

Trilobatin combats copper overload-induced cognitive impairment by blocking FDX1-dependent cuproptosis and restoring mitochondrial dynamics

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Abstract

Copper homeostasis disorder triggers neuronal cuproptosis and progressive cognitive degeneration, lacking effective natural therapeutic agents. Trilobatin (TLB), a dihydrochalcone from *Lithocarpus polystachyus* Rehd., was reported to inhibit FDX1, yet its systematic neuroprotective mechanism against copper overload remains unclear. Herein, chronic CuSO₄ gavage mouse model and CuSO₄-exposed HT22 hippocampal neurons were constructed. Behavioral tests confirmed TLB dose-dependently rescued copper-induced memory deficits. Mechanistically, TLB bidirectionally regulated copper transporters SLC31A1/ATP7B to reduce intracellular copper deposition, alleviated oxidative stress, restored mitochondrial fusion-fission balance and ultrastructural integrity. TLB suppressed FDX1-driven cuproptotic signaling, normalized disrupted TCA cycle and mitochondrial respiratory function. FDX1 siRNA knockdown further verified FDX1 as the indispensable mediator of TLB's neuroprotection. Collectively, TLB ameliorates copper overload-mediated hippocampal injury and cognitive dysfunction by inhibiting FDX1-dependent cuproptosis and restoring mitochondrial metabolism, offering a promising candidate for metal dyshomeostasis-related neurodegenerative diseases.

1. Introduction

Copper acts as an indispensable trace mineral that maintains homeostatic stability within the central nervous system through participation in neurotransmitter biosynthesis mitochondrial energy transduction and endogenous antioxidant defense cascades(Kunwar et al., 2026, Wong et al., 2026). Long term disruption of cerebral copper equilibrium drives progressive neuronal deterioration and this pathological process bears strong pathogenic relevance to a broad spectrum of cognitive degenerative illnesses that cover Wilson disease, sporadic Alzheimer disease and progressive memory loss associated with advanced age(Deloncle, 2026, Cheng et al., 2026, Kepp and Squitti, 2019). Chronic excessive copper intake via digestive routes facilitates abnormal copper accumulation within the hippocampus a brain region that governs the formation consolidation and retrieval of spatial learning and memory signals(Gao et al., 2025a, He et al., 2025). Clinical interventions available for copper overload linked cognitive complications remain limited and conventional therapeutic approaches rely largely on synthetic metal chelators that carry systemic adverse reactions poor blood brain barrier permeability and incapacity to resolve downstream intracellular damage cascades beyond copper clearance(Hong et al., 2026, Wang et al., 2023a). The unsatisfactory therapeutic outcomes of existing interventions can be attributed to the intricate multi layered molecular network activated by intracellular copper overload which proceeds through interconnected cell death and organelle dysfunction pathways that single target metal chelation fails to counteract comprehensively.

Cuproptosis represents a recently characterized metal triggered programmed cell death modality that serves as the central effector pathway underlying copper mediated neurotoxicity(Zhu et al., 2024, He et al., 2026). Excessive intracellular copper accumulation activates ferredoxin 1 (FDX1) which catalyzes the conversion of low toxic divalent copper ions into highly reactive monovalent copper species(Wang et al., 2026, Wang et al., 2023b, Chen et al., 2022). The reactive monovalent copper binds lipoylated enzymes

of the tricarboxylic acid cycle and triggers toxic aggregation of DLAT containing pyruvate dehydrogenase complexes(Zhang et al., 2025a). This toxic cascade concurrently amplifies mitochondrial reactive oxygen species generation disrupts physiological mitochondrial fission fusion balance and impairs the integrity of mitochondrial respiratory chains to trigger neuronal bioenergetic collapse and subsequent cognitive functional decline(Li et al., 2019, Chen et al., 2026). Cumulative preclinical evidence identifies FDX1 as the core upstream molecular switch that initiates full scale cuproptotic activation and targeted suppression of FDX1 related signaling cascades effectively alleviates mitochondrial structural and functional impairment induced by copper toxicity(Zhou and Peng, 2026, Feng et al., 2024a). Strategies designed to inhibit FDX1 and normalize disrupted mitochondrial dynamics have therefore emerged as promising directions for the management of copper overload associated neuronal injury.

Trilobatin (TLB) refers to a natural dihydrochalcone polyphenol mainly extracted from *Lithocarpus polystachyus* Rehd.(Gao et al., 2026), and previous studies have verified its multiple biological activities which include anti-oxidative effect(Hou et al., 2023), anti-neuroinflammation(Lin et al., 2025, Xie et al., 2024) and robust neuroprotective properties(Wu et al., 2025, Gao et al., 2025b, Wei et al., 2025). Intriguingly, our prior work has confirmed the direct inhibitory effect of TLB on FDX1(Wei et al., 2025). However, no systematic combined evidence from *in vivo* and *in vitro* models has clarified whether TLB can reverse cognitive deficits induced by chronic copper overload and whether its neuroprotective effects rely on the regulation of copper transporters and the suppression of FDX1 dependent cuproptosis and mitochondrial damage pathways. Therefore, we construct a chronic copper sulfate intragastric administration mouse model to simulate cognitive dysfunction induced by copper overload and a HT22 hippocampal neuron injury model treated with copper sulfate to address three core research questions. The first question explores whether trilobatin treatment improves spatial recognition novel object discrimination and long term spatial memory damaged by copper toxicity in mice. The second question examines whether trilobatin counteracts cerebral oxidative stress triggered by copper exposure and reconstructs balanced mitochondrial structure and kinetic homeostasis. The third question investigates whether trilobatin restores the balance of copper transporters and interrupts cuproptotic signaling centered on FDX1 and we conduct siRNA mediated gene silencing *in vitro* to verify FDX 1 as an indispensable functional mediator of trilobatin related protection.

This study establishes a complete mechanistic framework to illustrate that TLB alleviates neurotoxicity from copper overload through coordinated regulation of copper influx and efflux inhibition of FDX1 driven cuproptosis and restoration of mitochondrial bioenergetic function. Our research identifies FDX1 as a critical functional target of trilobatin and provides a novel natural polyphenol candidate for the treatment of neurodegenerative cognitive disorders caused by metal dyshomeostasis. This work also builds theoretical support for the development of neuroprotective phytochemical agents that target cuproptosis pathways.

2. Materials and Methods

2.1 Animals

Seventy male C57BL/6J mice aged 5–6 weeks with initial body weights ranging from 17 to 22 g were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China). All animals were housed in a specific pathogen-free (SPF) animal facility at Zunyi Medical University under standardized breeding conditions: ambient temperature maintained at 22–24°C, relative humidity controlled at 55–60%, a 12 h light-dark cycle, with free access to standard chow and sterilized drinking water. All mice underwent a 7-day adaptive feeding period prior to formal experimental operations. All animal experimental protocols strictly complied with the ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978). The experimental scheme was approved by the Animal Ethics Committee of Zunyi Medical University (Approval Certificate No. ZMU[2020]2-134). Terminal euthanasia was performed via deep anesthesia with sodium pentobarbital followed by rapid cervical dislocation to minimize animal suffering.

2.2 Experimental Grouping and Intervention Protocol

After a one-week acclimatization period, six-week-old male C57BL/6J wild-type mice were randomly divided into six groups: the control group, the control + TLB (10 mg/kg) group, the CuSO₄ group, the CuSO₄ + TLB (2.5 mg/kg) group, the CuSO₄ + TLB (5 mg/kg) group, and the CuSO₄ + TLB (10 mg/kg) group. The cognitive impairment model was established by administering CuSO₄ (5 mg/kg) via oral gavage. Subsequently, TLB was administered via oral gavage at doses of 2.5, 5, and 10 mg/kg once daily, while the control and model groups received an equivalent volume of normal saline. TLB were ground into fine powder and suspended in sterile normal saline before administration. Vehicle groups received equivalent volumes of 0.9% physiological saline. After finishing all behavioral tests at the experimental endpoint, mice were deeply anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Whole blood was collected via cardiac puncture for serum separation, while hippocampal and cortices were rapidly isolated, snap-frozen in liquid nitrogen and preserved at – 80°C for subsequent biochemical and transcriptomic detection.

2.3 Morris Water Maze (MWM) Test

- The MWM test was performed to assess spatial learning and memory abilities in mice (Zhang et al., 2025b). The apparatus consisted of an animal behavior tracking system, a video camera, and a black circular water pool (diameter: 120 cm; height: 50 cm). The pool was divided evenly into four quadrants, designated as quadrants I, II, III, and IV. Quadrant III was defined as the target quadrant, and a platform was placed at its center. The pool was filled with tap water to a depth of 1 cm above the platform, and the water temperature was maintained at 23 ± 1°C. At the start of each trial, the MWM system was activated, and a mouse was gently picked up and placed into the water facing the wall of any quadrant other than the target quadrant (quadrant III), with its head oriented upward. The recording was initiated simultaneously with the mouse's entry into the water, during which the surroundings were kept quiet and the mouse was allowed to swim freely for 60 s. If the mouse located the submerged platform and climbed onto it within 60 s, the time taken was recorded as the escape latency for that trial; the mouse was then allowed to remain on the platform for 10 s. If the mouse failed to find the platform within 60 s, it was guided to the platform and allowed to stay there

for 10 s, and its escape latency was recorded as 60 s. After each trial, the mouse was dried with a heater before being returned to its cage. The training was conducted three times per day for five consecutive days, with an inter-trial interval of at least 30 min for each mouse. The latency to reach the platform, the swim path, and the swim speed were recorded and analyzed. On the sixth day, a probe trial was conducted with the platform removed. Each mouse was placed into the water from the entry point in quadrant I, and the number of crossings over the former platform location and the time spent in the target quadrant were recorded within a 60-s period, to evaluate the integrity and strength of spatial memory.

2.4 Y-maze test

- The Y-maze test was conducted to evaluate spatial recognition memory in mice. The apparatus consisted of a Y-shaped maze with three identical arms (length: 30 cm, width: 10 cm, height: 20 cm). Mice were placed in the starting arm and allowed to freely explore one of the other arms for 8 min. Subsequently, the mice were returned to the starting arm and permitted to explore all three arms freely for 5 min, during which the number of entries into each arm was recorded. The percentage of spontaneous alternation was calculated using the following formula: Spontaneous alternation (%) = [number of alternations / (total arm entries – 2)] × 100%.

2.5 Novel object recognition (NOR) test

- The NOR test is a behavioral paradigm used to assess attention, memory, and discrimination abilities in mice. Mice were placed into the experimental apparatus and allowed to freely explore two identical objects for 5 min. On the following day, the mice were allowed to explore two identical objects for 5 min. Subsequently, the mice were removed from the apparatus, and the objects were cleaned with ethanol to eliminate olfactory cues. On the third day, one of the familiar objects (F) was replaced with a novel object (N). Exploration was defined as sniffing or touching the object with the nose or forepaws. The novel object recognition index was calculated using the following formula: Novel object recognition index (%) = [N / (N + F)] × 100%.

2.6 Molecular docking

- Molecular docking was performed to predict the binding affinity between FDX1 and PINK1. The protein–ligand docking tool Autodock Vina 1.2.2 was used to evaluate the molecular docking between FDX1 and PINK1. The three-dimensional structure of FDX1 (PDB ID: 3P1M) was obtained from the RCSB PDB database (<http://www.rcsb.org/>), and the three-dimensional structure of PINK1 (UniProt ID: Q99MQ3) was retrieved from the UniProt database (<https://www.uniprot.org/>). Molecular docking simulations were carried out using Autodock Vina 1.2.2, and the visualization of the models was performed with PyMOL software to display the best binding conformations (Feng et al., 2024b, Abraham et al., 2015, Van Der Spoel et al., 2005).

2.7 Molecular dynamics simulation

- Molecular dynamics (MD) simulations were conducted to explore the interaction between FDX1 and PINK1. The simulations were performed using Gromacs 2022.3 software. Potential energy data were incorporated into the topology files of the MD simulation system, with the topology files generated by the pdb2gmx command. A free MD simulation of 5,000,000 steps with a step size of 2 fs was carried out for protein–protein interactions, corresponding to a simulation duration of 100 ns. After the simulations were completed, comprehensive analyses were performed using the software to calculate the free energy landscape, root-mean-square deviation (RMSD), and root-mean-square fluctuation (RMSF).

2.8 Surface plasmon resonance

- Surface plasmon resonance (SPR) technology was employed to establish an interaction assay model between PINK1 and FDX1 proteins and to determine their binding interaction. Analyses were performed using a CM5 sensor chip (GE Healthcare, Uppsala, Sweden) and a Biacore X100 biosensor. PINK1 protein was diluted to 50 µg/mL with sodium acetate and immobilized on the flow channel of the CM5 sensor chip at a flow rate of 10 µL/min. The analyte FDX1 was serially diluted to several concentrations in a 96-well plate and then injected sequentially from low to high concentrations over the chip surface coupled with PINK1 protein at a flow rate of 30 µL/min for a contact time of 150 s. After each concentration injection, the chip surface was regenerated with 10 mM glycine-HCl (pH 2.0) for 5 min, and this procedure was repeated until all tested concentrations of the analyte had been completed. Finally, the data were globally fitted to a 1:1 Langmuir binding model using Biacore Insight Evaluation Software (Cytiva, Marlborough, MA, USA) to obtain the association and dissociation constants .

2.9 Cell culture and drug treatment

- HT22 mouse hippocampal neuronal cells were cultured in DMEM containing 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and 10% FBS at 37°C in 5% CO₂. At 80–90% confluence, cells were collected by centrifugation (1,000 × g, 3 min). The CuSO₄-induced injury model was established using CuSO₄ (10–90 µM), and TLB was applied at 7.5, 15, 30, and 60 µM; both compounds were dissolved in DMEM with DMSO ≤ 0.1% and co-administered to the cells.

2.10 Cell viability

- After treatment, cell viability was evaluated by the MTT assay. Cells were incubated with 20 µL of MTT (5 mg/mL) per well for 4 h at 37°C. The supernatant was discarded, and 150 µL of DMSO was added to dissolve the formazan crystals. After shaking at 37°C for 10 min, absorbance was measured at 490 nm using a microplate reader, and morphological changes were observed under an inverted microscope.

2.11 siRNA transfection in HT22 cells

- FDX1 expression was silenced by siRNA transfection. Cells were seeded at 60–80% confluence. Transfection complexes were prepared as follows: (1) 1.25 μL of 20 μM siRNA (final transfection concentration 50 nM per well in a 24-well plate, though 100 nM was used in the actual experiments) was mixed with 48.75 μL of serum-free medium (solution A); (2) 1.25 μL of Lipofectamine™ 3000 reagent was mixed with 48.75 μL of serum-free medium (solution B); (3) solution A and B were combined, gently mixed, and incubated at room temperature for 15–30 min. For transfection, the culture medium was replaced with 400 μL of serum-free medium 2 h before adding 100 μL of the complex mixture, and cells were incubated for 4–6 h, after which the medium was replaced with normal complete medium containing serum. Transfection efficiency was verified by immunofluorescence and western blotting. The siRNA sequences (sense/antisense) were as follows: 5'-UGUUAACAGUAUUUGGCA-3' and 5'-CCAAUACUGUUUUUAACAAA-3'; 5'-AAAUUACAUGGAGAAUUGUA-3' and 5'-CAAUUCUCCAUGUAAAUUUUC-3'; 5'-ACUAAUGUCAAUAGAUACAC-3' and 5'-GUAUCUAUUGACAUUUAGUUG-3'. Transfected cells were treated with 60 μM CuSO_4 and 30 μM TLB 24 h post-transfection, and protein levels were assessed by western blot at 48–96 h after transfection.

2.12 Mitochondrial superoxide detection in HT22 cells

- Mitochondrial superoxide was detected using Mito-SOX Red (a red fluorescent probe) and Mito-Tracker Green (a green fluorescent probe), following the manufacturer's instructions. The Mito-SOX Red working solution was prepared fresh at 5 μM by diluting the 5 mM stock in PBS, protected from light. The Mito-Tracker Green stock (1 mM in anhydrous DMSO) was stored at -20 or -80°C , and the working solution was prepared at 20–200 nM in culture medium and pre-warmed to 37°C before use. Cells seeded in 6-well plates were treated as designed, then incubated with 1 mL of Mito-SOX Red working solution per well for 20 min at 37°C , washed once with PBS, and further incubated with the pre-warmed Mito-Tracker Green working solution for 20 min at 37°C . After another PBS wash, cells were stained with DAPI for 5 min, washed again, mounted, and examined under a fluorescence microscope.

2.13 Mitochondrial bioenergetics assay

- To evaluate mitochondrial bioenergetics, the oxygen consumption rate (OCR) was measured using a Seahorse XFe96 extracellular flux analyzer. HT22 cells were cultured in Seahorse microplates and exposed to CuSO_4 (60 μM) and TLB (30 μM) for 24 h. OCR was then measured following sequential injections of oligomycin (2.5 μM), FCCP (1 M), and a mixture of rotenone and antimycin A (0.5 μM). OCR values were normalized to total protein content per well and expressed as pmol/min/ μg protein.

2.14 Fluorometric detection of cellular cuprous ions

- Cuprous ion levels in HT22 cells were measured using a commercial fluorometric assay. Before detection, reagents were equilibrated to room temperature, and substrate A was mixed with double-distilled water at a 1:9 volume ratio. Approximately 1×10^6 cells were homogenized in 200 μL of saline, and the supernatant was collected after centrifugation at $10,000 \times g$ for 10 min at 4°C . For the assay, 20 μL of the sample was added to each well of a microplate, followed by 100 μL of reagent A working solution and 100 μL of reagent B. The plate was shaken for 5 s and incubated at 37°C in the dark for 20 min. Fluorescence was then measured at an excitation wavelength of 395 nm and emission wavelength of 480 nm using a fluorescence microplate reader.

2.15 Enzyme-linked immunosorbent assay (ELISA)

- ELISA was performed to evaluate oxidative stress markers in mouse hippocampal tissues and key TCA cycle precursors, intermediates, and regulatory enzyme activities in HT22 cells. The oxidative stress markers measured included reactive oxygen species (ROS), malondialdehyde (MDA), catalase (CAT), glutathione (GSH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD). The TCA cycle-related parameters comprised pyruvate, α -ketoglutarate (α -KG), pyruvate dehydrogenase (PDH), citrate synthase (CS), isocitrate dehydrogenase (ICDH), α -ketoglutarate dehydrogenase (α -KGDH), and succinate dehydrogenase (SDH). Hippocampal tissues were homogenized and centrifuged at $3,000 \times g$ for 20 min at 4°C to obtain supernatants. For the assay, standard wells received 50 μL of serially diluted standards; sample wells received 40 μL of sample diluent followed by 10 μL of the test sample (final 5-fold dilution), while blank wells received neither sample nor enzyme reagent. Then, 100 μL of enzyme-labeled reagent was added to all wells except blanks, and the plate was sealed and incubated at 37°C for 60 min. After washing five times with wash buffer (prepared by 20-fold dilution of the concentrate), 50 μL each of chromogen solutions A and B were added sequentially, mixed, and incubated at 37°C in the dark for 15 min for color development. The reaction was terminated by adding 50 μL of stop solution, and the absorbance (OD) was read at 450 nm within 15 min, with the blank well used for zero calibration.

2.16 Western blot (WB) analysis

- WB was performed in accordance with the methodology outlined in a previous study. Hippocampal tissues were lysed in RIPA buffer containing PMSF and phosphatase inhibitors, homogenised, and centrifuged; mitochondrial proteins were additionally extracted using a commercial kit. Equivalent quantities of proteins were separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene fluoride membranes. The membranes were then blocked for 2 h in 5% skim milk in Tris-Buffered Saline Tween-20 (TBST). Subsequently, the membranes were incubated overnight at 4°C with primary antibodies against FDX1 (1:2000), DLD (1:2000), LIAS (1:2000), SLC31A1 (1:1000), ATP7B (1:1000), MTF1 (1:1000), DLAT (1:2000), GLS (1:1000), PDHB (1:2000), PDHA1 (1:2000), CDKN2A/p14ARF (1:1000), p-DRP1 (1:1000), DRP1 (1:1000), FIS1 (1:1000), MFN1 (1:2000), MFN2 (1:2000), OPA1 (1:1000), PINK1 (1:1000), and VDAC1 (1:2000). Following TBST washes, the membranes were incubated with

species-specific horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Representative bands were visualised using ECL reagent, and band intensities were quantified using ImageJ software. Each protein band was normalised to GAPDH (1:5000), β -tubulin (1:5000), or β -actin (1:5000) as loading controls, and data are presented as relative ratios to the control group.

2.3 Statistical analysis

Data are presented as mean $\pm$ standard deviation and were analyzed using SPSS 29.0 software. Statistical significance was determined using an unpaired two-tailed Student's t-test, one-way ANOVA followed by Tukey's post hoc test, or two-way ANOVA followed by Bonferroni's post hoc test, as appropriate. Body weight changes over time were analyzed using two-way repeated-measures ANOVA. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Trilobatin ameliorates copper sulfate-induced cognitive dysfunction

In this study, a mouse model of cognitive impairment was established by administering a CuSO_4 solution (5 mg/kg) via gastric intubation to investigate the effects of TLB on cognitive dysfunction in mice. We used behavioral tests (MWM test, Y-maze test, and NOR test) to assess the cognitive function of the mice (Fig. 1A, B). The Y-maze test was used to evaluate spatial recognition memory in mice. The results showed that, compared with the control group, mice in the CuSO_4 -treated group exhibited a significantly reduced spontaneous alternation rate; however, treatment with TLB (2.5, 5, 10 mg/kg) significantly reversed the changes induced by CuSO_4 . These results indicate that TLB can improve CuSO_4 -induced spatial recognition memory in the Y-maze in mice (Fig. 1C, D). The NOR test was used to evaluate the discriminative learning and memory function for novel objects in CuSO_4 -treated mice. The results showed that, compared with the control group, the novel object recognition index was significantly lower in the CuSO_4 -treated group; however, after treatment with TLB, the novel object recognition index in the mice increased significantly. These results indicate that TLB can improve the discriminative learning and memory function for novel objects in CuSO_4 -induced mice (Fig. 1E, F). The MWM test was used to evaluate the effects of CuSO_4 on

spatial learning and memory in mice (Fig. 1G). The results showed that, compared with the control group, mice in the CuSO_4 -treated group exhibited a significantly prolonged escape latency, while those in the TLB group exhibited a significantly shortened escape latency (Fig. 1H). Compared with mice in the control group, mice in the CuSO_4 -treated group spent significantly less time in the target quadrant and made significantly fewer platform crossings; whereas after treatment with TLB (5, 10 mg/kg), the time mice spent in the target quadrant was significantly prolonged, and the number of platform crossings

increased significantly (Fig. 1I, J). These results indicate that TLB can improve CuSO₄-induced spatial learning and memory abilities in mice.

3.2 Trilobatin Attenuates CuSO₄-Induced Oxidative Stress and Restores Mitochondrial Dynamics and Structural Integrity

To evaluate the protective effect of TLB against CuSO₄-induced oxidative damage, levels of oxidative stress indicators and antioxidant defense markers were examined. CuSO₄ exposure significantly elevated the levels of reactive oxygen species (ROS) and malondialdehyde (MDA) compared with the control group (Fig. 2A, B). Concurrently, antioxidant capacities including catalase (CAT), glutathione (GSH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) were markedly compromised (Fig. 2C-F). Importantly, TLB treatment (2.5, 5, and 10 mg/kg) dose-dependently reduced ROS and MDA accumulation while restoring the activities of CAT, GSH, GSH-Px, and SOD, highlighting its potent antioxidant capacity. Transmission electron microscopy (TEM) demonstrated that control mitochondria possessed well-defined membranes and intact cristae, whereas CuSO₄ challenge caused profound mitochondrial swelling and loss of cristae (Fig. 2G). Morphometric analysis confirmed a significant increase in mean mitochondrial size (Fig. 2H) alongside a marked reduction in mitochondrial numerical density (Fig. 2I) in response to CuSO₄. TLB co-treatment effectively alleviated these ultrastructural defects, suppressing pathological mitochondrial swelling and preserving mitochondrial density. We next investigated whether TLB influences mitochondrial fission machinery. Western blot analysis showed that CuSO₄ exposure significantly up-regulated the expression of fission-related proteins, as evidenced by increased p-DRP1/DRP1 ratio and FIS1 protein levels (Fig. 2J-L). TLB treatment markedly prevented the overactivation of p-DRP1 and FIS1, indicating that TLB effectively halts CuSO₄-induced hyper-fission. In contrast, mitochondrial fusion proteins MFN1, MFN2, and OPA1 were severely suppressed following CuSO₄ stimulation (Fig. 2M-P). TLB treatment significantly restored MFN1, MFN2, and OPA1 protein expression. Moreover, accumulation of the mitochondrial quality control marker PINK1 was markedly induced by CuSO₄ and reversed upon TLB administration (Fig. 2Q, R). Taken together (Fig. 2S), these findings demonstrate that CuSO₄ administration causes severe mitochondrial damage by disrupting fusion/fission dynamics and inducing oxidative injury, whereas TLB treatment achieves mitochondrial restoration and neuronal protection.

(GSH-Px) activity (n = 6). (F) Superoxide dismutase (SOD) activity (n = 6). (G) Representative images of mitochondrial ultrastructure (Magnification: 8000 times; Scale = 2µm; Magnification: 25000 times; Scale = 500 nm. The green arrow represents the mitochondria). (H) Number of mitochondria (n = 5). (I) Average of mitochondrial size (n = 5). (J) Representative immunoblots of mitochondrial fission proteins. (K) Quantification of p-DRP1 protein expression (n = 6). (L) Quantification of FIS1 protein expression (n = 6). (M) Representative immunoblots of MFN1, MFN2, and OPA1 proteins. (N) Quantification of MFN1 protein expression (n = 6). (O) Quantification of MFN2 protein expression (n = 6). (P) Quantification of

OPA1 protein expression (n = 6). (Q) Representative immunoblots of PINK1 proteins. (R) Quantification of PINK1 protein expression (n = 6). (S) Schematic summary illustrating TLB attenuates CuSO₄-Induced oxidative stress and restores mitochondrial dynamics and structural integrity. Data are expressed as mean ± SEM. **P < 0.01 vs. Control group; #P < 0.05, ##P < 0.01 vs. CuSO₄ group.

3.3 Trilobatin (TLB) Regulates Copper Transporters and Cuproptosis Signaling Pathways to Attenuate CuSO₄-Induced Toxicity

To investigate whether TLB alleviates copper toxicity by modulating intracellular copper transport, key copper influx and efflux proteins were measured. Western blot analysis demonstrated that CuSO₄ challenge markedly up-regulated the copper importer SLC31A1 (CTR1) (Fig. 3A, B) while down-regulating the copper exporter ATP7B (Fig. 3A, C). TLB co-treatment effectively reversed these changes by restoring ATP7B levels and repressing SLC31A1 expression, thereby counteracting intracellular copper accumulation. FDX1 is a master regulator of cuproptosis. As shown in Fig. 3D, E, CuSO₄ exposure significantly up-regulated FDX1 protein expression. Additionally, enzymes involved in protein lipoylation, DLD and LIAS, were examined (Fig. 3F-H). Parallel to FDX1, CuSO₄ administration significantly enhanced DLD and LIAS protein levels, indicating activation of the cuproptotic cascade. Treatment with TLB markedly suppressed the elevated expression of FDX1, DLD, and LIAS. Toxic accumulation of copper binds directly to lipoylated TCA cycle enzymes, leading to DLAT toxic aggregation and cuproptosis. Immunoblotting revealed that CuSO₄ significantly elevated the protein levels of DLAT, PDHB, and PDHA1 (Fig. 3M-P). Notably, TLB intervention significantly attenuated the aberrant accumulation of DLAT, PDHB, and PDHA1. Metal regulatory transcription factor 1 (MTF1) protects against metal toxicity. Exposure to CuSO₄ significantly inhibited MTF1 expression (Fig. 3I, J) and downregulated its protective downstream targets GLS and CDKN2A/p14ARF (Fig. 3I-L). TLB administration markedly restored the protein levels of MTF1, GLS, and CDKN2A. As schematically depicted in Fig. 3Q, CuSO₄ causes copper overload via SLC31A1 up-regulation and ATP7B down-regulation, triggering cuproptotic signaling (FDX1, LIAS, DLD, PDHA1, PDHB, DLAT) while suppressing MTF1/GLS/CDKN2A defenses. TLB intervention effectively reverses these alterations, leading to the inhibition of cuproptosis and improvement of cognitive function.

3.4 FDX1 Mediates the Protective Effects of Trilobatin Against CuSO₄-Induced Toxicity in HT22 Cells

To confirm the role and mechanism of TLB in CuSO₄-induced toxicity, we simulated a CuSO₄-induced toxicity model in vitro by treating HT22 cells with CuSO₄. Treatment with 60 μM CuSO₄ reduced cell viability to about 50% within 24 h; thus, CuSO₄ concentration of 60 μM was used to induce HT22 cell injury to mimic CuSO₄-induced toxicity (Figure S1A). Subsequently, TLB at various concentrations was administered simultaneously with 60 μM CuSO₄, and cell viability was assessed using the MTT assay.

The results showed that TLB at concentrations of 7.5, 15, 30, 60 μM concentrations of TLB all significantly increased cell viability following CuSO_4 treatment. Furthermore, there was no significant difference in the protective effects against CuSO_4 -induced HT22 cell damage between the 30 and 60 μM concentrations of TLB (Figure S1B). Therefore, the effective therapeutic concentrations of TLB were determined to be 7.5, 15, and 30 μM . Observation of HT22 cells under an inverted microscope revealed that CuSO_4 treatment caused a significant reduction in the number of HT22 cells, whereas TLB at concentrations of 7.5, 15, and 30 μM significantly increased cell numbers following CuSO_4 treatment (Figure S1C). These results indicate that TLB can inhibit CuSO_4 -induced damage to HT22 cells. To further verify whether TLB exerts its protective effect against CuSO_4 -induced damage in HT22 cells by targeting FDX1, a key protein involved in copper-mediated cell death, this study used siRNA transfection to knockdown FDX1 expression in HT22 cells and investigate the mechanism by which TLB protects against CuSO_4 -induced damage in HT22 cells. The results showed that transfection with 100 nM FDX1-siRNA reduced FDX1 protein levels to approximately 30% (Figure S2). Therefore, 100 nM FDX1-siRNA was selected for subsequent transfection experiments. CuSO_4 significantly increased intracellular cuprous ion levels, whereas FDX1 knockdown reduced intracellular cuprous ion levels, confirming that FDX1-mediated copper-induced cell death plays a critical role in CuSO_4 -induced damage to HT22 cells. TLB reduced intracellular cuprous ion levels in cells exposed to CuSO_4 , and FDX1 knockdown enhanced the effect of TLB in reducing cuprous ion levels in HT22 cells (Fig. 4A). To further verify the effect of TLB on CuSO_4 -induced oxidative stress in HT22 cells, the Mito-SOX Red staining method was used to detect changes in the levels of superoxide anion ($\text{O}_2^{\cdot-}$) in the mitochondria of HT22 cells. The results showed that, compared with the control group, the intensity of mitochondrial red fluorescence increased after CuSO_4 treatment, suggesting that CuSO_4 induced the formation of the superoxide anion $\text{O}_2^{\cdot-}$ in HT22 cells, while TLB treatment significantly reduced the production of mitochondrial superoxide anion $\text{O}_2^{\cdot-}$ in HT22 cells, and knocking down FDX1 expression in HT22 cells significantly enhanced the inhibitory effect of TLB on mitochondrial superoxide anion $\text{O}_2^{\cdot-}$ (Fig. 4B,C). These results indicate that TLB suppresses mitochondrial oxidative stress in HT22 cells by targeting FDX1. During copper-mediated cell death, Cu^{2+} is reduced by FDX1 to the more toxic Cu^+ , which can then modulate key regulatory enzymes of the TCA cycle, leading to disruption of mitochondrial energy metabolism. To further verify the effect of TLB on the TCA cycle in CuSO_4 -induced HT22 cells, the ELISA method was used to detect changes in the TCA cycle in HT22 cells before and after FDX1 knockdown. The results showed that, compared with the control group, CuSO_4 significantly increased intracellular levels of pyruvate and $\alpha\text{-KG}$ and inhibited the enzymatic activities of PDH, CS, ICDH, $\alpha\text{-KGDH}$, and SDH. However, TLB treatment markedly reversed these effects. Furthermore, FDX1 knockdown in HT22 cells significantly enhanced TLB's ability to reduce intracellular pyruvate and $\alpha\text{-KG}$ levels and to increase the enzymatic activities of PDH, CS, ICDH, $\alpha\text{-KGDH}$, and SDH (Fig. 4D-J). These results indicate that TLB improves CuSO_4 -induced TCA cycle disruption by targeting FDX1 (Fig. 4K). To investigate the effect of TLB on CuSO_4 -induced OCR in HT22 cells, the Seahorse energy metabolism analyzer was used to measure cellular oxygen consumption rate (OCR). Mitochondrial respiratory function was assessed by analyzing key indicators such as basal respiration,

ATP-related respiration, maximum respiratory capacity, and reserve respiration. The results showed that, compared with the control group, the CuSO₄ group exhibited significantly reduced OCR levels, with marked decreases in basal respiration, ATP-related respiration, maximum respiratory capacity, and reserve respiratory capacity. However, following TLB intervention, cellular OCR significantly rebounded, and the aforementioned respiratory function indicators were markedly improved. Furthermore, FDX1 knockdown in HT22 cells significantly enhanced the beneficial effect of TLB on OCR (Fig. 4L, M). These results indicate that TLB improves the oxygen consumption rate (OCR) of CuSO₄-induced HT22 cells by targeting FDX1, thereby enhancing cellular mitochondrial respiratory function.

4. Discussion

The principal findings of this study are summarized as follows: (1) TLB alleviates copper sulfate triggered cognitive deficits in mice by relieving oxidative stress and rebalancing mitochondrial fission and fusion dynamics within hippocampal neurons. (2) TLB remodels the expression profile of copper transporters and interrupts cuproptotic signaling cascades to mitigate copper sulfate mediated neuronal injury. (3) Mechanistic investigations confirm that FDX1 acts as an essential mediator to support TLB induced neuroprotection against copper toxicity. These results enrich the mechanistic understanding of TLB mediated neuroprotection for copper overload associated cognitive impairment (Fig. 9).

Most existing studies on metal neurotoxicity focus on the deposition of aluminum or iron while complete evidence that integrates behavioral detection mitochondrial observation and cuproptosis analysis to describe pathological cascades from oral copper overload remains insufficient. Three behavioral detection methods that include Y maze novel object recognition and Morris water maze collectively demonstrate that long term copper sulfate exposure severely impairs short term spatial recognition discriminative memory and long term spatial learning and such pathological phenotypes maintain tight coupling with hippocampal mitochondrial collapse and excessive activation of cuproptotic signaling. Our behavioral test results show that TLB exerts multi-layered neuroprotection rather than non specific clearance of free radicals because the compound can simultaneously correct excessive intracellular copper accumulation and block downstream cell death cascades dependent on FDX1 to break the self amplifying toxic loop initiated by excess copper. This dual regulatory characteristic distinguishes TLB from single target antioxidants and highlights its unique therapeutic potential for encephalopathy caused by metal imbalance.

Mitochondria serve as the primary subcellular target of copper cytotoxicity. Excessive copper induces overproduction of reactive oxygen species (ROS) and malondialdehyde while suppressing endogenous antioxidant enzymes (Tsai et al., 2026). It also causes mitochondrial ultrastructural damage pathological mitochondrial fission and weakened fusion processes to further trigger excessive accumulation of PINK1, which acts as a marker of irreversible mitochondrial damage (Wang et al., 2025). Our study combines oxidative stress biomarker detection, transmission electron microscopic observation of ultrastructure, expression profiling of fission and fusion proteins and analysis of mitochondrial quality control markers to describe the full spectrum of mitochondrial protective effects carried by TLB. Copper

intoxication upregulates fission mediators DRP1 and FIS1, and reduces the expression of fusion proteins MFN1, MFN2 and OPA1 to drive the fragmentation of dysfunctional mitochondria. Treatment with TLB significantly limits overactive fission signaling, recovers the abundance of fusion proteins, alleviates mitochondrial swelling and cristae rupture, and normalizes abnormal accumulation of PINK1.

SLC31A1 known as CTR1 functions as the main copper importer on plasma membranes and ATP7B facilitates the efflux of intracellular copper (Zhang et al., 2024). Copper overload creates a vicious positive feedback cycle because elevated CTR1 expression and repressed ATP7B expression jointly amplify the retention of copper inside cells (Du et al., 2025). TLB exerts bidirectional regulatory effects on these two transporters at transcriptional and translational levels as the compound inhibits copper import mediated by CTR1 and restores metal excretion driven by ATP7B to directly reduce the pool of labile cytoplasmic Cu^+ . Our previous published work has confirmed TLB as a direct inhibitor of FDX1 (Wei et al., 2025), which functions as the rate limiting initiator of cuproptosis to convert low toxicity Cu^{2+} into reactive Cu^+ , and trigger aggregation of lipoylated tricarboxylic acid enzymes and metabolic shutdown. *In vivo* immunoblot detection proves that TLB significantly downregulates the expression of FDX1, LIAS and DLD to reduce toxic accumulation of pyruvate dehydrogenase complex subunits PDHA1, PDHB and DLAT. TLB also restores metal responsive transcription factor 1 and its cytoprotective downstream targets GLS, CDKN2A and p14ARF to rebuild cellular defense systems against metal toxicity.

We perform loss of function experiments through transfection of FDX1 siRNA in HT22 hippocampal neurons to eliminate bias from simple correlation analysis and verify causal interaction between TLB and FDX1. Silencing of FDX1 alone partially rescues cytotoxicity induced by copper and combined treatment with TLB generates synergistic inhibitory effects on overproduction of mitochondrial superoxide, restoration of tricarboxylic acid cycle and recovery of mitochondrial oxygen consumption rate. These *in vitro* functional detection results definitively prove that the metabolic restoration effect of TLB on mitochondria fully relies on the inhibition of FDX1. Rigorous target verification represented by such loss of function assays surpasses traditional correlational analysis that only relies on western blot detection and establishes a complete signaling pathway which describes how TLB inhibits FDX1 to block cuproptosis, repair mitochondrial function and finally improve cognitive performance. Cuproptosis differs from classical oxidative cell death because the defining feature of cuproptosis refers to tricarboxylic acid cycle paralysis induced by aggregated lipoylated dehydrogenases (Liu et al., 2023, Li et al., 2023). Exposure to copper sulfate leads to accumulation of pyruvate and α -ketoglutarate and suppresses the activity of core tricarboxylic acid enzymes that include PDH, CS, ICDH, α -KGDH and SDH. Such metabolic changes are accompanied by universal decline of mitochondrial respiratory capacities that include basal respiration, ATP linked respiration, maximal respiration and spare respiration. Intervention with trilobatin reverses all metabolic abnormalities and silencing of FDX1 further amplifies the metabolic restoration effects of TLB.

Our mechanistic exploration mainly focuses on FDX1 signaling inside hippocampal neurons while researchers have not characterized cell type specific effects of trilobatin in astrocytes and microglia. Further exploration is required to clarify the combined effects of microglia mediated neuroinflammation

and copper transport. We only conduct in vitro silencing of FDX1 to verify protein function and construction of tissue specific FDX1 knockout mouse models is needed to consolidate in vivo evidence for target interaction. This research does not characterize direct molecular interactions between TLB and FDX1 or SLC31A1 through surface plasmon resonance cellular thermal shift assay or molecular docking so the precise structural basis of binding between TLB and target proteins remains unclear. Our copper toxicity model only simulates exogenous copper overload and additional verification is required to test the therapeutic efficacy of TLB against hereditary copper metabolic disorders such as Wilson disease.

In summary, the collective findings from this study demonstrate that TLB ameliorates hippocampal learning and memory deficits triggered by copper sulfate exposure. TLB remodels copper transporters bidirectionally to hinder intracellular copper accumulation in neurons while robustly suppressing FDX1 governed cuproptotic signaling cascades. The compound further mitigates oxidative stress, restores physiological mitochondrial fission and fusion dynamics, and recovers disrupted tricarboxylic acid cycle flux alongside compromised mitochondrial respiratory capacity. Our previously published work has validated TLB as a direct inhibitor of FDX1. The present study further establishes FDX1 as an essential functional mediator required for all neuroprotective effects exerted by TLB. This work uncovers a previously unrecognized mechanism through which TLB targets cuproptosis to attenuate neuronal damage arising from metal dyshomeostasis. It also provides robust preclinical evidence and establishes a novel research framework for developing neuroprotective therapeutics against cognitive degenerative disorders associated with disturbed copper balance.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

All animal care and experimental procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978), and all animal experiments were performed with the approval of the institutional ethics committee (approval number ZMU21-2203-538). Clinical trial number: not applicable.

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Author Contribution

G.L., W.R., H.L., J.G., G.L. and Y.W. conceived and designed research. G.L., W.R., Z. Y., F.H., W.Z., D.L., and N.C. conducted experiments. W.R. and X.W. contributed new reagents or analytical tools. W.R. and X.W. analyzed data. G.L. and W.R. wrote the manuscript. All authors read and approved the manuscript. Approval of the final version of the manuscript on behalf of all authors was done by J.G., G.L. and Y.W.. The authors declare that all data were generated in-house, that no paper mill was used and that no AI tool has been used for the generation of text or figures.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. KUNWAR D, NAMDEO KUMARJALISA, A. G., FLORA SJS (2026) Ceruloplasmin and neurodegeneration: A link between copper dysregulation, iron imbalance, and neuronal damage. *J Trace Elem Med Biol* 97. <https://doi.org/10.1016/j.jtemb.2026.127929>
2. WONG AYL, PRAKASH MYERSJW, MA G, PETROVA ABROOKJR, BULUT B, WHITE B, DE RMERZC, SOUZA M, WANG MAYNARDAG, POHL PYUAMY, WEITMAN NK, M., SILVERMAN, L. B., STEGMAIER, K., CHURCHMAN, L. S., COLE, P. D., BRADY, D. C., KANAREK N (2026) Copper depletion boosts CNS leukemia therapy by inhibiting nucleotide synthesis through impairment of mitochondrial complex IV activity. *Nat Cancer*. <https://doi.org/10.1038/s43018-026-01177-4>
3. DELONCLE R (2026) Conjecture for a free radical epimerization process in Alzheimer, Parkinson, Lewy body, amyotrophic lateral sclerosis, progressive Supranuclear Palsy and Creutzfeldt Jakob diseases. *Exp Neurol* 403. <https://doi.org/10.1016/j.expneurol.2026.115812>
4. CHENG YF, ZHAO YJ, CHEN C, ZHANG F (2026) Metallothionein and neurodegenerative diseases. *Neural Regeneration Res* 21:3449–3461. <https://doi.org/10.4103/Nrr.Nrr-D-25-00011>
5. KEPP KP, SQUITTI R (2019) Copper imbalance in Alzheimer's disease: Convergence of the chemistry and the clinic. *Coord Chem Rev* 397:168–187. <https://doi.org/10.1016/j.ccr.2019.06.018>
6. GAO JY, WU SM, YANG JX, YE T, YANG J, SHEN WH, HUANG CHENXW, LIN LPANGRQ, P, LIN, J. H., ZHOU, Y., WANG, W., TAN T (2025a) Comprehensive behavioral characterization and impaired hippocampal synaptic transmission in R1117X Shank3 mutant mice. *Translational Psychiatry* 15. <https://doi.org/10.1038/s41398-025-03505-1>
7. HE X, HU CAOZWCHENXY, LI J, WU JXJIAXH, J. C., LIU XC (2025) Resveratrol Alleviates the Prenatally Intermittent Hypoxia-Induced Cognitive Impairment in Male Offspring Mice Through Modulating the SIRT1/HIF-1 α Pathway. *Brain Behav* 15. <https://doi.org/10.1002/brb3.70413>

8. HONG JY, LIU YX, MEI PENGQ, Z. G., WANG GZ (2026) Cuproptosis and Aging: Molecular Mechanisms and Therapeutic Implications. *Aging Disease*. <https://doi.org/10.14336/Ad.2025.1305>
9. WANG Q, CHEN SUNJY, HOU TSONGSY, Y. J., FENG, L. N., FAN, C. D., LI MQ 2023a. Ferroptosis, Pyroptosis, and Cuproptosis in Alzheimer's Disease. *ACS Chem Neurosci*, 14, 3564–3587. <https://doi.org/10.1021/acscchemneuro.3c00343>
10. ZHU ZP, SONG M, LIANG RENJX, L. R., MAO, G. H., CHEN M (2024) Copper homeostasis and cuproptosis in central nervous system diseases. *Cell Death Dis* 15. <https://doi.org/10.1038/s41419-024-07206-3>
11. HE GT, ZHU HX, KANG PP, FENG LL, ZHU YUCH, LIU YL, GUO B, Z. X., YANG PP (2026) Bimetallic Peroxide Nanocomposites-Driven Redox Dyshomeostasis to Activate Sequential Cuproptosis and Pyroptosis for Amplified Tumor Immunotherapy. *Adv Sci* 13. <https://doi.org/10.1002/advs.202512470>
12. WANG FD, TAO LIUWY, NI CCHENLY, HUANG NJ, SHI LJOUYANGYY, LI QQWUDD, WANG CH, Y. F., MIN, J. X., XIE, L. W., LIU JX (2026) Copper and cuproptosis: mechanisms, biology, and roles in disease. *Sci Bull* 71:3136–3157. <https://doi.org/10.1016/j.scib.2026.05.007>
13. WANG EZ, ZENG ZHOUSJ, D. X., WANG R (2023b) Molecular regulation and therapeutic implications of cell death in pulmonary hypertension. *Cell Death Discovery* 9. <https://doi.org/10.1038/s41420-023-01535-6>
14. CHEN L, MIN, J., WANG F (2022) Copper homeostasis and cuproptosis in health and disease. *Signal Transduct Target Therapy*. 7<https://doi.org/10.1038/s41392-022-01229-y>
15. ZHANG Y, GU Y, ZHAN M, YANG L, WANG H (2025a) Targeting ferroptosis and cuproptosis in gastrointestinal cancers: molecular mechanisms, metabolic vulnerabilities, and therapeutic interventions. *Mol Biomed* 6. <https://doi.org/10.1186/s43556-025-00347-7>
16. LI N, YANG LIUFR, XIONG P, LI FYUQL, ZHOU JX, ZHANG ZG, S., WANG CY (2019) Aging and stress induced β cell senescence and its implication in diabetes development. *Aging-Us* 11:9947–9959. <https://doi.org/10.18632/aging.102432>
17. CHEN N, ZOU YAOL, WANG L, LI Q, LUO Y, ZHANG JZHANMZHOUKXIAOJTONGS, YANG H, L., GE L (2026) Cel-LDH dual-functional nanotherapy simultaneously promotes microglial efferocytosis and alleviates neuronal apoptosis for spinal cord injury recovery. *J Nanobiotechnol*. <https://doi.org/10.1186/s12951-026-04641-6>
18. ZHOU XT, PENG SP (2026) The cross-talk and interplay between ferroptosis and cuproptosis in tumor and therapeutics. *Cell Oncol* 49. <https://doi.org/10.1007/s13402-025-01138-6>
19. FENG Y, YANG ZB, WANG, J. P., ZHAO H (2024a) Cuproptosis: unveiling a new frontier in cancer biology and therapeutics. *Cell Communication Signal*. 22<https://doi.org/10.1186/s12964-024-01625-7>
20. GAO JM, YI Y, RAN W, CHENG Y, DENG WK, SHI DUANSY, WEI FG, ZHANG Y, Y. D., GONG QH (2026) Targeting astrocytic Nrf2 by Trilobatin alleviates lipopolysaccharide-induced depressive-like

- behaviors and cognitive impairment in mice: Mechanistic insights into gut microbiota and metabolites modulation. *Phytomedicine* 155. <https://doi.org/10.1016/j.phymed.2026.158118>
21. HOU FQ, WU XY, GONG MX, WEI JJ, YI Y, WEI Y, HE ZX, GONG, Q. H., GAO JM (2023) Trilobatin rescues fulminant hepatic failure by targeting COX2: Involvement of ROS/TLR4/NLRP3 signaling. *Phytomedicine* 120. <https://doi.org/10.1016/j.phymed.2023.155059>
 22. LIN M, LUO XIEDY, DONG YM, WEI L, GONG Y, ZHU QH, Y. Z., GAO JM (2025) Trilobatin, a Naturally Occurring GPR158 Ligand, Alleviates Depressive-like Behavior by Promoting Mitophagy. *J Agric Food Chem* 73:5163–5179. <https://doi.org/10.1021/acs.jafc.4c05431>
 23. XIE DY, LIN M, LUO YM, DONG L, WEI Y, GAO JM, ZHU YZ, GONG QH (2024) Trilobatin suppresses aging-induced cognitive impairment by targeting SIRT2: Involvement of remodeling gut microbiota to mediate the brain-gut axis. *Phytomedicine* 130. <https://doi.org/10.1016/j.phymed.2024.155744>
 24. WU XY, WEI JJ, RAN W, LIU DJ, YI Y, LIU GONGMX, LI XGONGQH, H. B., GAO JM (2025) The Gut Microbiota-Xanthurenic Acid-Aromatic Hydrocarbon Receptor Axis Mediates the Anticolitic Effects of Trilobatin. *Adv Sci* 12. <https://doi.org/10.1002/advs.202412234>
 25. GAO JM, LI WB, WANG CHENNNYIY, CHEN ZH, YUAN XZHAOYY, GAO ZL, GUO JPANYC, D. S., GONG QH 2025b. A Single-Molecule Pleuripotent Scaffold for Combinational Therapy of Alzheimer's Disease via Intranasal Administration. *Adv Sci*, 12. <https://doi.org/10.1002/advs.202511611>
 26. WEI JJ, ZHANG LANGZ, RAN WF, WEI W, LIU Y, ZHANG X, LI YDGONGQH, H. B., GAO JM (2025) Targeting FDX1 by trilobatin to inhibit cuproptosis in doxorubicin-induced cardiotoxicity. *Br J Pharmacol* 182:2409–2425. <https://doi.org/10.1111/bph.17468>
 27. ZHANG HC, YA J, DU SUNMY, X. B., REN, J. S., QU XG (2025b) Inhibition of the cGAS-STING pathway an endogenous copper ion-responsive covalent organic framework nanozyme for Alzheimer's disease treatment. *Chem Sci* 16:7215–7226. <https://doi.org/10.1039/d4sc07963a>
 28. FENG L, LI Y, LIN M, XIE D, LUO Y, ZHANG Y, HE Z, GONG Q, ZHUN ZY, GAO J (2024b) Trilobatin attenuates cerebral ischaemia/reperfusion-induced blood-brain barrier dysfunction by targeting matrix metalloproteinase 9: The legend of a food additive. *Br J Pharmacol* 181:1005–1027. <https://doi.org/10.1111/bph.16239>
 29. ABRAHAM MJ, MURTOLA T, PÁLL SCHULZR, HESS SSMITHJC, B., LINDAHL E (2015) GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* 1–2:19–25. <https://doi.org/https://doi.org/10.1016/j.softx.2015.06.001>
 30. VAN DER SPOEL D, LINDAHL E, HESS B, GROENHOF G, MARK AE, BERENDSEN HJ (2005) GROMACS: fast, flexible, and free. *J Comput Chem* 26:1701–1718. <https://doi.org/10.1002/jcc.20291>
 31. TSAI PK, LEE WUSW, CHANG YJ, LEE YT, HO SS, YANG YC, L. C., KUAN YH (2026) Integrated Multi-Endpoint Analysis of Soluble Copper(II) Chloride-Induced Oxidative Stress, Mitochondrial Dysfunction, and Genotoxicity in RAW264.7 Macrophages. *Biol Trace Elem Res* 204:5236–5247. <https://doi.org/10.1007/s12011-026-05027-3>

32. WANG SG, LIAO ZN, HAN ZHANGQY, LIU XY, C. Q., WANG J (2025) Mitochondrial dysfunction in Alzheimer's disease: a key frontier for future targeted therapies. *Front Immunol* 15. <https://doi.org/10.3389/fimmu.2024.1484373>
33. ZHANG SH, HUANG QB, JI T, LI QL, HU CY (2024) Copper homeostasis and copper-induced cell death in tumor immunity: implications for therapeutic strategies in cancer immunotherapy. *Biomark Res* 12. <https://doi.org/10.1186/s40364-024-00677-8>
34. DU F, TANG L, WANG F, JIANG X, LI JX, WANG, G. J., ZHANG Z (2025) Epigenetic modification of cuproptosis by non-coding RNAs in cancer drug resistance. *Mol Cancer* 24. <https://doi.org/10.1186/s12943-025-02434-3>
35. LIU L, WANG, Q., ZHOU, J. Y., ZHANG B (2023) Developing four cuproptosis-related lncRNAs signature to predict prognosis and immune activity in ovarian cancer. *J Ovarian Res.* 16 <https://doi.org/10.1186/s13048-023-01165-7>
36. LI Y, ZHOU DUYH, LUO YCHENQH, REN ZJ, CHEN YF, X. D., CHEN GA (2023) Iron and copper: critical executioners of ferroptosis, cuproptosis and other forms of cell death. *Cell Communication Signal* 21. <https://doi.org/10.1186/s12964-023-01267-1>

Figures

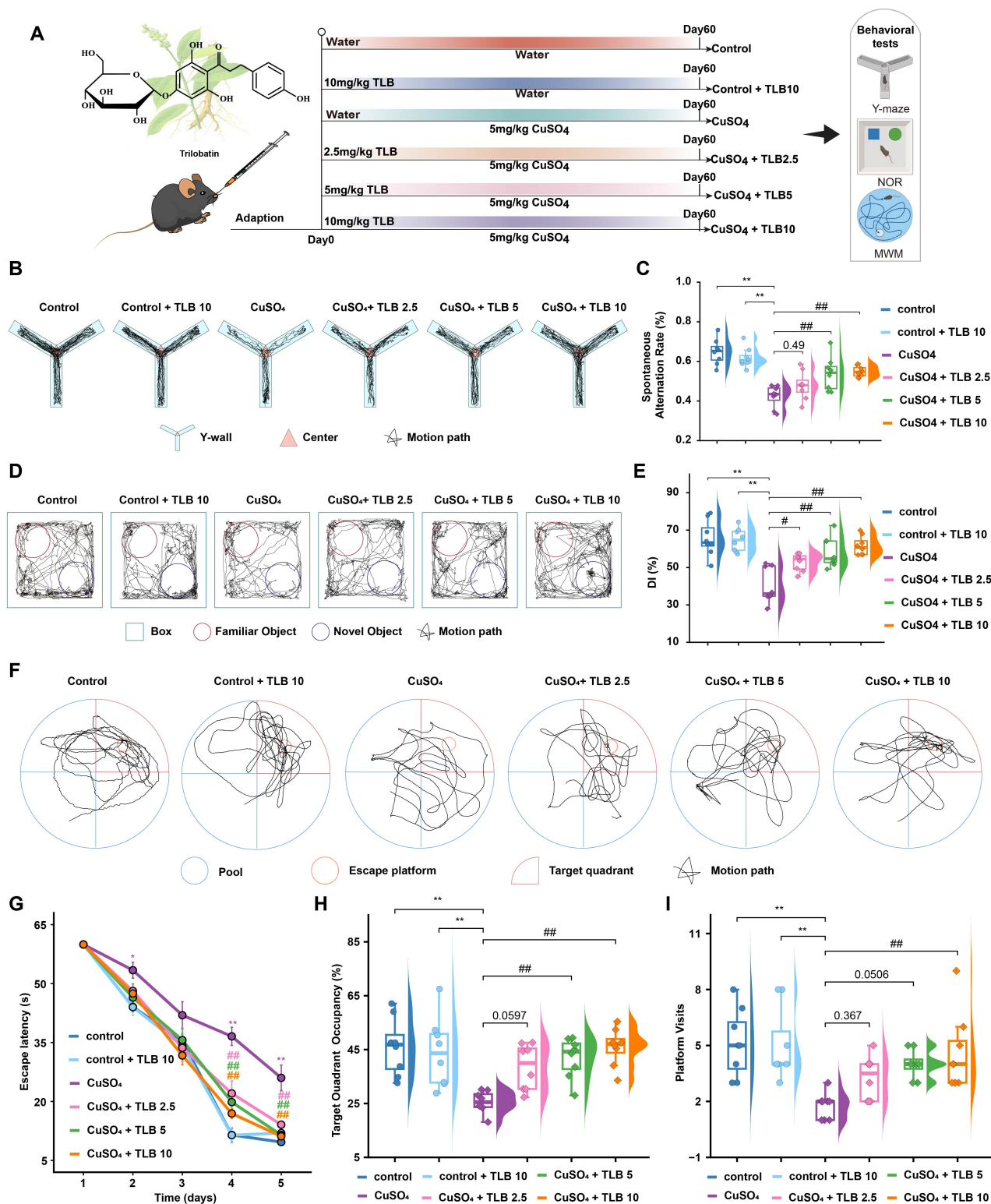


Figure 1

Trilobatin ameliorates copper sulfate-induced cognitive dysfunction. (A) Establishment of the CuSO₄-induced cognitive impairment model and experimental animal grouping and treatment procedures. (B) Object exploration trajectory diagram of Y-maze test. (C) Alternation percentage (n = 8). (D) Object exploration trajectory diagram of Novel object recognition test. (E) Quantitative map of discrimination index (n = 8). (F) Object exploration trajectory diagram of MWM test. (G) Escape latency (n = 8). (H) The

time spent in the target quadrant (n = 8). (I) Platform crossover number (n = 8). Data are expressed as mean ± SEM. **P < 0.01 vs. Control group; #P < 0.05, ##P < 0.01 vs. CuSO₄ group.

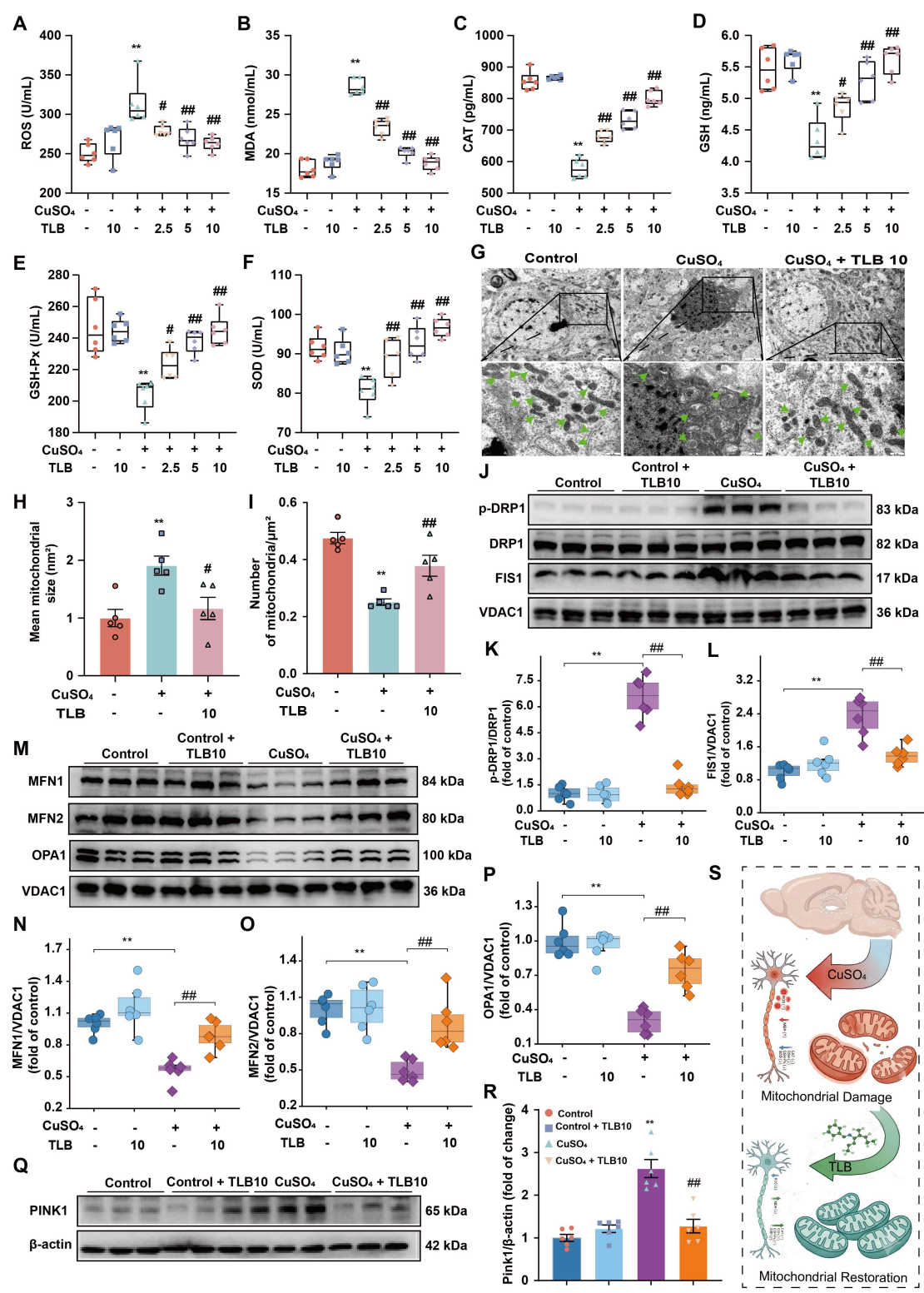


Figure 2

Trilobatin Attenuates CuSO₄-Induced Oxidative Stress and Restores Mitochondrial Dynamics and Structural Integrity. (A) Reactive oxygen species (ROS) level (n = 6). (B) Malondialdehyde (MDA) activity

(n = 6). (C) Catalase (CAT) activity (n = 6). (D) Glutathione (GSH) activity (n = 6). (E) Glutathione peroxidase

(GSH-Px) activity (n = 6). (F) Superoxide dismutase (SOD) activity (n = 6). (G) Representative images of mitochondrial ultrastructure (Magnification: 8000 times; Scale = 2 μ m; Magnification: 25000 times; Scale = 500 nm. The green arrow represents the mitochondria). (H) Number of mitochondria (n = 5). (I) Average of mitochondrial size (n = 5). (J) Representative immunoblots of mitochondrial fission proteins. (K) Quantification of p-DRP1 protein expression (n = 6). (L) Quantification of FIS1 protein expression (n = 6). (M) Representative immunoblots of MFN1, MFN2, and OPA1 proteins. (N) Quantification of MFN1 protein expression (n = 6). (O) Quantification of MFN2 protein expression (n = 6). (P) Quantification of OPA1 protein expression (n = 6). (Q) Representative immunoblots of PINK1 proteins. (R) Quantification of PINK1 protein expression (n = 6). (S) Schematic summary illustrating TLB attenuates CuSO₄-Induced oxidative stress and restores mitochondrial dynamics and structural integrity. Data are expressed as mean $\pm$ SEM. **P < 0.01 vs. Control group; #P < 0.05, ##P < 0.01 vs. CuSO₄ group.

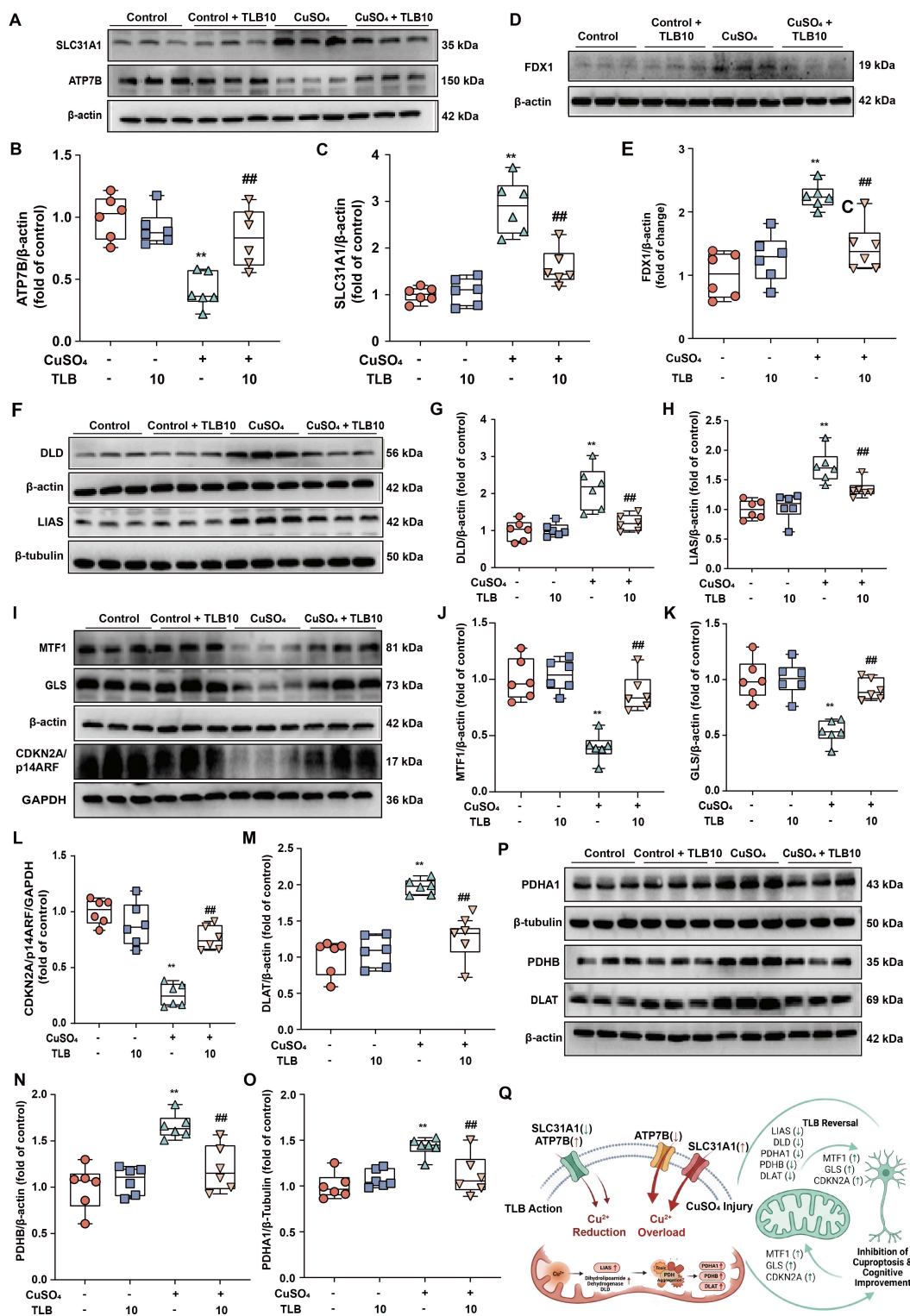


Figure 3

TLB Regulates Copper Transporters and Cuproptosis Signaling Pathways to Attenuate CuSO₄-Induced Toxicity. (A) Representative image of SLC31A1 and ATP7B proteins expression. (B) Quantification of SLC31A1 protein levels (n = 6). (C) Quantification of ATP7B protein levels (n = 6). (D) Representative image of FDX1 protein expression. (E) Quantification of FDX1 protein levels (n = 6). (F) Representative image of DLD and LIAS protein expression. (G) Quantification of DLD protein levels (n = 6). (H)

Quantification of LIAS protein levels (n = 6). (I) Representative images of the expression of cuproptosis-related proteins. (J) Quantification of MTF1 protein levels (n = 6). (K) Quantification of GLS protein levels (n = 6). (L) Quantification of CDKN2A/p14ARF protein levels (n = 6). (M) Quantification of PDHA1 protein levels (n = 6). (N) Quantification of PDHB protein levels (n = 6). (O) Quantification of DLAT protein levels (n = 6). (P) Representative images of the expression of pyruvate dehydrogenase complex-related proteins. (Q) Schematic summary illustrating TLB regulates copper transporters and cuproptosis signaling pathways to attenuate CuSO₄-Induced toxicity. Data are expressed as mean ± SEM. **P < 0.01 vs. Control group; #P < 0.05, ##P < 0.01 vs. CuSO₄ group.

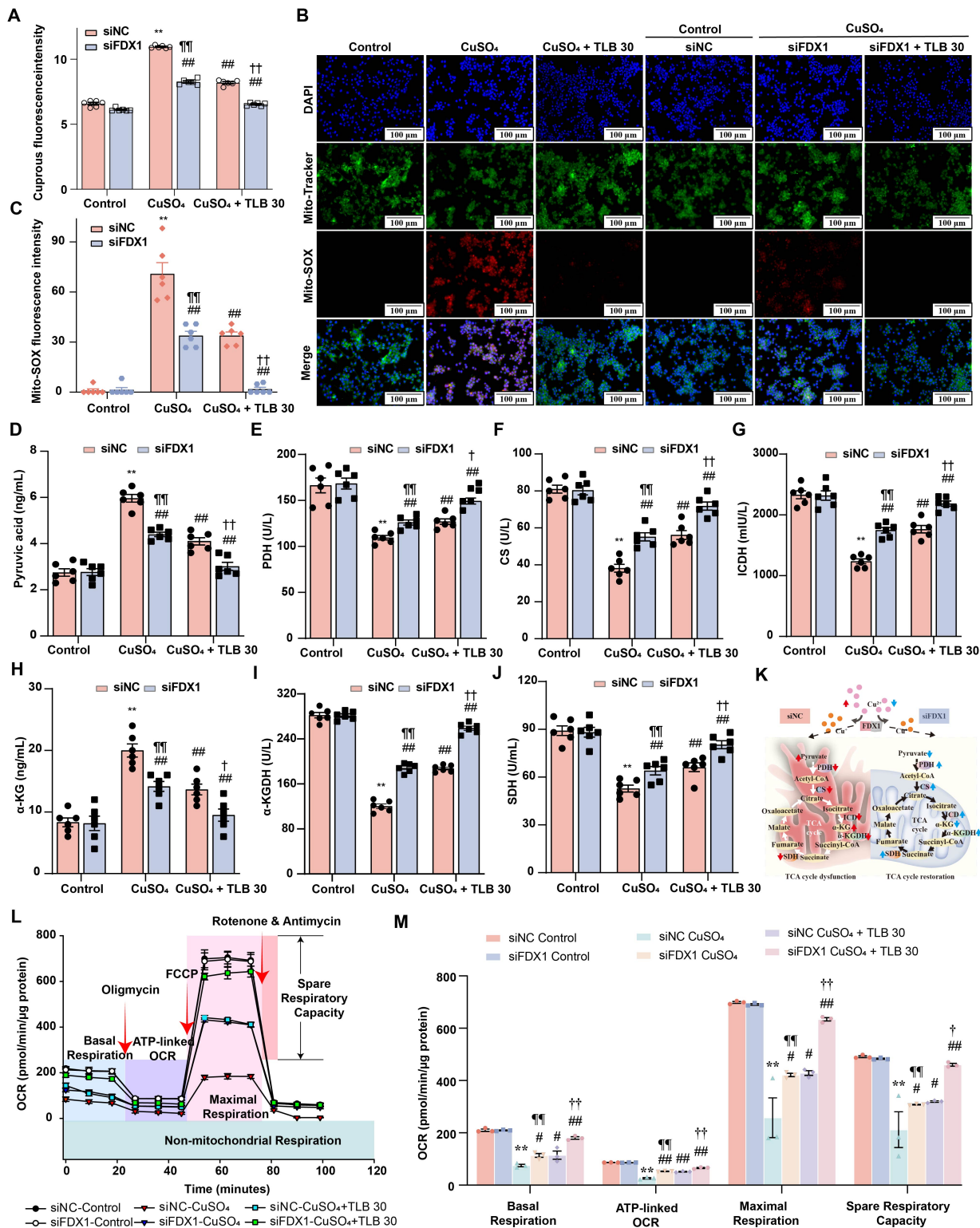


Figure 4

FDX1 Mediates the Protective Effects of Trilobatin Against CuSO₄-Induced Toxicity in HT22 Cells. (A) Cellular cuprous fluorescence intensity (n = 6). (B) Representative images of Mito-SOX Red staining (magnification: 200 times; scale bar = 100 μm). (C) Quantitative analyses of Mito-SOX Red fluorescence intensity (n = 6). (D) Pyruvic acid level (n = 6). (E) PDH activity (n = 6). (F) CS activity (n = 6). (G) ICDH activity (n = 6). (H) α-KG level (n = 6). (I) α-KGDH activity (n = 6). (J) SDH activity (n = 6). (K) Proposed

mechanism of CuSO₄-triggered mitochondrial TCA cycle disorder and TLB-mediated functional restoration after FDX1 knockdown. Data are expressed as mean ± SEM. **P < 0.01 vs. Control group; ##P < 0.01 vs. CuSO₄ group; ††P < 0.01 vs. siFDX1 group; †P < 0.05, ††P < 0.01 vs. CuSO₄+ siFDX1 group.

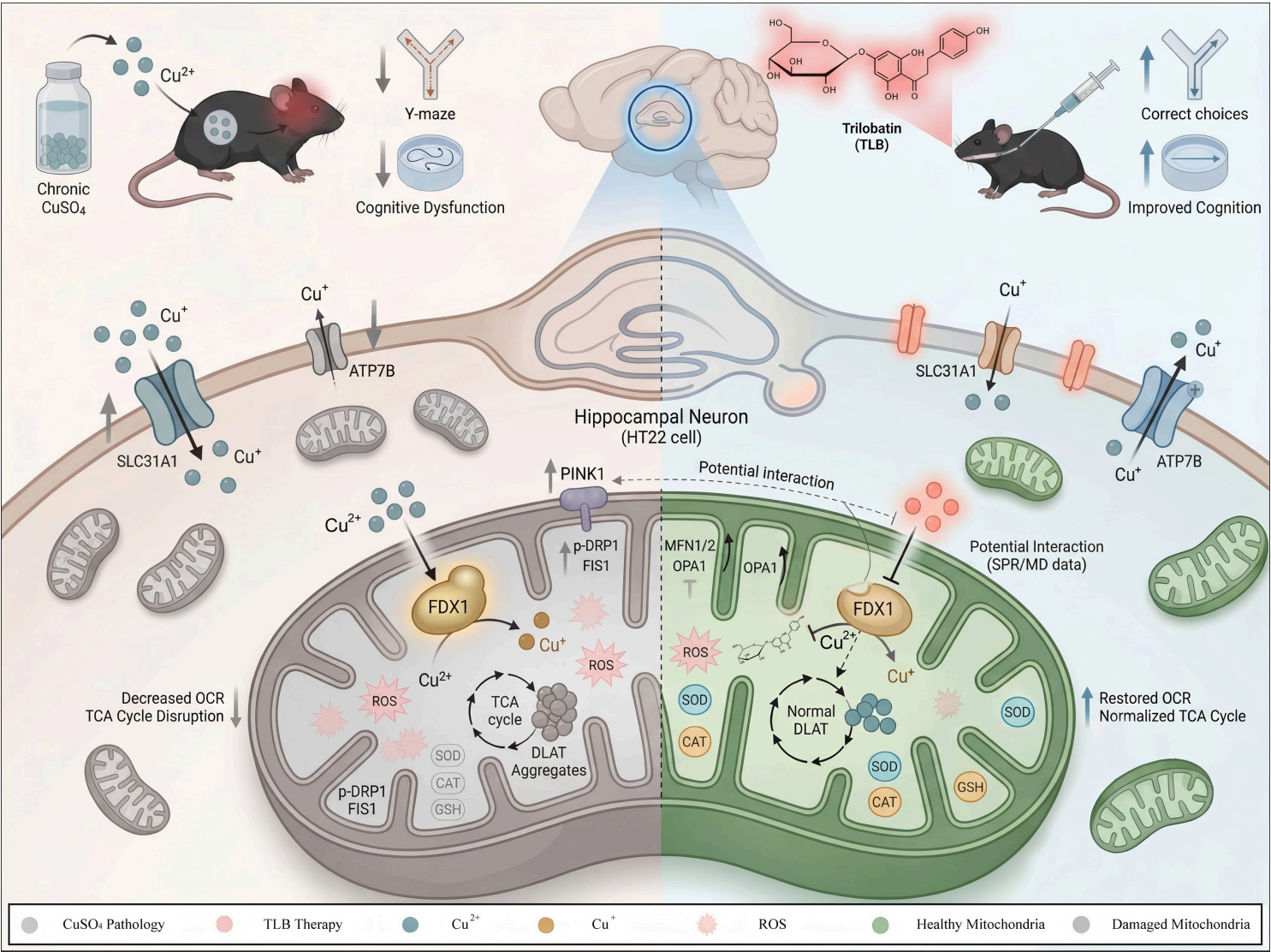


Figure 5

Figure 9. Proposed mechanistic model for TLB mediated neuroprotection against copper overload induced cognitive deficits. Copper sulfate intoxication elevates intracellular copper concentration in hippocampal neurons. Increased copper initiates FDX1 governed cuproptosis, breaks copper transporter balance, provokes oxidative stress and mitochondrial dysfunction to drive neuronal damage and cognitive impairment. Trilobatin regulates SLC31A1 and ATP7B to stabilize cellular copper pools and inhibits FDX1 dependent cuproptosis. Such actions mitigate oxidative burden, normalize mitochondrial dynamics and recover tricarboxylic acid cycle activity, thereby alleviating hippocampal neuronal injury and improving cognitive performance.

Supplementary Files

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