An INTERACT Submission

dualAAV9/SMABE Therapy

FOR SPINAL MUSCULAR ATROPHY

SPONSOR
STEVEN J. GRAY, PHD
UT SOUTHWESTERN MEDICAL CENTER

[redacted]

ON BEHALF OF

THE SOMATIC CELL GENOME EDITING CONSORTIUM
PROJECT: *PRECLINICAL GENOME EDITING FOR RARE NEUROLOGICAL DISEASES*
NIH GRANT # U19NS132304

April 30, 2024

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Abbreviation	Definition
AAV	Adeno-associated Virus
AAV2	Adeno-associated Virus, serotype 2
AAV9	Adeno-associated Virus, serotype 9
ABE	adenine base editor
AGE	Agarose Gel Electrophoresis
ALT	alanine aminotransferase
ASO	antisense oligonucleotide
AST	aspartate aminotransferase
BCA	Balanced chromosomal abnormalities
BCH	Boston Children's Hospital
BP	blood pressure
BW	body weight
C6T	position 6 of exon 7
CSF	Cerebrospinal fluid
ChAT	choline acetyltransferase
CHOP-INTEND	Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders
CMAP	compound muscle action potential
CMC	Chemistry Manufacturing and Controls
CND	caudal-neural differentiation
CNS	central nervous system
CoA	Certificate of Analysis
CRIM	Cross-Reactive Immunological Material
CRISPR	clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
ELISA	enzyme-linked immunosorbent assay
GE	Genome Editing core
GFAP	Glial fibrillary acidic protein
GFP	green fluorescent protein
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
HEK293	Human Embryonic Kidney 293 cells
HR	heart rate
ICV	intracerebroventricular
IP	intraperitoneal
IT	intrathecal
ITR	inverted terminal repeat
IU	Intrauterine
IV	intravenous

Abbreviation	Definition
mESC	mouse embryonic stem cells
MND	motor neuron differentiation
mRNA	messenger ribonucleic acid
MUNE	motor unit number estimation
NHP	non-human primate
NLS	nuclear localization signal
O2	oxygen
OSU	Ohio State University
PCR	polymerase chain reaction
PMBMCs	Peripheral Blood Mononuclear Cells
PMM	Preclinical Mouse Model core
PND	postnatal day
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
RO	retro-orbital
RR	respiratory rate
scAAV	self-complementary AAV
sgRNA	single-guide RNA
SMA	Spinal Muscular Atrophy
SMABE	Spinal Muscular Atrophy adenine base editor
SMN	survival motor neuron
SMN1	survival motor neuron 1
SMN2	survival motor neuron 2
SMNRT	survival motor neuron reverse transcriptase
SMNΔ7	Truncated protein from skipping of exon 7
Sp	Staphylococcus pyogenes
T	temperature
TNC	Translational Neuroscience Center
USP	ubiquitin specific protease
VIVO	Verification of in vivo off targets
VVR	Viral Vector Regulatory Core
WHV	Woodchuck Hepatitis Virus
WPRE	Post-transcriptional Regulatory Element
WT	wild-type

1. INTERACT MEETING BRIEFING DOCUMENT OUTLINE

1.1. Introduction

SMA is a progressive motor neuron disease and has historically been the leading genetic cause of infant mortality across all ethnic groups [1-3]. SMA is caused by the homozygous loss of the essential survival motor neuron 1 (*SMN1*) gene. One or more copies of the highly similar *SMN2* gene partially compensates for the loss of *SMN1* in SMA patients. However, *SMN1* differs from *SMN2* in that it contains a C•G-to-T•A change at position 6 of exon 7 (C6T) that leads to skipping of exon 7 during mRNA splicing [4, 5]. The resulting truncated SMNΔ7 protein is rapidly degraded in cells, causing SMN protein insufficiency that results in the loss of motor neurons, progressive paralysis, respiratory distress and ultimately death [6, 7]. Patients with the most common form of SMA, type I, have a >6-fold reduction in SMN protein levels and live to a median age of 18 months if untreated [8-11].

Upregulation of SMN protein can rescue motor function and substantially improve the prognosis of SMA patients [12-16]. The antisense oligonucleotide (ASO) nusinersen (Spinraza) and the small-molecule splicing modifier risdiplam (Evrysdi) both promote inclusion of exon 7 in spliced *SMN2* transcripts and result in ~1.5 to 2-fold upregulation of SMN protein levels in patients [17, 18]. The effect of these therapeutics is transient, however, and patients therefore require repeated treatment throughout their lifetimes [19-22]. Alternatively, self-complementary AAV (scAAV)-mediated gene complementation of SMN1 cDNA by the gene therapy onasemnogene abeparvovec-xioi (Zolgensma) leads to constitutive overexpression of SMN protein in transduced cells [23-25]. It is not known whether episomal AAV will permanently persist in motor neurons to provide life-long protection against SMN loss in patients. Moreover, endogenous SMN protein levels are subject to multiple levels of regulation that variably constrain its expression across tissues [26-29], and SMN overexpression has been observed in animals to cause aggregation, toxicity and tissue-specific pathologies [30-34]. A therapeutic modality that permanently restores endogenous SMN protein expression and preserves native gene regulation could thus offer substantial benefits over existing SMA therapies. Development of a gene editing-based therapeutic to rescue splicing defects of endogenous *SMN2* genes as described in this proposal can either complement or replace existing treatments for SMA in patients to enable a one-time treatment that permanently restores SMN expression to more physiologically normal levels which may improve patients' outcomes.

A therapeutic modality that permanently restores endogenous SMN protein expression and preserves native gene regulation could offer substantial benefits over existing SMA therapies. We have designed a bespoke adenine base editor (ABE) that enables efficient and precise single nucleotide conversion of *SMN2* exon 7 C6T (94±3.4% editing efficiency, 82±1.9% single nucleotide editing purity) to effectively and permanently restore wild-type SMN1 gene functionality at endogenous *SMN2* alleles in SMA cells.

1.2. Product Development

Preclinical proof-of-concept studies have been conducted with dualAAV9/SMABE, which is a bespoke adenine base editor (ABE) that is split across two separate AAV genomes. This ABE was shown capable of efficient and precise single nucleotide conversion of *SMN2* exon 7 C6T (94±3.4% editing efficiency, 82±1.9% single nucleotide editing purity) to effectively and permanently restore wild-type SMN1 gene functionality at endogenous *SMN2* alleles in SMA cells. The proof-of-concept studies have demonstrated therapeutically relevant levels of base editing in cultured cells as well as following neonatal ICV administration in mice. Experimental genome-wide unbiased off-target editing analysis of dual-AAV9/SMABE in cultured human cells revealed minimal Cas-dependent off-target editing.

Proposed IND enabling studies will include the following activities, which are summarized below but expanded in greater detail in subsequent sections.

- The kinetics of base editing will be quantified in a humanized mouse model.
- Off-target editing will be assessed longitudinally in mice over an 18-month timespan, to better understand whether off-target edits further accumulate over time.
- Pivotal dose-ranging efficacy studies will be conducted with dualAAV9/SMABE in the humanized SMA disease mouse model, alone and in combination with nusinersen, to further assess the additional benefit of dualAAV9/SMABE over that of nusinersen alone, and to establish the minimally effective dose.
- A GLP safety study will be conducted in NHPs in a manner modeling the proposed human treatment, to assess safety, confirm expected biodistribution patterns, and evaluate potential anti-ABE immune responses.

1.3. Purpose of the Meeting

To our knowledge, an AAV-delivered base editing therapeutic has not been advanced to clinical trials for a central nervous system disorder. We recognize that while this therapeutic strategy may have advantages over current SMA therapeutics, it also has unique safety and regulatory considerations. We'd like to discuss specific CMC concerns regarding the manufacture of the dual AAV vector therapeutic. We've also outlined a proposed set of IND-enabling pharmacology and toxicology studies, which we'd like early feedback on to set the direction of pending mouse proof-of-concept studies. We'd further like to discuss a clinical trial strategy, which we recognize has complex considerations due to the availability and nature of existing SMA therapeutics. The information provided here includes the background details of the key studies in addition to preliminary data and the plans for other investigations in consideration. Specific questions are provided below.

1.4. Questions

1.4.1. Chemistry Manufacturing and Controls (CMC)

The manufacturer and process for the clinical drug product has not yet been identified.

Q1: dualAAV9/SMABE is composed of two separate AAV9 vectors that are intended to be administered together, at the same time, at a 1:1 ratio. They would never be expected to be administered alone. Our position is that the combined dual vectors would be considered a single drug product. However, we would prefer to manufacture and vial each of the two vectors separately, combining them just prior to administration to the patient. Does the agency agree with this position and strategy? Are there any specific considerations that we should be aware of when taking this position?

Q2: Are the release testing plan and specifications listed in section 1.5.1.5 appropriate for this product to be used in the initial Phase I/II clinical trial?

Q3: Is the plan presented in section 1.5.1.9 appropriate, to bridge the nonclinical lots with the future clinical drug product based on analytical comparability and in vitro potency?

Q4: We plan to manufacture the nonclinical non-GMP drug product batches (for pivotal pharmacology and toxicology studies) in a different facility than the drug product, which will be manufactured under GMP quality. Although the facility manufacturing the GMP drug product may use a slightly different overall process than that for the nonclinical drug product batches, our strategy would be to conduct analytical comparability studies to assure that the critical quality attributes of the GMP drug product meet or exceed the specifications of the nonclinical drug product batches. Does the Agency agree with this strategy? Would the Agency provide any specific guidance towards acceptance criteria for the manufacture of non-GMP and GMP qualities, which we could follow with the respective vendors?

1.4.2. Pharmacology/Toxicology

Q5: Our primary pharmacology strategy is to establish a minimally effective dose and a prospect for direct benefit to conduct dose-ranging efficacy studies in a humanized mouse model of SMA, with dualAAV9/SMABE alone or in combination with nusinersen, using survival as the primary efficacy outcome. Are the nonclinical pharmacology studies proposed in section 1.5.2.2 appropriate to evaluate the prospect for direct benefit of our gene editing approach in the target pediatric SMA patient population?

Q6: The strategy for our pivotal toxicology studies is to conduct non-GLP and long-term safety assessments in mouse models, and further complement the safety data with a GLP non-human primate (NHP) study to specifically investigate the immune responses and biodistribution of our investigational drug product. Would the Agency consider the relevant nonclinical pharmacology and toxicology studies proposed in sections 1.5.2.3 (mouse studies) and 1.5.2.4 (NHP studies) appropriate to assess the safety of our gene editing approach to enable a human trial?

Q7: Our off-target editing strategy is to evaluate the prospect of this approach primarily using in vitro studies in human cellular models, and to complement it with long-term mouse studies to understand the general kinetics of short-term versus long-term off-target editing events. This is described in more detail in section 1.5.2.3. Is our plan to evaluate off-target editing sufficient to evaluate this risk?

1.4.3. Clinical

Q8: We recognize that the expressed base editor may be viewed as a foreign antigen and stimulate an anti-transgene immune response of this is not properly controlled. We proposed to incorporate a published immunomodulatory regimen consisting of corticosteroids, tacrolimus, and sirolimus appropriate for CRIM (-) individuals. Safety monitoring is focused on potential adverse immune responses. Would the FDA consider if the clinical immunosuppression regimen and safety monitoring strategy outlined in section 1.5.3 are suitable for this Phase I/II trial?

Q9: In brief, we plan to enroll subjects that are taking nusinersen according to normal treatment guidelines. Our rationale is that patients may receive additional benefit of dualAAV9/SMABE over nusinersen alone. During future evaluation and development of dualAAV9/SMABE, there may be the possibility to use dualAAV9/SMABE as a stand-alone treatment. Does the agency agree with the proposed patient population and enrollment strategy outlined in Section 1.5.3?

1.4.4. Cross-cutting/Other

Q10: Using the intrathecal route of administration of AAV9, germline cells are not expected to be transduced to any appreciable extent, making the risk of germline editing negligible. Our position is that a detailed germline transmission study would therefore not be necessary for our drug product at this stage of development (i.e., first-in-human studies). Does the agency agree with this position?

1.5. Data to Support Discussion

1.5.1. dualAAV9/SMABE Therapy: Chemistry Manufacturing and Controls

1.5.1.1. Biological Substance

DualAAV9/SMABE is composed of two recombinant serotype 9 adeno-associated virus vectors, encoding separate halves of the complete expression cassette. Half of the final product (SMABE-N) consists of an AAV9 capsid that is packaged with a recombinant AAV genome comprising an AAV2 inverted terminal repeat (ITR), the CBh promoter, a gene comprised of [*an N-terminally nuclear localization signal (NLS)-tagged ABE TadA* evolved deaminase enzyme linked to an N-terminal portion of Cas9 from Staphylococcus pyogenes (Sp) that is directly fused to the N-terminal half of the split DnaE intein from Nostoc punctiforme (Npu) linked to a C-terminal NLS*] [35], the Woodchuck Hepatitis Virus (WHV) Post-transcriptional Regulatory Element (WPRE) gamma expression enhancer, BGH polyadenylation signal, a U6 sgRNA expression cassette encoding the *SMN2* exon 7-targeting sgRNA, and WT AAV2 ITR. The other half of the final product (SMABE-C) consists of an AAV9 capsid that is packaged with a recombinant AAV genome comprising an AAV2 inverted terminal repeat (ITR), the CBh promoter, a gene comprised of [*an N-terminal NLS tagged to the C-terminal half of the split Npu DnaE intein that is fused to the C-terminal chimeric SpCas9 and Streptococcus macacae Cas9 (SpyMac) protein linked to a C-terminal NLS*], the WHV WPRE gamma enhancer, BGH polyadenylation signal, a U6 sgRNA expression cassette encoding the *SMN2* exon 7-targeting sgRNA, and WT AAV2 ITR. The final product will be a mixture of these 2 vectors at a 1:1 ratio. Following transduction of a cell with the 2 vectors, the encoded deaminase-SpyMacCas9 protein fuses into a full-length ABE protein via trans-splicing and excision of the Npu intein elements, and the guide RNA is expressed from both the SMABE-N and SMABE-C vectors. The full sequence of the plasmid will be provided in the IND.

- **ProductName:** dualAAV9/SMABE
- **Serotype:** AAV9 Serotype Capsid
- **Formulation:** Delivered in phosphate-buffered saline with 5% D-sorbitol and 0.001% pluronic F-68
- **Gene Insert (1st half):** SMABE-N

AAV2 ITR	CBh Promoter	ABE TadA*	N-term spCas9	Npu Intein	WPRE	BGH polyA	U6 sgRNA cassette	AAV2 ITR

- **Gene Insert (2nd half):** SMABE-C

AAV2 ITR	CBh Promoter	Npu Intein	Mid-spCas9	C-term Mac	WPRE	BGH polyA	U6 sgRNA cassette	AAV2 ITR

1.5.1.2. Biological Product:

In vivo and in vitro preclinical studies have demonstrated the function of dualAAV9/SMABE [4, 5]. The specific vector lot for clinical use will be evaluated for purity and potency before release for human injection.

Research grade dualAAV9/SMABE vector for preclinical testing was manufactured by either the UMass Chan Medical School Viral Vector Core or the UTSW Vector Core. The manufacturer and final manufacturing process that will be used to produce the toxicology batch(es) and the GMP clinical lot have not been decided.

The sterile dualAAV9/SMABE biological product will be provided in capped vials for aseptic delivery, and our plan is to separately vial and test the two SMABE-N and SMABE-C vectors that together comprise dualAAV9/SMABE. Our position is that the two vectors will never be used separately and will always be combined 1:1 prior to animal or clinical use, or prior to any analytical tests that directly assess their function. Thus, while they would be vialed and released separately, the 2 vectors would be considered one drug product. Each vector would be vialed in a 2-mL CZ vial (1 mL per vial); it will be stored and shipped at ≤ -60°C. The product will be an aqueous solution in pre-sterilized vehicle (phosphate buffered saline containing 5% W/V D-sorbitol and 0.001% F-68).

1.5.1.3. Description of Components Used in Manufacturing

1.5.1.3.1. Vector, Packaging and Helper Plasmid DNA

Three plasmids will be used for manufacturing each of the 2 AAV9 vectors that compose dualAAV9/SMABE. The packaging/helper plasmids pAAV9 and pALD-X80 (which provide the AAV and Adenoviral helper functions needed for AAV vector production in mammalian cell culture) will be used in the manufacture of both components of dualAAV9/SMABE. The 2 vectors that compose dualAAV9/SMABE will utilize different ITR plasmids, with the SMABE-N vector using pAAV-Cbh-TadA*-SpCas9-NpuN-Sg52 and the C-terminal vector using pAAV-Cbh-NpuC-SpyMac-Sg52.

1.5.1.3.2. Plasmid DNA Manufacturing, Testing and Qualification

GMP-sourced plasmid DNA (or equivalent) will be manufactured using standard methods for E. coli fermentation and plasmid purification. Testing and qualification of plasmid DNA lots will be performed prior to release for GMP manufacturing of the vector and will include assays of plasmid identity, sterility, endotoxin content, purity, host cell genomic DNA, protein and RNA. See below for lot release criteria for the plasmid raw materials (Table 1):

Table 1. Lot release criteria for the plasmid raw materials			
--	TEST	SPECIFICATION	ANALYTICAL METHOD
1	**Appearance**	Clear and colorless	Visual inspection
2	**Sterility**	Sterile	EP, Ph.Eur. 6th Ed Chap 2.6.1 USP<71> Direct Inoculation
3	**Mycoplasma**	Negative for the presence of Mycoplasma	PCR or Culture
4	**DNA concentration**	1 mg/mL	UV-Absorption
5	**DNA purity by A260/280**	1.8-2.0	UV-Scan
6	**Plasmid identity (1 enzyme)**	Co-migrates with DNA client reference DNA	Restriction digestion and AGE
7	**Plasmid identity with ITR**	Co-migrates with DNA client reference DNA	Restriction Digestion with XmaI (or SmaI) and Ahdh and AGE
8	**Plasmid identity**	Sequence homology	Sequencing (double stranded)
9	**Residual host RNA**	<5% w/w	Visual inspection after AGE
10	**DNA homogeneity (ccc monomer content)**	>80% supercoiled	Densitometry after AGE
11	**Endotoxin (LPS)**	≤100 E.U./mg DNA	Quantitative LAL assay (kinetic Turbidimetric)
12	**Bacterial Chromosomal DNA**	Not visible	Visual inspection after AGE
13	**Residual Host Protein**	<2% w/w	BCA assay (Bicinchoninic Acid)
14	**Osmolality**	Report	USP <785>
15	**pH**	8±0.5	USP <791>
16	**Residual KanR**	Report	ELISA
17	**Facility**	Uncontrolled environment	
18	**Documentation**	Documentation package and CoA	

1.5.1.4. dualAAV9/SMABE Vector Manufacturing.

We envision utilizing a manufacturing approach similar to that used by the UTSW Vector Core, involving triple transfection of HEK293 cells followed by harvest and filtration of a crude lysate including a DNase treatment. The crude lysate will likely be purified through affinity chromatography, following by a polishing step to remove empty particles using a density gradient or ion exchange chromatography. Final details of vector design, formulation, GMP manufacturing, lot-release assays, stability studies, and potency assay will be presented in a pIND meeting and/or in the IND submission.

1.5.1.5. Quality testing program for dualAAV9/SMABE

Each lot of dualAAV9/SMABE vector will be tested prior to product release, with its 2 components SMABE-N and SMABE-C tested separately. The proposed list of tests, methods, and release specifications are provided in Table 2. For the tests with "report value" release specification, it is our expectation that with additional manufacturing runs and a stronger manufacturing history of dualAAV9/SMABE using these production methods, appropriate release criteria values can be set.

Table 2. Quality testing program for dualAAV9/SMABE

TEST	METHOD	SPECIFICATION		Research Batch	Toxicology Batch	GMP Batch
Safety						
Sterility	EP 2.6.1 / 2.6.27	No growth			†	†
Bioburden	EP 2.6.12	< 10 CFU / 100 mL				†
Endotoxin	EP 2.6.14	< 0.2 EU / mL			†	†
Mycoplasma	EP 2.6.7 qPCR	None detected			†	†
Replication Competent AAV	Serial infection and analysis by PCR	Report Results				†
Adventitious Viruses	EP 2.6.16 In vitro cell culture	None detected				†
Purity						
Residual Host Cell DNA	EP 2.6.21 qPCR	Report Value			†	†
Residual Host Cell Protein	ELISA	Report Value			†	†
Residual Plasmid	EP 2.6.21 qPCR	Report Value				†
Vector Purity and Host cell protein	SDS-PAGE gel and Silver Staining	Number and position of the bands conform to reference		†	†	†
Residual E1A gene	EP 2.6.21 qPCR	Report Value				†
Strength / Dose						
Vector Genome Titer	ITR ddPCR EP 2.6.21; USP<1127>	≥8E13 vg/ml		†	†	†
Activity / Potency	TBD	Report Value		†	†	†
Identity / Characteristics						
Genomic Identity	Sequencing of Vector Transgene	100% match to reference sequence				†
Full:empty capsid ratio	TBD	Report Value		†	†	†
Particle aggregates	TBD	Report value			†	†
Appearance	Visual Exam	Clear colorless solution				†
pH	Potentiometric	Report Value			†	†
Osmolality	Freezing point	Report Value			†	†
Identity by Western Blot	SDS-PAGE and Western blot	VP1, VP2 and VP3 are detected			†	†

1.5.1.6. Product Stability Testing

The tests listed in *Table 3* will be performed at each time point using the methods listed.

Table 3. dualAAV9/SMABE Product Stability Testing

Test	M6	M12	M24	M36
Sterility		X	X	X
Vector genome titer (vg/mL)	X	X	X	X
Appearance	X	X	X	X
pH	X	X	X	X
Osmolality	X	X	X	X
Particle size distribution	X	X	X	X
Potency	X	X	X	X

1.5.1.7. In vitro Potency Assay

A product-specific potency assay will be utilized to quantify transgene expression following transduction of cells *in vitro*. To support the initial first-in-human trial, we envision testing the 2 principal components of dualAAV9/SMABE (SMABE-N and SMABE-C) separately for potency using a cell-based assay to quantify transgene mRNA expression following transduction of Lec2 cells. We reason that the sequence of each vector will be verified as part of its release testing, and if we quantify faithful expression of the transgene mRNA that will confirm that the AAV particles are fully capable of delivering their nucleic acid cargo to mediate transgene expression.

1.5.1.8. Device Compatibility

dualAAV9/SMABE will be assessed for compatibility with all conditions, devices, and materials utilized in all preclinical and proposed clinical studies. This includes preclinical animal and clinical human studies. The same materials (i.e., syringes, pumps, needles, tubing, etc.) will be used in conditions that simulate the intended use of dualAAV9/SMABE. We propose to use non-GMP dualAAV9/SMABE from the toxicology production lot for these studies. Following passage of dualAAV9/SMABE through the device, it will be assessed for titer and potency as well as endotoxin levels.

1.5.1.9. Comparability to prior preclinical lots of dualAAV9/SMABE.

As outlined in Table 4, critical attributes of the vectors used in all preclinical studies (i.e., vector from academic cores and the toxicology lot) will be directly compared to the GMP drug product and with each other. Our development plan will likely include the use of separate manufacturers (and potentially slightly different processes) for our pivotal toxicology lot and our GMP drug product. Our acceptance criteria for analytical comparability will use a strategy that all critical

quality attributes in the toxicology lot will need to be met or exceeded with the GMP drug product. Further, the potency of the lots will need to be shown equivalent.

1.5.2. Nonclinical Pharmacology and Toxicology

1.5.2.1. Completed Pharmacology Studies

Table 4 Summary of completed pharmacology studies

Study	Key results	Figure(s)
In vitro comparison of splicing rescue by top base editor candidates compared to nusinersen and risdiplam.	Base editing of *SMN2* exon 7 C6T results in complete rescue of *SMN* splicing to full-length SMN transcripts in Δ7SMA mESCs, on par with direct treatment with approved therapies such as risdiplam and nusinersen.	Fig 1
In vitro comparison of base editing to nusinersen and risdiplam.	Base editing of *SMN2* exon 7 C6T enabled a 4.5-fold and 1.5-fold greater increase in SMN protein levels compared to approved therapies such as risdiplam and nusinersen in Δ7SMA mESCs, respectively.	Fig 2
Editing efficiency of neonatal mice by ICV injection of dualAAV9/SMABE	87% and 47% conversion of *SMN2* exon 7 C6T among transduced cortical and lumbar spinal cord cells in Δ7SMA mice, respectively.	Fig 3
Transduction efficiency of neonatal mice by ICV injection of dualAAV9/SMABE	Transduction of 43% of lumbar spinal cord motor neurons in Δ7SMA.	Fig 4
In vivo efficacy studies in neonatal SMA mice by ICV injection, compared to Zolgensma or risdiplam	CMAP amplitudes were higher for dualAAV9/SMABE-treated mice compared to low-dose risdiplam-treated or untreated Δ7SMA mice, while CMAP amplitudes did not differ between heterozygotes, Zolgensma-treated mice, and dualAAV9/SMABE-treated animals. dualAAV9/SMABE treatment extended the median survival by 33%, from 17 to 22 days.	Fig 5
In vitro time course of *SMN2* splicing rescue by top base editing strategy, compared to nusinersen treatment	*In vitro*, splicing rescue of *SMN2* transcripts is not achieved until 7 days post-transfection, compared to ca. 3 days after nusinersen treatment.	Fig 6
In vivo combinatorial study with dualAAV9/SMABE + nusinersen	Combined treatment of SMA mice with dualAAV9/SMABE and nusinersen extended median survival to 77 days, versus 29 days for nusinersen alone.	Fig 7

Identification of a base editing strategy to correct SMN expression in vitro.
Current SMA drugs induce nonnative SMN protein levels, achieving ~ 1/3rd of native levels in the target tissue [23, 24, 30-32, 36], and require repeated dosing or may fade over time. The permanent and precise editing of endogenous *SMN2* genes that preserves native regulatory mechanisms and transcript levels governing SMN protein expression may circumvent limitations of existing SMA therapies [1, 4, 5, 22, 29, 37]. To develop a therapeutic that can restore normal regulation and

expression of SMN protein in SMA, we screened base editing strategies able to convert the *SMN2* C6T, which is located in exon 7 of the *SMN2* gene and differentiates insufficient *SMN2* genes from functional *SMN1* genes. Using computational predictions of genome editing outcomes, our lead "*D10"* editing strategy (out of 79 tested base editing strategies) converted *SMN2* exon 7 C6T with the highest efficiency (99%) and high single nucleotide editing precision (82%) in Δ7SMA mouse embryonic stem cells (mESCs) [38, 39]. Because base editing does not require the formation of double-strand DNA breaks, our validated lead strategy did not induce measurable indels. Although, we identified a sub-fraction of edited alleles that harbor bystander nucleotide changes, those have previously been shown to benefit splicing inclusion of exon 7 by improving protein binding at the exonic splicing enhancer and have no effect on SMN protein function because the amino acid sequence of exon 7 is not specifically required for SMN protein function [38, 40-42]. Off-target genome editing by base editors is rare [36] , as supported by our preliminary studies, and we discuss our detailed prior and ongoing off-target analysis in section *1.5.2.3. In vitro and In vivo off-target assessment*.

To assess functional rescue of cellular SMA phenotypes with our base editing strategy, we measured splicing of *SMN2* exon 7 following base editing of C6T. We observed 87% inclusion of *SMN2* exon 7 following base editing, compared to 9.0% at baseline in untreated Δ7SMA mESCs (see Fig. 1 A) [38]. Remarkably, these results are on par with, or exceed, maximum exon 7 inclusion of approved therapies such as nusinersen or risdiplam (see Fig. 1 A and B) and resemble comparable splicing ratios of *SMN1* genes (82% in U2OS cells) [38, 43, 44].

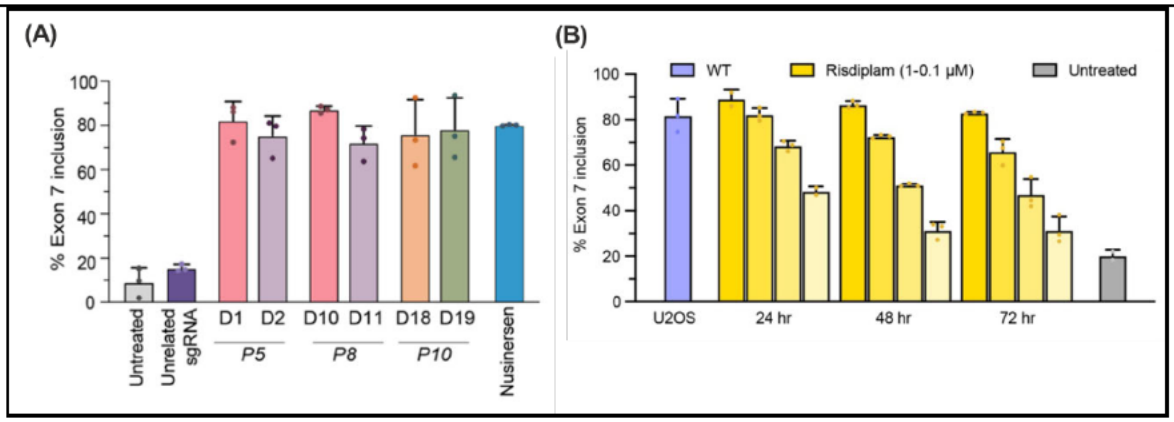

Figure 1. Exon 7 inclusion in SMN. Analysis of Exon 7 inclusion in SMN mRNA in (A) base editing-, (A) Nusinersen-, or (B) risdiplam-treated Δ7SMA mESCs compared to untreated Δ7SMA mESCs and wild-type human U2OS cells. Base editing of *SMN2* exon 7 C6T and target nucleotide position within the protospacer are referred to as *strategy D* and *P#*, respectively. Our lead base editor is *D10* strategy. The dark yellow to light yellow bars indicates risdiplam doses of 1.0, 0.5, 0.25, or 0.1 μM.

Normal levels of SMN protein are essential to the function, survival, and long-term health of all cells in the animal kingdom [45-48]. SMN protein levels are reduced by about 40-fold in Δ7 SMA compared to wild-type mESCs [49]. Importantly, base editing of *SMN2* exon 7 C6T resulted in the up-regulation of SMN protein by about 38-fold compared to untreated Δ7SMA mESCs (see Fig. 2 A) [38], indicating comparable SMN protein levels to those observed in wild-type mESCs [49]. Interestingly, base editing of *SMN2* exon 7 C6T enabled a 4.5-fold and 1.5-fold greater increase

in SMN protein levels compared to approved therapies such as risdiplam and nusinersen in Δ7SMA mESCs, respectively (see Fig. 2 A and B) [38]. Together, these data indicate that base editing of *SMN2* exon 7 C6T faithfully restores the genomic sequence, protein levels, and may ultimately maximize the long-term function of SMN in SMA patients.

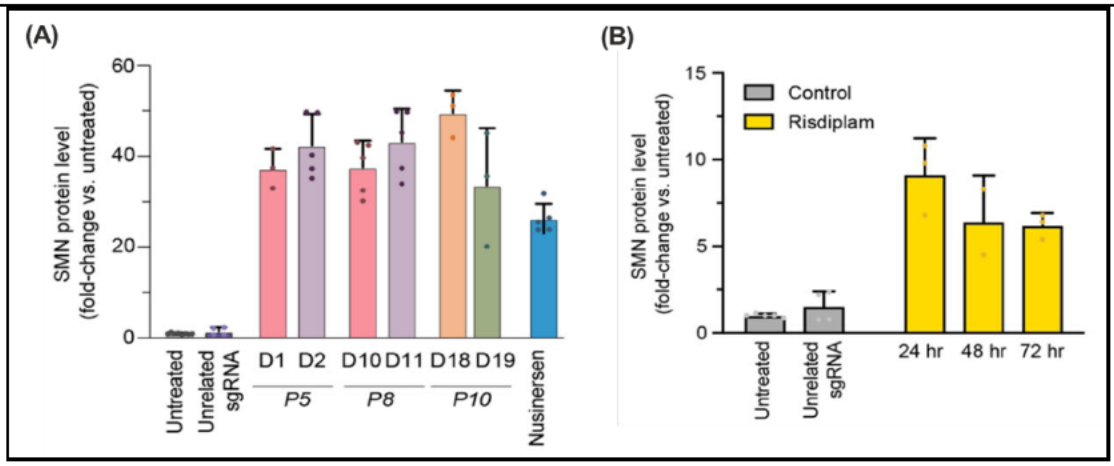

Figure 2. Western blotting analysis of SMN protein levels. SMN protein levels were measured in (A) base editing-, (A) Nusiernsen-, or (B) risdiplam-treated Δ7SMA mESCs, normalized to H3, and are shown relative to untreated Δ7SMA mESCs. Base editing of *SMN2* exon 7 C6T and target nucleotide position within the protospacer are referred to as strategy D and P#, respectively. Our lead base editor is *D10* strategy.

In vivo investigation of base editing efficacy in mice.

The adeno-associated virus serotype 9 (AAV9) has a well-established tropism for multiple neuronal populations of the CNS (e.g., cortical neurons) and the peripheral nervous system (e.g., spinal cord motor neurons) in mice (e.g., Δ7SMA) and human patients [50-52]. Importantly, Zolgensma that also utilizes an AAV9 based delivery to treat SMA patients, has shown efficient biodistribution to disease-relevant tissues, a proven safety profile, and is tolerated in the SMA patient population. Moreover, intracerebroventricular (ICV) or systemic injection in neonatal mice results in efficient transduction of numerous neurons including lumbar spinal cord motor neurons, which are both necessary and sufficient for the rescue of SMA disease phenotypes and lethality in mice and humans [15, 32, 53]. Therefore, we designed a dual AAV9 vector system to package our lead *D10* base editing strategy (ssAAV9-ABE, a.k.a. dualAAV9/SMABE) for *in vivo* delivery, which may enable *SMN2* exon 7 C6T conversion in SMA mouse models.

SMA neonates were ICV-injected at birth (P0) with ssAAV9-ABE (dualAAV9/SMABE, 2.7×10^{13} vg/kg) and AAV9-CBh-eGFP-KASH (2.7×10^{12} vg/kg, [54]), which serves as a viral transduction control. Although, this dose is significantly lower compared to FDA-approved doses administered to SMA patients in humans, our dose is comparable to those used for P0 ICV administration of Zolgensma in Δ7SMA mice, and other base editor AAVs that enable efficient genome editing in mice [32, 54]. We isolated nuclei of treated animals to measure base editing efficiency in either bulk nuclei or by sorting GFP-positive cells that are enriched for AAV9 transduced cells as previously described [54, 55]. Surprisingly, we observed 87% and 47% conversion of *SMN2* exon 7 C6T among transduced cortical and lumbar spinal cord cells,

respectively (see Fig 3 A and B). Similar to our observation in Δ7SMA mESCs (see section above, [38]), we observed high single-nucleotide precision for C6T (73%) and few indels (<0.4%) or bystander edits [38].

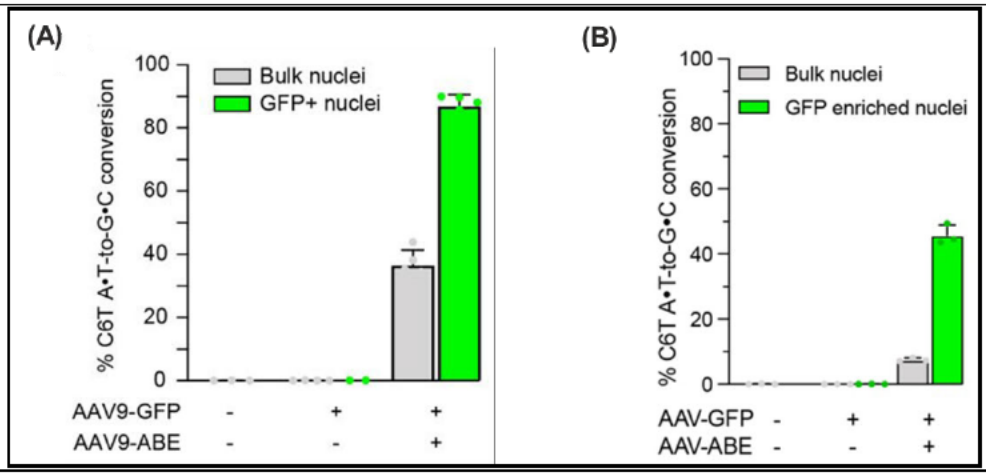

Figure 3. *In vivo SMN2* exon 7 C6T conversion in vivo. Base editing conversion of C6T in (A) cortical and (B) spinal cord bulk and GFP enriched nuclei of Δ7SMA mice treated with dualAAV9/SMABE (AAV9-ABE).

Consistent with previous studies, AAV9 transduction was observed throughout the spinal cord (see Fig. 4, A). Importantly, 43% of lumbar spinal cord motor neurons (ChAT positive cells) were transduced (see Fig. JJ B, dark green bar) and is supported by the transduction efficiencies (>20%) that have been previously observed to enable phenotypic rescue of Δ7SMA mice following ICV injection of Zolgensma [32].

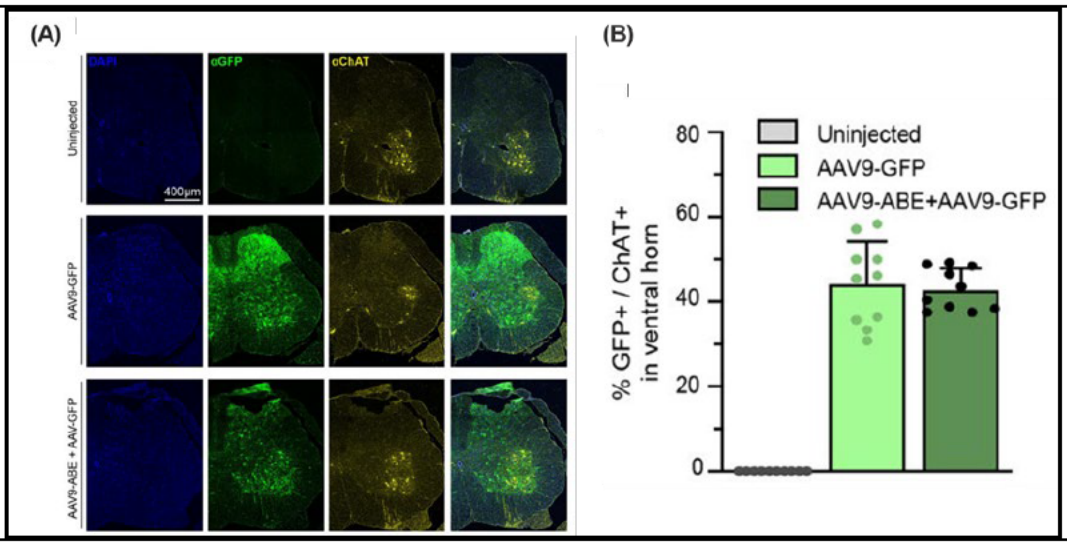

Figure 4. Transduction efficiency of spinal cord motor neurons. (A) Immunofluorescence of lumbar spinal cord sections from wild-type Δ7SMA mice at 25 weeks old, ICV injected at birth with ssAAV9-ABE, AAV9-GFP, or uninjected. GFP indicates transduction, ChAT labels spinal motor neurons in the ventral horn, DAPI stains all nuclei. (B) Quantification of GFP and ChAT double-positive cells within the ventral horn of the lumbar spinal cord.

Interestingly, the transduction efficiency of lumbar spinal motor neurons was equivalent to that of a 10-fold higher dose of AAV9-GFP alone (see Fig. 4 B, light green bar), revealing that our low-dose co-transduction of AAV9-GFP accurately represents cells that are transduced by our ssAAV9-ABE base editor, and suggesting a high degree of co-transduction by our dualAAV9/SMABE vectors. Collectively, dualAAV9/SMABE delivery via ICV injection in Δ7SMA neonates enables efficient and precise conversion of *SMN2* exon 7 C6T in the CNS with minimal undesired byproducts [38, 40, 41, 56].

Investigation of base editing to correct phenotypic defects in SMA mouse models.
In addition, we assessed whether motor function measurements are improved in ssAAV9-ABE treated Δ7SMA mice. Electrophysiological measurements including CMAP (compound muscle action potential) or MUNE (motor unit number estimation) assess function of motor neurons and/or motor neuron axons that innervate the muscle, a key feature of SMA and pre-clinical SMA models [57]. We compared outcomes in our ICV injected base editor with FDA-approved therapeutics for SMA including ICV injection of Zolgensma, and daily intraperitoneal (IP) injection of risdiplam (Evrysdi) at doses that were previously demonstrated to confer a survival benefit to these mice [32, 33]. CMAP amplitudes were higher for dualAAV9/SMABE-treated mice compared to risdiplam-treated or untreated Δ7SMA mice, while CMAP amplitudes did not differ between heterozygotes, Zolgensma-treated mice, and ssAAV9-ABE-treated animals (see Fig. 5 A). Concordantly, MUNE in dualAAV9/SMABE-treated SMA mice was also improved compared to untreated Δ7SMA animals [38]. Furthermore, dualAAV9/SMABE treatment extended the median survival from 17 to 22 days (see Fig. 5 B). Collectively, these data highlight that postnatal conversion of *SMN2* exon 7 C6T by ssAAV9-ABE improves motor phenotypes and survival in SMA mice.

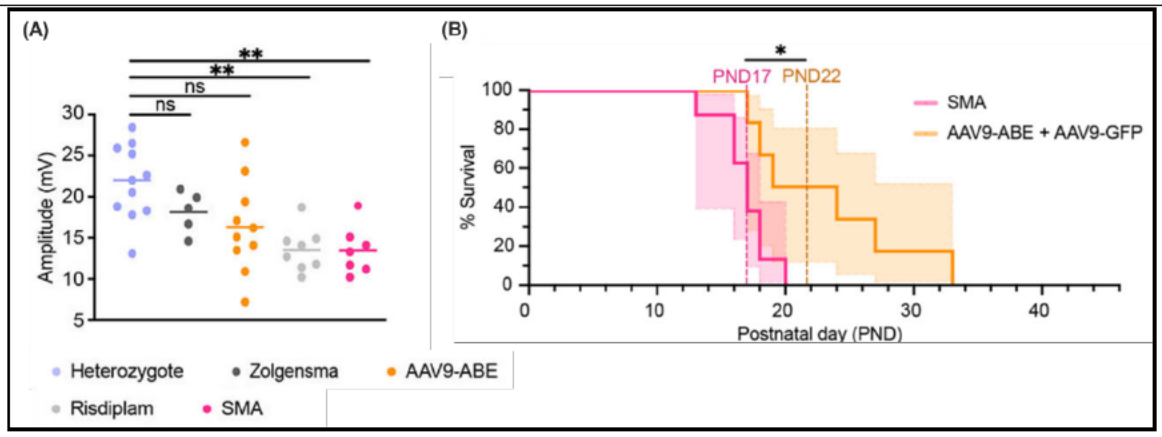

Figure 5. Behavioral efficacy in SMA treated mice. (A) Compound muscle action potential (CMAP) amplitude in heterozygotes (n=11), and Δ7SMA uninjected (n=7) mice to treated with Zolgensma (n=5), dualAAV9/SMABE (AAV9-ABE, n=10), and risdiplam (n=8) at PND12. Statistical significance was determined using Kruskal-Wallis one-way ANOVA. (B) Kaplan-Meier curve of uninjected (n=8) and dualAAV9/SMABE treated (AAV9-ABE, n = 6) Δ7SMA mice. Statistical significance was determined using Mantel-Cox test. Postnatal day (PND), *≤0.02, **≤0.01.

Development of a combinatory therapeutic in SMA mice.

In SMA type I patients, therapeutic intervention can meaningfully improve disease outcomes if administered in the first several months of life [58-61]. However, motor neuron loss and survival defects occur rapidly within a few days after birth in Δ7SMA mice, which is in part due to the higher rate of maturation of mice compared to humans in the first months of life [29, 62]. Thus, restoration of SMN protein levels using inducible transgenes demonstrates that high levels of SMN are required within the first 6 days after birth to improve survival defects in Δ7SMA mice, and a delay in delivery is strongly anti-correlated with survival [32, 62-66]. Similarly, survival correction declines precipitously when animals receive treatment (e.g., Zolgensma) past postnatal day 6 (see Fig. 6 A). Although P0 ICV dualAAV9/SMABE treatment significantly improved survival in Δ7SMA mice (see Fig. 5 B), the survival improvement mimics that achieved by scAAV9-SMN gene therapy in P7 Δ7SMA mice (see Fig. 6 A), suggesting that *SMN2* exon 7 C6T conversion requires time for its completion to occur. In agreement, complete mRNA rescue *in vitro* is not achieved until 7 days post-transfection of our base editor (D10P8) (see Fig. 6 B).

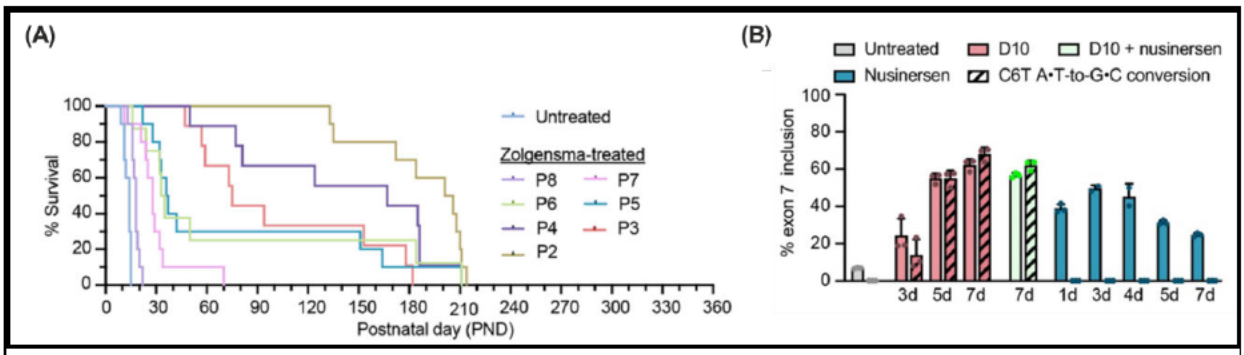

Figure 6. Time course of *SMN2* correction. (A) Kaplan–Meier curve of Δ7SMA neonates ICV injected with Zolgensma from Robbins et al. (87) at different postnatal days (P). (B) Time course exon 7 inclusion in Δ7SMA mESCs treated with the *D10* strategy and 20 μM nusinersen as indicated, showing the frequency of U-to-C conversion at C6T in spliced transcripts, measured by high-throughput sequencing of reverse transcribed SMN mRNA.

Delivery with the dual ssAAV9 ABE base editor requires completion of (1) second-strand synthesis of each ssAAV9-ABE genome [67, 68], (2) transcription and translation of the split-intein ABE protein segments, (3) assembly and trans-splicing of the split ABE protein, (4) RNP assembly and base editing of *SMN2*, (5) transcription of full length C6T-modified endogenous *SMN2* pre-mRNA driven by its native promoter, and (6) splicing and translation of corrected *SMN2* transcripts. Thus, the timing for *in vivo* base editing to impact and/or restore protein levels may require 1-3 weeks post-administration [69], which is slower than fast-acting splice-switching drugs or constitutive gene complementation for SMN such as Zolgensma [67, 68, 70] and surpasses the extremely short therapeutic window in Δ7SMA mice.

Transient SMA drug administration such as nusinersen can ameliorate SMA pathology and extend survival of Δ7SMA mice. We hypothesized that nusinersen may extend the unusually short therapeutic window of Δ7SMA mice and thereby, allow dualAAV9/SMABE mediated editing to occur prior to extensive irreversible SMA damage. Thus, we co-injected a 1μg dose of nusinersen

that has been previously shown to extend survival in P0 treated Δ7SMA mice [71] together with dualAAV9/SMABE. Nusinersen alone increased survival from 17 (median) to 29 days (median) in Δ7SMA mice. The combination of dualAAV9/SMABE with nusinersen further improved survival to 77 days (median) with 60% of Δ7SMA mice surviving past the nusinersen-only treated mice (see Fig. 7 A). Furthermore, assessment of motor strength and function revealed that nusinersen-treated Δ7SMA animals performed significantly worse than healthy heterozygous mice. In contrast, treatment of dualAAV9/SMABE with nusinersen showed no significant difference compared to healthy heterozygous mice (see Fig. 7 B). Notably, about 50% of mice only treated with nusinersen were already deceased by this age, and no untreated Δ7SMA mice survived long enough.

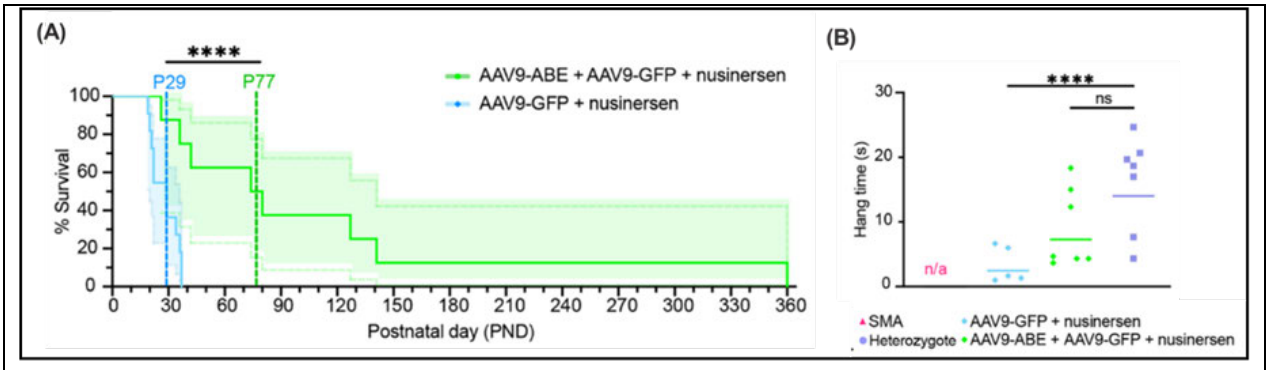

Figure 7. Combinatory treatment of ssAAV9-ABE and nusinersen in mice. (A) Kaplan–Meier curve of Δ7SMA neonates ICV injected with nusinersen (blue) or dualAAV9/SMABE (ssAAV9-ABE) + nusinersen (green). Graph line shading represents the 95% confidence interval. Statistical significance was determined using Mantel-Cox test. ****= 0.002 (B) The hang time of Δ7SMA mice on the inverted screen test at postnatal day 25. Statistical significance was determined using Kruskal-Wallis test. ****=0.007.

For a more complete behavioral assessment of our combination treatment, we subjected mice to open field testing of locomotor activity and exploratory behavioral phenotypes at postnatal day 40. No significant differences were observed across the open field measurements including distance traveled, velocity, duration, and counts of activities between dualAAV9/SMABE and nusinersen combination-treated and heterozygous mice [38]. No Δ7SMA mice treated with only nusinersen survived long enough to undergo this analysis. Collectively, neonatal ICV delivery of dualAAV9/SMABE alone enables survival extension in Δ7SMA mice, however, co-administration with nusinersen temporarily slows disease progression, broadens the narrow therapeutic window, and allows base editing to more effectively improve SMA defects in mice. Our initial proof-of-concept data demonstrate the compatibility of dualAAV9/SMABE with nusinersen as a treatment without adverse effects in animal behavior and highlight the potential for a clinical application of dualAAV9/SMABE in SMA patients already receiving an approved SMA treatment.

1.5.2.2. Proposed Pharmacology Studies

Introduction to proposed studies

An important consideration and limitation of our preclinical studies is that many SMA mouse models, including our Δ7 SMA mouse model, are incredibly severe with a lifespan in the range of 14 days. This raises challenges for evaluating AAV9-based therapeutics, where a lag time to achieve full protein expression in transduced cells competes with the severity and overall health of the model.

Our studies in the Δ7 SMA mouse model revealed that the co-administration of nusinersen and our optimized dual vector ssAAV9-ABE (base editor, henceforth dualAAV9/SMABE) provides a vital combinatory therapeutic approach to rescue SMA phenotypes including the output of functional motor units innervating muscle (CMAP), and survival in mice [38]. Nusinersen, which transiently allows for survival of motor neurons in mice, opens the therapeutic treatment window for genome editing in Δ7 SMA mice. Based on our previous investigations, the outlined studies intend to identify the optimal route of administration and dose in mice. The experimental strategy has been divided into several phases that (1) address more effectively outstanding questions and (2) collectively will provide an optimized administration of dualAAV9/SMABE in SMA mouse models for efficacy (see Fig. 8). The studies described will be performed at the Boston Children's Hospital (BCH), Translational Neuroscience Center (TNC) and The Jackson Laboratory (JAX) and will also make use of the resources of the Viral Vector Regulatory Core (VVR) at UT Southwestern Medical Center (UTSW), the Preclinical Mouse Model core (PMM) at JAX, and the Genome Editing (GE) cores for viral production, large scale maintenance and experimentation in mouse cohorts, and analysis of molecular and physiological phenotypes.

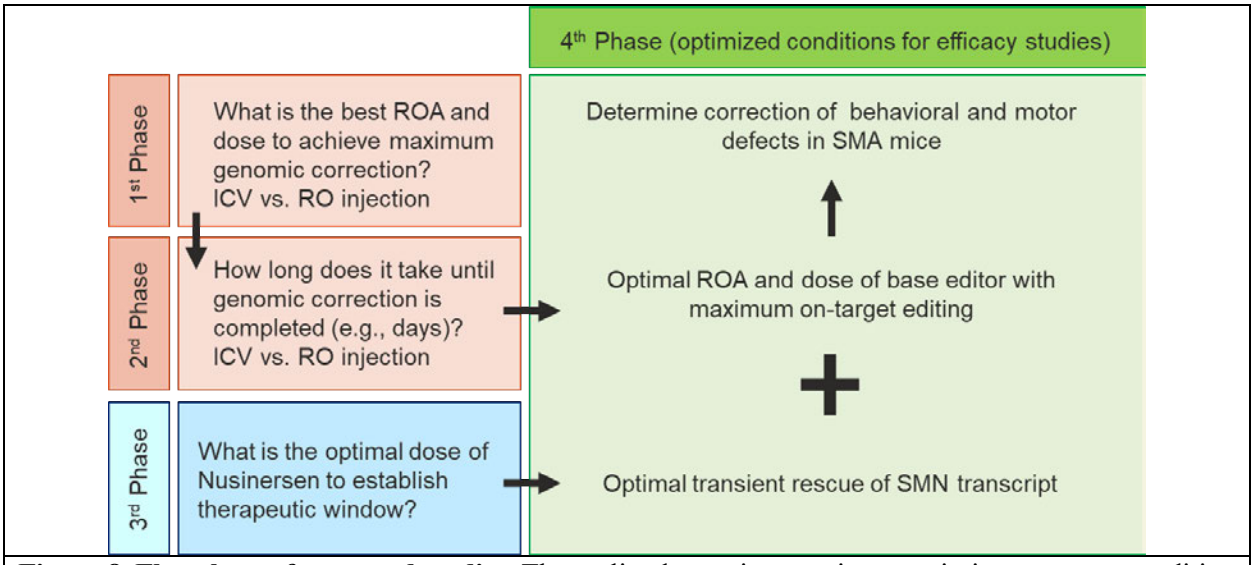

Figure 8. Flowchart of proposed studies. The outlined experiments aim to optimize our genome editing therapeutics (base editor, permanent correction of *SMN2* splicing) in combination with nusinersen (transient SMA treatment) in Δ7 SMA mouse model.

Phase 1: Optimal route and dose of dualAAV9/SMABE administration to maintain maximum editing correction in vivo.

In patients, therapeutic treatment of spinal motor neurons in the central nervous system (CNS) is required for efficacy, and intrathecal delivery of nusinersen directly to the CNS is sufficient to rescue SMA disease progression. The current AAV9-gene therapy (Zolgensma) is administered systemically to SMA patients, and the viral vector enables CNS, motor neuron transduction by crossing the blood-brain-barrier (BBB). Promising clinical trials on intrathecal delivery of Zolgensma for direct treatment of the CNS are currently in progress, and if successful this route of administration (ROA) may broaden the range of SMA patients eligible for treatment with an AAV-based therapy. The scAAV9-SMN gene therapy has an efficacy range of about 2-fold (1.8-3.3E13 vector genomes per kg of body weight, vg/kg) that enables comparable levels of rescue in Δ7 SMA mice by neonatal ICV injection [24]. In patients, systemic treatment with Zolgensma is determined by weight (1.1E14 vg/kg) and the ongoing intrathecal clinical trial uses a one-time dose of 1.2E14 vg.

Furthermore, Δ7 SMA mice exhibit a range of peripheral phenotypes related to the loss of SMN protein, including fatal cardiac dysfunction and necrosis of the extremities that are not seen in SMA patients [72-74]. The peripheral phenotypes may reduce lifespan of CNS-only treated Δ7 SMA mice, but can be overcome by systemic treatments [49].

Therefore, we will expand our proof-of-concept dualAAV9/SMABE (base editor) studies by performing neonatal ICV (CNS delivery) and RO (retro-orbital, systematic delivery) injections at three different doses per route, with a maximum ICV dose of $5x10^{11}$ vg per mouse (5 uL)and a maximum RO dose of $8x10^{11}$ vg per mouse (8 uL) and evaluate on-target genomic DNA (gDNA) editing efficacy (*SMN2* C6T conversion) in the CNS (e.g., cortex and spinal cord) and peripheral tissues (e.g., heart and liver) of non-phenotypic SMA mice to identify the degree of editing efficacy at lower doses (see Fig. 9). Based on our prior experience with ssAAV9 delivery of base editors we expect to see a minimal decrease in gDNA editing with a ~ 2 to 5-fold dilution but a drop in efficiency upon 10-fold dilution. Since our aim in this study is to compare tissue transduction and subsequent editing across tissues and timepoints, we will perform this study in non-symptomatic Δ7 SMA mice, which are homozygous for murine *SMN1* and for the human *SMN2* transgene that is targeted by the base editor, to avoid confounding due to SMA disease.

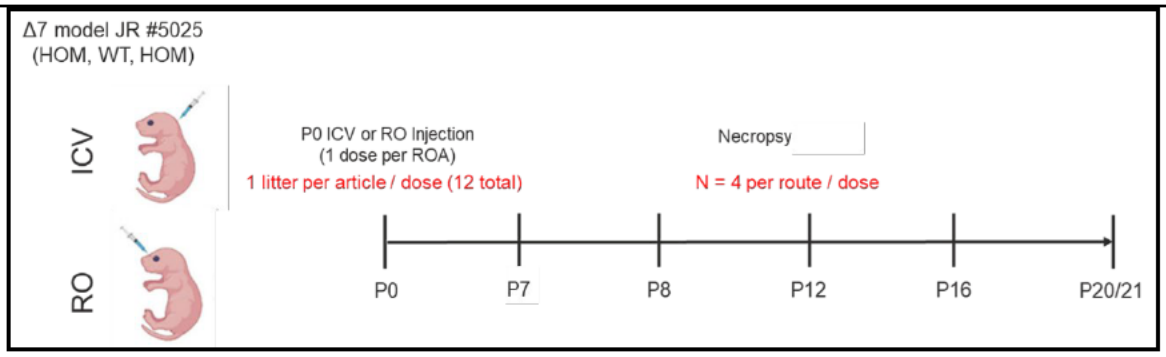

Figure 9. Comparison of dose and route of administration. At birth, mice will be injected with a range of doses either via ICV or RO injection. CNS and peripheral tissues will be collected at 4-weeks postinjection and analyzed for on-target editing correction.

Phase 2: Identification of the minimal treatment window to complete gDNA editing in vivo.
In our proof-of-concept studies, dualAAV9/SMABE-mediated correction of C6T in the CNS was completed by 4 weeks post injection (the earliest timepoint measured). Based on our extensive experience with genome editing agents, we anticipate that sufficient expression levels of the base editor and thereby, C6T correction in the CNS may be completed at earlier timepoints (e.g., within 1-2 weeks). To refine our understanding of the timeline in which dualAAV9/SMABE restores native wild-type SMN protein levels in transduced cells, we will assess genomic and transcriptomic *SMN2* correction at earlier timepoints than in our preliminary study.

Experiments described in *Phase 1* will allow us to identify the minimal dose by which either ROA maintains maximum on-target gDNA editing in the CNS and peripheral tissues. Subsequently, we will measure RNA expression of the base editor and assess genomic editing at earlier timepoints using the optimal dose of dualAAV9/SMABE for either ROA (see Fig. 10). This study will allow us to identify the time course of (1) base editor gene expression, (2) when gDNA editing is completed in the CNS (e.g., cortex and spinal cord) and thereby, (3) establish the minimal therapeutic window (minT) that is needed to support maximum dualAAV9/SMABE-mediated gDNA editing *in vivo*. To avoid confounding due to SMA disease, we will perform this study in non-symptomatic Δ7 SMA mice as in *Phase 1*.

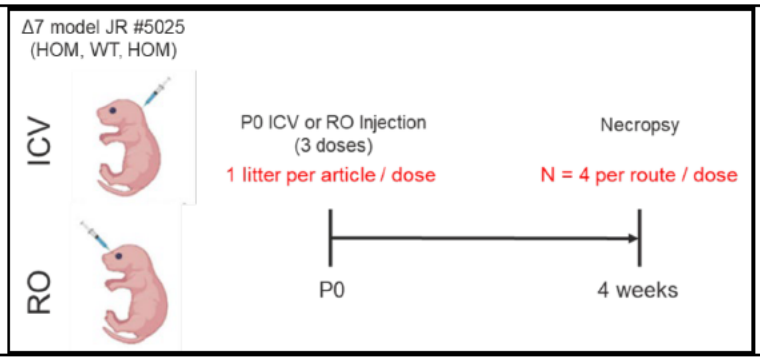

Figure 10. Comparison of route of administration and minimal time window to complete gDNA editing. At birth, mice will be injected either via ICV or RO injection. CNS tissues (e.g., cortex and spinal cord) will be collected at different time points post injection and analyzed for on-target editing correction.

Phase 3: Identification of nusinersen's window of efficacy to support gDNA editing completion in vivo.
Due to the severity and early disease onset of motor neuron degeneration in Δ7 SMA mice, co-administration with a transient SMA therapeutic (e.g. nusinersen) to widen the therapeutic window is necessary to support *in vivo* base editing rescue of Δ7 SMA mice. We have selected a dose-range between 1 µg to 20 µg of nusinersen based on our prior experience with administering this transient therapeutic in neonatal Δ7 SMA mice. *Phase 3* will allow us to determine the window of efficacy (effT) at different doses in mice (see Fig. 11) and thus, help us to determine the minimal treatment duration of nusinersen that is necessary to support maximum gDNA editing efficacy of dualAAV9/SMABE by neonatal ICV injection (minT, experiments described in the 2nd Phase). Additionally, establishing the effT at these doses will allow us to establish treatment protocols for

dualAAV9/SMABE administration in SMA mice at later timepoint ages, which may be informative for treatment in SMA patients who already receive treatment for their condition, or who have a later onset than type I patients.

We will inject neonates either once at birth (P0) or twice (P0 and P3) with different doses of nusinersen, then CNS tissues including cortex, brain and spinal cord will be collected at various time points. Tissue samples will be subjected to RNA analysis to assess the efficacy of nusinersen at exon 7 inclusion and FL-SMN transcript expression levels. To avoid confounding due to SMA disease, we will perform this study in non-symptomatic Δ7 SMA mice as in *Phase 1* and *2*.

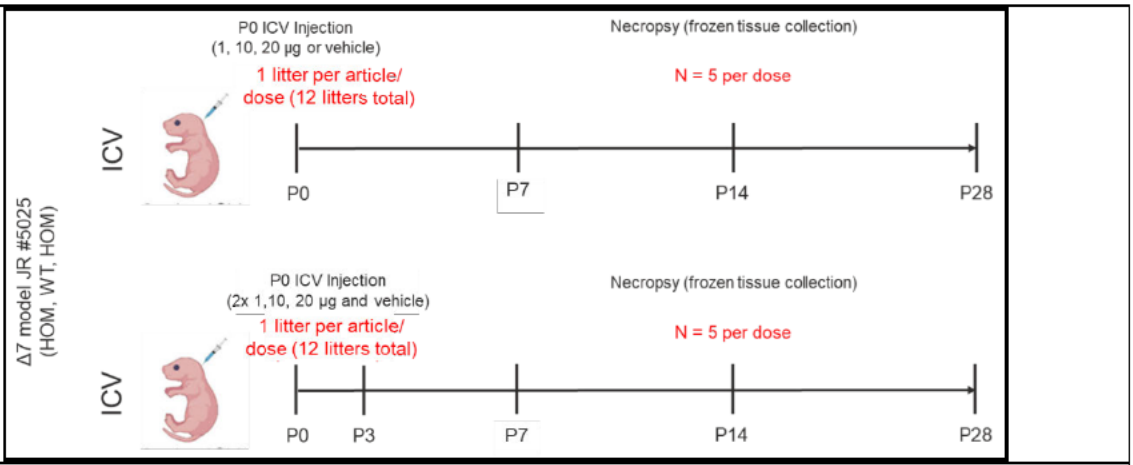

Figure 11. Identification of the window of efficacy of nusinersen. At birth, mice will be subjected to different doses of nusinersen, CNS tissues will be collected and analyzed for exon 7 inclusion. This will allow the identification of the dose that transiently rescues SMN expression and thereby, concordantly widens the therapeutic window for our dualAAV9/SMABE editor to complete gDNA editing.

Phase 4: Combination of dualAAV9/SMABE with nusinersen under optimized conditions for treatment of phenotypic Δ7 SMA mice.

Completion of the experiments described above (*Phase 1 to 3*) will define the (1) optimal route of administration (ICV or RO injection) of dualAAV9/SMABE, (2) optimal dose of dualAAV9/SMABE, (3) the minimum duration of dualAAV9/SMABE that is needed to complete maximum gDNA editing (minT), and (4) the optimized dose of nusinersen that enables transient rescue of SMN transcript and extend the therapeutic window to support maximum gDNA editing by dualAAV9/SMABE in mice.

Based on our proof-of-concept data, co-administration of nusinersen and dualAAV9/SMABE via ICV will extend lifespan in Δ7 SMA mice. However, if experiments described in *Phase 1* reveal that systemic delivery (RO injection) of dualAAV9/SMABE provides sufficient and comparable gDNA editing efficiency in the CNS relative to the editing efficiency observed via ICV injection, our studies would focus on using RO delivery of the dualAAV9/SMABE because gDNA editing in the CNS and peripheral organs (e.g., heart) may further improve extension of survival compared to our previously seen proof-of-concept studies.

Therefore, we will treat Δ7 SMA mice (both phenotypic and non-phenotypic) at the earliest treatment time point P0 with nusinersen (ICV) and dualAAV9/SMABE (ICV or RO) (see Fig. 12 A). Subsequently, the mice will undergo phenotyping for efficacy assessment including body weight, CMAP, and survival, along with tissue collection to evaluate gDNA editing and SMN transcript splicing analysis.

Since transient treatment with nusinersen can temporarily support SMN splicing (*Phase 3*) and promote motor neuron survival, thereby widening the treatment window, administration of dualAAV9/SMABE at a later time points may still be beneficial to nusinersen treated Δ7 SMA mice. Evaluating the efficacy of dualAAV9/SMABE in juvenile mice and in the setting of a later-stage combination treatment may be relevant to treatment in SMA patients already receiving treatment for their condition and/or for later onset SMA patients. Treatment of older mice may also allow more accurate dose-extrapolation relative to treatment in humans *via* our preferred intrathecal route of administration for clinical studies. Thus, if feasible, we will treat juvenile homozygous mutant Δ7 SMA mice treated with nusinersen at later timepoints (e.g., P7 or P21) with a range of dualAAV9/SMABE doses by direct CNS (IT, intrathecal) or systemic (RO) administration (see Fig. 12 B).

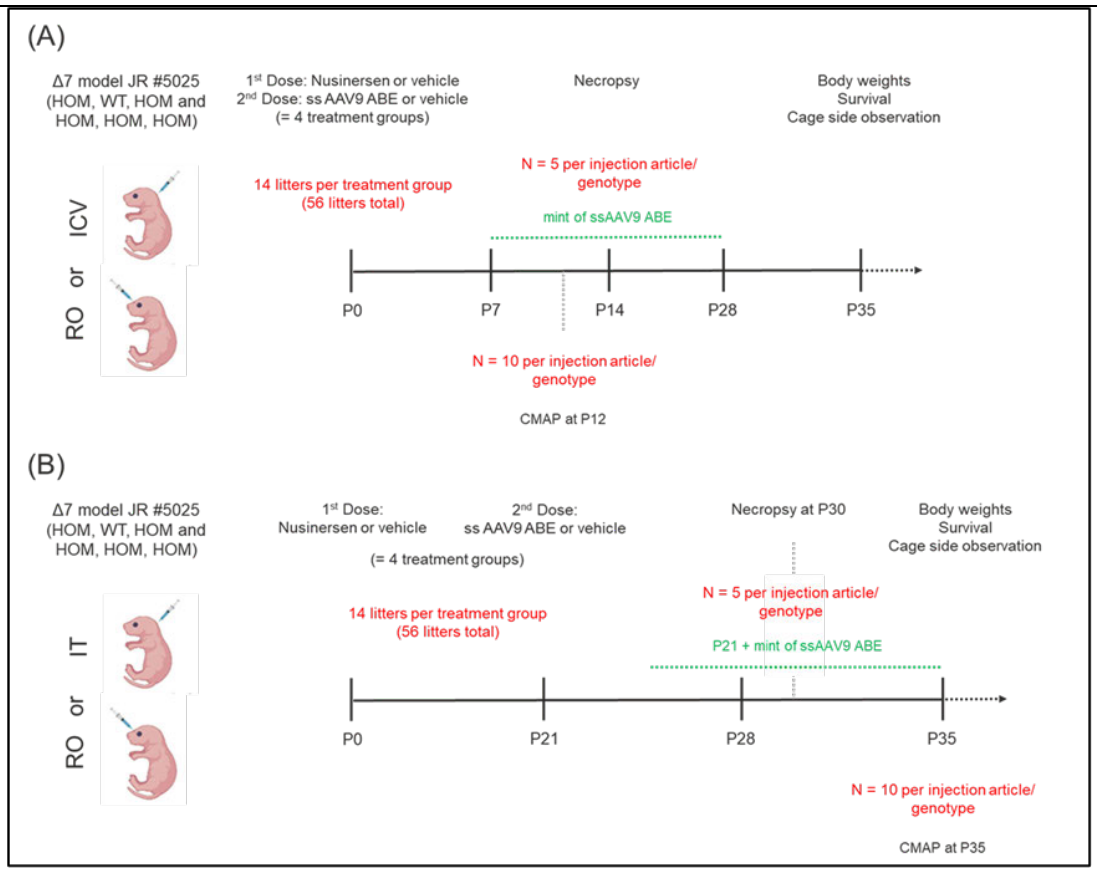

Figure 12. Combination of dualAAV9/SMABE and nusinersen for behavioral efficacy studies in Δ7 SMA mice. Optimized route of administration and dose of dualAAV9/SMABE (ssAAV9-ABE) and nusinersen, determined in *Phase 1* to *3*, will be injected in neonates (A) and in juvenile (B) Δ7 SMA mice and determine behavioral improvement.

In summary, we aim to (1) verify the efficacy of our lead dualAAV9/SMABE therapeutic in mice, (2) verify that extension of the therapeutic window by transient drug administration improves lifespan of dualAAV9/SMABE treated animals, (3) establish the minimum therapeutic window needed to support maximum rescue by dualAAV9/SMABE treatment, (4) establish optimal dosing of nusinersen that is necessary to support maximum efficacy of dualAAV9/SMABE in Δ7 SMA mice, (5) verify the compatibility (and synergy) of ssAAV9-ABE with nusinersen to support future combination treatment clinical trial design, and (6) assess rescue of molecular and physiological SMA phenotypes following co-administration of dualAAV9/SMABE with nusinersen in Δ7 SMA mice under optimized conditions.

Possible technological improvements

Lastly, we will also investigate how variants of the adenine deaminase component of the base editor, some of which were reported after the studies described above were underway, affect on-target and off-target editing properties of our base editing approach in order to identify an editing strategy with the most promising combination of efficient target editing of *SMN2* with minimized off-target editing. These adenine deaminase variants include one already used in the VERV-101/VERV-102 *in vivo* base editing clinical trial previously reviewed by FDA or the UK MHRA and Health Canada, as well as other current-generation deaminases. We would like to make the agency aware that if we identify variants of our lead candidate base editor that have equivalent on-target editing efficiency but a more favorable (i.e., less) off-target editing profile, we would seek further guidance from the agency about making a change to the more favorable adenine deaminase variant and any additional preclinical studies that would be necessary to enact that change.

1.5.2.3. In vitro and In vivo off-target assessment

Some base editors can induce off-target deamination in cells, including Cas-dependent off-target DNA editing and Cas-independent off-target DNA or RNA editing [40, 41, 46, 47, 75, 76]. Genomic and transcriptomic off-target deamination by ABEs without involvement of the Cas protein component is rare, and deaminase variants that further minimize these events have been reported [48, 75]. Nevertheless, our completed as well as planned pharmacology studies intend to further interrogate potential concerns for off-target events and minimize their occurrence however possible, as described in our approach and below in this section.

Table 5. Completed and Proposed studies to evaluate off-target editing

Study	Results / Purpose	Figure or status
In vitro study of human genomic off-targets in HEK293T cells	High on-target editing (49%) with minimal ABE editing activity at off-target site rank 19 (0.4%) which falls in an intergenic region of chromosome 15, and no base changes (>0.03% above that of untreated cells) at the remaining 21 top CIRCLE-seq nominated off-target loci. Overall, minimal off-target editing events of expected clinical significance in a human genetic background.	Fig 13
In vitro study of murine genomic off-targets in Δ7SMA mESCs	High on-target editing (95%) at *SMN2* with off-target ABE editing activity at only one non-coding locus (off-target site rank 5 in *Muc16*, 31%) of a gene that is not expressed in the CNS. No base changes (>0.03% above that of untreated cells) at the remaining 34 off-target loci.	Fig 14 A
In vivo study of murine genomic off-targets in Δ7SMA mice	High on-target editing (87%) at *SMN2* with off-target ABE editing activity at only two non-coding loci: off-target site rank 5 in *Muc16* (15%) and off-target site rank 15 (0.5%). No base changes (>0.03% above that of untreated cells) at the remaining four loci, measured in five mice across 14 weeks. No increased off-target editing events over time, and ca. 2-fold reduction in *in vivo* off-target editing compared to *in vitro.* These indicate a favorable *in vivo* off-target editing profile, and that outcomes observed in cell culture experiments are representative of those observed *in vivo.*	Fig 14 B
In vitro study of transcriptomic off-targets in Δ7SMA cells	No significant difference in trancriptome-wide A-to-I changes in mESCs and differentiated motor neurons and caudal neural lineages.	Fig 15
In vivo kinetics of off-target editing in mice following neonatal ICV and RO injection of dualAAV9/SMABE	Mice will be injected, then timed sacrifice of subsets of the cohort will be done across an 18-month timeframe. This study will aim to understand the long-term risks of off-target editing, based on whether off-target edits accumulate over time.	Fig 16

Completed pharmacology studies to assess off-target events.

To thoroughly investigate the off-target risks of our genome editing strategy, we assessed off-target editing in both the human genome, which is most relevant for future application of our therapeutic in patients, and in the mouse genome, to assess long-term off-target risk in vivo. First, we identified putative Cas-dependent genomic off-targets using CIRCLE-seq [45], an unbiased and sensitive empirical *in vitro* off-target detection method that relies on DSB formation by the Cas protein complexed with the guide RNA to capture and identify Cas-targeted sites. Potential off-target sites identified by CIRCLE-seq can then be sequenced in-depth in base-edited human cells to provide a sensitive genome-wide analysis of off-target base editing events [77, 78]. We generated purified ribonucleoprotein (RNP) complexes of our ABE editing strategy to treat human genomic DNA extracted from HEK293T cells *in vitro* and identified a short list of 54 off-target genomic cleavage events. Subsequently, we measured ABE editing activity at the top 23 CIRCLE-

seq-nominated loci in human cells (see Fig. 13) [38]. We achieved 49% C6T on-target editing at *SMN2* in HEK293T cells and observed minimal base editing at *SMN1* (0.15%). The significant reduction in base editor activity at the *SMN1* gene that shares near-complete overlap with the on-target *SMN2* gene demonstrates that our base editor:sgRNA combination is highly specific to the intended *SMN2* target. We detected minimal ABE editing activity at off-target site rank 19 (0.4%) which falls in an intergenic region of chromosome 15, and no base changes (>0.03% above that of untreated cells) at the remaining 21 potential off-target loci. Thus, ABE editing targets *SMN2* alleles with high efficiency without inducing any additional coding mutations and minimal (<1/100 relative to on-target at 1 locus) non-coding changes in the genome [38]. These data establish that our lead ABE editing therapeutic is highly specific to the *SMN2* on-target locus and does not result in off-target editing events of expected clinical significance.

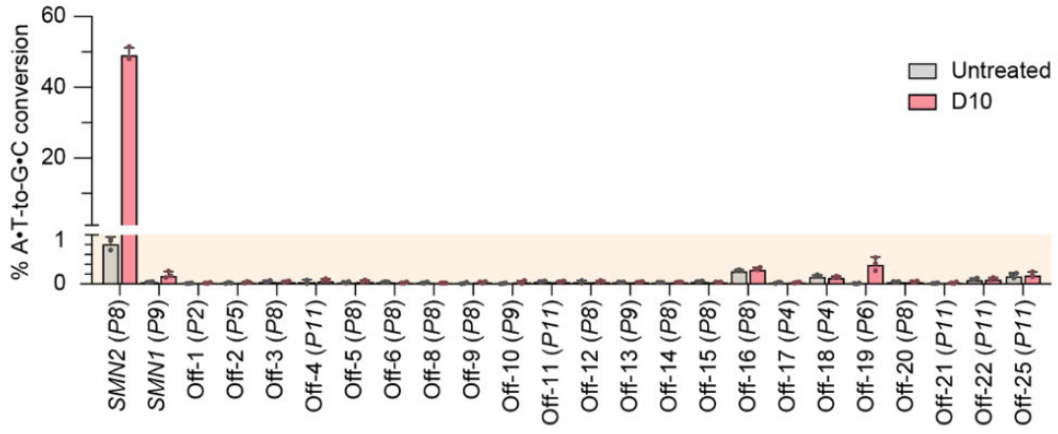

Figure 13. On- and off-target analysis in human HEK293T cells. On- and off-target indel frequency of base editing (Spy-mac nuclease and P8 sgRNA) in human cells.

In addition to the analysis in human cells described, we assessed the specificity of our ABE editing strategy in the mouse genome using CIRCLE-seq. We treated mouse genomic DNA extracted from NIH3T3 cells *in vitro* with ABE editing RNPs and analyzed rare off-target genomic cleavage events. We identified 108 candidate Spy-mac-dependent DNA off-targets that are predominantly at intergenic and intronic loci, as well as 4 coding loci (off-target rank 32, 33, 37 and 86). Next, we measured ABE editing at the top 35 nominated sites in Δ7SMA mESCs (see Fig. 14 A). We achieved 95% on-target editing at the *SMN2* transgene and only observed significant off-target editing at off-target site rank 5, which is located in an intron of the mucin 16 gene (*Muc16*, 31%) that is not expressed in the CNS [79]. *In vivo*, we observed 15% editing at the *Muc16* off-target locus, and 0.5% editing at the non-coding off-target site rank 15, compared to 87% on-target editing of *SMN2* in the CNS in five Δ7SMA mice that were injected at P0 with ssAAV9-ABE (see Figure 14 B) (79). Interestingly, animals ranged from 4 to 18 weeks of age and we observed no increase in off-target editing events over this timeframe [38]. Thus, off-target editing outcomes observed in cell culture experiments are representative of those observed *in vivo*.

Notably, our ABE editing strategy did not result in coding mutations in either the human or mouse genome and detected *in vivo* off-target editing was lower compared to our *in vitro* in cell culture studies (~ 2-fold lower at *Muc16*). The relative reduction in off-target editing observed *in vivo* is likely due to having lower copy number and expression levels of the base editor in transduced cells or *in vivo* gene silencing over time [30, 80, 81].

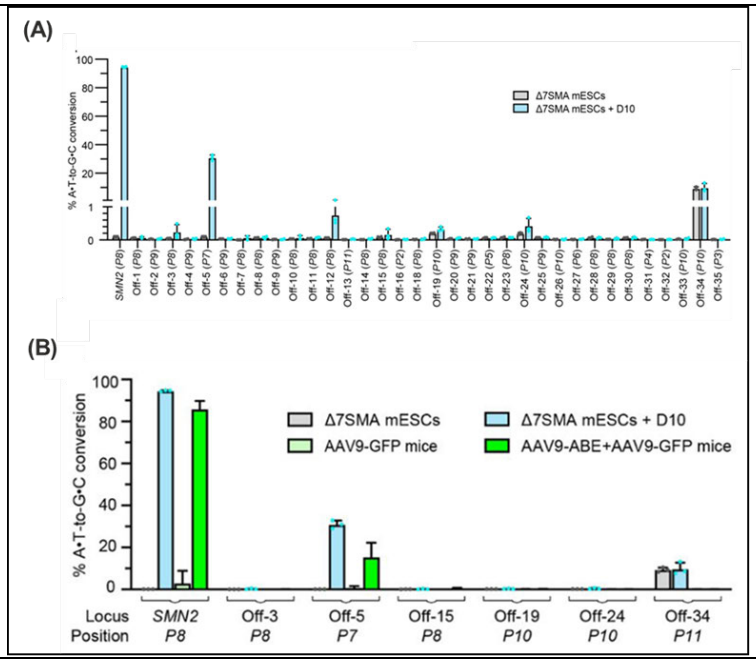

Figure 14. On- and off-target analysis in mouse cells. (A) On-target and off-target base editing of strategy D10 in Δ7SMA mESCs. Bars show editing of the highest edited nucleotide (P# shown in parenthesis) at each locus. (B) On-target and off-target editing following analysis of strategy D10 in Δ7SMA mESCs compared to DualAAV9/SMABE (AAV9-ABE) + AAV9-GFP treatment in Δ7SMA mice. Bars show editing of the most frequently edited nucleotide at each locus, with the P# position shown in parenthesis.

In addition to Cas-dependent off-target events which can be captured by CIRCLE-seq, base editors may induce Cas-independent editing events. These events are not strategy dependent, and prior studies have shown that RNA off-target adenine base editing *in vivo* is rare and typically indistinguishable from background A-to-I conversion, likely due to the low copy-number of ABE-expressing transgenes [30, 69]. Nevertheless, we investigated RNA off-target editing in Δ7SMA mESCs and differentiated neural lineages including motor neurons (MND) and caudal-neural (CND) differentiated cells, which stably produce ABE8e from low gene copy numbers similar to those resulting from AAV9 transduction [38]. Consistent with previous reports [69, 82], whole transcriptome sequencing did not detect accumulation of RNA A-to-I edits over background levels of endogenous A-to-I and A-to-G changes (see Fig. 15).

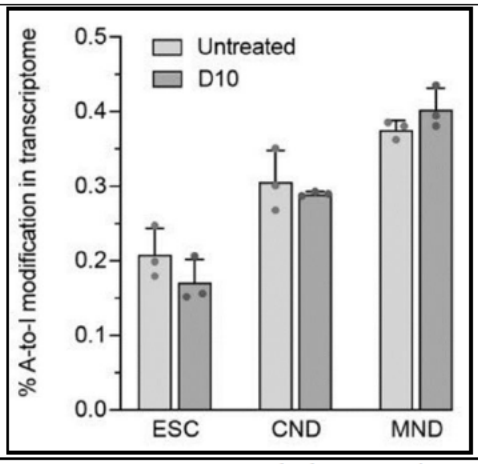

Figure 15. RNA off-target analysis in mouse cells. Whole transcriptome A-to-I RNA off-target editing analysis in Δ7SMAmESCs, differentiated CND, and differentiated MND stably expressing the D10 strategy.

Together, these data suggest (1) that CIRCLE-seq followed by cell culture validation of putative CRISPR off-targets reliably identifies prominent off-target loci, (2) cell-based determination of off-target editing is generally reflective of off-target frequencies observed *in vivo* and (3) we do not anticipate off-target editing by dualAAV9/SMABE to be of clinical or physiological significance in either patients or mice. Continued pre-clinical assessment and minimization of off-target editing is important to ensure the safety of a potential base editing therapeutic for the treatment of SMA in patients. Deaminase variants that further minimize potential off-target events have been reported [48, 75], and ongoing studies may also evaluate the efficacy and safety of these variants to improve the safety of our ABE strategy.

Planned pharmacology studies to assess off-target events.

Although base editing rarely induces off-target changes in the genome [36], prolonged and constitutive expression of base editors may increase the incidence of off-target editing events. In our preliminary studies, we did not observe any increase in off-target editing over the course of 14 weeks. Nevertheless, continued pre-clinical assessment of off-target editing is important to ensure the safety of a potential base editing and thus, tissues from aged mice will be examined for off-target and off-target events using for our optimized dose and route of administration of the dualAAV9/SMABE editor. Neonates will be injected by ICV or RO injection and aged up to 18 months of age. At various time points (see Fig. 16), serum samples to measure markers of toxicity (e.g., ALT, AST, and alkaline phosphatase) and tissues samples (e.g., cortex, spinal cord, heart, liver and DRG (dorsal rout ganglion)) will be collected, which will allow to determine both on-target and off-target gDNA and RNA editing in aged mice, respectively. Moreover, tissue samples such as brain, spinal cord, liver and DRGs of 12-month-old mice will be subject to histological analysis to further investigate potential *in vivo* toxicity defects.

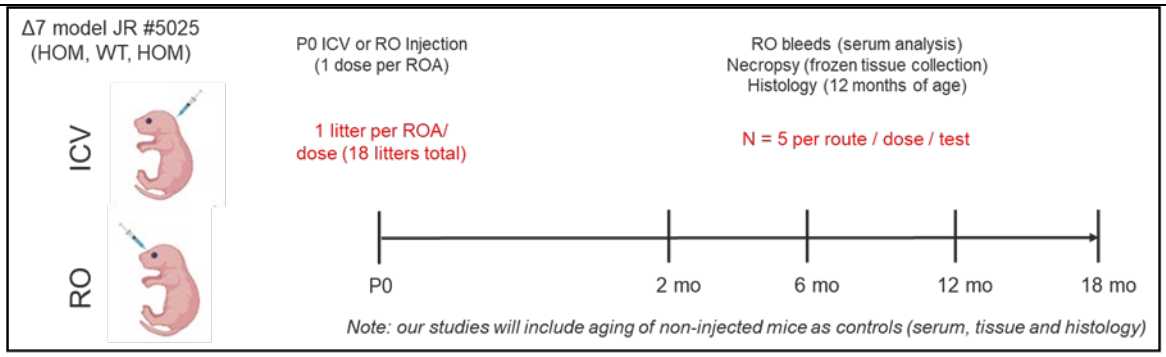

Figure 16. Biodistribution and toxicity analysis of ABE editing. At birth, mice will be injected either via ICV or RO injection. Tissue samples at different ages will be analyzed for gDNA and RNA editing (on-target and off-target events) and histological analysis will be performed on tissue samples from 12 months old mice.

1.5.2.4. Non-human primate GLP Toxicology and Biodistribution Studies

Biodistribution Studies

Our position is that the biodistribution of AAV9 following intrathecal administration is generally well characterized and known from existing literature. A recent review summarized key publications describing the biodistribution of AAV9 after intrathecal delivery in small and large animals [83]. Considering that the biodistribution of AAV9 vectors is well-established and translates reasonably well from rodents to primates, the only value of additional biodistribution studies for dualAAV9/SMABE would be to assess the possible impact of the transgene expression on the expected biodistribution pattern, which we view as a negligible concern. A limited biodistribution analysis will be incorporated into the GLP toxicology studies, to confirm that the vector biodistribution matches the expected pattern for AAV9 after intrathecal administration.

Toxicology Studies

Our overall strategy to assess preclinical toxicology of dualAAV9/SMABE is that the general toxicology profiles of AAV9 are reasonably well understood. Our major concerns are the possibility of off-target base editing, and the possibility of an immune response against the expressed base editors. The off-target base editing is best assessed against the background of a human genome, using cultured human cells. In vitro studies of limited duration may not fully capture the long-term risk of off-target effects, so to better understand that general issue we are relying on a longitudinal mouse study (see section 1.5.2.3) to assess whether the rate of off-targeted editing increases over time *in vivo*. To model the potential impacts of an immune response against the base editors, we are relying on a non-human primate (NHP) study conducted with the same immune management strategy that we are proposing in humans. A secondary benefit of the NHP study will be to assess whether appropriate biodistribution of the dual vectors is achieved and assess whether any known AAV9-related complications arise, such as DRG toxicity.

Non-human primate GLP toxicology and biodistribution study

As introduced above, the primary intent to this NHP study is to evaluate the biodistribution of dualAAV9/SMABE and evaluate potential immune responses against the ABE, in the context of a species and dosing paradigm that model our proposed human trial as closely as possible. Since degeneration of dorsal root ganglia in a known possible risk associated with intrathecal AAV9 administration, nerve conduction studies will be carried out to address the possible physiological consequences of this specific toxicity.

Male and female NHPs will be randomized into cohorts, with 2-4 NHPs per cohort, and dosed by a qualified laboratory technician. The animals will receive a single lumbar IT injection at doses of vehicle, $1x10^{14}$, or $2x10^{14}$ vg per NHP (total doses indicated; each dose will consist of half SMABE-N and half SMABE-C). The dosing cohorts and the rational for each are provided in the table below. A 1-mL sample of CSF will be withdrawn from each animal immediately prior to vector injection, to verify needle placement and avoid elevating intracranial pressure, and then maintained in a 15° head down Trendelenburg position for 30 minutes following the injection. All animals will receive IV methylprednisolone starting day 1 until the study termination (10 mg/kg/day on day 1 and 1 mg/kg/day thereafter), tacrolimus starting day 1 until the study termination (0.1 mg/kg, twice daily), and rapamycin starting 7 days prior to injection until the termination of the study (0.01 mg/kg, twice daily), to mimic the immunosuppression planned for human subjects. NHPs will be maintained until 90 days post-injection.

Table 6. Non-Human Primate Proposed GLP and Biodistribution Study

Treatment	Numbers of NHPs	Day 91 Necropsy	Rationale
Vehicle Control	1M/1F	1M/1F	Vehicle control group for baseline comparison
$1x10^{14}$ vg / NHP	1M/1F	2M/2F	Equivalent to the proposed human high dose group
$2x10^{14}$ vg / NHP	1M/1F	2M/2F	2x safety margin above the proposed human high dose group

Peripheral Blood Mononuclear Cells (PBMCs) will be collected at baseline, day 28, and termination to assess immune responses to AAV9 and ABE using an interferon gamma ELISpot. Peripheral nerve conduction studies will be carried out at baseline, day 29, and prior to necropsy. Histopathology will be conducted in key target tissues and where biodistribution will be highest: brain (motor cortex, cerebellum, brainstem), spinal cord (cervical, thoracic, lumbar), dorsal root ganglia (cervical thoracic, lumbar), bicep muscle, liver, and heart. Vector genome biodistribution and transgene RNA expression will be assessed for SMABE-N and SMABE-C in key tissues, to assess if the expected biodistribution pattern for AAV9 is achieved and that both halves of the transgene are expressed: brain (motor cortex, cerebellum, brainstem), spinal cord (cervical, thoracic, lumbar), dorsal root ganglia (cervical thoracic, lumbar), bicep muscle, liver, and heart. In situ hybridization (RNAscope) will be conducted against the C-terminal and N-terminal halves of the transgene in the brain motor cortex and spinal cord (cervical, thoracic, and lumbar) to assess the degree of dual expression (both halves) in motor neurons, which is the key target cell.

1.5.3. Clinical Program

Overview

This will be an open-label Phase I/II study to evaluate the safety of dualAAV9/SMABE, with secondary outcomes incorporated to explore potential benefits of the treatment beyond that of the existing standard of care. Our target population is patients with Type I, II or III Spinal Muscular Atrophy, who are currently taking nusinersen or risdiplam according to normal standard of care. Enrolled patients would receive a single intrathecal injection of dualAAV9/SMABE. Patients would also receive an immunosuppressive regimen to manage acute immune responses against the drug product, as well as to manage longer-term possible immune responses against the ABE.

Special considerations for this patient population

Zolgensma is an FDA-approved gene therapy product available to Type I SMA patients, which utilizes an AAV9 vector. Any patients that have received Zolgensma would be ineligible to receive dualAAV9/SMABE. Patients that receive dualAAV9/SMABE would likely be ineligible to receive any future AAV-based treatment, due to the development of persisting anti-AAV antibodies.

Immunological considerations, monitoring, and management

It is anticipated that a relatively high dose of dualAAV9/SMABE will be needed. In previous gene therapy studies, antigen specific T-cell responses to the AAV9 vector have been reported. This is an expected response between 2- and 12-weeks following gene transfer, even when administered IT. One possible consequence to such antigen specific T- cell responses is clearance of the transduced cells and loss of transgene expression.

Since all SMA patients express some full-length SMN, they are expected to be immunotolerant to the increased expression of SMN following editing vial dualAAV9/SMABE. However, the ABE itself will be a non-self protein that is potentially permanently expressed long-term, so a strategy will be in place to monitor for anti-ABE immune responses and to mitigate any potential adverse effects through an immunosuppressive regimen.

Potential neuroinflammation and expansion of T-cells will be monitored by collecting and examining CSF at the times of nusinersen administration, to measure cell counts and cytokine levels. This should occur every 4 months throughout the course of the trial. Reactive T-cells against AAV9 and ABE will be assessed by interferon-gamma ELISpot on peripheral blood monocytes at day 0, day 21, day 90, day 180, day 360, day 720, and at the 2-year follow-up.

To reduce the risk of the host immune response to the AAV9-based dualAAV9/SMABE, an immunosuppression regimen has been designed based the clinical trial of AAV9 gene transfer to CSF for giant axonal neuropathy [84]. IV methylprednisolone (10 mg/kg) will be administered the day of drug product administration. Prednisone/prednisolone will be maintained at 1 mg/kg/day x 3 month, then tapered gradually from months 3-5. Tacrolimus will be administered starting at the day of drug product administration at 0.1 mg/kg/day divided into twice daily dosing (goal level: 4-8 ng/mL); tacrolimus will be continued for 6 months and will be tapered from month 6-8. Sirolimus (goal level 4-8 ng/mL) will be administered starting 1 week prior to

dualAAV9/SMABE administration, continued until month 15, gradually tapered off from months 15-17.

Synopsis of the proposed Phase I/II Clinical Trial

Title	Phase I/II gene transfer clinical trial of dualAAV9/SMABE for treatment of subjects with Type I, Type II, or Type III Spinal Muscular Atrophy.
Number of Subjects	N = 3 low dose and 3-6 high dose (6-9 total)
Clinical Study Phase	Phase I/II trial
Number of Centers	TBD
Study Objectives	Primary outcome is safety Secondary outcome is initial efficacy, compared to standard-of-care treatment with nusinersen
Study Design	Open-label, single dose, dose-escalation clinical trial of dualAAV9/SMABE injected intrathecally.
Inclusion Criteria	• Birth – 12 years old • Confirmed diagnosis of Type I, II, or III SMA disease • Parent/legal guardian willing to accompany the participant to all study visits and who will provide permission for their child's participation. • Prescribed and taking nusinersen or risdiplam according to normal standard of care.
Exclusion Criteria	• Having been dosed with Zolgensma, or any other AAV-based approved or experimental treatment. • Need for ventilator support for more than 12 hours daily, signifying advanced disease • Inability to participate in the clinical evaluation • Presence of a concomitant medical condition that precludes lumbar puncture • Inability to be safely sedated in the opinion of the clinical anesthesiologist. • Active viral infection based on clinical observations, documented fever within the last 2 weeks, or confirmed positive viral testing within the last 2 weeks. • Concomitant illness or requirement for chronic drug treatment that in the opinion of the PI creates unnecessary risks for gene transfer • Serology consistent with exposure to HIV, or testing (PCR + serology) consistent with active hepatitis A, B or C infection • Any item which would exclude the individual from being able to undergo MRI according to local institutional policy • Any other situation that would exclude the individual from undergoing any other procedure required in this study • Individuals with cardiomyopathy or significant congenital heart abnormalities, clinically significant abnormal laboratory values based upon local institutional normal values, including, but not limited to: - White blood cell count < 3000/µL, hemoglobin < 11g/dL, or thrombocytopenia <

	80,000/µL. - Abnormal liver function (aspartate aminotransferase (AST) or alanine aminotransferase (ALT) or bilirubin greater than 3 times the upper limit of normal). • The presence of significant non-SMA related CNS impairment or behavioral disturbances that would confound the scientific rigor or interpretation of results of the study • Have received an investigational drug within 30 days prior to screening or plan to receive an investigational drug (other than gene therapy) during the study.
Study Procedures	Proposed Human Therapy: In Cohort 1 and 2, Type I SMA subjects will have a spinal needle inserted percutaneously at the lumbar level into the IT space of the spinal column. A volume of CSF approximately equal to the infusion volume is withdrawn from lumbar thecal sac. The vector solution is then infused at a rate of 1 mL per minute. Subject will remain 15-degree Trendelenburg (head down) for 1 hour following vector administration. Overall dose and dosing volume will be determined based on the results of the preclinical pharmacology and toxicology studies. An immunosuppression protocol will be incorporated following the management of CRIM (-) subjects as described for a gene therapy clinical trial for Giant Axonal Neuropathy [84]. Briefly, subjects will receive 4 months of prednisone followed by a taper. Subjects will also receive tacrolimus for 6 months followed by a taper, and sirolimus for 18 months followed by a taper. The procedure will be performed with an anesthesiologist or qualified physician present to administer sedation as needed. Subjects will stay in Pediatric ICU overnight. Physiologic monitoring in accord with the standards set by the American Society of Anesthesiologists will be utilized for all subjects while they are receiving analgesia/anesthesia and until they have fully recovered from its effects. Vital signs (BP, HR, RR, T, O2 saturation, telemetry) will be checked hourly while hospitalized after anesthesia.
Primary Outcome	Determination of safety based on the development of unacceptable toxicity. Unacceptable toxicity at any dose level will be defined as the occurrence of two or more unanticipated Grade III or higher treatment-related toxicities.
Secondary Outcomes	• Change in nerve conduction velocities on electromyography and nerve conduction studies (also to assess safety related to dorsal root ganglia toxicity). • Free from permanent ventilator support • For infants / early onset SMA patients up to the age of 2: - The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) - WHO milestone acquisition (e.g. sitting independently) • For older / later onset SMA patients over the age of 2: - Hammersmith functional motor scale expanded (HFMSE) - revised upper limb module (RULM)

Study Duration	Initial screening and evaluation will be performed up to 1-4 weeks before the administration of dualAAV9/SMABE. We will evaluate short-term safety over a two-year period. Individuals will be tested at baseline (-28 to -1 days) and return for follow up visits on days 7, 14, 30, 60, 90, 180, 270, 360, 540 and 720 for active monitoring. After the 24-month visit, they will be followed according to an annual monitoring plan.
Sample Size	This is a dose escalation study in six (6) – nine (9) subjects consisting of two cohorts. Cohort 1 will receive an IT infusion; Cohort 2 will receive escalating IT dose. Final doses are to be determined, based on the results of the planned pharmacology and toxicology studies. Dose escalation will only occur after review of safety information with the Data Safety Monitoring Board.
Statistical Analysis	This is a Phase I/II trial, with safety as the primary measure. Dose escalation will occur based on evaluation of dose-limiting toxicity as typically applied in a 2x3 design. Secondary outcomes include potential efficacy measures. Data of post-gene transfer monitoring will be compared with baseline data and analysed by paired t-tests with a significance level of $p = 0.05$.
Long-term follow-up	Safety follow-up will continue over a two-year period that incorporates the active phase of the protocol. Individuals will then transfer to an annual monitoring program where data will be collected from annual standard care visits for up to 5 additional years.

1.6. Participants

Name	Title	Affiliation
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

1.7. Suggested Meeting Dates

June 12, 2024
July 3, 2024
July 5, 2024

2. REFERENCES

1. Lefebvre, S., et al., *Identification and characterization of a spinal muscular atrophy-determining gene.* Cell, 1995. **80**(1): p. 155-165.
2. Roberts, D.F., J. Chavez, and S.D.M. Court, *The Genetic Component in Child Mortality.* Archives of Disease in Childhood, 1970. **45**(239): p. 33-38.
3. Sugarman, E.A., et al., *Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72 400 specimens.* European Journal of Human Genetics, 2012. **20**(1): p. 27-32.
4. Boda, B., et al., *Survival motor neuron SMN1 and SMN2 gene promoters: identical sequences and differential expression in neurons and non-neuronal cells.* European Journal of Human Genetics, 2004. **12**(9): p. 729-737.
5. Rochette, C., N. Gilbert, and L. Simard, *SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to Homo sapiens.* Human Genetics, 2001. **108**(3): p. 255-266.
6. Lorson, C.L., et al., *A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy.* Proceedings of the National Academy of Sciences, 1999. **96**(11): p. 6307-6311.
7. Monani, U.R., *A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2.* Human Molecular Genetics, 1999. **8**(7): p. 1177-1183.
8. Burnett, B.G., et al., *Regulation of SMN Protein Stability.* Molecular and Cellular Biology, 2009. **29**(5): p. 1107-1115.
9. Cho, S. and G. Dreyfuss, *A degron created by SMN2 exon 7 skipping is a principal contributor to spinal muscular atrophy severity.* Genes & Development, 2010. **24**(5): p. 438-442.
10. Cobben, J.M., et al., *Survival in SMA type I: A prospective analysis of 34 consecutive cases.* Neuromuscular Disorders, 2008. **18**(7): p. 541-544.
11. Vitte, J., et al., *Refined Characterization of the Expression and Stability of the SMN Gene Products.* The American Journal of Pathology, 2007. **171**(4): p. 1269-1280.
12. Hoy, S.M., *Onasemnogene Abeparvovec: First Global Approval.* Drugs, 2019. **79**(11): p. 1255-1262.
13. Kernochan, L.E., et al., *The role of histone acetylation in SMN gene expression.* Human Molecular Genetics, 2005. **14**(9): p. 1171-1182.
14. Kolb, S.J., et al., *Natural history of infantile-onset spinal muscular atrophy.* Annals of Neurology, 2017. **82**(6): p. 883-891.
15. Mendell, J.R., et al., *Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy.* New England Journal of Medicine, 2017. **377**(18): p. 1713-1722.
16. Ottesen, E.W., *ISS-N1 makes the first FDA-approved drug for spinal muscular atrophy.* Translational Neuroscience, 2017. **8**(1): p. 1-6.
17. d'Ydewalle, C., et al., *The Antisense Transcript SMN-AS1 Regulates SMN Expression and Is a Novel Therapeutic Target for Spinal Muscular Atrophy.* Neuron, 2017. **93**(1): p. 66-79.

18. Woo, C.J., et al., *Gene activation of SMN by selective disruption of lncRNA-mediated recruitment of PRC2 for the treatment of spinal muscular atrophy.* Proceedings of the National Academy of Sciences, 2017. **114**(8).
19. Blauw, H.M., et al., *SMN1 gene duplications are associated with sporadic ALS.* Neurology, 2012. **78**(11): p. 776-780.
20. Chaytow, H., et al., *Spinal muscular atrophy: From approved therapies to future therapeutic targets for personalized medicine.* Cell Reports Medicine, 2021. **2**(7): p. 100346.
21. Cucchiarini, M., H. Madry, and E.F. Terwilliger, *Enhanced expression of the central survival of motor neuron (SMN) protein during the pathogenesis of osteoarthritis.* Journal of Cellular and Molecular Medicine, 2014. **18**(1): p. 115-124.
22. Van Alstyne, M., et al., *Gain of toxic function by long-term AAV9-mediated SMN overexpression in the sensorimotor circuit.* Nature Neuroscience, 2021. **24**(7): p. 930-940.
23. Baranello, G., et al., *Risdiplam in Type 1 Spinal Muscular Atrophy.* New England Journal of Medicine, 2021. **384**(10): p. 915-923.
24. Chiriboga, C.A., et al., *Results from a phase 1 study of nusinersen (ISIS-SMN $_{Rx}$) in children with spinal muscular atrophy.* Neurology, 2016. **86**(10): p. 890-897.
25. Lefebvre, S., et al., *Correlation between severity and SMN protein level in spinal muscular atrophy.* Nature Genetics, 1997. **16**(3): p. 265-269.
26. Coovert, D., *The survival motor neuron protein in spinal muscular atrophy.* Human Molecular Genetics, 1997. **6**(8): p. 1205-1214.
27. Kariya, S., et al., *Requirement of enhanced Survival Motoneuron protein imposed during neuromuscular junction maturation.* Journal of Clinical Investigation, 2014. **124**(2): p. 785-800.
28. Patrizi, A.L., et al., *SMN protein analysis in fibroblast, amniocyte and CVS cultures from spinal muscular atrophy patients and its relevance for diagnosis.* European Journal of Human Genetics, 1999. **7**(3): p. 301-309.
29. Ramos, D.M., et al., *Age-dependent SMN expression in disease-relevant tissue and implications for SMA treatment.* Journal of Clinical Investigation, 2019. **129**(11): p. 4817-4831.
30. Armbruster, N., et al., *Efficacy and biodistribution analysis of intracerebroventricular administration of an optimized scAAV9-SMN1 vector in a mouse model of spinal muscular atrophy.* Molecular Therapy - Methods & Clinical Development, 2016. **3**: p. 16060.
31. Bevan, A.K., et al., *Systemic Gene Delivery in Large Species for Targeting Spinal Cord, Brain, and Peripheral Tissues for Pediatric Disorders.* Molecular Therapy, 2011. **19**(11): p. 1971-1980.
32. Meyer, K., et al., *Improving Single Injection CSF Delivery of AAV9-mediated Gene Therapy for SMA: A Dose–response Study in Mice and Nonhuman Primates.* Molecular Therapy, 2015. **23**(3): p. 477-487.
33. Ratni, H., et al., *Discovery of Risdiplam, a Selective Survival of Motor Neuron-2 (SMN2) Gene Splicing Modifier for the Treatment of Spinal Muscular Atrophy (SMA).* Journal of Medicinal Chemistry, 2018. **61**(15): p. 6501-6517.
34. Wurster, C.D. and A.C. Ludolph, *Nusinersen for spinal muscular atrophy.* Therapeutic Advances in Neurological Disorders, 2018. **11**: p. 175628561875445.

35. Zettler, J., V. Schütz, and H.D. Mootz, *The naturally split Npu DnaE intein exhibits an extraordinarily high rate in the protein trans-splicing reaction.* FEBS Lett, 2009. **583**(5): p. 909-14.
36. Thomsen, G., et al., *Biodistribution of onasemnogene abeparvovec DNA, mRNA and SMN protein in human tissue.* Nature Medicine, 2021. **27**(10): p. 1701-1711.
37. Groen, E.J.N., et al., *Temporal and tissue-specific variability of SMN protein levels in mouse models of spinal muscular atrophy.* Human Molecular Genetics, 2018. **27**(16): p. 2851-2862.
38. Arbab, M., et al., *Base editing rescue of spinal muscular atrophy in cells and in mice.* Science, 2023. **380**(6642): p. eadg6518.
39. Miller, S.M., et al., *Continuous evolution of SpCas9 variants compatible with non-G PAMs.* Nature Biotechnology, 2020. **38**(4): p. 471-481.
40. Cartegni, L., et al., *Determinants of Exon 7 Splicing in the Spinal Muscular Atrophy Genes, SMN1 and SMN2.* The American Journal of Human Genetics, 2006. **78**(1): p. 63-77.
41. Singh, N.N., E.J. Androphy, and R.N. Singh, *An extended inhibitory context causes skipping of exon 7 of SMN2 in spinal muscular atrophy.* Biochemical and Biophysical Research Communications, 2004. **315**(2): p. 381-388.
42. Wolstencroft, E.C., et al., *A non-sequence-specific requirement for SMN protein activity: the role of aminoglycosides in inducing elevated SMN protein levels.* Hum Mol Genet, 2005. **14**(9): p. 1199-210.
43. Hua, Y., et al., *Antisense Masking of an hnRNP A1/A2 Intronic Splicing Silencer Corrects SMN2 Splicing in Transgenic Mice.* The American Journal of Human Genetics, 2008. **82**(4): p. 834-848.
44. Singh, N.K., et al., *Splicing of a Critical Exon of Human Survival Motor Neuron Is Regulated by a Unique Silencer Element Located in the Last Intron.* Molecular and Cellular Biology, 2006. **26**(4): p. 1333-1346.
45. Briese, M., et al., *Deletion of smn-1, the Caenorhabditis elegans ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan.* Human Molecular Genetics, 2008. **18**(1): p. 97-104.
46. Monani, U.R., *Spinal Muscular Atrophy: A Deficiency in a Ubiquitous Protein; a Motor Neuron-Specific Disease.* Neuron, 2005. **48**(6): p. 885-895.
47. Schrank, B., et al., *Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos.* Proceedings of the National Academy of Sciences, 1997. **94**(18): p. 9920-9925.
48. Singh, R.N., et al., *Diverse role of survival motor neuron protein.* Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 2017. **1860**(3): p. 299-315.
49. Rodriguez-Muela, N., et al., *Single-Cell Analysis of SMN Reveals Its Broader Role in Neuromuscular Disease.* Cell Reports, 2017. **18**(6): p. 1484-1498.
50. Foust, K.D., et al., *Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes.* Nature Biotechnology, 2009. **27**(1): p. 59-65.
51. Hammond, S.L., et al., *Cellular selectivity of AAV serotypes for gene delivery in neurons and astrocytes by neonatal intracerebroventricular injection.* PLOS ONE, 2017. **12**(12): p. e0188830.

52. Mathiesen, S.N., et al., *CNS Transduction Benefits of AAV-PHP.eB over AAV9 Are Dependent on Administration Route and Mouse Strain.* Molecular Therapy - Methods & Clinical Development, 2020. **19**: p. 447-458.
53. Arnold, W., et al., *The neuromuscular impact of symptomatic SMN restoration in a mouse model of spinal muscular atrophy.* Neurobiology of Disease, 2016. **87**: p. 116-123.
54. Levy, J.M., et al., *Cytosine and adenine base editing of the brain, liver, retina, heart and skeletal muscle of mice via adeno-associated viruses.* Nature Biomedical Engineering, 2020. **4**(1): p. 97-110.
55. Swiech, L., et al., *In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9.* Nature Biotechnology, 2015. **33**(1): p. 102-106.
56. Cartegni, L. and A.R. Krainer, *Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1.* Nature Genetics, 2002. **30**(4): p. 377-384.
57. David Arnold, W., et al., *Electrophysiological biomarkers in spinal muscular atrophy: proof of concept.* Annals of Clinical and Translational Neurology, 2014. **1**(1): p. 34-44.
58. Dangouloff, T. and L. Servais, *Clinical Evidence Supporting Early Treatment Of Patients With Spinal Muscular Atrophy: Current Perspectives.* Therapeutics and Clinical Risk Management, 2019. **Volume 15**: p. 1153-1161.
59. De Vivo, D.C., et al., *Nusinersen initiated in infants during the presymptomatic stage of spinal muscular atrophy: Interim efficacy and safety results from the Phase 2 NURTURE study.* Neuromuscular Disorders, 2019. **29**(11): p. 842-856.
60. Strauss, K.A., et al., *Onasemnogene abeparvovec for presymptomatic infants with two copies of SMN2 at risk for spinal muscular atrophy type 1: the Phase III SPR1NT trial.* Nature Medicine, 2022. **28**(7): p. 1381-1389.
61. Tscherter, A., et al., *Evaluation of real-life outcome data of patients with spinal muscular atrophy treated with nusinersen in Switzerland.* Neuromuscular Disorders, 2022. **32**(5): p. 399-409.
62. Le, T.T., et al., *Temporal requirement for high SMN expression in SMA mice.* Human Molecular Genetics, 2011. **20**(18): p. 3578-3591.
63. Finkel, R.S., et al., *One-Time Intrathecal (IT) Administration of AVXS-101 IT Gene-Replacement Therapy for Spinal Muscular Atrophy: Phase 1 Study (STRONG) (2493).* Neurology, 2020. **94**(15_supplement): p. 2493.
64. Lutz, C.M., et al., *Postsymptomatic restoration of SMN rescues the disease phenotype in a mouse model of severe spinal muscular atrophy.* Journal of Clinical Investigation, 2011. **121**(8): p. 3029-3041.
65. Mercuri, E., et al., *Nusinersen versus Sham Control in Later-Onset Spinal Muscular Atrophy.* New England Journal of Medicine, 2018. **378**(7): p. 625-635.
66. Robbins, K.L., et al., *Defining the therapeutic window in a severe animal model of spinal muscular atrophy.* Human Molecular Genetics, 2014. **23**(17): p. 4559-4568.
67. Hauck, B., et al., *Intracellular Viral Processing, Not Single-Stranded DNA Accumulation, Is Crucial for Recombinant Adeno-Associated Virus Transduction.* Journal of Virology, 2004. **78**(24): p. 13678-13686.
68. Wang, Z., et al., *Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors in vitro and in vivo.* Gene Therapy, 2003. **10**(26): p. 2105-2111.

69. Davis, J.R., et al., *Efficient in vivo base editing via single adeno-associated viruses with size-optimized genomes encoding compact adenine base editors.* Nature Biomedical Engineering, 2022. **6**(11): p. 1272-1283.
70. McCarty, D., P. Monahan, and R. Samulski, *Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis.* Gene Therapy, 2001. **8**(16): p. 1248-1254.
71. Passini, M.A., et al., *Antisense Oligonucleotides Delivered to the Mouse CNS Ameliorate Symptoms of Severe Spinal Muscular Atrophy.* Science Translational Medicine, 2011. **3**(72).
72. Li, J.-J., et al., *Disruption of splicing-regulatory elements using CRISPR/Cas9 to rescue spinal muscular atrophy in human iPSCs and mice.* National Science Review, 2020. **7**(1): p. 92-101.
73. Madocsai, C., et al., *Correction of SMN2 Pre-mRNA splicing by antisense U7 small nuclear RNAs.* Molecular Therapy, 2005. **12**(6): p. 1013-1022.
74. Zhou, M., et al., *Targeted-Deletion of a Tiny Sequence via Prime Editing to Restore SMN Expression.* International Journal of Molecular Sciences, 2022. **23**(14): p. 7941.
75. Huang, T.P., et al., *Circularly permuted and PAM-modified Cas9 variants broaden the targeting scope of base editors.* Nature Biotechnology, 2019. **37**(6): p. 626-631.
76. Le, T.T., et al., *SMNΔ7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN.* Human Molecular Genetics, 2005. **14**(6): p. 845-857.
77. Tsai, S.Q., et al., *CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR–Cas9 nuclease off-targets.* Nature Methods, 2017. **14**(6): p. 607-614.
78. Zhao, Y., et al., *Sequence-specific inhibition of microRNA via CRISPR/CRISPRi system.* Scientific Reports, 2014. **4**(1): p. 3943.
79. Karlsson, M., et al., *A single–cell type transcriptomics map of human tissues.* Science Advances, 2021. **7**(31): p. eabh2169.
80. Das, A., et al., *Epigenetic Silencing of Recombinant Adeno-associated Virus Genomes by NP220 and the HUSH Complex.* Journal of Virology, 2022. **96**(4): p. e02039-21.
81. Greig, J.A., et al., *Loss of transgene expression limits liver gene therapy in primates.* 2022.
82. Rothgangl, T., et al., *In vivo adenine base editing of PCSK9 in macaques reduces LDL cholesterol levels.* Nature Biotechnology, 2021. **39**(8): p. 949-957.
83. Chen, X., et al., *Biodistribution of Adeno-Associated Virus Gene Therapy Following Cerebrospinal Fluid-Directed Administration.* Hum Gene Ther, 2023. **34**(3-4): p. 94-111.
84. Bharucha-Goebel, D.X., et al., *Intrathecal Gene Therapy for Giant Axonal Neuropathy.* N Engl J Med, 2024. **390**(12): p. 1092-1104.