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Small Leucine Zipper Protein (sLZIP) Negatively Regulates Skeletal Muscle Differentiation via Interaction with α-Actinin-4*[S]

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Background: Cooperation between transcription factors and myogenic regulatory factors is important for skeletal muscle differentiation.
Results: sLZIP inhibits expression of muscle-specific genes during myogenesis via disruption of an association between α-actinin-4 and MEF2.
Conclusion: A novel myogenesis regulatory mechanism of sLZIP is characterized.
Significance: sLZIP can be used as a therapeutic target for treatment of muscle diseases.

The small leucine zipper protein (sLZIP) plays a role in transcriptional regulation in various types of cells. However, the role of sLZIP in myogenesis is unknown. We identified α-actinin-4 (ACTN4) as a sLZIP-binding protein. ACTN4 functions as a transcriptional regulator of myocyte enhancer factor (MEF)2, which plays a critical role in expression of muscle-specific genes during skeletal muscle differentiation. We found that ACTN4 translocates to the nucleus, induces myogenic gene expression, and promotes myotube formation during myogenesis. The myogenic process is controlled by an association between myogenic factors and MEF2 transcription factors. ACTN4 increased expression of muscle-specific proteins via interaction with MEF2. However, sLZIP decreased myogenic gene expression and myotube formation during myogenesis via disruption of the association between ACTN4 and MEF2. ACTN4 increased the promoter activities of myogenic genes, whereas sLZIP abrogated the effect of ACTN4 on transcriptional activation of myogenic genes in myoblasts. The C terminus of sLZIP is required for interaction with the C terminus of ACTN4, based on deletion mutant analysis, and sLZIP plays a role in regulation of MEF2 transactivation via interaction with ACTN4. Our results indicate that sLZIP negatively regulates skeletal muscle differentiation via interaction with ACTN4 and that sLZIP can be used as a therapeutic target molecule for treatment of muscle hypertrophy and associated diseases.

The small leucine zipper protein (sLZIP),[2] a novel isoform of human LZIP, is located in the nucleus and functions as a transcription cofactor (1). sLZIP negatively regulates transactivation of the glucocorticoid receptor via recruitment of histone deacetylases (HDACs) to the glucocorticoid response element of target genes, leading to suppression of glucocorticoid receptor-mediated gene expression (1). sLZIP is involved in activation of matrix metalloproteinase (MMP)-9 transcription via direct binding to the cAMP-responsive element of the MMP-9 promoter, resulting in an increase in cell migration and invasion in cervical cancer cells (2). sLZIP also promotes phorbol 12-myristate 13-acetate-induced breast cancer cell migration via up-regulation of ADP-ribosylation factor 4 expression (3). These evidences indicate that sLZIP plays a critical role in transcriptional regulation in different cell types; however, its function remains obscure. α-Actinin-4 (ACTN4) was identified as a sLZIP-binding protein, and the roles of sLZIP and ACTN4 in skeletal muscle differentiation were studied.

ACTNs are actin-binding proteins that play multiple roles in different types of cells, including attachment to actin filaments in skeletal muscle cells (4). The members of the ACTN family share a number of structural features and regulatory regions (4). There are four unique types of ACTN genes (ACTN1, 2, 3, and 4) that encode for highly homologous proteins. ACTN2 and 3 are enriched in muscle cells, whereas ACTN1 and 4 are widely expressed in other cells (5, 6). In nonmuscle cells, ACTN1 and 4, which belong to the cytoskeletal isoforms, are found in actin filament bundles and adherent junctions and are involved in cell shape and motility (5, 6). ACTN2 and 3, which belong to skeletal, cardiac, and smooth muscle isoforms, are localized in the Z-disc and help in binding to actin filaments (7, 8). Unlike other ACTN isoforms, ACTN4 participates in regulation of phenotypes and functions in podocytes and is involved in differentiation, motility, and invasion of cancer cells (4, 9–11). However, the function of ACTN4 during myogenesis is unclear. It is preferentially localized in the nucleus upon induction of myogenesis, and its expression leads to disruption of HDAC7 and the myocyte enhancer factor (MEF) 2A, and enhancement of MEF2-mediated transcription (12–14). ACTN4 is evidently involved in muscle differentiation and regulation of gene expression.

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[S] This article contains supplemental Table S1 and Figs. S1–S3.
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[2] The abbreviations used are: sLZIP, small leucine zipper protein; ACTN, α-actinin; HDAC, histone deacetylase; MCK, muscle creatine kinase; MEF, myocyte enhancer factor; MRF, myogenic regulatory factor; MyHC, myosin heavy chain; qRT-PCR, quantitative real-time PCR.

Myogenesis is a process of muscle tissue formation from myoblasts (15). During myogenesis, the expression levels of members of the myogenic regulatory factor (MRF) gene families, including myosin heavy chain (MyHC), myogenin, MyoD, and muscle creatine kinase (MCK), are increased (16–18). The MEF2 family of MADS (MCM1, agamous, deficiens, and serum response factor) domain transcription factors cooperates with MRFs in promoting muscle differentiation (15, 16, 19). The MEF2 proteins are key transcription factors that control gene expression in myocytes. The MEF2 binding site, an AT-rich consensus sequence ((C/T)TA(A/T)$_4$TA(A/G)), is found in the promoters and enhancers of genes involved in myogenesis (19–21). Myogenin is an essential muscle-specific transcription factor that is involved in skeletal muscle development. It is required for terminal differentiation and fusion of myogenic precursor cells, which eventually become new fibers (17, 22). MCK is highly induced during skeletal muscle differentiation and plays a critical role in the energy metabolism of skeletal muscle tissue as the key enzyme in the phosphocreatine shuttle between mitochondria and myofibrils (23–25). The members of the MRF proteins are differentially regulated during skeletal muscle differentiation (16, 26, 27).

In this study, ACTN4 was identified as a sLZIP-binding protein and characterized as a regulator of skeletal muscle differentiation. ACTN4 enhances differentiation of myoblasts via interaction with MEF2, whereas sLZIP disrupts the association between ACTN4 and MEF2, resulting in suppression of myogenic gene expression and skeletal muscle differentiation.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen. Horse serum was purchased from Welgene Inc. Lipofectamine 2000 reagent was purchased from Invitrogen. Nickel-nitrilotriacetic acid beads were purchased from Bio-Rad. Glutathione-Sepharose 4B beads were from GE Healthcare. Silver nitrate, anti-MyHC, anti-Flag (M2), anti-MEF2D, and anti-myogenin antibodies were purchased from Sigma and BD Biosciences. Anti-glutathione *S*-transferase (GST), anti-ACTN4, anti-green fluorescence protein (GFP), anti-Myc, β-actin, and α-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Differentiation—Murine myoblast C2C12 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. When cells were 80–90% confluent, the proliferation medium was replaced by the differentiation medium containing 2% horse serum and 1% penicillin/streptomycin for 48 h.

Transfection and Western Blotting—C2C12 cells (5×10^5/well) were transfected with the ACTN4 expression plasmid (1 μg) for 24 h using Lipofectamine 2000 reagent and lysed in radioimmunoprecipitation assay lysis buffer. Equal amounts of protein (30 μg) were electrophoresed on 8% or 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the specific antibodies at 4 °C overnight. β-Actin was used as an internal control. The blots were then incubated with secondary antibody at room temperature for 1 h. The immune complex was detected using an ECL Plus Detection Kit (Pierce).

Preparation of His-sLZIP Protein—His-sLZIP protein was expressed in BL21 cells using the T7 isopropyl-β-D-thiogalactopyranoside-inducible system. IPTG-induced cells were disrupted by sonication, and cell lysates were clarified by centrifugation at 10,000 × *g* for 30 min. His-sLZIP protein was applied onto a nickel-nitrilotriacetic acid beads column, and the column was washed until the elute contained no materials.

His Pulldown Assay and Mass Spectrometry (LC-MS/MS)—For the pulldown experiment, His-tagged fusion protein-immobilized Sepharose beads were prepared. A total of 500 μl of purified sLZIP beads and myoblasts nuclear extracts were mixed with binding buffer and incubated at 4 °C for 4 h. The supernatant was removed, and the beads were washed four times with washing buffer. Each time the beads were incubated with washing buffer on a rotator for 3 min and collected. The samples were boiled in SDS sample loading buffer for 5 min, resolved by SDS-PAGE, and visualized by Coomassie Blue and silver staining. Proteins in silver-stained gels were analyzed and profiled using a Thermo Finnigan LCQ DeCa Xp Max mass spectrometer (Korea Basic Science Institute, Seoul, South Korea).

GST Pulldown Assay—Transfected cells were grown in 100-mm cell culture dishes to 70–80% confluence before medium was removed, then the cells were washed twice with ice-cold PBS and incubated for 5 min at 4 °C in radioimmunoprecipitation lysis buffer. Cells were collected, and lysates were cleared by centrifugation at 10,000 × *g* for 30 min at 4 °C. Approximately 500 μg of solubilized lysates was used for each pulldown assay using the GST beads, and an immunoprecipitation assay was performed according to the manufacturer's instructions. The immunoprecipitated proteins were analyzed by SDS-PAGE and probed with the antibody described in the assay.

RNA Isolation and Quantitative Real-time PCR—Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized with the use of 2 μg of total RNA through SuperScript reverse transcriptase (Bioneer, Daejeon, South Korea) with oligo(dT) primers. Quantitative real-time PCR (qRT-PCR) was performed with the LightCycler 480 using SYBR Green Master Mix (Roche Applied Science). The sequences of the primers used for qRT-PCR were as follows: myogenin forward, 5′-TTG CTC AGC TCC CTC AAC CAG GA-3′ and reverse, 5′-TGC AGA TTG TGG GCG TCT GTA GG-3′; MyoD forward, 5′-GAC CTG CGC TTT TTT GAG GAC C-3′ and reverse, 5′-CAG GCC CAC AGC AAG CAG CGA C-3′; MyHC forward, 5′-CCA TTC AGA GCA AAG ATG CAG G-3′ and reverse 5′-GCA TAACGC TCT TTG AGG TTG-3′. Each experiment was performed in three experimental replicates with three technical replicates within each experiment.

Luciferase Assay—C2C12 and HEK-293T cells transfected with the specific plasmid were washed with ice-cold PBS and lysed for 20 min on ice using 80 μl/well Luciferase Cell Culture Lysis Reagent (Promega). After centrifugation at 10,000 × *g* for 10 min at 4 °C, the supernatant was collected, and luciferase activity was analyzed using the Luminometer 20/20^n (Turner

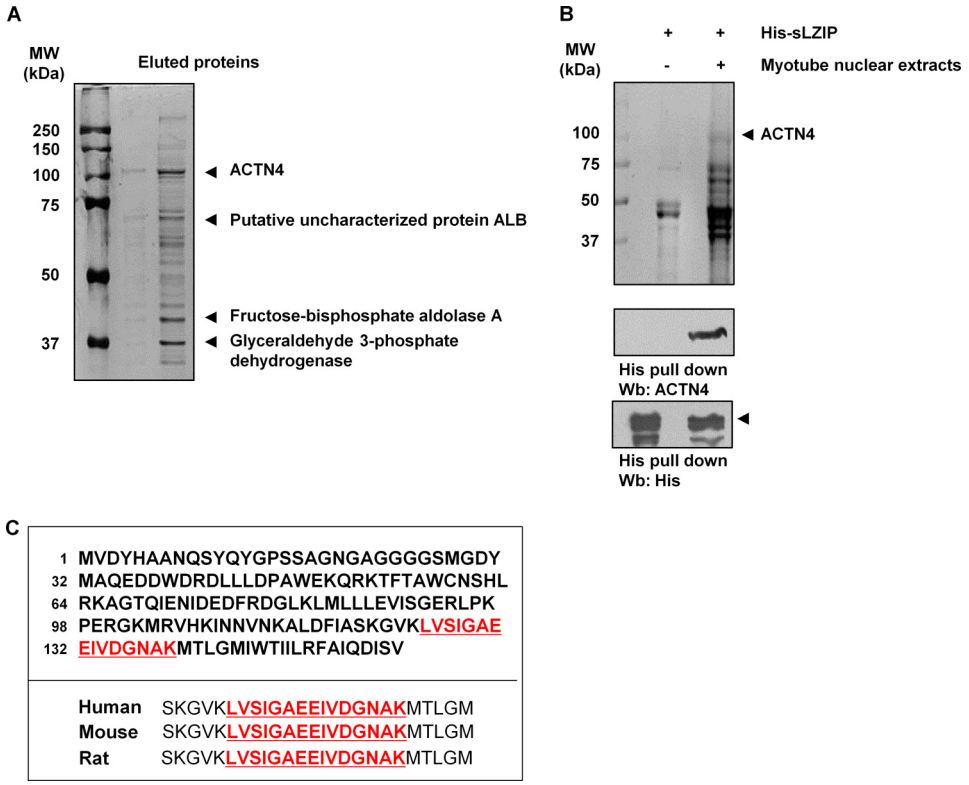

FIGURE 1. **Identification of ACTN4 as a novel sLZIP-binding protein.** *A*, protein was identified from silver-stained 10% SDS-polyacrylamide gel for automated LC-MS/MS. His pulldown assay was performed using purified His-sLZIP fusion proteins (His-sLZIP) in HeLa cells, and proteins were separated by 10% SDS-PAGE. The *arrowheads* indicate the candidate proteins that bind to His-sLZIP. *B*, His-sLZIP proteins were purified using nickel-nitrilotriacetic acid beads for a His pulldown assay in the absence or presence of differentiated C2C12 nuclear extracts. Equal volumes of purified fusion proteins and pulldown proteins were separated by 10% SDS-PAGE, followed by silver staining. Pulled down proteins were analyzed by Western blotting using anti-ACTN4 antibody. *Wb*, Western blotting. *C*, amino acid sequences of ACTN4 are shown. The peptide sequences obtained by LC-MS/MS are indicated in *red*.

Biosystems, Sunnyvale, CA). For normalization, pSV40-β-galactosidase was co-transfected with the luciferase reporter gene. Collected supernatants were assayed for β-galactosidase activity using the β-galactosidase enzyme assay system (Promega) and analyzed by a DU530 spectrophotometer (Beckman Instruments).

Immunofluorescence Microscopic Analysis—Cells were grown on coverslips. After 24 h, growth medium was changed with differentiation medium (2% horse serum containing DMEM), and cells were incubated for 3 days. Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 5 min. Cells were then incubated with 1% BSA for 1 h in 4 °C and the specific antibody at room temperature for 1 h. After washing with 1× PBS, cells were incubated with Texas Red-conjugated antibody at room temperature for 1 h. Coverslides were washed with PBS, mounted, and analyzed. All images were captured with an Axiovert 100 fluorescent microscope (Carl Zeiss, Jena, Germany).

Statistical Analysis—Data were expressed as the mean ± S.D. Analyses were performed with Student's *t* test using SPSS 12.0 software. A value of $p < 0.05$ was considered to be significant. $p = 0.05$ and $p = 0.001$ are designated by * and **, respectively.

RESULTS

Identification of ACTN4 as a Novel sLZIP-binding Protein in HeLa and C2C12 Cells—To investigate the function of sLZIP in cellular processes, sLZIP-binding proteins were identified. A His pulldown assay was performed in HeLa cells using His-tagged sLZIP purified from bacterial cell lysates. A number of proteins were identified as sLZIP-binding proteins. Protein bands were analyzed using LC-MS/MS, and four dominant proteins, including ACTN4, were identified (Fig. 1*A* and supplemental Table S1). The actin-binding protein ACTN4 translocates to the nucleus and probably participates in transcriptional regulation during myogenesis (12). C2C12 myoblasts are a commonly used model for *in vitro* skeletal muscle differentiation. Therefore, a His pulldown assay was performed using nuclear extracts of C2C12 cells. Results showed that sLZIP interacts with endogenous ACTN4 (Fig. 1*B*). Interaction between sLZIP and ACTN4 in the same samples was confirmed using Western blotting (Fig. 1*B*). The peptide sequences obtained using LC-MS/MS and the highly conserved region in mammalian cells are shown in Fig. 1*C*.

ACTN4 Enhances Differentiation of C2C12 Myoblasts—To investigate the role of ACTN4 in myogenesis, we examined the effect of ACTN4 on transcription of muscle-specific genes in C2C12 myoblasts. Results from a luciferase assay showed that ACTN4 increased the transcriptional activities of MCK and myogenin in a dose-dependent manner (Fig. 2*A*). Knockdown of ACTN4 (supplemental Fig. S1*A*) using shRNA for ACTN4 (sh-ACTN4) decreased the promoter activities of MCK and myogenin in a dose-dependent manner (Fig. 2*B*). C2C12 cells transfected with ACTN4 exhibited an increase in myotube for-

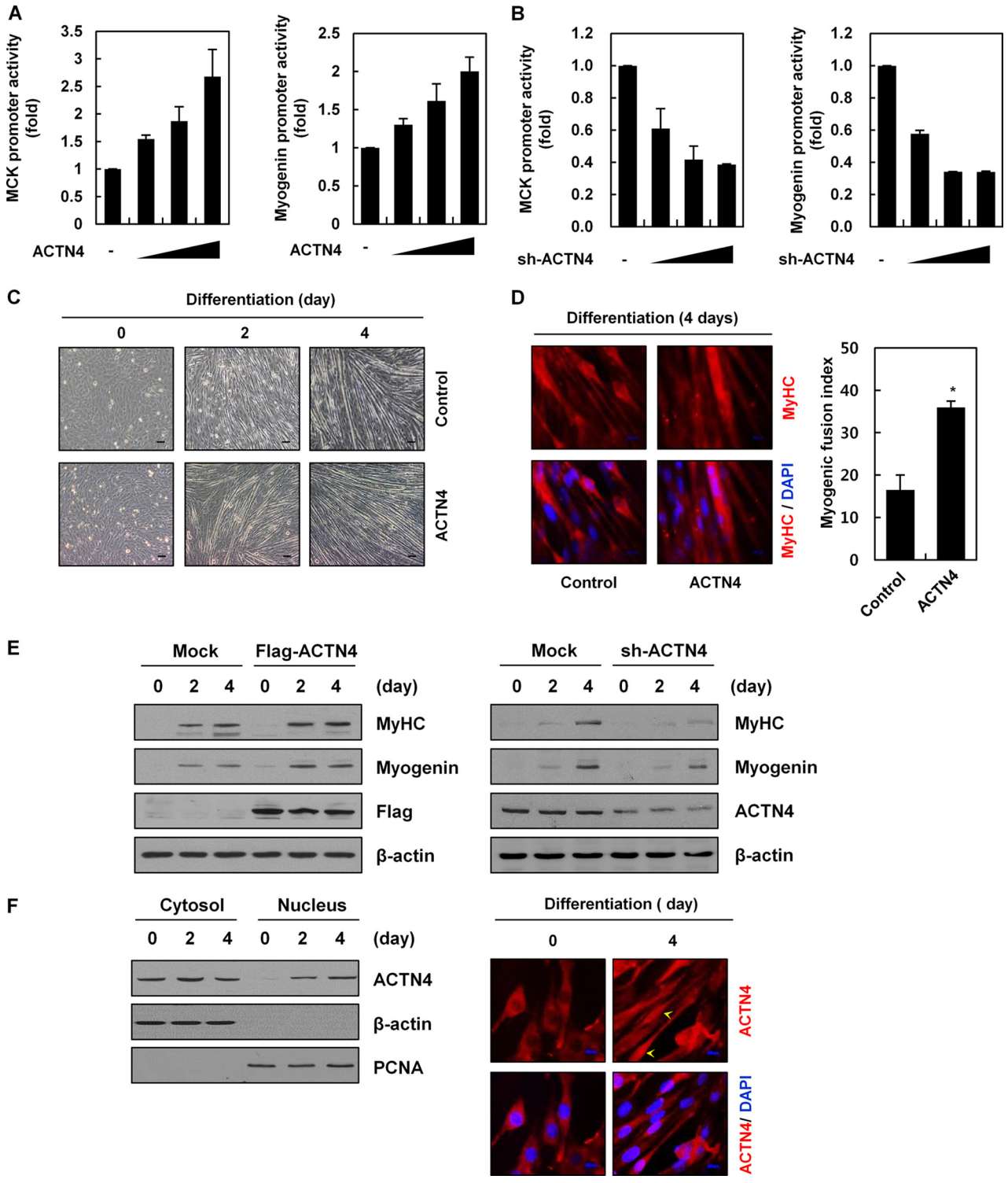

FIGURE 2. **ACTN4 enhances differentiation of C2C12 myoblasts.** *A* and *B*, C2C12 cells were transfected with the ACTN4 (0, 0.25, 0.5, and 1 μg) or sh-ACTN4 (0, 0.25, 0.5 and 1 μg) plasmids, and the MCK and myogenin luciferase reporter gene plasmids (0.5 μg). The luciferase activity was determined after 48 h of transfection. The luciferase activity was normalized to β-galactosidase activity, and the experiments were performed in triplicate. Data are expressed as the mean ± S.D. (*error bars*) and are presented as the relative luciferase activity. *C*, microscopy of undifferentiated proliferating myoblasts and differentiated myotubes in control (empty vector) and ACTN4-transfected C2C12 cells is shown. Cells were observed at same magnification, and the *scale bars* indicate 100 μm. *D*, C2C12 cells transfected with the empty vector (control) and ACTN4 (1 μg) were incubated in differentiation medium (DMEM supplemented with 2% horse serum) for 4 days, and the morphology was recorded using fluorescence microscopy against anti-MyHC antibody and DAPI. The myogenic fusion index was determined at 4 days of differentiation in control and ACTN4-transfected C2C12 myoblasts. *, $p < 0.05$. *Error bars*, S.D. *E*, lysates from C2C12 cells cultured in differentiation medium (0, 2, and 4 days) were immunoblotted with anti-FLAG, anti-MyHC, and anti-myogenin antibodies in ACTN4- (1.5 μg) and sh-ACTN4- (1.5 μg) transfected myoblasts. All experiments were repeated at least in triplicate with similar results. *F*, cytosolic and nuclear fractions were prepared, and ACTN4 expression levels were determined at the indicated time points using Western blotting. Cytosolic proteins were blotted for β-actin. Nuclear fraction was blotted for proliferating cell nuclear antigen (*PCNA*). Differentiated cells were fixed with 4% paraformaldehyde and immunofluorescence stained with DAPI and anti-ACTN4 antibody. *Arrowheads* indicate ACTN4 in the nucleus.

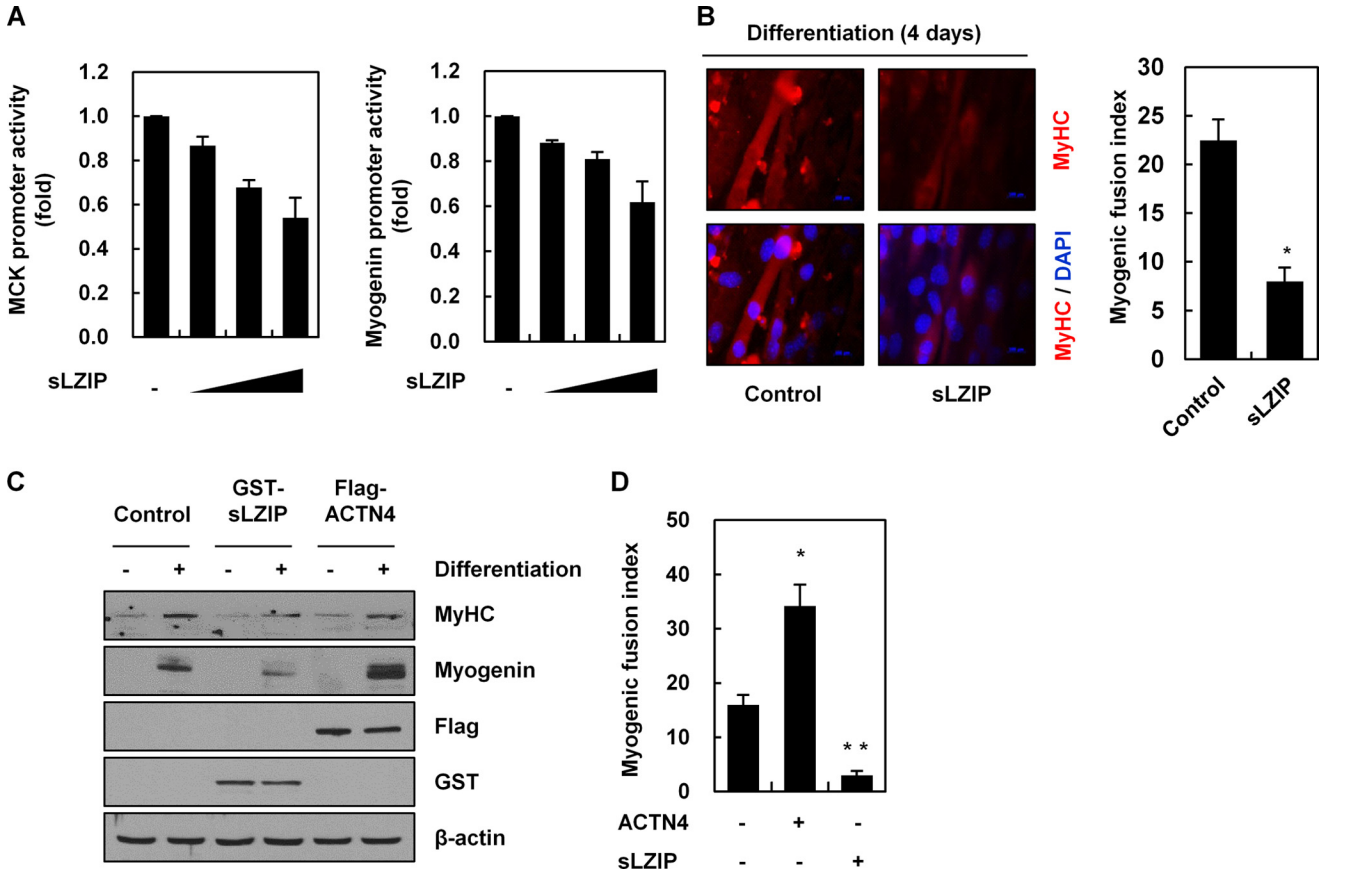

FIGURE 3. **sLZIP suppresses expression of muscle-specific proteins and myotube formation.** *A*, C2C12 cells were transfected with the sLZIP (0, 0.25, 0.5, and 1 μg) expression plasmid, and the MCK and myogenin luciferase reporter gene plasmids (0.5 μg). The luciferase activity was determined after 48 h of transfection. The luciferase activity was normalized to β-galactosidase activity, and the experiments were performed in triplicate. Data are expressed as the mean ± S.D. and are presented as the relative luciferase activity. *B*, C2C12 cells transfected with the empty vector (control) and sLZIP (1 μg) were incubated in differentiation medium for 4 days, and the morphology was recorded using fluorescence microscopy against anti-MyHC antibody and DAPI. The myogenic fusion index was determined at 4 days of differentiation in control and sLZIP-transfected C2C12 myoblasts. *, $p < 0.05$. *Error bars*, S.D. *C*, cell lysates of the empty vector (control), ACTN4- (1 μg), and sLZIP- (1 μg) transfected myoblasts were extracted from cells cultured in differentiation medium (0 and 3 days), and were immunoblotted. *D*, the myogenic fusion index was determined at 4 days of differentiation in control, sLZIP-, and ACTN4-transfected C2C12 myoblasts. All experiments were repeated at least in triplicate with similar results. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.

mation, compared with a control (Fig. 2*C*). This observation was confirmed using immunofluorescence staining. In cells transfected with ACTN4, the number of visible myotubes was increased, compared with a control (Fig. 2*D*). The number of nuclei per myotube was counted and is presented as the myogenic fusion index, which indicates that ACTN4 increased myotube formation by 2.2-fold (Fig. 2*D*). Because levels of MyHC and myogenin are increased during myogenic differentiation, we examined the effect of ACTN4 on expression of these marker proteins. ACTN4 increased the protein expression of MyHC and myogenin during myoblast differentiation, whereas knockdown of ACTN4 decreased expression of both proteins (Fig. 2*E*). These results indicate that ACTN4 is involved in skeletal muscle differentiation via transcriptional regulation of muscle-specific genes. Because localization of ACTN4 is important for transcriptional regulation, the location of ACTN4 during myoblast differentiation was identified. Results from cell fractionation showed that ACTN4 translocates from the cytoplasm to the nucleus as differentiation proceeds (Fig. 2*F*). This result was confirmed using immunofluorescence staining (Fig. 2*F*). These findings indicate that ACTN4 induces myogenic gene expression and promotes myotube formation via transcriptional regulation of target genes.

sLZIP Suppresses Expression of Muscle-specific Proteins and Myotube Formation—Because ACTN4 is involved in myotube formation and interacts with sLZIP, the role of sLZIP in the myogenic process was investigated. We first examined the effect of sLZIP on the promoter activities of MRF family genes. sLZIP reduced the promoter activities of MCK and myogenin in a dose-dependent manner (Fig. 3*A*). Myotube formation was decreased in C2C12 cells transfected with sLZIP compared with a control (Fig. 3*B* and supplemental Fig. S1*B*). The myogenic fusion index indicated that sLZIP suppresses myotube formation by 2.8-fold (Fig. 3*B*). These results indicate that sLZIP negatively regulates myogenesis. Because ACTN4-mediated myogenesis is associated with expression of the MRF family genes, we examined the effect of sLZIP on MRF gene expression during the myogenic process. Expressions of MyHC and myogenin were increased during the myogenic process, and ACTN4 up-regulated expression of these myogenic marker proteins (Fig. 3*C* and supplemental Fig. S1*C*). However, sLZIP decreased expression of MyHC and myogenin in differentiated myoblasts (Fig. 3*C* and supplemental Fig. S1*C*). These results were quantified using the myogenic fusion index. ACTN4 significantly enhanced C2C12 differentiation, whereas sLZIP suppressed myoblast differentiation, compared with a control (Fig.

3*D*). These results indicate that sLZIP suppresses expression of muscle-specific proteins and myoblast differentiation.

sLZIP Negatively Regulates Transcriptional Activation of MEF2 in C2C12 Cells—sLZIP is located in the nucleus and functions as a transcriptional regulator (1). Because ACTN4 is localized in the nucleus of myotubes and increases the transcriptional activity of MEF2 in C2C12 cells (14), we investigated the role of sLZIP in transactivation of MEF2. C2C12 cells were transfected with the MCK promoter coupled to the luciferase reporter gene, ACTN4, or sLZIP, and the promoter activity was determined. ACTN4 increased the MCK promoter activity in both complete and differentiation media; however, sLZIP decreased the ACTN4-induced promoter activity of MCK (Fig. 4*A*). MEF2 increased the MCK promoter activity, whereas knockdown of ACTN4 decreased MEF2-induced MCK promoter activity in a dose-dependent manner (Fig. 4*B*). However, knockdown of sLZIP (supplemental Fig. S2*A*) enhanced the MEF2-induced MCK transcriptional activity (Fig. 4*C*). The enhancer of the MCK promoter contains the $(TA(A/T)_4TA)$ sequence which is known as a MEF2 binding site, and this consensus sequence is found in the promoter region of numerous muscle-specific genes, including MyHC, myogenin, and MCK (20). Therefore, the 3× tandem repeats of the MEF2 binding site were generated coupled to the luciferase reporter gene ($MEF2^{\times 3}$-Luc) (supplemental Fig. S2*B*). The $MEF2^{\times 3}$-Luc was co-transfected with MEF2A, C, D, and ACTN4 into HEK-293T cells. Results from a luciferase assay showed that ACTN4 increased the luciferase activity of $MEF2^{\times 3}$, and the luciferase activity was enhanced in the presence of MEF2A, C, and D in HEK-293T cells (Fig. 4*D*). However, sLZIP repressed the luciferase activity of $MEF2^{\times 3}$ (Fig. 4*E*). When MEF2 and ACTN4 were co-transfected, the luciferase activity of $MEF2^{\times 3}$ was increased by 3.7-fold; however, sLZIP reduced the luciferase activity by ~2-fold (Fig. 4*E*). These results indicate that sLZIP is involved in regulation of myogenic gene expression via inhibition of the transcriptional activation of MEF2. These results were confirmed using qRT-PCR analysis. ACTN4 induced differentiation of myoblasts and increased the mRNA expressions of myogenin, MyoD, and MyHC (Fig. 4*F*). However, sLZIP suppressed the mRNA levels of the muscle-specific genes both in the presence and absence of ACTN4 (Fig. 4*F*), indicating that sLZIP suppresses expression of myogenic genes in C2C12 myoblasts.

The C Terminus of sLZIP Is Required for Interaction with the C Terminus of ACTN4—Because sLZIP is involved in regulation of ACTN4-mediated myogenic gene expression, the interaction domains of sLZIP and ACTN4 were investigated using a GST pulldown assay. As shown in Fig. 5*A* and supplemental Fig. S2*C*, sLZIP interacts with ACTN4. To analyze the interaction domain of ACTN4 with sLZIP, three ACTN4 deletion mutants were generated (Fig. 5*B*). mGST-sLZIP and a series of FLAG-tagged deletion mutants of ACTN4 were co-expressed in HEK-293T cells, and cell lysates were pulled down using GST-agarose beads. Results showed that the C-terminal region (480–911) of ACTN4 is required for interaction with sLZIP (Fig. 5*B*). Deletion mutants of sLZIP were also prepared and were used to examine the luciferase activity of $MEF2^{\times 3}$. sLZIP-F (1–354), sLZIP-C (229–354), and sLZIP-CC (296–354) repressed the luciferase activity of $MEF2^{\times 3}$ in HEK-293T cells (Fig. 5*C*). Results from a GST pulldown assay showed that sLZIP(296–354) interacts with ACTN4(480–911) (Fig. 5*D* and supplemental Fig. S2*D*). These results were confirmed using an $MEF2^{\times 3}$ luciferase assay. ACTN4(480–911) increased the luciferase activity of $MEF2^{\times 3}$ up to 70% of wild-type ACTN4, whereas other deletion mutants did not affect the luciferase activity (Fig. 5*E*). sLZIP(296–354) effectively decreased the luciferase activity of $MEF2^{\times 3}$ (Fig. 5*E*). These results suggest that the C terminus of sLZIP is required for interaction with the C terminus of ACTN4 and that sLZIP plays a role in regulation of MEF2 transactivation via interaction with ACTN4.

sLZIP Disrupts the Association between MEF2D and ACTN4—To investigate the regulatory mechanism of sLZIP in myoblast differentiation, the effect of sLZIP on the interaction between ACTN4 and MEF2D was investigated. We performed an immunoprecipitation assay using cells transfected with ACTN4, sLZIP, and MEF2D. Results showed that ACTN4 binds to MEF2D; however, sLZIP interferes with the binding between ACTN4 and MEF2D (Fig. 6*A*). The formation of complexes between ACTN4 and MEF2D and between ACTN4 and sLZIP was examined using an immunoprecipitation assay. ACTN4 increased the formation of ACTN4 and MEF2D complexes; however, sLZIP repressed the interaction between ACTN4 and MEF2D (Fig. 6*B*). These results indicate that sLZIP disrupts the association between ACTN4 and MEF2D. We examined whether sLZIP disrupts the endogenous interaction between ACTN4 and MEF2D in C2C12 cells. Results from an immunoprecipitation assay showed that ACTN4 interacts with MEF2D at the endogenous level (Fig. 6*C*). However, ectopically expressed sLZIP disrupted the association between ACTN4 and MEF2D in differentiated C2C12 cells (Fig. 6*C*). ACTN4 also interacted with MEF2A and C, and sLZIP disrupted the interaction between ACTN4 and MEF2A/C (supplemental Fig. S3). These results suggest that ACTN4 forms a complex with MEF2 and promotes myogenesis; however, sLZIP inhibits the myogenic process via interaction with ACTN4 (Fig. 6*D*).

DISCUSSION

Differentiation of myoblasts is a process of change in cellular morphology and organization. During this process, expression of muscle-specific genes is increased, and formation of myotubes occurs (16–18). Various transcription factors, including the MEF2 and the MRF gene families, are involved in myogenic differentiation and play critical roles in regulation of gene expression in myogenesis (15, 16). Knock-out studies have demonstrated that complex association between various muscle transcription factors occurs during myogenesis and that each transcription factor plays an important role in regulation of the gene expression pattern (28, 29).

In this study, we characterized a novel mechanism by which sLZIP plays a role in inhibition of myoblast differentiation and myotube formation. Regulation of gene expression by transcription factors comprises combinatorial events in that coordinated interactions of co-factors are required. In an effort to identify sLZIP-binding partners, we performed a His pulldown assay and LC-MS/MS and identified ACTN4 as a novel sLZIP-

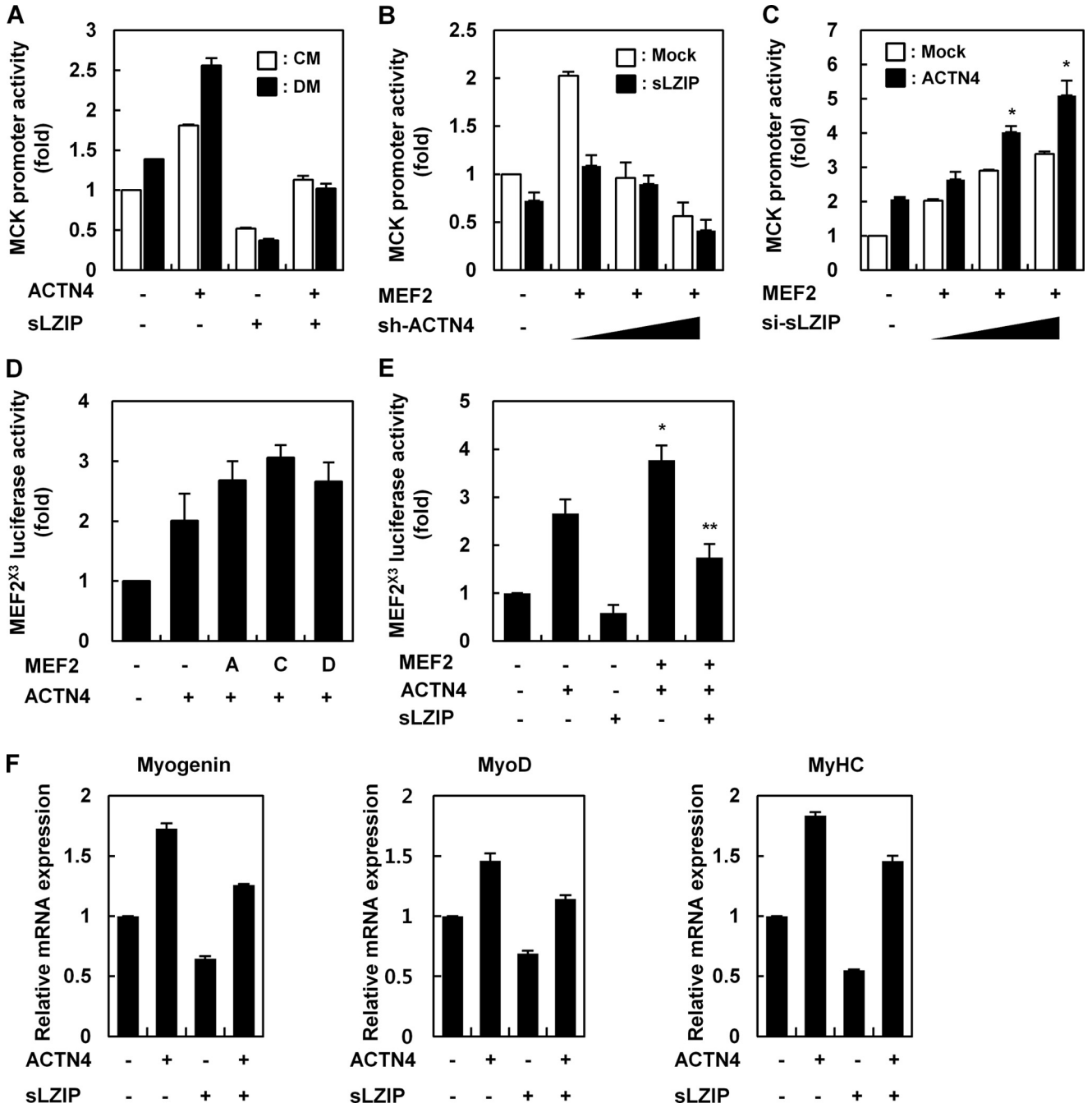

FIGURE 4. **sLZIP negatively regulates transcriptional activation of MEF2 in C2C12 cells.** *A*, the empty vector (control), ACTN4 (1 μg), and sLZIP (1 μg) expression plasmids were co-transfected with the MCK luciferase reporter gene plasmid (0.5 μg) into C2C12 cells and were assayed for luciferase activity after 48 h. *CM*, complete medium; *DM*, differentiation medium. *B*, C2C12 cells were transfected with 0.5 μg of MCK-Luc and sh-ACTN4 (0, 0.25, 0.5, 1 μg). *C*, HEK-293T cells were transfected with 0.5 μg of MCK-Luc and si-sLZIP (0, 25, 50, 100 nM). *, $p < 0.05$. *Error bars*, S.D. *D*, HEK-293T cells were transfected with the MEF2^{x3} reporter gene plasmid, combinations of MEF2A, C, D (1 μg) and ACTN4 (1 μg) as indicated. *E*, HEK-293T cells were transfected with the MEF2^{x3} reporter gene plasmid (0.5 μg), ACTN4 (1 μg), and sLZIP (1 μg), and MEF2 (1 μg) expression plasmids were co-transfected and were assayed for luciferase activity after 48 h. The luciferase activity was normalized to β-galactosidase activity, and the experiments were performed in triplicate. Data are expressed as the mean $\pm$ S.D. and are presented as the relative luciferase activity. *F*, C2C12 myoblasts were transfected with the empty vector (control), sLZIP (1 μg), and ACTN-4 (1 μg), and subjected to myogenic differentiation conditions for 3 days. Total RNA was isolated and used for reverse transcription and real-time PCR analysis. The relative expression levels were determined after normalization with β-actin level. All experiments were repeated at least three times with similar results. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.

binding protein in myotubes. The sLZIP-binding partner ACTN4 is apparently involved in MEF2 transcriptional regulation (14). Ectopic expression of ACTN4 increases the transcriptional activity of MEF2 by disrupting the interaction between MEF2 and HDAC7 (14). MEF2 proteins increase the expression of muscle-associated proteins, including MyHC, myogenin, MyoD, and MCK during muscle differentiation, and the binding sites of MEF2 proteins are localized in the promoter region of the myogenic genes (16, 19, 22). MEF2 activity is regulated by many signaling pathways in which MAPKs act directly on specific residues of MEF2A, C, and D, thereby augmenting MEF2 transactivating functions (30). However, hyperactivation of

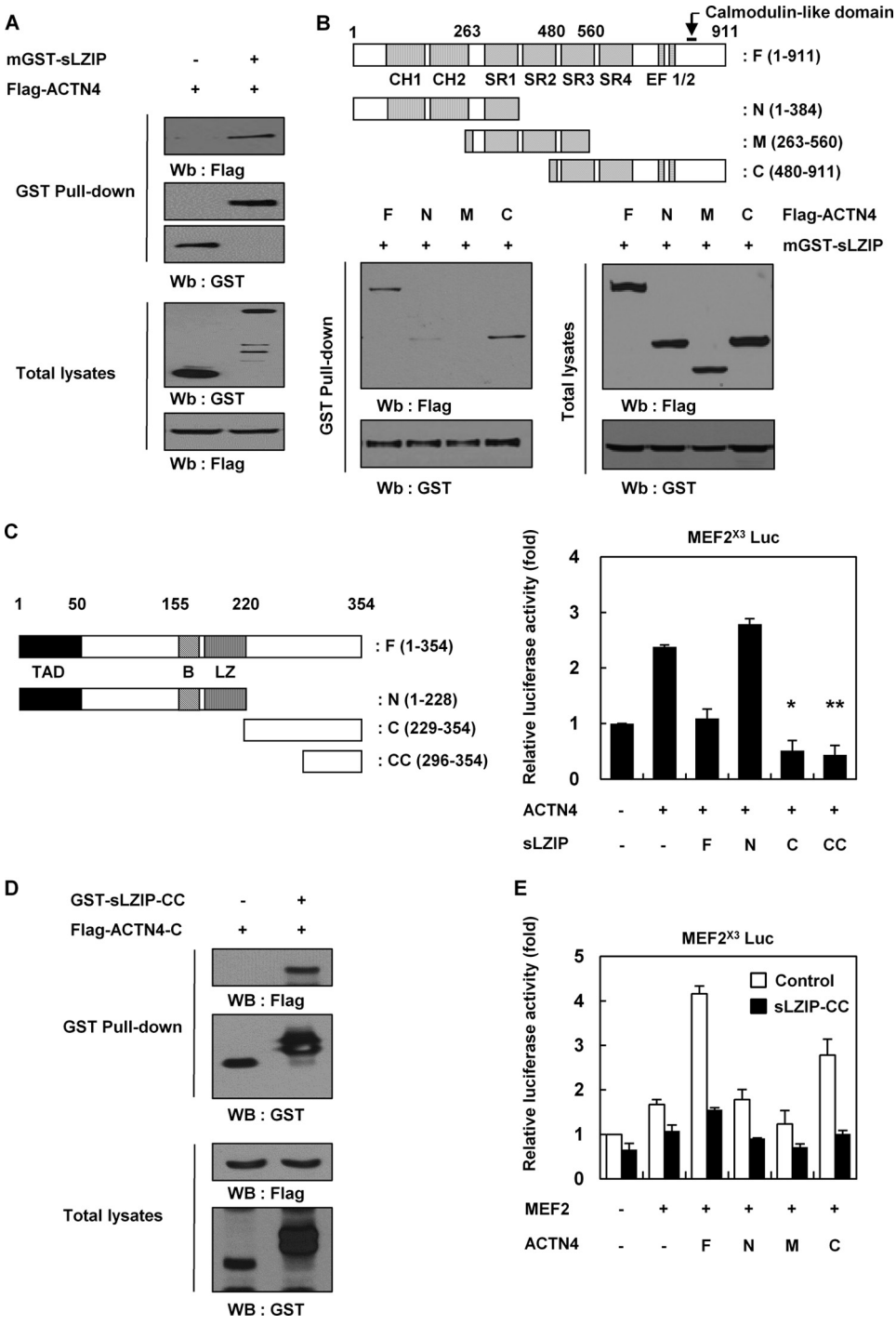

FIGURE 5. **The C terminus of sLZIP is required for interaction with the C terminus of ACTN4.** *A*, HEK-293T cells were transfected with sLZIP (4 μg) and ACTN4 (4 μg), and subjected to a GST pulldown assay and Western blotting against anti-FLAG and anti-GST antibodies. *B*, schematic represents ACTN4 deletion mutants. HEK-293T cells were transfected with mGST-sLZIP and FLAG-ACTN4 deletion mutants and subjected to a GST pulldown assay and Western blotting against anti-FLAG and anti-GST antibodies. *C*, schematic represents sLZIP deletion mutants. MEF2^{X3} luciferase assay was performed in HEK-293T cells. ACTN4 (1 μg) was co-transfected with sLZIP deletion mutants (1 μg) and the MEF2^{X3} luciferase reporter plasmids (0.5 μg). The luciferase activity was normalized to β-galactosidase activity, and the experiments were performed in triplicate. Data are expressed as the mean $\pm$ S.D. and are presented as the relative luciferase activity. *Error bars*, S.D. *D*, HEK-293T cells were transfected with mGST-mock (2 μg), mGST-sLZIP-CC (2 μg) and FLAG-ACTN4-C (2 μg) and subjected to a GST pulldown assay and Western blotting (*WB*) against anti-FLAG and anti-GST antibodies. *E*, MEF2^{X3} luciferase assay was performed in HEK-293T cells. sLZIP-CC (0.5 μg) was co-transfected with MEF2 (0.5 μg), ACTN4 deletion mutants (0.5 μg), and the MEF2^{X3} luciferase reporter plasmids (0.5 μg). The luciferase activity was normalized to β-galactosidase activity, and the experiments were performed in triplicate. Data are expressed as the mean $\pm$ S.D. and are presented as the relative luciferase activity.

MEF2-dependent transcription induces muscle disease, such as hypertrophy, by acting as a part of the transcriptional program (31, 32). ACTN4 also has a nuclear receptor interacting motif that functions either as a transcriptional regulator or as a co-activator. Recently, ACTN4 was shown to be a transcriptional regulator via direct interaction with an estrogen receptor (33).

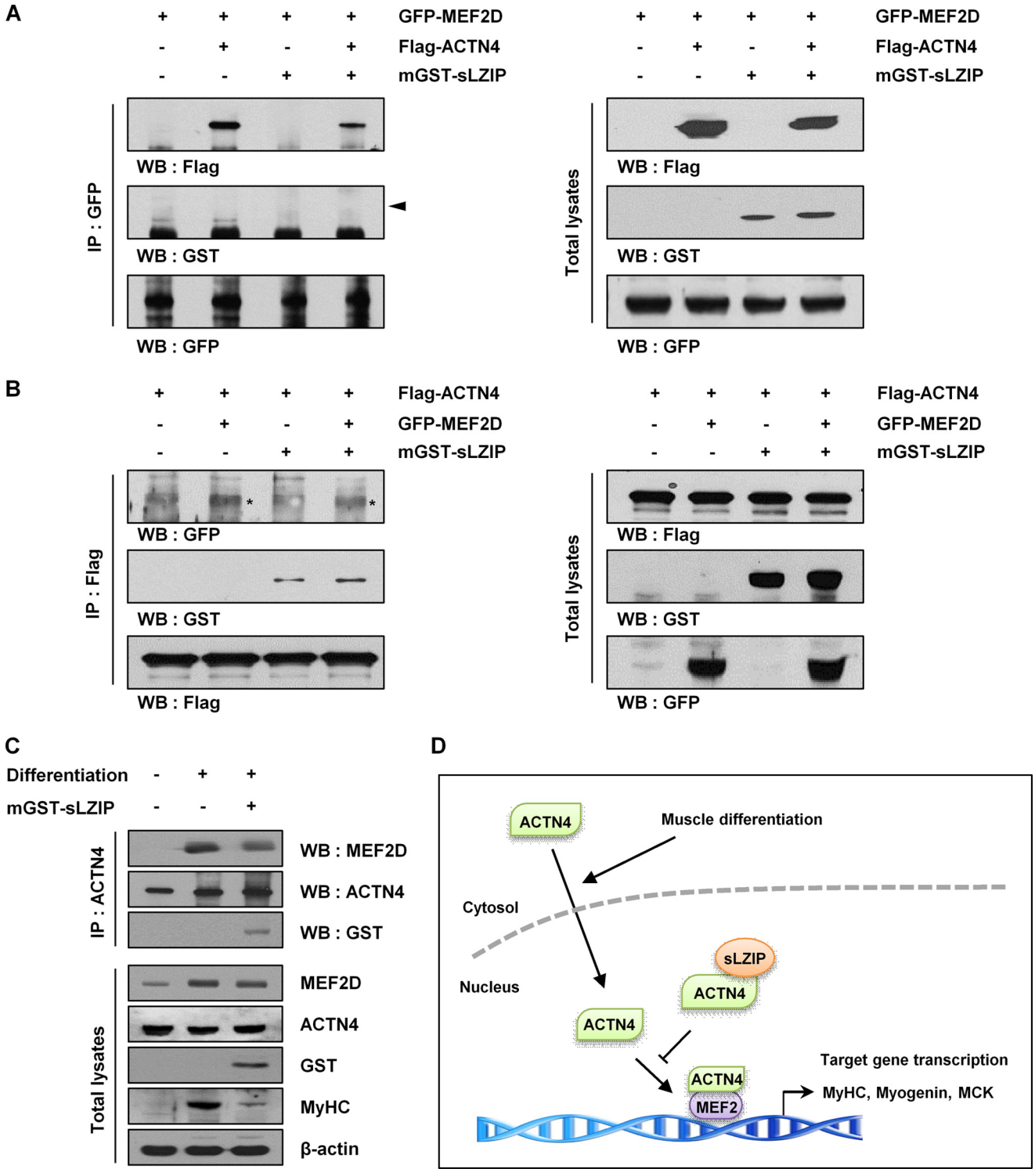

FIGURE 6. **sLZIP disrupts the association between MEF2D and ACTN4.** *A* and *B*, HEK-293T cells were transfected with FLAG-ACTN4 (2 μg), mGST-sLZIP (2 μg), and GFP-MEF2D (2 μg) and subjected to an immunoprecipitation (*IP*) assay. Result shown is representative of two independent experiments. *Arrowhead* indicates mGST-sLZIP. *C*, C2C12 cells were transfected with or without mGST-sLZIP (3 μg). Cells were differentiated for 3 days and subjected to an immunoprecipitation assay using anti-ACTN4 antibody. *D*, schematic represents the roles of sLZIP and ACTN4 in myoblast differentiation. During the myogenesis, ACTN4 translocates to the nucleus, binds to MEF2, and increases the transcriptional activity of MEF2, leading to expressions of MEF2-induced myogenic genes. sLZIP interacts with ACTN4 in the nucleus and disrupts the association between ACTN4 and MEF2, resulting in suppression of myogenic gene expression. Therefore, sLZIP functions as a negative regulator of MEF2-mediated gene expression and skeletal muscle differentiation.

Some nuclear receptors, including retinoic acid receptor, retinoid X receptor, and T3 receptors, are also involved in regulation of myogenesis (34–36). Thus, ACTN4 is a candidate for study as a myogenesis regulator. In this study, ACTN4 showed two important features that are required of a transcriptional co-activator: (i) translocation to the nucleus and (ii) direct interaction with transcription factors during myogenesis. ACTN4 is translocated to the nucleus during muscle differen-

tiation, indicating that ACTN4 probably functions as a transcriptional regulator during myoblast differentiation. ACTN4 induces expression of muscle-specific genes, including some known transcription factors that regulate myogenesis, and promotes myotube formation in C2C12 myoblast differentiation. Our results indicate that ACTN4 is also a co-regulator of myogenesis.

The roles of sLZIP and ACTN4 in myogenesis were investigated. C2C12 cells overexpressing ACTN4 exhibited an increase in MCK and myogenin promoter activities and the luciferase activity of MEF2$^{\times 3}$. However, sLZIP decreased these activities. A recent study showed that ACTN4 is required for the transcriptional activation of MEF2 through interaction with the C terminus of MEF2 (14). sLZIP disrupts the interaction between ACTN4 and MEF2, and sLZIP interacts with ACTN4 through the C terminus, resulting in suppression of ACTN4-induced myogenesis. Down-regulation of MEF2 activity is important in regulating muscle expansion and in preventing myogenesis (19, 30, 32). sLZIP inhibits myoblast differentiation via regulation of ACTN4 and MEF2. Therefore, sLZIP is a candidate for study as a therapeutic agent in muscle-associated diseases, such as hypertrophy.

It has been reported that expression of class II HDACs, which repress MEF2-dependent gene expression by binding to MEF2, is significantly reduced in skeletal muscles (12). During the muscle differentiation process, release of MEF2 from HDACs is required, which leads to disruption of the HDACs-MEF2 complex and enhancement of MEF2-mediated transcription (14, 28, 29). sLZIP increases the activities of HDACs, resulting in suppression of target gene expression (1). Therefore, sLZIP probably plays a role in regulation of HDAC activities in myogenesis. Although further study regarding the relationship between sLZIP and HDACs is needed, our findings demonstrate that sLZIP functions as a negative regulator in myoblast differentiation via interaction with ACTN4.

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