Mast cells and the neurofibroma microenvironment

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Short title – Mast cells and the neurofibroma microenvironment

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ABSTRACT:

Neurofibromatosis type 1 (NF1) is the most common genetic disorder with a predisposition to malignancy and affects one in 3500 persons worldwide.\textsuperscript{1,2} NF1 is caused by a mutation in the \textit{NF1} tumor suppressor gene that encodes the protein neurofibromin.\textsuperscript{3,4} Patients with NF1 suffer from cutaneous, diffuse, and plexiform neurofibromas, tumors comprised primarily of Schwann cells, blood vessels, fibroblasts, and mast cells. Studies from human and murine models that closely recapitulate human plexiform neurofibroma formation indicate that tumorigenesis necessitates \textit{NF1} loss of heterozygosity (LOH) in the Schwann cell. Additionally, our most recent studies with bone marrow transplantation and pharmacologic experiments implicate haploinsufficiency of \textit{Nf1 (Nf1\textsuperscript{+/-})} and c-kit signaling in the hematopoietic system as required and sufficient for tumor progression.\textsuperscript{5} Here, we review recent studies implicating the hematopoietic system in plexiform neurofibroma genesis, delineate the physiology of SCF-dependent hematopoietic cells and their contribution to the neurofibroma microenvironment, and highlight the application of this research toward the first successful, targeted medical treatment of a patient suffering from a non-resectable and debilitating neurofibroma. Finally, we emphasize the importance of the tumor microenvironment hypothesis, asserting that tumorigenic cells in the neurofibroma do not arise and grow in isolation.
INTRODUCTION:

Although several reports characterized NF1 or NF1-like syndromes as early as the 18th century,6-8 Friedrich von Recklinghausen did not publish his seminal, detailed case reports until 1882.9 Von Recklinghausen observed that neurofibromas contain elements of both neuronal and fibroblastic tissue. In 1911, H. Greggio reported his observation that mast cells infiltrate the neurofibroma.10,11 Vincent Riccardi later postulated a pivotal role for mast cells and melanocytes in NF1 pruritis, pigmentation defects, and, potentially, neurofibroma formation.12

Today, NF1 is recognized as a common and fully penetrant genetic disease demonstrating variable expressivity.11 The disease is transmitted in an autosomal dominant fashion as a mutation in NF1, a tumor suppressor gene encoding the protein Neurofibromin. Neurofibromin functions as a p21ras (Ras) guanosine tri-phosphotase (GTP) activating protein (GAP), accelerating the hydrolysis of Ras-GTP thousands of fold and functioning at least in part to negatively regulate multiple Ras-dependent cellular signaling pathways.11,13-17

Mutations of NF1 predispose patients to variable neuronal, hematopoietic, and skeletal pathologies, including myeloid leukemia, kyphoscoliosis, long bone pseudoarthrosis, and cutaneous, subcutaneous, and diffuse plexiform neurofibromas.11,18 Neurofibromas are pathognomonic for the disease. Cutaneous neurofibromas arise from small peripheral nerves during adolescence or adulthood and are observed in over 95% of NF1 patients (Figure 1, left panel).19 Plexiform neurofibromas, by contrast, appear in about 15-40% of NF1 patients and arise during early development from cranial nerves and proximal large peripheral nerve sheaths.
(Figure 1, right panel). These interdigitating tumors are composed of Schwann cells, fibroblasts, degranulating mast cells, and vascular cells.\textsuperscript{11,20} Though cutaneous neurofibromas have limited growth and a very low propensity for transformation, plexiform neurofibromas can constitute an early and lifelong source of disfigurement, disability, mortality, and potential transformation to a metastatic malignant peripheral nerve sheath tumor (MPNST).

As indicated by human tissue analyses and animal models, pleomorphic NF1 manifestations result from widespread $NF1$ heterozygosity and variable $Nf1$ LOH in multiple cell lineages. Malignant and non-malignant pathologies in NF1 can originate in neural crest derived tissue: LOH in Schwann cells permits neurofibroma formation, LOH in chromaffin cells initiates pheochromocytomas, and LOH in melanocytes produce pigmented lesions such as café-au-lait macules and Lisch nodules.\textsuperscript{21-23} NF1 morbidities also arise from other germ layers: LOH in skin-derived precursors (SKPs) leads to cutaneous neurofibroma formation, LOH in myeloid cells induces myelomonocytic leukemia, and LOH in glial cells permits astrocytoma formation.\textsuperscript{5,19,21,26,28-31} While NF1 pathologies arise from multiple germ layers, regionalized tissue may demonstrate increased reliance on NF1 GAP signaling. For example, a recent study has shown increased NF1 protein in astrocytes localized to the optic nerve, brain stem, and cerebellum compared to astrocytes in the neocortex. This study found that NF1 deficient astrocytes in the optic nerve, brain stem, and cerebellum, but not in the neocortex, demonstrate hyperfunctioning phenotypes, perhaps explaining the propensity for localized tumor formation in patients with neurofibromatosis type 1.\textsuperscript{32}
Recent studies have additionally implicated Nf1 haploinsufficient cell lineages in non-malignant but common manifestations of NF1, including altered osteoclast-osteoblast interactions in skeletal dysplasia, Nf1+/− endothelial cells in vascular infarcts, and Nf1+/− GABA neurons in learning deficiencies.24-27 As reviewed in this paper, haploinsufficiency of bone marrow derived cells within the neurofibroma microenvironment critically contribute to tumorigenesis. Broadly, these data emphasize that consideration of molecular mechanisms underlying specific NF1 morbidities requires consideration of intracellular signaling not only in single cell types nor in a single germ layer but within and between multiple cell systems. In the case of the plexiform neurofibroma, it is important to investigate signaling mechanisms within and interactions between the Nf1−/− Schwann cell, the Nf1+/− dermal tissue and vasculature, and the Nf1+/+ hematopoietic system.5
DISCUSSION:

A question of lineage: mouse modeling unravels a genetic riddle

A series of murine models have indispensably facilitated our understanding of the malignant and non-malignant manifestations of NF1. Though these models have provided insights into a range of disease phenotypes, we focus our discussion here on the models illuminating the mechanisms of plexiform neurofibroma formation.

The \textit{Nf1} heterozygote

The first \textit{Nf1} knockout mice, created by Jacks et al and Brannan et al, carried a targeted disruption of \textit{Nf1} exon 31 (\textit{Nf1}^{+/n31}).\textsuperscript{33,34} This mutation produces protein instability and subsequent degradation. Compared to wild-type (WT) controls, the \textit{Nf1}^{+/−} animals had a high predisposition to multiple cancers after one year of age, experienced a shorter overall lifespan, and occasionally exhibited characteristic NF1 tumors such as pheochromocytomas and myeloid leukemia. The leukemic cells and pheochromocytoma tissue demonstrated LOH in the WT \textit{Nf1} allele, consistent with the \textit{Nf1} tumor suppressor model. None of these mice, however, developed neurofibromas, Lisch nodules, or café-au-lait macules, hallmark characteristics of human NF1.

This failure to develop key symptoms in the \textit{Nf1}^{+/−} mouse posed a formidable problem – after all, humans born heterozygous at the \textit{NF1} locus ubiquitously suffer from a combination of cutaneous, diffuse, or plexiform neurofibromas. To explain this apparent disconnect, Jacks and Weinberg hypothesized that the reduced cell number, shorter lifespan, or disparate resistance to \textit{Nf1} mutations decreases the probability of a second-hit in the murine system. Problematically, \textit{Nf1}^{−/−} mice die at embryonic day 13.5...
due to cardiac developmental defects, complicating the analysis of a germ line second-hit. Although the *Nf1*+/− mouse disappointed as a model for neurofibroma formation, it propelled the stimulus for the development of additional murine models and created a tremendous resource for studying *Nf1*+/− dependent physiology in multiple cell lineages, including Schwann cells, fibroblasts, vascular cells, and hematopoietic cells.

**The *Nf1* chimera**

Following the observations from the *Nf1*+/− mouse, Cichowski et al devised a murine model to test the hypothesis that *Nf1* functions as a tumor suppressor and that LOH precedes tumor formation. They injected *Nf1*−/− embryonic stem (ES) cells into WT blastocysts, creating *Nf1*−/− chimeric mice. While high chimerism resulted in early death and low chimerism produced no phenotypic effect, all of the remaining animals in the experimental group (12/18) experienced myelodysplasia, neuromotor defects, and the formation of neurofibromas. The neurofibromas arose from multiple nerves – in the tongue, along the dorsal root, and within the limb muscle – and shared gross morphological, histological, and electron microscopic features with human neurofibromas. Neurofibroma tissue from chimeric animals carrying an *Nf1*−/− ES cell-linked β-galactosidase transgene demonstrated near uniform β-gal expression, suggesting tumor dependence on *Nf1* nullizygosity. However, this model relied on a large and unpredictable number of *Nf1*−/− admixed cells throughout the animal and precluded the assessment of admixed *Nf1*−/− and *Nf1*+/− cells – a genetic status expected in the human condition. While the chimera solidified the tumor suppressor effect of *Nf1*
in vivo and suggested the Schwann cell as the tumor cell of origin, the question of the exact genetic and cell conditions required for neurofibroma formation persisted.

**Impact of Nf1 haploinsufficiency in multiple cell lineages**

The studies described above demonstrated that LOH in tumorigenic cells concur with NF1 as a tumor suppressor gene. However, the slow growth of neurofibromas and the numerous and pleomorphic non-malignant manifestations observed in NF1 patients suggested an NF1 gene dosage effect. Genetic evidence for this dosage effect was first provided by the study of Nf1+/− mast cells and melanocytes, showing that Nf1 heterozygosity – and not solely nullizygosity – produced a stem cell factor (SCF)-dependent hyperactive phenotype (Figure 2). In this study, Ingram et al intercrossed Nf1+/− mice with c-kit mutant mice (W41/W41), a point mutation which compromises SCF-induced c-kit receptor tyrosine kinase activity by ~85%. (The W, or white spotting locus mutation, severely diminishes mast cell differentiation and compromises neural crest derived melanocyte migration, leading to a white coat color.) Dermal tissue and peritoneal fluid from both Nf1+/− and Nf1+/−; W41/W41 mice exhibited increased numbers of mast cells compared to WT and W41/W41 mice, respectively. Likewise, bone marrow from Nf1+/− and Nf1+/−; W41/W41 mice formed increased numbers of mast cell colonies in semi-solid media compared to WT and W41/W41 mice, respectively. SCF induced Nf1+/− and Nf1+/−; W41/W41 mast cell proliferation at nearly twice the rate as WT and W41/W41 cells, respectively, and both Nf1+/− and Nf1+/−; W41/W41 mast cells demonstrated increased SCF-dependent survival. Most strikingly, the Nf1+/−; W41/W41 animals demonstrated a black-white mottling of the W41/W41 mouse’s normally albino coat,
showing that \textit{Nf1} haploinsufficiency modulated the fates of both mast cells and the neural crest derived melanocytes, a lineage known to express high levels of neurofibromin. Thus, \textit{Nf1} haploinsufficiency partially or fully restored multiple loss-of-function phenotypes associated with the \textit{W} mutation \textit{in vitro} and \textit{in vivo}.

Concomitant investigations revealed \textit{Nf1} haploinsufficient phenotypes in other cell lineages. Atit et al demonstrated that \textit{Nf1} \textsuperscript{+/−} C57BL/6 mice, a strain typically resistant to chemical-induced carcinogenesis, uniformly developed papillomas subsequent to treatment with dimethylbenzanthracene and 12-O-tetradecanoyl-13-acetylphorbol.\textsuperscript{42} This papilloma formation showed \textit{Nf1} haploinsufficient keratinocytes aberrantly proliferating in response to chemical injury. Likewise, Bajenaru et al found hyperactivity in \textit{Nf1} haploinsufficient astrocytes, a pertinent discovery considering the predisposition of NF1 patients to low-grade astrocytomas.\textsuperscript{43,44} These studies demonstrated that \textit{Nf1} \textsuperscript{+/−} astrocytes proliferated more quickly than WT cells, grew autonomously \textit{in vitro}, and exhibited increased activation of Ras pathways.

Taken together, these data from \textit{in vitro} and \textit{in vivo} studies of \textit{Nf1} \textsuperscript{+/−} melanocytes, mast cells, keratinocytes, and astrocytes — all of which are lineages relevant to manifestations of NF1 — argued that genetic haploinsufficiency of a tumor suppressor could modulate cellular phenotypes.

**Generation of a conditional \textit{Nf1} knockout**

The early embryonic deaths of \textit{Nf1} \textsuperscript{+/−} mice generated using traditional homologous recombination prompted Zhu et al to create a conditional knockout murine model of NF1. In this model, \textit{loxP} sites flank \textit{Nf1}’s exon 31 and 32 (\textit{Nf1} \textsuperscript{flox/flox}), allowing
for Cre-recombinase mediated gene deletion. To test the hypothesis that \( Nf1 \) LOH in the Schwann cell results in neurofibroma formation, the authors crossed the \( Nf1^{lox/lox} \) mouse with a mouse expressing Cre protein under control of the Krox20 promoter (Krox20cre). Because Krox20 specifically promotes transcription in about 5-10% of Schwann cells, the \( Nf1^{lox/lox}; \) Krox20cre mouse provides limited Schwann cell \( Nf1 \) nullizygosity while circumventing the problem of embryonic lethality in the \( Nf1^{-/-} \) animal.

The \( Nf1^{lox/lox}; \) Krox20cre mouse, however, failed to develop the hallmark neurofibroma. The authors hypothesized that neurofibroma formation requires not only Schwann cell LOH but also haploinsufficiency in the microenvironment. To test this hypothesis, the authors intercrossed their \( Nf1^{lox/lox}; \) Krox20cre mouse with an \( Nf1^{+/-} \) mouse, creating the \( Nf1^{lox/-}; \) Krox20cre animal. In this model the Schwann cell loses both alleles of \( Nf1 \). Concurrently, all other cells remain functionally \( Nf1^{+/-} \) (i.e., \( Nf1^{lox/-} \)). The \( Nf1^{lox/-}; \) Krox20cre mouse develops normally until 10 to 12 months of age, at which time it experiences an enlargement of peripheral nerves along the spinal dorsal root ganglia which are phenotypically similar to human plexiform neurofibroma tissue. Histology demonstrated disassociated Schwann cells, pervasive collagen bundles, fibroblastic proliferation, and an abundant infiltration of mast cells. Of critical importance, these pathological features remain consistently absent in mice with \( Nf1^{+/-} \) Schwann cells and a WT background (\( Nf1^{lox/lox}; \) Krox20cre).

In this mouse model, Zhu et al demonstrated that neurofibroma formation requires not only LOH in the Schwann cells but also \( Nf1 \) haploinsufficiency in the supporting tissue. Importantly, the tumors in \( Nf1^{lox/-}; \) Krox20cre mice are infiltrated with an abundance of mast cells and are histologically reminiscent of human neurofibroma tissue.
tissue. Thereby, the \(Nf1^{\text{floxed}}\)-Krox20cre mouse recapitulates the human condition with 100% penetrance, granting NF1 scientists indispensable insights into neurofibroma pathogenesis, elegantly demonstrating microenvironment requirements for neurofibroma development, and providing foundational evidence implicating the \(Nf1^{+/-}\) mast cell (reviewed in \(^{21}\)).

**Interactions in the \(Nf1\) haploinsufficient microenvironment**

Following the creation of the \(Nf1^{\text{floxed}}\)-Krox20cre \textit{in vivo} plexiform neurofibroma model, subsequent discoveries further elucidated inter and intracellular mechanisms of tumor formation. It is well established that mast cells differentiate, proliferate, and secrete cytokines in response to SCF, a secreted ligand to the c-kit receptor tyrosine kinase. \(^{46}\) Importantly, SCF mRNA can be detected in neurofibroma tissue, and both normal and dysplastic Schwann cells can secrete SCF. \(^{20,39,47}\) Moreover, evidence in other experimental models have implicated inflammatory cells such as mast cells in the growth, vascularization, and spread of neoplastic conditions, \(^{48-55}\) and, as discussed above, Ingram et al had shown that \(Nf1^{+/-}\) mast cells are hypersensitive to SCF. \(^{36}\)

Given these data, NF1 investigators have increasingly sought to elucidate the interactions through which Schwann cells, mast cells, fibroblasts, and endothelial cells interact within the neurofibroma and to identify critical molecular and cellular events in neurofibroma pathogenesis.

**Schwann cells**
In 2001, Mashour et al showed that *Nf1*-/− Schwann cells directly promoted the proliferation of endothelial cells and fibroblastoid cells *in vitro*.56 This study implicated the abnormal secretion of fibroblast growth factor (FGF-2), platelet-derived growth factor (PDGF), and midkine (MK) from nullizygous Schwann cells as effectors of *in vitro* cellular mitogenesis. These initial data suggested the potential for Schwann cell-driven cellular growth. Subsequently, Yang et al provided mechanistic insight into the aberrant Schwann cell activity driving neurofibroma inflammation.57 This study found that *Nf1*-/− Schwann cells secrete pathological concentrations of SCF and, in turn, the secreted SCF potently recruits *Nf1*+/− mast cells. Schwann cells derived from *Nf1*-/− mouse embryos proliferated more quickly than WT cells, displayed an irregular morphology, and secreted approximately six times as much soluble SCF as *Nf1*+/− and WT Schwann cells. Conditioned media from *Nf1*-/− Schwann cells promoted chemotaxis of *Nf1*+/− mast cells at twice the rate as WT mast cells. Recombinant SCF reproduced these results *in vitro*, and the genetic disruption of c-kit or the addition of c-kit receptor blocking antibodies prevented mast cell chemotaxis in response to Schwann cell conditioned media. Taken in the context of *Nf1*<sup>flox/−</sup>/Krox20cre tumor model, these data suggested a potentially important interaction between the *Nf1*-/− Schwann cell and the *Nf1*+/− mast cell.

**SCF- and *Nf1*-dependent mast cell biochemistry**

SCF aberrantly secreted from *Nf1*-/− Schwann cells promotes a hyperactive SCF-responsive phenotype in *Nf1*+/− mast cells, and this *Nf1*-dependent mast cell pathophysiology stems from deregulated Ras signaling. In response to multiple distinct growth factors and cytokines, Ras activates to its guanine triphosphate (GTP)-bound state and initiates a series of signal transduction cascades.58-61 Neurofibromin, a highly-
conserved GAP encoded by 350kb of genomic DNA located on human chromosome 17q11.2 (chromosome 11 in mice) and closely related to the yeast gene products IRA1 and IRA2, converts active Ras from its GTP-bound state to its inactive guanine diphosphate (GDP)-bound state.\textsuperscript{3, 13, 16, 21, 62, 63} Loss of GAPs can induce neoplasia through increased cellular growth, proliferation, and migration.\textsuperscript{54} Loss of the NF1 GAP in hematopoietic cells, including but not limited to mast cells, increases the latency and potency of GTP-bound Ras and phosphorylated downstream effectors within the Raf-MEK-ERK and phosphoinositide-3-kinase (PI-3K)-Rac-Pak-P38 pathways.\textsuperscript{36, 57, 59, 64-70}

SCF-dependent c-kit signaling in the mast cell hinges specifically on K-ras pathways.\textsuperscript{67} Immunoprecipitation experiments indicate that SCF:c-kit activated Ras induces a cascade ultimately phosphorylating and activating p44/p42 (ERK1/2), p38, and AKT.\textsuperscript{36, 68, 71, 72} \textit{In vitro} studies of mast cells generated from the marrow of various knock-out mice and cells treated with chemical inhibitors of MEK, PI-3K, and p38 MAPK (PD98059, LY294002, and SB203580, respectively) indicate that the Raf-MEK-ERK pathway primarily modulates proliferation and cytokine synthesis while the PI-3K-Rac2-Pak-p38 pathway additionally modulates F-actin rearrangement and cellular motility.\textsuperscript{67, 68, 70-72} Biochemical studies show that the classical Raf-MEK-ERK cascade and the PI-3K-dependent cascade crosstalk through the activity of the p21 activated kinases (Paks). Pak phosphorylates Raf1 at serine 338, which primes Raf1 for increased phosphorylation of MEK at serine 217/222.\textsuperscript{68} Pak1 can also directly phosphorylate MEK at serine 298. Both of these events potentiate the phosphorylation of the extracellular regulated kinases, ERK1 and ERK2. ERK1 and ERK2 translocate to the nucleus where they phosphorylate pro-growth transcription factors such as Elk1 and
potentially regulate the G1- to S-phase transition.\textsuperscript{68,72-74} Additional data indicate that SCF-dependent activation of Rac2, the highly-expressed hematopoietic Rho GTPase isoform, phosphorylates Akt, which modulates the Bcl-2 family of proteins to prevent apoptosis, increasing mast cell survival.\textsuperscript{71} Because neurofibromin negatively regulates Ras-GTP and its multiple downstream targets, these data mechanistically explain aberrant function in the \textit{Nf1\textsuperscript{+/-}} mast cell, providing potential therapeutic targets along the Raf-MEK-ERK and PI-3K-Rac-Pak-p38 pathways. (Figure 3).

**Fibroblasts**

In 2006, Yang et al demonstrated another critical neurofibroma microenvironment interaction: SCF-stimulated \textit{Nf1\textsuperscript{+/-}} mast cells potentiate \textit{Nf1\textsuperscript{+/-}} fibroblast functions.\textsuperscript{75} Fibroblasts comprise a major cellular portion of the neurofibroma, and their secreted collagen accounts for nearly half of the dry tumor weight.\textsuperscript{76} Fibroblasts migrate, proliferate, and synthesize collagen in response to transforming growth factor beta (TGF-\beta). Yang et al demonstrated that \textit{Nf1\textsuperscript{+/-}} mast cells secreted 2.5 fold higher TGF-\beta than WT mast cells \textit{in vitro}.\textsuperscript{75} Likewise, \textit{Nf1\textsuperscript{+/-}} fibroblasts co-cultured with \textit{Nf1\textsuperscript{+/-}} mast cells demonstrated the greatest ability to contract collagen in an \textit{in vitro} lattice system, representing positive cooperation in the remodeling of the extracellular matrix. \textit{Nf1\textsuperscript{+/-}} mast cell conditioned media compared to WT conditioned media induced higher fibroblast bioactivity as measured by proliferation, migration, and collagen production. The study confirmed this heightened fibroblast bioactivity to be TGF-\beta dependent by neutralizing \textit{Nf1\textsuperscript{+/-}} mast cell conditioned media with TGF-\beta blocking antibody. These data supported the growing idea that the \textit{Nf1\textsuperscript{+/-}} mast cell is the critical effector in the
paracrine induction of neurofibroma pathogenesis. Interestingly, TGF-β dependent

\( Nf1^{+/+} \) fibroblast hyperactivity appears to result from increased kinase activity of c-abl secondary to increased Ras-GTP. Accordingly, imatinib mesylate (Gleevec©) inhibits both \textit{in vitro} collagen production and \textit{in vivo} fibroblast migration in the dermal tissue of mice given subcutaneous TGF-β or \( Nf1^{+/+} \) mast cell conditioned media. (Figure 4).

**An SCF/c-Kit signaling axis in the \( Nf1^{+/+} \) bone marrow cells is required for plexiform neurofibroma formation in a murine model**

In light of the conditional plexiform neurofibroma model (\( Nf1^{\text{flox/flox}}; \text{Krox20cre} \)) and the data implicating interactions between Schwann cells, mast cells, and fibroblasts, Yang et al tested the hypothesis that \( Nf1^{+/+} \) mast cells are the critical effectors of neurofibroma pathogenesis. The investigators transplanted bone marrow from \( Nf1^{+/+} \) mice into lethally irradiated \( Nf1^{\text{flox/flox}}; \text{Krox20cre} \) mice. An eGFP reporter gene carried by the donors ensured that engrafting hematopoietic cells – and not isolated host progenitor cells – had successfully reconstituted the recipient’s bone marrow. These transplants created mice with \(~10\%\) of Schwann cells \( Nf1 \) nullizygous, bone marrow cells \( Nf1 \) haploinsufficient, and all other cells functionally wild-type. Within six months, the \( Nf1^{\text{flox/flox}}; \text{Krox20cre} \) mice reconstituted with \( Nf1^{+/+} \) marrow developed neuromotor defects, weight loss, dorsal root ganglia thickening, and an increased mortality rate comparable to the tumorigenic \( Nf1^{\text{flox/flox}}; \text{Krox20cre} \) mice. By comparison, \( Nf1^{\text{flox/flox}}; \text{Krox20cre} \) mice reconstituted with WT marrow did not exhibit any of these defects. In a critical converse experiment, transplantation of WT bone marrow into the \( Nf1^{\text{flox/flox}}; \text{Krox20cre} \) mouse prevented the normally reliable tumorigenesis of the
conditional knockout model. These data validated the hypothesis that \( Nf1 \) haploinsufficiency in the marrow microenvironment is both required and sufficient for neurofibroma formation (Figure 5).

To test the hypothesis that SCF-recruited and stimulated mast cells are the principal hematopoietic effectors of the neurofibroma microenvironment, Yang et al next examined the effect of c-kit inhibition on tumor formation. They transplanted bone marrow harboring both the \( Nf1^{+/} \) mutation and two different c-kit gene mutations (\( W^{d1}/W^{d1} \) or \( W^{v}/W^{v} \)) compromising kinase activity 85% and 92-95%, respectively. \( Nf1^{\text{floxed/floxed}} \); Krox20cre mice reconstituted with \( Nf1^{+/+};W \) marrow failed to exhibit the neuromotor defects, dorsal root ganglia enlargement, and mast cell infiltration found in the \( Nf1^{\text{floxed/floxed}} \); Krox20cre neurofibroma model and the \( Nf1^{\text{floxed/floxed}} \); Krox20cre mouse reconstituted with \( Nf1^{+/+} \) marrow. Importantly, Southern blot analysis of genomic DNA isolated from the bone marrow and individual myeloid colonies (CFU-GM) isolated from the bone marrow of recipients confirmed greater than 95% \( Nf1^{+/+};W \) marrow (donor) engraftment efficiency. These data demonstrated that neurofibroma formation in a murine model depends on \( Nf1 \) LOH in the Schwann cell and c-kit-dependent \( Nf1 \) haploinsufficiency in the bone marrow.

Correspondingly, models utilizing other Schwann cell-limited Cre promoters (periostin-Cre, P0-Cre, and tamoxifen inducible PLPCre) require \( Nf1^{+/+} \) and c-kit dependent hematopoietic contributions.\(^5\) However, it should be noted that widespread \( Nf1^{+/+} \) deletion in glial cells at the appropriate murine developmental timing (E12.5), such as that driven by Dhh-Cre, permits plexiform and dermal neurofibroma formation despite a WT cellular background.\(^7\) This model may provide an important explanation for
sporadic plexiform neurofibromas occurring in individuals without genetic neurofibromatosis type 1.

**Pharmacological studies support SCF/c-kit axis as critical to the neurofibroma microenvironment**

Given the evidence that the hematopoietic system and the c-kit pathway contribute to the Krox20cre murine plexiform neurofibroma formation, Yang et al proceeded to treat a cohort of adult Nf1\(^{\text{flox/−}}\);Krox20cre mice with the tyrosine kinase inhibitor imatinib mesylate (Gleevec\(©\)). Imatinib mesylate potently inhibits the c-kit, PDGF-β, and bcr/abl receptor tyrosine kinases and currently carries FDA approval for the treatment of chronic myelogenous leukemia, other hematological malignancies, and some solid tumors.\(^7\) Mice were followed to an age at which they would be expected to have multiple plexiform neurofibromas, as evidenced by fluoridated deoxyglucose uptake in positron emission tomography (FDG-PET). Further volumetric analysis performed with FDG-PET CT showed that imatinib reduced tumor volume and metabolic activity in the Nf1\(^{\text{flox/−}}\);Krox20cre mice approximately 50%. By contrast, the placebo treated controls demonstrated a small increase in FDG-PET uptake. Histological samples from nerve roots of the treatment group demonstrated regularly patterned Schwann cells and tissue free of mast cell infiltrate while tissue from the placebo group demonstrated characteristic stigmata of neurofibromas.\(^5\)

Hypothetically, imatinib prevents mast cell proliferation, infiltration, and exacerbation of the nascent tumor by inhibiting the c-kit receptor tyrosine kinase and, subsequently, inflammation driven by Nf1 haploinsufficient mast cells. Further, imatinib
may reinforce its effect through inhibition of the PDGFR and c-abl tyrosine kinases, signaling molecules potentially important to vascular and fibroblastic aberrancies in

Imatinib mesylate reduces a highly morbid neurofibroma in a pediatric patient

Based on these murine experiments implicating c-kit dependent \( Nf1^{+-} \) marrow cells and the efficacy of imatinib, clinicians treated a three year old girl suffering from a highly vascularized, non-resectable, and progressively growing neurofibroma. The girl presented as an infant with several hallmark signs of NF1 and a histologically confirmed neurofibroma first appearing at six months of age. The tumor had progressively enlarged to encompass the left floor of her mouth, tongue, and mastoid bone and was encasing her carotid artery and jugular vein. At the time of treatment, the tumor had severely compressed her airway, leading to drooling, sleeplessness, and anorexia. After discussing the risks and potential benefits of experimental medical therapy with her physician and her parents, the patient received 350 mg/m\(^2\)/dose of imatinib mesylate. Following three months of treatment, magnetic resonance imaging revealed a remarkable 70% reduction in tumor volume, and the complications associated with the airway compression had resolved. After the tumor ceased its regression and appeared stable, treatment was terminated.\(^5\) The patient remains stable and in relatively good health (Figure 6). Encouraged by the mouse model and the successful medical treatment of this index patient, a phase II clinical trial involving NF1 patients suffering from highly morbid neurofibromas has been initiated.
Other cell lineages and future directions

While we have principally discussed interactions among Schwann cells and the hematopoietic microenvironment in the context of neurofibroma formation, other lineages in the microenvironment are clearly involved in tumor progression and are potential targets for drug treatment. Neo-angiogenesis is required for tumor expansion and metastasis in multiple human cancers,\textsuperscript{54,79} and previous data indicated that \textit{Nf1}^{-/-} Schwann cells can initiate neo-angiogenesis by secreting vascular endothelial growth factor (VEGF).\textsuperscript{80} Likewise, Munchhof et al demonstrated that \textit{Nf1}^{-/-} cultured Schwann cell media placed intradermally into \textit{Nf1}^{+/+} mice produced hyperactive angiogenic responses similar to the intradermal placement of VEGF and bFGF.\textsuperscript{28} \textit{In vitro}, VEGF and bFGF treatment of murine and human NF1 cultured ECs increased cell proliferation and migration and demonstrated hyperphosphorylation of ERK1 and ERK2. Addition of a MEK inhibitor (PD98059) diminished these effects, including the vascularization of matrigel plugs treated with VEGF, bFGF, or Schwann cell conditioned media and placed within the skin of experimental mice. Similarly, \textit{Nf1}^{+/-} VSMCs demonstrate increased migration and proliferation, providing an additional Ras-dependent mechanism enabling neoangiogenesis.\textsuperscript{26} However, many of the NF1-dependent biochemical mechanisms within non-neoplastic tissue such as the vasculature need further exploration.

In addition, while the experimental data to date demonstrate a clear role for c-kit/SCF signaling in the initiation of tumorigenesis, the exact processes within mast cells and potentially other c-kit responsive hematopoietic cells that promote this pathological progression still need to be defined. \textit{Nf1}^{+/-} mast cells secrete a number of matrix metalloproteinases, cytokines (e.g. IL-6, TNF-\( \alpha \), CCL2, CCL3, CCL4, and C5a), and
growth factors (e.g. NGF, VEGF, PDGF, bFGF, and TGF-β). These secreted factors – along with factors secreted by the Schwann cell – stimulate fibroblasts, endothelial cells (ECs), and smooth muscle cells, promoting extracellular matrix remodeling, collagen deposition, chemotaxis, neo-angiogenesis, and generalized inflammation.\(^5,20,36,46,57,67,68,72,75\) (also, unpublished). Whether a subset of these secreted molecules has a particularly seminal role in the initiation of tumorigenesis is an area of experimentation that may provide both more specific therapies and potentially inform other neoplastic conditions potentiated by inflammatory microenvironments.\(^52,53,55\)

Importantly, a deeper understanding of the cellular and biochemical mechanisms inducing tumor regression during imatinib treatment would directly inform – and perhaps improve – the medical management of human patients with established plexiform neurofibromas. Finally, while mast cell inflammation underpins the murine plexiform neurofibroma microenvironment and undoubtedly contributes to the human condition, we have not explored cellular events required for the transition to the malignant peripheral nerve sheath tumor. The continuing refinement of murine models, the use of mouse genetics, and advances in vivo experimental imaging are important tools that may help provide additional insights into these processes.
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AUTHORSHIP:

Contribution: K.S. conducted literature review and analysis, prepared the figures, and composed the paper; F.C.Y. offered critical insight and analysis; D.W.C. offered critical insight and analysis and contributed to manuscript preparation.

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REFERENCES:


FIGURE LEGENDS:

Figure 1: Examples of cutaneous and plexiform neurofibromas. Cutaneous neurofibromas growing on the chest and abdomen and an MRI of a large plexiform neurofibroma compressing the spinal column. Photographs courtesy of the Children's Tumor Foundation: www.ctf.org.

Figure 2: Effect of haploinsufficiency of Nf1 on coat color and total numbers of cutaneous and peritoneal mast cells. (A) Coat color pattern of a representative mouse from each of the following genotypes: +/++;+/+, Nf1+/-;+/+, Nf1+/--;+/+, W41/W41, and Nf1+/--;W41/W41. Haploinsufficiency at Nf1 partially corrects the coat color deficiency in mice homozygous for the W41 allele in a C57BL/6 genetic background. (B) Representative cytospins from peritoneal lavages stained for mast cells from individual mice of the four Nf1 and W genotypes. Peritoneal cells were stained with toluidine blue to quantify the total number of mast cells per peritoneal lavage. A higher magnification of a representative mast cell is shown in the inset of the wild-type mouse (original magnification: x200). Bar (inset) 10 μm. Bar (far right) 30 μm. (C) Representative ear biopsies stained for cutaneous mast cells from individual mice of the four Nf1 and W genotypes. Specimens were stained with hematoxylin-eosin to assess routine histology, and with Giemsa to identify mast cells. Ear biopsies were stained with Fontana-Masson to differentiate melanin-containing cells from mast cells. Cutaneous mast cells (Giemsa-positive, Fontana-Masson–negative) were quantitated in a blinded fashion by counting the distal 5 mm of ears. Black arrows indicate Giemsa-positive mast cells,

**Figure 3:** Schematic of SCF:c-kit signaling in the mast cell. Upon SCF binding at the c-kit RTK, c-kit dimerizes and autophosphorylates. This phosphorylation promotes the conversion of Ras-GDP to Ras-GTP, which activates PI-3K, MAPK, and Rho GTPase signaling pathways. NF1 potentiates the hydrolysis of Ras-GTP to Ras-GDP, the inactive form of Ras.

**Figure 4:** Potential cellular interactions in the plexiform neurofibroma microenvironment.

**Figure 5:** Transplant schematic.

**Figure 6:** Evaluation of imatinib mesylate efficacy in an index patient with a plexiform neurofibroma. Coronal MRI scans (T1 weighted images with gadolinium contrast and fat saturation) of the head and oropharynx of an NF1 patient with a plexiform neurofibroma before (panel 1) and 3 months following treatment with imatinib mesylate (panel 2). The region of the tumor in the respective images is indicated. Reprinted from Yang et al, “*Nf1*-Dependent Tumors Require a Microenvironment Containing *Nf1*+/− and c-kit-Dependent Bone Marrow,” *Cell*, 31 October 2008, with permission from Elsevier.
Figure 3
Schwann cells

NGF

Collagen
Fibronectin
Laminin

SCF

Mast cells

VEGF

FGF

MMPs

Heparin

Histamine

TGF-β

Tryptase

Collagen

Endothelial cells

Fibroblast
Figure 5

Bone marrow transplants

Control

WT  Nf1\(^{+/−}\)  Nf1\(^{+/−};\ W\)

WT

Krox20; Nf1\(^{flox/flox}\)
Schwann cells: Nf1\(^{−/−}\)
Fibroblasts: Nf1\(^{+/−}\)
Endothelial cells: Nf1\(^{+/−}\)
Hematopoietic cells: Nf1\(^{+/−}\)

Krox20; Nf1\(^{flox/flox}\)
Schwann cells: Nf1\(^{−/−}\)
Fibroblasts: WT
Endothelial cells: WT
Hematopoietic cells

Krox20; Nf1\(^{flox/flox}\)
Schwann cells: Nf1\(^{−/−}\)
Fibroblasts: Nf1\(^{+/−}\)
Endothelial cells: Nf1\(^{+/−}\)
Hematopoietic cells

Neurofibroma Formation

Yes
No
Yes
No
No
Mast cells and the neurofibroma microenvironment

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