



ARTICLE

Mdfi Regulates Myoblast Proliferation and Cell Cycle Progression through Cyclins

Yiren Jiao^{1,*}, Lingbo Cao², Jing Xu¹, Hongkai Sun¹, Jia Shi³, Xiaoli Ma¹, Zehao Wang^{1,*} and Baozhen Zhou^{1,*}

¹College of Medicine, Xi'an International University, Xi'an, China

²School of Geography and Tourism, Jiaying University, Meizhou, China

³National Engineering Research Center for Breeding Swine Industry, Guangdong Provincial Key Lab of Agro-Animal Genomics and Molecular Breeding, College of Animal Science, South China Agricultural University, Guangzhou, China

*Corresponding Authors: Yiren Jiao. Email: jiaoyr92@163.com; Zehao Wang. Email: zhwang9415@163.com;

Baozhen Zhou. Email: zhoubaozhen123@163.com

Received: 09 April 2026; Accepted: 24 July 2026; Published: 19 October 2026

ABSTRACT: Background: The inhibitor of MyoD family (*Mdfi*) has been characterized as a myogenic repressor that regulates transcription factor activity through cytoplasmic retention; however, its specific function in myoblast proliferation remains poorly understood. This study aimed to elucidate the precise role of *Mdfi* in regulating myoblast cell cycle progression and proliferation using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated gene editing. **Methods:** We employed the CRISPR/Cas9 system to construct *Mdfi*-knockout (*Mdfi*^{-/-}) C2C12 cell lines. Cell cycle distribution was analyzed by flow cytometry, proliferation was assessed by EdU incorporation assays, and molecular mechanisms were investigated through quantitative RT-PCR, Western blotting, and RNA sequencing (RNA-Seq). Differentially expressed genes (DEGs) between wild-type (WT) and *Mdfi*-overexpressing (*Mdfi*-OE) were identified and subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. **Results:** *Mdfi* overexpression significantly increased the proportion of cells in G1 ($p < 0.001$) phase while reducing S and G2 phase populations ($p < 0.001$), accompanied by decreased EdU-positive cells. Conversely, *Mdfi* knockout promoted cell cycle progression into S phase ($p < 0.05$) and enhanced proliferation. Mechanistically, *Mdfi* overexpression downregulated cyclin B1 (*Ccnb1*) ($p < 0.01$), cyclin D1 (*Ccnd1*) ($p < 0.05$), and proliferating cell nuclear antigen (*Pcna*) ($p < 0.05$), while upregulating the cyclin-dependent kinase inhibitor P21 ($p < 0.01$). RNA-Seq analysis identified 889 DEGs (FDR < 0.05 and $|\log_2FC| > 1$), with enrichment in cell cycle and calcium signaling pathways. **Conclusions:** *Mdfi* functions as a negative regulator of myoblast proliferation by inducing G1 phase arrest, potentially through modulation of cyclin D within the cyclin-CDK-P21 pathway. These findings advance our understanding of the molecular mechanisms governing muscle cell proliferation and identify *Mdfi* as a potential therapeutic target for muscle regeneration and repair.

KEYWORDS: Inhibitor of MyoD family (*Mdfi*); myoblasts; cell cycle arrest; clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9); cyclin; cyclin-dependent kinase inhibitor

1 Introduction

Skeletal muscle possesses remarkable regenerative capacity dependent on the precise coordination of satellite cell activation, proliferation, and differentiation [1]. Upon injury, quiescent muscle satellite cells (MuSCs) become activated, re-enter the cell cycle, and undergo extensive proliferation to generate

a sufficient pool of myogenic progenitors before terminal differentiation and fusion into multinucleated myotubes [2]. The transition from proliferation to differentiation is tightly controlled by cell cycle regulators, including cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs), which determine whether myoblasts continue dividing or exit the cell cycle to differentiate [3,4]. Dysregulation of this proliferative phase can impair muscle regeneration, leading to compromised tissue repair and functional deficits [5]. Understanding the molecular mechanisms that govern myoblast proliferation is therefore essential for developing therapeutic strategies to enhance muscle regeneration.

The inhibitor of MyoD family (*Mdfi*) represents a unique class of myogenic regulatory proteins containing the conserved I-mfa domain. Initially characterized as a transcriptional repressor that inhibits *MyoD* family members through cytoplasmic retention by masking their nuclear localization signals [6]. *Mdfi* plays a critical role in placenta development, and *Mdfi* knockout sharply reduces the number of trophoblast giant cells *in vivo* [7]. In the developing embryo, *Mdfi* anchors the bHLH transcription factor Hand1, which is essential for placentation and cardiac morphogenesis, in the nucleolus to inhibit its release and activation [8]. In osteoblasts, *Mdfi* regulates the osteogenesis controlled by 1,25-dihydroxy-vitamin D-3 [9]. *Mdfi* knockout increases the number of osteoclasts and osteopenic surface, while *Mdfi* negatively fine-tunes the dynamic range of Ca^{2+} release-activated Ca^{2+} (CRAC) channel through regulation of osteoclastogenesis [10]. *Mdfi* also interacts with Zic family proteins to translocate them to the cytoplasmic compartment and inhibits their transcriptional activity in vertebrate development [11]. The MyoD family inhibitor domain-containing protein (MDFIC) is a new binding partner of the cytoplasmic glucocorticoid receptor (GR) that modulates the transcription of GR and is also negatively regulated by GR [12]. However, despite these advances, the specific role of *Mdfi* in regulating myoblast proliferation and cell cycle dynamics remains largely unexplored.

To address this knowledge gap, the present study utilized clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9 (CRISPR/Cas9)-mediated genome editing to establish stable *Mdfi*-overexpressing (*Mdfi*-OE) and *Mdfi*-knockout (*Mdfi*^{-/-}) C2C12 myoblast cell lines. This approach enabled a precise investigation of *Mdfi* function in cell cycle progression and proliferation. We hypothesized that *Mdfi* regulates myoblast proliferation through modulation of key cell cycle regulators, potentially influencing the balance between self-renewal and differentiation. Our findings provide novel insights into the molecular circuitry controlling muscle cell expansion.

2 Materials and Methods

2.1 Cell Culture

The C2C12 mouse myoblast cell line (CRL-1772, American Type Culture Collection, ATCC, Manassas, VA, USA) was maintained in growth medium consisting of Dulbecco's Modified Eagle Medium (11965092, DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (A5256701, FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (10378016, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO₂. The C2C12 cells were identified by Short Tandem Repeat (STR) and were free from mycoplasma contamination. Cells were passaged when reaching 70–80% confluency using 0.25% trypsin-EDTA (25200072, Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cells were used within 20 passages from thawing to minimize genetic drift.

2.2 sgRNA Design and Plasmid Construction

Five pairs of single-guide RNA (sgRNA) oligonucleotides targeting different exons of the mouse *Mdfi* gene (Ensembl ID: ENSMUSG00000032717) were designed using the CRISPR design tool (<https://portals.>

broadinstitute.org/gppx/crispick/public) to minimize off-target effects (Table S1). The sgRNA sequences were cloned into the pX330-U6-Chimeric_BB-CbH-hSpCas9 plasmid (#42230, Addgene, Watertown, MA, USA) following standard protocols [13]. Briefly, complementary oligonucleotides were annealed by heating to 95°C for 5 min and gradually cooling to room temperature, then ligated into the BbsI (R0539S, New England Biolabs, Ipswich, MA, USA)-digested pX330 vector with T4 DNA ligase (M0202, New England Biolabs, Ipswich, MA, USA).

2.3 Transfection and T7EI Assay

C2C12 cells were seeded at 50–60% confluency in 6-well plates and transfected with pX330-sgRNA plasmids using Lipofectamine 3000 (L3000015, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. To evaluate CRISPR editing efficiency, genomic DNA was extracted 48 h post-transfection using the QuickExtract DNA Extraction Kit (D6945-02, Omega, Norcross, GA, USA). The target region was amplified by PCR using gene-specific primers (Table S1) and 2× High-Fidelity PCR Master Mix (M0531, New England Biolabs, NEB, Ipswich, MA, USA). The T7 Endonuclease I (T7EI) assay was performed by incubating 200 ng of purified PCR product with 10 units of T7EI (M0302S, New England Biolabs, Ipswich, MA, USA) at 37°C for 20 min. Cleavage products were separated on 1.5% agarose gels, and editing efficiency was calculated based on band intensities using ImageJ software (v1.54s, National Institutes of Health, Bethesda, MD, USA).

2.4 Generation of *Mdfl* Knockout Cell Lines

Based on T7EI screening results, sgRNA 3 and sgRNA 4 showing the highest editing efficiency were selected for stable cell line generation. C2C12 cells were co-transfected with pX330-sgRNA3 and pX330-sgRNA4 plasmids along with pEGFP-N1 as a transfection marker to indicate transfection efficiency. At 48 h post-transfection, cells were subjected to limiting dilution cloning in 96-well plates at a density of 0.5 cells per well to obtain monoclonal populations. Single-cell-derived clones were expanded and screened for *Mdfl* protein expression by Western blot analysis.

2.5 Construction of *Mdfl* Overexpression Cell Line

The *Mdfl*-overexpressing (*Mdfl*-OE) C2C12 cell line was previously constructed and validated in our earlier study [14]. The full-length mouse *Mdfl* coding sequence was amplified from C2C12 cDNA and cloned into a targeting vector (DONOR vector) for knock-in at the *Rosa26* locus. The targeting vector (*Mdfl* donor) contained left and right homologous arms flanking the *Rosa26* locus, the *Mdfl* expression cassette driven by the CAG promoter, and a neomycin resistance gene for selection. The MCP-ROSA26-CG01 vector with DCDON-SH02 (Wild Type) and the *Mdfl* donor were co-transfected into C2C12 cells, respectively. After G418 (10131035, Thermo Fisher Scientific, Waltham, MA, USA) selection (400 µg/mL), monoclonal cell lines were isolated by limiting dilution and screened for correct integration by PCR using primers specific for the junction regions (Table S1) between the inserted cassette and the *Rosa26* locus.

2.6 EdU Incorporation Assay

Cell proliferation was assessed using the Cell Light™ EdU Kit (C10310; Ribo Biotech Co., Guangzhou, China) according to the manufacturer's protocol. Briefly, C2C12 cells were seeded in 24-well plates at a density of 1×10^4 cells per well and cultured for 24 h. EdU (5-ethynyl-2'-deoxyuridine, 10 µM) was added to the culture medium and incubated for 2 h to allow incorporation into newly synthesized DNA. Cells were then fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 20 min,

and subjected to the click reaction with Alexa Fluor 594 azide for 30 min. The nuclei were counterstained with DAPI (C1006, Beyotime, Shanghai, China) at a concentration of 1 µg/mL for 30 min. Images were captured using the inverted fluorescence microscope (Axio Observer A1, Zeiss, Oberkochen, Germany). The proportion of EdU-positive cells was quantified from three randomly selected fields per well across three independent experiments using ImageJ software.

2.7 Cell Cycle Analysis by Flow Cytometry

Cell cycle distribution was analyzed by propidium iodide (PI) staining and flow cytometry. C2C12 cells were harvested 24 h after seeding at a density of 1×10^6 cells/mL in 6-well plates. After washing with PBS, the cells were digested with 0.25% trypsin, collected, and fixed in ice-cold 70% ethanol overnight at -20°C . Fixed cells were washed with PBS, treated with RNase A (100 µg/mL) at 37°C for 30 min, and stained with PI (50 µg/mL) for 30 min in the dark. Cell cycle profiles were acquired using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) with excitation at 488 nm and emission at 585 nm. Data were analyzed using FlowJo software (v10.8, BD Biosciences, San Jose, CA, USA). At least 10,000 events were collected per sample, and experiments were performed in triplicate.

2.8 RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted using RNAiso Plus (9109, Takara, Kusatsu, Shiga, Japan) according to the manufacturer's protocol. RNA purity and concentration were determined using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesized from 1 µg of total RNA using PrimeScriptTM RT reagent Kit with gDNA Eraser (RR047A, TaKaRa, Kusatsu, Shiga, Japan) with random primers. Quantitative PCR was performed using TB Green[®] Premix Ex TaqTM (Tli RNaseH Plus) (RR820A, TaKaRa, Kusatsu, Shiga, Japan) on a CFX96TM Optical Reaction Module (Bio-Rad, Hercules, CA, USA). The relative expression levels were calculated using the comparative CT ($\Delta\Delta\text{CT}$) method with β -actin as the internal reference gene. Primer sequences are listed in Table S2.

2.9 Western Blot Analysis

Protein samples were prepared by lysing cells in RIPA buffer (P0013C, Beyotime, Shanghai, China) supplemented with protease inhibitor cocktail (P1005, Beyotime, China). Protein concentrations were determined using the BCA Protein Assay Kit (P0010, Beyotime, China). Equal amounts of protein (30 µg per lane) were separated by 10% SDS-PAGE and transferred to PVDF membranes (FFP106-20pcs, Beyotime, China). Membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature, then incubated overnight at 4°C with primary antibodies against CCNB1, CCND1, P21, and β -actin (Table S3). After washing with TBST, membranes were incubated with HRP-conjugated secondary antibodies (Table S4) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) substrate (FD8030, FDBio Science, Hangzhou, China) and captured with a ChemiDoc imaging system (Bio-Rad, Hercules, CA, USA). Band intensities were quantified using ImageJ software and normalized to β -actin.

2.10 RNA Sequencing and Bioinformatics Analysis

Total RNA was extracted from Wild Type (PC1, PC2, PC3) and Mdfi-OE (PE1, PE2, PE3) C2C12 cells during the proliferation phase using RNAiso Plus (9109, Takara, Kusatsu, Shiga, Japan). RNA quality was assessed using an Agilent 2100 Bioanalyzer, and samples with RNA integrity numbers (RIN) > 8.0 were used for library preparation. Strand-specific RNA-seq libraries were prepared using the TruSeq Stranded

mRNA Library Prep Kit (RS-122-2101, Illumina, San Diego, CA, USA) and sequenced on the Illumina HiSeq platform to generate 150 bp paired-end reads.

Raw reads were filtered to remove adapter sequences and low-quality bases using Cutadapt (version 1.9.1) (Table S5). Clean reads were aligned to the mouse reference genome (GRCm38) using HISAT2 (v2.1.0) with default parameters. Gene-level quantification was performed using featureCounts (v2.0.1) with the GENCODE M25 annotation. Differential expression analysis was conducted using ‘DESeq2’ (v1.30.0) in R (v4.0.3). Genes with an adjusted *p*-value (FDR) < 0.05 and $|\log_2\text{FoldChange}| > 1$ were considered differentially expressed. Hierarchical clustering was performed using the ‘pheatmap’ package (1.0.13), and volcano plots were generated using ‘ggplot2’ (4.0.0). Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using ‘clusterProfiler’ (v3.18.0).

2.11 Statistical Analysis

Data are presented as mean $\pm$ standard error of the mean (S.E.M.) from at least three biological replicates. Prior to statistical analysis, data normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated by Levene’s test. For data satisfying the assumptions of parametric testing, comparisons between two groups were performed using an unpaired two-tailed Student’s *t*-test, whereas comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. A *p*-value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Construction of the *Mdfl*^{-/-} C2C12 Cell Line

To investigate the function of *Mdfl* in the development of C2C12 cells, we used the CRISPR/Cas9 system to construct the *Mdfl*^{-/-} C2C12 cell line. First, we designed five pairs of sgRNA oligos targeted to the *Mdfl* gene and constructed the all-in-one plasmid after denaturation, annealing, and renaturation (Fig. S1 and Table S1). Then, pX330-sgRNA was transfected into C2C12 cells. The T7EI enzyme digestion assay results showed that sgRNA 3, sgRNA 4, and sgRNA 3+4 have higher editing efficiency than all other groups in C2C12 cells, and lower molecular weight DNA fragments were produced in sgRNA 3, sgRNA 4, and sgRNA 3+4 groups (Fig. 1A). The sequencing results showed that various mutation forms exist in the *Mdfl*^{-/-} C2C12 cells, including insertion, deletion, and substitution (Fig. 1B). These results indicate that both sgRNA 3 and sgRNA 4 are efficient sgRNAs that can guide the Cas9 protein to mutate the *Mdfl* gene.

We chose sgRNA 3 and sgRNA 4 as efficient sgRNAs to produce *Mdfl*^{-/-} C2C12 cell lines. After transfecting C2C12 cells with sgRNA3 and sgRNA4, we used a limiting dilution assay to select the *Mdfl*^{-/-} monoclonal C2C12 cells. A total of 70 monoclonal clones were selected, and we measured the MDFI protein level by Western blot. The results show that three clones (clone 50, clone 60, and clone 70) had no MDFI expression at the protein level and were selected for further analyses (Fig. 1C). After comprehensive evaluation of cell morphology, cell viability, and other relevant characteristics, clone 60 was selected as the candidate *Mdfl*^{-/-} C2C12 cell clone for subsequent experiments. We examined the mutation in clone 60’s genome DNA by the T7EI assay. We found that the amplified fragment, which we used pX330-sgRNA-transfected C2C12 cells’ genome DNA as a template, was cleaved, resulting in the generation of lower molecular weight DNA fragments of ~300 bp and ~600 bp (Fig. 1D). The mRNA level of *Mdfl* was sharply reduced and almost equal to zero (Fig. 1E). The MDFI protein was not identified in clone 60 by Western blot (Fig. 1F). The results show that there is no MDFI expression in clone 60, and we have successfully constructed the *Mdfl*^{-/-} C2C12 cell line.

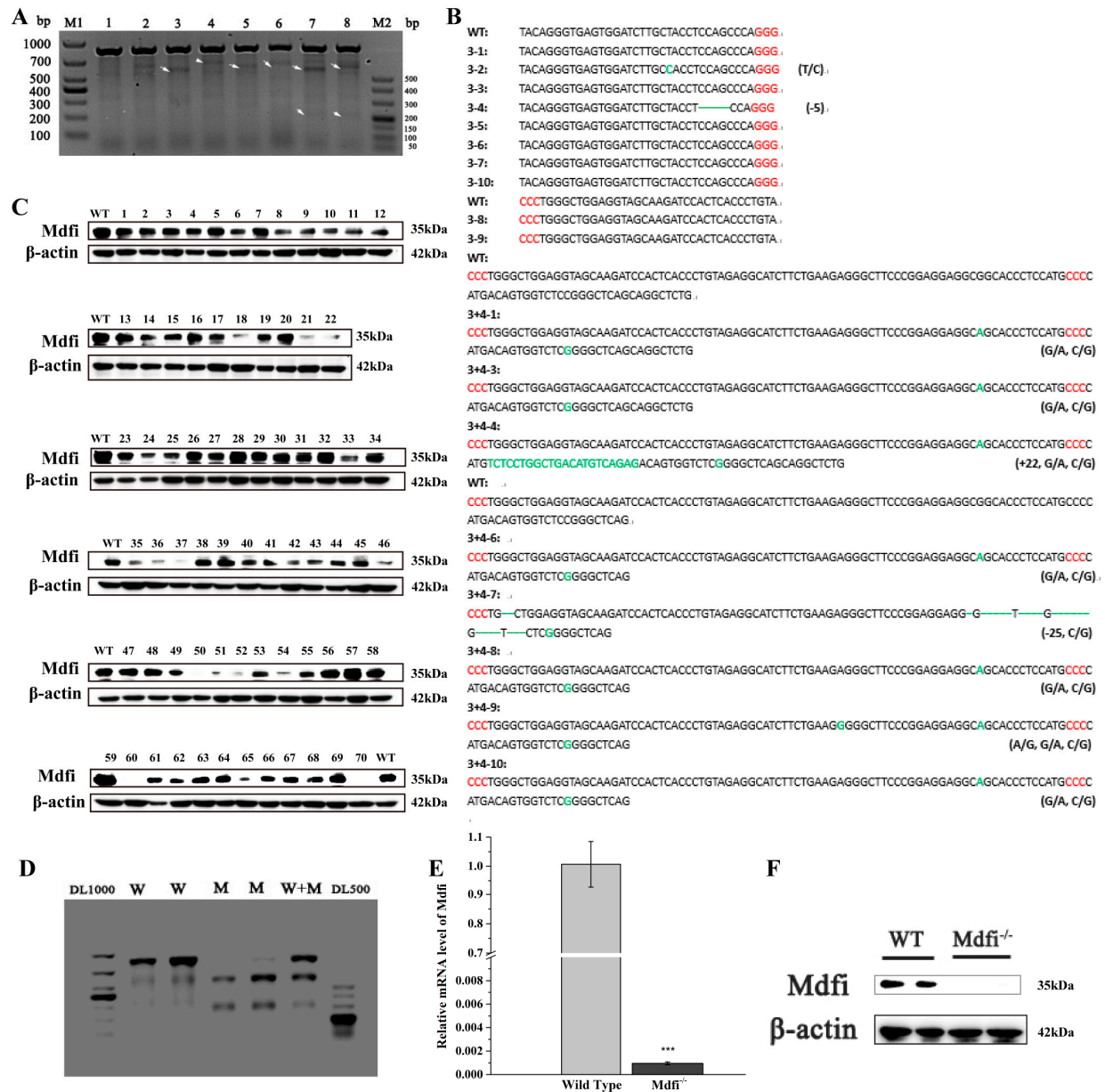


Figure 1: Generation of the *Mdfi*^{-/-} C2C12 cells. (A). T7EI assay was used to identify the mutation status of the *Mdfi* gene in C2C12 cells, which were transfected with pX330-sgRNA1, pX330-sgRNA2, pX330-sgRNA3, pX330-sgRNA4, pX330-sgRNA5, pX330-sgRNA2+ pX330-sgRNA4, pX330-sgRNA3+ pX330-sgRNA 4, and pX330-sgRNA5+ pX330-sgRNA4. The white arrow indicates the lower molecular weight DNA fragments. (B). DNA sequencing was used to identify the mutation status of *Mdfi* in C2C12 cells that were transfected with pX330-sgRNA3 or co-transfected with pX330-sgRNA3 and pX330-sgRNA4. +: insertion; -: deletion; /: substitution. (C). A limiting dilution assay was used to select the *Mdfi*^{-/-} monoclonal C2C12 cells after being transfected with pX330-sgRNA3 and sgRNA4. Western blot identified 70 monoclonal clones. WT: wild-type C2C12 cells. n = 3. (D). T7EI assay confirmed the *Mdfi* mutation of clone 60. W: Wild type. M: Mutant. W+M: Wild type + Mutant. (E). qRT-PCR measured the mRNA level of *Mdfi* in clone 60. (F). Western blot confirmed the absence of MDFI protein in clone 60. ****p* < 0.001. An unpaired two-tailed Student's *t*-test was used to assess statistical significance. Error bars represent the standard error of the mean (S.E.M.) from biological replicates.

3.2 *Mdfi* Inhibits the Entry of Myoblasts into the S and G2 Phases of the Cell Cycle

To investigate the role of *Mdfi* in C2C12 cell proliferation, we assessed cell cycle distribution 24 h after seeding. Flow cytometry analysis revealed that *Mdfi* overexpression significantly increased the proportion of cells in the G1 phase ($p < 0.001$), while reducing the proportions in both the S ($p < 0.001$) and G2 ($p < 0.001$) phases (Fig. 2A,B). In contrast, *Mdfi* knockout decreased the proportion of cells in the G1 phase ($p < 0.05$) while increasing the proportions in the S phase ($p < 0.05$) (Fig. 2C,D). Collectively, these results indicate that *Mdfi* suppresses C2C12 cell proliferation by inducing G1 phase arrest and inhibiting progression into the S and G2 phases.

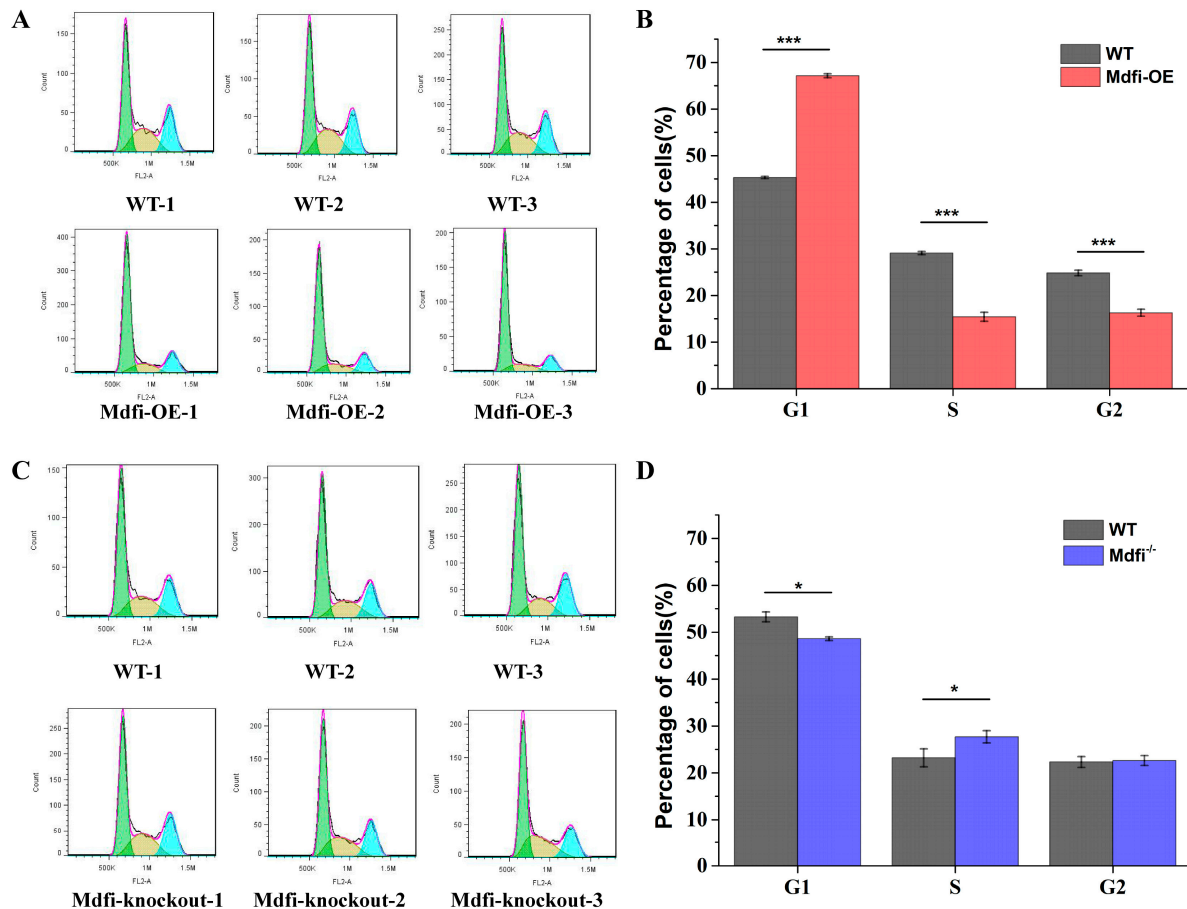


Figure 2: Effects of *Mdfi* on myoblast cell cycle progression. (A). Cell cycle distribution of wild-type (WT) and *Mdfi*-overexpressing (*Mdfi*-OE) cells analyzed by flow cytometry. The colored areas indicate different cell cycle phases: green for G1 phase, yellow for S phase, and cyan for G2 phase. $n = 3$. (B). Quantification of the proportions of cells at different cell cycle phases corresponding to (A). (C). Cell cycle distribution of wild-type and *Mdfi*-knockout cells analyzed by flow cytometry. The colored areas indicate different cell cycle phases: green for G1 phase, yellow for S phase, and cyan for G2 phase. $n = 3$. (D). Quantification of the proportions of cells at different cell cycle phases corresponding to (C). * $p < 0.05$. *** $p < 0.001$. An unpaired two-tailed Student's t -test was used to assess statistical significance. Error bars represent the standard error of the mean (S.E.M.) from biological replicates.

3.3 *Mdfi* Inhibits the Proliferation of Myoblasts

The effects of *Mdfi* overexpression and knockout on C2C12 cell proliferation were evaluated using EdU incorporation assays. The results showed that, compared with the WT group, *Mdfi* overexpression

significantly reduced the proportion of EdU-positive cells at 24 h ($p < 0.001$) during the proliferation phase (Fig. 3A,B). In contrast, Mdfi knockout markedly increased the percentage of EdU-positive cells at 24 h (Fig. 3C,D) ($p < 0.01$). These findings indicate that Mdfi suppresses the proliferation of C2C12 cells.

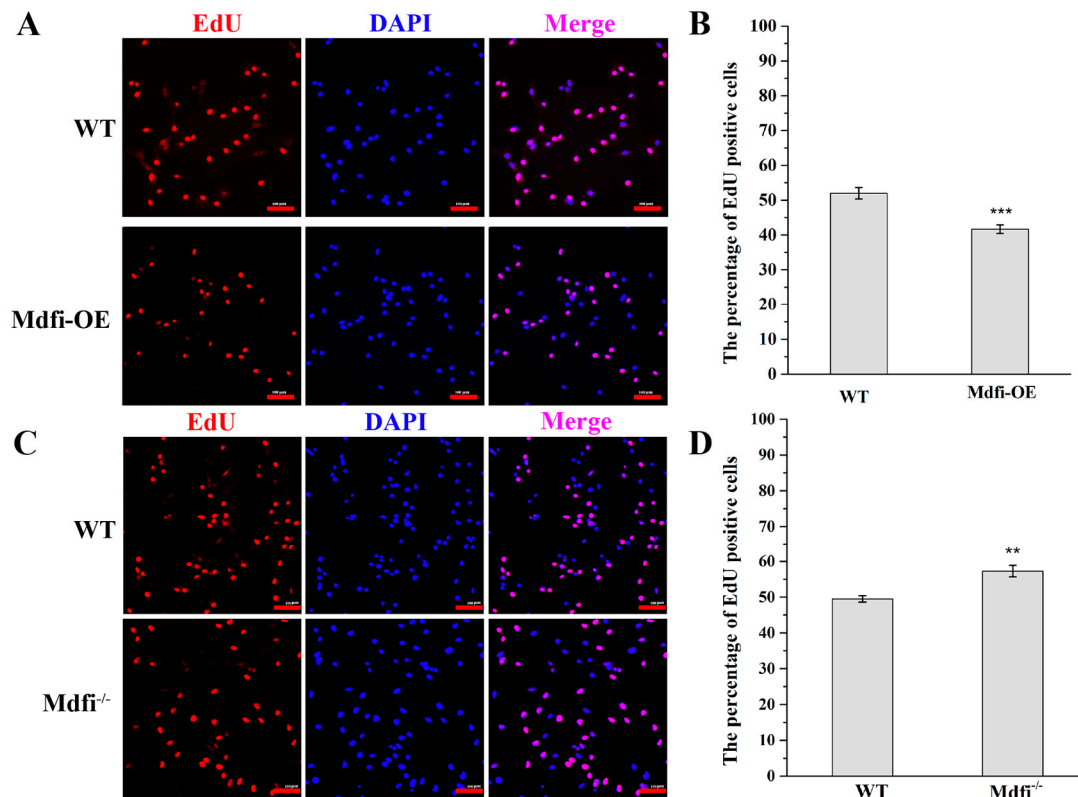


Figure 3: Mdfi inhibits the proliferation of C2C12 cells. (A). EdU staining of C2C12 cells in wild-type (WT) and Mdfi-overexpressing (Mdfi-OE) groups at 24 h of proliferation. EdU: red, the nuclei of proliferating cells; DAPI: blue, cell nuclei; Merge: EdU-positive cells. Scale bar: 100 μ m. (B). Quantification of EdU-positive cells corresponding to (A). $n = 3$. (C). EdU staining of C2C12 cells in WT and Mdfi-knockout (Mdfi^{-/-}) groups at 24 h of proliferation. Scale bar: 100 μ m. (D). Quantification of EdU-positive cells corresponding to (C). $n = 3$. ** $p < 0.01$. *** $p < 0.001$. An unpaired two-tailed Student's t -test was used to assess statistical significance. Error bars represent the standard error of the mean (S.E.M.) from biological replicates.

3.4 Mdfi Suppresses Cell Cycle Progression via Downregulation of Cyclins in C2C12 Cells

Cell cycle regulation is a critical determinant of cell proliferation. To elucidate the mechanism by which Mdfi regulates the proliferation of C2C12 cells, we examined the expression of cyclins and cyclin-dependent kinase inhibitor 1A (P21). qRT-PCR analysis revealed that Mdfi overexpression significantly downregulated the mRNA levels of *Ccnb1* ($p < 0.01$), *Ccnd1* ($p < 0.05$), and *Pcna* ($p < 0.05$), while markedly upregulating *P21* expression ($p < 0.01$) (Fig. 4A). In contrast, Mdfi knockout significantly increased the expression of *Ccnd1* ($p < 0.05$) and *Pcna* ($p < 0.05$), accompanied by a notable decrease in *P21* expression ($p < 0.01$) (Fig. 4B). Consistent with the mRNA results, Western blot analysis demonstrated that Mdfi overexpression reduced the protein levels of CCNB1 and CCND1, while increasing the protein level of P21 (Fig. 4C,D). In contrast, Mdfi knockout elevated CCND1 protein expression, while decreasing the protein level of P21. In addition, no significant change was observed in the protein level of CCNB1 (Fig. 4E,F). Collectively, these findings

suggest that *Mdfr* inhibits the proliferation of C2C12 cells by suppressing cell cycle progression, particularly the transition into the S and G2 phases, through downregulation of *Ccnb1* and *Ccnd1* expression.

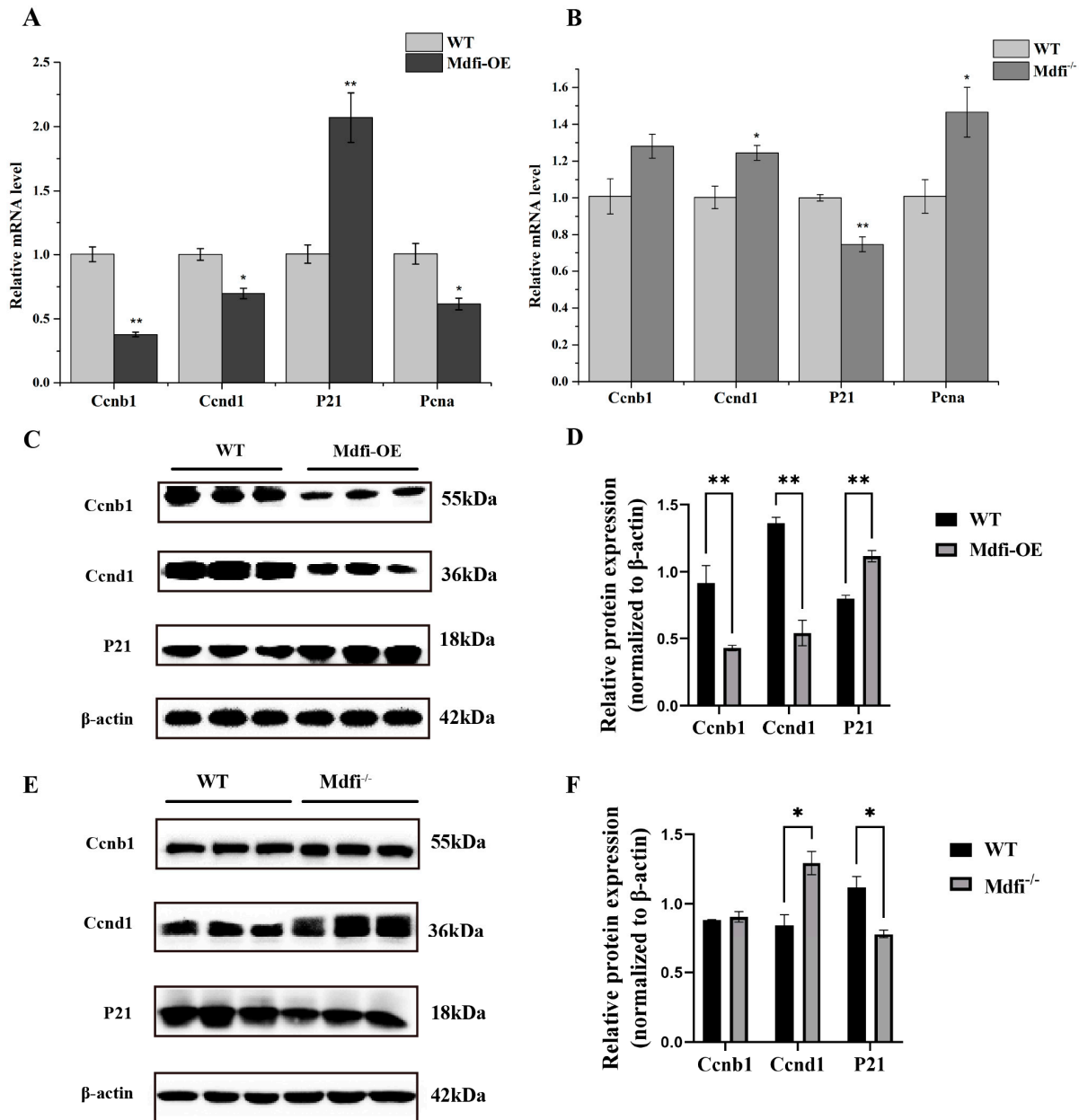


Figure 4: Effects of *Mdfr* on cyclin expression in myoblasts. (A). qRT-PCR analysis of genes in WT and overexpressing *Mdfr* C2C12 cells. (B). qRT-PCR detection of genes in WT and *Mdfr*-knockout C2C12 cells. (C). Western blot detection of cyclins in WT and overexpressing *Mdfr* C2C12 cells. (D). Quantitative analysis of protein expression levels shown in (C). $n = 3$. (E). Western blot detection of cyclins in WT and *Mdfr*-knockout C2C12 cells. (F). Quantitative analysis of protein expression levels shown in (E). $n = 3$. * $p < 0.05$. ** $p < 0.01$. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. An unpaired two-tailed Student's *t*-test was used to assess statistical significance. Error bars represent the standard error of the mean (S.E.M.) from biological replicates.

3.5 RNA-Seq Analysis Reveals the Transcriptional Regulation of Mdfi in Myoblast Proliferation

To investigate the regulatory role of Mdfi at the transcriptional level during myoblast proliferation, mRNA samples from wild-type (WT, PC) and Mdfi-overexpressing (Mdfi-OE, PE) myoblasts were collected for RNA sequencing and subsequent analysis. Correlation analysis demonstrated high reproducibility among biological replicates, with correlation coefficients exceeding 0.98 in the control group and 0.99 in the Mdfi-OE group (Fig. 5A). As shown in Table 1, during the proliferation stage, a total of 5139 DEGs were identified between the Mdfi-OE and control groups under the threshold of $p < 0.05$. When applying more stringent criteria ($FDR < 0.05$ and $|\log_2FC| > 1$), 889 DEGs were identified (Table S6). Among these, 608 genes were significantly upregulated in the Mdfi-OE group, with Mdfi itself exhibiting the highest fold change (Tables 2 and 3). Hierarchical clustering analysis of DEGs revealed a clear separation between the Mdfi-OE and WT groups, indicating distinct transcriptional profiles (Fig. 5B). Volcano plot analysis further showed that the number of upregulated genes was markedly higher than that of downregulated genes during proliferation (Fig. 5C).

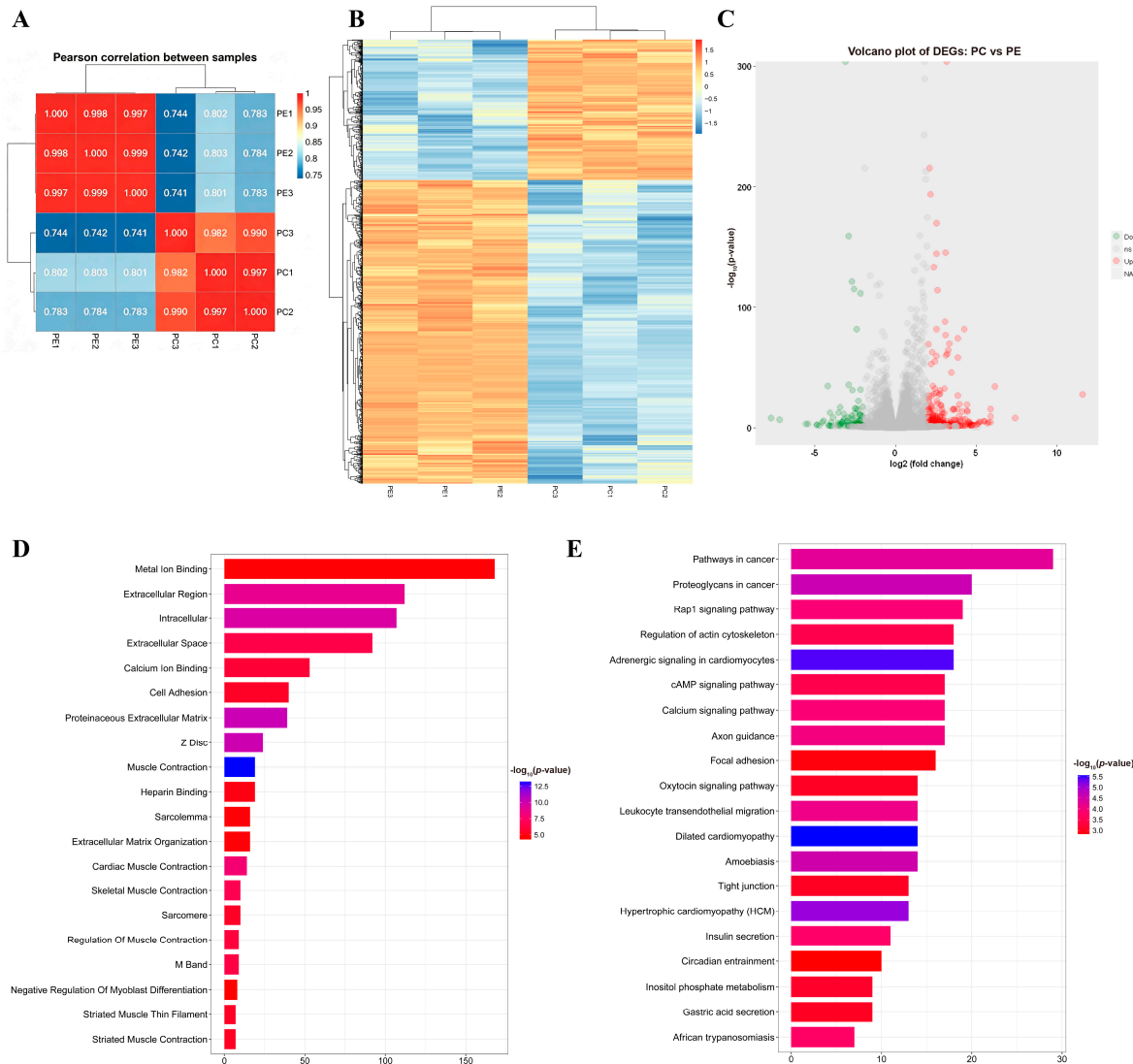


Figure 5: Cont.

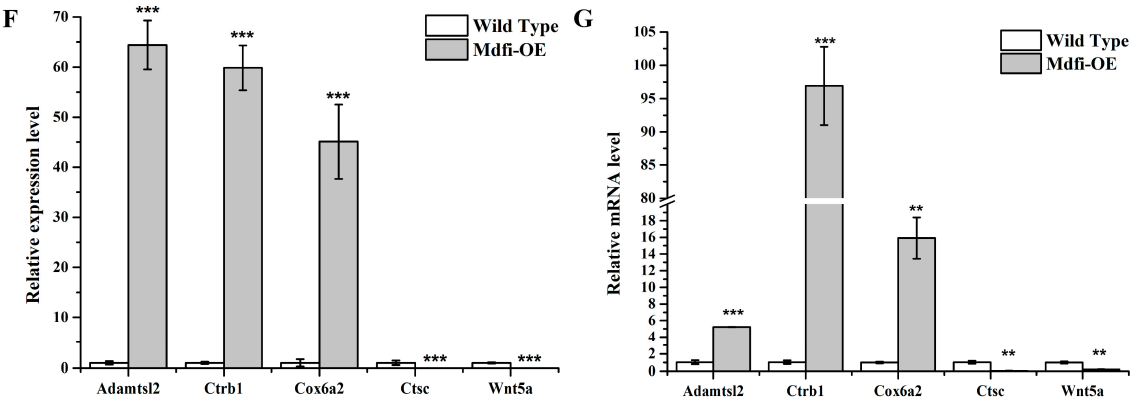


Figure 5: RNA-sequencing analysis of Mdfi in myoblasts. (A). Pearson correlation analysis between samples in the proliferative phase. (B). Clustering analysis heatmap of differentially expressed genes in PC vs. PE. (C). Volcano plot of differentially expressed genes in the PC vs. PE. (D). GO analysis of differentially expressed genes in the proliferative phase. (E). KEGG analysis of differentially expressed genes in the proliferative phase. (F). Relative expression of differentially expressed genes from sequencing data. $n = 3$, *** $p < 0.001$. (G). qRT-PCR identifies the mRNA level of differentially expressed genes. $n = 3$, ** $p < 0.01$, *** $p < 0.001$. An unpaired two-tailed Student's t -test was used to assess statistical significance. Error bars represent the standard error of the mean (S.E.M.) from biological replicates.

Gene Ontology (GO) enrichment analysis demonstrated that DEGs were primarily enriched in terms related to metal ion binding, extracellular region, intracellular components, extracellular space, calcium ion binding, and cell adhesion (Fig. 5D and Table S7). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that DEGs were mainly enriched in pathways in cancer, proteoglycans in cancer, the Rap1 signaling pathway, regulation of actin cytoskeleton, adrenergic signaling in cardiomyocytes, the cAMP signaling pathway, and the calcium signaling pathway (Fig. 5E and Table S8).

To further validate the reliability of the RNA-seq data, several DEGs were randomly selected for qRT-PCR validation during both proliferation and differentiation stages. The results showed that *Adamtsl2* ($p < 0.001$), *Ctrb1* ($p < 0.001$), and *Cox6a2* ($p < 0.01$) were significantly upregulated, whereas *Ctsc* ($p < 0.01$) and *Wnt5a* ($p < 0.01$) were significantly downregulated during proliferation, consistent with the RNA-seq results (Fig. 5F,G).

Table 1: Analysis of differentially expressed genes.

Group	Total Genes	DEG	Up-Regulated	Down-Regulated
WT vs. Mdfi-OE	5139	889	608	281

Table 2: Top 20 up-regulated differentially expressed genes in the proliferative phase.

Ensembl_ID	Gene	log ₂ FC	FDR
ENSMUSG00000032717	<i>Mdfi</i>	11.60289	4.91E−28
ENSMUSG00000019787	<i>Trdn</i>	7.428237	1.41E−08
ENSMUSG00000018893	<i>Mb</i>	6.158442	6.82E−35
ENSMUSG00000041986	<i>Elmod1</i>	5.913479	4.67E−10
ENSMUSG00000036040	<i>Adamtsl2</i>	5.903756	4.22E−05
ENSMUSG00000031957	<i>Ctrb1</i>	5.886448	4.94E−16
ENSMUSG00000079034	<i>Gm8300</i>	5.771384	7.02E−05

Table 2: Cont.

Ensembl_ID	Gene	log ₂ FC	FDR
ENSMUSG00000035277	<i>Arx</i>	5.702098	0.000395
ENSMUSG00000030785	<i>Cox6a2</i>	5.515281	8.65E−06
ENSMUSG00000004939	<i>Nmrk2</i>	5.494865	7.41E−06
ENSMUSG00000053093	<i>Myh7</i>	5.256415	2.56E−05
ENSMUSG00000038067	<i>Csf3</i>	5.15954	4.59E−05
ENSMUSG00000093847	<i>Eif1ad15</i>	5.136731	0.000859
ENSMUSG00000089756	<i>Zfp966</i>	5.071461	0.001113
ENSMUSG00000116765	NA	4.987232	0.005866
ENSMUSG00000111971	NA	4.870239	0.01018
ENSMUSG00000058163	<i>Gm5431</i>	4.822488	0.011026
ENSMUSG00000061825	<i>Ces2c</i>	4.796628	0.011729
ENSMUSG00000078495	<i>Zfp984</i>	4.718714	0.015556
ENSMUSG00000113212	NA	4.707537	0.016952

Note: NA, Not Available.

Table 3: Top 20 down-regulated differentially expressed genes in proliferative phase.

Ensembl_ID	Gene	log ₂ FC	FDR
ENSMUSG00000037999	<i>Arap2</i>	−7.70882	8.72E−09
ENSMUSG00000062184	<i>Hs6st2</i>	−7.187	1.93E−07
ENSMUSG000000021994	<i>Wnt5a</i>	−5.54683	0.000803
ENSMUSG00000000690	<i>Hoxb6</i>	−5.42111	0.001515
ENSMUSG00000030560	<i>Ctsc</i>	−4.89636	0.009437
ENSMUSG00000032263	<i>Bckdhh</i>	−4.84987	0.003185
ENSMUSG00000079164	<i>Tlr5</i>	−4.70695	0.015691
ENSMUSG00000028351	<i>Brinp1</i>	−4.50325	4.48E−05
ENSMUSG00000053279	<i>Aldh1a1</i>	−4.4408	2.62E−06
ENSMUSG00000029838	<i>Ptn</i>	−4.18315	3.50E−35
ENSMUSG00000026602	<i>Nphs2</i>	−4.11226	0.027283
ENSMUSG00000026321	<i>Tnfrsf11a</i>	−4.10862	0.024634
ENSMUSG00000056073	<i>Grik2</i>	−4.06172	6.14E−13
ENSMUSG00000034810	<i>Scn7a</i>	−4.02727	0.030702
ENSMUSG00000033491	<i>Prss35</i>	−3.99412	7.88E−05
ENSMUSG00000034459	<i>Ifit1</i>	−3.93696	0.040801
ENSMUSG00000034613	<i>Ppm1h</i>	−3.84891	7.57E−09
ENSMUSG00000044220	<i>Nkx2-3</i>	−3.56902	0.030114
ENSMUSG00000068735	<i>Trp53i11</i>	−3.56668	0.000143
ENSMUSG00000030666	<i>Calcb</i>	−3.53735	3.76E−13

4 Discussion

The proliferation, differentiation, migration, and fusion of myoblasts are necessary steps involved in muscle development and regeneration [15,16]. The precise regulation of myoblast proliferation is fundamental to skeletal muscle development and regeneration, ensuring adequate progenitor expansion while preventing premature differentiation or exhaustion of the stem cell pool. Our study establishes *Mdfi* as a critical negative regulator of myoblast proliferation through G1 phase arrest, mediated by downregulation of *Ccnd1*, *Ccnb1*, and *Pcna* coupled with upregulation of the CDK inhibitor *p21*. These findings align with recent observations that Calponin 3 (CNN3) regulates myoblast proliferation through similar mechanisms, where knockdown of CNN3 enhances proliferation by upregulating cyclin B1, cyclin D1, and PCNA while increasing S and G2/M phase fractions [17]. During bone development, deletion of *Mdfi* increased the number of osteoclasts

and the osteolytic area [10]. In our study, we found that Mdfi knockout increased the EdU-positive C2C12 cells, promoting the proliferation of C2C12 cells, which is consistent with the previous findings. In the breast cancer cell line T47D, ER60 protease induced G1/S cell arrest in breast cancer cells, and thereby inhibited the proliferation of cancer cells [18]. In our study, the flow cytometry results showed that Mdfi arrested C2C12 cells in G1 phase and inhibited the transformation of the cell cycle from G1 to S phase. The process of myoblast proliferation is the result of normal maintenance of the cell cycle. Cell cycle progression is highly conserved, and the temporal expression and regulation of cyclins, cyclin-dependent kinases (Cdks), cyclin kinase inhibitors (CKIs), and other cytokines maintain the process of cell cycle [19]. qRT-PCR and Western blot showed that overexpression of Mdfi could promote the expression of P21 and inhibit the expression of Ccnb1 and Ccnd1. Conversely, knocking out Mdfi also has corresponding results. Ccnd1 is the first expressed cyclin in the cell cycle. Ccnd1 interacts with cyclin-dependent kinase-4/6 (Cdk4/6) to form the active Ccnd1-Cdk4/6 complex and regulates the G1-phase of the cell cycle [20]. Ccna and cyclin B1 (Ccnb1) are required for mammalian cells to enter the M-phase [21]. P21 is a cell cycle inhibitor, which blocks the activity of the cyclin-Cdks complexes [22]. Otherwise, Mdfi has been reported to be hypermethylated in both colorectal cancer (CRC) [23] and non-small cell lung carcinoma by methylation sequencing assay [24]. In the colorectal cancer cell line SW480, the next-generation sequencing results showed that p53 activation increases the expression of Mdfi [25]. The consistency between these studies underscores the importance of cell cycle regulatory networks in controlling myoblast expansion and suggests that Mdfi may function within broader signaling pathways that coordinate proliferation and differentiation decisions.

The mechanistic insights from our RNA-Seq analysis further illuminate Mdfi's regulatory scope, revealing 889 differentially expressed genes enriched in pathways governing cell adhesion, calcium signaling, and cytoskeleton regulation. Notably, the upregulation of p21 represents a critical node in Mdfi-mediated cell cycle inhibition, consistent with established paradigms where p21 induction promotes irreversible cell cycle withdrawal during myogenic differentiation [26]. Previous studies have demonstrated that p21 overexpression induces cellular senescence programs in skeletal muscle, leading to atrophy and functional impairment, suggesting that precise temporal control of p21 expression is essential for maintaining muscle homeostasis [27]. Our findings regarding cell cycle regulation are consistent with previously published transcriptomic analyses of C2C12 proliferation [28,29]. Our observation that Mdfi knockout reduces p21 expression while promoting proliferation supports the notion that Mdfi fine-tunes the proliferative capacity of myoblasts, potentially influencing the transition from expansion to differentiation phases during regeneration.

The KEGG pathway enrichment analysis from our RNA-Seq data provides additional insights into the broader signaling networks influenced by Mdfi during myoblast proliferation. Notably, the Rap1 signaling pathway emerged as one of the most significantly enriched pathways among the differentially expressed genes. Rap1, a small GTPase belonging to the Ras family, has been increasingly recognized as a crucial regulator of skeletal muscle growth and development [30]. Rap1 geranylgeranylation is essential for maintaining mTORC2 activity in skeletal muscle, as simvastatin-induced impairment of Rap1 geranylgeranylation and function leads to mTORC2 inhibition, mitochondrial dysfunction, and cytotoxicity in C2C12 myoblasts and mouse skeletal muscle [31]. The Rap1 signaling pathway is one of the significantly activated pathways associated with sarcopenia in human skeletal muscle biopsies, suggesting that Rap1 may play an important role in the molecular mechanisms underlying age-related muscle mass and function deterioration [32]. This integrative ATAC-seq and RNA-seq study of Tianzhu white yak longissimus dorsi muscle identifies the Rap1 signaling pathway as one of the significantly enriched pathways among differentially expressed genes related to muscle growth and development, highlighting its potential role as a classical regulator of cellular proliferation and migration in skeletal muscle [33]. The enrichment of Rap1 pathway components in Mdfi-overexpressing cells

suggests that Mdfi may modulate myoblast proliferation not only through direct cell cycle regulation but also via integration with broader growth control networks.

The calcium signaling pathway represents another significantly enriched pathway in our transcriptomic analysis, which is particularly relevant given the central role of calcium dynamics in muscle stem cell activation and regeneration [34]. Spontaneous calcium transients manifest in the early stages of muscle regeneration, and perturbing calcium release from internal stores impairs the regenerative process by reducing the number of muscle progenitor cells and activated satellite cells [35]. The calcium-dependent phosphatase calcineurin is expressed in activated and proliferating satellite cells following injury, and inhibiting calcineurin activity impairs regeneration while leading to muscle fiber atrophy [36]. Calcium signaling plays a central and indispensable role in regulating skeletal muscle contraction, metabolism, and adaptive remodeling, and disrupted mitochondrial calcium uptake impairs muscle function and integrity during aging [37]. The orderly release of calcium from the endoplasmic reticulum (ER) is essential for regulating muscle excitation–contraction coupling, as well as for the development, maintenance, and regeneration of muscle cells [34,38]. Recent studies have demonstrated that elevated Mdfi levels promote myofiber-type transition through the calcium signaling pathway [39]. The enrichment of calcium signaling pathway genes in our Mdfi-overexpressing cells suggests that Mdfi may influence the calcium-dependent mechanisms that govern satellite cell activation and proliferation, potentially linking cell cycle control with calcium-mediated developmental signals.

Additionally, the cAMP signaling pathway was prominently enriched among the differentially expressed genes in our study. cAMP signaling is dynamically regulated during embryonic muscle development, *ex vivo* myogenesis, and muscle regeneration, with multiple studies demonstrating its essential role in satellite cell-mediated repair processes [40]. The cAMP-PKA pathway has been extensively studied in the context of skeletal muscle adaptation, contributing to myofiber size regulation, metabolic phenotype determination, and promotion of hypertrophic responses [41]. Notably, cAMP signaling participates in almost all essential cellular activities required for effective regeneration of adult skeletal muscle, including myoblast differentiation, migration, and fusion [42]. Activation of cAMP signaling leads to pronounced hypertrophic responses in skeletal myofibers and can promote regeneration in models of muscular dystrophy and acute muscle injury [43,44]. Pharmacological inhibition of cAMP–PKA–CREB1 signaling, restoration of cAMP–PKA–CREB1 signaling, and inhibition of phosphodiesterase 4 (PDE4) can restore the expression of mitochondrial-related genes, improve mitochondrial function, and alleviate skeletal muscle atrophy in male mice [45]. Blood vessel epicardial substance (BVES) functions as a negative feedback regulator of adenylyl cyclase 9 (ADCY9)–cAMP signaling in skeletal muscle and plays a critical role in maintaining muscle homeostasis [46]. The identification of cAMP pathway components among the genes regulated by Mdfi suggests potential crosstalk between Mdfi-mediated cell cycle control and cAMP-dependent growth regulation, which warrants further investigation to elucidate the precise molecular mechanisms underlying this interaction.

From a methodological perspective, our successful application of CRISPR/Cas9 technology to generate Mdfi-modified C2C12 cell lines highlights the utility of precise genome editing for dissecting gene function in muscle biology. The CRISPR/Cas9 system is a versatile and efficient gene-editing technology. The high editing efficiency observed in monoclonal clones demonstrates the robustness of this approach for functional studies in myogenic cells [47]. A long fragment of DNA knock-in at the Rosa26 site via the CRISPR/Cas9 system has been achieved in animals such as mice [48], rabbits [49], and goats [50]. In pig fibroblasts, EGFP was transferred to the Rosa26 site of pigs by CRISPR/Cas9 technology, which achieved a positive rate of 29.6% [51]. Using CRISPR/Cas9 plasmids co-transfected with GFP plasmids can identify the function of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in human airway epithelial cells [52]. Co-transfecting

pX330 with pEGFP-N1 was a practical way to indicate the transfection efficiency, and it can also be used for cell sorting [53]. In this study, we used the CRISPR/Cas9 system by co-transfecting pX330-sgRNA with pEGFP-N1 to edit the *Mdfr* gene in the C2C12 cells. Similarly, co-transfecting pX330-sgRNA with pEGFP-N1 has a high transfection efficiency in C2C12 cells, and the transfected cells can be used to identify the *Mdfr*^{-/-} monoclonal clones. Besides, the monoclonal clone sequencing results showed that about 50% (10/20) were genetically modified, indicating that the pX330 vector has high cleavage and gene editing efficiency in C2C12 cells (Fig. 1B). Recent comprehensive reviews recommend multi-method genome-wide analysis combining *in silico* prediction, biochemical assays, and cellular verification [54]. Our sgRNAs were designed using CRISPick, which now integrates with advanced prediction tools like CCLMoff—a transformer-based language model trained on 23 million RNA sequences that outperforms existing off-target prediction methods [55]. For experimental validation, recent studies in myogenic cells demonstrate that CRISPR/Cas9 achieves >60% editing efficiency in C2C12 myotubes with >90% protein reduction [56], and up to 93.8% editing in primary human myoblasts under optimized conditions [57]. Otherwise, in this study, we used a dual sgRNA strategy (sgRNA3 + sgRNA4) targeting different exons to increase knockout efficiency while reducing off-target probability [58,59]. These technical advances, combined with our functional characterization, position *Mdfr* as a potential therapeutic target for modulating muscle regeneration. Future investigations should explore whether manipulation of *Mdfr* expression can enhance muscle repair *in vivo*, particularly in contexts of acute injury or degenerative muscle diseases where accelerated progenitor expansion may be beneficial.

This study has several notable limitations. First, the research was conducted exclusively in the C2C12 immortalized mouse myoblast cell line, which may not fully recapitulate the complex physiological environment of primary muscle satellite cells or *in vivo* muscle regeneration. Second, while CRISPR/Cas9-mediated gene editing was employed, comprehensive off-target effect assessment was not performed. Third, the observed mRNA-protein divergence for certain cell cycle regulators suggests potential post-transcriptional regulation that was not mechanistically explored. Finally, the therapeutic potential of *Mdfr* modulation for muscle regeneration was discussed without direct *in vivo* evidence.

5 Conclusion

In summary, this study establishes *Mdfr* as a critical negative regulator of myoblast proliferation, potentially via the coordinated regulation of cyclin D within the cyclin-CDK-P21 axis and other signaling pathways. Our findings demonstrate that *Mdfr* enforces G1 phase arrest by downregulating *Ccnd1* and *Ccnb1* while upregulating the CDK inhibitor P21, thereby controlling the transition from proliferation to differentiation. The transcriptomic analysis further reveals that *Mdfr* influences multiple signaling networks—including Rap1, calcium, and cAMP pathways—that are essential for skeletal muscle development and regeneration. These results not only advance our understanding of the molecular mechanisms governing muscle cell dynamics but also position *Mdfr* as a potential therapeutic target for enhancing muscle repair in contexts of injury, aging, or degenerative diseases. Future studies utilizing *in vivo* models will be crucial to validate these findings and explore the translational potential of modulating *Mdfr* expression to optimize muscle regenerative outcomes.

Acknowledgement: None.

Funding Statement: This study was funded by the Initiation Funds for High-level Talents Program of Xi'an International University (XAIU202574, XAIU2025117, XAIU202573).

Author Contributions: The authors confirm their contribution to the paper as follows: study conception and design: Yiren Jiao, Zehao Wang and Baozhen Zhou; experimentation: Yiren Jiao, Lingbo Cao and Jia Shi; analysis and interpretation of results: Jing Xu, Hongkai Sun and Xiaoli Ma; draft manuscript preparation: Yiren Jiao. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The datasets presented in this study can be found in online repositories: <http://www.ncbi.nlm.nih.gov/bioproject/679162>.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Supplementary Materials: The supplementary material is available online at <https://www.techscience.com/doi/10.32604/biocell.2026.083738/s1>. Table S1. Sequence of sgRNA oligo pairs and the primer pairs for amplification of the targeting site. Table S2. Primer sequences used for qRT-PCR. Table S3. Primary Antibodies used in the study. Table S4. Secondary antibodies were used in the study. Table S5. Summary of RNA-seq data quality control and read alignment. Table S6. The list of DEGs in Mdfi-OE vs. WT. Table S7. The classified GO terms enriched by DEGs. Table S8. The classified KEGG pathways enriched by DEGs. Fig. S1. The schematic diagram of five sgRNA pairs located in the Mdfi gene. Red box: exons. Yellow box: UTR, the untranslated regions. Black lines: introns. The F1/R1 primer amplifies an Mdfi gene fragment containing the sgRNA1 target site. The F2/R2 primer amplifies an Mdfi gene fragment containing the sgRNA2, sgRNA3, sgRNA4, and sgRNA5 target sites.

References

- Englund DA, Jolliffe A, Aversa Z, Zhang X, Sturmlechner I, Sakamoto AE, et al. p21 induces a senescence program and skeletal muscle dysfunction. *Mol Metab*. 2023;67:101652. [CrossRef].
- Zhou S, Han L, Wu Z. A Long Journey before Cycling: Regulation of quiescence exit in adult muscle satellite cells. *Int J Mol Sci*. 2022;23(3):1748. [CrossRef].
- Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: A changing paradigm. *Nat Rev Cancer*. 2009;9(3):153–66. [CrossRef].
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature*. 1993;366(6456):701–4. [CrossRef].
- Forcina L, Cosentino M, Musarò A. Mechanisms Regulating Muscle Regeneration: Insights into the Interrelated and Time-Dependent Phases of Tissue Healing. *Cells*. 2020;9(5):1297. [CrossRef].
- Chen CM, Kraut N, Groudine M, Weintraub H. I-mf, a novel myogenic repressor, interacts with members of the MyoD family. *Cell*. 1996;86(5):731–41. [CrossRef].
- Kraut N, Snider L, Chen CM, Tapscott SJ, Groudine M. Requirement of the mouse I-mfa gene for placental development and skeletal patterning. *EMBO J*. 1998;17(21):6276–88. [CrossRef].
- Martindill DM, Risebro CA, Smart N, Franco-Viseras Mdel M, Rosario CO, Swallow CJ, et al. Nucleolar release of Hand1 acts as a molecular switch to determine cell fate. *Nat Cell Biol*. 2007;9(10):1131–41. [CrossRef].
- Tsuji K, Kraut N, Groudine M, Noda M. Vitamin D₃ enhances the expression of I-mfa, an inhibitor of the MyoD family, in osteoblasts. *Biochim Biophys Acta*. 2001;1539(1–2):122–30. [CrossRef].
- Ong EC, Nesin V, Long CL, Bai CX, Guz JL, Ivanov IP, et al. A TRPC1 protein-dependent pathway regulates osteoclast formation and function. *J Biol Chem*. 2013;288(31):22219–32. [CrossRef].
- Mizugishi K, Hatayama M, Tohmunda T, Ogawa M, Inoue T, Mikoshiba K, et al. Myogenic repressor I-mfa interferes with the function of Zic family proteins. *Biochem Biophys Res Commun*. 2004;320(1):233–40. [CrossRef].
- Oakley RH, Busillo JM, Cidlowski JA. Cross-talk between the glucocorticoid receptor and MyoD family inhibitor domain-containing protein provides a new mechanism for generating tissue-specific responses to glucocorticoids. *J Biol Chem*. 2017;292(14):5825–44. [CrossRef].
- Ma Y, Sun W, Zhao L, Yao M, Wu C, Su P, et al. Generation of an mESC model with a human hemophilia B nonsense mutation via CRISPR/Cas9 technology. *Stem Cell Res Ther*. 2022;13(1):353. [CrossRef].
- Huang B, Jiao Y, Zhu Y, Ning Z, Ye Z, Li QX, et al. Mdfi promotes C2C12 cell differentiation and positively modulates fast-to-slow-twitch muscle fiber transformation. *Front Cell Dev Biol*. 2021;9:605875. [CrossRef].

15. Esper ME, Saber J, Rudnicki MA. Satellite cells in Regeneration and disease. *Cold Spring Harb Perspect Biol.* 2026;18(2):a041474. [[CrossRef](#)].
16. Zhang H, Zhou M, Zhang Z, Wang Z, Shi R, Wang Y, et al. CHAMP1 is an essential regulator for human myoblast fusion and muscle development. *Nat Commun.* 2026;17(1):546. [[CrossRef](#)].
17. Nguyen MT, Ly QK, Ngo THP, Lee W. Calponin 3 regulates myoblast proliferation and differentiation through actin cytoskeleton remodeling and YAP1-mediated signaling in myoblasts. *Cells.* 2025;14(2):142. [[CrossRef](#)].
18. Lwin ZM, Yip GW, Chew FT, Bay BH. Downregulation of ER60 protease inhibits cellular proliferation by inducing G1/S arrest in breast cancer cells *in vitro*. *Anat Rec.* 2012;295(3):410–6. [[CrossRef](#)].
19. McGill CJ, Brooks G. Cell cycle control mechanisms and their role in cardiac growth. *Cardiovasc Res.* 1995;30(4):557–69. [[CrossRef](#)].
20. Gaffey MJ, Iezzoni JC, Meredith SD, Boyd JC, Stoler MH, Weiss LM, et al. Cyclin D1 (PRAD1, CCND1) and glutathione-S-transferase pi gene expression in head and neck squamous cell carcinoma. *Hum Pathol.* 1995;26(11):1221–6. [[CrossRef](#)].
21. Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G. Cyclin A is required at two points in the human cell cycle. *EMBO J.* 1992;11(3):961–71. [[CrossRef](#)].
22. Besson A, Dowdy SF, Roberts JM. CDK inhibitors: Cell cycle regulators and beyond. *Dev Cell.* 2008;14(2):159–69. [[CrossRef](#)].
23. Li J, Chen C, Bi X, Zhou C, Huang T, Ni C, et al. DNA methylation of CMTM3, SSTR2, and MDFI genes in colorectal cancer. *Gene.* 2017;630:1–7.
24. Ma H, Chen X, Hu H, Li B, Ying X, Zhou C, et al. Hypermethylation of MDFI promoter with NSCLC is specific for females, non-smokers and people younger than 65. *Oncol Lett.* 2018;15(6):9017–24. [[CrossRef](#)].
25. Hüntel S, Kaller M, Drepper F, Oeljeklaus S, Bonfert T, Erhard F, et al. p53-regulated networks of protein, mRNA, miRNA, and lncRNA expression revealed by integrated pulsed stable isotope labeling with amino acids in cell culture (pSILAC) and next generation sequencing (NGS) analyses. *Mol Cell Proteom.* 2015;14(10):2609–29. [[CrossRef](#)].
26. Guo K, Wang J, Andrés V, Smith RC, Walsh K. MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol Cell Biol.* 1995;15(7):3823–9. [[CrossRef](#)].
27. Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, et al. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science.* 1995;267(5200):1018–21. [[CrossRef](#)].
28. Guimarães D, Barrios NMF, de Oliveira AG, Rizo-Roca D, Jollet M, Smith JAB, et al. Concerted regulation of skeletal muscle metabolism and contractile properties by the orphan nuclear receptor Nr2f6. *J Cachexia Sarcopenia Muscle.* 2024;15(4):1335–47. [[CrossRef](#)].
29. Amin MS, Yu B, Wu D, Lu Y, Wu W, Wang J, et al. Zein-derived peptides from corn promote the proliferation of C2C12 myoblasts via crosstalk of mTORC1 and mTORC2 signaling pathways. *Foods.* 2024;13(6):919. [[CrossRef](#)].
30. Pizon V, Cifuentes-Diaz C, Mège RM, Baldacci G, Rieger F. Expression and localization of RAP1 proteins during myogenic differentiation. *Eur J Cell Biol.* 1996;69(3):224–35.
31. Sanvee GM, Hitzfeld L, Bouitbir J, Krähenbühl S. mTORC2 is an important target for simvastatin-associated toxicity in C2C12 cells and mouse skeletal muscle—Roles of Rap1 geranylgeranylation and mitochondrial dysfunction. *Biochem Pharmacol.* 2021;192:114750. [[CrossRef](#)].
32. Abdelrahman Z, Wang X, Wang D, Zhang T, Zhang Y, Wang X, et al. Identification of novel pathways and immune profiles related to sarcopenia. *Front Med.* 2023;10:928285. [[CrossRef](#)].
33. Li J, Chen Z, Bai Y, Wei Y, Guo D, Liu Z, et al. Integration of ATAC-seq and RNA-seq analysis to identify key genes in the Longissimus Dorsi muscle development of the Tianzhu white Yak. *Int J Mol Sci.* 2023;25(1):158. [[CrossRef](#)].
34. Tu MK, Levin JB, Hamilton AM, Borodinsky LN. Calcium signaling in skeletal muscle development, maintenance and regeneration. *Cell Calcium.* 2016;59(2–3):91–7. [[CrossRef](#)].
35. Tu MK, Borodinsky LN. Spontaneous calcium transients manifest in the regenerating muscle and are necessary for skeletal muscle replenishment. *Cell Calcium.* 2014;56(1):34–41. [[CrossRef](#)].
36. Chin ER. The role of calcium and calcium/calmodulin-dependent kinases in skeletal muscle plasticity and mitochondrial biogenesis. *Proc Nutr Soc.* 2004;63(2):279–86. [[CrossRef](#)].
37. Gherardi G, Weiser A, Bermont F, Migliavacca E, Brinon B, Jacot GE, et al. Mitochondrial calcium uptake declines during aging and is directly activated by oleuropein to boost energy metabolism and skeletal muscle performance. *Cell Metab.* 2025;37(2):477–95.e11. [[CrossRef](#)].

38. Li X, Zhao X, Qin Z, Li J, Sun B, Liu L. Regulation of calcium homeostasis in endoplasmic reticulum-mitochondria crosstalk: Implications for skeletal muscle atrophy. *Cell Commun Signal*. 2025;23(1):17. [[CrossRef](#)].
39. Lu T, Zhu Y, Guo J, Mo Z, Zhou Q, Hu CY, et al. MDFI regulates fast-to-slow muscle fiber type transformation via the calcium signaling pathway. *Biochem Biophys Res Commun*. 2023;671:215–24. [[CrossRef](#)].
40. Berdeaux R, Stewart R. cAMP signaling in skeletal muscle adaptation: Hypertrophy, metabolism, and regeneration. *Am J Physiol Endocrinol Metab*. 2012;303(1):E1–17. [[CrossRef](#)].
41. Dalle S, Schouten M, Meeus G, Slagmolen L, Koppo K. Molecular networks underlying cannabinoid signaling in skeletal muscle plasticity. *J Cell Physiol*. 2022;237(9):3517–40. [[CrossRef](#)].
42. Ifegwu OC, Awale G, Rajpura K, Lo KW, Laurencin CT. Harnessing cAMP signaling in musculoskeletal regenerative engineering. *Drug Discov Today*. 2017;22(7):1027–44. [[CrossRef](#)].
43. Tong T, Kim M, Park T. α -Ionone attenuates high-fat diet-induced skeletal muscle wasting in mice via activation of cAMP signaling. *Food Funct*. 2019;10(2):1167–78. [[CrossRef](#)].
44. Lautherbach N, Gonçalves DAP, Silveira WA, Paula-Gomes S, Valentim RR, Zanon NM, et al. Urocortin 2 promotes hypertrophy and enhances skeletal muscle function through cAMP and insulin/IGF-1 signaling pathways. *Mol Metab*. 2022;60:101492. [[CrossRef](#)].
45. Angelino E, Bodo L, Sartori R, Malacarne V, D'Anna B, Formaggio N, et al. Impaired cAMP-PKA-CREB1 signalling drives mitochondrial dysfunction in skeletal muscle during cancer cachexia. *Nat Metab*. 2025;7(12):2548–70. [[CrossRef](#)].
46. Li H, Wang P, Zhang C, Zuo Y, Zhou Y, Han R. Defective BVES-mediated feedback control of cAMP in muscular dystrophy. *Nat Commun*. 2023;14(1):1785. [[CrossRef](#)].
47. Lyu G, Li P, Lang W. A review of recent studies on CRISPR/Cas9-mediated genome editing in a variety of muscle-related genetic disorders. *J Transl Med*. 2025;23(1):1381. [[CrossRef](#)].
48. Chu VT, Weber T, Graf R, Sommermann T, Petsch K, Sack U, et al. Efficient generation of Rosa26 knock-in mice using CRISPR/Cas9 in C57BL/6 zygotes. *BMC Biotechnol*. 2016;16:4. [[CrossRef](#)].
49. Yang D, Song J, Zhang J, Xu J, Zhu T, Wang Z, et al. Identification and characterization of rabbit ROSA26 for gene knock-in and stable reporter gene expression. *Sci Rep*. 2016;6:25161. [[CrossRef](#)].
50. Wu M, Wei C, Lian Z, Liu R, Zhu C, Wang H, et al. Rosa26-targeted sheep gene knock-in via CRISPR-Cas9 system. *Sci Rep*. 2016;6:24360. [[CrossRef](#)].
51. Xie Z, Pang D, Wang K, Li M, Guo N, Yuan H, et al. Optimization of a CRISPR/Cas9-mediated knock-in strategy at the Porcine Rosa26 Locus in Porcine Foetal Fibroblasts. *Sci Rep*. 2017;7(1):3036. [[CrossRef](#)].
52. Bellec J, Bacchetta M, Losa D, Anegón I, Chanson M, Nguyen TH. CFTR inactivation by lentiviral vector-mediated RNA interference and CRISPR-Cas9 genome editing in human airway epithelial cells. *Curr Gene Ther*. 2015;15(5):447–59. [[CrossRef](#)].
53. Roy A, Goodman JH, Begum G, Donnelly BF, Pittman G, Weinman EJ, et al. Generation of WNK1 knockout cell lines by CRISPR/Cas-mediated genome editing. *Am J Physiol Renal Physiol*. 2015;308(4):F366–76. [[CrossRef](#)].
54. Angelini Stewart A, Ahrens-Nicklas RC, Tsai SQ, Musunuru K, Giannikopoulos P, Clelland CD. Measurement and clinical interpretation of CRISPR off-targets. *Nat Genet*. 2026;58(1):20–7. [[CrossRef](#)].
55. Du W, Zhao L, Diao K, Zheng Y, Yang Q, Zhu Z, et al. A versatile CRISPR/Cas9 system off-target prediction tool using language model. *Commun Biol*. 2025;8(1):882. [[CrossRef](#)].
56. Thürkau M, Lin S, Oliveri F, Grimm D, Platt RJ, Rüegg MA. Fast, multiplexable and efficient somatic gene deletions in adult mouse skeletal muscle fibers using AAV-CRISPR/Cas9. *Nat Commun*. 2023;14(1):6116. [[CrossRef](#)].
57. Goullée H, Taylor RL, Forrest ARR, Laing NG, Ravenscroft G, Clayton JS. Improved CRISPR/Cas9 gene editing in primary human myoblasts using low confluency cultures on Matrigel. *Skeletal muscle*. 2021;11(1):23. [[CrossRef](#)].
58. Fierro J Jr, DiPasquale J, Perez J, Chin B, Chokapone Y, Tran AM, et al. Dual-sgRNA CRISPR/Cas9 knockout of PD-L1 in human U87 glioblastoma tumor cells inhibits proliferation, invasion, and tumor-associated macrophage polarization. *Sci Rep*. 2022;12(1):2417. [[CrossRef](#)].
59. Hua Z, Xu K, Xiao W, Shu C, Li N, Li K, et al. Dual single guide RNAs-mediating deletion of mature myostatin peptide results in concomitant muscle fibre hyperplasia and adipocyte hypotrophy in pigs. *Biochem Biophys Res Commun*. 2023;673:145–52. [[CrossRef](#)].