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Functional study and epigenetic targets analyses of *SIRT1* in intramuscular preadipocytes via ChIP-seq and mRNA-seq

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ABSTRACT

The SIRT1 epigenetic regulator is involved in hepatic lipid homoeostasis. However, the role of SIRT1 in regulating intramuscular fat deposition as well as the pathways and potential epigenetic targets involved remain unknown. Herein, we investigate SIRT1 function, its genome-wide epigenetic target profile, and transcriptomic changes under SIRT1 overexpression during yak intramuscular preadipocytes differentiation. To this end, we analysed the relationship between SIRT1 and intramuscular fat content as well as lipid metabolism-related genes in longissimus dorsi tissue. We found that SIRT1 expression negatively correlates with intramuscular fat content as well as with the expression of genes related to lipid synthesis, while positively correlating with that of fatty acid oxidation-involved genes. SIRT1 overexpression in intramuscular preadipocytes significantly reduced adipose differentiation marker expression, intracellular triacylglycerol content, and lipid deposition. Chromatin immunoprecipitation coupled with high-throughput sequencing of H3K4ac (a known direct target of SIRT1) and high-throughput mRNA sequencing results revealed that SIRT1 may regulate intramuscular fat deposition via three potential new transcription factors (NRF1, NKX3.1, and EGR1) and four genes (MAPK1, RXRA, AGPAT1, and HADH) implicated in protein processing within the endoplasmic reticulum pathway and the MAPK signalling pathway in yaks. Our study provides novel insights into the role of SIRT1 in regulating yak intramuscular fat deposition and may help clarify the mechanistic determinants of yak meat characteristics.

Introduction

The yak (*Bos grunniens*) thrives well in hypoxic, cold, harsh environments as well as on the limited grassland resources of the Qinghai-Tibetan Plateau (QTP) and adjacent highland regions. Yak husbandry is a major traditional industry within the QTP, driving socioeconomic development. The Tibetan people primarily raise yaks for meat and milk, with the species being of considerable cultural importance. According to our update, the yak stock in 2021 amounted to approximately 18 million heads, accounting for approximately 18% of the beef cattle stock in China. Previous studies have shown that nutrients, such as proteins and polyunsaturated fatty acids, are present at higher levels in yak meat than in cattle meat[1], [2]. However, yak

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meat has a lower intramuscular fat (IMF) content [1], which seriously affects its taste, tenderness, colour, and flavour [3,4], thus limiting the sale and market development of yak products. The main factors that determine IMF content include breed, age, and nutrition [5-7]. Nutrient regulation [8,9] as well as common molecular mechanisms that affect gene and protein expression (acetylation [10], methylation, and phosphorylation [11]) are also implicated in IMF metabolism. The heritability of IMF deposition ranges from 0.36 to 0.54 [12,13], which is a low-to-moderate magnitude. Therefore, the regulation of IMF in yaks has become a key problem that required urgent solutions through research. Current research on yak IMF mainly focuses on the

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differences between yak and cattle through correlation analysis of lipid metabolism-related gene expression profiles and the study of intramuscular fatty acid composition. However, there is no report on the molecular mechanisms regulating IMF metabolism in yaks.

Intramuscular fat refers to the amount of fat located in skeletal muscle fibres. In livestock, IMF content is assessed as the marbling of meat and represents an important meat quality trait, which depends mainly on the size and number of intramuscular adipocytes as well as the muscle growth rate. It is a complex trait influenced by multiple genes or quantitative trait locus (QTLs). To date, 891 QTLs have been associated with IMF content in pigs (https://www.animalgenome.org/ cgi-bin/QTLdb/SS/index). However, most QTLs were determined via genome-wide association or other sequencing studies, without functional characterization. Various studies on the mechanism of IMF deposition in livestock have revealed genes involved in the deposition-regulation pathway. For bioinformatics example, through and colocalization analyses, in addition to RNAimediated loss-of-function screens, IRLnc has been verified as an IMF decomposition regulator that directly targets the nuclear receptor- subfamily-4-group-A-member-3 (Nr4a3) associated with insulin resistance [14]. Further, peroxisome proliferator-activated receptor γ (PPARy) [15], sterol regulatory element-binding transcription factor 1 (SREBF1) [16], and liver X receptor-alpha (LXR α) [17] are key transcription factors involved in IMF deposition, which coordinate lipogenic gene networks during lipid metabolism [18].

Histone deacetylases (HDACs) are enzymes that catalyse the deacetylation of histone amino-terminal tails, particularly H3 and H4 tails. As chromatin dynamics have major effects on gene expression and other nuclear processes, HDACs play an important role in various physiological and pathological processes. Mammalian sirtuins are a family of HDACs that deacetylate substrates ranging from histones to transcription regulators, in a nicotinamide adenine dinucleotide (NAD⁺) dependent manner. Silent mating information regulation 2 homolog 1 (*SIRT1*) is among the most extensively studied sirtuins, reported to regulate essential biological processes (such as cell differentiation [19], energy metabolism [20], and immune response [21]) implicated in various pathologies (such as diabetes [19,22], cardiovascular diseases [23], and cancer [24]). Mechanistic studies of SIRT1 suggest that it acts as a key regulator of mammalian lipid metabolism. It plays a role downstream of adipose triglyceride lipase during the regulation of hepatic lipid droplet catabolism and fatty acid oxidation in mice [25], limits preadipocyte hyperplasia through C-Myc deacetylation [26], and suppresses lipid accumulation by inhibiting PPARy[27] as well as SREBF1c signalling[28], while increasing PPARa[28] signalling in adipocytes. There is significant interest in identifying SIRT1-dependent molecular pathways relevant to adipogenesis. Previous studies of the transcriptomic landscape of muscle and adipose tissues have suggested that SIRT1 as a potential key regulator of IMF deposition in yaks [29]. However, the detailed mechanisms and signalling factors that mediate SIRT1-catalysed deacetylation remain poorly understood.

The present study aimed to explore the role of SIRT1 as a critical regulator and to identify signalling pathways controlling IMF deposition in yaks. We found that, even though the N-terminus of yak SIRT1 is encoded by a sequence that is 500 bp shorter than that of cattle, this shorter protein also functions as a negative regulator during yak intramuscular preadipocyte (YIMA) differentiation (Figure 2c). Moreover, chromatin immunoprecipitation sequencing (ChIP-seq) of H3 lysine 4 acetylation (H3K4ac) and mRNA-sequence (mRNA-seq) data revealed that large-scale chromatin state and gene expression changes occur during SIRT1 overexpression, mediating the attenuation of YIMA lipid deposition. Taken together, our study provides evidence for the regulatory role of SIRT1 in yak IMF deposition through the comprehensive analysis of the global epigenetic changes mediated SIRT1. These establish a basis for further clarifying the molecular mechanisms of yak IMF deposition.

Materials and methods

Animals and cells

Seventeen four-year-old male Maiwa yaks of similar weight from the Sichuan Zoige Alpine Wetland Ecosystem National Observation and Research Station of Southwest Minzu University were used for *SIRT1* expression analysis. Yaks were sacrificed for *longissimus dorsi-*, heart-, liver-, kidney-, lung-, adipose-, and rumen -tissue sample collection. Total RNA was extracted from yak tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The IMF content of *longissimus dorsi* tissue was determined according to the standard Soxhlet extraction method [16].

YIMAs were isolated from three male Maiwa yaks, as per previously described protocols for purification and authentication [30]. Briefly, oneyear-old yaks were humanely sacrificed, whereafter the longissimus dorsi muscle tissue was immediately collected and washed with phosphatebuffered saline. All visible connective tissue was removed, and the residual tissue was minced into pieces of approximately 1 mm³. Thereafter, the tissue was subjected to 2 h of digestion at 37°C in a shaking water bath, with 2.5 mg/mL type-II collagenase as the digestion buffer (Sigma Aldrich, St Louis, MO, USA) followed by a wash in Hank's balanced salt solutions buffer (Thermo Fisher Scientific, Waltham, MA, USA). The longissimus *dorsi* tissue digesta was centrifuged at $1,000 \times g$ for 5 min, and the supernatants were passed through a sterile 40-µm filter to isolate digested cells. The cells were then rinsed with DMEM (Thermo Fisher Scientific) and subjected to centrifugation twice at $1,500 \times g$ for 10 min. Thereafter, the cells were rinsed with basal medium and cultured in 5% CO₂ at 37°C. The culture medium was changed 1 h after plating, and 3 mL of fresh media was added to each dish to remove myoblasts, insoluble myofibrillar proteins, and other insoluble debris, as preadipocytes attach much earlier than myoblasts. The culture growth medium contained 90% basal DMEM/F12 (1:1) medium, 10% foetal bovine serum, and 100 U/mL penicillin/streptomycin. After contact inhibition, cells were incubated in growth medium supplemented with 50 µM oleic acid to promote differentiation for 2 d, followed by further differentiation maintained in growth medium supplemented with 5 mg/mL insulin for 2 d, then changed every 2 d until cells were harvested.

Generation of recombinant adenoviruses

The *SIRT1* gene was amplified using cDNA from the yak *longissimus dorsi* muscle tissue with the following primers: sense: 5'-CGCATGCGAGTTCTAGTG ACTG-3', antisense: 5'-CTGGTGGAACAATTTC TGTACCTG-3'. After bioinformatics analysis, the yak *SIRT1* gene sequence was submitted to GenBank, with accession number MT475828.1. The *SIRT1* coding sequence was cloned into a plasmid vector pAdEasy-EF1-MCS-3flag-CMV-EGFP, between the *Kpn*I and *Xho*I restriction sites. All the adenoviruses were packaged by Hanbio Technology (Shanghai) Co., Ltd, Shanghai, China.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Total RNA extraction from tissues and YIMAs was performed using TRIzol Reagent (Sigma-Aldrich) according to the manufacturer's instructions. First, 500 ng of total RNA was purified using the genomic DNA eraser enzyme (Takara Bio, Dalian, China), followed by quality and integrity determination, whereafter cDNA was produced with the PrimeScript RT Reagent Kit (RR047A, Takara Bio). Next, all quantitative polymerase chain reaction (qPCR) experiments were conducted using the SYBR Premix Ex Taq kit (Takara Bio) on a LightCycler96 Real-Time PCR system (Roche, Munich, Germany), using the primers listed in Table 1. Next, the relative expression values were normalized to the level of Glyceraldehyde-3-phosphate dehydrogenase (GADPH) and ubiquitously expressed transcript (UXT) and calculated using the $2^{-\Delta\Delta Ct}$ method. Finally, after amplification, the products were confirmed via the Sanger sequencing.

Oil Red O staining and cellular triacylglycerol assay

YIMAs were harvested, and subjected to the Oil Red O staining and cellular triacylglycerol (TAG) content determination as previously described for dairy goat mammary epithelial cells [31]. Finally, the intracellular

Gene	Accession number	Primer ^a (5'-3')	Size(bp)bp)	Efficiency[2]
SIRT1	MT475828.1	F:TGGGGTTTCTGTTTCTTGTGG	98	2.07
		R:CTTGAGGATCTGGAAGGTCTGG		
PPARγ	NM_181024.2	F:CGTGGACCTTTCTATGATGGA	117	2.04
		R:GCTCTTGGGAACGGAATG		
FASN	CR552737	F:ACCTCGTGAAGGCTGTGACTCA	92	1.99
		R:TGAGTCGAGGCCAAGGTCTGAA		
CPT1A	FJ415874.1	F:CATCCAGGCGGCAAGAG	117	2.03
		R:GAGCAGAGCGGAATCGT		
PGC1a	XM_024993060.1	F:GTACCAGCACGAAAGGCTCAA	120	2.05
		R:ATCACACGGCGCTCTTCAA		
C/EBPa	NM_176784.2	F:GTGGACAAGAACAGCAACGAGTA	138	2.08
		R:GCGGTCATTGTCACTGGTCAG		
aP2	XM_005897260	F:GGAAAGTCAAGAGCATCGTAAAC	110	2.01
		R:CACCATCTTATCATCCACGAGTT		
FABP3	DN518905	F:GAACTCGACTCCCAGCTTGAA	102	2.07
		R:AAGCCTACCACAATCATCGAAG		
GADPH	NM_001034034.2	F:CCACGAGAAGTATAACAACACC	120	2.04
		R:GTCATAAGTCCCTCCACGAT		
UXT	NM _001037471.2	F:TCGTGACAAGGTATATGAGCAG	156	2.10
		RITAGATCCGTGAAGTGTCTGG		

Table 1. Characteristics of primers used in RT-qPCR.

 ${}^{a}F$ = forward primer; R = reverse primer. [2] The PCR efficiency was determined by [10(-1/slope)]; the slope was obtained by the 7-point standard curve (with a minimum R² of 0.99).

Oil Red O was extracted using isopropanol and quantified by measuring the optical absorbance at 510 nm.

ChIP assays and high-throughput sequencing

Cells were harvested after incubation with Ad-SIRT1 or Ad-green fluorescent protein (Ad-GFP) for 4 d, and ChIP assays were performed using H3K4ac (ab176799, Abcam, Cambridge, England) as previously described [32], with little modifications. Briefly, cells were crosslinked with 1% formaldehyde (Roche) for 5 min, and crosslinking was stopped by adding 1 mL of 2 M glycine and incubating for 5 min at 20°C. Next, chromatin was sheared using Covaris M220 Focused-ultra-sonicator (Covaris, Woburn, MA, USA) to obtain DNA fragments of approximately 500 bp. Thereafter, 5 µg of H3K4ac antibody was used to pull down the tagged protein. The chromatin was then de-cross-linked overnight with proteinase K at 65°C, after which the DNA was purified using the MinElute PCR purification kit (Qiagen, Hilden, Germany). Subsequently, the libraries for high-throughput sequencing were constructed using an Illumina HiSeq 2500 sequencer in Novogene Biotech Co., Ltd. (Beijing, China). Two and three independent biological samples were used for ChIP-seq and mRNA-seq, respectively. Full read data were deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus repository under accession number PRJNA852401.

ChIP-seq analysis

The ChIP-seq assays raw data were quality checked using fastqc(http://www.bioinformatics. babraham.ac.uk/projects/fastqc/). Reads were trimmed using fastp (version 0.19.11), quality checked again via fastqc (version V0.11.5), and mapped to the yak reference genome (BosGru v2.0) with the Burrows Wheeler Aligner programme (bwa v0.7.12.r1039) (Illumina, San Diego, CA, USA). Next, peaks were called using the model-based analysis of ChIP-seq (v2.1.0) peak caller with default parameters and an FDR cut-off of 0.05. Thereafter, the chromosome distribution, peak width, fold enrichment, significance level, and peak summit number per peak were recorded. Peak-related genes were then identified.

Homer finMotifsGenome.pl (v4.9.1) software was used to identify the sequence motifs on yak genomics binding with the ChIP-seq peaks. The peak summit position around the transcription start site (TSS) of genes can predict the protein and gene interaction sites. Therefore, the nearest TSS of every peak as well as the distance between peaks and TSSs were determined using Chipseeker. Furthermore, the distribution of peak summits on different functional regions, such as the 5' untranslated region (UTR), protein-coding sequence (CDS), and 3'-UTR, was performed. The peak- related genes confirmed by Chipseeker were then subjected to Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genome (KEGG) enrichment analyses via the GOseq R package and KOBAS (v3.0) software (Kobas Technologies Ltd., London, England). In addition, the differential peak analyses were based on the fold enrichment of peaks from different experiments. A peak was determined as differential when the odds ratio between two groups was greater than 2. Using the same method, genes associated with different peaks were identified, and the log2foldchange of genes was calculated using DESeq2 software with the default parameters, whereafter GO and KEGG enrichment analyses were conducted.

ChIP-qPCR assays

The immunoprecipitated DNA was quantified via qPCR with the following the real-time PCR primers: yak ACACA forward: 5'-CTCTCTCTTCTGGAGCG TGG-3' and reverse: 5'- CGCCACAACTAGAGG GTCTG-3'; yak LPIN1 forward: 5'-CTGCTCAGG GCATTTTGCTC-3' and reverse: 5'-GAACCGTA AGGACGGTGTCA-3'; yak PRDM16 forward: 5'-AGCGATTCTGGGGGACCTCTG-3' and reverse: 5'-TCTGCTTGAGAGTCGTGTCTG-3'; yak FOXN1 forward: 5'-TGAAGTCCCTCAGAGGCTCG-3' and reverse: 5'- ACGCCACATTTAGCTTTGCC-3'; yak PPARD forward: 5'- GAGATGAGAGGCAAGG GTGG-3' and reverse: 5'-TTTTCGAGCCCTAA CCGTGG-3'. Fold enrichment was then calculated as: percentage input = $100*2(CT_{input sample} -$ CT_{immunoprecipitation sample}); CT = threshold cycle of PCR reaction, followed by normalization to indicated controls.

SIRT1 overexpression and mRNA-seq

YIMAs were harvested after incubation with Ad-SIRT1 or Ad-GFP for 2 d and subsequently sent to Novogene Biotech Co., Ltd. (Beijing, China) for transcriptome sequencing. The raw data processing as well as DEGs assays was performed refer to the previously study [29]. The sequence raw data can be obtained at NCBI with the accession number PRJNA852863.

Statistical analyses

The data are presented as the mean \pm standard error of mean (SEM). Differences between the means of the individual groups were assessed using a two-tailed Student's t-test using SPSS 22.0 software (SPSS, Chicago, IL, USA) and were considered statistically significant at P < 0.05.

Results

Relationship between *SIRT1* expression, IMF content, and key lipid metabolism genes

To the best of our knowledge, no information is available on the regulation of SIRT1 in yak IMF deposition. This prompted us to clone and analyse the CDS of SIRT1. To this end, a 1,738 bp fragment of the cDNA sequence containing 1,674 bp of the CDS, 71 bp of the 5' franking sequence, and 37 bp of the 3' franking sequence was amplified (Figure 1a). The detailed sequence information is available in GenBank under the accession number MT475828.1. Remarkably, the N-terminal of the CDS sequence of yak SIRT1 is 531 bp shorter than that in Bos taurus. To determine the distribution of SIRT1 in different yak tissues, total RNA was obtained from seven tissues of four-year-old yaks, whereafter RT-qPCR was performed. The results showed that SIRT1 is highly expressed in adipose and rumen tissues, moderately expressed in the longissimus dorsi muscle, liver, and kidneys, with low expression in the lungs and heart (Figure 1b). To investigate whether SIRT1 is involved in yak IMF deposition, we determined the relationship of SIRT1 expression with IMF content and key lipid metabolism gene. As depicted in Figures 1C-g, SIRT1 transcript levels were negatively correlated



Figure 1. Expression analysis of *SIRT1* in yak. (a) Cloning of the cDNA sequence of yak *SIRT1*. M: DNA Marker 3. (b) The mRNA expression level of *SIRT1* in different tissues. (c-g)The correlation of *SIRT1* expression and IMF content (c), *PPARy*(d), *FASN*(e), *CPT1A*(f) and *PGC-1a*(g) expression in *longissimus dorsi* of yak. (h) *SIRT1* expression during intramuscular adipogenesis. Values are presented as mean \pm SEM. Different lowercase letters in figures indicate significant differences among different tissues or groups during intramuscular adipogenesis.

with IMF content (R = -0.627, P = 0.007, Figure 1c), *PPARy* expression (R = -0.530, P = 0.029, Figure 1d), and fatty acid synthase expression (FASN, R = -0.781, P = 0.001, Figure 1e).However, SIRT1 expression was positively correlated with genes related to fatty acid oxidation (carnitine palmitoyl transferase 1A, CPT1A, R = 0.799, P = 0.001, Figure 1f; peroxisome proliferator-activated receptor gamma coactivator 1 alpha, $PGC-1\alpha$, R = 0.691, P = 0.002, Figure 1g). Expression analysis of SIRT1 in yak. (a) Cloning of the cDNA sequence of yak SIRT1. M: DNA Marker 3. (b) The mRNA expression level of SIRT1 in different tissues. (c-g)The correlation of SIRT1 expression and IMF content (c), PPARy(d), FASN(e), CPT1A(f) and PGC-1 α (g) expression in longissimus dorsi of yak. (h) SIRT1 expression during intramuscular adipogenesis. Values are presented as mean ± SEM. Different lowercase letters in figures indicate significant differences among different tissues or groups during intramuscular adipogenesis.

To better understand the potential role of *SIRT1* during yak adipocyte differentiation, we monitored *SIRT1* expression after adipogenic induction of YIMAs. We noted that *SIRT1* transcripts decreased by approximately 50% after 48 h of induction, reaching a plateau (Figure 1h).

Overexpression of *SIRT1* suppresses adipogenesis during YIMA differentiation

To further validate the function of SIRT1 in adipogenesis during YIMA differentiation, an adenovirus-mediated SIRT1 expression system was constructed (Figure 2a). RT-qPCR results showed a 200-fold increase of SIRT1 mRNA levels at day 0 of differentiation, which was 11-fold higher than that of the control until day 6 (Figure 2b). Overexpression of SIRT1 suppressed YIMA adipogenesis, as verified by the decreased amount of lipid droplet accumulation in Oil Red O staining and TAG content assays performed on day 4 (Figure 2c). Furthermore, we detected the expression of markers and adipogenesis-related genes during differentiation, with the results showing that PPARy and cytosine-cytosine-adenosineadenosine-thymidine enhancer-binding protein alpha (C/EBP α) were significantly downregulated during differentiation. A significant decrease in adipocyte lipid-binding protein (aP2) expression was detected only until day 6 (Figure 2d). In addition, the FASN and FABP3 genes, which play a key role in IMF deposition in cattle [33], were significantly downregulated during differentiation (Figure 2d). Taken together, these results showed that overexpression of SIRT1 suppresses YIMA



Figure 2. Overexpression of *SIRT1* decreased yak intramuscular preadipocyte differentiation. (a) Outline of the experimental protocol. (b) *SIRT1* expression level during differentiation after infection with Ad-GFP or Ad-SIRT1. (c) Oil Red O staining (magnification: 100×, upper panel) and quantitative assay (left lower panel), and TAG content determination (right lower panel) at day 4 after adipogenic induction. (d) *PPAR*_Y, *C/EBPa*, *aP2*, *FASN* and *FABP3* expression profile during intramuscular adipogenesis after infection with Ad-GFP or Ad-SIRT1. Values are shown as mean ±SEM for at least three biological replicates and repeated three times (n = 9). * *P* < 0.05, ** *P* < 0.01.Expression analysis of *SIRT1* in yak. (a) Cloning of the cDNA sequence of yak *SIRT1*. M: DNA Marker 3. (b) The mRNA expression level of *SIRT1* in different tissues. (c-g)The correlation of *SIRT1* expression and IMF content (c), *PPAR*_Y(d), *FASN*(e), *CPT1A*(f) and *PGC-1a*(g) expression in *longissimus dorsi* of yak. (h) *SIRT1* expression during intramuscular adipogenesis. Values are presented as mean ± SEM. Different lowercase letters in figures indicate significant differences among different tissues or groups during intramuscular adipogenesis.

differentiation, suggesting that *SIRT1* is a negative adipogenic regulator.

Genome-wide changes underlying H3K4ac deacetylation by *SIRT1* in differentiated YIMAs

H3K4ac is one of the most intensively studied histone marks involved in transcriptional regulation and is a direct target of *SIRT1* [34,35]. However, the understanding of genomic changes underlying H3K4 deacetylation by *SIRT1* remains elusive. Therefore, we examined the genome-wide enrichment of H3K4ac in intramuscular preadipocytes after overexpression of *SIRT1* and adipogenic differentiation at day 4. ChIP-seq

experiments identified 220,233 peaks, with 194,703 identified after overexpression of SIRT1 and 154,291 peaks in the control group (Figure 3a). The genomic binding profile under SIRT1 overexpression and control conditions were very similar, with approximately 61% of binding occurring in gene deserts as well as intergenic regions and the remaining 39% occurring in promoter and gene body regions (Figure 3b). Compared with the control group, peak-related gene analysis revealed 20,163 genes whose expression changed significantly under SIRT1 overexpression (Table S1). Further evaluation of these differentially expressed genes (DEGs) revealed significant enrichment in GO terms associated with cellular metabolic process (GO:0044237), cell (GO:0005623), cell part



Figure 3. Global function of H3K4ac in mediating *SIRT1* action. (a) Venn diagrams showing the numbers of H3K4ac binding peaks in YIMAs chromatin infection with Ad-GFP or Ad-SIRT1. (b) Genomic distribution of H3K4ac binding peaks in the Ad-GFP or Ad-SIRT1 treated group. (c-d) GO annotation (c) and KEGG pathway enrichment (d) of the significantly peak-related genes. Only the top 30 enriched GO terms are presented here.

(GO:0044464), binding (GO:0005488), and protein binding (GO:0005515) (Figure 3c) as well as ribosome (bom03010), cancer pathway (bom05200), Rap1 signalling pathway PI3K-Akt (bom04015), signalling pathway (bom04151), and protein processing in endoplasmic reticulum (bom04141) pathway terms (Figure 3d and Table S2).

Further analysis of the ChIP-seq list of peakrelated genes that were significantly altered between the two groups revealed several genes that are regulated as part of the lipid metabolism pathways. For example, the promoter region within 1 kb upstream of the TSS was significantly enriched in acetyl-CoA carboxylase alpha (ACACA), acyl-CoA synthetase long chain family member 1 (*ACSL1*), lipin 1 (*LPIN1*), and thyroid hormone responsive (*THRSP1*). The region within 1–2 kb upstream of the TSS was significantly enriched in PR domain-containing 16 (*PRDM16*), forkhead box K2 (*FOXK2*), and forkhead box N1 (*FOXN1*), while that, within 2–3 kb upstream was significantly enriched in peroxisome proliferatoractivated receptor delta (*PPARD*) and forkhead box protein O4 (*FOXO4*) (Figure 4a). As shown in Figure 4b, the qChIP results were consistent with those from ChIP-seq, confirming that the identification of differential peak-related genes in this study was reliable.

We sought to determine sequence motifs for DNA-binding proteins that were significantly enriched for H3K4ac after overexpression of *SIRT1*.



Figure 4. ChIP-seq peaks for lipid metabolism related genes (a) and CHIP-qPCR was conducted to validate ChIP-seq results for selected targets (b). Data were presented as the mean and standard error of two biological replicates and repeated three times.

The results revealed significant enrichment for 131 known motifs (Table S3 and S4), including binding motifs for known transcription factors, such as nuclear factor erythroid 2-related factor 1 (NRF1), E-box, SP1 transcription factor, BCL6 transcription repressor (Bcl6), retinoid X receptor gamma (RXRg). However, the analysis also revealed 31 highly

enriched motifs exhibiting a close match to the consensus sequences for early growth response 1 (EGR1), NK3 homeobox 1 (NKX3.1), and peroxisome proliferator-activated receptor gamma, coactivator-related 1 (PPRC1). The biological relevance of those motifs has not been well described in lipid metabolism. We found that there were 5,640 EGR1, 2,217 PRDM1, and 15,704,612 PPRC1 motifs in 4,034, 2,023, and 196,917 regions enriched for H3K4ac, respectively. These regions were further associated with 380, 131, and 1,656,074 putative target genes, respectively (Table 2).

DEGs in differentiated YIMAs after SIRT1 overexpression

Under a series of strict criteria, 706 significantly DEGs, including 323 up-regulated and 383 downregulated, were detected in the Ad-SIRT1 injection group relative to the Ad-GFP group (Figure 5a and 5b, Table S5-1). These DEGs, including SREBF1, INSIG1, FASN, and SCD, play significant roles in lipid metabolism as direct or indirect targets of SIRT1 (Table S5-1). GO and KEGG pathway enrichment analyses were carried out to identify the biological functions of DEGs using the yak reference genome. We obtained 17 enriched significant GO terms (Figure 5c, Table S5-2), including translation (GO:0006412), protein folding (GO:0006457), ribonucleoprotein complex (GO:1990904), and structural molecule activity (GO:0005198). KEGG pathway analysis revealed that these DEGs were significantly enriched in ribosome (bom03010) pathway (Figure 5d, Table S5-3). However, other pathways, such as Th17 cell differentiation (bom04659), focal adhesion (bom04510), insulin resistance (bom04931), fatty acid biosynthesis (bom00061), and Wnt signalling (bom04310), were not markedly enriched. Thus, *SIRT1* may, at least in part, be involved in IMF deposition through the regulation of genes related to above-listed pathways.

Combined analysis of ChIP-seq and mRNA-seq data

The ChIP-seq heatmap of all genes detected via mRNA-seq revealed that the signal densities on 3,000 bp upstream and downstream of TSS and gene body region were both significantly in the Ad-SIRT1 overexpression group compared with the control group (Figure 6a). The cumulative frequency of ChIP-seq signal density at the gene promoter (Figure 6b, left panel) and of the log2-foldchange of peak-related genes (Figure 6b, right panel) reinforce the notion that expression changes were correlated with the H3K4ac modification ratio during differentiation (Figure 6c). In addition, upon *SIRT1* overexpression (Table S6),

 Table 2. De novo motif discovery by HOMER in SIRT1-responsive H3K4ac regions.

Name	Score	p value	Motif	No. of Motifs in Increased Ad_S Regions	No. of Increased Ad_S Regions Including this Motif	No. of Assigned Genes
FXR2	0.85	1.00E- 153	奏奏会CCG奏奏会会CGG会	259,788	119,387	13,376
EGR1	0.99	1.00E- 64	ÇZSCCCCG ZS会会	5640	4034	380
PRDM1	0.88	1.00E- 50	ZZZZAGIGAAAGI	5478	4279	999
PPRC1	0.86	1.00E- 51	<mark>⋦CGCGÇG</mark> ≩⋛	15,704,612	196,917	1,556,074
ERF2	0.89	1.00E- 124	ġġ⊊≙GCCGCC ġġ	14,869	11,037	935
NKX3.1	0.92	1.00E- 02	ŞIAGGTCASTĢĢGICA	2217	2032	131
GFI1B	0.75	1.00E- 110	ZZZZAGATTC ŻŻŻŻ	11,863	11,367	2676
RDS1	0.71	1.00E- 76	奏奏をCGGCCG奏奏奏	41,542,805	196,917	4,141,268
LRF	0.67	1.00E- 42	╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤╤	43,121	37,984	2462

Analysis using HOMER for increased H3K4ac regions revealed 31 significant motifs(only nine of these motifs are presented here). The table also includes data about the number of de novo sequence motifs identified in increased H3K4ac regions in *SIRT1* overexpression cells and the total number of genes assigned to increased H3K4ac regions containing these motifs.



Figure 5. The functional annotation of the differentially expressed mRNAs at day 4 after adipogenic induction. (a-b) The volcano map (a) and heatmap (b) of the differentially expressed mRNAs after infection with Ad-GFP or Ad-SIRT1. (c-d) The GO annotation(c) and KEGG pathway enrichment (d) of the differentially expressed mRNAs.

593 genes exhibited reduced acetylation (244 had reduced mRNA levels, 349 had increased levels), and 2,206 genes exhibited increased acetylation (682 had reduced mRNA levels, 1,524 had increased levels). Further analysis confirmed that there were 519 significant DEGs in common between ChIP-seq and mRNA-seq data (Figure 6d, Table S7–1). GO enrichment analysis revealed that the 519 DEGs were significantly enriched in 40 terms(Table S7–2), such as cytoplasm (GO:0005737), negative regulation of biosynthetic process (GO:0009890), and transcription factor binding (GO:0008134). Furthermore, KEGG pathway analysis revealed that the DEGs were significantly enriched in ribosomes (bom03010), Protein processing in endoplasmic reticulum (bom04141), and mitogen-activated protein kinase (MAPK) signalling pathway (bom04010) (Figure 6e, Table S7-3). Further analysis of the 519 DEGs revealed several genes that have been previously shown to



Figure 6. The comprehensive analysis of ChIP-seq and mRNA-seq data after infection with Ad-GFP or Ad-SIRT1. (a) The ChIP-seq heatmap of all genes. Left panel: the signal density of each 3,000 bp upstream of the transcription start site (TSS) and downstream of transcription ending site (TES). Right panel: the signal density of each 3,000 bp upstream and downstream of TSS. (b) Cumulative frequency of ChIP-seq signal of the DEGs (left panel) and FPKM of differential peak-related genes (right panel). (c) Heatmap of ChIP-seq signal and expression changes. Left panel is the heatmap of the log2foldchange of ChIP-seq RPM in the differential peak region, the right panel is the heatmap of the log2foldchange of the mRNA-seq FPKM of the differentially peak-related genes. (d) The H3K4ac modification level (left panel) is correlated with the gene expression level (right panel). (f) Distribution of genes with a significant change in both ChIP-seq and mRNA-seq. (g) The KEGG pathway enrichment of the DEGs in common in ChIP-seq and mRNA-seq.

be regulated during lipid metabolism, including mitogen-activated protein kinase1 (*MAPK1*), RXR-alpha (*RXRA*), 1-acylglycerol-3-phosphate O-acyltransferase 1 (*AGPAT1*), and hydroxyacyl-CoA dehydrogenase (*HADH*).

Discussion

IMF content is among the most important determinants of meat quality characteristics, being positively correlated with tenderness, flavour, and juiciness. Yak meat is rich in nutrients but has a very low IMF content. The *SIRT1* class III histone deacetylase plays a critical role in lipid metabolism by modulating the activity of transcription factors or cofactors through protein deacetylation [36]. However, its local role in lipid metabolism, especially IMF metabolism in yaks, remains unclear. Previous studies revealed that mice with adipose tissue-specific *SIRT1* deficiency exhibit increased fat mass, impaired glucose tolerance, and increased exosome secretion [37]. Furthermore, transgenic mice overexpressing SIRT1 fed an atherogenic diet develop worse lipid profiles, in parallel to the deacetylation of transcription factor cAMP response element binding protein (CREB) [38]. SIRT1 promotes the binding of FOXO1 to PPARy and C/EBP protein by regulating FOXO1 deacetylation and then inhibits the transcriptional activity of PPARy, leading to decreased hepatic lipid synthesis [27]. Various studies have confirmed that SIRT1 acts as an upstream regulator for liver kinase B1 or AMPactivated protein kinase [39], as well as sterol regulatory element-binding protein 1c signalling [40]. Similarly, our findings showed that SIRT1 was negatively correlated with IMF content in the longissimus dorsi of yak, with its overexpression resulting in a significant down-regulation of adipocyte differentiation markers as well as lipid droplet formation. These results were further supported by the fact that *SIRT1* overexpression decreased cellular TAG content during YIMAs differentiation. Interestingly, we found that although the N-terminal of the SIRT1 CDS sequence is 531 bp shorter than that in *Bos taurus*, it still functions as a negative regulator of lipid synthesis during IMF deposition in yaks. We speculate that the missing region within the N-terminal alters deacetylation activity without significantly changing its function. In addition, this difference in yak *SIRT1* may be due to evolution during adaptation to the Qinghai-Tibet Plateau environment.

Epigenetic changes in histones H3 and H4 are well-known regulators of gene expression. While transcription factors serve as direct transcriptional activators, they may also be involved in the induction of epigenetic alterations within specific regions of the genome. Researchers performed ChIP-seq with SIRT1 and H4K16ac antibodies in cocaine-treated mouse coronal brains, revealing chronic cocaine use causes depletion of SIRT1 from most of the studied gene promoters, in concert with H4K16ac enrichment [41]. H3K4ac is among SIRT1 targets in breast cancer [35]. Unfortunately, we did not find a SIRT1 antibody suitable for ChIP in yaks. Therefore, we performed ChIP-seq with an H3K4ac antibody in order to analyse the mechanism through which SIRT1 regulates IMF deposition in the vak. Our analyses revealed that SIRT1 brings about genome-wide epigenetic alterations in YIMAs, which are correlated with differential gene expression. Furthermore, we identified enrichment of sequence motifs in H3K4ac regions showing changes in response to SIRT1 overexpression, among which were known motifs of transcription factors such as RXRg, PPRC1, and PRDM1, involved in lipid metabolism as well as new motifs, including NRF1, EGR1, and NKX3.1, whose role in lipid synthesis has not been clarified. NRF1 is a transcription factor that has been linked to mitochondrial regulation [42]. The mitochondria are the key organelles for fatty acid ß oxidation, and PGC-1a, a well-known coactivator of PPARy, is also serves as a coactivator of NRF1 [43]. Long protein isoforms of NRF1 negatively regulate PPARy2 and acts as master regulators of adipoadipogenesis genesis, suppressing [44].Therefore, we speculate that NRF1 is implicated in IMF deposition through its influence on fatty acid β-oxidation via the regulation of lipid metabolism related genes (including PGC-1 α and PPARy) in the yak. EGR1 is a multifaceted transcription factor involved in browning and insulin resistance [45,46]. The browning of $Egr1^{-/-}$ mice was associated with an up-regulation of beige adipocyte-associated genes, such as uncoupling protein 1, CCAAT-enhancer-binding protein beta, cell death-inducing DFFA Like Effector A, and cytochrome c oxidase subunit 8B [45,47]. NK3 homeobox 1 (NKX3.1), an androgen-regulated prostate-specific transcription factor, has been reported to cause cell cycle arrest at G1/S phase via direct binding to the promoter of FOXO1 [48]. FOXO1 is a crucial transcription factor involved in lipid metabolism [49]. Combined with the present ChIP-seq data, we speculate that SIRT1 regulates these transcription factors to influence IMF deposition in the yak, with NRF1, EGR1, and NKX3.1 representing key potential bridges in this process. However, future work is needed to confirm this notion.

Through combined analysis with mRNA-seq data, we identified 519 common DEGs, which were enriched in various pathways, such as protein processing in endoplasmic reticulum and MAPK pathways. The endoplasmic reticulum is the site where at least one-third of cellular proteins and lipids are synthesized, folded, and transported [50]. MAPK phosphorylates various enzymes and transcription factors to regulate diverse cellular processes, including apoptosis, differentiation and proliferation, as well as cellular lipid metabolism [51,52]. Studies have shown that MAPK is a key regulator of gene expression as well as of the phosphorylation of various factors related to lipogenesis, including *PPARy* and *C/EBP* α [53]. MAPK pathway inhibitor-treated bovine preadipocytes exhibited significant downregulation of FABP4, FASN, C/EBP α , and PPAR γ , indicating the MAPK pathway plays an important role in mediating the effects of SIRT5 during preadipocyte differentiation [54]. In addition, studies on the early stage of adipocyte differentiation revealed that after blocking MAPK/ERK signalling markedly reduced the number of lipid droplets in adipocytes in parallel to the downregulation of genes involved in lipid synthesis and adipocyte differentiation [55]. As a key regulator of hepatic lipid metabolism, knockdown of MAPK1 was shown to promote liver steatosis in C57/B6J mice [56]. RXRs are orphan receptors which can form homoand heterodimers with various nuclear transcription factors to then regulate lipid metabolism, cholesterol homoeostasis, and inflammatory responses. RXR-a is a member of RXR family, which forms a heterodimer with PPARy and can be activated by sterol. Single nucleotide polymorphism (SNP) previously revealed that SNP rs133517803 significantly affects back-fat thickness and total monounsaturated fatty acid content in cattle [57]. HADH and AGPAT1 are enzymes involved in fatty acid β-oxidation and TAG synthesis, respectively [58,59]. The present finding indicated that MAPK1, RXRA, AGPAT1, and HADH are directly or indirectly involved in lipid metabolism, thus being potential targets of SIRT1 implicated in yak IMF deposition.

In conclusion, we demonstrated that SIRT1 negatively regulates IMF content in the longissimus dorsi muscle of yaks. Further, overexpression of SIRT1 in YIMAs negatively affected lipid differentiation marker expression, significantly reducing lipid droplets in adipocytes as well as TAG content. We identified three transcription factors (NRF1, NKX3.1, and EGR1) and four potential genes (MAPK1, RXRA, AGPAT1, and HADH) that may play a crucial role in SIRT1-mediated regulation of IMF deposition in yaks. To our knowledge, this is the first molecular characterization of SIRT1 in the regulation of yak IMF deposition. Nevertheless, several limitations of the present study should be noted. There is no yak SIRT1 antibody available for ChIP. Thus, we only analysed the genes downstream of SIRT1 using an H3K4ac antibody in order to evaluate epigenetic regulation. Further studies are necessary to elucidate the epigenetic regulation mechanism of SIRT1 during IMF deposition in yaks.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data related to this article can be obtained in the Supplementary materials, contact the author for other requirements.

Institutional review board statement

All animal experiments were conducted according to the regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004) and approved by the Institution Animal Care and Use Committee of the Southwest Minzu University (Permit number:S2020-013), Chengdu, China.

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