SUPPORTING INFORMATION

Metal organic layers enabled cell surface engineering coupling biomembrane fusion for dynamic membrane proteome profiling

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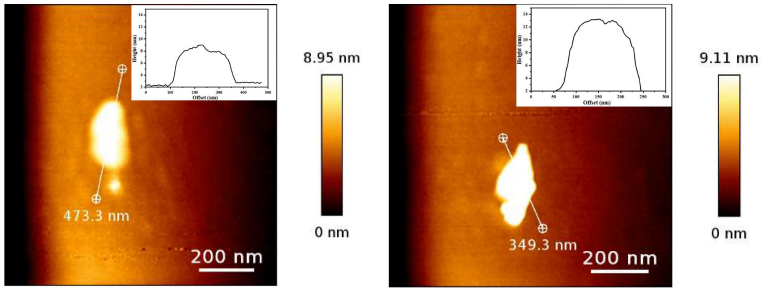

Figure S1. Tapping-mode AFM-topographic images (left) with its height profiles (right) showing thickness of the MOL-DpaZn material.

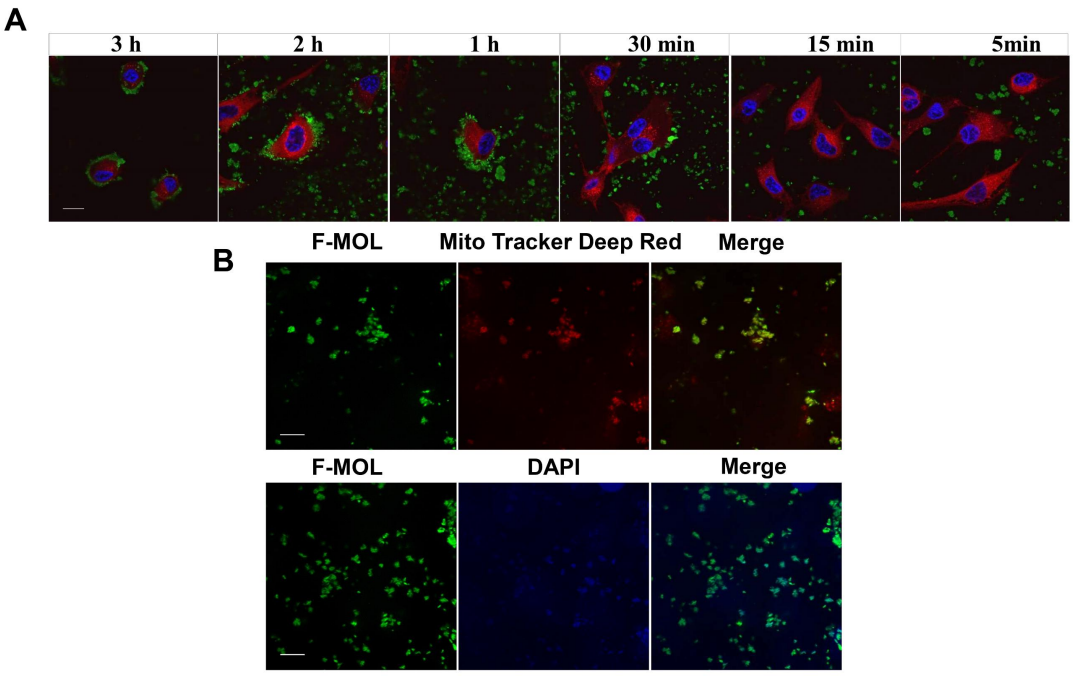

Figure S2. Confocal microscopic images showing the cell surface engineering coupling biomembrane fusion process. (A) Cell surface engineering of living HeLa cells: Cells were sequentially incubated with Hoechest 33342 (5 μg/mL, 30 min), DiI (10 μg/mL, 25 min) and F- MOL (0.1 mg/mL at different times). Scale bar: 10 μm. (B) The co-localization of F-MOL with nuclear and mitochondrial dye after mild lysis of pre-engineered HeLa cells might indicat appearance of biomembrane fusion. Scale bar: 10 μm.

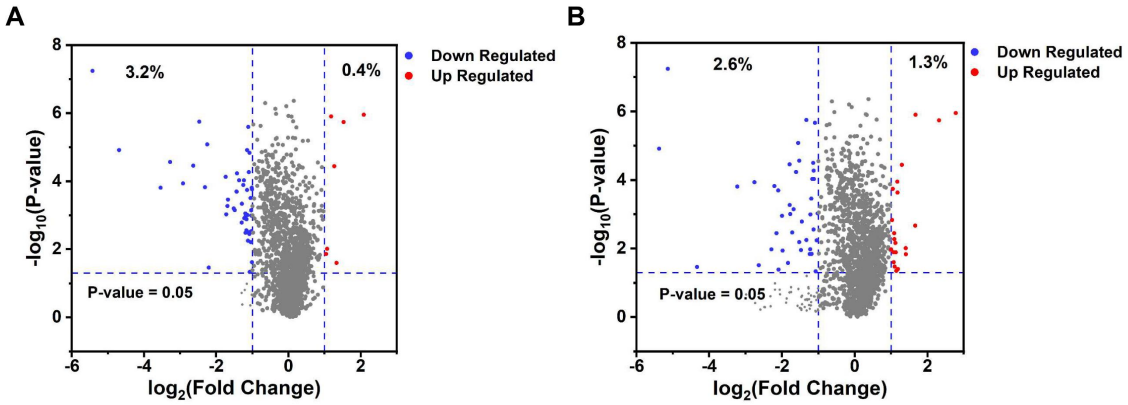

Figure S3. (A) Volcano plot of differential proteins after MOL-DpaZn treatment (0.1 mg/mL, 3 h). (B) Volcano plot of differential proteins after MOL-DpaZn treatment (0.1 mg/mL, 7 h).

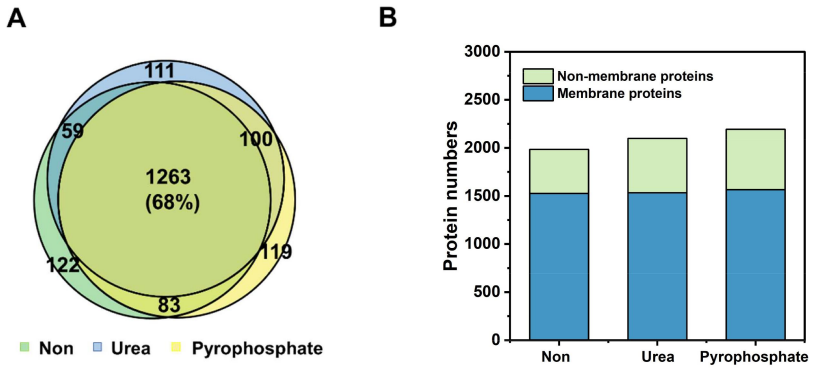

Figure S4. (A) Overlap of the identified membrane proteins in three groups, respectively. (B) Bar graphs indicated the number of membrane proteins identified in three groups, respectively. “Pyrophosphate” represented the enrichment of membrane proteins with the addition of 200 mM pyrophosphate in extraction buffer. “Urea” represented the enrichment of membrane proteins with an extra washing procedure towards MOL-DpaZn/biomembranes system using 1.0 M urea. “Non” represented the enrichment of membrane proteins without the above treatment, as the control group.

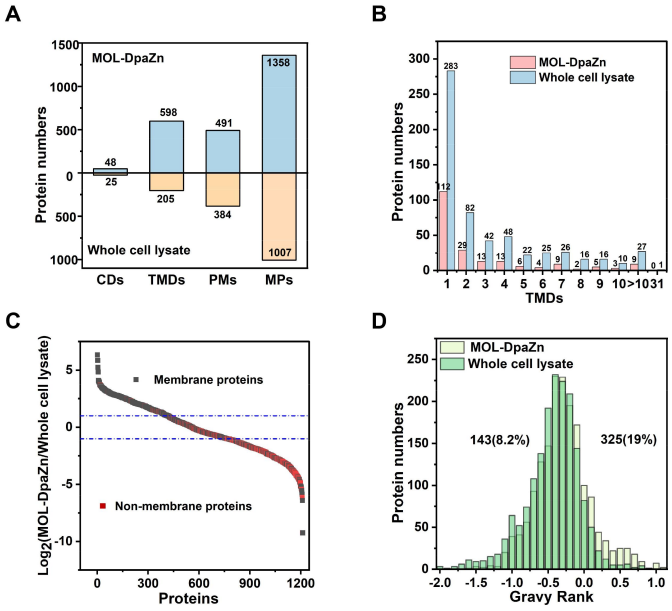

Figure S5. Comparison of membrane proteome data from whole cell lysate and E-fusion enriched sample (MOL-DpaZn). (A) Bar graphs indicated the identified numbers of proteins per LC-MS/MS run. (B) Comparison of TMD proteins predicted by TMHMM from whole cell lysate and MOL-DpaZn. (C) Ratio distribution of the identified proteins comparing the MOL-DpaZn sample with whole cell lysate. The intensity ratio was obtained from label-free MS quantitative results. (D) Evaluation of the protein hydrophobicity by GRAVY distribution. CDs: CD antigens; TMDs: TMD proteins; PMs: plasma membrane proteins; MPs: membrane proteins.

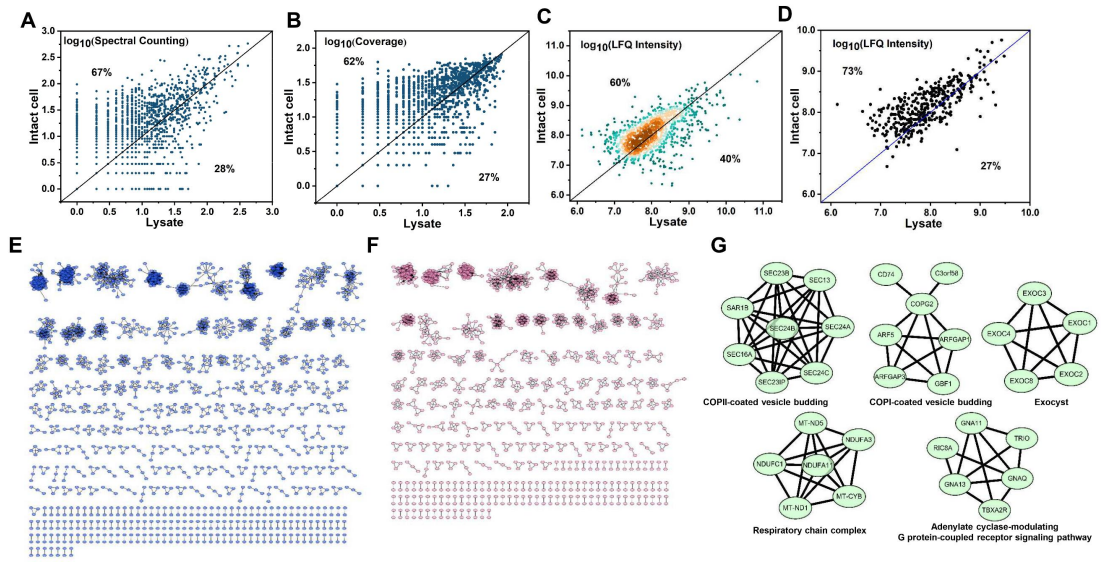

Figure S6. Enrichment in intact cells (E-fusion treatment) improves the quality of membrane protein identification and keeps more PPI protein-protein interactions (PPIs). Comparison of (A) spectral counting and (B) sequence coverage of the membrane proteins identified in both intact cell and lysate samples with three LC-MS/MS runs. Scatter plots indicated the LFQ intensity of the membrane proteins (C) and TMD proteins (D) identified in both intact cell and lysate samples. PPI networks obtained in intact cell sample (E) and lysate sample (F). (G) Membrane protein complexes obtained from the unique proteins identified in intact cell sample compared to the data in lysate. The clusters were visualized using Cytoscape software, version 3.7.1. The network was imported with the "confidence (score) cutoff" setting as 0.70 and "maximum additional interactors" setting as 0 by default. Clusters were obtained through "MCL cluster" APP with the inflation parameter setting as 6. Protein molecules were shown as nodes, and the edges indicated the interaction between proteins.

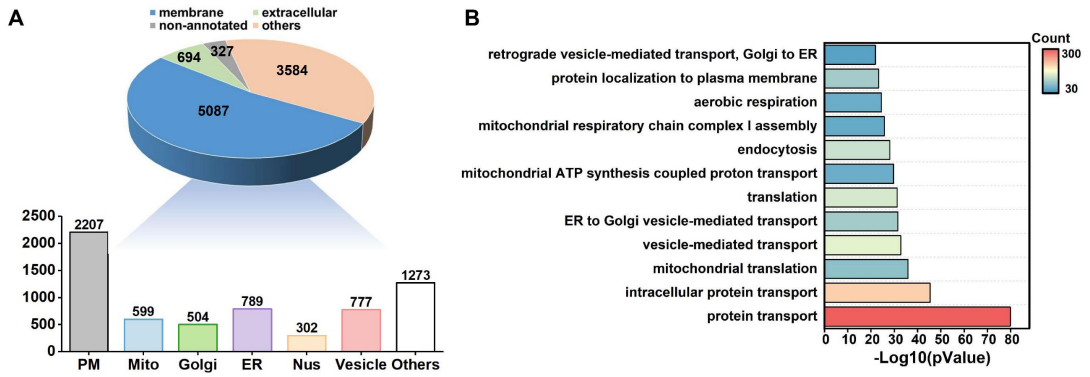

Figure S7. Deep coverage analysis of HeLa cell membrane proteome. (A) Distribution of the identified proteins. (B) Biological processes in GO enrichment analysis for the identified 5,087 membrane proteins.

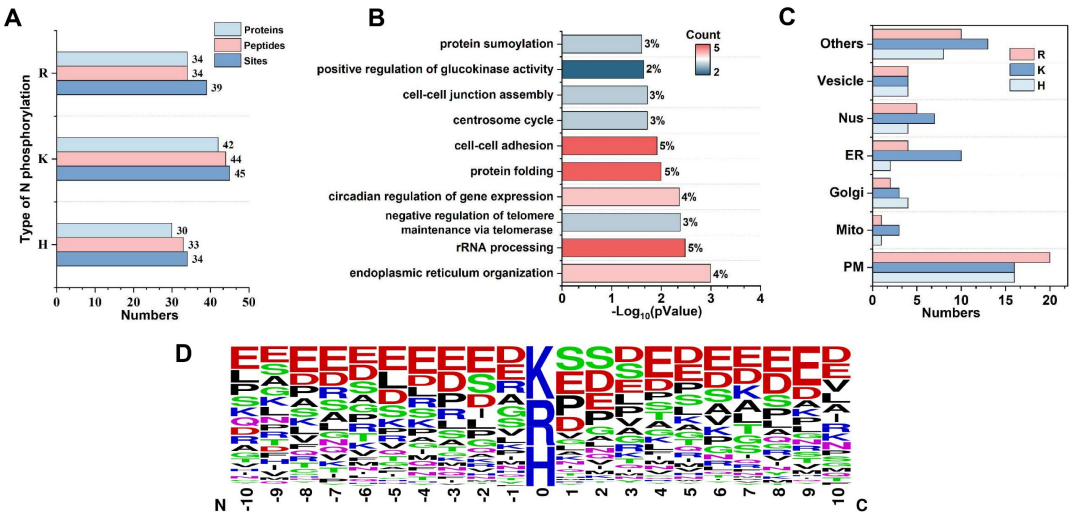

Figure S8. Membrane N-phosphoproteome profiling with three LC-MS/MS runs. (A) The number of identified N-phosphorylation membrane proteins, peptides and sites. (B) GO analysis results of N-phosphorylated membrane proteins. (C) Distribution of the identified sites. (D) Sequence motif analysis of identified N-phosphorylation sites using WebLogo.

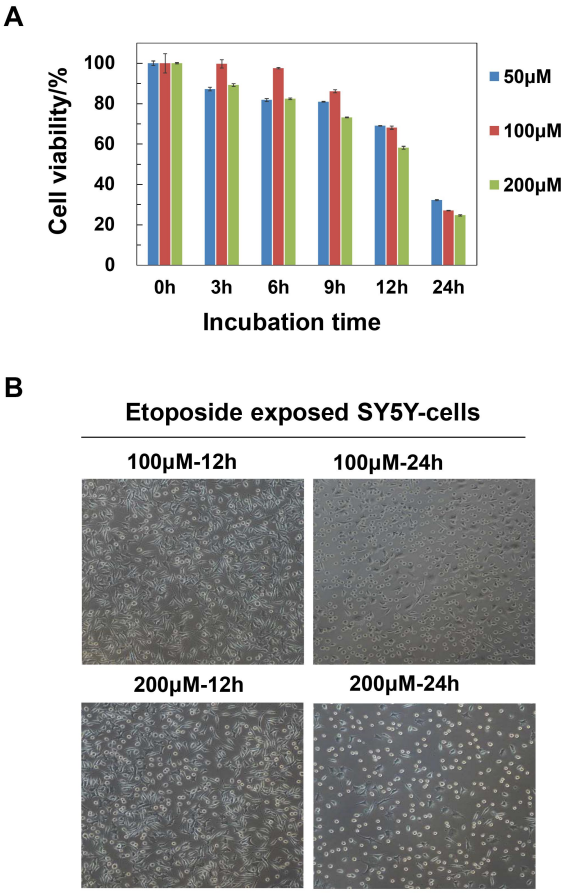

Figure S9. Apoptosis of SY5Y cells induced by etoposide. (A) Cytotoxicity tests of etoposide in SY5Y cells under different incubation conditions. (B) The morphological changes observed by microscopy on SY5Y cells treated with 100 μM and 200 μM etoposide for 12 and 24 h, respectively.

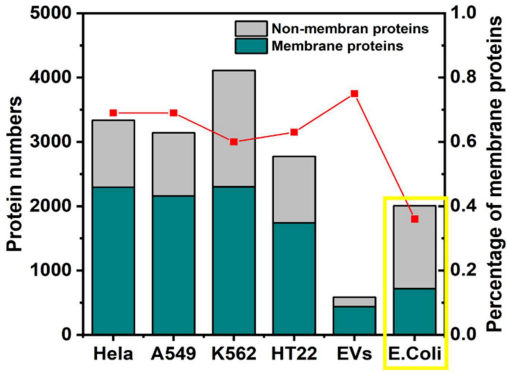

Figure S10. Bar graphs indicated the poor enrichment of membrane proteins in *E.Coli* and successful enrichment in extracellular vesicles (EVs) using E-fusion method.

Table S1. Comparison of identified membrane proteome from HeLa cells by E-fusion and kit method.

Method	MPs	TMD proteins	PPIs	Extraction yields
Kit	2392-67%	794	1585 nodes with 5598 PPIs	~150 μg
E-fusion	2357-67%	866	1578 nodes with 6754 PPIs	~300 μg

Experimental methods

Synthesis and characterization of MOL-DpaZn

(1) Reagents and Characterization

General chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), Tokyo Chemical Industry (Tokyo, JPN) and Aladdin (Shanghai, China) and were used without purification. Atomic force microscope images were collected on a NanoWizar (JPK, Germany) instrument. Electron microscopy images were obtained on a JSM-7800F scanning electron microscopy and JEM-2100 transmission electron microscopy. Zeta potential was measured using the Nano-ZS90 zetasizer (Malvern, UK) and contact angle was measured by DSA100 (Krüss, Hamburg, Germany). X-ray photoelectron spectroscopy (XPS) data was collected by a Thermo ESCALAB250Xi spectrometer with Al Kα radiation as the X-ray source (Thermo, Waltham, USA).

(2) Synthesis of two-dimensional (2D) Metal-Organic Layer $Zr_6O_4(OH)_4(BTB)_2(OH)_6(H_2O)_6$

The synthetic protocol was based on the reported literature[1]. To a solution of DMF (2.05 mL), HCOOH (0.88 mL) and H_2O (0.3 mL) was added a mixture of $ZrCl_4$ (14 mg) and H_3BTB (12.5 mg). White precipitates were collected by centrifugation after incubating at 120 °C for 48 h. The final washed MOL was dispersed in water for further functionalization.

(3) Synthesis of functionalized two-dimensional (2D) Metal-Organic Layer (MOL-DpaZn)

The DpaZn was synthesized according to a previous literature[2]. To functionalize the MOL with DpaZn, 10 mg of DpaZn was added into 5mL of ultrasonically dispersed MOL aqueous solution (6 mg/mL, 30 mg). The mixture, after another one-minute ultrasonic treatment, was incubated at room temperature overnight with end-over-end mixing. The yellow precipitate was re-dispersed in water for further usage after washing repeatedly by centrifugation in water. The material dispersion requires extensively ultrasonic treatment for about 5 min prior to each use.

(4) Fluorescent functionalization of MOL

To labeling the MOL with fluorescence, a solution of MOL (5 mg) in DMF (5 mL) was incubated with 20 mg of 5(6)-carboxyfluorescein at 85 °C for 24 h. The mixture is then repeatedly washed in DMF and water by centrifugation. The final MOL functionalized with 5(6)-carboxyfluorescein (F-MOL) was re-dispersed in water for further usage.

(5) Fluorescent confocal imaging of MOL/Hela engineering system

Living HeLa cells grown in 35-mm Petri dishes were sequentially incubated with Hoechst 33342 (5 μg/μL, 30 min, Beyotime, China), DiI (10 μg/μL, 25 min, Beyotime, China) and F-MOL (different concentrations and times) at 70-80% confluence. Cells were washed gently 3 times with PBS buffer to remove unbound dye after each incubation. Confocal fluorescent imaging was performed separately in 350 nm, 550 nm and 488 nm channels using a two-photon confocal microscope system (FV1000MPE).

(6) Fluorescent confocal imaging of MOL-DpaZn/biomembranes system

Living HeLa cells grown in 6-cm dishes were incubated with DAPI (1 μg/mL, 30 min, Beyotime, China) or Mito Tracker Deep Red (100 nM, 25 min, Thermo, USA), and then incubated with F-MOL (0.1 mg/mL, 3 h) at 70-80% confluence. Cells were washed gently 3 times with PBS buffer to remove unbound dye after each incubation. Then, the wrapping system was harvested by mild scraping and transferred into a 2 mL protein low-binding tube, followed by three more spin washes, each wash with 1 mL cold PBS at 500 g for 5 min. Subsequently, the obtained wrapping system was resuspended in 1.5 mL of ice-cold hypotonic buffer (10 mM NaCl, 10 mM Tris base, 1.5 mM $MgCl_2$, 1% cocktail (v/v), pH=7.4), and incubated on ice for 5min to improve the swelling of the cells. Three freeze-thaw treatments were performed for mild cell disruption. After centrifugation (1,000 g, 3 min), the obtained cell membrane sheets were suspended in PBS for following imaging. Confocal fluorescent imaging was performed separately in 350 nm (DAPI), 488 nm (F-MOL) and 640 nm (Mito Tracker Deep Red) channels using a two-photon confocal microscope system (FV1000MPE).

Cell and *E. coli* culture

HeLa, A549, HT22, K562 and SH-SY5Y cell lines were acquired from the cell bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (CTCC, Shanghai, China). HeLa, HT22 and SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and penicillin (100 U/mL)/streptomycin (100 μg/mL) (Gibco), while K562 and A549 cells were grown in RPMI-1640 media (Gibco) with 10% (v/v) fetal bovine serum and penicillin (100 U/mL)/streptomycin (100 μg/mL) (Gibco). All cells were maintained in a humidified 5% CO_2 incubator at 37 °C.

E. coli (strain K12) was cultured in Luria-Bertani medium (LB, 5 g/L yeast extracts, 10 g/L NaCl, and 10 g/L tryptone) at 37 °C overnight.

Isolation of extracellular vesicles (EVs)

To isolate vesicles secreted from SH-SY5Y cells, conditioned media (CM) were collected. After collection of 200 mL conditioned media, cell debris and large membrane particles were removed by sequential centrifugation at 500g for 10 min and then 4,000 g for 20 min. Then, the CM was filtered through a 0.22 μm syringe filter (Millipore) to remove large vesicles. Extracellular vesicles were collected after ultracentrifugation at 100,000 g for 1.5 h and then resuspended in PBS for further experiments.

Cytotoxicity and Proteomic perturbations analysis of E-fusion method

For cytotoxicity analysis, a CCK8 assay was performed according to the manufacturer’s protocol (C0037, Beyotime, China). HeLa cells (8,000 cells per 100 μL of medium) were plated in 96 well plates. After culturing for one day, cells were treated with MOL-DpaZn at different concentrations and further incubated for different times, followed by the addition of WST-8 (CCK-8 working solvent, 10 μL per well). After 4h incubation, the final OD value was measured at 450 nm through a microplate reader (Thermo Scientific Multiskan Go).

For proteomic perturbations analysis, the HeLa cells in DMEM at approximately

80% confluence were treated with/without MOL-DpaZn dispersion (0.1 mg/mL), and then continued to cultivate for 3 h, followed by washing with ice-cold PBS three times. Next, the cells were harvested by scraping, broken by sonication in 1% SDS/PBS (w/v) with 1% cocktail (v/v) and subjected to centrifugation (16,000 g, 10 min) to obtain the protein supernatant. The whole-cell proteins were then reduced, alkylated and digested using the FASP protocol. The lyophilized peptides were analyzed by a Dionex Ultimate 3000 RSLC nano LC system coupled to an LTQ Orbitrap Velos mass spectrometer with three technical replicates. The MS data were searched by MaxQuant (version 1.6.17) against the Uniprot_Homo Sapiens Swiss-Prot database (updated on 2022/01/06) in label-free mode. The proteins with fold change exceeded 2 were filtered out to be significantly differential proteins.

Optimization of membrane protein enrichment workflow

For optimization of incubation time, 0.1 mg/mL MOL-DpaZn materials dispersed in cell medium were incubated with HeLa cells for 0.5, 1, 3 h, respectively. Then cells were collected for subsequently extraction of membrane proteins and LC-MS/MS analysis.

To increase the extraction efficiency of membrane proteins, several cell lysis methods were performed as follows: (1) For strong sonication processing, the wrapped cells, resuspended in 1.5 mL of hypotonic buffer with 1% cocktail (v/v), were sonicated at 80 watts for 7 min (working for 5 s and pausing for 10 s). (2) For mild sonication processing, the wrapped cells, resuspended in 1.5 mL of hypotonic buffer with 1% cocktail (v/v), were sonicated at 20 watts for 2 min (working for 5 s and pausing for 10 s). (3) For freeze-thaw treatments, the wrapped cells, resuspended in 1.5 mL of hypotonic buffer with 1% cocktail (v/v), were frozen at -80 ℃ for 40 min and thawed at room temperature. Three cycles were performed. (4) For trypsin treatment, the wrapped cells were lysed in 1.5 mL of hypotonic buffer containing 80 μg of trypsin for 0.5 h at room temperature. (5) For homogenization processing, the wrapped cells, resuspended in 1.5 mL of hypotonic buffer with 1% cocktail (v/v), were transferred into a 1 mL tg tube dounce (Central Infusion Alliance, Kimble Chase

CSX8853030001, Mexico), and subjected to homogenization for 10 times on ice. HeLa cells incubated with 0.1 mg/mL MOL-DpaZn materials for 3 h were lysed through the above methods, respectively, and processed for subsequent LC-MS/MS analysis. Throughout the optimization process, the obtained peptides were analyzed by a Dionex Ultimate 3000 RSLC nano LC system coupled to an LTQ Orbitrap Velos mass spectrometer with three technical replicates. MS data were analyzed using the Mascot search engine in Proteome Discoverer (PD) software (Version2.5.0.400, Thermo Fisher Scientific) against the UniProt Homo Sapiens Swiss-Prot database (downloaded on January 07, 2022).

Identification of membrane proteome in HeLa cells

(1) Cell surface engineering of living HeLa cells using MOL-DpaZn materials

The HeLa cells in DMEM at 80% approximately confluence were co-incubated with MOL-DpaZn (0.1 mg/mL). After another 3-hour cultivation, three washes were performed with cold PBS to remove the excess MOL-DpaZn. The wrapping system was harvested by mild scraping and transferred into a 2 mL protein low-binding tube, followed by three more spin washes, each wash with 1 mL cold PBS at 500 g for 5 min. Subsequently, the obtained wrapping system was stored at -80℃ until further processing.

(2) Extraction of membrane proteins of MOL-DpaZn/HeLa system

The wrapped cells were resuspended in 1.5 mL of ice-cold hypotonic buffer, and incubated on ice for 5 min to improve the swelling of the cells. Three freeze-thaw treatments were performed for mild cell disruption. The cell membrane sheets were obtained after centrifugation (1,000g, 3min), while the supernatant was collected into a new 2 mL protein low-binding tube for another centrifugation (1,000g, 3min) to increase the recovery. The obtained membrane sheets wrapped by MOL-DpaZn were then sequentially washed three times with 1 mL of 1 M NaCl, three times with 1 mL of 0.1 M Na_2CO_3 (pH 11.5), once with 1 mL of 500 mM NH_4HCO_3 and once with 1 mL of 50 mM NH_4HCO_3. For membrane protein extraction, the washed wrapping system was resuspended in 100 μL of the ionic liquid extraction buffer

(1-dodecyl-3-methylimidazolium chloride (C12Im-Cl) buffer (10%, m/v)), sonicated for 10 min at 4℃ (working for 30 s and pausing for 30 s), and then centrifuged at 16,000 g for 5 min at 4℃. The extraction procedure was repeated three times for complete extraction. The combined 300 μL supernatant, containing membrane and membrane associated proteins, was carefully transferred to a new tube. After a BCA assay ((P0012, Beyotime, China)) for protein concentration, a certain amount of protein solution (~100 μg) was mixed with DTT (100 mM), boiled at 65 ℃ for 30 min, followed by FASP proteolytic digestion for LC-MS/MS analysis.

(3) Nano LC-MS/MS analysis

The digested peptides resuspended in 0.1% FA/H_2O were separated by the nano reversed-phase liquid chromatography (RPLC) on a 15 cm C18 capillary column packed in-house with ReproSil-Pur C18-AQ particles (1.9 μm, 120 Å), and analyzed using the LTQ Orbitrap Velos mass spectrometer coupled on-line to the Dionex Ultimate 3000 RSLC nano LC system. About 2 μg of peptides per sample were loaded onto the capillary column with 98% mobile phase A (2% ACN, 0.1% FA), 2% B (98% ACN, 0.1% FA) and eluted at a constant flow rate of 600 nL/min with the gradient of 0 min (2% B)-10 min (2% B) -10.1 min (6% B) -70 min (22% B) -82 min (40% B) -85min (95% B) -88 min (95% B) -88.1 min (2% B)-90 min (2% B). The MS parameters are shown as follows. The capillary temperature was maintained at 320 °C and the ion spray voltage was set as 2.3 kV. The LTQ Orbitrap Velos mass spectrometer was operated in positive ion mode and all MS/MS spectra were acquired in data-dependent mode. Full MS scanning ranging from m/z 350 to 1,500 with a mass resolution of 60,000 was performed in the Orbitrap mass analyzer for MS1 analysis at an AGC target of 1e6. The 20 most intense ions were fragmented, and tandem mass spectra were acquired with a scan rate normal mode. The dynamic exclusion time was 22 s, and the maximum allowed ion accumulation time was 500 ms for MS scans and 100 ms for MS/MS.

(4) Database search

Collected data were analyzed using the Mascot search engine in Proteome Discoverer (PD) software (Version2.5.0.400, Thermo Fisher Scientific). The MS

instrument mode was set as CID-ITMS. The precursor and fragment mass tolerances were set to 10 ppm and 0.5 Da, respectively. Peptides were fully tryptic with a maximum of two missed cleavage sites, oxidation (M) and acetyl (Protein N-term) were set as dynamic modification, carbamidomethyl (C) was set as the static modification. All extracted spectra were searched against the UniProt Homo Sapiens Swiss-Prot database (downloaded on January 07, 2022). The false discovery rate was controlled < 1% to filter peptides and proteins. Other settings were the same default as the conventional search. All data were combined from three technical replicates. Keratins were first removed from the data. A protein was defined as "membrane protein" when it was annotated as "membrane" in Gene Ontology cellular components or predicted to possess at least one transmembrane helix domain according to TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). We assigned membrane proteins to different organelle membranes according to GO cellular component annotation. Plasma membrane (PM) proteins contain "plasma membrane" or "cell surface". Endoplasmic reticulum membrane (ER) proteins contain "endoplasmic reticulum tubular network membrane" or "endoplasmic reticulum membrane" or "endoplasmic reticulum-Golgi intermediate compartment". Golgi membrane (Golgi) proteins contain "Golgi membrane" or "endoplasmic reticulum-Golgi intermediate compartment". Mitochondrial membrane (Mito) proteins contain "mitochondrial membrane" or "mitochondrial inner/outer membrane". Nuclear membrane (Nus) proteins contain "nuclear membrane/envelope" or "nuclear inner/outer membrane". Vesicle associated membrane (Vesicle) proteins contain "lysosomal/peroxisomal/endosome/vesicle membrane". Membrane proteins without the above annotations were classified as others.

Comparison of membrane proteome identified in MOL-DpaZn incubated lysate and intact cell samples

To obtain the MOL-DpaZn wrapped cell sample, 2 mg (0.1 mg/mL) of MOL-DpaZn materials were incubated with HeLa cells seeded in a 15-cm culturing dishe for 3 h, followed by three freeze-thaw treatments. To obtain the MOL-DpaZn

incubated lysate sample, 2 mg of MOL-DpaZn materials were incubated with HeLa cell lysate from ultrasonication for 30 min at room temperature with end-to-end rotation. The two samples were then subjected to the same centrifugation, washing and extraction processes as described above to obtain membrane proteins. After reduction, alkylation and digestion, the obtained peptides were analyzed by an EASY-nLC 1000 system (Thermo, USA) coupled to a Q-Exactive mass spectrometer (Thermo, USA) with three technical replicates. MS data were analyzed using the Mascot search engine in Proteome Discoverer (PD) software (Version2.5.0.400, Thermo Fisher Scientific) against the UniProt Homo Sapiens Swiss-Prot database (downloaded on January 07, 2022).

Reproducibility and universality of the membrane protein identification

(1) Membrane proteome analysis of different biological samples using E-fusion method

For the analysis of A549, HT22 and K562, the membrane protein enrichment processes were consistent with the above. For EVs, 0.1 mg/mL MOL-DpaZn material was incubated with EVs in HEPES buffer for 3 h at 4 ℃ with end-to-end rotation. For Escherichia coli, 0.1 mg/mL materials were incubated with the bacterial (OD_{600}=0.5) in LB medium for 1 h on the shaker at 37 ℃ with a speed of 200 rpm. After incubation, MOL-DpaZn wrapped EVs and *E. Coli* were obtained through centrifugation, and were then subjected to the same centrifugation, washing and extraction processes as described above to obtain membrane proteins. After digestion, the digested peptides were analyzed using the same LC-MS/MS system and search engine as the above comparison experiments.

(2) Membrane proteome analysis using different batches of MOL-DpaZn materials

HeLa cells were co-incubated with three batches of MOL-DpaZn materials which were prepared in different months, respectively, for membrane proteome analysis. The detailed membrane protein enrichment processes were consistent with the above. Finally, the digested peptides were analyzed using the same LC-MS/MS

system and search engine as the above comparison experiments.

Deep coverage analysis of HeLa membrane proteome

(1) Hp-RP Stage Tip fractionation of peptides

To increase the depth of proteome coverage, the membrane proteins extracted from MOL-DpaZn were further fractionated by a high pH RP-C18 column (Durashell, 5 μm, 100 Å, 2.3 × 150 mm i.d.) at a flow rate of 0.3 mL/min. The mobile phase A consisted of 2% ACN with 10 mM ammonium acetate, mobile phase B consisted of 80% ACN with 10mM ammonium acetate, and both were adjusted to pH 10.0 with 28% $NH_3 \cdot H_2O$. An 80min-seperation gradient (70min from 0 to 45% B, 5 min from 45 to 100% B, and maintain at 100% B for 5 min) was applied. Fractions were collected in one-minute increments and mixed into total 10 fractions at equal intervals. The 10 fractions were lyophilized and resolved in 0.1% FA/H_2O for LC-MS/MS analysis.

(2) Nano LC-MS/MS analysis

The digested and fractionated peptides were analyzed by LC-FAIMS-MS using an EasyNano 1200 LC system (Thermo Fisher Scientific, Waltham, MA, USA) with a high-field asymmetric waveform ion mobility spectrometry system (FAMIS, Thermo, USA) (-45 V and -65 V compensation voltage), and followed by tandem MS, an Orbitrap Exploris™ 480 mass spectrometer (Thermo, USA). Peptides were resuspended in 0.1% FA/H_2O and about 1 μg were loaded onto a 15 cm C18 capillary column packed in-house with Venusil XBP C18 silica particles (1.9 μm, 100 Å). The HPLC solvent A was 2% ACN, 0.1% FA, and the solvent B was 98% ACN, 0.1% FA. Fractionated samples were analyzed using a 65 min LC-MS/MS method with the following gradient: 0 min (12% B)-45 min (30% B) -51 min (38% B) -55 min (95% B) -59 min (95% B) -65min (95% B), and were eluted at a constant flow rate of 600 nL/min.

The MS parameters are shown as follows. The compensation voltages of FAIMS were set as -45 V and -65 V. The capillary temperature was maintained at 320 °C and the ion spray voltage was set as 2,250 V. Full MS scanning ranging from m/z 350 to

1,500 with a mass resolution of 60,000 was performed for MS1 analysis in positive ion mode. MS/MS spectra were acquired in data-dependent mode, and total cycle time was 2 s. The 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 tandem mass spectra were acquired with a mass resolution of 15,000 at m/z 200. The isolation window (m/z) was 1.6 and the dynamic exclusion time was 40 s. Data search was using the Mascot search engine in Proteome Discoverer (PD) software, the parameter settings were the same as above except that the precursor and fragment mass tolerances were set to 10 ppm and 20 mmu, respectively and the MS instrument mode was set as HCD-FTMS.

Profiling of membrane N-phosphoproteome

(1) Enrichment of membrane N-phosphopeptides

The obtained membrane proteins using E-fusion method from a 15-cm HeLa cell culturing dish were dissolved in 8 M urea (20 mM HEPES, containing 1% (v/v) cocktail, 1% (v/v) phosphatase). Then proteins were denatured in 25 mM DTT for 60 min at 37°C. Alkylation was performed in 50 mM IAA for 30 min at room temperature in the dark. The membrane protein solution was diluted to 1 M urea and subsequently added with trypsin (1:3, m:m) for digestion at 37 °C for 1.5 h to obtain peptides.

Activated SiO_2@DpaZn (~5 mg) was reconstituted with acetonitrile, then added to peptides (v:v=1:1). For direct enrichment, the final concentration of urea is 0.5 M (50% ACN). The mixture was shaken at 1,500 rpm for 1.5 hour at 4 °C. Washing steps were carried out with 200 μL of 50% ACN, 80% ACN, 30% ACN, 0.001% $NH_3 \cdot H_2O$, respectively. The phosphopeptides were eluted with 50 μL of 0.1% $NH_3 \cdot H_2O$. The N-phosphopeptides were centrifuged at 16,000 g for 30 min at 4°C

(2) Nano LC-MS/MS analysis

Mass spectrometry method was used as previously described[3]. Briefly, the HPLC solvent A was water, 0.1% FA, and the solvent B was 80% ACN, 0.1% FA. A 121 min gradient: 0 min (6% B)-90 min (29% B) -110 min (50% B) -111 min (95% B) -121

min (95% B), with the flow rate of 600 nL/min was used. An Orbitrap Fusion Lumos mass spectrometer (Thermo-Fisher, San Jose, CA, USA) was operated. The electro-spray voltage was 2.5 kV, and the heated capillary temperature was 320 °C. MS/MS spectra were acquired by data-dependent acquisition mode, and total cycle time was set to 3 s. Peptides with charge state ≥ 2 were selected for sequencing with HCD (collision energy 32%, max injection time 35 ms) and neutral-loss-triggered (Δ97.98) EThcD (ETD reaction time 50 ms, max ETD reagent injection time 200 ms, supplemental activation energy 25%, max injection time 35 ms) for fragmentation.

(3) Database search.

The MS data was searched by MaxQuant (version 1.6.3.3) against the Uniprot Homo Sapiens Swiss-Prot database (updated on 2023/03/20). The parameters of database searching included: up to three missed cleavages allowed for full tryptic digestion, precursor ion mass tolerance 10 ppm, product ion mass tolerance 20 mmu, carbamidomethylation (C) as a fixed modification, and oxidation (M), acetyl (protein N-term), phosphorylation (HRK) as variable modifications. The false discovery rate was controlled < 1% to filter peptides and proteins. Other settings were the same default as the conventional search. Three independent technical replicates were performed together.

The dynamic membrane proteome during apoptosis

(1) Cytotoxicity tests of etoposide

SY5Y cells were seeded in 96-well plate for using after 24 h. The medium was replaced by fresh DMEM with different concentrations of etoposide (50, 100, 200 μM) and incubated for different times (3, 6, 9, 12, 24 h) respectively. Next, 10 μL of WST-8 (CCK-8 working solvent) was added to each well and incubated for 2 h and the absorbance in 450 nm was measured by microplate reader (Thermo Scientific Multiskan Go).

(2) Immunoblotting analysis of caspase-3 and Bax

SY5Y cells were seeded in 10-cm culturing dishes for using after 24 h. The medium was replaced by fresh EMEM with etoposide (100 μM) and incubated for

additional 0, 3, 6, 9, 12, 24 h respectively. The cells were harvested and lysed in 1% SDS/DPBS (w/v) with 1% cocktail (v/v) through ultrasonication. The protein concentration of the obtained cell lysate was determined using a BCA protein assay. Then proteins were boiled and loaded for SDS-PAGE (12.5% gel). Gels were transferred to PVDF membranes, followed by block with 5% (m/v) skimmed milk in TBST (0.1% Tween-20, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0) for 1 h at room temperature. Next, the membranes were incubated with anti-caspase-3 and anti-Bax antibody respectively at 4°C overnight. After three washes with TBST, the membranes were incubated with HRP-conjugated secondary antibody for 30 min at room temperature and then were analyzed by ChemiDoc XRS+ system (BIO-RAD).

(3) Digestion, dimethyl labeling and high pH-RP fractionation

The enrichment of membrane proteins was conducted as above. After reduction and alkylation through filter aided sample preparation (FASP) method, the extracted proteins were digested in phosphate buffer (100 mM, pH=8.0) with trypsin (1:30, trypsin: protein, w/w) in 37℃ overnight. The peptide solution collected by centrifugation was then treated with 18 μL 4% CH_2O or CD_2O (v/v) (Cambridge Isotope Laboratories, USA) and 18 μL 0.6 M $NaBH_3CN$ or $NaBD_3CN$ (Sigma-Aldrich, USA) for 1 h at room temperature with end-over-end rotation for pseudo-isobaric dimethyl labeling. (Control group (etoposide treated 0 h): $^{13}CH_2O$+ $NaBH_3CN$ (30L); Sample group 1 (etoposide treated 6 h): CH_2O+ $NaBD_3CN$ (30H); Sample group 2 (etoposide treated 18 h): $^{13}CH_2O$+ $NaBD_3CN$ (32L)). Then the labeled peptide solution was combined in a ratio of 1:1 (m/m) for the following desalination and high pH-RP fractionation. 30 mg of durashell C18 packing (5 μm, 100 Å) (Agela technologies, USA) was filled into a Gilson 200-μL pipet tip with soild phase extraction C18 disk (Sigma-Aldrich, USA) on top. For desaltination and separation, water with $NH_3 \cdot H_2O$ (pH=10) and 80% (v/v) ACN with $NH_3 \cdot H_2O$ (pH=10) were used as the mobile phase A and B, respectively. After washing the tip with mobile phase B and mobile phase A for three times, the mixed peptide solution was loaded onto the C18 tip. Then the C18 tip was washed with mobile phase A for three times and eluted with mobile phases containing different ratios of ACN (6%, 9%,

12%, 15%, 18, 21%, 25%, 30%, 80%) in turn by centrifugation in 500 g for 5-10 min. The eluents were lyophilized for NanoLC-MS/MS.

(4) Nano LC-MS/MS analysis

The digested and fractionated peptides were resuspended in 0.1% FA/H_2O and about 1 μg were loaded onto a 15 cm C18 capillary column packed in-house with Venusil XBP C18 silica particles (5 μm, 100 Å) and analyzed by an Orbitrap Fusion Lumos mass spectrometer (Thermo-Fisher, San Jose, CA, USA). The HPLC solvent A was water, 0.1% FA, and the solvent B was 80% ACN, 0.1% FA. An 85 min gradient: 0 min (5% B)-10 min (12% B) - 28 min (20% B) -58 min (32% B) -77 min (48% B)-78min (95%B)- 85min (95%B), with the flow rate of 600 nL/min was used. The capillary temperature was maintained at 320 °C and the ion spray voltage was set as 2100 V. Full MS scanning ranging from m/z 350 to 1,500 with a mass resolution of 60,000 was performed for MS1 analysis in positive ion mode. MS/MS spectra were acquired in data-dependent mode, and total cycle time was 3 s. The 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 tandem mass spectra were acquired with a mass resolution of 15,000. The isolation window (m/z) was 1.6 and the dynamic exclusion time was 40 s.

(5) Database search and quantification

The MS data was searched by MaxQuant (version 1.6.5.0) against the Uniprot_Homo Sapiens Swiss-Prot database (updated on 2023/03/20). Each independent biological replicate, including 9 fractions, was performed separately. The parameters of database searching included: up to three missed cleavages allowed for full tryptic digestion, precursor ion mass tolerance 10 ppm, product ion mass tolerance 20 ppm, carbamidomethylation (C) as a fixed modification, and oxidation (M), dimethyl labeling (+30.04385 Da and +32.0564 Da) at N-terminal/lysine with neutral loss of ±2.01256 Da for isotope modifications as variable modifications. The false discovery rate was controlled < 1% to filter peptides and proteins. Other settings were the same default as the conventional search. The searched results were further quantified by extracting the intensity of a1 ion from spectra with pQuant software[4].

The median of isotope abundance ratio of a1 ion for all peptides was taken as the final ratio of proteins. For each replicate, proteins identified as reverse hits and contaminants were removed and proteins with leading group type were retained for further processing. Then the protein H/L ratios were normalized by dividing the median H/L ratio of the remained proteins. Proteins identified and quantified by three biological replicates were retained as the final membrane proteome.

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