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The histone demethylase KDM4B interacts with MyoD to regulate myogenic differentiation in C2C12 myoblast cells

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ABSTRACT

Enzymes that mediate posttranslational modifications of histone and nonhistone proteins have been implicated in regulation of skeletal muscle differentiation. However, functions of histone demethylases that could counter the actions of H3-K9 specific histone methyltransferases remain still obscure. Here we present evidences that KDM4B histone demethylase regulates expression of myogenic regulators such as MyoD and thereby controls myogenic differentiation of C2C12 myoblast cells. We demonstrate that expression of KDM4B gradually increases during myogenic differentiation and depletion of KDM4B using shRNA results in inhibition of differentiation in C2C12 myoblast cells, which is correlated with decreased expression of MyoD and myogenin. In addition, we find that KDM4B shRNA represses expression of MyoD promoter-driven luciferase reporter and exogenous expression of MyoD rescues myogenic potential in KDM4B-depleted myoblast cells. We further show that KDM4B interacts with MyoD, binds to MyoD and myogenin promoters in vivo, and finally, is involved in demethylation of tri-methylated H3-K9 on promoters of MyoD and myogenin. Taken together, our data suggest that KDM4B plays key roles in myogenic differentiation of C2C12 cells, presumably by its function as a H3-K9 specific histone demethylase.

1. Introduction

Differentiation of myogenic progenitor cells during embryonic development requires a series of events that are coordinated by a number of cell-type specific and ubiquitous transcription factors. Among those are myogenic regulatory factors (MRFs) that initiate and maintain myogenic differentiation program by orchestrating tightly controlled expression of genes required for skeletal muscle formation [1]. MyoD, a member of MRF family, is considered as a master regulator of myogenesis because of its ability to initiate myogenic differentiation program even in non-muscle lineage through activating muscle specific genes and another MRFs called myogenin [2,3]. Expression of MyoD in myoblast cells is tightly regulated to prevent premature differentiation of myoblast cells into myofibers and at the same time to maintain myogenic potential. Previous studies have shown that expression of endogenous MyoD is positively autoregulated by MyoD itself but also negatively regulated by transcription factors such as Msx1 [4–6].

In addition to MRFs, roles of histone modifying enzymes that mark local chromatin structure have been implicated in skeletal muscle differentiation [7]. Evidences suggest that acetylation levels of MyoD and histones on MyoD-responsive genes, which are determined by the actions of histone acetyltransferase (HAT) and histone deacetylase (HDAC), are correlated with myogenic differentiation [8,9]. Similarly, enzymes that determine levels of methylation of histones have also been shown to regulate myogenic differentiation. SET7 and SETD3, which can methylate H3-K4 and often mark actively transcribed region, interact with MyoD to target muscle specific genes and thereby promote differentiation of myoblast cells [10,11]. In contrast, Suv39h1 that target H3-K9 and the polycomb protein Ezh2 that mediates methylation of H3-K27 inhibit myogenic differentiation by repressing muscle specific genes, while another H3-K9 specific histone methyltransferase (HMTase) G9a targets nonhistone substrates such as MEF2D and MyoD to inhibit the formation of differentiated myofibers [12–15]. Actions of HMTases on muscle specific genes are known to be countered by histone demethylases (KDMs). UTX, an H3-K27 specific demethylase, counteract Ezh2 and LSD1 reverses the action of G9a on MEF2D, leading to the differentiation of C2C12 cells [14,16]. However, enzymes that mediate demethylation of tri-methylated H3-K9 in myogenic differentiation has not been tested extensively.

KDM4/JMJD2 histone demethylases (KDM4A, B, C, and D) are jumonji domain-containing enzymes that mediate demethylation of tri-methylated H3-K9 as well as H3-K36 [17–20]. The KDM4 family of proteins is implicated in many cellular processes

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including transcription, cell cycle regulation, and cellular differentiation [21]. KDM4C is preferentially expressed in undifferentiated ES cells. Its deletion affects global levels of di- and tri-methylated H3-K9 and leads to morphological change in ES cells [22]. In addition, loss of KDM4A results in down-regulation of genes for neural crest specification [23]. Recently, an isoform of KDM4A, which lacks N-terminal domain of KDM4A, has been shown to target myogenin to regulate skeletal muscle differentiation [24]. However, roles of other KDM4 histone demethylases in myogenic differentiation remain largely unknown. In this study, we investigated roles of KDM4 histone demethylases in myogenic differentiation by using stable cell lines expressing shRNAs specific to each member of KDM4 family of genes. We have found that KDM4B, which interacts with MyoD to regulate expression of myogenic regulatory factors including MyoD gene itself, plays critical roles in the differentiation of C2C12 myoblast cells.

2. Materials and methods

2.1. Plasmids

The plasmid containing full-length mouse KDM4B gene was purchased from Korea human gene bank (mMU001161). Coding sequences for KDM4B was amplified with a forward primer containing *BamH1* restriction site and a Flag epitope tag and a reverse primer with *XhoI* restriction site. Amplified products were digested with *BamHI* and *XhoI* and cloned into pcDNA3 vector and pLZRS-IRS-GFP retroviral vector. The MyoD expression vector was kindly provided by Dr. K.Y. Lee (Chonnam National University, Korea). The complete sequences of all PCR-amplified constructs were confirmed. The plasmid (pMyoD-luciferase containing F3/-2.5 fragment and *MyoD* promoter) used in reporter assay has been previously described [6].

2.2. Cell cultures, retroviral gene transfer and in vitro differentiation assays

Human 293T cells, mouse C2C12 myoblast cells, and mouse C3H 10T1/2 pluripotent mesenchymal cells were maintained in DMEM (WelGENE) supplemented with 10% fetal bovine serum (WelGENE) and antibiotics in humidified atmosphere with 5% CO_2 at 37 °C. Retroviral gene transfer and in vitro differentiation of C2C12 myoblast cells were carried out as previously described [6].

2.3. Establishment of stable cell lines expressing shRNA

C2C12 cells were grown to 30% confluency in 60 mm plates in DMEM supplemented with 10% fetal bovine serum and transfected with 5 μg of pLKO.1 (control) or pLKO.1 containing shRNA against mouse KDM4A, KDM4B, KDM4C, and KDM4D genes (purchased from Open Biosystems). Transfected cells were treated with puromycin (5 μg/ml) for 2 weeks and puromycin-resistant cells were tested for endogenous expression of KDM4 genes by quantitative real time RT-PCR. For each KDM4 family of genes, at least two shRNAs per each KDM4 genes were examined for their roles in myogenic differentiation. Established cells were maintained in the presence of 2.5 μg/ml of puromycin for further study. Sequence information of KDM4 shRNAs we used in this study can be found at http://www.broadinstitute.org/rnai/public/clone/search with the following IDs; KDM4A: TRCN0000103525 ~ TRCN0000103529, KDM4B: TRCN0000103535 ~ TRCN0000103539, KDM4C: TRCN0000103550 ~ TRCN0000103554, and KDM4D: TRCN0000103540 ~ TRCN0000103544.

2.4. Cell proliferation assay

To measure relative cell growth, 2×10^3 cells were seeded in 24-well plates and cultured in a CO_2 incubator at 37 °C. Cells were fixed with 10% formalin (Sigma) at indicated time points and stained with 0.1 % naphthol blue black (NBB) staining solution containing 9% acetic acid and 0.1 M sodium acetate for 30 min. After staining, cells were extracted with 200 μl of 50 mM NaOH and absorbance of each sample was measured at 595 nm. Experiments were performed at least three times with triplicate for each time point.

2.5. Western blot analysis, immunofluorescence and immunoprecipitation

Western blot, immunofluorescence, and immunoprecipitation assays were performed as previously described [6]. Antibodies used for Western blot analysis were monoclonal anti-Flag M2 (Sigma–Aldrich), monoclonal anti-Myc [6], monoclonal anti-Actin (Sigma), and monoclonal anti-myosin heavy chain (anti-MHC) (Developmental hybridoma). For immunofluorescence, monoclonal anti-MHC (Developmental hybridoma), and Alexa 488-conjugated secondary antibody (Molecular Probes) were used. Immunofluorescence was visualized with an Olympus IX71 inverted microscope equipped with U-RFL-T burner and TH4-200 halogen lamp. All images were taken with 20× objective and processed using Adobe Photoshop premium suite CS3 software.

2.6. Quantitative reverse transcription-PCR (qRT-PCR)

For quantitative reverse transcription PCR (qRT-PCR) analysis, total RNA was first isolated from C2C12 cells by using TRIzol solution (Invitrogen) and then further purified with RNeasy mini kit (Qiagen) following treatment with DNaseI (Promega) to eliminate genomic DNA. The cDNA synthesis was carried out by using superscript II cDNA synthesis kit as recommended by manufacturer (Invitrogen). Real-time PCR was carried out on ABI 7300 real-time PCR system (Applied Biosystems) with SYBR Green I and iTaq polymerase (Intron). Relative expression was first quantified using standard curve method and data were normalized to *GAPDH* mRNA. Primers for MyoD, myogenin, and GAPDH genes are previously described [6] and primer sets for KDM4 genes are as follows. KDM4A; 5′ – GAGGAAGACTGCTGCTTATGCTC – 3′ and 5′ – TCACATCCACTGGACTTCTTTCA – 3′, KDM4B; 5′ – TGCAGTCCCTGAGGTACGATT – 3′ and 5′ – CCCGACACTCTCTTCATACGC – 3′, KDM4C; 5′ – CCTTCAGCAGAGACACATTTCCT – 3′ and 5′ – TCCAAGATACTTTGCCCCATAGA – 3′, KDM4D; 5′ – TTCAAGACCTATTGGAACAGGAA – 3′ and 5′ – TGCCCAAAGTGTAGGTAGTTGAT – 3′.

2.7. Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) analysis was performed as previously described [6]. Briefly, sonicated chromatins were incubated with anti-Flag M2 agarose (Sigma–Aldrich) or anti-trimethyl H3-K9 antibody (Abcam). Immunoprecipitated DNA was analyzed by real-time PCR and relative enrichment was quantified as previously described [5,6]. Primer sets to amplify MyoD proximal regulatory region (PRR), MyoD core enhancer region (CER) and Actin are previously described [6] and primers to amplify MyoD DRR, myogenin promoter (−185 ~ −1), and myogenin distal region (−1565 ~ −1447) are as follows. MyoD DRR; 5′ – ACTAGCCAAGGGAGCTGAAATG – 3′ and 5′ – ACTGACCTGGAGAAGCACACAG – 3′, myogenin promoter; 5′ – CGGGGTACCCTGCAGGGT GGGGTGGGGGCAAAAGGAGAG – 3′ and 5′ – CCGCTCGAGGCCCCTCACGCCAACTGCTGGGTG – 3′, and myogenin distal; 5′ –

CGGGGTACCATTCTAGAGTTGTATGACGCAGGC – 3′ and 5′ – GGTCGATAAGGAGAAAGAG – 3′.

2.8. Reporter assay

C2C12 cells were grown to 50% confluency in 6-well plates in DMEM with 10% fetal bovine serum and transfected with 0.4 μg of pMyoD-luciferase containing F3/-2.5 fragment and *MyoD* promoter, 0.2 μg of pSV-β-Gal and 0.8 μg of KDM4B shRNA expression vector and/or 0.6 μg of myc-MyoD expression vector. For Fig. 3F and G, 0.6 μg of Flag-KDM4B expression vector was used for transfection. The total amount of plasmid DNA was adjusted to 2.0 μg by adding empty vector (pcDNA3). Cells were harvested at 24 h after transfection and analyzed for luciferase activity as recommended by manufacturer (Promega).

3. Results and discussion

3.1. KDM4 genes are differentially expressed during C2C12 myoblast differentiation

In order to investigate roles of KDM4 histone demethylases in myogenic differentiation, we first examined their expression in C2C12 myoblast cells and compared it with other cells with different differentiation potentials (Fig. 1A). We found that steady state levels of KDM4 mRNAs are significantly higher in C3H 10T1/2 pluripotent mesenchymal cells, which can be differentiated into not only muscle cells but also other mesenchymal cell types such as adipocytes and chondrocytes. On the other hand, NIH3T3 cells that are known to lack myogenic potential express KDM4 genes modestly but consistently lower than C2C12 myoblast cells. Next, we monitored dynamics of KDM4 expressions during C2C12 myoblast differentiation (Fig. 1B–E). Quantitative real-time RT-PCR analysis revealed that KDM4A, C, and D display similar expression patterns during differentiation. Their expressions are increased upon differentiation but gradually decreased as differentiation progress to form terminally differentiated myotubes (Fig. 1B, D, and E). Of particular interest, expression of KDM4B continuously increased during differentiation (Fig. 1C). These data demonstrate that KDM4 genes are differentially expressed during myogenic differentiation of C2C12 cells.

3.2. KDM4 histone demethylases are involved in regulation of myogenic differentiation

Next, we have established stable C2C12 myoblast cell lines that express shRNA against each of 4 KDM4 histone demethylases and tested for myogenic differentiation (Fig. 2A and B). While C2C12 cells with decreased KDM4A expression appeared to differentiate normally, cells depleted of KDM4B or KDM4C showed delayed myogenic differentiation, of which KDM4B depletion resulted in more severe delay in differentiation (Fig. 2B and C). In contrast, effect of KDM4D depletion on myogenic differentiation seemed to be opposite to those of KDM4B and KDM4C as cells with decreased KDM4D expression not only form more myotubes in serum-deprived condition but also show higher level of MHC expression than control C2C12 cells (Fig. 2B and C). These data suggest that KDM4 family of histone demethylases might be involved in regulation of myogenic differentiation in both positive and negative ways.

3.3. KDM4B is required for the differentiation of C2C12 myoblast cells

As shown in previous data, effect of KDM4B depletion on myogenic differentiation is more severe than other members of KDM4 demethylase family in C2C12 myoblast cells (Fig. 2). So we further investigated roles of KDM4B in myogenic differentiation by using immunofluorescence and subsequently measuring myogenic index (Fig. 3A). Consistent with previous results (Fig. 2B and C), myoblast cells depleted of KDM4B resulted in much less number of myotubes (Fig. 3A, left) as well as lower myogenic index at each time point after initiation of differentiation (Fig. 3B, right). Since shRNA used for KDM4B depletion targets 3′-UTR of KDM4B mRNA, we next tested whether KDM4B lacking 3′-UTR could rescue myogenic differentiation. Exogenous expression of KDM4B lacking 3′-UTR resulted in modest but significant expression of myosin heavy chain compared to KDM4B-depleted cells infected with control retrovirus (Fig. 3B). Next, we analyzed expressions of myogenic

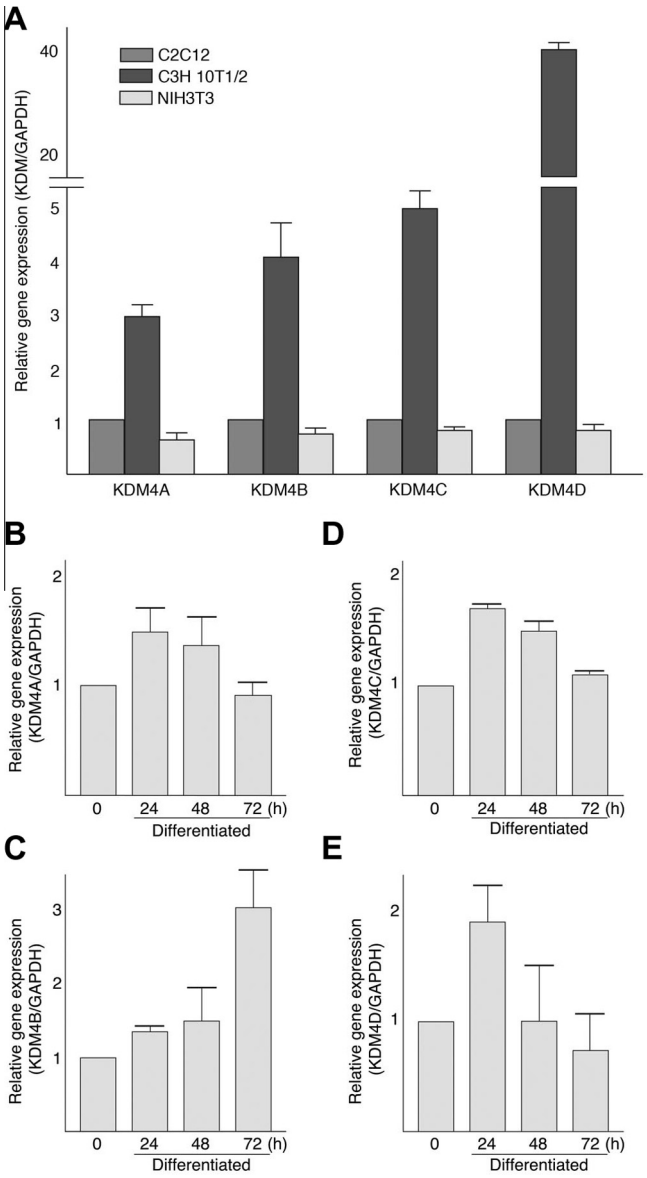

Fig. 1. KDM4 histone demethylases are differentially expressed during differentiation of C2C12 myoblast cells (A) Cells were grown to become sub-confluent in DMEM supplemented with 10% fetal bovine serum and total RNAs were prepared as described in Materials and methods. Expressions were quantified by quantitative RT-PCR using primers specific for indicated KDM4 family of genes. Relative expression was determined using standard curve method and then normalized to GAPDH. Error bars indicate standard deviation. (B–E) C2C12 myoblast cells were grown to become confluent and differentiation was induced by serum withdrawal. Cells were harvested at indicated time points and relative expressions of KDM4 histone demethylases were analyzed by using quantitative RT-PCR.

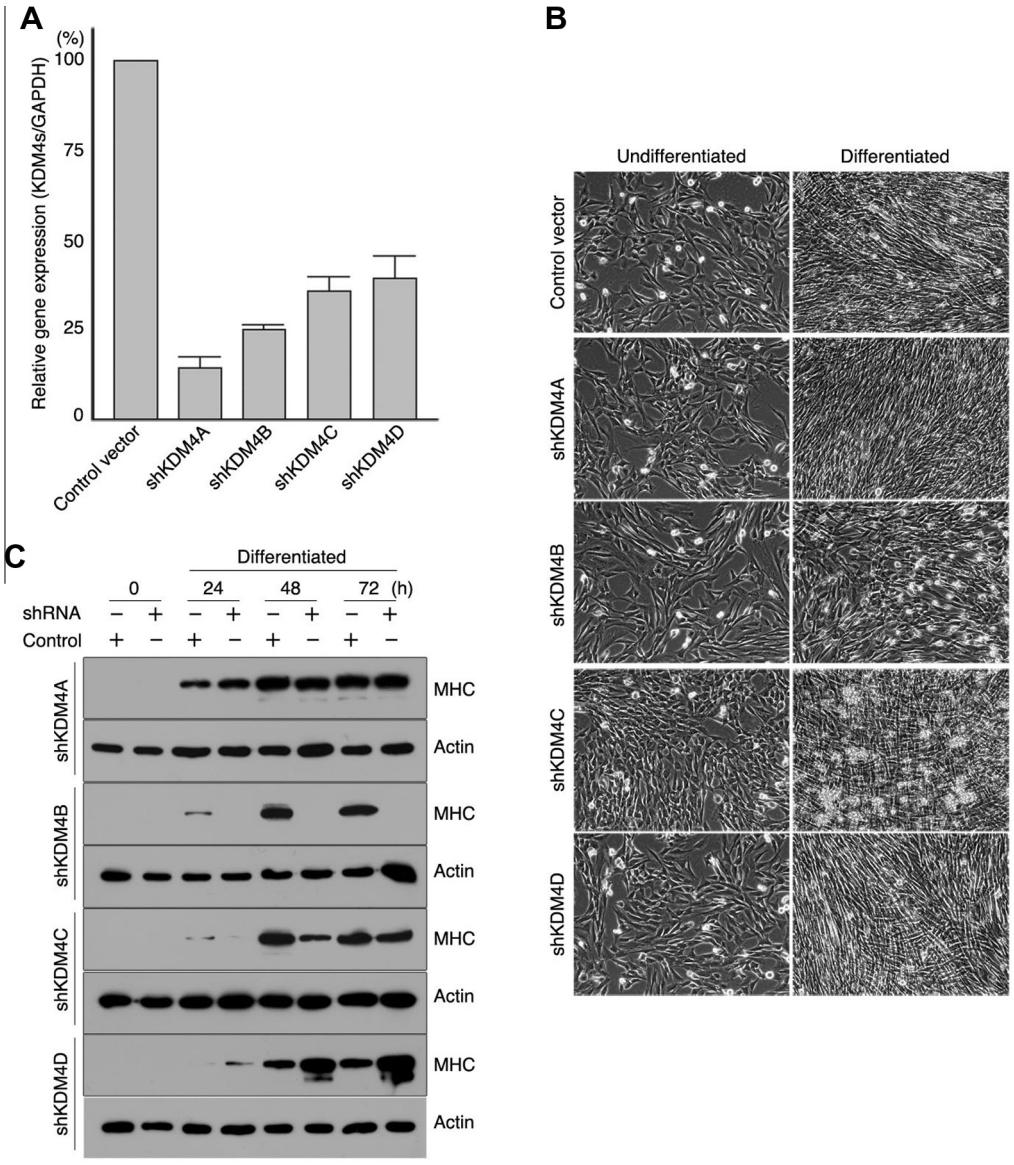

Fig. 2. KDM4 family of histone demethylases was involved in myogenic differentiation. (A) C2C12 myoblast cells that express shRNA against each of 4 KDM4 histone demethylases (KDM4A, 4B, 4C, and 4D) were established as described in Materials and methods. Levels of endogenous KDM4 genes in established cell lines were compared with control C2C12 myoblast cells by using quantitative RT-PCR. Experiments were performed at least three times for each cell lines. Error bars indicate standard deviation. (B and C) Effect of KDM4 depletion on differentiation of C2C12 myoblast cells was assessed by the appearance of terminally differentiated myotubes 72 h after serum withdrawal (B) and levels of myosin heavy chain at indicated time points (C). Shown are representative data obtained from at least three independent experiments.

regulatory factors in KDM4B-depleted C2C12 cells (Fig. 3C and D). Quantitative RT-PCR analysis revealed that steady state levels of MyoD mRNA as well as myogenin mRNA are significantly lower in KDM4B-depleted myoblast cells than control C2C12 cells even prior to differentiation. Interestingly, depletion of KDM4B did not seem to inhibit differentiation-dependent increases in expressions of MRFs, suggesting that effect of KDM4B depletion on myogenic differentiation is to delay it rather than completely block it (Fig. 3C and D). While depletion of KDM4B severely delayed myogenic differentiation as shown in previous data, it did not promote cell proliferation. Rather, depletion of KDM4B led to modestly slower cell proliferation than control C2C12 cells (Fig. 3E).

In contrast to the inhibitory effect of KDM4B depletion on myogenic differentiation, overexpression of KDM4B in normal C2C12 myoblast cells appeared to have little or no effect on differentiation up to 72 h in differentiation medium (Fig. 3F). Consistently, we were not able to observe any significant changes in levels of MyoD and myogenin mRNAs by the expression of KDM4B prior to and after induction of differentiation (Fig. 3G and H), suggesting that overexpression of KDM4B, at least in our experiments, did not perturb myogenic differentiation in normal C2C12 cells.

3.4. KDM4B interacts with MyoD to maintain myogenic potential of C2C12 myoblast cells

It is quite intriguing that depletion of KDM4B leads to the decreased level of endogenous MyoD in proliferating myoblast cells (Fig. 3C). So we next tested whether KDM4B acts on MyoD promoter by using MyoD-luciferase reporter plasmid (Fig. 4A and B). We found that transfection of KDM4B shRNA resulted in repression of MyoD-luciferase reporter in a dose dependent manner in C2C12 myoblast cells (Fig. 4A). Similar patterns of inhibition by KDM4B shRNA can be observed in C3H 10T1/2 cells, which unlike C2C12 cells does not express endogenous MyoD (Fig. 4B), implying

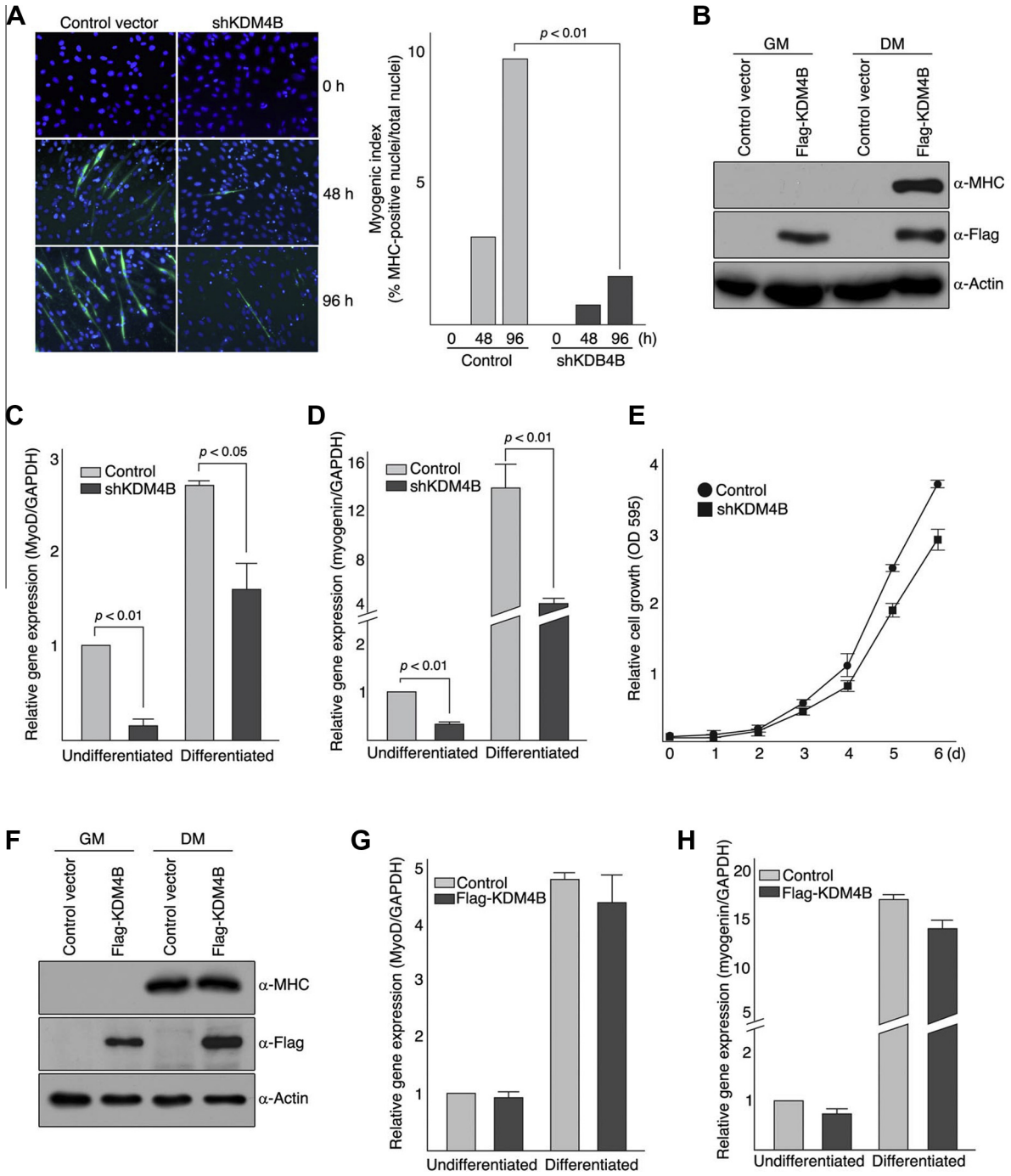

Fig. 3. Depletion but overexpression of KDM4B perturbs myogenic differentiation. (A) Cells were fixed at indicated time points after serum withdrawal and incubated with anti-MHC antibody followed by Alexa 488 conjugated secondary antibody. Immunofluorescence was visualized as described in Materials and methods (left). Myogenic index was calculated by % MHC-positive nuclei in myotubes per total nuclei (right). (B) Exogenous expression of KDM4B rescues myogenic differentiation in KDM4B-depleted C2C12 cells. KDM4B-depleted cells infected with retrovirus expressing Flag-KDM4B or control vector were harvested before and after serum withdrawal. Differentiation was assessed by the expression of myosin heavy chain. (C and D) Expressions of endogenous MyoD (C), and myogenin (D) were measured by quantitative RT-PCR as described in Materials and methods. (E) Knockdown of KDM4B has modest effect on cell growth. 2×10^3 cells stably expressing KDM4B shRNAs or control vector (pLKO.1) were grown in 24-well plates, harvested at indicated time points. Relative cell growth was measured by using NBB staining. (F–G) Overexpression of exogenous KDM4B does not promote myogenic differentiation. C2C12 myoblast cells were infected with retroviruses expressing Flag-KDM4B. Empty vector (pLZRS-IRES-GFP) was used as a control. After two consecutive days of infection, cells were harvested before or 72 h after initiation of myogenic differentiation. Myogenic differentiation was assessed by the expression of myosin heavy chain (F), MyoD (G), and myogenin (H) using Western blot analysis and quantitative RT-PCR, respectively. All experiments were performed at least three times with triplicate for each time point. Error bars indicate standard deviation.

that KDM4B is required for maintaining the level of MyoD expression in cells. In contrast, cotransfection of Flag-KDM4B and MyoD seemed to have no significant effect on the expression of MyoD-luciferase reporter (data not shown).

If inhibition of myoblast differentiation observed in KDM4B-depleted cells is caused by decreased level of endogenous MyoD, it is possible that exogenous expression of MyoD that is independent of MyoD promoter restore the myogenic potential in cells

depleted of KDM4B. Indeed, we found that retroviral infection of MyoD is sufficient for initiating myoblast differentiation in KDM4B-depleted cells, supporting the idea that KDM4B acts on MyoD promoter to maintain the level of endogenous MyoD (Fig. 4C). Next, we investigated whether KDM4B physically interacts with MyoD as MyoD is known to regulate its own expression. As shown in Fig. 4D, KDM4B could interact with MyoD in cells and moreover, we found that KDM4B binds to the promoters of MyoD and myogenin that contain MyoD target site (E box). However, no enrichment was observed in other regulatory elements of MyoD (DRR and CER) or distal region of myoenin promoter (Fig. 4E and F). Consistently, we observed that depletion of KDM4B led to increase in tri-methylation of H3-K9 on histones of MyoD proximal regulatory region (PRR) and myogenin promoter (Fig. 4G).

In summary, our present study identifies KDM4B histone demethylase as a regulator of myogenic differentiation in C2C12 myoblast cells. Differential expression during differentiation and inhibited differentiation by KDM4B depletion suggest its involvement in differentiation. Of particular interest, depletion of KDM4B resulted in significant decrease in steady state level of MyoD in proliferating myoblast cells and exogenous expression of MyoD as well as KDM4B rescued myogenic differentiation in cells depleted of KDM4B, indicating that function of KDM4B in myogenic differentiation is related to maintaining the level of key myo-

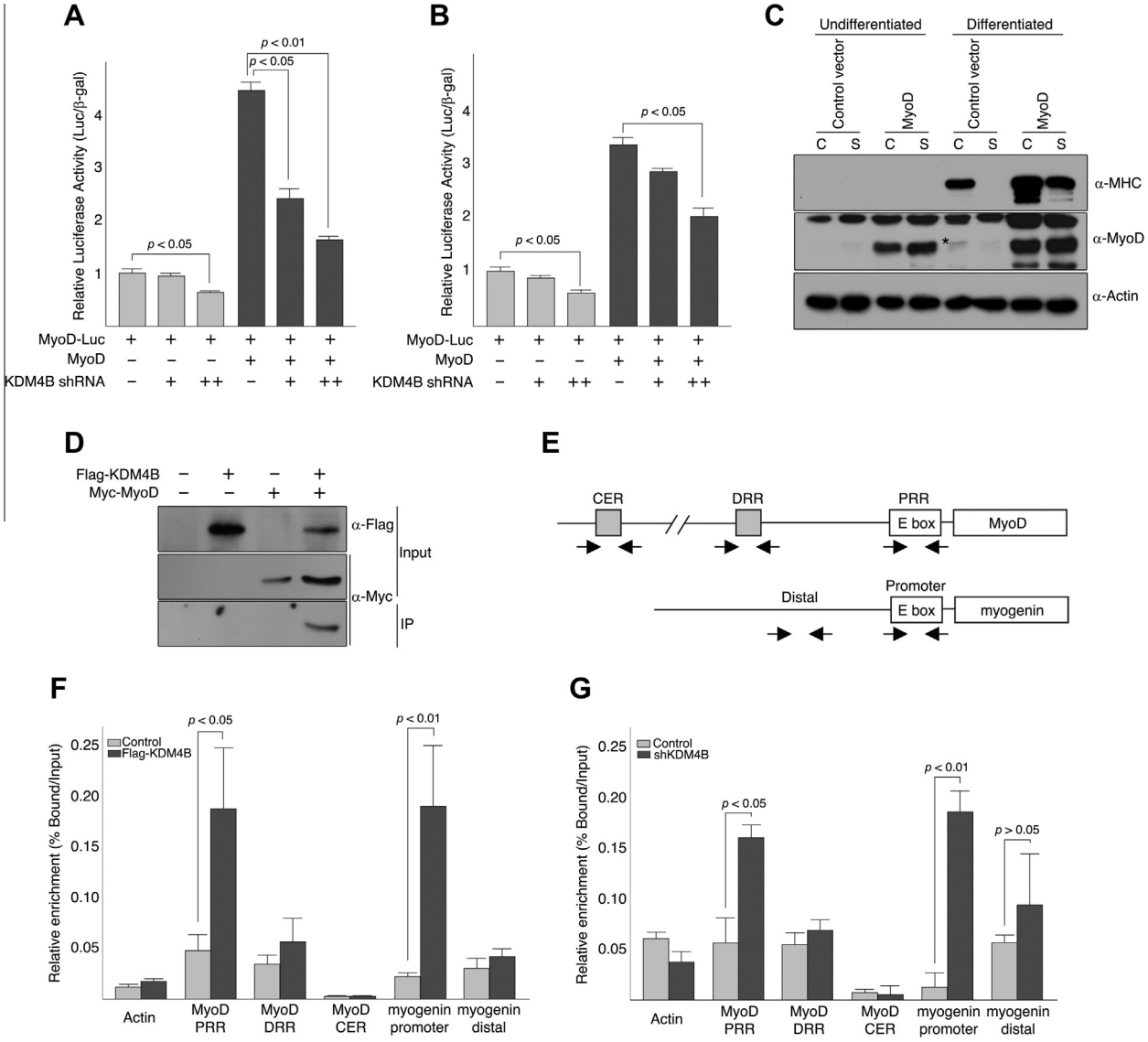

Fig. 4. KDM4B interacts with MyoD to regulate expression of MRFs in C2C12 cells. C2C12 myoblast cells (A) or C3H 10T1/2 mesenchymal cells (B) were transiently transfected for 24 h with plasmids expressing myc-MyoD and/or KDM4B shRNA together with a MyoD-luciferase reporter plasmid. For all reporter analysis, empty vector (pcDNA3) was added to adjust total amount of transfected DNA to 2.0 μg. Data are presented as relative luciferase activity to the control (empty vector); Shown are representative data of three independent experiments in triplicate, error bars indicate standard deviation. (C) Exogenous expression of MyoD rescues myogenic differentiation in KDM4B-depleted C2C12 cells. C2C12 cells expressing KDM4B shRNA (S) or normal C2C12 cells (C) were infected with retrovirus expressing MyoD. Empty vector was used as a control. Differentiation was assessed by myosin heavy chain expression using Western blot analysis. Single asterisk (*) shown in the middle panel indicates endogenous MyoD. (D) KDM4B interacts with MyoD. Human 293T cells were transfected with plasmids expressing Flag-KDM4B and/or myc-MyoD. Interactions were analyzed by immunoprecipitation and visualized by Western blot analysis following SDS–PAGE. (E) Schematic representation of MyoD (upper) and myogenin (lower) genes with amplicons analyzed by ChIP and quantitative RT-PCR. (F) Flag-KDM4B binds to promoters of MyoD and myogenin and (G) depletion of KDM4B in myoblast cells results in enrichment of tri-methylated H3-K9 on histones at MyoD and myogenin promoters. Chromatin Immunoprecipitation (ChIP) was done in C2C12 cells infected with retroviruses expressing Flag-KDM4B (F) or C2C12 cells depleted of KDM4B (G) along with control C2C12 cells. The empty vector (pLZRS-IRES-GFP) was used as a control. Immunoprecipitated DNA was analyzed by quantitative RT-PCR with specific primer sets described in Materials and methods. Shown are representative data of three independent experiments.

genic regulators such as MyoD and myogenin. This is also supported by our data showing physical interaction of KDM4B with MyoD, binding of KDM4B to the promoters of MyoD and myogenin, and the decreased demethylation of tri-methylated H3-K9 on histones of those promoters.

While expression of MyoD is important for determining myogenic potential in cells, studies have shown that level of MyoD is also important as it is believed to determine the timing and rate of differentiation in myoblast cells [3,25]. Recently, histone modifying enzymes have been suggested as regulators that determine the timing of myogenic differentiation because of their activities to mediate reversible modifications of histones and thereby regulate transcription of muscle-specific genes. Functions of histone acetyltransferases (HAT) and histone deacetylases (HDAC) are well described and suggested as a mechanism for initiation of myogenic differentiation [8,9]. In addition, an H3-K27 specific histone methylase Ezh2 and its counterpart UTX histone demethylase are also implicated in muscle differentiation [12,16]. However, histone demethylase that can counter the action of Suv39h1, which mediates tri-methylation of H3-K9 and inhibits myogenic differentiation, has not been reported [13]. Considering that KDM4B demethylates tri-methylated H3-K9 and its role in C2C12 myoblast cells appear to be opposite to Suv39h1 in many ways, it is tempting to speculate that balanced actions of KDM4B and Suv39h1 play critical role in maintaining myogenic potential as well as preventing premature differentiation, which remains to be determined.

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