Development of **Allele-Specific Gene-Silencing siRNAs** for MPZ-D61N in CMT1B Neuropathy

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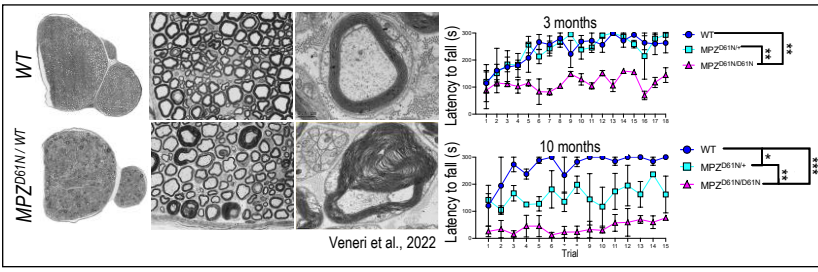
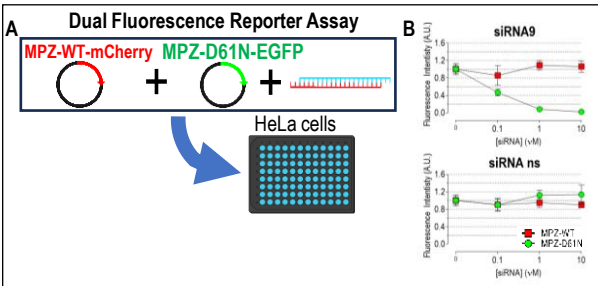
Charcot-Marie-Tooth disease (**CMT**) is a heterogeneous group of hereditary peripheral neuropathies causing both motor and sensory dysfunction. Despite CMT high prevalence (1:2500) and significant socio-economic burden, effective therapeutic options are currently lacking, and its management remains solely supportive and symptomatic. The CMT subtype 1B is caused by mutations in the **MPZ gene**, coding for a structural protein of peripheral myelin.

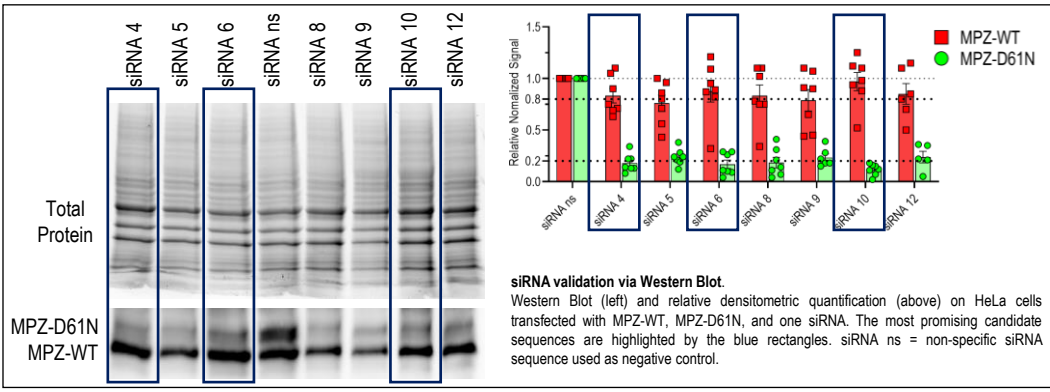
We recently generated a mouse model carrying the **D61N heterozygous mutation** in the **MPZ gene**. This mutation consists of a single nucleotide substitution and causes, in humans, a severe early-onset form of **CMT1B**, characterized by extensive **dys/demyelination**. The MPZ$^{D61N/WT}$ mouse model recapitulates several aspects of the human disease, and dorsal root ganglia (DRG) myelinating cultures prepared from MPZ$^{D61N/WT}$ embryos recapitulate myelination defects observed *in vivo*. In this study, we investigated the feasibility of short-interfering RNA (**siRNA**) treatment for CMT1B caused by the MPZ-D61N heterozygous mutation. To find effective siRNA sequences, a panel of 19 MPZ-D61N-specific siRNAs was tested by a dual-fluorescent reporter assay and Western blot analysis. The most promising sequences will be tested in a functional rescue experiment in DRG myelinating cultures from D61N heterozygous embryos.



MPZ$^{D61N/WT}$ model characterization. MPZ$^{D61N/WT}$ mice show evident abnormalities of the myelin sheaths (left), defective motor performance on the rotarod test (right), and impaired neurophysiology (not shown). Overall, MPZ$^{D61N/WT}$ mice show a much more severe phenotype compared to mice carrying a heterozygous loss-of-function MPZ mutation, indicating that the D61N mutation acts through a **dominant-negative** or **gain-of-function** mechanism.



siRNA screening.
(A) Schematic representation of the experimental protocol used to screen the 19 candidate siRNA sequences. HeLa cells were transfected with a plasmid carrying WT MPZ conjugated with mCherry, a plasmid carrying D61N MPZ conjugated with EGFP, and one of the 19 siRNAs. Fluorescence Intensity was used as a readout for silencing efficacy. (B) Screening results and (C) representative fluorescence images for one of the siRNA sequences showing high silencing efficacy and specificity. siRNA ns = non-specific siRNA sequence used as negative control.



siRNA validation via Western Blot.
Western Blot (left) and relative densitometric quantification (above) on HeLa cells transfected with MPZ-WT, MPZ-D61N, and one siRNA. The most promising candidate sequences are highlighted by the blue rectangles. siRNA ns = non-specific siRNA sequence used as negative control.

Conclusion and Future Perspectives

We identified several sequences that strongly suppressed the mutant protein, with a minor effect on the WT. We plan to perform an *ex vivo* rescue experiment on MPZ$^{D61N/WT}$ DRG cultures by delivering shRNA sequences using lentiviral vectors. If this treatment will improve the dysmyelinating phenotype of the mutant cultures, we will also perform an *in vivo* rescue experiment on MPZ$^{D61N/WT}$ mice using AAV shRNA expression vectors administered by intrathecal injection.

Our results will hopefully provide the proof of principle that allele-specific RNA interference has potential therapeutic efficacy for CMT subtypes caused by gain-of-function and dominant-negative mutations.