ROLE OF RAS PROTEINS IN MALIGNANT PERIPHERAL NERVE SHEATH TUMORS

NICOLE M. BROSSIER

DEPARTMENT OF CELL BIOLOGY

ABSTRACT

Malignant peripheral nerve sheath tumors (MPNSTs) arising in patients with neurofibromatosis type I (NF1) are null for the tumor suppressor neurofibromin, a negative regulator of signaling from both classic Ras (H-Ras, N-Ras and K-Ras) and R-Ras (R-Ras, R-Ras2/TC21, M-Ras/R-Ras3) subfamily members. Treatment of these tumors with Ras-targeted agents such as farnesyltransferase inhibitors has proven unsuccessful, likely due to the inability of these agents to successfully target all of the Ras isoforms regulated by neurofibromin. Thus, determining which Ras isoforms are critical for MPNST pathogenesis would be of therapeutic value. In this dissertation, we first review the clinical manifestations of NF1 and then describe transgenic models developed to study NF1-related tumorigenesis in the peripheral nervous system. We then examine the role of the classic Ras and R-Ras subfamilies in MPNST proliferation, survival and migration. We show that a dominant negative (DN) H-Ras mutant, which can inhibit activation of all three classic Ras isoforms, inhibits MPNST proliferation and survival but not migration. In contrast, a dominant negative R-Ras mutant, which is thought to inhibit R-Ras and R-Ras2 activation, inhibits MPNST proliferation and migration but not survival. We then utilize a mass spectrometric phosphoproteomics approach to identify the mechanistic basis for the differential effects of DN H-Ras and DN R-Ras on MPNST cells. Finally, we attempt to determine whether shRNA-mediation ablation a single classic Ras isoform can recapitulate the effects of pan-inhibition of the
classic Ras subfamily with DN H-Ras. We show that K-Ras ablation, but not N-Ras ablation, can reduce proliferation to a similar extent as DN H-Ras expression in at least one MPNST cell line. All together, this dissertation explains our current understanding of how neurofibromin loss promotes tumor formation in the peripheral nervous system and extends our knowledge about the contribution of neurofibromin-regulated Ras isoforms to this process.

Keywords: Nf1, MPNST, Ras, R-Ras, K-Ras, Phosphoproteomics
DEDICATION

This dissertation is dedicated to my father, who always believed I could do anything I set my mind to. Even though I completed this chapter of my life without you, I owe any success to the life lessons you taught me. I also dedicate this dissertation to my mother, Pamela Brossier, and my two best friends, Darcy Denner and Michelle Windsor. Without your constant support, I couldn’t have done it.
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**VARIABLE DEPENDENCE UPON K-RAS SIGNALING IN MALIG-NANT PERIPHERAL NERVE SHEATH TUMOR PROLIFERATION**

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INTRODUCTION

The autosomal dominant tumor predisposition syndrome known as neurofibromatosis type 1 (NF1) occurs in approximately 1 in 3500 newborns worldwide (1-3). Mutation or deletion of the NF1 tumor suppressor gene is found in 95% of these patients (4); the remainder of patients may have deletions in regions not examined during established screening procedures, such as the NF1 promoter or untranslated regions. The primary manifestation of NF1 is the development of multiple benign tumors of the peripheral nerve sheath called neurofibromas. Secondary manifestations of NF1 can include pigmentary lesions (Lisch nodules (5-7), café-au-lait macules and axillary freckling (8)), bony dysplasias (9) and learning disabilities (10, 11). These patients also have an increased incidence of certain malignancies, including juvenile myelomonocytic leukemias (12), pheochromocytomas (13), rhabdomyosarcomas (14), glioblastomas (15) and malignant peripheral nerve sheath tumors (MPNSTs) (16). Of these, MPNSTs are the most common; they typically arise by progression from benign neurofibromas.

Neurofibromas are composed of neoplastic Schwann cells intermingled with perineurial cells, mast cells, and fibroblasts (17). The disfiguring neurofibromas that arise within the dermis (dermal neurofibromas) have little malignant potential. In contrast, malignant progression is a major concern for patients with plexiform neurofibromas, which present as diffuse lesions involving multiple fascicles of large nerves or nerve plexuses (18). NF1 patients have an 8-13% lifetime risk of developing an MPNST (16); the risk may be as high as 30% for patients with symptomatic plexiform neurofibromas.
Unlike neurofibromas, MPNSTs are primarily composed of neoplastic Schwann-like cells, and these aggressive sarcomas often metastasize to the lungs, soft tissue, bones, liver, or brain. The primary treatment for MPNSTs is surgery, and few therapeutic options are available if complete surgical resection is not achieved. Radiotherapy does not increase long-term survival in patients with MPNSTs and currently available chemotherapeutic regimens are ineffective. With overall 5-year survival rates of only 34%, there is a clear need to identify new targets for chemotherapeutic intervention.

Loss of heterozygosity at the \textit{NF1} locus is found in neoplastic Schwann cells from both neurofibromas and MPNSTs arising in NF1 patients. Thus, these tumors are functionally null for neurofibromin, the gene product encoded by the \textit{NF1} gene. Neurofibromin is a GTPase activating protein (GAP) that negatively regulates the activity of both the classic Ras proteins (H-, N-, and K-Ras) and members of the R-Ras subfamily (R-Ras, R-Ras2/TC21, and R-Ras3/M-Ras). Accordingly, \textit{NF1}-deficient MPNSTs exhibit increased Ras activation. These observations suggest that inhibiting Ras signaling may be an effective means of treating MPNSTs. However, it has not yet been determined which Ras isoforms are critical for neoplastic Schwann cell survival, proliferation, and migration. These isoforms do not show equal responsiveness to Ras-targeted therapeutic agents such as farnesyltransferase inhibitors (FTIs), so determining which isoforms contribute to MPNST pathogenesis may help to guide future chemotherapeutic regimen choice. It is also unclear whether Ras signaling is equally important to the pathogenesis of sporadic MPNSTs, which represent at least one-third of all MPNST cases and often show intact neurofibromin.
In the first article of this dissertation (*Genetically Engineered Mouse Models Shed New Light on the Pathogenesis of Neurofibromatosis Type I-Related Neoplasms of the Peripheral Nervous System*), we review the clinical manifestations of NF1 and the known functions of neurofibromin in more detail. We then describe how transgenic mouse models have improved our understanding about tumorigenesis in the peripheral nervous system following neurofibromin loss. We also describe mouse models of PNS tumorigenesis driven by activated Ras mutants and discuss what this may reveal about Ras isoform specific roles in neurofibroma and MPNST development.

In the second article of this dissertation (*Classic Ras and R-Ras Subfamily Proteins Elicit Distinct Physiological Effects and Phosphoproteomic Alterations in Neurofibromin-Null Tumor Cells*), we attempt to determine the function of each subfamily of Ras proteins negatively regulated by neurofibromin. We utilize a dominant negative (DN) H-Ras mutant, previously demonstrated to inhibit activation of N-Ras and K-Ras as well as H-Ras, as a pan-inhibitor of the classic Ras subfamily. We similarly utilize a DN R-Ras mutant as a pan-inhibitor of the R-Ras subfamily isoforms. We show that DN H-Ras reduces MPNST proliferation and survival but not migration, whereas DN R-Ras reduces MPNST proliferation and migration but not survival. We then identify the changes in the MPNST phosphoproteome following expression of each mutant. This information revealed DN H-Ras and DN R-Ras effects on cellular proliferation may occur through different mechanisms. It also suggested a role for the phosphorylation of apoptosis-inhibitor 5 (API5) in DN H-Ras-induced reduction of MPNST cell viability. Further, it suggested that DN R-Ras may reduce MPNST migration by effects on
microtubules or vesicular trafficking, with Arf6 as a potential mediator of both of these effects.

In the third and final article of this dissertation (Variable Dependence upon K-Ras Signaling in Malignant Peripheral Nerve Sheath Tumor Proliferation), we attempt to determine whether ablation of a single classic Ras isoform can inhibit MPNST proliferation. We show that shRNA-mediated K-Ras ablation can inhibit proliferation of T265-2c cells but not ST88-14 MPNST cells, while N-Ras ablation does not affect proliferation in either line. We then show that K-Ras expression increased following N-Ras ablation in both lines, and ST88-14 cells showed increased K-Ras activation following N-Ras loss. This may indicate that cells compensate for N-Ras loss by increased K-Ras activation. It further suggests that the reverse compensation event may be ineffective at least in T265-2c cells.
GENETICALLY ENGINEERED MOUSE MODELS SHED NEW LIGHT ON THE PATHOGENESIS OF NEUROFIBROMATOSIS TYPE I-RELATED NEOPLASMS OF THE PERIPHERAL NERVOUS SYSTEM

by

NICOLE M. BROSSIER AND STEVEN L. CARROLL

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ABSTRACT

Neurofibromatosis type 1 (NF1), the most common genetic disorder affecting the human nervous system, is characterized by the development of multiple benign Schwann cell tumors in skin and large peripheral nerves. These neoplasms, which are termed dermal and plexiform neurofibromas respectively, have distinct clinical courses; of particular note, plexiform, but not dermal, neurofibromas often undergo malignant progression to form malignant peripheral nerve sheath tumors (MPNSTs), the most common malignancy occurring in NF1 patients. In recent years, a number of genetically engineered mouse models have been created to investigate the molecular mechanisms driving the pathogenesis of these tumors. These models have been designed to address key questions including: 1) whether NF1 loss in the Schwann cell lineage is essential for tumorigenesis; 2) what cell type(s) in the Schwann cell lineage gives rise to dermal neurofibromas, plexiform neurofibromas and MPNSTs; 3) how the tumor microenvironment contributes to neoplasia; 4) what additional mutations contribute to neurofibroma-MPNST progression; 5) what role different neurofibromin-regulated Ras proteins play in this process and 6) how dysregulated growth factor signaling facilitates PNS tumorigenesis. In this review, we summarize the major findings from each of these models and their limitations as well as how discrepancies between these models may be reconciled. We also discuss how information gleaned from these models can be synthesized to into a comprehensive model of tumor formation in peripheral nervous system and consider several of the major questions that remain unanswered about this process.
INTRODUCTION

Tumors of the peripheral nervous system (PNS)—neurofibromas, schwannomas and malignant peripheral nerve sheath tumors (MPNSTs)—cause considerable morbidity and mortality in afflicted individuals. This class of tumors is also common, representing 8.9% of the nervous system neoplasms resected in the United States between 2004 and 2006 [13]. While these tumors do occur sporadically, they are also often seen in association with the genetic disorders neurofibromatosis type I (NF1), neurofibromatosis type 2 (NF2), schwannomatosis, and Carney complex. Early transgenic modeling of these tumors thus focused on replicating the genetic defects seen in human patients with these disorders. This work provided insights into the role these mutated genes play in key signaling cascades, how they interact with other intratumoral abnormalities (e.g., aberrant growth factor signaling) and how their mutation enhances tumorigenesis via effects on the tumor microenvironment. These findings enabled the production of a second generation of genetically engineered murine (GEM) models that have further refined our understanding of tumorigenesis in the peripheral nervous system.

As the pathogenesis of NF1-related neoplasms (neurofibromas and MPNSTs) has been most extensively studied, we will focus on NF1-related GEM models in this review. We will first discuss the pathology of human NF1-related peripheral nerve sheath tumors, the genetic syndrome with which they are associated and our current understanding of the function(s) of the \textit{NF1} gene. We will then consider the mouse models that have been developed to investigate the mechanisms underlying NF1-related PNS tumorigenesis and the fundamental new insights that resulted from these models.
PATHOLOGY OF HUMAN PERIPHERAL NERVE SHEATH TUMORS AND THEIR ASSOCIATION WITH NF1

The Anatomy of Peripheral Nerve and Its Implications for the Pathogenesis of Peripheral Nerve Sheath Tumors

As peripheral nerve sheath tumors are derived from cells normally found in peripheral nerve, it is useful to first consider the composition and architecture of this complex tissue. The outermost layer of the nerve, the epineurium (Fig. 1), is composed of dense connective tissue and contains the highly anastamotic vascular supply of the nerve (the vasa nervorum). Within the epineurium, fascicles of nerve fibers are ensheathed by the perineurium, a dense concentric layer of specialized cells. Although perineurial cells were initially thought to be Schwann cell variants, it is now evident that these cells are not even of neural crest origin [36], being instead derived from the central nervous system (CNS) [44]. These perineurial cells, together with the neural vasculature, form a diffusion barrier (the “blood-nerve” barrier) that maintains endoneurial homeostasis. The compartment within the perineurium, the endoneurium, contains axons projecting into the periphery and their investing glia, the Schwann cells. The endoneurial space between axon-Schwann cell units contains collagen, fibroblasts, resident tissue macrophages and mast cells.

Neurofibromas are benign tumors that arise within peripheral nerve. Consistent with this origin, the cellular composition of a neurofibroma arising within a large nerve or nerve plexus (a plexiform neurofibroma) resembles a disordered version of the endoneurium (Fig. 2). These lesions contain large numbers of Schwann cell-like elements (referred to below as Schwann cells for simplicity’s sake; however, see Section 3.3 for a discussion of the origin of these cells) and fibroblasts that diffusely infiltrate
along the length of the nerve, separating and spreading apart entrapped axons. Large numbers of mast cells are also typically present in neurofibromas. This cellular composition, considered together with evidence indicating that Schwann cells are the neoplastic cell type within neurofibromas (see below), implies that the initial steps in neurofibroma formation occur within the endoneurium and that interactions with other cell types found in this microenvironment shape the course of tumor formation. Further, the early stages of neurofibroma growth are likely constrained and shaped by the perineurium.

In contrast, MPNSTs, the highly aggressive sarcomas that develop from plexiform neurofibromas, are overwhelmingly composed of cells with the morphologic, immunohistochemical (Fig. 3A, B) and ultrastructural characteristics of Schwann cells. Indeed, these observations, considered together with the observation that \textit{NF1} loss of heterozygosity (LOH) is found in Schwann cells but not other cell types intrinsic to neurofibromas, provide strong evidence that Schwann cells are the primary neoplastic cell type in both neurofibromas and MPNSTs. Interestingly, the conventional schwannomas arising in patients with schwannomatosis and NF2 (Fig. 3C) as well as the melanotic schwannomas occurring in Carney complex (Fig. 3D-F) are also composed of neoplastic Schwann cells. However, these benign lesions are composed almost exclusively of mature Schwann cells and lack other cellular components found in peripheral nerve. It is also exceedingly uncommon for schwannomas to undergo malignant progression. Considered together, these observations suggest that the mechanisms responsible for neurofibroma and MPNST pathogenesis are distinct from those driving the development of schwannomas.
Histologically, the GEM PNS tumors described below closely resemble their human counterparts. Neurofibromas from early GEM models (\(Nft^{\text{flo}};\) Krox20-Cre and \(Nft^{1+/-};\) \(Nft^{1-}\) chimeras; see section Initial Nfl Knockout Models) contain long spindle-shaped cells on a myxoid background with extensive mast cell infiltration and collagen deposition while lacking marked hypercellularity, nuclear atypia or frequent mitotic figures [15, 73, 97]. Staining for S100β, a marker of Schwann cells, was observed in some but not all of these tumors; electron microscopy was required to establish the presence of cells with morphologic features characteristic of Schwann cells in S100β-tumors. Based on these features, a panel of pathologists classified these tumors as GEM grade I neurofibromas [73]. Although not reviewed by this panel, the neurofibromas formed by the more recently developed GEM models discussed in this review appear to share these same histologic traits. Initial investigator-assigned designations of these GEM neurofibromas as plexiform, however, could not be confirmed by the panel [73].

This panel of pathologists was also cautious in their classification of the higher grade PNS lesions observed in early models (\(cis\)-linked \(Nft^{1+/-}/p53^{1+/-}\) mice). Although these GEM lesions share a number of histologic traits with human MPNSTs – including an association with a peripheral nerve or neurofibroma, high cellularity, brisk mitotic activity, nuclear pleomorphism and anaplasia – the panel recommended that they be classified as GEM grade III peripheral nerve sheath tumors (PNSTs) rather than as MPNSTs. The reason for this is that the panel felt that the term “malignant” was inappropriate due to a lack of information about the clinical course of these tumors at the time of their report [73]. While we recognize this point, we will refer to these GEM tumors as MPNSTs below for simplicity’s sake.
Clinical Characteristics of NF1 and Function of the Gene Mutated in this Disorder

NF1 is the most common genetic disease affecting the human nervous system, occurring in 1 in 3500 newborn infants. Manifestations of this autosomal dominant disease include learning disabilities, bony dysplasias, pigmentary lesions of the skin (café-au-lait macules, axillary freckling) and iris (Lisch nodules), and the development of a variety of tumor types (optic gliomas, glioblastomas, pheochromocytomas and juvenile myelomonocytic leukemia). As implied by the name of the disorder, however, neurofibromas are the hallmark lesion of NF1. It is widely recognized that distinct neurofibroma subtypes occur in NF1 patients and several classification schemes are currently in use for defining these subtypes. However, many investigators prefer to simply classify neurofibromas as dermal or plexiform variants as this has important clinical implications. Dermal neurofibromas arise in skin, typically developing in NF1 patients entering puberty or in women with NF1 that have become pregnant. Interestingly, dermal neurofibromas virtually never undergo malignant progression. In contrast, plexiform neurofibromas are often congenital. Individuals with plexiform neurofibromas also have an 8-13% lifetime risk that their tumors will progress to become MPNSTs, the most common malignancy developing in NF1 patients.

Linkage analyses were initially used to establish that the gene mutated in NF1 patients was localized to the long arm of human chromosome 17 [5, 70]. Transcription of this gene gives rise to a 13 kilobase mRNA [12, 85, 91] which encodes the 220 kDa (2,818 amino acid) tumor suppressor protein neurofibromin. Neurofibromin contains a domain homologous to the yeast GTPase activating proteins (GAPs) IRA1 and IRA2, which function as negative regulators of the yeast RAS1 and RAS2 proteins. As
predicted by this homology, neurofibromin’s GAP-related domain (GRD) stimulates the intrinsic GTPase activity of the mammalian Ras homologues, catalyzing the hydrolysis of Ras-GTP to Ras-GDP, which inactivates these small growth-promoting G-proteins (Fig. 4). In keeping with these \textit{in vitro} observations, Ras proteins are hyperactivated in nerve sheath tumors that have lost \textit{NF1}, and reintroduction of the NF1 GRD into tumor cells decreases Ras activation and slows tumor cell proliferation [4, 54, 91].

Although most studies of neurofibromin action have focused on its GRD domain, this domain is only a small portion of the protein (Fig. 4). Several other domains have been identified within neurofibromin, including a tubulin-binding domain (TBD) [7], a cysteine-serine-rich domain (CSRD) [32], a Sec14-homology domain (Sec14) [3], a pleckstrin homology domain (PH) [17] and a nuclear localization sequence (NLS) [79]. The TBD and CSRD may modulate neurofibromin’s ability to regulate Ras, as the GAP activity of neurofibromin is substantially reduced upon binding to tubulin [7] and is increased by CSRD phosphorylation [52]. At present, the function of the bipartite lipid binding and exchange motif created by the adjacent Sec14 and PH domains [17, 88] is unclear, as is the significance of the NLS. Neurofibromin also interacts with focal adhesion kinase (FAK) through a C-terminal region surrounding the NLS and can regulate FAK-mediated substrate adherence in serum deprived cells via an unknown mechanism [45]. Finally, neurofibromin modulates cAMP levels. Curiously, neurofibromin effects on cAMP levels are cell-type dependent, as neurofibromin loss elevates cAMP levels in Schwann cells [18, 38] and reduces them in astrocytes [20]. The mechanism responsible for these effects is unknown. However, since the neurofibromin
CSRD contains cAMP-dependent kinase (PKA) phosphorylation sites, it is reasonable to postulate that these differential effects result from interruption of a feedback loop.

**MOUSE MODELS OF PERIPHERAL NERVE SHEATH TUMORS**

*Initial Nf1 Knockout Models*

In 1994, the Copeland and Weinberg labs independently described knockout mice with null mutations of *Nf1* exon 31, a region that is often mutated in human NF1 patients. Both groups found that homozygous *Nf1^Δ31/Δ31* mice died by embryonic day 13.5 (E13.5) due to cardiac failure [8, 33]. Defects in renal, hepatic, and skeletal muscle development were also observed [8]. However, nervous system pathology was limited to enlargement of sympathetic ganglia secondary to neuronal hyperplasia [8] and, less commonly, exencephaly. The former phenomenon may reflect the prolonged proliferation [82] and enhanced neurotropin-independent survival [81] that occurs in embryonic sympathetic neurons following *Nf1* loss.

Interestingly, complete *Nf1* loss had quite different effects on Schwann cells. In keeping with neurofibromin’s role as a negative regulator of Ras, Ras activation was increased in cultured *Nf1^Δ31/Δ31* Schwann cells. As activated Ras generally promotes cell growth, the initial expectation was that these cells would show enhanced mitogenesis. However, *Nf1^Δ31/Δ31* Schwann cells instead demonstrated reduced proliferation in response to neuregulin-1 (NRG-1) stimulation and axonal contact [39], two classic Schwann cell mitogenic stimuli. It was thus proposed that Ras hyperactivation in these cells led to oncogene-induced senescence, similar to that previously observed when activated Ras mutants were introduced into Schwann cells in the absence of other
oncogenic signals [64]. However, it was unclear why NfI loss would produce senescence in Schwann cells but hyperplasia in sympathetic neurons, particularly given the absence of the latter finding in NF1 patients.

The phenotype of heterozygous NfIA31/+ mice only created more questions. Contrary to expectations, these mice did not develop neurofibromas, pigmentation defects, or Lisch nodules [8, 33]. However, approximately 15% of NfIA31/+ mice did develop adrenal tumors, many of which showed NfI LOH. These lesions were pheochromocytomas [33], a tumor type which is often observed in human NF1 patients but is very rare in wild-type mice. Interestingly, their pathogenesis was strain-specific, as the elevated pheochromocytoma incidence observed in NfIA31/+ mice on a mixed sv/129 x C57BL/6 genetic background disappeared when the NfIA31/+ allele was bred onto a sv/129 background [76]. Otherwise, the NfIA31/+ mice were indistinguishable from wild-type mice until after 12 months in age, when their survival declined sharply due to the development of a lymphomas, leukemias, lung adenocarcinomas, hepatomas, fibrosarcomas and adrenal tumors [33]. As these malignancies are also normally observed in older (>24 months) wild-type mice [9], this suggested that germline loss of a single NfI allele merely accelerated the development of tumors to which the mice were already predisposed.

One possible explanation for the lack of neurofibroma formation in the NfIA31/+ mice was that the acquisition of a second-hit mutation within one or more cell types in peripheral nerve was the rate limiting step for neurofibroma generation. To test this hypothesis, chimeric mice were generated by injecting NfI−/− embryonic stem cells into NfI+/− C57BL/6 blastocysts [15]. Although those animals with the highest degree of
chimerism died by one month of unknown causes, mice with an intermediate degree of chimerism developed multiple plexiform neurofibromas. Consistent with the hypothesis that \( Nf1 \) LOH in Schwann cells was required for neurofibroma development, the tumors were composed largely of \( Nf1^{+/} \) Schwann cells.

The presence of \( Nf1^{-/-} \) Schwann cells in the plexiform neurofibromas formed in the \( Nf1^{-/-};Nf1^{+/+} \) chimeric mice suggested that Schwann cell \( Nf1 \) LOH was required for neurofibroma generation. However, it did not establish that such LOH was sufficient for plexiform neurofibroma pathogenesis. To address that question, \( Nf1^{flox/flox} \) mice were bred to mice expressing Cre recombinase under the control of a Schwann cell-active promoter (\( Krox20-Cre \) mice) [97]. Peripheral nerves from these mice showed only mild Schwann cell hyperplasia with no evidence of neurofibroma formation, despite confirmation of Cre expression and \( Nf1 \) loss in Schwann cells from these animals. However, when conditional \( Nf1 \) ablation in Schwann cells occurred on an \( Nf1 \) heterozygous background (\( Nf1^{flox/-};Krox20-Cre \) mice), lesions with the histologic features of human neurofibromas developed in all of the animals by 1 year of age [97]. These findings painted a much more complex picture of the neurofibroma pathogenesis than had been previously appreciated. In particular, the discovery that \( Nf1 \) loss in Schwann cells only resulted in neurofibroma formation when all other cell types were \( Nf1 \) haploinsufficient suggested for the first time that the presence of susceptible non-neoplastic cell types in the tumor microenvironment was critical for neurofibroma development.
Mouse Models Probing the Role of the Tumor Microenvironment in Neurofibroma Formation

A key question that is still incompletely answered is precisely which cell types in the tumor microenvironment interact with neoplastic Schwann cells to promote neurofibroma pathogenesis. Although \( Nf1 \) haploinsufficiency in multiple cell types – including mast cells, fibroblasts and vascular elements – could contribute to this process, the most convincing work to date has focused on mast cells. These cells appear to function as critical intermediaries between \( Nf1^- \) Schwann cells and other \( Nf1^+ \) cell types in the microenvironment. \( Nf1^- \) Schwann cells secrete elevated levels of Kit ligand, a growth factor which activates the c-Kit membrane tyrosine kinase. \( Nf1^+ \) mast cells show increased c-Kit expression [19] and an enhanced chemotactic response to Kit ligand relative to wild-type mast cells [93], leading to increased recruitment of these cells into the nascent tumor. Kit ligand also induces enhanced activation, degranulation [14], and TGF-\( \beta \) secretion in \( Nf1^+ \) mast cells [92]. TGF-\( \beta \) in turn acts upon \( Nf1^+ \) fibroblasts and promotes increased production of collagen [92], a molecule which is found in abundance in neurofibromas.

An essential role for mast cells in neurofibroma formation has been elegantly demonstrated using bone marrow transplantation in knockout mice. As noted above, \( Nf1^{\text{lox/lox}};Krox20-Cre \) mice do not develop neurofibromas. However, when \( Nf1^{\text{lox/lox}};Krox20-Cre \) mice were lethally irradiated and transplanted with \( Nf1^+ \) bone marrow, they developed multiple plexiform neurofibromas that were infiltrated by donor mast cells [94]. In contrast, when donor marrow from \( Nf1^+ \) mice with hypoactive c-Kit receptors (\( Nf1^+; \text{c-Kit}^{W41/W41} \) mice) was used, no tumors formed, indicating that c-Kit signaling in bone marrow-derived elements was critical for neurofibroma formation.
Consistent with the hypothesis that \(Nf1^{+/--}\) mast cells are critically important for neurofibroma pathogenesis, no neurofibromas formed in lethally irradiated \(Nf1^{flox/-}\);\(Krox20\-cre\) mice transplanted with wild-type bone marrow, despite the presence of \(Nf1\) haploinsufficient fibroblasts and endothelial cells in the peripheral nerve [94]. Mast cell recruitment is also apparently important for the continued growth of existing neurofibromas. Treating eight-to-nine month old \(Nf1^{flox/-}\);\(Krox20\-Cre\) animals with established plexiform neurofibromas with 200mg/kg/day of the c-Kit inhibitor imatinib mesylate substantially reduced the volume of their dorsal root ganglia as well as mast cell recruitment and hypercellularity in nerve segments proximal to the dorsal root. This treatment also decreased proliferation and increased apoptosis within the plexiform neurofibromas [94]. Following this demonstration, 350mg/m\(^2\) imatinib mesylate was administered to a child with life-threatening airway compression produced by an unresectable plexiform neurofibroma. This treatment produced a 70% reduction in tumor volume [94], consistent with the hypothesis that recruitment of \(Nf1^{+/--}\) mast cells is critical for neurofibroma maintenance as well as formation.

Although it is clear that mast cell recruitment is essential for neurofibroma formation and that \(Nf1\) haploinsufficiency in other cell types cannot overcome this requirement, it remains to be determined whether \(Nf1\) haploinsufficiency in these other cell types promotes neurofibroma growth. It is also unclear what protumorigenic function(s) are performed by the recruited mast cells after their arrival in the nascent neurofibroma and whether these effects are directed at the neoplastic Schwann cells or other intratumoral cell types. At present, an entire series of protumorigenic interactions between the various
cell types composing a neurofibroma can be envisioned (Fig. 5). However, the existence and functional significance of most of these interactions remains to be determined.

**The Neurofibroma Cell-of-Origin Debate**

Although the neoplastic cells in plexiform neurofibromas clearly have schwannian characteristics, the initial Nf1 knockout models did not establish whether these neoplastic cells were derived from mature Schwann cells or a more primitive precursor (see Fig. 6 for an illustration of the stages of Schwann cell differentiation). To address this question, Nf1\(^{floxed}\) mice have been crossed to animals in which Cre expression was directed by promoters active at different stages in Schwann cell development. Elimination of Nf1 expression in neural crest cells, the earliest stage in Schwannian differentiation, was achieved by mating Nf1\(^{floxed}\) mice with Wnt1-Cre, Mpz-Cre, and Pax3-Cre animals. Although these mice had abnormal sympathetic ganglia and adrenal glands and died at birth, they did not develop neurofibromas [26]. Given the early death of these animals, it is conceivable that, had they survived, Nf1 ablation in neural crest cells would have ultimately resulted in the development of neurofibromas. However, Nf1\(^{floxed}\); Krox20-Cre mice do develop neurofibromas, and Krox20 is not expressed in neural crest cells, which argues that Nf1 loss in neural crest cells is not required for neurofibroma pathogenesis.

Mouse models in which Nf1 was ablated in Schwann cell precursors (SCPs; also known as neural crest stem cells) were more informative. For these experiments, 3.9Periostin-Cre (which is active in SCPs by E11) and P\(\gamma\alpha\)-Cre (expressed in SCPs beginning at E12.5) driver lines were created and bred to Nf1\(^{floxed}\) mice. While the majority of Nf1\(^{floxed}\); 3.9Periosin-Cre animals died by the 4\(^{th}\) postnatal week [35]
(possibly due to activation of the 3.9Periostin promoter in cardiac fibroblasts [61, 72]), \( Nf1^{flox-}; P_{\alpha}-Cre \) animals survived and formed neurofibromas by 15-20 months of age [35, 96]. Curiously, however, SCPs could not be isolated from the peripheral nerves of adult \( Nf1^{flox-}; P_{\alpha}-Cre \) mice, and SCPs from E13 \( Nf1^{-/-} \) mice did not generate tumors when transplanted into \( Nf1^{+/+} \) sciatic nerves [35]. Further, the proliferating cells in these neurofibromas were p75+, GFAP+ and BLBP- [35, 96], suggesting that mature non-myelinating Schwann cells rather than SCPs were the cell type giving rise to neurofibromas in this model. In keeping with this idea, hyperproliferative non-myelinating Schwann cells were found in the postnatal sciatic nerves of \( Nf1^{flox-}; P_{\alpha}-Cre \) mice prior to neurofibroma development [96].

However, this conclusion was inconsistent with the phenotype of a mouse model in which the Desert Hedgehog promoter drives Cre-mediated \( Nf1 \) ablation in SCPs at E12.5 (\( Nf1^{flox/flox}; Dhh-Cre \) mice) [89]. Unlike the tumors arising in \( Nf1^{flox-}; P_{\alpha}-Cre \) mice, neurofibromas developing in \( Nf1^{flox/flox}; Dhh-Cre \) mice contained numerous BLBP+ cells [89], suggesting that immature Schwann cells were the progenitors for these tumors. Interestingly, the development of neurofibromas in \( Nf1^{flox/flox}; Dhh-Cre \) mice occurred despite the presence of a wild-type \( Nf1 \) microenvironment; no evidence was found for Cre-mediated recombination in mast cells, endothelial cells or endoneurial fibroblasts [89], despite the fact that Dhh-expressing progenitors capable of differentiating into both Schwann cells and endoneurial fibroblasts have been found in peripheral nerve [36]. Given these contradictory results, it is not yet clear whether the neoplastic Schwann cells within plexiform neurofibromas are derived from mature nonmyelinating Schwann cells,
immature Schwann cells or both cell types. The possibility that these neoplastic Schwann cells arise from another source such as boundary cap cells also has not yet been ruled out.

Given the marked differences in the clinical behavior of dermal and plexiform neurofibromas, it is possible that the neoplastic cells in these neurofibroma subtypes are derived from distinct progenitors. Neural-crest derived precursor cells capable of both Schwannian and melanocytic differentiation, termed skin-derived precursors (SKPs) [46], are present in the dermis of adult mice. Consistent with the hypothesis that SKPs give rise to dermal neurofibromas, topical administration of tamoxifen to neonatal Nf1<sup>flac<sup>−</sup> ; CMV-CreERT<sup>2</sup>; Rosa26-LacZ(stop) mice results in dermal neurofibroma formation at the site of tamoxifen administration [46]. Further, SKPs isolated from these animals and treated <i>ex vivo</i> with tamoxifen to inactivate Nf1 were also capable of generating neurofibromas upon autologous subcutaneous transplantation into pregnant mice, indicating that these progenitors (and not other cell types residing in the dermis) were the cell of origin of the dermal neurofibromas.

Interestingly, Nf1<sup>−/−</sup> SKPs were also capable of forming plexiform neurofibromas when autografted into sciatic nerves (Le 2009). Thus, while SKPs residing in the dermis may be the cell of origin of dermal neurofibromas, these cells are apparently highly similar to the neurofibroma-initiating cells in peripheral nerve. Transcriptional profiling does not distinguish dermal and plexiform neurofibromas [56], which suggests that the distinct clinical behavior of these tumors primarily reflects differences in their microenvironment rather than their cell of origin. If this hypothesis is correct, the expansion of dermal neurofibromas during pregnancy, for example, may be due to hormonally-altered paracrine signaling from fibroblasts, melanocytes, or other cell types.
within the dermis feeding back on the \(Nf1^{+/−}\) progenitor cells rather than major differences in the \(Nf1^{+/−}\) progenitors themselves. This is consistent with the fact that pregnancy has a well-established effect on skin pigmentation and elasticity [77, 84].

However, if the cell of origin for dermal and plexiform neurofibromas is virtually identical save for their microenvironment niche, why do dermal neurofibromas not arise in the various \(Nf1^{\text{lox/−}}\) models discussed above? The experiments described above clearly demonstrate that mouse dermis is capable of giving rise to neurofibromas, so species-specific differences in skin susceptibility to neurofibroma formation are unlikely to explain this phenomenon. Clearly, more work is needed to understand these cells and their true relationship with the progenitor cells in the peripheral nerve.

*Mouse Models of Malignant Peripheral Nerve Sheath Tumor Pathogenesis*

Although most mouse models of NF1 developed to date have focused on plexiform neurofibroma pathogenesis, the transformation of these benign tumors into MPNSTs is of far greater concern to NF1 patients [55], especially given the lack of effective treatment options for these malignancies. However, modeling MPNST formation in mice has proven challenging, as the plexiform neurofibromas developing in mice with \(Nf1\) mutations only rarely progress to become MPNSTs. This may be due in part to the relatively short lifespan of mice, which could prevent murine \(Nf1^{+/−}\) Schwann cells from having adequate time to accumulate the additional tumor suppressor mutations driving MPNST pathogenesis. Alternatively, cells that acquire secondary mutations may persist in the latent phase of malignancy progression until the mice die from other causes.
Consequently, mice with null alleles of both *Nf1* and *p53* were generated to accelerate this process. As these genes are both located on mouse chromosome 11, tumorigenesis was compared in mice with mutant *Nf1* and *p53* alleles on opposite chromosomes (*trans Nf1*<sup>/+</sup>/<sup>+/p53</sup><sup>+/+</sup> mice) and in mice with mutant *Nf1* and *p53* alleles on the same copy of chromosome 11 (*cis-linked Nf1*<sup>/−</sup>/<sup>/−/p53</sup><sup>/−/−</sup> mice). Perhaps not surprisingly, these animals showed discordant phenotypes. *Trans Nf1*<sup>/+</sup>/<sup>+/p53</sup><sup>+/+</sup> animals died by 10 months of non-MPNST soft-tissue sarcoma types typically associated with *p53* loss of function. In contrast, 30% of the *cis*-linked *Nf1*<sup>/−</sup>/<sup>/−/p53</sup><sup>/−/−</sup> animals dying by 5 months of age were found to have MPNSTs [15]. Interestingly, these MPNSTs did not appear to arise in preexisting plexiform neurofibromas, suggesting that combined loss of *Nf1* and *p53* allowed MPNSTs to develop *de novo*.

MPNST formation also occurs in mice carrying a mutated *Nf1* gene in combination with other tumor suppressor mutations. In keeping with the observation that *CDKN2A* is commonly mutated in human MPNSTs [1, 6, 43, 58, 63, 67], 26% of *Nf1*<sup>+/−</sup> mice with simultaneous homozygous deletion of the *CDKN2A* locus (*Nf1*<sup>+/−</sup>; *p16<sup>ink4a</sup>-<sup>/−/p19<sup>arf</sup>-<sup>/−</sup>/−* mice) developed MPNSTs, while those with heterozygous deletions of both loci (*Nf1*<sup>+/−</sup>; *p16<sup>ink4a</sup>/−/p19<sup>arf</sup>-<sup>/−/−</sup> mice) developed MPNSTs at a much lower frequency [35]. As with MPNSTs arising in *cis*-linked *Nf1*<sup>/−</sup>/<sup>/−/p53</sup><sup>/−/−</sup> animals, the MPNSTs developing in *Nf1*<sup>+/−</sup>; *p16<sup>ink4a</sup>-<sup>/−/p19<sup>arf</sup>-<sup>/−/−</sup> mice did not appear to arise from a precursor neurofibroma. Neither *Nf1*<sup>/−</sup>; *p19<sup>arf</sup>-<sup>/−[41]</sup> nor *Nf1*<sup>/−</sup>; *p16<sup>ink4a</sup>-<sup>/−[35]</sup> animals showed a predisposition to MPNST development, indicating that deletion of both products encoded by the *CDKN2A* locus (and thus, dysregulation of both the *p53* and pRb pathways) is necessary to promote MPNST formation in the setting of *Nf1* heterozygosity.
The cell-of-origin debate was also revisited in MPNSTs derived from these models, as the absence of a benign precursor tumor raised the question of whether these malignancies were derived from a progenitor population distinct from that giving rise to neurofibromas. As at least some MPNST cells isolated from Nf1+/--; p16<sup>Ink4a−/-</sup>/p19<sup>Arf−/-</sup> and <i>cis</i>-linked Nf1+/--; p53<sup>+/−</sup> animals can grow as neurospheres capable of self-renewal [35], this work focused on the Schwann cell precursor stage of Schwannian development. However, SCPs did not show abnormal persistence or impaired differentiation in these animals and did not form tumors when transplanted into sciatic nerves. Moreover, MPNST cells from these animals, unlike SCPs, were not capable of differentiation along multiple lineages [35] and showed decreased levels of Sox10, a HMG-box factor critically important for maintaining the ability of precursor cells to give rise to glia and neurons [40].

Reduced Sox10 expression is also evident in human MPNSTs [47, 56, 57], together with a general downregulation of genes important for Schwannian differentiation [56]. At the same time, MPNSTs upregulate the expression of molecules characteristic of migrating neural crest cells such as Sox9 and Twist1 [56, 57]. In contrast, neurofibromas express a gene signature more characteristic of Schwann cell precursors or immature Schwann cells [56]. As human NF1-associated MPNSTs develop via malignant progression from neurofibromas, it is unlikely these differences in gene expression are due to neurofibromas and MPNSTs arising from distinct cell populations. Consequently, loss or suppression of Schwannian differentiation signals is apparently an important step in the progression to MPNSTs.
Neurofibroma and MPNST Formation in Conditional Ras Activation Mutants

As noted above, one of the best understood functions of neurofibromin is its ability to negatively regulate Ras action. What is not as widely appreciated is that neurofibromin regulates the activity of multiple Ras proteins from both the classic Ras (H-Ras, N-Ras, K-Ras) and R-Ras (R-Ras, R-Ras2/TC21, R-Ras3/M-Ras) families of small G-proteins. Further, it has not yet been established which Ras isoforms are expressed in neoplastic Schwann cells within human neurofibromas or MPNSTs or which of these molecules are critically important for tumorigenesis. However, mouse models in which constitutively active mutants of N-Ras or K-Ras are expressed in the Schwann cell lineage have been constructed for the purpose of determining whether this results in neurofibroma and/or MPNST pathogenesis.

Interestingly, distinct phenotypes were observed when activated N-Ras and K-Ras were expressed in the Schwann cell lineage. Mice expressing activated N-Ras in neural-crest derived cells (\(^{L_{SL}}Ne\)ras\(^{G12V/+}\); CAMK2-Cre) developed diffuse dermal neurofibromas [68] and hyperpigmented skin lesions similar to café-au-lait macules. Curiously, these skin lesions showed enhanced accumulation of pigment in melanocytes and large numbers of pigment-laden macrophages rather than an increase in melanocyte numbers [68], suggesting that pigment production rather than melanocyte proliferation was promoted by activated N-Ras. No plexiform neurofibromas or MPNSTs were observed in these animals.

In contrast, \(^{L_{SL}}Kr\)as\(^2B^{G12D/+}\); mGFAP-Cre mice did not develop tumors on a wild-type background. However, when bred onto a \(Pten^{\text{lox/lox}}\) background, they all developed multiple plexiform neurofibromas by 4 months of age [28]. Interestingly, neurofibroma
progression to MPNSTs, which was associated with loss of the remaining functional \textit{Pten} allele, also occurred in all animals by 7 months. This pattern of tumor incidence was not evident in \textit{Nf1}^{\text{flax/}} \textit{Pten}^{\text{flax/}}; \textit{mGFAP-Cre} mice, likely due to a requirement for LOH for both \textit{Nf1} and \textit{Pten} in the same cell; as these genes are located on separate mouse chromosomes, this is probably a highly uncommon event. Considered together, these findings suggest a role for PTEN in MPNST progression, consistent with previous reports that \textit{PTEN} deletion \cite{30} or silencing by promoter methylation \cite{37} is present in a subset of human MPNST samples.

So why does activation of N-Ras induce dermal neurofibroma formation directly, while plexiform neurofibroma generation in K-Ras2B-activated cells requires concomitant PTEN haploinsufficiency? One possible explanation is the Cre-driver lines used. \textit{CAMK2-Cre} mediates recombination in cells derived from the neural crest \cite{68}. Consequently, \textit{CAMK2-Cre} will activate expression of the mutated Ras allele earlier in development and in more cell types than will the \textit{mGFAP-Cre} driver, which is not active until the immature Schwann cell stage \cite{28}. This would increase the pool of cells capable of transformation, leading to more frequent development of neurofibromas in \textit{CAMK2-Cre} mice. Alternatively, cells at different stages in Schwannian development may differentially regulate the signaling pathways that are affected in these models. PTEN is a negative regulator of class I phosphoinositide-3-kinase (PI3K) signaling \cite{51}, a prosurvival cascade which can be activated by GTP-bound Ras \cite{42, 65, 66, 80, 90}. In many cell types, transformation by oncogenic Ras requires coordinate activation of PI3K and other Ras effectors such as Raf and RalGDS \cite{53, 83}. However, it is possible that Ras-induced transformation requires unrestrained PI3K activation in immature Schwann cells.
but not in neural crest cells and their immediate derivatives. Alternatively, cells at earlier developmental stages may downregulate PTEN, leading to heightened Ras-induced PI3K activation without the need for additional mutations. Finally, intrinsic differences in the ability of oncogenic K-Ras and N-Ras mutants to activate the PI3K pathway may also be responsible for the differential requirement for PTEN haploinsufficiency in these models. Although N-Ras and K-Ras activate PI3K signaling with approximately equal magnitude when overexpressed [29, 48, 65], studies with Ras molecules expressed at physiological levels indicate that K-Ras but not N-Ras is the major mediator of PI3K activation downstream of growth factors [49] and cytokines [95].

Manifestations of NF1 in Dysregulated Growth Factor Signaling Models

As noted above, when neurofibromin is lost in neoplastic Schwann cells, the rate of Ras inactivation is dramatically reduced, leading to accumulation of activated Ras. However, this begs the question of precisely what activates these Ras proteins in the first place. One likely possibility is that aberrant growth factor signaling performs this function in neoplastic Schwann cells. Indeed, aberrant expression of several growth factors and growth factor receptors has been identified in human neurofibromas and MPNSTs [11]. However, this hypothesis has been tested in transgenic mouse models only for the EGF receptor (EGFR, erbB1), the prototype of the erbB family of membrane tyrosine kinase receptors, and neuregulin-1 (NRG1), a growth factor that activates the other three members of the erbB kinase family (erbB2, erbB3, erbB4). Interestingly, these mouse models produced quite different outcomes.

A chemical carcinogenesis model of MPNST formation provided the first evidence implicating the erbB kinases in PNS tumor formation. Beginning more than four decades
ago, investigators noted that rats [23, 34], mice [2] and hamsters [10, 22] exposed in utero to the chemical carcinogen N-ethyl-N-nitrosourea (EtNU) developed peripheral nerve sheath tumors that satisfy modern diagnostic criteria for MPNSTs. These tumors frequently carried activating mutations of the ErbB2 (HER2, c-neu) membrane tyrosine kinase [59, 60]. Although erbB2 does not directly bind growth factors, it is the preferred heterodimerization partner for the other erbB receptors [27, 78] and facilitates ErbB heterodimer signal transduction [71]; mutated erbB2 can also homodimerize [69, 86, 87]. Thus, ErbB2 activation could trigger MPNST formation by dysregulating signaling pathways downstream of erbB2 homodimers, downstream of EGF or NRG1 or all three.

The potential importance of the observations in these rodent models was reinforced by the subsequent demonstration that amplification of ErbB2 [75] and EGFR [62, 63] occurs in at least some human MPNSTs.

To examine the impact of neuregulin signaling on PNS tumorigenesis, a transgenic mouse model was produced in which expression of the secreted NRG1 isoform GGFβ3 was directed by the Schwann cell-specific myelin protein zero (P0) promoter [31]. These animals developed Schwann cell hyperplasia that was evident by 1 month of age, followed by neurofibroma formation (unpublished observations) which progressed to MPNSTs by 6-10 months of age [31]. Importantly, this is one of only two reported transgenic models in which neurofibromas frequently progress to MPNSTs. Further, this malignant progression is associated with the mutation of additional tumor suppressor genes as is observed in human MPNSTs (unpublished data). Supporting the relevance of NRG signaling to human NF1-associated neurofibromas and MPNSTs, human neurofibromas, MPNSTs and MPNST cell lines express ErbB2, ErbB3, and/or ErbB4
together with multiple NRG-1 isoforms [74]. The erbB receptors expressed in these MPNST cell lines are constitutively activated and MPNST mitogenesis is profoundly inhibited by the pan-ErbB inhibitor PD168393 [74]. Further, stimulation of human MPNST cells with NRG-1β increases the migration and invasion of these cells [24].

The role of EGF receptor signaling in NF1-associated peripheral nerve sheath tumors has also been examined. EGFR is aberrantly expressed in many human neurofibromas and MPNSTs as shown by the fact that this molecule is not found in normal neonatal Schwann cells [21, 25]. In addition, the EGFR gene is amplified in a subset of human MPNSTs [62, 63] and EGF stimulation of serum-starved MPNST cells enhances their growth and survival [21]. To examine the contribution of this membrane tyrosine kinase to PNS tumorigenesis, transgenic mice were produced which over-expressed human EGFR in Schwann cells under the control of the CNPase promoter (CNP-hEGFR mice). These animals developed a hyperproliferative nerve phenotype with evidence of mast cell accumulation and fibrosis [50]. However, frank neurofibroma formation was exceedingly rare in these mice and was only observed at a very advanced age. Crossing CNP-hEGFR mice to Nf1+/− animals did not worsen the phenotype. However, Nf1+/− p53+/− mice did show enhanced survival on an EGFRwa-2 (EGFR hypomorphic mutation) background as compared to a wild-type background. As these animals develop many malignancies other than MPNSTs, it was unclear whether this increase in survival was due to a decrease in MPNST incidence or inhibition of the development of other tumor types that occur in these animals [50].

So why would NRG1 but not EGFR over-expression cause neurofibroma and MPNST formation? One possibility is that secreted NRG1 may affect multiple cell types
within the peripheral nerve, potentially invoking paracrine signaling analogous to that occurring in human neurofibromas, while EGFR over-expression has action that is limited to Schwann cells. Another possibility is that EGFR expression is primarily required for tumor progression rather than promoting the initial formation of neurofibromas. Finally, it is possible that different signaling pathways are activated downstream of the NRG1 and EGF receptors, allowing the former but not the latter to mimic \textit{Nf1} loss.

**SUMMARY**

The findings from the mouse models described above together with observations from human tumors suggest that neurofibroma pathogenesis and subsequent progression to become MPNSTs results from the accumulation of a series of molecular abnormalities (Fig. 7). In this scenario, the initial step in neurofibroma pathogenesis is loss of the remaining functional \textit{NF1} allele in a Schwann cell. It is likely that Ras surveillance mechanisms as well as additional tumor suppressors such as p53, p16\textsuperscript{NK4a}, p19\textsuperscript{Arf}, and pRb at least transiently maintain the \textit{NF1}\textsuperscript{+-} Schwann cell in a quiescent state, a suggestion which is consistent with the previous demonstration that senescent regions are present in neurofibromas [16]. Eventually, however, some unknown factor or confluence of factors stimulates a brief hyperproliferative period that is associated with paracrine recruitment of \textit{NF1}\textsuperscript{+-} mast cells and fibroblasts. Paracrine signaling during this period may also facilitate inactivation of the tumor suppressor proteins noted above via mechanisms that promote cellular growth in untransformed cells. As this would probably occur in individual cells or in small regions of the benign tumor, tumor expansion could still occur.
regionally while the bulk of the tumor was maintained in a growth arrested state. Loss or mutation of tumor suppressor genes such as \( p53 \), \( CDKN2A \) and \( Pten \) would allow the neoplastic Schwann cells to stably evade this senescence response, resulting in progression to MPNSTs. At this point, paracrine signaling would no longer be required to support the growth of the tumor, resulting in gradual overgrowth of the malignant cells until the untransformed mast cells and fibroblasts were no longer evident in the growing mass.

Although this scenario is consistent with the findings described in this review, it is clear that several key steps in this process remain poorly understood. Understanding these steps will require that we answer many questions such as how exactly how senescence fail-safes are evaded in neoplastic Schwann cells, what paracrine signaling molecules facilitate neurofibroma pathogenesis, what roles these paracrine signaling molecules play in this process and what additional mutations are responsible for neurofibroma-MPNST progression. Given the insights they have yielded thus far, new genetically engineered mouse models will undoubtedly play an important role in our efforts to answer these questions.

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Table 1. Genetically engineered mouse models of PNS neoplasia

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<td><strong>Early Models</strong></td>
<td></td>
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<tr>
<td>Nf1(^{Δ31/Δ31})</td>
<td>die by E13.5 due to cardiac failure</td>
<td>early death prevents observation of tumorigenic effects of Nf1 loss</td>
</tr>
<tr>
<td>Nf1(^{Δ31/+})</td>
<td>develop pheochromocytomas (15% incidence); show accelerated development of other non-NF1 tumors as compared to wild-type mice</td>
<td>no neurofibromas or MPNSTs observed</td>
</tr>
<tr>
<td>Nf1(^{-/-};Nf1^{+/-}) chimeras</td>
<td>multiple plexiform neurofibromas present in animals with intermediate level of chimerism</td>
<td>cannot control which cell types are Nf1(^{+/-}) and which are Nf1(^{-/-})</td>
</tr>
<tr>
<td>Nf1(^{flox/flox}); Krox20-Cre</td>
<td>Schwann cell hyperplasia</td>
<td>no neurofibromas or MPNSTs observed</td>
</tr>
<tr>
<td>Nf1(^{flox/-}; Krox20-Cre)</td>
<td>plexiform neurofibroma development by 1yr of age (demonstrating importance of both Nf1(^{+/-}) and Nf1(^{-/-}) cells in neurofibroma formation)</td>
<td>Krox20 promoter is expressed in Schwann cells and boundary cap cells, making it hard to identify a clear progenitor</td>
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<tr>
<th><strong>Tumor Microenvironment Models</strong></th>
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<tbody>
<tr>
<td>Nf1(^{flox/flox}); Krox20-Cre transplanted with Nf1(^{-/-}) bone marrow</td>
<td>developed plexiform neurofibromas infiltrated by donor mast cells (demonstrating importance of Nf1 haploinsufficiency in hematopoietic lineage for neurofibroma formation)</td>
<td>Other Nf1(^{+/-}) cell types within the hematopoietic lineage may contribute to neurofibroma development</td>
</tr>
<tr>
<td>Nf1(^{flox/flox}); Krox20-Cre transplanted with Nf1(^{-/-}); c-Kit(^{W41/W41}) bone marrow</td>
<td>no neurofibromas developed (demonstrating importance of c-Kit signaling in Nf1 haploinsufficient cells in the hematopoietic lineage)</td>
<td>Other Nf1(^{+/-}) cell types within the hematopoietic lineage may contribute to neurofibroma development</td>
</tr>
<tr>
<td>Nf1(^{flox/+}); Krox20-Cre transplanted with Nf1(^{+-/+}) bone marrow</td>
<td>no neurofibromas developed (demonstrating importance of Nf1 haploinsufficiency in hematopoietic lineage for neurofibroma formation)</td>
<td>Other Nf1(^{+/-}) cell types within the hematopoietic lineage may contribute to neurofibroma development</td>
</tr>
<tr>
<td>Nf1 ablation in Migrating Neural Crest</td>
<td>Nf1\textsuperscript{fluc/-}; Wnt1-Cre</td>
<td>died at birth; no neurofibromas developed</td>
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<td></td>
<td>Nf1\textsuperscript{fluc/-}; Mpz-Cre</td>
<td>died at birth; no neurofibromas developed</td>
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<tr>
<td></td>
<td>Nf1\textsuperscript{fluc/-}; Pax3-Cre</td>
<td>died at birth; no neurofibromas developed</td>
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<tr>
<th>Nf1 ablation in Schwann Cell Precursors</th>
<th>Nf1\textsuperscript{fluc/-}; 3.9Periostin-Cre</th>
<th>died by 4 weeks after birth; no neurofibroma development observed</th>
<th>early death prevents observation of tumorigenic effects of Nf1 loss</th>
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<tr>
<td></td>
<td>Nf1\textsuperscript{fluc/-}; P\textsubscript{0a}-Cre</td>
<td>plexiform neurofibroma formation observed by 15-20 months</td>
<td>due to broad P\textsubscript{0a} promoter expression in the Schwann cell lineage, a definitive cell of origin still could not be identified</td>
</tr>
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<td></td>
<td>Nf1\textsuperscript{fluc/-}; Dhh-Cre</td>
<td>plexiform and subcutaneous neurofibroma development (demonstrating that an Nf1\textsuperscript{+/-} microenvironment might not be strictly required for neurofibroma formation)</td>
<td>Dhh promoter expression in progenitor cells capable of differentiation into both Schwann cells and endoneurial fibroblasts</td>
</tr>
</tbody>
</table>

| Nf1 ablation in SKPs | Nf1\textsuperscript{fluc/-}; CMV-Cre\textsubscript{ERT2}; Rosa\textsubscript{26} | dermal neurofibromas generated ~6 months following topical tamoxifen administration | no MPNST formation |

<table>
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<tr>
<th>MPNST Formation Driven by Dual Tumor Suppressor Loss</th>
<th>\textit{trans}-linked Nf1\textsuperscript{+/-}; p53\textsuperscript{+/-}</th>
<th>developed non-MPNST sarcomas characteristic of p53 LOH</th>
<th>likely that ablation of Nf1 in non-SKP cells in dermis contributes to neurofibroma formation</th>
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<tr>
<td></td>
<td>\textit{cis}-linked Nf1\textsuperscript{+/-}; p53\textsuperscript{+/-}</td>
<td>developed MPNSTs (~30% incidence)</td>
<td>no neurofibroma precursor lesion</td>
</tr>
<tr>
<td>PNS Tumor Formation Driven by Ras Activation</td>
<td>Lsl^Nras^G12V/+; CAMK2-Cre</td>
<td>pigmented abnormalities of skin and dermal neurofibromas observed</td>
<td>expression of the CAMK2 promoter in the Schwann cell lineage has not been clearly defined</td>
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<tr>
<td></td>
<td>Lsl^Kras2B^G12D+; mGFAP-Cre</td>
<td>no obvious phenotype</td>
<td>no neurofibromas observed</td>
</tr>
<tr>
<td></td>
<td>Lsl^Kras2B^G12D++; Pten^flox/+; mGFAP-Cre</td>
<td>plexiform neurofibroma development by 4 months of age with progression to MPNSTs by 7 months</td>
<td>unclear how necessary Pten loss of function is to neurofibroma formation in the context of Nf1 loss</td>
</tr>
<tr>
<td>PNS Tumor Formation Driven by Dysregulated Growth Factor Signaling</td>
<td>P0-GGFβ3</td>
<td>neurofibroma formation with progression to MPNSTs by 6-10 months</td>
<td>not yet clear whether and how dysregulated NRG1 signaling interacts with neurofibromin loss</td>
</tr>
<tr>
<td></td>
<td>CNPase-EGFR</td>
<td>Schwann cell hyperplasia with mast cell recruitment and fibrosis</td>
<td>neurofibroma formation exceedingly rare at very advanced age; no MPNSTs observed</td>
</tr>
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**MPNST Formation Driven by Dual Tumor Suppressor Loss (Cont)**

| Nf1^+/− p16^Ink4a−/− | accelerated development of tumors characteristic of p16^Ink4a loss | no MPNST formation |
| Nf1^+/− p19^Arf−/− | accelerated development of tumors characteristic of p19^Arf loss | no MPNST formation |
| Nf1^+/− p16^Ink4a−/− p19^Arf−/− | developed MPNSTs (~30% incidence) | no neurofibroma precursor lesion |
Figure 1. Schematic illustrating the anatomy of normal peripheral nerve. Indicated are the outmost layer of nerve (the epineurium) and the vasa nervorum, the perineurium (which ensheathes bundles of nerve fibers and forms the blood-nerve barrier) and the endoneurium. The inset highlights the mixture of cell types present in the endoneurium including axons, myelinating and nonmyelinating Schwann cells, fibroblasts and mast cells.
Figure 2. Photomicrographs demonstrating the presence of multiple cell types in neurofibromas. Unlike other types of peripheral nerve sheath tumors, neurofibromas are composed of a complex mixture of multiple cell types normally present in peripheral nerve. (A) Hematoxylin and eosin stained section of a plexiform neurofibroma showing the typical loosely packed collection of spindled cells characteristic of these tumors. (B) Immunostains for S100β label the cytoplasm and nuclei of Schwann cell-like elements within a plexiform neurofibroma. (C) Axons, which are visualized here by their immunoreactivity for neurofilaments, are separated by neoplastic Schwann cells and other cellular elements recruited into the tumor. This demonstrates the infiltrative nature of these lesions. (D) Numerous mast cells, identifiable by their immunoreactivity for CD117 (c-Kit), are also present in this plexiform neurofibroma. Scale bars, 50 µm.
Figure 3. A comparison of the pathology of MPNSTs with that of conventional schwannomas and melanotic schwannomas, two other tumor types composed predominantly of neoplastic Schwann cells. (A) Hematoxylin and eosin stained section of an MPNST showing the high degree of cellularity and nuclear atypia characteristic of these neoplasms. The arrow indicates a mitotic figure. (B) Unlike plexiform neurofibromas, the benign precursors from which they arise, MPNSTs are overwhelmingly composed of neoplastic cells which in this panel are highlighted by their S100β immunoreactivity. (C) In contrast, this schwannoma resected from the VIIIth cranial nerve of an NF2 patient shows a lower degree of cellularity and relatively uniform “cigar-shaped” tumor cell nuclei. (D) Hematoxylin and eosin stained section of a melanotic schwannoma, a tumor type associated with Carney complex. Note the abundant deposits of brown pigment. (E) A Fontana stain highlights the melanin in a melanotic schwannoma as black deposits. (F) Unlike conventional schwannomas, melanotic schwannomas express antigens characteristic of melanocytes and melanomas. Shown is an immunostain for the melanoma marker HMB45 in this melanotic schwannoma. Magnification: A, C, D, E and F; 40x; B, 20x.
**Figure 4. Schematic illustrating key subdomains within neurofibromin, the tumor suppressor protein encoded by the NF1 gene.** Subdomains are indicated as follows: CSRD, cysteine/serine-rich domain; TBD, tubulin-binding domain; GRD, GAP-related domain; Sec14/PH, Sec14-homologous domain and pleckstrin homology domain; NLS, nuclear localization sequence. Numbers above each subdomain indicate the positions of the corresponding amino acids within the 2818 amino acid length of the neurofibromin protein. The amino acid position of the TBD is not indicated, as this is an 80 amino acid region found within the N-terminal GRD domain.
Figure 5. Schematic illustrating established and potential interactions between NF1<sup>−/−</sup> Schwann cells and other NF1 haploinsufficient cell types intrinsic to peripheral nerve. Established interactions are depicted with solid black arrows and font, while potential interactions are depicted with gray dashed arrows labeled with question marks. Established interactions include the elevated secretion of Kit ligand from NF1<sup>+/−</sup> Schwann cells, which is further increased upon loss of the remaining NF1 allele and which acts as a chemoattractant and activating factor for NF1<sup>+/−</sup> mast cells. Activated NF1<sup>+/−</sup> mast cells have also been shown to secrete elevated levels of TGFβ, which stimulates increased collagen deposition from NF1<sup>+/−</sup> fibroblasts.
Figure 6. Schwann cell development. Neural crest cells give rise to a series of cell types in the Schwann cell lineage, one or more of which can become a neurofibroma initiating cell (NIC) following biallelic NF1 loss. Plexiform NICs are thought to be derived from Schwann cell precursors or their more differentiated progeny in deep peripheral nerves; alternative origins such as boundary cap cell or satellite cells have also been proposed. Another progenitor population arising from the neural crest has been located in the dermis, and dermal NICs may arise instead from these skin-derived precursors (SKPs) or their progeny. Times indicated are the embryonic days (E) at which specific Schwann cell precursors appear in the mouse. Listed below each cell type are useful markers of specific developmental stages and promoters which are active at these stages (see text for further discussion). Note that promoters used to drive Cre-mediated recombination are often fragments of whole gene promoters and may have a separate designation (i.e. P₀α is a 1.1kb fragment of the P₀ promoter, and 3.9Periostin is a 3.9kb fragment of the periostin promoter).
Figure 7. Major events in the pathogenesis of a neurofibroma and its subsequent progression to become a MPNST. Illustrated are the changes leading to neurofibroma and MPNST development in GEM models of NF1. Transient hyperplasia is often observed in the peripheral nerves of these animals prior to neurofibroma development; it is unclear whether an \textit{Nf1\textsuperscript{+/+}} background is sufficient to generate this phenotype or whether biallelic loss of \textit{Nf1} in Schwann cells must also occur. Neurofibroma development, however, does depend upon biallelic loss of \textit{Nf1} in Schwann cells or their less differentiated precursors. Loss of the remaining functional \textit{Nf1} allele in the Schwann cell lineage triggers elaboration of paracrine signaling molecules that recruit other \textit{Nf1\textsuperscript{+/+}} cell types (including mast cells, fibroblasts, and other Schwann cells) from the peripheral nerve to the nascent tumor site. As not all \textit{Nf1\textsuperscript{+/+}} Schwann cells generate neurofibromas, there are likely other as-yet-undetermined events that must occur to the \textit{Nf1\textsuperscript{+/+}} Schwann cell or its microenvironment to trigger tumor formation. Once the neurofibroma has been established, additional mutations in tumor suppressor genes such as \textit{TP53} and \textit{p16\textsuperscript{INK4a}/p19\textsuperscript{ARF}} in \textit{Nf1\textsuperscript{+/+}} Schwann cells drive malignant progression. Comparisons with human neurofibromas and MPNSTs indicate that most of these events are relevant to the pathogenesis of the human counterparts of these murine tumors. However, the relevance of some changes seen in the murine tumor models (e.g., the initial phase of intraneural hyperplasia) remains to be determined. It should also be noted that other events associated with malignant progression in human MPNSTs (e.g., suppression of Sox10 expression and upregulation of Sox9 expression, altered epigenetic modification or miRNA regulation programs) have not yet been thoroughly explored in genetically engineered mouse models of peripheral nerve sheath tumors.
CLASSIC RAS AND R-RAS SUBFAMILY PROTEINS ELICIT DISTINCT PHYSIOLOGICAL EFFECTS AND PHOSPHOPROTEOMIC ALTERATIONS IN NEUROFIBROMIN-NULL TUMOR CELLS

by

NICOLE M. BROSSIER, STEPHEN BARNES, LANDON S. WILSON, STEPHANIE J. BYER, STEPHANIE N. BROSIUS, STEVEN L. CARROLL

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ABSTRACT

Inactivating mutations of the neurofibromatosis type 1 (NF1) gene and the resulting loss of neurofibromin promote the development of multiple tumor types in NF1 patients. Neurofibromin loss can also promote the development of many sporadic neoplasms. Hypothetically, small G-proteins from both the classic Ras (H-Ras, N-Ras, K-Ras) and the R-Ras (R-Ras, R-Ras2/TC21, R-Ras3/M-Ras) subfamilies can be activated following neurofibromin loss. However, the relative contributions these Ras subfamilies make to tumorigenesis are poorly understood, as are the signaling pathways mediating these responses. We have found that all of these Ras proteins (with the exception of M-Ras), are expressed and can be activated in neurofibromin-null malignant peripheral nerve sheath tumor (MPNST) cells. Dominant negative (DN) H-Ras and R-Ras mutants, which act as pan-inhibitors of their respective Ras subfamilies, both inhibit MPNST mitogenesis. However, only DN H-Ras induces MPNST cell apoptosis, while only DN R-Ras inhibits the migration of these lines. We subsequently utilized mass spectrometry to identify phosphoproteome changes downstream of these two mutants to determine which signaling pathways were responsible for these differential effects.

INTRODUCTION

The Ras family of small GTPases consists of three proto-oncogenes – H-Ras, N-Ras, and K-Ras – which share >85% sequence homology, leading to significant overlap in their function. These proteins are activated by GTP binding, a process catalyzed by guanine nucleotide exchange factors (GEFs) downstream of various stimuli (9, 20, 56) including extracellular mitogens. The duration of Ras activation is limited by GTPase
activating proteins (GAPs), which stimulate the intrinsic GTPase activity of Ras, triggering hydrolysis of GTP to GDP and subsequent Ras inactivation (71, 72). Under these controlled conditions, Ras proteins can mediate various phenotypic responses, including proliferation, survival, migration and differentiation, depending upon the cellular context. Activating mutations in Ras proteins disrupt this control by preventing GTP hydrolysis, thus decoupling Ras activation of downstream signaling cascades from appropriate upstream regulation. Ultimately, these changes can lead to cellular transformation, tumorigenesis and/or malignant progression.

Aberrant Ras activation also occurs following loss of the Ras GAP-related protein neurofibromin, the gene product of the neurofibromatosis type I (NF1) gene. Somatic mutation of NF1 or inappropriate degradation of the neurofibromin protein can contribute to tumorigenesis in sporadically occurring neoplasms such as glioblastomas (1, 10, 46), ovarian adenocarcinomas (2) and adult acute myelogenous leukemia (60). Furthermore, patients with germline mutations of the NF1 gene are prone to develop multiple types of tumors, including both benign and malignant tumors of the peripheral nerve sheath (11, 14). The Ras activation observed in these neoplasms differs from Ras activation produced by an activating mutation in one critical regard. As neurofibromin negatively regulates the activity of all three classic Ras proteins (5, 43, 77), neurofibromin loss can potentially result in the simultaneous activation of H-, N- and K-Ras.

Neurofibromin also inactivates proteins in the R-Ras subfamily (R-Ras, R-Ras2/TC21 and R-Ras3/M-Ras) of small G-proteins (52). It is likely that inappropriate activation of R-Ras subfamily members following neurofibromin loss also contributes to tumorigenesis, as constitutively active R-Ras subfamily mutants induce transformation in
vitro (18, 27, 61) and oncogenic mutations in R-Ras2 have been identified in human neoplasms (7, 16, 33). However, little is known regarding the signaling pathways and cellular functions regulated by hyperactivated R-Ras subfamily members in neurofibromin-null neoplasms. Further, much of the information that is available regarding the signaling pathways downstream of R-Ras subfamily proteins has been obtained by over-expressing constitutively active mutants; the interpretation of such experiments is complicated as Ras protein overexpression can lead to non-physiologic effector activation (31, 39) and phenotypic effects (30).

We hypothesized that hyperactivated R-Ras subfamily members promote the pathogenesis of neurofibromin-null tumors by eliciting physiologic effects distinct from those controlled by classic Ras proteins. We further hypothesized that these distinct physiologic effects reflects the regulation of distinct cytoplasmic signaling pathways downstream of classic Ras and R-Ras subfamily proteins. We tested these postulates using cells derived from malignant peripheral nerve sheath tumors (MPNSTs; aggressive Schwann cell-derived sarcomas that occur commonly in NF1 patients) as a model. We first determined which specific classic Ras proteins and R-Ras proteins were expressed and could be activated in these lines. Establishing the functional role of specific Ras proteins by ablating them individually was potentially problematic as other members of the same subfamily might compensate for such losses. Consequently, we next introduced dominant negative H-Ras and R-Ras mutants, which act as pan-inhibitors of their respective subfamilies, into MPNST cells and determined whether these mutants differentially affected proliferation, migration, and survival. Mass spectrometry-based
phosphoproteome analyses was then used to identify key signaling pathways potentially responsible for the differential effects of each dominant negative Ras mutant.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies directed against H-Ras (sc-520), N-Ras (sc-31) and the HA epitope (sc-805) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal antibody directed against K-Ras (MCA3223Z) was obtained from AbD Serotec (Kidlington, UK), and an R-Ras (H00006237_M01) mouse monoclonal antibody was purchased from Abnova (Walnut, CA). R-Ras2/TC21 antibodies were obtained from Santa Cruz (sc-833, sc-81931, and sc-166262), Abnova (H00022800-M01), Abcam (Cambridge, MA; ab96307) and R&D Systems (Minneapolis, MN; AF3605). Mouse monoclonal antibodies directed against GFP (G1546), GAPDH (RDI-TRK5G4-6C5) and the Myc epitope tag (#2276) were purchased from Sigma-Aldrich (St. Louis, MO), Fitzgerald Industries International (Concord, MA) and Cell Signaling Technology (Beverly, MA), respectively. Antibodies against phosphorylated forms of Erk1/2Thr202/Tyr204 (#9101), AktThr308 (#9275) and JNKThr183/Tyr185 (#9251) were obtained from Cell Signaling Technology. HRP-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

MPNST Cell Lines

We have previously described the source of the human MPNST cell lines used in this study (12, 66). All MPNST lines were maintained in Dulbecco’s Modification of Eagle’s
Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin and 10μg/ml streptomycin (DMEM-10). We routinely assessed the morphology and doubling times of all of these cell lines. Cells were also routinely tested for *Mycoplasma* infection. Karyotypic analysis of MPNST cells revealed only human chromosomes, eliminating the possibility of contamination with non-human cell lines.

**Immunoblotting**

Human MPNST lines were homogenized in HES buffer (40mM HEPES, 2mM EDTA, 500mM sucrose) containing protease inhibitor cocktail (Sigma #P8340, 1:100 dilution). Protein concentrations were measured using the DC Assay kit (Bio-Rad; Hercules, CA) per the manufacturer’s recommendations. Equal quantities of protein were resolved on 12% SDS polyacrylamide gels. Western blotting was performed as previously described (67). Antibodies were diluted in 1% nonfat dry milk in TBST [0.15M NaCl, 10mM Tris (pH 8.0), 0.05% Tween-20]. Blots were reprobed with an antibody directed against GAPDH to verify equal loading. Immunoreactive species were detected using SuperSignal Pico Chemiluminescence kit (Thermo Scientific; Rockford, IL).

**Conventional and Quantitative PCR**

RNA from MPNST cell lines was isolated using the Trizol reagent per manufacturer’s recommendations (Invitrogen; Carlsbad, CA). Oligo-dT primers (Invitrogen) were used with 2μg RNA template to create cDNA for conventional PCR, while random primers were used to create cDNA for real-time PCR. Conventional PCR was performed as
previously described (13) using primers designed from reference sequences for each GEF (Supplementary Table 1). To conduct real-time PCR, 2μl of cDNA template was mixed with 10μl of TaqMan 2x PCR Master Mix and 1μl TaqMan primers (Applied Biosystems; Carlsbad, CA) in a 20μl volume. Each reaction was run in triplicate. Amplification was monitored on the Applied Biosystems 7500 Real-Time PCR System and analyzed using Applied Biosystems Sequence Detection Software (version 1.4).

**Ras Dominant Negative Mutants and Epitope-Tagged Ras Expression Plasmids**

Plasmids expressing HA-epitope tagged dominant negative mutants of human H-Ras (S17N, #RASH00TND0) and R-Ras (S43N, #RASR00TND0) were obtained from the Missouri S&T cDNA Resource Center (Rolla, MO). Inserts from these plasmids were cloned into the *Kpn*I and *Eco*RV sites of the bidirectional doxycycline-inducible vector pBIG2i (68) to produce doxycycline-inducible DN H-Ras (pSLC703) and DN R-Ras (pSLC658) plasmids. The pEGFP-N1 plasmid was obtained from Clontech (Mountain View, CA), and the insert from this plasmid was cloned into *Bam*HI-*Not*I digested pBIG2i to yield a doxycycline-inducible GFP control plasmid (pSLC460).

Plasmids expressing Myc-tagged wild-type H-Ras (#RASH00MN00) and K-Ras (#RASK20MN00) and untagged wild-type N-Ras (#RASN000000), R-Ras (RASR000000), and R-Ras2/TC21 (TC21000000) were also obtained from the Missouri S&T cDNA Resource Center. Three single-nucleotide deletions observed in the latter plasmid were corrected using the QuikChange II Site-Directed Mutagenesis kit (Stratagene; Santa Clara, CA). Untagged wild-type sequences were PCR amplified using primers containing *Not*I or *Xho*I sites and the resulting PCR products cloned into pCR4-
Topo (Invitrogen; Carlsbad, CA). The resulting plasmids were digested with NotI and XhoI and the released inserts cloned into a NotI/XhoI digested Myc-tagged plasmid backbone to create Myc-tagged Ras expression vectors for N-Ras (pSLC816), R-Ras (pSLC891) and R-Ras2 (pSLC901).

**Ras Activation Assays**

Human MPNST lines transiently transfected with Myc-tagged wild-type Ras plasmids or Myc-tagged DN H-Ras control were lysed in magnesium-containing lysis buffer [25mM HEPES (pH 7.5), 150mM NaCl, 10mM MgCl$_2$, 1mM EDTA, 1% Igepal CA-630, 10% glycerol] supplemented with protease inhibitor (Sigma #P8340, 1:100 dilution) and HALT phosphatase inhibitor (Thermo Scientific #78420, 1:100 dilution) cocktails. Lysates were clarified by centrifugation at 14,000rpm for 10 minutes, and protein concentrations then measured using the DC Assay kit (Bio-Rad; Hercules, CA) per the manufacturer’s recommendations. Lysates were diluted to 0.5mg/ml protein. 500µl of this diluted protein was mixed with 20µl Ras Assay Reagent (Raf-1 Ras-binding domain agarose beads; Millipore, Lake Billerica, MA) and incubated for 30 minutes at 4°C on a rotary shaker. Cells were washed three times in magnesium-containing lysis buffer containing protease and phosphatase inhibitors. Following the final wash, Ras Assay Reagent beads were collected by centrifugation at 14,000 rpm for 10s. Beads were boiled for 15 minutes in 40µl 2x Stop Buffer (250mM Tris-HCl (pH 6.8), 5mM EDTA, 5mM EGTA, 2% SDS, 10% glycerol, 25mM dithiothreitol, 300µM bromophenol blue) prior to loading on 12% SDS-PAGE gels. Clarified lysate samples were run in parallel on 12% SDS-PAGE gels. Immunoblotting was performed as described above.
Transient and Stable Transfection of MPNST Cell Lines

Transient transfections of MPNST cells were performed in serum-free DMEM using Fugene 6 Transfection Reagent (Roche; Indianapolis, IN) as recommended by the manufacturer. To create stable cell lines containing doxycycline-inducible dominant negative Ras plasmids, transfected cells were split into tetracycline-free DMEM-10 supplemented with hygromycin (50-100μg/ml, empirically determined for each line). Clones were picked after two weeks and screened by immunoblotting for the HA-epitope tag; appropriate clones expressed this epitope when cultured in media supplemented with 2μg/ml doxycycline, but not when maintained in tetracycline-free media.

Cell lines stably transfected with doxycycline-inducible vectors were maintained in tetracycline-free DMEM-10 (DMEM, 10% tetracycline-free FBS, 10 U/ml penicillin, 10 μg/ml streptomycin) supplemented with the optimal concentration of hygromycin.

3H-Thymidine Proliferation Assays

Cells stably transfected with doxycycline-inducible vectors were detached, resuspended in tetracycline-free DMEM-10 with or without 2μg/ml doxycycline and then plated at 5,000 cells/well in 48-well plates. 48 hours post-plating, 0.5μCi of 3H-thymidine was added to each well. 24 hours later, cells were washed with ice-cold phosphate buffered saline (PBS) and then incubated in 10% trichloroacetic acid (TCA) for 1 hour on ice. Following additional PBS washes, cells were incubated in 0.3N NaOH for 1 hour at room temperature. Equal volumes of solution from each well were counted via scintillation, with 16 replicates counted per condition. Data was analyzed using Student’s t-test, with p-values <0.05 considered statistically significant.
Transwell Migration Assays

Cell lines stably transfected with doxycycline-inducible vectors were cultured for 48 hours in tetracycline-free DMEM-10 with or without 2μg/ml doxycycline to induce expression. Cells were then serum-starved in Schwann cell-defined medium (22, 23) with or without 2μg/ml doxycycline for an additional 24 hours. At the end of this starvation, cells were resuspended (100,000 cells/ml) in Migration Assay Buffer (MAB; DMEM supplemented with 0.1% fatty-acid free BSA) ±2μg/ml doxycycline. 40,000 cells were plated on Transwell filter inserts with 8μm pores (Becton Dickinson Labware; Franklin Lakes, NJ) that had been coated with poly-L-lysine and laminin. Cells were allowed to migrate for 6 hours, after which they were fixed and stained with crystal violet. Cells migrating to the filter undersurface were counted using a 20x objective, with 9 fields counted per filter. Three replicate filters were performed for each condition. Data was analyzed using Student’s t-test, with p-values <0.05 considered statistically significant.

Cell Viability (Calcein) Assays

MPNST lines stably transfected with doxycycline-inducible plasmids were plated at 20,000 cells per well in DMEM-10 overnight and then cultured in either DMEM-10 or DMEM alone for an additional 24 hours in the presence or absence of 2μg/ml doxycycline. After two rinses in Hanks’ Balanced Salt Solution (HBSS), cells were incubated for 30 minutes at room temperature in 200μl of 4ng/ml calcein acetomethoxy (AM) diluted in HBSS. Signals were measured using a fluorescent plate reader.
Mass Spectrometric Phosphoproteome Analysis

The Invitrogen SILAC (stable-isotopic labeling using amino acids in culture) Protein and Quantitation Media Kit with DMEM-flex (MS10030) was used to label MPNST cells stably transfected with doxycycline-inducible dominant negative Ras mutants with light ($^{12}$C$_6$) or heavy ($^{13}$C$_6$) L-lysine HCl. Cells were maintained in log-phase growth in SILAC Light or Heavy medium for five days under doxycycline-free conditions. 2µg/ml doxycycline was then added to Heavy-labeled cells only, and both Heavy and Light-labeled cells were cultured for an additional 48 hours. Phosphoproteins were then isolated using the Pierce Phosphoprotein Enrichment Kit (90003). In brief, cells were lysed in the provided lysis buffer supplemented with 1% CHAPS, 1:100 Sigma protease inhibitor cocktail (#P8340) and 1:100 HALT phosphatase inhibitor cocktail (Thermo Scientific #78420). Protein concentrations were determined and equal quantities of protein mixed together and incubated in the provided phosphoprotein enrichment column. The column was washed three times with lysis buffer. The provided elution buffer was then used to detach bound phosphoproteins from the Ga$^{2+}$ ion-metal affinity chromatography (IMAC column). Eluted fractions were concentrated using the provided ICON concentrator and then resolved by SDS-PAGE. Gels were stained with GelCode Blue Stain Reagent (Thermo Scientific) to visualize proteins bands. Bands were excised and destained overnight with a 1:1 mix of 100mM ammonium bicarbonate and acetonitrile. Following destaining, bands were incubated with 25mM dithiothreitol at 50$^\circ$C for 30 minutes to reduce disulfide bonds and then incubated with 55mM iodoacetamide for 30 minutes in the dark to alkylate free thiol groups. Bands were washed twice with 100mM ammonium bicarbonate for 30 min and then dried in a
SpeedVac (Savant). Trypsin digestion was performed overnight at 37°C using Promega (Madison, WI) Gold Mass Spectrometry Grade Trypsin at a final concentration of 12.5ng/µl. A 1:1 mixture of 5% formic acid and 50% aqueous acetonitrile was incubated with digested bands twice for 15 minutes to extract peptides. Peptides were pooled, dried, and then resuspended in 20µl of 0.1% formic acid prior to mass spectrometry analysis.

A 2µl aliquot of each digest was loaded at 2µl/min onto a 2 cm x 75 µm i.d. PepMap100 C<sub>18</sub> reverse-phase trap cartridge (Dionex, Sunnyvale, CA) using an Eksigent autosampler. The cartridge was washed for 4 minutes with 0.1% formic acid in ddH<sub>2</sub>O, after which the bound peptides were flushed onto a 15 cm x 75 µm i.d. PepMap100 C<sub>18</sub> reverse-phase analytical column (Dionex, Sunnyvale, CA) with a 40 minute linear (5-50%) acetonitrile gradient in 0.1% formic acid at 300 nl/min using an Eksigent Nano1D+ LC (Dublin, CA). The column was washed with 90% acetonitrile-0.1% formic acid for 15 minutes and then re-equilibrated with 5% acetonitrile-0.1% formic acid for 30 minutes. The protein digest was analyzed using an Applied Biosystems 5600 Triple-ToF mass spectrometer (AB-Sciex, Toronto, Canada). The IonSpray voltage was 2300 V and the declustering potential was 60 V. Ionspray and curtain gases were set at 10 psi and 20 psi, respectively. The interface heater temperature was 120ºC. Eluted peptides were subjected to a time-of-flight survey scan from 400-1250 m/z to determine the top twenty most intense ions for MS/MS analysis. Product ion time-of-flight scans at 50 msec were carried out to obtain the tandem mass spectra of the selected parent ions over the range from m/z 400-2000. Spectra were centroided and de-isotoped using Analyst software (Applied Biosystems; version TF). A β-galactosidase trypsin digest was used to establish
and confirm the mass accuracy of the mass spectrometer. ProteinPilot Software version 4.0 (Applied Biosystems) was used to determine the relative abundance of heavy versus light-labeled proteins from tryptic peptide spectra using the Paragon algorithm. Results were manually bias corrected in ProteinPilot, using the median H:L ratio of all proteins in the data set for a given experiment (after removing all contaminating proteins, reversed proteins, and proteins with only one quantifiable peptide) as a correction factor. Reported proteins were identified with ≥99% confidence by ProteinPilot (unused score ≥2) with a false discovery rate <1%. Network analyses were performed with Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) using only those proteins in which the |log₂ (heavy-to-light ratio)| >0.33. Fisher’s exact test was used to determine the probability that the changes in the networked proteins could occur by random chance. The –log of this p-value is reported as the IPA score.

RESULTS

Multiple Classic Ras and R-Ras Subfamily Proteins Are Expressed and Activated in Human MPNST Cells

To establish which proteins in the classic Ras and R-Ras subfamilies potentially contribute to MPNST pathogenesis, we examined the expression of all six neurofibromin-regulated Ras isoforms in a panel of eight human MPNST cell lines. Six of these lines were derived from neoplasms arising in NF1 patients (ST88-14, 90-8, NMS2, NMS2-PC, S462, T265-2c), while the other two originated from sporadic MPNSTs (STS-26T, YST-1). Antibody specificity was assessed by immunoblotting lysates of MPNST cells transiently transfected with Myc-tagged wild-type Ras isoforms (Figure 1A-B). Using this approach, we established that our antibodies against H-Ras, N-Ras, K-Ras and R-Ras
did not cross-react with other Ras proteins and were specific for their intended antigens. While none of the six antibodies for R-Ras2 that we tested specifically recognized only R-Ras2 (Figure 1B), we did identify an antibody that recognized both R-Ras and R-Ras2 but did not cross-react with H-Ras, N-Ras or K-Ras. As M-Ras mRNA was only detectable in a single MPNST cell line (see below), we did not assess the specificity of an M-Ras antibody.

With this panel of antibodies, we found that H-Ras, K-Ras, and N-Ras were present in all eight MPNST cell lines (Figure 2A). R-Ras expression was more variable, being found in five of the eight lines. The antibody recognizing both R-Ras and R-Ras2 labeled bands of the appropriate molecular weight in all 8 lines, with more intense bands evident in cell lines with demonstrated R-Ras expression (Figure 2A). To determine whether this change in intensity reflected the presence of both R-Ras and R-Ras2, we performed real-time PCR assays for R-Ras and R-Ras2 transcripts in this panel of MPNST cell lines. Although there was some variability in the levels of R-Ras2 transcripts detected, all eight lines showed expression that was greater than that seen in a normal human cerebellum control (Figure 2B). Levels of R-Ras mRNA were more consistent in the MPNST cell lines. Curiously, however, R-Ras transcripts were detected at similar levels in all eight MPNST cell lines, including the ones that lacked detectable R-Ras protein (YST-1, 90-8, NMS2); in addition, we noted that R-Ras protein was present in NMS2-PC cells, which were derived from a metastasis of the primary neoplasm used to establish the R-Ras deficient NMS2 line. M-Ras protein could not be detected by immunoblotting, and M-Ras mRNA was only present in one of the eight lines examined (Figure 2C). Considered together, these findings indicate that all eight MPNST
cell lines express H-Ras, K-Ras, N-Ras and R-Ras2, with five of them also expressing R-Ras protein. Our comparison of R-Ras protein and mRNA expression suggests that the lack of R-Ras protein in some lines might be due to a post-transcriptional mechanism that impedes R-Ras protein accumulation.

Ras proteins are activated upon binding GTP, a process that is catalyzed by guanine nucleotide exchange factors (GEFs) which are themselves activated by various stimuli (9, 20, 56) including extracellular mitogens. To ensure that each of the Ras isoforms expressed in MPNST cells could be activated, we examined the expression of the ten known Ras GEFs in our panel of cells. Transcripts encoding nine of these ten GEFs were detected in at least some MPNST cell lines (Figure 3A). Five of the GEFs – Sos1, Sos2, RasGRP3, BCAR3 and C3G – were universally expressed, while the other four [Ras GRF1, Ras GRF2, Ras GRP1 (CalDAG-GEF II), Ras GRP2 (CalDAG-GEF I)] displayed a more variable pattern of expression (Figure 3A). Sos1 and RasGRP3 are capable of activating both classic Ras and R-Ras isoforms, whereas Sos2 has only shown affinity to H-Ras, and BCAR3 and C3G have demonstrated the ability to activate R-Ras but not classic Ras isoforms (26, 47, 52). Considered together with the Ras expression data presented above, these data indicate that human MPNSTs are capable of activating members of both the classic Ras and the R-Ras subfamilies. It is also notable that the profile of Ras GEFs expressed in human MPNST cells differ in some regards from what has been previously found in Nf1 null murine Schwann cells (32). Although C3G and the BCAR3 murine homolog AND34 were present in these non-neoplastic glia, Nf1-/- Schwann cells, unlike MPNST cells, did not express Ras GRF1, Ras GRP1 or Ras GRP3. The other Ras GEFs we detected in MPNST cells were not examined in this earlier study.
To establish which classic Ras and R-Ras subfamily members are activated in neurofibromin-null MPNST cells, we examined Ras activation in lysates of ST88-14 cells transiently transfected with Myc epitope-tagged wild-type Ras plasmids or a Myc-tagged dominant negative H-Ras control. In these experiments, the Raf-1 Ras binding domain (RBD) affinity reagent, which only binds activated Ras, readily pulled down Myc-tagged wild-type H-Ras, N-Ras, and K-Ras (Figure 3B). In contrast, the Myc-tagged dominant negative H-Ras mutant – which is permanently locked in the inactive GDP-bound conformation (35) – did not bind this reagent. Myc-tagged wild-type R-Ras and R-Ras2 were also pulled down by the Raf-1 RBD reagent (Figure 3C). Thus, ST88-14 cells activate all of the classic Ras and R-Ras proteins that they express.

**H-Ras and R-Ras Dominant Negative Mutants Differentially Affect the Proliferation, Migration, and Survival of Human MPNST Cell Lines**

We next wished to determine the effects of broadly inhibiting the classic Ras or the R-Ras subfamilies in MPNST cells in order to assess the relative importance of each subfamily to MPNST pathogenesis. As no chemical inhibitors have been shown to effectively inhibit all members of one subfamily without affecting the other, we utilized dominant negative (DN) Ras mutants for this purpose. DN H-Ras has previously been demonstrated to inhibit the activation of H-Ras, N-Ras and K-Ras (44). DN R-Ras similarly inhibits the activation of both R-Ras and R-Ras2 (32). We therefore created ST88-14, T265-2c and STS-26T MPNST cell lines stably transfected with plasmids expressing HA epitope-tagged DN H-Ras, HA epitope-tagged DN R-Ras, or GFP (as a negative control) under the control of a doxycycline-inducible promoter. Maximal protein induction occurred in these lines 36-48 hours after addition of 2µg/ml
doxycycline (Figure 4A-B), and all lines demonstrated appropriate doxycycline-dependent induction of the target proteins (Figure 5A). Neither DNA synthesis (Figure 6A) nor migration (Figure 6B) of the MPNST parent lines was affected when their media was supplemented with 2μg/ml doxycycline, indicating that doxycycline alone had no effect on these experimental measures.

We first assessed the impact of DN Ras mutant expression on DNA synthesis in our MPNST cell lines using ³H-thymidine incorporation assays. We found that expression of the H-Ras DN mutant significantly reduced ³H-thymidine incorporation in ST88-14, T265-2c and STS-26T cells (Figure 5B). Interestingly, the R-Ras DN mutant also significantly inhibited DNA synthesis in all three MPNST cell lines (Figure 5B). Doxycycline-induced expression of GFP had no significant effect on MPNST proliferation (Figure 5B).

We next examined the effect Ras DN mutants exerted on MPNST migration using Transwell migration assays that were performed using experimental parameters we have previously optimized for MPNST cells (21). In contrast to the results described above for proliferation, we found that inducing the expression of the DN H-Ras mutant had no effect on the migration of ST88-14, T265-2c or STS-26T cells (Figure 5C). However, expression of the R-Ras DN mutant significantly inhibited the migration of all three of these MPNST cell lines (Figure 5C). Interestingly, the sporadic STS-26T MPNST cell line, which continues to express wild-type neurofibromin and does not show hyperactivation of the classic Ras proteins (6), showed only slight inhibition of ³H-thymidine incorporation in the presence of DN H-Ras and DN R-Ras (Figure 5B), while DN R-Ras inhibition of migration was comparable to that observed in ST88-14 and
T265-2c lines (Figure 5C). As with proliferation, doxycycline-induced expression of GFP had no significant effect on the migration of these MPNST cell lines (Figure 5C).

We also examined the effects that each Ras DN mutant exerted on the survival of MPNST cells. In serum-containing media, ST88-14 cell viability, as assessed by calcein cleavage, was not affected by induction of either DN R-Ras or GFP expression (Figure 7A). In contrast, induction of the DN H-Ras mutant resulted in a modest, but statistically significant reduction in the number of viable ST88-14 cells (Figure 7A, Figure 8A). The ability of DN H-Ras to reduce MPNST cell viability in serum-containing media was confirmed in T265-2c cells (Figure 8B). To determine whether inhibition of classic Ras signaling further enhanced cell death under conditions of cellular stress, we repeated this same experiment in serum-deprived ST88-14 and T265-2c cells. When cultured in serum-free DMEM, expression of the DN H-Ras mutant resulted in an exaggerated loss of cell viability in both MPNST cell lines (Figure 7B, Figure 8A-B) whereas induction of DN R-Ras or GFP expression had no effect. To determine whether the death of ST88-14 cells expressing DN H-Ras was apoptotic in nature, we next examined the cleavage of caspase-3, the major effector caspase expressed in the nervous system, in ST88-14 cells maintained in DMEM alone. We found that the accumulation of cleaved caspase-3 increased in parallel with the expression of the H-Ras DN mutant 12 and 24 hours post-induction (Figure 7C). In the presence of a 50μM concentration of the broad caspase inhibitor BAF, the DN H-Ras mutant had no effect on ST88-14 viability (Figure 7D). We conclude that inhibition of classic Ras protein signaling induces apoptosis in this MPNST line.
Expression of DN H-Ras Versus DN R-Ras Induces Distinct Changes in the Phosphoproteome

The findings described above indicate that DN H-Ras and DN R-Ras have similar effects on MPNST mitogenesis. However, DN H-Ras preferentially inhibits the survival of these cells whereas DN R-Ras preferentially inhibits their migration. This suggests that hyperactivated classic Ras proteins and R-Ras proteins regulate some overlapping signaling pathways in MPNST cells (in particular, ones controlling mitogenesis) as well as signaling pathways distinct to each Ras subfamily (pathways promoting migration for the R-Ras subfamily and pathways controlling survival for the classic Ras proteins).

To identify these differentially regulated pathways, we performed mass spectrometry-based differential phosphoproteome analyses in ST88-14 cells stably transfected with plasmids expressing DN H-Ras or DN R-Ras under the control of a doxycycline-inducible promoter. Uninduced cells were labeled with media containing a light (^{12}C_{6}) lysine isotope, while doxycycline-induced cells were labeled with media containing heavy (^{13}C_{6}) lysine isotope. Equal quantities of light- and heavy-labeled cell lysates were mixed together and enriched for phosphoproteins on a gallium (Ga^{2+}) immobilized metal ion affinity chromatography (IMAC) column, which binds proteins containing negatively charged phosphate groups (Figure 9A). In these experiments, we found that approximately 10% of the protein loaded onto the column bound to the matrix, which is consistent with estimates of the number of phosphorylated proteins present in the cytoplasm. Immunoblotting for representative phosphoproteins (phosphorylated Erk1/2\textsuperscript{Thr202/Tyr204}, Akt\textsuperscript{Thr308}, and JNK1-3\textsuperscript{Thr183/Tyr185}) confirmed that these proteins were enriched in the protein fraction binding to the IMAC column (Figure 9B).
Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was then used to determine the identity and relative abundance of each light-labeled versus heavy-labeled protein captured on IMAC based on the relative intensities of the detected heavy and light tryptic peptides (Figure 10). The phosphorylation status of most of the detected proteins did not change following induction of either DN H-Ras (Figure 11A) or DN R-Ras (Figure 11B), as indicated by the tight clustering of heavy-to-light (H:L) ratios around 1 (log₂(H:L) = 0) in these population of proteins. However, there was a subpopulation of proteins evident that both demonstrated substantial differences in phosphorylation after induction of either DN H-Ras or DN R-Ras (|log₂(H:L)| > 0.33) and met criteria for statistically significant detection (i.e., multiple peptides with similar H:L ratios from each protein were detected, providing statistical confidence that the H:L quantitation was accurate). Those proteins fell into three categories: those that changed following both DN H-Ras and DN R-Ras induction (Table 1), those that changed only upon DN H-Ras induction (Table 2), and those that changed only upon DN R-Ras induction (Table 3).

To identify cytoplasmic signaling networks potentially regulated by the classic Ras and R-Ras subfamilies, we applied Ingenuity Pathway Analysis (IPA) to the population of proteins whose levels changed (|log₂(H:L)| > 0.33) in response to induction of either DN H-Ras or R-Ras. Seven interconnected protein networks were identified that changed following induction of DN H-Ras (Table 4, Figure 11C). The highest ranked of these networks included proteins involved in RNA and protein synthesis, stability and modification (Table 4). Proteins involved in the regulation of actin cytoskeletal dynamics also appeared in the top three networks affected by DN H-Ras induction (Table 4), clustering in ways that suggested effects on cellular morphology and organization.
rather than on migration. Many of the detected actin dynamic proteins fell downstream of the RhoA/ROCK1 signaling axis, suggesting that DN H-Ras may enhance actin stress fiber formation through RhoA/ROCK1 rather than impacting dynamic actin structures in the lamellipodia. Other protein networks altered by DN H-Ras have been implicated in proliferation, cell death and metabolism (Table 4). Although not implicated by IPA as part of an affected cell death network, we noted that the apoptosis inhibitor 5 (API-5) protein showed significantly decreased phosphorylation following DN H-Ras (Table 2) but not DN R-Ras induction. API-5 phosphorylation is inversely correlated with levels of apoptosis (62), suggesting that DN H-Ras induced reduction in API-5 phosphorylation promotes the apoptosis observed following the expression of this DN mutant. Alternatively, the pro-apoptotic effects of DN H-Ras may reflect lower level changes in multiple factors rather than extreme changes in a single factor, as proteins in multiple DN H-Ras networks have secondary roles in apoptosis induction.

DN R-Ras induction caused changes in thirteen networks distinct from those affected by DN H-Ras; the seven top ranked networks are reported (Figure 11D, Table 5). Changes in the phosphorylation of proteins regulating actin dynamics were also observed downstream of DN R-Ras induction and, as observed in the DN H-Ras IPA analysis, these grouped into networks controlling cellular organization and morphology (Table 5). In contrast to DN H-Ras, however, IPA analysis of the DN R-Ras phosphoproteome identified networks involved in the control of cellular movement (Table 5) via their effects on microtubule dynamics. Vesicular transport and RNA trafficking proteins were more prominently altered by DN R-Ras (RNA post-transcriptional modification network; Table 5) than by DN H-Ras, as were proteins involved in lipid metabolism (data not
Proteins involved in protein synthesis, metabolism and proliferation were also altered by DN R-Ras induction; these proteins had some overlap with those affected by DN H-Ras induction, but distinct changes were also observed. Amino acid biosynthesis was also prominently affected by DN R-Ras (data not shown).

**DISCUSSION**

We have hypothesized that multiple members of the classic Ras and R-Ras subfamilies are hyperactivated in MPNST cells as a result of neurofibromin loss and that these Ras subfamilies promote tumorigenesis by activating distinct sets of cytoplasmic signaling pathways that elicit different physiologic effects. In keeping with this hypothesis, the majority of the MPNST cell lines we examined express five neurofibromin-regulated Ras proteins—H-Ras, N-Ras, K-Ras, R-Ras, and R-Ras2. These lines also express a complement of RasGEFs sufficient to activate each of these molecules, and indeed, we demonstrate that all of the Ras proteins expressed in MPNST cells can be activated. The effects of our H-Ras and R-Ras DN mutants indicate that both classic Ras and R-Ras subfamily proteins contribute to neoplasia, but do so by effecting distinct cellular functions—both subfamilies promote tumor cell mitogenesis, while only classic Ras proteins promote survival and only R-Ras family proteins enhance migration. Using phosphoproteomics, we have identified several signaling networks that are differentially regulated downstream of the classic Ras and R-Ras subfamilies, thereby providing a mechanistic explanation for the distinct effects of the DN H-Ras and R-Ras mutants. Considered together, these findings provide some intriguing insights into the mechanisms involved in the pathogenesis of neurofibromin-null neoplasms.
The complement of Ras proteins that we detected in our panel of human MPNST cell lines is similar to that previously reported in wild-type and Nf1⁻/⁻ mouse Schwann cells (32). Notably, however, a number of the GEFs that are expressed in human MPNST cells are not normally present in non-neoplastic murine Schwann cells. GEFs serve as key intermediates between cell surface signaling molecules such as growth factor receptors and Ras proteins. These upstream regulators of Ras signaling remain relevant even the context of neurofibromin loss, as evidenced by the ability of growth factor simulation to increase Ras activation in neurofibromin-null cells (6). Consequently, the presence of GEFs in neoplastic Schwann cells, but not their non-neoplastic counterparts, raises the question of whether the upstream signals driving Ras activation in MPNST cells are distinct from those operant prior to transformation. Given this, it will be of considerable interest to determine which of the GEFs identified in our studies are particularly important for Ras activation in MPNST cells and to establish whether specific growth factor receptors are essential for the activation of key GEFs.

We also demonstrated that all of the classic Ras proteins were activated in MPNST cells, contradicting previous findings that N-Ras and K-Ras, but not H-Ras, were activated in MPNST cells (45). As the authors of this study also could not detect H-Ras expression in ST88-14, 90-8 or STS-26T cells – all of which demonstrated H-Ras expression in our hands – this discrepancy may be explained by the relative specificity and sensitivity of the different H-Ras antibodies used in our two studies. It should also be noted that the spectrum of classic Ras proteins activated by neurofibromin loss in MPNST cells may differ from that observed in other cell types. For example, studies in Nf1⁻/⁻ astrocytes have shown that K-Ras is the sole activated classic Ras protein in these
glia (74), while activation of both N-Ras and K-Ras is observed in neurofibromin-null acute myelogenous leukemia cells (49). The reason for such differences is at present unclear, but may include factors such as the profile of GEFs expressed in different cell types.

We were also able to detect activation of both R-Ras and R-Ras2 with the Raf-1 RBD reagent, consistent with previous reports that activated R-Ras and R-Ras2 are capable of binding the truncated Raf-1 RBD (28, 65) even though the binding of these isoforms to full-length Raf-1 is controversial (28, 29, 42, 51, 63, 65, 75) and may be cell-type dependent. To our knowledge, this is the first direct examination of R-Ras protein activation in any neurofibromin-null cancer cells, although neurofibromin has been shown to inactivate these proteins in vitro (52) and R-Ras proteins have been implicated in the enhanced migration of Nf1-/- non-neoplastic Schwann cells (32). It should be noted that this examination occurred in ST88-14 cells, which express both R-Ras and R-Ras2. Three other MPNST cell lines – YST-1, 90-8 and NMS2 – had no detectable R-Ras expression, indicating that R-Ras2 would be the only functional R-Ras protein in these cells.

Our demonstration that a DN H-Ras mutant inhibits the proliferation and survival of MPNST cells implicates the classic Ras subfamily proteins in regulating these phenotypes. The combined effects of all three hyperactivated classic Ras proteins may contribute to hyperproliferation and resistance to apoptosis in these tumors; alternatively, some of these proteins may be primarily responsible for mitogenesis, while others promote tumor cell survival. In future studies, it will be important to ablate the expression of individual classic Ras proteins in MPNST cells and examine the effect this
has on mitogenesis and survival. We may find that specific classic Ras proteins are solely responsible for driving proliferation and preventing apoptosis, but given the fact that all three of these proteins are activated and their functions often overlap, a more likely possibility is that they are functionally redundant. Distinguishing between these possibilities will be critically important for the development of new therapeutic agents that effectively inhibit Ras action in MPNST cells.

Our observation that DN R-Ras, but not DN H-Ras, reduced the migration of MPNST cells is consistent with previous observations in Nf1−/− Schwann cells (32) and suggests that R-Ras subfamily members function similarly in neoplastic Schwann cells and their non-neoplastic counterparts. However, the finding that DN R-Ras inhibits MPNST mitogenesis is, to the best of our knowledge, the first evidence implicating R-Ras subfamily members in the proliferation of neurofibromin-null tumor cells. At present, we do not know whether R-Ras and R-Ras2 drive mitogenesis and migration through combinatorial effects or whether one of these R-Ras subfamily proteins drives proliferation while the other enhances migration. However, our observation that R-Ras2 is uniformly present in MPNST cells while R-Ras is more variably expressed suggests that R-Ras2 may be of primary importance. Of possible relevance to these possibilities is the surprising finding that the migration of STS-26T cells, which maintain intact neurofibromin and do not show Ras hyperactivation (6), was inhibited by DN R-Ras to an extent similar to that seen in our NF1-null cells, while their proliferation was relatively resistant to DN R-Ras. This raises the question of whether STS-26T cells have acquired an activating mutation in a single R-Ras subfamily member, rendering them sensitive to DN R-Ras mediated inhibition of migration but not proliferation. Such a circumstance
would argue that R-Ras and R-Ras2 exert distinct effects on MPNST mitogenesis and proliferation.

Our phosphoproteomics experiments suggest a mechanistic basis for the different effects of DN H-Ras and DN R-Ras on migration. Although we found that DN H-Ras induced profound changes in the phosphorylation of some proteins regulating cytoskeletal dynamics, our analyses of the physiologic effects of this mutant indicated that it did not affect migration. As an explanation for this apparent discrepancy, it should be noted that these proteins affected by DN H-Ras expression were grouped by IPA analysis into networks potentially affecting cellular morphology rather than cellular migration. The two cytoskeletal dynamic proteins whose phosphorylation status was most strongly and significantly affected by DN H-Ras induction were vimentin and cofilin. While vimentin has been implicated in a variety of cellular functions, its most well-studied roles are in maintenance of cell integrity (25, 34) and intracellular distribution of organelles (36, 69). Only a few of the vimentin phosphorylation sites identified from in vitro experiments have been validated in vivo, and phosphorylation at these residues has been linked to filament disassembly during mitosis (17, 70, 73), supporting a non-migratory role for the increase in vimentin phosphorylation observed following DN H-Ras induction. Cofilin phosphorylation, however, has a well-established role in migration—activation of cofilin by Ser-3 dephosphorylation at the leading edge of a cell is a critical early step in this process. However, this dephosphorylation must also be paired with phosphorylation/inactivation of cofilin in the cell body (50, 64) to spatially restrict cofilin activation for polarized cell motility. Global cofilin status is thus unlikely to correlate directly to migratory capacity (57), and so the increased cofilin
phosphorylation observed following DN H-Ras induction may reflect alterations in cofillin phosphorylation in the cell body that would not affect the function of this molecule in lamellipodia. Indeed, cofillin inhibition is necessary for RhoA-induced actin stress fiber formation (3, 41), and increased stress fiber formation has been previously reported in MPNST cells following treatment with farnesylthiosalicylic acid (6), another broad spectrum inhibitor of the classic Ras subfamily.

The phosphorylation of proteins controlling actin dynamics – among them cofillin and cortactin – were changed to a similar in MPNST cells expressing the DN R-Ras mutant. In contrast, the phosphorylation of microtubule subunits (i.e. tubulin alpha-1B and -1C chains) and binding proteins (i.e. dynactin subunits 1 and 3, kinesin light chain 1) appeared to be more strongly affected by DN R-Ras induction, and IPA analysis grouped these molecules into networks with suggested roles in cellular movement. This raises the possibility that DN R-Ras could reduce migration by impacting microtubule-dependent phenomenon such as selective microtubule stabilization at the leading edge of migrating cells, the reorientation of the microtubule-organizing center (MTOC) towards the leading edge, or directed vesicular transport towards the leading edge (76). The mediators of these R-Ras dependent events are less clear. R-Ras has previously been implicated in microtubule stability in hippocampal neuron growth cones (53, 54), but this was dependent upon its effects on PI3K/Akt signaling which did not appear to be affected by DN R-Ras expression in this experiment. Previous studies have linked the activation of integrin proteins, which can function both upstream (59) and downstream (15) of microtubule dynamics in migration, to the promotion of migration by activated R-Ras (8, 37, 55). As our phosphoproteomics protocol is geared towards examining cytoplasmic
proteins rather than integral membrane proteins, we were not able to directly assess the impact of impaired integrin activation by DN R-Ras on microtubule dynamics. However, our phosphoproteomics data did suggest a potential role for Arf6 in coordinating R-Ras dependent effects on microtubule and integrin function. The Arf6 GTP-binding protein promotes migration by enhancing post-endocytic trafficking of β1-integrin (19), Cdc42 (58) and Rac1 (4) to the leading edge of cells in a microtubule- and AP2-dependent manner (4, 38). It is also a critical mediator of R-Ras-induced Rac1 activation and migration (24) and plays a role in the regulation of kinesin and dynactin function (48). As Arf6 is regulated by GTP/GDP binding, its binding to the IMAC column would be unlikely to change based on its activation state, and indeed, phospho-enriched Arf6 levels do not change following DN R-Ras induction. However, phosphorylation of the phosphoinositide-activated Arf6 GTPase ACAP2 was significantly reduced following DN R-Ras induction. DN R-Ras induction also increased phosphorylation of the PI(4,5)P2 phosphodiesterases PLCβ3 and PLCβ4, which is expected to lead to impaired PLCβ function, elevated PI(4,5)P2 levels, and activation of ACAP2. DN R-Ras induction also leads to elevated phosphorylation of the RalBP1-associated Eps domain-containing protein 1 (Reps1). As R-Ras activation of Arf6 is dependent upon RalBP1 (24), and Reps1 is a RalBP1 effector that also localizes to AP-2 endocytic complexes (40), alteration of Reps1 phosphorylation downstream of DN R-Ras induction could also potentially inhibit Arf6 function.

Microtubule (DN R-Ras) and actin (DN H-Ras) dynamic proteins are also intimately involved in cytokinesis, suggesting that these changes might affect proliferation. Further, DN H-Ras and DN R-Ras both affected the phosphorylation of molecules involved in
protein synthesis, a process that is intimately intertwined with cellular proliferation (78, 79). Although we did not observe changes in the phosphorylation of nuclear proteins directly controlling mitogenesis in our phosphoproteomics experiments, this was not unexpected. As noted above, the phosphoproteomics methodology we employed is geared towards the analysis of cytoplasmic proteins—we have confirmed that our procedure does not lyse the nuclear envelope and consequently nuclear proteins were not represented in our lysates.

In conclusion, we have found that neurofibromin loss results in the activation of multiple members of the classic Ras and R-Ras subfamilies in neoplastic Schwann cells. Our observations suggest that these subfamilies serve different functions in MPNST cells despite an overlapping set of effector molecules, a hypothesis that is further supported by the differential effects of these mutants on MPNST proliferation, migration, and survival. Our phosphoproteomics analyses indicate that these Ras subfamilies also affect the phosphorylation of distinct collections of cytoplasmic signaling molecules which begins to explain the different physiologic effects of each subfamily. However, although the analyses presented in this manuscript provide useful and novel insights into the mechanisms operant in neurofibromin-null tumors, we would caution that this study represents only a beginning step in deciphering the cytoplasmic signaling cascades downstream of the classic Ras and R-Ras subfamilies. Studies with inhibitors such as the DN Ras mutants we employed examine the terminal consequences of inhibiting the signaling cascade—identifying the cytoplasmic signaling intermediates that are more proximal to each Ras protein will be essential as we move forward and will require a different experimental design. It will also be very important to establish the role of
individual classic Ras and R-Ras subfamily members and how they fit into the development of neurofibromin-null neoplasms. Answering questions such as these will provide the basic framework of information that is necessary for the future development of therapies that are effective against the growing number of tumor types that are realized to occur as a result of a loss of neurofibromin expression.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Neurological Diseases and Stroke (R01 NS048353 to S.L.C. and F30 NS063626 to N.M.B) and the National Cancer Institute (R01 CA122804 to S.L.C.).

We thank the Alabama Neuroscience Blueprint Core Center (P30 NS57098), the UAB Neuroscience Core Center (P30 NS47466), the UAB Targeted Metabolomics and Proteomics Laboratory (S10RR027822) and the UAB Heflin Center for Genomic Science for technical assistance. The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Department of Defense.

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# Table 3. Proteins significantly altered by DN R-Ras only

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Table 4. DN H-Ras IPA networks

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1. Red gene symbols represent proteins which showed increased binding to the IMAC column following DN H-Ras induction, while green gene symbols indicate proteins with decreased binding.

2. Bolded gene symbols represent proteins whose quantitation was deemed to be significantly different between uninduced and DN H-Ras induced ST88-14 cells by ProteinPilot.
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1. Red gene symbols represent proteins which showed increased binding to the IMAC column following DN R-Ras induction, while green gene symbols indicate proteins with decreased binding.

2. Bolded gene symbols represent proteins whose quantitation was deemed to be significantly different between uninduced and DN R-Ras induced ST88-14 cells by ProteinPilot.
Figure 1. Determination of classic Ras and R-Ras antibody specificity. (A) ST88-14 cells were transiently transfected with Myc-tagged dominant negative H-Ras or wild-type H-Ras, N-Ras, or K-Ras. Lysates of these cells were immunoblotted for classic Ras isoform expression using antibodies directed against H-Ras (sc-833, 1:1000), N-Ras (sc-30, 1:1000), or K-Ras (AbD Serotec MCA3223Z, 1:1000). Endogenous Ras (which migrates ~5kD below Myc-tagged isoforms) is not shown. (B) ST88-14 cells were transiently transfected with Myc-tagged dominant negative H-Ras or wild-type R-Ras or R-Ras2. Lysates of these cells were immunoblotted for R-Ras isoform expression using antibodies directed against R-Ras (Abnova H00006237_M01, 1:10,000) or R-Ras2 (Abnova H000022800_M01, 1:10,000; sc-833 1:1000; sc-81931 1:10,000; R&D Systems AF3605 1:1000; Abcam 96307 1:10,000). A sixth R-Ras2 antibody (sc-166232, 1:100) failed to recognize a band at the appropriate molecular weight (data not shown).
Figure 2. Human MPNSTs variably express classic Ras and R-Ras isoforms. (A) Lysates from 8 human MPNST cell lines probed with isoform-specific antibodies for the classic Ras and R-Ras proteins using GAPDH as a loading control. Antibody dilutions were as follows: H-Ras 1:500, N-Ras 1:1000, K-Ras 1:1000, R-Ras 1:10,000, R-Ras plus R-Ras2 (Abnova H000022800_M01) 1:10,000 and GAPDH 1:100,000. (B) Real-time PCR quantitation of R-Ras and R-Ras2 transcripts in MPNST lines versus the human cerebellum control tissue. (C) PCR showing M-Ras transcript expression in only 1 of 8 human MPNST lines.
Figure 3. Multiple Ras isoforms are activated in MPNST cells. (A) PCR showing expression of Ras guanine nucleotide exchange factors in human MPNST cell lines. No bands were seen in RT-samples (data not shown). (B) Raf-1 RBD assays performed on ST88-14 cells transiently transfected with Myc-tagged wild-type H-Ras, N-Ras and K-Ras plasmids or the Myc-tagged dominant negative H-Ras control plasmid. (C) Raf-1 RBD assays performed on ST88-14 cells transiently transfected with Myc-tagged wild-type R-Ras and R-Ras2 plasmids or the Myc-tagged dominant negative H-Ras control plasmid.
Figure 4. Dominant negative mutants reach maximal expression by 48hr post-induction with 2μg/ml doxycycline. (A) Expression of HA-tagged DN R-Ras in stably transfected ST88-14 cells challenged with different concentrations of doxycycline. (B) Expression of HA-tagged DN R-Ras in stably transfected ST88-14 cells at various timepoints after addition of 2μg/ml doxycycline.
Figure 5. Dominant negative H-Ras and R-Ras mutants both inhibit MPNST mitogenesis, but only DN R-Ras affects MPNST migration. (A) Relative expression levels of HA-tagged DN H-Ras, HA-tagged DN R-Ras, and GFP control in ST88-14, STS-26T, and T265 doxycycline-inducible stable cell lines in the presence or absence of 2µg/ml doxycycline 48hr post-induction. (B) Induction of DN H-Ras and DN R-Ras expression reduces 3H-thymidine incorporation at 48hr post-induction in the ST88-14, STS-26T, and T265 doxycycline-inducible stable cell lines. (C) Induction of DN R-Ras expression, but not DN H-Ras, reduces transwell migration in the ST88-14, STS-26T, and T265 doxycycline-inducible stable cell lines 48hr post-doxycycline addition.
Figure 6. Doxycycline does not reduce $^3$H-thymidine incorporation or migration in MPNST parent lines. (A) $^3$H-thymidine incorporation in ST88-14, STS-26T and T265 MPNST cell lines in the presence or absence of 2µg/ml doxycycline for 48hrs. (B) Migration in ST88-14 and STS-26T MPNST cell lines in the presence or absence of 2µg/ml doxycycline for 48hrs.
Figure 7. Dominant negative H-Ras, but not dominant negative R-Ras, induces apoptotic cell death under serum-deprived conditions. (A) Calcein cleavage modestly reduced by doxycycline induction of DN H-Ras (but not GFP or DN R-Ras) in ST88-14 cells cultured in DMEM-10 at 24hrs post-induction. (B) Calcein cleavage markedly reduced by doxycycline induction of DN H-Ras (but not GFP or DN R-Ras) in ST88-14 cells cultured in DMEM alone at 24hrs post-induction. (C) Immunoblotting shows increased cleaved caspase-3 (Cell Signaling 9661, 1:1000) upon induction of DN H-Ras in ST88-14 cells cultured in DMEM alone. Also shown is HA epitope expression to verify DN H-Ras induction and GAPDH as a loading control. (D) Doxycycline-induction of DN H-Ras fails to decrease calcein cleavage in serum-deprived conditions in the presence of the broad caspase inhibitor BAF (50μM) at 24hrs post-induction.
Figure 8. DN H-Ras further reduces viability under serum-free conditions at 36hr post-induction. (A) Calcein cleavage is significantly reduced in ST88-14 cells following 36hr doxycycline induction of DN H-Ras in both DMEM-10 and serum-free DMEM. As at 24hr, a larger decrease is observed in serum-free conditions. (B) Calcein cleavage is significantly reduced in T265-2c cells following 36hr doxycycline induction of DN H-Ras in both DMEM-10 and serum-free DMEM. A larger decrease is observed in serum-free conditions.
Figure 9. Phosphoprotein enrichment of stable-isotope labeled doxycycline-inducible cell lines. (A) Flow scheme of Phosphoprotein enrichment. Cells are labeled for 5 days in media containing light ($^{12}$C$_6$) or heavy ($^{13}$C$_6$) lysine stable isotopes. Cells are maintained in these media for an additional 2 days in the presence or absence of 2μg/ml doxycycline, then lysed in lysis buffer containing 1% CHAPS. Protein concentrations are determined and equal quantities of Light- and Heavy-labeled lysates are mixed together and incubated with the phosphoprotein enrichment column. After washing in lysis buffer, columns are eluted with elution buffer, and elution fractions are concentrated by centrifugation in iCON concentrators. (B) Immunoblotting for common phosphoproteins was used to verify phosphoprotein enrichment. Induction of dominant negative mutants was verified by immunoblotting for the HA epitope, and GAPDH was used as a loading control. Antibody dilutions were as follows: p-Erk1/2 1:1200, pAkt 1:500, pJnk1-3 1:500, HA epitope 1:50,000, GAPDH 1:100,000.
Figure 10. Sample MS and MS/MS spectra. MS spectra corresponding to the heavy- and light-labeled peptide LIFAGK from the ubiquitin-60S ribosomal protein L40 (sp|P62987|RL40_HUMAN) are shown. Relative peak intensities of all heavy- and light-labeled detected peptides are used to estimate relative quantitation of parent proteins. The MS/MS spectra of the LIFAGK peptide show the daughter b- and y-ions generated by collision with the neutral gas helium; these ions are used to confirm the peptide sequence.
Figure 11. Different networks of phosphorylated proteins affected by DN H-Ras compared to DN R-Ras. (A) Distribution of heavy (DN H-Ras) to light (uninduced) ratios of ST88-14 DN H-Ras stable cell. Plotted values are log$_2$(H:L). (B) Distribution of heavy (DN R-Ras) to light (uninduced) ratios of ST88-14 DN R-Ras stable cell. Plotted values are log$_2$(H:L). (C) IPA analysis identified seven networks affected by DN H-Ras induction with IPA scores >16. (D) IPA analysis identified thirteen networks affected by DN R-Ras induction with IPA scores >16; the seven top ranked networks are shown.
VARIABLE DEPENDENCE UPON K-RAS SIGNALING IN MALIGNANT PERIPHERAL NERVE SHEATH TUMOR PROLIFERATION

by

NICOLE M. BROSSIER, KATHRYN L. BAILEY, STEVEN L. CARROLL

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ABSTRACT

Neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs) arising in patients with the genetic disorder neurofibromatosis type I (NF1) show loss or mutation of both \textit{NF1} alleles in the Schwann cell lineage. The \textit{NF1} gene encodes the tumor suppressor neurofibromin, a molecule which can inactivate Ras family proteins (H-Ras, N-Ras, and K-Ras). It is currently unclear which of these Ras isoforms contributes most strongly to MPNST pathogenesis. We have previously demonstrated that a dominant negative (DN) H-Ras mutant capable of inhibiting the activation of H-Ras, N-Ras, and K-Ras proteins reduces the proliferation but not the migration of MPNST cells. Here, we show that shRNA-mediated ablation of K-Ras but not N-Ras reduces proliferation in T265-2c MPNST cells while ST88-14 MPNST cells are resistant to both N-Ras and K-Ras single-isoform loss. Increased activation of K-Ras is observed following N-Ras loss in the resistant line, indicating that compensatory activation of other classic Ras isoforms may be responsible for its insensitivity to K-Ras loss. As predicted by our DN H-Ras studies, the migration of both lines remained unaffected by ablation of either N-Ras or K-Ras.

INTRODUCTION

The Ras family of small GTPases consists of three proto-oncogenes – H-Ras, N-Ras, and K-Ras – which share $>$85\% sequence homology, leading to significant overlap in effector utilization and function. These proteins are activated by GTP binding, a process catalyzed by guanine nucleotide exchange factors (GEFs) downstream of various stimuli (1-3) including extracellular mitogens. The duration of Ras activation is limited by
GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity of Ras, triggering hydrolysis of GTP to GDP and subsequent Ras inactivation (4, 5). Under these controlled conditions, Ras proteins can mediate various phenotypic responses, including proliferation, survival, migration and differentiation, depending upon the cellular context.

Activating mutations in Ras proteins are often observed in human cancers. Despite the overlapping functions of these proteins, most human cancers show an association with mutation of only one Ras isoform, suggesting that Ras isoforms differ in their ability to promote tumorigenesis in specific tissues. The different transformation capacity of activated H-Ras, N-Ras and K-Ras isoforms in specific cell lineages in vivo (6-8) provides further support for this hypothesis. However, the impact of individual Ras proteins is less clear when Ras activation occurs via the loss of the GAP protein neurofibromin. Neurofibromin negatively regulates H-Ras, N-Ras and K-Ras activity (9-11), and neurofibromin-null cells display simultaneous hyperactivation of these isoforms (12, 13). As these isoforms respond differently to Ras-targeted therapeutic agents such as farnesyltransferase inhibitors (14-17), the contribution of individual Ras proteins to the development of neurofibromin-null neoplasms has obvious clinical implications.

Malignant peripheral nerve sheath tumor (MPNST) cells provide a good model system for studying multiple Ras isoform activation. These aggressive sarcomas often arise in patients with neurofibromatosis type I (NF1); in this context, they typically display biallelic loss of the $NFI$ gene (18), which encodes the neurofibromin protein. MPNSTs may also arise sporadically, and these typically do not display biallelic $NFI$ loss or profound Ras hyperactivation (19). We have previously demonstrated that a dominant negative (DN) H-Ras mutant, which can inhibit the activation of wild-type H-
Ras, N-Ras and K-Ras, inhibits mitogenesis of both sporadic and NF1-associated MPNST cells under nutrient-rich conditions and induces the death of these cells under serum-deprived conditions (see second article, Classic Ras and R-Ras Subfamily Proteins Elicit Distinct Physiological Effects and Phosphoproteomic Alterations in Neurofibromin-Null Tumor Cells). However, it was unclear whether simultaneous inhibition of all three isoforms was required to produce these effects.

We hypothesized that inhibition of a single hyperactivated Ras isoform would be sufficient to reduce MPNST proliferation. In this paper, we have used isoform-specific shRNAs to ablate N-Ras and K-Ras expression and then assessed the impact this had on MPNST mitogenesis.

**MATERIALS AND METHODS**

*Antibodies and Reagents*

H-Ras (sc-520) and N-Ras (sc-31) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies directed against K-Ras (MCA3223Z), the Myc epitope tag (#2276) and GAPDH (RDI-TRK5G4-6C5) were purchased from AbD Serotec (Kidlington, UK), Cell Signaling Technology (Beverly, MA) and Fitzgerald Industries International (Concord, MA), respectively. Jackson ImmunoResearch Laboratories (West Grove, PA) supplied HRP-conjugated secondary antibodies.
Ras Doxycycline-Inducible shRNA Lentiviruses

Lentiviruses containing bi-directional tetracycline-inducible cassettes expressing shRNAs targeted against N-Ras (pSLC 748, 749, 750), K-Ras (pSLC 751, 752, 753) or nonsense sequences (pSLC 727) were created as previously described (20). Briefly, shRNA sequences were designed using the RNAi Codex algorithm (http://katahdin.cshl.org:9331/portal/scripts/main2.pl), and cloned into the pen_TTGmiRc2 entry vector (ATCC #MBA-253). The daughter vectors were then recombined with the pSLIK-hygro vector (ATCC #MBA-237) using LR clonase II reaction mix (Invitrogen; Carlsbad, CA). The resulting bi-directional tetracycline-inducible lentiviral plasmid was co-transfected with pLP1, pLP2, and pLP/VSVg helper plasmids (Invitrogen) into 293FT packaging cells (Invitrogen) using Polyfect (Qiagen; Germantown, MD). Lentivirus-containing conditioned media was collected and stored at -80°C until use. All ATCC vectors were obtained with aid from the UAB Protein Interaction Core, supported by P30 NS47466.

Stable Transduction of MPNST Cell Lines

The sources of the human MPNST cell lines used in this study have been previously described (21, 22). To create stable cell lines containing doxycycline-inducible shRNA plasmids, cells were transduced by culturing in lentivirus-containing media supplemented with 6μg/ml Polybrene (Sigma-Aldrich; St. Louis, MO). After three days, cells were split into fresh DMEM supplemented with 10% tetracycline-free fetal bovine serum (TF DMEM-10), 10U/ml penicillin, 10μg/ml streptomycin, and the optimal concentration of hygromycin selection antibiotic (50-100μg/ml; determined empirically for each line).
Colonies were picked after two weeks and screened by immunoblotting. Colonies with appropriate shRNA expression demonstrated knockdown of the targeted protein when cultured in media supplemented with 2μg/ml doxycycline, but not in tetracycline-free media.

Cell lines stably transfected with doxycycline-inducible vectors were maintained in TF DMEM-10 supplemented with the optimal concentration of hygromycin.

3H-Thymidine Proliferation Assays

Stable cell lines were detached, resuspended in TF DMEM-10 and plated at 3,000 cells/well in 48-well plates. 2μg/ml doxycycline was then added to wells in which shRNA expression was to be induced. Fresh TF DMEM-10 ±2μg/ml doxycycline was added every 48 hours. 96 hours post-plating, 0.5μCi of 3H-thymidine was added to each well. Following an overnight incubation, cells were washed once in ice-cold phosphate-buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 4.3mM Na2HPO4, 1.47mM KH2PO4 at pH 7.4) and then incubated in 200μl ice-cold 10% trichloroacetic acid (TCA) for 1hr on ice. Samples were subsequently washed in PBS and incubated in 200μl 0.3N NaOH for 1hr at room temperature. Equal volumes of NaOH-dissolved samples were counted by scintillation. Sixteen replicates were counted per condition and statistical significance was determined using Student’s t-test, with p-values < 0.05 considered significant.

Transwell Migration Assays

Stable cell lines were cultured in poly-L-lysine and laminin coated flasks for 72 hours in TF DMEM-10 ± 2μg/ml doxycycline to induce shRNA expression. Fresh TF DMEM-
10 ±2μg/ml doxycycline was added every 48 hours. After 72 hours, cells at 80% confluency were serum-starved overnight in Schwann cell-defined medium (23, 24) ± 2μg/ml doxycycline. Cells were then detached, resuspended in migration assay buffer (MAB; DMEM supplemented with 0.1% fatty acid-free BSA) and plated in triplicate at 40,000 cells/well onto 8μm pore Transwell filters (Becton Dickinson Labware; Franklin Lakes, NJ) pre-coated with 0.1mg/ml poly-L-lysine (Sigma #P5899) and 10μg/ml natural mouse laminin (Invitrogen #23017-015). 2μg/ml doxycycline was added to both the upper and lower chambers for doxycycline-induced cells. After 6 hours, cells were fixed and stained with an ethanol-crystal violet solution. The filter undersurface was visualized with bright-field microscopy under a 20x objective, and nine fields were counted per filter. Statistical significance was determined with Student’s t-test, with p-values < 0.05 considered significant.

**Immunoblotting**

Cells were lysed in magnesium-containing lysis buffer (MLB; 25mM HEPES (pH 7.5), 150mM NaCl, 10mM MgCl₂, 1mM EDTA, 1% Igepal CA-630, 10% glycerol) containing protease inhibitor cocktail (Sigma #P8340, 1:100 dilution). Samples were clarified by centrifugation at 14,000rpm, for 10 minutes at 4°C. The DC Assay kit (Bio-Rad; Hercules, CA) was used to determine protein concentrations. Equal quantities of protein were loaded onto 12% SDS polyacrylamide gels. We have previously described our Immunoblotting protocol (25). Antibodies were diluted in 1% nonfat dry milk in TBST (0.15M NaCl, 10mM Tris [pH 8.0], 0.05% Tween-20). Antibody dilutions were as follows: H-Ras 1:1000, N-Ras 1:1000, K-Ras 1:1000, GAPDH 1:100,000. The
SuperSignal Pico Chemiluminescence kit (Thermo Scientific; Rockford, IL) was used to visualize immunoreactive species.

**Ras Activation Assays**

Human MPNST stable cell lines transiently transfected with Myc-tagged wild-type Ras plasmids were lysed in MLB containing protease inhibitor cocktail (Sigma #P8340, 1:100 dilution) and HALT phosphatase inhibitor cocktail (Thermo Scientific #78420, 1:100 dilution). Samples were clarified by centrifugation at 14,000 rpm for 10 minutes at 4°C, and protein concentrations then measured using the DC Assay kit. Lysates were diluted to 0.5 mg/ml protein, and 1 ml of this diluted protein mixed with 20 µl Ras Assay Reagent (Raf-1 Ras-binding domain agarose beads; Millipore, Lake Billerica, MA) and incubated for 30 minutes at 4°C on a rotary shaker. Cells were washed three times in MLB containing protease and phosphatase inhibitors. Following the final wash, Ras Assay Reagent beads were collected by centrifugation at 14,000 rpm for 10 seconds. Beads were boiled for 15 minutes in 40 µl 2x Stop Buffer (250 mM Tris-HCl (pH 6.8), 5 mM EDTA, 5 mM EGTA, 2% SDS, 10% glycerol, 25 mM dithiothreitol, 30 µM bromophenol blue) prior to loading on 12% SDS-PAGE gels. Clarified lysate samples were run in parallel on 12% SDS-PAGE gels. Immunoblotting was performed as described above.
RESULTS

*K-Ras Ablation Inhibits Proliferation in T265-2c but Not in ST88-14 MPNST Cells*

To determine whether inhibition of a single classic Ras isoform could affect the proliferation of MPNST cells, we utilized shRNAs to specifically ablate the expression of H-, N-, or K-Ras. In preliminary experiments, we were unable to obtain robust knockdown of classic Ras isoforms with transient transfection of shRNA-expressing plasmids. Consequently, we stably transduced two MPNST cell lines derived from neoplasms arising in NF1 patients (ST88-14 and T265-2c cells) with lentiviruses containing doxycycline-inducible shRNA cassettes. Optimal target knockdown was observed in stable lines following incubation for 4-5 days in media supplemented with 2μg/ml doxycycline (data not shown). As expected, control shRNA expression did not affect H-Ras, N-Ras, or K-Ras expression (Fig. 1A, 2A). Three different shRNAs directed against H-Ras failed to reduce target protein levels (data not shown). Consequently, we focused our attention on N-Ras and K-Ras. The shRNAs targeting these Ras isoforms appropriately distinguished between different Ras proteins as doxycycline-induction of K-Ras shRNA reduced K-Ras expression without affecting N-Ras or H-Ras protein levels, while N-Ras shRNA expression produced no alterations in K-Ras or H-Ras expression (Fig. 1A, 2A).

We next examined the effects of N-Ras or K-Ras ablation on MPNST mitogenesis. We found that knockdown of N-Ras expression did not affect proliferation in either ST88-14 (Fig. 1B) or T265-2c (Fig. 2B) cells. K-Ras knockdown was similarly ineffective in reducing the proliferation of ST88-14 cells (Fig. 1B). In contrast, doxycycline-induced expression of two different shRNAs targeting K-Ras reduced the
proliferation of T265-2c cells (Fig. 2B). Further, the extent to which the K-Ras shRNAs inhibited T265-2c mitogenesis correlated with the degree of K-Ras knockdown—shK-Ras2, which more potently reduced K-Ras levels (Fig. 2A), inhibited T265-2c proliferation more profoundly than shK-Ras1 (Fig. 2B).

We also examined the effect that knockdown of N-Ras and K-Ras expression exerted on the migration of ST88-14 and T265-2c cells in Transwell migration assays. Migration of both ST88-14 and T265-2c cells was unaffected by N-Ras or K-Ras ablation (Fig. 1C, 2C). This finding is consistent with our previous demonstration that expression of a dominant negative H-Ras (DN H-Ras) mutant, which inhibits the action of H-Ras, N-Ras and K-Ras, similarly fails to reduce MPNST migration (see Chapter 2).

*N-Ras Ablation Upregulates Activation of K-Ras in ST88-14 Cells*

K-Ras expression is much higher in T265-2c cells than in ST88-14 cells (Fig. 3A and Chapter 2). This suggested that ablation of K-Ras expression might have no effect on ST88-14 mitogenesis because this cell line is not dependent on K-Ras for proliferation. Alternatively, ST88-14 cells might evade the consequences of K-Ras loss by compensatory upregulation of either H-Ras or N-Ras signaling. We found that neither H-Ras nor N-Ras protein levels are increased in response to K-Ras loss (Fig. 1A, 2A). In contrast, N-Ras loss resulted in increased K-Ras expression in both ST88-14 (Fig. 1A) and T265-2c (Fig. 2A) cells. To test the hypothesis that compensation for N-Ras loss in ST88-14 cells instead involves enhanced activation of K-Ras, we transiently transfected ST88-14 cells with a plasmid directing the expression of Myc-tagged K-Ras. Using Raf-1 RBD pulldown assays, we found that N-Ras loss increased the level of activation of
Myc-tagged K-Ras (Fig. 3B). These observations suggest that compensatory activation of K-Ras contributes to the ability of ST88-14 cells to evade the deleterious effects of loss of N-Ras expression. This may indicate that while MPNST cells can compensate for N-Ras loss by increased K-Ras expression and activation, they cannot compensate for K-Ras loss by increasing N-Ras expression.

DISCUSSION

Variable Dependence on K-Ras for Proliferation of MPNST Cells

In this paper, we demonstrate that K-Ras ablation inhibits the proliferation of T265-2c but not ST88-14 MPNST cells (Fig. 1B, 2B). This differential effect was surprising, as both of these lines showed reduced mitogenesis in the presence of DN H-Ras (submitted), which acts as a pan-inhibitor of all three classic Ras isoforms. Since ST88-14 proliferation is still dependent upon classic Ras signaling but does not require either N-Ras or K-Ras, H-Ras may play a more important role in these cells. Although we were unable to test this hypothesis directly with shRNA-mediated ablation of H-Ras, ST88-14 cells express H-Ras at a higher level than most other MPNST cell lines examined (see Chapter 2). Further, ST88-14 cells are sensitive to inhibition by the farnesyltransferase inhibitor (FTI) BMS-186511 (26); FTIs are potent inhibitors of H-Ras activation but are much less effective at inhibiting N-Ras and K-Ras activation (14, 15). However, FTIs also inhibit the post-translational modification and localization of other farnesylated proteins, including the Ras superfamily members Rap1 (27), Rap2 (28) and Rheb (29), so no definite conclusion about the importance of H-Ras isoform signaling in ST88-14 proliferation can be drawn from FTI studies alone.
At this time, it is unclear whether the K-Ras independent proliferation observed in ST88-14 cells is the exception or the rule for MPNSTs. ST88-14 cells show very low K-Ras expression relative to other MPNST cell lines examined (see Chapter 2), making it likely that these cells would be the outlier if K-Ras dependent proliferation was directly correlated to K-Ras expression level. However, two cell lines are insufficient to demonstrate such a correlation, so these studies should be repeated in at least one other high K-Ras and one other low K-Ras MPNST cell line. If these studies do not support a correlation, it is possible that alternative expression of effector molecules or scaffolding proteins underlies the insensitivity of ST88-14 cells to K-Ras loss.

Compensatory activation of other Ras isoforms in ST88-14 cells but not T265-2c cells could also explain the relative resistance of ST88-14 cells to K-Ras loss.

If the factors that control sensitivity to K-Ras loss in MPNSTs can be identified, it may be possible to determine which MPNST patients would benefit from K-Ras targeted therapy. However, no currently available therapeutic agents are capable of selective inhibition of K-Ras, and siRNA-based K-Ras therapies are far from clinical trials. Thus, it may be better to utilize inhibitors capable of inhibiting all three classic Ras proteins. One such inhibitor is salirasib (farnesylthiosalicylic acid), which displaces activated Ras from active signaling microdomains within the inner surface of the cell membrane (30-33) rather than interfering with its post-translational modification. This agent reduces cell viability (34) and invasiveness (35) of MPNST cells in vitro, and is well tolerated clinically (36). However, the in vivo efficacy of salirasib has yet to be determined. Although this reagent is capable of inhibiting all classic Ras isoforms in vitro (30-33), this may not be attainable in vivo when administered at clinically tolerated doses.
N-Ras Loss Does Not Affect Proliferation of MPNST Cells

N-Ras ablation does not affect the proliferation of either of the MPNST cell lines examined (Fig. 1B, 2B), possibly due the ability of MPNST cells to compensate for N-Ras loss by increasing K-Ras expression (Fig. 1A, 2A) and activation (Fig. 3B). It is unclear if N-Ras loss has other phenotypic consequences in MPNST cells. Murine models support a role for both N-Ras and K-Ras in NF1-related tumors, as neurofibromas can be generated by the introduction of either activated N-Ras (37) or activated K-Ras (38) alleles into the Schwann cell lineage. However, activated N-Ras has only been demonstrated to induce the formation of dermal neurofibromas, which as expected did not progress to form MPNSTs (37). Recent results suggest that dermal neurofibromas and plexiform neurofibromas arise from different progenitor populations (39), so it is possible that activated N-Ras preferentially initiates neurofibroma formation in the progenitor population located in the dermis. If this is the case, it would be expected that plexiform neurofibromas and their MPNST derivatives would not be affected by N-Ras loss. Examining the proliferation of dermal and plexiform neurofibroma cells after N-Ras and K-Ras loss may help support this hypothesis.

However, it is also possible that plexiform neurofibromas did not form in the activated N-Ras model because the promoter used to drive expression of this mutant was not active in the peripheral nerve progenitor population. If this is indeed the case, it is possible that N-Ras loss could affect MPNST cells in a distinct manner from K-Ras loss. In particular, the effect of N-Ras loss on survival of serum-deprived MPNST cells should be investigated, as DN H-Ras induces apoptosis in MPNST cells under nutrient poor
conditions (submitted) and the anti-apoptotic properties of the N-Ras isoform have been well established (7, 40-42).

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Figure 1. Neither N-Ras nor K-Ras knockdown affects proliferation or migration of ST88-14 cells. (A) ST88-14 cells stably transduced with doxycycline-inducible shRNA lentiviruses were maintained for 5 days in TF DMEM-10 with or without 2μg/ml doxycycline. Immunoblotting for classic Ras proteins shows specificity of K-Ras and N-Ras shRNAs. GAPDH was used as a loading control. (B) Neither N-Ras nor K-Ras shRNA ablation reduces 3H-thymidine incorporation in ST88-14 cells. (C) Neither N-Ras nor K-Ras shRNA ablation reduces migration in ST88-14 cells.
Figure 2. K-Ras knockdown, but not N-Ras knockdown, affects proliferation of T265-2c cells. (A) T265-2c cells stably transduced with doxycycline-inducible shRNA lentiviruses were maintained for 5 days in TF DMEM-10 with or without 2μg/ml doxycycline. Immunoblotting for classic Ras proteins shows specificity of K-Ras and N-Ras shRNAs. GAPDH was used as a loading control. (B) Induction of K-Ras shRNA, but not N-Ras shRNA, reduces ³H-thymidine incorporation in ST88-14 cells. Two shRNAs targeting different sequences in the K-Ras isoform are shown. (C) Neither N-Ras nor K-Ras shRNA ablation reduces migration in T265-2c cells.
Figure 3. K-Ras activation is increased following shRNA-mediated ablation of N-Ras in ST88-14 cells. (A) MPNST cell lines (ST88-14, STS-26T, T265-2c) and non-MPNST cancer lines (Daoy, MCF-7, SKN-MC) were immunoblotted for H-Ras, N-Ras, and K-Ras isoforms. (B) ST88-14 cells stably transduced with doxycycline-inducible shRNAs were transiently transfected with Myc-tagged wild-type K-Ras and cultured for 3 days in DMEM-10 with or without 2μg/ml doxycycline. Activation of the ectopically expressed Myc-tagged K-Ras isoform was assessed with the Raf-1 RBD assay.
CONCLUSIONS

In this dissertation, we describe the need to understand which neurofibromin-regulated Ras proteins contribute to MPNST pathogenesis. We furthermore demonstrate that the classic Ras subfamily and R-Ras subfamily proteins may contribute to this process in distinct ways. While both classic Ras subfamily proteins and R-Ras subfamily proteins appear to promote proliferation, only classic Ras subfamily proteins promote survival in nutrient-poor conditions and only R-Ras subfamily proteins promote MPNST migration. We also demonstrate that individual Ras isoforms may have distinct roles in this process, as K-Ras loss in T265-2c cells reduces proliferation to a similar extent as pan-classic Ras inhibition with DN H-Ras. These studies have important implications for the use of Ras-targeted therapeutics in MPNSTs.

Classic Ras and R-Ras Subfamily Proteins in Survival, Proliferation and Migration – Clinical Implications for Tumor Debulking and Metastasis

Inhibition of classic Ras proteins but not R-Ras proteins inhibited MPNST survival in vitro, suggesting that therapeutic agents targeted at classic Ras proteins may be more efficacious at tumor debulking than those targeted at R-Ras proteins. However, this effect was fairly modest under nutrient rich conditions, only becoming substantial when cells were stressed by serum deprivation. Although poor perfusion due to leaky vasculature often creates an environment of nutrient and oxygen deprivation in tumor cells in vivo, these conditions are neither pervasive nor constant. Instead, they exist
within microregions of the tumor (25, 26), and the perfusion of these regions may fluctuate over time (27). Thus, total serum deprivation is unlikely to occur within the tumor bed for any significant period of time, and poor perfusion would limit delivery of a therapeutic agent to any persistently deprived tumor microregions. This in turn suggests that inhibition of classic Ras proteins may induce apoptosis in only a small percentage of tumor cells \textit{in vivo}, resulting in modest tumor debulking. It would be interesting to determine whether concurrent inhibition of both classic Ras and R-Ras proteins enhanced the apoptotic effects of classic Ras inhibition, as this would indicate that targeting both subfamilies of Ras proteins might be more effective in causing tumor debulking \textit{in vivo}.

Targeting both subfamilies of Ras proteins would likely be needed to effectively impair tumor proliferation, as both classic Ras and R-Ras subfamily proteins were found to mediate MPNST proliferation \textit{in vitro}. Although impeding tumor proliferation would not be sufficient to cause tumor regression, it might be sufficient to maintain the tumor volume at a static level for a period of time, particularly if tumor debulking can first be accomplished by surgical resection and post-operative radiotherapy. This suggests that therapeutic agents directed against both classic Ras and R-Ras proteins may be useful as adjuvant therapy for MPNSTs, potentially improving quality of life and/or survival in cases where complete surgical resection cannot be achieved.

Targeting R-Ras subfamily proteins, on the other hand, may be more beneficial at preventing tumor metastasis. MPNSTs have a high rate of distal metastasis (28, 29), resulting in significant morbidity and mortality in afflicted patients. To form metastases, tumor cells must invade through the basement membrane of their parent tissue, extravasate into tissue capillaries, travel through the vasculature, intravasate through the
capillary walls at a secondary site and proliferate at this secondary site (30). Although cellular motility alone does not predict metastatic potential, cellular migration is an essential part of the metastatic process. Our demonstration that MPNST migration depends upon R-Ras proteins suggests that inhibiting these proteins may impede MPNST metastasis, particularly as R-Ras proteins have previously been linked to metastasis of other tumor types (31, 32). This therapeutic strategy would likely be most beneficial in patients who present with non-metastatic disease where complete surgical resection cannot be achieved.

In total, our data suggests that a therapeutic agent against classic Ras and R-Ras subfamily proteins may be most useful as adjuvant therapy to impaire tumor regrowth and metastasis after surgical resection and post-operative radiotherapy. However, much more preclinical work will be required to find a compound with efficacy against both classic Ras and R-Ras subfamily proteins.

**Therapeutic Agents Targeted Against Ras Proteins**

Most of the existing Ras inhibitors function by inhibiting post-translational modification (PTM) of Ras proteins, which inhibits their subsequent trafficking to the plasma membrane. These include inhibitors of isoprenylation at the CAAX motif (farnesyltransferase inhibitors (FTIs) and geranylgeranyl-transferase inhibitors (GGTIs)), inhibitors of CAAX proteolysis (Reel inhibitors), and inhibitors of methylation at the exposed C-terminal cysteine residue (Icmt inhibitors). However, the enzymes inhibited by these agents are critical for PTM of many proteins other than Ras, and none have yet been shown to inhibit all of the Ras proteins negatively regulated by neurofibromin.
The earliest Ras-targeted agents, FTIs, were predicted to inhibit all three classic Ras isoforms. Instead, they were found to effectively reduce only H-Ras activity, as N-Ras and K-Ras can be geranylgeranylated in the presence of FTIs (33, 34), allowing them to escape inhibition by FTI monotherapy. R-Ras2 is also a good substrate for both the farnesyltransferase and the geranylgeranyltransferase enzymes (35), and R-Ras seems to be preferentially geranylgeranylated (36), making it likely that these two isoforms would also escape inhibition by FTIs. Thus, FTI treatment seems unlikely to benefit MPNST patients.

Because the classic Ras isoforms are preferentially farnesylated, single therapy GGTIs should not affect their activity, and indeed, many believe that clinical response to GGTI therapy in other cancer types is due to inhibition of the Rho GTPase proteins (37-39) rather than Ras. Targeting both farnesyltransferase and geranylgeranyltransferase – either with FTI/GGTI combination therapy or with dual prenylation inhibitors (DPIs) such as L-778,123 – may be more effective at inhibiting Ras activity, and many in vitro studies support the higher efficacy of these treatments as cancer therapeutic agents (40-42). However, at least some preclinical studies have found that therapeutic agents targeting GGT, either alone or in combination with FTIs, have dose-limiting toxicities below the level required to effectively inhibit K-Ras isoprenylation (43), which may limit their utility in the clinical management of NF1-related tumors.

Ras isoprenylation can also be reduced by the cholesterol-lowering statin drugs, whose inhibition of the HMG coA reductase enzyme lowers levels of mevalonate, a critical precursor for the synthesis of both the farnesyl and geranylgeranyl isoprenoid groups (44, 45). Statins as therapeutic agents for NF1-related phenotypes have only
recently been investigated, and while these compounds have shown promise in preclinical models for NF1-related learning deficits (46), MPNST growth (47, 48) and bone-healing defects (49), their clinical performance so far has been disappointing (50). As with FTIs and GGTIs, this may be partly due to differential activity against specific Ras isoforms. While a definitive study of statin inhibition of specific Ras isoforms has not yet been conducted, it seems reasonable to hypothesize that in the context of limited farnesyl and geranylgeranyl groups, those proteins with the highest affinity for the FTase and GGTase enzymes would be preferentially isoprenylated. As K-Ras has been shown to have a higher affinity for FTase than H-Ras and N-Ras (51, 52), and K-Ras isoprenylation has been shown to be unaffected by concentrations of lovastatin that reduced RhoA and C isoprenylation (53), it seems likely that K-Ras at least could escape inhibition by lower-dose statin regimens by this mechanism. Alternatively, mevalonate may be preferentially shuttled into synthesis of farnesyl groups, making farnesylated proteins more resistant to statin therapy than geranylgeranylated proteins. Regardless, the statin concentration required to effectively inhibit all of the Ras proteins negatively regulated by NF1 may be impossible to attain in a clinical setting (54).

Rce1 and Icmt inhibitors have been more recently described. Agents targeting Rce1 have been shown to cause mislocalization of Ras in yeast (55), but no information is yet available about the effects of Rce1 inhibitors on Ras isoform localization or anti-cancer properties in mammalian cells. Cysmethynil, a small molecule inhibitor of Icmt, has been shown to cause Ras mislocalization (56) and induce autophagic cell death in both in vitro (57) and in vivo (58) models of mammalian cancer. However, it is unclear whether
this compound will be equally effective at inhibiting all of the neurofibromin-regulated Ras proteins.

Following the failure of Ras PTM inhibitors to live up to their initial promise, investigators began to devise alternative strategies for targeting Ras proteins. Only one alternative agent has been currently described: farnesylthiosalisylic acid (FTS). FTS has been proposed to mimic the structure of the farnesylated classic Ras proteins, allowing it to occupy active signaling microdomains within the plasma membrane and inhibit Ras function by displacing Ras proteins from these regions (59, 60). Although it has been shown to affect H-Ras (60, 61), N-Ras (62), and K-Ras (63, 64) localization, its effect on the normally geranylgeranylated R-Ras proteins has yet to be determined. As the factors controlling Ras microdomain localization are not well understood, it seems possible that the presence of geranylgeranyl group rather than a farnesyl group could alter the microdomain localization of R-Ras proteins, allowing them to resist inhibition by FTS. Nonetheless, FTS has been shown to normalize classic Ras-GTP levels and inhibit growth of MPNST cells in xenograft models (65). Interestingly, FTS has also been shown to cause dissociation of the mTOR-raptor complex (66) and inhibit some functions of mast cells (67). As the mTOR signaling cascade has been implicated in MPNST pathogenesis (68, 69), and mast cells have well-studied roles in neurofibroma formation (70-72), this agent may ameliorate NF1-related phenotypes in ways other than targeting Ras hyperactivation in neoplastic Schwann cells. However, its efficacy in a clinical setting has yet to be determined.

Clearly, more work is needed to understand the specificity of the described agents for the neurofibromin-regulated classic Ras and R-Ras proteins. It seems unlikely that a
single agent will effectively inhibit all of these proteins, suggesting that combinatorial therapy may prove more effective in treating NF1-related tumors. However, combination therapy predicted to affect both classic Ras and R-Ras protein activity has thus far proved to have far more profound side effects than monotherapy (43), leading to an undesirable therapeutic index for these combined regimens. This may be because these agents have broad effects within the Ras superfamily of GTPases, allowing them to inhibit many molecules other than classic Ras and R-Ras proteins. Thus, new therapeutic agents may be required to effectively target neurofibromin-regulated Ras proteins in MPNSTs.

**Targeting Individual Ras Proteins Clinically**

The demonstrated ability of K-Ras loss to reduce proliferation in T265-2c cells suggests another therapeutic avenue for MPNST cells – inhibition of individual Ras proteins. If a subset of MPNSTs are dependent upon K-Ras activation for proliferation, it is possible that inhibiting K-Ras alone would be as effective in these tumors as joint inhibition all of the classic Ras proteins. Likewise, if shRNA-ablation of either R-Ras or R-Ras2 can be found to reduce proliferation and/or migration to the same extent as DN R-Ras, single R-Ras protein inhibition might be beneficial in MPNST treatment. This also raises the possibility that combined therapy against classic Ras and R-Ras proteins could be achieved by inhibition of just two proteins, which might produce fewer side effects that combinatorial therapy capable of inhibiting all five neurofibromin-regulated Ras proteins expressed in MPNST cells. However, finding a chemotherapeutic agent or combination of agents to inhibit both proteins may still prove challenging. Directly
ablating these proteins with RNAi may be more feasible in the long term if RNAi-based therapeutic agents hold up to the early promise they have demonstrated in preclinical and clinical trials (73).

Obviously, much more work is needed to confirm the effects of single classic Ras or R-Ras protein loss on MPNST cells as well as to establish whether molecular subtypes of these tumors characterized by dependence upon different Ras proteins exist.

**Future Directions**

Although these studies have yielded valuable information about the roles of Ras proteins in MPNST cells, further avenues of investigation remain. Studies of the phenotypic consequences of K-Ras loss should be expanded into more MPNST cell lines given the differential effects of K-Ras ablation on ST88-14 and T265-2c cells. The effect of N-Ras and K-Ras loss on MPNST survival should also be examined, as pan-inhibition of classic Ras proteins was found to induce apoptosis in MPNST cells. Single-isoform ablation studies of R-Ras proteins should also be performed; it may be possible that the R-Ras subfamily affects proliferation and migration through distinct isoforms. Finally, the role of R-Ras proteins in stimulating migration through microtubule-dependent events deserves further investigation. Determining whether R-Ras subfamily inhibition affects microtubule stability, post-endocytic microtubule-dependent trafficking or both would be an important first step in this process.
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APPENDIX A

INSTITUTIONAL REVIEW BOARD FOR HUMAN USE EXEMPTION FORM
DATE:  5/22/06

MEMORANDUM

TO:    Nicole Brossier
        Principal Investigator

FROM:  Sheila Moore, CIP
        Director, IRB

RE:    Request for Determination—Human Subjects Research
        IRB Protocol #N071106003 – Regulation of Ras Isoforms in Malignant
        Peripheral Nerve Sheath Tumors

An IRB Member has reviewed your application for Designation of Not Human Subjects
Research for above referenced proposal.

The reviewer has determined that this proposal is not subject to FDA regulations and is
not Human Subjects Research. Note that any changes to the project should be resubmitted to the
Office of the IRB for determination.

SM/hw
APPENDIX B

MENTOR LETTER CONCERNING MODIFICATIONS TO IACUC APPROVAL
UNDER APN1002S0363
October 24, 2011

UAB Graduate School

To Whom It May Concern:

Nicole Brossier obtained IACUC approval for her dissertation project in 2008 based on predicted animal experiments. However, these animal experiments were not performed and her project was completed using only human cell lines. Thus, her IACUC approval was allowed to lapse, as approval for animal studies was no longer relevant.

Best Wishes,

[Signature]

Steven L. Carroll, M.D., Ph.D.
Professor of Pathology, Cell Biology and Neurobiology
Director, UAB Division of Neuropathology
Director, UAB Brain Resource Program
Director, UAB Cellular and Molecular Neuropathology Core