Targeting the 5′ untranslated region of SMN2 as a therapeutic strategy for spinal muscular atrophy

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Spinal muscular atrophy (SMA) is a neuromuscular disorder caused by mutations in the survival motor neuron 1 (SMN1) gene. All patients have at least one copy of a paralog, SMN2, but a C-to-T transition in this gene results in exon 7 skipping in a majority of transcripts. Approved treatment for SMA involves promoting exon 7 inclusion in the SMN2 transcript or increasing the amount of full-length SMN by gene replacement with a viral vector. Increasing the pool of SMN2 transcripts and increasing their translational efficiency can be used to enhance splicing correction. We sought to determine whether the 5′ untranslated region (5′ UTR) of SMN2 contains a repressive feature that can be targeted to increase SMN levels. We found that antisense oligonucleotides (ASOs) complementary to the 5′ UTR of SMN2 increase SMN mRNA and protein levels and that this effect is due to inhibition of SMN2 mRNA decay. Moreover, use of the 5′ UTR ASO in combination with a splice-switching oligonucleotide (SSO) increases SMN levels above those attained with the SSO alone. Our results add to the current understanding of SMN regulation and point toward a new therapeutic target for SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by loss-of-function mutations in the survival motor neuron 1 (SMN1) gene.1 Although SMN1 is a ubiquitously expressed gene, SMA is primarily a disease of lower motor neurons. Denervation results in symmetrical muscle weakness, often within weeks or months of birth.2 SMN1 encodes the SMN protein, which has a well-characterized function in small nuclear ribonucleoprotein (snRNP) assembly.3,4 Other cellular processes where SMN is likely involved include axonal mRNA transport and local translation and endocytosis, which may account for the motor neuron vulnerability in SMA.5–8

A complete absence of SMN protein results in embryonic lethality.9 All SMA patients have at least one copy 10,11 of an SMN1 gene paralog, SMN2, which arose from a duplication of the SMN locus on chromosome 5.1 SMN2 does not fully compensate for the loss of SMN1; due to a C-to-T transition that results in exon 7 skipping in a majority of transcripts, only 10 percent to 20 percent of SMN2 mRNAs encode the fully functional SMN protein.12–14 Clinically, disease severity therefore correlates with SMN2 copy number and full-length SMN2 transcript level.15,16

Increasing the level of SMN, via targeting SMN2 or via gene therapy,17 has been a primary therapeutic strategy for SMA. Nusinersen is an antisense oligonucleotide (ASO) that increases the proportion of SMN2 transcripts containing exon 7.18–20 Another SMN splice modifier, risdiplam, is a small molecule that has the advantage of being orally bioavailable.21,22 Targeting splicing as a means of increasing SMN levels has a ceiling effect determined by the abundance of SMN2 transcripts in cells. Increasing the total pool of SMN2 transcripts and increasing the translational efficiency of these transcripts are two strategies to overcome the ceiling effect associated with the splice-switching strategy.

To identify a new target, we looked in the SMN2 5′ untranslated region (5′ UTR). It is known that regulatory motifs within 5′ UTRs influence gene expression by controlling transcript stability, translational efficiency, and subcellular localization.23,24 This occurs through
a dynamic interplay between cis-acting elements (i.e., the primary sequence and secondary structures of 5′ UTRs) and trans-acting factors (i.e., RNA-binding proteins and noncoding RNAs). We sought to determine whether the 5′ UTR of SMN2 contains a repressive feature that limits its expression, of which targeting could increase SMN levels. We identified sequences in the 5′ UTR that can be targeted with an ASO, which through binding to the 5′-most (ASO #1) end of the SMN2 transcript, increases SMN levels by stabilizing SMN2 mRNA. We found that the 5′ UTR ASO used in combination with a splice-switching oligonucleotide (SSO) augments SMN above levels achieved with an SSO alone. Our results add to the current understanding of SMN2 mRNA turnover and point toward a new therapeutic target for SMA that can be pursued as a combinatorial therapy.

RESULTS

Targeting the 5′ end of SMN2 with ASOs increases SMN protein levels

The 5′ UTR of SMN2 contains a start codon 157 nucleotides upstream of the canonical SMN translation initiation codon. Nearly one-half of all human transcripts contains upstream open reading frames (uORFs), features that may attenuate translation of the main protein coding sequence.28 Recently, it was shown that ASO binding at start codons in 5′ leader sequences can prevent translation initiation at uORFs and promote translation of primary ORFs.29 In addition, ASOs have been used to increase translation of mRNAs containing other types of 5′ UTR inhibitory elements, such as G-quadruplexes or hairpin structures.30,31

We designed a series of overlapping 2′-O-methyl (2′-MOE) ASOs in 2-nucleotide increments across the 5′ region of the SMN2 transcript, including the uORF associated with the putative start codon (Figure 1A). ASOs in which all bases contain the 2′-OMe modification operate via steric hindrance rather than RNase H-mediated RNA degradation and can therefore be used to increase gene expression. Table S1 shows the sequence of each ASO. Transfection of ASOs targeting the 5′ UTR in SMA patient-derived fibroblasts resulted in increased SMN protein levels compared to the level of SMN in untransfected patient cells or those treated with a nontargeting control (NTC) ASO (Figures 1B and 1C). Stepping 5′ to 3′ across the UTR, there is a downward trend in the ASO effect on SMN expression, indicating that the critical target region is close to the 5′ terminal cap. We decided to use ASO #1 in the experiments that follow since it demonstrated the largest biological effect, with an average 2.7-fold increase in SMN protein levels. We tested this ASO #1 in a second SMA cell line (Figure S1) to confirm that its effects are not cell-line specific. We found a similar trend using a 5′ UTR phosphorodiimide morpholino oligomer (PMO) conjugated to a cell-penetrating peptide (pPMO) in motor neuron-like cells chemically differentiated from SMA patient-derived induced pluripotent stem cells (iPSCs; Table S2; Figure S2).32,33

We also tested an ASO with the same sequence as ASO #1 but with 2′-O-(2-methoxyethyl) (2′-MOE)-modified bases. 2′-MOE-modified ASOs are known to undergo less nonspecific protein binding and are among the most widely used in clinical trials.34,35 We found that treating human SMA fibroblasts with the 5′ UTR 2′-MOE increases SMN protein levels 3.7-fold (Figures 2A and 2B). The increased efficacy observed in Figure 2 compared to Figure 1 is likely attributable to the shift from 2′-OMe to 2′-MOE chemistry.

Studies in fibroblasts derived from Taiwanese SMA mice, which are null for mouse Smn but contain a 115-kb human DNA sequence containing SMN2,36 showed no effect of 5′ UTR ASO treatment (Figures S3A and S3B). However, the 5′ UTR ASO is effective in mouse embryonic fibroblasts (MEFs) containing the PAC 215P15-derived human SMN2 transgene37,38 (Figures S3C and S3D). This could be due to differences in the background genetics of the two mouse strains.

The SMN protein is part of a large protein complex where it associates with Gemin proteins. Previous investigation demonstrated that levels of Gemin6 and Gemin8, which are core components of the SMN complex, correlate with SMN expression levels.39 After seeing the effect of the ASO on SMN levels, we sought to determine whether Gemin levels are also increased. If so, this would indicate functional correction of the SMN deficiency with ASO treatment. Indeed, by immunoblot, we found the increase in SMN to be accompanied by
1.9- and 3.4-fold increases in Gemin6 and Gemin8, respectively (Figures 2A and 2B).

The 5' UTR ASO increases SMN2 mRNA levels in fibroblasts by stabilizing transcripts

We next performed qRT-PCR to determine whether SMN2 transcript levels increase following ASO treatment. Compared to untreated cells or cells treated with a NTC ASO, the 5' UTR ASO increases total SMN mRNA levels in both SMA patient fibroblasts and carrier fibroblasts (Figures 3A and 3D). Total SMN mRNA levels were measured with primers spanning the exon 2a-2b junction and are thus irrespective of exon 7 inclusion.

Transcripts containing exon 7 are the most therapeutically relevant, as they encode the full-length SMN protein. This prompted us to check levels of the full-length and Δ7 SMN isoforms by RT-PCR and by qRT-PCR. To our surprise, the 5' UTR ASO led to a shift toward a full-length transcript (with exon 7 inclusion) in patient fibroblasts (Figures 3B and 3C). In carrier fibroblasts from an unaffected individual with higher baseline SMN levels, the ASO increases steady-state mRNA without affecting the ratio of full-length to total transcripts (Figure 3D). Thus, we suspect that the increased level of total SMN mRNA is a direct effect of the 5' UTR ASO, whereas increased exon 7 inclusion is more likely due to SMN feedback (caused by an increased pool of snRNPs) in states of SMN deficiency rather than a primary mechanism of action of the ASO.

A higher steady-state level of mRNA could either be due to an increased rate of transcription or to a decreased rate of RNA decay. To distinguish between the two possibilities, we pulsed cells with a uridine analog, 5-ethynyl uridine (EU), to measure newly transcribed SMN2. Biotinylating the EU allowed us to isolate and quantify only those RNAs transcribed during the 1-h pulse via qRT-PCR. We confirmed assay specificity by blocking transcription with actinomycin D and observing an increase in cycle threshold (Ct) values of three to nine cycles for GAPDH and SMN, indicating decreased transcript levels (Table S5). As a positive control, we used SMA carrier fibroblasts that have one copy of SMN1 and five copies of SMN2 and thus should transcribe more SMN than our patient fibroblasts, which contain two copies of SMN2 and no SMN1. With this RNA-labeling method, we saw no significant difference in nascent SMN2 transcript levels among cells treated with the 5' UTR ASO, cells treated with the NTC ASO, or untreated patient cells (Figure 4A). This indicates that the higher steady-state level of SMN2 is not due to increased transcription but is due instead to slower RNA turnover. Indeed, by treating cells with actinomycin D and collecting RNA at different time points, we found that SMN2 transcripts are significantly more stable in cells treated with the 5' UTR ASO (Figure 4B).

The SMN2 UORF is not readily translated and does not reduce SMN levels

When present, a uORF stop codon may be processed like a premature termination codon (PTC), subjecting the mRNA to nonsense-mediated decay. ASO-mediated inhibition of the uORF could thus explain
the increased SMN2 transcript stability. To better understand the mechanism of action of the 5' UTR ASO, we designed reporter constructs to study the effects of the uORF on SMN levels. In addition to a construct with enhanced green fluorescent protein (EGFP) under the control of the wild-type SMN2 5' UTR, constructs were made with the following: (1) a mutation to remove the uORF start codon, (2) a mutation that strengthens the sequence context (Kozak sequence) surrounding the uORF start codon, and (3) a frameshift mutation that extends the uORF coding sequence and places it in-frame with GFP (Figure 5A). The latter reporter was designed in order to be able to observe uORF translation initiation, since the uORF peptide is too small to detect by standard techniques.

The expression of these reporters in HEK293T cells showed that removing the upstream start codon (mutating ATG to ACG) does not increase EGFP levels (Figures 5B and 5C), suggesting that the uORF does not have a significant influence on gene expression. There is, however, a decreased EGFP signal when a guanine is present in the +4 position ("uORF optimized" reporter). We can infer from this that the nonoptimized, native uORF is not functional. Finally, the larger protein encoded by the "frame shift" reporter was only detectable at very low levels, indicating that ribosomes do not often engage the uORF (Figure S4).

To rule out a lack of uORF effect being due to an artifact of the reporter system (e.g., use of a non-endogenous transcription start site), we aligned publicly available ribosome profiling data to the SMN2 locus (Figure 5D). Sequencing reads corresponding to SMN1 and/or SMN2 cannot be accurately mapped to standard reference genomes because multi-mapping reads are discarded or randomly distributed between the paralogs. Thus, we used a custom reference genome in which only the SMN2 sequence is present. In the ribosome profiling data, the absence of ribosome-protected fragments mapping to the SMN2 uORF was in line with our reporter assay findings and supports the
conclusion that the uORF in SMN2 is not a meaningful regulator of SMN2 expression in fibroblasts or in HEK293T cells.

A combinatorial therapeutic approach further increases levels of the SMN protein

Novel strategies to complement splice modulation of SMN2 may be especially useful for those with SMA who have low SMN2 copy numbers. The SSO and the 5' UTR ASO were designed to target distinct RNA processes, leading us to investigate whether a combination of these two ASOs overcomes the ceiling effect associated with the SSO. We tested the 5' UTR ASO and a SSO that targets the ISSN1 sequence in SMN2 separately and jointly. We found that concurrent use of the two ASOs in SMA patient fibroblasts increases SMN protein levels significantly more than use of the SSO alone (Figures 6A and 6B). We speculate that the combined treatment is not significantly different from the 5' UTR ASO alone because of the shift toward the full-length transcript seen with 5' UTR ASO treatment (depicted in Figure 3C).

DISCUSSION

Previously, histone deacetylase (HDAC) inhibitors were shown to increase SMN2 levels.45–47 HDAC inhibitors are not specific to the SMN2 gene, however, and transcriptionally activate a broad array of genes. Some of these nonspecific changes in gene expression may benefit the SMA phenotype, but other changes may be harmful.48,49 Thus, a method for increasing SMN production in a more specific way that complements the splice-switching approach is therapeutically desirable. Here, we show that an ASO, in three different chemistries, with sequence complementary to the 5' UTR of SMN2, increases SMN mRNA and protein levels in human fibroblasts and motor neuron-like cells. Based on our experiments in MEFs, future preclinical studies should use the Burghes and colleagues'50 SMNdelta7 SMA mouse model.

In addition to the SMN protein, we show that levels of at least two SMN-associated proteins (Gemin6 and Gemin8) increase with ASO treatment. This is likely because as SMN levels increase, there are more SMN complexes to which Gemin6 and Gemin8 can bind, and this confers stability. Details about the stoichiometry of proteins in the SMN complex are unknown. The 5' UTR ASO may thus be useful in future experiments to study how proteins such as Gemin6 and Gemin8 are incorporated into the SMN complex, as well as in studying other pathways in which the SMN protein is involved more generally.

Our initial hypothesis was that by blocking translation of the uORF, the 5' UTR ASO promotes translation of the primary ORF. However, with multiple techniques, we found that the SMN2 uORF is not normally translated. This may be due to the short distance between the 5' cap and the uORF start codon (7 nucleotides) or to the weak sequence context surrounding the start codon (T at the +4 position).

Instead, the 5' UTR ASO stabilizes SMN2 mRNA. Although it has been established that the SMN protein is degraded by the E3 ubiquitin ligase mind bomb 1 and the proteasome, the process through which SMN transcripts are degraded is less clear.51 A high-throughput screen identified a quinazoline compound that inhibits the mRNA decapping enzyme DcpS and increases SMN2 promoter activity in cell-based assays.52 Follow-up studies found that this small molecule increases survival and motor function in SMA mice.53–56 Whereas it is known that DcpS hydrolyzes cap structures from mRNA fragments that are generated by extensive 3' to 5' exonuclease decay, the specific mechanism through which the quinazoline compound increases SMN2 expression is unknown.
The idea that the 5′ UTR ASO operates via a mechanism related to decapping is compelling since the ASO is complementary to the SMN2 sequence immediately adjacent to the 5′ cap. However, we did not find a significant difference in the ASO’s ability to upregulate SMN levels in cells in which decapping factors were knocked down (Figure S5). We cannot rule out this mechanism of action entirely since it is possible that with the knockdown of individual enzymes, there is compensation by other RNA decay machinery.

We found that with the increase in SMN mRNA levels, there is a trend toward an increase in the ratio of full-length to exon 7-excluded transcripts in patient fibroblasts. Other compounds that increase levels of SMN have been shown to increase exon 7 inclusion, including an HDAC inhibitor and an ASO that knocks down the antisense strand of SMN (called SMN-AS1). Unlike these two compounds, the 5′ UTR ASO works by a transcription-independent mechanism, and we propose that the shift in splicing is due to SMN autoregulation. The SMN complex is required for spliceosome biogenesis, and it is possible that the transcriptomic changes accompanying an increase in SMN protein levels include modulation of its own alternative splicing. This hypothesis is supported by our observation that there is no change in exon 7 inclusion in carrier fibroblasts treated with the 5′ UTR ASO, where baseline SMN levels (and spliceosome levels) are not low enough to perturb splicing.

Two papers have been published describing the antisense transcript, SMN-AS1. The long noncoding RNA SMN-AS1 is transcribed from SMN intron 1 but binds directly to the SMN transcription start site region. Here, it recruits PRC2 and reduces transcription of SMN2. Due to the proximity in binding locales, we wondered whether the 5′ UTR ASO reduces SMN-AS1 activity. However, we found no difference in the transcription rate of SMN2 with ASO treatment, indicating that the mechanism of action is likely independent of SMN-AS1.

Experiments are ongoing to elucidate the mechanism of action of the 5′ UTR ASO. We cannot yet rule out the possibility that the increase in SMN levels is due to off-target engagement, such as electrostatic interactions or sequence-specific interactions between the ASO and proteins. The determination of the mechanism of action of the 5′
UTR ASO may reveal that there are other genes for which expression can be increased using a similar strategy. For example, the upregulation of utrophin via its 5′ UTR may be used for the treatment of Duchenne muscular dystrophy.60 For now, our results add to the current understanding of SMN regulation and point toward a new therapeutic target for SMA.

MATERIALS AND METHODS

ASO synthesis

Three types of ASO were used in this study: (1) fully modified with 2′-OMe bases and phosphorothioate linkages; (2) fully modified with 2′-MOE bases and phosphorothioate linkages; and (3) PMOs. All 2′-OMe and 2′-MOE ASOs were purchased from Integrated DNA Technologies (IDT). PMOs were purchased from Gene Tools and subsequently conjugated to Pip9b2 as described previously.61,62 ASO sequences are provided in Table S1.

Cell culture

SMA patient (Coriell; GM00232 and GM03813) and carrier (Coriell; GM03814) fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and maintained in a 37°C incubator with 5% CO₂. Fibroblast transfections were performed using RNAiMAX transfection reagent (Invitrogen). For each well of a 6-well plate, ASOs or small interfering RNA (siRNA) were complexed with 7.5 μL RNAiMAX in 300 μL Opti-MEM and added to cells at 70% confluency. The next day, media were changed to remove transfection reagents, and cells were harvested 2 days post-transfection (unless stated otherwise in the figure legends). The siRNAs used in these transfections were the following: DCP2 (Dharmacon; 167227), DCPS (Dharmacon; 28960), DXO (Dharmacon; 1797), NUDT3 s22028 (Thermo Fisher Scientific), NUDT16 (Dharmacon; 131870), SMN1/SMN2 (Thermo Fisher Scientific; s446415), XRN2 (Thermo Fisher Scientific; s22412), Negative Control No. 1 (Thermo Fisher Scientific; 4390843), and siGENOME Non-Targeting siRNA #2 (Dharmacon; D-001210-02-05).

HEK293T cells (ATCC) were cultured in DMEM, supplemented with 10% FBS, and maintained in a 37°C incubator with 5% CO₂. Plasmids were transfected using Lipofectamine 3000 (Invitrogen). Each well of a 6-well plate was transfected with 1 μg DNA, complexed with 3.75 μL Lipofectamine 3000 reagent and 4 μL P300 reagent in 250 μL Opti-MEM. Media were changed 24 h later, and cells were harvested 2 days after transfection.

iPSCs were derived from fibroblasts grown from skin biopsies collected from type 2 or 3 SMA patients attending the Oxford Motor Neuron Disorders Clinic (under ethical approval granted by the South Wales Research Ethics Committee; ref 12/WA/0186). These were reprogrammed in the James Martin Stem Cell Facility, University of Oxford, using the method indicated in Table S2. Type I SMA iPSCs were a gift of Dr. Jeroen Pasterkamp, University of Utrecht. iPSCs were differentiated into motor neuron-like cells as described previously.32,33 Briefly, the iPSCs were grown on Matrigel. They were then induced using equal volumes of DMEM/F12 and neurobasal media supplemented with N2, B27, ascorbic acid (0.5 mM), 2-mercaptoethanol (50 mM), compound C (1 mM), and Chir99021 (3 mM). After 4 days in culture, media were further supplemented with retinoic acid (1 mM) and smoothened agonist (500 nM). The following day, the media were changed to media without compound C and Chir99021. The cells were then cultured for 4–5 additional days before being split 1:3 using Accutase. Rock inhibitor was added for 24 h. After splitting, the media were supplemented with growth factors brain-derived neurotrophic factor (BDNF; 10 μM), glial cell line-derived neurotrophic factor (GDNF; 10 μM), N-[N-(3,5-difluorophenacetyl)-L-ala-nyl]-S-phenylglycine t-butyl ester (DAFT) (10 mM),...
and laminin (0.5 mg/mL) for 7 days. DAPT and laminin were then
removed from the media, and the neurons remained in culture until
day 28. Neurons were treated with ASOs (with no transfection re-
agents) on day 24 and again on day 26, before collection on day 28.

Cloning
To test the effect of the upstream start codon on gene expression, re-
porter constructs were created using the pBI-CMV4 bidirectional
promoter vector (Takara Bio). The plasmid backbone was double di-
gested with BglII and EcoRI and gel extracted using the NucleoSpin
Gel and PCR Clean-Up Kit (Takara Bio). Then, DsRed2 was replaced
with PCR-amplified mCherry using the In-Fusion HD Cloning Plus
Kit (Takara Bio). Mini-prepped mCherry plasmid was double di-
gested with BamHI and NotI for insertion of the reporter protein cod-
ing sequence. As a template for the reporter, we used a gBlocks Gene
Fragment (IDT) containing the 5' UTR of SMN2 followed by the cod-
ing sequence of EGFP followed by exon 2 through exon 3 of the hu-
mnan beta-globin gene (HBB). The well-characterized HBB exon 2,
intron 2, exon 3 splice junction was included to make the reporters
more sensitive to endogenous gene regulation. This feature is impor-
tant since one of the means through which uORFs downregulate gene
expression is promoting nonsense-mediated decay, a process that re-
quires the presence of an exon junction complex. The gBlocks Gene
Fragment was PCR amplified using CloneAmp HiFi PCR Premix
(Takara Bio) and 500 nM each primer, where the forward primer con-
tained the desired uORF mutation(s). Primer sequences are provided
in Table S7. One of the reporters (frame shift) required a second
gBlocks Gene Fragment in order to obtain a plasmid with the desired
mutation (Table S6). These PCR products and digested plasmids were
gel extracted and cloned using the In-Fusion HD Cloning Plus Kit
(Takara Bio), as above.

For all cloning work, One Shot TOP10 Chemically Competent E. coli
(Invitrogen) was transformed with In-Fusion reaction products. Plas-
mids were extracted from bacterial cultures using the QIAprep Spin
Miniprep Kit (QIAGEN) and tested by restriction enzyme digest.
Selected clones were expanded and plasmids extracted using the Hi-
Speed Plasmid Maxi Kit (QIAGEN). The sequences of inserted DNA
fragments were verified in all plasmids by Sanger sequencing (GENE-
WIZ). Details of primers used for sequencing are provided in Table S8.

Immunoblotting
Lysates were prepared in radioimmunoprecipitation assay (RIPA)
buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40
[IGEPAL], 1% sodium deoxycholate, 0.1% sodium dodecyl sul-
fate [SDS]), supplemented with Halt Protease and Phosphatase Inhib-
itor Cocktail (Thermo Fisher Scientific) or Complete Protease Inhibitor
Cocktail (Roche). After lysing on ice, samples were centrifuged (15 min,
14,000 × g, 4°C) to remove pelleted material. Protein concentrations
were determined by the Bradford assay using Protein Assay Dye Re-
agent Concentrate (Bio-Rad). Samples were then prepared in RIPA
and 4× sample loading buffer (H2O, Tris-HCl, 40% glycerol, 0.08 g/
ml SDS, 5% [v/v] 2-mercaptoethanol, bromophenol blue). Proteins
were resolved on Novex 4%–20% Tris-Glycine WedgeWell Gels (Invi-
trogen) and transferred to a 0.45-µm polyvinylidene fluoride (PVDF)
membrane. The amount of protein loaded per lane in micrograms is
indicated in the relevant figure legend. Membranes were blocked
with 5% (w/v) milk in Tris-buffered saline and 0.1% Tween (TBST)
before incubation with primary antibodies at the indicated dilutions:
mouse anti-SMN (BD Biosciences; 610647, 1:1,000 dilution), anti-
Gemin6 (Abcam; ab88290, 1:500 dilution), anti-Gemin8 (Abcam;
ab46778, 1:1,000 dilution), rabbit anti-alpha tubulin (Abcam, ab4074,
1:5,000 dilution; or Cell Signaling Technology, 2144, 1:2,000), rabbit
anti-heat shock protein (HSP)90 (Cell Signaling Technology; 4874,
1:5,000 dilution), rabbit anti-GFP (Abcam; ab290, 1:10,000 dilution),
and mouse anti-mCherry (Abcam; ab125096, 1:2,000 dilution). The membranes
were then incubated with either IRDye or horseradish peroxidase
(HRP) secondary antibodies and detected on a LI-COR Odyssey
or a Bio-Rad ChemiDoc XRS+ imaging system, respectively. Pri-
mary antibodies and secondary antibodies were incubated overnight
at 4°C or 1 h at room temperature with shaking and were followed by
three washes with TBST. Densitometric analysis of protein signal was
done using ImageJ software.

RNA stability assay
For mRNA stability assays, fibroblasts were transfected with 600 nM
2'-OMe ASOs as described above. 2 days post-transfection, fibroblasts
were treated with media containing 5 µg/mL actinomycin D (Sigma-
Aldrich). The cells were then collected in 0.5 mL TRIzol at the indi-
cated time points after treatment with actinomycin D. After adding
100 µL chloroform, the samples were vortexed and centrifuged
(15 min, 12,000 × g, 4°C). The supernatant was transferred to a new
tube, to which 1.5 volumes of 100% ethanol were added. The samples
were then pipetted into columns, and RNA purification continued
according to the miRNeasy Mini Kit manual (QIAGEN). Total RNA was
converted into cDNA using the High-Capacity cDNA Reverse Transcription
Kit (Applied Biosystems). qRT-PCRs were performed in triplicate using the QuantStudio 6 Flex Real-
Time PCR System (Applied Biosystems). 10 µL qRT-PCR reactions
contained Power SYBR Green (Applied Biosystems) with 200 nM
of each target primer and CDNA diluted in nuclease-free water (to
a concentration for which primers have 90%–110% efficiency).
Primer sequences are provided in Table S3.

Transcription assay
Transcription was measured using the Click-iT Nascent RNA Cap-
ture Kit (Invitrogen). In summary, 3 days after ASO transfection,
SMA fibroblasts were pulsed with 0.5 mM EU for 1 h and then
collected in 0.5 mL TRIzol. 100 µL chloroform was added to each
sample, which was then vortexed and centrifuged (15 min,
12,000 × g, 4°C). The supernatant was transferred to a new tube,
and 1.5 volumes of 100% ethanol were added. The samples were
then pipetted into columns, and RNA purification continued accord-
ing to the RNeasy Mini Kit manual (QIAGEN).

750 ng purified RNA was used in each Click reaction. Subsequently,
400 ng biotinylated RNA was mixed with 25 µL magnetic bead sus-
pension for each binding reaction. After washing away unbound
RNA, on-bead cDNA synthesis was performed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) in a 50-μL final reaction volume. 500 ng total RNA (pre-biotinylation) was also converted to cDNA in a 15-μL reaction using the SuperScript VILO cDNA Synthesis Kit, which represents steady-state mRNA. qRT-PCRs were performed as 20 μL reactions in triplicate. Reactions contained Power SYBR Green (Applied Biosystems), 200 nM of each target primer, 1 μL cDNA, and nuclease-free water. cDNA of total RNA was diluted 1:4 in nuclease-free water, whereas cDNA of biotinylated RNA was undiluted. qRT-PCR plates were run on the StepOnePlus Real-Time PCR System (Applied Biosystems).

**Splice isoform analysis**

SMN2 exon 7 splicing was qualitatively assessed by gel electrophoresis of RT-PCR products. First, RNA was extracted, and cDNA was converted according to the protocol described above for the RNA stability assay. The cDNA from ASO-treated samples was used as template for PCR. The reaction was performed with PCR Master (Roche) and with primers situated in exon 5 and exon 8 of SMN2. Primer sequences are provided in Table S4. Amplicons were resolved on 2% agarose gels. To ensure that the full-length amplicon did not sequester the Δ7 amplicon, a test run was performed in which samples were diluted, denatured by heating to 95°C for 5 min, and then immediately placed on ice until being loaded on a gel. The results were consistent between heated and unheated samples, so the extra denaturing and steps were omitted. Exon 7 splicing was quantitatively assessed for the same samples using the qRT-PCR protocol described for the RNA stability assay.

**Bioinformatics**

Ribosome profiling sequencing data from GEO sample accessions (GEO: GSM1047584, GSM1047585, and GSM3566399) were downloaded in the fastq format using the fastq-dump command supported by the NCBI SRA Toolkit (https://www.ncbi.nlm.nih.gov/sra). The FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to inspect the quality of the sequence data. To clip adaptor sequences that may be present and to remove low-quality sequences, the Trimmomatic tool (http://www.usadellab.org/cms/?page=trimmomatic) was used with the following command-line specifications: “ILLUMINACTION:TruSeq3-SE-2.fa:2:30:10 HEADCROP:11 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:15.” Reads were mapped to a custom reference genome. The custom reference genome was constructed by deleting all SMN gene annotations from human reference genome hg19 and then, to the modified hg19, adding the 215P15 clone sequence as a separate contig, as described previously.4 For reference mapping against the customized version of the human genome, the RNA sequencing (RNA-Seq) tool, supported in the CLCbio Genomics Workbench (version v.11.2) was used under default parameters. Mapped reads were visualized using Integrative Genomics Viewer (IGV) version 2.4.8 (http://software.broadinstitute.org/software/igv/).

**MEF isolation and culture**

Mouse work was performed in the Biomedical Sciences Unit at the University of Oxford as authorized by the UK Home Office (Animal Scientific Procedures Act 1986). Taiwanese SMA mice were bred and maintained as described on The Jackson Laboratory website and as described previously.6,63 MEFs were isolated from strain FVB.Cg-Smm1^+/HungTg(SMN2)2Hung/J (Jackson Laboratory; 005058), crossed with strain FVB.129P2(B6)-Smn1^+/Hung/J (Jackson Laboratory; 031678), using a method described previously.24

After 2 additional days of culturing in MEF culture medium, the cells were plated for ASO transfection. For the MEF lines with sufficient cell counts, duplicate wells were plated (one for transfection with the 5′ UTR ASO and one for transfection with the NTC ASO). Single wells were plated for the MEF lines with fewer cells. The MEFs were transfected with ASO using RNAi MAX as described above. 3 days later, the MEFs were collected in RIPA buffer and subsequently assayed by immunoblotting.

KO/D7;SMN2- and KO/F7-immortalized MEFs37 shared by the Burghes lab were transfected with ASO and immunoblotted 2 days post-transfection.

**Statistics**

Data were analyzed in Microsoft Excel and GraphPad Prism 8. In experiments without an untreated condition, all sample values are shown as a fold difference relative to the untreated samples. For these figures, error bars show standard error of the mean (SEM) of the fold differences. One-way ANOVA with Dunnett’s multiple comparisons test was used to determine statistical significance.

In experiments without an untreated condition, all samples were expressed as a fold difference relative to the NTC samples. For these figures, error bars show propagated error. In short, when normalizing the protein of interest to the loading control, the error associated with each signal intensity value was divided by the mean signal intensity value, and this fraction was squared. The values for the two proteins were summed, and then the square root was calculated. The resulting error value was used in a second, identical round of error computation in order to propagate it through the fold-difference calculation. Statistical significance was determined by t test between the normalized signal intensity values for the two sample groups.

For the RNA stability assay, we used a linear mixed model with total SMN mRNA level as the outcome variable and hours post-actinomycin D treatment, group, and their interaction as independent variables. Sample was specified as a random intercept. The significant interaction statistic was tested using a likelihood ratio test between the full model and a reduced no-interaction model and reported using a chi-square statistic and p value. If significant, the slopes for each group were then compared to each other and were Bonferroni corrected for 3 comparisons.

**Data presentation**

Graphs were made using GraphPad Prism 8. Some schematics were created using BioRender (BioRender.com). Figures were assembled in Microsoft PowerPoint and Adobe Illustrator.
SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.12.027.

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AUTHOR CONTRIBUTIONS
A.M.W., C.G., K.H.F., S.A., K.T., S.M.H., and C.R. designed the experiments. M.B. helped in experimental set-up. A.M.W., G.G.H., K.C., S.M.H., and S.A. conducted the experiments. A.M.W., K.H.F., C.G., K.J., C.R., and M.J.W. analyzed and interpreted the data. A.M.W. wrote the first draft, and K.H.F., C.G., K.T., S.M.H., S.A., and C.R. contributed to the final version of the manuscript.

DECLARATION OF INTERESTS
A patent application was led for the 5′ UTR ASOs described in this manuscript. M.J.W. is a founder of and shareholder in PepGen.

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