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Histone methyltransferase Setd2 is critical for the proliferation and differentiation of myoblasts

Xin Yi [a,b,1], Ye Tao [b,1], Xi Lin [a], Yuan Dai [b], Tingli Yang [b,1], Xiaojing Yue [c], Xuejun Jiang [a], Xiaoyan Li [a], Ding-sheng Jiang [d], Kelsey C. Andrade [b], Jiang Chang [b,*]

[a] Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, China
[b] Texas A&M University Health Science Center, Institute of Biosciences and Technology, Houston, TX 77030, USA
[c] Department of Obstetrics and Gynecology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China
[d] Division of Cardiothoracic and Vascular Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

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ABSTRACT

Skeletal muscle cell proliferation and differentiation are tightly regulated. Epigenetic regulation is a major component of the regulatory mechanism governing these processes. Histone modification is part of the epigenetic code used for transcriptional regulation of chromatin through the establishment of an active or repressive state for genes involved in myogenesis in a temporal manner. Here, we uncovered the function of SET domain containing 2 (Setd2), an essential histone 3 lysine 36 trimethyltransferase, in regulating the proliferation and differentiation of myoblasts. Setd2 was silenced in the skeletal muscle myoblast cell line, C2C12, using the CRISPR/CAS9 system. The mutant cells exhibited defect in myotube formation. The myotube formation marker, myosin heavy chain (MHC), was downregulated earlier in Setd2 silenced cells compared to wild-type myoblasts during differentiation. The deficiency in Setd2 also resulted in repression of Myogenin (MyoG) expression, a key myogenic regulator during differentiation. In addition to the myoblast differentiation defect, decreased proliferation rate with significantly reduced levels of histone 3 phosphorylation, indicative of cell proliferation defect, were observed in the Setd2 silenced cells; suggesting an impaired proliferation phenotype. Furthermore, compromised G1/S- and G2/M-phase transition and decreased expression levels of major regulators of cell cycle G1/S checkpoints, cyclin D1, CDK4, CDK6, and cyclin E2 were detected in Setd2 silenced cells. Consistent with the cell cycle arrested phenotype, cyclin-dependent kinase inhibitor p21 was upregulated in Setd2 silenced cells. Together, this study demonstrates an essential role of Setd2 in myoblast proliferation and differentiation, and uncovers Setd2-mediated molecular mechanism through regulating MyoG and p21.

1. Introduction

Myoblast proliferation and differentiation are two major components in myogenesis and skeletal muscle development. The process is a tightly regulated. Undifferentiated myoblasts undergo a multiple-step process including proliferation, cell cycle exit, differentiation, and fusion of mononucleated myocyte to form multinucleated myotubes. The process of myogenesis is precisely regulated in which transcriptional regulation plays crucial roles. Myogenic regulatory factors (MRFs), such as basic helix-loop-helix (bHLH) transcription factors MyoD [1], MyoG [2,3], Myf5 [4], and MRF4 [5–7] function coordinately at different stages of myogenesis. MyoD and Myf5 are required for the myogenic progenitors to differentiate into myoblasts - the myogenic cells, whereas MyoG is indispensable for the myoblasts to differentiate into myocytes and subsequent myotube formation. MRF4 is involved in both the commitment of myoblast lineage and the maintaining of differentiated myocyte state.

In addition to the transcriptional regulation, accumulating evidence has begun to reveal epigenetic factors as important regulatory components in myogenesis. Histone acylation, deacylation and methylation have been reported to be involved in the regulation of myogenesis. Histone methyltransferase families PcG (trimethylates histone 3 lysine 27 and introduces repressive H3K27me3 mark) and TrxG (trimethylates histone 3 lysine 4 and introduces activated H3K4me3 mark) have been reported to be critical to establish the chromatin state that permit or block essential genes' expression in myogenesis in a time-dependent manner (reviewed in Moresi v. et al [8]). Therefore, continual identification of new epigenetic regulatory mechanism is highly significant.

Histone 3 lysine 36 trimethylation (H3K36me3) is an essential histone modification that modulates the chromatin state to activate gene expression [9]. SET domain containing 2 (Setd2) is a major

* Corresponding author at: Texas A&M University Health Science Center, Institute of Biosciences and Technology, 2121 W. Holcombe Blvd., Houston, TX 77030.
E-mail address: jchang@ibt.tamhsc.edu (J. Chang).
[1] These authors contributed equally to this work.

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methyltransferase responsible for H3K36me3 [10]. Setd2 mutations have been detected in multiple human cancers [11–23]. In addition, Setd2-mediated RNA splicing regulation and impaired DNA repair have been reported in cancer studies [24–29]. Genetic silencing of *Setd2* resulted in severe vascular defects and embryonic lethality, suggesting an essential role of Setd2 in vascular genesis and mouse development [30]. Due to the large number of genes that are regulated by Setd2 through its ability to establish active chromatin state, and the complicated nature of myogenic regulation that requires master regulators to switch on the gene expression machinery, a role of Setd2 in regulating myogenesis could be possible.

In this study, Setd2 was silenced in the skeletal muscle myoblast model cell line C2C12 by CRISPR/CAS9 technique. We found that genetic silence of *Setd2* abolished myotube formation. Decreased MyoG and early downregulation of MHC, marker of myotube formation, were detected in the differentiating Setd2 knockout (KO) cells. In addition to the myoblast differentiation defect, Setd2 KO myoblasts also exhibited decreased proliferation rate and compromised G1/S and G2/M phase transition. Decreased expression levels of major regulators of cell cycle G1/S checkpoint, cyclin D1, CDK4, CDK6, and cyclin E2 were detected in Setd2 KO cells. In consistent with the cell cycle arrest phenotype, cyclin-dependent kinase inhibitor p21 was upregulated in Setd2 KO cells. Taken together, we report a new function of H3K36 trimethylase Setd2 in regulating the proliferation and differentiation of myoblasts, and suggest MyoG and p21 as potential downstream effectors of Setd2 in these processes.

2. Materials and methods

2.1. Generation of stable Setd2$^{-/-}$ mouse myoblasts by CRISPR/CAS9 genome editing

Crispr/CAS9 genome editing was performed following the protocol described in Ran, F.A. et al [31]. In brief, Setd2$^{-/-}$ stable mouse myoblasts were produced by transfection of C2C12 cells with plasmid expressing mouse Setd2 sgRNA and Cas9 nuclease (pSpCas9(BB)-2A-GFP (PX458), Addgene plasmid# 48138). The target sequence of mouse Setd2 was located at exon 3. After positive GFP cell detection, single-cell colonies were isolated by flow cytometry. The colonies were cultured for two weeks before individual genotyping was applied on each colony in order to screen for positive clones.

PCR products were generated from amplifying ~500 bp genomic regions that surrounding CRISPR/Cas9 mediated genomic editing locus. PCR products were sequenced and presence of superimposed traces (indels in chromatograms) indicated mutation in the genomic region. Sequences of both alleles of the genomic region were decoded by Indelligent software [32] followed by sequence analysis. Sequences with protein coding frame shift and premature stop codon shortly after the genomic editing locus were considered as silencing mutations. Cell colonies with both alleles of silencing mutation were considered as Setd2$^{-/-}$ C2C12 cell lines which were further verified by western blotting for the silencing of Setd2 protein.

Potential off-target sequences of Setd2 sgRNA were identified using the CRISPR design tool (http://tools.genome-engineering.org) against the mouse genomic sequence. We selected the top three potential off-target sites (score ≥ 0.3, number of mismatches ≤ 4) as our candidate off-target sites. For each candidate off-target site, we PCR-amplified an ~500 bp fragment centered on the candidate site from genomic DNA isolated from C2C12 WT cell line and from Setd2 KO cell line. The PCR products were sequenced and found no mutations at any of these sites.

The forward and reverse primers for Setd2 sgRNA are:

D001F forward primer: CACCG AATGAACTGGGATTCCGACG
D001R reverse primer: AAACCGTCGGAATCCCAGTTCATTC
For verification, the following primers were used:
On target
Forward primer: ATGATTCTGAACGGCGCTAC
Reverse primer: TGTTGCCACATCAGCTTCTG
Off target
OT1-F forward primer: CTCCTCCCTGCTGCTAGATG
OT1-R reverse primer: CCAAAGTGAGCAACCCTCAT work with OT1-F
OT2-F forward primer: GGGACATCCTCCAAAGCATA
OT2-R reverse primer: AACATGGATGCTGGGTTTTC work with OT2-F
OT3-F forward primer: GGGCTGTGCATATCAGGTCT
OT3-R reverse primer: CCCTAAGCCTTGCAGTACGA work with OT3-F

2.2. Cell culture, myoblast differentiation, and fusion index calculation

C2C12 mouse myoblasts (from ATCC) were cultured in either growth medium (GM) or differentiation medium (DM). To grow the C2C12 myoblasts and keep them from differentiation GM was used which consisted of Dulbecco's modified Eagle's medium (DMEM) and 20% fetal bovine serum (FBS). To make C2C12 cell undergo myogenic differentiation, the cells at 70% confluence were switched to DM which contained DMEM and 2% heat-inactivated horse serum. To measure fusion index, cells incubated in DM for 5 days were fixed in methanol and stained with antibody against myosin heavy chain (MHC) (MF-20, Developmental Studies Hybridoma Bank, which labels the myotubes), after which pictures were captured randomly at more than 5 different spots. For the obtained images, the number of nuclei within the myotubes and the total number of nuclei were counted using Adobe Photoshop software and the fusion index was calculated as the percentage of nuclei incorporated into myotubes vs. total number of nuclei. Images of myoblasts from differentiation were taken using phase-contrast microscopy.

2.3. Immunofluorescence assay and TUNEL staining

Cells on a glass coverslip were briefly fixed using 4% paraformaldehyde and permeabilized using phosphate-buffered saline containing 10% FBS and 0.2% Triton X-100. The cells were then treated with a blocking solution, phosphate-buffered saline with 5% BSA and 0.1% Triton X-100, before incubations with antibodies were performed. The following primary antibodies were used: MHC (MF-20, Developmental Studies Hybridoma Bank), Ki67 (9449, Cell Signaling). The secondary antibody used was goat anti-mouse IgG antibody coupled with Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Nuclei were visualized by DAPI (4′,6′diamidino-2-phenylindole) staining (Vectashield H-1200, Vector Laboratories, Inc.), and images were acquired by fluorescence microscopy. TUNEL staining was performed per the instruction of the manufacturer (Roche, Cat# 11684795910).

2.4. Western blotting

Protein samples for Western blot analysis were extracted and separated as described previously [33]. The following primary antibodies for the following targets were used: MHC (MF-20, Developmental Studies Hybridoma Bank), MyoG (ab82843, Abcam), Setd2 (Everest Biotech, EB08118), H3K36me3 (ab9050, Abcam), Histone H3 (ab7970, Abcam), p-His (sc-8656-R, Santa Cruz Biotechnology), CyclinD1 (2922, Cell Signaling), CyclinE2 (4132, Cell Signaling), CDK2 (2546, Cell Signaling), CDK4 (12790, Cell Signaling), CDK6 (13331, Cell Signaling), p21 (sc-397, Santa Cruz Biotechnology), LaminB1 (13435, Cell Signaling), cleaved Caspase 3 (sc-22171, Santa Cruz Biotechnology), cleaved PARP (5625, Cell Signaling), and GAPDH (sc-20357, Santa Cruz Biotechnology). The secondary goat anti-mouse and anti-rabbit IgG antibodies coupled with horseradish peroxidase (Invitrogen, Carlsbad, CA) were used. Histone was used as the internal control for H3K36me3 levels. Lamin B1 was used as the internal control for nuclear protein and GAPDH was used to verify even protein loading. Equal protein loading