A Potent and Selective Anti-Glutathione Peroxidase 4 Nanobody as Ferroptosis Inducer

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Supporting Information

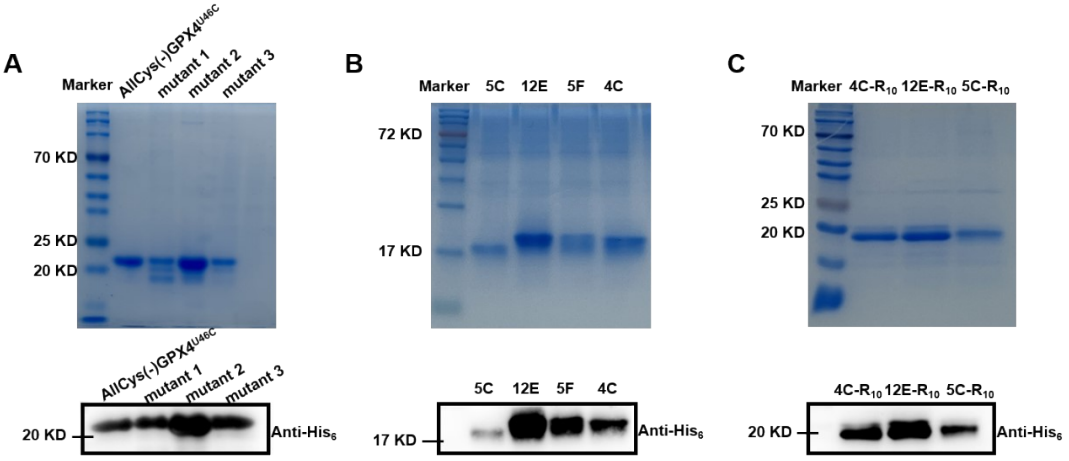

Figure S1. SDS-PAGE and western blot (WB) of GPX4 mutants (A), NBs (B) and NBs-R10 (C) after protein expression and purification.

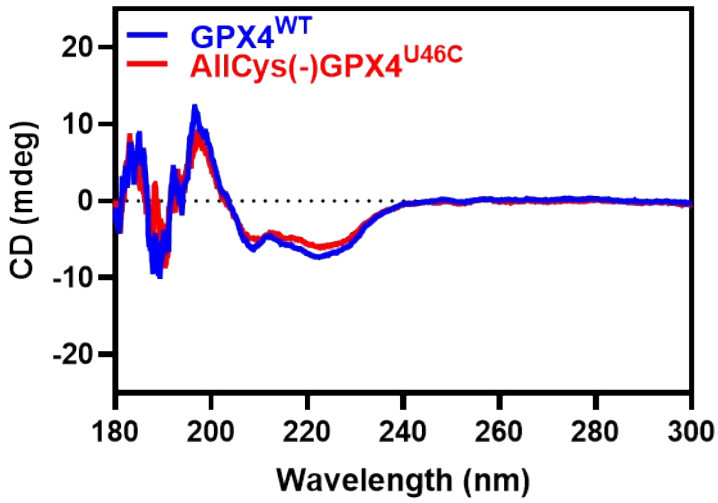

Figure S2. Circular dichroism spectra of AllCys(-)GPX4^{U46C} and recombinant GPX4^{WT} protein.

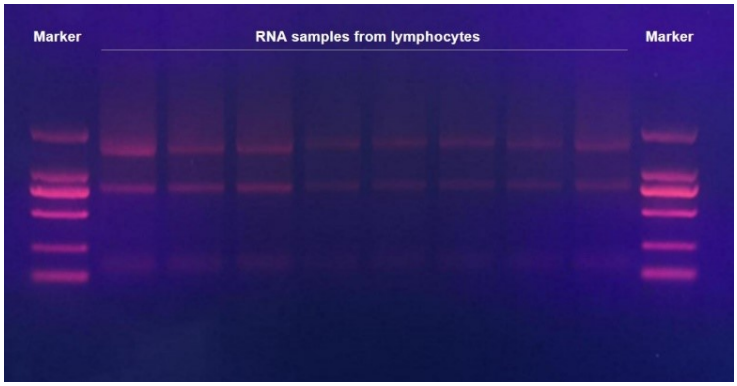

Figure S3. Agarose gel plot of RNA samples extracted within blood lymphocytes.

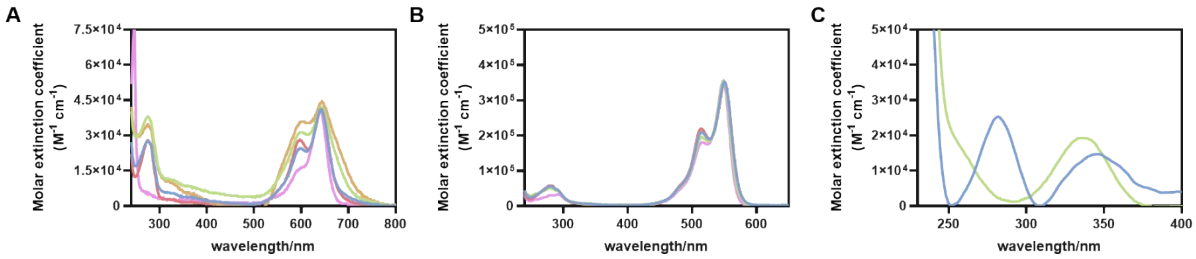

Figure S4. The absorption spectra (normalized to molar extinction coefficient) of NB-Cy5 (A), NB-Cy3 (B) and His-tag antibody-Tb conjugates (C). A-B, blue: 4C; green: 5C; brown: 5F; red: 12E; pink: pure dyes. C, blue: His-tag antibody-Tb; green: Tb-NHS.

Table S1. Concentrations and conjugation ratios of NB-Cy5 conjugates.

Sample	[NBs]/μM	[Cy5]/μM	Cy5/NBs
4C	29.0	39.0	1.3
12E	68.8	91.7	1.3
5C	6.2	6	1.0
5F	17.5	17.3	1.0

Table S2. Concentrations and conjugation ratios of NB-Cy3 conjugates.

Sample	[NBs]/μM	[Cy3]/μM	Cy3/NBs
4C	48.9	55.9	1.1
12E	57.2	59.4	1.0
5C	5.9	8.9	1.5
5F	17.8	22.1	1.2

Table S3. Concentrations and conjugation ratios of His-tag antibody-Tb conjugate.

Sample	[antibody]/μM	[Tb]/μM	Tb/antibody
His-tag antibody	0.95	6.8	7.2

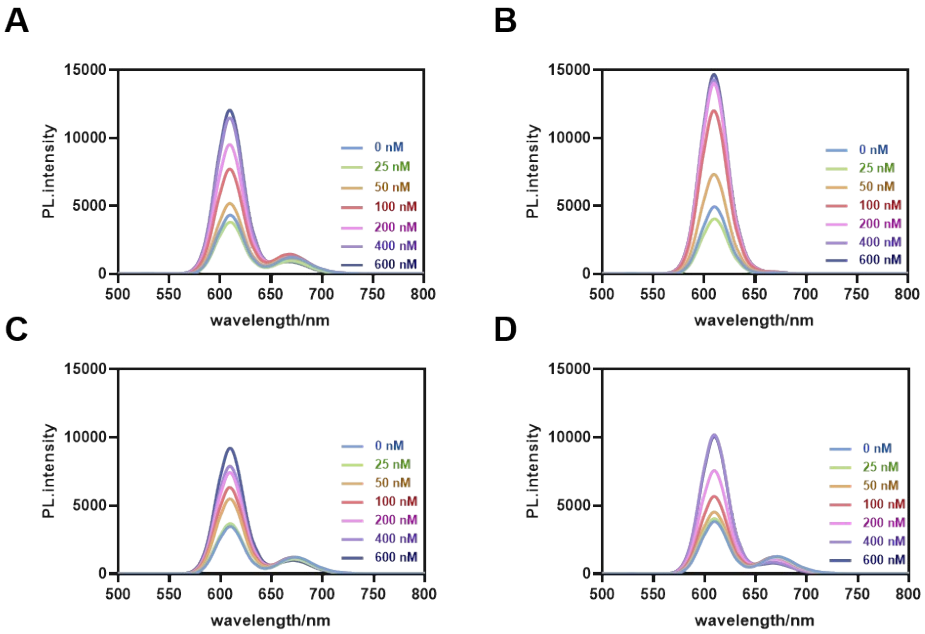

Figure S5. Fluorescence spectra of NB-on-QD displacement assays (QD-to-Cy5 FRET). A-D represents 4C, 5C, 5F, and 12E, respectively. 3 nM of QD605, 60 nM of NBs-Cy5 and different concentrations of GPX4 (0-600 nM) were incubated for 2 hrs at 37 °C.

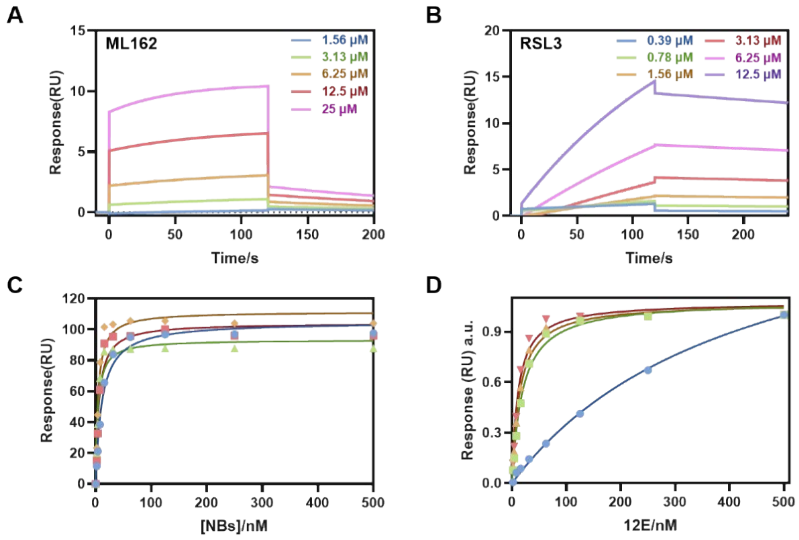

Figure S6. A-B, Kinetic binding curves of GPX4 with ML162 and RSL3 via SPR. C, Saturation binding curves of GPX4 with NBs via SPR. Blue:12E (Kd=11.2 nM); green: 5F (Kd=4.1 nM); brown: 5C (Kd=4.3 nM): red: 4C (Kd=5.8 nM). D, Saturation binding curves of 12E with GPX4 mutants via SPR. Blue: GPX4 mutant 1 (Kd=425.9 nM); green: GPX4 mutant 2 (Kd=19.0 nM); brown: GPX4 mutant 3 (Kd=13.9 nM); red: AllCys(-)GPX4^{U46C} (Kd=11.2 nM). Kd was obtained by fitting the maximum response of SPR angle with the 'One site-specific binding' model in Prism 8.0. The fitting results are as follows: 12E: Y=105X/(11.15+X); 5F: Y=93.4X/(4.1+X); 5C: 111.6X/(4.3+X); 4C: 104.3X/(5.8+X); GPX4 mutant 1: Y=1.84*X/(425.9+X); GPX4 mutant 2: Y=1.085*X/(19.0+X); GPX4 mutant 3: Y=1.07*X/(13.9+X); AllCys(-)GPX4^{U46C}: Y=1.08*X/(11.2+X).

Table S4. Association rate constant (ka) and dissociation rate constant (kd) of GPX4 to NB or RSL3/ML162.

Sample	ka (1/Ms)	kd (1/s)
GPX4-ML162	5.9×10^{2}	5.6×10^{-3}
GPX4-RSL3	4.4×10^{2}	6.8×10^{-4}
GPX4-12E	1.1×10^{6}	2.5×10^{-4}
GPX4-4C	4.8×10^{6}	8.5×10^{-4}
GPX4-5C	8.4×10^{6}	1.9×10^{-3}
GPX4-5F	1.1×10^{7}	3.6×10^{-3}

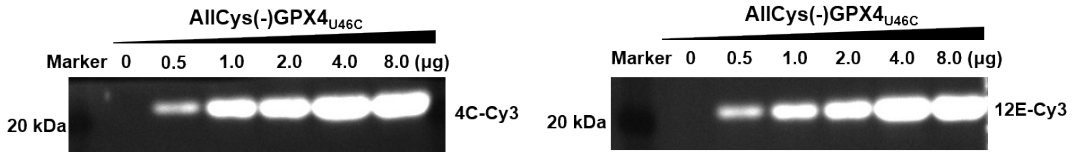

Figure S7. 4C-Cy3 and 12E-Cy3 for selective staining of GPX4 in WB. Imaging of 0, 0.5, 1.0, 2.0, 4.0 and 8.0 μg GPX4 utilizing 4C-Cy3 (left) and 12E-Cy3 (right). 4C-Cy3 and 12E-Cy3 can detect as low as 0.5 μg of GPX4.

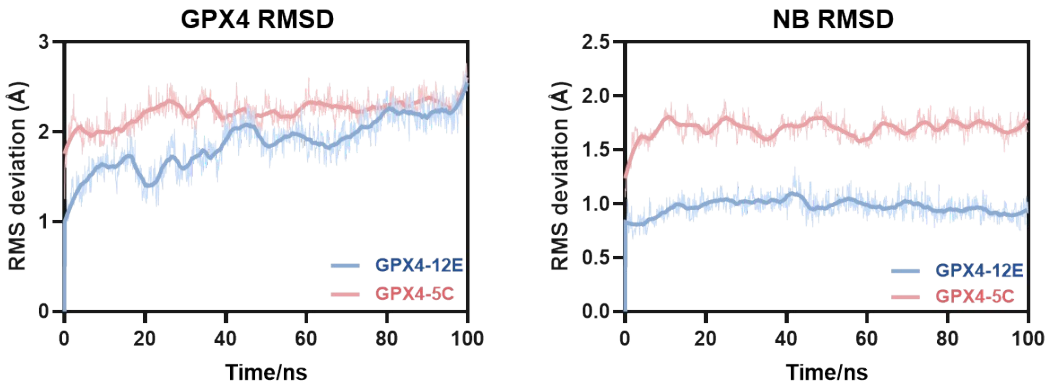

Figure S8. The GPX4 RMSD (left) and NB RMSD (right) of GPX4-12E or GPX4-5C complexes.

Table S5. Conjugation ratios of NB-R10-Cy5 conjugates.

Sample	[NBs-R10]/μM	[Cy5]/μM	Cy5/NBs-R10
12E-R10	23.2	32.7	1.4
4C-R10	21.9	30.0	1.4

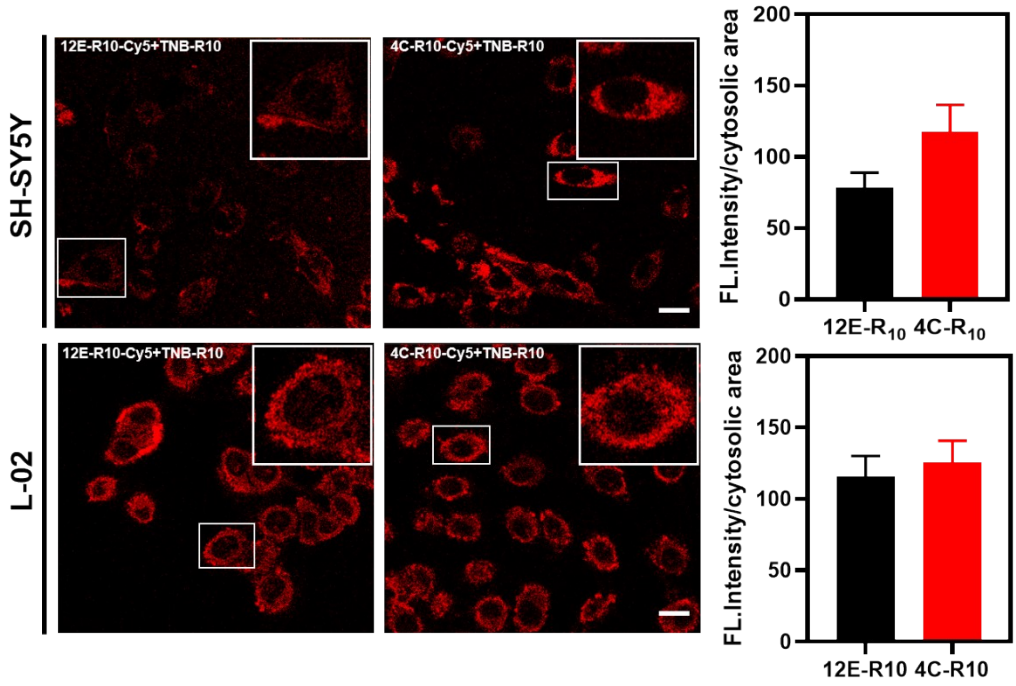

Figure S9. CLSM images of SH-SY5Y and L-02 cells incubated with 5 μM 12E-R10-Cy5/4C-R10-Cy5 in the presence of 10 μM TNB-R10 for 1 h at 37 ℃. Excitation: 581 nm; Emission: 580-650 nm; scale bar: 20 μm. Individual cytosolic fluorescence intensity of the CLSM images were processed with Image J software.

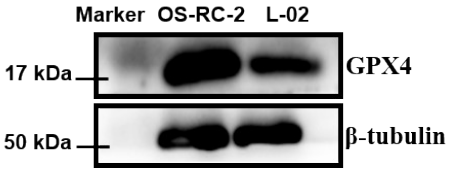

Figure S10. WB analysis of GPX4 level in OS-RC-2 and L-02 cells.

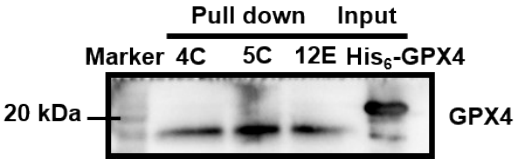

Figure S11. Proteome profiling of 4C-R10, 12E-R10 and 5C-R10 on OS-RC-2 cells by WB. Purified His_6-GPX4 protein was added as a reference.

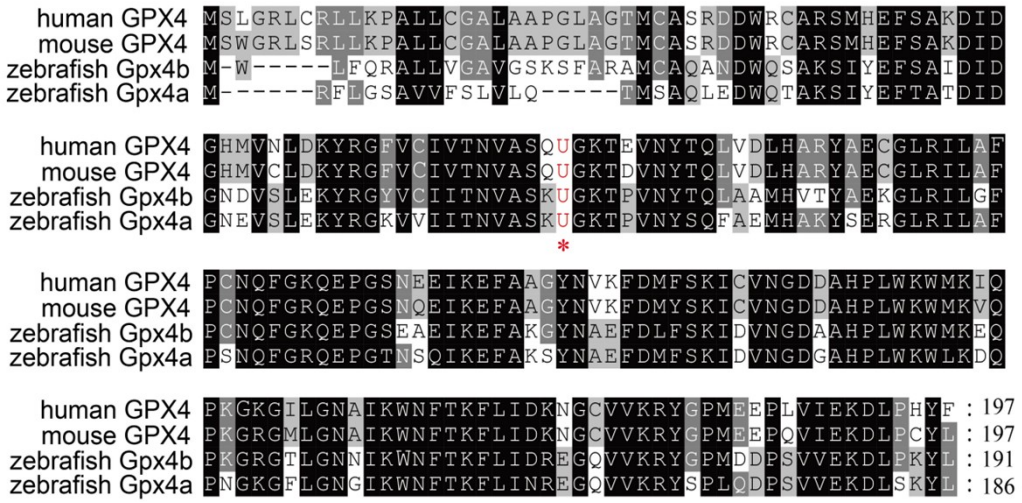

Figure S12. Amino acid sequence alignment of human, mouse, and zebrafish GPX4/Gpx4. Identical amino acids were indicated in black and similar amino acids were indicated in grey. The Sec residue (U) was indicated with an asterisk and highlighted in red. The accession numbers are as follows: human GPX4 NP_002076.2, mouse GPX4 NP_032188.3, zebrafish Gpx4b NP_001025241.2, zebrafish Gpx4a NP_001007283.2.

Materials and Methods

Materials and instruments

Tris (hydroxymethyl) aminomethane (T8060), PBS buffer (P1000), and penicillin-streptomycin liquid (P1400) were provided by Solarbio (Beijing, China). QD605-PEG-NH_2 was provided by Wuhan Jiayuan Quantum Dots (Wuhan, China). Cy5-azide (777323) was provided by Sigma-Aldrich (St. Louis, USA). Cy5-NHS ester (C595956) was provided by Aladdin (Shanghai, China). The Cy3-NHS ester (11020) was obtained from the Lumiprobe Corporation (Cockeysville, MD, USA). $C^{11}BODIPY^{581/591}$ (217075-36-0) was provided by Cayman (Ann Arbor, Michigan).

Sensor Chip CM5 (29104988) was provided by Cytiva (Marlborough, USA). Ni-NTA agarose (20503ES60) was provided by Yeasen Biotechnology (Shanghai, China). Zeba Spin Desalting Columns (89883) were provided by Thermo Fisher Scientific (Waltham, USA). TNB-R10 was provided by GenScript Biotech (Nanjing, China). RNA extraction solution (G3013) was provided by Servicebio (Wuhan, China).

Incomplete and complete Freund's adjuvants were provided by Sigma-Aldrich (St. Louis, USA). Auxiliary phage M13KO7, restriction enzyme, and T4 DNA ligase were provided by New England Biolabs (Massachusetts, UK). Rabbit anti-camelid V_HH antibody-HRP (anti-V_HH-HRP) was provided by GenScript (Nanjing, China). Rabbit GPX4 antibody and were provided by Epizyme Biotech (Shanghai, China). And rabbit His-tag antibody were provided by Univ (Shanghai, China). HEK293T, SH-SY5Y, OS-RC-2 and L-02 cells were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academic of Science (Shanghai, China). Fetal bovine serum was provided by PAN-Biotech GmbH (Aidenbach, Germany). Dulbecco's Modified Eagle Medium (L110KJ) was provided by BasalMedia (Shanghai, China). RPMI1640 (CR-31800) was provided by Cienry (Zhejiang, China). IP lysate (P0013) was provided by Beyotime Biotechnology (Shanghai, China). Chemically competent BL21(DE3) *E. coli* cells (BC201-01) were provided by Biomed (Hebei, China).

RNA extraction kit was provided by Gbcbio Technologies (Guangzhou, China). The first-strand cDNA synthesis kit was provided by Takara Corporation (Dalian, China). The gel extraction and PCR purification kit were provided by Tiangen Biotech (Beijing, China). The Chip CM5 amino coupling kit (BR-1000-50) was provided by Cytiva (Marlborough, USA). Glutathione peroxidase detection kit (NADPH, S0056), Glutathione peroxidase detection kit (DTNB, S0057S), MDA detection kit (S0131S), LDH detection kit (WST-8, C0018S), GSH and GSSG assay kit (S0053) and rapid silver staining kit (P0017S) were produced by Beyotime Biotechnology (Shanghai, China). $GPX4^{WT}$ (26906) was purchased from Cayman (Ann Arbor, Michigan). Cell Counting Kit (40203ES76) and streptavidin-magnetic beads (47503ES03) were provided by Yeasen Biotechnology (Shanghai, China). SweScript All-in-One RT SuperMix for qPCR kit (G3337) was produced by Servicebio (Wuhan, China).

The circular dichroism spectra were measured on JASCO circular dichroism spectrophotometers J-810 (Japan, Tokyo). PCR was performed on a DNA Engine PCR amplifier. DNA was characterized using a Mini-Sub cell GT (Bio-Rad, 1664400EDU, US) and GelDoc Go Gel imaging system (Bio-Rad, 12009077, US). Electroporation was performed on a Gene Pulser Xcell (Bio-Rad, 1652660). The fluorescence (FL) intensity was measured on a multifunctional plate reader Tecan Spark (Männedorf, Switzerland) and fluorescence spectrophotometer Techcomp FL970 (Shanghai, China). Fluorescence images were acquired with a Leica SP8 confocal microscope (Wetzlar, Germany). Absorption spectra was measured on an ultraviolet spectrophotometer HITACHI U3900 (Tokyo, Japan). SPR experiment was implemented with Biacore T200. WB experiments were implemented with Tanon 5200 Multi (Shanghai, Chain).

Expression and purification of AllCys(-)$GPX4^{U46C}$ and GPX4 mutants proteins

The amino acid sequence of the AllCys(-)$GPX4^{U46C}$ was as follows: MGSSHHHHHHSSGLVPRGSHMLEAASRDDWRAARSMHEFSAKDIDGHMVN LDKYRGFVSIVTNVASQCGKTEVNYTQLVDLHARYAEAGLRILAFPSNQFGK QEEPGSNEEIKEFAAGYYNVKFDMFSKIAVNGDDAHPLWKWMKIQPKGKGI

LGNAIKWNFTKFLIPKNGAVVKRYGPMEEPLVIEKPLPHYF.

The amino acid sequence of the GPX4 mutant 1 was as follows:

MGSSHHHHHHSSGLVPRGSHMLEAASRDDWRAARSMHEFSAKDIDGHMVN
LDKYRGFVSIVTNVA**AA**CG**A**T**A**VNYTQLVDLHARYAEAGLRILAFPSNQFGK
QEEPGSNEEIKEFAAGYYNVKFDMFSKIAVNGDDAHPLWKWMKIQPKGKGI
LGNAIKWNFTKFLIPKNGAVVKRYGPMEEPLVIEKPLPHYF.

The amino acid sequence of the GPX4 mutant 2 was as follows:

MGSSHHHHHHSSGLVPRGSHMLEAASRDDWRAARSMHEFSAKDIDGHMVN
LDKYRGFVSIVTNVASQCGKTEVNYTQLVDLHARYAEAGLRILAFPSNQFGK
QEEPGSNEEIKEFAAGYYNVKFDMFSKIAVNGDDAHPLWKWMKIQPKGKG**A**
AGNAI**AA**NFTKFLIPKNGAVVKRYGPMEEPLVIEKPLPHYF.

The amino acid sequence of the GPX4 mutant 3 was as follows:

MGSSHHHHHHSSGLVPRGSHMLEAASRDDWRAARSMHEFSAKDIDGHMVN
LDKYRGFVSIVTNVASQCGKTEVNYTQLVDLHARYAEAGLRILAFPSNQFGK
QEEPGSNEEIKEFAAGYYNVKFDMFSKIAVNGDDAHPLWKWMKIQPKGKGI
LGNAIKWNFTKFLIPKNGAVVKRYG**AAA**EPLVIEKPLPHYF.

The *E. coli*-preferred coding sequence (GenSmart™ Codon Optimization) of GPX4 was cloned into the pET-15b plasmid with the Xhol/BamHl digestion site (custom-clone of Ginsery) and the pET-15b-GPX4 plasmid was transformed into chemically competent BL21(DE3) *E. coli* cells. A single transformed colony was picked from a Luria-Bertani (LB)-ampicillin agar plate and inoculated into medium containing ampicillin (0.1 $mg \cdot mL^{-1}$), and the culture was incubated overnight at 37 °C. Then the cells were expanded in 150 mL LB medium containing ampicillin and incubated at 37 °C until the optical density at 600 nm went up to 0.6. When cultures were cooled to 10 °C in ice water, the expression of GPX4 was induced using 0.2 mM IPTG for 16 hrs at 18 °C with continuous shaking at 220 rpm. *E. coli* cells were collected by centrifugation at 8,000 rpm for 15 min at 4 °C, washed once with 1× PBS, and cell pellets were stored

at -80 °C.

Cell pellets were resuspended in Lysis buffer (20 mM Tris, 200 mM NaCl, 1 mM Phenyl methane sulfonyl fluoride (PMSF), 10% glycerol, pH 8.0) and sonicated using an ultrasonic crusher. After sonication, the samples were centrifuged at 10,000 rpm at 4 °C for 30 min and the insoluble part was discarded. The lysates were then loaded on 15 mL Ni-NTA agarose and purification column. The columns were washed with 1× PBS and 20 mM imidazole, and then the proteins were eluted with 1× PBS containing increasing concentrations of imidazole (20-300 mM). The GPX4 protein was eluted with 300 mM imidazole and the purified proteins were split and stored at -20 °C. WB analysis of AllCys(-)GPX4^{U46C} and GPX4 mutants were performed with His-tag antibody.

CD spectra of AllCys(-)GPX4^{U46C} and GPX4^{WT} protein

AllCys(-)GPX4^{U46C} and GPX4^{WT} proteins were diluted to 0.2 mg·mL^{-1} in 1× PBS. Circular dichroism spectra were recorded from 180 to 300 nm.

Construction of the phage display NB library

A healthy Bactrian camel was immunized with 500 μL GPX4 (1 mg·mL^{-1}) emulsified with 500 μL Freund's adjuvant. Specifically, the first immunization was conducted with a complete adjuvant and boost immunizations with an incomplete adjuvant at intervals of 3 weeks. After six times of immunization, 100 mL fresh blood was collected for isolation of PBMC (Peripheral Blood Mononuclear Cell) and extraction of total RNA to perform reverse transcription. The V_HH gene was amplified using double-nested PCR. The pComb3xss phagemid vector and V_HH genes were digested with SfiI restriction enzyme and ligated by T4 DNA ligase. The ligation products were purified and transformed into E. coli TG1 competent cells. After that the nanobody phage libraries for biological screening were obtained by culturing and infecting E. coli TG1 competent cells with the M13KO7 helper phage. The experimental camel is still alive, and this part of the experiment follows animal ethical guidelines.

Biopanning of GPX4 phage clone

After the phage library was constructed, the high-affinity anti-GPX4 NBs were selected from the phage library using the biopanning method. Briefly, 120 μL·well^{-1} of GPX4 (100 μg·mL^{-1}) was coated in three wells of a microtiter plate at 4 °C overnight, and then blocked with 2% BSA at 4 °C for 2 hrs. Then, the 120 μL·well^{-1} phage library was added to the coated well at 37 °C for 1 h. After incubating, the three wells were washed 5 times with 0.1% 1× PBST and 5 times with 1× PBS to remove unbound phage. The bound phages were eluted by 10 mg·mL^{-1} trypsin at 37 °C for 30 min. The 10 μL of eluent was diluted to determine the output titer, and the other eluent was amplified for the next biopanning. In the following second, third, and fourth rounds, a coated concentration of GPX4 was carried out (50, 25, and 12.5 μg·mL^{-1}). After the biopanning was finished, several clones were randomly picked up to test the binding with GPX4 by ELISA. The positive clones were sequenced for further analysis.

Expression and purification of NBs and NBs-R10

The amino acid sequences of selected NBs and NBs-R10 were as follows:

12E:

EVQLQESGGGSVQAGGSLRLSCAASGDTSSRYYMGWFRQAPGKEREVVAGF
TSMDGSTSYADSVKGRFTMSQDNAKNTVYLQMDSLKVEDTAMYYCATGRA
WAAGRPAYGPFDLRRYNYWGQGTQVTVGQHHHHHHGAYPYDVPDYAS

4C:

EVQLEESGGGSVQAGGSLRLSCAAPGDTSSRYYMGWFRQAPGKEREGVAGF
STGLGRTTYADSVKGRFTISQDNAKNTIYLQMNSLKVEDTAMYYCATGRAW
AAGSPMYGPFDSRRYNYWGQGTQVTVGQHHHHHHGAYPYDVPDYAS

5C:

EVQLESGGGSVQAGGSLRLSCAASGDTSSRYYMGWFRQAPGKERSGVAGIS
GGTGSAYYADSVKGRFTISQDNAKNMVYLQMNSLKVEDTAMYYCATGRA
WAAGRPMYGPFDSRRYNYWGQGTQVTVSSGQHHHHHHGAYPYDVPDYAS

5F:

EVQLVQSGGGSVQAGGSLRLSCTASGDTSSRHYMGWFRQAPGKEREGVAGI
SVGLGSTHYADSVKDRFTISQDNAKNTVYLQMNSLKVEDTAMYYCATGRP
WAAGRPAYGPFDSRRYNYWGQGTQVTVSSGQHHHHHHGAYPYDVPDYAS

12E-R10:

EVQLQESGGGSVQAGGSLRLSCAASGDTSSRYYMGWFRQAPGKEREVVAGF
TSMDGSTSYADSVKGRFTMSQDNAKNTVYLQMDSLKVEDTAMYYCATGRA
WAAGRPAYGPFDLRRYNYWGQGTQVTVSSSGSGSGRRRRRRRRRR

4C-R10:

EVQLEESGGGSVQAGGSLRLSCAAPGDTSSRYYMGWFRQAPGKEREGVAGF
STGLGRTTYADSVKGRFTISQDNAKNTIYLQMNSLKVEDTAMYYCATGRAW
AAGSPMYGPFDSRRYNYWGQGTQVTVSSSGSGSGRRRRRRRRRR

5C-R10:

EVQLESGGGSVQAGGSLRLSCAASGDTSSRYYMGWFRQAPGKERSGVAGIS
GGTGSAYYADSVKGRFTISQDNAKNMVYLQMNSLKVEDTAMYYCATGRA
WAAGRPMYGPFDSRRYNYWGQGTQVTVSSSGSGSGRRRRRRRRRR

The *E. coli*-preferred coding sequence (NovoPro, https://www.novoprolabs.com/tools/codon-optimization) of NBs was cloned into the pComb3xss phagemid vector, and the NBs-R10 were cloned into the pET-28a (+) plasmid. The expression and purification of NBs and NBs-R10 followed the same procedure as that of GPX4 mutant. WB analysis of NBs and NBs-R10 were performed with His-tag antibody.

Conjugation of NBs to Cy dyes and His-tag antibody to CoraFluor

The CoraFluor with a like NHS eater (Cora-like-NHS, Tb-NHS) was synthesized following a previously reported method[1, 2]. Cy5/Cy3-NHS and Tb-NHS were dissolved to 10 mM in anhydrous DMF. Four times excesses of Cy5/Cy3-NHS was mixed with

NBs and eight times excesses of Tb-NHS was mixed with His-tag antibody in 100 mM carbonate buffer (pH 9.0). The samples were rotated at 75 rpm and incubated overnight at 4 °C. The conjugates were purified twice with 1×PBS buffer by 7k Zeba Spin Desalting Columns. The concentrations of Cy3/Cy5 dyes, Tb and NBs and His-tag antibody were determined by the respective absorbances at 550 nm/650 nm, 340 nm and 280 nm, respectively.

Synthesis of RSL3-Cy5 and ML162-Cy5

RSL3-yne and ML162-yne were synthetized following the protocol of ref. 3[3], and then 100 μM RSL3-yne or ML162-yne was reacted with 100 μM Cy5-azide, 0.5 μL triethylamine and 50 μM CuI for 24 hrs at RT with vigorous shaking. RSL3-Cy5 or ML162-Cy5 was used in FRET assay without further purifications.

Photophysical properties of Cy3/Cy5/Tb probes

Absorption spectra and emission spectra of QD605, Cy5, Cy3 and Tb samples were recorded in 1× PBS and Reaction buffer (20 mM Tris, 50 mM NaCl, 3 mM TCEP, 0.1% Tween, pH 8.0), respectively.

Affinity test of GPX4 with NBs by NB-on-QD displacement assay (QD-to-Cy5 FRET)

NB-Cy5 (120 nM) was incubated with QD605 (6 nM) in 60 μL Reaction buffer for 1 h at RT. Then 60 μL GPX4 with different concentrations (0 nM-600 nM) were added and incubated for 2 hrs at 37 °C. The fluorescence spectra of the samples were measured with an excitation wavelength at 450 nm.

SPR measurements

CM5 chip was firstly activated using an amino coupling kit and then AllCys(-)GPX4^{U46C} or three of GPX4 mutants in sodium acetate (pH 5.0) buffer was injected onto the CM5 chip surface at a flow rate of 10 μL·min^{-1} for 15 min. The chip was then blocked with ethanolamine, and after that different concentrations of NBs (1.95 nM to

500 nM) were injected and allowed to bind for 60s, and the dissociation time was set to be 600s. Different concentrations of ML162/RSL3 (0-25 μM) were injected and allowed to bind for 120s, and the dissociation time was set to be 120s. The chip with GPX4 was regenerated with glycine (pH 2.5) for 30s at the end of each run. The maximum responses of SPR angles with different concentrations of NBs were collected and plot into dose-response curves which was then fitted with the 'One site-specific binding' model in Prism 8.0 to estimate the affinity of NBs to GPX4.

4C-Cy3 and 12E-Cy3 for selective staining of GPX4 in WB

GPX4 was resolved on a precast 15% NuPAGE Bis-Tris gel for SDS-PAGE and was then transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% skim milk in TBS-T buffer for 2 hrs at RT. It was washed three times with TBS-T and incubated with 5 μg 4C-Cy3 or 12E-Cy3 (5 mL, 66 nM) for 12 hrs at 4 °C. The images were collected with Tenon 5200 Multi gel imager.

Investigation of the binding site of NBs to GPX4 by Cy3-to-Cy5 FRET

50 nM NB-Cy3, 50 nM RSL3/ML162-Cy5, and different concentrations of GPX4 (0 nM-200 nM) were mixed and incubated in 40 μL Reaction buffer for 2 hrs at 37 °C. The FL intensities of Cy3 and Cy5 were measured in 384-well plate with the multifunctional microplate reader TECAN Spark with an excitation filter at 495 ± 5 nm and emission filters at 550 ± 5 nm for Cy3 channel and 665 ± 4 nm for Cy5 channel, respectively. The FRET ratio was calculated by dividing the intensity of the Cy5 acceptor (I_{Cy5}) by that of the Cy3 donor (I_{Cy3}) (Equation (1)). The relative FRET ratio represented the relative change in FRET ratio compared to that in the absence of GPX4 (Equation (2)).

$$FRET\ ratio = \frac{I_{Cy5}}{I_{Cy3}} \quad (1)$$

$$Relative\ FRET\ ratio = \frac{FRET\ ratio}{FRET\ ratio\ (0)} \quad (2)$$

Biomolecular modeling

The crystal structure for GPX4 was retrieved from Protein Data Bank (PDB ID: 7U4N). The structure of NBs was predicted with the state-of-the-art AlphaFold2 using ColabFold[4, 5]. To explore protein-protein interactions, PIPER was utilized to fulfill protein-protein docking of GPX4 and NBs[6]. To decide the best combination mode, we sorted and compared the output conformations with RMSD less than 0.05, based on potential energy, PIPER pose energy and PIPER pose score. The analysis of docking results was carried out using molecular graphics program PyMol[7].

Molecular dynamics simulation was conducted by using the force field ff19SB[8]. The predicted structures of 12E and 5C in complex with GPX4 were prepared by AmberTools workflow with slight changes[9]. Specifically, a time step of 1 fs was set for the minimization and equilibration stage, followed by the production stage with 4 fs as the integration time step. Periodic boundary conditions (PBC) were used in the MD simulations. The aqueous environment was simulated by centrally positioning the complexes in periodic rectangular box, maintaining a minimum distance of 10 Å away from the outer residues, so that the complexes would not interact with their periodic images, preventing the inaccurate interactions and results. Moreover, a physiological ionic concentration at 0.15M was set to simulate a physiological ionic concentration. Subsequently, restrained molecular dynamic simulation for minimization and equilibration and unstrained molecular dynamic simulation were conducted. The generation of trajectories was at a temperature of 303.15 K. VMD was used to calculated different properties to investigate the flexibility and stability[10].

Affinity test of GPX4 mutants with 12E by TR-FRET

10 nM His-tag antibody-Tb was incubated with different concentrations of GPX4 (0, 2.5, 5, 10, 20, 40, 80, 160, 320 nM) in 26 μL buffer for 30 min at 37 °C (20 mM Tris, 50 mM NaCl, 3 mM TCEP, 0.1% BSA, 0.1% Tween, pH 8.0). Then 26 μL 12E-Cy5 of 20 nM was added and incubated for 30 min at 37 °C, and the detection was based on time-gated FRET from Tb to Cy5. The time-gated intensities of Tb and Cy5 were

measured in 384-well plate with the TRF mode of multifunctional microplate reader TECAN Spark (0-400 μs time window) with an excitation filter at 340 ± 10 nm and emission filters at 550 ± 5 nm for Tb channel and 665 ± 4 nm for Cy5 channel, respectively. The FRET ratio was calculated by dividing the intensity of the Cy5 (I_{Cy5}^{TG}) by that of the Tb (I_{Tb}^{TG}) (Equation (3)). The relative FRET ratio represents the relative change in the FRET ratio compared to that in the absence of a GPX4 (Equation (2)).

$$FRET\ ratio = \frac{I_{Cy5}^{TG}}{I_{Tb}^{TG}} \tag{3}$$

Cell Culture

HEK293T and SH-SY5Y were cultured in DMEM with 10% fetal bovine serum and 1×penicillin-streptomycin solution. OS-RC-2 and L-02 were cultured in RPMI-1640 with 10% fetal bovine serum and 1×penicillin-streptomycin solution. All cells were cultured in 5% CO_2 and at 37 °C.

Confocal microscope imaging

Imaging of CPP-appended NB-R10: OS-RC-2, SH-SY5Y or L-02 cells were cultured in 15 mm confocal dishes at 37 °C and in 5% CO_2 for 12 hrs. 5 μM 12E-R10-Cy5/4C-R10-Cy5 and 10 μM TNB-R10 in complete medium were added and incubated for 1 h at 37 °C. Cells were then washed three times with complete mediums and visualized with Leica TCS SP8 confocal microscope. The fluorescence of Cy5 was excited with a 581 nm laser and detected from 580 nm to 650 nm.

Imaging of $C^{11}BODIPY^{581/591}$: OS-RC-2 cells were treated with 5 μM 12E-R10, 4C-R10 (both with the assists of 10 μM TNB-R10) or 1 μM RSL3 for 2 hrs at 37 °C, respectively. After that 1 μM $C^{11}BODIPY^{581/591}$ were added and incubated for 30 min. The fluorescence of $C^{11}BODIPY^{581/591}$ in reduced state was excited with a 581 nm laser and detected from 590 nm to 650 nm, and that in oxidized state was excited with a 488 nm laser and detected from 500 nm to 550 nm.

Cell viability assays

Cell viability assays of 12E-R10 and 4C-R10 on OS-RC-2: OS-RC-2 cells in logarithmic growth phase were collected and digested and approximately 5,000 cells in 80 μL of complete medium with or without the presence of ferrostatin-1 (1.5 μM) were seeded per well in 96-well plates. Cells were incubated overnight at 37 °C and in 5% CO_2. Then different concentrations of 12E-R10 or 4C-R10 (100, 75, 50, 25, 12.5, 6.25, 5, and 0 μM) and TNB-R10 additive (50 μM) in 20 μL of complete medium were added and incubated for 48 hrs at 37 °C and in 5% CO_2. Cell viability assays for 72 hrs' incubation of 12E-R10, 4C-R10, RSL3 and ML162 on L-02 were also tested following the same protocol. Cell viabilities were determined by CCK-8 assay.

Western blot analysis of GPX4 level in OS-RC-2 and L-02 cells

L-02 cells and OS-RC-2 cells were digested using trypsin and collected by centrifugation at 1,200 rpm for 5 min. Cell pellets were lysed using IP lysate for 30 min at 4°C, cell lysate centrifuged at 12,000 rpm for 10 min, and total protein concentration was determined by the BCA kit. The cell lysate was loaded with 50 μg of total protein, and then lysates were separated on 15% Bis-Tris gel for SDS-PAGE. Proteins were transferred to PVDF membrane, which were incubated overnight at 4°C with anti-GPX4 antibody. The membrane was washed 5 times with 1× TBST, and then it was incubated with goat anti-rabbit secondary antibody for one hour at room temperature, finally the membrane was treated with ECL detection reagent, the data were collected by Tanon 5200 Multi.

12E-R10 induced ferroptosis in OS-RC-2 cells

OS-RC-2 cells were seeded in T25 culture flasks at a density of 1×10^6 cells/flask and cultured for 24 hrs. OS-RC-2 cells were treated with different concentrations of 12E-R10/4C-R10 with 10 μM TNB-R10 and RSL3 for 4 hrs (in WB assay and GSH assay, 12E-R10 treated cells for 24 hrs). After that, cells were washed three time with 1× PBS and collected. The cellular sediments were lysed with IP lysate at 4 °C for 30 min and

centrifuged at 12,000 rpm for 10 min at 4 °C. Subsequently, the MDA assay, endogenous GPX4 activities assay, PTGS2 mRNA analysis, GSH assay and WB analysis of GPX4 levels were test.

MDA assay: the MDA in the cell lysates were determined according to the instructions of the MDA detection kit. Endogenous GPX4 activities assay: the activities of GPX4 in the lysates were determined according to the instructions of glutathione peroxidase detection kit (NADPH). *PTGS2 mRNA* analysis: total RNAs of OS-RC-2 cells were extracted according to the instructions of RNA extraction solution. cDNA was synthesized using the SweScript All-in-One RT SuperMix for qPCR kit. The RT-qPCR primer sequences were as follows:

PTGS2 forward primer: 5'-GGGTTGCTGGTGGTAGGAATG-3'

PTGS2 reverse primer: 5'-CATAAAGCGTTTGCGGTACTCAT-3'

GAPDH forward primer: 5'-GGAAGCTTGTCATCAATGGAAATC-3'

GAPDH reverse primer: 5'-TGATGACCCTTTTGGCTCCC-3'

GSH assay: the GSH was determined according to the instructions of the GSH and GSSH assay kit. GPX4 levels: the specific procedure for WB is the same as the one used for analyzing GPX4 levels in OS-RC-2 and L-02 cells.

LDH analysis: after the cell treated with 12E-R10 and TNB-R10 for 24 hrs, the LDH releaser was added to the untreated cell group as a positive control, and the LDH concentration in each group was determined with the LDH detection kit. The LDH concentration was normalized by the value of the positive control.

Exogenous GPX4 activity assay

7.5 μM $GPX4^{WT}$ and 7.5 μM 4C-R10/12E-R10/RSL3 were mixed in 10 μL Reaction buffer at 120 rpm for 1.5 hrs at 37 °C. GPX4 activity was determined according to the instructions of a glutathione peroxidase detection kit (DTNB).

Proteome profiling

Two million OS-RC-2 cells were lysed with 80 μL IP lysate for 30 min at 4 °C and then the cell lysate was centrifuged at 12,000 rpm for 10 min and the supernatant was collected for subsequent experiments. 12E-R10, 5C-R10 and 4C-R10 were labeled with biotin-NHS (with the same labeling protocol of NB-Cy3 conjugate), and the biotin labeled 12E-R10, 5C-R10 and 4C-R10 were mixed separately with streptavidin-magnetic beads for 2 hrs at RT with continuous rotating at 75 rpm. The magnetic beads were washed 5 times with 1×PBS to remove free 12E-R10-biotin 12E-R10, 5C-R10-biotin and 4C-R10-biotin. Magnetic beads conjugated with 12E-R10, 5C-R10 or 4C-R10 were resuspended in 120 μL PBS and were incubated with 80 μL of OS-RC-2 cell lysate for 12 hrs at 4 °C with continuous rotating at 75 rpm. Then they were washed again 5 times with 1× PBS, and 0.1 M glycine (pH 2.8) was added to elute the potential proteins pulled down by NBs. The eluate samples were analyzed with SDS-PAGE (silver staining) and WB.

Effects of NBs on early developmental morphology of zebrafish

Embryos obtained through natural hybridization of wild zebrafish strain (*Danio rerio*) were preserved in a standard embryo culture medium at 28.5 °C according to a previously described protocol[11]. The staging of embryos strictly adhered to established methodologies[12]. Zebrafish was reared in circulating freshwater at 28 °C and neutral pH with a light cycle of 14 hrs and a dark cycle of 10 hrs. All experimental protocols were approved by and conducted in accordance with the Ethical Committee of Experimental Animal Care, Ocean University of China. 1 nL of 7 μM 12E, 4C, RSL3 or ML162 in 1× PBS was microinjected separately into zebrafish embryos at the one-cell stage and the morphology of zebrafish embryos was observed at 26 hpf.

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