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Inhibition of MTERF4 disrupts mitochondrial function and attenuates amyloidogenic APP processing via HSDL2-mediated apoptosis and autophagy

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CITATION

Cao Z, Feng X, Wang L, Wang X. Inhibition of MTERF4 disrupts mitochondrial function and attenuates amyloidogenic APP processing via HSDL2-mediated apoptosis and autophagy. *Psycho-Oncologie*. 2025; *Psycho-Oncology Research in China*: 4571.

ARTICLE INFO

Received:

Accepted:

Available online:

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Abstract: Abnormal proteolytic cleavage events and mitochondrial dysfunction are implicated in the neuropathology of Alzheimer's disease (AD). Mitochondrial transcription factor 4 (MTERF4) is essential for mitochondrial ribosome assembly and translational regulation. In this study, we investigated the role of MTERF4 in mitochondrial dysfunction and amyloid precursor protein (APP) processing. MTERF4 knockdown (KD) significantly reduced adenosine triphosphate (ATP) production, mitochondrial DNA (mtDNA) copy number, and mitochondrial membrane potential (MMP) in HEK293-APPswe cells. To uncover the fundamental mechanisms, RNA sequencing (RNA-seq) analysis was conducted to screen for differentially expressed genes (DEGs). Notably, we demonstrated an upregulation of Hydroxysteroid dehydrogenase-like protein 2 (HSDL2), consistent with RNA-seq analysis, which has been implicated in apoptosis and autophagy regulation. Flow cytometry (FCM) analysis demonstrated that MTERF4 KD significantly reduced apoptosis, and subsequent experimental validation revealed that this effect was mediated via the Bcl-2/Bax/Caspase-3 pathway. Furthermore, MTERF4 KD notably raised the protein levels of Beclin-1 and ATG5, elevated the LC3 II/LC3 I ratio, and decreased P62 protein expression, thereby promoting autophagy. Additionally, MTERF4 KD resulted in reduced levels of APP, ADAM10, and secreted A β 42. Moreover, MTERF4 KD reduced both α - and β -cleavage of APP. In conclusion, our study highlights the crucial role of MTERF4 in mitochondrial dysfunction and its regulatory effect on HSDL2 expression in HEK293-APPswe cells. MTERF4 inhibition suppresses apoptosis via the Bcl-2/Bax/Caspase-3 pathway and attenuates APP processing by enhancing autophagy. These findings indicate that MTERF4 is critically involved in AD pathogenesis and may serve as a potential therapeutic target.

Keywords: Alzheimer's disease; MTERF4; mitochondrial dysfunction; amyloid precursor protein; apoptosis; autophagy

1. Introduction

Alzheimer's disease (AD) is a complex and progressive neurodegenerative disorder, marked by memory impairments and multiple cognitive deficits. The hallmark neuropathological features of AD include intracellular neurofibrillary tangles, formed by abnormal tau in limbic and cortical association areas, as well as extracellular senile plaques composed of β -amyloid (A β) in the neocortex [1]. Although the exact etiology of AD remains largely unknown, several factors contribute to its pathogenesis, including the accumulation of reactive oxygen species (ROS), the presence of the *APOE4* allele, and calcium dysregulation [2].

Amyloid precursor protein (APP) metabolism primarily occurs through two classical proteolytic pathways [3]. The amyloid pathway involves the cleavage of APP into a C-terminal fragment of 99 amino acids (C99) and soluble APP- β (sAPP β) by β -secretase; C99 is further cleaved into amyloid precursor protein intracellular C-terminal domain (AICD) and A β . The non-amyloid pathway involves the cleavage of APP into sAPP α and C83 by α -secretase; C83 is subsequently cleaved by γ -secretase, generating P3 and AICD [4]. The balance between A β formation and clearance is primarily regulated by β -site APP Cleaving Enzyme 1 (BACE1) and a disintegrin and a metalloproteinase 10 (ADAM10, α -secretase) [5].

A β binds to mitochondrial proteins, affecting mitochondrial biogenesis and ultimately causing mitochondrial dysfunction [6]. The interaction between A β and mitochondria disrupts mitochondrial membrane permeability, resulting in a loss of mitochondrial membrane potential (MMP) and elevated ROS levels. Previous studies have implicated MTERF4 can form a stoichiometric complex with NOP2/Sun RNA methyltransferase 4 (NSUN4) and play a crucial role in facilitating the recruitment of NSUN4 to the large ribosomal subunit [7]. MTERF4 has also been identified as a regulatory factor in mitochondrial dysfunction within a neuronal model of Parkinson's disease [8]. Mitochondria are the center of the intrinsic apoptotic pathway and impaired mitochondrial function can activate intrinsic apoptotic pathways via Bcl-2, Bax, caspases, and ROS, ultimately contributing to neurodegeneration [9]. A decline in autophagic efficiency results in the accumulation of A β in the mitochondrial membrane and the release of cytochrome c, which can trigger a signaling cascade that ultimately induces cell death [10].

Our previous work showed that overexpression of MTERF4 enhances the amyloidogenic processing of APP [11]. However, its precise role in AD-related mitochondrial biology and proteolysis of APP remains largely unknown. In this study, we investigate how inhibition of MTERF4 may offer therapeutic potential in modulating disease progression by regulating apoptosis and autophagy. Our findings elucidate the effects of MTERF4 knockdown (KD) on mitochondrial function, apoptotic pathways, and APP metabolism. These results offer novel insights into the contribution of MTERF4 to AD progression.

2. Materials and methods

2.1. Plasmid construction

We induced MTERF4-specific KD using short-hairpin RNA (shRNA) interference. shRNAs targeting human MTERF4 were synthesized and obtained from GenePharma (Shanghai, China), as previously described [8]. The shRNA oligonucleotides were annealed and subcloned into the pGPU6/green fluorescent protein (GFP)/Neo vector following the manufacturer's protocol.

2.2. Cell culture and transfection

HEK293-APP_{swe} cells (HEK293 cells stably transfected with the human APP Swedish K595N/M596L double mutation) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained at 37 °C in a

humidified incubator with 5% CO₂. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS, PAN, Aidenbach, Germany), 1 % penicillin-streptomycin (P/S), and 1 mg/mL geneticin (G418; Sangon, Shanghai, China). Plasmid constructs and control vectors were transfected into HEK293-APPswe cells using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA).

2.3. Western blotting

After the indicated treatments, cells were collected and lysed in a lysis buffer (KeyGen Biotech, Shanghai, China). Equal amounts of protein were separated by 10 % SDS-PAGE and transferred onto nitrocellulose membranes (Millipore Sigma, Burlington, MA, USA). The membranes were incubated overnight at 4 °C with the following primary antibodies: MTERF4 (1:1000, Abcam, Cambridge, UK), ADAM10 (1:500, Abcam), BACE1 (1:500, Abcam), C-terminal APP (1:1000; Sigma-Aldrich), BAX (1:500; Cell Signaling Technology [CST], Danvers, MA, USA), Bcl-2 (1:500; CST), Caspase-3 (1:500; CST), LC3 (1:500; CST), ATG5 (1:500; CST), Beclin-1 (1:500; CST), P62 (1:500; CST), HSDL2 (1:500; CST), GAPDH (1:1000; CST), α -Tubulin (1:1000; CST). The membranes were then incubated for 1 hour with HRP-conjugated secondary antibodies (1:5000; Abcam). Protein bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Millipore).

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using the TRIpure Total RNA Extraction Kit (Sangon Biotech, Shanghai, China). Complementary DNA (cDNA) was synthesized using the PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan). All primers were synthesized by TsingKe Biotech (Beijing, China) and are listed in Table 1. qRT-PCR was then performed using the SYBR Premix Ex Taq Kit (TaKaRa) on a LightCycler 96 instrument (Roche, Basel, Switzerland).

2.5. Estimation of mitochondrial DNA (mtDNA) copy number

Total DNA was extracted from cells using the Wizard Genomic DNA Purification Kit (TsingKe Biotech, Beijing, China). The mtDNA copy number was quantified by qRT-PCR using the SYBR Green Master Mix (TaKaRa) on a LightCycler 96 instrument (Roche). The relative mtDNA copy number was normalized to that of *thymidine kinase 2* (TK2) levels [19].

2.6. Adenosine triphosphate (ATP) analysis

ATP levels were quantified using an ATP Assay Kit (Beyotime Biotech, Shanghai, China). HEK293-APPswe cells were lysed completely and centrifuged at 15,000 rpm for 10 min at 4 °C. Subsequently, 20 μ L of the supernatant was mixed with 100 μ L of ATP working solution in each detection well. Bioluminescence was measured using a microplate reader (Tecan, Männedorf, Switzerland). ATP levels were normalized to the total protein content in each sample.

2.7. MMP measurement

MMP was evaluated using an MMP Assay Kit with tetramethylrhodamine ethyl ester (TMRE; Beyotime). Briefly, HEK293-APPswe cells were incubated with 100 nM TMRE at 37 °C for 20 min and then washed gently with pre-warmed PBS to remove residual extracellular fluorescence. Nuclear morphology was examined after Hoechst 33342 staining. Fluorescence images were captured using an inverted fluorescence microscope (Leica DMI8, Wetzlar, Germany).

2.8. Human A β 42 quantitation

The concentration of human A β 42 in the culture medium was quantified using a specific sandwich enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen). After transfection, the supernatant was collected and treated with a protease inhibitor cocktail to prevent protein degradation. The optical density (OD) at 450 nm was determined for each well using a microplate reader (Tecan).

2.9. RNA sequencing and bioinformatics analysis

Total RNA was extracted from cells using the TRIpure Total RNA Extraction Kit (Sangon Biotech) 24 hours after transfection. cDNA libraries were then constructed and sequenced on the Illumina sequencing platform by Metware Biotechnology Co., Ltd (Wuhan, China). Differentially expressed genes (DEGs) were identified using DESeq2 software (version 1.38.3), with Benjamini-Hochberg correction applied for multiple testing adjustments. Significance thresholds for differential expression were determined based on adjusted *p*-values and log₂ fold changes. For functional enrichment analysis, the hypergeometric test was applied, and Gene Ontology (GO) enrichment was conducted using GO terms. Gene set enrichment analysis (GSEA) was performed using the ClusterProfiler R package (version 4.6.0).

2.10. Cell cycle and apoptosis assay

The cells from each group were trypsinized, washed with cold phosphate-buffered saline (PBS), and fixed in 70 % cold ethanol at 4 °C overnight. The cells were then resuspended in propidium iodide (PI)/RNase staining buffer (BD Biosciences, San José, CA, USA) and incubated for DNA staining. Cellular DNA content was measured using a FACSCalibur flow cytometer (BD Biosciences). Apoptosis was quantified by flow cytometry following staining with the Annexin V-FITC/PI kit (BD Biosciences).

2.11. Cell proliferation assay

Cell viability was evaluated with the Cell Counting Kit-8 (CCK-8) assay (Sangon). Cells were plated in 96-well plates at a density of 2000 cells per well. After transfection, CCK-8 reagent was diluted and added to each well, followed by incubation at 37 °C for 2 h. Absorbance at 450 nm was measured using a microplate reader (Tecan).

2.12. Statistical analysis

The data are expressed as the mean $\pm$ standard error of the mean (SEM) derived from a minimum of three independent experiments. Statistical analyses were performed using SPSS software (version 19.0, IBM Corp., Armonk, NY, USA). A *p*-value < 0.05 was considered statistically significant compared to the control group.

3. Results

3.1. MTERF4 plays a crucial role in mitochondrial function

To investigate the role of MTERF4 in cell functions, HEK293-APPsw cells were transfected with either a vector control or a recombinant plasmid. Western blot analysis revealed a significant reduction in MTERF4 protein expression compared with the control at both 24 and 48 h post-transfection (**Figure 1A**). Consistently, qRT-PCR analysis demonstrated a significant decrease in MTERF4 mRNA expression in transfected cells compared with NC at both time points (**Figure 1B**). These results confirmed that MTERF4 was efficiently knocked down in transfected cells. Given the crucial role of MTERF4 in regulating mitochondrial translation, we next quantified mtDNA levels in NC and MTERF4 KD cells. Our results demonstrated a significant reduction in mtDNA levels in MTERF4 KD cells at both 24 and 48 h (**Figure 1C**). Furthermore, ATP quantification assays revealed a significant decrease in cellular ATP levels in MTERF4-KD cells at both time points (**Figure 1D**). As expected, MMP levels were significantly reduced in MTERF4 KD cells relative to NC cells (**Figure 1E**). Taken together, these findings indicate that MTERF4 KD leads to mitochondrial dysfunction.

3.2. Pathways involved in the effects of MTERF4 KD

To elucidate the impact of MTERF4 KD on cellular function and identify the associated biological pathways, transcriptomic sequencing was conducted to analyze DEGs between control and MTERF4 KD cells. As shown in **Figure 2A**, Venn diagram analysis identified 187 significantly enriched genes in the MTERF4 KD groups, with 117 genes downregulated and 70 genes upregulated (FDR < 0.05 , fold change > 1.5). Hierarchical clustering analysis further revealed distinct gene expression profiles in the MTERF4 KD group compared with the control group (**Figure 2B**). As shown in **Figure 2C**, three major GO categories, including biological processes, cellular components, and molecular functions of the transcripts, were significantly enriched in response to MTERF4 KD.

3.3. MTERF4 KD induces HSDL2 expression and regulates cell cycle progression

RNA-seq analysis revealed that MTERF4 KD stimulates the expression of Hydroxysteroid dehydrogenase-like protein 2 (HSDL2). To validate the RNA-seq findings, we quantified HSDL2 expression at both the mRNA and protein levels using qRT-PCR and Western blot analysis. The results confirmed a significant increase in HSDL2 expression in MTERF4-KD cells at both the mRNA and protein levels (**Figure 3A,B**). To further investigate the impact of MTERF4 KD on cell

cycle progression, we performed a flow cytometric (FCM) analysis. MTERF4 KD reduced the proportion of cells in G0/G1 phase while increasing the percentage in S phase (**Figure 3C,D**). Furthermore, we assessed whether MTERF4 KD affects cell proliferation using the CCK-8 assay. Our results indicated that MTERF4 KD did not significantly alter proliferative capacity at 24 or 48 h post-transfection (**Figure 3E**).

3.4. Cell apoptosis is reduced by inhibition of MTERF4

RNA-seq analysis indicated that MTERF4 KD is involved in apoptosis regulation. To further investigate this, we performed FCM analysis to quantify apoptotic cell populations. As shown in **Figure 4A**, MTERF4 KD significantly decreased apoptosis levels at 24 and 48 h post-transfection. To elucidate the molecular mechanisms underlying apoptosis suppression, we examined the expression levels of key apoptotic regulators, including Bcl-2, Bax, and Caspase-3, via western blotting. As shown in **Figure 4B,C**, the expressions of Caspase-3 and Bax were significantly downregulated, whereas Bcl-2 expression was upregulated in MTERF4 KD cells. These findings were consistent with the RNA-seq analysis, providing further evidence that inhibition of MTERF4 suppresses apoptosis.

3.5. MTERF4 KD modulates the expression of autophagy-related biomarkers

Since the mitochondrial outer membrane serves as a platform for recruiting autophagy-related proteins, and autophagy inhibition disrupts intracellular APP transport, we next examined the expression levels of key autophagy markers [12]. Western blot analysis revealed that MTERF4 KD significantly upregulated Beclin-1 and ATG5 protein levels at 24 and 48 h post-transfection (**Figure 4D–G**). In addition, the LC3II/LC3I ratio was upregulated in MTERF4 KD cells after 24 and 48 h, while P62 protein expression was significantly decreased. These findings suggest that MTERF4 KD enhances autophagy by modulating key autophagy-related markers.

3.6. Inhibition of MTERF4 impairs the amyloidogenic pathway of APP

Given our previous finding that MTERF4 overexpression enhances APP amyloidogenic processing, we next investigated whether MTERF4 KD influences APP cleavage and A β metabolism. Western blot analysis revealed that MTERF4 KD significantly decreased APP and ADAM10 protein levels at 48 hours post-transfection (**Figure 5A,B**). However, MTERF4 KD did not induce significant alterations in BACE1 protein levels. qRT-PCR analysis confirmed that MTERF4 knockdown suppressed *ADAM10* gene expression at the transcriptional level but had no significant effect on *APP* and *BACE1* gene expression (**Figure 5C–E**). Moreover, as indicated by decreased levels of C99 and C83 fragments, MTERF4 KD attenuated both α - and β -cleavage of APP (**Figure 5G,H**). As shown in **Figure 5F**, A β 42 peptide levels were significantly reduced in the MTERF4 KD group at 48 h, highlighting the role of MTERF4 in regulating A β metabolism.

4. Discussion

AD is the most common neurodegenerative condition and represents a primary contributor to dementia on a global scale [13]. Our previous study demonstrated that elevated MTERF4 expression promotes the amyloidogenic pathway of APP. This finding suggests that MTERF4 deficiency may disrupt APP metabolism and affect cellular functions. Therefore, this study aimed to systematically investigate the effects of MTERF4 inhibition on mitochondrial function and APP metabolism. To evaluate mitochondrial function, we measured mtDNA content, MMP, and ATP production. Additionally, we examined key markers of apoptosis and autophagy, as well as the mRNA and protein expression levels of APP, ADAM10, BACE1, and A β 42 in HEK293-APPswe cells. Our findings highlight the pivotal role of MTERF4 in APP processing, mitochondrial biogenesis, and cellular homeostasis.

Mitochondrion-targeted strategies have been proposed as potential therapeutic approaches for AD, with several demonstrating efficacy in mitigating cellular abnormalities in various animal and cellular models [14]. Previous studies have demonstrated that MTERF4 forms a stoichiometric complex with NSUN4 and is localized to mitochondria [15]. Furthermore, MTERF4 deficiency in brown adipose tissue has been shown to reduce mitochondrial oxidative phosphorylation protein levels, leading to impaired assembly of mitochondrial complexes due to decreased translation of mtDNA-encoded proteins [16]. In this study, we observed that MTERF4 KD led to a decrease in mtDNA copy number, a reduction in MMP, and diminished ATP production, aligning with previously reported findings [8].

We performed RNA-seq to investigate DEGs and the signaling pathways enriched in these DEGs. A total of 187 DEGs were identified, including 70 upregulated and 117 downregulated genes, mapped to the reference genome. The GO analysis indicated that MTERF4 inhibition affected multiple biological processes in HEK293-APPswe cells, including the intrinsic apoptotic signaling pathway, cellular iron ion homeostasis, and the regulation of signaling receptor activity, etc. HSDL2 belongs to the short-chain dehydrogenase/reductase (SDR) family and is localized in mitochondria, associated with AD dementia, neuropathological changes, and progressive cognitive decline [17]. HSDL2 depletion has been reported to impair cell proliferation and motility, while promoting cell cycle arrest and apoptosis [18]. Additionally, HSDL2 downregulation significantly suppresses autophagy and exacerbates oxidative stress [19]. Guided by insights from RNA-seq data, we then confirmed significant upregulation of *HSDL2* mRNA and protein levels following MTERF4 KD. Given these findings, we hypothesized that HSDL2 serves as a key downstream effector in the MTERF4 signaling pathway. To further elucidate the role of MTERF4 in cellular function regulation, we examined cell cycle progression and proliferation in HEK293-APPswe cells. FCM analysis revealed that MTERF4 KD reduced the G0/G1 phase cell population and increased the S phase cell proportion. A previous study demonstrated that MTERF4 is essential for cell proliferation in HeLa cells [20]. However, our CCK-8 assay results indicated that MTERF4 KD had no significant impact on HEK293-APPswe cell proliferation.

HSDL2 functions at the intersection of autophagy and apoptosis, highlighting its intricate interplay with these two cellular processes. Our findings revealed that

MTERF4 KD is involved in the regulation of apoptosis in HEK293-APPswe cells, as confirmed by Western blot analysis. Specifically, MTERF4 KD suppressed Bax expression, elevated Bcl-2 levels, and reduced Caspase-3 activity at 24 and 48 h post-transfection, ultimately reducing apoptosis. Defects in the autophagy pathway have been implicated in AD pathogenesis, leading to the accumulation of toxic A β aggregates, increased cellular apoptosis, and progressive degeneration [21]. Given the critical role of autophagy in A β clearance, we examined whether MTERF4 KD could activate autophagy. Our results demonstrated that Beclin-1 and Atg5 protein levels, as well as the LC3-II/LC3-I ratio, were significantly increased, whereas P62 protein expression was reduced following MTERF4 KD. By clearing misfolded protein aggregates, autophagy helps maintain normal cellular function and prevents apoptosis induction [22].

Building on our previous finding that MTERF4 overexpression enhances the amyloidogenic pathway of APP, we investigated whether MTERF4 KD could influence APP cleavage [11]. Our results demonstrated that MTERF4 KD significantly reduced APP and ADAM10 protein levels and attenuated both α - and β -cleavage of APP in HEK293-APPswe cells. The amyloidogenic processing of APP by β - and γ -secretases generates A β 40 and A β 42 peptide monomers, which subsequently aggregate into amyloid fibrils that form senile plaques [23]. Consistently, we observed that A β 42 peptide levels were significantly reduced in the MTERF4 KD group at 48 hours post-transfection.

In conclusion, our study suggests that MTERF4 serves as a key regulator of mitochondrial biogenesis. We demonstrated that MTERF4 inhibition induces HSDL2 expression, suppresses apoptosis, promotes autophagy, and attenuates the amyloidogenic processing of APP (**Figure 6**). Additionally, our findings suggest that HSDL2 may act as a critical downstream effector in the MTERF4 signaling pathway. This work has important implications for elucidating the underlying mechanisms of AD, highlighting MTERF4 as a potential key player in AD pathogenesis and a promising therapeutic target.

Author contributions: Conceptualization, XW; methodology, ZC; formal analysis, LW; investigation, ZC; data curation, XF; writing—original draft preparation, ZC; writing—review and editing, XW; supervision, XW; funding acquisition, XW. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Natural Science Foundation of Chongqing (cstc2019jcyj-msxmX0294); the Science and Technology Research Program of Chongqing Municipal Education Commission (KJQN202000444); and the Basic Research and Frontier Exploration Project of Yuzhong District (20180116).

Conflict of interest: The authors declare no conflict of interest.

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Figure 1. MTERF4 plays a crucial role in mitochondrial function. (A) Western blot analysis of MTERF4 protein levels. (B-C) Relative mRNA expression levels of *MTERF4* and the mtDNA copy number were quantified using qRT-PCR. (D) Intracellular ATP levels were significantly reduced in MTERF4 KD cells compared with NC. (E) Representative fluorescence images of MMP were captured using an inverted fluorescence microscope. Scale bar: 50 μ m. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

Figure 2. Pathways involved in the effects of MTERF4 knockdown. (A) Volcano plot depicting the significant DEGs between NC and MTERF4 KD cells. (B) Hierarchical clustering heatmap illustrating distinct gene expression patterns. (C) Bar plots representing GO enrichment analysis.

Figure 3. Inhibition of MTERF4 upregulates HSDL2 expression and modulates the cell cycle. (A) Western blot analysis of HSDL2 protein levels. (B) qRT-PCR analysis of HSDL2 mRNA levels. (C) Flow cytometry analysis of cell cycle distribution. (D) Histograms showing the distribution of cells across the G0/G1, S, and G2/M phases. (E) CCK-8 assay of cell proliferation. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ vs. control.

Figure 4. Effects of MTERF4 knockdown on apoptosis and autophagy. (A) Flow cytometry analysis of apoptotic cells. (B-C) Western blot analysis of apoptosis-related markers, including Bax, Bcl-2, and Caspase-3. (D-E) Western blot analysis of Beclin-1 and P62 protein levels. (F-G) Western blot analysis of ATG5 and LC3 protein levels. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

Figure 5. Inhibition of MTERF4 impairs the amyloidogenic processing of APP. (A-B) Western blot analysis of full-length APP, ADAM10, and BACE1 protein levels (C-E) Relative mRNA expression levels of *APP*, *ADAM10*, and *BACE1* were determined by qRT-PCR. (F) ELISA quantification of human A β 42 peptide levels in the culture media. (G-H) Western blot analysis of C99 and C83 protein levels. Data are presented as mean $\pm$ SD. * $p < 0.05$ vs. control.

Figure 6. Schematic illustration of the major findings in the present study. MTERF4 inhibition induces HSDL2 expression, suppresses apoptosis via the Bcl-2/Bax/Caspase-3 pathway, and attenuates APP processing by enhancing autophagy.