Supplementary Materials for

Engineered Extracellular Vesicle-Delivered CRISPR/Cas9 for Radiotherapy Sensitization of glioblastoma

Authors: Xiao Liu[†,‡*], Zhengcong Cao[†*], Weizhong Wang[‡*], Cheng Zou[†], Yingwen Wang[†], Luxiang Pan[†], Bo Jia[‡], Kuo Zhang[†], Wangqian Zhang[†], Weina Li[†], Qiang Hao[†], Yingqi Zhang[†], Wei Zhang[†], Xiaochang Xue[ᘔ], Wei Lin[‡#], Meng Li[†#] and Jintao Gu[†#].

[†]State Key Laboratory of Cancer Biology, Biotechnology Center, School of Pharmacy, The Fourth Military Medical University, Xi'an, 710000, China.

[‡]Department of Neurosurgery, Xijing Hospital, Xi'an, 710000, China.

[ᘔ]The Key Laboratory of Medicinal Resources and Natural Pharmaceutical Chemistry, The Ministry of Education, College of Life Sciences, Shaanxi Normal University, Xi'an, 710000, China.

[*]These authors contributed equally to this work

[#]Corresponding author

Jintao Gu, MD., State Key Laboratory of Cancer Biology, Biotechnology Center, School of Pharmacy, Fourth Military Medical University, Xi'an, 710000, China (gujintao@fmmu.edu.cn)

Meng Li, MD., State Key Laboratory of Cancer Biology, Biotechnology Center, School of Pharmacy, Fourth Military Medical University, Xi'an, 710000, China (limeng@fmmu.edu.cn)

Wei Lin, MD., Department of Neurosurgery, Xijing Hospital, Xi'an, 710000, China (linwei@fmmu.edu.cn)

Materials

Colony formation and soft agar assays

LN229 and GBM02 cells (1×10^3 per well) were seeded into a 12-well plate in DMEM supplemented with 3% Noble agar (BD Biosciences) and 10% FBS on top of a 0.6% agar DMEM medium. After incubation for 3 weeks, formed colonies with a diameter of at least 500 μm were counted under a digital microscope. The data are means ± SD from triplicate wells.

Immunoblot

Total protein was extracted using lysis buffer from cells and concentrations were determined using a BCA protein assay kit (Thermo Scientific, USA). After being boiled for 10 minutes in a loading buffer, the protein samples were separated using SDS-PAGE and then transferred onto PVDF membranes. Block with 5% skimmed milk, incubate with primary antibody overnight at 4°C, wash 3 times with TBST, and incubate with secondary antibody. Finally, the membranes were examined with a Tanon 5200 imaging system (Tanon, Shanghai, China).

Histology and immunohistochemistry (IHC)

Tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Sections were then processed for H&E and IHC. Use primary antibodies specific for GPX4, GSS, 4-HNE and PTGS2.

In vivo safety evaluation

Ten female BALB/c mice were randomly divided into two groups. One group received an intravenous injection of Ang/TAT-sgGSS-EVs and the other group was treated with saline. Blood samples and major organ tissues were collected at 24 h after the last administration, for hematologic and histochemistry

analysis. The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr) and blood urea nitrogen (BUN) levels were analyzed by Chemistry Analyzer (Hitachi 7080). Major organs such as brain, heart, lung, liver, spleen, and kidney were fixed with paraformaldehyde for 48 h and embedded in paraffin. Levels of five major inflammatory cytokines in healthy mice treated with or without Ang/TAT-sgGSS-EVs were detected by ELISA kit. All animal studies were performed in accordance with protocols approved by the Ethical Committee and Institutional Review Board of Fourth Military Medical University.

Bioinformatics analyses of public glioma database

The gene expression in human glioma and patient survival information were analyzed using gene-profiling data from the TCGA, Gravendeel, Rembrandt and CGGA database through GlioVis (http://gliovis.bioinfo.cnio.es/).

The Cancer Genome Atlas (TCGA), initiated by National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI) in 2006, molecularly characterized over 20,000 primary cancer and matched normal samples spanning 33 cancer types, generated over 2.5 petabytes of genomic, epigenomic, transcriptomic, and proteomic data. Chinese Glioma Genome Atlas (CGGA) database contains clinical and sequencing data of over 2,000 brain tumor samples from Chinese cohorts, and is equiped with a user-friendly web application for data storage and exploration. Gravendeel and Rembrandt database are two public online databases for glioma.

Kyoto Encyclopedia of Genes and Genomes (KEGG) is an online database specialized in analyzing the metabolic pathways of gene products in cells and the functions of these gene products. KEGG enrichment analysis is used to screen out genes and pathways that have significantly changed in the experimental group. Gene Set Enrichment Analysis (GSEA) is an enrichment analysis based on gene sets, which results reveal whether the overall trend of all genes in a gene set is up or down.

EVs isolation

FBS used in the present study was depleted of EV through overnight centrifugation at 120,000 g. The EVs were incubated in the fluorescent dye DID working fluid (1:300, dilute with PBS) for 20 min at 37℃, then ultracentrifuged at 120,000×g, 90 min to remove the unbounded dye. After being washed twice in PBS with 120,000×g centrifugation, the labeled EVs were resuspended in PBS prior to use.

Preparation of Ang/TAT-EVs

To establish the brain tumor-targeting and cell-penetrating dual-functional EVs simultaneously expressing the Ang peptide (TFFYGGSRGKRNNFKTEEYC) and TAT peptide (YGRKKRRQRRRC), we fused the targeting peptides Ang and TAT to the extra-extracellular vesicles N terminus of Lamp2b protein reported to be present abundantly in EV membranes, generating Ang-Lamp2b-FLAG and TAT-Lamp2b-HA fragments. Then, the fragments were inserted into the lentiviral vector pLVX-IRES-Puro (Ang-Lamp2b-FLAG) and pLVX-IRES-G418 (TAT-Lamp2b-HA), respectively. The Ang-Lamp2b-FLAG and TAT-Lamp2b-HA were infected into the 293T cells with a lentivirus transfection system. Subsequently, infected cells were subjected to pressure screening using puromycin and G418 to ensure that each cell was infected with both viruses. Then the culture medium was replaced with serum-free DMEM for 48h and EVs were isolated using ultracentrifugation.

Preparation of Ang/TAT-sgGSS-EVs

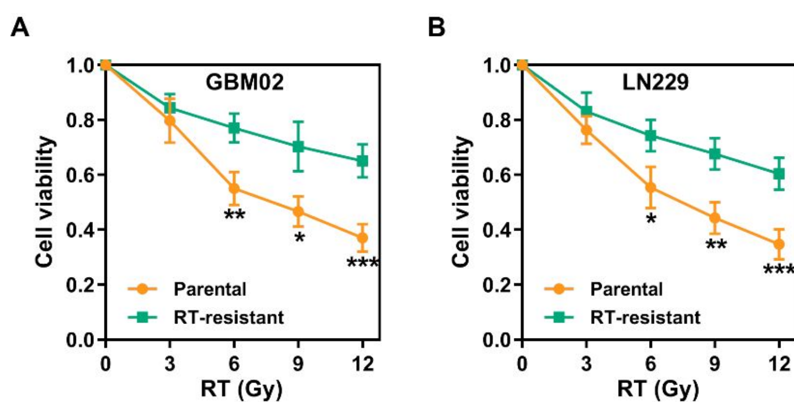
To prepare Ang/TAT-sgGSS-EVs, the pellet was resuspended in electroporation buffer (1.15 mM potassium phosphate (pH 7.2), 25 mM potassium chloride, and 21% OptiPrep working solution). The suspended EV were then filtered through 0.22-μm filters. To prepare EV ribonucleoprotein (RNP) complexes, Cas9 proteins were mixed well with sgRNA to from RNP complexes. Then, RNP complexes were added to EV at a weight ratio of 1:3, and the mixture was electroporated to form EV RNP complexes. After electroporation at 110 V for 4 ms, with an interval for 10 ms, and pulse duration for 50 ms at 25 V, pulse separation at 50 ms, and 10 pulses by Gene Pulser Xcell (Bio-Rad), EVs were centrifuged at 800 g for 5 min, 3000 g for 15 min, and 10,000 g for 1 hour, respectively. The resulting supernatant was subjected to ultracentrifugation at 100,000 g, 4°C for 2 hours and the precipitation was resuspended in cold PBS solution.

Encapsulation efficiency of electroporation

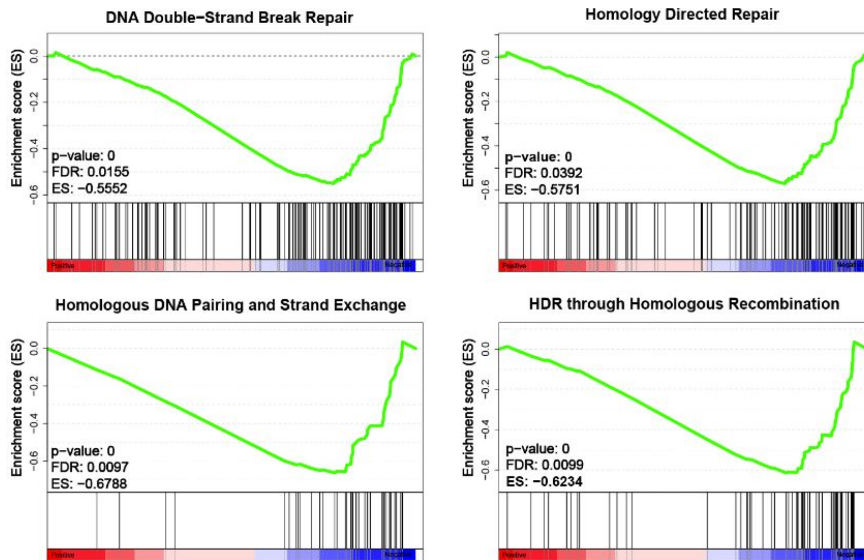
To assess the encapsulation efficiency of electroporation, we generated a standard curve via immunoblot. The gray value was plotted on the y-axis, while the grams of Cas9 protein were plotted on the x-axis, and a corresponding formula was obtained (Y=0.02092X-0.2050). Then, we added 400 ng of Cas9 protein to the electroporation reaction systems. After electroporation, we separated the EVs according to the above method and extracted the EVs protein.

T7EI assays, Sanger sequencing and NGS

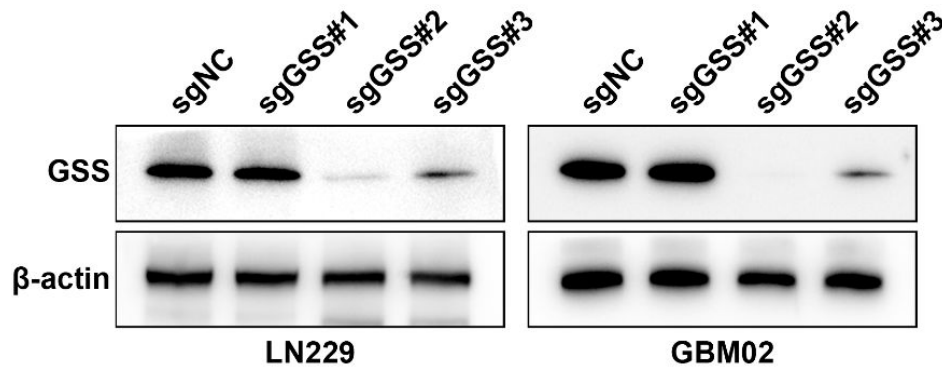
The T7EI experiment was used to evaluate the editing efficiency of target genomic loci. The cells or tissues were collected to extract the DNA through the Cell/Tissue DNA Isolation Mini Kit (Tsingke Biotechnology). PCR amplifies each specific target locus using a mini DNA extraction kit (Tsingke Biotechnology). Afterward, a standard T7EI assay was performed according to the manufacturer's protocol. Briefly, 200 ng of purified polymerase chain reaction (PCR) product was denatured and re-annealed in 2 μl NEBuffer 2 (10x) using the following protocol: 95 °C for 5 minutes; 95° to 85°C at -2 °C/s; 85°~25°C, -0.1°C/s; then store at 4°C. Then add 1 μl T7EI to the annealed PCR product and incubate at 37°C for 1 hour. Products were analyzed on 2% agarose gels and imaged on a Bio-Rad imaging system.



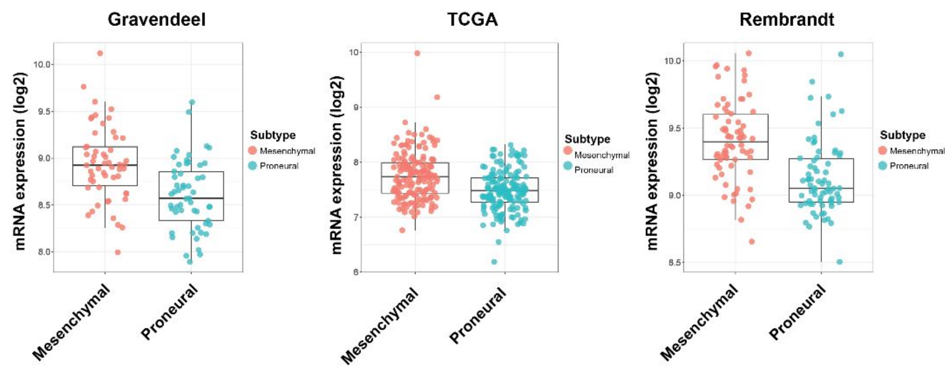
Supplementary Figure 1. (A-B). Detection of cell viability after radiation treatment in GBM02 and LN229 cells.



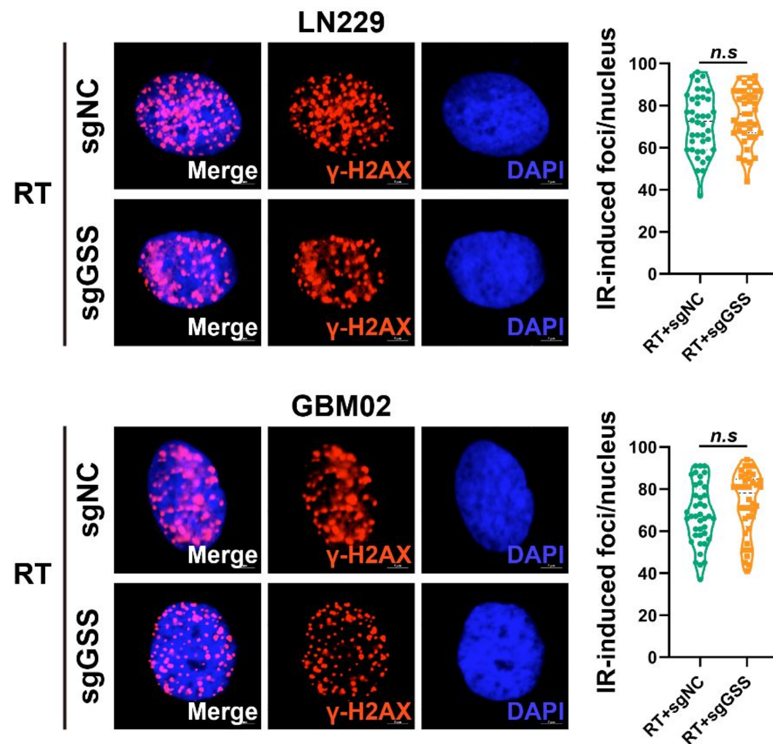
Supplementary Figure 2. GSEA analysis of DNA damage repair related pathways.



Supplementary Figure 3. Western blot was used to detect the knockout efficiency of sgGSS.

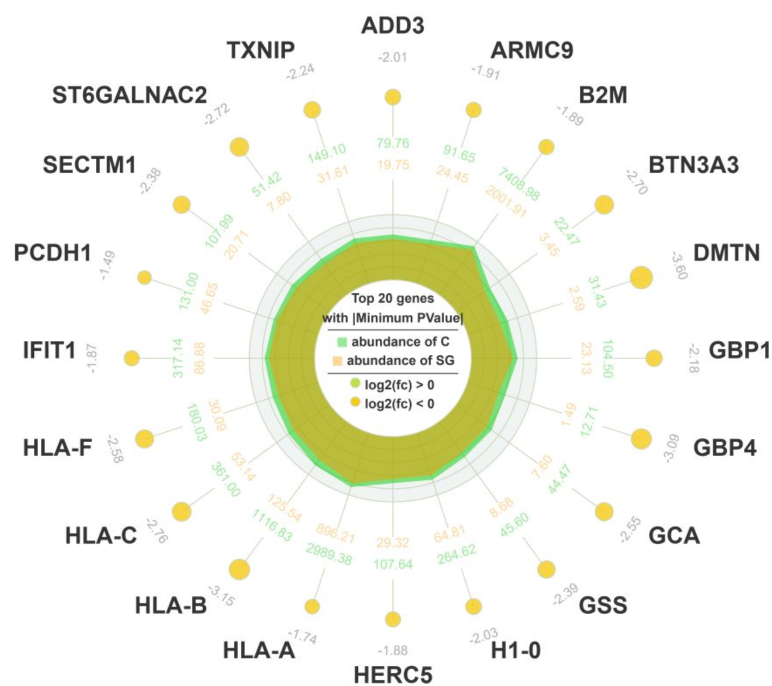


Supplementary Figure 4. GSS expression level in mesenchymal (MES) and proneural (PN) subtypes glioma.

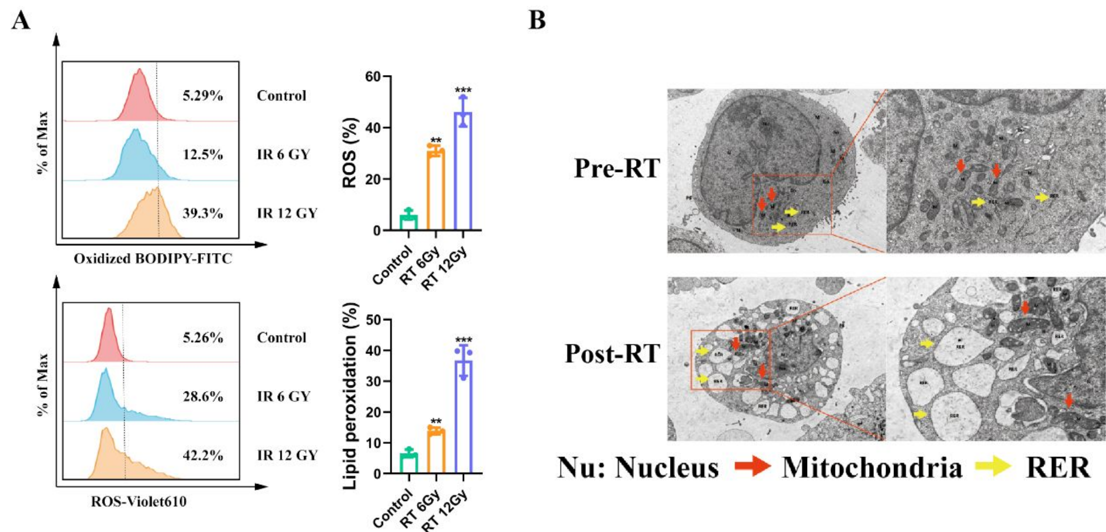


Supplementary Figure 5. Representative immunofluorescence images showing staining of phospho-H2AX foci (red) and nuclei counterstained with DAPI (blue) in the indicated cells.

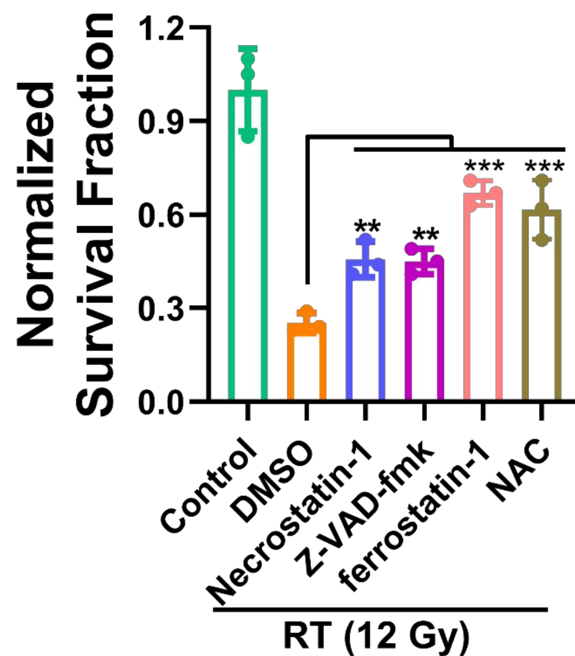
Supplementary Figure 6. The effect of GSS deletion on DNA damage response signaling.



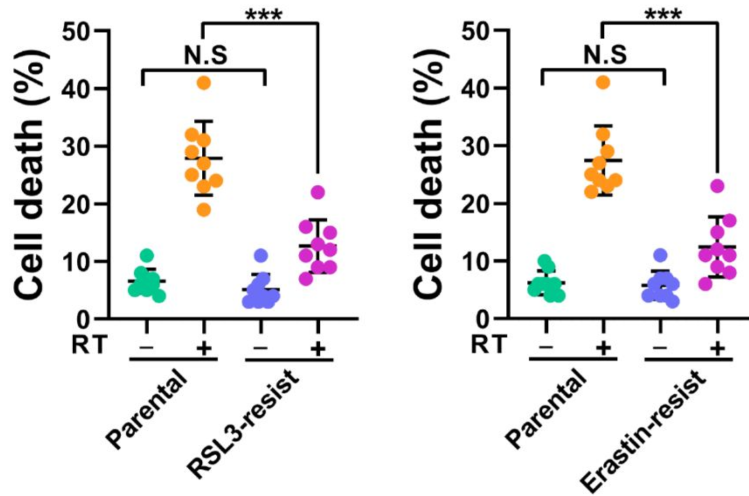
Supplementary Figure 7. The effects of GSS deletion on global transcriptional patterns in LN229 cells.



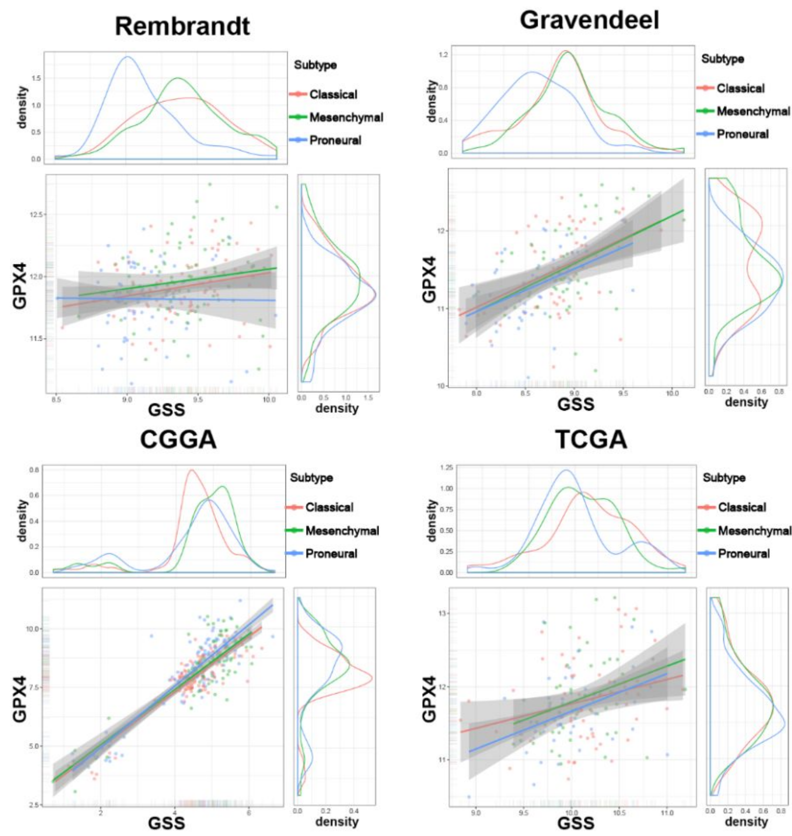
Supplementary Figure 8. (A) RT induced lipid peroxidation and ROS production in the tested LN229 cells. **(B)** Transmission electron microscopy images of LN229 cells without RT (control) or at 24 h after exposure to 6 Gy of RT. Nu, nucleus; red arrows, mitochondria; yellow arrows, autophagosomes.



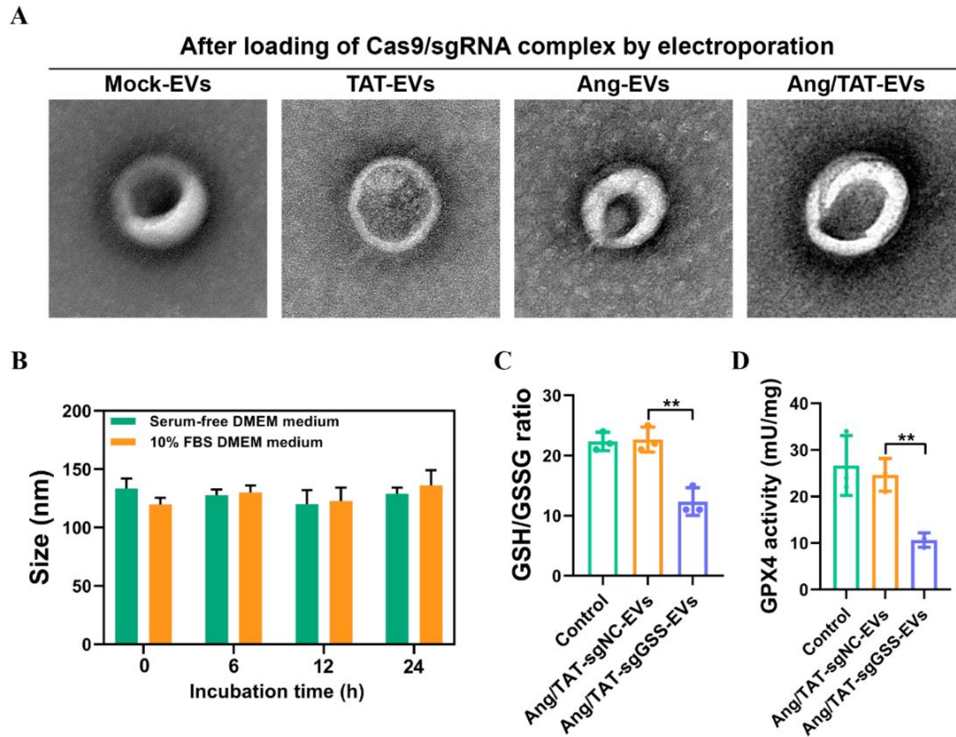
Supplementary Figure 9. Survival assay in LN229 cells that were pretreated with 2 μM necrostatin-1s, 2 μM Z-VAD-fmk, 2 μM ferrostatin-1, 2 mM N-acetyl-L-cysteine, or DMSO for 24 h followed by exposure to 12 Gy of RT.



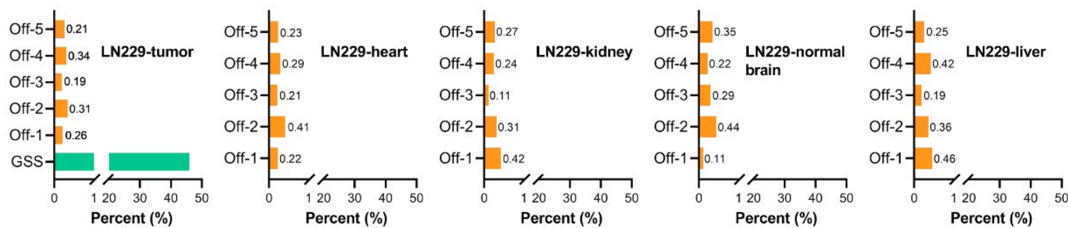
Supplementary Figure 10. RSL3/erastin-resistant LN229 cells were resistant to radiotherapy.



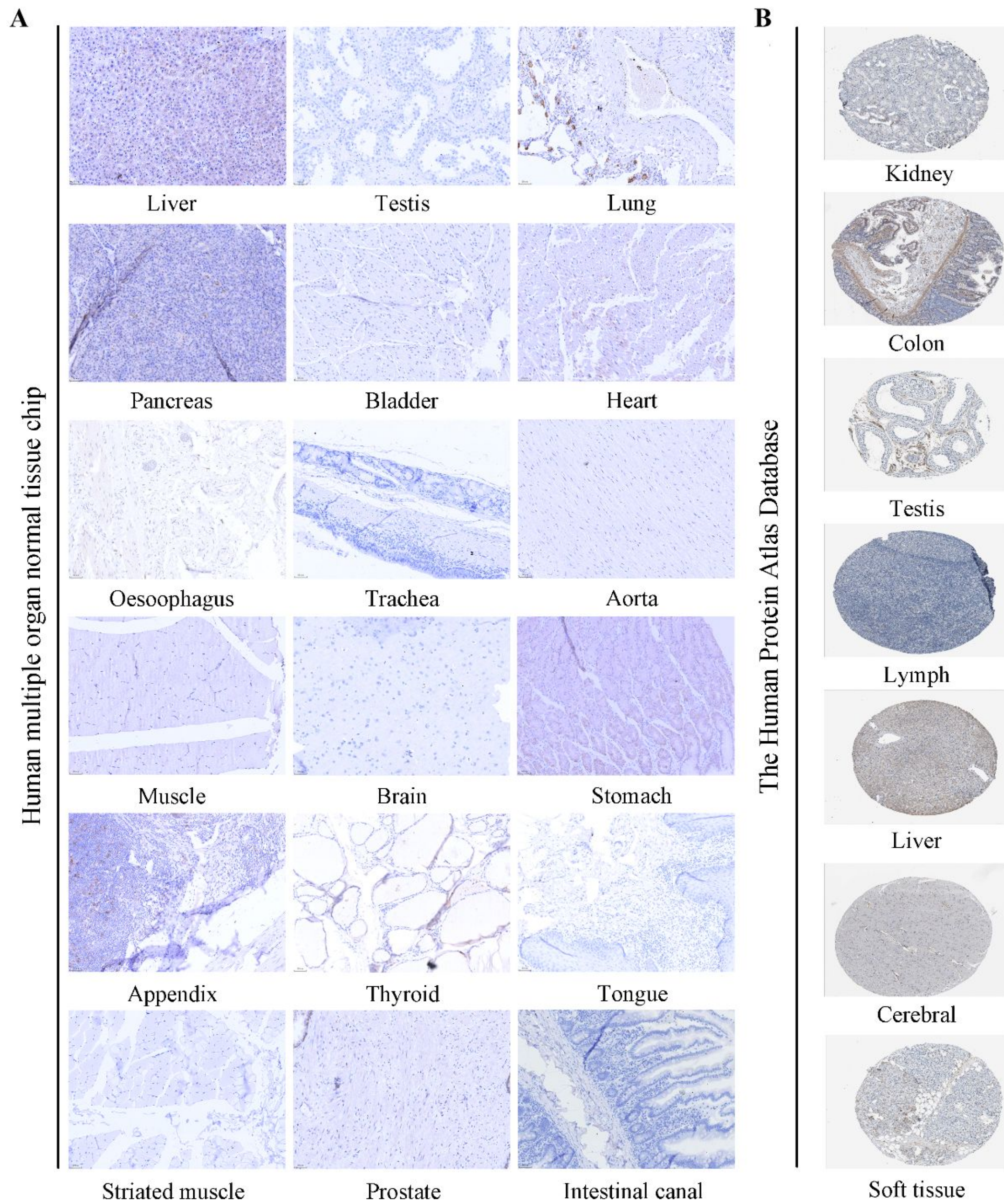
Supplementary Figure 11. GPX4 expression was positively correlated with GSS level in Gravendeel, Rembrandt, TCGA and CGGA databases.



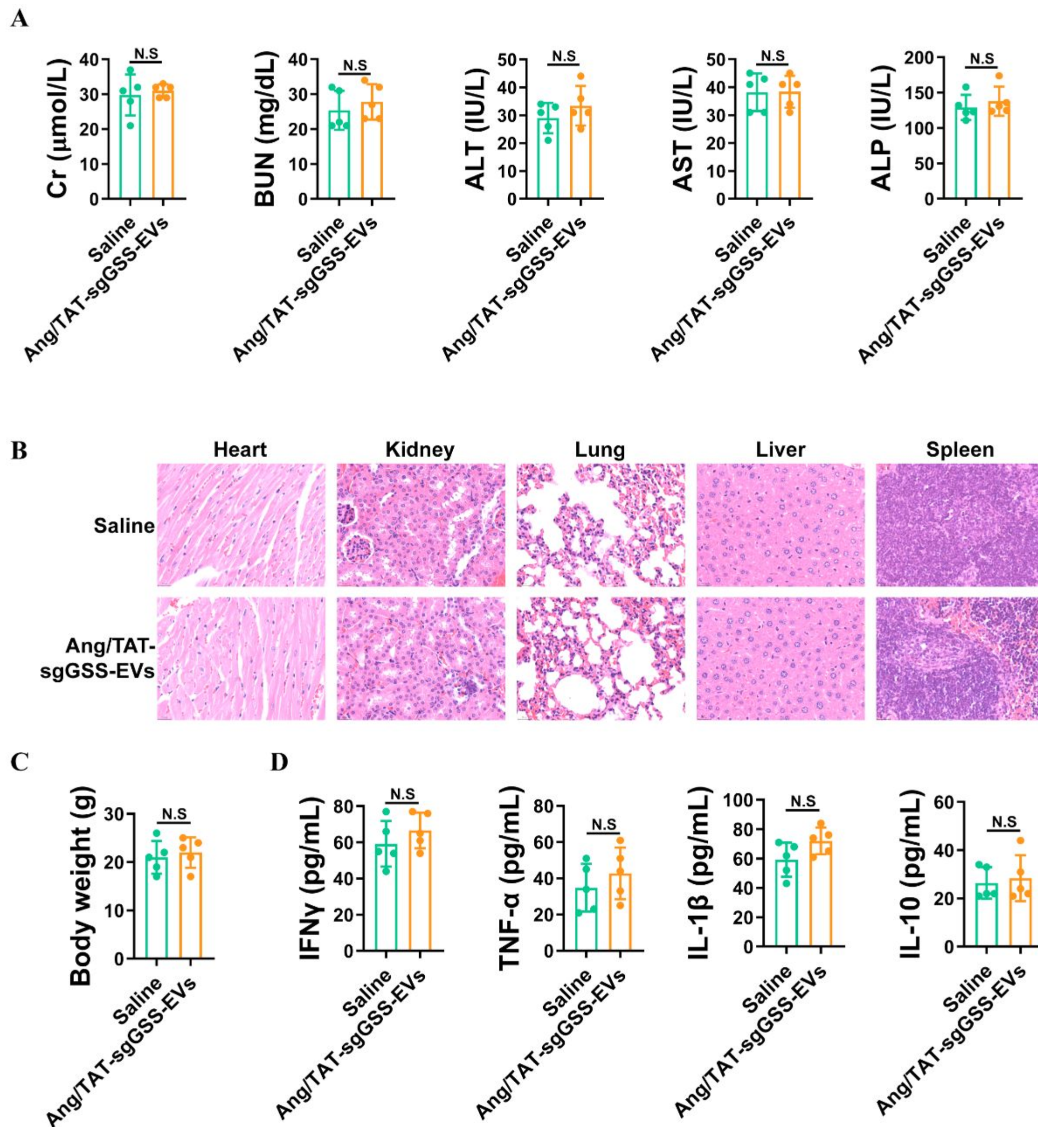
Supplementary Figure 12. (A) Detection of the size and morphology of EVs after loading of Cas9/sgRNA complex by electroporation. **(B)** The particle size variation of Ang/TAT-EVs in the culture DMEM medium with or without serum. **(C)** The effect of Ang/TAT-sgGSS-EVs on the ratio of GSH/GSSG. **(D)** The effect of Ang/TAT-sgGSS-EVs on the activity of GPX4.



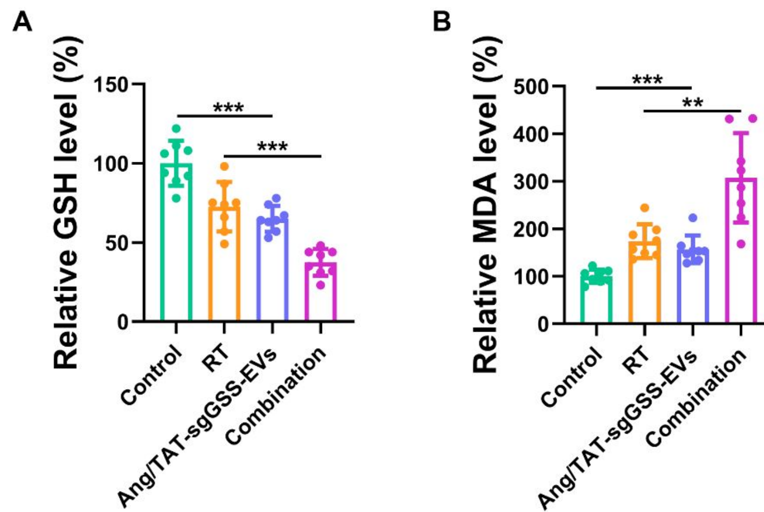
Supplementary Figure 13. Mutation frequencies of off-target sites (tumor, normal brain tissue, liver, and kidney) in LN229 tumor–bearing mice treated with Ang-sgGSS-EVs (1.5 mg of Cas9 equiv./kg). Each value was determined from a single deep sequencing library prepared from genomic DNA.



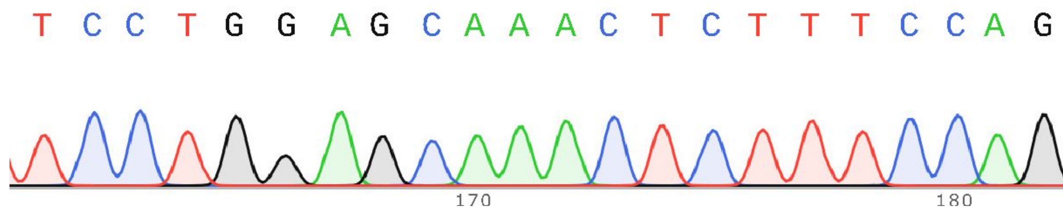
Supplementary Figure 14. (A) The expression of LRP-1 receptor in 18 normal systemic organs as detected by Human multiple organ normal tissue chip. **(B)** The expression of LRP-1 in The Human Protein Atlas (http://www.proteinatlas.org).



Supplementary Figure 15. (A) Blood biochemistry analysis and blood parameter analysis of healthy BALB/c mice treated with Ang-sgGSS-EVs or saline. Data are presented as means ± SD (n = 5). N.S. represents non-significance. **(B)** H&E staining of the liver, Heart, Lung, Spleen and Kidney sections from the mice after the specified treatments. **(C)** Body weight changes of healthy BALB/c mice treated with Ang/TAT-sgGSS-EVs or Saline. **(D)** Levels of four major inflammatory cytokines in healthy mice treated with Ang/TAT-sgGSS-EVs or Saline.



Supplementary Figure 16. (A) Relative level of GSH. **(B)** The levels of lipid peroxidation products (MDA).



Supplementary Figure 17. Sequencing results of the GSS gene in GSC05.

Supplementary Table 1. Sequences of sgRNAs used in this study.

Nucleic Acid ID	Sequences (5'-3')
sgGSS-1	**GGTCTCTGGACCAAGACCGA**
sgGSS-2	**GGAAAGAGTTTGCTCCAGGA**
sgGSS-3	**CCTCTCTAATAATCCCAGCA**

Supplementary Table 2. Off-target sequences.

Off-target sequences	Off-target（Human）	AGAATAATTTTGCTCCAGGA
	Off-target（Human）	GGAAAGGGTCTGCTCGAGGA
	Off-target（Human）	TTAAAGTGTTGGCTCCAGGA
	Off-target（Human）	AAAAAGATTTTCCTCCAGGA
	Off-target（Human）	AGAAGGAGTTTAATCCAGGA