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RESEARCH ARTICLE

An InDel in the promoter of ribosomal protein S27-like gene regulates skeletal muscle growth in pigs

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Highlights

- *RPS27L* is identified as a novel regulator of skeletal myogenesis in pigs.
- A 13-bp promoter InDel in *RPS27L* modulates its expression by altering MYOD and TCF3 binding.
- The insertion and deletion alleles are specific to Chinese local breeds and Western commercial breeds, respectively. This InDel is associated with meat production traits.

Abstract

Genetic improvement of meat production traits has always been the primary goal of pig breeding. Geographical isolation, natural and artificial selection led to significant differences in the phenotypes of meat production traits between Chinese local pigs and Western commercial pigs. Comparative genomics and transcriptomics analysis provided powerful tools to identify genetic variants and genes associated with skeletal muscle growth. However, the number of available genetic variants and genes are still limited. In this study, a comprehensive comparison of transcriptomes showed that ribosomal protein S27-like (*RPS27L)* gene was highly expressed in skeletal muscle and up-regulated in Chinese local pigs when compared with Western commercial pigs. Functional analysis revealed that overexpression of *RPS27L* promoted myoblast proliferation and repressed differentiation in pig skeletal muscle cells. Conversely, the knockdown of *RPS27L* led to the inhibition of myoblast proliferation and the promotion of differentiation. Notably, a 13-bp insertion-deletion (InDel) mutation was identified within the *RPS27L* promoter, inserted in Chinese local breeds and predominantly deleted in Western commercial breeds. Luciferase reporter assay suggested this InDel modulated *RPS27L* expression by influencing transcription factor 3 (TCF3) and myogenic differentiation antigen (MYOD) binding to the promoter. Furthermore, a positive correlation was observed between *RPS27L* expression and backfat thickness. Association studies demonstrated this InDel was significantly associated with the body weight of pigs at the age of 240 d. Together, our results suggested that *RPS27L* was a regulator of skeletal muscle development and growth, and was a candidate marker for improving meat production traits in pigs. This study not only provided a biomarker for animal breeding, but also was helpful for understanding skeletal muscle development and muscular disease in humans.

Keywords: pig, InDel, *RPS27L*, skeletal muscle, growth and development

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1. Introduction

Pigs are one of the most important farm animals for meat production worldwide, while also provide an attractive biomedical model for human diseases (Groenen *et al.* 2012; Prather 2013; Yang *et al.* 2014; Zeng *et al.* 2024) and a donor for xenotransplantation (Längin *et al.* 2018; Yan *et al.* 2018). Due to geographical divergence, local adaptation, natural and artificial selection, significant genetic and phenotypic divergences have developed between Chinese local pigs and Western commercial pigs (Ai *et al.* 2015; Li *et al.* 2017). Chinese local breeds such as Tongcheng, Luchuan and Guizhou pigs generally have slower growth rates, less lean meat productivity, and poor feed efficiency, but have comparatively higher levels of intramuscular fat and tolerance to roughage in their diets than Western commercial breeds such as Landrace, Yorkshire and Duroc pigs (Tang *et al.* 2007; Ai *et al.* 2015).

Comparative genomics and transcriptome analysis provide powerful tools to identify genetic variants, regulatory elements and genes associated with economic traits such as growth rates in pigs (Tang *et al.* 2007; Li *et al.* 2017; Kojima *et al.* 2018; Zhao *et al.* 2018). For example, a mutation in intron 3 of the insulin-like growth factor 2 (*IGF2*) gene was the causative mutation regulating muscle growth in pigs (Van Laere *et al.* 2003). Additionally, a splice mutation in the phosphorylase kinase subunit G1 (*PHKG1*) gene has been shown to increase glycogen content and thereby reduced pork quality (Ma *et al.* 2014). Besides variants in the genic regions, recent studies also suggest that mutations in *cis*-regulatory elements may affect gene expression in a spatio-temporal manner to drive phenotypic diversity (Shapiro *et al.* 2004; Kvon *et al.* 2016; Berger *et al.* 2018; Naval-Sanchez *et al.* 2018; Feigin *et al.* 2019). For instance, a structural variant in the promoter region of myosin heavy chain 3 (*MYH3*) influences muscle fiber-type composition and intramuscular fat content (Cho *et al.* 2019). Additionally, an enhancer-modulating single-nucleotide polymorphism (SNP) that is associated with up-regulation of the troponin C1, slow skeletal and cardiac type (*TNNC1*) gene might increase the proportion of slow skeletal muscle fibers and affect meat quality (Liu *et al.* 2022).

The ribosomal protein S-27-like (*RPS27L*) gene is an evolutionarily conserved ribosomal protein with 84-amino acid protein in ribosome 40S small subunit. Previous studies reported that *RPS27L* played important roles in various biological processes, such as tumorigenesis (Sun *et al.* 2020), cancer (Xiong *et al.* 2011), and apoptosis (Xiong *et al.* 2014). In pigs, studies have reported that *RPS27L* is located in a quantitative trait locus (QTL) interval associated with body weight (Guo *et al.* 2008; Rothammer *et al.* 2014). By integrating analysis of dynamic transcriptome and genome re-sequencing data, we uncovered transcriptomic diversity of skeletal muscle between the Tongcheng (a Chinese local pig breed) and Landrace (a Western commercial pig breed) pigs, and found that the *RPS27L* gene was differentially expressed between these two breeds. This suggests that *RPS27L* gene may be involved in the diversity of skeletal muscle phenotype between Chinese local pigs and Western commercial pigs. It drives us to explore the function of the *RPS27L* gene in skeletal muscle and the molecular mechanism of regulating meat production traits in pigs.

In this study, we used our own and public large-scale transcriptome data to explore the expression of the *RPS27L* across different tissues and developmental stages in Chinese local pigs and Western commercial pigs. The function and regulation mechanism of *RPS27L* in myogenesis were studied in skeletal muscle cells. Using population re-sequencing data, we identified a novel functional 13-bp insertion in the promoter of *RPS27L* in Chinese local pigs. Association studies demonstrated that *RPS27L* was associated with meat production traits. Overall, our study indicated that *RPS27L* was a new regulator of muscle growth, which could be used as a marker for pig breeding. These findings were also helpful for understanding skeletal muscle development and muscular disease in humans.

2. Materials and methods

2.1. Experimental design

In this study, publicly available RNA-seq data from Chinese local pigs and Western commercial pigs were firstly used to analyze the expression of *RPS27L* across different tissues, breeds and developmental stages (postnatal days 30, 180, and 300) (Zhou *et al.* 2021). The RNA-seq data from Tongcheng (a Chinese local breed) and Landrace (a Western commercial breed) pigs were used to analyze the expression profile of *RPS27L* gene in skeletal muscle (*longissimus dorsi*) across 27 developmental time points (from embryonic day 33 (E33) to postnatal day 180 (D180)) Yang *et al*. 2021). The expression pattern of *RPS27L* were further verified by real-time quantitative PCR (RT-qPCR). The function and regulation mechanism of *RPS27L* in myogenesis were studied in skeletal muscle cells. Whole genome sequencing data in a wide panel of 953 individuals from 81 Chinese local breeds and Western commercial breeds were used to identify variants in or around the genic region of *RPS27L*. Lastly, molecular experiments in skeletal muscle cells and association analyses with pig economic traits were used to assess whether these variants are associated with skeletal muscle growth and meat production traits.

2.2. Transcriptome and whole genome resequencing datasets

The publicly available RNA-seq data from NCBI (Bioproject PRJNA612825) (Zhou *et al*. 2021) was used for gene expression analysis, which consisted samples from five organs/tissues (skeletal muscle, adipose, liver, hypothalamus, and pituitary) from three developmental time points (postnatal days 30, 180, and 300) across four pig breeds (Yorkshire, Meishan, Wuzhishan, and Tibetan). The gene expression matrix of skeletal muscles across 27 developmental stages was used to analyze the expression abundance (RPKM value) of *RPS27L* during skeletal muscle development (Yang *et al*. 2021). The whole genome resequencing data were used to identify variants of *RPS27L* and to analyze the allele frequency (AF) in Chinese local pigs and Western commercial pigs (Wang *et al.* 2014; Xie *et al.* 2021).

2.3. Populations and association studies

A F_1 crossbred population of 67 pigs from Luchuan (a Chinese local breed)×Duroc (a Western commercial pig breed), was used to analyze the association between the insertion-deletion (InDel) in the promoter of *RPS27L* and average backfat thickness at 6th to 7th rib. Another F_2 resource population, which was constructed by intercrossing Western commercial Yorkshire boars and Chinese local Min sows (Wang *et al.* 2014; Xie *et al.* 2021), was used to analyze the association of InDel with economic traits in a population of 557 pigs. The mixed linear model under restricted maximum likelihood (REML) estimation was used for the association studies after taking into account the family relationships among samples.

2.4. RNA preparation and RT-qPCR

The *longissimus dorsi* muscle was isolated from Landrace, Duroc, Tongcheng, Luchuan and Guizhou pigs slaughtered at 180 d (D180) of age, and 3 biological replicates were obtained from each breed. Total RNAs were extracted by using TRIzol reagent (TaKaRa, Japan), cDNA synthesis from RNA was performed using the HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, China) according to the manufacturer's instructions. RT-qPCR was performed using the ChamQ SYBR qPCR Master Mix (Without ROX) (Vazyme, China). RT-qPCR data were analyzed using the ΔΔCt method, and individual gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression or 18s *RNA* expression. The primer sequences used in this study are listed in Appendix A.

2.5. Cell culture

Satellite cells from pig skeletal muscle were maintained in our laboratory. Pig skeletal muscle cells (SKMs) were bought from Guangzhou Xinyuan Technology Co., Ltd., China. Cells were cultured in growth medium: DMEM (Gibco, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO_2.

2.6. Plasmid construction, RNA interference and transfection

To construct the *RPS27L*-overexpression vector (*RPS27L-OV*), the full-length sequence of *RPS27L* was cloned into the pcDNA3.1 vector (Genecreate Biotech, China), and the mock vector lacking the *RPS27L* sequence was used as a negative control (*RPS27L-NC*). The same process was used to obtain the myogenic differentiation antigen (MYOD) overexpression vector (*MYOG-OV*) and transcription factor 3 (TCF3) overexpression vector (*TCF3-OV*). To knock down *RPS27L*, positive small interfering RNAs (siRNAs, si-1, si-2, si-3) and a negative control (si-NC) were synthesized (RiboBio Biotech, China). siRNA sequences used in this study is listed in Appendix A. All transient transfections were performed using Lipofectamine 3000 according to the manufacturer's instructions (Invitrogen, USA).

2.7. Cell proliferation and differentiation

To examine cell proliferation, 10 μL Cell Counting Kit-8 (CCK-8) reagent (Beyotime, Shanghai, China) was added to each of 25 wells in a 96-well plate containing cells and incubated at 37°C for 45 min. The absorbance of each sample at 450 nm was measured using a microplate reader (Tecanspark, Switzerland), with samples measured at each of the following time point: 0, 24, 36, 48, and 72 h after transfection, according to the manufacturer's instructions. Five independent replicates were measured at each time point.

Cell proliferation was also assessed using the Cell-Light ethynyl-2′-deoxyuridine (EdU) DNA Cell Proliferation Kit (Beyotime, China). Cells were seeded in a 12-well plate and were transfected when they reached 50% confluence. After cultivating with GM for 24 h, the cells were incubated in 50 μmol L^{-1} EdU for 1 h. Fixed with 1 mL 4% paraformaldehyde for 15–30 min and permeabilized with 0.5% Triton X-100 for 10–15 min. The Click reaction mixture was then prepared according to the manufacturer's instructions, which was added to each well. After incubation at room temperature in the dark for 30 min, the cells were stained with DAPI at a dilution of 1:1,000 for nuclear staining. The number of EdU-stained cells was observed using a fluorescence microscope.

As for cell differentiation, myotubes were required firstly. To induce myotube formation, the growth medium needs to be replaced with differentiation medium containing 2% heat-inactivated horse serum (BI, USA) and 1% PS for 4 d, when myoblasts grew to 80–90% confluency of satellite cells from pig skeletal muscle.

2.8. Cell cycle assay

Flow cytometry analysis of the cell cycle was performed on a BD Accuri C6 flow cytometer (BD Biosciences, USA) as previously described (Schiaffino and Reggiani. 1996), and data were processed using the ModFit LT 5.0 software (Verity Software House, USA).

2.9. Western blotting

Total proteins respectively extracted from immortalization of SKMs and satellite cells from pig skeletal muscle followed as previously described (Yang *et al.* 2015, 2019). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were blocked with 5% skim milk for 2 h at room temperature, and subsequently probed with primary antibodies overnight at 4°C. The following dilutions were used for each antibody: PCNA (1:1,000, CST, 2586S, USA), KI67 (1:1,000, Novus, NB500-170, USA), CCNA2 (1:1,000, Abcam, ab137769, USA), MYOG (1:1,000, Affinity, DF8273, China) and β-actin (1:1,000, CST, 4970, USA), β-tublin (1:1,000, Santa Cruz, sc-58882, USA). Afterwards, the membranes were washed with PBS-Tween and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:1,000, Proteintech, China). Protein bands were detected after treatment with SuperSignal West Femto agent (Thermo Scientific, USA).

2.10. Luciferase reporter assay

To generate luciferase reporter constructs for *RPS27L*, the *RPS27L* promoters containing the *Q* and *q* alleles were chemically synthesized and cloned into a pGL3-Basic (Promega, USA) vector, respectively. The *RPS27L* luciferase reporter constructs and internal control (pRL-SV40) vectors were co-

transfected into SKMs, respectively. Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega, USA). Each transfected well was assayed in triplicate. Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well.

2.11. Statistical analysis

For the wet experiments, statistical analysis was performed with Prism 7 software (GraphPad Software, San Diego, CA, USA). A two-tailed Student *t*-test was used to analyze the statistical significance between two genotypes. Data are expressed as mean±SEM unless otherwise noted, and the level of significance was set at $P<0.05$.

3. Results

3.1. The expression profiling of *RPS27L* across tissues, developmental stages and breeds

We first explored the expression of *RPS27L* in various tissues across Chinese local and Western commercial pig breeds. Using a public RNA-seq dataset (BioProject, PRJNA612825) that covers five tissues (skeletal muscle, adipose, liver, hypothalamus, and pituitary) at three postnatal stages (D30, D180, and D300) in three Chinese local breeds (Meishan, Wuzhishan and Tibetan) and one Western commercial breed (Yorkshire), we found that *RPS27L* was much higher expressed in skeletal muscles (leg and dorsal muscles) than in other tissues. In skeletal muscles, the expression of *RPS27L* in pigs from China was much higher than in Yorkshire pigs (Fig. 1-A). Furthermore, using our time-series RNA-seq data in skeletal muscle of Tongcheng and Landrace pigs across 27 developmental stages from E33 to D180, we found that the expression of *RPS27L* in Tongcheng was significantly higher than in Landrace pigs at adult period (D80–D180) (Fig. 1-B). The expression difference of *RPS27L* in skeletal muscle between Chinese local pigs and Western commercial pigs were further confirmed by RT-qPCR (Fig. 1-C). These results suggested that *RPS27L* was highly expressed in skeletal muscle and differentially expressed between Chinese local pigs and Western commercial pigs.

3.2. *RPS27L* overexpression promotes proliferation but inhibits differentiation in pig skeletal muscle cells

To investigate the function of *RPS27L* in myogenesis, we

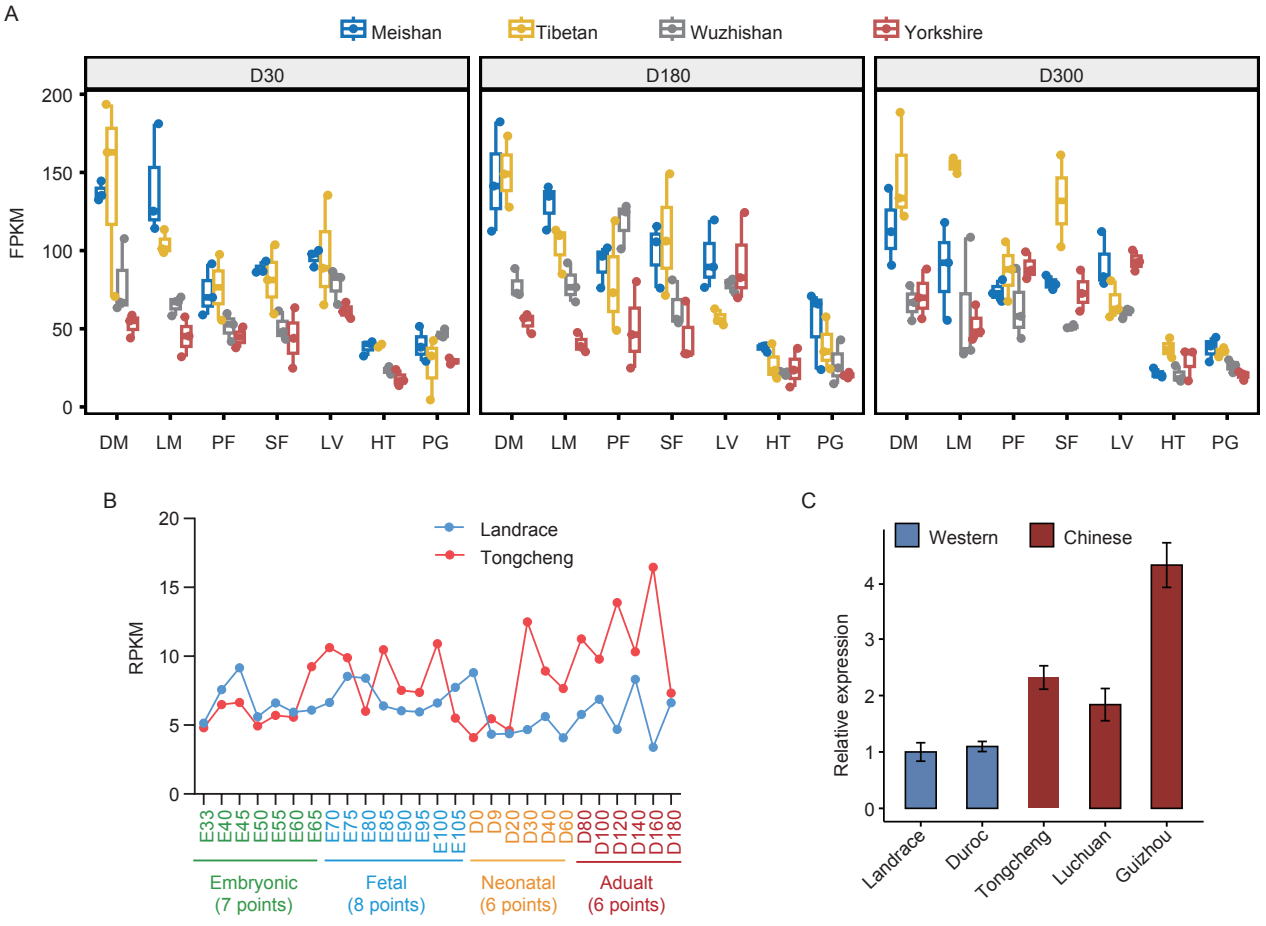


Fig. 1 The expression abundance of *RPS27L* in skeletal muscle across breeds, tissues and development stages. A, transcriptome data showed that the spatial-temporal expression of ribosomal protein S27-like (*RPS27L*) across 5 tissues from 3 postnatal stages (D30, D180 and D300) in four pig breeds (Meishan, Wuzhishan, Tibetan and Yorkshire). B, comparison analysis of *RPS27L* expression between Tongcheng and Landrace pigs. C, the validation of differential expression of *RPS27L* between Western commercial and Chinese local pig breeds using RT-qPCR. DM, dorsal muscles; LM, leg muscles; PF, perirenal fat; SF, subcutaneous fat; LV, liver; HT, hypothalamus; PG, pituitary gland. Data are mean±SE (n=3).

firstly constructed pcDNA3.1-*RPS27L* plasmid (*RPS27L-OV*) to overexpress *RPS27L* and the empty plasmid *(RPS27L-NC)* was used as the negative control. RT-qPCR results showed *RPS27L* was significantly overexpressed in SKMs (Fig. 2-A). Both CCK-8 assay and EdU incorporation assay showed that *RPS27L* overexpression exhibited higher proliferation activity compared with the negative control group (Fig. 2-B and C). Cell cycle analysis indicated that *RPS27L* overexpression reduced the number of cells entering G0/G1 phase and increased the number of cells entering S phase (Fig. 2-D and E). Additionally, *RPS27L* overexpression strongly enhanced the expression of proliferation markers such as KI67, PCNA, CDK6 and N-RAS at both mRNA (Fig. 2-F) and protein levels (Fig. 2-G and H). Notably, *RPS27L* overexpression in myotubes repressed the expression of myogenic markers such as *MYOD*, *MYOG* and *MYHC* at mRNA level (Fig. 3-I), while reduced MYOG protein level (Fig. 2-J and K). Collectively, these findings indicated that overexpression of *RPS27L* could promote myoblast proliferation but inhibit differentiation.

3.3. *RPS27L* knockdown inhibits myoblast proliferation and promotes differentiation

To further validate the function of *RPS27L* in myogenesis, siRNAs were designed to knock down *RPS27L* in porcine SKMs. The expression of *RPS27L* was effectively knocked down by all the three siRNAs (Fig. 3-A). Two of the siRNAs (si-1 and si-2) were then selected to launch the following experiments. Both CCK-8 and EdU incorporation assays showed that the proliferation activity was reduced when *RPS27L* was knocked down (Fig. 3-B and C). Cell cycle analysis indicated that *RPS27L* knockdown increased the proportion of cells entering G0/G1 phase and reduced the proportion of cells entering S phase (Fig. 3-D and E). In addition, *RPS27L* knockdown significantly reduced the expression of KI67, PCNA, N-RAS, CCNA2 and CCND1 at both mRNA (Fig. 3-F) and protein levels (Fig. 3-G). Meanwhile, RT-qPCR results showed the expression of MYOD, MYOG and MYHC were up-regulated at mRNA (Fig. 3-H) and protein level (Fig. 3-I) when myotubes were formed after induced by differentiation medium. Overall, these findings demonstrated that *RPS27L* knockdown can inhibit myoblast proliferation but promote myogenic differentiation.

3.4. Identification of an InDel in the promoter of *RPS27L*

Variations in *cis*-regulatory elements could alter gene expression and influence the phenotypic diversity (Roscito *et al.* 2018; Feigin *et al.* 2019). To analyze the effect of *cis*-regulatory variants of *RPS27L* on skeletal muscle phenotypes, the whole genome re-sequencing data of Landrace and Tongcheng pigs (Yang *et al.* 2021) were analyzed. Five InDel polymorphisms and 25 SNPs with AF differences (ΔAF>0.9) were identified between the two breeds, including a 13-bp novel InDel (chr1:108,880,114, Suscrofa11.1) in the promoter of *RPS27L*. The insertion allele (*Q* allele) was fixed in Togncheng, while the deletion allele (*q* allele) was found in Landrace pigs, which was further confirmed by Sanger sequencing (Fig. 4-A).

We further investigated the allele type of the InDel in different Chinese local and Western commercial pig breeds. Comparative analysis across the 13 pig reference genomes deposited in Ensembl database suggested that the promoter of *RPS27L* was the *q* allele type in 7 Western commercial breeds, and was the *Q* allele type in 6 Chinese local breeds (Fig. 4-B). Using our own and publicly available re-sequencing data, we further detected the AF of this InDel in a wide panel of 953 individuals from 81 Chinese local and Western commercial breeds. In the 903 successfully genotyped pigs, we found that the *Q* allele was absent in most Western commercial populations (AF=5.98%), such as in Landrace, Duroc and Large white pigs, while fixed in almost all the Chinese local pigs (AF=90.36%), such as in Tongcheng, Meishan and Luchuan pigs (Fig. 4-C). These results suggested that the allele was widely existed in Chinese local breeds, but largely skipped in Western commercial breeds.

3.5. The InDel affects the expression of *RPS27L* by affecting TCF3 and MYOD binding

The luciferase reporter assay suggested that the constructs containing the *Q* sequence fragment showed almost 9 times higher reporter activity than the *q* construct in satellite cells from pig skeletal muscle (Fig. 5-A), indicating that this InDel affected the expression of *RPS27L*. We used MEME Suite (v5.5.3, https://meme-suite.org) to predict potential transcription factors (TFs) that could bind with the InDel, leading to the identification of a putative TCF3 binding motif (Fig. 5-B). The basic helix-loop-helix E-protein TCF3 was reported to dimerize with myogenic regulatory factors (Londhe and Davie 2011). Reports demonstrated that TCF3 was able to heterodimerize with MYOD *in vivo* (Lluís *et al.* 2005; Lingbeck *et al.* 2008). We used TRRUST (V2, https://www.grnpedia.org/trrust/) to detect other TFs that could share targets with TCF3, which led to the prediction of MYOD (P=1.52×10^{-8}, Fig. 5-C), a myogenesis regulator (Weintraub *et al.* 1991; Rudnicki *et al.* 1993). To facilitate the comparison of the effects of the transcription factors, we utilized the empty PGL3 vector as the negative control group. The luciferase activities of both control groups were normalized to 1. The luciferase reporter assay in SKMs showed that *TCF3-OV* significantly increased the luciferase activity of *Q* allele compared with that of *q* allele (Fig. 5-D). Similar results were obtained from *MYOD-OV*, as it shared the similar motif with TCF3 (Fig. 5-D). In addition, when *TCF3-OV* and *MYOD-OV* constructs were co-transfected with the InDel respectively, these two TFs synergistically promoted luciferase activity, leading the activity of *Q* allele was much higher than *q* allele (Fig. 5-D). Collectively, these findings demonstrated that *Q* sequence had a strong binding site specific to TCF3 and MYOD.

3.6. The InDel is potentially associated with the body weight and backfat thickness

Previous studies reported that *RPS27L* was located in a QTL interval significantly associated with body weight in pigs (Guo *et al.* 2008; Rothammer *et al.* 2014). In a crossbred F_1

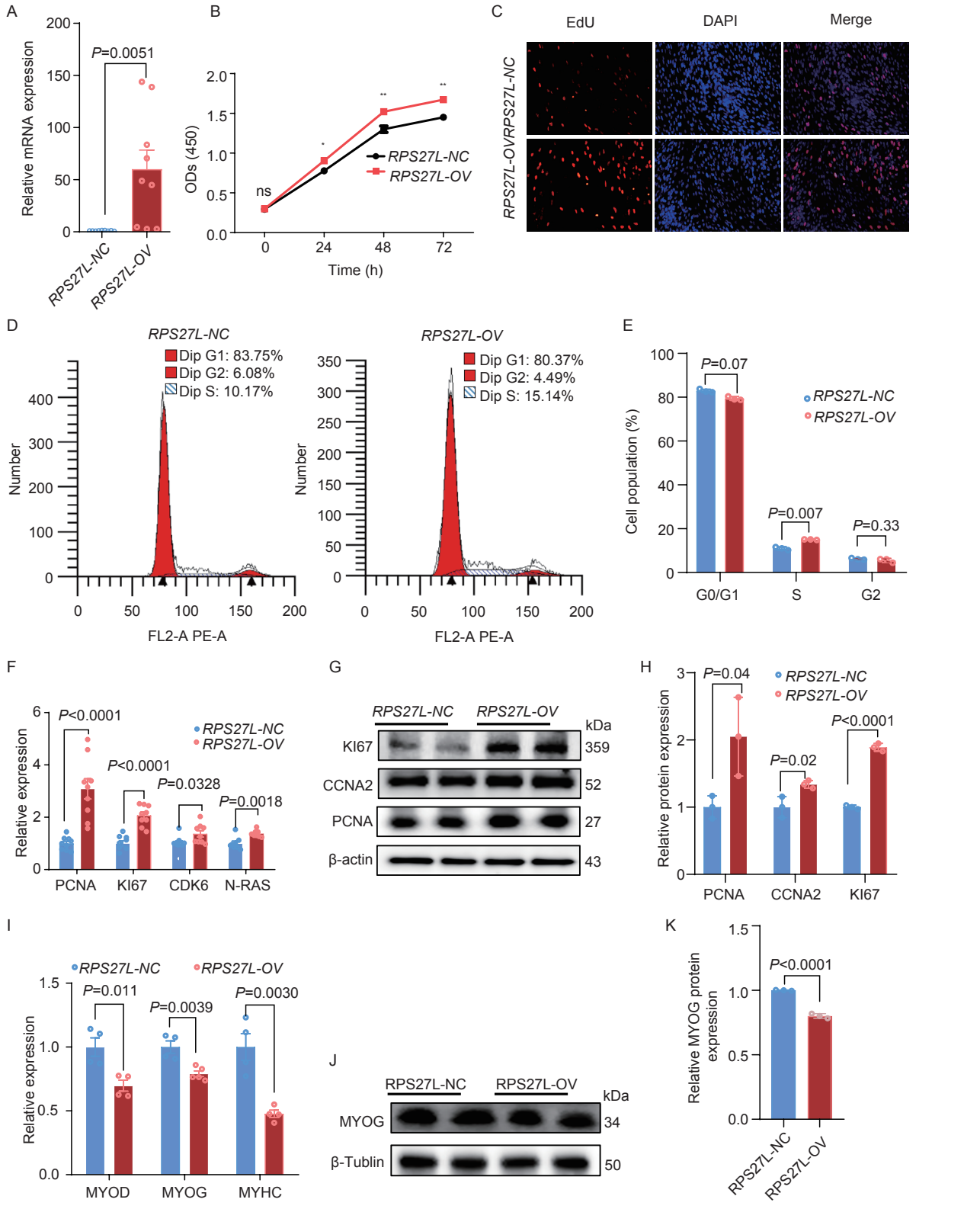


Fig. 2 *RPS27L* overexpression facilitates myoblast proliferation but inhibits differentiation in porcine skeletal muscle cells. A, *RPS27L* was overexpressed in porcine skeletal muscle cells (SKMs) when transfected with *RPS27L* overexpressed vector (*RPS27L-OV*) compared with the negative control vector (*RPS27L-NC*). B, cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) assay in SKMs when transfected with *RPS27L-OV* or *RPS27L-NC*. C, using ethynyl-2′-deoxyuridine (EdU) assay to assess cell proliferation in SKMs when transfected with *RPS27L-OV* or *RPS27L-NC*. EdU staining (red) for positive cells; DAPI staining (blue) for cell nuclei. D and E, the cell cycle was analyzed using flow cytometry after transfection with *RPS27L-OV* or *RPS27L-NC* in SKMs. F–H, the expression level of proliferation markers was detected at mRNA (F), and protein level (G) and H, after transfecting with *RPS27L-OV* or *RPS27L-NC* plasmids in SKMs. I–K, the expression level of myogenic markers was detected at transcription (I) and protein (J and K) in satellite cells from pig skeletal muscle after transfected with *RPS27L-OV* or *RPS27L-NC* plasmids. MYOD, myogenic differentiation antigen; MYOG, myogenin; MYHC, myosin heavy chain. Data are mean±SE (n≥3). ns, not significant, *, P<0.05, **, P<0.01 and ***, P<0.001.

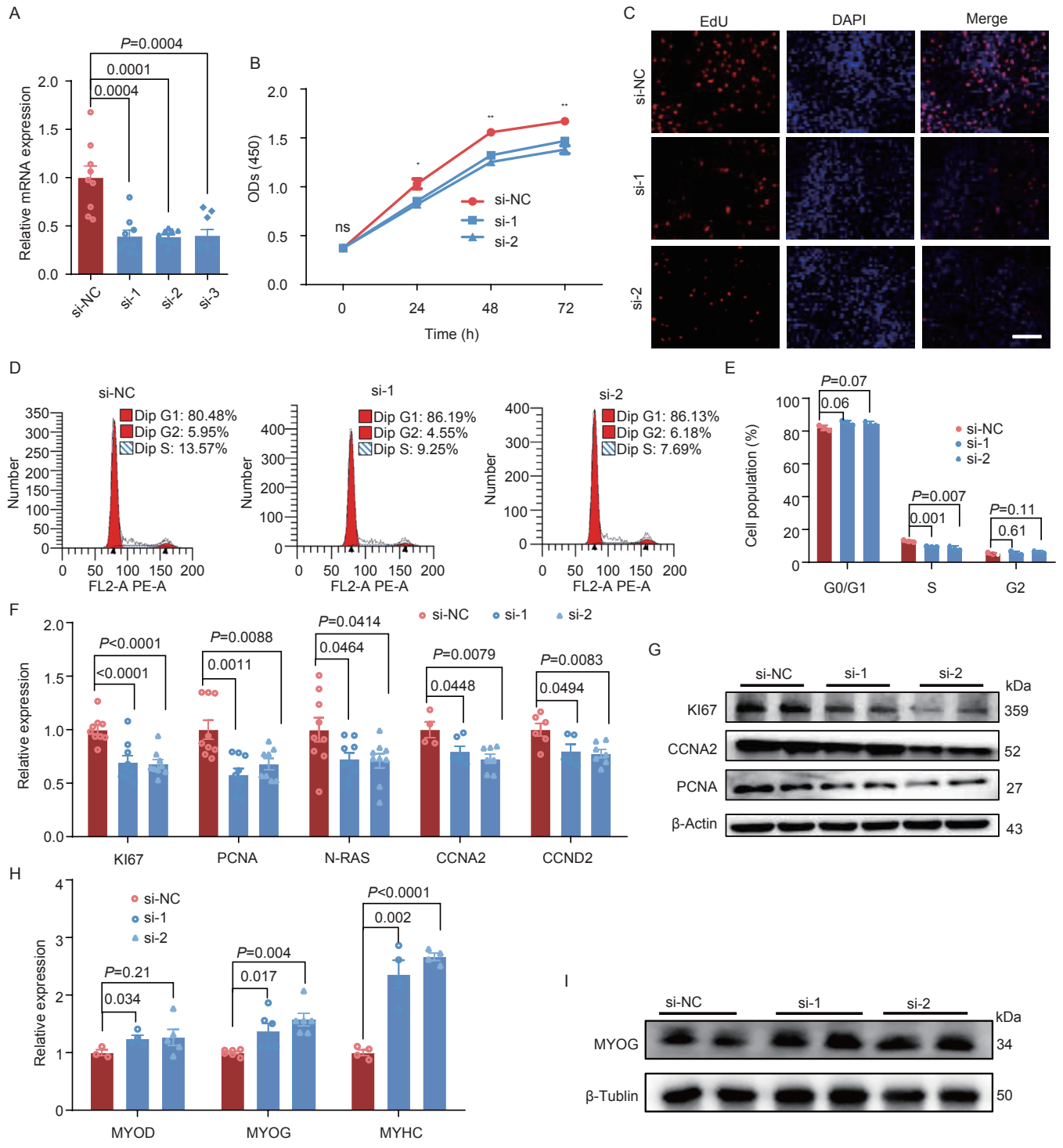


Fig. 3 *RPS27L* knockdown inhibits myoblast proliferation but promotes myogenic differentiation. A, *RPS27L* was knocked-down by small interfering RNAs (siRNAs) in porcine skeletal muscle cells (SKMs). B, cell proliferation was assessed by CCK-8 assay when transfected with si-*RPS27L* or si-NC in SKMs. C, EdU assay was used to assess cell proliferation when transfected with si-*RPS27L* or si-NC. EdU staining (red) for positive cells; DAPI staining (blue) for cell nuclei in SKMs. D and E, the cell cycle was analyzed using flow cytometry after transfecting with si-*RPS27L* or si-NC in SKMs. F and G, the expression level of proliferation markers was detected at mRNA (F) and protein level (G) after transfection with si-*RPS27L* or si-NC in SKMs. H and I, the expression level of myogenic markers was detected at mRNA (H) and protein level (I) after transfecting with si-*RPS27L* or si-NC in satellite cells from pig skeletal muscle. MYOD, myogenic differentiation antigen; MYOG, myogenin; MYHC, myosin heavy chain. Data are mean±SE (n≥3). ns, not significant, *, P<0.05, **, P<0.01.

population from Luchuan×Duroc, we observed the expression level of *RPS27L* mRNA was significantly positively correlated with backfat thickness at 6th to 7th rib (Pearson's R=0.27, P=0.024; Fig. 6-A). In a F_2 resource population constructed by intercrossing Yorkshire boars and Min (a native Chinese breed) sows (Wang *et al.* 2014; Xie *et al.* 2021), we

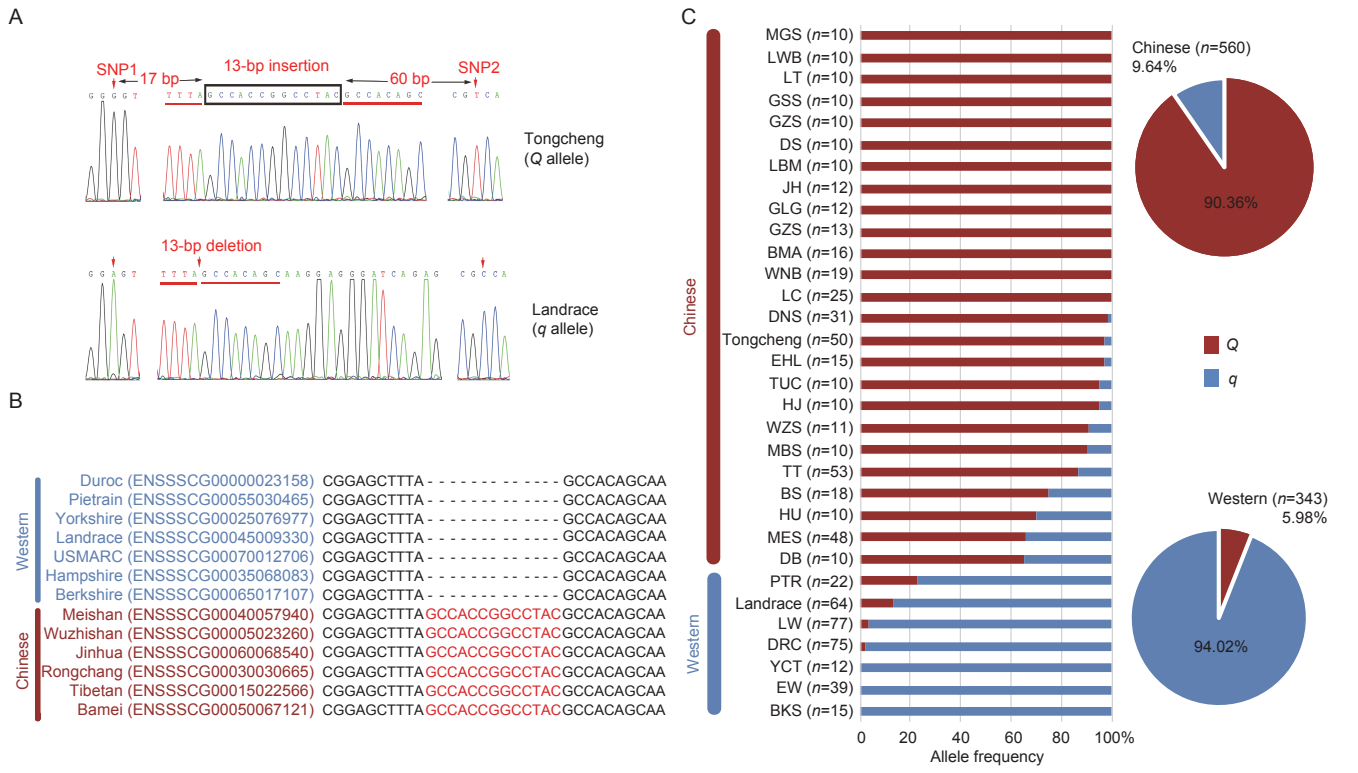


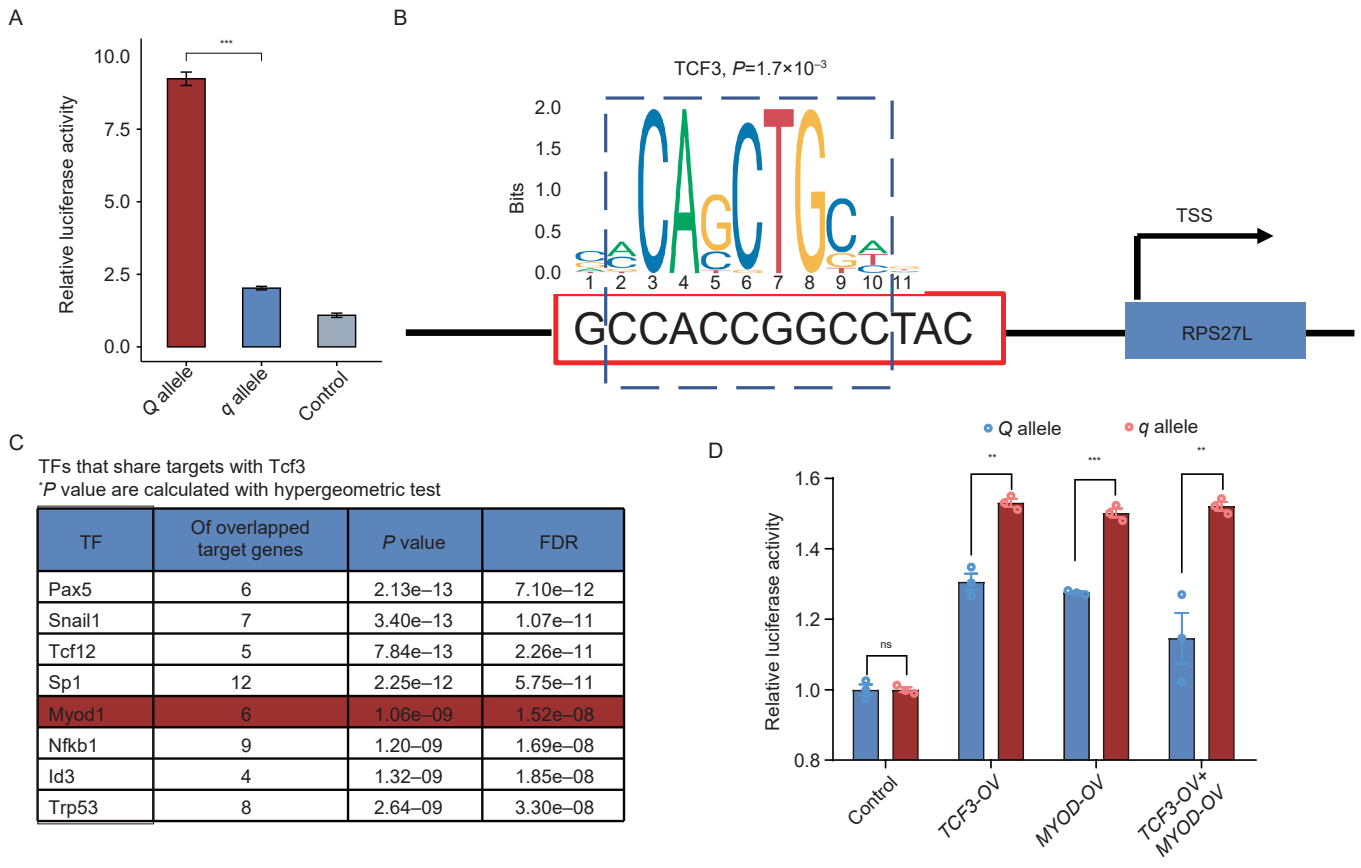
Fig. 4 Identification of a 13-bp InDel in the promoter of *RPS27L*. A, validation of the three variants, with one insertion-deletion (InDel) and two single-nucleotide polymorphisms (SNPs) in the promoter of *RPS27L* by sanger sequencing. B, comparison of the sequences around the InDel (chr1:108880114, Suscrofa 11.1) in 13 pig genomes deposited in ensembl database. C, allele frequency (AF) of the InDel in different Chinese local breeds and Western commercial breeds. MGS, Mingguang small-ear; LWB, Laiwu black; LT, Lantang; GSS, Guangdong small-ear spotted; GZS, Guanzhuang spotted; DS, Dongshan; LBW, Large black-white; JH, Jinhua; GLG, Gaoli gongshan; GZS, Guizhong spotted; BMA, Bama; WNB, Wannan black; LC, Luchuan; DNS, Diannan small-ear; EHL, Erhualian; TUC, Tunchang; HJ, Huanjiang; WZS, Wuzhishan; MBS, Minbei spotted; TT, Tibetan; BS, Baoshan; HU, Huai; MES, Meishan; DB, Debao; PTR, Pretriain; LW, Large white; YCT, Yucatan; EW, European wild; BKS; Berkshire. The number of pigs in each breed is in parentheses. Breeds with less than 10 individuals are not shown.

observed the average body weight at 240 days of age in pigs with the *qq* genotype (average 109.63 kg, *n*=411) was significantly higher than that of pigs with the *QQ* genotype (average 103.68 kg, *n*=36) (Fig. 6-B; *P*=0.03), whereas no significant differences were found in pigs between *QQ* and *Qq* genotypes and between *qq* or *Qq* genotypes (*P*>0.05). Overall, evidence indicated that *RPS27L* was a negative regulator of muscle growth, and the functional *cis*-regulatory element could potentially contribute to the muscle mass in pigs.

4. Discussion

Chinese local pigs and Western commercial pig populations showcase distinctive genotypes and phenotypes, stemming from separate domestication events (Fang *et al.* 2012; Zhang *et al.* 2019). Comparative transcriptome analysis has unveiled numerous differentially expressed genes between these two populations at various developmental stages. Many of these genes have been functionally validated, underscoring the efficacy of this approach in identifying candidate genes associated with economically important traits (Yang *et al.* 2021). In the prior research, a comparison of skeletal muscle transcriptome between Landrace and Tongcheng pigs across 27 developmental stages highlighted that *SATB2* and lncRNA-*XLOC_036765* regulated myoblast migration and proliferation, respectively (Yang *et al.* 2021). In this study, an elevated expression of the *RPS27L* gene was observed in the skeletal muscle of Chinese local pigs compared to that of Western commercial pigs. Additionally, *RPS27L* was also higher expressed in skeletal muscle than in other tissues. Subsequent functional analyses revealed the roles of *RPS27L* in promoting myoblast proliferation while inhibiting differentiation in SKMs. These results demonstrate the regulation of *RPS27L* in myogenesis, suggesting *RPS27L* functions as a potential regulator of skeletal muscle development. As the development of skeletal muscle directly determines the performance of muscle growth traits. In addition, as the largest endocrine tissue, skeletal muscle can secrete peptides that regulate inter-tissue communication, such as liver and adipose tissue, to mediate the whole-body energy balance (Hu *et al.* 2023), thus orchestrates growth associated traits. Given the regulatory role of *RPS27L* in skeletal muscle development, we propose that *RPS27L* may also be associated with key growth traits such as backfat thickness and body weight.

Genomic variants, especially within *cis*-regulatory



TF	Of overlapped target genes	*P* value	FDR
Pax5	6	2.13e–13	7.10e–12
Snail1	7	3.40e–13	1.07e–11
Tcf12	5	7.84e–13	2.26e–11
Sp1	12	2.25e–12	5.75e–11
Myod1	6	1.06e–09	1.52e–08
Nfkb1	9	1.20–09	1.69e–08
Id3	4	1.32–09	1.85e–08
Trp53	8	2.64–09	3.30e–08

Fig. 5 The 13-bp novel InDel was a functional *cis*-regulatory variants. A, activities of two alleles (*Q* and *q*) by the luciferase reporter assay in satellite cells from pig skeletal muscle. Two tailed *t*-test, ***, $P<0.001$. B, potential TCF3 binding motif was predicted around the 13-bp insertion sequence. C, transcription factors (TFs) that could share targets with TCF3 were predicted by MEME Suite 5.5.3 (https://meme-suite.org). D, luciferase activities of *TCF3-OV* and *MYOD-OV* constructs. FDR, false discovery rate; TSS, transcription start site. Two tailed *t*-test, data are mean±SE ($n=3$). ns, $P>0.05$; *, $P<0.05$, **, $P<0.01$ and ***, $P<0.001$.

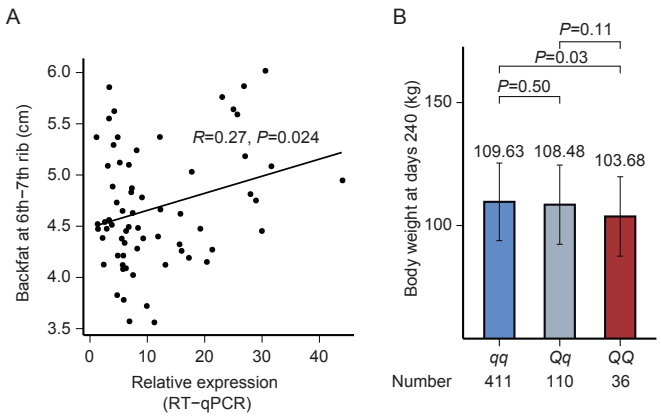


Fig. 6 The InDel is potentially associated with the body mass and backfat thickness in pigs. A, the mRNA expression of *RPS27L* was significantly positively associated with backfat thickness at 6th to 7th rib in Luchuan×Duroc F_1 population ($P=0.024$, $n=67$). B, barplot showing the association of the InDel with body weight of pigs at 240 days of age in a Large White×Min F_2 population. The error bars depict the mean±SD. The *P*-value was calculated by mixed linear model under restricted maximum likelihood (REML) estimation considering the family relationships among samples.

elements, wield significant influence over phenotypic diversity by modulating gene expression (Wittkopp and Kalay 2012). Such variations provide a rich resource for comprehending the impact of genomic variants on traits of economic importance in pigs (Liu *et al.* 2022). For instance, our recent investigation, involving the re-sequencing of Luchuan and Duroc, identified a SNP capable of influencing the enhancer activity of the *TNNC1* gene, thereby affecting muscle fiber-types and meat quality (Liu *et al.* 2022). The variants in lncRNA-MEG3 are reported to be associated with meat production traits (Yao *et al.* 2023). In the present study, a novel 13-bp InDel in the promoter region of *RPS27L* was identified. The insertion allele of this InDel was nearly fixed in Chinese local pigs but absent in their Western commercial counterparts, suggesting its potential selection during domestication and breeding. Furthermore, luciferase reporter assay suggested this insertion allele (*Q*) showed a strong binding site specific to TCF3 and MYOD. Notably, MYOD serves as a pivotal regulator during myogenic differentiation (Weintraub *et al.* 1991; Rudnicki *et al.* 1993). Moreover, a positive expression correlation between *RPS27L* and backfat thickness was validated. An association study supported that *RPS27L* was associated with body weight. Collectively, these results underscored the functional significance of this InDel as

a *cis*-regulatory variant governing muscle growth. However, it should mention that there are no available antibodies for TCF3 and MYOD ChIP-qPCR or -Seq analysis in pigs right now, further studies are needed to valid the direct interactions between this InDel and TCF3/MYOD. Meanwhile, the *Q* sequence corresponds to the E-box sequence, suggesting the binding of other potential TFs on this site.

5. Conclusion

Overall, our investigation highlights *RPS27L* as a novel muscle growth regulator, potentially serving as a molecular marker for pig breeding to enhance meat production traits. However, it is important to emphasize that further studies are needed to thoroughly elucidate the regulatory mechanisms of *RPS27L* in pig skeletal muscle development.

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Declaration of competing of interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical approval

All animal procedures were performed according to the protocols of the Chinese Academy of Agricultural Sciences and the Institutional Animal Care and Use Committee.

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