Supplemental Information

SHP-2/*PTPN11* mediates gliomagenesis driven by *PDGFRA* and *Ink4a/Arf* aberrations in mice and humans

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RNA Interference

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Figure S I

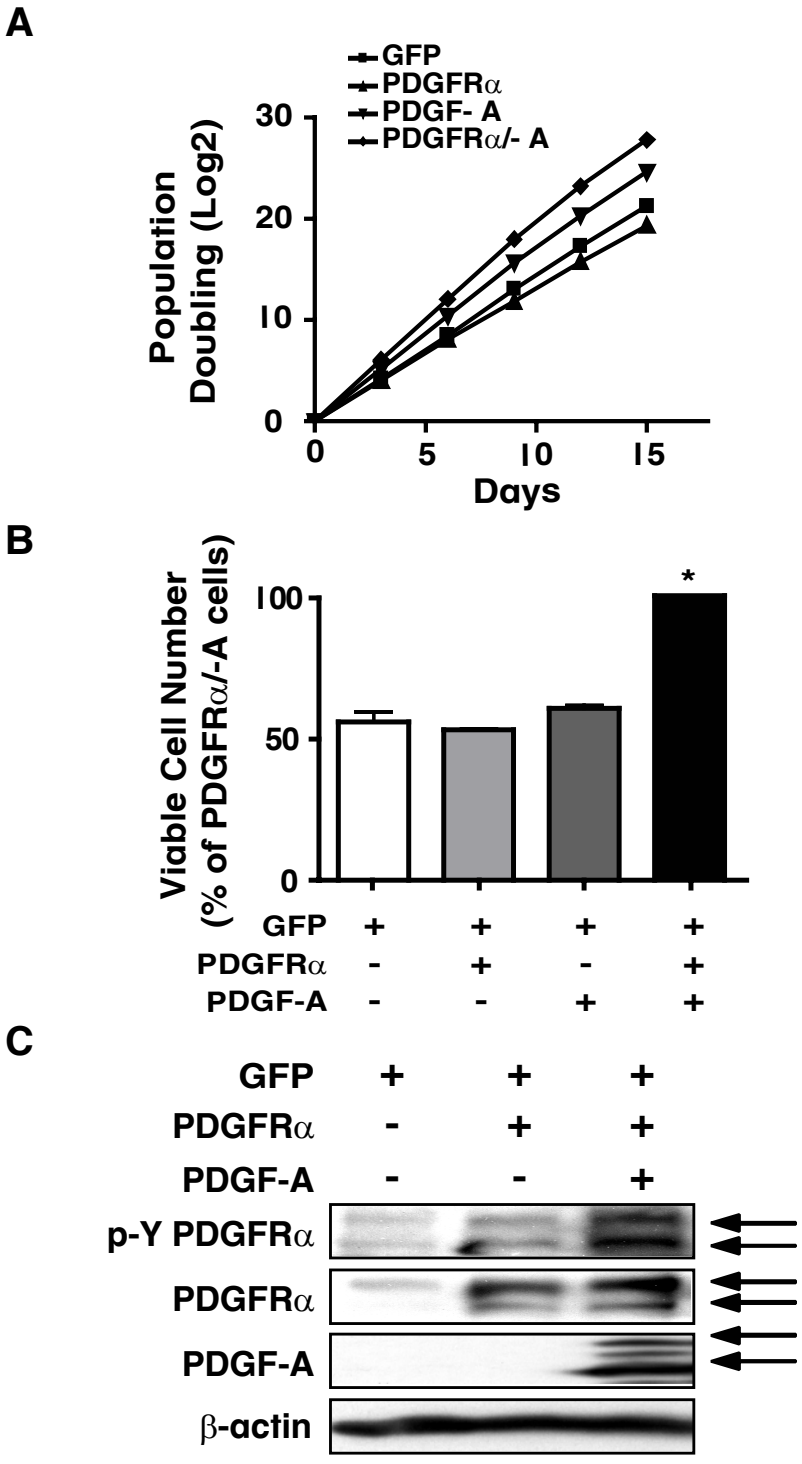
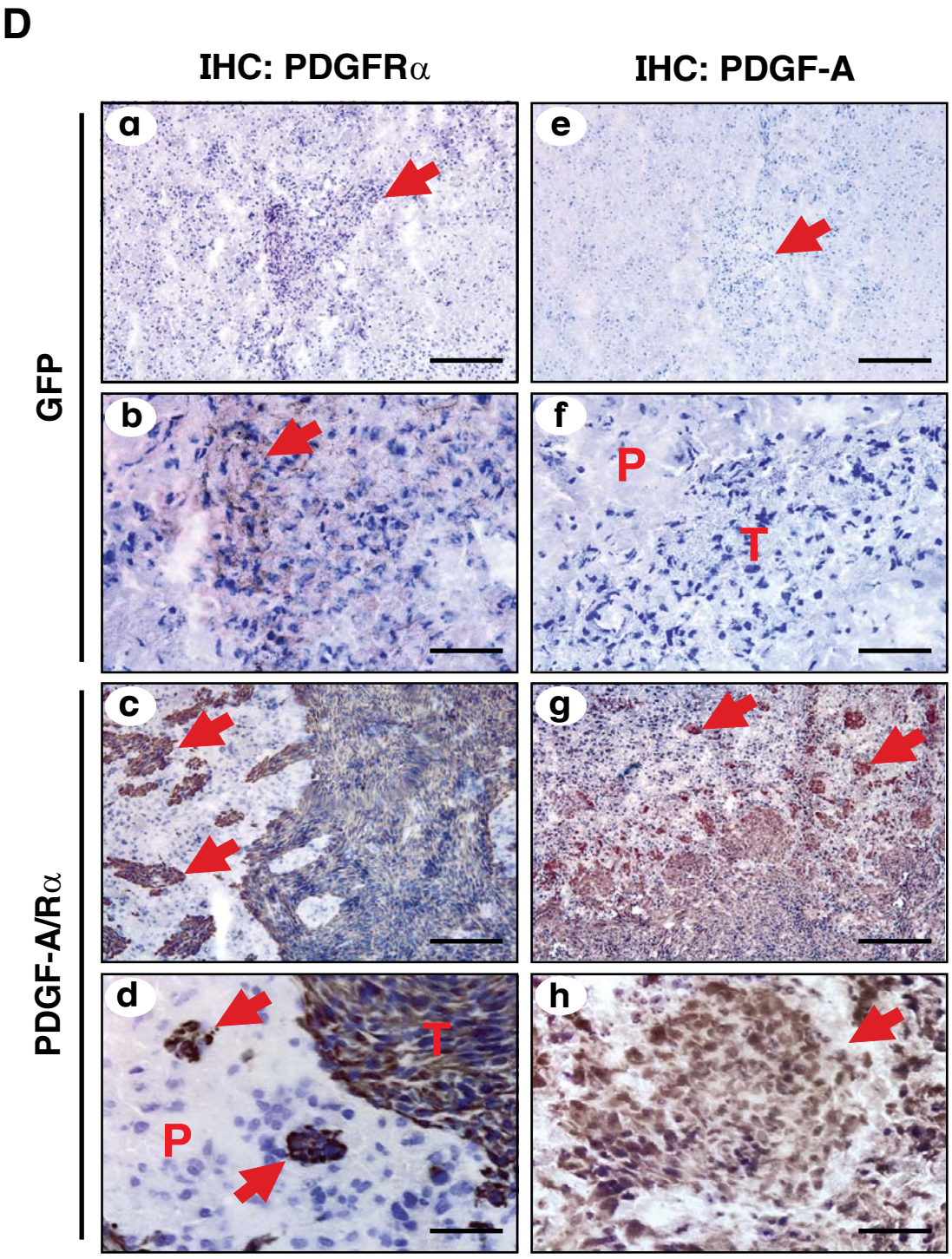
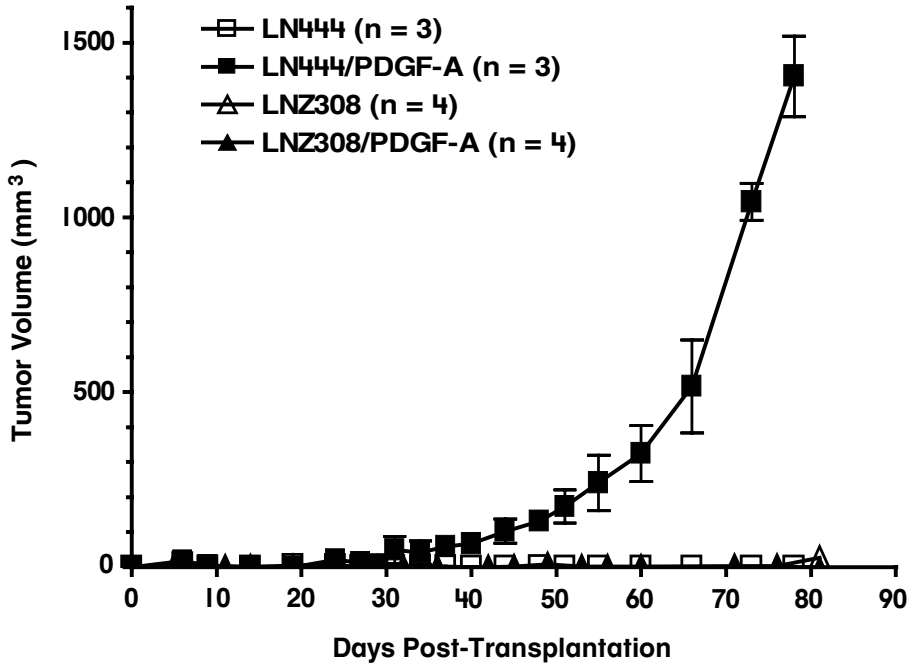

A
GFP
PDGFRα
PDGF- A
PDGFRα/- A
Population Doubling (Log2)
30
20
10
0
0
5
10
15
Days
B
Viable Cell Number (% of PDGFRα/-A cells)
100
50
0
*
GFP + + + +
PDGFRα - + - +
PDGF-A - - + +
C
GFP + + +
PDGFRα - + +
PDGF-A - - +
p-Y PDGFRα
PDGFRα
PDGF-A
β-actin

Figure S I



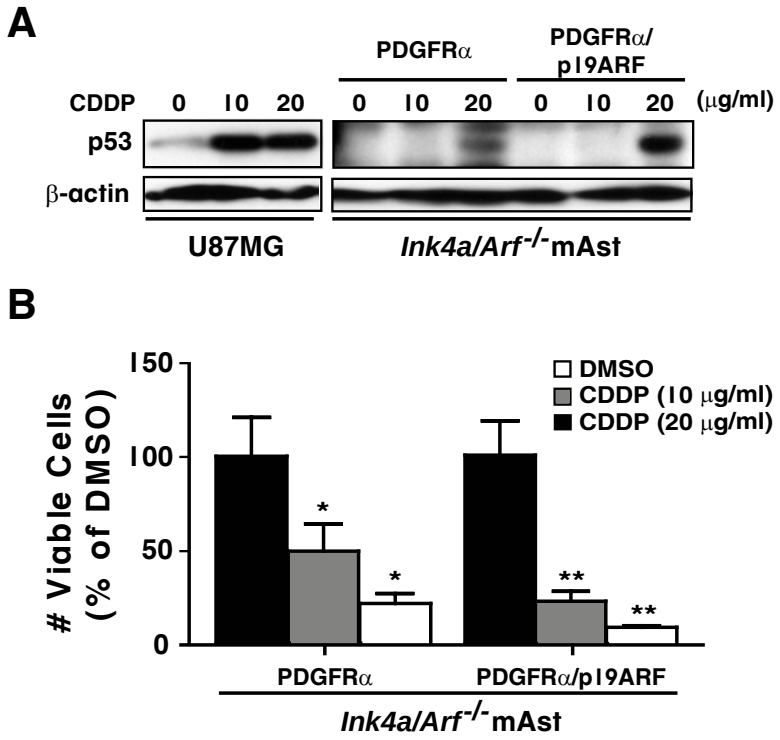
Supplemental Figure 1. *In vitro* and *in vivo* characterization of *Ink4a/Arf* $^{-/-}$ mouse astrocytes overexpressing PDGFRα and/or PDGF-A. (**A**) Growth of *Ink4a/Arf* $^{-/-}$ mouse astrocytes *in vitro*. 50,000 of various cells with similar passage numbers were seeded in triplicate wells of 6-well plates, counted every three days, and re-seeded. Population doubling was calculated by dividing the total cell number by 50,000 and converting it to a $\log_2$ value. No significant difference in cell proliferation was found among these cells. (**B**) Viability of *Ink4a/Arf* $^{-/-}$ mouse astrocytes *in vitro*. 3,000 of various cells were seeded in triplicate wells of a 96-well plate, followed by a 48-hour serum starvation. The number of viable cells was estimated using the WST-1 reagent and presented as a percentage to the number of viable PDGFRα/-A cells. Data are shown as mean ± s.d. *, $P < 0.001$, one-way ANOVA followed by Newman-Keuls post hoc test. (**C**) IB analysis of tissue lysates from s.c. tumors derived from *Ink4a/Arf* $^{-/-}$ GFP control, PDGFRα, and PDGFRα/-A mAst. Various s.c. tumors were snap-frozen, weighed, homogenized, and lysed in lysis buffer before IB analysis. Arrows, PDGFRα and PDGF-A proteins run as doublet bands. β-actin was used as a loading control. (**D**) Immunohistochemical (IHC) staining of brain tumors derived from *Ink4a/Arf* $^{-/-}$ GFP or PDGFRα mAst using anti-PDGFRα (panels *a*-*d*) and anti-PDGF-A (panels *e*-*h*) antibodies. PDGFRα was weakly expressed (panels *a* and *b*) whereas PDGF-A proteins (panels *e* and *f*) were not detected in the *Ink4a/Arf* $^{-/-}$ GFP control tumor. In contrast, high levels of expression of PDGFRα (panels *c* and *d*) and PDGF-A (panels *g* and *h*) were maintained in the *Ink4a/Arf* $^{-/-}$ PDGFRα/-A tumor in the brain of mice. Arrows in panels *a* and *b*, GFP control tumor lesions; the arrow in *b* indicates low level of endogenous PDGFRα staining. Arrows in panels *c*, *d*, *g* and *h*, positive staining of PDGFRα (*c* and *d*) and PDGF-A (*g* and *h*) in tumor cell clusters invading the brain parenchyma. T, tumor mass; P, normal brain parenchyma. Scale bars represent 200 μm in panels *a*, *c*, *e*, and *g*; 50 μm in panels *b*, *d*, *f*, and *h*. Data in (**A**) to (**D**) are representatives of two to three independent experiments with similar results.

Figure S2



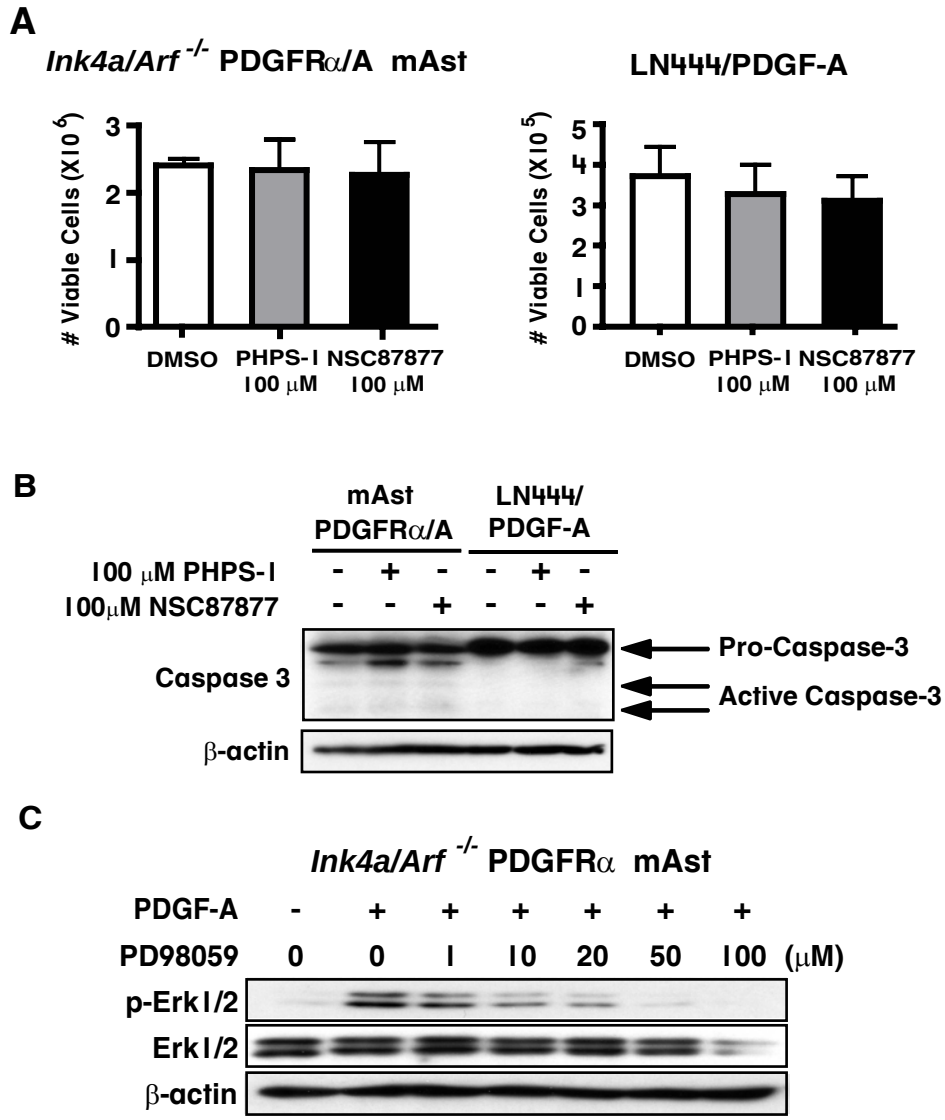
Supplemental Figure 2. Exogenous PDGF-A expression enhances growth of *INK4A/ARF*-null LN444 but not *INK4A/ARF*-wt LN-Z308 tumors in the flanks of mice. Parental human glioma cells were inoculated into the left flank of mice, whereas glioma cells expressing PDGF-A were implanted into the right flank of the same animals. Three to four mice were used in each group. Tumor volumes were estimated [volume = $(a^2 \times b) / 2$, $a < b$] (1) at indicated times after implantation. Data are shown as mean ± s.e.m. and representative from two independent experiments.

Figure S3



Supplemental Figure 3. Cisplatin induces p53 expression and cell death in *Ink4a/Arf*$^{-/-}$ PDGFRα-expressing mAst in the presence of p19ARF. (**A**) U87MG or *Ink4a/Arf* $^{-/-}$ mAst expressing PDGFRα or PDGFRα/p19ARF were serum-starved for 24 hours followed by incubation with cisplatin (CDDP) at indicated concentrations for an additional 24 hours. Cells were then lysed for IB analyses. Cisplatin was able to induce p53 expression at a concentration of 10 μg/ml for U87MG and 20 μg/ml for *Ink4a/Arf* $^{-/-}$ mAst expressing PDGFRα/p19ARF. β–actin proteins were used as loading control. (**B**) An equal number of PDGFRα or PDGFRα/p19ARF-expressing *Ink4a/Arf* $^{-/-}$ mAst were cultured in triplicate wells in the presence of DMSO, 10 μg/ml, or 20 μg/ml CDDP for 48 hours before Trypan Blue exclusion assay for cell viability. *, $P < 0.01$; **, $P < 0.0001$, compared with DMSO control. Data are shown as mean ± s.d. and representative of two independent experiments.

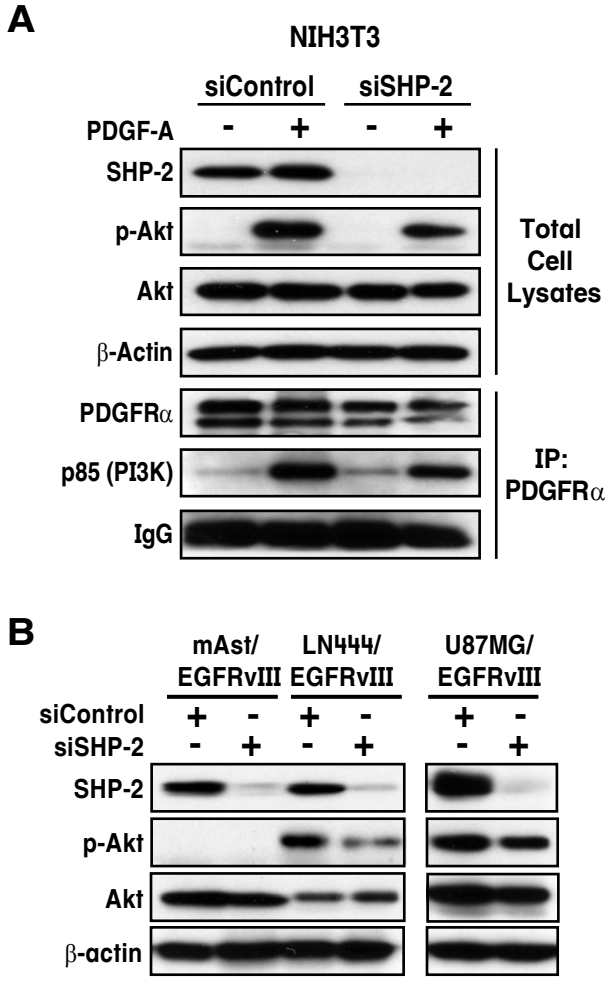
Figure S4



Supplemental Figure 4. Effects of SHP-2 inhibitors on *Ink4a/Arf* $^{-/-}$ mAst and LN444 cell viability and a MEK inhibitor on p-Erk1/2 expression in *Ink4a/Arf* $^{-/-}$ mAst. (**A**) The same number of PDGFRα/PDGF-A-coexpressing *Ink4a/Arf*$^{-/-}$ mAst or LN444 cells was separately cultured in the presence of DMSO, 100 μM PHPS-1 or NSC87877 for 48 hours. Trypan Blue exclusion assay was then performed to determined cell viability. No significant difference was found between inhibitors- and DMSO-treated groups of both types of cells. Data are presented as mean ± s.d. (**B**) PDGFRα/PDGF-A-coexpressing *Ink4a/Arf*$^{-/-}$ mAst or LN444 cells were separately cultured in the presence or absence of 100 μM PHPS-1 or NSC87877 for 48 hours. Cells were then lysed for IB analysis using an anti-caspase-3 antibody. No detectable caspase-3 cleavage was seen in both types of cells. β-actin was used as a loading control. (**C**) *Ink4a/Arf* $^{-/-}$ mAst expressing PDGFRα were serum-starved for 24 hours followed by incubation with PD98059 at indicated concentrations for an additional 24 hours. Cells were then stimulated with

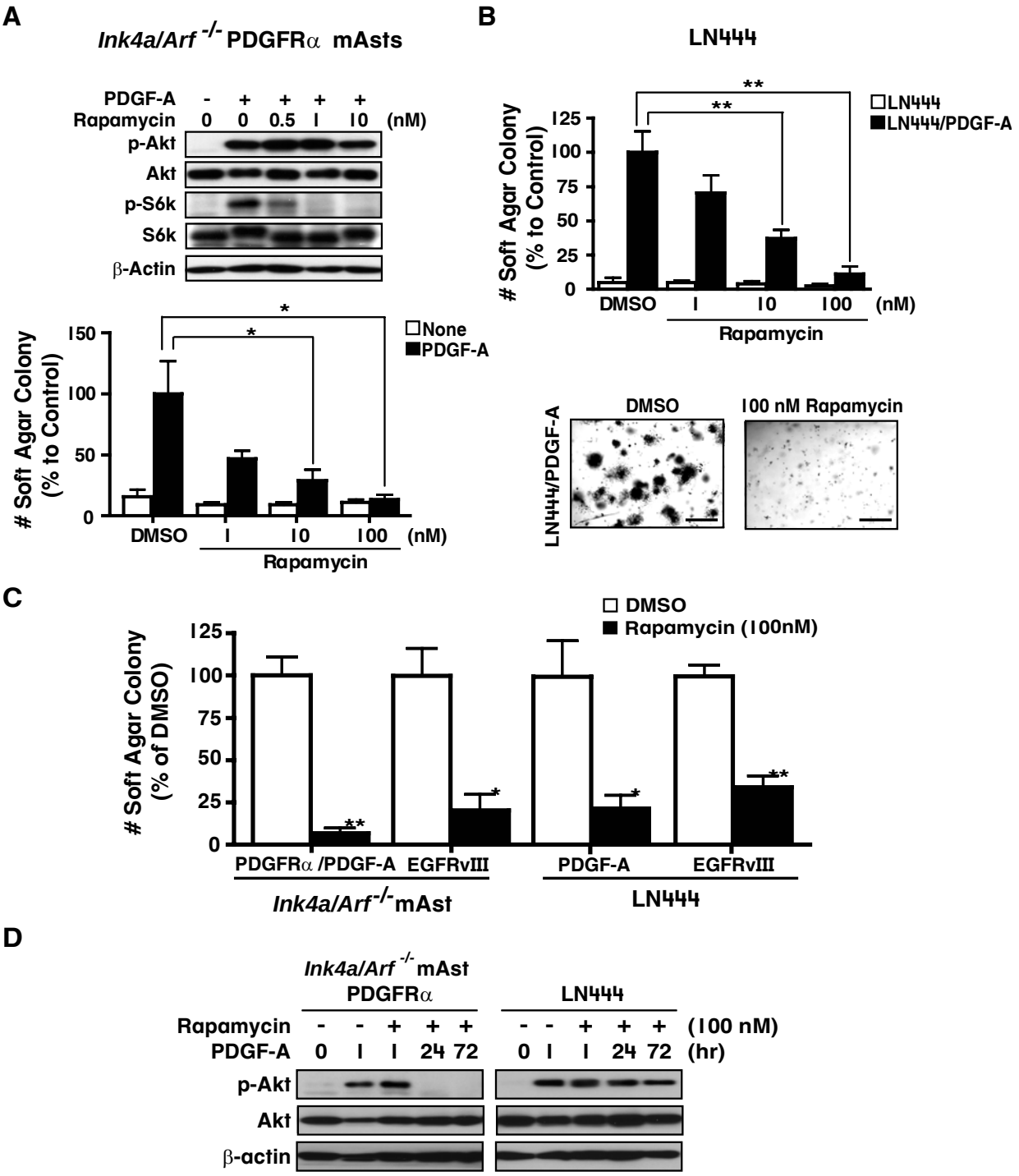
50 ng/ml PDGF-A for 5 min before IB analyses. PD98059 was able to inhibit Erk1/2 phosphorylation at a concentration of 10 μM. Total Erk1/2 and β-actin proteins were used as loading controls. Results from (**A**) to (**C**) are representative of two independent experiments.

Figure S5



Supplemental Figure 5. The impacts of SHP-2 knockdown in NIH3T3, *Ink4a/Arf* $^{-/-}$ EGFRvIII mAst, LN444/EGFRvIII, and U87MG/EGFRvIII cells. (**A**) Association of PI3K to PDGFRα and downstream Akt phosphorylation in NIH3T3 cells were only moderately affected by SHP-2 knockdown. IB analysis of NIH3T3 mouse fibroblasts that were transfected with control or SHP-2 siRNA for 48 hours, serum-starved for an additional 24 hours followed by 50 ng/ml PDGF-A stimulation. Various whole-cell lysates or immunoprecipitates pulled down by an anti-PDGFRα antibody were subjected to IB analysis using indicated antibodies. Corresponding total proteins, β-actin, or total pulled-down IgG were used as loading controls for induced protein phosphorylation (2) or associations (IP followed by IB). (**B**) *Ink4a/Arf* $^{-/-}$ EGFRvIII mAst, LN444/EGFRvIII, and U87MG/EGFRvIII cells were transfected with control or SHP-2 siRNA for 48 hours, and then lysed for IB analysis using indicated antibodies. β-actin was used as a loading control.

Figure S6


A
Ink4a/Arf -/- PDGFRα mAsts
PDGF-A - + + + +
Rapamycin 0 0 0.5 1 10 (nM)
p-Akt
Akt
p-S6k
S6k
β-Actin
Soft Agar Colony (% to Control)
None
PDGF-A
DMSO 1 10 100 (nM)
Rapamycin
B
LN444
LN444
LN444/PDGF-A
Soft Agar Colony (% to Control)
DMSO 1 10 100 (nM)
Rapamycin
DMSO
100 nM Rapamycin
LN444/PDGF-A
C
DMSO
Rapamycin (100nM)
Soft Agar Colony (% of DMSO)
PDGFRα /PDGF-A
EGFRvIII
PDGF-A
EGFRvIII
Ink4a/Arf -/- mAst
LN444
D
Ink4a/Arf -/- mAst
PDGFRα
LN444
Rapamycin - - + + + - - + + + (100 nM)
PDGF-A 0 1 1 24 72 0 1 1 24 72 (hr)
p-Akt
Akt
β-actin

Supplemental Figure 6. Rapamycin inhibits PDGFRα-promoted cell transformation. (**A**) *Upper panel*, IB analysis of whole-cell lysates from serum-starved PDGFRα-expressing *Ink4a/Arf* $^{-/-}$ mAst that were pre-incubated in the presence or absence of rapamycin at indicated concentrations for 24 hours followed by stimulation with or without PDGF-A for 5 min. Corresponding total proteins or β-actin were used as loading controls for induced protein phosphorylation. *Lower panel*, bar graph, quantification of soft agar assays. Cells were grown in triplicates in soft agar with or without rapamycin at indicated concentrations. PDGF-A-expressing mAst in 10% of total cells were included as a source of PDGF-AA in these experiments. (**B**) Soft agar assay of LN444 and LN444/PDGF-A glioma cells treated with indicated concentration of rapamycin. *Upper panel*, bar graph, quantification of the soft agar assay. *Lower panel*, representative images of LN444/PDGF-A cells treated with DMSO control or 100 nM rapamycin. Data are presented as percentage to the controls in mean ± s.d. and are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$, Student's *t* test. (**C**) Soft agar assay of PDGFRα/PDGF-A- or EGFRvIII-expressing *Ink4a/Arf* $^{-/-}$ mAst or LN444 cells treated with 100 nM rapamycin. Cells were grown in triplicates in soft agar with or without rapamycin at indicated concentrations. Data are presented as percentage to the controls in mean ± s.d. and are representative of two independent experiments. *, $P < 0.005$; **, $P < 0.001$, Student's *t* test. (**D**) IB analysis of whole-cell lysates from serum-starved PDGFRα-expressing *Ink4a/Arf* $^{-/-}$ mAst that were pre-incubated in the presence or absence of 100nM rapamycin for 24 hours followed by stimulation with PDGF-A for the indicated times. Total Akt or β-actin were used as loading controls for induced protein phosphorylation. Data are representative of two independent experiments.

Supplemental Table 1. Immunohistochemical Staining of PDGFRα, PDGF-A, p-AKT, p-S6 and p-SHP-2 in Human glioma Tissues

Number	Grade	Histology	PDGFRα	PDGF-A	p-AKT	p-S6	p-SHP-2
J7	4	Glioblastoma	++	++	-	-	-
J10	3	Anaplastic Astrocytoma	++	++	+	+	+
J16	3	Anaplastic Oligodendroglioma	+++	+++	++	+	+
J44	2	Diffuse Astrocytoma	+++	++++	++++	+++	++
J57	2	Diffuse Astrocytoma	++	++	+	+	+
J69	4	Glioblastoma	+++	+++	+	++	++
J72	4	Glioblastoma	++	+++	+++	+++	+++
J80	4	Glioblastoma	++	++	+	++	-
J84	4	Glioblastoma	+++	+++	+	+	+
J94	2	Oligodendroglioma	++	++	++	-	-
J152	4	Glioblastoma	+	++	+	+ / -	++
J156	4	Glioblastoma	+	+	+	++	+
J158	4	Glioblastoma	+++	++	+++	+	++
J159	4	Glioblastoma	+++	+ / -	+++	+	++
J161	4	Glioblastoma	++	+	++	++	++
J163	4	Glioblastoma	++	+++	++	+++	+
J165	3	Anaplastic Astrocytoma	++	++	++	++	-
J171	3	Anaplastic Oligodendroglioma	+++	++	++	++	++++
J182	1	Pilocytic Astrocytoma	+ / -	+ / -	-	-	+
J183	1	Pilocytic Astrocytoma	+ / -	+ / -	+	++	+
J196	3	Anaplastic Astrocytoma	+++	++	+++	++	++++
J200	3	Anaplastic Oligodendroglioma	++	+ / -	++	+	-
J211	4	Glioblastoma	+	+	+	+	+
J212	4	Glioblastoma	+++	++	++	+++	+
J216	4	Glioblastoma	+	++	++	++	++
J233	4	Glioblastoma	+	+ / -	-	-	-
J235	4	Glioblastoma	+	++	+	++	+
JKU01	4	Glioblastoma	+ / -	++	+ / -	+	+
JKU02	4	Glioblastoma	++	+ / -	+ / -	+ / -	+ / -
JKU03	4	Glioblastoma	++	+ / -	+	+	+
JKU04	4	Glioblastoma	++	+	+	+	++
JKU05	4	Glioblastoma	++	++	++	+	+
JKU06	3	Anaplastic Astrocytoma	+++	++++	+	+ / -	++
JKU07	4	Glioblastoma	++	++	+	+ / -	++
JKU08	4	Glioblastoma	+++	++	+	+	+
JKU09	4	Glioblastoma	+	+++	+	+	+
JKU10	4	Glioblastoma	++	+++	++	+	+

++++	Strong signals in most tumor cells (>75%)
+++	Moderate signals in most tumor cells (~50%)
++	Moderate signals in some tumor cells (<25%)
+	Low signals in few tumor cells (<5%)
+ / -	Low or no signals in few tumor cells (<1%)
-	No detectable signals in all tumor cells (0%)

Supplemental Experimental Procedures

Antibodies and Reagents

The following antibodies and reagents were used in this study: rabbit anti-PDGFRα (sc-338, IHC, 1:50), rabbit anti-PDGF-A (sc-128, IHC, 1:50), rabbit anti-p16 (sc-759), and goat anti-β-actin (sc-1616) antibodies were from Santa Cruz Biotechnology Inc., Santa Cruz, CA; rabbit anti-phospho-p44/42 MAP Kinase (Thr202/Tyr204, #9101), rabbit anti-p44/42 MAP Kinase (#9102), mouse anti-phospho-Akt (Ser473, #4051), rabbit anti-phospho-AKT (Ser473, #4060, for IHC, 1:50), rabbit anti-AKT (#9272), rabbit anti-phospho-p70 S6 Kinase (Thr389, #9205), rabbit anti-p70 S6 Kinase (#9202, IHC, 1:50), rabbit anti-phospho-S6 (Ser235/236, #4858), rabbit anti-phospho-RB (Ser807/811, #9308), mouse anti-Myc-Tag (#2276), and rabbit anti-phopho-SHP-2 (Tyr542, #3751, IHC, 1:50) antibodies were from Cell Signaling Technology, Danvers, MA; mouse anti-nestin (MAB353), rabbit anti-GFAP (AB5804), rabbit anti-NG2 (AB5320), mouse anti-phosphotyrosine (clone 4G10, #05-321) and rabbit anti-PI3K p85 (#6-497) antibodies were from Millipore, Temecula, CA; a mouse anti-neuronal class III β-tubulin (clone TUJ1, MMS-435P) was from Covance, Richmond, CA; a mouse anti-SHP-2 (610621) antibody was from BD Biosciences, San Jose, CA; a mouse anti-P14ARF (P2610) was from Sigma-Aldrich, St. Louis, MO; a mouse anti-p53 antibody (OP33) was from EMD Chemicals, Gibbstown, NJ; a rabbit anti-caspase-3 antibody was from Stressgen, Ann Arbor, MI; a rabbit anti-Ki67 antigen (NCL-Ki67p, IHC, 1:200) antibody was from Leica Microsystems Inc., Bannockburn, IL; Matrigel™ Basement Membrane Matrix, Growth Factor Reduced (GFR) (#356230) was obtained from BD Biosciences, San Jose, CA; LY294002 (#440202) (3), Insolution™ SU6656 (#572636), PP2 (#529573) (4, 5), SHP1/2 PTPase Inhibitor NSC87877 (#565851) (6) and MEK inhibitor PD98059 were from EMD Chemicals, Gibbstown, NJ; PHPS-

1 sodium salt hydrate (P0039) (7) was from Sigma-Aldrich, St. Louis, MO. Rapamycin/Sirolimus (NSC-226080) was from NCI Developmental Therapeutic Program, National Institute of Health, Bethesda, MD; CDK inhibitor PD0332991 (S1116) was from Selleck Chemicals, Houston, TX; Cisplatin (C3374) was from LKT Laboratories, Inc., St. Paul, MN. All secondary antibodies were from Vector Laboratories (Burlingame, CA) or Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were from Invitrogen, Sigma-Aldrich, or Fisher Scientific.

Retroviral and Lentiviral Constructs and infections

The retroviral vector pMXI-*gfp* was a gift from Dr. R. Pieper at the University of California, San Francisco (8). cDNA's of wild-type and various PDGFRα mutants were described previously (2, 9). cDNA insert encoding wild-type PDGFRα (a gift from Dr. Carl-Henrik Heldin at Uppsala University, Uppsala, Sweden) was excised from a pcDNA3 vector and subcloned into a BamHI site of the pMXI-*gfp* retroviral vector. To generate retroviral vectors encoding various PDGFRα mutants, cDNA inserts were subcloned from either pLNCX2 (for R627, F731/42, F572/74, F988, F1018, and Y720 PDGFRα mutants) or pCS2 (for F720 and Y731/42 PDGFRα mutants) vectors into the NotI-SnaBI sites of the pMXI-*gfp* retroviral vector. To generate retroviral vectors encoding PDGF-A, cDNA insert encoding PDGF-A was excised from pcDNA3 vector and subcloned into a BamHI-NotI site of pMXI-*gfp* or into an EcoRI site of a pMSCV (Murine Stem Cell Virus)-dsred2 retroviral vector (a gift from Dr. T. Cheng at the University of Pittsburgh, Pittsburgh). For experiments of p16INK4a (a gift from Dr. L. Chin, Dana-Farbar Cancer Institute, Boston) and p19ARF (a gift from Dr. Y. Zheng, Cincinnati Children's Hospital Medical Center, Cincinnati) re-expression, retroviral vectors pBabe-Puro-

p16INK4a and pMIEG3-*egfp-p19ARF* were used. For shRNA experiments, the lentiviral vectors pLKO.1-shCDKN2A (6 clones) and pLKO.1-shPTPN11 (5 clones) were purchased from Thermo Scientific, Huntsville, AL.

Retroviruses and lentiviruses were produced by co-transfecting various cDNA and packaging plasmids into 293T or Phoenix™ (Orbigen Inc., San Diego, CA) cells using Lipofectamine 2000™ reagent according to manufacturer's instruction (#52758, Invitrogen). Forty-eight hours after transfection, the supernatants containing viruses were filtered by a 0.45-μm syringe filter (Fisher) and added into the culture media supplemented with 8 μg/ml polybrene. Forty-eight hours after the infection, transduced human glioma cells or primary astrocytes were harvested and re-plated in DMEM containing 10% FBS and 2 μg/ml puromycin or 300 μg/ml hygromycin for drug selection or sorted by Fluorescence-activated Cell Sorting (FACS) for GFP expression. Expression of exogenous PDGFRα and its various mutants in the resultant cell populations was validated by immunoblotting (IB) and GFP expression by FACS.

RT-PCR

Total RNAs from various human glioma cell lines were extracted using RNeasy Mini Kit (Qiagen). Approximately 5 μg of total RNAs were used to synthesize the first strand cDNA by Superscript™ II Reverse Transcriptase (Invitrogen). The following primers were used for the PCR reactions: *INK4A* forward, 5'-CAA CGC ACC GAA TAG TTA-3'; reverse, 5'-AGC ACC ACC AGC GTG TC-3'; actin forward, 5'-CGG GAA ATC GTG CGT GAC AT-3'; reverse, 5'-GGA GTT GAA GGT AGT TTC GTG-3'. The resulting PCR products were then analyzed using 2% agarose gel electrophoresis.

RNA Interference.

SHP-2 specific siRNA (5'-AAG GAC AUG AAU AUA CCA AUA-3') (10) and a scrambled siRNA control were obtained from Dharmacon (Lafayette, CO). Transient transfection of siRNAs was performed using Effectene® Reagent (Qiagen) according to manufacturer's instructions. Cells were transfected with 100 nM siRNAs for 24 hours, recovered in 10% FBS / DMEM for 48 hours prior to further analyses. Knockdown of SHP-2 was validated by IB using a mouse anti-SHP-2 antibody.

Mouse glioma Xenografts

Experiments of glioma xenografts were performed as previously described (11). For intracranial glioma xenograft experiments, human glioma cells or mAst were harvested and re-suspended at 1×10^5 cells /μl in PBS solution and placed on ice until injection. Anesthetized 6-week-old female athymic *nu/nu* mice (Taconic Farms Inc., Hudson, NY) were placed on a stereotactic frame with ear bars. A burr hole was drilled 2 mm lateral and 1.5 mm anterior to the Bregma. Approximately 3×10^5 cells in a 3 μl volume (for mAst) or 5×10^5 cells in a 5 μl volume (for human glioma cells) were then injected 3 mm below the skull into the striatum of the brain. Mice were then monitored every 3 days. When neuropathological symptoms developed due to tumor burdens in the brain, the mice were euthanized. Brains of mice were then removed, embedded in Optimum Cutting Temperature (OCT) compound (Tissue-Tek) at -80°C, and cryo-sectioned at 5-μm thickness. Sections of brains were stored at -80°C until histological analysis. For s.c. tumor xenograft experiments, 5 million of various types of cells in 100-μl PBS were mixed with equal volume of growth factor-reduced Matrigel™ and injected into flanks of mice using a 1-ml

syringe with a 30-gauge needle (Bekton Dickinson). Tumor volumes were measured every 3 days with a caliper, estimated as ($a^2 \times b$) / 2, $a < b$ (1), and analyzed with GraphPad Prism version 4.00 for Windows (GraphPad Software inc., La Jolla, CA). Mice were euthanized before s.c. tumors reached 2000 mm^3 in volume or when pathological symptoms developed due to tumor burdens. All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of University of Pittsburgh, Pittsburgh, PA.

Immunoprecipitation (IP) and Immunoblotting (IB)

For IP experiments, cells were serum-starved for 24-48 hours and lysed in a lysis solution (20mM Tris-HCl pH 7.4, 50mM NaCl, 0.5% Triton X-100, 2% NP-40, 1mM $CaCl_2$, 1mM $MgCl_2$, and Complete™ EDTA-free protease inhibitor cocktail, cat# 11836170001, Roche). In some experiments, cells were pre-incubated with or without indicated inhibitors for 24 hours and then stimulated by 50 ng/ml PDGF-A for 5 min prior to lysis. The cell extracts were then centrifuged, and proteins in the supernatants were quantified. Approximately 1 μg of indicated primary antibody was added to a lysate preparation containing 1 mg total protein. The mixtures were then incubated at 4°C overnight on a rotator. The protein-antibody complex was pulled down by rProtein G Agarose beads (15920-010, Invitrogen), washed 3 times with the lysis solution, and analyzed in a SDS-PAGE. For IB analysis, immunoprecipitated proteins or total cell lysates containing ~30 μg of total proteins were separated in a SDS-PAGE gel under a reducing condition. The separated proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad), blocked by 5% (w/v) nonfat dry milk (Bio-Rad) in PBS, and probed with indicated primary antibodies at 4°C overnight. Proteins of interest were then visualized by incubating the membrane with indicated peroxidase-labeled secondary antibodies

at room temperature for 30 min followed by detection with enhanced chemiluminescence (ECL, Amersham Bioscience) reaction following manufacturer's instructions.

Cell Proliferation and Viability Assays

Cell proliferation and viability were determined as previously described (12). Briefly, 50,000 cells were seeded in 10% FBS / DMEM, split, counted, and re-seeded every 3 days in a 6-well plate. Population doubling was calculated by dividing the total cell number by the cells seeded (50,000 cells), using $\log_2$ versus the days in culture to determine the proliferation rate of each type of cells.

Cell viability was assessed by a colorimetric assay using a WST-1 reagent (Roche) according to manufacturer's instruction. Briefly, 3000 cells were seeded in triplicate wells of a 96-well microplate and incubated in a serum-free medium for 48 hours at 37°C and 5% CO_2. A WST-1 reagent was then added to the media (10 μl/well) and incubated for an additional 2 hours. Light absorption of samples in each well was measured at a wavelength of 450 nm in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). Wells containing only culture media were used as a background control for all the samples measured. Trypan Blue Exclusion assay was performed as previously described (13). Briefly, cells were harvested and mixed with an equal volume of Trypan Blue dye. The number of live (appearing bright under light microscope) cells was then estimated from a total number of 200 cells from the harvested cells. The data were then analyzed using GraphPad Software.

References

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