

***lncING2-DT* suppresses proliferation and promotes differentiation of porcine skeletal muscle satellite cells through an *ING2*-dependent mechanism**

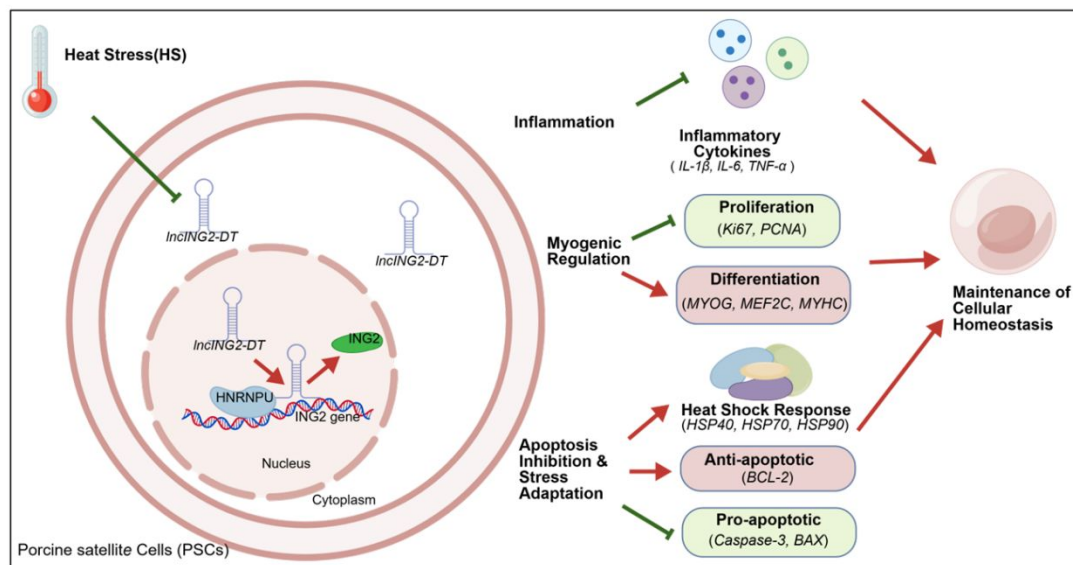
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ABSTRACT

Heat stress (HS) severely impairs skeletal muscle development, yet the regulatory roles of long non-coding RNAs (lncRNAs) in this process remain largely unclear. In

this study, RNA-seq was performed on porcine soleus (SOL), gastrocnemius (GAS), and tibialis anterior (TA) muscles under HS and control conditions, revealing multiple differentially expressed lncRNAs. Among them, ENSSSCG00000048143 (which we named *lncING2-DT*) was consistently down-regulated in all three muscle types of the HS treatment group compared with the control group, and was selected for further investigation. Functional analyses in porcine skeletal muscle satellite cells (PSCs) showed that *lncING2-DT* inhibited cell proliferation while promoting myogenic differentiation under normal conditions. Mechanistically, *lncING2-DT* positively regulated the expression and promoter activity of its neighboring gene *ING2*, and co-transfection experiments demonstrated that the inhibitory effect of *lncING2-DT* on PSC proliferation was partially dependent on *ING2*. RNA pull-down and mass spectrometry (MS) identified multiple *lncING2-DT*-interacting proteins, among which HNRNPU was identified as a candidate interacting protein that may participate in *lncING2-DT*-mediated regulation of *ING2*. Under HS conditions, *lncING2-DT* expression was markedly reduced, accompanied by impaired myogenic differentiation, enhanced proliferation, apoptosis, and inflammatory responses. Notably, overexpression of *lncING2-DT* alleviated HS-induced cellular dysfunction by promoting differentiation, enhancing heat shock responses, and suppressing excessive apoptosis and inflammation. Collectively, these findings identify *lncING2-DT* as a critical regulator of PSC homeostasis and stress adaptation, providing novel insights into the molecular mechanisms underlying HS-induced skeletal muscle dysfunction.

KEYWORDS: *lncING2-DT*; Skeletal muscle; Proliferation; *ING2*; HNRNPU; Heat stress

INTRODUCTION

Pigs are a critically important agricultural animal in China with significant economic value. Genetic improvement in pigs holds immense significance for the development of China's meat industry. With global warming, extreme heatwaves during summer have become increasingly frequent, making HS a prominent challenge in pig

farming. HS has become a major challenge in intensive animal husbandry, adversely affecting growth and reproduction (Gonzalez-Rivas et al., 2020; Prates, 2025). Due to the absence of functional sweat glands, pigs are susceptible to HS during hot summers and during transport, leading to economic losses in the swine industry (Mayorga et al., 2019; Most & Yates, 2021).

HS has been demonstrated to negatively affect skeletal muscle growth, primarily through the induction of oxidative stress and disruption of cellular homeostasis (Fennel et al., 2022; Marchant et al., 2023; Most & Yates, 2021). This imbalance leads to increased production of reactive oxygen species, which in turn impairs protein turnover by enhancing proteolysis and autophagy-mediated degradation while inhibiting protein synthesis (Lei et al., 2025; Yang et al., 2024). In addition, studies have reported that HS can lead to mitochondrial dysfunction, myofibrillar disruption, and centralization of myonuclei (Machiela et al., 2020).

These stress-induced alterations are tightly regulated at the transcriptional and post-transcriptional levels, suggesting that gene regulatory mechanisms play a critical role in mediating skeletal muscle adaptation to HS. Among these regulatory layers, lncRNAs, a class of transcripts longer than 200 nucleotides with little or no protein-coding potential (Herman et al., 2022), have emerged as important modulators of stress-responsive gene expression. Increasing evidence indicates that lncRNAs participate in cellular responses to environmental stress and may play important regulatory roles in HS adaptation (Hu et al., 2023a; Kitajima et al., 2023; Tabury et al., 2022). Therefore, exploring lncRNA-mediated regulatory networks may provide important insights into skeletal muscle responses to HS.

Skeletal muscle is the largest tissue within the body (Chal & Pourquié, 2017), not only supporting movement and metabolic functions (Della Guardia et al., 2024; Sun et al., 2021), but also dynamically responding to developmental signals, injury, and environmental stress (Dumont et al., 2015). Precise regulation of myoblast proliferation and differentiation is crucial for muscle growth and regeneration (Yamakawa et al., 2020). In recent years, lncRNAs have been identified as key regulators of diverse biological processes, including chromatin remodeling, transcriptional regulation, and

post-transcriptional control (Chen et al., 2017; Wang et al., 2015; Yu et al., 2017; Zhao et al., 2018). Growing evidence indicates that lncRNAs participate in muscle development and regeneration by interacting with transcription factors, chromatin-modifying factors, and RNA-binding proteins (RBPs) (Chen et al., 2024; Chen et al., 2017; Gong et al., 2015; Liang et al., 2018; Liu et al., 2019; Militello et al., 2018; Wang et al., 2016).

Although several muscle-related lncRNAs have been reported, relatively few studies have explored how lncRNAs coordinate myogenic processes under HS conditions. Therefore, the molecular mechanisms underlying lncRNA-mediated regulation of skeletal muscle adaptation to HS remain poorly understood. Identifying novel lncRNAs and elucidating their mechanisms of action may provide important insights into muscle physiology and offer potential molecular targets for improving muscle health and productivity.

In this study, we focused on a lncRNA identified in porcine skeletal muscle. This lncRNA is located upstream of *ING2* gene and is transcribed from the antisense strand relative to *ING2*. Notably, its transcription initiation site overlaps with the *ING2* promoter, a defining characteristic of divergent transcripts (DTs)—a class of lncRNAs transcribed in the opposite direction of adjacent protein-coding genes that often play regulatory roles in gene expression (Bruford et al., 2020). Based on these positional and transcriptional features, the lncRNA was designated *lncING2-DT*. *ING2* is a member of the inhibitor of growth family and has been implicated in chromatin remodeling, cell cycle regulation, apoptosis, and stress-related cellular responses (Archambeau et al., 2019; Bua et al., 2013). These biological functions suggest that the *lncING2-DT/ING2* axis may play an important role in maintaining skeletal muscle homeostasis under environmental stress conditions. However, it remains unknown whether *lncING2-DT* regulates myoblast proliferation and differentiation under normal and HS conditions, and how it functionally interacts with its neighboring gene *ING2* under these conditions. We investigated its effects on cell proliferation and differentiation at 37°C and under HS conditions, analyzed its regulatory relationship with *ING2*, and explored its interacting proteins through RNA pull-down assays and MS analysis. Our findings

reveal *lncING2-DT* as a novel regulator of myoblast fate, providing new insights into the integrated role of chromatin remodeling, RBPs, and stress response pathways in muscle biology.

MATERIALS AND METHODS

Ethics Statement

All research procedures involving animals were carried out according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in March 2017) and approved by the animal ethical and welfare committee (AEWC) of Sichuan Agricultural University under permit DKY-20240664.

Analysis of RNA-seq Data

For RNA-seq analysis, tissue samples were collected from 2-month-old female DLY (Duroc × Landrace × Yorkshire) pigs (approximately 24 kg). Animals in the HS group were exposed to elevated temperature using a heating blower, maintaining a temperature range of $41.5 \pm 0.5^{\circ}\text{C}$ for 3 h, while the control (NC) group was kept under normal temperature conditions (25°C). A total of 5 biological replicates were included in the HS group and 3 in the NC group. All samples were grouped into HS and NC conditions for subsequent transcriptomic analysis.

All annotated protein-coding genes (PCGs) and lncRNAs in the reference genome (Sscrofa11.1, release 109) were utilized for gene annotation and subsequent transcript quantification. Gene-level expression was estimated as transcripts per million (TPM) using the high-speed transcript quantification tool Kallisto (v.0.43.0) with parameters (--bias --rf-stranded). Mapped read counts for each gene were obtained by employing the tximport (v.1.6.0) R package with default settings. We considered a PCG to be detected/expressed if its expression level was greater than 0.5 TPM in at least one sample. For lncRNAs, we used a cutoff of 0.1 TPM in at least one sample.

Based on the counts of each lncRNA, differential lncRNA expression analysis was performed using edgeR (v.3.22.5) (Robinson et al., 2010), with a false discovery rate

(FDR)<0.05 and $|\log_2\text{fold change}|\geq 1$ as the cutoffs for statistical significance (Supplementary Table S1).

The prediction of lncRNA function was based on PCGs located near the lncRNA or correlated with its expression levels (Supplementary Table S2). Cis-target genes were defined as PCGs located within 100 kb upstream and downstream of each lncRNA genomic locus (Chai et al., 2023).

Weighted gene co-expression network analysis (WGCNA) was performed using the R package WGCNA (Langfelder & Horvath, 2008) (v.1.74). Expression profiles of 11,549 mRNAs and 1,190 lncRNAs, including the target lncRNA *lncING2-DT* (ENSSSCG00000048143), were used to construct the co-expression network. The final expression matrix was checked using goodSamplesGenes, and sample clustering was used to inspect potential outliers. An unsigned co-expression network was constructed using the blockwiseModules function. The soft-thresholding power was selected 14 (with a correlation coefficient threshold of 0.8), TOMType="unsigned", minModuleSize=60, mergeCutHeight=0.25. Module eigengenes were calculated to summarize module-level expression patterns. Module-treatment relationships were first evaluated by correlating module eigengenes with the HS indicator. And module eigengenes were analyzed using the linear model module eigengene ~ treatment + tissue, and *P* values for the treatment term were adjusted using the Benjamini-Hochberg method. For *lncING2-DT*-centered analysis, Pearson correlations between *lncING2-DT* and module mRNAs were calculated using both raw expression values and tissue-adjusted residuals. Tissue-adjusted correlations were obtained by regressing each transcript on tissue and correlating the resulting residuals. High-confidence *lncING2-DT*-associated mRNAs were defined as mRNAs satisfying $|r|\geq 0.8$ and FDR<0.01 in both raw and tissue-adjusted correlation analyses.

Candidate target genes were first converted to their human orthologs and used as inputs, taking all the annotated PCGs in the reference human genome as the background. Next, the obtained homologous genes were uploaded to the Metascape web server (<https://metascape.org>) for gene ontology (GO) analysis, including the molecular function and biological process categories, and Kyoto Encyclopedia of Genes and

Genomes (KEGG) pathway analysis. Genes that exhibited a Benjamini-adjusted P value < 0.05 for a given pathway were considered to be significantly enriched in that pathway.

PSCs Culture and HS Exposure

PSCs were purchased from iCell Bioscience (China). PSCs were maintained in skeletal muscle cell complete medium (PriMed-iCell-018, iCell Bioscience Inc., China), supplemented with 10% fetal bovine serum (iCell Bioscience Inc., China), 1% penicillin/ streptomycin (iCell Bioscience Inc., China), and 1% supplement (iCell Bioscience Inc., China). When PSCs reached 90% confluence, they were transferred to DMEM supplemented with 5% horse serum (Gibco, USA) to induce differentiation. Control cells were cultured at 37°C under 5% CO₂. Cells incubated at 42°C for 6h under 5% CO₂ constituted the HS group (Hu et al., 2024; Kim et al., 2020; Park et al., 2023).

Total RNA Extraction and Quantitative Reverse Transcription PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription was performed using the HiScript® III RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, China) following the manufacturer's instructions. RT-qPCR analysis was performed on at least three independent biological replicates using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) on a CFX96 real-time PCR system (Bio-Rad, USA). β -actin served as the housekeeping gene, and relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study are detailed in Supplementary Table S4.

Separation of Nuclear and Cytoplasmic RNA

Total RNA was isolated from the cytoplasm and nuclei of PSCs using a cytoplasmic and nuclear RNA purification kit (Norgen, USA) according to the manufacturer's instructions. cDNA synthesis and qPCR were performed as previously described. Quality control was performed using the nuclear housekeeping gene *U6* and the cytoplasmic housekeeping gene *GAPDH*. The specific steps are as follows: After

washing the PSCs culture plates with PBS, ice-cold lysis buffer was directly added to the plates. Following cell lysis, the lysate was transferred to microcentrifuge tubes and centrifuged at maximum speed for 3min. The supernatant containing cytoplasmic RNA was transferred to a new tube, while the pellet containing nuclear RNA was retained in the original tube. Binding solution was then added to both the supernatant and pellet, thoroughly mixed to resuspend the material. RNA samples were subsequently adsorbed onto centrifugal columns via centrifugation. The columns were washed twice with 400 μ L of wash buffer, followed by elution of fractionated RNA from the columns using 50 μ L of elution buffer. This procedure was repeated three times.

Plasmid Construction and Transfection

For the *lncING2-DT* overexpression and knockdown experiments, cells were seeded in 12-well plates. At 80% confluence (Li et al., 2023), *lncING2-DT* plasmid (the full-length sequence of *lncING2-DT* was inserted into the plasmid), the pEGFP-N1 empty plasmid, small interfering RNA (siRNA), and siRNA - negative control were added.

For lncRNA overexpression, cells were divided into a control group (Control), an empty vector group (PcDNA3.1+), and an overexpression group (PclncING2-DT). For the knockdown experiment, cells were divided into a control group (Control), a negative control group (Si-NC), and a knockdown group (SiRNAs). To knock down *lncING2-DT*, siRNA targeting *lncING2-DT* (Si-lncING2-DT-1, Si-lncING2-DT-2, Si-lncING2-DT-3) and control siRNA were synthesized by Sangon Biotech (Sangon, China). Cells were transfected using Lipofectamine 3000 (Invitrogen, USA) following the manufacturer's instructions (with a vector concentration of 500 ng per well). Medium was changed 6h post-transfection. 24h after medium change, cells were harvested to measure transfection efficiency and perform RT-qPCR assays. All siRNA sequences used in this study are detailed in Supplementary Table S5.

EdU Detection

For EdU (5-ethynyl-2'-deoxyuridine) detection, 48h post-transfection, PSCs were

incubated in 50 μ M 5-ethynyl-2'-deoxyuridine (EdU) (Beyotime, China) for 2h. Cells were then fixed in 4% paraformaldehyde for 30min and stained using the C10310 EdU Apollo In Vitro Imaging Kit (Beyotime, China). The ratio of EdU-labeled cells to Hoechst 33342-stained cells was determined by analyzing images from three randomly selected fields of view under a fluorescence microscope (TE2000-U; Japan). ImageJ v1.8.0 software (Schneider et al., 2012) was used to quantify EdU-positive cells. Each group comprised three biological replicates (n=3).

CCK-8 Assay

At 12, 24, 36, and 48h post-transfection, add 10 μ L of CCK-8 working solution (Vazyme, China) to PSCs seeded in 96-well plates. After incubation at 37°C for 2h, absorbance at 450 nm was measured using a fluorescence/multimode microplate reader (Thermo Varioskan LUX, USA). Each group comprised three biological replicates (n=3).

Immunofluorescence Assay

PSCs were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15min, and permeabilized with 0.2% Triton X-100 for 10min at room temperature. After washing with PBS, the cells were blocked with a protein-free rapid blocking buffer (Servicebio, China) for 1h at room temperature. The cells were then incubated overnight at 4°C with a Myosin heavy chain rabbit monoclonal antibody (1:500; A4963, ABclonal, China). After three washes with PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (1:200; GB25303, Servicebio, China), diluted in PBS containing 3% bovine serum albumin, for 1h at room temperature in the dark. Following three additional washes with PBS, the cells were mounted using an antifade mounting medium containing DAPI (Servicebio, China). Fluorescence images were acquired using a fluorescence microscope (TE2000-U; Nikon, Japan). The fusion index was calculated as the percentage of nuclei within MyHC-positive multinucleated myotubes relative to the total number of nuclei in each visual field: Fusion index (%) = (number of nuclei within

MyHC-positive myotubes/total number of nuclei)×100.

Dual Luciferase Reporter Assay

When cell confluence reached approximately 80%, the *ING2* promoter and dual luciferase reporter vector were co-transfected into PSCs with the TK normalization reporter plasmid. After 36h of incubation, firefly and renin luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega, USA).

RNA Pull-Down and Mass Spectrometry Analysis

Biotin-labeled RNA probes (target probes and negative controls, 2-5 µg, 300-1000 bp) were denatured at 90°C for 2min, cooled on ice, and then refolded at room temperature in RNA structural buffer for 20min to form secondary structures. Structured probes were incubated with streptavidin magnetic beads at 25°C for 30min to prepare probe-magnetic bead complexes. Whole-cell lysates were extracted using RIP buffer containing PMSF, protease inhibitors, and DTT. Following DNase digestion and agarose bead pre-clearing, the RNA probe-magnetic bead complexes were incubated at 25°C for 2h in RIP buffer supplemented with RNase inhibitor, EDTA, EGTA, and poly(dI·dC). Thoroughly wash beads with NT2 buffer. Elute bound RNA-protein complexes at 37°C for 2h using urea-CHAPS buffer. Denature eluted proteins and separate via SDS-PAGE. Simultaneously, resolve associated proteins via gel electrophoresis and visualize with silver staining. Bound proteins were also identified by mass spectrometry (TSINGKE, China).

Statistical Analysis

All experimental data were obtained from at least three independent experiments. Values are shown as the mean standard error of the mean (SEM). SPSS (v.19.0) statistics software was used for statistical analysis. The normality of the data distribution was assessed prior to analysis, and the data met the assumptions for parametric testing. Student's t-test was used for individual comparisons. A significance level of <0.05 was considered to be statistically significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

Screening of differentially expressed lncRNAs in porcine skeletal muscle under HS

To investigate lncRNA expression changes in pig skeletal muscle after HS, we performed RNA-seq on HS and control groups. After HS, we identified 13, 6, and 2 significantly up-regulated lncRNAs, and 12, 5, and 4 significantly down-regulated lncRNAs in SOL, GAS, and TA muscle tissues, respectively (Figure 1A-1D; Supplementary Table S1).

To further investigate the potential functions of these differentially expressed lncRNAs, we identified protein-coding genes located within 100 kb upstream and downstream of both up-regulated and down-regulated lncRNAs as their target genes. In SOL, GAS, and TA, up-regulated lncRNAs predicted 54, 12, and 9 target genes, while down-regulated lncRNAs predicted 52, 17, and 19 target genes, respectively (Supplementary Table S2). GO analysis revealed that target genes of up-regulated lncRNAs were mainly enriched in monocarboxylic acid metabolic process, organophosphate catabolic process, mRNA metabolic process (Figure 1E). Target genes of down-regulated lncRNAs were primarily enriched in muscle structure development, cellular response to cytokine stimulus, regulation of transforming growth factor beta receptor signaling pathway (Figure 1F).

ENSSSCG00000048143 (*lncING2-DT*) was identified as the only lncRNA that was consistently downregulated across all three muscle tissues under HS (Figure 1G). The full length of *lncING2-DT* is 1902 nt. *ING2*, located in close genomic proximity to *lncING2-DT* (562 bp downstream), is known to play an important role in cell cycle regulation and myogenesis (Eapen et al., 2012; Larrieu et al., 2010). Based on its consistent differential expression and potential functional relevance, *lncING2-DT* was selected for subsequent functional and mechanistic analyses.

To further predict the potential function of *lncING2-DT*, WGCNA was performed to construct co-expression modules. Hierarchical clustering and dynamic tree-cutting analysis identified 12 distinct co-expression modules based on similar expression

patterns (Figure 1H; Supplementary Figure S1A, B). *lncING2-DT* was assigned to module-1, which showed a significant negative correlation with HS, suggesting its potential involvement in stress-adaptive regulatory processes (Figure 1I; Supplementary Figure S1C; Supplementary Table S3). A total of 334 high-confidence mRNAs co-expressed with *lncING2-DT* within module-1 were selected for downstream functional analysis. Functional enrichment analysis of these genes revealed significant enrichment in biological processes associated with cellular stress responses, redox regulation, myogenic differentiation, and mitochondrial function (Figure 1J).

Overlap enrichment analysis further showed that the 334 high-confidence mRNAs associated with *lncING2-DT* in module-1 were significantly enriched among the union set of HS-responsive DEGs identified across GAS, SOL, and TA tissues, with 60 observed overlapping genes ($\text{FDR}=1.55 \times 10^{-21}$). These overlapping genes were mainly enriched in cell differentiation and oxidative stress response pathways (Supplementary Figure S1D).

Combined with expression profiling, the significant downregulation of *lncING2-DT* under HS suggests that it may be associated with cellular stress responses, metabolic homeostasis, and muscle development, thereby contributing to the maintenance of skeletal muscle homeostasis under stress conditions.

***lncING2-DT* inhibits myoblast proliferation**

To assess the effect of *lncING2-DT* on PSCs proliferation, we performed knockdown and overexpression experiments. Three specific siRNAs (Si-*lncING2-DT*-1, Si-*lncING2-DT*-2, Si-*lncING2-DT*-3) were used to silence *lncING2-DT* in PSCs. The most effective siRNA was Si-*lncING2-DT*-1, which was used in subsequent experiments (Figure 2A). The knockdown group showed a higher proportion of EdU-positive cells than the control group, suggesting that *lncING2-DT* knockdown increases mitotic activity (Figure 2B). CCK-8 assays indicated that knockdown significantly promoted cell proliferation rates (Figure 2C). After knocking down *lncING2-DT*, the mRNA expression levels of proliferation-related markers, including *Ki67*, *CCNB1*, and

CDK1, were significantly increased compared with the Si-NC group (Figure 2D). In contrast, *lncING2-DT* overexpression reduced the proportion of EdU-positive cells and proliferation rates compared with the negative control group, accompanied by decreased expression of *Ki67*, *CCNB1*, and *CDK1* (Figure 2E–2H). These results collectively indicate that *lncING2-DT* inhibits PSCs proliferation under normal conditions.

***lncING2-DT* promotes myoblast differentiation**

To further investigate the effect of *lncING2-DT* on myoblast differentiation, we examined the expression of myogenic differentiation markers *MYOG* and *MEF2C* after knockdown and overexpression of *lncING2-DT*. RT-qPCR results showed that *lncING2-DT* knockdown significantly decreased the expression levels of *MYOG* and *MEF2C*, whereas their expression levels were significantly increased after *lncING2-DT* overexpression (Figure 3A, B).

Immunofluorescence staining showed that the myoblast fusion index was significantly increased in the *lncING2-DT* overexpression group compared with the control group. In contrast, myotube formation was markedly reduced in the *lncING2-DT* knockdown group, and the fusion index was significantly decreased (Figure 3C). These results demonstrate that *lncING2-DT* positively regulates myogenic differentiation of PSCs under normal conditions.

***lncING2-DT* functions through an *ING2*-dependent mechanism**

We hypothesize that *lncING2-DT* exerts its function by regulating the expression of its target gene *ING2*. Subcellular localization analysis revealed that *lncING2-DT* was present in both nucleus and cytoplasm, suggesting its potential involvement in both transcriptional and post-transcriptional regulation (Figure 4A). This provides a spatial basis for its interaction with the nuclear protein *ING2*. Combined with RNAinter (Kang et al., 2022) prediction results, this further suggests a potential interaction between *lncING2-DT* and *ING2* (Figure 4B).

We found that overexpression of *lncING2-DT* in PSCs significantly increased

ING2 mRNA levels, while knockdown significantly decreased *ING2* expression (Figure 4C). Similarly, luciferase reporter assays showed that *lncING2-DT* enhanced the transcriptional activity of the *ING2* promoter, whereas silencing *lncING2-DT* inhibited this activity (Figure 4D, E). Given these results, we demonstrate that *lncING2-DT* positively regulates *ING2* expression.

To investigate the relationship between *lncING2-DT* and *ING2* in regulating PSC proliferation, we performed *ING2* knockdown in PSCs overexpressing *lncING2-DT*. Knockdown of *ING2* significantly increased *Ki67* expression, indicating that *ING2* suppresses PSC proliferation. Notably, overexpression of *lncING2-DT* attenuated the increase in *Ki67* expression induced by *ING2* knockdown, although the levels were not fully restored to baseline (Figure 4F–H). These results suggest that *lncING2-DT* partially counteracts the effect of *ING2* knockdown and may regulate PSC proliferation through both *ING2*-dependent and -independent mechanisms.

Identification of interacting proteins and potential mediating factors of *lncING2-DT*

LncRNAs typically regulate target genes through interactions with specific proteins (Wang et al., 2025). To explore the potential mechanism by which *lncING2-DT* regulates *ING2*, we performed RNA pulldown assays in PSCs to identify proteins interacting with *lncING2-DT*. Silver staining results showed abundant and clear protein bands in the *lncING2-DT* RNA pull-down group, while the negative control group exhibited only a few bands, indicating that target *lncING2-DT* effectively enriched multiple binding proteins (Figure 5A). MS identification results revealed 873 proteins in the *lncING2-DT* RNA pull-down group and 535 in the NC group (Supplementary Table S6), with 275 overlapping proteins between the two groups, suggesting that the differential proteins are specific binding partners of *lncING2-DT* (Figure 5B).

GO enrichment analysis indicated that the specific binding proteins of *lncING2-DT* were mainly enriched in biological processes and molecular functions such as cytoplasmic translation initiation, chromatin structural components, and protein binding (Figure 5C). KEGG pathway analysis showed significant enrichment in

signaling pathways including cell cycle, PI3K-Akt signaling pathway, and endocytosis. These results suggest that *lncING2-DT* may participate in processes such as cell proliferation and differentiation through specific interactions with proteins related to cell cycle and chromatin regulation (Figure 5D).

Given the close regulatory association between *lncING2-DT* and *ING2* observed in our earlier results, we sought to investigate whether *lncING2-DT* may modulate *ING2* through interacting proteins. Therefore, we selected several candidate proteins potentially binding to *lncING2-DT* via MS scores, including LOC100302368, RPS5, SRSF1, and HNRNPU (Figure 5E). Among these candidates, HNRNPU is a typical nuclear RNA-binding protein involved in chromatin structure maintenance and gene transcription regulation (Ma et al., 2025; Sapir et al., 2022; Sapir & Reiner, 2023; Zhang et al., 2021), suggesting that it may act as a molecular mediator linking *lncING2-DT* to transcriptional control. Based on this hypothesis, we further assessed whether *ING2* could potentially interact with HNRNPU or other RNA-binding proteins. We used the catRAPID website to predict proteins that can interact with *ING2*. The results showed that *ING2* could interact with various RBPs, including HNRNPU, a candidate protein that may bind to *lncING2-DT* (Figure 5F). These results suggest that *lncING2-DT* may indirectly regulate *ING2* stability or transcriptional activity through its interaction with HNRNPU.

***lncING2-DT* regulates PSC proliferation and differentiation under HS**

To further investigate the role of *lncING2-DT* under HS, PSCs were exposed to HS conditions, under which *lncING2-DT* expression was markedly down-regulated compared with normal conditions (Figure 6A). Under HS conditions, PSC proliferation and myogenic differentiation were significantly inhibited, as evidenced by reduced proliferation activity and decreased expression of myogenic differentiation markers (Figure 6B, C). Immunofluorescence staining of MyHC further demonstrated that HS markedly impaired myotube formation and reduced myotube fusion ability compared with normal conditions (Figure 6D). These findings suggest that HS exerts an overall inhibitory effect on PSC growth and differentiation. To further determine whether

lncING2-DT participates in the regulation of PSC fate under HS conditions, knockdown and overexpression experiments were subsequently performed (Figure 6E, F). Functionally, *lncING2-DT* overexpression under HS significantly suppressed the expression of proliferation-related genes *Ki67* and *PCNA*, while knockdown of *lncING2-DT* produced the opposite effect (Figure 6G). Concurrently, *lncING2-DT* overexpression markedly increased the expression of myogenic differentiation markers (*MYOG*, *MEF2C*, and *MYHC*), whereas *lncING2-DT* silencing significantly attenuated their expression under HS conditions (Figure 6H, I). Consistent with the transcriptional results, MyHC immunofluorescence staining further demonstrated that *lncING2-DT* overexpression enhanced myotube formation and myotube fusion under HS conditions, whereas *lncING2-DT* knockdown further impaired myogenic differentiation and inhibited myotube formation (Figure 6J).

HS induces cell apoptosis and enhances inflammatory responses (Gillette et al., 2025; Liu et al., 2024; Qin et al., 2021). Therefore, we further investigated the regulatory effects of *lncING2-DT* on apoptosis and inflammation in PSCs under HS. The results indicate that *lncING2-DT* overexpression enhanced the expression of heat shock genes (*HSP40*, *HSP70*, and *HSP90*), while *lncING2-DT* knockdown reduced their induction in response to HS (Figure 6K). Moreover, overexpression of *lncING2-DT* under HS decreased the expression of pro-apoptotic genes (*Caspase-3* and *BAX*) and increased the anti-apoptotic gene *BCL-2*, whereas *lncING2-DT* silencing resulted in the opposite expression pattern (Figure 6L). Similarly, inflammatory cytokine genes (*IL-1 β* , *IL-6*, and *TNF- α*) were significantly downregulated by *lncING2-DT* overexpression and upregulated upon *lncING2-DT* knockdown under HS conditions (Figure 6M).

Collectively, *lncING2-DT* contributes to maintaining the myogenic differentiation potential and cellular homeostasis of PSCs by participating in the regulation of the balance between proliferation and differentiation, while positively modulating heat shock defense pathways and suppressing excessive apoptosis and inflammatory responses. This finding reveals *lncING2-DT* as an important functional molecule that is regulated during the HS response and provides a new theoretical basis for further

elucidating the molecular mechanisms by which HS affects skeletal muscle development and regeneration.

DISCUSSION

***lncING2-DT* is a novel divergent lncRNA regulating proliferation and differentiation of PSCs**

In this study, we identified *lncING2-DT* as a novel divergent lncRNA transcribed from the promoter region of *ING2* and demonstrated its dual role in suppressing proliferation while promoting myogenic differentiation in PSCs. These findings are consistent with accumulating evidence that promoter-associated divergent lncRNAs are widely distributed across mammalian genomes (Lepoivre et al., 2013). Functionally, these lncRNAs regulate the expression of neighboring PCGs, contribute to cell cycle homeostasis, and participate in differentiation processes (Chellini et al., 2020; Hung et al., 2011; Luo et al., 2016). Due to their transcription start sites being located in close proximity to the promoters of PCGs, these lncRNAs often exert cis-regulatory effects by modulating local chromatin architecture (Wang et al., 2020), influencing the recruitment of transcriptional complexes (Engreitz et al., 2016), or altering epigenetic modification states (Statello et al., 2021). Thus, the genomic location and functional effects of *lncING2-DT* support its classification as a promoter-associated divergent lncRNA involved in the regulation of PSC proliferation and myogenic differentiation.

Mechanistically, our data indicate that *lncING2-DT* positively regulates the expression and promoter activity of *ING2*. Given its genomic proximity and divergent transcription pattern, *lncING2-DT* may function in cis to modulate the chromatin state of the *ING2* locus. Divergent lncRNAs have been reported to regulate neighboring gene transcription by recruiting chromatin modifiers or enhancing promoter accessibility (Engreitz et al., 2016). Therefore, it is plausible that *lncING2-DT* facilitates *ING2* transcription through chromatin remodeling or transcriptional activation mechanisms.

In addition, RNA pull-down assays and MS analysis identified multiple *lncING2-DT*-interacting proteins enriched in RNA binding and chromatin-associated functions,

implying that *lncING2-DT* may participate in broader transcriptional and post-transcriptional regulatory networks. Notably, under HS conditions, *lncING2-DT* was significantly downregulated in both skeletal muscle tissue and PSCs, and maintaining its expression during HS was sufficient to suppress excessive cell proliferation and promote myogenic differentiation. Collectively, these findings indicate that *lncING2-DT* is an important regulatory factor governing the proliferation and myogenic differentiation of PSCs under basal conditions. Moreover, it is markedly regulated during HS and may play a role in modulating the stress adaptability of skeletal muscle.

***lncING2-DT* regulates myogenic cell fate through an *ING2*-dependent regulatory axis**

Previous studies have demonstrated that *ING2* is involved in chromatin remodeling, DNA repair, and cell cycle regulation, playing a critical role in maintaining cellular homeostasis (Eapen et al., 2012; Goeman et al., 2008; Ricordel et al., 2021; Ythier et al., 2010; Zhou et al., 2016). In this study, our findings position *lncING2-DT* upstream of *ING2* within a regulatory axis that modulates PSC proliferation and differentiation. The positive correlation between *lncING2-DT* and *ING2* expression, combined with co-transfection experiments, supports the notion that *ING2* serves as a key downstream effector mediating the biological functions of *lncING2-DT*.

***lncING2-DT* interacts with multiple RNA-binding and chromatin-associated proteins**

Among high-confidence candidate proteins, LOC100302368, RPS5, SRSF1, and HNRNPU were identified as potential key binding partners. Notably, HNRNPU is a well-known nuclear matrix protein that bridges chromatin and RNA, acting as a scaffold protein for lncRNA-mediated gene regulation (Fan et al., 2018; Hu et al., 2023b; Ma et al., 2025); SRSF1 plays a vital role in precursor mRNA splicing and mRNA export (Qi et al., 2021; Sandoval-Castellanos et al., 2023; Sanford et al., 2009); whilst RPS5, as a structural component of ribosomes (Qiu et al., 2023; Ramakrishnan & White, 1992), may link *lncING2-DT* to translational regulation. Collectively, these findings suggest that *lncING2-DT* may form ribonucleoprotein complexes with multiple RBPs, coordinating myogenic cell growth and stress adaptation processes by

regulating the expression and stability of genes such as *ING2* and *Ki67*. Although further studies are required to validate these interactions and define their molecular consequences, our data provide a foundation for understanding how *lncING2-DT* integrates into chromatin-associated regulatory pathways.

***lncING2-DT* modulates myogenic differentiation and cellular stress responses under HS**

Previous studies have demonstrated that environmental stressors, such as elevated temperatures, induce significant alterations in the skeletal muscle transcriptome, disrupting the equilibrium between cellular proliferation and differentiation (Hao et al., 2016; Reith et al., 2022). In addition, increasing evidence has shown that lncRNAs are important regulators of skeletal muscle development, with several lncRNAs reported to modulate myoblast proliferation and differentiation through epigenetic regulation or interactions with transcription factors. However, most of these studies have focused on basal myogenesis under normal physiological conditions, and only a limited number of reports have investigated the involvement of lncRNAs in skeletal muscle responses to HS. Consistent with this notion, our data show that *lncING2-DT* is significantly downregulated in skeletal muscle tissues and PSCs under HS conditions.

Further analysis revealed that HS treatment significantly inhibited the myogenic differentiation of PSCs, suggesting that HS stimulation may exert detrimental effects on skeletal muscle regeneration. In this context, our functional experiments demonstrated that although the overall expression of *lncING2-DT* was downregulated under HS conditions, its regulatory functions in suppressing cell proliferation and promoting myogenic differentiation were retained. Knockdown of *lncING2-DT* further exacerbated HS-induced impairment of differentiation, whereas overexpression of *lncING2-DT* partially alleviated the adverse effects of HS on the myogenic differentiation of PSCs, indicating that the downregulation of *lncING2-DT* may represent one of the key molecular events underlying HS-mediated differentiation defects.

In addition to regulating myogenic cell fate, *lncING2-DT* modulated key stress-response pathways under HS conditions. Overexpression of *lncING2-DT* enhanced the

expression of heat shock genes (e.g., *HSP70*) while attenuating the expression of inflammatory and apoptotic markers, including *IL-6* and *Caspase-3*, whereas *lncING2-DT* knockdown exacerbated these stress-associated responses. These findings indicate that *lncING2-DT* not only governs myogenic differentiation but also plays a protective role in maintaining cellular homeostasis during acute thermal stress. This aligns with recent proposals that specific lncRNAs can finely regulate cellular stress adaptation responses through multi-level control of gene expression (Lv et al., 2022; Trewin et al., 2022; Yao et al., 2025).

Positional conservation and regulatory relevance of *lncING2-DT*

Although lncRNAs are generally characterized by limited primary sequence conservation, functionally important lncRNAs often exhibit conservation at the level of genomic position, transcriptional orientation, or regulatory association with neighboring PCGs (Amaral et al., 2018). The *lncING2-DT* sequence exhibits low sequence conservation but maintains positional conservation in humans and mice, transcribing divergently from the promoter region of the evolutionarily conserved gene *ING2* (Supplementary Figure S2).

ING2 is highly conserved across species. Its core structural domains, including the PHD zinc finger and ING domains, show over 80% sequence identity from yeast to mammals. In addition, its key functions—such as mediating epigenetic regulation via H3K4me3 binding, participating in chromatin remodeling, regulating the p53-dependent tumor suppressor pathway, and responding to cellular stress—are also evolutionarily conserved (He et al., 2005; Taheri et al., 2022). The strong evolutionary conservation of *ING2* suggests that the regulatory architecture of this locus, rather than the nucleotide sequence of *lncING2-DT*, is under selective constraint. This raises the possibility that the *lncING2-DT-ING2* regulatory axis may be functionally conserved across mammals, although this requires further investigation. From a comparative physiology perspective, such conserved lncRNA–gene regulatory modules may contribute to shared mechanisms underlying skeletal muscle adaptation to environmental stress across species. This positional and functional association supports the biological relevance of *lncING2-DT* and aligns with emerging evidence that

promoter-associated lncRNAs frequently fine-tune the expression of adjacent genes involved in fundamental cellular processes (Lepoivre et al., 2013).

Limitations

This study has several limitations that should be acknowledged. First, the candidate lncRNA was identified based on bulk RNA-seq data from skeletal muscle tissues, whereas functional validation was performed in PSCs. Given that bulk RNA-seq captures averaged signals from heterogeneous cell populations, caution is warranted when extrapolating tissue-level transcriptomic findings to cell-specific functions. Nevertheless, PSCs represent the primary myogenic cell population responsible for muscle growth and regeneration, supporting their relevance as an *in vitro* model. Future studies using single-cell transcriptomics or *in vivo* approaches will be necessary to better resolve the cell-type-specific roles of *lncING2-DT* under HS.

Second, the functional characterization of *lncING2-DT* was restricted to *in vitro* cellular models, and *in vivo* validation is currently lacking. Therefore, its physiological relevance at the organismal level under HS conditions remains to be established. Future studies employing animal models, including gene knockout or overexpression systems, will be essential to confirm its role in muscle development and stress adaptation.

Finally, HS induces global transcriptional reprogramming and reshapes RNA–protein interaction networks, which may complicate direct mechanistic interpretation (Park et al., 2024; Pessa et al., 2024). Future investigations integrating chromatin-level analyses and stress-specific interactome profiling will be important to further elucidate the molecular mechanisms by which *lncING2-DT* regulates myogenesis and cellular adaptation under HS.

CONCLUSION

In summary, our study identifies *lncING2-DT* as a previously uncharacterized regulator of skeletal muscle cell homeostasis under both physiological and heat-stress conditions. Functional experiments demonstrated that *lncING2-DT* suppresses myoblast proliferation while promoting differentiation, and attenuates apoptosis and excessive inflammatory responses under HS conditions. Mechanistically, *lncING2-DT*

exhibits consistent expression patterns with its neighboring gene *ING2* and positively regulates its expression, suggesting that *lncING2-DT* functions, at least in part, through an *ING2*-dependent mechanism. RNA pull-down assays and MS further revealed that *lncING2-DT* interacts with a broad set of RNA-binding and chromatin-associated proteins. These findings uncover *lncING2-DT* as an important heat-responsive lncRNA that participates in chromatin- and transcription-related regulatory pathways through protein interactions, thereby modulating myoblasts proliferation, differentiation, and stress adaptation. This work provides new mechanistic insights into lncRNA-mediated muscle stress biology and identifies *lncING2-DT* as a potential molecular target for improving muscle resilience under thermal stress.

DATA AVAILABILITY

The transcriptomic data used in this study is available in the Sequence Read Archive under accession number PRJNA1367776.

SUPPLEMENTARY DATA

This article contains supporting information (Supplementary Figure S1-S2 and Supplementary Table S1-S6).

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

R.Z. and H.C. contributed equally to this work. R.Z. and L.J. designed the experiments. X.T. and G.Z. improved the methodology. D.Z. and Z.H. designed the bioinformatics analyses. H.C. and D.Z. performed the data analysis. R.Z. conducted cell culture experiments. S.W. and H.R. conducted the gene quantification experiment. R.Z. drafted the paper. L.J., S.S., and M.L. revised the paper. L.J., X.Z., and A.J. acquired the funding. All authors read and approved the final version of the manuscript.

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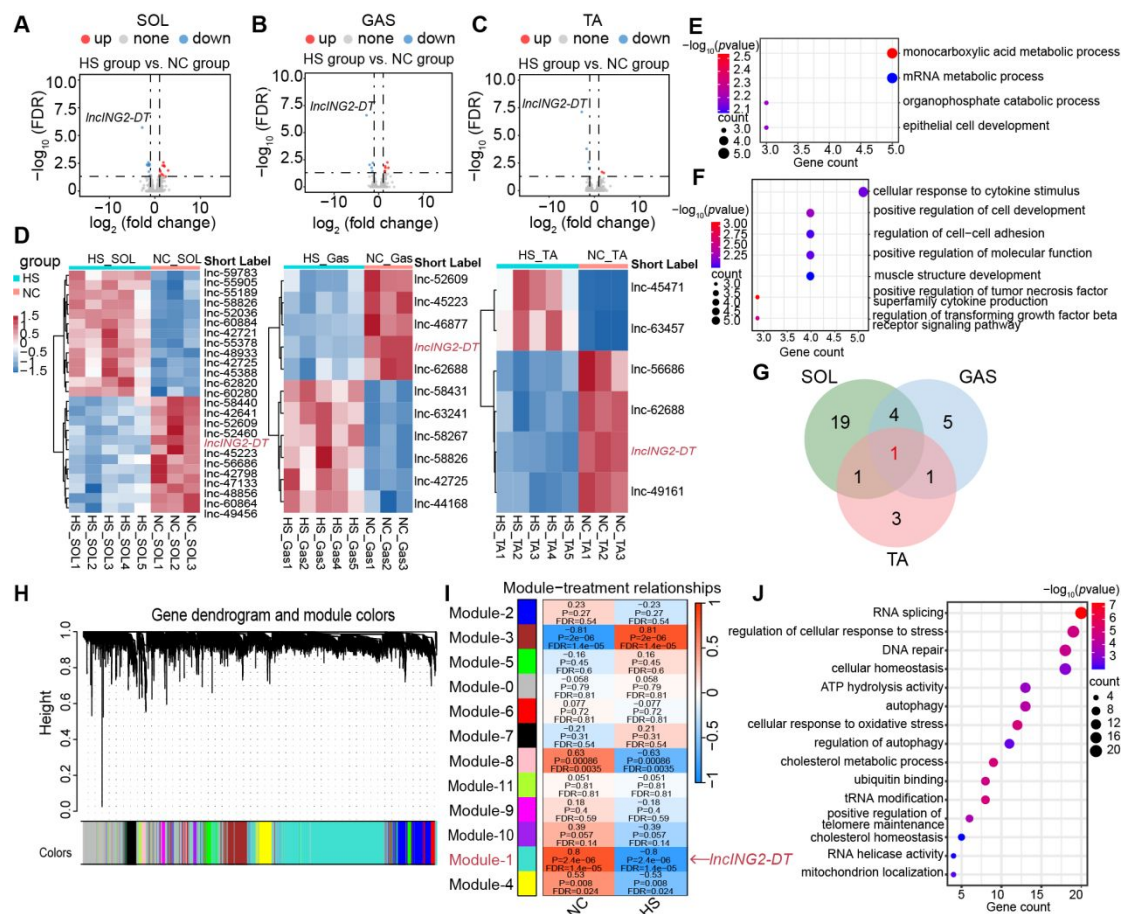


Figure 1 Screening of differentially expressed lncRNAs in porcine skeletal muscle under HS

A: Volcano plots of differentially expressed lncRNAs in the SOL. Positive values indicate upregulation; negative values indicate downregulation. Volcano plot showing the distribution of gene based on log₂fold change (x-axis) and -log₁₀(FDR) (y-axis).

B: Volcano plots of differentially expressed lncRNAs in the GAS. Positive values indicate upregulation; negative values indicate downregulation. Volcano plot showing the distribution of gene based on log₂fold change (x-axis) and -log₁₀(FDR) (y-axis).

C: Volcano plots of differentially expressed lncRNAs in the TA. Positive values indicate upregulation; negative values indicate downregulation. Volcano plot showing the distribution of gene based on log₂fold change (x-axis) and -log₁₀(FDR) (y-axis).

D: Heatmap of differentially expressed lncRNAs between the NC and HS groups in SOL, GAS, and TA muscle. Rows represent individual lncRNAs and columns represent samples. Ensembl IDs were simplified as lnc-XXXXX for clarity; full ID mappings are

853 listed in Supplementary Table S1.

854 E: GO and KEGG pathway analysis of 75 target genes significantly upregulated by
855 lncRNA in GAS, SOL, and TA.

856 F: GO and KEGG pathway analysis of 88 target genes significantly downregulated by
857 lncRNA in GAS, SOL, and TA.

858 G: Venn diagram showing the differentially expressed lncRNAs identified in the SOL,
859 GAS, and TA muscles.

860 H: Gene cluster dendrograms using certain module colors and variations in topological
861 overlap.

862 I: Module-treatment associations of GAS, SOL, TA tissue under HS. Each row
863 corresponds to a module epigene, while each column corresponds to a temperature
864 treatment. Each cell contains the corresponding correlation and *P* value. The table is
865 colour-coded by correlation according to the colour legend.

866 J: GO and KEGG pathway analysis of 334 high-confidence genes ($|r| \geq 0.8$ and
867 $FDR < 0.01$) in the module-1.

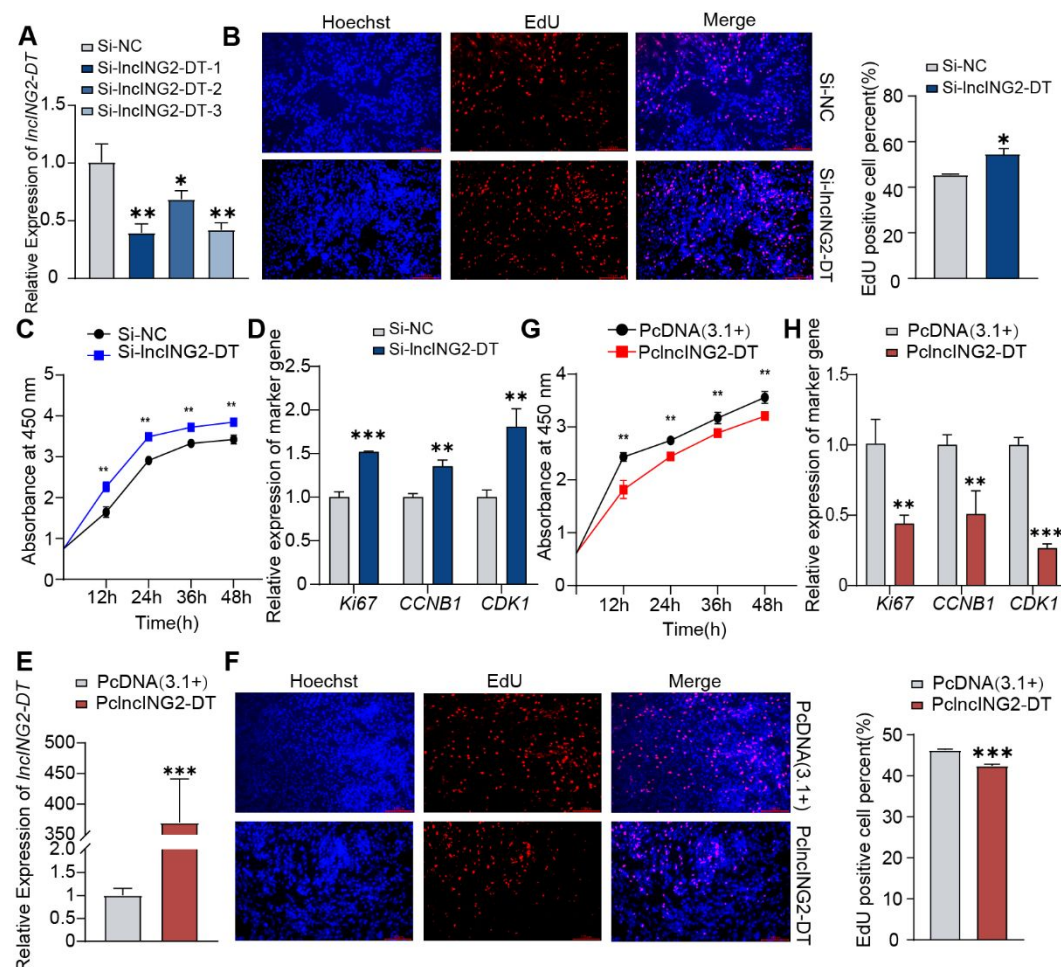


Figure 2 *IncING2-DT* inhibits myoblasts proliferation

A: Detection of *IncING2-DT* knockdown efficiency.

B: EdU staining assays after *IncING2-DT* knockdown. Red: 5-ethynyl-2'-deoxyuridine staining; blue: Hoechst 33342 staining. Scale bar: 100 μ m.

C: CCK-8 assays of PSCs after *IncING2-DT* knockdown.

D: Relative mRNA expression levels of proliferation marker genes (*Ki67*, *CCNB1*, and *CDK1*) in PSCs after *IncING2-DT* knockdown.

E: Detection of *IncING2-DT* overexpression efficiency.

F: EdU staining assays after *IncING2-DT* overexpression. Red: 5-ethynyl-2'-deoxyuridine staining; blue: Hoechst 33342 staining. Scale bar: 100 μ m.

G: CCK-8 assays of PSCs after *IncING2-DT* overexpression.

H: Relative mRNA expression levels of proliferation marker genes (*Ki67*, *CCNB1*, and *CDK1*) in PSCs after *IncING2-DT* overexpression.

Note: Data are expressed as mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

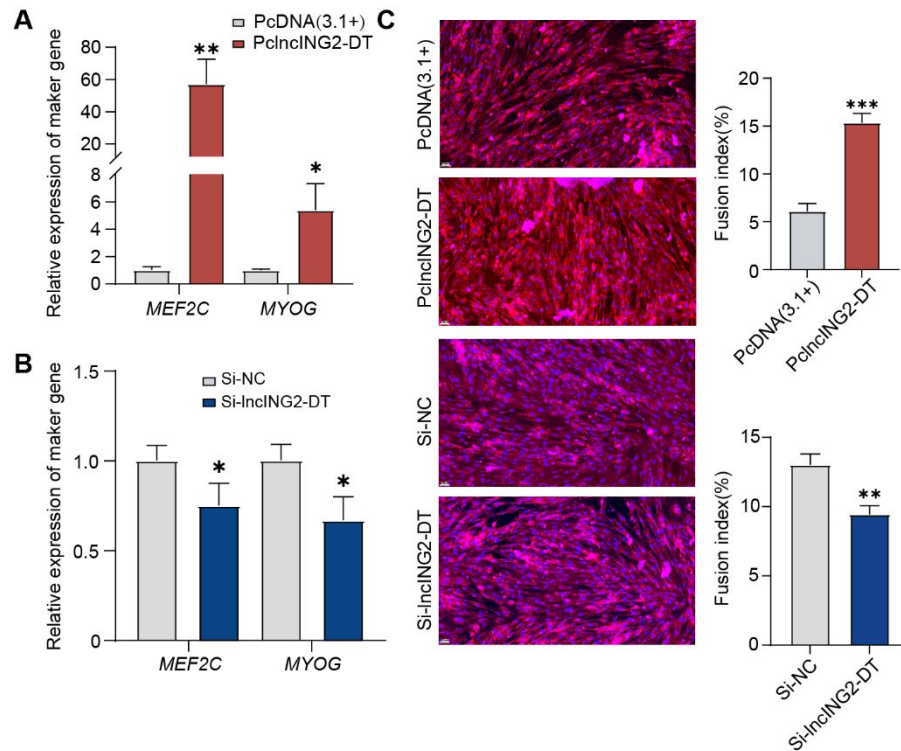


Figure 3 *lncING2-DT* promotes myoblast differentiation

A: Relative mRNA expression levels of myogenic differentiation marker genes (*MEF2C*, *MYOG*) in PSCs after *lncING2-DT* overexpression.

B: Relative mRNA expression levels of myogenic differentiation marker genes (*MEF2C*, *MYOG*) in PSCs after *lncING2-DT* knockdown.

C: Left: Immunofluorescence staining of PSCs differentiated for 36h. Red, myosin heavy chain (MyHC); blue, DAPI. Right: Quantification of fusion index for myoblasts.

Scale bar: 50 μ m.

Note: Data are expressed as mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

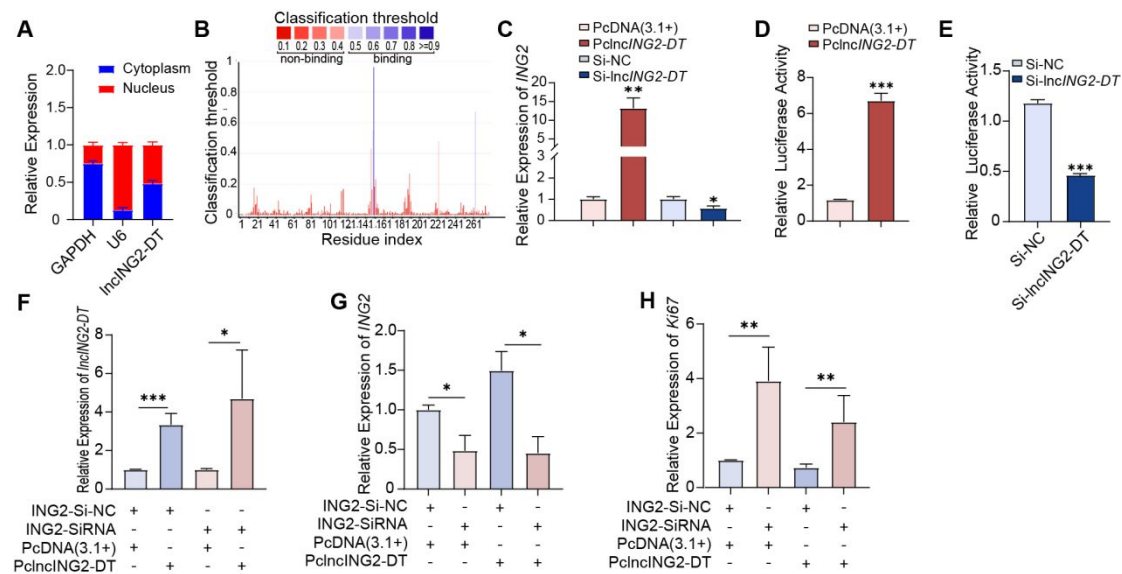


Figure 4 *lncING2-DT* functions through an *ING2*-dependent mechanism

A: Localisation detection of *lncING2-DT* in PSCs. *GAPDH* served as an internal reference for cytoplasmic localisation, while *U6* served as an internal reference for nuclear localisation.

B: Predicted potential binding sites between *lncING2-DT* and *ING2*. The horizontal axis represents the sequence position, and the vertical axis indicates the interaction score at each position, reflecting the predicted binding propensity. A classification threshold of 0.5 was applied; regions with scores > 0.5 were considered potential binding sites, while scores between 0 and 0.4 were considered non-binding.

C: mRNA expression levels of *ING2* in PSCs following overexpression and knockdown of *lncING2-DT*.

D: Effect of *lncING2-DT* overexpression on luciferase activity in the *ING2* promoter construct.

E: Effect of *lncING2-DT* knockdown on luciferase activity in the *ING2* promoter construct.

F: PSCs transfected with *ING2* siRNA, followed by transfection with the *lncING2-DT* overexpression vector after 24h. Transfection efficiency for *lncING2-DT* overexpression analysed via RT-qPCR after a further 24h.

G: Analysis of *ING2* mRNA expression levels via RT-qPCR 24h after transfecting PSCs with *ING2* siRNA followed by *lncING2-DT* overexpression vector.

914 H: PSCs were transfected with *ING2* siRNA, followed by transfection with the
915 *lncING2-DT* overexpression vector after 24h. *Ki67* mRNA expression levels were
916 analysed by RT-qPCR after a further 24h.

917 Note: Data are expressed as mean $\pm$ SEM; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

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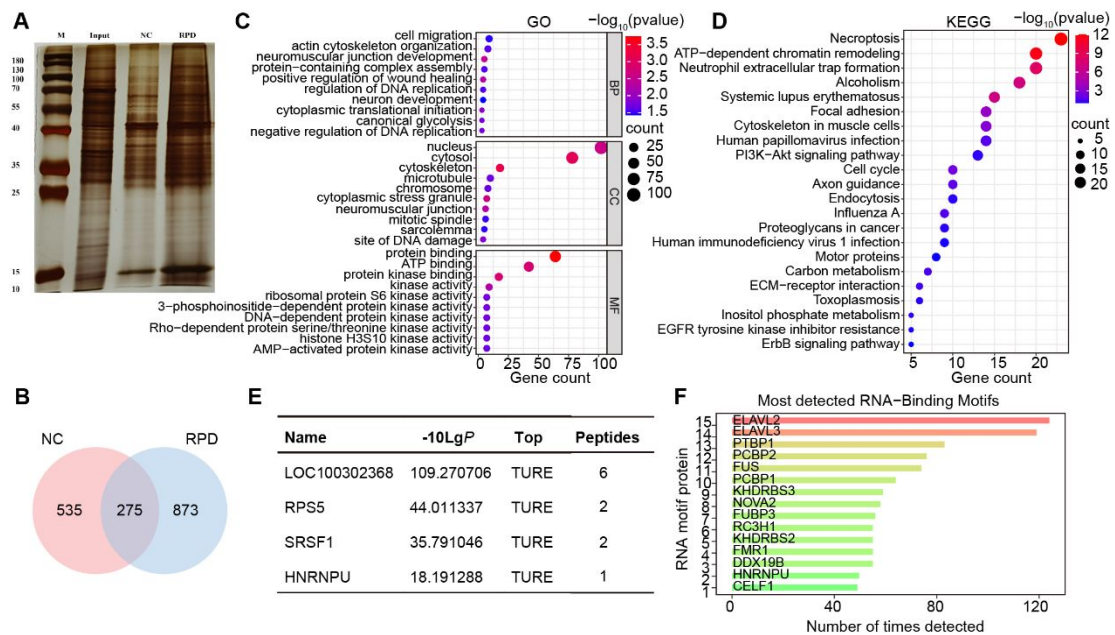


Figure 5 Identification of interacting proteins and potential mediating factors of *lncING2-DT*

A: Proteins precipitated in the RNA pull-down assay were separated by SDS-PAGE and subsequently silver-stained.

B: Statistics for *lncING2-DT*-binding proteins identified in control and RNA pull-down groups. The Venn diagram illustrates overlap between RBPs identified in control versus RNA pull-down groups.

C: GO analysis results for 873 RBPs identified in PSCs.

D: KEGG analysis results for 873 RBPs identified in PSCs.

E: Top proteins interacting with *lncING2-DT* from PSCs, identified by MS analysis.

F: CatRAPID (http://service.tartagialab.com/page/catrapid_omics2_group) predicted proteins interacting with *ING2*.

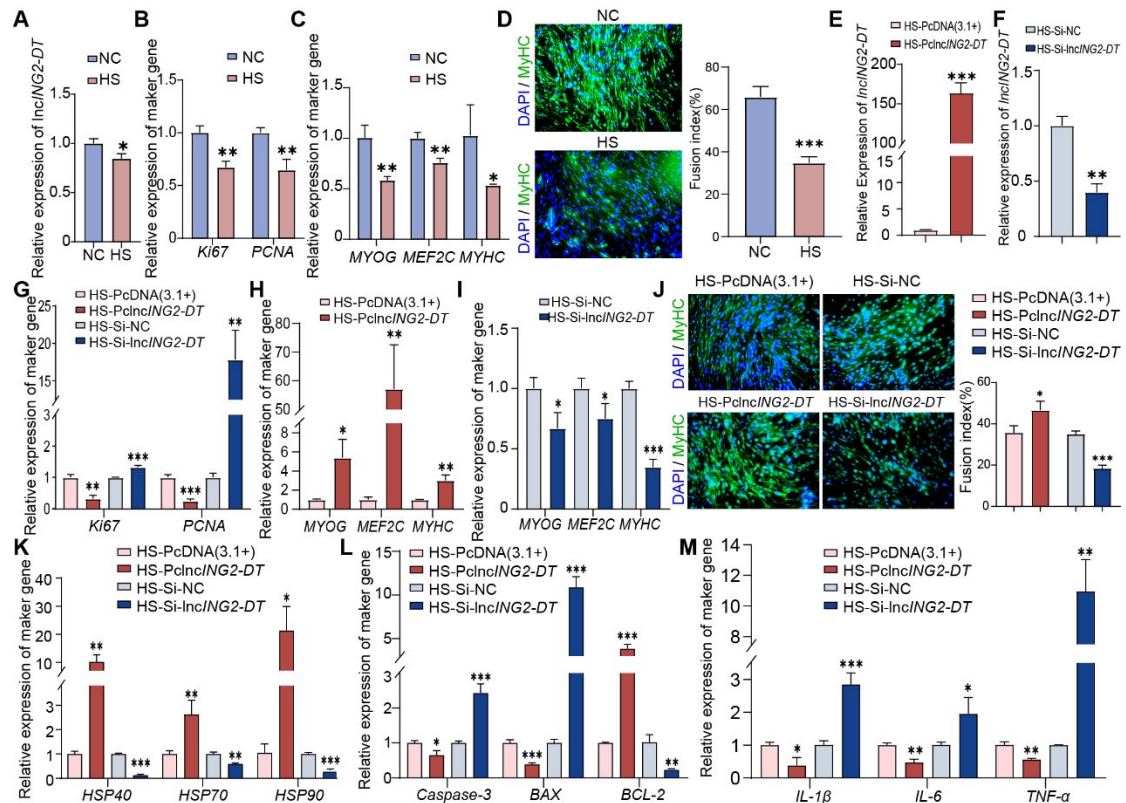


Figure 6 *IncING2-DT* regulates PSC proliferation and differentiation under HS

A: Relative expression of *IncING2-DT* in PSCs under normal control (NC) and HS conditions.

B: Relative mRNA expression levels of proliferation marker genes (*Ki67*, *PCNA*) in PSCs under NC and HS conditions.

C: Relative mRNA expression levels of myogenic differentiation marker genes (*MYOG*, *MEF2C*, and *MYHC*) in PSCs under NC and HS conditions.

D: Left: Immunofluorescence staining of PSCs differentiated for 4 days. Green, myosin heavy chain (MyHC); blue, DAPI. Right: Quantification of fusion index for myoblasts. Scale bar: 50 μ m.

E: Detection of *IncING2-DT* overexpression efficiency under HS conditions (42°C, 6h).

F: Detection of *IncING2-DT* knockdown efficiency under HS conditions (42°C, 6h).

G: Relative mRNA expression levels of proliferation-related genes (*Ki67* and *PCNA*) following *IncING2-DT* overexpression or knockdown under HS conditions.

H: Relative mRNA expression levels of myogenic differentiation markers (*MYOG*, *MEF2C*, and *MYHC*) in PSCs with *IncING2-DT* overexpression under HS conditions.

I: Relative mRNA expression levels of myogenic differentiation markers (*MYOG*, *MEF2C*, and *MYHC*) in PSCs with *lncING2-DT* knockdown under HS conditions.

J: Left: Immunofluorescence staining of PSCs transfected with *lncING2-DT* after HS. Green, myosin heavy chain (MyHC); blue, DAPI. Right: Quantification of fusion index for myoblasts. Scale bar: 50 μ m.

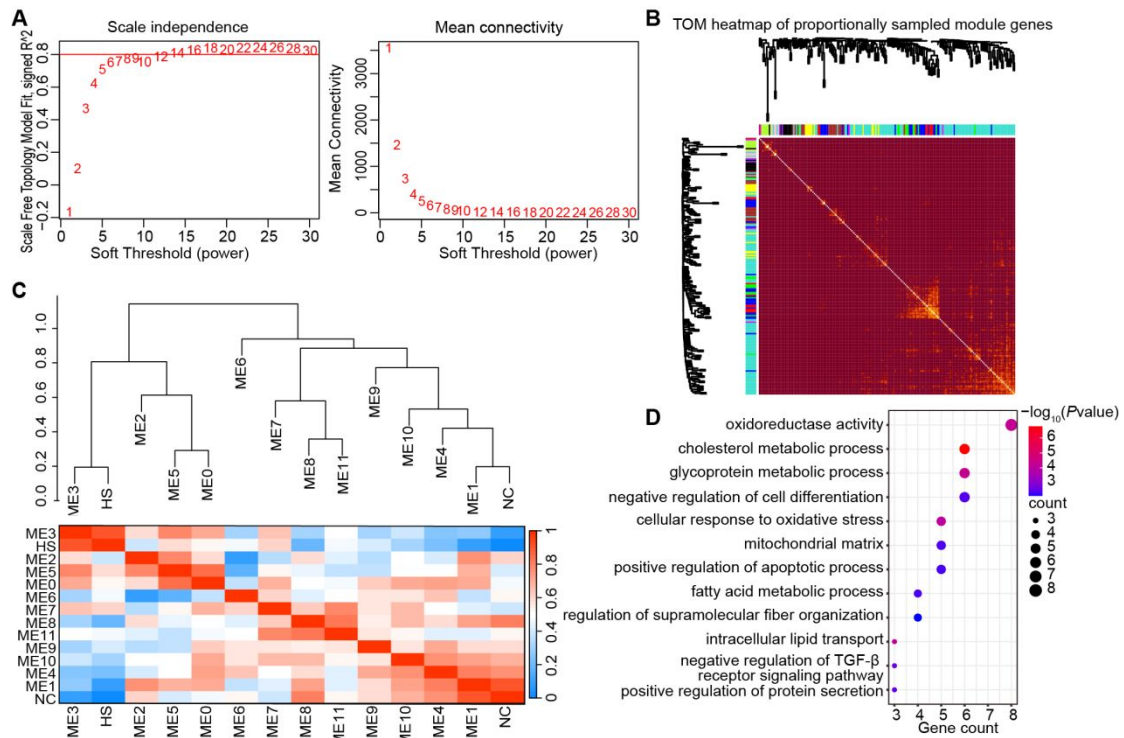
K: Relative mRNA expression levels of heat shock genes (*HSP40*, *HSP70*, and *HSP90*) following *lncING2-DT* overexpression or knockdown under HS conditions.

L: Relative mRNA expression levels of apoptosis-related genes (*Caspase-3*, *BAX*, and *BCL-2*) in PSCs after *lncING2-DT* overexpression or knockdown under HS conditions.

M: Relative mRNA expression levels of inflammatory cytokine genes (*IL-1 β* , *IL-6*, and *TNF- α*) following *lncING2-DT* overexpression or knockdown under HS conditions.

Note: Data are expressed as mean $\pm$ SEM; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Supplementary Materials



Supplementary Figure S1 WGCNA reveals co-expression modules and identifies HS-responsive genes in pig skeletal muscles.

A: A soft threshold of $\beta=14$ was used to define the adjacency matrix, and scale-free topologies were investigated.

B: Clustering dendrogram of module genes with corresponding topological overlap matrix.

C: Heatmaps for gene network visualization.

D: GO and KEGG pathway analysis of the 60 genes overlapping between the module-1 and DEGs.

Supplementary Table S1 Differentially expressed lncRNAs in porcine SOL, GAS, and TA muscles under HS compared with NC.

Supplementary Table S2 Predicted cis-target genes of differentially expressed lncRNAs in porcine SOL, GAS and TA muscles under HS.

Supplementary Table S3 Composition of WGCNA co-expression modules and module assignment of *lncING2-DT*

981 **Supplementary Table S4 Primer sequences used for RT-qPCR analysis in this**
 982 **study.**

Target genes	Sequences of primers
β -Actin-F	AAGGACCTCTACGCCAACAC
β -Actin-R	CTGGCTGATCCACATCTGCT
lncING2-DT-F	TTGTCCACCCTCCCGCTCTTG
lncING2-DT-R	AAGCCCTAGCCGACGTTTAATGC
ING2-F	CAGCAGCAGCAGCAACTCTACTC
ING2-R	CAACTCTCGCAGCACGGACAC
Ki67-F	GGACGCTTCGACAAAAGCTG
Ki67-R	TCTTCCGTAAAGCCTGGTGC
PCNA-F	GGCCCGCGTCGTTGTGATTC
PCNA-R	CGCTTCCAGCACCTTCTTCAGG
CCNB1-F	AATCCCTTCTTGTGGTTA
CCNB1-R	CTTAGATGTGGCATACTTG
CDK1-F	CAGCTCGCTACTCAACTCCA
CDK1-R	GAGTGCCCAAAGCTCTGAAA
GAPDH-F	ACCCAGAAGACTGTGGATGG
GAPDH-R	AAGCAGGGATGATGTTCTGG
MYOG-F	CATCCAGTACATCGAGTGCCT
MYOG-R	ACTGTGATGCTGTCCACGAT
MYHC-F	G TTCAGAGAAAGGCATCCCAAA
MYHC-R	GAGAGTGACCGACACCACAAGTG
MEF2C-F	TTACCAGGTGAGACCAGCAGA
MEF2C-R	GTGTCCAGCCACAACAGTTTG
HSP40-F	CTCTCATAAGCGGCTGAACC
HSP40-R	GCTGGAATGTTGTTGGAGGT
HSP70-F	GCAACGTGCTGATCTTCGAC
HSP70-R	CAGCCTGTTGTCGAAGTCCT
HSP90-F	GTCGAAAAGGTGTTGTGTCG
HSP90-R	TTTGCTGTCCAGCCGTATGT
Caspase3-F	GCAGTTTTATTTGCGTGCTTC
Caspase3-R	TCCGTCTCAATCCCACAGTC
BAX-F	AATTGGCTTGGTCTGTAT
BAX-R	CGGTGCTGATGGTATGTG
BCL-2-F	CATGCGTATTTATATTTG
BCL-2-R	CTCTGCTGCTTGCTGCTA
TNF- α -F	CGCCACGTTGTAGCCAATGT
TNF- α -R	CAGATAGTCGGGCAGGTTGATCTC
IL-1 β -F	TAGTACCTGAACCCGCCAAG
IL-1 β -R	GGTGAGAGATTTGCAGCTGG
IL6-F	ACCGGTCTTGTGGAGTTTCA
IL6-R	GCATTTGTGGTGGGGTTAGG

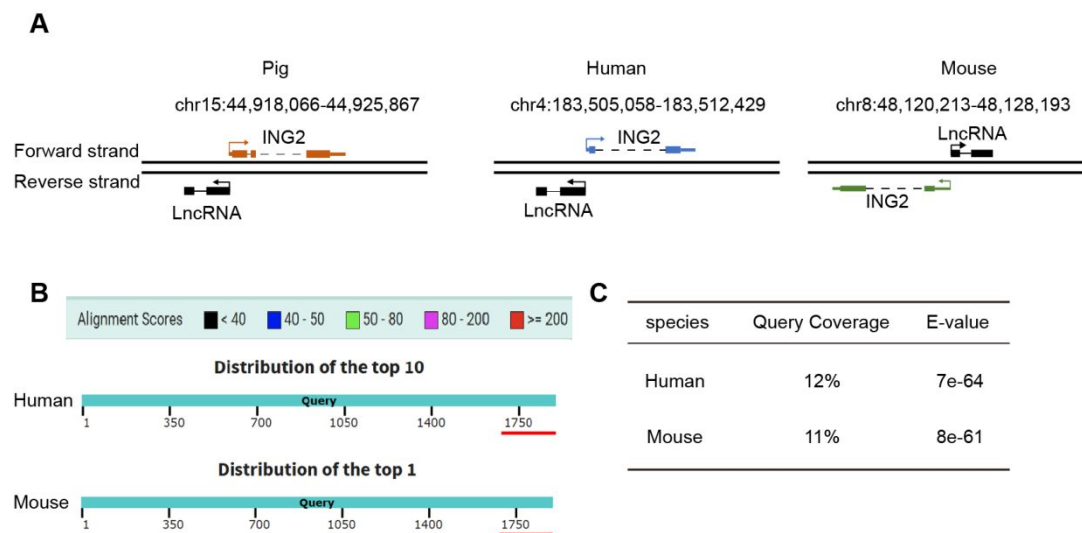
U6-F	TTGGCAGCCCATGTGCTAAT
U6-R	ACTGACTGCTTCACGAATTTACA

983 **Supplementary Table S5 siRNA sequences used for knockdown of *lncING2-DT* in**
984 **this study.**

genes	Sequences
LncING2-DT-1-s	GAUGGACUCAGUUUGUAUA
LncING2-DT-1-a	UAUACAAACUGAGUCCAUC
LncING2-DT-2-s	CGAAGAUUGUCAUGUGCUA
LncING2-DT-2-a	UAGCACAUGACAAUCUUCG
LncING2-DT-3-s	GGUGUAAAGAGAACUAAUA
LncING2-DT-3-a	UAUUAGUUCUCUUUACACC
Si-NC-s	UUCUCCGAACGUGUCACGU
Si-NC-a	ACGUGACACGUUCGGAGAA

985 **Supplementary Table S6 Proteins identified by mass spectrometry in the *lncING2-***
986 ***DT* RNA pull-down groups.**

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988



Supplementary Figure S2 Conservation of *lncING2-DT*'s genomic position and sequence alignment results

A: Schematic of *ING2* and *lncING2-DT* for pigs (orange), humans (blue), and mice (green) from Ensembl genome browser. Schemas are not drawn to scale. Blocks with colors (orange, blue, and green) represent exons, and the dotted line represents intronic regions. Arrow shows the direction of transcription; short line represents no transcripts found in this species.

B: BLASTn alignment distribution of *lncING2-DT* against the pig (*Sus scrofa*) and mouse (*Mus musculus*) genomes using the NCBI BLAST platform (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The *x-axis* indicates the length of the query lncRNA sequence (1-1902 bp). The cyan bar represents the full-length query sequence, while colored segments denote aligned regions, with colors transitioning from dark to red to indicate increasing alignment scores and sequence similarity.

C: Summary of BLAST alignment results showing query coverage and statistical significance across species.

lncING2-DT 通过依赖 *ING2* 的机制抑制猪骨骼肌卫星细胞的增殖并促进其分化

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摘要

热应激(Heat stress, HS)严重损害骨骼肌发育, 但长链非编码 RNA(long non-coding RNA, lncRNA)在这一过程中的调控作用仍不清楚。本研究对热应激组 and 对照组猪比目鱼肌(soleus, SOL)、腓肠肌(gastrocnemius, GAS)和胫骨前肌(tibialis anterior, TA)进行 RNA-seq, 鉴定出多个差异表达 lncRNA。其中, ENSSSCG00000048143(本研究命名为 *lncING2-DT*) 在热应激组的 3 种肌肉中均较对照组一致下调, 因此被选作进一步研究对象。猪骨骼肌卫星细胞(porcine skeletal muscle satellite cells, PSCs)中的功能分析表明, 在正常条件下, *lncING2-DT* 可抑制细胞增殖并促进成肌分化。机制研究显示, *lncING2-DT* 正向调控其邻近基因 *ING2* 的表达及启动子活性; 共转染实验进一步表明, *lncING2-DT* 对 PSCs 增殖的抑制作用部分依赖于 *ING2*。RNA pull-down 结合质谱分析鉴定出多种与 *lncING2-DT* 相互作用的蛋白, 其中 HNRNPU 被确定为候选互作蛋白, 可能参与 *lncING2-DT* 对 *ING2* 的调控。在热应激条件下, *lncING2-DT* 表达显著降低, 同时伴随成肌分化受损、细胞增殖增强以及细胞凋亡和炎症反应加剧。值得注意的是, 过表达 *lncING2-DT* 可通过促进成肌分化、增强热休克反应并抑制过度凋亡和炎症, 缓解热应激诱导的细胞功能障碍。综上, 本研究鉴定了 *lncING2-DT* 作为维持 PSCs 稳态和应激适应的关键调控因子, 为阐明热应激诱导骨骼肌功能障

1034 碍的分子机制提供了新的认识。

1035 **关键词：** *lncING2-DT*; 骨骼肌; 增殖; *ING2*; HNRNPU; 热应激

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