

The 67-bp Variable Duplication in the Bovine ADIPOQ Promoter Is Associated with Fat-Related Carcass Traits and Modulates Promoter Activity through a HES5-Responsive Motif

Yufu Li

Northwest A&F University

Qunli Li

Northwest A&F University

Peidong Qu

Northwest A&F University

Aixin Li

Northwest A&F University

Chuanying Pan

Northwest A&F University

Ningbo Chen

Northwest A&F University

Enhui Jiang

Northwest A&F University

Xianyong Lan

lanxianyong79@nwfau.edu.cn

Northwest A&F University

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Abstract

Background:

The *ADIPOQ* gene is involved in lipid metabolism, energy homeostasis, and fat deposition in cattle. Although previous studies have suggested that regulatory variants in the *ADIPOQ* promoter may contribute to phenotypic variation, the functional and phenotypic significance of the 67-bp variable duplication in the bovine *ADIPOQ* promoter remains incompletely understood. This study aimed to characterize the regulatory effect of this duplication and evaluate its association with carcass-related traits in Gaoqing Black cattle.

Results:

Analysis of public cattle transcriptomic data showed that *ADIPOQ* had an adipose-enriched expression pattern. Genotyping of 461 Gaoqing Black cattle identified three genotypes at the 67-bp duplication locus, with 1D/1D being predominant, 1D/2D present at a low frequency, and 2D/2D rare. Dual-luciferase reporter assays in 293T and CHO cells showed that increasing copy number of the 67-bp duplication reduced *ADIPOQ* promoter activity, indicating a copy number-dependent repressive effect. Transcription factor prediction and functional validation suggested that HES5 may contribute to this repression through a binding site within the duplicated sequence, and mutation of the predicted binding motif abolished the HES5-associated inhibitory effect. Association analyses further showed that, compared with 1D/1D cattle, 1D/2D males had lower brisket fat weight and shank meat weight, while 1D/2D females had lower carcass fat weight.

Conclusions:

The 67-bp variable duplication in the bovine *ADIPOQ* promoter acts as a functional regulatory variant that suppresses promoter activity and is associated with selected carcass and fat-related traits in Gaoqing Black cattle. These findings provide insight into the regulatory architecture of *ADIPOQ* and suggest that this promoter variant may be useful for understanding genetic variation in fat deposition-related traits in cattle.

1. Background

Carcass composition and fat deposition are particularly valued in cattle markets for meat eating quality [1, 2]. In particular, fat-related carcass traits such as brisket fat, carcass fat, intermuscular fat content, and marbling score are closely associated with carcass yield, meat flavor, juiciness, and consumer preference [3–5]. Therefore, understanding the genetic mechanisms underlying fat deposition and carcass composition is important for beef cattle breeding.

Adiponectin is a hormone generated from adipocytes and encoded by the *ADIPOQ* gene [6]. Due to its roles in lipid metabolism and energy regulation, *ADIPOQ* has been considered a candidate gene for fat deposition and production traits in livestock [7–10]. In cattle, adiponectin mRNA is highly expressed in adipose tissues and is upregulated during adipocyte differentiation of preadipocytes derived from bovine stromal-vascular cells [11]. Several studies have revealed that genetic variation in or around *ADIPOQ* may be associated with economically relevant traits such as productive traits, growth traits, reproductive traits, and carcass-related phenotypes [11–13].

Transcriptional regulation is a primary mechanism by which genetically encoded information is converted into phenotypic variation [14]. Genetic variants in regulatory regions are important contributors to inter-individual variation in gene expression [15]. Therefore, sequence variation in the promoter region of *ADIPOQ* may contribute to individual differences in *ADIPOQ* transcription and downstream fat deposition-related traits. A 67-bp variable duplication in the bovine *ADIPOQ* promoter has been reported to affect promoter activity in a copy number-dependent manner and to be associated with decreased *ADIPOQ* mRNA expression in cattle ovarian samples [13, 16]. Such promoter duplications may alter transcriptional regulation by changing the copy number, spatial arrangement, or accessibility of transcription factor (TF) binding motifs [17–19].

However, the transcriptional mechanism and phenotypic relevance of this 67-bp *ADIPOQ* promoter duplication remain insufficiently defined. Gaoqing Black cattle are characterized by favorable meat quality and distinctive fat deposition characteristics, providing a suitable population for evaluating whether this promoter duplication contributes to variation in carcass fat deposition and partition traits [20]. Because carcass fat deposition and partition traits are economically important in beef cattle production, characterizing the functional effect of this promoter variant may improve our understanding of the genetic regulation of *ADIPOQ* and its potential contribution to carcass trait variation in Gaoqing Black cattle. Here, we aimed to define the transcription factor-dependent mechanism by which the 67-bp *ADIPOQ* promoter repeat regulates *ADIPOQ* promoter activity and to evaluate whether this regulatory variant contributes to variation in carcass fat deposition and partition traits in Gaoqing Black cattle.

2. Methods

2.1 Tissue Sample and Data Collection

To systematically investigate the genetic determinants of beef quality features, we collected muscle tissue samples from 461 Gaoqing Black cattle at slaughter. The cattle were sourced from two commercial farms with similar management practices: Shandong Yangxin Yiliyuan Halal Meat Company (Binzhou City, Shandong Province, China) and Shandong Kai-yuan Animal Husbandry Company (Zhaoyuan City, Shandong Province, China), where standard feeding regimens, housing conditions, and health monitoring were applied. All animals entered the fattening phase at 10 to 11 months of age and were slaughtered at 26 to 28 months of age under standardized commercial slaughter procedures. The cattle were slaughtered as part of routine commercial production at a licensed abattoir. Biological

samples and carcass phenotype records were collected after slaughter. No animals were euthanized or experimentally sacrificed specifically for the purpose of this study, and no anaesthetic agents or injection dosages were administered by the research team for euthanasia or sacrifice.

The collected data include growth and carcass weight traits, muscle development traits, carcass cut yield traits, fat-related carcass traits, and organ/by-product traits. Growth and carcass weight traits included body weight before slaughter, carcass weight, left and right carcass side weight. Muscle development traits included loin eye area, loin eye length, and loin eye width. Carcass cut yield traits included striploin weight, tenderloin weight, ribeye weight, chuck roll weight, brisket meat weight, shank meat weight, top blade cut weight, and several commercial beef cuts. Fat-related carcass traits mainly included brisket fat weight, carcass fat weight, interleaved fatty beef weight, special marbled beef weight, rib belly weight, brisket fascia weight, and rib meat weight. Organ and by-product traits included tongue weight, tail weight, rectum weight, large intestine weight, small intestine weight, and heart-associated tissues. All measurements were performed by trained personnel following standardized operating procedures, with calibrated instrumentation and duplicate recordings to ensure data reliability. This approach was designed to provide reliable phenotyping for subsequent genetic association analyses.

The work followed the guidelines of the "Management of Laboratory Animal Affairs" established by the Ministry of Science and Technology, with ethical approval from the Ethics Committee of Northwest A&F University (DK2022032).

2.2 Extraction of Genomic DNA

Approximately 2 g of muscle tissue was collected from each animal, and a subsample was used for genomic DNA extraction. Genomic DNA was extracted using the phenol–chloroform method [21]. To ensure the quality of the extracted DNA, we utilized a NanoDrop 1000 spectrophotometer to measure the A260/A280 ratio of each DNA sample [22]. Subsequently, the DNA samples were diluted to a concentration of 50 ng/μL and stored at – 40°C until further use.

2.3 PCR Amplification and Agarose Gel Electrophoresis

Genomic DNA extracted from tissue samples of Gaoqing Black cattle was used as the PCR template. A primer pair was designed to generate a short amplicon spanning the 67-bp duplication in the *ADIPOQ* promoter, thereby enhancing the differentiation of genotypes on agarose gels. PCR amplification was performed using Bio-Engineering Taq PCR Mix premix (B639295). The PCR products were separated by 3% agarose gel electrophoresis, and the genotypes of individual DNA samples were determined according to the band patterns. The primer sequences, PCR reaction conditions, and amplification parameters are provided in Table 1. The individual-level genotypes for this 67-bp variable duplication were submitted to the Genome Variation Map under accession number GVM001518.

Table 1
Bovine *ADIPOQ* gene promoter region amplification primers

Primer	Sequence (5'–3')	Tm(°C)	Product size (bp)	Function
<i>ADIPOQ</i> -F1	GTCTTCTGTCGCTGTTGT	50.2	335/402	PCR
<i>ADIPOQ</i> -R1	AACTTGAATCAGTCGTCCTT	52.4		

2.4 Construction of *ADIPOQ* Promoter Reporter Constructs

ADIPOQ promoter reporter vectors containing different 67-bp duplication haplotypes were generated using the pGL3-Basic vector. For the 1D and 2D constructs, promoter fragments were amplified from Gaoqing Black cattle genomic DNA carrying the corresponding genotypes. To maintain consistency with the previous study that first characterized this 67-bp variable duplication, we used the reported primer pair that amplifies a 484-bp *ADIPOQ* promoter fragment [13]. Restriction sites for Mlu I and Hind III were introduced at the 5' ends of the forward and reverse primers, respectively (Table 2). The amplified fragments were digested and ligated into the pGL3-Basic vector. Other engineered constructs, including the 0D deletion construct and transcription factor binding-site mutant constructs, together with transcription factor expression vectors, were synthesized by Sangon Biotech. All constructs were confirmed by Sanger sequencing before use in transfection experiments.

Table 2
Primers for truncated fragments of the *ADIPOQ* gene promoter region

Name	Sequence (5'–3')	Product size	Tm (°C)
pGL3-1D-F	GCG ACGCGTagaaatgttcctcacctcagt	484 bp	69.7
pGL3-2D-F	GCG ACGCGTagaaatgttcctcacctcagt	551 bp	69.7
R	CCCAAGCTT tctcggtactcatggggac		65.6

Conserved bases are shown in bold, and restriction enzyme sites are underlined.

2.5 Prediction of Transcription Factors

Potential transcription factors (TFs) capable of binding to the 67-bp variable duplication sequence were systematically predicted based on the AnimalTFDB database (<http://bioinfo.life.hust.edu.cn/AnimalTFDB4/>) and the JASPAR database (<http://jaspar.genereg.net/>). To ensure the reliability of the prediction results, only TFs with Q-value < 0.01 were retained from AnimalTFDB, while high-confidence candidates with a Relative Binding Score > 10 were selected from JASPAR.

2.6 Plasmid Extraction

Plasmid DNA was extracted using the EndoFree Plasmid Mini Kit (CW BIO, Jiangsu, China) according to the manufacturer's instructions. Briefly, bacterial cultures grown in a shaking incubator for 24 h were collected and centrifuged at 10,000 rpm for 5 min, and the supernatant was discarded. The bacterial

pellet was resuspended in 500 μ L of RB buffer containing RNase A until no visible clumps remained. Subsequently, 500 μ L of LB buffer was added, and the mixture was gently inverted 4–6 times to ensure complete cell lysis, with the lysis step controlled within 5 min. Then, 700 μ L of NB buffer was added, followed by gentle inversion 4–6 times, and the mixture was incubated at room temperature for 2 min.

The lysate was centrifuged at 12,000 rpm for 5 min, and the supernatant was carefully transferred to a spin column, followed by centrifugation at 12,000 rpm for 1 min. The column was then washed with 650 μ L of WB buffer and centrifuged at 12,000 rpm for 1 min, after which the flow-through was discarded. An additional centrifugation at 12,000 rpm for 2 min was performed to remove residual wash buffer. Finally, the spin column was placed into a clean 1.5 mL centrifuge tube, and 30 μ L of ddH₂O was added to the center of the membrane. After incubation at room temperature for 1 min, plasmid DNA was eluted by centrifugation at 12,000 rpm for 1 min. The concentration and purity of the extracted plasmid DNA were determined, and the samples were stored at -20°C until further use.

2.7 Construction of Dual-Luciferase Reporter Vectors

The plasmids pGL3-Basic, pcDNA3.1, and pRL-TK used in this study were preserved in our laboratory, and all plasmids had been previously verified to ensure their integrity. Different fragments of the bovine *ADIPOQ* gene were amplified by PCR with introduced restriction endonuclease recognition sites.

After separation and purification by agarose gel electrophoresis, the amplified products from the *ADIPOQ* gene promoter region were digested with Mlu I and Hind III, and subsequently ligated into the pGL3-Basic vector digested with the same enzymes. To ensure the reliability of the experimental results, all recombinant plasmids were screened by colony PCR and further confirmed by sequencing to verify the correctness and orientation of the inserted fragments.

2.8 Dual-Luciferase Reporter Assay

The experiments were performed using two cell lines preserved in our laboratory: CHO cells and 293T cells. Cells were revived and passaged one week prior to the experiments. Cells were seeded in 24-well plates at a density of 5×10^4 cells per well (293T cells) or 8×10^4 cells per well (CHO cells) and cultured to reach 70–80% confluence at the time of transfection.

Lipofectamine 2000 was used as the transfection reagent. The relative promoter activities of the constructed vectors were determined using a dual-luciferase reporter gene assay system and were expressed as the ratio of firefly luciferase activity to Renilla luciferase activity. At 36 h after transfection, cells were lysed using cell lysis buffer. Luciferase activities were subsequently measured under light-protected conditions using a microplate reader with the Dual-Luciferase Reporter Assay Kit (11402ES60) from Yeasen Biotechnology.

2.9 Data Analysis

The population genotype frequencies, allele frequencies, and Hardy–Weinberg equilibrium (HWE) were analyzed using the chi-square test. Observed heterozygosity (H_o), expected heterozygosity (H_e),

effective number of alleles (Ne), and polymorphism information content (PIC) were calculated using a genetic diversity index calculator (<http://www.msrcall.com/Gdical.aspx>) [23].

The association analysis between genotype and carcass-related traits was performed using SPSS 27.0 software (IBM Corp., Armonk, NY, USA). Considering the potential influence of sex on carcass composition and fat deposition, genotype–phenotype association analyses were conducted separately in male and female Gaoqing Black cattle. For body weight and carcass weight traits, associations between genotypes and phenotypic traits were analyzed using a general linear model as follows: $Y_{ij} = \mu + G_i + e_{ij}$, where Y_{ij} represents the phenotypic value of the j -th individual with the i -th genotype, μ represents the overall mean, G_i represents the fixed effect of genotype, and e_{ij} represents the random residual [24].

For carcass cut yield traits, fat-related carcass traits, and organ/by-product traits, slaughter weight was included as a covariate to adjust for differences in final body weight. The model was as follows: $Y_{ij} = \mu + G_i + \beta W_i + e_{ij}$, where W_i represents slaughter weight and β represents the regression coefficient for slaughter weight. Entry weight was further used as an auxiliary covariate in sensitivity analyses to evaluate whether the genotype–phenotype associations were affected by initial body weight. Homogeneity of variance among genotype groups was assessed before group comparisons. All data are presented as means $\pm$ standard error of the mean (SEM). A value of $p < 0.05$ was considered statistically significant. Multiple comparisons among genotypes were adjusted using the Bonferroni correction.

For the dual-luciferase reporter assay, Renilla luciferase activity was used as the internal control, and relative promoter activity was expressed as the ratio of Firefly luciferase activity to Renilla luciferase activity. Statistical analysis of the dual-luciferase assay data was performed using SPSS 27.0, and figures were generated using GraphPad Prism 8.

3. Results

3.1 ADIPOQ shows an adipose-enriched expression pattern in cattle

We first analyzed the multi-tissue expression profile of *ADIPOQ* using the public CattleGTEx dataset. *ADIPOQ* expression data were available for 94 tissues comprising 26,414 RNA-seq samples. After excluding tissues with fewer than five samples, tissues were ranked according to median $\log_2(\text{TPM} + 1)$ values. *ADIPOQ* exhibited an adipose-enriched expression pattern, with the highest levels detected in visceral fat, subcutaneous fat, and other adipose depots (Fig. 1a). Expression was also observed in several urogenital, endocrine, vascular, neural, and muscle-related tissues. Given the adipose-enriched expression pattern of *ADIPOQ*, fat-related carcass traits were emphasized when interpreting the subsequent association results.

3.2 Genotyping of the 67-bp variable duplication of ADIPOQ reveals low polymorphism in Gaoqing Black cattle

To explore the association between the 67-bp variable duplication and fat-related carcass traits, we next genotyped the duplication in the *ADIPOQ* promoter of Gaoqing Black cattle (Fig. 1b).. PCR genotyping identified three genotypes of the 67-bp duplication in the *ADIPOQ* promoter region within this population, including 1D/1D, 1D/2D, and 2D/2D (Fig. 1c, Table 3). Representative samples with different genotypes were selected for Sanger sequencing validation (Fig. 1d). Among the 461 individuals analyzed, the 1D/1D genotype was predominant, with a frequency of 0.907, whereas the frequencies of the 1D/2D and 2D/2D genotypes were 0.089 and 0.004, respectively. The frequencies of the 1D and 2D alleles were 0.952 and 0.048 respectively, indicating that the 2D allele was present at a relatively low frequency in the Gaoqing Black cattle population.

Table 3

Different genotype frequencies and population genetic parameters of the 67-bp variable duplication in the promoter region of the *ADIPOQ* gene in Gaoqing Black cattle

Sample size	Genotypic frequencies			Population parameters				HWE
	1D/1D	1D/2D	2D/2D	Ho	He	Ne	PIC	<i>p</i> values
461	0.907	0.089	0.004	0.089	0.167	1.202	0.153	<i>p</i> > 0.05

The genotype distribution did not significantly deviate from Hardy–Weinberg equilibrium ($p > 0.05$). Genetic diversity analysis revealed low genetic variability at this locus, with observed heterozygosity (H_o) of 0.089 and expected heterozygosity (H_e) of 0.167. The polymorphism information content (PIC) was 0.153, and the effective number of alleles (N_e) was 1.202, indicating limited genetic diversity within the population. These results indicate that this locus exhibits low genetic diversity and represents a low-polymorphism locus in Gaoqing Black cattle.

3.3 The 67-bp variable duplication suppresses *ADIPOQ* promoter activity in a copy number-dependent manner

Given the low frequency of the 2D allele and the low polymorphism of this locus in Gaoqing Black cattle, we next examined whether different copy numbers of the 67-bp duplication affected *ADIPOQ* promoter activity. We generated three luciferase reporter constructs carrying different copy numbers of the 67-bp duplication (Fig. 2a). Specifically, the pGL3-0D vector contained a promoter fragment lacking the 67-bp duplication sequence, with a total length of 404 bp; the pGL3-1D vector contained the native single-copy 67-bp duplication sequence, with a total length of 471 bp; and the pGL3-2D vector contained two copies of the 67-bp duplication sequence, with a total length of 538 bp. All constructs were confirmed by Sanger sequencing before functional assays.

The constructed pGL3-0D, pGL3-1D, and pGL3-2D reporter vectors were co-transfected with the internal control plasmid pRL-TK into 293T and CHO cells, respectively. Dual-luciferase reporter assays showed that *ADIPOQ* promoter activity differed significantly among constructs carrying different copy numbers of the 67-bp variable duplication. In 293T cells, compared with pGL3-2D, pGL3-1D showed a significant

increase in promoter activity by $20.12 \pm 5.60\%$ ($p < 0.05$), whereas pGL3-0D exhibited a further increase of $422.50 \pm 61.49\%$ compared with pGL3-1D ($p < 0.001$) (Fig. 2b, Supplementary Table 1). Pearson correlation analysis confirmed a significant negative correlation between the copy number of the 67-bp variable duplication and promoter activity ($r = -0.879$, $p < 0.001$) (Fig. 2c).

The results in CHO cells were highly consistent with those observed in 293T cells. Specifically, the activity of pGL3-1D was $50.53 \pm 24.87\%$ higher than that of pGL3-2D ($p < 0.05$), and the activity of pGL3-0D was further increased by $664.02 \pm 126.41\%$ compared with pGL3-1D ($p < 0.001$) (Fig. 2d, Supplementary Table 1). Correlation analysis also revealed a significant negative correlation between repeat copy number and promoter activity ($r = -0.896$, $p < 0.001$) (Fig. 2e).

Notably, pGL3-0D exhibited markedly higher promoter activity than the 1D and 2D constructs in both cell lines, suggesting that the 67-bp repeat sequence suppresses basal *ADIPOQ* promoter activity in vitro.

3.4 HES5 mediates copy number-dependent repression of the *ADIPOQ* promoter

To further elucidate how the 67-bp variable duplication affects *ADIPOQ* promoter activity, potential TF binding sites within this region were predicted using both the Animal TFDB and JASPAR databases. Using a Q-value < 0.01 as the screening threshold, 264 candidate TFs were identified from AnimalTFDB, whereas 50 candidate TFs were obtained from JASPAR (Supplementary Table 2). Venn analysis showed that 43 TFs were commonly predicted by both databases (Fig. 3a).

Among these overlapping candidates, HES5 was selected for further validation because it belongs to the HES family of transcriptional repressors, which was consistent with the observed suppressive effect of the 67-bp variable duplication on *ADIPOQ* promoter activity. (Fig. 3a, b). Because the predicted HES5 binding site was located within the 67-bp variable duplication sequence, the 0D construct contained no predicted HES5 binding site, whereas the 1D and 2D constructs contained one and two predicted HES5 binding sites, respectively (Fig. 3c). This variation in binding-site copy number served as a basis to figure out whether HES5 mediates the regulation of *ADIPOQ* promoter activity that is dependent on copy number.

To clarify the effect of the 67-bp variable duplication sequence on the transcriptional regulatory function of HES5, the repressive effect of HES5 was compared across constructs carrying different copy numbers of the duplication. The results showed that, in the empty vector control group, the basal promoter activity of pGL3-0D was significantly higher than that of pGL3-1D and pGL3-2D ($p < 0.001$), which was consistent with the results of previous independent transfection assays (Fig. 3d). After co-transfection with the HES5 expression vector, both the 1D and 2D constructs exhibited significant transcriptional repression ($p < 0.05$), whereas no significant inhibitory effect of HES5 was observed on the 0D construct ($p > 0.05$) (Fig. 3d). This result confirmed that the binding of HES5 to the *ADIPOQ* promoter region is sequence-specific. Furthermore, the inhibitory efficiency of HES5 on the 2D construct

was significantly greater than that on the 1D construct ($p < 0.01$) (Fig. 3d), suggesting that the number of binding sites may influence the strength of transcriptional repression.

To precisely verify the predicted HES5 binding site, the predicted HES5 binding motif (E-box sequence: CACGTG) and its flanking region, comprising a total of 10 bp, were systematically mutated (Fig. 3e). The wild-type 1D sequence 'GGCACGTGTC' was mutated to 'TTTCATAAAA' to disrupt the predicted HES5 binding motif. Co-transfection assays showed that, when the mutant 1D construct (1D-mut) was co-transfected with HES5, no significant difference in luciferase activity was observed compared with the construct transfected alone ($p = 0.47$). In contrast, the luciferase activity of the wild-type 1D construct was significantly repressed by HES5 ($p < 0.01$) (Fig. 3f). These results further confirmed that the predicted 10-bp region plays a decisive role in HES5-mediated transcriptional repression, and that the inhibitory effect of HES5 on the *ADIPOQ* promoter depends on the integrity of this specific binding site. HES5-mediated repression provides an in vitro mechanistic explanation for the copy number-dependent repression of *ADIPOQ* promoter activity by the 67-bp duplication.

3.5 Association of the 67-bp duplication-associated regulatory variant with carcass and fat-related traits in Gaoqing Black cattle

Given the adipose-enriched expression pattern of *ADIPOQ* and the HES5-mediated repressive effect of the 67-bp duplication on *ADIPOQ* promoter activity, we next investigated whether this regulatory variant was associated with carcass and fat-related traits in Gaoqing Black cattle. Because carcass composition and fat deposition differ substantially between male and female cattle, association analyses were performed separately by sex, with particular attention to fat-related carcass traits.

Association analyses between different genotypes of the 67-bp variable duplication and 70 carcass-related traits were performed separately in male and female Gaoqing Black cattle (Supplementary Table 3). Among the fat-related carcass traits, brisket fat weight in males was significantly higher in individuals with the 1D/1D genotype (3.44 ± 1.06 kg) than in those with the 1D/2D genotype (2.10 ± 0.28 kg) ($p = 0.011$; Fig. 4a, Table 4). In females, carcass fat weight was significantly higher in individuals with the 1D/1D genotype (91.54 ± 1.94 kg) compared with the 1D/2D genotype (84.03 ± 2.29 kg) ($p = 0.015$; Fig. 4b, Table 5). In contrast, no significant genotype-associated differences were observed for other fat-related carcass traits, including rib meat weight, special marbled beef weight, brisket fascia weight, interleaved fatty beef weight, and rib belly weight (Supplementary Table 3).

Among the carcass cut yield traits, shank meat weight in males was significantly higher in the 1D/1D genotype group (17.16 ± 0.75 kg) than in the 1D/2D genotype group (13.89 ± 0.82 kg) ($p < 0.001$; Fig. 4c, Table 4). No significant associations were detected between the 67-bp variable duplication and the remaining carcass-related traits and muscle development traits, organ/by-product traits analyzed in this study (Supplementary Table 3).

Collectively, these results suggest that the 67-bp variable duplication in the *ADIPOQ* promoter may be associated with selected fat-related carcass traits and carcass cut yield traits in sex-stratified analyses

of Gaoqing Black cattle, with 1D/2D individuals showing lower values than 1D/1D individuals for the significant traits.

Table 4
Association analyses between different genotypes of the 67-bp variable repeat and carcass-related traits in Gaoqing Black cattle (Male cattle)

Carcass-related traits	1D/2D	1D/1D	p-value
Shank meat weight (kg)	13.89 ± 0.82 (n = 9)	17.16 ± 0.75 (n = 74)	< 0.001
Brisket fat weight (kg)	2.10 ± 0.28 (n = 8)	3.44 ± 1.06 (n = 77)	0.011

Table 5
Association analyses between different genotypes of the 67-bp variable repeat and carcass-related traits in Gaoqing Black cattle (Female cattle)

Carcass-related traits	1D/2D	1D/1D	p-value
Carcass fat weight (kg)	84.03 ± 2.29 (n = 24)	91.54 ± 1.94 (n = 201)	0.015

4. Discussion

This study systematically constructed luciferase reporter gene vectors carrying different copy numbers of the 67-bp variable duplication and, combined with transcription factor overexpression experiments, explored its regulatory role on *ADIPOQ* transcriptional activity. The use of 293T and CHO cells, although heterologous, represents a standard and widely accepted approach to assess the intrinsic transcriptional regulatory activity of promoter variants in a controlled manner; the highly consistent copy-number-dependent effects observed in both cell lines strengthen the robustness of our conclusions. The results revealed that the 67-bp variable duplication inhibits *ADIPOQ* promoter activity in a dose-dependent manner, and further elucidated the regulatory mechanisms of HES5 TFs in this process (Figure 5).

Figure 5. Proposed regulatory mechanism of the 67-bp variable duplication in the bovine *ADIPOQ* promoter.

The illustration was created using BioRender.com.

Previous studies have identified the 67-bp variable duplication in the bovine *ADIPOQ* promoter and mainly examined its associations with growth-related, milk, or ovarian traits in cattle [13, 16, 25]. In contrast, the present study focused on Gaoqing Black cattle, a local beef cattle population characterized by a snowflake-like marbling pattern, fine texture, and relatively uniform fat distribution. Large-scale genotyping confirmed the presence of the 67-bp variable duplication in Gaoqing Black cattle (Figure 1c, d), but the 2D allele occurred at a low frequency and the locus showed a low level of polymorphism

(Table 3). This allele distribution may reflect the breed-specific genetic background of Gaoqing Black cattle and may also be related to long-term selection for meat quality and carcass fat deposition characteristics. Although the 2D allele occurred at a low frequency, the genotype distribution did not deviate from HWE. This result indicates that the low frequency of the 2D allele was not accompanied by a significant distortion of genotype proportions in the analyzed population [26]. Therefore, the presence of the 2D allele at a relatively low frequency in Gaoqing Black cattle and other reported cattle populations may reflect population history, genetic drift, artificial selection, or functional constraint [27, 28].

Variable duplication sequences in promoter regions can function as cis-regulatory elements by altering transcription factor binding, chromatin accessibility, or local epigenetic states [29, 30]. In the present study, *ADIPOQ* promoter activity decreased progressively as the copy number of the 67-bp variable duplication increased (Figure 2). Specifically, the pGL3-0D construct showed the highest basal promoter activity, whereas the 1D and 2D constructs showed markedly reduced activity in both 293T and CHO cells. This copy number-dependent decrease indicates that the 67-bp variable duplication functions as a dosage-sensitive regulatory element rather than a passive sequence insertion. Therefore, variation in duplication copy number may directly modulate *ADIPOQ* transcriptional output by changing the regulatory architecture of its promoter. Combined with transcription factor prediction analysis, the 67-bp duplication sequence was predicted to contain binding sites for HES5 (Figure 3a). Dual-luciferase reporter assays showed that HES5 mediated transcriptional repression (Figure 3d). Notably, HES5-mediated repression followed the same copy-number-dependent pattern, with the 2D construct showing a stronger inhibitory response than the 1D construct. HES family TFs are classical basic helix-loop-helix transcriptional repressors and are well-known downstream effectors of Notch signaling [31, 32]. These factors repress target gene transcription by binding to specific cis-regulatory motifs and recruiting Groucho/TLE family corepressors, which can further engage chromatin-repressive machinery such as histone deacetylase-containing complexes [33, 34]. Together, these findings extend the current understanding of upstream transcriptional regulation of *ADIPOQ* by identifying HES5 as a potential mediator of the copy number-dependent repression driven by the 67-bp variable duplication.

Given that the 67-bp duplication altered *ADIPOQ* promoter activity and introduced HES5-mediated copy-number-dependent repression, this variant may have downstream phenotypic consequences in cattle. Because *ADIPOQ* showed adipose-enriched expression and is closely related to lipid metabolism, we further evaluated whether this repeat-associated regulatory variant was associated with carcass composition traits, particularly fat-related carcass traits, in Gaoqing Black cattle. In the present study, sex-stratified association analyses showed that the 67-bp repeat-associated variant was significantly associated with selected carcass traits in Gaoqing Black cattle. Notably, 1D/2D individuals showed lower brisket fat weight in males and lower carcass fat weight in females than 1D/1D individuals. This pattern is consistent with the promoter activity assays, in which increased repeat copy number reduced *ADIPOQ* promoter activity. Because *ADIPOQ* was predominantly expressed in adipose-related tissues, these results suggest that the repeat-expanded allele is associated with reduced fat-related carcass traits, potentially through transcriptional repression of *ADIPOQ*, although direct in vivo evidence is needed to confirm this mechanistic link.

It is noteworthy that the observed association, the allele linked to lower *ADIPOQ* promoter activity correlating with reduced fat-related carcass traits, appears to contrast with the well-established inverse relationship between circulating adiponectin levels and body fat mass commonly reported in human and rodent studies of obesity and metabolic syndrome [35–37]. However, in the context of beef cattle production, *ADIPOQ* has been repeatedly implicated as a candidate gene positively associated with fat deposition and marbling. For instance, genetic variants in the *ADIPOQ* promoter region have been associated with increased marbling scores in Hanwoo cattle [12], and polymorphisms near the gene have shown links to carcass fat traits in Angus cattle [9]. These apparent discrepancies may reflect species-specific differences in *ADIPOQ* function, the distinction between local autocrine/paracrine effects in adipose depots versus systemic circulating adiponectin, depot-specific biology (e.g., brisket fat versus overall adiposity), or the consequences of long-term artificial selection for meat quality in beef breeds. Our findings are therefore consistent with a net positive contribution of *ADIPOQ* to selected fat-related carcass traits in Gaoqing Black cattle, aligning with its established role as a candidate gene for fat deposition in livestock [7–10]. This comparison highlights the importance of studying regulatory variants in production animals rather than extrapolating directly from metabolic disease models.

However, significant associations were not observed for all fat-related carcass traits. Several factors may contribute to this pattern, including biological differences among fat depots, variation in local tissue microenvironments, and the limited statistical power caused by the low frequency of the 2D allele. Different fat depots may respond differently to changes in *ADIPOQ* transcription because they differ in developmental origin, metabolic activity, and endocrine sensitivity. Therefore, larger and independent cattle populations are needed to further validate the association between this 67-bp duplication and fat-related carcass traits. Together, these findings provide new insight into the transcriptional regulation of bovine *ADIPOQ* and suggest that the 67-bp duplication may serve as a candidate genetic marker for fat-related carcass traits in beef cattle.

5. Conclusions

In this study, we functionally characterized a 67-bp variable duplication in the promoter region of bovine *ADIPOQ*. The duplication showed a copy number-dependent regulatory effect on *ADIPOQ* promoter activity, with increased duplication copy number leading to reduced promoter activity. Functional analyses further indicated that HES5 may mediate transcriptional repression through its binding site within the 67-bp duplication sequence. In Gaoqing Black cattle, association analyses showed that this duplication was associated with shank meat weight and brisket fat weight in male cattle, and with carcass fat weight in female cattle. Overall, these findings provide new evidence that the 67-bp variable duplication may contribute to phenotypic variation in Gaoqing Black cattle by modulating *ADIPOQ* promoter activity through HES5-mediated transcriptional repression. This variant warrants further investigation as a candidate locus for carcass and fat-related trait improvement in this local beef cattle population.

Abbreviations

ADIPOQ

Adiponectin gene

293T

Human embryonic kidney 293T cells

CHO

Chinese hamster ovary

He

Expected heterozygosity

HES5

Hes family bHLH transcription factor 5

Ho

Observed heterozygosity

HWE

Hardy-Weinberg equilibrium

Ne

Effective number of alleles

PCR

Polymerase chain reaction

PIC

Polymorphism information content

SEM

Standard error of the mean

TF

Transcription factor.

Declarations

Ethics approval and consent to participate: All procedures involving animals were reviewed and approved by the Ethics Committee of Northwest A&F University (Approval No. DK2022032). The study was conducted in accordance with the relevant institutional guidelines and the national regulations for the care and use of animals issued by the Ministry of Science and Technology of China. Written informed consent was obtained from the animal owners or their authorized representatives for the use of animals, biological samples, and associated production records in this study. No animals were euthanized or experimentally sacrificed specifically for the purpose of this study.

Consent for publication: Not applicable.

Availability of data and materials: Availability of data and materials: The variation data reported in this paper have been deposited in the Genome Variation Map (GVM) in National Genomics Data Center,

Beijing Institute of Genomics, Chinese Academy of Sciences and China National Center for Bioinformation, under accession number GVM001518.

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Authors' contributions: CP, EJ and XL conceived and designed the study. YL, QL, PQ, AL, XL and EJ performed the experiments and contributed to data analysis. QL, XL and NC curated the data. EJ, XL and NC provided resources. YL, PQ, XL and QL drafted the manuscript. YL, EJ and XL reviewed and edited the manuscript. YL, AL, XL and NC prepared the figures and other visualizations. EJ and XL supervised the study. EJ administered the project. XL acquired funding. YL and QL contributed equally to this work. All authors read and approved the final manuscript.

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Figures

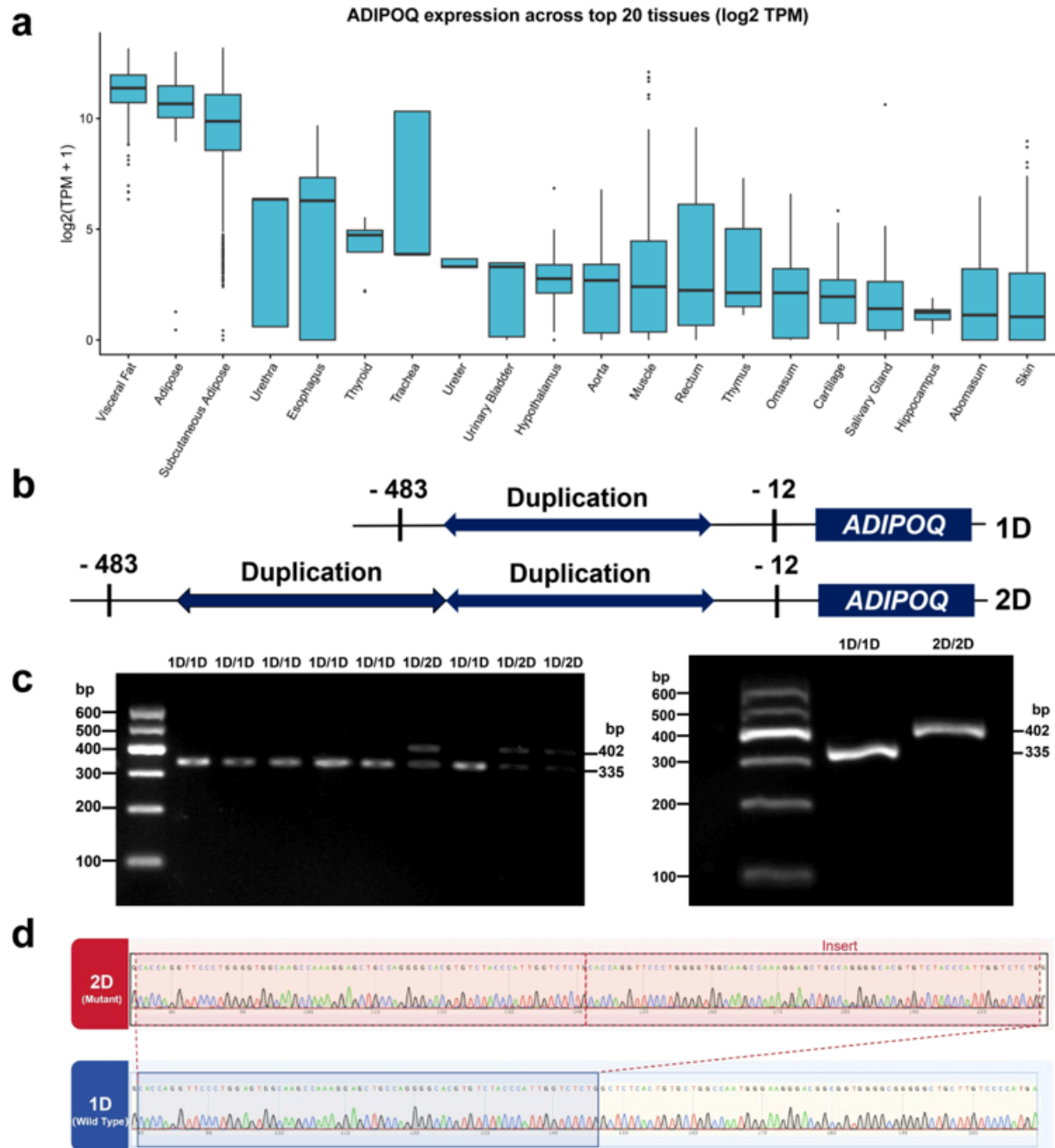


Figure 1

Characterization of the *ADIPOQ* promoter 67-bp variable duplication in Gaoqing Black cattle: (a) Tissue expression profile of *ADIPOQ* based on CattleGTEx data; (b) Schematic representation of the 67-bp variable duplication in the *ADIPOQ* promoter; (c) PCR genotyping of the 67-bp variable duplication; (d) Sanger sequencing validation of the 1D and 2D alleles.

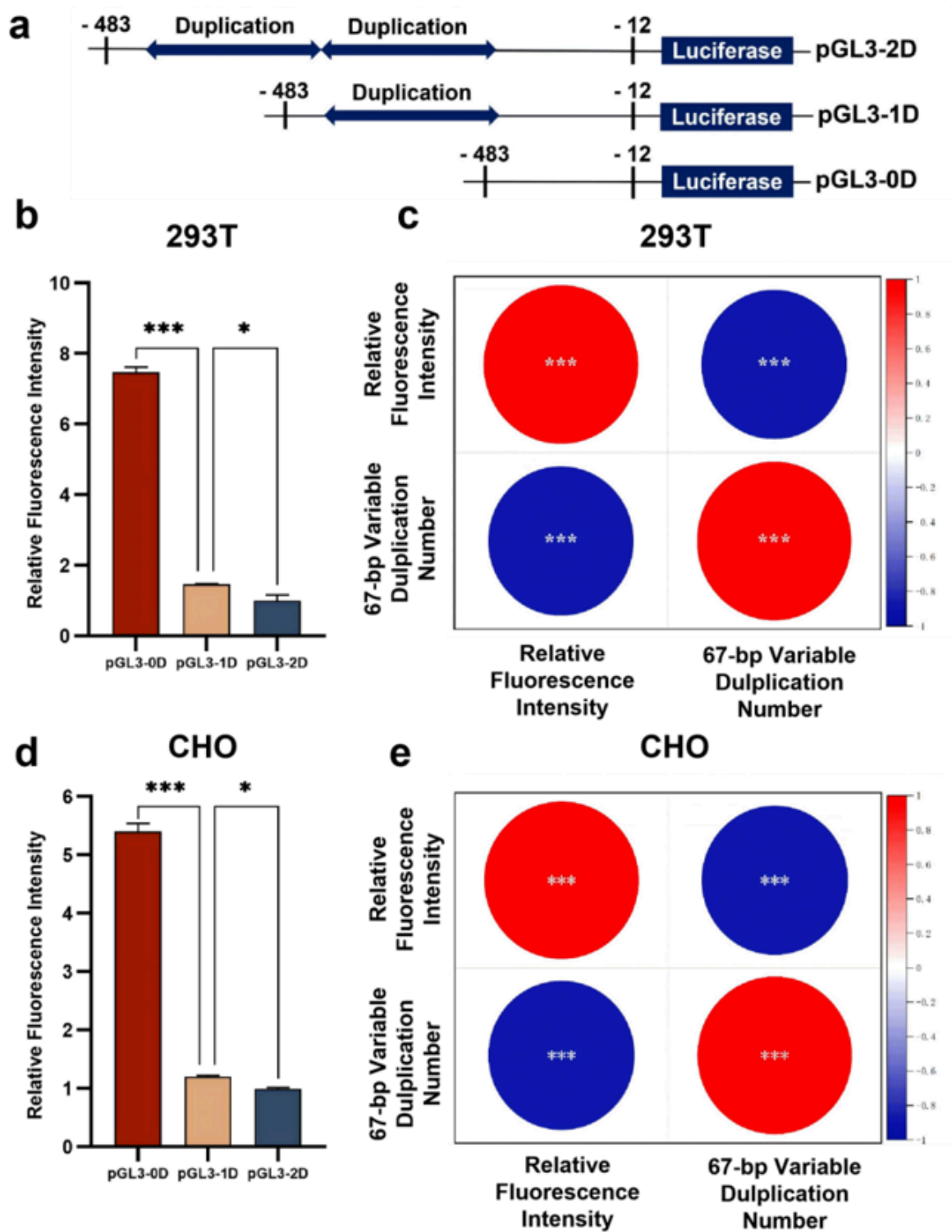


Figure 2

Dual-luciferase assay results of constructs carrying different copy numbers of the 67-bp duplication in the *ADIPOQ* promoter region: (a) Schematic diagram of the construction of truncated vectors for different copy number variants in the promoter region of the bovine *ADIPOQ* gene; (b) Relative luciferase activity of different constructs in 293T cells; (c) Correlation analysis between 67-bp copy number and luciferase activity in 293T cells. (d) Relative luciferase activity of different constructs in CHO cells; (e)

Correlation analysis between 67-bp copy number and luciferase activity in CHO cells. Differences in relative luciferase activity among groups were evaluated using a two-tailed t-test. Error bars represent mean $\pm$ standard error of the mean (SEM). * and *** indicate $p < 0.05$ and $p < 0.001$, respectively.

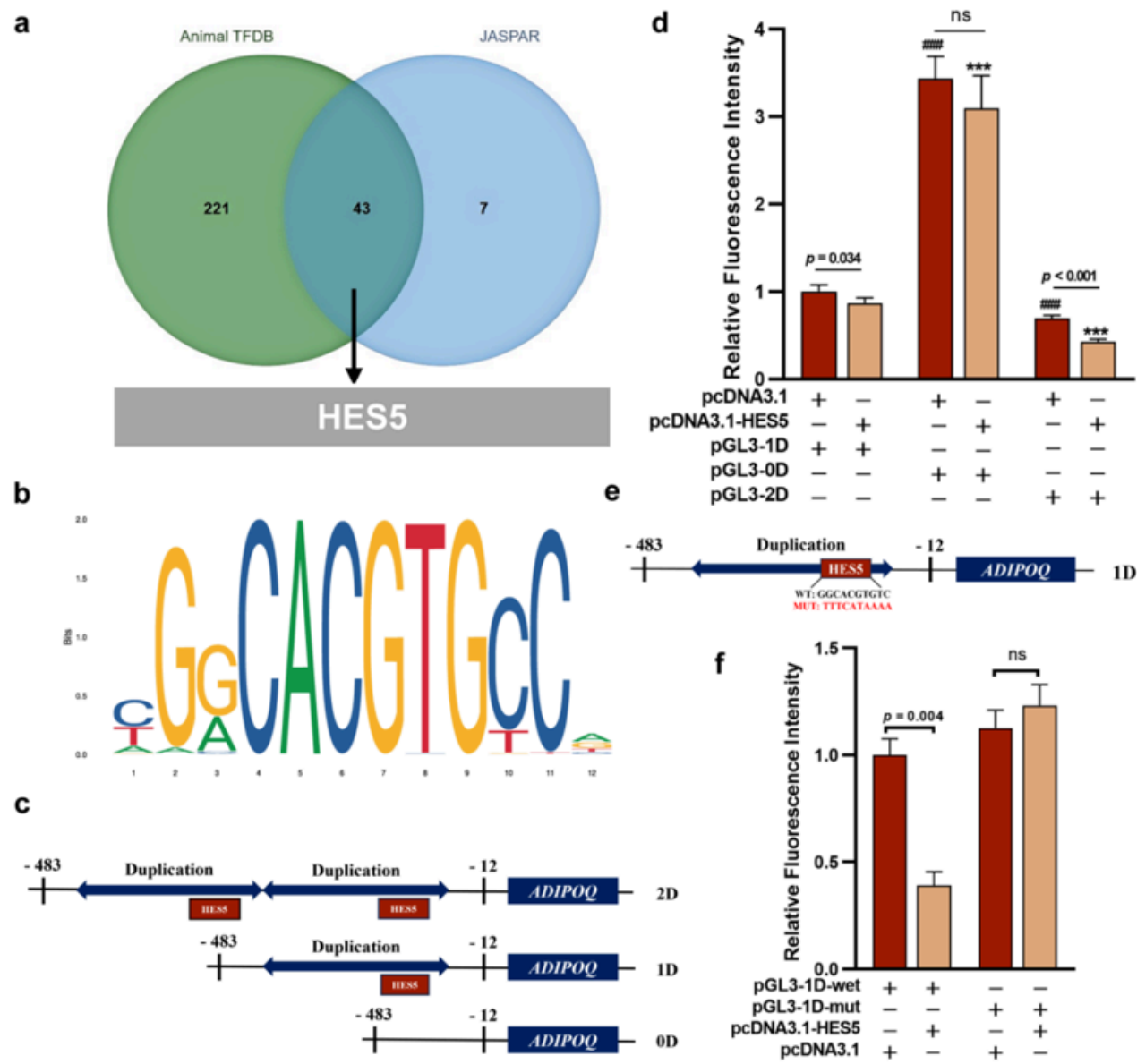


Figure 3

Analysis of the interaction between the bovine *ADIPOQ* promoter region and the HES5 transcription factor: (a) Screening results of potential TFs; (b) Sequence characteristics of the HES5 binding motif; (c) Schematic diagram of HES5 binding sites in different haplotypes of the *ADIPOQ* promoter; (d) Effect of the 67-bp variable duplication on HES5-mediated repression of the *ADIPOQ* promoter activity; ### indicates $p < 0.001$ compared with the pcDNA3.1 + pGL3-1D/G group; *** indicates $p < 0.001$ compared

with the pcDNA3.1-HES5 + pGL3-1D/G group; (e) Mutation of HES5 Binding Site; (f) Functional validation of HES5 binding site by mutation analysis, supporting that disruption of the motif abolishes HES5-mediated transcriptional repression. Differences in relative promoter activity between groups were evaluated using a two-tailed t-test. Data are presented as mean $\pm$ standard error of the mean (SEM). ns, not significant ($p > 0.05$).

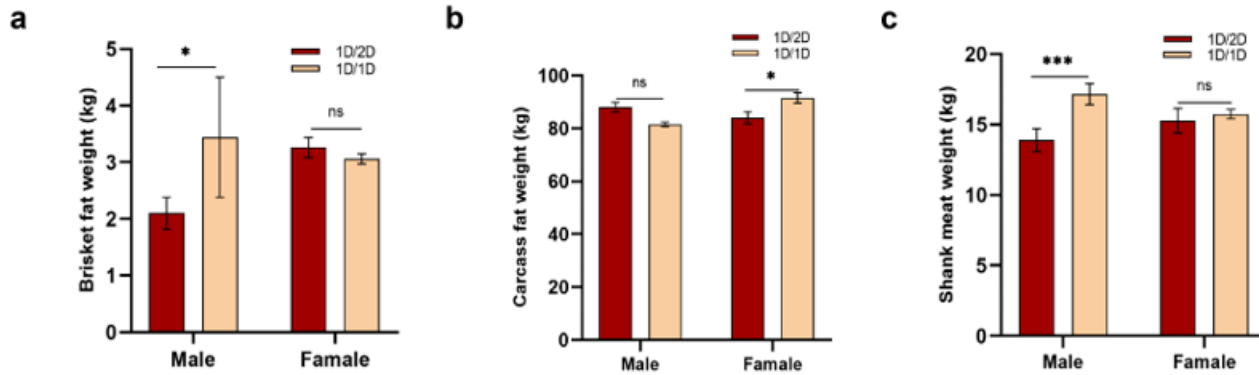


Figure 4

Carcass-related traits significantly associated with the 67-bp variable duplication in Gaoqing Black cattle. Association analyses were performed separately in male and female cattle. The bar plots show differences between the 1D/1D and 1D/2D genotypes for three carcass and fat-related traits: brisket fat weight (a), carcass fat weight (b), and shank meat weight (c). Data are presented as mean $\pm$ SEM. Statistical significance was evaluated using genotype-based association analysis within each sex group. ns, not significant; * $p < 0.05$; *** $p < 0.001$.

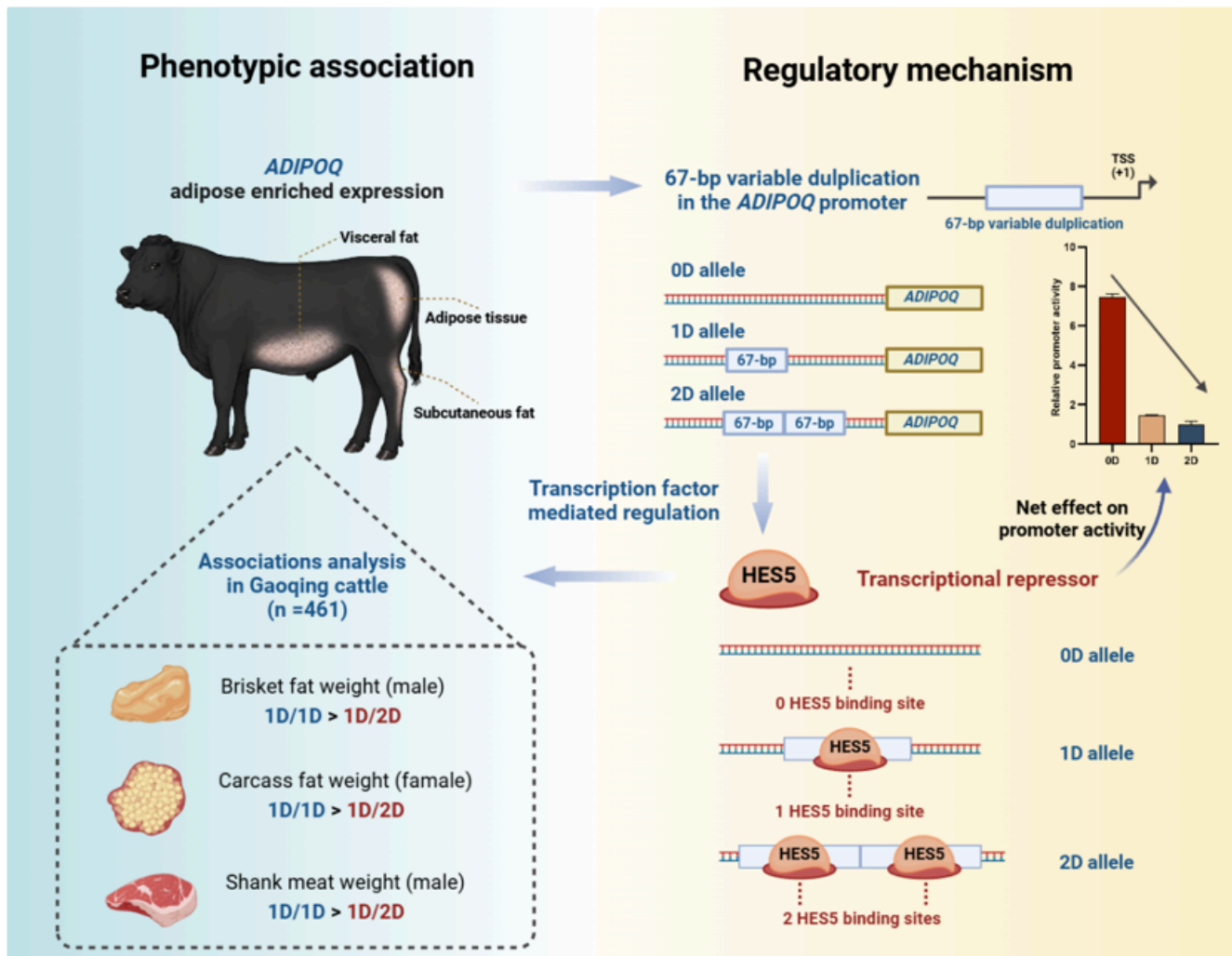


Figure 5

Proposed regulatory mechanism of the 67-bp variable duplication in the bovine *ADIPOQ* promoter.

The illustration was created using BioRender.com.

Supplementary Files

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