ABSTRACT

Patients carrying an inactive NF1 allele develop tumors of Schwann cell origin called neurofibromas (NF). Genetically engineered mouse models have significantly enriched our understanding of plexiform forms of NFs (pNF). However, this has not been the case for cutaneous neurofibromas (cNF), observed in all NF1 patients, as no previous model recapitulates their development. Here, we show that conditional Nf1 inactivation in Prss56-positive boundary cap cells leads to bona fide pNFs and cNFs. This work identifies subepidermal glia as a likely candidate for the cellular origin of cNFs and provides insights on disease mechanisms, revealing a long, multistep pathologic process in which inflammation-related signals play a pivotal role. This new mouse model is an important asset for future clinical and therapeutic investigations of NF1-associated neurofibromas.

SIGNIFICANCE: Patients affected by NF1 develop numerous cNFs. We present a mouse model that faithfully recapitulates cNFs, identify a candidate cell type at their origin, analyze the steps involved in their formation, and show that their development is dramatically accelerated by skin injury. These findings have important clinical/therapeutic implications.

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common human genetic disorder caused by heterozygous loss of function of the tumor suppressor gene NF1, encoding neurofibromin 1. It predisposes patients to develop benign nerve sheath tumors, termed neurofibromas (NF). Although about 20% of NF1 patients suffer from plexiform NFs (pNF) along peripheral nerve tracts, with significantly higher incidence at nerve roots, all of them develop cutaneous NFs (cNF) localized at nerve terminals in the skin (1). cNFs typically appear at puberty and tend to increase in number throughout life, so that they may reach thousands. Although of benign character, these tumors can be disfiguring and painful, thus significantly affecting quality of life. All NFs are composed of a mixture of Schwann cells (SC) with other nerve fiber elements, such as axons, fibroblasts, mastocytes, macrophages, and endothelial cells. Despite this marked cellular heterogeneity, NFs are known to arise from cells belonging to the SC lineage in which the remaining functional copy of NF1 is mutated, resulting in complete NF1 loss of function and permanent hyperactivation of the RAS proto-oncogene and its downstream effectors (1). However, the specific cell type within the SC lineage in which the second-hit mutation occurs is still controversial. Although nonmyelinating SCs (nmSC) were initially considered the primary pathogenic target, several recent studies suggest that at least pNFs arise as a result of NF1 mutation at earlier stages in the SC lineage, possibly as early as neural crest stem cells (2).

Recently, significant efforts have been made to develop genetically engineered mouse (GEM) models to study NF1-associated NFs (2). These genetic tools have allowed major advances in the understanding of the mechanisms implicated in pNF formation. However, as none of these models recapitulate the development of cNFs, their origin and pathogenic mechanisms remain largely unknown. There is therefore an urgent need to develop new animal models that recapitulate cNFs and allow their experimental analysis.

As possible candidates for the cells at the origin of paraspinal pNFs (ppNF), neural crest-derived boundary cap (BC) cells, which are transiently located at the embryonic nerve root entry/exit zone, have attracted particular attention. Using the Krox20/Egr2 gene as a marker for BC cells,
we have demonstrated that Krox20-expressing BC cells give rise to the entire SC component of the nerve roots, precisely where ppNFs develop (3). Furthermore, conditional inactivation of Nfi1 in Krox20-expressing cells gives rise to ppNFs, but not cNFs (4). This suggests that Nfi1 inactivation in the Krox20-positive BC cell lineage leads to ppNFs and that cNFs do not originate from these cells. Recently, we have identified another BC cell marker, Prss56, and developed a mutant allele, Prss56<sup>−/−</sup>, in which the Cre recombinase coding sequence is inserted into the locus (5). In vivo fate-mapping analyses with this allele indicated that BC derivatives migrate into the nerve roots and, along peripheral nerves, into nerve terminals in the skin, precisely the two locations where NFs are observed (6). These observations led us to hypothesize that the lineage of Prss56-expressing BC cells might be the target for Nfi1 mutation in both plexiform and cutaneous forms of NFs.

In the present study, we have generated and characterized mice carrying biallelic Nfi1 deletions in Prss56-expressing BC cells and their progeny, by introducing Prss56<sup>lox/lox</sup> and the Cre-activatable reporter R26<sup>tdTom</sup> alleles into Nfi1-floxed backgrounds (Nfi1<sup>fl/−</sup> or Nfi1<sup>−/−</sup>), susceptible to loss of function upon Cre recombination. This approach allows conditional deletion of Nfi1 in Prss56-expressing BC cells and in vivo tracing of mutant cells due to permanent expression of the fluorescent reporter. We found that both types of mutants develop numerous cNFs and pNFs. As inactivation of Nfi1 in the peripheral nervous system (PNS) is restricted to Prss56-expressing BC cells and their derivatives, this population is clearly directly involved in the development of NFs. Our model allows a detailed analysis of cNF development, leading to critical observations on the etiology, progression, and properties of the disease that are of prime importance for clinical and therapeutic applications.

RESULTS

**Prss56-expressing BC Cells Give Rise to Glial and Nonglial Derivatives in Nerve Roots and Skin Nerve Terminals**

We have previously shown that derivatives of Prss56-expressing BC cells migrate into nerve roots and nerve terminals in the skin (6). Here, using Prss56<sup>lox/lox</sup>R26<sup>tdTom</sup> mice, we present a detailed characterization of tdTomato (TOM)-traced cells in these two structures. As expression of Prss56 was never detected in nerve roots nor in the skin, we consider that all traced cells in these tissues correspond to BC derivatives (ref. 6; Supplementary Fig. S1A). Consistent with our previous report (6), numerous traced cells were present along the adult dorsal and ventral nerve roots and in the dorsal root ganglia (DRG), but there were only a few in the proximal segment of the spinal nerves (Fig. 1A). In the trunk of the sciatic nerve, TOM staining was absent from SCs (S100-positive; S100<sup>+</sup>) and remained restricted to a small subset of axons (TUJ1<sup>+</sup>; Supplementary Fig. S1B). We performed IHC analyses in the nerve roots with SC marker S100 and neural crest lineage marker SOX10 (Fig. 1B–D). All traced cells were derived from the neural crest, whereas only a subset of the SOX10<sup>+</sup> population was traced (Fig. 1C). Quantification in cervical dorsal nerve roots indicated that 76.1% ± 10.2% (mean ± SD) of the SOX10<sup>+</sup> cells originated from Prss56-expressing BC cells. Most traced cells were S100<sup>+</sup> and correspond to myelinating SCs (mSC) and nmSCs, according to their morphology and organization with respect to axons. The level of S100 expression was much weaker in nmSCs than in mSCs (Fig. 1B and D).

In addition to SCs, we observed a small subset of TOM<sup>+</sup> endoneurial cells that lacked S100 immunoreactivity (Fig. 1B), possibly corresponding to neural crest–derived endoneurial fibroblasts (eFB; ref. 7). To explore this possibility, we immunolabeled adult nerve roots with TOM, a generic fibroblast marker, PDGFRα, and CD34, recently identified as a putative eFB marker in human and rat peripheral nerves (8). Whereas numerous cells expressing these markers were observed in the endoneurial space, no overlap with TOM labeling was observed (Supplementary Fig. S1C and S1D). Further studies will therefore be required to characterize the identity of this TOM<sup>+</sup>S100<sup>−</sup> population. Finally, we have never observed TOM<sup>+</sup> perineurial fibroblasts (Fig. 1B; Supplementary Fig. S1E).

In the adult skin, traced cells were found in the dermis (Fig. 1E) and hypodermis (subcutis; Fig. 1F), but were absent from the epidermis (Fig. 1G). In the dermis, they showed a highly heterogeneous distribution, with patches aligned with some, but not all, nerves and nerve terminals (Fig. 1E and G). IHC analysis with S100 revealed that most of these cells correspond to nmSCs (Fig. 1G). Nevertheless, we also observed a few traced mSCs (S100<sup>+</sup>/MBP<sup>+</sup>) enwrapping rare large-diameter fibers, mainly located in the lower dermis or innervating hair follicles (Supplementary Fig. S1F). Dermal traced nmSCs exhibited broad heterogeneity and could be classified into four categories: (i) typical nerve-associated nmSCs unshathing small-caliber axons (Remak SCs; Fig. 1G); (ii) hair follicle-associated glia unshathing lanceolate nerve endings (Fig. 1G and H); (iii) glia associated with the arrector pili muscle (Fig. 1G and I); and (iv) terminal glia associated with nociceptive free nerve endings (Fig. 1G and J; Supplementary Fig. S1G). This latter population, residing directly beneath the epidermis (thus further referred to as subepidermal SCs), is characterized by a complex multipolar morphology and remains largely uncharacterized, although it has been identified in the human hairy skin (9, 10). Analysis of large skin fragments also revealed the presence of pigmented TOM<sup>+</sup> and KIT<sup>+</sup> melanocytes, in a subset of hair follicle bulbs (Fig. 1K, K′; Supplementary Fig. S1H, H′). Finally, although BC derivatives are absent from the trunk of peripheral nerves, they populate subcutaneous nerve branches, where they correspond to a subset of mSCs, nmSCs, and eFBs (Fig. 1F; ref. 6).

Together, our observations indicate that Prss56-expressing BC cells participate in the development of nerve roots and cutaneous/subcutaneous innervation as a source of glial and nonglial derivatives.

**Conditional Nfi1 Knockout in BC Cells Leads to Skin Lesions and Progressive Limb Paralysis**

Because BC derivatives populate nerve roots and nerve terminals in the skin, two highly favored sites for NF development, we explored whether Nfi1 knockout in Prss56-expressing BC cells was sufficient to promote formation of NFs in the mouse. We transferred the Prss56<sup>lox/lox</sup>R26<sup>tdTom</sup> alleles into Nfi1<sup>floxed/−</sup> or Nfi1<sup>−/−/−</sup> backgrounds to generate Prss56<sup>lox/lox</sup>,
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Figure 1. Tracing of Prss56-positive BC cell derivatives in the adult PNS. A–D, Analysis of BC cell derivatives (TOM+) in the adult nerve roots of Prss56Cre, R26tdTom mice. A, Transverse section through the cervical spinal cord reveals the presence of numerous TOM+ cells along the dorsal and ventral nerve roots and in the DRG. The inset shows a transverse section through the dorsal root. B–D, Most nerve root TOM+ cells express S100 (B, D) and SOX10 (C) and correspond to SCs (full arrowheads in B and D). Note the presence TOM+/S100− endoneurial cells (open arrowheads in D) and TOM− perineurial fibroblasts (see also Supplementary Fig. S1C). Spc, spinal cord; DR, dorsal root; VR, ventral root; DRG, dorsal root ganglion; Nu, nuclear labeling.

E–K, Analysis of BC cell derivatives in the dorsal skin of adult Prss56Cre, R26tdTom mice. E and F, Dorsal (epidermal) and ventral (hypodermal) views of the back skin showing direct TOM fluorescence. Note the patch-like distribution of TOM+ cells. G, Transverse section of the skin immunolabeled with TOM and an SC marker S100. TOM+ cells are associated with dermal and hypodermal innervation (arrowheads in G and arrow in F, respectively) and correspond to nmSCs, including lanceolate glia (G, H), arrector pili glia (G, I), and subepidermal glia (G, J). I and J are dorsal views of whole-mount skin colabeled with TOM and axonal marker TUJ1. K and K’, TOM+ pigmented melanocytes within a hair follicle. TOM staining merged with a bright field image (K’) reveals melanin granules (dark pigment). Epi, epidermis; der, dermis; hyp, hypodermis; HF, hair follicle. Scale bars, 50 μm (A–D, G–K’), 1 mm (E, F).
analyses, as the phenotypes in the spinal cord at the cervicothoracic level. In subsequent DRGs, often accompanied by tissue masses compressing logic analysis of Nf1-KO mice revealed enlarged nerve roots/
required the sacrifice of the animals. Gross anatomic–patho-
Skin lesions were typically accompanied by pruritus that and were often accompanied by progressive limb paralysis. (Supplementary Table S1). The symptoms included lethargy
flox/–KO–mutant spinal cord with the (Supplementary Fig. S1A).

The use of the two genetic backgrounds (Nf1–flox/– or Nf1–flox/flox) for the analysis of the phenotypes associated with the targeted mutation is important, because in some of the existing Nf1 GEM models, tumorigenicity requires inactivation of both Nf1 alleles in the cells that become neoplastic, along with inactivation of one allele in the tumor environment (4). As controls (CTRL), we used Prss56Cre/R26TOM,Nf1–flox/+ littermates, which carry only a heterozygous Nf1 alleles in the cells that become neoplastic, along
Both types of Nf1-KO mutants were born at the expected Mendelian frequency and all developed identical symptoms, at 10 ± 4.5 months and 13.1 ± 3.9 months (mean ± SD) of age for Nf1–flox/– and Nf1–flox/flox backgrounds, respectively (Supplementary Table S1). The symptoms included lethargy
and numerous skin lesions, mainly localized on the neck, and were often accompanied by progressive limb paralysis. Skin lesions were typically accompanied by pruritus that required the sacrifice of the animals. Gross anatomic–pathologic analysis of Nf1-KO mice revealed enlarged nerve roots/DRGs, often accompanied by tissue masses compressing the spinal cord at the cervicothoracic level. In subsequent analyses, as the phenotypes in Nf1–flox/– and Nf1–flox/flox backgrounds appeared to be similar apart from the mean age of appearance, animals from both backgrounds were used interchangeably, except when explicitly indicated.

BC-Derived nmSCs Constitute the Major Pathogenic Cell Type in Plexiform Neurofibroma

Gross dissection of Nf1-KO-mutant spinal cord with the attached nerve roots revealed the presence of tumors that are largely TOM+, localized at the thoracic and/or cervical nerve roots (Fig. 2A–C). All remaining nerve roots showed signs of marked hyperplasia (Fig. 2A–C). The number of tumors varied from 1 to 4 and often affected contralateral sides. Severe spinal cord compression was frequent and likely accounted for the observed paralysis (Fig. 2C and D). Paraspinal lesions contained abnormal accumulations of disorganized, slender TOM+ cells that expressed low or no S100, likely corresponding nmSCs (Fig. 2E–G*). In the mutants, unlike controls, most TOM+ cells coexpressed SOX2, a marker of precursor/immature SCs (Supplementary Fig. S2A–S2B*). In contrast, TOM+ mSCs, enwrapping large-caliber axons, expressed high levels of S100 and showed no obvious abnormalities (Fig. 2E–G*). We also observed increased infusion of KIT+ mas-
tocytes and IBA1+ macrophages (both nontraced), which are always detected in human pNFs (Supplementary Fig. S2C–S2F*). Ultrastructure analysis of paraspinal tumors revealed a substantial expansion of an interstitial compartment filled with collagen-rich extracellular matrix and accumulation of abnormal Remak bundles (Fig. 2H–K). Mutant nmSCs (identified by continuous basal lamina) showed profound morphologic abnormalities, including partial or complete dissociation from axons, and extension of numerous cyto-
plasmic protrusions surrounding collagen fibrils or myeli-
nated fibers (Fig. 2I–K).

In addition, subcutaneous nerves containing traced BC derivatives were markedly enlarged, and localized enlargements (ballooning) were often observed beneath the skin lesions (Fig. 2L). Subcutaneous lesions were very similar to paraspinal tumors in their histology and ultrastructure (Fig. 2M–P) and contained large numbers of traced, S100+ nmSCs with disrupted morphologies and axonal interactions, intermixed with numerous nontraced, PDGFRα+ fibroblasts. Fibroblasts present in the peripheral part of a tumor had typical characteristics of perineurial fibroblasts, such as patchy basal lamina and the presence of pinocytic vesicles (Supplementary Fig. S2G). However, unlike the control nerves, where perineurial cells form a multilayered, tightly compacted protective sheath, the perineurial compartment of the Nf1-KO nerves appeared much thicker and contained a high number of loosely organized fibroblasts intermingled with numerous SC processes and large amounts of collagen (Fig. 2O and P). Despite these abnormalities, nerve integrity seemed to be preserved. Finally, as in paraspinal lesions, there was an increased influx of mastocytes and an even more pronounced infiltration of macrophages in the tumor masses (Supplementary Fig. S2H and S2I).

Together, our observations support the previously proposed role of BC-derived nmSCs in the formation of ppNFs and a similar involvement in the development of subcutaneous tumors.

Nf1 Inactivation in Prss56-Positive Cells Leads to the Development of Diffuse cNFs

In Nf1-KO mice, the first macroscopically detectable cutaneous symptoms were areas of hair thinning on the neck, which rapidly evolved into pruritus (Fig. 3A and B). After shaving the hair, we frequently observed additional smaller lesions (with or without scabs) containing well-circumscribed, dense aggregates of traced cells (Fig. 3B). Such dense clusters of TOM+ cells were never observed in control skin. Although affected skin regions were significantly thicker than nonaffectected skin from the same individual, their basic architecture, including integrity of hair follicles, remained preserved. IHC analyses of lesioned areas indicate that (i) the diffuse accumulation of traced cells is restricted to the dermis; (ii) traced cells correspond to S100+ mSCs. However, the level of S100 was significantly lower than in rare mSCs. These nmSCs appear highly disorganized and extend numerous slender cytoplasmic processes (Fig. 3C and D*). Their SC identity is further supported by expression of other known nmSC/immature SC markers, including p75NTR, NCAM, and L1, as well as SOX2 (Supplementary Fig. S3A–S3D); (iii) mutant nmSCs express high levels of phosphorylated ERK1/2, a downstream effector
Figure 2. Targeted Nf1 inactivation in the BC cell lineage gives rise to paraspinal and subcutaneous pNFs. A, Global view of control (left) and Nf1-KO mutant (right) spinal cords with attached nerve roots from 11-month-old animals. Paraspinal tumors are present in the mutant at the cervical and thoracic levels (arrowheads). B and C, Higher magnifications of the control (B) and Nf1-KO-mutant (C) thoracic regions showing enlargement of the mutant nerve roots (full arrowheads) and adjacent DRGs (empty arrowheads) caused by accumulation of TOM+ cells. D, Section through this region showing compression of the spinal cord by the TOM+/S100+ tumor mass. E-G, Increasing magnifications through a neurofibroma immunolabeled for TOM, S100, and TUJ1. Strongly S100+ mSCs (always in contact with large-caliber axons) are intermixed with randomly organized TOM+ cells that are weakly S100− (full arrowheads in G−G′) or S100− (empty arrowheads in G−G′). Ultrastructure analysis of control (H) and Nf1-KO (I–K) nerve roots revealed an enlargement of the extracellular compartment and disruption of Remak bundles (arrowhead) in the mutant. Normal Remak SCs are shown in H (arrowhead and inset). Mutant nmSCs ensheath fewer axons or are devoid of axonal contact and extend slender protrusions that enwrap collagen fibrils (J) or myelinated fibers (K). nmSCs are pseudocolored in red in (H, J, and K). L, Subcutaneous nerve showing the local enlargement corresponding to a subcutaneous plexiform NF (spNF; arrowhead). M–O, Sections through an spNF immunolabeled for TOM, S100, and PDGFRα (fibroblasts) reveal expansion of traced SCs and non-traced fibroblasts. N–O correspond to the boxed area in M. P, Electron microscopy micrograph of the spNF shows disrupted Remak bundles (arrows) and abnormal perineurial sheath (arrowheads). Spc, spinal cord; DR, dorsal root; DRG, dorsal root ganglion; m, myelinating Schwann cell; a, axon. Scale bars, 1 mm (A–C, L), 50 μm (D–F, M–O), 1 μm (H–K, P).
Figure 3. Mice with Nf1 loss of function in BC cell derivatives develop diffuse cutaneous NFs. A, 19-month-old Nf1-KO mutant showing pruritic skin lesions (arrows) at cervical and thoracic levels. B, Numerous small lesions present on the back skin (arrowheads) showing direct TOM fluorescence (inset). C–G′, IHC analysis of cNF reveals accumulations of TOM+ and S100+ SCs with aberrant morphologies (C, D′), and heavy infiltration of KIT+ mastocytes (E, E′), IBA1+ macrophages (F, F′) and PDGFRα+ fibroblasts (G, G′). Control skin is shown on the bottom left of each figure. Insets in E–G′ represent low magnifications of the corresponding images with TOM labeling. Dotted lines indicate the upper tissue limits and the boundaries between dermis and hypodermis.

KIT staining within hair follicles (HF) in C corresponds to melanocytes. H, Dense collagen accumulation in cNF revealed by sirius red staining (red). I, Extrafollicular pigmentation in cNF. The inset shows higher magnification of TOM+ and TOM− pigmented cells (dark granules). Dotted lines encircle hair follicles containing pigmented hair shafts. J and K, Electron microscopy micrographs showing SC cytoplasmic protrusions (arrowheads in J) unsheathing isolated axons (a) and/or collagen fibrils (inset in J). K, Immunoelectron micrograph of mutant SC (pseudocolored in red) without axonal contact at the level of the soma. Dark granules correspond to TOM staining. Scale bars, 5 mm (B), 100 μm (C–I), 1 μm (J, K).
of RAS (Supplementary Fig. S3E); (iv) numerous nontraced KIT+ mastocytes, IBA1+ macrophages, and PECAM1+ blood vessels infiltrate the lesion (Fig. 3E and F; Supplementary Fig. S3F); (v) the dermal fibroblast population (nontraced and defined by PDGFRα expression) is drastically enlarged (Fig. 3G and G’) and accompanied by heavy deposition of collagen (Fig. 3H); (vi) both traced and nontraced pigmented cells, presumably melanocytes, are often concentrated in extracellular dermis within the lesion (Fig. 3I).

Ultrastructural analysis of the Nf1-KO skin lesions confirmed that numerous nmSCs with severely affected morphologies are present in the dermis. These SCs form a dense network of cytoplasmic extensions that often enwrap isolated axons and/or are devoid of axonal contact, and enclose clusters of collagen fibrils, forming apparent collagen pockets (Fig. 3J and K). Immunoelectron microscopy confirmed that most of these cells express TOM and correspond to mutant nmSCs (Fig. 3K). Finally, numerous fibroblasts (identified by morphology and the absence of basal lamina) and collagen fibers were observed at the level of the lesions (Supplementary Fig. S3G). Because endoneurial and perineurial fibroblasts are rare or absent in the dermis, we assume that this accumulation of fibroblasts mainly corresponds to recruitment and/or proliferation of mesodermally derived dermal population (nontraced). Lack of basal lamina and pinocytic vesicles, a typical characteristic of perineurial fibroblasts, further supports their dermal identity. Although in control skin, dermal fibroblasts are rare, of round shape, with few ribosomes and poorly developed endoplasmic reticulum (ER), in the Nf1-KO mutant dermis, they often form long cytoplasmic extensions with or without collagen pockets and contain numerous ribosomes and highly developed ER (Supplementary Fig. S3G–S3I), supporting their engagement in the active synthesis of collagen. Finally, in addition to infiltration by mastocytes and macrophages, ultrastructural analysis revealed accumulations of polymorphonuclear neutrophils, which were only sporadically observed in control skin (Supplementary Fig. S3J). In conclusion, all morphologic, cellular, and molecular characteristics associated with the cutaneous lesions are highly similar to those of diffuse human cNFs (11). These data indicate that the loss of Nf1 in dermal BC derivatives is sufficient to promote development of cNFs. This is correlated with activation of the ERK signaling pathway in mutant SCs.

Development of cNFs Involves Progressive Alterations of the Dermis

Although patients with NF1 develop cNFs during adulthood after a long asymptomatic period, it is unclear whether these tumors appear suddenly in the adult skin or whether they slowly evolve from microtumoral loci already present at younger ages. To investigate this issue, we compared the skin of Nf1-KO mice and control littermates from embryonic day E13.5, when the first traced BC derivatives settle in the skin, until 6 months after birth, when most mutants do not show obvious cutaneous manifestations. For this study, we used only Prs65+R26tdTom,Nf1fl/+ mutants which, similar to NF1 patients, harbor heterozygous germline Nf1 mutation along with homozygous Nf1 knockout in the SC lineage. The inactivation of Nf1 in BC cells around E11 did not affect the migration of BC-derived progenitors along the nerves to the skin nor the number of traced SC precursors or immature SCs in the embryonic skin (Supplementary Fig. S4A–S4F). This suggests that the early specification and migration of the SC lineage is not affected in the absence of Nf1. At birth, however, the number of traced SCs in Nf1-KO was nearly double that in control skin (Fig. 4A–C), although all mutant SCs remained in contact with axons and did not show any morphologic abnormalities. Intraperitoneal 5-ethyl-2’-deoxyuridine (EdU) injection into pregnant females at E18.5, followed by analysis of newborn skin, revealed a small but significant (P = 0.036) increase in the proportion of proliferating SCs (EdU+/TOM+) in Nf1-KO (22.6% ± 1.37%) compared with control animals (18.19% ± 1.89%, mean ± SEM). Slow SC proliferation is later maintained in cNFs, as immunolabeling of cNFs from 10-month-old Nf1-KO mutants against the mitotic marker phospho-histone-H3 (pH3) indicates that a small proportion of traced cells undergo mitosis (Supplementary Fig. S3K). This is in line with clinical observations demonstrating rare mitoses in human NFs (12). Overall, our data suggest that inactivation of Nf1 provides a small proliferative advantage to neonatal mutant SCs.

As BC derivatives include a subset of skin melanocytes, we compared the number of cells coexpressing TOM and the melanocyte lineage marker TRP2 between Nf1-KO and control, newborn animals. Although we observed no difference in the number of traced melanoblasts within the hair follicles, the extrafollicular (dermal) fraction was substantially increased in Nf1-KO skin (Fig. 4D–F). Interestingly, there was already enhanced melanocytogenesis in embryonic mutant skin at E15.5 (Supplementary Fig. S4G–S4L). At P9, when melanoblast migration into developing hair follicles is complete in control skin, numerous double-positive (TOM+/TRP2+) cells were still observed in the mutant dermis. Conversely, there was no difference in the numbers of traced follicular melanocytes (Supplementary Fig. S4M), suggesting a selective expansion of the extrafollicular melanocyte fraction in the mutant.

We next investigated whether the elevated SC content in mutant newborn skin is accompanied by modifications of the other cell types. Although Nf1-KO skin had a slight (1.1-fold) elevation in the number of KIT+ mastocytes compared with controls (Fig. 4G), the densities of PDGFRα+ fibroblasts (Fig. 4H and I) and of IBA1+ macrophages (Fig. 4J) were comparable between the two groups.

Analysis of the postnatal back skin between P0 and 3 months of age revealed a further progressive increase in the numbers of TOM+ SCs in Nf1-KO mutants compared with controls (Fig. 4K–M; Supplementary Fig. S4N). Although at 3 months most traced SCs were still well aligned with axons, many appeared hypertrophic and extended numerous cytoplasmatic protrusions that detached from axons (Fig. 4N–O’). In some cases, we observed an entire SC soma “escaping” from the nerve bundle (Fig. 4O’). These abnormal morphologies preferentially occurred in the upper dermis (Supplementary Fig. S5A–S5D). In addition, we observed a dramatic increase in the density of dermal fibroblasts, as revealed by labeling for PDGFRα and increased density of nuclear staining (Fig. 4P and Q). However, as in the newborn...
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**Figure 4.** Nf1 loss of function in BC cells promotes proliferation of dermal Schwann cell and melanocyte lineages. A–J, Comparison of control and Nf1-KO newborn skin immunolabeled for TOM, TUJ1 (nerves), TRP2 (melanocytes; A, B, D, E), and PDGFRα (fibroblasts; H and I) show expansion of traced BC derivatives, including SCs (nerve-associated TOM+/TRP2− cells; D and E, arrows) and melanocytes (nerve-free TOM+/TRP2+ cells; D and E, arrowheads), but not of dermal fibroblasts in the mutant dermis. Insets in D and E show higher magnifications of the indicated area. C, F, G, and J, Quantification of traced SCs (C), traced follicular and extrafollicular melanocytes (F), nontraced KIT+ mastocytes (G), and IBA1+ macrophages (J) in control versus Nf1-KO P0 skin. K–O″, Comparison of 3-month-old control and Nf1-KO–mutant skin immunolabeled for TOM and TUJ1 (K and L) reveals further expansion and morphologic abnormalities of the traced SC population in the mutant. M, Quantification of traced SCs in the control and Nf1-KO dermis. N and O, Dorsal views of clarified skin at the level of subepidermal nerve plexus. O′ and O″ show higher magnifications of mutant subepidermal SCs. Note numerous cytoplasmic extensions (arrowheads) and an SC soma (arrow) detached from the adjacent axon bundle. Asterisks indicate SC somas. P and Q, Staining of PDGFRα+ dermal fibroblasts reveal a dramatic expansion of this population in the mutant dermis. R, Quantification of nontraced KIT+ mastocytes in the control and Nf1-KO dermis. Data are represented as mean values ± SD. Each data point corresponds to a number of cells per field of view. Fold change (FC) differences are given for each comparison. Scale bars, 50 μm. **, P < 0.01; ***, P < 0.001.
skin, the number of mastocytes was only slightly (1.4-fold) higher in Nf1-KO dermis than in controls (Fig. 4R), whereas the density of macrophages was unaffected (Supplementary Fig. S4O and S4P).

It is surprising that despite their increased number, mutant SCs mostly remain in contact with axons for up to 3 months. This observation raises the possibility of an increase in the density of cutaneous innervation to cope with elevated SC numbers. To examine innervation density, we performed whole-mount immunostaining of the 3-month-old Nf1-KO and control skin with antibodies against TOM and the pan-axonal marker β-III-tubulin (TUJ1). The density of innervation appeared higher in the regions of Nf1-KO skin corresponding to patches of traced SCs as compared with controls or regions of mutant skin poor in traced cells (Supplementary Fig. S5E–S5G). This suggests that enhanced local innervation in the mutant skin is linked to SC hyperplasia.

To investigate if enhanced innervation might result from increased sensory DRG neurogenesis, we compared the numbers of traced sensory neurons in Nf1-KO mutant and control newborn DRGs from the same A-P level (Supplementary Fig. S5H). There was no significant difference in the number of traced sensory neurons, suggesting that enhanced innervation is rather due to branching of nerve terminals in the mutant dermis.

We next analyzed the skin of 6-month-old Nf1-KO mice, which still did not show any visually detectable abnormalities. However, numerous microscopic lumps were detected upon tactile examination. Histologic analysis of the skin revealed SC hyperplasia along with the presence (over the entire back skin) of multiple small (up to 1 mm) aggregates of disorganized nmSCs, positive for TOM and S100 (Fig. 5A–C), which were intermixed with numerous nontraced fibroblasts, mastocytes, macrophages, and polymorphonuclear neutrophils, all present in typical cNFs. Quantification of distinct cell populations was performed by electron microscopy (Fig. 5D). Interestingly, the presence of inflammation-related cells, such as neutrophils, mastocytes, and macrophages, in these microlesions suggests these cell types are involved in the development and/or progression of tumors rather than being a simple consequence of pruritus-related inflammation. Ultrastructural characteristics of nmSCs in the microlesions were also typical of classic cNFs and included abnormal extension of cytoplasmic processes that enclosed isolated axons and/or groups of collagen fibrils, or were devoid of axonal contact (Fig. 5E and F). Whole-mount immunostaining of the mutant dermis confirmed accumulation of traced SCs with profound morphologic aberrations (Fig. 5G–I). SC hypertrophy in the upper dermis was far more severe than in 3-month-old mutants, and some cells seemed to lose contact with axons. In the lower dermis, most mutant SCs had detached from axons and extended multiple filipodia-like structures. Together, these data indicate that the microlesions, apart from their size, share essential characteristics with bona fide cNFs and, consequently, we refer to them as micro-cNFs.

Utrastructural examination of dermal innervation also revealed that intact Remak bundles, containing small groups of tightly packed axons, were extremely rare in the mutant dermis. Instead, we commonly observed numerous isolated axons that were separated by large quantities of collagen and individually sheathed by small fragments of the SC cytoplasm (Fig. 5F). This suggests decompaction of nerve bundles. Moreover, we observed a 2-fold increase in axon numbers between control and Nf1-KO dermis (Fig. 5D). Immunolabeling with CGRP, a marker of peptidergic nociceptive neurons (13), further supported an increased density of dermal innervation in micro-cNFs compared with control dermis (Supplementary Fig. S5L–S5L). Overall, these data point toward a marked decompaction of nerve bundles combined with increased branching of axon terminals in the mutant dermis.

If micro-cNFs constitute a precursor stage of full-blown tumors, it would be expected that the number of tumors arising in older individuals should be much larger than what was actually observed (typically up to a few well-demarcated lesions per mutant at the cervical or cervicothoracic level). As mutant mice had to be sacrificed as soon as pruritic lesions appeared, it is likely that most of the developing tumors did not have enough time to reach a macroscopically detectable stage. Indeed, this idea is supported by the observation of a unique Nf1-KO individual that survived until 20 months of age without developing pruritus. In this case, dozens of raised skin bumps containing dense clusters of traced cells were observed all over the back skin. Analysis of their cellular composition with the set of markers described above confirmed their cNF identity (Supplementary Fig. S3L–S3N). This strongly supports the hypothesis that many of the micro-cNFs observed in the 6-month-old skin could evolve into full-blown diffuse cNFs in older animals.

**Skin Injury Accelerates the Development of cNFs**

In NF1 patients, surgical and laser ablation of cutaneous tumors are routinely used for tumor resection. However, several case reports suggest that skin injury (trauma) might promote development of additional cNFs (14). To test whether an injury favors the appearance of tumors, small skin incisions were made at the thoracic level on 3-month-old Nf1-KO mutants (n = 6) and control individuals (n = 4). These animals were sacrificed at 10 months of age and their skin was analyzed and compared with that of uninjured age-matched Nf1-KO mutants (n = 6). All injured Nf1-KO mutants developed large, mostly nonpruritic lesions at the site of injury (Fig. 6A–D). Both types of lesions, at the injury site or distant from it, contained dense accumulations of TOM+ cells (Fig. 6A-b, d, f, g). Thoracic and lumbar lesions were histologically identical (except for their size) and corresponded to typical diffuse cNFs (Fig. 6A-d, g, i–k). No abnormalities in the distribution or densities of traced cells were ever observed in injured control animals (Fig. 6A-c). Moreover, we did not observe any morphologically visible skin lesions at the thoracic or lumbar level in 10-month-old uninjured Nf1-KO mutants (Fig. 6A-e). The accumulation of traced cells in this region was limited and corresponded to micro-cNFs (Fig. 6A-e’, h). Furthermore, in our original Nf1-KO cohort (Supplementary Table S1), morphologically visible lesions were rare at the thoracic level (4 animals out of 26) and found only in aging individuals (17.5 ± 3.6 months).
Figure 5. Six-month-old Nf1-KO–mutant skin contains numerous micro-cNFs. A and B, Transverse sections through Nf1-KO–mutant (A) and control (B) skin reveal local accumulations of TOM+ cells in the mutant dermis. The dotted lines indicate upper tissue limits and the boundaries between the dermis (Der) and the hypodermis (Hyp). C, High-magnification view of the mutant dermis shows abnormal morphology of TOM+/S100+ SCs. D, Quantification of the relative abundance of different cell types in micro-cNFs and control skin. The data are represented as mean values measured per 0.026 μm² ± SEM (***, P < 0.0001). Fold-change differences are given for each comparison. SC unit refers to SC soma or fragment of SC cytoplasm (>2 μm). E and F, Electron micrographs showing typical nonmyelinated nerve fibers in the control dermis (E) and a disrupted Remak bundle in a micro-cNF (F). Arrow indicates the perikaryon of a mutant SC devoid of axonal contact, and arrowheads indicate abnormal cytoplasmic extensions, often enwrapping pairs or single axons separated by collagenous extracellular matrix. a, axon; Nu, cell nucleus. G–H, Global (G, H) and higher-magnification (G′, H′) views of the mutant skin in the upper and lower dermis showing aggregates of TOM+ SCs with abnormal morphologies. Most SCs extend abnormal cytoplasmic protrusions (arrowheads) or appear to be detached from the axons (arrows). Scale bars, 50 μm (A–C, G–H′), 1 μm (E, F).
Finally, although the thickness of the skin in the uninjured mutants in the lumbar region was typical of early-stage micro-NFs (Fig. 6A-h), it was increased in the same region in injured mutants (Fig. 6A-g), consistent with cNF stage. Skin thickness reached an unprecedented level in the thoracic region of the injured mutants, with a dramatic expansion of the number of traced cells (Fig. 6A-f).

Wound healing following skin injury is a highly dynamic process that involves soluble mediators, extracellular matrix components, resident cells, and infiltrating leukocytes, which differentially participate in three sequential phases: inflammation, proliferation, and remodeling (15). Initially, neutrophils accumulate in wound sites, followed by a large influx of macrophages that augment inflammatory response. Recent studies highlighted the role of nerves and SCs as pivotal elements promoting skin repair (16). As soon as 2 days after injury, SCs detach from sectioned nerves, proliferate, and begin to invade the wound bed, where they support extracellular matrix remodeling via paracrine signaling. This delamination is transient, as 2 weeks later many SCs at the site of injury are associated with nerves. To investigate the impact of skin injury on BC-derived Nf1-KO SCs, we analyzed TOMEM cells at 2, 4, 7, and 14 days after injury (dpi) in 3-month-old control (Prss56^Cre+, R26^Tom^) and Nf1-KO animals (Fig. 6B).

During the peak of the inflammation phase (2–4 dpi), traced Nf1-KO and control SCs from the damaged nerves were similarly distributed into the wound bed (Fig. 6B-a–d). However, by day 7, the number of Nf1-KO SCs drastically increased as compared with control SCs, and this phenomenon was even more pronounced at 14 dpi (Fig. 6B-e–h). Rapid expansion of the mutant SC lineage was also supported by the observation that the majority of Nf1-KO SCs expressed the proliferation marker PH3 at 7 dpi (inset in Fig. 6B-f). In addition, detailed analysis of 14 dpi mutant wound revealed the presence of morphologically abnormal Nf1-KO SCs, similar to those observed in cNFs (Fig. 6B-i). At this stage, numerous Nf1-KO SCs were still dissociated from the axons, in contrast to control SCs (Fig. 6B-g, h). Furthermore, macrophage and fibroblast, but not mastocyte, densities were increased in Nf1-KO as compared with control wounds, suggesting enhanced inflammatory response in the mutant.

To identify candidate factors involved in the abnormal behavior of Nf1-KO SCs and their microenvironment upon injury, and possibly in the acceleration of cNF development, we analyzed the distribution of mRNAs by high-throughput sequencing in wounded (14 dpi) and intact skin from Nf1-KO and control mice (n = 3). Prss56 mRNA was undetected across all compared samples, excluding the possibility of de novo activation of this gene in the skin upon injury. Among the 9,627 genes found to be differentially transcribed (P < 0.05; fold change (FC) > 4x) between injured and uninjured control skin, 13.4% were upregulated and 86.6% were downregulated. Among the 4,112 genes differentially transcribed between injured and uninjured Nf1-KO skin, 34% were upregulated and 66% were downregulated (Fig. 6C-a). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses performed on the upregulated transcripts identified “Cytokine–cytokine receptor interaction,” “ECM-receptor interaction,” “Complement and coagulation cascades,” “Protein digestion and absorption,” and “Focal adhesion” among the top-ranked pathways in injured versus uninjured skin comparisons, in both Nf1-KO and control (Fig. 6C-b, c; Supplementary Table S2). As expected, many genes from these lists have previously been implicated in wound healing and tissue repair (17). Remarkably, similar to the wounded Nf1-KO skin, “Cytokine–cytokine receptor interaction” has also been reported as the most enriched pathway in mouse neurofibroma-derived SCs and macrophages (18), suggesting the involvement of similar paracrine/autocrine signaling pathways.

More specifically, among the cytokines identified as significantly upregulated in injured versus uninjured Nf1-KO skin, Ccl2 (FC = 10.5x), Ccl5 (FC = 17.4x), Cxcl12 (FC = 22.6x), Cxcl5 (FC = 37.2x), Cxcl12 (FC = 11.3x), Cxcl11 (FC = 7.2x), Il1b (FC = 9.2x), and Pd (FC = 13.4x) have previously been reported as overexpressed in human and/or mouse neurofibromas compared with healthy nerves (18, 19). Furthermore, 39% of the upregulated genes in this KEGG category were specifically activated in Nf1-KO and not in control animals, suggesting the existence of differences in injury-induced inflammatory response and tissue repair between mutant and control mice (Supplementary Table S2). This observation is in line with the increased density of mutant SCs and macrophages observed in Nf1-KO wound beds. Of particular interest, Ccl5, which encodes a potent chemoattractant for immune effector cells, including macrophages, and has been reported as upregulated in both neurofibroma Nf1+SCs and macrophages (18), was among the genes specifically activated in Nf1-KO wounds. Its receptors, Cor5 and Cor1, were upregulated in both Nf1-KO and control wounds, although with a higher FC in mutant (8.8x and 12.6x) than in control (4.4x and 6.7x) skin. This makes Ccl5 an interesting candidate for being involved in early development of cNFs. Cxcr1 was also found specifically upregulated in Nf1-KO wounds (FC = 5.8x). It encodes a macrophage and fibroblast chemokine receptor known to promote macrophage and fibroblast accumulation in the wound site, as well as collagen deposition (20). Furthermore, CX3CR1-mediated signaling has recently been implicated in Nf1-related optic glioma formation (21). Finally, the mRNA levels of Ccl2, its receptor Ccr2, and Cdx3, which are also implicated in macrophage recruitment, were more strongly increased in Nf1-KO (10.5x, 13.2x, and 23.8x) than in control (6x, 4.8x, and 4.8x) skin comparisons.

Together, our findings demonstrate that skin injury promotes cNF development. The injury has a rapid impact on Nf1-KO SC proliferation, morphology, and interactions with axons. RNA-sequencing (RNA-seq) analysis of the skin supports the implication of inflammation-related signals in the acquisition of an invasive, protumorigenic SC phenotype and provides several gene candidates susceptible to play key roles in this process.

**Subepidermal Glia Are the Likely Cell Type at the Origin of cNF**

It is striking that mice subjected to targeted Nf1 inactivation in Krox20+ BC cells or Dhb+ SC precursors develop pNFs, but never diffuse cNFs (22). A likely explanation is that the BC derivatives that migrate into the skin and participate in development of cNFs do not activate these two genes. To test this hypothesis, in vivo fate mapping and characterization of derivatives of either Krox20- or Dhb-expressing progenitors in the adult skin was achieved by morphologic and
molecular analysis of TOM\(^+\) cells from Krox20\(^{-}\)R26\(^{flox}\) and Dhh\(^{-}\)R26\(^{flox}\) adult animals. In Krox20\(^{-}\)R26\(^{flox}\) mice, the TOM\(^+\) cells in the skin correspond to the rare S100\(^+/\)MBP\(^+\) mSCs, NG2\(^+\) pericytes, and previously described keratinocytes and hair follicle cells (23, 24) (Fig. 7A and B\(^+\)). Notably, traced nMSCs were never observed. Expression of TOM in mSCs is likely to reflect Krox20 locus activation in mSC precursors around E15.5 (23), rather than a BC origin of these cells.

Analysis of Dhh\(^{Cre}\)R26\(^{flox}\) adult skin revealed that a majority of SCs were traced in the hypodermis (Fig. 7C). In the dermis, TOM\(^+\) cells corresponded to various glial and non-glial derivatives (Fig. 7D–G). However, subepidermal glial, unsheathing nociceptive fibers at the dermis/epidermis boundary and melanocytes were not traced, in stark contrast to the situation in Prss56\(^{Cre}\)R26\(^{flox}\) skin (Fig. 7D, H, and I).

Together, these observations indicate that Prss56-, Krox20-, and Dhh-traced populations give rise to overlapping and distinct types of glial populations in the skin (summarized in Fig. 7J). Among them, the only cell type uniquely derived from Prss56\(^+\) cells is the subepidermal glia. As diffuse cNFs do not develop upon conditional Nf1 loss in Krox20- and Dhh-traced lineages, subepidermal SCs are likely to be the cell type at the origin of cNFs.

**DISCUSSION**

Several mouse models of NFI have been previously developed with two major objectives: to recapitulate the various aspects of the human disease and to provide cues on the cellular origins of the different types of neurofibromas. These objectives have been so far only partially fulfilled, as diffuse cNFs are not recapitulated in these models (2). In the present study, we performed targeted inactivation of Nf1 in Prss56-positive BC cells and observed the development of bona fide plexiform and cutaneous NFs. Comparison with previous models, together with an intrinsic tracing system, allows us to suggest a precise hypothesis on the cell type at the origin of cNFs. Furthermore, follow-up of mutant animals from birth until the appearance of cNFs has allowed a detailed description of disease progression and revealed decisive steps in tumor development. Understanding these steps is likely to affect the search for therapeutic molecules against cNFs.

Finally, the discovery that the skin trauma might stimulate the systemic progression of micro-cNFs into full-blown cNFs is also of clinical relevance.

**Figure 6.** Skin injury promotes development of cNFs in NFI-KO mice. A, Comparison of NFI-KO and control skin at 7 months after injury. (a) Dorsal skin from a 10-month-old injured NFI-KO mutant (b) and control (c) injured mice (dotted line indicates the site of incision) (d–e) Lumbar skin from a 10-month-old injured NFI-KO mouse (d, e) and an age-matched uninjured NFI-KO mutant (e, s). Note in (d) the presence of numerous pale spots (arrowheads) (d, e). Higher magnification of the lumbar skin with direct TOM fluorescence. Arrowheads indicate aggregates of TOM\(^+\) cells, related to pale spots in (d, f–h). Transverse sections through thoracic (f) and lumbar (g) skin from the injured NFI-KO animal and lumbar skin from the uninjured mutant (h). Dense accumulations of TOM\(^+\) cells are observed in the dermis, both at sites close to (f) and distant from (g, arrowheads) the injury. The skin from uninjured NFI-KO mutants contains only microscopic lesions, corresponding to micro-cNFs (arrowhead in h and magnified view in the inset). (i–k) Injury sites show cell composition typical of cNFs, with accumulation of TOM\(^+\)/S100\(^+\) SCs with aberrant morphologies (i). KIT\(^+\) mastocytes, IBA1\(^+\) macrophages (j) and PDGFR\(^{a}\) fibroblasts (k). Dotted line in (f–h) boundary between dermis and hypodermis. B, Time-course analysis of wounded control (CTRL) and NFI-KO skin. (a–h) Tracing of wild-type (a, c, e, g) and NFI-KO (b, d, f, h) TOM-SCs at 2, 4, 7, and 14 day after injury (dpi). The arrowheads indicate the injury site. Inset in (f) shows coexpression of TOM and proliferation marker Ph3. Nu; nuclear staining (g, h). High-magnification images of (g) and (h) showing TOM and axonal (TUJ1) staining. (i–p) Immunolabelling of control (i, l, m, n) and NFI-KO (k, l, o, p) wound sites for S100 (SCs, i, k), PDGFR\(^{a}\) fibroblasts (j, l), IBA1 (macrophages, m, o), and KIT (mastocytes, n, p). Scale bars, 5 mm (A–h, c, d, e, s). 500 µm (A–f, h), 50 µm (A–i, k). 100 µm (B, C, D, F, G, I, J, K, M). C, Differential expression of genes between injured and uninjured skin and pathway enrichment analysis. (a) Significantly upregulated and downregulated genes (P < 0.05 and FC > 4) between injured (14 dpi) and uninjured skin in control and NFI-KO animals. (b, c) Top-ranked enriched KEGG pathways among the upregulated genes in injured versus uninjured comparisons in NFI-KO (b) and control (c) skin.
Origin and Development of Cutaneous Neurofibromas

**A**

Injured Nf1-KO vs. Injured CTRL

**B**

CTRL vs. Nf1-KO

**C**

Upregulated and Downregulated Genes

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**Research.**

Figure 7. Tracing of the derivatives of Krox20<sup>Cre</sup>, R26<sup>Tom</sup> and Dhh<sup>Cre</sup>, R26<sup>Tom</sup> cells in the adult skin. A–B″, Transverse sections of Krox20<sup>Cre</sup>, R26<sup>Tom</sup> skin stained with TOM and glial markers (A, A′) and vascular markers, PECAM (endothelial cells, B–B″), and NG2 (pericytes, B′). All traced S100<sup>+</sup> SCs are MBP<sup>+</sup> and therefore correspond to mSCs (arrowheads in A, A′). Note that S100<sup>−</sup>/MBP<sup>−</sup> nmSCs, including Remak SCs, lanceolate and subepidermal glia, are not traced (arrows in A, A′). TOM<sup>+</sup> cells are also present in the epidermis (keratinocytes, arrow in B), inside the hair follicle (HF), and along capillaries (arrowheads in B). C–G: Transverse sections of Dhh<sup>Cre</sup>, R26<sup>Tom</sup> skin stained for TOM, S100 (C–F), MBP (C), K15 (keratinocytes), and TUJ1 (axons, G, G′). TOM<sup>+</sup> cells correspond to a subset of mSCs (C, full arrowheads), Remak SCs (D, full arrowhead), arrector pili glia (E, arrowhead), together with arrector pili muscle (D, open arrowhead) and other muscle fibers (C, open arrowhead), lanceolate glia (F, arrowheads), and Merkel cells of the touch dome (G, arrowhead). No subepidermal glia are traced (D, arrows). G′, A higher magnification of the area indicated in G. H and I, Dorsal view of the subepidermal region from Prss56<sup>Cre</sup>, R26<sup>Tom</sup> (H) and Dhh<sup>Cre</sup>, R26<sup>Tom</sup> (I) adult skin. Note numerous TOM<sup>+</sup> SCs associated with the subepidermal neuronal plexus (TUJ1-positive) in Prss56<sup>Cre</sup>, R26<sup>Tom</sup> dermis and absence of TOM labeling at this level with the Dhh<sup>Cre</sup> driver (arrowheads point to cell bodies of subepidermal SCs, empty arrowhead indicates traced Merkel cells). J, Table summarizes the types of derivatives in the adult skin traced with the three Cre drivers. Scale bars, 50 μm.
defasciculation and branching. This stage also involves an accumulation of fibroblasts, due to accretion and/or proliferation, and of collagen fibers presumably synthesized by these fibroblasts. At 6 months (Supplementary Fig. S6D, stage 3), numerous micro-cNFs are present all over the back skin, which share all the characteristics of full-blown cNFs, apart from their size. They contain dense aggregates of mutant SCs that show altered morphologies and frequent loss of contact with the axons. These aggregates are massively infiltrated by fibroblasts, mastocytes, macrophages, and neutrophils. Inflammation is therefore a major hallmark of this stage. From 12 months of age (Supplementary Fig. S6D, stage 4), morphologically visible, diffuse cNFs are often associated with pruritus. On the basis of these observations, we believe that tumor progression in cNFs involves three decisive transitions: (i) an increase in the level of proliferation of SCs during the late embryonic period; (ii) initiation of morphologic changes of SCs and the accumulation of fibroblasts between stages 1 and 2, establishing a pretumorous state; and (iii) loss of SC axonal contact and the establishment of an inflammatory process that corresponds with the progression to a tumorous state, between stages 2 and 3. The transition between stages 3 and 4 may simply be a slow increase in the tumor size.

Interestingly, before stage 2, the elevation of the SC number appears to be compensated by increased branching of nerve bundles, allowing the supernumerary SCs to maintain appropriate contacts with axons. This observation also raises the possibility that the defasciculation/branching phenomenon might actually be induced by the excess of SCs. It is possible that this putative compensatory mechanism has limitations and, when overridden, SCs can no longer interact with axons, possibly favoring a protumorigenic phenotype. The branching capacity of nerve terminals in the upper dermis might be related to the absence of perineurium in this layer. Increased skin innervation might explain the skin hypersensitivity and neuropathic pain reported in approximately 10% of patients with NF1 (25). If confirmed, this link could be used to predict sites of cNF development. Intriguingly, skin trauma is also accompanied by active proliferation of SCs, their dissociation from nerves, and local increase of density of innervation, further supporting the impact of peripheral glia and/or inflammation on axonal branching (16).

Presence of highly innervated microscopic neurofibromas, often associated with hair follicle apparatus, was reported in clinically asymptomatic skin of NF1 patients (ref. 26 and F. Rice, personal communication). In our model, despite an identical cellular composition of diffuse cNFs, their association with skin adnexa cannot be firmly established due to a higher density of hair follicles present in the mouse skin. More generally, the fact that micro-cNFs in the asymptomatic skin of NF1 patients (ref. 26 and F. Rice, personal communication) often associated with hair follicle apparatus, was reported as highly expressed in human and/or mouse neurofibromas. Soluble factors attract particular attention, as they might promote development of cNFs both locally and remotely. Modulation of the activity of such factors and/or of their receptors might constitute an interesting option to impede cNF growth.

systematic development of cNFs in our mouse model makes it a powerful tool for the screening of candidate therapeutic molecules. Furthermore, the availability of the Cre-inducible fluorescent reporter that allows identification of Nf1-mutant cells allows a continuous survey of the impact of the drug.

The similarity between patients and mice at the “pathologic” level is surprising in view of their differences at the genetic level. Previous studies suggested that NF1 heterozygosity in the cellular microenvironment (particularly in mastocytes) was necessary for initiation and development of cNFs (27). However, more recent studies performed in mice and humans suggest that heterozygosity in mastocytes is dispensable for tumor development (28). Our observations are consistent with these latter findings, as NFs develop in both NF1+/− and wild-type backgrounds. Another difference is that all BC cell derivatives have undergone a homozygous loss of NF1 in our model, whereas this condition is sporadic in humans. A key factor might be the difference in lifetime of both species. Only a minority of the possible tumors might develop during the short lifetime of the mouse, an interpretation supported by the dramatic density of tumors observed in a 20-month-old mutant animal. In addition to NFs, all NF1 patients develop a number of pigmentary lesions, including café au lait macules that correspond to accumulation of epidermal melanocytes. In our model, BC-derived melanocytes carry biallelic Nf1 loss, whereas all other melanocytes are either NF1+/− or wild-type (TOM). Although café au lait macules do not develop in mice, due to the absence of melanocytes in the interfollicular epidermis, we observed accumulation of Nf1-KO melanocytes in the dermal compartment starting from late embryogenesis. Moreover, we noticed marked accumulation of Nf1-KO and wild-type pigmented melanocytes in cNFs. This is in line with the presence of pigmented cNFs in NF1 patients. In conclusion, NF1-associated dysregulation of melanocytogenesis and/or melanin synthesis is a common feature in NF1 patients and in our model.

Finally, we found that local mechanical skin injury promotes the development of cNFs, not only at the site of the injury, but also at distant sites. Wounding Nf1-KO skin triggers massive local proliferation of denervated Nf1−/− SCs within the first week after surgery, suggesting the involvement of inflammation-related signals. Consistently, transcriptome profiling of wounded versus intact control and Nf1-KO skin led to the identification of several candidate molecules linked to inflammation that are specifically over-expressed in Nf1-KO skin upon injury and may play a role in this activation of SCs. Some of them have been previously reported as highly expressed in human and/or mouse neurofibromas. Soluble factors attract particular attention, as they might promote development of cNFs both locally and remotely. Modulation of the activity of such factors and/or of their receptors might constitute an interesting option to impede cNF growth.

**METHODS**

**Animals**

Mice used in this study were housed in a temperature- and humidity-controlled vivarium on a 12-hour dark–light cycle with free access to food and water. The following mouse lines were used and genotyped...
as described in the original publications: Nf1fl/fl and Nf1+/− (4), Prss56+/− (6), R26loxP (29), Ddx2+/− (30), and Kras2f+/− (31). We bred Prss56+/−, R26loxP mice with the Nf1fl/fl mice to obtain the F1 generation (Prss56+/−, R26loxP/Nf1fl/fl); we bred F1 mice with Nf1fl/fl and Nf1+/− mice to obtain Prss56+/−, R26loxP/Nf1fl/fl and Prss56+/−, R26loxP/Nf1+/− mutants, respectively. Prss56+/−, R26loxP/Nf1fl/fl animals were used as controls. For embryo analysis, we established timed pregnancies with the morning of a vaginal plug defined as E0.5. All animal manipulations were approved by a French Ethical Committee (APAFIS#1882-2015092215426712) and were performed according to French and European Union regulations.

Skin Incisions and Tissue Processing

Three-month-old control and Nf1-KO mice were anesthetized with isoflurane. Dorsal skin at the lateral thoracic level was shaved and disinfected with 70% ethanol. Preoperative analgesia was applied using subcutaneous injection of buprenorphine (0.1 mg/kg). Five-millimeter-long full-thickness incisions were performed unilaterally with surgical microscissors and sutured. Animals were perfused with 4% paraformaldehyde (PFA) on days 2, 4, 7, and 14 after surgery, and 7 months after surgery. The back skin was dissected and processed for IHC analysis. The 14 dpi wounds and unwounded skin of three Nf1-KO and three control animals were excised postmortem, cleaned of subcutaneous fat, and homogenized in RNLYS lysis buffer for subsequent RNA isolation (RNeasy Micro Kit, Qiagen).

Electron Microscopy

For standard ultrastructure analysis, mice were perfused with 4% PFA/1.6% glutaraldehyde (Polysciences) in 0.1 mol/L phosphate buffer. Dorsal skin, nerve roots, and subcutaneous nerves were dissected and post-fixed in the same solution overnight at 4°C, followed by embedding in Epon as previously described (6). Semi-thin sections (1 μm) were stained with toluidine blue, and ultrathin sections were stained with Uranyl Acetate and analyzed using a Jeol microscope.

Statistical Analysis

Statistical analyses were carried out using two-tailed Student t tests or nonparametric Mann–Whitney tests (for quantification of the melanocyte lineage and electron microscopy-based counting), and P values considered significant are indicated by asterisks as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Statistical analyses and scatter plots were generated using the GraphPad Prism 6.0 package. The data are represented as mean values ± SD or SEM.

Analysis of Cell Proliferation

Pregnant mice were intraperitoneally injected with EdU at the dose of 20 mg per kg of body mass at embryonic age E18.5, and newborn pups were collected 24 hours later. Three Nf1-KO and two control littermates were decapitated, and their skin was dissected and fixed overnight in 4% PFA. EdU detection was performed on 20-μm-thick cryosections using the Click-IT Plus EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions, followed by immunolabeling for TOM and TRP2. Numbers of proliferating Schwann cells (TOM+/TRP2+/EdU+) were normalized against the total number of traced (TOM+/TRP2+) SCs. Quantifications were performed on 4 distant sections from each individual (~24 fields per sample) using 25× magnification under a TCS SP5 confocal microscope. The two-tailed Mann–Whitney test was used for statistical analysis. P values < 0.05 were considered statistically significant.

Library Preparation, RNA-seq, and Data Analysis

cDNA libraries, RNA-seq library preparation, and Illumina sequencing were performed at the Ecole normale supérieure genomic core facility (Paris, France). Ten nanograms of total RNA was amplified and converted to cDNA using SMART-Seq v4 Ultra Low Input RNA kit (Clontech). An average of 200 pg of amplified cDNA was used for library preparation using the Nextera XT DNA kit (Illumina). Libraries were multiplexed by 38 on 3 high-output flow cells. Sequencing reads (75 bp) were performed on a NextSeq 500 device (Illumina). The analyses were performed using the Eoulsan pipeline (32), including read filtering, mapping, alignment filtering, read quantification, normalization, and differential analysis. Prior to mapping, poly-N read tails were trimmed, reads ≤40 bases and with mean quality ≤30 were discarded. Reads were then aligned against the Mus Musculus genome from Ensembl version 91 using STAR (version 2.5.2; ref. 33). Alignments from reads matching more than once on the reference genome were removed using Java version of SamTools (34). To compute gene expression, Mus musculus GFF3 genome annotation version 91 from the Ensembl database was used. All overlapping regions between alignments and referenced exons were counted using HTSeq-count 0.9.3 (35). The sample counts were normalized and then statistically analyzed using DESeq 1.8.3. P value <0.05 and FC > 2 were used as cutoff criteria to select differentially expressed genes (DEG). The RNA-seq gene-expression data and raw Fastq files are available on the GEO repository (www.ncbi.nlm.nih.gov/geo/) under accession number GSE117781.

Pathway overrepresentation analysis was performed using the Webgestalt web server (http://www.webgestalt.org). DEG sets were queried against the KEGG database using FDR q < 0.05 cutoff to select significantly enriched pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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