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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.

Keywords: Chromatin/Differentiation/Histone modifications/Myogenesis/Transcriptional regulation

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 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 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~ +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% CO2 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% CO2 and 37°C condition. Primary myoblasts were isolated according to [29]. Briefly, 12 to 14 weeks adult rat was sacrificed and gastrocnemius muscles were dissociated. Muscles were cut into small pieces in the present of DMEM containing 1% PS and 1% L-Gln and processed to digestion using 0.1% pronase at 37°C for 1 hour with general shaking every 5 minutes. Minced solution was centrifuged at 2k rpm 3 minutes and re-suspended with DMEM containing 10% FBS, 1% PS and 1% L-Gln and filtered with 70 μm nylon mesh. Single cell was collected and re-suspended 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-coated for 30-45 minutes 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 hours after transfection and added into C2C12 cells in the presence of polybrene (8 μg/ml) for further 24 hours. 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 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 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, 6h, 1d and 2d of C2C12 cells were first harvested with L-RIPA (RIPA with 0.1% Triton X-100). After 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.

2.7. Semi-quantitative real-time polymerase chain reaction (qPCR)

The RNA of C2C12 cells was isolated by TRIzol reagent according to the manufacture's instruction of reverse transcription kit (Applied Biosystems). Reverse transcription polymerase chain reaction (RT-PCR), genes of interest were amplified by specific primers: *SH2B1* (Forward:

5'TTCGATATGCTTGAGCACTTCCGC 3' and Reverse: 5'GCCTCTTCTGCCCCAGGATGT 3'), *SH2B2* (Forward: 5'GTCAGCAACACCTAATGAATG 3' and Reverse: 5'GCCACTGTAGGAGTAACCAAC 3'), *SH2B3* (Forward: 5'CATCATCTTTGAGGTGGGAG 3' and Reverse: 5' TCTGCTCAGGAGGTGTCACT3'), *MYOG* (Forward: 5' GACTCCCCACTCCCCATTCACATA 3' and Reverse: 5'GGCGGCAGCTTTACAAACAACACA 3') and *GAPDH* (Forward: 5'ACCACAGTCCATGCCATCAC 3' and Reverse: 5' TCCACCACCCTGTTGCTGTA 3'). For qPCR, genes of interest were amplified by specific primers: *IGF1* (Forward: 5'AGACAGGCATTGTGGATGAG 3'and Reverse: 5' TTCAGTGGGGCACAGTACAT 3'), *IGF2*: (Forward: 5' CTGATGGTTGCTGGACATCT 3' and Reverse: 5' TCCAGGTGTATATTGGAAG 3'), *SH2B1α* (Forward: 5' CGGCAGCAGGAACGGAG 3' and Reverse: 5' AGGATGTGGGGGATCTGTCC 3'), *SH2B1γ* (Forward: 5' GTCTGCAGGGGAGGAGG 3' and Reverse: 5' TGCTCCCGGCCTCACTT 3') and *GAPDH* (Forward: 5' TGAAGCAGGCATCTGAGGG 3' and Reverse: 5' CGAAGGTGGAAGAGTGGGAG 3'). Interested genes were amplified by TaqMan techniques: *SH2B1β* (Forward: 5' ATGTTGTCCTTGTCAGCTATGT 3', Reverse: 5' GCTCCGTTACACAGTCACTC 3' and Probe: 5' FAM/AGCAGGGCC/ZEN/GGGAGCA/IABkFQ 3'); *GAPDH* (Forward: 5' AATGGTGAAGGTCGGTGTG 3', Reverse: 5' GTGGAGTCATACTGGAACATGTAG 3' and Probe: Predesign Mm.PT.39a.1). All primers and probes for TaqMan experiments were synthesized by Integrated DNA Technologies Inc. (Coralville, IW). All results of PCR and qPCR were normalized to *GAPDH*.

2.8. Luciferase assays

For the promoter activity, C2C12 cells were co-transfected with 4RE-tk-Luc or *MYOG*-Luc and vectors encoding GFP, Flag-MyoD, and Myc-SH2B1β. Luciferase activities were examined using a luciferase assay system (Promega) and GFP fluorescence was measured with the VICTOR3 multi-label plate reader

(PerkinElmer). The firefly luciferase activity was normalized to the GFP fluorescence. For detecting promoter reporter activity in SH2B1 reducing cells, C2C12-shLacZ and C2C12-shSH2B1 cells were co-transfected with 4RE-tk-Luc or *MYOG*-Luc and vectors encoding GFP, Flag and Flag-MyoD. Luciferase activities were determined and normalized as described above.

2.9. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed according to [30] with modifications. For MyoD occupancy, briefly, C2C12 cells were cross-linked and the cross-linked DNA was then sonicated to generate 200 to 500 bp on a Bioruptor sonicator (Diagenode). The sonicated protein-DNA complexes were immunoprecipitated with 2 µg anti-MyoD, SH2B1, histone H1 and IgG antibodies followed by protein A-agarose beads incubation to pull down protein-DNA complexes. DNA was eluted, reverse-crosslinked and then purified. For analyzing histone modifications, cross-linked DNA was generated as described above and next processed to MNase digestion [0.5 µl, (2000 gel units/µl)] by using SimpleChIP® Plus Enzymatic Chromatin IP Kit (Cell Signaling) according to manufacturer protocol. Digested chromatin was then sonicated on a Bioruptor sonicator (Diagenode). The protein-DNA complexes were immunoprecipitated with anti-H3K4me3, H3K9me3, H3K27me3 antibodies and pulled down by ChIP-Grade Protein G Magnetic Beads (Cell Signaling). The immunoprecipitated DNA and input control were analyzed by qPCR using *Power* SYBR Green PCR Master Mix and ABI StepOnePlus Real-Time PCR System. Specific primers were used to amplify *MYOG* promoter and enhancer regions (Primer 1: Forward 5’ AGCCTTTTCCGACCTGATGG 3’ and Reverse 5’ CCCCATCATAGAAGGGGGC 3’; Primer 2: Forward 5’ GAGGGTTTAAATGGCACCCAG 3’ and Reverse 5’ ATACAGCTCCATCAGGTCGGA 3’; Primer 3: Forward 5’ GAATCACATGTAATCCACTGGA 3’ and Reverse 5’ ACACCAACTGCTGGGTGCCA 3’; Primer 4: Forward 5’ GACTAGAACGGGGTTGGAGG 3’ and Reverse: 5’ TCACCTGCTCCCTCTTCCTT

3'; Primer 5: Forward 5' AAGAAAAGACATCTCCACCCAGG 3' and Reverse 5' CCAAACTGCTTCTATTTCTGCCTC 3'), *IGF2* promoter P3 (Forward: 5' TACGATTCGTGCTGTGCAGG 3', Reverse: 5' CAACATACCAGGGTGGGCTT 3'), *IGF2* enhancer E-box 1 (Forward: 5' TTCTGGAAGGGGCCTCCTAA 3' and Reverse: 5' GGTGAAGACAGGCCAAGATGA 3'), *IGF2* enhancer E-box 2 (Forward: 5' TGTGTTCCGAGCCTCCTTTG 3' and Reverse: 5' TGCCATCCTTCATCACCCTC 3') and *GAPDH* promoter (Forward: 5' AGAGAGGGAGGAGGGGAAATG 3' and Reverse: 5' AACAGGGAGGAGCAGAGAGCAC 3'). Specific primers were used to amplify *SH2B1* promoter region (TSS, Forward: 5' GAGCTAAGCGCGGGGAC 3' and Reverse: 5' CGAGTCCGGAGAGGAAAGAG 3'; +0.5 k, Forward: 5' TCTCTTCTGCCAAGACGGC 3' and Reverse: 5' TGGAATGTCAGAACTAAGCCAAGA 3'; +1 k, Forward: 5' TGACAACTGTAGCAGGTAGCG 3' and Reverse: 5' GTTTTGGACTCAAGCCCCAG 3'; +1.5 k, Forward: 5' GCGGTAGACTCTGAGTTCTGG 3' and Reverse: 5' TCCAGAGGGCTCTACAAGGT 3'; +2 k, Forward: 5' CCTGGCCTGGGCTGAGATA 3' and Reverse: 5' CAGCCAGAAGACACGGGATG 3'). Results were normalized to non-specific binding to IgG and shown as fold enrichment.

2.10. Restriction endonuclease digestion

Restriction Endonuclease digestion was followed by [31] with modifications. Isolated nuclei of C2C12-shLacZ and C2C12-shSH2B1 cells at GM and 2d were incubated in 150 µl of AluI or BanI digestion buffer (NEBuffer 2.1 and Cutsmart® Buffer, respectively) with 1 unit/µl of AluI or BanI (New England Biolabs) overnight. 2X Proteinase K digestion buffer (200 mM NaCl, 100 mM Tris-HCl, 2 mM EDTA, 1% SDS, pH 7.5) was added for 2 hours at 55°C. Samples were then added with 100 µl AluI or BanI digestion buffer, 100 µl of 2X proteinase K digestion buffer and 100 µg of proteinase K 37°C overnight. DNA was

isolated and 50 ng of DNA was subjected to PCR analysis with specific primers targeting *IGF2* enhancer or *MYOG* promoter region (*IGF2* enhancer 1, Forward: 5' CTTCCAGACTCATCAAGAATA 3' and Reverse: 5' GAACAACTGTGGGGACCAAAG 3'; 2, Forward: 5' ATTGCAGGCAGTGGGTGGA 3' and Reverse: 5' ATAGAAATGCCTCTTAAGAGT 3'; 3, Forward: 5' GGCTTCCCGCCATCTCGA 3' and Reverse: 5' TGGGGTTAGGAGCAGCTGT 3'; 4, Forward: 5' AAGGAGGATTTAGCTCGGGAG 3' and Reverse: 5' CTGGGGTCCGGCTCACAT 3'; 5, Forward: 5' ATGTGACCCGGACCCCAGGCC 3' and Reverse: 5' GACAGGCCTTGTGTTCTTGCA 3' as well as *MYOG* promoter: Forward: 5' TCTCTGGGTTCATGCCAGCAGGG 3' and Reverse: 5' CGAAGCCCTGAAGGTGGACAG 3'). *MAP2* promoter region (For AluI, Forward: 5' ACCTCCACTTACACTGCTGCCTTCT 3' and Reverse: 5' CCCCATCCAGTCCATCTGTGGTGT 3' as well as BanI, Forward: 5' TGCAGTCTCCTGTAAATGGGTCAC 3' and Reverse: 5' TGTCCAAGAAGGCAGCAGTGTA 3') served as an undigested control.

2.11. Micrococcal nuclease (MNase) digestion assays

Isolated nuclei of C2C12-shLacZ and C2C12-shSH2B1 cells were washed twice and re-suspended with MNase digestion buffer (50 mM, Tris-HCl, pH 7.5; 25 mM KCl). The suspension was separated into several fractions according to the time points. 0.01 unit/sample of MNase and final concentration of 1 mM $CaCl_2$ were added to initiate the reaction. The reaction was processed at 37°C at different time intervals and stopped by adding final concentration of 2 mM EGTA and 50 mM EDTA on ice 5 minutes. DNA was purified and analyzed by electrophoresis.

2.12. Duolink in situ proximity ligation assay (PLA)

PLA was performed according to the manufacturer's instructions. Briefly, C2C12 and C2C12 transfected with SH2B1β deleting NES cells were fixed, permeabilized, incubated in blocking buffer containing 1% BSA/PBS and followed by incubation with anti-SH2B1 and histone H1 at 4°C overnight. Cells were then incubated with Duolink PLA rabbit plus and mouse minus probes. After incubation, ligation and amplification were followed by using Duolink detection reagent red. DAPI staining marks the location of the nucleus. Fluorescence signals were detected by LSM 780 confocal fluorescence microscope with a 100X oil objective with z-stack series. Images were analyzed by ZEN 2012 software (Zeiss) and shown in maximum intensity projection.

2.13 Glutathione S-transferase (GST) pull down assay

GST and GST-SH2B1β proteins were pulled down via glutathione-Sepharose 4B beads (GE Healthcare) in BL21 strain expressing indicated plasmids. Beads conjugated with GST-SH2B1β were incubated with lysates of C2C12 cells at GM or 2d for 2 hours at 4 °C. The pull down proteins were then analyzed by western blotting.

2.14. Statistical analysis

All results were expressed as means ± S.E.M. The statistical analysis for all results was performed using the paired Student's *t*-test. The statistical significance (*) is defined as P value < 0.05.

3. Results

3.1. β splicing variant of SH2B1 is the major variant during myogenesis

To investigate the role of SH2B1 in myogenesis, the expression levels of SH2B1 during myogenesis were determined using C2C12 cells as a model system [32]. C2C12 cells can be kept as undifferentiated myoblasts when cultured at low density in growth medium (GM) and differentiated to form multinucleated myotubes within two days of differentiation (2d). The mRNA levels of SH2B1 from GM to four days after differentiation (4d) were determined by reverse transcription polymerase chain reaction (RT-PCR) use the same pair of primers. β isoform of SH2B1 appeared to be the major splicing variant and its expression was up-regulated during myogenesis. SH2B1α and δ were down-regulated during differentiation whereas SH2B1γ remained unchanged (Fig. 1A). qPCR was also performed with similar results. SH2B1β was increased approximately 3-fold whereas SH2B1γ was increased 1.5-fold and SH2B1α was decreased (Fig. 1B). SH2B1δ was not able specifically amplified due to its high sequence similarity to other SH2B1 splicing variants. The expression levels of the other two SH2B family members, SH2B2 and SH2B3, during myogenesis were also examined. SH2B2 level increased by 1d then reduced to basal level from 2d to 4d (Fig. 1C). In contrast to SH2B1 and SH2B2, the expression of SH2B3 was reduced during myogenesis (Fig. 1D). The protein levels of SH2B1 during myogenesis were similarly increased during myogenesis of C2C12 cells (Fig. 1E) as well as of primary myoblasts (Fig. 1F). These data indicate that the expression of SH2B1β is increased during myogenesis.

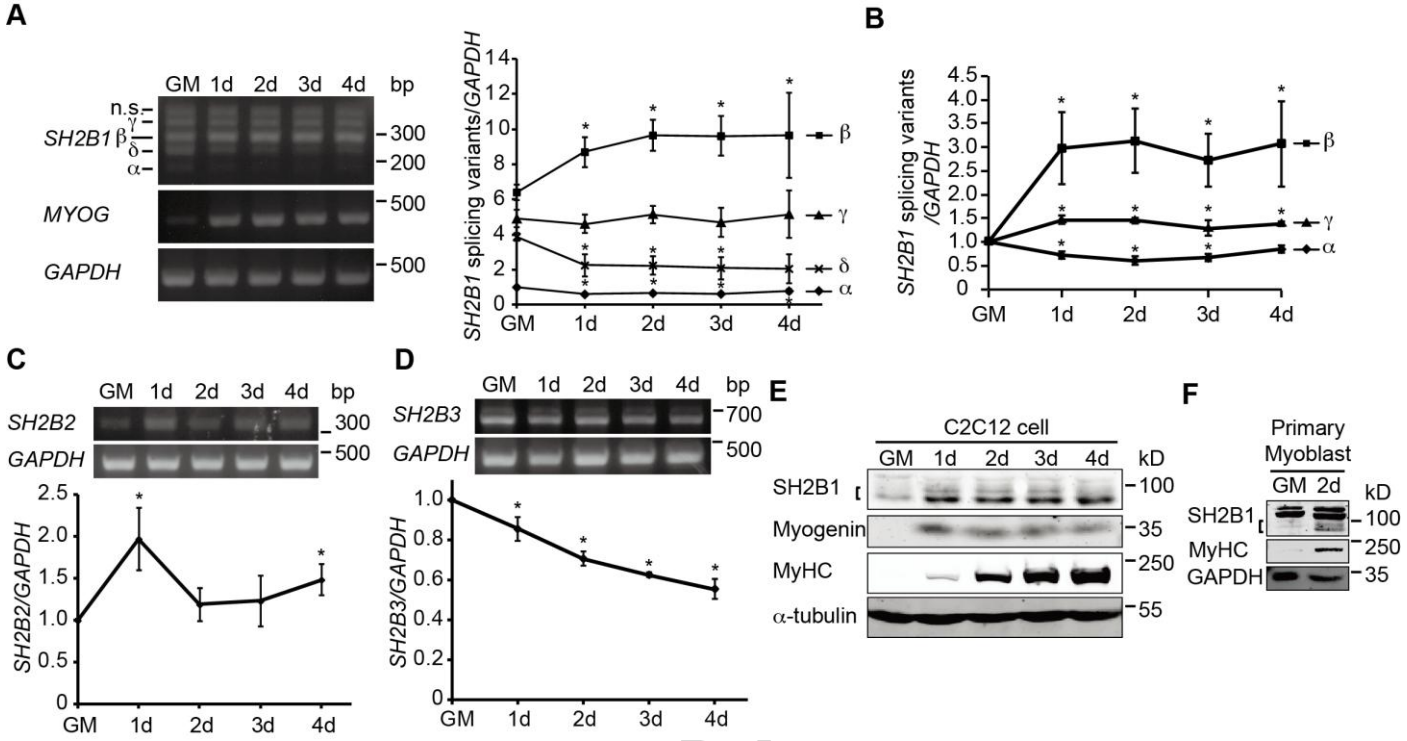

Fig. 1. The expression of SH2B family members during myogenesis. (A), (C) and (D) The levels of SH2B1 splicing variants, SH2B2, and SH2B3 were compared using RT-PCR. *MYOG* is a differentiation marker and *GAPDH* is an internal control. n.s.: non-specific band. (B) The levels of SH2B1 splicing variants were analyzed by qPCR. (E) The protein levels of SH2B1 were compared in C2C12 cells at undifferentiated (GM) and during differentiation days 1-4 (1d-4d). Myosin heavy chain (MyHC) is another myogenic differentiation marker and α-tubulin was used as a loading control. (F) The levels of SH2B1 were examined in primary myoblasts at GM and 2d. GAPDH was used as a loading control. Values are means ± S.E.M from at least three independent experiments. *: $P<0.05$ using paired Student's *t*-test compared to GM.

3.2. SH2B1 is required for myotube formation

To analyze the function of SH2B1 during myogenesis, C2C12 cells transiently expressing short hairpin RNAs (shRNAs) that target LacZ (shLacZ) or SH2B1 (shSH2B1#1-#3) were established. The protein levels of SH2B1 in C2C12-shSH2B1 cells were reduced compared to control C2C12-shLacZ cells (Fig. 2A). Concomitantly, the levels of myogenesis markers, Myogenin and Myosin heavy chain (MyHC), were also reduced (Fig. 2A). shSH2B1#3 showed the best knockdown efficacy at both GM and 2d (Fig. 2A), and thus was used to generate stable cell line for the following experiments. The expression of SH2B1 in the established C2C12-shSH2B1 cell line was 70% less than that in C2C12-shLacZ stable cell line (Fig. 2B). To examine whether reduction of SH2B1 affects myotube formation, C2C12-shLacZ and C2C12-shSH2B1 cells were differentiated and the fusion index was quantified to represent differentiated multi-nuclear muscle cells [33]. As shown in Fig. 2C, the fusion index of C2C12-shSH2B1 cells was significantly lower than that in C2C12-shLacZ cells at 2d, suggesting that SH2B1 is required for myogenesis. The effect of reducing SH2B1 on primary myoblasts was also determined. As shown in Fig. 2D-E, reduction of SH2B1 decreases the length of myotubes as well as the levels of MyHC in primary myoblasts at 2d which is consistent with the results from C2C12 cells. In line with this finding, overexpression of SH2B1β promoted myogenesis by increasing Myogenin and fusion index in C2C12 cells, the length of myotubes and the levels of MyHC in primary myoblasts (Fig. 2F-I). To determine whether the reduction of myogenesis resulted from knockdown of SH2B1, rescue experiments were performed. As shown in Fig. 2J-K, shSH2B1 reduced the fusion index and the length of primary myoblast whereas re-expression of SH2B1β rescued the fusion index and the length of primary myoblasts to the similar level in control shLacZ cells. To examine the possibility that SH2B1 may regulate exit of cell cycle to modulate myogenesis, cell numbers of C2C12-shLacZ and C2C12-shSH2B1 cells were quantified at the indicated time points. There was no obvious difference of cell proliferation between C2C12-shLacZ and C2C12-shSH2B1 cells (Supplementary Fig. S1A). Consistent

with this result, the levels of G1 to S phase regulator, Cyclin D1, in C2C12-shLacZ and C2C12-shSH2B1 cells were similarly reduced during myogenesis (Supplementary Fig. S1B). These results suggest that SH2B1 is required for myogenesis and may regulate myogenesis through a mechanism other than controlling the cell cycle progression.

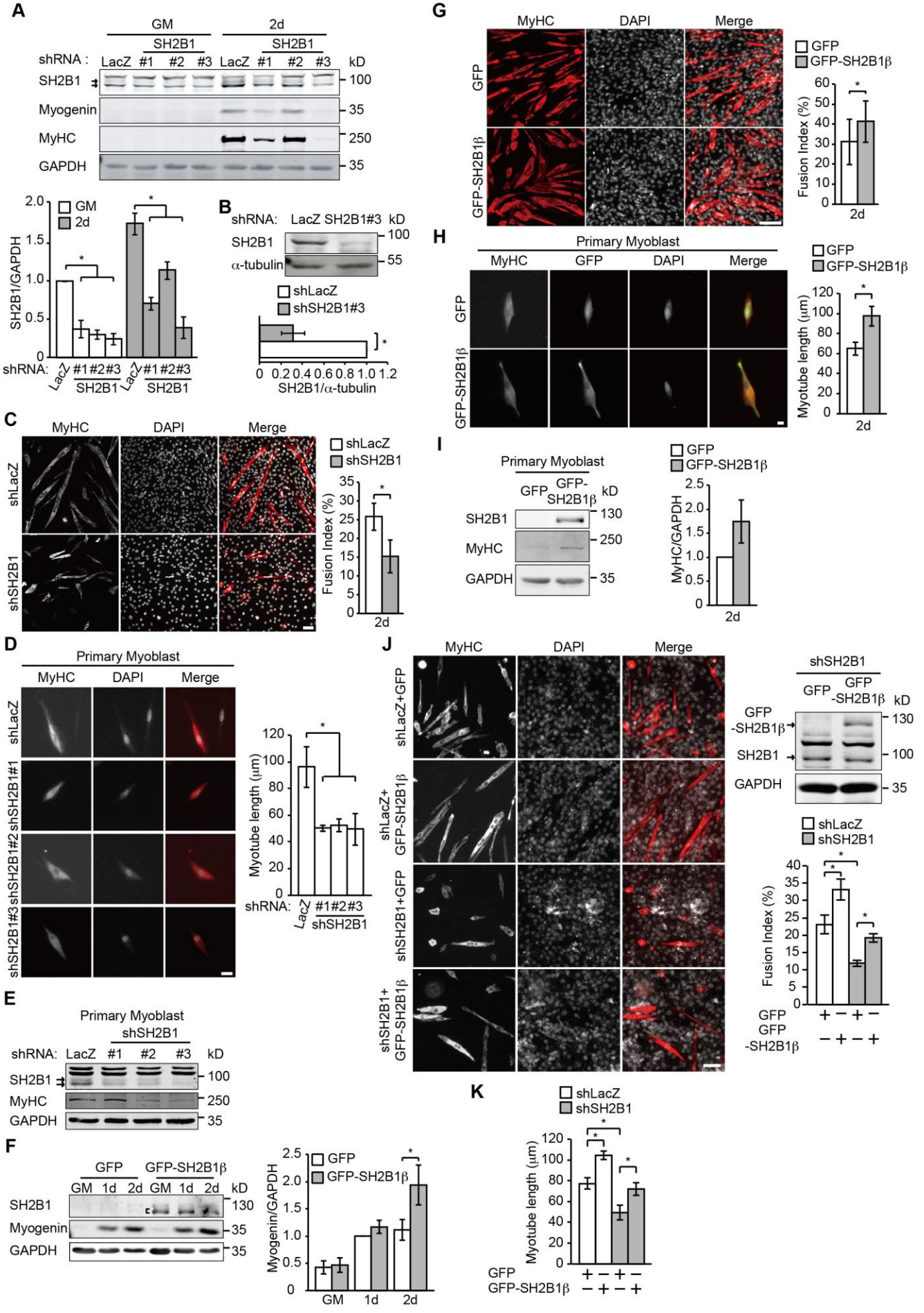

Fig. 2. SH2B1 is required for the formation of myotubes. (A) The levels of SH2B1 were compared among C2C12 cells infected by lentivirus expressing three shRNAs targeting SH2B1 (shSH2B1#1-#3) or one targeting LacZ (shLacZ). (B) The levels of SH2B1 in C2C12 cells stably expressing shLacZ (C2C12-shLacZ) and shSH2B1#3 (C2C12-shSH2B1) cells were compared. (C) Representative images and fusion index of differentiated C2C12-shLacZ and C2C12-shSH2B1 cells at 2d were shown. (D) Representative images and the length of differentiated primary myoblasts infected with shLacZ and shSH2B1#1-#3 at 2d were shown. (E) The levels of SH2B1 and MyHC were compared at 2d among primary myoblasts infected with shLacZ and shSH2B1#1-#3. (F) The levels of Myogenin in C2C12 cells overexpressing GFP (C2C12-GFP) and C2C12 cells overexpressing GFP-SH2B1β (C2C12-SH2B1β) were analyzed by western blotting. (G) Representative images and fusion index of differentiated C2C12-GFP and C2C12-GFP-SH2B1β cells at 2d were shown. (H) Representative images and the length of differentiated primary myoblasts transfected with GFP and GFP-SH2B1β at 2d were shown. (I) The levels of MyHC were compared at 2d between primary myoblasts transfected with GFP or GFP-SH2B1β. (J) Representative images and fusion index of C2C12 cells at 2d were shown. C2C12-shLacZ and shSH2B1 cells were transfected with GFP or GFP-SH2B1β and differentiated. (K) Primary myoblasts were first infected with lentivirus expressing shLacZ and shSH2B1, then transfected with GFP or GFP-SH2B1β followed by differentiation. The length of differentiated primary myoblasts at 2d was shown. Scale bar in (C), (G) and (J) is 100 μm and in (D) and (H) is 20 μm. Color: Red, MyHC; green, GFP; white, DAPI. Values are means ± S.E.M from three independent experiments. *: $P<0.05$, paired Student's *t*-test.

3.3. SH2B1 is required for chromatin condensation during myogenesis

During myogenesis, chromocenters (heterochromatin foci) gradually cluster together as global chromatin reorganizes, showing as larger puncta and reduced number of chromocenters [34]. To determine whether SH2B1 regulates chromatin re-organization, the number of chromocenter was compared between C2C12-shLacZ and C2C12-shSH2B1 at 2d. The major population of cells contained around 16~20 foci/cell in control cells at 2d but the numbers were mostly around 31~35 foci/cell for C2C12-shSH2B1 cells (Fig. 3A). This result suggests that SH2B1 may participate in overall chromatin re-organization during myogenesis. To identify candidate binding partners of SH2B1, SH2B1 was immunoprecipitated at different differentiation stages of myogenesis followed by analysis via Thermo Q-Exactive liquid chromatograph-tandem mass spectrometry. According to the proteomics data, 181, 142, 37 and 50 proteins were identified in the SH2B1-containing complexes at GM, 6h, 1d and 2d (complete list was shown in the Supplementary File S2). Among these proteins, several proteins such as actin, SH2B1 and Filamin-A have been reported previously to interact with SH2B1 (Fig. 3B) [35-37]. Interestingly, SH2B1 was found associated with histone H1 variants and this association decreased during myogenesis (Fig. 3B). This result was confirmed by reverse co-immunoprecipitation with anti-histone H1 and probe for the existence of SH2B1 (Fig. 3C and Supplementary Fig. S3A). *In vitro* GST pull down assays and co-immunoprecipitation of Myc-SH2B1β and histone H1 confirmed their interaction (Fig. 3D-E). In addition, co-immunoprecipitation between Flag-tagged histone H1 variants and Myc-SH2B1β showed stronger interaction between SH2B1 and histone H1a (Supplementary Fig. S3B). Furthermore, PLA assays were performed to determine the *in vivo* interaction between SH2B1 and histone H1. C2C12 cells were transfected with or without GFP-SH2B1β(ΔNES), SH2B1 mutant lacking nuclear export sequences. Cells were fixed and followed by antibodies incubation, oligonucleotide adapter ligation and amplification (Materials and Methods 2.12). The interactions between endogenous SH2B1 and histone H1 as well as

GFP-SH2B1β(ΔNES) and histone H1 were shown as fluorescence puncta in the nucleus, supporting their *in vivo* interaction (Supplementary Fig. S3C). We thus postulate that SH2B1 may regulate the dynamics of linker histone proteins to alter chromatin structure. In line with this possibility, micrococcal nuclease (MNase) digestion of genomic DNA from C2C12-shSH2B1 cells displayed more DNA fragments compared to C2C12-shLacZ at 2d, indicating that shSH2B1 delays chromatin condensation during differentiation (Fig. 3F). The fact that MNase targets to linker region between nucleosomes and knocking down SH2B1 increased the sensitivity of chromatin to MNase supports a possibility that SH2B1 regulates chromatin conformation through interacting with the histone linker protein H1 (Fig. 3 and Supplementary Fig. S3). These data reveal a possibility that SH2B1-histone H1 complex may regulate chromatin structure.

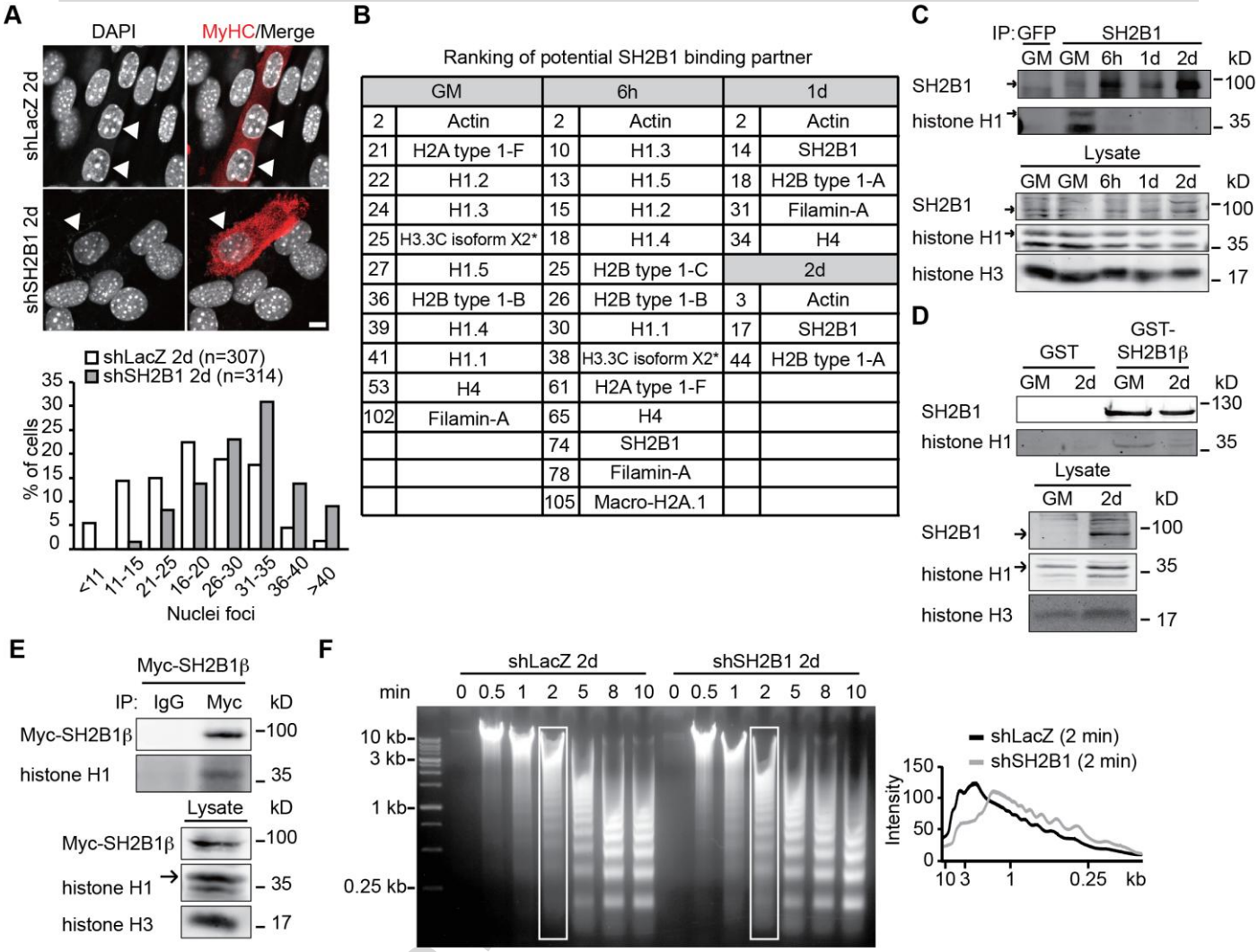

Fig. 3. SH2B1 is required for chromatin condensation during myogenesis. (A) Representative images of chromocenters in C2C12-shLacZ and C2C12-shSH2B1 cells at 2d were shown. Scale bar: 10 μm. (B) Potential SH2B1 binding partner from proteomics analysis were shown. Smaller numbers for the hit proteins represent the higher enrichment of the indicated protein. *H3.3C isoform X2 is a predicted protein which NCBI reference sequence is XP_894986.1. (C) Nuclear lysates from C2C12 cells were immunoprecipitated using anti-SH2B1 antibody and immunoblotted for the indicated proteins. (D) GST and GST-SH2B1β were incubated with nuclear lysates of C2C12 cells at GM and 2d and the pull down complexes were analyzed by immunoblotting. (E) Nuclear lysates from C2C12 cells transfected with Myc-SH2B1β were immunoprecipitated using anti-Myc or anti-IgG antibodies at GM and immunoblotted for the indicated proteins. (F) C2C12-shLacZ and C2C12-shSH2B1 cells at 2d were subjected to MNase digestion, DNA fragments were analyzed via agarose gel. Representative images from three independent experiments and band quantification were shown.

3.4. SH2B1 is required for the expression of IGF2, transcriptional activity of MyoD and histone modifications of IGF2

During development, the overall chromatin gradually condenses over time. Nonetheless, the local structure of differentiation genes opens to promote tissue differentiation. Our data thus far support a role of SH2B1 in regulating global chromatin condensation. We next examined whether SH2B1 may regulate local chromatin opening to promote the expression of myogenic genes. The expression of *IGF2*, an essential gene for myogenesis [10], was compared by qPCR between C2C12-shLacZ and C2C12-shSH2B1 cells. As shown in Fig. 4A, expressions of both *IGF1* and *IGF2* were increased during myogenesis in C2C12-shLacZ cells. On the other hand, shSH2B1 robustly reduced the expression of *IGF2* but not *IGF1* during myogenesis. In mouse, the expression of *IGF2* is controlled by its promoter regions (P1 to P3), adjacent to exon 1 to 3 [38]. In the skeletal muscle, *IGF2* promoter P3 as well as its enhancer contribute to the expression of *IGF2* (Fig. 4B) [31]. Thus, the accessibility of chromatin at *IGF2* enhancer region of C2C12-shLacZ and C2C12-shSH2B1 cells were analyzed by endonuclease digestion followed by PCR analysis using specific primer sets (Fig. 4C). Condensed chromatin is resistant to endonuclease digestion so that designated DNA fragments can be obtained via PCR. We found that chromatin around *IGF2* enhancer was more accessible to endonuclease digestion, suggesting a more opened configuration during myogenesis (shLacZ GM and 2d) (Fig. 4C), which is consistent with published result [31]. In contrast, the endonuclease cutting was inhibited surrounding *IGF2* enhancer in C2C12-shSH2B1 cells at the position of primer pairs #2 and #3 during myogenesis (shSH2B1 GM and 2d) (Fig. 4C), indicative of a less accessible chromatin structure. Histone H1 facilitates chromatin condensation and epigenetic silencing of genes [39]. To test whether SH2B1 directly or indirectly affects chromatin condensation through regulating histone H1 binding to the active transcription sites, histone H1 ChIP was performed. At the promoter of *IGF2*, a decline of histone H1 binding was found during myogenesis (Fig. 4D). In contrast, the level of histone H1 retained for

shSH2B1 cells, suggesting that, without SH2B1, histone H1 could not be removed from *IGF2* promoter. Concomitantly, the occupancy of SH2B1 was also decreased from *IGF2* promoter during myogenesis (Fig. 4E), indicating the removal of SH2B1-histone H1 complexes from *IGF2* promoter. Reorganization of chromatin structure often accompanies with changes in histone modifications. In fact, at the enhancer of *IGF2*, the level of histone repressive marks, H3K9me3 and H3K27me3, were decreased during myogenesis. In contrast, knocking down SH2B1 resulted in elevated H3K9me3 but not H3K27me3 (Fig. 4F). On the other hand, at the promoter region of *IGF2*, increase of H3K4me3 active mark and decrease of H3K9me3 and H3K27me3 were observed during myogenesis. shSH2B1 inhibited the increase of H3K4me3, increased H3K9me3 and did not affect H3K27me3 (Fig. 4G). These data indicate that SH2B1 is required for the change of histone H3K4me3 and H3K9me3 modifications at *IGF2* gene region. Furthermore, the findings that SH2B1 facilitates the removal of histone H1 and the decline of H3K9me3 is consistent with the positive correlation between histone H1 deposition and H3K9me3 modification in embryonic stem cells [40, 41].

The activation of IGF signaling induces PI3K-AKT pathway [16]. The fact that SH2B1 is required for the induction of *IGF2* during myogenesis suggests the involvement of SH2B1 in PI3K-AKT signaling. To this end, C2C12-shLacZ and C2C12-shSH2B1 cells were differentiated and the effect on PI3K-AKT pathway was determined. As shown in Fig. 4H, phospho-AKT (pAKT) was increased from 6h to 2d during myogenesis whereas the elevation of pAKT was blocked in C2C12-shSH2B1 cells. In contrast, phospho-ERK1/2 (pERK1/2) was not affected by knocking down SH2B1. These results indicate that SH2B1 is required for the expression of *IGF2* and IGF2-PI3K-AKT signaling during myogenesis.

Myogenesis determining transcription factor, MyoD, lies downstream of IGF signaling. To determine whether SH2B1 affects the activity of MyoD, luciferase reporter assays using 4RE-tk-Luciferase (4RE-tk-Luc) construct, containing four MyoD binding E-boxes sites, were performed to determine the transcriptional activity of MyoD with or without overexpression of SH2B1β or MyoD. Overexpression of MyoD increased luciferase activity of 4RE-tk-Luc construct 7-fold whereas co-expressing SH2B1β

enhanced MyoD-induced activity for additional 1.3-fold (Fig. 4I, left panel). MyoD-induced luciferase activity of 4RE-tk-Luc reporter was reduced in C2C12-shSH2B1 cells compard to that in C2C12-shLacZ cells (Fig. 4I, right panel). These results suggest that SH2B1 regulates *IGF2* but not *IGF1* through MyoD, which is consistent with a previous study showing that overexpression of MyoD increased the expression of *IGF2* but not *IGF1* [42].

A target gene of MyoD is *IGF2* of which expression was reduced by shSH2B1 and the transcriptional activity of MyoD was also modulated by SH2B1 (Fig. 4A and 4I). Two putative MyoD binding sites at the enhancer region of *IGF2* were reported [31]. Thus, the effect of SH2B1 on MyoD at the enhancer region of *IGF2* was investigated by ChIP assays. MyoD-chromatin complexes from C2C12-shLacZ and C2C12-shSH2B1 cells at GM as well as 2d were immunoprecipitated using anti-MyoD antibody and the occupancy of MyoD at enhancer region of *IGF2* was determined via qPCR. ChIP assay results showed that shSH2B1 decreased the binding of MyoD to both E-boxes at *IGF2* enhancer region at 2d (Fig. 4J). These results demonstrate that SH2B1 is required for MyoD binding at the enhancer region of *IGF2* and thus regulates *IGF2* transcription.

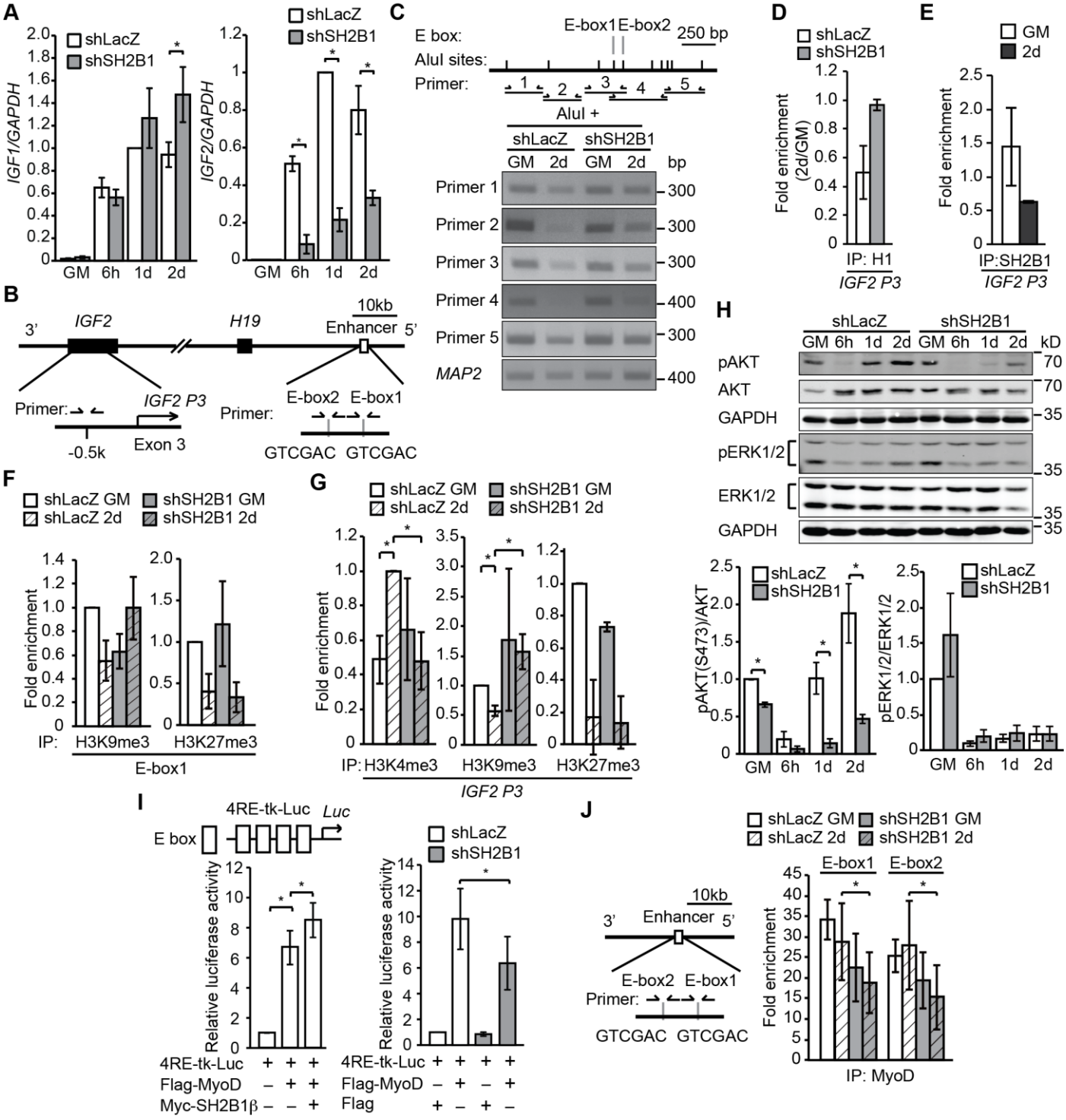

Fig. 4. SH2B1 is required for MyoD occupancy and histone modifications at *IGF2* gene region. (A) mRNA of *IGF1* and *IGF2* in C2C12-shLacZ and C2C12-shSH2B1 cells were quantified using qPCR. (B) Diagram of mouse *IGF2* promoter/enhancer regions. (C) Diagram of mouse *IGF2* enhancer region was modified according to [26]. Nuclei from C2C12-shLacZ and C2C12-shSH2B1 cells at GM and 2d were digested with AluI followed by PCR. *MAP2* promoter served as an undigested control. (D-G) and (J) Cross-linked DNA from C2C12, C2C12-shLacZ and C2C12-shSH2B1 cells at GM and 2d were subjected to ChIP assays using anti-IgG, MyoD, histone H1, SH2B1, H3K4me3, H3K9me3 and H3K27me3 antibodies. ChIP-isolated DNA was analyzed by qPCR using specific primers. (H) Levels of pAKT and pERK1/2 in C2C12-shLacZ and

C2C12-shSH2B1 cells were determined via western blotting. (I) C2C12 cells, C2C12-shLacZ and C2C12-shSH2B1 cells were transfected with the indicated constructs and luciferase activity was measured. Data in (D-G) and (J) were normalized to IgG and shown as fold enrichment and data in (D) was presented as relative level in 2d compared to in GM (2d/GM). Values are means ± S.E.M from at least three independent experiments, except that (D) and (E) were from two experiments and values are means ± S.D. *: $P<0.05$, paired Student's *t*-test.

3.5. SH2B1 regulates MYOG expression through histone modifications and MyoD occupancy

The fact that SH2B1 promotes the transcriptional activity of MyoD as well as the occupancy of MyoD at *IGF2* enhancer (Fig. 4) suggests that SH2B1 could potentially regulate other target genes of MyoD. One of these genes is *MYOG*, a master transcription factor for myotube formation. Protein levels of Myogenin and MyHC were induced during myogenesis and the induction was reduced in C2C12-shSH2B1 cells (Fig. 5A). This reduction was not through reduced expression of MyoD (Fig. 5A). To examine whether SH2B1 also regulates Myogenin expression through chromatin remodeling, promoter of *MYOG* in control and C2C12-shSH2B1 cells during myogenesis was analyzed. According to BanI digestion, chromatin around *MYOG* promoter opened during myogenesis but shSH2B1 resulted in a more closed chromatin state (Fig. 5B). Similar to reduced histone H1 occupancy at *IGF2* promoter during myogenesis (Fig. 4D), a decline of histone H1 at the promoter of *MYOG* was detected. On the other hand, the level of histone H1 did not change for shSH2B1 cells (Fig. 5C). A decrease of SH2B1 was also observed during myogenesis at the promoter of *MYOG* (Fig. 5D), consistent with the function of SH2B1-histone H1 complex in de-condensation of local chromatin structure. At the promoter region of *MYOG*, an increase of H3K4me3 but a decrease of H3K9me3 and H3K27me3 were observed during myogenesis (Fig. 5E). shSH2B1 repressed the accumulation of H3K4me3 and increased the level of H3K9me3; whereas H3K27me3 modification at the same region remained unchanged (Fig. 5E). Given that SH2B1 regulates MyoD transcriptional activity (Fig. 4I) and MyoD is known to bind to *MYOG* promoter, the *MYOG* promoter activity was examined. *MYOG*-Luciferase construct (*MYOG*-Luc) containing ~1.5 kb promoter of *MYOG* was used for luciferase reporter assays. There are two MyoD-binding E-boxes within the *MYOG*-Luc construct. Overexpressing MyoD increased luciferease activity of *MYOG*-Luc construct. Co-expressing SH2B1β and MyoD further enhanced MyoD-induced luciferase activity of *MYOG*-Luc 2.1-fold (Fig. 5F, left panel). MyoD-induced *MYOG* promoter activity was reduced in C2C12-shSH2B1 cells compared to control

cells (Fig. 5F, right panel). Interestingly, in the absence of MyoD, shSH2B1 decreased luciferase activity of *MYOG*-Luc, suggesting that SH2B1 could also regulate activity of *MYOG*-Luc independent of MyoD (Fig. 5F, right panel).

Moreover, the effect on the occupancy of MyoD at the *MYOG* promoter and enhancer region by SH2B1 was determined during myogenesis. To this end, ChIP assays were performed. The occupancy of MyoD at the two E-boxes of the *MYOG* promoter (primer sets #2 and #3) as well as enhancer (-4.5 kb and -6.5 kb, primer sets #4 and #5) regions were increased during myogenesis. The increased MyoD binding was inhibited in C2C12-shSH2B1 cells except for the -4.5 kb region (primer sets #4) (Fig. 5G). These data suggest that SH2B1 is required for the increase of H3K4me3 and the decline of H3K9me3 modifications at promoter and MyoD occupancy at both promoter and enhancer regions of *MYOG*. In addition, a novel distal E-box (-6.5k) site was identified, suggesting a large relaxation region of the *MYOG* gene region.

SH2B1 was up-regulated during myogenesis (Figs. 1 and 5A). It is possible that SH2B1 itself is also a target of MyoD. We next determine whether MyoD binds to 5' [from transcription start site (TSS: +1 to ATG)] region of *SH2B1* gene. There are eight E-boxes in this region. Five E-box regions were analyzed further based on the report that MyoD prefers binding to GC-rich centered E-box during myogenesis [43]. ChIP assays showed that the binding of MyoD on the E-box at +0.5 kb and +1 kb of *SH2B1* was increased at 1d during myogenesis (Fig. 5H). These results demonstrate for the first time that *SH2B1* is a novel target of MyoD during myogenesis.

Together, we have identified SH2B1 as a novel positive regulator of myogenesis. SH2B1 interacts with histone H1, modulates global-local chromatin states and regulates epigenetic changes of myogenic genes. *SH2B1* gene is also a target of MyoD, revealing a positive IGF2-MyoD-SH2B1 loop during myogenesis.

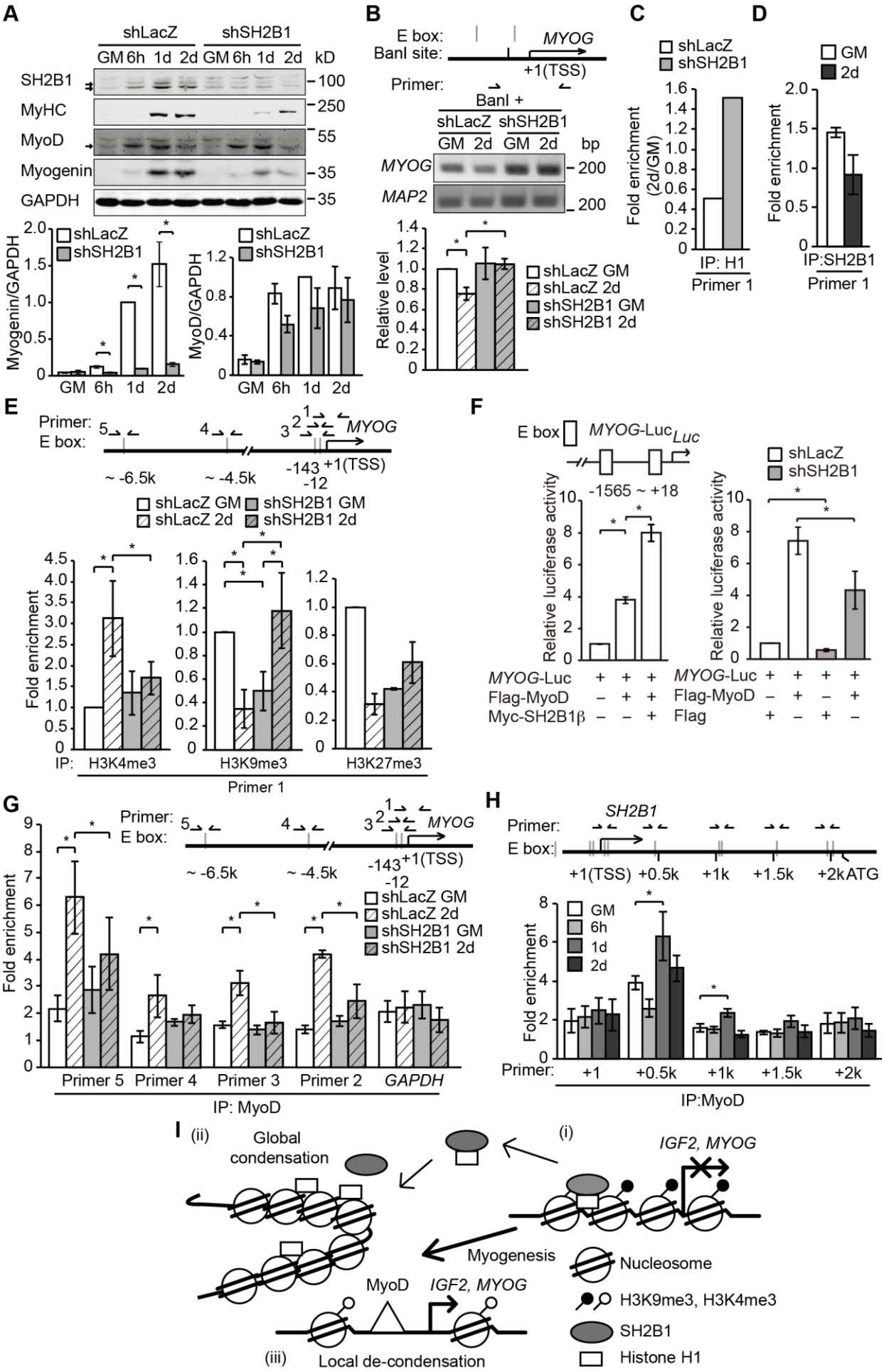

Fig. 5. SH2B1 is required for MyoD occupancy, histone modifications and expression of *MYOG*. (A) The levels of SH2B1, MyHC, MyoD, and Myogenin were compared via western blotting between C2C12-shLacZ and C2C12-shSH2B1 cells at different differentiation stages. (B) Schematic diagram of mouse *MYOG* promoter region. The nuclei from C2C12-shLacZ and C2C12-shSH2B1 cells at GM and 2d were processed to restricted enzyme digestion. Digested DNA was analyzed by PCR. *MAP2* promoter served as undigested control. (C-E) and (G) Schematic diagram of mouse *MYOG* promoter and enhancer regions were shown. Cross-linked DNA from C2C12, C2C12-shLacZ and C2C12-shSH2B1 cells at GM and 2d were subjected to ChIP assays by using anti-IgG, MyoD, SH2B1, histone H1, H3K4me3, H3K9me3 and H3K27me3 antibodies. The ChIP DNA was analyzed by qPCR using specific primers targeting to mouse *MYOG* promoter/enhancer regions. (F) C2C12 cells were transfected with the indicated constructs and luciferase activity was measured (left panel). C2C12-shLacZ and C2C12-shSH2B1 cells were transfected with the indicated constructs and the luciferase activity was measured (right panel). (H) Schematic diagram of mouse *SH2B1* promoter region was shown. Cross-linked DNA from C2C12 cells at GM, 6h, 1d and 2d were subjected to ChIP assays using anti-IgG and MyoD antibodies. The ChIP DNA was analyzed by qPCR using specific primers targeting to mouse *SH2B1* promoter region. (I) Schematic model of how SH2B1 regulates myogenesis. (i) In undifferentiated state, interaction between SH2B1 and histone H1 maintains the condensed chromatin state of *IGF2* and *MYOG* genes. (ii) During myogenesis, SH2B1 removes histone H1 away from the region of myogenic genes and places histone H1 on the regions that should be condensed. (iii) As a result, at the enhancer/promoter of *IGF2* and *MYOG*, H3K9me3 is decreased; H3K4me3 is elevated and local chromatin opens, recruiting of MyoD and thus activates *IGF2* and *MYOG* genes. Data in (C)-(E) and (G) were normalized to IgG and shown as fold enrichment; data in (C) was presented as relative level in 2d compared to GM (2d/GM); data in (E) were normalized to shLacZ at GM. Values are means ± S.E.M from at least three independent experiments, except that (C) was from one and (D) from two experiments. *: $P<0.05$, paired Student's *t*-test.

4. Discussion

The eukaryotic DNA organizes into chromatin making use of histones as structural components. While significant amount of work have investigated the role of histone H3.3 and H3.1 during transcriptional activation, the function of histone H1 is under-studied. Although the *in vitro* and *in vivo* data have not been completely consistent, the mainstream belief suggests that histone H1-mediated chromatin compaction reduces accessibility of transcription factors to the promoters/enhancers [44-47]. In addition, histone H1 was shown to regulate histone H3 modifications through several histone H3 modifiers [48]. Nonetheless, the exact interplay among histone H1 deposition, H3 modifications, and transcription factor accessibility to chromatin is not clear. Histone chaperones can escort different histone variants for dynamic distribution, and thus define chromatin landscapes for cell fate determination. Considering there are seven isoforms of H1 in somatic cells, it seems logical that additional chaperones are yet to be identified. The current study provides evidence that the scaffolding protein SH2B1 is required for global chromatin condensation, removal of histone H1, histone H3 modifications and transcriptional activity of MyoD binding during myogenesis. Histone H1 chaperones would regulate distribution of histone H1 to nucleosomes and help with constructing the suitable chromatin structure [47]. A known histone H1 chaperone, SET or TAF-I, binds to histone H1, regulates the level of histone H1 and the chromatin structure at the promoter of *interferon (IFN)-stimulated gene 15* [49]. The fact that binding of SH2B1 to histone H1 is required for the removal of histone H1 from myogenic genes raises an intriguing possibility that SH2B1 is a potential H1 chaperone. SH2B1 interacts with histone H1 at undifferentiated state to maintain the compacted chromatin at the enhancer/promoter of *IGF2* and *MYOG*. In response to differentiation signal, SH2B1 removes histone H1 from region of myogenic genes, allowing the access of histone modifiers and transcription factors to these regions, and then deposit H1 to regions that need to be condensed. By doing so, at the enhancer/promoter of *IGF2* and *MYOG*, histone H3K9me3 modification is decreased, H3K4me3 modification is increased, and local chromatin opens, leading to increased MyoD occupancy and activation of myogenic genes (Fig. 5I). The distribution of

histone H1 was positively correlated to the H3K9me3 in mouse and human embryonic stem cells [40, 41]. Our data also showed that knock-down of SH2B1 increased the level of H3K9me3 and histone H1 at the promoter and/or enhancer regions of *IGF2* and *MYOG* (Figs. 4F, 4G and 5E). These findings suggest that, without SH2B1, histone H1 cannot be removed and thus interferes with the changes of H3K9me3, possibly through preventing the access of lysine demethylase 4 (KDM4) family members. We showed that SH2B1 preferentially bound to histone H1a (Supplementary Fig. S3C). A previous study showed that inhibition of transcription elongation factor b (P-TEFb) complex resulted in de-phosphorylation of histone H1a, prevented removal of histone H1 from the promoter of myogenic genes and reduced myogenesis [50]. Thus, it is possible that SH2B1 participates in phosphorylation of histone H1a and thus promotes the removal of histone H1a from myogenic gene region.

SH2B1 is required for MyoD activity, enhancer occupancy, and the expression of *IGF2* but not *IGF1* (Fig. 4). These results are in line with a study showing that overexpressing MyoD increased the expression of *IGF2* but not *IGF1* expression [42]. While promoters of *IGF1* and *IGF2* do not contain MyoD binding sites, according to the ENCODE database and published results [31], the enhancer region of *IGF2* contains at least two known MyoD binding sites. Thus, the differential regulation of SH2B1 on the expression of *IGF2* versus *IGF1* may result from enhancer regulation and depend on MyoD binding.

Given that SH2B1 regulates the expression of *IGF2* through promoting MyoD binding activity, we asked whether SH2B1 could rescue the myogenesis defect of RD cells. RD cells are originated from human rhabdomyosarcomas (RMS) that is common sarcoma in children cancer. RD cells fail to differentiate into myotubes in part due to the poor transcriptional activity of MyoD [51]. To this end, RD cells were transfected with GFP or GFP-SH2B1β, and the percentages of MyHC-positive cells and levels of MyHC were analyzed at 4d after differentiation. Overexpressing SH2B1β enhanced the differentiation of RD cells at 4d (Supplementary Fig. S4). Limited by the poor differentiation property of RD cells, the enhancement is not dramatic but significant. While our findings suggest that SH2B1 regulates transcriptional activity of

MyoD, the enhancement of SH2B1 on the 1.5kb *MYOG* promoter-luciferase was higher than that for the four E box-luciferase (Figs. 4I and 5F). The 1.5kb *MYOG* promoter contains only two MyoD binding sites. Thus, SH2B1 potentially affects other transcriptional components that bind to the *MYOG* promoter region. In line with this possibility, in C2C12-shSH2B1 cells, *MYOG*-Luc activity was reduced significantly in the absence of MyoD overexpression (Fig. 5F). In addition to the promoter region, MyoD also bound to the enhancer (-4.5 kb and -6.5 kb) of *MYOG* at 2d. Based on the ENCODE database [52], MyoD binds to -6.5 kb at 1d and 5d but only binds to -4.5 kb at 1d. These data indicate that the E-box at the -6.5 kb region might be the major enhancer that contributes to the *MYOG* expression during myogenesis and the E-box at the- 4.5 kb region may contribute to early expression of *MYOG*. SH2B1 mainly regulates MyoD binding on both the promoter and the -6.5 kb of the enhancer regions (Fig. 5G).

SH2B1 has been shown to participate in neurogenesis and adipogenesis [23, 24]. However, the regulation of *SH2B1* gene and protein levels has not been characterized. The level of SH2B1 mRNA was increased 3-fold during myogenesis whereas the protein level was increased much higher (Fig. 1). This result raises a possibility that, in addition to being a target of MyoD (Fig. 5H), SH2B1 may be subjected to post-translational regulation to promote its own protein stability during myogenesis. Knockout mice of SH2B1 became obese, developed insulin-resistant and then diabetes with changed energy expenditure [27]. These physiological outcomes could in part result from reduced muscle mass, impaired muscle maintenance and regeneration due to lack of SH2B1.

5. Conclusions

Together, our findings suggest that SH2B1 is required for configuring global and regional chromatin structures, epigenetic modifications and MyoD binding to promoter/enhancer of *IGF2* and *MYOG*, during myogenesis

Authors' contribution

L.C., K.W.C., and C.F. K. conceived and designed the experiments. K.W.C., Y.J.C., Y.L.L., and C.M.Y. performed the experiments. L.C., K.W.C., and M.W.Y.C. analyzed the data. L.C. and K.W.C. wrote the manuscript.

Acknowledgements

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

- SH2B1 fine-tunes global and regional chromatin structures and promotes myogenesis.
- SH2B1 regulates epigenetic modification and enhances MyoD transcriptional activity to control expressions of myogenic genes.
- Identify a novel distal E-box (-6.5k) MyoD binding site for *MYOG* gene.
- SH2B1 binds to histone H1 and is required for the removal of histone H1 at *IGF2* and *MYOG* promoters.
- *SH2B1* is a novel target of MyoD.