

Key role of SMN/SYNCRIP and RNA-Motif 7 in spinal muscular atrophy: RNA-Seq and motif analysis of human motor neurons

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Spinal muscular atrophy is a motor neuron disorder caused by mutations in *SMN1*. The reasons for the selective vulnerability of motor neurons linked to SMN (encoded by *SMN1*) reduction remain unclear. Therefore, we performed deep RNA sequencing on human spinal muscular atrophy motor neurons to detect specific altered gene splicing/expression and to identify the presence of a common sequence motif in these genes. Many deregulated genes, such as the neurexin and synaptotagmin families, are implicated in critical motor neuron functions. Motif-enrichment analyses of differentially expressed/spliced genes, including neurexin2 (*NRXN2*), revealed a common motif, motif 7, which is a target of SYNCRIP. Interestingly, SYNCRIP interacts only with full-length SMN, binding and modulating several motor neuron transcripts, including SMN itself. SYNCRIP overexpression rescued spinal muscular atrophy motor neurons, due to the subsequent increase in SMN and their downstream target *NRXN2* through a positive loop mechanism and ameliorated SMN-loss-related pathological phenotypes in *Caenorhabditis elegans* and mouse models. SMN/SYNCRIP complex through motif 7 may account for selective motor neuron degeneration and represent a potential therapeutic target.

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Abbreviations: AAV9 = adenovirus-associated vector serotype 9; DAVID = database for annotation, visualization, and integrated discovery; GO = gene ontology; hnRNP = heterogeneous nuclear ribonucleoprotein; iPSC = induced pluripotent stem cell; NMJ = neuromuscular junction; SMA = spinal muscular atrophy; SMN = survival motor neuron; RBP = RNA binding protein; RNA-Seq = RNA sequencing

Introduction

Spinal muscular atrophy (SMA) is a severe genetic neuromuscular disease with early onset and represents the most common genetic cause of infant mortality (Pellizzoni *et al.*, 1998; Faravelli *et al.*, 2015). Affected children present atrophic muscular masses, weak and hypotonic limbs, and life-threatening respiratory complications (Pellizzoni *et al.*, 1998; Faravelli *et al.*, 2015). SMA is caused by mutations in the survival motor neuron 1 gene (*SMN1*), which impairs the function and survival of lower motor neurons in the spinal cord (Lefebvre *et al.*, 1995), but why deficiency in this ubiquitously expressed protein primarily affects motor neurons is unclear.

The majority of current SMA therapeutic approaches, such as nusinersen, the only compound recently approved by the FDA/EMA (www.curesma.it), are focused on increasing the levels of full-length SMN protein (Pellizzoni *et al.*, 1998; Foust *et al.*, 2010; Faravelli *et al.*, 2015). However, SMN-independent approaches to target downstream pathological events can be valuable as complementary strategies, particularly in the symptomatic phase of the disease. Furthermore, understanding the complex series of mechanisms and pathways responsible for the effect of SMN deficiency is crucial for monitoring therapeutic efficacy and allowing further therapeutic progress, including the development of novel effective cures.

SMN plays a key role in RNA splicing (Pellizzoni *et al.*, 1998), but its precise function is not completely clear. The absence of SMN causes improper assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs), leading to impaired splicing. SMN may also play a direct role in splicing, interacting transiently with components of the splicing machinery, acting prior to their assembly into the spliceosome (Mourelatos *et al.*, 2001), suggesting a direct molecular link between SMN, pre-mRNA splicing, and other RNA processing events that could be impaired when levels of full-length SMN are reduced.

Many studies have documented the association between SMN and several RNA binding proteins (RBPs) that are not part of the SMN complex, such as HuD or heterogeneous nuclear ribonucleoprotein (hnRNP) R, fragile X mental retardation protein (FMRP), and amyotrophic lateral sclerosis-related proteins FUS and TDP43. These factors are involved in the splicing, transport, stability, and translation of mRNAs, among other aspects of RNA metabolism (Pellizzoni *et al.*, 1998).

Alterations in gene expression and splicing have been shown in tissues derived from SMA mice (Zhang *et al.*, 2008, 2013; Lotti *et al.*, 2012; Maeda *et al.*, 2014) and patient samples (Corti *et al.*, 2012; Ng *et al.*, 2015). However, analyses of the correlation between SMN defects, specific gene expression, and splicing alterations in human SMA motor neurons, and studies of common motif sequences present in these altered genes are still lacking. This study could suggest a mechanistic link between

aberrant transcripts and the motor neuron selective dysfunction observed in SMA.

To investigate these aspects, we performed RNA sequencing (RNA-Seq) of SMA motor neurons derived from patients' induced pluripotent stem cells (iPSCs) and healthy lines and we identified SMA-specific molecular changes, including gene expression and splicing alterations in transcripts related to cytoskeletal, axonal, and synaptic functions, such as neurexin 2 (*NRXN2*), a neuron-specific gene encoding a presynaptic cell adhesion protein involved in motor neuron survival and function. Other groups have already observed reduced expression and altered alternative splicing of *NRXN2* in transgenic SMA mice (See *et al.*, 2014). In the present study, the overexpression of *NRXN2* was shown to improve human SMA motor neuron survival and increase motor axon length, suggesting a role as a modifier gene.

Motif enrichment analysis of differentially expressed/spliced genes in SMA motor neurons identified motif 7 as one of the most significantly enriched. Notably *NRXN2* also possesses motif 7, which is a common target sequence for RBPs, particularly hnRNPs. Among these, hnRNPQ (SYNCRIP), which is highly homologous to hnRNPR, recognizes a large number of key motor neuron genes and is likely implicated in misregulation of their gene expression/splicing (Mourelatos *et al.*, 2001; Rossoll *et al.*, 2002). This observation goes along with the finding that SYNCRIP protein directly interacts only with full-length SMN and not with the non-functional ~~7~~ truncated form, the most prevalent SMN mutant found in SMA patients (Mourelatos *et al.*, 2001; Rossoll *et al.*, 2002). Importantly, SYNCRIP has been studied as a splicing modulator of SMN, promoting the inclusion of exon 7 in *SMN2*, probably by activating the upstream 3rd splice site (Chen *et al.*, 2008). Overall, this suggests that the disruption of SMN-SYNCRIP-dependent RNA pathways causes specific downstream molecular defects, including deregulation of *NRXN2*, which can contribute to motor neuron death and axonal impairment. Interestingly, the overexpression of SYNCRIP rescued the phenotype in our SMA cells due to the subsequent increase in SMN and *NRXN2*, demonstrating the presence of a positive loop among SYNCRIP, SMN, and *NRXN2*. Noteworthy, the overexpression of SYNCRIP also rescued the neurodegenerative phenotype observed *in vivo* in a *C. elegans* model (Gallotta *et al.*, 2016). This study produced novel insights into the role of SMN loss in mRNA processing and its selective effects on human SMA-motor neurons, highlighting novel targets for therapeutic strategies.

Materials and methods

Induced pluripotent stem cell lines

The studies involving human samples were conducted in compliance with the Code of Ethics of the World Medical

Association (Declaration of Helsinki) and with national legislation and institutional guidelines. After obtaining informed consent, fibroblasts were generated from dermal biopsies (Eurobiobank) as described previously (ethical committee approval at the IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico) (Corti *et al.*, 2012; Rizzo *et al.*, 2016, 2017). The iPSCs were generated by non-viral transduction with six reprogramming factors: OCT4, SOX2, NANOG, LIN28, c-Myc, and KLF4 (Supplementary Table 1; Corti *et al.*, 2012). iPSC colonies with embryonic stem cell-like morphology were expanded and detached for analysis. iPSCs were grown on Matrigel® (BD Biosciences) with E8 media (Life Technologies). All cell cultures were maintained at 37°C in 5% CO₂.

Differentiation of iPSCs into motor neurons

We generated spinal motor neurons using a multistage differentiation protocol (Corti *et al.*, 2012; Rizzo *et al.*, 2016, 2017). Wild-type and SMA iPSCs were plated with neuronal medium (DMEM/F12; Gibco, Invitrogen) supplemented with MEM non-essential amino acids, N2, and heparin (2 mg/ml, Sigma-Aldrich). After 10 days, we added retinoic acid (0.1 mM, Sigma-Aldrich) for neural caudalization. On Day 17, we collected the posteriorized neuroectodermal cells. These clusters were suspended for 1 week in the same medium with retinoic acid (0.1 mM) and sonic hedgehog (SHH; 100–200 ng/ml, R&D Systems, Inc.). On Day 24, we supplemented the medium with other growth factors e.g. brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and IGF1 (10 ng/ml, Peprotech). To achieve greater cell purity, motor neurons were enriched using a gradient centrifugation method (Corti *et al.*, 2012). To monitor the proper acquisition of a motor neuron phenotype, cells were transduced with a lenti-Hb9::GFP construct (Marchetto *et al.*, 2008), fixed, and stained for quantification using established neuronal and motor neuron markers. For genetic modification, cultures were infected with lentivirus-NRXN2 (Origene RC219788, Accession number NM_015080), lentivirus-SYNCRIP, lentivirus-SMN, or null vector, which was used as a negative control (Simone *et al.*, 2014).

SH-SY5Y transfection

The human SH-SY5Y neural cell line was cultured in DMEM high glucose medium containing 10% foetal bovine serum (FBS), 2.5 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all purchased from Euroclone). The cells were transfected with 15 nM SYNCRIP siRNA (ThermoFisher, ID s20564) or with select negative control (ThermoFisher, ID 4390843), which was used as a negative control, and harvested after 48 h.

Immunocytochemistry and phenotypic analysis of iPSCs and motor neurons

Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with Triton 0.25%, and then blocked with 10% bovine serum albumin in phosphate-buffered saline and

0.3% Triton™ X-100 for 1 h at room temperature. We incubated the cells with primary antibodies to NANOG (1:100, Abcam), SSEA-3 (1:100, Covance), ChAT (1:200, Chemicon and Millipore), SMI32 (1:500, Covance), hnRNP-Q (1:100, Sigma), and NRXN 2a (1:100, Abcam) overnight, and then with anti-rabbit, anti-mouse, or anti-goat Alexa Fluor® 488 or 594 (1:400; Life Technologies) secondary antibody for 1.5 h at room temperature. Negative controls were performed for all stains. Microphotographs were taken with a LEICA LCS2 confocal microscope.

Motor neuron morphometric analysis

Phenotypic analysis was performed as described previously (Corti *et al.*, 2012; Nizzardo *et al.*, 2015; Allodi *et al.*, 2016). We quantified the motor neurons by determining cells positive for motor neuron marker and counting 10 randomly selected fields/well. Morphometric and axon length analyses were performed by measuring soma diameter and length distance between two points (one point from the soma and one on the distal axon) (Corti *et al.*, 2012). All quantification analyses were carried out in a double-blind fashion. For all imaging, we used a confocal LEICA SP2.

RNA analysis

Selected cells were treated with proteinase K for 1 h at 50°C before the addition of TRIzol® LS (Life Technologies) for RNA extraction. DNase treatment using Turbo DNA-free (Ambion) was performed to remove contaminating genomic DNA. The RNA analysis was processed by DNA vision as follows. A sample quality control was performed on an Agilent 2100 Bioanalyzer and quantity checked by Nanodrop. Next, the TrueSeq mRNA library was constructed, followed by Library Quality Control on the Agilent 2100 Bioanalyzer. Sequencing was performed on a HiSeq 2000, two samples per lane with a 2 x 100 cycles strategy.

RNA-Seq reads mapping

The raw data generated by sequencing were quality-checked using the FastQC tool and cleaned of low quality reads, adaptor sequences, and other contaminants. Quality was judged in terms of read length distribution, Phred score distribution, and nucleotide frequencies obtained from FastQC statistics. STAR software (Dobin *et al.*, 2013) was then used to align the reads to the reference *Homo sapiens* Ensembl GRCh37 genome (Flicek *et al.*, 2014). Parameters included the removal of non-canonical junctions, the generation of the XS strand attribute for all alignments that contain splice junctions, and the sorting of the output files in bam format. The annotation provided in Illumina iGenomes repository was given as an input to the STAR routine, as well as all other routines requiring an annotation file.

Alternative splicing differential analysis

To detect the differential usage of exons between patients and controls, data were processed using the rMATS tool (Shen *et al.*, 2014). The tool was run in paired-end data processing mode, with declared length of reads of 101 bp and with a

junction anchor length of 4. The statistical model of rMATS calculated the inclusion level difference (ILD), *P*-value, and false discovery rate (FDR) for each skipping exon from a list obtained from both data and annotation. Exons were then separated on the basis of three conditions: (i) enhanced exons ($|\text{ILD}| \leq 0$, $|\text{ILD}| \leq 4 \text{ median}|\text{ILD}|$, $\text{FDR} \leq 0.05$); (ii) silenced exons ($|\text{ILD}| \geq 0$, $|\text{ILD}| \geq 4 \text{ median}|\text{ILD}|$, $\text{FDR} \leq 0.05$); and (iii) invariant exons ($|\text{ILD}| \leq 5 \text{ median}|\text{ILD}|$, $\text{FDR} \leq 0.50$).

Motif discovery

The enhanced and silenced cassette exons were analysed to identify sequence patterns inside both exons and flanking sequences that discriminate cassette exons differentially included in patients. The genomic sequences were retrieved for enhanced, silenced, and control exons and their flanking upstream and downstream introns using GeCo++ library (Cereda *et al.*, 2011). The flanking intronic regions were selected for 150 bp fixed length and location 10–160-bp upstream and +10/+160 bp downstream from the exon. For each sequence, the frequency distribution of all possible tetramers was estimated and collected in a matrix. Projective non-negative matrix factorization was then applied to identify a small set of distributions (motifs) that approximate the full matrix. The factorization rank *r* corresponds to the number of desired motifs. For each sequence, we then calculated the Battacharyya coefficient between the corresponding distribution and each motif representing a set of *r* features. At this point, each cassette exon was characterized by a set of 3*r* features comprising upstream intronic, exonic, and downstream intronic sequence features.

This set of features was then used as variables in logistic regressions to identify the parameters of two distinct models and distinguish controls from enhanced and silenced exons. We evaluated the performance of the models by 10-fold cross-validation AUROC (area under the receiver operating characteristic). The entire procedure was repeated for increasing number of motifs (*r*) until no AUROC improvement. A manuscript with a full description of this method is in preparation.

The obtained motifs were then compared to a set of 119 vertebrate RBP motifs selected from a publicly available database (Ray *et al.*, 2013). Again, we used Battacharyya coefficients to measure the similarity between one of our motifs and the tetramer distributions obtained for each of the Ray dataset position weight matrices. Significant similarities were obtained through 200 000 random permutations of the tetramer distributions. The proportion of permutations, resulting in a higher Battacharyya coefficient than the observed one, were used as empirical *P*-value for the comparison. Bonferroni correction was applied to take into account the fact that we performed 119 (Ray dataset) \times 15 (our motifs) comparisons.

Gene and transcript quantification and differential expression

The files containing data alignments to genome assembly were processed by Cufflinks tools (Trapnell *et al.*, 2013) to compute the gene and transcript expression levels. Cuffquant routine was run first on each individual sample with the ‘-u’ flag, supporting correction of multiple reads (i.e. reads mapped at

multiple loci within the genome). Next, the Cuffnorm routine was run to normalize the quantified expression with respect to possible differences in library size, sequencing depth, and/or transcript length. The resulting normalized abundances, measured as fragments per kilobase of transcript per million mapped reads (FPKM), were further used to build a dendrogram plot of all samples and replicates. The results of the Cuffquant routine were further employed for the differential expression analysis between patients with SMA and controls. Thus, the Cuffdiff2 routine was run with the option of multiple reads correction. Differentially expressed genes were then separated into three sets on the basis of three conditions: (i) significant upregulation in patients with SMA ($\log_2\text{fold-change} \geq 0$, $q \leq 0.05$); (ii) statistically significant downregulation in patients with SMA ($\log_2\text{fold-change} \leq 0$, $q \leq 0.05$); and (iii) lack of significance between patients and controls.

Functional annotation analysis

The web-based functional annotation analysis tool Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang *et al.*, 2009) was applied to evaluate the list of significantly differentially used exons, and to obtain a set of enriched functional annotations in three domains: biological process (BP), cellular component (CC), and molecular function (MF). For each exon, the corresponding gene was considered. The complete set of genes, inclusive of all significantly up- and downregulated exons, was provided as the target ($N_t = 4175$) and tested against all genes, named background ($N_b = 29\,237$).

The analysis was repeated for the differentially expressed genes ($P \leq 0.05$). The complete set of significantly up- and downregulated genes was provided as the target ($N_t = 1795$) and tested against all genes, named background ($N_b = 16\,122$).

In all analyses, the classification stringency was set to high. We also used gene set enrichment analysis (GSEA) as a bioinformatics tool (Subramanian *et al.*, 2005). Enrichment for up- or downregulated sets of genes from the REACTOME pathway and GO (gene ontology) term database was performed by running GSEA against the test statistic-ranked list of genes in the experiment. Ranking was based on the Cuffdiff 2-derived test statistic. All REACTOME and GO term gene sets with 415 members in the MSigDB package ‘c2.all.v3.0.symbols.gmt’ and ‘c5.all.v4.0.symbols.gmt’, respectively, were downloaded from <ftp://gseaftp.broadinstitute.org/>.

RBP motif enrichment of differentially expressed genes

Among the 29 237 genes analysed, we only kept those for which the most representative transcript (i.e. the one with the highest Cuffquant estimated mean FPKM) was fully annotated as coding [i.e. both the 3⁰ and 5⁰ untranslated region (UTR) and coding sequence (CDS) were present]. A subset of 13 853 genes was obtained with the most representative coding transcript for each, 1385 from differentially expressed genes.

Motif enrichment was performed in differentially expressed genes as follows. For each transcript and motif, a profile was calculated separately for the 5⁰ UTR, CDS, and 3⁰ UTR sequences. Profiles were obtained by evaluating the tetramer

frequency distribution in 50-bp sliding windows at each position along each sequence and by assigning each position its similarity with the motif (Bhattacharyya coefficient). A motif was then considered present in a profile if its maximum window similarity was 40.7.

Considering the set of 5[′] UTR profiles derived from differentially expressed genes (D5[′]UTR), we merged all of the 5[′] UTR profiles into a unique profile, then starting from a random position we mapped a number of consecutive intervals equal to the number of D5[′]UTRs of matching length. We counted the number of motifs containing intervals and compared it to the number of motifs containing D5[′]UTRs. We repeated this step 10 000 times and obtained the empirical enrichment *P*-value as the proportion of iterations that gave a number of motifs containing intervals equal or greater to the number of motifs containing D5[′]UTR. The entire procedure was repeated for CDS and 3[′] UTR profiles derived from differentially expressed genes.

Other bioinformatics analysis

For RNA-Seq data analysis, we used two bioinformatics tools that query for enriched GO terms: gene-annotation enrichment analysis with DAVID (Huang *et al.*, 2009) and GSEA (Subramanian *et al.*, 2005). Enrichment for sets of genes from the REACTOME pathway and GO term database was performed by running GSEA against the test statistic-ranked list of genes in the experiment. Ranking was based on the Cuffdiff 2-derived test statistic. All REACTOME and GO term gene sets with 415 members in the MSigDB package ‘c2.all.v3.0.symbols.gmt’ and ‘c5.all.v4.0.symbols.gmt’, respectively, were downloaded from <ftp://gseafp.broadinstitute.org/>.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from cells using the RNeasy[®] Mini Kit (Qiagen) and concentrations measured by a Nanodrop spectrophotometer. Only samples with ratios between 1.8 and 2.0 were analysed further. A total of 1.5 mg of total RNA was reverse-transcribed using the Ready-To-Go kit[™] (GE Healthcare). Reverse-transcribed material (5 ng for each sample) was amplified using the TaqMan[®] Universal PCR Master Mix (Applied Biosystems) and appropriate probes to evaluate gene expression and exons (probe ID available upon request, Supplementary Table 2) in the 7500 Real Time PCR System (Software 2.01, Applied Biosystems). Expression levels were normalized to the average level of housekeeping gene *18S* and referred to the relevant control samples.

Western blot analysis

Western blot analysis was performed as described previously (Nizzardo *et al.*, 2014a; Rizzo *et al.*, 2016). Briefly, cells were sonicated on ice for 10 min in buffer supplemented with protease and phosphatase inhibitor cocktail (Pierce) (Nizzardo *et al.*, 2014a; Rizzo *et al.*, 2016). Alternatively, 20 mg of frozen brain were homogenized in 0.4 ml of protein sample buffer containing 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 6.8), and 0.1 M DTT. A total of 50 mg was

separated via 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to a nitrocellulose membrane and incubated with primary antibodies overnight at 4°C. The primary antibodies used in these experiments were: NFs (1:1000, Sigma), MMP14 (ThermoFisher, 1:1000), SYT13 (1:1300, Proteintech), NRXN1 (1:2000, Abcam), NRXN2a (1:500, Abcam), SMN (1:1000, BD), STMN2 (1:2000, Proteintech), PLP1 (1:800, Sigma), and hnRNP-Q (1:1000, Sigma). The blots were then incubated in secondary antibody: polyclonal anti-rabbit (1:2700, Dako) and polyclonal anti-mouse (1:3200, Dako). The immune complexes were revealed by chemiluminescence assay (Amersham). The nitrocellulose membrane was stripped and reprobed with anti-actin (1:1000, Sigma) as a loading control. Densitometry was performed using ImageJ software.

In vivo experiments in the SMA *C. elegans* model

Nematodes were grown and handled following standard procedures in uncrowded conditions at 20°C on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* strain OP50 (Brenner, 1974). The wild-type animals were strain N2, variety Bristol. The transgenic strains were: NA1330 *gbls4* [GBF109 *punc-25::smn-1(RNAi)*; GB301 *pchs-2::GFP*] III; NA1678 *gbEx612a* [GB301 *pchs-2::GFP*]; NA1252 *gbEx540a* [GBF322 *punc-119::dsRED*; *pelt-2::RFP*]; EG1285 *oxIs12* [*punc-47::GFP*; *lin-15(+)*] X; NA1355 *gbls4* III, *oxIs12* III (Gallotta *et al.*, 2016). EG2185 and N2 were provided by the *Caenorhabditis* Genetics Center (CGC) funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The following strains were obtained by genetic crosses: NA2052 *gbls4* III, *gbEx540a*, *oxIs12* X; NA2017 *gbls4* III, *gbEx540a*. The rescue construct (GBF362 *punc-119::hrp-2*) for pan-neuronal expression of *hrp-2* was created by PCR fusion (Hobert, 2002) of *unc-119* promoter with *hrp-2*, both amplified from genomic sequences. All primer sequences are available upon request. Germline transformation was accomplished as described by Mello *et al.* (1991) by injecting a DNA mixture containing the transgenic construct at 2 ng/μl (lower concentration, LC) and 20 ng/μl (higher concentration, HC) into the gonad of NA1330 *gbls4* III adult animals together with a phenotypic marker for selection of the transgenic progeny. We used pJM371 *pelt-2::RFP* (RFP expression in the nuclei of intestinal cells) as the co-injection marker at 30 ng/μl, a kind gift of Prof. J.D. McGhee (University of Calgary) (McGhee *et al.*, 2009). Two independent transgenic lines were examined for each concentration: NA2078 *gbls4* III, *gbEx656a* [GBF362 *punc-119::hrp-2* LC; *pelt-2::RFP*]; NA2079 *gbls4* III, *gbEx656b* [GBF362 *punc-119::hrp-2* LC; *pelt-2::RFP*]; NA2075 *gbls4* III, *gbEx655a* [GBF362 *punc-119::hrp-2* HC; *pelt-2::RFP*]; NA2076 *gbls4* III, *gbEx655b* [GBF362 *punc-119::hrp-2* HC; *pelt-2::RFP*]. Genetic crosses were made to transfer transgenes to the appropriate genetic background to obtain NA2082 *gbls4* III, *gbEx655a*, *oxIs12* X; NA2080/2081 *gbls4* III, *gbEx656a/b*, *oxIs12* X. In all cases, the presence of the desired transgenes was verified by phenotyping the clonal F2s using a dissecting microscope equipped with epifluorescence. Two independent clones with the same genotype were examined after each cross, and the mean of the two clones has been reported in

the results. Animals were immobilized in 0.01% tetramisole hydrochloride (Sigma-Aldrich) on 4% agar pads and visualized using a Zeiss Axioskop microscope. The phenotypes of dying motor neurons were scored by counting the number of ventrally located D-type motor neurons acquiring fluorescence, which we previously demonstrated to be specific for *smn-1* silencing, a late sign of apoptosis, visible in the absence of any motor neuron-specific expression of GFP, different from endogenous autofluorescence in the intestine, and never observed in control animals (Gallotta *et al.*, 2016). The degenerative phenotype was scored by counting the number of visible, and hence viable, D-type motor neurons expressing GFP due to a motor neuron-specific promoter (*punc-47::GFP*) (McIntire *et al.*, 1997). Microscopes used for scoring and imaging were equipped with epifluorescence and DIC Nomarski optics. Epifluorescence and confocal images were collected with a Leica TCS SP8 AOBS microscope, using 40x and 20x objectives, respectively. Epifluorescence images were reconstructed using the Tile Scan function of the Leica LAS X program, which automatically assembles a picture by taking a number of adjoining images of the sample and merges them digitally to obtain a larger view. Confocal images were collected using a 20x objective on a Leica TCS SP8 AOBS confocal microscope. Well-fed, young adult animals were used for the backward movement assay (McIntire *et al.*, 1993) to test D-type motor neuron function. The assay was performed blindly on 6-cm diameter NGM plates seeded with bacteria. Using an eyelash, the animal was touched first on the tail to induce forward movement and then on the head to test for backward movement. Defective movement was scored when animals were unable to fully move backward. For each data set, the percentage of defective animals among the total number of tested animals was calculated.

In vivo experiments in the SMA mouse model

The SMA^{Δ7} mouse transgenic model was used. Heterozygous mice (*Smn*^{+/-}, *hSMN2*^{+/+}, *SMN*^{Δ7}^{+/+}, Jackson Laboratories) were bred and pups identified by genotyping (Le *et al.*, 2005). All animal experiments were approved by the University of Milan and Italian Ministry of Health review boards. Homozygous affected pups were cryoanaesthetized and injected intracerebroventricularly as described previously (Nizzardo *et al.*, 2014b) on postnatal Day 1 (P1) with 10¹¹ particles of adenovirus-associated vector serotype 9 (AAV9) vector expressing *Syncrip* or null vector (SignaGen Laboratories). The brain (*n* = 3/group) was collected and harvested at P7 for western blot analysis. Disease onset, progression, survival, and motor function (righting test) were monitored after treatment (*n* = 5/group) as described previously (Nizzardo *et al.*, 2014b). All tests were performed blinded to the mouse genotype and treatment. Intercostal muscles were collected, frozen on dry ice at P10 (*n* = 3/group), cryosectioned (20 μm), and stained for neuromuscular junction (NMJ) detection and counting. All sections were saturated with 10% bovine serum albumin and 0.3% Triton X-100 for 1 h at room temperature before incubation with rabbit Neurofilament Medium (NF-M, Millipore 1:250) overnight at 4°C. The next day, the slides were incubated with Alexa Fluor[®] 488 (1:1000; Life Technologies) and a-bungarotoxin

555 (1:500, Life Technologies). A minimum of 100 NMJs from each muscle were randomly selected and the number of denervated/degenerated NMJs was determined for each muscle group in each animal based on NF-M/a-BTX staining.

Statistical analysis

Statistical analyses were carried out in StatsDirect for Windows (version 2.6.4) or GraphPad Prism 5 software. Two-tailed, unpaired Student's *t*-test was used to compare two groups. Differences in axonal length were investigated by the Kolmogorov-Smirnov test (http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html). The Kaplan–Meier log rank test and logistic regression analysis were used to compare lifespans. Contingency and chi-squared tests were used to determine differences in the righting test and NMJ innervation. All experiments were carried out in at least triplicate. One-way ANOVA with Kruskal-Wallis test was used for *C. elegans* data related to statistical analyses.

The experimental results are provided as mean ± standard error of the mean (SEM) or mean standard deviation (SD). The null hypothesis was rejected at the 0.05 level.

Data availability

The data that support the findings of this study are openly available in GEO at <https://www.ncbi.nlm.nih.gov/geo/> reference number GSE108094.

Results

Motor neurons generated from SMA patient iPSCs present reduced cell survival and axonal length in culture

We previously generated iPSCs from type 1 SMA patients and healthy subject fibroblasts using a non-viral, non-integrating method (Supplementary Table 1; Corti *et al.*, 2012; Nizzardo *et al.*, 2014a). Obtained iPSCs exhibited markers of pluripotency (Fig. 1A) and were able to differentiate into motor neurons using established protocols (Fig. 1B) (Corti *et al.*, 2012; Nizzardo *et al.*, 2015).

Differentiated cells expressed motor neuron-specific transcription factors, such as spinal cord progenitor markers HB9, ISLET1, and OLIG2 and pan-neuronal markers TuJ1, neurofilament, and MAP2. The majority of these HB9/ISLET1-positive neurons expressed choline acetyltransferase (ChAT) and were positive for motor neuron marker SMI-32, demonstrating a mature motor neuron phenotype (Fig. 1C). The *in vitro* differentiation protocol yielded a mixed cell population that included non-motor neuron cells. Given the limited availability of surface markers to isolate and purify motor neurons, we applied a physical strategy based on gradient centrifugation. After selection, immunocytochemistry revealed that the percentage of ChAT + SMI32 + cells was 89.6 ± 8.4% for cells derived from wild-type iPSCs and 87.6 ± 7.7% for cells

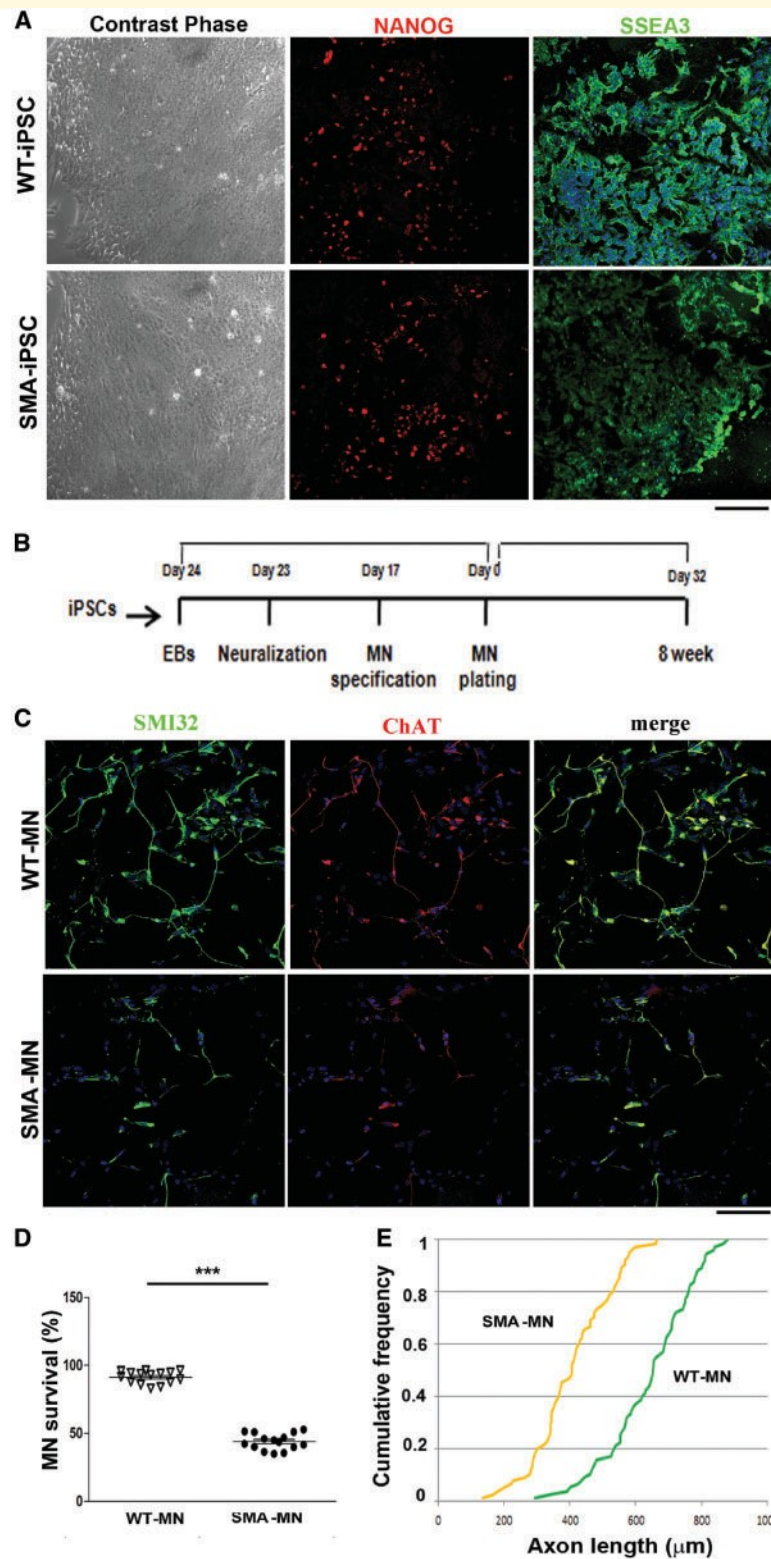


Figure 1 SMA iPSCs and the derived motor neurons express typical specific cell type markers. (A) Left: Wild-type and SMA iPSCs showed the typical pluripotent stem cell colony morphology under the contrast phase microscope. Right: The cells express the pluripotency markers NANOG (red) and SSEA3 (green). Nuclei are labelled with DAPI (blue). Scale bar = 75 μm . (B) Experimental outline for wild-type and SMA motor neuron differentiation. (C) Immunocytochemistry of wild-type and SMA motor neurons. The cells were positive for typical motor neuron markers SMI32 (green) and ChAT (red) (merge, yellow signal). Nuclei are labelled with DAPI (blue). Scale bar = 75 μm . (D) Eight weeks after differentiation, SMA motor neuron survival was significantly reduced compared to wild-type. *** $P \leq 0.0001$, Student *t*-test. Error bars represent SEM. (E) After 8 weeks of culture, SMA motor neurons exhibited significantly reduced axon elongation with respect to wild-type. $P \leq 0.001$, Kolmogorov-Smirnov test, five independent experiments.

derived from SMA-iPSCs. Less than 1% of cells differentiated from iPSCs expressed the astrocyte marker GFAP (data not shown). At 8 weeks, we observed a reduction in the number of motor neurons and axonal length in the SMA-iPSC cultures compared to wild-type-iPSCs ($P \leq 0.0001$, Fig. 1D; $P \leq 0.001$, Fig. 1E), which is in line with previous reports that SMA iPSC-derived motor neurons exhibit reduced survival in long-term culture (Ebert *et al.*, 2009; Nizzardo *et al.*, 2015).

RNA sequencing of SMA motor neurons shows specific changes in key motor neuron function

We performed massively parallel deep RNA sequencing targeting 40M clusters per sample to evaluate global gene expression in SMA type I and wild-type motor neurons to identify differentially expressed/spliced transcripts in SMA motor neurons. The analysis revealed a downregulation of 1084 genes and upregulation of 808 genes out of the whole set of 24 691 genes tested (fold change cut-off = 1.5; $P \leq 0.01$; Supplementary Table 3). We identified the 30 transcripts most increased and decreased in abundance at an FDR of 5% (Fig. 2A). The web-based functional annotation analysis tool DAVID was applied to evaluate the RNA-Seq data and obtain a set of enriched GO terms (Supplementary Tables 4 and 5). The most relevant GO terms are provided in Supplementary Fig. 1.

Interestingly, in the Reactome analysis we observed a significant reduction in transcripts related to specific neural assets in SMA motor neurons, including axon-related proteins [i.e. stathmin 2 (*STMN2*), proteolipid protein 1 (*PLP1*)], ion channels, particularly potassium channel, and synapses [i.e. synaptotagmin 13 (*SYT13*), and neurexin (*NRXN*) 1, 2, and 3] (Fig. 2B). An upregulation of neurofilament heavy polypeptide (*NEFH*) was detected (Table 1).

Cross-checking our RNA-Seq data (Fig. 2B) with data published in the literature, we found sets of genes differentially expressed between SMA and wild-type motor neurons that are likely involved in SMA vulnerability. We validated some of the genes by western blot and quantitative PCR (qPCR) analysis (Fig. 2C–G). We detected a reduction in glutamate ionotropic receptor AMPA-type subunit 1 (*GRIA1*) and, even though not significant, *GRIA2* and *GRIA4*, which encode core subunits of AMPA-type glutamate receptors that are critical for glutamatergic excitatory synapses (Zhang *et al.*, 2013). We also found dysregulation of IGF-related pathways, detecting the downregulation of insulin-like growth factor 1 (*IGF1*) and one of its substrates, insulin receptor substrate 4 (*IRS4*), in motor neurons, as well as the downregulation of *IGF2*. Both *IGF1* and *IGF2* play a significant role in SMA motor neuron survival, as we recently demonstrated (Allodi *et al.*, 2016). The pathology of SMA has been linked to the endoplasmic reticulum (ER) stress pathway and apoptosis (Ng

et al., 2015), and we evaluated whether some of these genes were deregulated. Some heat shock proteins, unfolded protein response (UPR), and apoptosis genes were upregulated, suggesting that activation of the UPR pathway in SMA motor neurons leads to hyperactivation of the pro-apoptotic branches of the UPR pathway. In terms of mRNA metabolism, we detected the dysregulation of some proteins involved in mRNA splicing and processing, including the upregulation of muscle blind-like protein 1 (*MBLN1*), the key gene involved in splicing dysregulation in myotonic dystrophy, and the downregulation of heterogeneous nuclear ribonucleoprotein c-like 1 (*hNRNPCL1*), RNA binding motif protein 47 (*RBM47*), and epithelial splicing regulatory protein 2 (*ESRP2*). This alteration can further promote transcriptional/splicing alterations. We also confirmed the downregulation of some key transcripts altered in other SMA models, such as chondrolectin (*CHODL1*) (Gavrilina *et al.*, 2008; Zhang *et al.*, 2008; Baumer *et al.*, 2009), neuritin (*NRN1*) (Akten *et al.*, 2011), and cellular retinoic acid-binding protein 1 (*CRABP1*), further supporting the potential role of these genes as downstream targets of RNA processing dysfunction induced by SMN deficiency.

Differential splicing analysis

SMN plays a crucial role in the biogenesis of spliceosomal snRNPs; thus, its deficiency is expected to impact splicing (Pellizzoni, 2007; Neuenkirchen *et al.*, 2008). Therefore, we started our analysis by searching for cassette exons that demonstrate a significant difference in the level of inclusion between SMA and wild-type motor neurons (alternative splicing differential analysis). A total of 12 144 cassette exons were deregulated in terms of the inclusion level; 2402 and 1035 exhibited a significant decrease and increase in inclusion, respectively, in patient samples with respect to the controls (Supplementary Table 6). Functional annotation analysis of genes containing both significantly silenced and enhanced cassette exons revealed that the most represented GO terms were in the categories ‘Molecular Function’, ‘Biological Process’, and ‘Cellular Component’ (Table 2, Supplementary Tables 4 and 7, and Supplementary Fig. 2).

Interestingly, the genes with the greatest degree of differential splicing, analysed with Reactome, belonged to functional classes considered responsible for SMA pathogenesis, including axon guidance and synapses (Supplementary Table 9). In the vast majority of cases, there was reduced inclusion of an exon.

Among differentially spliced genes, we identified genes involved in motor neuron diseases and function. In particular, *PIP5K1C* is the causative gene of Lethal Congenital Contracture Syndrome type 3 (LCCS3), a neurological syndrome in which patients present with rather selective atrophy of the spinal cord anterior horn, similar to SMA patients (Narkis *et al.*, 2007). *RAC1* has been implicated in motor neuron disease pathogenesis (D’Ambrosi *et al.*,

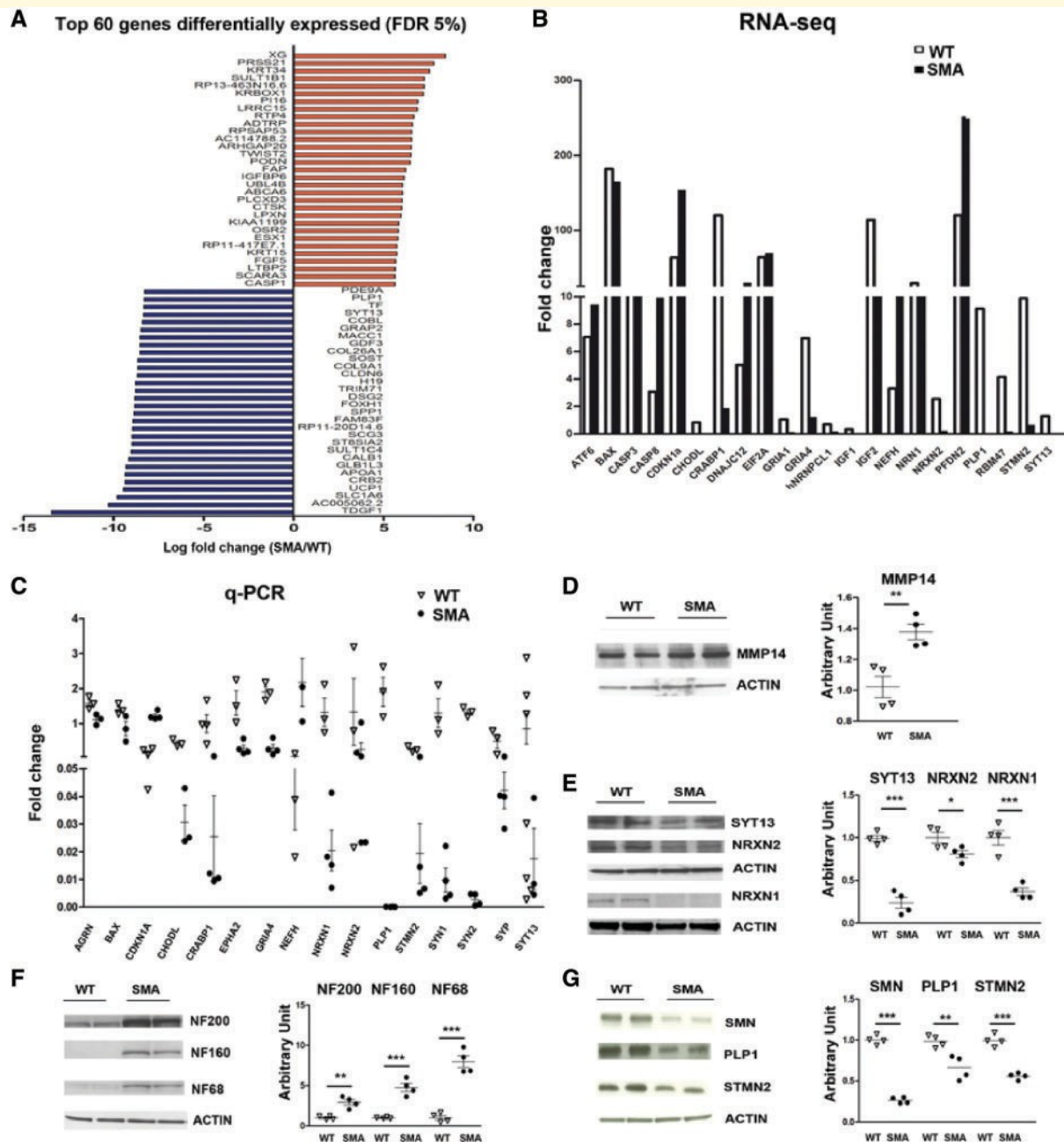


Figure 2 Transcriptional analysis of SMA versus wild-type-motor neurons by RNA-Seq analysis shows deregulation of specific mRNAs involved in motor neuron functions. (A) Top 60 genes (based on fold change) differentially expressed in SMA versus wild-type motor neurons with an FDR of 5%. (B and C) Comparison of RNA-Seq and quantitative RT-PCR data for a selected number of transcripts in wild-type and SMA motor neurons. *STMN2* (** $P \leq 0.001$), *PLP1* (* $P \leq 0.05$), *NEFH* (* $P \leq 0.05$), *NRXN1* (* $P \leq 0.05$), *SYN1* (* $P \leq 0.05$), *SYN2* (** $P \leq 0.0001$), *EPH2A* (* $P \leq 0.05$), *SYP* (* $P \leq 0.05$), *CRABP1* (** $P \leq 0.01$), *CDKN1A* (** $P \leq 0.0001$), *CHODL* (* $P \leq 0.05$), *GRIA4* (** $P \leq 0.01$). Student's *t*-test. Error bars represent \pm SEM from three independent experiments. (D–G) Representative image of western blot of selected proteins in SMA motor neurons compared to wild-type motor neurons: *MMP14*: ** $P \leq 0.01$; *SYT13*: *** $P \leq 0.0001$; *NRXN2*: * $P \leq 0.05$; *NRXN1*: *** $P \leq 0.001$; *NF200*: ** $P \leq 0.01$; *NF160*: *** $P \leq 0.001$; *NF68*: *** $P \leq 0.001$; *SMN*: *** $P \leq 0.0001$, *PLP1*: ** $P \leq 0.01$; *STMN2*: *** $P \leq 0.001$, Student's *t*-test. Data are presented as mean \pm SEM from three independent experiments.

2014). Furthermore, *SINJ1* and *RIMS1* were found as alternative spliced genes specifically in motor neurons in a pathway dependent from FUS protein, a key gene in amyotrophic lateral sclerosis (ALS) (Honda *et al.*, 2013).

We also identified genes that were previously detected as misplished in rodent SMA-motor neurons (Zhang *et al.*, 2013), such as microtubule affinity regulating kinase 2 (*MARK2*), calcium/calmodulin dependent protein kinase II

Table 1 Selected enriched gene sets obtained with Reactome analysis that included terms related to axon-related proteins, synapses, and potassium channels

Category	Gene	Expression
Axon-related protein	NEFH	Up
	<i>STMN2</i>	Down
	<i>PLP1</i>	Down
	<i>NCAN</i>	Down
	<i>RGMA</i>	Down
	<i>UNC5A</i>	Down
Synapsis	<i>NRXN1</i>	Down
	<i>NRXN2</i>	Down
	<i>NRXN3</i>	Down
	SYT7	Up
	SYT9	Down
	SYT11	Up
	SYT13	Down
	VAMP8	Down
	SYCP2	Down
	RIMS4	Down
	BSN	Down
	SNAP91	Down
	STXBP1	Up
Potassium channels	KCNA3	Down
	KCNA5	Down
	KCNC1	Down
	KCND2	Down
	KCNFI	Down
	KCNGB1	Down
	KCNJ10	Down
	KCNK3	Down
	KCNN1	Down
	KCNN2	Down
	KCNQ3	Up
	KCNC4	Up
	KCNN4	Up

The selected genes upregulated and downregulated in SMA motor neurons are shown in bold and italics, respectively.

delta (*CAMK2D*), agrin (*AGRN*) (Zhang *et al.*, 2013), which is critical for NMJ maintenance, and M-phase phosphoprotein 9 (*MPHOSPH9*) (Supplementary Table 9). Some of these genes with altered splicing were validated by RT-PCR in our SMA motor neurons (Supplementary Fig. 7A).

Overall, our data confirm the role of SMN in splicing modulation, particularly for genes related to axon guidance and synapses.

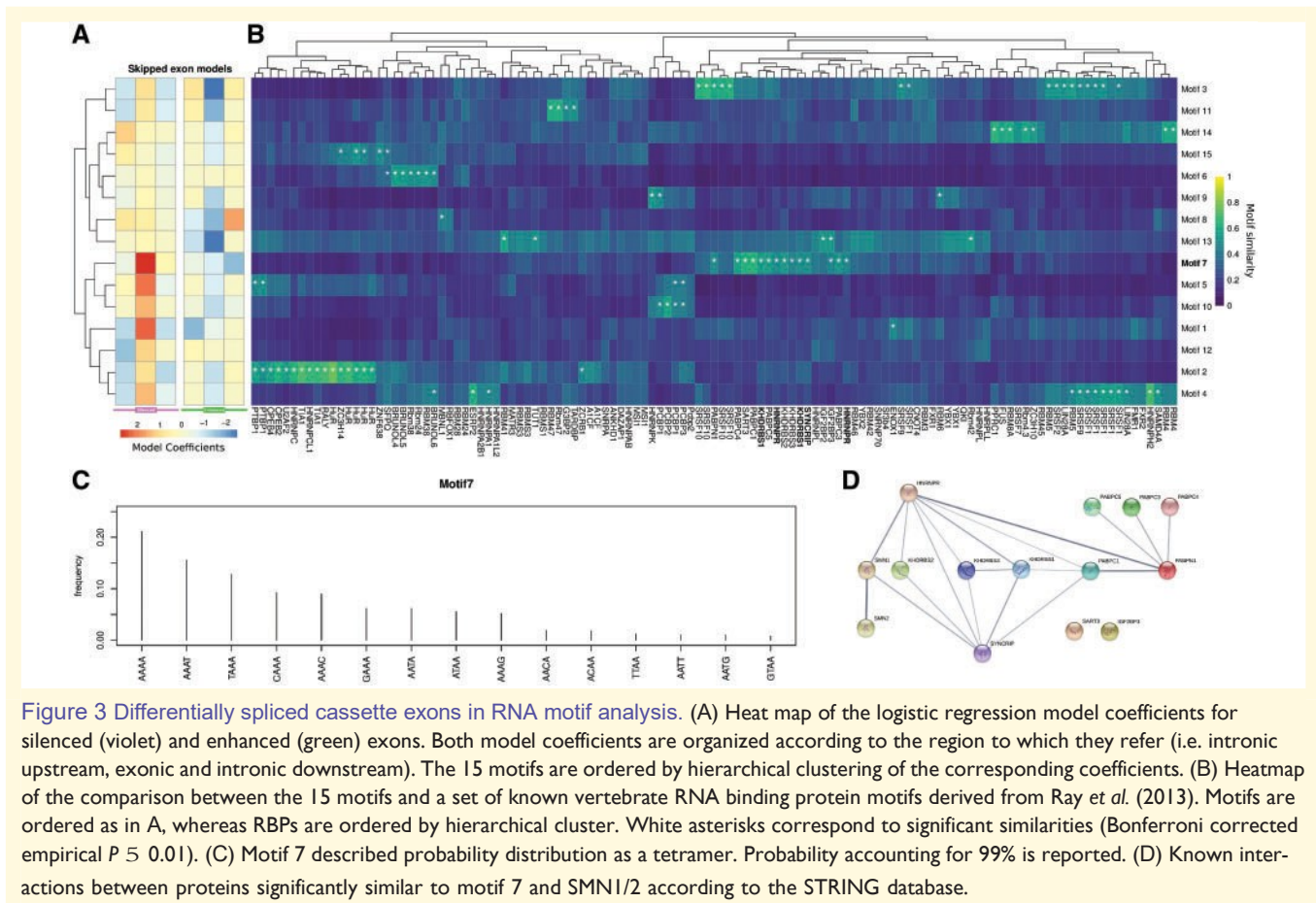
RNA binding motif analysis

We searched for common RNA sequence motifs (i.e. tetramer distributions) that could predict either silenced or enhanced exons in the whole set of analysed cassette exons. In logistic regression, we found 15 motifs (Supplementary Fig. 3) with an area under the curve (AUC) of 0.629 and 0.623 for enhanced and silenced

Table 2 GO terms of differentially spliced genes in SMA-motor neurons versus wild-type motor neurons ($P \leq 0.05$) using DAVID analysis

GO category	GO terms
Molecular function	RNA binding
	Protein binding
	Cytoskeletal protein binding
	Actin binding
	Tubulin binding
	Microtubule plus-end binding
	Protein serine/threonine kinase activity
	Rab GTPase activator activity
	RNA methyltransferase activity
Biological process	Cell division
	Cell cycle, differentiation, secretion and proliferation
	Regulation of translation and signal transduction
	RNA and snRNA
	Synaptosome
	Flotillin complex
Cellular component	Membranes
	Intracellular organelles
	Mitochondrion and mitochondrial membrane
	Cell junctions
	Microtubules
	Endoplasmic reticulum
	Endocytic vesicle

cassette exons, respectively. Each model assigned three different weights to each motif in exonic, upstream, and downstream intronic regions (Fig. 3A and B). In particular, motif 7 (Fig. 3C) in the silenced exonic region had the highest positive weight, suggesting an enrichment in these regions. We hypothesized that the altered expression of transcript variants among multiple genes is dependent on the interaction of common RBPs with SMN. To examine this possibility, the 15 motifs were compared to a set of 119 vertebrate RNA binding protein motifs derived from a previous work (Ray *et al.*, 2013) using a similarity measure. We completed an inventory of the common motifs and their interacting RBPs (Fig. 3B). Significant similarities were observed, particularly for motif 7, to a group of polyA binding proteins involved in splicing and other mRNA processing, transport, and transcription (Glisovic *et al.*, 2008). Among these, hnRNPQ (SYNCRIP), HNRNPR, and KHDRBS1 have been described as interacting with the full-length SMN protein (Rossoll *et al.*, 2002; Pagliarini *et al.*, 2015; Geuens *et al.*, 2016). The interaction between these polyA binding proteins and SMN are illustrated in Fig. 3D. SYNCRIP emerged as one of the top RBPs regulating the splicing of dysregulated transcripts in SMA cells. SYNCRIP is an SMN-interacting protein partner highly related to hnRNPR (Rossoll *et al.*, 2002). In our experiments, one of the most frequent motifs with highly probable interaction with SYNCRIP is the motif identified as 7 (Fig. 3C). This finding indicates a possible link between the loss of the interaction between SYNCRIP and full-length SMN and inefficient pre-mRNA splicing downstream.



Given the possible role of RBPs in transcription and post-transcriptional regulation other than splicing, we wanted to test whether enrichment of these motifs can be detected in differentially expressed genes. Thus, we devised a motif enrichment procedure to test the coding, 5', and 3' UTR sequences of differentially expressed genes for motif enrichment (Supplementary Table 8). We found one motif enriched in 5' UTRs, none in CDSs, and six in 3' UTRs (including motif 7). No motif was depleted in 5' UTRs, four in CDS, and three in 3' UTRs. Remarkably, some genes with high motif 7 scores in 3' UTRs exhibited axonal and synaptic functions, including *NRXN1*, *NRXN2*, and *SYT13*.

NRXN2 protects vulnerable SMA patient motor neurons from degeneration

Among the deregulated genes with motif 7, we focused our attention on *NRXN2* (Fig. 5A), which has already been associated with SMA in animal models and is relevant for motor neuron survival and function (See *et al.*, 2014). The reduction in *NRXN2* was further confirmed by qPCR ($P \leq 0.05$, Fig. 4B).

To confirm the role of *NRXN2* in SMA pathogenesis, we investigated whether the upregulation of *NRXN2* can halt the degeneration of human patient-specific SMA iPSC-derived motor neurons. SMA motor neurons (monitored by Hb9::eGFP) were transfected with a vector encoding the human cDNA for *NRXN2*, confirming its upregulation after infection ($P \leq 0.01$ Fig 4C and D). SMA motor neurons present apparent cell autonomous degeneration *in vitro* after 8 weeks of culturing (Corti *et al.*, 2012; Allodi *et al.*, 2016), and in the present study overexpression of *NRXN2* in SMA-motor neurons significantly improved their survival ($P \leq 0.0001$, Fig. 4E and F) and increased neurite length compared to *null*-treated SMA motor neurons ($P \leq 0.001$, Fig. 4G). These data suggest that *NRXN2* can rescue the disease phenotype in a human SMA model.

SYNCRIP protects vulnerable SMA motor neurons, increasing SMN and NRXN2 expression

We investigated whether the upregulation of SYNCRIP could rescue the expression levels of *NRXN2*, which harbours motif 7, halting the degeneration of human patient-specific SMA iPSC-derived motor neurons (Fig. 5).

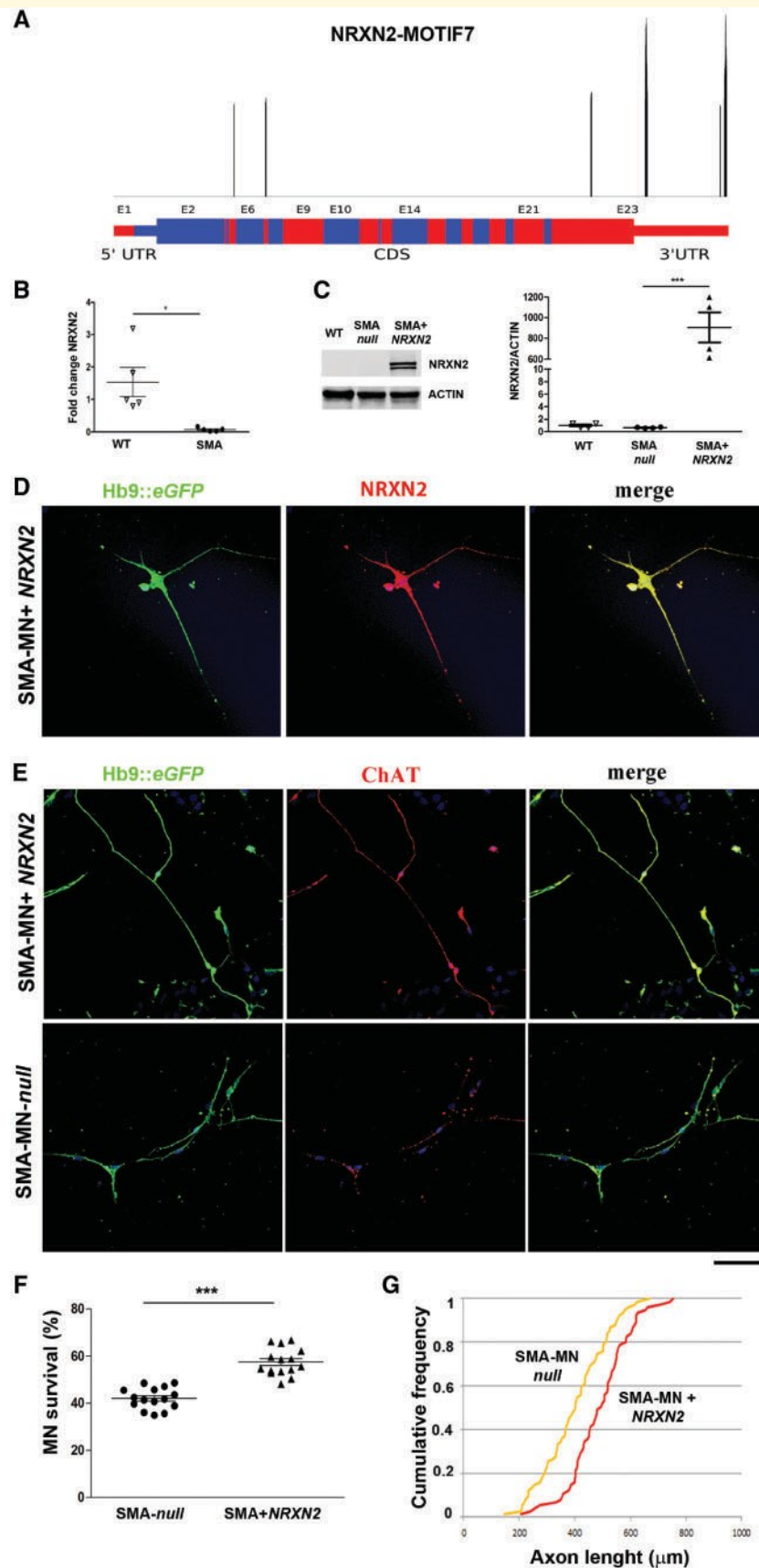


Figure 4 NRXN2 protects human SMA-motor neurons from degeneration. (A) Significant motif 7 peaks along the NRXN2 transcript (RefSeq ID NM_015080). Only significant peaks are reported (Bonferroni corrected $P \leq 0.01$). Red and blue shades represent exon boundaries, thinner lines the UTRs. (B) Quantitative PCR analysis showed a significant decrease in the expression of NRXN2 transcript in SMA-motor

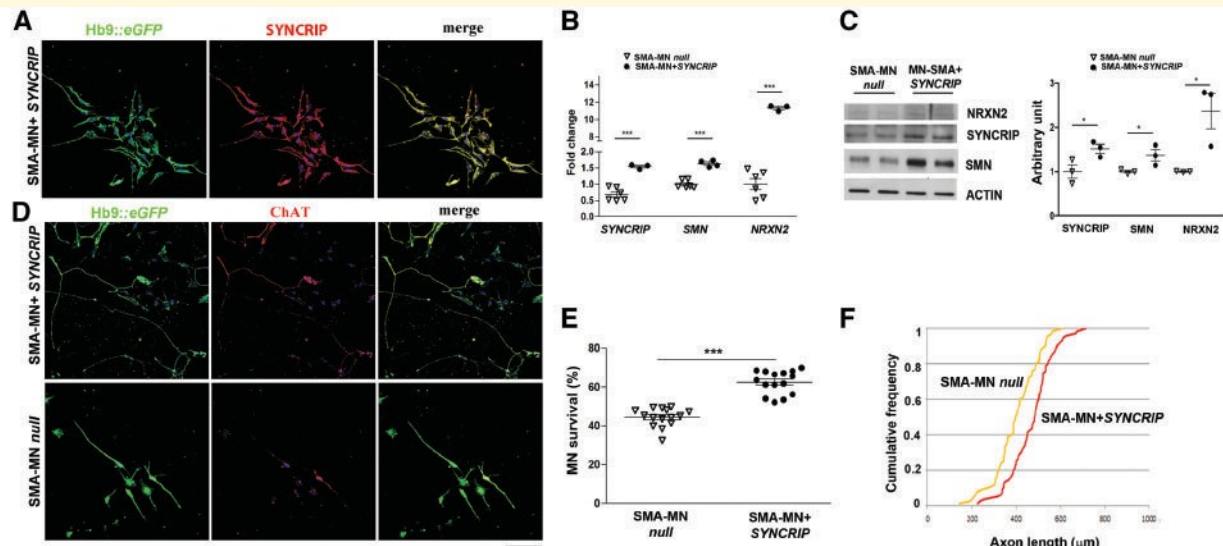


Figure 5 SYNCRIP protects human SMA motor neurons from degeneration by increasing NRXN2 and SMN levels.

(A) Representative images of SMA motor neurons after SYNCRIP transfection (Hb9::eGFP, green; SYNCRIP, red). Nuclei are labelled with DAPI (blue). (B) q-PCR analysis showed a significant increase in the expression of SYNCRIP, SMN, and NRXN2 transcripts in SMA-motor neurons compared to null SMA-motor neurons. $***P \leq 0.001$, student t-test. Data are presented as mean \pm SEM from three independent experiments. (C) Western blot representative image of SYNCRIP, SMN, and NRXN2 in SMA motor neurons after SYNCRIP overexpression compared to SMA-null cells. Western blot analysis revealed an increased level of SYNCRIP ($*P \leq 0.05$), SMN ($*P \leq 0.05$), and NRXN2 ($*P \leq 0.05$) in SMA motor neurons after SYNCRIP overexpression compared to SMA-null cells. Data are presented as mean \pm SEM of three independent experiments. (D) Representative images of SMA motor neurons (Hb9::eGFP, green; ChAT, red) with and without SYNCRIP transfection. Nuclei are labelled with DAPI (blue). (E) The number of SYNCRIP SMA-motor neurons in long-term culture was significantly increased compared to SMA motor neurons null. SYNCRIP overexpression was protective to motor neurons. $***P \leq 0.0001$, Student t-test. Data are presented as mean \pm SEM of five independent experiments. (F) At 8 weeks, SMA motor neurons overexpressing SYNCRIP showed longer axons than SMA motor neurons null. $P \leq 0.001$, Kolmogorov-Smirnov test. Scale bar = 75 μ m.

SMA motor neurons (monitored by Hb9::eGFP) were transfected with a vector expressing the human cDNA for SYNCRIP (Fig. 5A and B). A subsequent increase in NRXN2 was detected by both qPCR ($P \leq 0.0001$, Fig. 5B) and western blot ($P \leq 0.05$, Fig. 5C), confirming our hypothesis of SYNCRIP having a role in its expression. SYNCRIP has also been described to modulate the splicing of SMN2, promoting the inclusion of exon 7 (Chen *et al.*, 2008). In line with this finding, we demonstrated increased expression of SMN in SYNCRIP-treated SMA-motor neurons by q-PCR ($P \leq 0.001$, Fig. 5B) and western blot ($P \leq 0.05$, Fig. 5C). Overexpression of SYNCRIP in SMA-motor neurons significantly improved their survival ($P \leq 0.0001$, Fig. 5D and E) and increased neurite length compared to null-treated SMA motor neurons ($P \leq 0.001$,

Fig. 5F). Therefore, the missing interaction between SMN and SYNCRIP could be the cause of NRXN2 reduction, contributing to the SMA phenotype. Our hypothesis is that the complex SYNCRIP/SMN can bind NRXN2 3'UTR through motif 7 regulating its mRNA and protein expression.

To validate the hypothesis of a direct interaction between SMN, SYNCRIP, and NRXN2, we downregulated SYNCRIP with siRNA in the human neural SH-SY5Y cell line. SYNCRIP silencing induced a significant reduction in SMN levels, as expected (mRNA $P \leq 0.001$, protein $P \leq 0.05$), and a marked decrease in NRXN2 levels in q-PCR and western blot analysis ($P \leq 0.001$, Supplementary Fig. 5A–C), confirming the presence of a positive loop among the three proteins (Supplementary Fig. 5D).

Figure 4 Continued

neurons compared to wild-type. $*P \leq 0.05$, Student's t-test. Data are presented as mean \pm SEM from three independent experiments. (C) Representative image of NRXN2 western blot in wild-type motor neurons, SMA motor neurons null and SMA-NRXN2. NRXN2 overexpression in SMA motor neurons was confirmed with respect to SMA null ($***P \leq 0.01$, Student's t-test). Data are presented as mean \pm SEM from three independent experiments. (D) Representative images of SMA-motor neurons after NRXN2 transfection (Hb9::eGFP, green; NRXN2, red). Nuclei are labelled with DAPI (blue). (E) Representative images of SMA-motor neurons (ChAT, red; Hb9::eGFP, green) with and without NRXN2 transfection. Nuclei are labelled with DAPI (blue). (F) The number of NRXN2-treated SMA motor neurons in long-term culture was significantly increased compared to SMA motor neurons null. NRXN2 overexpression was protective to motor neurons. $***P \leq 0.0001$, Student t-test. Data are presented as mean \pm SEM of five independent experiments. (G) At 8 weeks, SMA-motor neurons overexpressing NRXN2 had longer axons than SMA motor neurons null. $P \leq 0.001$, Kolmogorov-Smirnov test. Scale bar = 75 μ m.

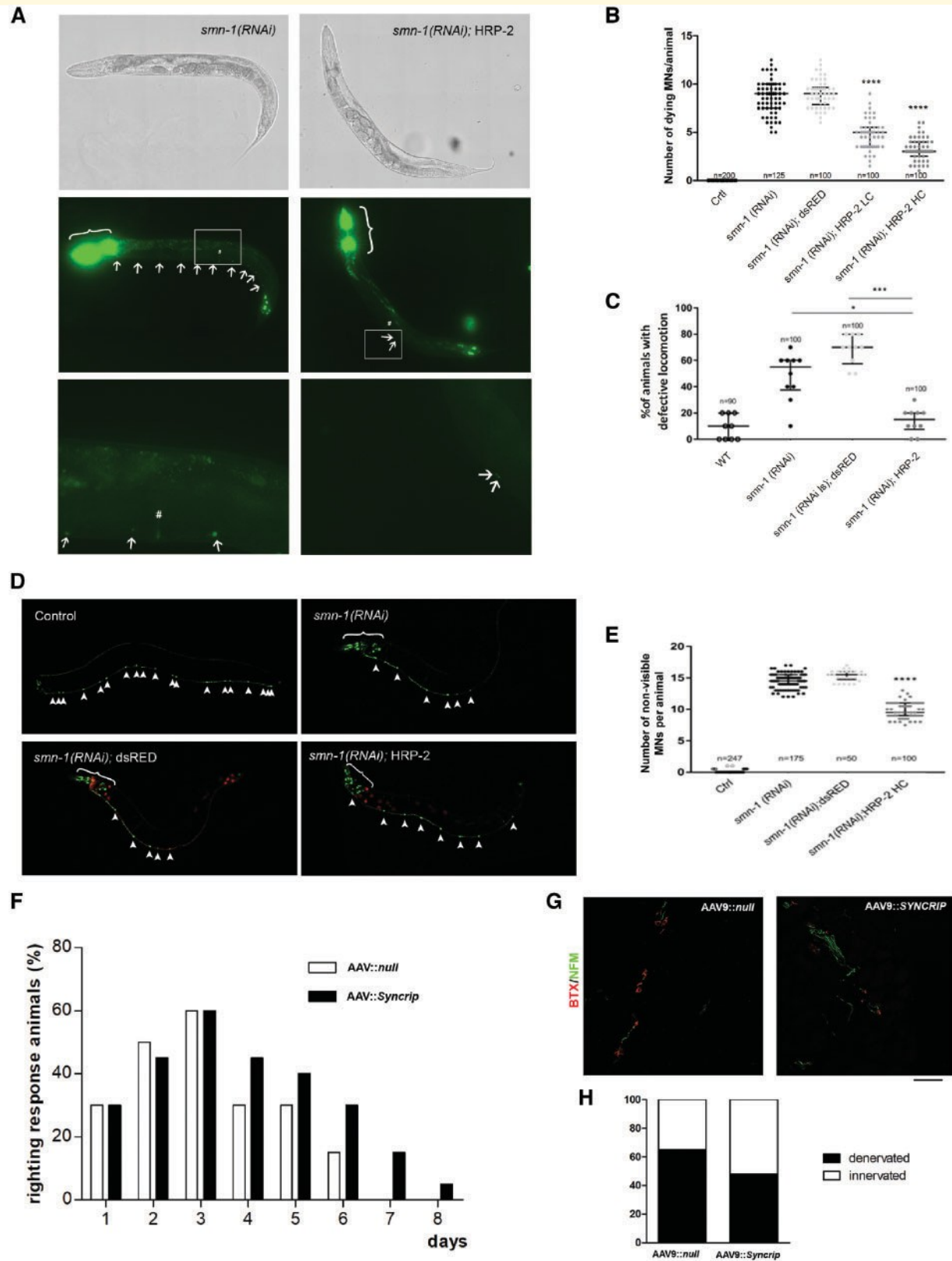


Figure 6 SYNCRIP ameliorated the disease phenotype in *in vivo* models of SMA. (A–E) *C. elegans*; (F–H) mouse model. (A) Transgenic animals expressing HRP-2 in all neurons and silenced for *smn-1* (right) present fewer dying neurons (arrows) than *smn-1*(RNAi) animals (left). In all panels, anterior is left and ventral is down. The animals were observed using brightfield (top) or epifluorescence (middle and bottom). The top and middle panels were reconstructed using the Tile Scan function of the Leica LAS X program. Middle: The co-injection marker was expressed in the pharynx and the nuclei of the most posterior intestinal cells (brackets). Bottom: Enlargements of the areas outlined in the middle panels around the vulva (#). (B) Quantification of dying motor neurons in non-silenced control (Ctrl), *smn-1*(RNAi), *smn-1*(RNAi)

Intiguingly, we found that even *SYT13* and *STMN2* expression, deregulated in SMA, directly correlate with *SYNCRIP* expression (Supplementary Fig. 4, $P \leq 0.0001$), suggesting that the interaction between SMN and *SYNCRIP* may account for the observed dysregulation of other key motor neuron genes in SMA.

Syncrip overexpression ameliorates pathological phenotype in SMA *in vivo* models

We next investigated if overexpression of *Syncrip* in SMA animal models could improve the disease phenotypes. First, we used *C. elegans* depleted of *smn-1* in D-type motor neurons (Gallotta *et al.*, 2016). The *SYNCRIP* homolog in *C. elegans* is *hrp-2* (NM_060648.3), a gene expressed in all tissues, including D-type motor neuron (Blazie *et al.*, 2017), during embryogenesis, larval development, and in adult animals (Kinnaid *et al.*, 2004). The protein is localized in the nuclei and has nucleic acid binding activity, and its depletion causes defects in embryonic and larval development, fertility (Kinnaid *et al.*, 2004), lifespan (Heintz *et al.*, 2017), and splicing (Kabat *et al.*, 2009; Heintz *et al.*, 2017). To test whether *hrp-2* genetically interacts with *smn-1* (*Smn1* homologue), we expressed *hrp-2* under the control of a pan-neuronal promoter (*punc-119::hrp-2*, referred to as HRP-2), in *smn-1(RNAi)* knocked-down *C. elegans*. The *smn-1(RNAi)* animals used in this work are transgenic for D-type motor neurons with specific silencing of *smn-1* at a very high dose (Gallotta *et al.*, 2016). These animals are viable and fertile but with motor neuron degeneration, followed by their death and strong impairment in backward locomotion (Gallotta *et al.*, 2016).

Overexpression of HRP-2 at two different concentrations (low and high) halved the number of dying motor neurons per animal, from 8.6 in *smn-1(RNAi)* to 4.7 in *smn-1(RNAi);HRP-2 LC* and 3.3 in *smn-1(RNAi);HRP-2 HC* ($P \leq 0.0001$ in both cases, Fig. 6A and B). The rescue obtained is dose-dependent and gene-specific because the expression of an unrelated protein under the same conditions, such as dsRED fluorescent protein, did not rescue this phenotype (nine dying motor neurons/animal). Therefore, we used the most effective concentration (high concentration) to determine whether HRP-2 was able to rescue another earlier phenotype caused by *smn-1(RNAi)*, neuronal degeneration. HRP-2 partially rescued the disappearance of viable motor neurons expressing GFP; 51.7% of the 19 expected neurons degenerated in *smn-1(RNAi);HRP-2 HC* compared to 76.5% in *smn-1(RNAi)* animals and 80.7% in *smn-1(RNAi);dsRED* ($P \leq 0.0001$ in both cases, Fig. 6D and E). Finally, we tested the ability of HRP-2 to rescue the lack of *smn-1* at the functional level. Notably, the locomotion defect with *smn-1(RNAi)* was fully rescued by HRP-2 expression, reducing the number of animals with an abnormal locomotion behaviour from 48% in *smn-1(RNAi)* and 68% in *smn-1(RNAi);dsRED* to 14% in *smn-1(RNAi);HRP-2 HC* ($P = 0.0303$ and $P = 0.0001$, respectively), which is similar to that observed in wild-type animals (9%, $P \leq 0.05$, Fig. 6C). Our results demonstrate that HRP-2 plays a conserved role in the SMN pathway in neurons, and its overexpression can rescue neuron degeneration and death, and the locomotion defects caused by *smn-1* silencing.

We also evaluated the effect of *Syncrip* overexpression in a more complex *in vivo* SMA model, SMAD7, which presents a severe SMA phenotype type 1, characterized by loss

Figure 6 Continued

expressing dsRED (negative control), and HRP-2 at low (LC) and high (HC) concentrations. **** $P \leq 0.0001$ between *smn-1(RNAi);HRP-2 LC* and HC versus *smn-1(RNAi)* and *smn-1(RNAi);dsRED* (one-way ANOVA with Kruskal-Wallis test). Each dot represents the number of apoptotic fluorescently-labeled dying motor neurons in a single animal. The median with interquartile range is shown. *n* is the total number of animals observed from two independent transgenic lines. (C) Knock-down of *smn-1* in D-type motor neurons leads to a locomotion defect not observed in wild-type (WT). This defect is rescued by HRP-2 but not by dsRED expression. * $P = 0.0303$, *smn-1(RNAi);HRP-2* versus *smn-1(RNAi)* and **** $P = 0.0001$ *smn-1(RNAi);HRP-2* versus *smn-1(RNAi);dsRED* (one-way ANOVA with Kruskal-Wallis test). Each dot represents the percentage of animals with a defective backward, among 10 animals tested. The median with interquartile range is shown. *n* is the total number of animals tested from at least two independent transgenic lines. (D) Transgenic animals expressing HRP-2 in all neurons and silenced for *smn-1* (bottom right) present more visible/viable neurons labelled with GFP (arrowheads) than *smn-1(RNAi)* (top right) or *smn-1(RNAi);dsRED* animals (bottom left). All 19 motor neurons are visible in the ventral cord in control animals (top left). Motor neurons are visible because of the expression of GFP in the ventral cord, transgene *oxls12[punc-47::GFP]*. Additional cells express GFP in the head because of the expression of the *pchs-2::GFP* co-injection marker (brackets, top right and bottom). Red fluorescence is visible in all neurons in dsRED animals (bottom left) or in the intestinal nuclei because of *pelt-2::RFP* co-injection marker (bottom right). (E) Quantification of the percentage of degenerating neurons in wild-type (Ctrl), *smn-1(RNAi)*, and *smn-1(RNAi)* expressing dsRed or *hrp-2*. The *smn-1* knock-down induced the degeneration of motor neurons, and this defect was partially rescued by HRP-2 expression. **** $P \leq 0.0001$ *smn-1(RNAi);HRP-2* versus *smn-1(RNAi)* and *smn-1(RNAi);dsRED* (one-way ANOVA with Kruskal-Wallis test). Each dot represents the number of degenerated motor neurons in a single animal, among 19 that are visible in the wild-type. The median with interquartile range is shown. *n* is the total number of animals observed from two independent transgenic lines. (F) The righting reflex was significantly improved in SMA mice injected ICV at P1 with AAV9::Syncrip compared to AAV9::null SMA mice beginning on Day 4. Notably, after P6, no AAV9::null SMA mice were able to right, whereas a percentage of SMA mice overexpressing *Syncrip* maintained the righting reflex function. ** $P \leq 0.01$, contingency chi-squared test ($X^2 = 23.76$), $n = 5$ mice/group. (G and H) Quantification of presynaptic NF-M (green) and postsynaptic a-bungarotoxin (red) signal in intercostal muscles showed a significant increase in NMJ innervation in AAV9::Syncrip SMA mice compared to AAV9::null SMA mice. * $P \leq 0.05$, contingency chi-squared test ($X^2 = 5.879$), $n = 100$ NMJs analysed for each animal, three mice/group.

of motor neurons and neuromuscular function, NMJ denervation, and dramatically reduced survival (Le *et al.*, 2005). We intracerebroventricularly injected SMA mice at P1 with AAV9, a vector with a particular tropism for the CNS (Foust *et al.*, 2009), carrying *Syncrip*. *Syncrip* overexpression was able to improve righting performance in SMA mice (Fig. 6F, $P \leq 0.01$, $n = 5$, contingency and $X^2 = 23.76$) and increase neuromuscular junction innervation (Fig. 6G, $P \leq 0.05$, $n = 3$, contingency and $X^2 = 5.879$). However, the treatment did not show significant results in terms of survival elongation (data not shown).

As demonstrated by western blot (Supplementary Fig. 6), we detected significantly higher levels of SYNCRIP ($P \leq 0.05$), as expected, but also SMN and NRXN2 (both $P \leq 0.05$) in the brains of treated mice, confirming *in vivo* the hypothesis of a positive loop among these three genes.

Discussion

SMA is a severe, disabling, inherited neurological disease characterized by motor neuron degeneration (Pellizzoni *et al.*, 1998) and SMN protein depletion. Nusinersen, the first approved therapeutic compound, became available at the end of 2016 (Finkel *et al.*, 2016) (www.curema.org), even if it is unclear whether this approach can be resolute for all types of patients. The implementation of successful therapy requires better understanding of the molecular mechanisms underlying the disease, particularly regarding the selectivity of motor neuron loss. Patient-specific iPSCs represent a novel tool for *in vitro* disease modelling and therapeutic discovery in neurodegenerative diseases that involve a specific neuronal population (Frattini *et al.*, 2015). Here, we differentiated SMA patient iPSCs into motor neurons, confirming their reduced survival and axonal length in culture. The role of SMN in mRNA processing is well established (Pellizzoni *et al.*, 1998), even if the mechanisms of this function are still not completely elucidated.

To understand mRNA-related dysregulation linked to selective motor neuron vulnerability, we performed whole RNA-Seq on SMA and wild-type motor neurons, targeting 40 M clusters per sample. The gene expression profiles clearly distinguished the two groups, specifically 1084 downregulated and 808 upregulated genes. Among the most deregulated gene sets, we observed a significant reduction in transcripts linked to specific neural assets in SMA motor neurons, including axon-related proteins (*STMN2* and *PLP1*), ion channels, particularly potassium channel, and synapses (i.e. *SYT13* and *NRXN1*, *NRXN2*, *NRXN3*). We validated the relevant genes by quantitative PCR and/or western blot analysis, including genes critical for motor neuron function, such as *GRIA1*, *GRIA12*, *GRIA14* and *IGF1/IGF12*. Remarkably, we confirmed the downregulation of some key transcripts already found to be altered in other SMA models (Pellizzoni *et al.*, 1998), such as *CHODL1*, *NRN1*, and *CRABP1*.

Based on the known role of SMN in the biogenesis of spliceosomal snRNPs (Pellizzoni *et al.*, 2002), an effect of SMN deficiency on splicing is expected. Therefore, we analysed the exon alternatively spliced in SMA motor neurons compared to wild-type motor neurons. We found 12 144 deregulated cassette exons belonging to genes involved in motor neuron diseases and function, such as *MARK2*, *CAMK2D*, *AGRN*, and *MPHOSPH9*.

We determined whether differentially spliced genes share some common gene sequence motifs, which could predict either silenced or enhanced exons in the whole set of cassette exons analysed. We found 15 significant motifs and compared them to the most complete set of known RBP motifs (Ray *et al.*, 2013) to detect significant binding probabilities. Interestingly, motifs identified in differentially spliced exons, particularly motif 7, match those of known RBPs involved in mRNA splicing, transcription, and post-transcriptional regulation. As these processes could have an effect in determining the level of gene expression, we evaluated the enrichment of these motifs in differentially expressed genes. Strikingly, some genes with high motif 7 enrichment in the 3' UTR exhibit axonal and synaptic functions, including *NRXN1*, *NRXN2*, and *SYT13*.

We focused our attention on *NRXN2*, which is associated with axonogenesis, synaptogenesis, and synaptic function and is relevant for motor neuron survival and function. *NRXN2*, a neuron-specific gene encoding a presynaptic cell adhesion protein that associates with postsynaptic proteins, such as neuroligin, is involved in Ca^{2+} -evoked neurotransmitter release (Ushkaryov *et al.*, 1992; Missler *et al.*, 2003). Moreover, reduced expression and decreased inclusion of exon 12 has been shown in SMN-deficient zebrafish embryos. *Nrxn2a* knock-down in zebrafish embryos led to motor axon branching defects, as well as reduced Ca^{2+} influx into presynaptic terminals, resembling SMA disease (See *et al.*, 2014). Reduced expression and altered alternative splicing of *Nrxn2a* mRNA have also been identified in both the spinal cord and motor neurons of mammals, particularly in severe transgenic SMA mice (See *et al.*, 2014).

Here, we detected for the first time reduced expression of *NRXN2* and decreased inclusion of exon 12 in human SMA motor neurons. Furthermore, our *in vitro* experiments showed that *NRXN2* upregulation was protective for SMA motor neurons, improving survival and increasing neurite length. Based on these data, we hypothesize that *NRXN2* alteration can account for the axon and pre-synaptic defects at neuromuscular endplates in SMA pathophysiology, representing a potential non-SMN therapeutic target.

The RBPs with a higher probability of binding motif 7, the polyA binding protein, include SYNCRIP (hnRNPQ), which was described previously as directly interacting with RG-rich domains of full-length SMN protein (Mourelatos *et al.*, 2001). Interestingly, SYNCRIP interacts only with full-length SMN, and not with truncated or mutant SMN

forms identified in SMA; this may account for the inefficient pre-mRNA expression and splicing in SMA patients (Mourelatos *et al.*, 2001). On the other hand, SYNCRIP upregulation has been described in pauci-symptomatic siblings of SMA patients, supporting its role as a protective gene (Pellizzoni *et al.*, 1998). Finally, SYNCRIP is a splicing modulator of SMN, promoting the inclusion of exon 7 in *SMN2*, probably by activating the use of its upstream 3' splice site (Chen *et al.*, 2008). Intriguingly, SYNCRIP emerged as one of the top RPBs with high probability of interacting with motif 7, which is also present in *NRXN2* mRNA. We demonstrated that SYNCRIP upregulation rescued *NRXN2* levels, suggesting that it can stabilize the *NRXN2* mRNA and increase its expression by interacting with motif 7. Furthermore, increased expression of SMN protein was found in SYNCRIP-treated SMA-motor neurons, confirming the ability of SYNCRIP to enhance SMN levels (Chen *et al.*, 2008). We demonstrated that SYNCRIP overexpression increased *in vitro* SMA motor neuron survival and axonal outgrowth, likely due to the upregulation of SMN and downstream target *NRXN2*. Finally, we demonstrated that SYNCRIP overexpression rescued neuron survival and function *in vivo* in a *C. elegans* SMA model and ameliorated the pathological phenotype in *SMN*^{Δ7} mice. These data highlighted its conserved role in the SMN pathway in neurons, making SYNCRIP a promising therapeutic target and modifier in SMA. We hypothesized the presence of a positive loop among SYNCRIP, SMN, and *NRXN2*, which was further demonstrated in our experiments on SH-SY5Y cells in which SYNCRIP was silenced and in the brain of mice overexpressed for *Syncrrip* with AAV9. Because motif 7 is present in other motor neuron altered genes, such as *SYT13*, we propose that the interaction between SMN and SYNCRIP is crucial in SMA, regulating the expression of several motor neuron genes. Furthermore, this hypothesis is supported by the correlation detected between *SYNCRIP* expression and the expression of two other key motor neuron genes deregulated in SMA *SYT13* and *STMN2*.

Overall, we performed the first detailed analysis of the correlation between SMN defects, specific gene expression, and splicing alterations in human SMA motor neurons, identifying common motif sequences in altered genes. This study led us to identify non-SMN therapeutic targets, such as *NRXN2* and especially SYNCRIP. Moreover, we demonstrated that the SMN/SYNCRIP interaction is likely responsible for the altered mRNA processing of several key motor neuron genes in SMA, contributing to motor neuron degeneration, and that its modulation can be crucial to modifying the SMA phenotype. The therapeutic approach presented in this study can also be combined with the already approved SMN-targeted therapy (nusinersen), to complement the beneficial effect and in particular when it is not completely successful, for example in patients who are already symptomatic.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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