Electronic Supplementary Information

Vanadates inhibit non-small cell lung cancer through modulation of ferroptosis mediated by CBS-CPS1 axis

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Supplementary Methods

Cell lines and reagents

The human non-small cell lung cancer cell lines A549, H460, PC9 cells and human normal lung epithelial cell line BEAS-2B cells were obtained from the American Type Culture Collection (ATCC) and authenticated based on the Short Tandem Repeat (STR) analysis. All the cell lines were cultured at 37 °C in a humidified 5% CO2 incubator in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco). Vanadyl sulfate ($VOSO_4$) and sodium metavanadate ($NaVO_3$) were purchased from Sigma-Aldrich and instantly dissolved in PBS for in vitro and in vivo studies. Cisplatin was purchased from Sigma-Aldrich. Aminooxyacetic acid hemihydrochloride (AOAA) was purchased from MCE and dissolved to 10 mmol/L with DMSO as stock solutions for in vitro studies.

Nude mice xenograft tumor model

Female BALB/c nude mice (6-8 weeks old) were used for subcutaneous tumorigenesis experiments. Tumor cells were inoculated subcutaneously in the axilla of nude mice. The maximum diameter (a) and minimum diameter (b) of the tumors were measured using vernier calipers, and the tumor volume was calculated according to the formula $0.52 \times ab^2$. The mice were divided into a variety of groups exposed to different treatments when the tumor volume reached 100 mm^3. Tumor growth was terminated by execution of the mice according to the actual tumor growth. Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University approved all the experimental procedures (2021-KY-0856). Efforts have been made to minimize animal suffering.

Cell viability assay

Cells were seeded at a density of 5×10^3 cells/well in 96-well plates in RPMI-1640 medium containing 10 % FBS. After 24 h, cells were exposed to the indicated concentrations of $VOSO_4$ and $NaVO_3$ for another 72 h. Then 10 μL of CCK-8 reagent (Vazyme) was added to each well for 1 h at 37 °C, and the absorbance (OD) was read at a wavelength of 450 nm using a multifunctional microplate reader (SpectraMax iD3, MD). The IC_{50} values were calculated by concentration-response curve fitting using the SPSS statistics software (v25).

EdU cell proliferation assay

The EdU cell proliferation assay was performed according to the manufacturer's instructions (C10310-1, RIBOBIO). NSCLC cells were seeded in 12-well plates at a density of 2×10^5 cells/well. After incubation for 24 h, cells were treated with $VOSO_4$, $NaVO_3$, $VOSO_4$ + DFO, or $NaVO_3$ + DFO with PBS as the control and cultured in RPMI-1640 medium containing 10% FBS for another 72 h. Then, cells were performed with the EdU fluorescent label, 4 % paraformaldehyde fixation, and Apollo staining. Finally, the proportion of EdU-labeled cells was observed under the inverted fluorescence microscope (IX73, Olympus).

Lipid ROS measurement by flow cytometry

Lipid ROS levels in NSCLC cells treated with $NaVO_3$ or $VOSO_4$ alone or vanadium and 20 μM DFO combination were detected by flow cytometry using the BODIPY 581/591 C11 fluorescent probe (ThermoFisher Scientific). Cells were seeded at a density of 5×10^5 cells/well in 6-well plates in RPMI-1640 medium containing 10% FBS. After incubation with vanadium compounds for 48 h, cells were stained with BODIPY 581/591 C11 probe for 30

min at 37 °C. Then, cells were washed with PBS twice and subsequently analyzed through the FITC channel of flow cytometry (FACSCelesta, BD Biosciences).

Detection of Ferrous ion levels

The content of ferrous ions in A549, H460, and PC9 cells was determined using FerroOrange fluorescent probe (F374, Dojindo Laboratories). Cells were inoculated in the confocal dishes and cultured at 37 °C for 24 h. After treatment of vanadium compounds and DFO for 72 h, 1 μM FerroOrange working solution was added and incubated for 30 min at 37 °C. The intensity of fluorescence excited by ferrous ions was measured using laser scanning confocal microscopy (LSM 980, ZEISS).

GSH content detection

GSH contents in NSCLC cells under the treatment of vanadium compounds were detected by the GSH assay kit (S0053, Beyotime). In brief, cells were washed with PBS, and protein removal reagent M solution was added to the cell precipitate. The samples were then frozen and thawed twice quickly using liquid nitrogen and a 37 °C water bath. The supernatant was taken partly for total glutathione determination and partly for the GSSG detection. Finally, the concentration of GSH per mg of protein was calculated according to the formula (GSH = Total Glutathione – GSSG×2).

MDA content

The concentration of MDA in the vanadium compounds-treated control, CBS KD group, and CBS OE group were detected according to the manufacturer's instructions (A003-4-1, Nanjing Jiancheng Bioengineering Institute). 1 mL of extract solution was added to the collected cell precipitates and ultrasonically broken in an ice bath. The cells were centrifuged at 4 °C and 12,000 rpm for 10 min and the supernatant was then transferred to a fresh EP tube. Working solution (clarifying agent: reserve solution: color developer = 0.2: 3: 1) was added and centrifuged at 4,000 rpm for 10 min. Then, the empty 96-well plate was scanned at 530 nm. Subsequently, 0.25 mL of supernatant from each tube was dispensed to the 96-well plate. The absorbance of each well was quantified using a multifunctional microplate reader (SpectraMax iD3, MD). The MDA content within cells was calculated according to the formula outlined in the instruction manual.

ICP-MS for iron and vanadium content detection

A549 cells were seeded into 10 cm cell culture dishes for overnight culturing. Subsequently, cells were cultured for another 72 h under treatment of 8 μM $VOSO_4$, 8 μM $NaVO_3$, 8 μM $VOSO_4$ + 10 μM DFO, or 8 μM $NaVO_3$ + 10 μM DFO. After washing with PBS twice, cells with an equivalent number in each group were collected for further ICP-MS analysis. The cell precipitates were pyrolyzed by concentrated nitric acid (225711, Merck). After centrifugation, the supernatant was used for the next dilution and finalization. 103Rh was used as the internal standard. The intracellular contents of 51 V and 56 Fe were calculated from the related standard curves.

Bulk RNA-Seq assay

Illumina sequencing was performed to prepare the synthetic cDNA library. The derived clean reads were annotated via mapping to the sequenced human genome using the HISAT2 software. Next, differential expression analysis between the two groups was performed by calculating the FPKM of the genes in each sample using the DESeq R package (v1.18.0). The genes with the adjusted *p*-value of < 0.05 and $|\text{FoldChange}| \geq 1.5$,

revealed by DESeq, were designated as differentially expressed genes (DEGs). Finally, these differential genes in all samples were analyzed using cluster analysis. Subsequently, the identified DEGs were subjected to Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using the DAVID bioinformatics resources (v6.8). The GO terms and pathways with the adjusted *p*-value < 0.05 were regarded as significantly differential signaling pathways.

qRT-PCR

The expression of target genes was further verified by quantitative real-time PCR (qRT-PCR). Total RNA of cells cultured in 6-well plates was extracted using TRIzol reagent (Invitrogen). The purity and integrity of total RNA were detected by NanoDrop 2000 (ThermoFisher Scientific). The total RNA was used to synthesize cDNAs using HiScript III RT SuperMix for qPCR (Vazyme) according to the manufacturer's instructions. Then, the samples of qRT-PCR were prepared using ChamQ SYBR qPCR Master Mix (Vazyme) in 20 μL reaction volume and detected by QuantStudio 5 (Applied Biosystems). GAPDH was used as the internal reference, and the relative expression of target mRNA was calculated by $2^{-\triangle\triangle Ct}$ in three replications. The primers used are listed in Table S1.

Western blot analysis

Cells were washed twice with cold PBS and then lysed with RIPA buffer at 4 °C for 30 min. The whole-cell lysate was collected, ultrasonicated, and centrifuged at 12,000 rpm for 10 min. The concentration of supernatant was measured using a standard BCA kit (P0012, Beyotime). Proteins were separated using SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk for 60 min, the membrane was firstly incubated with the indicated primary antibodies overnight at 4 °C, and then with the corresponding horseradish peroxidase-labeled secondary antibody. Protein levels were measured using an ECL kit and images were acquired using the Chemiluminescence System (Amersham Imager 680, GE). The proteins were immunoblotted with antibodies (1:1000) against CBS (14787-1-AP, Proteintech), CPS1 (18703-1-AP, Proteintech), GPX4 (ab125066, Abcam) and xCT/SLC7A11 (HA600098, HUABIO).

Lentiviral infection and construction of stable KD and OE cell lines

shRNA sequences targeting CBS and CPS1 were synthesized and detailed in Table S2. Annealed oligo pairs were cloned into the pLKO.1 vector, whereas CDS sequences overexpressed CBS and CPS1 were respectively inserted into the pLVX-IRES-Puro-3×Flag vector and pCDH-CMV-IRES-HA vector. Plasmids containing the shCBS and shCPS1 sequences were constructed using standard molecular cloning techniques. Lentiviruses were produced by cotransfecting HEK293T cells with the packaging plasmid psPAX2, envelope plasmid pMD2.G, and shRNA or overexpression plasmids. Viral supernatants were harvested at 48 h and 72 h post-transfection, filtered through a 0.45 μm filter, and concentrated using a 100 kDa ultrafiltration device. Target NSCLC cells were infected with the viral supernatant at 80% confluence in fresh medium containing 1 μL polybrene (10 mg/mL). Stably transfected A549 and H460 cells were then selected using 4 μg/mL and 8 μg/mL puromycin, respectively.

Colony formation assay

The NSCLC cells transfected with CBS KD and OE were seeded in 6-well plates at 500 cells/well. After 10-14 days of incubation, colonies were fixed with 4% paraformaldehyde and stained with 0.25% crystal violet at room temperature. The colony number was measured by ImageJ software (v1.42q).

Wound healing assay

The wound healing assay was performed to detect cell migration viabilities of CBS KD and OE A549 and H460 cells. When cells reached 90% confluence in 6-well plates, cells were subjected to serum-free medium for 24 h. Then, two lines were scraped using a sterile plastic pipette tip in each cultured well. After washing twice with the warm serum-free medium to remove cellular debris, cells were kept incubating in serum-free RPMI-1640 medium at 37 °C. Images were acquired using the microscope at time points of 0 h, 24 h, and 48 h (IX73, Olympus).

Cellular thermal shift assay

Cellular thermal shift assay (CETSA) was performed to detect the efficiency of vanadium compounds binding to CBS. Cells were collected from the control group and vanadium compounds treated group and resuspended with PBS. The cells were aliquoted into 10 portions. Gradient heating treatment was conducted using a gradient temperature PCR instrument (BioRad). Proteins were subsequently extracted from the heated cells by repeated freezing and thawing methods. Western Blot analysis was performed, and alterations in protein expression were visualized using the Chemiluminescence System (Amersham Imager 680, GE).

Untargeted metabolomics analysis

To determine the relative levels of intracellular metabolites in NSCLC cells under treatment of vanadium compounds, metabolite extracts were prepared and analyzed by LC-MS/MS. The control and $VOSO_4$ or $NaVO_3$-treated cells were plated at a density of 2×10^6 cells/dish in 100 mm dishes for 72 h. Cells were trypsinized and resuspended in 1 mL of pre-cooled extraction solution (methanol/acetonitrile/water = 2/2/1) at 80 °C. The cell suspension underwent ultrasonication and freeze-thaw cycles, repeated three times. The lysate was transferred to a new tube after centrifugation at 14,000×g for 20 min. The supernatant was collected and then dried using vacuum centrifugation. The dried samples were stored at 80 °C until further LC-MS/MS analysis.

For metabolites analysis, evaporated samples were reconstituted in water containing 0.1% formic acid (FA), followed by acetonitrile containing 0.1% FA. Then, the samples were separated by the Agilent 1290 Infinity UPLC system in Shanghai Applied Protein Technology Co., Ltd (Shanghai, China). Mobile phase A was 25 mM ammonium formate containing 0.08% FA. Mobile phase B (MPB) was acetonitrile including 0.1% FA. The flow rate was 250 µL/min, and the injection volume was 1 µL. The gradient conditions were as follows: MPB linearly changed from 90% to 70% at 12 min, decreased to 50% at 18 min, then decreased to 40% at 25 min, followed by a linearly change from 40% to 90% at 30 min, held at 90% MPB for 7 min. The total run time was 37 min. Data were acquired using QTRAP 5500 (AB SCIEX) mass spectrometry in positive and negative ionization modes, and MRM mode was used to detect the ion pairs. The raw files were imported to MultiQuant software to extract the peak area and retention time, and the calibrated data were analyzed by SIMCA-P software (v13.0) for OPLS-DA statistical analysis.

Hematoxylin and eosin (HE) staining

Tumor tissue slides were subjected to an in situ hybridizer at 70 °C for 30 min, followed by prompt immersion in xylene twice, each for 5 min. Gradient hydration was achieved using 100%, 90%, 80%, and 70% ethanol. Subsequently, the sections were dried and stained with hematoxylin for 5 min, followed by differentiation with acid alcohol for 10 s. They were then counterstained with 0.5% eosin for 10 s to 15 s. Dehydration was performed using 80%, 90%, 95%, and 100% ethanol, followed by clearing in xylene. After drying, the slides were sealed with drops of neutral gum and allowed to cure for 24 h before examination under a microscope (BX51, Olympus).

Immunohistochemical analysis

Immunohistochemistry (IHC) was utilized to analyze the protein expression in stripped tumor tissues. Briefly, paraffin-embedded sections were subjected to a heating treatment at 60 °C for 4 h, followed by deparaffinization. Antigen retrieval was achieved through heat-induced citric acid (pH 6.0) buffer treatment for 23 minutes at 95 °C. Endogenous peroxidase activity was inhibited by incubating the tissues with 3% hydrogen peroxide at room temperature for 25 min. Subsequently, the slides were blocked with 3% BSA for 30 min at room temperature and incubated with the primary antibody of Ki67 (GB111499, Servicebio) and CBS (14787-1-AP, Proteintech) overnight at 4 °C. Then, the slides were incubated with horseradish peroxidase-conjugated secondary antibodies for 50 min. Images were scanned using the 3Dhistech Pannoramic Viewer (v1.15.4).

Transmission electron microscopy

The stripped xenograft tumor tissues (2 mm × 2 mm) were fixed in an electron microscope fixation solution containing 2.5% glutaraldehyde and 0.1 mM phosphate buffer for 2 h at room temperature and then transferred to 4 °C for storage. After dehydration, the tissues were embedded in an epoxy resin. Ultrathin sections were sliced using an ultramicrotome and placed on a copper mesh (300 mesh). The morphology of the mitochondria in vanadium-treated cells was evaluated using a transmission electron microscope (HT-7700, Hitachi).

Co-immunoprecipitation-MS

The regular CO-IP assay was executed according to the instruction of Flag tag Nanoab Magnetic Beads (FNM-25-500, NuoyiBio). The CBS-overexpressed A549 cells with Flag-tag were harvested and lysed with 1 mL lysis buffer at 4 °C for 30 min. Total protein was added to the pre-treated Flag tag-Nanoab-Magnetic Beads at 4 °C overnight. The eluted protein was used in part for silver staining of bands separated by SDS-Tris-glycine gel and immunoblot with antibody against Flag Tag (30504ES50, Yeasen), and in part for subsequent mass spectrometry identification. The protein was pre-treated by trypsin enzyme and desalted with a C18 column. The peptides were analyzed on the LC-MS/MS instrument (EASY-nLC 1200 and Q Exactive HF-X, ThermoFisher Scientific) and proteins with a minimum of one unique peptide were quantitatively analyzed.

Double immunofluorescence labeling assay

A double immunofluorescence labeling assay was used to detect the positional relationship between CBS and CPS1 proteins in NSCLC cells and the presence of interaction. The procedures of colocalization were mainly according to the manufacturer's instructions (AFIHC023, AiFang biological). In brief, cells seeded in confocal dishes were initially fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.3% Triton X-100 for 20 min. Peroxidase blocking solution was added into cells for 15 min at room temperature and then blocked with 3% BSA for 30 min. After incubating the cells with the specific primary antibody against CBS and CPS1 overnight at 4 °C and Polymer-HRP secondary antibody for 1 h, tyramide signal amplification (TSA) fluorescent dye (TYR-570 and TYR-520) was added for 5 min. DAPI staining was performed after antibody elution and fluorescence localization was observed through the confocal laser scanning microscopy (LSM 980, ZEISS).

Statistical and data analysis

Each experiment was repeated three times under identical conditions. The results were expressed as mean ± standard error of the mean (SEM). The statistical analysis of the results was performed using GraphPad Prism software (v8.0). One-way ANOVA and the student's t-test were performed to compare the values of the test and

control samples. The differences with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered to be statistically significant**.**

Table S1 The nucleotide sequences of primers used for qRT-PCR.

Gene Name	Forward primer (5’-3’)	Reverse primer (5’-3’)
GPX4	CGGAATTCATGAGCCTCGGCCGCCTTTG	CCGCTCGAGGAAATAGTGGGGCAGGTCCT
CBS	GTAATCCTGGGAATGGTGACG	GAGGCGGATCTGTTTGAACTG
CPS1	ATTCCTTGGTGTGGCTGAAC	ATGGAAGAGAGGCTGGGATT
GAPDH	CGCTCTCTGCTCCTCCTGTTC	ATCCGTTGACTCCGACCTTCAC

Table S2 The sequence of shRNA oligo sequences.

Gene Name	Sense (5’-3’)
shCBS	CCGTCAGACCAAGTTGGCAAA
shCPS1	CGTACTTCAATCAATGTTGTT

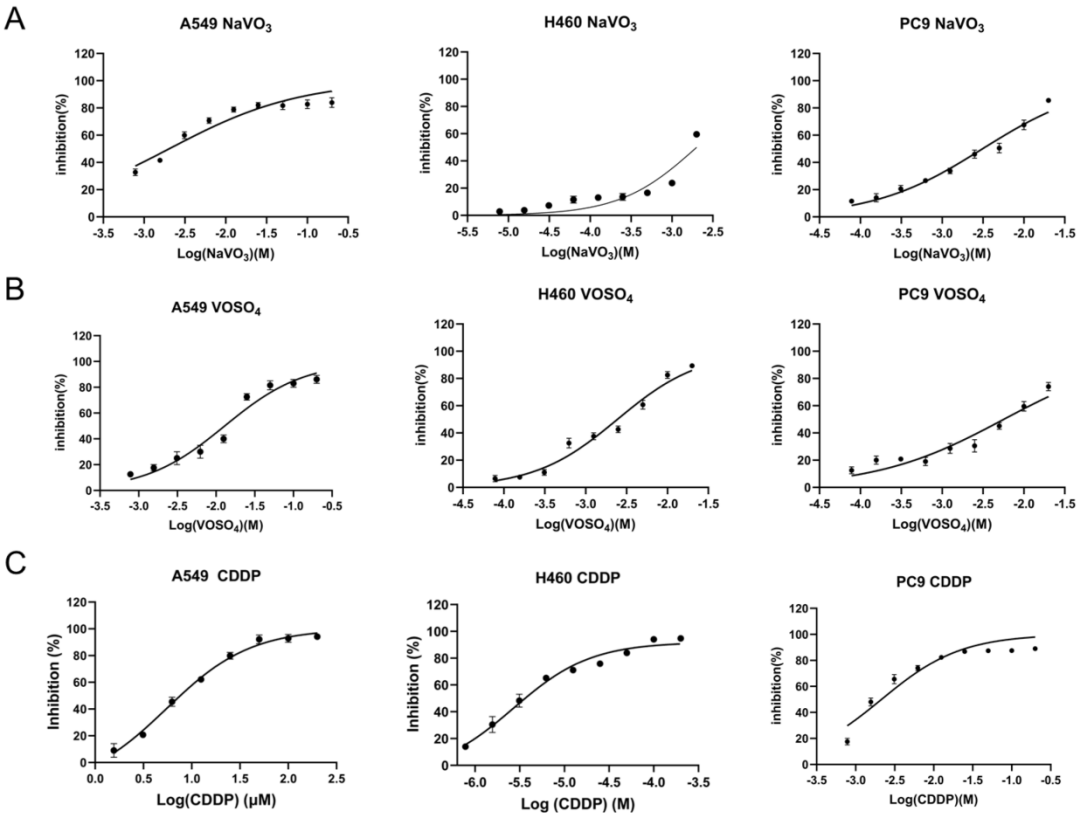

Fig. S1 The growth curves of A549, H460 and PC9 cells treated with different concentrations of vanadyl sulfate (IV), sodium metavanadate (V) and cisplatin were detected by CCK8 assay.

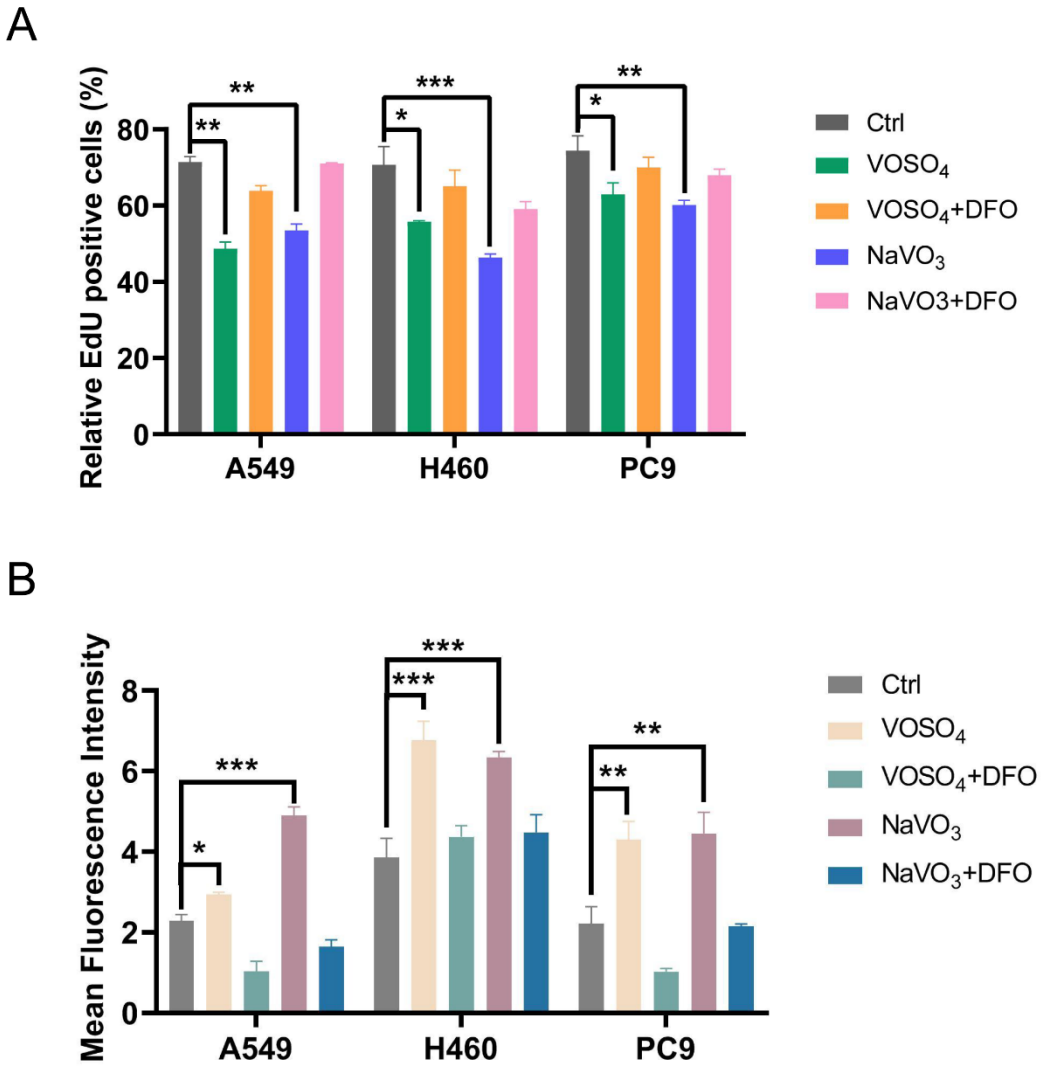

Fig. S2 The quantitative analysis of cell proliferation and ferrous ion levels in NSCLC cells treated with $VOSO_4$, $NaVO_3$, or in combination with DFO. (A) Quantification of the relative percentage of EdU-positive cells using ImageJ software. (B) The mean fluorescence intensity after FerroOrange staining was analyzed by ImageJ software.

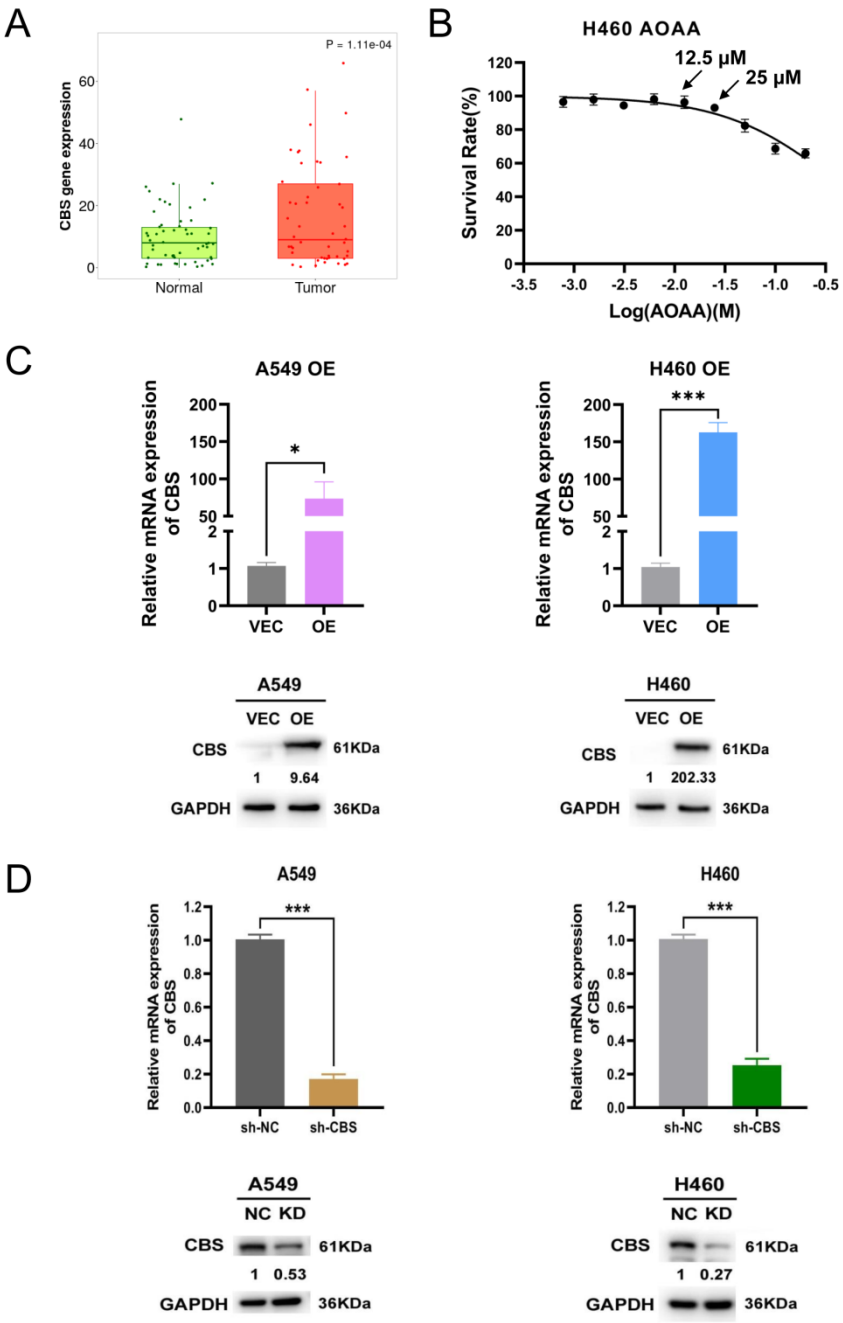

Fig. S3 Characterization of CBS expression in lung adenocarcinoma and the validation of the efficiency of knockdown/overexpression of CBS. (A) The boxplot of CBS expression analysis across lung cancer. (B) The concentration of AOAA screened by CCK8 assay did not affect cell growth. qRT-PCR (C) and western blot analysis (D) for detecting mRNA transcription level and protein expression level of *CBS* in A549 and H460 cells transfected with CBS-overexpression plasmid with empty vector as the control.

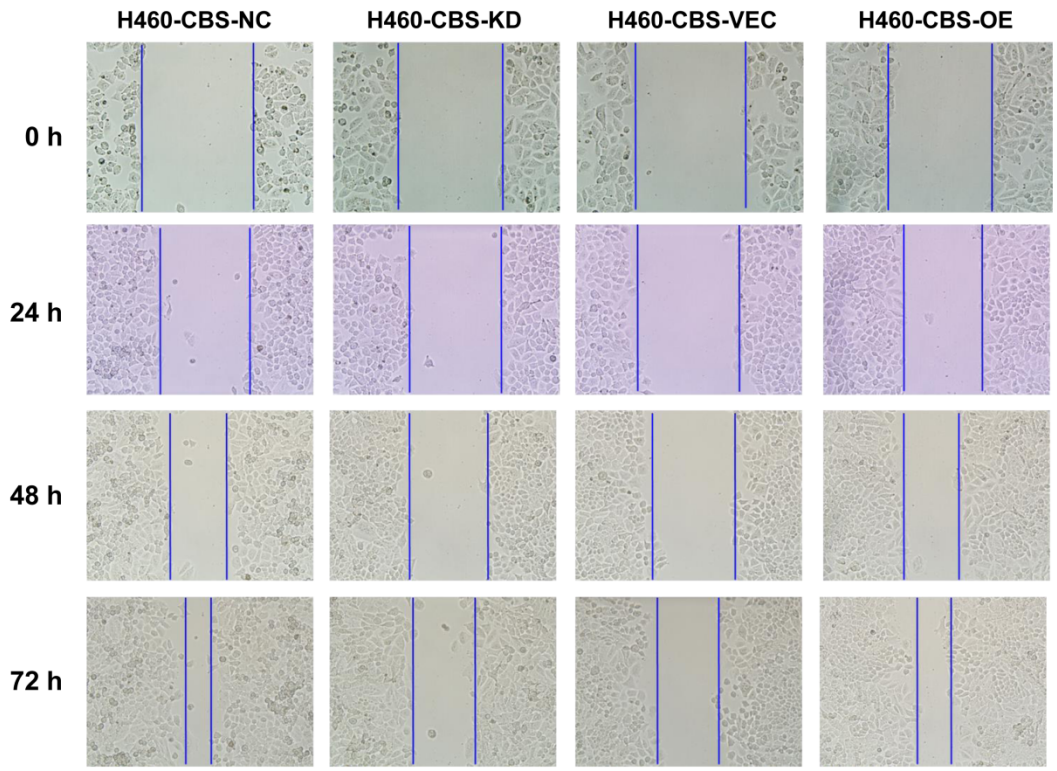

Fig. S4 The migration of H460 cells transfected with shRNA and overexpression plasmid were examined by wound healing assay.

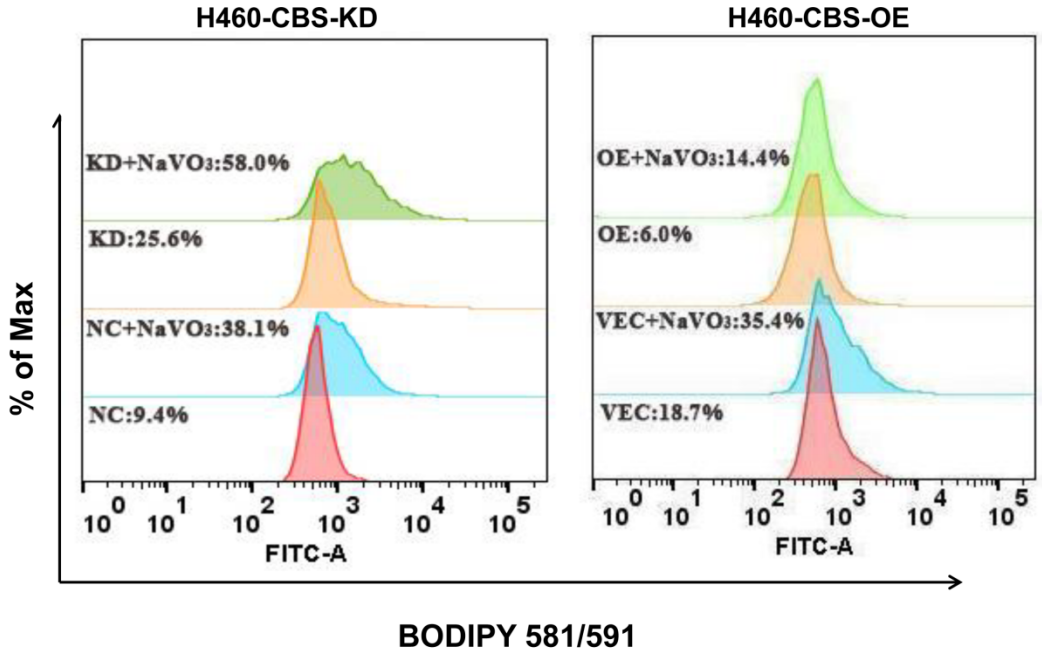

Fig. S5 Lipid ROS levels were measured in CBS KD and OE H460 cells after $NaVO_3$ treatment for 48 h by BODIPY 581/591-C11 staining and flow cytometry analysis.

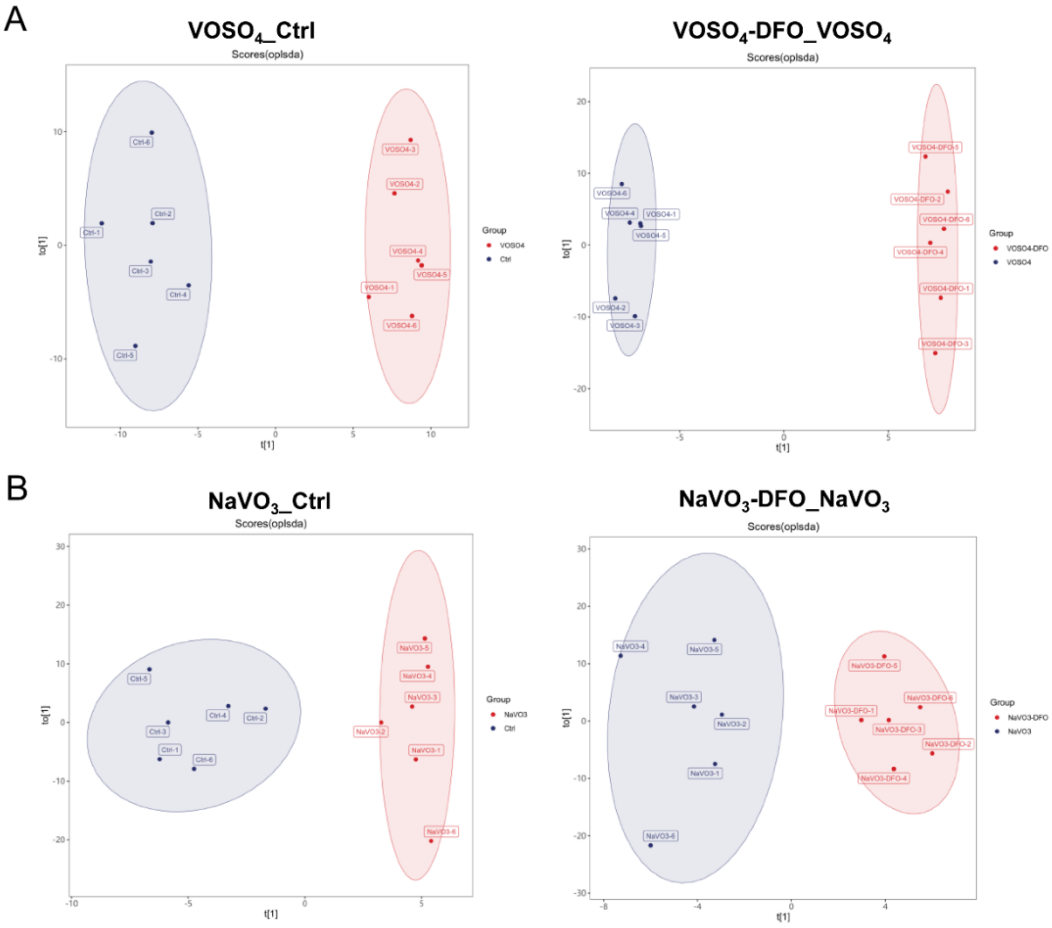

Fig. S6 The OPLS-DA models of different groups. OPLS-DA score plots between (A) the $VOSO_4$ treated group and the control group (left) and between the $VOSO_4$ + DFO combined treatment group and the $VOSO_4$ exposure group (right), and (B) $NaVO_3$ group versus the control group (left) and $NaVO_3$ + DFO group versus $VOSO_4$ group (right).

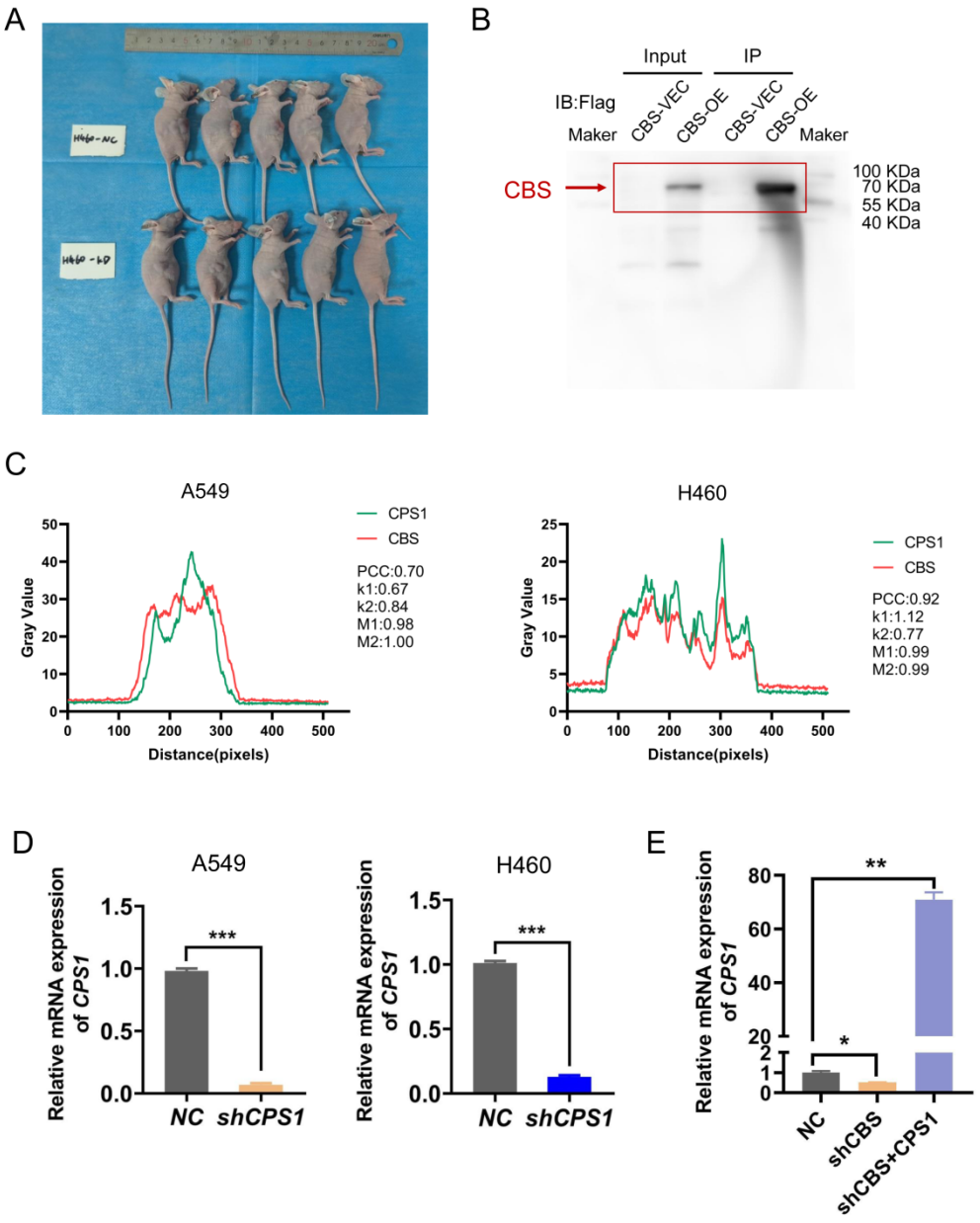

Fig. S7 The identification of the target for CBS. (A) The representative images of mice xenograft tumors after transplantation of shCtrl and shCBS cells. (B) The CBS expression in the input group and the immunoblotting group extracted from A549 cells infected with empty vector and CBS overexpression plasmid were detected by western blot assay. (C) The colocalization analysis of CBS and CPS1 immunofluorescence was performed by Plot Profile of Image J. (D) Validation of the efficiency of *CPS1* knockdown in A549 and H460 cells by qRT-PCR. (E) qRT-PCR was used to detect the transcription levels of *CPS1* in A549 cells transfected with shCtrl, shCBS, and shCBS+CPS1 vectors.