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Mouse Models of Neurofibromatosis 1 and 2[1]

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Abstract

The neurofibromatoses represent two of the most common inherited tumor predisposition syndromes affecting the nervous system. Individuals with neurofibromatosis 1 (NF1) are prone to the development of astrocytomas and peripheral nerve sheath tumors whereas those affected with neurofibromatosis 2 (NF2) develop schwannomas and meningiomas. The development of traditional homozygous knockout mice has provided insights into the roles of the *NF1* and *NF2* genes during development and in differentiation, but has been less instructive regarding the contribution of *NF1* and *NF2* dysfunction to the pathogenesis of specific benign and malignant tumors. Recent progress employing novel mouse targeting strategies has begun to illuminate the roles of the *NF1* and *NF2* gene products in the molecular pathogenesis of NF-associated tumors.
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Introduction

The neurofibromatoses comprise two distinct clinical conditions, neurofibromatosis 1 (NF1) and neurofibromatosis 2 (NF2). Each of these disorders shares the common feature of benign and malignant tumor predisposition; however, the tumor types and clinical manifestations are markedly different [1]. In addition, the genetic basis for NF1 is clearly distinct from that of NF2, in that the *NF1* and *NF2* genes encode unique and markedly dissimilar gene products, neurofibromin and merlin, located on chromosomes 17 and 22, respectively. Significant progress toward elucidating the molecular pathogenesis of NF1 and NF2 has resulted from the identification of their associated genes; however, our ability to design targeted therapies for these disorders is heavily dependent on the development of relevant and accurate preclinical mouse models of the specific clinical features of NF1 and NF2. This review will focus on the development and refinement of mouse models for NF1 and NF2.

Clinical Disorders

Neurofibromatosis 1

Neurofibromatosis 1 is the most common autosomal dominant disorder affecting the nervous system. Affected individuals are prone to the development of both benign and malignant tumors, including peripheral nerve sheath tumors (neurofibromas and malignant peripheral nerve sheath tumors [MPNSTs]), astrocytomas (gliomas), leukemias, and pheochromocytomas [2]. Aside from the tumor phenotypes, individuals with NF1 also exhibit pigmentary abnormalities, including hyperpigmented skin macules (café-au-lait macules), skinfold freckling, and iris hamartomas (Lisch nodules). In addition, children with NF1 often have skeletal defects (long bone deformities, scoliosis, sphenoid wing dysplasia) and learning disabilities. The most common tumor in *NF1* is the benign peripheral nerve sheath tumor (neurofibroma) composed of Schwann cells, fibroblasts, and mast cells [3]. The onset of these tumors typically coincides with the onset of puberty and they continue to appear throughout adulthood. These tumors do not transform into malignant tumors, but carry a tremendous cosmetic burden. Approximately 25% to 30% of individuals with NF1 will develop a more extensive neurofibroma (plexiform neurofibroma) that is thought to represent a congenital lesion. These tumors, although also benign, may grow to enormous proportions, develop a rich blood supply, and transform into aggressive MPNSTs. Within the central nervous system (CNS), the most common tumor is the optic pathway glioma, seen in 15% of children with NF1 [4]. This low-grade pilocytic astrocytoma is rarely fatal, but can lead to visual loss and hypothalamic dysfunction. In contrast to the neurofibroma, the growth of these tumors appear to be limited to the first decade of life, with rare examples of continued growth in adulthood.

Although rare, myeloid malignancies, such as myelodysplastic syndrome (MDS) and juvenile chronic myeloid leukemia (JCML), are overrepresented in children with NF1 [5]. Boys tend to be affected more often than girls. Pheochromocytoma, a tumor of the adrenal gland, is more common in individuals with NF1. Despite its association

Abbreviations: ERM, ezrin-radixin-moesin; GAP, GTPase-activating protein; MPNST, malignant peripheral nerve sheath tumor; NF1, neurofibromatosis type 1; NF2, neurofibromatosis type 2; WHO, World Health Organization

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with NF1, it affects less than 1% of individuals with the disorder.

Children with NF1 also frequently exhibit specific learning disabilities [6]. Approximately 40% to 60% of children with NF1 will manifest below average performance on standardized IQ tests and demonstrate difficulties with traditional learning paradigms. These children are not retarded, but have specific deficits in learning.

Neurofibromatosis 2

Neurofibromatosis type 2 (NF2), also called bilateral acoustic neurofibromatosis, is a dominantly inherited genetic disorder characterized by the development of bilateral vestibular schwannomas, schwannomas of other cranial, spinal, and cutaneous nerves, as well as cranial and spinal meningiomas and ependymomas [1,2,7]. The incidence of NF2 is much lower than NF1, affecting approximately 1/40,000 individuals (vs 1/3,000 for NF1). Bilateral vestibular schwannomas and multiple spinal schwannomas are diagnostic of NF2. These benign Schwann cell neoplasms (World Health Organization [WHO] grade I) develop in the majority of NF2 patients and are the hallmark of the disease. Occasionally, schwannomas may also arise on other sensory nerves, including the fifth (trigeminal) cranial nerve and spinal dorsal roots. Most vestibular schwannomas are located at the internal auditory meatus or within the internal auditory canal and slowly grow toward the cerebellopontine angle to result in increased intracranial pressure, cranial nerve dysfunction, or cerebellar herniation. Malignant progression of schwannomas to MPNSTs is a rare event.

Multiple meningiomas are the second most common tumor in NF2, occurring in nearly half of all affected individuals. These benign neoplasms (WHO grade I), composed of neoplastic arachnoidal cap cells, also grow slowly and elicit neurological signs and symptoms by compression of adjacent structures. Because of their tendency to invade brain tissue, recur after resection, and spread along the leptomeninges to involve multiple regions, some meningiomas represent a therapeutic challenge.

Other CNS tumors include ependymomas and astrocytomas. Occurrence of gliomas is less frequent in NF2 than in NF1 patients and most of these neoplasms are morphologically benign intramedullary spinal tumors. Dysplastic CNS lesions, such as glial microhamartomas, schwannosis, meningioangiomatosis and ependymal ectopias are also encountered in the context of NF2.

Pigmentary abnormalities are uncommonly seen in patients with NF2. About 60% to 80% of NF2 patients develop juvenile posterior subcapsular lens opacities. Therefore, lens changes are considered a valuable, early marker of NF2 in individuals at risk.

NF1 Gene and Function

The identification of the *NF1* gene ushered in a new era of molecular understanding of the pathogenesis of this disorder. The *NF1* gene on chromosome 17q11.2 spans 350 kilobases (kb) of genomic DNA and encodes an 11- to 13-kb mRNA [8-11]. The protein product, neurofibromin, is a large 220- to 250-kDa cytoplasmic protein expressed predominantly in neurons, Schwann cells, oligodendrocytes, astrocytes, leukocytes and the adrenal medulla (Figure 1) [12-14]. Neurofibromin has been shown to associate with the actin and microtubule cytoskeleton, but lacks conventional binding motifs for known protein–protein associations [15-17]. Analysis of the predicted *NF1* amino acid sequence revealed a small central region of the protein with sequence similarity to a family of proteins involved in the inactivation of RAS (GTPase-activating protein or GAPs) [18]. RAS is an important intracellular signaling molecule, which mediates cell proliferation in some cells (e.g., astrocytes and leukocytes) and differentiation in other cell types (e.g., neurons and Schwann cells) [19]. The association of the RAS molecule with guanosine phosphates dictates RAS activity: RAS is active when bound to GTP and inactive when associated with GDP (Figure 1). RAS-GAP molecules, like neurofibromin, function as negative regulators of RAS, by accelerating the intrinsic GTPase activity of RAS, thereby converting RAS from an active GTP-bound form to inactive RAS-GDP. Because RAS promotes cell proliferation and transformation in some cells, neurofibromin is believed to function as a tumor suppressor by inactivating RAS mitogenic signaling.

Individuals with NF1 are born with one mutated or nonfunctional *NF1* gene. Similar to the retinoblastoma paradigm, tumors in NF1 form only when the one remaining wild-type *NF1* allele is inactivated by somatic mutation [20]. This "two-hit" hypothesis has been validated in tumor tissues from individuals with NF1, in which both the somatic and germline *NF1* mutations were identified, resulting in no neurofibromin expression (Figure 2). In agreement with the proposed function of neurofibromin as a RAS

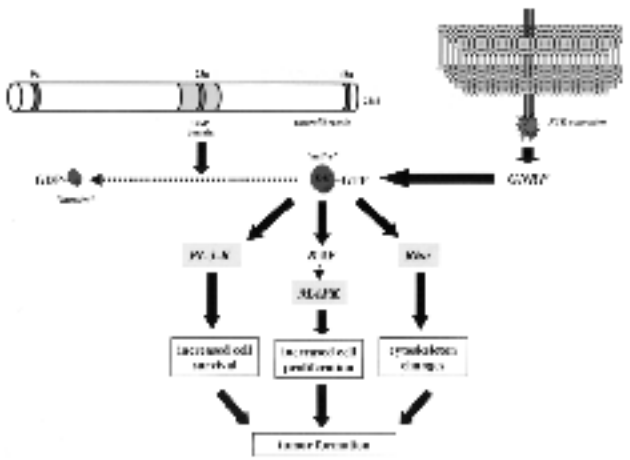

Figure 1. *Neurofibromin, the product of the NF1 gene, encodes a 2818 amino acid protein that contains a central region with RAS GTPase-activating protein (GAP) activity. The GAP domain is denoted by the shaded area. In addition to this functionally important domain, neurofibromin contains three alternatively spliced exons (9a, 23a, and 48a). Neurofibromin, like other GAP molecules, accelerates the conversion of active GTP-bound RAS to the inactive GDP-bound form. Guanosine nucleotide replacing factors (GNRFs) reactivate RAS by exchanging GDP for GTP.*

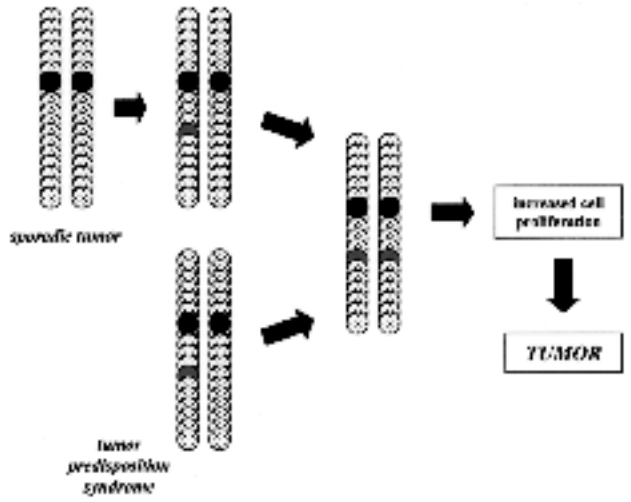

Figure 2. *Knudson "two-hit" hypothesis for tumor suppressor genes. In sporadic cancers, there is sequential inactivation of both copies of a given tumor suppressor gene, whereas in individuals with an inherited cancer syndrome, only one additional genetic "hit" is required. Because individuals with NF1 or NF2 start life with a germline mutation in the NF1 or NF2 genes, respectively, the only genetic event required for tumor formation is inactivation of the one remaining functional NF1 or NF2 gene. Loss of NF1 or NF2 expression results in increased cell proliferation that predisposes to tumor formation.*

regulator, loss of neurofibromin in NF1-associated tumors is associated with elevated levels of activated RAS [21-25].

The *NF1* gene has three alternatively spliced exons, 9a, 23a, and 48a, which are differentially expressed in specific tissue types. Exon 9a–containing neurofibromin is restricted to neurons of the forebrain beginning in early postnatal development [26]. Neurofibromin containing exon 48a is only expressed in muscle tissues [27]. It is not known what functions are gained or lost by the insertion of exons 9a or 48a. Lastly, exon 23a inserts a unique 21 amino acids into the GAP domain of neurofibromin and results in a neurofibromin protein with reduced GAP activity [28]. *NF1*-23a mRNA is found in many tissues during development and in the adult [29].

NF2 Gene and Function

The identification of germline mutations in NF2 patients provided the genetic evidence that this condition is caused by germline alterations in the *NF2* gene [30,31]. The majority of germline and somatic mutations in the *NF2* gene, resulting in either a premature termination codon, a splicing alteration, or a frameshift, lead to the production of a truncated protein. In-frame deletions and missense mutations have also been found, suggesting that the alteration of particular functional domains may abolish the function of the *NF2* protein. In tumors from patients with NF2, mutations in both *NF2* alleles have been identified, concomitant with loss of *NF2* protein expression. These observations indicate that *NF2* is a tumor suppressor gene, although the detailed mechanism by which *NF2* mutation leads to transformation of Schwann cells is largely unknown.

The product of the *NF2* gene has been termed merlin [31] or schwannomin [30]. Analysis of the predicted protein sequence indicates that merlin belongs to the band 4.1 family of cytoskeleton-associated proteins and exhibits the greatest similarity to a subset of proteins in this family, notably the ezrin, radixin, and moesin (ERM) proteins [32]. The *NF2* gene encodes a 595 amino acid protein with 17 exons (Figure 3*A*). Alternative splicing yields at least two merlin isoforms, type 1 (lacking exon 16) and type 2 that contains exon 16, but lacks exon 17 sequences, owing to a premature termination signal within exon 16 [33]. These *NF2* isoforms are conserved in mouse [34] and rat [35]. There are three predicted regions with potential functional significance: The FERM domain (residues 1–302) and the alpha helical region (residues 303–479) are shared among all ERM family members whereas the carboxyl terminal domain of merlin is unique and lacks a conventional actin binding motif, found in other ERM proteins. Merlin is expressed in Schwann cells, neurons, lens fibers, blood vessels, and leptomeningeal cells in the adult, but exhibits a more widespread tissue expression pattern during embryonic development. The FERM homology domain of merlin appears to be the main determinant that localizes the protein to the plasma membrane [36]. Mutations that lead to an interstitial deletion in this domain have been observed both in the germline of NF2 patients and in sporadic schwannomas, meningiomas, and mesotheliomas. Mutant proteins lacking exon 2 or exons 2 and 3 do not interact with the plasma membrane, and are diffusely distributed in the cytoplasm [36,37].

Merlin, like other ERM proteins, exists in two conformations, dictated by its ability to form an intramolecular

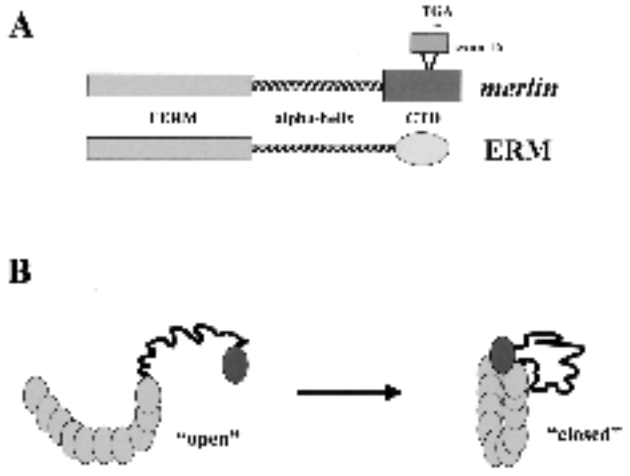

Figure 3. *Structure of the NF2 gene product, merlin. (A) Merlin is structurally related to the ERM subfamily of Protein 4.1 molecules, including ERM. Merlin contains an amino terminal FERM domain and a central predicted alpha helical domain followed by a unique carboxyl terminal region (CTD) that is not conserved among ERM family members. The conventional ERM actin-binding region is not contained within the CTD of merlin. The NF2 gene has an alternatively spliced exon (exon 16) that encodes 11 unique residues followed by a termination codon, which results in a truncated protein lacking exon 17 sequences. (B) Merlin exists in "open" and "closed" conformations that may be dictated by phosphorylation. In the "open" conformation, merlin is defective as a negative growth regulator, but, in the closed self-associated form is active and binds to CD44 to mediate growth suppression.*

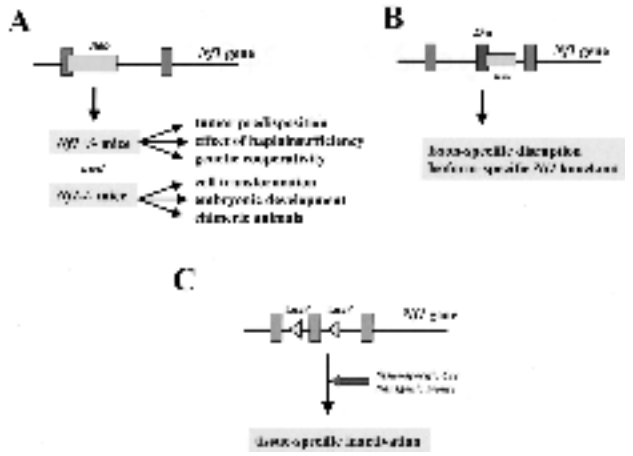

Figure 4. *Strategies for Nf1 mouse modeling. (A) Traditional Nf1+/− mice were generated in which one allele of the murine Nf1 gene is inactivated by the insertion of a neomycin (neo) cassette. Nf1+/− mice can be studied for tumor predisposition, effects of haploinsufficiency, and genetic cooperativity. In addition, Nf1+/− mice can be intercrossed to generate Nf1−/− mice to analyze the effect of neurofibromin loss on cell transformation and embryonic development. Nf1−/− cells can be used to generate chimeric Nf1 mice. (B) Nf1 mutant mice can also be generated in which specific exons are deleted. In this regard, the effect of ablation of a particular Nf1 alternatively spliced exon can be studied. Alternatively, mice can be generated in which a stop codon is inserted into the alternatively spliced exon to ablate Nf1 expression in tissues that express that particular Nf1 isoform. (C) Tissue-specific Nf1 inactivation can be accomplished by the use of Cre/LoxP technology. Briefly, LoxP recombinatorial sequences are inserted into noncoding regions of the Nf1 gene to produce phenotypically normal Nf1^{flox} mice. Tissue-specific inactivation is mediated by the expression of the bacteriophage Cre recombinase from a tissue-specific promoter (e.g., synapsin-I promoter).*

complex. Merlin forms an intramolecular association using residues in the extreme carboxyl terminus (exon 17) to bind to a region at the end of the FERM domain (residues 302–308) [38-40]. In addition to the N-term:C-term intramolecular complex, merlin also forms another intramolecular association within the FERM domain, which is disturbed by mutations within exons 2 and 3 [40,41]. Failure to form either an N-term:C-term or N-term:N-term association leads to an inactive growth suppressor protein (Figure 3*B*). The crystal structure of the merlin FERM domain has been recently determined and provides physical evidence for the biochemical studies demonstrating that intramolecular associations within the FERM domain are important for merlin function [42].

In addition, there is a good correlation between the ability of the various merlin isoforms to form intramolecular interactions and the growth suppressive activity of these molecules. In particular, merlin isoform I, which is the isoform able to inhibit growth when exogenously expressed in rat schwannoma cells, forms both intramolecular interactions [38,40]. However, merlin isoform II, which lacks the region encoded by exon 17 required for proper folding, does not affect schwannoma cell growth. Similarly, some rare merlin isoforms or naturally occurring missense mutants with variant N-terminal regions likewise exhibit a constitutively "open" conformation and lack growth-suppressive activity [43,44].

Merlin folding is also dictated by the phosphorylation state of merlin: Hyperphosphorylation of merlin leads to protein unfolding and is associated with an "inactive" molecule whereas the "active" merlin growth suppressor is underphosphorylated and associates with specific interactors to modulate cell growth [45]. In keeping with the proposed function of merlin in signal transduction events beginning at the plasma membrane, merlin interacts with the transmembrane protein CD44. Merlin associates with the cytoplasmic tail of CD44 preferentially in its hypophosphorylated "active" form [45]. Under growth-permissive conditions, merlin becomes phosphorylated and inactive and does not associate with CD44. In situations permissive for growth arrest, merlin is hypophosphorylated and can bind to CD44 to abrogate CD44 mitogenic signaling. Recent work demonstrated that merlin is phosphorylated in a Rac1/cdc42-dependent fashion [46-48]. The discovery of the link between a well-known signaling pathway and merlin represents an important step toward understanding the regulation of merlin growth suppression.

One approach to defining how merlin functions involves the identification of molecules that specifically interact with merlin and could potentially transduce its growth inhibitory signal. Several merlin interacting proteins have been identified, including βII-spectrin [49], CD44 [50], sodium–hydrogen exchange regulator factor (NHE-RF) [51], schwannomin-interacting protein-1 (SCHIP-1) [52], hepatocyte growth factor–regulated tyrosine kinase substrate (HRS) [53], actin [43], and β1-integrin [54]. NHE-RF interacts with all ERM family members [55] and is therefore unlikely to be a specific merlin signaling molecule. SCHIP-1

Table 1. *Nf1* Mouse Models.

Genotype	Phenotype	Reference
Nf1−/−	Embryonic lethality Double outlet right ventricle Exencephaly	[61-64]
Nf1+/−	Tumor development: pheochromocytoma, leukemia, lymphoma	[62]
	Astrogliosis; increased astrocyte proliferation	[75,76]
	Impaired spatial learning and memory	[80,81]
Neuronal *Nf1* conditional knockout	Astrogliosis, growth retardation	[86]
Astrocyte *Nf1* conditional knockout	Increased astrocyte proliferation	Bajenaru et al., unpublished results Zhu et al., unpublished results
Nf1 exon 23a knockout	Impaired spatial learning and contextual discrimination, delayed motor skill acquisition	[85]
Nf1−/− chimera	Plexiform neurofibromas, myelodysplasia, neuromotor defects	[82]
Nf1+/−:*p53*+/−	Malignant peripheral nerve sheath tumors	[82,83]
	Malignant astrocytomas	[84]

has no known function to date. The interaction between merlin and HRS is intriguing, given the observation that hepatocyte growth factor (HGF) is one of the most potent mitogenic factors for Schwann cells. HRS interacts with merlin better in merlin's "open" conformation and HRS overexpression in rat schwannoma cells has the same effect on cell growth as merlin overexpression [56]. It is not known whether merlin growth suppression requires HRS.

In addition to growth suppression, merlin is involved in actin cytoskeleton–mediated processes, such as motility, spreading, and attachment. Human schwannoma cells deficient in *NF2* expression have dramatic alterations in the actin cytoskeleton, which can be partially reversed by attenuation of Rho pathway activity [57] and by wild-type merlin reexpression [58]. Similarly, overexpression of wild-type merlin in rat schwannoma cells results in decreased cell attachment and motility as well as abnormalities in actin cytoskeleton organization during cell spreading [59]. Support for a role of merlin in modulating actin cytoskeleton–mediated processes also derives from experiments on tumors from $Nf2^{+/-}$ mice, which were shown to be highly motile and metastatic [60].

NF Mouse Modeling

A number of distinct approaches have been taken to develop models for the tumors seen in individuals affected with NF1 (Figure 4 and Table 1). Initial studies focused on the generation of mice with a targeted mutation in the *Nf1* gene. Second-generation models included *Nf1*−/− chimeric mice and *Nf1* exon-specific knockout mice. Recently, tissue-specific inactivation of *Nf1* has yielded important insights into the function of neurofibromin in specific cell types and the consequence of *Nf1* loss on tumorigenesis in NF1. Similar approaches have been taken for the study of NF2 (Figure 5 and Table 2).

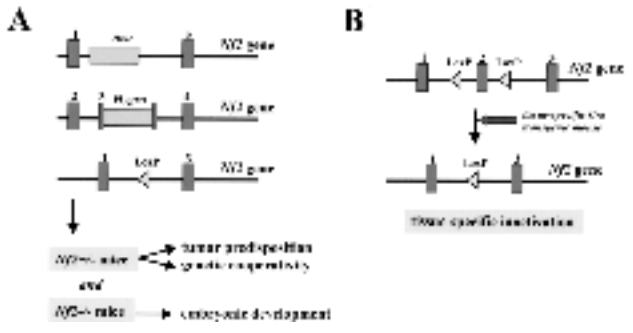

Figure 5. *Strategies for Nf2 mouse modeling. (A) Nf2+/− mice were generated by using three different targeting vectors. In the Nf2 mutant allele generated by McClatchey and associates [88] the 3′ part of exon 2 up to the 5′ part of intron 3 has been replaced by the selection marker leading to a message that has deleted exons 2 to 4. Giovannini and coworkers [87] generated two different mutant Nf2 alleles. One mutant allele, $Nf2^{KO3}$, was generated by an insertional mutation in exon 3 leading to a message in which exon 3 is deleted. The other mutant allele, $Nf2^{\Delta 2}$, carried an in-frame deletion of exon 2. (B) As for Nf1, tissue-specific Nf2 inactivation can be accomplished by the use of Cre/LoxP technology [91]. LoxP recombinatorial sequences are inserted into noncoding regions flanking exon 2 of the Nf2 gene to produce phenotypically normal $Nf2^{flox}$ mice. Tissue-specific inactivation is mediated by the expression of the bacteriophage Cre recombinase from a tissue-specific promoter (e.g., P0 promoter) or by direct injection of adenoviral Cre.*

Table 2. *Nf2* Mouse Models.

Genotype	Phenotype	Reference
Nf2−/−	Embryonic lethality	[88]
Nf2+/−	Tumor development: osteogenic tumors, fibrosarcoma, liver tumors	[60,92]
P0-Sch-Δ(39–121) transgenic mice	Schwann cell hyperplasia, Schwann cell tumors (schwannoma, malignant peripheral nerve sheath tumors)	[87]
Schwann cell *Nf2* conditional knockout	Schwann cell hyperplasia, Schwann cell tumors (schwannoma, malignant peripheral nerve sheath tumors)	[92]
Leptomeningeal cell *Nf2 conditional* knockout	Meningioma	[107]
Schwann cell *Nf2* conditional knockout; *Nf1*+/−	Schwann cell hyperplasia, Schwann cell tumors (schwannoma, neurofibroma, malignant peripheral nerve sheath tumors)	Niwa-Kawakita et al., unpublished results, 2002

Traditional Nf1 Knockout Mice

Traditional gene-targeted studies initially focused on the development of mice in which the murine *Nf1* gene was disrupted. Mice heterozygous for a mutation in the *Nf1* gene (*Nf1*+/−) are viable and fertile, whereas those homozygous for an *Nf1* mutation die during embryonic development. The embryonic lethality resulting from homozygous inactivation of the *Nf1* gene reflects the critical role of neurofibromin during tissue development and organogenesis. *Nf1*−/− embryos die of heart failure and edema secondary to a developmental cardiac vessel defect in which the aortic and pulmonary outflow vessels remain fused (double outlet right ventricle) [61,62]. In addition, some of these mice exhibit neural tube closure defects (exencephaly) and endocardial cushion abnormalities [63,64]. The death of *Nf1*−/− embryos between E12.5 and 13.5 precludes an analysis of the role of the *Nf1* gene in postnatal tissues and cell types affected in adults and children with NF1.

Studies on *Nf1*-deficient cell types have demonstrated that loss of neurofibromin is associated with increased cell proliferation or survival, increased RAS pathway activation, and tumorigenesis. *Nf1*−/− Schwann cells exhibit activation of RAS and but do not initially hyperproliferate *in vitro* [65]. After serum removal, individual clones of hyperproliferating cells emerge that lose expression of myelin P0 and display angiogenic and invasive properties [66]. Neurofibromin-deficient fibroblasts also exhibit abnormal cell proliferation and cannot form perineurium *in vitro* [67]. In hematopoietic progenitor cells, neurofibromin loss is associated with increased cell proliferation in response to multiple mitogenic cytokines, increased RAS pathway

signaling, and chronic myeloid leukemia [22,68,69]. This hyperactivation of RAS is mediated in part through Rac2, which provides cross talk between phosphoinositide 3-kinase (PI-3K) and the Raf-MAPK pathways to modulate cell fate [70]. Lastly, in neurons, activation of RAS is associated with increased cell survival and not proliferation. Sympathetic ganglion neurons deficient in *Nf1* expression display neurotrophin-independent survival [71] that is associated with hyperactivation of RAS and its downstream effector, PI-3K [72,73].

Nf1+/− Mice

Analysis of *Nf1+/−* mice has yielded some important insights into NF1 pathophysiology. *Nf1+/−* animals are prone to tumor development. By 15 to 18 months of age, *Nf1+/−* mice die with leukemias and pheochromocytomas [61,62]. Loss of the wild-type *Nf1* gene can be demonstrated in these tumors, fulfilling the two-hit hypothesis proposed by Alfred Knudson for retinoblastoma. In addition, *Nf1+/−* mice are more susceptible to carcinogen-induced pigmentation and papilloma formation [74].

Nf1+/− mice also demonstrate subtle cell growth abnormalities. Mice heterozygous for a targeted mutation in the *Nf1* gene demonstrate 50% more brain astrocytes with no effects on other glia populations [75]. In contrast, mice heterozygous for a targeted mutation in another RAS GAP molecule, p120-Gap, do not manifest increased astrocyte numbers. *Nf1+/−* astrocytes have a cell-autonomous growth advantage *in vitro* associated with increased activation of the downstream RAS effectors, AKT and MAPK [76]. Genetic cooperativity between *Nf1* and the p53 and retinoblastoma cell cycle regulators can be seen in mice doubly heterozygous for targeted mutations in *Nf1* and *p53* as well as *Nf1* and *RB*. *Nf1+/−* astrocytes also display abnormalities in cytoskeleton-associated processes, including motility, attachment and actin cytoskeleton organization during cell spreading, supporting the notion that some of the nontumor phenotypes in NF1 may result from additional alterations in cell physiology, not strictly due to increased cell proliferation [77]. Similarly, *Nf1+/−* fibroblasts proliferate faster *in vitro* than wild-type fibroblasts, display abnormalities in collagen deposition, and exhibit abnormal wound healing *in vivo* [78]. As observed with fibroblasts and astrocytes, *Nf1+/−* melanocytes and mast cells demonstrate increased cell proliferation and RAS pathway activation [79].

Nf1+/− mice exhibit spatial learning deficits reminiscent of the specific learning disabilities in children with NF1 [80]. The mechanism for these learning deficits appears to be related to increased GABA-mediated inhibition and specific defects in long-term potentiation (LTP) [81]. Decreasing RAS function in *Nf1+/−* mice reverses both the inhibition and LTP deficits, suggesting that brain dysfunction in NF1 might be a direct consequence of abnormal RAS activation.

Nf1+/− mice develop malignant tumors when bred with mice heterozygous for a mutation in another tumor suppressor gene, *p53*. *Nf1+/−;p53+/−* mice are prone to the development of high-grade MPNSTs that are histologically similar to their human counterparts [82,83]. The spectrum of tumors in these *Nf1+/−;p53+/−* mice can be modulated by the genetic background of the mouse [84].

Exon-Specific Nf1 Knockout Mice

Mice in which exon 23a has been selectively ablated are viable, fertile, and do not succumb to tumors, as reported for *Nf1+/−* mice. Interestingly, loss of exon 23a results in mice with significant abnormalities in spatial learning and memory, similar to *Nf1+/−* mice [85]. This phenotype is unexpected given the lack of *Nf1* mRNA containing exon 23a in cultured neurons [29], but clearly argues that exon 23a is important for neurofibromin's function in cognition, memory and learning.

Exon 9a knockout mice have recently been generated by Alcino Silva and his colleagues (Y. Elgersma and A. Silva, personal communication). Mice in which *Nf1* is inactivated in exon 9a–expressing neurons, produced by the insertion of a termination codon in exon 9a, demonstrate deficits in long-term potentiation and spatial learning, as well as a slight increase in astrocyte number. In contrast, ablation of only exon 9a–containing *Nf1* results in mice with normal learning, but a similar increase in astrocyte number. These results suggest that whereas loss of neurofibromin in neurons results in learning deficits, exon 9a itself does not appear to be essential for learning and memory in mice.

Nf1 Chimeric Mice

A limitation to the conventional *Nf1* knockout strategy is the inability to study the growth advantage and tumorigenic properties of a small population of cells deficient in neurofibromin expression in the context of the whole mouse. Two approaches have been taken to circumvent this problem. The first involves the generation of chimeric mice in which the developing embryo is composed of a small number of *Nf1−/−* cells. The chimeric approach allows one to more accurately model the human condition, because tumors arising in individuals with NF1 involve small numbers of cells or clones in which somatic inactivation of *NF1* results in loss of neurofibromin expression. Chimeric mice composed in part of *Nf1−/−* cells develop neurofibromas, reminiscent of plexiform neurofibromas seen in patients with NF1 [82]. Similarly, adoptive transfer of *Nf1−/−* myeloid cells into an irradiated recipient normal host induces myeloproliferative disease through hypersensitivity to granulocyte–macrophage colony-stimulating factor (GM-CSF) [68].

Conditional Nf1 Knockout Mice

A second approach involves the generation of tissue-specific conditional knockout mice. Recently, mice have been generated by Luis Parada and colleagues in which the *Nf1* gene is conditionally disrupted using Cre-*LoxP* technology. In this approach, specific exons of the *Nf1* gene are flanked by *LoxP* recombinatorial sequences placed in noncoding introns. These flox*Nf1* mice are phenotypically

indistinguishable from wild-type mice because the insertion of these *LoxP* sequences does not disrupt *Nf1* gene expression. Inactivation of the *Nf1* gene occurs in the presence of the bacteriophage Cre recombinase enzyme, which can be expressed *in vitro* by adenoviral delivery or *in vivo* using tissue-specific promoters in transgenic mice. Several *Nf1* conditional knockout mice have been generated including those with neuron-specific, astrocyte-specific, and Schwann cell–specific *Nf1* inactivation. Ablation of *Nf1* in neurons using the synapsin-I promoter results in viable, growth-retarded pups that exhibit massive brain astrogliosis and abnormalities in cortical development [86]. The astrogliosis in this model appears to be reactive in response to abnormal neuronal function and is not related to increased astrocyte proliferation. In contrast, astrocyte-specific *Nf1* inactivation appears to be insufficient for astrocytoma formation, but does confer a significant growth advantage for astrocytes *in vitro* and *in vivo* (Bajenaru, Zhu, Parada, and Gutmann, unpublished observations). It is not clear whether the failure to generate astrocytomas reflects additional genetic events or other cooperating factors necessary for brain tumor formation in NF1.

Traditional Nf2 Knockout Mice

Two different groups have reported the development of *Nf2* knockout mice [87,88]. In the two studies, similar gene-targeting approaches were used, with the primary differences relating to how much of the endogenous *Nf2* gene was deleted during the targeting process (Figure 5). In our approach to model more closely human hereditary (NF2-related) and sporadic schwannomas in the mouse, we have initially generated two mouse lines carrying different *Nf2* mutant alleles [87]. The first mutant allele, $Nf2^{KO3}$, was generated by an insertional mutation in exon 3. This $Nf2^{KO3}$ allele differed from the mutant allele described by McClatchey and associates [88], here called "$Nf2^{KO2-3}$." In the latter, the 3′ part of exon 2 up to the 5′ part of intron 3 has been replaced by the selection marker leading to a message that lacks exons 2 to 3. The second mutant allele, $Nf2^{\Delta 2}$, carried an in-frame deletion of exon 2. Both the $Nf2^{KO3}$ and $Nf2^{\Delta 2}$ alleles mimicked two different, naturally occurring, human mutant *NF2* alleles and allowed us to compare the phenotypic effects of these two human *NF2* mutations in mice. Because the three different targeting approaches resulted in germline *Nf2* homozygous mutant mice, which were not viable, it is likely that all have *Nf2* null alleles. *Nf2* null mice die early during embryonic development of a failure to initiate gastrulation as a result of an absence of organized extraembryonic ectoderm [88]. The underlying defect in these $Nf2^{-/-}$ embryos does not appear to be related to cell proliferation abnormalities in the embryo itself, but rather a failure to produce the extraembryonic structures required to generate a mesoderm-inducing signal from the embryo proper. The inability of the developing *Nf2*-deficient embryo to generate or respond to important differentiation cues suggests a role for the *Nf2* protein in cell–cell signaling events critical for extraembryonic formation.

Heterozygosity for any of the three mutant alleles leads to a high incidence of bone tumors showing loss of the wild-type *Nf2* allele. However, differences were observed with respect to the grade of malignancy. Metastases of osteosarcoma were found less frequently in $Nf2^{KO3/+}$ and $Nf2^{\Delta 2/+}$ mice in comparison to the mice described by McClatchey and associates [60] (29% vs. 95%, $P<0.0001$, in case of $Nf2^{KO3/+}$ mice). The different time windows of the histologic analyses may account for this difference, because mice were followed up to 24 months in our study and up to 30 months in the study of McClatchey and colleagues. The finding of osteomas in $Nf2^{KO3/+}$ and $Nf2^{\Delta 2/+}$ mice contrasts with the absence of benign bone tumors described by McClatchey [60]. This difference might be explained by the use of different genetic backgrounds. Regardless, our data indicate that *Nf2* mutant alleles lacking only exon 2 or exon 3 do not permit normal embryonic development and lack tumor-suppressor properties. Thus, both exon 2 and exon 3 carry sequences that are essential for *Nf2* function and regardless of the mutation type, all three hemizygous mice do not show clinical features of human NF2. In particular they demonstrate a tumor spectrum that differs significantly from that observed in NF2 patients. Therefore, these animals do not accurately recapitulate the cognate human genetic disease.

Schwannomas and Schwann Cell Hyperplasia Are Induced by Overexpression of a Mutant Nf2

When transiently expressed in various cell types, mutant proteins corresponding to naturally occurring *NF2* mutations demonstrate distinct subcellular localizations [36,89,90]. C-terminal deletion mutants of various lengths remain located at the cell membrane. In contrast, mutants with an intact C-terminal domain but with a deleted or altered N-terminal domain are mislocalized mainly to the perinuclear cytoplasmic region. Such mislocalization was observed for a mutant protein modeled from naturally occurring mutations where exons 2–3 are deleted without a frameshift, Sch-Δ(39–121) [36].

To develop a system by which to identify functional domains of the *NF2* protein that may play a role in merlin growth suppression, we have generated transgenic mice expressing various *Nf2* mutants under the control of the Schwann cell-specific P0 promoter [91], including a mutant schwannomin modeled from a naturally occurring mutation, Sch-Δ(39–121) and a mutant schwannomin prototypic for C-terminal deletion mutants, Sch-ΔCter. Mice expressing Sch-Δ(39–121) showed a high prevalence of Schwann cell–derived tumors and Schwann cell hyperplasia, whereas those expressing Sch-ΔCter were normal [87]. These results indicate that a subset of mutant *NF2* alleles observed in patients may encode products with dominant-negative properties when overexpressed in specific cell lineages. However, the endogenous expression level of a mutant *Nf2* allele may not be sufficient to elicit this "potential" oncogenic effect. Indeed, proteins generated from endogenous mutant alleles in $Nf2^{KO3/+}$ and $Nf2^{\Delta 2/+}$ mice are hardly detectable and recent data demonstrate that mutant products of

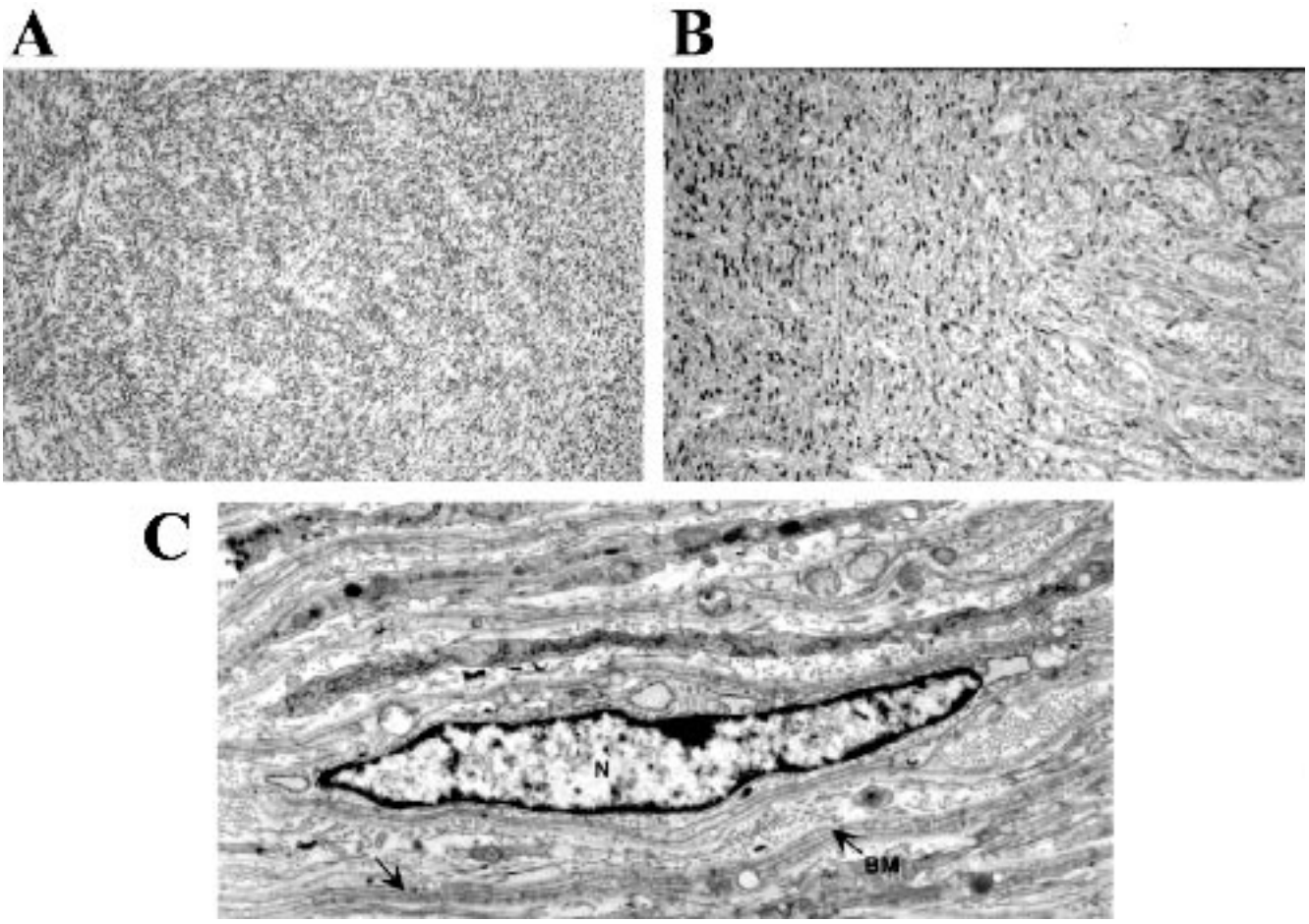

Figure 6. *Schwannomas and Schwann cell hyperplasia in P0CreC;Nf2$^{flox2/flox2}$ mice. (A) Schwannoma in the uterus of a P0CreC;Nf2$^{flox2/flox2}$ mouse. The tumor appeared as a rather uniform Schwann cell growth extending from the corpus uteri to the uterine horns in combination with (B) Schwann cell hyperplasia in the peripheral part of the myometrium showing strong S-100 protein immunoreactivity. (C) Ultrastructural examination of the tumor demonstrated Schwann cells (Schwann cell nucleus denoted by N) with long, thin cytoplasmic processes (arrow), which was variably associated with a basement membrane (BM).*

endogenous alleles are efficiently degraded by the ubiquitin-dependent proteasome pathway (Gautreau et al., submitted for publication, 2002).

Conditional Nf2 Knockout Mice

To examine the consequences of *Nf2*-deficiency in selected cell types of adult mice, we have generated a conditional *Nf2* allele (*Nf2*^{flox2}) [92]. Upon successive matings with transgenic P0-Cre mice, the *Nf2* conditional mutant mice recapitulate several features observed in NF2 patients, specifically Schwann cell hyperplasia, Schwann cell tumors, cataracts, and cerebral calcifications (Figure 6). All these manifestations have been exclusively observed in mice with both alleles of the *Nf2* gene inactivated in Schwann cells and in a subset of neural crest cells. Meningioma, the second tumor hallmark of human NF2, was not observed in these mice, suggesting that this tumor originates from cells that do not express the P0 protein. To determine whether *Nf2* disruption is also sufficient for meningioma formation, we inactivated *Nf2* in homozygous conditional knockout mice by adenoviral Cre delivery. Mice with arachnoidal cell Cre-mediated excision of *Nf2* exon 2 developed a range of meningioma subtypes histologically similar to the human tumors [107].

Conditional *Nf2* knockout mice also provide a powerful tool for investigating genetic interactions and tumorigenic cooperativity. In an attempt to assess the cooperativity of the *Nf2* gene with other cancer-associated genes, we and others have crossed *Nf2* mutant (germline or conditional) mice to other strains of knockout mice. In such crosses, if the offspring containing both parental oncogenic alleles develop tumors faster or of a different type than either tumor-prone parent, then genetic cooperativity is operative. The identification of cooperating tumor suppressor genes, which synergize with *Nf2* loss, can provide important mechanistic insights into *Nf2*-associated tumorigenesis pathways. Table 2 describes the results from some representative crosses performed to date. Remarkably, some crosses result in accelerated tumorigenesis in the bitransgenic offspring compared to either parent. For example, mice carrying two mutant *Nf2* (*conditional*; *P0*) and one *Nf1* null allele(s) not only show accelerated tumorigenesis, but also exhibit novel tumor types (e.g., neurofibroma) not seen in the parental mutant mice.

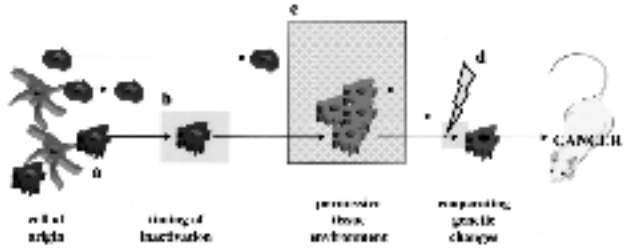

Figure 7. *Development of future mouse models for NF1-associated tumors. The formation of tumors or cancer requires a number of events, both genetic and environment, that culminate in tumorigenesis. First, Nf1 inactivation must occur in the correct cell of origin at the proper developmental time. In some cell types, the timing of inactivation might be critical. Second, in the brain, some regions might be permissive for tumor development. This permissiveness may reflect genetic factors, regional differences in cellular composition, and brain environment. Third, cooperating genetic changes may influence tumor expansion. These genetic changes might be subtle genetic alterations, not previously thought to be conventional initiating events for tumorigenesis, or modifying loci that promote tumor formation.*

Future Approaches

The failure of *Nf1*+/− and *Nf2*+/− knockout mice to develop tumor spectra similar to the human conditions reflects the major limitation of first-generation knockout modeling strategies. The generation of mouse models that accurately mimic the human disease requires additional sophisticated approaches, such as tissue-specific and conditional mutations. However, the failure of astrocyte-specific knockout mice to develop astrocytomas indicates that even the newest generation of mouse model has a number of limitations (Figure 7).

First, NF1-associated astrocytomas typically occur in specific brain regions (optic nerve, chiasm, hypothalamus, and brainstem). It is possible that not all regions of the brain are permissive for NF1-associated brain tumor formation. In this regard, pilocytic astrocytomas in NF1 typically occur within the optic pathway, whereas in the general population, the most frequent brain location for a pilocytic astrocytoma is the cerebellum. Astrocytes from different regions of the brain have been reported to have differing responses to agents that elevate cyclic AMP [93], regional specificity in gap junction coupling [94], differences in glutamate and serotonin uptake [95], and distinctive growth patterns [96]. Future studies aimed at inactivating *Nf1* specifically in cerebellar or optic nerve astrocytes might be useful in determining the importance of regional differences in astrocyte biology or brain environment.

Second, the cell of origin of the pilocytic astrocytoma may not be a type 1 fibrillary astrocyte, based on recent studies demonstrating gene expression similarities between pilocytic astrocytomas and oligodendrocyte–astrocyte precursors [103]. Pilocytic astrocytomas represent a unique grade of glial neoplasm that does not progress to a fibrillary astrocytoma. Several studies have highlighted these inherent differences, both with regard to genetic changes and glial cell protein expression patterns [97-101]. For instance, pilocytic astrocytomas express the unique PEN5 epitope shared with cells and tumors of an oligodendrocyte lineage [102]. This marker is not expressed in fibrillary astrocytes or astrocytomas. Recent studies from our laboratory have demonstrated expression of oligodendrocyte-associated transcripts, such as PLP, PMP-22, MBP, and oligodendrocyte myelin glycoprotein (OMgP) in pilocytic astrocytomas, suggesting that these tumors have at least some gene expression features of oligodendrocyte-type 2 astrocyte (O2A) cells [103]. In this regard, it may be necessary to inactivate *Nf1* in the correct progenitor cell, perhaps an O2A lineage cell, to generate NF1-associated astrocytomas in mice.

Third, NF1-associated astrocytomas do not develop in the context of a genetically normal (*Nf1*+/+) brain environment. In contrast, these tumors develop in a brain environment in which the rest of the brain is *Nf1*+/−. In the neurofibroma, it has been suggested that *Nf1*+/− mast cells and fibroblasts play a role in tumorigenesis. Another environmental effect is the contribution of modifying genes to tumor development. In the case of glioblastoma, Reilly et al. observed genetic background-specific effects on the spectrum of tumors in *Nf1*+/−;*p53*+/− mice [84]. Further work will be required to formally address this genetic environment effect as well as the mechanisms that underlie the modifier gene effect on tumor spectrum.

Lastly, it is possible that NF1-associated astrocytomas only form when other cooperating genetic alterations are present. It is unlikely that these additional genetic events are the common alterations seen in high-grade astrocytomas, like p53 loss [101], despite the observation that high-grade glioblastomas form in mice doubly heterozygous for mutations in both *Nf1* and *p53* [84]. Work is ongoing in many laboratories using gene and protein expression profiling technologies to identify subtle cooperating factors that promote or inhibit tumorigenesis. The characterization of these additional proteins may provide additional targets for rational drug design for the effective management of tumors in NF1.

In the case of NF2 modeling, the refinement of current targeting approaches has allowed the generation of relevant models of NF2-related tumor development and provides powerful tools for investigating meningioma progression and for the preclinical evaluation of potential therapeutic interventions. In particular, the availability of arachnoid cell–specific *Nf2* conditional mutant mice will greatly facilitate the elucidation of the critical genetic factors that influence meningioma development and progression.

Recently, inactivation of Protein 4.1B (or DAL-1), a second protein 4.1–related tumor suppressor, was identified as an early event in meningioma tumorigenesis [104,105]. In contrast to *NF2*, Protein 4.1B loss occurs in meningiomas and not in schwannomas [104], and overexpression of Protein 4.1B suppresses meningioma cell line proliferation, but has no effect on schwannoma cell line growth *in vitro* [106]. Further work will need to be performed to determine the relationship of merlin loss to Protein 4.1B expression as well as the contribution of Protein 4.1B to meningioma pathogenesis in the mouse. The generation of a preclinical mouse model for meningiomas through the targeted

disruption of the Protein 4.1B and *Nf2* genes could facilitate the assessment of potential targeted therapies for this common brain tumor in NF2.

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