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ZBTB18-mediated STAT1 transcriptional repression contributes to bovine myogenesis, implying an association with oxidative myofiber formation and beef eating quality

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ABSTRACT

Skeletal muscle development, orchestrated by muscle stem cells (MuSCs), is a critical determinant of meat yield and quality in cattle. Transcription factors (TFs) are pivotal regulators of MuSCs fate, yet their specific roles in meat quality remain largely unexplored. In this study, we integrated ATAC-seq and RNA-seq analyses to delineate the chromatin accessibility landscape and transcriptomic dynamics during bovine MuSCs myogenesis, identifying *ZBTB18* as a core TF dynamically regulated in this process. *ZBTB18* expression was higher in muscles enriched with oxidative myofibers and positively correlated with beef eating quality-related parameters. Functional assays demonstrated that *ZBTB18* inhibits MuSCs proliferation while promoting myogenic differentiation, with a specific bias toward oxidative myofiber formation. Mechanistically, we established that *ZBTB18* acts as a transcriptional repressor of *STAT1*. Our findings unveil *ZBTB18* as a novel regulator of bovine myogenesis via transcriptional repression of *STAT1*. Notably, *ZBTB18* expression is significantly associated with oxidative myofiber formation and beef eating quality. These findings also provide a strategic target for precision breeding to enhance meat quality in cattle.

1. Introduction

The development of skeletal muscle fundamentally determines the efficiency and quality of meat production (Zhang, Pan, Feng, et al., 2022). Muscle growth and regeneration are driven by muscle stem cells (MuSCs). Muscle stem cells first differentiate into myoblasts, which subsequently fuse to form multinucleated myofibers and further differentiate into mature myofibers (Wang et al., 2018). Although the number of myofibers is largely determined at birth, postnatal myofiber hypertrophy and myofiber type conversion, regulated by muscle stem cell activity, are crucial for determining final meat yield and quality (Qaisar et al., 2016). It is well known that the proliferation and differentiation of MuSCs also involve the regulation of transcription factors, with some myogenic regulatory factors (MRFs) playing a leading role, such as *MyOD1*, *Myf5*, and *MRF4* (Kassar-Duchossoy et al., 2004; Vicente-Garcia et al., 2022). Studies have shown that *Pax7* is a hallmark gene for MuSCs to maintain stemness (Abou-Khalil et al., 2015; Peault et al., 2007), and *Pax7* and *Pax3* affect the early stability, migration and myogenic differentiation of MuSCs (de Morree et al., 2019). However, the broader upstream transcriptional networks associated with skeletal muscle development have not yet been fully mapped.

Recent advances in multi-omics, particularly the integration of ATAC-seq and RNA-seq technologies, have identified key transcription factors (TFs) involved in processes such as lipid deposition (Xu et al., 2022), neurological diseases (Guo et al., 2023), and cancer (Bai et al., 2024). Notably, regarding skeletal muscle development, previous studies have revealed that *ZNF384*, *KLF6*, *EGR1* and *RHOB* promote myogenic differentiation in myoblasts (Cai et al., 2023; Li et al., 2022).

Abbreviations: MuSCs, muscle stem cells; TFs, transcription factors; GM, bovine MuSCs at proliferation phase; DM, bovine MuSCs at myogenic differentiation phase; ATAC-seq, the assay for transposase-accessible chromatin with high-throughput sequencing; RT-qPCR, Real-time Quantitative Reverse Transcription PCR; FBS, fetal bovine serum; HS, horse serum; WB, Western Blotting; EdU, 5-Ethynyl-2′-deoxyuridin.

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Integrated multi-omics analyses have become a powerful tool for elucidating the transcriptional regulatory mechanisms of myogenesis, providing a reliable technical framework for identifying core regulatory factors.

ZBTB18, also known as *RP58*, or *ZNF238*, was first discovered as a transcription repressor, and is highly conserved in mice and humans (Hemming et al., 2019; Okado, 2021). It was the first protein reported to be associated with chromatin condensation, and it has been proved to have the function of sequence-specific transcriptional repression (Aoki et al., 1998; Meng et al., 2000). In previous studies, *ZBTB18* has been shown to play an important role in neurological diseases (Kvarnung et al., 2020). However, in the research of muscle development, only studies have shown that *ZBTB18* can promote mouse muscle development by targeting *ID2* and *ID3* (Yokoyama et al., 2009). By integrating ATAC-seq and transcriptomic data, we found that *ZBTB18* may be a key transcription factor in the myogenic differentiation of MuSCs. However, the function of *ZBTB18* in bovine myogenesis, particularly its potential role in guiding the early specialization of muscle fiber types, remains entirely unknown.

Here, we hypothesized that *ZBTB18* is a critical regulator of bovine MuSCs fate and meat quality. By integrating ATAC-seq and RNA-seq, we identified *ZBTB18* as a differentiation-enriched TF. We subsequently correlated its expression with beef quality traits, defined its dual role in regulating MuSCs proliferation and myogenic differentiation, and elucidated *STAT1* as its direct functional downstream target. Our work bridges fundamental myogenesis with agricultural application, providing novel insights and a potential target for improving meat quality through genetic strategies.

2. Materials and methods

2.1. Ethical standards

All animal procedures complied with the institutional animal welfare guidelines and were strictly approved by the Institutional Animal Care and Use Committee at the College of Animal Science and Technology, Guangxi University (Approval No. Gxu-2021-087). No human subjects were involved.

2.2. Cell culture

Muscle tissues from Guangxi cattle at approximately 90 days of gestation were collected from a local abattoir (Nanning, China) for MuSCs isolation. Bovine MuSCs were isolated from the fetal longissimus thoracis muscle utilizing a sequential type I collagenase and trypsin digestion method, as previously described (Zhang, Pan, Zou, et al., 2022). The isolated MuSCs were cultured in proliferation medium consisting of high-glucose DMEM, 20% fetal bovine serum (FBS), and 1% penicillin-streptomycin premix (100×) (all purchased from Gibco, Waltham, MA, USA). When cell confluence reached 90%, the cells were switched to a differentiation medium (DMEM supplemented with 2% horse serum) and cultured for up to 4 days to induce myogenic differentiation. All cultures were maintained in a humidified environment at 37 °C and 5% CO_2.

2.3. Total RNA extraction and purification

Total RNA from tissues and cultured cells was extracted using TRIzol™ reagent (Life Technologies, Mt. Waverley, VIC) according to the manufacturer's protocol. RNA concentration and purity (A260/A280 ratio) were quantified using an ND-1000 UV–Vis spectrophotometer (Agilent, Santa Clara, CA, USA). Samples were stored at −80 °C prior to cDNA synthesis.

2.4. Combined analysis of ATAC-seq and RNA-seq data

To map chromatin accessibility dynamics, ATAC-seq libraries were constructed using MuSCs in the proliferation phase (GM) and on day 4 of differentiation (DM). Sequencing was executed by Annoroad Gene Technology (Beijing, China) according to previously detailed procedures (Buenostro et al., 2015; Su et al., 2024).

To systematically screen for critical myogenic TFs, we integrated the ATAC-seq differential accessibility peaks with our previously published RNA-seq dataset (Zhang, Pan, Zou, et al., 2022). The obtained ATAC-seq differential gene set was compared with the RNA-seq differential gene set, and the three parts of genes that were simultaneously differentially expressed and differentially open, only differentially expressed but not differentially open, and only differentially open but not differentially expressed were screened. Among them, genes that were simultaneously differentially expressed and differentially open were used as our candidate genes. Then Motif enrichment analysis of TFs was performed for gene groups that were significantly up-regulated in DM cells and gene groups that were significantly up-regulated in GM cells, respectively. Finally, the enrichment ranking of the core TFs obtained was performed.

2.5. Bovine muscle collection and meat quality evaluation

For mature meat quality evaluation, five male Guangxi cattle at 36 months of age were euthanized and slaughtered. Longissimus thoracis muscle tissues from Guangxi cattle and Simmental cattle at fetal and adult stages were also collected, which were only used for gene expression analysis and did not involve meat quality-related detection. Muscle samples encompassing distinct anatomical regions (scapular transverse, semitendinosus, pectoralis, forelimb, hindlimb, longissimus thoracis, and gluteus) were rapidly excised. Evaluated macroscopic phenotypes included pH, shear force, lightness (L*), redness (a*), yellowness (b*), myofiber density, and myofiber diameter. The detailed determination protocol was referred to the previous study (Boukha et al., 2011). According to the beef quality grading (NY/T676–2010, Ministry of Agriculture of the People's Republic of China), the indexes of high quality beef are low shear force, high L^* value and a^* value, low b^* value, small myofiber diameter and high density.

2.6. Real-time quantitative reverse transcription PCR (RT-qPCR)

The cDNA was prepared according to the HiScript®III RT Super Mix (ABclonal, RK20408, Wuhan, China) for qPCR. Quantitative PCR experiments were performed on an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The cDNA samples ($N = 3$) were prepared according to the requirements of the ChamQ Universal SYBR qPCR Master Mix (ABclonal, RK21219, Wuhan, China). The relative expression level of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method using β-actin as the reference gene. The detail of primer information in this study are provided in Table S1 of Supplementary tables in the Data availability.

2.7. Cell viability and proliferation assays

Cellular viability and DNA synthesis rates were determined using the Cell Counting Kit-8 (Vazyme, A311–01, Nanjing, China) and the 5-Ethynyl-2′-deoxyuridine incorporation assay (RiboBio, C10310–1, Guangzhou, China), respectively, strictly adhering to the manufacturers' guidelines.

2.8. Vector construction and cell transfection

The full-length coding sequences (CDS) of *ZBTB18* and *STAT1* were PCR-amplified and cloned into the pcDNA3.1+ backbone to generate overexpression vectors (pcDNA3.1-ZBTB18 and pcDNA3.1-STAT1). A

targeted shRNA against *ZBTB18* (shZBTB18) was constructed using the psi-LVRU6GP vector (GeneCopoeia, Guangzhou, China). For routine functional assays, logarithmic-phase MuSCs were transfected with respective plasmids using Lipofectamine™ 3000 (Thermo Fisher Scientific, USA). Cells were harvested at least 24 h post-transfection for downstream phenotyping.

2.9. Flow cytometry

Cell cycle progression and apoptosis were quantified via flow cytometry (FACSCanto II, BD Biosciences, USA). According to the manufacturer's protocol, harvested cells were processed using the Cell Cycle Testing Kit (Multisciences, Hangzhou, China). Apoptosis rates were determined utilizing the Annexin V-FITC/PI Apoptosis Kit (Multisciences); cells were suspended in binding buffer and co-incubated with Annexin V-FITC and propidium iodide (PI) for 10 min in the dark prior to acquisition.

2.10. Immunofluorescence

The cell samples were washed twice in PBS buffer and fixed with 100 μL 4% paraformaldehyde at room temperature for 45 min. After washing, the cells were permeabilized with 1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin (BSA). Cells were incubated overnight at 4 °C with MyHC antibody (1100, ABclonal, A12964SP, Wuhan, China), Pax7 antibody (1100, ABclonal, A7335, Wuhan, China) and ZBTB18 antibody (1100, ABclonal, Wuhan, China). Following PBS washes, cells were incubated with fluorophore-conjugated secondary antibodies for 2 h at room temperature. Nuclei were counterstained with 10 μg/mL DAPI (ABclonal, RM02978, Wuhan, China) for 10 min. The fusion index was calculated as the percentage of nuclei residing within multinucleated (≥2 nuclei) myosin-positive syncytia.

2.11. Western blotting (WB)

Total cellular protein was extracted using RIPA lysis buffer supplemented with 1% PMSF (Beyotime, P0013B, ST506, Shanghai, China). Protein concentrations were normalized using a BCA Protein Assay Kit (Beyotime, P0012, Shanghai, China). Equal amounts of protein lysates were resolved via 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were probed overnight at 4 °C with primary antibodies against CDK2, PCNA, MyHC, ZBTB18, and β-actin (Abcam, Cambridge, MA, USA). Following secondary antibody incubation, immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and quantified via a ChemiDoc XRS+ imaging system (Bio-Rad, USA).

2.12. Dual-luciferase reporter system assay

Seven potential *ZBTB18* binding sites were predicted in the *STAT1* promoter region (a 1809-bp fragment upstream of the transcription initiation site) by online software (http://jaspar.genereg.net). The fragment was amplified and ligated into the pGL3-Promoter luciferase reporter vector (Table S2 of Supplementary tables in the Data availability), which was recorded as pGL3-STAT1. pcDNA3.1-ZBTB18 (experimental group) or the empty pcDNA3.1 (control group) was co-transfected into 293 T cells with pGL3-STAT1 and the internal control pRL-TK, respectively. For validation, pGL3-Basic and pGL3-Promoter reporters were also co-transfected under the same conditions. For transfection in a 24-well plate, the amounts per well were 500 ng reporter plasmid, 500 ng pcDNA3.1-ZBTB18 (or pcDNA3.1), and 50 ng pRL-TK. All transfections were performed in triplicate, and results were confirmed by at least three independent biological replicates. Twenty-four hours after transfection, dual-luciferase activity was measured according to the instructions for the Dual-Luciferase Report Gene Assay Kit (GeneCopoeia, LF004, Guangzhou, China).

2.13. Statistical analysis

To account for potential variability among individual animals, a linear mixed model was also applied, with muscle type as a fixed effect and animal ID as a random effect. Correlation analysis was conducted between *ZBTB18* expression and meat quality parameters or myofiber type markers. All experiments in this study were performed in at least three biological replicates. The quantitative data in the figures are presented as mean ± SEM. One-way ANOVA performed with GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze significant variations of different treatments. $P < 0.05$ was considered significant difference and marked with *, $P < 0.01$ and $P < 0.001$ were considered extremely significant difference and marked with ** and ***, respectively, or bars with different letters indicated a significant difference ($P < 0.05$).

3. Results

3.1. Bovine MuSCs have the phenotypic characteristics of stem cells and myogenic differentiation ability

Bovine MuSCs derived from fetal muscle and cultured in vitro exhibited a characteristic spindle-shaped morphology and retained high proliferation potential (Fig. 1A, B), as evidenced by immunofluorescence detection of the MuSCs-specific marker Pax7 (Fig. 1C). Upon exposure to myogenic differentiation medium for 4 days, MuSCs underwent differentiation into myotubes, with subsequent fusion into multinucleated myotubes (Fig. 1B). The differentiation process was further supported by immunofluorescence detection of MyHC, a key marker of myogenic commitment (Fig. 1C).

Concurrently, the relative gene expression levels in both GM and DM cells were analyzed. RT-qPCR revealed that mRNA levels of proliferation-related genes (*PCNA*, *BCL-2*, *CDK2*, and *CyclinD1*) were markedly elevated in GM cells relative to DM cells (Fig. 1D, $P < 0.001$). In contrast, mRNA expression levels of myogenic differentiation-related genes (*MyOD1*, *MyOG*, and *MyHC*) were significantly upregulated in DM cells (Fig. 1E, $P < 0.001$). Consistent with these transcriptional changes, WB results demonstrated that the protein expression levels of proliferation-related genes CDK2 and PCNA were significantly higher in GM cells compared to DM cells (Fig. 1F, G, $P < 0.01$). Additionally, MyHC exhibited significantly higher expression in DM cells (Fig. 1F, G, $P < 0.001$). Overall, these bovine MuSCs demonstrate the defining characteristics of stem cells, including self-renewal capacity, proliferative ability, and myogenic differentiation potential.

3.2. Identification of chromatin openness and TFs during bovine MuSCs myogenic differentiation

To characterize the chromatin accessibility landscape during bovine MuSCs proliferation and myogenic differentiation, we integrated ATAC-seq with RNA-seq data for a comprehensive analysis of functional TFs. The results revealed dynamic changes in ATAC signal peaks during myogenic differentiation (Fig. 2A), with a total of 234,955 open chromatin regions identified (Fig. 2B). These open peaks were predominantly distributed in introns, gene intervals, promoters, and coding regions (Fig. 2C). Notably, chromosomal heterogeneity in ATAC signal intensity was observed, with distinct patterns of open chromatin across different chromosomes (Fig. 2D). By combining ATAC-seq, RNA-seq, and gene annotation, we demonstrated a strong correlation between gene expression and chromatin accessibility during myogenic differentiation (Fig. 2E). Furthermore, specific open peak regions associated with proliferation and differentiation—termed differentially accessible regions (DARs)—were identified (Fig. 2F). Among 2296 differentially expressed genes, 158 DARs were detected, of which 101 (63.92%) exhibited increased chromatin accessibility during myogenic differentiation (Fig. 2G). Motif analysis of promoter-associated DARs revealed

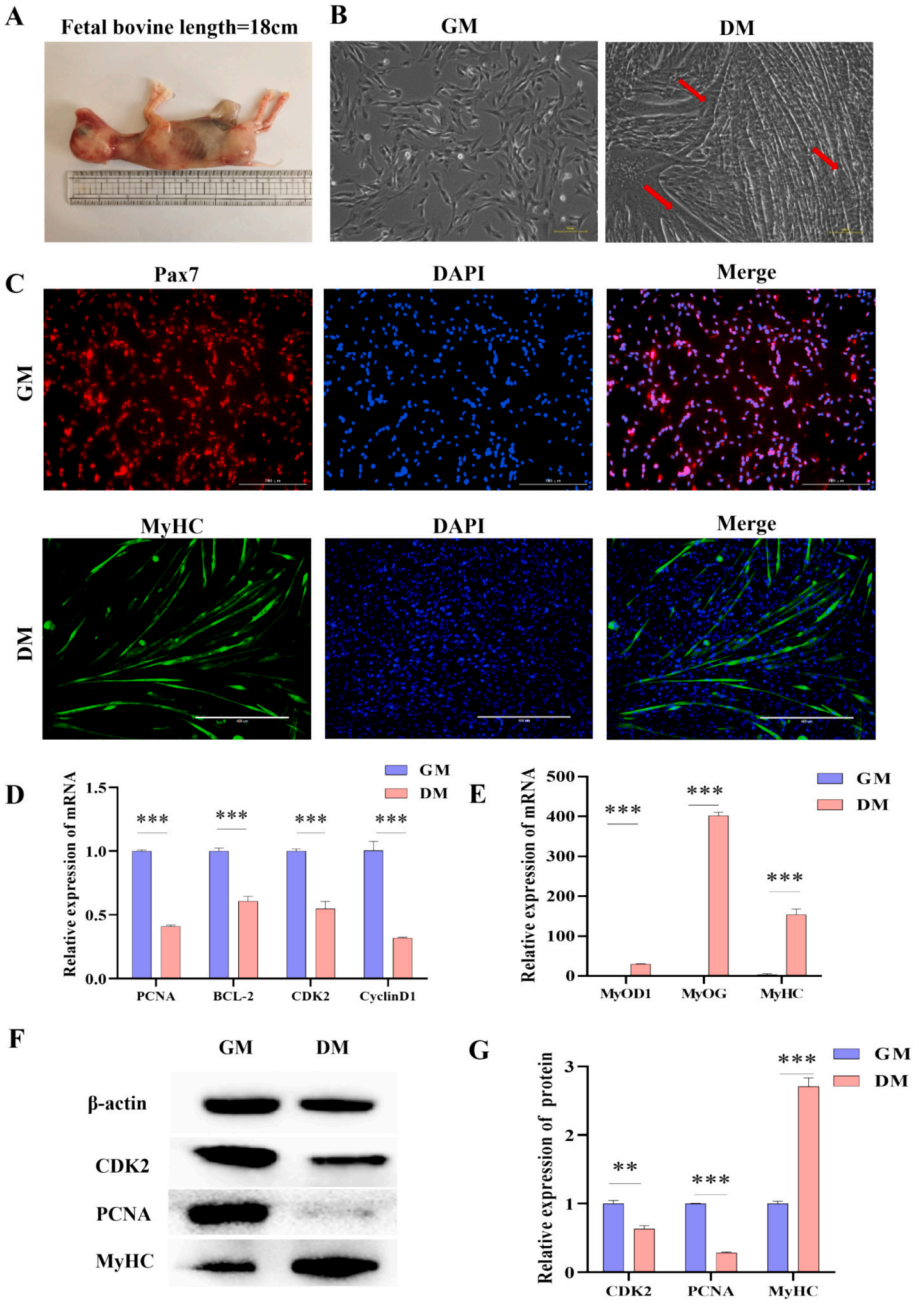

Fig. 1. Identification of proliferation and differentiation characteristics of bovine MuSCs. (A) Fetal cattle used to isolate and culture MuSCs. (B) Cellular morphological characteristics of MuSCs during the proliferation (GM) and differentiation (DM) phases. (C) Immunofluorescence determined the GM cells expressed MuSCs marker gene *PAX7* and the DM cells expressed myogenic differentiation marker gene *MyHC*. (D, E) RT-qPCR determined the mRNA expression level of proliferation-related genes (*PCNA*, *BCL-2*, *CDK2* and *CyclinD1*) and myogenic differentiation-related genes (*MyOD1*, *MyOG*, and *MyHC*) in GM cells and DM cells. (F, G) WB determined the protein expression levels of CDK2, PCNA and MyHC in GM cells and DM cells. The data are expressed as the means ± SEMs of three individuals. $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered extremely significant difference. Scale bar: 400 μm. The red arrow points to the myotubes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1047 enriched TFs (Data availability: GM-motif and DM-motif) critical for MuSCs development. Functional enrichment sequencing identified key candidate TFs: *KLF5*, *ZBTB18*, *MyOD1*, and *Twist2* for myogenic differentiation (Fig. 2H), and *Eip74EF*, *TEAD4*, *Barhl1*, and *ELK4* for proliferation (Fig. 2I). Given the established roles of *KLF5* and *MyOD1* in muscle development, we prioritized *ZBTB18* as a novel regulator of myogenic differentiation for further investigation.

3.3. The expression pattern of ZBTB18 is related to beef quality

To date, the association between *ZBTB18* and beef quality remains unexplored. To investigate this relationship, we evaluated meat quality parameters in multiple cattle muscles, including the transverse scapular, semitendinosus, pectoralis, forelimb, hindlimb, longissimus thoracis, and gluteus muscles. Among the seven muscles examined, we observed significant differences in meat quality parameters (Fig. 3A–D). The hindlimb, longissimus thoracis, and pectoralis muscles exhibited lower shear force, higher a* values, smaller myofiber diameter, and higher

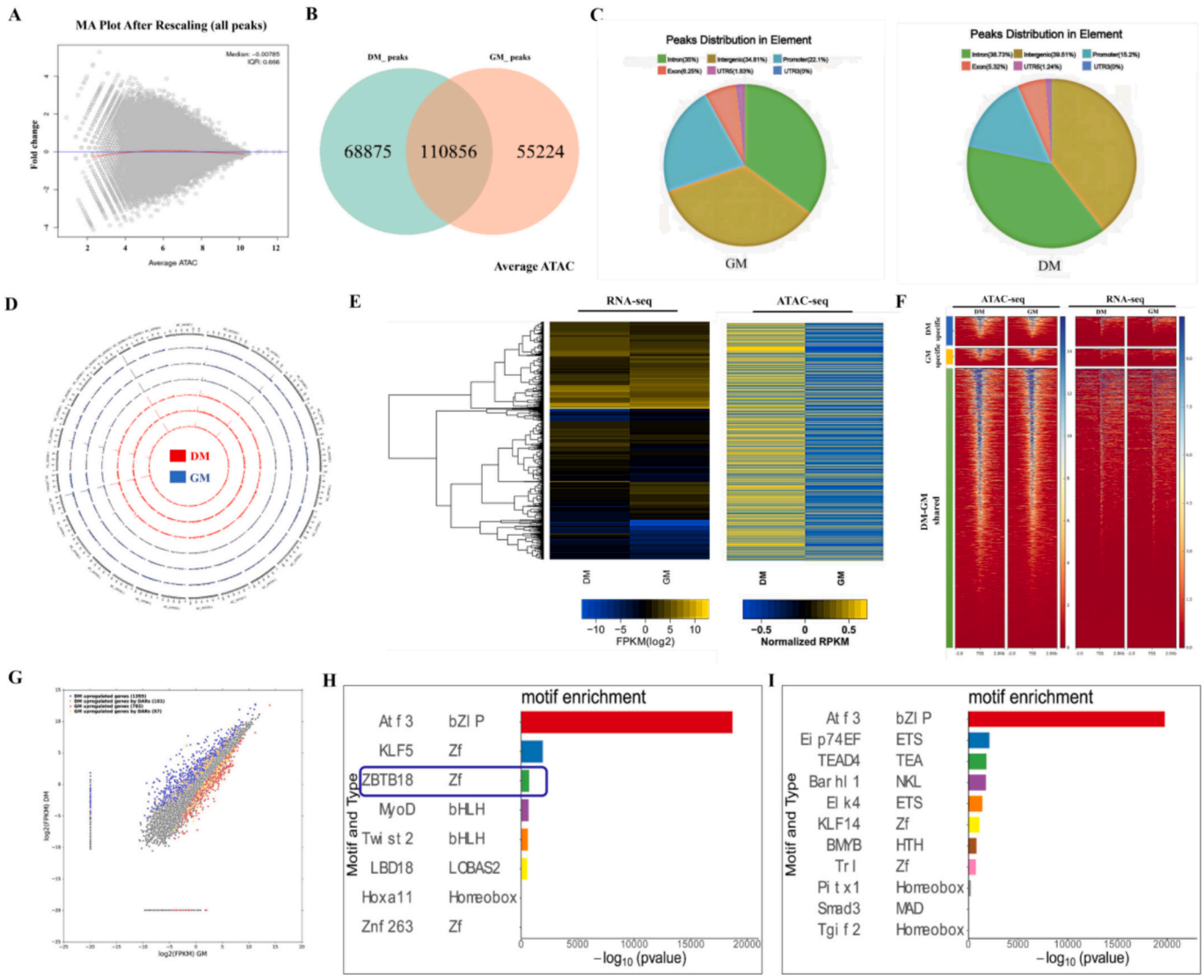

Fig. 2. Chromatin openness mapping of bovine MuSCs in proliferation and myogenic differentiation phase, and integrated analysis of ATAC-seq and RNA-seq data. (A) ATAC signal peaks during the myogenic differentiation of bovine MuSCs. (B) Overlapping analysis of open peaks in proliferation (GM) and myogenic differentiation (DM) groups. (C) Genome-wide distribution of all open peaks. (D) The degree of openness of the ATAC signal peaks on different chromosomes. (E) Integrated ATAC-seq and RNA-seq data to analyze the the correlation between chromatin openness and myogenic differentiation. (F) Hierarchical clustering heat map of DARs. (G) Correlation analysis between DAR and myogenic differentiation of MuSCs. (H) Enrichment analysis of TFs associated with myogenic differentiation. (I) Enrichment analysis of TFs associated with proliferation.

myofiber density compared to the forelimb and gluteus muscles. In contrast, the gluteus and forelimb muscles showed higher shear force and b* values, indicative of more glycolytic properties. These variations reflect the normal physiological specialization of muscles with different functions. Oxidative myofiber markers (*MyHC I* and *MyHC IIa*) were predominantly enriched in the pectoralis and hindlimb muscles, while the glycolytic myofiber marker (*MyHC IIb*) was primarily enriched in the gluteus muscle (Fig. 3E–H). Notably, *ZBTB18* expression levels were significantly higher in the pectoralis, longissimus thoracis, and hindlimb muscles compared to the forelimb and gluteus muscles (Fig. 3I).

(A) Determination of beef quality in different body parts of Guangxi cattle (L*: lightness, a*: redness, b*: yellowness). Different lowercase letters in the same column indicate a statistically significant difference ($P < 0.05$) (B) Masson staining of muscle tissues from different body parts of Guangxi cattle. (C) The myofiber density of muscle tissues from different body parts of Guangxi cattle. (D) The myofiber diameter of muscle tissues from different body parts of Guangxi cattle. (E–I) The relative expression levels of *MyHC I, MyHC IIa*, *MyHC IIx*, *MyHC IIb* and *ZBTB18* in muscle tissues. The data are expressed as the means ± SEMs of three individuals. Different letters in the table or on the bars indicated a significant difference ($P < 0.05$). Scale bar: 100 μm.

3.4. *ZBTB18 inhibits the proliferation of bovine MuSCs*

To elucidate the functional role of *ZBTB18* in bovine MuSCs proliferation, we first characterized its spatiotemporal expression. *ZBTB18* mRNA and protein levels were significantly upregulated in differentiated myoblasts (DM) compared to proliferating myoblasts (GM) (Fig. 4A–C, $P < 0.001$). Immunofluorescence staining further confirmed its nuclear localization in DM cells (Fig. 4D). Subsequently, gain-of-function and loss-of-function experiments were conducted to validate its role.

To investigate the role of *ZBTB18* in bovine MuSCs proliferation, a recombinant overexpression vector encoding *ZBTB18* was transfected into MuSCs (Fig. 4E, $P < 0.001$). Following overexpression, the following observations were made: EdU staining revealed a significant

A

Position	pH	Shear force (N/mm^2)	Lightness (L^*)	Redness (a*)	Yellowness (b*)
Scapularis muscle	6.93^{a}±0.33	37.98^{bc}±3.79	30.91^{b}±0.18	12.21^{a}±0.76	7.57^{ab}±0.52
Semitendinosus	6.47^{ab}±0.11	33.16^{c}±5.63	28.03^{b}±0.82	10.08^{a}±1.46	6.63^{bc}±0.40
Pectoralis	6.74^{ab}±0.20	38.15^{bc}±9.07	31.55^{ab}±2.33	10.35^{a}±0.37	5.36^{d}±0.69
Forelimb	6.81^{a}±0.02	49.29^{b}±6.47	35.28^{a}±2.20	11.91^{a}±1.42	8.90^{a}±0.39
Hindlimb	6.84^{a}±0.05	30.31^{c}±2.71	29.81^{b}±0.62	8.66^{a}±0.98	6.57^{bc}±0.11
Longissimus thoracis	6.45^{ab}±0.29	32.84^{c}±0.34	35.81^{a}±2.13	9.42^{a}±1.22	5.06^{d}±0.94
Gluteus	6.26^{b}±0.45	62.60^{a}±1.49	30.99^{b}±0.91	11.30^{a}±2.81	7.24^{ab}±0.83

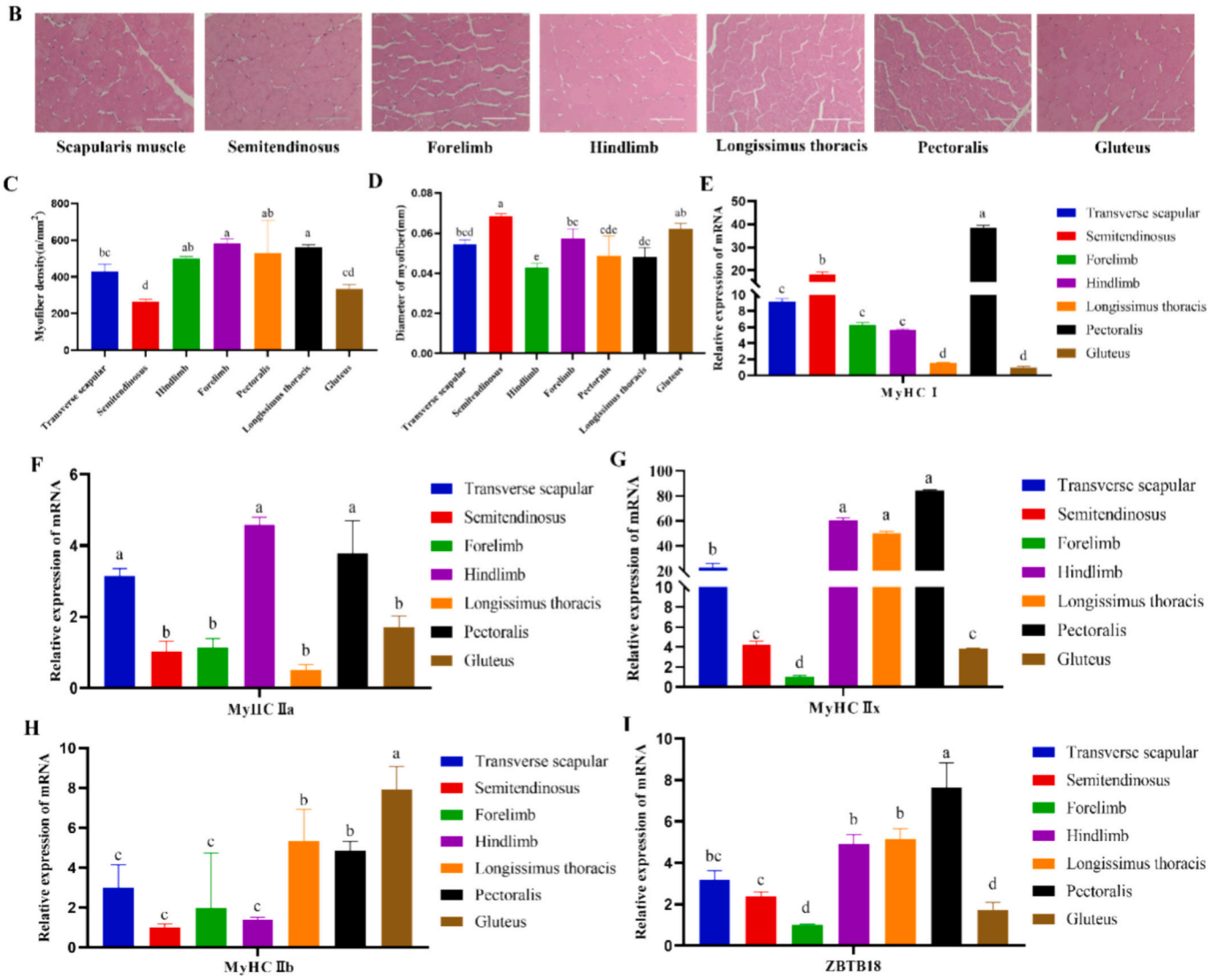

Fig. 3. Correlation analysis between *ZBTB18* and beef quality.

reduction in the proportion of proliferating cells (Fig. 4F, G, $P < 0.01$). Cell-cycle analysis demonstrated a marked increase in G0/G1 phase populations and a corresponding decrease in S and G2 phase populations (Fig. 4H, I, $P < 0.05$). Notably, CCK-8 assays confirmed a significant decline in cell viability (Fig. 4J, $P < 0.05$). Additionally, RT-qPCR and WB analyses showed that *ZBTB18* overexpression downregulated mRNA levels of *PCNA*, *CyclinD1*, and *BCL-2* (Fig. 4K, $P < 0.01$), and reduced protein expression of PCNA and CDK2 (Fig. 4L–M, $P < 0.05$).

Conversely, the effects of *ZBTB18* silencing on bovine MuSCs proliferation were evaluated using RNAi experiments (Fig. 5A, $P < 0.01$).

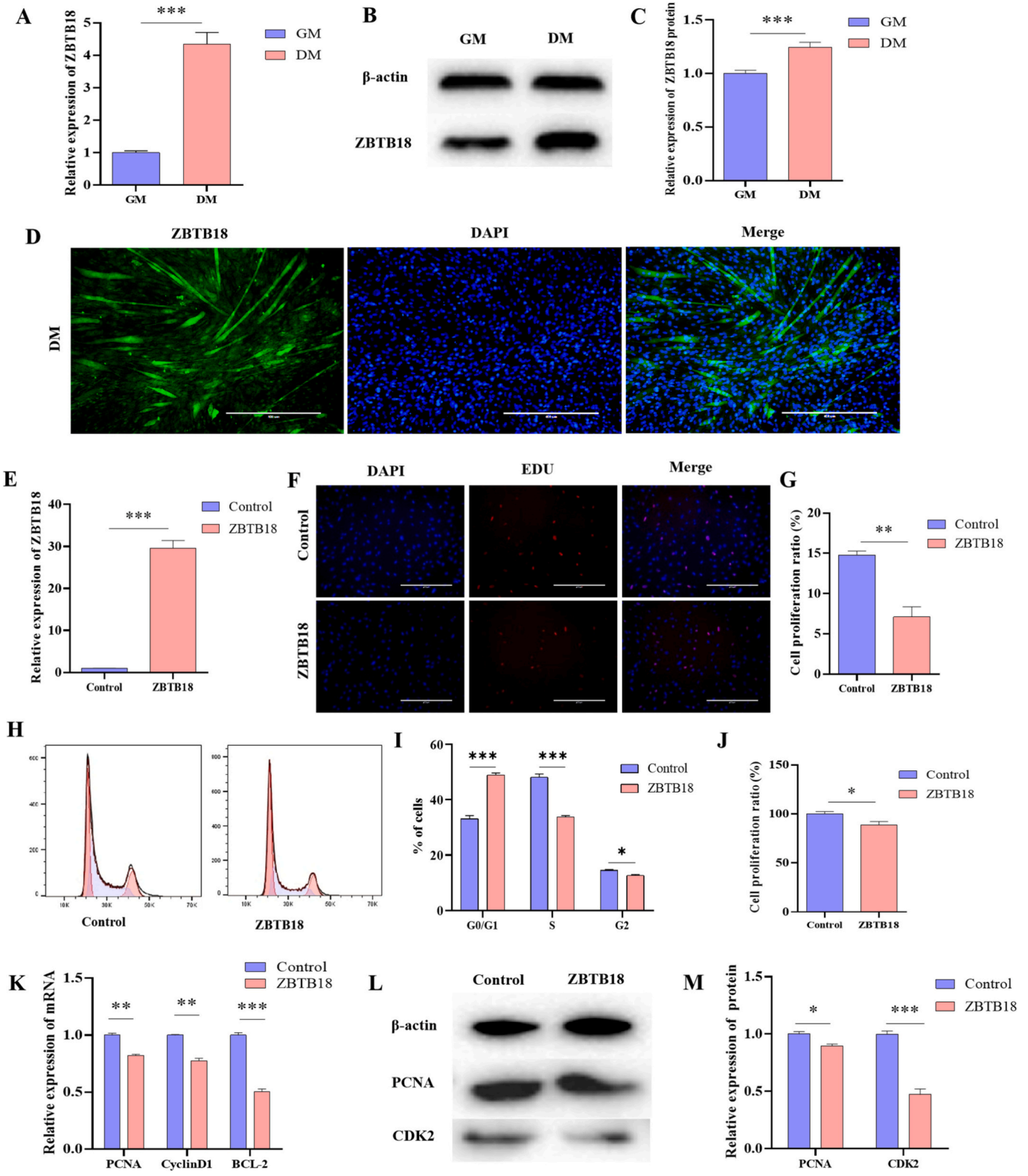

Fig. 4. Overexpression of *ZBTB18* inhibits proliferation of MuSCs. (A) The relative mRNA expression level of *ZBTB18* in GM and DM of MuSCs. (B, C) The relative protein expression level of ZBTB18 in GM and DM of MuSCs. (D) Immunofluorescence determined the localization of ZBTB18 protein in DM cells. (E) RT-qPCR determined the overexpression efficiency of *ZBTB18*. (F, G) EdU assay assessed proliferation of MuSCs. (H, I) Cell cycle assays of MuSCs. (J) CCK-8 assay assessed the viability of MuSCs. (K) The relative mRNA expression levels of *PCNA*, *CyclinD1* and *BCL-2* in MuSCs. (L, M) The relative protein expression levels of PCNA and CDK2 in MuSCs. The data are expressed as the means ± SEMs of three individuals. $^{*}P < 0.05$ was considered significant difference, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered extremely significant difference. Scale bar: 400 μm.

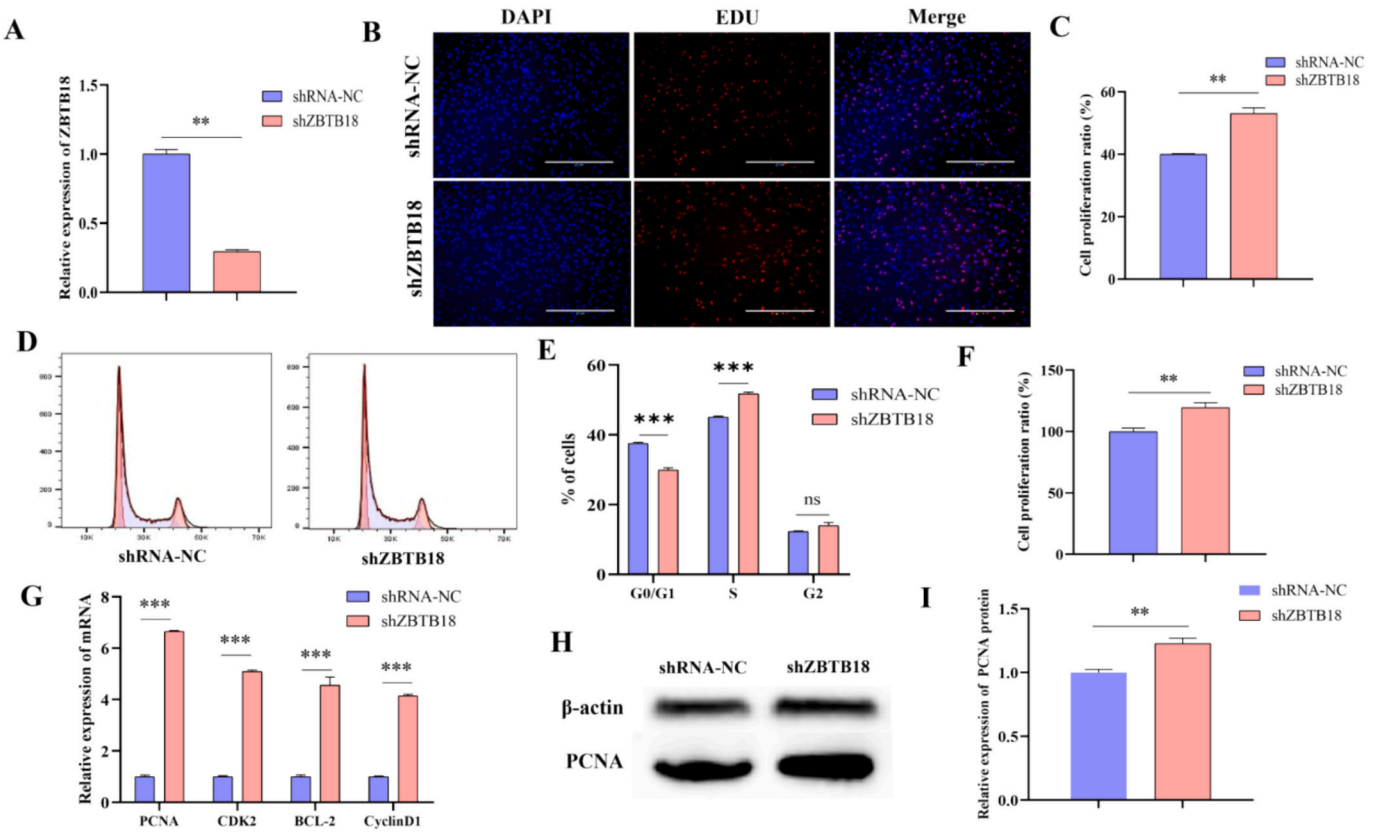

Fig. 5. Knockdown of *ZBTB18* promotes proliferation of MuSCs. (A) RT-qPCR determined the silencing efficiency of *ZBTB18*. (B, C) EdU assay assessed proliferation of MuSCs. (D, E) Cell cycle assays of MuSCs. (F) CCK-8 assay assessed the viability of MuSCs. (G) The relative mRNA expression levels of *PCNA*, *CDK2*, *BCL-2* and *CyclinD1* in MuSCs. (H, I) The relative protein expression level of PCNA in MuSCs. The data are expressed as the means ± SEMs of three individuals. ns on the bar indicates no significant difference. $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered extremely significant difference. Scale bar: 400 μm.

The silencing group exhibited the following outcomes compared to the control: EdU assays revealed significantly increased cell proliferation (Fig. 5B, C, $P < 0.01$). Notably, flow cytometry analysis demonstrated reduced G0/G1 phase populations and elevated S phase populations (Fig. 5D, E, $P < 0.001$). And, CCK-8 assays also revealed significantly increased cell proliferation (Fig. 5F, $P < 0.01$), alongside enhanced cell viability. Additionally, RT-qPCR and WB analyses showed upregulation of cell proliferation- and cell cycle-related genes at both mRNA (*PCNA*, *CyclinD1*) and protein (PCNA, CDK2) levels (Fig. 5G–I, $P < 0.01$).

Collectively, these findings demonstrate that *ZBTB18* acts as a negative regulator of bovine MuSCs proliferation by modulating cell-cycle progression and suppressing key proliferative pathways. This study provides a molecular basis for understanding muscle development and highlights the potential of *ZBTB18* as a target for improving beef production efficiency.

3.5. *ZBTB18 promotes the oxidative myofiber differentiation of bovine MuSCs*

To elucidate the functional role of *ZBTB18* in myogenic differentiation, we transfected MuSCs with a recombinant overexpression vector encoding *ZBTB18*. Immunofluorescence staining revealed a significant increase in the number of MyHC-positive myotubes following *ZBTB18* overexpression, along with enhanced myotube fusion rates (Fig. 6A, B, $P < 0.001$). Consistent with this, RT-qPCR and WB analyses showed significantly higher mRNA expression of *MyOG*, *Myf5,* and *MyHC* and elevated protein levels of MyHC in the overexpression group compared to the control (Fig. 6C–E, $P < 0.05$). Furthermore, *ZBTB18* overexpression markedly increased the mRNA and protein expression of MyHC IIx and MyHC IIa, but significantly decreased those of MyHC IIb, with no notable effect on MyHC I expression (Fig. 6F–H). These results indicate that *ZBTB18* biases the myogenic differentiation program toward intermediate and oxidative myofiber identities.

Conversely, RNAi-mediated silencing of *ZBTB18* markedly impaired myogenic differentiation, as evidenced by a pronounced reduction in MyHC-positive myotubes and a significant decrease in myotube fusion rates (Fig. 7A, B, $P < 0.001$). Accordingly, both mRNA (*MyOG*, *Myf5*, and *MyHC*) and protein (MyHC) levels were significantly downregulated (Fig. 7C–E, $P < 0.05$). These findings were inversely correlated with the overexpression results. Importantly, *ZBTB18* silencing significantly reduced the expression of MyHC IIx and MyHC IIa and increased that of MyHC IIb, without affecting MyHC I (Fig. 7F–H). These results further support the conclusion that silencing *ZBTB18* inhibits the myogenic differentiation program toward the phenotypes of intermediate and oxidative muscle fibers.

Collectively, these data demonstrate that *ZBTB18* acts as a positive regulator of oxidative myofiber differentiation in bovine MuSCs.

3.6. *ZBTB18 targets STAT1 to regulate myogenic differentiation of bovine MuSCs*

Previously, the downstream target genes of *ZBTB18* in myogenic differentiation of bovine MuSCs remained undefined. Gene Transcription Regulation Database (GTRD, http://gtrd.biouml.org/) and the Cistrome Data Browser (Cistrome DB, http://cistrome.org/db) were used to predict the potential target genes of *ZBTB18*. Through the bioinformatic integration of two databases, 68 potential target genes were identified (Fig. 8A), and their functional relevance to muscle development was analyzed. Subsequently, *STAT1* was selected as the candidate target gene of *ZBTB18* for further validation (Fig. 8A). The JASPAR

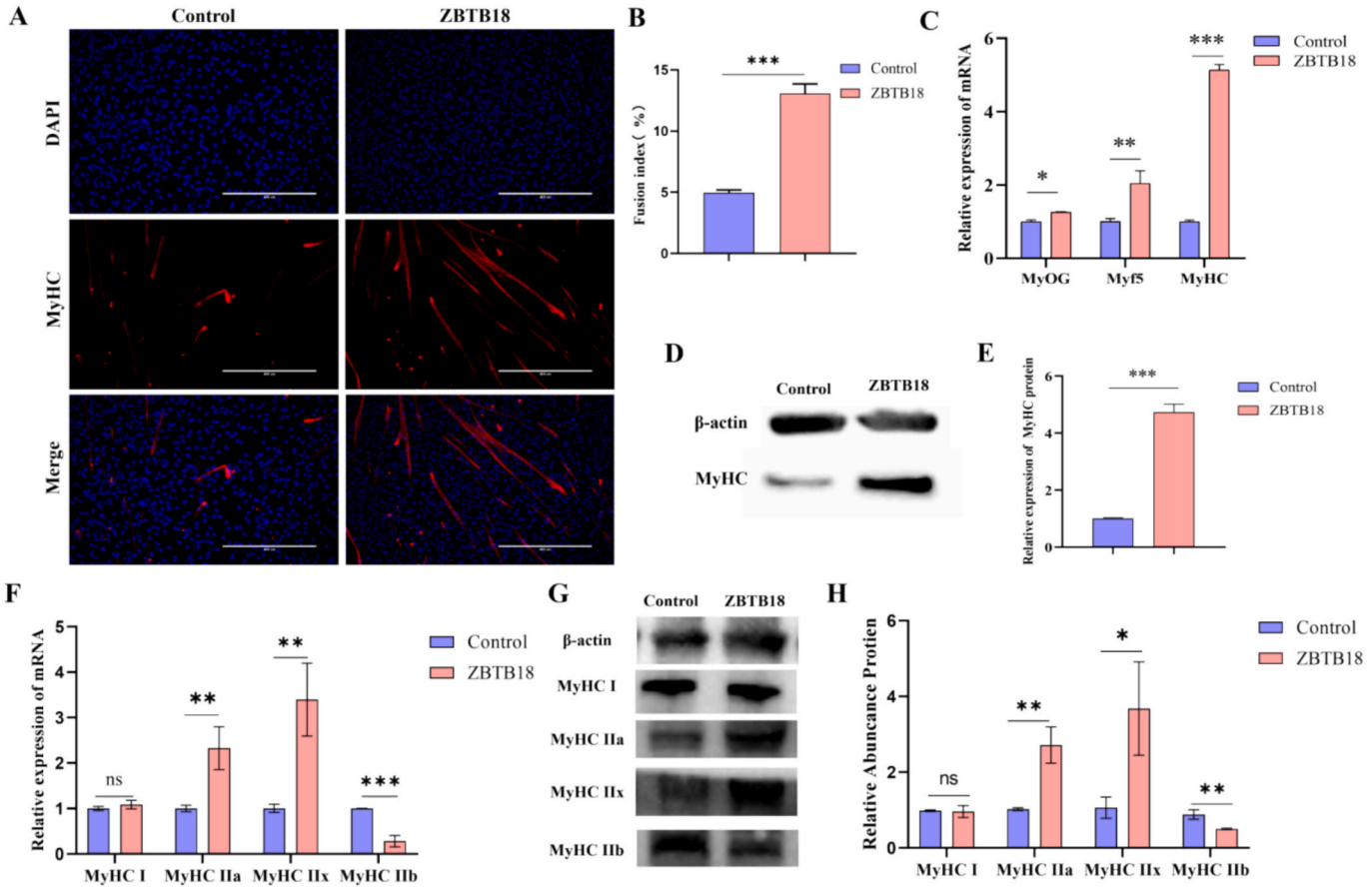

Fig. 6. Overexpression of *ZBTB18* promotes oxidative myofiber differentiation of MuSCs. (A) Number of myotubes labeled with MyHC immunofluorescence during myogenic differentiation after *ZBTB18* overexpression in MuSCs. (B) Fusion index is calculated as the percentage of nuclei in myosin+ myotubes. Structures containing at least two nuclei are considered as a myotube. (C) The relative mRNA expression levels of *MyOG*, *Myf5* and *MyHC* during myogenic differentiation. (D, E) The relative protein expression level of MyHC during myogenic differentiation. (F) The relative mRNA expression levels of *MyHC I*, *MyHC IIa*, *MyHC IIx* and *MyHC IIb* during myogenic differentiation after *ZBTB18* overexpression in MuSCs. (G, H) The relative protein expression level of MyHC I, MyHC IIa, MyHC IIx and MyHC IIb. The data are expressed as the means ± SEMs of three individuals. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ were considered extremely significant difference. Scale bar: 400 μm.

database predicted the presence of *ZBTB18* binding sites in the *STAT1* promoter region, indicating a potential direct regulatory interaction between them (Table S3 of Supplementary tables in the Data availability).

To validate this hypothesis, a Dual-Luciferase Reporter Assay was performed to investigate the targeting relationship between *STAT1* and *ZBTB18*. The results demonstrated that *ZBTB18* overexpression significantly suppressed *STAT1* transcriptional activity compared to the control vector (Fig. 8B, $P < 0.001$). Subsequently, RT-qPCR was employed to validate the regulatory effect of *ZBTB18* on *STAT1* expression levels. Consistent with the reporter assay, *STAT1* expression was markedly decreased following *ZBTB18* overexpression and significantly upregulated upon *ZBTB18* knockdown (Fig. 8C, $P < 0.001$). Collectively, these findings demonstrate that *ZBTB18* directly targets the *STAT1* promoter to modulate its expression.

To further validate the role of *STAT1* in MuSCs myogenic differentiation, we first compared its expression levels in GM and DM cells, as well as in fetal and adult bovine longissimus thoracis muscles. The results revealed that *STAT1*, *ZBTB18*, and *MyHC* exhibited a consistent expression pattern, with elevated levels in DM cells and muscle tissues (Fig. 7D–G, $P < 0.05$). These findings indicate that *STAT1*, like *ZBTB18*, was closely associated with MuSCs myogenic differentiation. To directly assess the functional impact of *STAT1*, a *STAT1* overexpression vector was constructed and validated (Fig. 8H, $P < 0.001$). Following *STAT1* overexpression, the number of MyHC-positive myotubes was significantly reduced, and myotube fusion rates were markedly decreased (Fig. 8I, J, $P < 0.001$). Additionally, the expression of key myogenic differentiation markers—*MyOD1*, *MyoG* and *MyHC*—was markedly downregulated (Fig. 8K, $P < 0.001$). Collectively, these results demonstrate that *STAT1* suppresses MuSCs myogenic differentiation, whereas *ZBTB18* promotes differentiation by inhibiting *STAT1* transcriptional activity.

4. Discussion

As the primary drivers of muscle formation, MuSCs directly regulate meat yield and meat quality traits. Identifying molecular regulators of beef quality and delineating their mechanisms in MuSCs proliferation/myogenic differentiation are therefore essential for precision breeding. To address this, we integrated ATAC-seq and RNA-seq during bovine MuSCs proliferation and myogenic differentiation phases, mapping chromatin accessibility landscapes and transcriptomic dynamics. This multi-omics approach revealed *ZBTB18* as a key TF modulating muscle development. Notably, *ZBTB18* expression strongly correlated with superior meat quality metrics. Functional validation confirmed that it has a dual effect of inhibiting the proliferation of bovine MuSCs and promoting myogenic differentiation. Mechanistically, *ZBTB18* represses the transcription of its downstream target *STAT1*, which likely represents a key step in directing early oxidative myofiber formation.

Although the roles of canonical MRFs in muscle development are

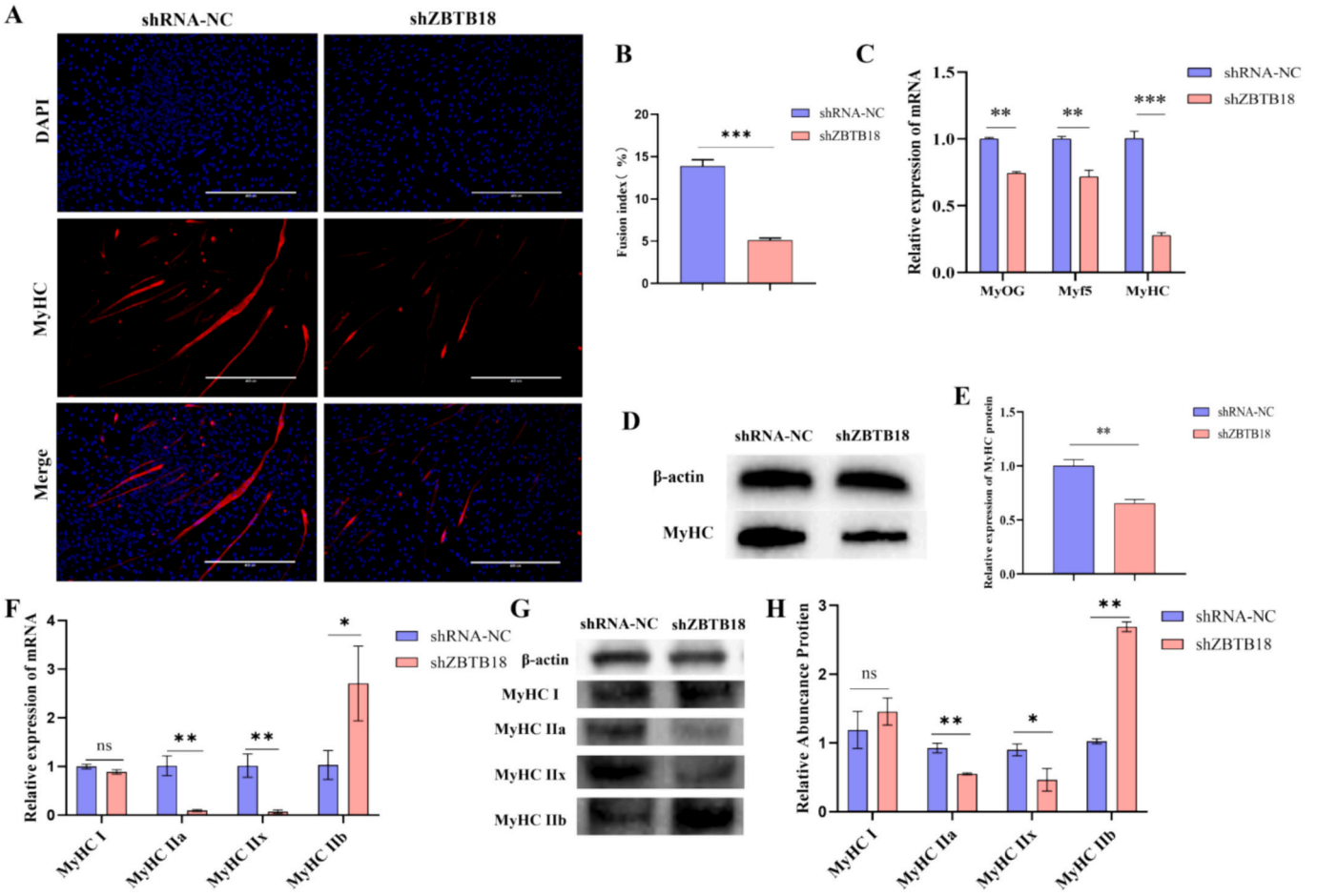

Fig. 7. Knockdown of *ZBTB18* inhibits oxidative myofiber differentiation of MuSCs.
(A) Number of myotubes labeled with MyHC immunofluorescence during myogenic differentiation after *ZBTB18* silencing in MuSCs. (B) Fusion index. (C) The relative mRNA expression levels of *MyOG*, *Myf5* and *MyHC* during myogenic differentiation. (D, E) The relative protein expression level of MyHC during myogenic differentiation. (F) The relative mRNA expression levels of *MyHC I*, *MyHC IIa*, *MyHC IIx* and *MyHC IIb* during myogenic differentiation. (G, H) The relative protein expression level of MyHC I, MyHC IIa, MyHC IIx and MyHC IIb. The data are expressed as the means ± SEMs of three individuals. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ were considered extremely significant difference. Scale bar: 400 μm.

well-characterized, the functions of many core TFs remain elusive. Recent advances highlight functionally diverse core TFs: *Tcf12* acts as an essential coordinator of human muscle regeneration through *MyOD1* synergy (Wang, Liao, et al., 2022), whereas *Sox11*—though differentiation-enriched—is functionally redundant in regeneration (Oprescu et al., 2023). Conversely, *Osr1* depletion disrupts murine myogenic differentiation, underscoring its non-redundant role (Kotsaris et al., 2023). Integrated single-cell RNA-seq, ATAC-seq, and functional validation recently identified *EGR1* and *RHOB* as key regulators of porcine embryonic myogenesis (Li et al., 2022). Similarly, ATAC-seq/RNA-seq analyses have uncovered core TFs governing bovine myoblast proliferation and differentiation (Wang, Li, et al., 2022), though systematic functional verification remains limited. In this study, we mapped genome-wide chromatin accessibility landscapes in bovine MuSCs during proliferation and myogenic differentiation phases. ATAC-seq revealed 55,224 proliferation-specific and 68,875 differentiation-specific accessible regions. Integrated analysis with RNA-seq identified 2296 differentially expressed genes and 158 DARs. Motif enrichment analysis of promoter-associated DARs predicted 1047 transcription factors implicated in MuSCs development, including 525 myogenesis-related candidates. *KLF5*, *ZBTB18*, *MyOD1*, and *Twist2* emerged as the most significantly enriched factors, suggesting their roles as core myogenic regulators. Given KLF5's established function in mammalian muscle development (Hayashi et al., 2016; Zhang et al., 2020; Zhang, Zhang, Chen, et al., 2022), We prioritized *ZBTB18*, which is the second-most enriched yet underexplored factor, for mechanistic investigation in bovine myogenesis.

Transcription factors have been demonstrated to be major regulators of meat quality. Key transcription factors such as *MEF2*, *MyOD1*, *FOXO1*, and *FOXO4* modulate meat attributes by orchestrating muscle fiber-type conversion (Hughes et al., 1993; Kitamura et al., 2007; Wu et al., 2001). Similarly, *MSTN* (myostatin) acts as a critical negative regulator of muscle growth, with recent studies confirming that *MSTN* mutations alter pork quality via impacts on leanness, fat deposition, and tenderness (Gao et al., 2024). In this study, we pioneer the discovery that *ZBTB18* expression positively correlates with beef quality. Regional analysis of Guangxi cattle revealed distinct physiological specializations across muscle types. Specifically, the hindlimb, pectoralis, and longissimus thoracis exhibited favorable meat quality parameters, such as lower shear force and higher a* values, whereas the forelimb and gluteus demonstrated contrasting characteristics. This variation reflects physiological specialization rather than a strict quality hierarchy. Consistently, *ZBTB18* expression was elevated in cuts exhibiting favorable meat quality parameters, specifically the hindlimb, pectoralis, and longissimus thoracis, when compared to the forelimb and gluteal muscles. Notably, *ZBTB18* levels exhibited a positive correlation with oxidative/intermediate myofiber content and a negative correlation with glycolytic myofibers. Furthermore, *ZBTB18* promotes the

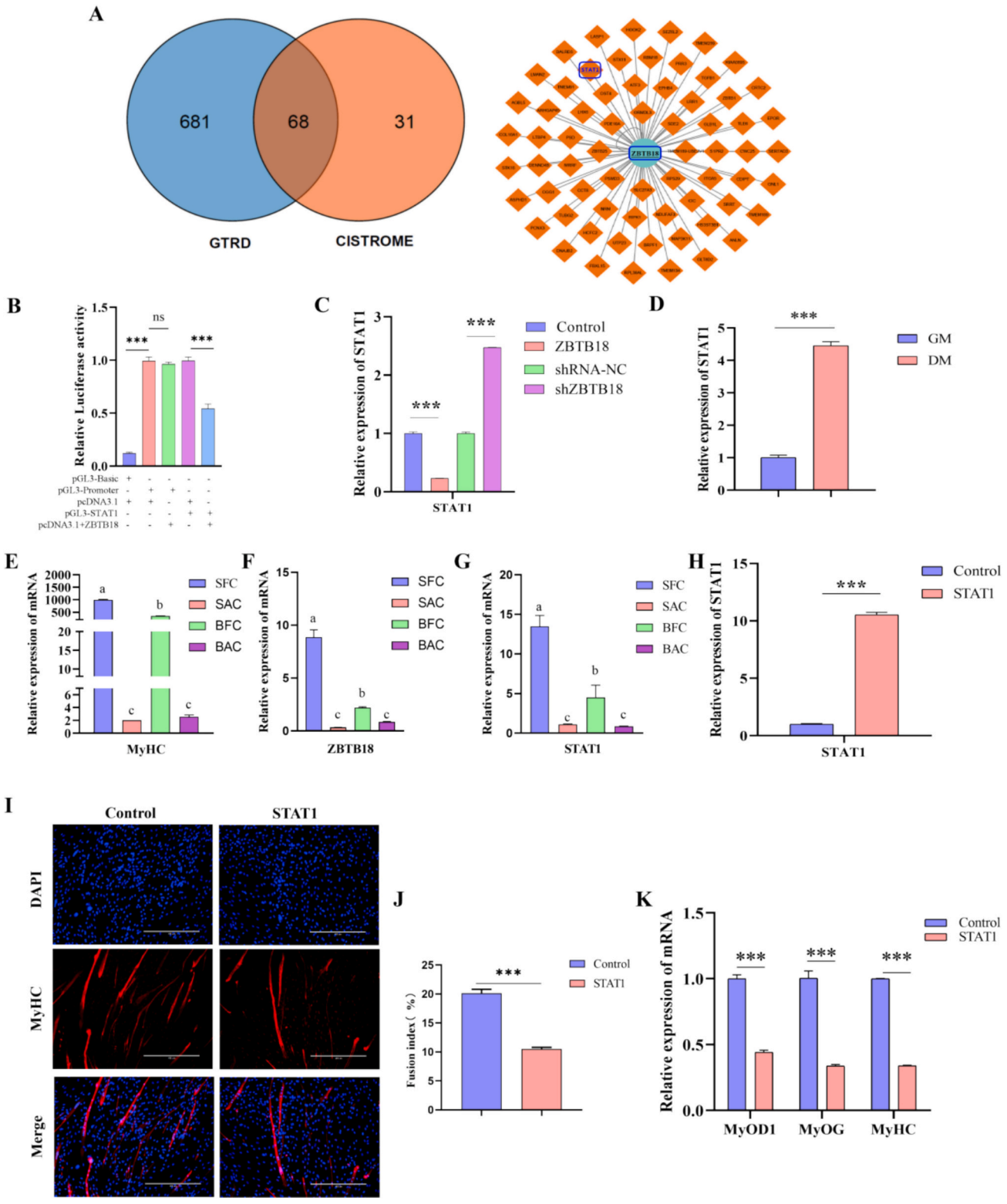

Fig. 8. *ZBTB18* affects MuSCs myogenic differentiation by targeting *STAT1*.

(A) Overlapping analysis potential target genes of *ZBTB18* through two different online prediction software GTRD and Cistrome DB. (B) Verification of the targeting relationship between *ZBTB18* and *STAT1* by dual-luciferase reporter assay. (C) The relative mRNA expression level of *STAT1* after *ZBTB18* overexpression or silencing. (D) The relative mRNA expression level of *STAT1* in GM and DM of MuSCs. (*E*-G) Expression trend analysis of *STAT1*, *ZBTB18* and *MyHC* in longissimus thoracis muscle of fetal and adult Guangxi local cattle (fetus: BFC; adult: BAC) and Simmental cattle (fetus: SFC; adult: SAC). (H) RT-qPCR determined the over-expression efficiency of *STAT1*. (I) Number of myotubes labeled with MyHC immunofluorescence during myogenic differentiation after STAT1 overexpression in MuSCs. (J) Fusion index. (K) The relative mRNA expression levels of *MyOD1*, *MyOG* and *MyHC* during myogenic differentiation. The data are expressed as the means ± SEMs of three individuals. Different letters in the table or on the bars indicated a significant difference ($P < 0.05$). ns on the bar indicates no significant difference. ***$P < 0.001$ were considered extremely significant difference. Scale bar: 400 μm.

generation of intermediate and oxidizing myofibers subtypes. Given that oxidative myofibers enhance meat quality through higher mitochondrial density (Lee et al., 2010), and myoglobin content—yielding brighter redness and improved tenderness (Raj et al., 2010; Xin et al., 2018), *ZBTB18* may serve as a key candidate gene for precision breeding of high-quality beef.

MuSCs exhibit two defining characteristics: sustained proliferation to maintain the stem cell pool, and commitment to myogenic differentiation (Wu & Yue, 2024). The balance between these processes critically regulates skeletal muscle development. Previous studies established that *P2Y1R/P2Y2R* stimulate MuSCs proliferation to drive muscle growth (Wang et al., 2023), and *mTOR/Fzd7* jointly promote both proliferation and differentiation (Xu & Velleman, 2023). In this study, functional genomics approaches revealed that *ZBTB18* may be a novel regulator of bovine MuSCs fate determination. Loss-of-function assays demonstrated that *ZBTB18* knockdown increased proliferation while impairing differentiation. Conversely, gain-of-function experiments confirmed its dual role: suppressing proliferation while accelerating myogenic differentiation. This pro-differentiation/anti-proliferation function positions *ZBTB18* as a key molecular switch for myogenic progression and promising candidate for precision breeding strategies targeting meat yield and quality in beef cattle.

STAT1, a 91-kDa TF, mediates cellular responses to cytokines and growth factors via the JAK-STAT signaling pathway (Sun et al., 2007). While prior studies established STAT1's role in regulating MuSCs proliferation/differentiation, its functional impact remains complex: *STAT1* deficiency paradoxically accelerates MuSCs differentiation and muscle regeneration, potentially through immunomodulatory mechanisms involving pro−/anti-inflammatory factors (Gao et al., 2012). Our mechanistic investigations reveal that *ZBTB18* suppresses *STAT1* transcriptional activity correlating with inverse expression patterns. Further experiments showed that *STAT1* inhibits bovine MuSCs myogenic differentiation, contrasting ZBTB18's pro-differentiation role. Then *ZBTB18* targets *STAT1* to orchestrate myogenic differentiation. These findings establish a novel ZBTB18-STAT1 regulatory relationship in bovine myogenesis.

In this study, we have preliminarily elucidated the role of *ZBTB18* in bovine MuSCs. However, there are limitations to extrapolating these findings to adult meat traits. Adult meat quality is regulated by multiple factors that cannot be replicated in fetal MuSCs culture systems, including nutritional status, endocrine regulation (e.g., growth hormone, insulin-like growth factors), growth rate, intramuscular fat formation, connective tissue remodeling, and postnatal myofiber maturation (e.g., myofiber type conversion and metabolic adaptation). Therefore, although *ZBTB18* provides profound insights into the regulatory mechanisms of MuSCs proliferation, myogenic differentiation, and early myofiber type differentiation, it is insufficient to fully explain the multiple determinants of adult beef quality phenotypes. Future studies should include in vivo genetic manipulation experiments and research on co-culture systems encompassing both adipogenic and myogenic lineages to bridge the gap between fetal myogenic models and adult carcass traits, and to validate the translational potential of *ZBTB18* in precision breeding.

5. Conclusion

In conclusion, our study indicates *ZBTB18* as a pivotal regulator of bovine MuSCs biology. It functions by curbing proliferation and steering differentiation toward an oxidative phenotype, which may ultimately influence meat quality. The finding that *ZBTB18* represses *STAT1* provides a potential mechanistic explanation for these effects. These findings not only advance our fundamental understanding of myogenesis but also open new avenues for improving meat quality in cattle through molecular breeding strategies targeting the *ZBTB18* pathway. Future work, including in vivo validation in animal models, will be crucial to fully harness the potential of this axis for agricultural biotechnology.

Authors contribution

Y.D., R.Z. and J.W. designed the study. Y.D., M.L. and R.Z. drafted the manuscript. M.L., R.Z., Q.A. and K.C. perform the experiments and analyzed the data. J.W., Y·W., C.Z. and L.Z. helped perform the experiments and analyzed the data. M.L., R.Z. and K.C. collected samples and analyzed the data. Y.W supervised the project.

CRediT authorship contribution statement

Mingjun Liang: Writing – original draft, Formal analysis. **Ruimen Zhang:** Writing – original draft, Methodology, Formal analysis. **Qiang An:** Data curation, Formal analysis. **Kehe Cen:** Data curation, Formal analysis. **Lingxiu Zou:** Data curation, Formal analysis. **Chaoxia Zou:** Data curation, Formal analysis. **Yingming Wei:** Data curation, Formal analysis, Project administration, Supervision. **Jingwei Wei:** Conceptualization, Data curation, Formal analysis, Methodology. **Yanfei Deng:** Writing – original draft, Conceptualization.

Consent

All the authors read and approved the final manuscript. This article has not yet been cited by other publications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2026.100423.

Data availability

Primers for RNAs and binding sites of *ZBTB18* can be found in Supplementary tables. TFs enriched in MuSCs development can be found in GM-motif and DM-motif. The datasets presented in this study can be found in online repositories (CNCB, https://www.cncb.ac.cn/). The names of the accession number and repositories for ATAC-seq can be found below: accession number CRA019336, https://ngdc.cncb.ac.cn/gsub/submit/gsa/subCRA031146/finishedOverview.

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