SUPPLEMENTARY INFORMATION

A photo-oxidation driven proximity labeling strategy enables profiling of mitochondrial proteome dynamics in living cells

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Table of Contents

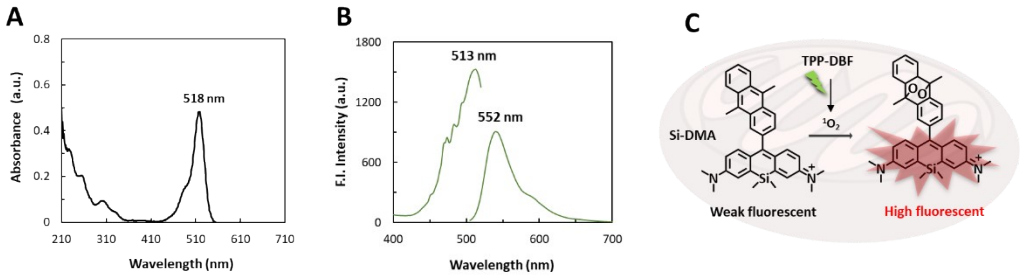


Figure S1. Characterization of TPP-DBF. (A) UV-visible absorption spectrum of TPP-DBF measured in 95% ethanol with 0.01 M KOH. (B) Fluorescence spectrum of TPP-DBF measured in DPBS. (C) Molecular structure and $^1O_2$ detection mechanism of Si-DMA.

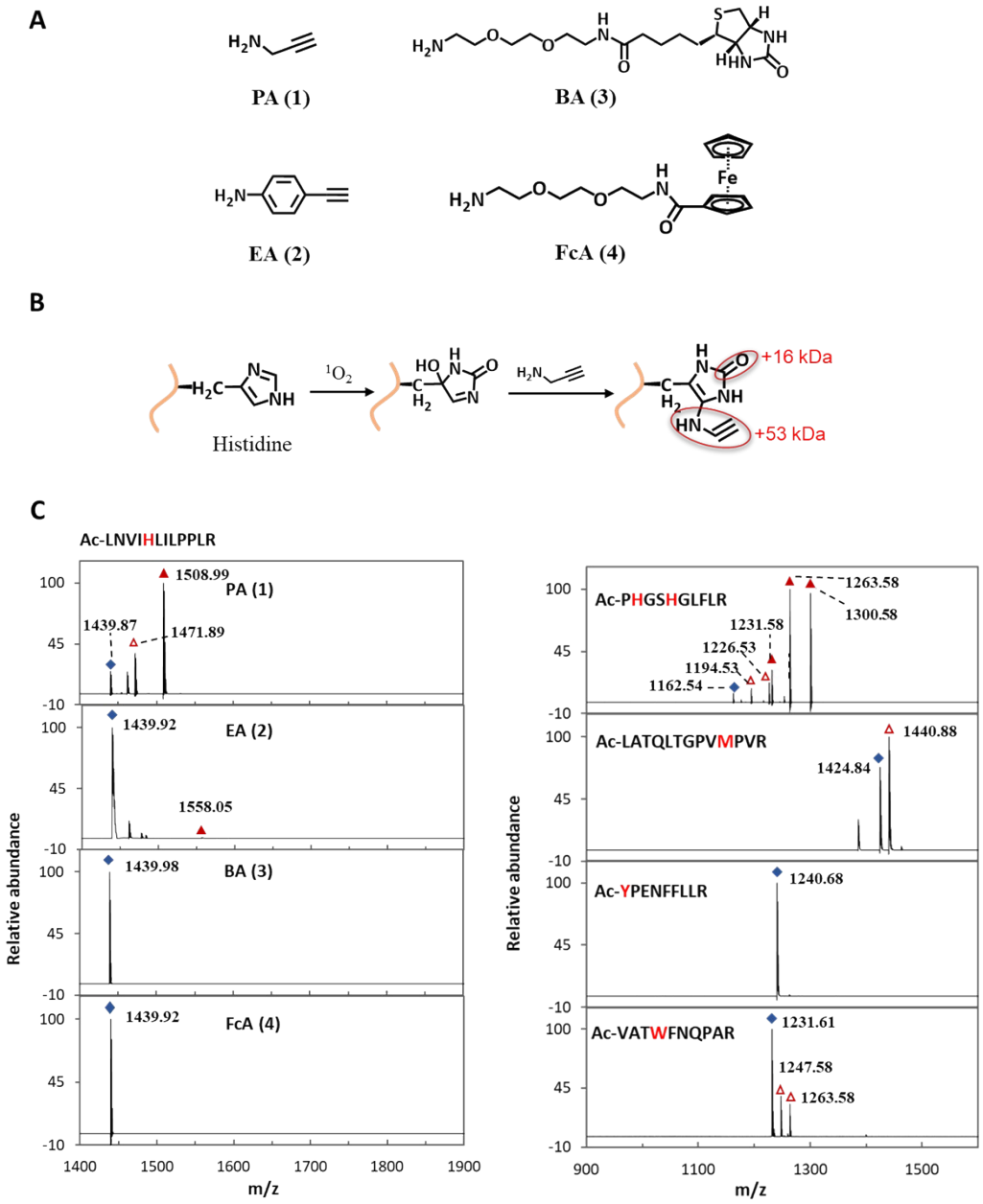


Figure S2. Photo-oxidation driven labeling of peptides in vitro. (A) Molecular structure of capture probes (CP). PA: propargylamine; EA: 4-ethynylaniline; BA: biotin-PEG_2-amine; FcA: ferrocene-PEG_2-amine. (B) Modification mechanism of PA addition (take histidine as an example). (C) MALDI-TOF/TOF spectra of peptide labeled by capture probes (PA, EA, BA, FcA) (left); MALDI-TOF/TOF spectra of peptides labeled by PA (right). ◆ : molecular ion peak; △ : oxidation peak; ▲ : PA-addition peak. Labeling condition: solution of peptides (0.5 mg/mL) in DPBS was mixed with DBF (1 mM) and capture probes (50 mM), then illuminated for 10min by homemade green light irradiation device.

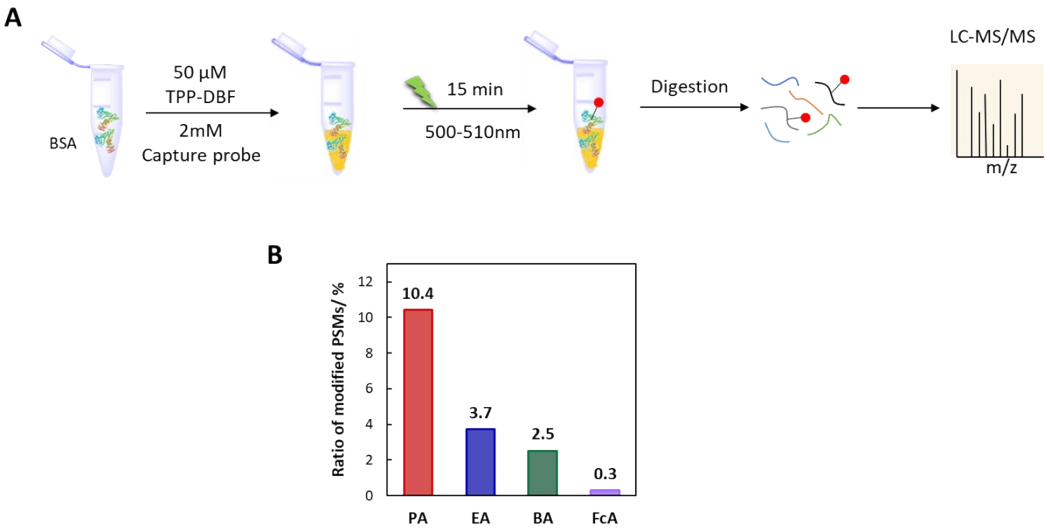


Figure S3. Photo-oxidation driven labeling of proteins in vitro. (A) Schematic of bovine serum albumin (BSA) photo-oxidation driven labeling for LC-MS/MS. (B) LC-MS/MS characterization of BSA labeled by capture probes for modification ratio of PSMs except for oxidation modification.

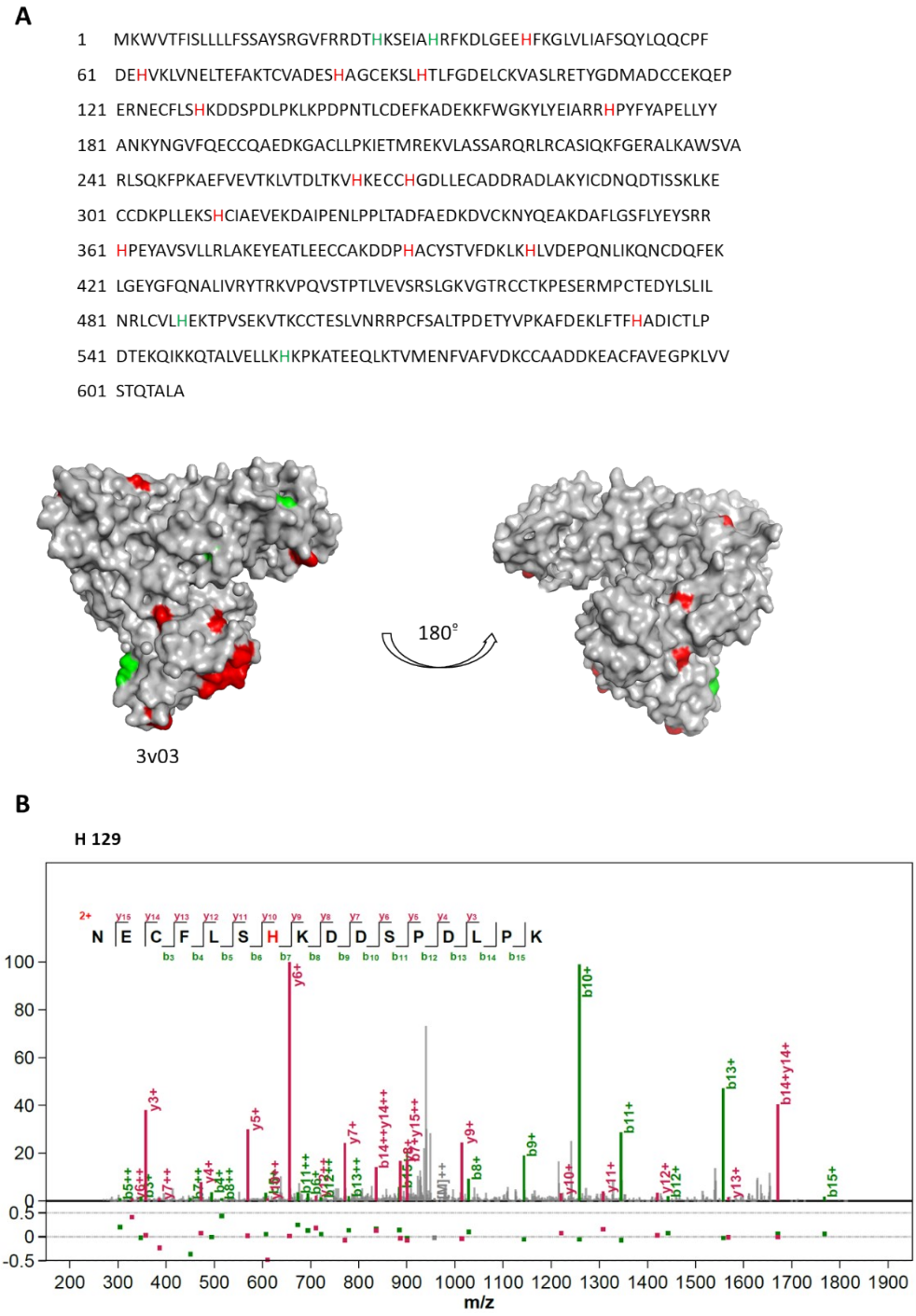


Figure S4. Photo-oxidation driven labeling of BSA with PA in vitro characterized by LC-MS/MS. (A) Amino acid sequence and crystal structure of BSA (visualized by PyMOL). Red residues: PA-labeled histidine residues; Green residues: PA-unlabeled histidine residues. (B) MS/MS spectra of labeled peptides containing PA-modified histidine residues on BSA (take H129 as an example).

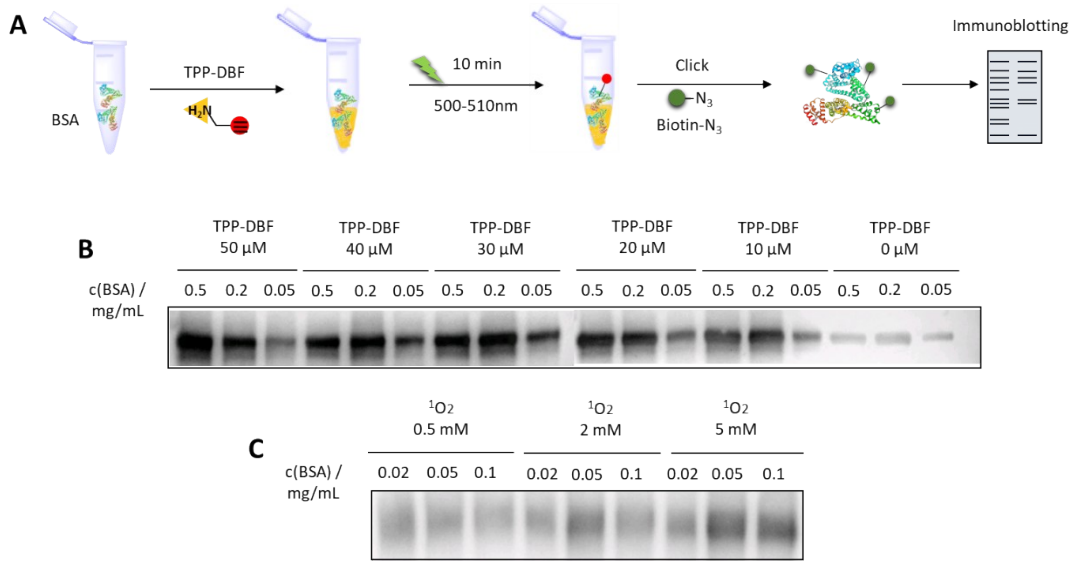


Figure S5. Photo-oxidation driven labeling of proteins in vitro. (A) Schematic of BSA photo-oxidation driven labeling by PA for immunoblotting. (B) Immunoblotting analysis of BSA labeled by TPP-DBF and PA (1 mM) with irradiation of green light for 10 min. (B) Immunoblotting analysis of BSA labeled by PA (1 mM) and $^1O_2$ which was produced via reaction of NaClO and H_2O_2 (biotin was probed with streptavidin-horseradish peroxidase conjugate).

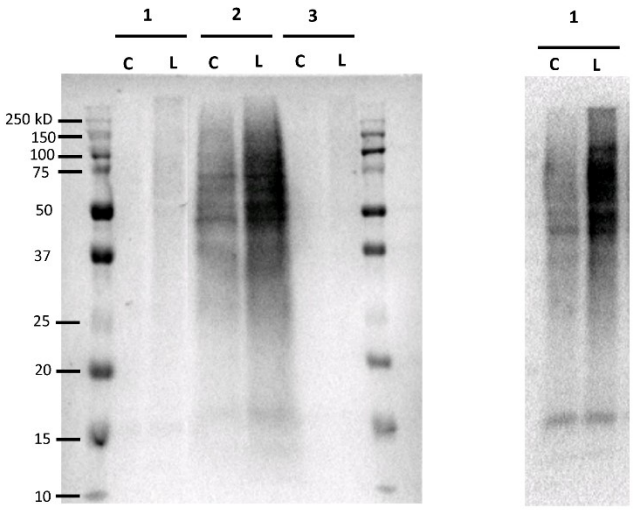


Figure S6. Photo-oxidation driven labeling of proteins in vivo. Immunoblotting analysis of proteins labeled by photo-oxidation driven labeling strategy (10 μM TPP-AcDBF and 2 mM PA with 0 min or 10 min of irradiation by green light) in HeLa cells before and after enrichment by streptavidin agarose beads. Exposure time enhanced image of band 1/2 (right). C: control (0 min of irradiation); L: light (10 min of irradiation); 1: before enrichment; 2: after enrichment; 3: supernatant after enrichment.

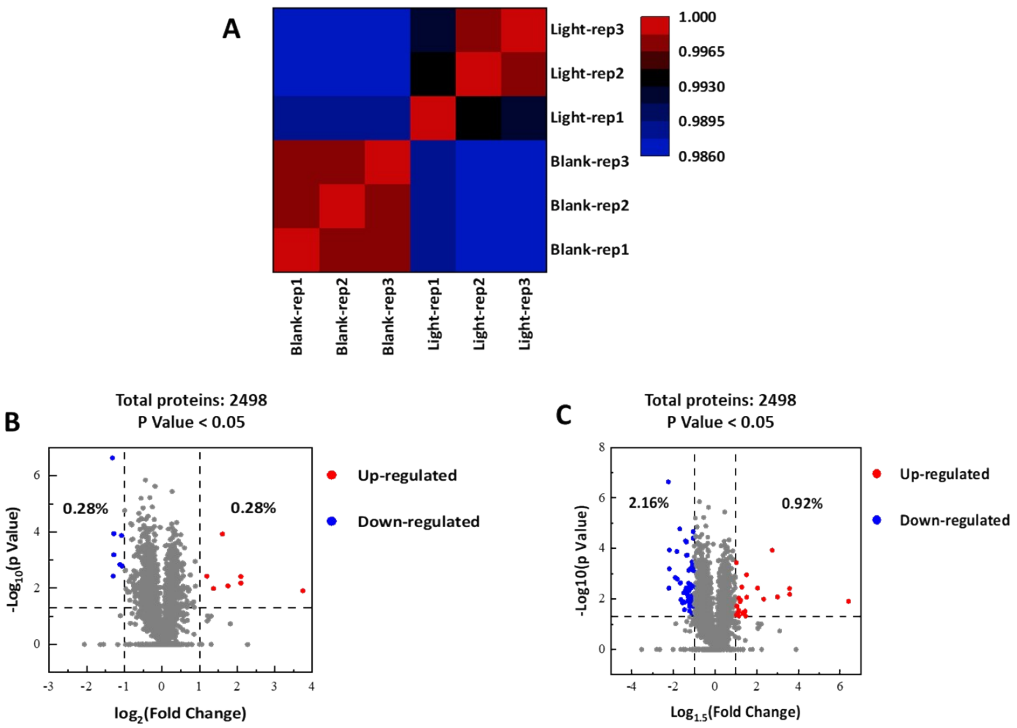


Figure S7. Proteomic perturbation of photo-oxidation driven labeling strategy in HeLa cells. (A) Pearson correlation coefficients between different groups. Blank: without TPP-AcDBF, PA nor irradiation; Light: with TPP-AcDBF, PA and irradiation. (B)(C) Volcano plot of differential proteins with ratio-L/B≧2 or ratio-L/B≧1.5.

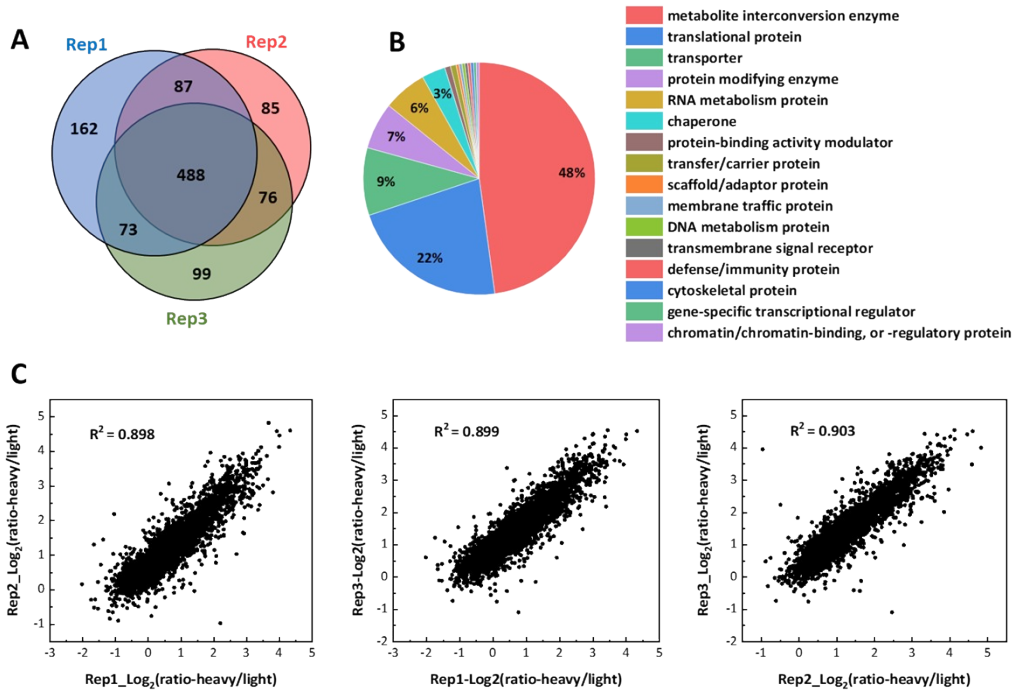
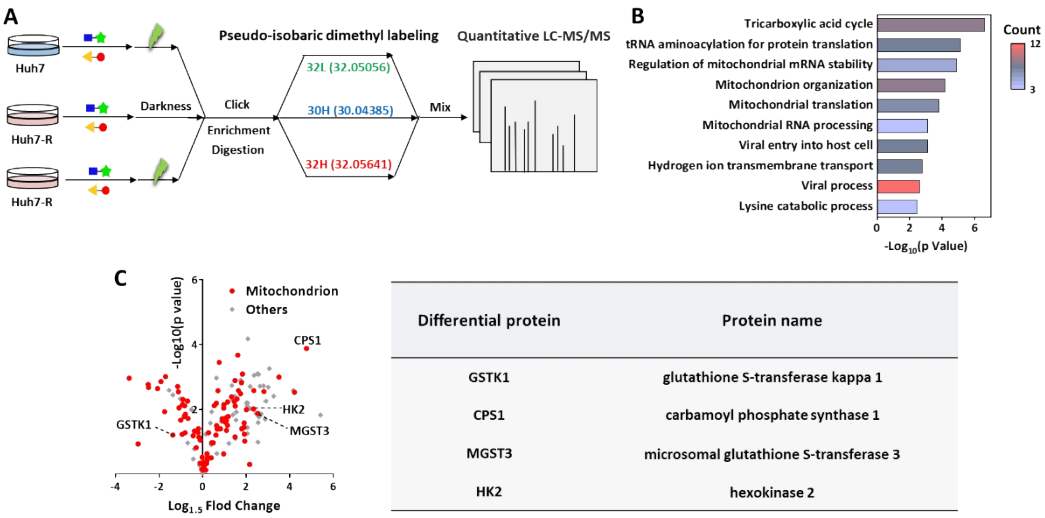


Figure S8. Photo-oxidation driven labeling mitochondrial proteome in HeLa cells. (A) Venn diagram of labeled proteome in three independent biological replicates. (B) Class of identified mitochondrial proteins (those identified proteins hold various and pivotal roles in biological processes). (C) Correlation plots of identified proteins between three independent biological replicates.



Differential protein	Protein name
GSTK1	glutathione S-transferase kappa 1
CPS1	carbamoyl phosphate synthase 1
MGST3	microsomal glutathione S-transferase 3
HK2	hexokinase 2

Figure S9. Mitochondrial proteome identification and quantification in Huh7-R cells. (A) Workflow of mitochondrial proteome profiling in Huh7-R cells. (B) Biological processes in GO enrichment analysis for identified proteins in Huh7-R cells. (C) Volcano plot of quantified proteins in Huh7-R cells comparing with those in Huh7 cells.

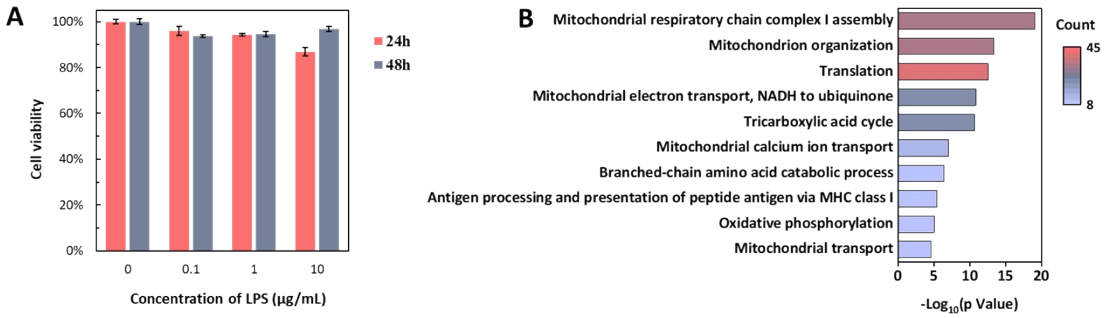


Figure S10. Dynamic mitochondrial proteome revealing of HMC3 cells stimulated by LPS. (A) Cytotoxicity tests of LPS in HMC3 cells under different incubation conditions. (B) Biological processes in GO enrichment analysis for identified proteins in HMC3 cells.

Table S1: Mitochondrial proteome profiling in HeLa cells by integrating photo-oxidation driven proximity labeling strategy and quantitative mass spectrometry. Cellular localization classification of identified proteins was according to the MitoCarta 3.0 database, the Uniprot_Homo Sapiens Swiss-Prot database (proteins with the Gene Ontology cell component annotation containing "mitochondria" or "mitochondrion" were assigned as mitochondrial proteins) and literatures.

	Rep1-30H/30L	**Rep2-30H/30L**	**Rep3-30H/30L**	**Overlap**
Identified proteins	810	736	736	**488**
Mitochondrial proteins	411	408	414	**310**
False positive proteins	396	323	319	**177**
Un-assigned proteins	3	5	3	**1**
Proportion of mitochondrial proteins / %	50.74	55.43	56.25	**63.52**

Table S2: Comparison of identified mitochondrial proteome by our and other methods. ORMs: organelle-localizable reactive molecules[1]. MRMs: mitochondria-localizable reactive molecules. CAT-Prox: a bioorthogonal and photocatalytic decaging-enabled proximity labeling strategy[2]. Proximity labeling based on engineered enzymes: APEX[3] and TurboID[4]. (Our method, ORMs and CAT-Prox was performed in HeLa cells, APEX and TurboID was performed in HEK 293T cells).

Method	**Ours**	**ORMs-Total (5 MRMs)**	**CAT-Prox**	**APEX**	**TurboID**
Identified proteins	**488**	375	258	588	314
Mitochondrial proteins	**310**	259	182	495	209
Ratio of mitochondrial proteins / %	**63.52**	69.07	70.54	84.18	66.56

Table S3: Differential mitochondrial proteins with >1.5-fold change of HMC3 cells in group stimulated by LPS for 48 h comparing with those in group stimulated for 24 h.

Differential proteins	Protein name	$Log_{1.5}$ ratio-48h/24h (average)
PCK2	Phosphoenolpyruvate carboxykinase	2.07
GPT2	Glutamate pyruvate transaminase 2	2.03
MRPS14	28S ribosomal protein S14	1.91
IDH3G	Isocitrate dehydrogenase [NAD] subunit gamma	1.55
CHCHD2	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2	1.44
FPGS	Folylpolyglutamate synthase	1.34
ACSF2	Medium-chain acyl-CoA ligase ACSF2	1.23
LONP1	Lon protease homolog	1.22
ACOT9	Acyl-coenzyme A thioesterase 9	1.21
PRORP	Mitochondrial ribonuclease P catalytic subunit	1.09
LRPPRC	Leucine-rich PPR motif-containing protein	1.01
MTHFD2	Bifunctional methylenetetrahydrofolate dehydrogenase	1.00
PON2	Serum paraoxonase/arylesterase 2	-1.32

Experimental methods

Cell culture

Human HeLa, Huh7 and HMC3 cell lines were acquired from the cell bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (CTCC, Shanghai, China). Huh7-R cells were established by long-term exposure to low dose of sorafenib (1 μM) which was gradually reaching a higher dose of sorafenib (up to 10 μM) in months. HeLa cells were cultured in Minimum Essential Medium (MEM) (Gibco, Grand Island, NY, USA) with 10% (v/v) fetal bovine serum (FBS) (Gibco) and penicillin (100U/mL)/streptomycin (100μg/mL) (Gibco) at 37 ℃ and with 5% CO_2. Huh7 and Huh7-R cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% (v/v) fetal bovine serum and penicillin (100 U/mL)/streptomycin (100 μg/mL) at 37 ℃ and with 5% CO_2. HMC3 cells were cultured in Minimum Essential Medium (MEM) containing non-essential amino acid (NEAA) (Procell, Wuhan, China) with 10% (v/v) fetal bovine serum and penicillin (100 U/mL)/streptomycin (100 μg/mL) at 37 ℃ and with 5% CO_2.

Green light irradiation device

Green light irradiation device was homemade with wavelength peak of 500-510nm and lumen of approximately 6000 lux.

Quantify singlet oxygen production of TPP-DBF by Si-DMA

In vitro

TPP-DBF and singlet oxygen fluorescent probe Si-DMA (Dojindo, JPN) were mixed in 0.5 mL DPBS (Gibco)/MeOH (Merck, Germany) (1:1) with the final concentration of 2 μM. After illuminated by homemade green light irradiation device for 0, 5, 10, 15, 20, 25, 30 min, the fluorescence intensity of the solution at 660 nm was measured by fluorescence spectrophotometer (Cary Eclipse, Agilent, USA) respectively.

In vivo

HeLa cells were seeded in 35 mm^2 glass-based confocal dishes (Nest, China) and used for imaging when reaching 60-80% confluence. The cells were washed with HEPES-

buffered saline (HBS) buffer (20 mM HEPES, 107 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11.5 mM Glucose, pH 7.4), and incubated in fresh HBS buffer with TPP-AcDBF (10 μM) for 1 h at 37 ℃. Cells without TPP-AcDBF was used as control. After 30 min, Si-DMA (50 nM) and Hoechst 33342 (5 μg/mL) (Sigma-Aldrich, USA) were added to the cells and continued to incubate for an additional 30min at 37 ℃. Then the cells were washed three times with HBS buffer and illuminated by homemade green light irradiation device for 10 min. Cells were imaging by andor live cell confocal imaging platform (Nikon Instruments, JPN) with a 60X objective. The fluorescence images were acquired separately in 405nm (Hoechst 33342) and 640 nm (Si-DMA) channels (images in 640 nm channel were acquired first).

Photo-oxidation driven labeling of peptides and proteins in vitro

Photo-oxidation driven modification of peptides

A solution of peptides (Synpeptide, China) in DPBS (0.5 mg/mL) was mixed with DBF (1 mM) and capture probes (50 mM), then illuminated for 10 min by homemade green light irradiation device. After samples were crystallized with DHB substrate on the sample plate, MALDI-TOF spectra were recorded by Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Germany) equipped with a 200-Hz smart-beam 1 laser at 355 nm.

Photo-oxidation driven modification of proteins

Identified by LC-MS/MS

A solution of BSA in DPBS (0.5 mg/mL) was mixed with TPP-DBF (50 μM) and capture probes (PA, EA, BA and FcA) (2 mM), and then illuminated by homemade green light irradiation device for 15 min. The mixture was precipitated in acetone overnight at -20 ℃ and precipitated BSA was redissolved in 1% SDS after air-dried. BSA solution was mixed with dithiothreitol (DTT) (100 mM), boiled at 95 ℃ for 5 min. Following steps were carried out with filter aided sample preparation (FASP) method. The protein solution was transferred to the filter membrane (MWCO 10000) (Vivacon 500, Sartorius, German), and centrifugated in 15000 g at 20 ℃ to remove supernatant. After washing once with urea (8 M), 200 μL iodoacetamide (20 mM) was

added to the filter membrane and incubated for 30 min at room temperature in the dark. Then the filter membrane was washed with urea (8 M) and ammonium bicarbonate (ABC) solution (50 mM) for three times respectively. Proteins were digested in 100μL ABC solution (10 mM) with trypsin (1:25-1:50, trypsin: protein, w/w) (Promega, USA) in 37 ℃ for 12-16 h. In the end, peptide solution was collected and lyophilized for LC-MS/MS analysis on an Ultimate 3000 HPLC system (Dionex, USA) coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo, USA). The MS data was searched by pFind (version 3.1.5) against uniport_BSA database (amino acid sequence of BSA) with $M_{CP}+M_O-2M_H$ and $M_{CP}+2M_O$ in histidine (H), $M_{CP}-M_H$ in methionine (M), $M_{CP}+M_O$ in tyrosine (Y), $M_{CP}+M_O$ in tryptophan (W) and $2M_O$ in cysteine (C) and HMYW as variable modifications.

Characterized by immunoblotting analysis

A solution of BSA in DPBS (2 mg/mL) was mixed with TPP-DBF (50 μM) and PA (1mM), and then illuminated by homemade green light irradiation device for different time (0, 5, 10, 15, 20, 30 min). Groups without TPP-DBF or PA were used as controls. The samples were boiled at 95 ℃ for 5 min, added freshly premixed click solution (200 μM azo biotin-azide, 100 μM $CuSO_4$, 800 μM THPTA ligand (Tris (3-hydroxypropyltriazolylmethyl) amine), 2.5 mM sodium ascorbate), and incubated at 37 ℃ for 3 h in the dark. The solutions were mixed with 6 × protein loading buffer (1/5, v/v) (TransGen, China) and boiled again for SDS-PAGE (7.5% gel, Shanghai epizyme biomedical technology, China). After gels were transferred to PVDF membranes (Bio-Rad, USA), membranes were blocked by 3% BSA and incubated with streptavidin-horseradish peroxidase conjugate (Thermo, USA) in TBST (150 mM NaCl, 10 mM Tris, 0.05% (v/v) Tween 20, pH 7.2-7.5) solution for 30 min at room temperature. Then samples were analyzed by ChemiDoc XRS+ (Bio-Rad, USA) for chemiluminescence and detection.

Solutions of BSA in DPBS with different concentrations (0.05, 0.2, 0.5 mg/mL) were mixed with various concentrations of TPP-DBF (50, 40, 30, 20, 10, 0 μM) and PA (1 mM), then vortexed and illuminated by homemade green light irradiation device for 10

min. Subsequent sample treatment was same as above.

Solutions of BSA in DPBS with different concentrations (0.02, 0.05, 0.1 mg/mL) were mixed with PA (1 mM) and various concentrations of NaClO and H_2O_2 (0.5, 2, 5 mM), then vortexed and incubated for 10min in the dark. Subsequent sample treatment was same as above.

Live cells imaging of TPP-AcDBF subcellular localization

HeLa cells were seeded in glass-based confocal dishes (35 mm^2) and used for imaging when reaching 60-80% confluence. The cells were washed with HBS buffer, and incubated in fresh HBS buffer with TPP-AcDBF (10 μM) for 1 h at 37℃. After 30 min, Mito Tracker Deep Red FM (100 nM) (Thermo, USA) and Hoechst 33342 (5 μg/mL) were added to the cells and continued to incubate for an additional 30 min at 37 ℃. Then the cells were washed three times with HBS buffer, and imaging by andor live cell confocal imaging platform with 60X and 100X objective. The fluorescence images were acquired separately in 405 nm (Hoechst 33342), 488 nm (TPP-AcDBF) and 640 nm (Mito Tracker Deep Red FM) channels.

CLSM imaging of proximity labeling in situ by TPP-AcDBF and PA

HeLa cells were seeded in glass-based confocal dishes (35 mm^2) and used for imaging when reaching 60-80% confluence. The cells were washed with HBS buffer, and incubated in fresh HBS buffer with 10 μM TPP-AcDBF for 10 min at 37 ℃. After washing three times, the cells were added fresh HBS buffer with 5 mM PA and incubated for 5 min at 37 ℃. Then the cells were illuminated for 0 min or 10 min by homemade green light irradiation device at room temperature and incubated for another 5 min in the dark at 37 ℃. Sample without light was used as control. The cells were washed three times with DPBS, and stained with Mito Tracker Deep Red FM (150 nM) for 30 min. After washing again with DPBS, the cells were fixed and permeabilized for 30 min with 4% formaldehyde and 0.1% Triton X-100 in DPBS at room temperature, respectively. Then the cells were added with freshly premixed click solution (10 μM Azide-fluor 488, 1 mM $CuSO_4$, 4 mM THPTA ligand, 10 mM sodium ascorbate), and

incubated for 1 h at 37 ℃ in the dark. The cells were washed with 0.1% Triton X-100 and DPBS for three times respectively. Next the cells were incubated with Hoechst 33342 (2 μg/mL) for 5 min in the dark. After washing three times with DPBS, the cells were imaging by andor live cell confocal imaging platform with a 100X objective. The fluorescence images were acquired separately in 405 nm (Hoechst 33342), 488 nm (Fluor 488) and 640 nm (Mito Tracker Deep Red FM) channels.

HeLa cells were seeded in 10-cm culturing dishes (Nest, China) and used when reaching 80-90% confluence. The cells were treated with TPP-AcDBF and PA as above. Sample without irradiation was used as control. Cells were collected and lysed to obtain protein solution. Then the samples were boiled, added freshly premixed click solution (200 μM azo biotin-azide, 100 μM $CuSO_4$, 800 μM THPTA ligand, 2.5 mM sodium ascorbate) and incubated at 37 ℃ for 3 h in the dark. The mixture was precipitated in acetone overnight at -20 ℃ to remove excess click reagents. Precipitated protein was air-dried and redissolved in 1% SDS for SDS-PAGE (12.5% gel). A part of protein solution was diluted to 0.2% SDS and added streptavidin agarose beads (Thermo, 20353, USA) for enrichment by incubating 3 h at room temperature with end-to-end rotation. The supernatant was acquired by centrifugation. After washing away nonspecific adsorption, labeled proteins were eluted with 1% SDS and boiled at 95 ℃ for 5 min. The protein solution, supernatant and eluent were conducted for immunoblotting analysis as above.

Optimization of incubation conditions of TPP-AcDBF

HeLa cells were seeded in 10-cm culturing dishes and used when reaching 80-90% confluence. The cells were washed with HBS buffer, and incubated in fresh HBS buffer with TPP-AcDBF (5 or 10 μM) for different time at 37 ℃. Then cells were washed three times with HBS buffer. Sample without TPP-AcDBF was used as control. The cells were collected, washed and adjusted to same amount, followed by lysis and centrifugation to obtain clear solution. Standard solutions of TPP-DBF with linear gradient were prepared. 100μL of standard solutions and sample solutions were added to 96-well plate (Nest, China) with three replicates. The absorption value of solutions

at 510 nm was measured by microplate reader (Multiskan go, Thermo, USA) to calculate the intracellular concentration of TPP-DBF in HeLa cells. The volume of a single HeLa cell was reported as 2.4 pL[5].

HeLa cells were seeded in 10-cm culturing dishes and used for labeling when reaching 80-90% confluence. The cells were washed with HBS buffer, and incubated in fresh HBS buffer with TPP-AcDBF (same condition as above). After washing three times, the cells were added fresh HBS buffer with 2 mM PA and incubated for 5 min at 37 ℃. Then the cells were illuminated for 0 min or 10 min by homemade green light irradiation device at room temperature and incubated for another 5 min in the dark at 37 ℃. Samples without illumination was used as controls. Proteins were extracted, biotinylated, enriched and digested as follows. The samples illuminated or not were treated parallelly and analyzed by an EASY-nLC 1000 system (Thermo, USA) coupled to a Q-Exactive mass spectrometer (Thermo, USA). The MS data was searched by MaxQuant (version 1.6.5.0) against the Uniprot_Homo Sapiens Swiss-Prot database (updated on 06/20/2021, 20360 proteins) in label-free mode. The proteins with ratio (light/control) ≥ 2 were filtered out to be labeled proteins.

Proteomic perturbation of photo-oxidation driven labeling in living cells

HeLa cells were seeded in 10-cm culturing dishes and used when reaching 80-90% confluence. The cells were washed with HBS buffer after removal of culture medium, and incubated in fresh HBS buffer with 10 μM TPP-AcDBF for 10 min at 37 ℃. Then cells were washed three times, added fresh HBS buffer with 2 mM PA and incubated for 5min at 37 ℃. After that the cells were illuminated for 0 min or 10 min by homemade green light irradiation device at room temperature and incubated for another 5 min in the dark at 37 ℃. Samples without TPP-AcDBF, PA and illumination were used as controls. The cells were washed three times with DPBS and harvested in pre-cold DPBS. The cell pellet was collected by centrifugation in 500 g at 4 ℃ and washed twice with DPBS. The cell pellet was subsequently resuspended in lysis buffer (1% SDS (w/v), 1% cocktail (v/v), 138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4-$7H_2O$), and lysed by ultrasonication (Scientz-IID, China) for 3min (10 s

intervals every 5 s) in an ice bath to acquired cell lysate. The protein concentration of cell lysate was determined by a BCA assay. A certain amount of protein solution (~200 μg protein each group) was mixed with DTT (100 mM), boiled at 95 ℃ for 5 min. Following steps were carried out with FASP as above. The lyophilized peptides were analyzed by an EASY-nLC 1000 system coupled to a Q-Exactive mass spectrometer with three technical replicates. The MS data was searched by MaxQuant (version 1.6.5.0) against the Uniprot_Homo Sapiens Swiss-Prot database (updated on 06/20/2021, 20360 proteins) in label-free mode. The proteins with fold change exceeded 2/1.5 were filtered out to be significantly differential proteins.

Identification of mitochondrial proteome in HeLa cells

Mitochondrial proteins labeling

HeLa cells were seeded in 10-cm culturing dishes and used for labeling when reaching 80-90% confluence. The cells were washed with HBS buffer after removal of culture medium, and incubated in fresh HBS buffer with 10 μM TPP-AcDBF for 10 min at 37 ℃. Then cells were washed three times, added fresh HBS buffer with 5 mM PA and incubated for 5 min at 37 ℃. After that the cells were illuminated for 0 min or 10 min by homemade green light irradiation device at room temperature and incubated for another 5min in the dark at 37 ℃. The cells were washed three times with pre-cold DPBS. Samples without illumination were used as controls.

Proteins biotinylation and enrichment

The cells were harvested in pre-cold DPBS and collected by centrifugation in 500 g at 4 ℃ followed by twice washing with DPBS. The cell pellet was subsequently resuspended in lysis buffer, and lysed by ultrasonication for 3 min (10 s intervals every 5 s) in an ice bath. The cell lysate was boiled at 95 ℃ for 5 min and diluted with DPBS to 0.2% SDS. Then the solution was mixed with freshly premixed click solution (200 μM azo biotin-azide, 100 μM $CuSO_4$, 800 μM THPTA ligand, 2.5 mM sodium ascorbate) and incubated at 37 ℃ for 3 h in the dark. After that the mixture was precipitated in acetone overnight at -20 ℃ to remove excess click reagents. The protein pellet was collected by centrifugation in 2000 g at 4 ℃ and air-dried after washing

twice with pre-cold acetone.

The protein pellet was redissolved in 0.2% SDS/DPBS. The protein concentration of the solution was determined by a BCA assay so as to dilute the solutions to same concentration. ~ 1.5 mg protein (2-3 mg/mL) each group was used for enrichment. Streptavidin agarose beads (Thermo, 20353, USA) were centrifuged (1500 g, 3 min) to remove stock solution and washed by DPBS for three times. 45 μL beads were added to each sample solution to enrich biotin-labeled proteins by incubating 3 h at room temperature with end-to-end rotation. After enrichment, supernatant was removed by centrifugation (1500 g, 3 min) and beads were washed with 0.2% SDS/DPBS (×3), 1% SDS/DPBS (×2), DPBS (×1), 4 M NaCl + 0.2% Tween-20 + 50 mM Tris (pH=8.0) (×2), 0.1M $NaCO_3$ + 50 mM Tris(pH=8.0) (×2), 2 M Urea + 50mM Tris(pH=8.0) (×2), PBS (×2) at room temperature in turn. Then the enriched proteins were eluted with 100μL eluent (300 mM $Na_2S_2O_4$, 6 M urea, 2 M thiourea, 20 mM HEPES) by incubating for 30 min at 37 ℃ with gentle shaking and followed by another elution.

Digestion, pseudo-isobaric dimethyl labeling and high pH-RP fractionation

Reduction, alkylation and digestion were carried out with filter aided sample preparation (FASP) method. The elution was transferred to the filter membrane (MWCO 10000), and centrifugated in 15000 g at 20 ℃ to remove supernatant. After washing three times with ABC solution (50 mM), 200μL tris(2-carboxyethyl)phosphine (TCEP) (10mM) was added and incubated for 2 h at 37 ℃. TCEP solution was removed. The filter membrane was sequentially added 200 μL IAA (20 mM) and incubated for 30 min at room temperature in the dark. Then the filter membrane was washed three times with ABC solution (50 mM), once with phosphate buffer (100 mM, pH=8.0) respectively. Proteins were digested in 100 μL phosphate buffer with trypsin (1:25-1:50, trypsin: protein, w/w) in 37 ℃ for 12-16 h. In the end the membrane was washed once with 40 μL phosphate buffer and peptide solution was collected by centrifugation.

To each peptide solution was added 16 μL 4% CH_2O or $^{13}CH_2O$ (v/v) (Cambridge Isotope Laboratories, USA) and 16 μL 0.6 M $NaBH_3CN$ or $NaBD_3CN$ (Sigma-Aldrich, USA) (Control group: $^{13}CH_2O$+ $NaBH_3CN$ (light); Sample group: CH_2O+ $NaBD_3CN$

(heavy)). The mixture was vortexed and incubated for 1h at room temperature with gentle shaking. Then the labeled peptide solution was combined in a ratio of 1:1 (v/v) for the following desalination and high pH-RP fractionation. 10 mg durashell C18 packing (5 μm, 100 Å) (Agela technologies, USA) was filled into a T-400 tip with soild phase extraction C18 disk (Sigma-Aldrich, USA) on top. Two mobile phases (A: H_2O with $NH_3 \cdot H_2O$ (pH=10) and B: 80% (v/v) ACN with $NH_3 \cdot H_2O$ (pH=10)) were used to desaltination and separation. After C18 tip was washed with B mobile phase (activization) and A mobile phase (equilibration) for three times each, the mixed peptide solution was loaded onto C18 tip by centrifugation in 500 g for 10 min. Then the C18 tip was washed with A mobile phase for three times and eluted with mobile phases containing different ratios of acetonitrile (6%, 9%, 12%, 15%, 18, 21%, 25%, 30%, 80%) in turn by centrifugation in 500 g for 5-10 min. The eluents were merged to three fractions (6%+15%+25%, 9%+18%+30%, 12%+21%+80%) and lyophilized for NanoLC-MS/MS.

Nano LC-MS/MS analysis

Fractions of enriched peptides were analyzed on an Easy-nLC HPLC system (Thermo, USA) coupled to high-field asymmetric waveform ion mobility spectrometry system (FAMIS, Thermo, USA) with compensation voltage of -45 V and -65 V, and followed by tandem MS, an Orbitrap Exploris™ 480 mass spectrometer (Thermo, USA). The HPLC capillary column (150 μm i.d. × 25 cm) was packed in-house with 1.9 μm, 100 Å Venusil XBP C18 silica particles (Dr. Maisch GmbH, Germany). Two mobile phases were (A) 2% (v/v) ACN with 0.1% (v/v) FA and (B) 98% (v/v) ACN with 0.1% (v/v) FA. The peptides were separated by a gradient of 120 min (from 6% to 28% B in 85min, from 28% to 38% B in 17 min, from 38% to 100% B in 8 min, keeping at 100% B for 10 min) with the flow rate of 600 nL/min. MS1 analysis was performed in full scan ranging from m/z 300 to 1500 with a mass resolution of 60,000 at positive ion mode. The spray voltage was set to 2200 V and the ion transfer tube temperature was 320 °C. MS/MS spectra were acquired in data-dependent mode with total cycle time of 2 s in which highest intensity precursor ions of 2-7 charge stage were selected for sequencing in the HCD collision mode with normalized collision energy of 30%. The MS/MS

spectra of each precursor ion were scanned twice for identification and quantification dividually using orbitrap detector. The qualitative scan was operated in auto scan range mode with a mass resolution of 15000 and the quantitative scan was performed in defined scan range from m/z 50 to 250 with a mass resolution of 30000. The isolation window (m/z) was 1.6. The dynamic exclusion time was 40s.

Database search and quantification

MS raw files were analyzed by MaxQuant (version 1.6.5.0) against the Uniprot_Homo Sapiens Swiss-Prot database (updated on 06/20/2021, 20360 proteins). The MS/MS spectra were searched with a precursor mass tolerance of 10 ppm and a product ion mass tolerance of 20 ppm and up to 2 missed cleavages were allowed for full tryptic digestion. Carbamidomethyl (C) was set as a fixed modification and variable modifications were set to oxidation (M) and dimethyl labeling (+ 30.04385 Da) at N-terminal/lysine for isotope modifications. The FDR for peptide spectral matches (PSMs), peptides, and proteins was controlled at 0.01 to filter the spectra based a target-decoy database strategy. The searched results were further quantified by extracting the intensity of a1 ion from spectra with pQuant software[6]. The median of isotope abundance ratio of a1 ion for all peptides was taken as the final ratio of proteins. Three independent biological replicates were performed separately.

For each replicate, proteins identified as reverse hits and contaminants were removed and proteins containing more than 2 unique peptides with quantified heavy/light (H/L) ratios were retained for further processing. The proteins with explicit mitochondrial annotation were sorted out according to the MitoCarta 3.0 database, the Uniprot_Homo Sapiens Swiss-Prot database (updated on 06/20/2021, proteins with the Gene Ontology cell component annotation containing “mitochondria” or “mitochondrion” were assigned as mitochondrial proteins) and literatures. Then the protein H/L ratios were normalized by dividing the median H/L ratio of non-mitochondrial proteins. Proteins with H/L ratio ≥ 2 were filtered out to be labeled proteome. Proteins identified by three biological replicates were retained as the final mitochondrial proteome.

Identification and quantification of mitochondrial proteome in Huh7-R cells

Sorafenib-resistant and sensitive Huh7 cells were seeded in 10-cm culturing dishes and used for labeling when reaching 80-90% confluence. The cells were treated as protocol above. Sorafenib-resistant Huh7 cells with illumination was sample group, while sorafenib-resistant Huh7 cells without illumination (group 1) and sensitive Huh7 cells with illumination (group 2) were used as controls. Pseudo-isobaric dimethyl labeling after digestion: Control group 1: CH_2O+ $NaBD_3CN$ (30H, light); Control group 2: $^{13}CH_2O$+ $NaBD_3CN$ (32L, medium); Sample group: CD_2O+ $NaBH_3CN$ (32H, heavy). Fractions of peptides were analyzed on an Easy-nLC 1200 system (Thermo, USA) followed by an Orbitrap Fusion Lumos mass spectrometer (Thermo, USA). The MS/MS spectra of each precursor ion were scanned twice for identification with ion trap detector and quantification with orbitrap detector dividually. Data processing was same as above. Variable modifications were set to oxidation (M) and dimethyl labeling (+ 30.04385 Da and + 32.0564 Da) at N-terminal/lysine with neutral loss of ±2.01256 Da for isotope modifications when searching database. Three independent technical replicates were performed separately. Proteins identified by two or more replicates were retained as the final mitochondrial proteome.

Delineation of mitochondrial proteome dynamics in LPS-stimulated microglia cells

Cytotoxicity tests of LPS

HMC3 cells were seeded in 96-well plate for using after 24 h. The medium was replaced by fresh EMEM with different concentrations of lipopolysaccharide (LPS, 0, 0.1, 1, 10 μg/mL) and incubated for additional 24h and 48h respectively. Then the cell viability was determined by cell counting kit-8 (Beyotime, China). 10 μL WST-8 was added to each well of plate and the absorbance in 450 nm was measured by microplate reader after incubating for 2 h at 37 ℃.

Immunoblotting analysis of NLRP3

HMC3 cells were seeded in 10-cm culturing dishes for using after 24 h. The medium was replaced by fresh EMEM with or without LPS (1 μg/mL) and incubated for additional 24 h and 48 h respectively. The cells were collected and lysed in 1%

SDS/DPBS (w/v) with 1% cocktail (v/v) and ultrasonicated to acquired cell lysate. After a BCA assay for protein concentration, proteins were boiled and loaded for SDS-PAGE (7.5% gel). Gels were transferred to PVDF membranes, followed by block with 5% (m/v) milk and incubated with anti-NLPR3 antibody at 4 °C overnight. After washing three times with TBST, the membranes were incubated with HRP-conjugated secondary antibody for 30 min at room temperature and then were analyzed by ChemiDoc XRS+ for chemiluminescence and detection.

Nano LC-MS/MS analysis

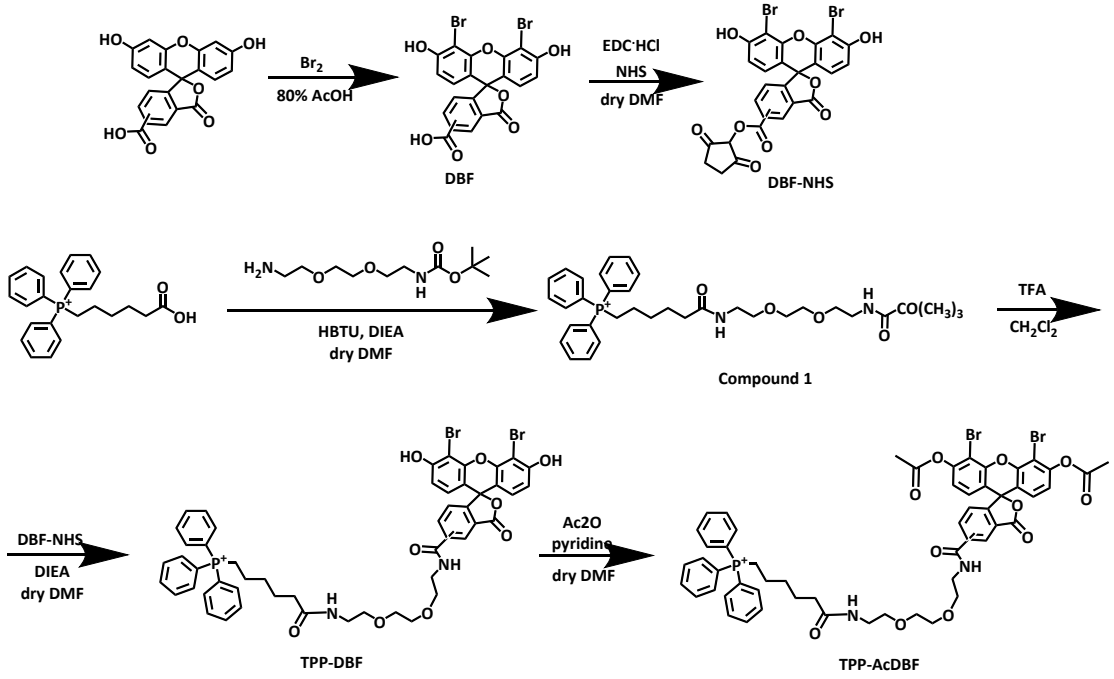
The extraction, biotinylation, enrichment and digestion of proteins were conducted as above. Pseudo-isobaric dimethyl labeling: Control group 1 (LPS-48h, no illumination): $^{13}CH_2O$+ $NaBH_3CN$ (30L); Control group 2 (LPS-0h, illumination): CH_2O+ $NaBD_3CN$ (30H); Sample group 1 (LPS-24h, illumination): $^{13}CH_2O$+ $NaBD_3CN$ (32L); Sample group 2 (LPS-48h, illumination): CD_2O+ $NaBH_3CN$ (32H). Then peptides were mixed in a ratio of 1:1 (v/v) and fractionated as above to six fractions (6%+25%, 9%+30%, 12%+80%, 15%, 18%, 21%). Each fraction was analyzed on an Easy-nLC HPLC system coupled to FAMIS in compensation voltage of -45 V and -65 V, and followed by tandem MS, an Orbitrap Exploris™ 480 mass spectrometer with a gradient of 90 min (from 3% to 8% B in 5min, from 8% to 30% B in 58min, from 30% to 40% B in 12min, from 40% to 100% B in 5min, keeping at 100% B for 10min). Data search and quantification were same as above except that the variable modifications were set to oxidation (M) and dimethyl labeling (+30.04385 Da and +32.0564 Da) at N-terminal/lysine with neutral loss of ±2.01256 Da for isotope modifications when searching database. Three independent biological replicates were performed separately. Proteins identified by two or more biological replicates were retained as the final mitochondrial proteome.

Preparation and characterization of probes

General procedures for probes synthesis

All used chemical reagents and organic solvent were purchased from commercial suppliers, including Sigma-Aldrich (St. Louis, MO, USA), Tokyo Chemical Industry

(Tokyo, JPN), Aladdin (Shanghai, China), J&K Scientific (Beijing, China), Damao (Tianjin, China) and Kermel (Tianjin, China) without further purification. Chromatographic purification of compound was conducted using semipreparative chromatography with a DAC-HB C18 column (10μm, 50 × 300 mm) (NP7000C serials pump, NU3000C serials UV-Vis detector, Hanbon Sci&Tech, China) in linear gradient and mobile phases were (A) ACN and (B) H_2O containing 0.1% (v/v) TFA. Nuclear magnetic resonance ($^1H/^{13}C$-NMR) was detected by a bruker AVANCE III HD 400 MHz spectrometer (German) in deuterated solvent and spectra were processing with MestReNova (Mestrelab Research, USA) software. Multiplicities were referred by the following abbreviation: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Molecular weight was determined by orbitrap LTQ (Thermo, USA) with electrospray ionization source. UV-visible absorption spectrum measurement was performed on Cary 60 UV-Vis spectrophotometer (Agilent, USA). Fluorescence spectrum was measured by Quanta Master 400 steady-state/transient fluorescence spectrometer (Horiba, JPN).



Synthesis of DBF

To a suspension of 5(6)-carboxyfluorescein (377.0 mg, 1 mmol, 1e.q.) in 80% acetic acid (6 mL) was added dropwise bromine (103 μL, 2 mmol, 2 e.q.) in 80% acetic acid

(1.2 mL) and stirred at room temperature overnight in darkness. The mixture was added water to precipitate crude product, the precipitate was collected, washed with water and dried under vacuum. The residue was purified by semipreparative chromatography (A:B = 70:30 (0 min)→35:65 (35 min); DBF eluted at 25 min). The product was lyophilized to yield DBF (278 mg, 52%) as red amorphous.

^{1}H NMR (400 MHz, MeOD) δ 8.60 (s, 0.5H), 8.39 (dd, J = 8.0, 1.4 Hz, 0.5H), 8.34 (dd, J = 8.0, 1.1 Hz, 0.5H), 8.11 (d, J = 8.0 Hz, 0.5H), 7.80 (s, 0.5H), 7.36 (d, J = 8.0 Hz, 0.5H), 6.71 (dd, J = 8.8, 1.8 Hz, 2H), 6.63 (dd, J = 8.7, 3.9 Hz, 2H).

ESI-MS: Calcd for $C_{21}H_{10}Br_2O_7$ $[M+H]^+$ 535.12, found 535.12 $[M+H]^+$.

Synthesis of compound 1

To a solution of (5-carboxypentyl) triphenylphosphonium bromide (110.2 mg, 0.24 mmol, 1 e.q.) in dry DMF (5 mL) was added N-(tert-Butoxycarbonyl)-2,2'-(ethylenedioxy)diethylamine (72.7 mg, 0.29 mmol, 1.2 e.q.), O-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate (HBTU) (100.3 mg, 0.26 mmol, 1.1 e.q.) and N-ethyldiisopropylamine (DIEA) (125 μL, 0.72 mmol, 3 e.q.). After stirring overnight at room temperature, the mixture was purified by semipreparative chromatography (A:B = 70:30 (0 min)→30:70 (30 min), compound 1 eluted at 18 min). The product was lyophilized to yield compound 1 (115.2 mg, 79%) as colorless oil.

^{1}H NMR (400 MHz, MeOD) δ 7.92 (t, J = 7.6 Hz, 3H), 7.86-7.80 (m, 6H), 7.79-7.75 (m, 6H), 3.62 (s, 4H), 3.56-3.49 (m, 4H), 3.35 (dd, J = 10.2, 4.3 Hz, 4H), 3.22 (t, J = 5.7 Hz, 2H), 2.20 (t, J = 7.0 Hz, 2H), 1.65 (ddd, J = 22.6, 16.3, 7.2 Hz, 6H), 1.44 (s, 9H).

ESI-MS: Calcd for $C_{35}H_{48}N_2O_5P^+$ $[M]^+$ 607.75, found 607.73 $[M]^+$.

Synthesis of TPP-DBF

To a solution of compound 1 (70 mg, 0.12 mmol) in dichloromethane (1 mL) was added trifluoroacetic acid (1 mL, 5.7 mmol). After stirring for 2 h at room temperature, the mixture was evaporated under reduced pressure. The residue was resuspended in dichloromethane, evaporated the solvent and repeated this process for three times. Tert-

butoxycarbonyl of compound 1 was removed and the product was used in next reaction without further purification.

To a solution of DBF (40.4 mg, 0.076 mmol, 1 e.q.) in dry DMF (1mL) was added N-hydroxysuccinimide (15.7 mg, 0.136 mmol, 1.8 e.q.) and N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC-HCl) (28.7 mg, 0.150 mmol, 2 e.q.). After stirring for 1h at room temperature in darkness, the mixed solution was added compound 2 (1.5 e.q.) in dry DMF (5 mL) and DIEA (66 μL, 0.38 mmol, 5 e.q.). The reaction solution was stirring for 1-1.5h at room temperature in darkness. Then the mixture was purified by semipreparative chromatography (A:B = 60:40 (0 min)→30:70 (30 min), TPP-DBF eluted at 14 min). The product was lyophilized to yield TPP-DBF (64.6 mg, 83%) as bright yellow solid.

Compound 1 without tert-butoxycarbonyl: ^{1}H NMR (400 MHz, MeOD) δ 7.91 (t, *J* = 7.5 Hz, 3H), 7.82 (t, *J* = 6.3 Hz, 6H), 7.79-7.74 (m, 6H), 3.73-3.69 (m, 2H), 3.67 (s, 4H), 3.55 (t, *J* = 5.8 Hz, 2H), 3.36 (d, *J* = 5.5 Hz, 4H), 3.16-3.11 (m, 2H), 2.20 (t, *J* = 7.0 Hz, 2H), 1.74-1.56 (m, 6H).

ESI-MS: Calcd for $C_{30}H_{40}N_2O_3P^+$ $[M]^+$ 507.63, found 507.64 $[M]^+$.

TPP-DBF: ^{1}H NMR (400 MHz, MeOD) δ 8.47 (s, 0.5H), 8.23 (dd, J = 8.1, 1.6 Hz, 0.5H), 8.16 (dd, J = 8.0, 1.4 Hz, 0.5H), 8.11 (d, J = 8.0 Hz, 0.5H), 7.87 (dd, J = 6.9, 1.9 Hz, 3H), 7.81-7.74 (m, 6H), 7.73 (dt, J = 6.6, 4.0 Hz, 6H), 7.65 (s, 0.5H), 7.35 (d, J = 8.0 Hz, 0.5H), 6.72 (dd, J = 8.8, 1.4 Hz, 2H), 6.64 (dd, J = 12.0, 8.8 Hz, 2H), 3.72 (d, J = 5.0 Hz, 2H), 3.68-3.64 (m, 2H), 3.64-3.60 (m, 4H), 3.59-3.49 (m, 4H), 2.13 (dt, J = 19.0, 7.0 Hz, 2H), 1.70-1.60 (m, 2H), 1.20 (t, J = 7.0 Hz, 6H).

ESI-MS: Calcd for $C_{51}H_{48}Br_2N_2O_9P^+$ $[M]^+$ 1023.73, found 1023.50 $[M]^+$.

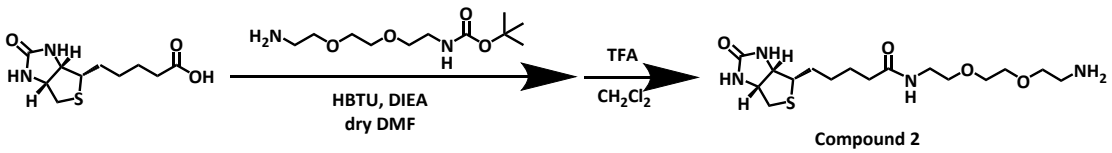
Synthesis of TPP-AcDBF

To a solution of TPP-DBF (10.1 mg, 9.9 μmol, 1 e.q.) in dry DMF (1mL) was added acetic anhydride (9.5 μL, 100 μmol, 10 e.q.) and pyridine (29 μL, 360 μmol, 36 e.q.). The mixture was stirring for 2 h at room temperature in darkness and purified by semipreparative chromatography (A:B = 60:40 (0 min)→20:80 (30 min), TPP-DBF eluted at 19 min). The product was lyophilized to yield TPP-DBF (8.3 mg, 76%) as white solid.

^{1}H NMR (400 MHz, MeOD) δ 8.48 (s, 0.5H), 8.21 (dd, J = 8.1, 1.6 Hz, 0.5H), 8.17 (dd, J = 8.0, 1.3 Hz, 0.5), 8.13 (d, J = 8.1 Hz, 0.5H), 7.83 (dd, J = 7.1, 1.7 Hz, 3H), 7.78-7.71 (m, 6H), 7.71-7.66 (m, 6H), 7.65 (s, 0.5H), 7.38 (d, J = 8.1 Hz, 0.5H), 7.04 (dd, J = 8.7, 4.4 Hz, 2H), 6.92 (dd, J = 11.4, 8.7 Hz, 2H), 3.72-3.65 (m, 2H), 3.65-3.60 (m, 2H), 3.59-3.49 (m, 4H), 3.44 (dt, J = 18.0, 5.4 Hz, 4H), 3.19 (t, J = 5.5 Hz, 2H), 2.36 (d, J = 5.9 Hz, 6H), 2.12 (dt, J = 20.6, 7.0 Hz, 2H), 1.67-1.46 (m, 6H).

^{13}C NMR (101 MHz, MeOD) δ 174.18, 168.22, 168.20, 166.46, 150.95, 150.89, 148.68, 148.64, 134.85, 133.45, 133.39, 133.35, 133.30, 130.15, 130.12, 130.02, 129.99, 127.33, 127.20, 119.87, 118.91, 117.99, 117.52, 117.44, 106.04, 105.99, 81.46, 81.30, 69.82, 69.20, 69.09, 68.94, 39.62, 38.88, 38.79, 34.98, 29.56, 24.47, 21.86, 19.19.

ESI-MS: Calcd for $C_{55}H_{52}Br_2N_2O_{11}P^+$ $[M]^+$ 1107.8052, found 1107.8066 $[M]^+$.



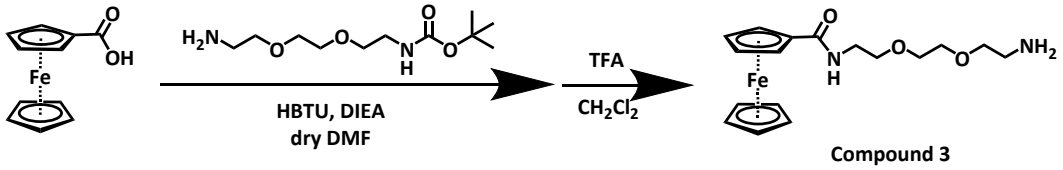
Synthesis of compound 2 (BA)

To a solution of biotin (40.2 mg, 0.16 mmol, 1 e.q.) in dry DMF (2 mL) was added N-(tert-Butoxycarbonyl)-2,2'-(ethylenedioxy)diethylamine (49.3 mg, 0.20 mmol, 1.2 e.q.), HBTU (68.5 mg, 0.18 mmol, 1.1 e.q.) and DIEA (90 μL, 0.52 mmol, 3 e.q.). After stirring overnight at room temperature, the mixture was purified by semipreparative chromatography (A:B = 85:15 (0 min)→65:35 (20 min)→65:35 (10 min), product eluted at 26 min). The product was lyophilized and dissolved in dichloromethane (1.5 mL), then trifluoroacetic acid (0.5 mL, 6.48 mmol) was added. After stirring for 2 h at room temperature, the mixture was evaporated under reduced pressure. The residue was resuspended in dichloromethane, evaporated the solvent and repeated this process for three times to obtain compound 2 as colorless oil.

^{1}H NMR (400 MHz, MeOD) δ 4.50 (dd, J = 6.8, 4.8 Hz, 1H), 4.30 (dd, J = 7.0, 4.3 Hz, 1H), 3.69 (d, J = 4.7 Hz, 2H), 3.66 (s, 4H), 3.55 (d, J = 5.4 Hz, 2H), 3.36 (d, J = 10.3 Hz, 2H), 3.24 – 3.17 (m, 1H), 3.12 (s, 2H), 2.93 (dd, J = 12.7, 4.7 Hz, 1H), 2.70 (d, J =

12.7 Hz, 1H), 2.22 (t, J = 7.0 Hz, 2H), 1.68 (tdd, J = 20.4, 13.7, 6.7 Hz, 4H), 1.45 (dd, J = 14.1, 6.8 Hz, 2H).

ESI-MS: Calcd for $C_{16}H_{30}N_4O_4S$ $[M+H]^+$ 375.51, found 375.39 $[M+H]^+$.



Synthesis of compound 3 (FcA)

To a solution of ferrocenecarboxylic acid (41.2 mg, 0.18 mmol, 1 e.q.) in dry DMF (2mL) was added N-(tert-Butoxycarbonyl)-2,2'-(ethylenedioxy)diethylamine (52.5 mg, 0.21 mmol, 1.2 e.q.), HBTU (72.6 mg, 0.19 mmol, 1.1 e.q.) and DIEA (90 μL, 0.52 mmol, 3 e.q.). After stirring overnight at room temperature, the mixture was purified by semipreparative chromatography (A:B = 60:40 (0 min)→30:70 (30 min), product eluted at 21 min). The product was lyophilized and dissolved in dichloromethane (1.5 mL), then trifluoroacetic acid (0.5 mL, 6.48 mmol) was added. After stirring for 2 h at room temperature, the mixture was evaporated under reduced pressure. The residue was resuspended in dichloromethane, evaporated the solvent and repeated this process for three times to obtain compound 3 as rufous oil.

^{1}H NMR (400 MHz, MeOD) δ 4.81 (s, 1H), 4.22 (s, 4H), 3.69 (s, 4H), 3.67 – 3.61 (m, 4H), 3.51 (d, J = 4.2 Hz, 4H), 3.10 (s, 4H).

ESI-MS: Calcd for $C_{17}H_{24}FeN_2O_3$ $[M+H]^+$ 361.24, found 361.22 $[M+H]^+$.

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