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***Basic Study***

***ALKBH5* suppresses autophagic flux *via* N6-methyladenosine demethylation of *ZKSCAN3* mRNA in acute pancreatitis**

Zhang T *et al*. *ALKBH5* suppresses autophagy in AP

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**Author contributions:** Zhu S and Huang GW conceived, designed and refined the study protocol; Zhang T finished the experiments, collected and analyzed the data and drafted the manuscript; Zhu S and Huang GW reviewed and revised the manuscript. All authors had access to the study data and reviewed and approved the final manuscript. Zhu S and Huang GW contributed equally to this work as co-corresponding authors. The reasons for designating Zhu S and Huang GW as co-corresponding authors are twofold. First, co-corresponding authors jointly conceived the overall design of the study and revised the manuscript. Second, they jointly provided financial support for the study. In summary, we believe that designating Zhu S and Huang GW as co-corresponding authors accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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**Abstract**

BACKGROUND

Increasing evidence has demonstrated that N6-methyladenosine (m6A) RNA modification plays an essential role in a wide range of pathological conditions. Impaired autophagy is a critical hallmark of acute pancreatitis (AP).

AIM

To explore the role of the m6A modification of *ZKSCAN3* in the regulation of autophagy in AP.

METHODS

The AP mouse cell model was established by cerulein-treated mouse pancreatic acinar cells (MPC-83), and the results were confirmed by the levels of amylase and inflammatory factors. Autophagy activity was evaluated by specific identification of the autophagy-related microstructure and the expression of autophagy-related genes. *ZKSCAN3* and *ALKBH5* were knocked down to study the function in AP. A m6A RNA binding protein immunoprecipitation assay was used to study how the m6A modification of *ZKSCAN3* mRNA is regulated by *ALKBH*.

RESULTS

The increased expression of amylase and inflammatory factors in the supernatant and the accumulation of autophagic vacuoles verified that the AP mouse cell model was established. The downregulation of *LAMP2* and upregulation of *LC3-II/I* and *SQSTM1* demonstrated that autophagy was impaired in AP. The expression of *ZKSCAN3* was upregulated in AP. Inhibition of *ZKSCAN3* increased the expression of *LAMP2* and decreased the expression of the inflammatory factors, *LC3-II/I* and *SQSTM1*. Furthermore, *ALKBH5* was upregulated in AP. Knockdown of *ALKBH5* downregulated *ZKSCAN3* expression and restored decreased autophagic flux in AP. Notably, the bioinformatic analysis revealed 23 potential m6A modification sites on *ZKSCAN3* mRNA. The m6A modification of *ZKSCAN3* mRNA was significantly decreased in AP. Knockdown of *ALKBH5* increased the modification of *ZKSCAN3* mRNA, which confirmed that *ALKBH5* upregulated *ZKSCAN3* expression in a m6A-dependent manner.

CONCLUSION

*ALKBH5* inhibits autophagic flux through m6A demethylation of *ZKSCAN3* mRNA in AP, thereby aggravating the severity of the disease.

**Key Words:** Acute pancreatitis; Autophagy; *ZKSCAN3*; N6-methyladenosine; *ALKBH5*

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**Core Tip:** Acute pancreatitis (AP) is a common emergency in digestive system. Impaired autophagy is one of important pathogenic mechanisms of AP, however, its regulatory mechanism remains unclear. N6-methyladenosine modification and *ZKSCAN3* are crucial regulatory factors of autophagy, but their roles in AP are not well-defined. This study confirmed that the demethylase *ALKBH5* can inhibit autophagy flux by upregulating *ZKSCAN3*, thereby exacerbating the inflammatory severity of AP. The findings of this study provided new insights into the autophagy regulation mechanism and offered a novel direction for early intervention in AP.

**INTRODUCTION**

Acute pancreatitis (AP) is one of the most common digestive emergencies. The global prevalence and incidence of AP are approximately 76/100000 and 34/100000, respectively, and the number of new cases is increasing at an annual rate of 3%[1-3]. With the progression of therapeutic concepts and interventions, the prognosis of AP has significantly improved. However, due to the unclear pathogenesis of AP, clinicians are still unable to effectively intervene specifically in local or systemic inflammation. The pathogenesis of AP is complex and multifactorial and induces significant and sustained pathological disruption[4,5]. Therefore, further study of the mechanism underlying the progression of AP will provide insight into the development of future therapeutic strategies.

Autophagy is a highly conserved catabolic process in which abnormal biomolecules and organelles are degraded and degradation products are recycled. Autophagy plays an important role in maintaining cellular homeostasis. The entire autophagy process is defined as autophagic flux, and disrupted integrity of the process is called impaired autophagy[6]. Studies have shown that impaired autophagy plays an important role in the development of various diseases, such as neurodegeneration, inflammation, infection, tumors, and metabolic disorders[7-9]. In recent years, the important role of autophagy in AP has been gradually recognized. The basal level of autophagy in the mouse exocrine pancreas is significantly greater than that in the endocrine pancreas and other organs[10]. In experimental pancreatitis, interfering with the expression of upstream regulatory molecules or autophagy-related genes can induce inflammatory changes in exocrine pancreatic cells[11]. Impaired autophagy in AP manifests as activation of the initial stage but blockade of the degradation stage, resulting in harmful factors such as abnormal zymogen granules and disrupted organelles that cannot be effectively degraded[10]. Although impaired autophagy can mediate abnormal zymogen activation, inflammation, and cell death in pancreatic acinar cells[12], the specific regulatory mechanism involved is still unclear.

*ZKSCAN3* is a zinc finger DNA-binding protein that simultaneously contains KRAB and SCAN domains; it is also a recognized inhibitory factor of autophagy[13,14]. Studies have shown that *ZKSCAN3* can inhibit the transcription of numerous autophagy-related genes, such as *LC3* and *WIPI2*, thereby suppressing a series of autophagy steps in various diseases[15,16]. However, the role of *ZKSCAN3* in autophagy in AP has not yet been determined.

The N6-methyladenosine (m6A) modification of RNA plays an important role in the autophagy regulatory network. This process is reversible and involves mainly methyltransferases, demethylases, and methylated RNA-binding proteins[17,18]. ALKBH5 is a crucial demethylase that plays a key role in various diseases[19,20]. In ovarian cancer, the overexpression of ALKBH5 promotes the formation of the BCL-2-Beclin1 complex, and inhibits autophagy[21]. In silica-related pneumonia, *ALKBH5* can mediate autophagic flux blockade through the Slam7 pathway[22]. However, in myocardial ischemia-reperfusion injury, *ALKBH5* plays a role in promoting autophagic flux[23]. Although the role of m6A modification in impairing autophagy has been demonstrated in various tumors and inflammatory diseases, there is no experimental research on m6A modification in AP. Recent bioinformatics studies have shown that decreased m6A levels are related to the occurrence of severe AP[24], but whether this change is related to *ALKBH5*-mediated impaired autophagy in AP is unclear.

Clarifying the regulatory mechanism of autophagy in AP is crucial for early intervention. However, research on the autophagy and its regulatory mechanism in AP has not been illustrated. Therefore, in this article we aimed to explore the role and mechanism of action of *ALKBH5* in *ZKSCAN3* regulated autophagy. We verified the results at the cellular level through a series of molecular biology experiments, which provided a novel perspective on the research of pathogenesis and molecular mechanism of AP and highlighted new targets for therapeutic intervention.

**MATERIALS AND METHODS**

***Cell culture***

Mouse pancreatic acinar cells (MPC-83) were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in a 37 °C incubator with 5% CO2. The control groups were not treated, and the AP groups were pretreated with cerulein (10 nM) for 24 h.

***Cell transfection***

MPC-83 cells were seeded in 6-well plates and maintained at 37 °C and 5% CO2. *ALKBH5* and *ZKSCAN3*-siRNA (50 nM) (RiboBio, Gunagzhou, China) were transfected into MPC-83 cells. After 48 h of transfection, the cells were treated with cerulein (10 nM) for 24 h.

***Quantitative real-time RT-PCR***

Total RNA was extracted from cells using the TRIzol method. All mRNAs were reverse transcribed using the PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa, Kyoto, Japan). Reverse transcription and quantitative real-time RT-PCR were performed with SYBR® Premix Ex Taq™ (TaKaRa, Kyoto, Japan). The results were normalized to that of β-actin and calculated via the relative quantification (2-ΔΔCt) method. The primers used were purchased from Sangon Company (Table 1).

***ELISA***

The supernatant of MPC-83 cells was collected. The levels of interleukin 6 (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α were assessed using ELISA kits (Neobioscience, Shenzhen, China).

***Western blot***

Cell lysates were prepared using lysis buffer composed of 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% NP-40. The lysates were centrifuged to collect the supernatants. An equal amount of protein was denatured in SDS sample buffer and separated on 8% or 10% polyacrylamide gels based on the molecular weight of the target proteins. The separated proteins were then transferred to a PVDF membrane. The membranes were blocked with 5% nonfat milk in TBST (TBS containing 0.05% Tween 20), incubated with primary antibodies, and subsequently incubated with secondary antibodies conjugated to alkaline phosphatase. Protein expression was detected by chemiluminescence. The antibodies used were against *ALKBH5* (ab195377, Abcam, Britain), *ZKSCAN3* (ab223477, Abcam, Britain), *LC3* (Proteintech, Wuhan, China), *LAMP-2* (Proteintech, Wuhan, China) and *SQSTM1* (Proteintech, Wuhan, China).

***Immunofluorescence***

After cell fixation, the cells were treated with 0.2% Triton X-100 at room temperature. The cells were then blocked with blocking solution. Subsequently, the cells were treated with primary and secondary antibodies. DAPI dye was added to the cells, which were subsequently incubated in the dark. The cells were mounted on slides using anti-fade mounting medium, and fluorescence was observed using a fluorescence microscope. The antibodies used were against *LC3* (Proteintech, Wuhan, China) and *LAMP-2* (Proteintech, Wuhan, China).

***Transmission electron microscopy***

The specimens were cut and fixed in a 2.5% glutaraldehyde solution with Millonig's phosphate buffer (pH = 7.3). The samples were washed three times with Millonig's phosphate buffer at 10-minute intervals. The dehydration process was performed at room temperature using a graded series of acetone (50%, 70%, and 90%) at 10-min intervals, followed by two washes with 100% acetone at 15-min intervals. The samples were then soaked and embedded in a mixture of acetone and resin (1:1) for 12 h, followed by polymerization overnight at 37 °C using 100% resin. To solidify the sample resin, the specimens were further polymerized at 37 °C overnight, followed by an additional 12 h at 60 °C. Ultrathin sections of 50-100 nm were obtained from the specimens using an ultramicrotome and a diamond knife. The sections were then stained with 3% uranyl acetate and lead nitrate, after which they were examined and photographed using a Hitachi HT-7700 electron microscope.

***M6A RNA binding protein immunoprecipitation assay***

The M6A RNA binding protein immunoprecipitation kit was purchased from RiboBio. RNA was fragmented using RNA fragmentation buffer. Magnetic beads for m6A were prepared using magnetic beads A/G and an anti-m6A antibody. RNA immunoprecipitation was conducted by mixing the fragmented RNA with anti-m6A magnetic beads. The RNA was washed with elution buffer to remove it from the magnetic beads.

***Website for m6A site prediction***

Potential m6A binding sites on *ZKSCAN3* mRNA were analyzed via a website (<http://www.cuilab.cn/sramp/>).

***Statistical analysis***

All the statistical analyses were performed in GraphPad Prism 8. Independent sample *t* tests were used to compare the means of two samples, while one-way ANOVA was used for analyzing and comparing the means of more than two groups of samples. *P* values < 0.05 were considered to indicate statistical significance. The experimental results are presented as the mean ± SD.

**RESULTS**

***Impaired autophagy in the AP mouse cell model***

To construct a cell model of AP, MPC-83 cells were treated with 10 nM cerulein for 24 h. The levels of amylase and the inflammatory factors IL-1β, IL-6, and TNF-α in the supernatant were measured *via* ELISA. The results showed that the levels of amylase and inflammatory factors were significantly greater in the cerulein-treated group (Figure 1A and B), indicating that the AP cell model was successfully established.

The expression levels of autophagy-related marker proteins were detected by western blotting, which showed that the ratio of *LC3B-II/I* was increased in the AP group, indicating an increase in autophagosomes. The expression of *LAMP-2* was decreased in the AP groups, indicating impaired lysosomal synthesis. The expression of the selective autophagy receptor *SQSTM1* was increased, indicating inhibited substrate degradation (Figure 1C). Transmission electron microscopy (TEM) revealed the accumulation of circular autophagic vacuoles in the AP group, indicating impaired degradation and accumulation of autophagosomes and autolysosomes (Figure 1D). Furthermore, immunofluorescence staining revealed that *LC3* was significantly increased in the AP groups (Figure 1E), while *LAMP-2* expression was decreased (Figure 1F). These results demonstrated that autophagosome formation is activated, while lysosomal synthesis and function are impaired, leading to decreased substrate degradation efficiency and accumulation of autophagic vacuoles, suggesting impaired autophagy.

***ZKSCAN3 is upregulated and promotes the release of inflammatory factors in AP***

To investigate the role of *ZKSCAN3* in AP, qPCR, and western blot were used to detect the expression levels of *ZKSCAN3*. The results showed that the mRNA and protein expression levels of *ZKSCAN3* were significantly increased in the AP group (Figure 2A and B). Three different siRNAs were used to knock down the expression of *ZKSCAN3*, and siRNA-2 had the most significant interference effect (Figure 2C and D). Subsequent experiments were performed using siRNA-2 to knock down *ZKSCAN3*. After the inhibition of *ZKSCAN3*, the cells were treated with cerulein to construct the AP cell model. The levels of inflammatory factors IL-1β, IL-6, and TNF-α in the knocking down (KD) group were significantly lower than those in the negative control (NC) group (Figure 2E). These results suggest that *ZKSCAN3* is upregulated and promotes the release of inflammatory factors in the AP mouse cell model.

***ZKSCAN3 impaired autophagic flux in AP***

To investigate the role of *ZKSCAN3* in autophagic flux in AP, western blot was used to detect the expression of autophagy marker proteins (Figure 3A). The *LC3B-II/I* ratio was decreased in the KD group, demonstrating the increased clearance of autophagolysosomes. The expression of *LAMP-2* increased, suggesting a reduction in lysosomal biogenesis impairment, and the expression of *SQSTM1* decreased, indicating an improvement in substrate degradation efficiency. TEM revealed a significant reduction in autophagosome accumulation in the KD group (Figure 3B). Immunofluorescence staining revealed decreased expression of *LC3* in the KD group (Figure 3C) and increased expression of LAMP-2 (Figure 3D). These results suggest that *ZKSCAN3* inhibits autophagic flux in AP and that KD *ZKSCAN3* expression can impair the blockade of autophagic flux.

***ALKBH5 is upregulated in AP and promotes the release of inflammatory factors***

M6A methylation is widely involved in autophagy and contributes to the pathogenesis of human disease. The expression and function of *ALKBH5*, a primary m6A demethylase in AP, have not yet been determined. We detected *ALKBH5* expression in the AP mouse cell model by qPCR and western blot analysis. The level of *ALKBH5* was upregulated in the AP mouse cell model (Figure 4A and B). Furthermore, three different siRNAs were used to knock down *ALKBH5* expression in MPC-83 cells, and siRNA-3 had the most effective interference effect (Figure 4C and D); therefore, siRNA-3 was used for subsequent experiments. The expression of the inflammatory factors IL-1β, IL-6, and TNF-α was significantly reduced (Figure 4E). These results suggest that *ALKBH5* was upregulated in the mouse AP cell model and promoted the release of inflammatory factors.

***ALKBH5 inhibited autophagic flux by promoting ZKSCAN3 expression***

In the AP mouse cell model, knockdown of *ALKBH5* downregulated the mRNA and protein expression of *ZKSCAN3* (Figure 5A), indicating that *ALKBH5* promotes *ZKSCAN3* expression. Furthermore, the expression of *LC3B-II/I* and *SQSTM1* decreased, while *LAMP-2* expression was increased (Figure 5B), indicating that the knockdown of *ALKBH5* rescued the blockade of autophagic flux in AP. TEM confirmed that autophagic vacuole accumulation was reduced after the expression of *ALKBH5* was inhibited (Figure 5C). Immunofluorescence revealed that the immunoreactivity of the *LC3* protein decreased (Figure 5D), while the immunoreactivity of the *LAMP-2* protein increased in the AP mouse cell model transfected with the *ALKBH5* target siRNA (Figure 5E). These results suggested that *ALKBH5* promoted *ZKSCAN3* expression, resulting in the blockade of autophagic flux in AP.

***ALKBH5 regulated ZKSCAN3 expression in a m6A-dependent manner***

Considering that *ALKBH5* is a well-known m6A demethylase, we further investigated the role of m6A modification in the regulation of *ZKSCAN3* by *ALKBH5*. Biological software analysis revealed 23 potential m6A binding sites on *ZKSCAN3* mRNA, including 6 highly credible sites, 7 highly credible sites, 6 moderately credible sites, and 4 sites with low credibility (Table 2, Figure 6A). Additionally, we constructed a secondary structure diagram of highly credible m6A binding sites (Figure 6B).

To confirm the role of m6A modification in the relationship between *ALKBH5* and *ZKSCAN3*, MeRIP-qPCR was performed with specific primers aimed at identifying potential m6A sites, and the enrichment of m6A-modified *ZKSCAN3* mRNA in the AP group was significantly lower (Figure 7). This finding suggested that *ALKBH5* can decrease the m6A modification of *ZKSCAN3*.

**DISCUSSION**

This study is the first to reveal the regulatory roles of *ZKSCAN3* and m6A modification in impairing autophagy in AP. We found that *ALKBH5* upregulated *ZKSCAN3* expression by demethylating *ZKSCAN3* inhibited autophagy, and promoted the release of inflammatory factors in a mouse cell model of AP.

Impaired autophagy is one of the key pathogenic mechanisms in AP; this process affects the functions of various organelles, such as mitochondria and the endoplasmic reticulum, and disrupts the homeostasis of acinar cells[12,25]. Usually, autophagy degrades dysfunctional mitochondria during AP. Inhibition of autophagic flux by knocking out the *ATG5* and *ATG7* genes impaired the clearance of damaged mitochondria, further affecting generation the efficiency of ATP generation in acinar cells[26,27]. Moreover, autophagy maintains the stability of endoplasmic reticulum function. Knocking out the IκB kinase α gene leads to impaired autophagy, and the accumulated *SQSTM1* further causes the accumulation of misfolded proteins in the endoplasmic reticulum, triggering endoplasmic reticulum stress and ultimately inducing AP[28]. Therefore, impaired autophagy may trigger or exacerbate other cellular pathological factors in AP. Furthermore, other pathological factors can also induce impaired autophagy. In arginine-treated mice, abnormal mitochondrial membrane leads to disrupted energy metabolism, which inhibits autophagic flux[29]. In ethanol-induced AP, endoplasmic reticulum stress causes folding and transport disorders of autophagy-related proteins[30]. Therefore, autophagy is interconnected with other pathological events during AP. Early autophagy-related intervention may help alleviate the malignant cycle caused by pathological factors.

*ZKSCAN3* is currently recognized as a key autophagy inhibitor[15,31]. It affects the progression of various diseases by inhibiting autophagic flux. In hepatocellular carcinoma (HCC), *ZKSCAN3* inhibits autophagy, leading to decreased degradation of local adhesion proteins and reducing the metastasis of HCC[32]. In addition, impaired autophagy mediated by *ZKSCAN3* is closely related to sepsis-induced immunosuppression[33]. However, the role of *ZKSCAN3* in the pathogenesis of AP is still uncertain. Our study first confirmed the high expression of *ZKSCAN3* in cerulein-treated MPC-83 cells and the inhibitory effect on autophagy.

*ZKSCAN3* functions mainly through nucleoplasmic translocation; when activated, it moves into the nucleus to suppress the transcription of autophagy related genes[15]. However, the upstream regulatory mechanism of *ZKSCAN3* is unclear. A study revealed that SIRT1 deacetylates the lysine residues of *ZKSCAN3* and promotes its shuttling between the nucleus and cytoplasm[34]. PKC and BRAF inhibitors can activate the inhibition of *ZKSCAN3* *via* phosphorylation[35,36]. Although a few studies have revealed the upstream molecular mechanisms of *ZKSCAN3*, there is no related research focusing on this gene in AP.

As an important component of epigenetics, m6A modification plays an important regulatory role in autophagy. METTL3 promotes the binding of the RNA-binding protein HNRNPD to the precursor mRNA of TFEB, thus inhibiting autophagy[23]. In HCC, loss of METTL3 increases the stability of the FOXO3 mRNA 3'-UTR modification through a YTHDF1-dependent mechanism and activates autophagy[37]. Moreover, in testicular stromal cells, human chorionic gonadotropin activates autophagy flow by upregulating the expression of *ALKBH5* and inhibiting the translation of the m6A-mediated protein PPM1A, thereby increasing testosterone secretion[38]. Therefore, m6A modification is widely involved in the regulation of autophagy in physiological and pathological processes. Among different types of diseases, the same type of m6A modification has different effects on autophagy, which is related to downstream molecular targets and the pathological and physiological stages of disease[39]. Currently, the regulatory role of m6A modification in autophagy has been well documented in various disorders[40], but its role in AP has rarely been studied. Bioinformatics study has shown that m6A-modified noncoding RNAs may participate in the pathological changes observed in AP, but there is still a lack of relevant experimental evidence[24]. This research is the first to demonstrate that *ALKBH5* upregulates the expression of *ZKSCAN3* by demethylation, thereby inhibiting autophagy in AP.

This study has several limitations. First, the experimental subjects were cell models, and further *in vivo* animal experiments need to be conducted. In addition, there are significant differences in homology between animal and human tissues. However, due to the lack of human pancreatic exocrine cell lines and a stable extraction method, experimental research on AP cannot be performed in depth in human tissue[41,42]. In clinical practice, identifying pancreatic, peripancreatic or infected necrotic tissues is difficult due to pancreatic juice corrosion or infection. Therefore, human pancreatic exocrine cell lines and tissues are essential for mechanistic research on AP in the future.

**CONCLUSION**

In summary, we first revealed the important roles of *ZKSCAN3* and m6A modification in AP. In cerulein-treated MPC-83 cells, *ALKBH5* upregulates *ZKSCAN3* expression by demethylation, thereby inhibiting autophagic flux and aggravating the severity of AP. The results obtained in this study provide important insights into the mechanism of autophagy regulation in AP and offer reference value for future in-depth exploration and early intervention.

**ARTICLE HIGHLIGHTS**

***Research background***

The incidence of acute pancreatitis (AP) is increasing annually, and its mortality rate is high. Impaired autophagy is a key factor in the occurrence and development of AP. Therefore, it is crucial to clarify the regulatory mechanism of autophagy in AP.

***Research motivation***

Evidence has shown that *ALKBH5* and *ZKSCAN3* can regulate autophagy in a variety of diseases, but there are no relevant studies on AP.

***Research objectives***

We aimed to explore the regulatory functions and mechanisms of autophagy mediated by *ALKBH5* and *ZKSCAN3* in AP.

***Research methods***

The AP mouse cell line was constructed with cerulein, and the levels of inflammatory factors were detected *via* ELISA. Similarly, the expression of *ALKBH5*, *ZKSCAN3* and autophagy-related proteins was detected *via* qPCR, western blot, and immunofluorescence. Microscopic manifestations of autophagy in the cell model were observed *via* transmission electron microscopy. Additionally, RNA binding protein immunoprecipitation was used to analyze the interaction between *ALKBH5* and *ZKSCAN3*.

***Research results***

The expression of *ALKBH5* and *ZKSCAN3* was upregulated in the AP model, and the trend toward increased expression of autophagy-related genes suggested that autophagic flux was blocked in AP. Autophagy was improved by inhibiting the expression of *ALKBH5* and *ZKSCAN3*. *ZKSCAN3* mRNA has m6A binding sites, and *ALKBH5* can upregulate its expression by demethylating *ZKSCAN3*, which inhibits autophagic flux, thereby aggravating inflammation in AP.

***Research conclusions***

*ALKBH5* suppresses autophagic flux by demethylating the m6A site on *ZKSCAN3* mRNA, consequently promoting the onset and progression of AP.

***Research perspectives***

We proved that *ALKBH5* inhibits autophagy by upregulating *ZKSCAN3*, thereby promoting the occurrence and development of AP and providing new ideas for future research on autophagy regulation and early drug intervention in AP.

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**Footnotes**

**Institutional review board statement:** The study was reviewed and approved by the Institutional Review Board at Xiangya Hospital of Central South University.

**Institutional animal care and use committee statement:** All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Xiangya Hospital of Central South University.

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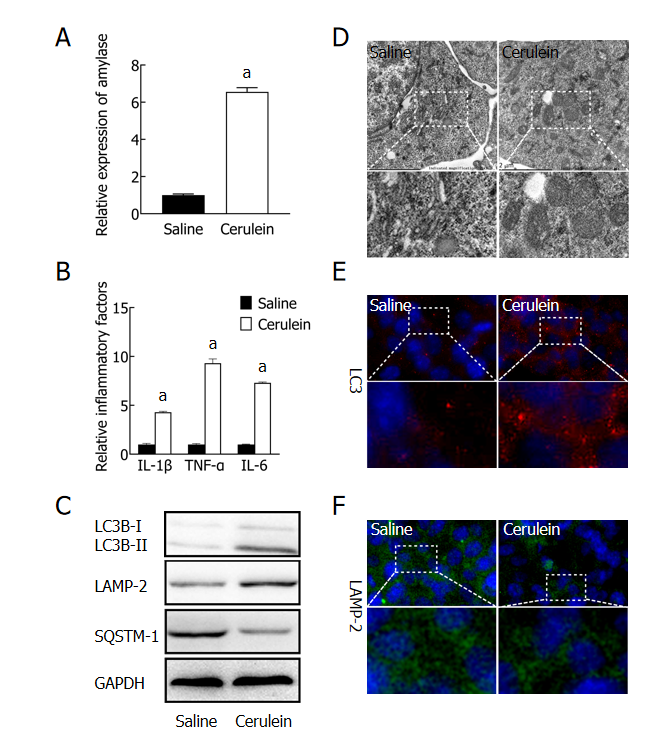
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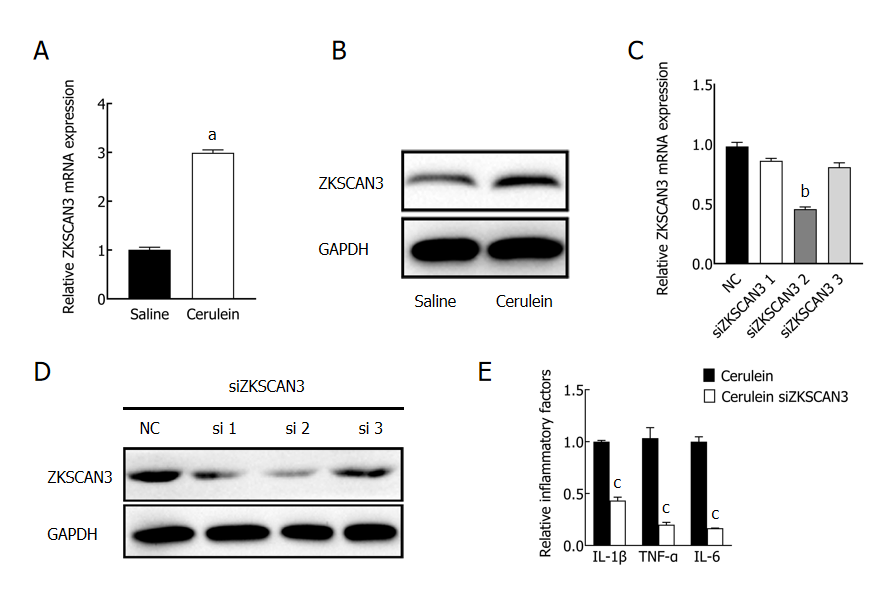
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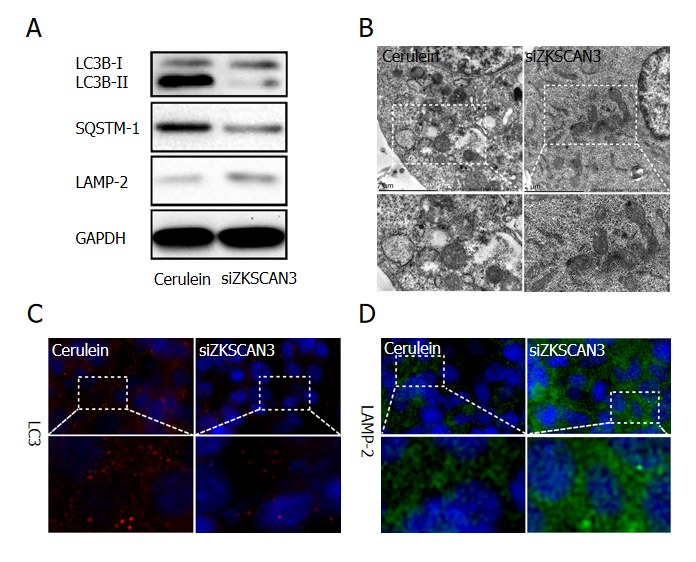
**Figure Legends**



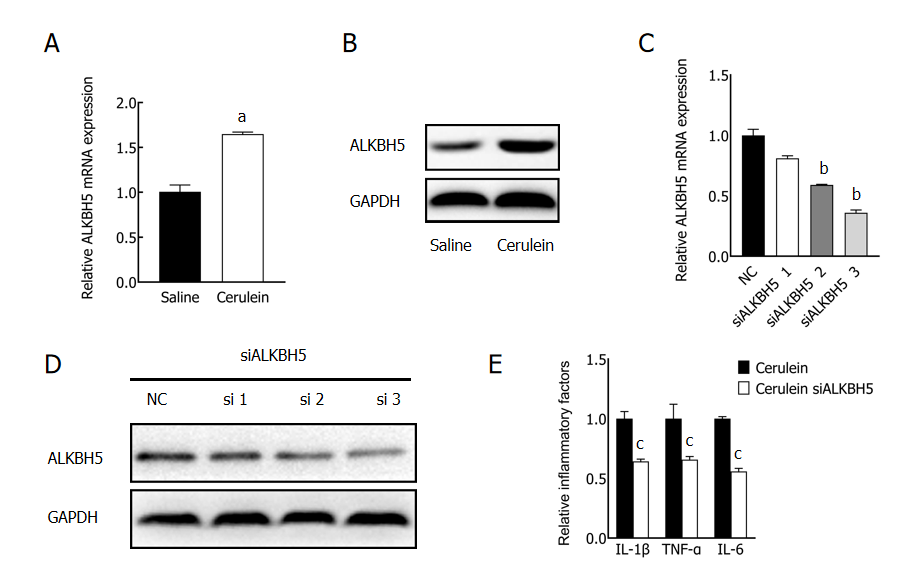
**Figure 1 Impaired autophagy in a mouse acute pancreatitis cell model.** A: The levels of amylase in the supernatant were detected by ELISA; B: The levels of inflammatory factors in the supernatant were detected by ELISA; C: The expression of autophagy-related proteins was detected by western blot; D: The microstructure of intracellular autophagy was observed by transmission electron microscopy; E: The expression of *LC3* was detected by immunofluorescence (magnification × 800); F: The expression of *LAMP-2* was detected by immunofluorescence (magnification × 800). a*P* < 0.05 *vs* saline group. TNF: Tumor necrosis factor; IL: Interleukin.



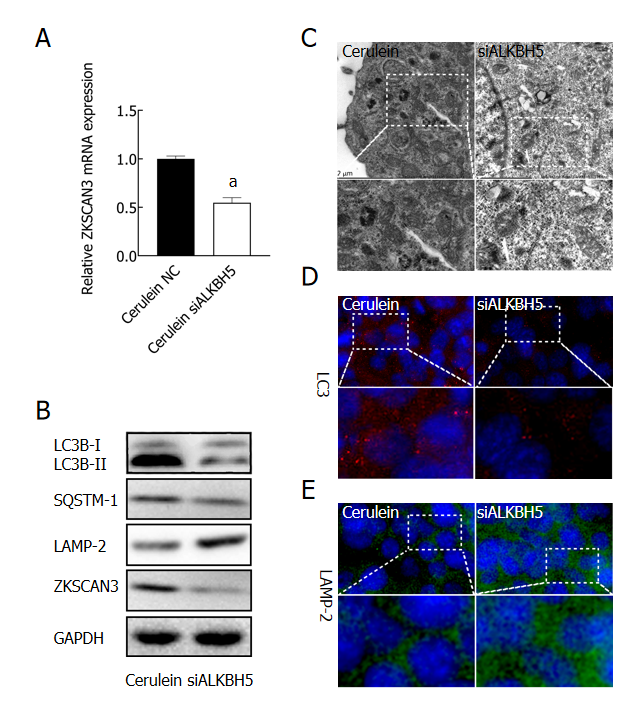
**Figure 2 The expression and function of *ZKSCAN3* in acute pancreatitis.** A: The expression of *ZKSCAN3* mRNA was detected by qPCR; B: The expression level of *ZKSCAN3* protein was detected by western blot; C: The expression level of *ZKSCAN3* mRNA treated with three different siRNAs was detected by qPCR; D: The expression level of *ZKSCAN3* protein in the MPC-83 cell line treated with three different siRNAs; E: After interfering with the expression of *ZKSCAN3*, the expression level of inflammatory factors in the mouse acute pancreatitis cell model was detected by ELISA. a*P* < 0.05 *vs* saline group; b*P* < 0.05 *vs* negative control group; c*P* < 0.05 *vs* cerulein group. NC: Negative control. TNF: Tumor necrosis factor; IL: Interleukin.



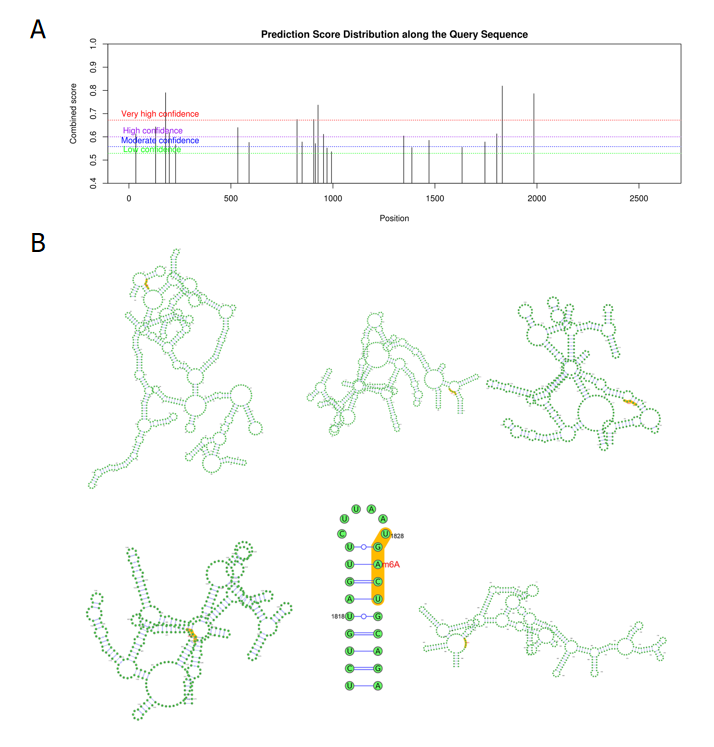
**Figure 3 *ZKSCAN3* inhibits autophagic flux in acute pancreatitis.** A: The expression of autophagy related proteins in the negative control group and knockdown group was detected by western blot; B: The extent of autophagy was observed by transmission electron microscopy; C: The expression of *LC3* was detected by immunofluorescence (magnification × 800); D: The expression of *LAMP-2* was detected by immunofluorescence (magnification × 800).



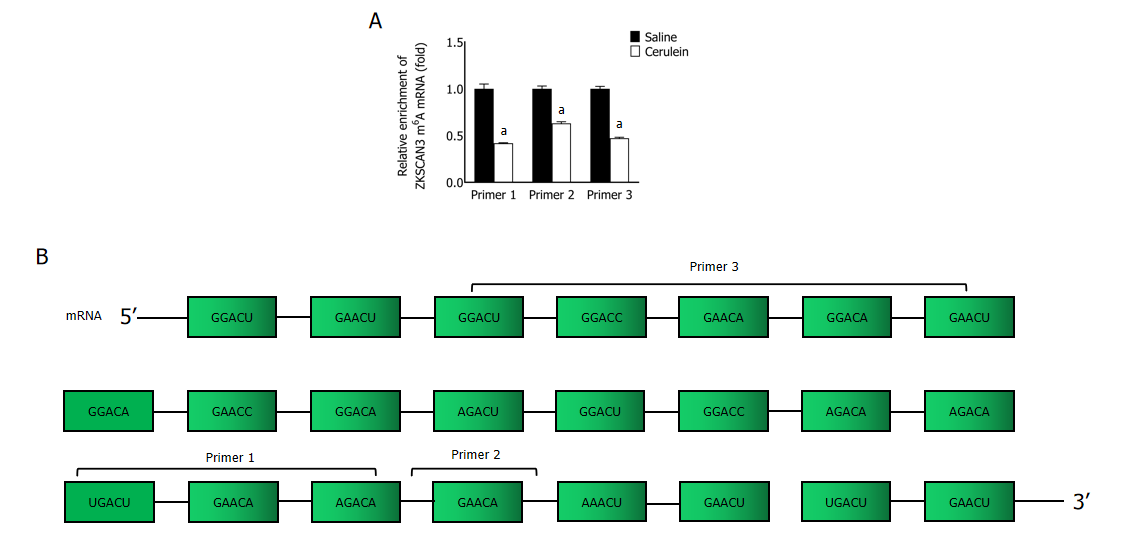
**Figure 4 The expression and function of *ALKBH5* in acute pancreatitis.** A: qPCR was used to detect *ALKBH5* mRNA expression in acute pancreatitis (AP) group and control group; B: The expression level of the *ALKBH5* protein in AP group and control group was detected by western blot; C: qPCR was used to detect *ALKBH5* mRNA expression in the MPC-83 cell line treated with different siRNAs; D: western blot was used to detect *ALKBH5* protein expression in MPC-83 cell line treated with three different siRNA; E: After interfering with the expression of *ALKBH5*, the expression level of inflammatory factors in the mouse AP cell line was detected by ELISA. a*P* < 0.05 *vs* saline group; b*P* < 0.05 *vs* negative control group; c*P* < 0.05 *vs* cerulein group. NC: Negative control; TNF: Tumor necrosis factor; IL: Interleukin.



**Figure 5 *ALKBH5* promoted the expression of *ZKSCAN3* and inhibited autophagic flux.** A: After interfering with *ALKBH5* expression, the expression level of *ZKSCAN3* mRNA was detected by qPCR; B: The expression levels of *ZKSCAN3* protein and autophagy related proteins were detected by western blot; C: Autophagic microstructure was observed by transmission electron microscopy; D: *LC3* was detected by immunofluorescence (magnification × 800); E: *LAMP-2* was detected by immunofluorescence (magnification × 800). a*P* < 0.05 *vs* negative cerulein group. NC: Negative control.



**Figure 6 Prediction of m6A binding sites in *ZKSCAN3*.** A: Potential m6A binding sites on *ZKSCAN3* mRNA were analyzed *via* a website (http://www.cuilab.cn/sramp/); B: Diagram of the secondary structure of highly credible m6A binding sites. m6A: N6-methyladenosine.



**Figure 7 Enrichment levels of m6A modifications on *ZKSCAN3* mRNA.** A: qPCR was used to detect the m6A modification level of *ZKSCAN3* mRNA; B: Schematic diagram of the sequences of primers used for *ZKSCAN3* mRNA. a*P* < 0.05 *vs* saline group.

**Table 1 Primer sequences for qPCR**

|  |  |
| --- | --- |
| **Genes** | **Sequence** |
| *ALKBH5* | Forward 5’- CTTTGCTTCGGCTGCAAGTT -3’ |
|  | Reverse 5’- CCGGCGTTCCTTAATGTCCT -3’ |
| *ZKSCAN3* | Forward 5’- CAGAGTAGGGTGGAAAGCC -3’ |
|  | Reverse 5’- AAGGTATGAAGGTCGGGTG -3’ |
| Primer 1 | Forward 5’- CCAGGCGGTTCTATTGC -3’ |
|  | Reverse 5’- TGGCTTTCCACCCTACTCT -3’ |
| Primer 2 | Forward: 5’- CAGAGTAGGGTGGAAAGCC-3’ |
|  | Reverse 5’- AGGTATGAAGGTCGGGTG-3’ |
| Primer 3 | Forward 5’- TGGTTCGGGATGGCTAG-3’ |
|  | Reverse 5’- AACAGCACTGCCTTGGAG-3’ |
| β-actin | Forward 5’- GTGGCCGAGGACTTTGATTG-3’ |
|  | Reverse 5’- CCTGTAACAACGCATCTCATATT-3’ |

**Table 2 m6A sites of *ZKSCAN3* mRNA**

|  |  |  |  |
| --- | --- | --- | --- |
| **Number** | **Position** | **Sequence context** | **Confidence** |
| 1 | 34 | GUGCCCCGCCCCCCGGGGUCGGACUUUCGACACUUUUGUGACUGC | High |
| 2 | 131 | ACAGCUACAGUGAAACGGGAGAACUGCUUGGUUCGGGAUGGCUAG | High |
| 3 | 180 | UCAAGGGAAAGCACAACCUUGGACUCACACUCUGCAGAGGACCAG | Very high |
| 4 | 198 | UUGGACUCACACUCUGCAGAGGACCAGAUGGAGCUACUGGUCAUA | High |
| 5 | 229 | AGCUACUGGUCAUAAAGGUGGAACAAGAAGAGGCCUCCCCCUUGG | Moderate |
| 6 | 534 | GUGGCGCUGCUGGAGUACUUGGACAGGCAGCUGGAUGACACACCU | High |
| 7 | 589 | CAGAUGAUGACGAUGGGCAGGAACUCCUUUGCUCCAAGGCAGUGC | Moderate |
| 8 | 824 | CCCAGUCCUUUCCCCCAGAUGGACAGAGCAGGAUUCAUCUCAGAU | Very high |
| 9 | 849 | GAGCAGGAUUCAUCUCAGAUGAACCUCUACAAAGAUGGAAUGCAG | Moderate |
| 10 | 906 | AGCCUGGUUUCCCUGGAUCAGGACAUGCAGACUAAGGUUAGGGAC | Very high |
| 11 | 914 | UUCCCUGGAUCAGGACAUGCAGACUAAGGUUAGGGACUUGCCUCG | Moderate |
| 12 | 927 | GACAUGCAGACUAAGGUUAGGGACUUGCCUCGAGCUGAAGAAUAC | Very high |
| 13 | 954 | CCUCGAGCUGAAGAAUACAGGGACCAAAAGCCUGAGCAGACAGUG | High |
| 14 | 971 | CAGGGACCAAAAGCCUGAGCAGACAGUGUGCUUCCUGGGUGAAGA | Low |
| 15 | 993 | ACAGUGUGCUUCCUGGGUGAAGACACUGUCCCGAUUCCUACAGGU | Low |
| 16 | 1347 | GAAAAGCCCUACGAGUGUGAUGACUGUGGGAAAACCUUCACUCAG | High |
| 17 | 1387 | CUCAGAGCUGCAGCCUCCUUGAACAUCACAGAAUUCACACUGGGG | Low |
| 18 | 1471 | GGCGUAGCUCACAUCUUCUGAGACAUCAGAGGACCCAUACUGGGG | Moderate |
| 19 | 1633 | GUAGGAUUACAAGCCUUAUUGAACACCAAAAAGUACACACUGGUG | Low |
| 20 | 1745 | GAGAAGACACACGGGGAAGAAAACUUCUGUCACAGUGACCCCUGC | Moderate |
| 21 | 1803 | GUUGGUGUUCAACUGUCAUUGAACUGAAGCCACUCUGUAGUUCUU | High |
| 22 | 1830 | AGCCACUCUGUAGUUCUUAAUGACUGCAGAAGUCAUAGGCUGGGG | Very high |
| 23 | 1985 | ACAAGAGUCCUCACCCAUUGGAACUAAAUGGGCUUCCUGACUGUC | Very high |