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J Invest Dermatol. Author manuscript; available in PMC 2010 May 3.

Published in final edited form as:
J Invest Dermatol. 2000 June ; 114(6): 1093–1100. doi:10.1046/j.1523-1747.2000.00994.x.

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

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Abstract

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*-tetradecanoyl-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.

Keywords

carcinogenesis; keratinocyte; melanocyte; phorbol ester; Ras

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

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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 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-

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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

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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 CO_2. 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 ethylendiamine 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).

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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 cm^2. 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 1*B*, *C*; Table I). Most wild-type mice did not develop pigmentation in response to DMBA (Fig 1*A*), 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 1*D*). In contrast, skin from pigmented patches of affected wild-type and *Nf1*+/− mice showed large numbers of hair follicles filled with melanin pigment (Fig 1*E*, *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 1*H*, *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 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 2*B*) and pigmented (Fig 2*C*) regions was indistinguishable from that observed when skin was treated with DMBA alone (Fig 1*D*, *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 2*A*, Table II). By the end of 24 wk, nine of 12 (75%) of *Nf1*+/− animals (Fig 3*A*) had developed papillomas. Most animals had one to three tumors; tumor ranged in volume from 1 to 80 mm^3 (Fig 3*B*). 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.

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Papillomas were analyzed histologically in hematoxylin and eosin stained paraffin sections (Fig 2*D*). Papillomas showed epithelial hyperplasia (*e* in Fig 2*E*) and trapped sebaceous glands with follicular cysts (*s* and *c*, respectively, Fig 2*F*) 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 4*A*, *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 4*B*, *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.

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 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 ≈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.

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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 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

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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*. Mangues *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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Abbreviations

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

Acknowledgments

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. Radhika Atit was the recipient of a University of Cincinnati Dean's Distinguished Dissertation Fellowship. This work was supported by NIH-ES01203 (to D.W.) and NIH-NS28840 and the DOD through grants to NR.

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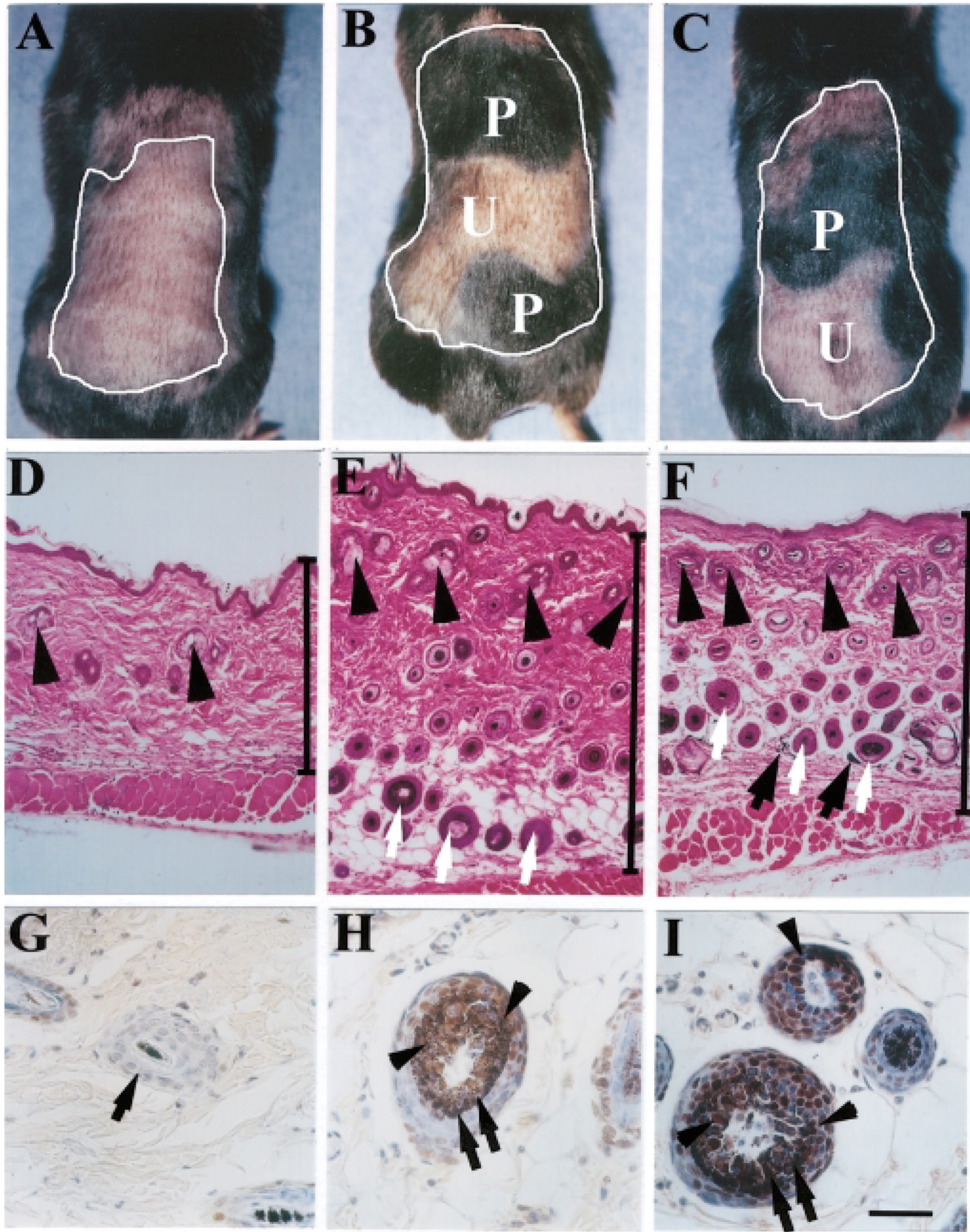

Figure 1. Gross appearance and histology of DMBA treated wild-type and *Nf1*+/− skin
(*A–C*) Show gross photographs of shaved dorsum of mice 2 mo after initiation with two doses of 40 μg of DMBA. Treated areas are outlined in white. (*A*) Representative of a wild-type animal after initiation; this animal 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

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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 hematoxlyin counterstain of sections from the regions shown in *D–F*,*H*,*I* melanin is present in follicles (*black arrows*). *Scale bar*: (*D–F*) 50 µm; (*G–I*) 10 µm.

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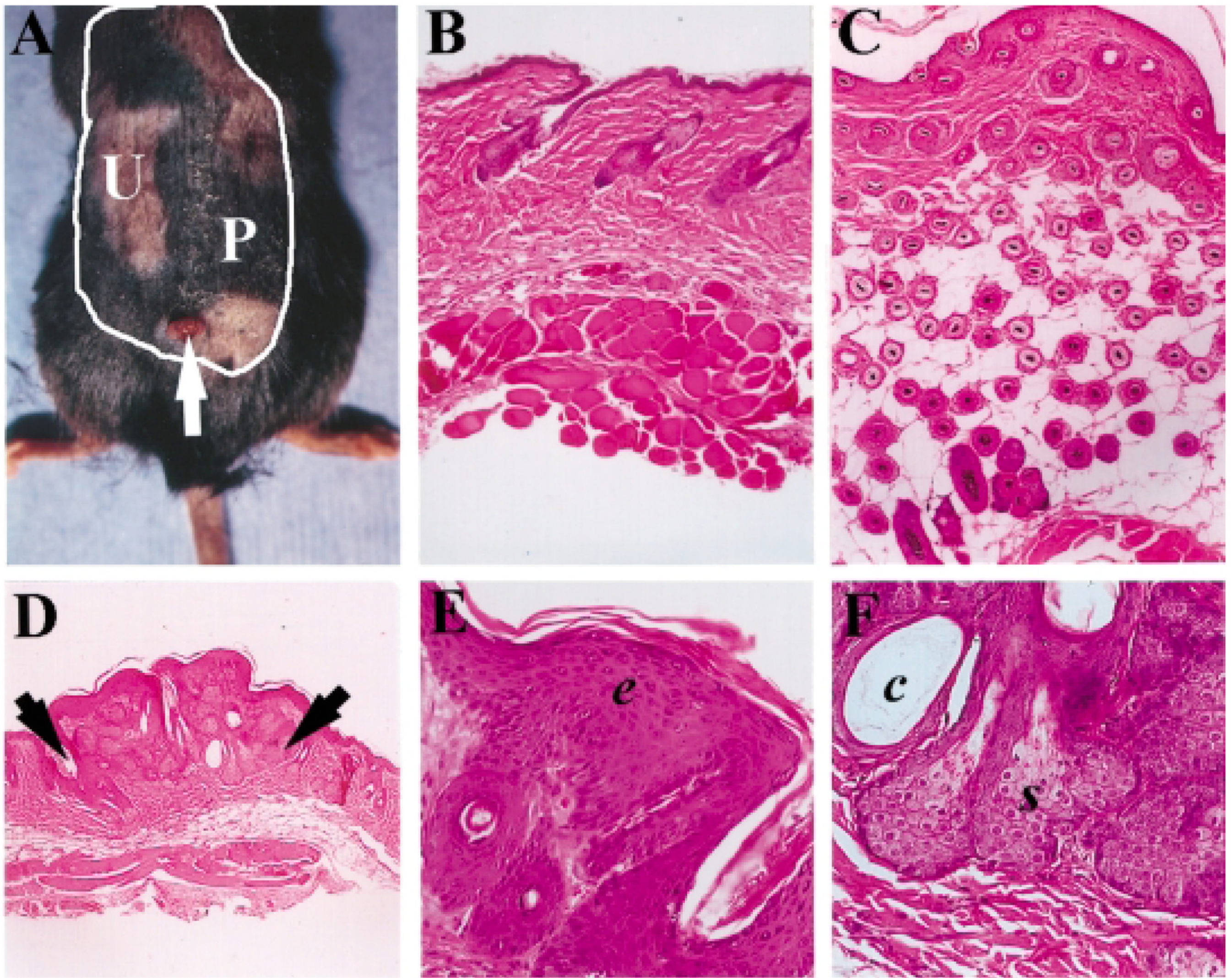

Figure 2. Gross and histologic appearance of skin and papillomas from *Nf1*+/− mice after tumor promotion

(*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.

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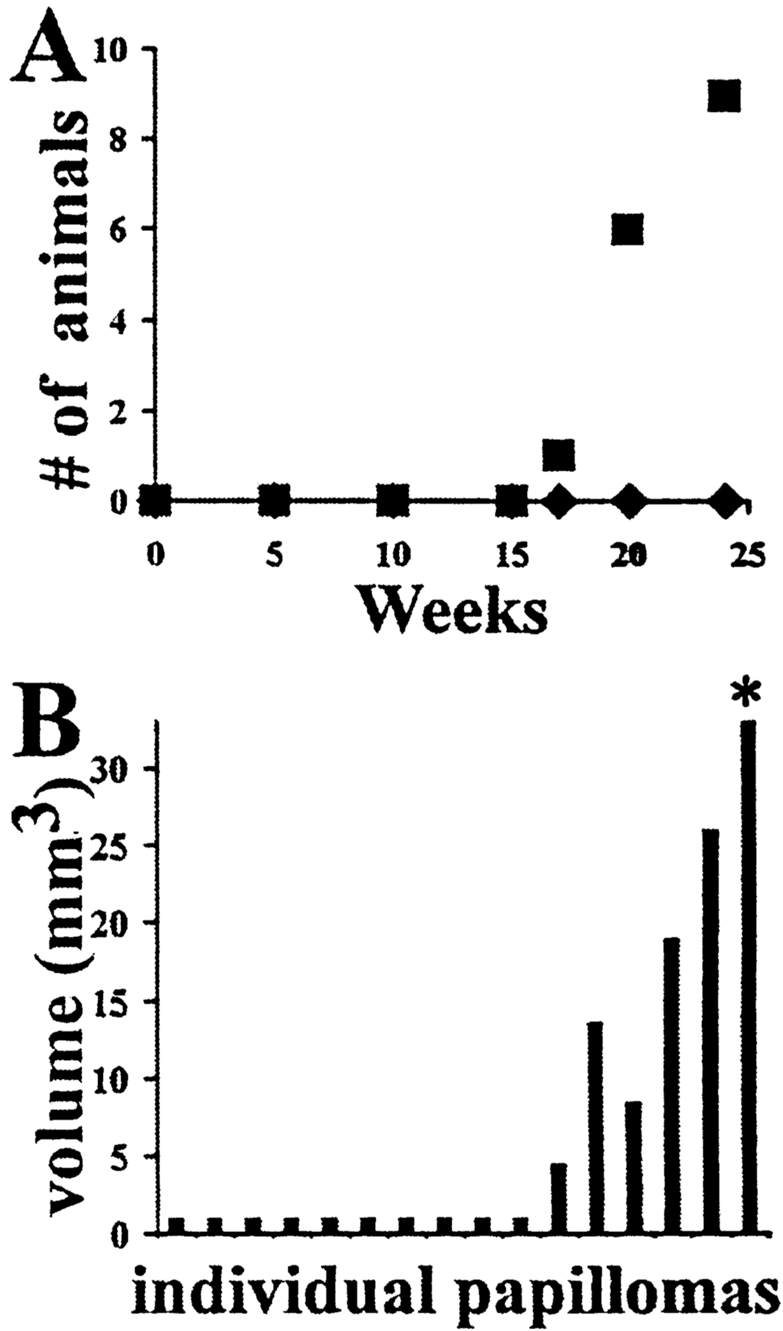

Figure 3. Papilloma formation in *Nf1*+/− mice

(*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 mm^3 papilloma.

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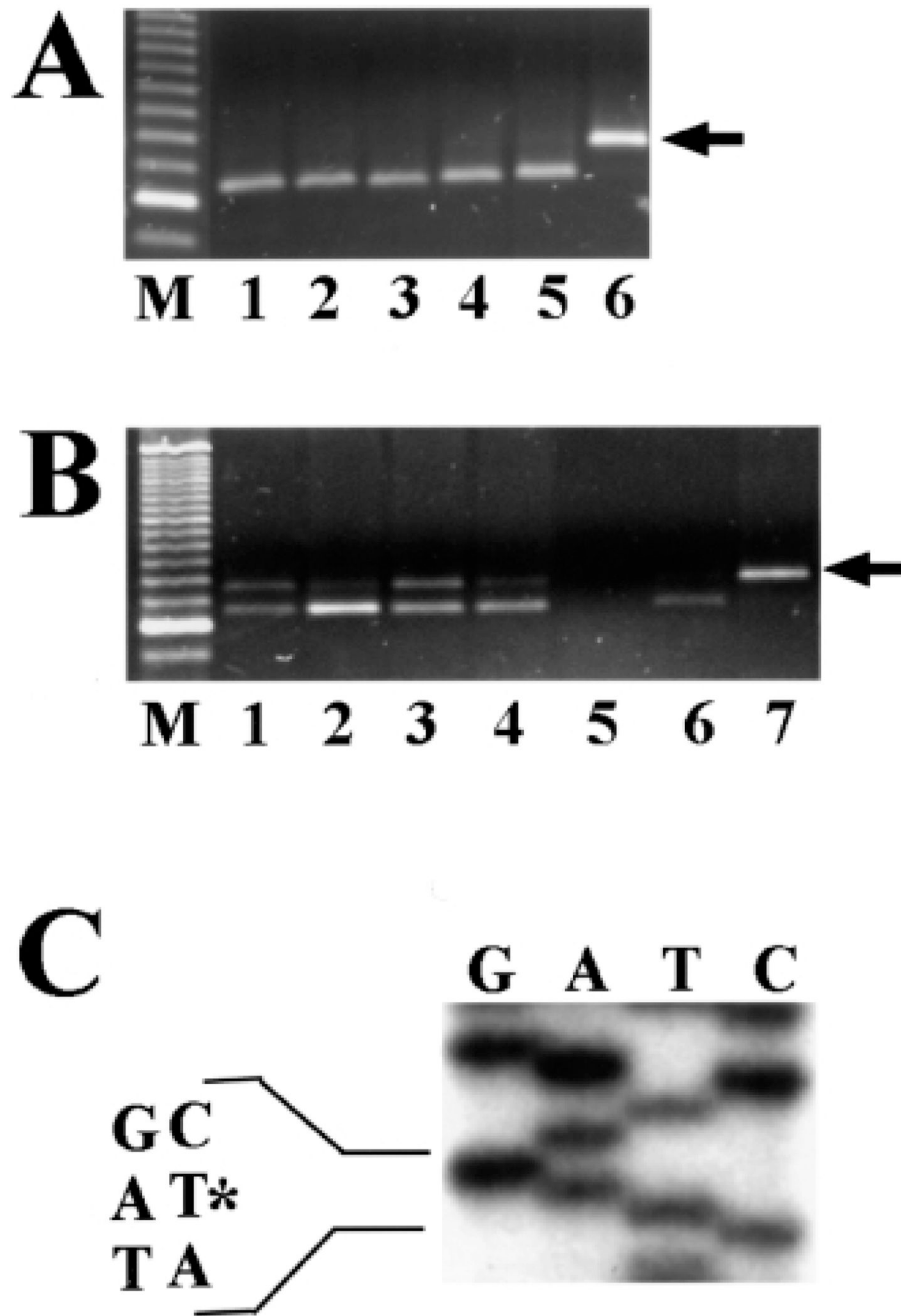

Figure 4. Mutational analysis of codon 61 from the H-*ras* gene in treated skin and papillomas

(*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.

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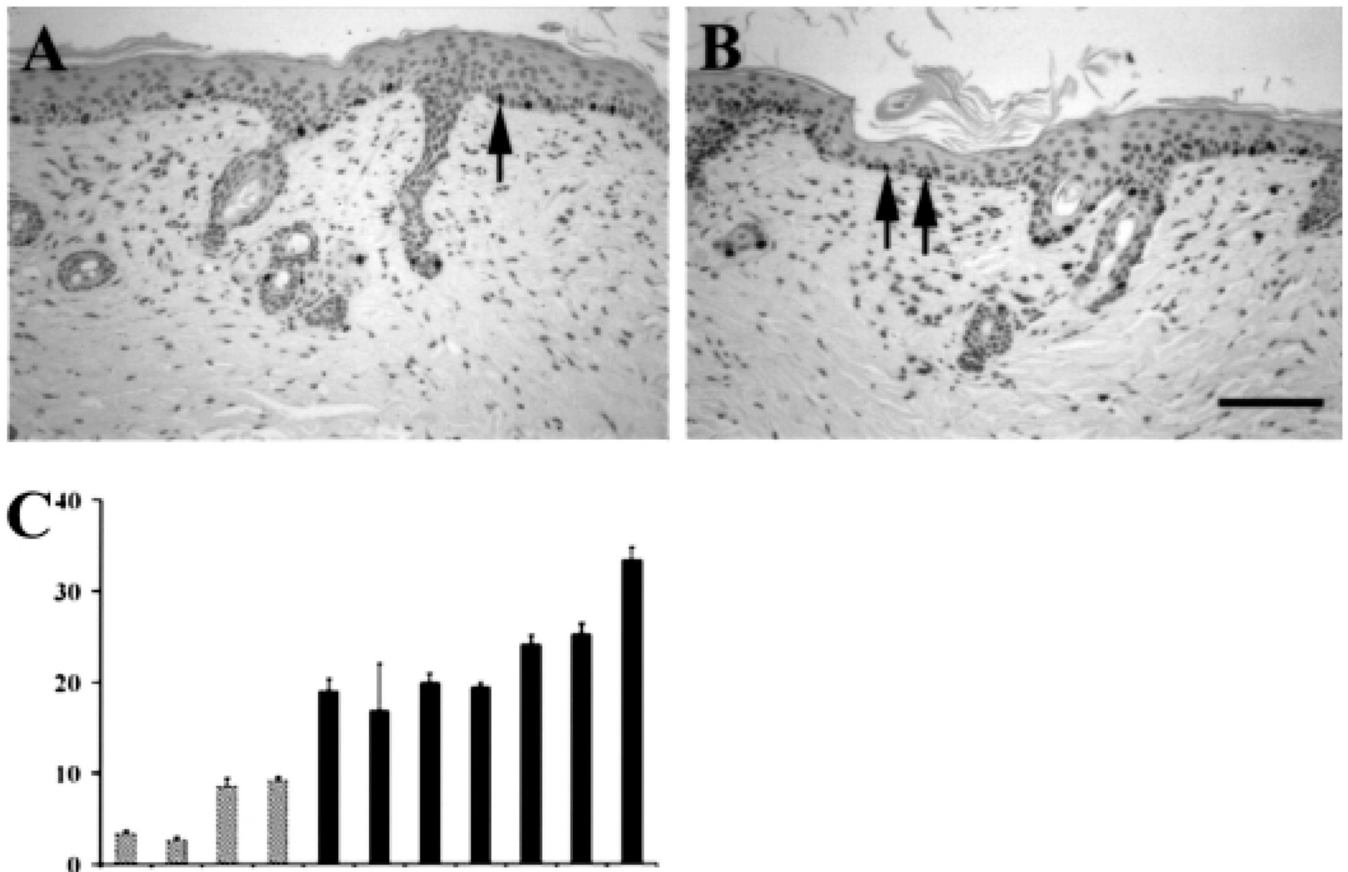

Figure 5. TPA exposure results in enhanced epidermal labeling index in *Nf1*+/− mice

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 μg). Arrows designate positively stained basal keratinocytes. *Scale bar*. 10 μm. (*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).

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Table I

Incidence of pigmentation after treatment with skin carcinogens

Treatment[a]	*Nf1*+/+	% affected	*Nf1*+/−	% 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

[a]C57BL/6 male mice were treated with 200 µl topical acetone vehicle, TPA (0.8 µg, three times per week), DMBA (40 µg, two treatments), or DMBA + TPA (DMBA 40 µg, two treatments followed by TPA, 0.8 µg, three times per week).

[b]Animals exhibited that exhibited one or more patches of pigmentation larger than 1 cm^2 were scored as positive. When patches appeared, they were always larger than 1 cm^2.

[c]The first three groups were scored by 4 mo after initiation of experiments.

[d]The DMBA + TPA group was scored 6 mo after initiation of treatment.

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Table II

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

Specimen[a]	**Genotype**	**Mutant H-*ras* positive**[b]
Unpigmented skin	*Nf1*+/+	0/4
Nf1+/−	0/5	
Pigmented skin	*Nf1*+/+	0/4
Nf1+/−	0/5	
Papillomas	*Nf1*+/−	6/6

[a]Following 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.

[b]DNA 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*.