

1 **miR-1/206 down-regulates splicing factor Srsf9 to promote myogenesis**

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45 **Abstract:**

46 Background Myogenesis is driven by specific changes in the transcriptome that occur during the
47 different stages of muscle differentiation. In addition to controlled transcriptional transitions,
48 several other post-transcriptional mechanisms direct muscle differentiation. Both alternative
49 splicing and miRNA activity regulate gene expression and production of specialized protein
50 isoforms. Importantly, disruption of either process often results in severe phenotypes as reported
51 for several muscle diseases. Thus, broadening our understanding of the post-transcriptional
52 pathways that operate in muscles will lay the foundation for future therapeutic interventions.

53 Methods We employed bioinformatics analysis in concert with the well-established C2C12 cell
54 system for predicting and validating novel miR-1 and miR-206 targets engaged in muscle
55 differentiation. We used reporter gene assays to test direct miRNA targeting and studied C2C12
56 cells stably expressing one of the cDNA candidates fused to a heterologous, miRNA-resistant 3'
57 UTR. We monitored effects on differentiation by measuring fusion index, myotube area, and
58 myogenic gene expression during time course differentiation experiments.

59 Results Gene ontology analysis revealed a strongly enriched set of putative miR-1 and miR-206
60 targets associated with RNA metabolism. Notably, the expression levels of several candidates
61 decreased during C2C12 differentiation. We discovered that the splicing factor Srsf9 is a direct
62 target of both miRNAs during myogenesis. Persistent Srsf9 expression during differentiation
63 impaired myotube formation and blunted induction of the early pro-differentiation factor
64 myogenin as well as the late differentiation marker sarcomeric myosin, Myh8.

65 Conclusions Our data uncover novel miR-1 and miR-206 cellular targets and establish a
66 functional link between the splicing factor Srsf9 and myoblast differentiation. The finding that

67 miRNA-mediated clearance of Srsf9 is a key myogenic event illustrates the coordinated and
68 sophisticated interplay between the diverse components of the gene regulatory network.

69 **Keywords:** miR-1; miR-206; Srsf9; Srp30c; skeletal muscle; myogenesis; cellular
70 differentiation; miRNA

71 **Background:**

72 A complex network of integrated transcriptional and post-transcriptional regulatory
73 mechanisms control skeletal muscle gene expression. Overall, skeletal muscle displays one of the
74 most tissue-specific splicing profiles, and changes in alternative splicing trigger proper temporal
75 gene expression patterns during myogenesis [1–7]. Moreover, biochemical and biophysical
76 properties of many components of the contractile machinery can be fine-tuned by selectively
77 expressing specialized isoforms. Regulated splicing is orchestrated by the combinatorial
78 interaction between *cis*-regulatory elements and *trans*-acting factors. This process efficiently
79 creates functionally diverse proteins from a single gene; for instance, the fast troponin T primary
80 transcript can be alternatively spliced into 64 functionally distinct isoforms [8]. The critical
81 importance of alternative splicing in skeletal muscle is even more apparent when this process is
82 misregulated, as occurs in a number of muscle disorders [1]. For instance, perturbations in
83 alternative splicing ultimately result in an inability to transition from a fetal to an adult splicing
84 pattern in myotonic dystrophy 1 [9,10].

85 Members of both hnRNP and SR protein families play important roles during muscle
86 development [11–13]. SR proteins constitute a highly conserved group of splicing factors
87 required for both constitutive and alternative splicing [14,15]. They are structurally characterized
88 by the presence of an N-terminal RNA recognition motif (RRM) and a C-terminal

89 arginine/serine-rich domain (RS domain), which interacts with other cellular factors during
90 spliceosome assembly and splice site selection. A number of reports underscore the vital
91 importance of certain SR protein *in vivo*. For example, global SR gene knockouts exhibit
92 embryonic lethality [13,16–19], and Srsf10 (Srp38) knockout embryos die mid- to late-gestation
93 of multiple defects affecting both cardiac and skeletal muscle [13,19]. Finally, while conditional
94 cardiac Srsf1 knockout animals die postnatally from defective juvenile-to-adult heart remodeling
95 [18], conditional cardiac knockouts of Srsf2 (SC35) and Srsf4 (Srp75) are viable but develop
96 dilated and hypertrophic cardiomyopathies, respectively [20,21].

97 While alternative splicing modulates the expression of different gene isoforms, miRNAs can
98 control their cellular levels by base-pairing with miRNA response elements (MREs) present in
99 target mRNAs. Formation of this duplex can subsequently block translation or trigger
100 degradation of the mRNA. Importantly, disrupted miRNA processing in muscle leads to a
101 perinatal lethal phenotype characterized by muscle hypoplasia, abnormal myofiber organization,
102 and increased cell death [22]. Several specific miRNAs are enriched in skeletal muscle,
103 including miR-1 and miR-206, which share a common seed sequence. These miRNAs suppress
104 myoblast proliferation and promote muscle differentiation in both animal and cell culture models
105 [23–27]. Both miR-1 and miR-206 are minimally expressed in proliferating myoblasts, but
106 ectopic expression or inhibition can force or prevent differentiation, respectively [24–27].
107 Notably, miR-1 may also act as a key myogenic fate determinant as forced expression in HeLa
108 cells shifts their transcriptome to a typical muscle profile [28].

109 miRNAs also control myogenesis by modulating the cellular concentration of several
110 splicing factors. For example, miR-133a regulates nPTB, miR-30-5p targets MBNL, and miR-
111 222 regulates Rbm24 [12,29,30]. In addition, the RNA helicases Ddx5 and Ddx17, which

112 cooperate with the splicing factors hnRNP H/F and the transcription factor MyoD to regulate
113 transcription of myogenic genes in C2C12 mouse myoblasts, are direct miR-1 and miR-206
114 targets [31].

115 Through a combination of *in silico* predictions and correlated myogenesis functional analyses
116 in C2C12 cells, we report herein that miR-1 and miR-206 targeting of the transcript encoding the
117 SR protein Srsf9 is a significant myogenic event. The data presented highlight the cellular
118 relationship between miRNA expression and levels of splicing factors that control the temporal
119 production of specialized isoforms during muscle differentiation.

120 **Methods:**

121 **RNA isolation, cDNA synthesis, and qPCR:** Total RNA was isolated with TRI Reagent (MRC
122 TR 118) according to the manufacturer's protocol. Tissue samples were homogenized directly
123 into TRI reagent using a dispersion tool (IKA T10 Basic S1) while cultured cells were washed
124 with PBS and scraped directly into TRI reagent. All qPCR was performed on a Bio-Rad CFX96
125 attached to a C1000 thermocycler. miRNA expression was assessed by TaqMan-based qPCR
126 (ThermoFisher, Part Number 4427975; Assay IDs: sno202 = 001232, miR-206 = 000510, miR-1
127 = 002222). miRNAs were reverse transcribed with a TaqMan MicroRNA Reverse Transcription
128 Kit (ThermoFisher, 4366596) and qPCR was performed with TaqMan Universal PCR Master
129 Mix, No AmpErase UNG (ThermoFisher, 4324018), all according to the manufacturer's
130 instructions. Relative expression was analyzed with the $\Delta\Delta C_T$ method. mRNA expression was
131 assessed with SYBR Green-based qPCR. All primer sequences are listed in Supplementary Table
132 1. Total RNA was reverse transcribed with random hexamer primers using the Superscript II
133 Reverse Transcriptase kit (ThermoFisher, 18064-022). qPCR was performed with SYBR Green

134 PCR Master Mix (ThermoFisher, 4312704) according to the manufacturer's instructions.
135 Relative expression was analyzed with the Pfaffl standard curve method.

136 **RNA sequencing:** Total RNA from 3 independent growing myoblast cultures before
137 differentiation (BD1, BD2, BD3) and 3 independent day 6 myotube cultures after differentiation
138 (AD1, AD2, AD3) was oligo dT-selected and reverse transcribed with random hexamers,
139 followed by second strand synthesis, adapter ligation, and 5' end selection. Libraries were
140 sequenced with an Illumina HiSEQ. 210 million paired end reads were aligned to the mm9
141 version of the mouse genome downloaded from UCSC Genome Browser. Supplementary Table
142 2 presents reads per million aligned. BAM files are available upon request.

143 **miR-1/206 target selection and gene ontology (GO) analysis:** TargetScan 7.2 was queried to
144 retrieve the set of predicted miR-1/206 mouse target genes [32]. This was crossed with a list of
145 all mRNAs in the RNA-Seq dataset that decreased by 1.3-fold or more during differentiation,
146 regardless of statistical significance. These permissive criteria were chosen to be as inclusive as
147 possible for potential miR-1/206 targets in C2C12. This list was analyzed with DAVID
148 (Database for Annotation, Visualization and Integrated Discovery version 6.8 [33–35]) to
149 uncover enriched GO clusters of related gene sets. The functional clustering tool was used with
150 default settings and the mouse genome as background. Clusters were ranked by descending
151 Enrichment Score. Enrichment score is the $-\log_{10}$ of the geometric mean of the p-values of all
152 individual terms in the cluster. A cutoff of 0.05 or below for the geometric mean of p-values,
153 which corresponds to an Enrichment Score of greater than 1.3, was chosen.

154 **Cloning and mutagenesis:** All primer sequences are listed in Supplementary Table 1. miRNA
155 overexpression constructs were generated by cloning either the stem loop region (miR-450a-1
156 and miR-1) or a larger 1 kilobase region encompassing the stem loop (miR-206; the stem loop

157 sequence alone was insufficient for proper processing and subsequent targeting; data not shown)
158 into pcDNA3.1(-) through standard molecular cloning techniques. miR-450a-1 and miR-1 stem
159 loops were assembled with primers through overlap extension PCR and cloned into the
160 EcoRI/BamHI sites of the plasmid while the 1 kb miR-206 locus was PCR-amplified from
161 mouse genomic DNA and inserted between the HindIII and XhoI sites of the plasmid. 3' UTR
162 reporter constructs were generated in psiCheck2, a dual luciferase plasmid where UTRs are
163 cloned downstream of Renilla luciferase and firefly luciferase serves as an internal control. All
164 sequences were cloned between the XhoI and NotI sites. Positive controls for miR-450a-1, miR-
165 1, and miR-206 (2x450a-1, 2x1, and 2x206) were constructed by introducing 2 repeats (spaced
166 by 2 A residues) of the mature miRNA antisense sequence downstream of Renilla luciferase
167 using annealed complementary oligonucleotides. 3' UTRs were PCR-amplified from mouse
168 genomic DNA and inserted into the plasmid using standard molecular cloning techniques. The
169 Srsf9 MRE was mutated by replacing the natural MRE with the reverse sequence through inverse
170 PCR of the entire psiCheck2-Srsf9 wild-type 3' UTR plasmid. iProof high fidelity DNA
171 polymerase (Bio-Rad, 172-5302) was used according to the manufacturer's instructions with
172 phosphorylated primers. pEGFP-Srsf9 (a C-terminally GFP-tagged Srsf9 expression construct)
173 was generated by amplifying Srsf9 cDNA from C2C12 myoblast cDNA and inserting it between
174 the EcoRI and BamHI sites of pEGFP-N1 with standard molecular cloning techniques. A C-
175 terminal GFP tag in a related vector, pEGFP-N3, has been shown to be tolerated by other SR
176 proteins [36]. All clones were verified by Sanger sequencing.

177 **Cell culture, transfection, and stable cell line generation:** C2C12 cells were grown as
178 myoblasts in Growth Medium (GM): high glucose DMEM (Invitrogen 11960069) supplemented
179 with 20% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL

180 streptomycin, and 1 mM sodium pyruvate. Myotubes were differentiated by changing media to
181 Differentiation Medium (DM): high glucose DMEM supplemented with 5% adult horse serum, 2
182 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, and 1 mM sodium
183 pyruvate. When differentiating, DM was refreshed every day to prevent media acidification. For
184 luciferase assays, cells were plated in triplicate in 6-well dishes at a density of 50,000 cells/well
185 24 hours before transfection. Cells were transfected 24 hours after plating using the transfection
186 reagent *TransIT-LT1* (Mirus, MIR 2305) according to the manufacturer's instructions. When
187 ectopically expressing a miRNA along with a 3' UTR-linked reporter gene, cells were harvested
188 24 hours post-transfection. When differentiating cells for endogenous induction of miRNAs, Day
189 0 timepoints were collected 24 hours post-transfection and differentiation was triggered at the
190 same time for later timepoints. At harvest, cells were washed twice with phosphate-buffered
191 saline (PBS) solution and stored in an ultralow freezer in order to process all timepoints together
192 at the end of the experiment. For stable cell line generation, C2C12 cells were transfected with
193 plasmids (either pcDNA3.1(-), pEGFP-N1, or pEGFP-N1-Srsf9) using *TransIT-LT1*. 24 hours
194 post-transfection, G418 antibiotic selection was initiated by adding GM supplemented with 250
195 µg/mL G418. Selection was continued until control untransfected cells all died and cells were
196 split whenever necessary to keep density below 60% to prevent spontaneous differentiation. Cell
197 lines were maintained as pools to minimize potential genomic locus insertional effects.

198 **Tissue collection:** Animal work was reviewed by the University of Colorado Boulder
199 Institutional Animal Care and Use Committee and approved under protocols 1002.07 and
200 1002.08. Wild-type C57Bl/6 mice were bred and housed at the University of Colorado Boulder
201 under standard conditions. Adult animals were anesthetized by inhaled isoflurane and sacrificed
202 by cervical dislocation followed by pneumothorax. Embryos were anesthetized on ice and

203 sacrificed by decapitation. Whole hindlimbs were collected for embryonic timepoints and males
204 and females were equally represented (sex was determined by PCR for the Sry gene using
205 genomic DNA from the tail as template and primers against β -glucuronidase on chromosome 5
206 as positive control; see Supplementary Table 1 for primer sequences). Adult soleus was collected
207 from 6 month-old male mice. Tissue samples were flash frozen in liquid nitrogen and stored in
208 an ultralow freezer. Animal numbers for 15.5 dpc, 17.5 dpc, 19.5 dpc, and adult were 4, 6, 6, and
209 4, respectively.

210 **Luciferase assays:** A Dual-Luciferase Reporter Assay System (Promega, E1960) was used
211 according to the manufacturer's instructions with a Turner Designs TD-20/20 luminometer.
212 Briefly, frozen cells were lysed in 1X Passive Lysis Buffer and reporter gene activities were
213 measured first in LARII reagent (firefly luciferase internal control) and second in Stop & Glo
214 reagent (Renilla luciferase experimental reporter gene). Read times for both were 10 seconds.
215 Renilla/firefly ratios were compared.

216 **Western blotting:** Cell lysates were prepared in ice cold RIPA buffer (50 mM Tris pH 8.0, 1
217 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 140 mM NaCl)
218 with 1X cOmplete EDTA-free Protease Inhibitor Cocktail (Roche, 11873580001). Total protein
219 concentration was measured with a BCA protein assay kit (Pierce, 23250) and 20 μ g total protein
220 was resolved by 10% PAGE and transferred to nitrocellulose membrane. Standard western
221 blotting techniques were employed with all antibodies diluted in TBS/0.1% tween-20/4% nonfat
222 dry milk. 1:2,000 α -GFP (Santa Cruz Biotechnology, sc-8334, rabbit polyclonal) and 1:5,000 α -
223 Gapdh (Cell Signaling Technology, 2118S, rabbit monoclonal 14C10) were used as primary
224 antibodies and horse radish peroxidase-linked goat anti-rabbit (Jackson ImmunoResearch, 111-
225 035-144) was used as secondary.

226 **Immunostaining and microscopy:** Cells were seeded at a density of 100,000 cells per well in 6-
227 well dishes on glass coverslips that had been silanized, gelatinized, and glutaraldehyde-
228 crosslinked to promote adhesion of myotubes. Cells were harvested by washing once in 37°C
229 PBS + 0.4% glucose and fixed 5' at room temperature in 3% paraformaldehyde diluted in PBS.
230 Paraformaldehyde was quenched with 50 mM ammonium chloride. Fixed cells were
231 permeabilized 5' at room temperature in PBS/0.1% triton X-100 (Permeabilization Solution
232 (PS)) and blocked in Blocking Solution (BS: PS/1% BSA/1% normal goat serum) while rocking.
233 Myosin heavy chain was probed with F59 as primary antibody (Developmental Studies
234 Hybridoma Bank; custom production from hybridoma cells) and AlexaFluor568-linked goat anti-
235 mouse IgG1 as secondary antibody (Invitrogen, A-21124) diluted 1:200. DNA was stained with
236 300 nM 4',6-diamidino-2-phenylindole (DAPI; Sigma D9542). All imaging (for GFP,
237 AlexaFluor568, and DAPI) was performed on a Nikon TE2000 inverted fluorescent microscope
238 with a 20X objective connected to a Nikon DS-QiMc-U3 camera controlled through the NIS-
239 Elements AR software version 4.00.03. Exposure times for a given channel were kept constant
240 for all slides. Raw images were processed with the Image5D plugin in ImageJ using the same
241 settings for a given channel across all images.

242 **Nuclear fusion index and myotube area calculation:** Fusion indices were calculated from the
243 percent of total nuclei residing in syncytia (syncytium defined as a cell with 2 or more nuclei). 8-
244 10 non-overlapping 20X fields of view were analyzed and averaged for each cell line. The
245 number of nuclei in syncytia was tabulated and divided by the total nuclei in each field. Myotube
246 area per field of view was calculated as follows. Red (myosin) and blue (DNA) channels for a
247 given field of view were individually opened in ImageJ. Each was inverted and thresholded (the
248 same threshold for a given channel was applied to all images) to create a binary black and white

249 mask. The Analyze Particles function was run on each myosin and DNA image and the area of
250 the myosin image was divided by the area of the DNA image (to normalize for potential
251 differences in cell density across cell lines and across different fields of view). A representative
252 set of thresholded image masks is displayed in Supplementary Figure 1.

253 **Graphing and statistical analysis:** All graphing was done with GraphPad Prism and statistical
254 significance was assessed by one-way ANOVA with a Dunnett's post-test to compare to the
255 control condition or a Tukey's post-test to compare all pairs as noted for multiple comparisons.
256 Data are presented as means with error bars representing standard error of the mean. To assess
257 potential differences in myogenin and perinatal myosin heavy chain expression over a
258 differentiation time course, nonlinear regression analysis was used to fit quadratic curves to the
259 data points for GFP control and Srsf9-GFP cell lines. Curve fits were compared to determine
260 whether they were statistically different. A p-value cutoff of 0.05 was the minimum for
261 significance. Other cutoffs are as noted in the figure legends.

262 **Results:**

263 **Splicing factors are enriched amongst predicted miR-1/206 targets and they decrease in**
264 **expression during myotube formation.** In order to identify functional categories of putative
265 miR-1/206 targets in myogenesis, we took a combined *in silico* and *in vitro* cell culture model
266 approach. We reasoned that miR-1/206 target genes would be expressed in myoblasts as they are
267 poised to differentiate but that their concentrations would be reduced during the differentiation
268 process by the increasing levels of miR-1/206. To this end, in both C2C12 myoblasts and day 6
269 myotubes we initially measured the amounts of miR-1 and miR-206 by qPCR as well as the
270 global changes in their mRNA transcriptomes by RNA-Seq (Supplementary Table 2). These
271 analyses showed that while miR-1 and miR-206 levels are very low in myoblasts, both miRNAs

272 are robustly expressed in differentiated myotubes, which have both the molecular and
273 morphological characteristics of mature muscle (Supplementary Figure 2, Panels A and B). This
274 also confirms previous measurements of miR-1 and miR-206 in differentiating C2C12 cells [26].
275 Since miRNAs often exert mild effects on individual targets [37,38], we used the RNA-Seq
276 dataset to assemble a list of genes down-regulated by 1.3-fold or more during differentiation. We
277 then retrieved the set of 896 predicted miR-1/206 targets from TargetScan and found that the
278 levels of 354 decreased during myogenesis (Supplementary Table 3).

279 In order to evaluate whether particular functional categories are enriched in this gene set, we
280 employed the DAVID functional annotation clustering tool, which groups functional categories
281 with similar gene sets from multiple different annotation sources to capture biological themes. Of
282 the 354 candidate C2C12 miR-1/206 targets, 351 had associated DAVID IDs. We found 21
283 clusters (Supplementary Table 4) with an enrichment score greater than 1.30 (indicating an
284 average p-value of below 0.05 for all terms in the cluster). While the top two clusters were
285 related to DNA binding and transcriptional regulation and included known targets such as E2f5
286 [39], Pax3 [40], and Sox9 [41], both the third and fifth clusters included genes related to RNA
287 metabolism. Since regulated splicing plays a central role in promoting myogenesis [4,6,7], we
288 investigated whether miR-1/206 control muscle differentiation by specifically targeting RNA
289 processing factors.

290 **mRNAs encoding SR protein family members are miR-1/206 targets during myogenesis.**

291 Our bioinformatics analysis predicted mRNAs encoding four of these proteins, Srsf1 (ASF/SF2),
292 Srsf3 (Srp20), Srsf9 (Srp30c), and the SR-related protein Tra2b, as miR-1/206 targets. Since
293 Srsf1 has an established critical role in heart development [18], we focused our attention on the
294 remaining candidates. We assayed miRNA targeting in C2C12 myoblasts, which, as already

295 noted, have extremely low levels of both miR-1 and miR-206. We co-transfected cells with
296 constructs expressing a reporter Renilla luciferase gene fused to the mouse 3' UTRs of our RNA
297 candidates and either miR-1 or miR-206 overexpression plasmids. We determined the specificity
298 of miR-1 or miR-206 by measuring the targeting activity of miR-450a-1, a non-myogenic
299 miRNA which does not have overlapping predicted targets with miR-1/206. Luciferase
300 quantification showed that this miRNA did not cross-react with the selected SR 3' UTRs or with
301 3' UTRs carrying tandem copies of the complete reverse complement of either miR-206 (2x206)
302 or miR-1 (2x1) (data not shown). However, it efficiently down-regulated the positive control
303 construct 2x450a-1, constructed similarly to 2x206 and 2x1 (Supplementary Figure 3). To
304 determine the sensitivity of the assay, we next assessed the activity of miR-1/206 on the 2x206
305 and 2x1 constructs as well as on the 3' UTR of *Ccnd1*, previously identified as a miR-206 target
306 in C2C12 cells [42]. We also monitored promoter interference causing potential transcriptional
307 repression of the reporter construct using a miRless construct, which expresses the Renilla
308 luciferase RNA without miRNA target sites. Figure 1A shows the result of this analysis with
309 each luciferase signal measured in the presence of miR-1 or miR-206 normalized to the same
310 constructs co-transfected with the control miRNA, miR-450a-1 (see Materials and Methods).
311 The data, presented as fold change versus miR-450a-1 control, show that miRless expression did
312 not change appreciably in the presence of miR-1 or miR-206 and that miR-1 and miR-206
313 efficiently targeted 2x1 and 2x206 along with the positive control *Ccnd1* (Figure 1A).
314 Furthermore, both miR-1 and miR-206 also reduced luciferase activity by targeting the 3' UTRs
315 of *Srsf9* and *Tra2b* fused to the reporter gene while they did not have any effect on the *Srsf3*
316 construct (Figure 1B). While statistically significant, the negative regulation of the *Tra2b* 3'
317 UTR was modest in magnitude. Thus, we focused our next set of experiments only on *Srsf9*

318 activity and its potential role in muscle differentiation. We substantiated the specific miR-1/206
319 targeting of the Srsf9 3' UTR by MRE mutagenesis. When we reversed the orientation of the
320 predicted MRE in the Srsf9 3' UTR, which preserves positioning of any unrecognized flanking
321 elements, this mutant construct (termed Srsf9 MRE Rev) restored reporter gene activity to levels
322 measured in the presence of the control miR-450a-1 (Figure 1C). Taken together, these results
323 establish that expression of the miR-1/206 family can directly modulate the level of Srsf9 in
324 C2C12 myoblasts.

325 To broaden the data obtained in myoblasts, we next surveyed the activity of the reporter
326 construct during C2C12 differentiation which exposes the Srsf9 3' UTR to physiological
327 concentrations of both miR-1 and miR-206. Time course analysis showed an inversely
328 proportional relationship between the decreased reporter luciferase activity and the concomitant
329 increase in miR-1 and miR-206 expression occurring during the activation of the C2C12
330 differentiation program (Figure 1D; Supplementary Figure 2). Interestingly, we observed a
331 similar pattern for the Ccnd1 reporter construct (Figure 1D). Consistently, the mRNA expression
332 levels of Srsf9 and Ccnd1 measured six days after differentiation were significantly reduced
333 (Figure 2A), which is also in accordance with the RNA-Seq data. We also queried a quantitative
334 pSILAC mass spectrometry dataset that compiles protein level changes in HeLa cells 8 hr after
335 miR-1 overexpression. The median fold change for Srsf9 peptides was -3.71, which supports
336 direct miR-1 targeting [37,43].

337 We extended this analysis to embryonic limb formation in the developing mouse. As shown
338 in Figure 2B, we found that both miR-1 and miR-206 expression, which increased between 3-
339 and 5-fold as embryogenesis progressed, peaked during the transition to mature adult muscle. In
340 agreement with the inverse correlation observed in the C2C12 analysis, the mRNA levels of

341 Srsf9 and Ccnd1 showed a modest decline in expression from embryonic day 15.5 to day 19.5
342 but a marked decrease from the embryonic to adult mouse transition (Figure 2C).

343 **The inability to down-regulate Srsf9 expression during myogenesis results in impaired**
344 **differentiation.** Finally, we investigated whether the down-regulation of Srsf9 expression plays
345 an important role in controlling the sequential myogenic pathway. To this end, we generated
346 C2C12 stable cell lines expressing a GFP-tagged version of Srsf9 fused to a heterologous 3'
347 UTR containing the SV40 polyA signal as well as GFP only or empty vector controls. After
348 selection, we maintained pools of stable transfectants and measured transgene RNA and protein
349 expression during differentiation. qPCR and western blot analysis carried out with GFP-specific
350 primers and antibody revealed that both Srsf9-GFP and GFP were stably expressed in our cell
351 pools (Supplementary Figure 4 A,B). More importantly, time course analysis showed that the
352 presence of the heterologous 3' UTR stabilized the level of Srsf9-GFP mRNA throughout cell
353 differentiation (Supplementary Figure 4C). Moreover, we found that, as revealed by co-
354 localization with DAPI stain, Srsf9-GFP was properly localized in the nucleus of the majority of
355 the cells, while the GFP control showed diffuse fluorescence signal (Figure 3A). To determine
356 potential functional repercussions caused by sustained Srsf9 expression, we first calculated the
357 fusion index, a method frequently used to quantify the extent of C2C12 differentiation. Scoring
358 of the number of nuclei residing in a fused syncytium (defined as a cell with 2 or more nuclei) at
359 day 6 of differentiation showed that the average fusion index of cells expressing Srsf9-GFP was
360 significantly lower than the ones expressing either GFP or the empty vector control (Figure 3B).
361 Since Srsf9-GFP differentiated myotubes imaged at day 6 appeared to have reduced width
362 compared to controls, we measured whether the myotube-positive area of each field of view was
363 statistically smaller when normalized to DNA-positive area, a control for potential differences in

364 cell density. After imaging the different cell pools stained with DAPI and a myosin heavy chain
365 antibody (considered a terminal marker of muscle differentiation), we created cell masks by
366 imposing equal intensity thresholds to the acquired images and then divided the myosin area by
367 the DAPI area derived from the binary derivatives. As shown in Figure 3C, the stable expression
368 of the Srsf9-GFP construct, which is refractory to miR-1/206 targeting activity, resulted in a
369 significant reduction of myotube area, a clear indication of impaired differentiation. Supporting
370 this observation, induction of the perinatal myosin isoform (Myh8), the most expressed myosin
371 in mammalian skeletal muscle during the early perinatal period [44] and one of the most
372 abundant myosins expressed in differentiated C2C12 (Supplementary Table 2), was significantly
373 blunted in Srsf9-GFP cells (Figure 3D). This is indicated not only by decreased expression at
374 individual timepoints but also by statistically different curves fit to each series when comparing
375 GFP control and Srsf9-GFP cells. Finally, as the myogenesis program is fundamentally
376 orchestrated at the transcriptional level, we measured induction of the muscle regulatory factor
377 myogenin which is robustly expressed at early myogenic timepoints and then decreases as
378 myotubes approach terminal differentiation. Although both the GFP controls and the Srsf9-GFP
379 cells induced myogenin relative to D0 growing myoblast expression levels, this induction was
380 much weaker in the Srsf9-GFP cells (Figure 3E). Since the series could still be fit to a quadratic
381 curve, with myogenin expression in Srsf9-GFP cells peaking mid-time course and then declining
382 at day 6, we believe the data do not support a simple delay of differentiation. Taken together,
383 these data strongly suggest that proper progression throughout the steps of muscle differentiation
384 requires temporal Srsf9 down-regulation.

385 **Discussion**

386 In this report we used a computational analysis of differentiating C2C12 myoblasts to
387 identify miR-1/206 targets relevant to myogenesis. We found that the 3' UTRs of several RNA-
388 binding proteins, which belong to the SR splicing factor family, were highly enriched in our
389 bioinformatics assessment. Accordingly, we discovered that down-regulation of one of its
390 members, Srsf9, controls myoblast differentiation. One limitation of our computational method is
391 that it predicts miR-1/206 targets based on the decrease of their RNA levels; targets that are
392 translationally inhibited but not regulated at the mRNA level, such as Hdac4 and Igf1 [25,45,46],
393 escaped our analysis. However, several genome-wide studies on miRNAs, which include miR-1,
394 have shown good correlation between RNA and protein levels [28,38].

395 miR-1 and miR-206 are highly conserved members of the myomiR family that also includes
396 miR-133a/b, miR-208a/b, miR-486, and miR-499. This ensemble of miRNAs, specifically
397 expressed in cardiac and skeletal muscle, governs muscle differentiation, maintenance, and
398 plasticity [47]. To date, several reports have shown that miR-1/206 control the expression levels
399 of genes implicated in transcription (Pax3, Pax7, Hdac4; [24,40,45,48,49]) and cellular
400 proliferation (Pola1; [26]). In this study, we found that down-regulation of the splicing factor
401 Srsf9 by miR-1/206 targeting is also essential for proper C2C12 differentiation. Srsf9 cooperates
402 with several other RNA binding proteins to repress or activate regulated splicing. It modulates
403 the inclusion of SMN exon 7 [50], the alternative splicing of glucocorticoid receptor beta and
404 gonadotropin-releasing hormone [51,52], inclusion/exclusion of tau exons 2 and 10 [53,54], and
405 generation of the hnRNP A1^B isoform [55,56]. Notably, the main hnRNP A1 isoform regulates
406 the alternative splicing of pyruvate kinase-M (PK-M). While the PK-M1 isoform is highly
407 enriched in differentiated myotubes, myoblasts express the PK-M2 isoform which gives them a
408 proliferative advantage [57]. Thus, it is tempting to speculate that, by controlling the shift in

409 pyruvate kinase isoform expression, the combined activity of Srsf9 and miR-1/206 during
410 differentiation lays the groundwork for metabolic adaptation. Moreover, it has been proposed
411 that Srsf9 can interact cooperatively or compete with other splicing regulators for binding to
412 high-affinity sites present in alternatively spliced pre-mRNAs [56]. Therefore, even a small
413 decrease in Srsf9 cellular concentration could lead to profound changes in global splicing
414 patterns. The SR protein cellular function is still not completely defined. Global mapping of
415 RNA targets determined by uv crosslinking and immunoprecipitation (CLIP/iCLIP) has been
416 carried out for Srsf1, 2, 3, and 4, and the eight drosophila SR homologs [36,58,59] but not for
417 Srsf9. These studies performed in mouse embryo fibroblasts (MEFs) and P19 embryonal
418 carcinoma cells revealed intriguing findings. For example, i) specific alternative splicing patterns
419 apparently controlled by a single SR protein actually hinge upon an intricate network of
420 relationships with many other SR proteins; ii) SR proteins can control gene expression by
421 binding intronless transcripts as well as ncRNAs. Since no such map is available for Srsf9, it is
422 difficult to predict through which molecular pathways Srsf9 exerts control over myoblast
423 differentiation. Srsf9 high-affinity binding sites have been identified from a randomized pool of
424 RNA sequences by a SELEX approach [60]. However, a computational survey of SELEX-
425 derived consensus sequences, which are short and degenerate, revealed frequent over-
426 representation in RNAs [61].

427 miR-1 and miR-206 have both been proposed as tumor suppressors. Interestingly, Srsf9
428 depletion reduces viability of prostate cancer cells [62]. Moreover, in two human bladder cancer
429 cell lines, Srsf9 down-regulation by miR-1 overexpression resulted in significant reduction in
430 cell proliferation, migration, and invasion [63]. Accordingly, siRNA-mediated Srsf9 knockdown
431 promoted apoptosis [63,64]. These data strongly suggest that in bladder cancer cells, the tumor

432 suppressive activity of miR-1 triggers apoptosis through direct Srsf9 inhibition. However, we did
433 not observe any impact on cell survival when Srsf9 expression was stabilized during C2C12
434 differentiation.

435 **Conclusions**

436 We report here that miR-1/206 target the Srsf9 3' UTR during C2C12 myoblast
437 differentiation. This is the first report showing a direct correlation between this member of the
438 SR protein family and muscle maturation. We demonstrate that Srsf9 down-regulation is
439 necessary to achieve robust cell fusion and muscle-specific gene expression. Based on the
440 complex web of functional interactions occurring amongst the RNA splicing proteins, we
441 suggest that the persistence of Srsf9 alters several alternative splicing events and impairs proper
442 production of specific protein isoforms driving myoblast maturation. The data presented also
443 emphasize the importance of maintaining appropriate miRNA-mediated gene repression in adult
444 muscle to avoid re-expression of fetal genes that could cause muscle disease.

445 **List of abbreviations**

446 DAPI = 4',6-diamidino-2-phenylindole; DAVID = Database for Annotation, Visualization, and
447 Integrated Discovery; dpc = days post coitum; FC = fold change; GFP = green fluorescent
448 protein; MRE = miRNA response element; PBS = phosphate-buffered saline; qPCR =
449 quantitative PCR; RNA-Seq = RNA sequencing; SR protein = serine/arginine-rich protein; TBS
450 = Tris-buffered saline; UTR = untranslated region

451 **Declarations**

452 Ethics approval All animal work was approved by the University of Colorado Boulder
453 Institutional Animal Care and Use Committee under protocols 1002.07 and 1002.08

454 Availability of data All data generated or analyzed during this study are included in this
455 published article and its supplementary files.

456 Competing interests The authors declare that they have no competing interests.

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459 Authors' contributions KKB and LAL conceived and designed the study. KKB performed
460 bioinformatics analyses, luciferase assays, and stable cell line generation and accompanying
461 analysis, analyzed data, and co-wrote the manuscript. MB was a major contributor to data
462 analysis and co-wrote the manuscript. EKP performed mutagenesis on reporter gene constructs
463 and performed luciferase assays. MMP assisted with cloning of reporter gene constructs. All
464 authors read and approved the final manuscript.

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469 **Figure Legends:**

470 **Figure 1. Srsf9 is a direct target of miR-1 and miR-206.** (A) miR-206 and miR-1 target 3'
471 UTRs with cognate MREs in C2C12 myoblasts. Cells were transfected with reporter constructs
472 and expression constructs for miR-206, miR-1, or the control miRNA, miR-450a-1. Renilla
473 luciferase linked to two tandem copies of the reverse complement of miR-206 or miR-1 (2x206
474 and 2x1, respectively) or to the 3' UTR of known miR-1/206 target Ccnd1 were positive
475 controls. A 3' UTR with no MREs in the empty reporter construct (miRless) was the negative

476 control. Cells were harvested 24 hr after transfection. The graph presents fold changes (FC) in
477 firefly-normalized Renilla luciferase activity for miR-206- or miR-1-transfected vs. miR-450a-1-
478 transfected cells. The dashed line at $y=1$ represents miR-450a-1-transfected levels. ** = $p \leq 0.01$;
479 *** = $p \leq 0.001$ vs miR-450a-1 **(B)** 3' UTRs from Srsf9 and Tra2b but not Srsf3 are sensitive to
480 miR-206 and miR-1 activity. C2C12 myoblasts were transfected with reporter constructs
481 containing the Srsf3, Srsf9, or Tra2b 3' UTRs along with miRNA expression constructs. Data
482 were collected and analyzed as in A. **(C)** Srsf9 is a direct target of miR-1/206. The predicted
483 MRE in the Srsf9 3' UTR was reversed in the reporter construct to abolish miR-1/206 binding.
484 C2C12 myoblasts were transfected and analyzed as in Panel A. **(D)** The Srsf9 3' UTRs is
485 sensitive to endogenous myogenic cues. 3' UTR reporter constructs were transfected into C2C12
486 myoblasts which were then differentiated for 0, 1, 2, or 4 days. Firefly-normalized Renilla
487 luciferase activity was measured. Fold changes for differentiating vs. Day 0 myoblasts were
488 calculated and normalized to the miRless control. The dashed line at $y=1$ represents Day 0 levels.
489 * = $p \leq 0.05$; ** = $p \leq 0.01$ vs D0 For all panels, N = 3 independent cultures.

490 **Figure 2. Srsf9 mRNA levels are inversely proportional to miR-1/206 levels.** **(A)** Srsf9
491 mRNA levels decrease in differentiating C2C12 cells. Expression was assessed by qPCR in
492 growing myoblasts (Day 0) and in Day 6 myotubes. Gapdh was the reference gene. Expression
493 levels in the differentiating samples were normalized to myoblast levels (indicated by the dashed
494 line). Myogenin (MyoG) was the positive control as its expression increases during
495 differentiation. * = $p \leq 0.05$; ** = $p \leq 0.01$ vs. D0 N = 3 independent cultures for all genes and
496 timepoints. **(B)** miR-206 and miR-1 levels increase during mouse hindlimb skeletal muscle
497 development. Expression was assessed by qPCR and normalized to sno202 in whole hindlimb
498 for developmental timepoints and in the soleus from 6 month-old male mice. Fold change

499 relative to 15.5 dpc is presented. N for 15.5 dpc, 17.5 dpc, 19.5 dpc, and adult was 4, 6, 6, and 4,
500 respectively. *** = $p \leq 0.001$ vs. 15.5 dpc (C) Srsf9 and Ccnd1 mRNAs decrease during mouse
501 hindlimb skeletal muscle development. Expression was assessed by qPCR with 18S rRNA as the
502 reference gene. Expression levels were normalized to 15.5 dpc (indicated by the dashed line). * =
503 $p \leq 0.05$; ** = $p \leq 0.01$ vs 15.5 dpc Ns were the same as in Panel B.

504 **Figure 3. mir-1/206-resistant Srsf9-GFP expression blunts myogenic differentiation.** (A)
505 C2C12 cell lines stably expressing GFP alone (GFP control) or GFP-tagged Srsf9 linked to a
506 heterologous miR-1/206-resistant 3' UTR (Srsf9-GFP) were differentiated for 6 days,
507 immunostained, and imaged (20X objective). Myosin heavy chain, a marker of terminal
508 differentiation, was visualized with the F59 antibody and an AlexaFluor568-linked secondary
509 antibody. DNA was visualized by DAPI staining and GFP or Srsf9-GFP was visualized by GFP
510 autofluorescence. Scale bars denote 6.5 μm . (B) Srsf9-GFP persistence reduces fusion. The
511 fusion index of Srsf9-GFP cells was significantly lower than either pC-Empty cells (an empty
512 expression plasmid) or GFP control cells. 8-10 non-overlapping fields of view were analyzed for
513 each cell line. * = $p < 0.05$ vs pC-Empty; ### = $p < 0.001$ vs GFP control. (C) Srsf9-GFP
514 persistence reduces myotube size. Myotube area was assessed by calculating the myosin-positive
515 area of D6 myotubes. The myosin-positive area/DAPI-positive area was calculated for 8-10 non-
516 overlapping fields of view. Myosin positive area was significantly lower than both pC-Empty
517 and GFP control cell lines. * = $p \leq 0.05$ vs pC-Empty; # = $p < 0.05$ vs GFP control (D) Srsf9-
518 GFP persistence reduces myosin mRNA expression. mRNA levels of perinatal myosin (Myh8)
519 were assessed by qPCR at Days 0, 1, 2, 4, and 6 of differentiation. Statistically different
520 quadratic curves were fit to GFP control and Srsf9-GFP series. (E) Srsf9-GFP persistence
521 reduces MyoG induction. MyoG mRNA levels were measured by qPCR as in Panel D.

522 Statistically different quadratic curves were fit to GFP control and Srsf9-GFP. For D and E:
523 asterisks next to the Srsf9-GFP legend denote $p < 0.0001$ for the curve fit comparison.

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- 688 **Supplementary Figures**
- 689 **Supplementary Figure 1.** Supplementary Figure 1.tif

690 **Representative masked images used for D6 myotube area calculation. (A)** Myosin heavy
691 chain, a marker of terminal differentiation, was visualized with the F59 antibody and an
692 AlexaFluor568-linked secondary antibody. **(B)** DNA was visualized by DAPI staining. **(C)** The
693 binary myosin mask corresponding to Panel A. **(D)** The binary DNA mask corresponding to
694 Panel B.

695 **Supplementary Figure 2.** Supplementary Figure 2.tif

696 **miR-1 and miR-206 levels increase during C2C12 differentiation.** Expression was assessed
697 by qPCR and normalized to sno202 in growing myoblasts (D0) and days 1, 2, 4, and 6 of
698 differentiation. Fold changes vs D0 are presented. * = $p < 0.05$; *** = $p < 0.001$

699 **Supplementary Figure 3.** Supplemental Figure 3.tif

700 **Non-myogenic miR-450a-1 is processed and active when ectopically expressed in myoblasts.**
701 C2C12 myoblasts were co-transfected with a reporter construct containing two tandem copies of
702 the reverse complement of miR-450a-1 (2x450a-1) in the 3' UTR along with expression
703 constructs for miR-206, miR-1, or miR-450a-1. Cells were harvested 24 hours later. Firefly-
704 normalized Renilla luciferase activity was equivalent between miR-206 and miR-1 expression
705 but significantly down-regulated in the presence of miR-450a-1. *** = $p < 0.001$ vs miR-206;
706 ### = $p < 0.001$ vs miR-1 N = 3 independent cultures for each.

707 **Supplementary Figure 4.** Supplementary Figure 4.tif

708 **miR-1/206-resistant Srsf9-GFP expression does not change during differentiation of stable**
709 **cell lines. (A)** GFP or Srsf9-GFP mRNA levels in the corresponding stable C2C12 cell lines
710 were compared to a negative control C2C12 cell line (pC-Empty) incorporating the empty
711 expression vector pCDNA3.1(-). Expression was assessed by qPCR with GFP-specific primers

712 and normalized to 18S rRNA levels. Fold change relative to pC-Empty is presented. N = 3
713 independent cultures for each. **(B)** Srsf9-GFP protein is expressed in the stable cell line.
714 Expression in pC-Empty, GFP, and Srsf9-GFP cells was assessed by western blot with a GFP-
715 specific antibody. **(C)** Srsf9-GFP mRNA levels are stable during differentiation of the Srsf9-GFP
716 cell line. Expression was assessed by qPCR as in A. There is no statistical difference amongst the
717 time points.

718 **Supplementary Table 1.** Supplementary Table 1.xlsx

719 **Primer sequences.** All primer sequences are presented 5' → 3'.

720 **Supplementary Table 2.** Supplementary Table 2.xlsx

721 **C2C12 RNA-Seq data.** mRNA from C2C12 day 6 myotubes (AD1, AD2, AD3) and
722 proliferating myoblasts (BD1, BD2, BD3) was sequenced with a paired end protocol on an
723 Illumina HiSEQ. The table presents reads per million aligned to the mouse mm9 genome.
724 Column A is the UCSC Gene Symbol and Columns B-E list chromosomal coordinates and
725 coding strand designation.

726 **Supplementary Table 3.** Supplementary Table 3.xlsx

727 **TargetScan-predicted miR-1/206 targets that decrease during C2C12 differentiation.**

728 Predicted mouse miR-1/206 targets from TargetScan v. 7.2 were crossed with all mRNAs that
729 decreased by 1.3-fold or greater during C2C12 differentiation. This filtered the list of all 896
730 candidate targets to 354 that were down-regulated at the mRNA level. This is the list that was
731 used for functional clustering gene ontology analysis with the DAVID database. Fold change
732 from D0 to D6 is presented along with MRE scores from TargetScan.

733 **Supplementary Table 4.** Supplementary Table 4.xlsx

734 **Functional Clustering output for DAVID analysis of 354 candidate miR-1/206 targets.**

735 Candidate miR-1/206 targets were uploaded to DAVID and the functional clustering tool was

736 applied to the gene set. 21 GO term clusters of related gene sets had an enrichment score greater

737 than 1.3, corresponding to an average p-value of less than 0.05 for all GO categories in a cluster.

738 Clusters are ranked by descending Enrichment Score and include all output columns from

739 DAVID.





