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Supporting Information

A mitochondria-targeted anticancer copper dithiocarbamate amplifies immunogenic cuproptosis and macrophage polarization

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1. Experimental Procedures

1.1 Reagents

Copper(II) bis(diethyldithiocarbamate) (CuET), 2-cyano-3-(1-phenylindol-3-yl)acrylate (UK-5099), ferrostatin-1 (Fer-1), necrostatin-1 (Nec-1), 4-dimethylaminopyridine (DMAP), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), carbon bisulfide, and bovine serum albumin (BSA) were purchased from Aladdin Reagent Co., Ltd (Shanghai, China). Copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) and sodium hydroxide (NaOH) were purchased from Kelong Chemical Reagent Factory (Chengdu, China). 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) was purchased from Adamas Reagent Ltd (Shanghai, China). 2-(Ethylamino)ethanol, dimethyl fumarate, tranilast, 3,4-methylenedioxy-β-nitrostyrene (MNS), belnacasan (VX-765), lenalidomide and (3-carboxypropyl)triphenylphosphonium bromide (TPP-COOH) were bought from Shanghai Macklin Biochemical Co., Ltd. (3-Chlorophenyl)hydrazonomalononitrile (CCCP) and doxorubicin (DOX) were obtained from Shanghai yuanye Bio-Technology Co., Ltd. Lipopolysaccharide (LPS) was purchased from Beijing Solarbio Science & Technology Co., Ltd

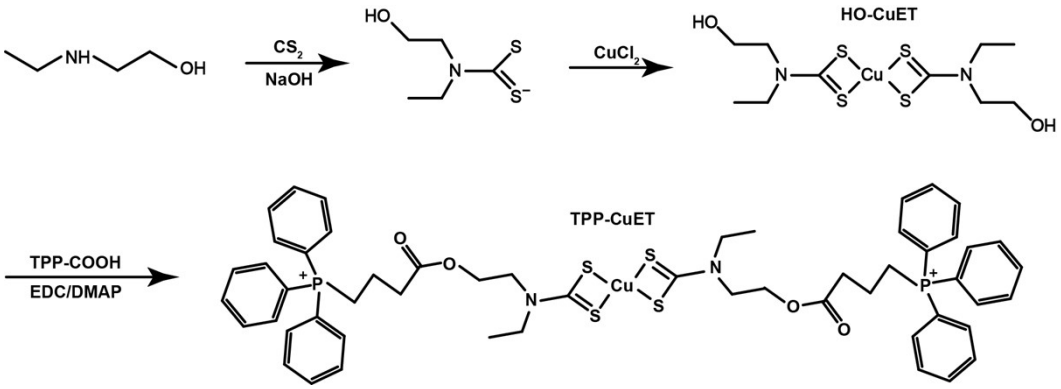
1.2 Cell culture

4T1, B16 and CT26 cells were grown in RPMI media with 10% fetal bovine serum (FBS, Cell-Box) supplemented with 1% penicillin-streptomycin. HepG2 and RAW 264.7 cells were incubated in MEM and DMEM medium (Gibco) containing 10% fetal bovine serum supplemented with 1% penicillin-streptomycin (Gibco), respectively. For the glucose, galactose experiments, RPMI media without glucose (Thermo Fisher Scientific, 11879020) was supplemented with 10% fetal bovine serum, 100 μg/ml of uridine (Beyotime Biotechnology, ST1735), 1 mM sodium pyruvate (Beyotime Biotechnology, C0331) and either 10 mM glucose (Beyotime Biotechnology, ST491) or 10 mM galactose (Biosharp, BS917). All the above cells were cultivated in a humidified incubator with 5% CO_2 at 37 °C, and grown to about 80% confluence before splitting or harvesting.

1.3 Synthesis of TPP-CuET

Synthesis of HO-CuET. N-ethyl-N-ethanolamine (0.98 mL, 10 mmol) was reacted with CS2 (0.72 mL, 12 mmol) in ice ethanol with NaOH (399.9 mg, 10 mmol) for 2 h to obtain a pale-yellow solution. Then, $CuCl_2$ (1.0 g, 6 mmol) was added and the reaction continued overnight. HO-CuET was obtained after washing with water, rotary evaporation, and vacuum drying (yield: 87%).

Synthesis of TPP-CuET. TPP-COOH (1.8 g, 4.2 mmol), EDC (996.8 mg, 5.2 mmol), and DMAP (171.1 mg, 1.4 mmol) were dissolved in dichloromethane (DCM) and magnetically stirred at room temperature for 1 h. HO-CuET (784.1 mg, 2 mmol) was added and the mixture was stirred for 48 h and the reaction process was monitored by thin-layer chromatography. After multiple extractions of water and saturated salt solution to remove excess reactants, black solid product TPP-CuET (yield: 94%) was obtained by removing the solvent through rotary evaporation.



Scheme S1. Synthetic route of TPP-CuET.

1.4 Measurement of partition coefficient

The partition coefficient (log *P*) of TPP-CuET were measured using the shake-flask technique, as described previously [1, 2]. The n-octanol and aqueous phase (PBS, 10 mM, pH 7.4) were mutually saturated for 24 h. The standard curves of TPP-CuET in aqueous (n-octanol saturated PBS) and oil (PBS saturated water n-octanol) phases were measured using UV-vis spectrophotometry at 435 nm. An appropriate amount of TPP-CuET was dissolved in a mixture of saturated n-octanol and PBS of equal volume, and fully shaken at room temperature to achieve the partitioning equilibrium of the solute. After separation of the equilibrated phases (in a centrifuge at 3000 rpm for 10 min) the concentrations of TPP-CuET in aqueous (C_w) and oil (C_o) phases were determined using UV-vis spectrophotometry. The log $P_{O/W}$ was measured using the following equation.

$$\text{Log } P_{o/w} = \log ([C_o]/[C_w])$$

1.5 Immunoblot analysis

4T1 cells (5×10^5) were incubated with CuET (2.5 μM) or TPP-CuET (4.5 μM) for 12 h. After washing twice with ice-cold PBS, cells were lysed on ice for 30 minutes in RIPA lysis buffer (Beyotime Biotechnology, P0013B) supplemented with phenylmethanesulfonyl fluoride (PMSF, Beyotime Biotechnology, ST506) protease inhibitor. Cell fragments were removed by centrifugation at 4 °C for 10 minutes at 14000 rcf and the supernatant was collected and quantified by bicinchoninic acid (BCA) assay kit (Biosharp, BL521A). After appropriate dilution, proteins were fractionated by SDS-PAGE gradient gel and transferred onto PVDF membranes (MerckMillipore). Subsequently, the membranes were incubated at room temperature for 2 h in tris-buffered saline with Tween 20 (TBST) containing 5% skim milk, and then incubated overnight with antibodies against DLAT (Abcam, ab172617) and β-actin (Affinity, AF7018) at 4 °C. Afterwards, PVDF membranes were washed 3 times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature

before washing again with TBST. The protein bands were detected with ECL detection reagent (Biosharp) and imaged on a ChemiScope 6100 (Clinx Science Instruments Co., Ltd, China).

1.6 Cytotoxicity assay

The cytotoxicity of CuET and TPP-CuET was evaluated via a typical MTT method. Briefly, cells (1×10^4 per well) were seeded in 96-well plates and incubated overnight. The culture medium was then replaced with different concentrations of drug containing medium and incubated for 24 h, followed by treatment with MTT solution (0.5 mg/mL) for 4 h. For the insoluble CuET and TPP-CuET, 10 mM stock solutions in dimethyl sulfoxide (DMSO) were prepared and sequentially diluted with culture medium to experimental concentrations. Finally, the supernatant was carefully removed and the formazan was fully dissolved in DMSO, and the absorbance of the solution at 570 nm was measured using a microplate reader (Synergy H1, BioTek, USA). In the chemical rescue experiments, death inhibitors were added 16 h before the addition of cell death inducing copper compounds.

1.7 Cellular distribution

To investigate the cellular uptake and intracellular distribution of CuET and TPP-CuET, cell mitochondria isolation kit (Beyotime Biotechnology, C3601) and inductively coupled plasma-mass spectrometer (ICP-MS) were combined for analysis. Specifically, 4T1 cells were seeded in 10 cm dishes at a density of 2×10^6 cells per dish. After incubating overnight, the cells were treated with CuET (2.5 μM) or TPP-CuET (4.5 μM) for 12 h, at which concentrations comparable cell viability were obtained. Subsequently, the cells were harvested and counted using a haemocytometer, and mitochondria were extracted according to the manufacturers' instructions. The resulting samples were digested with ultrapure nitric acid at room temperature overnight and then boiled for 2 h. After diluting to about 10 mL with ultrapure water, the Cu contents were determined by ICP-MS (ICP-MS 7900, Agilent Technologies, USA).

1.8 JC-1 Assay

4T1 cells were incubated with CuET (2.5 μM) or TPP-CuET (4.5 μM) for 24 h, and CCCP (10 μM) was added 30 min before the end of the experiment. According to the manufacturer's protocol of the JC-1 mitochondrial membrane potential assay kit (Yeasen, 40705ES03), treated cells were stained for 20 min and washed three times with PBS. Cells were then analyzed by confocal laser scanning microscope (CLSM, LSM880 Zeiss, Germany) and flow cytometry (BD LSRfortessa, USA).

1.9 Cell and mitochondrial morphology

After incubation with CuET (2.5 μM) or TPP-CuET (4.5 μM) for 24 h, 4T1 cells were harvested. The cell suspension was centrifuged (1000 rpm) for 5 min and the supernatant was carefully removed. The cell pellet was then prefixed with a 3% glutaraldehyde, and postfixed in 1% osmium tetroxide. Next, after dehydration in series acetone, infiltration and embedding in Epox 812, the semithin sections were cut by ultramicrotome and mounted onto copper grids. Ultrathin sections were then cut with diamond knife and stained with uranyl acetate and lead citrate. Sections were observed by a transmission electron microscope (JEM-1400-FLASH, JEOL, Japan).

1.10 Cytolytic death

Cell lytic death was validated using a lactate dehydrogenase (LDH) release assay kit (Beyotime Biotechnology, C0016) and annexin V-FITC/PI staining (Beyotime Biotechnology, C1062) experiments [3]. 4T1 cells seeded in 96-wells plate were incubated different concentrations of CuET or TPP-CuET for 24 h. The level of LDH in the supernatant was monitored using LDH release assay kit based on a diaphorase catalyzed INT chromogenic reaction. For annexin V-FITC/PI staining assay,

cells seeded in 6-well plate were treated with CuET (2.5 μM) or TPP-CuET (4.5 μM) for 24 h and then collected for flow cytometry analysis.

1.11 ATP release

4T1 cells seeded in white 96-wells plate were incubated with DOX (1 μM), CuET (2.5 μM) or TPP-CuET (4.5 μM) for 24 h. The ATP levels in the supernatant were detected by an ATP assay kit (Beyotime Biotechnology, S0026) based on luciferin-luciferase reaction. The chemiluminescence of the samples was monitored by a multifunctional microplate reader (Synergy H1, BioTek, USA).

1.12 Surface CRT expression

4T1 cells seeded in glass bottom culture dishes were incubated with DOX (1 μM), CuET (1.2 μM) or TPP-CuET (2.0 μM) for 12 h. After carefully removing the culture medium and washing three times with PBS, 4% paraformaldehyde was added and fixed overnight at 4 °C. Subsequently, cells were washed and blocked at room temperature with 10% rabbit serum (Solarbio, SL034) for 20 minutes, followed by incubation with AF488-conjugated calreticulin (CRT) antibodies (Bioss, bs-5913R-AF488) at 37 °C for 2 h. After counterstaining with Hoechst 33342, CRT immunofluorescence was photographed with CLSM.

1.13 HMGB1 release

4T1 cells were seeded into glass bottom culture dishes and 6-well plates for CLSM observation and flow cytometry analysis, respectively. After incubation with DOX (1 μM), CuET (1.2 μM) or TPP-CuET (2.0 μM) for 12 h, cells were fixed overnight at 4 °C in 4% paraformaldehyde. Cells for CLSM observation were permeabilized with 0.1% Triton X-100 (Beyotime Biotechnology, Triton X-100) at room temperature for 20 min. Cells for flow cytometry analysis were permeabilized in 90% methanol at -20 °C for 20 min. Subsequently, the cells were fixed at room temperature with goat serum (Bioss, C-0005) for 20 min and incubated with high-mobility group box 1 (HMGB1) antibodies (Bioss, 0664R) at room temperature for 2 h. After washing 3 times with PBS, AF488-conjugated secondary antibodies (Yeasen Biotechnology, 33106ES60) were added and incubated at 37 °C for 90 min. Thereafter, the cells were applied for flow cytometry analysis or for CLSM observation after counterstaining with Hoechst 33342.

1.14 Macrophage polarization

In vitro macrophage polarization induction experiment was adapted from previous report [4]. The conditional medium was prepared as follows: after incubation with CuET (2.5 μM) or TPP-CuET (4.5 μM) for 12 h, the culture medium was replaced with fresh, drug-free culture medium to incubate 4T1 cells for another 12 h, thereby obtaining a conditioned medium.

For flow cytometry assay, RAW 264.7 cells were incubated with the aforementioned conditioned medium or LPS (1 μg/mL) for 12 h. Then, the cells were collected, centrifuged, washed with PBS, and blocked at room temperature with 3% BSA in PBS for 40 min. After staining with APC-conjugated CD86 antibodies (BD Pharmingen, 558703) at 4 °C for 1 h, the cells were washed, resuspended, and tested on a flow cytometer.

For transwell migration assay, the 20% high concentration serum conditioned medium of each group or LPS (1 μg/mL) prepared by supplementing serum was added to the lower chamber of transwell plates (Corning, 3422). RAW 264.7 cells were resuspended in a 5% low serum medium and then seeded onto the upper chamber with 8 μm micropores. After 12 h of incubation, the macrophages of upper chamber were stained with crystal violet ammonium oxalate solution (0.1%, Biosharp, BL802A) and migrated cells were observed under a microscope and counted by ImageJ.

For quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay, RAW 264.7 cells were incubated with the aforementioned conditioned medium or LPS (1 μg/mL) for 12 h. After washing with cold BPS, Trizol (Beyotime

Biotechnology, R0016) was added to extract RNA. The quality and quantity analysis of the extracted RNA was performed on NanoDrop 2000 spectrophotometer (Thermo Fisher, USA). Then, Evo M-MLV RT Mix Kit (Accurate Biotechnology, AG11728) was used for reverse transcription. qPCR was conducted by using SYBR Green Pro Taq HS qPCR Kit (Accurate Biotechnology, AG11701). The primer sequences used for target gene amplification are listed in Table S1.

Table S1. Primers used for RT-qPCR.

Gene	Primer sequence
β-actin-F	CATCCGTAAAGACCTCTATGCCAAC
β-actin-R	ATGGAGCCACCGATCCACA
IL-1β-F	TTCCTGAACTCAACTGTGAAATGC
IL-1β-R	TGTTGATGTGCTGCTGCGAG
TNF-α-F	GCCAGGAGGGAGAACAGAAACTC
TNF-α-R	GGCCAGTGAGTGAAAGGGACA

1.15 mRNA sequencing

Total RNA was extracted by Trizol reagent according to the manufacturer's protocol. RNA purity and quantification were evaluated using a NanoDrop 2000 spectrophotometer. RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then the libraries were constructed using VAHTS Universal V6 RNA-seq Library Prep Kit according to the manufacturer's instructions. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China).

The libraries were sequenced on an Illumina Novaseq 6000 platform and 150 bp paired-end reads were generated. Raw reads of fastQ format were firstly processed and the low-quality reads were removed to obtain clean reads. The clean reads were mapped to the reference genome using HISAT2, FPKM of each gene was calculated and the read counts of each gene were obtained by HTSeq-count. PCA analysis were performed using R (v 3.2.0) to evaluate the biological duplication of samples.

Differential expression analysis was performed using the DESeq25. Q value < 0.05 and $|\log_2$ foldchange $| > 0.5$ was set as the threshold for significantly differential expression gene (DEGs). Hierarchical cluster analysis of DEGs was performed using R (v 3.2.0) to demonstrate the expression pattern of genes in different groups and samples.

Based on the hypergeometric distribution, GO6, KEGG7 pathway and Reactome enrichment analysis of DEGs were performed to screen the significant enriched term using R (v 3.2.0), respectively. R (v 3.2.0) was used to draw the column diagram, the chord diagram and bubble diagram of the significant enrichment term. Utilizing a predefined gene set, GSEA software was used for Gene Set Enrichment Analysis (GSEA).

2. Supplementary results

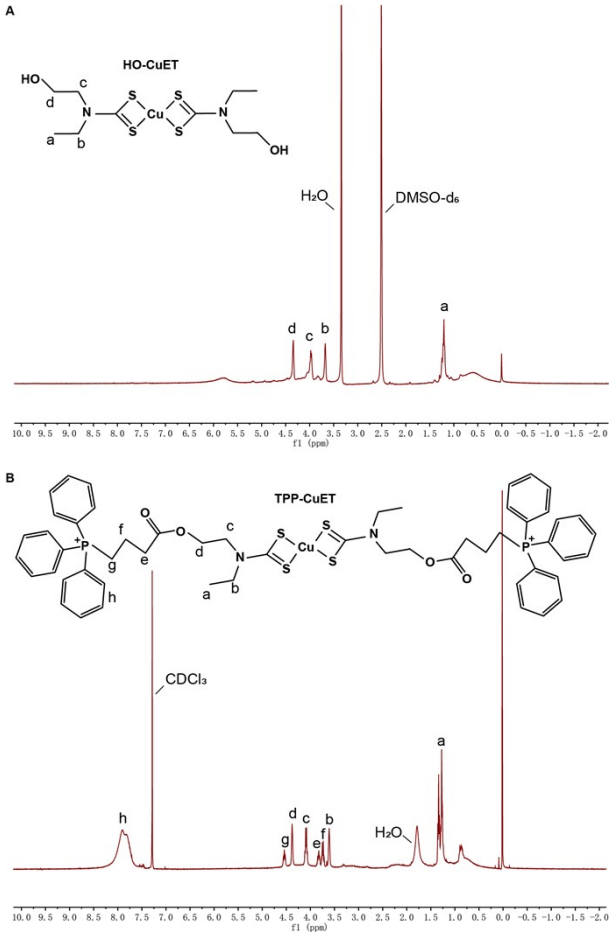


Figure S1. [1]HNMR spectra of HO-CuET in dimethyl sulfoxide-d6 and TPP-CuET in chloroform-d. Due to the paramagnetism of Cu(II) center, the nuclear longitudinal and transverse relaxation times of the magnetic resonance are shortened, resulting in low-intensity resonances overlapping with other peaks in backbone [5].

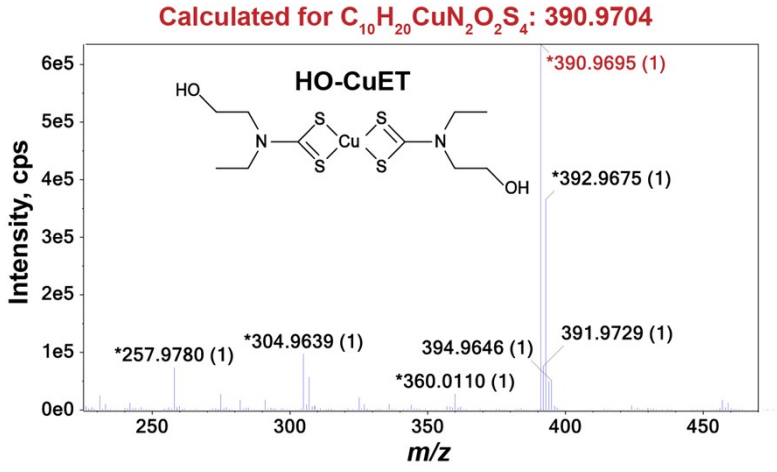


Figure S2. High-resolution mass spectrum of HO-CuET.

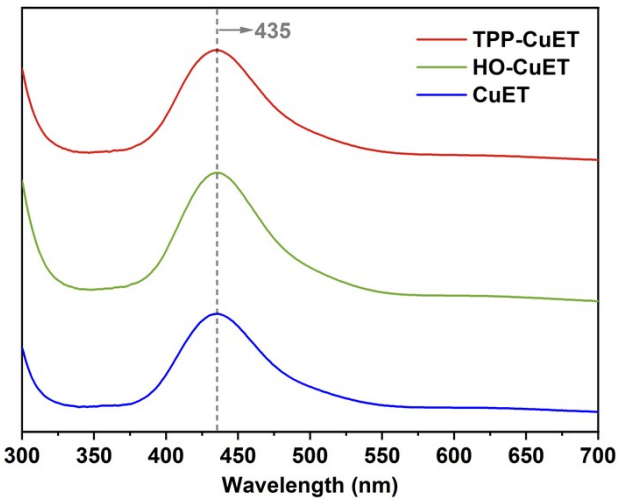


Figure S3. UV-vis spectra of CuET, HO-CuET, and TPP-CuET.

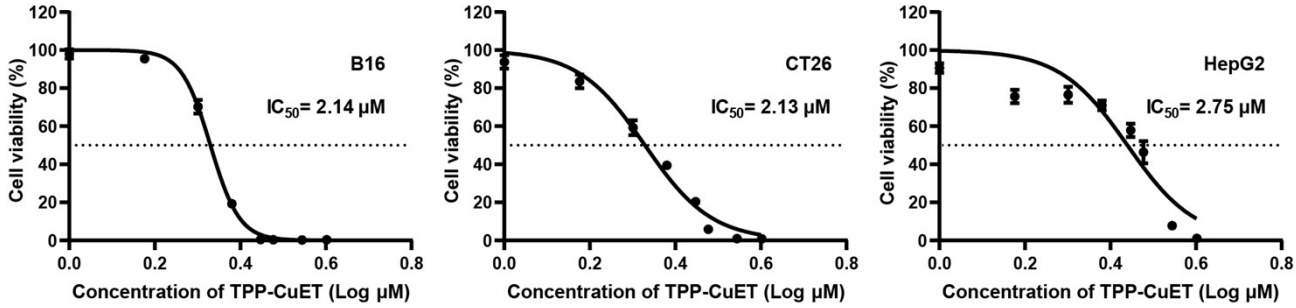


Figure S4. Dose-response curves of various tumor cell lines upon TPP-CuET treatment for 24 h (n = 4, mean ± S.D.).

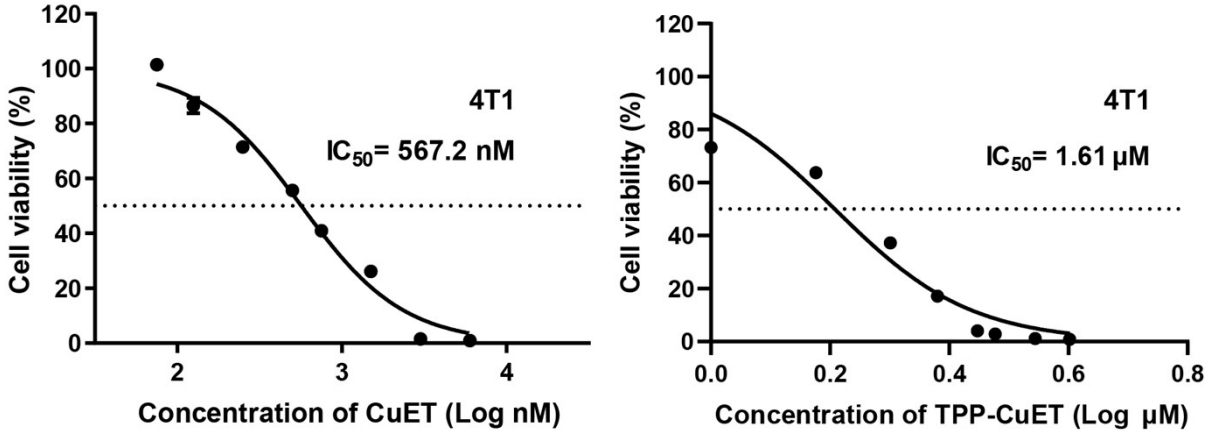


Figure S5. Dose-response curves of 4T1 cells upon CuET or TPP-CuET treatment for 24 h (*n* = 4, mean ± S.D.).

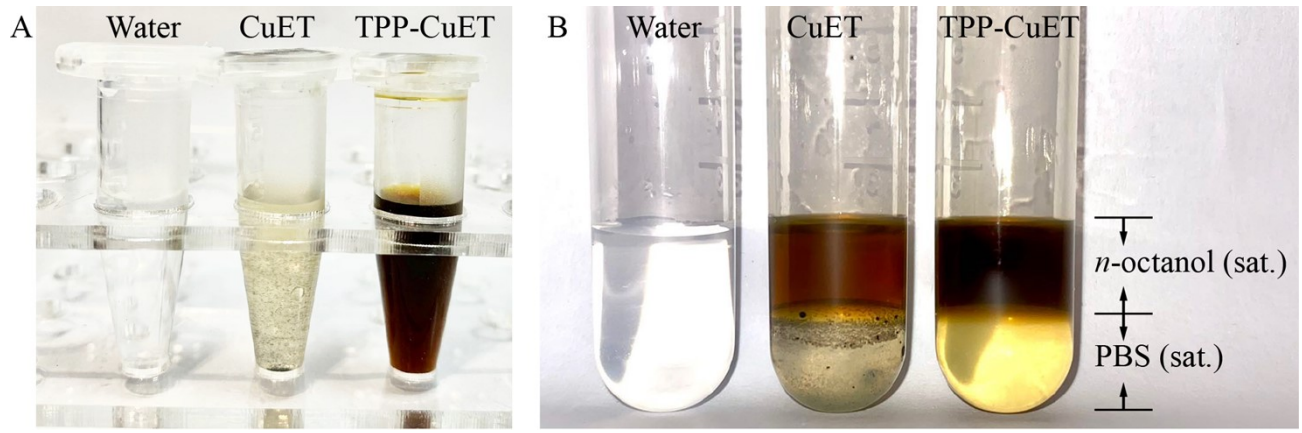


Figure S6. (A) Picture of 1mL of water added to 3 μmol of CuET or TPP-CuET. (B) Distribution of 3 μmol of CuET or TPP-CuET in a mixture of equal amounts of saturated n-octanol and PBS.

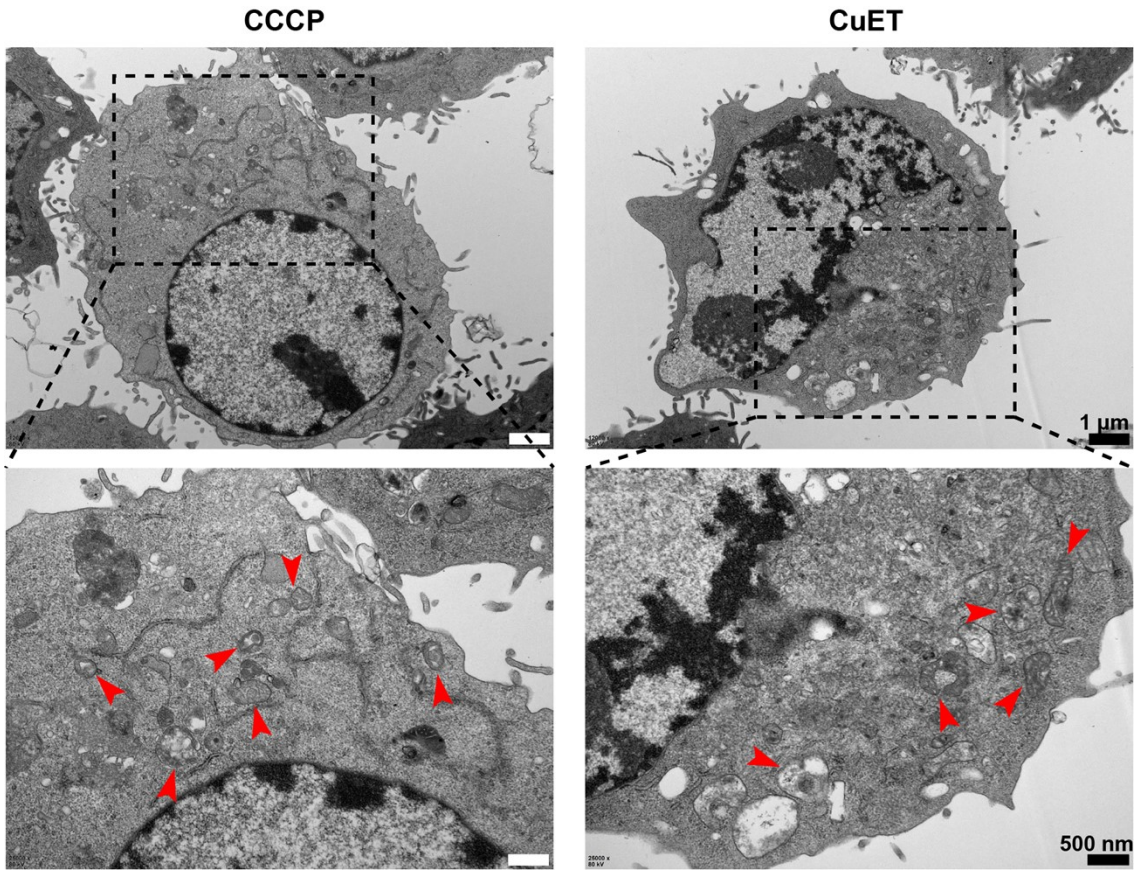


Figure S7. Bio-TEM images displaying mitochondrial morphology of 4T1 cells treated with CCCP or CuET. Red arrows indicate damaged mitochondria.

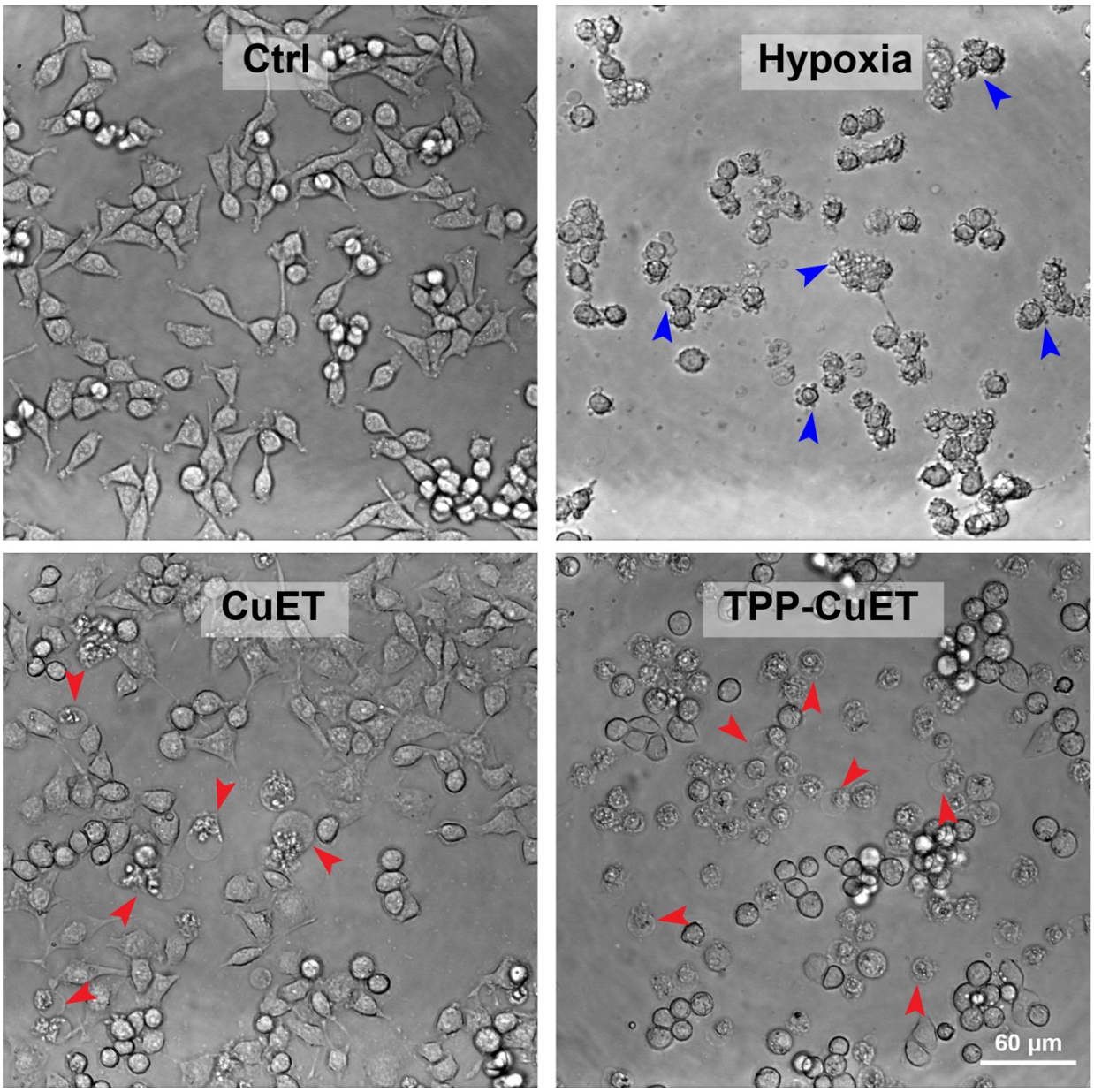


Figure S8. Photographs of 4T1 cells treated with hypoxia, CuET, or TPP-CuET for 24 h. Blue arrows indicate apoptotic cells with intact cell membranes while red arrows indicate swollen and ruptured cell membranes.

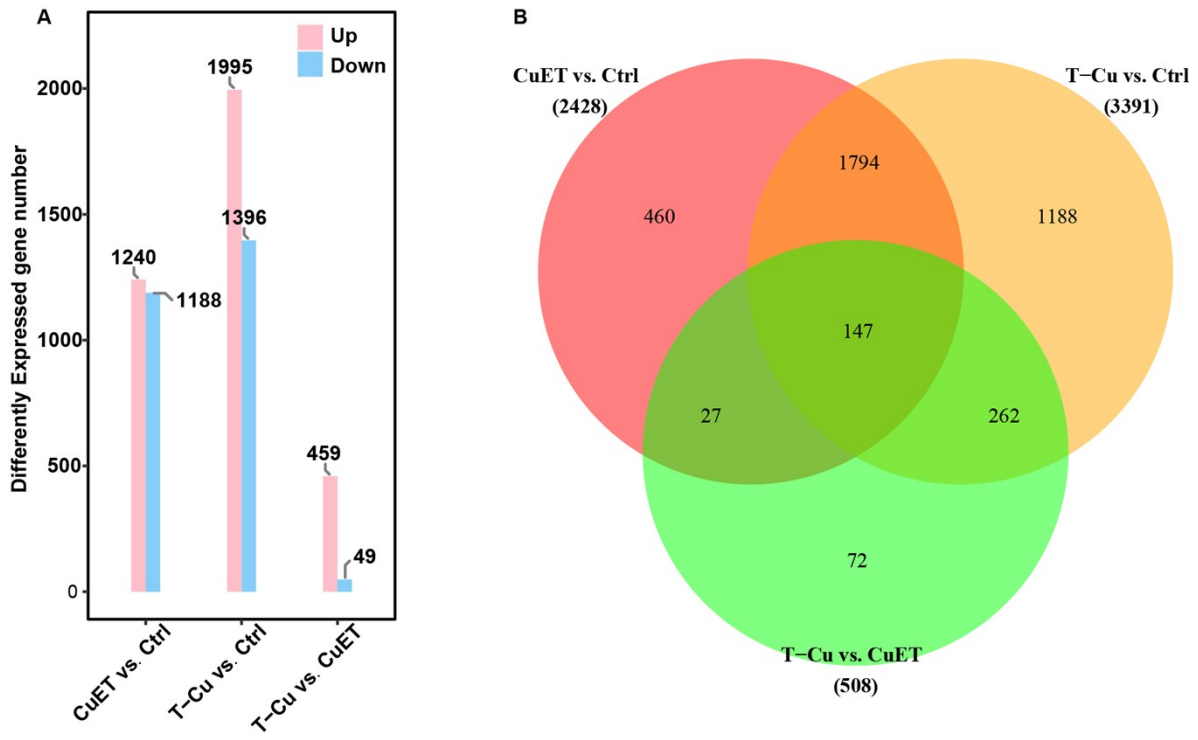


Figure S9. Statistic results of (A) upregulated and downregulated genes in 4T1 cells, as well as (B) shared and unique DEGs among different treatment groups.

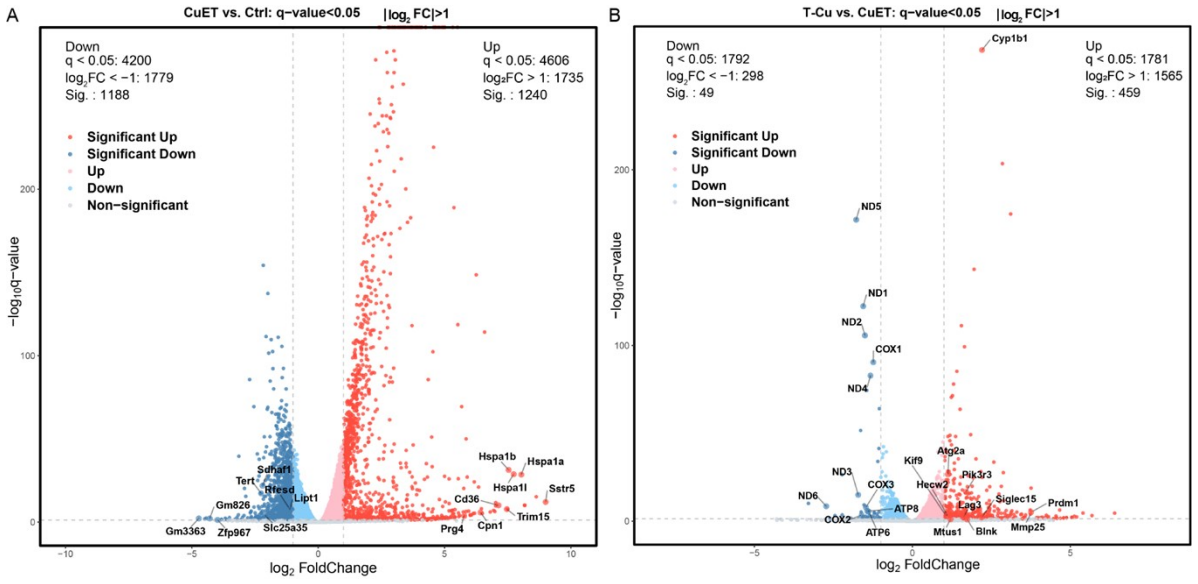


Figure S10. Volcano plots of DEGs in (A) CuET compared to the control, and (B) TPP-CuET compared to CuET.

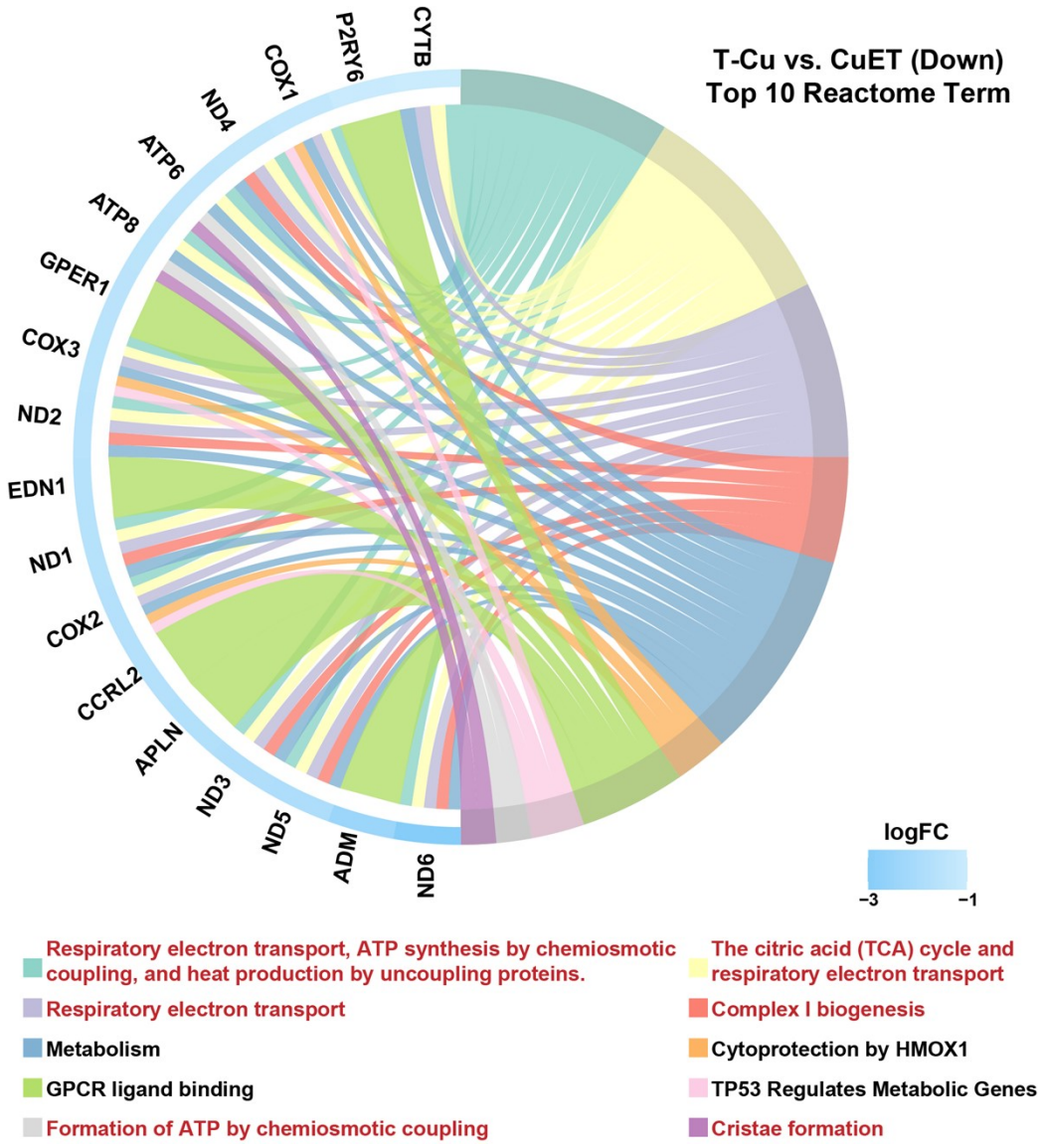


Figure S11. Reactome enrichment of top 10 downregulated genes and corresponding terms of TPP-CuET compared to CuET.

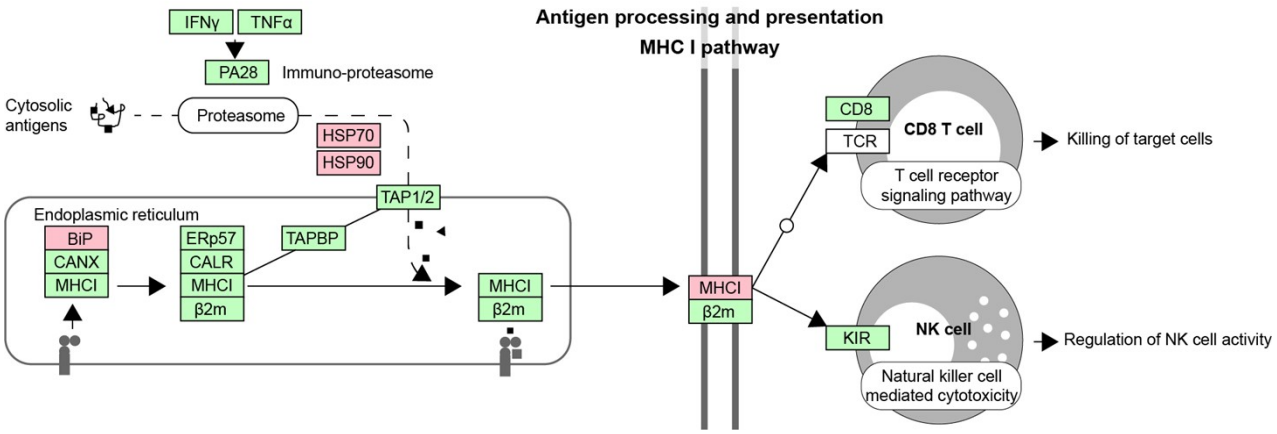


Figure S12. KEGG map of MHC I antigen processing and presentation pathway of CuET group.

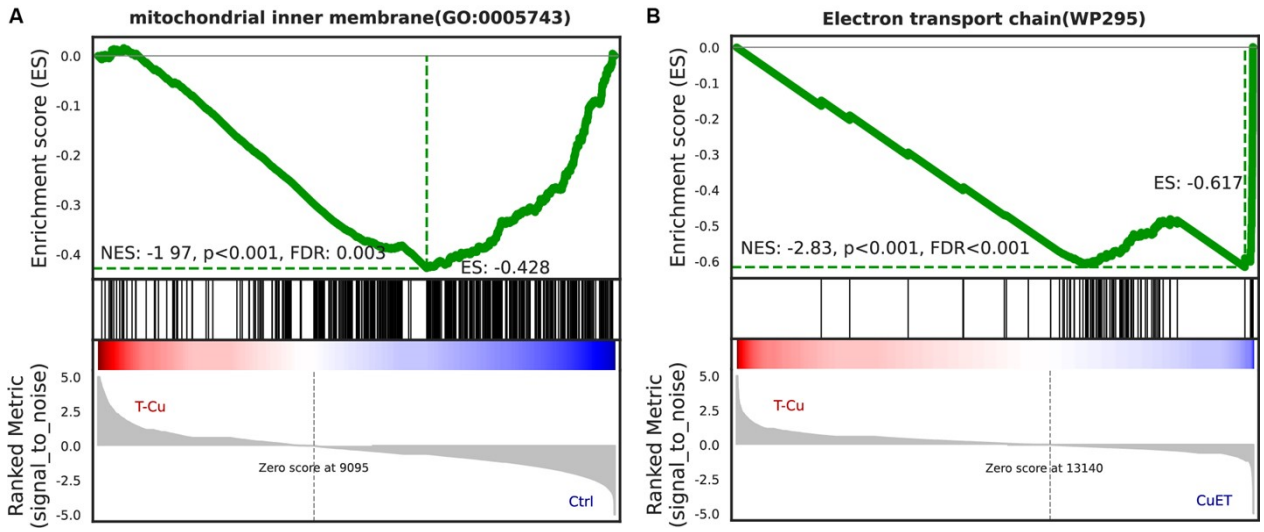


Figure S13. (A) Negative enrichment of mitochondrial inner membrane in TPP-CuET treated 4T1 cells compared to control and (B) electron transport chain in TPP-CuET treated cells in comparison with CuET via GSEA.

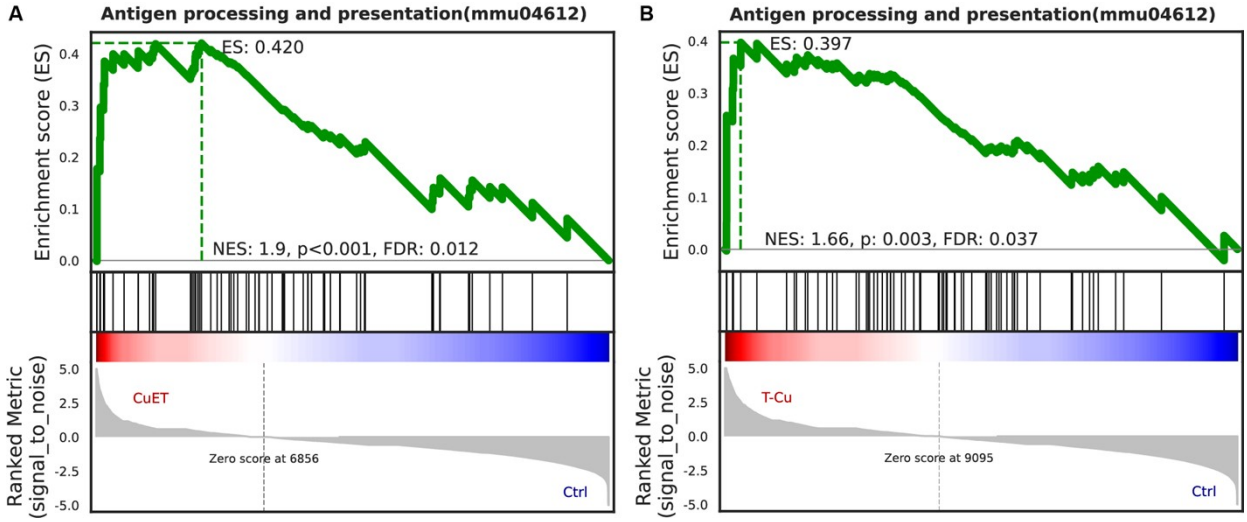


Figure S14. Positive enrichment of antigen processing and presentation in (A) CuET and (B) TPP-CuET treated 4T1 cells in comparison with the control via GSEA.

3. References

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4. Author Contributions

Y. Lu performed most of the described experiments, analyzed the data and wrote the manuscript. X. Fan assisted in the RT-qPCR experiment. Q. Pan helped analyze the data. Y. Pu and B. He supervised the project and revised the manuscript.