Chen et al _Inventory of Supplemental Materials

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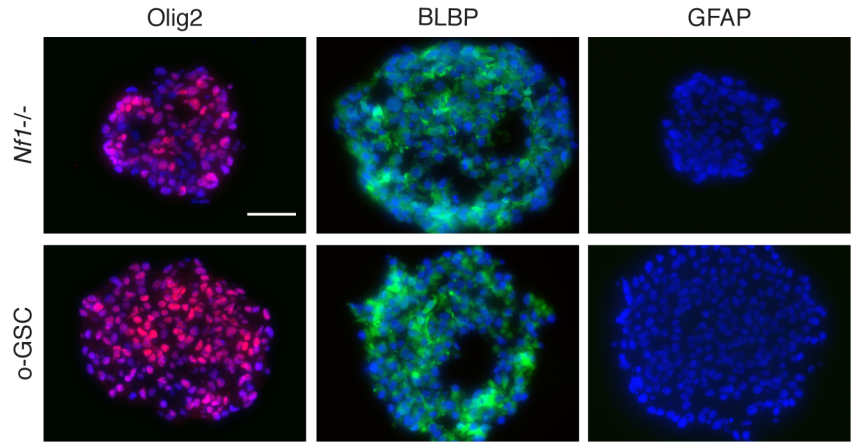
Supplemental References

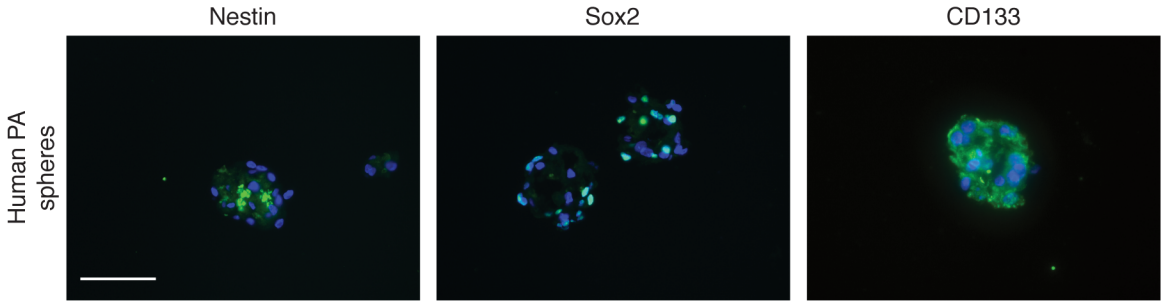
A



B

	Olig2	BLBP	GFAP
Nf1-/- NSCs	74%	99%	0%
o-GSC	96%	99%	0%

C



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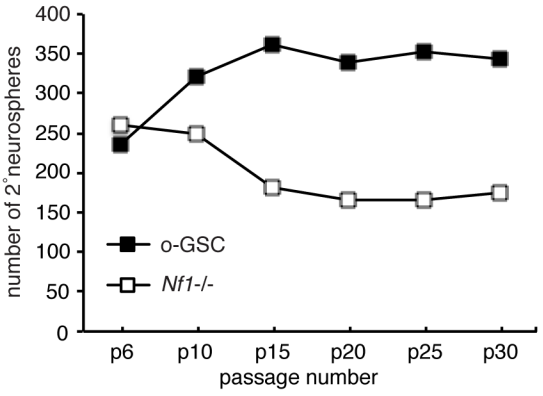

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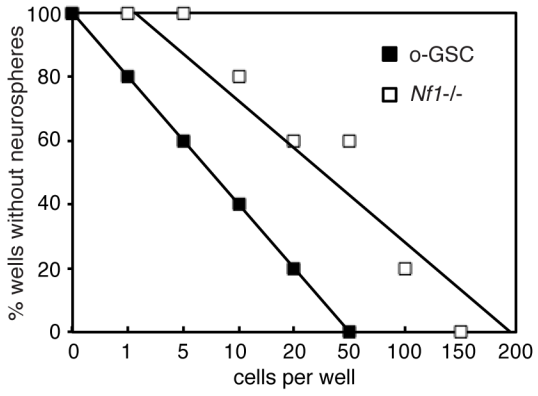


Figure S1. Long-term culture of o-GSCs reveals no loss of stem cell function. (**A**) *Nf1-/-* TVZ NSCs and o-GSCs both express Olig2 and BLBP, but not GFAP. (**B**) Quantitation of cell type-specific markers. (**C**) Spheres from human PA tumors express Nestin, Sox2, and CD133.

(**D**) Whereas o-GSCs exhibit increased numbers of secondary neurospheres with continued passage (>passage 6), reduced numbers were observed in *Nf1*-/- TVZ NSCs. (**E**) Fewer o-GSCs were required to generate one neurosphere by limiting dilution assay (passage 15). Scale bars, 100 μm.

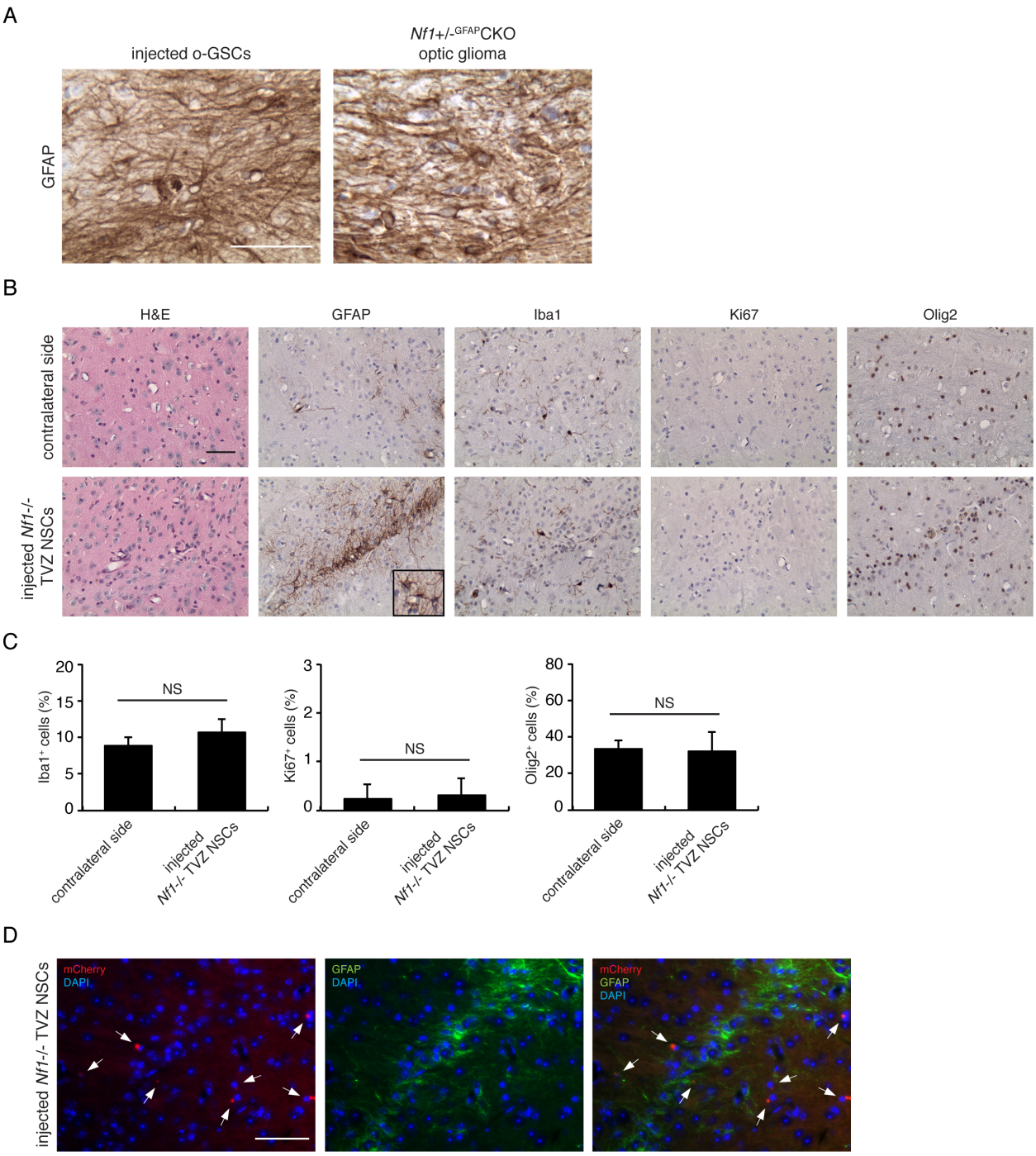


Figure S2. CD133-negative *Nf1*-deficient TVZ NSCs do not form glioma-like lesions following transplantation *in vivo*. (**A**) A representative high-power magnification image of GFAP-expressing cells in a glioma-like lesion following o-GSC injection reveals similar astrocyte morphology to those found in the parental *Nf1* GEM optic gliomas. (**B, C**) Increased numbers of glial fibrillary acidic protein (GFAP)-immunoreactive cells were found in the brainstems of *Nf1*+/-

mice 6 months following the injection of *Nf1*-deficient TVZ NSCs. The $GFAP^+$ cells present at the injection sites had a stellate morphology (inset) typical of reactive astrocytes. Minimal changes in the numbers of $Iba1^+$ and $Olig2^+$ cells were observed. Importantly, only rare $Ki67^+$ cells were identified at the injection sites. The contralateral uninjected sides were used as reference controls. (**D**) mCherry-labeled cells were detected at the injection sites; however, the majority of the $GFAP^+$ cells were negative for mCherry immunostaining. Scale bars, 50 μm. NS, not significant.

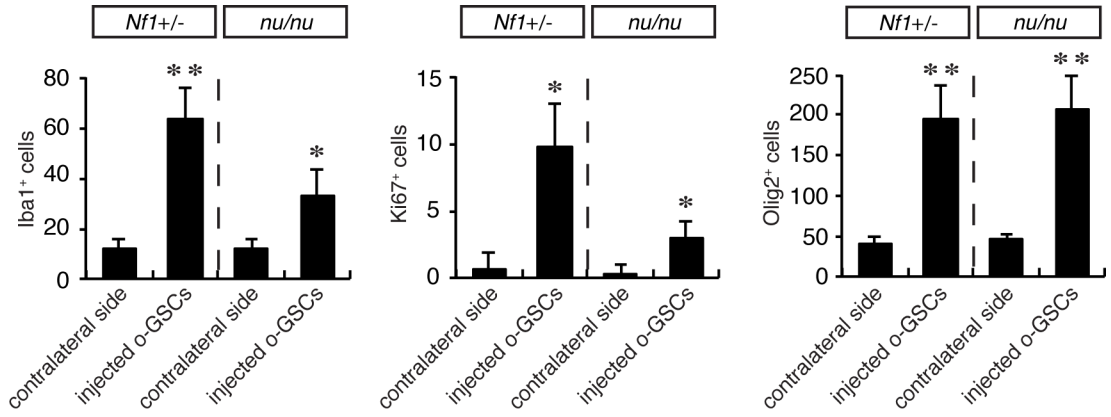


Figure S3. The impact of the tumor microenvironment on o-GSC-induced gliomagenesis.

Increased numbers of Iba1$^+$ and Ki67$^+$ cells per surface area (0.1 mm^2) were observed following o-GSC injection into the brainstems of *Nf1*+/- mice relative to athymic (*nu/nu*) mice. Similar numbers of Olig2$^+$ cells were detected. (*) $p<0.05$; (**) $p<0.01$.

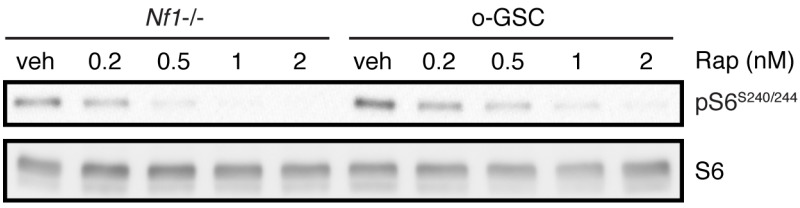


Figure S4. Rapamycin treatment decreases mTOR activation. *Nf1*-/- TVZ NSCs and o-GSCs show dose-dependent inhibition of mTOR activation following rapamycin treatment.

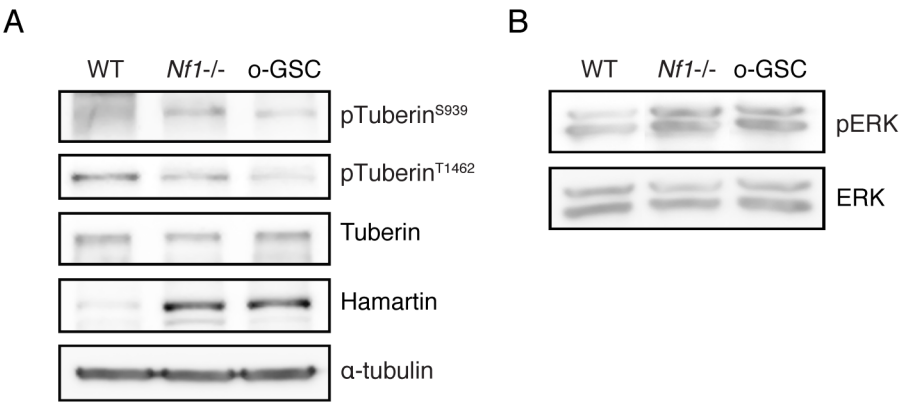
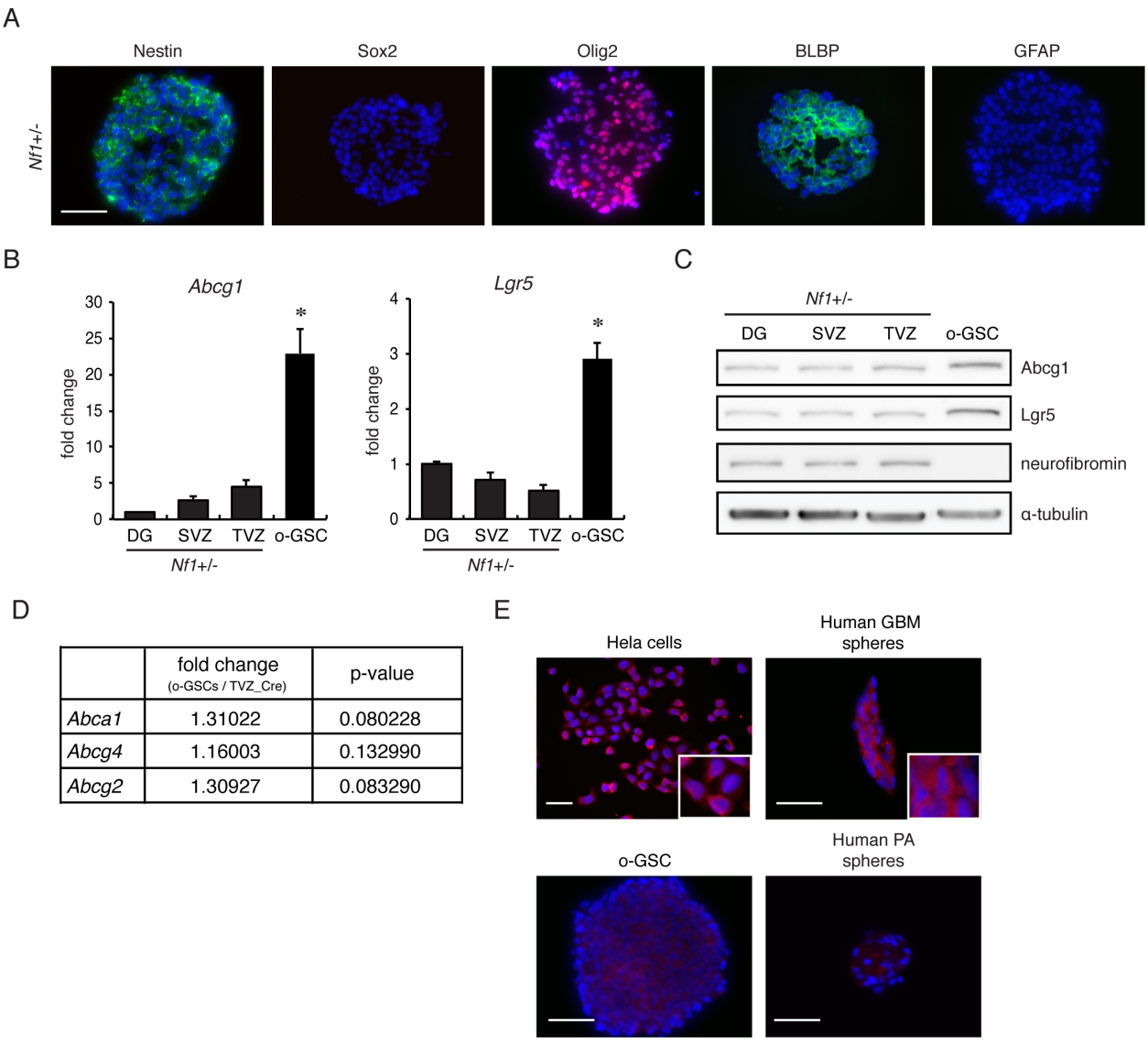


Figure S5. RAS pathway signaling defects in o-GSCs. (A) Decreased tuberin phosphorylation (Ser^{939} and Thr^{1462}) was observed in o-GSCs relative to WT controls, while hamartin expression was increased. (**B**) *Nf1*-/- TVZ NSCs and o-GSCs show similar levels of ERK activation.



	fold change (o-GSCs / TVZ_Cre)	p-value
Abca1	1.31022	0.080228
Abcg4	1.16003	0.132990
Abcg2	1.30927	0.083290

Figure S6. o-GSCs exhibit increased Abcg1 expression. (**A**) Neurospheres generated from the TVZ of 3-month-old *Nf1*+/- mice were positive for nestin (green), Olig2 (red) and BLBP (green) expression, but negative for GFAP and Sox2 expression. (**B**) qRT-PCR analysis and (**C**) Western blotting demonstrated that Abcg1 and Lgr5 are highly expressed in o-GSCs relative to *Nf1*+/- NSCs from different brain regions. (**D**) RNA expression of other ATP-binding cassette (ABC) transporters (*Abca1*, *Abcg4*, *Abcg2*) were similar in o-GSCs relative to *Nf1*-/- NSCs. (**E**) In contrast to human GBM spheres, nearly absent Abcg2 protein expression was found in o-

GSCs and human PA spheres. HeLa cells were used as a positive control for ABCG2 immunostaining. Scale bars, 100 μm.

Supplemental Experimental Procedures

Sectioning of neurospheres

Neurospheres were fixed in 4% paraformaldehyde for 15 min followed by washing twice in PBS. Neurospheres were cryoprotected with 30% sucrose in 0.1 M phosphate buffer at 4°C overnight (von Holst et al., 2006). Fixed neurospheres were embedded in OCT compound and 10 µm sections generated on a microtome.

NSC self-renewal

10 single neurospheres from each genotype were trypsinized and plated into individual wells of ultra-low binding 24 well plates with defined NSC medium containing N2, B27 supplement, EGF and FGF. After 7 days, the number of resulting secondary neurospheres was counted. Supplements were added every 3 days.

Limiting dilution assay

Limiting dilution analyses were performed as previously described (Dasgupta and Gutmann, 2005).

TUNEL staining

Neurospheres were trypsinized and plated onto 50 µg/mL poly-D-lysine-coated and 10 µg/mL fibronectin-coated 24-well plates in defined NSC culture medium containing N2, B27 and growth factors. After 24 hrs, cells were fixed in 4% paraformaldehyde. TUNEL labeling was performed using a fluorescence-based *in situ* cell death detection kit (Roche Diagnostics). The percent of TUNEL-positive cells was determined as a percent of the total cell number ($DAPI^+$ cells).

Table S1. List of genes differentially expressed at least >5-fold in o-GSCs relative to *Nf1*-deficient (TVZ_Cre) NSCs.

RNA microarray (Fold Change)

Gene Symbol		FC (o-GSCs/TVZ_Cre)	*p value*
Hoxa4	homeobox A4	*55.63*	*0.0007*
Dmp1	dentin matrix protein 1	43.02	0.0017
Tmem204	transmembrane protein 204	31.98	0.0003
Neurod1	neurogenic differentiation 1	*28.13*	*0.0001*
Foxd1	forkhead box D1	20.93	0.0001
Arpp21	Cyclic AMP-regulated phosphoprotein, 21	20.79	0.0043
Gabra2	Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 2	19.75	0.0016
Bves	blood vessel epicardial substance	19.60	0.0003
Casp12	Caspase 12	16.95	0.0018
Kcnj15	potassium inwardly-rectifying channel, subfamily J, member 15	16.45	0.0002
Zfp616	zinc finger protein 616	16.20	0.0010
Hoxa5	homeobox A5	*15.74*	*0.0035*
Cgnl1	cingulin-like 1	15.41	0.0043
Abcg1	ATP-binding cassette, sub-family G, member 1	15.14	0.0010
Hoxa3	homeobox A3	14.77	0.0002
Gria4	glutamate receptor, ionotropic, AMPA4 (alpha 4)	*14.70*	*0.0010*
St3gal6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	14.14	0.0002
Enpep	glutamyl aminopeptidase	13.22	0.0032
Kcnip3	Kv channel interacting protein 3, calsenilin	12.72	0.0046
Pcdh10	protocadherin 10	12.02	0.0047
Cml2	camello-like 2	11.65	0.0008
Pappa	pappalysin 2	11.19	0.0026
Lipk	lipase, family member K	11.11	0.0016
Gabrd	gamma-aminobutyric acid (GABA) A receptor, subunit delta	11.00	0.0011
Nodal	Nodal	10.75	0.0031

Fam70a	family with sequence similarity 70, member A	10.10	0.0035
Kif5a	kinesin family member 5A	*10.09*	*0.0033*
Lpl	lipoprotein lipase	9.30	0.0014
Htr3b	5-hydroxytryptamine (serotonin) receptor 3B	8.86	0.0001
Kndc1	kinase non-catalytic C-lobe domain (KIND) containing 1	8.83	0.0001
Polh	polymerase (DNA directed), eta (RAD 30 related)	8.77	0.0004
Pcdh15	protocadherin 15	8.77	0.0035
Speer5-ps1	spermatogenesis associated glutamate (E)-rich protein 5, pseudogene 1	8.75	0.0042
Apob48r	Apolipoprotein B48 receptor	8.48	0.0018
Car6	carbonic anhydrase 6	8.22	0.0004
Stom	stomatin	7.69	0.0006
Jph3	junctophilin 3	7.67	0.0018
Pcdh17	protocadherin 17	7.67	0.0027
Bcas1	breast carcinoma amplified sequence 1	7.65	0.0004
Atad2	ATPase family, AAA domain containing 2	7.25	0.0038
Map6d1	MAP6 domain containing 1	7.19	0.0025
Car10	carbonic anhydrase 10	7.11	0.0007
Nav3	neuron navigator 3	*6.99*	*0.0009*
Pdgfd	platelet-derived growth factor, D polypeptide	6.87	0.0008
Mbd5	methyl-CpG binding domain protein 5	6.73	0.0029
Lgr5	leucine rich repeat containing G protein coupled receptor 5	6.71	0.0019
Endod1	endonuclease domain containing 1	6.40	0.0040
Alcam	activated leukocyte cell adhesion molecule	*6.38*	*0.0033*
Fgf12	fibroblast growth factor 12	*6.30*	*0.0049*
Hgf	hepatocyte growth factor	*6.21*	*0.0048*
Mgat4a	mannoside acetylglucosaminyltransferase 4, isoenzyme A	6.12	0.0040
Cox8b	cytochrome c oxidase, subunit VIIIb	6.05	0.0046
Tac1	tachykinin 1	5.68	0.0049

Cys1	cystin 1	5.40	0.0048
Grid2	glutamate receptor, ionotropic, delta 2	5.35	0.0017
Olfm2	olfactomedin 2	5.31	0.0049
Fam63a	family with sequence similarity 63, member A	5.30	0.0034
Sox12	SRY-box containing gene 12	5.22	0.0013
Cela1	chymotrypsin-like elastase family, member 1	5.21	0.0017

Table S2. List of genes differentially expressed at least <5-fold in o-GSCs relative to *Nf1*-deficient (TVZ_Cre) NSCs.

RNA microarray (Fold Change)

Gene Symbol		FC (o-GSCs/TVZ_Cre)	*p value*
Fbln2	fibulin 2	-703.24	0.0024
Otx2	Otx2 opposite strand transcript 1	-486.93	0.0002
Car8	carbonic anhydrase 8	-41.96	0.0001
Serpinh1	serine (or cysteine) peptidase inhibitor, clade H, member 1	-38.77	0.0010
Aldh1l1	Aldehyde dehydrogenase 1 family, member L1	-34.52	0.0011
Aqp4	aquaporin 4	-33.56	0.0044
Pcp4l1	Purkinje cell protein 4-like 1	-30.75	0.0018
Slc25a31	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31	-29.42	0.0002
Nebl	nebulette	-26.39	0.0015
Rgs16	regulator of G-protein signaling 16	-25.91	0.0045
Zcchc12	zinc finger, CCHC domain containing 12	-23.39	0.0005
Kcne1l	potassium voltage-gated channel, Isk-related family, member 1-like	-20.38	0.0045
Trpm3	transient receptor potential cation channel, subfamily M, member 3	-19.20	0.0035
Pitx2	paired-like homeodomain transcription factor 2	-18.39	0.0001
Emid1	EMI domain containing 1	-18.04	0.0049
Ccdc151	coiled-coil domain containing 151	-17.93	0.0002
Stk33	serine/threonine kinase 33	-15.29	0.0045
Tgfb3	transforming growth factor, beta 3	-14.73	0.0008
Eya2	eyes absent 2 homolog (Drosophila)	-14.64	0.0017
Prdxdd1	PrdX-deacylase domain 1	-14.49	0.0021
Gria1	glutamate receptor, ionotropic, AMPA1 (alpha 1)	-14.34	0.0013
Flywch2	FLYWCH family member 2	-14.25	0.0014
Sulf1	sulfatase 1	-13.39	0.0007
Nrn1	neuritin 1	-13.39	0.0001

Rbp1	retinol binding protein 1, cellular	-13.25	0.0000
H19	H19 fetal liver mRNA	-12.33	0.0003
Igf1	insulin-like growth factor 1	-12.19	0.0001
Gbp2	guanylate binding protein 2	-11.75	0.0000
Peg3	paternally expressed 3	-11.30	0.0001
Crx	cone-rod homeobox containing gene	-11.29	0.0041
Ctsc	cathepsin C	-11.16	0.0004
Parvb	Parvin, beta	-10.94	0.0020
Nkain2	Na+/K+ transporting ATPase interacting 2	-10.89	0.0004
Fam101a	family with sequence similarity 101, member A	-10.18	0.0019
Psg19	pregnancy specific glycoprotein 19	-10.17	0.0028
Foxa1	Forkhead box A1	-9.81	0.0026
Iqcg	Leucine-rich repeats and calponin homology (CH) domain containing 3	-9.74	0.0024
Spef2	sperm flagellar 2	-9.69	0.0034
Rbp3	retinol binding protein 3, interstitial	-9.69	0.0000
Cldn1	claudin 1	-9.62	0.0021
Kcnf1	potassium voltage-gated channel, subfamily F, member 1	-9.59	0.0038
Itih5	inter-alpha (globulin) inhibitor H5	-9.43	0.0036
Cxcr4	chemokine (C-X-C motif) receptor 4	-9.26	0.0013
Mmp14	matrix metallopeptidase 14 (membrane-inserted)	-9.22	0.0006
Ripk3	receptor-interacting serine-threonine kinase 3	-9.07	0.0001
Prdm16	PR domain containing 16	-8.79	0.0009
Agtrap	angiotensin II, type I receptor-associated protein	-8.73	0.0008
Thbs2	thrombospondin 2	-8.70	0.0034
Cybrd1	cytochrome b reductase 1	-8.39	0.0002
Tmem146	transmembrane protein 146	-8.33	0.0005
Cgref1	cell growth regulator with EF hand domain 1	-8.28	0.0001
Ereg	epiregulin	-8.11	0.0010
Ptx3	pentraxin related gene	-8.09	0.0043

Mpg	N-methylpurine-DNA glycosylase	-7.89	0.0008
Fbxw27	F-box and WD-40 domain protein 27	-7.86	0.0023
Rpp25	ribonuclease P 25 subunit	-7.83	0.0012
B9d1	B9 protein domain 1	-7.69	0.0047
Pkp2	plakophilin 2	-7.65	0.0021
Mfap2	microfibrillar-associated protein 2	-7.57	0.0027
A2m	alpha-2-macroglobulin	-7.37	0.0005
Vasn	vasorin	-7.37	0.0037
Emcn	endomucin	-7.05	0.0050
Bcl11b	B-cell leukemia/lymphoma 11B	-7.02	0.0047
Gng2	guanine nucleotide binding protein (G protein), gamma 2	-6.74	0.0036
Mdk	midkine	-6.73	0.0025
Cnn2	calponin 2	-6.64	0.0039
Dzip1	DAZ interacting protein 1	-6.63	0.0047
Cx3cl1	chemokine (C-X3-C motif) ligand 1	-6.57	0.0050
Naprt1	nicotinate phosphoribosyltransferase domain containing 1	-6.48	0.0024
Anxa1	annexin A1	-6.27	0.0008
Emx2	empty spiracles homolog 2	-6.24	0.0031
Myom1	myomesin 1	-6.19	0.0031
Tox2	TOX high mobility group box family member 2	-6.14	0.0013
Tppp3	tubulin polymerization-promoting protein family member 3	-6.10	0.0004
Thrsp	thyroid hormone responsive SPOT14 homolog	-5.95	0.0044
Npnt	nephronectin	-5.71	0.0003
Afap1	actin filament associated protein 1	-5.59	0.0006
Loxl1	lysyl oxidase-like 1	-5.53	0.0031
Ezr	Ezrin	-5.52	0.0034
Gabbr1	gamma-aminobutyric acid (GABA) B receptor, 1	-5.43	0.0031
Gbp1	guanylate binding protein 1	-5.27	0.0015
Col5a2	collagen, type V, alpha 2	-5.25	0.0010
Klhdc8b	kelch domain containing 8B	-5.23	0.0006

Wdr78	WD repeat domain 78	-5.18	0.0031
Wnt5a	wingless-related MMTV integration site 5A	-5.18	0.0048
Eya4	eyes absent 4 homolog	-5.16	0.0039
Hoxc8	homeobox C8	-5.05	0.0037

Table S3. Plasmids.

Construct	**Source**
mCherry-FUW	Dr. Joshua Rubin, Washington University
shAbcg1 NM_009593.1-2038s1c1	The Genome Institute at Washington University
shAbcg1 NM_009593.1-1161s1c1	The Genome Institute at Washington University
shGFP	The Genome Institute at Washington University

Table S4. Antibodies.

Antibody	Host	Source	Dilution
Tuj-1 (ICC)	Mouse	Covance	1:1000
O4 (ICC)	Mouse	Chemicon	1:1000
GFAP (ICC)	Mouse	Millipore	1:500
GFAP (IHC)	Rat	Invitrogen	1:200
Nestin (ICC)	Mouse	Abcam	1:500
Sox2 (ICC)	Mouse	Abcam	1:500
BLBP (ICC)	Rabbit	Millipore	1:500
Olig2 (IHC, ICC)	Rabbit	Millipore	1:500
CD133 (ICC)	Rat	eBioscience	1:100
CD133 (ICC)	Mouse	Biorbyt	1:400
A2B5 (ICC)	Mouse	A2B5 clone 105 hybridoma (ATCC)	1:50
CD15 (ICC)	Mouse	STEMCELL	1:100
CD49f (ICC)	Mouse	Thermo	1:100
Iba1 (IHC)	Rabbit	Wako	1:1000
Ki67 (IHC, IF, ICC)	Mouse	BD Pharmingen	1:500
mCherry (IF)	Rabbit	Abcam	1:250
Lgr5 (WB, ICC)	Rabbit	Abcam	WB 1:1000 ICC 1:500
Lgr5 (IHC)	Rabbit	Abcam	1:100
Abcg1 (WB, IHC, ICC)	Rabbit	GeneTex	WB 1:2000 IHC 1:50 ICC 1:400
phospho-S6 $^{Ser240/244}$ (WB)	Rabbit	Cell Signaling	1:5000
phospho-RSK^{Thr573} (WB)	Rabbit	Cell Signaling	1:1000
phospho-Tuberin^{Ser939} (WB)	Rabbit	Cell Signaling	1:1000
phospho-Tuberin^{Thr1462} (WB)	Rabbit	Cell Signaling	1:1000

phospho-ERK $^{Thr202/Tyr204}$ (WB)	Rabbit	Cell Signaling	1:8000
phospho-MEK1/2 $^{Ser217/221}$ (WB)	Rabbit	Cell Signaling	1:1000
phospho-CRAF ^{Ser338} (WB)	Rabbit	Cell Signaling	1:1000
S6 (WB)	Rabbit	Cell Signaling	1:8000
Tuberin (WB)	Rabbit	Cell Signaling	1:1000
RSK (p90-RSK)	Rabbit	Cell Signaling	1:1000
ERK (WB)	Rabbit	Cell Signaling	1:1000
MEK1 (IP, WB)	Mouse	Cell Signaling	IP 1:50 WB 1:3000
MEK1/2 (WB)	Rabbit	Cell Signaling	1:1000
CRAF (WB)	Rabbit	Cell Signaling	1:500
Caspases (WB)	Rabbit	Cell Signaling	1:1000
Cleaved PARP (WB)	Rabbit	Cell Signaling	1:500
BiP (WB)	Rabbit	Cell Signaling	1:1000
CHOP (WB)	Mouse	Cell Signaling	1:1000
Abcg2 (ICC)	Mouse	Abcam	1:100
Neurofibromin (WB)	Rabbit	Santa Cruz	1:250
α-tubulin (WB)	Mouse	Sigma	1:20,000

WB: Western Blot, IP: Immunoprecipitation, IHC: Immunohistochemistry,

IF: immunofluorescence, ICC: Immunocytochemistry

Table S5. Primers.

Gene	Accession #	Forward primer	Reverse primer
Lgr5	NM_010195	5'-CCACAGCCTGGAGACTTTAGATT-3'	5'-TGTTGTTGCTGTGGAATCCTAGT-3'
Abcg1	NM_009593	5'-TGCGAGAGGGCATGTGTGAC-3'	5'-GGAGGCGGAGTCCTCTTCAG-3'
H3f3a (house-keeping)	NM_008210	5'-CGTGAAATCAGACGCTATCAGAA-3'	5'- TCGCACCAGACGCTGAAAG -3'

Supplemental References

Dasgupta, B., and Gutmann, D.H. (2005). Neurofibromin regulates neural stem cell proliferation, survival, and astroglial differentiation in vitro and in vivo. J Neurosci *25*, 5584-5594.

von Holst, A., Sirko, S., and Faissner, A. (2006). The unique 473HD-Chondroitinsulfate epitope is expressed by radial glia and involved in neural precursor cell proliferation. J Neurosci *26*, 4082-4094.