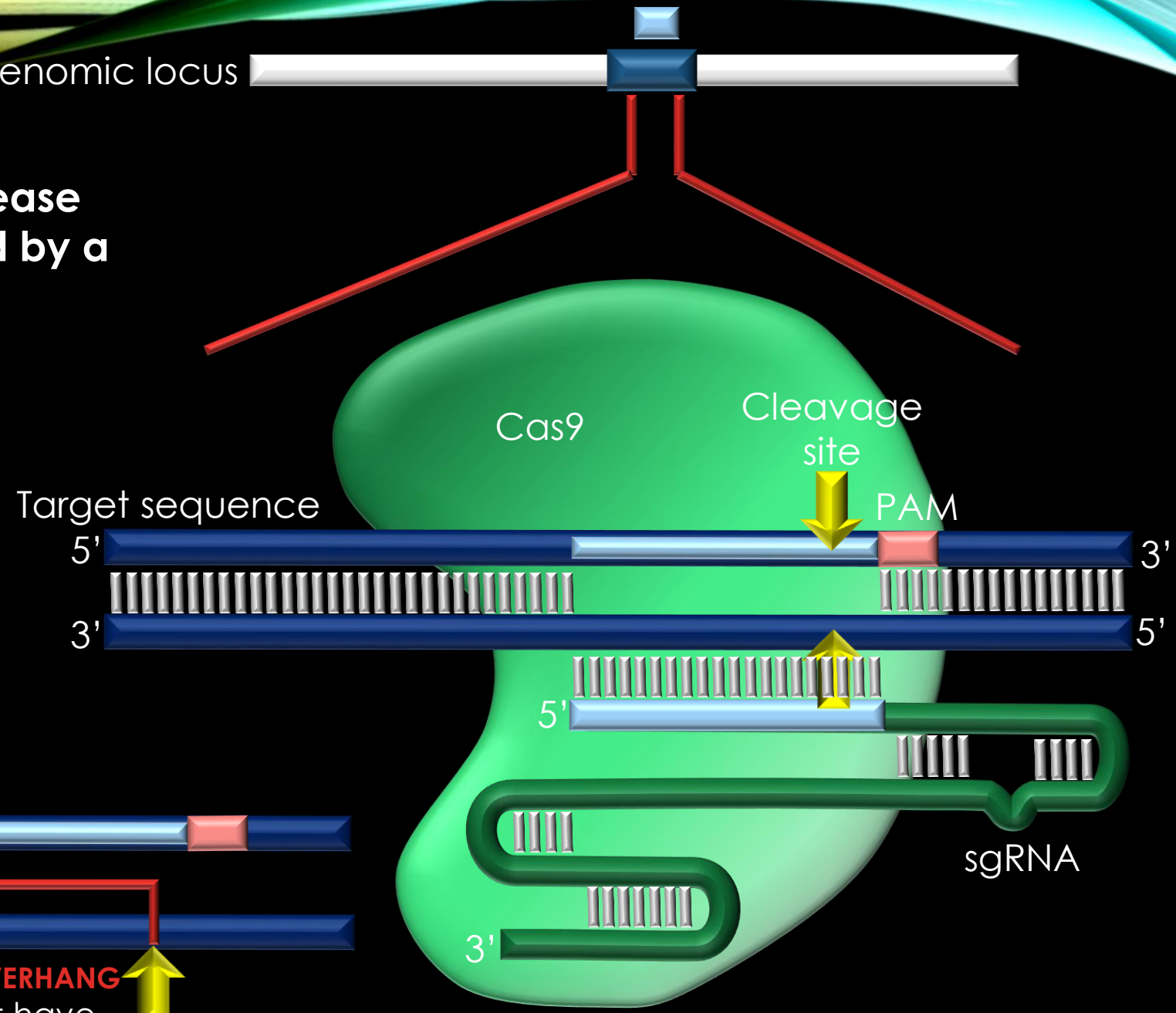
CRISPR/Cas9

CRISPR/Cas9 type II is an endonuclease that can cut DNA where it is allowed by a single guide RNA (crRNA+tracrRNA)

We choosed to perform genome editing with CRISPR/Cas 9 NICKASE drived by a couple of sgRNA
It cut only the base-paired strand but not the other one

HIGHER SPECIFICITY!





SgRNAs sequences and probability of off-target have been designed by tools.genome-engineered.org

Adapted from Ran et al, *Nature Protocols*, 2013

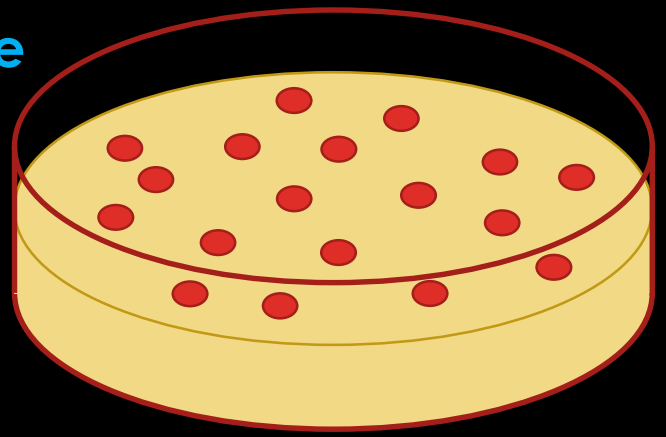
In vitro experiments: HEK 293 CELLS

3 types of rPE-HPhA-LCC DNAvector

CRISPR/Cas9-1° couple of sgRNAs-eGFP

+

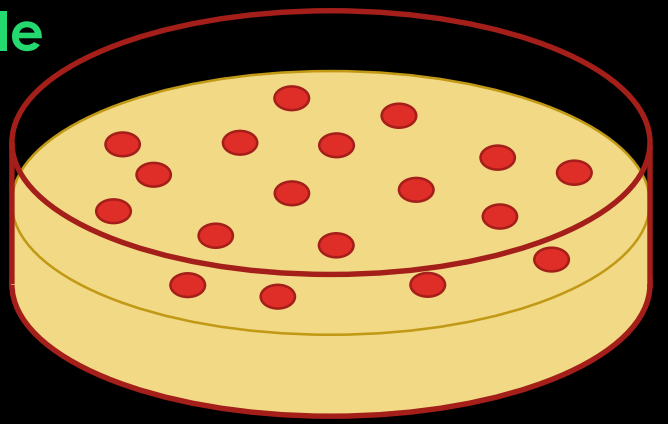
intron5-Exon6-intron6-exon7-intron 7 T-PURO^{R}



CRISPR/Cas9-2° couple of sgRNAs-eGFP

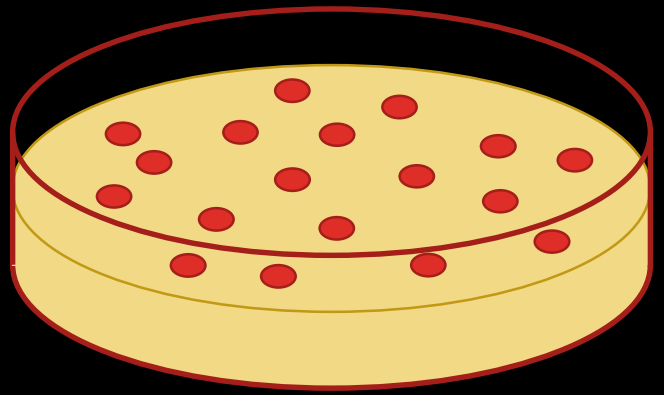
+

intron5-Exon6-intron6-exon7-intron 7 T-PURO^{R}



TO CHOOSE WHICH ONE IS THE BEST

Untreated HEK 293 cells

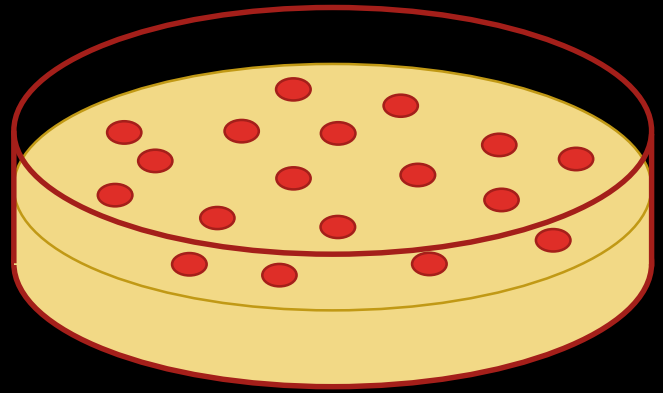


Selection is performed by:

- PURO assay
- eGFP assay

As control

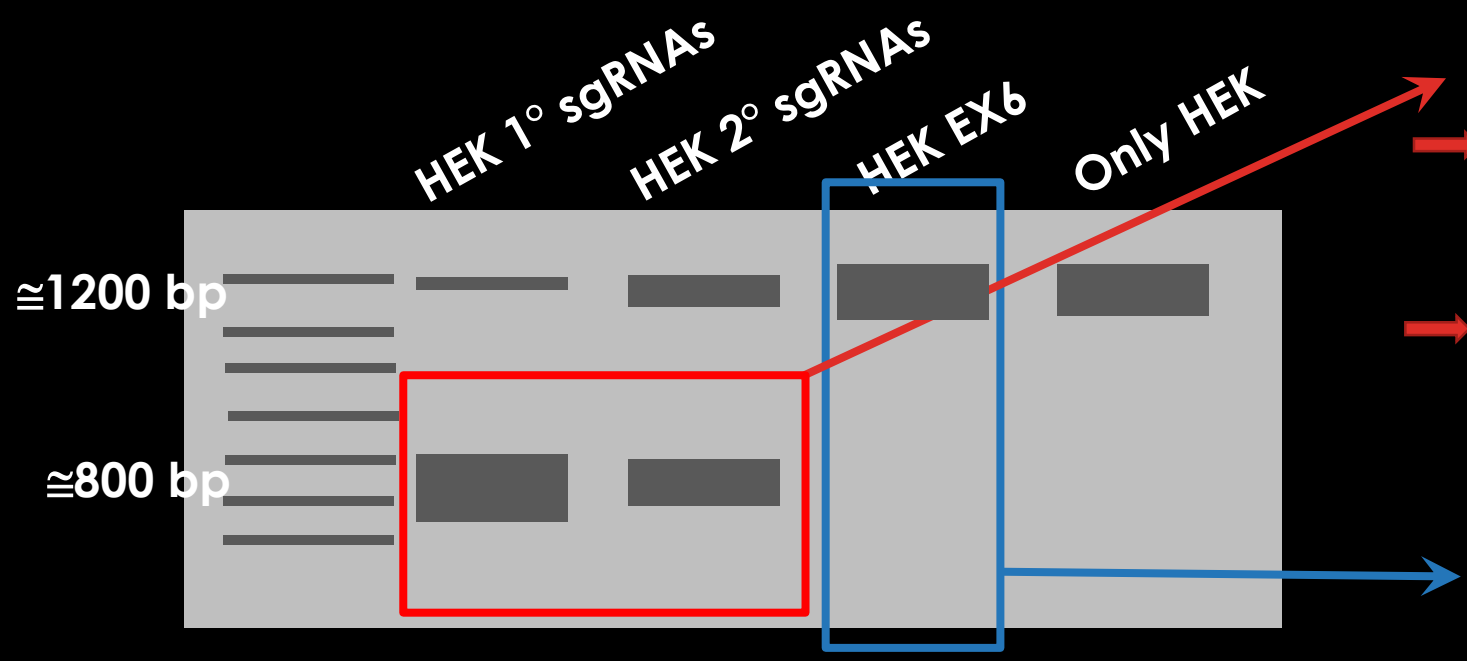
intron5-Exon6-intron6-exon7-intron 7 T-PURO^{R}



Has the recombination occurred?

DNA EXTRACTION → PCR amplification

The insertion of our gene will be detected by CLEAVAGE BY *SalI*



SOUTHERN BLOT WITH PROBE AGAINST EXON 6

These bands tell us that

- **YES! RECOMBINATION HAS HAPPENED**
- **1° couple of sgRNAs is more efficient than 2° one**

Eventualy traces in this lane would have ment insertion of the LCC DNA directly in the genome: then the replacement is not due to CRISPR/Cas9

Going further: the *in vivo* model

Adapted from G. Bonne et al, *Human Molecular Genetics* 2005

We chose *H222p knock in* mouse model that shows the same phenotype of our disease.[Ref foto]

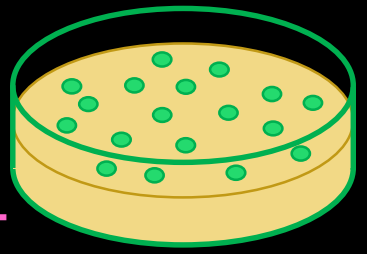
- **normal embryonic development and sexual maturity**
  - **at adulthood reduced locomotion activity with abnormal stiff walking posture**
    - **die by 9 months of age**

In vivo extperiments have been developed with an *ex vivo* approach to boost the possibility of **EFFECTIVE gene editing**

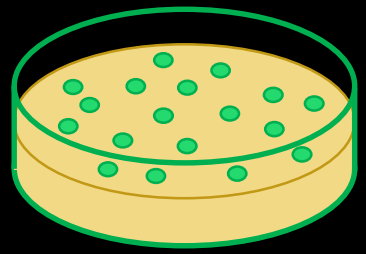
Isolation of mesoangioblasts from juvenile *Lmna* *H222P/H222P* mice [17] → **Construction of specific rPE-HPhA-LCC DNA vectors**

CRISPR/Cas9 1° couple sgRNAs-eGFP +

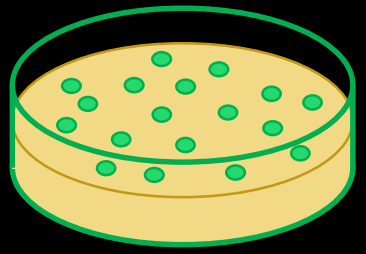
Intron5 Truncated -exon 6-intron6-exon7-intron 7-PURO^R



Intron5 Truncated -exon 6-intron6-exon7-intron 7-PURO^R



Untreated cells



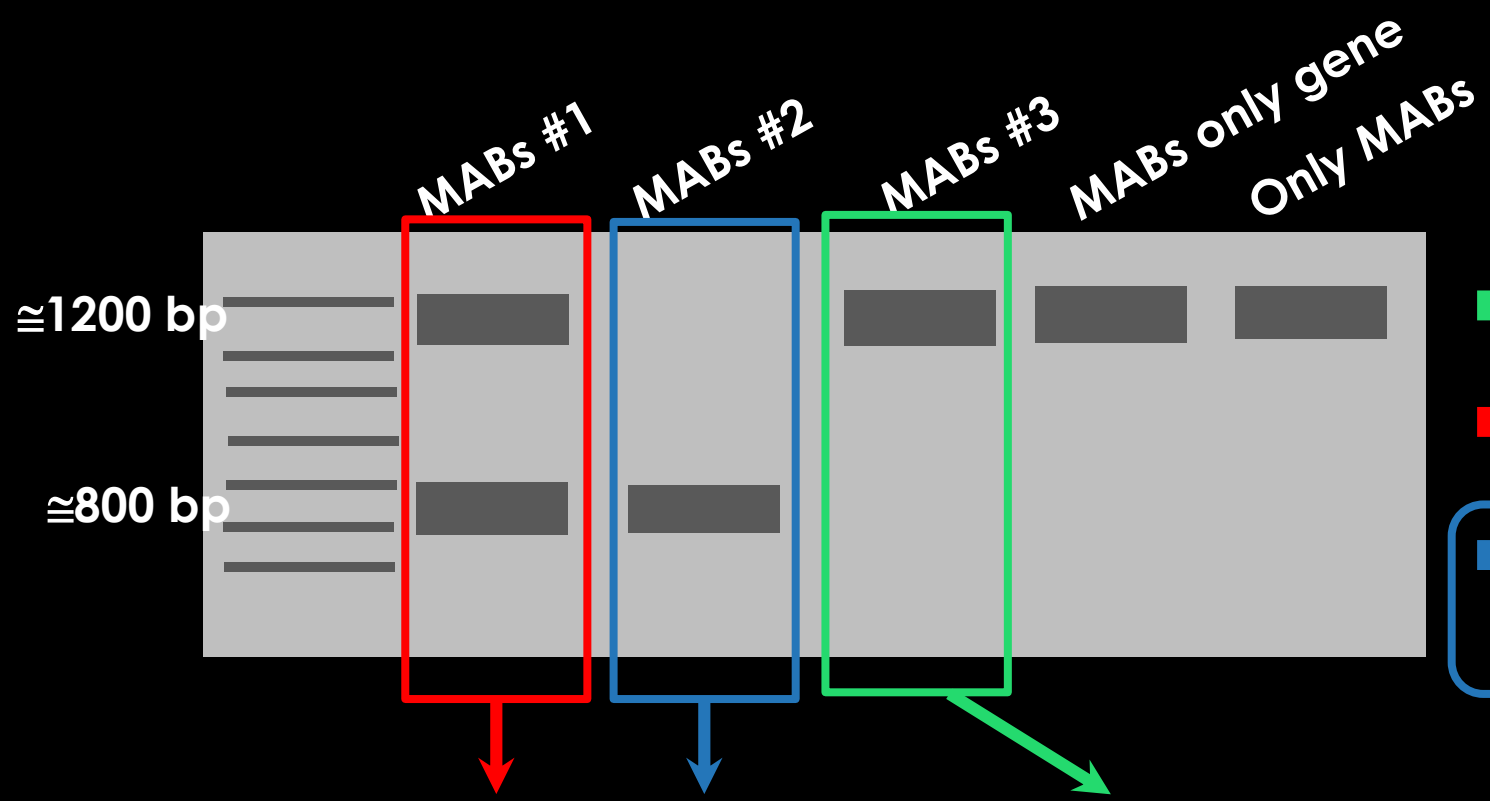
CONTROLS

SELECTION BY eGFP EXPRESSION&PURO RESISTANCE

CLONAL EXPANSION

SINGLE COLONY ANALYSIS

- Isolation and amplification of the fragment with PCR
- Digestion with SalI
- **Southern Blot with probe against exon 6**

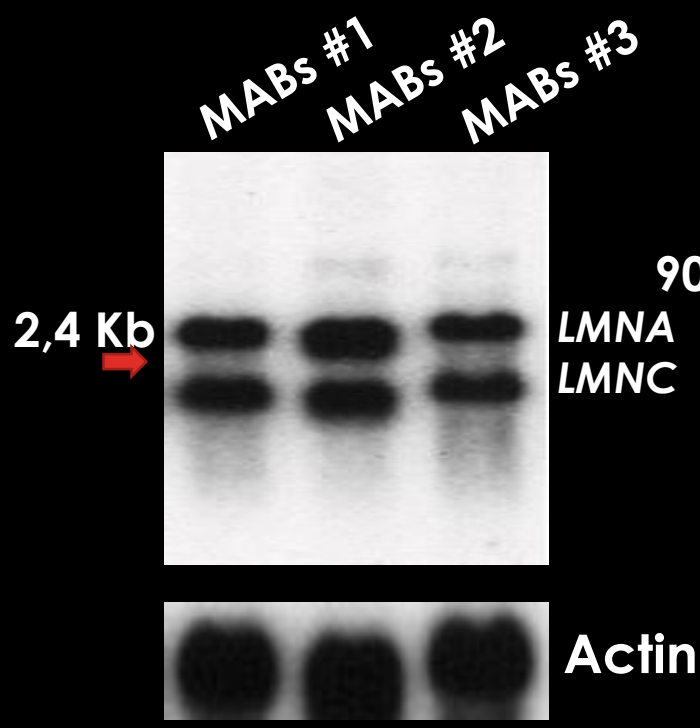


The lenght of the fragment tell us if recombination has happend:

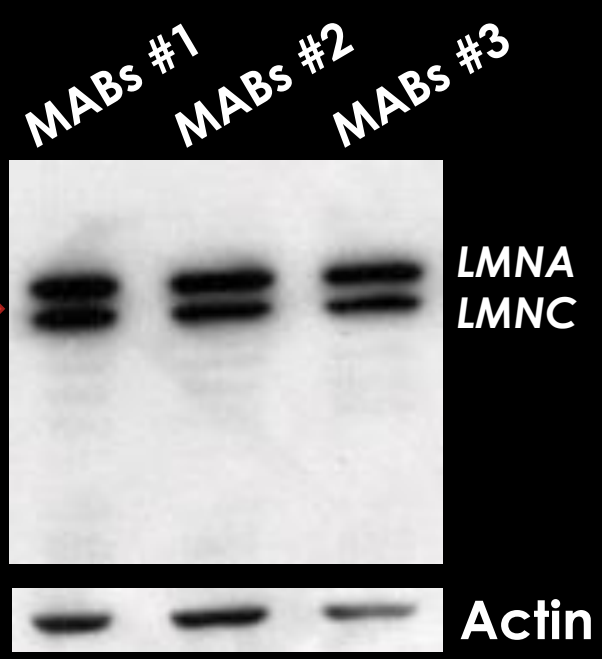
- 1 band at ≅1200: no cleavage
  NO RECOMBINATION
- 2 bands: half fragment cleft
  1 ALLELE RECOMBINATED
- 1 band at ≅800: all fragments cleft
  BOTH ALLELES RECOMBINATED

WE NEED THESE COLONIES

NORTHERN BLOT ASSAY SHOWS NO DIFFERENT FEATURES AMONG 3 SAMPLES…

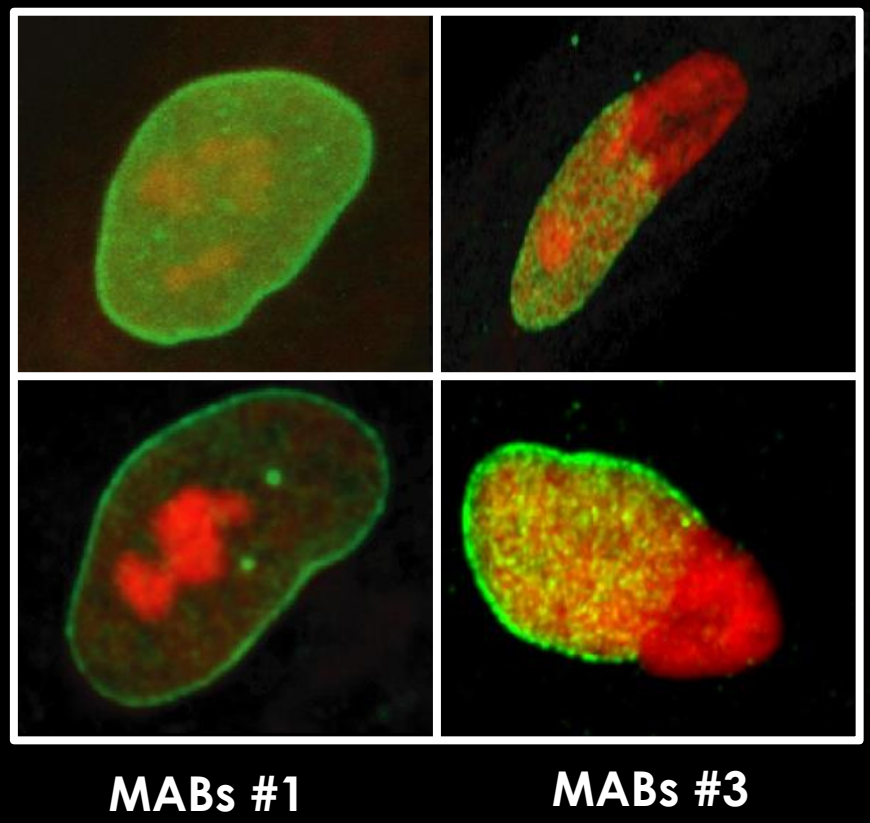


…AND WESTERN BLOT TOO



Adapted from Human molecular genetics Oxford University press

While immunostaining assay shows relevant differences among the three clonal populations



Adapted from Hutchinson et al APS JOURNAL July 2006

AND GENE SEQUENCING OF POSITIVE COLONIES

CLONAL EXPANSION OF HOMOZYGOUS MABs COLONY

TNFa treatment pre-injection [18]

INJECTION OF ENGINEERED MABs IN MICE VIA FEMORAL ARTERY [17-19]

WE EXPECT TO SEE RESCUING OF PHENOTYPE IN MICE

LOCOMOTION FUNCTIONALITY ASSAY

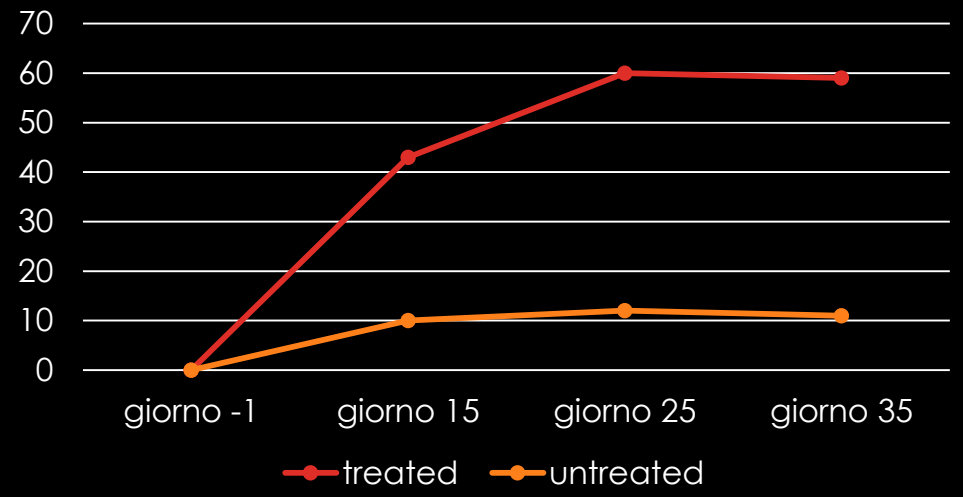
Running on wheels [17]

Adapted from https://www.mouseclinic.de

Forced run on the rotarod (at a fixed speed 1.6 m/min for 4 min, 2 min run, 1 min rest, 2 min run)

Adapted from https://www.mouseclinic.de

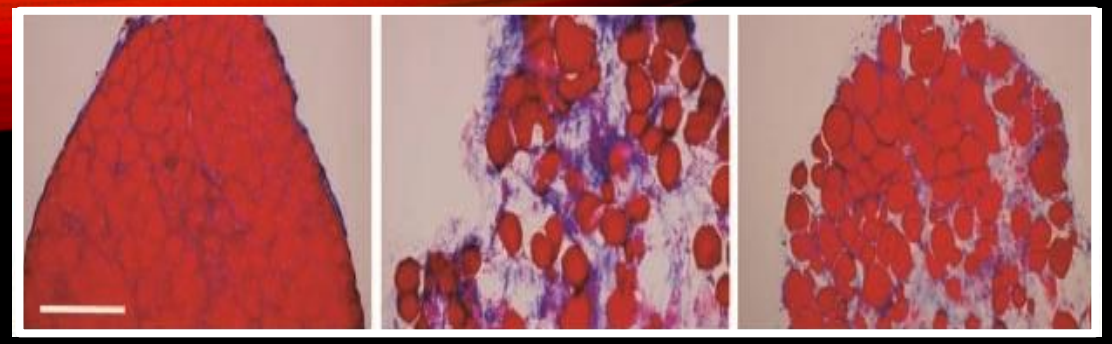
Time to exhaustion on a treadmill test



Adapted from Tedesco et al, *Science Translational Medicine*, 2012

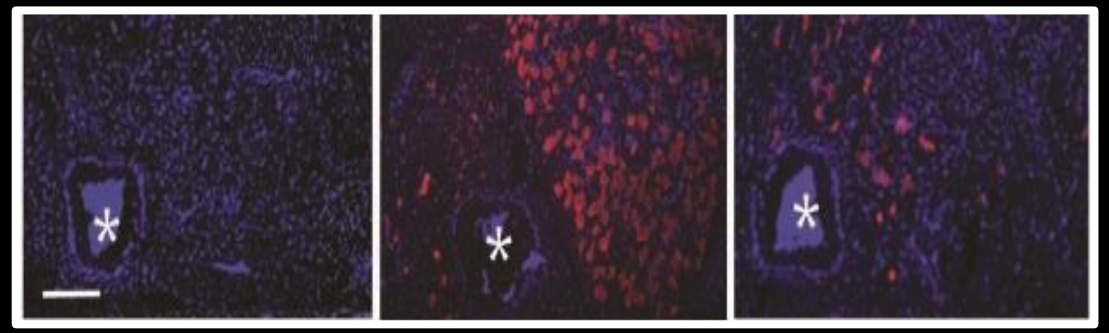
HISTOLOGICAL ASSAYS

CONTROL UNTREATED TREATED



AZAN-MALLORY TRICHROME STAINING

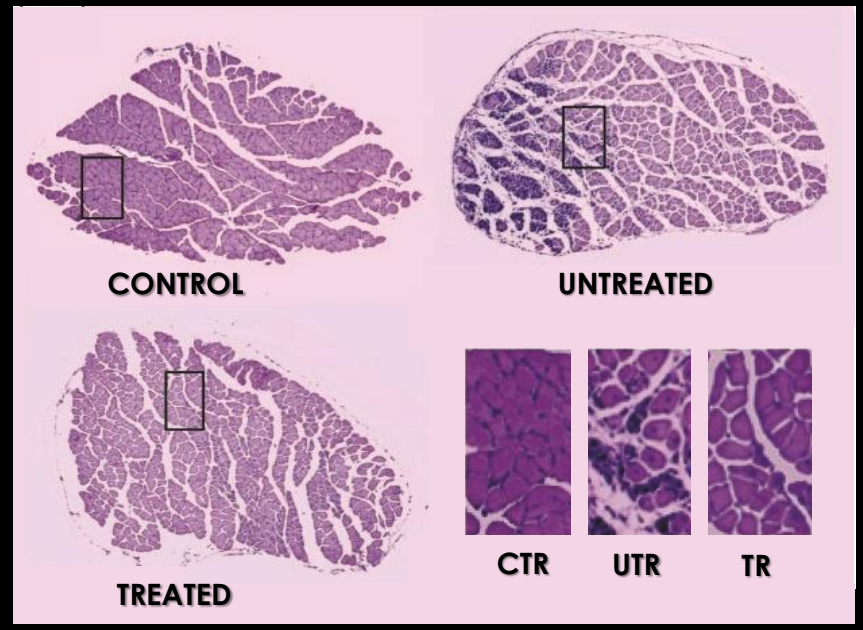
Tibialis anterioris



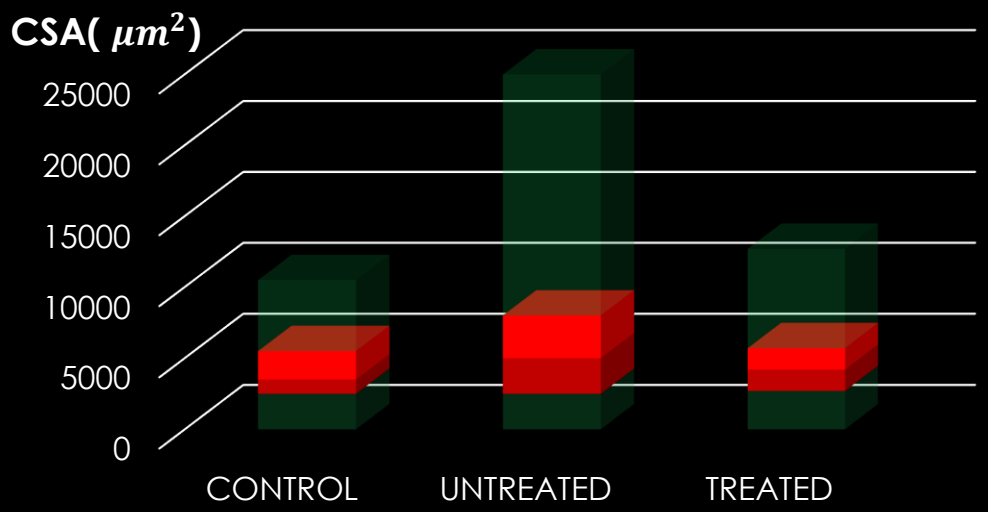
FLUORESCENT IMMUNOSTAINING

Blue dye injection into the tail vein
nuclei in blue by DAPI staining
tibial bone stared

HAEMATOXYLIN/EOSIN STAINING



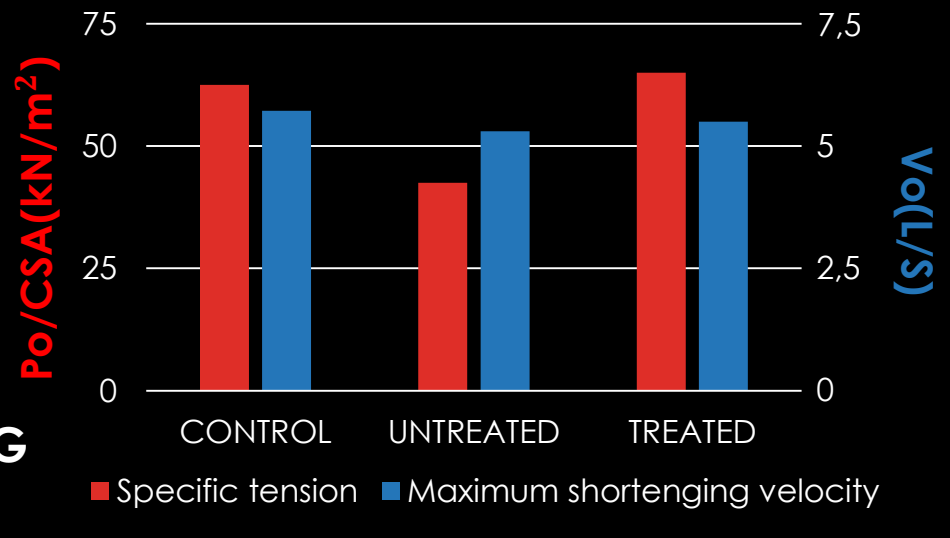
Soleus muscle



SINGLE MYOFIBERS MEASUREMENTS

Cross-sectional areas

MEASUREMENT OF MUSCLE FORCE&SHORTENING



AND OF COURSE GENE SEQUENCING!

…SO WHAT ABOUT THE FUTURE?

- Testing many couples of CRISPR/Cas9 and eventually testing a strategy with two couples of nickases
- Instead of measoangioblasts, using iPSC for *ex vivo* therapy [19]
- Repeating the experiments with a larger group of animal models and going further with other ones, such as dogs [20]

Pitfalls&solutions

- If the engraftment doesn't work?
- If CRISPR/Cas9 offtarget probability is too high?
- If HDR probability is too low?

- *In vivo* delivery with lipoplex [10]
- Screening many couple of sgRNAs
- Delivery a ss oligo DNA that boost HDR [21]

Cost$

	€	Company
DNA	Purchased from	
HPhA cDNA	Yan Feng (Jilin University) [6]	
PE cDNA	Guoli Zhang (Changchun, China)[6]	
Plasmids		
pET26b	59	Addgene
pNN9	59	Addgene
***Escherichia coli* strains**		
JM109	53	Promega
BL21	129	New England Biolabs
W2NN	Purchased from R. Slavcev and N.Nafissi [8]	
Animal models		
H222P mice	2365+ Mainteinence	Jackson Laboratory
wild type mouse	45+ Mainteinence	Jackson Laboratory
Cell cultrures&Maintainence		
HEK 293	575	Addgene
HEK 293 medium	39	Addgene
Megacells DMEM	33,50	Sigma-aldrich
Restriction enzymes	≅60/RE	N.E.B.
CRISPR/Cas9 encoding plasmid (bacteria agar plate)	59	Addgene

	€	Company
PCR kit	200	Qiagen
PCR primers	25/oligo	Jena Bioscience
Cloning kit	330	Addgene
Antibiotics	≅400	Sigma-aldrich
Southern Blot Reagents	≅ 1000	Thermo Scientific
Northern Blot Reagents	≅ 1000	T.S.
Probe labeling kit	345,50	T.S.
Western Blot reagents	≅ 1500	T.S
Antibodies	≅600/ab	T.S
Secondary antibodies	≅150/ab	T.S
Immunostaining reagents	≅1000	Sigma-aldrich
Fluorescent Probe	≅200/pr	T.S.
Histologial staining Reagents	≅400	Sigma-aldrich
Plasmidic Dna purification kit	150-200/kit	Qiagen
Genimic DNA purification kit	400	Qiagen

Whole project cost estimated about 20000 for 1 year work

COSTS COULD BE REDUCED WITH COLLABORATIONS

References

1 Kind, J., Pagie, L., de Vries, S. S., Nahidiazar, L., Dey, S. S., Bienko, M.,van Steensel, B. et al (2015). Genome-wide Maps of Nuclear Lamina Interactions in Single Human Cells. Cell, 163(1), 134–47.

2 Camozzi, D., Capanni, C., Cenni, V., Mattioli, E., Columbaro, M., Squarzoni, S., & Lattanzi, G. (2014). Diverse lamin-dependent mechanisms interact to control chromatin dynamics: Focus on laminopathies. Nucleus (Austin, Tex.), 5(5), 1–14.

3 Zaremba-Czogalla, M., Dubińska-Magiera, M., & Rzepecki, R. (2011). Laminopathies: The molecular background of the disease and the prospects for its treatment. Cellular and Molecular Biology Letters (Vol. 16).

4 Ostlund, C., Bonne, G., Schwartz, K., & Worman, H. J. (2001). Properties of lamin A mutants found in Emery-Dreifuss muscular dystrophy, cardiomyopathy and Dunnigan-type partial lipodystrophy. Journal of Cell Science, 114 (Pt 24), 4435–4445.

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