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Plexiform neurofibroma genesis: questions of *Nf1* gene dose and hyperactive mast cells

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Abstract

Purpose of review—Tumorigenic cells can co-opt normal functions of non-malignant hematopoietic cells, promoting tumor progression. Recent mouse and human studies indicate that mast cells underpin inflammation in the plexiform neurofibroma microenvironment of neurofibromatosis type 1. In this model, *Nf1* homozygous deficient Schwann cells recruit hyperactive mast cells, promoting tumorigenesis. Here, we discuss the importance of *Nf1* gene dosage, delineate hematopoietic contributions to the plexiform neurofibroma microenvironment, and highlight applications to human treatment.

Recent findings—Previous studies found that plexiform neurofibroma formation in a mouse model requires biallelic loss of *Nf1* in Schwann cells and an *Nf1* heterozygous cellular background. Now, transplantation and pharmacological experiments have indicated that tumor formation specifically requires *Nf1* heterozygosity of c-kit dependent bone marrow.

Summary—Neurofibromatosis type 1 results from autosomal dominant mutations of the *NF1* tumor suppressor gene. While unpredictable second-hit mutations in the remaining *NF1* allele precede local manifestations such as tumor formation, human and mouse data indicate that *NF1/Nf1* gene haploinsufficiency modulates cellular physiology and disease pathogeneses. In particular, *Nf1* haploinsufficient mast cells demonstrate multiple gain-in-functions, and mast cells permeate neurofibroma tissue. Transplantation experiments have shown that these aberrant mast cells critically underpin the tumor microenvironment. Using these findings, clinicians have medically treated a patient with a debilitating plexiform neurofibroma.

Keywords

mast cells; NF1; c-kit; tumor microenvironment; neurofibroma

Introduction

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen's disease, is a pandemic genetic disorder affecting approximately one in 3000–3500 persons worldwide

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Conflict of Interest
The authors declare that no conflict of interest exists.

[1,2]. NF1 results from autosomal dominant mutations in the *NF1* tumor suppressor gene, which encodes neurofibromin, a $p21^{ras}$ (Ras) guanosine tri-phosphatase (GTP) activating protein (GAP) [3,4*,5*]. Among other non-malignant manifestations, individuals with NF1 frequently develop cutaneous, subcutaneous, and/or plexiform neurofibromas comprised of irregular Schwann cells, fibroblasts, and mast cells [6,7**].While individuals with NF1 are born with one mutated *NF1* allele in all cells (i.e. germ-line mutation), loss of the residual normal *NF1* allele (i.e. loss of heterozygosity) in tissues such as Schwann cells precedes tumor formation, consistent with *NF1*'s categorization as a tumor suppressor gene [7**]. Similarly, in a mouse model mimicking the human condition, plexiform neurofibroma development requires $Nf1^{-/-}$ Schwann cells [8], analogous to *NF1* loss of heterozygosity (LOH) in humans. Although biallelic gene loss precedes neurofibroma formation in both humans and mice, mouse models have additionally demonstrated that tumor formation requires *Nf1* haploinsufficiency ($Nf1^{+/-}$) of cellular components within the tumor microenvironment [8,9]. Recently, bone marrow transplantation experiments have shown that tumor formation specifically requires the inflammatory contributions of $Nf1^{+/-}$ and c-kit dependent bone marrow and, arguably, hyperactive mast cells [10**]. Here, we discuss gene dosage in NF1 pathogeneses, argue that interactions between tumorigenic Schwann cells, deregulated mast cells, and the microenvironment critically underpin plexiform neurofibroma formation, and show how these findings have led to the successful medical treatment of a patient with a debilitating plexiform neurofibroma.

Nf1 gene dose modulates NF1 pathogeneses

In humans and in mice, NF1 manifestations result from a combination of ubiquitous *NF1*/*Nf1* heterozygosity and unpredictable *NF1*/*Nf1* LOH in different cell lineages. These genetic doses dictate both generalized and variable localized maladies. For example, LOH in Schwann cells precedes plexiform neurofibroma formation while LOH in chromaffin cells induces pheochromocytoma, two disparate yet frequently encountered NF1 symptoms (Table 1)[10**,11,12**,13**,14,15,16*,17*,18*,19*]. Moreover, regions of particular tissues may rely more heavily on neurofibromin than other unaffected regions. For example, astrocytes of the optic nerve, brain stem, and cerebellum express more NF1 and hyperactively proliferate in its absence when compared to astrocytes from the neocortex, suggesting why gliomas in NF1 patients arise more frequently in these locations [20*].

Despite the persistent requirement for *Nf1* LOH in tumor cells of origin, *Nf1* haploinsufficiency also alters cell fate and function. These phenomena have been reported in mast cells and melanocytes [21,22], neurons [17*], non-dysplastic astrocytes [23], osteoblasts and osteoclasts [18*,24,25], endothelial cells [19*,26], smooth muscle cells [27], keratinocytes [28], and fibroblasts [29]. *Nf1* haploinsufficient cells typically demonstrate gain-in-functions secondary to deregulated Ras signaling. While aberrancies in these lineages may underpin generalized NF1 symptoms such as skeletal dysplasia, learning deficiencies, and vascular pathologies, a mouse model of plexiform neurofibroma formation demonstrates that localized tumorigenesis depends on *Nf1* haploinsufficiency in supporting tissue.

Plexiform neurofibroma formation requires *Nf1* haploinsufficiency

Concordant with these data showing *Nf1* gene dosage effects in multiple cell lineages, a plexiform neurofibroma mouse model depends on not only a population of $Nf1^{-/-}$Schwann cells but also an *Nf1* haploinsufficient cellular background [8,9]. *Nf1* homozygous deficiency is embryonically lethal due to heart malformations, and $Nf1^{-/-}$chimeric mice but not $Nf1^{+/-}$ mice develop neurofibromas [30,31,32]. In light of these data, Zhu et al created a Schwann cell-specific *Nf1* conditional knockout on an *Nf1* haploinsufficient background. In this mouse, *loxP* sites, the 34 base pair recognition sequence for the Cre recombinase

enzyme, flank *Nf1*'s exon 31 and 32 on one allele (e.g. $Nf1^{flox/\backslash}$), and the other allele carries a traditional "knockout" *Nf1* mutation (thus, $Nf1^{flox/-}$). This circumvents the problem of embryonic lethality while allowing tissue specific, Cre-directed exon deletion and genetic level inhibition. In the plexiform neurofibroma tumor model, the mouse carries the Cre recombinase transgene under control of the *Krox20* gene promoter element ($Nf1^{flox/-}$; Krox20cre), which expresses Cre recombinase with specificity in 10–20% of Schwann cells [8,33]. While mice with $Nf1^{-/-}$ Schwann cells but wild-type (WT) cellular backgrounds ($Nf1^{flox/flox}$; Krox20cre) never develop neurofibromas, mice with additionally heterozygous backgrounds ($Nf1^{flox/-}$; Krox20cre) reliably form dorsal root ganglia tumors comprised of irregular Schwann cells, fibroblasts, and mast cells. These findings grossly and histologically mimic plexiform neurofibromas found in individuals with NF1.

With the insights of the $Nf1^{flox/-}$; Krox20cre model, the question arises – which haploinsufficient cells and/or cellular interactions underlie neurofibroma genesis? The $Nf1^{+/-}$ mast cell seems a likely candidate: mast cells infiltrate neurofibroma tissue in large numbers, neurofibroma tissue and Schwannoma-derived Schwann cells express high levels of stem cell factor (SCF) messenger RNA [34,35], SCF/c-kit signaling critically regulates mast cell hematopoiesis and physiology [36], and, generally, mast cells and other inflammatory cells have been increasingly implicated in neoplasia [37,38,39]. Likewise, mast cells can synthesize and secrete matrix metalloproteinases, pro-inflammatory cytokines such as IL-6, TNF-α, and CCL2-4, and mitogens such as NGF, VEGF, and PDGF. These secreted factors modulate the differentiation and growth of multiple cell types, effect extracellular matrix remodeling, and induce collagen deposition, angiogenesis, and generalized inflammation [40,41].

Moreover, studies of *Nf1* haploinsufficientmast cells and melanocytes provided initial evidence that haploinsufficiency of a tumor suppressor gene can substantially regulate lineage-specific cell fate and function [21]. Ingram et al found that an intercrossed $Nf1^{+/-}$ genotype corrects multiple deficiencies of the W^{41}/W^{41} mouse, a mutation compromising c-kit receptor tyrosine kinase activity by ~85%. This mutation ablates mast cell cytogenesis and produces a predominantly white coat due to melanocyte dysfunction [42]. However, $Nf1^{+/-};W^{41}/W^{41}$ mice show partially restored dermal mast cell numbers, elevated bone marrow-derived mast cell colonies, increased SCF-induced proliferation and survival, and biochemical hyperactivity in c-kit signaling pathways.

Plexiform neurofibroma formation requires $Nf1^{+/-}$ and c-kit^{+} marrow

In accordance with the above observations, recent transplantation experiments have indicated that *Nf1* haploinsufficient and c-kit-dependent bone marrow is required for plexiform neurofibroma formation [10**]. First, $Nf1^{+/-}$ marrow transplanted into lethally irradiated $Nf1^{flox/flox}$; Krox20cre mice (which do not develop tumors) induces tumor formation similar to the tumorigenic $Nf1^{flox/-}$; Krox20cre mouse model. Second, transplantation of WT bone marrow into the $Nf1^{flox/-}$; Krox20cre mouse abolishes tumor formation in the normally reliable tumor model. In the first experiment, the mouse carries $Nf1^{-/-}$ Schwann cells, $Nf1^{+/-}$ marrow, and WT fibroblasts, vascular cells, neurons, etc. In the second experiment, the mouse carries $Nf1^{-/-}$ Schwann cells, WT marrow, and $Nf1^{+/-}$fibroblasts, vascular cells, neurons, etc. Therefore, we can conclude that $Nf1^{+/-}$ bone marrow, combined with $Nf1^{-/-}$ Schwann cells, is required and sufficient for plexiform neurofibroma formation [10**].

Furthermore, genetic inhibition of c-kit (i.e. *W* mutations), the principal molecular effector of mast cell development, protects against the tumorigenic potential of $Nf1^{+/-}$marrow. Marrow derived from $Nf1^{+/-}$ mice intercrossed with W^{41} or W^{sh} mice and transplanted into $Nf1^{flox/flox}$; Krox20cre mice does not induce tumor formation as in $Nf1^{flox/flox}$; Krox20cre

mice reconstituted with *Nf1*$^{+/-}$ marrow. Thus, we can additionally conclude that plexiform neurofibroma formation in a mouse model requires Schwann cell *Nf1* deficiency, marrow *Nf1* haploinsufficiency, and marrow expressing high functioning c-kit receptors. Given these direct data and other inferences, the *Nf1*$^{+/-}$ mast cell arises as the premier culprit promoting the plexiform neurofibroma microenvironment [10**].

Other recent studies have additionally sought to pinpoint the developmental stage at which *Nf1*$^{-/-}$ Schwann cells and/or glial progenitors become tumorigenic. While *Nf1*$^{-/-}$neural crest stem cells do not induce tumors when transplanted into the peripheral nerves of *Nf1*$^{+/-}$ mice [43**], tumors do arise in mice with *Nf1*$^{-/-}$ non-myelinating Schwann cells (i.e. Remak bundles) on an *Nf1*$^{+/-}$ background (*Nf1*$^{flox/-}$; P0-Cre) [44**], deepening the observations from the Krox20-Cre plexiform neurofibroma model. Alternately, widespread and early (E12.5) biallelic loss of *Nf1* in glia (*Nf1*$^{flox/flox}$; Dhh-Cre) permits dermal and plexiform neurofibroma formation despite a WT cellular background [13**]. Thus, developmental timing of *Nf1* genetic loss critically influences tumor formation. While Cre promoters controlling *Nf1* genetic loss in a limited subset of Schwann cells (e.g. Krox20-Cre, P0-Cre, and Periostin-Cre (unpublished)) underlie the models closely recapitulating tumor formation of genetic NF1, the widespread and early glial loss induced by Dhh-Cre provides an apt model for spontaneous, congenital neurofibroma formation. Tellingly, mast cells infiltrate tumor tissue in all of the aforementioned models.

Nf1$^{+/-}$ mast cells and the microenvironment hypothesis

Several *in vitro* and in *vivo* studies have suggested mechanisms through which the *Nf1*$^{+/-}$ mast cell influences the plexiform neurofibroma microenvironment. In the current hypothesis, *Nf1*$^{-/-}$ Schwann cells co-opt and activate *Nf1*$^{+/-}$ mast cells through secreted SCF, causing mast cells to differentiate, proliferate, degranulate, and synthesize/secrete cytokines. These activities, in turn, induce continued Schwann cell expansion, aberrant fibroblast bioactivity, the in-growth of new vasculature, and the perpetuation of marrow-based inflammation (Figure 1). As evidence, *Nf1*$^{-/-}$ Schwann cells release pathological concentrations of SCF under normal culture conditions, and this SCF provides a chemotactic and activating signal most potently for *Nf1*$^{+/-}$ mast cells [45]. *Nf1*$^{-/-}$ Schwann cell-conditioned media drives *Nf1*$^{+/-}$ mast cell chemotaxis at approximately twice the rate of WT mast cells, an effect duplicated with recombinant SCF and ablated with genetic and pharmacologic disruption of c-kit. In turn, activated *Nf1*$^{+/-}$ mast cells secrete high levels of TGF-β, inducing *Nf1*$^{+/-}$ and WT fibroblasts to migrate, proliferate, and synthesize collagen, a protein comprising nearly half the dry weight of the tumor [29,46]. Likewise, mast cells potentiate Schwann cell-axonal disassociation in peripheral nerve injury and neuronal tumor models [47]. And, as described above, SCF-activated mast cells produce a host of inflammatory and mitogenic factors with potential yet relatively unexplored roles in the plexiform neurofibroma microenvironment.

Biochemically, *Nf1*- and SCF-dependent mast cell pathophysiology results from deregulated Ras signaling. Multiple ligand-receptor interactions, including SCF binding at the c-kit receptor tyrosine kinase, induce Ras to its guanine triphosphate (GTP)-bound state. Ras-GTP initiates multiple downstream signaling networks [48,49]. Ras-GTP is converted to its inactive guanine diphosphate (GDP)-bound state Neurofibromin, a highly-conserved GAP encoded by 350kb of genomic DNA on human chromosome 17q11.2 (chromosome 11 in mice)[50,51,52,53]. In SCF-stimulated mast cells, *Nf1* haploinsufficiency increases Ras-GTP activity and potentiates the phosphorylation and activity of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI-3K) pathways, which involve activated p44/p42 (ERK1/2), p38, Akt, and RacGTPases [21,54,55]. Genetic and chemical inhibition of MEK, PI-3K, and p38 suggest a primarily proliferative role for the Ras-Raf-MEK-ERK cascade and a primarily chemotactic role for the PI-3K-Rac-Pak-p38 cascade[21,55,56,57].

Hypothetically, ERK nuclear translocation and activity regulate the G1- to S-transition while p38 regulates cytoskeletal rearrangement and motility [55,58,59]. However, these pathways directly crosstalk through the p21 activated kinases (Paks), which phosphorylate Raf and MEK, further potentiating ERK activity [55]. Additionally, PI-3K-Rac2 activates Akt, which increases mast cell survival through anti-apoptotic pathways [57]. These data mechanistically validate *Nf1*$^{+/-}$ mast cell gain-in-function, and they demonstrate putative, specific targets for NF1 disease management.

Translation to the clinical treatment of human plexiform neurofibromas

In light of the data implicating the *Nf1*$^{+/-}$ mast cells in tumor formation and their dependence on hyperactive c-kit pathways, pharmacological c-kit inhibition should modulate disease course. Concordantly, Imatinib mesylate (Gleevec©), a potent inhibitor of c-kit, PDGF-β, and the bcr/abl receptor tyrosine kinases [60*], successfully reduces existing plexiform neurofibroma volume and *de novo* genesis in the *Nf1*$^{flox/-}$;Krox20cre mouse model [10**]. While imatinib inhibits c-kit-dependent mast cell activity, the drug may also contribute through simultaneous inhibition of PDGFR and cabl, signaling molecules involved in *Nf1*-dependent angiogenesis [27] and fibroblast activity, respectively [29].

From the results of drug treatment in the mouse model, clinicians have used imatinib to treat a three year old with NF1. This patient presented with hallmarks of NF1 at six months old and developed a progressive, non-resectable, and debilitating plexiform neurofibroma encasing her carotid artery and jugular vein. After three months of treatment with 350 mg/m^2/dose of imatinib mesylate, the tumor decreased in volume by 70%. Sleeplessness, fatigue, and drooling associated with airway compression resolved, and the patient remains stable and relatively healthy without further imatinib treatment [10**]. Now, a phase II clinical trial of imatinib in plexiform neurofibroma management is in its final stages.

Conclusions

As observed in the mast cell and other cell types, *Nf1* gene dose modulates cell fate and physiology. While some NF1 pathologies – such as myeloid leukemia [14,61,62,63,64] and early developmental glial tumors [13**] – require *Nf1* LOH in only the tumor cell of origin, *Nf1* haploinsufficiency in mast cells critically underpins the inflammatory microenvironment of the plexiform neurofibroma. These data directly compare to mouse models of at least two other NF1 conditions. One, *Nf1*-dependent optic glioma formation requires *Nf1* deficiency in astrocytes and *Nf1* haploinsufficiency in surrounding brain tissue, most likely the microglia [11,65]. Two, dermal neurofibroma formation requires *Nf1* deficiency in skin-derived precursor cells and haploinsufficiency in the surrounding dermal tissue [12**]. Despite these data from murine models, we recognize that, in humans, variable and, as yet, largely undefined modulators independent of the NF1 locus can contribute to heterogeneous disease manifestations and may explain family-linked predispositions to certain symptoms [66*].

Many questions concerning the plexiform neurofibroma microenvironment persist. The cellular actors downstream of mast cell hyperactivity need further exploration, and specific molecules modulating *Nf1*-dependent tumor development continue to emerge. For example, recent research indicates PTEN protects against neurofibroma development and its transformation to malignancy [67*]. Likewise, microarray and RNA data indicate that dermal and plexiform neurofibromas aberrantly express Sox9, a transcription factor normally expressed in maturing – but not fully differentiated – Schwann cells [68*]. Moreover, data from the study of gliomagenesis have revealed that enhanced NF1 proteosomal degradation – and not exclusively genetic loss – can instigate tumor genesis [69*]. Thus, novel mechanistic discoveries remain to be made across the breadth of NF1

pathology. Much like the cellular and molecular discoveries showing the critical contribution of the mast cell to the plexiform neurofibroma, further discovery of *Nf1*-dependent cells and molecules will impel effective medical therapies targeted at novel pathogenic effectors.

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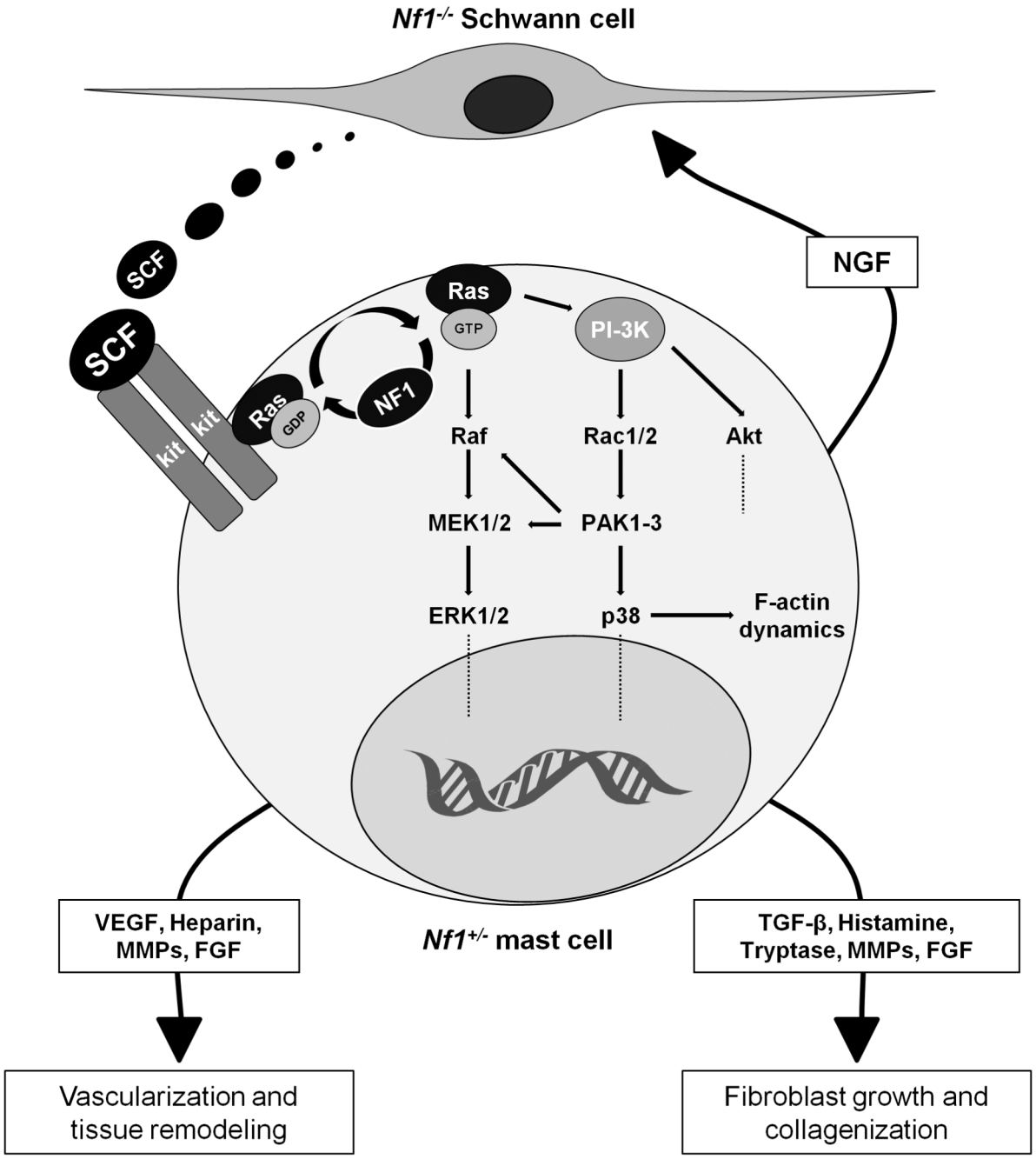

Figure 1.

Proposed neurofibroma microenvironment interactions. In this model, the *Nf1*$^{-/-}$ Schwann cell secretes pathological concentrations of SCF, the ligand for the c-kit receptor tyrosine kinase on the *Nf1*$^{+/-}$ mast cell. C-kit dimerization activates Ras-Raf-MEK-ERK and PI-3K-Rac-Pak-p38 signaling pathways, which promote mast cell proliferation, survival, migration, and cytokine synthesis/secretion. These pathways crosstalk through Pak phosphorylation of Raf and MEK and are negatively regulated through NF1 GAP activity. Secreted products such as VEGF, TGF-β, NGF, and MMPs promote tumor vascularization, collagen deposition, Schwann cell expansion, and extracellular matrix remodeling, respectively. These products initiate and promote tumor growth.

Table 1

NF1 symptoms and genetic requirements.

This table relates the known genetic requirements for the development of specific NF1 symptoms. The data in this figure derive principally, although not exclusively, from mouse models. Events marked “unclear” indicate that the studies came from subjects either *Nf1/NF1* heterozygous or *Nf1* deficient in multiple lineages. Thus, contributions of the genetic background cannot be ruled out.

Neoplasia	Cell of origin	Associated events	Ref
Plexiform neurofibromas	*Nf1*$^{-/-}$ Schwann cells	*Nf1*$^{+/-}$ mast cells	10**
CNS Gliomas	*Nf1*$^{-/-}$ astrocytes	*Nf1*$^{+;/-}$ microglia	11
Dermal neurofibromas	*Nf1*$^{-/-}$ skin precursors	*Nf1*$^{+/-}$ background	12**
Early glial tumors	*Nf1*$^{-/-}$ glia	None	13**
Myeloid leukemia	*Nf1*$^{-/-}$ myeloid cells	None	14
Pheochromocytomas	*Nf1*$^{-/-}$ chromaffin cells	Unclear	15
Other symptoms			
Café-au-lait macules	*Nf1/Nf1*$^{-/-}$ melanocytes	Unclear	13**, 16*
Learning deficits	*Nf1*$^{+/-}$ neurons	Unknown	17*
Osteoporosis	*Nf1*$^{+/-}$ osteoblasts *Nf1*$^{+/-}$ osteoclasts	Unknown	18*
Vascular pathologies	*Nf1*$^{+/-}$ endothelia *Nf1*$^{+/-}$ smooth muscle	Unknown	19*