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SH2B1 modulates chromatin state and MyoD occupancy to enhance expressions of myogenic genes

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ABSTRACT

As mesoderm-derived cell lineage commits to myogenesis, a spectrum of signaling molecules, including insulin growth factor (IGF), activate signaling pathways and ultimately instruct chromatin remodeling and the transcription of myogenic genes. MyoD is a key transcription factor during myogenesis. In this study, we have identified and characterized a novel myogenic regulator, SH2B1. Knocking down SH2B1 delays global chromatin condensation and decreases the formation of myotubes. SH2B1 interacts with histone H1 and is required for the removal of histone H1 from active transcription sites, allowing for the expressions of myogenic genes, *IGF2* and *MYOG*. Chromatin immunoprecipitation assays suggest the requirement of SH2B1 for the induction of histone H3 lysine 4 trimethylation as well as the reduction of histone H3 lysine 9 trimethylation at the promoters and/or enhancers of *IGF2* and *MYOG* genes during myogenesis. Furthermore, SH2B1 is required for the transcriptional activity of MyoD and MyoD occupancy at the enhancer/promoter regions of *IGF2* and *MYOG* during myogenesis. Together, this study demonstrates that SH2B1 fine-tunes global-local chromatin states, expressions of myogenic genes and ultimately promotes myogenesis.

1. Introduction

The interplay between histone modifications and chromatin accessibility by transcription factors orchestrates the sequential gene expressions during development. Myogenesis is initiated from the determination of muscle progenitor cells to myoblasts followed by differentiation and fusion of myoblasts into myotubes [1,2]. During myogenesis, chromatin gradually condenses in myoblasts whereas the local chromatin structure at the myogenic genes opens allowing for the access of histone modifiers and transcription factors [3–5]. Among the myogenic gene network, the expression of *MYOG*-encoded Myogenin is regulated by the binding of a key transcription factor, MyoD at the promoter [6]. In addition, chromatin immunoprecipitation (ChIP) followed by deep sequencing has identified several MyoD binding sites at the distal regulatory regions of *MYOG* suggestive of additional regulation at the enhancer region [7,8]. MyoD and Myogenin are downstream effectors of insulin like growth factors (IGFs) [9]. In response to IGF1 and 2, myoblasts initiate differentiation, resulting in activation of signaling and expression of myogenic genes [10–13]. *IGF1*- or *IGF2-null* mice show defect in myogenesis as well as regeneration of skeletal muscle [10,12,14], suggesting that activation of phosphoinositide 3-kinase (PI3K)-AKT pathway by IGF1 and 2 is critical for the myotube formation [15–17]. Concomitant with MyoD binding, histone modifications shape the epigenome and contribute to the expression of myogenic genes. Nonetheless, the interplay among chromatin remodeling, transcription factor binding, and histone modification during myogenesis remains to be clarified.

In a search to identify novel regulators of myogenesis, SH2B1 was found required for the differentiation of primary myoblasts and C2C12 cells. SH2B1 belongs to SH2B family members, including SH2B1, SH2B2 and SH2B3, and has been characterized as a scaffolding protein. Cellular SH2B1 binds to several receptor tyrosine kinases (RTKs), regulates downstream signaling of RTKs, shuttles between the cytoplasm and the nucleus, regulates transcription, and has been implicated in neurogenesis, adipogenesis, and cardiac hypertrophy [18–25]. Human subjects with SH2B1 mutations have been reported to show social isolation and aggression [26]. Knockout mice of SH2B1 develop obesity and diabetes [27]. While evidence suggests that SH2B1 is required for proper leptin and insulin regulation [24], presumably in neuronal and muscle

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cells, its role in muscle physiology is unclear. In this study, we examine how SH2B1 may regulate myogenesis.

2. Materials and methods

2.1. Reagents

Polyclonal antibody of SH2B1 was a generous gift from Professor Christin Carter-Su at the University of Michigan and described previously [28]. Anti-MyHC (MF20) and anti-Myogenin (F5D) antibodies were purchased from Developmental Studies Hybridoma Bank at the University of Iowa. Anti-α-tubulin, anti-MyoD, anti-GFP and anti-histone H1 antibodies, protein G-agarose beads as well as Bicinchoninic acid assay (BCA) reagents and bovine serum albumin (BSA) were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-Flag antibody was purchased from GeneTex (Irvine, CA). Anti-AKT, anti-phospho-AKT(S473), anti-phospho-ERK1/2 and mouse IgG antibodies were purchased from Cell Signaling (Danvers, MA). Anti-ERK1/2 and anti-GAPDH antibodies as well as powder of F-10 medium, micrococcal nuclease (MNase), DNase I and pronase were purchased from Sigma-Aldrich (St. Louis, MO). Anti-H3K4me3 and anti-H3K9me3 antibodies were purchased from Active Motif (Carlsbad, CA). Anti-Cyclin D1 and negative control normal rabbit IgG were purchased from Abcam (Cambridge, United Kingdom). Anti-H3K27me3 and Myc antibodies were purchased from Merck Millipore (Billerica, MA). Powder of Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), horse serum (HS), L-glutamine (L-Gln), antibiotic-antimycotic (AA), penicillin-streptomycin (PS), lipofectamine 2000, Alexa Fluor 700 goat anti-mouse, 647 goat anti-mouse and 488 donkey anti-rabbit IgG secondary antibody, 4′,6-diamidino-2-phenylindole (DAPI), Prolong gold and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). IRDye800CW-labeled anti-rabbit secondary antibody was purchased from LI-COR Bioscience (Lincoln, NE). Fibroblast growth factor 2 (FGF2) was purchased from ProSpec (Rehovot, Israel). PLA kit was purchased from Olink Bioscience (Uppsala, Sweden).

2.2. Plasmids

The pEGFP-C1 vector, GFP-SH2B1β, GFP-SH2B1β-deleting nucleus export sequence (ΔNES), Myc and Myc-SH2B1β constructs were generous gifts from Dr. Christin Carter-Su [28]. The 4RE-tk-luciferase plasmid (containing four E-boxes) (4RE-tk-Luc), *MYOG* promoter region (−1565 to +18) luciferase plasmid (*MYOG*-Luc) and MyoD conjugated with flag tag (Flag-MyoD) were gifts from Dr. Shen-Liang Chen at National Central University, Taiwan. Mouse histone H1a, H1c, H1d and H1e in pCMV-Entry vector were purchased from Origene (Rockville, MD).

2.3. Cell culture and isolation of primary myoblasts

293T cells were purchased from American Type Culture Collection. C2C12 cells (60083) and RD cells (60113) were purchased from Bioresource Collection and Research Center of Food Industry Research and Development Institute (Taiwan). C2C12, RD and 293T cells were maintained in DMEM containing 10% FBS, 1% AA, and 1% L-Gln and grown at 37 °C under 5% CO_2 condition. C2C12 cells were maintained at 30% confluency in growth (10% FBS) medium as undifferentiated cells (GM). For differentiation into myotubes, 90% confluency of C2C12 and RD cells were shifted into differentiation medium (DM) containing DMEM supplemented with 2% HS, 1% AA, and 1% L-Gln in the 5% CO_2 and 37 °C condition. Primary myoblasts were isolated according to [29]. Briefly, 12 to 14 week old adult rat was sacrificed and gastrocnemius muscles were dissociated. Muscles were cut into small pieces in the presence of DMEM containing 1% PS and 1% L-Gln and processed to digestion using 0.1% pronase at 37 °C for 1 h with general shaking every 5 min. Minced solution was centrifuged at 2k rpm for 3 min and resuspended with DMEM containing 10% FBS, 1% PS and 1% L-Gln and filtered with 70 μm nylon mesh. Dissociated cells was collected and resuspended with F-10 medium containing 5 ng/ml FGF2 and 0.5 μg/ml heparin and finally seeded on collagen-coated dishes at 37 °C under 5% CO_2 condition. Cells were passaged twice to enrich primary myoblasts with pre-plating on dishes without collagen-coating for 30–45 min before transferring onto collagen-coated dishes.

2.4. Knockdown of endogenous SH2B1 via RNA interference

pLKO.1-shRNA lentiviral plasmids containing oligonucleotides targeting mouse SH2B1 [clone ID TRCN0000247808 (#1), 0000247810 (#2), 0000247809 (#3)] and LacZ (clone ID TRCN0000072236, 0000231717) were obtained from the National Core Facility at the Institute of Molecular Biology, Genomic Research Center, Academic Sinica, Taiwan. Lentivirus containing pLKO.1-shLacZ and shSH2B1 (#1–#3) were prepared from 293T cells that were co-transfected with pCMVΔR8.91, pMD.G and pLKO.1-shLacZ or shSH2B1 (#1–#3). Medium containing lentivirus was harvested 24 h after transfection and added into C2C12 cells in the presence of polybrene (8 μg/ml) for a further 24 h. C2C12 cells were then subjected to puromycin (5 μg/ml) selection for at least 10 days.

2.5. Immunoblotting, immunoprecipitation, immunofluorescence staining and fusion index

Cells were harvested by radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA and 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 10 ng/ml aprotinin, and 10 ng/ml leupeptin (A + L). Protein samples were determined by BCA assay and an equal amount of protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by incubating with the indicated primary antibodies and IRDye-conjugated secondary antibodies. Signal was detected by Odyssey infrared imaging system (LI-COR Biosciences). For immunoprecipitation, isolated nuclei were dissolved in lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.5% *N*-lauroylsarcosine, 10 μg/ml DNase I, 1 mM PMSF, 1 mM Na_3VO_4 and 10 ng/ml A + L) and incubated with the specific antibody followed by protein A or G-agarose beads pull down. The immunoprecipitated proteins were analyzed by western blotting. For immunofluorescence staining, cells were fixed, permeabilized, incubated in blocking buffer (1% BSA/PBS) and then with antibodies. The fusion index was calculated as followed: the number of nuclei (≥3) in myotubes divided by the total number of nuclei of MyHC positive cells. Chromocenters were identified as DAPI-positive foci in the nucleus. Total cell numbers were indicated in Fig. 3A from three independent experiments (each >100 cells were counted). Images were taken by a Carl Zeiss Observer Z1 microscope, LSM 510 and 780 confocal microscopes and analyzed by ZEN 2012 software (Zeiss).

2.6. Proteomic analysis using liquid chromatograph-tandem mass spectrometry

Cell lysates (Cytosol) at GM, 6 h, 1d and 2d of C2C12 cells were first harvested with L-RIPA (RIPA with 0.1% Triton X-100). After being centrifuged, the pellets were then dissolved in lysis buffer described in immunoprecipitation and incubated with the anti-SH2B1 antibody. SH2B1 protein complexes were pulled down by protein A-agarose beads and analyzed via SDS-PAGE. Gel bands were purified and proteomic analysis was performed by Mithra Biotechnology Inc. (http://www.mithracro.com/) in Taiwan.