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Supplemental information

Iron oxide-coupled CRISPR-nCas9-based genome editing assessment in mucopolysaccharidosis IVA mice

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Supplemental Information

Methodology S1. Analysis of mono-keratan sulfate by LC/MS-MS. Briefly, ~50 mg of frozen tissue was weighed and transferred into homogenization tubes (OMNI International, Kennesaw, GA, USA) on dry ice containing 1 mL cold acetone (Fisher Scientific, Waltham, MA, USA). After homogenization, acetone was removed, and pellets were dried completely using a vacuum centrifuge (SAVANT AES1010, Ramsey, MN, USA). 200 µL 0.5 N NaOH was added, and samples were incubated at 50 ºC for 2 hours. 100 µL 1 N HCl was added, and the pH was adjusted to around 7 using litmus paper (Fisher Scientific, Waltham, MA, USA) and. NaCl was then included in each tube to make a 3 M solution. After centrifugation at 10,000 rpm at RT, the upper phase was transferred to a new clean tube, and 83.3 µL 1 N NaOH was added. A wash with 1.3% potassium acetate in 100 % ethanol was done, and the resulting pellet was washed once with 1 mL of cold 80 % ethanol (Fisher Scientific, Waltham, MA, USA). Pellets were dried at RT and resuspended into 50 µL Tris-HCl (pH 7.0).

Samples were placed into 96-well Omega 10K molecular weight cutoff (MWCO) filter paper (Pall Corporation, Port Washington, NYC, USA) on a 96-well receiver plate. A cocktail containing 50 mM Tris-HCL (pH 7.0), 5 µg/mL chondrosine as internal standard (IS), 1 mU heparitinase, and 1 mU keratanase II was added to each well. After overnight incubation at 37 ºC in a water bath, the plate was centrifuged at 2500 rpm for 15 minutes, and A Hypercarb column (2.0 mm inner diameter, 50 mm long, 5-µm particles; ThermoFisher Scientific, Waltham, MA, USA) was used for disaccharide isolation. The mobile phase consisted of a gradient elution of 5 mM ammonium acetate (pH 11.0) to a 100 % acetonitrile solution. The mass spectrometer was operated with electrospray ionization in the negative ion mode (Agilent Technologies, Palo Alto, CA, USA). Each disaccharide was identified using m/x: IS 354.3 to 193.1 and mono-sulfated KS 462 to 97. A running time of 5 min per sample in 10 µL injection volume was used.

Table S1. sgRNA sequences used for targeting the ROSA26 *locus*. Lowercases in forward and reverse primers correspond to the 5' overhangs for ligation of sgRNA with *Bbs*I or *Bsa*I in AIO-mCherry plasmid (Add gene: #74120).

Orientation	Forward oligo (5' to 3')	Reverse oligo (5' to 3')
Sense	accgTAAGCATGCTCTAACAGGCC	aaacGGCCTGTTAGAGCATGCTTA
Antisense	accgCACAAGAGTAGTTACTTGGC	aaacGCCAAGTAACTACTCTTGTG

Table S2. Off-target sequences predicted. The green letters correspond to the PAM sequence (NGG), while the red ones represent mismatches. Note that seven of ten off-target predicted sequences contain four mismatches instead of three.

***Mus musculus genomes*-mm10-GRCm38** **Antisense sgRNA sequence: CACAAGAGTAGTTACTTGGC**			
Assembly	**Chromosome**	**Genomic position**	**Off-target sequence**
NC_000074.6	8	122348063	GCCCAGAGTAGTTACTTGGCTGG
NC_000083.6	17	11427276	CGCAAGAGGAGTTACATGGCTGG
NC_000069.6	3	44819048	CACAAGATTAGTTAATTAGCAGG
NC_000083.6	17	56568798	GGCAAGAGTTGTCACTTGGCTGG
NC_000081.6	15	38342742	CAACAGATTAATTACTTGGCTGG
NC_000073.6	7	16604293	AAGAGGAGTACTTACTTGGCAGG
NC_000068.7	2	47748397	CAGCAGAGGAGATACTTGGCTGG
NC_000068.7	2	127874345	GACAACAGTAGTTCGTTGGCTGG
NC_000070.6	4	67284961	CAGAAGATAAGTTAGTTGGCTGG
NC_000067.6	1	130513568	CAGAAGAGGAGAAACTTGGCTGG

Table S3. Oligos used in this work. **OT.** Off-target.

Purpose	Oligo's name	Sequence
T7 assay	T7_Fw	CTGAGGCAGGGTCTCACTATG
	T7_Rv	GGTCAGAGAGTCTTGCCTGCA
Off-target	OT1_Fw	AGCAGCAAGCTGAGCTGTCT
	OT1_Rv	TTGTGCTGTCTGGCAAGCACC
	OT2_Fw	GCAAGTGTGCCCATGACAAA
	OT2_Rv	TGCCTAGGCTCCTGCAGTG
	OT3_Fw	GGGTGTTTGTCCTTTCTG
	OT3_Rv	GCACATCAATACCCCTGTACA
	OT4_Fw	CTGCTGTACATCTGATGCTCTG
	OT4_Rv	GGATCAAGATGAACTCACACC
	OT5_Fw	AACTCAGTGCCTCCAGCC
	OT5_Rv	GGTTCCTTAGGCTACTTCC
	OT6_Fw	GGGAGAGATCACTTTGTATGG
	OT6_Rv	TTGGACTGTAGTCCCTCCAG
	OT7_Fw	GGAGGATGGAATAGGGCAT
	OT7_Rv	GTAGCACTGCTAATCTGCC
	OT8_Fw	CCTCTGAGTTTGCCTTTTGG
	OT8_Rv	GTCAATAAGCCATCTCTTGG
	OT9_Fw	GGCAATCGAAAGAGATCCCAC
	OT9_Fw	TCAGCATTCGGCAACCTCA
	OT10_Fw	TATCCTGCCTAAACCTGACCT
	OT10_Fw	CTTCACCACTTATGCCCAAC
Homologous recombination	bGH_Fw	CTGTGCCTTCTAGTTGCC
	ROSA26_HR_Rv	GAGGCTGTTGGTACTAGTG

Table S4. Pathological scoring in knee joint sections from MTOL mice after 12 weeks post-edition with the IONPs-coupled CRISPR/nCas9 system.

Bone	Structure	Finding	MTOL strain		
			WT	Untreated	IONPs/Donor/CRISPR
Tibia	GP	Vacuolization	0.00 ± 0.00	3.00 ± 0.00	**2.87 ± 0.03***
		Column structure	0.00 ± 0.00	2.89 ± 0.06	2.47 ± 0.15
	AC	Vacuolization	0.00 ± 0.00	3.00 ± 0.00	**2.87 ± 0.03***
		Column structure	0.00 ± 0.00	3.00 ± 0.00	2.77 ± 0.09
Femur	GP	Vacuolization	0.00 ± 0.00	2.92 ± 0.08	2.92 ± 0.08
		Column structure	0.00 ± 0.00	3.00 ± 0.00	2.85 ± 0.08
	AC	Vacuolization	0.00 ± 0.00	3.00 ± 0.00	2.93 ± 0.07
		Column structure	0.00 ± 0.00	3.00 ± 0.00	2.88 ± 0.07
Ligament		Vacuolization	0.00 ± 0.00	3.00 ± 0.00	2.96 ± 0.04
Meniscus		Vacuolization	0.00 ± 0.00	2.92 ± 0.08	2.96 ± 0.04

Scores for vacuolization and column structure were assigned between 0-3 as follows: no storage (0), slight storage (1), moderate storage (2), and abundant storage (3). Slides from WT (n= 3), untreated (n= 3), and IONPs/Donor/CRISPR (n= 5) MTOL mice were scored in a double-blinded manner three times. * $p \leq 0.05$. Two-way ANOVA test. **GP:** Growth plate. **AC:** Articular cartilage.

Figure S1. Indel contribution – ROSA26 *locus*. Notice that indels are represented mainly by deletions.

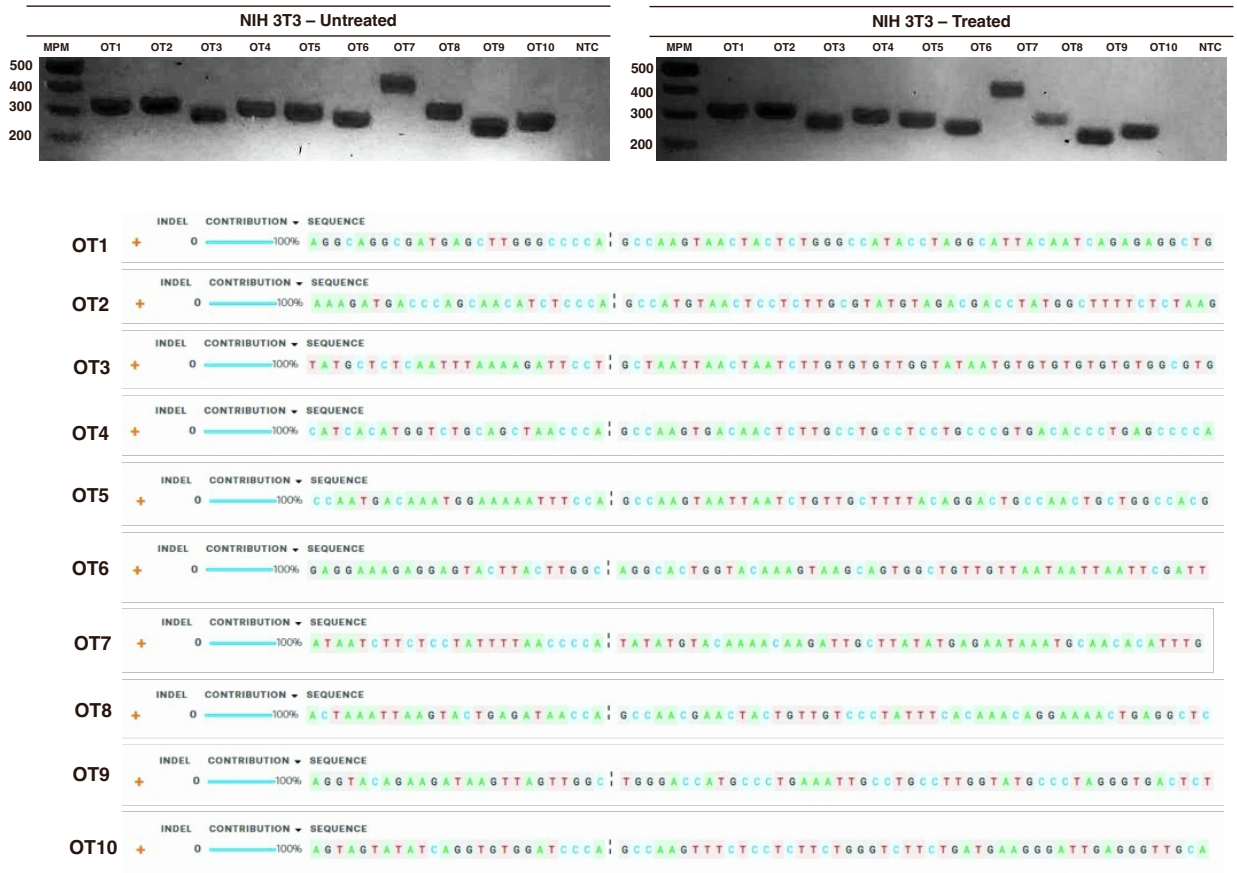

Figure S2. nCas9 off-target cutting assessment into the ROSA26 *locus.* The upper panel shows a 2% w/v agarose gel for 10 predicted sequences from FACS-sorted untreated and CRISPR/nCas9-treated 3T3 cells. The lower panel shows the bioinformatic analysis of electropherograms for each off-target. Non-off targets were detected at any predicted sites. The dashed line represents the nCas9 cut site. Analysis was performed on ICE Analysis from Synthego v2.0.

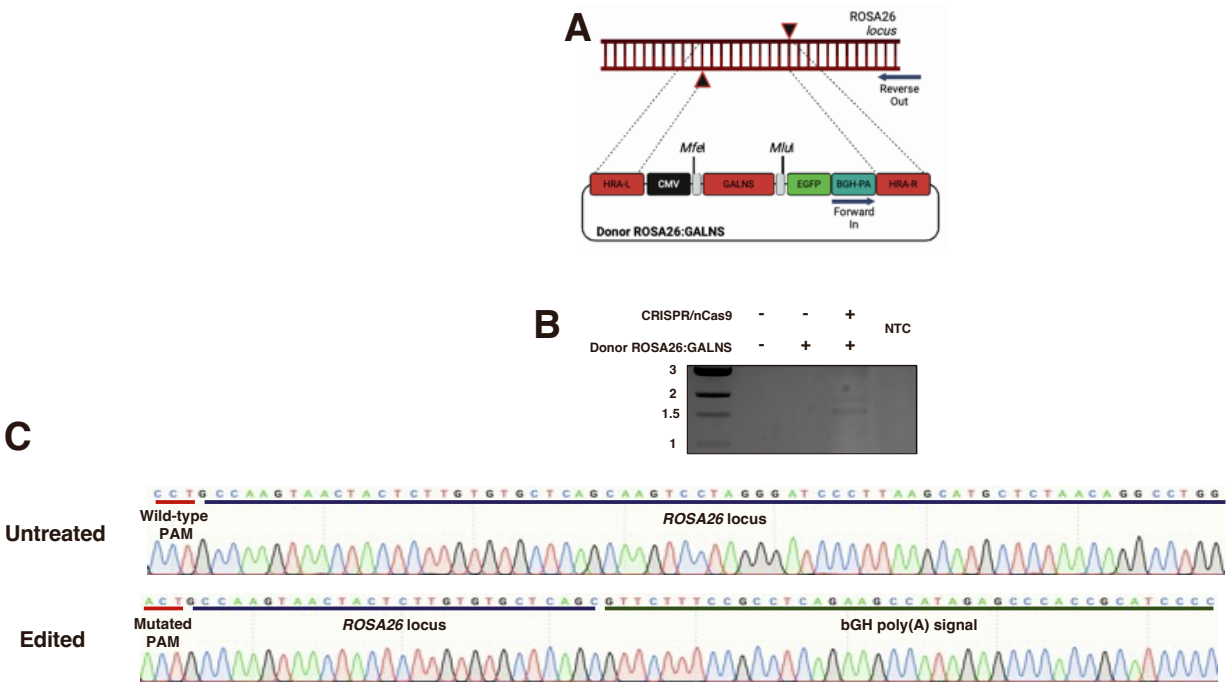

Figure S3. Expression cassette knock-in into the ROSA26 *locus*. **A.** Molecular strategy to determine the knock-in of the Donor ROSA26:GALNS into ROSA26 *locus*. Note that reverse primer is outside HR's left arm, and only a successful HR arises in a PCR product. **B.** Agarose gel shows a band of 1.5 kb. No PCR products are achieved when Donor ROSA26:GALNS is transfected without CRISPR/nCas9 plasmids. **C.** Electropherograms from PCR product obtained in b. The reverse sequence is shown. Notice that the PAM sequence was intentionally mutated in the HA arm of the Donor template to avoid re-cuts from Cas9 upon knock-in.

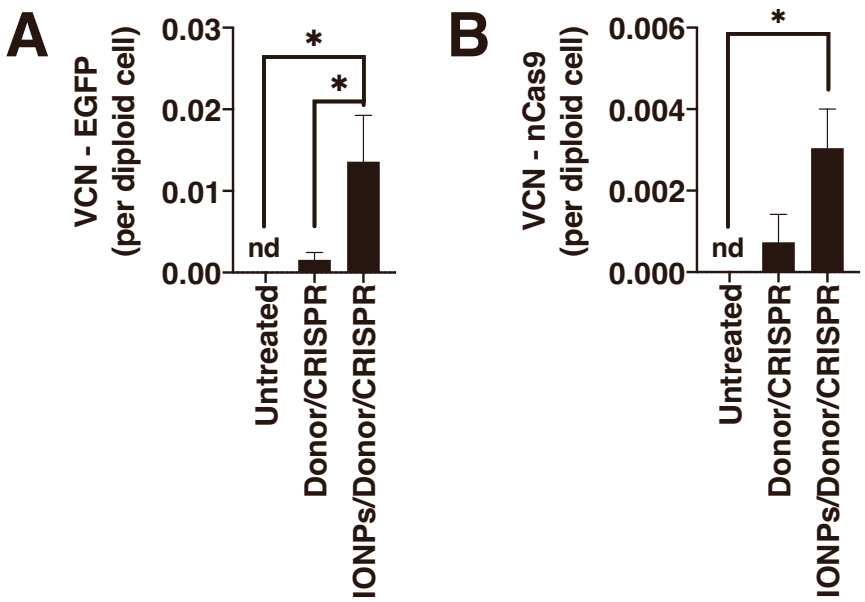

Figure S4. Vector copy number assays. VCN was assessed in liver samples from untreated (n= 3), Donor/CRISPR (n= 3) and IONPs/Donor/CRISPR (n= 5) treated mice for the donor (**A**, using EGFP as target) and nCas9 (**B**) plasmids persistence. * $p \leq 0.05$. Two-way ANOVA test.

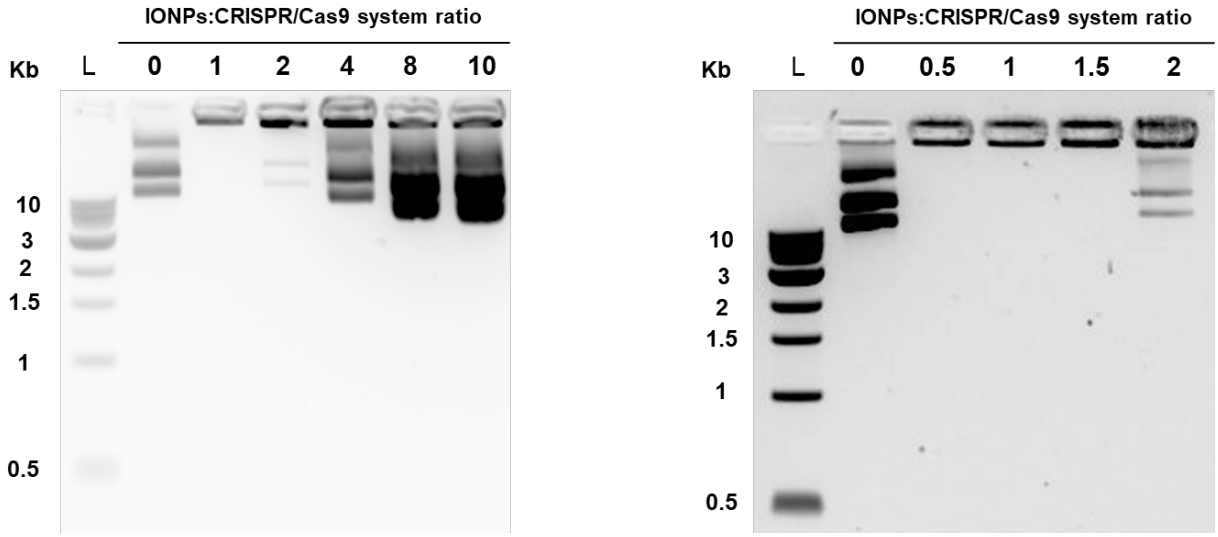

Figure S5. IONPs loading capacity assays. The agarose gels show several ratios of IONPs:pDNA. Ratios refer to the different amounts of pDNA tested. Note that 1.5 was the maximum loading capacity of these IONPs, corresponding to 1 μg IONPs/1.5 μg pDNA.

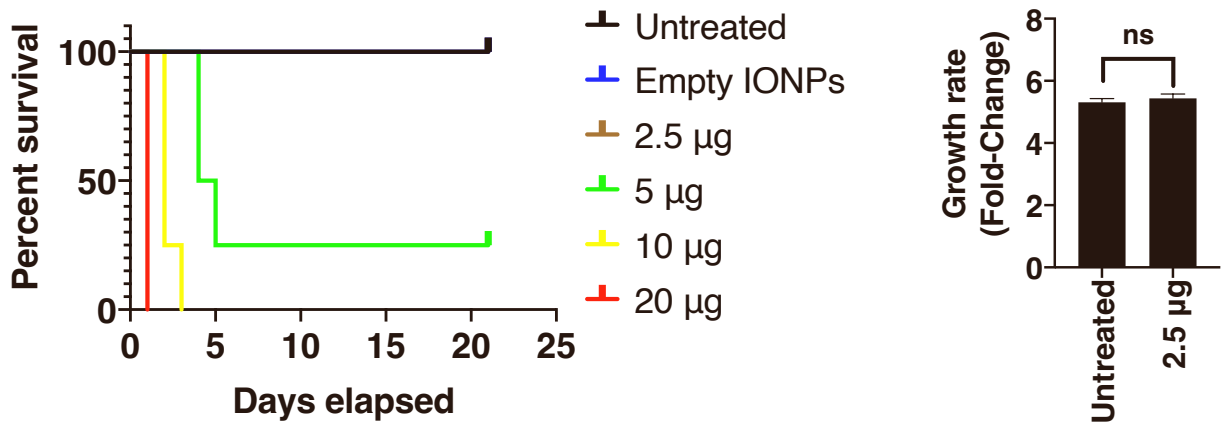

Figure S6. Dose-response curves in MTOL mice. Upon IV administration of several IONPs/CRISPR/nCas9 formulations, only 2.5 µg was safe. No changes in the growth rate were found between untreated and treated mice (n=4) after 21 days post-injection by using such a dose.

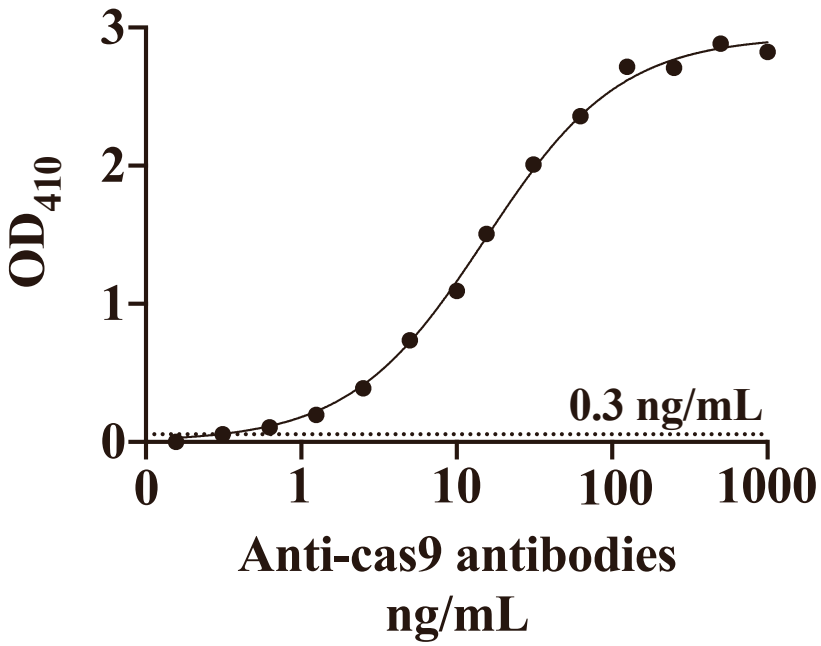

Figure S7. The figure shows a standard curve obtained by incubating spnCas9 protein with serial dilutions of mouse monoclonal antibody raised against the N-terminal region of Cas9 of *Streptococcus* protein. Note that the limit of antibody quantification was 0.3 ng/mL.