

Membrane organization and tumorigenesis—the NF2 tumor suppressor, Merlin

Andrea I. McClatchey^{1,3} and Marco Giovannini²

¹Massachusetts General Hospital, Center for Cancer Research and Harvard Medical School, Department of Pathology, Charlestown, Massachusetts 02129, USA; ²Inserm U674, Fondation Jean Dausset-CEPH et Institut Universitaire d'Hématologie, Paris, France

The *NF2* tumor-suppressor gene was cloned more than a decade ago, but the function of its encoded protein, Merlin, remains elusive. Merlin, like the closely related ERM proteins, appears to provide regulated linkage between membrane-associated proteins and the actin cytoskeleton and is therefore poised to function in receiving and interpreting signals from the extracellular milieu. Recent studies suggest that Merlin may coordinate the processes of growth-factor receptor signaling and cell adhesion. Varying use of this organizing activity by different types of cells could provide an explanation for the unique spectrum of tumors associated with *NF2* deficiency in mammals.

The identification of the genetic defect responsible for the familial cancer syndrome Neurofibromatosis type 2 (*NF2*) nearly 15 years ago yielded the unexpected prospect that the encoded tumor suppressor was a cytoskeleton-associated protein (Rouleau et al. 1993; Trofatter et al. 1993). This was in marked contrast to the known and now “classic” tumor suppressors p53, Rb, and NF1 that function either to directly control the cell cycle machinery in the nucleus, or in the case of NF1, to directly negatively regulate mitogenic Ras signaling (Sherr 2004). Several factors have rendered progress in defining the molecular basis of *NF2*-associated tumorigenesis slow, including the rare incidence of *NF2* in humans, the paucity of *NF2*^{-/-} cell lines and technical challenges in studying the *NF2*-encoded protein, Merlin, which is a novel type of tumor suppressor. However, recent advances, highlighted by the study of *NF2* function in several different model organisms have begun to yield a model of Merlin function that offers important lessons about the origins and progression of cancer and provides novel insight into some basic biological principles. In

this review we have highlighted recent advances in understanding the pathology and molecular biology of *NF2* with particular emphasis on the broader impact that the study of *NF2* may have on the fields of tumor, cellular, and molecular biology.

The incidence of *NF2* in humans is rare (fewer than one in 25,000 individuals) and the symptoms and phenotypes associated with *NF2* are unusual and restricted (Evans et al. 2005). The hallmark of the disease is the development of Schwann cell tumors (schwannomas) on or around the vestibular branch of both eighth cranial nerves. Most *NF2* patients go on to develop multiple schwannomas that are associated with other cranial nerves and spinal nerve roots, cranial and spinal meningiomas and, less frequently, intraspinal ependymomas. In contrast to other major human malignancies, these are benign, slow-growing tumors that respond poorly to chemotherapeutic intervention and cause significant morbidity. Indeed, the current standard of treatment remains local tumor control by repeated surgeries and radiation, which are often accompanied by damage of nerves and CNS structures (Evans et al. 1992).

Biallelic inactivation of the *NF2* gene can also be identified in most sporadically occurring schwannomas (Stemmer-Rachamimov et al. 1997) and a large fraction of sporadically occurring meningiomas (Ruttledge et al. 1994; Lomas et al. 2005). In addition to tumors that are associated with familial *NF2*, *NF2* mutations are frequently found in sporadic mesotheliomas of the lung lining that are associated with asbestos exposure (Bianchi et al. 1995; Sekido et al. 1995). More recently, *NF2* mutations have been reported in some sporadic thyroid carcinomas, hepatocellular carcinoma cell lines, and perineurial tumors, revealing additional cell types affected by *NF2* loss (Lasota et al. 2001; Pineau et al. 2003; Sheikh et al. 2004). The generation and study of *Nf2*-mutant strains of mice has further expanded the range of cancers associated with *NF2* mutation and cell types affected by *NF2* deficiency (see below). However, *NF2* mutations have not yet been reported in many common/prevalent human cancers. As discussed below, these data raise the important question of whether there is a biological and/

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³Corresponding author.

E-MAIL mcclatch@helix.mgh.harvard.edu; FAX (617) 726-7808.

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or genetic basis for the unique spectrum of spontaneous tumors associated with *NF2* mutation in humans and mice.

A novel type of tumor suppressor

Initial studies of the function of the *NF2*-encoded tumor suppressor, Merlin (also known as Schwannomin), were modeled after studies of other tumor suppressors and of the closely related ERM proteins (Ezrin, Radixin, and Moesin). Early studies confirmed, as for other tumor suppressors, that overexpression of Merlin can block both cell proliferation and oncogene-induced transformation (Tikoo et al. 1994; Lutchman and Rouleau 1995). Indeed, Merlin can negatively regulate cyclin D1 levels, consistent with the cell cycle arrest observed when Merlin is overexpressed (Xiao et al. 2002). However, given its predominant localization to the membrane:cytoskeleton interface, Merlin is not likely to directly control the cell cycle machinery. Instead, Merlin, like the ERM proteins, appears to provide regulated linkage between membrane-associated proteins and the actin cytoskeleton and thereby to function in membrane organization. Merlin is therefore a novel type of tumor suppressor poised to function in receiving and interpreting signals from the extracellular milieu.

Model organisms

The identification of *NF2* homologs from other species and the functional characterization of their encoded proteins in other model organisms provides a valuable complement to the functional characterization of the mammalian *NF2* protein. Genes similar to the human *NF2* gene can be identified in many other metazoans, including invertebrates (*Drosophila melanogaster*, *Anopheles gambiae*, *Caenorhabditis elegans*, *Xenopus laevis*), fish (*Danio rerio*, *Oryzias latipes*), and birds (*Gallus gallus*). No *NF2* homolog has been identified in *Saccharomyces cerevisiae* (yeast), suggesting that Merlin function is specific to the evolutionary branching of metazoans. The high degree of homology among metazoan *NF2* gene products is complemented by the fact that they can be used interchangeably in functional studies, i.e., the human protein can function reliably in mouse and *Drosophila* model systems (LaJeunesse et al. 1998; Giovannini et al. 1999).

Several *Nf2* mutant alleles that mimic naturally occurring human *NF2* mutations have been engineered in the mouse, allowing a comparison of the phenotypic effects of these mutations in an animal model. Three different targeting approaches resulted in germline *Nf2* homozygous mutants that were not viable (McClatchey et al. 1997; Giovannini et al. 2000). *Nf2* null embryos fail early during embryonic development without initiating gastrulation (McClatchey et al. 1997). The underlying defect in *Nf2*^{-/-} embryos is not due to cell proliferation abnormalities in the embryo itself, but rather, the ab-

sence of extra-embryonic structures that are required to generate a mesoderm-inducing signal. Notably, Merlin function is also required for normal development in the fruitfly, *D. melanogaster* and in the flatworm *C. elegans* (Fehon et al. 1997; J. Gervais, J. Satterlee, and A.I. McClatchey, unpubl.).

In the mouse, heterozygosity for any of the three mutant *Nf2* alleles leads to a high incidence of bone tumors that exhibit loss of the wild-type *Nf2* allele (McClatchey et al. 1998; Giovannini et al. 2000). Differences in the grade of malignancy observed in these tumors might be explained by the use of different genetic backgrounds. Indeed, some studies have indicated that *Nf2* loss contributes to tumor metastasis (see below; McClatchey et al. 1997). Notably, *Nf2*^{+/-} mice are collectively predisposed to developing a variety of other tumor types at lower frequency, suggesting a broader role for *Nf2* loss in tumorigenesis. None of the three strains of *Nf2* heterozygous mutant mice spontaneously develop the classic clinical features of human *NF2*. However, consistent with the identification of *NF2* mutations in asbestos-induced mesotheliomas in humans, *Nf2*^{+/-} mice do show increased sensitivity to the carcinogenic effects of asbestos fibers. Intraperitoneal injection of asbestos fibers induces the formation of malignant mesotheliomas at a higher frequency in *Nf2*^{+/-} mice than in wild-type mice (Fleury-Feith et al. 2003). Importantly, asbestos-induced tumors in *Nf2*^{+/-} mice exhibit loss of the wild-type *Nf2* allele. These mice should be useful for further studies of the molecular pathogenesis, genetic modification, and environmental initiation of mesothelioma development.

Although *Nf2* heterozygous mutant mice that genetically mimic human *NF2* patients do not spontaneously develop schwannomas, inactivation of both *Nf2* alleles specifically in Schwann cells (SCs) does yield manifestations of human *NF2*. Upon mating with transgenic *PO-Cre* mice, in which the Cre recombinase is expressed specifically in SCs and in a subset of neural crest cells, *Nf2* conditional mutant (*PO-Cre;Nf2*^{fllox2/fllox2}) mice develop SC hyperplasia, SC tumors, cataracts, and cerebral calcifications (Fig. 1A,B; Giovannini et al. 2000). Moreover, mice with biallelic inactivation of *Nf2* specifically in arachnoid cells develop a range of meningioma subtypes that are histologically similar to the human tumors (Kalamarides et al. 2002). Therefore, the lack of spontaneous schwannoma and meningioma development in *Nf2*^{+/-} mice is not due to a fundamental difference in the function of mouse Merlin or in the biology of mouse Schwann and arachnoid cells, but instead to differences in the probability of loss of the wild-type allele in the cells from which schwannomas and meningiomas originate.

The growth-suppressing function of Merlin is conserved in other species. For example, somatic mosaicism analysis reveals that clones of *Mer* homozygous mutant epithelial cells in the *Drosophila* eye hyperproliferate, providing a powerful system for carrying out genetic screens to identify modifiers of *Nf2* deficiency (LaJeunesse et al. 2001). Recent analyses of the chicken Merlin

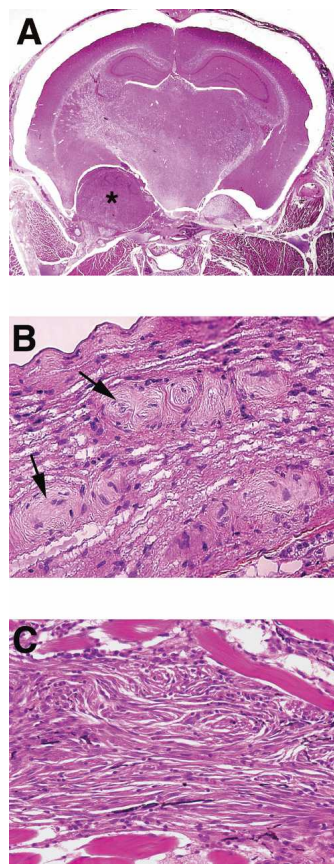


Figure 1. Loss of Merlin leads to SC hyperproliferation and tumorigenesis in animal models. (A) Hematoxylin and eosin (H&E)-stained section of frank schwannoma emanating from the trigeminal nerve of a *P0-Cre;Nf2^{flox2/flox2}* mouse (*). (B) H&E-stained section depicting SC hyperplasia in the sciatic nerve of a *P0-Cre;Nf2^{flox2/flox2}* mouse. (C) Malignant peripheral nerve sheath tumor in *Nf2a*-mutant zebrafish. Zebrafish bearing a germline mutation in one allele of the *NF2a* gene develop spindle cell tumors that have features in common with nerve sheath tumors from other species (image kindly provided by K. Edepli) (Amsterdam et al. 2004).

homolog also confirm its function as a negative regulator of proliferation in vitro and in vivo (Chen et al. 2004a,b). The study of the *Drosophila* Merlin protein has also provided key insight into the regions of Merlin that are required for its localization and function (LaJeunesse et al. 1998). This also led to the identification and analysis of the so-called “Blue Box” mutant, *Mer^{ABB}*, which is known to act in a dominant-negative manner and therefore to interfere with the activity of the wild-type protein. Ectopic expression of *Mer^{ABB}* in the wing results in increased proliferation (LaJeunesse et al. 1998). Expression of the murine analog of *Mer^{ABB}* induces transformation of cultured NIH3T3 fibroblasts (Johnson et al. 2002). Interestingly, SC hyperplasia and tumors were observed in transgenic mice that express a patient-derived mutant version of Merlin lacking amino acids 39–121 in SCs, indicating that this isoform, when overexpressed, may also have dominant-negative properties

(Giovannini et al. 1999). Therefore, when overexpressed, certain mutant versions of Merlin exhibit oncogenic properties. However, in human tumors, where expression of mutant *NF2* alleles is controlled by endogenous regulatory sequences, the same mutant Merlin isoforms must be unstable, resulting in a loss of function rather than a dominant oncogenic effect (Gautreau et al. 2002). Delineation of the regions of Merlin that are necessary for the dominant negative activity of overexpressed Merlin may provide novel mechanistic insight into Merlin function.

The different strains of *Nf2* mutant mice also provide powerful tools for investigating genetic cooperativity in tumorigenesis. The identification of tumor suppressor or other gene mutations that cooperate with *Nf2* loss can provide important mechanistic insight into *Nf2*-associated pathways. Dramatic cooperativity between the *Nf2* and *p53* tumor suppressor gene mutations is displayed by *Nf2^{+/-};p53^{+/-}* *cis* mice that carry heterozygous *Nf2* and *p53* mutations on the same chromosome; these mice develop multiple osteosarcomas early in life (McClatchey et al. 1998). Notably, *Nf2^{+/-};p53^{+/-}* *trans* mice develop fewer tumors with longer latency; therefore genetic linkage of cancer predisposing mutations can profoundly influence tumorigenesis. In conditional *Nf2* mutant mice, *p53* heterozygosity can also markedly increase the incidence of SC-derived malignant peripheral nerve sheath tumors (MPNSTs) (Robanus-Maandag et al. 2004). Importantly, the timing of *Nf2* inactivation seems to determine in which neural crest-derived cell type the growth advantage occurs since, in contrast to *P0-Cre;Nf2^{flox2/flox2};p53^{+/-}* mice, *P0-Cre;Nf2^{flox2/+};p53^{+/-}* *cis* mice develop osteogenic but not SC tumors. This difference in tumor spectrum could be simply explained by the fact that *P0-Cre;Nf2^{flox2/flox2};p53^{+/-}* mice do not require an additional event for biallelic inactivation of *Nf2* and SC hyperproliferation; in the *P0-Cre;Nf2^{flox2/+};p53^{+/-}* *cis* mice, spontaneous loss of the wild-type *Nf2* allele may occur more rapidly in osteoblasts than in SCs. Alternatively, this difference could reflect cell-type-specific consequences of sequential versus simultaneous loss of *Nf2* and *p53* function. Perhaps *Nf2* deficiency leads to *p53*-dependent growth arrest or apoptosis in OBs but not SCs. Finally, early biallelic *Nf2* loss in *P0-Cre;Nf2^{flox2/flox2};p53^{+/-}* mice could specifically target a population of SC progenitors that are particularly sensitive to loss of Merlin function.

More recently, the role of *NF2* and *p53* mutations in SC tumorigenesis has been emphasized by the observation of MPNST development in *NF2* (Amsterdam et al. 2004) and *p53* (Berghmans et al. 2005) mutant zebrafish (Fig. 1C). Although *p53* mutations are found in human MPNSTs, it is interesting to note that in *NF2* patients, benign SC tumors initiated by biallelic *NF2* gene inactivation do not progress into MPNSTs (see below). Indeed, malignant transformation of a schwannoma is an exceedingly rare event and analysis of the few cases reported in the literature show that none of the patients had *NF2* (Woodruff et al. 1994; McMenamin and Fletcher 2001).

Regulation of Merlin

Structure

Many studies have established parallels between Merlin and the ERMs. Central to Merlin function is the fact that like the ERM proteins, Merlin can undergo regulated intramolecular self-association (Gonzalez-Agosti et al. 1996; Sherman et al. 1997; Gronholm et al. 1999; Nguyen et al. 2001). Several recent structural studies have yielded complementary models of Merlin and ERM tertiary conformation. The 1.9 Å crystal structure of the complexed N-terminal FERM (Four-point-one, Ezrin, Moesin, Radixin) and C-terminal tail domains of mammalian Moesin reveals that the C-terminal tail adopts an extended conformation to interact with the trilobed FERM domain, masking potential binding sites of other proteins (Pearson et al. 2000). Most (~81%) of the residues that are conserved between Merlin and the ERM proteins map to this interface, indicating that Merlin likely adopts a similar conformation. Indeed, the structure of the isolated Merlin FERM domain is very similar to that of the ERMs (Shimizu et al. 2002). Although most human *NF2* mutations identified are deletions or truncating mutations that effect complete loss of function, missense mutations do occur and are distributed throughout the FERM domain and C-terminal tail, suggesting that Merlin function is highly dependent upon the tertiary configuration of the entire protein (Pearson et al. 2000). Mutations that disrupt self-association likely also alter Merlin localization and protein interactions. Like the ERMs, Merlin self-association is regulated, at least in part, by phosphorylation (see below).

Post-translational modification

An increasing number of studies have examined the regulation of Merlin by phosphorylation. It was recognized early on that the two major isoforms of Merlin visualized by SDS-PAGE and immunoblotting represent differentially phosphorylated species (Shaw et al. 1998). Although Merlin is phosphorylated on multiple residues, phosphorylation of a single serine residue, S518, causes the mobility shift and has therefore been best studied (Shaw et al. 2001; Kissil et al. 2002; Xiao et al. 2002; Rong et al. 2004). Available data suggest that S518 phosphorylation weakens self-association and inactivates the growth-suppressing function of Merlin. Phosphorylation of Merlin at S518 is regulated by specific cell culture conditions, including cell density, cell:substrate attachment, and growth factor availability (Shaw et al. 1998). The best-studied stimulus for Merlin S518 phosphorylation is activation of the small GTPase Rac1 (Shaw et al. 2001). Rac-induced phosphorylation of Merlin S518 appears to be mediated by the major Rac effector p21-activated kinase (Pak) (Kissil et al. 2002; Xiao et al. 2002). Interestingly, S518 can also be phosphorylated in a Pak-independent manner by protein kinase A (PKA) (Alfthan et al. 2004), suggesting that disparate stimuli regulate Merlin. Similarly, multiple signals appear to induce phosphorylation of a conserved C-terminal threonine

residue in the ERM C-terminal tail (Matsui et al. 1998, 1999; Pietromonaco et al. 1998; Hipfner et al. 2004). Virtually nothing is known about the regulation or functional consequences of phosphorylation of other Merlin residues. Finally, phosphatidylinositol 4,5-bisphosphate (PIP₂) binding to the FERM domain is thought to cooperate with phosphorylation in weakening self-association during ERM activation; the mapped ERM PIP₂-binding sites are conserved in Merlin, suggesting that PIP₂ binding may also play an important role in regulating Merlin function (Barret et al. 2000). Collectively, these studies suggest that the cell may integrate multiple strategies to fine-tune the localization and activity of Merlin.

Subcellular localization

If Merlin's ability to organize membrane domains is central to its function as a tumor suppressor, then it is critical to define where Merlin is localized within the cell. This has been particularly challenging due to the low levels of endogenous Merlin in most cell types and to the insolubility of a substantial pool of Merlin. As a consequence, many studies have examined the localization of exogenous (overexpressed), often epitope-tagged Merlin, which may not fully mirror the endogenous protein. In *Drosophila* epithelia, endogenous Merlin is concentrated in the subapical junctional region, where it overlaps with the more apically localized Moesin (Bretscher et al. 2002). Similarly, in the *C. elegans* intestinal epithelium, exogenous *nfm-1*, the Merlin paralog, localizes basolaterally relative to *erm-1* (Gobel et al. 2004). In cultured mammalian cells, endogenous and exogenous Merlin is concentrated in actin-rich structures such as membrane ruffles in isolated cells and along cell:cell boundaries as they form, consistent with its role in mediating contact-dependent inhibition of proliferation (see below; Gonzalez-Agosti et al. 1996; Maeda et al. 1999; Lallemand et al. 2003). Although the localization of Merlin in polarized mammalian epithelial cells has not been reported, Merlin localizes to synaptic junctions in cultured polarized neurons (Gronholm et al. 2005). In addition to concentrated localization to cell protrusions and cell:cell boundaries, some Merlin also exhibits diffuse localization throughout the membrane or cytoplasm and sometimes a punctate distribution that has been attributed to localization to intracellular vesicles or membrane lipid rafts (McCartney and Fehon 1996; Stickney et al. 2004). Indeed, vesicular and/or lipid raft localization would be consistent with a number of lines of recent evidence suggesting a role for Merlin in the endocytic trafficking of membrane receptors (see below). Finally, Merlin has recently been reported to localize to the nucleus in cultured cells under certain conditions (Kressel and Schmucker 2002; Muranen et al. 2005). It is not yet clear whether Merlin has an activity in the nucleus. However, the sum of the available data are consistent with the notion that Merlin normally carries out its growth-suppressing activity from the cell periphery; perhaps Merlin is removed from the periphery and sequestered in the

nucleus during conditions of exponential proliferation. Conversely, Merlin could help to sequester growth-promoting factors outside of the nucleus during conditions of growth arrest.

Pathways/activities

The NF2 field is at a critical juncture, passage of which will require innovative approaches to delineating the molecular basis of Merlin's function as a tumor suppressor. Many Merlin-interacting proteins have been reported—most of which also interact with the ERM proteins—and many cellular and molecular activities have been attributed to Merlin. However, follow-up functional studies of many of these putative interactors have not been forthcoming, perhaps due to the aforementioned technical challenges associated with isolating and localizing Merlin. The fact that a substantial fraction of Merlin is insoluble and that Merlin solubility is regulated, present biochemical challenges to ascribing functional significance to specific Merlin-associated proteins. Nevertheless, the Merlin-interacting proteins and phenotypic consequences of Merlin overexpression can be organized into several pathways that appear to be controlled by Merlin. Indeed, a key question is whether Merlin performs multiple independent functions, one of which is critical in tumor suppression, or whether Merlin controls a specific combination of pathways that collectively impart tumor suppression.

Rho GTPases

The function of Merlin and the ERM proteins has been linked to that of the Rho family of small GTPases that control actin cytoskeleton remodeling (for review, see Bretscher et al. 2002; McClatchey 2003). Central to the functional relationships between Merlin/ERMs and Rho GTPases are reciprocal modes of regulation. Thus, in addition to the role of Rac in regulating Merlin phosphorylation described above, overexpression of Merlin negatively regulates Rac-dependent signaling and *Nf2*^{-/-} cells display phenotypes that are observed in cells expressing activated Rac alleles (Shaw et al. 2001). Similarly, in addition to Rho-dependent ERM phosphorylation, loss of the single *Drosophila* ERM protein Moesin, leads to elevated Rho signaling (Speck et al. 2003). Although the mechanism whereby Merlin/ERMs control Rho/Rac activity is not yet clear, Merlin/ERMs have been shown to physically interact with Rho guanine-dissociation inhibitor (RhoGDI) (Takahashi et al. 1997; Maeda et al. 1999). Like the ERMs, Merlin interaction with RhoGDI could counteract RhoGDI-mediated inhibition and sequestration of Rho or Rac in the cytosol, thereby effecting activation of Rho or Rac signaling. Alternatively, recent evidence suggests that Merlin can bind to and directly negatively regulate Pak itself (Kissil et al. 2003). This latter model predicts that Merlin does not regulate Rac-effector pathways that are Pak independent.

Actin remodeling

A key aspect of Merlin function that must be delineated is its functional association with the actin cytoskeleton. Merlin, like the ERMs, localizes to specific regions of cortical actin remodeling (den Bakker et al. 1995; Gonzalez-Agosti et al. 1996; Sainio et al. 1997). In contrast to the ERMs, Merlin does not have an actin-binding domain at the C terminus, but instead may bind actin directly via an N-terminal domain (Brault et al. 2001; James et al. 2001). Alternatively, interaction of Merlin with the actin cytoskeleton may occur indirectly via the cytoskeletal proteins β II spectrin, paxillin or the ERM proteins themselves (Scoles et al. 1998; Gronholm et al. 1999; Meng et al. 2000; Nguyen et al. 2001; Fernandez-Valle et al. 2002). Loss of Merlin function in SCs and keratinocytes yields marked changes in the morphology of the actin cytoskeleton (Pelton et al. 1998; Lallemand et al. 2003; A. Chan, D. Lallemand, A.I. McClatchey, and M. Giovannini, unpubl.). Similarly, loss of ERM function can lead to dramatic alterations in the cortical actin cytoskeleton (Speck et al. 2003). Although these phenotypes could be an indirect consequence of altered RhoGTPase activity as described above, Merlin may also directly control actin cytoskeleton remodeling. In fact, it has recently been reported that Merlin and the ERMs can directly interact with and inhibit the function of N-WASP, which normally controls activation of the actin nucleator Arp2/3 (Manchanda et al. 2005). The Arp2/3 complex nucleates actin subunits and drives actin filament branching in specialized cortical domains such as the membrane ruffle and adherens junction (AJ) where Merlin is concentrated (Bershadsky 2004).

Contact inhibition/cell:cell adhesion

Several studies suggest that Merlin can control contact-dependent inhibition of proliferation (Morrison et al. 2001; Johnson et al. 2002; Lallemand et al. 2003). Indeed, the key consequence of Merlin deficiency in cultured primary cells of several types is their failure to undergo contact-dependent inhibition of proliferation (Lallemand et al. 2003). Merlin phosphorylation and levels are regulated by cell density and Merlin localizes to nascent boundaries between cells as they form, suggesting a role in cell:cell communication (Shaw et al. 1998; Lallemand et al. 2003). Merlin may regulate contact-dependent inhibition of proliferation through interaction with the hyaluronic acid receptor CD44 in some cell types (Morrison et al. 2001). Alternatively, Merlin associates with and is required for the formation of stable cadherin-containing AJs between cells of several types (Lallemand et al. 2003). In this setting, Merlin appears to stabilize the final AJ structure in association with the actin cytoskeleton. The mechanistic basis of contact-dependent inhibition of proliferation of any cell type is poorly understood and the study of Merlin may provide novel insight into this important biological phenomenon. Moreover, it is well established that defective cell:cell communication can contribute to both tumor initiation and metas-

tasis, providing an explanation for the tumorigenic and metastatic consequences of *Nf2* deficiency in humans and mice.

Growth-factor/membrane-receptor signaling

Several lines of evidence suggest that Merlin can regulate receptor tyrosine kinase activity and perhaps trafficking. For example, genetic interactions between a *Mer* mutation and EGFR pathway mutations have been documented in *Drosophila* (LaJeunesse et al. 2001). In addition, Merlin has been reported to physically interact with several proteins with established roles in growth-factor receptor signaling. Thus, Merlin can form a ternary complex with a novel protein dubbed Magicin and Grb2, an adaptor that coordinates receptor tyrosine kinase and Ras signaling (Wiederhold et al. 2004). Merlin can also interact with EBP50/NHE-RF1, which can, in turn, interact with Erbin; both proteins are PDZ-domain containing adaptors that have been implicated in the membrane distribution of receptor tyrosine kinases (Murthy et al. 1998; Kolch 2003; Lazar et al. 2004; Rangwala et al. 2005). Similarly, Ezrin has been reported to interact with Lin-7/Pals1, which controls ErbB2 membrane distribution in *C. elegans* (Shelly et al. 2003; Cao et al. 2005). Moreover, Merlin can inhibit EGFR internalization and signaling upon cell:cell contact (M. Curto and A.I. McClatchey, unpubl.). A role for Merlin in later stages of endocytosis is suggested by its reported ability

to interact with Hepatocyte Growth-Factor Receptor Substrate (HRS), which controls lysosomal trafficking of membrane receptors including the EGFR (Scoles et al. 2000). The localization of Merlin to vesicular structures under some conditions in *Drosophila* and mammalian cells together with its reported localization to lipid rafts further supports a role in membrane receptor trafficking (McCartney and Fehon 1996; Stickney et al. 2004). Regulation of growth-factor receptor surface availability and endocytosis would be a plausible mechanism whereby Merlin could control cell proliferation. The study of Merlin function could thus provide a novel insight into growth-factor receptor signaling and trafficking in normal cells and suggest novel avenues of growth-factor receptor deregulation in tumors.

Different complexes for different purposes or coordination of signaling from multiple membrane complexes?

If endogenous Merlin does control multiple different pathways as described above, this could reflect independent functions or a role for Merlin in coordinating signaling from multiple membrane complexes. Perhaps the accumulation of growth inhibitory signals from multiple Merlin-associated membrane complexes ultimately reaches a threshold that halts proliferation (Fig. 2). This threshold could vary in different cell types or contexts. For example, Merlin could stabilize large, actin-cytoskeleton-associated membrane-signaling complexes such as

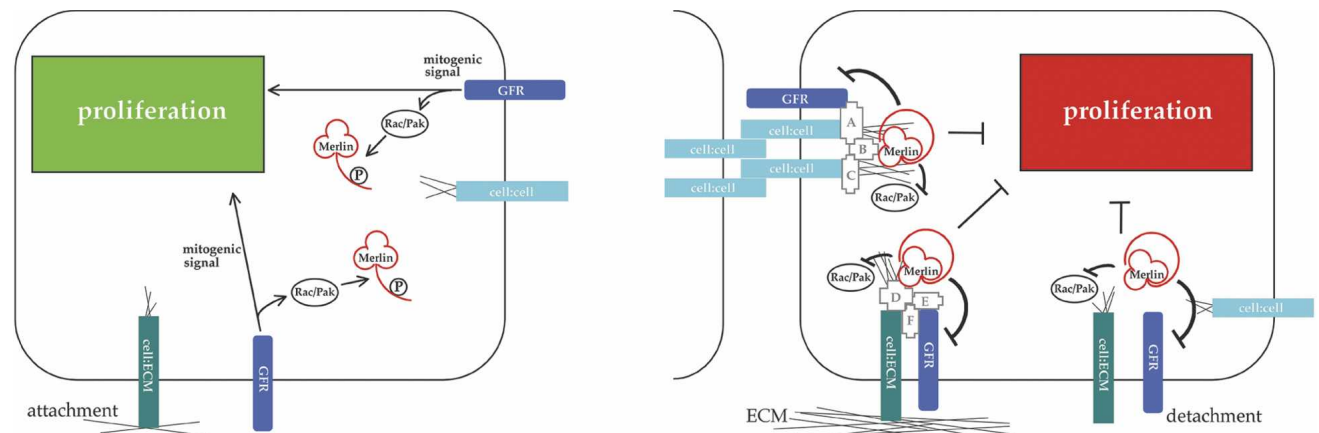


Figure 2. Merlin-organized complexes prevent mitogenic signaling and contact-dependent inhibition of proliferation. (Left) At low cell density in cell culture, Merlin is predominantly hyperphosphorylated and inactive and mitogenic signaling proceeds. Similarly, upon cell reattachment to certain ECM substrates, a pool of Merlin is rapidly phosphorylated. (Right) At high cell density in cell culture, Merlin is hypophosphorylated, self-associated, and active in mediating contact-dependent inhibition of proliferation. Merlin is recruited to nascent cell:cell boundaries where it appears to stabilize AJs between cells, perhaps by inhibiting Rac/Pak signaling and/or stabilizing the actin cytoskeleton. Under these conditions, Merlin may inhibit signaling from AJ-associated growth-factor receptors. Hypophosphorylated Merlin can also interact with cell:ECM receptors such as CD44, which binds to hyaluronic acid (HA); increased CD44:HA interaction with increased cell density leads to high levels of active Merlin, which may inhibit associated growth-factor receptors. Notably, complete cell detachment also leads to hypophosphorylation and activation of Merlin. Perhaps inhibited mitogenic signaling from multiple complexes accumulates, reaching a threshold that halts proliferation. The study and manipulation of cell:cell contact in two dimensions in cell culture is nonphysiological; in vivo cells in a tissue are contacting other cells and can override contact-dependent inhibition of proliferation under certain normal conditions or in the context of a tumor. Merlin may coordinate the receipt of physical information from the extracellular milieu with the receipt and processing of mitogenic stimuli.

the AJ by locally inhibiting Rac–Pak signaling and/or WASP-mediated actin filament protrusion. Increasing evidence suggests that growth-factor receptors physically associate with and are regulated by AJs (Takahashi and Suzuki 1996; Lampugnani et al. 2003; Qian et al. 2004). Merlin could also organize CD44-containing complexes that coordinate cell:ECM attachment and proliferation control; in addition to Merlin and Ezrin, CD44 can be found in complexes containing growth-factor receptors (Morrison et al. 2001; Cavallaro and Christofori 2004). A fundamental mechanism by which growth-factor receptors are regulated is via endocytosis. Both cell:ECM and cell:cell junctions are dynamic structures that also undergo regulated endocytic turnover. Perhaps by combining the turnover of adhesion structures with growth-factor receptors, the cell can coordinate cell adhesion stability with inhibition of mitogenic signaling—Merlin could function to integrate the two. This would be consistent with the observation that Merlin both controls AJ stability and prevents EGFR internalization (M. Curto and A.I. McClatchey, unpubl.) and with the reported role for Erbin in controlling both AJ formation and proliferation in SCs (Rangwala et al. 2005). Indeed, the study of Merlin could provide novel insight into the poorly understood mechanism whereby cells coordinate growth-factor receptor signaling and AJ turnover.

Schwann, arachnoid, ependymal, and mesothelial cells—unique properties?

Most tumor-suppressor genes including *NF2* are expressed broadly, providing little explanation for the restricted tumor spectrum exhibited by heterozygous mutant carriers. The predisposition of humans and mice that carry heterozygous tumor-suppressor gene mutations to certain types of tumors could be due to (1) cell-type-specific differences in the temporal occurrence of obligatory genetic events such as loss of the wild-type *Nf2* allele or cooperating mutations; (2) cell-autonomous differences in dependence of various cell types upon Merlin for proliferation control, perhaps due to differential expression of key components of a particular tumor-suppressor pathway such as associated proteins, regulators, downstream targets or proteins with partially or fully redundant functions; or (3) non-cell-autonomous differences in the contribution of surrounding heterozygous mutant tissue to tumorigenesis as has been demonstrated for NF1-associated tumors (Zhu et al. 2002).

Indeed, there is ample evidence for cell-type-specific consequences of *Nf2* deficiency. For example, although several types of *Nf2*^{-/-} cells hyperproliferate in vivo, hyperproliferation is not a consequence of *Nf2* deficiency in *Nf2*-null mouse embryos as described above (McClatchey et al. 1997). Similarly, in the fly and in mosaic *Nf2*^{-/-} ↔ wild-type mouse embryos *Nf2* deficiency leads to defects in many tissues, but hyperproliferation in only certain contexts in vivo (Fehon et al. 1997; LaJeunesse et al. 1998; MacDougall et al. 2001; A.I. McClatchey, unpubl.). In some types of cultured cells (i.e., keratinocytes and SCs), but not others (fibroblasts, osteoblasts),

loss of Merlin yields dramatic alterations in actin cytoskeleton morphology (Pelton et al. 1998; Lallemand et al. 2003; A. Chan, D. Lallemand, A.I. McClatchey, and M. Giovannini, unpubl.). Finally, stable AJs do not form at all in cultured *Nf2*^{-/-} fibroblasts, osteoblasts, SCs, and keratinocytes, while more subtle defects in AJ stability and cell:cell communication are apparent in other *Nf2*^{-/-} epithelial cells (A.I. McClatchey and M. Curto, unpubl.). Defective cell:cell communication is a consequence of *Nf2* deficiency in many different cell types. Are Schwann, arachnoid, ependymal, and mesothelial cells particularly dependent upon Merlin for integrating cell:cell communication with inhibition of cell proliferation?

SCs are neural crest-derived cells that undergo a sophisticated program of movement and differentiation as they associate with and insulate peripheral axons. Neural crest cells that migrate from the dorsal neural tube to the sensory ganglia and to positions along emerging peripheral axon bundles become immature SCs (for review, see Garratt et al. 2000). Both immature and mature SCs assume an exquisite degree of compartmentalization that is coordinated by intra- and intercellular junctions (Salzer 2003). Dividing immature SCs first loosely ensheath and then progressively segregate clusters of axons from one another with extensive cytoplasmic processes. The initial extension of SC processes along axons and communication between adjacent SCs appear to be mediated by N-cadherin; this association is required for contact-dependent signaling in SC proliferation and differentiation (Salzer et al. 1980a,b; Garratt et al. 2000). The mature peripheral nervous system contains both myelinating and nonmyelinating SCs. Nonmyelinating SCs continue to invest multiple unmyelinated axons. In contrast, myelinating SCs stretch to wrap a single axon multiple times, forming intracellular AJs between adjacent loops of membrane at the paranode and between internodal layers at Schmidt-Lanterman incisures (Fig. 3A; Salzer 2003). At the paranode, the terminal loops are attached to the axonal membrane via septate-like junctions that compartmentalize the axonal surface (for review, see Peles and Salzer 2000; Denisenko-Nehrbass et al. 2002). Merlin has been reported to localize to both the paranode and Schmidt-Lanterman incisures of myelinating SCs (Scherer and Gutmann 1996). Ultrastructural examination of sciatic nerves in *P0-Cre; Nf2*^{flox2/flox2} mice revealed markedly abnormal myelination, although it is not clear whether these abnormally myelinating cells are the precursors of schwannoma development (Giovannini et al. 2000). In fact, human schwannomas do not commonly contain compact myelin (Erlandson and Woodruff 1982). As discussed below, a key issue for the study of NF2-associated tumorigenesis is defining the type of SC that gives rise to a schwannoma.

SCs are critically dependent upon signaling via the ErbB (EGFR) family of receptors for their survival, proliferation, and differentiation (for review, see Garratt et al. 2000). In general, ErbB-expressing SCs and their precursors respond to axonally derived neuregulin, the major functional ErbB ligand in the peripheral nervous sys-

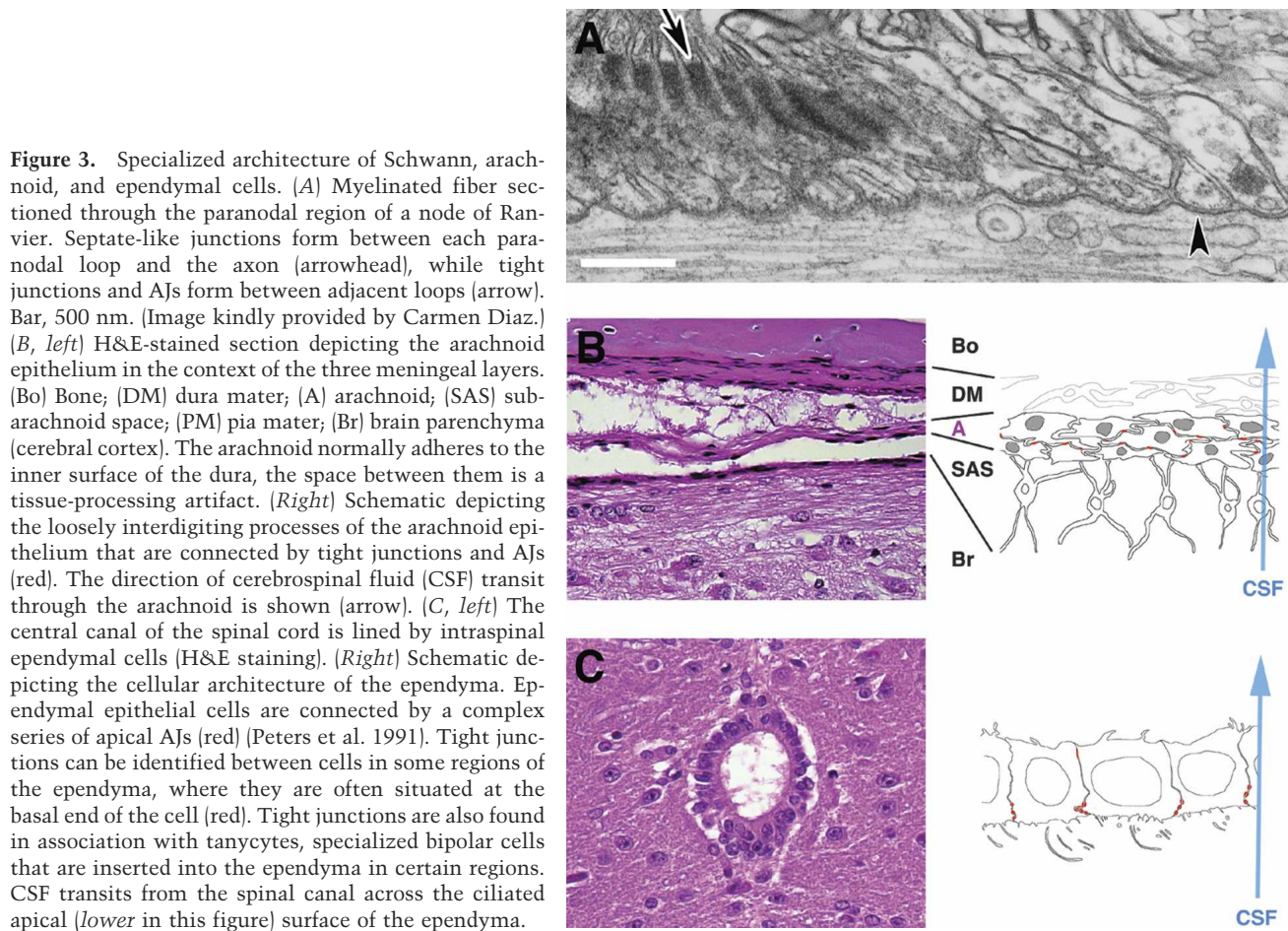


Figure 3. Specialized architecture of Schwann, arachnoid, and ependymal cells. (A) Myelinated fiber sectioned through the paranodal region of a node of Ranvier. Septate-like junctions form between each paranodal loop and the axon (arrowhead), while tight junctions and AJs form between adjacent loops (arrow). Bar, 500 nm. (Image kindly provided by Carmen Diaz.) (B, left) H&E-stained section depicting the arachnoid epithelium in the context of the three meningeal layers. (Bo) Bone; (DM) dura mater; (A) arachnoid; (SAS) sub-arachnoid space; (PM) pia mater; (Br) brain parenchyma (cerebral cortex). The arachnoid normally adheres to the inner surface of the dura, the space between them is a tissue-processing artifact. (Right) Schematic depicting the loosely interdigitating processes of the arachnoid epithelium that are connected by tight junctions and AJs (red). The direction of cerebrospinal fluid (CSF) transit through the arachnoid is shown (arrow). (C, left) The central canal of the spinal cord is lined by intraspinal ependymal cells (H&E staining). (Right) Schematic depicting the cellular architecture of the ependyma. Ependymal epithelial cells are connected by a complex series of apical AJs (red) (Peters et al. 1991). Tight junctions can be identified between cells in some regions of the ependyma, where they are often situated at the basal end of the cell (red). Tight junctions are also found in association with tanycytes, specialized bipolar cells that are inserted into the ependyma in certain regions. CSF transits from the spinal canal across the ciliated apical (lower in this figure) surface of the ependyma.

tem. In fact, ErbB signaling regulates myelin thickness by controlling the number of successive wraps made by individual SCs (Garraff et al. 2000; Michailov et al. 2004). Reduced ErbB signaling effects hypomyelination (fewer wraps) and excess ErbB signaling effects hypermyelination (more wraps). In this unique setting, intracellular communication between wraps and growth-factor receptor signaling must be coordinated and may be particularly sensitive to Merlin loss.

Much less is known about the molecular control of proliferation and cell:cell communication in the developing and mature meninges and ependyma. The arachnoid membrane lies between the outer dura and inner pia mater, collectively forming the meningeal layers that cover the brain and spinal cord (Fig. 3B; Yamashima 1996). Meningiomas tend to arise from arachnoid cells (also termed meningotheial cells) located within arachnoid villi, specialized projections that have an important role in the transport of cerebrospinal fluid (Louis et al. 2000). It has been reported that the number of E-cadherin-containing AJs is decreased in many meningiomas (Tohma et al. 1992; Yamashima et al. 1992). In fact, NF2 patients predominantly develop a particular type of meningioma known as the fibroblastic meningioma that lacks strong epithelial characteristics (Heinrich et al. 2003).

The ependymal epithelium lines the ventricles of the brain and central canal of the spinal cord and is a remnant of the proliferative ventricular zone that gives rise to most neurons and glia of the central nervous system during development (Fig. 3C; Peters et al. 1991). Together with the choroid plexus, these cells form the brain:cerebrospinal fluid (CSF) interface; in contrast to the major function of the adjacent choroid plexus in CSF secretion, the ependyma plays an important role in CSF transport (Del Bigio 1995). NF2 mutations are associated with intraspinal ependymomas rather than intracranial ependymomas (Ebert et al. 1999). Notably, the spinal, but not cranial ependyma is capable of proliferative regeneration following injury (Nag 1997).

Both the arachnoid and ependyma are simple epithelia that are in contact with and involved in transport of CSF, which is a source of certain growth factors (Redzic and Segal 2004). The arachnoid is often described as a "delicate" epithelium. Ultrastructurally, normal arachnoid cells are characterized by loosely interdigitating cellular processes connected by junctional complexes; between the cellular processes are tunnels of extracellular space (Fig. 3B; Peters et al. 1991; Yamashima 1996). Ependymal cells form a simple ciliated cuboidal epithelium connected by apical adherens and gap junctions (Fig. 3C; Lagunowich et al. 1992; Lippoldt et al. 2000). Notably,

some studies have concluded that the ependyma is a "leaky" epithelium, perhaps due to the lack of tight junctions between cells in some regions of the ependyma (Gotow and Hashimoto 1982). Interestingly, the mesothelial lining also performs a critical active function in fluid transport across the peritoneal and pleural cavities. Therefore, like the arachnoid and ependyma, the mesothelia are designed to allow the passage of fluid. All three epithelia must both delimit a fluid-filled space and allow active transit of fluid. Could the histoarchitectural requirements of such epithelia be particularly sensitive to loss of Merlin? Could Merlin be particularly important for preventing signaling from growth-factor receptors that are exposed to a constant source of ligand in cells with loose or permeable intercellular junctions?

The most consistent phenotype associated with *Nf2* deficiency in cultured cells is loss of contact-dependent inhibition of proliferation. In a developing organism, cells in the context of a tissue are all in contact with other cells, but can divide and, therefore, override contact-dependent inhibition of proliferation at certain times. It is likely that cells in most adult tissues integrate multiple inhibitory signals to maintain contact-dependent inhibition of proliferation; perhaps certain cell types rely more heavily upon Merlin for this function. In mice and perhaps in humans, loss of *Nf2* is sufficient for hyperproliferation of SCs and arachnoid cells in vivo, although at least one additional mutation is likely required for frank tumor formation. Animal models of *Nf2* deficiency in the ependyma have not yet been described. Notably, *Nf2*-associated schwannomas, meningiomas, and ependymomas are benign tumors that do not progress to malignancy, perhaps reflecting physiological loss of contact-dependent inhibition of proliferation in vivo. In contrast, osteosarcomas and other tumors that spontaneously develop with longer latency in *Nf2*^{+/-} mice are malignant and metastatic, likely reflecting the cooperative loss of *Nf2* function and that of other growth inhibitory signals. Benign, slow-growing tumors represent a special therapeutic challenge, as standard chemotherapeutic strategies target rapidly dividing cells. In addition, it is well established that cells from malignant tumors are often particularly sensitive to the induction of apoptosis, a feature that is exploited by many chemotherapeutic agents (Johnstone et al. 2002).

Future directions/perspectives

There are many exciting directions and challenges ahead for the study of *Nf2*-associated tumorigenesis and the molecular function of Merlin. The study of *Nf2* function in animal models has extended our view of Merlin beyond its role in tumor suppression and revealed that Merlin function is essential in many different types of cells. In fact, functional studies of Merlin have already provided new insight into fundamental biological processes such as contact-dependent inhibition of proliferation, cell:cell communication, receptor trafficking, actin cytoskeleton remodeling, and membrane organization.

The study of *Nf2*-mutant mice suggests that loss of

Merlin function could play an unexpectedly broad role in tumorigenesis and metastasis. Studies of tumorigenesis in these mice have revealed that both genetic and biological parameters can dictate the spectrum of spontaneous tumors associated with heterozygous *Nf2* mutation. Thus, linkage of cancer-predisposing mutations, temporal control of *Nf2* loss, and cell-type-specific phenotypic consequences of *Nf2* deficiency can each profoundly impact spontaneous tumor development in *Nf2*^{+/-} mice. These principles are broadly applicable to the study of tumorigenesis initiated by other cancer-predisposing mutations.

A key goal for the study of NF2-associated tumorigenesis will be the determination of what cell type gives rise to the schwannoma. It has been suggested that schwannomas express key markers of immature SCs (Hung et al. 2002). However, some studies have indicated a surprising capacity of differentiated (myelinating) SCs to dedifferentiate (Harrisingh et al. 2004). So, does loss of Merlin cause progressive dedifferentiation of a mature SC or proliferation of a SC progenitor? A detailed histological and ultrastructural analysis of the initiation and progression of schwannomas in *Nf2*-mutant mice and parallel comparison to human NF2-mutant schwannomas should help to answer this question. Similarly, comparative analyses of the development of meningiomas in NF2-mutant mice and humans together with the development and analysis of models of ependymoma will provide valuable insight into the pathogenesis of these tumors.

The identification of additional genetic mutations that supplant cooperate with *Nf2* loss may provide important insight into the signaling pathways that are deregulated by *Nf2* loss. For example, while the vast majority of sporadic schwannomas exhibit biallelic *Nf2* inactivation, *Nf2* mutations have been identified in only a subset of sporadic meningiomas (Rutledge et al. 1994; Lomas et al. 2005). This suggests the existence of another locus, which, when mutated somatically, can cause meningioma development. Mathematical modeling of vestibular schwannoma development in NF2 patients suggests that in addition to loss of the wild-type *Nf2* allele, one other mutational event may be necessary for the development of this tumor (Woods et al. 2003). In addition, the low incidence and late onset of schwannomas, in contrast to the high incidence of SC hyperplasia in *PO-Cre;Nf2*^{fllox2/fllox2} mice strongly suggest that an additional mutation is required for the progression of SC hyperplasia to tumor formation (Giovannini et al. 1999, 2000; Stemmer-Rachamimov et al. 2004). Documentation of biallelic NF2 inactivation in preneoplastic lesions (hyperplastic SCs and tumorlets) of NF2 patients also supports this hypothesis (Stemmer-Rachamimov et al. 1998).

Many strategies will be exploited to continue the investigation of the molecular mechanism of Merlin function. It will be critical to precisely define the subcellular localization of Merlin in different cell types under different conditions and to functionally validate each candidate Merlin-interacting protein. The identification of

higher order Merlin-containing complexes and examination of how such complexes respond to the loss of Merlin will provide important insight into how Merlin coordinates the physical and signaling properties of membrane complexes. In addition to further genetic analyses in *Drosophila* and investigation of *Nf2*^{-/-} cells from mutant mice, new studies of Merlin orthologs in other species that exploit the unique strengths of each model system will provide powerful, complementary strategies for mechanistic studies of Merlin function. Collectively, the study of NF2-associated tumorigenesis in mammals, together with basic cellular and molecular studies in other model systems and in cell culture will facilitate the identification of potential therapeutic targets and the development of therapeutic strategies for NF2. Further molecular investigation of this novel tumor suppressor will continue to provide new insight into how cells organize their interface with the extracellular environment to coordinate positional information and proliferation control and how disorganization of this interface contributes to tumorigenesis and metastasis.

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