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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Humans with mutations in the <i>NF1</i> gene develop benign peripheral nerve tumors comprised mainly of Schwann cells (neurofibromas) and hyperpigmented spots on the skin (CALM). Mice with mutations of the <i>Nf1</i> gene fail to develop CALM or neurofibromas. We cut the sciatic nerve of <i>Nf1/nf1</i> mice and induced frequent pigmented spots and rare tumors. We hypothesized that nerve lesion could create an environment triggering abnormal behavior of heterozygous cells including Schwann cells. We tested this hypothesis using nerve grafting. Our data shows mutant Schwann cells form pigment cells in the wound environment. In addition, using mutant mice we show that the environment suppresses pigmentation via the IL3/GMCSF shared receptor β^c ; this suppressive effect is lost in <i>Nf1</i> mutants. We also wounded <i>Nf1/nf1</i> mice using chemical carcinogenesis and obtained increased pigmentation and keratinocyte tumors, further substantiating our hypothesis that a wound environment can trigger features of human NF1 disease. Finally, using transgenic mice, we proposed testing whether Ras activation in peripheral nerve Schwann cells is necessary and/or sufficient to promote wound-associated phenotypes of <i>Nf1/nf1</i> mice. We identified a promoter that drives robust Schwann cell expression, cloned GRD and Ha-V12Ras into CNPase promoter-driven plasmids, identified founder mice and for Ras, confirmed overexpression and began phenotype analysis.					
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Introduction:

Our goal is to understand the development of benign manifestations of NF1, especially hyperpigmentation and neurofibromas. A link between wounding and both pigmentation and tumor formation was hypothesized for NF1 patients (Riccardi, 1990). Involvement of wounding or wound repair mechanisms in carcinogenesis may be prevalent (Dvorak, 1986; Martins-Green et al., 1994). Therefore, based on anecdotal evidence, we hypothesized that wounding mice with a single mutant *Nf1* allele might cause pigmentation and/or tumor formation. Indeed, transection of the sciatic nerve in mice with a single mutant *Nf1* allele led to consistent hyperpigmentation and rare tumor formation. A major goal of this grant was to clarify the mechanism(s) underlying these effects. We showed that glial cells within the nerve are sufficient to mimic this phenotype, and that we can explain the pigmentation by migration of glial cells from the nerve into the epineurium where they are exposed to melanogenic factors. In addition, using mutant mice we show that the environment suppresses pigmentation via the IL3/GM-CSF shared receptor β^c and this suppressive effect is lost in *Nf1* mutants. Thus the adult nerve glial cell phenotype is maintained after nerve injury by response to cytokines that act via β^c receptor, through neurofibromin. We have also manufactured new Ras-GTP expressing mice to test in the wounding assay, to begin to test if *Nf1* effects can be explained by Ras-GTP elevation only. We extended our studies on wounding *Nf1/nf1* mutant mice by exposing them to DMBA + TPA in a chemical carcinogenesis assay. We showed that *Nf1/nf1* mutant mice respond abnormally in this paradigm, demonstrating both benign tumor formation (papillomas) and increased pigmentation. We are convinced, based on these studies, that wounding is sufficient to promote features of human NF1 disease in *Nf1/nf1* mutant mice.

(6) Body:

STATEMENT OF WORK (REVISED AS DIRECTED FOLLOWING 1998 REORT)

Wounding-Induced Manifestations of Type 1 Neurofibromatosis

Task 1, Analyze *Nf1/nf1* mice after nerve lesion.

- a. Lesion nerves by crush, nerve cut or nerve cut with deflection and analyze pigmentation (complete)
- b. Crush nerves of adult mice and define abnormalities in proliferation (complete).
- c. Wound nerves of 30 neonatal mice and define abnormalities (completed).
- d. Paralyze 20 mice with ricin and cut nerves; analyze for pigmentation (completed).

Task 2, Develop nerve grafting experiments for assessment of contribution of specific cell types to the *Nf1/nf1* wound-induced phenotype.

- a. Carry out graft surgeries on recipient mice and complete analysis of phenotypes of grafted cells (completed).
- b. *In vitro* analysis of mutant cells for migratory potential (completed).
- c. *In vivo* analysis of contribution of a receptor tyrosine kinase to the wound-induced phenotype (completed).

Task 3, Test if another wounding paradigm, chemical carcinogen exposure, stimulates increased pigmentation or tumor formation in *Nf1/nf1* mice.

- a. Apply DMBA or DMBA and TPA to wild type and mutant mice (completed).
- b. Analyze resultant skin spots and tumors for Ras mutations (completed).
- c. Analyze skin for abnormal TPA response (completed).

Task 4, Develop transgenic mice with altered Ras-GTP levels in Schwann cells.

- a. Make and sequence v-Ha-*ras* and *GRD-1* and *GRD-2* constructs (completed).
- b. Test each construct in Schwann cells (completed) and inject each DNA into blastocysts (completed).
- c. Obtain founder animals; breed to obtain F₁ generation (completed).
- d. Characterize phenotype of three transgenic strains; breed *GRD-1* and *GRD-2* strains to *Nf1/nf1* mice (completed).
- e. Confirm overexpression and begin phenotypic analysis of 20 v-Ha-*ras* expressing mice (completed).
- f. Lesion nerves of *GRD-1* and 20 *GRD-2* -expressing mice and Ras double transgenics and complete analysis (in progress).

Tasks 1 & 2: Define steps in wound healing that precede and correlate with hyperpigmentation; develop nerve grafting experiments to assess the contribution of specific cell types to the *Nf1/nf1* wound-induced phenotype.

The neural crest gives rise to numerous cell types, including Schwann cells, neurons and melanocytes. The extent to which plasticity remains in adult neural crest-derived cells has not been previously tested. We report that cutting adult mouse sciatic nerve induces pigmentation around nerve fascicles, among muscle bundles and in the hypodermis. Unexpectedly, pigmented cells derive from within adult nerve, as even cells from nerve fragments grafted into tyrosinase null albino mice become pigmented. Pigmentation defects are pervasive in patients with neurofibromatosis type 1. Mice hemizygous for *Nf1* mutations show enhanced pigmentation after nerve lesion, and form rare pigmented and unpigmented tumors. Enhanced pigmentation results from the both grafted nerve and the host environment. Purified *Nf1* mutant glial cells [$S100^+$ - $p75^{NGFR^+}$ - $GFAP^+$ - $EGFR^+$ or $S100^+$ - $p75^{NGFR^+}$ - $GFAP^+$ - $EGFR^-$] mimic the nerve response and show enhanced migration and secretion of chemotactic factors. The NF1 protein, neurofibromin, is a Ras-GAP that acts downstream of a few defined receptor tyrosine kinases including the shared common receptor for GM-CSF, IL3 and IL5 (β^c). Using mutant mice we show that the environment suppresses pigmentation via β^c and this suppressive effect is lost in *Nf1* mutants. Thus the adult nerve glial cell phenotype is maintained after nerve injury by response to cytokines that act via the β^c receptor, through neurofibromin.

Introduction

Cells derived from the neural crest differentiate into numerous derivatives including pigment cells of the skin and iris called melanocytes, sensory and sympathetic neurons of the peripheral nervous system and peripheral nerve glial cells, called satellite cells and Schwann cells (LeDourain and Kalcheim, 1999). Studies suggest that the fates of neural crest cells, as with other embryonic cell types, become progressively more restricted as development proceeds. Neural crest cells that contribute to the dorsal root ganglion (DRG) cells normally develop into neurons, satellite cells or Schwann cells but not pigment cells. It is unclear to what extent developmental restrictions can be reversed, or whether small numbers of unrestricted cells persist in adult nerve.

Melanocytes and Schwann cells can arise from a partially restricted bipotential, Schwann cell-melanocyte precursor that is present transiently during development (Dupin et al., 2000). Pioneering studies showed that avian DRG cells and spinal nerve cells could be transiently stimulated to make pigment (Nichols et al., 1977a,b; Ciment et al., 1986; Nataf et al., 2000; Stocker et al., 1991). The development of pigmented cells in peripheral ganglion cultures might indicate the presence of a stem cell population. Embryonic day 14 rat peripheral nerves contain cells that can form neurons, glia, and smooth muscle/mesenchymal cells (Morrison et al., 1999), even though most neural crest cells make a transition from a precursor population to S100⁺O4⁺ Schwann cells at this time (Dong et al., 1995; 1999). However, differentiated melanocytes from embryo day 7 avian skin can lose melanocytic properties and exhibit glial markers (Dupin et al., 2000). This is believed to result from transdifferentiation, loss of differentiation properties followed by acquisition of markers specific to a different cell phenotype (see Eguchi and Kodama, 1993 for review). Neither

stem cells nor transdifferentiation of Schwann cells has been demonstrated in adult peripheral nerve.

Neurofibromatosis type 1 (NF1) patients develop neurofibromas, disfiguring peripheral nerve tumors containing mainly Schwann cells but sometimes also pigmented cells (Fetsch et al., 2000). These patients also exhibit extensive skin hyperpigmentation (Riccardi, 1992). *Nf1* is expressed in neural crest cells and Schwann cells (Daston et al., 1992; Daston and Ratner, 1992; Stocker et al., 1995). We hypothesized that if progenitor cells in NF1 patients were defective or increased in number, or if neural crest-derived cells were prone to transdifferentiation, then glial cells from *Nf1* mutant mice (Jacks et al., 1994; Brannan et al., 1994) might show an increased propensity to become pigmented cells.

The *NF1* gene product, neurofibromin is an essential Ras-GAP in certain cell types. Neurofibromin lies downstream of particular tyrosine kinase receptors (reviewed in Cichowski and Jacks, 2001). Ras-GTP levels, in response to particular cytokines and growth factors, are heightened in *Nf1* mutant cells (Vogel et al., 1995; Kim et al., 1995; Lakkis et al., 1999; Wehrle-Haller et al., 2001). In *Nf1* mutant haematopoietic cells response to GM-CSF and to IL3, which signal through a common β receptor known as β -common (β^c), is enhanced (Bollag et al., 1996; Largaespada et al., 1996; Zhang et al., 1998; Ingram et al., 2000; Birnbaum et al., 2000). We hypothesized that neurofibromin-dependent changes in the GM-CSF and IL3 signaling pathway might be relevant to peripheral nerve cells, because these two cytokines are up-regulated following nerve lesion (Saada et al., 1996).

Injury to adult peripheral nerve causes loss of differentiated Schwann cell phenotypes (reviewed in Fawcett and Keynes, 1990; Scherer, 1997). We used sciatic nerve injury to test whether peripheral nerve cells can differentiate along alternative pathways, because wounding partially re-capitulates the developmental cytokine environment (e.g. Grotendorst,

1992). We demonstrate that wounding causes pigmentation of nerve derived glial cells. We identify a novel molecular pathway involving the β^c receptor, acting via *Nf1*, that normally suppresses melanogenesis after injury in adult mice.

Materials and Methods

Mouse strains: Male wild type and *Nf1* heterozygous (+/-) mice backcrossed onto the C57Bl/6 background; they were derived and genotyped as described (Brannan et al., 1994). Mice null for *Nf1* die in utero. Mice null for ϵ were on the C57Bl/6 background and were genotyped as described (Nishinakamura et al., 1995). In some experiments albino C57Bl/6 mice with a spontaneous mutation in tyrosinase C57Bl/6J-TYR^{C-2J} were used (Jackson Labs).

Morphology of the embryonic and adult sciatic nerve: Pregnant mice at day 18 - 20 of gestation were anaesthetized by inhalation of metafane, embryos removed and transcardially perfused for 5 minutes with EM fixative. Mice at postnatal day 0 (P0), P1, P3, P7 and P30 were anaesthetized with sodium pentobarbital and perfused, as were 10-month-old mice. Sciatic nerves were dissected, post fixed and processed for semithin sections and EM; (n=3 each age group/genotype) were evaluated.

Nerve wounding: Wild type and *Nf1/nf1* mice (3 - 4 months old) were anaesthetized with avertin (0.5-0.8 ml i.p.). Skin overlying the sciatic nerve was cut, muscles parted and sciatic nerve exposed. After surgeries, muscles were realigned and the skin closed with surgical staples. Animals were allowed to survive 7 days to one year after nerve surgery, then anaesthetized with sodium pentobarbital and perfused with 4% paraformaldehyde. Sciatic nerve, skin or muscle were dissected, post fixed and processed for paraffin embedding and light microscopy, postfixed in glualdehyde and processed for electron microscopy or cryopreserved and sectioned. Nerve crush: Sciatic nerve 2 mm medial from the sciatic notch was crushed by mechanical pressure three times with a sharp pair of #5 Dumont tweezers. Animals were evaluated after 6 (n = 9/genotype) or 10 weeks (n = 9/genotype) post crush. Nerve Transection: The sciatic nerve was cut 2 mm medial to the sciatic notch and cut ends of the nerve sutured together using prolene sutures (7-0). Animals were allowed to survive for 1 (n = 48/genotype), 3 (n = 22/genotype) or 6 months (n = 6/genotype) or 1 year (n=4/genotype) post surgery. Nerve Transection and Deflection: The sciatic nerve was cut 2 mm medial to the sciatic

notch and cut ends deflected to opposite sides then secured under muscle masses. Animals were allowed to survive for 1 (n = 15/genotype) or 3 months (n = 15/genotype).

Quantitation of pigmentation: For counting pigmented patches, after perfusion animals skin was removed and the gross thigh area was viewed under a dissecting microscope (Wild) at 40X magnification. Pigmented patches appeared as collections of streaks on the fascia overlying muscle and nerve that likely represent clones of differentiated melanocytes. Each streak was counted in each animal. This analysis represents an underestimate of total melanocytes.

To measure melanosomes electron micrographs were generated of hypodermal pigmented cells and skin melanosomes, and >235 melanosomes traced and areas measured on a Zidas imaging pad.

Histology and immunocytochemistry: 5-6 um thick paraffin sections were cut and processed for H & E or Gomori's Trichrome. For immunostaining, these sections were stained with polyclonal rabbit anti S-100 (DAKO; 1:5000) to mark Schwann cells, or mouse monoclonal anti neurofilament (15GI, 1:1) or polyclonal anti neurofilament (NF 178.3, 1:500; a gift of L. Parysek) to mark axons. BrdU labeling was as described by Weiler and Farbman (1997). Paraffin sections were processed using an anti-BrdU kit (Zymed). The number of BrdU positive nuclei counted in triplicate sections from each animal. To stain macrophages, unfixed nerves were sectioned on a cryostat and stained with F4/80 as described (Perry et al., 1995).

Nerve grafts: Adult mice were anaesthetized and small sciatic fragments (2 – 2.5 mm) removed. Nerves were rinsed in DMEM, incubated in DMEM with 10% FBS and 10ug/ml Hoechst 33342 dye (Sigma) for 1 hr at 37°C, rinsed in DMEM and chilled on ice for 30 minutes. A second mouse (recipient) was anesthetized, sciatic nerve exposed, nerve transected and Hoechst dye labeled nerve fragment grafted between the cut ends of the recipient sciatic nerve by suturing one end of recipient nerve to donor labeled nerve on each side. This method is based on that of Aguayo (Aguayo et al.

1979). The grafted nerve was then transected and each end of the dye labeled nerve fragment was deflected. Animals (n=9/genotype) were sacrificed 30 days post-surgery, fixed by perfusion and the pigmented area excised, cryoprotected, sections cut and then analyzed for pigmentation and dye labeled nuclei.

Cell grafting: Wild type, *Nf1*^{+/-} and *Nf1*^{-/-} Schwann cells were prepared from E12.5 DRG (Kim et al, 1995) and used at passage 2 - 3. Morphologically transformed hyper-proliferative cells derived from *-/-* Schwann cells (*-/-*-TXF) were purified as described (Kim et al, 1997) and used at passage 2 - 4. Wild type and *Nf1*^{-/-} fibroblasts were prepared from E12.5 torsos as described (Atit et al., 1999) and used at passage 2. 20,000 cells were plated onto matrigel coated transwell filters (Kim et al., 1997) for 48h then labeled with Hoechst dye (see above). Control filters processed in parallel were analyzed to ensure that cells were labeled prior to grafting. Filters were grafted around the transected area of the nerve (n=4 per cell type into wild type and n=4 into heterozygous mice). After 30 days, animals were fixed by perfusion and cryostat sections from pigmented areas evaluated for pigmentation and for dye positive nuclei.

Characterization of *Nf1*^{-/-} TXF cells: Cells plated onto LabTek slides were immunostained after fixation in 4% paraformaldehyde with rat anti-mouse P75 NGFr (1:10; the kind gift of D. Anderson, Caltech), rabbit anti-S-100 (Dako; 1:200), rabbit anti-GFAP (Dako; 1:1000) and mouse anti-smooth muscle actin (SMA, Sigma). Cells were rinsed, incubated in FITC labeled secondary antibody (1:200; Jackson Labs), rinsed and coverglassed. Immunostaining was visualized by fluorescence microscopy.

Migration assay: Conditioned medium: 2×10^6 cells Schwann cells of designated genotype were plated onto poly-L-lysine coated plastic 60mm dishes, cells incubated in serum-free N2 medium for 48h, medium collected, centrifuged to remove cells and debris, and stored frozen in aliquots. Cells were collected after trypsinization and volume of conditioned medium normalized to cell number.

Transwell assay: Transwell membranes containing 8 μ m pore size polycarbonate membrane (2 cm² transwell unit, Costar) were coated with poly-L-lysine and 30,000 Schwann cells plated, in 200 μ l, onto the upper well in N2 medium with 0.1% BSA and 0.1% fetal bovine serum. The lower well contained the same medium with 50% conditioned medium. After 10-15 hrs. media was aspirated, cells removed from the upper chambers using cotton tipped sticks and cells fixed with -20°C 95% EtOH for 5 minutes. Membranes were washed with 100% EtOH, air dried then hemotoxylin stained. After dehydration and clearing in xylene filters were mounted onto cover glass slides, coverslipped and cells counted using bright field microscopy.

Results

To test the hypothesis that trauma can alter the differentiation of neural crest-derived cells in peripheral nerves, we examined the effects of three injury paradigms (nerve crush, nerve cut and suture, and nerve cut with deflection of cut ends) on pigmentation.

Severe nerve injury induces pigmentation around peripheral nerves. One month after adult mouse sciatic nerves were cut and cut ends re-sutured ($n = 48/\text{genotype}$).

Pigmented cells were observed on gross dissection; clusters of highly pigmented cells extended up to 1 cm proximal and 1 cm distal to the transection site (Fig. 1). Wild type mice consistently showed hardly detectable groups of pigmented cells (Fig. 1A). *Nf1* mutant mouse littermates showed slightly enhanced pigmentation response (Fig. 1B). Increase in pigmentation and difference between wild type and mutant animals was easily detectable after nerve cut and deflection ($n = 15/\text{genotype}$) (Fig. 1C, D). Of the 15 mutant animals subjected to nerve cut with deflection, 8 showed the massive pigmentation documented in Fig. 1D; 0 of 15 wild type mice showed this response. When nerves are cut and deflected, axons in the distal stump degenerate, indicating that axons are not required for the pigmentation.

Pigmented cell clusters were also detectable on the ventral portion of the dermis (Fig. 1G) and on the ventral surface of the *Nf1/nf1* nerve (Fig. 1H). Wild type animals following nerve cut and deflection rarely showed pigment in these locations (not shown). Semi-thin plastic sections showed that pigmented cells in skin were subjacent to the hypodermis (Fig. 1E). Electron microscopy confirmed that the pigmented cells were melanocytes (Fig. 1F), with characteristic developing and mature melanosomes. The size of the melanosomes in these cells was not different from that of melanosomes from skin hair follicles in wild type animals indicating that the cells were melanocytes, not melanophages (not shown).

Mice were studied at various times after nerve cut and re-suture. Increased pigmentation was detectable by 7d after nerve cut; pigmentation reached maximal levels at 21 – 30 days. Variable amounts of pigmentation were detected 3 months after injury. In some animals dramatic melanogenesis was observed while in others little pigmentation was present. By 6 – 12 months after injury pigmentation was no longer present (not shown).

Nerve crush does not initiate pigmentation. Crush injury causes a transient breach of the blood nerve barrier and severs axons; Schwann cells proliferate, myelin is digested by resident and recruited macrophages, and proximal stumps of neurons regrow axons that regenerate and reinnervate distant targets. Sciatic nerves analyzed after crush injury did not show nerve abnormality 6 or 10 weeks after injury as assessed by gross analysis or H&E staining of paraffin sections. No differences between wild type and *Nf1/nf1* mutants were evident in Schwann cell proliferation after crush injury, assessed by staining with anti-BrdU (not shown) and myelin degradation proceeded on schedule. No pigmented cells were noted on gross or microscopic inspection indicating that a crush injury is not sufficient to cause the pigmentation response. Thus the *Nf1/nf1* response is an amplified response to wounding, and the response is modulated by the severity of the injury.

To determine if increased macrophage numbers might explain why the cut-and-deflected nerves showed increased melanogenesis as compared nerve crush, the F4\80 antibody was used to stain macrophages in distal nerve segments 7 days after injury when macrophage numbers are at their peak (Perry et al., 1995). Similar staining intensity was observed in the distal stumps of crushed and cut nerves (not shown) indicating that altered macrophage numbers is unlikely to explain the differences observed.

To determine if increased pigmentation in *Nf1* mutants results from peripheral nerve abnormality, sciatic nerve structure was analyzed at embryonic day 18 through postnatal day 30 in plastic sections and by electron microscopy. No abnormalities were detected in nerves of mutant

animals (not shown). Significantly, no pigmented cells were noted within or surrounding the nerve. These data extend and are consistent with previous studies (Jacks et al., 1994; Brannan et al., 1994; Cichowski et al., 1999).

Tumor formation after nerve lesion: Unpigmented and pigmented tumors were occasionally associated with lesioned *Nf1* nerves. Unpigmented tumors were detected in 3/22 mice studied at three months after nerve cut. Peripheral nerve tumors never developed in wild type mice, with or without wounding. Unpigmented tumors were at least 0.5 cm distant from the lesion sites and associated with nerves (Fig. 2A). In sections, these tumors were unencapsulated and diffusely invasive (Fig. 2D), with significant amounts of Gomori's trichrome positive matrix (Fig. 2E), S-100-positive [presumed Schwann cells] (60%) (Fig. 2F), S-100 negative cells (40%) (methyl Green-H) and a few neurofilament-positive axons (Fig. 2G). The high ratio of S100-positive cells to neurofilament-positive axons suggests that many S100-positive cells were free of axons, as reported for human neurofibromas. In 3/22 mice 3 months after nerve cut, and 2/15 mice after cut and deflection, heavily pigmented tumors were detected proximal or distal to the lesion sites (Fig. 2B). No pigmented tumors were evident in wild type littermates after nerve injury. In semi-thin plastic sections, pigmented tumors had capsules (Fig. 2C). These tumors have not been studied further.

Pigmented cells that arise after nerve injury are derived from the adult nerve. Collectively the data presented above suggested the hypothesis that pigmented cells result from abnormal response of endogenous melanocytes to wound cytokines. An alternative possibility is that cells within the adult nerve migrate away from the nerve and become pigmented. To distinguish between these possibilities, nerve fragments from wild type and mutant animals were labeled with Hoechst dye, a fluorescent nuclear marker, then grafted into wild type or mutant host animals. Nerves were then lesioned and pigmented cells evaluated for the presence of the marker dye 30 days later (Figs 3A, 5A). Surprisingly, we observed numerous examples of cells containing dye-labeled nuclei with

adjacent cytoplasm containing pigment granules. Two representative examples are shown in Figs 3B-D and 3E-G. Not all dye labeled cells became pigmented, indicating that only some grafted cells developed into mature melanocytes containing pigment. These data strongly suggest that some cells derived from the adult nerve form pigment in the wound environment.

To rule out the possibility that dye labeled cells were simply in close proximity to pigmented cells, wild type or *Nf1* adult nerve fragments were grafted from black C57Bl/6 mice into albino mice that carried a null allele for the tyrosinase (*tyr*) gene (Fig. 4A). The albino mice shown in Figure 4A are white because their melanocytes cannot make pigment. Therefore if any black pigment arises in the mutant mice after a nerve graft, pigmented cells must derive from the graft. The mutation causing the lack of pigment in these mice arose on the C57Bl/6 background, making them syngenic with the *Nf1* mice. Thirty days after nerve injury mice were evaluated for pigmentation. Black patches were visible in all recipient albino mice in muscle overlying nerve (Fig. 4C,D), and in adjacent skin (Fig. 4B), supporting the view that pigmented cells derive from nerve. Wild type nerve fragments grafted into the albino mice also resulted in appearance of pigmented cells, but as anticipated no black patches were present when nerve was cut and deflected in albino mice without nerve graft (not shown, $n = 3$). Thus cells with the potential to form melanocytes exist in adult wild type and *Nf1* mutant nerve.

In experiments in which C57Bl/6 nerve was grafted into albino mice, the pigmentation phenotype was not as dramatic as observed when the mutant nerve was transected and deflected in animals with a normal tyrosinase gene, as shown in Fig.1. This observation suggested the possibility that cells within the nerve in conjunction with a mutant environment amplify the pigmentation response. To test this a series of grafting experiments were carried out in which Hoescht labeled nerve fragments, 2mm in length, were grafted into wild type or mutant animals. The percent of dye-labeled cells containing pigment (in fascia around nerve and around muscles) was expressed as a

percent of total dye-labeled cells. As shown in Fig. 5A the percent of dye-positive cells containing pigment in wild type to wild type grafts was extremely low; no pigmented cells were detected in 10,000 dye labeled cells analyzed. In contrast when graft alone or host alone was *Nf1* heterozygous 6.3% and 5% of the dye labeled cells contained pigment granules, respectively. Strikingly when both graft and host were *Nf1* heterozygous a ten-fold increase in dye and pigment-laden cells was observed; 55.2% of labeled cells were pigmented. The data confirm a role for both nerve-derived cells and for the host environment in the pigmentation phenotype.

Glial peripheral nerve cells retain the ability to become pigmented. To identify nerve-derived cell populations that might contribute to the pigmentation response, Schwann cells or fibroblasts were cultured on filters and then grafted into recipient *Nf1* heterozygous animals, around cut and re-sutured nerves. The filter provided a solid support and allowed implantation of a similar number of cells per genotype. Thirty days after implantation blocks of tissue containing pigmented cells were removed for sectioning and analysis. Neither wild type Schwann cells nor fibroblasts generated detectable numbers of dye positive, pigmented cells (Fig. 5B). *Nf1* mutant (-/-) fibroblasts also did not produce dye positive pigmented cells (not shown). These results suggested that another cell population in the nerve contributes to the pigmented cell population. In an attempt to identify such a cell we used Schwann cells from *Nf1* mutant animals that became pigmented with increased frequency. All *Nf1* Schwann cell preparations tested yielded dye-labeled pigmented cells. *Nf1* heterozygous and null Schwann cells purified from DRG neurons showed 4.5% and 7.4% dye-positive pigmented cells, respectively. A population of rapidly proliferating cells was previously identified in dorsal root ganglion cultures from *Nf1*^{-/-} and to a lesser extent from cultures from *Nf1*^{+/-} embryos (Kim et al., 1997). When these cells, designated “-/-TXF”, were grown on filters and grafted around cut nerves of host animals numerous examples of dye labeled pigmented cells were observed in each of four host animals; an average of 12.1% of the dye labeled cells were

pigmented. The -/-TXF cells were different from the *Nf1* +/- cells in a Student's t-test (2-tail, 2-sample unequal variance; $p=0.03$). The *Nf1* -/- cells showed a trend toward difference from the -/-TXF cells ($p=0.10$) but did not reach statistical significance. This series of experiments demonstrates that glial cells, not fibroblasts, have the potential to become pigmented in the wound environment. In addition loss of function mutations at *Nf1* in glial cells increase the percent of cells that form pigment in the wound environment.

Immunohistochemical characterization of -/-TXF peripheral nerve cells that become

pigmented. The -/-TXF population was characterized by staining cells with antibodies known to recognize wild type Schwann cells or wild type fibroblasts. The -/-TXF cells expressed p75 and S100, characteristic markers for Schwann cells, and higher levels of GFAP, another glial cell marker, than did wild type mouse Schwann cells (Fig. 6, see inset in 6H). Embryonic fibroblasts expressed smooth muscle actin (SMA), and low levels of S100 [expressed by some fibroblast and muscle cells], but not detectable levels of p75 or GFAP. The -/-TXF cells were negative for SMA. In three independent preparations the percent of -/-TXF cells expressing S100, p75 and GFAP was defined. In each preparation, every cell expressed all three markers (not shown). This marker analysis demonstrates that -/-TXF cells are within the glial cell lineage. The high percentage of cells expressing all three antigens rules out the possibility that a small but significant portion of the cells have characteristics different from -/-TXF cells.

In vitro abnormalities in migration and secretion are detectable in Schwann cells mutant at *Nf1*. In vitro tests were carried out to determine if cell autonomous defects in *Nf1* mutant cells might account for aberrant migration of nerve-derived cells into muscle and skin. We used Schwann cells and medium conditioned by Schwann cells to avoid possible confounding variables due to cell proliferation using the *Nf1* -/-TXF population. Medium conditioned by wild type or *Nf1* mutant

Schwann cells was collected, normalized for cell number used to generate the medium, and used as an attractant in in vitro cell migration assays (Fig. 7). Medium conditioned by wild type Schwann cells provided a very weak chemoattractant for wild type Schwann cells, allowing an average of 7 cells per 4 fields to migrate through the filter. In contrast, medium conditioned by mutant Schwann cells provided a much more robust stimulus for migration of wild type cells. An average of 21 wild type cells migrated (per 4 fields) in response to *Nf1*^{+/+} medium and 53 cells migrated/ 4 fields in response to *Nf1*^{-/-} medium. Furthermore *Nf1*^{-/-} mutant cells showed more than 28-fold enhanced migration as compared to wild type cells. The enhanced response of *Nf1*^{-/-} cells to wild type medium was enhanced a further 8-fold enhanced in response to *Nf1*^{-/-} medium. *Nf1*^{+/+} cells were intermediate in migration as compared to wild type and *Nf1*^{-/-} cells. These results were consistent among 15 independent experiments in which replicates differed by less than 10%. Entry into S-phase of wild type and mutant Schwann cells exposed to mitogen begins at 24h, so neither doubles during a 13h assay, excluding cell proliferation as a cause of altered migration. The data demonstrate that loss of *Nf1* by Schwann cells has dual effects on migration, a cell autonomous increase in migration and increased secretion of migration-promoting factor(s).

Suppression of pigmentation occurs through the β^c subunit of the GM-CSF/IL5 receptor. We hypothesized that the GM-CSF/IL3 receptor β^c effect might underlie heightened response of *Nf1* mutant cells to nerve lesion. We tested the effects of β^c loss by crossing *Nf1* mutant mice with those null for loss of β^c (Nishinakamura et al., 1995). Data shown in Figure 8A represent quantitation of the number of pigmented patches visible on muscles in wild type and mutant for *Nf1*, β^c or both. Loss of β^c increased pigmentation in mice to levels comparable with *Nf1* mutation. Conversely, in the absence of β^c the effects of *Nf1* mutation were completely reversed. These data show that β^c is necessary for the enhanced pigmentation in *Nf1* mutants.

Discussion

The major conclusion of this study is that cells in adult rodent nerves retain the capacity to form pigment cells. We have identified a signaling pathway that suppresses melanogenesis after adult nerve injury. This pathway requires the IL-3/GMCSF receptor β^c (Figure 8B), as peripheral nerve melanogenesis is enhanced in the β^c knockout mouse. GMCSF, IL-5 and IL-3 use the β^c receptor. Each associates with a unique α subunit that confers binding (reviewed in Bagley et al., 1997). One or more of these ligands may suppress melanogenesis through activation of β^c . While further experiments will be required to define relevant ligand(s), GMCSF may play a key role as it does in the hematopoietic system upstream of *Nf1* signaling (Birnbaum et al., 2000).

In mice hemizygous for *Nf1* mutation pigmentation-induced injury was greatly increased, just as in β^c mutants. This finding defines a role for the normal *Nf1* gene in suppression of the melanogenic phenotype. *Nf1* mutation appears to act downstream of β^c because loss of β^c and mutation at *Nf1* together inhibited the melanogenesis enhanced by either alone. The NF1 gene product, neurofibromin, is a Ras-GTPase activating protein (Ras-GAP) (reviewed in Cichowski and Jacks, 2001). Effects of *Nf1* mutation on nerve pigmentation could be Ras-mediated. In hematopoietic cells GM-CSF stimulates Ras activation, and Ras activation in response to GM-CSF or IL-3 is augmented in cells lacking *Nf1* (Bollag et al., 1996; Largaespada et al. 1996). Schwann cells lacking *Nf1* have elevated Ras-GTP (Kim et al., 1995). Loss of β^c is predicted to cause decreased Ras signaling in cells, because the receptor that normally couples to Ras activation is missing. Decreased neurofibromin, in contrast, is predicted to cause increased Ras-GTP or longer duration Ras-GTP signals after receptor mediated signaling. However, both mutants show increased pigmentation after nerve injury. One interpretation of these data is that precise levels of Ras-GTP in peripheral nerve cells are required after nerve injury to maintain Schwann cell phenotype. Loss of β^c

and mutation at *Nf1* together could be envisioned to normalize Ras signaling (add together a positive and negative effect on Ras signaling, see ? in Fig. 8B), thus normalizing pigmentation.

We cannot completely exclude the possibility that cells with the capacity to form pigment are present in the epineurium and are induced to pigment in the wound environment. However, our evidence argues that these cells are insufficient to explain wound-induced melanogenesis, because pigmentation is greatly enhanced when endoneurial cells can escape the endoneurium (after nerve cut and deflection) and basal lamina constraining endoneurial cells is lost. In addition, the fact that purified glial cells grafted into the wound environment become pigmented argues strongly against a sole requirement for epineurial pigment-forming cells.

Our data support a model in which adult sciatic nerve cells migrate from the endoneurium into epineurial fascia and the hypodermis, then become pigmented. Pigmentation does not occur after nerve crush injury, because axons and glial cells remain in basal lamina tubes, but does result when peripheral nerve is cut and cells escape from nerve ends (Fawcett and Keynes, 1990; Sunderland, 1991). A pathway out of the endoneurium is important because normal cells cannot penetrate a basal lamina (Erickson, 1987). In addition, GM-CSF and IL3 released by nerve fibroblasts after injury (Saada et al., 1996) may actively prevent melanogenesis by endoneurial cells. Our *in vitro* experiments demonstrate increased migration of *Nf1* mutant Schwann cells; once mutant cells escape the endoneurium they are predicted to show increased migration into an environment permissive for melanogenesis, containing endothelins, bFGF, SCF, α -MSH and/or other factors that regulate melanogenesis.

When host mice are hemizygous for *Nf1* mutation, we observed an increase in pigment formation by cells in wild type nerve grafts. This effect cannot be due to pigmented

cells from the host *Nf1* nerve outside the graft, as we scored only dye-labeled (grafted) cells for pigment. The environmental effect might result from abnormal secretion by *Nf1* mutant cells, acting on grafted cells. Factor-producing cells could be in the nerve outside the graft, and/or in denervated skin or muscle. Factors could influence migration and/or melanogenesis of grafted cells. *Nf1* mutant Schwann cells do increase expression of several chemotactic factors (Mashour et al., 2001). One plausible factor is stem cell factor (c-kit ligand); *Nf1* loss partially rescues melanocyte pigmentation defects caused by c-kit mutation (Ingram et al., 2000) and this factor enhances melanocyte migration in an *Nf1*-dependent manner (Wehrle-Haller et al., 2001).

When wild type adult nerve fragments are grafted into wild type, *Nf1* mutant, or tyrosinase null mice, pigmented cells develop. Therefore adult nerves must contain cells with the potential to become pigmented. However, grafting purified cultured wild type Schwann cells did not result in detectable numbers of graft-derived pigmented cells, indicating that less than 1 in 10,000 wild type Schwann cells becomes pigmented. It is possible that cells with the potential to pigment in wild type nerves are stem cells that do not survive under culture conditions favoring embryonic Schwann cell development. It seems more likely that pigmented cells may de-differentiate from Schwann cells and re-differentiate (transdifferentiate) at low incidence in the wound environment, because we showed that uniformly S100, p75 and GFAP-positive cells (glial cells) can become pigmented.

The increase in pigmented cells present after nerve wounding in *Nf1* mutants may reflect increased plasticity of *Nf1* mutant Schwann cells and/or the presence of an abnormal glial cell population. *Nf1*^{-/-}-TXF cells characterized here may be abnormal Schwann cell progenitors. They are like progenitors in cobblestone-like growth patterns and expression of p75 (Dong et al., 1995; 1999). They differ from neural stem cells and Schwann cell progenitors as they can be maintained in

neuregulin (Kim et al., 1997), usually a sufficient signal for Schwann cell differentiation (Shah et al., 1994). They express GFAP, normally highly expressed in differentiated only in non-myelinating Schwann cells, yet express the epidermal growth factor receptor, normally expressed by neural crest cells but not Schwann cells (DeClue et al., 2000). The *Nf1* mutation alters hematopoietic progenitors (Zhang et al., 1998; Ingram et al., 2000; Birnbaum et al., 2000). *Nf1* mutation is also associated with defects in developing neurons (Brannan et al., 1994; Lakkis et al., 1999; Vogel et al., 1995). We have no *in vivo* evidence that within adult peripheral nerve progenitor cells exist, which would require identification of precursor-specific markers.

Abnormal plasticity of *Nf1* mutant Schwann cells, or their precursors, could be relevant to peripheral nerve tumorigenesis. Mice heterozygous for *Nf1* and for *p53* mutations develop malignant peripheral nerve tumors that express neural crest markers (Cichowski et al., 1999; Vogel et al., 1999). In neurofibromas a few cells, like *Nf1* TXF cells, express the epidermal growth factor receptor and S100 (DeClue et al., 2000). The mechanism underlying the rare tumor formation in our study is unknown, as is its relevance to human NF1 disease. However, our data support the hypothesis that when the cytokine environment is permissive, as it is after wounding, both tumors and aberrant pigmentation can occur. In humans with NF1, pigmented cells within neurofibromas occur and hyperpigmented skin frequently overlies plexiform neurofibromas of childhood (Fetsch et al., 2000; B. Korf, personal communication). Both could result from differentiation of a stem cell population to glial and melanocyte derivatives, or through transdifferentiation.

Our findings show that pigmentation can be induced by nerve trauma in mice and that glial cells within adult nerve become pigmented. Pigmentation after nerve injury is normally suppressed through a pathway involving the IL-3/GM-CSF receptor β^c and *Nf1*.

Relationship to Tasks and Data not submitted:

In the midterm report we described data showing that Schwann cells mutant at *Nf1* have increased secretion of melanogenic factors. Reviewers of our submitted manuscript advised us to remove that data as it distracted from the focus of the paper. It is not yet published.

Task 3, Test if another wounding paradigm, chemical carcinogen exposure, stimulates increased pigmentation or tumor formation in *Nf1/nf1* mice.

- a. Apply DMBA or DMBA and TPA to wild type and mutant mice (completed).
- b. Analyze resultant skin spots and tumors for Ras mutations (completed).
- c. Analyze skin for abnormal TPA response (completed).

Rationale:

Neurofibromatosis type 1 patients, but not mice heterozygous for mutations in the *Nf1* tumor suppressor (*Nf1*^{+/-}), develop hyperpigmented spots and benign tumors. *Nf1* mice were exposed to DMBA or DMBA + TPA to test for co-operation between *Nf1* mutation and carcinogen exposure. DMBA caused C57Bl/6 *Nf1*^{+/-} mice to develop pigmented skin patches, with hair follicles in anagen phase, at 2-fold increased frequency. Papillomas developed in 75% of *Nf1*^{+/-} mice treated with DMBA and TPA but never in wild type mice or *Nf1*^{+/-} mice treated with TPA only. Loss of *Nf1* does not circumvent the requirement for activating mutations in c-H-*ras* in this model; although neurofibromin is a GAP for Ras all papillomas analyzed had mutations in codon 61 of H-*ras*. *Nf1*^{+/-} mutants showed significant sustained increases in proliferation of keratinocytes in response to TPA. Thus, the *Nf1* gene is a modifier of carcinogen-induced phenotypes that acts by stimulating TPA-mediated pathways, co-operating with activating *ras* mutations in keratinocytes. The data are consistent with a role for *Nf1* in epidermal cell carcinogenesis and keratinocyte dysfunction in NF1 patients.

Neurofibromatosis type 1 (*NF1*) is a common inherited autosomal dominant human disease, affecting 1 in 3500 individuals worldwide (reviewed in Huson, 1998). *NF1* patients heterozygous for mutations in the *NF1* gene are predisposed to develop benign and malignant tumors, and to pigmentation defects. Pigmentation defects include patches of hyperpigmented skin called cafe-au-lait macules (Crowe and Schull, 1953; Benedict et al., 1968; Takahashi, 1976; Frenk and Marazzi, 1984) found in all *NF1* patients by five years of age (Korf et al., 1992). *NF1* patients also develop axillary and inguinal freckling (Crowe et al., 1964; Friedman and Birch, 1997), and patches of retinal melanocytes (Lisch nodules) (Lewis and Riccardi, 1981; Huson et al., 1987). While it is not understood how mutations at the *NF1* locus in specific skin cell type(s) cause these *NF1* skin manifestations, a role for the *NF1* gene product, neurofibromin, in the skin is suggested by this aberrant pigmentation.

The expression pattern of neurofibromin is consistent with *NF1* function in the skin. In adult human skin neurofibromin is readily detectable in melanocytes and keratinocytes, and at much lower levels in fibroblasts (Malhotra and Ratner, 1994; Hermonen et al., 1995). Wounding up-regulates neurofibromin expression in human skin fibroblasts (Yla-Outinen et al., 1998). In rodents, neurofibromin is expressed at very low levels in keratinocytes, melanocytes, and fibroblasts of the adult skin (Daston and Ratner, 1992), but shows high expression in keratinocytes during embryonic and early postnatal life as keratinocytes are differentiating (Malhotra and Ratner, 1994). Thus, the major cell types in the skin express neurofibromin and could be affected by loss of function at *NF1*. Melanocytes from *NF1* patients are subtly abnormal (Kaufmann et al., 1991); keratinocytes have not been studied.

NF1 is considered to be a tumor suppressor gene, as loss of heterozygosity at *NF1* has been demonstrated in *NF1* patient malignant tumors, in neurofibromas, and in myeloid

disease (Legius; 1993; Sawada et al., 1996; Side et al., 1998; Serra et al., 1997). NF1 patients are at 4-fold increased risk for malignant disease (Sorenson et al., 1986; Zoller et al., 1997). In a population-based study in Sweden, 16% of adult NF1 patients developed carcinomas, suggesting possible increased risk for epidermal tumors (Zoller et al., 1997).

Heterozygous *Nf1* mice do not spontaneously develop benign tumors or the pigment abnormalities seen in human NF1 patients; *Nf1* null mice die *in utero* so are unavailable for analysis (Brannan et al., 1994; Jacks et al., 1994). Wounding has been hypothesized to serve as a triggering event of neurofibroma formation and café-au-lait macules in human NF1 (Riccardi, 1992). To begin to test this hypothesis, we used mice with targeted mutations in the *Nf1* gene and found aberrant skin fibroblast function after excisional skin wounding (Atit et al., 1999). Like wounding, skin carcinogens elicit an inflammatory response and induce skin cells to proliferate (reviewed in DiGiovanni, 1992; Scribner and Suss, 1978).

We tested if treatment of *Nf1* mutant skin with dimethylbenzanthracene (DMBA) increases pigmentation. Topical application of DMBA induces patches of pigmentation in susceptible strains of mice (Klaus and Winkelmann, 1965; Forbes et al., 1965; Kanno et al., 1987). DMBA treated skin shows increased proliferation of normally dormant DOPA-inactive melanocytes and enhanced melanogenic activity (Tsambaos et al., 1989); melanin is transferred to adjacent keratinocytes leading to visible pigmentation.

We also challenged *Nf1* mutant mice in the two-stage carcinogenesis model, in which skin is initiated with a potent carcinogen like DMBA and then exposed to multiple treatments with a tumor promoter such as wounding or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Deelman et al., 1927; DiGiovanni, 1992). Susceptible strains of mice develop papillomas of the skin (Boutwell et al., 1964; Slaga et al., 1989).

Ras activation is an important step in tumor progression in response to skin carcinogens. After DMBA exposure, activating mutations in the c-Ha-*ras* proto-oncogene at codon 61 develop in keratinocytes (Quintinalla et al., 1986; Nelson et al., 1992). Activating mutations in *ras* can serve as an initiating event in the two stage tumorigenesis model, because targeting of the v-Ha-*Ras* gene to follicular keratinocytes causes development of papillomas at a very high frequency, after promotion with wounding or TPA (Leder et al., 1990; Hansen and Tennant 1994a,b). Targeting Ras to melanocytes results in increased pigmentation and susceptibility to melanoma (Powell et al., 1995; Gause et al., 1997).

The molecular pathways regulated by neurofibromin are not completely understood. Neurofibromin functions as a Ras-GTPase activating protein (Ras-GAP) in some mammalian cell types (De Clue et al., 1992; Basu et al., 1992; Kim et al., 1995, 1997; Largaespada et al., 1996; Bollag et al., 1996; reviewed in Kim and Tamanoi, 1998). Neurofibromin also has ill-defined non-Ras functions in mammalian cells (Johnson et al., 1994; Atit et al., 1999). In *Drosophila*, NF1 regulates a cyclic AMP-dependent protein kinase A pathway in a Ras-Raf independent manner (The et al., 1997; Guo et al., 1997). Functional effects *in vivo* of *Nf1* mutations in skin could occur through either of these pathways, or others.

We show in this report that DMBA treatment leads to an increased frequency of pigmented spots in *Nf1*^{+/-} as compared to wild type mice. Furthermore, initiation with DMBA followed by promotion with TPA stimulates papilloma formation in *Nf1*^{+/-} C57Bl/6 mice but not wild type mice. The data are particularly striking as the C57Bl/6 strain is noted for resistance to chemical carcinogens (Kiguchi et al., 1997; Reiners, 1984). The increase in papilloma incidence in *Nf1*^{+/-} mice suggests co-operation between Ras activation and *Nf1* inactivation. Our data indicate that *Nf1* is a strong modifier of responses induced by skin carcinogens.

Materials and Methods:

Chemicals and Reagents:

7,12-dimethylbenz[a]anthracene (DMBA), 12-*O*-tetradecanoyl-13-acetylphorbol (TPA), BrdU were purchased from Sigma Chemicals Co. (St. Louis, MO). Biotinylated monoclonal anti-PCNA and anti-BrdU was from Zymed Laboratories Inc. (San Francisco, CA).

Animals:

C57Bl/6 wild-type female mice (breeders) were obtained from Harlan (Indianapolis, IN). *Nf1* +/- mice were generated by targeting one allele of the *Nf1* gene (Brannan et al., 1994). For genotyping, DNA from a toe clip was isolated and the presence of the targeted allele determined by PCR as described in Brannan et al. (1994). Wild type and *Nf1*+/- mice were obtained by mating *Nf1*+/- C57Bl/6 males to C57Bl/6 breeder females. Mice were maintained on a 12-h light/dark cycle in a temperature and humidity-controlled room and were provided with reverse osmosis water and rodent chow (Purina, St. Louis, MO) ad libitum. Male mice were treated as described at 9-12 weeks of age. Dorsal skin hair was carefully shaved with surgical clippers one day before topical dosing was begun and mice with skin in anagen phase at the initial shaving were excluded from the analysis. Mice were housed four per cage until 20 weeks of tumor promotion treatment, or until papillomas formed. Subsequently mice were individually housed in polystyrene cages to avoid injury to newly formed papillomas.

Treatments:

Mice were initiated by application of 40ug of DMBA in 200ul acetone on Day 1 and Day 10. Mice were observed for 2-4 months after initiation. One week after initiation was complete one group of mice received topical applications of 0.2 ml of 6.4 nM TPA in acetone (0.8ug) delivered using a micropipette. Mice received TPA three times per week for 24 weeks. Control mice received 0.2 ml of acetone vehicle on the same schedule. Dorsal skin was shaved once every 4-6 weeks to maintain exposure of the skin to the treatments. The incidence of pigmentation and skin papillomas was recorded weekly. Tumor data are expressed as the percentage of mice with papillomas (tumor incidence) and the average number of papillomas per mouse (tumor multiplicity).

Histology:

Mice were euthanized in a chamber filled with CO₂. Treated skin from pigmented and unpigmented regions, and papilloma tissue with adjacent skin was excised from the dorsum. Specimens were fixed in 10% formalin, embedded in paraffin, and 6 microns sections cut and stained with hematoxylin and eosin. Some sections were immunostained with biotinylated anti-PCNA or anti-BrdU per directions from the manufacturer. Hair follicle number was counted on a light microscope in at least 5 different fields/section. Multiple sections were analyzed from two different specimens of treated skin per mouse.

H-ras codon 61 mutational analysis:

DNA was isolated from unpigmented and pigmented regions of the treated skin of three wild type animals and five *Nf1*^{+/-} animals after an overnight lysis in buffer (100mM Tris, 0.2% SDS, 200mM NaCl, 5mM EDTA) containing proteinase K (100ug/ml). DNA was precipitated in isopropanol and resuspended in TE. Genomic DNA was isolated

from papilloma laden mouse skin using Puregen reagents (Gentra Systems, Minneapolis, MN) and proteinase K. DNA was quantitated by UV spectroscopy at 260 nm. For *ras* mutational analysis, all PCR primers were based on the mouse *H-ras* genomic sequence of Brown et al. (1988). The enriched polymerase chain reaction (EPCR) method described in detail by Mitchell and Warshawsky (1998) was used to screen for codon 61 mutations in treated skin and papilloma-derived DNA. Amplification of a normal allele gives a 135 bp band and the allele with the codon 61 mutation gives a 162 bp fragment. Normal mouse liver DNA was used as the negative control and an *H-ras* codon 61 CAA-CGA DNA fragment was used as the positive control (Mitchell and Warshawsky, 1998). Reverse cyclic dideoxy sequencing was used to confirm the codon 61 mutations (Mitchell and Warshawsky, 1998).

Epidermal labeling index after TPA treatment

Wild type or *Nf1*^{+/-} mice were treated 4 times over two weeks with acetone, 0.8µg TPA, or 4µg TPA, and sacrificed 48 hours after the last treatment. In other experiments animals were treated once with acetone, 0.8µg TPA, or 4µg TPA, and sacrificed 48 hours after treatment. One hour before sacrifice, mice were injected intraperitoneally with 100mg/kg BrdU (Sigma) in saline. Two to three specimens from the treated area of the dorsal skin were collected into 10% normal buffered formalin, and processed for paraffin embedding. Immunohistochemistry to detect BrdU-labeled cells was carried out on deparaffinized sections according to manufacturer's instructions; sections were counterstained with hematoxylin. The percent of labeled basal keratinocytes (labeling index) was calculated after counting 1500 to 2000 basal keratinocytes per mouse as described in



Naito et al. (1987).

Results

Increased incidence of pigmentation in *Nf1*^{+/-} skin after treatment with DMBA:

Twenty-two wild-type mice and 30 *Nf1*^{+/-} littermates were treated with DMBA on day 1 and day 10. Within three weeks after initiation, most *Nf1*^{+/-} (n=26/30) mice showed large patches of pigmentation, all greater than 1 cm². This phenotype was absent in untreated mice, and in mice treated with acetone only (n = 5-9/ genotype; Table).

<u>Treatment</u>	<u><i>Nf1</i>^{+/+}</u>	<u>% affected</u>	<u><i>Nf1</i>^{+/-}</u>	<u>% affected</u>
Acetone	0/5	0	0/9	0
TPA	0/8	0	0/15	0
DMBA	8/22	36	23/30	76
DMBA+TPA	6/6	100	12/12	100

Table. Incidence of pigmentation after treatment with skin carcinogens.

C57Bl/6 male mice were treated with 200μl topical acetone vehicle, TPA (0.8ug, 3 times per week), DMBA (40ug, 2 treatments), or DMBA+TPA (DMBA 40ug, 2 treatments followed by TPA, 0.8ug, 3 times per week). Animals exhibited that exhibited one or more patches of pigmentation larger than 1cm² were scored as positive. When patches appeared, they were always larger than 1cm². The first three groups were scored by 4 months after initiation of experiments. The DMBA+TPA group was scored 6 months after initiation of treatment.

Pigmented patches were also absent in wild type or *Nf1*^{+/-} exposed to TPA alone, even after 2 months of treatment (Table). Pigmented patches remained grossly visible for at least 4 months (Fig. 9B and C; Table). Most wild type mice did not develop pigmentation in response to DMBA (Fig. 9A), but 8 of 22 wild type mice did (Table). Thus, a significant increase in the incidence of pigmentation was observed in *Nf1*^{+/-} mice in response to DMBA.

Skin was analyzed by histology to define skin components contributing to the pigmentation. Wild type and *Nf1*^{+/-} skin had normal histology outside pigmented areas, with hair follicles in the resting (telogen) phase (Fig. 9D). In contrast, skin from pigmented patches of affected wild type and *Nf1*^{+/-} mice showed large numbers of hair follicles filled with melanin pigment (Fig. 9E and F, white arrows). Skin sections from wild type and *Nf1*^{+/-} mice were immunostained with anti-PCNA, a proliferation marker, to confirm that the hair follicles in pigmented spots were in anagen phase. The visible brown precipitate marks proliferating follicular keratinocytes (Fig. 9H and I). Hair follicles were found in multiple stages of differentiation in which different compartments of the follicle contain proliferating cells (not shown).

Estimates of hair follicle numbers were obtained from sections of unpigmented and pigmented skin of 4-6 wild type and *Nf1*^{+/-} mice two months after initiation (Fig. 10). Normal-appearing skin of wild type and *Nf1*^{+/-} mice had similar low numbers of hair follicles/field (Fig. 10A). When pigmented skin was analyzed (Fig. 10B) much higher numbers of follicles were present, but the numbers were indistinguishable in the two genotypes. Thus, visible pigmented spots are indistinguishable at the gross and histological levels between the wild type and *Nf1*^{+/-} groups. These data suggest that the *Nf1* gene is a modifier of the pigmentation response to DMBA in the C57Bl/6 strain of mice.

Papilloma formation in *Nf1*^{+/-} mice after DMBA and TPA treatment:

In a second group of mice, dorsal skin was initiated with DMBA and then promoted with multiple treatments of TPA to determine if *Nf1*^{+/-} skin would respond differentially to a tumor promoter. All animals in the wild type (n=6/6) and *Nf1*^{+/-} (n=12/12) groups developed large patches of pigmented skin (Table) after exposure to DMBA + TPA. Skin histology

from unpigmented (Fig. 11B) and pigmented (Fig. 11C) regions was indistinguishable from that observed when skin was treated with DMBA alone (Fig. 9D, F).

The C57Bl/6 mouse strain is extremely resistant to tumor formation in response to the DMBA and TPA regimen (Reiners et al., 1984; Naito et al., 1987; reviewed in DiGiovannini 1992; Kiguchi et al., 1997). In an initial study, none of 6 wild type mice analyzed developed papillomas. Yet, after 20 weeks of promotion, papillomas began to arise on the dorsum of most *Nf1*^{+/-} mice, which were littermates of the wild type mice analyzed (Fig. 12A). By the end of 24 weeks, 9/12 (75%) of *Nf1*^{+/-} animals (Fig. 10A) had developed papillomas. Most animals had 1-3 tumors; tumor ranged in volume from 1 to 80mm³ (Fig. 12B). In a second experiment none of 12 wild type mice developed papillomas in response to DMBA + TPA whereas 9 of 12 mutants developed papillomas by 20 weeks after promotion (not shown). None of 8 wild type or 15 *Nf1*^{+/-} mice developed papillomas in response to TPA alone. Acetone alone did not provoke papillomas in one wild type or 4 *Nf1*^{+/-} mice.

Papillomas were analyzed histologically in hematoxylin and eosin stained paraffin sections (Fig. 11D). Papillomas showed epithelial hyperplasia (*e* in Fig. 11E) and trapped sebaceous glands with follicular cysts (*s* and *c* respectively, Fig. 11F) characteristic of papillomas. Trapped sebaceous glands were a significant component of most of the papillomas examined. The hypothesis that the sebaceous glands are part of the neoplastic process was considered but excluded because the whole structure of nearby hair follicles was well preserved. It appears that sebaceous glands are trapped in the papillomas in *Nf1*^{+/-} mice even at stages when the papillomas are large (C. Conti, MD Anderson Cancer Center, Smithville, TX, personal communication). The reason for this is not known. Larger papillomas showed hyperkeratosis (not shown). These data demonstrate that loss of one *Nf1*

allele dramatically increases papilloma incidence in C57Bl/6 *Nf1*^{+/-} mice treated with DMBA + TPA. Thus, the *Nf1* gene acts as a modifier of the papilloma phenotype.

Ras mutational analysis of pigmented skin and papillomas

The c-H-*ras* gene is a target for activating mutations induced by DMBA and TPA (Quintanilla, 1986). In mouse skin papillomas, mutations in >90% of the tumors are detected at codon 61 of c-H-*ras* (reviewed in DiGiovannini, 1992). Because neurofibromin can act as a GAP for the Ras proteins, it seemed possible that *ras* gene mutations that frequently are detected in papillomas might not be detected in *Nf1* mutants. To test this idea, we used an enriched PCR analysis designed by David Warshawsky and Kent Mitchell of the University of Cincinnati (see Methods). We carried out the analysis in collaboration with them. Treated mouse skin from unpigmented and pigmented regions from three different *Nf1*^{+/+} mice and five different *Nf1*^{+/-} mice did not show the c-H-*ras* codon 61 mutation (Fig. 13A lanes 1-4). We analyzed the DNA from 6 of the largest *Nf1*^{+/-} skin papillomas for c-H-*ras* codon 61 mutations. Results showed that 6 of 6 independent tumor specimens analyzed contain cells with the A to T transversion at codon 61 from the c-H-*ras* gene (Figure 13B, lanes 1-4). Thus, presence of *ras* mutations in addition to functional inactivation of a single allele of *Nf1* is required to overcome the resistance of C57Bl/6 mouse strain to papilloma formation.

Epidermal labeling index after TPA treatment of epidermis

We tested whether loss of *Nf1* alters keratinocyte proliferation in response to TPA, accounting for altered susceptibility of mutant animals to papilloma formation. Mice were treated with TPA once, or four times over two weeks. Forty-eight hours after the last TPA

treatment, mice were injected with BrdU for 1 hour. *In vivo* labeling indices of basal intra-follicular epidermal keratinocytes were calculated in tissue sections. Labeling indices were similar in wild type and mutant animals either untreated or treated with acetone. We also did not observe a significant difference between genotypes in labeling index after a single treatment with 0.8 μ g or 4 μ g of TPA (not shown). Even after 4 treatments of 0.8 μ g TPA over two weeks, no difference in labeling index was observed between genotypes. Because wild type mice had a high degree of proliferation ($37 \pm 5.3\%$, $n=4$) under these conditions, we treated two sets of mice with a higher dose (4 μ g) of TPA over two weeks. Similar epidermal hyperplasia (7-9 layers) was observed in animals of both genotypes. Under these conditions a difference between the wild type and *Nf1* $\pm$ keratinocytes was revealed (Fig. 12). A consistent increase in epidermal labeling index was detected in mutant mice (7/7) as compared to 4 wild type animals. This difference was significant ($p < .0001$; Student's *t*-test). Our results on sections from wild type mice can be compared with Naito et al. (1987), and Kiguchi et al. (1997), who showed a $10.8 \pm 2.1\%$ and $\sim 8\%$ labeling index using this protocol on C57Bl/6 mice. Values for individual wild type and mutant animals are shown in Fig. 13C. The results show that *Nf1* $\pm$ keratinocytes abnormally sustain proliferation in response to TPA.

Discussion

We demonstrated a cooperating effect of mutation in a single *Nf1* allele with activating *ras* mutations to override the genetic resistance of C57Bl/6 mice to skin tumor promotion. Thus, *Nf1* is one of the long-elusive modifier genes for epithelial tumorigenesis in mice. Our data are consistent with a role for *NF1* in human epidermal carcinogenesis. Indeed, regions of LOH including *NF1* have been reported in breast, ovarian and esophageal cancer (Wertheim et al., 1996; Dunn et al., 1999). In addition, *NF1* message is down-regulated in epithelial ovarian cancer (Iyengar et al., 1999) and neurofibromin expression is down-regulated in urinary bladder transitional cell carcinogenesis (Aaltonen et al., 1999).

DMBA-treated *Nf1*^{+/-} mice were twice as likely as wild type mice to develop pigmented skin patches. NF1 patients develop pigmented patches with greatly increased frequency as compared to normal humans (Riccardi, 1992; Korf et al., 1992). In humans, melanocytes and epidermal keratinocytes in café-au-lait macules contain "macromelanosomes" (Benedict et al., 1968; Takahashi, 1976; Malhotra and Ratner, 1994). In the mouse pigment was in keratinocytes in hair follicles, not in the epidermis, with no evidence for macromelanosomes. The storage of pigment and the location of the pigmented cells are different in mouse and human skin (Miller et al., 1993), possibly accounting for these differences.

DMBA was required for pigmented patch formation; patches were elicited neither by acetone nor by TPA alone. DMBA may have direct effects on skin cells, or act as a mutagen (reviewed in DiGiovanni, 1992; Quintanilla et al., 1986). Increased numbers of patches in *Nf1* mutants could be explained if mutants have increased numbers of stem cells that are targets for DMBA-induced mutation, or if the *Nf1* mutation increases the frequency of mutations in the stem cell population. However, wild type and mutant mice treated with

DMBA each had cells with the potential to form pigmented patches, because DMBA + TPA treatment caused patches to develop in all mice. Therefore we speculate that DMBA-initiated cells with reduced neurofibromin are more likely to proliferate and form pigmented patches in the absence of TPA than are wild type cells.

Keratinocytes and/or melanocytes may be responsible for increased pigmentation in response to DMBA. It is unlikely that pigmentation is due solely to elevated Ras-GTP in melanocytes. We failed to detect *H-Ras* mutation in pigmented patches, even though the assay used detects such mutations in as few as 1 in 10^8 cells (Quintinalla et al., 1986; Mitchell and Warshawsky, 1998), and Greisser et al. (1995) failed to find altered Ras-GTP levels in melanocytes from NF1 patients. Also, while hyperpigmentation does result in mice in which melanocytes express *v-H-Ras* (Powell et al., 1995), melanogenesis is present in hair bulbs and cells within the dermis and not almost exclusively associated with hair follicles as in *Nf1* mice.

In the mouse, proliferation of follicular keratinocytes correlates with melanogenesis in the anagen phase of the hair follicle cycle (reviewed in Slominiski et al., 1993). Melanin produced in follicular melanocytes is transferred to precortical keratinocytes (Chase, 1954; reviewed in Slominski et al., 1993), leading to visible pigmentation. Our analysis of DMBA-treated pigmented patches revealed skin with features of the anagen phase of the hair cycle (Hansen, et al., 1984; Miller et al., 1993). Pigmented skin contained numerous large hair follicles with actively dividing follicular keratinocytes, significantly increased dermal thickness, and numerous enlarged sebaceous glands. We therefore hypothesize that the target cell of the pigmentation response stimulated by DMBA is the keratinocyte (or its stem cell).

Increased papilloma formation in *Nf1* mutants is also consistent with the affected cell being the keratinocyte or its precursor. Initiated cells proliferate during tumor promotion,

eventually forming papillomas with expansion of the epidermal keratinocyte population (Scribner et al., 1978; DiGiovanni, 1992). Both the initiated papilloma precursor and the pigmented spot precursor may be follicular bulge cells (Binder et al., 1997).

C57Bl/6 is a tumor- promoter resistant mouse strain (reviewed in DiGiovanni, 1992). While no previous studies utilized exactly the doses of DMBA and TPA used here, and none used male mice, using female mice investigators reported no papillomas (Kiguchi et al., 1997) or few papillomas (Reiners et al., 1984) in wild type C57Bl/6 mice treated with these carcinogens. Others reported low numbers of papillomas (Chouroulinkov et al., 1988; O'Brien et al., 1997). In our experiments none of the wild type animals, littermates of mutants and backcrossed at least 10 generations onto the C57Bl/6 background, treated with DMBA and TPA developed tumors. In contrast, papillomas arose in 75% of *Nf1* heterozygous mice treated using this paradigm, demonstrating that loss of one *Nf1* allele is sufficient to increase the frequency of papilloma formation in a resistant strain of mouse. The question of whether complete loss of *Nf1* is required for the papilloma formation described here remains open. Preliminary experiments (not shown) have failed to show LOH using PCR analysis. However, DMBA is a point mutagen and point mutations affecting *Nf1* cannot be excluded.

Mutational analysis revealed activating H-*Ras* mutations at codon 61 in all (n=6) of tested papillomas. These data are consistent with previous reports that have shown 90% of papillomas tested have an A to T transversion mutation in codon 61 of the c-H-*ras* gene (Quintanilla et al., 1986). Indeed, activated *ras* targeted to keratinocytes can serve as an initiating event in skin carcinogenesis models (Roop et al., 1986; Leder et al., 1990; Greenhalgh et al., 1993; Hansen and Tenant, 1994a,b; Brown et al., 1998). It is believed that populations of initiated cells expand during the promotion phase and manifest as papillomas

(DiGiovanni, 1992). We therefore have shown that functional inactivation of at least a single allele of the *Nf1* gene cooperates with activating mutations in the *ras* gene to enhance the papilloma phenotype in *Nf1*^{+/-} mice on a resistant genetic background.

The co-operative effects between *Ras* and *Nf1* that we have defined in keratinocytes could act downstream of Ras or independent of Ras. Neurofibromin may function in non-Ras pathways in keratinocytes, just as it appears to do in several other cell types (Johnson et al., 1994; Griesser et al., 1997; Guo et al., 1997; Kim et al., 1997; Atit et al., 1999).

Alternatively, decreased levels of neurofibromin may increase Ras-GTP in keratinocytes, increasing signaling downstream of Ras-GTP and providing an effect additive with mutationally activated *H-Ras*. The idea that Ras-GTP higher than achieved by a single mutated *H-Ras* allele can contribute to epidermal tumorigenesis is consistent with results of Bremmer et al. (1994) who showed that most papillomas carrying mutant *H-Ras* alleles are trisomic for chromosome 7, increasing the copy number of mutant *H-Ras*. Manges et al. (1998) showed that *Nf1*^{+/-} mice over-expressing N-Ras driven by the MMTV promoter are at increased risk for developing lymphomas. Just as in our study, loss of *Nf1* co-operates with increased Ras activity (from over expression or activating mutations) to increase tumor incidence.

Nf1 mutant mouse keratinocytes show sustained proliferation in response to TPA. This result is consistent with data showing that strains of mice susceptible to papilloma formation have sustained epidermal proliferation in response to TPA (Naito et al., 1987; Kiguchi et al., 1997). TPA activates PKC, which is essential for regulation of genes involved in keratinocyte differentiation (Dlugosz and Yuspa, 1993, 1994; Lee et al., 1998). Our data suggest that neurofibromin normally down-regulates TPA-mediated signaling pathways in

keratinocytes. Our finding that *Nf1* hemizygous mouse keratinocytes have increased proliferative potential likely accounts for the observed papilloma formation.

Like mechanical wounding, topical application of skin carcinogens injures the skin and induces a wound-healing response (Scribner, 1978). Riccardi hypothesized a role for injury in pigmentation defects and tumor formation in NF1 patients (Riccardi, 1992). We have demonstrated that mutation in a single *Nf1* allele in mice alters the susceptibility of skin to pigmentation and tumors induced by carcinogens. Our data are consistent with a role for keratinocytes, and perhaps injury, in the abnormal skin pigmentation characteristic of NF1 patients.

Acknowledgements:

We thank Drs. George Mashour and Andreas Kurtz (Georgetown University) for sharing information concerning DMBA treatment of *Nf1* mutant mice. We also thank Dr. Stuart Yuspa (NCI) for suggesting use of *in vivo* labeling and Dr. Claudio Conti (University of Texas MD Anderson Cancer Center) for analysis of sections of papillomas and Laura James (Shriner's Hospital, Cincinnati) for statistical analysis.

Task 4: Develop transgenic mice with altered Ras-GTP levels in Schwann cells to test the hypothesis that this alteration is necessary and/or sufficient to explain the nerve transection induced phenotype observed with *Nf1/nf1* mice.

Several lines of evidence suggest that increased levels of Ras-GTP contribute to cellular abnormalities that underlie features of type 1 neurofibromatosis disease. For example, NF1-deficient Schwann cells derived from a knock-out animal model have characteristics similar to those derived from neurofibromas, including elevated levels of Ras-GTP. In addition, some of the abnormal phenotypes found in *Nf1*-deficient mouse Schwann cells can be reversed by an inhibitor of Ras activation, FPTI (Kim, et al., 1997). These observations support the idea that a normal function of neurofibromin in Schwann cells is regulation of Ras-GTP levels. *Nf1/nf1* heterozygous mouse nerves appear normal, however, upon nerve lesion they form rare Schwann cell tumors and show hyperpigmentation (see above). We postulate that altered Ras-GTP levels in Schwann cells are involved in the development of wound-related phenotypes in *Nf1/nf1* mice. To test this hypothesis, we have chosen to utilize Schwann cell-specific gene promoters to drive expression of activated Ras and the GAP-related domain (GRD) of neurofibromin in transgenic mice. Mice expressing constitutively active Ras may mimic the *Nf1* mutant mice, whereas expressing GRD may rescue the mutant phenotype.

4A. Creating Schwann cell-specific Ras and GRD constructs.

Previously, the P0 promoter was the only Schwann cell-specific gene promoter available. Because we have experienced difficulty expressing transgenes with this promoter, we have chosen an alternative promoter derived from the 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) gene to drive expression in Schwann cells. CNP expression is restricted to oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous

system. Unlike P₀ expression levels which fluctuate dependent upon myelination events, and in regeneration, CNP is expressed at a relatively constant level throughout development of the peripheral nervous system and after nerve injury (Edwards and Braun, 1988; Stahl, et al., 1990). Therefore, the CNP upstream sequence may serve as general, glial cell-specific promoter not dramatically influenced by Schwann cell differentiation. This promoter drove expression of a β -galactosidase-neomycin phosphotransferase fusion gene, called GEO, both *in vitro* and *in vivo* (Chandross, et al., 1999). To validate this construct in our laboratory, we transiently transfected primary rat Schwann cells and stained with the β -galactosidase antibody; the construct expressed well. In contrast we were never able to detect expression of β -galactosidase after transient transfection of cultured Schwann cells with a P₀-LacZ construct. Having this positive control, we have chosen to repeat the experiments described for the P₀-Ras and P₀-GRD constructs in the original grant utilizing the CNP promoter.

Driving expression of the GRD and Ras transgenes with the CNP promoter promoter will be a major advantage in the wounding experiments. Following nerve injury, P₀ expression is downregulated (Gupta, et al., 1988). If the introduced transgene is under the control of the P₀ promoter, it may not be expressed at a high enough level at this critical moment. In other words, by the time the transgene is expressed, events leading to the wounding-induced phenotype may have already occurred unaffected by the introduced gene. Therefore, absence of a change in phenotype induced by P₀-driven mutant Ras and GRD may not necessarily indicate the Ras pathway is not involved. Unlike the P₀ gene, CNP expression is slightly upregulated in response to nerve injury (LeBlanc, et al., 1992). Therefore, CNP-driven transgenes will be more likely to be expressed at sufficient levels after wounding.

Both isoforms of the GRD of human *NF1*, GRD1 and GRD2, were subcloned downstream of the functional CNP promoter fragment. Transcript level and GAP activity of GRD2 is reduced *in vivo* compared to GRD1 (Huynh, et al., 1994), however, the type 2 transcript predominates in differentiating Schwann cells (Gutmann, et al., 1993). Therefore, transgenic mice expressing GRD1 and GRD2 may have different phenotypes. Creating the CNP-GRD1 and CNP-GRD2 constructs was similar to the protocol described in our original grant for cloning downstream of the P₀ promoter. Both GRD cDNA sequences were amplified by PCR to include a 5' HindIII site, a 3' NcoI site, and a 5' HA tag for subsequent detection with an HA antibody. The HA tag was chosen because, unlike the myc antibody required to observe transgene expression in our original P₀ constructs, the HA antibody was not raised in mice. Therefore, less non-specific background signal should be detected in the transgenic mice with the HA antibody. The CNP-GEO plasmid was digested with HindIII and NcoI to remove the GEO sequence, allowing for directional cloning of the GRD PCR products downstream of the CNP promoter. Transformed bacterial colonies were screened by colony PCR followed by restriction mapping and sequencing. Extensive screening was required to identify positive clones, possibly due to an inherent susceptibility of the GRD fragment to rearrangement.

A mutated version of Ras, Ras12V (Capon et al., 1983), was also subcloned into a CNP expression vector. The Ras12V mutant activates all known pathways downstream of Ras. Because there is an internal HindIII site in the Ras cDNA, an alternate cloning strategy was conducted to create CNP-Ras12V. The original CNP constructs we received contained limited restriction sites for subcloning. Therefore, to accomplish the cloning of the Ras12V and to facilitate future cloning endeavors a CNP expression vector was created. We chose the pCMV-Script expression vector (Stratagene) as a template for several reasons: 1) The

multiple cloning site (MCS) contains 15 restriction sites providing a simple cloning strategy for several different fragments. 2) Downstream of the MCS lies a termination sequence that can be utilized by the inserted gene. 3) The vector size is small (~ 4 kb), aiding in transfection efficiency. 4) The neomycin resistance gene is controlled by both a prokaryotic and a eukaryotic promoter, allowing for selection with kanamycin in bacterial cells and G418 in mammalian cells.

The CMV promoter was removed from pCMV-Script with *VspI* and *SacI* and replaced with a 50 bp polylinker, created by dual primed extension of oligonucleotides containing 8 additional restriction sites. This polylinker allowed for insertion of the CNP promoter while preserving restriction sites within the MCS. The modified Script construct was cut with *Psp1406I*, filled out with Klenow to create a blunt end, and then digested with *SpeI*. A pSKII vector containing the CNP promoter region was digested with *HindIII*, filled out with Klenow to create a blunt end, and then cut with *XbaI* to remove the functional promoter fragment used in CNP-GEO. *SpeI* and *XbaI* share compatible ends, allowing for semi-directional cloning of the CNP promoter into the Script backbone. The Ras cDNA sequences were amplified with primers containing a 5' *ClaI* site followed by an HA tag and a 3' *XhoI* site. PCR products were digested and cloned into these sites in the MCS, between the CNP promoter and the termination sequence. Positive clones were confirmed by restriction mapping and sequencing.

3B. Test Ras and GRD constructs *in vitro* and inject into blastocysts to create transgenic mice.

To show the CNP constructs were functional, transient transfections into primary rat Schwann cells were conducted. Expression was detected with the HA antibody. These constructs were then prepared for *in vivo* studies. The functional expressing unit containing

the CNP promoter followed by the inserted gene sequence and the termination sequence was excised and purified for injection into blastocysts.

Seven injections into 120 embryos each were performed with the CNP-GRD1 and CNP-GRD2 constructs. Pups were characterized by PCR for the presence of the introduced transgene resulting in only six possible founders. Breeding was initiated to obtain an F1 generation for each putative founder. Two of the three CNP-GRD1 possible founders transmitted the transgene to their offspring, as determined by PCR. The low percentage of founder mice was surprising. In contrast, 12 of 65 embryos carried the transgene at embryo day 12.5, suggesting that lethality was associated with transgene expression.

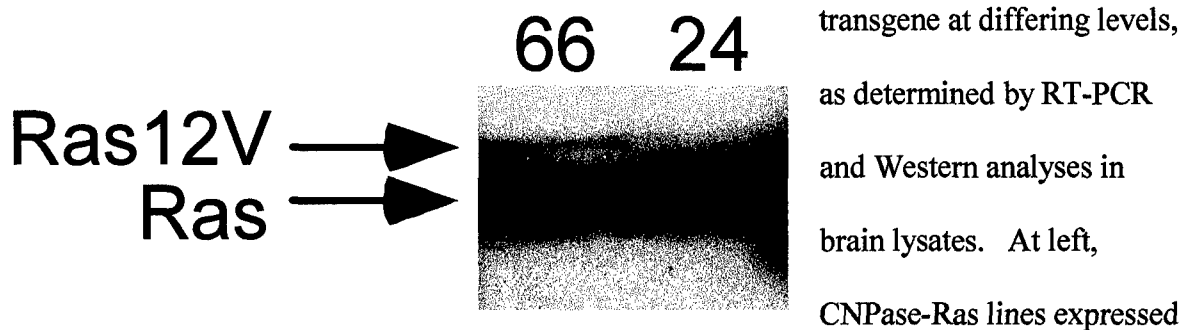
Two founders containing CNP-GRD2 were identified and transmitted the transgene. Expression of the transgene was verified in all five lines by RT-PCR amplification in brain mRNA. One CNP-GRD1 line was bred to homozygosity to increase transgene expression level. Protein analysis in tissue specimens and cultured cells failed to detect GRD protein in these mice. Furthermore, sequencing of genomic DNA from each of the five lines shows mutation that caused deletion of the HA-tag and in two lines deletion of the initiation methionine. We surmise that mutation occurred *in vivo*, but the mechanism of the effect is unknown.

Thus two factors appear to have made generation of GRD mice unsuccessful. First, GRD expression is likely lethal. Second, transgene rearrangement *in vivo* may interfere with protein expression.

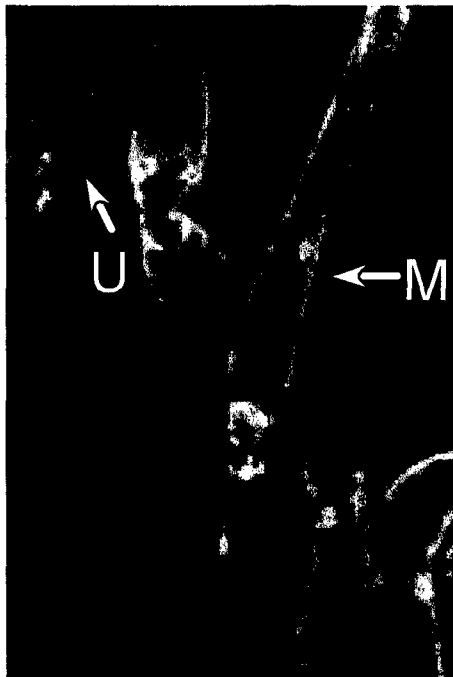
In spite of our failure to detect GRD protein, one of the lines was mated to *Nf1* to test if low levels of expression might rescue NF1 lethality. None of 22 pups born were *Nf1*^{-/-} GRD⁺. None of 13 embryos (day 14.5) were *Nf1*^{-/-} GRD⁺. Based on these results we are sacrificing all these GRD mice.

3C. Characterize phenotypes of the transgenic strains and breed strains to *Nf1/nf1*

mice. Eight putative founders carrying the CNP-Ras12V transgene were obtained. Seven of the putative founders transmitted the transgene to offspring; three of the seven expressed the



the transgene on a Western blot using an anti-H,N,K-Ras antibody. The mutant protein runs slower than normal Ras, as described by other investigators. The figure shows line #66, an expressing line, in comparison to a line that does not express mutant protein (#24). The slower running species (12V) was also detected with an anti-HA antibody (not shown).



To confirm expression of Ras by Schwann cells, we made teased nerve preparations from adult transgenic mouse nerves. Nerves were fixed and stained with a commercial anti-HA antibody followed by fluorescent secondary antibody. Anti-HA reactivity (red) was found at the cell membranes of myelinated (M) and unmyelinated (U) fibers, external to anti-S100 marking of Schwann cell cytoplasmic staining (green). The unstained channel reflects the axon and myelin sheath of the myelinated fibers.

Thus the overexpressed mutant Ras localizes to the plasma membrane.

In addition, preliminary electron microscopy demonstrates that elevated Ras activity in Schwann cells alters nerve organization. Specifically, myelin sheaths appear thicker than normal, and unmyelinated fiber bundles are disorganized. This phenotype will be assessed in additional CNP-Ras12V animals.

3D. Lesion nerves of CNP-Ras12V transgenic mice and analyze phenotype.

Once we complete analysis of the Ras12V expressing in mice, we will lesion nerves. After a one to three month recovery period, nerves will be analyzed for wounding response and compared to wild type.

3E. Lesion nerves of GRD expressing mice and *Nf1/nf1*-GRD double transgenic mice and analyze phenotype. We were not able to do these experiments as we do not have convincing GRD expression.

Key Research Accomplishments

- We now understand how pigmentation arises after nerve lesion in *Nf1/nf1* mice. The defect has two parts. One is a cell autonomous defect in mutant Schwann cells that at least in part can be ascribed to increased cell migration. The second is a non-cell autonomous effect of mutant environment mediated through a GM-CSF receptor-signaling pathway. This work is submitted for publication.
- We showed that *Nf1/nf1* mice developed aberrant pigmentation and benign keratinocyte tumors after chemical carcinogenesis. This study published and is consistent with our hypothesis that wounding abnormally affects *Nf1/nf1* mice and, significantly, demonstrates that effects of *Nf1* mutation synergizes with Ras activation in cells.
- We made a new CNPase promoter cassette and cloned all constructs into it; all constructs were tested *in vitro* and injected into blastocysts; we developed new mice expressing the V12-Ha-Ras in Schwann cells.

Reportable outcomes:

Publications resulting from the research effort:

1. Atit, R.A., Mitchell, K., Nguyen, L, Warshawsky, D. and Ratner, N. (2000) The neurofibromatosis type 1 (*Nf1*) tumor suppressor is a modifier of carcinogen-induced pigmentation and papilloma formation in C57Bl/6 mice. *J. Invest. Dermatol.*, 114:1093-1100.
2. Ratner, N. and Daston, M.M. (2001) Neurofibromatosis type 1 and Neurofibromatosis type 2: Genetic and cellular mechanisms of peripheral nerve tumor formation. *Glial Cell Development: Basic Principles and Clinical Relevance* Jessen, K.R. and Richardson, W.D. Oxford University Press, in press.
3. Rizvi, T.A., Ling, B., Sarangarajan, R., Sidani, A., Huang, Y.N., Boissy, R. Largesprada, D. and Ratner, N. (2001) Adult nerve injury induces pigmentation by adult nerve cells, submitted.

Abstracts:

1. Ratner, N., Atit, R. Kim, H.A., Ling, B., DeClue, J.A., Crowe, M. and Rizvi, T.A. (1998) Neurofibromatosis type 1: Genetic and cellular mechanisms of peripheral nerve tumor formation. *J. Cancer Res. Clin. Oncol.*, 124:S15.
2. Rizvi, T., Atit, R.A., and Ratner, N. (1999) Transdifferentiation of Neurofibromatosis type-1 mutant Schwann Cells into Melanocytes following wounding of *Nf1/nf1* mouse nerve. *Soc. Neurosci.*, 25, 740a.
3. Ratner, N., Atit, R.A., and Rizvi, T. (1999) Transdifferentiation of Neurofibromatosis type-1 mutant Schwann Cells into Melanocytes following wounding of *Nf1/nf1* mouse nerve. *J. Neurotrauma*
4. Ratner, N., Atit, R., Sherman, L.S., Crowe, M., Cox, A.D. and Wenstrup, R. (1999) Evidence in support of Ras-GTP dependent and independent abnormalities in *NF1*-mutant cells revealed by a new *in situ* Ras-activation assay and by skin wounding. *Medizinische Genetik*, 11: 483a- 484a.
5. Oral Presentation of data at the International Consortium for the Molecular Biology of NF1 and NF2; MIT, Cambridge, MA, 1999; Aspen, CO, 2000; US-Japan Conference on Phakomatoses, Boston, MA 2000

Patents: None

Development of animal models:

CNPase-V12-Ha-Ras transgenic mouse lines (3)
CNPase-V12-Ha-Ras mice on an *Nf1* +/- background

Funding applied for based on work supported by this award:

DOD 1999 (not funded)
NIH 1999 (not funded)
NIH 2000 (funded)
MS 2000 (funded)
DOD 2001, under review

Research Opportunities based on experience/training via this award:

Radhika Atit: Trained as a graduate student, obtained Ph.D. degree from UC, now working in a Hughes-funded laboratory (L. Nieswander) at Sloan-Kettering-Institute, New York
Radhika's salary was from other sources her experiments were paid by this award.

Shyra Miller: Current postdoc, now funded to analyze the Ras transgenics by a fellowship from the National MS Society

Jason Bowersock and Patricia Fulkerson were undergraduates assisting with this project. For each it was a first research experience. Jason is now starting Medical School at University of Dayton while Patty is entering her third year as an MSTP student at University of Cincinnati.

Personnel Paid:

Nancy Ratner (CV attached), PI

Tilat Rizvi (CV attached), co-PI

Shyra Miller, (postdoc)

Genotyping:

Jason Bowersock

Patricia Fulkerson

Technical help (animal colony):

Yuan Huang

Lam Nguyen

Kenyon Ogburn

Technical help (EM technician):

Maureen Fitzgerald

Conclusions

Importance and Implications of the studies: Our studies tested the hypothesis that wounding can initiate features of human NF1 in transgenic mice. We demonstrated that wounding does cause features of human NF1 in mutant mice. We also defined relevance of the GM-CSF receptor-signaling cascade to the effect. By extrapolation of these results in mice to humans, some pigmentation in NF1 patients could result from transdifferentiation of Schwann cells into pigment-forming melanocytes. Abnormal plasticity of *Nf1* mutant glial cells may also be relevant to tumorigenesis in NF1, and be regulated through the same signaling cascade. In this light it is remarkable that myeloid tumor formation in *Nf1* mice is regulated via GM-CSF.

It appears plausible that Ras regulation is a major, if not the sole, function of the NF1 protein in vertebrates. The experiments we conducted have revealed that Ras-GTP expression is not synonymous with *Nf1* mutation. First, our CNPase Ras-GTP mice (with predicted high Ras-GTP) have a different phenotype from *Nf1* mice (with high Ras-GTP). Second, Ras-GTP elevation via mutation synergizes with *Nf1* mutation effects on skin carcinogenesis. These results could be ascribed to Ras-GTP dosage effects and/or non-Ras effects of NF1.

Changes to future studies to address the problem: We generated an important new reagent in mice overexpressing v-Ha-Ras specifically in Schwann cells, but failed to generate stable GRD-expressing mice. Studies are underway to analyze the CNPase-12V-Ha-Ras mice, 12V-Ha-Ras x *Nf1* +/- mice, and to generate mice that stably express the *Nf1*-GRD.

So What? Mouse models of neurofibromatosis type 1 are urgently needed. Models can be used to test proposed therapies, and to suggest new pathways for therapeutic intervention. Our studies generated two models that partially mimic NF1 disease. In one, nerve wounding, we found that a cytokine signaling pathway is important: therapeutics based on this pathway may be useful in NF1 patients. In a skin carcinogenesis model we showed that *Nf1* loss modifies skin

tumor formation. The data are consistent with a role for NF1 in skin cancer, as well as the cancers associated with familial NF1.

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Appendices

FIGURE 1: Hyperpigmentation in C57Bl/6 adult mice after nerve injury: A - D: Gross photographs of mice hind limb areas 1 month after nerve transection and/or deflection. Skin has been removed. (A): Wild type mouse with nerve cut shows little pigmentation (arrows). (B): *Nf1/nf1* mouse with nerve cut shows slightly more pigmentation compared to A. (C): Wild type mouse with nerve transection and deflection of the nerve shows some pigmentation. (D): *Nf1/nf1* mouse with nerve transection and deflection shows dramatic clusters of highly pigmented cells (arrowheads). (E): Toluidine blue stained plastic section of skin from the lesion area of a *Nf1*^{+/-} mouse 3 months after nerve cut. Clusters of pigmented cells present in the fascia underneath the hypodermis are within the black box. Bar = 100 μ m. This area was evaluated at the EM level and is shown in (F), showing cytoplasmic pigment granules characteristic of a melanocyte (magnification X 2700). (G): Gross photograph of a ventral view of mutant mouse skin over lesioned area 1 month after nerve transection showing pigmentation (arrows). (H): In a mutant mouse 1 month after nerve transection, spots of pigmentation extended ventral to the sciatic nerve (arrows).

FIGURE 2. Pigmented and unpigmented tumors in *Nf1/nf1* mutant mice three months after nerve transection: (A) Hindlimb portion of a fixed mutant mouse, three months after nerve cut (skin has been removed). An unpigmented tumor, delineated by arrows, is on the left side of the midline of the body. Pigmentation is evident in muscle lateral to the tumor. (B): Hindlimb portion of an *Nf1*^{+/-} mouse with a pigmented tumor, delineated by arrowheads. Arrows point to pigmented spots adjacent to the tumor. (C): Toluidine blue stained semithin plastic section showing the pigmented tumor in B, with capsule. Bar = 200 μ m. (D) A non-pigmented tumor in paraffin section after H and E staining, showing tumor infiltration between muscles and lack of a capsule. (E) Gomori's trichrome staining of an adjacent section reveals that this tumor is full of collagen rich

matrix and highlights collagen-rich bundles infiltrating nearby muscle (arrows). Bar D and E = 200 μ m. (F): Anti-S-100 staining (brown, DAB reaction product) of an adjacent section of the same tumor as D and E, at higher magnification. The section was counterstained with Methyl Green to show S-100-negative cells. (G): Anti-neurofilament staining of an adjacent section reveals the presence of axons within the tumor. Bar F and G = 20 μ m.

FIGURE 3: Cells from adult nerve can become pigmented: (A): Schematic diagram of nerve grafting technique. 1.: 1-2 mm sciatic nerve fragments from adult mice were labeled with Hoechst dye in vitro. 2: Hoechst labeled nerve fragments were transplanted into recipient adult mouse nerve by suturing the dye labeled nerve fragment between the cut ends of the host nerve. 3.: The dye labeled nerve fragment was cut and cut ends deflected. (4): 30 days later, animals were sacrificed, areas of pigmentation cut on a cryostat and dye positive cells evaluated for pigmentation. (B-D): Cryosection from a mouse in which an *Nf1*^{+/-} nerve segment was grafted into an *Nf1*^{+/-} host. Dye-positive blue cells are detectable within the endoneurium (END) and adjacent epineurium (Epi). Pigmented cells (arrowheads) are in the epineurium. (B): Hoechst labeled cells (blue nuclei, arrows) in endoneurium and epineurium were visualized with a DAPI filter. (C): Note black (pigmented) cells visualized by bright field microscopy. (D): Bright field and DAPI. (E-G): Cryosections of labeled cells at higher magnification; sections were taken from muscle distant from the transection site, 1 month after dye labeled nerve graft and nerve transection. These are from a different animal from B – D. Both donor and recipient were *Nf1/nf1*. B – D, bar = 100 μ m. (E): Hoechst labeled cells (blue nuclei) in the muscle using a DAPI filter. Arrow represents the area of pigmented cells seen in F and G. (F): Black, pigmented cells shown in bright field. (G):

Bright field and DAPI showing blue nucleus closely surrounded by pigment granules. E – G, bar = 20µm.

FIGURE 4: Pigmentation in an albino mouse (*tyr*^{-/-}) after a nerve graft from C57Bl/6 *nfl/nfl* mouse. (A): Dorsal view of the white skin of an albino host, showing absence of black hairs, 1 month after grafting of a 2.0 mm segment of nerve from a C57Bl/6 *Nf1/nf1* mouse, followed by cut and deflection of the grafted nerve. The skin remains white indicating absence of pigmented melanocytes in this mutant. (B): Ventral side of the skin overlying the graft region showing pigmented spots in the skin. C and D: Two examples of pigmented cells (brown/black pigment granules) in cryosections within muscle overlying the grafted nerve segment after staining of sections with H and E to highlight nuclei and cytoplasm. In (C), note clusters of pigmented cells (arrows). In (D), note individual cells with visible nuclei and pigment granules in the cytoplasm (arrows). C and D, bar = 10µm.

FIGURE 5: Hyperpigmentation after nerve and cell grafting: In A, 2 mm lengths of sciatic nerve from animals of designated genotype (under x axis) were Hoeschst labeled then grafted into recipient hosts of designated genotype (under arrows). Two – four thousand dye-labeled cells were counted in sections from each animal. Each section was analyzed using bright field optics then DAPI filter and fluorescence optics for pigmented cells surrounding labeled nuclei and total pigmented cells. Data are shown as an average of results from 2 animals for each condition except *Nf1*^{+/-} into *Nf1*^{+/-} for which 4 animals were used. The percentage of pigmented dye-labeled nuclei for each animal was similar (*Nf1*^{+/+} into *Nf1*^{+/+} = 0, 0; *Nf1*^{+/+} into *Nf1*^{+/-} = 2, 5; *Nf1*^{+/-} into *Nf1*^{+/+} = 5, 9; *Nf1*^{+/-} into *Nf1*^{+/-} = 44.6, 46.4, 57, 53.9). In B, wild type fibroblasts (Fb) or wild type Schwann cells (SC), *Nf1* heterozygous (SC^{+/-}) or null (SC^{-/-}) or *Nf1*^{-/-}TXF (-/-TXF) were dye labeled and grafted around

lesion areas of *Nf1* heterozygous host animals (+/-). Results shown are averages of results from sections taken from 3 recipient animals for +/+ cells, 2 animals for *Nf1* +/- and *Nf1* -/- Schwann cells and for *Nf1* -/-TXF 5 animals. Analysis was as in (A). The percentage of pigmented dye-labeled nuclei for each animal was similar (*Nf1*+/+ Schwann cells and fibroblasts into *Nf1* +/- = 0, 0, 0; *Nf1* +/- Schwann cells into *Nf1* +/- = 4.9, 4.0; *Nf1* -/- Schwann cells into *Nf1* +/- = 6.8, 7.8; *Nf1* -/- TXF into *Nf1* +/- = 8.3, 17.1, 9.3, 13.5).

FIGURE 6: Characterization of -/-TXF glial cells: Photomicrographs of immunostained cells. +/+ Schwann cells (SC) and fibroblasts (Fib) were used as positive and negative controls. A, D, G and J represent as -/- TXF Schwann cells; B, E, H and K are +/+ Schwann cells while C, F, I and L are -/- fibroblasts [with the same pattern of expression as +/+ fibroblasts, not shown here]. A-C shows anti-P75 NGFR; D-F, anti-S-100; G-I, anti-GFAP and J-L, anti-smooth muscle actin (SMA) immunostaining. The -/- TXF Schwann cells are p75NGFR, S-100 and GFAP positive. +/+ Schwann cells are also positive for p75NGFR and S-100, but show much weaker staining for GFAP (visible in only the boxed inset in 6H, a longer exposure than 6H, surrounding inset). Exposure times for G, H and I were matched. Fibroblasts are strongly positive for SMA. Bar = 50µm.

FIGURE 7: In vitro migration assays using mouse Schwann cells and Schwann cell conditioned medium. Conditioned media was collected from wild type (+/+ medium), *Nf1* +/- (+/- medium) and *Nf1* -/- (-/- medium) mouse Schwann cells. Conditioned medium from cells of each genotype, or N2 serum-free medium alone (control) was added to +/+, *Nf1* +/- and *Nf1* -/- mouse Schwann cells [as designated under the X axis] plated onto polycarbonate membranes. Designated conditioned media were added to the lower chambers. After 13 hours, filters were fixed and cells

that migrated through the filter counted. This experiment is representative of results from five independent experiments.

FIGURE 8: The *Nf1* mutant phenotype is rescued by loss of the β^c receptor. (A) Nerves were cut and deflected in groups of animals of the genotypes designated under the x-axis. Three weeks after surgery the number of visible pigmented patches/streaks was counted in muscle. Statistical analysis was using Student's t-test and for each pair-wise comparison is designated by lines connecting each two groups. (B) A model depicts the pathway that inhibits of pigmentation after nerve injury. In the left panel after injury levels of cytokines, including GM-CSF and IL-3, increase (Saada et al., 1996). Cytokines binds α subunits, which interact with β^c , resulting in activation of downstream signaling including Ras activation (Bagley et al., 1997), maintenance of Schwann cell differentiation and inhibition of most melanogenesis. The *Nf1* Ras-GAP mediate the β^c signaling, likely by modulating the duration or extent of Ras signaling, but perhaps in addition through other signaling cascades. In the middle panels, when β^c is absent or *Nf1* is mutant, melanogenesis is increased. On the right, when β^c is absent and *Nf1* is mutant in affected cells we postulate (?) that Ras activation is close to wild type levels, so that wild type levels of pigmentation are observed.

Fig. 9: Gross appearance and histology of DMBA treated wild type and *Nf1*^{+/-} skin.

A, *B*, and *C*, show gross photographs of shaved dorsum of mice two months after initiation with two doses of 40 μ g of DMBA. Treated areas are outlined in white. *A* is representative of a wild type animal after initiation; this animal shows no large pigmented spot. Other, affected, animals with large pigmented spots are shown in *B* and *C*. The mouse shown in *B* is wild type; in *C* is a heterozygous mouse. Pigmented areas are designated (P) and

unpigmented areas designated (U). *D*, *E*, and *F* show H&E-stained sections of skin, all at the same magnification. *D* shows initiated skin from an unpigmented area of *Nf1*^{+/-} skin, identical in histology to wild type skin. *E* and *F* show sections from pigmented region of wild type and *Nf1*^{+/-} animals shown in *B* and *C*, respectively. White arrows point to hair follicles filled with pigment; sweat glands are pointed out with black arrowheads. *F* shows a section from a pigmented region with dermal pigmentation that was not cell-associated (black arrow). *G*, *H*, *I* show anti-PCNA immunostaining (brown precipitate shown by black arrowheads) with hematoxylin counterstain of sections from the regions shown in *D*, *E*, *F*. *H*, *I* melanin is present in follicles (black arrows). Bar = 50µm *D-F*, Bar = 10µm *G-I*.

Fig. 10: Numbers of hair follicles are increased in pigmented skin regions in both wild type and *Nf1* mutant mice. Numbers of hair follicles were counted in unpigmented, *A* and pigmented, *B* areas of skin 2 months after initiation. Each bar represents averages of the number of hair follicles in 8-10 fields from at least 5 sections of individual wild type or *Nf1*^{+/-} mice.

Fig. 11: Gross and histological appearance of skin and papillomas from *Nf1*^{+/-} mice after tumor promotion. *A*, gross appearance of a representative affected *Nf1*^{+/-} mouse after initiation and 24 weeks of tumor promotion with 0.8µg of TPA three times per week. Hair was gently shaved to reveal pigmented areas, P and unpigmented areas, U. White arrowheads point to a papilloma on the dorsum of this mouse. *B-F*, are photographs of H&E stained sections. *B*, *C* show the histological appearance of unpigmented, *B* and pigmented, *C* skin from the mouse shown in *A*. Note the marked expansion of hair follicles in *C*. *D* shows a section through a typical exophytic papilloma with epidermal hyperplasia, centrally located

follicular cysts and trapped sweat glands. At higher magnification, keratinocyte hyperplasia (e) is shown in *E* and follicular cysts (c) in *F*, as are the sebaceous glands prominent in most of the papillomas generated. Bar=50 μ m *B*, *C*; Bar = 125 μ m *D*; Bar = 10 μ m *E*, *F*.

Fig. 12: Papilloma incidence and volume in *Nf1*^{+/-} mice. (*A*) shows the incidence of papilloma development over weeks after promotion with TPA. Squares represent papillomas in *Nf1*^{+/-} mice; diamonds represent wild type mice, which never developed papillomas. (*B*) shows volumes of papillomas in *Nf1*^{+/-} mice 24 weeks after promotion with TPA. * = a 80mm³ papilloma.

Fig. 13: Mutational analysis of codon 61 from the *H-ras* gene in treated skin and papillomas

A, *B*, PCR amplification of the A→T transversion at codon 61 from the *H-ras* gene. Presence of the top band (162 bp) indicates the presence of the mutation (arrow). *A*, from pigmented skin (lanes 1, 3) and unpigmented skin (2, 4) from the treated area of wild-type (lanes 1, 2) and *Nf1*^{+/-} (lanes 3,4) animals. Lane 5 and 6 represent negative control and positive controls respectively and M = marker. Data is representative of that collected from wild type (n=4) and *Nf1*^{+/-} (n=5) individual mice. *B*, Papillomas were isolated 24 weeks after tumor promotion with TPA. Lanes 1-4 show results of 4 representative papillomas analyzed from individual animals. Lane 5 shows a water control, lane 6 and 7 represent negative and positive controls respectively. M = marker. *C*, autoradiogram showing reverse cyclic dideoxy sequencing of the mutation within the second base of codon 61 (asterisk) from the *H-ras* gene. All 6 papillomas positive for mutations in the PCR screen were confirmed by sequencing.

FF

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EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

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Professor (1997 -); Associate Professor (1992-1997); Assistant Professor (1987-92); Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati College of Medicine, Cincinnati, OH; Sabbatical (2/95-8/95), Department of Molecular Genetics, University of Cincinnati

PEER REVIEW

1988 - Present Member, Scientific Advisory Board, National Neurofibromatosis Foundation (Co-Chair, 1992-94)

1991 Member, NIH Consensus Panel: Acoustic Neuroma

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HONORS

Burroughs Welcome Fellow of the Life Sciences Research Foundation, 1983-1985

Young Investigator Award, National Neurofibromatosis Foundation, 1986-1988

Harry Weaver Scholar, National Multiple Sclerosis Society, 1987-1992

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SOCIETY MEMBERSHIPS: AACR, American Society of Cell Biology, Society for Neuroscience, American Society of Neurochemistry

Peer-Reviewed Publications (selected):

Ratner, N., Hong, D., Lieberman, M.A., Bunge, R.P. and Glaser, L. (1988) The neuronal cell surface molecule mitogenic for Schwann cells is a heparin-binding protein. *Proc. Natl. Acad. Sci. USA* 85:6992-6996.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME Tilat Aziz Rizvi		POSITION TITLE Research Assistant Professor	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(S)	FIELD OF STUDY
Meerut College, Meerut, India	B.S.	1975	Zoology, Botany, Chemistry
Meerut College, Meerut, India	M.S.	1977	Zoology
All India Inst. of Medical Sciences, New Delhi, India	Ph.D.	1989	Neuroanatomy

RESEARCH AND PROFESSIONAL EXPERIENCE:

POSITIONS

- 1982-89 Research Fellow, All India Institute of Medical Sciences, New Delhi, India
 1989-93 Postdoctoral Assistant, Dept. of Cell Biology, Neurobiology & Anatomy, Univ. of Cincinnati College of Medicine
 1993-present Research Assistant Professor, Dept. of Cell Biology, Neurobiology & Anatomy, Univ. of Cincinnati College of Medicine

HONORS

- 1986 Dr. Dharam Narain Gold Medal for the best paper in Neuroanatomy, Anatomical Society of India, 1986
 1987 Awarded a special Travel Grant from Governor of Hamburg for presenting a paper in "Vth World Congress on Pain" in Hamburg
 1987 Selected for a training course on "Advanced Methods in Neuroanatomy" in Budapest
 1990-93 Academic Challenge Award, Dept. of Anatomy & Cell Biology, Univ. of Cincinnati

PUBLICATIONS

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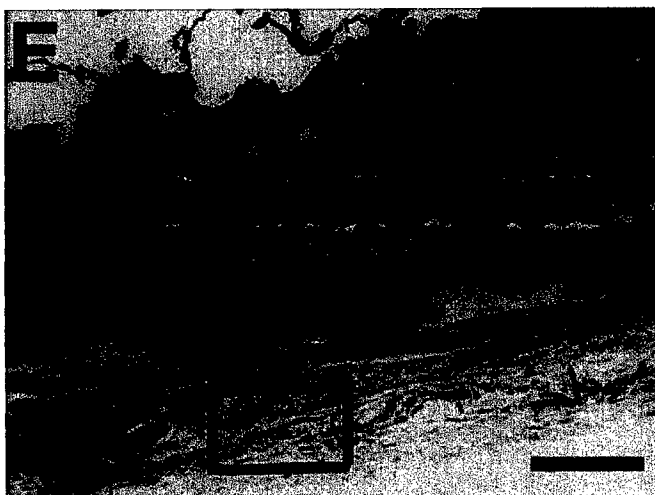
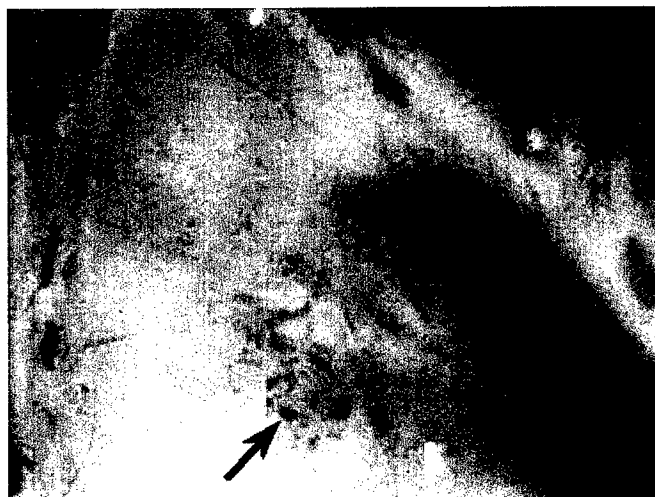


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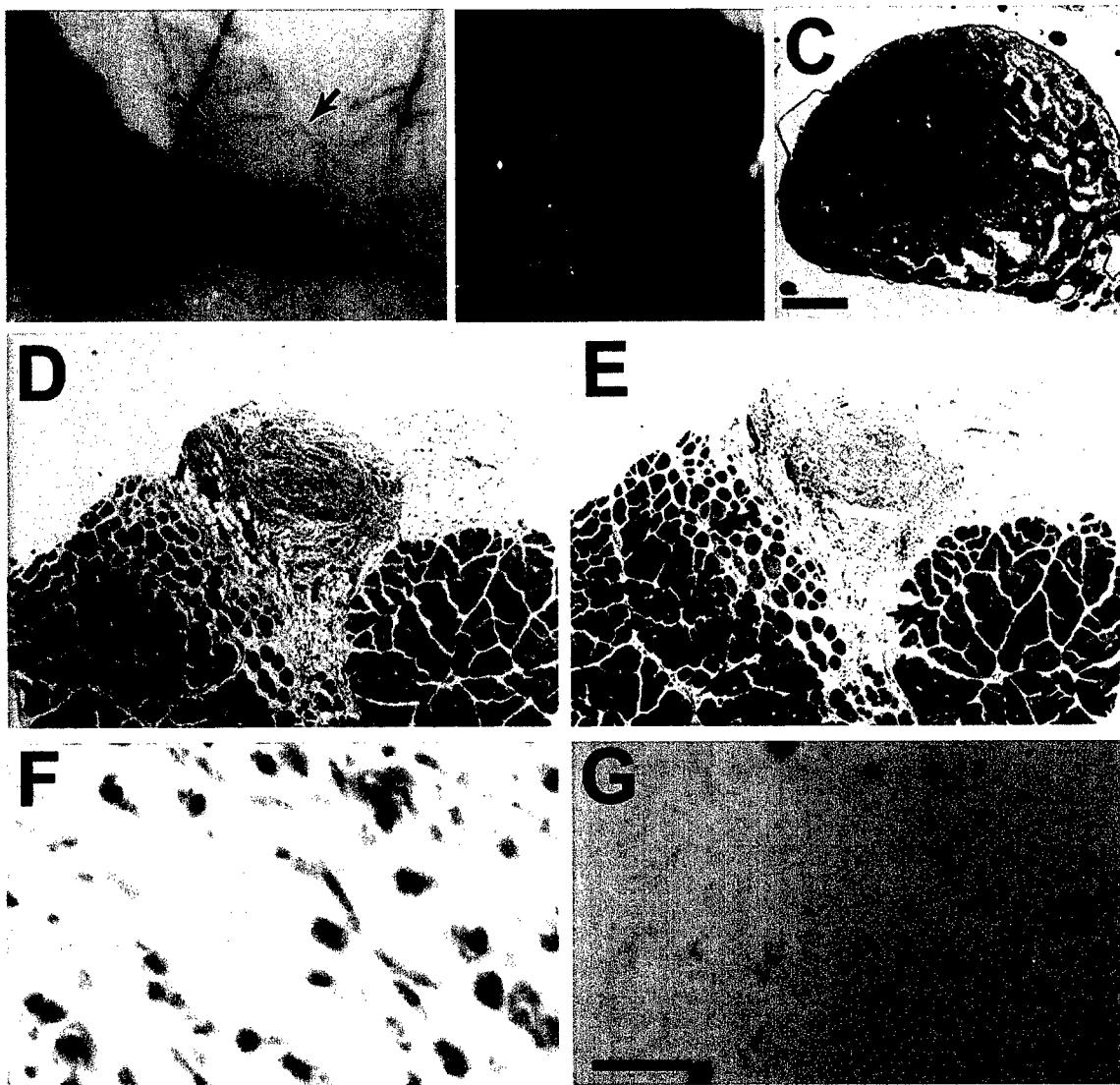
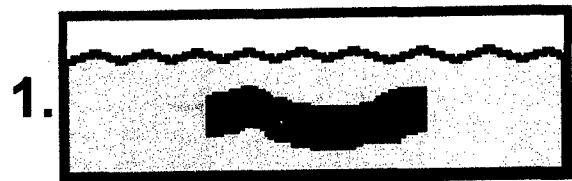
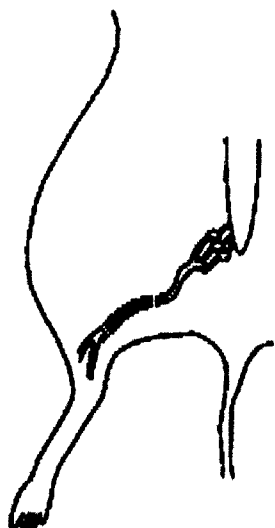


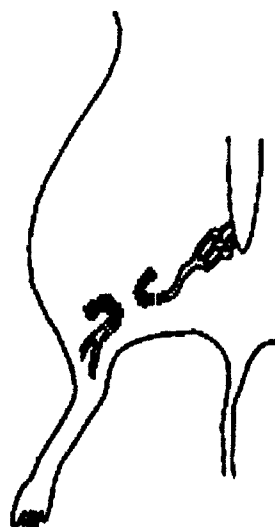
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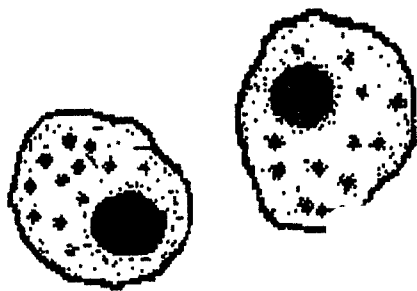
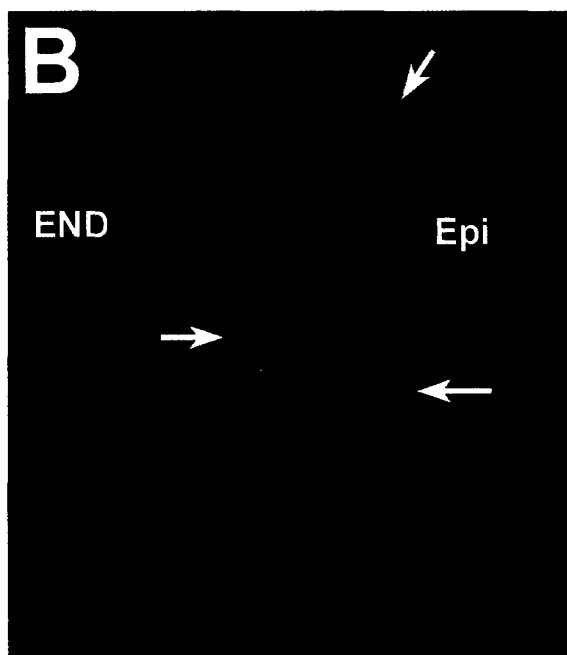
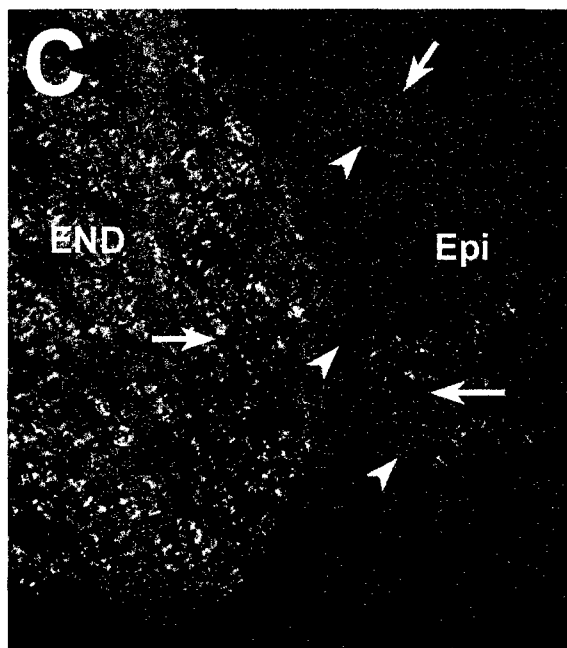
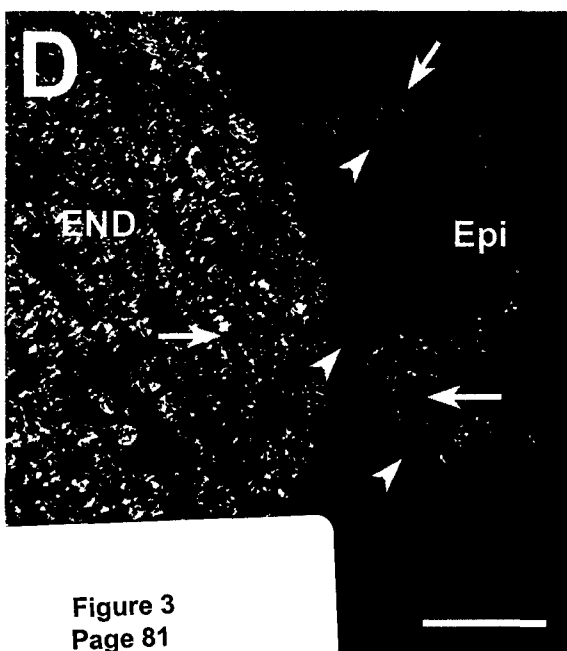
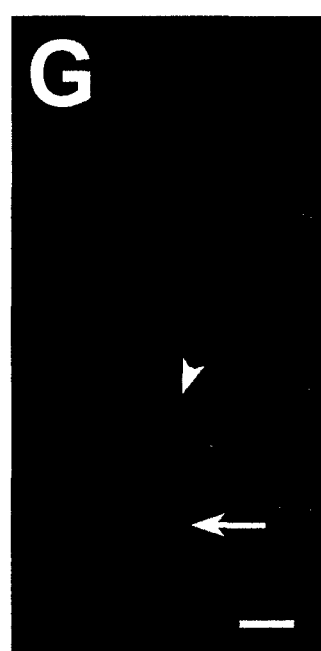
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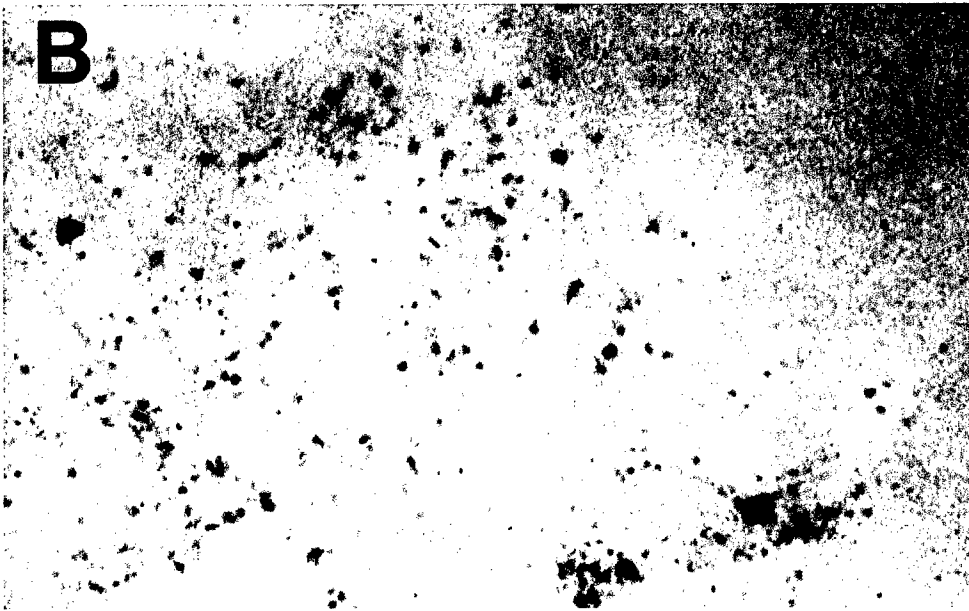
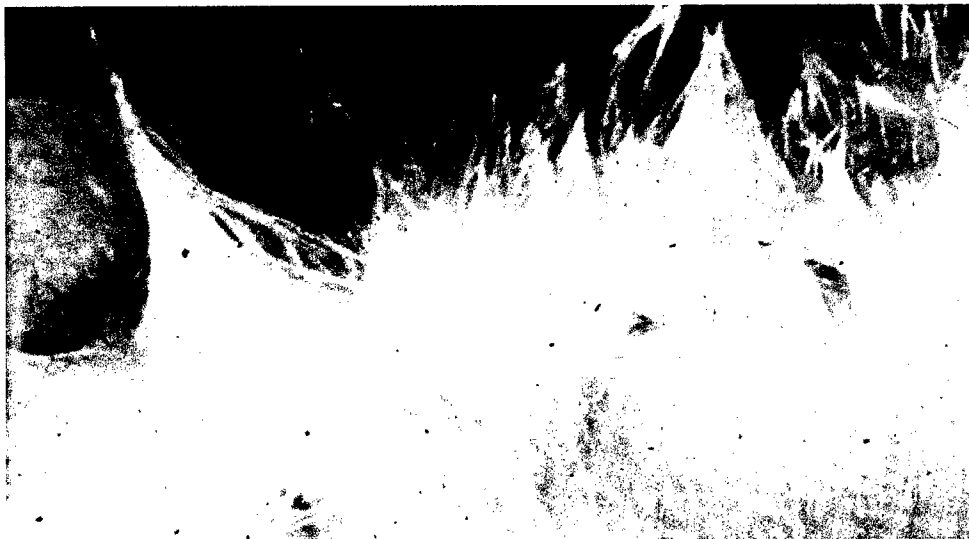


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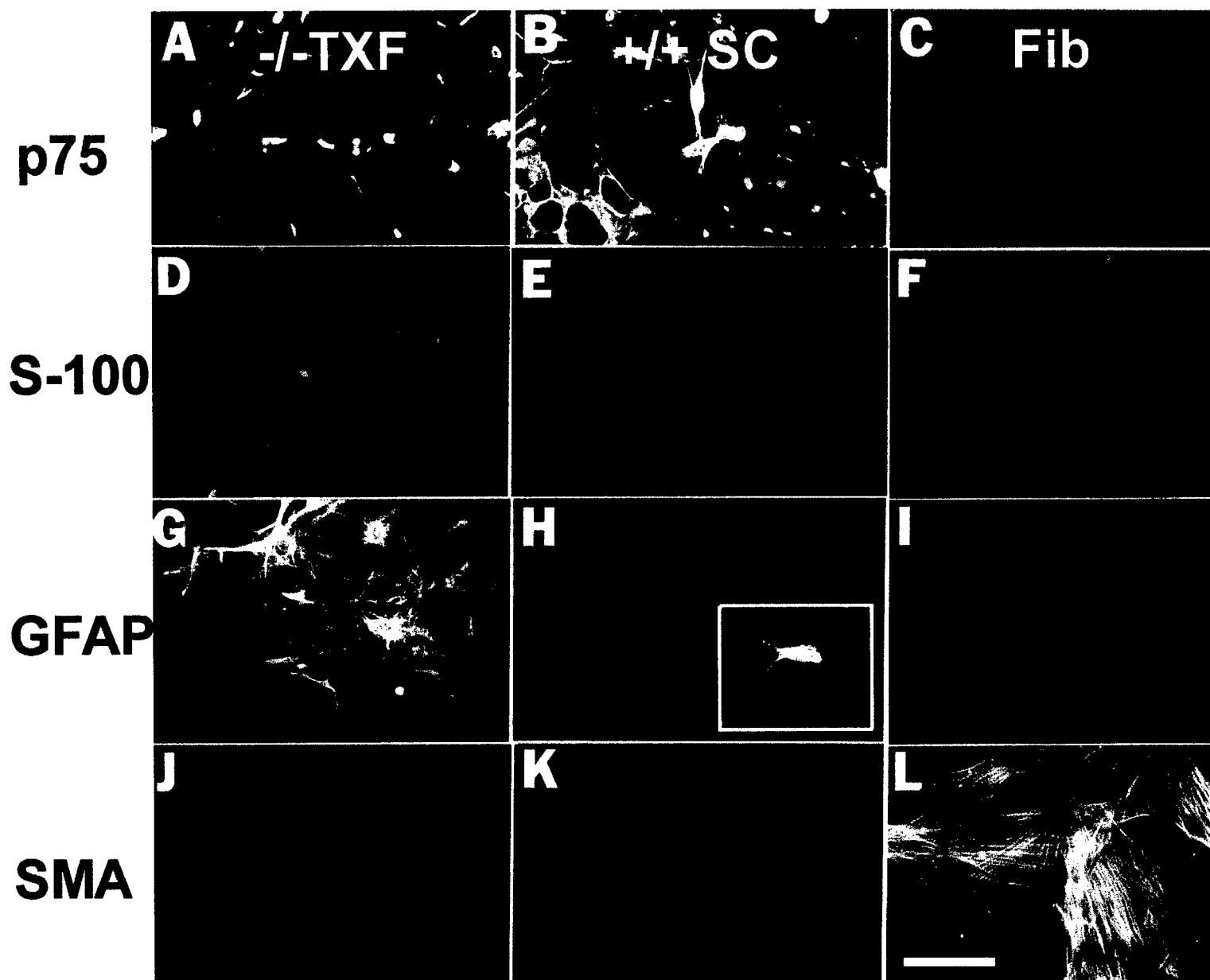


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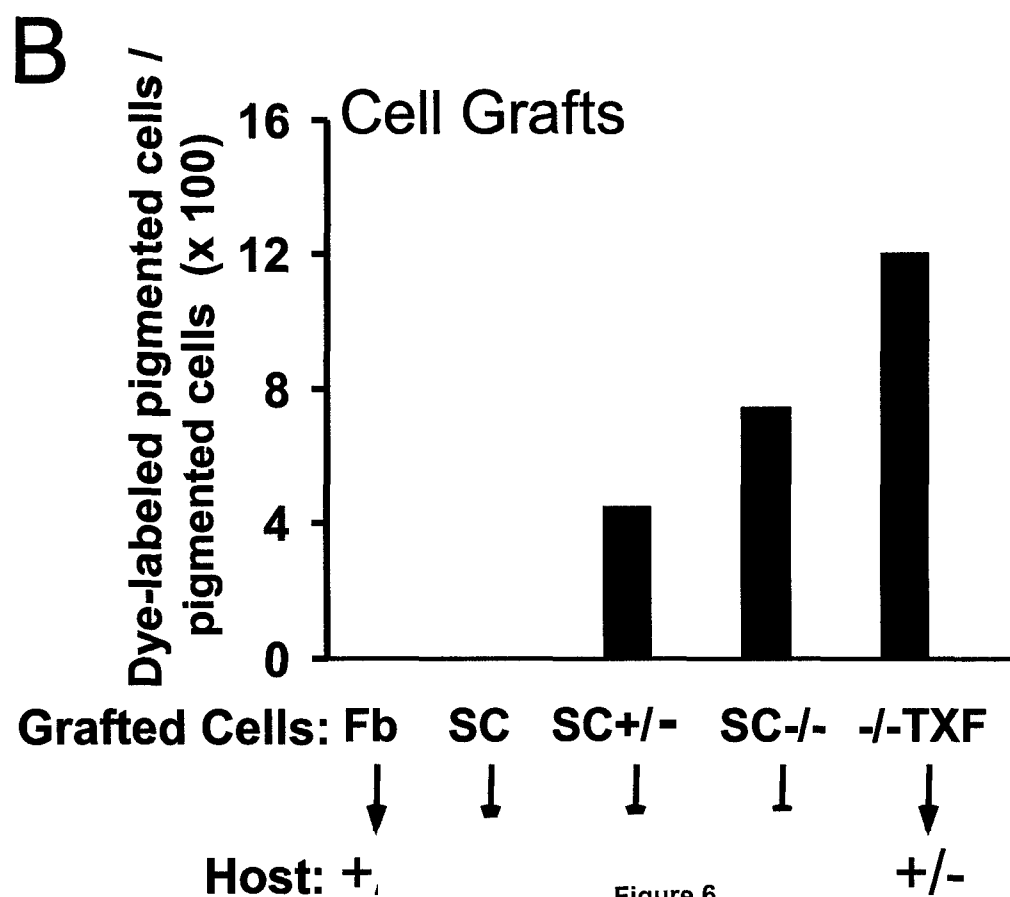
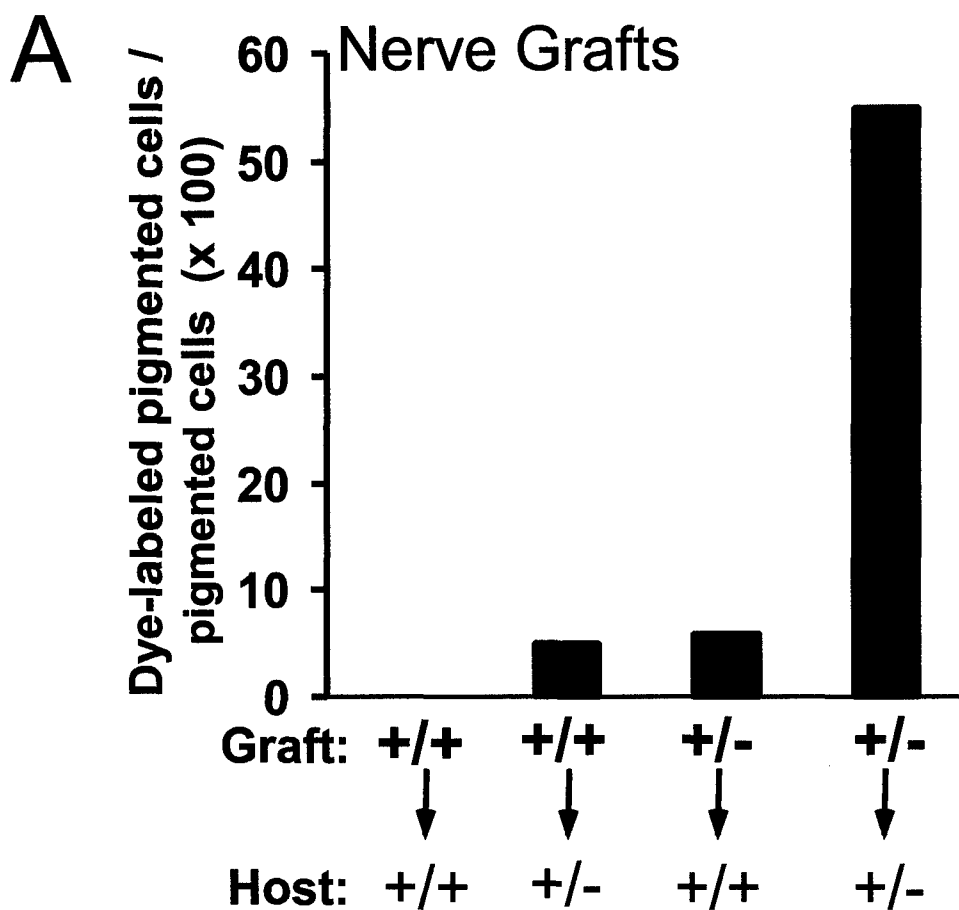


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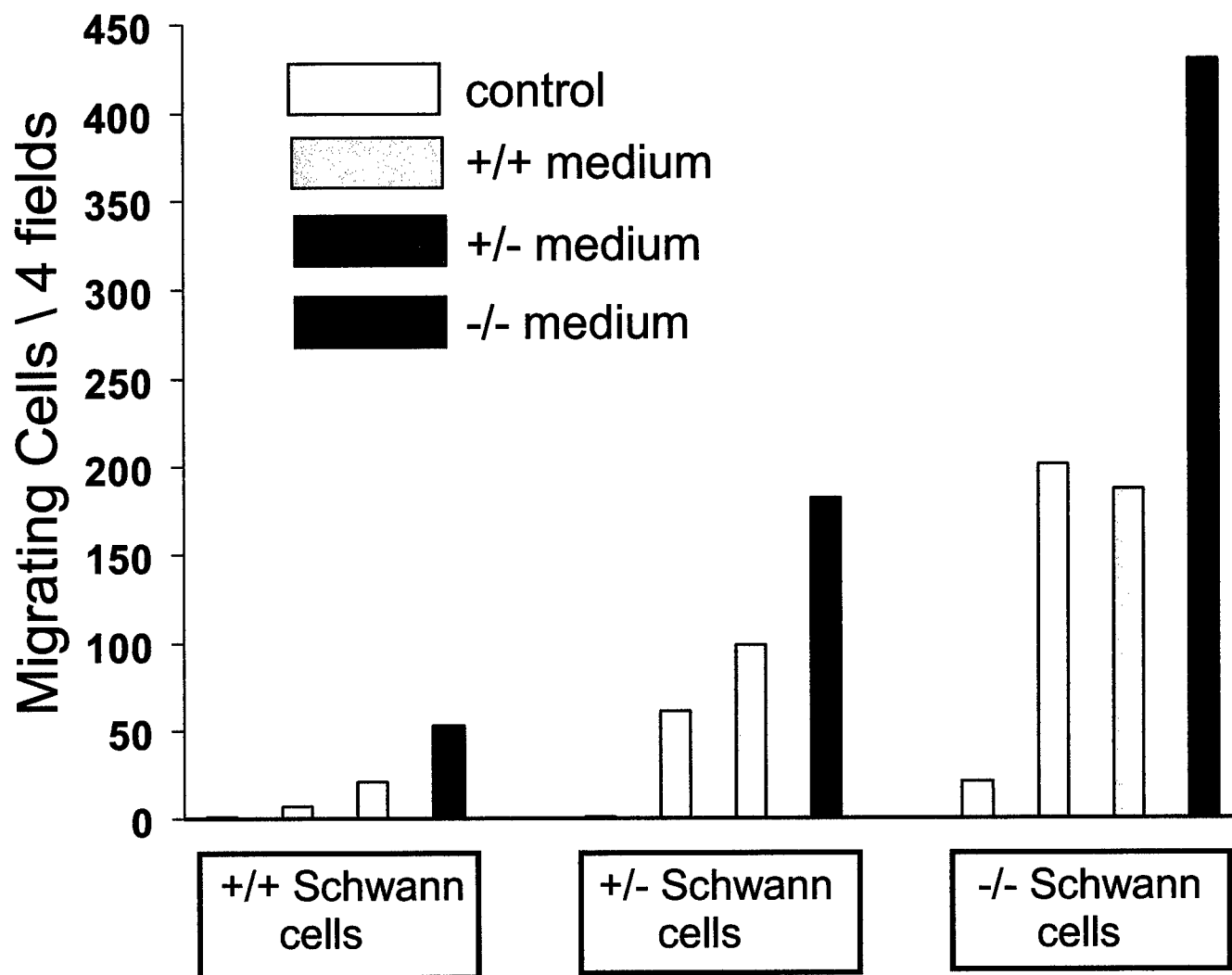
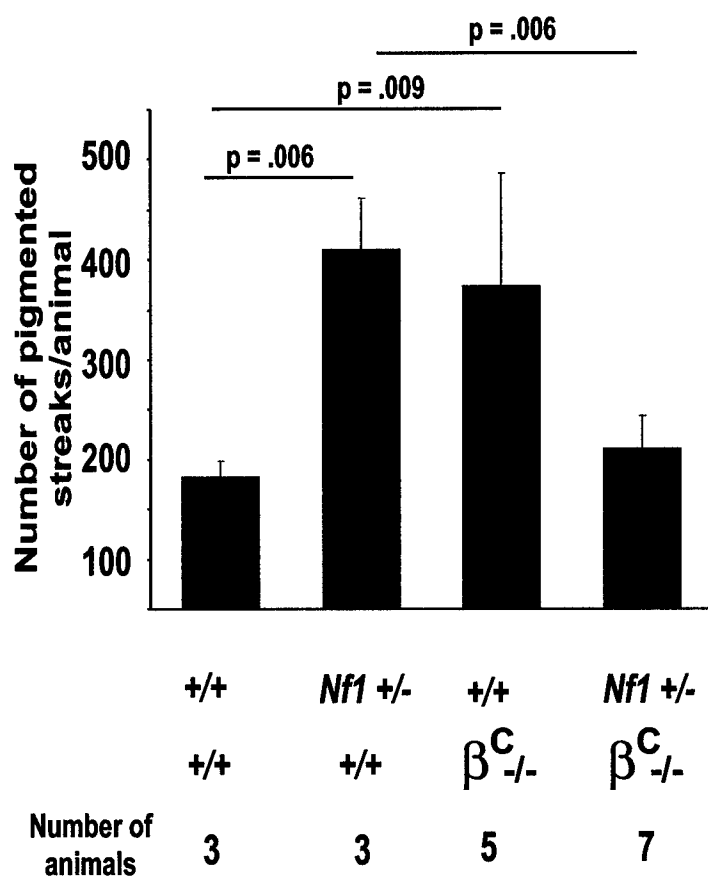
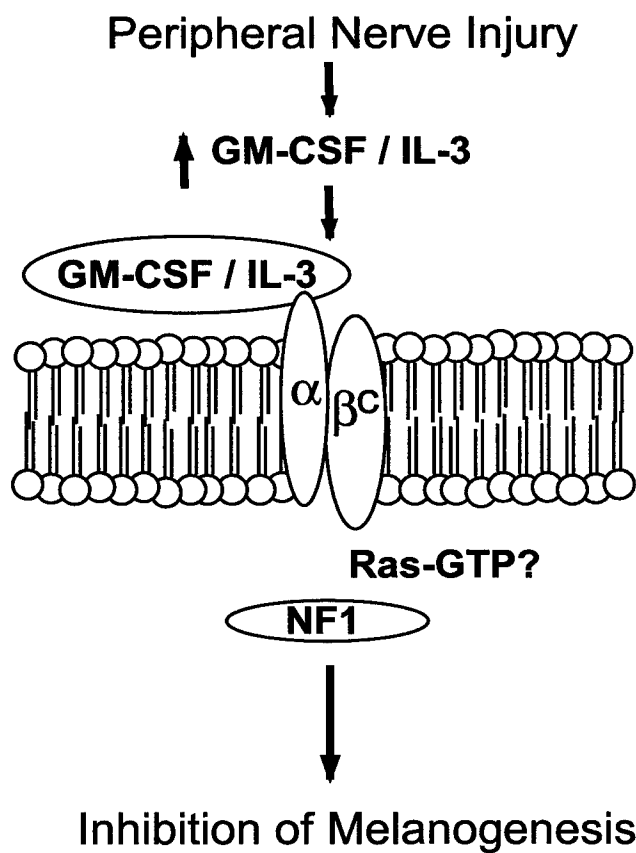


Figure 7
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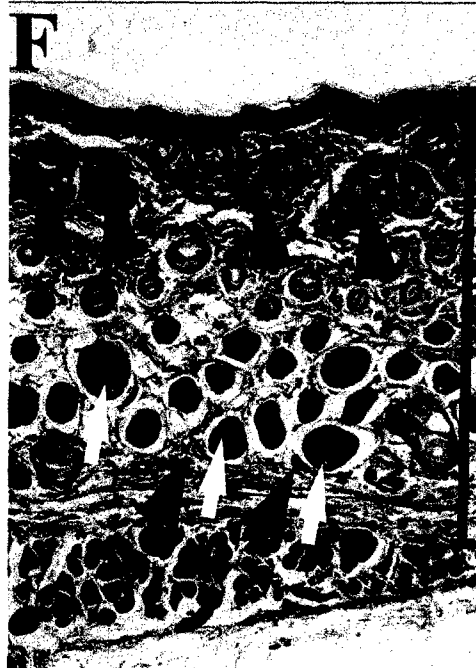
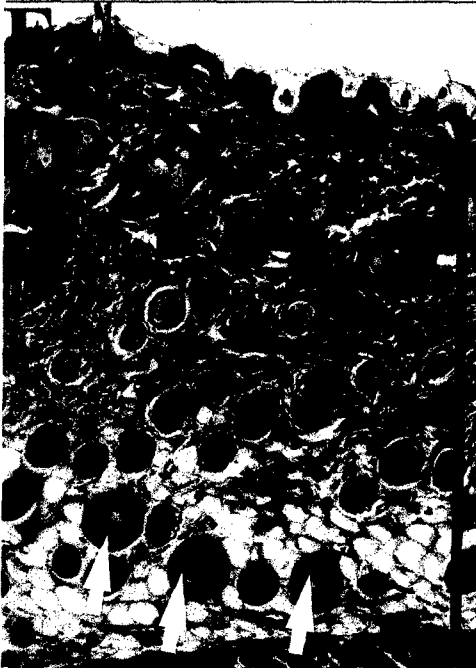
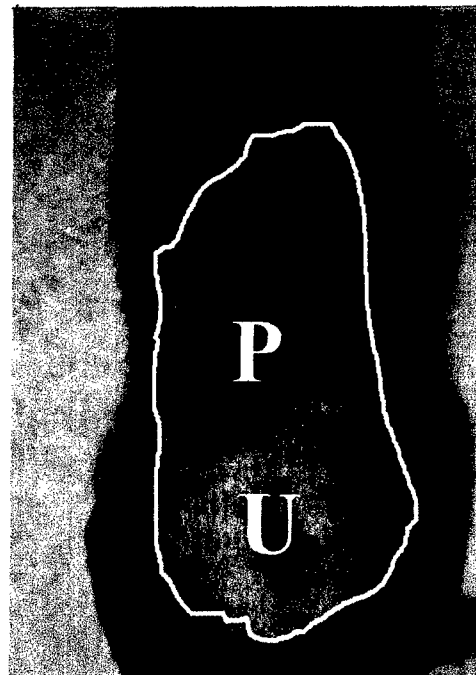
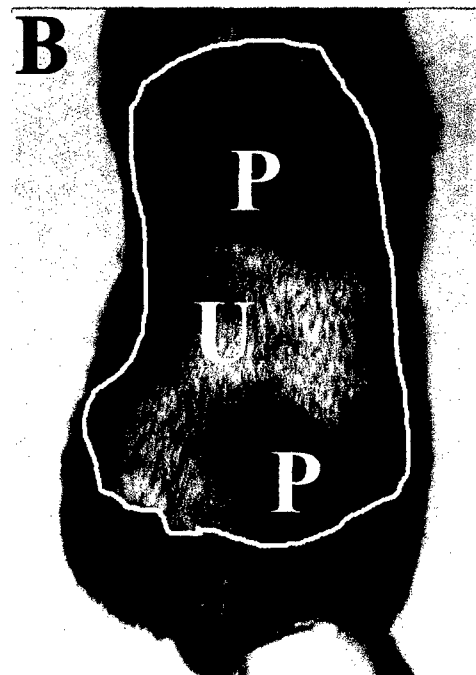


Figure 9
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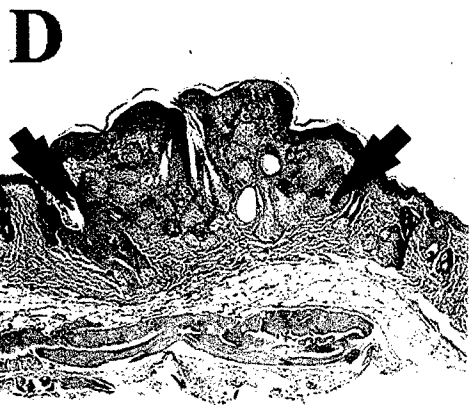
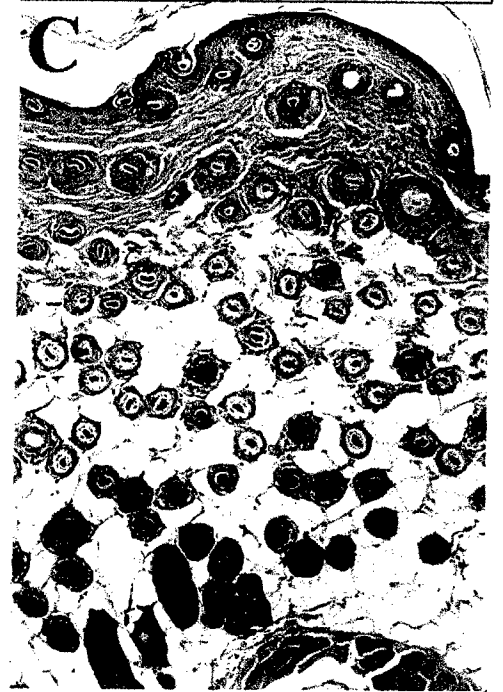
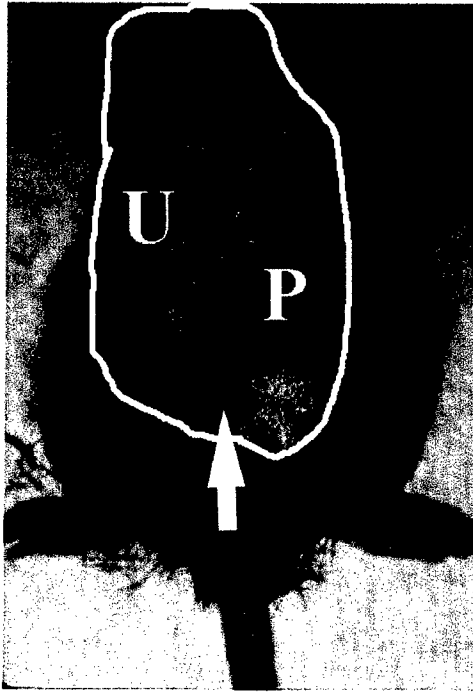


Figure 10
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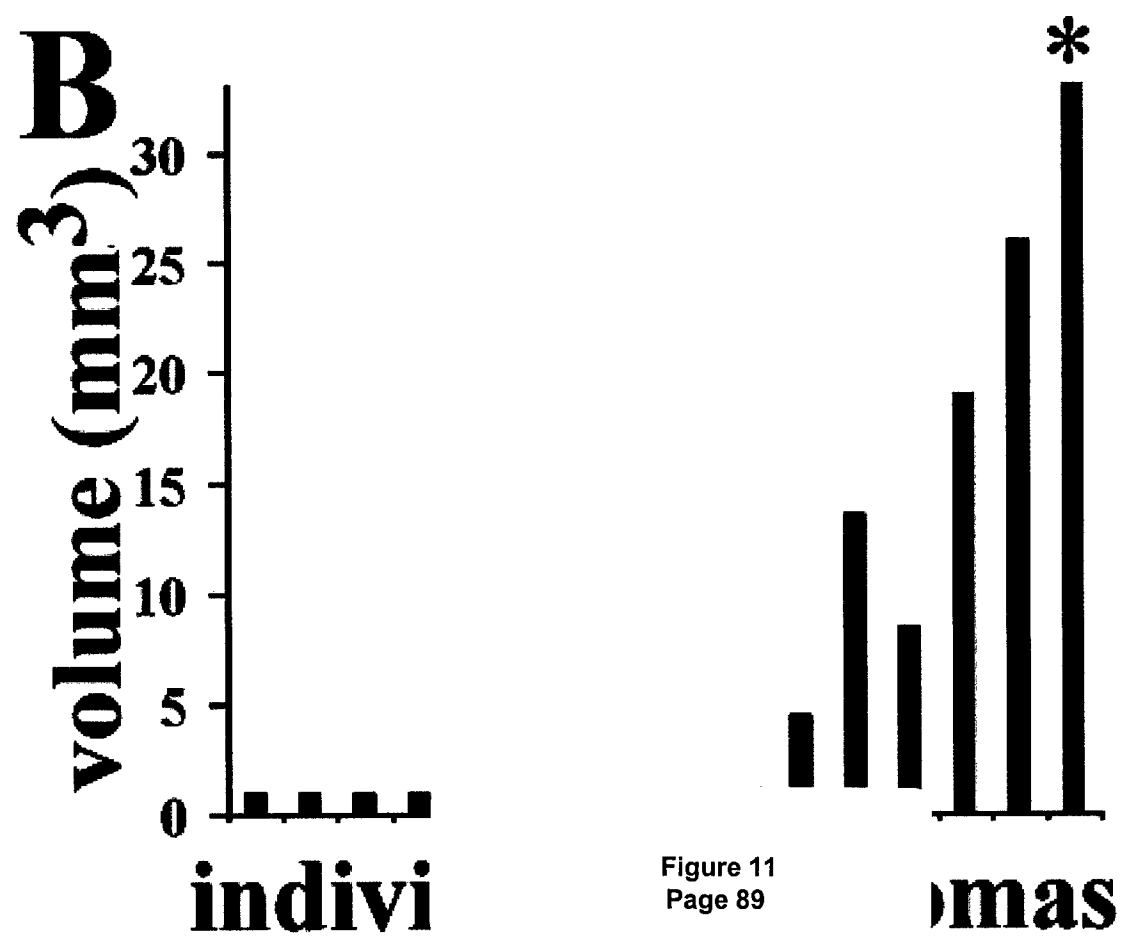
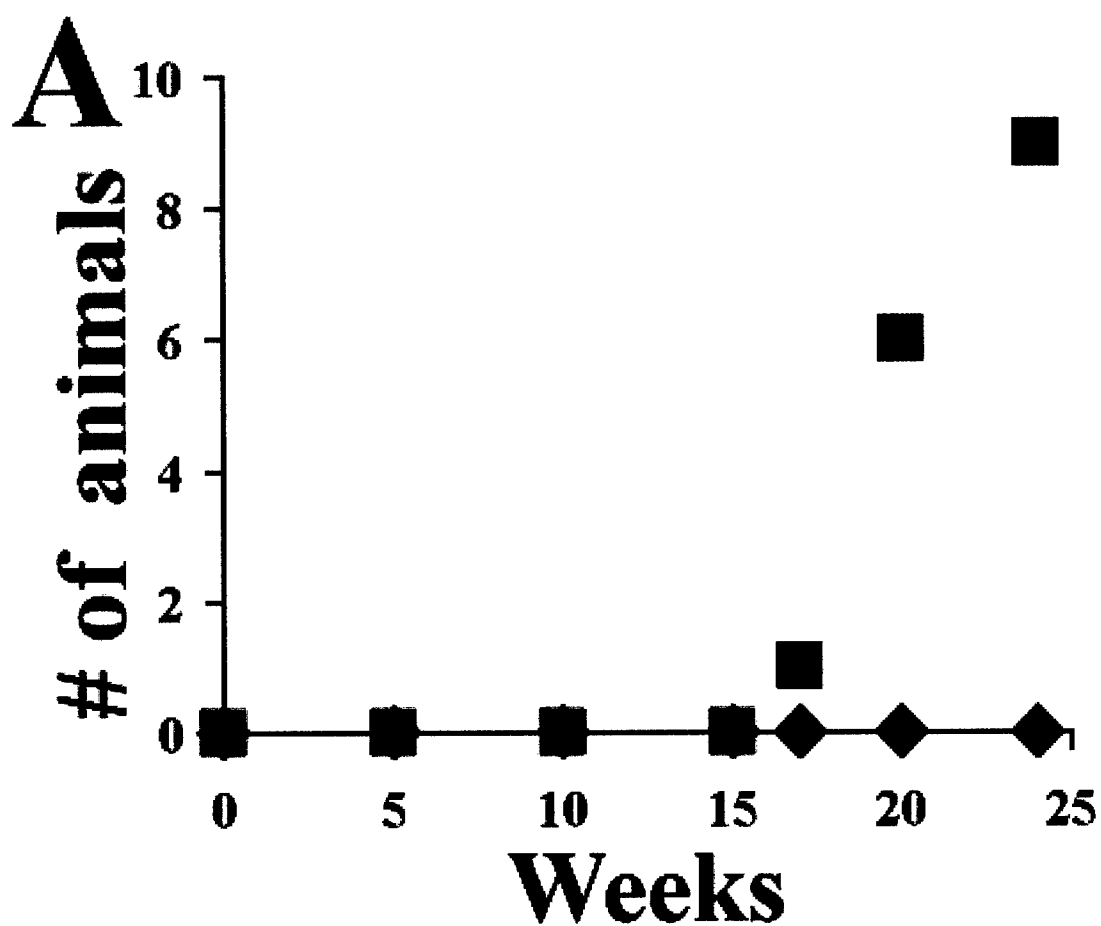
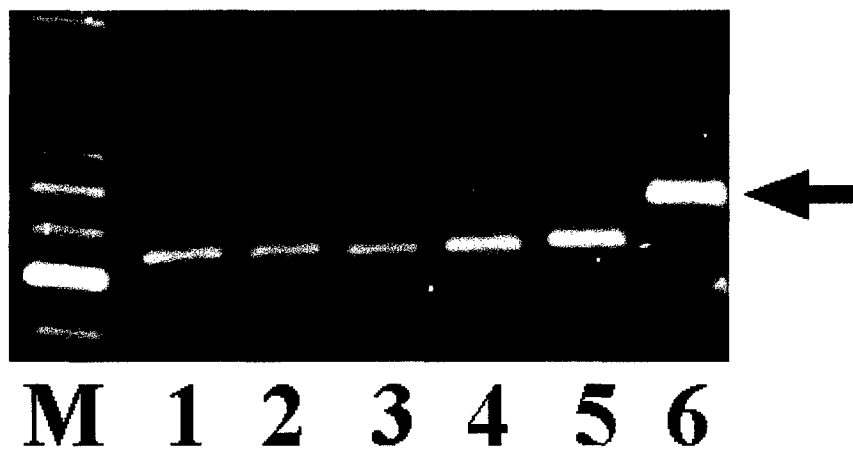
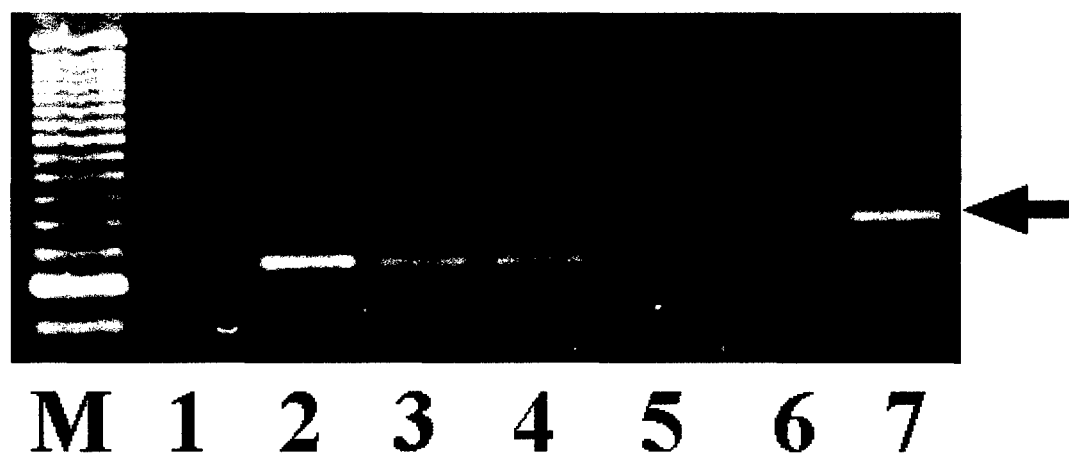
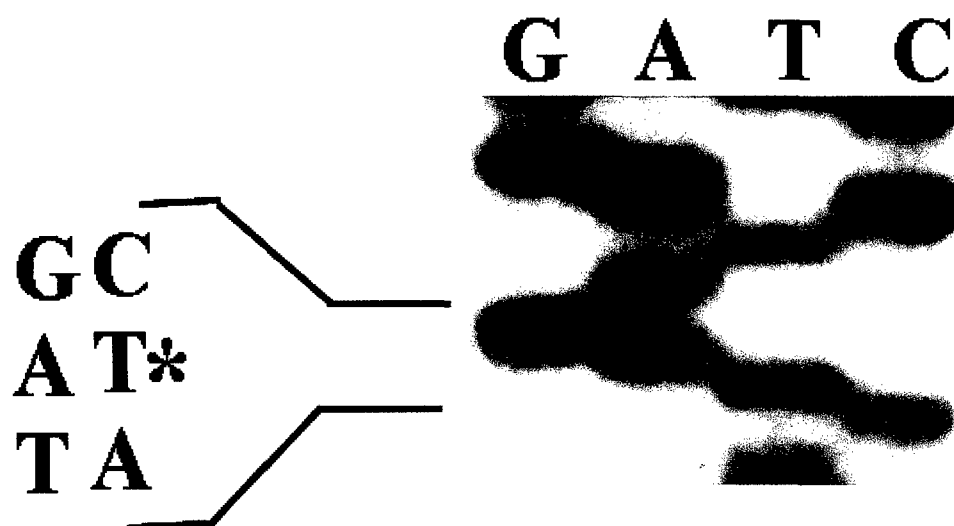


Figure 11
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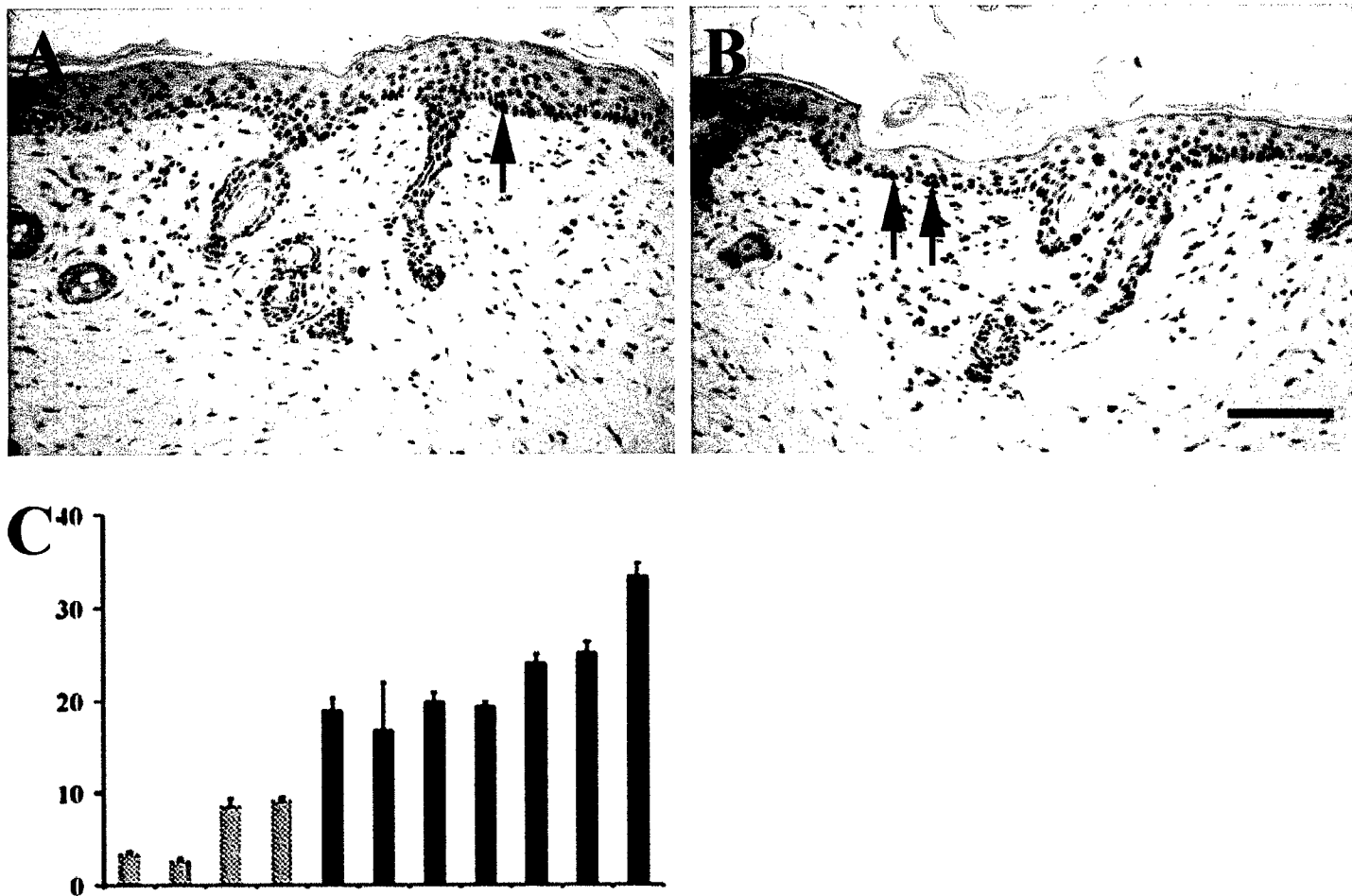


Figure 13
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The Neurofibromatosis Type 1 (*Nf1*) Tumor Suppressor is a Modifier of Carcinogen-Induced Pigmentation and Papilloma Formation in C57BL/6 Mice

Radhika P. Atit,* Kent Mitchell,‡ Lam Nguyen,† David Warshawsky,‡ and Nancy Ratner*†

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There is increasing evidence implicating the human *NF1* gene in epithelial carcinogenesis. To test if *NF1* can play a part in skin tumor formation, we analyzed effects of the skin cancer initiator dimethylbenzanthracene and/or the tumor promoter 12-O-tetradecanoyl-13-acetylphorbol on mice heterozygous for null mutations in *Nf1* (*Nf1*^{+/-}). Mice were on the C57BL/6 background, noted for resistance to chemical carcinogens. *Nf1*^{+/-} mice (18 of 24) developed papillomas after treatment with dimethylbenzanthracene and 12-O-tetradecanoyl-13-acetylphorbol; papillomas did not develop in wild-type C57BL/6 mice nor *Nf1*^{+/-} mice treated with 12-O-tetradecanoyl-13-acetylphorbol alone. All papillomas analyzed (six of six) had mutations in codon 61 of *H-ras*, demonstrating strong cooperation between the *Nf1* GTPase activating protein for Ras, neurofibromin, and Ras-GTP. After exposure to 12-O-tetradeca-

noyl-13-acetylphorbol, *Nf1*^{+/-} keratinocytes showed significant, sustained, increases in proliferation, implicating *Nf1* in phorbol ester responsive pathways. Thus, *Nf1* levels regulate the response of keratinocytes to 12-O-tetradecanoyl-13-acetylphorbol. *Nf1*^{+/-} mice also showed a 2-fold increase in the development of pigmented skin patches stimulated by dimethylbenzanthracene; patches were characterized by hair follicles in anagen phase, implicating keratinocytes in the aberrant hyperpigmentation. Our results show that mutation in the *Nf1* gene causes abnormal keratinocyte proliferation that can be revealed by environmental assaults such as carcinogen exposure. The data support a plausible role for *NF1* mutation in human epithelial carcinogenesis. **Key words:** carcinogenesis/keratinocyte/melanocyte/phorbol ester/Ras. *J Invest Dermatol* 114:1093-1100, 2000

Neurofibromatosis type 1 (NF1) is a common inherited autosomal dominant human disease, affecting 1 in 3500 individuals worldwide (reviewed in Huson, 1998). NF1 patients heterozygous for mutations in the *NF1* gene are predisposed to develop benign peripheral nerve tumors, learning disabilities, bone abnormalities, certain malignant tumors, and pigmentation defects. Pigmentation defects include patches of hyperpigmented skin called café-au-lait macules, found in all NF1 patients by 5 y of age, axillary and inguinal freckling, and patches of retinal melanocytes called Lisch nodules (Takahashi, 1976; Lewis and Riccardi, 1981; Frenk and Marazzi, 1984; Korf, 1992; Friedman and Birch, 1997; Huson, 1998). Although pigmentation defects are used as diagnostic criteria for NF1 disease, their pathogenesis is not understood.

Pathogenesis of many NF1 disease manifestations is believed to involve loss of *NF1* function. *NF1* is considered to be a tumor suppressor gene, because loss of heterozygosity at *NF1* has been demonstrated in malignant tumors, neurofibromas, and myeloid disease in NF1 patients (reviewed in Side and Shannon, 1998). NF1 patients are at an overall 4-fold increased risk for malignant disease (Zoller *et al*, 1997). Whereas NF1 patients have not been noted for high risk for epithelial malignancies, in a population-based study in Sweden 16% of adult NF1 patients developed carcinomas, suggesting possible increased risk for epithelial tumors (Zoller *et al*, 1997). *NF1* dysfunction may also occur in sporadic epithelial tumors. Regions of loss of heterozygosity including *NF1* have been reported in breast, ovarian, and esophageal cancer (Wertheim *et al*, 1996; Dunn *et al*, 1999). *NF1* message is downregulated in epithelial ovarian cancer (Iyengar *et al*, 1999) and neurofibromin expression is downregulated in urinary bladder transitional cell carcinogenesis (Aaltonen *et al*, 1999). This recent evidence supports a function for *NF1* in epithelial cell tumorigenesis.

The expression pattern of neurofibromin has been well documented and is consistent with *NF1* function in the skin. Neurofibromin is readily detectable in melanocytes and keratinocytes of adult human skin, with much lower levels in fibroblasts (Malhotra and Ratner, 1994; Hermonen *et al*, 1995). Neurofibromin is expressed at very low levels in keratinocytes, melanocytes, and fibroblasts in adult rodent skin (Daston and

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Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; NF1, neurofibromatosis type 1; RasGAP, GTPase activating protein for Ras; Ras-GTP, activated GTP-bound Ras.

Ratner, 1992), but shows high expression in differentiating keratinocytes (Malhotra and Ratner, 1994). *In vitro*, melanocytes from NF1 patients are subtly abnormal (Kaufmann *et al*, 1991; Griesser *et al*, 1995); keratinocytes have not been studied. Thus, the major cell types in the skin express neurofibromin and could be affected by loss of function at *NF1*.

Wounding has been hypothesized to serve as a triggering event of human NF1 phenotypes including neurofibroma formation and café-au-lait macules (Riccardi, 1992). Indeed, wounding upregulates neurofibromin expression in human skin fibroblasts (Yla-Outinen *et al*, 1998). As heterozygous *Nf1* mice do not spontaneously develop benign tumors or the pigment abnormalities seen in human NF1 patients (Brannan *et al*, 1994; Jacks *et al*, 1994), these mice can be used to test if wounding might induce features of human NF1 disease. *Nf1* null mice die *in utero* so are unavailable for analysis. In support of the hypothesis that mice heterozygous for a null mutation in the *Nf1* gene are abnormal after wounding, aberrant skin fibroblast proliferation and collagen deposition are evident after excisional skin wounding (Atit *et al*, 1999). In this study, we carried out experiments to test if *Nf1* mice are differentially sensitive to the skin cancer initiator 7,12-dimethylbenz[*a*]anthracene (DMBA) or tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Like wounding, skin carcinogens applied to mouse skin elicit an inflammatory response and induce skin cells to proliferate (reviewed in Scribner and Suss, 1978; DiGiovanni, 1992). Susceptible strains of mice develop skin papillomas (Boutwell, 1964; Slaga, 1989). This study uses the C57BL/6 mouse strain, noted for resistance to chemical carcinogens (Reiners *et al*, 1984; Kiguchi *et al*, 1997).

We challenged *Nf1* mutant mice with DMBA; topical application of DMBA induces patches of pigmentation in susceptible strains of mice (Klaus and Winkelmann, 1965). DMBA-treated skin shows increased proliferation of normally dormant DOPA-inactive melanocytes and enhanced melanogenic activity (Tsambaos *et al*, 1989); melanin is transferred to adjacent keratinocytes leading to visible pigmentation. We also challenged *Nf1* mutant mice in the two-stage carcinogenesis model, in which skin is initiated with a potent carcinogen like DMBA and then exposed to multiple treatments with a tumor promoter such as wounding or TPA (Deelman, 1927; DiGiovanni, 1992).

Ras activation is an important step in tumor progression in response to skin carcinogens. After DMBA exposure, activating mutations in the c-Ha-ras proto-oncogene at codon 61 develop in keratinocytes (Quintinalla *et al*, 1986). Activating mutations in *ras* serve as an initiating event in the two-stage tumorigenesis model, because targeting of the v-Ha-Ras gene to follicular keratinocytes causes development of papillomas at a very high frequency, after promotion with wounding or TPA (Leder *et al*, 1990). The *Nf1* gene product, neurofibromin, is itself a Ras-GTPase activating protein (Ras-GAP) (Basu *et al*, 1992; DeClue *et al*, 1992; Kim *et al*, 1995, 1997; Bollag *et al*, 1996; Largaespada *et al*, 1996; reviewed in Kim and Tamanoi, 1998). If Ras activation due to loss of *Nf1* occurs in skin keratinocytes, we reasoned that Ras mutations might not be found in DMBA- and TPA-induced papillomas in *Nf1* mice.

Neurofibromin also has ill-defined non-Ras functions in mammalian cells (Johnson *et al*, 1994; Atit *et al*, 1999). In *Drosophila*, NF1 regulates a cyclic adenosine monophosphate-dependent protein kinase A pathway in a Ras-Raf independent manner (Guo *et al*, 1997). Functional effects *in vivo* of *Nf1* mutations in skin could occur through these or other pathways.

We demonstrate here that the response of *Nf1*^{+/−} mice to chemical carcinogens differs from that of wild-type mice in several important ways. *Nf1*^{+/−} mice showed increased frequency of pigmented spots in response to DMBA and increased papilloma formation in response to DMBA plus TPA. All tested papillomas had activating Ras mutations. *Nf1*^{+/−} keratinocytes sustained increased epidermal bromodeoxyuridine (BrdU) labeling after TPA treatment, demonstrating that neurofibromin levels regulate the response of keratinocytes to

TPA. The data suggest cooperation between Ras activation and *Nf1* inactivation, and indicate that *Nf1* is a strong modifier of responses induced by skin carcinogens. Our findings indicate that specific environmental conditions reveal effects of mutations at *Nf1* in the mouse skin, and are consistent with a role for *NF1* mutations in human epithelial carcinogenesis.

MATERIALS AND METHODS

Chemicals and reagents DMBA, TPA, and BrdU were purchased from Sigma (St Louis, MO). Biotinylated monoclonal anti-proliferating cell nuclear antigen and anti-BrdU was from Zymed Laboratories (San Francisco, CA).

Animals C57BL/6 wild-type female mice (breeders) were obtained from Harlan (Indianapolis, IN). *Nf1*^{+/−} mice were generated by targeting one allele of the *Nf1* gene (Brannan *et al*, 1994). For genotyping, DNA from a toe clip was isolated and the presence of the targeted allele determined by polymerase chain reaction (PCR) as described in Brannan *et al* (1994). Wild-type and *Nf1*^{+/−} mice were obtained by mating *Nf1*^{+/−} C57BL/6 males to C57BL/6 breeder females. *Nf1*^{+/−} mice were backcrossed at least 10 generations on to the C57BL/6 prior to experimentation. Mice were maintained on a 12 h light/dark cycle in a temperature and humidity-controlled room and were provided with reverse osmosis water and rodent chow (Purina, St Louis, MO) ad libitum. Male mice were treated as described at 9–12 wk of age. Dorsal skin hair was carefully shaved with surgical clippers 1 d before topical dosing was begun and mice with skin in anagen phase at the initial shaving were excluded from the analysis. Mice were housed four per cage until 20 wk of tumor promotion treatment, or until papillomas formed. Subsequently mice were individually housed in polystyrene cages to avoid injury to newly formed papillomas.

Treatments Mice were initiated by application of 40 µg of DMBA in 200 µl acetone on days 1 and 10. Mice were observed for 2–4 mo after initiation. One week after initiation was complete one group of mice received topical applications of 0.2 ml of 6.4 nM TPA in acetone (0.8 µg) delivered using a micropipette. Mice received TPA three times per week for 24 wk. Control mice received 0.2 ml of acetone vehicle on the same schedule. Dorsal skin was shaved once every 4–6 wk to maintain exposure of the skin to the treatments. The incidence of pigmentation and skin papillomas was recorded weekly. Tumor data are expressed as the percentage of mice with papillomas (tumor incidence) and the average number of papillomas per mouse (tumor multiplicity).

Histology Mice were killed in a chamber filled with CO₂. Treated skin from pigmented and unpigmented regions, and papilloma tissue with adjacent skin was excised from the dorsum. Specimens were fixed in 10% formalin, embedded in paraffin, and 6 µm sections cut and stained with hematoxylin and eosin. Some sections were immunostained with biotinylated anti-proliferating cell nuclear antigen or anti-BrdU per directions from the manufacturer. Hair follicle number was counted on a light microscope in at least five different fields/section. Multiple sections were analyzed from two different specimens of treated skin per mouse.

H-ras codon 61 mutational analysis DNA was isolated from unpigmented and pigmented regions of the treated skin of three wild-type animals and five *Nf1*^{+/−} animals after an overnight lysis in buffer (100 mM Tris, 0.2% sodium dodecyl sulfate, 200 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA)) containing proteinase K (100 µg per ml). DNA was precipitated in isopropanol and resuspended in Tris-EDTA (TE). Genomic DNA was isolated from papilloma laden mouse skin using Puregen reagents (Gentra Systems, Minneapolis, MN) and proteinase K. DNA was quantitated by ultraviolet spectroscopy at 260 nm. For *ras* mutational analysis, all PCR primers were based on the mouse *H-ras* genomic sequence of Brown *et al* (1998). The enriched PCR method described in detail by Mitchell and Warshawsky (1998) was used to screen for codon 61 mutations in treated skin and papilloma-derived DNA. Amplification of a normal allele gives a 135 bp band and the allele with the codon 61 mutation gives a 162 bp fragment. Normal mouse liver DNA was used as the negative control and an *H-ras* codon 61 CAA-CGA DNA fragment was used as the positive control (Mitchell and Warshawsky, 1998). Sequencing confirmed the codon 61 mutations (Mitchell and Warshawsky, 1998).

Epidermal labeling index after TPA treatment Wild-type or *Nf1*^{+/−} mice were treated four times over 2 wk with acetone, 0.8 µg TPA, or 4 µg TPA, and killed 48 h after the last treatment. In other experiments animals were treated once with acetone, 0.8 µg TPA, or 4 µg

TPA, and killed 48 h after treatment. One hour before killing, mice were injected intraperitoneally with 100 mg per kg BrdU (Sigma) in saline. Two to three specimens from the treated area of the dorsal skin were collected into 10% normal buffered formalin, and processed for paraffin embedding. Immunohistochemistry to detect BrdU-labeled cells was carried out on deparaffinized sections according to manufacturer's instructions; sections were counterstained with hematoxylin. The percent of labeled basal keratinocytes (labeling index) was calculated after counting 1500–2000 basal keratinocytes per mouse as described in Naito *et al* (1987).

Table I. Incidence of pigmentation after treatment with skin carcinogens

Treatment ^a	<i>Nf1</i> +/+	% affected	<i>Nf1</i> +/-	% affected
Acetone ^c	0/5	0	0/9	0
TPA ^c	0/8	0	0/15	0
DMBA ^c	8/22 ^b	36	23/30	76
DMBA + TPA ^d	6/6	100	12/12	100

^aC57BL/6 male mice were treated with 200 μ l topical acetone vehicle, TPA (0.8 μ g, three times per week), DMBA (40 μ g, two treatments), or DMBA + TPA (DMBA 40 μ g, two treatments followed by TPA, 0.8 μ g, three times per week).

^bAnimals exhibited that exhibited one or more patches of pigmentation larger than 1 cm² were scored as positive. When patches appeared, they were always larger than 1 cm².

^cThe first three groups were scored by 4 mo after initiation of experiments.

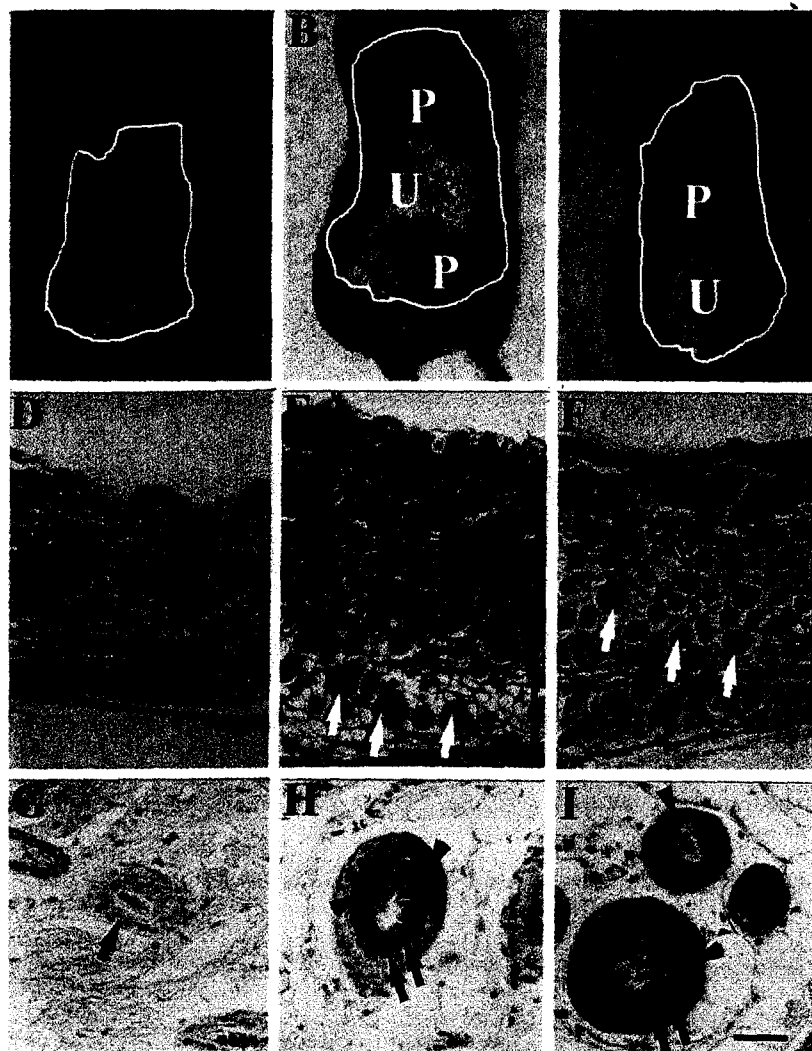
^dThe DMBA + TPA group was scored 6 mo after initiation of treatment.

RESULTS

Increased incidence of pigmentation in *Nf1*+/- skin after treatment with DMBA Twenty-two wild-type mice and 30 *Nf1*+/- littermates were treated with DMBA on days 1 and 10. Within 3 wk after initiation, most *Nf1*+/- (n = 23/30) mice showed large patches of pigmentation, all greater than 1 cm². This phenotype was absent in untreated mice, and in mice treated with acetone only (n = 5–9/genotype; **Table I**). Pigmented patches were also absent in wild type or *Nf1*+/- exposed to TPA alone, even after 2 mo of treatment (**Table I**). Pigmented patches remained grossly visible for at least 4 mo (**Fig 1B, C; Table I**). Most wild-type mice did not develop pigmentation in response to DMBA (**Fig 1A**), but eight of 22 wild-type mice did (**Table I**). Thus, a significant increase in the incidence of pigmentation was observed in *Nf1*+/- mice in response to DMBA. Skin was analyzed by histology to define skin components contributing to the pigmentation. Wild-type and *Nf1*+/- skin had normal histology outside pigmented areas, with hair follicles in the resting (telogen) phase (**Fig 1D**). In contrast, skin from pigmented patches of affected wild-type and *Nf1*+/- mice showed large numbers of hair follicles filled with melanin pigment (**Fig 1E, F**, white arrows). Skin sections from wild-type and *Nf1*+/- mice were immunostained with anti-proliferating cell nuclear antigen, a proliferation marker, to confirm that the hair follicles in pigmented spots were in anagen phase. The visible brown precipitate marks proliferating follicular keratinocytes (**Fig 1H, I**). Hair follicles were found in multiple stages of

Figure 1. Gross appearance and histology of DMBA treated wild-type and *Nf1*+/- skin.

(A–C) Show gross photographs of shaved dorsum of mice 2 mo after initiation with two doses of 40 μ g of DMBA. Treated areas are outlined in white. (A) Representative of a wild-type animal after initiation; this animal shows no large pigmented spot. Other, affected, animals with large pigmented spots are shown in B and C. The mouse shown in B is wild type; in C is a heterozygous mouse. Pigmented areas are designated (P) and unpigmented areas designated (U). (D–F) Hematoxylin and eosin-stained sections of skin, all at the same magnification. (D) Initiated skin from an unpigmented area of *Nf1*+/- skin, identical in histology to wild-type skin. (E, F) Sections from pigmented region of wild-type and *Nf1*+/- animals shown in B and C, respectively. White arrows point to hair follicles filled with pigment; sweat glands are pointed out with black arrowheads. (F) A section from a pigmented region with dermal pigmentation that was not cell-associated (black arrow). (G–I) Anti-proliferating cell nuclear antigen immunostaining (brown precipitate shown by black arrowheads) with hematoxylin counterstain of sections from the regions shown in D–F. H, I melanin is present in follicles (black arrows). Scale bar: (D–F) 50 μ m; (G–I) 10 μ m.



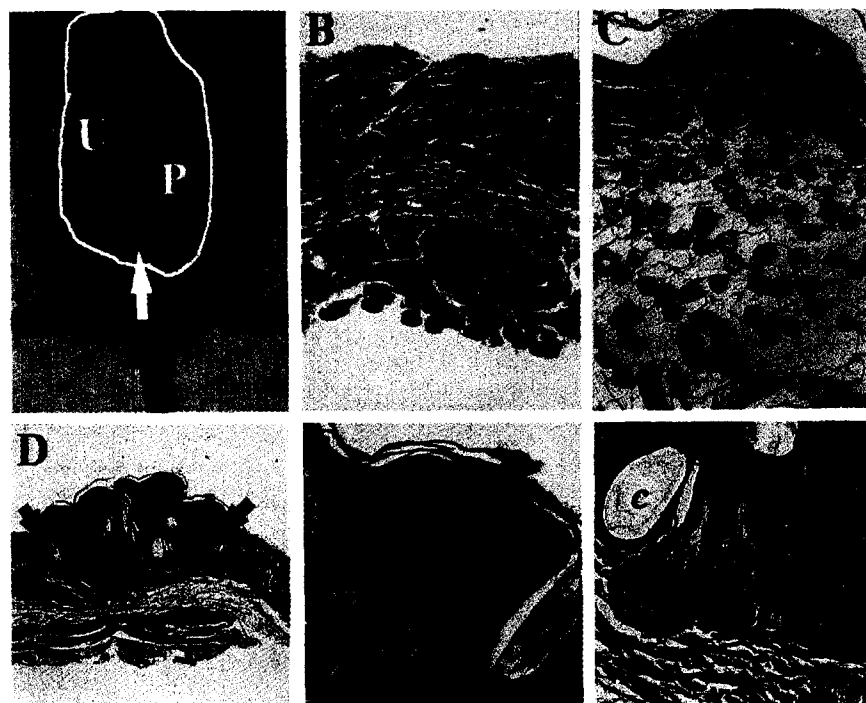


Figure 2. Gross and histologic appearance of skin and papillomas from *Nf1*^{+/-} mice after tumor promotion. (A) Gross appearance of a representative affected *Nf1*^{+/-} mouse after initiation and 24 wk of tumor promotion with 0.8 µg of TPA three times per week. Hair was gently shaved to reveal pigmented areas, P and unpigmented areas, U. White arrowheads point to a papilloma on the dorsum of this mouse. (B–F) Photographs of hematoxylin and eosin-stained sections. (B,C) The histologic appearance of unpigmented, B and pigmented, C skin from the mouse shown in A. Note the marked expansion of hair follicles in C. (D) A section through a typical exophytic papilloma with epidermal hyperplasia, centrally located follicular cysts and trapped sweat glands. Black arrows indicate the interface between the papilloma and adjacent normal skin. At higher magnification, keratinocyte hyperplasia (e) is shown in E and follicular cysts (c) in F, as are the sebaceous glands (s) prominent in most of the papillomas generated. Scale bar: (B, C) 50 µm; (D) 125 µm; (E, F) 10 µm.

differentiation in which different compartments of the follicle contain proliferating cells (not shown).

Estimates of hair follicle numbers were obtained from sections of unpigmented and pigmented skin of wild-type and *Nf1*^{+/-} mice 2 mo after initiation. Follicles were counted in five sections from each mouse. Eight to 10 fields were counted in each section. Normal-appearing skin of wild-type and *Nf1*^{+/-} mice had similar low numbers of hair follicles per field [8.1 ± 0.6 follicles/field wild type ($n = 4$); 8.0 ± 0.23 follicles per field heterozygotes ($n = 5$)]. When pigmented skin was analyzed much higher numbers of follicles were present [44.8 ± 3.6 follicles per field wild type ($n = 4$); 58.3 ± 5.1 follicles per field heterozygotes ($n = 6$)], but the numbers were similar in the two genotypes. Thus, visible pigmented spots are indistinguishable at the gross and histologic levels between the wild-type and *Nf1*^{+/-} groups. These data suggest that the *Nf1* gene is a modifier of the pigmentation response to DMBA in the C57BL/6 strain of mice.

Papilloma formation in *Nf1*^{+/-} mice after DMBA and TPA treatment In a second group of mice, dorsal skin was initiated with DMBA and then promoted with multiple treatments of TPA to determine if *Nf1*^{+/-} skin would respond differentially to a tumor promoter. All animals in the wild-type ($n = 6/6$) and *Nf1*^{+/-} ($n = 12/12$) groups developed large patches of pigmented skin (Table I) after exposure to DMBA + TPA. Skin histology from unpigmented (Fig 2B) and pigmented (Fig 2C) regions was indistinguishable from that observed when skin was treated with DMBA alone (Fig 1D, F). The C57BL/6 mouse strain is extremely resistant to tumor formation in response to the DMBA and TPA regimen (Reiners *et al*, 1984; Naito *et al*, 1987; reviewed in DiGiovanni, 1992; Kiguchi *et al*, 1997). In an initial study, none of six wild-type mice analyzed developed papillomas. Yet, after 20 wk of promotion, papillomas began to arise on the dorsum of most *Nf1*^{+/-} mice, which were littermates of the wild-type mice analyzed (Fig 2A, Table II). By the end of 24 wk, nine of 12 (75%) of *Nf1*^{+/-} animals (Fig 3A) had developed papillomas. Most animals had one to three tumors; tumor ranged in volume from 1 to 80 mm³ (Fig 3B). In a second experiment none of 12 wild-type mice developed papillomas in response to DMBA + TPA whereas nine of 12 mutants developed papillomas by 20 wk after promotion (not shown). None of eight wild-type or 15 *Nf1*^{+/-} mice

developed papillomas in response to TPA alone. Acetone alone did not provoke papillomas in one wild-type or four *Nf1*^{+/-} mice.

Papillomas were analyzed histologically in hematoxylin and eosin stained paraffin sections (Fig 2D). Papillomas showed epithelial hyperplasia (e in Fig 2E) and trapped sebaceous glands with follicular cysts (s and c, respectively, Fig 2F) characteristic of papillomas. Trapped sebaceous glands were a significant component of most of the papillomas examined. The hypothesis that the sebaceous glands are part of the neoplastic process was considered but excluded because the whole structure of nearby hair follicles was well preserved. It appears that sebaceous glands are trapped in the papillomas in *Nf1*^{+/-} mice even at stages when the papillomas are large (C. Conti, MD Anderson Cancer Center, Smithville, TX, personal communication). The reason for this is not known. Larger papillomas showed hyperkeratosis (not shown). These data demonstrate that loss of one *Nf1* allele dramatically increases papilloma incidence in C57BL/6 *Nf1*^{+/-} mice treated with DMBA + TPA. Thus, the *Nf1* gene acts as a modifier of the papilloma phenotype.

Ras mutational analysis of pigmented skin and papillomas The *c-H-ras* gene is a target for activating mutations induced by DMBA and TPA (Quintanilla *et al*, 1986). In mouse skin papillomas, mutations in >90% of the tumors are detected at codon 61 of *c-H-ras* (reviewed in DiGiovanni, 1992). Because neurofibromin can act as a GAP for the Ras proteins, it seemed possible that *ras* gene mutations that frequently are detected in papillomas might not be detected in *Nf1* mutants. To test this idea, we used an enriched PCR analysis (see Materials and Methods). Treated mouse skin from unpigmented and pigmented regions from three different *Nf1*^{+/-} mice and five different *Nf1*^{+/-} mice did not show the *c-H-ras* codon 61 mutation (Fig 4A, lanes 1–4). We analyzed the DNA from six of the largest *Nf1*^{+/-} skin papillomas for *c-H-ras* codon 61 mutations. Results showed that six of six independent tumor specimens analyzed contain cells with the A to T transversion at codon 61 from the *c-H-ras* gene (Fig 4B, lanes 1–4). Thus, presence of *ras* mutations in addition to functional inactivation of a single allele of *Nf1* is required to overcome the resistance of the C57BL/6 mouse strain to papilloma formation.

Table II. Detection of mutant *H-ras* genes in mouse treated skin after exposure to DMBA and TPA

Specimen ^a	Genotype	Mutant <i>H-ras</i> positive ^b
Unpigmented skin	<i>Nf1</i> ^{+/+}	0/4
<i>Nf1</i> ^{+/-}	0/5	
Pigmented skin	<i>Nf1</i> ^{+/+}	0/4
<i>Nf1</i> ^{+/-}	0/5	
Papillomas	<i>Nf1</i> ^{+/-}	6/6

^aFollowing initiation with DMBA and 24 wk of tumor promotion with TPA, unpigmented and pigmented regions of skin and papillomas were excised from animals of designated genotype.

^bDNA was isolated from the samples and analyzed by enriched PCR analysis (see *Materials and Methods*). Reverse cycle sequencing was performed on all the samples testing positive in the PCR analysis. Only the six papilloma samples demonstrate A→T transversion in codon 61 of *c-H-ras*.

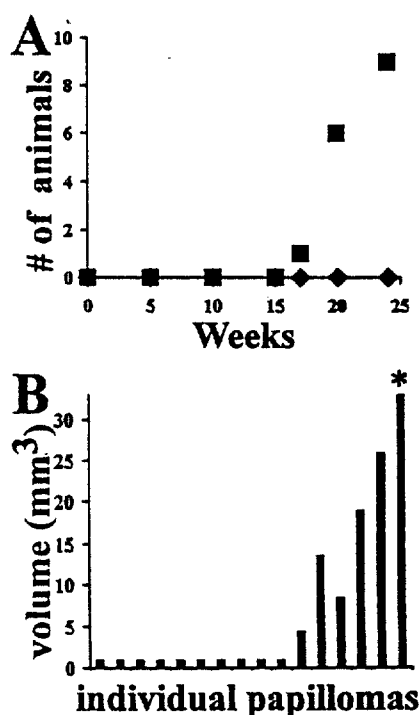


Figure 3. Papilloma formation in *Nf1*^{+/-} mice. (A) The number of animals that developed papillomas after the indicated number of weeks of promotion with TPA. In this experiment, 12 animals of each genotype were treated. Similar results were obtained in a second experiment. Squares represent papillomas in *Nf1*^{+/-} mice; diamonds represent wild-type mice, which never developed papillomas. (B) Volumes of papillomas in *Nf1*^{+/-} mice 24 wk after promotion with TPA. *80 mm³ papilloma.

Epidermal labeling index after TPA treatment of epidermis We tested whether loss of *Nf1* alters keratinocyte proliferation in response to TPA, accounting for altered susceptibility of mutant animals to papilloma formation. Mice were treated with TPA once, or four times over 2 wk. Forty-eight hours after the last TPA treatment, mice were injected with BrdU for 1 h. *In vivo* labeling indices of basal intrafollicular epidermal keratinocytes were calculated in tissue sections. Labeling indices were similar in wild-type and mutant animals either untreated or treated with acetone. We also did not observe a significant difference between genotypes in the labeling index after a single treatment with 0.8 µg or 4 µg of TPA (not shown). Even after four treatments of 0.8 µg TPA over 2 wk, no difference in

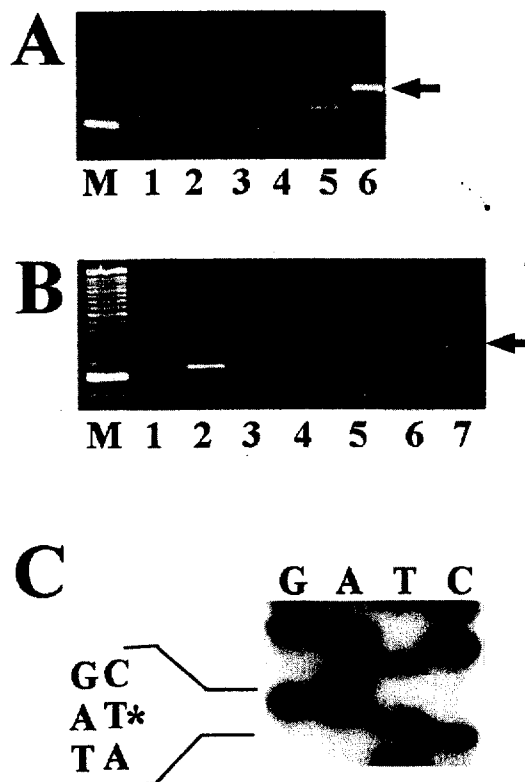


Figure 4. Mutational analysis of codon 61 from the *H-ras* gene in treated skin and papillomas. (A,B) PCR amplification of the A→T transversion at codon 61 from the *H-ras* gene. Presence of the top band (162 bp) indicates the presence of the mutation (arrow). (A) Pigmented skin (lanes 1 and 3) and unpigmented skin (lanes 2 and 4) from the treated area of wild-type (lanes 1 and 2) and *Nf1*^{+/-} (lanes 3 and 4) animals. Lanes 5 and 6 represent negative control and positive controls, respectively, and M = marker. Data are representative of that collected from wild-type (n = 4) and *Nf1*^{+/-} (n = 5) individual mice. (B) Papillomas were isolated 24 wk after tumor promotion with TPA. Lanes 1–4 show results of four representative papillomas analyzed from individual animals. Lane 5 shows a water control, lanes 6 and 7 represent negative and positive controls, respectively. M = marker. (C) Autoradiogram showing sequencing analysis, showing a mutation (A to T transversion) within the second base of codon 61 (asterisk) of the *H-ras* gene. All six papillomas that tested positive for mutations in the PCR screen were confirmed by sequence analysis.

labeling index was observed between genotypes. Because wild-type mice had a high degree of proliferation ($37 \pm 5.3\%$, n = 4) under these conditions, we treated two sets of mice with a higher dose (4 µg) of TPA over 2 wk. Similar epidermal hyperplasia (seven to nine layers) was observed in animals of both genotypes. Under these conditions a difference between the wild-type and *Nf1*^{+/-} keratinocytes was revealed (Fig 5). A consistent increase in the epidermal labeling index was detected in mutant mice (seven of seven) as compared with four wild-type animals. This difference was significant ($p < 0.0001$; Student's t-test). Our results on sections from wild-type mice can be compared with Naito *et al* (1987) and Kiguchi *et al* (1997), who showed a $10.8 \pm 2.1\%$ and $\approx 8\%$ labeling index using this protocol on C57BL/6 mice. Values for individual wild-type and mutant animals are shown in Fig 5(C). The results show that *Nf1*^{+/-} keratinocytes abnormally sustain proliferation in response to TPA.

DISCUSSION

This study was designed to determine if mice heterozygous for an *Nf1* null allele are differentially sensitive to the skin cancer initiator DMBA or the tumor promoter TPA. Using a mouse strain relatively resistant to chemical carcinogens we demonstrate a role for *Nf1* in carcinogen-induced pigmentation and papilloma

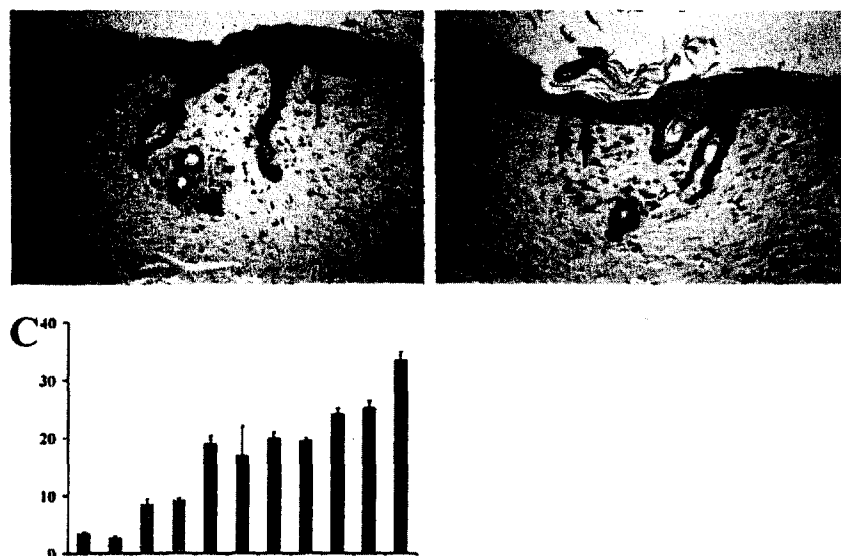


Figure 5. TPA exposure results in enhanced epidermal labeling index in *Nf1*^{+/-} mice. Anti-BrdU staining of typical skin sections from wild-type (A) and *Nf1*^{+/-} (B) mice 48 h after the last of four treatments with TPA (4.0 μ g). Arrow designate positively stained basal keratinocytes. Scale bar: 10 μ m. (C) Data from mice in two individual experiments are shown. The percent of BrdU-labeled basal keratinocytes was counted. Each bar (hatched = wild type; solid = *Nf1*^{+/-}) represents the mean percent labeled cells in two to three sections from each mouse. Error bars show standard error. Wild-type and mutant mice were significantly different ($p < 0.0001$; Student's *t*-test).

formation. The results provide evidence that the *Nf1* gene can contribute to abnormalities of melanocytes and keratinocytes under specific environmental conditions. Mechanistically, we have demonstrated a cooperating effect of mutation in a single *Nf1* allele with activating *ras* mutations to override the genetic resistance of C57BL/6 mice to skin tumor promotion. We have also shown that *Nf1* levels regulate the response of keratinocytes to TPA-stimulated proliferation. Each of these points is discussed below.

DMBA-treated *Nf1*^{+/-} mice were twice as likely as wild-type mice to develop pigmented skin patches. This finding is intriguing, as human NF1 patients develop pigmented patches with greatly increased frequency as compared with normal humans (Korf *et al*, 1992; Riccardi, 1992); indeed, pigmented patches are one of the most common phenotypes associated with human NF1. The pigmented patches we observed in mice do not have the same morphology as human café-au-lait macules. In human café-au-lait macules pigment is in melanocytes and epidermal keratinocytes, which can contain "macromelanosomes" (Takahashi, 1976). In the mouse, pigment was in keratinocytes in hair follicles in anagen phase, not in the epidermis, with no evidence for macromelanosomes. The storage of pigment and the location of the pigmented cells are different in mouse and human skin (Miller *et al*, 1993), possibly accounting for these differences.

DMBA-treated pigmented patches contained skin with features of the anagen phase of the hair cycle (Hansen *et al*, 1984; Miller *et al*, 1993), with numerous, large, hair follicles with actively dividing follicular keratinocytes, significantly increased dermal thickness, and numerous enlarged sebaceous glands. During mouse anagen phase melanin produced in follicular melanocytes is transferred to precortical keratinocytes, leading to visible pigmentation (Chase, 1954; reviewed in Slominski and Paus, 1993). We hypothesize that a target cell of the pigmentation response stimulated by DMBA is the keratinocyte (or its stem cell), which then indirectly affects melanogenesis by melanocytes. Another major finding of this study is increased papilloma formation in *Nf1* mutants. This result is consistent with the affected cell being the keratinocyte or its precursor. Initiated cells proliferate during tumor promotion, eventually forming papillomas with expansion of the epidermal keratinocyte population (Scribner and Suss, 1978; DiGiovanni, 1992). Both the initiated papilloma precursor and the pigmented spot precursor may be follicular bulge cells (Binder *et al*, 1997).

This study demonstrates that mutation in a single *Nf1* allele overcomes the genetic resistance of the C57BL/6 mouse strain to skin tumor promotion (reviewed in DiGiovanni, 1992). No previous studies utilized exactly the doses of DMBA and TPA used here, and none used male mice. Using female C57BL/6 mice treated with DMBA and TPA investigators reported no papillomas

(Kiguchi *et al*, 1997) or few papillomas (Reiners *et al*, 1984; Chouroulinkov *et al*, 1988; O'Brien *et al*, 1997). In our experiments no wild-type animals, littermates of mutants and backcrossed at least 10 generations on to the C57BL/6 background, treated with DMBA and TPA developed tumors whereas papillomas arose in 75% of similarly treated *Nf1* heterozygous mice. We cannot rule out the possibility that a gene tightly linked to *Nf1*, from the 129/SV background and present in congenic mice, contributes to the susceptibility of the *Nf1* heterozygous mice to papilloma formation. It is more likely that loss of one *Nf1* allele is sufficient to increase the frequency of papilloma formation in a resistant strain of the mouse. This result would indicate a dose effect of *Nf1* in keratinocytes. The question, however, of whether complete loss of *Nf1* is required for the papilloma formation described here remains open. Keratinocytes in six papillomas maintained neurofibromin expression in tissue sections using immunohistochemistry, and preliminary experiments have failed to show loss of heterozygosity using PCR or southern blot analysis (not shown); however, DMBA is a point mutagen so point mutations affecting *Nf1* cannot be excluded.

Mutational analysis revealed activating *H-Ras* mutations at codon 61 in all ($n = 6$) of tested papillomas. Thus even though neurofibromin can function as a Ras-GAP, and may increase Ras-GTP in keratinocytes, *Nf1* mutation does not obviate the demonstrated requirement for Ras mutation in this model system (Quintanilla *et al*, 1986; Roop *et al*, 1986; Leder *et al*, 1990; Greenhalgh *et al*, 1993; Brown *et al*, 1998). Whereas we cannot exclude the possibility that *Ras* mutations are absent in smaller, developing papillomas, it appears that functional inactivation of a single allele of the *Nf1* gene cooperates with activating mutations in the *ras* gene to enhance the papilloma phenotype in *Nf1*^{+/-} mice on a resistant genetic background. The cooperative effects between *Ras* and *Nf1* that we have defined in keratinocytes could act downstream of Ras or independent of Ras. Neurofibromin may function in non-Ras pathways in keratinocytes, just as it appears to do in several other cell types (Johnson *et al*, 1994; Griesser *et al*, 1995; Guo *et al*, 1997; Kim *et al*, 1997; Atit *et al*, 1999). Alternatively, decreased levels of neurofibromin may increase Ras-GTP in keratinocytes, increasing signaling downstream of Ras-GTP and providing an effect additive with mutationally activated *H-Ras*. The idea that Ras-GTP higher than achieved by a single mutated *H-Ras* allele can contribute to epidermal tumorigenesis is consistent with the results of Bremner *et al* (1994) who showed that most papillomas carrying mutant *H-Ras* alleles are trisomic for chromosome 7, increasing the copy number of mutant *H-Ras*. Manges *et al* (1998) showed that *Nf1*^{+/-} mice overexpressing N-Ras driven by the MMTV promoter are at increased risk for

developing lymphomas. As in our study, loss of *Nf1* cooperates with increased Ras activity (from overexpression or activating mutations) to increase tumor incidence.

Nf1 mutant mouse keratinocytes showed sustained proliferation in response to TPA. This result is consistent with data showing that strains of mice susceptible to papilloma formation have sustained epidermal proliferation in response to TPA (Naito *et al*, 1987; Kiguchi *et al*, 1997). The 4 mg dose of TPA is unlikely to have caused differential toxicity; this dose was identical to the dose used by previous investigators, and resulted in this study and in prior experiments in epidermal hyperplasia (Naito *et al*, 1987; Kiguchi *et al*, 1997). TPA activates protein kinase C, which is essential for regulation of genes involved in keratinocyte differentiation (e.g., Lee *et al*, 1998). Neurofibromin may normally downregulate TPA-mediated signaling pathways in keratinocytes. It is also possible that TPA increases the duration of Ras activation by effects on Ras exchange proteins such as RasGRP via a phorbol ester-responsive domain (Tognon *et al*, 1998). Increased Ras activation could in this way synergize with effects of decreased *Nf1*. In either case, our finding that *Nf1* hemizygous mouse keratinocytes have increased proliferative potential likely accounts for the observed papilloma formation.

Like mechanical wounding, topical application of skin carcinogens injures the skin and induces a wound-healing response (Scribner and Suss, 1978). Riccardi (1992) hypothesized a role for injury in pigmentation defects and tumor formation in NF1 patients. We have demonstrated here that mutation in a single *Nf1* allele in mice alters the susceptibility of skin to pigmentation and tumors induced by carcinogens. Our data are consistent with a role for keratinocytes, and perhaps injury, in the abnormal skin pigmentation characteristic of NF1 patients. In addition, chromosome 17 loss in the region of human *NF1* is frequently observed in carcinomas, as is downregulation of neurofibromin expression (Hermonen *et al*, 1995; Wertheim *et al*, 1996; Aaltonen *et al*, 1999; Dunn *et al*, 1999; Iyengar *et al*, 1999). Our experiments using a mouse model support the idea that *NF1* could play a part in human epithelial carcinogenesis.

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Evidence in support of Ras-GTP dependent and independent abnormalities in NF1-mutant cells revealed by a new *in situ* Ras-activation assay and by skin wounding.

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Patients with mutations in the *NF1* gene develop benign peripheral nerve tumors (neurofibromas) and, rarely, malignant peripheral nerve sheath tumors (MPNST). The *NF1* gene is believed to act as a tumor suppressor in malignancies including MPNST. We have developed model systems to study Schwann cells and fibroblasts, the two major cell types within neurofibromas, from human tumors and from transgenic mice with *Nf1* mutations. The *NF1* gene encodes neurofibromin, postulated to function in part as a GTPase activating protein (GAP) that converts the active, GTP-bound Ras proto-oncogene into inactive, GDP-bound Ras. To determine which neurofibroma cell type has altered Ras-GTP regulation, we developed a new assay for activated GTP-bound Ras. A fusion protein comprised of GST and the Ras-binding domain of the serine-threonine kinase Raf1 (RBD-GST) was used as a probe for Ras-GTP *in situ*. Bound RBD-GST was detected using an anti-GST antibody. In NIH 3T3 cells, the assay detected overexpressed, constitutively activated K-, N- and H-Ras and insulin-induced endogenous Ras-GTP. In dissociated neurofibroma cells from *NF1* patients, Ras-GTP was elevated in Schwann cells but not fibroblasts. Twelve to 62% of tumor Schwann cells showed elevated Ras-GTP, revealing neurofibroma Schwann cell heterogeneity. Furthermore, Ras-GTP was elevated in all mouse *Nf1*^{-/-} Schwann cells, but never in *Nf1*^{-/-} mouse fibroblasts. Schwann cell Ras-GTP signal was greatly diminished after exposure to 1 μ M of the L-744,832 farnesyl transferase inhibitor. Thus aberrant Ras activity is characteristic of only some neurofibroma Schwann cells but not fibroblasts. This study suggests that Schwann cells are likely to be targets of FPTI in clinical trials.

Fibroblasts make up 20-60% of cells within neurofibromas, and manufacture much of the collagen-rich matrix characteristic of neurofibromas. Thus even though increased Ras activation could not be detected in mutant fibroblasts, we tested if loss of the *NF1* gene (*nf1*) compromises fibroblast function. Trauma has been proposed trigger neurofibroma formation. Skin wounding was performed in *nf1* knockout mice. The pattern and amount of collagen-rich granulation bed tissue, manufactured by fibroblasts, was grossly abnormal in 60% of *nf1*^{+/-} wounds. *Nf1*^{+/-} skin wound fibroblasts also proliferated past the normal wound maturation phase; this *in vivo* effect was potentiated by muscle injury. *In vitro*, *nf1*^{+/-} fibroblasts showed higher proliferation in 10% serum than *nf1*^{+/+} fibroblasts. Macrophage-conditioned media or epidermal growth factor potentiated *nf1*^{+/-} fibroblast proliferation *in vitro*, demonstrating abnormal response of mutant fibroblasts to wound cytokines. *Nf1*^{-/-} fibroblasts also deposited significantly more collagen than did normal cells. We conclude that *nf1* is a key regulator of fibroblast responses to injury, and that *nf1* mutation in mouse fibroblasts causes abnormalities characteristic of human neurofibromas. As described above, no significant abnormalities in Ras activation were detected in *NF1* mutant fibroblasts. Ras activation is known to cause a decrease in collagen production by fibroblasts, in contrast to the excess collagen deposition by the mutant cells. Therefore the wound-induced fibroblast phenotypes we have identified are most likely due to loss of *NF1* function(s) other than the ability of neurofibromin to modulate Ras.

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TRANSDIFFERENTIATION OF NEUROFIBROMATOSIS-1 MUTANT SCHWANN CELLS INTO MELANOCYTES FOLLOWING WOUNDING OF *Nf1/nf1* MOUSE NERVE: Nancy Ratner*, Radhika Atit and Tilat Rizvi,
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Patients with type 1 neurofibromatosis (NF1) develop peripheral nerve tumors and cafe-au-lait macules, hyperpigmented spots on the skin. Mice heterozygous for targeted mutations at *Nf1* fail to develop hyper-pigmentation or nerve tumors. Wounding has been proposed as an initiating event in human NF1. To test this hypothesis, we cut the sciatic nerve in *Nf1/nf1* mice. Cut ends of transected sciatic nerve were either rejoined with sutures or pulled proximally and distally. Mice (22/genotype) were evaluated 30 days after surgery. All injured *Nf1/nf1* animals developed patches of pigmented cells in muscles overlying the nerve, identified as melanocytes by electron microscopy. Pigmentation was more intense in animals with deflection as compared to transection suggesting that axons are not required for the effect; wild type animals had little or no pigmentation. A few *Nf1/nf1* mice developed tumors 3 months after nerve injury. 4/28 developed unencapsulated unpigmented tumors while 5/28 developed encapsulated pigmented tumors. Thus, wounding of the sciatic nerve in mice with a single mutant *Nf1* allele causes features resembling human NF1.

To define mechanisms underlying wound-induced hyperpigmentation, purified mutant *Nf1* Schwann cells were labeled with Hoechst dye and grafted around transected wild type nerves. Of 4000 labeled cells counted in 6 animals, 68 contained pigment granules; of pigmented cells counted, 12.6% were dye-positive. Thus *Nf1* mutant Schwann cells appear to transdifferentiate into pigmented melanocytes. We tested if the *Nf1* environment contributes to the phenotype. Schwann cells wild type or mutant at *Nf1* were transplanted into wild type or heterozygous hosts. Pigmentation was maximal when both host and graft were mutant. Thus environmental factor(s) and Schwann cell abnormalities appear to contribute to lesion-induced hyperpigmentation in *Nf1* mutants. Supported by NIH NS-28840 and a grant from the DOD.

TRANSDIFFERENTIATION OF NEUROFIBROMATOSIS-1 MUTANT SCHWANN CELLS INTO MELANOCYTES FOLLOWING WOUNDING OF *Nf1/nf1* MOUSE NERVE: Tilat Rizvi* Radhika Atit and Nancy Ratner, Dept Cell Biology, Neurobiology & Anatomy, Univ. Cincinnati. Coll. Med., Cincinnati, OH 45267-0521

Patients with type 1 neurofibromatosis (NF1) develop peripheral nerve tumors and cafe-au-lait macules, hyperpigmented spots on the skin. Mice heterozygous for targeted mutations at *Nf1* fail to develop CALM or nerve tumors. To test if wounding causes NF1 manifestations, we cut the sciatic nerve in *Nf1/nf1* mice. Cut ends of transected sciatic nerve were rejoined with sutures pulled proximally and distally in deflection experiments. *Nf1/nf1* and wild type mice (22/genotype) were evaluated 30 days after surgery. All injured *Nf1/nf1* animals develop patches of pigmented cells in muscles overlying the nerve, identified as melanocytes by EM. Pigmentation was more intense in animals with deflection as compared to animals with transection suggesting that axons are not required for the effect; wild type animals had little or no pigmentation.

Purified mutant *Nf1* Schwann cells were labeled with Hoechst dye and grafted around transected wild type nerves. Of 4000 labeled cells counted in 6 animals, 68 contained pigment granules; of pigmented cells counted, 12.6% were dye-positive. Thus *Nf1* mutant Schwann cells can transdifferentiate into pigmented melanocytes. We also tested if the *Nf1*-mutant environment contributes to the phenotype. Schwann cells wild type or mutant at *Nf1* were transplanted into wild type or heterozygous mice. Pigmentation was maximal when both host and graft were mutant. Thus environmental factor(s) and cell autonomous Schwann cell abnormalities contribute to lesion-induced hyperpigmentation in *Nf1* mutants.

A few *Nf1/nf1* mice developed tumors by 3 months after nerve injury. 4/28 developed unencapsulated unpigmented tumors while 5/28 developed encapsulated pigmented tumors. Thus, wounding of the sciatic nerve in mice with a single mutant NF1 allele is sufficient to cause disruption of tissue organization with features resembling human NF1. Supported by NIH NS-28840 and a grant from the DOD.

Neurofibromatosis type 1: Genetic and cellular mechanisms of peripheral nerve tumor formation.

N. Ratner, R. Atit, H. A. Kim, B. Ling, J. A. DeClue¹, M. Crowe, and T. A. Rizvi; Department of Cell Biology, Neurobiology, and Anatomy, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA; ¹National Cancer Institute, Bethesda, MD, USA.

Neurofibromatosis type 1 (NF1) is among the most common inherited human diseases, affecting 1:3500 humans worldwide. The NF1 protein is a RAS-GTPase activating protein, positioning NF1 in important intracellular signaling pathways. Patients with mutations in the *NF1* gene develop benign peripheral nerve tumors (neurofibromas) and, rarely, malignant peripheral nerve sheath tumors (MPNST). The *NF1* gene is believed to act as a tumor suppressor in malignancies including MPNST.

We have developed model systems to study Schwann cells and fibroblasts, the two major cell types within neurofibromas, from transgenic mice with *Nf1* mutations. In spite of increased levels of Ras-GTP, mutant Schwann cells hypo-proliferate. *Nf1* mutant Schwann cells do transform readily under appropriate medium conditions, suggesting that loss of *Nf1* facilitates Schwann cell transformation. Using this mouse model system we have begun to define molecular changes that correlate with the Schwann cell transformation. We then tested if specific changes are also detected in human neurofibromas or malignant peripheral nerve sheath tumors. Alterations in tyrosine kinase growth factor receptor levels in mutant cells correlate with transformation in both mouse and human systems, providing new potential interventions in NF1-related peripheral nerve tumors. Whether Schwann cell abnormalities are sufficient to cause neurofibroma formation is not known. Fibroblasts make up 20-60% of cells within neurofibromas, and manufacture the collagen-rich matrix characteristic of neurofibromas. We have recently demonstrated that fibroblasts from transgenic mice with *Nf1* mutations are abnormal. *Nf1* mutant fibroblasts deposit increased levels of collagen and hyper-proliferate as compared to wild type cells. Furthermore, *in vivo* experiments show that wounding induces dysfunction of fibroblasts mutant at *Nf1*. Our data implicate multiple cell types mutant at *NF1*, non-NF1 related events, and environmental changes in the pathogenesis of NF1 peripheral nerve tumors.

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DEPARTMENT OF THE ARMY
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REPLY TO
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