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14. ABSTRACT

This report describes the sixth year of research, and the first under this award, by a Consortium of investigators who have been continuously funded by this Program to develop, characterize, and utilize strains of mice that accurately model tumors found in persons with NF1 and NF2. This Consortium has generated many novel models of NF1 and NF2-associated tumors and has exploited these strains to investigate biologic and preclinical questions. In this fund year, the Consortium organized a scientific conferences on the on the use of mouse models of NF-associated tumors to test new therapies, which resulted in a review article that addressed this general topic with specific reference to NF. The investigators have collaborated closely with each other and have shared expertise and reagents extensively. This NF Consortium is a member of the Mouse Models of Human Cancer Consortium of the National Cancer Institute and is participating fully in the activities of the group.

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INTRODUCTION

Benign and malignant tumors are a major cause of morbidity and mortality in individuals afflicted with NF1 and NF2. The *NF1* and *NF2* genes function as tumor suppressors in humans and mice. Although a great deal has been learned about the genetics, biochemistry, and cell biology of NF1 and NF2-associated tumors, it has proven difficult to translate these advances into new treatments. The development of accurate, well-characterized mouse models of NF-associated tumors NF1 and NF2 represent an invaluable resource for bringing improved treatments to NF patients. The overall purpose of this consortium, which has been in existence for 6 years, is to develop such models so that they will serve as permanent resources for the scientific community. These efforts are timely for a number of reasons.

First, advances in gene targeting technologies have made it feasible to introduce many types of alterations into the mouse germline. Indeed, the members of this research consortium developed the initial strains of *Nf1* and *Nf2* mutant mice, which provided major insights into a number of the complications seen in human NF1 and NF2 patients. Since the inception of this consortium effort in 2000, we have made substantial progress by engineering conditional mutant alleles of both *Nf1* and *Nf2* and in using these to create tractable new models for biologic and preclinical studies. Second, investigating cells from these *Nf1* and *Nf2* mutant mice has provided numerous fundamental insights that are directly relevant to understanding deregulated growth in *NF1* and *NF2*-deficient human cells. Genetic analysis of human and murine tumors has provided compelling evidence that *NF1/Nf1* and *NF2/Nf2* function as tumor suppressor genes (TSGs) *in vivo.* Biochemical data have suggested target proteins and pathways for rational drug design. The improved mouse models developed by this consortium also provide invaluable platforms for rigorous preclinical trials of these innovative approaches. Third, new therapies are urgently needed for many of the tumors that arise in individuals with NF1 and NF2. The current treatments for neurofibroma, optic nerve glioma, vestibular schwannoma, and for NF1 and NF2-asociated malignancies are frequently ineffective and carry a substantial risks of long-term morbidities. This consortium is highly complementary to the ongoing efforts of the NF Clinical Trials Consortium that is supported by this Program, which involve investigating novel treatments in NF patients, as the models that we have created facilitate testing novel agents and approaches in a controlled preclinical setting. The quantity of drug required, expense, time to obtain data, and potential liability are all either greatly reduced or eliminated in mouse models. These strains therefore facilitate testing a wide range of new therapies that might benefit NF patients. Finally, the Mouse Models of Human Cancer Consortium (MMHCC) of the National Cancer Institute (NCI) is providing an opportunity for interactions among ~20 research groups that are working to develop, validate, and enhance models of a variety of human cancers. NF is the only inherited cancer predisposition represented within the MMHCC as a discrete disease entity. Our group was admitted to the MMHCC in 2000 and has been participating in its activities. Drs. Jacks, Parada, and Shannon are members of the MMHCC Steering Committee, with Dr. Parada serving as the designated representative of the NF Consortium. Dr. Jacks was Co-Chair of the Steering Committee from the inception of the MMHCC until 2002, and Dr. Shannon served as Co-Chair from 2002-2005. Thus, this award has provided the NF research community with an exceptional level of representation within the mouse cancer modeling community. The MMHCC is spearheading efforts in areas such as building repositories, devising pathologic classification schemes, imaging mouse tumors, and stimulating interactions with

industry in the area of preclinical therapeutics that are of general importance to NF research. Our work under this award focuses on the two overall technical objectives (aims) listed below.

(1) To enhance existing strains of *Nf1* and *Nf2* mutant mice and to develop new *in vivo* models of NF-associated tumors. We will fully characterize lesions that arise in these mice, focusing on how closely they reproduce the phenotypic, genetic, and biochemical alterations seen in comparable human tumors.

(2) To perform *in vitro* and *in vivo* studies to elucidate biochemical pathways underlying the proliferative advantage of *Nf1* and *Nf2*-deficient cells as a way of identifying molecular targets for therapeutic interventions.

These technical objectives included a series of studies that were organized under specific subheadings in our application. For clarity, this Progress Report follows the same general format.

BODY

Background

Tumor Spectrum in NF1 and NF2 Patients. Persons with NF1 are predisposed to benign neurofibromas, optic nerve gliomas, and to specific malignant neoplasms. Individuals with NF1 typically develop multiple neurofibromas that can result in cosmetic, orthopedic, and neurologic disabilities. Optic nerve gliomas are another vexing clinical problem. Although histologically benign, these tumors frequently cause visual impairment or blindness because of their anatomic location. The malignant neoplasms seen in NF1 patients include astrocytoma, malignant peripheral nerve sheath tumor (MPNST), pheochromocytoma, and juvenile myelomonocytic leukemia (JMML). NF2 affects 1 in 40,000 persons worldwide. Other NF2-related tumors include meningiomas, gliomas, and ependymomas.

Production and Characterization of *Nf1* and *Nf2* Mutant Mice. Drs. Jacks and Parada independently disrupted *Nf1* by inserting a neomycin *(neo)* cassette into exon 31 (1, 2). Homozygous *Nf1* mutant ($Nf1^{-/-}$) embryos die *in utero* with cardiac anomalies, which precludes the use of these mice to study important aspects of NF1 pathology, including the formation of many tumor types. To circumvent this problem, Dr. Parada's lab harnessed *Cre-loxP* technology to create a conditional *Nf1* allele (3). Importantly, the Parada's lab showed that the $Nf1^{flox}$ allele functions as a wild-type allele in spite of harboring *loxP* sites and a *neo* gene within its intronic sequences. The $Nf1^{flox}$ allele is readily recombined *in vivo* to make a null allele through co-expression of *Cre* recombinase. Drs. McClatchey, Jacks, and Giovannini used gene targeting to disrupt the *Nf2* locus (4, 5). Homozygous *Nf2* mutant embryos failed without initiating gastrulation. Although heterozygous *Nf2* mutant mice are cancer prone, these animals do not develop schwannoma or meningioma. To circumvent the early embryonic-lethal phenotype associated with homozygous inactivation of *Nf2* and to test the hypothesis that the tumor spectrum might be modulated by the rate of the loss of the normal allele in specific tissues, Dr. Giovannini and his colleagues generated a conditional mutant *Nf2* allele (6). As expected, mice homozygous for the $Nf2^{flox2}$ mutant allele ($Nf2^{flox2/flox2}$) were viable and fertile suggesting that the introduction of *loxP* sites did not hamper *Nf2* expression. Induced expression of *Cre* recombinase

in *Nf2*$^{flox2/flox2}$ mice results in biallelic inactivation of *Nf2* in specific tissues. A long standing research goal of this Consortium has involved exploiting these conditional mutant alleles of *Nf1* and *Nf2* to develop tractable models of NF-associated tumors for use in our labs and by other investigators. A list of investigators who have received these mice appears in the Reportable Outcomes section.

Models of NF1 and NF2-Associated Tumors. In work published prior to date, the participants in this consortium reported the phenotypic and biologic features of NF1-associated mouse tumor models of MPNST/Triton tumor, astrocytoma, JMML, plexiform neurofibroma, and chemotherapy-induced leukemia, sarcoma, and breast cancer (7-12) and of NF2-associated tumors such as vestibular Schwannoma and meningioma (5, 6). These data are also described in detail in previous reports.

Progress Report

Technical Objective (Aim) 1: To produce and characterize models of NF-associated tumors.

Signal Transduction in Primary *Nf1*-Deficient Hematopoietic Cells. The Shannon lab generated strains of mice that develop a fatal myeloproleferative disorder (MPD) that models JMML by conditionally expressing oncogenic *Kras* (*Mx1-Cre, LSL-Kras*^{G12D}) (13, 14) or ablating *Nf1* (*Mx1-Cre, Nf1*$^{flox/flox}$) (15) in hematopoietic cells. Mutant *Kras* is expressed from its endogenous promoter in *Mx1-Cre, LSL-Kras*^{G12D} mice by Cre-mediated excision of a "loxP-stop-loxP" (LSL) transcriptional repressor. These *Kras*^{G12D} mice develop a fatal MPD with 100% penetrance that progresses rapidly with leukocytosis, anemia, splenomegaly, and death at a median age of 105 days. Bone marrow cells demonstrate profound hypersensitivity to the granulocyte-macrophage colony stimulating factor (GM-CSF) and form abnormally large myeloid progenitor colonies in methylcellulose cultures stimulated with low concentrations of myeloid growth factors. In another model of JMML that was developed under an award from this Program, the conditional mutant *Nf1* allele generated by Dr. Parada was inactivated by *Mx1-Cre*-mediated excision. *Nf1* mutant mice also develop MPD, but the disease course is less aggressive with almost all mice surviving for 6 months and about 15% living longer than 1 year (15). Myeloid progenitors from *Mx1-Cre, Nf1*$^{flox/flox}$ mice are hypersensitive to GM-CSF, but the pattern of growth is less abnormal than in *Kras* mice. However, biochemical investigation of bone marrow cells from *Mx1-Cre, Nf1*$^{flox/flox}$ and *Mx1-Cre, LSL-Kras*^{G12D} mice with MPD surprisingly revealed minimally elevated levels of the activated (phosphorylated) forms of the major Ras effectors MEK, ERK, and Akt. There are a number of potential explanations for these data including: (1) the experimental conditions used to starve and stimulate cells could have masked aberrant activation of effector cascades; (2) it is possible that primary *Nf1*-deficient cells respond to hyperactive Ras by remodeling signaling networks over time; and/or (3) hyperactive signaling within a minor subpopulation of immature myeloid cells might not be detected in lysates of whole bone marrow or spleen. The Shannon lab has been investigating these possibilities over the past year, which has included a focused effort to adapt fluorescence activated cytometric (FACS) methodologies to analyze phosphorylated signaling molecules in subpopulations of hematopoietic stem and progenitor cells.

Developing Biochemical Signatures of *Nf1* Mutant Bone Marrow Cells. The Shannon lab first assessed different conditions for assaying signaling in wild-type (WT) and *Mx1-Cre, Nf1*$^{flox/flox}$ bone marrow from mice with MPD. Incubating BM cells in IMDM containing 1% BSA for 2.5 hours before stimulation uncovered dramatic differences between WT and *Nf1* mutant cells without affecting cell appearance or survival (data not shown). Serum unexpectedly interferes with the ability of cells to down-regulate Ras signaling *in vitro* and obscures differences between WT and *Nf1* mutant cells. To obtain a broad view of how *Nf1* inactivation modulates the signaling network in response to GM-CSF, the Shannon lab interrogated multiple proteins commonly implicated in growth control **(Fig. 1A).** Under optimized conditions bone marrow cells from *Mx1-Cre, Nf1*$^{flox/flox}$ mice were starved in IMDM/BSA and stimulated with GM-CSF, 10% serum, or both to assess their specific contributions. Among a large number of signaling molecules, MEK, Akt, STAT5, p38, JNK, p70^{S6K} (S6K) and p90^{RSK} (RSK) were selected as "nodal" proteins for detailed investigation. Many of these molecules show enhanced phosphorylation in *Nf1* mutant cells, which are strongly modulated by the stimulating conditions used (GM-CSF alone, serum alone, or both). Interestingly, and in contrast to *Kras* mutant cells (data not shown), cells from *Mx1-Cre, Nf1*$^{flox/flox}$ mice with MPD cells did not display increased basal levels of any signaling protein. However, these cells showed higher levels of pMEK and pSTAT5 in response to GM-CSF; slightly higher phosphorylation of RSK and S6K in response to serum, and marked hyper-phosphorylation of RSK, Akt, MEK and S6KK when exposed to both serum and G-GCF **(Fig. 1B).** These data demonstrate that comparing the effects of multiple stimulating conditions on a group of nodal proteins provides more complete information on how loss of *Nf1* perturbs signaling in diseased cells. Examining an entire network has important implications for therapeutic strategies using small molecule inhibitors, which have traditionally focused on the use of agents such as rapamycin that inhibit a single biochemical target downstream of hyperactive Ras.

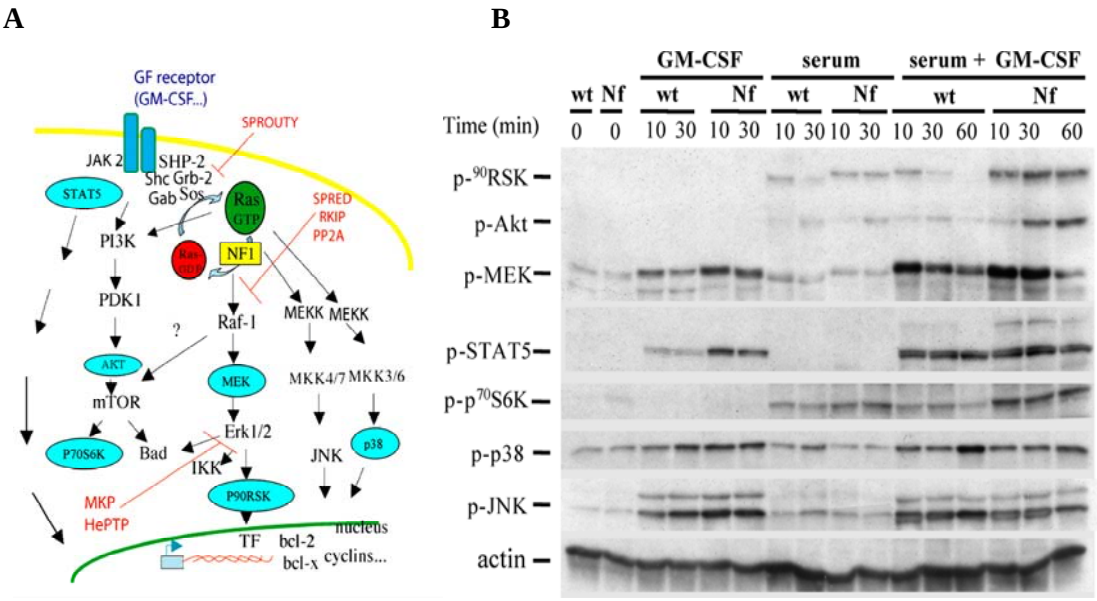


Figure 1. ***Multi-signaling protein analysis.*** *A) Tentative GM-CSF signaling network. In blue the selected nodal proteins. B) Comparison of the nodal phospho-proteins in unsorted BM cells from wild-type (wt) and Nf1 mutant mice. After 2.5 h of serum starvation, cells were stimulated with GM-CSF, serum, or both. Cells were collected at the indicated time points, lysed, and subjected to Western blotting analysis for the indicated phospho-proteins.*

Attenuated Ras Signaling in a Subset of *Nf1* Mutant Bone Marrow Cells. The experiments presented in the previous section were performed in *Mx1-Cre, Nf1*$^{flox/flox}$ mice that died with overt MPD. However, a group of mice that typically had long survival and developed relatively mild MPD as evidenced by lower leukocyte counts and less severe splenomegaly surprisingly showed attenuated RSK and Akt signaling, which was dramatic in some cases (**Fig. 2**). Attenuation was also observed in Mac1+ cells and in cultured macrophages from these mice (data not shown). Together, these provocative data raise the possibility that some *Nf1* mutant mice adapt to hyperactive Ras signaling by dynamically remodeling signaling networks *in vivo*. The Shannon lab is pursuing their unexpected observations, which first necessitates generating mice on a uniform F1 C57Bl/6 x 129Sv background. These crosses have been now completed and cohorts of *Mx1-Cre, Nf1* compound mutant mice are being aged.

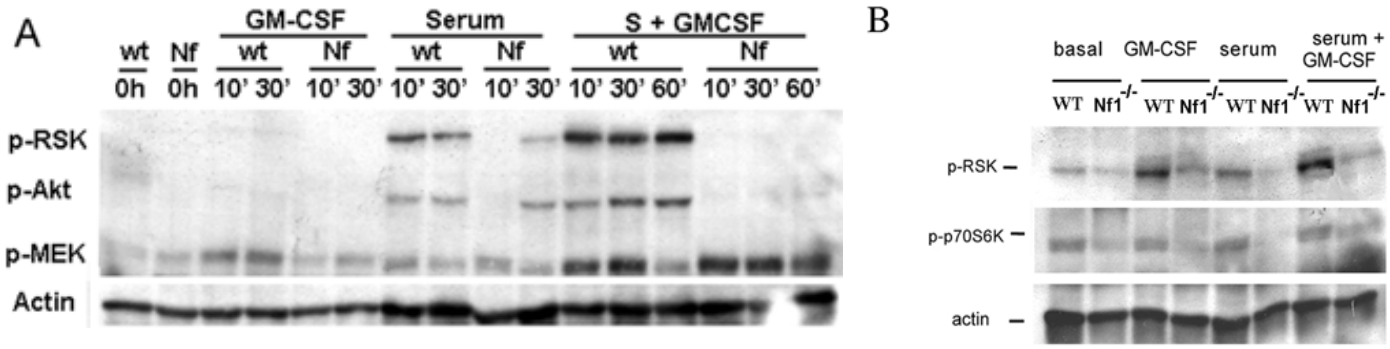


Figure 2. ***Attenuated Signaling in Nf1***$^{-/-}$ ***mice****. A) This experiment reflects Nf1*$^{-/-}$ *bone marrow cells that show dramatic attenuation in RSK and Akt phosphorylation. B) Cultured macrophages from Mx1-Cre, Nf1*$^{flox/flox}$ *with attenuated signaling profile also showed an attenuated signaling profile.*

Multiparameter FACS Analysis of Hematopoietic Stem/Progenitor Cells in *Kras* and *Nf1* Mice with MPD. Despite the differences observed between WT and *Nf1* mutant bone marrow cells, Western blot analysis of whole tissues cannot discriminate among discrete lineages. This is a major limitation as the cell populations found in diseased tissues frequently differ substantially from their normal counterparts. For example, the bone marrows of *Mx1-Cre, Nf1*$^{flox/flox}$ mice with MPD are almost entirely effaced by myeloid lineage cells with loss of normal lympohcyte and erythroid populations. Unfortunately, Western blotting is impractical for interrogating the activation status of signaling molecules in small subpopulations of stem/progenitor cells because these cells are rare (1-2% of total bone marrow). The Shannon lab has therefore been working to adpat FACS-based methodologies to measure phosphorylated signaling proteins in murine hematopoietic cells. These studies in *Kras* mutant mice are supported by NIH grant R01 CA72614. The Shannon lab first validated the ability of phospho-specific antibodies used for FACS analysis to detect changes in phosphorylation induced by exposing a Ba/F3 cells to interleukin-3 (IL-3), and showed that FACS analysis correlated with Western blotting (data not shown). They used bone marrow cells from *Mx1-Cre, Kras*^{G12D} mice with MPD for initial "proof of concept" studies because aberrant signaling is more severe and uniform in this strain that in *Nf1*-deficient mice. Adapting this methodology for use in mouse bone marrow required considerable optimization (rehydrating surface proteins, blocking non-specific antibody interactions, and performing multiple washes gave the best results). The experimental protocol available through http://itsa.ucsf.edu/~kmslab/resources reliably detects the lineage-specific

surface markers Mac1, Gr1, CD3, CD4, CD8, B220, and TER119, as well as the stem and progenitor cell marker c-kit. Cells defined by expression of c-kit and no (or low) expression of mature lineage markers (c-kit^{+} lin$^{-/dim}$) comprise 1-2% of nucleated marrow cells and are enriched for hematopoietic stem and progenitor cells. Although still diverse, this population is accessible for phospho-specific FACS analysis and allows comparison of signaling in WT and *Kras*^{G12D} cells with similar immunophenotypic and functional properties (**Fig. 3A**). The Shannon lab found that 3-10% of both WT and *Mx1-Cre, Kras*^{G12D} c-kit^{+} lin$^{-/dim}$ cells formed myeloid progenitor colonies in methylcellulose, and that the c-kit^{+} lin$^{-/dim}$ population included 85-94% of these cells within the marrow (data not shown).

Phospho-Protein Profiles in c-kit^{+} lin$^{-/dim}$ Cells. An important observation is that c-kit^{+} lin$^{-/dim}$ cells displayed a different phospho-protein profile than whole bone marrow with FACS analysis. Under unstimulated conditions pERK levels were similar in ungated WT and *Mx1-Cre, Kras*^{G12D} bone marrow cells but higher in gated c-kit^{+} lin$^{-/dim}$ cells from *Mx1-Cre, Kras*^{G12D} mice compared to the same subset in WT. (**Fig. 3B**). In response to GM-CSF the difference in pERK levels between *Mx1-Cre, Kras*^{G12D} mice compared to WT was bigger in c-kit^{+} lin$^{-/dim}$ cells from than in ungated cells (**Fig. 3B**). As with pERK, basal levels of pSTAT5 and pS6 were elevated in c-kit^{+} lin$^{-/dim}$ cells from *Mx1-Cre, Kras*^{G12D} mice compared to WT. Furthermore *Mx1-Cre, Kras*^{G12D} marrow exhibits a greater proportion of cells that respond to GM-CSF with a prolonged activation of all three signaling proteins (**Fig. 3B**).

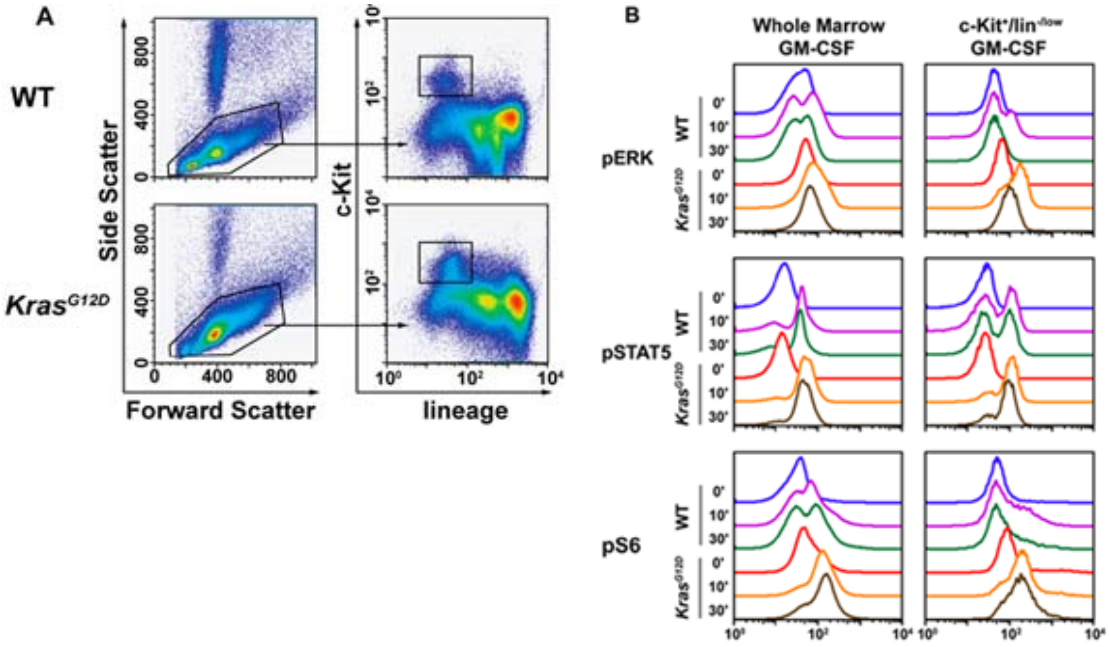


Figure 3. ***Intracellular FACS analysis demonstrates that c-kit^{+}lin$^{-/dim}$ cells have a distinct signaling profile that varies with cytokine stimulus***. *Panel A. c-kit^{+}lin$^{-/dim}$ cells were isolated by first gating live, non-neutrophil cells based on scatter properties and then gating cells that expressed c-kit and had no or low expression of the mature lineage markers. Serum- and cytokine-starved whole bone marrow from WT and Mx1-Cre, Kras^{G12D} mice was stimulated with GMCSF (10 ng/ml) for 10 or 30 minutes. Levels of phosphorylated ERK, STAT5, and S6 were measured in the whole marrow (Panel B left) and in the c-kit^{+}lin$^{-/dim}$ subset (Panels B right) Fluorescence intensity is depicted on a log scale, which tends to make differences appear smaller.*

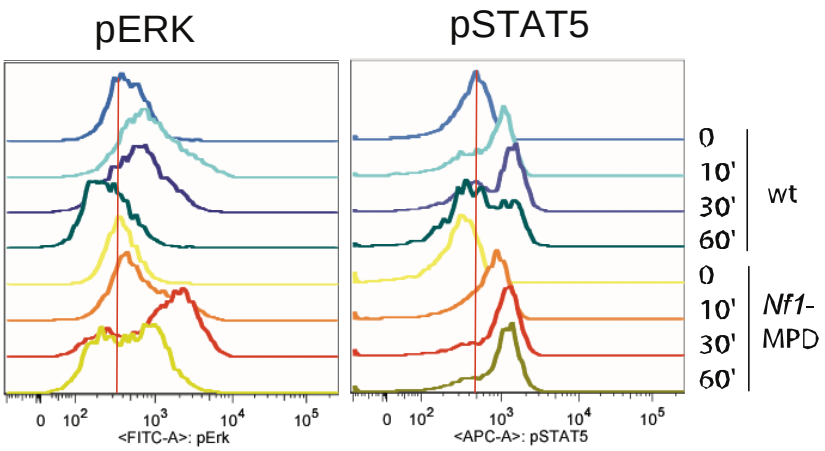


Figure 4. ***Phospho-signaling analysis of WT and Nf1 mutant c-kit^{+} lin$^{-/dim}$ cells***. *Nf1 mutant cells show delayed activation of pERK in response to GM-CSF that is prolonged compared to WT cells (left panel). Basal pSTAT5 levels are lower in Nf1-/- cells but remain elevated for longer after stimulation (right panel).*

The Shannon lab recently applied this technology to investigate signaling in c-kit^{+} lin$^{-/dim}$ bone marrow cells from *Nf1* mice with MPD. Preliminary studies support the idea that *Nf1* inactivation has distinct effects on cellular signaling networks. Whereas unstimulated c-kit^{+} lin$^{-/dim}$ cells from *Kras* mutant mice show elevated levels of phosphorylated ERK, this is not true of *Nf1*-deficient cells (**Fig. 4**). *Nf1* mutant cells also showed a distinct pattern of pERK and pSTAT5 activation in response to GM-CSF with maximal levels of phosphorylation achieved after 30 minutes. Importantly, these levels remain elevated 60 minutes after stimulation in Nf1 mutant, but not WT c-kit^{+} lin$^{-/dim}$ cells (**Fig. 4**). The Shannon lab is currently developing reagents to interrogate other signaling components by FACS in primary bone marrow cells. A major goal of the Shannon lab in next 2 fund years is to characterize how *Nf1* loss perturbs the overall architecture of Ras signaling networks in primary hematopoietic stem/progenitor cells, and to investigate the effects of specific inhibitors.

Local and Temporal Control of *Nf1* Inactivation. The Parada lab extended their neurofibroma model (8) by adding a *p53* mutation to the SC-specific ablation of *Nf1*. The resultant mice initially appeared normal but began exhibiting a phenotype reminiscent of the *Krox20-Cre;*

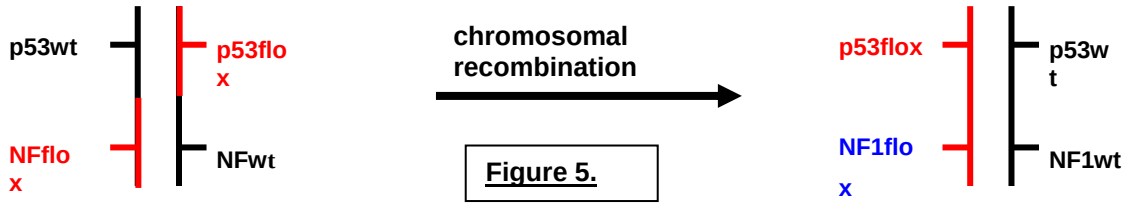


Figure 5.

Floxed alleles in trans when crossed to wt, generate pups floxed at either locus but never at both loci.

Floxed alleles in cis when crossed to wt, breed true for both alleles such that pups are either wt or floxed at both alleles.

Nf1^{flox} mice around 4 months of age. Necropsy and histologic analysis of these *Nf1^{flox}; p53-/-* mice revealed accelerated appearance of neurofibromas. The use of laser capture microdissection (LCM) permitted isolation and molecular analysis of benign and malignant tumor samples, which provided preliminary indication that only the malignant tumor samples show *p53* LOH. Confirming these initial data has been complicated by the appearance of p53-related sarcomas and lymphomas at higher rates than previously observed. To circumvent this problem, the Parada lab has generated a mouse strain that carries a recombinant chromosome harboring both the

Nf^{flox} and a *p53*^{flox} allele in *cis* (**Figs. 5 and 6**). These mice now provide us with a means to continue the proposed studies with greater facility, reducing the breeding and the confounding effects of the germline *p53* mutations.

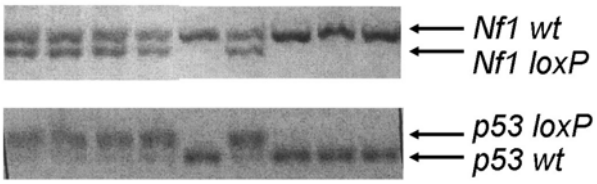


Figure 6. *Representative PCR analysis on loxP-Nf1: loxP-p53 cis mice indicate that both loxP alleles are present at the same mice in a litter derived from transNFflox;p53flox mice bred with wild type mice (see **Fig. 5**), confirming cis position of the loxP alleles in two tumor suppressor genes.*

As described below, the Parada lab will further compare the MPNSTs arising in this sequential model but exploiting this new and vastly improved mouse lines (**Fig. 5**) with the MPNSTs that they previously derived in the original MPNST model (7, 16).

The Parada lab has generated many cell lines from *cis Nf1; p53* tumors, and they will also make new lines from the present tumors for comparative molecular and genomic analysis as discussed below.

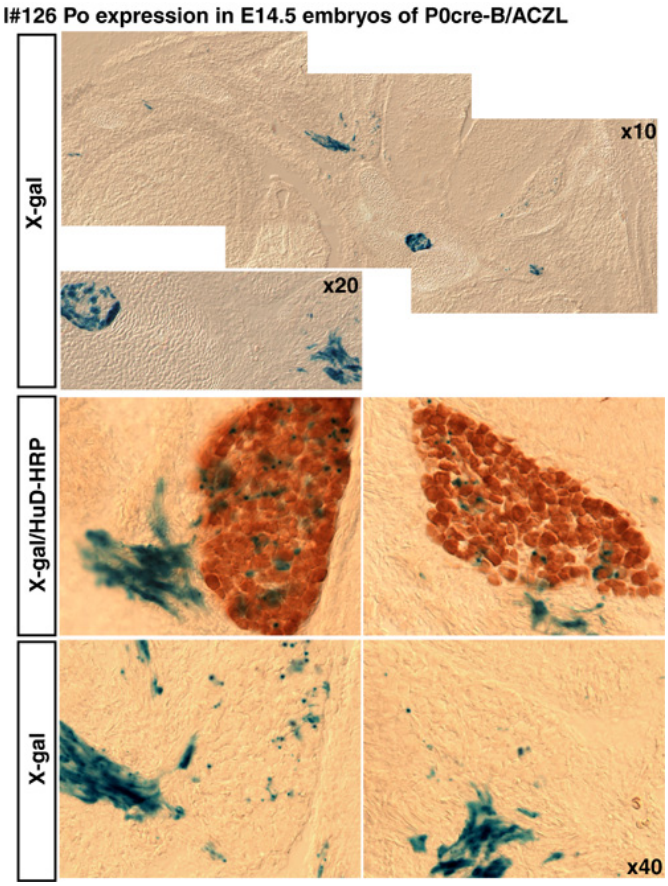


Figure 7. *Cell type-specific Cre-mediated recombination in mouse embryos.* *flox LacZ indicator mice were crossbred with P0 CreB transgenic mice. E14.5 embryos were isolated and cryosections stained with X-Gal and antibosdies anti-HuD, a neuronal nuclear marker showing Schwann cell-specific (non-neuronal) Cre recombination.*

The *Krox20-Cre* transgene has been valuable for ablating *Nf1* in SC and generating plexiform neurofibroimas and, subsequently, *Nf1; p53* mutant MPNSTs (8). However, this system presents obstacles as well. *Krox20-Cre* is expressed in the hindbrain during development and subsequently in many non-neural tissues. Consequently, the mice are sickly and do not grow well. Therefore, a Cre recombinase with tighter expression profile is highly desirable. To achieve this goal, Drs. Giovannini and Parada are using the P0-Cre-B strain generated by the Giovannini lab that shows a pattern of Cre expression restricted to Schwann cell (SC) precursors (**Fig. 7 above**). Body size/weight of P0-CreB; $Nf1^{flox/flox}$ and P0-CreB; $Nf1^{flox/-}$ mice was not statistically different from that of control $Nf1^{flox/flox}$ and $Nf1^{flox/-}$ littermates (data not shown). Hypertrophic nerves were found at high frequency in both P0-CreB; $Nf1^{flox/flox}$ (12 of 16, 75.0%) and P0-CreB; $Nf1^{flox/-}$ (12 of 12, 100.0%) mice starting at 6 months of age. To compare the phenotypic abnormalities within a similar time window, two P0-CreB; $Nf1^{flox/flox}$ and two P0-CreB; $Nf1^{flox/-}$ mice were sacrificed at the age of 3 and 6 months. Whereas hypertrophic nerves were not observed in 3-month-old mice, all 6-month-old mice of both genotypes had hypertrophic nerves.

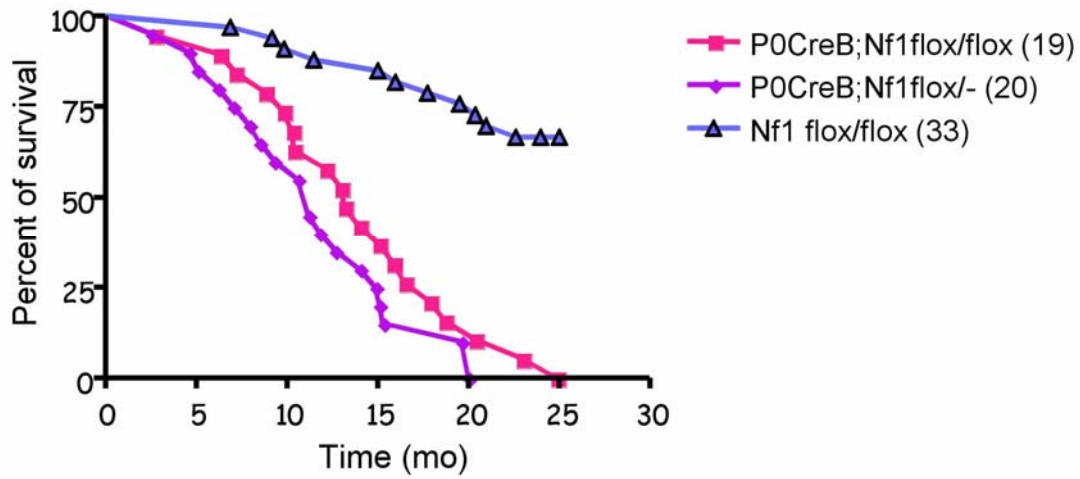


Figure 8. *Survival of Nf1 conditional (P0-CreB) mutant mice.*

P0-CreB; $Nf1^{flox/flox}$ and P0-CreB; $Nf1^{flox/-}$ mice were monitored for 24 months for the appearance of tumors. P0-CreB; $Nf1^{flox/flox}$ and P0-CreB; $Nf1^{flox/-}$ mice died starting at 3 months of age and exhibited similar survival (Kaplan-Meier Test: p=0.20) (**Fig. 8**). Tumors were classified based on criteria set forth in a 2003 Consensus Conference that was organized through the efforts of this consortium (17). Nine of the 16 P0-CreB; $Nf1^{flox/flox}$ mice and 10 of the 12 P0-CreB; $Nf1^{flox/-}$ mice developed GEM I neurofibromas at median ages of 14.1 and 12.7 months, respectively. GEM III PNST lesions (MPNST) developed in 4 of 16 P0-CreB; $Nf1^{flox/flox}$ mice and in 2 of 12 P0-CreB; $Nf1^{flox/-}$ mice (**Fig. 9**).

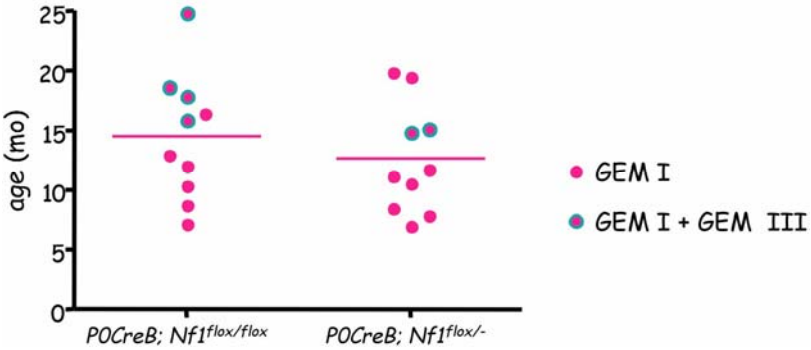


Figure 9. *Average time of GEM PNST development in Nf1 conditional P0-CreB mutant mice.*

Macroscopically, the multiple GEM I neurofibromas (1 to 4 per mouse) usually originated from the cranial nerves (5 of 9 tumors in P0-CreB; *Nf1*$^{flox/flox}$ and 8 of 10 tumors in P0-CreB; *Nf1*$^{flox/-}$), and from dorsal root ganglia (5 of 9 tumors in P0-CreB; *Nf1*$^{flox/flox}$ and 7 of 10 tumors in P0-CreB; *Nf1*$^{flox/-}$). GEM III PNST (MPNST) (1 to 2 per mouse) arose in the brachial plexus (1 of 4 tumors in P0-CreB; *Nf1*$^{flox/flox}$ and 1 of 2 tumors in P0-CreB; *Nf1*$^{flox/-}$), from dorsal root ganglia (1 of 2 tumors in P0-CreB; *Nf1*$^{flox/-}$), and also developed in the dermal layer of the skin (3 of 4 in P0-CreB; *Nf1*$^{flox/flox}$). The dermal tumors were located in the tail (2 P0-CreB; *Nf1*$^{flox/flox}$ mice) (**Fig. 10**) and in the ear (1 P0-CreB; *Nf1*$^{flox/flox}$ mouse). GEM III PNSTs were small in size (as were the GEM I neurofibromas), only slightly fibrotic, and contained multiple mitotic cells. All 24 peripheral nerve tumors of P0-CreB;*Nf1*$^{flox/flox}$ and P0-CreB; *Nf1*$^{flox/-}$ mice that were analyzed displayed biallelic *Nf1* inactivation (i.e. Cre/LoxP-induced deletion of exons 31-32). Eight GEM I neurofibromas from 6 P0-CreB; *Nf1*$^{flox/flox}$ mice, and 3 GEM I neurofibromas from 3 P0-CreB; *Nf1*$^{flox/-}$ mice were characterized by immunohistochemistry. All of the tumors showed S100 protein staining. Four GEM III PNSTs from 3 P0-CreB; *Nf1*$^{flox/flox}$ mice and one lesion from a P0-CreB; *Nf1*$^{flox/-}$ mouse also demonstrated S100 protein staining. Mast cell infiltration was present in GEM I neurofibromas of both genotypes, but not in GEM III PNSTs (**Fig. 10**). Bone marrow mast cell (BMMC) cultures were established from P0-CreB; *Nf1*$^{flox/+}$ and P0-CreB; *Nf2*$^{flox2/+}$ mice to determine the status of the conditional *Nf1* mutant allele in these cells. Genomic DNA was extracted and the *Nf1*$^{\Delta 31\text{-}32}$ and *Nf2*$^{\Delta 2}$ alleles were screened by PCR. The recombined *Nf1*$^{\Delta 31\text{-}32}$ and the *Nf2*$^{\Delta 2}$ alleles were not detected in BMMC from P0-CreB; *Nf1*$^{flox/+}$ and P0-CreB; *Nf2*$^{flox2/+}$ mice, respectively. These data indicate that tumorigenesis is not associated with loss of *Nf1* function within infiltrating hematopoietic cells in this model.

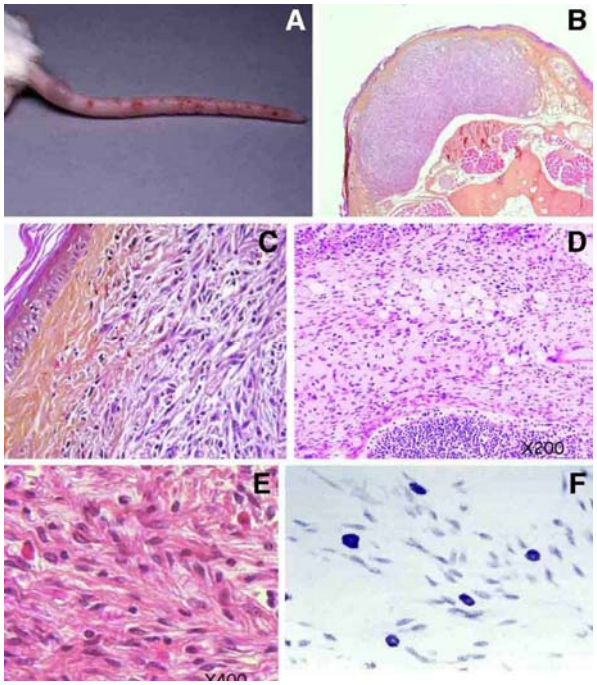


Figure 10. ***PNSTs in Nf1 conditional P0-CreB mutant mice***. *(A) GEM II MPNST in the tail of a P0CreB; Nf1*$^{flox/-}$ *mouse. (B-C) histological section of the tumor in (A). (B) 1.25X and (C) 20X. (D-E) GEM I Neurofibroma in a P0CreB; Nf1*$^{flox/flox}$ *mouse (F) Blue Toluidine staining showing mast-cells infiltrating the tumor.*

Construction of Drug-Inducible Cre Mice to Locally and Temporally Control Deletion or Reactivation of the *Nf2* Gene. The Jacks lab has generated tamoxifen-inducible Cre mice (Rosa26-CreER^{T2}) that permit local ablation at desired time points. These Rosa26-CreER^{T2} were crossed to *Nf2*^{flox} mice to generate Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice. A single intraperitoneal injection of 3 mg/40 g body weight of tamoxifen into pregnant female mice at E8.5 resulted in neural tube defects in the subset of embryos with the genotype Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$. Thus, acute inactivation of *Nf2* in development can recapitulate the tissue fusion defects observed in Nestin-Cre^{p}; *Nf2*$^{flox/1lox}$ mice (see below). The Jacks lab has also injected adult Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice with a single intraperitoneal tamoxifen dose of 4.5 mg/40 g body weight. At 3 months post injection, these mice appear healthy; however, their coat color has changed from solid black to patches of black and red. To date, no tumors have been detected. The Rosa26-CreER^{T2} mice will be instrumental for several planned studies by the Jacks lab to investigate the requirement for the *Nf1* and *Nf2* tumor suppressors in adult tissue and to develop novel tumor models utilizing the ability to control timing and region of *Nf1* or *Nf2* loss which have not previously been possible (see below).

Beyond neurofibromatosis, the Rosa26-CreER^{T2} mice developed as part of this consortium will have broad utility for generating novel mouse models of cancer and other human diseases. For example, in a separately funded project in the Jacks lab, Rosa26-CreER^{T2} mice were intercrossed with mice homozygous for a conditionally active WT *p53* allele (LSL-*p53*). The LSL-*p53* homozygous mice are phenotypically null for *p53* and therefore susceptible to tumor formation. Upon Cre-mediated recombination, WT *p53* is expressed. A cohort of Rosa26-CreER^{T2}; LSL-*p53*/LSL-*p53* mice were monitored for tumor formation. After these tumors were assessed by MRI imaging, the mice were given tamoxifen (1 mg/day x 4 days) intraperitoneally. Follow-up MRI studies demonstrated that thymic lymphomas and sarcomas regress in response to *p53* reactivation. In the case of the lymphomas, this was due to rapid apoptosis. This experiment elegantly shows that loss of *p53* function is required for tumor maintenance as well as tumor initiation, validating therapeutic strategies to reactivate *p53* in established tumors. A similar strategy can now be applied to *Nf2* to address whether loss of *Nf2* function is required for tumor maintenance and to assess how tumor cells respond to reactivation of *Nf2*. This information is critical to planning rational therapeutic approaches for NF2-associated tumors.

Generation and Characterization of Mouse Models of Ependymoma. Brain tumors are the second most frequent malignancy of childhood and are the leading cause of death from childhood cancer. Ependymomas are particularly common in young children, accounting for 6-12% of pediatric brain tumors, and this tumor type occurs at increased frequency in children with NF2. Although ependymomas are slow growing and histologically classified as WHO grade II/IV, the 5-year progression free survival is only 50%, with children under two years of age having a particularly dismal prognosis.

Since mutation of the *NF2* gene is the only well documented genetic alteration in human ependymomas, the Jacks lab is generating mouse models of ependymoma in which the initiating event is loss of *Nf2*. Previous work from the Jacks lab has shown that *Nf2*$^{-/-}$ mice die in early embryogenesis due to defects in the extraembryonic tissues, and that *Nf2*$^{+/-}$ mice develop malignant tumors but not the tumor types characteristic of the human disease (schwannoma, meningioma, and ependymoma). Therefore, the Jacks lab used the conditional *Nf2*^{flox} allele generated by Dr. Giovannini, and the transgenic Nestin-Cre and *h*GFAP-Cre mice in which Cre

is expressed within ependymal cells but also within other cells of the body and/or brain. Nestin-Cre expression is detected in the neuroepithelium as early as E8.5, and is expressed in a mosaic pattern throughout the body of the embryo. *h*GFAP-Cre expression is restricted to neurons and glial cells, and is detected as early as E14.5. The Jacks lab previously showed that Nestin-Cre; *Nf2*$^{flox/flox}$ mice in which the neural tube has closed (70% of all mutants) showed diffuse hyperproliferation of ependymal cells around the ventricles and central canal of the spinal cord. A single Nestin-Cre; *Nf2*$^{flox/flox}$ mouse that survived 11 days past birth had a focal lesion arising from the wall of the third ventricle in which the ependymal cells formed tubules resembling the true rosettes of human ependymomas. This lesion appears to represent the earliest stage of ependymoma. By contrast, the Jacks lab has generated 25 *h*GFAP-Cre; *Nf2* $^{flox/flox}$ mice, and to date none of the mice analyzed histologically have shown evidence of ependymal cell hyperproliferation. These studies suggest that loss of *Nf2* in neuroepithelial cells (precursors to ependymal cells) is a more efficient way to generate ependymomas. However, the early lethality of Nestin-Cre; *Nf2*$^{flox/flox}$ mice precludes its use as an ependymoma model.

Since *h*GFAP-Cre; *Nf2* $^{flox/flox}$ mice do not develop ependymoma, the Jacks lab has investigated whether mutations in other genes may cooperate with *Nf2* inactivation in tumor initiation. Toward this end, cohorts of 25 *h*GFAP-Cre; *Nf2* $^{flox/flox}$; *p53*$^{flox/flox}$ mice and 25 *h*GFAP-Cre; *Nf2* $^{flox/flox}$; *APC*$^{flox/flox}$ mice have been generated, as both *p53* and *APC* mutations are implicated in the pathogenesis of human ependymomas. None of these mice, which have been aged as long as 18 months, have developed ependymomas. Based on reports in the literature that loss of *Nf2* may synergize with oncogenic mutations in *Kras*, the Jacks lab also generated 25 *h*GFAP-Cre; *Nf2* $^{flox/flox}$; *Kras*^{G12D} mice. *Kras*^{G12D} mice conditionally express oncogenic K-Ras^{G12D} in the presence of Cre; thus, in these mice, cells that lack *Nf2* also express K-Ras^{G12D}. The Jacks lab have seen evidence of ependymal hyperproliferation in these mice by 6 weeks of age (**Fig. 11**), suggesting that *h*GFAP-Cre; *Nf2* $^{flox/flox}$; *Kras*^{G12D} mice may yield a tractable model of ependymoma. In addition, these mice develop widespread tumor formation within cranial nerves (**Fig. 12; next page**), which the Jacks lab is in the process of characterizing. Together these results suggest that expressing K-Ras^{G12D} in the context of *Nf2* inactivation may be a novel strategy for modeling the tumors seen in NF2 patients. The Jacks lab is currently generating Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$; *Kras*^{G12D} mice to study the role of interaction between these mutations in tumor formation in other tissues.

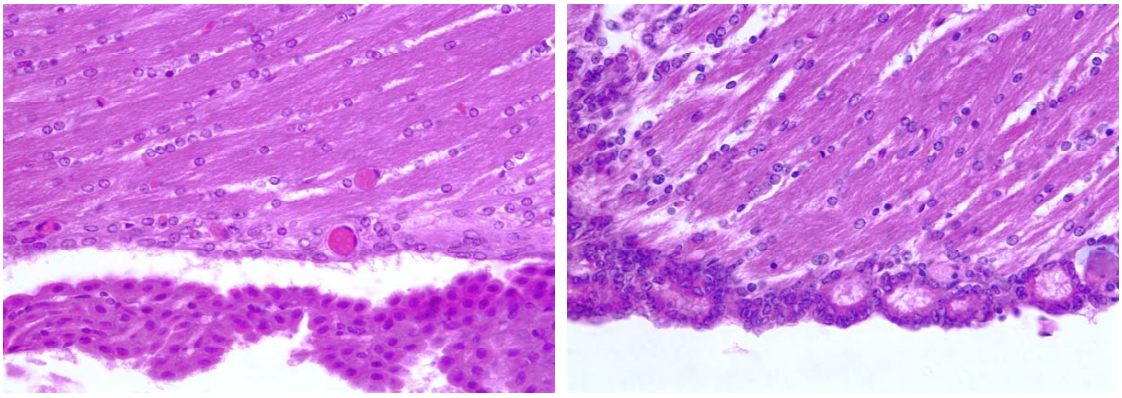


Figure 11. *Ependymal hyperplasia in hGFAP-Cre;K-RasG12D*$^{2lox/+}$*;Nf2*$^{2lox/2lox}$ *mice at 6 weeks of age (Control on the left, hGFAP-Cre;K-RasG12D*$^{2lox/+}$*;Nf2*$^{2lox/2lox}$ *on the right)*

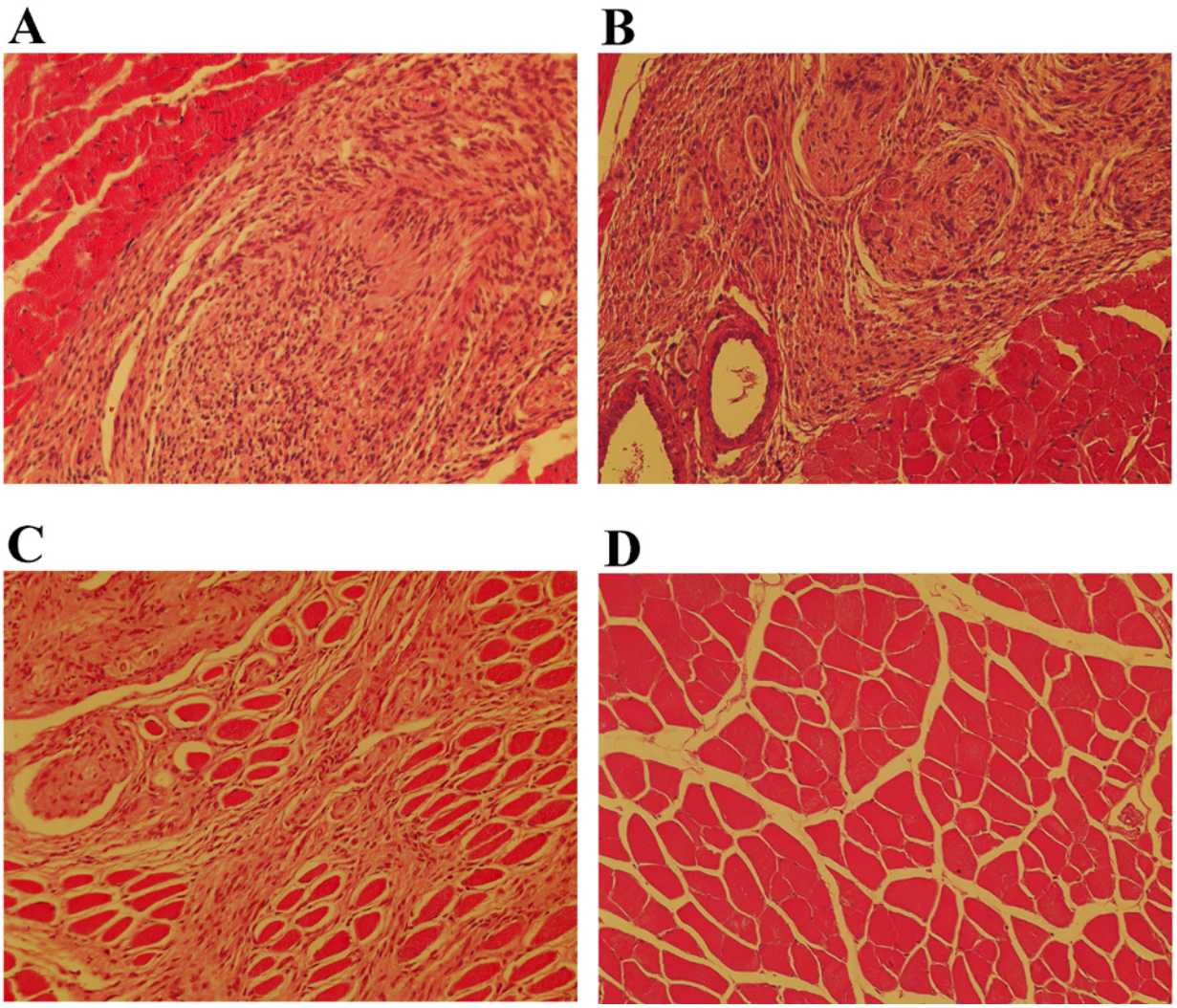


Figure 12. ***Loss of Nf2 and oncogenic K-rasG12D may synergize to result in tumor formation. A, B****. hGFAP-Cre;Nf2* $^{flox/flox}$*;K-RasG12D*$^{flox/+}$ *mice demonstrated widespread hyperplasia and tumor formation within cranial nerves that was not observed in hGFAP-Cre;Nf2* $^{flox/flox}$ *mice or control Nf2* $^{flox/flox}$ *mice (not shown). hGFAP-Cre;K-RasG12D*$^{flox/+}$ *mice also demonstrated hyperplasia within trigeminal nerves to a lesser degree than that observed in hGFAP-Cre;Nf2* $^{flox/flox}$*;K-RasG12D*$^{flox/+}$*mice; however, additional studies are underway to confirm these results and better characterize the lesions.* ***C.*** *In addition to the cranial nerve findings, every hGFAP-Cre;Nf2* $^{flox/flox}$*;K-RasG12D*$^{flox/+}$ *mouse analyzed thus far has demonstrated widespread hyperplasia and tumor formation within peripheral nerves such as those shown coursing through muscle in the head.* ***D.*** *Muscle taken from a control Nf2* $^{flox/flox}$ *mouse is shown for comparison.*

Investigating the cooperation between conditional loss of *Nf2* and other tumor suppressors in the formation of ependymomas is complicated by the presence of disease manifestations that limit the lifespan of the animals using either *h*GFAP-Cre or Nestin-Cre. As a result, it would be ideal to have a method to specifically drive Cre within ependymal cells. Given the location of ependymal cells along the ventricles of the brain, the Jacks lab is currently investigating a method to deliver viral Cre by stereotactic injection. Alternatively, a higher frequency of recombination in ependymal cells may be achieved by administering tamoxifen intraventricularly ultilizing the Rosa26-CreER^{T2} strain. Compound *p16*^{Ink4a}*p19*$^{Arf,flox/flox}$; *Nf2* $^{flox/flox}$ and

PTEN$^{flox/flox}$; *Nf2* $^{flox/flox}$ mice are being generated for this project. In addition, the Jacks lab is examining how *p16Ink4ap19Arf* and *PTEN* loss influence ependymal hyperplasia on the *Nf2* $^{flox/flox}$; *Kras*^{G12D} strain background.

A Mouse Model to Study Adult Onset Tumors in NF1. Significant advances have been made by members of this consortium in modeling nervous system tumors including MPNST/Triton tumors and plexiform neurofibromas that typically arise congenitally in NF1 patients. How *Nf1*-loss results in tumor formation in adulthood, however, has not been substantially addressed. This issue is of particular importance to patients given that subcutaneous neurofibromas, which typically arise during puberty, may result in significant disfigurement due to their presence in large numbers and discomfort from intense pruritis. These tumors also appear to differ fundamentally from congenital neurofibromas as they lack the ability to undergo malignant transformation. A mouse model of subcutaneous neurofibromas will be crucial for gaining a better understanding of factors leading to tumor initiation and potential mechanisms of prevention. In addition, the ability to contrast congenital with adult onset neurofibromas in a mouse model may yield insight into the mechanisms of malignant transformation. Of particular interest is whether developmental properties of the cell of origin may dictate the ultimate malignant potential of the tumor. Neurofibromas are thought to arise from SCs; however, it remains possible that congenital tumors may arise from SC precursors or stem cell population present only during early development.

The Jacks lab is currently generating a mouse model that will allow careful control of the timing of *Nf1*-loss by crossing Rosa26-CreER^{T2} mice with conditional *Nf1* $^{flox/+}$ and *Nf1*$^{+/-}$ mice to generate Rosa26-CreER^{T2}; *Nf1*$^{flox/-}$ mice. These mice will effectively be heterozygous *Nf1* mutant, which has previously been shown by Dr. Parada to be required for formation of neurofibromas. *Nf1* will then be lost in sporadic cells following tamoxifen administration. Neurofibromas will be induced at different stages of development by administering tamoxifen either systemically or through the skin by dissolving in DMSO.

Harnessing Bioluminescence to Detect NF-Associated Tumors *in Vivo*. The Jacks laboratory has access to an imaging system (NightOwl system from Berthold Technologies) that allows non-invasive detection and monitoring of tumor development in living animals. The Giovannini laboratory has recently acquired the IVIS100 System from Xenogen to perform omplementary work using analogous reporter strains and imaging equipment. These systems will allow them to determine the natural history of tumor development *in vivo* and would be a first step toward using NF-based models for therapeutic trials. *In vivo* expression of luciferase, the light-emitting enzyme of the firefly *Photinus pyralis*, has allowed the non-invasive detection of tumors in mice by other investigators. Luciferase can be measured in live animals using cooled ultra-sensitive charge coupled device (CCD) cameras for low light imaging. Both groups are using conditional activatable alleles of a luciferase reporter to target expression to tumor cells. Dr. Giovannini is focusing on the LucRep allele generated in the laboratory of Anton Berns (18). LucRep is a transgene in which luciferase is expressed by an actin promoter carrying a floxed transcriptional stop element. Dr. Giovannini generated *Nf2*$^{fl/fl}$; LucRep^{+} mice and injected them intravenously with an adeno-Cre suspension to test the efficiency of the system *in vivo*. Widespread activation of the luciferase reporter was observed starting at day 1 after adeno-Cre injection. The liver appeared the preferred target site after inoculation of the adeno-Cre virus, which is consistent

with data showing that nearly 100% of hepatocytes can be transduced *in vivo* when a large number of adenoviral particles are injected intravenously.

In work supported by other funding sources, the Jacks lab has generated a mouse carrying a distinct conditional activatable luciferase reporter gene. In this strain, transcription of the luciferase gene is driven by the CAGGS promoter (a fusion of the chicken β-actin promoter with CMV enhancer elements) inserted into the *Rosa26* locus to ensure that it resides in a region of open chromatin for consistent high-level expression. As with the LucRep strain, luciferase expression is Cre-dependent due to the presence of a stop element that is flanked by LoxP recombination sites. Both groups are intercrossing luciferase reporter mice with $Nf2^{fl/fl}$ and Cre-expressing mice to direct both *Nf2* mutation and activation of luciferase in the same cells. This strategy will allow the Giovannini and Jacks groups to readily monitor tumor initiation and progression by performing bioluminescence imaging. The testing of the two reporter strains and two imaging systems will allow for more rapid optimization of this technology and applying it for preclinical testing of new treatments for NF2-associated tumors.

Technical Objective (Aim) 2: Consequences of Nf1 and Nf2 Inactivation and Therapeutic Target Identification

Upstream Adapter Molecules as Therapeutic Targets in NF1 Disease. Based on the important role of hyperactive cytokine signaling networks in promoting the aberrant growth of *Nf1*-deficient cells including myeloid leukemia, MPNST, and neurofibroma (19-21), interfering with specific growth factor-activated signaling modules upstream of Ras might selectively block the growth of *Nf1*-deficient cells. Gab2 is an adapter protein contributes to Ras activation by associating with activated hematopoietic growth factor receptors. Whereas homozygous *Gab2* mutant mice are phenotypcially normal, hematopoietic cells from these animals are resistant to the MPD that is induced by retroviruses encoding Bcr-Abl. These observations raise the possibility that Gab2 is required to induce MPD in *Nf1*-deficient mice by linking activated cytokine receptors to Ras, but is dispensable for normal hematopoiesis. The Shannon lab is pursuing a genetic approach to test this hypothesis, which involves intercrossing *Mx1-Cre*, $Nf1^{flox/flox}$ and *Gab2* mutant mice. They will carry out correlative studies using the Western blotting and the phospho-FACS methodology described above and well-established techniques for enumerating myeloid progenitor colonies over a range of growth factor concentrations (15, 22, 23). The lab performed a series of crosses to generate the desired *Mx1-Cre*, $Nf1^{flox/flox}$, *Gab+/-* mice. These studies have progressed well and a cohort of $Nf1^{flox/flox}$ mice is being genotyped at the *Mx1-Cre* and *Gab2* loci to identify founders. If these studies show that Gab2 is required for MPD, this would identify adapter proteins upstream of Ras as a new class of potential therapeutic targets for treating the complications of NF1 disease.

Effects of Inhibiting p120GAP on the Growth of *Nf1*-Deficient Cells. The problem of how to selectively inhibit the growth of *Nf1* mutant cells led the Shannon lab to consider novel strategies. Most mammalian cells express two major GTPase activating proteins – neurofibromin and p120GAP, which have distinct binding affinities and catalytic activities (24, 25). *Nf1*-deficient cells only produce p120GAP and presumably rely upon this protein to control Ras activity. This raised the possibility that normal cells would tolerate transient inhibition of p120GAP because they retain neurofibromin, whereas this might induce catastrophic Ras activation in *Nf1*-deficient cells. The reverse is also possible – that *Nf1* mutant cells will

proliferate more vigorously if p120GAP is ablated. The Shannon lab is exploiting siRNA technology to test these alternative models in cells from *Nf1* mutant mice. Three siRNA constructs that target different regions of the p120GAP mRNA were developed and cloned into the murine stem cell virus (MSCV) retroviral backbone. Mouse embryonic fibroblasts (MEF) were then infected with these vectors, which resulted in variable reductions in p120GAP levels (**Fig. 13**). Knocking down p120GAP expression reduced proliferation in MEFs (**Fig. 14A**). Moreover, β galactosidase staining suggests that this is due to increased levels of senescence. Interestingly, the effect of p120GAP knockdown does not appear to be significantly different between WT and *Mx1-Cre* $Nf1^{flox/flox}$ MEFs (data not shown). Consistent with these results, knocking down p120GAP expression markedly reduced the growth of myeloid progenitor colonies from both WT and *Mx1-Cre* $Nf1^{flox/flox}$ hematopoietic cells over a range of GM-CSF concentrations (**Fig. 14B).** The inhibitory effects of p120GAP siRNAs observed to date are observed in both WT and *Nf1* mutant cells. The Shannon lab recently infected *Kras* mutant MEFs with the p120GAP siRNA, and the initial results revealed that this also inhibits proliferation. Together, the data obtained to date suggest that p120GAP is both a negative regulator of Ras signaling and plays an essential role in transducing proliferative signals, perhaps by interacting with Ras-GTP. If this idea is true, it might explain why *NF1* functions as a tumor suppressor gene in tumorigenesis, while *GAP* does not. The Shannon lab will pursue these provocative data during the next year. A high priority will be to investigate downstream effectors of Ras-GTP such as ERK, MEK, and S6 to ascertain if reducing p120GAP levels impairs signaling. The lab will also express a p120GAP siRNA to assess the effects on the ability of WT and *Nf1* mutant hematopoietic cells to reconstitute hematopoiesis in irradiated recipient mice.

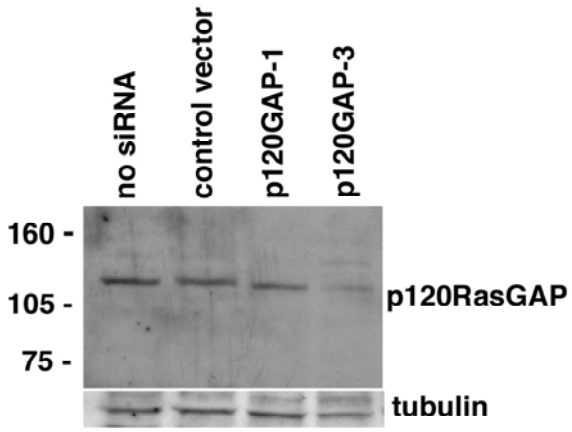


Figure 13. ***Reducted expression of p120GAP in NIH 3T3 cells infected with an MSCV-retrovirus encoding a siRNA against p120GAP (p120GAP-3).*** *The empty vector and a construct directed against a different p120GAP sequence (p12oGAP-1) had no effect on protein expression levels as detected by Western blotting.*

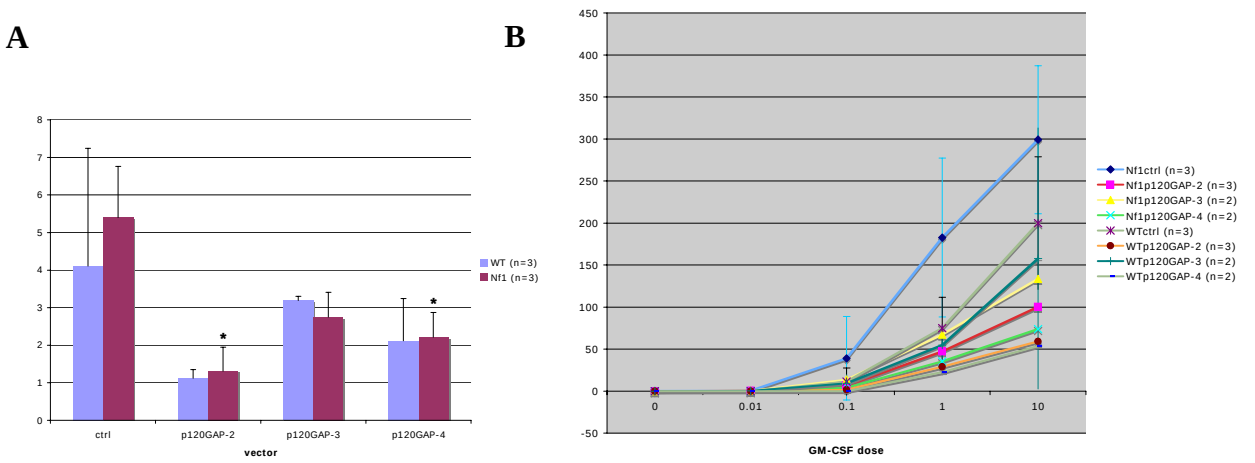


Figure 14. ***Reducted growth of WT and Nf1-/- Cells Expressing siRNAs Against p120GAP.*** *Panel A. Proliferation of MEFs. Panel B. Growth of CFU-GM colonies.*

Combining Mouse Models, Functional Genomics, and Cell Lines to Identify and Target Potential Therapeutic Pathways in Neurofibroma and Optic Glioma. The Parada lab has exploited recombinant adenoviral (Ad) vectors to neutralize growth of *cis Nf1; p53* MPNST cell lines and transplanted tumors (data not shown). These studies established the dominance of these two genetic lesions in tumor initiation and early progression. However, intermediate and late stage cell lines could overcome the growth-inhibitory effects of wild type *p53* and dominant negative MEK or ERK (not shown). Subsequent analyses suggested that one cause of this resistance could be due to properties of the Ad transduction system. First, it is virtually impossible to infect all cells, leading to the possibility of the emergence of uninfected cells. Second, gene expression diminishes as early as three weeks resulting in reemergence of cells that may have been in a static phase. Finally, it is likely that over time, additional genetic and epigenetic changes occur in tumor cells resulting in activation of tumor progression pathways that may render the block of the initiating *p53* and *Nf1* mutations ineffective.

To examine these possibilities in a more systematic way, the Parada lab proposed to utilize existing MPNST cell lines as well as the mouse models being generated in aim 1 as RNA sources for comparative microarray studies. Their rationale was based on the availability and to establish a baseline. However, more detailed signal transduction experiments using these lines has revealed perplexing heterogeneity (**Fig. 15**). Further analysis of downstream effectors including, ERKs, Akt, and Gsk3β indicates similar variability in downstream effectors (not shown and Parada and colleagues, in preparation). Consequently, they have decided to turn their attention more physiologically relevant models to study the genomic portrait of the process of benign to malignant transformation.

Therefore, they will continue to focus on neurofibromas, and neurofibrosarcomas derived from use of the *cis* $Nf1^{flox}$*;* $p53^{flox}$ mice described in Fig. 5. It is anticipated that use of these mice coupled with tamoxifen-inducible Cre mouse lines will result in generally healthier and more fertile mice, which will facilitate obtaining sufficient tumor material for well controlled microarray experiments. A P0-CreERT2 mouse line has imported from the laboratory of Dr. Ueli Suter in Switzerland. This well characterized line permits tamoxifen mediated temporal activation of the Cre transgene in neural derived tissues. These mice were rederived at the UT

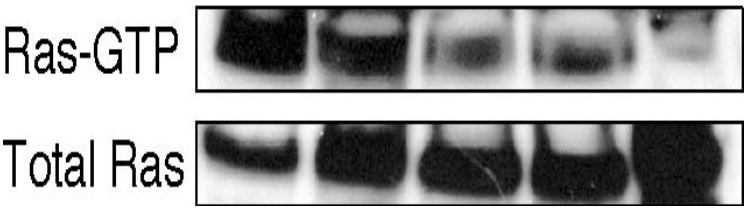


Figure 15. *Ras-GTP pull-downs from five independently isolated malignant peripheral nerve sheath tumor lines derived from cisNF-;p53- mice reveal a spectrum of variability of Ras activation.*

Southwestern facility and being intercrossed with *cis* $Nf1^{flox}$; $p53^{flox}$ mice. These mice will be used to generate neurofibromas and neurofibrosarcomes in a time-controlled fashion that obviates that need for stochastic loss of heterozygosity as loss of one (*cis* $Nf1^{flox/+}$; $p53^{flox/+}$) or both (*cis* $Nf1^{flox/flox}$; $p53^{flox/flox}$) tumor suppressor alleles can be induced with tamoxifen treatment. This will facilitate control of timing of tumor initiation and we permit deriving cell lines from tumors initiated at different times in development. Ultimately such lines would be of great use in the comparative genomic analysis and also in the adoption of siRNA mediated knockdown experiments to test potentially interesting tumor promotion pathways.

Spatiotemporal Control of *Nf2* the Tumor Suppressor During Tissue Fusion. Because $Nf2^{-/-}$ mouse embryos die early in gestation due to an extraembryonic defect, the consequences of *Nf2* loss in the embryo proper were unknown. The lack of such data limited our understanding of the normal function of merlin, and of how loss of merlin function contributes to tumorigenesis. To examine merlin's role in development broadly, and especially in nervous system development, the Jacks lab has again taken advantage of Cre/Lox technology. Through a series of crosses, they have generated mutant embryos of the genotype Nestin-Cre^{p}; $Nf2^{flox/1lox}$.

Nestin-Cre^{p}; $Nf2^{flox/flox}$ mice exhibit a range of neural tube defects, including craniorachischisis, a condition in which the entire neural tube fails to close. Strikingly, tissue fusion fails at several other sites within the mutant embryos, suggesting that there may be a global requirement for merlin in tissue fusion. To understand the molecular basis of this failure in tissue fusion, the Jacks lab studied neural tube closure in detail. Histologically, the mutant neuroepithelium exhibited foci of ectopic epithelial detachment. Loss of adherens junctions was noted in these foci by immunofluoresence for β-catenin and by electron microscopy. These findings suggest that ectopic epithelial detachment resulting in a paucity of cells was the primary reason for failure of tissue fusion.

By *in situ* hybridization, the Jacks lab found that *Nf2* is dynamically regulated during tissue fusion, with decreased levels of *Nf2* at the leading front prior to fusion and increased levels across the fused tissue bridge. Together these data suggest a new model of tissue fusion in which the dynamic regulation of *Nf2*, and thereby cell-cell adhesion, restricts physiologic detachment and detachment-induced apoptosis (anoikis) to the leading front.

After neural tube closure, the neuroepithelial (NE) cells and NE-derived radial glia that line the ventricles [ventricular zone (VZ) cells] do not assemble *de novo* apico-lateral junctional complexes (ALJCs, adherens junctions plus tight junctions). Instead, inheritance of existing ALJCs is determined by the orientation of cleavage during cell division. Therefore, the neuroepithelium before and after neural tube closure offers a unique setting in which the requirement for merlin in ALJC assembly versus maintenance can be assessed *in vivo.*

The Jacks lab examined the VZ in the subset of Nestin-Cre^{p}; $Nf2^{flox/1lox}$ embryos in which the neural tube closed and remained closed during late gestation and found that it had a biphasic appearance. There were discrete clumps of disorganized, loosely attached VZ cells that protruded

into the ventricular space, surrounded by well-organized, pseudostratified VZ cells. Electron microscopy showed an absence of ALJCs within the disorganized clumps, and immunofluoresence for the TJ component, Z0-1, and the AJ components, N-cadherin and β-catenin, further confirmed the lack of ALJCs. Although present, the apical band of actin was fragmented across the surface of the clumps. Interestingly, electron microscopy demonstrated the presence of ALJCs composed of distinct tight junctions and adherens junctions within the well-organized VZ. By immunofluoresence, ZO-1, N-cadherin, β-catenin and actin were properly localized. Thus, despite the fact that by late gestation there was no detectable merlin remaining in the brains of Nestin-Cre^{p}; *Nf2*$^{flox/1lox}$ embryos, the ALJCs in the well-organized regions of the VZ were retained.

These data in combination with Nestin-Cre1^{p} reporter analysis showing asynchronous recombination indicates that NE cells that lose *Nf2* early in gestation fail to assemble ALJCs and are prone to detaching, whereas NE cells and NE-derived radial glia that lose *Nf2* later in gestation are able to maintain existing ALJCs. This finding that merlin is required for the assembly but not the maintenance of ALJCs is completely unexpected based on the prior *in vitro* studies and contrasts sharply with the phenotype of mouse embryos in which core components of the junctional complex are deleted.

Characterizing the Role of *Nf2* in Wound Healing. The results of the Jacks laboratory demonstrating a role for merlin in regulating tissue fusion raises the possibility that it may also be important in normal wound healing, which occurs by similar mechanisms. Moreover, merlin is both a target and a regulator of Rac, a crucial protein for cell motility, migration, and proliferation that is also implicated in tumor progression. Prior work from the Jacks lab demonstrated that *Nf2*$^{-/-}$ MEFs had features consistent with hyperactivation of Rac, and displayed an enhanced migratory response in an *in vitro* wound healing assay resulting in wound closure 2-3 times faster than control MEFs Together, these data suggest that wounds deficient in *Nf2* may close faster as a result of increased migration, but have increased apoptosis and poor strength due to inability to form appropriate cell-cell junctions. To test these hypotheses, a cohort of Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice have been generated. These animals will be treated with tamoxifen (intraperitoneal dose of 4.5 mg/40 g body weight) and tested in a standard wound healing assay. Wounds of experimental animals will be compared to Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice that receive vehicle alone and *Nf2*$^{flox/flox}$ mice given the same dose of tamoxifen.

The results of this study should yield insights not only into the mechanisms of wound healing, but also to the role of *Nf2* in metastasis. Metastasis requires disruption of cell-cell contacts, migration, and proliferation; and Rac-signaling plays a key role in this process. Previous studies by Drs. McClatchey and Jacks demonstrated a relationship between loss of *Nf2* and increased metastatic potential of tumor cells.

Investigating a Potential Role of Injury in NF2 Tumor Initiation. The presence of bilateral vestibular Schwannomas is diagnostic for NF2, and mutations in *Nf2* are frequently seen in sporadic cases of these tumors. While tumors affecting the spinal roots, cranial nerves V, IX, or X occur, they are much less common, raising the possibility that cranial nerve VIII is particularly susceptible to tumor formation. Tumors are thought to arise from LOH at the *Nf2* locus, but an additional inciting event may be required. One hypothesis, given the tortuous path that cranial nerve VIII follows through the skull, is that injury may play a role in stimulating tumor formation. In order to test this hypothesis, the Jacks lab has generated a cohort of Rosa26-

CreER^{T2}; *Nf2*$^{flox/flox}$ mice, which will be injected with tamoxifen to induce loss of *Nf2*, then tested in injury models relevant to the most common tumors seen in NF2 patients. Both skin incision and sciatic nerve crush injury will be used to test induction of peripheral schwannomas, and stereotactic brain injury will be used to test induction of ependymomas and meningiomas. Similar injuries will be performed on control mice, Rosa26-CreER^{T2}; *Nf2*$^{flox/flox}$ mice administered vehicle alone and *Nf2*$^{flox/flox}$ mice given the same dose of tamoxifen. Given the evidence of a potential synergism between oncogenic *Kras*^{G12}and *Nf2*-loss in generating ependymal hyperplasia and cranial nerve tumors (discussed above), Rosa26-CreER^{T2}; *Nf2* $^{flox/flox}$; *Kras*^{G12D} mice will also be evaluated in these injury studies.

Relationship of *Nf2* Loss and PAK Activation with Tumorigenesis. Based on the regulation of PAK activation by merlin, the Jacks Lab has been developing reagents to examine the importance of PAK kinases in transformation and tumorigenesis. This has been accomplished through an RNAi approach. Five shRNAs against four different PAK family members were generated, and knockdown efficiency was tested in a *Kras*-transformed cell line (LKR13) by Western blotting. In the case of PAK1, the Jacks lab obtained very efficient silencing with two out of five shRNA constructs (**Fig. 16**). Generally, 1-3 shRNAs out of 5 produce a significant silencing effect, so this is not an unexpected result. Unfortunately, due to a lack of suitable antibodies against other PAK family members (PAK3, PAK4, and PAK6), the assessment of knockdown of other family members has not been possible. RT-PCR assays will be used to verify knockdown efficiency in these cases. These shRNAs will be useful in testing the effects of PAK inhibition in *Nf2*-deficient cells in culture and *Nf2*-mutant tumors.

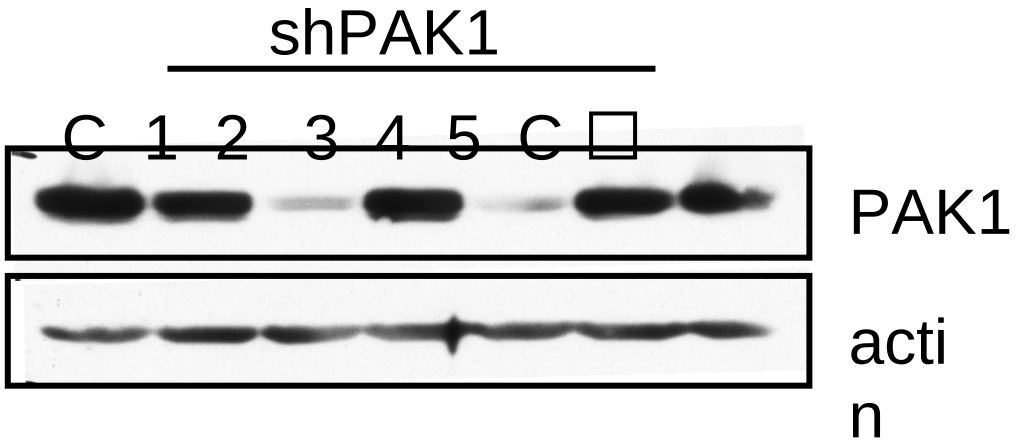
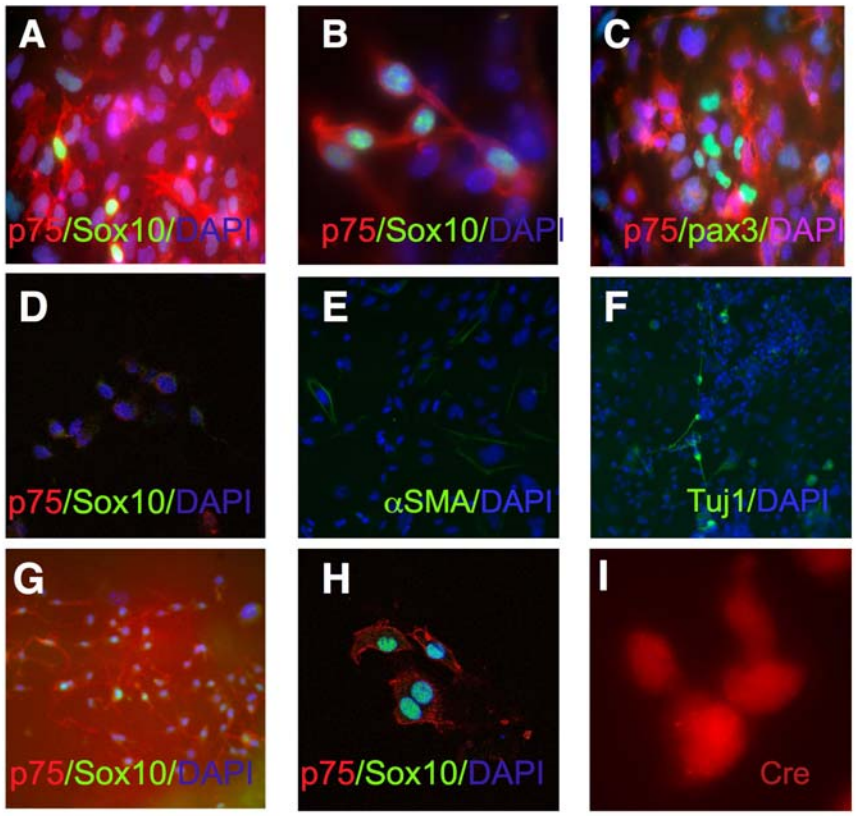


Figure 16. ***Western blot using a PAK1 antibody to assess knockdown efficiency in the LKR13 cell line.*** Very efficient silencing with two out of five shRNA constructs was obtained (C = controls).

Genetic and Epigenetic Factors Modulating the Tumor Spectrum Promoted by Different Combinations of *Nf2* and *p53* Mutant Alleles. Dr. Giovannini has investigated whether the developmental stage of neural crest-derived cells may determine their susceptibility to *Nf2* loss. *Ex vivo* cell cultures show that a minority of the cells are positive for neural crest cells (NCC) markers in E9.5 mouse embryos. To obtain a pure population of NCCs from mouse embryos, Dr. Giovannini first used an isolation strategy based on the presence of the NCC marker $p75^{LNGFR}$. This approach had been used previously to isolate SCs from embryos and adult mice (26). The lab utilized an array of lineage restricted markers, such as $p75^{LNGFR}$ and Sox10 for NCCs, calcineurin A for bone cells, αSMA for smooth muscle cells, Tuj1 for neurons, and GFAP for glial cells to characterize the cells present at the early stages of mouse development. During mouse development, neurolation starts at the embryonic day 8 (E8) - this process is followed by the progressive closure of the neural tube and the delamination of the neural crest, which occur at about E9.5. Therefore, to study whether the p75-positive cells are a unique cell population tracing back to NCCs, mouse embryos were dissected at E9.5. The embryos were dissociated in a special medium designed for NCCs (27), the cells were fixed by 4% PFA after 3 hours, and immunofluorescence was performed with the markers described above (**Fig. 17, A-C**). Dr. Giovannini and collaborators found that these cultures contain about 80% of p75-positive cells, but only a small percentage of them are also positive for Sox10. About 5% of cells from E9.5 mouse embryos were positive for both p75 and Sox10. Pax3, another NCC marker, was only expressed by cells that were negative for p75. These data confirm that some NCCs are pre-committed before starting their migration, as recently reviewed by Harris and Erickson (28). Finally, at this developmental stage, a few cells expressed makers found on glial cells, neurons, and for smooth muscle cells indicating that NCCs quickly differentiate into several cell types. *Ex-vivo* cell cultures from E9.5 embryos reach confluence in about 3 days when plated at 15000 cell/cm^2. Interestingly, all cells positive for both p75 and Sox10 are lost after 2-3 passages, (**Fig. 17, D-F**). Muscle cells and neurons, labelled respectively by αSMA and Tuj1, are mainly present in these cell cultures. Some cells are GFAP positive, but they do not show the classical bipolar morphology of glial cells. Based on these results Dr. Giovannini speculates that NCCs accomplish their cell fate by differentiating into various cell types *in vitro*, as they do *in vivo.* Preliminary results suggest that loss of *Nf2* may provide a powerful selective advantage to the most undifferentiated NCC-like cells (**Fig. 17, G-I**).

Figure 17 (next page). ***$Nf2^{-/-}$ cell cultures from E9.5 embryos are enriched in cells positive for the NCC markers p75 and Sox10.*** *(A) Ex vivo E9.5 embryos cell cultures show that, amongst a majority of p75 positive cells, only a few of them are truly NCCs. (B) At higher magnification, the double-stained p75/Sox10 cells show a typical undifferentiated morphology. (C) Pax3 positivity, an early marker of NCCs, indicates that some NCCs loose the p75 marker as soon as they become committed to their own fate. (D) Shortly after plating in vitro, NCCs become almost undetectable and differentiated cell types rise up. After two passages cells become negative for both p75 and Sox10. (E-F) Differentiated cells can be identified by specific markers such as αSMA for smooth muscle cells and Tuj1 for neurons. (G-H) In $Nf2^{-/-}$ NCC cultures p75- and Sox10-positive cells can be clearly identified while differentiated cells are not detected. (I) In these cultures Nf2 loss is induced by Cre recombinase. All the cells have been successfully infected by the AdenoCre virus and they express the Cre recombinase.*



To test whether loss of *Nf2* has a role in the differentiation and proliferation of NCCs, Dr. Giovannini and collaborators inactivated the *Nf2* gene in cell cultures obtained from E9.5 embryos carrying conditional knockout *Nf2* alleles and infected with an adenovirus expressing the Cre recombinase. The cultures were infected at subconfluency, split, and fixed when confluent. Besides a small increase in the speed of the cell cycle, the most remarkable phenotype in $Nf2^{-/-}$ NCC cultures was the maintenance of p75 and Sox10 positive cells together with the absence of differentiated cells. SCs, as NCC, are double-positive for p75 and Sox10 (29); however, $Nf2^{-/-}$ cells in these cultures did not show the typical SC morphology and were GFAP-negative. Therefore, based on the specific labelling and their morphology, these p75/Sox10 positive cells can be classified as NCC-like cells. Moreover, embryonic SCs (ESCs) from 12.5 embryos seem to acquire a multipotent cell fate potential after loss of *Nf2* **(Fig. 18; next page)** reminiscent of the totipotency described in the literature for NCCs (30).

Dr. Giovannini's lab previously developed a technique to isolate ESCs from E12.5 embryos, which is based on the presence of the p75 membrane marker and the selective action of neuregulin1 (NRG1) to induce SCs differentiation. To test whether early-derived cells from NCCs are able to accomplish their fate once they have lost *Nf2*, ESCs were treated with different grow factors, including BMP4. Wild type ESCs were not affected in their commitment to respond to the various growth factors; however, in $Nf2^{-/-}$ cells exposure to BMP4 can rescue their totipotency and give rise to mix population of neurons and smooth muscle cells. Altogether, these results show that *Nf2* may play a role in the patterning of NCCs and that loss of *Nf2*

reduces the differentiative capacities of NCCs, resulting in a proliferative advantage of the most undifferentiated NCC like cells.

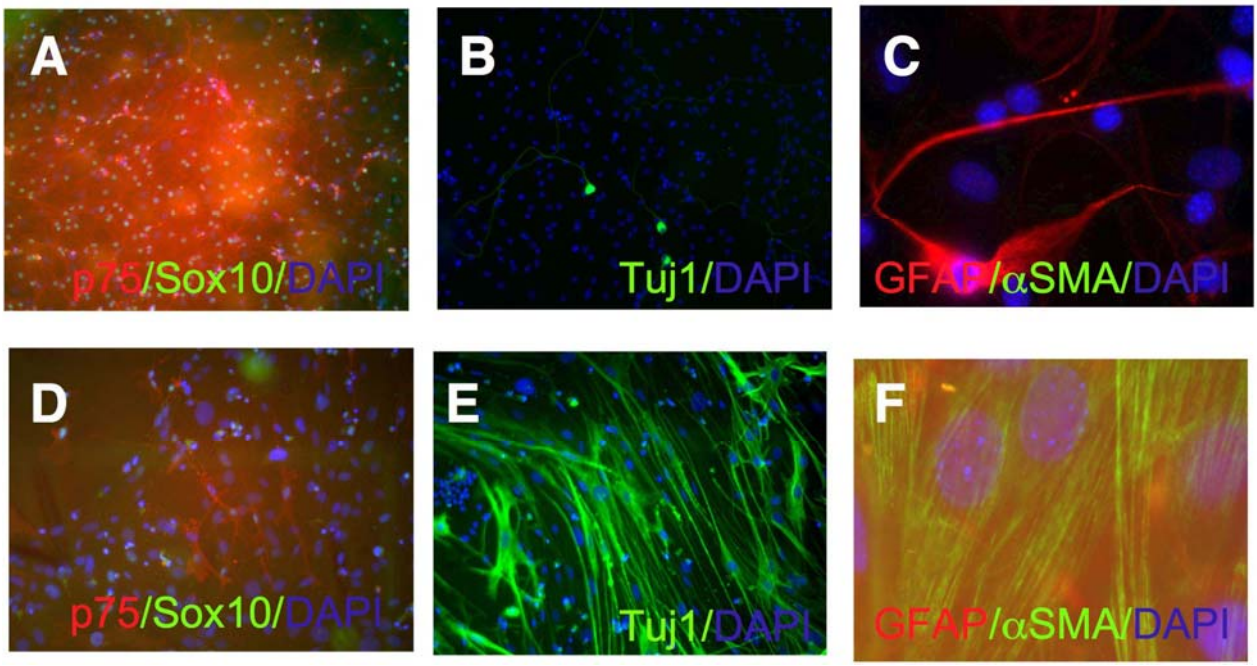


Figure 18. ***Embryonic Schwann cells (ESCs) that have deleted Nf2 acquire the capacity to differentiate in neural and muscle cells after stimulation with the growth factor BMP4.*** (A and C) *WT ESCs show their fate determination by the expression of typical markers such as p75 and GFAP, even after treatment with any kind of growth factors. (B) Treating ESCs with BMP4 only few cells are labelled for the neuronal markerTuj1. (D-F) ESCs that lose Nf2 are back to a stage of high responsiveness to the growth factor BMP4, giving either neural and smooth muscle cells, meanwhile GFAP or p75 positive cells are reduced.*

In addition, loss of *Nf2* in a committed cell, such the ESC, may revert an established cell fate, resulting in cells with NCC-like responses to growth factors. Dr. Giovannini is now investigating the role of *Nf2* during the induction, migration, and differentiation of NCCs. Three families of signalling molecules are known to regulate the growth and differentiation of NCCs: transforming growth factor β (TGFβ), fibroblast growth factors (FGF), and *Wnt*s (31). Cell culture experiments revealed the responsiveness of these cells to several instructive growth factors of different families of signaling molecules. Amongst these factors neuregulin1 (NRG1) isoforms induce the generation of peripheral glia, BMP2/4 promotes sensory neurogenesis, and TGFß1 leads to muscle cell differentiation. The lab of Dr. Giovannini is now able to generate pure populations of NCCs from E9.5 mouse embryos by explanting neural tubes (**Fig. 19; next page**). This system allows comparisons of the proliferation potential and differentiation of WT versus *Nf2* mutant NCCs in response to specific ligands and the effects of genetic manipulations on these processes. Dr. Giovannini will purse these questions over the next 2 fund years, and I currently isolating NCCs *Wnt-1*$^{cre/+}$*; Nf2*$^{flox2/flox2}$ embryos.

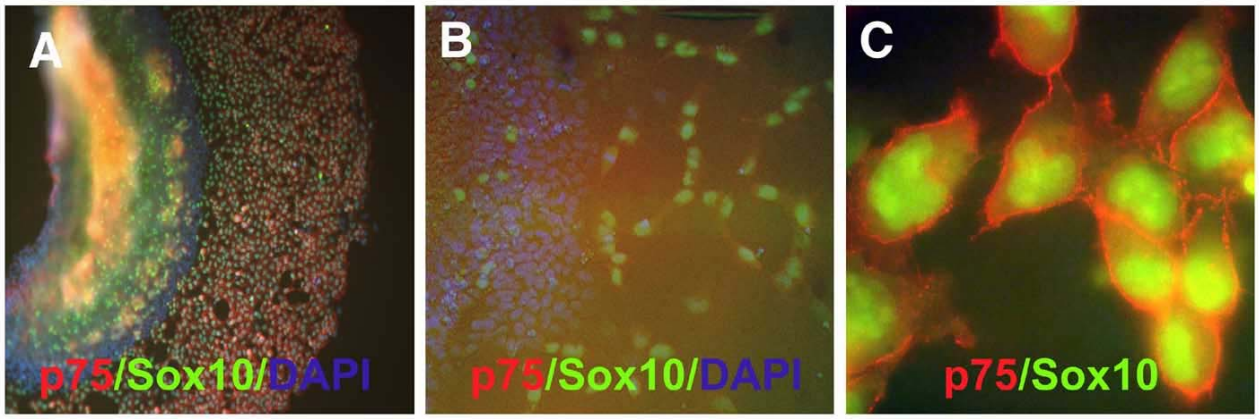


Figure 19. ***NCCs migrating from the neural tube give rise to a homogeneous cell population.*** (*A) cultures are derived from neural tubes of E9.5 mouse embryos. (B) Sox10 labels migrating NCCs fr neural tube. (C) High magnification of A shows the homogeneity of cell shapes of NCCs from neur explants.*

Investigation of ErbB Signaling in *Nf2*$^{-/-}$ SC. EGFR (ErbB1) is the prototype of the ErbB family of tyrosine kinase receptors (ErbB1-4 ; (32). SCs express multiple ErbB family members and are dependent upon ErbB signaling for proliferation and survival. Moreover, SCs display complex and exquisite coordination between ErbB receptor signaling and inter- and intracellular adhesion. The McClatchey lab initially studied primary *Nf2*$^{-/-}$ SCs isolated from the dorsal root ganglia (DRG) of 12.5 day old Nf2$^{lox/lox}$ embryos. Pooled SCs from multiple embryos were infected with experimentally determined titers of Ad-Cre or Ad-GFP. Recombination of the conditional allele was confirmed by PCR and loss of Merlin by western blot (not shown). Their analysis of *Nf2*$^{-/-}$ SCs reveals that, like other *Nf2*$^{-/-}$ cell types, they fail to undergo contact-dependent inhibition of proliferation specifically in the presence of neuregulin1 (NRG/GGF), the major ErbB ligand for SCs; if NRG is replaced with other growth factors such as PDGF, IGF or serum, *Nf2*$^{-/-}$ SCs do undergo contact-dependent inhibition of proliferation (**Fig. 20; next page**). Importantly, treatment of *Nf2*$^{-/-}$ cells with pharmacologic EGFR inhibitors (gefitinib and compound 56) yields dramatic reversion of their morphologic phenotype, restoration of contact-inhibition, a marked reduction of active (phosphorylated) MAPK and AKT and increased levels of active phosphorylated ErbB2. This is surprising given the accepted specificity of these inhibitors for EGFR and the fact that SCs are thought to express no or very little EGFR and instead predominantly express ErbB2 and ErbB3. However, the following observations suggest that they may express low levels of functional EGFR: 1) EGF stimulation of primary SCs yields increased membrane phosphotyrosine; 2) RT-PCR reveals the presence of EGFR mRNA in WT and Nf2$^{-/-}$ SCs; 3) Low levels of EGFR can be detected by western blot; and most importantly 4) EGFR inhibitors yield phenotypic reversion of Nf2$^{-/-}$ SCs. The McClatchey lab is currently testing the hypothesis that persistent EGFR signaling drives tumorigenesis in the absence of *Nf2* by crossing tumor-prone conditional *Nf2*-mutant mice to wa-2/wa-2 mice that harbor hypomorphic EGFR alleles (33).

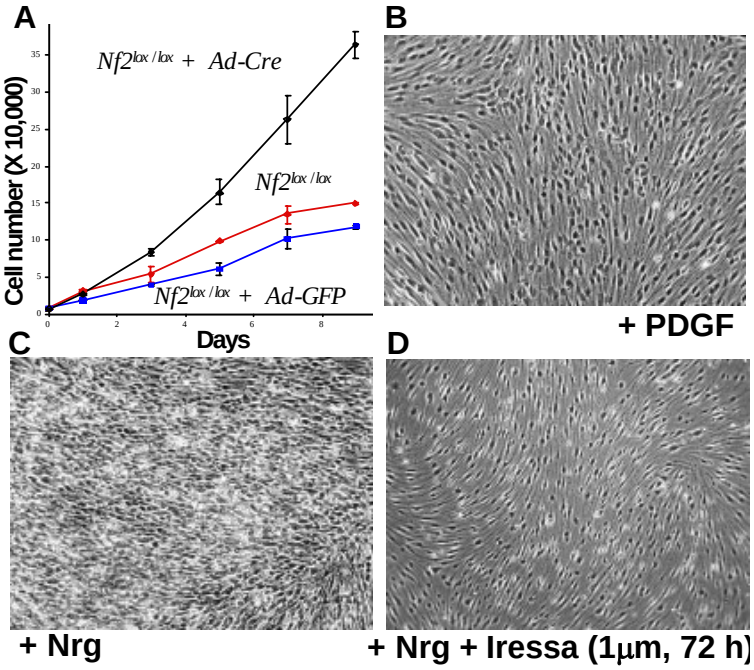


Figure 20. ***(A)*** *$Nf2^{-/-}$ Schwann cells fail to undergo contact-dependent inhibition of proliferation. Growth curves show $Nf2^{lox/lox}$ SCs infected with Ad-Cre, Ad-GFP or mock infected.* ***(B,C)*** *The overproliferation of $Nf2^{-/-}$ SCs occurs in the presence of Nrg (**C**) but not PDGF (**B**) and is reversed by the addition of 1um Iressa (**D**).*

Expressing WT and Mutant Versions of Merlin in *$Nf2^{-/-}$* SCs ***in vitro*** and ***in vivo***. Dr. McClatchey's lab has begun to examine the consequences of re-expressing WT and mutant versions of merlin in primary, transformed and tumor-derived *$Nf2^{-/-}$* SCs. Initial experiments confirm that reintroduction of WT *Nf2* via adenoviral expression in primary *$Nf2^{-/-}$* SCs restores contact-dependent inhibition of proliferation, providing the foundation for reintroducing mutant versions of merlin. Indeed, the McClatchey lab has generated a large panel of merlin mutants that contain missense mutations that alter candidate and established phosphorylation sites, membrane localization determinants and residues involved in effector binding. The ongoing testing of the behavior of these mutants in other cell types provides a framework for examining their function in SCs. The localization of each mutant in SCs is being examined, as well as its ability to restore contact-dependent inhibition of proliferation, to restore stable AJs and to alter the surface availability and signaling from the EGFR. The McClatchey lab will also examine the consequences of re-expressing WT and mutant *Nf2* in spontaneously transformed *$Nf2^{-/-}$* SCs and in *$Nf2^{-/-}$* SC tumor cell lines (see below).

The McClatchey lab previously found that SC tumors spontaneously arise in *$Nf1^{+/-}$;$Nf2^{+/-}$ cis* mice. In these mice individual tumors develop after several months of age and exhibit loss of both WT *Nf1* and *Nf2* alleles; in this model mutation of *Nf1* and perhaps at least one other gene cooperate with Nf2 loss in SC tumorigenesis. Mounting evidence indicates that some tumors acquire a dependence upon an activated oncogene for their survival, resulting in the phenomenon of 'oncogene addiction' (34). Indeed, several studies now suggest that sustained expression of an initiating oncogenic mutation is required for tumor maintenance *in vivo*. This information is critical for guiding therapeutic strategies. Few studies have investigated whether re-expression of a tumor suppressor can revert the tumorigenic process after the accrual of additional mutations. The McClatchey lab has generated a novel *Nf2* allele that allows conditional *Nf2* re-expression from the endogenous *Nf2* promoter after Flp-mediated removal of an frt-Stop-frt cassette. These mice have already been generated and the allele extensively characterized. To complement the studies of Ad-mediated *Nf2*-reintroduction described above, the McClatchey lab is generating

Nf2$^{-/frt}$ SCs and SC-derived tumor cells and infecting them with Ad-Flp^{ERT2} (provided by Sue Dymecki, Harvard Medical School; (35); in this system tamoxifen treatment leads to Flp-mediated restoration of *Nf2* expression. This work could set the stage for future studies of the consequences of Flp-mediated *Nf2*-re-expression to SC tumorigenesis *in vivo*.

Introduction of Signaling Molecules into SCs. Dr. McClatchey's laboratory previously generated transgenic P0-tv-a mice in which expression of tv-a in SCs renders them susceptible to infection by avian retroviruses engineered to express signaling molecules of interest. Detailed examination of SCs derived from these mice revealed mosaic expression of the tv-a receptor, however, precluding their use for efficient and homogeneous introduction of certain signaling molecules *in vitro* or *in vivo*. Instead, Dr. McClatchey's laboratory will carry out these experiments by introducing certain activated and dominant negative alleles of EGFR, E-cadherin, N-cadherin, Rac and PAK into the purified SC cultures described above by lentiviral infection. The McClatchey lab has already generated and is currently testing lentiviruses expressing several different activating EGFR mutations. The consequences of expressing each signaling molecule on WT and *Nf2*$^{-/-}$ SC proliferation and differentiation will be monitored with particular attention to whether expression in WT cells recapitulates the effects of *Nf2*-deficiency. If warranted, *in vivo* analyses will subsequently be performed using conditional Cre-LoxP lentiviral transgenesis.

Analysis of Mammalian Expanded Localization. As a critical step toward delineating the function of mammalian Expanded, Dr. McClatchey's lab has generated and characterized a rabbit polyclonal antibody that recognizes the mouse Expanded protein. To do so, a central fragment of mouse Expanded fused to glutathione-S-transferase (GST), was produced and purified from *E. coli* BL21 cells. This recombinant protein was then used to immunize two rabbits. Anti-Expanded polyclonal antibodies were affinity purified from the rabbit serum by passing it over a GST column followed by an antigen column. The eluted antibody was tested and found to recognize both exogenous and endogenous Expanded.

Dr. McClatchey's lab has used this polyclonal antibody to assess the localization of endogenous Expanded in epithelial cells by indirect immunofluorescence (**Figure 21; next page**). They found that Expanded is concentrated at regions of cell-cell contact and exhibits a punctate cytoplasmic distribution in mouse epithelial cells. This is markedly similar to the reported localization of *Drosophila* Expanded in epithelial tissues (36). Also as in *Drosophila*, merlin can co-localize with Expanded at regions of cell-cell contact, although the cytoplasmic distribution of Merlin is distinct from that of Expanded. Moreover, Expanded localization is apparently not dependent upon the presence of merlin as the staining pattern for Expanded is identical in WT and *Nf2*$^{-/-}$ cells. Initial studies indicate that EGF stimulation does not grossly alter Expanded localization; this is interesting given Dr. McClatchey's studies that establish a relationship between merlin and EGFR signaling. However, Expanded does associate with cell surface proteins; ongoing studies aim to identify Expanded-associated membrane proteins.

In the studies described above, Expanded appears to localize to a discrete apical portion of the cell-cell boundary in epithelial cells. Therefore Dr. McClatchey's lab utilized confocal microscopy to examine the localization of Expanded relative to specific cell junction components and to thereby better define the junctional distribution of Expanded. These studies revealed that Expanded does not colocalize with Desmoplakin, a marker of desmosomes, which are positioned basal to the apical junctional complex in these cells. In contrast, Expanded does colocalize with ZO-1, a marker of tight junctions; ongoing experiments aim to examine the localization of

Expanded relative to adherens junction components (**Fig. 21**). Together these studies indicate that as in *Drosophila*, mammalian Expanded localizes to the apical junctional region.

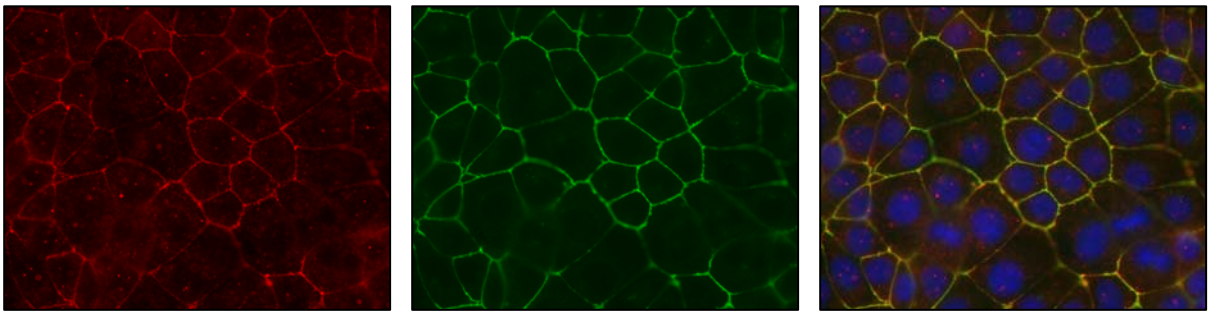


Figure 21. ***Mammalian Expanded localizes to the apical junctional region.*** *Colocalization of Expanded (left, red) and ZO-1 (middle, green), a marker of tight junctions and the apical junctional region, in mouse epithelial cells. The merge is shown in yellow (right). Nuclei are stained with DAPI.*

Phenotypic Consequences of Mammalian Expanded Under- and Over-Expression. Dr. McClatchey's lab has found that overexpression of Expanded does not yield overt phenotypic consequences in WT or *Nf2*$^{-/-}$ cells. However, their initial investigation of the consequences of downregulating mouse *expanded* expression using lentiviral-based shRNAs suggests that the consequences of Expanded downregulation may be dependent upon the presence of *Nf2*. Thus, *Nf2*-expressing MEFs, immortalized fibroblasts and ovarian epithelial cells (OECs), all fail to tolerate downregulation of *expanded*. In each case the cells detach and apparently die within 3-4 days after infection, or immediately after being passaged following infection. This may reflect apoptosis or defective cell-ECM or cell-cell adhesion that results in detachment from the plate and subsequent cell death. To pursue these possibilities, Dr. McClatchey's lab is monitoring the proportion of TUNEL-positive cells and the levels of cleaved caspase-3 after Expanded downregulation. They are also examining the ability of the Expanded-deficient cells to adhere to different substrates (i.e. fibronectin, collagen, laminin, matrigel) and measuring their ability to aggregate in suspension and in three-dimensional matrix.

In stark contrast to WT cells, *Nf2*-deficient cells (immortalized fibroblasts and epithelial cells) infected with lentiviral shRNAs targeting Expanded survive and display no obvious phenotype. Immunoflourescence microscopy reveals that the apical junctional complex (adherens junctions and tight junctions) and actin cytoskeleton appear similar in the cells infected with lentiviruses expressing Expanded-targeted versus control shRNAs. Moreover, downregulation of Expanded in *Nf2*$^{-/-}$ cells did not alter their rate of proliferation, density at saturation or their migration to close 'scrape wounds' in cell culture. However, Dr. McClatchey's lab did observe a striking difference between infected and control cells upon EGTA-mediated Ca^{2+}-depletion and disruption of cell-cell junctions. While control cells uniformly detach from one another and remain attached to the plate as individual cells during the time course, the infected cells round up more rapidly and nonuniformly, revealing large patches of visible plastic. Infected cells eventually detach from the plate in sheets during the time course. To determine whether the infected cells harbor cell-ECM or cell-cell contact defects, these cells will be evaluated by replating and aggregation assays as described above. Dr. McClatchey's lab will also examine the effect of Expanded overexpression in the Ca^{2+}-depletion assay to determine whether

elevated levels of Expanded affect junction disassembly. By isolating biotinylated membrane fractions, Dr. McClatchey will also look at the surface levels of key adhesion molecules in infected versus control cells. Taken together, these studies suggest a role for mammalian Expanded in cell-cell communication. This is particularly interesting given Dr. McClatchey's previous work establishing a role for Merlin in cell junction stability and very recent work linking *Drosophila* Expanded to the atypical cadherin and tumor suppressor, Fat (37-40).

Investigating the Role of Mammalian Expanded in Hippo/Warts Signaling. Recent studies in *Drosophila* suggests that Expanded and merlin, together, function upstream of the Hippo and Warts kinases (41). Dr. McClatchey's lab has begun to determine whether mammalian Expanded and Merlin also function in this pathway; initial studies do not support a role for mammalian Expanded and Merlin in mammalian Hippo/Warts signaling. The *Drosophila* studies demonstrated that overexpression of fly Expanded and Merlin activates the Hippo/Salvador/Warts pathway as measured by phosphorylation of Warts, however, Dr. McClatchey's lab was unable to detect changes in phosphorylation of mammalian Mst2 (Hippo) or Lats1 (Warts) in cells overexpressing Expanded, Merlin or both proteins. The *Drosophila* studies also suggested that Expanded and Merlin regulate their own expression through a negative feedback loop. However, elevated levels of mouse Expand protein or mRNA are not apparent in *Nf2*-deficient cells relative to WT cells. Similarly, gross changes in the levels of endogenous Expanded are not apparent upon Merlin or Expanded overexpression. Finally, cells overexpressing Expanded, Merlin, or both proteins do not exhibit obvious changes in the localization of YAP (Yorkie), a transcriptional co-activator that is negatively regulated by activation of the Hippo signaling pathway in *Drosophila*. Phosphorylation by Warts is thought to sequester Yorkie in the cytoplasm, thereby inactivating it. In summary, these data suggest that the relationship between Expanded/Merlin and the Hippo signaling pathway identified in *Drosophila* may not be conserved in mammals. A continued investigation of this relationship is ongoing.

Generation of an Expanded Mutant Allele. To generate a valuable tool for delineating the molecular function of mammalian Expanded and to investigate the function of Expanded in tumorigenesis, Dr. McClatchey's lab has designed and engineered a conditional mutant allele of mouse *expanded*. This *expanded* mutant allele features loxP sites flanking exon 2 and a promoterless β-geo cassette flanked by frt sites that is designed to report endogenous *expanded* expression *in vivo*. Expression of the Cre- recombinase will yield excision of exon 2, resulting in an immediate frame shift and a presumably null allele; Flp-mediated recombination will remove the β-geo cassette. The engineered targeting construct was electroporated into J1 and W4 embryonic stem (ES) cells. Two correctly targeted ES cell clones were identified out of 250 drug-resistant clones screened by Southern blot analysis and PCR. These two clones were injected into blastocysts and transplanted into recipient female mice yielding 11 chimeric progeny. These chimeras are currently being bred to test for germline transmission of the Ex^{lox} allele. Key future studies include determining whether Expanded is required for normal development, isolating and examining primary *Expanded*-deficient cells, determining whether *Expanded* heterozygous mutant mice are cancer prone, and uncovering genetic and biochemical interactions between Expanded and *Nf2* in growth control and tumorigenesis.

Other Activities

Workshop on Preclinical Therapeutics in Mouse Models of NF-Associated Tumors. Dr. Shannon organized a workshop entitled *"Barriers and Solutions in the Use of Mouse Models to Develop Therapeutic Strategies for NF1 and NF2-Associated Tumors"* with Drs. David Gutmann (Washington U.) and Kim Hunter-Schaedle (Children's Tumor Foundation) that achieved one of the goals of the previous award to this consortium (DAMD17-02-1-0638). This meeting, which was held at the Banbury Conference Center, Cold Spring Harbor National Laboratory from November 3-5, emphasized an informal "think tank" format with extensive discussion. In addition to prominent NF researchers, the attendees included outstanding investigators such as Scott Lowe, David Tuveson, and Eric Holland. The attendees identified a number of practical strategies to achieve the goal of using mouse models of NF-associated tumors to facilitate the goal of bringing new treatments to patients. A review article summarizing many of the general principles discussed at the meeting and how these principles are being applied to develop and evaluate new treatments for tumors in individuals with NF1 and NF2 disease was published earlier this year (42).

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KEY RESEARCH ACCOMPLISHMENTS

(a) The investigators continue to extensively share expertise and reagents to pursue common research goals.

(b) The NF Modeling Group is participating in the activities of the MMHCC and is contributing to achieving the goals of this national cancer research initiative.

(c) We found that pharmacologic inhibition of EGFR (using gefitinib, compound 56) reverts the phenotypic consequences of *Nf2*-deficiency in Schwann cells.

(d) An antibody that recognizes mouse Expanded was developed and tested.

(e) A conditional *Expanded*-mutant allele was generated by homologous targeting in mouse embryonic stem cells; chimeric Ex^{lox}/+√wild-type animals have been generated and are being evaluated for germline transmission of the Ex^{lox} allele.

(f) We generated a recombinant mouse strain harboring both an $Nf1^{flox}$ and a $p53^{flox}$ allele on the same chromosome for studies of tumorigenesis.

(g) We have developed a robust model for MPNST. These mice are now being placed in preclinical trials for prevention and tumor arrest therapies.

(h) We have developed a robust model for NF1 associated optic glioma. These mice are now being placed in preclinical trials for prevention and tumor arrest therapies.

(i) We have developed a robust model for NF1 associated astrocytoma. These mice are now being placed in preclinical trials for prevention and tumor arrest therapies.

(j) A new method was developed for isolating mitotically active Schwann cells that can also be used for the purification/enrichment of early NCC-derived cells.

(k) We have demonstrated a role for *Nf2* in promoting tissue fusion in embryogenesis.

(l) We have observed cooperative tumorigenic effects of Nf1 loss and oncogenic Kras expression in a model of ependymona.

(m) A novel strain of drug-inducible Cre mice was constructed that will permit regulated deletions between loxP elements. These mice are being harnessed to inactivate of the *Nf2* gene in specific tissues at defined time points.

(n) The biochemical consequences of *Nf1* inactivation in primary cells was investigated by Western blotting and, in preliminary studies, by phospho-FACS analysis of bone marrow cells from *Mx1-Cre* $Nf1^{flox/flox}$ mice.

(o) Studies of MEFs and bone marrow cells support the idea that p120GAP functions both as a negative regulator of Ras signaling and to promote growth.

(p) Strains of mutant mice have been shared widely with the NF research community (see list below in Reportable Outcomes). Through these collaborative experiments, the scientific value of this consortium has extended well beyond the studies being pursued in the participant's laboratories.

REPORTABLE OUTCOMES

Note: The list of research articles supported by the previous awards has been updated to reflect work that was in revision, under review, or in preparation at the time of the final report in November 2005. Most of the work published in 2006 was a direct result of our previous funding under this Program with a minor component supported by the current award. We anticipate continued productivity in 2007, which will be reflected in publications that are directly attributable to the current award.

(a) Research Articles and Reviews from Previous Consortium Awards (2000 – 2005)

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(b) Model Development and Distribution to the Research Community

Studies conducted to date have established a number of novel models of NF1 and NF2-associated tumors and have generated several new strains of mice. *Nf1* and *Nf2* mutant mice have been deposited in the MMHCC Repository where they are readily available to the research community. In addition, the participants in this Consortium have provided strains directly to the investigators listed below.

Karlene Reilly (National Cancer Institute)
Jeffrey DeClue (National Cancer Institute)
Jonathan Epstein (University of Pennsylvania)
D. Wade Clapp (Indiana University)
David Guttmann (Washington University)
David Largaespada (University of Minnesota)
Jeffrey Lawrence (UCSF)
Alcino Silva (UCLA)
Gerard Karsenty (Baylor)
Shaojun Tang (UC Irvine)
Shalom Avraham (Beth Israel)
James Bieker (Mount Sinai, New York)
Abhijit Guha (Labatt Brain Tumor Research Center, Tornoto)
Andreas Kurtz, (Harvard)

Jim Gussella (Harvard)
Dan Haber (Harvard)
Antonio Chiocca (Harvard)
Isidro Sanchez-Garcia (IBMCC)
Victor Tybulewicz (National Institute for Medical Research, London)
Lindsay Hinck (UC Santa Cruz)
Keqiang Ye (Emory University School of Medicine)
Lynda Chin (Dana Farber Cancer Institute)
Joseph Testa (Fox Chase Cancer Center)
Nancy Ratner (U. of Cincinnati)
Stefan Mundlos (U. of Berlin)
Juha Peltonen (U. of Helsinki, Finland)
Warren Pear (University of Pennsylvania)
David Beebe (Washington University)
Filippo Giancotti (MSKCC, New York)
Joe Kissil (Wistar Institute, Philadelphia)
Long Sheng Chang (Ohio State University, Columbus)
Cristina Fernandez Valle (University of Central Florida, Orlando)
Silvia Espejel (University of California, San Francisco)
Karen Cichowski, (Harvard)
Sean J. Morrison, (University of Michigan)
John J. Ryan, (Virginia Commonwealth University)
Isa Hussaini, (University of Virginia)
William Pu, (Children's Hospital, Boston)
Filippo G. Giancotti (Sloan-Kettering Institute for Cancer Research)
David Wilkes (Cornell University Medical College)
Ivan Radovanovic (Geneva)
Brian Weiss (University of Cincinnati)
Arturo Alvarez-Buylla (University of California, San Francisco)
Jonathan Chernoff, (Fox Chase Cancer Center
Laurent Elefteriou (UT San Antonio)
David Kaplan (Toronto)
Hong Wu (UCLA)
Ugur Ozerdem (La Jolla Insitiute)
David Ingram (Indiana U)
Takayuki Harada (Tokyo)
Alison Lloyd (London)
Junichi Sadoshima (New Jersey Medical School)

(c) Employment and Research Opportunities

This award has provided salary support for technical personnel and for trainees in each of participating labs.

CONCLUSIONS

During the sixth year of its existence, this consortium made progress in accomplishing its primary goal of generating and characterizing mouse models of NF1 and NF2-associated tumors. Novel strains have been developed and reported, innovative strategies were deployed to make optimal use of these resources, and our recent research has provided a number of new insights regarding how the *Nf1* and *Nf2* gene products regulate cellular processes such as survival, adhesion, proliferation, and self-renewal and biochemical signaling networks. The investigators continue to collaborate closely and have shared expertise and reagents extensively. The goals of the next fund year are to continue working to achieve the Technical Objectives of this project.