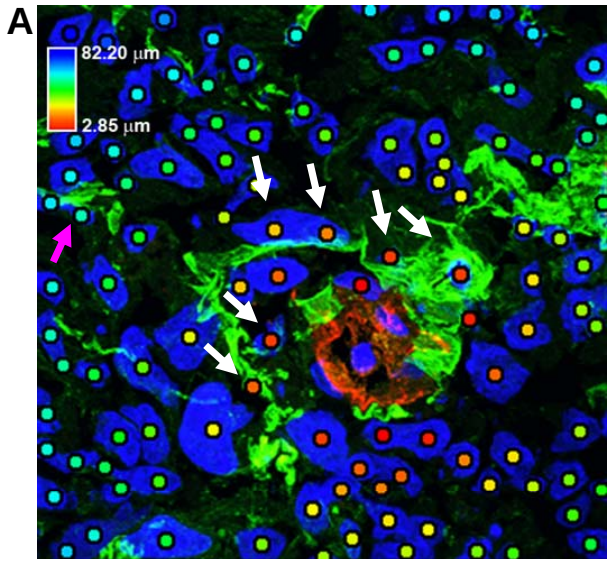
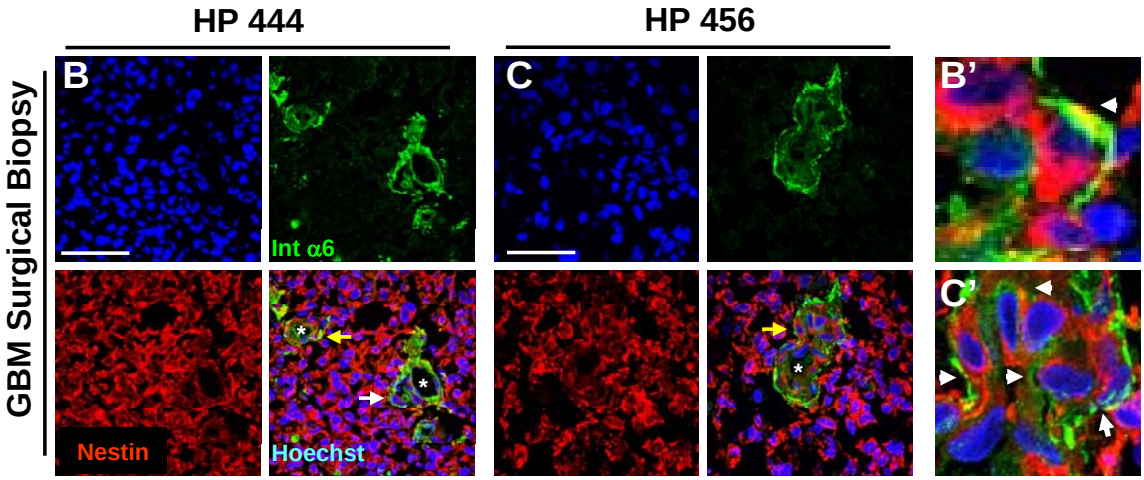
Lathia et al., Supplementary Information

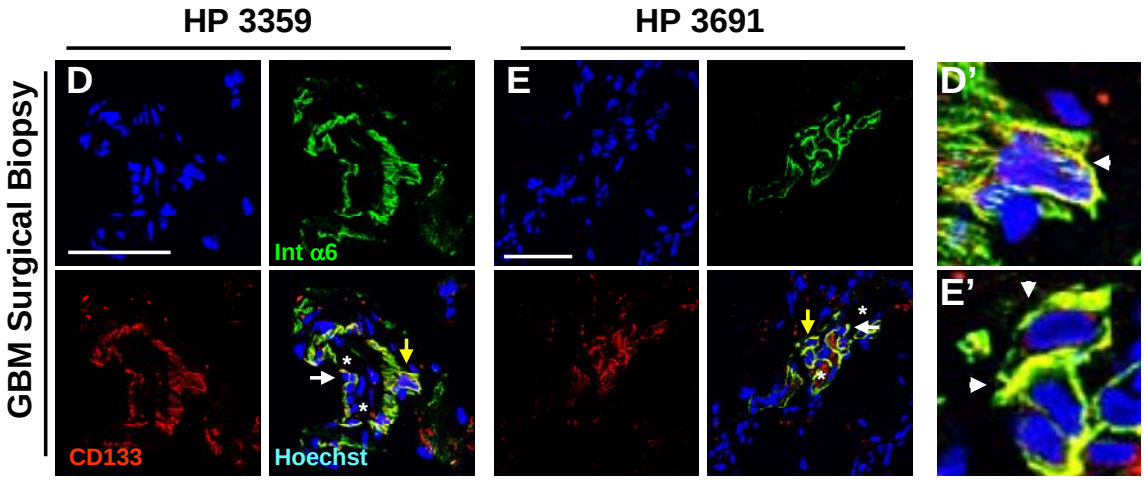
Supplementary Figure 1, related to Figure 1



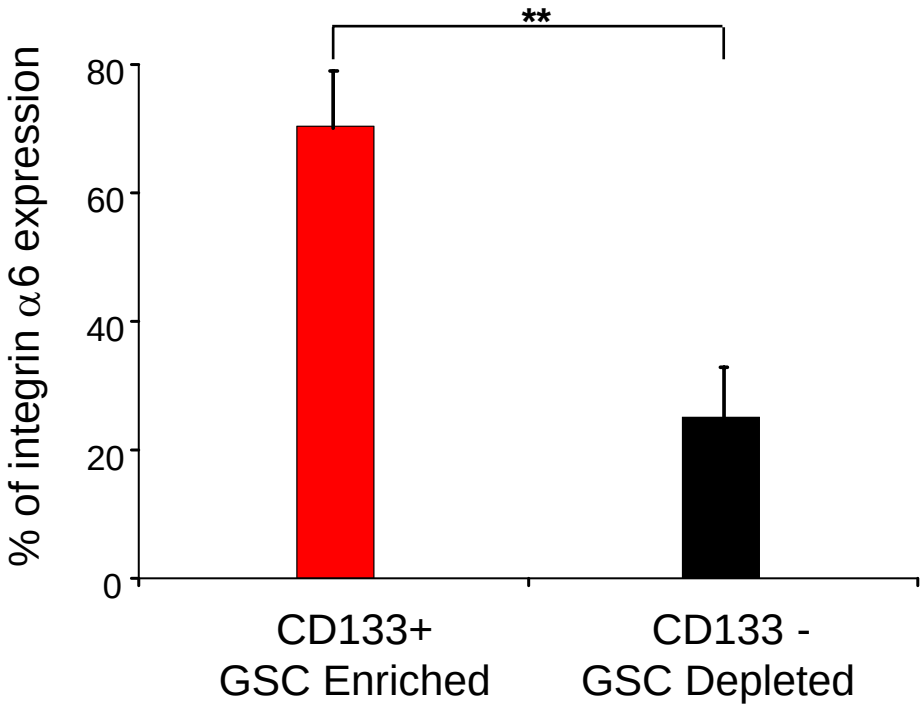
Distance (μm)	Integrin a6+ cell	Analyzed cell
5	60%	10%
10	68%	22%
15	77%	32%
25	80%	51%

*** p < 0.001

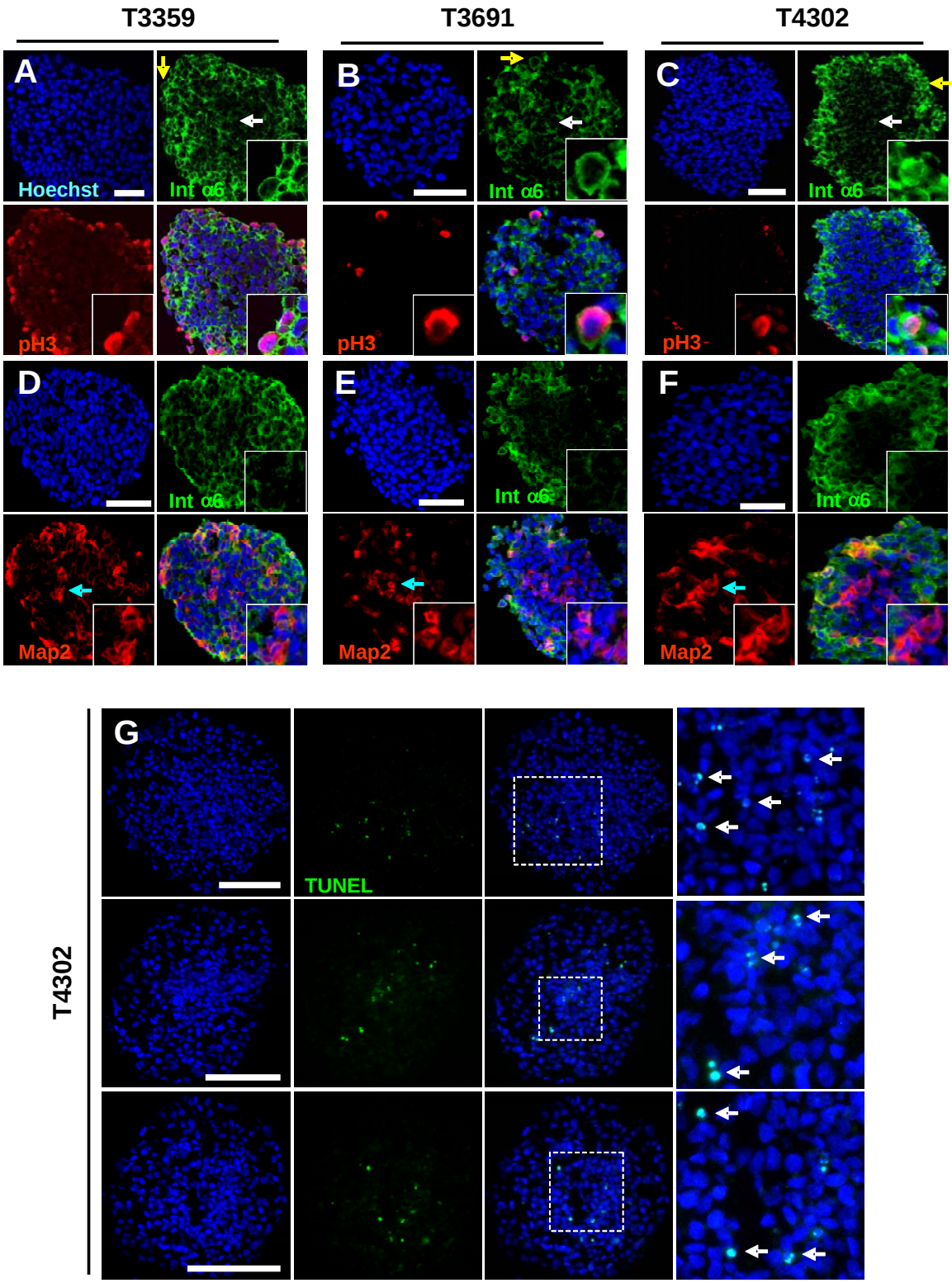




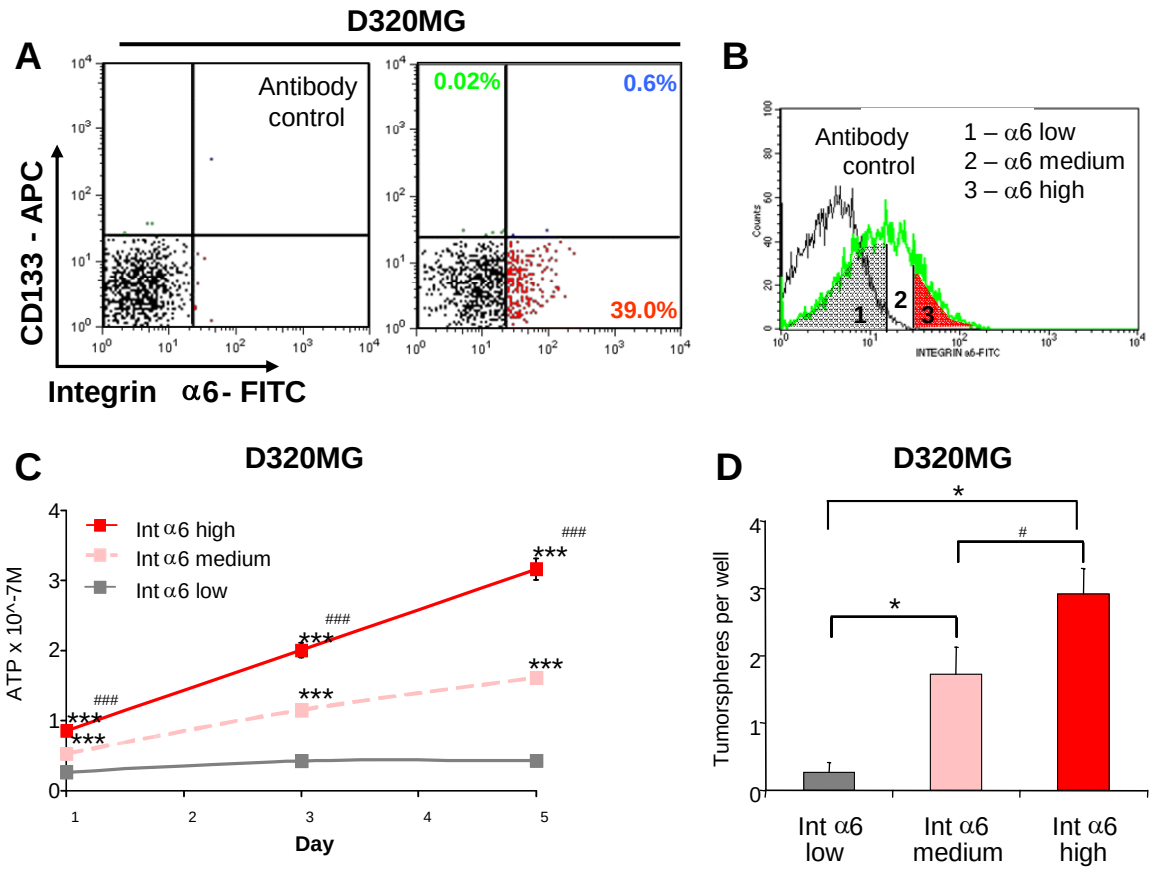
Supplementary Figure 2, related to Figure 2



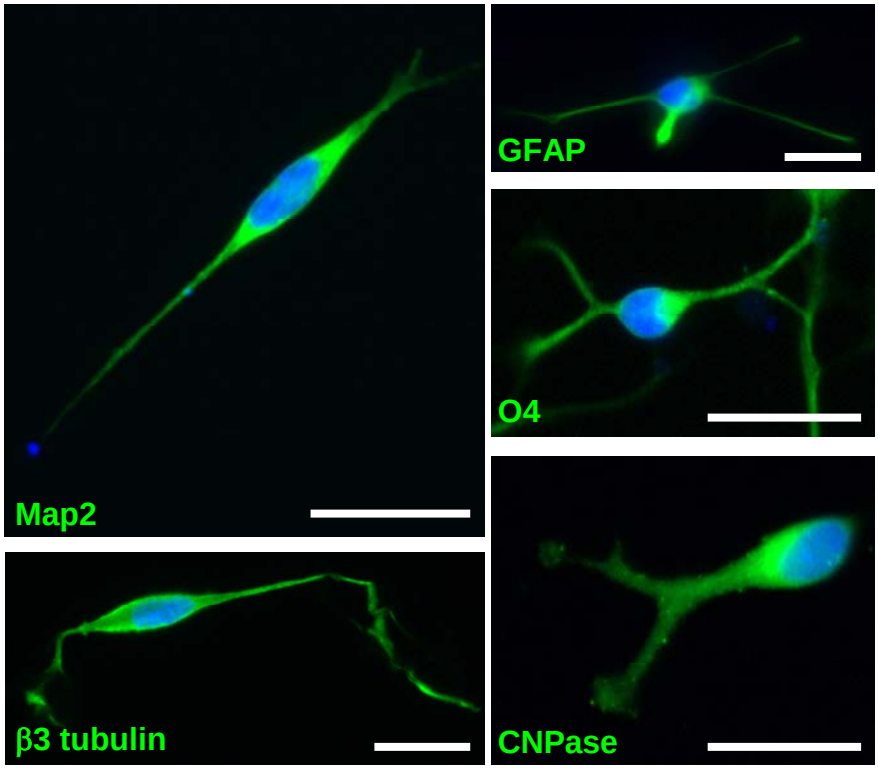
Supplementary Figure 3, related to Figures 3 and 4



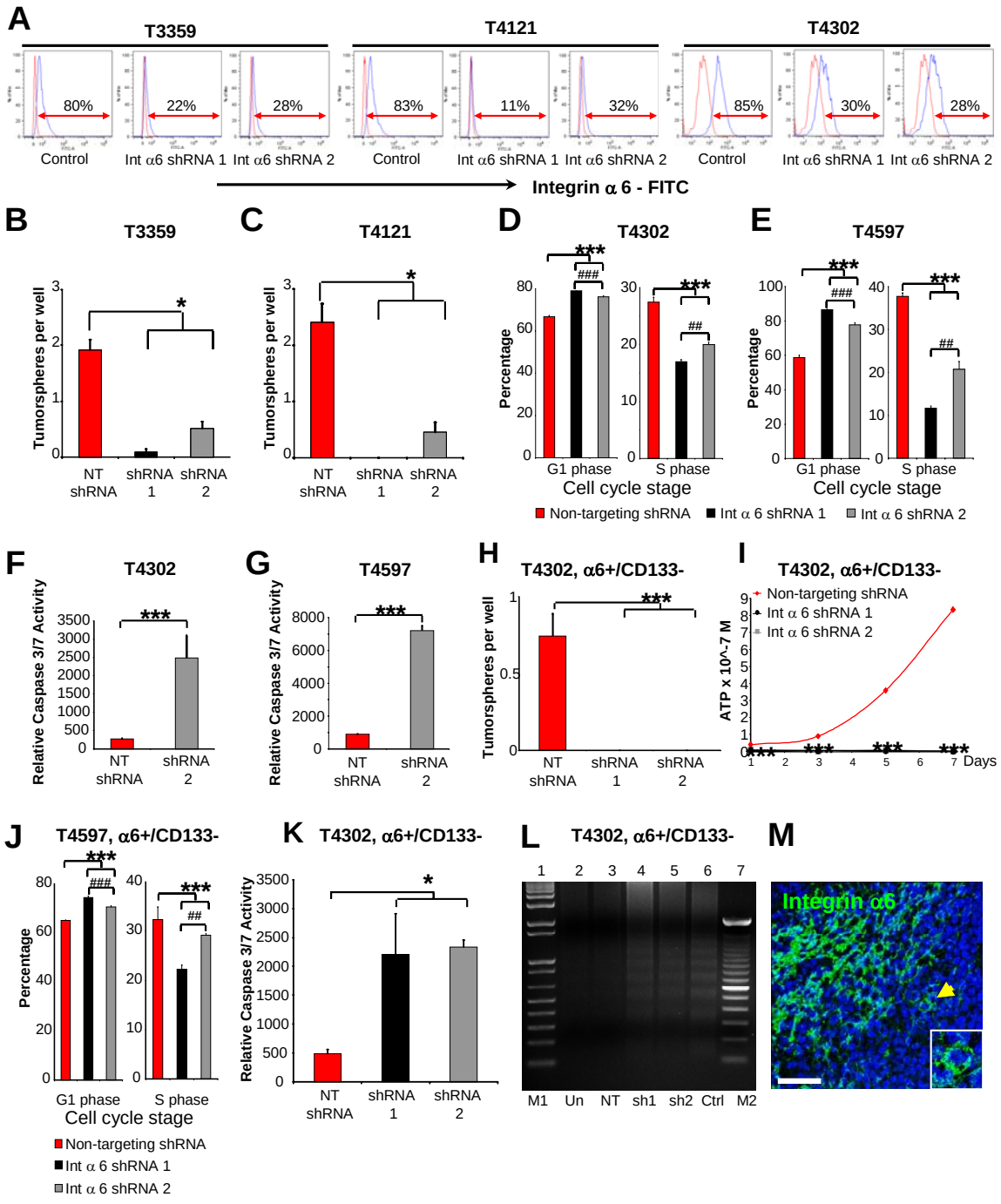
Supplementary Figure 4, related to Figure 5



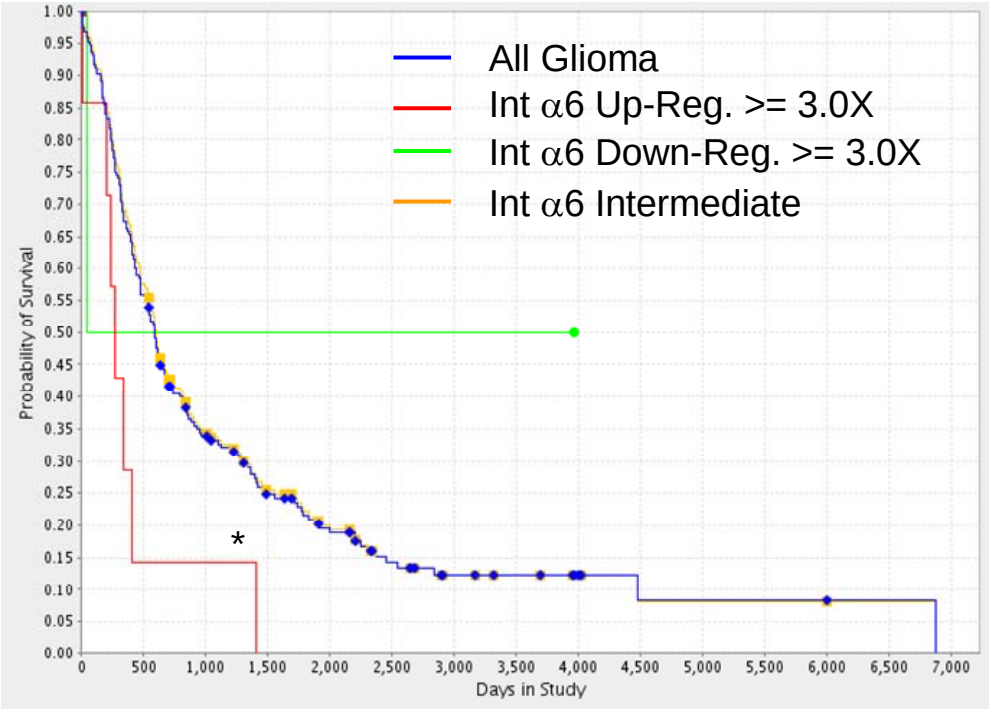
Supplementary Figure 5, related to Figure 5



Supplementary Figure 6, related to Figure 7



Supplementary Figure 7, related to Figure 8



Supplementary Figure 1, related to Figure 1 – Integrin α6 + cells are preferentially located in the perivascular compartment in GBM biopsy specimens and co-express nestin

Summary of immunofluorescence analysis of GBM surgical biopsies (**A**) double stained with int α6 (green) and CD31 (red) calculated based on 6 specimens (n=118 intα6 positive cells and 275 total cells, ***, $p < 0.01$ with ANOVA comparison between populations). Example analyzed micrograph (**A**, right) shows several intα6 positive cells (white arrows) located adjacent to a blood vessel and an int α6 positive cell (pink arrow) not adjacent to the blood vessel. Immunostaining analysis of GBM surgical biopsies (HP 444, HP 456) shows that co-expression of int α6 (green) and nestin (red) is maintained in the perivascular compartment while nestin expression appears to be non-perivascular cells (**B, C**). Blood vessels marked with an "*," regions of interest marked with a white arrow, and enlarged regions of interest marked with yellow arrow and shown in **B'-C'**. Immunostaining analysis of GBM surgical biopsies (HP 3359 and HP 3691) passaged as xenografts and used for analysis shows that co-expression of intα6 (green) and CD133 (red) is maintained in the perivascular compartment (**D, E**). Blood vessels marked with an "*," regions of interest marked with a white arrow, and enlarged regions of interest marked with yellow arrow and shown in **D'-E'**. All nuclei counterstained with Hoechst in blue. Scale bar represents 50 μm.

Supplementary Figure 2, related to Figure 2 – Integrin α6 co-segregates with CD133 in GBM cells

The majority of CD133 + cells (red) are int α6 + as compared to CD133 - cells (black) displayed as a bar graph calculated based on flow cytometry analysis (data contained in Supplementary Table 1, n=7, +/- S.E.M., **, $p < 0.01$).

Supplementary Figure 3, related to Figures 3 and 4 – Integrin α6 co-segregates proliferating cells in the periphery of tumorspheres but not in the center which contains differentiated and dead cells

Immunostaining analysis of cryosections from tumorspheres generated from GSC enriched populations (T3359, T3691, T4302) show two distinct regions of expression, the peripheral region and the inner region. Photomicrographs show int α6 (green) is co-expressed with the m-phase marker phospho histone H3 (pH3, red, **A, B, C**) in the peripheral region (yellow arrow) but not in the inner region (white arrow). The center of the tumorspheres which contains differentiated cells as assessed by a neuronal marker (Map2, positive center cell marked with blue arrow) expression (red, **C, D, E**) is int α6 low. Furthermore, the center of tumorsphere contains dead cells as assessed by TUNEL staining (**G**, white box indicates enlarged area in right panels). All nuclei counterstained with Hoechst in blue. Scale bar represents 50 μm.

Supplementary Figure 4, related to Figure 5 – Selection of cells based on integrin α6 expression levels in the absence of CD133 enriches for a population which display GSC properties

Flow cytometry plots (**A**) show int α6 expression levels in the xenograft D320MG, which has a low level on CD133 expression. GSC enrichment was based on the adjusted

intα6 expression alone as shown on this histogram (**B**), region 1 contains low levels of intα6 (α6 low, black), region 2 represents the lower 50% of int α6 expression level (α6 medium, red/white), and region 3 represents the upper 50% of int α6 expression level (int α6 high, red). (**C**) Cell enrichment based on intα6 expression level correlates a greater cell proliferation profile as assessed using the cell titer assay. ***, $p<0.001$ with ANOVA comparison to int α6 low cells at the same timepoint; ###, $p<0.001$ with ANOVA comparison of int α6 high to int α6 medium cells at the same timepoint. (**D**) Cell enrichment based on int a6 expression level correlates with a greater ability to form tumorspheres *, $p<0.05$ with ANOVA comparison to int α6 low expressing cells; #, $p<0.05$ with ANOVA comparison to int α6 medium cells.

Supplementary Figure 5, related to Figure 5 – Integrin α6 + cells are multipotent and capable of giving rise to all three major central nervous system lineages

Immunostaining analysis of int α6 + cells derived from xenografted GBMs (T4302, T4121 data not shown) demonstrate that int α6 high cells have the capacity to give rise to all three central nervous system lineages (green): neurons (indicated by Map2 and β3 tubulin), astrocytes (indicated by GFAP), and oligodendrocytes (indicated by O4 and CNPase). All nuclei counterstained with Hoechst in blue. Scale bar represents 10 μm.

Supplementary Figure 6, related to Figure 7 – Integrin α6 knockdown can be achieved using lentiviral shRNA constructs and compromises GSC properties in CD133 + and integrin α6 + /CD133 - GSCs

Int α6 was knocked down in GSCs using two separate lentiviral delivered shRNA constructs (shRNA 1 targeting exon 14 and shRNA 2 targeting exon 2). Flow cytometry analysis (**A**, xenografted tumor specimens T3359, T4121, T4302) show int α6 expression levels (blue) were reduced in targeting constructs but not in a control non-targeting construct. Percentage of int α6 + cells was calculated relative to an antibody control (red). Int α6 was knocked down in GSCs (T3359, 4121, T4302, T4597) using two separate lentiviral delivered shRNA constructs (black, grey). Self-renewal was impaired in T3359 (**B**) and T4121 (**C**) xenografted cells targeted with the lentivial shRNA constructs (shRNA1, shRNA2) directed against intα6 in comparison to non-targeting control (NT shRNA) as assessed by tumorsphere formation assays. ***, $p<0.001$ with ANOVA comparison to non-targeting control shRNA. In addition, cell cycle changes (increase in G1 and decrease in S-phases) were detected when T4302 (**D**) and T4597 (**E**) xenografted cells were targeted with the lentivial shRNA constructs directed against intα6 in comparison to a non-targeting control as assessed by flow cytometry cell cycle based analysis. ***, $p<0.001$ with ANOVA comparison to non-targeting control shRNA, ###, $p < 0.001$ and ## $p < 0.01$ with ANOVA comparison to shRNA1. Cell death was also increased when T4302 (**F**) and T4597 (**G**) xenografted cells were targeted with the lentivial shRNA construct (shRNA2) directed against int α6 in comparison to a non-targeting control (NT shRNA) as assessed by Caspase 3/7 activity. ***, $p<0.001$. Intα6 was knocked down in intα6 +/CD133 - GSCs (T4302, T4597) using two separate lentiviral delivered shRNA constructs (black, grey). Self-renewal was impaired in T4302 (**H**) xenografted cells targeted with the lentivial shRNA constructs (shRNA1, shRNA2) directed against intα6 in comparison to non-targeting control (NT shRNA) as assessed

by tumorsphere formation assays. ***, p<0.001 with ANOVA comparison to non-targeting control shRNA. Knockdown of int α6 using two separate lentiviral shRNA constructs results in a decreased cell proliferation profile as assessed by the cell titer assay in T4302 (**I**) xenograft tumor cells. ***, p<0.001 with ANOVA comparison to non-targeting shRNA at the same timepoint. In addition, cell cycle changes (increase in G1 and decrease in S-phases) was detected when T597(**J**) xenografted cells were targeted with the lentivial shRNA constructs directed against int α6 in comparison to a non-targeting control as assessed by flow cytometry cell cycle based analysis. ***, p<0.001 with ANOVA comparison to non-targeting control shRNA, ###, p < 0.001 and ## p < 0.01 with ANOVA comparison to shRNA1. Cell death was also increased when T4302 (**K**) xenografted cells were targeted with the lentivial shRNA constructs (shRNA1, shRNA2) directed against intα6 in comparison to a non-targeting control (NT shRNA) as assessed by flow cytometry cell cycle based analysis. *, p<0.05 with ANOVA comparison to non-targeting control shRNA. Induction of cell death upon intα6 targeting was also confirmed by a DNA fragmentation assay (**L**) on T4302 xenograft tumor cells (M1=1kb ladder plus from Invitrogen, Un = uninfected control, NT = non-targeting shRNA control, sh1=shRNA1, sh2=shRNA2, Ctrl = staurosporine control loaded at 1:10 dilution, M2=100bp ladder from Invitrogen). Immunostaining analysis (**M**) for intα6 shows positive cells (yellow arrow indicated inset) in a tumor generated from *in vivo* intracranial transplantation of 5000 CD133 + GSC (T3359) infected with int α6 shRNA targeting construct (shRNA 1). Scale bar represents 50 μm.

Supplementary Figure 7, related to Figure 8 – Elevated integrin a6 expression in GBMs correlates with poor patient prognosis

Kaplan-Meier survival plot for glioblastoma patients with differential tumor int α6 expression calculated by NCI REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) bioinformatics database (https://caintegrator.nci.nih.gov/rembrandt/). The log-rank *P*-value for significance of difference of survival between intα6 up-regulated group and int α6 intermediate group was 0.0127. The log-rank *P*-value for significance of difference of survival between int α6 up-regulated group and all other groups was 0.0129 (* p < 0.05). Numbers of samples in group were 7 (int α6 Up-Regulated), 2 (int α6 Down-Regulated), and 184 (int α6 Intermediate).

Supplementary Table 1, related to Figure 2

CD133	-	+	+	-	Total	-
Integrin α6	-	-	+	+	-	Total
Tumor ID						
T3691	56.0	3.0	9.6	31.5	12.5	41.1
T3359	54.4	6.9	9.5	29.2	16.4	38.7
T0322	85.6	5.2	3.2	6.1	8.4	9.2
T0007	73.3	0.9	5.8	19.9	6.8	25.8
T0166	79.8	8.1	3.7	8.4	11.8	12.1
CCF1518	77.3	0.8	11.3	10.6	12.1	21.9
CCF1585	98.8	0.0	0.1	1.1	0.1	1.2

Supplementary Table 2, related to Figure 6

	T08-0387 Integrin α6 Sorted Cells		
	Cell number	Incidence	Median Survival
Integrin α6 low	100	0/3	N.A.
	500	2/4	N.A.
	1000	5/5	60
Integrin α6 high	100	2/5	N.A.
	500	5/5	41**, ##
	1000	5/5	35**

	CCF 1966 Integrin α6 Sorted Cells		
	Cell number	Incidence	Median Survival
Integrin α6 low	100	0/4	N.A.
	1000	2/5	N.A.
	10000	3/4	42.5
Integrin α6 high	100	3/5	56*
	1000	4/5	34†
	10000	4/4	24.5

Supplementary Table 3, related to Figure 7

	T3359 CD133+ Lentivirus Infected Cells		
	Cell number	Incidence	Median Survival
Non-Targeting Control	1000	4/4	37.4
	5000	5/5	34
Integrin α6 shRNA 1	1000	4/5	62**
	5000	5/5	48*
Integrin α6 shRNA 2	1000	4/5	54*
	5000	4/5	61*

Supplementary Table 4, related to Figure 8

	T4597 α6+/CD133- Antibody Treated Cells		
	Cell number	Incidence	Median Survival
Isotype control antibody	1000	5/5	28**
α6 integrin blocking antibody	1000	5/5	46
	T4302 α6+/CD133- Antibody Treated Cells		
	Cell number	Incidence	Median Survival
Isotype control antibody	1000	5/5	36*
α6 integrin blocking antibody	1000	5/5	45

Supplementary Table 1, related to Figure 2 – Summary of integrin α6 and CD133 flow cytometry analysis

Summary of flow cytometry analysis based on int α6 and CD133 expression on GBM xenografts (T3691, T3359, T0322, T0007, T0166) and primary surgical GBM biopsies (CCF1518, CCF1585). Percentage positive was calculated relative to relevant antibody controls.

Supplementary Table 2, related to Figure 6 – Summary of in vivo intracranial transplantation of integrin α6 high and low cells

Summary of incidence and median survival of intracranial transplantation of limiting dilutions of int α6 high or low cells from tumor specimens T08-0387 and CCF1966. † $p < 0.067$, * $p < 0.05$ and ** $p < 0.01$ with log-rank analysis of survival curves for same cell concentration and ## $p < 0.01$ with log-rank analysis of survival curves of T08-0387 500 intα6 high vs 1000 int α6 low cells). N.A. represents median survival not achieved.

Supplementary Table 3, related to Figure 7 – Summary of in vivo intracranial transplantation of GSCs infected with integrin α6 shRNA targeting or control non-targeting constructs

Summary of incidence and median survival of intracranial transplantation of 5000 or 1000 T3359 CD133 + infected with int α6 shRNA targeting constructs of a control non-targeting construct. * $p < 0.05$ and ** $p < 0.01$ with log-rank analysis of survival curves for same cell concentration as compared to non-targeting control.

Supplementary Table 4, related to Figure 8 – Summary of in vivo intracranial transplantation of GSCs treated with an integrin α6 blocking antibody

Summary of incidence and median survival of intracranial transplantation of 1000 T4597 of T4302 int α6 + /CD133 - cells treated with an int α6 blocking antibody or isotype control antibody. * $p < 0.05$, ** $p < 0.01$ with log-rank analysis of survival curves for same cell concentration. N.A. represents median survival not achieved.

Supplementary Experimental Procedures

Isolation of Glioblastoma Stem Cells

All tumor specimens were derived from primary glioblastoma surgical biopsy specimens obtained from patients undergoing resection for newly diagnosed or recurrent GBM in accordance with protocols approved by the Duke University Medical Center or Cleveland Clinic Foundation Institutional Review Boards. Written consent to utilize excess tissue for research was obtained from each patient, and de-identified tissues were used for all studies. Tumors were dissociated to single cells by a papain dissociation kit (Worthington Biochemical) as per manufacturer's protocol and cultured in vitro prior to use using previously reported culturing methods (Lee et al., 2006a; Li et al., 2009). Cells under passage 10 were used to all analysis and the majority of cells used were immediately after dissociation.

PCR analyses

Total RNA was extracted from CD133-positive and CD133-negative cell fractions using the RNeasy kit (Qiagen), and reverse transcribed into cDNA by iScript cDNA synthesis kit (BioRad). Reverse transcription PCR was done using cDNA transcribed from RNA as described above. Following the manufacturer's cDNA amplification protocol, a 55°C annealing temperature was used. PCR products were verified by melting curves. As a control, no cDNA was added to the reaction to ensure accuracy. The PCR products were separated on a 1.5% agarose gel and imaged using ethidium bromide and a transilluminator.

Immunofluorescence

For immunostaining analysis at the single cell level, CD133-positive and CD133-negative fractions were separated by magnetic beads and plated on tissue culture plastic chamber slides (Nunc) for 8 hours to allow sufficient attachment. Cells were then fixed with 4% PFA for 15 minutes at room temperature, washed 3 times with PBS, and then blocked with a PBS based solution containing 10% normal goat serum (Sigma) and 0.1% triton x-100 (Sigma). Cells were incubated overnight with the appropriate antibody (1:250): rat monoclonal anti-integrin α6 (MAB1378, Millipore), rabbit polyclonal anti-CD133 (ab19898, abcam or CD133/1, Miltenyi), or rabbit polyclonal Olig2 (kind gift from Drs. John Alberta and Charles Stiles, Harvard University). Cells were washed 3 times with PBS and incubated with the appropriate secondary antibody (1:250): goat anti-rat Alexa 488 IgG or goat anti-rabbit Alexa 568 IgG (Invitrogen). Nuclei were counterstained with hoescht 33342 (1:1000 dilution from a 5mg/ml stock solution, Invitrogen). For immunostaining analysis on tumorspheres sections, tumorspheres were fixed as described above and cryoprotected with 30% sucrose prior to embedding in OCT (Sakura). Frozen sections of 10 microns were cut using a Leica (CM1900) cryostat. Immunostaining, as described above, was done by blocking sections and incubating with the appropriate primary antibody (1:100): anti-integrin α6, mouse monoclonal anti-integrin β1 (MAB1959, Millipore), mouse monoclonal anti-MAP2 (Sigma), anti-CD133, anti-Olig2, rabbit polyclonal anti-phospho histone H3 (06-570, Millipore), rabbit polyclonal anti-nestin (ab5968, abcam), or rabbit polyclonal anti-pan laminin (L-9393, Sigma). Sections were washed 3 times with PBS

and incubated with the appropriate secondary antibody (1:250): goat anti-rat Alexa 488 IgG, goat anti-rabbit Alexa 568 IgG (Invitrogen), or goat anti-mouse Alexa 568 IgG2a (Invitrogen). Nuclei were counterstained with Hoechst 33342 (1:1000 of a 5mg/ml stock solution, Invitrogen). The TUNEL assay was done per manufacturer's protocol (17-141, Millipore). For immunostaining analysis on human GBM surgical biopsy specimens, 10 micron sections frozen sections were obtained from the Duke University Brain Tumor Center Tissue Bank. Sections were fixed for with 4% PFA for 10 minutes at room temperature and processed as described in the section above. Differentiation studies was conducted by allowing whole tumorspheres to attach to tissue culture plastic chamber slides (Nunc) and cultured for a week in media void of EGF and bFGF or in DMEM containing 10% fetal calf serum (Sigma). After cells had attached, spread out, and undergone distinct morphological changes, they were fixed in 2% PFA, washed 3 times in PBS and processed as described above with the following antibodies: mouse monoclonal anti-Map2 (1:400, M9942, Sigma), mouse monoclonal anti-β3 tubulin (1:1000, T5076, Sigma), mouse monoclonal anti-GFAP (1:400, 610565, BD), mouse monoclonal anti-O4 (1:400, MAB345, Millipore), and mouse monoclonal anti-CNPase (1:400, C5922, Sigma). Appropriate goat secondary antibodies were used as described above. Nuclei were counterstained with Hoescht 33342 (1:1000 dilution from a 5mg/ml stock solution, Invitrogen). All imaging done using a Leica SP-5 confocal microscope as described previously (Wang et al., 2008b) and images were processed and assembled in photoshop (Adobe). For integrin α6 perivascular assessment, confocal microscopy images comprised of integrin α6 positive cells (nuclei counterstained with Hoechst 33342) and blood vessels (labeled with an antibody against CD31) were imported into Image-Pro Plus (v6.1, Media Cybernetics, Silver Spring, MD). Proximity analysis of cell nuclei to blood vessels for each image was performed in an automated fashion (batch mode) using customized visual basic Image-Pro Plus macros. Briefly, cell nuclei and Alexa 568 blood vessels channels were extracted from each image. The cell nuclei channel was "flattened" to remove any uneven illumination followed by a spectral filter to equalize brightness of pixels within each nucleus. The nuclei were then segmented using intensity and morphological thresholds and touching nuclei were split using a watershed filter. Subsequently, the user was prompted to draw a region of interest (ROI) around each vessel present within a given image. Each ROI was then converted to single pixel width perimeter (Sobel filter) forming a point boundary for each vessel. Finally a minimum distance was calculated between the centroid coordinate of each nucleus to the set of perimeter points around each vessel, exporting each cell distance to Excel. For a visual representation of distance profiles, the calculated distances were mapped to a LUT with nuclei centroids pseudo-colored to represent their respective distance to the nearest vessel boundary.

Growth curve and tumorsphere formation assays

All FACS sorted cell fractions of interest were plated at a density of 1000 cells/well in a 96-well plate in at least triplicate for growth curve analysis. Cell number was measured for every other day and normalized to 1 x 10^{-7} M of ATP using the CellTiter-Glo assay kit (Promega). For tumorsphere formation assessment, 24- or 96-well plates were seeded at a density of 1 or 10 cells per condition. Reported numbers represent a minimum of 8 wells per condition. Ten days after plating, tumorspheres containing more than 20 cells

were scored. For studies with the integrin α6 blocking antibody (GoH3, MAB1378, Millipore), doses of either 1 μg/ml or 10 μg/ml were used and compared to a rat isotype control antibody (1μg/ml Invitrogen).

Lentiviral shRNA construct production

All lentiviral constructs used were manufactured as previously reported (Li et al., 2009, Wang et al., 2008). In short, using lipofectamine 2000 (invitrogen), 293FT cells were co-transfected with packaging vectors psPAX2 and pCI-VSVG (Addgene) and lentiviral vectors directing expression of shRNA specific to integrin α6 (TRCN0000057774 (shRNA 1) and TRCN0000057775 (shRNA 2)) or a nontargeting control shRNA (SHC002) (Sigma) to produce virus. Media on the 293FT cells was changed 18 hours after transfection and two days later, viral supernatants were collected, filtered, and used or frozen at -80°C.

Caspase 3/7 assay

For caspase 3/7 assays, CD133-positive or integrin α6-positive/CD133-negative cells were infected for 2 days, selected for 2 days and sorted using flow cytometry based on negative PI incorporation. Sorted fractions were plated in triplicate, allowed to recover overnight, and measured three separate times after the caspase 3/7 substrate (Promega) was added. Relative luciferase was calculated based on cell numbers from three separate wells normalized to 1 x 10^{-7} M of ATP calculated using the CellTiter-Glo assays as described above.

Cell cycle analysis

Following integrin α6 knockdown, 500,000 cells from each treatment group were filtered through a 30μm filter before fixation in 3 mL of 70% Ethanol at 4 degrees overnight. After fixation, the cells were pelleted and resuspended in 0.5mL of PBS. In order to remove any traces of free RNA that could bind with propidium iodide and interfere with data interpretation, each sample was incubated with 5 μL of RNAse A (10 μg/mL) for 30 minutes at 37°C. The RNAse was then inactivated by incubation for 5 minutes at 4°C. Following addition of 10 μL of PI, cells were analyzed by flow cytometry using a FACScan for DNA content.

Detection of apoptosis by DNA fragmentation

Apoptosis was assessed in the GSCs infected with lentiviral shRNA knock down constructs after 2 days of puromycin selection (total of 4 days post infection) by the DNA fragmentation assay. The assay was performed as described in (Benhar et al., 2008) with slight modifications. In brief, the cells were pelleted from suspension culture and washed with PBS. The pellet was then lysed using 1 ml of lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0), and the solution was centrifuged for 20 min at 20,000 g. The protein content was measured using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) and was used to standardize the number of cells processed for each condition. All volumes were adjusted to 500 μl and treated with 20 μg RNAse A (Invitrogen) for 2 hours at 37°C. Following this, each sample was treated with 20 μg Proteinase K (Roche) for 1 hour at 37°C. DNA was precipitated from the samples by adding 1 volume of isopropanol and incubating at -20°C for 12 hours. The

DNA was pelleted by centrifuging at 20,000 g for 20 min. After removal of the isopropanol the pellets were redissovled in 50 µl buffer TE. A 30 µl aliquot of the solution was separated by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. A staurosporine positive control (Sigma, loaded at a 1:10 dilution) was used and for size comparisons, 1kb and 100 bp ladders were used (Invitrogen).

In vivo tumor initiation analysis

In vivo tumor initiation analysis was done as previously described (Bao et al., 2006b, Wang et al., 2008). For integrin α6 high vs low studies, cells (10000, 1000, 500, or 100 cells) were transplanted after 2 hours of recovery post-FACS. For integrin α6 shRNA studies, cells were used 4 days after selection and cells (5000 or 1000) were transplanted into the brains of BALB/c nu/nu mice. For integrin α6 blocking antibody studies, cells were incubated with integrin a6 blocking antibody (10 μg/ml) or isotype control antibody for five days prior to transplantation (1000 cells) into the brains of BALB/c nu/nu mice. Brains of euthanized mice were collected, fixed in 4% PFA, paraffin embedded, sectioned, and subjected to hematoxylin and eosin staining.

Supplementary References

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