

Original Article

Presenilin 1 is located in the mitochondrial inner membrane and regulates mitochondrial cristae junction protein arrangement and cristae formation in HEK293 cells

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Received 23 October 2025 Accepted 30 March 2026 Published 24 April 2026

Abstract

Presenilin 1 (PS1), a key pathogenic factor in familial Alzheimer's disease, is implicated in the regulation of mitochondrial functions, yet its precise sub-mitochondrial localization and underlying mechanisms remain poorly understood. In this study, we generate *PS1*-knockout cell lines to investigate the role of PS1 in mitochondrial structure and function. Our results indicate that PS1 is directly localized in the mitochondrial inner membrane. *PS1* deficiency leads to reduced ATP production, impaired mitochondrial respiration capacity, decreased mitochondrial membrane potential, disrupted Ca^{2+} homeostasis, and elevated ROS accumulation. Moreover, loss of *PS1* leads to abnormal mitochondrial cristae structure. Further analysis reveals that PS1 interacts with mitochondrial inner membrane proteins. Its absence promotes ATAD3A oligomerization and disrupts its arrangement at mitochondrial cristae junctions, leading to expansion of the mitochondria-associated membrane and instability of mitochondrial DNA. Our findings demonstrate that PS1 acts as a central regulator of mitochondrial cristae morphogenesis by modulating protein interaction networks at cristae junctions, thereby illuminating fundamental molecular mechanisms contributing to mitochondrial dysfunction in Alzheimer's disease.

Key words presenilin 1, mitochondrial dysfunction, ATAD3A, mitochondrial DNA

Introduction

Alzheimer's disease (AD), the predominant form of dementia, manifests as progressive deterioration of cognitive and mnemonic functions [1]. While most cases occur sporadically, approximately 1%–5% of early-onset AD arises from autosomal dominant mutations, predominantly in the *PSEN1* gene encoding presenilin 1 (PS1) [2,3]. As a nine-transmembrane protein with self-cleavage activity, PS1 is primarily localized on the endoplasmic reticulum (ER) and mitochondria-associated membranes (MAMs), and constitutes the catalytic core of the γ -secretase complex, which executes the terminal cleavage of amyloid precursor protein (APP) to generate amyloid- β (A β) peptides [4,5]. The pathological accumulation of neurotoxic A β species, culminating in amyloid plaque deposition, represents a hallmark neuropathological feature of AD [1,2]. Beyond its canonical role in A β production, PS1 regulates multiple cellular processes. It has been confirmed that PS1 is

involved in calcium ion (Ca^{2+}) homeostasis by interacting with IP3R [6], and SERCA2b [7] functions as a passive calcium channel on the ER. AD-associated PS1 mutations perturb ER calcium homeostasis, and PS1 dysfunction disrupts mitochondrial-ER Ca^{2+} crosstalk [8] and enhances the function of MAMs [9]. Additionally, PS1 governs lysosomal acidification and calcium dynamics by controlling glycosylation of the v-ATPase V0a1 subunit [10]. Furthermore, emerging evidence indicates that PS1 is directly localized in mitochondria and mediates mitochondrial functions [11–13]. Wang *et al.* [14] demonstrated that PS1 could interact with mitochondrial FKBP38 and regulate apoptotic pathways. Recently, studies have shown that pathogenic PS1 variants display detrimental effects on mitochondrial functions [13]. Notably, mitochondrial dysfunction has emerged as a convergent pathological mechanism across neurodegenerative disorders, with impaired bioenergetics, oxidative stress, and aberrant organellar dynamics being consistently

observed in AD models and postmortem brains from AD patients [15–17]. Despite decades of investigation into A β -centric pathways, the molecular etiology linking PS1 dysregulation to mitochondrial failure remains poorly defined, particularly regarding non-canonical γ -secretase-independent mechanisms.

Mitochondria are double-membrane organelles in which the inner mitochondrial membrane (IMM) forms a highly dynamic and compartmentalized structure essential for energy production, metabolic regulation, and apoptotic control [15,18,19]. In neurons, mitochondrial IM integrity is indispensable for sustaining ATP-dependent neurotransmission, Ca²⁺ homeostasis, and survival pathways critical to these long-lived postmitotic cells [15,19]. Structural damage to mitochondria is strongly implicated in AD pathogenesis [19]. The IMM achieves functional specialization through cristae invaginations, creating two morphologically and biochemically distinct domains: the boundary membrane (continuous with the outer membrane) and cristae membrane (folded inward), interconnected by cristae junctions. Respiratory chain supercomplexes are enriched in cristae membranes, whereas protein translocases such as TIM complexes predominantly localize to boundary membranes [20,21]. Cristae junctions, stabilized by the MICOS complex, OPA1, and ATAD3A, are essential for maintaining IMM structural integrity [22,23]. These protein assemblies cooperatively regulate cristae morphology, directly determining mitochondrial functional efficiency.

ATAD3A is a multi-pass transmembrane protein spanning both mitochondrial membranes [24]. It participates in maintaining inner membrane architecture, stabilizing mitochondrial DNA (mtDNA), and regulating the formation of MAMs to mediate Ca²⁺ flux at mitochondria-ER contact sites [25–27]. Furthermore, recent studies have demonstrated increased ATAD3A oligomerization in AD patients and models, correlating with MAMs expansion and mtDNA instability [28,29]. ATAD3A further serves as a scaffolding factor critical for the assembly of inner membrane proteins and structural organization of the mitochondrial inner membrane [27]. While ATAD3A is critical for organizing inner membrane protein networks, its essential role in maintaining mitochondrial inner membrane ultrastructure and initiating cristae biogenesis remains elusive.

In this study, we investigated the role of presenilin-1 (PS1) in mitochondrial function and architecture. In HEK293 cells, we identify that PS1 is localized in the mitochondrial inner membrane, and *PS1* deficiency induces mitochondrial vacuolization and impairs mitochondrial function, manifesting as reduced ATP production, disrupted calcium homeostasis, accumulated ROS, and loss of mitochondrial membrane potential. Notably, *PS1* deletion increases ATAD3A protein level and promotes its oligomerization, disrupting the interaction network of mitochondrial inner membrane proteins. These results advance our understanding of PS1-associated mitochondrial dysfunction in AD, underscoring the critical role of PS1 in preserving mitochondrial homeostasis through functional interplay with key regulators such as ATAD3A.

Materials and Methods

Plasmid construction

The lentiCRISPR v2-*PS1* gRNA plasmid was constructed by cloning the *PS1*-gRNA (5'-GCCACGCAGTCCATTCAGGG-3') into the lentiCRISPR v2 vector (52961; Addgene, Watertown, USA). The control

gRNA plasmid was generated using the control gRNA sequence (5'-GGCGAGGGCGATGCCACCTACGG-3'). Both constructs were verified by sequencing (Genewiz, Tianjin, China). The pcDNA3.1(+) N4Flag-PS1-wt plasmid was constructed by amplifying the *PS1* sequence from cDNA using primers and cloning it into the *Bam*HI/*Xho*I-digested pcDNA3.1(+)N4Flag vector (established in our laboratory). The pCMV-N3Myc-MFN2, pCMV-N3Myc-TIM23, and pCMV-N3Myc-MIC19 plasmids were constructed by amplifying the corresponding sequences from cDNA using specific primers and cloning the sequences into the *Hind*III/*Xho*I-digested pCMV-3Tag-7 vector (240202; Agilent Technologies, Santa Clara, USA), respectively. The pFX-mito-EGFP plasmid (159096; Addgene), expressing EGFP targeted to the mitochondrial inner membrane via the COX8 signal peptide, was used for mitochondrial fluorescence labeling. SPLICS Mt-ER Long P2A plasmid (164107; Addgene) was used for mitochondrial-associated membrane (MAM) labeling. siRNAs were used for ATAD3A knockdown: ATAD3A-siRNA-1, 5'-UGCACAUU UAGGAUAUGCUTT-3'; ATAD3A-siRNA-2, 5'-CAGGCAGGUGAU GUCUUUGTT-3'; and nc-siRNA, 5'-UUCUCCGAACGUGUCAC GUTT-3'. The sequences of the primers used in this study are listed in Table 1.

Cell culture and transfection

HEK293 cells (iCell-h243; Cellverse, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (CM10013; Macgene, Beijing, China) supplemented with 10% fetal bovine serum (S711-001S; Lonsera, Suzhou, China) and 1% penicillin-streptomycin (P1400; Solarbio, Beijing, China). The *PS1*-gRNA-lentiCRISPR v2 plasmid was transfected at a 1:2 ratio using Lipofectamine 2000 (11668019; Thermo Fisher, Waltham, USA) or PEI MAX (24765; Polyscience, Warrington, USA). After 48 h, cells were selected with puromycin (1 μ g/mL, A1113803; Gibco, Grand Island, USA). When the cells reached 80%–90% confluence, they were trypsinized, diluted to 0.5 cells/100 μ L, and plated at 100 μ L per well in 96-well plates. Single-cell clones were expanded, and *PS1* knockout was confirmed by western blot (WB) analysis.

Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (CA1210; Solarbio). Cells were seeded in 12-well plates at an identical density and cultured overnight. Then, each well was transfected with the indicated plasmids or the corresponding empty vector at a plasmid to PEI MAX ratio of 1:2. After transfection for 6 h, the culture medium was changed to complete culture medium. At 24 h after transfection, the cells were incubated with CCK-8 (1:10 in culture medium) for 1 h at 37°C. To assess the background signal, control wells of HEK293 and *PS1* knockout (*PS1*KO) cells were set up without the addition of CCK-8. Absorbance was measured at 450 nm using a microplate reader, corrected by subtracting the background absorbance, and normalized to the control group.

Dead cell staining

Dead cells were assessed using a Hoechst 33342/PI double staining kit (CA1120; Solarbio). Cells were incubated with a staining solution containing Hoechst 33342 and PI diluted 1:1000 in culture medium (DMEM with 10% FBS and 1% penicillin-streptomycin) for 30 min at 37°C under 5% CO₂. After washing twice with PBS, cells were maintained in phenol red-free medium (CM15020; Macgene)

Table 1. The sequences of primers used in this study

Primer	Sequence (5'→3')	Category
PS1-wt-F	GATCCTCGAGCTACATATTCACCAACCACACCATTGTTGAGGAG	Cloning
PS1-wt-R	GATCCTCGAGCTAGATATAAAATTGATGGAATGCTAATTGGTC	Cloning
MFN2-F	GATCAAGCTTATGTCCCTGCTCTTCTCTCGATGCAACTCT	Cloning
MFN2-R	GATCCTCGAGCTATCTGCTGGGCTGCAGGTACTGGTGTGT	Cloning
TIM23-F	GATCAAGCTTATGGAAGGAGGCGGGGAAGCGGCAACAAA	Cloning
TIM23-R	GATCCTCGAGTCAGAGTGACTGTTGGAGCAAGGAGCCTTT	Cloning
MIC19-F	GATCGGATCCATGGGTGGGACCACCAGCACCCGCGGGTCACCTT	Cloning
MIC19-R	GATCCTCGAGTTATCCTCCCTTCTCAAGCATGCTCTGTTTGGCAT	Cloning
12s-qF	TAGAGGAGCCTGTTCTGTAATCGA	qPCR
12s-qR	TGCGCTTACTTTGTAGCCTTCAT	qPCR
MLH1-qF	GTAGTCTGTGATCTCCGTTT	qPCR
MLH1-qR	ATGTATGAGGTCCTGTCCTA	qPCR
LM-qF	CCCACAGTTTATGTAGCTTACCTCCTCA	qPCR
LM-qR	TTGATTGCTGTACTTGCTTGTAAGCATG	qPCR
SM-qF	CTACACAATCAAAGACGCCC	qPCR
SM-qR	GAATTGTGTAGGCGAATAGG	qPCR
LN-qF	GAACGTCTTGCTCGAGATGTGATGAAGGAG	qPCR
LN-qR	TCTCCACCCATACTGGCAAACTTAAGCC	qPCR
SN-qF	TCACATTGTAGCCCTCTGTG	qPCR
SN-qR	ACACAATAGCTCTTCAGTCTG	qPCR

and imaged on an IX73 inverted microscope (Olympus, Tokyo, Japan). Dead cells were identified by bright co-staining with both blue (Hoechst 33342) and red (PI) fluorescence.

Live cell imaging

For live cell imaging, HEK293 or PS1KO cells were seeded on glass bottom cell culture dishes (801001; Cell-NEST, Wuxi, China) and transfected with indicated plasmids or the control plasmids via Lipofectamine 2000 or PEI MAX. The culture medium was refreshed 6 h after transfection. At 48 h after transfection, the cells were used for live cell imaging to measure mitochondrial functions.

Mitochondrial membrane potential measurement

Cells were incubated with 100 nM TMRM (T668; Thermo Fisher) in DMEM at 37°C and 5% CO₂ for 10 min. As a negative control for TMRM staining, cells were incubated with 20 μM FCCP (HY-100410; MedChem Express, Monmouth Junction, USA) at 37°C and 5% CO₂ for 30 min before TMRM staining. After staining, the cells were washed with PBS three times. The medium was changed to DMEM without phenol red, and all samples were imaged on the IX73 inverted microscope.

Mitochondrial Ca²⁺ measurement

Cells were incubated with 2 μM Rhod 2 AM dye (21064; AAT Bioquest, Sunnyvale, USA) at 37°C and 5% CO₂ for 30 min. After incubation, the cells were washed three times with PBS, and the medium was changed to DMEM without phenol red. All samples were imaged on the IX73 inverted microscope.

Mitochondrial ROS measurement

For mitochondrial ROS measurement, cells were incubated with 1 μM MitoSOX Red (M36008; Thermo Fisher) at 37°C and 5% CO₂

for 30 min. After incubation, the cells were washed three times with PBS, and the medium was changed to DMEM without phenol red. All samples were imaged on the IX73 inverted microscope.

All live-cell fluorescence images from these experiments were analyzed using ImageJ software under a consistent threshold. The fluorescence intensity was averaged to the cell number and normalized to the control group.

Immunofluorescence analysis

For immunofluorescence analysis, HEK293 or PS1KO cells were seeded at the appropriate density on cell culture plates within glass coverslips. After overnight culture at 37°C and 5% CO₂, the cells were transfected at a 1:2 ratio using PEI MAX, and the culture medium was changed after 6 h. After 48 h, the cells were fixed for 30 min using 4% paraformaldehyde (G1101; ServiceBio, Wuhan, China) at room temperature (RT). Then, the cells were permeabilized using 0.3% Triton X-100 in PBS at RT for 30 min. After permeation, the cells were blocked with 10% goat serum in PBS (SL038; Solarbio) at RT for 1 h. After blocking, the cells were incubated with antibody at 4°C overnight. Coverslips were washed with PBS three times for 5 min each at RT. The cells were incubated with fluorescently coupled secondary antibody for 2.5 h at 37°C. After washing with PBS three times for 5 min each at RT, the coverslips were mounted with DAPI-containing mounting medium (ab104139; Abcam, Cambridge, UK). Immunofluorescence images were taken with an LSM900 laser confocal microscope (Zeiss, Jena, Germany). For super-resolution capture, the Airyscan 2 model was chosen with a resolution option. All fluorescence images were analyzed using ImageJ software. Mitochondrial morphology was analyzed using the MitochondriaAnalyzer v2.3.1 plugin with default threshold settings [30]. Mitochondria that were completely fragmented and exhibited perinuclear

clustering were defined as punctation. The MAMs area was quantified in ImageJ using uniform thresholding. The antibodies used in this study are listed in Table 2.

Mitochondrial isolation

Mitochondria were isolated from cells as described previously [31]. For cell mitochondria isolation, 90 dishes of PS1KO cells were transfected with Flag-PS1-wt plasmid at a 1:2 ratio using PEI MAX. After 48 h, the cells were harvested, resuspended in isolation buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, and 30 mM Tris-HCl, pH 7.4), and homogenized with a Teflon glass homogenizer (Wheaton Dounce, Millville, USA). The homogenate was centrifuged twice at 600 g for 5 min at 4°C to collect the supernatant. The supernatant was then centrifuged at 7000 g for 10 min. The pellet (crude mitochondria) was washed twice with starting buffer

(225 mM mannitol, 75 mM sucrose, and 30 mM Tris-HCl, pH 7.4) and resuspended in MRB (250 mM mannitol, 5 mM HEPES, pH 7.4, and 0.5 mM EGTA). The supernatant containing the ER and cytosolic fractions was centrifuged at 20,000 g and 4°C for 30 min. The pellet was discarded, and the obtained supernatant was centrifuged at 100,000 g for 1 h at 4°C. The resulting pellet is the ER fraction, and the supernatant is the cytosolic fraction. The pellet was resuspended in lysis buffer (with protease inhibitor cocktail) for subsequent WB analysis.

For mouse brain mitochondria isolation, 6-month-old wild-type mice were sacrificed after anesthetization, and the brain was collected. The brain was removed and immediately rinsed three times with ice-cold start buffer (225 mM mannitol, 75 mM sucrose, and 30 mM Tris-HCl, pH 7.4). The brain tissue was then minced and homogenized in isolation buffer (225 mM mannitol, 75 mM sucrose,

Table 2. Antibodies used in this study

Antibody	Dilution	Source	Identifier
Rabbit anti-Flag	1:2000 for WB 1:200 for IF 1:100 for IEM	Thermo Fisher	PA1-984B
Rabbit anti-Myc tag	1:2000 for WB 1:200 for IF 1:200 for co-IP	Bethyl Laboratories	A190-105A
Rabbit anti-TIM23	1:1000 for WB 1:100 for IF	Proteintech	11123-1-AP
Rabbit anti-PS1-NTF	1:1000 for WB	Cell Signaling Technology	87146
Rabbit anti-PS1-CTF	1:1000 for WB	Abcam	ab76083
Mouse anti-Calnexin	1:2000 for WB	Abcam	ab22595
Mouse anti-ACSL4	1:1000 for WB	Santacruz	sc-271800
Mouse anti-Tubulin	1:5000 for WB	Sigma	T5168
Rabbit anti-ATAD3A	1:1000 for WB 1:100 for co-IP	ABclonal	A8230
Rabbit anti-MIC60	1:2000 for WB	ABclonal	A14107
Rabbit anti-MFN2	1:1000 for WB	Santa Cruz	sc-50331
Rabbit anti-TFAM	1:1000 for WB	Proteintech	22586-1-AP
Mouse anti-OPA1	1:1000 for WB 1:100 for co-IP	BD Bioscience	612607
Mouse anti-TOM20	1:1000 for WB	BD Bioscience	612278
Mouse anti-Actin	1:5000 for WB	Proteintech	20536-1-AP
Mouse anti-DNA	1:100 for IF	Progene	61014
Rabbit anti-TOM70	1:1000 for WB	Proteintech	14528-1-AP
Mouse anti-VDAC1	1:1000 for WB	Abcam	ab14734
Mouse anti-IP3R-II	1:1000 for WB	Santa Cruz	sc-398434
Mouse anti-cytochrome c	1:1000 for WB	Santa Cruz	sc-13156
Peroxidase AffiniPure® goat anti-mouse IgG(H+L)	1:20,000 for WB	Jackson ImmunoResearch Laboratories	115-035-003
Peroxidase AffiniPure® goat anti-rabbit IgG(H+L)	1:20,000 for WB	Jackson ImmunoResearch Laboratories	111-035-003
CoraLite594-conjugated goat anti-rabbit IgG(H+L)	1:800 for IF	Proteintech	SA00013-4
CoraLite488-conjugated goat anti-mouse IgG(H+L)	1:800 for IF	Proteintech	SA00013-1
12 nm Colloidal Gold AffiniPure® goat anti-rabbit IgG(H+L) (EM grade)	1:25 for IEM	Jackson ImmunoResearch Laboratories	111-205-144

0.1 mM EGTA, and 30 mM Tris-HCl, pH 7.4) at a ratio of 4 mL per gram of tissue using a Teflon-glass homogenizer with 20 up-and-down strokes. The homogenate was centrifuged at 700 *g* for 5 min at 4°C. The supernatant was collected and centrifuged at 700 *g* for 5 min at 4°C. The resulting supernatant was further centrifuged at 9000 *g* for 10 min at 4°C. The pellet obtained represented the crude mitochondrial fraction, and the supernatant was collected for ER isolation as previously described. After washing twice with start buffer, the crude mitochondria were resuspended in MRB. The suspension of cell or mouse brain crude mitochondria was layered over Percoll medium (225 mM mannitol, 25 mM HEPES, pH 7.4, 1 mM EGTA, and 30% Percoll) and centrifuged at 95,000 *g* for 30 min at 4°C. The mitochondrial fraction (bottom layer) and MAMs fraction (middle layer) were collected, resuspended in MRB at a 1:10 ratio, and centrifuged twice at 9000 *g* for 10 min at 4°C. The mitochondria and MAMs were lysed in cell lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail) for subsequent WB analysis. Protein concentrations of all fractions were determined by BCA assay for subsequent WB analysis. Wild-type C57BL/6JNifdc mice were obtained from Vital River (Beijing, China). All experimental procedures were conducted in accordance with the animal ethics guidelines of Shandong University (ethics approval number: ECSBMSSDU2024-2-247).

Sub-mitochondrial fraction

For sub-mitochondrial compartment fractionation [32], purified mitochondria were resuspended in isolation buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, and 30 mM Tris-HCl, pH 7.4) containing digitonin (0.12 mg of digitonin per mg of mitochondria) and incubated with rotation at 4°C for 15 min. Subsequently, a threefold volume of isolation buffer was added, and the mixture was centrifuged at 9000 *g* and 4°C for 10 min to yield an initial supernatant (Supernatant 1) and a pellet (Pellet 1). Supernatant 1 was ultracentrifuged at 144,000 *g* and 4°C for 1 h. The resulting pellet (Pellet 2) was lysed with an appropriate volume of lysis buffer as the outer mitochondrial membrane (OMM). The supernatant (Supernatant 2) was concentrated by a TCA Protein Precipitation kit (C510011; Sangon Biotech, Shanghai, China) as an inter-membrane space protein (IMS). Pellet 1 was resuspended in isolation buffer and subjected to sonication on ice (3 s/2 s on/off, total 30 s). The sonicated sample was centrifuged at 6500 *g* for 10 min at 4°C, and the resulting supernatant (Supernatant 3) was further ultracentrifuged at 144,000 *g* for 1 h at 4°C. The pellet (Pellet 4), representing the inner membrane fraction, was lysed in an appropriate volume of lysis buffer, while the supernatant (Supernatant 4) contained matrix proteins and was concentrated using the TCA Protein Precipitation kit for subsequent analysis.

Mitochondrial alkali assay

Crude mitochondria were treated with 0.1 M Na₂CO₃ on ice for 30 min. After centrifugation at 16,000 *g* for 15 min at 4°C, the supernatant (nonmembrane fraction) was collected, and the pellet (mitochondrial membrane fraction) was washed with MRB and resuspended in cell lysis buffer for WB analysis.

Mitochondrial proteinase K assay

Crude mitochondria were diluted to 1 µg/µL in MRB. Proteinase K (final concentration 800 ng/µL) was added with or without 0.2% Triton X-100 and incubated at RT for 15 min. The reaction was

terminated by adding PMSF, and samples were analyzed by WB analysis.

BCA assay

Protein concentrations were determined using a BCA Protein Assay kit (CW0014S; CWBIO, Taizhou, China) according to the manufacturer's instructions. Briefly, BSA standards (0–2 mg/mL) were prepared in the same lysis buffer used for samples. The BCA working reagent was prepared by mixing Reagent A and Reagent B at a ratio of 50:1. Twenty microliters of each standard or sample were dispensed into a 96-well plate in triplicate, followed by addition of 150 µL of working reagent. After incubation at 37°C for 30 min, the absorbance at 562 nm was measured using a microplate reader. Protein concentrations were calculated from the standard curve.

WB analysis

Cells were lysed in cell lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, and protease inhibitor cocktail) for 30 min at 4°C, followed by centrifugation at 16,000 *g* for 10 min at 4°C. The protein concentration was determined by BCA assay. Samples were mixed with 5× loading buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 0.5% BPB, 50% glycerol, and 5% β-mercaptoethanol), heated at 50°C for 8 min, and subjected to 10% SDS-PAGE at 30 mA for 2 h. For ATAD3A oligomerization detection, samples were mixed with 5× loading buffer without β-mercaptoethanol and analyzed by SDS-PAGE. Proteins were then transferred onto 0.45 µm PVDF membranes at 300 mA for 90 min. After a brief wash with TBST, the membranes were blocked with 5% non-fat milk in TBST for 1 h at RT. The membranes were incubated overnight at 4°C with primary antibodies diluted in 1% BSA in TBST. After three washes with TBST (10 min each), the membranes were incubated with HRP-conjugated secondary antibodies diluted in 0.2% non-fat milk for 1 h at RT, followed by three additional TBST washes (10 min each). Immunoreactive bands were visualized using the SuperPico ECL Chemiluminescence kit (E422-01; Vazyme, Nanjing, China) and a Tanon 5200 imaging system (Tanon, Shanghai, China). Band intensities were quantified using ImageJ software after background subtraction, and the integrated density of each band was measured. The values were normalized to the loading control and further normalized to the control group. The antibodies used in this study are listed in Table 2.

mtDNA copy number analysis

Total cellular DNA was extracted using a cell DNA isolation kit (DC102; Vazyme). Quantitative real-time PCR (qPCR) was performed on a LightCycler 96 instrument (Roche, Basel, Switzerland) using the SuperStar Universal SYBR Master Mix (CW3360M; CWBIO). mtDNA was amplified with primers targeting the mitochondrial 12S *rRNA* gene, nuclear DNA was amplified using primers targeting *MLH1* gene as an internal control. The mtDNA copy number was calculated using the 2^{−ΔΔCt} method, where ΔCt = Ct(12S) – Ct(MLH1). All values were normalized to the control group. The primers used in this study are listed in Table 1.

DNA repair assay

Cells were treated with 150 µM H₂O₂ for 30 min at 37°C, and the mtDNA damage level was quantified through comparative ampli-

fication efficiency analysis of long-range mtDNA fragments (10 kb), as described previously [33]. Total cellular DNA was extracted at various time points using the cell DNA isolation kit. DNA damage and repair were assessed by qPCR using primers for large and small mitochondrial and nuclear fragments. The extent of DNA damage and repair was calculated by comparing the copy numbers of large fragments to small fragments using the $2^{-\Delta\Delta C_t}$ method. qPCR was performed using the SuperStar Universal SYBR Master Mix on an LC960 instrument (Roche). The primer sets used were: large mitochondrial fragment (LM-F/LM-R), small mitochondrial fragment (SM-F/SM-R), large nuclear fragment (LN-F/LN-R), and small nuclear fragment (SN-F/SN-R). The primers used in this study are listed in Table 1.

Immunoprecipitation and co-immunoprecipitation

For the identification of PS1-interacting proteins, PS1KO cells were reconstituted with Flag-tagged wild-type PS1 (Flag-PS1wt). Cells were lysed in cell lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, and protease inhibitor cocktail) for 30 min at 4°C, followed by centrifugation. The supernatant was incubated overnight with Anti-DYKDDDDK Tag magnetic beads (B26102; Selleck, Shanghai, China). The beads were then washed five times with cell lysis buffer, resuspended in 1× loading buffer, heated at 50°C for 8 min, and analyzed by WB analysis. To explore PS1-interacting proteins, the Flag-PS1wt immunoprecipitate and a negative control were subjected to LC-MS/MS (Genechem, Shanghai, China) for identification, followed by Gene Ontology (GO) enrichment analysis for Cellular Component (CC) terms using Metascape [34] to specifically profile mitochondrial proteins that interact with PS1. For more information, see the manufacturer's website (<https://metascape.org/>).

For co-immunoprecipitation (co-IP) experiments, cells were lysed as described above. One-tenth of the supernatant was mixed with 5× loading buffer and heated at 50°C for 8 min as the input sample. The remaining supernatant was pre-cleared with Protein A/G magnetic beads (B23202; Selleck) for 20 min at 4°C. After removal of the pre-clearing beads, the supernatant was incubated overnight with the corresponding antibody, followed by incubation with fresh Protein A/G magnetic beads for 2 h at 4°C. The beads were washed five times with cell lysis buffer, resuspended in 1× loading buffer, and heated at 50°C for 8 min. Both input and IP samples were analyzed by WB analysis. All co-IP experiments were performed in at least three independent replicates, and the IP efficiency was normalized to the control group in each experiment. The antibodies used in this study are listed in Table 2.

Transmission electron microscopy

For transmission electron microscopy (TEM), PS1KO cells re-expressing Flag-tagged wild-type PS1 were collected by centrifugation, fixed in electron microscopy fixative (G1102; Servicebio) at 4°C, washed with 0.1 M phosphate buffer (pH 7.4), embedded in 1% agarose, and postfixed with 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 2 h, washed and dehydrated through a graded ethanol series (30%, 50%, 70%, 80%, 95%, and 100%) followed by two changes of acetone. Samples were infiltrated with EMBed 812 resin using graded acetone-resin mixtures (1:1 and 1:2) and pure resin, embedded in pure resin, and polymerized at 60°C for 48 h. Ultrathin sections (60–80 nm) were collected on Formvar-coated 150-mesh copper grids and stained with 2% uranyl acetate and 2.6% lead

citrate. Samples were imaged using an HT7800/HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

Immunoelectron microscopy

For immuno-electron microscopy (IEM), PS1KO cells re-expressing Flag-tagged wild-type PS1 were collected, fixed in immunoelectron microscopy fixative (G1124; Servicebio) at 4°C in the dark, and washed with ice-cold 0.1 M phosphate buffer (pH 7.4). The fixed cells were embedded in 2% low-melting-point agarose (A8350; Solarbio), dehydrated through a graded ethanol series (30% to 100%) at 4°C to −20°C, and infiltrated with LR White resin (14385; HaideBio, Beijing, China) using graded mixtures of ethanol and resin, followed by pure resin changes. Samples were embedded in capsules, degassed under vacuum, and polymerized under UV light at −20°C for > 48 h. Ultrathin sections (70–80 nm) were collected on formvar-coated nickel grids, blocked with 1% BSA (A8851; Solarbio), and incubated overnight at 4°C with primary anti-Flag antibody diluted (1:100) in 1% BSA/TBS, followed by gold-conjugated secondary antibody (1:25 diluted) incubation (20 min at RT, then 1 h at 37°C). After washing, the grids were stained with 2% uranyl acetate for 8 min in the dark, dried, and observed under the HT7800 transmission electron microscope, with 12-nm gold particles indicating specific labeling.

Mitochondrial oxygen respiration capacity assay

Mitochondrial oxygen respiration capacity was assessed using the XF Cell Mito Stress Test kit (103015-100; Agilent Technologies). A total of 1500 cells were seeded in XFp 24-well microplates with 250 μL of growth medium (DMEM + 10% FBS + 1% penicillin-streptomycin) per well and cultured overnight. The oxygen consumption rate (OCR) was measured using the Agilent Seahorse XFp24 instrument, with sequential additions of oligomycin (1.5 μM), FCCP (1.5 μM), and rotenone/antimycin A (0.5 μM). Basal respiration was defined as the OCR before the addition of any modulators. ATP production was calculated by subtracting the OCR after oligomycin treatment from the basal OCR. Proton leak was determined as the OCR remaining after oligomycin treatment minus the non-mitochondrial respiration (residual OCR after rotenone/antimycin A treatment). After testing, the cells in each well were lysed on ice for 15 min using lysis buffer. The protein concentration of each lysate was determined by BCA assay, and the obtained oxygen consumption rate (OCR) values were normalized to the total protein content.

Peptide inhibitor

To inhibit ATAD3A oligomerization, we synthesized the peptide inhibitor DA1 (GRKKRRQRRRPQ-GG-EDKRKT-NH₂) and the comparative control peptide TAT (GRKKRRQRRRPQ-GG-NH₂) [29]. Peptides were synthesized at Genechem (Shanghai, China). The purity was assessed as > 90% by mass spectrometry. PS1KO cells were cultured with 1 μM DA1 for at least 48 h to decrease ATAD3A oligomerization.

Statistical analysis

Data are presented as the mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad Prism 8.0 software. Student's *t*-test was used for comparisons between two groups, whereas one-way analysis of variance (one-way ANOVA) was applied for comparisons among multiple groups. $P < 0.05$ was

considered statistically significant.

Results

PS1 is located in the mitochondrial inner membrane

Although earlier studies have reported the presence of PS1 in the mitochondrial membrane, its specific localization, whether on outer or inner mitochondrial membrane, remains unclear. To address this, we used density gradient centrifugation to isolate mitochondria, MAM, and ER membrane fractions from mouse brain and from PS1KO cells re-expressing Flag-PS1wt, and then assessed PS1 distribution in these fractions. As shown in Figure 1A,B, PS1 was primarily detected in the ER and MAM fractions; moreover, full-length (FL) PS1, N-terminal (NTF) PS1 and C-terminal (CTF) PS1 could also be detected in mitochondrial fractions. Confocal microscopy further confirmed the partial co-localization of PS1 with TIM23, a marker of the mitochondrial inner membrane (Figure 1C). To further delineate the sub-mitochondrial localization of PS1, we performed an alkali assay, which collected mitochondrial membrane and nonmembrane fractions, confirming PS1 distribution in the mitochondrial membrane fraction (Figure 1D). Addi-

tionally, PS1 could not be digested by proteinase K when the mitochondrial membrane structure was intact but could be digested in the presence of both Triton-X 100 and proteinase K (Figure 1E), supporting its localization in the mitochondrial inner membrane. To definitively establish the sub-mitochondrial localization of PS1, we performed organelle fractionation, which verified its specific residency within the inner mitochondrial membrane (Figure 1F). Finally, through immunoelectron microscopy, we directly visualized PS1 in the mitochondrial inner membrane (Figure 1G-I). Collectively, these results suggested that PS1 could localize in the mitochondrial inner membrane.

PS1 deficiency impairs mitochondrial function

Mitochondria are essential organelles in eukaryotic cells, playing central roles in energy metabolism, ROS production, and Ca^{2+} homeostasis regulation [15,16,18,19,35]. Having demonstrated the localization of PS1 in the mitochondrial inner membrane, we next evaluated the impact of PS1 on mitochondrial function. PS1 knockout HEK293 cell lines were generated using CRISPR/Cas9, and the absence of PS1 protein was confirmed by WB analysis

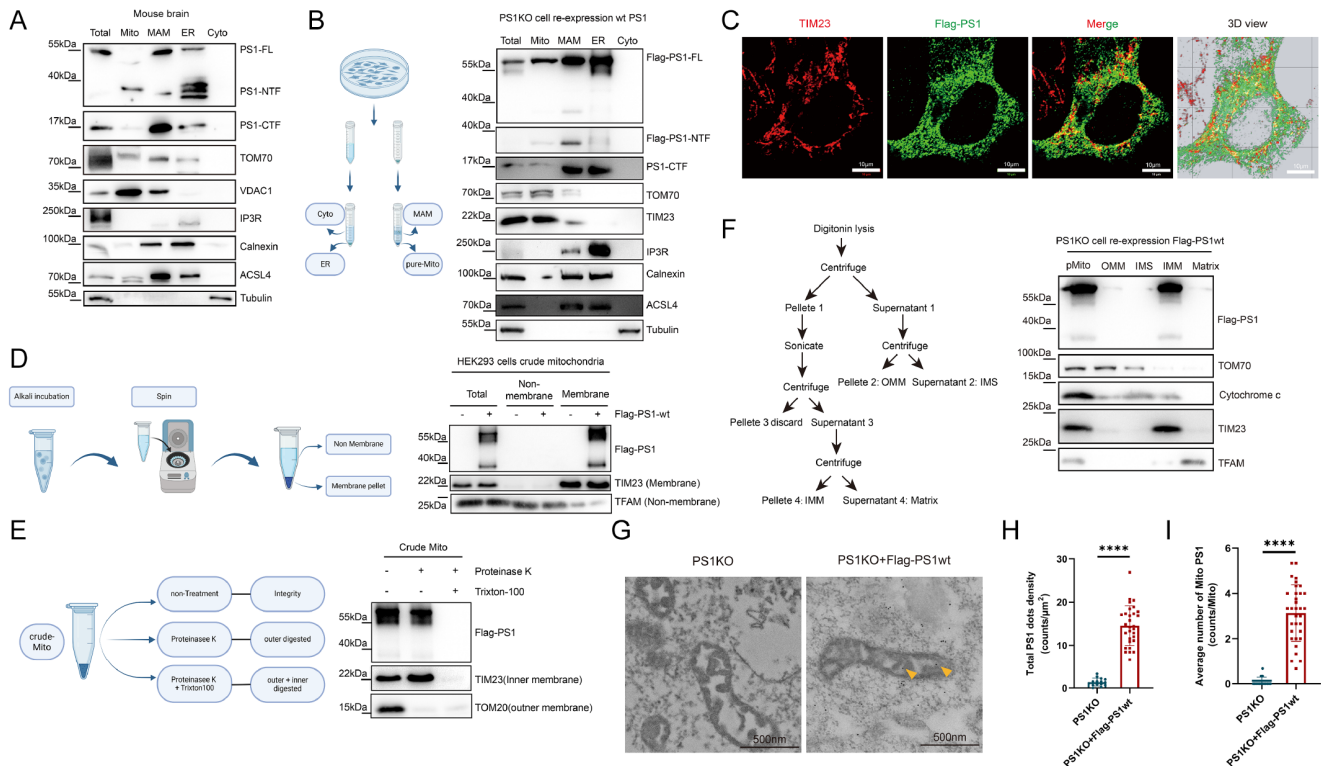


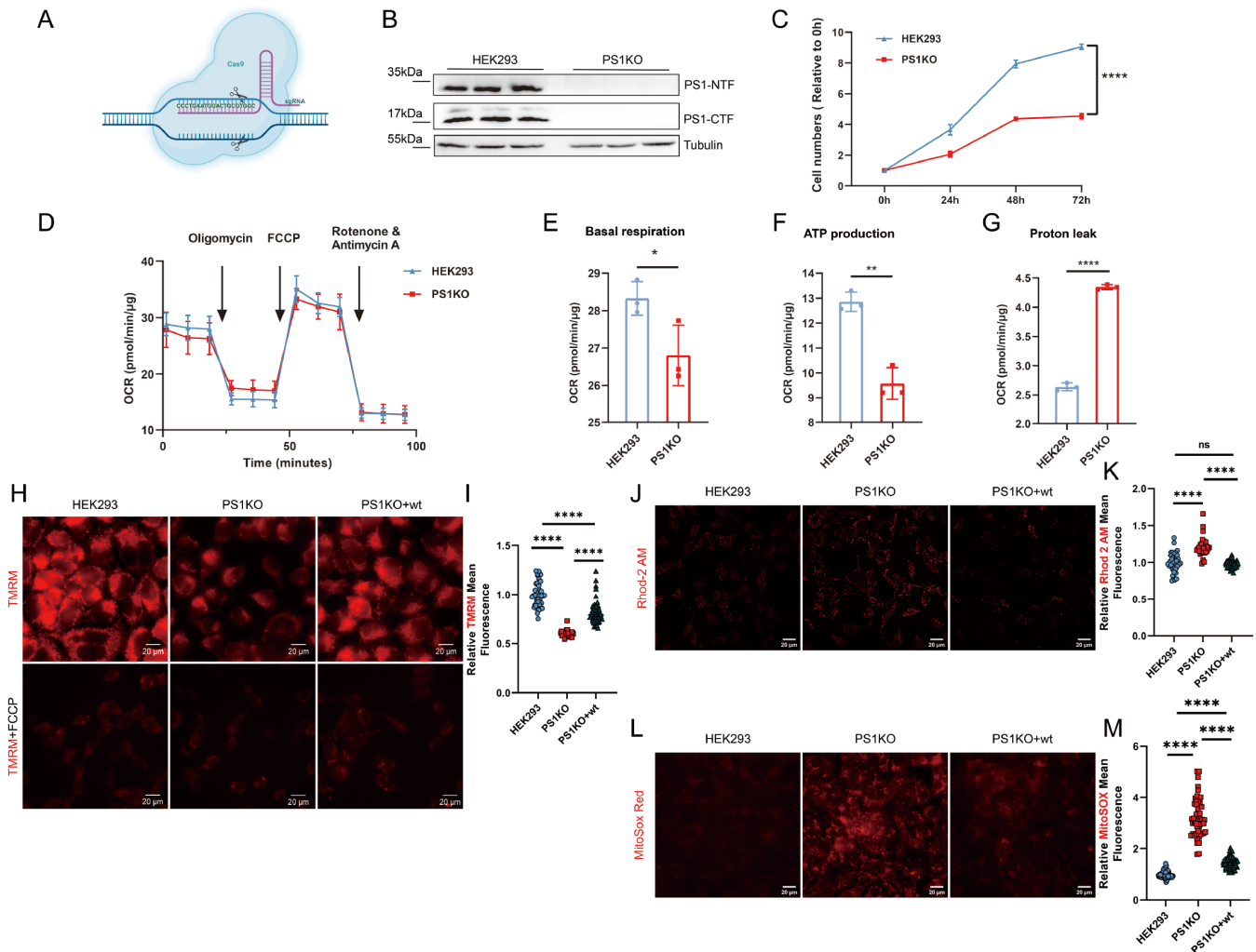
Figure 1. PS1 is located in the mitochondrial inner membrane (A,B) Subcellular fractions isolated from mouse brain (A) or HEK293 cells (B) were analyzed by WB analysis to determine PS1 localization. Total: total cell lysate; Mito: pure mitochondrial; MAM: mitochondrial-associated membrane; ER: endoplasmic reticulum; Cyto: cytosol. Markers: ACSL4 (MAMs), IP3R & Calnexin (ER), VDAC1, TOM70 & TIM23 (Mitochondria), Tubulin (Cytosol). (C) Immunofluorescence shows co-localization of PS1 and the mitochondrial inner membrane protein TIM23. Scale bar: 20 μm . (D) Crude mitochondria were prepared and subjected to alkali extraction. The resulting fractions were then analyzed by WB analysis to assess mitochondrial membrane and non-membrane proteins. (E) Crude mitochondria from HEK293 cells were incubated with proteinase K in the presence or absence of Triton X-100, followed by WB analysis. (F) WB analysis of sub-mitochondrial fractions indicates the inner membrane-localized PS1. Left: Schematic diagram of sub-mitochondrial fractionation by digitonin lysis. Right: pMito, pure mitochondria; OMM, outer mitochondrial membrane; IMS, inter-membrane space; IMM, inner mitochondrial membrane. Markers: TOM70 (OMM), Cytochrome c (IMS), TIM23 (IMM), and TFAM (matrix). (G) Immunoelectron microscopy shows that PS1 is located in the mitochondrial inner membrane. The arrow indicates the PS1 golden particles. Images were acquired at 25,000 $\times$ magnification. Scale bar: 500 nm. (H) Total density of PS1-specific immunogold particles (counts per μm^2). (I) Average number of PS1-labeled gold particles per mitochondrion (mitochondrial PS1 dots per mitochondrion). Analysis information: PS1KO cells, 16 fields of view; PS1KO + Flag-PS1wt cells, 30 fields of view. Data are presented as the mean $\pm$ SD. **** $P < 0.0001$.

(Figure 2A,B). CCK-8 assay showed significantly reduced proliferation of PS1KO cells (Figure 2C). Seahorse analysis revealed that *PS1* deficiency significantly reduced basal respiration and ATP production while increasing proton leak (Figure 2D–G). PS1KO cells displayed a significant decrease in mitochondrial membrane potential, which was rescued by re-expressing Flag-PS1wt (Figure 2H,I). Using mitochondrial-targeted Ca^{2+} and ROS probes, we observed elevated mitochondrial Ca^{2+} level and enhanced mitochondrial ROS generation in PS1KO cells (Figure 2J–M), indicating disrupted Ca^{2+} homeostasis and ROS production. Re-expression of Flag-PS1wt in PS1KO cells attenuated these abnormalities in Ca^{2+} and ROS. In summary, *PS1* deficiency compromises mitochondrial

bioenergetic metabolism, resulting in suppressed cell proliferation, reduced ATP production, disrupted mitochondrial calcium homeostasis, and excessive ROS accumulation. These findings underscore the role of PS1 in regulating mitochondrial physiology.

Loss of *PS1* leads to abnormal mitochondrial morphology and vacuolation

Maintenance of functional competence in mitochondria depends on their structural integrity [19]. To determine the effects of *PS1* deficiency on mitochondrial structural consequences, we investigated mitochondrial morphology in PS1KO cells using super-resolution imaging with inner membrane-targeted fluorescent



probes. The results demonstrated that mitochondria in PS1KO cells exhibited a significant reduction in mitochondrial area (Figure 3A,B) and total branch length (Figure 3A,C), an increase in vacuolated mitochondria (Figure 3A,D), and a decreased aspect ratio (Figure 3A,E). The re-expression of Flag-PS1wt restored these morphological defects (Figure 3A–E). We then performed ultrastructural and morphological analyses using transmission electron microscopy, which revealed pronounced abnormalities in the mitochondrial inner membrane of PS1KO cells, including a decreased number of cristae and swollen cristae morphology; and notably, re-expression of wild-type PS1 in PS1KO cells significantly ameliorated these cristae defects (Figure 3F–I). Together, these findings demonstrate that *PS1* deficiency disrupts both global mitochondrial architecture and the ultrastructure of the inner membrane, thereby providing a structural basis for the accompanying functional impairments.

Loss of *PS1* promotes mitochondrial inner membrane protein ATAD3A oligomerization

To explore the molecular mechanism underlying mitochondrial inner membrane damage upon *PS1* deficiency, we identified PS1-interacting proteins through immunoprecipitation coupled with mass spectrometry. GO cellular component (CC) enrichment analysis using Metascape [34] (<https://metascape.org/>) revealed that a number of interactors were mitochondrial proteins (Figure

4A), with the majority residing in the inner membrane (Figure 4B–D). Since mitochondrial membrane proteins are essential for cristae arrangement, we selected several for further validation. co-IP confirmed that PS1 interacts with the mitochondrial inner membrane proteins OPA1, MIC60, ATAD3A and TIM23, as well as the mitochondrial matrix protein TFAM (Figure 4C), all of which contribute to the maintenance of mitochondrial inner membrane architecture. In contrast, PS1 did not interact with the outer mitochondrial membrane proteins TOM70 and MFN2 (Figure 4C). We next assessed whether *PS1* deficiency alters mitochondrial protein expression. WB analysis revealed that loss of *PS1* led to decreased MFN2 and TIM23 but increased ATAD3A in total cell lysate (Figure 4D,E). ATAD3A is a mitochondrial membrane protein with C-terminus in the mitochondrial inner membrane and N-terminus spanning the outer membrane. It interacts with the MICOS complex and TFAM to maintain normal inner membrane structure and mtDNA stability [22,24,28]. In AD models, ATAD3A level changes impair mitochondrial and MAMs function [28,29]. We further focused on ATAD3A and found that *PS1* deficiency not only increased its protein level but also promoted its oligomerization, as detected by SDS-PAGE under non-reducing conditions (without β -mercaptoethanol) (Figure 4F–H). ATAD3A oligomerization has been implicated in mitochondrial dysfunction in AD, suggesting that mitochondrial damage induced by *PS1* deficiency may be associated with increased ATAD3A levels and oligomerization.

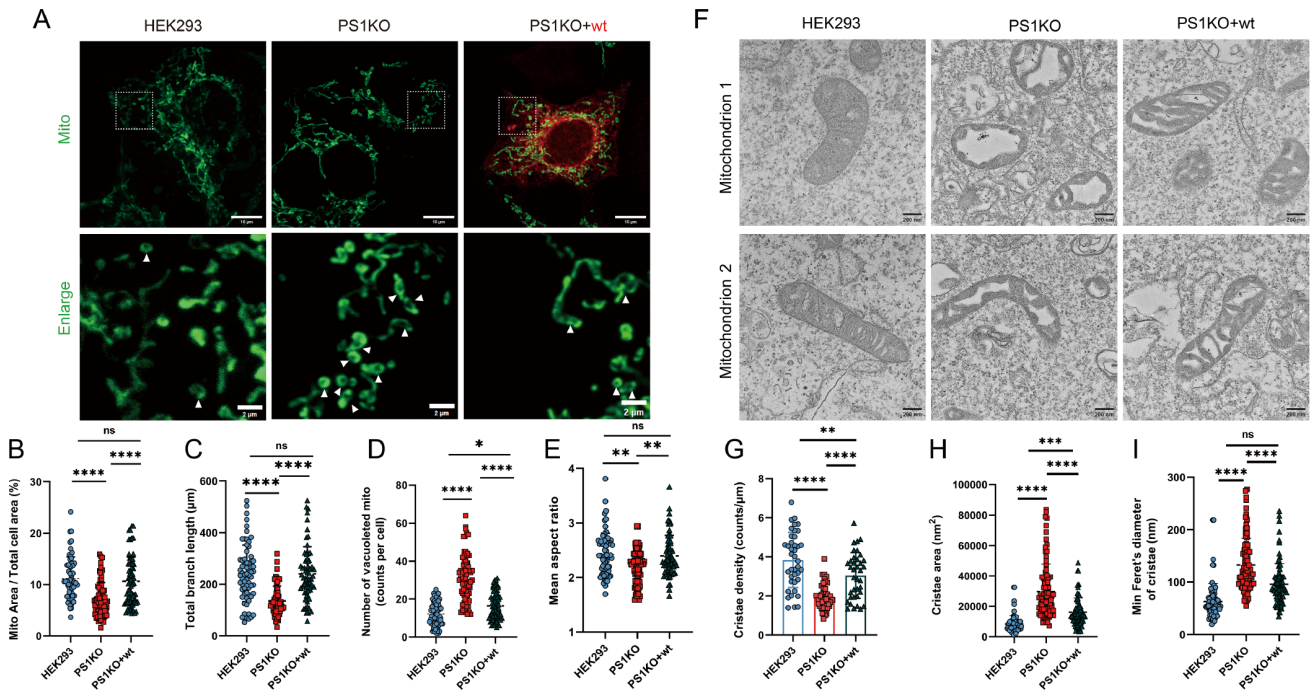


Figure 3. Loss of *PS1* leads to mitochondrial vacuolation and fragmentation (A) Mitochondrial morphology was analyzed by Mito-EGFP labeling. White arrows indicate vacuolated mitochondria. Scale bar: 2 μ m. (B–E) Quantification of mitochondrial morphology. (B) Percentage of mitochondrial area relative to total cell area per cell. (C) Total branch length of single-cell mitochondria. (D) Number of vacuolated mitochondria per cell. (E) Mean aspect ratio of cell mitochondria. Analysis information: HEK293 cells, $n = 58$; PS1KO cells, $n = 76$; PS1KO + wt cells, $n = 70$. (F) Transmission electron microscopy revealed swelling of the inner mitochondrial membrane in PS1KO cells. The upper and lower panels are from the same experiment; the lower image shows that although tubular mitochondria are still present in PS1KO cells, they exhibit damaged cristae architecture. Scale bar: 200 nm. (G) Cristae density of mitochondria (number of cristae per mitochondrial perimeter). (H) The mitochondrial cristae area was increased in PS1KO cells. (I) The Min Feret's diameter of cristae is elongated in PS1KO mitochondria, indicating a swollen cristae morphology. Analysis information: HEK293 cells, $n = 40$ and mitochondria; PS1KO cells, $n = 43$ and mitochondria; PS1KO + wt cells, $n = 37$ and mitochondria. Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant.

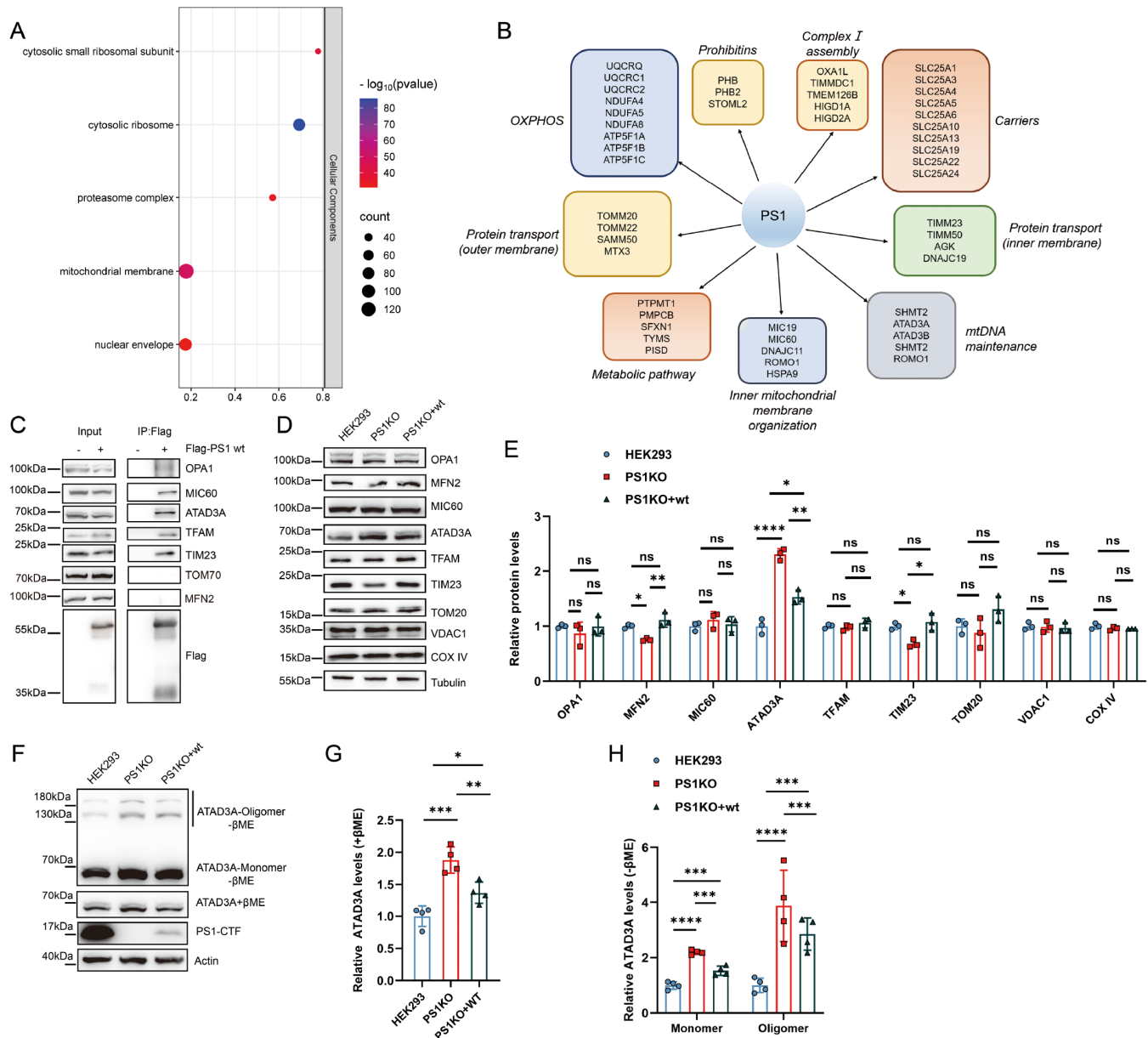


Figure 4. Loss of PS1 promotes mitochondrial inner membrane protein ATAD3A oligomerization (A,B) GO-cell compartment analysis indicated that mitochondrial membrane proteins interact with PS1. (C) Co-IP confirmed the interaction between PS1 and mitochondrial inner membrane and matrix proteins but not with the outer mitochondrial membrane proteins TOM70 or MFN2. (D) Analysis of mitochondrial protein alterations in total cell lysates by WB analysis. (E) Quantification of mitochondrial protein levels in total cell lysate. (F) ATAD3A protein levels in total cell lysate were determined by WB analysis with anti-ATAD3A antibody in the presence or absence of β -mercaptoethanol (β ME). (G,H) Quantification of ATAD3A monomer and oligomer levels. Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant.

ATAD3A oligomerization in PS1 knockout cells leads to expanded MAM area and mtDNA repair dysfunction

Given that ATAD3A oligomerization has been shown to promote the expansion of MAM structure and disrupt mitochondrial and ER function [22,27,36], we asked whether the MAM structure is altered in PS1KO cells. Using the SPLICS Mt-ER Long P2A plasmid for labeling, we observed a significant increase in the MAM area in PS1KO cells (Figure 5A,B). Since mtDNA integrity is closely linked to mitochondrial structure, we also examined mtDNA copy number and found it to be significantly reduced in PS1KO cells (Figure 5C). Re-expression of wild-type PS in PS1KO cells restored MAMs and

mtDNA copy number to level comparable to those in wild-type HEK293 cells (Figure 5A–C). Consistent with this, immunostaining with an anti-DNA antibody revealed fewer mtDNA nucleoids in PS1KO cells (Figure 5D,E). We next evaluated the sensitivity of PS1KO cells to oxidative stress. Following H_2O_2 -induced DNA damage, PS1KO cells exhibited defective mtDNA repair (Figure 5F, G), whereas nuclear DNA repair was unaffected and completed within one hour of recovery (Figure 5H).

To determine whether the mitochondrial structural defects, MAM expansion, and mtDNA damage in PS1KO cells are caused by the decrease in MFN2/TIM23 or by the elevation and oligomerization of

ATAD3A, we performed rescue experiments by overexpressing MFN2 or TIM23 and by knocking down *ATAD3A* with siRNA in PS1KO cells. We first found that overexpression of MFN2 or TIM23 in PS1KO cells induced severe cytotoxicity and reduced cell viability, whereas *ATAD3A* knockdown had no obvious effect on survival (Supplementary Figure S1A–F). Correspondingly, overexpression of MFN2 or TIM23 failed to rescue the decreased mtDNA copy number (Figure 6A) or the loss of mitochondrial membrane potential (Figure 6B,C) in PS1KO cells. In contrast, *ATAD3A* knockdown in PS1KO cells significantly increased mtDNA copy number (Figure 6A) and recovered mitochondrial membrane potential (Figure 6B,C). Regarding mitochondrial morphology, MFN2 overexpression in HEK293 cells predominantly induced the expected hyperfused, elongated mitochondrial morphology, with only a minor fraction of cells exhibiting fragmented mitochondria (Supplementary Figure S1J,K). In contrast, MFN2 overexpression in PS1KO cells led to severe mitochondrial aggregation and fragmentation (Figure 6D–F and Supplementary Figure S1J,K), suggesting that *PS1* deficiency may disrupt the balance of mitochondrial fusion and fission, rendering cells susceptible to MFN2-induced cytotoxicity. *ATAD3A* knockdown, however, did not induce mitochondrial fragmentation (Figure 6D–F and Supplementary Figure S1J,K). Additionally, *ATAD3A* knockdown markedly reduced the expanded MAM area, while overexpression of MFN2 or TIM23 further enlarged MAMs area (Figure 6G,H). Collectively, these results indicate that the mitochondrial structural damage, MAMs expansion, and mtDNA impairment in PS1KO cells are primarily driven by the upregulation and oligomerization of ATAD3A, rather than by the reduction of MFN2 or TIM23.

Considering the effects of ATAD3A oligomerization on MAMs regulation and mtDNA maintenance [22,24,28,29,37], we hypothesized that *PS1* deficiency impairs mitochondrial functions through increased level and oligomerization of ATAD3A. To confirm this, we generated a peptide DA1, corresponding to residues 113–118 of the C-terminal region of ATAD3A, which interacts with the ATAD3A coiled-coil domain and decreases its oligomerization [28,29]. Treatment with DA1, but not the control TAT peptide, significantly decreased the oligomerization of ATAD3A in PS1KO cells (Figure 6I,J) and restored mtDNA copy number (Figure 6K). Furthermore, DA1 treatment also normalized the MAM area in PS1KO cells (Figure 6L,M) and restored mitochondrial morphology (Figure 6N,O).

Together, these findings indicate that *PS1* deficiency could increase oligomerization of ATAD3A, which in turn drives the MAMs expansion area and directly compromises mitochondrial structure and mtDNA maintenance.

Loss of *PS1* disturbs mitochondrial cristae junction protein arrangement

The maintenance of intact cristae structure requires proper assembly of protein complexes in the mitochondrial inner membrane, such as the MICOS complex, and the interaction between MICOS and other proteins, such as OPA1 and ATAD3A [19,22,38]. To investigate the mechanism by which ATAD3A oligomerization impairs mitochondrial function in PS1KO cells, we further performed co-IP to assess the interactions between ATAD3A and components of the MICOS complex, OPA1 and other partners in both PS1KO and PS1-re-expressing cells. We found that *PS1* deficiency increased the interactions of ATAD3A with MIC60,

MIC19 and OPA1 (Figure 7A–F), while leaving the ATAD3A-ATPase ATP5A interaction unchanged (Figure 7G,H). Re-expression of Flag-PS1wt restored these interactions to normal levels (Figure 7A–F). Similarly, DA1 treatment, which ameliorates mitochondrial and MAM dysfunction in PS1KO cells, also normalized the exaggerated interaction between ATAD3A and MIC60, MIC19, and OPA1 (Figure 7I,J). Based on these results, we propose a model in which mitochondrial inner membrane-localized PS1 coordinates proper protein arrangement. Loss of *PS1* leads to increased ATAD3A level and oligomerization, which in turn disrupts its physiological binding with cristae junction proteins such as MIC60, MIC19, and OPA1, thereby compromising the structural integrity of the mitochondrial inner membrane (Figure 7K). These data indicate that PS1 itself, by orchestrating the proper arrangement of cristae junction proteins, functions as a pivotal upstream regulator of mitochondrial inner membrane architecture. Hence, targeting PS1 activity or its downstream cristae organization may provide novel therapeutic strategies for counteracting mitochondrial damage in Alzheimer's disease.

Discussion

In this study, we demonstrated that PS1 localizes in the mitochondrial inner membrane and regulates the arrangement of cristae junction architecture. Loss of *PS1* induced mitochondrial vacuolization and dysfunction, characterized by reduced mitochondrial membrane potential, impaired oxidative phosphorylation, and decreased ATP production, accompanied by mitochondrial calcium overload and ROS accumulation. Further mechanistic investigation revealed that these structural and functional deficits arise from upregulation and oligomerization of the key mitochondrial protein ATAD3A. Specifically, *PS1* knockout elevated ATAD3A protein expression, promoted its oligomerization, expanded MAM areas, and compromised mtDNA stability. These findings uncover a γ -secretase-independent role of PS1 in modulating mitochondrial morphology and function, potentially representing a critical mechanism underlying mitochondrial dysfunction in AD pathogenesis.

A key finding of this study is that PS1 localizes in the mitochondrial inner membrane and plays an important role in maintaining cristae morphology. Although the mitochondrial localization of PS1 has been previously suggested [11], we provide substantial evidence that a fraction of PS1 resides specifically in the mitochondrial inner membrane. We propose that PS1, ATAD3A and MICOS form a supercomplex within the inner membrane. In this model, inner membrane-localized PS1 may help stabilize ATAD3A, thereby contributing to cristae junction integrity. Loss of *PS1* could release ATAD3A from the inner membrane, promoting its oligomerization at the outer membrane and MAM regions and enhancing its interaction with MIC60 and Mic19-core components of the MICOS complex (Figure 7). ATAD3A, which spans both mitochondrial membranes, is a known regulator of mitochondrial inner membrane dynamics [24,26,27]. Reduced inner membrane retention of ATAD3A and its aberrant binding to MICOS components likely disrupt cristae junction architecture and inner membrane morphology.

It has been demonstrated that ATAD3A participates in diverse mitochondrial functional processes, including nucleoid organization, cholesterol metabolism, and mitochondrial translation [25]. For instance, it interacts with mitochondrial transcription factor A

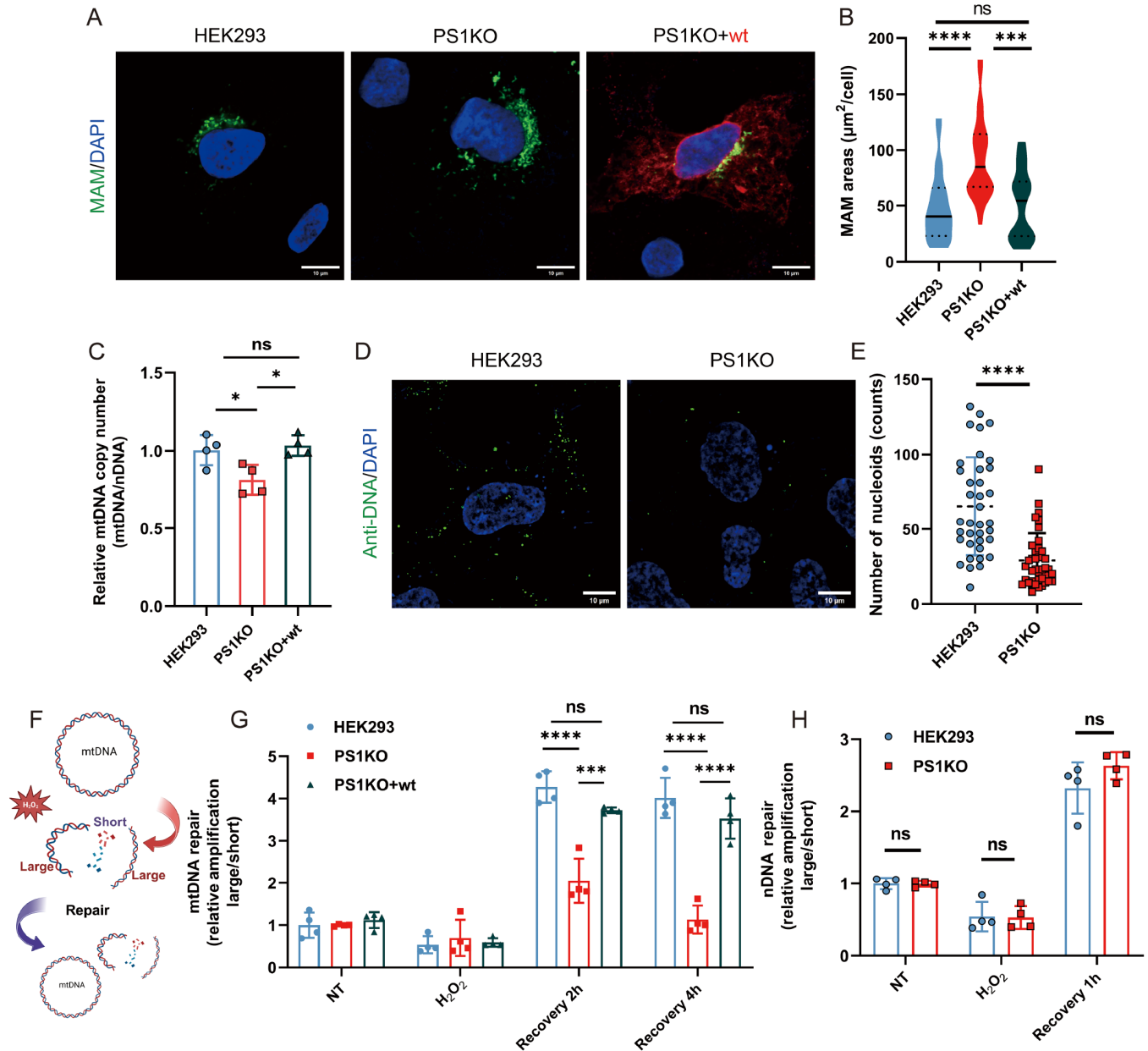


Figure 5. PS1 deficiency leads to an expanded MAM area and mtDNA repair dysfunction (A) MAM area was determined using the SPLICS Mt-ER L plasmid. Scale bar: 10 μm . (B) Quantification of the MAM area. Analysis information: $n = 32$ cells. (C) Mitochondrial DNA copy number was analyzed by quantitative PCR. (D) Mitochondrial DNA nucleoids were determined by anti-DNA staining. Scale bar: 10 μm . (E) Anti-DNA puncta were quantified. Analysis information: HEK293 cells, $n = 37$; PS1KO cells, $n = 40$. (F) Schematic of H_2O_2 -induced DNA disruption and repair. (G) Mitochondrial DNA repair assay by using quantitative PCR. (H) Nuclear DNA repair assay by using quantitative PCR. Data are presented as the mean $\pm$ SD. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant.

(TFAM) to regulate nucleoid dynamics and mtDNA transcription while also mediating respiratory chain complex assembly [22,37]. ATAD3A is also functionally linked to mitochondria-associated ER membranes (MAMs) and regulates mitochondrial Ca^{2+} homeostasis [24,29,39]. Thus, the disrupted mitochondrial Ca^{2+} handling and mtDNA maintenance observed in PS1-deficient cells stem from increased ATAD3A levels and oligomerization. These results underscore the central role of ATAD3A in mediating mitochondrial dysfunctions induced by PS1 deficiency. Notably, elevated ATAD3A

oligomerization has been reported in AD patients and models, highlighting its potential pathogenic relevance [28,29]. The precise mechanisms by which PS1 regulates ATAD3A, however, require further investigation.

Our study also reveals a novel γ -secretase-independent function of PS1, which may contribute to AD progression. PS1 undergoes autoproteolytic processing to form NTF and CTF fragments, which together constitute the catalytic core of the γ -secretase complex [40], which is responsible for APP cleavage and A β generation.

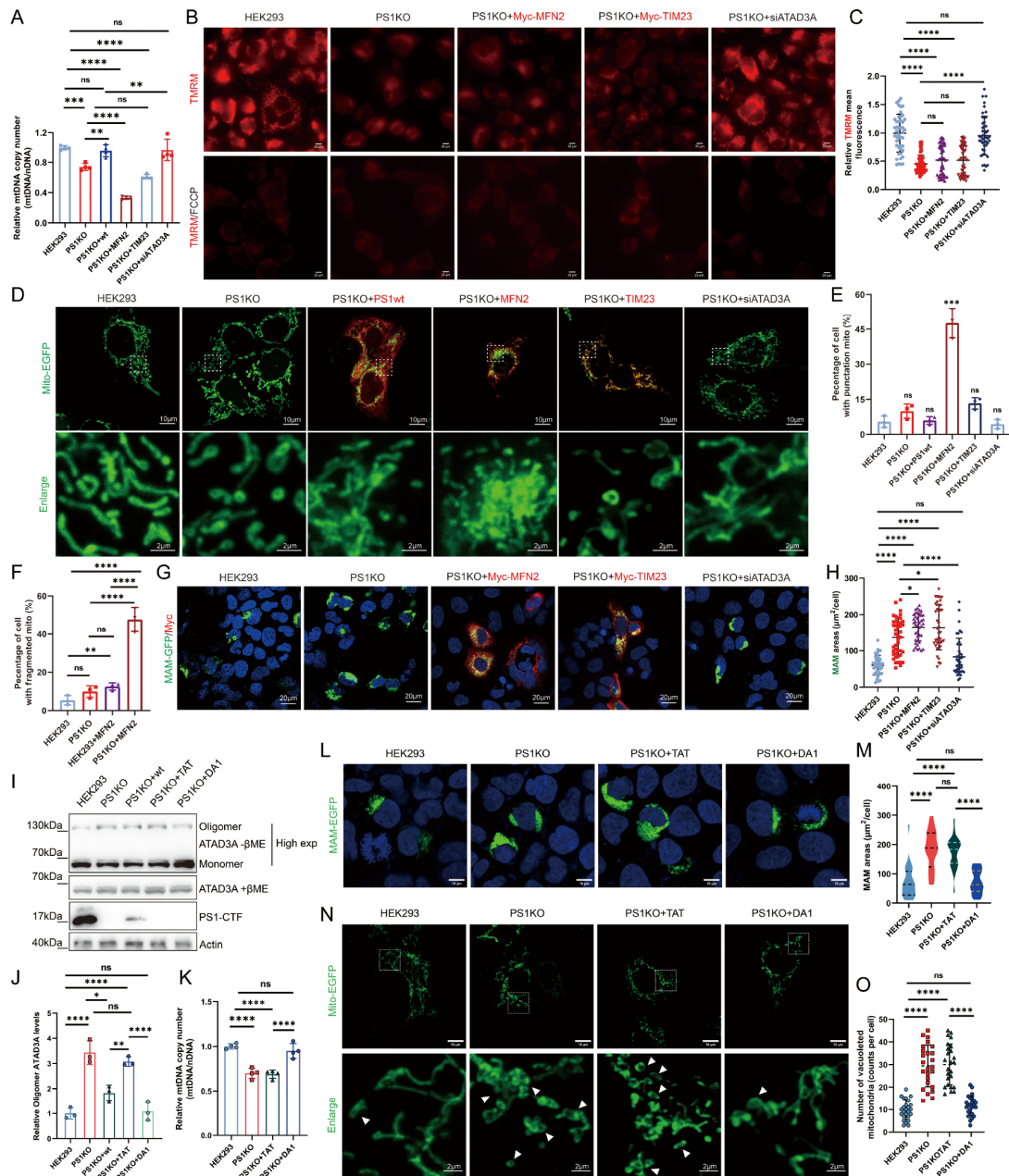


Figure 6. Inhibition of ATAD3A oligomerization reverses MAM expansion and mitochondrial dysfunction caused by PS1 deficiency (A) Quantification of mtDNA copy number in PS1KO cells following overexpression of MFN2 or TIM23 or knockdown of ATAD3A. (B) Measurement of mitochondrial membrane potential by TMRM staining in PS1KO cells following overexpression of MFN2 or TIM23 or knockdown of ATAD3A. Scale bar: 20 μ m. (C) Quantification of relative TMRM fluorescence. TMRM fluorescence was normalized to HEK293. Analysis information: HEK293 cells, $n = 720$ and 40 fields of view; PS1KO cells, $n = 644$ and 56 fields of view; PS1KO + MFN2 cells, $n = 506$ and 46 fields of view; PS1KO + TIM23 cells, $n = 564$ and 47 fields of view; PS1KO + siATAD3A cells, $n = 675$ and 45 fields of view. (D) Mitochondria in PS1KO cells were visualized by Mito-EGFP after overexpression of MFN2 or TIM23 or knockdown of ATAD3A. Scale bar: 10 μ m. (E) Quantification of the percentage of cells with punctate mitochondria. (F) Summary of cells with punctate morphology in HEK293 and PS1KO groups (data integrated from Figure 5D and Supplementary Figure S1J). Data are representative of three independent experiments. (G) MAM area was determined using the SPLICS Mt-ER L plasmid and quantified in PS1KO cells following overexpression of MFN2 or TIM23 or knockdown of ATAD3A. Scale bar: 20 μ m. (H) Quantification of the MAMs area. Analysis information: HEK293 cells, $n = 40$; PS1KO cells, $n = 44$; PS1KO + MFN2 cells, $n = 40$; PS1KO + TIM23 cells, $n = 41$; PS1KO + siATAD3A cells, $n = 42$. (I) PS1KO cells were treated with DA1 peptides or the control peptide TAT (1 μ M each) for 48 h. ATAD3A oligomerization was analyzed by WB analysis. (J) Quantification of ATAD3A oligomerization. (K) Mitochondrial DNA copy number of peptide-treated PS1KO cells was analyzed by quantitative PCR. (L) PS1KO cells were treated with peptides (DA1/TAT, 1 μ M each) for 48 h, and the MAM area was labeled by transfection with the SPLICS Mt-ER L plasmid. Scale bar: 10 μ m. (M) Quantification of the MAMs area. $n = 25$ cells. (N) PS1KO cells were treated with peptides (DA1/TAT, 1 μ M each) for 48 h, and mitochondria were labeled by transfection with Mito-EGFP plasmids. Scale bar: 10 μ m. Enlarge, Scale bar: 2 μ m. (O) Quantification of vacuolated mitochondria. Analysis information: $n = 25$ cells. Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant.

Mutations in PS1 represent the most common genetic cause of AD [5,41]. Here, we demonstrate that PS1 interacts with mitochondrial proteins and regulates mitochondrial functions independently of its role in γ -secretase activity. Mitochondrial dysfunction is a well-established feature of AD, characterized by impaired energy metabolism, disrupted calcium homeostasis, altered mitochondrial dynamics, and ROS accumulation [15,18,19,42]. A β and its precursor protein APP have been shown to disrupt mitochondrial energy metabolism and dynamics. For instance, pathogenic A β_{42} accumulates in mitochondria and impairs mitochondrial energy metabolism [43]. Neuronal mitochondria exhibit fragmented networks in AD, accompanied by defective mitophagy, leading to the accumulation of damaged mitochondria and subsequent neuronal death [17,44]. Additionally, mtDNA damage, which compromises bioenergetics and can trigger immune responses and apoptosis, is increasingly implicated in AD [18,45,46]. Recent studies have

highlighted the importance of MICOS assembly in both physiological and pathological contexts. [47] We speculate that PS1 may facilitate proper MICOS organization, and its γ -secretase-independent functions also contribute to AD pathogenesis. Our results thus establish a novel link between PS1 and mitochondrial dysfunction, offering a novel perspective on AD progression.

Several limitations of this study should be noted. First, we focused on the loss of PS1 and its effects on mitochondrial structure and function. Since PS1 mutations are frequently identified in AD patients, future studies should examine how disease-associated PS1 mutations affect mitochondrial integrity and function. Second, as both NTF and CTF fragments of PS1 were detected in mitochondria, their individual roles in regulating cristae architecture warrant further investigation. Additionally, the exact mechanism by which ATAD3A oligomerization impairs mitochondrial inner membrane integrity remains to be clarified.

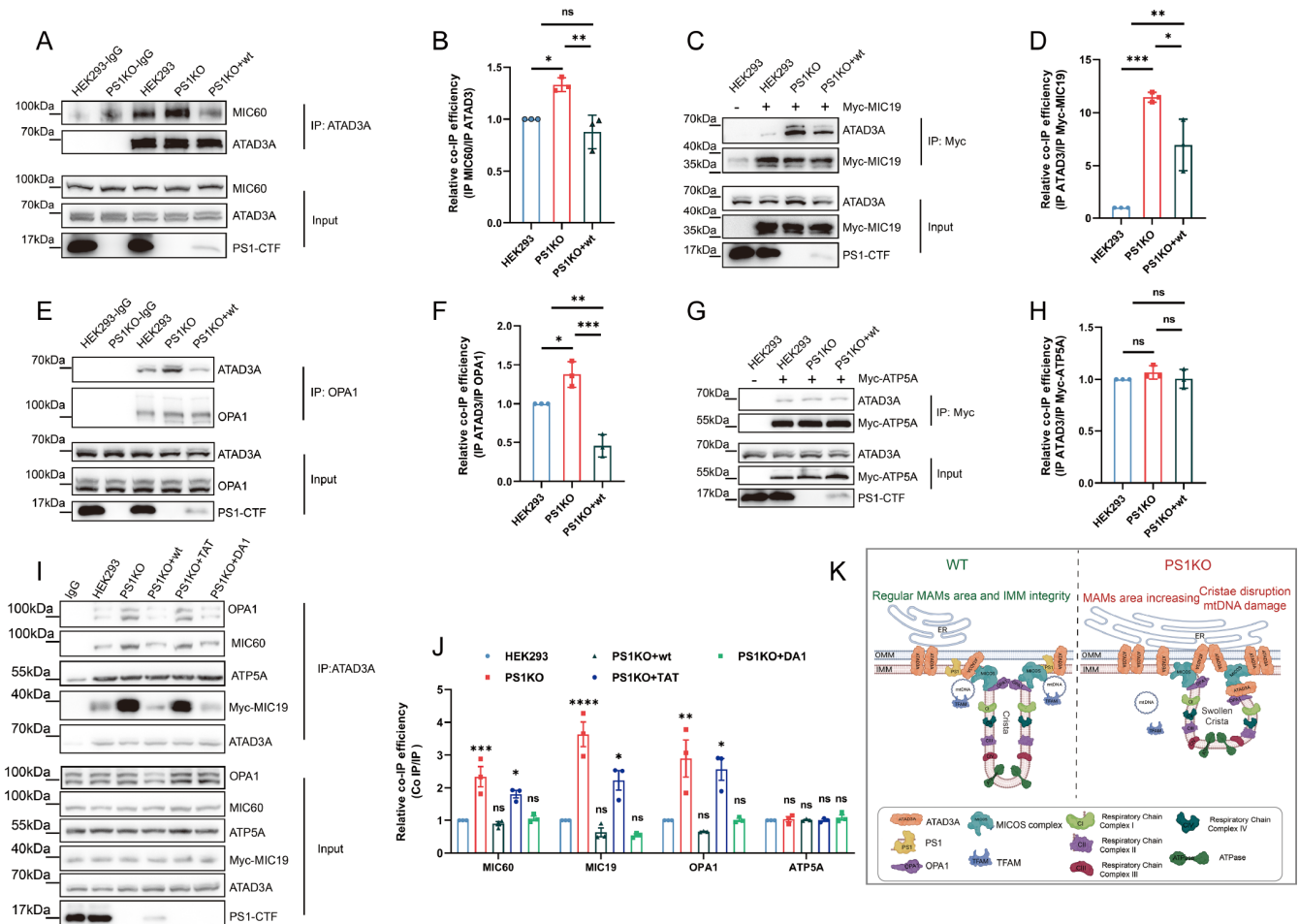


Figure 7. Loss of PS1 disturbs mitochondrial cristae junction protein arrangement (A–F) The interaction between ATAD3A and mitochondrial cristae proteins was determined by co-IP. (A) The increased interaction between ATAD3A and MIC60 in PS1KO cells. (B) Quantification of the binding efficiency between ATAD3A and MIC60. (C) The increased interaction between ATAD3A and MIC19 in PS1KO cells. (D) Quantification of the binding efficiency between ATAD3A and MIC19. (E) The increased interaction between ATAD3A and OPA1 in PS1KO cells. (F) Quantification of the binding efficiency between ATAD3A and OPA1. (G) The interaction between ATAD3A and ATPase subunit ATP5A was not changed. (H) Quantification of the binding efficiency between ATAD3A and ATP5A. (I) PS1KO cells were treated with DA1/TAT peptides for 72 h (DA1/TAT, 1 μ M each), and the interaction between ATAD3A and MIC60, OPA1, MIC19, and ATP5A was analyzed by co-IP. (J) Quantification of the binding efficiency between ATAD3A and mitochondrial membrane proteins. (K) A summary scheme shows that loss of PS1 disturbs mitochondrial cristae junction protein arrangement and resulted in swollen cristae. Data are from three independent biological replicates of co-immunoprecipitation. For each replicate, the co-IP efficiency was calculated relative to the control HEK293 cells. Data are presented as the mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. ns, not significant.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Data Availability

Data can be accessed upon reasonable request from the corresponding authors.

Author Contribution

Pan You performed the experiments and wrote the manuscript. Pengjin Zhu performed the data analysis. Hui Yu revised the manuscript. Luwen Wang designed the experiments and revised the manuscript. Bo Su designed the experiments, wrote and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Acknowledgement

We thank the Translational Medicine Core Facility of Shandong University for consultation and instrument availability (Zeiss LSM900 confocal microscope) that supported this work.

Funding

This work was supported by the grants from the Shandong Natural Science Foundation (Nos. ZR2019JQ24, ZR2023YQ064, and ZR2022MC200).

Conflict of Interest

The authors declare that they have no conflict of interest.

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