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### **► To cite this version:**

Virginie Lecomte, Emmanuelle Meugnier, Vanessa Euthine, Christine Durand, Damien Freyssenet, et al.. A new role for sterol regulatory element binding protein 1 transcription factors in the regulation of muscle mass and muscle cell differentiation.: SREBP-1a and -1c in muscle cells. *Molecular and Cellular Biology*, 2010, 30 (5), pp.1182-98. 10.1128/MCB.00690-09 . inserm-00817651

**HAL Id: inserm-00817651**

**<https://inserm.hal.science/inserm-00817651>**

Submitted on 25 Apr 2013

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1 **A new role for SREBP-1 transcription factors in the regulation of muscle mass**  
2 **and muscle cell differentiation**

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26 Running title : SREBP-1a and -1c in muscle cells

27

28 Word count "Materials and Methods" : 1250

29 Word count "Introduction + Results + Discussion" : 3767

30 Abstract

31 The role of the transcription factors SREBP-1a and SREBP-1c in the regulation of  
32 cholesterol and fatty acid metabolism has been well studied, however little is known  
33 about their specific function in muscle. In the present study, analysis of recent  
34 microarray data from muscle cells overexpressing SREBP1 suggested that they may  
35 play a role in the regulation of myogenesis. We then demonstrated that SREBP-1a  
36 and -1c inhibit myoblast to myotube differentiation, and also induce in vivo and in  
37 vitro muscle atrophy. Furthermore, we have identified the transcriptional repressors  
38 BHLHB2 and BHLHB3 as mediators of these effects of SREBP-1a and -1c in muscle.  
39 Both repressors are SREBP-1 target genes, and they affect the expression of  
40 numerous genes involved in the myogenic program. Our findings identify a new role  
41 for SREBP-1 transcription factors in muscle, thus linking the control of muscle mass  
42 to metabolic pathways.

## 43 **Introduction**

44 The Sterol Regulatory Element Binding Proteins (SREBP) transcription factors  
45 belong to the basic helix-loop-helix leucine zipper family of DNA binding proteins.  
46 The three isoforms identified so far in mammalian tissues are coded by two distinct  
47 genes, *Srebf1* and *Srebf2*, and vary in structure, regulation, and functions (14).  
48 SREBP-1a and SREBP-1c proteins are produced by alternative promoter usage of  
49 the *SREBF1* gene and are key actors of the regulation of genes related to lipid  
50 metabolism, especially those involved in lipogenesis and triglyceride deposition. In  
51 contrast, SREBP-2 has been more closely associated to cholesterol synthesis and  
52 accumulation (20, 52).

53 In agreement with these known functions, the SREBP-1 proteins are strongly  
54 expressed in tissues with high lipogenic capacities, like liver and adipose tissues.  
55 However, significant expression has been also reported in skeletal muscle, both in  
56 vivo and in vitro in cultured muscle cells (12, 13, 18). In muscle, SREBP-1 expression  
57 is induced by activation of the PI3K/Akt and the MAP kinase pathways by insulin and  
58 growth factors (6, 12, 18, 28, 38), suggesting additional functions of these  
59 transcription factors in a tissue with a low rate of lipid synthesis. Using microarray  
60 analysis to characterize the role of SREBP-1a and -1c in skeletal muscle, we have  
61 recently identified some of their potential target genes in primary cultures of human  
62 myotubes overexpressing SREBP-1a or SREBP-1c (43). In this study we found that  
63 SREBP-1a and -1c regulate more than one thousand genes, indicating that they are  
64 potentially involved in the regulation of a large variety of biological functions in  
65 muscle cells. Quite unexpectedly, we observed a dramatic reduction in the  
66 expression of a number of muscle-specific genes and markers of muscle

67 differentiation in cells overexpressing SREBP-1 proteins. This led us to investigate  
68 their potential role in the regulation of myogenesis and muscle development.

69         The early stages of muscle development are regulated by muscle-specific  
70 basic helix-loop-helix transcription factors (e.g. MYF5, MYOD1, MYOG (myogenin)  
71 and MYF6 (MRF4)), which are also involved in the differentiation of satellite cells  
72 during regeneration process in adult muscle. Recently, the transcriptional factor  
73 BHLHB3 was shown to inhibit in vitro muscle cell differentiation by interacting with  
74 MYOD1 (2). BHLHB3 (also named DEC1/SHARP1) is a transcriptional repressor  
75 closely related (97% homology in amino acid sequence in the bHLH domain) to  
76 BHLHB2 (also named Stra13/DEC2/SHARP2). They both repress the expression of  
77 target genes by binding to E-Box sequences as well as through protein-protein  
78 interactions with other transcription factors (review in (51)). BHLHB2 and BHLHB3  
79 genes are widely expressed in both embryonic and adult tissues and their expression  
80 is regulated in cell type-specific manner in various biological processes, including  
81 circadian rhythms (19), hypoxia (35) or cellular differentiation (7). Their involvement  
82 in the regulation of developmental processes during embryogenesis has been largely  
83 studied (4, 7, 24, 34, 44). We demonstrate here that BHLHB2 and BHLHB3 mediate  
84 negative effects of SREBP-1 transcription factors on myogenesis, acting both at the  
85 myoblast and myotube stages. The SREBP-1 mediated effects on BHLHB2 and  
86 BHLHB3 activity thus defines a novel negative regulation pathway in skeletal muscle  
87 cell development.

## 88 **Materials and Methods**

89

90 **Culture of human skeletal muscle cells.** Muscle biopsies were taken from healthy  
91 lean subjects during surgical procedure, with the approval of the Ethics Committee of  
92 Lyon Hospitals. Myoblasts were purified and differentiated myotubes were prepared  
93 according to the procedure previously described in detail (11).

94

95 **Expression vectors and generation of recombinant adenoviruses.** For the  
96 construction of expression vector encoding BHLHB2, a verified sequence I.M.A.G.E.  
97 clone (cloneID 4860809) was purchased from Geneservice (Cambridge, UK) and  
98 subcloned into the pcDNA 3.1 expression vector (Invitrogen). The expression vector  
99 encoding BHLH3 was generated by PCR amplification and ligated into PCDNA3.1.  
100 Expression vector encoding the dominant-negative form of SREBP-1 (ADD1-DN) is a  
101 generous gift of Dr. B. Spiegelman (Dana-Farber Cancer Institute/Harvard Medical  
102 School, Boston, USA) (27). Recombinant adenoviral genomes carrying the human  
103 BHLHB2 or BHLHB3 or ADD1-DN were generated by homologous recombination in  
104 the VmAdcDNA3 plasmid (a gift from Dr S. Rusconi, Fribourg, Switzerland) and  
105 amplified as described previously (9, 12).

106 Construction of expression vectors encoding mature nuclear forms of human  
107 SREBP-1a (named pCMV-hSREBP1a) and SREBP-1c (named pCMV-hSREBP1c)  
108 was described previously (12). A fragment of the pIRES plasmid (Clontech, Mountain  
109 View, CA, USA) containing the IRES and EGFP sequence was cloned into pCMV-  
110 hSREBP1a and pCMV-hSREBP1c to obtain pCMV-hSREBP1a-IRES-GFP and  
111 pCMV-hSREBP1c-IRES-GFP. Recombinant adenoviruses expressing  
112 simultaneously nuclear form of either SREBP-1a or SREBP-1c and GFP as a

113 marker, were generated by homologous recombination in the VmAdcDNA3 plasmid  
114 and amplified.

115

116 **Overexpression of Human SREBP-1a, SREBP-1c, BHLHB2 or BHLHB3 in**  
117 **human muscle cells.** The construction of recombinant adenoviruses encoding  
118 nuclear SREBP-1a and SREBP-1c was described previously (12). Human muscles  
119 cells were infected as myoblasts or myotubes. Myoblasts were grown in six-well  
120 plates. Myoblasts at 70% confluence, or myotubes after 5 days of differentiation,  
121 were infected for 48 h with the recombinant adenovirus encoding BHLHB2 or  
122 BHLHB3 or nuclear forms of SREBP-1a or SREBP-1c, or GFP as a control.

123

124 **Inhibition of BHLHB2 and BHLHB3 expression in human muscle cells.** Inhibition  
125 of BHLHB2 and BHLHB3 expression was performed by RNA interference using small  
126 interfering RNA (siRNA) against BHLHB2 and against BHLHB3 (Qiagen). A  
127 rhodamine labeled GFP-22 siRNA was used as control. Myoblasts at 70%  
128 confluence were transfected with siRNAs using the Hiperfect transfection reagent  
129 (Qiagen, Courtaboeuf, France), according to the manufacturers protocol.

130

131 **In vivo overexpression of Human SREBP-1a, SREBP-1c, BHLHB2, BHLHB3 in**  
132 **mice tibialis anterior muscles.** All animal procedures were conducted according to  
133 the national guidelines for the care and use of laboratory animals. Adult (12-14 week-  
134 old) BALB/c male mice (Harlan, France) were subjected to adenoviral delivery  
135 according to the procedure described by Sapru et al. (45). Briefly, right Tibialis  
136 Anterior muscles of mice were injected with  $10^{10}$  infectious unit of recombinant  
137 adenovirus expressing either SREBP-1a/GFP, SREBP-1c/GFP, BHLHB2 or

138 BHLHB3. As a control, the contralateral tibialis anterior muscles were also injected  
139 with  $10^{10}$  infectious units of recombinant adenovirus expressing GFP. Mice were  
140 sacrificed seven days after injection. Tibialis anterior muscle was removed and  
141 immediately snap-frozen in liquid nitrogen. Ten-micron sections were cut and every  
142 tenth section collected onto glass slides for examination under fluorescence  
143 illumination using an Axiovert 200 microscope, an Axiocam MRm camera and  
144 Axiovision 4.1 image acquisition software (Carl Zeiss, Göttingen, Germany). Muscle  
145 fibers size and fluorescence intensity were measured using NIH ImageJ software.

146

147 **Protein expression analysis by immunocytofluorescence.** Cells were fixed in  
148 10% formaldehyde and permeabilized with 0.1% Triton X-100. Non specific binding  
149 sites were blocked with 1% bovine serum albumin in PBS 1X for 1hour at room  
150 temperature. Cells were then incubated overnight at 4°C with specific primary  
151 antibodies (anti- TNNI1, C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-  
152 myogenin, F5D; Developmental Studies Hybridoma Bank, University of Iowa, Iowa  
153 City, IA). Detection was achieved using Alexa 555 donkey anti-goat and goat anti-  
154 mouse IgG (Molecular Probes, Invitrogen).

155 Cells were mounted with Vectashield with DAPI Fluoprep mounting medium (H1200;  
156 Vector Laboratories, Peterborough, England) and examined by fluorescence  
157 microscopy with an Axiovert 200 microscope, an Axiocam MRm camera and  
158 Axiovision 4.1 image acquisition software (Carl Zeiss, Göttingen, Germany). Area of  
159 TNNI1 immunostained differentiated myotubes was measured using NIH ImageJ  
160 software.

161



162 **Protein expression analysis by Western Blotting.** Classical western-blot  
163 experiments were performed according to (12). After transfer, gels were stained with  
164 Coomassie Blue. Membranes were then incubated overnight at 4°C with the following  
165 specific primary antibodies : anti-SREBP1 (H160), anti-MYOD1 (M316), anti-MEF2C  
166 (E17), anti-MYOG (M225), anti-TNNI1 (C-19), and anti-TNNI2 (C-19) from Santa  
167 Cruz Biotechnology, (Santa Cruz, CA, USA) ; anti-BHLHB2 (M01; 5B1) and anti-  
168 BHLHB3 from Abnova (M01; 4H6) (Taipei, Taiwan).

169 The signal was detected using a horse-radish peroxidase-conjugated secondary  
170 antibody and revealed with the enhanced chemiluminescence system (Pierce,  
171 Rockford, IL, USA). Signal was quantified using NIH ImageJ software. Intensity of  
172 Coomassie Blue staining was used to normalize the total amount of proteins.

173

174 **Quantification of mRNAs by real-time RT-PCR.** Total RNA was isolated using the  
175 Trizol reagent (Invitrogen, Courtaboeuf, France) according to manufacturer's  
176 instructions. First-strand cDNAs were synthesized from 500 ng of total RNAs in the  
177 presence of 100 U of Superscript II (Invitrogen) and a mixture of random hexamers  
178 and oligo(dT) primers (Promega). Real-time PCR assays were performed with Rotor-  
179 Gene™ 6000 (Corbett Research, Mortlake, Australia). A list of the primers and real-  
180 time PCR assay conditions are available upon request ([lefai@univ-lyon1.fr](mailto:lefai@univ-lyon1.fr)). The  
181 results were normalized using RPLP0 or HPRT mRNA concentration, measured as  
182 reference gene in each sample.

183

184 **Chromatin immuno-precipitation (ChIP) assay.** The ChIP experiments were  
185 performed as previously described (43) using the ChIP It Express Enzymatic Kit from  
186 Active Motif (Rixensart, Belgium), according to the manufacturer's instructions. ChIP

187 products were analyzed by quantitative and classical PCR using specific primers for  
188 BHLHB2 and BHLHB3 promoter (PCR primers are available on request).

189

190 **Construction of reporter plasmids, and BHLHB2 and BHLHB3 promoter**

191 **activity.** A human genomic clone (NR5-IH18RS) which contains NotI flanking regions

192 corresponding to the BHLHB2 promoter was obtained from Pr. E. R. Zabarovsky

193 (Microbiology and Tumour Biology Center and Center for Genomics and

194 Bioinformatics, Karolinska Institute, Stockholm, Sweden) (Zabarovsky ER, 2000).

195 The -408/+75 (according to the transcription starting site) fragment was then

196 subcloned into the luciferase reporter gene vector pGL3-Enhancer (Promega) to

197 obtain pB21 (-408/+75). The -951/-407 fragment was generated by PCR and ligated

198 into pB21 to obtain pB22 (-951/+75). The constructs pB23 (-264/+75) and pB26 (-

199 187/+75) were generated by deletion of pB21. To obtain pB32, two genomic

200 fragments, corresponding to the -940/-289 and -524/+238 regions of the BHLHB3

201 gene, were generated by PCR and combined to obtain the -940/+238 fragment into

202 pGL3-E vector. Mutations of the SRE motifs were performed as described (12).

203 Mutagenesis was performed to replace bases 2, 4 and 6 of each identified SRE by

204 thymidine residues (Quick Change Mutagenesis Kit, Qiagen).

205 Transfection studies were carried out on myoblasts or myotubes plated in 12-well

206 plates as previously described (12). Firefly and Renilla luciferase activities (Dual

207 luciferase reporter assay system; Promega) were measured using a Centro LB 960

208 Luminometer (Berthold Technology, Thoiry, France).

209

210 **Microarray analysis of myotubes overexpressing BHLHB2 and BHLHB3.** The

211 procedure used to obtain and analyze microarray data has previously been described

212 (43). Briefly, Total RNA extracted from BHLHB2 and BHLHB3 overexpressing  
213 myotubes were hybridized on oligonucleotide microarrays produced by the French  
214 Genopole Network (RNG) consisting of 25,342 oligonucleotides of 50-mers printed  
215 on glass slides. Only spots with recorded data on the 8 slides (4 for BHLHB2 and 4  
216 for BHLHB3) were selected for further analysis. With these selection criteria, 12,825  
217 spots were retrieved. Data were analyzed using the one-class significance analysis  
218 of microarray (SAM) procedure. Microarrays data are available in the GEO database  
219 under number GSE12947 and following the link:  
220 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi>.

221

222

## 223 **Results**

### 224 **SREBP-1a and -1c down-regulate muscle specific genes in human myotubes**

225 We recently reported that adenovirus-mediated expression of the mature nuclear  
226 forms of either SREBP-1a or SREBP-1c triggered the regulation of more than 1300  
227 genes in human differentiated myotubes (43). Using FATiGO ([www.babelomics.es](http://www.babelomics.es))  
228 to analyze these microarray data, three Gene Ontology (GO) classes showed  
229 significant over-representation in the list of genes found to be regulated in the  
230 presence of SREBP-1 proteins when compared to their representation in the human  
231 genome: “muscle contraction” (GO 0006936, adjusted p value = 2.84 e-5), the  
232 subclass “striated muscle contraction” (GO 0006941, adjusted p value = 2.46 e-5)  
233 and “muscle development” (GO 0007517, adjusted p value = 6.27 e-5). The  
234 corresponding genes with fold-change values upon SREBP-1a or -1c expression are  
235 listed in Table 1. These genes encode transcription factors involved in muscle  
236 differentiation (i.e. MYOD1, MYOG, MEF2C), as well as a large number of muscle  
237 contraction proteins (i.e. heavy and light chains of myosin, troponins, titin). Most of  
238 them were down-regulated in the presence of SREBP-1a or -1c (28 of 38 for “muscle  
239 contraction” and 26 of 39 for “muscle development”).

240

### 241 **Transcriptional repressors BHLHB2 and BHLHB3 are SREBP-1 target genes**

242 The SREBP-1s microarray data obtained on differentiated myotubes contain two  
243 bHLH family members that are up regulated upon SREBP-1s overexpression. The  
244 transcriptional repressors BHLHB2 and BHLHB3 show an about 2-fold increase in  
245 their expression levels (supplementary data of (43)). Since recent report indicated  
246 that BHLHB3 is a potent inhibitor of muscle cell differentiation (2), we decided to  
247 focus on these factors. To assess SREBP-1a and -1c effects on BHLHB2 and

248 BHLHB3 expression, we overexpressed nuclear SREBP-1 in human primary muscle  
249 cells at both myoblasts and myotubes stages, and also in vivo in mouse tibialis  
250 anterior muscle. As shown in figure 1A and 1B, overexpression of SREBP-1 in  
251 myoblasts, myotubes and mouse muscle induced significant increases in both  
252 BHLHB2 and BHLHB3 mRNA and proteins levels in all situations. As a control, we  
253 verified that overexpression of ADD1-DN, a dominant-negative mutant of SREBP-1  
254 (27), does not significantly affects BHLHB2 and BHLHB3 expression levels in  
255 cultured muscle cells.

256

257 The promoter sequences of the human BHLHB2 and BHLHB3 genes contain  
258 putative SRE motifs for SREBP-1 binding (located at -839/-830 and -32/-23 for  
259 BHLHB2; -651/-642 and +43/+52 for BHLHB3 relative to the respective transcription  
260 start sites). Additionally, a degenerate motif was identified at -248/-238  
261 (TCACAGGGT) in the BHLHB2 promoter. To investigate whether SREBP-1a and -1c  
262 increase BHLHB2 and BHLHB3 expression through promoter activation, we  
263 performed gene reporter experiments in muscle and non-muscle cell lines transiently  
264 transfected with SREBP-1a/-1c expressing plasmids. Measurements of luciferase  
265 activities confirm that overexpression of SREBP-1 proteins strongly increases both  
266 BHLHB2 and BHLHB3 promoters activities in myoblasts, myotubes and non muscle  
267 HepG2 cells (Figure 2 A and B, left). Activation of the promoters in non-muscle cells  
268 excluded the participation of additional muscle-specific factors in the induction of  
269 BHLHB2 and BHLHB3 by SREBP-1 proteins. To assess the involvement of the  
270 identified putative SREs in both promoters, we performed mutations and deletions of  
271 the various sites (Figure 2 A and B, right). Concerning the BHLHB2 promoter,  
272 deletion of the distal motif as well as mutation of the proximal motif did not modify

273 enhancement of promoter activity by SREBP-1 proteins, whereas the deletion of the  
274 SRE-like motif suppressed SREBP-1 activation. Concerning the BHLHB3 promoter,  
275 mutation of either distal or proximal SREs suppressed promoter activation, showing  
276 that they are both are involved in the response to SREBP-1. Finally, chromatin  
277 immunoprecipitation (ChIP) experiments further confirmed that SREBP-1 proteins  
278 directly bind the BHLHB2 and BHLHB3 promoters (Figure 2C).

279 We then conclude that transcriptional repressors BHLHB2 and B3 are new direct  
280 target genes of SREBP-1, the expression of which is increased by SREBP-1 binding  
281 on their promoters.

282

### 283 **Overexpression of BHLHB2 and BHLHB3 in myotubes**

284 We performed microarray analysis in human primary myotubes overexpressing either  
285 BHLHB2 or BHLHB3 after adenovirus infection. FATIGO analysis revealed that the  
286 same biological processes identified after SREBP-1 overexpression (“muscle  
287 contraction”, “striated muscle contraction” and “muscle development”) were  
288 significantly enriched (adjusted p values < 0.05) in the lists of regulated genes. We  
289 found that BHLHB2 and BHLHB3 down-regulated 69 and 65 genes with muscle  
290 annotation, respectively (Table 2). Furthermore, the comparison with the SREBP-1  
291 microarray data showed that a large proportion (34%) of the muscle-specific genes  
292 that were down-regulated by SREBP-1 expression were also down-regulated by  
293 BHLHB2/B3 overexpression.

294 Overlapping down-regulated genes for the two GO biological processes “muscle  
295 development” and “muscle contraction” are represented in Figure 3. Among genes  
296 involved in muscle differentiation, MYOD1, MYOG and MEF2C show a decrease in  
297 their expression upon both SREBP-1 and BHLHB2/B3 overexpression.

298

299 **SREBP-1a and -1c inhibit myoblast differentiation**

300 Because the expression of specific markers of muscle differentiation was decreased  
301 in myotubes overexpressing SREBP-1, we first examined the expression of the four  
302 studied transcription factors during the differentiation of human primary muscle cells  
303 (Figure 4 A). All four present a similar pattern of expression with an increase during  
304 proliferation and a decrease after induction of differentiation. To further examine  
305 whether SREBP-1 could directly affect myogenic differentiation, primary human  
306 myoblasts were thus infected with recombinant adenoviruses expressing GFP,  
307 SREBP-1a, or SREBP-1c. After 48 hours, SREBP-1 expressing myoblasts showed a  
308 dramatic decrease in MYOD1, MYOG and MEF2C levels (Figure 4B). When the cells  
309 were induced to differentiate (medium change and serum starvation) for five days,  
310 only Ad-GFP infected cells underwent differentiation (Figure 4C). The presence of  
311 SREBP-1 totally blocked the differentiation of myoblasts into myotubes.

312 To determine the implication of BHLHB2 and/or BHLHB3 in this process, human  
313 primary myoblasts were infected with recombinant adenovirus expressing either  
314 BHLHB2 or BHLHB3. As shown in Figure 4D, 48 hours of BHLHB2 and BHLHB3  
315 overexpression also induced a marked decrease in the expression of muscle  
316 regulatory factors (MYOD1, MYOG and MEF2C). After 5 days of differentiation, we  
317 observed a dramatic decrease in the number and the size of polynucleated cells,  
318 correlated with the reduced expression of myogenin and troponin (figure 4E).

319 To finally demonstrate the involvement of BHLHB2 and BHLHB3 in the effects of  
320 SREBP-1 on myoblasts, SREBP-1 overexpressing myoblasts were transfected with  
321 siRNA against GFP (control), BHLHB2, or BHLHB3, resulting in a partial gene  
322 extinction of BHLHB2 and BHLHB3 expression (Figure 5A). As shown in Figure 5B,

323 inhibition of either BHLHB2 or BHLHB3 can restore, at least partially, the expression  
324 of MYOD1, MYOG and MEF2C proteins that are down-regulated upon SREBP-1  
325 overexpression. Depletion of BHLHB2/B3 was sufficient to restore differentiation and  
326 myogenin and troponin expression in cells overexpressing SREBP-1 (Figure 5C).  
327 Altogether, these data led us to propose that SREBP-1a and -1c block myoblasts to  
328 myotubes differentiation via an increase in BHLHB2 and BHLHB3 expression, the  
329 latter repressing the expression of MRFs.

330

### 331 **SREBP-1a and -1c induce atrophy of differentiated myotubes**

332 We next examined the consequences of nuclear accumulation of SREBP-1 proteins  
333 in differentiated muscle cells. To confirm and expand the microarray data, we  
334 measured the expression levels of several transcription factors and sarcomeric  
335 protein genes using quantitative PCR in primary myotubes overexpressing the  
336 SREBP-1 factors for 48 hours. Figure 6 shows that both SREBP-1a and 1c  
337 decreased the expression of myogenic regulatory factors (MYOD1, MYOG, and  
338 MEF2C) (Figure 6A). A significant reduction in the mRNA levels of muscle contractile  
339 proteins (TTN, TNNI1, TNNI2, and MYL1) was also observed. These data were  
340 further confirmed at the protein level (Figure 6B). Therefore, the mature forms of  
341 SREBP-1a and -1c clearly induced a dramatic decrease in the expression of major  
342 actors of skeletal muscle function, involved in either formation or contractility.

343 Direct observation of myotubes overexpressing SREBP-1 showed a decrease in cell  
344 surfaces. Troponin immunostaining confirmed a considerable reduction in sarcomeric  
345 protein content (Figure 6C). Cell sizes measurements showed that SREBP-1 proteins  
346 induced an approximately 6-fold decrease in cell surface (Figure 6D). These  
347 observations indicated thus that nuclear accumulation of SREBP-1 led to myotube



348 atrophy, with a severe decrease in the expression of muscle regulatory factors and  
349 sarcomeric proteins. To assess whether the observed SREBP1-induced atrophy  
350 involved known atrophic factors, we measured the mRNA levels of FBXO32  
351 (Atrogin1), SMURF1 (MuRF-1/TRIM63) and FOXO1. As shown in Figure 6A, with the  
352 exception of SMURF1, the expression of these factors was reduced in the presence  
353 of SREBP-1a and -1c. The up-regulation of SMURF1 mRNA however, is in  
354 agreement with our previous microarray data (43).

355

356 As observed with SREBP-1a and -1c, infection of fully differentiated myotubes with  
357 adenoviruses expressing BHLHB2 or BHLHB3 strongly repressed the expression of  
358 myogenic factors (MYOD1, MYOG, and MEF2C) and sarcomeric proteins (MYL1,  
359 TNNI1, and TTN) (Figure 7A). Overexpression of BHLHB2 and BHLHB3 also  
360 provoked the atrophy of muscle cells (Figure 7B), as evidenced by cell size  
361 measurements indicating a greater than 60% reduction in myotube areas (Figure  
362 7C). However, in contrast to SREBP-1, BHLHB2 and BHLHB3 overexpression  
363 induced a marked decrease in SMURF1 expression level (Figure 7A).

364 To confirm the involvement of BHLHB2 and BHLHB3 in the atrophic effect of  
365 SREBP-1 on differentiated myotubes, SREBP-1 overexpressing myotubes were  
366 transfected with siRNA against GFP, BHLHB2, or BHLHB3. As shown in Figure 7D,  
367 gene extinction of either BHLHB2 or BHLHB3 restores the expression of troponin.  
368 Depletion of BHLHB2/B3 also restored, at least partially, the size of myotubes, with a  
369 greater effect of BHLHB3 silencing (Figure 7E).

370

371 Altogether, these data indicated that, as observed for inhibition of myoblast  
372 differentiation, the transcriptional repressors BHLHB2 and BHLHB3 are directly  
373 involved in the atrophy induced by SREBP-1 in differentiated myotubes.

374

375 **SREBP-1a and -1c promote skeletal muscle atrophy *in vivo***

376 To investigate the effects of SREBP-1 factors on muscle phenotype *in vivo*, we  
377 overexpressed SREBP-1a or SREBP-1c in limb muscle of mice using recombinant  
378 adenovirus. Adenoviruses expressing either GFP only, or both SREBP-1a and GFP  
379 (or SREBP-1c and GFP) were generated using dual expression properties of  
380 constructs containing an IRES element (26). Twelve week-old BALB/c male mice  
381 were separated into two groups and adenoviral suspensions were injected in tibialis  
382 anterior muscle with  $10^{10}$  infectious units of recombinant adenoviruses expressing  
383 only GFP (Ad-GFP) in the left limb of all animals and either SREBP-1a and GFP (Ad-  
384 1a/GFP, first group) or SREBP-1c and GFP (Ad-1c/GFP, second group) in the right  
385 limb. Animals were sacrificed seven days after injections and tibialis anterior muscles  
386 were removed for analysis. When comparing the two groups, no differences were  
387 found in GFP-only expressing muscles of the left limbs (weight, fiber sizes, and  
388 fluorescence intensity); we thus considered the data concerning Ad-GFP infected  
389 muscles as a unique set. As shown in Figure 8A, tibialis anterior weight showed a  
390 significant decrease of 17.5% (SREBP-1a/GFP vs. GFP, n=7, p=0.001) and 18.6%  
391 (SREBP-1c/GFP vs. GFP, n=7, p= 0.002) when expressing either of the SREBP-1  
392 proteins. When performing similar experiment with intramuscular injection of  
393 recombinant adenoviruses overexpressing either BHLHB2 or BHLHB3, muscle  
394 weight showed a decrease of 17.1 % (BHLHB2 vs. GFP, n=7, p=0.001) and 24,8 %

395 (BHLHB3 vs. GFP, n=7, p=0.001) respectively (Figure 8A). We next examined fiber  
396 size in histological sections of treated muscles. Quantitative analysis revealed a  
397 significant decrease in average cross-sectional area (CSA) of myofibers for both  
398 SREBP-1a (mean  $\pm$  SEM =  $1998.3 \pm 19.7 \mu\text{m}^2$ ) and SREBP-1c (mean  $\pm$  SEM  
399 =  $1950.2 \pm 21.0 \mu\text{m}^2$ ) compared to GFP (mean  $\pm$  SEM =  $2378.6 \pm 21.7 \mu\text{m}^2$ , p<0.001  
400 for both) (Figure 8B). Size distribution of muscle fiber CSA was different between  
401 GFP-only and SREBP-1/GFP expressing muscles, the latter presenting a marked  
402 displacement of distribution towards smaller sizes of fibers (Figure 8C).  
403 Representative histological sections are shown in Figure 8D with the expected  
404 mosaic pattern of fluorescence. Because of the dual expression strategy,  
405 fluorescence intensities in the muscle fibers of the right limbs reflect the level of  
406 expression of the SREBP-1 recombinant proteins. We therefore examined fiber CSA  
407 as a function of the fluorescence distribution (Figure 8E). While uninfected fibers  
408 (lowest fiber fluorescence category) showed similar myofiber CSA means, the  
409 reduction in mean fiber CSA of Ad-1a/GFP and Ad-1c/GFP infected fibers increased  
410 with fluorescence intensity, reaching a maximum around 20% reduction of mean  
411 CSA when compared to Ad-GFP infected fibers.

412

## 413 **Discussion**

414 SREBP-1a and SREBP-1c are bHLH transcription factors first identified as  
415 adipocyte determination and differentiation factors (49). Their functions have been  
416 extensively studied in hepatocytes and in mouse liver. By activation of specific target  
417 genes involved in lipogenesis, SREBP-1 increase triglycerides synthesis, and to a  
418 lesser extent cholesterol synthesis (8, 20, 21, 47). SREBP-1c was also shown to  
419 mediate the action of insulin on the expression of lipogenic genes in liver (16).  
420 SREBP-1 proteins are also expressed in skeletal muscle (13, 38, 39) and in cultured  
421 muscle cells (12, 18). In this report we identified a new role for these transcription  
422 factors and demonstrated that both SREBP-1a and SREBP-1c can block myoblast to  
423 myotube differentiation, and also induce myotube atrophy in vitro and in vivo.

424 The results of the present study also demonstrate that the transcriptional  
425 repressors BHLHB2 and BHLHB3 are SREBP-1 target genes and that they mediate  
426 the observed SREBP-1 action on human muscle cell. Both BHLHB2 and BHLHB3  
427 have been involved in the regulation of differentiation and growth of several cell  
428 types. BHLHB2 promotes the differentiation of trophoblast stem cells to trophoblast  
429 giant cells (22), induces neuronal differentiation of pheochromocytoma P19 cell (7)  
430 and promotes chondrocyte differentiation of ATDC5 cells (46). BHLHB2 can also  
431 block adipocyte differentiation through direct transcriptional repression of  
432 PPARgamma gene expression (53). Concerning muscle cells, BHLHB2 is expressed  
433 in embryonic and adult skeletal muscle cells, and has been recently proposed as a  
434 possible regulator of satellite cell activation since BHLHB2 knockout mice exhibit  
435 increased cellular proliferation and degenerated myotubes during muscle  
436 regeneration process (48). BHLHB3 mRNA is expressed in proliferating C2C12 cells  
437 and is down-regulated during myogenic differentiation (2). Moreover, its

438 overexpression blocks myoblast to myotube differentiation in C2C12 cells, through  
439 either E-Box occupancy, direct interaction with MYOD1 protein, or both (3).

440 In the present work we have demonstrated that both BHLHB2 and BHLHB3  
441 can inhibit muscle cell differentiation and induce myotube atrophy, reproducing the  
442 observed SREBP-1 effects in cultured muscle cells, notably a marked decrease in  
443 the expression of muscle specific transcription factors and sarcomeric proteins.  
444 Furthermore, silencing of BHLHB2 and BHLHB3 protein levels using siRNA fully  
445 restored the myogenic differentiation process in the presence of SREBP-1, and  
446 rescued, even if not completely, myotubes from atrophy induced by SREBP-1  
447 overexpression. These data therefore establish a novel regulatory pathway of muscle  
448 cell differentiation implicating SREBP-1, BHLHB2 and BHLHB3. Interestingly, it is  
449 also known that the transcriptional repressors BHLHB2 and BHLHB3 can antagonize  
450 each other's effects (3, 32), and the scheme of this novel pathway can be completed  
451 with a negative feedback loop that has recently been described, in which both  
452 BHLHB2 and BHLHB3 inhibit SREBP-1c expression in a HIF-dependent mechanism  
453 (10).

454

455 Muscle differentiation is under the control of two families of transcription  
456 factors, named Muscle Regulatory Factors (MRFs): the myogenic basic helix-loop-  
457 helix (bHLH) proteins (i.e. MYF5, MYOD1, MYOG and MYF6), and the myocyte  
458 enhancer factor2 (MEF2) family of MADS domain-containing proteins (i.e. MEF2A,  
459 2B, 2C, and 2D) (5, 40). Moreover, the myogenic bHLH factors interact with MEF2  
460 proteins to cooperatively activate muscle specific genes (36). We have demonstrated  
461 here that nuclear accumulation of SREBP-1 proteins led to a coordinated inhibition of  
462 the expression of the MRF in myoblasts. This decrease, which results from

463 BHLHB2/B2 transcriptional repressors activation, is sufficient to explain the blockade  
464 of differentiation. How BHLHB2/B3 repress the expression of MRF remains to be  
465 precisely examined, but this may occur through competitive binding to E-Box on MRF  
466 promoters. Moreover, a direct interaction of the transcriptional repressors with MRF  
467 proteins may participate in the inhibition of differentiation, as already demonstrated  
468 with BHLHB3 and MYOD1 in C2C12 cells (3).

469

470 Overexpression of SREBP-1 proteins, and also of BHLHB2/B3, induces both  
471 in vitro and in vivo myotube atrophy. The maintenance of muscle protein content  
472 results from intricately regulated anabolic and catabolic pathways. Examining genes  
473 regulated by both transcription factors reveals that MRFs and sarcomeric proteins  
474 are jointly down-regulated, whereas only SREBP-1 induce SMURF-1, an actor of the  
475 proteolytic pathway. The ubiquitin proteasome system has been described as the  
476 main regulator of muscle atrophy (30), and the role of SMURF1, FBXO32 (atrogin-1)  
477 and FOXO1 in this process has been recently reviewed (37). The marked reduction  
478 in sarcomeric protein, the induction of myotube atrophy, and the in vivo muscle  
479 wasting observed in the presence of SREBP-1 proteins could also have resulted from  
480 activation of this pathway. As the reversion of atrophy by BHLHB2/B3 silencing is  
481 only partial, a specific action of SREBP-1 proteins on the ubiquitin proteasome  
482 system involving other effectors than BHLHB2/B3 might thus be considered.  
483 Nevertheless, a significant part of the atrophic effect is due to BHLHB2/B3 action,  
484 through inhibition of sarcomeric proteins expression. This decrease in protein  
485 synthesis may be due to a direct action of BHLHB2/B3 on contractile protein  
486 promoters, or may also involve the decrease in MRFs expression. MRFs are still  
487 expressed in differentiated myotubes (50), and participate in the expression of

488 sarcomeric proteins (31). Whether MRFs are involved in the maintenance of the full  
489 differentiated phenotype is still debated, but a combined decrease in MRFs expression  
490 in differentiated myotubes may affect muscle protein synthesis and thus participate in  
491 the observed atrophy. Further studies are needed to characterize this atrophic process  
492 in terms of fiber type change, mitochondrial content, and oxidation capacity.

493

494 The control of the amount of SREBP-1 proteins in the nucleus involves  
495 regulation at several levels, including SREBP-1 gene expression, proteolytic  
496 cleavage in the endoplasmic reticulum, nuclear import and activation/degradation  
497 within the nucleus (for review see (42)). It has been recently demonstrated that  
498 SREBP-1 expression is enhanced through the PKB/mTOR pathway, and could  
499 participate in the regulation of cell size through the control of lipid and cholesterol  
500 metabolism (41). The inflammatory cytokine TNF-alpha, which is known to induce  
501 muscle atrophy (33) has been shown to increase SREBP-1 levels in hepatocytes  
502 (15). Growth factors like insulin and IGF-1 are potent inducers of SREBP-1  
503 expression in various cell types and tissues (1, 13, 38). In muscle, SREBP-1c nuclear  
504 content can be dramatically increased by insulin through activation of both the  
505 PI3K/PKB (38) and the MAPK (28, 38) pathways. Furthermore, the SREBP-1  
506 proteins can control and enhance their own expression in human muscle cells (12).  
507 Due to the major and clearly demonstrated role of insulin, growth factors and the  
508 PI3K/PKB signaling pathway on muscle development and hypertrophy (23, 29), the  
509 atrophic effect of SREBP-1 proteins overexpression demonstrated in the present  
510 work likely represents a negative feedback loop to control muscle hypertrophy. In the  
511 same context, it is also interesting to notice that SREBP-1a and -1c enhance the  
512 expression of the p55 subunit of the PI3K (25, 43), which is regarded as a positive

513 regulator of the PI3K/PKB pathway (17). The SREBP-1 proteins may thus regulate  
514 the hypertrophic effects of growth factors not only negatively through induction of the  
515 BHLHB2 and BHLHB3 repressors, but also positively through the control of  
516 PI3K/PKB signaling pathway. Further investigations are required to study the impact  
517 of SREBP-1 on signaling pathways in skeletal muscle cells.

518

519 In summary, the data presented here identify a new role for the SREBP-1  
520 transcription factors in the regulation of myogenesis and muscle tissue maintenance.  
521 As SREBP-1a and -1c are master regulators of fatty acids and cholesterol synthesis,  
522 this new function can justify to consider them as integrators of signals coming from  
523 growth factors, inflammation and nutritional status toward a control of muscle mass. It  
524 will therefore be of particular interest to further study these transcription factors in  
525 pathological situations inducing muscle wasting, but also in metabolic diseases  
526 where abnormalities in SREBP-1 have already been reported such as insulin-  
527 resistance and type 2 diabetes.

528



529 **Acknowledgments**

530 This work was supported by the Programme National de Recherche sur le Diabète  
531 (grant to E. Lefai). V. Lecomte is supported by a doctoral fellowship from the  
532 Fondation pour la Recherche Médicale.

533 The authors thank Annie Durand, Cyrille Debard, Christine Durand and Aurelie  
534 Granjon for technical assistance and Pr. E.R. Zabarowsky for the generous gift of the  
535 human genomic clone containing the BHLHB2 promoter region.

537

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- 709  
710

711 **Figure Legends**

712

713 **Figure 1: BHLHB2 and B3 are up-regulated upon SREBP-1 overexpression**

714 (A) mRNA levels of BHLHB2 and BHLHB3 in myoblasts, myotubes and mouse TA  
715 muscle overexpressing GFP, SREBP-1a, SREBP-1c or ADD1-DN. (B) Protein levels  
716 of BHLHB2 and BHLHB3 in myotubes overexpressing GFP, SREBP-1a, SREBP-1c  
717 or ADD1-DN. Illustrative immunoblot on the left and quantification on the right.  
718 Coomassie blue (Coom) staining was used to normalize the total amount of proteins.  
719 Results are presented as mean  $\pm$  SEM. \*  $p \leq 0,05$  ; \*\*  $p \leq 0,001$  (n= 3).

720

721 **Figure 2: BHLHB2 and B3 are SREBP-1 target genes**

722 BHLHB2 (A, left panel) and BHLHB3 (B, left panel) promoter activity in myoblasts  
723 (Mb), myotubes (Mt), and HEPG2 cells co-transfected with reporter gene plasmid  
724 pB22 or pB32 and expression vectors encoding either human SREBP-1a (pCDNA-  
725 hSREBP1a) or SREBP-1c ( pCDNA-hSREBP1c) , or empty pCDNA3 as control. On  
726 the right panels, relative luciferase activity of constructs harboring mutations of SRE  
727 motifs identified in either BHLHB2 (A) or BHLHB3 (B) promoters. (C) Recruitment of  
728 SREBP1 on BHLHB2 and BHLHB3 promoters determined by ChIP experiments  
729 carried on insulin-treated HEK 293 cells. ChIP products were analysed by  
730 quantitative and classical PCR. Results are presented as mean  $\pm$  SEM. \*  $p \leq 0,05$  ; \*\*  
731  $p \leq 0,001$  (n=4).

732

733 **Figure 3: Common SREBP-1, BHLHB2 and B3 down-regulated muscle genes**

734 Venn diagram representing the distribution of SREBP-1, BHLHB2 and BHLHB3  
735 down- regulated genes corresponding to « Muscle development » (GO 0007517) (A)  
736 and to « Muscle contraction » (GO 0006936) (B). Overlapping genes are listed on the

737 right.

738

739 **Figure 4: SREBP1 and BHLHB2/B3 inhibit human myoblasts differentiation**

740 (A) mRNA levels of SREBP-1a, SREBP-1c, BHLHB2 and BHLHB3 in human primary  
741 muscle cells showing an increase during proliferation and a decrease after induction  
742 of differentiation. (B) mRNA levels of myogenic factors (MYOD, MEF2C, MYOG) in  
743 myoblasts overexpressing GFP, SREBP1a, or SREBP1c. (C) Representative phase  
744 contrast images of myoblasts overexpressing GFP, SREBP1a, or SREBP1c after 5  
745 days of differentiation (Scale bar = 100 $\mu$ m). (D) mRNA levels of myogenic factors  
746 (MYOD, MEF2C, MYOG) in myoblasts overexpressing GFP, BHLHB2, or BHLHB3.  
747 (E) Representative images of myoblasts overexpressing GFP, BHLHB2, or BHLHB3  
748 after 5 days of differentiation (Scale bar = 100 $\mu$ m); Myogenin (MYOG) and Troponin  
749 I1 (TNNI1) immunostaining (red), with DAPI staining (blue), were performed to  
750 assess differentiation state. Results are presented as mean  $\pm$  SEM. \*  $p \leq 0,05$  ; \*\*  
751  $p \leq 0,001$ , (n=3).

752

753 **Figure 5: SREBP-1 inhibit human myoblasts differentiation through BHLHB2/B3**  
754 **repressors**

755 Human myoblasts were infected for 48 h with recombinant adenoviruses encoding  
756 SREBP-1a, or SREBP-1c, or GFP and co-transfected for 72h with siRNA against  
757 BHLHB2 or BHLHB3 or both, or with siRNA against GFP as control. Representative  
758 immunoblot of BHLHB2 and BHLHB3 (A) and MYOD1, MYOG and MEF2C (B) in  
759 myoblasts transfected with siRNA against GFP (lanes 1) BHLHB2 (lanes 2) or  
760 BHLHB3 (lanes 3), and quantification of the protein levels (right panels). Coomassie  
761 blue (Coom) staining was used to normalize the total amount of proteins. Results are

762 presented as mean  $\pm$  SEM. \*  $p \leq 0,05$  ; \*\*  $p \leq 0,001$ , (n=3). (C) Representative images  
763 of myoblasts overexpressing GFP, SREBP-1a or SREBP-1c, and transfected with  
764 siRNA against GFP (line 1) BHLHB2 (line 2) BHLHB3 (line 3) and both BHLHB2 and  
765 BHLHB3 (line 4) after 5 days of differentiation (Scale bar = 100 $\mu$ m); Myogenin  
766 (MYOG, left) and Troponin I1 (TNNI1, right) immunostaining (red), with DAPI staining  
767 (blue) were performed to assess differentiation state.

768

### 769 **Figure 6: SREBP-1 induce human myotubes atrophy**

770 Human myotubes were infected for 48 h with recombinant adenoviruses encoding  
771 GFP, SREBP-1a, or SREBP-1c. (A) mRNA levels of myogenic factors (MYOD1,  
772 MEF2C, MYOG), sarcomeric proteins (MYL1, TNN, TNNI1 or TNNI2), and atrogenic  
773 factors (FOXO1, FBXO32, SMURF1) (n= 6 in each group). (B) Protein levels of  
774 SREBP1, MYOD1, MYOG, MEF2C, TNNI1 and TNNI2; Coomassie blue (Coom)  
775 staining was used to normalize the total amount of proteins (n= 4 in each group). (C)  
776 Representative images of myotubes overexpressing GFP, SREBP-1a, SREBP-1c  
777 and ADD1-DN (Scale bar = 100 $\mu$ m); upper panels: phase contrast images; lower  
778 panels: immunostaining with TNNI1 antibody (red) and DAPI staining (blue). (D)  
779 Measurement of the area of myotubes overexpressing GFP, SREBP-1a and SREBP-  
780 1c stained with TNNI1 antibody (n= 3 in each group).

781 Results are presented as mean  $\pm$  SEM. \*  $p \leq 0,05$  ; \*\*  $p \leq 0,001$ .

782

### 783 **Figure 7: SREBP-1 induce myotubes atrophy through BHLHB2/B3 repressors**

784 Human myotubes were infected for 48 h with recombinant adenoviruses encoding  
785 GFP, BHLHB2, or BHLHB3. (A) mRNA levels of myogenic factors (MYOD1, MEF2C,  
786 MYOG), sarcomeric proteins (MYL1, TNN, TNNI1), and atrogenic factors (FOXO1,



787 FBXO32, SMURF1). (B) Representative images of myotubes overexpressing GFP,  
788 BHLHB2 and BHLHB3. Upper panels: phase contrast, lower panels: immunostaining  
789 with TNNI1 antibody (red) and DAPI staining (blue) (Scale bar = 100 $\mu$ m). (C)  
790 Measurement of the area of myotubes overexpressing GFP, BHLHB2 or BHLHB3  
791 immunostained with TNNI1 antibody (n= 3 in each group). Results are presented as  
792 mean  $\pm$  SEM. \*  $p \leq 0,05$  ; \*\*  $p \leq 0,001$ . (D) Representative images of myotubes  
793 overexpressing GFP, SREBP-1a or SREBP-1c, and transfected with siRNA against  
794 GFP (lane 1), BHLHB2 (lane 2), and BHLHB3 (lane 3) for 48 hours (Scale bar =  
795 100 $\mu$ m). (E) Measurement of the area of myotubes overexpressing GFP, SREBP-1a  
796 and SREBP-1c and transfected with siRNA against GFP, BHLHB2 or BHLHB3.  
797 Myotubes were stained with TNNI1 antibody (n= 3 in each group).  
798 Results are presented as mean  $\pm$  SEM. \*  $p \leq 0,05$  ; \*\*  $p \leq 0,001$ .

799

### 800 **Figure 8: In vivo overexpression of SREBP-1 leads to muscle atrophy**

801 Tibialis Anterior (TA) muscles of mice were injected with recombinant adenovirus Ad-  
802 GFP, Ad-SREBP-1a/GFP, Ad- SREBP-1c/GFP, Ad-BHLHB2 or Ad-BHLHB3. (A) TA  
803 weight 7 days after adenoviral infection (n= 7 in each group). (B) Mean CSA of TA  
804 fibers. (C) Distribution of mean CSA of TA muscle fibers (n=4 in each group). (D)  
805 Representative images of TA sections, expressing GFP, or SREBP-1a and GFP, or  
806 SREBP-1c and GFP: DAPI staining (blue) and GFP fluorescence (green) (Scale bar  
807 = 100  $\mu$ m). (E) Distribution of CSA of TA muscle fibers as a function of myofiber  
808 fluorescence. Results are presented as mean  $\pm$  SEM. \*\*  $p \leq 0,001$ ; \*\*\*  $p \leq 0,0001$   
809 (n=4).

810 **Table 1: Muscle specific SREBP-1 target genes**

811 Listing of 1300 SREBP-1 targets genes identified previously (43) was analysed using  
 812 FATIGO+. Three GO classes were found to be statistically over-represented: muscle  
 813 contraction (GO 0006936, adjusted p value = 1.66 e-4), striated muscle contraction  
 814 (GO 0006941, adjusted p value = 7.29 e-5) and muscle development (GO 0007517,  
 815 adjusted p value = 6,51 e-5).  
 816

Symbol	LLID	Fold 1A	Fold 1C	Name
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**GO 0006936 : Muscle contraction (Adjusted p value : 2.84 e-5)**

ADRB2	154	-2.48	-2.61	Adrenergic, beta-2-, receptor, surface
ALDOA	226	2.06		Aldolase A, fructose-bisphosphate
ATP1A1	476	-2.27		ATPase. Na+/K+ transporting. alpha 1 polypeptide
ATP1A2	477	-2.31		ATPase. Na+/K+ transporting. alpha 2 (+) polypeptide
ATP2A2	488	-1.86		ATPase. Ca++ transporting. cardiac muscle. slow twitch 2
CACNG1	786	-7.42		Calcium channel. voltage-dependent. gamma subunit 1
CASQ2	845	-3.45		Calsequestrin 2 (cardiac muscle)
CHRNA1	1134	-1.96		Cholinergic receptor. nicotinic. alpha 1 (muscle)
DTNA	1837	-4.02	-3.01	Dystrobrevin, alpha
EDNRA	1909	-3.21		Endothelin receptor type A
FXYD1	5348		3.31	FXYD domain containing ion transport regulator 1 (phospholemman)
GAL	2660	4.28		Galanin
GJA1	2697	-4.83	-2.02	Gap junction protein. alpha 1. 43kDa (connexin 43)
HRC	3270	-2.72		Histidine rich calcium binding protein
ID2B	84099	-1.60		Inhibitor of DNA binding 2B. dominant negative helix-loop-helix protein
KBTBD10	10324		-1.60	Kelch repeat and BTB (POZ) domain containing 10
KCNH2	3757	-1.73		Potassium voltage-gated channel. subfamily H (eag-related). member 2
MRCL3	10627		-1.63	Myosin regulatory light chain MRCL3
MYBPC1	4604	-4.62		Myosin binding protein C. slow type
MYBPC2	4606	14.91	10.04	Myosin binding protein C. fast type
MYBPH	4608	-19.98		Myosin binding protein H
MYH2	4620	-6.45		Myosin. heavy polypeptide 2. skeletal muscle. adult
MYH3	80184	16.48		Myosin. heavy polypeptide 3
MYH6	4624		3.47	myosin. heavy polypeptide 6. cardiac muscle. alpha
MYH8	4626	-8.82	-2.39	Myosin. heavy polypeptide 8. skeletal muscle. perinatal
SCN7A	6332	2.64		Sodium channel. voltage-gated. type VII. alpha
SLC6A8	6535	-1.47		Solute carrier family 6 (neurotransmitter transporter, creatine), member 8
SMPX	23676	-8.55		Small muscle protein, X-linked
SNTA1	6640	1.70		Syntrophin, alpha 1 (dystrophin-associated protein A1)
SSPN	8082	-3.15		Sarcospan (Kras oncogene-associated gene)
TNNC2	7125	-2.83		Troponin C type 2 (fast)
TNNI1	7135	-2.32		Troponin I type 1 (skeletal. slow)
TNNI2	7136	-3.07		Troponin I type 2 (skeletal. fast)
TNNT2	7139	-1.88		Troponin T type 2 (cardiac)
TNNT3	7140	-2.83		Troponin T type 3 (skeletal. fast)
TPM1	7168	-4.54		Tropomyosin 1 (alpha)
TPM3	7170		-2.44	Tropomyosin 3
TTN	7273	1.92	2.93	Titin

**GO 0007517 : Muscle development (Adjusted p value : 6.27 e-5)**

ACTG1	71	-2.86	-2.37	Actin, gamma 1
AEBP1	165	2.29		AE binding protein 1
CAV3	859	-1.94		Caveolin 3

COL5A3	50509	-1.53		Collagen. type V. alpha 3
COL6A3	1293	1.40		Collagen. type VI. alpha 3
CSRP2	1466	-4.25		Cysteine and glycine-rich protein 2
CUGBP2	10659	-1.80		CUG triplet repeat, RNA binding protein 2
DSCR1	1827	-2.18	-1.78	Down syndrome critical region gene 1
EVC	2121	-1.50		Ellis van Creveld syndrome
FHL1	2273	-4.99		Four and a half LIM domains 1
FXYD1	5348		3.31	FXYD domain containing ion transport regulator 1 (phospholemman)
GDF8	2660	-6.20	-1.98	Growth differentiation factor 8 (myostatin)
HBEGF	1839	5.22	4.10	Heparin-binding EGF-like growth factor
HDAC5	10014	2.35		Histone deacetylase 5
HDAC9	9734	-2.15		Histone deacetylase 9
HSBP2	3316	-3.37		Heat shock 27kDa protein 2
ITGA7	3679	-2.40		Integrin. alpha 7
ITGB1BP2	26548	-4.72		Integrin beta 1 binding protein (melusin) 2
KRT19	3880	2.50		Keratine 19
MEF2C	4208	-6.73	-3.45	Myocyte Enhancer Factor 2C)
MLLT7	4303	1.96	3.40	Myeloid/lymphoid or mixed-lineage leukemia
MRAS	22808	-3.86	-2.03	Muscle RAS oncogene homolog
MYH6	4624		3.47	Myosin. heavy polypeptide 6. cardiac muscle. alpha
MYH10	4628	2.28		Myosin, heavy polypeptide 10, non-muscle
MYL1	4632	-4.16		Myosin. light polypeptide 1. alkali; skeletal. fast
MYL4	4635		2.28	Myosin. light polypeptide 4. alkali; atrial. embryonic
MYOD1	4654	-2.76		Myogenic differentiation 1
MYOG	4656	-7.42		Myogenin (myogenic factor 4)
NRD1	4898	3.97	2.59	Nardilysin (N-arginine dibasic convertase)
SGCD	6444	-5.09		Sarcoglycan. delta (35kDa dystrophin-associated glycoprotein)
SGCG	6445	-2.42		Sarcoglycan. gamma (35kDa dystrophin-associated glycoprotein)
SIX1	6495	-3.88	-2.85	Sine oculis homeobox homolog 1 (Drosophila)
SMAD3	4088	-1.82	-1.78	SMAD, mothers against DPP homolog 3
SNTA1	6640	1.70		Syntrophin, alpha 1 (dystrophin-associated protein A1)
TEAD4	7004	-3.54		TEA domain family member 4
TMOD1	7111	-3.01		Tropomodulin 1
TNNT2	7139	-1.88		Troponin T type 2 (cardiac)
TTN	7273	1.92	2.93	Titin
VAMP5	10791	-3.03		Vesicle-associated membrane protein 5 (myobrevin)

818 **Table2: Muscle specific BHLHB2/B3 target genes**

819 Microarray analysis was performed on human primary muscle cells overexpressing  
 820 either BHLHB2 or BHLHB3. Listing of BHLHB2/B3 identified target genes was  
 821 analysed using FATIGO+. Biological processes “muscle contraction”, “striated  
 822 muscle contraction” and muscle development” show significant enrichment (adjusted  
 823 p value < 0.05).  
 824

LLID	Symbol	Fold B2	Fold B3	Gene name
58	ACTA1	-2,89	-4,55	Actin, alpha 1, skeletal muscle
70	ACTC	-1,78	-2,75	Actin, alpha, cardiac muscle
88	ACTN2	-1,69		Actinin, alpha 2
89	ACTN3	-2,67	-3,27	Actinin, alpha 3
203	AK1	-1,56	-2,19	Adenylate kinase 1
10930	APOBEC2		-1,76	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 2
57679	ALS2	1,75		Amyotrophic lateral sclerosis 2 (juvenile)
130540	ALS2CR12	1,41		Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 12
26287	ANKRD2	-1,52		Ankyrin repeat domain 2 (stretch responsive muscle)
316	AOX1	1,51		Aldehyde oxidase 1
487	ATP2A1	-1,83		ATPase, Ca <sup>++</sup> transporting, cardiac muscle, fast twitch 1
444	ASPH		1,57	Aspartate beta-hydroxylase
79888	AYTL2	1,80		Acyltransferase like 2
8678	BECN1	1,36		Beclin 1 (coiled-coil, myosin-like BCL2 interacting protein)
779	CACNA1S	-1,36	-1,68	Calcium channel, voltage-dependent, L type, alpha 1S subunit
782	CACNB1	-2,85		Calcium channel, voltage-dependent, beta 1 subunit
786	CACNG1	-2,06	-5,29	Calcium channel, voltage-dependent, gamma subunit 1
823	CAPN1	1,37		Calpain 1, (mu/l) large subunit
84698	CAPS2	1,34		Calcyphosine 2
859	CAV3	-1,73	-3,44	Caveolin 3
928	CD9	1,96		CD9 molecule
1013	CDH15		-2,07	Cadherin 15, M-cadherin (myotubule)
50937	CDON	-1,59	-2,79	Cdon homolog (mouse)
1072	CFL1	-1,38		Cofilin 1 (non-muscle)
1134	CHRNA1	-1,71		Cholinergic receptor, nicotinic, alpha 1 (muscle)
1146	CHRNG	-2,34	-2,31	Cholinergic receptor, nicotinic, gamma
1152	CKB	-1,42		Creatine kinase, brain
1158	CKM	-2,33	-2,90	Creatine kinase, muscle
1160	CKMT2	-4,09	-3,86	Creatine kinase, mitochondrial 2 (sarcomeric)
50509	COL5A3	-1,74	-1,62	Collagen, type V, alpha 3
1339	COX6A2	-1,55		Cytochrome c oxidase subunit VIa polypeptide 2
1410	CRYAB	-2,02	-2,12	Crystallin, alpha B
1674	DES	-1,58	-1,80	Desmin
25891	DKFZP586H2123	1,35		Regeneration associated muscle protease
1760	DMPK	-1,97	-2,84	Dystrophia myotonica-protein kinase
1837	DTNA		1,89	Dystrobrevin, alpha
1838	DTNB		-1,49	Dystrobrevin, beta
8291	DYSF		-1,59	Dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)
1917	EEF1A2	-1,98	-2,26	Eukaryotic translation elongation factor 1 alpha 2
112399	EGLN3			Egl nine homolog 3 (C. elegans)
2027	ENO3	-1,82	-1,91	Enolase 3 (beta, muscle)
114907	FBXO32	2,07		F-box protein 32
2281	FKBP1B		2,16	FK506 binding protein 1B, 12.6 kDa
2318	FLNC		-2,30	Filamin C, gamma (actin binding protein 280)
2308	FOXO1	-1,46	-2,20	Forkhead box O1
2660	GDF8	2,18		Gap junction protein, alpha 1, 43kDa (connexin 43)
93626	GNA11	1,34		guanine nucleotide binding protein (G protein), alpha 11 (Gq class)
2997	GYS1	-1,83		Glycogen synthase 1 (muscle)
9759	HDAC4	-1,57	-1,69	Histone deacetylase 4

3270	HRC	-1,58	-1,68	Histidine rich calcium binding protein
3679	ITGA7		-1,59	Integrin, alpha 7
10324	KBTBD10	1,86		Kelch repeat and BTB (POZ) domain containing 10
3939	LDHA	-1,55		Lactate dehydrogenase A
6300	MAPK12	-1,97	-2,85	Mitogen-activated protein kinase 12
4151	MB	-1,90	-1,52	Myoglobin
10150	MBNL2	1,57		Muscleblind-like 2 (Drosophila)
4208	MEF2C		-1,95	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)
22808	MRAS		-2,11	Muscle RAS oncogene homolog
23164	M-RIP		-1,84	Myosin phosphatase-Rho interacting protein
103910	MRLC2	1,57		Myosin regulatory light chain MRLC2
136319	MTPN	1,76		Myotrophin
4604	MYBPC1	-1,69		Myosin binding protein C, slow type
4608	MYBPH		-2,69	Myosin binding protein H
4620	MYH2	-1,76		Myosin, heavy polypeptide 2, skeletal muscle, adult
80184	MYH3 (CEP290)	-1,69	-3,82	Myosin, heavy polypeptide 3
4624	MYH6	-1,41	-1,71	myosin, heavy polypeptide 6, cardiac muscle, alpha
4625	MYH7	-1,70	-3,28	Myosin, heavy chain 7, cardiac muscle, beta
8735	MYH13	-1,42		Myosin heavy chain 13
4632	MYL1	-1,41		Myosin, light polypeptide 1, alkali; skeletal, fast
4633	MYL2	-1,81	-2,23	Myosin, light chain 2, regulatory, cardiac, slow
4634	MYL3	-1,69	-1,72	Myosin, light chain 3, alkali; ventricular, skeletal, slow
4636	MYL5	-1,84		Myosin, light chain 5, regulatory
93408	MYLC2PL	1,31		Myosin light chain 2, precursor lymphocyte-specific
85366	MYLK2	1,33		Myosin light chain kinase 2, skeletal muscle
53904	MYO3A	1,48		Myosin IIIA
4645	MYO5B	-1,54		myosin VB [GDB]
4654	MYOD1	-1,71		Myogenic differentiation 1
4656	MYOG	-1,91	-5,79	Myogenin (myogenic factor 4)
9172	MYOM2	-3,03	-3,11	Myomesin (M-protein) 2, 165kDa
9499	MYOT	-2,95	-3,67	Myotilin
84665	MYPN	-1,60		Myopalladin
4692	NDN		1,44	Necdin homolog (mouse)
4703	NEB		-1,72	Nebulin
84033	OBSCN	-1,39	-2,46	Obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF
55229	PANK4	-1,32		Pantothenate kinase 4
5081	PAX7	-1,42		Paired box 7
5213	PFKM	-1,48		Phosphofructokinase, muscle
5224	PGAM2	-2,57	-2,60	Phosphoglycerate mutase 2 (muscle)
64091	POPDC2		-1,36	Popeye domain containing 2
64208	POPDC3		-1,78	Popeye domain containing 3
10891	PPARGC1A	-2,32		Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
4659	PPP1R12A	1,45		Protein phosphatase 1, regulatory (inhibitor) subunit 12A
53632	PRKAG3	-2,16	-6,45	Protein kinase, AMP-activated, gamma 3 non-catalytic subunit
89970	RSPRY1	-1,43		Ring finger and SPRY domain containing 1
6415	SEPW1	-1,40	-1,48	Selenoprotein W, 1
6444	SGCD	-1,61	-1,39	Sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)
6445	SGCG		-1,96	Sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein)
6526	SLC5A3	-1,93		sodium/myo-inositol cotransporter 1
6535	SLC6A8		-1,59	Solute carrier family 6 (neurotransmitter transporter, creatine), member 8
6586	SLIT3		-3,95	Slit homolog 3 (Drosophila)
6641	SNTB1		-1,92	Syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)
8878	SQSTM1	1,52		Sarcospan (Kras oncogene-associated gene)
8082	SSPN		-1,69	Sarcospan (Kras oncogene-associated gene)
6840	SVIL	1,52	-1,39	Titin-cap (telethonin)
23345	SYNE1		1,40	Supervillin
8557	TCAP	-1,80		Spectrin repeat containing, nuclear envelope 1

7004	TEAD4	-2,37	-4,18	TEA domain family member 4
7111	TMOD1		-2,13	Tropomodulin 1
29766	TMOD3	1,66	1,49	Tropomodulin 3 (ubiquitous)
7134	TNNC1		-1,59	Troponin C type 1 (slow)
7135	TNNI1	-2,46	-2,34	Troponin I type 1 (skeletal, slow)
7136	TNNI2	-1,40	-1,52	Troponin I type 2 (skeletal, fast)
7139	TNNT2	-1,48	-2,12	Troponin T type 2 (cardiac)
7140	TNNT3	-2,07	-2,13	Troponin T type 3 (skeletal, fast)
57159	TRIM54		-1,79	Tripartite motif-containing 54
84675	TRIM55	-1,61	-2,56	Tripartite motif-containing 55
84676	TRIM63	-3,91	-4,12	Tripartite motif-containing 63
7273	TTN	-1,90	-4,98	Titin
81622	UNC93B1	-1,78		Unc-93 homolog B1 (C. elegans)
7431	VIM	1,82		Vimentin

825

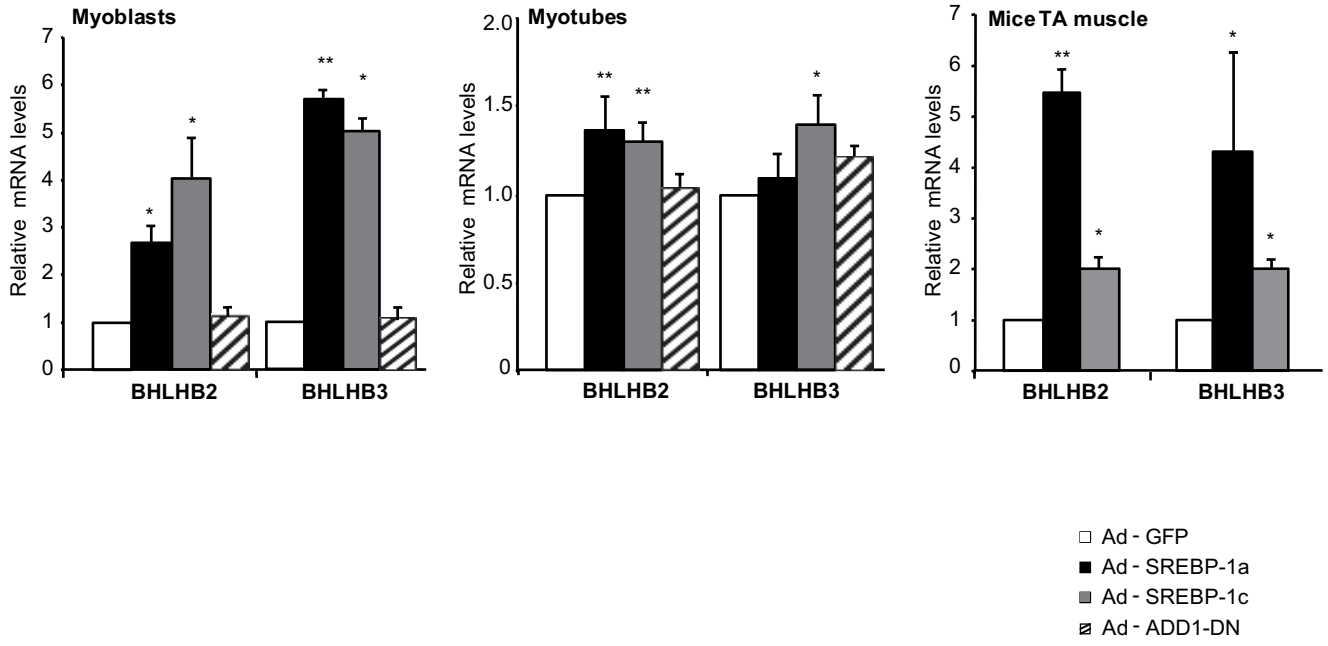
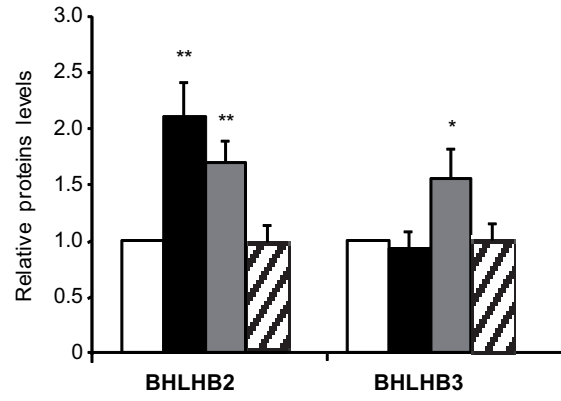
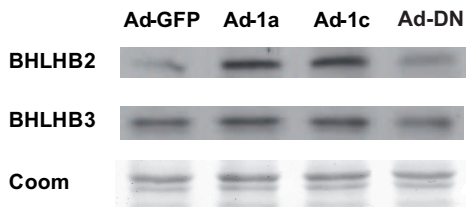
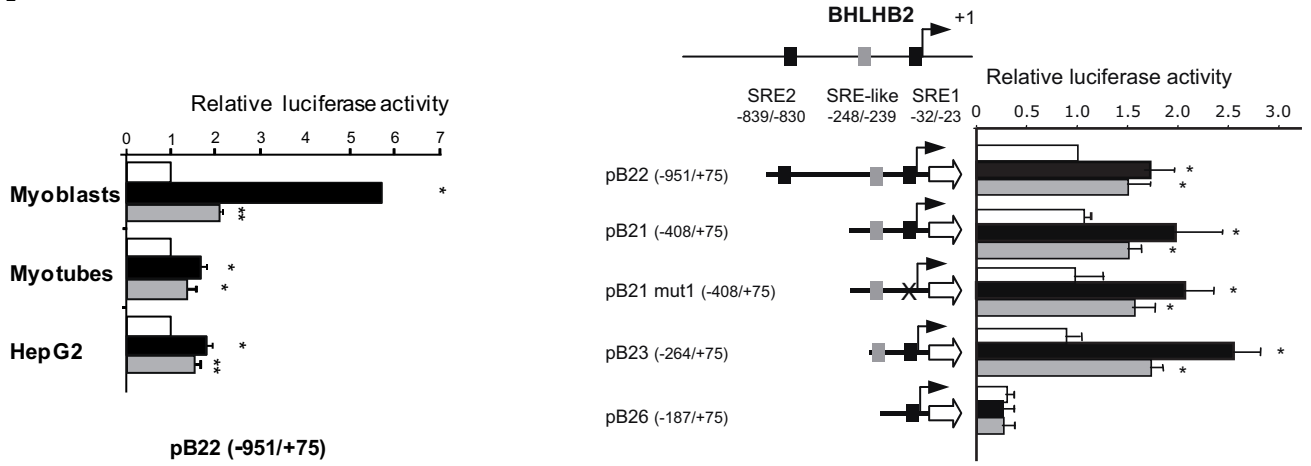
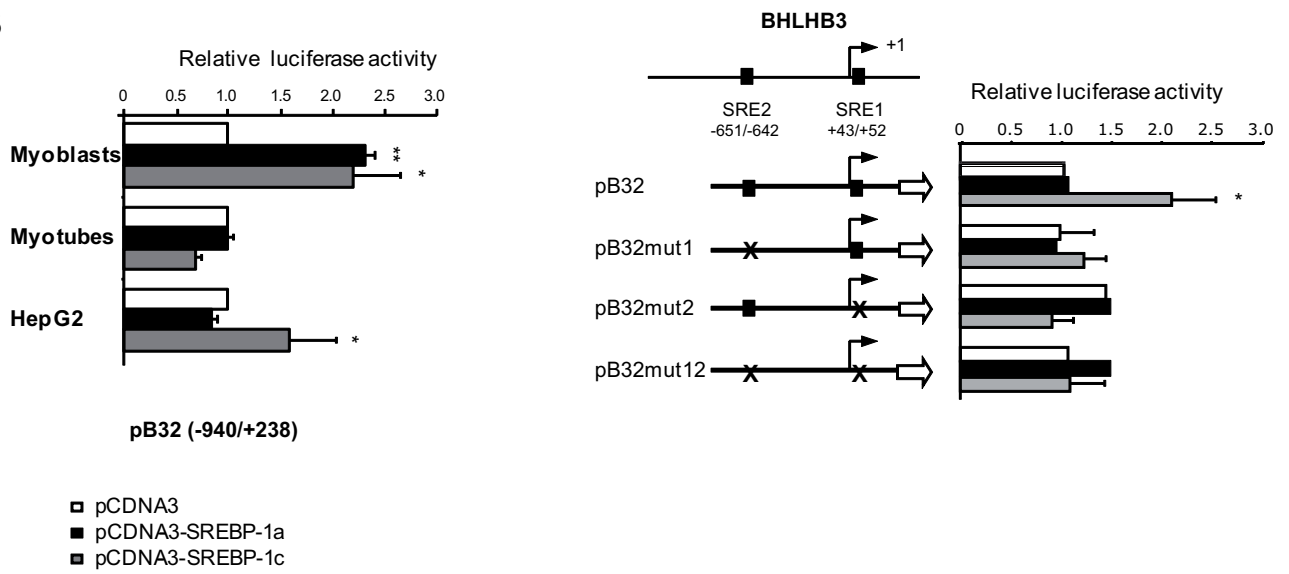
**A****B**

Figure 1

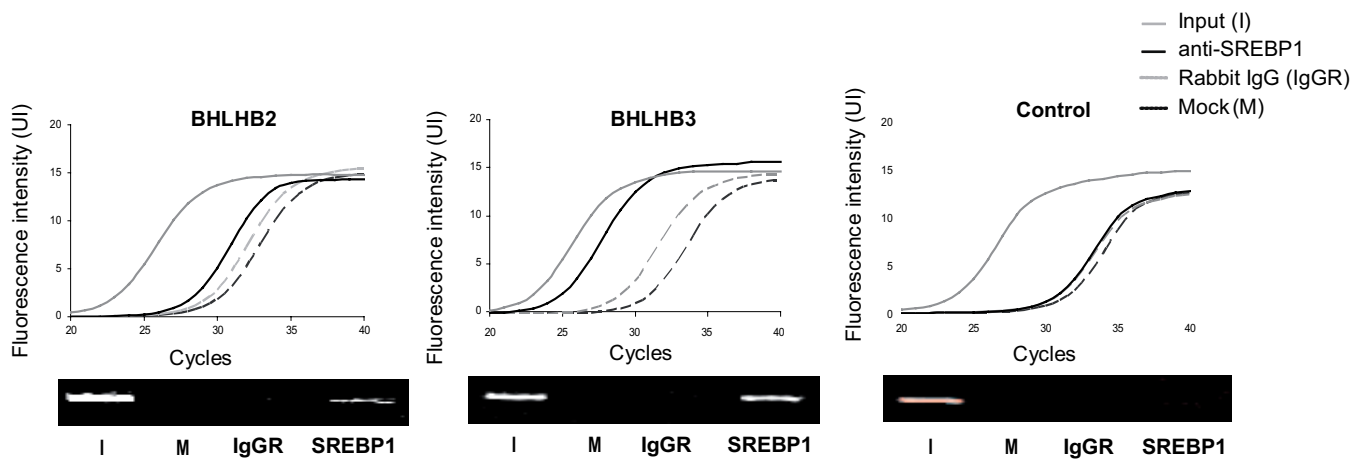
**A**



**B**



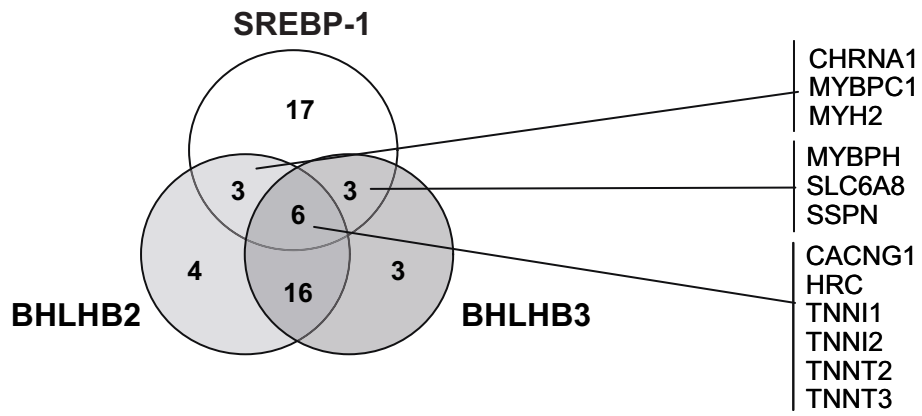
**C**



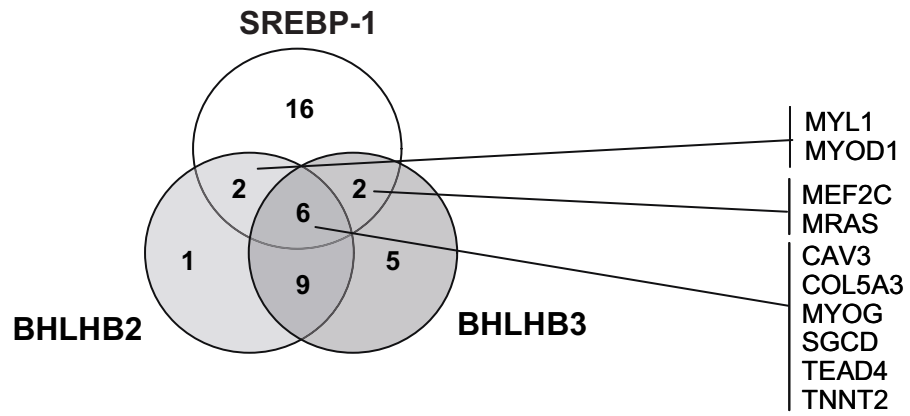
**Figure 2**



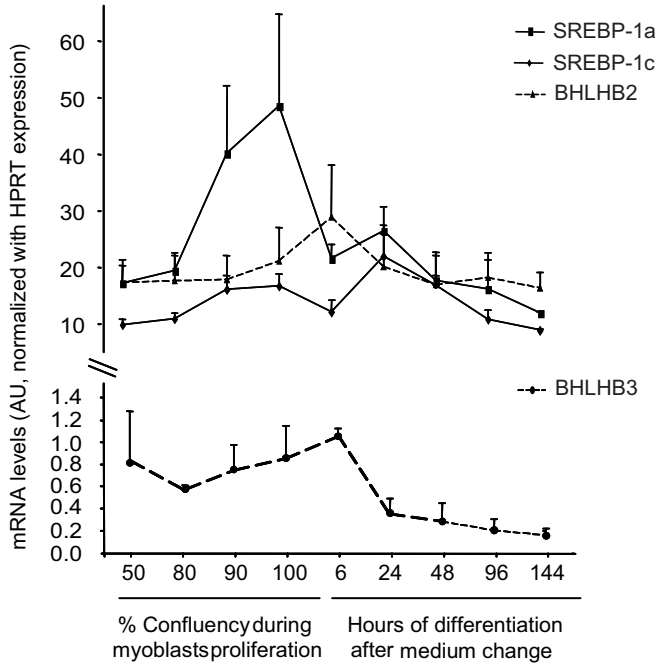
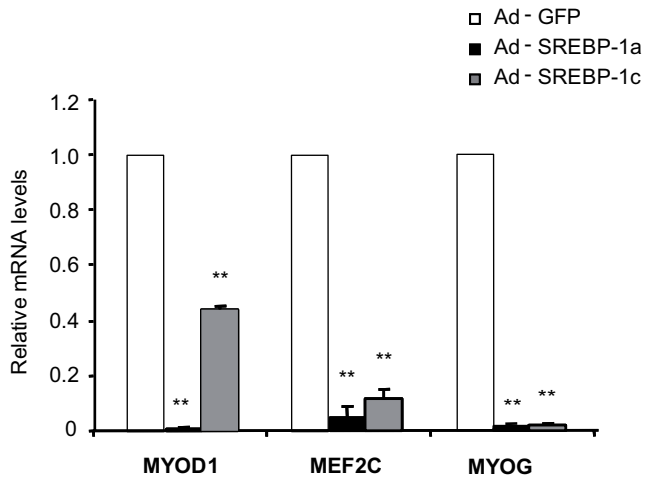
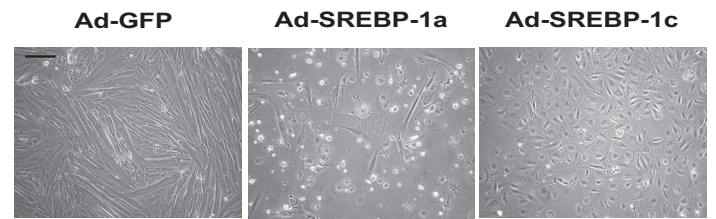
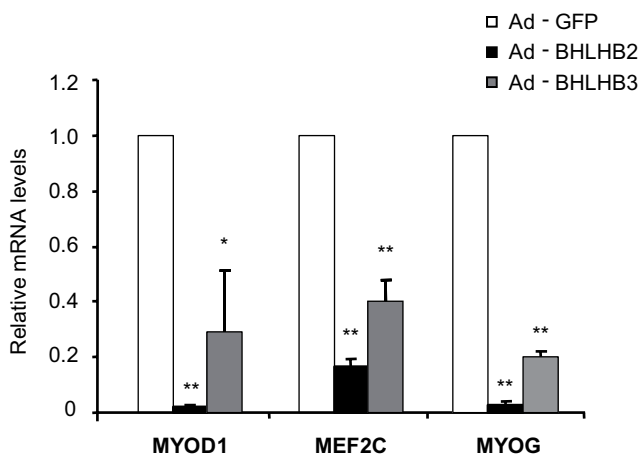
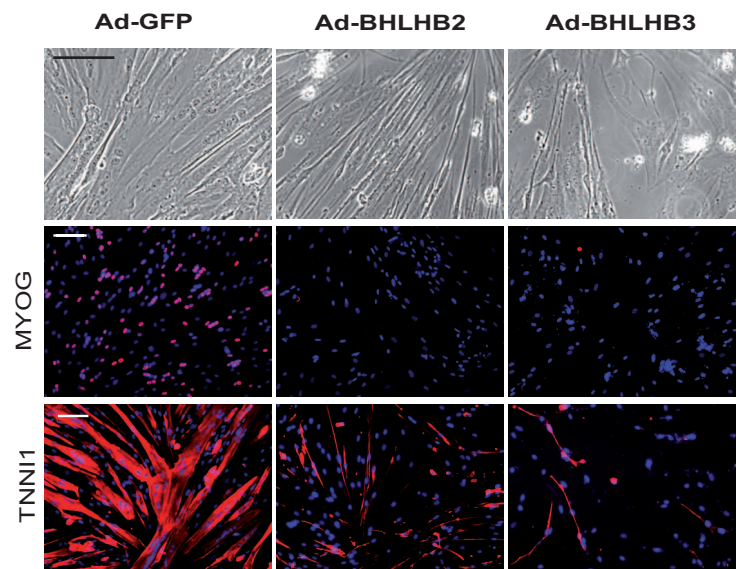
**A**

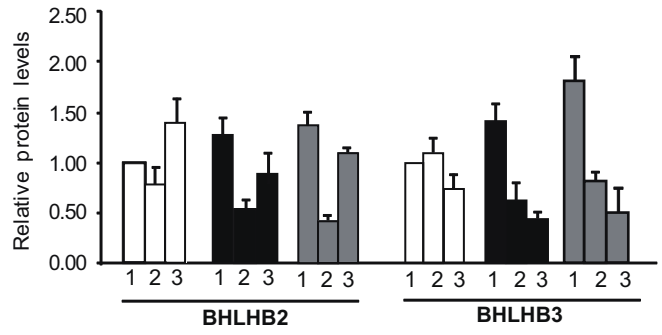
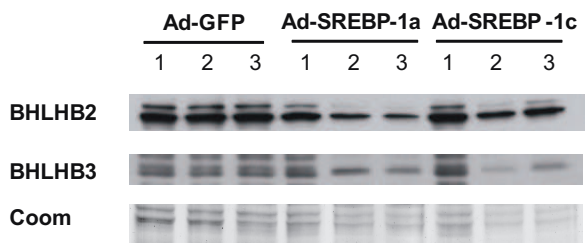


**B**

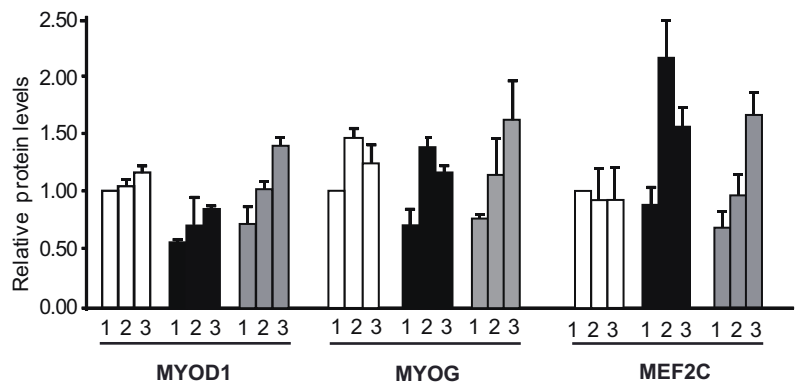
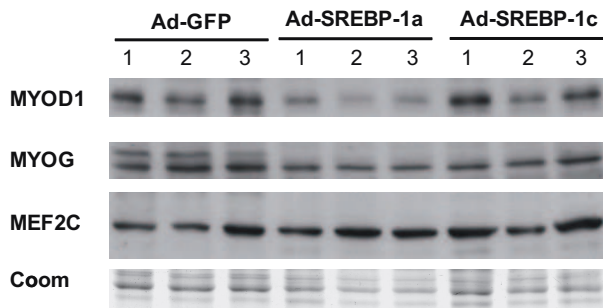
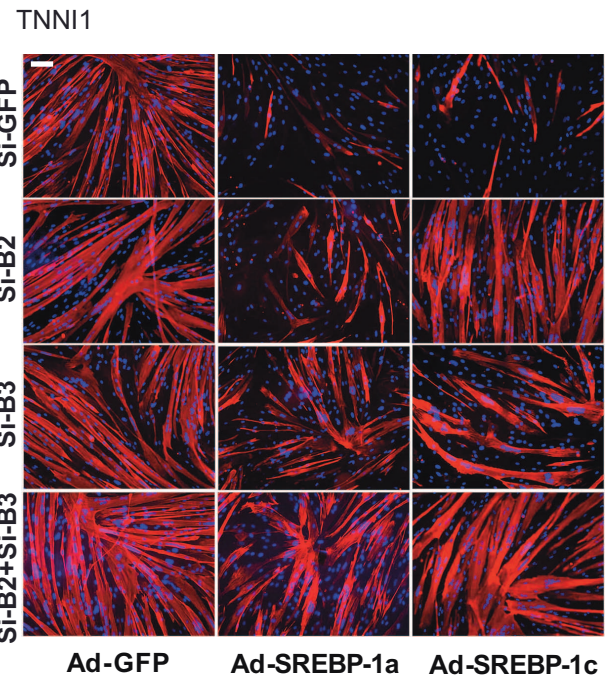
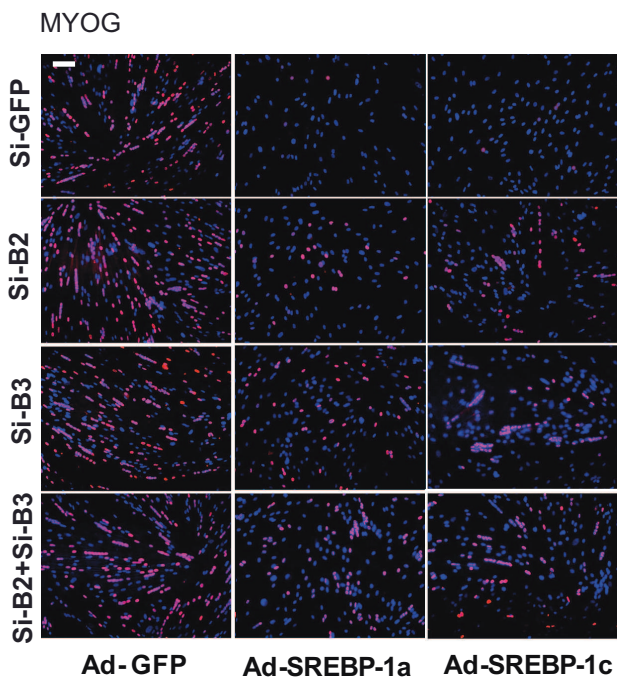


**Figure 3**

**A****B****C****D****E****Figure 4**

**A**

□ Ad - GFP  
 ■ Ad - SREBP-1a  
 ■ Ad - SREBP-1c

**B****C****Figure 5**

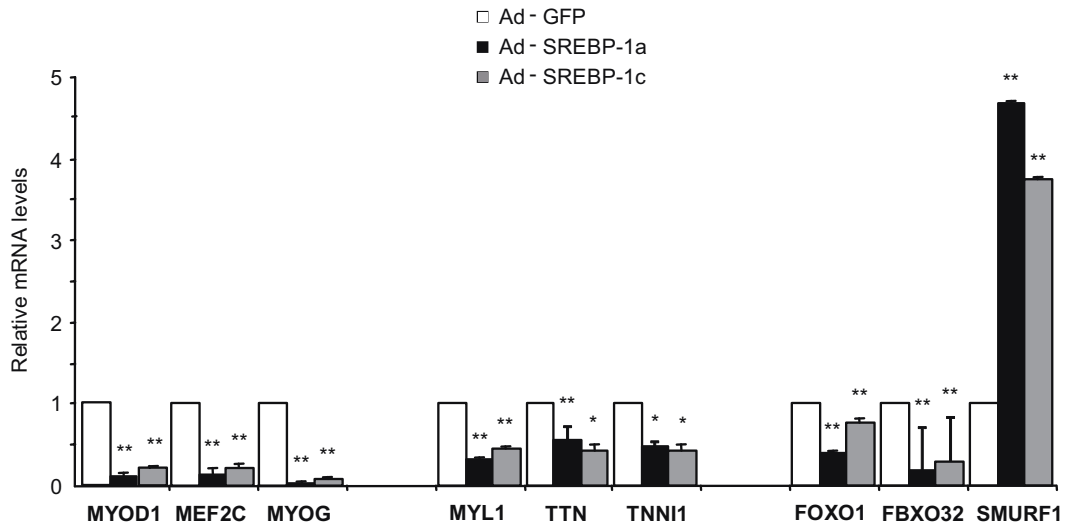
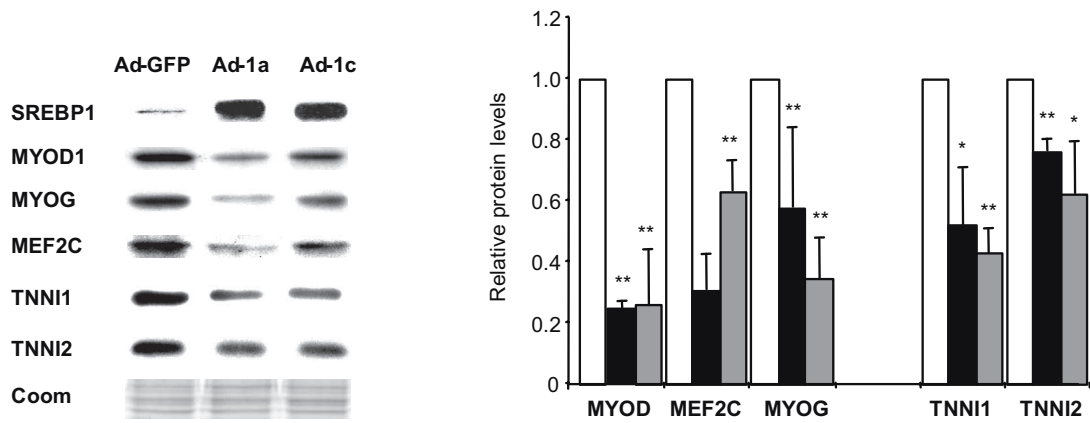
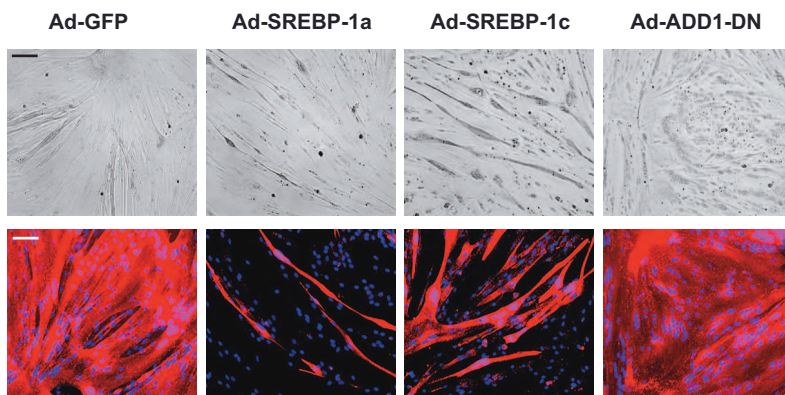
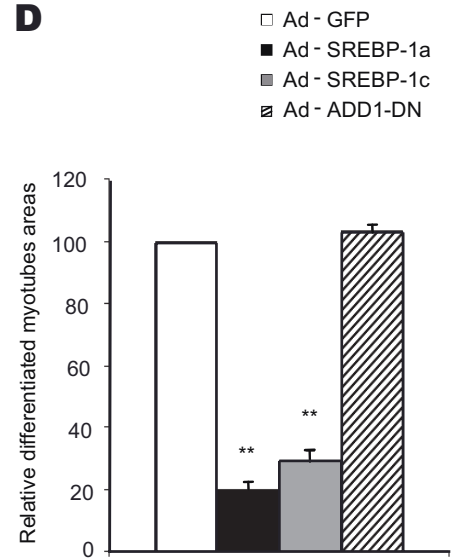
**A****B****C****D**

Figure 6

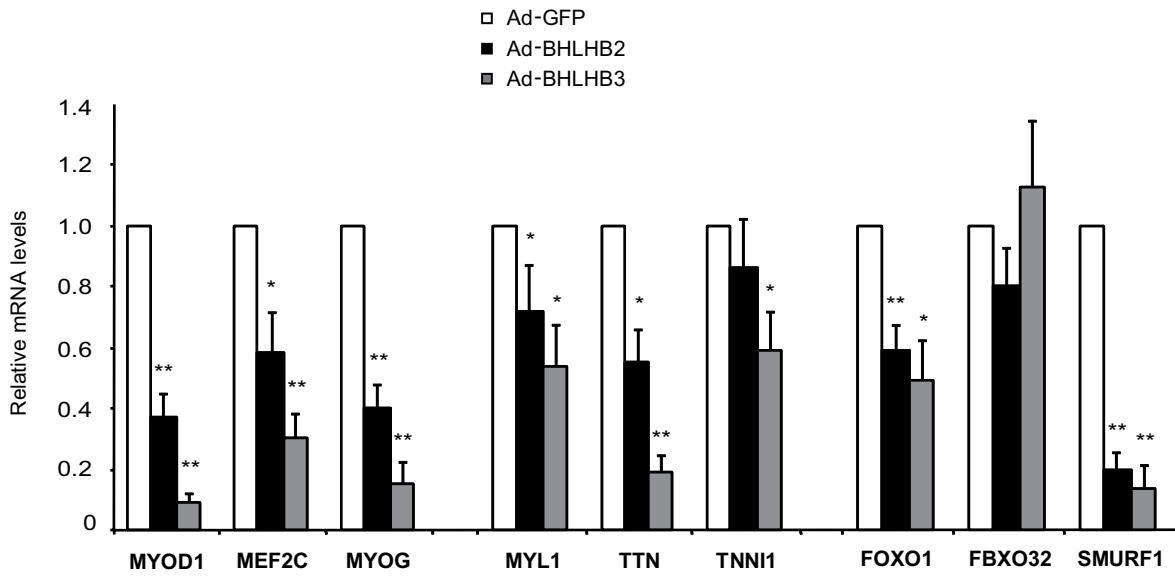
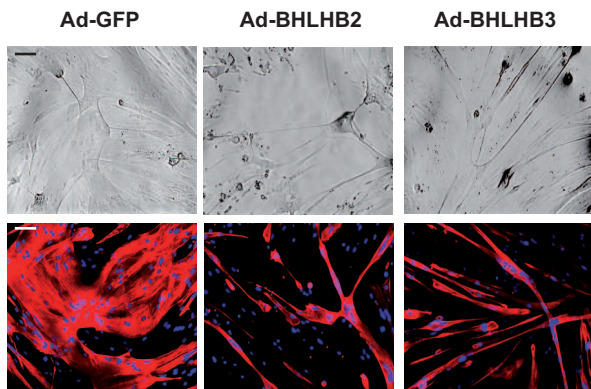
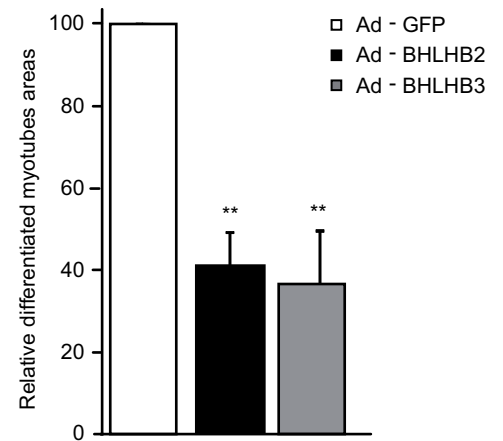
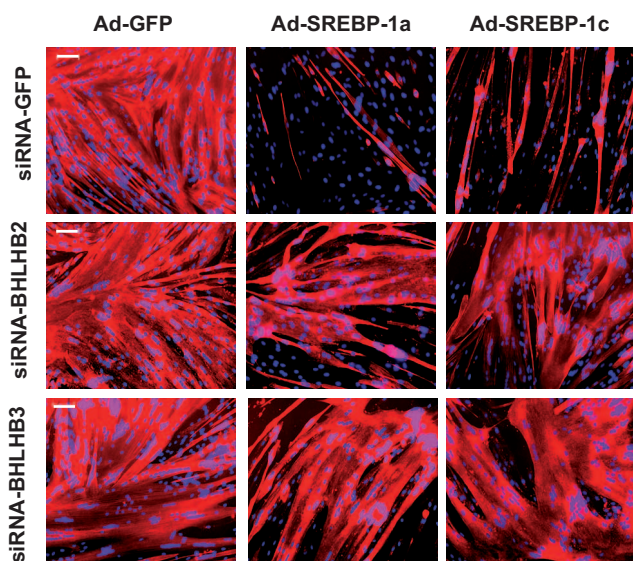
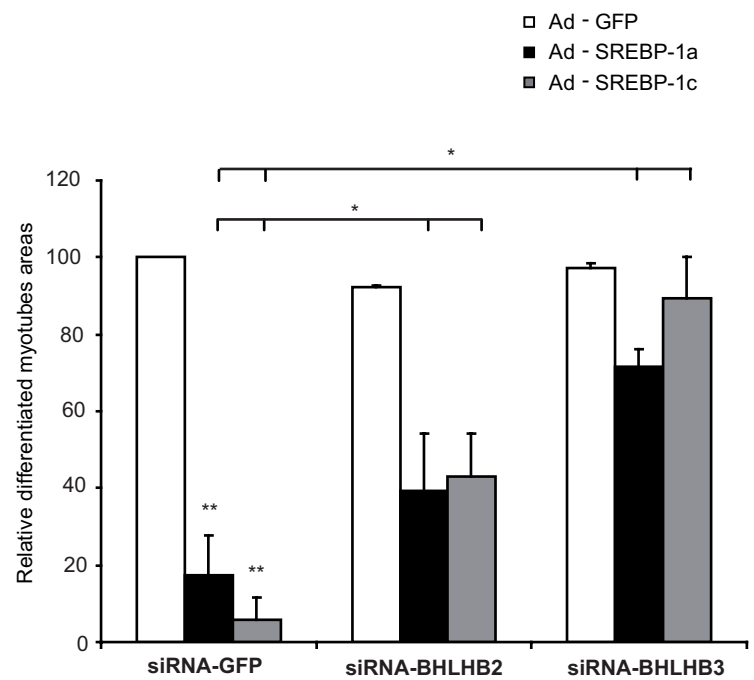
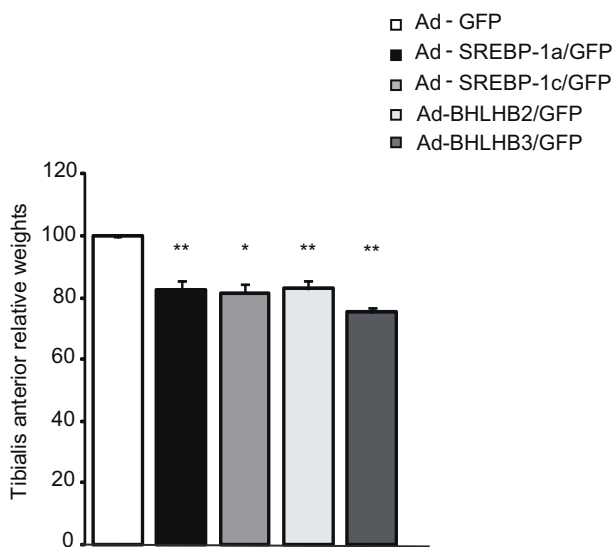
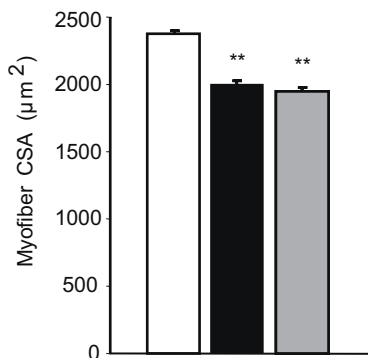
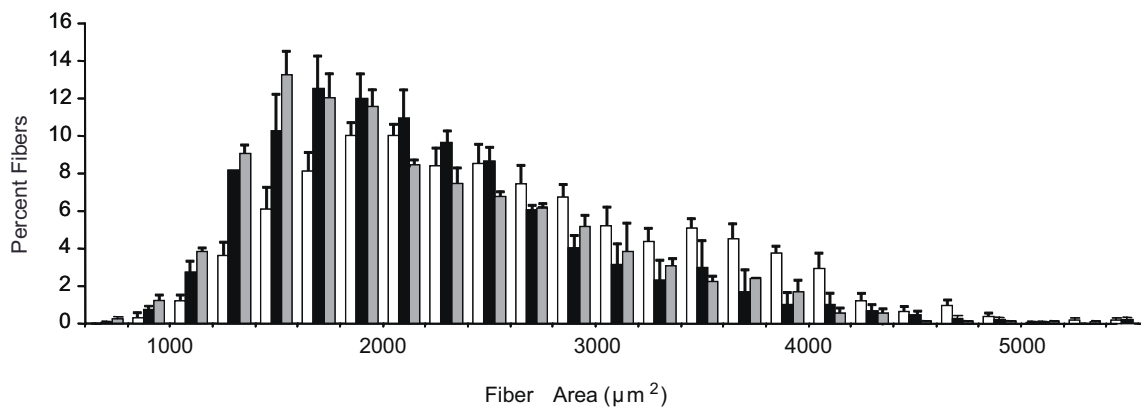
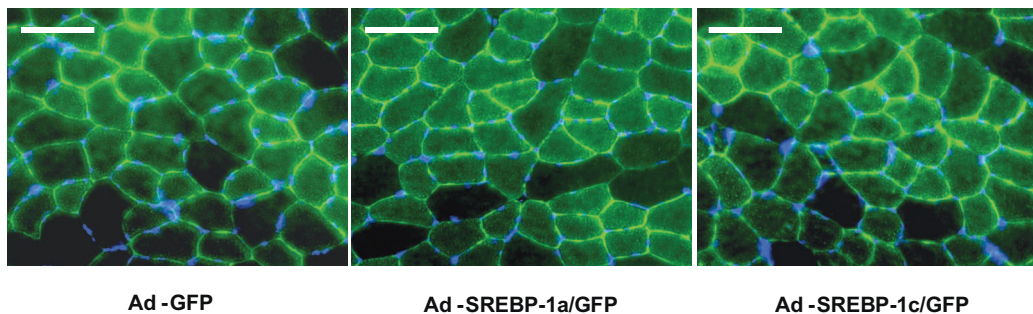
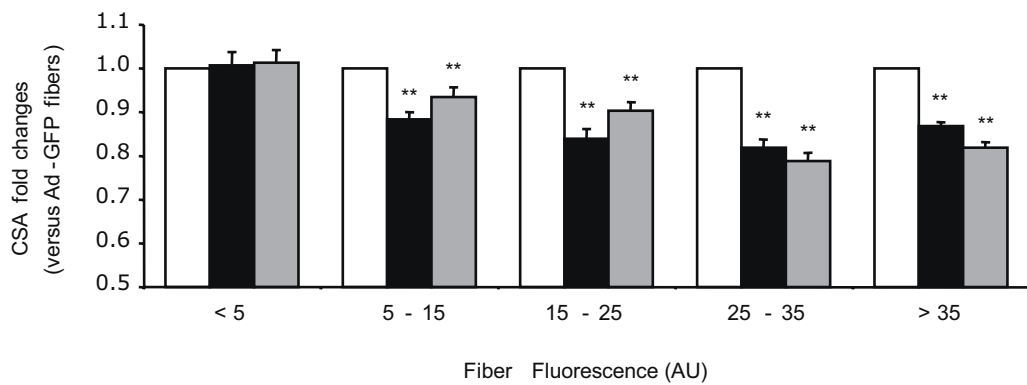
**A****B****C****D****E**

Figure 7

**A****B****C****D****E****Figure 8**