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Supplementary Materials for

Blood-brain barrier–penetrating single CRISPR-Cas9 nanocapsules for effective and safe glioblastoma gene therapy

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Materials

Acrylate guanidine was synthesized as we have described previously. (*Advanced Materials*, 2019, 31, 1903277) N,N'-bis(acryloyl) cystamine, N,N'-methylene bisacrylamide, chloral hydrate, D-luciferin potassium salt were supplied by J&K (Scientific, Beijing, China), acrylate polyethylene glycol (acryl-PEG, M_n = 2000 Da), and acrylate polyethylene glycol succinimidyl carboxymethyl ester (acryl-PEG-NHS, M_n = 2000 Da) were purchased from Jenkem Technology (Beijing, China). Angiopep-2 (TFFYGGSRGKRNNFKTEEYC) was obtained from China Peptide Co., Ltd. (Suzhou, China). Lipofectamine was purchased from Invitrogen (USA), polyethyleneimine was supplied by Sigma (USA), cell culture media, antibiotics, and fetal calf serum were purchased from Gibco BRL (Gaithersberg, MD, USA). PLK1 siRNA sequence: 5'-UGA AGA AGA UCA CCC UCC UUA dTdT-3' (sense); 5'-UAA GGA GGG UGA UCU UCU UCA dTdT-3' (antisense). T7 RNA polymerase, EnGen Cas9 NLS, *S. pyogenes*, and BstAP were purchased from New England Biolabs (NEB, Ipswich, MA, USA).

Characterization

Nanocapsule size and zeta potential were determined at 25 °C using dynamic light scattering (DLS; Zetasizer Nano-ZS, Malvern Instruments) in triplicate measurements. The structure of the nanocapsules with or without glutathione (GSH, 10 mM) treatment was examined by transmission electron microscopy (TEM; JEM-2010HT, Japan). 10 μL of nanocapsules solution was deposited onto a glow-discharged carbon-coated copper grid. After 10 min, the grid was washed with two drops of distilled water, and a drop of 1% uranyl acetate stain was added to the grid. The grid was then dried and visualized by TEM.

Synthesis of Cas9 nanocapsules

Cas9 (16.5 μg) and sgRNA (4.0 μg) were mixed at a molar ratio of 1:1.2 in 500 μL HEPES buffer (10 mM, pH 7.4) and incubated at room temperature for 5 min. 5 μg of acrylate guanidine dissolved in dimethylsulfoxide solution (1 mg/mL) was added and reacted for 5 min, followed by addition of the degradable crosslinker cysteamine bisacrylamide and angiopep-2 decorated PEG-acryl (Ang-PEG-acryl). The latter reagent was prepared by reacting angiopep-2 (132 μg, 0.06 μmol) with NHS group of the NHS-PEG-acryl (44 μg, 0.02 μmol) via an amidation reaction. The molar ratio of Ang-PEG-acryl, guanidine, and cysteamine bisacrylamide was 1:1:1. Polymerization was initiated by adding 3 μL of ammonium persulfate (1 mg/mL) and 1% (v/v) N, N, N', N'-tetramethylethylenediamine. The polymerization was allowed to proceed for 90 min at 4 °C. The mixture was then centrifuged, followed by the PBS (10 mM, pH 7.4) re-dispersion/washing to remove unreacted monomers, initiators and Ang-PEG-acyl. Non-degradable control nanocapsules were fabricated similarly, except that the non-degradable crosslinker hexamethylene diacrylate was used.

Cell culture

U87MG, U251, GL261 and CT2A cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone). CSC2 and 83NS

GSCs were cultured in DMEM/F-12 supplemented with B27 (Invitrogen), EGF (10 ng/mL, R&D Systems), and bFGF (5 ng/mL, R&D Systems).

Flow cytometry and confocal microscopy

Flow cytometry and confocal microscopy. Alexa Fluor 647-labeled Cas9 (AF647-Cas9) protein and scrambled sgRNA were used for flow cytometry and confocal microscopy studies. For flow cytometry, U87MG, U251 and CT2A cells were seeded in a 24-well plate at 2×10^5 cells/well for 24 h. After that, the medium was replaced, and ANC_{SS}(Cas9/sgRNA), ANC (Cas9/sgRNA), NC_{SS}(Cas9/sgRNA), or free Cas9/sgRNA were added (AF647-Cas9: 20 nM), and the cells were incubated at 37 °C for 4 h. The cells were then collected, washed three times with PBS, and analyzed by flow cytometry. Fluorescence histograms were recorded with a BD FACS Calibur flow cytometer (Becton Dickinson, USA) with excitation 647 nm and emission at 666 nm, and were analyzed based on 10,000 gated events using Cell Quest software. U87MG cells cultured in 500 μL PBS were used as a control.

For confocal microscopy, U87MG, U251 and CT2A cells cultured on microscope slides in 24-well plates at 1×10^5 cells/well that were incubated with ANC_{SS}(Cas9/sgRNA), ANC(Cas9/sgRNA), NC_{SS}(Cas9/sgRNA), or free Cas9/sgRNA (AF647-Cas9: 20 nM) at 37 °C. After 4 h incubation, the culture medium was removed, and the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min and washed with PBS. The cytoskeleton was stained with Alexa 488-phalloidin (Sigma, USA) for 30 min. Cell nuclei were stained with Hoechst 33342 for 10 min and washed three times with PBS. Fluorescence images were obtained using a confocal microscope (Zeiss 880, 63× magnification).

Animal models

All animal handling protocols and experiments were approved by the Medical and Scientific Research Ethics Committee of Henan University School of Medicine (PR China) (HUSOM-2020-177). BALB/c nude mice (female, 6-8 weeks) were purchased from Charles River Laboratories. Orthotopic U87MG glioblastoma bearing mouse model were established with a high success rate of nearly 100% via implantation of minced glioblastoma tissue into the left striatum of BALB/c nude mice, as we previously reported. (*Advanced Materials*, 2020, 32, 200416) Briefly, U87MG-Luc cells (1×10^7) suspended in 50 μL of 0.9% NaCl were injected into the flank of nude BALB/c mice. When the subcutaneous tumor grew to ~200 mm^3, the mouse was sacrificed to harvest the subcutaneous tumor. Subsequently, ~2 mg of minced subcutaneous tumor tissue was implanted into the left striatum (2 mm lateral to the bregma and 3 mm deep) of anesthetized (chloral hydrate, 80 mg) animals using a 24# trocar and a custom-made propeller. The burr hole was filled with bone wax (Johnson & Johnson International, Brussels, Belgium) and the scalp was closed with tissue glue (3M Animal Care Products, St Paul, Minnesota, USA). The GL261, 83NS and CSC2 GSCs models were established similarly, except GL261, 83NS or CSC2-Luc cells (1×10^5) were transplanted instead of tumor tissues. 83NS and CSC2 GSCs models were established in previous work. (*PLOS Biology*, 2015, 13, e1002152; *Oncotarget*, 2014, 5, 6756-6769) The growth of the tumor was monitored by bioluminescence using an IVIS (Lumina III; Caliper, MA, USA), starting 10 min after the mice were injected with chloral hydrate (5% w/v) at a dose of 62.5

mg/kg combined with the luciferase substrate D-luciferin potassium (15 mg/mL in PBS) at 75 mg/kg.

Pharmacokinetics

Alexa Fluor 647-labeled Cas9 protein and scrambled sgRNA were used for pharmacokinetics, *ex vivo* imaging, and biodistribution studies. ANC_{SS}(Cas9/sgRNA), ANC(Cas9/sgRNA), NC_{SS}(Cas9/sgRNA) or free Cas9/sgRNA (1.5 mg Cas9 equiv./kg) in 200 μL of PBS were *i.v.* injected into BALB/c mice via the tail vein (n=3). At prescribed time points post-injection, 50 μL of blood was removed from the eye socket of each mouse. The blood samples were immediately dissolved in 0.1 mL of lysis buffer (1% Triton X-100). Alexa Fluor 647-Cas9 was extracted via centrifugation (14.8k rpm, 30 min). The Alexa Fluor 647 level in the supernatant was determined by a multifunctional microplate reader (SpectraMax i3x). Blood circulation followed a typical two-compartment model: a rapid decline in the distribution phase and long period elimination phase. The half-lives of the two phases ($t_{1/2},\alpha$ and $t_{1/2},\beta$) were calculated using Origin 8 SR6 software by fitting the experimental data using an exponential decay 2 model: $y = A1 \times \exp(-x/t1) + A2 \times \exp(-x/t2) + y0$, then taking $t_{1/2},\alpha = 0.693 \times t1$ and $t_{1/2},\beta = 0.693 \times t2$.

Statistical analysis

Results were analyzed by using GraphPad Prism software. Differences between two groups were assessed using unpaired t tests. For comparisons of three or more groups, differences among means will be analyzed using one-way analysis of variance (ANOVA) with the different treatments as the independent factor followed by Bonferroni (Dunn) post-hoc testing will test pair-wise comparisons between means. The level of statistical significance was set at $p < 0.05$. $*p < 0.05$ was considered significant, and $**p< 0.01$, $***p< 0.001$ and $****p<0.0001$ were considered highly significant. All data were expressed as mean ± SD unless otherwise indicated.

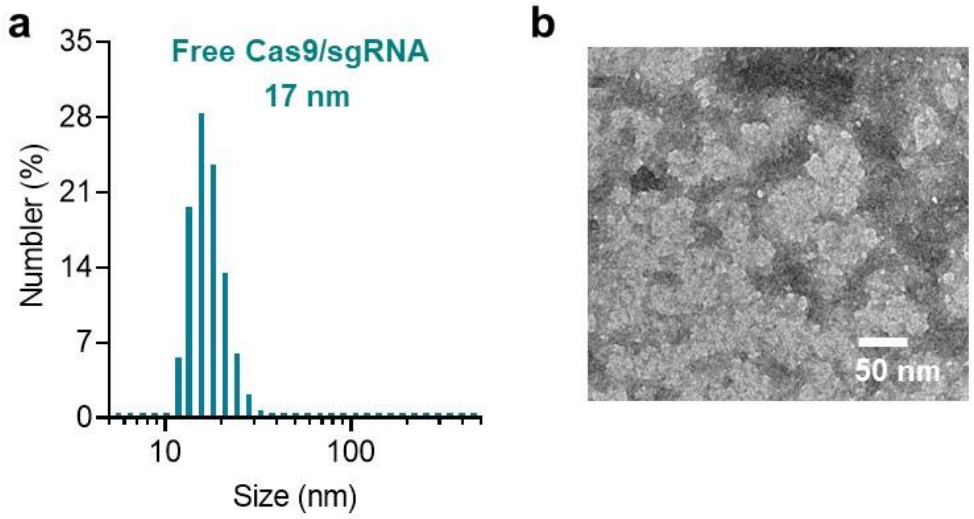
Supplementary Table 1. Size, size distribution, and surface charge of the nanocapsules or free Cas9/sgRNA.

Particle	Diameter (nm)	PDI	Zeta potential (mV)
ANC_{SS}(Cas9/sgRNA)	31	0.25	+ 4.1
ANC (Cas9/sgRNA)	35	0.23	+ 3.3
NC_{SS}(Cas9/sgRNA)	33	0.27	+ 2.4
Free Cas9/sgRNA	17	0.56	- 22.8

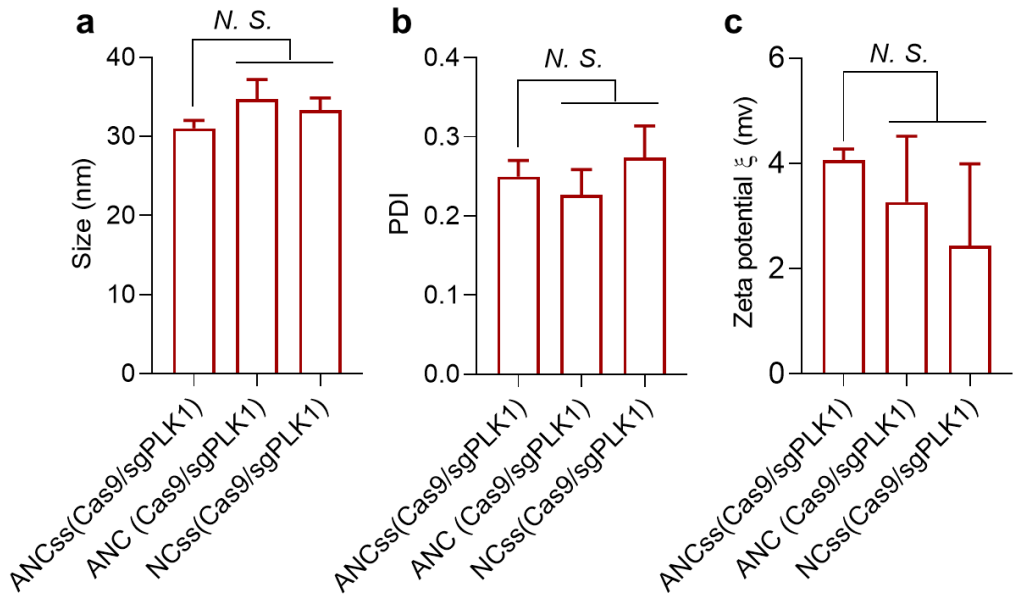
Supplementary Table 2. Sequencing of primers.

		Sequence
Primers for sgRNA transcription template	T7 pro-gRNA-Luc F	5'-TAATACGACTCACTATAGGGATAAATAACGCGCCCAACACGTTTTAGAGCTAGAAATAGCAAG-3'
	T7 pro-gRNA-PLK1 F	5'-TAATACGACTCACTATAGGGTACCTACGGCAAATTGTGCT TTTTAGAGCTAGAAATAGCAAG-3'
	T7 pro-scramble-gRNA F	5'-TAATACGACTCACTATAGGGCACGGGCAGCTTGCCGGGTTTTAGAGCTAGAAATAGCAAG-3'
	Constant-R	5'AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3'
Off-target sequences	Off-Target 1	TCCCAGCGGCAAACTGTGCT
	Off-Target 2	TTTTTACTGCAAATTGTGCT
	Off-Target 3	CACCAACGACAATTTGTGCT
	Off-Target 4	CACCCACGGCAGACTGTGCT
	Off-Target 5	AACCTACAGCCTATTGTGCT
	Off-Target 1 (mouse)	CAACTCCTGCAAATTGTGCT
	Off-Target 2 (mouse)	TTCCTCTGGCTAATTGTGCT
	Off-Target 3 (mouse)	TGCCTAAGGCAAAAGGTGCT
	Off-Target 4 (mouse)	TAAGTACAGCATATTGTGCT
	Off-Target 5 (mouse)	TAGCTTCTGCAAGTTGTGCT
Primers	PLK1-F	GAGAAGGGGTGCTGCGAATG
	PLK1-R	CTTTCTCCCAGCCTCCTCCAA
	Off-Target 1-F	AGTTCAAGTTCAGGGCCAGG
	Off-Target 1-R	CGGTTTTCAGCACACCATCC
	Off-Target 2-F	TGCCAATTGCACGTTTTAGGT

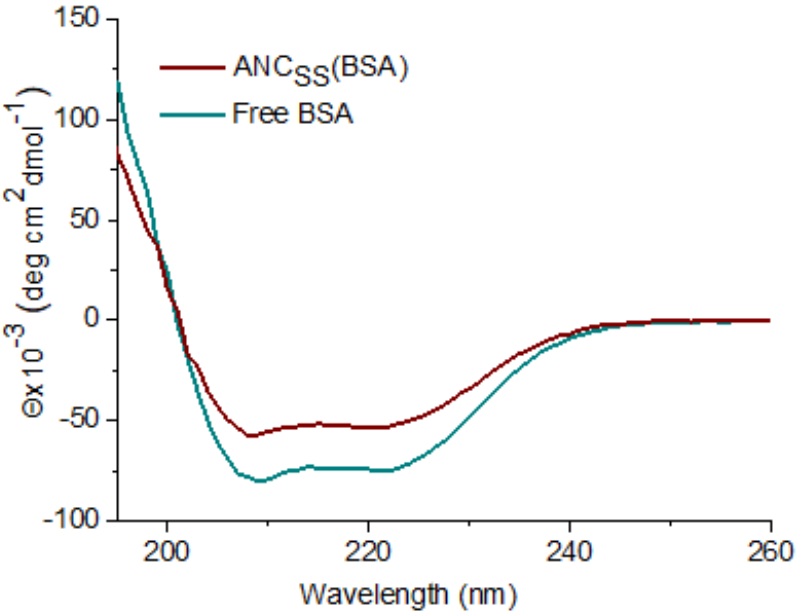
	Off-Target 2-R	AAGCTGTAGACCCTTTTTACG
	Off-Target 3-F	TGAATAGGTGGTGGTGGGAGTA
	Off-Target 3-R	AGCATGTGATATCAGTCCCCAC
	Off-Target 4-F	TTCTTTCAAGCCCAAGGGGG
	Off-Target 4-R	TGTGCCACTGTGATTCCTCC
	Off-Target 5-F	CCCCCACAGTTTCGTTTCCT
	Off-Target 5-R	GCGTGTACATCCCTGTTTGC
	Off-Target 1 (mouse)-F	ATAGACCACAGAAAGCGCCC
	Off-Target 1 (mouse)-R	CCAGGATCTGTCAGTGGCTG
	Off-Target 2 (mouse)-F	CTCAAAGCCACGCAAAGACC
	Off-Target 2 (mouse)-R	ATCAAGGACCTGTGGCTCTC
	Off-Target 3 (mouse)-F	TCAGCCCTCCTTGCTAGACT
	Off-Target 3 (mouse)-R	ACTTCCAGTTGGCCTTGCAT
	Off-Target 4 (mouse)-F	CTTCTACTCCGCACAGCCA
	Off-Target 4 (mouse)-R	TGCAGGACAGACGTTTGGAAAT
	Off-Target 5 (mouse)-F	AGATAAACAAGGCCTGCGGA
	Off-Target 5 (mouse)-R	GGGTGATTCCCACAGCACTAA



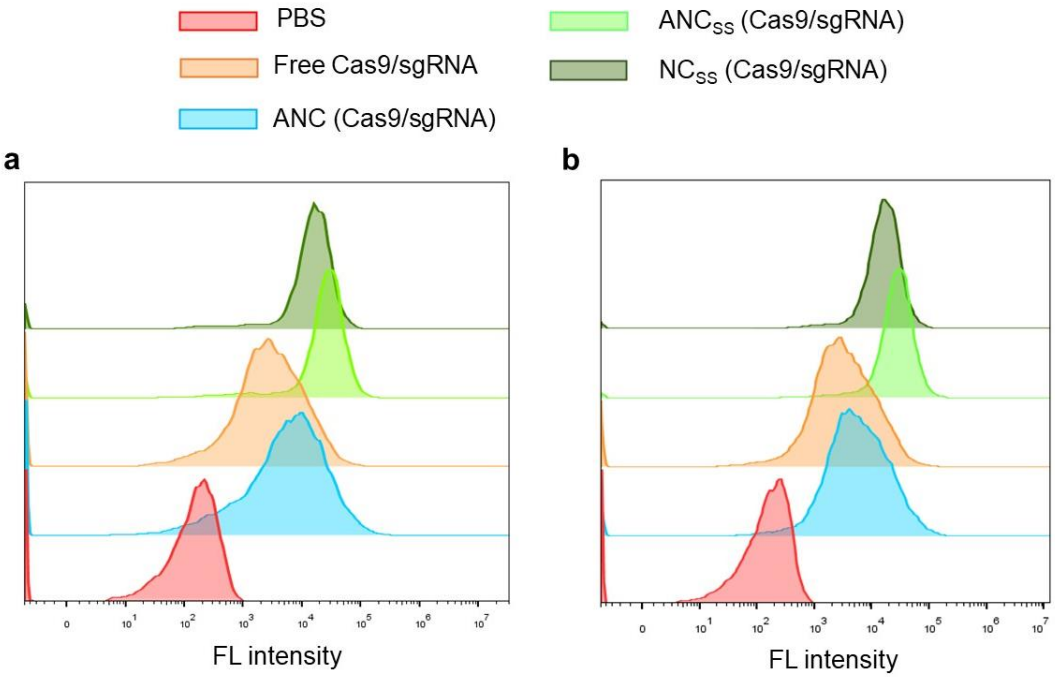
Supplementary Figure 1. (**a**) Size distribution of free Cas9/sgRNA determined by DLS. (**b**) TEM picture of free Cas9/sgRNA.



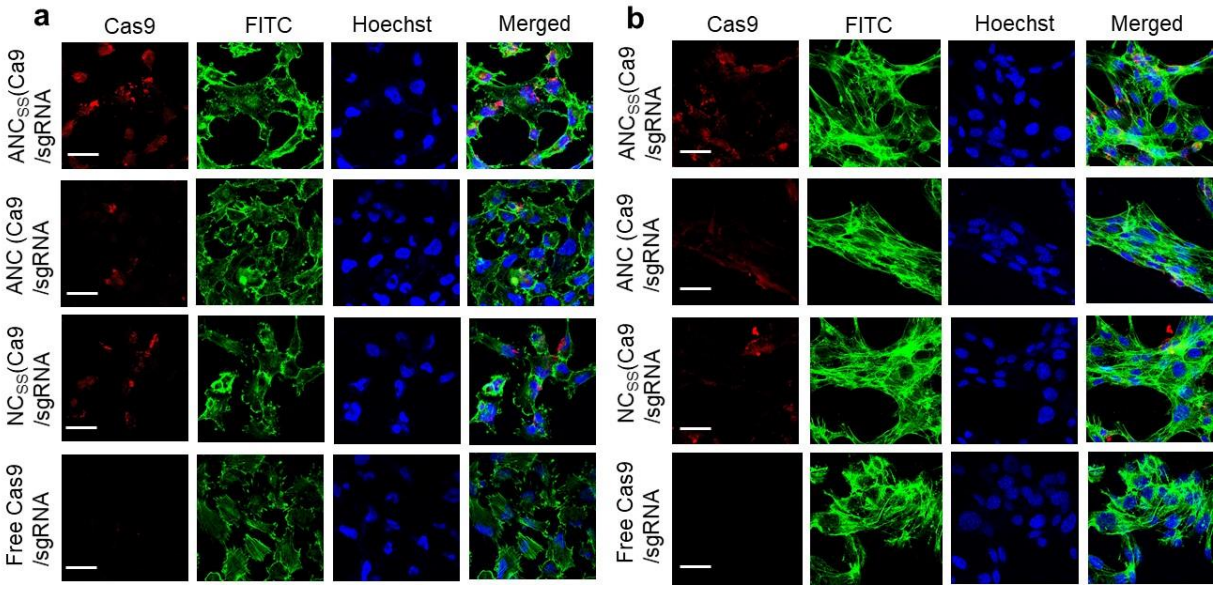
Supplementary Figure 2. (a) Size, **(b)** PDI and **(c)** zeta potential of ANC_{SS}(Cas9/sgRNA), ANC (Cas9/sgRNA) and NC_{SS}(Cas9/sgRNA) nanocapsules. Data are mean ± SD (n=3, *N. S.* indicates non-significance).



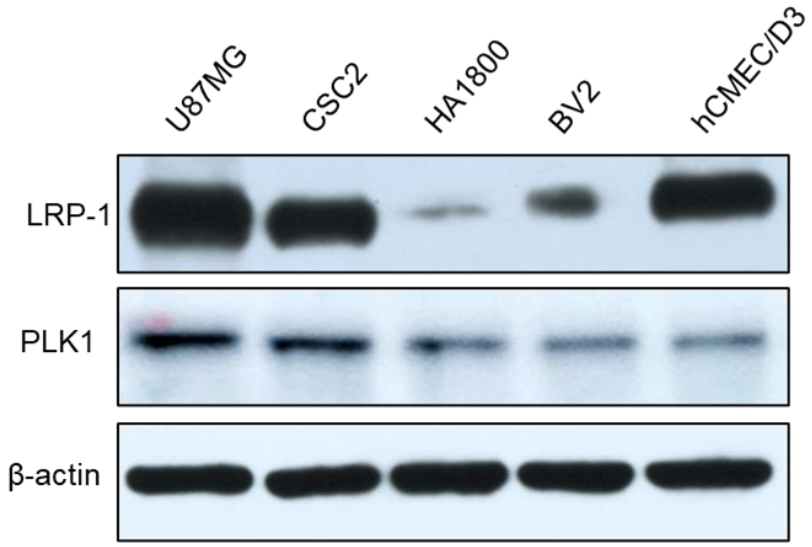
Supplementary Figure 3. Circular dichroism spectra of ANC_{SS}(BSA) and free BSA.



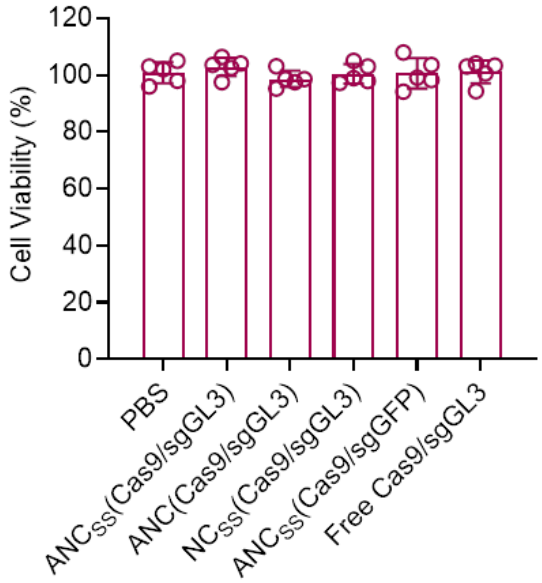
Supplementary Figure 4. Flow cytometry of **(a)** U251 cells and **(b)** CT2A cells following 4 h incubation with ANC_{SS}(Cas9/sgRNA) or controls. The AF647-Cas9 concentration was 20 nM.



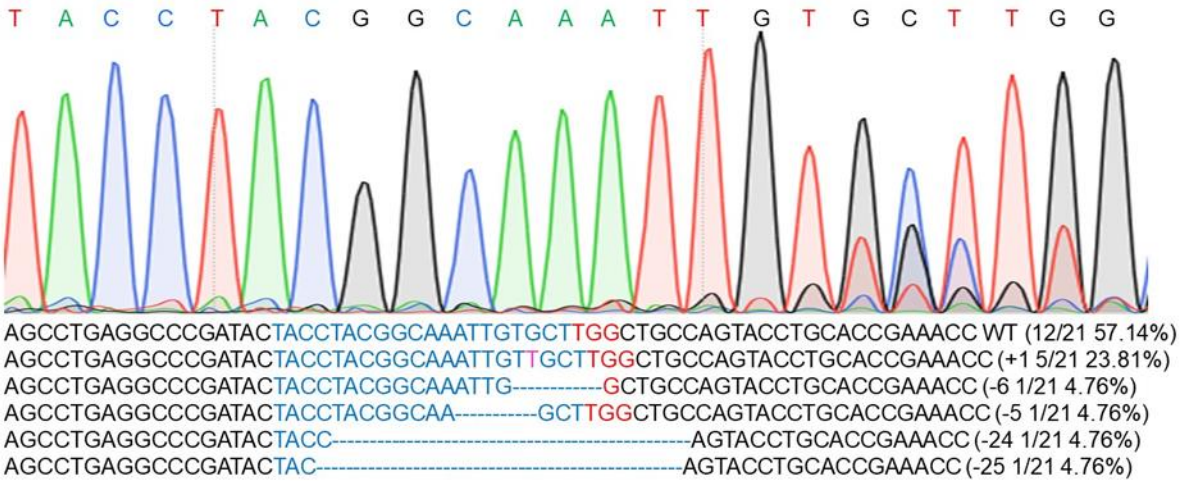
Supplementary Figure 5. CLSM images of **(a)** U251 and **(b)** CT2A cells following 4 h incubation with ANC_{SS}(Cas9/sgRNA) or controls. The AF647-Cas9 concentration was 20 nM. Scale bar: 20 μm. Cas9 was labeled with AF647 (Red), the cytoskeleton was stained with Alexa Fluor™ 488 (Green), and the nuclei was stained with Hoechst 33342 (Blue).



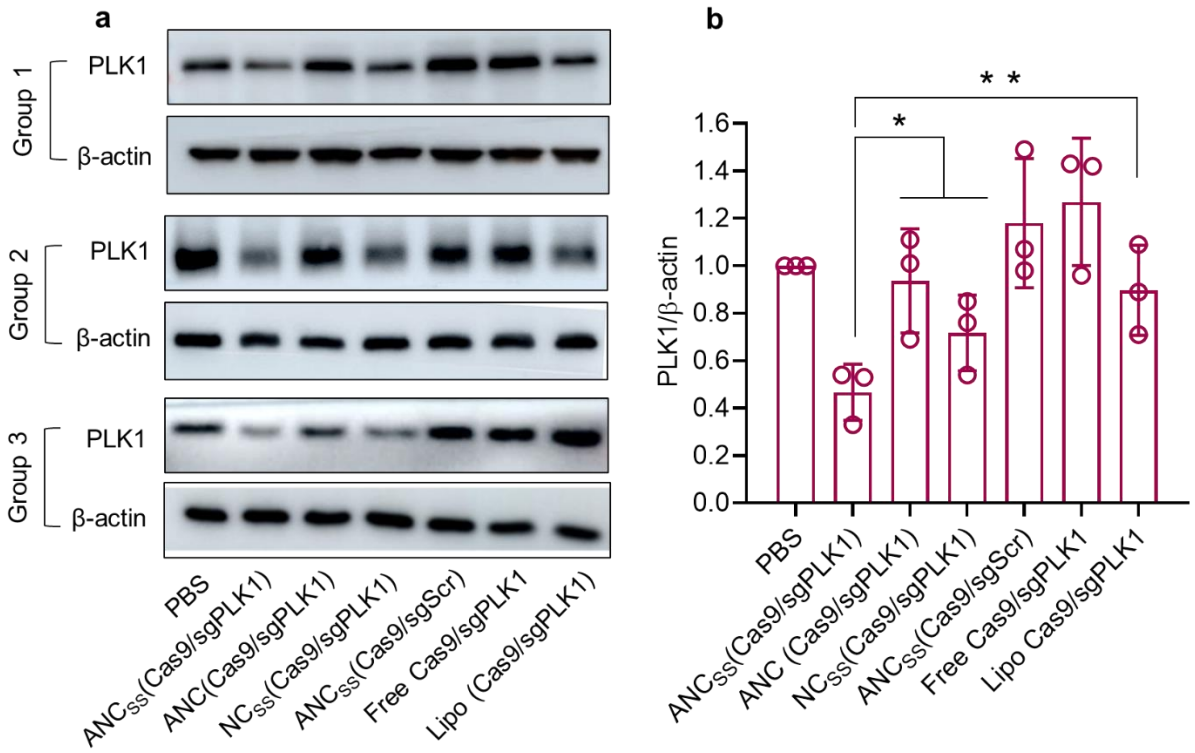
Supplementary Figure 6. LRP-1 and PLK1 protein expression in U87MG human glioblastoma cells, CSC2 glioblastoma stem cells (GSCs), normal glial cell HA1800, astrocytes cell BV2 and endothelial cell hCMEC/D3, respectively.



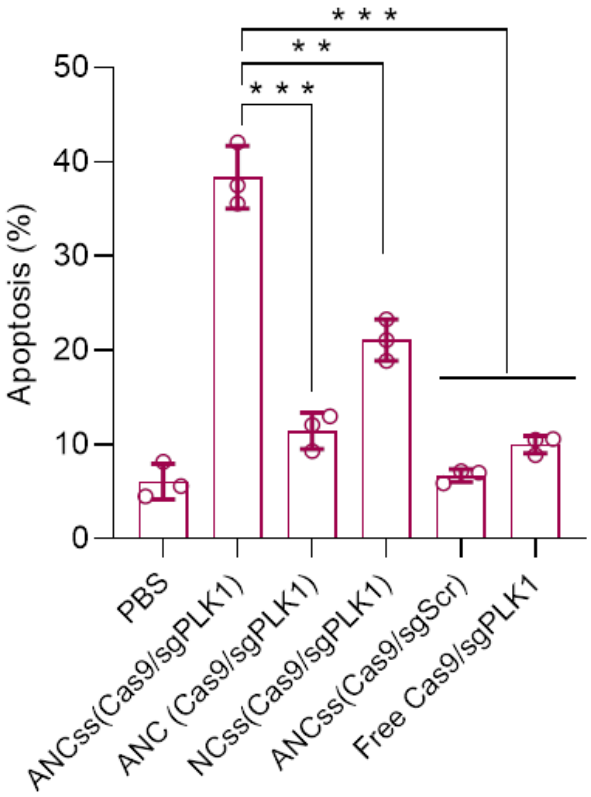
Supplementary Figure 7. Cell viability of U87MG cells after 72 h incubation with ANC_{SS}(Cas9/sgRNA) or controls. Data were normalized to cells incubated with PBS. Data are mean ± SD (n=5).



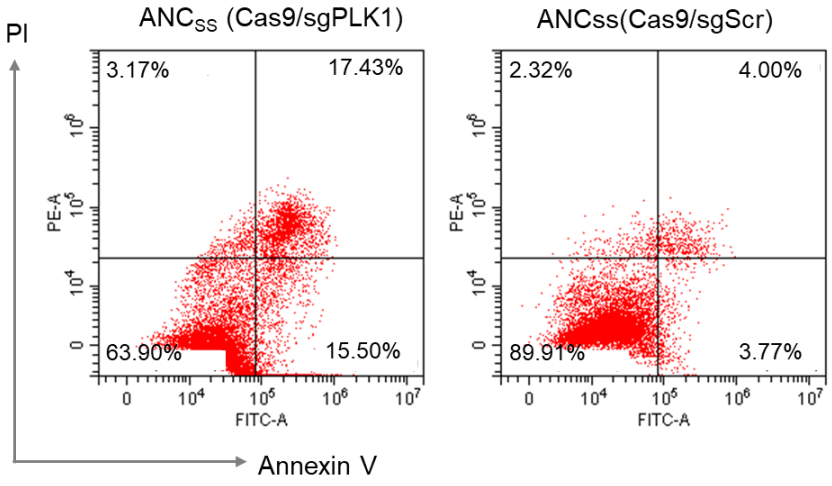
Supplementary Figure 8. Sequencing results of PCR amplicon of the targeted sites in U87MG cells treated with ANC_{SS}(Cas9/sgPLK1).



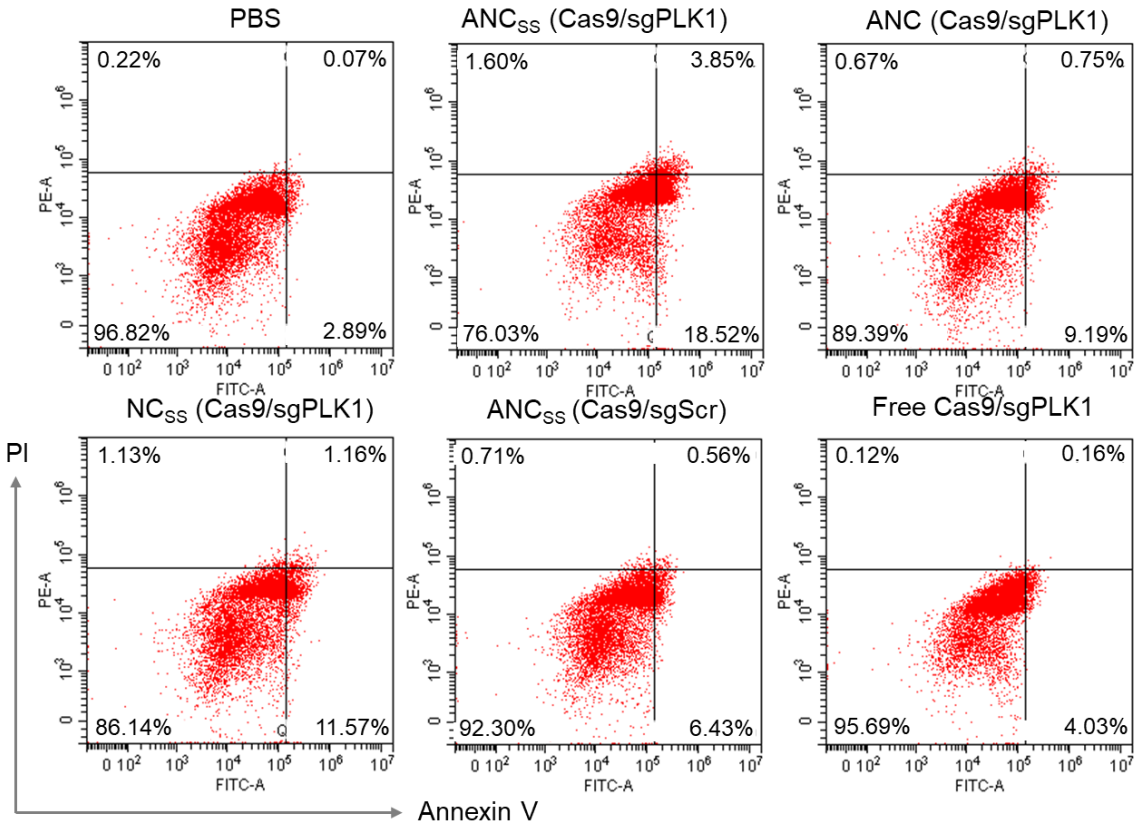
Supplementary Figure 9. (a) Expression levels of PLK1 in U87MG cells after 72 h incubation with ANC_{SS}(Cas9/sgPLK1) or controls. **(b)** Quantification of PLK1 expression relative to β-actin. Data are presented as mean ± SD (n=3, *p<0.05, **p<0.01).



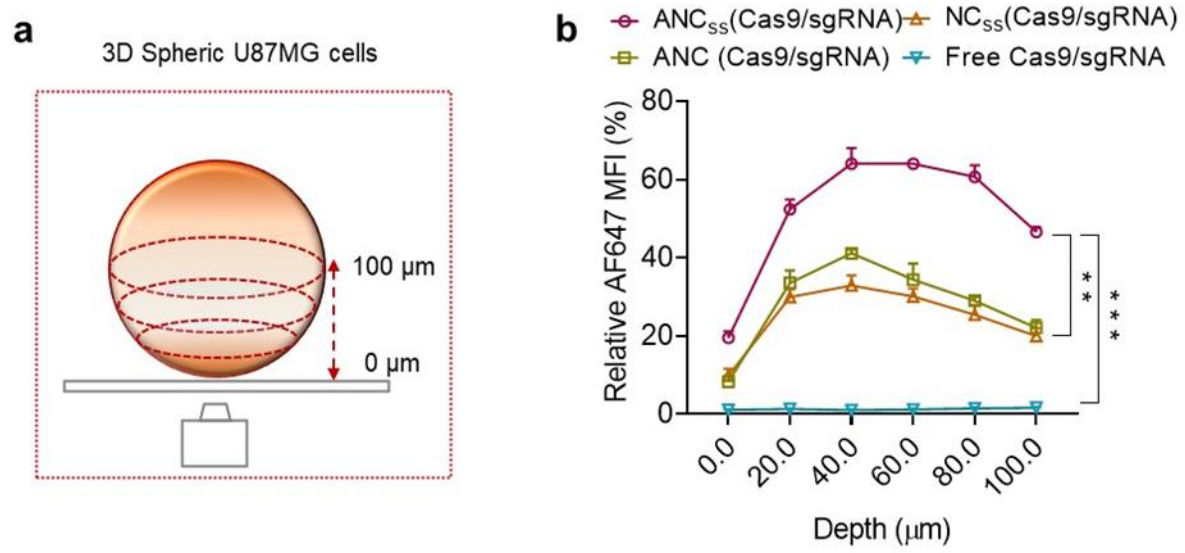
Supplementary Figure 10. Quantitative analysis of apoptosis assay in U87MG cells after 72 h incubation with ANC_{SS}(Cas9/sgRNA) and other controls. The Cas9 concentration was 20 nM. Data are mean ± SD (n=3, **p<0.01, ***p<0.001).



Supplementary Figure 11. Apoptosis assay of U87MG cells incubated with ANC_{SS}(Cas9/sgPLK1) and non-functional ANC_{SS}(Cas9/sgScr) for 72 h. The cells were pretreated with PLK1 siRNA loaded polyethyleneimine transfection agent for 48 h. The siRNA and Cas9 concentrations were 100 nM and 20 nM, respectively.

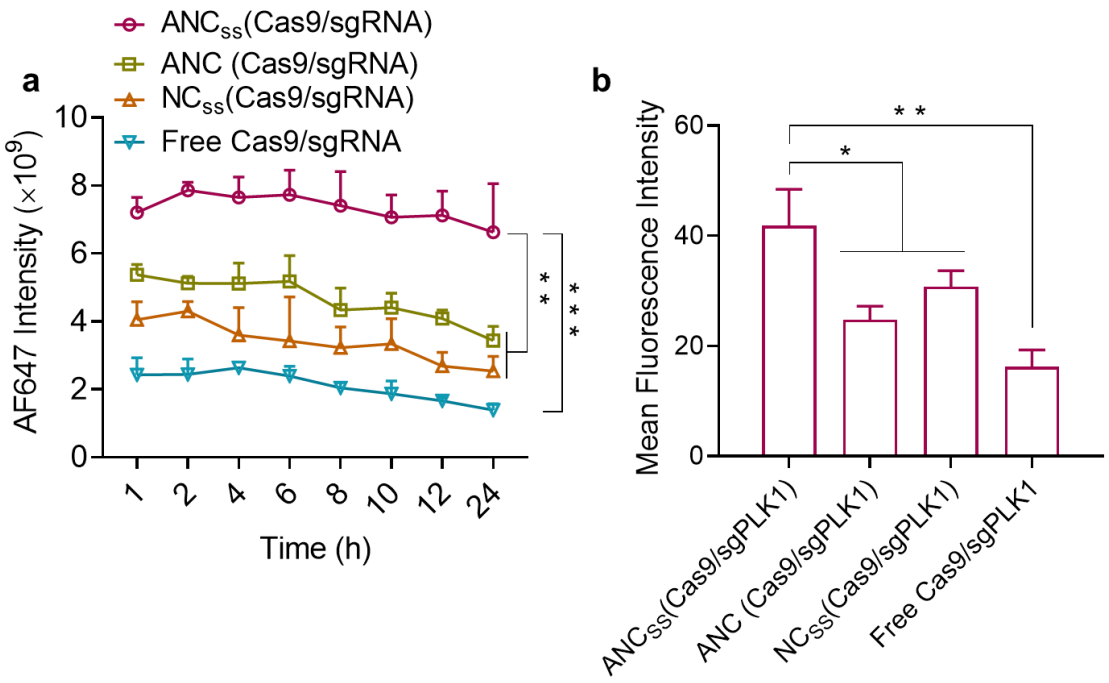


Supplementary Figure 12. Apoptosis assay of U251 cells after 72 h incubation with ANC_{SS}(Cas9/sgRNA) and other controls. The Cas9 concentration was 20 nM.

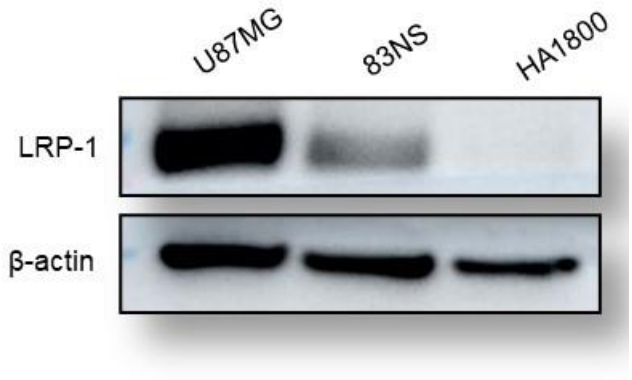


Supplementary Figure 13. **(a)** Schematic of the 3D spheric U87MG models. **(b)** Quantification of the relative AF647-Cas9/sgRNA mean fluorescence intensity in each slice

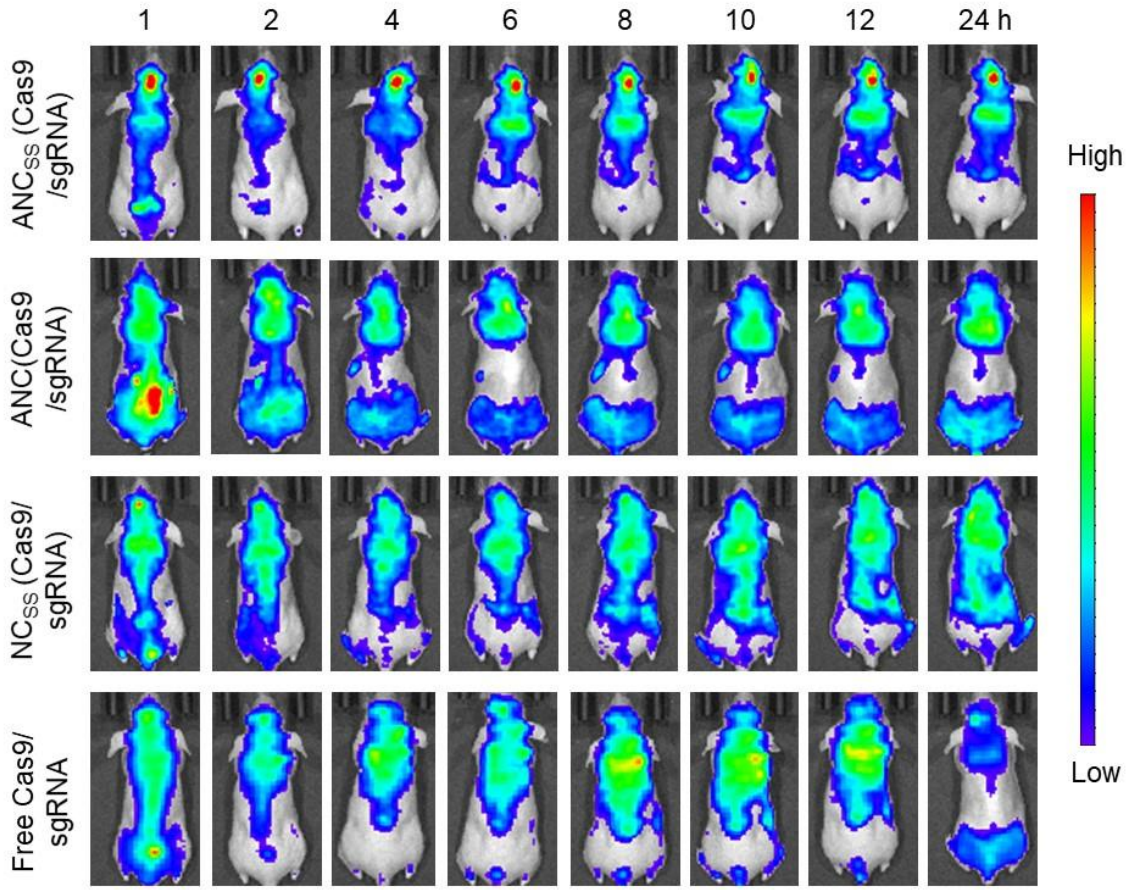
in Z-stack of U87MG tumor spheroid for ANC_{SS}(Cas9/sgRNA), ANC (Cas9/sgRNA), NC_{SS}(Cas9/sgRNA) or free Cas9/sgRNA treatments. Data are mean ± SD (n=3, $^{**}p<0.01$, $^{***}p<0.001$).



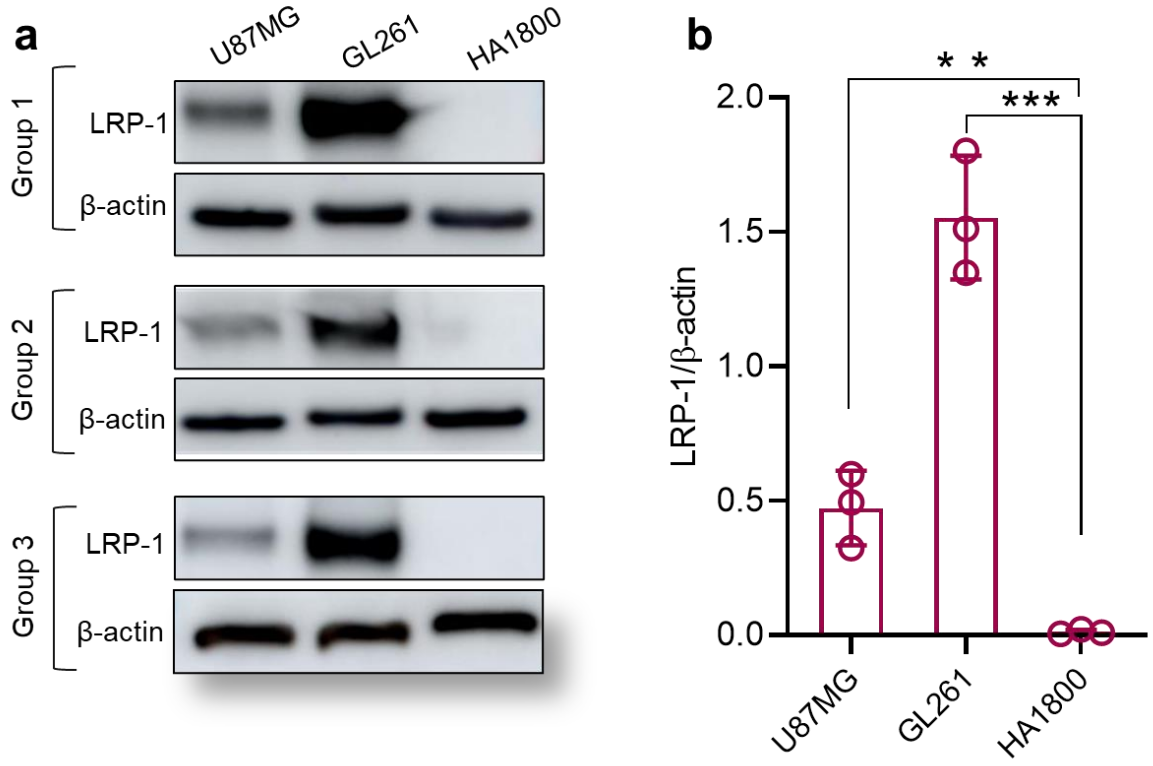
Supplementary Figure 14. Quantification analysis of fluorescence in *in vivo* imaging **(a)** and **(b)** penetration towards U87MG orthotopic bearing mice treated with nanocapsules. Data are mean ± SD (n=3, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).



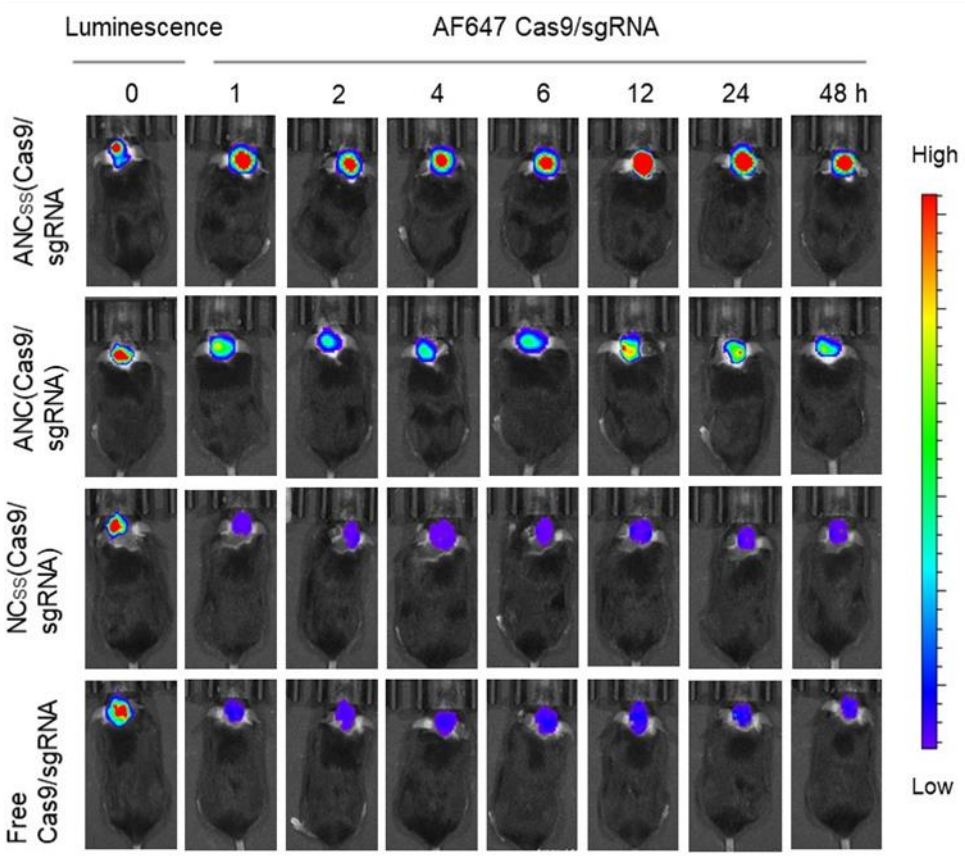
Supplementary Figure 15. LRP-1 protein expression in U87MG, 83NS and HA1800 glial cells.



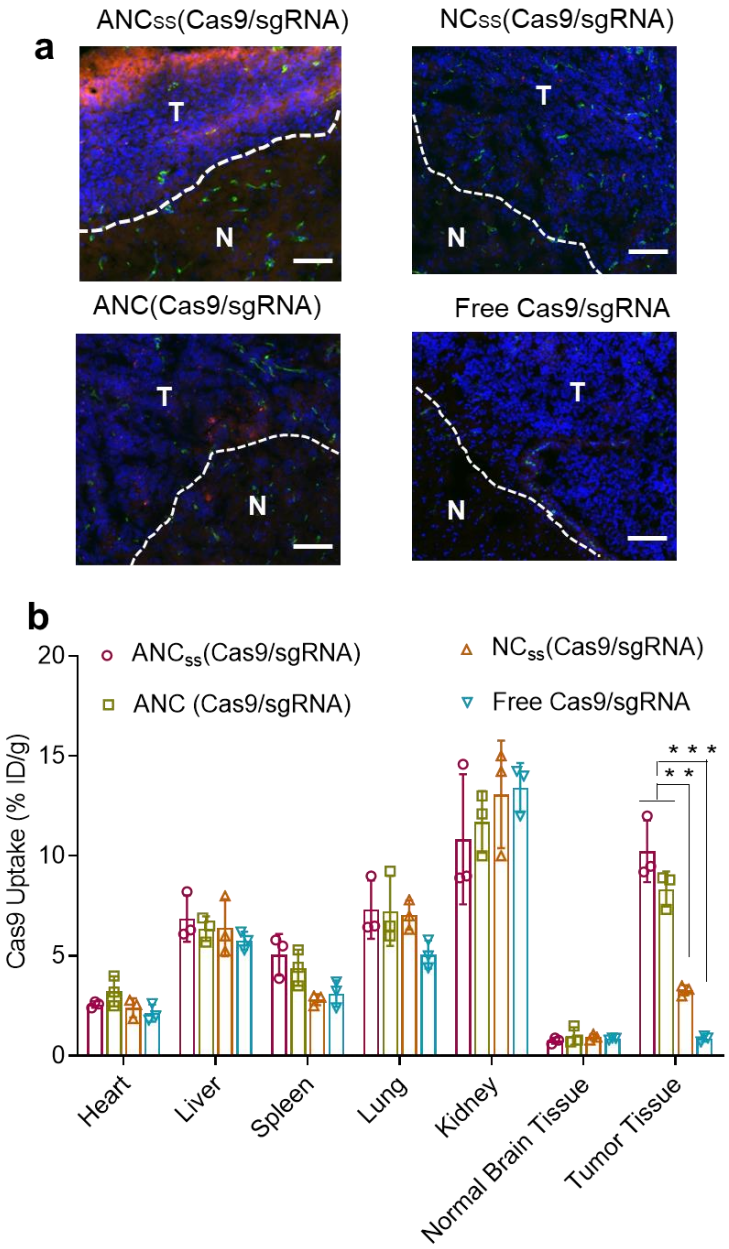
Supplementary Figure 16. Fluorescence images of orthotopic glioblastoma stem cells (GSCs) 83NS tumor-bearing nude mice at different time points following the injection of ANC_{SS}(Cas9/sgRNA) nanocapsules or controls (1.5 mg Cas9 equiv./kg).



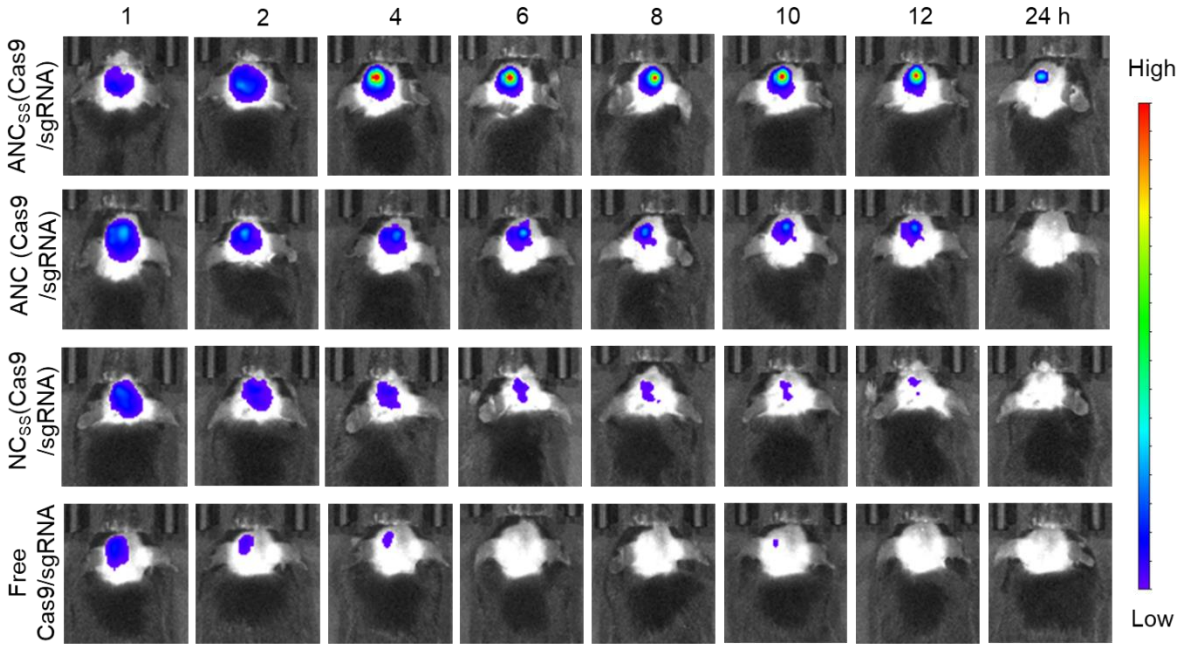
Supplementary Figure 17. **(a)** LRP-1 protein expression and **(b)** quantification in U87MG human GBM, GL261 mouse GBM and HA1800 normal glial cells, respectively. Data are mean ± SD (n=3, **p<0.01, ***p<0.001).



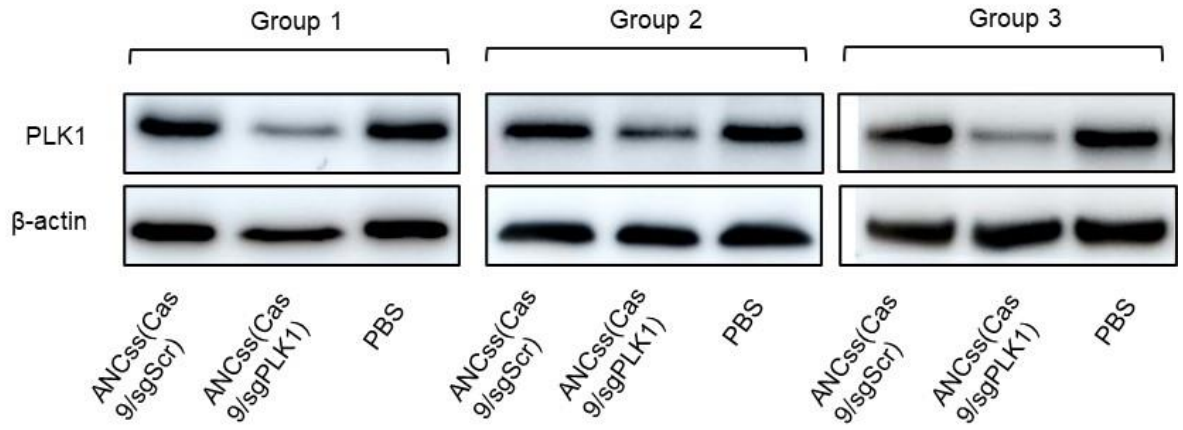
Supplementary Figure 18. Fluorescence images of orthotopic GL261-Luc glioblastoma tumor-bearing C57BL/6 mice at different time points following the injection of ANC_{SS}(Cas9/sgRNA) nanocapsules or controls (1.5 mg AF647-Cas9 equiv./kg).



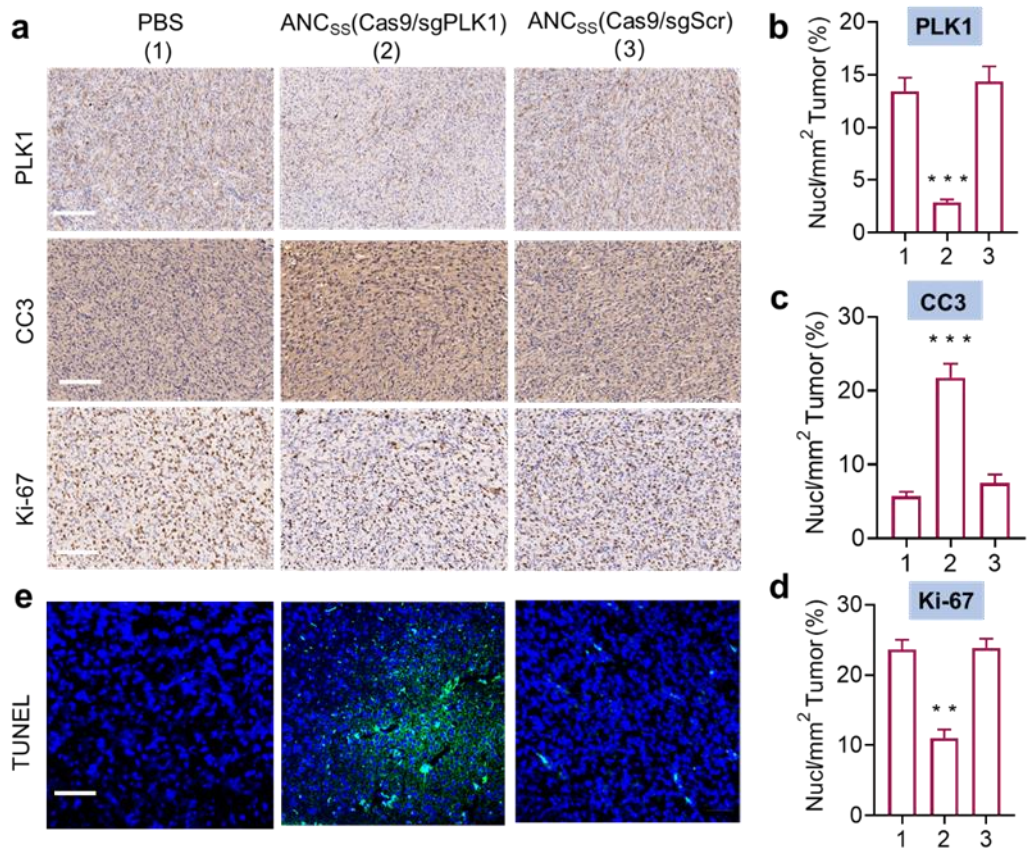
Supplementary Figure 19. (a) Tumor penetration of ANC_{SS}(Cas9/sgRNA) and controls observed by CLSM. Tumor sections were obtained from GL261-Luc bearing mice at 12 h post tail vein injection (1.5 mg Cas9 equiv./kg). Nuclei were stained with DAPI (blue) and blood vessels with CD31 (green); AF647-Cas9 (red). Dotted lines marked the tumor boundary in the brain of mouse model. N: normal brain tissue, T: tumor. Scale bars, 100 μm. **(b)** Quantitation of AF647-Cas9 accumulation in different organs. Levels of AF647-Cas9 fluorescence were determined by fluorescence spectroscopy and expressed as % ID/g. Data are mean ± SD (n=3, $^{**}p<0.01$, $^{***}p<0.001$).



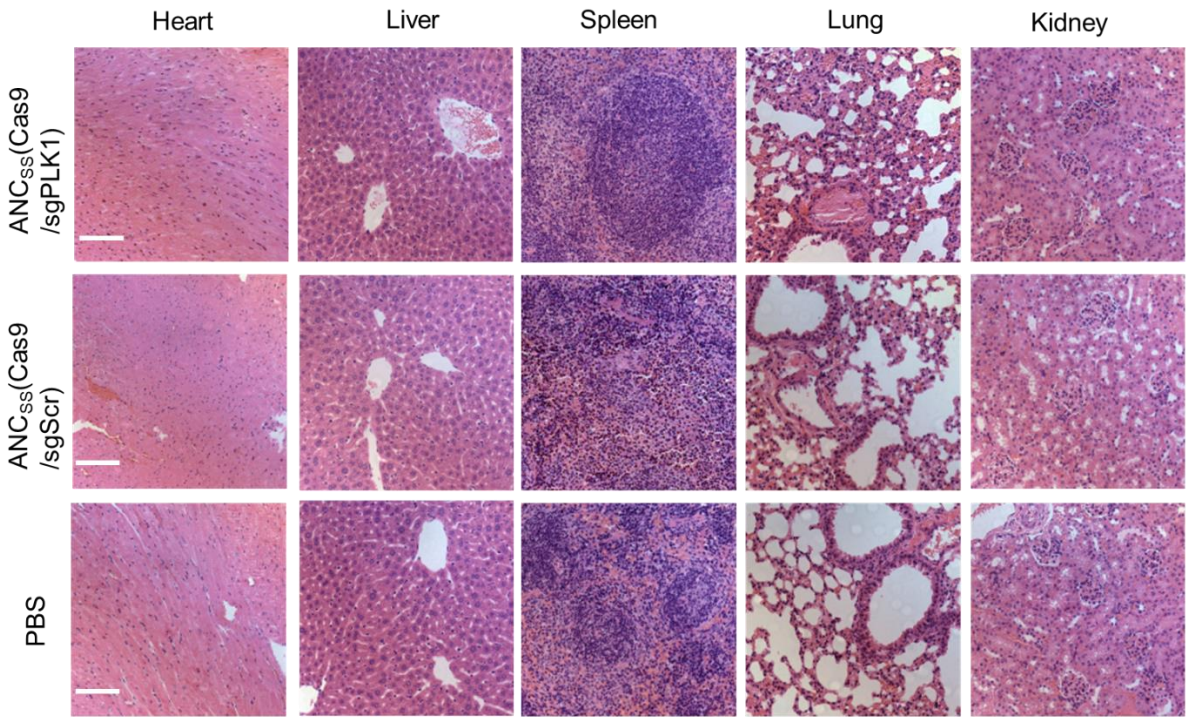
Supplementary Figure 20. Fluorescence images of orthotopic luciferase free GL261 glioblastoma tumor-bearing C57BL/6 mice at different time points following injection of ANC_{SS}(Cas9/sgRNA) nanocapsules or controls (1.5 mg Cas9 equiv./kg).



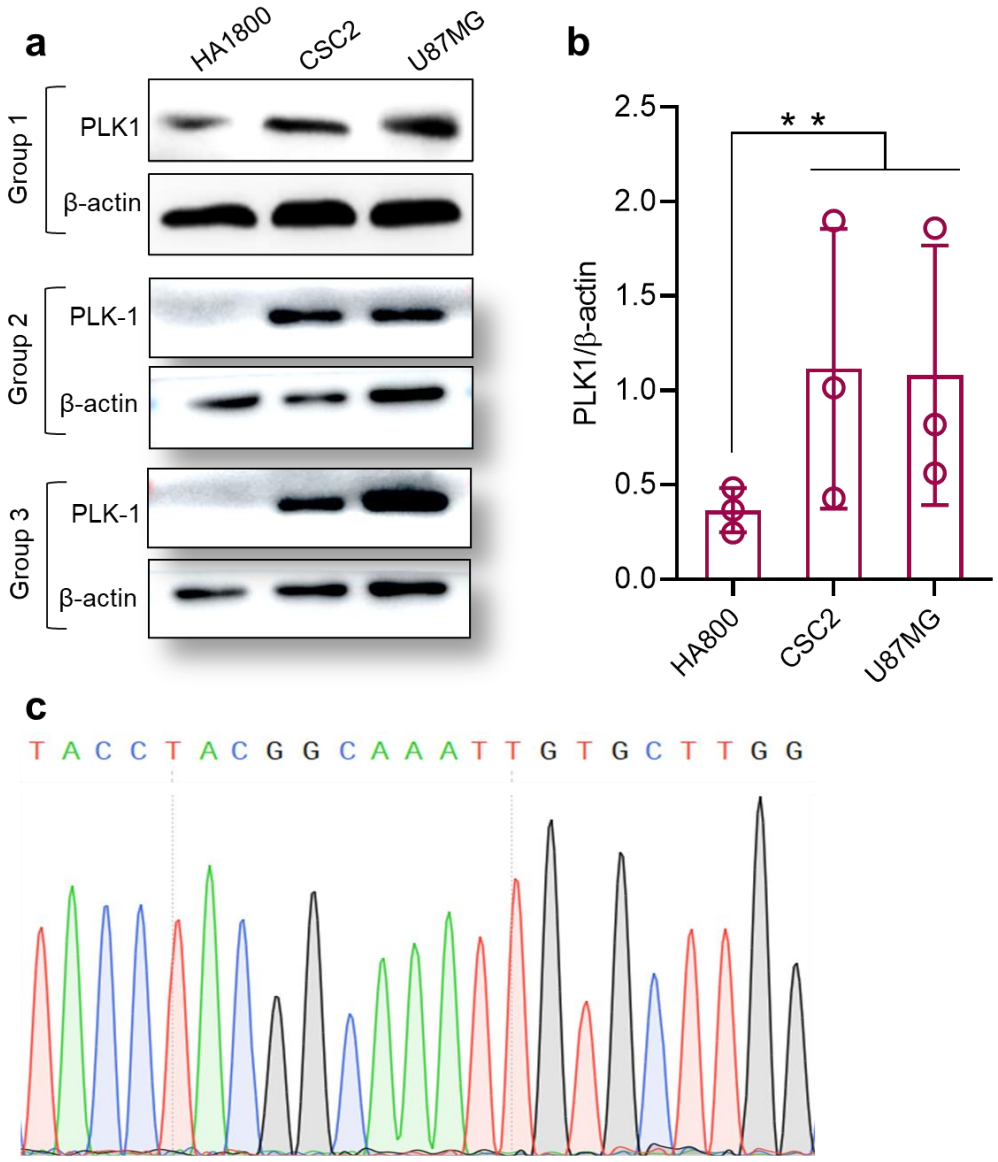
Supplementary Figure 21. Western blot of PLK1 protein expression in tumor tissues excised on day 20 from U87MG-Luc bearing mice receiving different treatments (n=3).



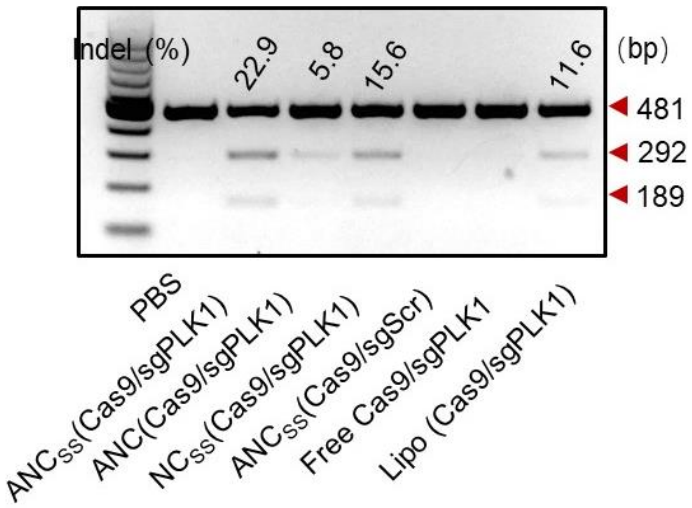
Supplementary Figure 22. **(a)** Tumor slices excised from orthotopic U87MG-Luc human glioblastoma tumor-bearing nude mice following treatment with ANC_{SS}(Cas9/sgPLK1), ANC_{SS}(Cas9/sgScr) (1.5 mg Cas9 equiv./kg), or PBS and stained for PLK1, cleaved caspase 3 (CC3), or for the proliferation marker Ki-67. Quantification of the number of tumor cells stained positive for PLK1 **(b)**, CC3 **(c)**, or Ki-67 **(d)**. Signal intensity was quantified from over 300 cells in tumors of mice using Image J. **(e)** TUNEL apoptosis staining of tumor slices. Scale bars are 200 µm for PLK1, CC3, and Ki-67, and 100 µm for TUNEL, respectively. Data are mean ± SD (**p<0.01, ***p<0.001).



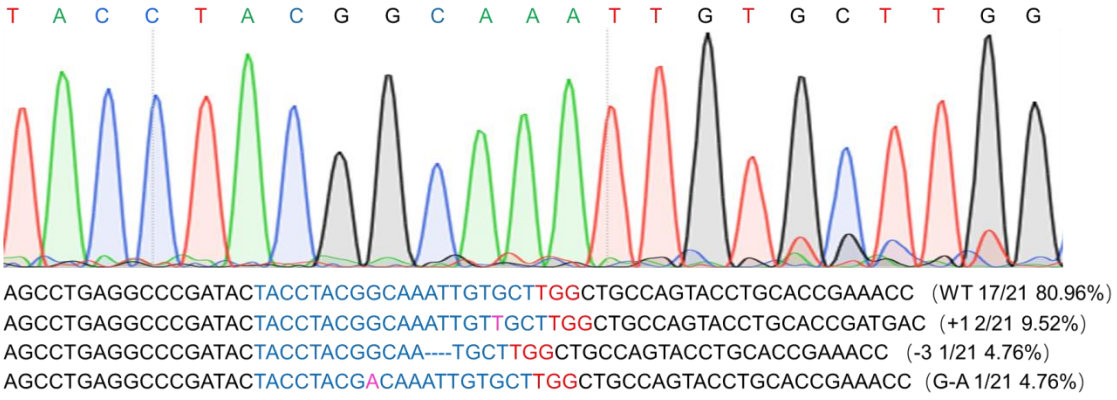
Supplementary Figure 23. Optical images of H&E-stained sections of heart, liver, spleen, lung, and kidney of orthotopic U87MG-Luc tumor bearing nude mice following treatment with different formulations. Scale bar: 200 μm.



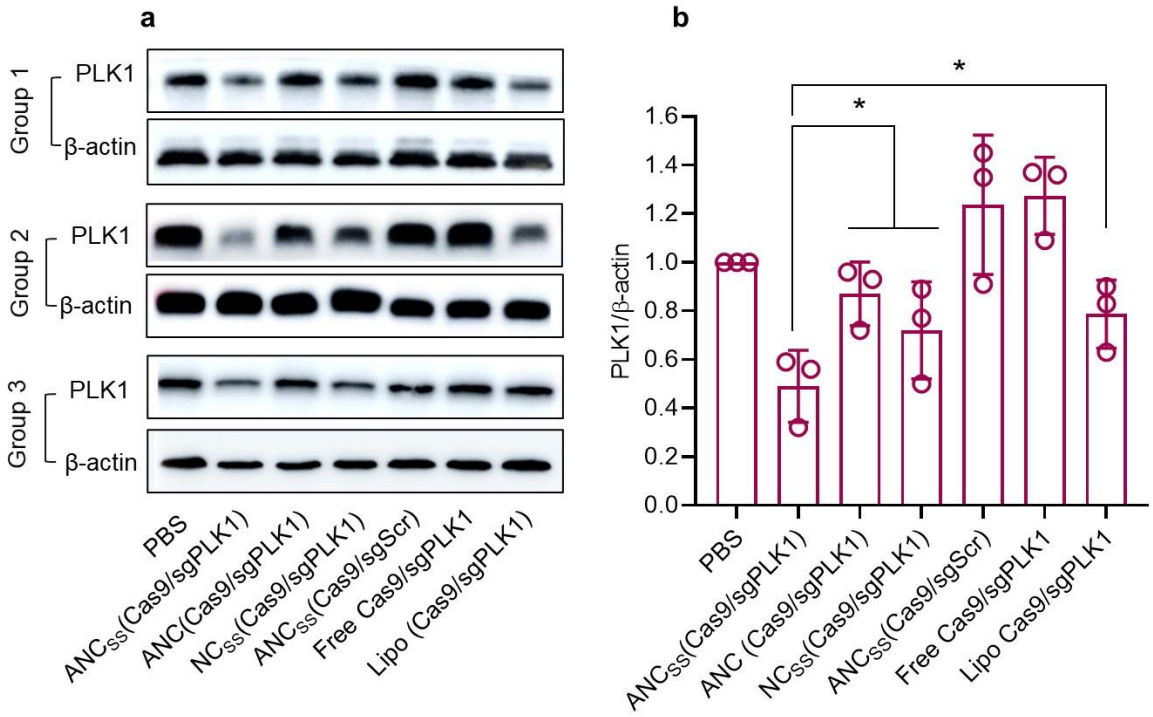
Supplementary Figure 24. **(a)** LRP-1 protein expression and **(b)** quantification in U87MG human GBM, GL26 GBM and HA1800 normal glial cells, respectively. Data are mean ± SD (n=3, ***p*<0.01). (**c**) Sequencing results of the PLK1 gene in CSC2 cells.



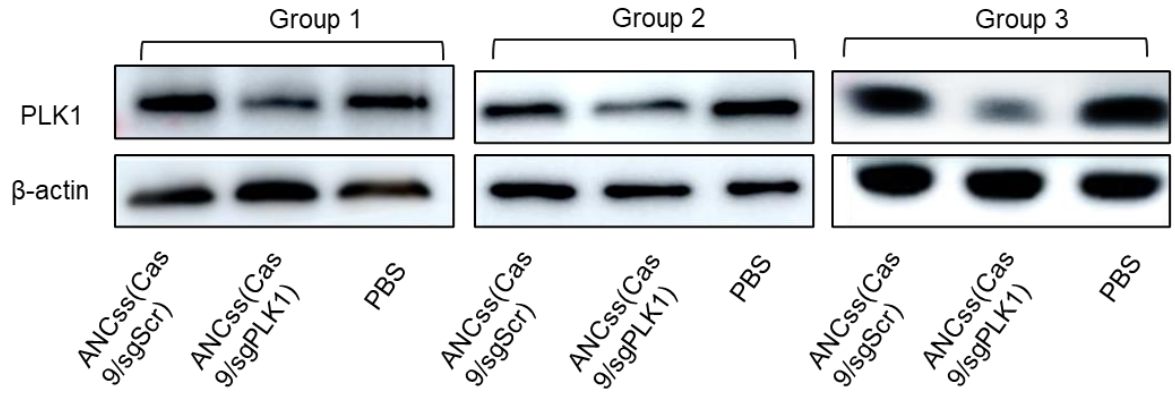
Supplementary Figure 25. PLK1 gene indels in CSC2 cells transfected with ANC_{SS}(Cas9/sgPLK1), ANC(Cas9/sgPLK1), NC_{SS}(Cas9/sgPLK1), ANC_{SS}(Cas9/sgScr), or free Cas9/sgPLK1 for 48 h.



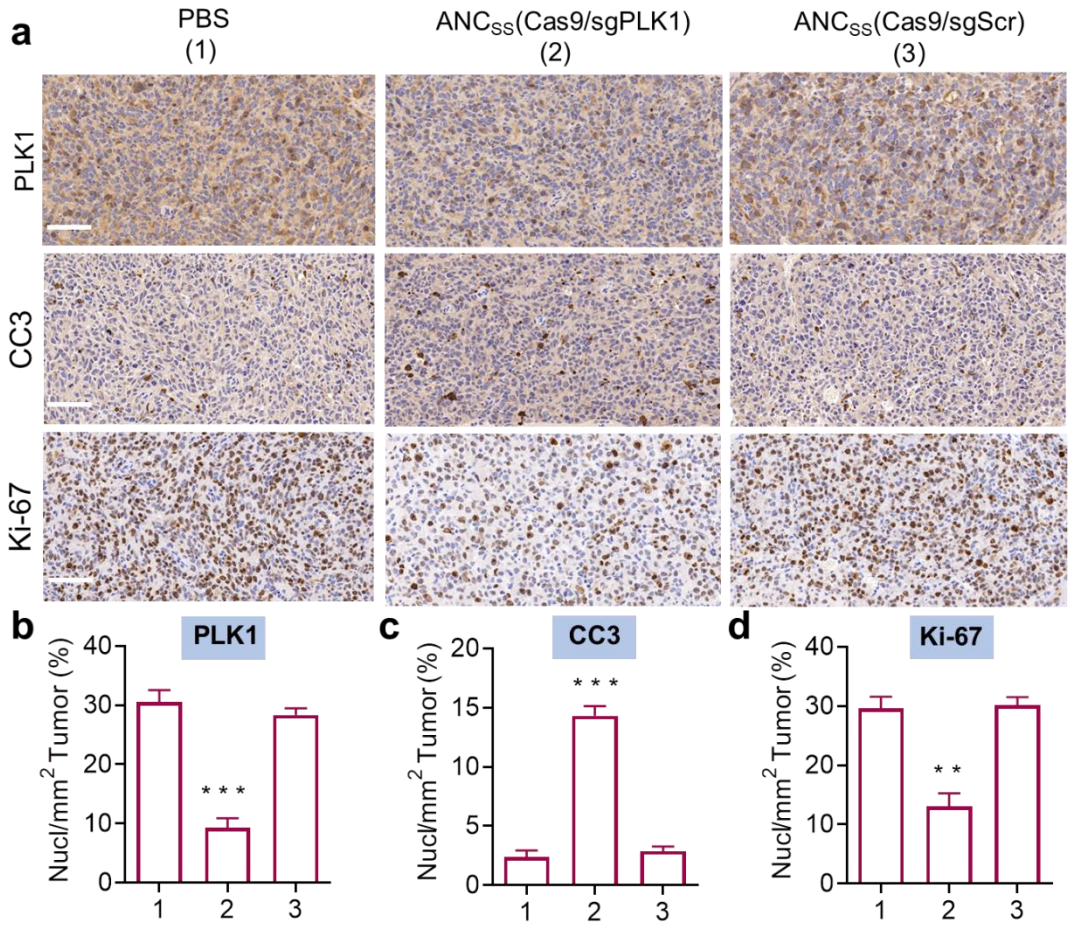
Supplementary Figure 26. Sequencing results of PLK1 gene editing in CSC2 GSCs cells treated with ANC_{SS}(Cas9/sgPLK1).



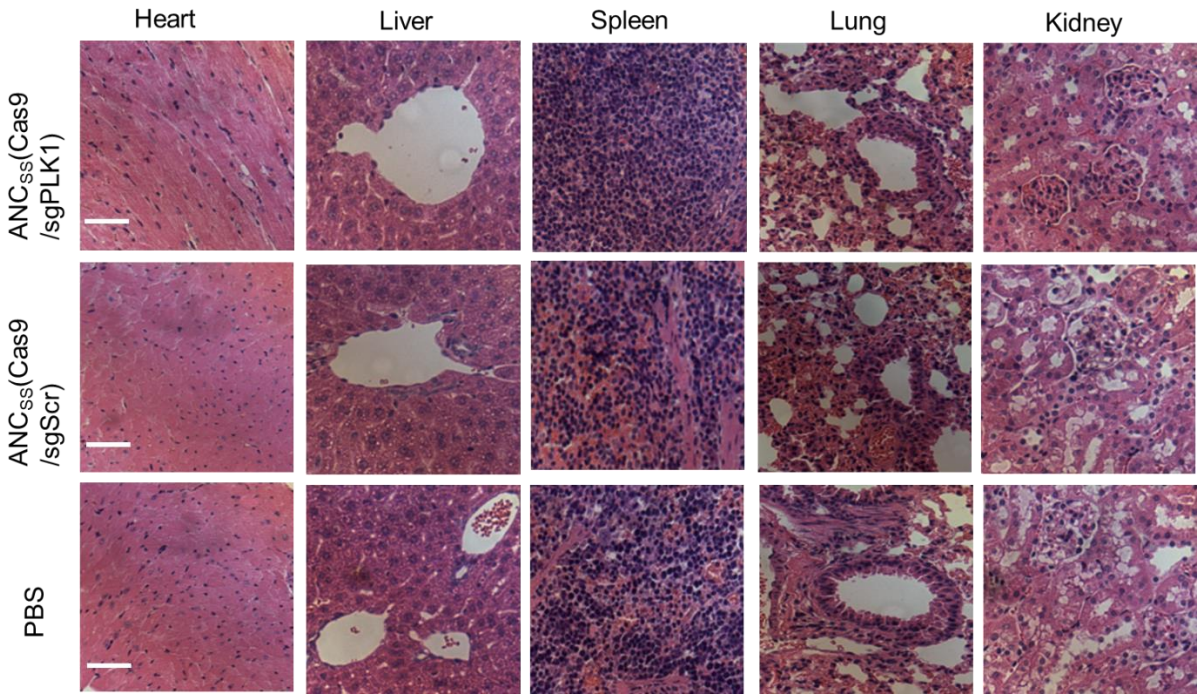
Supplementary Figure 27. (**a**) Expression levels of PLK1 in CSC2 GSCs cells after 72 h incubation with ANC_{SS}(Cas9/sgPLK1) or controls. (**b**) Quantification of PLK1 expression relative to β-actin. Data are presented as mean ± SD (n=3, **p*<0.05).



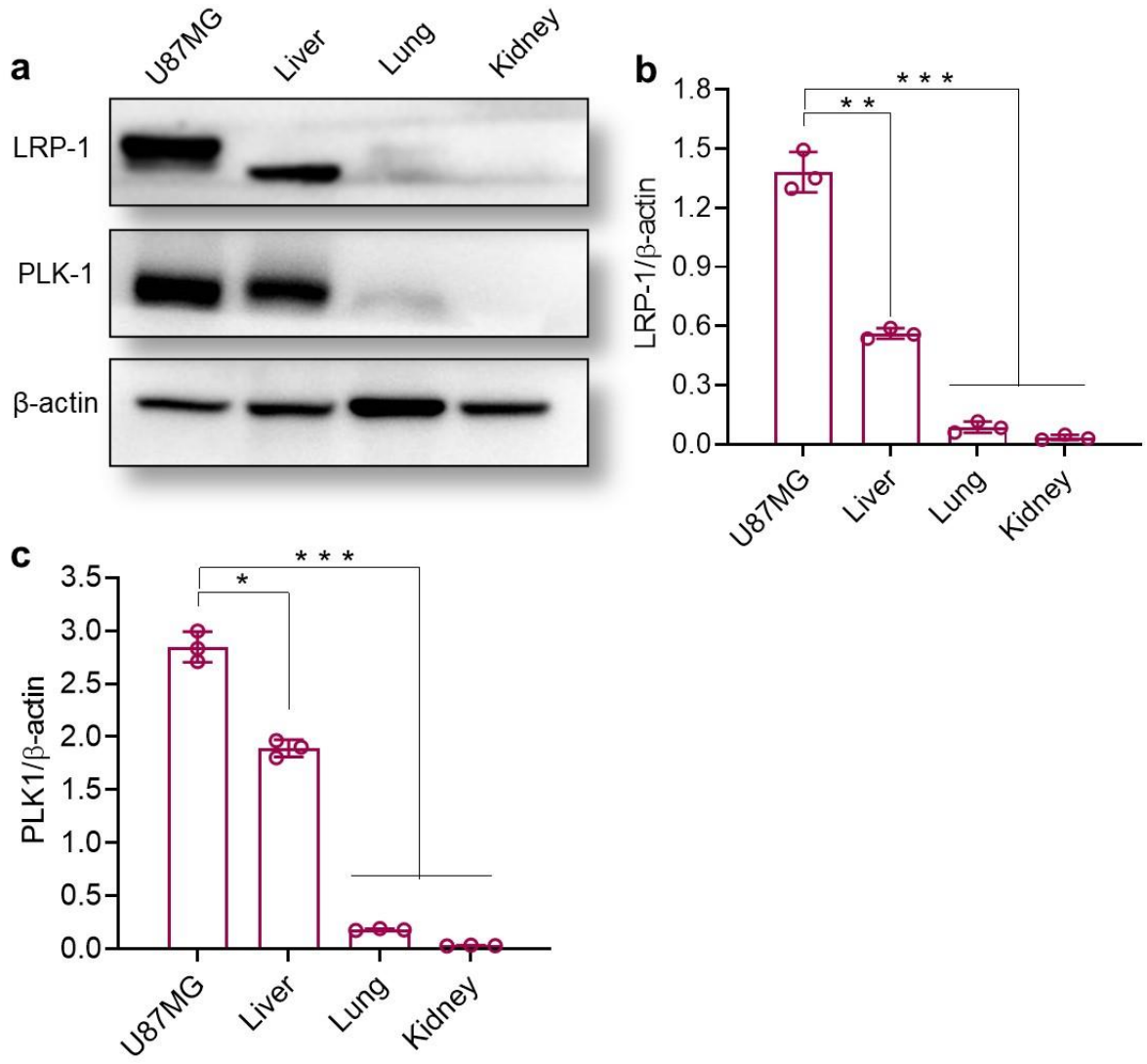
Supplementary Figure 28. PLK1 protein expression in tumor tissues excised on day 20 post tumor implantation from the CSC2 GSCs tumor-bearing mice receiving different formulations (n=3).



Supplementary Figure 29. (**a**) Tumor slices excised from orthotopic CSC2-Luc GSCs tumor-bearing nude mice following treatment with nanocapsules. The slices were stained for PLK1, cleaved caspase 3 (CC3), or proliferation (Ki-67). Quantification of number of tumor cells that express PLK1 (**b**), stained CC3 (**c**), or Ki-67 (**d**) in mice treated with ANC_{SS}(Cas9/sgPLK1), ANC_{SS}(Cas9/sgScr) (1.5 mg Cas9 equiv./kg), or PBS. Signal intensity was quantified from over 300 cells in tumors of mice using Image J. Scale bars are 150 µm. Data are mean ± SD (**p<0.01, ***p<0.001).



Supplementary Figure 30. Optical images of H&E-stained sections of heart, liver, spleen, lung and kidney of orthotopic CSC2-Luc GSCs tumor-bearing nude mice following treatment with different formulations. Scale bar: 100 μm.



Supplementary Figure 31. (a) LRP-1 and PLK1 protein expression in U87MG glioblastoma cells, liver, lung and kidney respectively. Quantitation of **(b)** LRP1 and **(c)** PLK1 protein expression relative to β-actin, respectively. Data are mean ± SD (n=3, *p<0.05, **p<0.01, ***p<0.001).