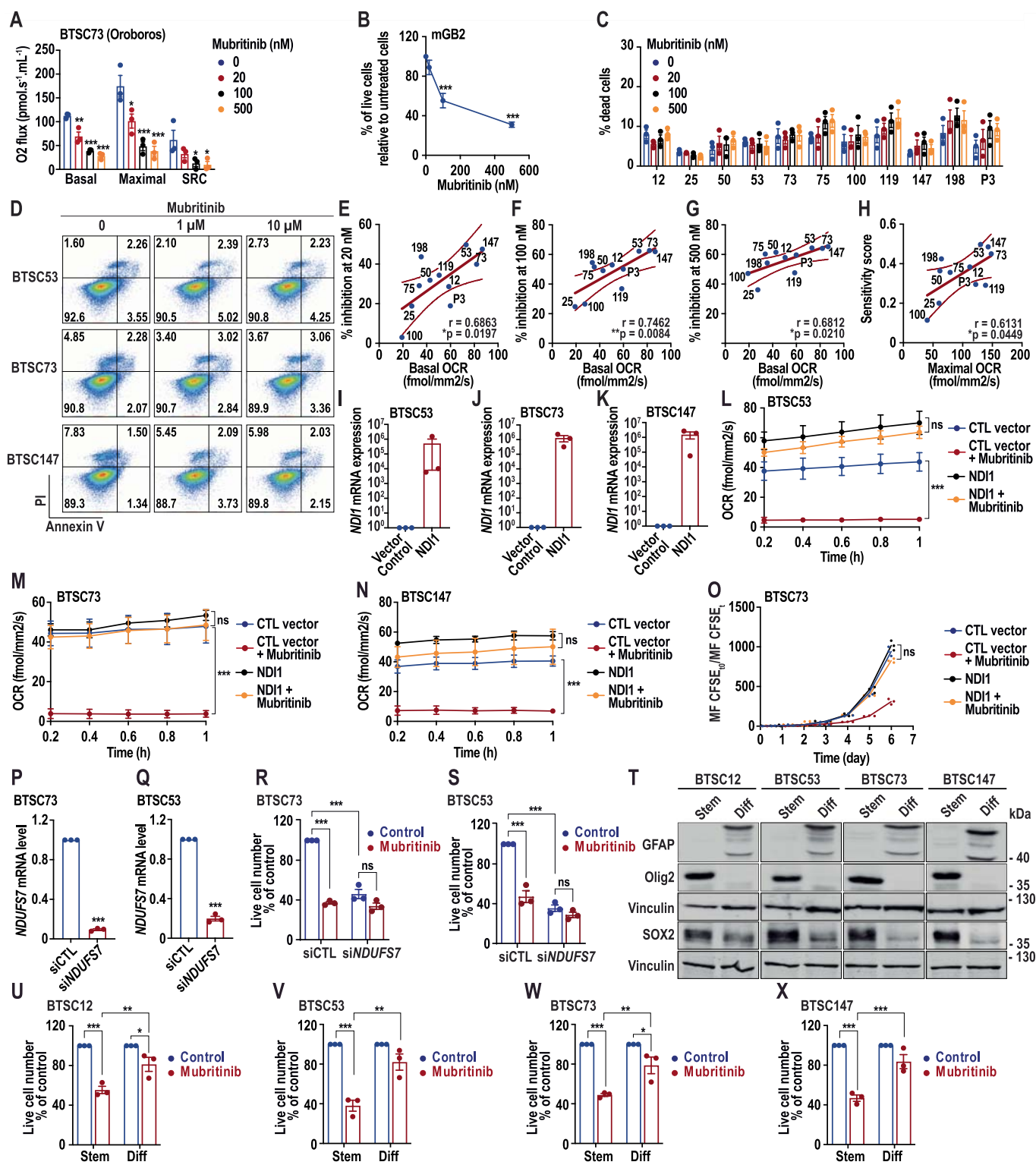


Expanded View Figures

Figure EV1. Mubritinib decreases the proliferation of patient- and murine-derived BTSCs without inducing cell death.

(A) Basal mitochondrial respiration, maximal mitochondrial respiration and spare respiratory capacity (SRC) were measured in BTSC73 following mubritinib treatment using the high-resolution respirometer Oroboros. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Dunnett's test vs control. $^{**}p_{\text{basal}}(20) = 0.0012$; $^{***}p_{\text{basal}}(100) = 2.9\text{e-}5$; $^{***}p_{\text{basal}}(500) = 1.1\text{e-}5$, $^{*}p_{\text{maximal}}(20) = 0.0228$, $^{***}p_{\text{maximal}}(100) = 0.0009$, $^{***}p_{\text{maximal}}(500) = 0.0006$, $^{*}p_{\text{SRC}}(100) = 0.0488$, $^{*}p_{\text{SRC}}(500) = 0.0377$. (B) The murine glioblastoma cells, mGB2, were exposed to increasing concentrations of mubritinib (0 to 500 nM), followed by live cell counting. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Dunnett's test vs control. $^{***}p_{\text{mubritinib } 100} = 0.0009$, $^{***}p_{\text{mubritinib } 500} = 4.4\text{e-}5$. (C) The percentages of dead cells (PI positive) were measured by PI staining followed by flow cytometry in BTSCs treated with increasing concentrations of mubritinib for 7 days. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Dunnett's test vs control. p -values are higher than 0.05 for all conditions. (D) The percentages of dead cells (PI positive) and early apoptotic cells (Annexin V positive and PI negative) were measured by Annexin V/PI double staining followed by flow cytometry in BTSCs treated with increasing concentrations of mubritinib for 7 days. (E-G) Pearson correlation analysis was performed between basal OCR and the percentage of live cells in BTSC following 7 days treatment with mubritinib at 20 nM (E) 100 nM (F) and 500 nM (G). (H) Pearson correlation analysis was performed between maximal OCR and sensitivity score to mubritinib following 7 days of treatment. (I-K) mRNA levels of *NDI1* gene were assessed by RT-qPCR in BTSC53 (I), BTSC73 (J) and BTSC147 (K) transduced with control or *NDI1* vector. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. (L-N) Basal OCR was measured by Resipher system in BTSC53 (L), BTSC73 (M) and BTSC147 (N) expressing the control (CTL) vector or *NDI1* following treatment with vehicle control or 500 nM mubritinib. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. Two-way ANOVA followed by Tukey's test. BTSC53 (L): $^{***}p_{\text{CTL vs mubritinib}} = 1.8\text{e-}12$, $p_{\text{NDI1 vs NDI1 + mubritinib}} = 0.1702$. BTSC73 (M): $^{***}p_{\text{CTL vs mubritinib}} = 4.9\text{e-}13$, $p_{\text{NDI1 vs NDI1 + mubritinib}} = 0.6586$. BTSC147 (N): $^{***}p_{\text{CTL vs mubritinib}} = 1\text{e-}10$, $p_{\text{NDI1 vs NDI1 + mubritinib}} = 0.0828$. (O) Proliferation curve was generated using the CFSE assay in BTSC73 expressing the CTL vector or *NDI1* following treatment with vehicle control or 500 nM mubritinib. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. Two-way ANOVA followed by Tukey's test. $^{*}p_{3d}(\text{CTL vs mubritinib}) = 0.0267$, $^{*}p_{4d}(\text{CTL vs mubritinib}) = 0.0265$, $^{*}p_{5d}(\text{CTL vs mubritinib}) = 0.0312$, $^{**}p_{6d}(\text{CTL vs mubritinib}) = 0.0011$, $p_{\text{NDI1 vs NDI1 + mubritinib}} > 0.05$ at all the time points. (P, Q) mRNA levels of *NDUFS7* gene were assessed by RT-qPCR in BTSC73 (P), BTSC53 (Q) electroporated with either siCTL or si*NDUFS7*. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. Unpaired two-tailed t test. $^{***}p_{\text{BTSC73}} = 5\text{e-}8$, $^{***}p_{\text{BTSC53}} = 4.8\text{e-}6$. (R, S) BTSC73 (R) and BTSC53 (S) were electroporated with either siCTL or si*NDUFS7* and treated with 500 nM of mubritinib for 7 days, followed by live cell counting. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Tukey's test. BTSC73 (R): $^{***}p_{\text{siCTL vs siCTL + mubritinib}} = 1.6\text{e-}6$, $^{***}p_{\text{siCTL vs siNDUFS7}} = 5.3\text{e-}6$, $p_{\text{siNDUFS7 vs siNDUFS7 + mubritinib}} = 0.0690$. BTSC53 (S): $^{***}p_{\text{siCTL vs siCTL + mubritinib}} = 3\text{e-}5$, $^{***}p_{\text{siCTL vs siNDUFS7}} = 6.8\text{e-}6$, $p_{\text{siNDUFS7 vs siNDUFS7 + mubritinib}} = 0.5667$. (T) BTSCs and differentiated progeny counterparts (Diff) were subjected to immunoblotting using the antibodies indicated on the blots. Vinculin is used as loading control. (U-X) The number of live stem and differentiated cells from BTSC12 (U), BTSC53 (V), BTSC73 (W) and BTSC147 (X) was measured following 500 nM mubritinib treatment. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Tukey's test. BTSC12 (U): $^{***}p_{\text{stem control vs mubritinib}} = 0.0002$, $^{*}p_{\text{diff control vs mubritinib}} = 0.0426$, $^{**}p_{\text{stem mubritinib vs diff mubritinib}} = 0.0078$. BTSC53 (V): $^{***}p_{\text{stem control vs mubritinib}} = 9.4\text{e-}5$, $^{**}p_{\text{stem mubritinib vs diff mubritinib}} = 0.0010$. BTSC73 (W): $^{***}p_{\text{stem control vs mubritinib}} = 0.0001$, $^{*}p_{\text{diff control vs mubritinib}} = 0.0322$, $^{**}p_{\text{stem mubritinib vs diff mubritinib}} = 0.0049$. BTSC147 (X): $^{***}p_{\text{stem control vs mubritinib}} = 5.1\text{e-}5$, $^{***}p_{\text{stem mubritinib vs diff mubritinib}} = 0.0007$.



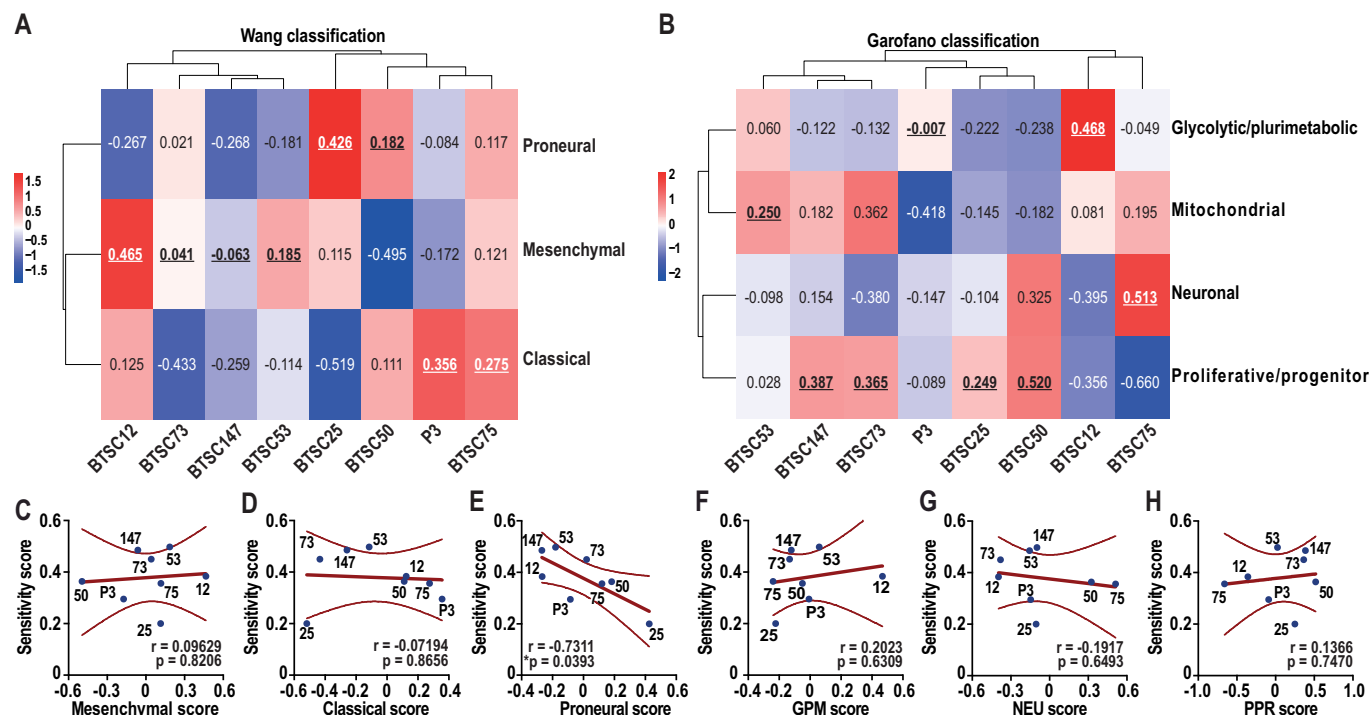


Figure EV2. Transcriptional classification scores of BTSCs and correlation analysis with sensitivity to mubritinib.

(A, B) Heatmaps illustrating the hierarchical clustering of genes related to Wang (A) and Garofano (B) glioblastoma transcriptional subtypes. Dominant transcriptional subtype score is underlined and highlighted in bold. (C–H) Pearson correlation analysis was performed between the BTSC sensitivity score to mubritinib following 7 days of treatment and mesenchymal (C), classical (D), proneural (E), glycolytic/plurimetabolic (GPM) (F), neuronal (NEU) (G) and proliferative/progenitor (PPR) (H) transcriptional subtype signature scores.

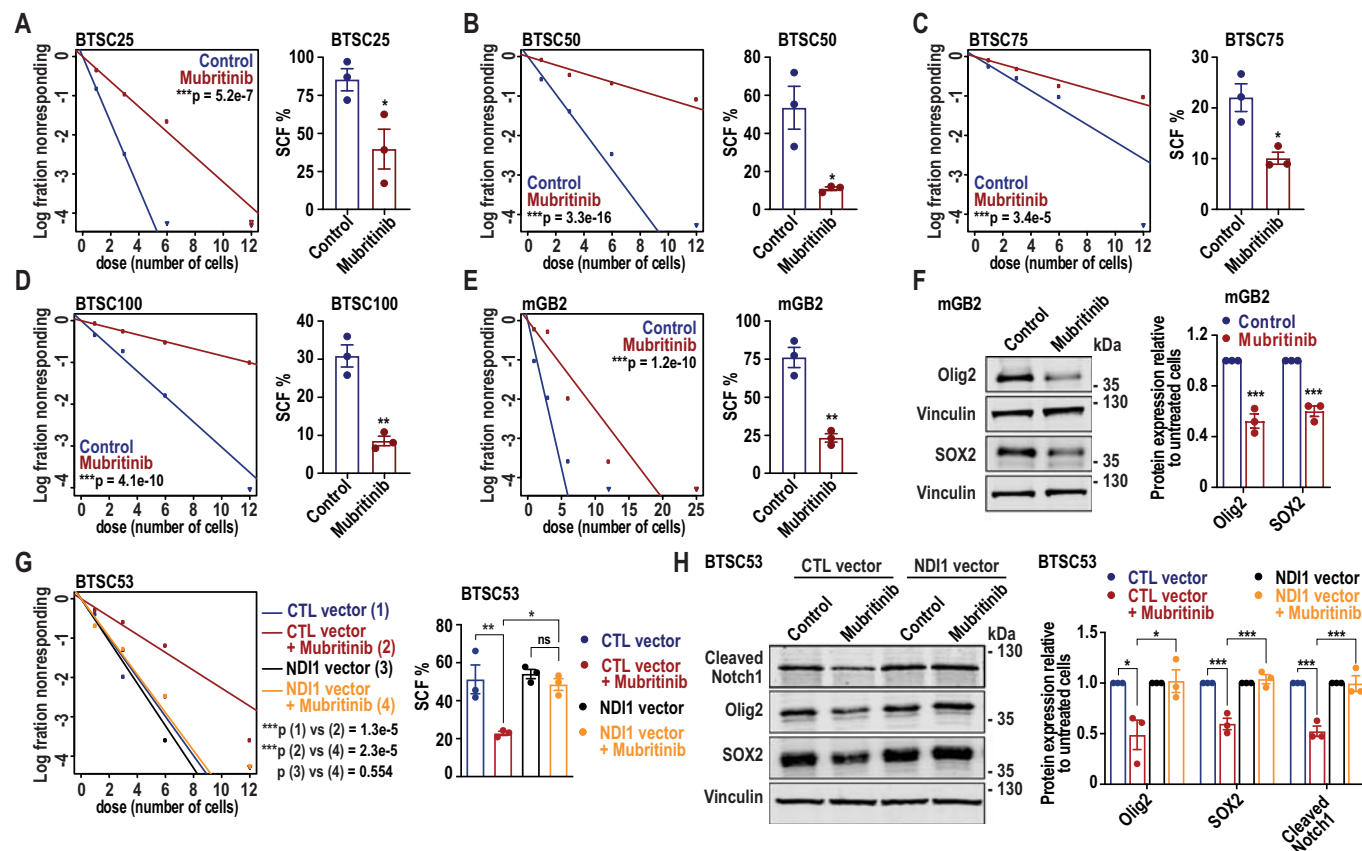


Figure EV3. Validation of the effect of mubritinib on the stemness of murine-derived and additional patient-derived BTSCs.

(A–D) BTSC25 (A), BTSC50 (B), BTSC75 (C) and BTSC100 (D) were subjected to ELDA to estimate the SCF, 21 days following treatment with 500 nM mubritinib or vehicle control. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. Chi-square test for ELDA plots and unpaired two-tailed t test for SCF were used. $*p_{BTSC25} = 0.038$, $*p_{BTSC50} = 0.0196$, $*p_{BTSC75} = 0.016$, $**p_{BTSC100} = 0.0022$. (E) The murine-derived BTSCs (mGB2) were subjected to ELDA to estimate the SCF, 7 days following treatment with 500 nM mubritinib or vehicle control. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. Chi-square test for ELDA plots and unpaired two-tailed t test for SCF were used. $**p_{mGB2} = 0.0018$. (F) mGB2 cells were treated for 4 days with 500 nM mubritinib or vehicle control and subjected to immunoblotting using the antibodies indicated on the blots. Vinculin was used as loading control. Densitometric quantifications of Olig2 and SOX2 protein levels normalized to their corresponding loading controls are presented. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Dunnett's test vs vehicle control. $***p_{Olig2} = 0.0003$, $***p_{SOX2} = 0.0007$. (G) BTSC53 transduced with the control (CTL) or NDI1 vector were treated with vehicle control or 500 nM mubritinib and subjected to ELDA to estimate the SCF, 21 days following treatment. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. Chi-square test for ELDA plots and one-way ANOVA followed by Tukey's test for SCF were used. $**p_{CTL \text{ vs mubritinib}} = 0.0069$, $*p_{mubritinib \text{ vs NDI1 + mubritinib}} = 0.0121$, $p_{NDI1 \text{ vs NDI1 + mubritinib}} = 0.8008$. (H) BTSC53 transduced with the CTL or NDI1 vector were treated with vehicle control or 500 nM mubritinib for 4 days and subjected to immunoblotting using the antibodies indicated on the blots. Vinculin was used as loading control. Densitometric quantifications of cleaved Notch1, Olig2 and SOX2 protein levels normalized to their corresponding loading controls are presented. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Tukey's test. $*p_{Olig2 \text{ (CTL vs mubritinib)}} = 0.0174$, $*p_{Olig2 \text{ (mubritinib vs NDI1 + mubritinib)}} = 0.0142$, $p_{Olig2 \text{ (NDI1 vs NDI1 + mubritinib)}} = 0.9986$, $***p_{SOX2 \text{ (CTL vs mubritinib)}} = 0.0002$, $***p_{SOX2 \text{ (mubritinib vs NDI1 + mubritinib)}} = 0.0001$, $p_{SOX2 \text{ (NDI1 vs NDI1 + mubritinib)}} = 0.8669$, $***p_{Cleaved \text{ Notch1 (CTL vs mubritinib)}} = 0.0004$, $***p_{Cleaved \text{ Notch1 (mubritinib vs NDI1 + mubritinib)}} = 0.0004$, $p_{Cleaved \text{ Notch1 (NDI1 vs NDI1 + mubritinib)}} > 0.9999$.

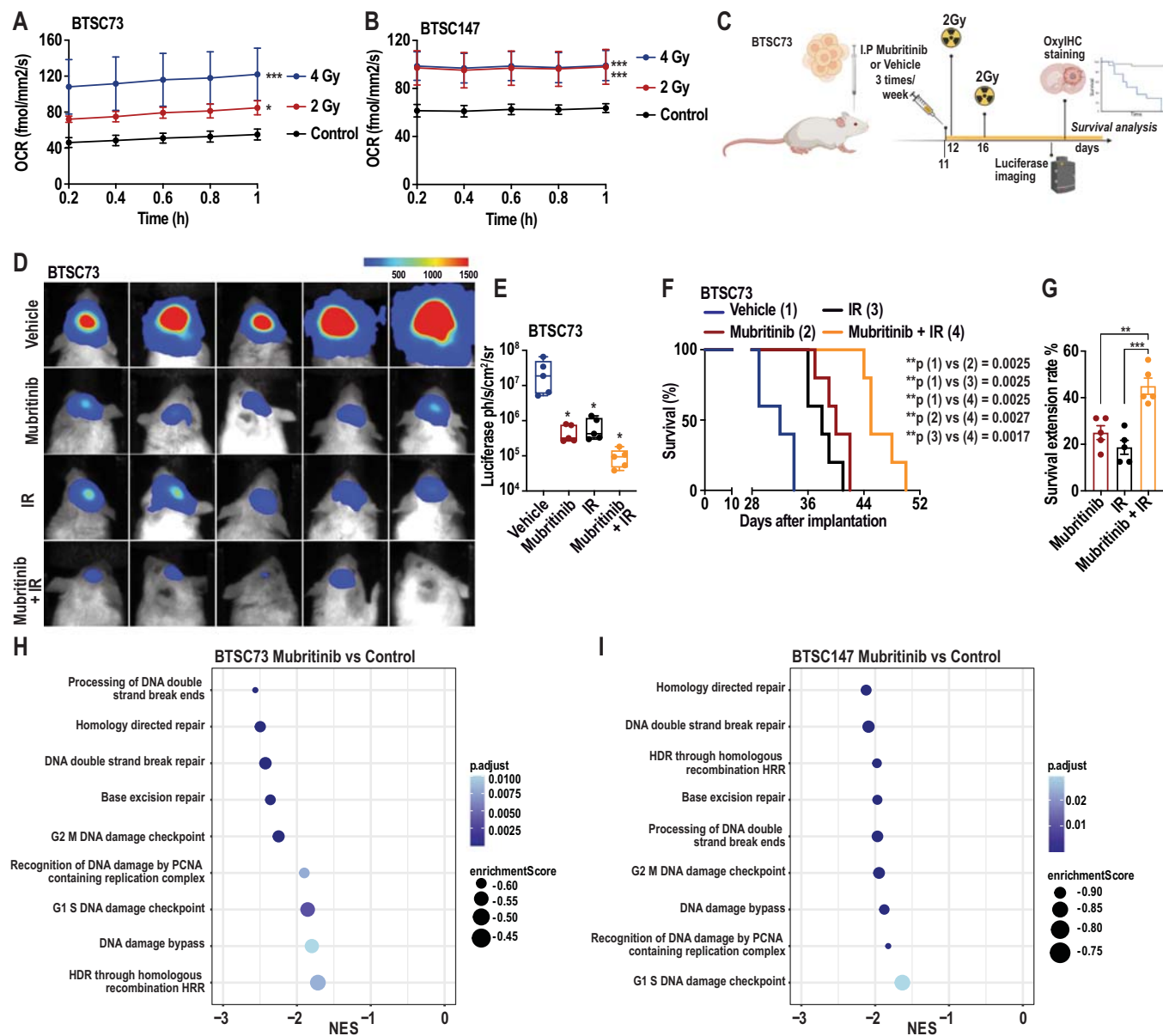


Figure EV4. Mubritinib sensitizes GB tumours to ionizing radiation.

(A, B) 2 Gy or 4 Gy irradiated BTSC73 (A) BTSC147 (B) were subjected to real-time Resipher analysis to measure the basal OCR 16 h following irradiation. Data are presented as the means ± SEM, n = 3 independent biological experiments. Two-way ANOVA followed by Dunnett's test vs vehicle control. BTSC73 (A): *p_{control} vs 2 Gy = 0.0357, ***p_{control} vs 4 Gy = 6e-6. BTSC147 (B): ***p_{control} vs 2 Gy = 6.8e-6, ***p_{control} vs 4 Gy = 3.3e-6. (C) Schematic diagram of the experimental procedure in which luciferase-expressing BTSC73 cells were intracranially injected into RAGY2C^{-/-} mice. 11 days after implantation, the mice were randomized into 2 groups: vehicle control or mubritinib (6 mg/kg). Mice were treated 3 times per week (Monday, Wednesday and Friday). For IR groups, 24 h after the first mubritinib injection, mice received IR (2 Gy). On day 16, mice were subjected to another cycle of IR at 2 Gy. (D, E) Bioluminescence images (D) and quantification of luciferase activity (E) are presented. Data are presented as box plots showing 25th and 75th percentiles (box), median (centre line), minima and maxima (whiskers), n = 5 mice. One-way ANOVA followed by Tukey's test. *p_{vehicle} vs mubritinib = 0.0244, *p_{vehicle} vs IR = 0.0258, *p_{vehicle} vs mubritinib + IR = 0.0222. (F) KM survival plot was graphed to evaluate mice lifespan in each group, mice were collected at end stage (log-rank test, n = 5 mice). (G) Survival extensions of mice bearing BTSC73-derived tumours treated with mubritinib, IR, or mubritinib + IR relative to those treated with the vehicle control were calculated. Data are presented as the means ± SEM, n = 5 mice. One-way ANOVA followed by Tukey's test. **p_{mubritinib} vs IR + mubritinib = 0.0020, ***p_{IR} vs IR + mubritinib = 0.0002. (H, I) Gene set enrichment analysis of deregulated genes in BTSC73 (H) and BTSC147 (I) treated with 500 nM of mubritinib for 24 h demonstrates enrichment of gene sets corresponding to DNA repair pathways. Permutation test was used to calculate p-values, which were then corrected using the Benjamini-Hochberg method to obtain adjusted p-values (p.adjust).

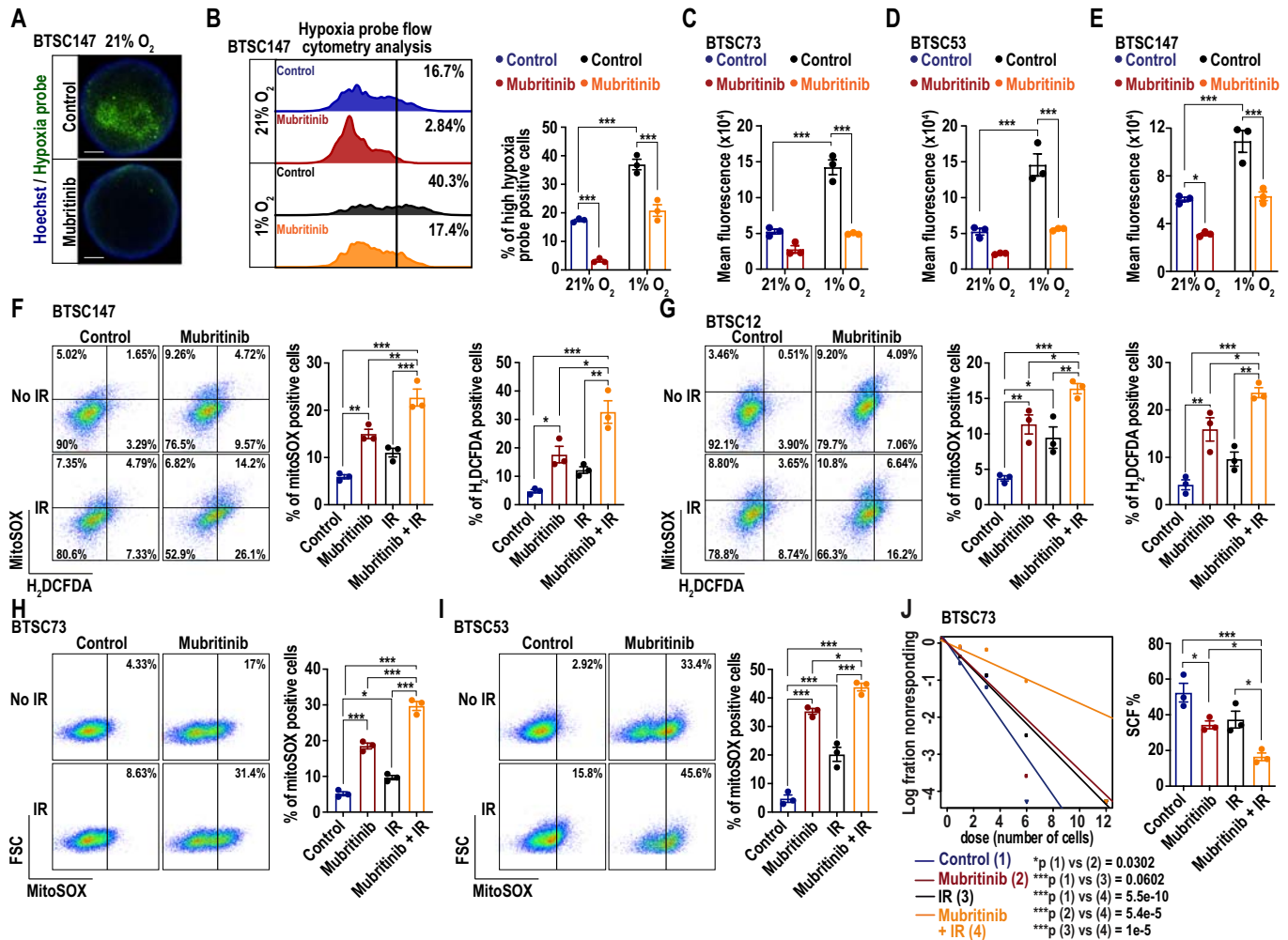


Figure EV5. Mubritinib alleviates tumour hypoxia and enhances oxidative stress to sensitize GB tumours to IR.

(A) BTSC147 tumourspheres were treated with 500 nM mubritinib and subjected to labelling with hypoxia probe (green) followed by live fluorescent imaging. Nuclei were stained by Hoechst. Scale bar = 100 μ m. (B) BTSC147 tumourspheres were treated with 500 nM mubritinib under normoxic (21% O₂) or hypoxic (1% O₂) conditions and subjected to labelling with hypoxia probe followed by flow cytometric analysis. The percentages of high hypoxia probe-positive cells are shown. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Tukey's test. *** $p_{\text{control 21\% O}_2 \text{ vs mubritinib 21\% O}_2} = 0.0005$, *** $p_{\text{control 21\% O}_2 \text{ vs control 1\% O}_2} = 7.4 \times 10^{-7}$, *** $p_{\text{control 1\% O}_2 \text{ vs mubritinib 1\% O}_2} = 0.0002$. (C-E) BTSC73 (C), BTSC53 (D) and BTSC147 (E) tumourspheres were treated with 500 nM mubritinib under normoxic or hypoxic conditions and subjected to labelling with hypoxia probe followed by flow cytometric analysis. The mean fluorescence of hypoxia probe is shown. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Tukey's test. BTSC73 (C): *** $p_{\text{control 21\% O}_2 \text{ vs control 1\% O}_2} = 2.8 \times 10^{-5}$, *** $p_{\text{control 1\% O}_2 \text{ vs mubritinib 1\% O}_2} = 2.2 \times 10^{-5}$. BTSC53 (D): *** $p_{\text{control 21\% O}_2 \text{ vs control 1\% O}_2} = 0.0002$, *** $p_{\text{control 1\% O}_2 \text{ vs mubritinib 1\% O}_2} = 0.0002$. BTSC147 (E): * $p_{\text{control 21\% O}_2 \text{ vs mubritinib 21\% O}_2} = 0.0148$, *** $p_{\text{control 21\% O}_2 \text{ vs control 1\% O}_2} = 0.0006$, *** $p_{\text{control 1\% O}_2 \text{ vs mubritinib 1\% O}_2} = 0.0009$. (F, G) BTSC147 (F) and BTSC12 (G) tumourspheres were treated with 500 nM mubritinib, IR 2 Gy, or combination of both for 3 days and subjected to labelling with H₂DCFDA and MitoSOX followed by flow cytometric analysis. The percentages of H₂DCFDA or MitoSOX positive cells are presented. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Tukey's test. BTSC147 (F): ** $p_{\text{MitoSOX (control vs mubritinib)}} = 0.0023$, *** $p_{\text{MitoSOX (control vs mubritinib + IR)}} = 3.1 \times 10^{-5}$, *** $p_{\text{MitoSOX (mubritinib vs mubritinib + IR)}} = 0.0065$, *** $p_{\text{MitoSOX (IR vs mubritinib + IR)}} = 0.0004$. * $p_{\text{H}_2\text{DCFDA (control vs mubritinib)}} = 0.0287$, *** $p_{\text{H}_2\text{DCFDA (control vs mubritinib + IR)}} = 0.0002$, * $p_{\text{H}_2\text{DCFDA (mubritinib vs mubritinib + IR)}} = 0.0129$, ** $p_{\text{H}_2\text{DCFDA (IR vs mubritinib + IR)}} = 0.0020$. BTSC12 (G): ** $p_{\text{MitoSOX (control vs mubritinib)}} = 0.005$, * $p_{\text{MitoSOX (control vs IR)}} = 0.0237$, *** $p_{\text{MitoSOX (control vs mubritinib + IR)}} = 0.0002$, * $p_{\text{MitoSOX (mubritinib vs mubritinib + IR)}} = 0.0453$, ** $p_{\text{MitoSOX (IR vs mubritinib + IR)}} = 0.0089$. ** $p_{\text{H}_2\text{DCFDA (control vs mubritinib)}} = 0.0040$, *** $p_{\text{H}_2\text{DCFDA (control vs mubritinib + IR)}} = 0.0001$, * $p_{\text{H}_2\text{DCFDA (mubritinib vs mubritinib + IR)}} = 0.0386$, ** $p_{\text{H}_2\text{DCFDA (IR vs mubritinib + IR)}} = 0.0013$. (H, I) BTSC73 (H) and BTSC53 (I) tumourspheres were treated with 500 nM mubritinib, IR 2 Gy, or combination of both for 3 days under hypoxic conditions (1% O₂) and subjected to labelling with MitoSOX followed by flow cytometric analysis. The percentages of MitoSOX positive cells are presented. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. One-way ANOVA followed by Tukey's test. BTSC73 (H): *** $p_{\text{control vs mubritinib}} = 1.7 \times 10^{-5}$, * $p_{\text{control vs IR}} = 0.0226$, *** $p_{\text{control vs mubritinib + IR}} = 1.3 \times 10^{-7}$, *** $p_{\text{mubritinib vs mubritinib + IR}} = 6.4 \times 10^{-5}$, *** $p_{\text{IR vs mubritinib + IR}} = 7.7 \times 10^{-7}$. BTSC53 (I): *** $p_{\text{control vs mubritinib}} = 4.5 \times 10^{-6}$, *** $p_{\text{control vs IR}} = 0.0006$, *** $p_{\text{control vs mubritinib + IR}} = 6.6 \times 10^{-7}$, * $p_{\text{mubritinib vs mubritinib + IR}} = 0.0241$, *** $p_{\text{IR vs mubritinib + IR}} = 3.2 \times 10^{-5}$. (J) BTSC73 treated with 500 nM mubritinib, irradiated with 2 Gy, and subjected to ELDA, under hypoxic condition (1% O₂), to estimate the SCF. Data are presented as the means $\pm$ SEM, $n = 3$ independent biological experiments. Chi-square test for ELDA plots and one-way ANOVA followed by Tukey's test for SCF were used. * $p_{\text{control vs mubritinib}} = 0.0419$, *** $p_{\text{control vs mubritinib + IR}} = 0.0007$, * $p_{\text{mubritinib vs mubritinib + IR}} = 0.0435$, * $p_{\text{IR vs mubritinib + IR}} = 0.0206$.