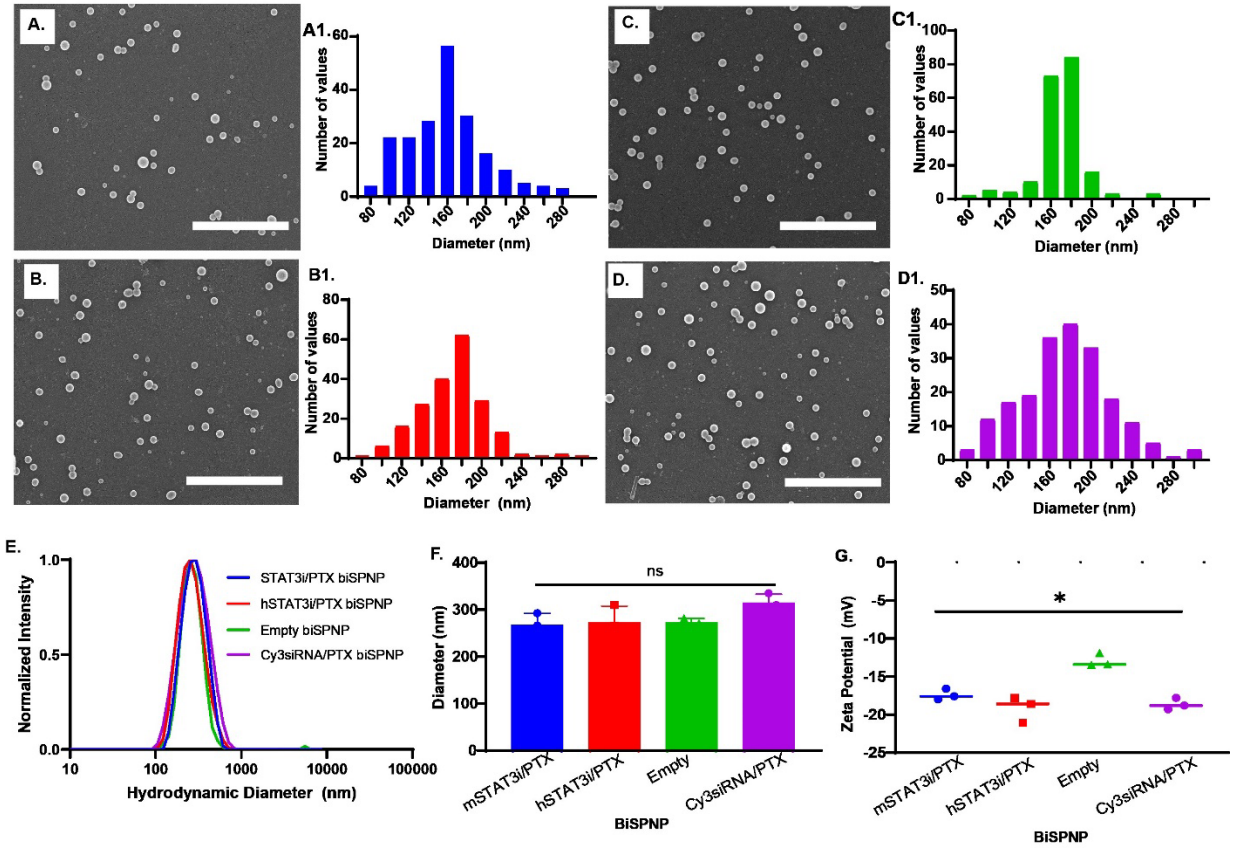
# SUPPLEMENTARY INFORMATION

## Bicompartmental Nanoparticles Overcome Drug Incompatibility to Eradicate Glioma and Induce Long-Lasting Immunological Memory

Ava Mauser*, Kaushik Banerjee*, Julio Zelaya, Anzar A. Mujeeb, Luis A. Ortiz-Rodríguez, Sophia Lee, Jingyao Gan, Isabel Waibel, Albert Chang, Anthony Berardi, Anna Schwendeman, Pedro Lowenstein, Julie S. Biteen, Maria Castro**, Joerg Lahann**

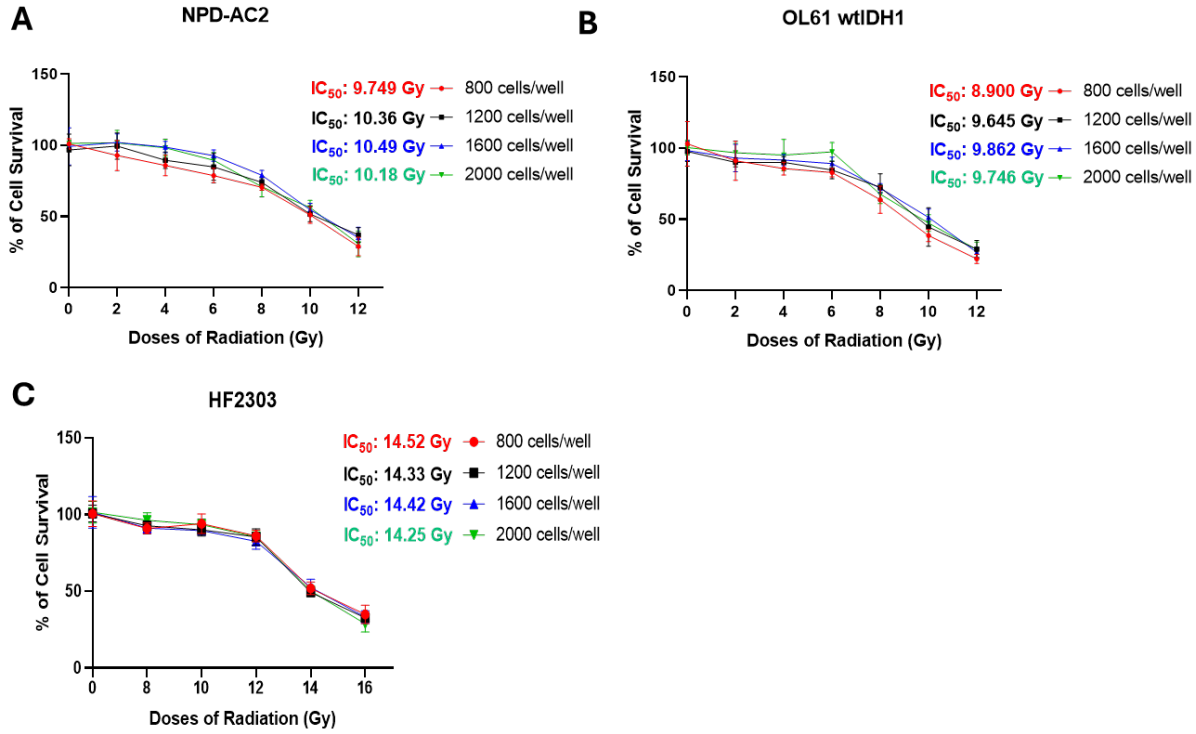
**Supplementary Table 1.** Size information of Cy3siRNA/PTX biSPNPs at 37 ˚C during the release study compared with Cy3siRNA/PTX biSPNPs stored at 4 ˚C.

| Day | Temperature (ºC) | Intensity Diameter (nm) | Number Diameter (nm) | PDI |
|---|---|---|---|---|
| 0 | 4 | 310 | 219 | 0.37 |
| 5 | 4 | 337 | 283 | 0.30 |
| 5 | 37 | 373 | 294 | 0.35 |
| 14 | 4 | 384 | 287 | 0.30 |
| 14 | 37 | 369 | 311 | 0.29 |

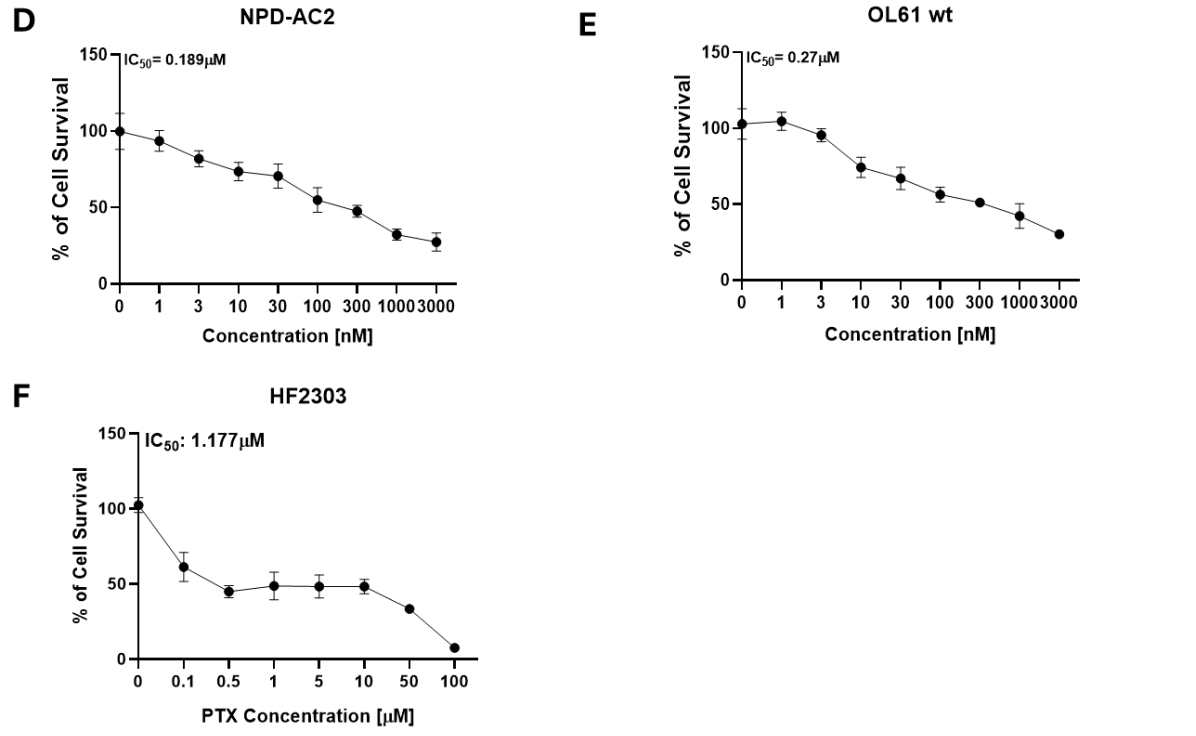



**Supplementary Figure 1**: Comparison of the mouse STAT3i/PTX biSPNPs (mSTAT3i/PTX biSPNPs), human STAT3i/PTX biSPNPs (hSTAT3i/PTX), empty biSPNPs, and Cy3siRNA/PTX biSPNPs. **A.** SEM micrograph of mSTAT3i/PTX biSPNPs (scale bar = 2 micron) and the corresponding diameter distribution of 200 analyzed biSPNPs (A1). **B.** SEM micrograph of hSTAT3i/PTX biSPNPs (scale bar = 2 micron) and the corresponding diameter distribution of 200 analyzed biSPNPs (B1). **C.** SEM micrograph of Empty biSPNPs (scale bar = 2 micron) and the corresponding diameter distribution of 200 analyzed biSPNPs (C1). **D.** SEM micrograph of Cy3siRNA/PTX biSPNP (scale bar = 2 micron) and the corresponding diameter distribution of 200 analyzed biSPNPs (D1). **E.** The hydrodynamic diameter of all groups measured via DLS. **F.** The peak diameter from the DLS spectra in E are not statistically different ($p>0.05$, Kruskal-Wallis test). **G.** The zeta potential of all biSPNP groups in water is significantly different (*$p<0.05$, Kruskal-Wallis test).

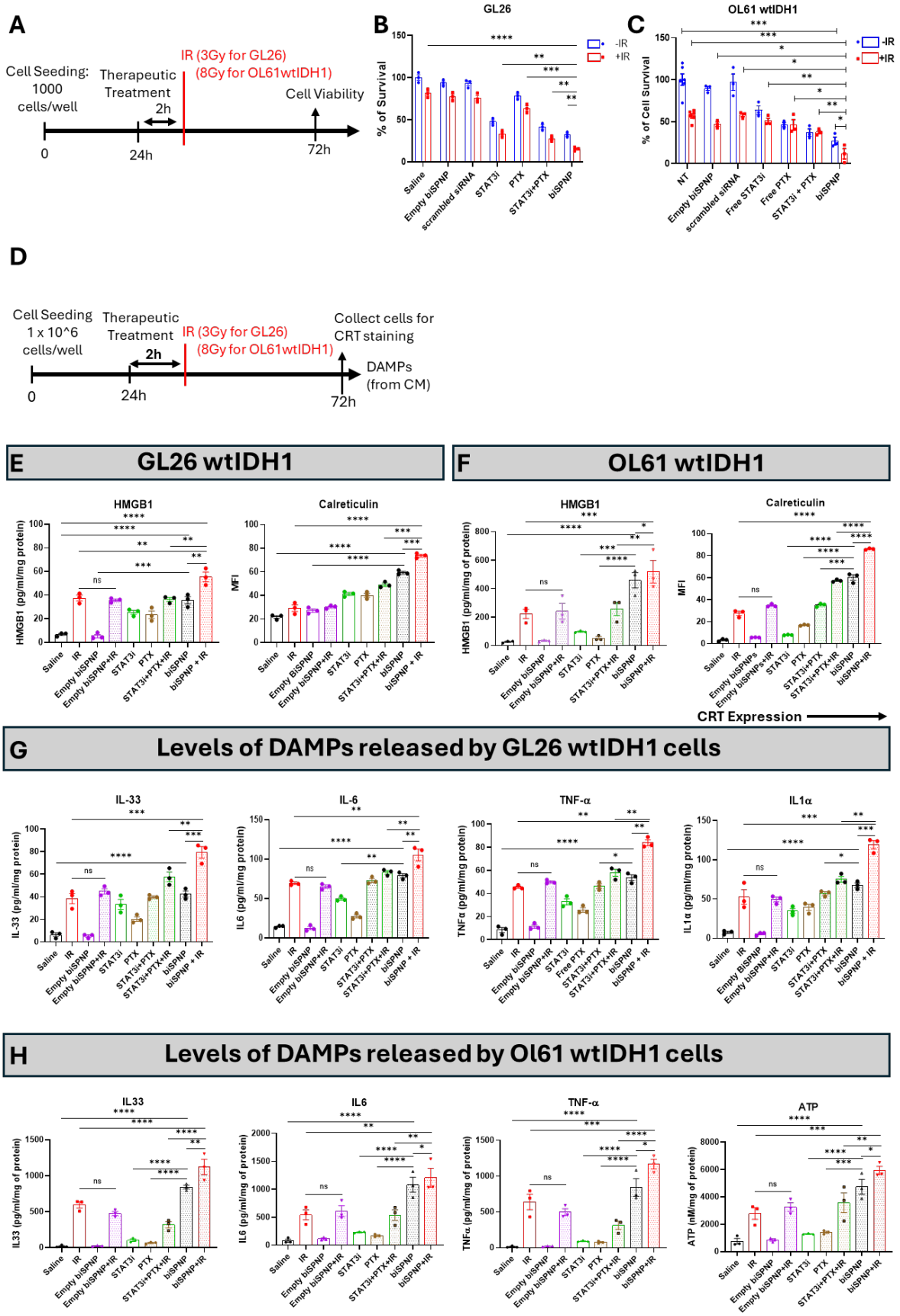
## Dose-response curve for radiation in NPD AC2 cells, HF2303 and OL61 wtIDH1 cells



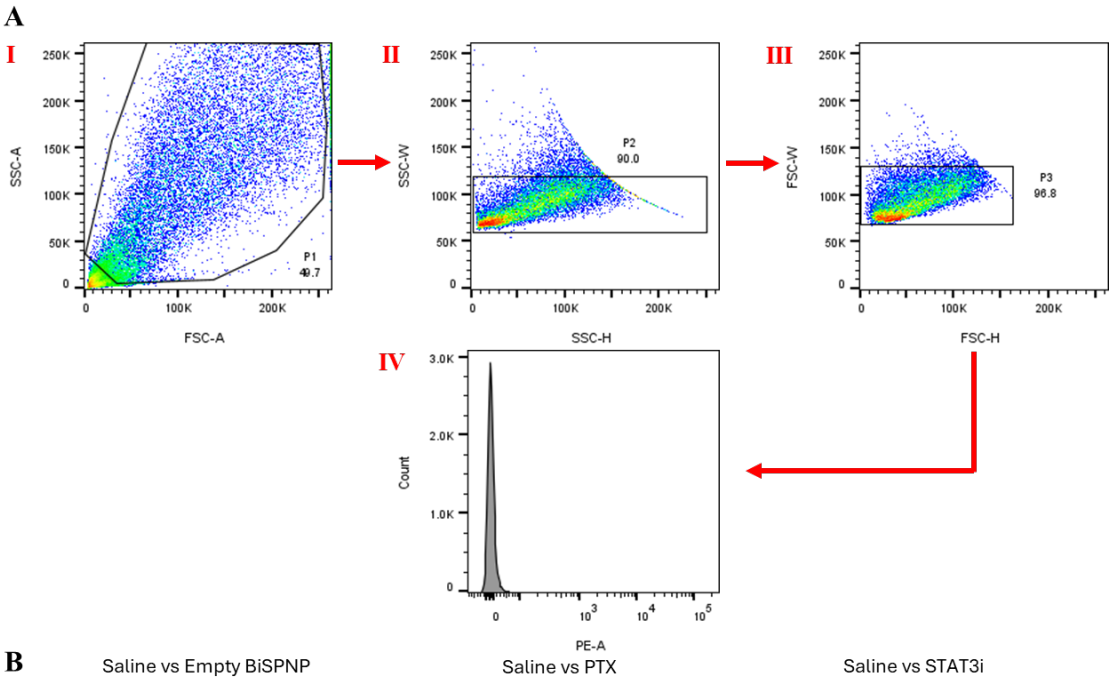

## Determination of IC50 doses of Paclitaxel in NPD AC2 cells, HF2303 and OL61 wtIDH1 cells

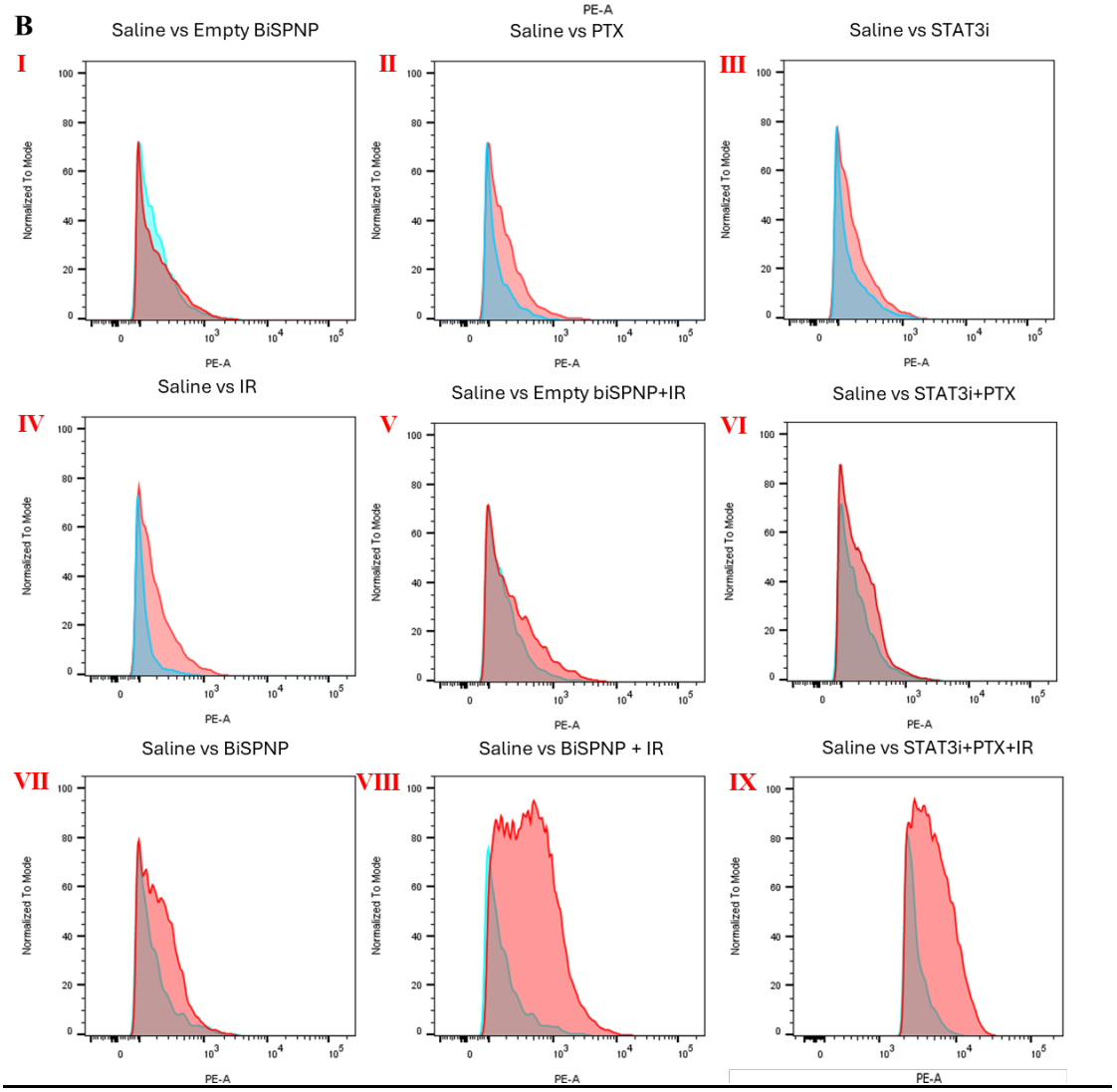


**Supplementary Figure 2**: Determination of $IC_{50}$ doses of radiation and paclitaxel in mouse and human high-grade glioma cells. Dose-response curve for irradiation (IR) in mouse genetically engineered (A) NPD AC2, (B) OL61 wtIDH1 and human patient-derived GBM (C) HF2303 cells. Dose-response curve for paclitaxel (PTX) on mouse genetically engineered (D) NPD AC2, (E) OL61 wtIDH1 and human patient-derived GBM (F) HF2303 cells.

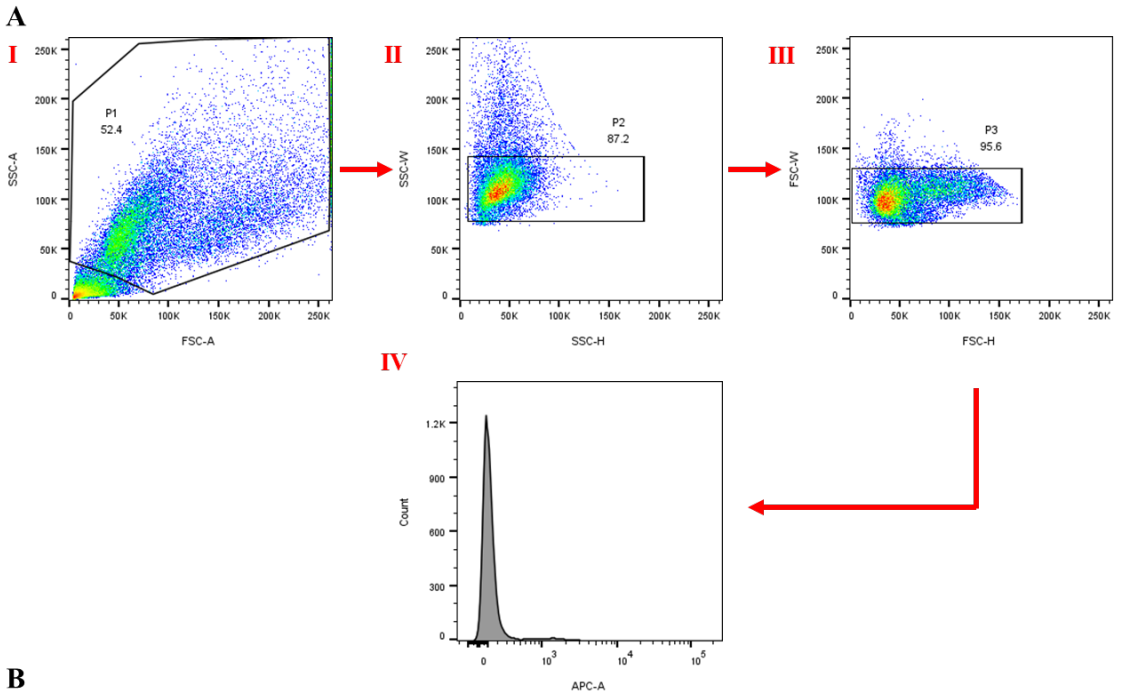

A
Cell Seeding: 1000 cells/well
Therapeutic Treatment 2h
IR (3Gy for GL26) (8Gy for OL61wtIDH1)
Cell Viability
0
24h
72h
B
GL26
% of Survival
-IR
+IR
Saline
Empty biSPNP
scrambled siRNA
STAT3i
PTX
STAT3i+PTX
biSPNP
C
OL61 wtIDH1
% of Cell Survival
NT
Empty biSPNP
scrambled siRNA
Free STAT3i
Free PTX
STAT3i + PTX
biSPNP
D
Cell Seeding 1 x 10^6 cells/well
Therapeutic Treatment 2h
IR (3Gy for GL26) (8Gy for OL61wtIDH1)
Collect cells for CRT staining
DAMPs (from CM)
0
24h
72h
E GL26 wtIDH1
F OL61 wtIDH1
HMGB1
HMGB1 (pg/ml/mg protein)
Calreticulin
MFI
CRT Expression
G Levels of DAMPs released by GL26 wtIDH1 cells
IL-33
IL-33 (pg/ml/mg protein)
IL-6
IL6 (pg/ml/mg protein)
TNF-α
TNFα (pg/ml/mg protein)
IL1α
IL1α (pg/ml/mg protein)
H Levels of DAMPs released by Ol61 wtIDH1 cells
IL33
IL33 (pg/ml/mg of protein)
IL6
IL6 (pg/ml/mg of protein)
TNF-α
TNFα (pg/ml/mg of protein)
ATP
ATP (nM/mg of protein)

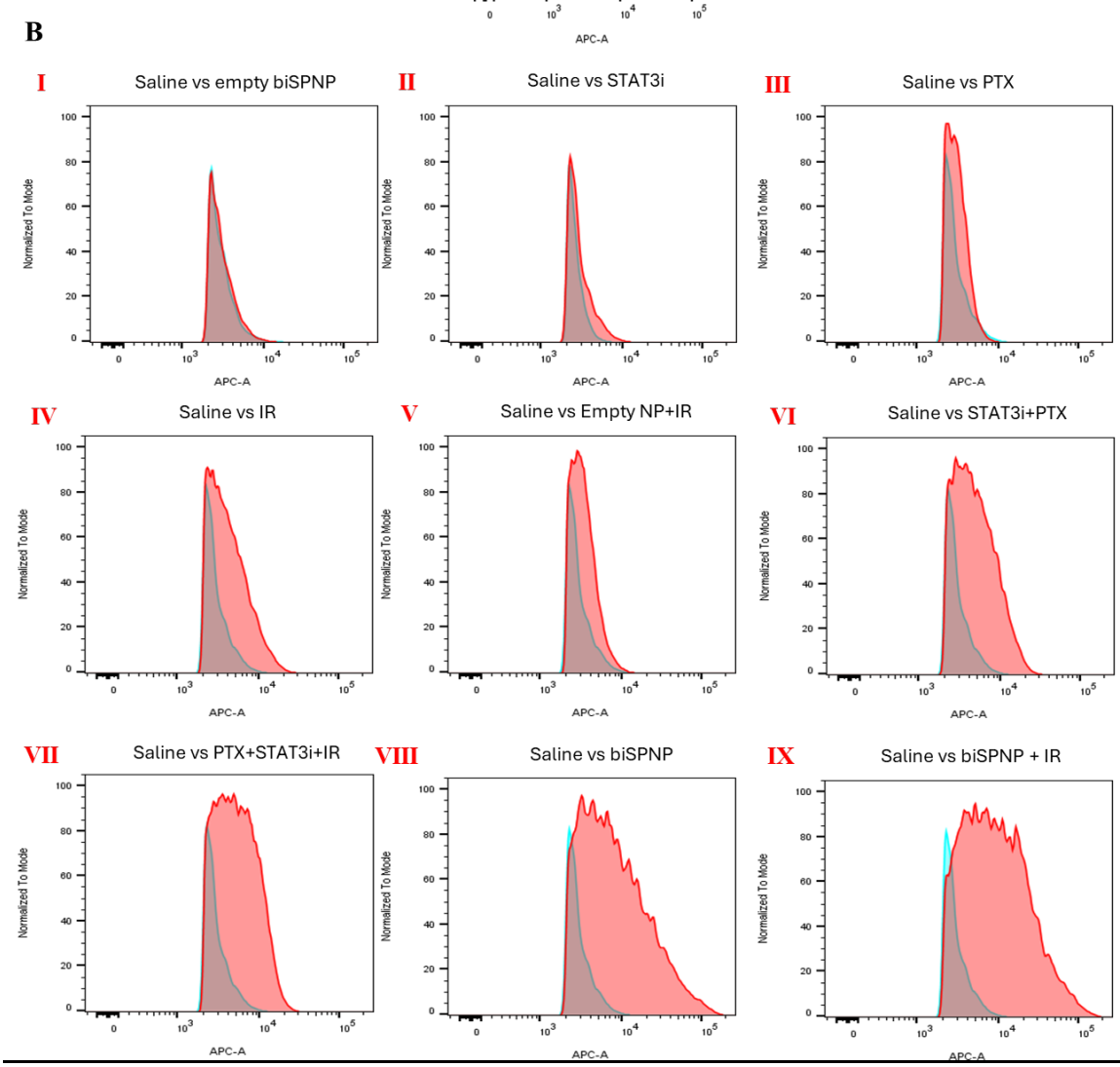
**Supplementary Figure 3: Targeting STAT3 and depolymerization of tubulin through STAT3i/PTX biSPNPs enhance radiosensitivity and immunogenic cell death in murine high-grade glioma cells. (A)** Schematic shows the timeline of the in vitro application of STAT3i/PTX biSPNPs and/or radiation in GL26 and GEMM mouse HGG cells. Cells were treated with either STAT3i/PTX biSPNPs, empty biSPNPs, scrambled siRNAs, free STAT3 siRNA (STAT3i), free paclitaxel (PTX), alone or in combination with radiation at their respective $IC_{50}$ doses (3Gy and 8Gy for GL26 and OL61wtIDH1 cells, respectively) for 72 h. Cells were pretreated with the respective compounds 2h prior to irradiation. Bar plot shows the % viable **(B)** GL26 and **(C)** OL61 wtDIH1 cells after treatment either with saline, biSPNP, empty biSPNP, scrambled siRNA, free-STAT3 siRNA, free-PTX, alone or in combination with IR. **(D)** Timeline of treatment to assess Calreticulin expression and other ICD markers released by murine glioma cells following STAT3i/PTX biSPNPs treatment. Bar diagrams represent levels of ICD markers, i.e., HMGB1 release and surface expression of Calreticulin in **(E)** mouse GL26 and **(F)** GEMM OL61 wtIDH1 cells after treatment with saline, PTX, STAT3i, free (STAT3i+PTX), empty biSPNPs, and STAT3i/PTX biSPNPs alone or in combination with IR. Quantitative ELISA shows the levels of DAMPs as ICD markers. i.e., IL33, IL6, TNFα, and IL1α in the **(G)** GL26 wt and **(H)** OL61 wtIDH1 HGG cells after treatment either with biSPNP, empty biSPNP, free PTX, free STAT3 siRNA, and free (STAT3i+PTX) with and without combination with IR at their respective $IC_{50}$ doses and analyzed after 72h. The levels of DAMPs released by the cells were normalized based on their respective protein concentrations. All the statistical analyses were done through an unpaired t-test. Bars represent mean ± SEM (n = 3 or 6 biological replicates). ns= not significant, *p<0.05 **p< 0.01, ***p< 0.001, ****p< 0.0001. MFI: mean fluorescence intensity.


A
I
II
III
IV
SSC-A
FSC-A
SSC-W
SSC-H
FSC-W
FSC-H
P1
49.7
P2
90.0
P3
96.8
Count
PE-A


B
I
Saline vs Empty BiSPNP
II
Saline vs PTX
III
Saline vs STAT3i
IV
Saline vs IR
V
Saline vs Empty biSPNP+IR
VI
Saline vs STAT3i+PTX
VII
Saline vs BiSPNP
VIII
Saline vs BiSPNP + IR
IX
Saline vs STAT3i+PTX+IR
Normalized To Mode
PE-A

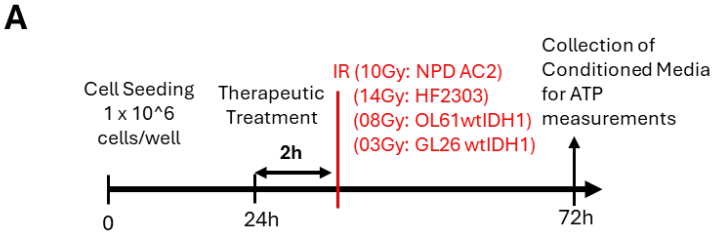
**Supplementary Figure 4: STAT3i/PTX biSPNPs enhance the surface expression of Calreticulin in NPD AC2 cells. (A)** Sequential gating strategy for flow cytometry analysis of Calreticulin expression in NPD AC2 cells. **[I]** Cells were gated to exclude cellular debris. **[II-III]** doublet discrimination gating was performed to filter out cellular aggregates prior to analysis. **[IV]** PE+ cells were gated to identify Calreticulin positive NPD AC2 GBM cells. **(B)** Surface expression of Calreticulin in NPD AC2 cells were determined following biSPNP treatment (at $IC_{50}$ values) alone or in combination with 10Gy of IR. Representative histograms and bar diagrams display calreticulin's expression levels following different therapeutic treatments (In histograms: blue = non-treated and red =treated) compared to non-treated controls. Cells were also treated with combined (STAT3i+PTX), free-PTX, free-STAT3i, and empty biSPNPs alone or in combination with IR at their respective $IC_{50}$ values within the same experimental condition and analyzed after 72h. MFI= mean fluorescence intensity. ns= non-significant, *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001; unpaired t-test. Bars represent mean ± SEM (n= 3 biological replicates).

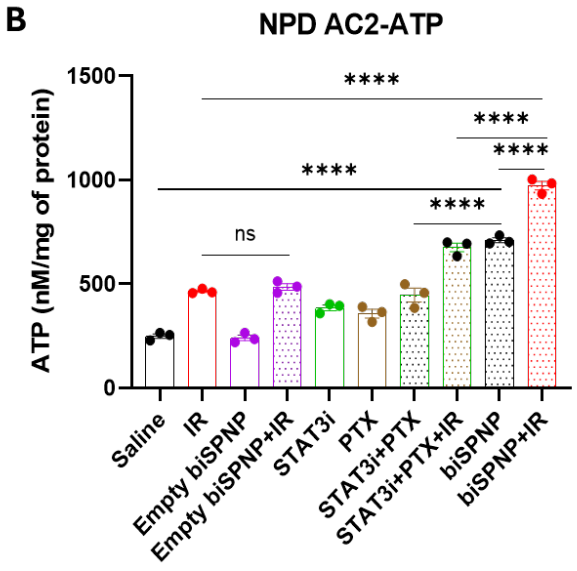

A
I
P1
52.4
SSC-A
FSC-A
II
P2
87.2
SSC-W
SSC-H
III
P3
95.6
FSC-W
FSC-H
IV
Count
APC-A

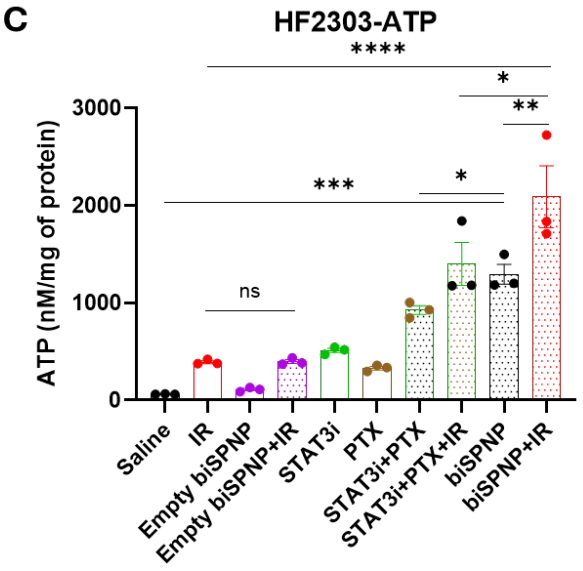

B
I
Saline vs empty biSPNP
II
Saline vs STAT3i
III
Saline vs PTX
IV
Saline vs IR
V
Saline vs Empty NP+IR
VI
Saline vs STAT3i+PTX
VII
Saline vs PTX+STAT3i+IR
VIII
Saline vs biSPNP
IX
Saline vs biSPNP + IR
Normalized To Mode
APC-A

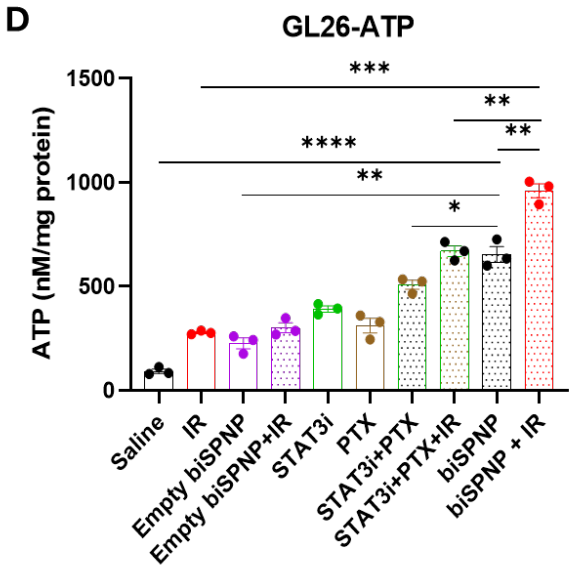
**Supplementary Figure 5: STAT3i/PTX biSPNPs enhance the surface expression of Calreticulin in human patient-derived glioma cells. (A)** Sequential gating strategy for flow cytometry analysis of Calreticulin expression in human HGG, HF2303 cells. **[I]** Cells were gated to exclude cellular debris. **[II-III]** doublet discrimination gating was performed to filter out cellular aggregates prior to analysis. **[IV]** APC+ cells were gated to identify Calreticulin positive HF2303 GBM cells. **(B)** Surface expression of Calreticulin in HF2303 cells were determined following biSPNP treatment (at $IC_{50}$ values) alone or in combination with 14 Gy of IR. Representative histograms and bar diagrams display calreticulin's expression levels following different therapeutic treatments (In histograms: blue = non-treated and red =treated) compared to non-treated controls. Cells were also treated with free (STAT3i+PTX), free PTX, free STAT3i, and empty biSPNPs alone or in combination with IR at their respective $IC_{50}$ values within the same experimental condition and analyzed after 72h. MFI = mean fluorescence intensity. Ns = non-significant, *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001; unpaired t-test. Bars represent mean ± SEM (n = 3 biological replicates).

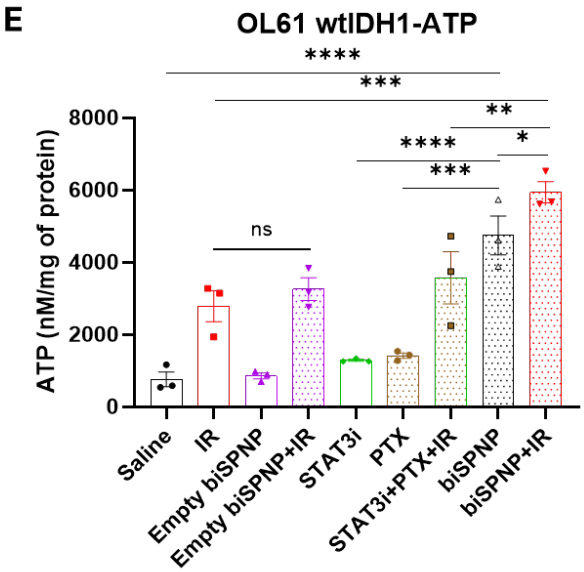
# Levels of ATP released by mouse and human high-grade glioma cells



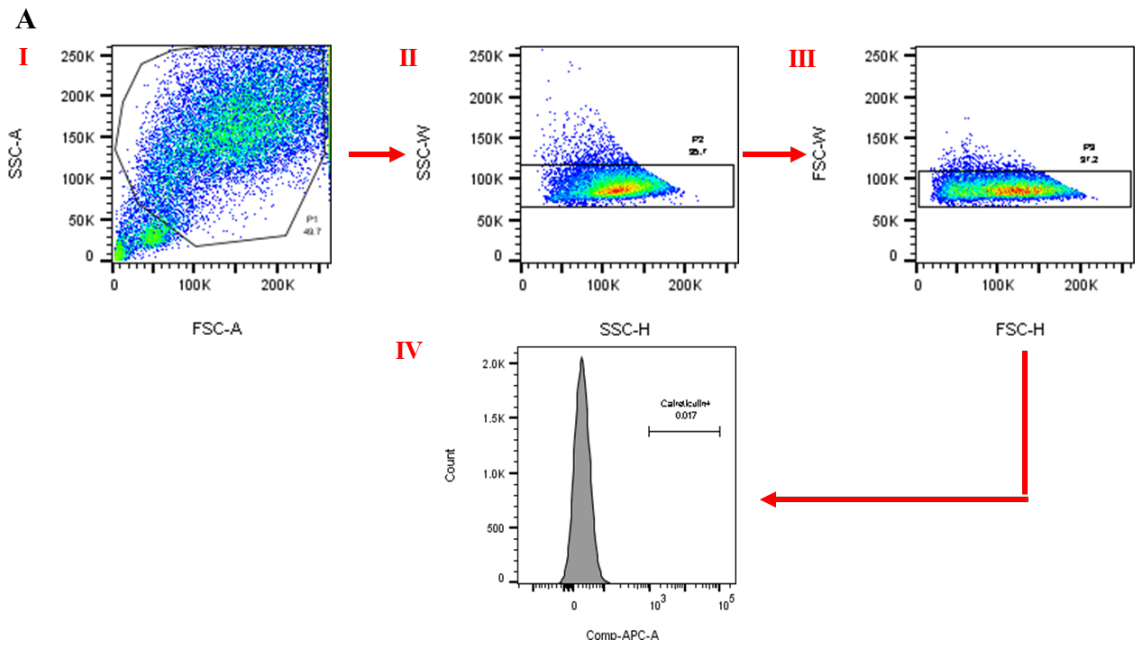



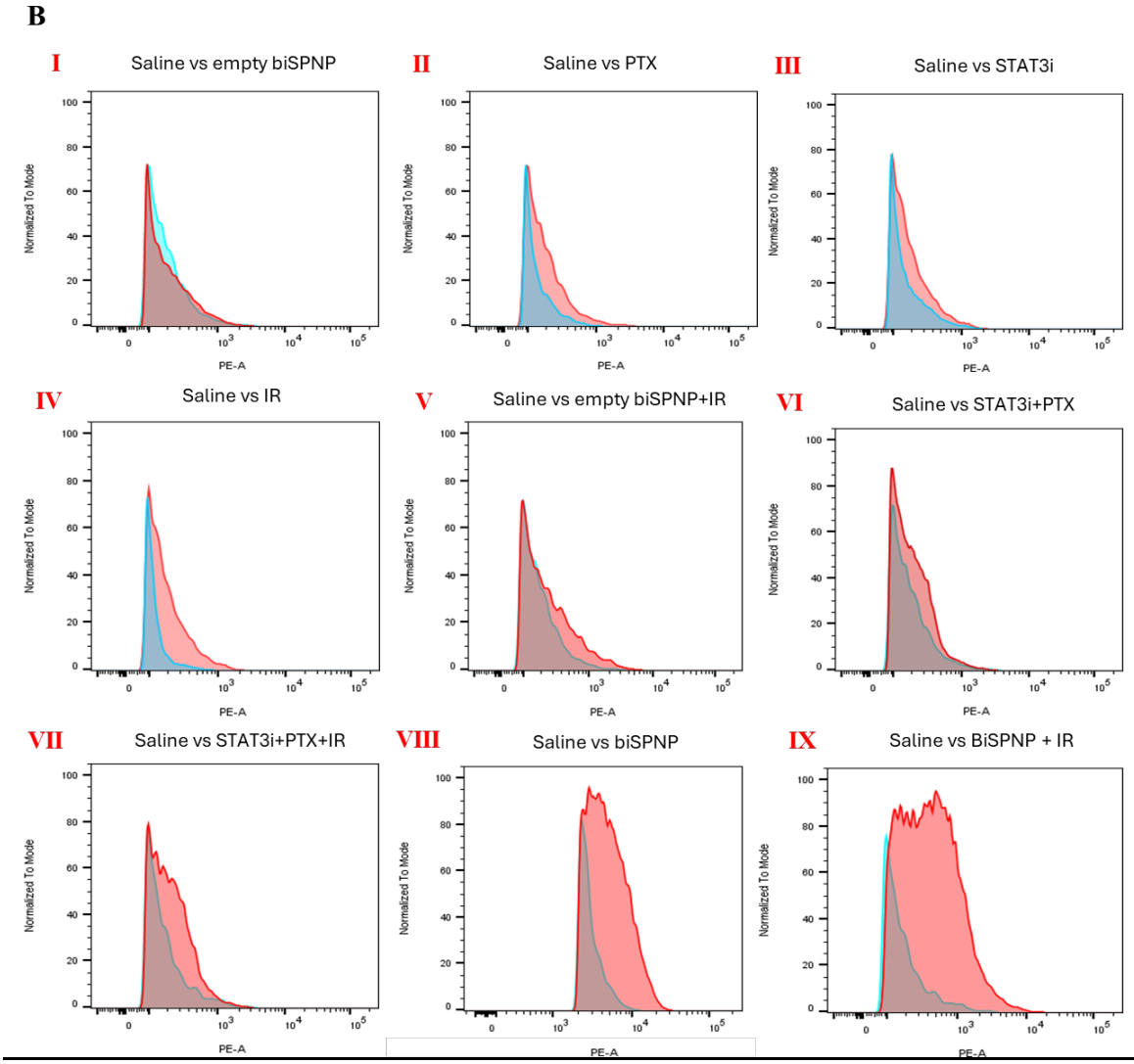



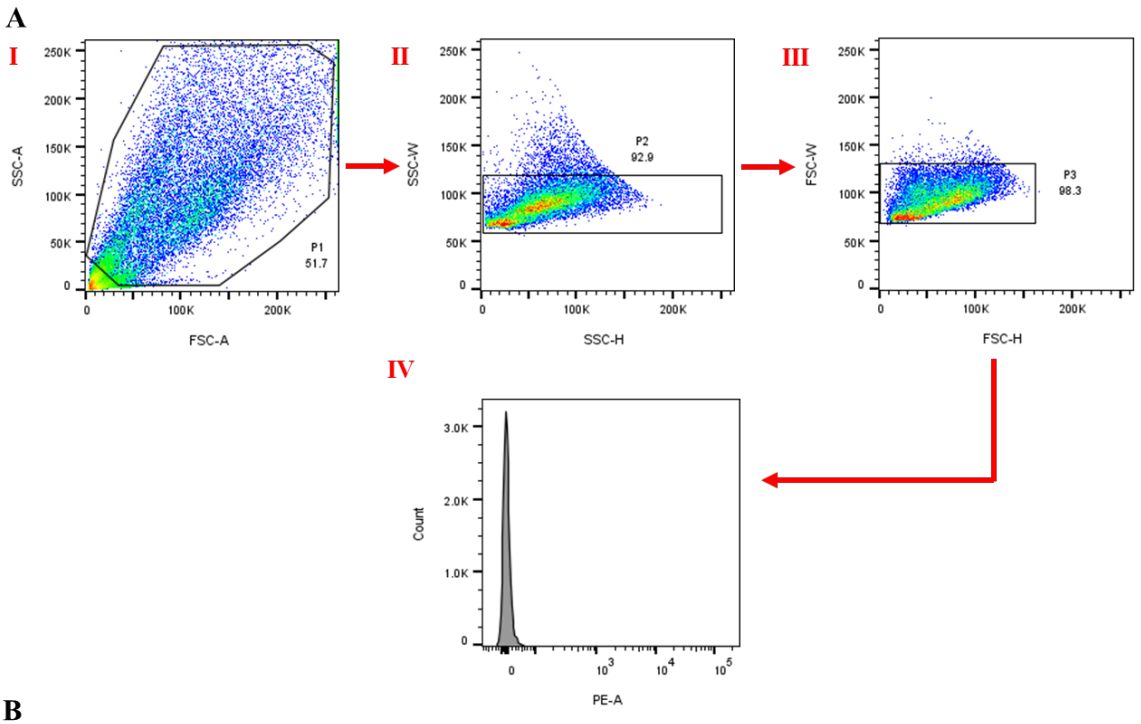



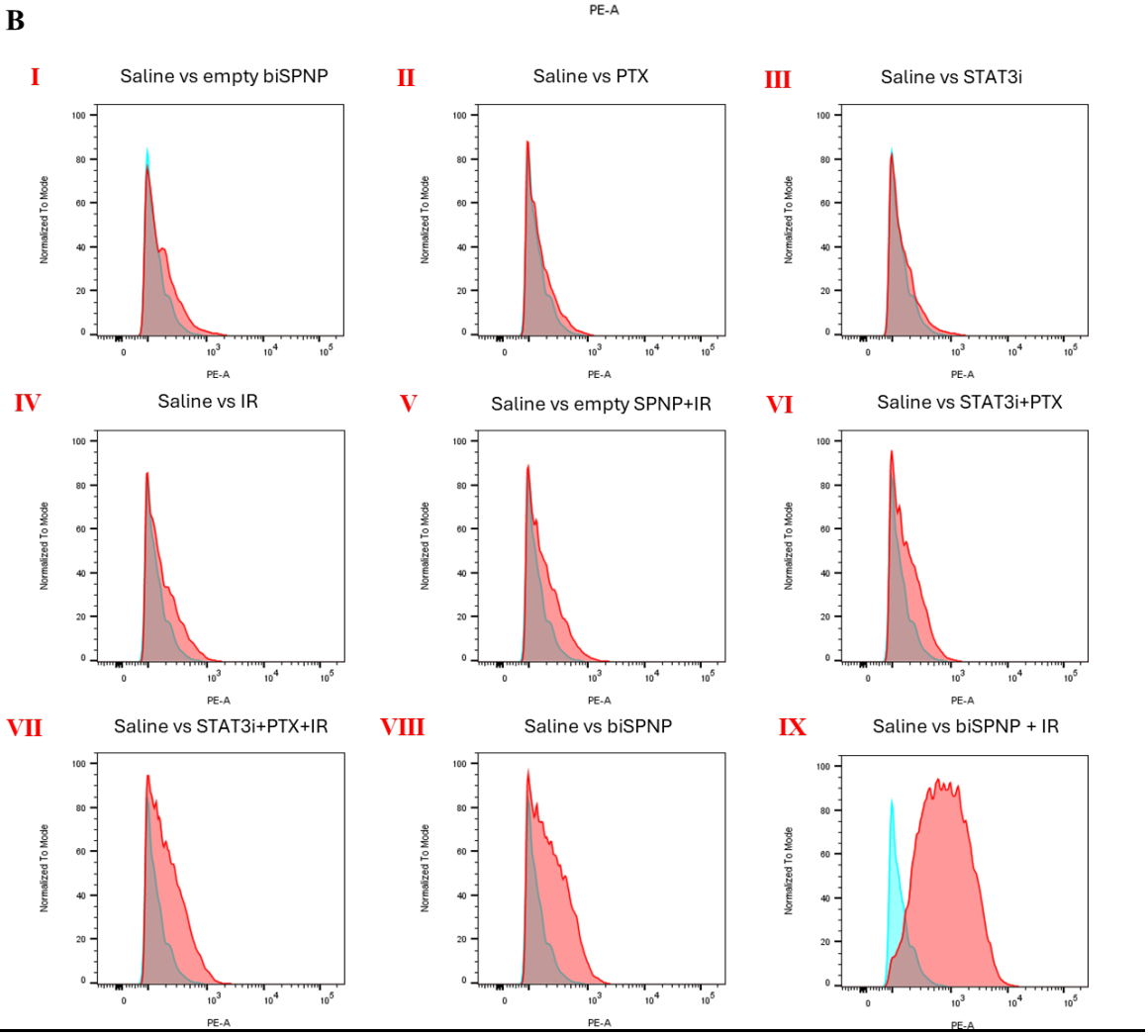
**Supplementary Figure 6: Targeting STAT3 and inhibiting tubulin depolymerization through STAT3i/PTX biSPNPs increase the release of ATP as a key regulator of ICD in murine and human patient-derived HGG cells. (A)** Schematic shows the timeline of the in vitro application of STAT3i/PTX biSPNPs and/or radiation in GEMM mouse and human patient-derived HGG cells. Cells were treated with either STAT3i/PTX biSPNPs, empty biSPNP, scrambled siRNAs, free STAT3 siRNA (STAT3i), free paclitaxel (PTX), free (STAT3i + PTX) with and without ionizing radiation at their respective $IC_{50}$ doses (3Gy, 8Gy, 10Gy, and 14Gy for GL26, OL61 wtIDH1, NPD AC2, and HF2303 cells) for 72 h. Cells were pretreated with the respective compounds 2h prior to irradiation. Bar plots represent the level of ATP as an important ICD marker released from **(B)** NPD AC2, **(C)** HF2303, **(D)** GL26 wt, and **(E)** Ol61 wtIDH1 high-grade glioma cells after treatment either with biSPNP, empty biSPNP, free PTX, free STAT3i, free (STAT3i+PTX) or in combination with IR at their respective $IC_{50}$ doses and analyzed after 72h. The levels of ATP released by the respective cells were normalized through its protein concentration. All the statistical analyses were done through an unpaired t-test. Bars represent mean ± SEM (n = 3 biological replicates) ns= non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.


A
I
II
III
IV
SSC-A
FSC-A
SSC-W
FSC-W
FSC-H
SSC-H
Count
Comp-APC-A
Calreticulin+
0.017


B
I
Saline vs empty biSPNP
II
Saline vs PTX
III
Saline vs STAT3i
IV
Saline vs IR
V
Saline vs empty biSPNP+IR
VI
Saline vs STAT3i+PTX
VII
Saline vs STAT3i+PTX+IR
VIII
Saline vs biSPNP
IX
Saline vs BiSPNP + IR
Normalized To Mode
PE-A

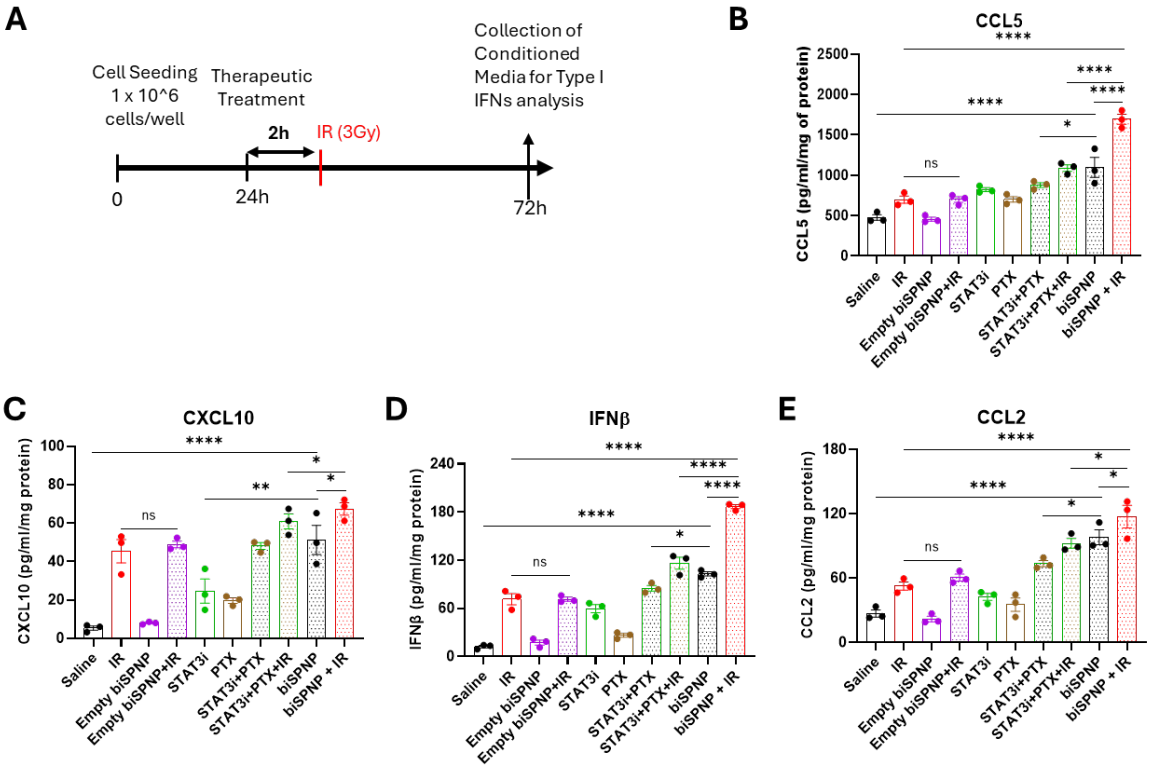
**Supplementary Figure 7: STAT3i/PTX biSPNPs enhance the surface expression of Calreticulin in mouse GL26 wt high-grade glioma cells. (A)** Sequential gating strategy for flow cytometry analysis of Calreticulin expression in GL26 wt cells. **[I]** Cells were gated to exclude cellular debris. **[II-III]** doublet discrimination gating was performed to filter out cellular aggregates prior to analysis. **[IV]** PE+ cells were gated to identify Calreticulin-positive GL26 wt glioma cells. **(B)** Surface expression of Calreticulin in GL26 cells were determined following biSPNP treatment (at $IC_{50}$ values) alone or in combination with 3 Gy of IR. Representative histograms and bar diagrams display calreticulin's expression levels following different therapeutic treatments (In histograms: blue = non-treated and red = treated) compared to non-treated controls. Cells were also treated with free (STAT3i+PTX), free PTX, free STAT3i, and empty biSPNPs alone or in combination with IR at their respective $IC_{50}$ values within the same experimental condition and analyzed after 72h. MFI= mean fluorescence intensity. ns= non-significant, *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001; based on an unpaired t-test. Bars represent mean ± SEM (n = 3 biological replicates).


A
I
SSC-A
FSC-A
P1
51.7
II
SSC-W
SSC-H
P2
92.9
III
FSC-W
FSC-H
P3
98.3
IV
Count
PE-A

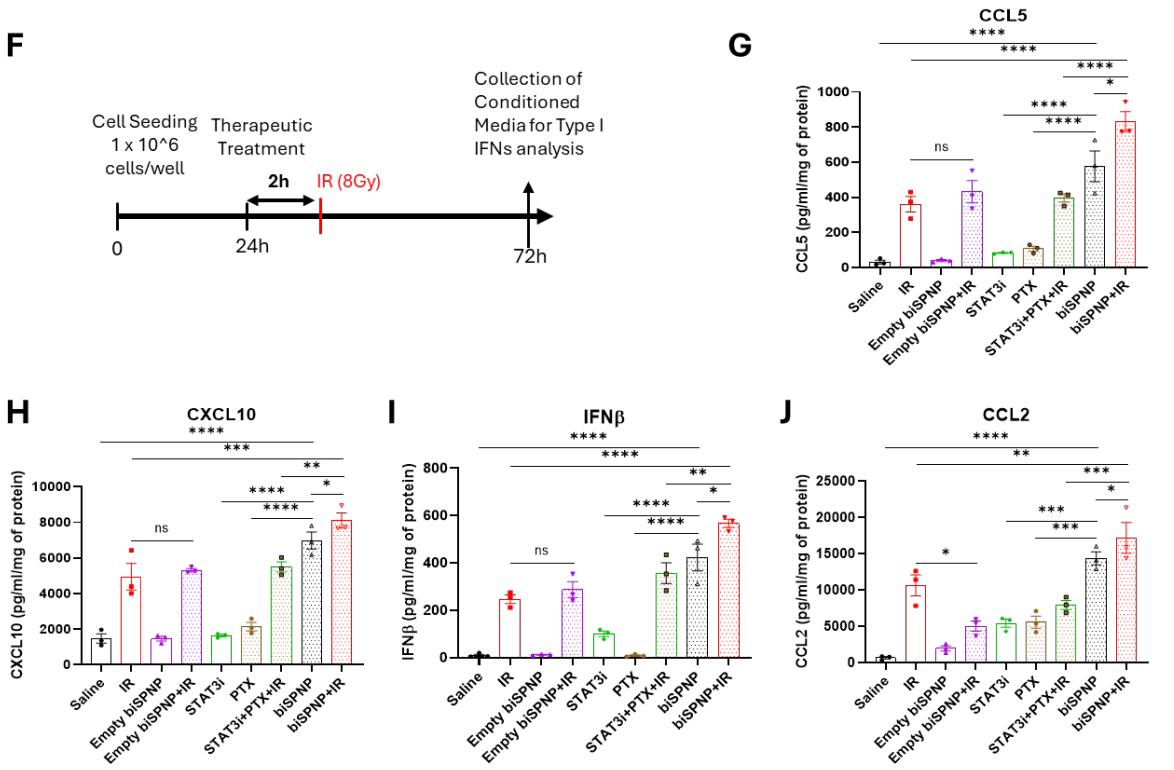

B
I Saline vs empty biSPNP
II Saline vs PTX
III Saline vs STAT3i
IV Saline vs IR
V Saline vs empty SPNP+IR
VI Saline vs STAT3i+PTX
VII Saline vs STAT3i+PTX+IR
VIII Saline vs biSPNP
IX Saline vs biSPNP + IR
Normalized To Mode
PE-A

**Supplementary Figure 8: STAT3i/PTX biSPNPs enhance the surface expression of Calreticulin in genetically engineered mouse OL61 wtIDH1 cells. (A)** Sequential gating strategy for flow cytometry analysis of Calreticulin expression in OL61 wtIDH1 cells. **[I]** Cells were gated to exclude cellular debris. **[II-III]** doublet discrimination gating was performed to filter out cellular aggregates prior to analysis. **[IV]** PE+ cells were gated to identify Calreticulin-positive OL61 wtIDH1 glioma cells. **(B)** Surface expression of Calreticulin in OL61 wtIDH1 cells was determined following biSPNP treatment (at $IC_{50}$ values) alone or in combination with 8 Gy of IR. Representative histograms and bar diagrams display calreticulin's expression levels following different therapeutic treatments (In histograms: blue = non-treated and red =treated) compared to non-treated controls. Cells were also treated with free (STAT3i+PTX), free PTX, free STAT3i, and empty biSPNPs alone or in combination with IR at their respective $IC_{50}$ values within the same experimental condition and analyzed after 72h. MFI= mean fluorescence intensity. ns= non-significant, *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001; based on an unpaired t-test. Bars represent mean ± SEM (n= 3 biological replicates).

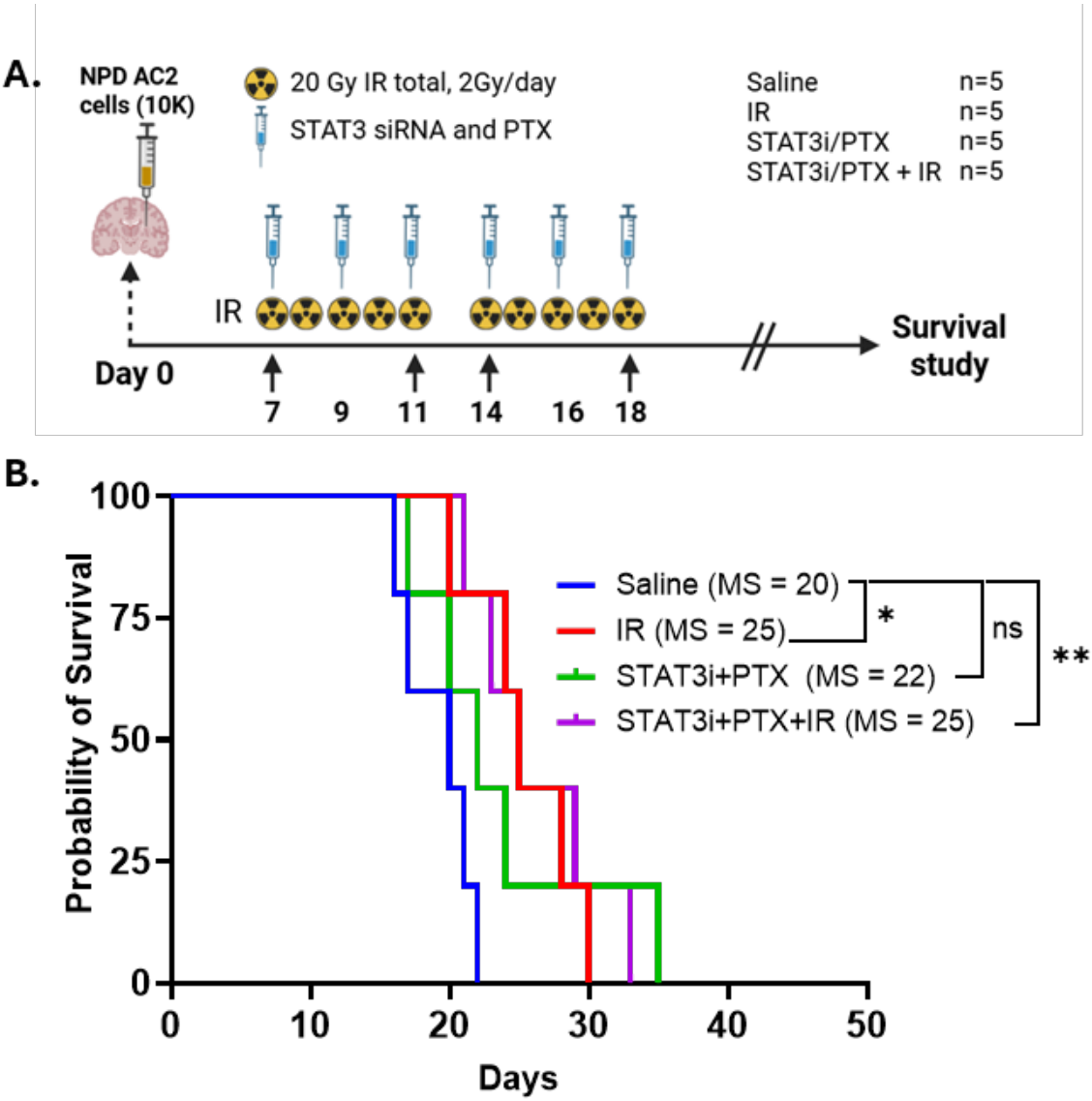
## Levels of Type I IFNs released by GL26 wtIDH1 cells




## Levels of Type I IFNs released by Ol61 wtIDH1 cells



**Supplementary Figure 9: In vitro inhibition of STAT3 and inhibition of tubulin depolymerization through STAT3i/PTX biSPNPs increase the release of Type I IFNs in murine HGG cells.** The schematic illustrates the timeline for the in vitro application of STAT3i/PTX biSPNPs and/or radiation in **(A)** GL26 wt and genetically engineered mouse HGG **(F)** Ol61 wtIDH1 cells. Cells were treated for 72 hours with either STAT3i/PTX biSPNPs, empty biSPNPs, scrambled siRNAs, free STAT3 siRNA (STAT3i), free paclitaxel (PTX), free (STAT3i + PTX), with and without ionizing radiation at their respective $IC_{50}$ doses (3 Gy for GL26 and 8 Gy for Ol61 wtIDH1). Pretreatment with the respective compounds was conducted 2 hours before irradiation. **(B-E)** Quantitative ELISA shows the levels of Type I IFNs as pro-immunogenic markers i.e., CCL5, CXCL10, IFNβ, and CCL2, released by GL26 HGG cells after treatment either with biSPNP, empty biSPNP, free-PTX, free-STAT3 siRNA, free-(STAT3i+PTX) or in combination with IR at their respective $IC_{50}$ doses and analyzed after 72h. **(G-J)** Quantitative ELISA shows the levels of Type I IFNs as pro-immunogenic markers i.e., CCL5, CXCL10, IFNβ, and CCL2, released by mouse genetically engineered HGG, OL61 wtIDH1 cells after treatment either with biSPNP, empty biSPNPs, free PTX, free STAT3i, free (STAT3i+PTX) with and without IR at their respective $IC_{50}$ doses and analyzed after 72h. The levels of Type I IFNs released by the cells were normalized through its respective protein concentration. All the statistical analysis were done through unpaired t test. Bars represent mean ± SEM (n = 3 biological replicates) ns= non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



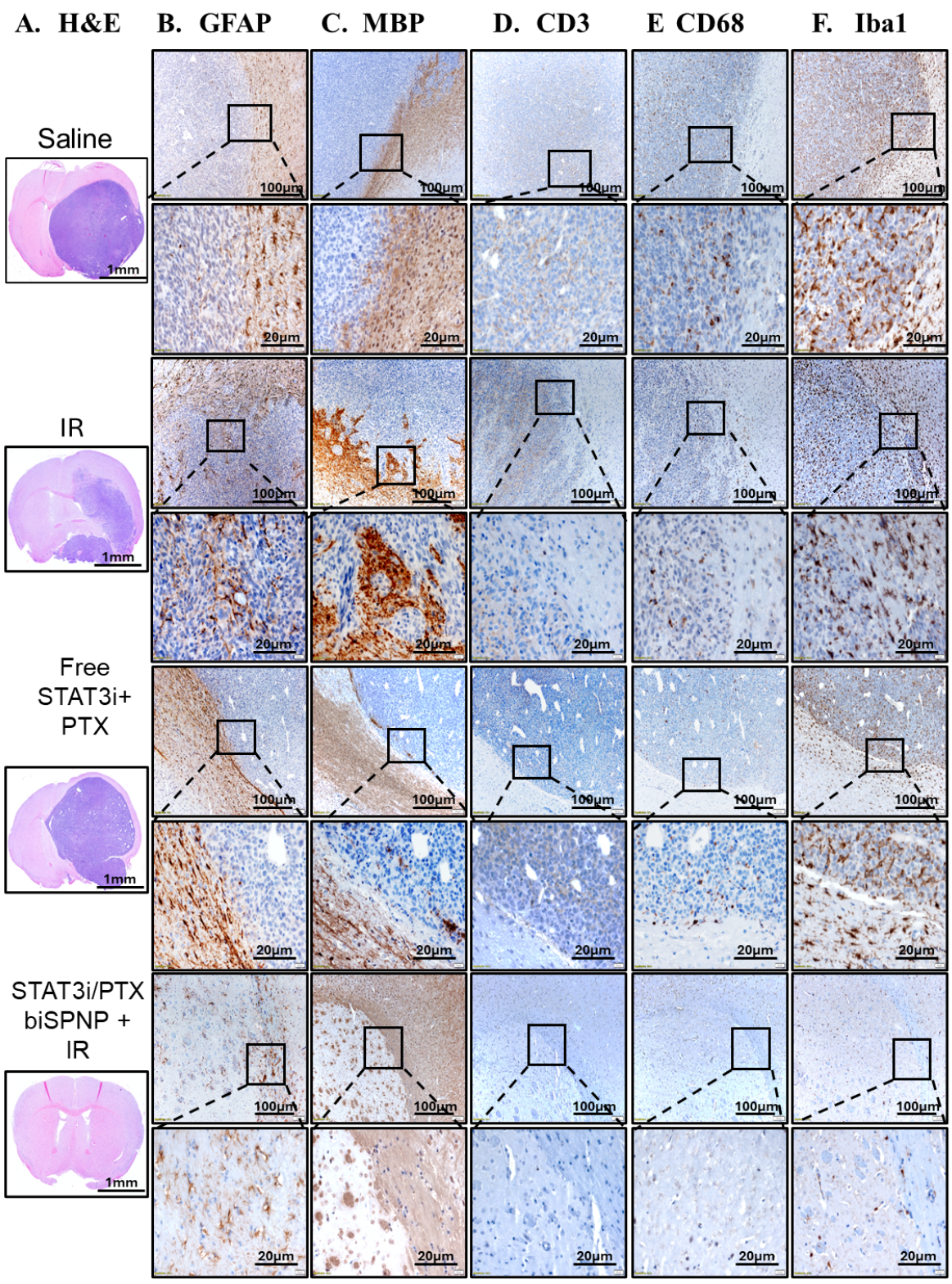

**Supplementary Figure 10: Efficacy of Free STAT3i+PTX in glioma-bearing mice**. **A.** Illustration depicting the treatment schedule of free (STAT3i+PTX) with and without IR. After tumor implantation on day 0, treatment began on day 7 and ended on day 18. The groups tested were saline, free STAT3i+PTX combination, IR, and free STAT3i+PTX combination with IR, each with n=5 mice per group. **B.** Kaplan-Meier survival curves for groups treated with (saline, Free STAT3i+PTX, Free STAT3i+PTX +IR). Ns = not significant, *p<0.05, **p<0.01; log-rank (Mantel-Cox) test.

**Supplementary Table 2.** P-values of compared treatment groups (saline, free STAT3i+PTX, IR, free STAT3i+PTX + IR) using log-rank (Mantel-Cox) Kaplan Meier survival analysis. Significance levels were defined as not significant (ns, $p>0.05$), *$p<0.05$, **$p<0.01$.

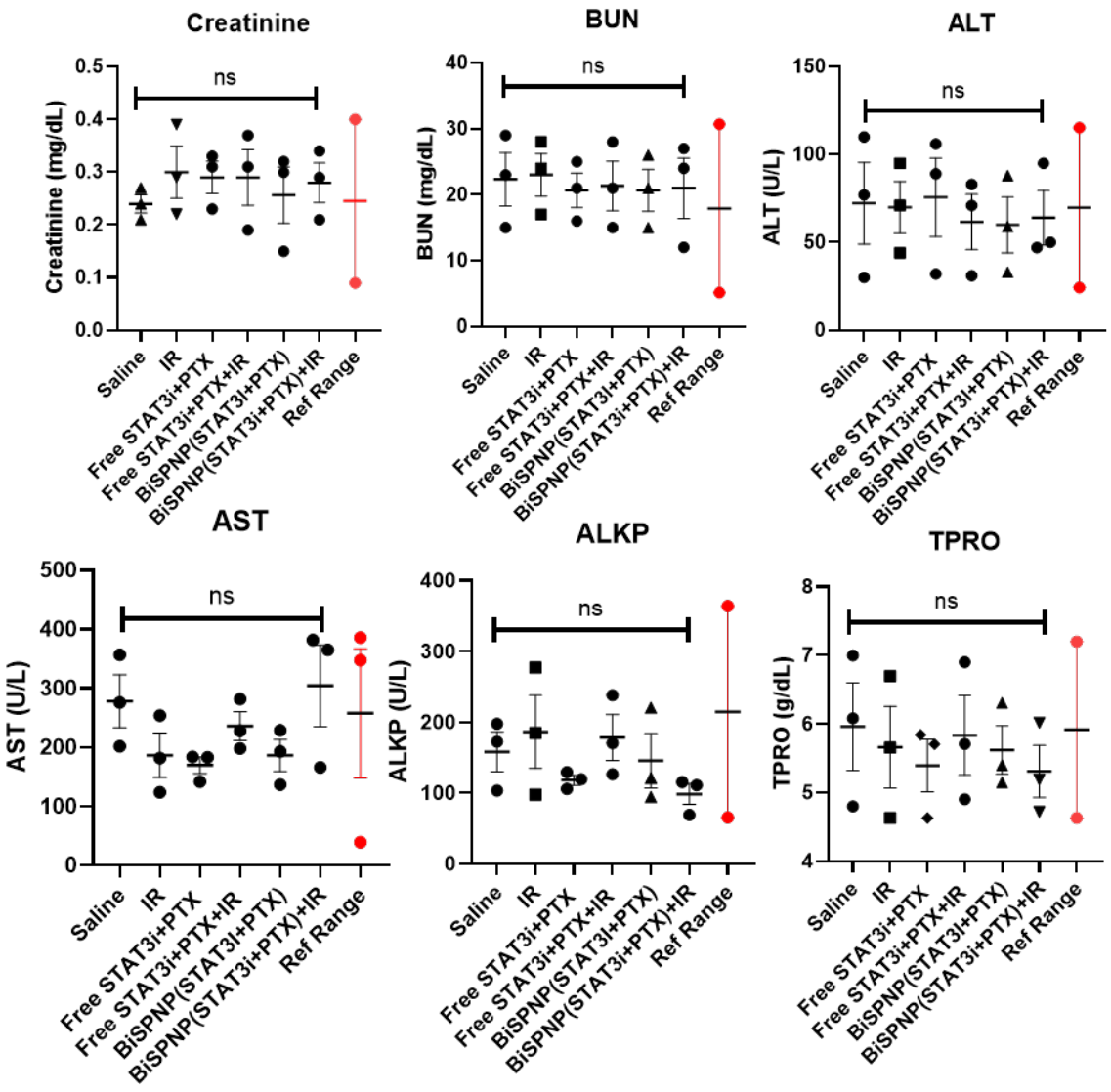
| Treatment groups | P value | Significance |
|---|---|---|
| Saline (MS=20) vs IR (MS=25) | P value=0.0143 | * |
| Saline (MS=20) vs Free STAT3i+PTX (MS=22) | P value=0.143 | ns |
| Saline (MS=20) vs Free STAT3i+PTX+IR +IR (MS=25) | P value=0.0088 | ** |
| Free STAT3i+PTX (MS=22) vs Free STAT3i+PTX +IR (MS=25) | P value=0.7638 | ns |

**Supplementary Table 3.** P-values of compared treatment groups (saline, STAT3i/PTX biSPNP, IR, STAT3i/PTX biSPNP + IR) using log-rank (Mantel-Cox) Kaplan Meier survival analysis. Significance levels were defined as not significant (ns, $p>0.05$), *$p<0.05$, **$p<0.01$.

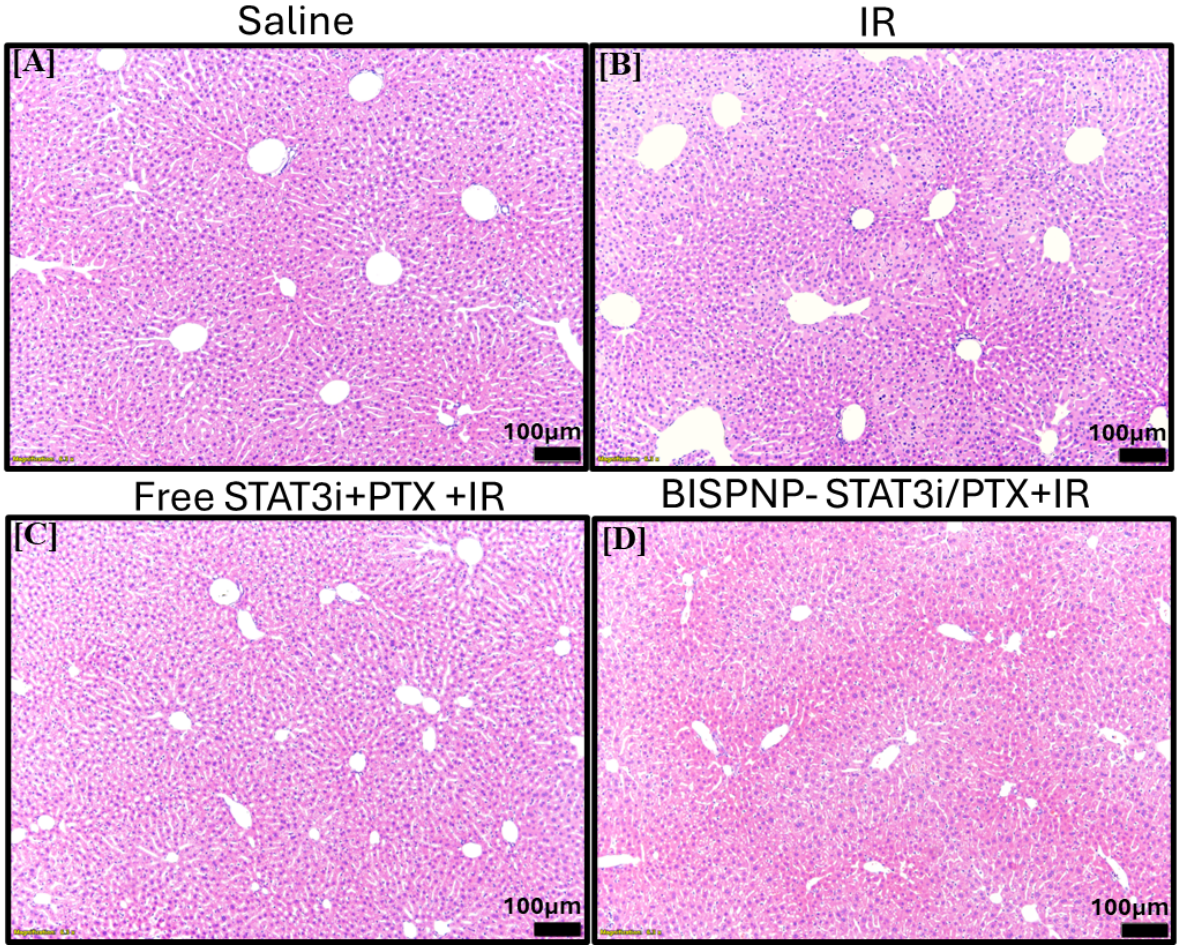
| Treatment groups | P value | Significance |
|---|---|---|
| Saline (MS=18) vs IR (MS=21) | P value=0.0843 | ns |
| Saline (MS=18) vs STAT3i/PTX biSPNP (MS=23) | P value=0.0026 | ** |
| Saline (MS=18) vs STAT3i/PTX biSPNP+IR (MS=43.5) | P value=0.0013 | ** |
| STAT3i/PTX biSPNP (MS=23) vs STAT3i/PTX biSPNP+IR (MS=43.5) | P value=0.3265 | ns |


A. H&E
B. GFAP
C. MBP
D. CD3
E CD68
F. Iba1
Saline
IR
Free STAT3i+ PTX
STAT3i/PTX biSPNP + IR
1mm
100μm
20μm

**Supplementary Figure 11: Histopathological and immunohistochemical analysis of brain sections from different treatment groups. A.** H&E staining of 5 μm paraffin-embedded brain sections from saline, IR, free STAT3i+PTX + IR, and long-term survivors from STAT3i/PTX biSPNPs + IR treatment groups (scale bar = 1 mm). **B.** Paraffin-embedded 5 μm brain sections for each treatment groups were stained for glial fibrillary acidic protein (GFAP), **C.** myelin basic protein (MBP), **D.** CD3 (T cells), **E.** CD68 (activated macrophages/microglia), and **F.** Iba1 (microglia/macrophages). Representative images from an experiment consisting of three independent biological replicates are displayed.




**Supplementary Figure 12. Mouse serum biochemical analysis following treatment with STAT3i/PTX biSPNPs in combination with IR.** Glioma-bearing mice treated with STAT3i/PTX biSPNPs in combination with radiation (IR) exhibit normal serum biochemical parameters compared to saline-treated control animals. Serum was collected from tumor-bearing mice treated with saline, IR, free STAT3i+PTX, free STAT3i+PTX +IR, STAT3i/PTX biSPNPs, or STAT3i/PTX biSPNPs + IR. For each treatment group, levels of Creatinine, blood urea nitrogen (BUN), Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALKP), and Total Protein (TPRO), were quantified. The reference range is indicated in red. The levels of different serum biochemical parameters between the treatment groups were compared and were found non-significant when compared to the control group. $p > 0.05$ (n=3 biological replicates).




**Supplementary Figure 13: Figure Histopathological assessment of livers from tumor-bearing mice treated with STAT3i/PTX biSPNPs.** H&E staining of 5µm paraffin-embedded liver sections from Saline (A), IR (B), free STAT3i+PTX+IR(C), STAT3i/PTX biSPNPs (D) treatment groups. In each treatment group. There are no differences in the hepatocytes and the stromal central and portal areas between the control saline group and the different treatment groups. Representative images from an experiment consisting of 5 independent biological replicates are displayed. Black scale bars = 100 µm.