Electronic supplementary information

Identification of Skp1 as a target of mercury sulfide for neuroprotection

Mei-Mei Zhao[a,#], Lu-Di Li[b,#], Mi-Mi Yang[b,#], Lu Yao[a], Qi Wang[b,c,d,*], Ke-Wu Zeng[a,*]

[a] State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, 100191, China.

[b] Department of Toxicology, School of Public Health, Peking University, Beijing, 100191, China.

[c] Key Laboratory of State Administration of Traditional Chinese Medicine for Compatibility Toxicology, Beijing, 100191, China.

[d] Beijing Key Laboratory of Toxicological Research and Risk Assessment for Food Safety, Beijing, 100191, China.

[#] The authors contributed equally to this work.

Corresponding authors: ZKW@bjmu.edu.cn (K. Zeng), wangqi@bjmu.edu.cn (Q. Wang).

Table of Contents

Experimental methods

1. Target protein identification of HgS

1.1 Preparation of HgS molecular probe (bio-HgS)

The detailed synthesis process and NMR spectra was shown in Fig.S1 and Fig.S2, respectively.

Step 1: Thiomersal (6.0 g, 14.8 mM) in N, N-dimethylformamide (DMF, 120 mL) was added to a mixture of N-hydroxysuccinimide (1.9 g, 16.3 mM), 1-ethyl-(3-dimethylaminopropyl) carbamide diimide hydrochloride (EDC, 3.4 g, 17.7 mM) and triethylamine (Et_3N, 2.2 g, 22.2 mM). The mixture was stirred at room temperature (RT) overnight. The reaction was quenched by the addition of water, followed by extraction with ethyl acetate. Organic phase was washed by saturated salt and dried with anhydrous sodium sulfate. Then, the mixture was filtered and concentrated to obtain solid compound I (thiomersal active ester, 3.0 g, yield: 44.3%). The crude product was used in the next step directly.

Step 2: Biotin (5.0 g, 20.5 mM) in DMF (150 mL) was added to a mixture of N-hydroxysuccinimide (2.5 g, 22.1 mM) and EDC (4.7 g, 24.6 mM). The mixture was stirred overnight at RT. After removing the solvent by reduced pressure distillation and isopropyl alcohol recrystallization, the compound II (biotin active ester, 5.6 g, yield: 80%) was obtained.

Step 3: 3, 6, 9-trioxazundecyl-1, 11-diamine (1.8 g, 9.4 mM) was dissolved in tetrahydrofuran (THF, 60 mL), then biotin active ester (2.3 g, 6.7 mM) was added and stirred at RT for 1 h. The reaction liquid was spun dry to afford crude compound III (2.5 g).

Step 4: Compound III was dissolved in THF (50 mL), followed by addition of compound I (2.9 g, 6.0 mM). The mixture was stirred at RT for 3 h. The HgS molecular probe (1 g, yield: 21.7%) was separated by thin-layer chromatography (TLC; dichloromethane:methanol = 3:1, Rf = 0.6).

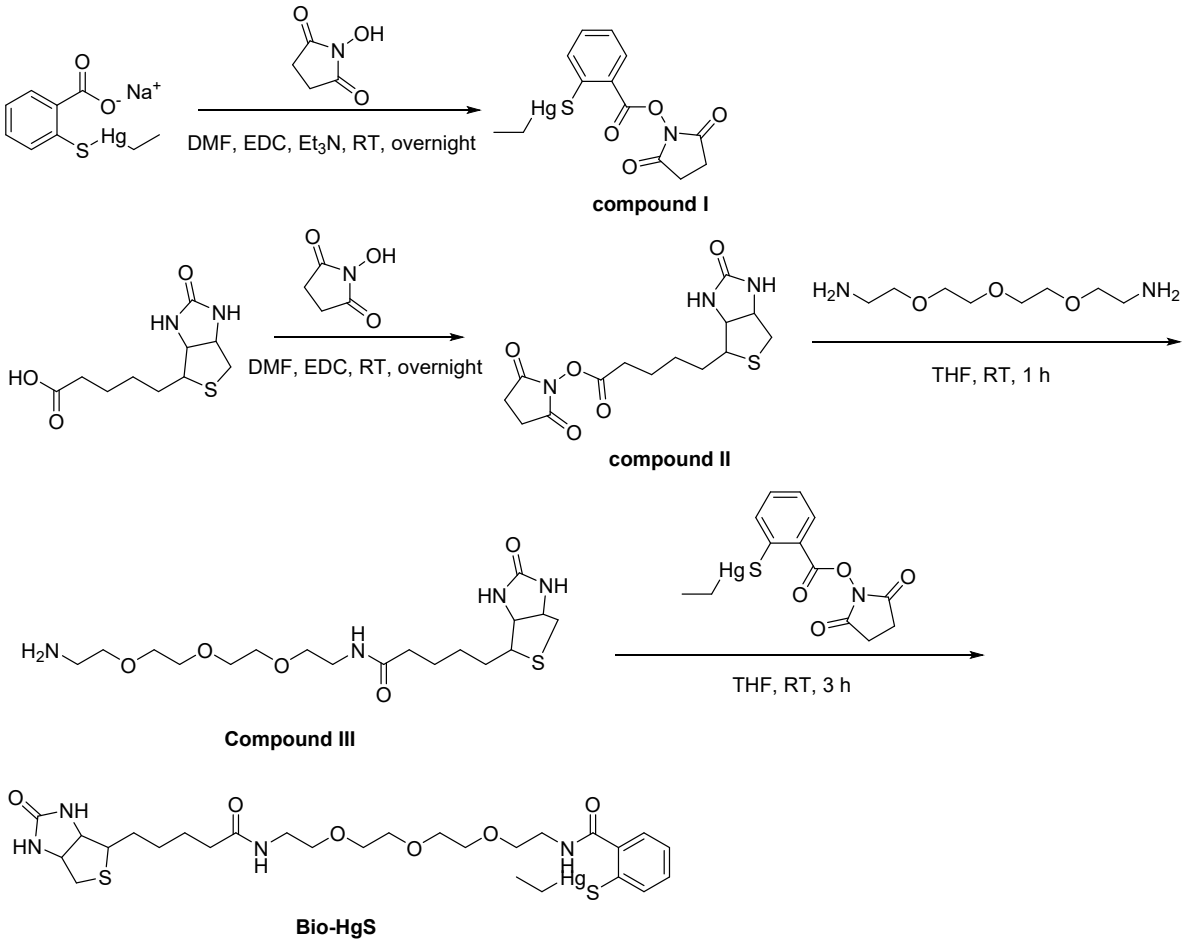


Fig. S1 Schematic synthesis route of bio-HgS.

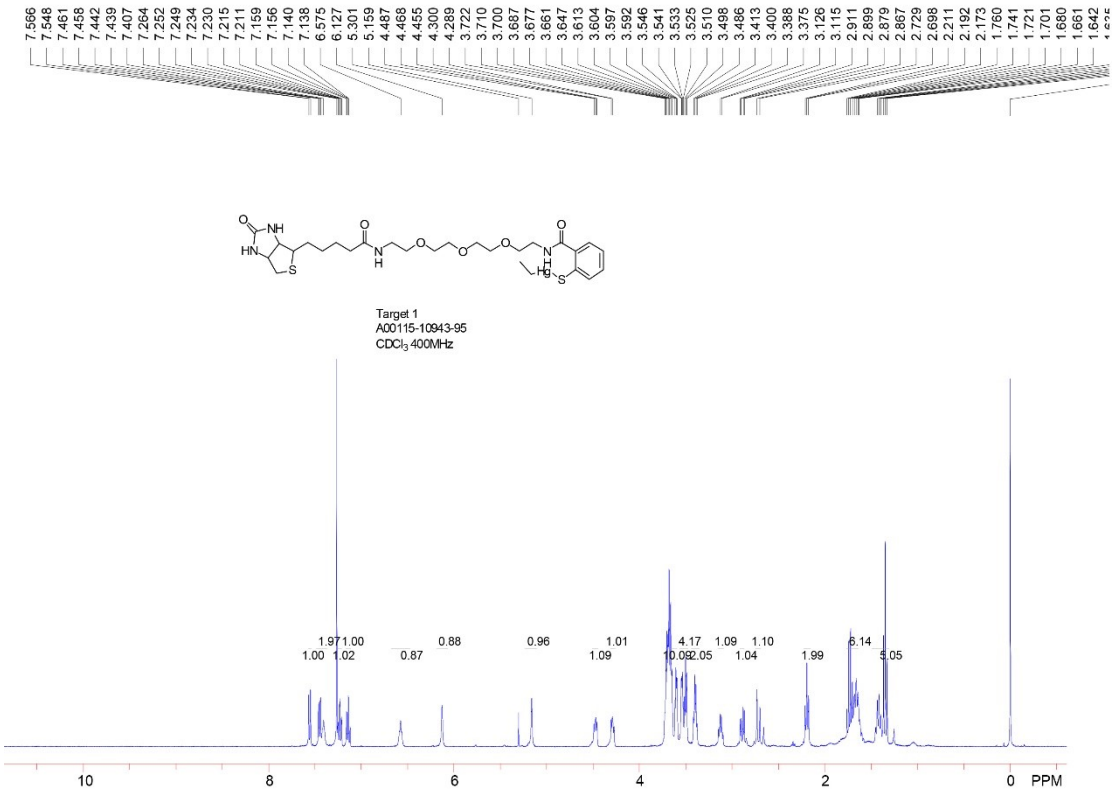


Fig. S2 NMR spectra of bio-HgS.

1.2 Intracellular localization analysis of Bio-HgS

Neuro-2a cells were treated with or without Bio-HgS (100 μM) for 12 h, followed by fixation with 4% paraformaldehyde for 30 min at room temperature. After being washed with PBS three times, cells were permeabilized with 0.5% Triton X-100 for 30 min, blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature, and incubated with streptavidin-FITC (1:200) overnight at 4 °C. Then, cells were stained with 4’,6-diamidino-2-phenylindole (DAPI). Image acquisition was achieved using a SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Streptavidin-FITC was detected at an emission wavelength of 530 nm and excitation wavelength of 488 nm, and DAPI was detected at an emission wavelength of 610 nm and excitation wavelength of 514 nm.

1.3 Pull-down assay for target protein identification

Avidin agaroses were incubated with an excess of Bio-HgS (400 μM) for 2

h at room temperature. Then, immobilized beads were washed with PBS six times and kept at 4 ℃ before use. The assay consisted of three groups: (A) binding group, (B) thimerosal competition group, and (C) 2-ethylbenzoic acid competition group. Specifically, Sprague-Dawley rat brain lysates were pre-mixed with thimerosal or 2-ethylbenzoic acid individually at 4 ℃ for 1 h and incubated with 100 μL of bio-HgS coupled avidin agarose in PBS overnight at 4 ℃. After washing with 0.01% Triton X-100 in PBS, beads were trypsin-digested, and then analyzed by LC-MS/MS using a nano-HPLC-tandem LTQ-Orbitrap Velos pro mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) for target identification. We chose the proteins satisfied both fold change (binding group / competition group) > 2 and *p*-value < 0.05 as the differential proteins of (group A *vs.* group B) or (group A *vs.* group C).

2 Binding verification of HgS with target protein

2.1 Competitive pull-down assay

The rat pheochromocytoma PC12 cells were lysed with NP-40 lysis buffer and then centrifuged at 4 °C to collect supernatant. For pre-treatment group, the supernatant fluid was pre-mixed with thimerosal or HgS for 12 h individually and incubated with bio-HgS coupled avidin agarose beads for 12 h. For post-treatment group, the lysates was pre-incubated with bio-HgS coated beads for 12 h and then further incubated with thimerosal or HgS for 12 h for competitive binding. For active sulfhydryl compounds treatment groups, PC12 cell lysates was pre-incubated with DTT (1 mM) and β-mercaptoethanol (BME, 1 mM) for 2 h, followed by incubation with HgS beads at 4 °C for 12 h. Subsequently, the beads were boiled in 50 μL of 2 × protein loading buffer for 10 min. Samples were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibody (Abcam, Cambridge, UK).

2.2 Cellular thermal shift assay (CETSA)

For the cell CETSA experiments, PC12 cells were treated with or without HgS (200 μM) for 2 h, and then heated individually at different temperatures of 41°C, 44°C, 47°C, 50°C, 53°C, 56°C, 59°C and 62°C for 3 min, following two liquid nitrogen freeze-thaw cycles. The cell lysates were collected and analyzed by SDS-PAGE followed by immunoblotting for Skp1.

2.3 Surface plasmon resonance (SPR) Assay

Firstly, recombinant Skp1 protein was expressed and purified. The DNA sequences encoding Skp1 were cloned into the Nde I/Xho I restriction sites of the pET-28a vector with His-tag. Recombinant plasmid was transformed into Escherichia coli BL21 (DE3) cells. Cells were cultured in Luria Broth (LB) medium at 37 °C to an OD600 of 0.8 and expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16 °C overnight. Harvested cells were resuspended in lysis buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM DTT, 1 mM EDTA) and then lysed by sonication. Cell debris were removed by centrifugation and the supernatant was loaded onto a 5 mL His column equilibrated in lysis buffer. Nonspecific binding protein was washed with binding buffer, and recombinant Skp1 was collected with elution buffer (same as the lysis buffer but with 250 mM imidazole). The concentration of Skp1 was quantified by the BCA protein assay reagent.

Subsequently, interaction between thimerosal or 2-ethylbenzoic acid with Skp1 was quantitatively analyzed using the Biacore T200 system (GE Healthcare, Boston, MA). Briefly, the CM5 sensor chip was activated using sulpho-NHS/EDC chemistry. The chip was subsequently immobilized with Skp1 protein (30 μg/mL) in sodium acetate buffer (pH 4.0) and blocked with ethanolamine. Various concentrations of thimerosal or 2-ethylbenzoic acid (0.39 μM, 0.78 μM, 1.56 μM, 3.13 μM, 6.25 μM, 12.5 μM, 50 μM, 100 μM, 200 μM) were injected at a flow rate of 30 μL/min in PBS. The data were collected and analyzed with the Biacore evaluation software (T200 Version 1.0).

2.4 LC-MS/MS analysis

Extracted peptides were separated using an EASY-LC system and conducted by nano-liquid chromatography linear trap quadrupole mass spectrometry (LC-LTQ-MS). The mixtures were directly injected by autosampler, bound onto a trapping column, and eluted with the following gradient: 2%−40% B for 70 min; 40%−95% B for 5 min; 95% B for 20 min (solvent A: 0.1% formic acid in H2O, solvent B: 0.1% formic acid in acetonitrile). The eluent was introduced to the mass spectrometer at a flow rate of 300 μL/min. Full scan MS spectra (m/z 350−2000) were acquired in the Orbitrap analyzer with a resolution of 60000. The top 15 most abundant precursor ions from each MS scan with charge states of at least 2 were selected for MS/MS scans in the linear ion trap analyzer with collision-induced dissociation (CID) of 35% collision energy. MS data were analyzed with Proteome Discoverer (1.4) software with the SEQUEST search engine (Thermo Fisher Scientific) using the following criteria: taxonomy, human; enzyme, trypsin; missed cleavage sites, 2; precursor mass tolerance as 10 ppm, fragment mass tolerance as 0.6 Da; and the false discovery rate (FDR) at 0.01.

2.5 Molecular Docking

The crystal structure of Skp1 was obtained from the RCSB Protein Data Bank (PDB) database with PDB code 5XYL. The two-dimensional structure of HgS was acquired from the PubChem compound database and inverted into a three-dimensional structure according to the energy minimization principle. Molecular docking was carried out using the AutoDock 4.2 software. Taking the midpoint of Cys62 and Cys120 as the center of the box, the docking box sizes were set to 50, 50 and 50 Å along the X, Y and Z axes, and the lattice spacing was set to 0.375a. The docking conformation with the minimum energy was selected as the docking mode and visualized by PyMol software.

3 Biological functions of target protein

3.1 siRNA transfection

HEK293T and Neuro-2a cells were cultured in 6-well plates until 80% confluence and washed with Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, Waltham, MA, USA) before transfection. Specific *SKP1*/*Skp1* or negative control siRNA (GenePharma, Suzhou, Jiangsu, China) was transfected into cells using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The siRNA sequences were listed in Table S1.

Table S1. The siRNA sequences for transfection.

SiRNA	Sequence	
SKP1 (Homo)	Forward	5'-GCAAGACUGUUGCCAAUAUTT-3'
	Reverse	5'-AUAUUGGCAACAGUCUUGCTT-3'
Negative control (Homo)	Forward	5'-UUCUCCGAACGUGUCACGUTT-3'
	Reverse	5'-ACGUGACACGUUCGGAGAATT-3'
Skp1 (Mus)	Forward	5'-CCAAACAAUCUGUGACUAUTT-3'
	Reverse	5'-AUAGUCACAGAUUGUUUGGTT-3'
Negative control (Mus)	Forward	5'-UUCUCCGAACGUGUCACGUTT-3'
	Reverse	5'-ACGUGACACGUUCGGAGAATT-3'

3.2 Ubiquitin-protease system (UPS) report assay

The plasmid pEGFP-N1-Ubiquitin (Ubi-GFP) was constructed by Biogot technology (Nanjing, Jiangsu, China). HEK293T cells at the density of 70% were transiently transfected with plasmid Ubi-GFP using Lipofectamine 2000 Transfection Reagent. After 4 h, the cells were transferred with *SKP1* or negative control siRNA and incubated for 36 h. Subsequently, HEK293T cells were treated with HgS or cinnabar (50 μM and 100 μM) for 24 h and then imaged at 488/519 nm for green fluorescence by the inverted fluorescence microscope (IX73, Olympus, Japan).

3.3 FM1-43 staining

FM1-43 staining assay was used to evaluate the synaptic vesicle exocytosis of primary cortical neurons. FM1-43 is a styryl fluorescent dye that

can be taken up into synaptic vesicles by endocytosis and subsequently released into the extracellular medium by exocytosis [1]. Thus, the synaptic vesicle exocytosis can be measured by the changes in fluorescence intensity. Primary cortical neurons were obtained from the cerebral cortex tissue of 16-day-old ICR mice and cultured in the neurobasal medium (2% B-27, 1% penicillin, 1% streptomycin, and 1% glutamine). On the 12th day of culture *in vitro*, primary cortical neurons were exposed to 50 μM HgS for 24 h. Then, neurons were washed with Tyrode solution and incubated with Honest 33258 solution (5 μg/mL) at 37℃ for 1 h. After that, neurons were washed with PBS buffer and incubated with 60 mM high-potassium Tyrode solution containing 4 μM FM1-43 for 1 min to stimulate synaptic vesicle exocytosis. Finally, neurons were washed with Tyrode solution to remove excess dye and stimulated with 90 mM high-potassium Tyrode solution. The synaptic vesicle exocytosis was measured as the decrease in fluorescence intensity. The images were captured under a confocal microscope (Leica Microsystems, Wetzlar, Germany) with continuous shooting mode for 10 min with 2 s intervals.

3.4 *C. elegans* strains, culture condition and HgS exposure

Wild-type N2 *C. elegans*, *SKP1* homologous gene *skr-1* knockout transgenic VC1241 *C. elegans*, and *Escherichia coli* OP50 (*E. coli* OP50) used in this study were provided by *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, USA). *C. elegans* were maintained on the nematode growth medium (NGM) plates seeded with *E. coli* OP50 at 20 ℃. Gravid hermaphrodites were washed and lysed using the bleaching buffer (0.5 M NaOH, 2% HClO) to obtain synchronized L1 larvae. During the exposure, inactivated *E. coli* OP50 by ^{60}Co radiation was used to avoid the potential metabolism of HgS by bacteria [2]. HgS was dissolved in K-medium (52 mM NaCl, 32 mM KCl) and diluted to the concentrations of 250, 500, and 1000 $\mu g \cdot mL^{-1}$, respectively. Age-synchronized L1 larvae of N2 or VC1241 were

placed on the NGM plates and exposed to different concentrations of HgS for 72 h in a 20 ℃ incubator.

3.5 Locomotion behavior assay of C. elegans

The frequencies of body bend and head thrash are common and effective indicators to evaluate the locomotion behavior of *C. elegans*. After exposure to HgS for 72 h, the body bends and head thrashes of N2 and VC1241 *C. elegans* were counted within 20 sec. A body bend was defined as a change in the direction of the posterior pharynx along the Y-axis if the nematode moved along the X-axis. A head thrash was defined as an alteration in the direction of bending at the middle of the body [3]. Twenty nematodes were examined per treatment, and the experiments were performed in triplicate.

4 Statistical analysis

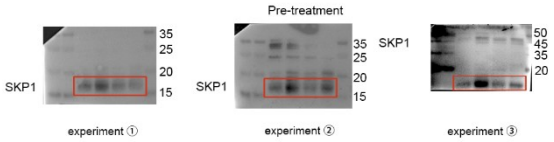
Data were presented as mean ± standard deviation. Student *t* test was used to compare the difference between two groups. ANOVA accompanied by Dunnett-t test was applied to compare differences among multiple groups. A value of $P < 0.05$ was considered statistically significant. Results analysis and graphing were performed with SPSS version 22.0 (SPSS, USA) and GraphPad Prism version 7.0 (GraphPad Software, USA), respectively.

References

1. C. W. Lu, Y. C. Huang, K. M. Chiu, M. Y. Lee, T. Y. Lin and S. J. Wang, *Int J Mol Sci*, 2021, **22**.
2. Z. H. Ji, W. J. Zhang, Y. Z. Li, N. Zhang, S. Gao, G. J. Li and Q. Wang, *Journal of toxicology*, 2022, **36**, 275-279+291.
3. J. Ju, Q. Ruan, X. Li, R. Liu, Y. Li, Y. Pu, L. Yin and D. Wang, *Environ Sci Pollut Res Int*, 2013, **20**, 1823-1830.

Original Blots

Fig 2A Competitive pull-down



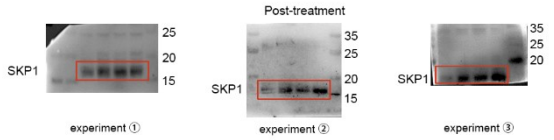


Fig 2B Control

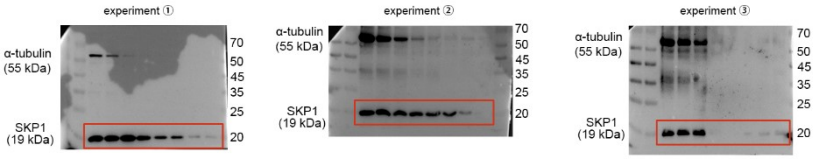


Fig 2B HgS

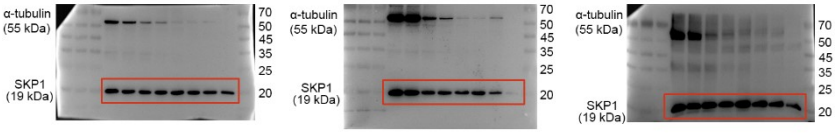


Fig 3E Sulfhydryl compounds competition

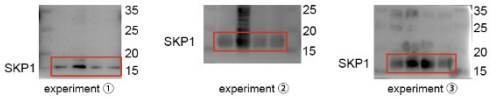


Fig 4C CO-IP

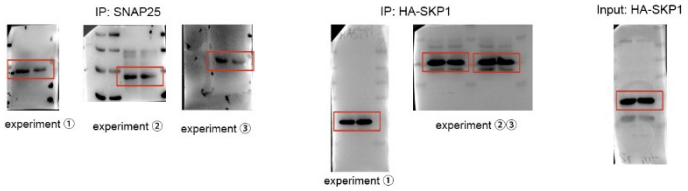


Fig 4D SNAP25

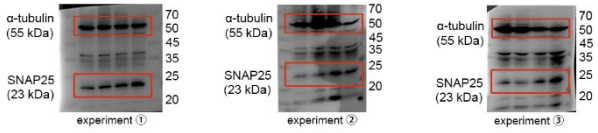


Fig 4E SNAP25

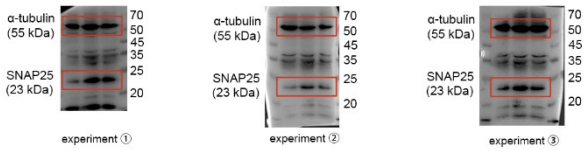


Fig 4D CyclinE

Fig 4E CyclinE

Fig 4D C-JUN

Fig 4E C-JUN

Fig 4D C-MYC

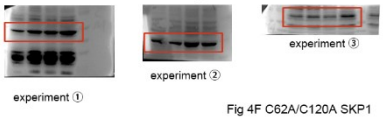


Fig 4E C-MYC

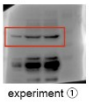


Fig 4F C62A/C120A SKP1