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

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Abstract

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

Introduction

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

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

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

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

Materials and Methods

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

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

Construction of expression vectors encoding mature nuclear forms of human SREBP-1a (named pCMV-hSREBP1a) and SREBP-1c (named pCMV-hSREBP1c) was described previously (12). A fragment of the pIRES plasmid (Clontech, Montain View, CA, USA) containing the IRES and EGFP sequence was cloned into pCMV-hSREBP1a and pCMV-hSREBP1c to obtain pCMV-hSREBP1a-IRES-GFP and pCMV-hSREBP1c-IRES-GFP. Recombinant adenoviruses expressing 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

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

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

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

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

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

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

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

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

Construction of reporter plasmids, and BHLHB2 and BHLHB3 promoter activity. A human genomic clone (NR5-IH18RS) which contains NotI flanking regions corresponding to the BHLHB2 promoter was obtained from Pr. E. R. Zabarovsky (Microbiology and Tumour Biology Center and Center for Genomics and Bioinformatics, Karolinska Institute, Stockholm, Sweden) (Zabarovsky ER, 2000). The -408/+75 (according to the transcription starting site) fragment was then subcloned into the luciferase reporter gene vector pGL3-Enhancer (Promega) to obtain pB21 (-408/+75). The -951/-407 fragment was generated by PCR and ligated into pB21 to obtain pB22 (-951/+75). The constructs pB23 (-264/+75) and pB26 (-187/+75) were generated by deletion of pB21. To obtain pB32, two genomic fragments, corresponding to the -940/-289 and -524/+238 regions of the BHLHB3 gene, were generated by PCR and combined to obtain the –940/+238 fragment into pGL3-E vector. Mutations of the SRE motifs were performed as described (12). Mutagenesis was performed to replace bases 2, 4 and 6 of each identified SRE by thymidine residues (Quick Change Mutagenesis Kit, Qiagen).

Transfection studies were carried out on myoblasts or myotubes plated in 12-well plates as previously described (12). Firefly and Renilla luciferase activities (Dual luciferase reporter assay system; Promega) were measured using a Centro LB 960 Luminometer (Berthold Technology, Thoiry, France).

Microarray analysis of myotubes overexpressing BHLHB2 and BHLHB3. The procedure used to obtain and analyze microarray data has previously been described

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

Results

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

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

Transcriptional repressors BHLHB2 and BHLHB3 are SREBP-1 target genes

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

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

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

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

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

Overexpression of BHLHB2and BHLHB3 in myotubes

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

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

SREBP-1a and -1c inhibit myoblast differentiation

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

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

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

inhibition of either BHLHB2 or BHLHB3 can restore, at least partially, the expression of MYOD1, MYOG and MEF2C proteins that are down-regulated upon SREBP-1 overexpression. Depletion of BHLHB2/B3 was sufficient to restore differentiation and myogenin and troponin expression in cells overexpressing SREBP-1 (Figure 5C).

Altogether, these data led us to propose that SREBP-1a and -1c block myoblasts to myotubes differentiation via an increase in BHLHB2 and BHLHB3 expression, the latter repressing the expression of MRFs.

SREBP-1a and -1c induce atrophy of differentiated myotubes

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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Figure Legends

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

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

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

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

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

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

right.

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

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

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

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

762 presented as mean ± SEM. * p≤ 0,05 ; ** p≤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 = 100µm); 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 = 100µm); 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 ± SEM. * p≤ 0,05 ; ** p≤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,

FBXO32, SMURF1). (B) Representative images of myotubes overexpressing GFP, BHLHB2 and BHLHB3. Upper panels: phase contrast, lower panels: immunostaining with TNNI1 antibody (red) and DAPI staining (blue) (Scale bar = 100µm). (C) Measurement of the aera of myotubes overexpressing GFP, BHLHB2 or BHLHB3 immunostained with TNNI1 antibody (n= 3 in each group). Results are presented as mean ± SEM. * p≤ 0,05 ; ** p≤0,001. (D) Representative images of myotubes overexpressing GFP, SREBP-1a or SREBP-1c, and transfected with siRNA against GFP (lane 1), BHLHB2 (lane 2), and BHLHB3 (lane 3) for 48 hours (Scale bar = 100µm). (E) Measurement of the area of myotubes overexpressing GFP, SREBP-1a and SREBP-1c and transfected with siRNA against GFP, BHLHB2 or BHLHB3. Myotubes were stained with TNNI1 antibody (n= 3 in each group).
Results are presented as mean ± SEM. * p≤ 0,05 ; ** p≤0,001.

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

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

Table 1: Muscle specific SREBP-1 target genes

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

Symbol	LLID	Fold 1A	Fold 1C	Name
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)

817

Table2: Muscle specific BHLHB2/B3 target genes

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

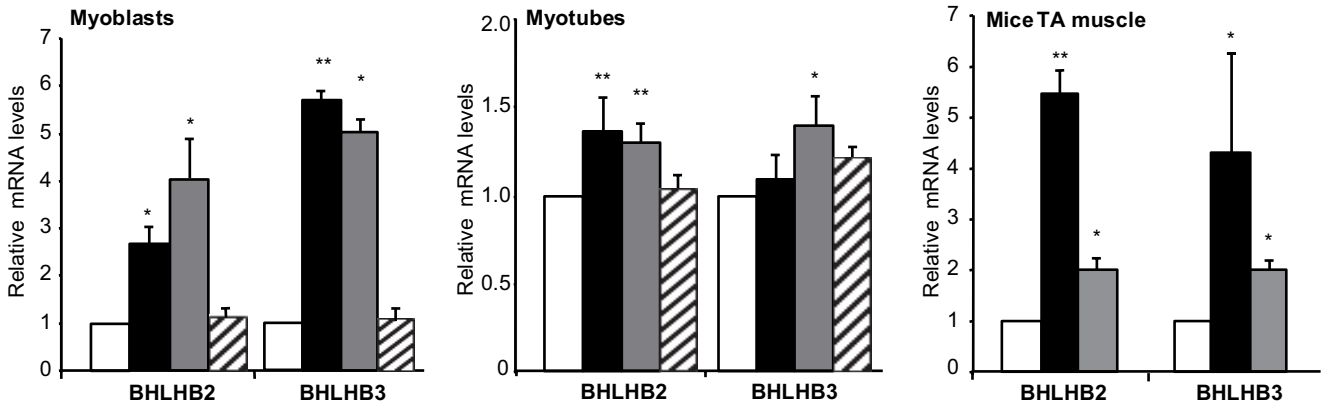
LLID	Symbol	Fold B2	Fold B3	Gene name
58	ACTA1	-2,89	-4,55	Actin, alpha 1, skeletal muscle
70	ACTC	-1,78	-2,75	Actin, alpha, cardiac muscle
88	ACTN2	-1,69		Actinin, alpha 2
89	ACTN3	-2,67	-3,27	Actinin, alpha 3
203	AK1	-1,56	-2,19	Adenylate kinase 1
10930	APOBEC2		-1,76	Apolipoprotein B mRNA editing 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++ 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/I) 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

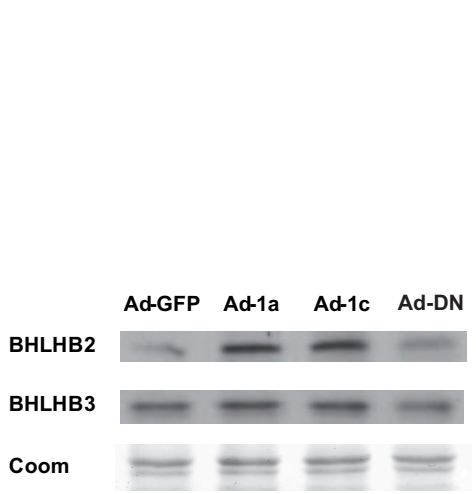
7004	TEAD4	-2,37	-4,18	TEA domain family member 4
7111	TMOD1		-2,13	Tropomodulin 1
29766	TMOD3	1,66	1,49	Tropomodulin 3 (ubiquitous)
7134	TNNC1		-1,59	Troponin C type 1 (slow)
7135	TNNI1	-2,46	-2,34	Troponin I type 1 (skeletal, slow)
7136	TNNI2	-1,40	-1,52	Troponin I type 2 (skeletal, fast)
7139	TNNT2	-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

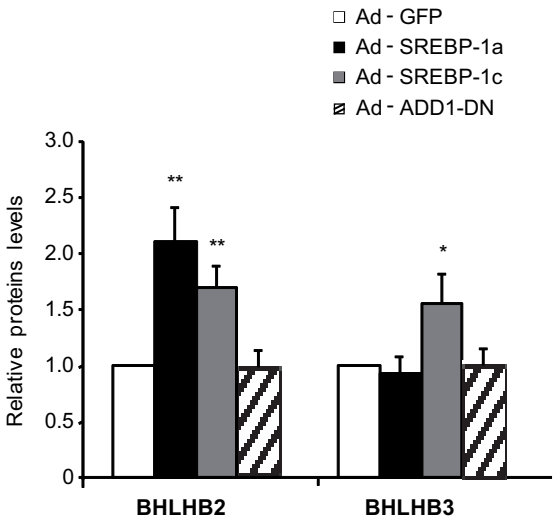
825

A


Myoblasts
Relative mRNA levels
BHLHB2
BHLHB3
Myotubes
Relative mRNA levels
BHLHB2
BHLHB3
Mice TA muscle
Relative mRNA levels
BHLHB2
BHLHB3

B


Ad-GFP
Ad-1a
Ad-1c
Ad-DN
BHLHB2
BHLHB3
Coom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Ad - SREBP-1a
Ad - SREBP-1c
Ad - ADD1-DN
Relative proteins levels
BHLHB2
BHLHB3

Figure 1

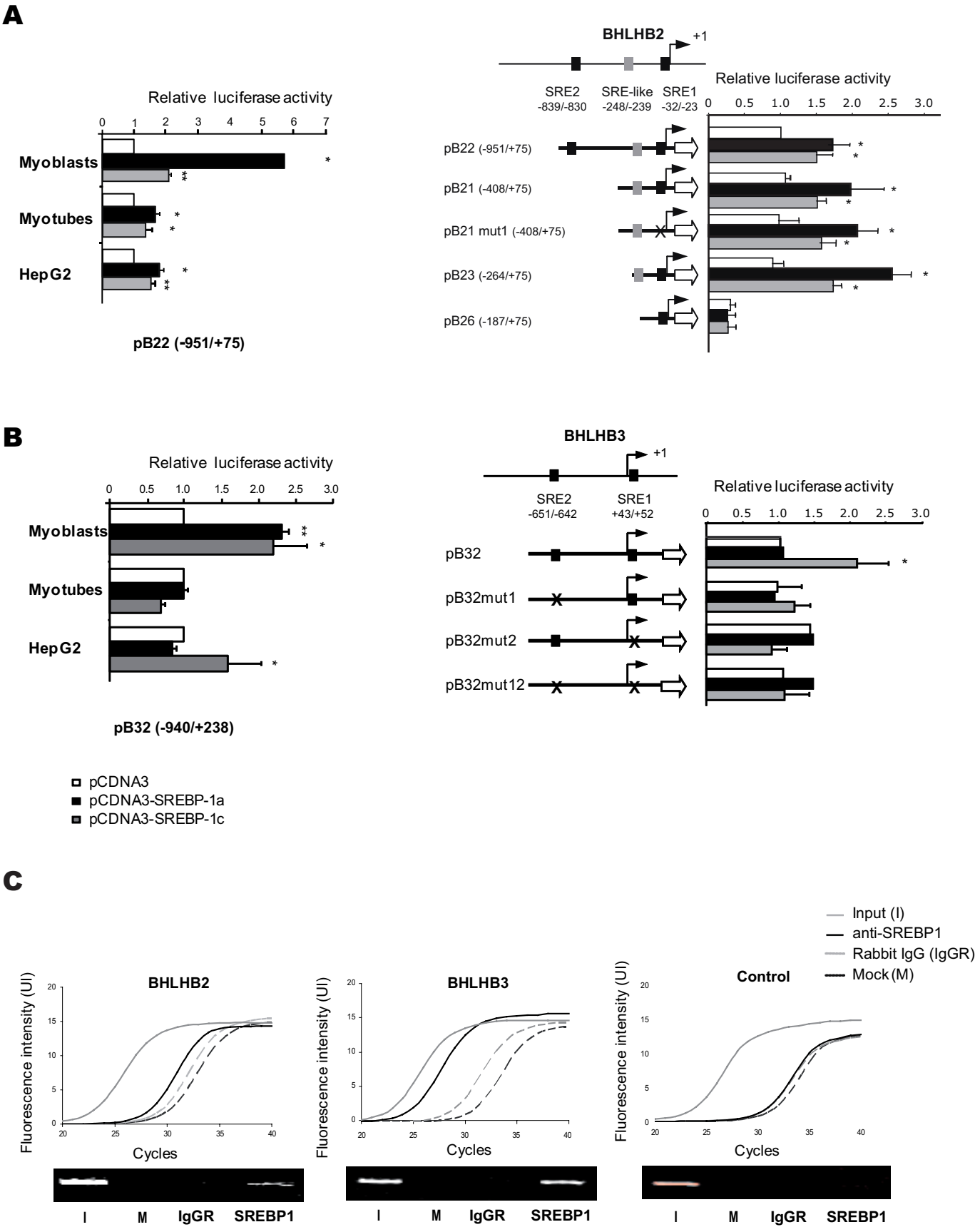
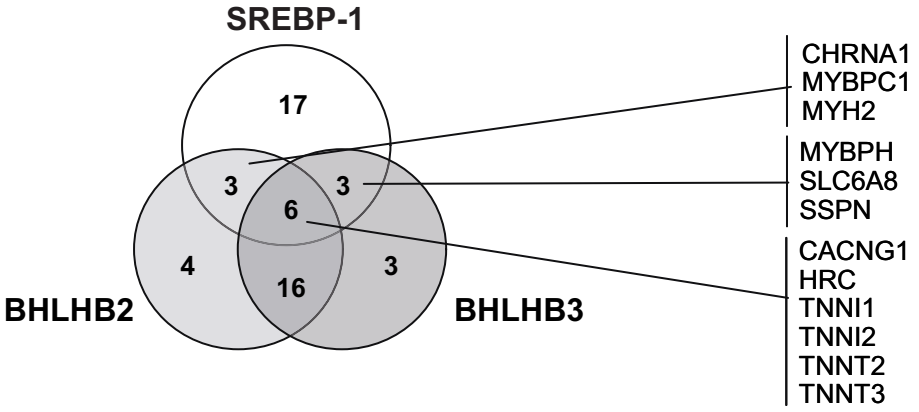

A
Relative luciferase activity
Myoblasts
Myotubes
HepG2
pB22 (-951/+75)
BHLHB2
+1
SRE2
-839/-830
SRE-like
-248/-239
SRE1
-32/-23
pB22 (-951/+75)
pB21 (-408/+75)
pB21 mut1 (-408/+75)
pB23 (-264/+75)
pB26 (-187/+75)
B
Relative luciferase activity
Myoblasts
Myotubes
HepG2
pB32 (-940/+238)
BHLHB3
+1
SRE2
-651/-642
SRE1
+43/+52
pB32
pB32mut1
pB32mut2
pB32mut12
pCDNA3
pCDNA3-SREBP-1a
pCDNA3-SREBP-1c
C
Input (I)
anti-SREBP1
Rabbit IgG (IgGR)
Mock (M)
BHLHB2
BHLHB3
Control
Fluorescence intensity (UI)
Cycles
I
M
IgGR
SREBP1

Figure 2

A


SREBP-1
17
3
3
6
4
16
3
BHLHB2
BHLHB3
CHRNA1
MYBPC1
MYH2
MYBPH
SLC6A8
SSPN
CACNG1
HRC
TNNI1
TNNI2
TNNT2
TNNT3

B

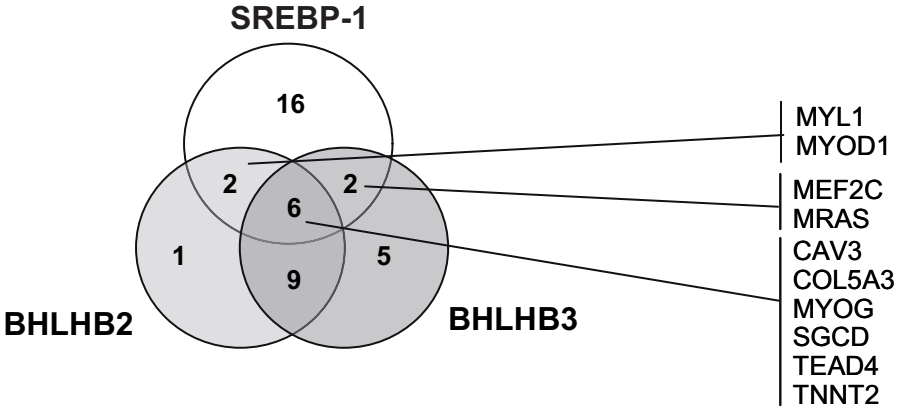

SREBP-1
16
2
2
6
1
9
5
BHLHB2
BHLHB3
MYL1
MYOD1
MEF2C
MRAS
CAV3
COL5A3
MYOG
SGCD
TEAD4
TNNT2

Figure 3

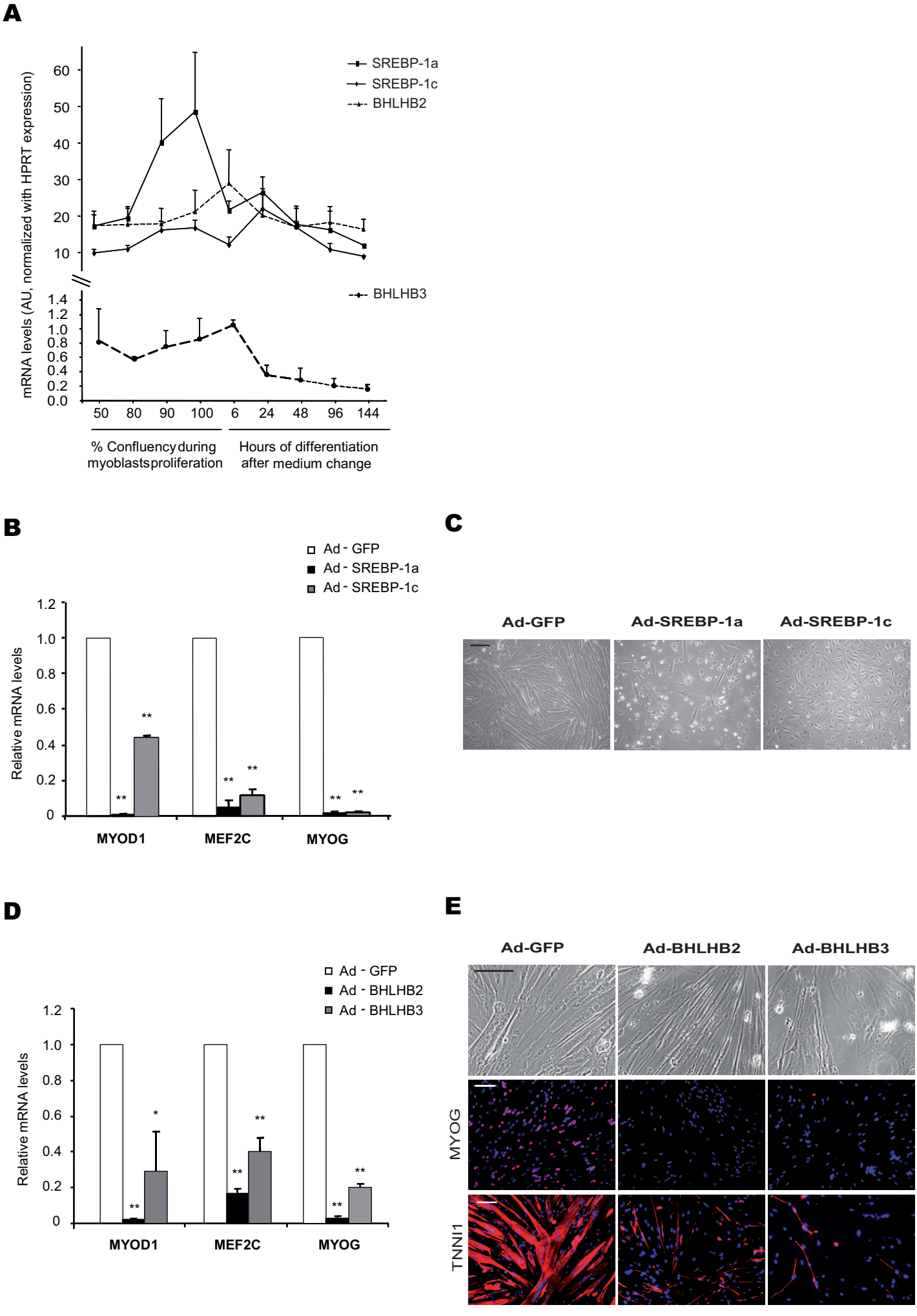

A
SREBP-1a
SREBP-1c
BHLHB2
BHLHB3
mRNA levels (AU, normalized with HPRT expression)
% Confluency during myoblasts proliferation
Hours of differentiation after medium change
B
Ad - GFP
Ad - SREBP-1a
Ad - SREBP-1c
Relative mRNA levels
MYOD1
MEF2C
MYOG
C
Ad-GFP
Ad-SREBP-1a
Ad-SREBP-1c
D
Ad - GFP
Ad - BHLHB2
Ad - BHLHB3
Relative mRNA levels
MYOD1
MEF2C
MYOG
E
Ad-GFP
Ad-BHLHB2
Ad-BHLHB3
MYOG
TNNI1

Figure 4

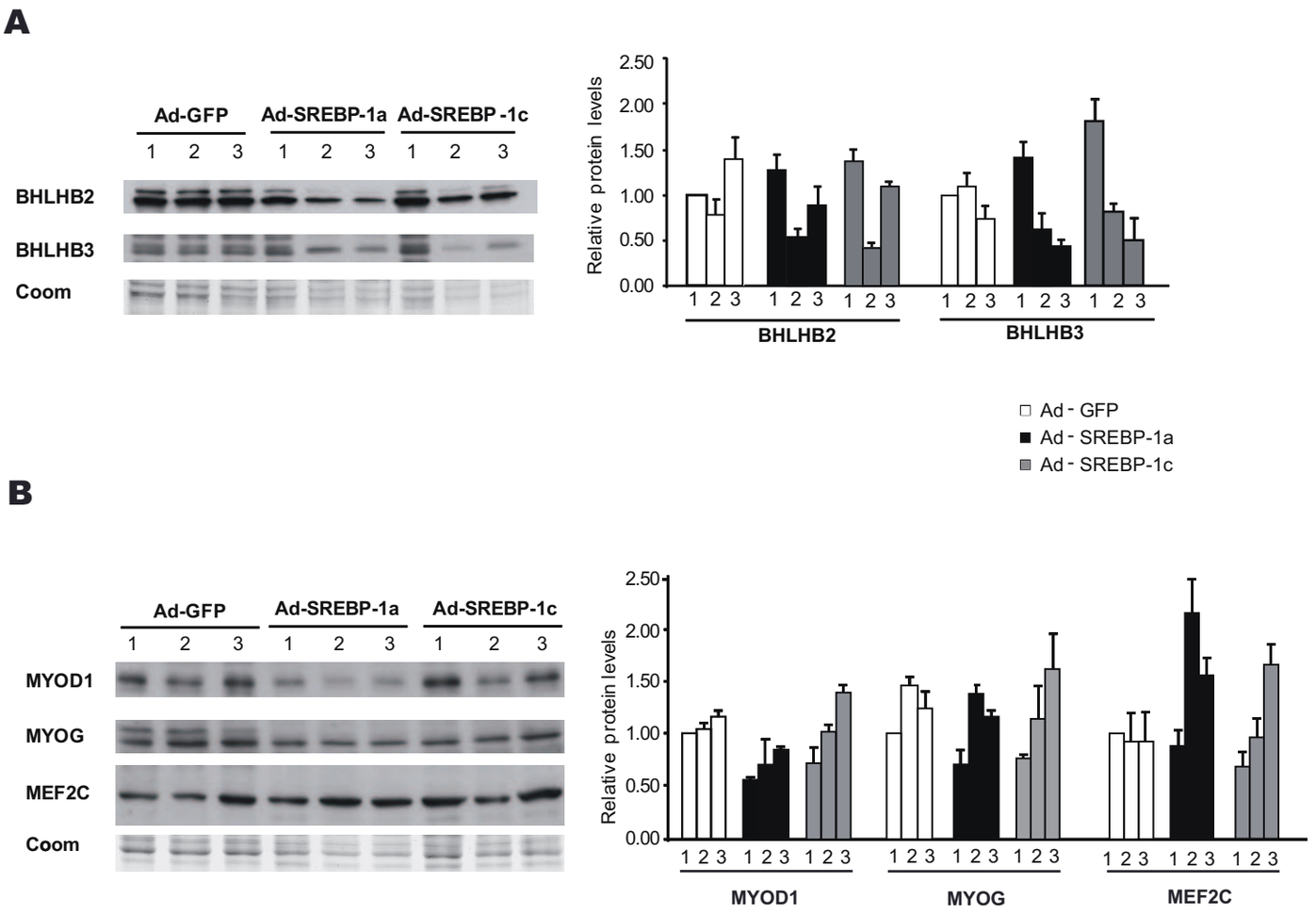

A
Ad-GFP
Ad-SREBP-1a
Ad-SREBP -1c
1 2 3 1 2 3 1 2 3
BHLHB2
BHLHB3
Coom
Relative protein levels
2.50
2.00
1.50
1.00
0.50
0.00
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
BHLHB2
BHLHB3
Ad - GFP
Ad - SREBP-1a
Ad - SREBP-1c
B
Ad-GFP
Ad-SREBP-1a
Ad-SREBP-1c
1 2 3 1 2 3 1 2 3
MYOD1
MYOG
MEF2C
Coom
Relative protein levels
2.50
2.00
1.50
1.00
0.50
0.00
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
MYOD1
MYOG
MEF2C
C
MYOG
TNNI1
Si-GFP
Si-B2
Si-B3
Si-B2+Si-B3
Ad-GFP
Ad-SREBP-1a
Ad-SREBP-1c

Figure 5

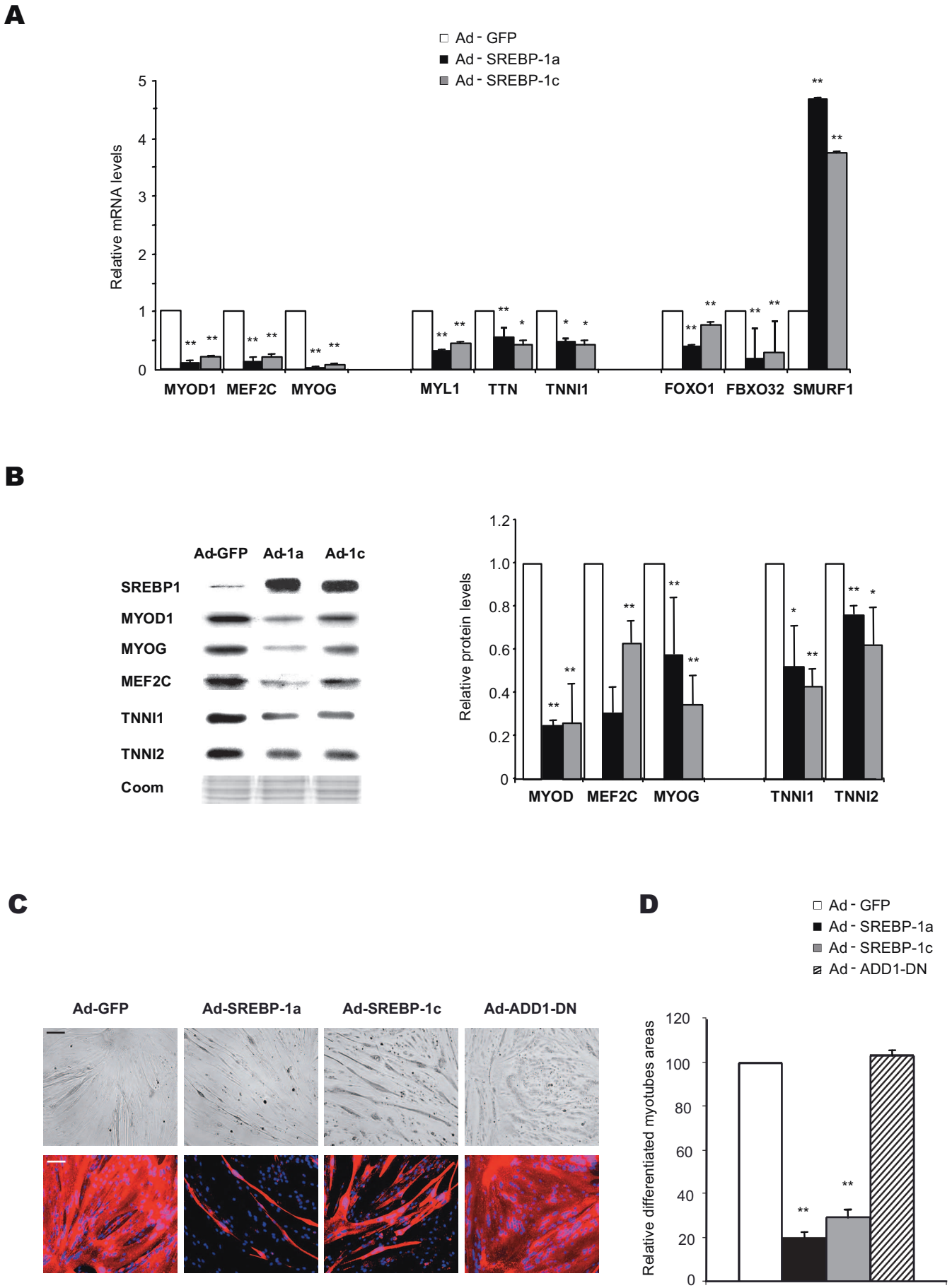

A
Ad - GFP
Ad - SREBP-1a
Ad - SREBP-1c
Relative mRNA levels
MYOD1
MEF2C
MYOG
MYL1
TTN
TNNI1
FOXO1
FBXO32
SMURF1
B
Ad-GFP
Ad-1a
Ad-1c
SREBP1
MYOD1
MYOG
MEF2C
TNNI1
TNNI2
Coom
Relative protein levels
MYOD
MEF2C
MYOG
TNNI1
TNNI2
C
Ad-GFP
Ad-SREBP-1a
Ad-SREBP-1c
Ad-ADD1-DN
D
Ad - GFP
Ad - SREBP-1a
Ad - SREBP-1c
Ad - ADD1-DN
Relative differentiated myotubes areas

Figure 6

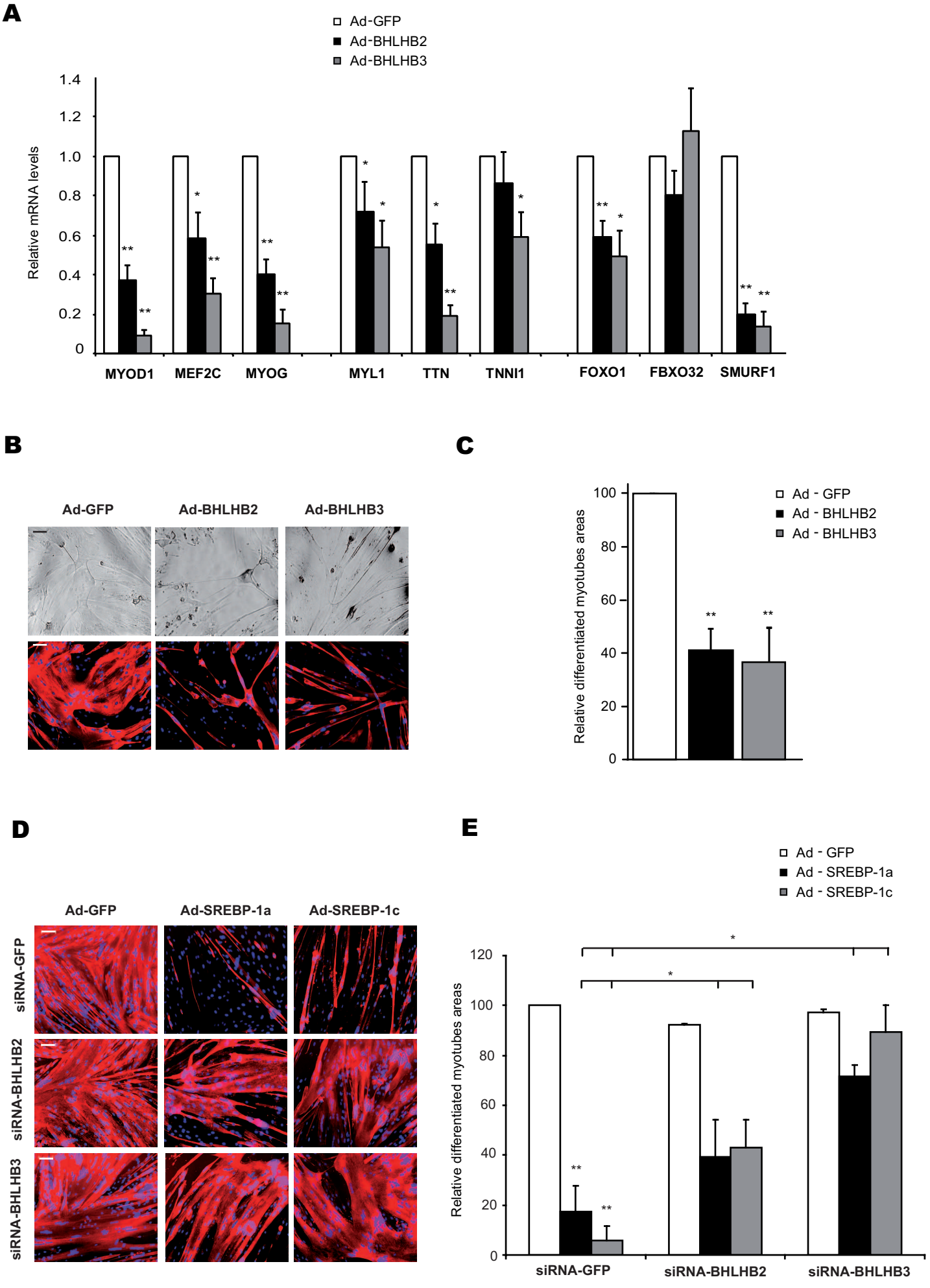

A
Ad-GFP
Ad-BHLHB2
Ad-BHLHB3
Relative mRNA levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
MYOD1
MEF2C
MYOG
MYL1
TTN
TNNI1
FOXO1
FBXO32
SMURF1
B
Ad-GFP
Ad-BHLHB2
Ad-BHLHB3
C
Ad - GFP
Ad - BHLHB2
Ad - BHLHB3
Relative differentiated myotubes areas
100
80
60
40
20
0
D
Ad-GFP
Ad-SREBP-1a
Ad-SREBP-1c
siRNA-GFP
siRNA-BHLHB2
siRNA-BHLHB3
E
Ad - GFP
Ad - SREBP-1a
Ad - SREBP-1c
Relative differentiated myotubes areas
120
100
80
60
40
20
0
siRNA-GFP
siRNA-BHLHB2
siRNA-BHLHB3

Figure 7

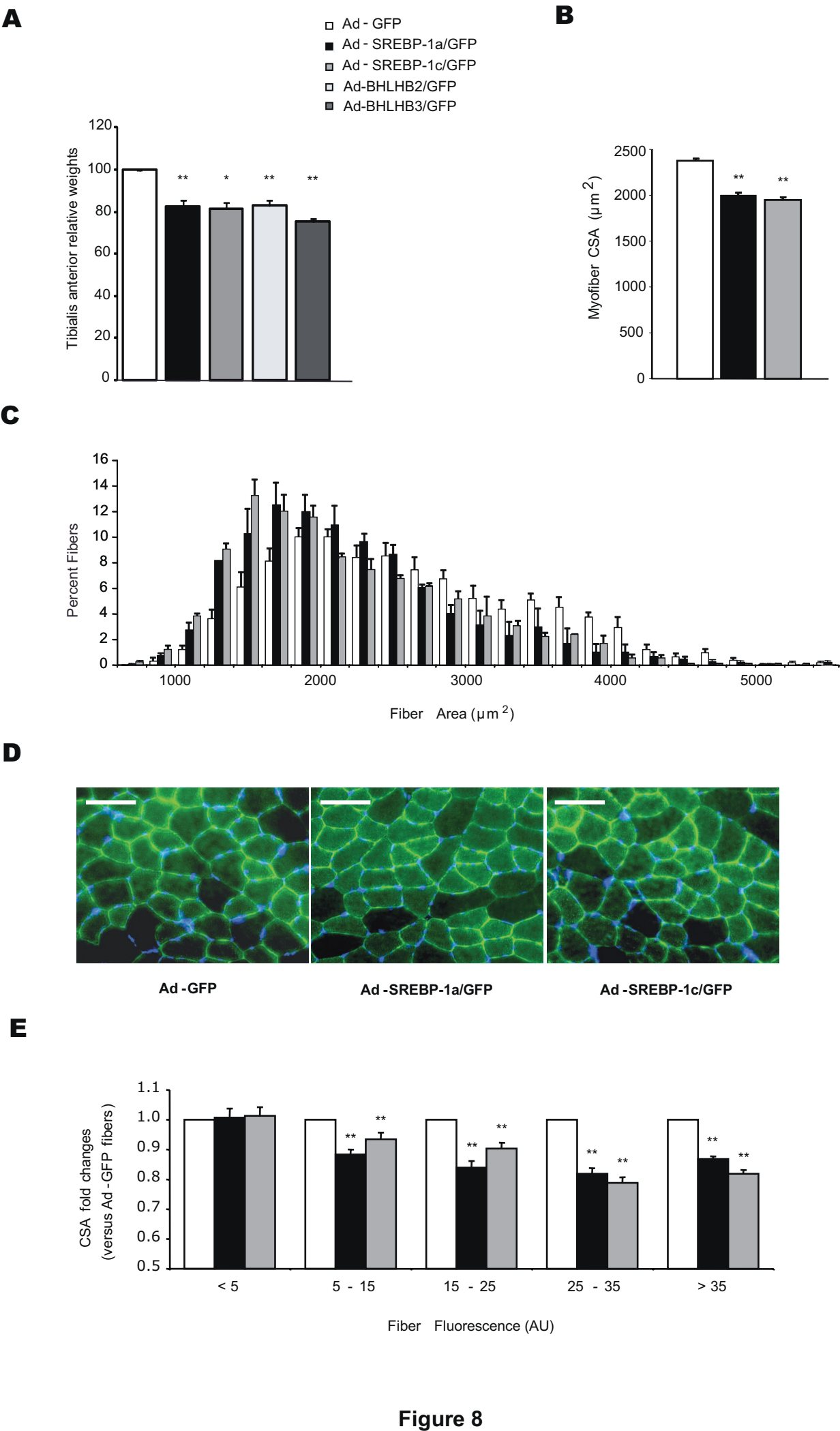

A
Ad - GFP
Ad - SREBP-1a/GFP
Ad - SREBP-1c/GFP
Ad-BHLHB2/GFP
Ad-BHLHB3/GFP
Tibialis anterior relative weights
120
100
80
60
40
20
0
**
*
**
**
B
Myofiber CSA (µm 2)
2500
2000
1500
1000
500
0
**
**
C
Percent Fibers
16
14
12
10
8
6
4
2
0
1000
2000
3000
4000
5000
Fiber Area (µm 2)
D
Ad -GFP
Ad -SREBP-1a/GFP
Ad -SREBP-1c/GFP
E
CSA fold changes
(versus Ad -GFP fibers)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
**
**
**
**
**
**
**
**
< 5
5 - 15
15 - 25
25 - 35
> 35
Fiber Fluorescence (AU)

Figure 8