Charcot Marie Tooth type 1A (CMT1A)

Silencing of PMP22 promoter 2 using a CRISPR/dCas9 combined with methyltransferase (DNMT3A)

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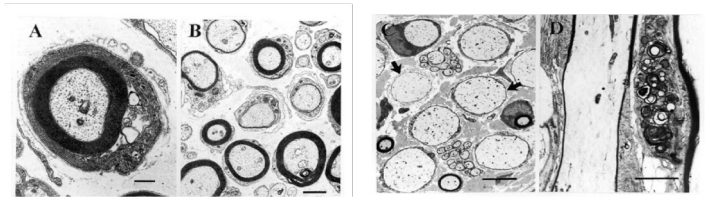
Aim of the project

Use of CRISPR-dCas9-DNMT3A to perform an epigenetic silencing of the Promoter 2 of PMP22 in order to restore the axonal degeneration and recover the wild type phenotype

Background

Molecular basis of the disease:

- PMP22 aggregates
- Dys Demyelination
- Onion Bulb formation
- Secondary axonal degradation

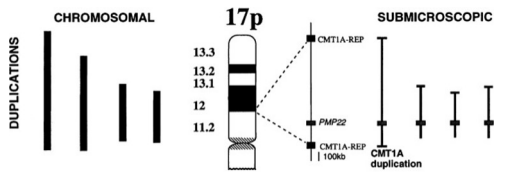


A. Uncompacted myelin outside of the myelin sheath
B. Hypomyelination of large axons
C. Thin myelin around large or lack of myelin around large axons
D. Macrophage indicating active demyelination

Electron micrographs from the sciatic nerve of C3 mouse

Duplication of *PMP22*

Peripheral Myelin Protein 22 (PMP22) gene on the 17p11.2-12.
Overload of the Endoplasmic Reticulum (ER)



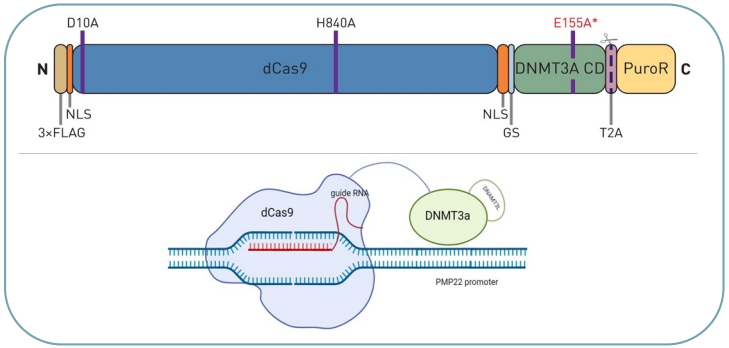
Materials and Methods

C3 mice
PMP22dup Schwann cells

→ Copy number of *hPMP22*: 3-4
→ NCV: 25 m/s
→ Phenotype: mid demyelination

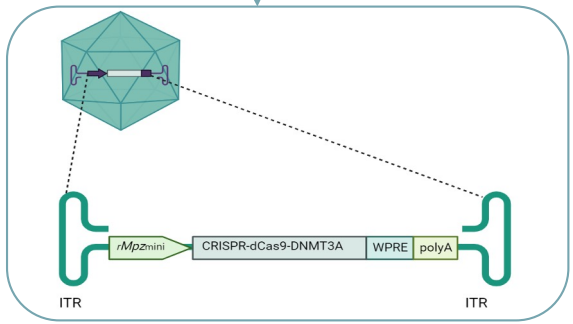
CRISPR-dCas9-DNMT3A

→ 2 sgRNA



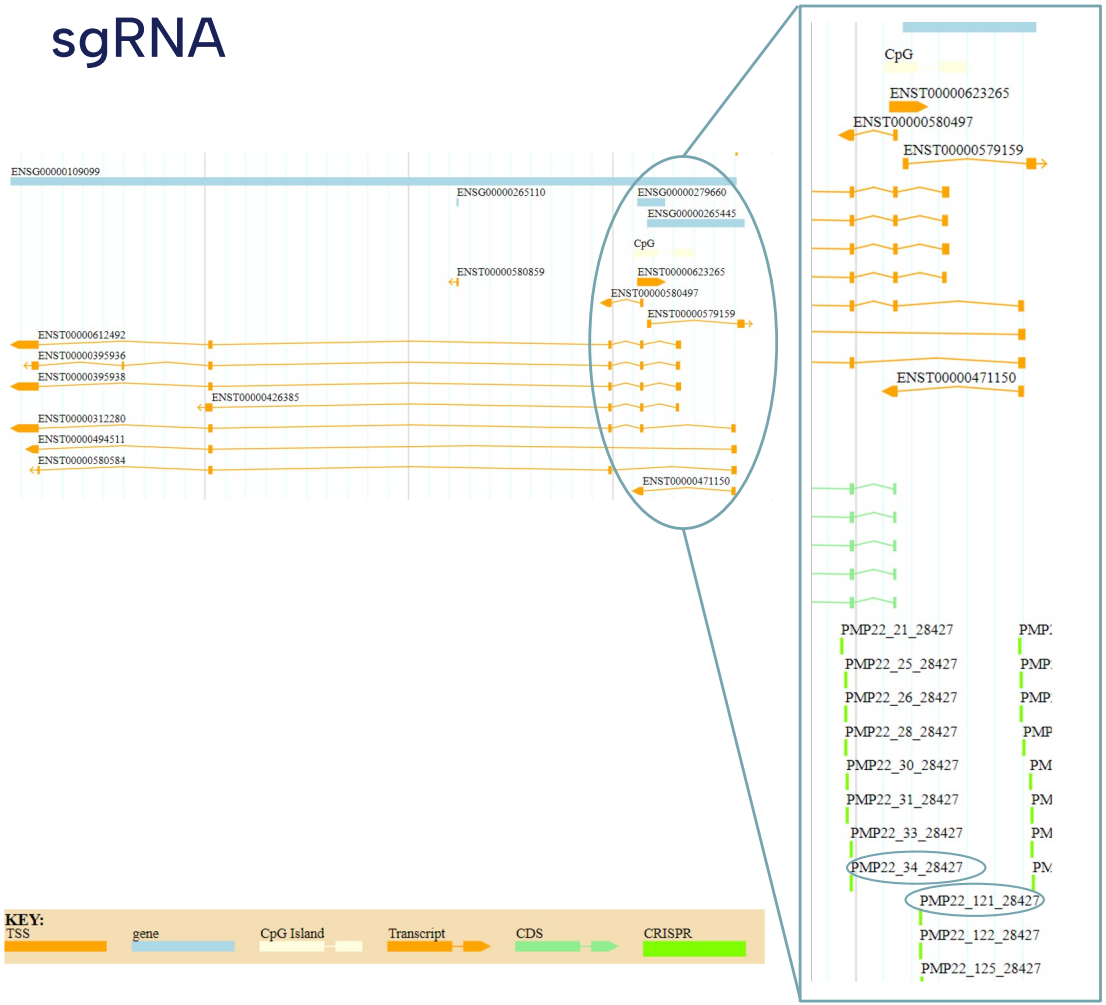
AAV 2/9

→ MPZ promoter-SC specific
→ Intrathecal lumbar injection



Adapted from Vojta et al. Nucleic acids research, 2016.

sgRNA



sgRNA 34

PMP22_34_28427	GTGGACATTTCCTGA GGAAG NGG	S A E	ENSG00000109099::P MP22

```
Target:       |29832*|AGAAACAGTGGTGGACATTTCCTGAGGAAGAGGTGCTACAGT|29875*|
Matchstring:                MMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMM
Query:                      GTGGACATTTCCTGAGGAAGNGG
```

sgRNA 121

```
Target:       |1052*|AGTCTGCACCCCAGGCACTCAAAGCCAGGACACGAACCCCAA|1095*|
Matchstring:                MMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMM
Query:                      CCNGGCACTCAAAGCCAGGACAC
```

PMP22_121_28427	GTGTCCTGGCTTTGA GTGCC NGG	S A E	ENSG00000265445::R P11-849N15.1

S: Specificity score
A: Annotation score
E: Efficiency score

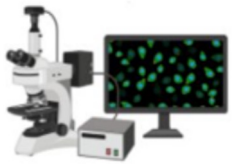
In vitro

Vector biodistribution and cytotoxicity

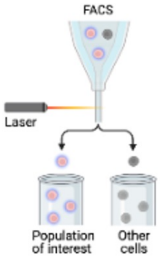
→SC-WT
→SC-PMP22dup
→SC-PMP22dup-mock
→sgRNA 121
→sgRNA 34

C3 mouse

Immunofluorescence assay
AAV2/9-eGFP
+
AAV2/9-eGFP-CRISPR dCAS9-DMT3A

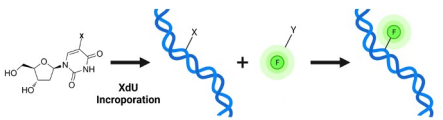


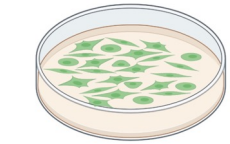
Facs analysis



MTT assay

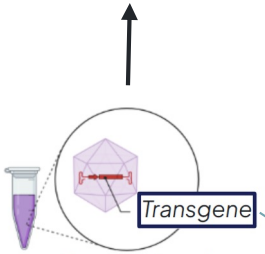
BrdU incorporation assay





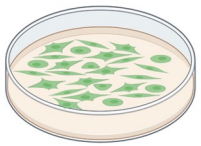
Schwann Cell seeding

DAY 0	DAY 7	DAY 8	DAY 10	DAY 11



CRISPR-dCas9/DNMT3A

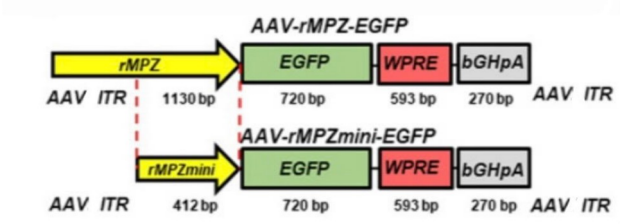
Seeding Transduced SC (TSc)



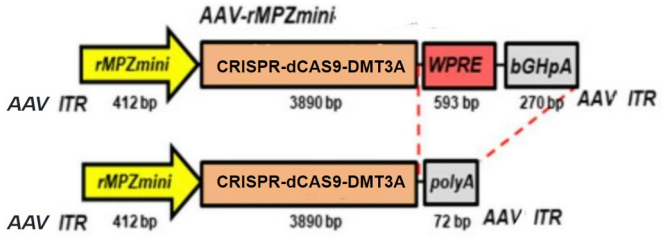
→COBRA
→qPCR
→Western blot
→Immunocytochemical analysis

What is the system of delivery?

AAV2/9 mock vector → high tropism for Schwann cells



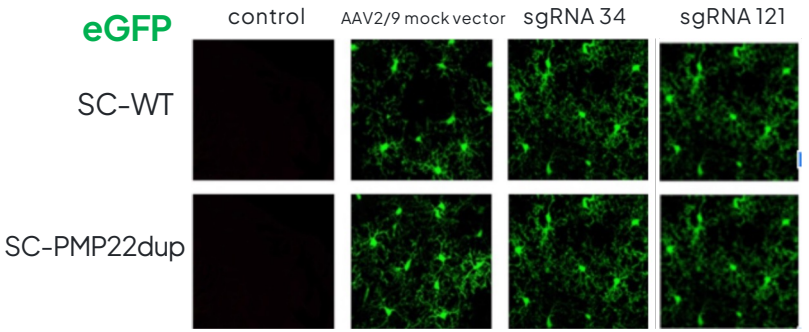
Therapeutic vector → **CRISPR-dCAS9-DMT3A**

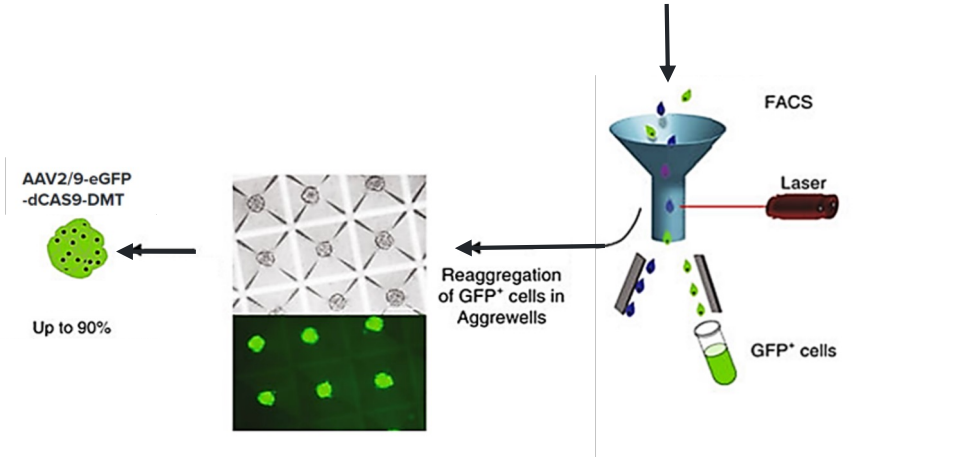


Adapted from Georgiou E. et al., 2023

How to test AAV2/9 efficiency in vitro ?

Immunofluorescence assay

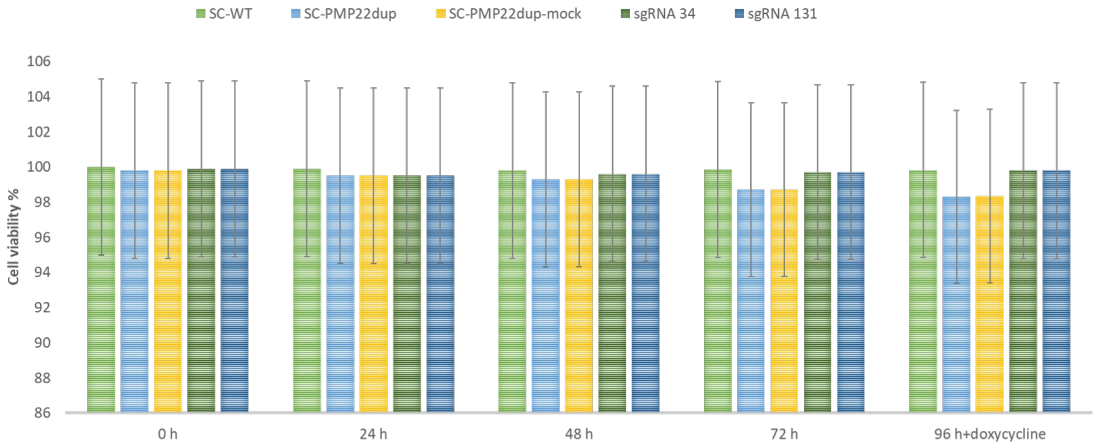




Adapted from Gopika G. Nair et al. Nat Cell Biol. 2019

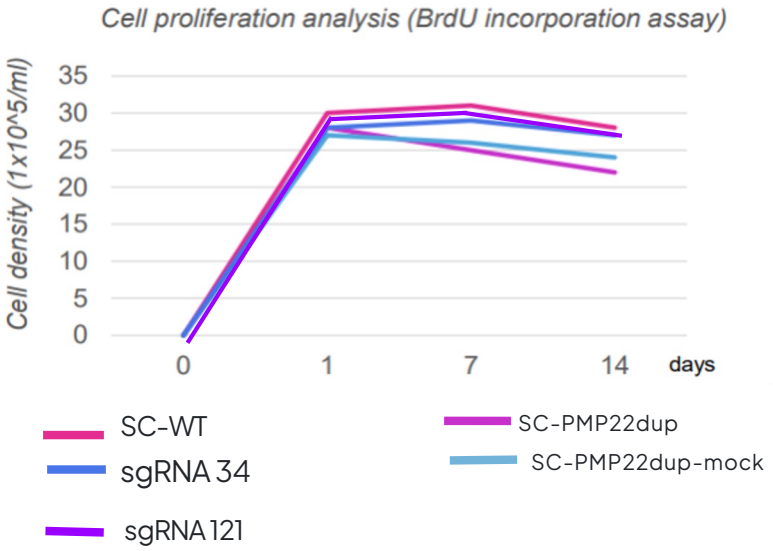
How to test the non-cytotoxicity of the treatment in vitro?

MTT assay



Adapted from Gao, F. et al. 2023

BrdU incorporation assay



Adapted from Crane AM et al., Methods Mol Biol. 2013

EXPECTED RESULTS: in vitro

Does Methylation downregulate expression?

Fig 1 **COBRA**

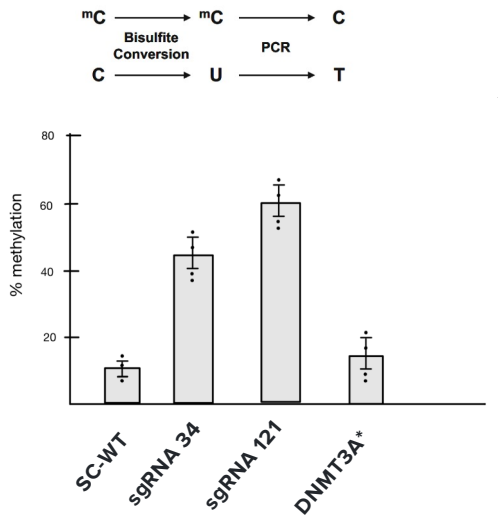


Fig 2 **WESTERN BLOT**

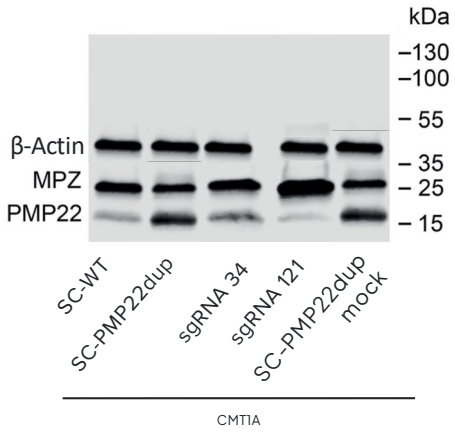
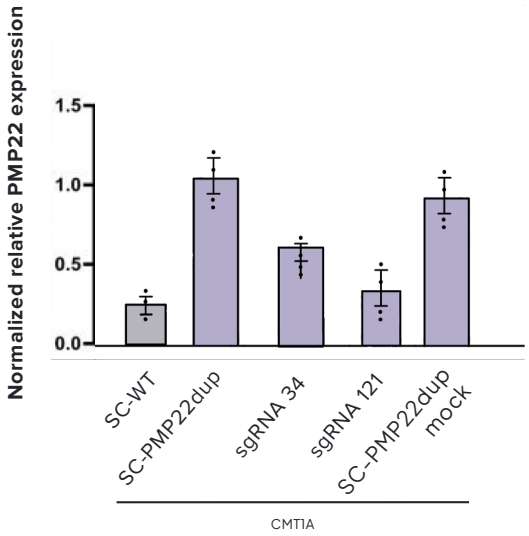


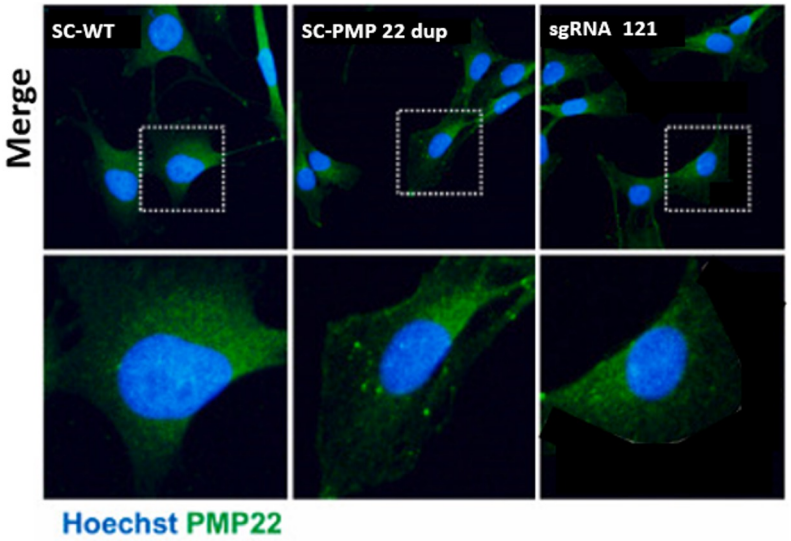
Fig 3 **qPCR**

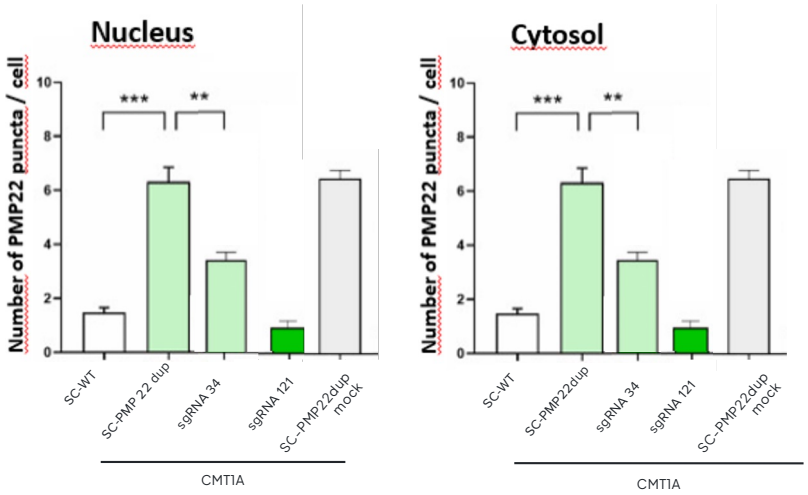


Adapted from Gautier et al. Nature communications, 2021

Accumulation of *PMP22*

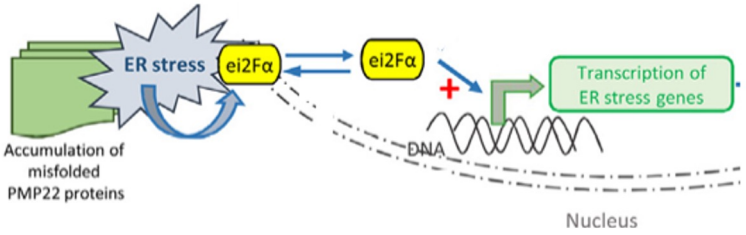
→ PMP22 aggregates

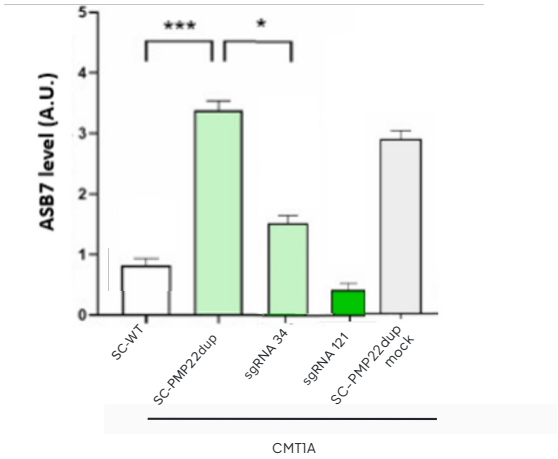




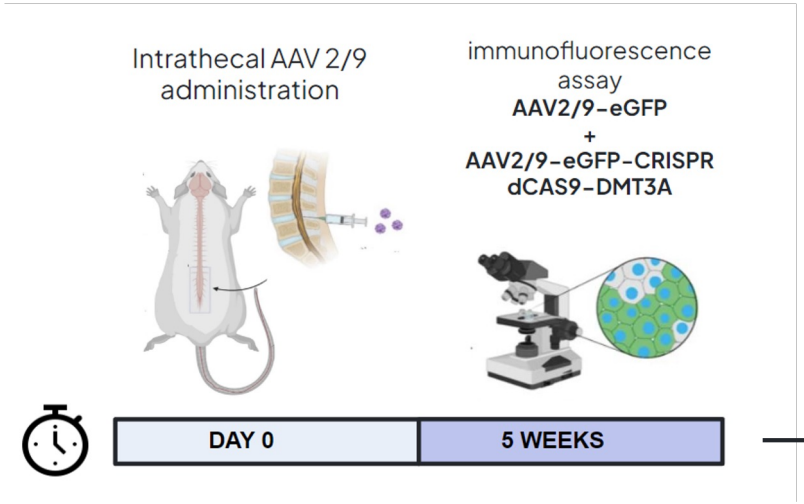
→ ER stress

→ increase ASB7 expression



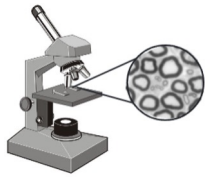


In vivo



Histological
onion bulb on axon

SC- PMP22 dup
sgRNA



Western blot

PMP22
MPZ
comparison
physiological
pathological

EMG

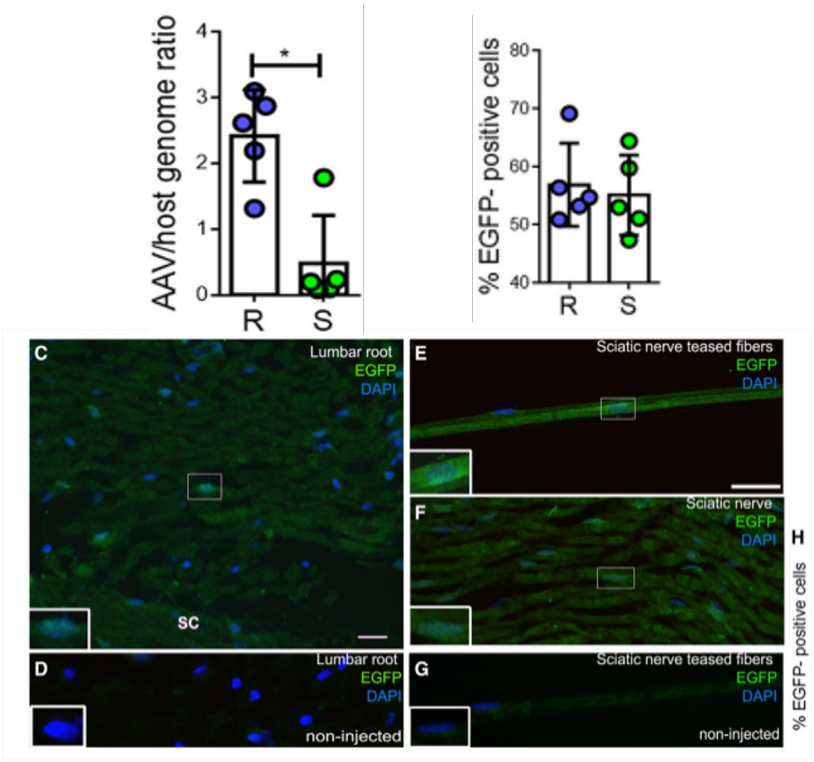
sc - PMP22dup
sgRNA34-121
SC- WT

DAY 1 | DAY 3 | up to 12 weeks

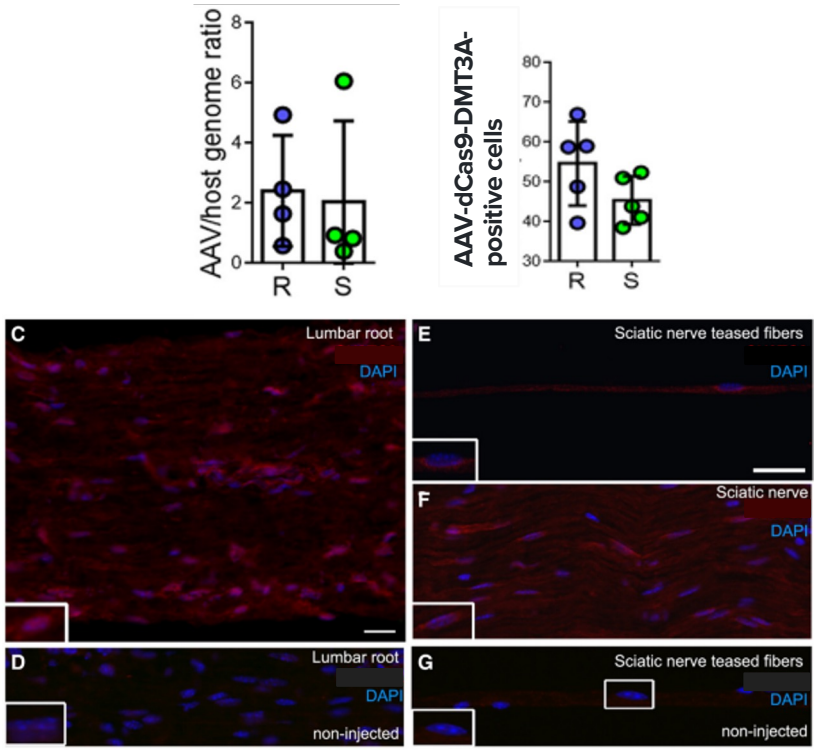
Behavioral tests
→ Randall Selitto test
→ Grip test
→ Rotarod test

In vivo

Biodistribution of the mock **AAV2/9-EGFP** in PNS 5 weeks following lumbar intrathecal injection



Biodistribution of the therapeutic vector **AAV2/9-CRISPR-dCas9-DMT3A** in PNS 5 weeks following lumbar intrathecal injection



Adapted from Georgiou E. et al., 2023 Molecular Therapy

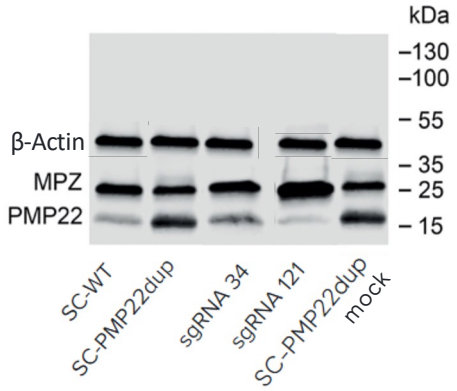
How to check the treatments efficiency ?

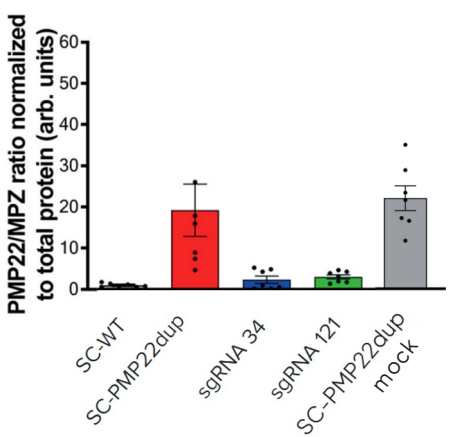
WESTERN BLOT

Myelin protein zero (MPZ):

→ expressed by Schwann cells
→ main structural component of the myelin.

Pmp22 was upregulated relative to the myelin marker Mpz in CMT1A, resulting in higher expression of Pmp22



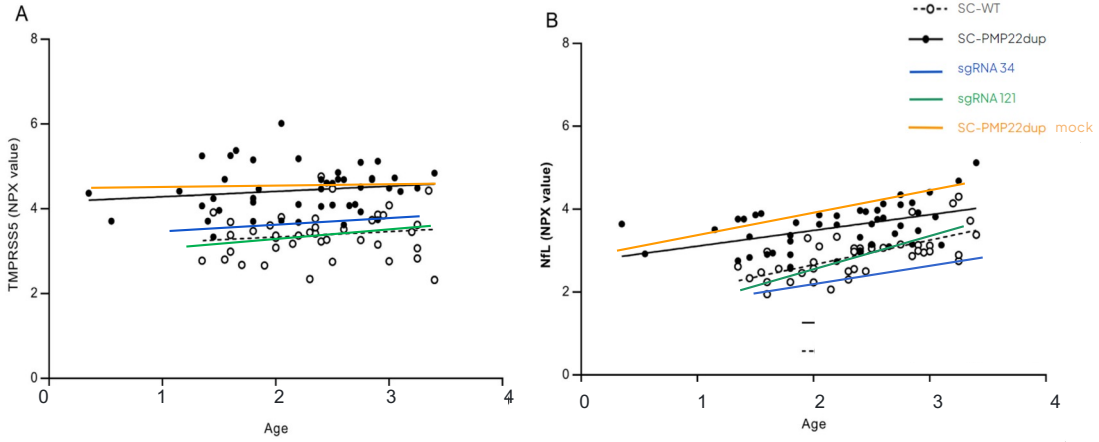


Adapted from Gautier et al. Nature communications, 2021

Blood concentration values
NPX (Normalized Protein eXpression)

High NPX value equals a high protein concentration.
Circulating Biomarker:

- Nf-L (marker for axonal degeneration) ;
- TMPRSS5 (biomarker for myelinating).



Adapted from Hongge Wang, et al. 2020

Histological

In CMT1A the PMP22 overexpression causes decreased myelination,
recovery of axon myelination after treatment

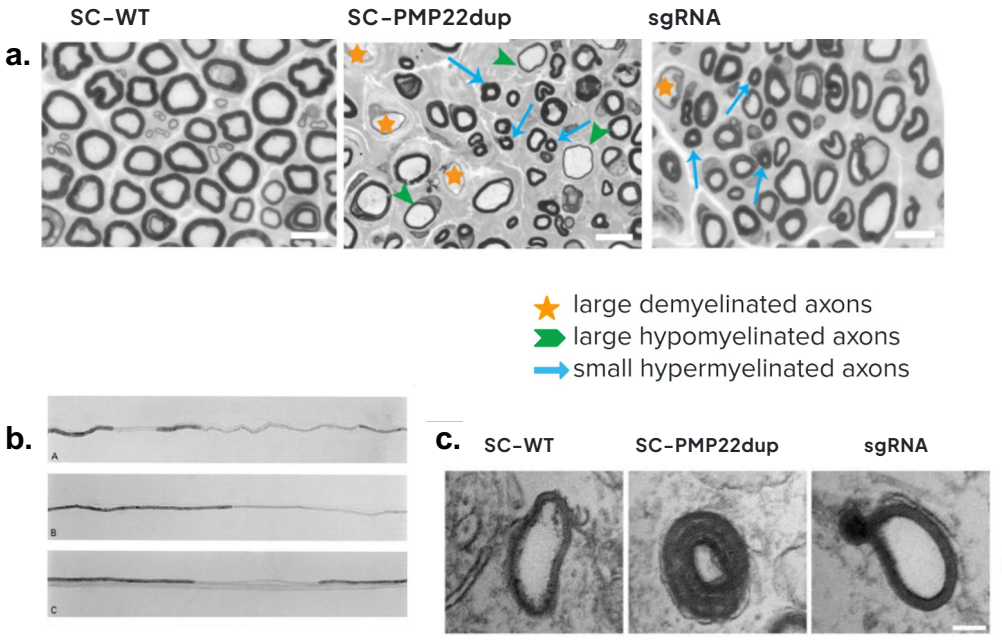


Fig 1

Adapted from Gautier et al. nature communications, 2021

Electromyography

The loss of myelin in CMT1A causes a delay in impulse transmission

After the treatment we can see a recovery of the impulse, due to the correct reformation of the myelin around axons

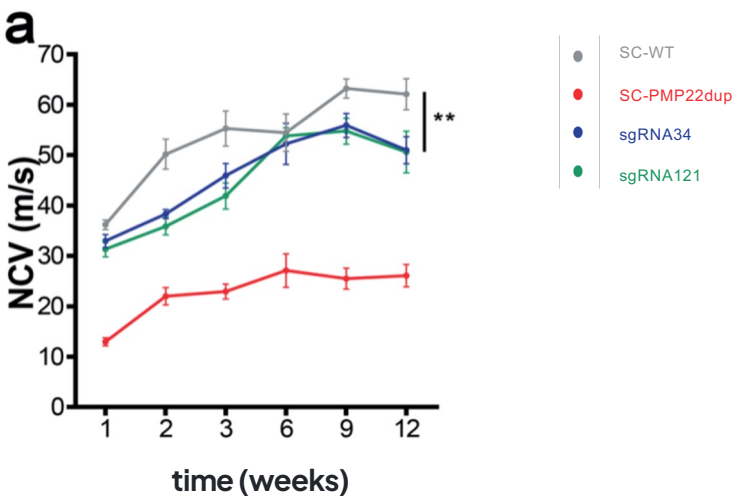
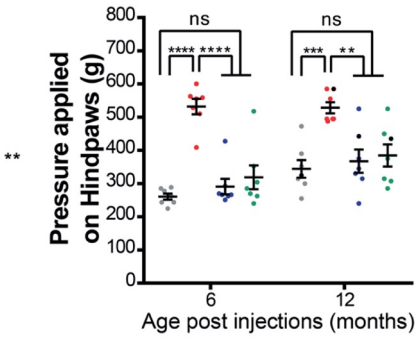


Fig 2

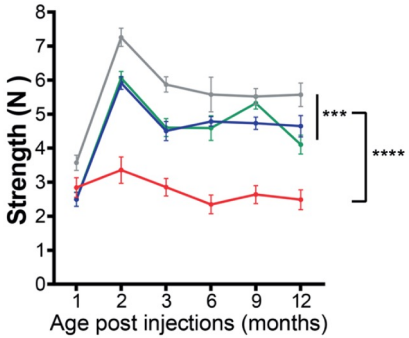
Adapted from Gautier et al. nature communications, 2021

Does the treatment restore motor and sensitive defects?

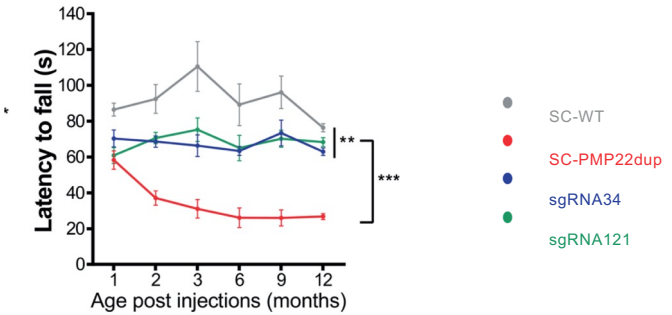
Randall Selitto test
(gram)



Grip test
(Newton)



Rotarod test
(second)



Adapted from Gautier et al. Nature communications, 2021

Pitfalls and Solutions

1) Off-target of CRISPR-dCas9

→ Use of multiple sgRNA

1) Ineffectiveness of treatment in late stages
Due to excessive PMP22 aggregate accumulation

→ Early diagnosis thanks to circulating biomarkers (NFL, TMPRSS5)

3) Translating this strategy into the Clinic

→ developing strategies to overcome the immunological barrier in humans

Conclusion

→ **Downregulation of PMP22** after Methylation of Promoter 2

→ No **Onion Bulb** formation

→ Recovery of **axon myelination** after treatment

→ Recovery of **motor and sensitive functions** as strength, coordination and pain resistance.

Budget and Materials

Materials	Costs
C3 mice PMP22dup Schwann cells + control animals + mice stabulation	595€ (x10) + 110 (x3) + 10.000€
Culture medium supplements (DMEM, FBS, GlutaMAX, Penicillin + Strepromicine, ViraDuctin AAV Transduction Kit)	600€
Packaging plasmid AAV 2/9	600€
MTT Assay Kit (Cell proliferation)	499€
eBioscience™ BrdU Staining Kit for Flow Cytometry FITC	724€
COBRA (validated Methylated Analysis Primer Set, 300 reactions)	745,6€
WB Analysis kit + Antibody (Anti-PMP22, MPZ)	200€
RT-qPCR kit and equipments (Thermo Fisher Scientific)	1200€
Monoclonal Antibody (NF-L; TMPRSS5)	500€
AssayLite Multi-color Conjugated Antibodies Flow Cytometry (FACS Analysis kit)	595€
Immunofluorescence assay (anti-PMP22; GFP)	330€
CRISPR-dCAS9-DNMT3A + 2sgRN + Cas9 protein	3500€
Research team	150.000€/year

TOTAL COST OF 325.773,6€
FOR A TOTAL OF **2 YEARS** OF RESEARCH

References

- Kagiava A, Richter J, Tryfonos C, Leal-Julià M, Sargiannidou I, Christodoulou C, Bosch A, Kleopa KA. Efficacy of AAV serotypes to target Schwann cells after intrathecal and intravenous delivery. Sci Rep. 2021 Dec 2;11(1):23358. doi: 10.1038/s41598-021-02694-1.

-Marina Stavrou , Kleopas A. Kleopa: CMT1A current gene therapy approaches and promising biomarkers. N.R.R. 2022 Nov 25; doi: 10.4103/1673-5374.361538.

-Hongge Wang, Matthew Davison et al. Transmembrane protease serine 5: a novel Schwann cell plasma marker for CMT1A. ANA.2020; 7(1): 69–82. doi: 10.1002/acn3.50965

-Boe SG, Antonowicz NM, Leung VW et al. (2010). High inter-rater reliability in analyzing results of decomposition based quantitative electromyography in subjects with or without neuromuscular disorder. J Neurosci Methods 1992: 138–145

- Recapitulating endocrine cell clustering in culture promotes maturation of human stem-cell-derived β cells. Gopika G. NairJennifer S. Liu[...]Matthias HebrokNature Cell Biology (2019)

-Jennifer A. TracyPeter J. Dyck[...]P. James B. Dyc. Onion-bulb patterns predict acquired or inherited demyelinating polyneuropathy Muscle and Nerve (2019). doi:10.1002/mus.26452

-Bilichak, Andriy, and Igor Kovalchuk. “The Combined Bisulfite Restriction Analysis (COBRA) Assay for the Analysis of Locus-Specific Changes in Methylation Patterns.” In Plant Epigenetics, edited by Igor Kovalchuk, 1456:63–71. Boston, MA: Springer US, 2017. https://doi.org/10.1007/978-1-4899-7708-3_5.

-Li, Jun, Brett Parker, Colin Martyn, Chandramohan Natarajan, and Jiasong Guo. “The PMP22 Gene and Its Related Diseases.” Molecular Neurobiology 47, no. 2 (April 2013): 673–98. https://doi.org/10.1007/s12035-012-8370-x.

-Moore, Lisa D, Thuc Le, and Guoping Fan. “DNA Methylation and Its Basic Function.” Neuropsychopharmacology 38, no. 1 (January 2013): 23–38. https://doi.org/10.1038/npp.2012.112.

-Park, Hanseul, Jaein Shin, Yunkyung Kim, Takashi Saito, Takaomi C. Saido, and Jongpil Kim. “CRISPR/DCas9-Dnmt3a-Mediated Targeted DNA Methylation of APP Rescues Brain Pathology in a Mouse Model of Alzheimer’s Disease.” Translational Neurodegeneration 11, no. 1 (September 15, 2022): 41. https://doi.org/10.1186/s40035-022-00314-0.

-Stavrou, Marina, and KleopasA Kleopa. “CMT1A Current Gene Therapy Approaches and Promising Biomarkers.” Neural Regeneration Research 18, no. 7 (2023): 1434. https://doi.org/10.4103/1673-5374.361538.

-Van Lent, Jonas, Leen Vendredy, Elias Adriaenssens, Tatiana Da Silva Authier, Bob Asselbergh, Marcus Kaji, Sarah Weckhuysen, Ludo Van Den Bosch, Jonathan Baets, and Vincent - Timmerman. “Downregulation of PMP22 Ameliorates Myelin Defects in IPSC-Derived Human Organoid Cultures of CMT1A.” Brain 146, no. 7 (July 3, 2023): 2885–96. https://doi.org/10.1093/brain/awac475.

-Vojta, Aleksandar, Paula Dobrinić, Vanja Tadić, Luka Bočkor, Petra Korać, Boris Julg, Marija Klasić, and Vlatka Zoldoš. “Repurposing the CRISPR-Cas9 System for Targeted DNA Methylation.” Nucleic Acids Research 44, no. 12 (July 8, 2016): 5615–28. https://doi.org/10.1093/nar/gkw159.

-Georgiou E, Kagiava A, Sargiannidou I, Schiza N, Stavrou M, Richter J, Tryfonos C, Heslegrave A, Zetterberg H, Christodoulou C, Kleopa KA. AAV9-mediated SH3TC2 gene replacement therapy targeted to Schwann cells for the treatment of CMT4C. Mol Ther. 2023 Nov 1;31(11):3290-3307. doi: 10.1016/j.ymthe.2023.08.020. Epub 2023 Aug 28. PMID: 37641403; PMCID: PMC10638072.

-Gao, F.; Zhang, Y.; Wu, D.; Luo, J.; Gushchina, S.; Bo, X. Combination of Engineered Expression of Polysialic Acid on Transplanted Schwann Cells and in Injured Rat Spinal Cord Promotes Significant Axonal Growth and Functional Recovery. *Neuroglia* 2023, *4*, 222-238. https://doi.org/10.3390/neuroglia4040016

-Guo C, Ma X, Gao F, Guo Y. Off-target effects in CRISPR/Cas9 gene editing. Front Bioeng Biotechnol. 2023 Mar 9;11:1143157. doi: 10.3389/fbioe.2023.1143157. PMID: 36970624; PMCID: PMC10034092.

-Gautier, Benoit, Helene Hajjar, Sylvia Soares, Jade Berthelot, Marie Deck, Scarlette Abbou, Graham Campbell, et al. “AAV2/9-Mediated Silencing of PMP22 Prevents the Development of Pathological Features in a Rat Model of Charcot-Marie-Tooth Disease 1 A.” Nature Communications 12, no. 1 (April 21, 2021): 2356. https://doi.org/10.1038/s41467-021-22593-3.