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RESEARCH ARTICLE

Alternative Polyadenylation of *Psmd14* Mrna Modulates Neuronal Apoptosis and Impacts Aggressive Behavior in Pigs

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A B S T R A C T

Porcine aggressive behavior is a complicated trait that is harmful to animal welfare. Alternative polyadenylation (APA) plays a significant role in various biological processes. However, the relationship between APA and aggressive behavior in pigs remains poorly understood. In our study, we conducted transcriptome sequencing on brains (frontal lobe, parietal lobe, temporal lobe, hypothalamus and pituitary gland) from pigs exhibiting the most and least aggressive behavior. A combination of transcriptomic and molecular data revealed that APA-modulated *PSMD14* was associated with aggression in pigs. In particular, the expression of cleavage stimulation factor subunit 2 (*CSTF2*) was higher in the brains of the highly aggressive pigs than in the least aggressive pigs. We found that *CSTF2* bound to the motif of GUGGA on the *PSMD14* gene, leading to cleavage results in a shortened 3'UTR of *PSMD14*, the phenomenon observed in the most aggressive pigs. By contrast, the least aggressive pigs with lower *CSTF2* expression, exhibited a lengthened 3'UTR. The dual luciferase reporter system indicated that this elongated 3'UTR contains a binding site for miR-29-5a. Binding of miR-29-5a downregulated *PSMD14* expression, leading to subsequent apoptosis upregulation. In summary, we identified a potential regulatory pathway governing porcine aggressive behavior initiated by the *CSTF2*-mediated APA network (*CSTF2-PSMD14*-miR-29-5a axis). This study provides a theoretical foundation for investigating the role of APA in regulating aggressive behavior in pigs.

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INTRODUCTION

Aggressive behavior is common in animals, which mainly benefits individuals in their competition for resources and the attainment of a higher social rank. However, in the production of farm animals, emphasis is placed on the quality and quantity of animal products. Aggressive behavior can lead to a significant decline in animal welfare, which in turn affects the quality of animal products (Tubert *et al.*, 2012; Guo *et al.*, 2022; Lin *et al.*, 2024). Genetic factors are the main regulators of aggressive behavior, including common genomic variations, and interactions between genes and the environment (Roy *et al.*, 2018; Rodriguez-Morales *et al.*, 2022; Zhang *et al.*, 2025). Research on the molecular mechanisms regulating aggressive behavior is still limited. A recent study, by integrating the transcriptome and metabolome of the HPA-axis, identified that the GABA-related pathways play an important role in the aggressive behavior of pigs. However, more extensive research is needed on aggressive behavior, especially in commercial pig production, to further deepen our understanding of aggressive behavior and better guide pig genetic breeding (Zhang *et al.*, 2025).

Genomic variations and transcriptional alterations are the focus of attention for animal breeders. Association analyses at the whole-genome level have been widely applied in GWAS and TWAS studies, which have greatly advanced the process of animal breeding. It should be noted that alternative polyadenylation (APA), as a common post-transcriptional regulatory mechanism, has received

relatively little attention. APA can affect the stability and translation efficiency of mRNA by regulating changes in mRNA structure, ultimately leading to changes in the expression levels of proteins that perform biological functions, which is a more direct regulatory mode (Yu *et al.*, 2006; Guvenek *et al.*, 2022). In the nervous system, APA is believed to be related to the physiological activities of neurons and synaptic plasticity, and these are closely associated with the regulation of aggressive behavior (Epstein *et al.*, 2014). However, to date, there has been no research on the role of APA in regulating the aggressive behavior of pigs.

PSMD14 (also known as POH1 or Rpn11) is a member of the deubiquitinating enzyme family, based on its ability to regulate protein degradation. It plays an essential role in basic cellular functions such as cell proliferation, apoptosis, and cellular stress (Guo *et al.*, 2016; He J *et al.*, 2019; Spasskaya *et al.*, 2020). However, most current studies focus on its deubiquitination ability, and there is little understanding of the regulation of *PSMD14* itself, especially the post-transcriptional regulatory mechanism. Moreover, it is still unknown whether *PSMD14* is involved in the regulation of pig's aggressive behavior.

To explore this knowledge gap, we conducted a study in which we first observed aggressive behavior in 160 pigs. Transcriptome analysis of multiple brain regions was then performed in pigs exhibiting varying degrees of aggressive behavior. Further integrative analysis of APA and differentially expressed genes based on transcriptome analysis revealed that *PSMD14* expression may be regulated by APA. Subsequently, we performed a series of molecular experiments to determine how APA regulates *PSMD14* expression, with the aim of exploring whether APA of *PSMD14* has the potential to regulate aggressive behavior in pigs.

MATERIALS AND METHODS

Animals and RNA-Seq: The pigs' behavioral observations were the same as described in a previous study (Zhang *et al.*, 2025). Briefly, a two-stage mixing was applied to a cohort of 160 weaned pigs. The four most and four least aggressive pigs were selected based on consistent behavioral observations. Upon dissection, five brain tissues (frontal lobe (FL), parietal lobe (PL), temporal lobe (TL), hypothalamus (HP), and pituitary gland (PG)) were harvested and preserved in a -80°C freezer. Total RNA was extracted using the Trizol method, following the manufacturer's protocol (Vazyme, Nanjing, China). Libraries were pooled and sequenced on the Illumina NovaSeq-6000 platform (San Diego, CA, USA) using the PE150 strategy. The reads were aligned to the pig reference genome (Sscrofa 11.1) using HISAT2 (v 2.2.1), and featureCounts was used to generate the gene expression matrix (Liao *et al.*, 2014; Kim *et al.*, 2019). DESeq2 was used to identify differentially expressed genes (DEGs) between groups, with thresholds of fold change≥1.5 or ≤0.67 and $P<0.05$, and visualizations were generated with OmicStudio (Lyu *et al.*, 2023). Additionally, a public transcriptomic dataset was incorporated, representing three si-PSMD14 conditions and their corresponding controls (Yang *et al.*, 2023).

Transcriptome-based analysis of alternative polyadenylation in the 3'UTR: DaPars (https://github.com/ZhengXia/dapars) was used to calculate the percentage change in distal poly(A) site usage index (PDUI, percentage change in distal poly (A) site usage index) — a measure of proximal and distal PAS usage. The ΔPDUI (calculated as the average PDUI of one group minus the other) was used to assess 3'UTR length variations between groups. An APA locus was considered statistically significant if FDR (false discovery rate)<0.05 and $|\Delta PDUI|>0.3$. The IGV tool was used for visualization and analysis of 3'UTR length and abundance (Thorvaldsdóttir *et al.*, 2012).

Cell culture and transfection: Porcine neuronal cells (PNCs) were isolated and cultured from 15-day-old piglets using enzymatic digestion. PNCs were maintained in DMEM/F-12 medium (Gibco, Thermo Scientific, USA), supplemented with 15% fetal bovine serum (FBS, Gibco, Thermo Scientific, USA) and 1% penicillin-streptomycin, at 37°C in a 5% CO_2 incubator, with medium changes every two days. For porcine kidney-15 (PK15) cells, the culture medium consisted of 89% high-glucose DMEM (Gibco, Thermo Scientific, USA), 10% FBS, and 1% penicillin-streptomycin. miR-29-5a mimics and small interfering RNA (siRNA) targeting *CSTF2*, along with their respective controls, were synthesized by Shanghai Generay (Generay, Shanghai, China). Transfection was performed using the SuperKine™ Lipo3.0 transfection reagent (Abbkine, Wuhan, China) following the manufacturer's instructions.

Quantitative real-time PCR (qPCR) and Gel electrophoresis: Total RNA was extracted from cells and tissues using the Trizol method, followed by cDNA synthesis using a reverse transcriptase kit (Vazyme, Nanjing, China). qPCR was performed in 20μL reactions (2μL cDNA, 10μL SYBR Mix, 0.4μL each primer, 7.2μL H_2O) on a QuantStudio 5 system: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Each sample was analyzed in triplicate (technical replicates), and relative expression was normalized to the housekeeping gene *GAPDH*. To validate APA isoforms, we designed primers to amplify a 74-bp fragment within the distal 3'UTR region unique to the lengthened *PSMD14* isoform. cDNA from frontal lobe tissues of the most (FLM) and least (FLL) aggressive pigs was used as template. PCR amplification products were confirmed by agarose gel electrophoresis, and band intensities were quantified using ImageJ.

Immunofluorescence assay: PNCs were cultured on coverslips until reaching 70% confluence, then washed once with PBS and fixed with 4% paraformaldehyde (Beyotime, Shanghai, China) for 15 minutes. After two additional PBS washes, cells were permeabilized with 0.5% Triton X-100 for 20 minutes and blocked with QuickBlock™ blocking buffer (Beyotime, Shanghai, China) for 1 hour. Cells were then incubated for 1 hour at room temperature with primary antibodies targeting Ionized Calcium Binding Adaptor Molecule 1 (anti-IBA1, Affinity #DF6442, 1:200), Glial Fibrillary Acidic Protein (anti-GFAP, Affinity #DF6040, 1:200), Neurofilament Heavy Chain Protein (anti-NEFH, Affinity #DF13211, 1:200), and Myelin Basic Protein (anti-

MBP, Affinity #AF4085, 1:200). After PBS washes, cells were incubated with a fluorescein-conjugated goat anti-rabbit secondary antibody, and nuclei were stained with DAPI. Confocal laser microscopy (Zeiss, Jena, Germany) was used to assess staining intensity, which correlated with protein expression levels.

Dual-Luciferase reporter gene assay: The miRanda (http://www.bioinformatics.com.cn/local_miranda) software was used to predict miRNAs targeting the lengthened region of the *PSMD14* 3'UTR, identifying miR-29-5a as a strong candidate. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay Kit (Vazyme, Nanjing, China) on a dual-luciferase assay system (Promega, WI, USA).

Cell apoptosis analysis: Apoptosis was assessed using Annexin V-FITC and PI staining by flow cytometry following the Annexin V-FITC Apoptosis Detection Kit (Elabscience, Wuhan, China). Apoptosis rates were quantified using FlowJo (v 10.6.2) software. Additionally, the TUNEL assay was used to evaluate cell apoptosis (Apexbio, Houston, USA).

Statistical analysis: Data are presented as mean±SEM from at least three independent biological replicates. Normality was assessed using the Shapiro−Wilk test, and homogeneity of variance was evaluated using Levene's test. For comparisons between two groups, a two-tailed unpaired Student's t-test was used. For comparisons among three or more groups, one-way ANOVA followed by Tukey's post hoc test was performed. Statistical significance was defined as $P<0.05$. All analyses were conducted using GraphPad Prism 8.0.1.

RESULTS

Differential gene expression analysis in brain tissues of pigs with varying aggression levels: Principal component analysis (PCA) clearly separated samples into five distinct brain regions (Fig. 1A). DEGs were identified in all five regions between the most and least aggressive pigs, with the HP showing the largest transcriptional divergence (Fig. 1B). A Venn diagram analysis identified 27 DEGs shared across all brain regions, which are linked to endocrine regulation and calcium reabsorption (Fig. 1C, D). Weighted gene co-expression network analysis (WGCNA) was conducted to identify candidate gene modules linked to aggressive behavior in pigs. WGCNA revealed that MEgreen module showed the strongest association with the hypothalamus in pigs from the high aggression hypothalamus (HPM) group (Fig. 1E). Considering the hypothalamus plays a critical role in regulating aggressive behavior, we further investigated the functions of genes within the MEgreen module (Flanigan *et al.*, 2020). A scatter plot analysis confirmed a significant correlation between the MEgreen module and the HPM group (Fig. 1F), while pathway enrichment revealed significant involvement in neuroactive ligand–receptor interactions and cerebrospinal fluid circulation (Fig. 1G, H).

Comprehensive analysis of APA sites linked to aggressive behavior across multiple tissues: Using DaPars, we identified APA sites in 40 transcriptomic samples. In this analysis, 3'UTR lengthening events were defined as cases where the mean PDUI value of the least aggressive group exceeded that of the most aggressive group, while the opposite was classified as shortening events. Similarly, the PCA plots showed distinct tissue-specific genetic signatures (Fig. 2A). The largest number of APA fragment ranged from 300 to 500 bp, while fragments<300 bp were the least frequent (Fig. 2B). Comparative analysis based on APA events between the most and least aggressive pigs revealed the highest number of differential APA events in the frontal lobe ($|PDUI\_Group\_diff|\geq0.3$, $FDR<0.05$) (Fig. 2C). A Manhattan plot was used to show the genomic locations of the APA sites ranked by |PDUI_Group_diff| (Fig. 2D). APA-related genes in the frontal lobe were enriched in proteasome, pyruvate metabolism, and carbon metabolism pathways (Fig. 2E). Protein-protein interaction (PPI) network identified *PSMD14* as a hub gene in this network (Fig. 2F).

APA patterns of *PSMD14* in pigs with varying aggression levels: To identify genes whose expression may be regulated by APA, we analyzed 1,135 genes exhibiting altered expression and 102 genes undergoing APA regulation, and four genes that simultaneously underwent both changes (*PSMD14*, *GTF3C1*, *IFI44L*, and *PLXNB1*) (Fig. 3A). The scatter plot of |PDUI_diff| values in the frontal lobe showed different APA patterns (Fig. 3B). Interestingly, only *PSMD14* showed a significant negative correlation between |PDUI_diff| and gene expression ($R=-0.92$, $P<0.01$), indicating the APA of *PSMD14* was associated with its expression (Fig. 3C). *GTF3C1*, *IFI44L*, and *PLXNB1* showed no significant correlations ($P>0.05$), implying their expression changes may not be driven by APA. We focused on *PSMD14* as a candidate gene regulated by APA and conducted further validation. IGV showed that proximal APA site usage was lower in the least aggressive pigs (FLL) than in the most aggressive pigs (FLM) in the frontal lobe, which resulted in the increased expression of a 198 bp 3'UTR region (Fig. 3D). Moreover, we designed specific primers targeting the extended 3'UTR. Gel electrophoresis confirmed significantly upregulated expression in the FLL group compared to the FLM group, consistent with DaPars and IGV results (Fig. 3E).

Reduced *PSMD14* expression promotes cellular apoptosis: Based on DaPars analysis, sequences of both the common 3'UTR and lengthened 3'UTR of *PSMD14* were retrieved from the NCBI database. Two *PSMD14* expression vectors were constructed: (1) A Common-type vector lacking the proximal APA site; (2) A Lengthened-type vector containing the proximal APA site (Fig. 4A, B). Sanger sequencing confirmed that Common-type and Lengthened-type pcDNA3.1 vectors contained the complete coding region of the *PSMD14* gene. The Lengthened-type vector included an additional 198 bp APA fragment (Figure 4C, D).

Immunofluorescence showed the presence of diverse cell markers within the isolated PNCs (Fig. 5A). To assess the impact of APA-mediated *PSMD14* regulation, we transfected two vectors into PNCs and PK15 cells. qPCR showed that the Lengthened type of vector significantly

reduced *PSMD14* expression (Fig. 5B). To investigate the gene function of *PSMD14*, we reanalyzed the publicly available dataset containing the *PSMD14* interference experiment (si-PSMD14) (Yang *et al.*, 2023). When *PSMD14* expression was reduced, DEGs were mainly enriched to MAPK signaling pathway, apoptosis pathway and FoxO signaling pathway (Fig. 5C). Interestingly, apoptosis-related genes were found to exhibit significant changes (Fig. 5D). qPCR confirmed that downregulation of *PSMD14* expression indeed significantly increased the expression of apoptotic marker genes (*Caspase3*, *Caspase7, P53*) (Fig. 5E). Flow cytometry results also showed that knockdown of *PSMD14* (Lengthened type) significantly increased the apoptosis rate (Fig. 5F-H). The TUNEL assay further supported these findings, showing that the lengthened type vector increased the number of apoptotic cells (Fig. 5I, J). These results suggest that APA-mediated changes in *PSMD14* expression affect apoptosis.

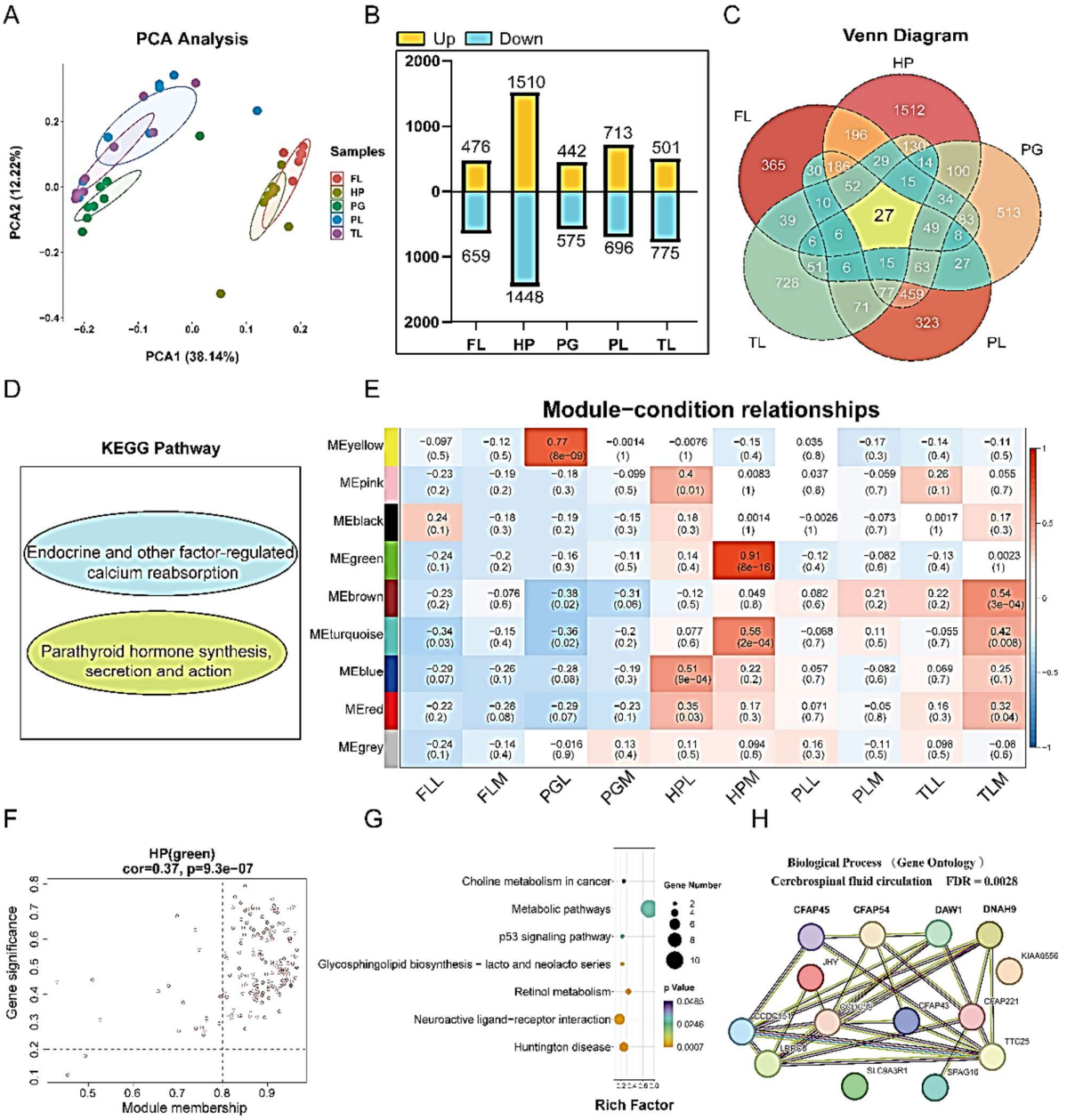

Fig. 1: Transcriptomic analysis of differentially expressed genes (DEGs) in multiple brain regions of pigs with varying aggression levels. **(**A) Principal Component Analysis (PCA) revealed distinct tissue-specific clustering patterns across brain regions. (B) Quantitative DEG analysis identified significant differences in gene expression across brain regions, categorizing DEGs into upregulated (UP) and downregulated (DOWN) groups. **(**C) Venn diagram analysis identified 27 DEGs consistently differentially expressed across all five brain regions. **(**D) KEGG pathway enrichment analysis of these 27 common DEGs highlighted key biological pathways associated with aggression. (E) Transcriptomic data were divided into ten groups based on aggression level (L: the least aggressive pigs; M: the most aggressive pigs) and brain region, showing associations between co-expressed gene modules and each group. (F) Scatter plot analysis revealed a significant correlation (cor=0.78, P<0.01) between the MEgreen module and the hypothalamus of the most aggressive pigs (HPM group). (G) KEGG pathway enrichment analysis of MEgreen module genes highlighted pathways related to neuroactive ligand-receptor interaction and Huntington disease. (H) Protein-Protein Interaction (PPI) analysis showed that these genes in MEgreen module are primarily involved in "Cerebrospinal fluid circulation" pathways.

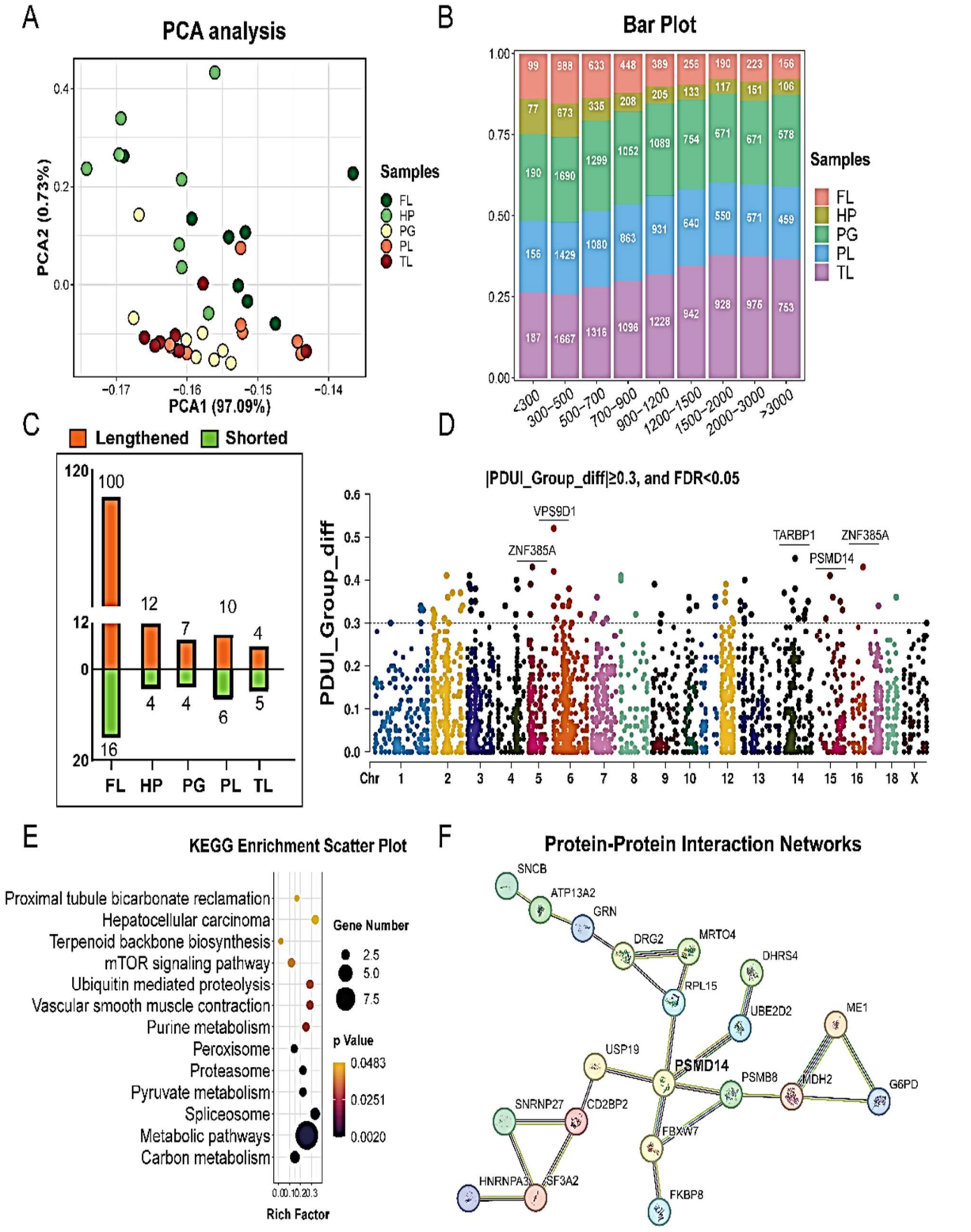

Fig. 2: Comprehensive analysis of alternative polyadenylation (APA) sites linked to aggressive behavior across multiple brain regions. (A) PCA plot demonstrating that APA site information retains tissue-specific genetic signatures, with distinct clustering observed among brain regions. (B) Bar chart depicting the distribution of APA site numbers and APA fragment lengths across different tissues. (C) Number of differential APA sites identified in each tissue using stringent selection criteria (|PDUI_Group_diff|≥0.3, FDR<0.05). (D) Manhattan plot illustrating genome-wide APA events, based on absolute differences in the PolyA Usage Difference Index (PUDI) between the frontal lobe of the least (FLL) and most aggressive pigs (FLM). The top five candidate genes, corresponding to significant APA sites, are highlighted. (E) KEGG pathway enrichment analysis of differential APA genes identified in the frontal lobe, revealing key biological pathways. (F) Protein-Protein Interaction (PPI) analysis of differential APA genes in the frontal lobe, highlighting PSMD14 as a central hub within the network.

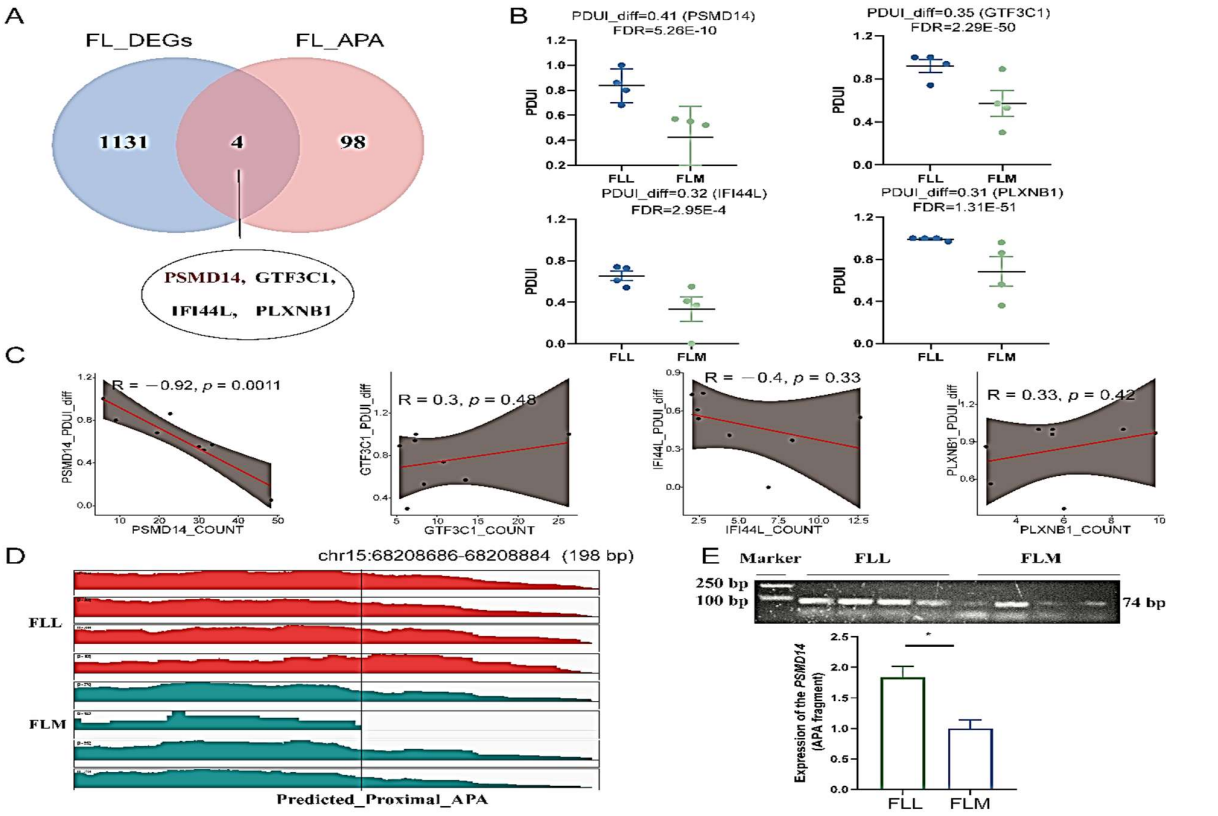

Fig. 3: Distinct APA patterns of *PSMD14* in pigs with different aggressive behaviors. (A) Venn diagram analysis identified four overlapping genes (*PSMD14*, *GTF3C1*, *IFI44L*, and *PLXNB1*) between DEGs and APA-associated genes in the frontal lobe. (B) Scatter plot showing differential PUDI values of these four genes between FLL and FLM. (C) Correlation analysis revealed a strong negative correlation (R=-0.92, P<0.05) between *PSMD14* expression levels and its PUDI_diff value, while no significant correlations were observed for the other genes. (D) Integrative Genomics Viewer (IGV) visualization confirmed differential APA site usage of *PSMD14* in pigs with varying aggression levels. (E) Gel electrophoresis validation showed significantly higher expression of the lengthened *PSMD14* 3'UTR fragment (74 bp) in FLL pigs compared to FLM pigs (P<0.05), aligning with DaPars and IGV predictions. Data are presented as mean±SEM. *P<0.05, **P<0.01.

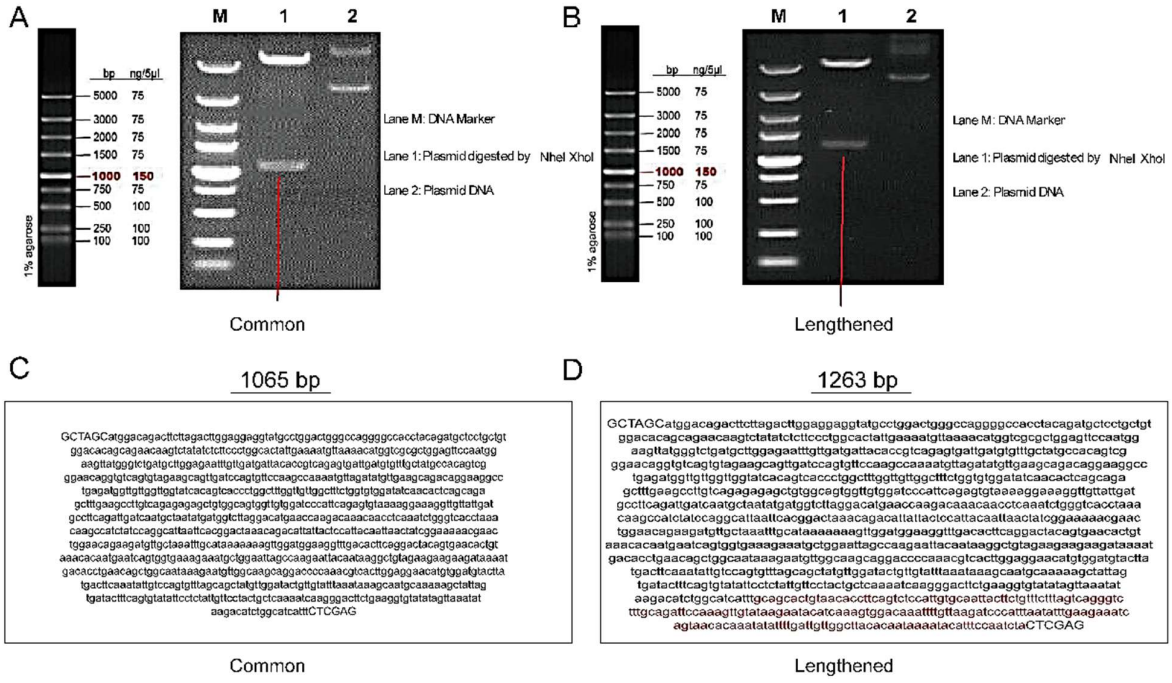

Fig. 4: Construction and validation of *PSMD14* gene vectors with distinct APA patterns. (A) and (B) Gel electrophoresis validation of the Common-type and Lengthened-type *PSMD14* gene vectors. Lane M: DNA marker; Lane 1: Digestion product after NheI and XhoI treatment, with the red line indicating the target fragment; Lane 2: Undigested pcDNA3.1 vector. (C) and (D) Sequencing results of the Common-type and Lengthened-type vectors, respectively. In (D), the red text highlights the 198 bp 3'UTR extension present in the Lengthened-type vector.

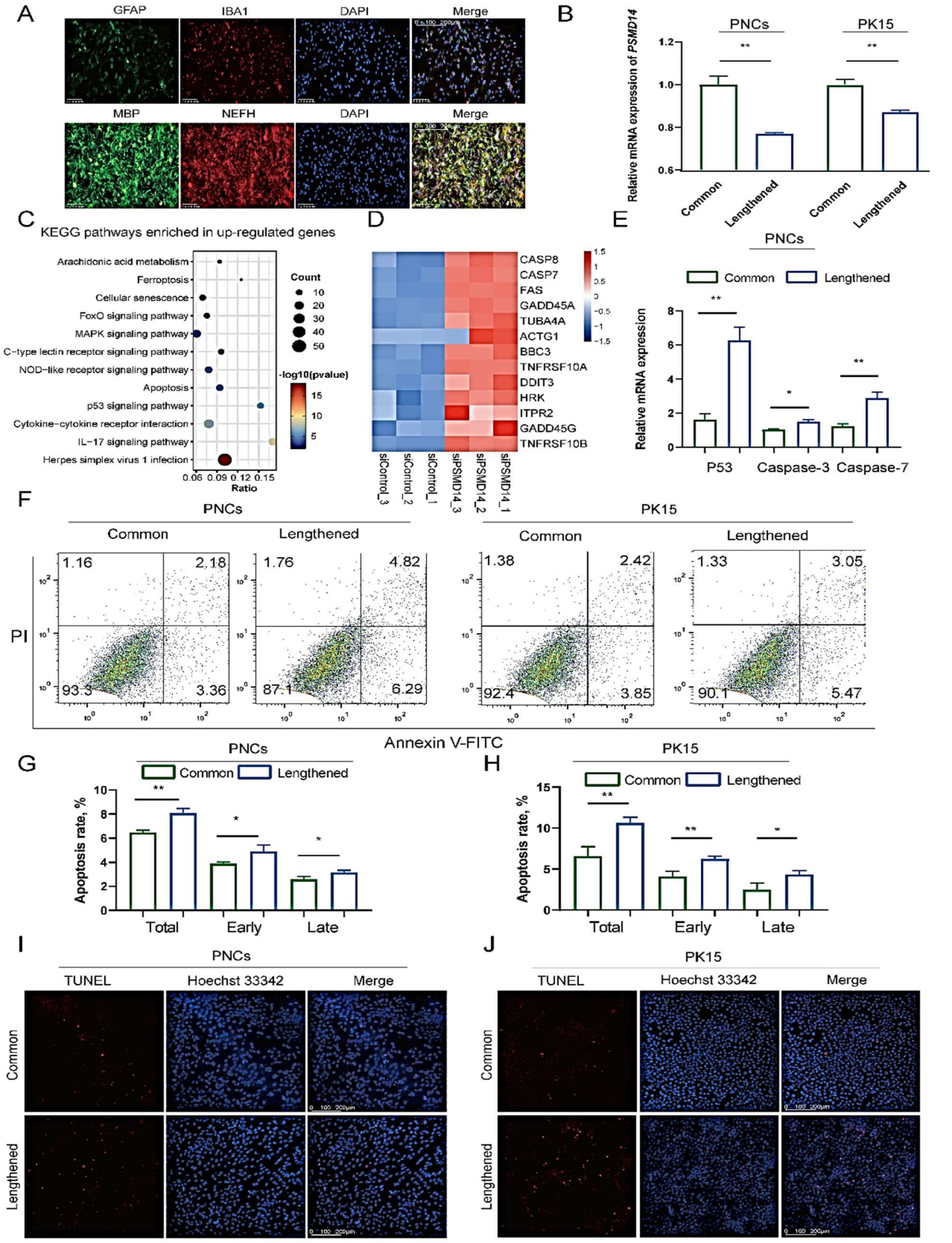

Fig. 5: Reduced *PSMD14* expression promotes cellular apoptosis. (A) Immunofluorescence analysis showing that porcine neural cells (PNCs) express multiple neuronal markers, confirming their identity. (B) qPCR analysis demonstrating that transfection with the Lengthened-type vector significantly reduces *PSMD14* expression compared to the Common-type vector. (C) Analysis of a public transcriptomic dataset (si-PSMD14 vs. si-NC) identified DEGs, with KEGG pathway enrichment analysis revealing that *PSMD14* knockdown leads to upregulation of genes involved in the FoxO signaling pathway, apoptosis pathway, and P53 signaling pathway. (D) Heatmap visualization showing widespread upregulation of apoptosis-related genes following *PSMD14* knockdown. (E) qPCR analysis confirming that transfection with the Lengthened-type vector significantly upregulates apoptosis-related genes (*P53*, *Caspase3*, *Caspase7*) compared to the Common-type vector. (F – H**)** Flow cytometry analysis demonstrating that apoptosis levels in PNCs and PK15 cells were significantly higher in cells transfected with the Lengthened-type vector compared to the Common-type vector. (I – J) TUNEL assay results showing that cells transfected with the Lengthened-type vector exhibited a higher proportion of apoptotic cells, indicated by red fluorescence. Data are presented as mean±SEM. *P<0.05, **P<0.01.

***PSMD14* expression downregulation through its interaction with miR-29-5a:** A role of the 3'UTR is its binding to miRNAs, which typically bind to the 3'UTR through their seed sequences, generally leading to the degradation of the mRNA (Griesemer *et al.*, 2021). Sequence analysis of the APA region revealed a binding site to the seed region of ssc-miR-29-5a (hereafter miR-29-5a) (Fig. 6A). To confirm this interaction, we performed dual-luciferase reporter assays, including miR-29-5a mimics and corresponding controls, a wild-type 198 bp APA region, and a mutant-type lacking the miR-29-5a binding site. miR-29-5a significantly reduced luciferase activity when bound to the Lengthened-type fragment, while other combinations had no effect, confirming a direct interaction between miR-29-5a and the Lengthened-type sequence (Fig. 6B).

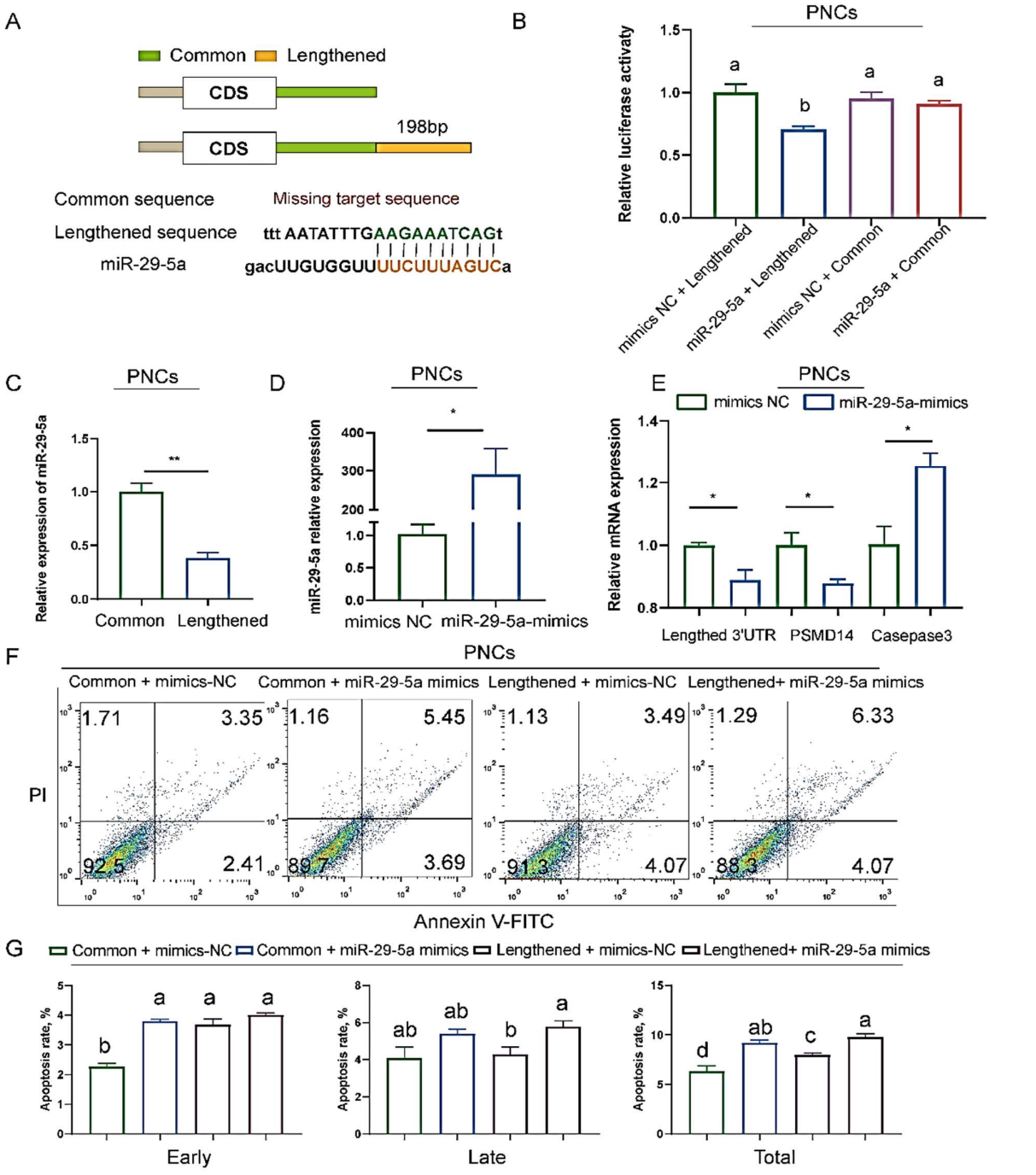

Fig. 6: APA patterns in PSMD14 regulate its expression via interaction with miR-29-5a. (A) miRanda prediction shows that miR-29-5a binds to the 3'UTR of the lengthened APA isoform of PSMD14, whereas the Common-type vector lacks this binding site. (B) Dual-luciferase reporter assay confirms that miR-29-5a directly interacts with the lengthened APA isoform of PSMD14, leading to a significant reduction in luciferase activity. (C) qPCR analysis reveals that overexpression of the Lengthened-type APA vector significantly reduces miR-29-5a expression compared to the Common-type vector. (D) qPCR validation shows that transfection with miR-29-5a mimics significantly increases miR-29-5a expression levels in cells. (E) Compared to mimics-NC, transfection with miR-29-5a mimics significantly reduces expression of the lengthened APA fragment and PSMD14, while significantly increasing Caspase3 expression. (F) and (G) Flow cytometry analysis shows increased apoptosis levels in cells co-transfected with APA vectors and miR-29-5a mimics or their controls. Data are presented as mean±SEM. *P<0.05, **P<0.01.

To validate these findings, we measured miR-29-5a expression in PNCs overexpressing either the Common-type or Lengthened-type vector. Cells overexpressing the Lengthened-type vector showed significantly reduced miR-29-5a levels (Fig. 6C). Further, transfection of miR-29-5a mimics into PNCs efficiently increased miR-29-5a levels, downregulated both the 198 bp Lengthened region and *PSMD14* expression, and upregulated the apoptosis-related gene *Caspase3* (Fig. 6D, E). Flow cytometry analysis of co-transfected cells revealed that: in Common-type vector-transfected cells, miR-29-5a mimics significantly increased early and total apoptosis rates compared to mimics-NC (Fig. 6F, G). In Lengthened-type vector-transfected cells, miR-29-5a mimics significantly increased late and total apoptosis rates. The lower total apoptosis rates in the Lengthened + miR-29-5a mimics group (compared to the Common + miR-29-5a mimics group) may result from degradation of the exogenous Lengthened-type vector upon miR-29-5a binding (Fig. 6F, G).

***CSTF2* regulates alternative polyadenylation of *PSMD14* via 3'UTR binding:** Cleavage Stimulation Factors (CSTFs) are critical regulators of eukaryotic mRNA 3'UTR polyadenylation. Expression of *CSTF1*, *CSTF2*, *CSTF3*, and *CSTF2T* was consistently higher in the most aggressive pigs, with *CSTF2* showing the most significant differential expression in the frontal lobe, pituitary gland, and temporal lobe (Fig. 7A). Using our

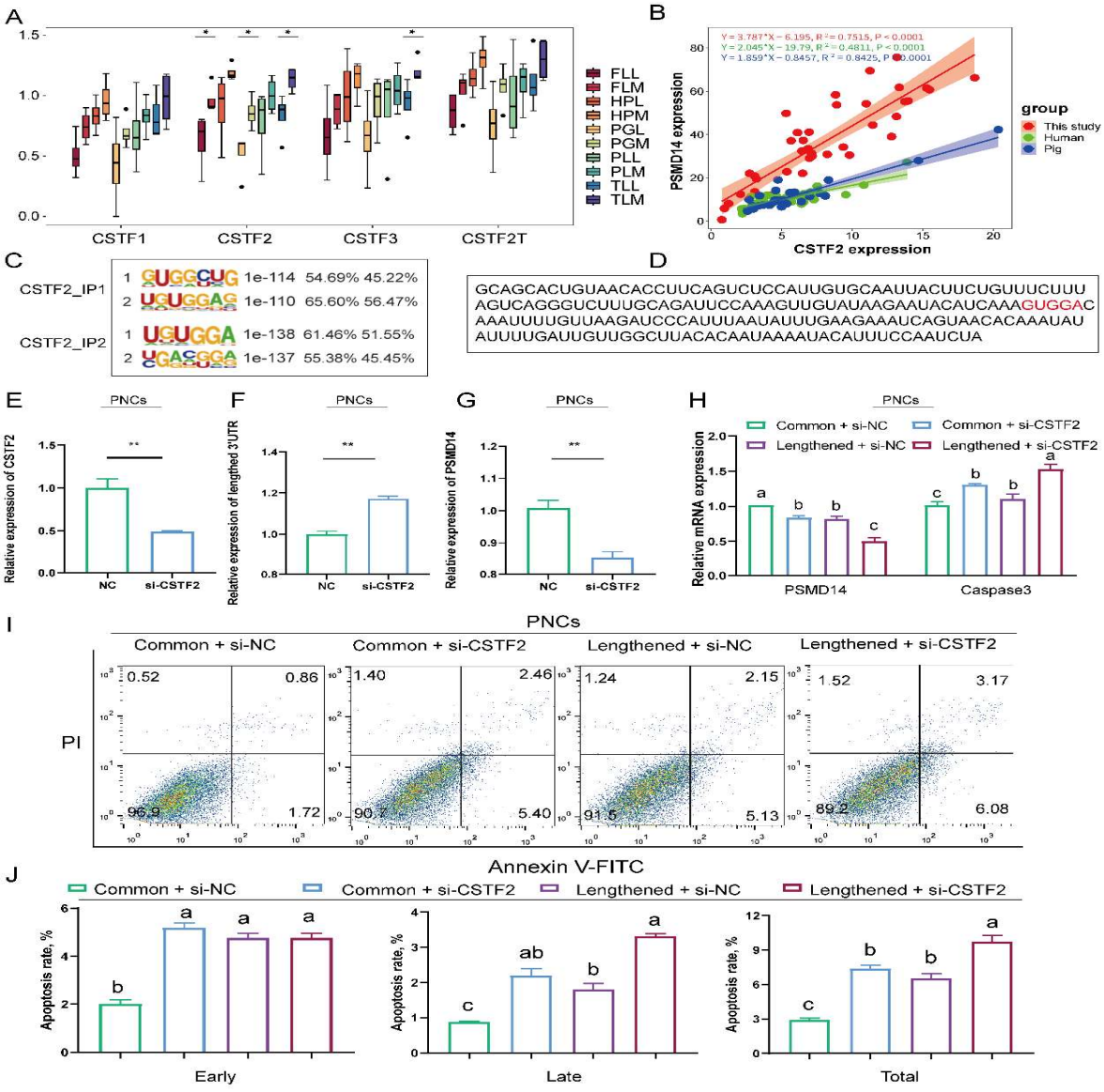

Fig. 7: CSTF2 regulates the APA pattern of PSMD14 by binding to Its 3'UTR. (A) Expression analysis of CSTF family genes in brain tissues of pigs with different aggression levels. CSTF2 expression was significantly higher in the frontal lobe, pituitary gland, and temporal lobe of the most aggressive pigs compared to the least aggressive pigs. (B) Correlation analysis in this study, as well as in two large-cohort public datasets (including human and pig studies), revealed a significant positive correlation between CSTF2 and PSMD14 expression. (C) Immunoprecipitation (IP) experiments demonstrated that CSTF2 binds to GU-rich sequences, identifying GUGGA as a high-affinity binding site. (D) Sequence analysis showed that the lengthened APA fragment of PSMD14 contains the high-affinity CSTF2 binding site (GUGGA, highlighted in red). (E) qPCR validation confirmed that si-CSTF2 significantly reduced CSTF2 expression. (F) CSTF2 knockdown resulted in a significant increase in the lengthened APA fragment of PSMD14. (G) CSTF2 knockdown significantly decreased PSMD14 expression, indicating its role in APA-mediated regulation. (H) qPCR analysis of PSMD14 and Caspase3 expression in cells co-transfected with APA vectors, si-CSTF2, and its control (Common + si-NC, Common + si-CSTF2, Lengthened + si-NC, Lengthened + si-CSTF2). (I) and (J) Flow cytometry analysis revealed that si-CSTF2 significantly increased apoptosis levels in co-transfected cells. Data are presented as mean±SEM. *P<0.05, **P<0.01.

experimental data and the public multi-tissue transcriptomic datasets, correlation analysis revealed a significant positive correlation between *CSTF2* and *PSMD14* expression across all datasets (Fig. 7B) (Zhenyang Zhang *et al.*, 2025). *CSTF2* functions as an RNA-binding protein, with immunoprecipitation (IP) experiments identifying GUGGA as its binding motif (Fig. 7C)(Xu *et al.*, 2023; Yang *et al.*, 2023). The presence of this motif in lengthened APA fragments suggests that *CSTF2* may regulate APA patterns via direct binding (Fig. 7D). To determine whether *CSTF2* can regulate APA patterns of *PSMD14*, we designed siRNA targeting *CSTF2* and verified the knockdown efficiency (Fig. 7E). Interestingly, decreased *CSTF2* expression was accompanied by a marked increase in the expression of the Lengthened APA fragment and a concomitant downregulation of the *PSMD14* (Fig. 7F, G). When co-transfected with the two APA pattern plasmids and si-CSTF2, we found that the combination of the Lengthened vector and si-CSTF2 significantly decreased the expression of *PSMD14* and increased the expression of apoptosis-related gene *Caspase3* (Fig. 7H). Flow cytometry analysis showed that the apoptosis rate was significantly increased after the down-regulation of *CSTF2* expression (Fig. 7I, J).

DISCUSSION

Aggressive behavior in pigs is a complex trait regulated by multiple factors. However, there are few studies on the molecular genetic basis of porcine aggressive behavior. In this study, we selected pigs with differential aggressive behavior for transcriptome sequencing across multiple brain regions. Integrated analysis of APA and DEGs based on transcriptome sequencing identified *PSMD14* as a potential aggressive behavior-related gene and regulated by APA. The fact that the APA pattern and expression changes of *PSMD14* gene did not occur in all brain regions highlights the advantage of our multi-tissue transcriptome sequencing. It also underscores the coordination of multiple brain regions and the central role of the hypothalamus in the regulation of porcine aggressive behavior. Previous studies mainly focused on specific brain regions (such as HP or FL) to explore the regulatory roles in aggressive behavior (Flanigan *et al.*, 2020; Eusebi *et al.*, 2021). Clearly, our multi-brain region method provides a comprehensive perspective for an understanding of the neural circuitry regulation of aggressive behavior.

In this study, we found that the brain tissue of pigs with the most aggressive behavior expressed higher *CSTF2*, which is an important regulator in regulating APA mechanisms. *CSTF2* is known to bind to GU-rich sequences of pre-mRNA, thereby shortening the 3'UTR length, consistent with previous studies (Masoumzadeh *et al.*, 2022). *CSTF2* was able to bind to specific sites of *PSMD14* resulting in a change in its APA pattern, as shown the shortened 3'UTR isoforms of *PSMD14* in the most aggressive behavior pigs.

Studies have shown that shorter 3'UTRs generally increase the stability and translation of mRNA (Griesemer *et al.*, 2021). In addition to the stability regulation mediated by length and structure, a more important regulation mode of 3'UTRs is the binding of miRNAs. This binding is an important step in post-transcriptional regulation to achieve spatiotemporal expression specificity. The 3'UTR of *PSMD14* contains a miR-29-5a binding site, and binding to miR-29-5a reduces *PSMD14* expression, which may be associated with altered aggressive behavior in pigs. *PSMD14* acts as a deubiquitinase to modulate protein stability and other signaling pathways, though its specific roles in neural circuits and aggression remain to be fully explored. In our study, APA fragments were mainly between 300 and 500 bp in length, highlighting the range of APA regulation of pig behavior. APA was also identified as a candidate genetic marker for key pig traits, supporting its potential in genomic breeding (Meng *et al.*, 2021; Zhao *et al.*, 2023; Han *et al.*, 2024; Liu *et al.*, 2024; Sun *et al.*, 2024).

APA is a heritable and tissue-specific regulatory mechanism, which in itself provides potential for genomic selection, and becomes a genetic marker for breeding key economic traits in pigs. However, there are still some technical and cost constraints. Most APA studies are based on RNA-seq data, which can simultaneously obtain the expression data of transcripts and APA information. However, this method is less efficient in capturing the 3' end of the transcript and may cause a certain false positive rate. Using targeted sequencing at the 3' end would provide more accurate results, but some critical genetic information would be lost. In addition, there are few bioinformatics tools for detecting APA, and their accuracy still needs improvement. This limits how reliably and consistently APA can be measured across different studies. To address these limitations, we combined transcriptomic DEG analysis with APA profiling to improve bioinformatics accuracy and used molecular validation to identify candidate APA events.

Overall, aggressive behavior in pigs involves coordinated regulation of multiple brain regions. For the first time, we performed transcriptome sequencing of multiple brain regions in pigs with different aggressive behaviors, and integrated DEGs and APA analysis to discover candidate markers regulating aggressive behaviors in pigs. Our findings show that elevated *CSTF2* expression in the most aggressive pigs promotes *PSMD14* 3'UTR shortening, blocks miR-29-5a binding, and increases *PSMD14* expression. These findings suggest *CSTF2* and *PSMD14* APA isoforms as candidate genetic markers for aggression in pigs, though further work is needed to clarify *PSMD14*'s downstream proteins and the functions of the APA-related genes identified in this study.

Conclusions: This study integrated DEGs and APA analyses from transcriptomic data to investigate candidate markers of aggression in pigs. We found that *CSTF2* exhibited higher expression in the most aggressive pigs than in the least aggressive pigs. *CSTF2* bound to the *PSMD14* pre-mRNA to promote 3'UTR shortening in the most aggressive pigs. The longer 3'UTR isoforms enhanced miR-29-5a binding and reduced *PSMD14* expression in the least aggressive pigs. Downregulation of *PSMD14* promoted apoptosis in porcine primary neurons, suggesting a potential link between APA and aggressive behavior. Our findings suggest that APA regulates aggressive behavior in pigs via the *CSTF2-PSMD14*-miR-29-5a axis. These findings suggest that APA could represent a regulatory factor in porcine aggressive behavior, and the APA pattern

of *PSMD14* may serve as a potential marker for aggression in pigs.

Conflicts of interest: The authors declare that they have no competing interests.

Ethical statement: All procedures were approved by the Nanjing Agricultural University Institutional Animal Care and Use Committee (NJAULLSC2021030, approval date: 2021-03-02).

Authors contribution: BZ and CZ conceived and designed the study. CZ, ML, XC, QX and HY collected the sample and data. CZ, JC, SL, YD, ZW and HB visualized the results. CZ, CX, WG, JF, Muhammad A, and Muhammad M performed molecular biology experiments. CZ wrote the original draft. BZ and Allan P. S reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Data availability: The data that support the study findings are available from the authors upon request.

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