

**Figure 5. Targeting the Akt Pathway Decreases the Neurosphere Formation Efficiency of CD133+ Cells.** CD133+ cells isolated from an established D456MG pediatric glioblastoma xenograft (A) or a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice (B) were plated at an approximate density of 20 cells per well and treated with the indicated concentration of the AktIII inhibitor. The number of neurospheres per well was quantified over three time points. The number of neurospheres per well significantly decreased in the presence of Akt inhibitor. Representative images of neurospheres photographed at day 9 are shown and demonstrate a clear qualitative difference in size between those grown in the control conditions and those treated with the Akt inhibitor. \* $p < 0.001$  with ANOVA comparison of AktIII inhibitor treatment to the DMSO treated control on the same day.

**Figure 6. Targeting the Akt Pathway Decreases CD133+ Cell Migration and Invasion.** CD133+ cells isolated from an established D456MG pediatric glioblastoma xenograft were plated in serum-free media in the upper chambers of uncoated inserts (A-C) or Matrigel coated inserts (D-F) and were allowed to migrate toward 2% FBS for 48 hours. The migrating or invading cells were then stained and quantified with ImageJ, demonstrating both migration (A) and invasion (D) decrease in CD133+ cells with increasing concentrations of Akt inhibitor. When the percent change from baseline migration (B) or invasion (E) was calculated, CD133+ cells exhibited a greater sensitivity to the effects of Akt inhibitor. Representative images of migrating (C) or invading cells (F) are shown. \*,  $p < 0.05$  with ANOVA comparison of AktIII inhibitor treated CD133+ cells to DMSO treated control CD133+ cells;  $\approx$ ,  $p < 0.05$  with ANOVA comparison of AktIII inhibitor treated CD133- cells to DMSO treated control CD133- cells; #,  $p < 0.001$  with ANOVA comparison of CD133+ cells to similarly treated CD133- cells.

**Supplemental Figure 1. CD133+ Brain Tumor Cells Express Neural Stem Cell Markers.** CD133+ cells isolated from an established D456MG pediatric glioblastoma xenograft were fixed in 4%

*paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) for 15 minutes at room temperature and washed 3 times with PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS for 15 minutes at room temperature before blocking cells in 5% normal goat serum (NGS) in PBS at room temperature for 30 minutes. Cells were incubated for 24 hours at 4°C with the indicated primary antibody (red) diluted in PBS with 5% NGS. Sox2, Olig2, and Oct4 were obtained from R&D Systems and used at a concentration of 1:500, 1:200, and 1:200 respectively. Nestin was obtained from Santa Cruz Biotechnology and used at a concentration of 1:500. After washing 3 times with PBS, cells were incubated with rhodamine-conjugated goat anti-mouse (Abcam) or Alexafluor568-conjugated donkey anti-goat (Invitrogen) secondary antibodies diluted in PBS with 5% NGS for 1 hour at room temperature. After washing 3 times with PBS, cells were mounted in Antifade glycerol mounting medium containing 4'6-diamidino-2-phenylindole (DAPI, blue).*

***Supplemental Figure 2. CD133- Brain Tumor Cells Lack Neural Stem Cell Marker Expression.***

*CD133- cells isolated from an established D456MG pediatric glioblastoma xenograft were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) for 15 minutes at room temperature and washed 3 times with PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS for 15 minutes at room temperature before blocking cells in 5% normal goat serum (NGS) in PBS at room temperature for 30 minutes. Cells were incubated for 24 hours at 4°C with the indicated primary antibody (red) diluted in PBS with 5% NGS. Sox2, Olig2, and Oct4 were obtained from R&D Systems and used at a concentration of 1:500, 1:200, and 1:200 respectively. Nestin was obtained from Santa Cruz Biotechnology and used at a concentration of 1:500. After washing 3 times with PBS, cells were incubated with rhodamine-conjugated goat anti-mouse (Abcam) or Alexafluor568-conjugated donkey anti-goat (Invitrogen) secondary antibodies diluted in PBS with 5% NGS for 1 hour at room temperature. After washing 3 times with PBS, cells were mounted in Antifade glycerol mounting medium containing 4'6-diamidino-2-phenylindole (DAPI, blue).*

**Supplemental Figure 3. The Akt pathway is preferentially targeted by an Akt inhibitor in CD133+ cells.** CD133+ and CD133- cells were isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice (A, B) or an established D456MG pediatric glioblastoma xenograft (C, D). Lysates from CD133- and CD133+ cells treated with 50  $\mu$ M Akt III inhibitor for the indicated times were analyzed by Western (A, C) and the relative intensity of phospho-Akt to tubulin calculated using ImageJ (B, D). The levels of phospho-Akt are lower in CD133+ cells compared to CD133- cells at baseline and further decrease with Akt with a maximum decrease in the phospho-Akt in CD133+ cells at 2 hours.

**Supplemental Figure 4. Differential Akt Activation in Attached and Unattached Brain Tumor Cells.** CD133+ and CD133- cells were isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice. Short term CD133+ and CD133- cultures were both changed to neurobasal medium supplemented with EGF/FGF overnight before plating. For unattached cells, CD133+ and CD133- cells were trypsinized and plated in neurobasal media with EGF and FGF. For attached cells, CD133- cells were trypsinized and plated in DMEM with 10% FBS. Once the DMEM-plated cells were attached to the plates (6 hours later), cells were harvested and lysed. Total cell lysates were analyzed by Western (A) and the relative intensity of phospho-Akt to tubulin calculated using ImageJ (B).

**Supplemental Figure 5. Targeting Akt Preferentially Decreases CD133+ Brain Tumor Cell Growth in a Concentration Dependent Manner.** CD133+ and CD133- cells isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice were treated with the indicated concentrations of AktIII (A), Akt II (B), Ly290042 (C), or Rapamycin (D) to inhibit different

components of the Akt signaling pathway. Cell growth was measured 24 (C) or 48 hours (A, B, D) after inhibitor treatment using the Cell Titer Glo assay (Promega) according to the manufacturer's instructions. The data for each concentration were standardized to the percent of DMSO treated controls for the same cell type. \*,  $p < 0.001$  with ANOVA comparison of inhibitor treated CD133+ cells to DMSO treated control CD133+ cells;  $\approx$ ,  $p < 0.001$  with ANOVA comparison of inhibitor treated CD133- cells to DMSO treated control CD133- cells; #,  $p < 0.05$  with ANOVA comparison of CD133+ cells to similarly treated CD133- cells.

**Supplemental Figure 6. Targeting the Akt Pathway Results in Preferential Induction of CD133+ Cell Apoptosis.** CD133+ and CD133- cells isolated from an established D456MG pediatric glioblastoma xenograft were treated with the indicated concentration of AktIII inhibitor for 24 hours, trypsinized, labeled with an Annexin V kit according to manufacturer's instructions and analyzed by FACS. Apoptosis was induced in CD133+ cells at significantly higher levels than CD133- cells with increasing concentrations of Akt inhibitor. \* $p < 0.001$  with ANOVA comparison of AktIII treated CD133+ cells to DMSO control treated CD133+ cells

**Supplemental Figure 7. Targeting Akt Decreases CD133+ Cell Migration and Invasion.** CD133+ cells isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice were plated in serum-free media in the upper chambers of uncoated inserts (A-C) or Matrigel coated inserts (D-F) and allowed to migrate toward 2% FBS for 48 hours. The migrating or invading cells were then stained and quantified with ImageJ, demonstrating a trend towards decreased migration (B) and a significant decrease in invasion (D) in CD133+ cells with increasing concentrations of AktIII inhibitor. When the percent change from baseline migration (B) or invasion (E) was calculated, CD133+ cells exhibited a greater sensitivity to the effects of Akt inhibitor. Representative images of

migrating (C) or invading cells (F) are shown. **\*\*** $p < 0.001$  with ANOVA comparison to the control of the same cell type. #,  $p < 0.05$ ; ##,  $p < 0.001$  with ANOVA comparison to similarly treated CD133- cells.

**Supplemental Figure 8. Akt inhibition has little impact on VEGF secretion.** CD133- and CD133+ cells isolated from a T3359 glioblastoma patient specimen passaged short-term in immunocompromised mice were plated at  $2.5 \times 10^5$  per mL of media. After 24 hours all of the media was replaced with fresh Neurobasal media and treated with increasing concentrations of AktIII inhibitor. The conditioned media was harvested after an additional 24 hours and cellular VEGF secretion assayed with ELISA. Although CD133+ cells exhibited significantly more VEGF secretion than CD133- cells, consistent with our prior work (##,  $p < 0.001$  with ANOVA comparison to similarly treated CD133- cells), there was no significant decrease in VEGF secretion by either the CD133+ or CD133- cells with increasing concentrations of AktIII inhibitor.

















