SI Appendix

Spatiotemporally resolved subcellular phosphoproteomics

Authors:

Yanjun Liu[1†], Ruxin Zeng[1†], Ruixuan Wang[1], Yicheng Weng[2,3], Ruixiang Wang[2,3], Peng Zou[1,3,4,5*], Peng R. Chen[1,2,3*]

Affiliations:

[1]Synthetic and Functional Biomolecules Center, Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China.

[2]Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, 100871, China.

[3]Peking-Tsinghua Center for Life Sciences, Peking University, Beijing, 100871, China.

[4]PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing, 100871, China

[5]Chinese Institute of Brain Research, Beijing, 102206, China

*Correspondence to: pengchen@pku.edu.cn (P.R.C), zoupeng@pku.edu.cn (P.Z)

†These authors contributed equally to this work.

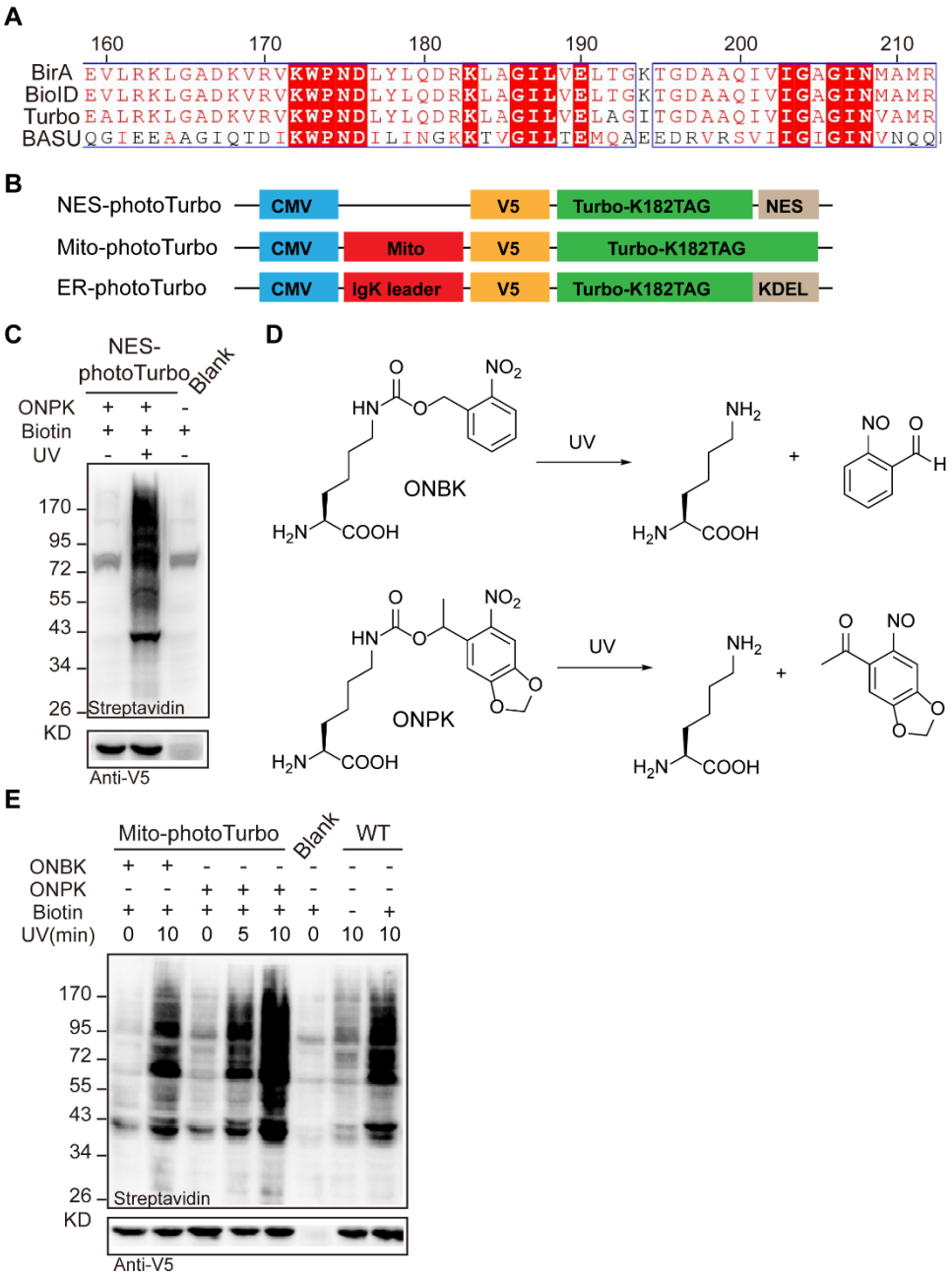


Figure S1. PhotoTurbo activation and UAA selection. A, Sequence alignment of BirA, BioID, Turbo and BASU. B, Schematic illustration of plasmid constructions. Cytosol location sequence: C-terminus-PLERLTLD; ER lumen location sequence: N-terminus (Igk leader sequence)-METDTLLLWVLLLWVPGSTGD, C-terminus-KDEL; Mitochondrial matrix location sequence: MLATRVFSLVGKRAISTSVCVRAH. V5-tag sequence: GKPIPNPLLGLDST. C,

Cytoplasmic localized photoTurbo activation by UV irradiation. D, Structure and photolysis reaction of ONBK and ONPK. E, Comparison photolysis efficiency of ONBK with ONPK on photoTurbo system.

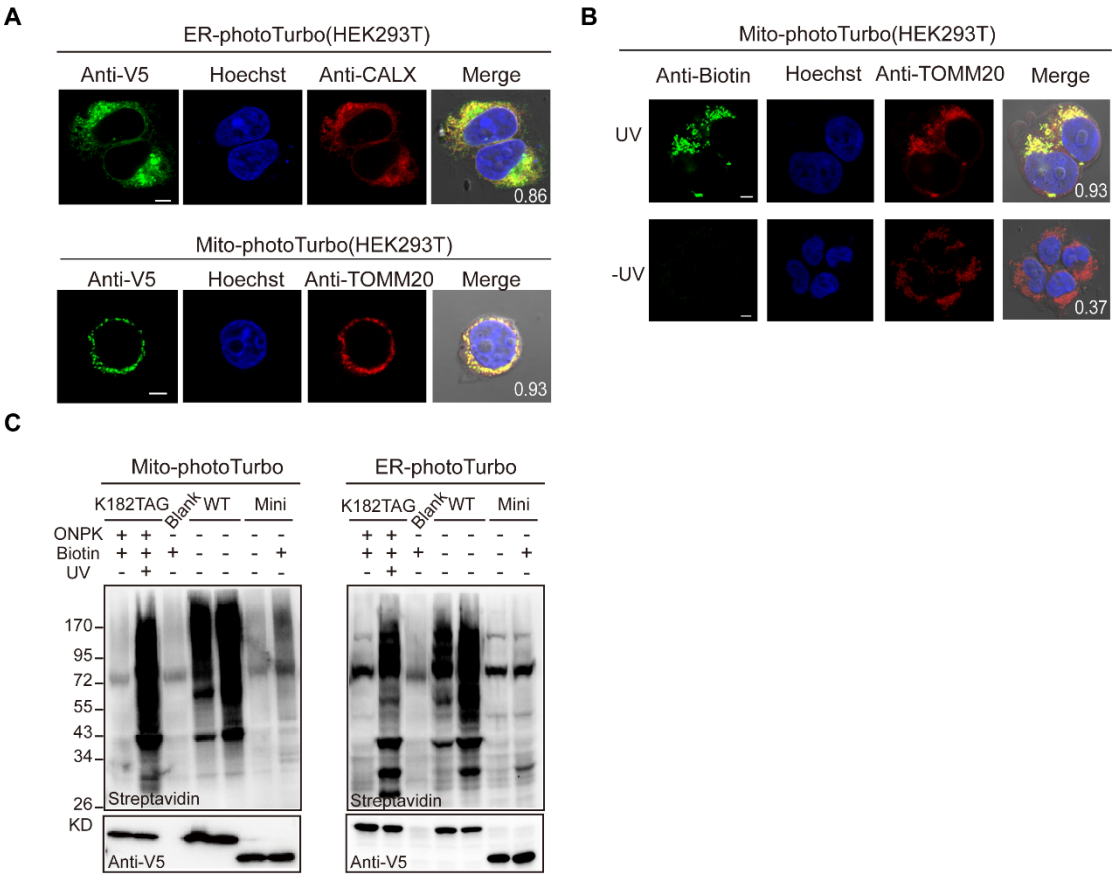


Figure S2. Subcellular photoTurbo activation. A, Validation of the subcellular localization of PhotoTurbo in mitochondrial matrix and ER lumen of HEK 293T cells by fluorescence imaging. TOMM20 and CALX were used as the mitochondria and ER marker respectively. Pearson's R values are shown in the corners. Scale bars, 5μm. B, Fluorescence imaging of photoTurbo activity in mitochondrial matrix of HEK293T. Green is the biotinylation signal to verify the labeling efficiency of photoTurbo, and red is the mitochondrial marker-TOMM20. UV, 5 min. Scale bars, 5 μm. Pearson's R values of Biotin and TOMM20 are shown in the corners. C, Comparison labeling efficiency of photoTurbo, Turbo, mini-Turbo in mitochondrial matrix and endoplasmic reticulum lumen.

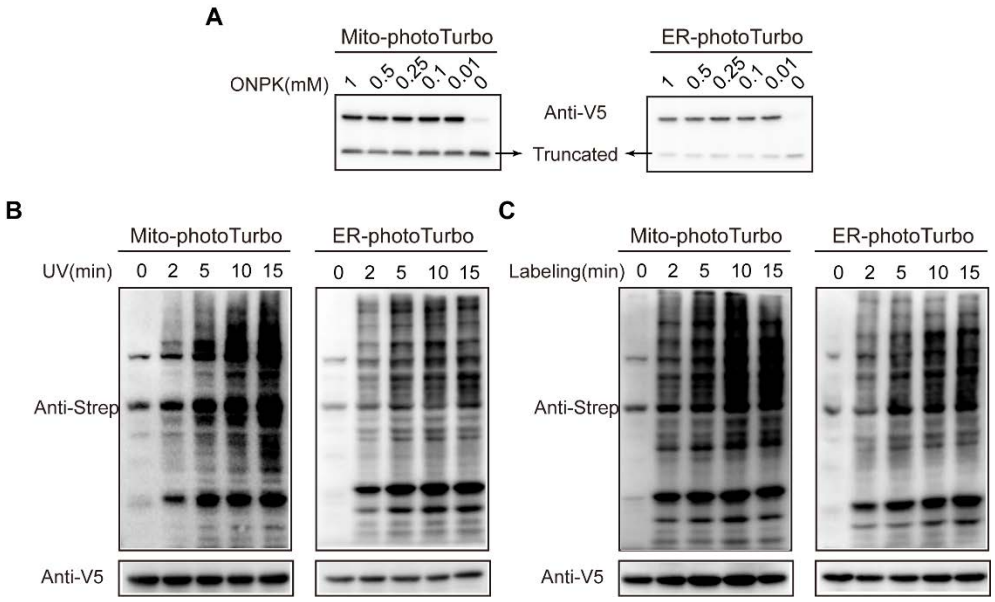


Figure S3. Optimization of photoTurbo for proteomic experiments. A, Optimization of concentration of ONPK for photoTurbo system. "Truncated" band represented the peptide from 1-182 residues of Turbo with no catalytic function. B, Optimization of UV irradiation time for photoTurbo system. C, Optimization of labeling time for photoTurbo system.

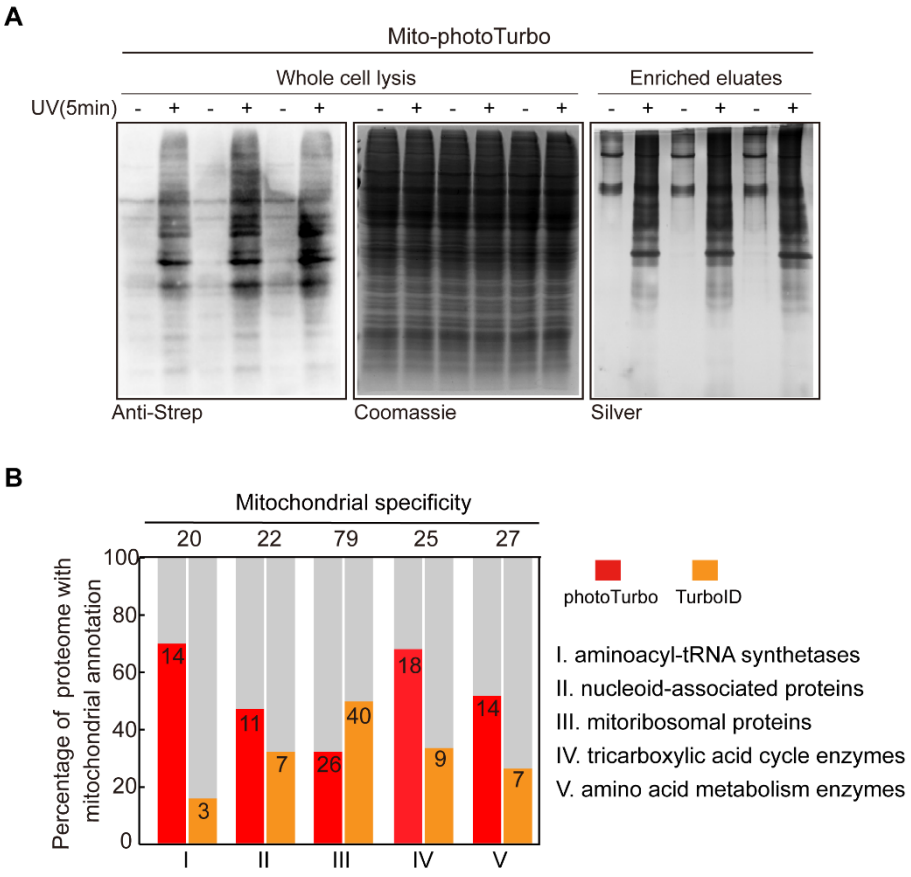


Figure S4. Additional data for the mitochondrial matrix proteomic experiment. A, Western blot and gel characterization of proteomic samples from **Figure 2A**. 2.5% of each whole cell lysate was used for streptavidin blotting (left). Coomassie staining (middle) shows equal loading of samples. Right: 5% of streptavidin beads were boiled in SDS buffer to elute biotinylated proteins. Eluted proteins were separated on SDS-PAGE and detected by silver stain. B, Analysis of depth of coverage. Five groups of well-established mitochondrial matrix proteins (I to V) were crossed with our and TurboID proteomic list.

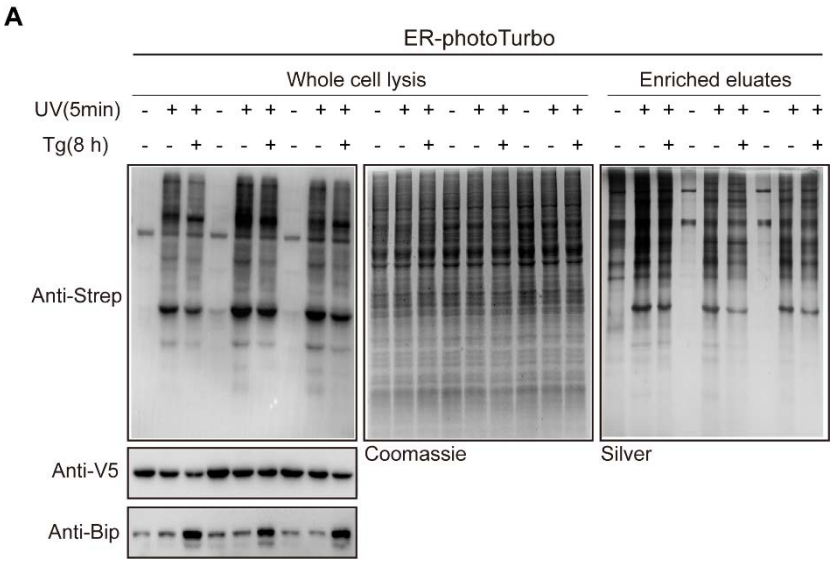


Figure S5. MS related data of ER lumen proteome experiment. A, Western blot and gel characterization of proteomic samples from ER lumen proteome. 2.5% of each whole cell lysate was used for streptavidin blotting (left). Coomassie staining (middle) shows equal loading of samples. Right: 5% of streptavidin beads were boiled in SDS buffer to elute biotinylated proteins. Eluted proteins were separated on SDS-PAGE and detected by silver stain.

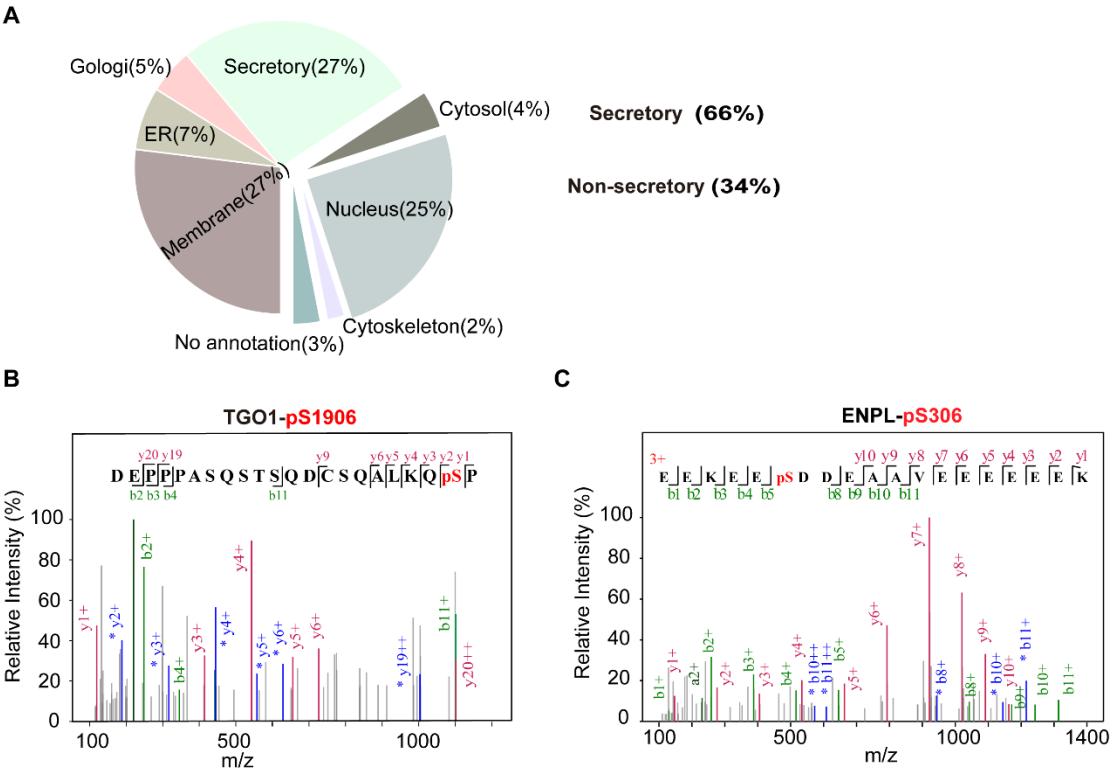


Figure S6. MS/MS analysis of ER phosphoproteome. A, Specificity analysis of ER phosphoproteome across three qualitative proteomes. B-C, MS/MS spectrum of peptides of known substrates of Fam20C in ER lumen.

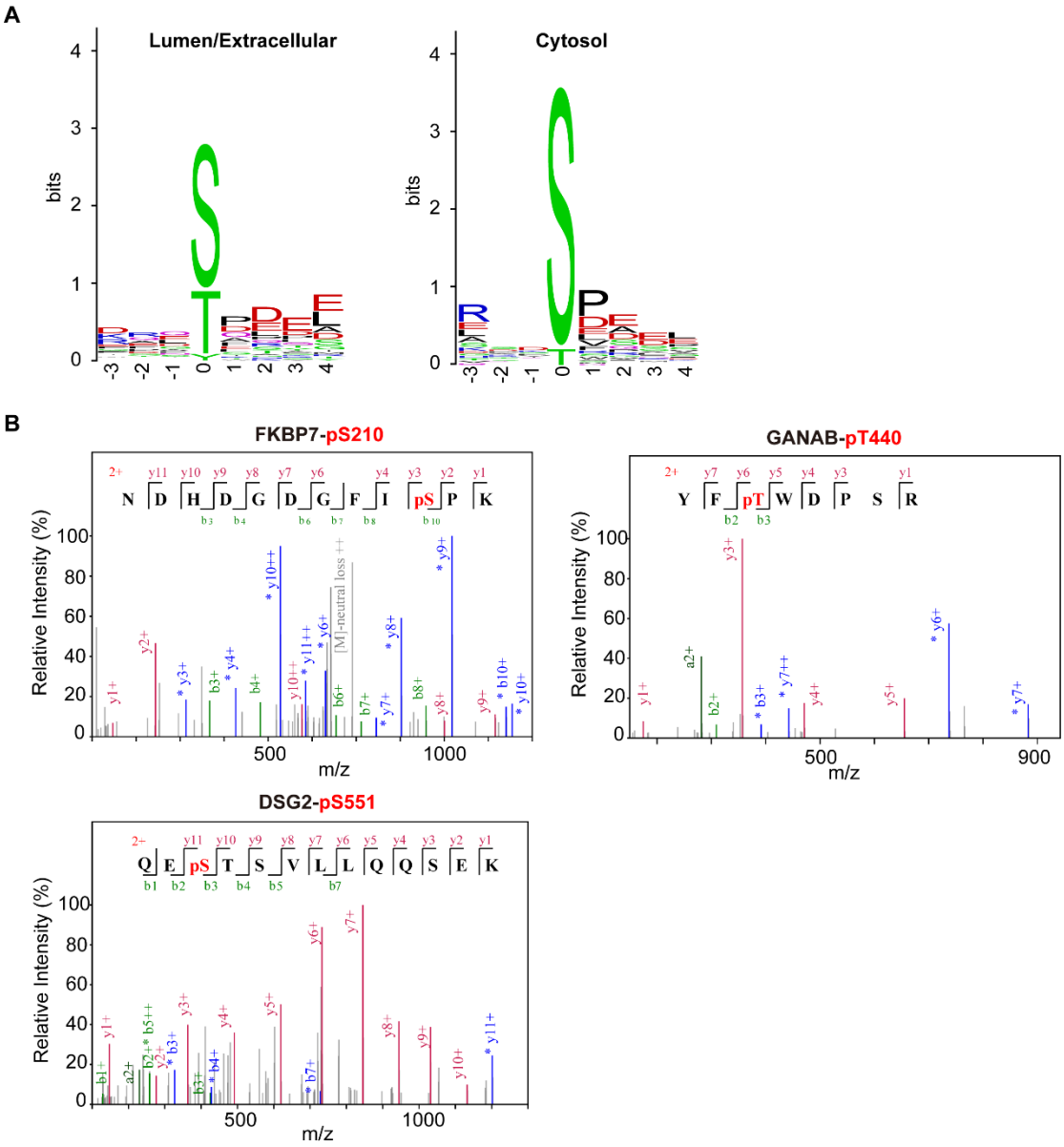


Figure S7. Motif analysis and MS/MS spectrum of identified phosphopeptides in ER lumen. A, Phosphopeptide motif analysis of phosphosites detected in the ER phosphoproteome. Only phosphopeptides predicted to reside in the secretory pathway and having explicit structure information were analyzed (filtered by Uniprot database). Lumen/Extracellular or cytosol represent phosphosites toward ER lumen/extracellular or cytoplasmic of these analyzed proteins. B, Part of MS/MS spectrum of newly identified phosphosites in ER lumen.

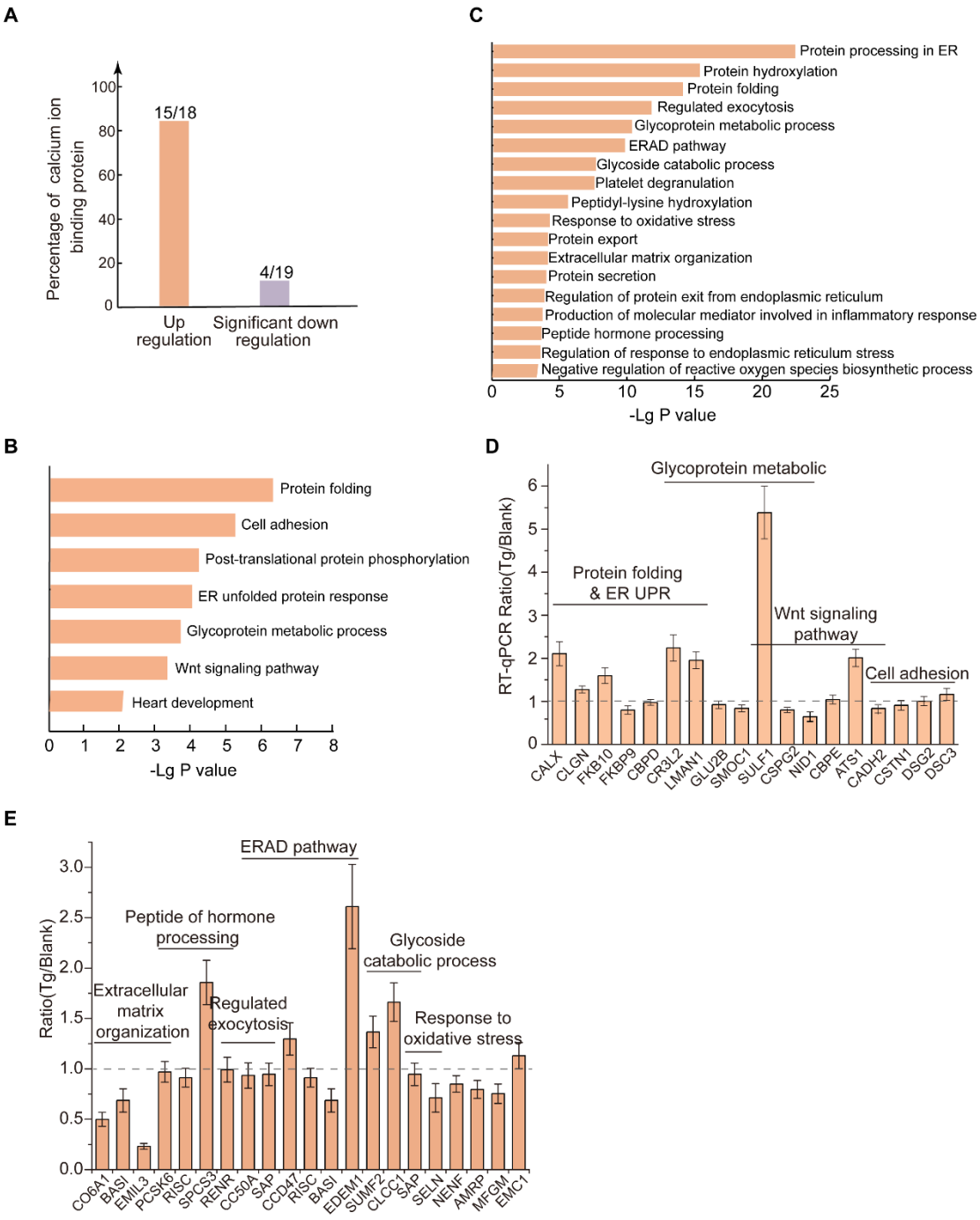


Figure S8. Additional analysis for the ER lumen proteomic experiments. A, Percentage of Ca^{2+} binding protein in up/down regulated proteins under ER stress. B, Gene ontology (GO) biological process analysis of the up-regulated proteins(R>1.5, P<0.05) with Metascape. C, Gene ontology (GO) biological process analysis of the down-regulated proteins(R<0.67, P<0.05) with Metascape. D, RT-qPCR for 18 up-

regulated proteins (R>1.5, P<0.05) under ER stress. E, RT-qPCR for 19 significant down-regulated proteins (R<0.5, P<0.05) under ER stress.

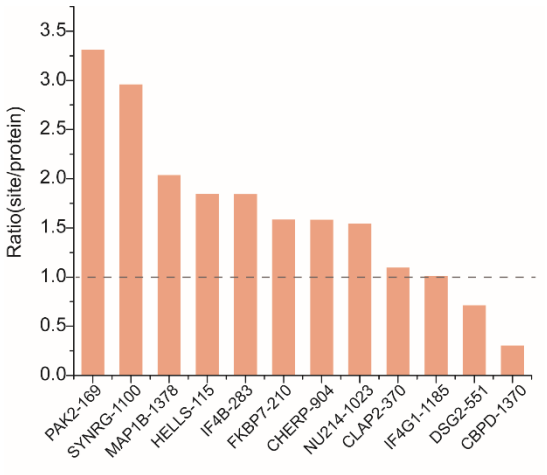


Figure S9. Additional analysis for the ER lumen phosphoproteome. The correction of phosphorylation level of partial phosphorylated proteins which coincide with ER proteome under ER stress.

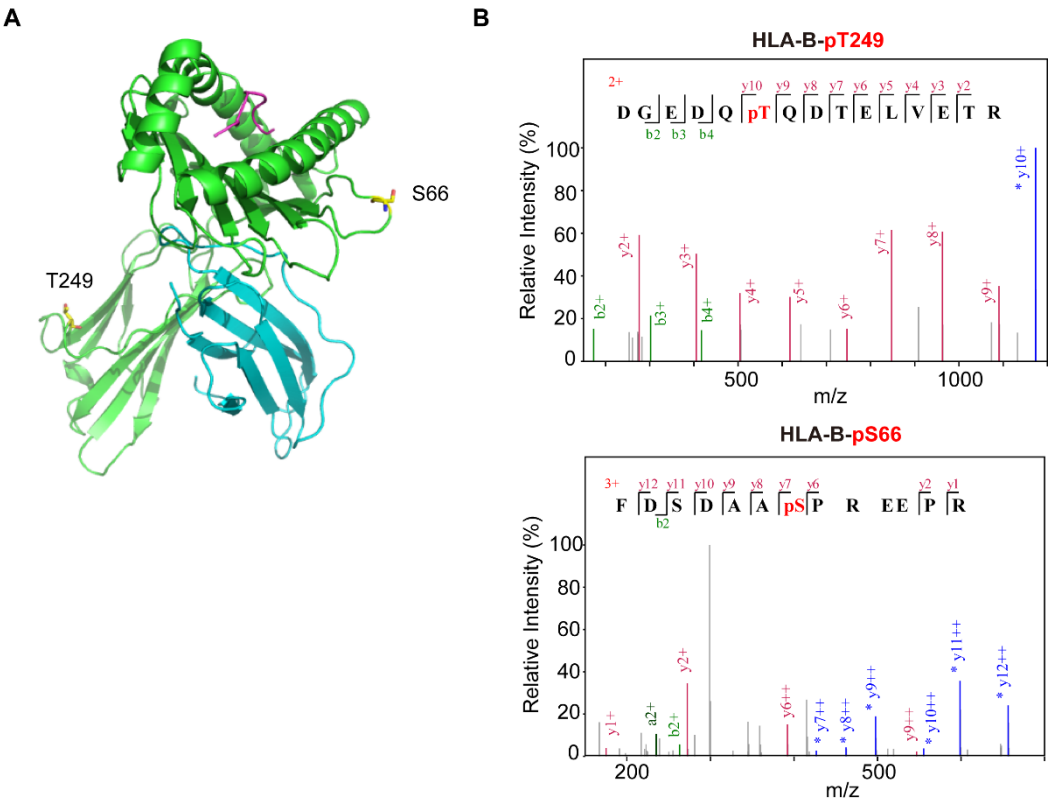


Figure S10. Structure and MS/MS spectrum of HLA-B. A, Location of S66 and T249 on HLA-B (PDB: 1a1m). B, MS/MS spectrum of S66 and T249 of HLA-B.

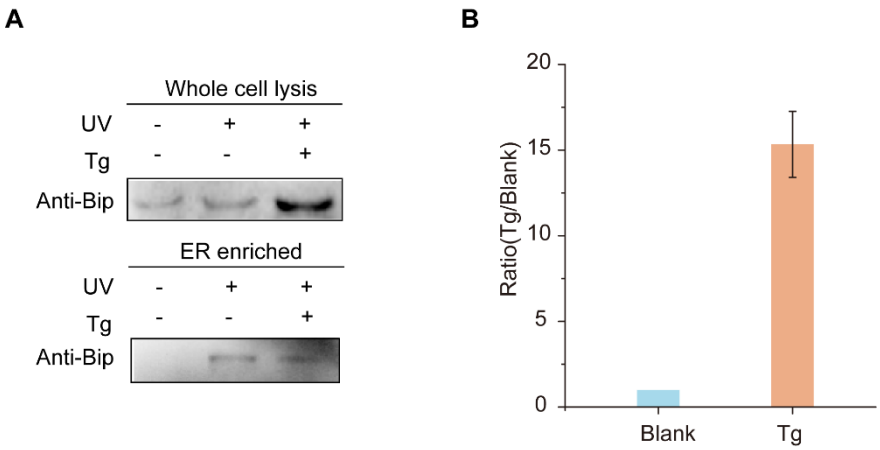


Figure S11. Additional data for Bip translocation experiment. A, Western blot characterization of expression level of Bip under ER stress. B, RT-qPCR for transcription level of Bip under ER stress.

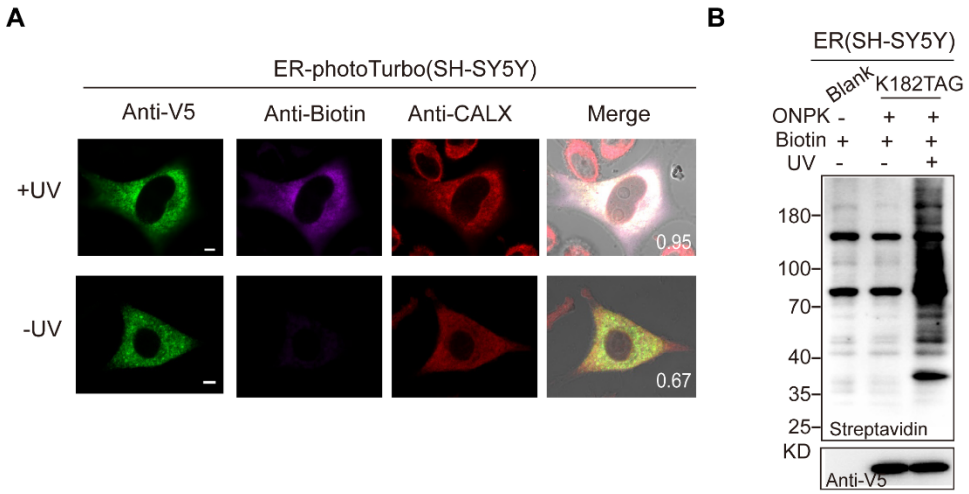


Figure S12. ER-photoTurbo activation in SH-SY5Y. A, Fluorescence imaging of photoTurbo location and the activation activity in ER lumen of SH-SY5Y. Anti-V5 (in green) was used to visualize photoTurbo expression, magenta is biotinylation signal to verify the labeling efficiency of photoTurbo, and red is the ER marker-CALX. UV, 5 min. Scale bars, 5μm. Pearson's R value of Biotin and CALX: 0.95(UV), 0.67(-UV). B, Photo-activation efficiency of photoTurbo for proximity-dependent labeling of proteins in ER lumen of SH-SY5Y.

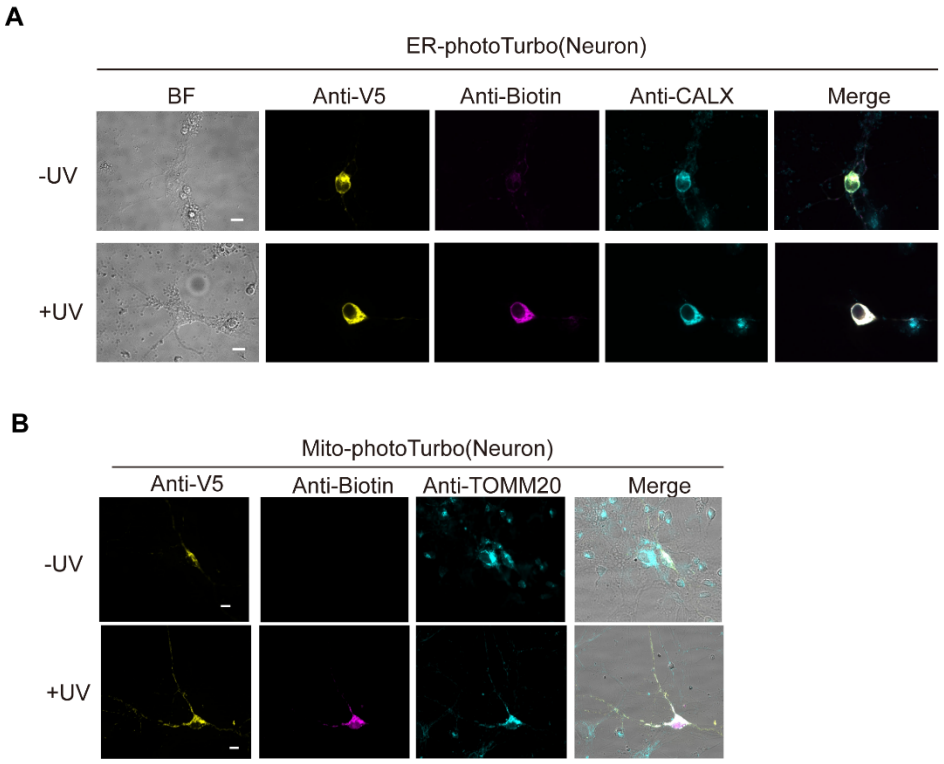


Figure S13. PhotoTurbo activation in primary neurons. A, Validation of the subcellular localization and protein labeling of photoTurbo in ER lumen of neuron cells by fluorescence imaging. CALX was used as the ER marker. B. Validation of the subcellular localization and protein labeling of photoTurbo in mitochondria matrix of neuron cells by fluorescence imaging. TOMM20 was used as the mitochondria marker. Scale bars, 10μm.

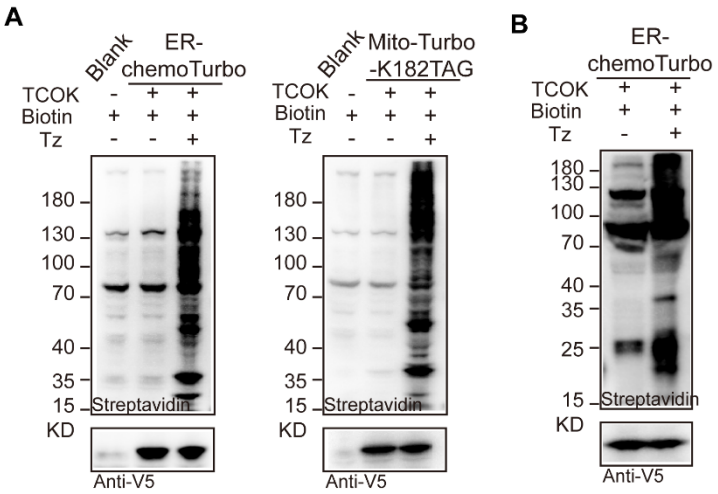


Figure S14. Chemically activatable Turbo (chemoTurbo) for subcellular proteins labeling. A, The catalytic K182 residue on Turbo was replaced by chemically caged lysine analogue-TCOK resulted in masking the proximity labeling activity until the addition of Dimethyl-tetrazine (DM-Tz) in living cells. Chemo-activation efficiency of chemoTurbo for proximity-dependent labeling of proteins in mitochondria and ER lumen of mice. B, Blotting of excised HEK293T samples.

SI Appendix Methods

Cell transfection and ONPK incorporation. To prepare the DNA-Lipofectamine complexes, 1 μg of the plasmid encoding photoTurbo and 1 μg of the plasmid encoding 4xtRNA$^{Pyl}_{CUA}$-ONPK-RS (CUA, the anticodon of the amber codon; Pyl, pyrrolysine) pair were diluted in 50 μL Opti-MEM then added 4 μL P3000 regent. Next, the diluted DNA were added into 50 μL Opti-MEM with 4 μL Lipofectamine 3000 reagent and incubated for 15 min. Then the DNA-lipid complexes were added into 1 mL DMEM with 10% FBS containing 100 μM ONPK. Subsequently, this mixture was added into HEK293T or SH-SY5Y cells cultured in 12-well plates with 60-70% confluence and cultured for another 24 h before the following experiments.

Construct stable cell line. For preparation of the lentivirus, the plasmids of pLX304-tdTomato-P2A-ONPK-RS (0.5 μg), dR8.91 (0.5 μg) and pVSV-G (0.4 μg) were diluted in 50μL Opti-MEM then added 3 μL P3000 regent, followed by addition into 50 μL Opti-MEM with 3 μL Lipofectamine 3000 reagent and incubation for 15 min. Then, the plasmid-lipid complexes were added into HEK293T cells cultured in 12-well plates with 60-70% confluence. The culture medium containing prepared lentivirus was collected and filtered through a 0.45 μm filter after 48 h. After that, 0.4 mL of the collected medium was added to a fresh HEK293T cells at 70-80% confluence in 12-well plates for another 48 h incubation, followed by replacing with fresh medium containing 5 μg ml^{-1} blasticidin (Selleck, S7419) every day for selection. After a week, cells expressing ONPK-RS were sorted by flow cytometry.

Photo-activation of Turbo and biotin labeling in living mammalian cell. Before activation, we diluted 100 mM biotin DMSO stock directly into cell culture medium to

a final concentration of 100 μM (HEK293T) or 500 μM (SH-SY5Y). Cells expressing photoTurbo were subjected to UV irradiation in the cell culture medium. The plate or dish was put on the ChemiDoc XRS+ and irradiated from the bottom by UV light at intensity of 0.5 milliwatt per cm^2 for 5 min. Next, cells were incubated at 37 ℃ for 10 min (HEK293T) or 20 min (neuron culture). Labeling was then stopped by exchanging the cell culture medium to ice-cold PBS. For negative control, we omitted the UV irradiation.

Optimize experiment conditions of photoTurbo. For optimizing UV irradiation time, each 3.5 cm dish was put on the ChemiDoc XRS+ and irradiated for different time (0/2/5/10/15 min) separately. Then the dish was incubated at 37 ℃ for 10 min. For optimizing labeling time, all the dishes were irradiated for 10 min and incubated at 37℃ for different time (0/2/5/10/15 min) separately. Labeling was stopped by exchanging the cell culture to ice-cold PBS and the samples were collected for subsequent western blotting.

Dissociated Rat Neuron Culture. For primary cortical neuron culture, sterile 12-mm glass coverslips in 24-well plate or 35mm dish were pre-incubated with poly-D-lysine (Sigma®) solution at 37°C with 5% CO_2 for 24 h. Then the coverslips or dishes were washed by ddH_2O and added Laminin Mouse Protein (Gibco™) solution. After overnight incubation at 37°C, they were washed twice by ddH_2O and dry at room temperature. Cortical tissue of postnatal day 0 rat were isolated and cut into small pieces, and incubated with 5mL Trypsin-EDTA (0.25%, Gibco™) at 37°C under 5% CO_2 for 15 min. The digestion was stopped by carefully replacing the supernatant with 1 mL

DMEM containing 10% FBS. The fragments were dispersed by gently pipetting for 1min and the supernatant was diluted with neuron culture medium (Neurobasal™ Medium, B-27™ Supplement, GlutaMAX™ Supplement and Penicillin-streptomycin) to appropriated cell density. For imaging sample, 1mL suspension contained 8-10×10^4 cell was added to each well of 24-well plated. For western blotting sample, 3mL suspension contained 6-7×10^5 cell was added to each 35mm dish. At 4 days in vitro, half of the neuron culture medium was replaced with fresh medium for transfection.

Expression of photoTurbo in primary rat neuron cultures. Rat cortical neurons were transfected at DIV8. To prepare DNA-Lipofectamine complexes, 2 μL Lipofectamine 3000 regent per well (24-well) was diluted into 25 μL Neuron Basal. Then another 25 μL Neuron Basal with 500 ng hSyn-V5-photoTurbo plasmid and 500 ng plasmid encoding ONPK–RS−$tRNA^{Pyl}_{CUA}$ pair was added into diluted Lipo 3000 and incubated for 15 min. Most of the preconditioned growth medium neurons cultured in was removed and set aside, the DNA-Lipid complexes were mixed with 450 μL Neuron Basal and added to the neurons. After 45 min, all the medium was removed, and replaced with 1 mL media with 100 μM ONPK and 100 μM biotin, which containing half of preconditioned growth medium and new medium. Subsequently, the neurons were incubated at cell incubator until DIV12 before the following experiments.

Fluorescence imaging of photoTurbo. HEK293T cells or SH-SY5Y cells were plated into an eight-well chamber and transfected with indicated plasmids. Before fixation with 4% PFA, cells were permeabilized with 0.1% Triton-X100, and blocked with 5% FBS. For immunostaining, fixed cells were incubated with primary antibody for 1 h at

room temperature. After washed with PBST for three times, cells were incubated with secondary antibody for another 1 h. Hoechst was used to stain nucleus in the last step. Images of stained samples were also visualized on LSM 700 laser scanning confocal microscope (Zeiss). For rat primary neuron imaging, buffer or regent were dissolved in Tyrode's Buffer. The glass coverslips with neuron were washed by Tyrode's Buffer. Neuron sample were imaging on an inverted fluorescence microscope (Nikon-TiE) equipped with a 40X 1.3 NA oil immersion objective lens, two scientific CMOS cameras (Hamamatsu ORCA-Flash 4.0 v2), a spinning disk confocal unit (Yokogawa CSU-X1), and five laser lines (Coherent OBIS).

Co-localization analysis of fluorescence imaging. The images were first imported into Image J software (https://imagej.net/Fiji), and divided into different channels by color. Cell body was selected for further analysis by using polygon selections tool and the Pearson Correlation Coefficient was calculated by Color 2 tool of Image J.

Sample preparation for proteomics. For each sample, HEK293T cells were grown as monolayer in DMEM (10% FBS) in cell incubator at 37°C under 5% CO_2. Mitochondrial matrix samples were generated in a 10-cm dish by transfecting cells with 10 μg of the two plasmids. ER lumen samples were grown in 15-cm dish and generated by transfecting HEK 293T cell lines stably expressing ONPK-RS with 20 μg plasmid encoding $tRNA^{Pyl}_{CUA}$ and photoTurbo. Each sample was preincubated with 100 μM biotin and irradiated by UV light for 10 min, followed by labeling for 10 min (HEK293T) or 20 min (SH-SY5Y). The labeling reaction was then stopped by exchanging the cell culture to ice-cold PBS. Cells were collected by gently pipetting the PBS on to the cells

and centrifuging at 800 g for 5 min. The cell pellets were then resuspended by 1 mL lysis buffer (50 mM pH7.4 Tris·HCl, 150 mM NaCl, 1% TritonX-100, EDTA free protease inhibitor: Bimake B14001, 5% Glycerin) and lysed by sonicating on ice. The lysates were clarified by centrifugation at 12,000 g for 10 min at 4 °C. Next, by adding CH_3OH and $CHCl_3$ to supernatant at a ratio of 4:1:4, proteins were precipitated out and washed three times using ice-cold CH_3OH. After drying the protein precipitate slightly, 2% SDS/PBS was mixed with it for protein redissolution, followed by diluting to 0.2% SDS/PBS solution.

To enrich biotinylated proteins from proteomic samples, 100 μL streptavidin-coated magnetic beads (Pierce) were washed three times with PBS buffer, then incubated with 0.2% SDS/PBS solution containing 10 mg protein for each sample. After 2-3 hours of rotation at room temperature, the beads were washed once with 10 mL 0.2% SDS/PBS buffer, three times with 5 mL PBS buffer and three times with ddH_2O. The beads were then transferred to a new 1.5 mL tube for subsequent experiment.

For proteomic samples, 1% of the lysate was removed before enrichment and used to estimate the protein concentration in lysates by Pierce BCA Protein Assay Kit (Thermo Fisher), as well as to verify the photoTurbo expression and biotinylation by Western blotting. After enrichment, 10% of beads were removed and boiled in 50 μL 1x protein loading buffer with 2 mM biotin for eluting biotinylated proteins. The eluted proteins were then separated on an SDS-PAGE gel and stained by silver stain kit (ProteoSilver^{TM}, PROTSIL1-1KT) to confirm successful enrichment of biotinylated samples.

On-bead trypsin digestion of biotinylated proteins. To prepare samples for mass spectrometry analysis, the beads bounded with biotinylated protein were incubated with 6 M urea/PBS contain 10 mM DTT at 37 ℃ for shaking. After 30 min, IAA was added to the mixture as a final concentration of 20 mM and shaken at 35℃ for another 30 min. Next, the supernatant was removed and the beads was washed four times with 100 mM TEAB buffer. The final volume of TEAB was removed and 100 μL 100 mM TEAB buffer containing 1 μg trypsin was added to the sample. Then the sample was shaken at 37 ℃ for 16 h incubation.

Dimethyl labeling and fractionation of peptides. After digestion, the samples were directly labeled with dimethyl regents (for mitochondria sample, -UV group was 0-plex and +UV group was 6-plex; for ER lumen sample, the -UV was 0-plex, +UV was 4-plex and +UV group with Tg treatment was 8-plex; for SH-SY5Y and mice samples, -UV group was 0-plex, +UV group was 4-plex). 4 μL (39.688 mg/mL) $NaBH_3CN$ or $NaBD_3CN$ was added to each sample and vortexed to mix, followed by addition of 4 μL 4% HCHO, DCDO or $D^{13}CDO$ to corresponding sample. The dimethyl labeling reactions were incubated at room temperature for 30 min and quenched by adding 16 μL 1% $NH_3{\cdot}H_2O$. 8 μL HCOOH was then added to each sample and the beads were removed. The supernatant was combined to a new tube and evaporated to dryness in a vacuum concentrator. At last, samples were fractionated by Pierce ™ High Reversed-Phase Peptide Fractionation Kit (84868).

Liquid chromatography and mass spectrometry. Enriched peptides were reconstituted in 0.1% formic acid and loaded onto a 100 μm × 2 cm pre-column. Online

chromatographic separation was completed using a 75 μm × 15 cm capillary column in-house packed with 4 μm C18 bulk material (InnosepBio), and connected to an EASY-nLC 1000 system (Thermo Fisher Scientific). Peptides were chromatographically separated by a 70-min gradient from 3 to 100% solvent B (A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile) at a flow rate of 300 nl min^{-1} and subject to MS/MS analysis on a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific). The MS/MS analysis was operated under the positive-ion mode with a full-scan *m*/*z* range from 350 to 1,800 and a mass resolution of 70,000. MS/MS fragmentation was performed in a data-dependent mode, of which TOP 20 most intense ions are selected for MS2 analysis with a resolution of 17,500 using the collision mode of HCD. The MS1 advance gain control (AGC) target was set to 3×10^6 ions and the maximum ion time was set to 100 ms. MS2 scans were recorded with an AGC target of 1×10^5 ions with a maximum ion time of 50 ms, isolation width of 2.0 m/z, normalized collision energy of 28%, dynamic exclusion time of 20 s.

Mass spectrometry data analysis. All raw data were processed within the MaxQuant software package (version 1.6.1.0). Data were searched against the *Homo sapiens* database downloaded from UniProt (www.uniprot.org) with default parameters: an initial mass tolerance of 20 ppm and a final mass tolerance of 6 ppm for precursor mass and the minimum peptide length was five amino acids. The enzyme specificity was set to trypsin allowing N-terminal to proline and two missed cleavages. Cysteine modification by IA (carbamidomethyl) was searched as a fixed modification. Methionine oxidation, N-terminal acetylation and modification of adducts were

searched as variable modifications. The MS/MS tolerance was set to 50 ppm and the top MS/MS peaks per 100 Da was set to 10. The peptide and protein false discovery rates were set to 0.01.

Profiling ER proteome under ER stress. For ER stress samples, cells were grown and transfected similar to ER lumen samples. After transfection for 18 h, the cell culture medium was exchanged to DMEM containing 100 μM biotin and 500 nM thapsigargin(Tg). After 8 h incubation, the sample was irradiated, labeled and a series of proteomic sample were prepared as described above. For negative control, we omitted the Tg. The occurrence of ER stress could be proved by anti-Bip blotting.

Pulse-chase experiment for Bip translocation. HEK293T cells expressing ER restricted photoTurbo were treated with 500 nM Tg for 4 h, and exposed to 365-nm light for 10 min to label a cohort of ER luminal proteins for another 4 h. Then, cells were lysed to isolate mitochondria fraction mitochondrion extraction kit (Abbike KTP4003). After digested by 100 μg/mL protease K in storage buffer (Abbike KTP4003) for 15 min at 4°C and stopped by addition of 7 mM PMSF, biotinylated proteins of mitochondria fraction were enriched according to the previous proteome process for anti-Bip blotting.

RT-qPCR analysis of RNA under ER stress. HEK293T cells were treated with 500 nM thapsigargin (Tg) for 8 h followed by RNA extraction. The RNA (2 μg) was then reverse transcribed with random primers and MultiScribe Reverse Transcriptase (Applied Biosystems, 4368814) in a 20 μL reaction according to the manufacturer's instructions. For each qPCR reaction, 0.11 μL cDNA was used as the template. The

templates were mixed with primers and PowerUp SYBR Green Master Mix (Life Technologies, A25742). Then the qPCR reactions were quantified by ABI StepOne Plus system. Ct values were averaged from at least three replicate measurements. Negative controls with Tg omitted were processed in the same manner as the sample. Since GAPDH expression does not change significantly during ER stress, GAPDH is used as endogenous control to normalize for differences in cDNA input. The fold changes of gene expression (eg. Bip) during ER stress can be calculated with the following formula:

$$Fold\ change = 2^{-(Ct\_Bip\_Tg - Ct\_Bip\_Control)+(Ct\_GAPDH\_Tg - Ct\_GAPDH\_Control)}$$

Profiling ER lumen phosphorylation proteome. For each sample, four 15-cm dishes of HEK293T cells were generated, followed by the biotinylated protein enrichment and digestion. The digestive solution was then used for next enrichment. Before phosphopeptide enrichment, Ti-IMAC beads needed to be prepared in advance. 30 g $Ti(SO_4)_2$ was added to a 250 mL flask and 150 mL pure water was added to dissolve with ultrasound. Next, 300 mg GTP material was added to the flask, dispersed with ultrasound for 5-10 min and stirred at room temperature overnight. The homogenized material was centrifuged at 20000 g for 3-5 min and the supernatant was discarded. The material was then washed five times with 0.1% TFA aqueous solution, once with 200 mM NaCl/0.1% TFA aqueous solution, twice with ddH_2O, and centrifuged 3-5 min at 15000 g for each washing. The digestive solution and loading buffer (80% ACN/6% TFA aqueous solution) were mixed in a volume ratio of 1:1, then according to mass ratio of 25:1 of beads and proteins, the Ti-IMAC beads was added to the mixture. After 30 min rotation at room temperature, the beads were washed once with 50% ACN/6%

TFA/200 mM NaCl aqueous solution, twice with 30% ACN/0.1% TFA aqueous solution. For eluting phosphopeptide, elution buffer was added to the beads and shaken for 15 min. Then the beads were sonicated for 15 min in ice bath and the supernatant was collected in a new tube. The beads can be mixed with elution buffer again and shaken for 5 min. The two supernatants were mixed and centrifuged to discard remaining beads. Then the eluent was evaporated to dryness in a vacuum concentrator and desalted on C18 Stage Tips.

Liquid chromatography and mass spectrometry for phosphoproteome. The samples were analyzed by nanospray LC-MS/MS on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific) coupled to an UltiMate 3000RSLC nano system (Thermo Scientific). Samples were loaded onto a C18 loading column (100 μm × 2 cm, packed in-house), and a C18 separating capillary column (75 μm × 15 cm, packed in-house). Solvent A was 0.1% formic acid in water. Solvent B was 0.1% formic acid in 80% acetonitrile. The flow rate was 4 μL/min for loading and 300 nL/min for eluting. The gradient was 2%-44% B in 70 min, an increase to 99% B in 5 min, then held for 10 min at 99% B and back to 5% B in 1 min. The mass spectrometer was operated in data-dependent mode with a full MS scan (350–1,600 M/z) in FT mode at a resolution of 60,000 in positive-ion mode. MS/MS fragmentation was performed at a resolution of 15000 using collision mode of HCD only for precursor ions with 2-7 charges with a cycle time of 3 seconds. Automatic gain control (AGC) targets were 4×10^5 ions for Orbitrap scans and 5×10^4 for MS/MS scans. The dynamic exclusion duration was set to 15 s.

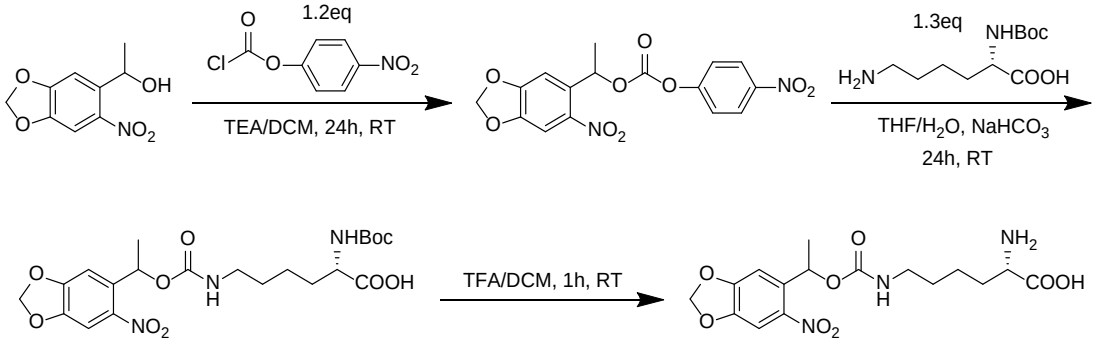
Analysis of mass data spectrometry of ER lumen phosphorylation proteome. Raw MS data files were processed according to the previous proteome analysis with a modification that phosphorylation (STY) was added as variable modifications. Processed data was uploaded in the Perseus software (1.5.8.5) to perform subsequent analysis. Reverse sequences and potential contaminants were removed from the total matrix, phospho-sites with localization probability > 0.75, score diff > 5, with 3 valid intensity value in at least one biological replicate group were used for further analysis. Intensities of phosphor-sites were log2 transformed. Missing values were replaced with values from normal distribution (width = 0.3, down shift = 1.8). P-values were from Student's T-test and phospho-sites with $p < 0.05$ were considered as Turbo-ID enriched sites.

ChemoTurbo Activation in Mice. Nu-Nu nude mice (male, 5-6 weeks) were purchased from Vital River Laboratories, China. All protocols were approved by the Institutional Animal Care and Use Committee of Peking University accredited by AAALAC International. HEK293T cells were cultured in 10cm dishes, cotransfected with Mito/ER-Turbo-K182TAG and TCOK-RS in DMEM/10% FBS supplemented with 200 μM TCOK and cells were grown for another 18 h. Cells were then digested, centrifuged, and resuspended in PBS. These cells (~1×10^7 cells/50 μL) were then subcutaneously injected into the mice legs. 50 μL DM-Tz of 300 mM concentration (equal to 66 mg/kg body weight for an~25 g mouse) was then intravenously injected into mice via tail vein after 2 h of the cell injection and biotin (100 μL, 200mM) was

intraperitonelly injected. For another 4 h, cells were extracted, followed by analysis of biotinylation level by western blotting or enrichment for proteomics.

Supplementary Notes 1

Chemical synthesis of ONPK. 1.41 g 4-nitrophenyl carbonochloridate was dissolved in 20mL DCM and dropwise added to 20mL DCM containing 1.24 g 1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethanol. Then 2mL TEA was dropwise added in to the mixture and stirred at room temperature overnight. The reaction solution was evaporated to remove the solvent and redissolved in 30mL THF. 1.20 g N^{α}-Boc-lysine was dissolved in saturated $NaHCO_3$ solution and the THF solution was dropwise added to it in ice bath. After overnight reaction, the reaction solution was extracted with ethyl acetate and purified by column chromatography to get N^{α}-Boc-ONPK. The deprotection was carried out by TFA in DCM solution at room temperature for 1h. The The reaction product was subsequently evaporated and redissolve in MeOH (5 mL) and precipitated into Et_2O (250 mL) to obtain ONPK.



Supplementary Notes 2

RT-qPCR primer sequence:

Entry name		
CADH2_HUMAN	F	AGCCAACCTTAACTGAGGAGT
	R	GGCAAGTTGATTGGAGGGATG
CBPD_HUMAN	F	CACGACAGAGGCTGTATCAAC
	R	ATGGACACCCGGATTATCAGA
CR3L2_HUMAN	F	CAGAGAAGAGTGTGTCAATGGAG

	R	CTGGTGGTAATGTGGGTGAAG
CALX_HUMAN	F	TCCATGACAAGACCCCTTATACG
	R	ACCACAGATTGGTCAACCAGTA
DSG2_HUMAN	F	CTAACAGGTTACGCTTTGGATGC
	R	GTGAACACTGGTTCGTTGTCAT
ATS1_HUMAN	F	ACTGGAAGCATAAGAAAGAAGCG
	R	AATTCTGCCATCGACTGGTCT
GLU2B_HUMAN	F	TCAGGTCAACGATGACTATTGC
	R	CCCGGTTGGAGGGGATATACA
CSPG2_HUMAN	F	GTAACCCATGCGCTACATAAAGT
	R	GGCAAAGTAGGCATCGTTGAAA
CLGN_HUMAN	F	GTTCCTCCTATCAAACCTCCCA
	R	TCCGTGTCTTCATTCCAGTCAT
DSC3_HUMAN	F	CCTCATCCGGTCAAGTGATCC
	R	AGTGTGTCTTGTCTTCGATACCT
SMOC1_HUMAN	F	TCAGGTTCAGTCACCGACAAG
	R	TCCTGGTCACACGAATAGACTT
CSTN1_HUMAN	F	ATGGCACCAACGTGAAAAAGT
	R	GATGACCGTGGCTTTGTAGGA
NID1_HUMAN	F	TCTCTTTCCAGCCTAGTAGCG
	R	CACGGCAGGAACTTGGTTG
FKB10_HUMAN	F	TACAGTAAGGGCGGCACTTAT
	R	GAGGACGTGAAAGACCAGCG
FKBP9_HUMAN	F	GACGGCCAGAAGTTCGACTC
	R	CGTTTACGCACATCCCAACAA
CBPE_HUMAN	F	CTTGGCCCAGTACCTATGCAA
	R	ACCAGTCCTTGAGTTCACCAG
SULF1_HUMAN	F	GATCCCCGAGGTTCAGAGGA
	R	GGTGTAGTCACAAAGGCATTGA
LMAN1_HUMAN	F	AGTGTAGGAGATCGAGAGCTAAG
	R	AGTTCTTGTTGAGTAATCTGCCC
SPCS3_HUMAN	F	AGAGGTGATAATCCGAAGCTGC
	R	CCTGTTTCCCTTGAGACCATTT
EMC1_HUMAN	F	GACAGCAATATGTTGGGAAGGT
	R	TCTCCCCAGTTCGGGAATTTAAT
SAP_HUMAN	F	ATGCAAAGACGTTGTCACCG
	R	GGGAGGTAGGAGTCCACTATCT
CLCC1_HUMAN	F	CGCCAAGATGGAGCCATTAAA
	R	TGTAACTGCAAGTGCCTTTGTT
CCD47_HUMAN	F	TCTCCTCAACGGGTCATAATCA
	R	GGTTCACTCTCAGTATCTCCCT
EDEM1_HUMAN	F	GCTACGACAACTACATGGCTC
	R	GACTTGGACGGTGGAATCTTT

SUMF2_HUMAN	F	CAGAACAACTACGGGCTCTATG
	R	CAGTGACCCTAGAAGGCTTTTC
RISC_HUMAN	F	GATCTCAAACCACGGAAAACCA
	R	GGCACCACTACCATTCACATAA
NENF_HUMAN	F	AGATCAGCCCATCTACTTGGC
	R	CTTCCCCGTCAAGGCATTG
PCSK6_HUMAN	F	GCTGCCGGTCGGAAATGAA
	R	GTCGTAGCTGGCGTAGGAAT
AMRP_HUMAN	F	CGGAGGGTCAGGTCGTTTC
	R	CAACTTCTCCATGCGGAACTC
MFGM_HUMAN	F	CCTGCCACAACGGTGGTTTAT
	R	CACATTTCGTCTCACAGTGGTT
EMIL3_HUMAN	F	GGGGATGAGCTTACGAGGC
	R	ATGTCCAACCTCAGACAGCAT
BASI_HUMAN	F	GAAGTCGTCAGAACACATCAACG
	R	TTCCGGCGCTTCTCGTAGA
RENR_HUMAN	F	AAATTGGCCTATACCAGGAGAGC
	R	ATGAAACAGGTTACCCACTGC
HLAC_HUMAN	F	CCATGAGGTATTTGTGGACCG
	R	TCTCGGACTCTCGTCGTCG
CO6A1_HUMAN	F	ACAGTGACGAGGTGGAGATCA
	R	GATAGCGCAGTCGGTGTAGG
SELN_HUMAN	F	CCTGACCCTAGCGAGGAGAC
	R	GGCTGTCCAGTTTCGGAGG
CC50A_HUMAN	F	CAAAACCATCGTCGTTACGTGA
	R	GTTGGCAATAGCTCCACAAGG

Supplementary Notes 3

Antibody information for WB:

Name	Catalog	Company	Dilution
V5	ab27671	abcam	1:1000
High Sensitivity Streptavidin-HRP	21130	Pierce	1:6000
CALX	ab22595	abcam	1:10000
TOMM20	ab186734	abcam	1:1000
BiP	3177S	Cell Signaling Technology	1:1000
HSP60	ab190828	abcam	1:5000
Anti-Mouse	7076S	Cell Signaling Technology	1:5000
Anti-Rabbit	7074S	Cell Signaling Technology	1:5000

Antibody information for IF:

Name	Catalog	Company	Dilution
V5	ab27671	abcam	1:1000
Streptavidin Alexa Fluor™ 568 conjugate	S11226	Invitrogen	1:1000
Streptavidin PE Conjugate	12-4317-87	eBioscience	1:500
CALX	ab22595	abcam	1:500
TOMM20	ab186734	abcam	1:500
Anti-Mouse Alexa Fluor™ 488	A-11017	Invitrogen	1:500
Anti-Rabbit Alexa Fluor™ 633	A-21072	Invitrogen	1:500
DAPI	D1306	Invitrogen	1:5000 (5mg/mL stock)