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The Loss of *Nf1* Transiently Promotes Self-Renewal but Not Tumorigenesis by Neural Crest Stem Cells

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SUMMARY

Neurofibromatosis is caused by the loss of *neurofibromin* (*Nf1*), leading to peripheral nervous system (PNS) tumors, including neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs). A long-standing question has been whether these tumors arise from neural crest stem cells (NCSCs) or differentiated glia. Germline or conditional *Nf1* deficiency caused a transient increase in NCSC frequency and self-renewal in most regions of the fetal PNS. However, *Nf1*-deficient NCSCs did not persist postnatally in regions of the PNS that developed tumors and could not form tumors upon transplantation into adult nerves. Adult *P0a-Cre*$^{+}$*Nf1*$^{fl/-}$ mice developed neurofibromas, and *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ and *Nf1/p53*$^{+/-}$ mice developed MPNSTs, but NCSCs did not persist postnatally in affected locations in these mice. Tumors appeared to arise from differentiated glia, not NCSCs.

INTRODUCTION

Neurofibromatosis type 1 is among the most prevalent disorders of the nervous system and is characterized by the formation of tumors throughout the PNS. It is an autosomal dominant disorder caused by mutations in the *neurofibromin* (*Nf1*) tumor suppressor, which encodes a GTPase activating protein that negatively regulates Ras signaling (Rubin and Gutmann, 2005). Patients typically inherit one *Nf1* mutant allele in the germline, and somatic mutations inactivate the other allele in cells that go on to form tumors. Tumors include discrete dermal neurofibromas that are associated with individual nerves as well as larger plexiform neurofibromas that arise from dorsal root ganglia (DRGs), spinal nerve roots, or nerve plexuses (Riccardi, 1999). In addition to these benign tumors, neurofibromatosis patients can also develop MPNSTs, which often bear additional mutations in *p53* and/or *Ink4a/Arf* (Agesen et al., 2005; Menon et al., 1990; Perrone et al., 2003). Neurofibromas and MPNSTs contain a mixture of normal and neoplastic cells including hyperproliferative Schwann cells as well as fibroblasts and other nerve components in addition to inflammatory cells.

A long-standing question relates to the cell of origin for neurofibromas and MPNSTs. Schwann cells are the most prevalent cell type in these tumors and have bialleleic *Nf1* mutations (Rubin and Gutmann, 2005). This suggests that these tumors arise from Schwann cells or their progenitors. Nonetheless, important questions remain regarding the stage of Schwann cell development that is rendered tumorigenic by *Nf1* deficiency. Mature Schwann cells fail to become hyperproliferative upon *Nf1* deletion or Ras activation (Kim et al., 1995). In contrast, conditional deletion of *Nf1* from fetal nerve progenitors using *Krox20-Cre* leads to plexiform neurofibromas in spinal nerve roots (Zhu et al., 2002).

SIGNIFICANCE

Cancers are often proposed to arise from stem cells that have been transformed by mutations that inappropriately activate self-renewal mechanisms. Neurofibromas and MPNSTs arise from neural crest-derived cells and sometimes arise congenitally, raising the question of whether they arise from NCSCs. We found that *Nf1* transiently inhibits the expansion of NCSCs during midgestation but that *Nf1*-deficient NCSCs appear to acquire normal differentiated fates, do not become tumorigenic, and do not persist postnatally. Instead, MPNSTs and plexiform neurofibromas appeared to arise from differentiated glia that began proliferating inappropriately postnatally or during adulthood. Cancer and benign tumors in the PNS can, therefore, arise from differentiated glia.

Krox20 is expressed by nerve NCSCs (T. Iwashita and S.J.M., unpublished data) in addition to Schwann cells and their committed progenitors (Topilko et al., 1994; Zhu et al., 2002). Fate mapping of *Krox20-Cre* expressing cells demonstrates that these cells undergo multilineage differentiation in DRGs (Maro et al., 2004; Zhu et al., 2002). These observations raise the question of whether neurofibromas and MPNSTs arise from NCSCs or from differentiated Schwann cells.

The neural crest is a heterogeneous population of progenitors that migrates from the dorsal neural tube in early to midgestation and gives rise to the neurons and glia of the PNS. NCSCs are a subset of neural crest cells and are defined by the ability of individual cells to self-renew and to undergo multilineage differentiation into neurons, glia, and myofibroblasts (Morrison et al., 1999; Stemple and Anderson, 1992). Postmigratory NCSCs have been found in all regions of the fetal PNS including peripheral nerves, sympathetic chain, DRGs, and gut (enteric nervous system) (Bixby et al., 2002; Morrison et al., 1999). In peripheral nerves, NCSCs give rise to myelinating and nonmyelinating Schwann cells as well as endoneurial fibroblasts (Joseph et al., 2004), cell types that are present in neurofibromas.

NCSCs persist throughout adult life in the gut (Kruger et al., 2002). However, in other regions of the PNS, including those that develop neurofibromas, NCSCs terminally differentiate by late gestation and cannot be detected postnatally (Kruger et al., 2002). Nonetheless, NCSCs could still be rendered tumorigenic by *Nf1* mutations as plexiform neurofibromas and MPNSTs can arise from mutations that occur during fetal development. Some tumors are even evident in patients at birth (Rubin and Gutmann, 2005). Other tumors develop around puberty or during adulthood. Thus, if NCSCs are rendered tumorigenic by *Nf1* deficiency, then deletion of *Nf1* from fetal NCSCs should lead to a sustained expansion of these cells, and their postnatal persistence, such that they can give rise to neonatal or adult tumors.

Many cancers appear to arise from mutations that transform normal stem cells by inappropriately activating self-renewal pathways (Pardal et al., 2003; Reya et al., 2001). *Nf1* inhibits the proliferation, survival, and glial differentiation of CNS stem cells (Dasgupta and Gutmann, 2005) and the proliferation of CNS glial progenitors (Zhu et al., 2005b). These effects of *Nf1* on CNS stem cells and glial progenitors appear to explain the astrocytomas that arise in neurofibromatosis patients (Zhu et al., 2005a). Although PNS tumors are more common than CNS tumors in neurofibromatosis, the origin of PNS tumors remains unknown.

We found that *Nf1*-deficient NCSCs from fetal DRGs, sciatic nerves, and sympathetic ganglia exhibited increased frequency, proliferation, self-renewal, and gliogenesis. To test whether these effects were sustained, we examined mice in which *Nf1* was conditionally deleted from the PNS using *Wnt1-Cre*, *P0a-Cre*, or *3.9Periostin-Cre*. We did not detect the persistence of *Nf1*-deficient NCSCs in the postnatal DRG, sciatic nerve, or sympathetic ganglia and NCSCs in the postnatal gut were not affected by *Nf1* deficiency. Yet *P0a-Cre*$^{+}$*Nf1*$^{fl/-}$ mice developed plexiform neurofibromas from adult nerves (Zheng et al., 2008 [this issue of *Cancer Cell*]). NCSCs also did not persist postnatally in the DRGs, sciatic nerves, trigeminal nerve, brachial plexus, or sympathetic ganglia of *Nf1/p53*$^{+/-}$ mice or *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice. Yet, these mice developed MPNSTs as adults. NCSCs are, therefore, not rendered tumorigenic by *Nf1* deficiency. Instead, plexiform neurofibromas and MPNSTs appear to arise from differentiated glia.

RESULTS

Nf1 Regulates NCSC Frequency throughout Much of the Developing PNS

Germline *Nf1*$^{-/-}$ embryos die from a cardiac defect by embryonic day (E)14.5 (Brannan et al., 1994; Jacks et al., 1994). Therefore, to test whether *Nf1* regulates NCSC function, we cultured NCSCs from various regions of the PNS from E13 *Nf1*$^{-/-}$ embryos and littermate controls. NCSC frequency was assessed as the percentage of cells from each region of the PNS that could form self-renewing neurospheres that underwent multilineage differentiation (Iwashita et al., 2003; Molofsky et al., 2005) (see Figure S1 available with this article online). The sympathetic chain, DRG, and sciatic nerve from *Nf1*$^{-/-}$ embryos contained a significantly higher percentage of cells (3- to 6-fold higher; $p < 0.05$) that formed multipotent neurospheres in culture compared to littermate controls (Figures 1A–1C). In contrast, *Nf1*-deficiency did not increase the frequency of gut cells that formed multipotent neurospheres. This suggests that *Nf1* negatively regulates the frequency of NCSCs from many, but not all, PNS regions.

To test whether the increased frequency of *Nf1*$^{-/-}$ NCSC colonies was attributable to an increased frequency of NCSCs in vivo or increased survival by NCSCs in culture, we assayed the frequency of p75$^{+}\alpha_4^{+}$ cells among uncultured DRG, sciatic nerve, sympathetic chain, and gut cells by flow-cytometry. p75$^{+}\alpha_4^{+}$ cells from the fetal PNS are enriched for NCSCs (Iwashita et al., 2003; Molofsky et al., 2005). Freshly dissociated *Nf1*$^{-/-}$ DRG, sciatic nerve, and sympathetic chain all had significantly ($p < 0.05$) higher frequencies of p75$^{+}\alpha_4^{+}$ cells (Figures 1A–1C). In contrast, we did not observe a significantly higher frequency of p75$^{+}\alpha_4^{+}$ cells in the gut of *Nf1*$^{-/-}$ mice (Figure 1D). These data suggest that NCSC frequency is increased in vivo at E13 by *Nf1* deficiency.

Nf1 Regionally Regulates NCSC Proliferation and Self-Renewal in the Developing PNS

In PNS regions where *Nf1* negatively regulated NCSC frequency, *Nf1* also negatively regulated proliferation from NCSCs in culture. Multipotent neurospheres formed by NCSCs from DRG, sciatic nerve, and sympathetic chain were significantly larger in the absence of *Nf1* (Figures 1A–1C). All cultures were initiated with very low densities of cells (1000 to 5000 cells per 35 mm dish forming up to 30 neurospheres per dish) so as to minimize fusion between neurospheres. To ensure that the increased size of colonies reflected increased proliferation by *Nf1*$^{-/-}$ NCSCs, we also cultured these cells adherently. Adherent multilineage colonies cultured at clonal density from DRG and sympathetic chain were also significantly larger in the absence of *Nf1* (Figure S2). Increased proliferation contributed to the increased size of NCSC colonies in the absence of *Nf1* as we detected a significantly increased rate of BrdU incorporation into *Nf1*$^{-/-}$ colonies but no decrease in the frequency of cells undergoing cell death (Figure S2I). In contrast, adherent NCSC colonies cultured from the gut were not significantly larger nor did they exhibit an increased rate of BrdU incorporation (Figure S2I).

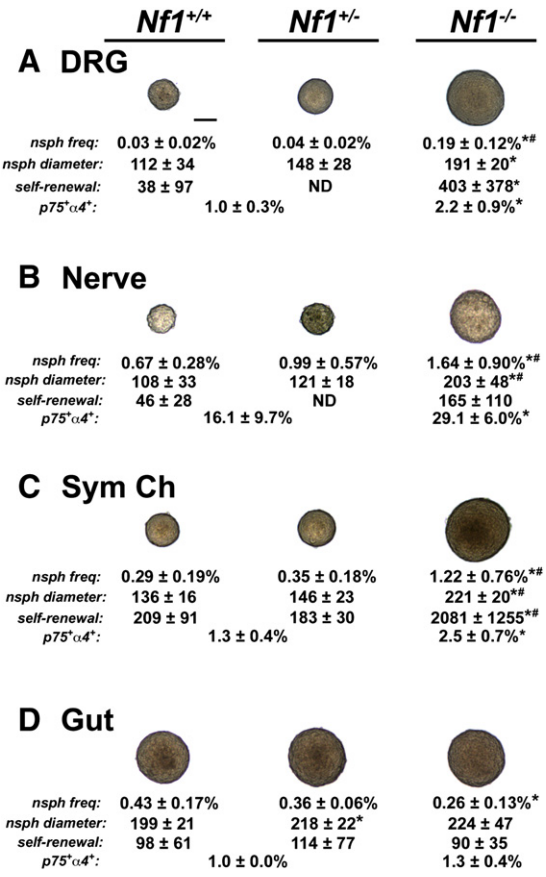


Figure 1. *Nf1* Negatively Regulates the Frequency, Proliferation, and Self Renewal of NCSCs in Most Regions of the Fetal PNS

DRG (A), sciatic nerve (B), sympathetic chain (C), and gut (D) were dissected from E13 $Nf1^{+/+}$, $Nf1^{+/-}$, and $Nf1^{-/-}$ mouse embryos. Dissociated cells were plated into nonadherent cultures at low density (1000 to 2500 cells/35 m well for most tissues; 5000 for DRG). Neurospheres were cultured non-adherently for 10 days, followed by 4 days in adherent cultures before being stained for neurons, glia, and myofibroblasts. Typical neurospheres are shown along with the percentage of freshly dissociated cells that formed neurospheres that underwent multilineage differentiation (nsph freq), the diameter of these neurospheres, and the number of multipotent secondary neurospheres generated per primary neurosphere upon subcloning (self-renewal) (*$p < 0.05$ versus wild-type; #$p < 0.05$ versus heterozygous). The scale bar (100 μm) in (A) applies to all panels. The frequency of $p75^{+}\alpha_4{}^{+}$ cells among freshly dissociated cells was significantly increased in DRG (A), sciatic nerve (B), and sympathetic chain (C), but not gut (D). Culture data represent 8 to 12 experiments and flow-cytometry data represent 4 to 7 experiments per tissue. All statistics are mean ± SD.

To test whether the increased proliferation of NCSCs also reflected increased self-renewal, we dissociated primary neurospheres and subcloned them into secondary cultures to determine the number of multipotent daughter neurospheres that arose per primary neurosphere. Secondary neurospheres were always differentiated to assess multipotency (Figure S1). When cultured from the DRG, nerve, or sympathetic chain, $Nf1^{-/-}$ neurospheres gave rise to 4 to 10 times as many multipotent daughter neurospheres as compared to control neurospheres (Figures 1A–1C). In contrast, we detected no increase in self-renewal by $Nf1^{-/-}$ gut neurospheres (Figure 1D). *Nf1* negatively regulates NCSC self-renewal in most regions of the developing PNS.

Increased Ras Signaling, Gliogenesis, and Growth Factor Sensitivity by *Nf1*-Deficient NCSCs

$Nf1^{-/-}$ cells exhibit increased sensitivity to growth factors that signal through the Ras pathway (Vogel et al., 1995). To test whether this was the case in NCSCs, we cultured NCSCs from the gut, sciatic nerve, DRG, and sympathetic chain of E13 $Nf1^{-/-}$ and littermate control embryos. Western blot demonstrated increased phosphorylated Erk (pErk) in the $Nf1^{-/-}$ colonies, consistent with increased Ras signaling in these cells (Figure 2A). Gut neural crest cells also exhibited increased pErk in the absence of *Nf1* (Figure 2A) despite the fact that these cells did not exhibit increased proliferation. The increased Ras signaling by $Nf1^{-/-}$ NCSCs suggests that these cells may exhibit increased responses to growth factors that signal through Ras, including factors that regulate survival, like FGF2, and gliogenesis, like Neuregulin (Nrg1). Indeed, addition of a short pulse of Nrg1 to NCSC cultures increased pErk levels in wild-type cells and further increased the elevated levels observed in $Nf1^{-/-}$ cells (Figure 2B).

To test whether *Nf1*-deficiency affected the differentiation of NCSCs, we cultured neurospheres from E13 $Nf1^{-/-}$ and control littermate embryos, then replated them in adherent cultures and stained the colonies for neurons, glia, and myofibroblasts. Multipotent $Nf1^{-/-}$ colonies exhibited much more exuberant gliogenesis (Figures 2E and 2H) as compared to control colonies (Figures 2C, 2D, 2F, and 2G). These colonies exhibited a dramatic increase in the absolute numbers of glia per colony without exhibiting decreases in the numbers of neurons or myofibroblasts per colony (data not shown). Similar results were obtained with nerve NCSCs, but we did not generally observe increased gliogenesis by cultured gut NCSCs (data not shown). This indicates that *Nf1* negatively regulates gliogenesis from NCSCs, though we do not know whether this reflects effects on glial lineage determination, proliferation, or survival.

$Nf1^{-/-}$ NCSCs also survived under adverse culture conditions in which FGF2 and chick embryo extract concentrations were reduced to levels that were non-permissive for the survival of wild-type NCSCs. $Nf1^{+/+}$ and $Nf1^{+/-}$ sympathetic chain or DRG progenitors formed only rare colonies that contained small numbers of myofibroblasts in this medium, while $Nf1^{-/-}$ cells formed multilineage colonies (Figure 2), albeit at a reduced frequency as compared to standard medium (Figures 2L and 2M; *$p < 0.05$).

Nf1-Deficient NCSCs Do Not Persist Postnatally outside of the Gut

To test whether *Nf1* deficiency leads to an ongoing postnatal expansion of NCSCs, we conditionally deleted *Nf1* from neural crest cells by crossing *Wnt1-Cre* mice (Danielian et al., 1998) with $Nf1^{fl}$ mice (Zhu et al., 2001). *Wnt1-Cre*$^{+}Nf1^{fl/-}$ mice die at birth (Gitler et al., 2003), so cells were cultured from E17 to E19 embryos. We were never able to culture multilineage NCSC colonies from the sciatic nerve or DRG of *Wnt1-Cre*$^{+}Nf1^{fl/-}$ embryos or controls (Table 1). We were able to culture rare NCSC colonies from the sympathetic chain and gut of *Wnt1-Cre*$^{+}Nf1^{fl/-}$ embryos and littermate controls, though their

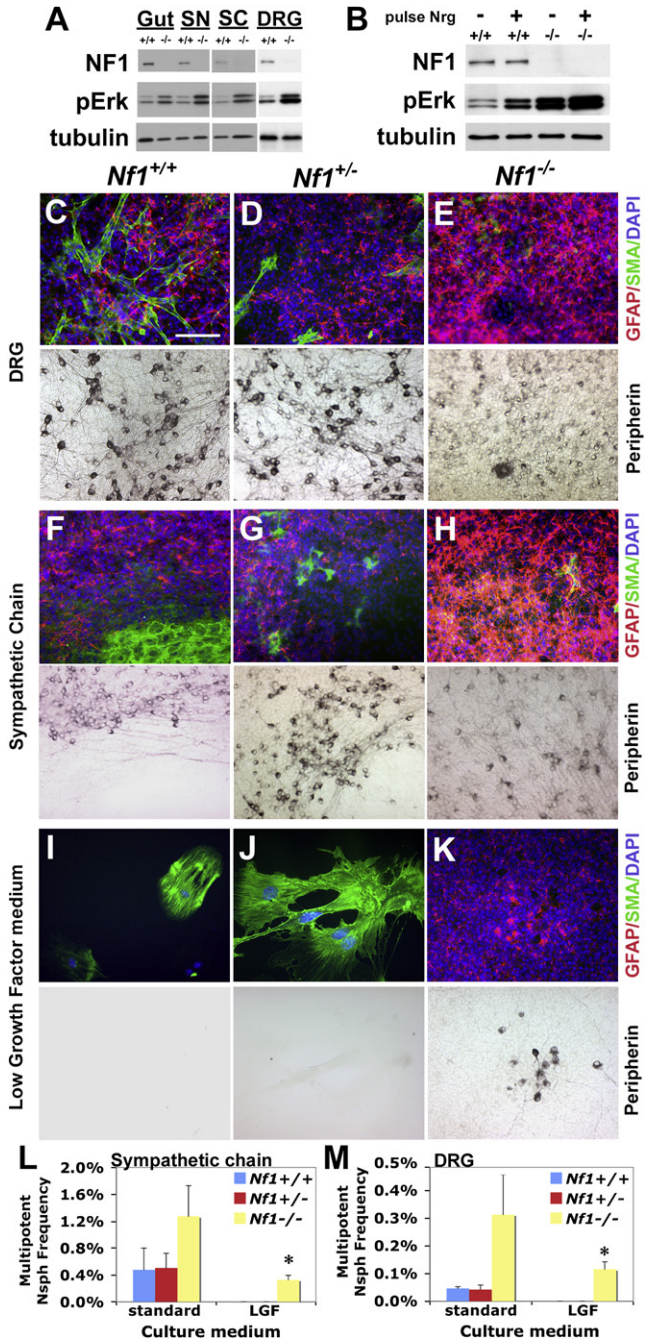


Figure 2. *Nf1*-Deficient NCSCs Exhibit Increased Ras Signaling, Increased Gliogenesis, and Increased Survival in Low Growth Factor Cultures

(A) Cell lysates from $Nf1^{+/+}$ and $Nf1^{-/-}$ neurospheres were analyzed for NF1 and phosphorylated Erk 42/44 (pErk) protein levels. (B) When $Nf1^{+/+}$ and $Nf1^{-/-}$ NCSC cultures from sympathetic chain were pulsed with Nrg-1, pErk levels increased in both $Nf1^{+/+}$ and $Nf1^{-/-}$ cells, but were highest in $Nf1^{-/-}$ cells. NCSCs cultured from E13 $Nf1^{+/+}$, $Nf1^{+/-}$, and $Nf1^{-/-}$ DRGs (C–E) and sympathetic chain (F–H) underwent multilineage differentiation, forming peripherin$^+$ neurons, GFAP$^+$ glia, and SMA$^+$ myofibroblasts. More gliogenesis (red) was observed from $Nf1^{-/-}$ NCSCs from DRG (E) and sympathetic chain (H). $Nf1^{+/+}$ and $Nf1^{+/-}$ cells from sympathetic chain (I–J and L) and DRG (M) failed to form multilineage colonies in low growth factor cultures (LGF), while $Nf1^{-/-}$ cells continued to do so ([K]; SMA+ myofibroblasts were present in this colony outside of the field of view) ([L and M]; *$p < 0.05$ versus control cultures). Brightfield (peripherin) and fluorescence (GFAP, SMA) images always represent the same field of view within a single colony. The scale bar (100 μm) in (C) applies to all panels. Data represent 3–5 independent experiments and error bars represent SD.

Table 1. *Nf1* Deficiency Does Not Lead to the Persistence of Expanded Numbers of NCSCs into Late Gestation or Postnatally

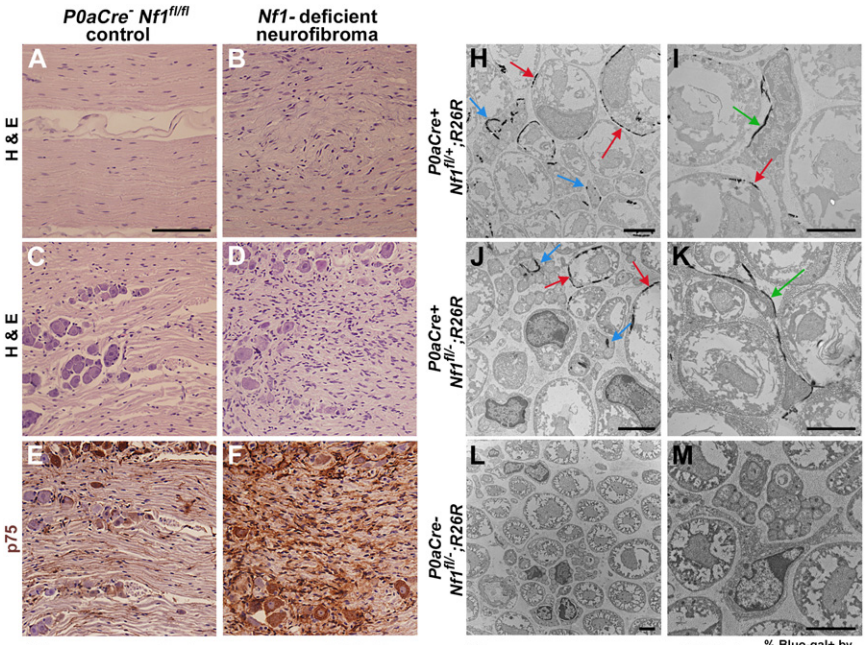
E17 to E19	Cells that Formed Multipotent Neurospheres	
	Littermate Controls	*Wnt1-Cre*$^+$*Nf1*$^{fl/-}$
DRG	0 ± 0%	0 ± 0%
Sciatic nerve	0 ± 0%	0 ± 0%
Sympathetic chain	0.03 ± 0.03%	0.05 ± 0.04%
Gut	0.08 ± 0.10%	0.08 ± 0.10%
P30 to P60	**Cells that Formed Multipotent Neurospheres**	
	Littermate Controls	*3.9Periostin-Cre*$^+$*Nfl*$^{fl/-}$
DRG	0 ± 0%	0 ± 0%
Sciatic nerve	0 ± 0%	0 ± 0%
Sympathetic chain	0 ± 0%	0 ± 0%
Gut	0.8 ± 0.6%	1.1 ± 0.8%

Tissues were dissected, dissociated, and added to nonadherent cultures. Neurospheres were replated to test multilineage differentiation into neurons, glia, and myofibroblasts. We were unable to detect NCSCs from the DRGs, sciatic nerves, or sympathetic chain of young adult (P30 to P60) *3.9Periostin-Cre*$^+$*Nfl*$^{fl/-}$ mice or littermate controls. All data represent mean ± SD for 3 to 5 independent experiments per PNS region.

frequency was similar in $Nf1^{+/+}$ and $Nf1^{-/-}$ embryos (Table 1). The expansion of $Nf1^{-/-}$ NCSCs at E13 does not lead to the inappropriate expansion or persistence of $Nf1^{-/-}$ NCSCs at later stages of development.

To test when $Nf1^{-/-}$ NCSCs are lost from the developing PNS, we analyzed *Wnt1-Cre*$^+$*Nf1*$^{fl/-}$ embryos and littermate controls at E13, E15, and E17–19. Like germline *Nf1*-deficient mice (Figure 1), we observed increased NCSC frequencies in E13 *Wnt1-Cre*$^+$*Nf1*$^{fl/-}$ embryos as compared to littermate controls in the sympathetic chain, sciatic nerve, and DRG, but not in the gut (Figures S3A–S3D). Nonetheless, the frequency of *Nf1*-deficient NCSCs declined in all regions of the PNS by E15 and declined further by E17. By E17–19, *Nf1*-deficient multipotent neurospheres could only be detected in the sympathetic chain and gut and did not differ in frequency as compared to littermate controls. We confirmed that *Nf1* was efficiently deleted from NCSCs in these experiments by genotyping individual neurospheres cultured from the sympathetic chain, sciatic nerve, DRG, and gut of E13 *Wnt1-Cre*$^+$*Nf1*$^{fl/-}$ embryos: all of the 25 neurospheres exhibited deletion of the *Nf1*^{fl} allele (Figure S3H). These data suggest that *Nf1*-deficient NCSCs differentiate during late gestation according to a similar time course as wild-type NCSCs.

To independently test whether *Nf1* deficiency leads to the postnatal persistence of NCSCs, we generated *3.9Periostin-Cre*$^+$*Nf1*$^{fl/-}$ mice. *3.9Periostin-Cre* conditionally deletes throughout the Schwann cell lineage after E11, at a later stage of development than *Wnt1-Cre* (Lindsley et al., 2007). We also observed an increase in the frequency of NCSCs within the sympathetic chain and sciatic nerve, but not the gut, of E15 *3.9Periostin-Cre*$^+$*Nf1*$^{fl/-}$ mice as compared to littermate controls (Figures



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	Fraction sorted	*P0aCre-Nf1fl/fl* control	*Nf1*-deficient neurofibroma
DRG	unfractionated	0 ± 0%	0 ± 0%
	p75+	0 ± 0%	0 ± 0%
Sciatic Nerve	unfractionated	0 ± 0%	0 ± 0%
	p75+	0 ± 0%	0 ± 0%
Trigeminal Nerve	unfractionated	0 ± 0%	0 ± 0%
	p75+	0 ± 0%	0 ± 0%
Brachial Plexus	unfractionated	0 ± 0%	0 ± 0%
	p75+	0 ± 0%	0 ± 0%
Sympathetic Ch	unfractionated	0 ± 0%	0 ± 0%
	p75+	0 ± 0%	0 ± 0%
Gut	p75+	10 ± 6%	4 ± 3%

N

	Genotype	Total # of cells counted	% Bluo-gal+ by Electron Microscopy
myelinating Schwann cells	*P0aCre+Nf1fl/+;R26R*	928	35.8%
	P0aCre+Nf1fl/-;R26R	525	35.0%
	P0aCre-Nf1fl/-;R26R	487	0.0%
non-myelinating Schwann cells	*P0aCre+Nf1fl/+;R26R*	161	40.4%
	P0aCre+Nf1fl/-;R26R	78	39.7%
	P0aCre-Nf1fl/-;R26R	109	0.0%
endoneurial fibroblasts	*P0aCre+Nf1fl/+;R26R*	63	55.6%
	P0aCre+Nf1fl/-;R26R	52	59.6%
	P0aCre-Nf1fl/-;R26R	48	0.0%

Figure 3. Adult *P0aCre*$^{+}$*Nf1*$^{fl/-}$ Mice Developed Plexiform Neurofibromas but NCSCs Appeared to Differentiate Normally and Did Not Persist Postnatally

P0aCre^{+} *Nf1*-deficient mice developed plexiform neurofibromas in adult peripheral nerves (B) and DRGs (D), marked by increased cellularity and disorganization relative to control nerves (A) and DRGs (C). Neurofibromas also exhibited increased p75 (F) staining relative to control nerves (E). Multipotent neurospheres arose from control and *P0aCre*$^{+}$ *Nf1*-deficient gut, but not from other regions of the adult PNS (G). Neural crest progenitors in developing nerves were fate-mapped by staining sciatic nerves from postnatal day 20 *P0aCre*$^{+}$*Nf1*$^{fl/+}$*R26R* (H and I), *P0aCre*$^{+}$*Nf1*$^{fl/-}$*R26R* (J and K), and *P0aCre*$^{-}$*Nf1*$^{fl/-}$*R26R* (L and M) pups with bluo-gal (Joseph et al., 2004). We detected no defects in nerve development in *Nf1* mutant mice. A similar percentage of endoneurial fibroblasts (green arrows) as well as myelinating (red arrows) and nonmyelinating (blue arrows) Schwann cells were bluo-gal+ in *P0aCre*$^{+}$*Nf1*$^{fl/-}$*R26R* nerves as compared to littermate controls (N). No bluo-gal staining was observed in *P0aCre*$^{-}$*Nf1*$^{fl/-}$*R26R* negative control nerves (L and M). The scale bar (100 μm) in (A) applies to panels (A)–(F). Scale bars in (H)–(M) equal 2.5 μm. (n = 3–6 mice/genotype for [A]–[G] and 2 mice/genotype for [H]–[N]). All statistics are mean ± SD.

S3E–S3G). Genotyping of individual neurospheres showed that *Nf1* was efficiently deleted from these cells: 30 out of 30 neurospheres exhibited deletion of the *Nf1*^{fl} allele (data not shown).

Most *3.9Periostin-Cre*$^{+}$*Nf1*$^{fl/-}$ mice died by 4 weeks after birth. Of 32 expected *3.9Periostin-Cre*$^{+}$*Nf1*$^{fl/-}$ mice, only 6 survived more than 4 weeks after birth. In these surviving mice, NCSCs did not persist into adulthood in sciatic nerve, DRG, or sympathetic chain in either *3.9Periostin-Cre*$^{+}$*Nf1*$^{fl/-}$ mice or littermate controls (Table 1). As expected, NCSCs did persist postnatally in the guts of *3.9Periostin-Cre*$^{+}$*Nf1*$^{fl/-}$ mice and littermate controls, but there was no difference in the frequency of gut NCSCs in these mice (Table 1). Genotyping of individual neurospheres cultured from the guts of adult *3.9Periostin-Cre*$^{+}$*Nf1*$^{fl/-}$ mice showed that *Nf1* was efficiently deleted from these cells: 35 out of 35 neurospheres exhibited deletion of the *Nf1*^{fl} allele (data not shown). The expansion of NCSCs that was observed at E13 in germline and conditional *Nf1*$^{-/-}$ embryos was therefore only transient: *Nf1*$^{-/-}$ NCSCs did not persist postnatally in regions of the PNS where plexiform neurofibromas form.

NCSCs Do Not Persist Postnatally in Mice that Develop Plexiform Neurofibromas

We never detected PNS tumors in the limited number of *3.9Periostin-Cre*$^{+}$*Nf1*$^{fl/-}$ mice that survived into adulthood (two mice were analyzed at 3 weeks of age and six from 4 to 8 weeks of age). To study mice that consistently survived into adulthood after *Nf1* deletion, we conditionally deleted *Nf1* using *P0a-Cre*, which deletes in trunk neural crest by E11.5 and in peripheral nerves by E12.5 (Giovannini et al., 2000). P0 is expressed by early migrating and multipotent neural crest cells (Hagedorn et al., 1999; Lee et al., 1997). We sacrificed six *P0a-Cre*$^{+}$ *Nf1*-deficient mice and six littermate controls between 15 and 20 months of age and analyzed sciatic nerves, DRGs, trigeminal nerves, and brachial plexi for plexiform neurofibromas. All six of the *P0a-Cre*$^{+}$ *Nf1*-deficient mice, but none of the littermate controls, exhibited neurofibromas (Figures 3A–3F). Classic features of plexiform neurofibromas were evident in each case, including grossly enlarged peripheral nerves and DRGs, with increased cell density, nerve disorganization, and expression of p75 and S100 within the tumors (Figures 3B, 3D, and 3F).

To assess whether NCSCs persist into adulthood in these mice, we dissociated and plated DRG, sciatic nerve, trigeminal nerve, brachial plexus, sympathetic chain, and gut cells from the *P0a-Cre*$^{+}$ *Nf1*-deficient mice and littermate controls into culture as described above. In each case, we sorted both unfractionated cells as well as p75^{+} cells into culture. p75^{+} cells from the gut of *P0a-Cre*$^{+}$ *Nf1*-deficient mice and littermate controls formed multipotent neurospheres in culture (Figure 3G). However, no neurospheres formed in culture from any other region

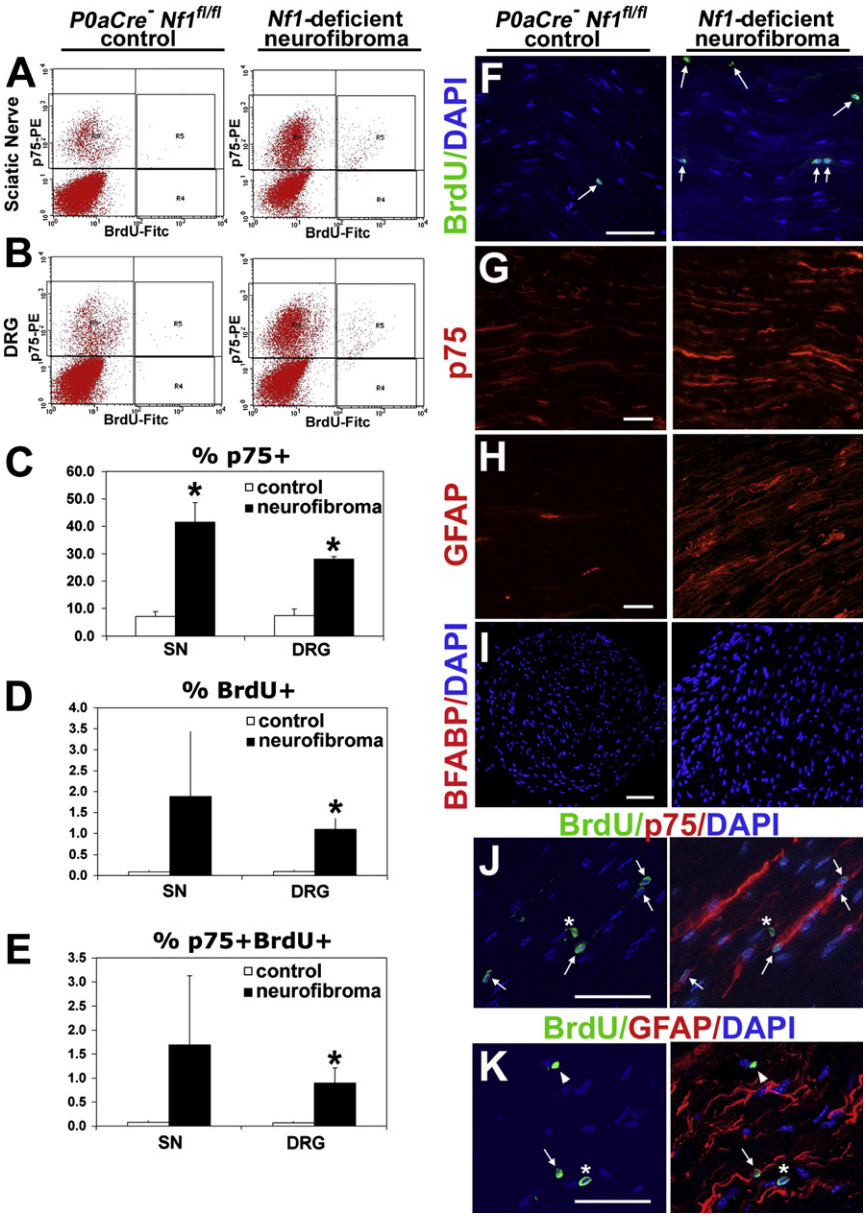


Figure 4. Proliferating Cells within Plexiform Neurofibromas Were p75+ and Expressed Markers of Nonmyelinating Schwann Cells

Sciatic nerves (A, C–E, and F–K) and DRGs (B–E) were dissected from adult *P0aCre*$^{+}$ *Nf1*-deficient mice with plexiform neurofibromas and littermate controls. Mice were administered BrdU for 4 days prior to being sacrificed. By flow-cytometry, neurofibromas contained significantly higher frequencies of p75+ cells (A–C), BrdU+ cells (A, B, and D), and p75+BrdU+ cells (A, B, and E). The vast majority of dividing cells within neurofibromas were p75+ (upper right quadrant of plots in [A] and [B]). Immunofluorescence in sections confirmed the increased frequency of BrdU+ cells ([F]; arrows), p75+ cells (G), and GFAP+ cells (H) in neurofibromas (right column) as compared to control nerves (left column). Normal nerves and neurofibromas lacked BFABP staining (I). Most BrdU+ cells costained with p75 (J), and many costained with GFAP (K), markers of nonmyelinating Schwann cells. In (J) and (K), double-positive cells are indicated with arrows, while possible double-positive cells are indicated with asterisks, and cells that are only BrdU positive are indicated with arrowheads. Scale bars in (F)–(K) equal 50 μm. Error bars represent SD.

of the PNS. These results demonstrate that we were able to culture adult NCSCs from the guts of these mice, but that NCSCs did not persist postnatally in regions of the PNS affected by neurofibromas.

NCSCs Appear to Differentiate Normally in the Nerves of Mice that Develop Neurofibromas

We fate-mapped neural crest progenitors in developing nerves by generating *P0a-Cre*$^{+}$*Nf1*$^{fl/-}$*R26R*$^{+}$ mice and littermate controls. In these mice, the fate of *P0a-Cre* expressing progenitors can be followed based on LacZ expression (R26R). Sciatic nerves were dissected from postnatal day 20 mice of each genotype, stained with bluo-gal, and analyzed by electron microscopy (Joseph et al., 2004). We detected no gross abnormalities in the size, composition, or histology of these postnatal nerves from *P0a-Cre*$^{+}$*Nf1*$^{fl/-}$*R26R*$^{+}$ mice as compared to littermate controls. Bluo-gal+ endoneurial fibroblasts as well as myelinating and nonmyelinating Schwann cells arose with similar frequencies from *P0a-Cre*$^{+}$*Nf1*$^{fl/-}$*R26R* mice as compared to *P0a-Cre*$^{+}$*Nf1*$^{fl/+}$*R26R* controls (Figures 3H–3N). These data suggest that *Nf1*-deficient neural crest progenitors acquired appropriate fates in developing peripheral nerves. Consistent with this, the only abnormality in *P0a-Cre*$^{+}$*Nf1*$^{fl/-}$ nerve development detected in a companion paper was the presence of rare nonmyelinating Schwann cells that were associated with abnormal numbers of axons (Zheng et al., 2008). These data suggest that *Nf1*-deficiency did not grossly alter nerve development or the differentiated fates of neural crest progenitors.

Nonmyelinating Schwann Cells Proliferate within Plexiform Neurofibromas

To address what cells were proliferating within the neurofibromas that arose after *Nf1* deletion with *P0a-Cre*, we analyzed the neurofibromas by flow-cytometry and immunohistochemistry. We observed a significant increase (4.0- to 6.4-fold; $p < 0.01$) in the frequency of p75+ cells within the plexiform neurofibromas in sciatic nerves and DRGs as compared to normal nerves and DRGs from littermate controls (Figures 4A–4C). By administering BrdU to mice for 4 days before analysis, we also found that these p75+ cells were much more likely to be dividing in neurofibromas as compared to normal tissue from littermate

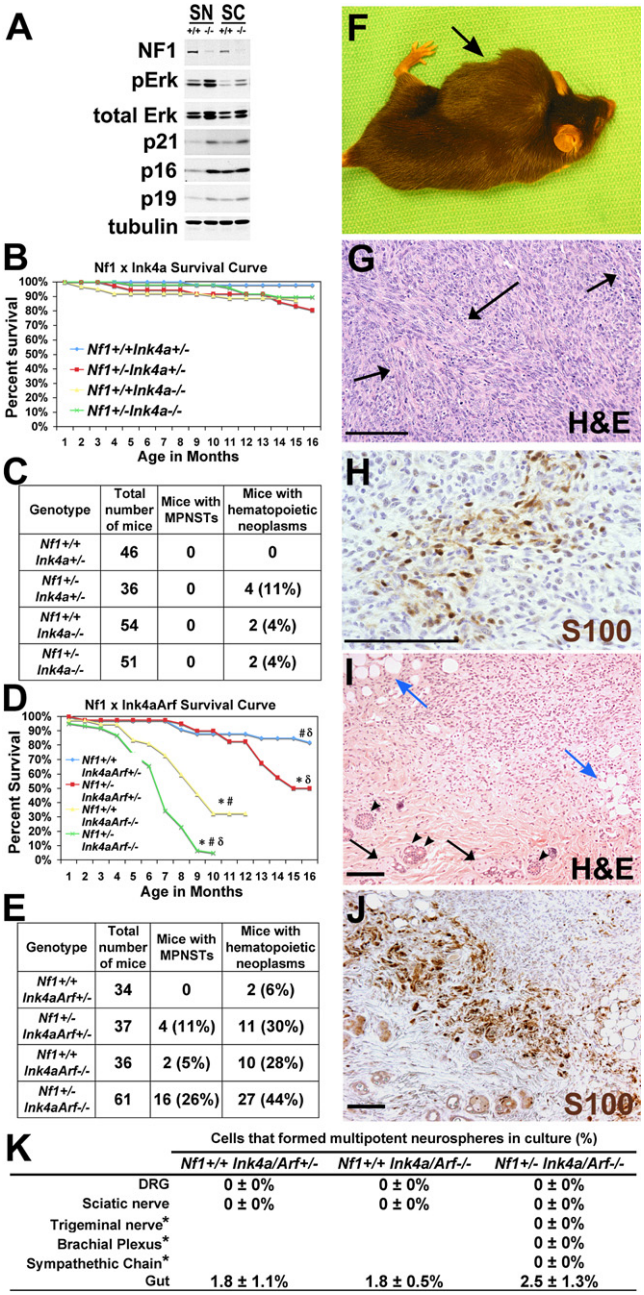


Figure 5. *Ink4a/Arf* Deficiency, but Not *Ink4a* Deficiency, Collaborates with *Nf1* Mutations to Generate MPNSTs without the Postnatal Persistence of NCSCs

(A) $Nf1^{+/+}$ and $Nf1^{-/-}$ neural crest cells cultured from sciatic nerve (SN) and sympathetic chain (SC) were analyzed by western blot. (B) *Ink4a* deficiency did not significantly affect the survival of $Nf1^{+/-}$ mice. (C) We detected no MPNSTs among $Nf1^{+/-}Ink4a^{-/-}$ mice or littermate controls. (D) The lifespan of $Nf1^{+/-}$ mice was significantly ($p < 0.05$) decreased by *Ink4a/Arf* deficiency as compared to littermate controls (* versus $Nf1^{+/+}Ink4aArf^{+/-}$; # versus $Nf1^{+/-}Ink4aArf^{+/-}$; δ versus $Nf1^{+/+}Ink4aArf^{-/-}$). Each line in (B) and (D) represents a cohort of 34–61 mice per genotype (see numbers in [C] and [E]). $Nf1^{+/-}Ink4a/Arf^{-/-}$ mice developed MPNSTs (E–J) as early as P30 but more commonly after 4–6 months of age. Tumors were typically large ([F], arrow). Paraffin sections exhibited classic features of MPNSTs including fascicular patterns of tightly packed spindle cells ([G], arrows) with hyperchromatic nuclei and frequent mitoses (G). These tumors contained $S100^{+}$ cells (H), a marker used in the diagnosis of MPNSTs. Some tumors were found embedded in the dermis (I and J) surrounding fat cells ([I], blue arrows), sebaceous glands ([I], arrows), and hair follicles ([I], arrowheads). Dermal tumors stained more intensely for S100 (J) than MPNSTs outside of the dermis (H). Scale bars in (G)–(J) equal 100 μm. (K) Multipotent neurospheres consistently arose from $Nf1^{+/-}$ *Ink4a/Arf* $^{-/-}$ and control guts, but not from other regions of the adult PNS (*these tissues were analyzed relative to age-matched wild-type controls). Statistics represent mean ± SD for 3–6 independent experiments.

controls (Figures 4A, 4B, 4D, and 4E). Indeed, virtually all of the dividing cells (BrdU+ cells) within neurofibromas were p75+ (Figures 4A and 4B). Since we could not culture NCSCs from the nerves or DRGs of these adult mice, these data suggest that nonmyelinating Schwann cells were proliferating within neurofibromas, as nonmyelinating Schwann cells also express p75 (Jessen et al., 1990).

To test whether the proliferating p75+ cells within plexiform neurofibromas expressed other markers of nonmyelinating Schwann cells, we stained tumor sections with antibodies against BrdU, p75, GFAP, and BFABP. We observed increased numbers of BrdU+ cells (Figure 4F), p75+ cells (Figure 4G), and p75+BrdU+ cells (Figure 4J) in neurofibromas as compared to normal nerves, just as we had observed by flow cytometry (Figures 4A–4E). We also observed greatly increased numbers of GFAP+ cells in neurofibromas (Figure 4H) and at least some of these GFAP+ cells also appeared to be BrdU+ (Figure 4K), though this was difficult to assess because GFAP is expressed mainly in cell processes while BrdU is nuclear. Since GFAP is expressed by nonmyelinating Schwann cells, but not by primitive glial progenitors in E13 fetal nerves (which include NCSCs and restricted Schwann cell precursors) (Jessen and Mirsky, 2005; Jessen et al., 1990; Morrison et al., 1999), these data suggest that GFAP+ nonmyelinating Schwann cells contribute to the growth of neurofibromas. Neither normal nerves nor neurofibromas exhibited detectable BFABP (also known as BLBP or Fabp7) staining (Figure 4I). Primitive glial progenitors in embryonic nerves express BFABP, but adult nonmyelinating Schwann cells do not (Britsch et al., 2001; Jessen and Mirsky, 2005). The presence of large numbers of p75+, GFAP+, and BFABP− cells in neurofibromas, at least some of which are dividing, suggests that nonmyelinating Schwann cells contribute to the growth of neurofibromas.

Nf1 and *p53* Mutations Lead to MPNSTs but Not to the Postnatal Persistence of NCSCs

$Nf1/p53^{+/-}$ mice formed MPNSTs by 6 months of age (Figures S4A–S4C) as previously reported (Cichowski et al., 1999; Vogel et al., 1999). To test whether these mice maintained postnatal NCSCs, we cultured dissociated cells from DRG, sciatic nerve, trigeminal nerve, sympathetic chain, and gut of adult $Nf1/p53^{+/-}$ mice. Gut cells from $Nf1/p53^{+/-}$ mice and littermate controls formed NCSC colonies in culture, but *Nf1/p53* heterozygosity did not affect the frequency or growth of these cells (Figure S4D). We did not detect cells from the DRG, sciatic nerve, trigeminal nerve, or sympathetic chain of $Nf1/p53^{+/-}$ mice or littermate controls that could form NCSC colonies (Figure S4D). *Nf1* and *p53* mutations did not affect the ability of fetal or adult NCSCs to form colonies in culture or to undergo multilineage differentiation (Figure S5A and S5E), so these mutations did not prevent us from

detecting NCSCs in culture. *Cis* deletion of *Nf1* and *p53* leads to the formation of MPNSTs during adulthood without promoting the postnatal persistence of NCSCs, suggesting that MPNSTs do not arise from NCSCs.

We also examined DRGs, sciatic nerves, trigeminal nerves, brachial plexi, and sympathetic ganglia from 3- to 6-month-old *Nf1/p53*$^{+/-}$ mice prior to the formation of MPNSTs to assess whether there were any abnormalities in PNS development. Except for a single *Nf1/p53*$^{+/-}$ brachial plexus that exhibited hyperproliferation, we were unable to distinguish *Nf1/p53*$^{+/-}$ tissues from wild-type tissues by histology or marker expression (Figure S6). We also did not detect any increase in the frequency of p75+ cells in adult *Nf1/p53*$^{+/-}$ tissues prior to the development of MPNSTs (Figure S6S). The failure to detect significantly increased numbers of p75+ cells in adult *Nf1/p53*$^{+/-}$ tissues prior to the development of tumors supports the conclusion that these tumors do not arise from the postnatal persistence of expanded populations of NCSCs.

Ink4a and *Arf* Deficiency Cooperate with *Nf1* Heterozygosity to Yield MPNSTs

To test whether the postnatal persistence of NCSCs might be inhibited by induction of p16^{Ink4a} or p19^{Arf} expression in *Nf1* mutant NCSCs we performed western blots on cultured NCSCs from the sciatic nerve and sympathetic chain. In both cases, we observed increased p19^{Arf} expression by *Nf1*$^{-/-}$ cells (Figure 5A). We also observed an increase in p16^{Ink4a} expression by *Nf1*$^{-/-}$ sciatic nerve cells, though the effect on p16^{Ink4a} expression in sympathetic chain cells was not as clear. Consistent with the known role for *p53* mutations in the formation of MPNSTs, we also observed a consistent increase in p21^{Cip1} expression by *Nf1*$^{-/-}$ NCSCs (Figure 5A). These observations raised the possibility that increased p16^{Ink4a} and p19^{Arf} expression by *Nf1*$^{-/-}$ NCSCs might retard the formation of MPNSTs.

To test this, we first generated mice bearing mutations in *Nf1* and *Ink4a* (leaving *Arf* intact). We aged cohorts of *Nf1*$^{+/-}$*Ink4a*$^{-/-}$ mice as well as various genotypes of littermate controls for up to 16 months. Overall mortality was low (Figure 5B). We never detected any MPNSTs or neurofibromas in these mice, though we did observe some hematopoietic neoplasms, particularly lymphoma (Figure 5C). The lack of grossly evident PNS tumors in these mice suggested that *Ink4a* deletion is not sufficient for tumorigenesis in an *Nf1* heterozygous background. *Nf1*$^{+/-}$ mice were previously generated on an *Arf*-deficient background and also did not develop neurofibromas or MPNSTs (King et al., 2002). These observations are consistent with genetic analyses of MPNSTs in patients, which usually exhibit loss-of-function mutations in both the Rb and p53 pathways (Agesen et al., 2005; Perrone et al., 2003).

To develop a mouse model that more faithfully recapitulated the mutations observed in human MPNSTs, we generated mice bearing mutations in *Nf1* and *Ink4a/Arf* (lacking both *Ink4a* and *Arf*). We aged cohorts of *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice as well as various genotypes of littermate controls for up to 16 months. Virtually all *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice died by 10 months of age, more quickly than littermates with other genotypes (Figure 5D). *Nf1*$^{+/+}$ *Ink4a/Arf*$^{+/-}$ control mice failed to develop MPNSTs or neurofibromas, but 26% of *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice had to be euthanized due to the formation of MPNSTs (Figures 5E and 5F). Most of the MPNSTs developed by *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice became grossly evident at 4 to 6 months of age. These MPNSTs tended to develop on the shoulders, ribs, or legs of mice, close to DRGs and peripheral nerves. Much lower rates of MPNSTs were observed among *Nf1*$^{+/-}$*Ink4a/Arf*$^{+/-}$ mice (Figure 5E), and only after 1 year of age. These statistics may underestimate the true frequency of MPNSTs, as smaller tumors may have gone undetected in mice that died due to other causes.

Histology of the MPNSTs revealed typical features including fascicular patterns of tightly packed spindle cells with hyperchromatic nuclei and frequent mitoses (Figure 5G) as well as S100 staining (Figure 5H). Five of the MPNSTs observed in *Nf1*$^{+/-}$ *Ink4a/Arf*$^{-/-}$ mice were confined to the dermis and epidermis (Figure 5I), were much smaller than the more typical tumors imaged in Figure 5F, and stained more intensely for S100 (Figure 5J). These tumors may actually be dermal neurofibromas, though they were characterized as MPNSTs because the frequent mitotic figures and invasiveness were more consistent with a malignancy. These data demonstrate that *Ink4a/Arf* deficiency leads to the formation of MPNSTs in an *Nf1*$^{+/-}$ background.

In addition to forming MPNSTs, we observed a significant frequency of hematopoietic neoplasms among *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$, *Nf1*$^{+/-}$*Ink4a/Arf*$^{+/-}$, and *Nf1*$^{+/+}$*Ink4a/Arf*$^{-/-}$ mice (Figure 5E). These included mainly lymphomas and histiocytic neoplasms, but we observed some acute myeloid leukemias as well as some mice with myeloproliferative disease.

NCSCs Did Not Persist Postnatally in *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ Mice

To test whether *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice maintained postnatal NCSCs, we cultured dissociated cells from DRG, sciatic nerve, trigeminal nerve, brachial plexus, sympathetic chain, and gut of adult *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice and littermate controls. Gut cells from *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice and controls formed multilineage NCSC colonies in culture, but we did not detect a significantly increased frequency of NCSCs in *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice (Figure 5K). *Nf1* deficiency also did not affect the size of gut NCSC colonies (data not shown). We did not detect cells from any other region of the PNS of *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice or littermate controls that could form multipotent neurospheres (Figure 5K). *Nf1* and *Ink4a/Arf* mutations did not affect the ability of fetal or adult NCSCs to form colonies in culture or to undergo multilineage differentiation (Figures S5B and S5F), so these mutations did not prevent us from detecting NCSCs in culture.

We also examined DRGs, sciatic nerves, trigeminal nerves, and sympathetic ganglia from 3- to 6-month-old *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice prior to the formation of MPNSTs to assess PNS development. We were unable to distinguish *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ tissues from wild-type tissues by either histology or marker expression and did not detect a significant increase in the frequency of p75+ cells in adult *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ tissues prior to the development of MPNSTs (Figure S6). The failure to detect significantly increased numbers of p75+ cells in adult *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ tissues prior to the development of tumors supports the conclusion that these tumors do not arise from the postnatal persistence of expanded populations of NCSCs.

Clonogenic MPNST Cells Did Not Resemble NCSCs

To test whether clonogenic MPNST cells resembled NCSCs, tumors from *Nf1*$^{+/-}$*Ink4aArf*$^{-/-}$ mice or *Nf1/p53*$^{+/-}$ mice were

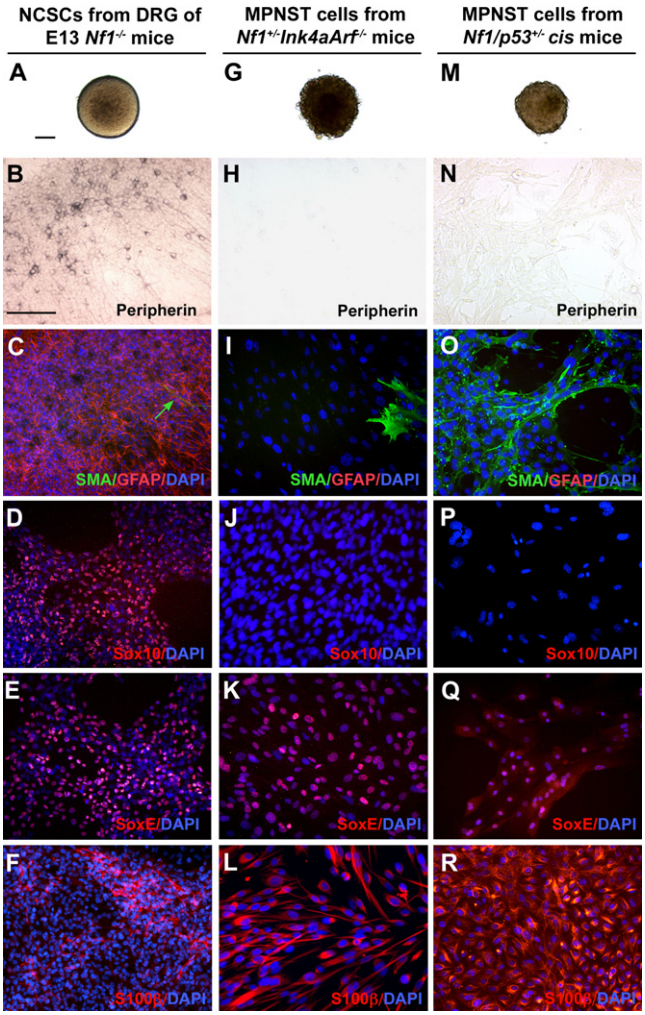


Figure 6. Self-Renewing Spheres Grew from *Nf1*$^{+/-}$*Ink4a/Arf* $^{-/-}$ and *Nf1/p53*$^{+/-}$ MPNSTs but Did Not Form Colonies that Resembled NCSCs Colonies

Some MPNST cells from *Nf1*$^{+/-}$*Ink4aArf*$^{-/-}$ mice and *Nf1/p53*$^{+/-}$ mice formed self-renewing spheres in culture. While all NCSCs generated peripherin^{+} neurons (B), GFAP^{+} glia (C), and SMA^{+} myofibroblasts ([C]; arrow), MPNST colonies from *Nf1*$^{+/-}$*Ink4aArf*$^{-/-}$ and *Nf1/p53*$^{+/-}$ mice failed to generate peripherin^{+} neurons (H and N) or GFAP^{+} glia (I and O). In contrast to *Nf1*$^{-/-}$ NCSC colonies (D), MPNST colonies exhibited little or no Sox10 staining (J and P). MPNST colonies typically contained cells with a glial morphology (data not shown) as well as SMA^{+} myofibroblasts (I and O). Like *Nf1*$^{-/-}$ NCSC colonies (E and F), most cells within MPNST colonies were SoxE^{+} (K and Q) and S100β^{+} (L and R). These represent typical colonies from six independent *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ MPNSTs and four independent *Nf1/p53*$^{+/-}$ MPNSTs. The scale bar (100 μm) in (A) applies to (A), (G), and (M) while the scale bar (100 μm) in (B) applies to all other panels.

dissociated and cultured in the conditions we use for NCSCs. Some *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ MPNST cells and *Nf1/p53*$^{+/-}$ MPNST cells formed spheres in culture (Figures 6G and 6M). These sphere-forming cells had self-renewal potential, giving rise to an average of 164 ± 133 and 104 ± 21 daughter spheres, respectively, when subcloned after 10 to 12 days of primary culture. These sphere-forming cells were unlikely to arise from normal cells in the tumors because we were unable to culture sphere-forming cells from normal adult nerves in control mice (Figure 5K). All *Nf1*$^{-/-}$ (Figures 6A–6F) and wild-type (Figures 2C and 2F) NCSC colonies formed peripherin^{+} neurons (Figure 6B), smooth muscle actin^{+} (SMA^{+}) myofibroblasts (Figure 6C), and GFAP^{+} glia (Figure 6C). Moreover, E13 *Nf1*$^{-/-}$*Ink4a/Arf*$^{-/-}$ and *Nf1/p53*$^{-/-}$ NCSCs also underwent multilineage differentiation, as did adult gut *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ and *Nf1/p53*$^{+/-}$ NCSCs (Figure S5). These data demonstrate that *Nf1* deficiency, with or without additional mutations in *p53* or *Ink4a/Arf*, does not alter the ability of NCSCs to undergo multilineage differentiation. In contrast, the *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ MPNST and *Nf1/p53*$^{+/-}$ MPNST colonies in the same culture conditions rarely generated peripherin^{+} neurons and never GFAP^{+} glia (Figure 6). Clonogenic MPNST cells fail to undergo multilineage differentiation characteristic of NCSCs.

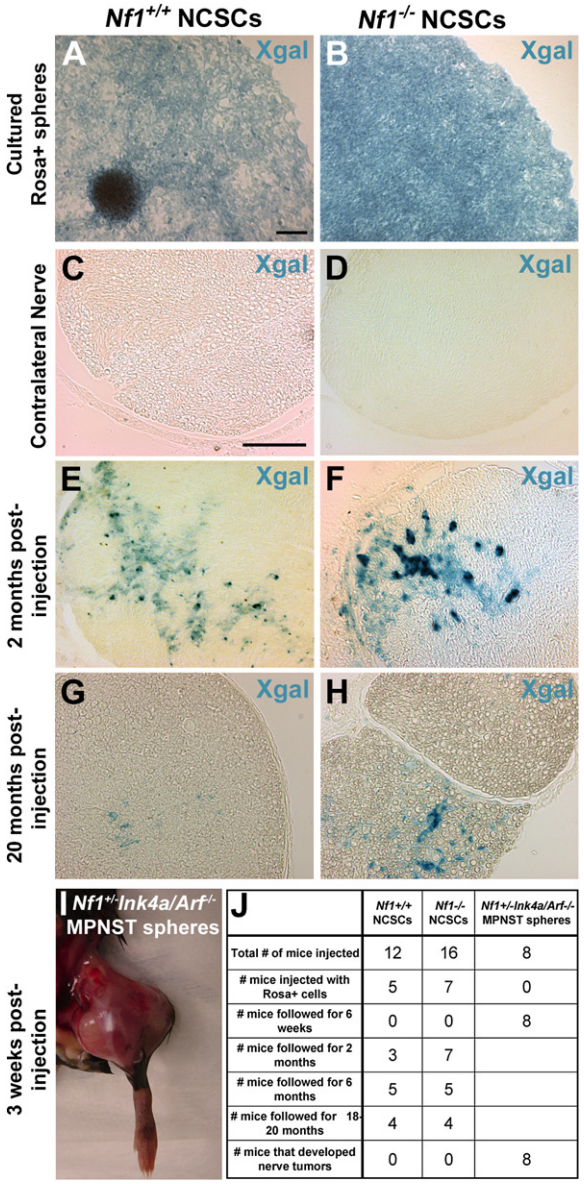
While cells within NCSC colonies were Sox10^{+} as expected (Kim et al., 2003), MPNST cells stained weakly or not at all for Sox10, as previously reported (Levy et al., 2004; Miller et al., 2006) (Figure 6). Apart from the lack of GFAP staining, most cells in MPNST colonies otherwise resembled glia, as they were small, spindle-shaped cells that stained positively for SoxE (Sox8/9/10) and S100β (Figure 6). Increased Sox9 expression is typical of MPNSTs, and a subset of MPNSTs express S100β (Miller et al., 2006; Takeuchi and Uchigome, 2001). MPNST cells thus formed colonies that consistently differed from wild-type (Figures 2C and 2F), *Nf1*$^{-/-}$ (Figures 6A–6F), *Nf1*$^{-/-}$*Ink4aArf*$^{-/-}$ (Figure S5B), and *Nf1/p53*$^{-/-}$ (Figure S5A) NCSCs, though these cells did resemble Schwann cells based on morphology and expression of S100β and SoxE.

Nf1-Deficient NCSCs Are Not Tumorigenic

To test whether *Nf1*-deficient NCSCs could form tumors, we cultured NCSCs from E13 *Nf1*$^{+/+}$ or *Nf1*$^{-/-}$ mice or MPNST cells from *Nf1*$^{+/-}$*Ink4a/Arf*$^{-/-}$ mice, then transplanted these cells into the sciatic nerves of adult *Nf1*$^{+/-}$ mice. In some experiments, the *Nf1* mutant allele was bred onto a Rosa background so that we could monitor the engraftment of the transplanted cells. All mice (8/8) injected with 50,000 MPNST cells developed large tumors within 2 to 6 weeks of injection (Figures 7I and 7J). None of 12 mice injected with 50,000 to 100,000 *Nf1*$^{+/+}$ cells or 16 mice injected with 50,000 to 100,000 *Nf1*$^{-/-}$ cells developed tumors despite being monitored for up to 20 months after injection (Figure 7J). The *Nf1*$^{+/+}$ and *Nf1*$^{-/-}$ cells did engraft within nerves as indicated by the presence of LacZ expressing cells (Figures 7E–7H). However, these LacZ+ donor cells never formed tumors (Figures 7G and 7H). Together, our data indicate that NCSCs are not rendered tumorigenic by *Nf1* deficiency, but rather infrequent differentiated glia (such as nonmyelinating Schwann cells) begin proliferating inappropriately and form tumors in adults.

DISCUSSION

If NCSCs give rise to plexiform neurofibromas and MPNSTs, then *Nf1*-deficient NCSCs would be expected to persist in expanded numbers throughout late gestation and into the postnatal period. However, we did not detect the postnatal persistence of NCSCs in DRG, sympathetic chain, trigeminal ganglion, brachial plexus, or peripheral nerve of conditional *Nf1*-deficient mice (Table 1), even in mice that went on to develop plexiform neurofibromas (Figure 3G). The expansion of *Nf1*-deficient



J	Nf1+/+ NCSCs	Nf1-/- NCSCs	Nf1+/-Ink4a/Arf-/- MPNST spheres
Total # of mice injected	12	16	8
# mice injected with Rosa+ cells	5	7	0
# mice followed for 6 weeks	0	0	8
# mice followed for 2 months	3	7	
# mice followed for 6 months	5	5	
# mice followed for 18-20 months	4	4	
# mice that developed nerve tumors	0	0	8

Figure 7. *Nf1*-Deficient NCSCs Are Not Tumorigenic In Vivo

We transplanted 50,000 MPNST cells or 50,000–100,000 $Nf1^{+/+}$ or $Nf1^{-/-}$ NCSCs into the nerves of adult $Nf1^{+/-}$ mice. NCSCs were grown as spheres from various regions of the E13 PNS of $Nf1^{+/+}$ and $Nf1^{-/-}$ mice that had been bred to Rosa mice in some experiments to allow the tracking of cells with Xgal staining (A and B). NCSC spheres were replated to adherent cultures, dissociated to single cell suspensions, and injected into the sciatic nerves of adult $Nf1^{+/-}$ mice. Contralateral nerves never showed X-gal+ cells (C and D) but nerves injected with Rosa+ cells consistently showed engraftment (E–H). MPNST spheres from $Nf1^{+/-}Ink4aArf^{-/-}$ mice were also dissociated and injected into the sciatic nerves of adult $Nf1^{+/-}$ mice (I). Tumors were never observed in mice injected with $Nf1^{+/+}$ or $Nf1^{-/-}$ neural crest cells, even after 20 months follow-up (E–J). MPNST cells gave rise to large tumors (I) in all recipients within 2–6 weeks after injection (J). The scale bar (100 μm) in (A) applies to (A) and (B) and the scale bar (100 μm) in (C) applies to (C)–(H).

NCSCs occurred only transiently during midgestation. Like wild-type NCSCs, *Nf1*-deficient NCSCs became increasingly rare in late gestation and failed to persist postnatally, precluding them from participating in tumorigenesis (Figure S3). NCSCs also did not persist postnatally in $Nf1^{+/-}Ink4aArf^{-/-}$ or $Nf1/p53^{+/-}$ mice (Figure 5K; Figure S4), despite the fact that they went on to develop MPNSTs as adults (Figure 5; Figure S4). Since NCSCs did not persist postnatally in regions of the PNS that formed plexiform neurofibromas or MPNSTs during adulthood, NCSCs could not have given rise to these tumors.

Our inability to detect *Nf1*-deficient NCSCs postnatally in regions of the PNS that developed tumors did not simply reflect altered differentiation or survival of these cells in culture. In all experiments, we were able to detect the postnatal persistence of both wild-type and *Nf1*-deficient NCSCs from the adult gut. This positive control demonstrated that we were able to culture adult NCSCs from each of the genetic backgrounds we studied. Moreover, *Nf1* deficiency increased the survival and proliferation of NCSCs in culture, making them easier, not more difficult, to grow (Figure 2). Furthermore, *Nf1* deficiency, with or without *p53* or *Ink4a/Arf* deficiency, did not alter the ability of NCSCs to undergo multilineage differentiation (Figure 2; Figure 6; Figure S1; Figure S5).

Nf1-deficient NCSCs appeared to differentiate normally in peripheral nerves during late gestation (Figure 3). We did not detect any gross alterations in nerve development in P20 *Nf1* mutant mice by electron microscopy (Figure 3). Rather, hyperplasia and increased frequencies of p75+ cells were not observed in $P0a\text{-}Cre^{+}Nf1^{fl/-}$ mice until around 3 months of age (Zheng et al., 2008). We also did not detect any gross alterations in PNS development or a significant increase in the frequency of p75+ cells in PNS tissues from $Nf1^{+/-}Ink4aArf^{-/-}$ or $Nf1/p53^{+/-}$ mice during early adulthood prior to the formation of tumors (Figure S6). When combined with the observation that *Nf1*-deficient NCSCs were lost from the late gestation PNS according to a similar time course as wild-type NCSCs (Figure S3), and the observation that *Nf1*-deficient NCSCs failed to form tumors after transplantation into adult $Nf1^{+/-}$ peripheral nerves (Figure 7), our data suggest that *Nf1*-deficient NCSCs terminally differentiate during late gestation and are long gone by the time tumors arise in the adult PNS.

Our data instead suggest that infrequent differentiated glia, such as nonmyelinating Schwann cells within peripheral nerves, begin proliferating inappropriately in the postnatal period and give rise to plexiform neurofibromas. The dividing cells within plexiform neurofibromas were almost exclusively p75+ (Figures 4A, 4B, and 4J). Many of these cells had a phenotype similar to nonmyelinating Schwann cells (and dissimilar to fetal NCSCs) as they were also GFAP+ and BFABP− (Figure 4). Proliferating cells within MPNSTs also expressed some differentiated glial markers as well as having a glial morphology (Figure 6).

Why would deletion of *Nf1* in fetal nerve progenitors lead to the formation of tumors that do not become evident until adulthood in mice (Zhu et al., 2002)? A cosubmitted manuscript by Zheng and colleagues concludes that abnormal differentiation of some nonmyelinating Schwann cells in the absence of *Nf1* leads to their association with unusually large numbers of axons (Zheng et al., 2008). These bundles degenerate postnatally, leading to inflammation that precedes Schwann cell hyperproliferation and the formation of plexiform neurofibromas. These observations suggest that *Nf1*-deficient Schwann cells differentiate perinatally and do not become hyperproliferative until early adulthood when their behavior is modified by epigenetic (i.e., inflammation,

hormones, and nerve damage) or genetic (i.e., secondary mutations) triggers.

Our results do not address the origin of dermal neurofibromas. Typical benign dermal neurofibromas did not arise in any of the mice we studied. Neural progenitors have been cultured from adult dermis and at least some of these are neural crest derived (Fernandes et al., 2004; Wong et al., 2006). While these cells express markers similar to NCSCs, the progenitors cultured from trunk skin have little capacity to make neurons (Wong et al., 2006), in contrast to the NCSC populations we have characterized (Bixby et al., 2002; Kruger et al., 2002; Morrison et al., 1999). More work will be required to identify the in vivo cells that give rise to the dermally derived neural progenitors. Nonetheless, this is a different neural progenitor population, in a different location, than the NCSC populations that we characterized in this study.

It is interesting that NF1 negatively regulates the frequency, self-renewal, growth factor sensitivity, and gliogenesis of NCSCs in most regions of the PNS but not in the gut. The basis for this regional difference in NF1 function is not clear. Nonetheless, these results are consistent with the observation that neurofibromatosis patients seem more likely to develop tumors from peripheral nerves, DRGs, and sympathetic ganglia than from the gut (Fuller and Williams, 1991). The failure of gut neural crest progenitors to exhibit increased proliferation or gliogenesis after *Nf1* deletion may partly explain this clinical observation.

While many cancers may arise from the transformation of stem cells, our results indicate that NCSCs are not rendered tumorigenic by mutations in *Nf1*. Benign tumors and cancers of the PNS can arise from differentiated glia.

EXPERIMENTAL PROCEDURES

All experiments using mice were performed in accordance with approved protocols by the University Committee on the Use and Care of Research Animals (UCUCA). Several *Nf1* alleles were used in these studies including $Nf1^{-}$ (germline mutant) mice (Jacks et al., 1994), $Nf1^{fl}$ mice for conditional deletion (Zhu et al., 2001), and $Nf1/p53^{-}$ *cis* mutant mice (Cichowski et al., 1999). For conditional deletion of *Nf1* we used *Wnt-1-Cre*$^{+}$ (Danielian et al., 1998), *3.9Periostin-Cre*$^{+}$ (Lindsley et al., 2007), and *P0a-Cre*$^{+}$ (Giovannini et al., 2000) mice. Compound mutant mice were generated by mating $Nf1^{+/-}$ mice with $Ink4a^{-/-}$ mice (Sharpless et al., 2001) or $Ink4aArf^{-/-}$ mice (Serrano et al., 1996).

Isolation of NCSCs

Timed pregnant matings of $Nf1^{+/-}$ mice were set up to obtain E13 $Nf1^{-/-}$ embryos. DRGs (cervical, thoracic, and lumbar), sciatic nerve, sympathetic chain, and gut (including stomach, small intestine, and hindgut) were dissected from E12.5 to E13.5 $Nf1^{+/+}$, $Nf1^{+/-}$, and $Nf1^{-/-}$ littermates and collected in ice-cold Ca, Mg-free HBSS. Tissues were dissociated for 4 min at 37°C in 0.05% trypsin/EDTA (Invitrogen, Carlsbad, California; diluted 1:10 in Ca, Mg-free HBSS) with 0.25 mg/ml type IV collagenase (Worthington, Lakewood NJ) then quenched with staining medium: L15 medium containing 1 mg/ml BSA (Sigma A-3912, St. Louis, MO), 10 mM HEPES (pH 7.4), 1% penicillin/streptomycin (BioWhittaker, Walkersville, MD), and 25 mg/ml deoxyribonuclease type 1 (DNase1, Sigma D-4527). Cells were centrifuged, resuspended in staining medium without DNase, triturated, filtered through nylon screen (45 μm, Sefar America, Kansas City, MO) to remove aggregates, counted by hemocytometer, and added to culture. In some experiments, cell suspensions were stained with antibodies against p75 (Ab 1554; Chemicon) and α_4 integrin (Becton Dickinson, San Jose, CA) for analysis by flow-cytometry (Bixby et al., 2002). In some experiments, cells were stained for BrdU using a flow-cytometry kit (BD PharMingen; cat. no. 559619). Flow cytometry was performed with a FACSVantage SE-dual laser, three-line flow-cytometer (Becton Dickinson).

See Supplementary Data for details regarding dissociation of adult cells, genotyping, cell culture, immunohistochemistry, western blots, nerve injections, and other methods.

Supplemental Data

The Supplemental Data include six supplemental figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/2/129/DC1/.

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