Analysis of the Function of the *Nf2* Tumor Suppressor Protein, Merlin

by

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A.B., Molecular Biology/Biochemistry and Religion
Dartmouth College, 1998

Submitted to the Department of Biology
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Massachusetts Institute of Technology

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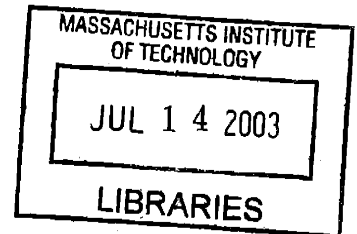
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Analysis of the Function of the *Nf2* Tumor Suppressor Protein, Merlin

by

Kristen C. Johnson

Submitted to the Department of Biology on June 6, 2003 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

ABSTRACT

The Neurofibromatosis type 2 tumor suppressor gene (*NF2*) is mutated in inherited and sporadically occurring central nervous system tumors. The *NF2* encoded protein, merlin, shares close sequence similarity in its amino-terminal domain to members of the band 4.1 family of membrane-cytoskeletal linkers. Similarities between merlin and this family suggest a role for merlin in regulating cytoskeletal function. Thus, *NF2* may be a novel type of tumor suppressor gene that mediates its tumor suppressor function through interactions with the actin cytoskeleton. However, the molecular and cellular functions of this tumor suppressor gene were largely unknown when the work described here began.

Mutational analysis of *Nf2* in flies has lead to the identification of a dominant-negative allele, which harbors mutations in the amino-terminal domain of the protein. The work presented here demonstrates that expression of a murine analog of this amino-terminal mutant of *Nf2* (termed, $Nf2^{BBA}$) leads to complete transformation of NIH3T3 fibroblasts in culture. Cells that express $Nf2^{BBA}$ display disruptions of the actin cytoskeleton, lack of contact inhibition of growth, and anchorage-independent growth. In addition, *Nf2*-deficient mouse embryo fibroblasts (MEFs) exhibited similar contact inhibition and cell-matrix adhesion defects to $Nf2^{BBA}$ expressing cells. $Nf2^{BBA}$ cells continue to cycle under normal growth inhibitory conditions, such as serum withdrawal, and exhibit high levels of the cell cycle regulator, cyclin D1. Elevated levels of cyclin D1 are necessary for cellular transformation following $Nf2^{BBA}$ expression. Nevertheless, the exact mechanism by which $Nf2^{BBA}$ results in cellular transformation remains elusive.

Recently published studies have revealed that merlin may regulate members of the RhoGTPase family, as absence of *Nf2* expression in fibroblasts leads to many phenotypes reminiscent of overactive Rac, such as increased membrane ruffling and increased activity of the c-jun N-terminal kinase (JNK). Our work has extended to the analysis of the role of merlin in the regulation of the Rac pathway. Using rat schwannoma cells and *Nf2*-deficient MEFs, we have demonstrated that merlin exerts its inhibitory effects downstream of Rac, through a direct interaction with the p21 activated kinase, Pak. We demonstrate that in the absence of merlin, Pak is active and hyperphosphorylated, and, conversely, when merlin is overexpressed, Pak activity is diminished. The N-terminal half of merlin binds to the functionally conserved Rac/Cdc42 interaction binding (CRIB) domain of Pak. Several models for merlin regulation of Pak activity will be discussed. Finally, the identification of Pak as a kinase that is misregulated in the absence of *NF2* may lead to possible avenues for therapeutic intervention.

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Bono P, Cordero E, Johnson K, Borowsky M, Ramesh V, Jacks T, Solomon F, and Hynes RO. (2003) "Layilin, a novel hyaluronan receptor, interacts with merlin and ERM proteins." In preparation.

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Kissil JL, Johnson KC, Eckman MS, Jacks T. (2002) "Merlin phosphorylation by p21-activated kinase 2 and effects of phosphorylation on merlin localization." *Journal of Biological Chemistry,* 277(12):10394-9.

Becker KB, Stephens KC, Davey JC, Schneider MJ, Galton VA. (1997) "The type 2 and type 3 iodothyronine deiodinases play important roles in coordinating development in Rana catesbeiana tadpoles." *Endocrinology*,138(7):2989-97.

PRESENTATIONS

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Kristen Johnson, Joseph Kissil, Reuben Shaw, Tyler Jacks. The role of the Nf2 tumor suppressor protein, merlin, in epithelial cell morphology and motility. NNFF International Consortium for the Molecular Biology of NF1 and NF2. June, 2000. Aspen, CO.

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Chapter 1

Introduction

Most cancers arise following the disruption of the normal function of genes involved in cell cycle progression. Proto-oncogenes, such as *Ras* and *c-myc*, which are often hyperactivated or overexpressed in human cancers, have defined roles as positive regulators of cell proliferation. On the other hand, tumor suppressor genes, such as *p53* and *RB*, whose functions are lost in many human cancers, clearly negatively regulate molecules and pathways that are critical for cell proliferation. Together, these cancer genes regulate signaling pathways that converge on transcription factors in the nucleus, thereby ultimately having a direct role in the regulation of the cell cycle. Nevertheless, in the last decade, cancer genes have been identified that have no clear link to progression through the cell cycle. For the most part, the role of such genes in tumorigenesis has been poorly understood. The projects described herein detail the functional analysis of a novel type of tumor suppressor gene, *NF2*, which may mediate its growth inhibition through associations with the cytoskeleton and modulation of Rac-dependent pathways.

The disease and cloning of the *NF2* gene

Neurofibromatosis Type 2 (NF2) is a rare inherited cancer disorder that is characterized by neoplastic and dysplastic lesions of Schwann cells (schwannomas and schwannosis), meningeal cells (meningiomas and meningioangiomatosis) and glial cells (gliomas and glial microhemartomas). These are benign tumors that endanger the patient because of compression of vital structures in the nervous system rather than progression to malignancy. Severe morbidity (average lifespan of 40 years) is associated with this disorder, which affects approximately 1 in 40,000 individuals. NF2 displays autosomal dominant transmission and most patients present in their twenties with hearing loss or imbalance due to the bilateral growth of schwannomas of the eighth cranial (auditory) nerve, the hallmark lesion in this disease (Martuza

and Eldridge 1988; Evans et al. 1992; Short et al. 1994). To date, there is no effective therapeutic treatment for this disease beyond tumor resection. Therefore, molecular analysis of the function of the *NF2* gene and its protein product will be critical to our understanding and treatment of this disease.

Early analysis suggested that the gene responsible for this disease was characteristic of a classic tumor suppressor gene: transmission of a mutated gene through the germline of NF2 patients predisposed to tumor formation, which was initiated following somatic deletion or mutation of the remaining wild-type copy of the gene (Rouleau et al. 1987; Seizinger et al. 1987; Rouleau et al. 1990). Molecular cytogenetic studies of familial and sporadic schwannomas, and NF2-related meningiomas and ependymomas, and linkage analysis of NF2-affected individuals within families led to the discovery of a link between loss/mutation of chromosome 22 and presence of the disease (Menon et al. 1990). By 1993, loss of heterozygosity (LOH) analysis in tumors and recombination analysis allowed scientists to clone the *NF2* gene located at 22q12 (Rouleau et al. 1993; Trofatter et al. 1993). Interestingly, inactivating mutations in the *NF2* gene have been identified in about 30% of malignant mesotheliomas, tumors of the tissues lining the plural, peritoneal, or pericardial cavities usually caused by asbestos exposure (Bianchi et al. 1995; Sekido et al. 1995; Deguen et al. 1998a). Because NF2 patients are not predisposed to this tumor type, mutation of *NF2* in mesotheliomas is thought to be a progression step, rather than an initiating event. In addition, the presence of *NF2* mutations not in only benign, but also in malignant, disease indicates that the function of this gene is likely very cell type/context-dependent.

The *NF2* gene spans 110kb and is composed of 17 exons. Although the *NF2* gene encodes multiple alternatively spliced transcripts, there are two main isoforms of *NF2* that differ

in their alternative splicing of the last two exons (Bianchi et al. 1994; Haase et al. 1994; Hara et al. 1994; Pykett et al. 1994). Isoform I contains exons 1-15 and 17, and isoform II contains exons 1-16 (Figure 1). *NF2* mutations in patients have been found in all parts of the gene except in the alternatively spliced exons, with the most frequent genetic alterations localized in exons 1-8. Mutations can be nonsense or framshift, which often alter splice junctions and/or result in protein truncations and more severe phenotypes, or missense, which tend to result in milder phenotypes (Parry et al. 1996; Ruttledge et al. 1996). However, the clinical course of the disease varies widely between and within families, even among patients with similar mutations (Evans et al. 1992). Therefore, it is clear that there must be additional factors that regulate the phenotypic expression of the mutant *NF2* genes.

The *NF2* gene is widely expressed in most human and mouse tissues, including the brain (Claudio et al. 1995; Huynh et al. 1996; den Bakker et al. 1999). Following the cloning of the *NF2* gene, its predicted protein product was determined to be a member of the band 4.1 family of membrane-cytoskeletal linker proteins. Within this highly conserved family, the *NF2*-encoded protein has most similarity to the ERM proteins (ezrin, radixin and moesin) and was thus termed "merlin" for Moesin, Ezrin, Radixin-LIke proteiN (alternatively called schwannomin). The general structure of merlin and the ERM proteins consists of a tri-lobed globular amino-terminal domain followed by an alpha-helical domain with a proline-rich region and a charged carboxy-terminal domain (Pearson et al. 2000; Kang et al. 2002; Shimizu et al. 2002) (Figure 2). Merlin is most similar to the ERM proteins in the N-terminal FERM (Four point one, ERM) domain, where it shows 61% amino acid identity with the prototypical member, ezrin (Figure 3). However, the similarity between merlin and the ERM proteins drops off significantly outside of

the FERM domain; merlin is less than 30% identical to the alpha-helical and charged C-terminal domains of the ERM proteins.

Overall, merlin is only 45% identical to the ERM proteins and lacks a conserved C-terminal actin binding site. Thererfore, merlin is thought to possess functional properties distinct from that of the ERMs. Furthermore, although the FERM domains of merlin and the ERMs are highly similar, structural analysis of the merlin FERM domain has revealed that most of the divergent residues are found clustered on the surface, indicating that these distinct residues might serve to specify binding of proteins particular to merlin's function as a tumor suppressor (Kang et al. 2002; Shimizu et al. 2002). In addition, while merlin is a well-established tumor suppressor gene, mutation in the ERM proteins has not been identified in any human cancer syndromes. Perhaps the paucity of disease-related mutations in the ERMs is due to functional compensation among the family members, which are ~75% identical to one another. In general, the ERMs are thought to be growth and motility promoting, whereas merlin is thought to function in an opposing manner. Nevertheless, despite these suppositions, scientists have learned much about merlin over the past decade by exploring its similarities with members of the ERM family. However, none of these studies has clearly identified how merlin's membrane-cytoskeleton function translates into tumor suppression.

The ERM family

Much information regarding the regulation of membrane cytoskeleton dynamics, including the determination of cell shape, adhesion, motility, cytokinesis, endocytosis and the integration of membrane transport with signaling has been appreciated as a result of extensive research on the ERM family of proteins. As membrane-cytoskeletal linkers, the ERMs have

been established as critical regulators at the cell cortex (Bretscher et al. 2002). The ERMs are one of the most conserved protein families in evolution, with one to three family members present in organisms ranging from parasites to humans. However, interestingly, no FERM-domain containing proteins have been identified in the yeast *S. cerevisiae* or any other unicellular organism, indicating that these proteins likely evolved specifically in response to mutlicellularity, and not merely as a constituent of the cytoskeleton. Genetic study of the function of ERMs in mammals is complicated by the fact that there are three family members. The extremely high degree of identity between the family members suggests that they arose from gene duplication and that their functions are likely redundant or at the very least, overlapping. This hypothesis is supported by the lack of a phenotype in moesin knockout mice (Doi et al. 1999). It would be useful to have triple knockout ERM mice (although likely embryonic lethal) and cells in order to understand more fully the role of this family in normal cellular processes. Nevertheless, to date, investigators have utilized expression of constitutively active and dominant-negative ERM proteins to begin to dissect the function(s) of this protein family in mammals.

The ERM proteins were originally identified independently as components of actin-rich structures, such as microvilli and lamellipodia (membrane ruffles) at the surface of various cell types (Bretscher 1983; Tsukita and Hieda 1989; Lankes and Furthmayr 1991; Sato et al. 1992). In general, these proteins act as a link between filamentous actin in the cell cortex and membrane proteins on the cell surface. The FERM domain of the ERM proteins is responsible for membrane protein binding, and the C-terminal region contains an actin binding domain (Algrain et al. 1993; Turunen et al. 1994). The activity of the ERM proteins is highly regulated by strong intramolecular interactions (Gary and Bretscher 1995; Magendantz et al. 1995). Closed

monomers, which exhibit a FERM domain-to-tail self-association as demonstrated through structural studies, are found in the cytoplasm (Berryman et al. 1995; Bretscher et al. 1995). Both the membrane protein-binding (FERM) domain as well as the actin-binding domain is masked in the inactive monomeric conformation (Gary and Bretscher 1995; Magendantz et al. 1995; Reczek and Bretscher 1998). Activation stimulates a switch from this dormant state to that of an open monomer, in which the membrane protein-binding sites in the FERM domain and the actin-binding sites in the C-terminal region are unmasked (Figure 4) (Bretscher et al. 1995; Gautreau et al. 2000). Interestingly, the ERM proteins not only homo-oligomerize, but they have also been found to hetero-oligomerize with one another and with merlin both *in vitro* and *in vivo*** (Gary and Bretscher 1993; Andreoli et al. 1994; Gonzalez-Agosti et al. 1999). Following growth factor stimulation, the ERM proteins are tyrosine-phosphorylated and found associated with the membrane and cytoskeleton in an oligomeric form (Krieg and Hunter 1992; Crepaldi et al. 1997). However, the physiological function of these higher order oligomers has not yet been established.

Activation, allowing the ERMs to link the membrane to the cytoskeleton, has been attributed primarily to the Rho small GTP-binding protein signaling pathway. Phosphorylation of the ERM proteins on a conserved C-terminal threonine residue (T567 in ezrin, T564 in radixin, and T558 in moesin) inhibits its self-association, thereby resulting in an active, open conformation of the molecule which is localized to actin-rich membrane structures (Nakamura et al. 1996; Huang et al. 1998; Matsui et al. 1998; Hayashi et al. 1999). ERM phosphorylation is stimulated by lysophosphatidic acid (LPA) activation of Gα subunits downstream of heterotrimeric G protein-coupled receptors (Machesky and Hall 1997; Matsui et al. 1998; Shaw

** See "Binding partners for merlin" section that follows.

et al. 1998a). The activated Gα subunits act on Rho GTP/GDP exchange factors (GEFs), which promote the conversion of inactive Rho-GDP to active Rho-GTP.[¥] Rho-GTP activates Rho kinase and stimulates the synthesis of PIP2, both of which have been found to be directly important for ERM activation (Nakamura et al. 1999; Yonemura et al. 2002). Through this Rho-ERM pathway, LPA and serum induces the formation of cell surface structures, such as filopodia, lamellipodia and microvilli, and, also, the formation of cell adhesion sites, such as focal adhesions and stress fibers (Shaw et al. 1998a). In addition, protein kinase C theta (PKCθ) and PKCα (downstream of hepatocyte growth factor, HGF, in epithelial cells) have been shown to phosphorylate the C-terminal threonine, thus activating the ERMs and resulting in cell surface protrusions and cell motility (Pietromonaco et al. 1998; Simons et al. 1998; Ng et al. 2001). Expression of a consitutively-active mutant of ezrin with an aspartic acid substitution mimicking phosphorylation at the threonine site (T567D) results in cortical actin changes in the absence of Rho or PKC stimulation (Huang et al. 1999; Gautreau et al. 2000). Conversely, cell surface extensions disappear with the expression of a T567A ezrin molecule, even in the presence of activated Rho or PKC, thereby indicating a critical role for phosphorylation of the ERMs in initiating cell surface changes.

The FERM domain of the activated ERM proteins interacts with membrane proteins in two ways: (1) direct binding to the cytoplasmic domain of single pass adhesive transmembrane proteins, and (2) indirect binding to multipass membrane proteins via scaffolding proteins. Interactions between the hyaluronic acid receptor, CD44, and the ERM proteins have been well documented and principally regulate directional cell motility (Tsukita et al. 1994). In addition, individual ERMs have also been shown to interact with intracellular adhesion molecule (ICAM)-

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[¥] The Rho family of proteins, which include Rac and Cdc 42, are small GTP binding proteins that regulate many aspects of the actin cytoskeleton, as well as cell signaling and proliferation. Due to the importance of Rac in merlin function, as argued later, these proteins will be discussed in greater detail in later sections of the introduction.

1, 2 and 3 and CD43. Interactions between ICAM-2 and ezrin are critical for interleukin-2 (IL-2) activation of natural killer (NK) cells; recruitment of ICAM-2 to uropods, specialized cellular protrusions required for cell motility, is dependent upon ezrin, as cells that lack ezrin maintain a diffuse ICAM-2 distribution throughout the cell (Helander et al. 1996). It is important to note that the direct interaction between the ERMs and membrane proteins appears to be very cell-type specific and may be exhibited principally in cells that necessitate cell surface protrusions for adhesive purposes, such as T cells.

In addition to binding directly to membrane proteins, ezrin interacts with two scaffolding proteins, Exchanger 3 kinase A regulatory protein (E3KARP) and ERM binding phosphoprotein 50 (EBP50), that are themselves bound through PDZ domains to the cytoplasmic face of multipass membrane proteins, such as Na+/H+ exchange factor 3 (NHE3), cystic fibrosis transmembrane conductance regulator (CFTR), and β2 adreneric receptor (β2AR). EBP50 (also known as NHERF, NHE regulator factor) was originally cloned in a screen to identify proteins that confer PKA regulation on NHE3 via phosphorylation (Weinman et al. 1995). More recent studies have indicated that the interaction between ezrin and EBP50 and E3KARP serves to mediate the regulation of NHE3 through ezrin's recruitment of PKA to this complex (Dransfield et al. 1997). In the case of β2AR and CFTR, as well as NHE3 and perhaps other membrane proteins, it appears that binding by EBP50 and E3KARP may regulate the recycling of these receptors via endocytic trafficking (Cao et al. 1999; Moyer et al. 1999). Mutation of the EBP50 binding site in CFTR results in a mislocalized receptor. A similar phenotype has been observed with β2AR. These data suggest that an EBP50/ERM/F-actin connection may be important for receptor recycling. However, how this complex participates in the membrane traffic pathway

has yet to be determined. Finally, the E3KARP/EBP50/ERM/F-actin linkage may be important in restricting certain proteins to plasma membrane subdomains.

In addition to the requirement for ERMs in cell shape, motility, and regulation of receptor function/endocytic trafficking, there appears to be a requirement for ERMs in several other contexts, such as signaling, transformation and cell survival. Recent studies have demonstrated that the ERMs are positive regulators of the Rho signaling pathway, acting upstream of Rho pathway activation. The prospect that ERMs act both downstream and upstream of Rho provides evidence for a feed-forward mechanism of Rho autoregulation. Two mechanisms for ERM regulation of Rho have been proposed. First, the FERM domain of activated ERMs can interact with Rho guanine nucleotide dissociation inhibitors (RhoGDIs), negative regulators of the Rho GTPases that sequester them in the cytoplasm in an inactive state (Takahashi et al. 1997). In cultured fibroblasts, expression of the FERM domain of radixin, which strongly binds RhoGDI, led to potent activation of Rho and the consequent formation of stress fibers. Secondly, in a yeast two-hybrid assay, investigators found that the FERM domain of ezrin interacts strongly with a region of the C terminus of hamartin, the product of the tuberous sclerosis 1 (TSC1) tumor suppressor gene (Lamb et al. 2000). Hamartin exhibits some of its tumor suppressor activity as a regulator of cell-matrix adhesion; inactivation of hamartin results in rapid cell retraction and progressive detachment from the substratum as a result of loss of focal adhesions. In another experiment, hamartin was shown to act upstream of Rho; overexpression of full-length or an N-terminal domain of hamartin led to rapid activation of Rho and formation of stress fibers and focal adhesions by an unknown mechanism (Lamb et al. 2000). Interaction of endogenous hamartin with ezrin was required for activation of Rho by serum or by LPA. Therefore, the

ERMs may mediate their upregulation of Rho activity via RhoGDI or hamartin, or, more likely, a complex interplay of these and other mechanisms.

Despite the fact that the ERM proteins have not been found mutated in human cancer, expression of ezrin is required for transformation downstream of the Net/Dbl Rho exchange factors in cultured fibroblasts (Tran Quang et al. 2000). In addition, in concert with its role in cell shape and motility, ezrin has been found to be overexpressed in invasive cells derived from human tumors, indicating a possible role for the ERMs in tumor progression. Interestingly, the ERMs have also been found to regulate cell survival, perhaps another mechanism for their promotion of cell proliferation. Phosphorylation of ezrin on Y353 is required for epithelial cell survival via the PI3K pathway (Gautreau et al. 1999). In T lymphocytes, the binding of ezrin to CD95 (APO/Fas) resulted in susceptibility to CD95-dependent apoptosis (Parlato et al. 2000). Finally, in addition to acting as a positive regulator of Rho signaling, ezrin has recently been shown to interact with and induce activation of focal adhesion kinase (FAK), a critical regulator of and docking site for signaling molecules at focal adhesions (Poullet et al. 2001).

In summary, the ERM family is important in the regulation of many cellular functions related to the actin cytoskeleton. In addition, recent studies have linked the ERMs to many signaling pathways, indicating that this family may have a broader role in the regulation of cell proliferation in response to the appropriate cellular cues, such as cell-matrix adhesion. As will be evident in the following sections, the ERM proteins and their protein interactors have been informative with respect to studies of merlin. Although it is clear that the ERMs and merlin function in opposing manners, the fact that they heterodimerize might indicate that merlin may mediate some of its functions by binding and inactivating the ERMs or, conversely, that the

ERMs mediate their growth-promoting activity by binding and inactivating merlin. Because the tumor suppressor function of merlin is still unclear, studies of these mechanisms are difficult.

Regulation of merlin expression, self-association, and activity

Merlin expression and activity are regulated by a variety of mechanisms. In mouse fibroblasts, expression of merlin is increased under conditions of (G1) cell cycle arrest, such as cell-cell contact, growth factor deprivation, and loss of adhesion (Shaw et al. 1998c). Interestingly, merlin was shown to migrate as two distinct species on a western blot, with the slower-migrating species being a phosphorylated form of the protein. Under conditions of cell confluency and serum-withdrawal, the levels of phosphorylated and unphosphorylated merlin species increased significantly. Furthermore, loss of adhesion resulted in almost complete dephosphorylation of merlin, which was reversed upon re-plating of the cells, suggesting that merlin phosphorylation may be responsive to cell spreading or changes in cell shape.

In addition to merlin expression's being regulated by conditions of cell stress, merlin is regulated by self-association, like the ERM proteins. Interestingly, an early study in rat schwannoma cells revealed that tumor growth inhibition by merlin depends on an interdomain association occurring either in cis or in trans between the N- and C-terminal domains (Sherman et al. 1997b). These studies and others revealed that isoform II likely exists in an open conformation, lacking the critical C-terminal residues required to mediate interaction with the N-terminal domain, whereas merlin isoform I was capable of interdomain interactions. Indeed, the amino acids encoded by exon 17, the final exon of isoform I, that is absent in isoform II, have been shown to be critical for mediation of N- and C-terminal interactions (Gonzalez-Agosti et al. 1999; Gronholm et al. 1999). Further data revealed that an intradomain interaction within the N-

terminal region of merlin is required for N/C-terminal interactions (Gutmann et al. 1999a). Many mutations in the FERM domain corresponding to those found in NF2 patients disrupt these N/N and N/C-terminal interactions, likely leading to abnormal behaviors of the protein. Interestingly, merlin is capable of forming heterotypic interactions with the ERM proteins in a head-to-tail fashion as shown by both *in vitro* and *in vivo* experiments[††] (Huang et al. 1998; Gonzalez-Agosti et al. 1999; Gronholm et al. 1999; Nguyen et al. 2001). Finally, self-association of merlin (and the ERMs) has been shown to mask binding sites for membrane proteins, such as NHERF (Gonzalez-Agosti et al. 1999). In summary, inter- and intra- molecular homo- and hetero-typic interactions between merlin and the ERM proteins may serve to regulate their functions.

Recent evidence suggests that merlin self-association and function may be regulated by phosphorylation (Figure 5). As noted above, increased levels of the hypophosphorylated form of merlin correlated with conditions of growth arrest, whereas phosphorylation was induced following replating of cells held in suspension. Therefore, it has been hypothesized that the hypophosphorylated form of merlin is "active" as a growth suppressor, whereas the phosphorylated form may be "inactive". Shaw and colleagues have recently reported that the activated small GTP-binding proteins, Rac or Cdc42, but not Rho, could stimulate phosphorylation of merlin on a C-terminal serine residue (Ser518) (Shaw et al. 2001). More recently, studies have demonstrated that the p21-activated kinase, Pak, is responsible for the direct phosphorylation of merlin at this site (Kissil et al. 2002; Xiao et al. 2002). Interestingly, merlin was able to be phosphorylated by active Pak1, active Pak2 or wild-type Pak6 (Xiao et al. 2002). Furthermore, *in vitro* and *in vivo* binding experiments suggest that Ser518 phosphorylation affects the ability of merlin to form intramolecular interactions (Shaw et al.

[††] Discussed later under "Binding partners for merlin".

2001). Thus, these data confirm the hypothesis that phosphorylated merlin is likely in an open, inactive conformation, whereas hypophosphorylated merlin is likely closed and active. Phosphorylation of merlin is also associated with a shift in subcellular localization from an insoluble to a soluble fraction[‡‡] (Shaw et al. 2001; Kissil et al. 2002).

In addition to phosphorylation, it appears that merlin (or merlin mutants) may be regulated by different mechanisms in the cell. Interestingly, in studies of many sporadic schwannomas and meningiomas, where no *NF2* gene mutations were identified, no merlin expression was detected (Gutmann et al. 1997; Lee et al. 1997; Stemmer-Rachamimov et al. 1997). Thus, these data indicate that perhaps post-translational regulation leads to loss of merlin function in these tumors. Merlin is cleaved by calpain proteases (Kimura et al. 1998), a large family of calcium-dependent neutral cysteine proteases (Sorimachi et al. 1997). Treatment of human fibroblasts with calpain inhibitors led to an overall increase in merlin levels (Kimura et al. 1998). *In vitro* experiments suggested that calpain cleavage of merlin between amino acids 295 and 299 resulted in 35kD proteolytic fragments of the protein which were not detected in cells in culture, perhaps due to degradation. Interestingly, many tumor samples with no detectable merlin protein expression, but expressing full-length copies of a wild-type *NF2* mRNA, including cells derived from a sporadic meningioma, showed increased activity of calpain (Kimura et al. 1998). In such cells, inhibition of calpain activity led to an increase in merlin expression, indicating that calpain-mediated degradation contributes to the absence of merlin in meningiomas without *NF2* mutation – perhaps a novel pathway for tumor formation. Accordingly, treatment of meningioma cells with calcium agonists (which inhibit calpain activity) resulted in a diminished proliferative capacity, indicating the potential importance of

[‡‡] More detailed information on the Rho GTPases and Pak and other effects of phosphorylation on merlin function and information will be presented in future sections.

this pathway in CNS tumor development (Jensen et al. 1995a; Jensen et al. 1995b). In fact, an examination of the expression of merlin proteins in meningioma and schwannoma tumor samples with *NF2* mutations predicted to cause protein truncation also revealed a lack of detectable merlin protein (Den Bakker et al. 2001). These studies and results from the calpain studies suggested that truncated forms of merlin are likely unstable and perhaps degraded in the cell. Further studies revealed that such pathogenic *NF2* mutations that lead to misfolding of merlin are targeted for degradation via the ubiquitin-proteasome pathway (Gautreau et al. 2002). In addition, mutant, but not wild-type, merlin was shown to be efficiently degraded in fibroblasts derived from $Nf2^{\Delta 39\text{-}121/+}$ mutant mice. Interestingly, these studies revealed that ubiquitination and degradation of merlin mutants was specific, as mutant ERM proteins were not degraded. Importantly, the ubiquitin-proteasome pathway is functional in Schwann cells, and, therefore, in combination with calpain proteolysis may contribute to complete loss of merlin function in NF2 patients and sporadically occurring NF2-related tumors.

Cellular localization of the *Nf2* tumor suppressor protein, merlin, and interactions with the cytoskeleton

Sequence similarity between merlin and the ERM proteins suggested that merlin might localize to the cell membrane in regions rich in actin structures. Early imunofluorescence studies of merlin in COS and human cells demonstrated a colocalization between merlin and elements of the actin cytoskeleton at the membrane, supporting the hypothesis that merlin may act as a "membrane organizing element" (den Bakker et al. 1995; Gonzalez-Agosti et al. 1996). Further localization studies in Schwann cells revealed that although localization of merlin to membrane structures, such as ruffles, appears to be mediated through the N-terminal FERM domain, proper

localization requires the entire protein (Scherer and Gutmann 1996; Xu et al. 1998). Overexpression of merlin leads to its localization in many actin-rich structures, such as ruffling edges (lamellipodia), filopodia, and cell/substrate adhesion points (Schmucker et al. 1997; Shaw et al. 1998b). Mutational analysis revealed that most disruptions of the FERM domain (corresponding to patient mutations) disturbed the connection between merlin and membrane and actin cytoskeleton structures (Deguen et al. 1998b). Finally, Brault and colleagues have extended our understanding of merlin localization through their demonstration that both membrane localization and actin association requires strong intradomain interactions within the FERM domain (amino acids 19-314) that are disrupted upon mutation (Brault et al. 2001). Collectively, these studies suggest that loss of activity of several naturally occurring merlin mutants may be due to disruption of the folding of the N-terminal domain, leading to loss of both membrane localization and actin association.

Although merlin does not share the C-terminal actin-binding domain with the ERM proteins, recent evidence suggests that merlin binds actin directly through discreet domains in its N-terminal region and indirectly through a domain near the C terminus. Initially, merlin was shown to associate with polymerized actin *in vitro* by virtue of an amino-terminal actin-binding domain including residues 178-367 (Xu and Gutmann 1998). Interestingly, the location of this actin-binding domain implies that actin binding is not affected by several naturally occurring NF2 patient mutations or alternatively spliced isoforms. Further delineation of actin-binding domains in merlin revealed that amino acids 1-27 and 280-323 (at opposing ends of the FERM domain) likely harbor the actin binding sites and that intramolecular interactions might mask these actin-binding sites in the context of full-length merlin (Brault et al. 2001; James et al. 2001). Yet, other studies employing detergent extraction techniques suggest merlin association

with the actin cytoskeleton is mediated through a high affinity site located between amino acids 29-131 and a lower affinity site between amino acids 321-470 (den Bakker et al. 2000). The importance of each of these identified sites for actin-merlin association requires further investigation.

Merlin has been shown to interact indirectly with the actin cytoskeleton through a linker protein, βII-spectrin/fodrin. Initial studies suggested that the interaction between merlin and βII-spectrin was masked in the context of full length isoform I, which only interacted weakly with βII-spectrin compared to isoform II interactions (Scoles et al. 1998). Further characterization of the binding between these two proteins has established a C-terminal interaction domain on merlin and confirmed that self-association of merlin isoform I denies βII-spectrin access to this binding site (Neill and Crompton 2001; Scoles et al. 2002a). Finally, one report suggests that merlin can interact with microtubules *in vitro* and that, like binding to βII-spectrin, the conformation of merlin is critical to its ability to participate in this interaction (Xu and Gutmann 1998; Gutmann et al. 2001b). The practical significance of merlin interaction with the actin cytoskeleton is highlighted by the cellular effects of disruption of merlin function.

In addition to interactions and colocalization with the actin cytoskeleton, two reports suggest that merlin may localize to the nucleus. Although almost all studies of *NF2* have focused on the activities of the two main isoforms of merlin, several other expressed isoforms have been identified that are expressed in a tissue/developmental-specific manner (Bianchi et al. 1994; Haase et al. 1994; Hara et al. 1994; Pykett et al. 1994). Alternative splicing of *NF2* reveals a nuclear localization signal (NLS) and results in nuclear localization of the encoded protein (Schmucker et al. 1999). Interestingly, this phenomenon has also been observed in other band 4.1 family members (Luque et al. 1998). More recently, expression of a murine *Nf2* allele

lacking exon 2 (Δ2) resulted in localization of the encoded merlin protein in the nucleus, indicating that perhaps sequences in exon 2 acted as a cytoplasmic retention factor (Kressel and Schmucker 2002). In fact, fusion of merlin exon 2 sequences to GFP demonstrated that exon 2 could retain GFP exclusively in the cytoplasm. Further examination of the *Nf2* sequence revealed a nuclear export sequence (NES) in exon 15, that, when deleted, lead to complete retention of Δ2 merlin in the nucleus. However, despite the identification of isoform-specific localization of merlin to the nucleus, there has been no demonstrated role for merlin at this site.

Merlin function

Early studies of merlin function focused mainly on the cellular and molecular properties of overexpressed wild-type and mutant merlin in different cell types. Consistent with its assignment as a tumor suppressor protein, several studies have demonstrated that a general feature of merlin overexpression is inhibition of cell proliferation. The first manifestation that merlin behaved as a tumor suppressor protein in cells in culture followed overexpression of merlin in oncogenic Ras-transformed NIH3T3 cells (Tikoo et al. 1994). Overexpression of wild-type merlin resulted in a reversal of anchorage-independent growth and a restoration of contact inhibition of growth in the malignant cells. Interestingly, overexpression of the N-terminal half of merlin resulted in a partial suppression of the malignant phenotype in the Ras-transformed NIH3T3s, indicating the importance of this domain for merlin function. Following that study, Lutchmann and colleagues demonstrated that overexpression of wild-type merlin in normal NIH3T3 cells inhibits cell proliferation at high cell densities, as the cells exhibited a three-fold slower growth rate than mock-transfected controls (Lutchman and Rouleau 1995). Treatment of merlin-transfected cells with antisense oligos complementary to merlin sequences resulted in a

reversal of the inhibition of cell proliferation, indicating the specificity of the effect of merlin on this phenotype. Reintroduction of merlin isoform I into rat schwannoma cells, which have low levels of endogenous merlin, resulted in a suppression of cell proliferation *in vitro* and *in vivo* (Sherman et al. 1997b). Interestingly, this was one of the first studies to demonstrate the difference between the activities of the two main isoforms of merlin, as merlin isoform II was incapable of suppressing the growth of these cells. Now, we are aware that these two isoforms have unique inter- and intra- molecular interactions that likely result in unique functional properties (Scoles et al. 2002a). Finally, recently, Schulze and colleagues have demonstrated an anti-proliferative, and, perhaps, pro-apoptotic, role for merlin in human schwannoma cells, as reintroduction of merlin via retroviral transduction into *NF2*-deficient schwannoma cells led to a significant decrease in proliferation, accompanied by a slight increase in apoptosis (Schulze et al. 2002). Not surprisingly, in every cell system studied thus far, expression of merlin has been shown to be growth-suppressive. Interestingly, the suppression of Ras transformation by merlin suggests that merlin may function to inhibit cell proliferation in a pathway downstream of Ras (see later discussion).

In addition to longer-term, anti-proliferative effects, overexpression of wild-type merlin in different cell types was shown to cause alterations in cytoskeletal dynamics, including cell morphology, cell motility, and cell attachment (den Bakker et al. 1995; Koga et al. 1998; Gutmann et al. 1999b). Support for the role of merlin in cell-matrix interactions comes from studies of cells following expression of merlin proteins with mutations corresponding to those found in NF2 patients. Koga and colleagues demonstrated that while expression of wild-type merlin in fibroblasts results in submembranous and perinuclear punctate cytoplasmic staining and elongation of cellular processes, expression of a truncation mutant lacking exons in the

FERM domain demonstrates a lack of membrane staining and a significant decrease in cell adhesion (Koga et al. 1998). In addition, a comprehensive study of many merlin mutants in three different cell types revealed that most mutations lead to altered cell adhesion and increased detergent solubility, and, therefore, support a role for wild-type merlin in interaction with the cytoskeleton and cell-matrix attachment (Stokowski and Cox 2000). Many of the mutations examined altered the ability of the protein to self-associate, indicating the importance of self-association for merlin function. Also, decreased cell adhesion was observed in Schwann-like cells following introduction of antisense oligonucleotides complementary to merlin (Huynh and Pulst 1996). These results have led to a general speculation that disruption of cytoskeletal organization downstream of *NF2* mutation is likely involved in the development of tumors.

Merlin and signaling

Several studies have indicated a role for merlin in the regulation of particular signaling pathways. *NF2*-deficient human schwannoma cells exhibit elevated basal proliferation and increased spreading on many different substrates (Pelton et al. 1998). In addition, increased membrane ruffling and aberrant stress fibers were prominent abnormalities observed in these cells compared to normal Schwann cells, which implicates aberrant activation of the Rac and Rho pathways, respectively. In order to assess whether RhoGTPase pathways played a role in the phenotypes demonstrated by *NF2*-deficient schwannoma cells, dominant-negative (dn) forms of the Rho proteins were expressed. Expression of dnRho lead to a reversal of the aberrant stress fibers, and expression of dnRac lead to reversal of the increased membrane ruffling phenotype (Pelton et al. 1998). Further studies with these cells revealed that the cytoskeletal phenotypes were specific to schwannoma cells with mutations in *NF2*, as *NF1*-deficient cells did not display

any of the same phenotypes (Pelton et al. 1998). In addition, the phenotypes in the *NF2*-deficient schwannoma cells were specific to *NF2* loss, as tat-mediated merlin reintroduction into these cells rescued the cytoskeletal defects and restored normal actin organization (Bashour et al. 2002). Thus, although the mechanisms have yet to be defined, early studies of the effects of loss of merlin function in Schwann cells implied that merlin expression might be critical for regulation of RhoGTPase pathways.

More recent studies have supported a role for merlin regulation of the Rac pathway, specifically. *Nf2*-deficient mouse embryo fibroblasts (MEFs) exhibit several phenotypes that are consistent with misregulation of Rac pathways, including increased membrane ruffling in response to adhesion and platelet-derived growth factor (PDGF)-stimulation, and increased cell motility in a wounding assay (Shaw et al. 2001). Importantly, an analysis of the function of Rho GTPases in wound healing of fibroblast layers revealed that while Rho activity is necessary to maintain adhesion, Cdc42 and Rac were more critical in promoting motility (Nobes and Hall 1999), thus supporting the hypothesis that misregulation of Rac (or Cdc42) pathways are responsible for the increase in motility in the absence of merlin. In addition to phenotypes indicative of misregulation of the Rac pathway, *Nf2*-/- MEFs exhibited higher basal levels of phosphorylated (active) c-jun N-terminal kinase (JNK)/ stress activated protein kinase (SAPK). Additional stimulation of these cells with serum or ultraviolet light (UV) further induced JNK activity. Reporter assays demonstrated that, when compared to wild-type MEFs, *Nf2*-deficient MEFs had increased activity of the AP-1 transcription factor (Shaw et al. 2001), which is known to be upregulated in response to JNK activation (Ip and Davis 1998). A very recent study confirms that the Rac pathway is misregulated in *NF2*-/- human schwannoma cells, as the authors demonstrated increased levels of activated Rac, increased membrane localization of Rac

and its effector, Pak, and increased activated levels of the downstream kinase, JNK (Kaempchen et al. 2003). Contrary to this study, experiments in fibroblasts suggested that Rac-GTP levels were normal under conditions of loss of merlin function [Chapter 4 and (Shaw et al. 2001)]. It is not clear whether the differences observed here are due to cell type differences or assay conditions. Nevertheless, taken together, these phenotypes are consistent with a role for merlin in the regulation of the Rac pathway.

Additionally, to test the effects of merlin expression on the Rac pathway, experiments were performed that demonstrated that overexpression of wild-type merlin could efficiently inhibit Rac-induced anchorage-independent growth of cells in soft agar. Finally, overexpression of merlin resulted in downregulation of JNK and AP-1 activity in cycling cells. Inhibition of other signaling molecules, such as the extracellular regulated kinase (ERK), was negligible under these conditions of merlin overexpression. Inhibition of AP-1 transcriptional activity by merlin was dependent upon the state of merlin phosphorylation, as a phosphorylation-defective mutant of merlin (S518A) inhibited Rac-induced AP-1 reporter activity as well or better than the wild-type protein, whereas a phosphorylation-mimicking mutant of merlin (S518D) was compromised in its ability to mediate such inhibition (Shaw et al. 2001). Intriguingly, these data imply that merlin functions to regulate the Rac pathway *and* that its function is regulated by the Rac pathway via Pak. Thus, negative regulation of merlin via Rac-dependent phosphorylation may relieve merlin's inhibition on the Rac pathway, thereby resulting in a feed-forward mechanism of Rac activation. This model is reminiscent, but opposite, of that seen with the ERMs, where Rho-dependent activated ERMs positively feedback on the Rho pathways causing increases in Rho activity. In summary, although a clear link has been made between loss of merlin function and

upregulation of Rac pathway activity, the identity of the step in the pathway at which merlin acts is unknown.

Several recent studies have implicated merlin in the negative regulation of various signaling pathways downstream of Ras. In particular, overexpression of merlin was shown to inhibit Ras upregulation of cyclin D1 in Ras transformed NIH3T3 cells via downregulation of AP-1 and E2F-1 dependent transcription (Kim et al. 2002a). Interestingly, although previous studies suggest that overexpression of merlin has a neglible effect on ERK phosphorylation in cycling cells [(Shaw et al. 2001) and Chapter 3], one study showed that serum-induced ERK activity is inhibited by merlin, perhaps via its N-terminal domain (Lim et al. 2003). Further studies of the effects of merlin overexpression have revealed a regulatory role for merlin on yet another transcription factor pathway, that of nuclear factor kappa B (NFκB), a protein involved in regulation of transcription downstream of mitogenic, anti-apoptotic, and inflammatory signals (Kim et al. 2002b). As with the inhibition of ERK activity, the N-terminal region of merlin was shown to be important for NFκB inhibition in various reporter assays. Interestingly, NFκB likely plays an important role in tumorigenesis, as it is aberrantly activated in many cancers (Rayet and Gelinas 1999), and its transcriptional activation is required for Ras transformation *in vitro* (Finco et al. 1997). Activation of Rac in NIH3T3 cells induces the activity of NFκB and its binding to sites in the cyclin D1 promotor, suggesting a critical role for NFκB in cell cycle regulation through cyclin D1 and Rac (Joyce et al. 1999). Thus, these data provide another point of convergence between merlin and the Rac pathway. The important details of such signaling cascades will be discussed in further detail in later portions of the introduction.

Binding partners for merlin

Several studies have been initiated to understand better the role of merlin in cytoskeletal function and signaling with a focus on the identification of cellular interacting proteins. Although merlin has been shown to interact with many cellular proteins, including adhesion molecules, scaffolding proteins, signaling molecules, and cytoskeletal proteins (as detailed above), very little is understood concerning the functional consequences of these interactions (Figure 6). The homology between merlin and the ERM proteins in the FERM domain has led to the identification of some of the binding partners for merlin. However, it is becoming more apparent that merlin may bind to a set of proteins distinct from those shared with the ERMs. It is perhaps these interactions that distinguish merlin function from that of the ERM proteins. Further investigation into all of these interactions is warranted in order to comprehend more completely the physiological function of merlin.

Like the ERM proteins, merlin has been shown to bind directly or indirectly to several integral membrane proteins. Probably one of the most well-characterized merlin interactors is the hyaluronic acid (HA) receptor, CD44. Early immunofluorescence analysis in a variety of cell types demonstrated colocalization of merlin with CD44 (and the ERMs) in membrane protrusions, and showed that merlin expression induced clustering of CD44 to discreet regions of the membrane (Sainio et al. 1997). In addition, the authors demonstrated direct binding between CD44 and merlin using *in vitro* methods (Sainio et al. 1997). More recent studies indicate that merlin binding to CD44 may provide a molecular switch that specifies either cell growth arrest or proliferation in rat schwannoma cells (Morrison et al. 2001). At high cell density, merlin becomes hypo-phosphorylated, binds the cytoplasmic tail of CD44, and inhibits cell proliferation in response to the interaction between the extracellular domain of CD44 and its substrate, HA, a

mucopolysaccharide that surrounds cells. At low cell density, merlin is phosphorylated, growth permissive, and exists in a complex with the ERMs and CD44. Although the mechanisms of downstream cell cycle regulation are not understood, these data imply that loss of merlin leads to uncontrolled cell proliferation, and at high cell densities, this deregulation results in observable contact inhibition defects. Interestingly, CD44 has been implicated in tumorigenesis in two opposing manners (Herrlich et al. 2000). First, overexpression of high molecular weight splice variants of CD44 have been observed, for example in NF2-derived schwannoma cell lines (Sherman et al. 1997a), indicating that an increased presence of this receptor might allow for amplified presentation of growth factors to their cognate receptors on the cell surface, a normal behavior of CD44 in certain cell types and contexts. Secondly, loss of CD44 has been associated with tumorigenesis, for example in prostate cancer (Kallakury et al. 1996), invoking a model of loss of critical CD44 function at confluency via merlin tumor suppressor function. Finally, CD44 has been shown to interact with Tiam-1, a Rac-specific GDP/GTP exchange factor (GEF) (Bourguignon et al. 2000). Some forms of HA have been shown to stimulate the activity of Rac (via CD44 and Tiam-1) causing membrane ruffling and increased cell motility in a variety of cell types (Oliferenko et al. 2000). Indeed, these data place merlin at another site critical for the regulation of Rac function. Taken together, these data imply that CD44 may be critical to merlin's mediation of contact inhibition. However, remarkably, CD44-deficient fibroblasts do not exhibit any contact inhibition defects [KCJ and TJ, unpublished results and (Lallemand et al. 2003)], indicating that the consequences of the interactions between CD44 and merlin are likely very cell context-specific.

In addition to binding to CD44, merlin has been shown to interact with other integral membrane proteins. Merlin relocalizes from a soluble to an insoluble fraction upon

differentiation of Schwann cells in culture, and this shift in localization coincides with its partial colocalization with β1 integrin, a receptor that mediates contacts with the extracellular matrix (Obremski et al. 1998). In addition, adhesion of Schwann cells to basal lamina results in β1 integrin clustering and the assembly of a signaling complex containing β1 integrin, focal adhesion kinase (FAK) and paxillin, signaling scaffold proteins localized mainly to focal adhesions, and merlin, as demonstrated by immunofluorescence and co-immunoprecipitation experiments (Taylor et al. 2003). In subconfluent, adherent cells, this complex was found at many actin-rich sites, including filopodia, membrane ruffles, and focal contacts. Interestingly, studies by Clark and colleagues demonstrate that several adhesion-dependent morphological and signaling changes downstream of integrin engagement require RhoGTPases (Clark et al. 1998). In addition, Rac recruitment to the membrane and its subsequent interaction with effectors is enhanced by local integrin stimulation (Del Pozo et al. 2002). Although the function of the interaction between merlin and β1 integrin has not been established, it is likely that merlin binding to the cytoplasmic face of this receptor helps to regulate adhesion-dependent "outside-in" integrin signaling, perhaps via the RhoGTPases, that normally promotes cell proliferation and cytoskeletal changes (Hynes 2002).

The interaction of merlin with the Na^+/H^+ exchanger regulatory factor (NHERF) allows merlin to be indirectly linked to many transmembrane proteins. NHERF was originally discovered in a screen to identify binding partners and regulators of the Na^+/H^+ exchanger 3 (NHE3) (Yun et al. 1998). However, more recent research suggests that it has an expanded role bringing together membrane-bound ion transporters and receptors and non-membrane proteins in order to regulate cell metabolism and growth (Voltz et al. 2001). Although the consequences of the interaction are still under investigation, the FERM domain of merlin has been demonstrated

to interact directly with the C-terminal domain of NHERF, in a way that demands an open conformation of the merlin molecule (Murthy et al. 1998; Gonzalez-Agosti et al. 1999). Again, here, merlin is poised at a site where many extracellular signals are integrated and relayed to the nucleus via signaling cascades.

Interestingly, experiments in Schwann cells have revealed that merlin can directly interact with, and be recruited to, the membrane by paxillin, a focal adhesion-associated protein that acts as a point of convergence for signals resulting from growth factor stimulation and extracellular matrix (ECM) adhesion (Fernandez-Valle et al. 2002). The merlin-paxillin interaction is not surprising given the recent identification of merlin as part of a complex localized to the cytoplasmic face of β1 integrin, which contains paxillin (see discussion above) (Taylor et al. 2003). The binding of merlin to paxillin is partially mediated through amino acids encoded by exon 2 of merlin (aa 50-70), and pathogenic mutations in this region lead to a significant reduction in the interaction between the two proteins (Fernandez-Valle et al. 2002). The primary function of paxillin as a molecular adaptor/scaffold protein is to provide many docking sites at the plasma membrane for an array of signaling and structural proteins, thereby directly linking activities on the outside of the cell to the actin cytoskeleton and signaling on the inside of the cell (Schaller 2001). Therefore, it is possible that merlin may mediate its tumor suppressor function at the cytoplasmic face of membrane receptors via the inhibition of a single or perhaps many signaling pathways.

Very recently, merlin has been demonstrated to colocalize and interact with β-catenin, a protein involved in the formation and maintainence of adherens junctions, thereby placing merlin at the cytoplasmic face with yet another membrane-bound protein complex (Lallemand et al. 2003). Classically, adherens junctions were thought of as specialized forms of cadherin-based

adhesive contacts that were important for stable maintenance of cell-cell contacts and tissue organization and cohesion (Yap et al. 1997). Indeed, disruption or loss of E-cadherin activity is associated with tumor progression and invasion in many epithelial cancers (Semb and Christofori 1998). Interestingly, more recent studies suggest that adherens junction-based cadherins can function as ligand-activated signaling receptors, as RhoGTPases have been shown to localize to the cytoplasmic face of cadherin-based cell-cell contacts (Braga 2000; Yap and Kovacs 2003). Within minutes of cadherin adhesion, either by cell-cell contact or *in vitro* stimulated cadherin ligation by a ligand-coated substrate, the activity of Rac is upregulated, perhaps via a PI3K-dependent mechanism, and the activated Rac is recruited to newly forming cell-cell contacts (Nakagawa et al. 2001; Noren et al. 2001; Kovacs et al. 2002). In fact, the critical importance of Rac at these sites of adhesion is highlighted by studies in which expression of dominant-negative Rac affects the initiation of newly forming contacts (Ehrlich et al. 2002). Fascinatingly, these data place merlin at a site of cell-cell contact signal integration where the function of Rac appears to be very important. The finding that loss of merlin expression leads to contact inhibition defects and upregulation of the Rac pathway, and that *Nf2*-deficient fibroblasts and keratinocytes have disrupted adherens junctions is consistent with a function for merlin at such sites (Shaw et al. 2001; Lallemand et al. 2003). Although the mechanisms are still unclear, it is likely that these findings will lead to further investigations of merlin function at cell-cell junctions and, thus, a better understanding of the tumor suppressor role of this protein.

Significant effort has been placed into understanding the interactions between merlin and the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS). Like merlin, overexpression of HRS suppresses the proliferation of rat schwannoma cells (Gutmann et al. 2001a). The binding between HRS and merlin was mapped using several *in vitro* methods; the

C-terminal half of merlin was shown to interact with the C-terminal domain of HRS in a region that is distinct from that required for HRS suppression of growth (Scoles et al. 2000; Sun et al. 2002). As with most binding partners studied thus far, interaction between merlin and HRS required that merlin be in an open conformation, and point mutations in merlin that overlap with the binding domain reduced its affinity for HRS (Gutmann et al. 2001a; Scoles et al. 2002a). In addition, in Schwann cells, merlin co-localized with HRS in endosomes, a site of potential importance for HRS growth-suppressing activity (Scoles et al. 2000). In fact, HRS has been demonstrated to be a powerful regulator of receptor tyrosine kinase trafficking to the degradation pathway in both mammals and flies (Lloyd et al. 2002; Raiborg et al. 2002; Shih et al. 2002). Recent studies have demonstrated a role for merlin and HRS in signal transducers and activators of transcription (STAT) signaling, as both merlin and HRS were capable of inhibiting Stat3 activation (phosphorylation) in a human schwannoma cell line, which additionally resulted in reduced transcription from a STAT-responsive promoter (Scoles et al. 2002b). Also, expression of merlin led to decreases in Stat3 and Stat5 phosphorylation in a rat schwannoma cell line. Finally, although the exact mechanism is still under investigation, the binding of merlin to HRS might be *required* for the tumor suppressor function of merlin, as expression of merlin in HRS-deficient MEFs is not growth inhibitory (Sun et al. 2002).

In vitro and *in vivo* experiments have demonstrated the binding of the N-terminal region of merlin to the RhoGDIs (Maeda et al. 1999). The binding of active RhoGTPases to their effectors leads to actin reorganization, gene expression and cell growth (Bishop and Hall 2000). However, the availability of active RhoGTPases is tightly regulated by several cellular proteins, including those that stimulate GTP loading (GEFs), those that catalyze GTP hydrolysis (GAPs) and RhoGDIs that antagonize both GEFs and GAPs (Olofsson 1999). RhoGDIs maintain Rho

proteins in a soluble, inactive state in the cytosol by shielding their hydrophobic isoprenoid moiety required for membrane targeting, and, therefore, act to regulate both the GDP/GTP exchange cycle as well as the membrane association/dissociation cycle (Keep et al. 1997). Interestingly, activated ERMs have been shown to bind to RhoGDI, catalyzing a dissociation of Rho from its inhibitor and an activation of downstream Rho-dependent pathways (Takahashi et al. 1997). Although the physiological consequences of the merlin-RhoGDI interaction have not yet been elucidated, it is interesting that merlin is again localized to a site/protein important for the regulation of the RhoGTPases.

Finally, given the strength of their N-terminal sequence homology and colocalization in various cell types, it was not surprising that merlin and the ERM protein, ezrin, were shown to heterodimerize (Gronholm et al. 1999). As expected, the heterotypic binding of merlin and ezrin involved interaction between the amino- and carboxy-termini, as demonstrated by *in vitro* binding experiments. Remarkably, the C-terminal half of merlin has a higher affinity for the FERM domain of ezrin than it does for its own FERM domain (Nguyen et al. 2001). In addition, merlin can co-immunoprecipitate with ezrin, although *in vitro* studies suggested that strong heterodimerization of merlin isoforms I and II may prevent heterodimerization between either isoform and ezrin (Meng et al. 2000). Hence, the authors of these studies suggest that merlin might mediate its tumor suppressor function by modulating the activity of the ERM proteins. However, studies suggesting that the molar ratio of merlin-to-ERM proteins in cells is only 0.05-0.14, argues against such a hypothesis (Maeda et al. 1999). Nevertheless, whether the interactions between merlin and the ERM proteins are important for their function needs further investigation.

NF2 in other organisms

An *NF2* tumor suppressor gene orthologue has been identified in mice, in the worm, *C. elegans,* and in the fly, *Drosophila melanogaster.* More recently, two *NF2*-like genes have been cloned and mutants studied in zebrafish (K. Lai, J. Lees, and N. Hopkins, pers. comm.). Interestingly, all of these organisms, as well as the sea urchin and several parasites, have at least one ERM protein in their genome. An *NF2* orthologue has yet to be identified in sea urchins or parasites. In all organisms where it has been studied, inactivating mutations of *NF2* have led to overproliferation phenotypes. Studies of *NF2* in other organisms have allowed tremendous insight into the molecular and genetic function behind this complex tumor suppressor gene.

NF2 in mice

The mouse orthologue of *NF2* is located on chromosome 11 in a region of synteny conservation with human chromosome 22q and its encoded protein shares 98% amino acid identity with human merlin (Claudio et al. 1994a; Claudio et al. 1994b; Haase et al. 1994). Homozygous deletion of *Nf2* in the mouse using homologous recombination techniques in embryonic stem cells resulted in embryonic lethality between days 6.5 and 7 (McClatchey et al. 1997). The *Nf2*-/- embryos fail to develop to term due to a collapsed extraembryonic region, which displays a severely disorganized ectodermal layer. The embryo proper appears to develop normally to this stage, but is unable to initiate gastrulation and, therefore, lacks a distinct mesodermal cell layer. Studies of chimeric mice demonstrated that merlin expression is not required cell autonomously for the establishment of the mesoderm, thereby indicating that the primary requirement for merlin in embryonic development is in the extraembryonic tissues.

In order to develop a murine model for NF2, McClatchey and colleagues went about characterizing mice heterozygous for mutation at the *Nf2* locus. Surprisingly, unlike the benign nervous system lesions in humans, *Nf2*+/- mice developed highly malignant sarcomas later in life (age of onset 10-30 months) (McClatchey et al. 1998). The majority of the most common tumors, osteosarcoma, fibrosarcoma and hepatocellular carcinoma, displayed LOH at the *Nf2* locus. 90% of the osteosarcomas metastasized to distant organs, including the liver and lung. In order to determine possible cooperative effects between *Nf2* and other known cancer genes, this mouse was crossed to a *p53* +/- mouse. *p53* is a tumor suppressor gene located on the same chromosome as *Nf2* in mice. Therefore, mice were generated in two configurations: cis, with both mutant alleles on one chromosome and trans, with the two mutant alleles on opposite chromosomes. Mice carrying *Nf2* and *p53* mutant alleles in both configurations died earlier than *p53*+/- or *Nf2*+/- mice, with the cis configuration resulting in the shortest tumor latency (< 5 months). The tumors found in the double heterozygous cis mice had undergone LOH of both alleles, likely indicating loss of the entire wild-type chromosome 11. Finally, whereas *p53*-deficient mice rarely have metastatic tumors, most of the *Nf2*+/-;*p53*+/- mice developed highly metastatic lesions. These studies, along with the fact that *Nf2* is a member of a family of cytoskeletal-linked proteins, indicated that perhaps, in certain contexts, *Nf2* may function to regulate cell morphology and/or motility and in its absence lead to malignant tumorigenesis.

In order to more accurately model the human disease, Giovannini and colleagues developed two mouse strains that contained disruptions of the *Nf2* tumor suppressor gene targeted specifically to Schwann cells. The first model created took advantage of a mutation, interstitial deletion of exons 2 and 3 (SchΔ39-121), observed in the germline of NF2 patients and in sporadic schwannomas, meningiomas and mesotheliomas and expressed it as a transgene

under the P0 promoter (Giovannini et al. 1999). P0 is a major structural protein of peripheral myelin and a minimal promoter of this gene can direct Schwann cell-specific transgene expression beginning as early as embryonic day 9 in the mouse (Messing et al. 1992). Mutant proteins lacking exons 2 and 3 have been shown to be localized diffusely in the cytoplasm, rather than at the cell membrane, and their overexpression affects cell adhesion (Deguen et al. 1998b; Koga et al. 1998). Interestingly, expression of the SchΔ39-121 transgene had a dominant oncogenic effect in mice. Even in the presence of endogenously expressed wild-type merlin, expression of the transgene led to formation of Schwann cell tumors and/or Schwann cell hyperplasia in half of the mice. The origin of the cells in the tumor was confirmed by S-100 and LNGFR (Low affinity Nerve Growth Factor Receptor) immunoreactivity, two Schwann cell markers. In addition, the authors showed that expression of another merlin transgene, consisting only of the FERM domain had no effect on survival or tumor formation in wild-type mice.

Giovannini's second mouse model of *NF2* took advantage of the conditional cre recombinase – loxP system in order to circumvent embryonic lethality and conditionally biallelically inactivate *Nf2* using a P0-cre transgene (Giovannini et al. 2000). These mice, like those previously described carrying a P0-SchΔ39-121 transgene, developed Schwann cell hyperplasia and Schwann cell tumors, as well as cataracts and osseous metaplasia, all characteristics of human *NF2*. Finally, the same *Nf2-conditional* mice were used in an experimental system, in which *Nf2* recombination was targeted to the leptomeninges via direct adenoviral-cre recombinase (ad-cre) injection into the cerebral spinal fluid (CSF) (Kalamarides et al. 2002). Using this method, the investigators created the first mouse model for meningioma and demonstrated that inactivation of both *Nf2* alleles is the rate-limiting step for tumor development. Interestingly, injection of ad-cre to *Nf2-conditional* mice with a *p53* +/-

background had no effect on meningioma development, indicating that loss of *p53* is not a critical event in the development of this tumor type. These *Nf2* mouse models demonstrate the development of schwannomas by a mechanism that is functionally equivalent to that seen in NF2 patients, and thus, provide powerful *in vivo* model systems and sources for genetically altered cells useful for dissecting the tumor suppressor function of merlin and the progression steps of the disease, and for testing possible therapeutic avenues for treatment.

NF2 in flies

In addition to mouse models of NF2, significant advances have been made in the understanding of merlin function from studies in the fly, *Drosophila melanogaster*. Interestingly, there is only one ERM protein, Dmoesin, and one *Nf2* protein, Dmerlin, encoded by the fly genome. Dmoesin was shown to be continuously associated with the plasma membrane in localization studies (McCartney and Fehon 1996). Recent genetic analysis reveals a requirement for *Dmoesin* in oogenesis, as disruption of *Dmoesin* expression results in severe cell shape defects (Polesello et al. 2002). On the other hand, Dmerlin, which is 49% identical to human merlin, is localized in punctuate structures at the membrane and in the cytoplasm, and has been shown to be associated with endocytic compartments (McCartney and Fehon 1996). As with mammals, *Dmerlin* is essential in D*rosophila*, as loss-of-function alleles lead to larval lethality (Fehon et al. 1997). Interestingly, human *NF2* rescues *Dmerlin* lethality, implying functional conservation throughout evolution. Using the FLP/FRT system, mosaic expression of an otherwise lethal *Dmerlin* allele was induced (LaJeunesse et al. 1998). As in humans and mice, disruption of *Dmerlin* expression led to hyperplasia. In addition, expression of viable hypomorphic merlin allele, Mer^3, led to sterility and broadening wing phenotypes.

Many studies of Dmerlin have focused on the regions of greatest difference between the FERM domain of Merlin and the ERMs. Accordingly, a region of seven amino acids (170-YQMTPEM-177) that displays exact identity between human, mouse, and fly merlin, but diverges in the ERMs, was identified (termed, "blue box") (McCartney and Fehon 1996) (Figure 2). Using mutants in this domain and other alleles, a merlin lethality rescue experiment was employed in order to genetically dissect Dmerlin function. Whereas wild-type *Dmerlin* (Mer^+) could rescue the lethality associated with expression of Mer^4, a *Dmerlin* lethal allele, neither an allele with deletion nor alanine substitution of the blue box domain (ΔBB and BBA, respectively) was able to rescue Mer^4 lethality. Therefore, these data implied that the blue box region of the FERM domain is essential to merlin function. Interestingly, expression of ΔBB in the background of wild-type merlin had an antimorphic effect – increased wing cell proliferation. Co-expression of ΔBB and wild-type merlin in cultured cells resulted in retention of wild-type merlin at the membrane, where ΔBB was localized, further implicating this allele as a dominant-negative. In addition, expression of Mer^3, which harbored a missense mutation at Met^{177}, led to a significant reduction in the rescue of Mer^4 lethality, further proving the importance of these sequences for merlin function.

Interestingly, studies also revealed that expression of a truncation mutant of *Dmerlin* lacking the final 35 amino acids ($Mer^{1\text{-}600}$) rescued Mer^4 lethality even better than Mer^+. Furthermore, expression of shorter portions of the N-terminal region (aa 1-375, and aa 1-350) partially rescued lethality, whereas expression of C-terminal alleles provided no rescue. These data indicated that the growth suppressive activity of Dmerlin resides in the N-terminal half of the protein and that the very C-terminal residues of the protein likely have a negative regulatory function. Intriguingly, these studies are in contrast to observations with mammalian merlin

where the final amino acids of the protein were shown to be essential for merlin's ability to function as a growth suppressor (Sherman et al. 1997b).

Finally, more recent studies have revealed that the *Drosophila* band 4.1 member *expanded* interacts genetically with *Dmerlin* (McCartney et al. 2000). *Expanded*, which has no identified vertebrate homolog, exhibits a loss of function phenotype that is remarkably similar to merlin loss-of-function (Boedigheimer et al. 1993). Recessive loss of function of either *Merlin* or *expanded* dominantly enhanced the phenotypes associated with mutation in the other. Coordinate loss of function in both of these alleles revealed that they function together to regulate proliferation and differentiation. In addition, Merlin and expanded interact biochemically, as they colocalize in *Drosophila* tissues and cells and physically interact through the FERM domain of expanded and the C-terminal half of Merlin. Most recently, a screen to identify dominant second-site modifiers of merlin loss-of-function (or dominant-negative) phenotypes has revealed several candidates (LaJeunesse et al. 2001). The most interesting gene identified was *blistered (bs),* a *Drosophila* homolog of the serum response factor (SRF), which is required for differentiation of the adult wing and is regulated by the epidermal growth factor receptor (EGFR) signaling pathway (Guillemin et al. 1996; Montagne et al. 1996; Roch et al. 1998). Mutation in *blistered* led to an enhancement of the dominant-negative phenotype in ΔBB mutants, indicating that these two genes probably function in a common pathway in wing development. Interestingly, these and other data imply that merlin may function antagonistically in EGFR signaling in the fly. However, further studies are required in order to determine the significance of the genetic interactions identified by this screen. In summary, we have learned much about the function of merlin from genetic and biochemical studies in *Drosophila*. It is

likely that more insight into the molecular basis of NF2 will arise following avenues such as those described here.

Role for small GTPase proteins in regulating the cell cycle, actin cytoskeleton, and transformation

Ras

Ras is often referred to as a "universal relay molecule", as it is involved in integrating signals from diverse stimuli through transmembrane receptors to the nucleus. Ras activation can lead to diverse downstream effects, including proliferation or differentiation, depending upon the cell type and upstream activator (receptor) (Marshall 1995). The biological activity of Ras is highly regulated by a GDP/GTP cycle. Though the mechanisms are not entirely clear, the key process in the generation of Ras-GTP (the active form) occurs by stimulation of guanine nucleotide exchange following receptor stimulation. This exchange is catalyzed by a group of proteins termed guanine nucleotide exchange factors (GEFs). Conversely, Ras is inactivated through stimulation of its intrinsic GTPase activity via GTPase activating proteins (GAPs). In addition, full activation of Ras requires a post-translational farnesyl modification that targets it to the membrane (Hancock et al. 1990).

Ras mediates its effects on cell proliferation through the activation of a cascade of kinases (Figure 7). Such signaling cascades result in the translocation of a kinase into the nucleus that phosphorylates and stimulates the activity of a transcription factor(s) responsible for expression of genes that promote progression through the G1-to-S phase transition. The most well-studied of these signaling cascades downstream of Ras is the Raf/ MAP or ERK kinases (MEK)/ERK cascade (Marshall 1995). ERK1/2 translocates into the nucleus where it

phosphorylates the Ets family of transcription factors, e.g., Elk1, part of a ternary complex that binds the serum response element (SRE) causing the transcription of c-fos. In addition, ERK phosphorylates the c-jun transcription factor that together with c-fos constitutes the AP-1 transcription factor, which stimulates the transcription of many genes responsible for cell cycle progression, including cyclin D1. Hyperactivation of this pathway causes different downstream effects depending upon the cell type. Whereas in NIH3T3 fibroblasts, hyperactivation of MEK leads to transformation, in PC12 neuronal cells, hyperactivation of MEK leads to differentiation (Cowley et al. 1994).

There have been many studies providing much evidence for Ras effector pathways in addition to Raf. In addition to stimulation of ERK activity, activated Ras was shown to upregulate the JNK and p38 mitogen activated protein kinase (p38 MAPK) cascades (Minden et al. 1995; Olson et al. 1995). Because activation of Raf only affects ERK activity, it was assumed that another Ras effector must mediate the effects of JNK and p38 MAPK. Shortly thereafter, the Ras effector MEK kinase 1 (MEKK1) was identified and linked to the upregulation of JNK (Lange-Carter et al. 1993; Russell et al. 1995). Activation of Raf is also incapable of promoting all cellular functions of Ras activation, for example transformation of some epithelial cells (Oldham et al. 1996). In addition, Ras proteins with point mutations in the effector binding domains, thereby only allowing activated Ras to bind to one of its effectors, proved that the coordination of multiple pathways contribute to the complete cellular effect of Ras activation (White et al. 1995; Khosravi-Far et al. 1996).

Two important Ras effector pathways, the Ral guanine nucleotide dissociation stimulation (RalGDS) and phosphoinositide-3 kinase (PI3K) pathways, lead to various downstream effects, including cell cycle progression and cytoskeletal changes. RalGDS was

cloned in 1994 and found to be a GEF for the RalGTPase (Hofer et al. 1994; Spaargaren and Bischoff 1994), a protein involved in the regulation of phospholipase D (Jiang et al. 1995) and the promotion of cytoskeletal arrangements (Cantor et al. 1995). In 1994, PI3K, which stimulates the production of a second messenger by the catalyzing the conversion of PI-4,5-P2 (PIP2) to PI-3,4,5-P3 (PIP3), was identified as a Ras effector necessary for actin cytoskeletal arrangements downstream of Ras activation (Rodriguez-Viciana et al. 1994). Early studies revealed that increases in PI3K activity lead to an increase in the activity of the Rho GTPase, Rac, and its effect(s) on the cytoskeleton (Wennstrom et al. 1994; Hawkins et al. 1995). Later studies revealed that PI3K had a downstream target, protein kinase B (PKB)/Akt (Hemmings 1997), that was important for both the inhibition of apoptosis (Datta et al. 1997; del Peso et al. 1997) as well as activation of Rho and Ras GEFs (Han et al. 1998; Nimnual et al. 1998). Interestingly, these data place a Ras downstream effector, PI3K, upstream of Ras, as PI3K was shown to activate a Ras GEF. Although the molecular mechanisms by which these pathways function in the cell is still unclear, and there are indeed many more pathways involved then those discussed above, ongoing research will continue to reveal a complex web of interactions downstream of Ras activation. Recent studies demonstrate the existence of multifaceted macromolecular machines that are held together by scaffolding proteins and contain upstream activators, downstream effectors, regulators, and, perhaps, even final targets. Such macromolecular entities are likely targeted to specific subcellular locales where their activity translates into functional consequences.

Mutations in *Ras*, which make it insensitive to GAP activity, result in a protein that is constitutively active and is capable of transforming many cell types. In fact, highlighting the importance of this protein in the functioning of the cell, studies have revealed a high frequency

of *Ras* mutations in human cancer (Bos 1989). Not only is the *Ras* gene itself mutated, but several other factors along the Ras pathway have been associated with many human tumors. Deletion of GAP genes, such as the Neurofibromatosis type 1 (*NF1*) gene, results in constitutive activation of Ras, and, in the case of NF1, tumors of the peripheral nervous system (Bollag et al. 1996; Weiss et al. 1999). Growth factor receptor overexpression (or amplification) also results in increased signaling through the Ras pathway. Indeed, mutations in *EGFR* and *erbB2* have been found in breast carcinomas (Mendelsohn and Baselga 2000). Finally, mutation or amplification of Ras effectors has been found in many cancer types. For example, deletion of phosphatase and tensin homolog deleted on chromosome ten (*PTEN*), a phosphatase whose function, converting PIP3 to PIP2, opposes the function of PI3K, is detected in 30-40% of all human tumors (Simpson and Parsons 2001). The plethora of mutations in the Ras pathway identified in human tumors implies that proper regulation of this protein and its downstream effectors is of utmost importance for prevention of abnormal cellular growth.

The RhoGTPase family[§§]

Given the increasing importance of the RhoGTPase pathways, particularly Rac, in merlin function, a discussion of these proteins in cytoskeletal function, signaling and tumorigenesis is warranted. Much effort has been put towards determining the relative importance of various effector pathways in the control of cellular processes downstream of Ras activation (Figure 7). These studies reveal a complex situation with varying roles for different pathways depending upon the cell type. Much current research has focused on the Rho family of small GTPases and most studies have concluded that these proteins are key downstream targets of Ras (Van Aelst

[§§] Although the major RhoGTPases will be discussed in general terms, this section will heavily focus on Rac, because of its recently demonstrated importance in the regulation of and by merlin.

and D'Souza-Schorey 1997). In fact, the RhoGTPases, Rho, Rac and Cdc42 are all required for serum-induced G1 progression and Ras-mediated transformation, thereby highlighting their importance for regulation of cellular behaviors (Khosravi-Far et al. 1995; Olson et al. 1995; Qiu et al. 1995; Qiu et al. 1997). Although, interestingly, these proteins are only weakly transforming on their own, indicating that other effectors of the Ras pathway are needed for full transformation (Khosravi-Far et al. 1995; Roux et al. 1997). Indeed, expression of activated RhoGTPases and activated Raf together in fibroblasts leads to efficient cellular transformation (Khosravi-Far et al. 1995). Although it is uncommon, overexpression of some Rho proteins have been observed in cancers (Fritz et al. 1999), and overexpression of RhoC was associated with increased metastatic potential in a tumor model in mice (Clark et al. 2000).

The Rho family of proteins has been implicated in a variety of cell biological behaviors. The earliest studies implicated these proteins in cell morphology and the cytoskeleton, and it is likely that they are the major mediators of these effects downstream of Ras activation in the cell. However, it is clear that the RhoGTPases likely have a more expansive role in regulating gene expression, cell proliferation, and cell survival.

RhoGTPases and the actin cytoskeleton

The function of the RhoGTPase family of proteins began to be revealed early in the 1990s. Using Swiss3T3 cells, Ridley and colleagues demonstrated that Rho was required for the assembly of stress fibers and focal adhesions in response to growth factors (serum or lysophospatidic acid, LPA) (Ridley and Hall 1992). In addition, Rac was shown to regulate growth factor (PDGF) induced membrane ruffling in Swiss3T3s (Ridley et al. 1992). Importantly, these initial studies revealed a link between growth factor receptors and the

cytoskeleton via RhoGTPase signaling. Later studies revealed that Cdc42 was required for the assembly of filopodia at the periphery of the cell (Kozma et al. 1995; Nobes and Hall 1995). Experiments demonstrated a hierarchical relationship among the RhoGTPases in Swiss3T3 cells where activation of Cdc42 promotes activation of Rac which promotes activation of Rho (Nobes and Hall 1995). Although this hierarchy is not conserved in all cell types, there appears to be significant crosstalk between the different RhoGTPases in many different cellular contexts.

Finally, Nobes and Hall beautifully demonstrated that actin filaments found in Rac and Cdc42-induced lamellipodia and filopodia were associated with multimolecular focal complexes at the cytoplasmic face of integrin adhesion complexes, which were distinct from Rho-induced focal adhesions (Nobes and Hall 1995). Despite the fact that the function of these focal contacts is still unclear (Machesky and Hall 1997), the biological implications of these findings are broad ranging. Studies continue to indicate that Rho GTPases are important wherever actin is required to guide cellular processes. In fact, the Rho GTPases have already been implicated in many actin-driven processes, including, cell motility, axonal guidance, cytokinesis, and cell shape and polarity changes (Hall 1998).

Rho GTPases and gene transcription

The JNK/SAPK and p38 MAPK cascades are known to control gene transcription downstream of many cell stress stimuli, such as UV light and osmotic shock (Treisman 1996). Many groups have reported that Rac (and Cdc42) can activate these kinase pathways, much like Ras activates the Raf/MEK/ERK pathway (Coso et al. 1995; Minden et al. 1995). However, the exact role for RhoGTPases in JNK/p38 MAPK activation or the mechanisms by which this activation occurs are still largely unknown. In addition, the Rho proteins have been reported to

activate SRF- and NFκB-dependent transcription. As mentioned above, the Rho proteins are required for progression through the G1/S-phase transition of the cell cycle, and for transformation downstream of activated Ras. However, there is much debate as to whether the requirement of the Rho proteins for these processes is a result of their downstream effects on the actin cytoskeleton and integrin adhesion or more direct effects on gene transcription (Yamamoto et al. 1993; Olson et al. 1995; Joneson et al. 1996; Lamarche et al. 1996).

Regulation of the Rho GTPases

As with Ras, the Rho proteins are controlled via a GDP/GTP cycle. Over 35 GEFs and many GAP proteins have been identified that function to regulate the activity of the Rho proteins (Whitehead et al. 1997). Some GEFs have been shown to function broadly as regulators for all of the Rho proteins, but others have been identified that specifically regulate only one Rho protein. In addition, the Rho family of proteins are negatively regulated by other proteins, RhoGDIs, which function to retain inactive GDP-bound Rho proteins in the cytosol (Fukumoto et al. 1990; Ueda et al. 1990). Additional experiments suggested that RhoGDI can also bind the GTP-bound form of the Rho proteins, thereby indicating a role for this protein in both regulation of GTP exchange and in its hydrolysis (Hart et al. 1992; Chuang et al. 1993).

The activation of the Rho proteins is spatially and temporally controlled, and it is thought that the GEFs may play a major role in such controls. All RhoGEFs share two common motifs: a Dbl homology (DH) domain which specifies their exchange activity and a plecstrin homology (PH) domain whose function is less well-defined. The PH domain may mediate GEF binding to phospholipids thereby affecting subcellular localization and/or it may regulate the exchange activity via intramolecular associations. Given that GEFs have only been characterized as

performing a single task catalyzing GTP/GDP exchange, it is puzzling why there are so many Rho GEFs. Interestingly, only 2 GEFs have been identified for Ras. Studies with the Rac-specific GEF, T-cell invasion and metastasis gene, Tiam1, have begun to answer some of these questions (Michiels et al. 1995). Tiam activation of Rac resulted in poor induction of JNK activity but significant induction of the activity of a downstream effector, Pak (Zhou et al. 1998). However, overexpression of activated Rac stimulates the activities of both of these kinases (Coso et al. 1995; Minden et al. 1995). Therefore, perhaps GEFs provide both spatial (through their PH domain) and temporal (downstream of growth factor activation) regulation of the RhoGTPases which might help to specify directed signaling toward different components of downstream pathways. It is likely that further studies of this nature will continue to reveal specificity of GEF regulation.

Rac has been shown to be activated by Ras-dependent (Ridley et al. 1992; Rodriguez-Viciana et al. 1997) and independent mechanisms (Nobes and Hall 1995) downstream of growth factor activation. A study revealed that wortmannin, a PI3K inhibitor, prevented the formation of membrane ruffles in cells following PDGF stimulation, thereby indicating a role for PI3K upstream of Rac activation of ruffling (Wennstrom et al. 1994). It is thought that PI3K links growth factor receptors (through its p85 subunit) to activation of Rac through its production of PIP3 and the recruitment of GEFs via their PH domain. Specifically, PI3K activation was shown to recruit vav (Han et al. 1998), a broad RhoGEF, and sos (Nimnual et al. 1998), primarily a RasGEF, but also shown to be active for Rac when found in a ternary complex (Scita et al. 1999). Additionally, Tiam1 was identified as a GEF specific for Rac, as it was able to promote GTP loading on Rac *in vitro* and membrane ruffling when overexpressed in cells in culture (Habets et al. 1994; Michiels et al. 1995). Activation of Tiam1 was induced upon its localization

to the membrane, which occurred presumably through the binding of its PH domain to the lipid products of PI3K (Michiels et al. 1997; Sander et al. 1998).

Rho GTPases and cell-matrix adhesion

In addition to growth factors, normals cells depend upon adhesion to the extracellular matrix for growth survival and differentiation (Guadagno and Assoian 1991). The effects of the extracellular matrix on cellular behavior are mediated by the integrin adhesion receptors, a large class of heterodimeric integral membrane proteins that bind to various extracellular matrix molecules. Binding of integrins to the extracellular matrix (ECM) results in intracellular, "outside-in" signaling and the activation of the Rho GTPases (Price et al. 1998; Ren et al. 1999). Conversely, the activities of the Rho GTPases can affect the ECM-binding activity of the integrins through Rho protein effects on the cytoskeleton, termed "inside-out" signaling. Much of this signaling occurs in the context of macromolecular complexes at the cytoplasmic face of points of adhesion, such as focal contacts and focal adhesions (Nobes and Hall 1995). The components of these adhesion complexes are under intense investigation, but likely consist of Rho GTPases, RhoGEFs, kinases, such as focal adhesion kinase (FAK), adaptors, such as paxillin, and actin-binding proteins, such as vinculin and talin. Therefore, cell attachment can modulate the activities of the RhoGTPases in response to serum growth factors. Interestingly, the promotion of G1 progression by Rac correlates with its membrane ruffling activity, rather than its activation of signaling cascades, such as JNK (Joneson et al. 1996; Lamarche et al. 1996). Therefore, these data indicate that cell adhesion likely triggers the levels of Rac activation required for a cell cycle response. In fact, in endothelial cells, the coordination of growth factors and the binding of integrins to fibronectin caused activation of Rac via sos and

ERK-independent post-transcriptional upregulation of the levels of the cell cycle control protein, cyclin D1 (Mettouchi et al. 2001). In addition, del Pozo and colleagues revealed that the activation of Rac by serum was severely attenuated when cells were placed in suspension (del Pozo et al. 2000). In suspended cells, Rac was not properly localized to the membrane and therefore was demonstrated to be unable to bind to its effector Pak (del Pozo et al. 2000). In summary, linkages between integrins and the ECM are important for modulating the efficiency of growth factor signaling through the GTPases. Deregulation of ECM-integrin-GTPase signaling can result in adhesion-independent proliferation, a hallmark of transformed cells.

Effectors of Rho proteins

Significant effort has been placed into experiments designed to determine the mechanisms by which activated RhoGTPases mediate their diverse downstream effects. Yeast two-hybrid experiments have been very useful for identifying Rac, Cdc42 and Rho interacting proteins. Rac and Cdc42 have been shown to interact with proteins containing a conserved Cdc42/Rac interaction binding (CRIB) domain, a motif which exists in many, but not all of their effectors (Burbelo et al. 1995). The most common mechanism of effector activation by Rac is through its binding and disruption of intramolecular autoinhibitory interactions in the effector protein leading to the exposure of functional domains. Effector proteins have been characterized which carryout Rho-dependent activities on the actin cytoskeleton and that mediate actin-independent activities of the RhoGTPases. Although many effector proteins have been identified to date, their roles remain largely unknown. Here, I will highlight some of the best studied effectors that regulate both actin-dependent and independent cellular behaviors.

Probably one of the best studied effectors is the Rac/Cdc42 specific effector p21 activated kinase (Pak)*** (Manser et al. 1994). The interaction between Pak and Rac/Cdc42 is mediated through the CRIB domain of Pak. Pak mediates many effects on the actin dynamics, motility and adhesion via phosphorylation of proteins important for actin polymerization and focal complex formation (Edwards et al. 1999; Sanders et al. 1999). In addition, Pak may mediate some Rac-dependent effects on the cell cycle via JNK, although this activity is heavily debated (Bagrodia et al. 1995b; Zhang et al. 1995; Brown et al. 1996; Teramoto et al. 1996b; Tapon et al. 1998). Phosphatidyl inositol 4-phosphate, 5-kinase (PI-4,5-K) has been identified as a Rac target that is likely to play a central role in actin polymerization (Hartwig et al. 1995). The product of this kinase, PIP2, is known to affect actin filament assembly, as it is essential for the release of capping proteins from the ends of actin filaments, an activity that is required for actin polymerization (Hartwig et al. 1995). Additionally, IQGAP is a scaffolding effector of both Rac and Cdc42 which may regulate cell-cell adhesion through actin polymerization and sequestration of the adherens junction (AJ) protein, β-catenin. Oligomerization of IQGAP has been shown to crosslink F-actin in vitro, an activity that requires Cdc42-GTP (Erickson et al. 1997; Fukata et al. 1997). In addition, free IQGAP sequesters β-catenin away from α-catenin and other AJ proteins, which leads to destabilized AJs (Kuroda et al. 1996). Therefore, Rac and Cdc42 may play a role in the maintenance of cell-cell contacts by binding to IQGAP preventing it from sequestering β-catenin away from AJs (Kuroda et al. 1999).

Early studies revealed that activation of Rac led to increases in oxygen radical production in the cell (Knaus et al. 1991). This effect was later shown to be mediated through Rac regulation of the NADPH oxidase complex, a specialized enzyme of phagocytic cells that

*** The importance of Pak signaling will be addressed in detail later in the introduction.

generates oxygen radicals (ROS) to kill internalized microorganisms (Chanock et al. 1994). Rac mediates its regulation of this complex through binding and unmasking the catalytic domain of $p67^{phox}$, a cytoplasmic component of the NADPH complex (Abo et al. 1991; Diekmann et al. 1994; Nisimoto et al. 1997). Interestingly, although it was first assumed that the Rac-NADPH connection was specific to the function of phagocytic cells, later studies revealed a broader importance for this connection, as production of ROS by Rac correlates with upregulation of NFκB transcription factor activity (Sulciner et al. 1996). Finally, Rac (and Cdc42) likely interact with a number of effectors to mediate upregulation of JNK and p38 MAPK. Mixed lineage kinases (MLKs) and MEKKs have been shown to interact with Rac through their CRIB domains and strongly upregulate JNK activity (Teramoto et al. 1996a; Fanger et al. 1997; Nagata et al. 1998). In fact, expression of a dominant-negative MEKK leads to a significant decrease in Rac-mediated JNK activation (Fanger et al. 1997). In summary, there are many effectors that mediate various activities downstream of activation of the RhoGTPases. The significance and contribution of each of these pathways in the control of cytoskeletal and cell cycle behaviors is far from being understood. Elucidation of the biochemical pathways that underlie Rho GTPase activation will have a significant impact on the understanding of normal cellular behaviors as well as aberrant cellular activities, such as those leading to cancer.

Pak

The p21-activated kinases (Pak 1, 2, and 3; group I Paks) are serine/threonine kinases which are molecular targets for the small GTP binding proteins Rac and Cdc42 (Jaffer and Chernoff 2002). Members of the Pak family are highly similar to the Ste20 protein kinase, which participates in the pheromone/mating factor signaling pathway in budding yeast (Leberer

et al. 1997). Currently, there are six identified Pak proteins that vary in their tissue expression patterns (Manser et al. 1994; Abo et al. 1998; Sells et al. 1999; Dan et al. 2002; Lee et al. 2002). Extensive study on the group I Paks, Paks 1-3, has revealed their important roles in actin cytoskeleton reorganization, cell motility, gene expression, and more recently, apoptosis (Kumar and Vadlamudi 2002). Less is understood about the group II Paks, Pak 4-6, which have been demonstrated to have both overlapping and distinct roles from the group I Paks. Sequence divergence, protein structural differences, and differences in regulation by the Rac/Cdc42 GTPases, indicate that these two groups may differ in their recruitment and activation of downstream effectors, which may specify their function.

Interestingly, growing evidence has implicated expression of activated group I Paks in anchorage-independent growth and invasiveness, for example, in human mammary epithelial cancer cells (Bagheri-Yarmand et al. 2001). In addition, studies using dominant-negative alleles of Pak demonstrate that Pak1 is necessary for high efficiency transformation downstream of Ras, but that Pak activation alone is not sufficient for transformation (Tang et al. 1997; Tang et al. 1998; Tang et al. 1999). In certain cell types, Ras activates Pak, and Pak potentiates signaling through ERK (Tang et al. 1999; Chaudhary et al. 2000; Eblen et al. 2002). Note, however, that the role of Paks 1-3 in transformation remains under debate (Joneson et al. 1996; Lamarche et al. 1996; Westwick et al. 1997). Pak inhibition of Ras transformation seems to be very cell-type-specific with most observations being recorded in Rat1 fibroblasts (Tang et al. 1997). Surprisingly, there seems to be no link between Ras and Pak activation in NIH3T3 cells (Tang et al. 1998). Finally, recent studies demonstrate that expression of Pak 4 (group II) is found elevated in many human tumor cell lines (Callow et al. 2002) and Pak 4 is the only family

member thus far that alone will induce cellular transformation when overexpressed as an activated kinase (Qu et al. 2001).

Pak activity is regulated by a variety of mechanisms. Structural studies indicate that, in the absence of G protein binding, group I Pak kinase activity is repressed by an intramolecular interaction between N-terminal regulatory and C-terminal catalytic domains. Rac/Cdc42 binding to the N-terminal CRIB (Cdc42/Rac interacting binding) domain of the Paks releases this repression and allows for autophosphorylation (Thr423) of the C-terminal catalytic domain and, thus, kinase activation in an adhesion-dependent manner (Zenke et al. 1999; del Pozo et al. 2000; Lei et al. 2000). In addition to activation by small G proteins, Pak has been shown to be regulated by lipids, such as sphingosine (Bokoch et al. 1998), and adaptor protein/kinase complexes, such as paxillin, Nck, a GEF, beta-Pak interacting exchange factor (βPIX), and a GAP, p95 paxillin kinase linker (p95PKL), and that act by recruiting Pak from the cytoplasm to focal adhesions at the cell membrane (Manser et al. 1998; Turner et al. 1999; Brown et al. 2002). Stimulation through receptor tyrosine kinases as well as sensing of adhesion via integrins can affect Pak recruitment to the membrane, and, thus, its activation (Hashimoto et al. 2001).

Pak has many downstream effectors which mediate its regulation of different cellular processes. Pak exerts its function on cytoskeletal dynamics through a variety of known actin remodeling proteins. Activation of Pak leads to phosphorylation and activation of LIM kinase (LIMK) which phosphorylates and inactivates the actin depolymerizing protein, cofilin, leading to an inhibition of actin depolymerization and increased membrane ruffling (Edwards et al. 1999). Active Pak phosphorylates and inactivates myosin light chain kinase (MLCK) resulting in a decrease in phosphorylated MLC, which results in the disassembly of stress fibers and focal adhesions and a likely increase in cell motility (Sanders et al. 1999). In addition, filamin

(Vadlamudi et al. 2002) and paxillin (Hashimoto et al. 2001), both signaling scaffolds for cytoskeletal rearrangements, are regulated Pak phosphorylation and binding. Finally, Pak promotes gene transcription and cell survival by directly phosphorylating kinases of the Raf/MEK/ERK signaling cascade, including Raf (Chaudhary et al. 2000) and MEK (Frost et al. 1997), by phosphorylation and inactivation of the pro-apoptotic protein, Bad (Schurmann et al. 2000), and by upregulating the activities of JNK and the NFκB transcription factor (Bagrodia et al. 1995a; Zhang et al. 1995; Frost et al. 2000).

Cell cycle progression through regulated expression of cyclin D1

In order for a cell to change from a normal to a transformed state, dysregulation of the cell cycle must occur. Cyclin D1, whose expression oscillates throughout the cell cycle, is a key factor that regulates G1/S transitions. Transcription of cyclin D1 is induced downstream of growth factor stimulation (Matsushime et al. 1991). Later studies revealed that growth factor-induced cyclin D1 transcription is dependent upon Ras (Aktas et al. 1997). Indeed, upregulation of cyclin D1 levels are necessary for Ras transformation (Filmus et al. 1994; Liu et al. 1995). However, cyclin D1 overexpression is not sufficient to mediate Ras-induced transformation (Liu et al. 1995; Pruitt et al. 2000). Multiple elements in the cyclin D1 promotor have been identified that facilitate both Raf-dependent and Raf-independent stimulation of transcription. Although the Raf/MEK/ERK pathway is important for the stimulation of cyclin D1 transcription via the Ets-2 transcription factor (Albanese et al. 1995; Lavoie et al. 1996; Cheng et al. 1998), reports indicate that many pathways are important, as Raf pathway stimulation of cyclin D1 is not sufficient for the sustained levels required for cell cycle progression (or transformation) (Gille and Downward 1999). Indeed, the JNK pathway, the RalGDS pathway, and the PI3K pathway

have all been shown to be important for transcriptional regulation of cyclin D1 (Albanese et al. 1995; Henry et al. 2000; Pruitt et al. 2000). In addition, post-transcriptional regulation of cyclin D1 occurs downstream of PI3K activation through enhanced translation of cyclin D1 mRNA (Muise-Helmericks et al. 1998) and increased stability through Akt inhibition of glycogen synthase kinase 3 beta (GSK3β), which phosphorylates cyclin D1 priming it for degradation (Diehl et al. 1998).

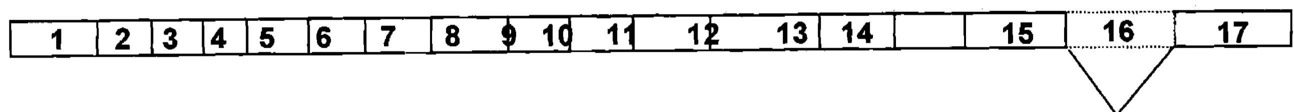
The RhoGTPases are important for the progression of the cell through the G1 phase in response to growth factors (Olson et al. 1995). Early studies showed that the RhoGTPases could upregulate the activity of several transcription factors, including SRF and NFκB, both of which are known to regulate cyclin D1 transcription (Hill et al. 1995; Perona et al. 1997). Indeed, activated Rac leads to an increase in cyclin D1 levels (Westwick et al. 1997), and dominant-negative Rac inhibits Ras induction of increased cyclin D1 levels (Gille and Downward 1999). Rac has been demonstrated to regulate the transcription of cyclin D1 by upregulation of various transcription factors through many upstream pathways. In one report, induction of AP-1 activity downstream of Rac was shown to be dependent upon JNK (Westwick et al. 1997), and in other reports Rac stimulation of Pak leads to an upregulation of the Raf/MEK/ERK pathways and increases in AP-1 and Ets-2 transcription factor activities (Frost et al. 1997). Rac has also been demonstrated to upregulate the activity of the NFκB transcription factor, which binds to sites on the cyclin D1 promoter (Sulciner et al. 1996). Rac can mediate its upregulation of this transcription factor in many ways: via its effector, Pak (Joyce et al. 1999; Frost et al. 2000), via activation of inhibitor of kappa B (IκB) kinase (IκK) which results in inhibition of NFκB activity (Cammarano and Minden 2001), or via production of reactive oxygen species (Sulciner et al. 1996; Page et al. 1999). Finally, recent studies reveal that Rac can affect cyclin D1 levels via a

post-transcriptional mechanism following integrin-ECM engagement (Mettouchi et al. 2001). The regulation of cyclin D1 downstream of Ras and RhoGTPase activity is far from being understood, and the relative importance of each of the pathways to the resulting cyclin D1 transcription downstream of GTPase activation is currently unknown.

Perspectives

Based on its localization and binding partners, the Neurofibromatosis type 2 tumor suppressor protein, merlin, likely functions at the interface of the membrane and cytoskeleton. Loss of *NF2* expression in humans, mice and flies leads to hyperplasia. Loss of merlin expression (or expression of merlin mutants) in cultured cells leads to cytoskeletal defects associated with contact inhibition and loss of cell adhesion. Recent data implicating Rac-dependent Pak regulation of merlin and, conversely, merlin regulation of Rac activity likely places this molecule at the center of many macromolecular complexes important for the regulation of cell adhesion, cell-cell contact and cytoskeletal rearrangements downstream of growth factor stimulation and integrin engagement. However, the detailed mechanisms by which merlin controls cell proliferation have yet to be identified. Here, I present data that expression of a dominant-negative allele of merlin ($Nf2^{BBA}$), based on homology to *Drosophila* blue box mutants, causes complete cellular transformation of fibroblasts. Therefore, as in flies, the amino acids encoded by the blue box in mammals are critical for proper merlin function. These data and my demonstration of the effects of loss of *Nf2* expression in primary mouse embryo fibroblasts confirm the importance of merlin in regulating cell proliferation, contact inhibition and cell adhesion. In addition, I show that expression of the $Nf2^{BBA}$ allele of merlin leads to drastic upregulation of cyclin D1 protein and transcript levels, and that elevated cyclin D1 levels

are necessary for transformation downstream of BBA merlin expression. However, the mechanism(s) by which disregulated merlin leads to cyclin D1 upregulation remains elusive, as many signaling pathways normally associated with cyclin D1 upregulation are normally regulated in these cells. Secondly, I show that merlin mediates its inhibition of the Rac pathway downstream of Rac, through a direct interaction with Pak. I demonstrate that the interaction is mediated between the FERM domain of merlin and the CRIB domain of Pak. Loss of merlin expression leads to upregulation of Pak activity, and, conversely, overexpression of merlin leads to downregulation of Pak activity. Finally, I explore the effect of merlin overexpression on the activity of downstream effectors of Pak in an effort to identify specific pathways that are regulated by merlin in the cell.



Isoform 2 (exons 1-16)

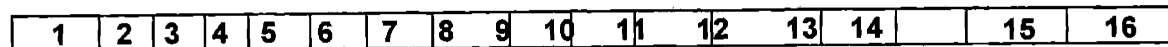


Figure 1. Schematic diagram of the two major isoforms of *NF2*. *NF2* has 17 exons. Isoform I is the result of alternative splicing around exon 16, resulting in a 595 amino acid protein. This isoform has been demonstrated to be capable of self-association. Isoform II lacks the splicing of exon 16, and, thus, terminates with a stop codon within exon 16. The isoform II encoded protein is 590 amino acids. Isoform II cannot form head-to-tail associations. Therefore, it is thought that the ability to self-associate requires sequences encoded by exon 17.

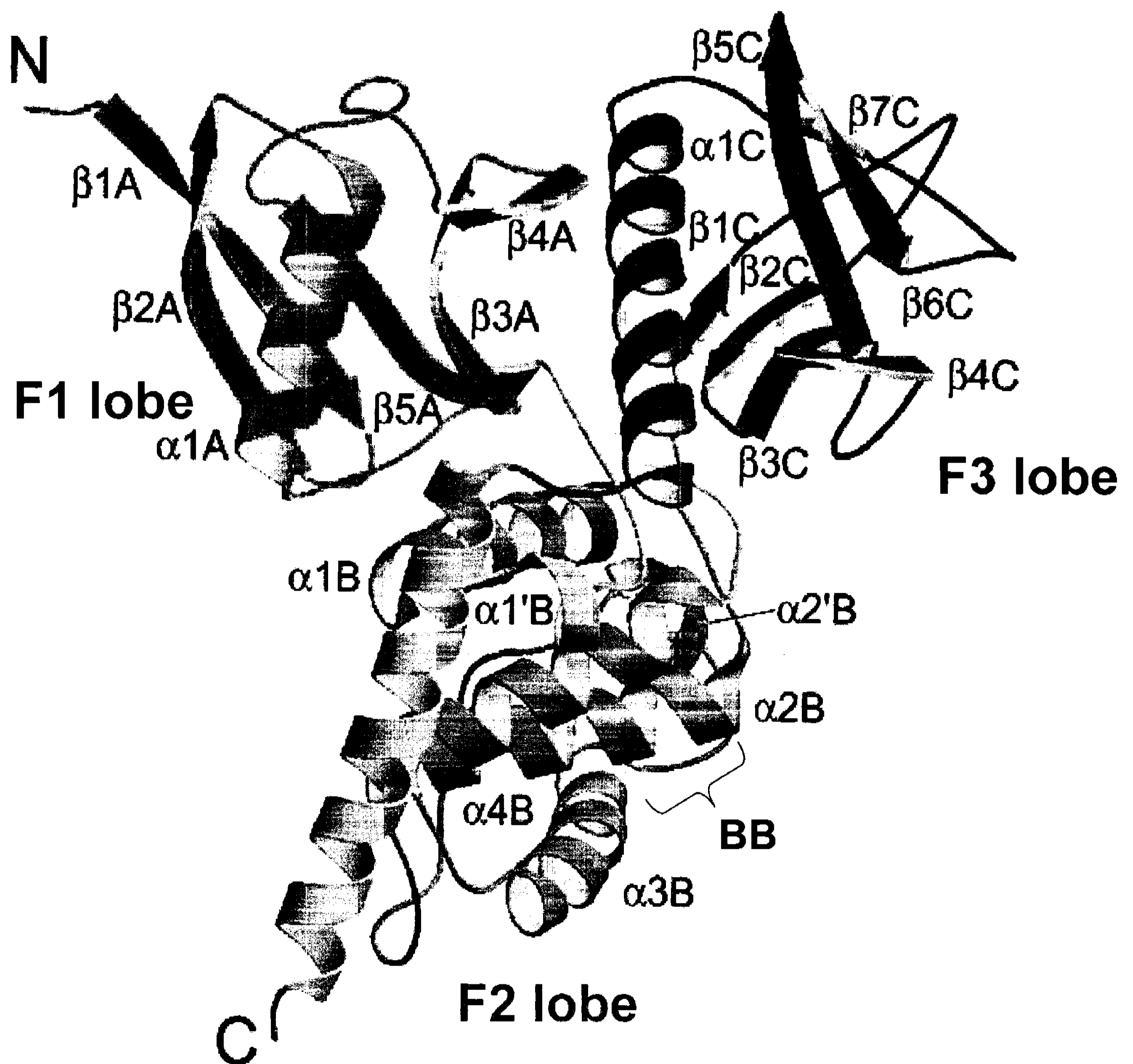


Figure 2. Merlin FERM domain structure. Schematic representing the structure of the merlin FERM domain based on crystal structure studies. The three lobes of the "cloverfleaf structure" are indicated F1, F2, and F3. BB: blue box domain; N: amino terminus; C: carboxy terminus; α: alpha helical region; β: beta-sheet region. (Adapted from Shimizu et al., 2002.)

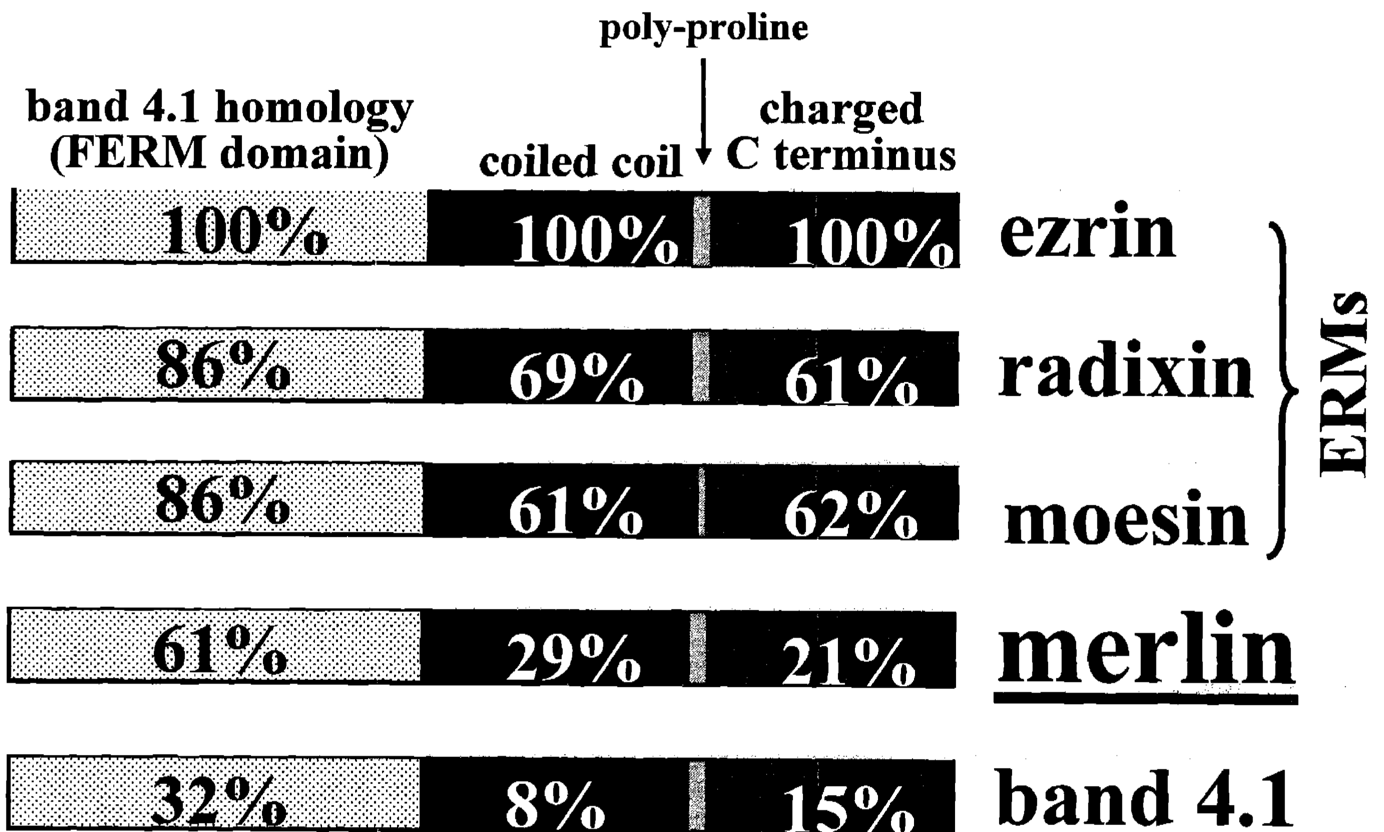


Figure 3. The band 4.1 family of proteins. Schematic diagram of the essential domains of merlin, the ERM proteins, and band 4.1. Percent identity to ezrin, the prototypical ERM, is indicated for each of the subdomains. Note: the highest degree of identity falls in the amino-terminal FERM domain.

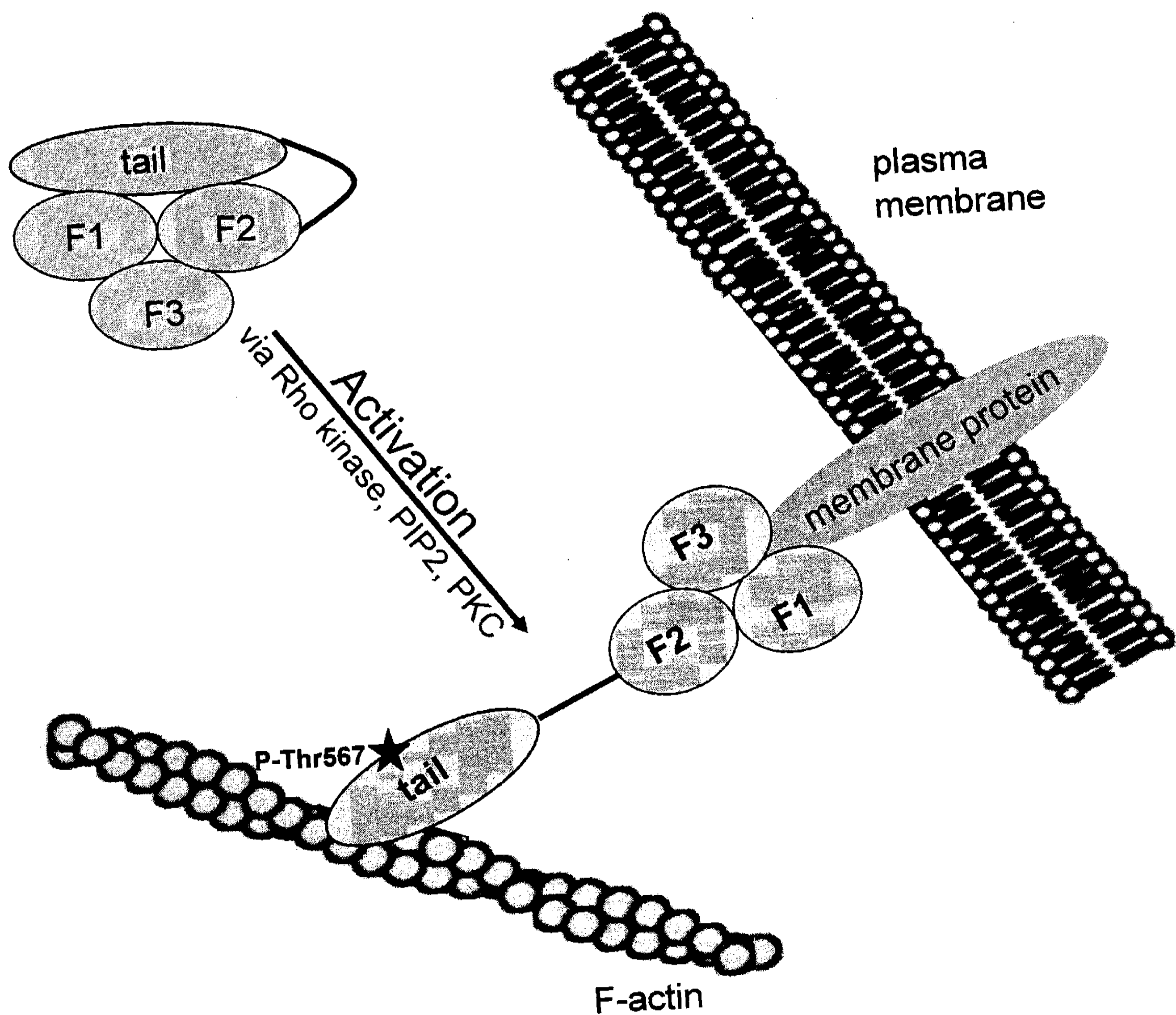


Figure 4. Regulation of the ERMs. Schematic depiction of the mechanism of ERM function as a membrane-cytoskeletal linker protein. Here, ezrin is shown in an inactive state, in which the trilobed FERM domain is bound to the C-terminal tail, in the cytosol. Rho kinase, PIP2 or PKC activates ezrin by phosphorylating (★) a C-terminal threonine residue (Thr567) leading to opening of the molecule. The opening of ezrin reveals binding sites in the FERM domain for membrane proteins and a C-terminal actin binding site, allowing ezrin to link the membrane protein to the cytoskeleton. F1, F2, F3 represent the three different lobes of the trilobed FERM domain.

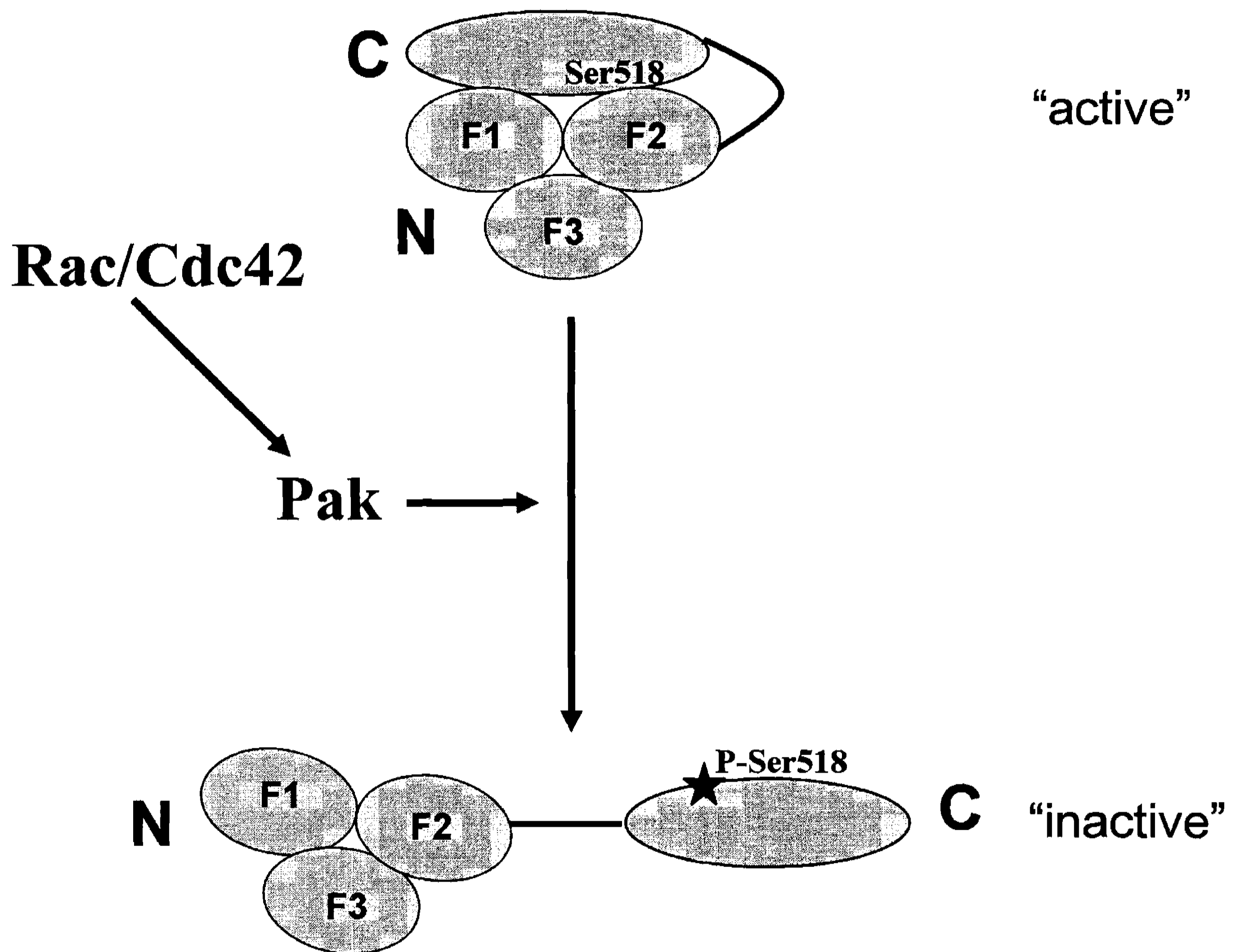


Figure 5. Regulation of merlin. Schematic diagram of Rac-dependent regulation of merlin. Merlin exists in a hypophosphorylated, closed, active conformation until it is phosphorylated by Pak downstream of Rac or Cdc42 activation. Phosphorylation (★) occurs on a C-terminal serine residue (Ser518) of merlin leading to opening and inactivation of the molecule. F1, F2, F3 represent the three different lobes of the trilobed FERM domain.

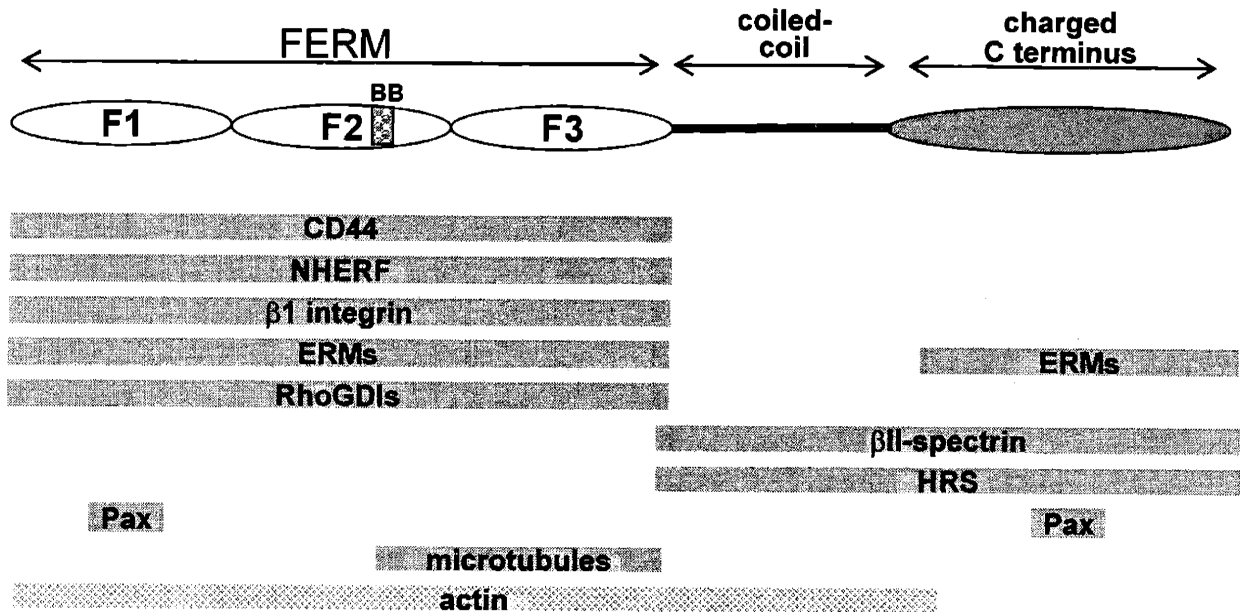


Figure 6. Merlin binding partners. Illustration of the relative location of binding of several merlin binding partners. Interestingly, most proteins interact with the N-terminal half of merlin. F1, F2, F3 represent the three different lobes of the trilobed FERM domain. BB indicates the location of the conserved blue box domain.

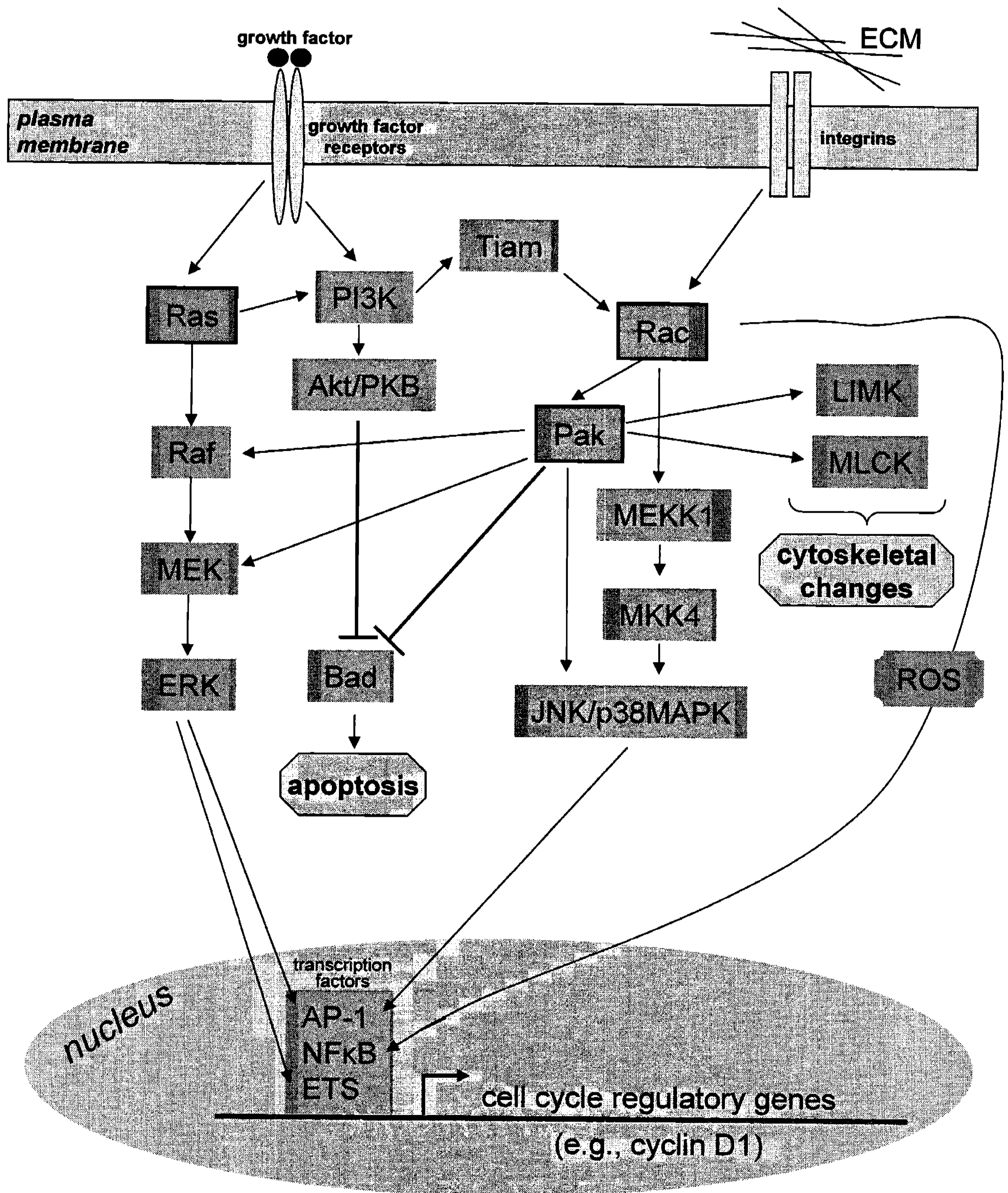


Figure 7. Ras/Rac/Pak pathways in the cell downstream of growth factor stimulation and cell-matrix adhesion. Simplified diagram of the Ras and Rac pathways leading to cell cycle progression. Molecules highlighted here are those that are important for the work presented in this thesis. Note, however, that there are many more effector proteins and pathways downstream of Ras and Rac that are not shown. It is evident from this *simplified* diagram that these pathways are extremely complex and much crosstalk occurs between kinases at various levels. ROS: reactive oxygen species; ECM: extracellular matrix.

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Chapter 2

Cellular transformation by a FERM domain mutant of the *Nf2* tumor suppressor gene

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Abstract

Mutations in the *Nf2* tumor suppressor gene lead to tumor formation in humans and mice and cellular overproliferation phenotypes in *Drosophila*. The *Nf2*-encoded protein, merlin, shares close sequence similarity in its amino terminus to members of the band 4.1 family of membrane-cytoskeletal linkers. Similarities between merlin and this family suggest a role for merlin in regulating cytoskeletal function. However, the mechanism of the tumor-suppressing activity of merlin is not yet understood. Mutational analysis of *Nf2* in flies has lead to the identification of a dominant-negative allele, which harbors mutations in the amino terminus of the protein. Here, we report that expression of a murine analog of this amino-terminal mutant of *Nf2* leads to complete transformation of NIH3T3 fibroblasts in culture. Cells that express this *Nf2* mutant allele display disruptions of the actin cytoskeleton, lack of contact inhibition of growth, and anchorage-independent growth. Finally, fibroblasts that express this mutant *Nf2* allele form tumors when injected into nude mice.

Introduction

Neurofibromatosis type 2 (NF2) is an inherited autosomal dominant disorder in which patients typically develop bilateral vestibular schwannomas and other tumors of the central nervous system (CNS) (Martuza and Eldridge, 1988). The *Nf2* gene was positionally cloned in 1993 and identified as a tumor suppressor gene found mutated in the germline of NF2 patients as well as in many sporadic tumors, including meningiomas, schwannomas and ependymomas (Rouleau *et al.*, 1993; Trofatter *et al.*, 1993; Twist *et al.*, 1994). Mice heterozygous for a mutation in *Nf2* were created in an effort to model the human disease. These animals do not develop CNS tumors but rather a range of highly metastatic sarcomas, which have undergone loss of heterozygosity at the *Nf2* locus (McClatchey *et al.*, 1998). Targeted mutation of the mouse *Nf2* gene in Schwann cells induces schwannomas (Giovannini *et al.*, 1999; Giovannini *et al.*, 2000).

The *Nf2* protein, merlin, is related to the band 4.1 family of membrane-cytoskeletal linkers (Rouleau *et al.*, 1993; Trofatter *et al.*, 1993). Within this family, merlin is most similar to ezrin, radixin, and moesin (the ERM proteins), which are implicated in cytoskeletal remodeling and formation of membrane ruffles and microvilli (Franck *et al.*, 1993). Sequence similarity between the ERM proteins and merlin lies primarily in the conserved N-terminal FERM (4.1, ERM) domain. Merlin shares certain properties with the ERM family, including subcellular localization to cortical actin structures and binding to the integral membrane hyaluronic acid receptor CD44 (Sainio *et al.*, 1997; Tsukita *et al.*, 1994). However, despite some sequence and functional overlap, it is clear that merlin has distinct properties not shared with the ERM proteins.

Similarities between merlin and the ERM proteins suggest that the growth-regulatory capabilities of merlin may be due to effects on cytoskeletal organization and/or function. A number of laboratories have demonstrated that overexpression of wild-type merlin can inhibit cell proliferation, reduce cell motility, and disrupt the actin cytoskeleton during cell adhesion and spreading (Gutmann *et al.*, 1999b; Lutchman and Rouleau, 1995; Sherman *et al.*, 1997). Studies using a rat schwannoma model system suggest that merlin helps to mediate contact inhibition of growth through interactions with the cytoplasmic tail of CD44 and signals from the extracellular matrix (Morrison *et al.*, 2001). These studies and the highly metastatic phenotype of tumors in *Nf2* +/- mice suggest a role for merlin in cell-cell and cell-matrix contacts, most likely associated with the processes of cell migration or invasion, and argue that merlin may regulate intracellular pathways important for both proliferation and actin cytoskeleton processes.

In order to elucidate the role of merlin in tumorigenesis, the effects of loss of merlin function both on an organismal level and in tissue culture have been examined. Several groups have attempted to create dominant-negative alleles of merlin by deleting regions of the conserved FERM domain. Giovannini and colleagues created a transgenic mouse with a mutant allele of *Nf2* in which exons 2 and 3 are deleted (Giovannini *et al.*, 1999). This allele shows a dominant effect on Schwann cell proliferation: the presence of one mutant *Nf2* allele (without further loss of the wild-type *Nf2* allele) promotes tumorigenesis in these transgenic animals. In tissue culture, Koga and colleagues show that nuclear microinjection of a mutant *Nf2* cDNA lacking exon 2 sequences alters subcellular localization of merlin and induces loss of adhesion in an SV40-transformed fibroblast cell line (Koga *et al.*, 1998). Importantly, deletions of exons in the FERM domain of *Nf2* are found in human NF2 patients and have been associated with early tumor onset and poor prognosis (Ruttledge *et al.*, 1996).

Significant information regarding *Nf2* function has come from studies in *Drosophila*. LaJeunesse and colleagues have made a dominant-negative allele of *Drosophila Merlin* (Mer) by deleting (ΔBB) or substituting alanine (BBA) for a seven amino acid stretch in the FERM domain (designated "blue box", a region which is completely conserved between fly and mammalian merlin, but not in the ERM proteins) (LaJeunesse *et al.*, 1998). When these "blue box" mutants ($Mer^{\Delta BB}$ or Mer^{BBA}) are overexpressed in the fly wing, the same phenotype is observed as with somatic loss of function of merlin: overproliferation of wing epithelial cells. Functional studies suggest that these alleles interfere directly with the activity of wild-type merlin protein. These findings, combined with those discussed above, suggest that mutations in the FERM domain of merlin may disrupt cell-to-cell or cell-to-matrix interactions resulting in changes in cell proliferation, morphology, and motility.

Following these studies and the identification of the highly conserved "blue box" region of the *Drosophila* Merlin FERM domain as potentially critical to its role as a growth suppressor, we studied the equivalent mutant in the context of mammalian *Nf2*. Expression of a murine "blue box" mutant cDNA, $Nf2^{BBA}$, in NIH3T3 fibroblasts in tissue culture caused complete transformation of the cells resulting in actin cytoskeleton abnormalities, loss of contact inhibition, growth in soft agar and tumor formation in nude mice.

Results

Expression of Nf2^{BBA} in NIH3T3 cells causes adhesion defects

In order to examine the effects of the BBA mutation (alanine substitution of codons 177-183 in murine *Nf2*) in mammalian cell culture, NIH3T3 cells were infected with recombinant retroviruses carrying the wild-type *Nf2* cDNA, mutant *Nf2* cDNA, or an empty vector as a control. Infected cell populations were designated 3T3-BABE, 3T3-Nf2^{WT}, and 3T3-Nf2^{BBA}, for NIH3T3 cells infected with an empty-vector control, wild-type *Nf2*, and *Nf2* "blue box" mutant (BBA). After 3 days of selection in puromycin, cell lysates were prepared to assay for merlin overexpression. Both wild-type merlin (Nf2^{WT}) and FERM domain "blue box" mutant merlin (Nf2^{BBA}) were overexpressed compared to endogenous merlin in the empty-vector (BABE) control cells (Figure 1). Overexpression levels of merlin in wild-type and mutant infected NIH3T3 cells were 5-10-fold higher than endogenous merlin levels in vector control cells. Infected NIH3T3 cells were used one passage immediately following selection for all the experiments described below.

NIH3T3 cells expressing Nf2^{BBA} displayed an extreme deficiency in cell adhesion. Most notably, at high cell densities, these cells would detach from the plate, making any further analysis difficult. To promote the attachment of these cells, fibronectin-coated tissue culture plates were employed. Initial characterization indicated that 3T3-Nf2^{BBA} cells remained adherent at all cell densities following this treatment. Thus, all infected cells for all described experiments were plated on fibronectin-coated plates.

3T3-Nf2^{BBA} cells have abnormal shape and a poorly organized actin cytoskeleton

Staining with Oregon Green-phalloidin to examine the actin cytoskeleton revealed that

3T3-Nf2^{BBA} cells had a very poorly organized cytoskeleton when compared to 3T3-BABE and 3T3-Nf2^{WT} cells, which possessed cytoskeletons with well-organized actin stress fibers (Figure 2). In addition, as visualized under immunofluorescence and phase light microscopy, 3T3-Nf2^{BBA} cells were found to have small cell bodies with many protrusions that tended to cross over nearby cells. Even at lower densities, the 3T3-Nf2^{BBA} cells grew on top of one another.

3T3-Nf2^{BBA} cells do not cease to divide at confluency

Disorganization of the cytoskeleton is often associated with cellular transformation. Therefore, we proceeded to test these cells for other properties of transformation. In order to determine the effect of expression of Nf2^{BBA} on the proliferation rate of NIH3T3 cells, the accumulation of 3T3-Nf2^{BBA} cells over time in culture was compared to that of 3T3-Nf2^{WT} and 3T3-BABE cells. Cells were counted beyond the point at which control cells reached confluency, in order to document the continued accumulation of the 3T3-Nf2^{BBA} cells. Whereas the number of 3T3-Nf2^{WT} and 3T3-BABE cells reached a plateau by day 6 due to contact inhibition, the 3T3-Nf2^{BBA} cells continued to accumulate beyond this point (Figure 3*a*). In the first three days of the experiment, the proliferation rate of the 3T3-Nf2^{BBA} cells was comparable to that of the 3T3-Nf2^{WT} and 3T3-BABE cells. Yet, as the cell populations became more dense, and the proliferation rate of the 3T3-Nf2^{WT} and 3T3-BABE cells began to slow, the 3T3-Nf2^{BBA} cells continued to accumulate at the same rate (days 4-8). Overall, 3T3-Nf2^{BBA} cells do not appropriately respond to growth inhibitory cues upon cell-cell contact. Overexpression of wild-type merlin (3T3-Nf2^{WT}) resulted in a small, but statistically significant ($p < 0.017$), decrease in the proliferation rate of the cells in the second half of the experiment (days 3-6) in 4 out of 6 cell lines tested (Figure 3*a* and data not shown). In addition, as shown in Figure 3*a*, the cells

overexpressing wild-type merlin did not plateau at as high a saturation density as the 3T3-BABE cells. These data are consistent with previous data describing a growth-inhibitory effect of merlin overexpression (Lutchman and Rouleau, 1995).

To characterize further the cell proliferation phenotype, we assayed the cell cycle profile of the infected cells either in log-phase growth or after being left at confluence for several days. Normal cells undergo a G0/G1 cell cycle arrest when they reach confluency due to contact inhibition. 3T3-$Nf2^{BBA}$ cells did not undergo the expected G0/G1 cell cycle arrest that was observed in the 3T3-$Nf2^{WT}$ and 3T3-BABE cells (Figure 3*b*). Flow cytometric analysis of 3T3-$Nf2^{WT}$ and 3T3-BABE cells grown in serum revealed that about 25% of exponentially growing control cells were in the S phase of the cell cycle, whereas under the same conditions, close to 40% of the 3T3-$Nf2^{BBA}$ cells were in S-phase. The proliferation curve demonstrates that all the infected cell populations have the same doubling rate at the time of cell cycle analysis (equivalent to days 1-2 of the proliferation assay). Therefore, based on the flow cytometric analysis of exponentially growing cells, 3T3-$NF2^{BBA}$ cells spend proportionately longer in S-phase, and, correspondingly, less time in the G2/M phases of the cell cycle. During exponential growth, only 16% of 3T3-$NF2^{BBA}$ cells are in the G2/M phase of the cell cycle versus ~33% of the control cells (3T3-BABE and 3T3-$NF2^{WT}$). A high S-phase percentage persisted in the 3T3-$Nf2^{BBA}$ cells even after they had reached a confluent state, confirming that these cells continued to cycle beyond the point that contact inhibition signals arrested the control cells.

Focus formation and anchorage-independent proliferation

On the basis of the assays described above, 3T3-$Nf2^{BBA}$ cells share many properties with transformed cells. In order to characterize the transformation phenotype further, we examined

the behavior of these cells in two common assays for transformation: focus formation on a monolayer of cells and anchorage-independent growth. When compared to control cells, the 3T3-Nf2^{BBA} cells formed a dense multilayer of cells on the tissue culture dish as demonstrated by giemsa staining of cells after two weeks in culture (data not shown). In order to visualize this phenotype more clearly, we diluted the infected NIH3T3 cells 1:100 into uninfected NIH3T3s before plating. Under these conditions, 3T3-Nf2^{BBA} cells formed distinct foci on top of the confluent monolayer of cells after just 2 weeks in culture, whereas the control cells formed few to no foci in the same assay (Figure 4). In addition, infected cells cultured at high cell density were fixed and their nuclei stained with DAPI. Under a fluorescent microscope, 3T3-Nf2^{BBA} cells exhibited many layers of overlapping nuclei, indicating that these cells were piling up on one another (data not shown). Control cells displayed no nuclear overlap in the cell monolayer.

Transformed cells often possess the ability to grow in anchorage-independent conditions. The ability of cells to adhere to the substratum of a dish can be denied by embedding the cells in soft agar before plating. Transformed cells will proliferate and form colonies of cells in the layer of soft agar, whereas normal cells will not, because they require adhesion to the substratum to proliferate. When 3T3-Nf2^{BBA} cells were subjected to a soft agar assay, they proliferated in an anchorage-independent manner, efficiently forming colonies in the soft agar matrix (Figure 5). 3T3-BABE and 3T3-Nf2^{WT} cells did not form colonies when subjected to the same assay.

3T3-Nf2^{BBA} cells form tumors in nude mice

To assess further the tumorigenicity of 3T3-Nf2^{BBA} cells, $1x10^6$ cells from at least three independently-derived cell lines of each type were injected into the flanks of nude mice and tumor formation was scored 4 weeks post injection (Table 1). Small tumors were visible at sites

injected with 3T3-$Nf2^{BBA}$ cells as early as 1 week post-injection, whereas no tumors were visualized when 3T3-$Nf2^{WT}$ and 3T3-BABE cells were injected. 3T3-$Nf2^{BBA}$-derived tumors increased in size over the duration of the experiment, until the tumor masses were harvested for histological examination 4-6 weeks post-injection (data not shown). There was no tumor growth detected at any of the sites injected with 3T3-$Nf2^{WT}$ or 3T3-BABE cells at the time of sacrifice.

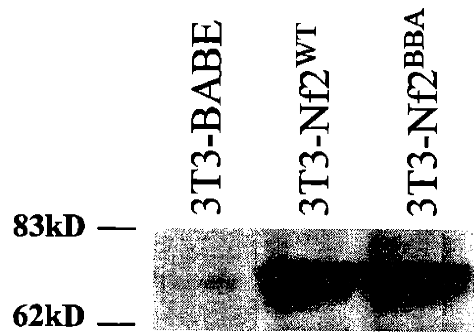


Figure 1. Overexpression of wild-type and BBA mutant merlin in NIH3T3 fibroblasts. Western blot of cell lysates prepared from retrovirally-infected NIH3T3 cells following selection. Merlin (~70kD) is detected using the sc331 antibody. 3T3-BABE = NIH3T3 cells infected with retroviruses carrying pBABE-puro empty vector; 3T3-$Nf2^{WT}$ = NIH3T3 cells infected with retroviruses carrying wild-type *Nf2* cDNA; 3T3-$Nf2^{BBA}$ = NIH3T3 cells infected with retroviruses carrying BBA mutant *Nf2* cDNA.

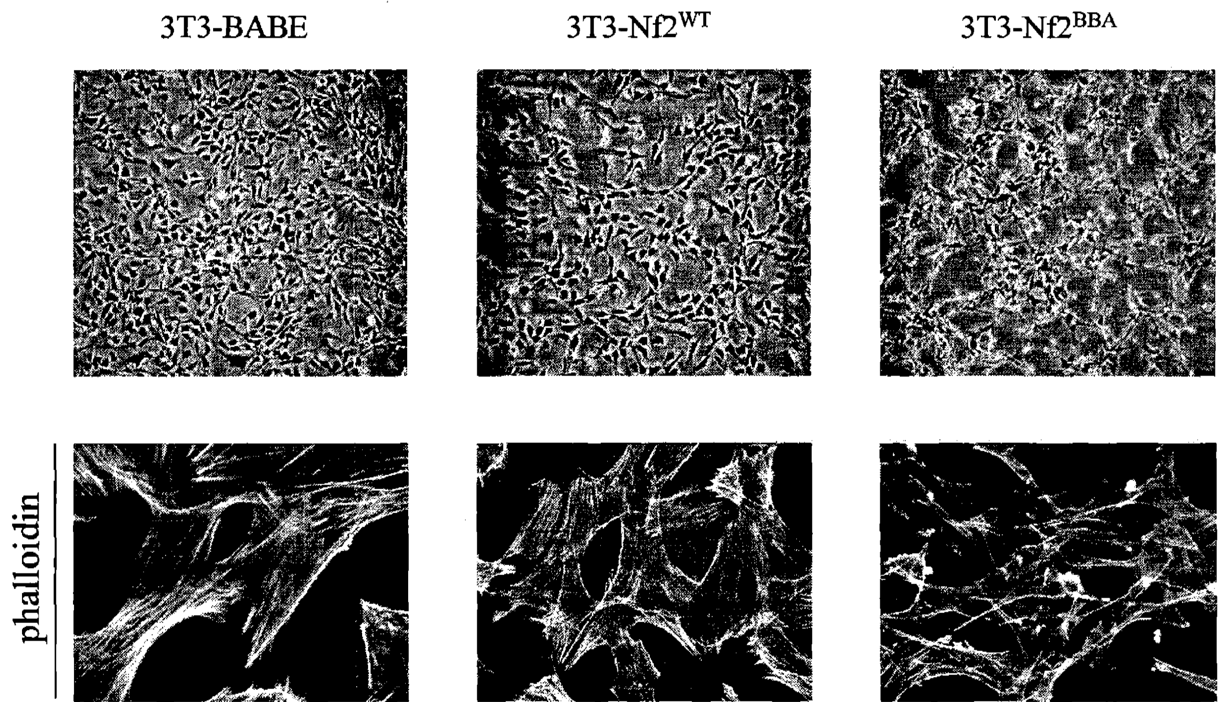
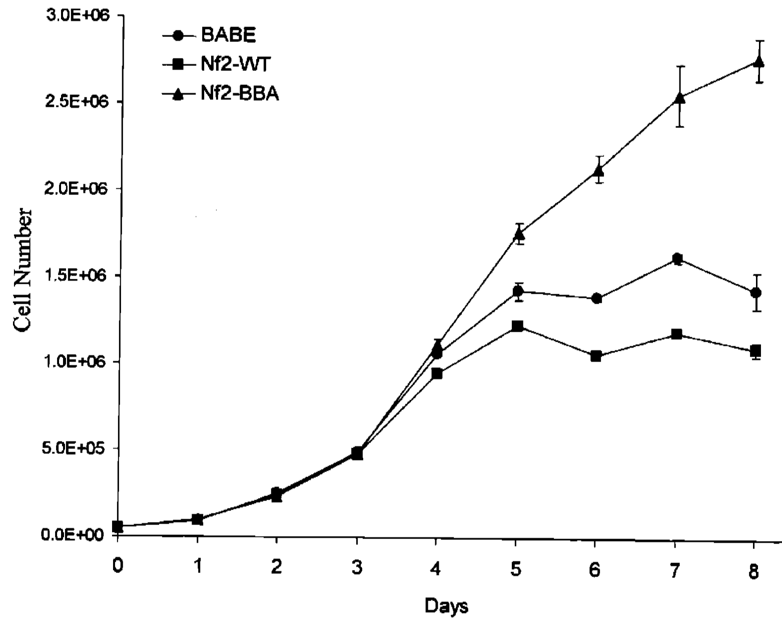


Figure 2. Cell shape and cytoskeletal abnormalities of 3T3-$Nf2^{BBA}$ cells. *Top panels:* Representative photomicrographs of infected NIH3T3 cells (40x magnification). *Bottom panels:* Actin cytoskeleton in infected NIH3T3 cells as detected by phalloidin staining (200x magnification).

a



b

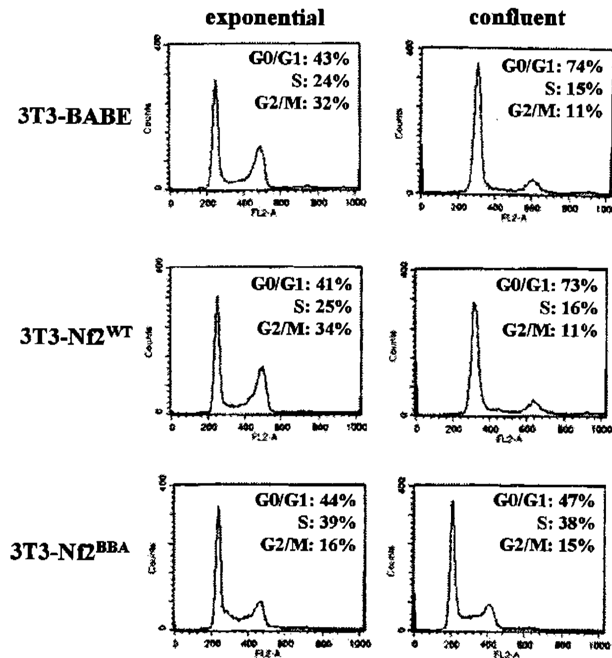


Figure 3. Continued accumulation of 3T3-Nf2^{BBA} cells beyond confluence. ***(a)*** Proliferation curves for infected NIH3T3 cells. Data shown are from one infected cell line of each type but are representative of data obtained from 6 independently-derived cell lines. ***(b)*** Cell cycle analysis of infected NIH3T3 cells grown exponentially ("exponential"; left column) or left at confluence for three days ("confluent"; right column). Flow cytometry results are representative of three independent experiments.

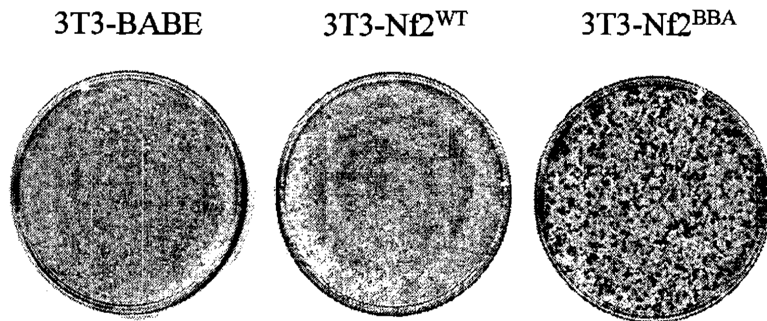


Figure 4. Focus formation on a monolayer of cells. Infected NIH3T3 cells were diluted 1:100 in uninfected NIH3T3 cells and cultured for 2 weeks. Focus formation on top of the monolayer of cells was visualized with Giemsa stain.

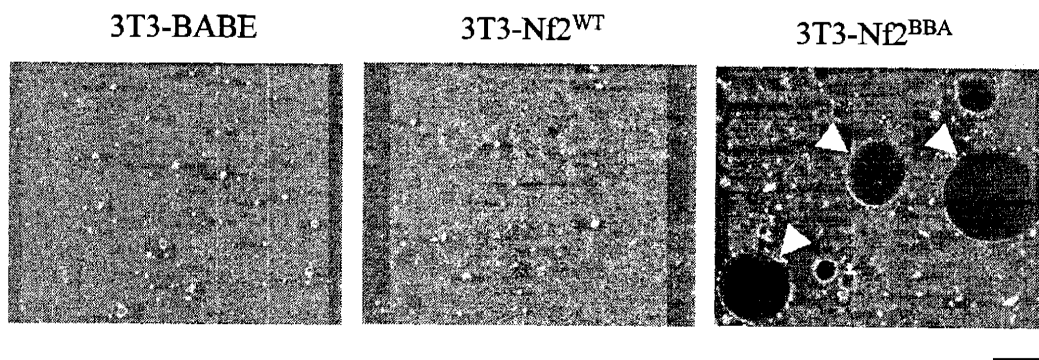


Figure 5. Soft agar colony formation. Representative photomicrographs of colony formation following a 3-week incubation of infected NIH3T3 cells in soft agar. *Arrowheads* (in Nf2^{BBA} panel) indicate colonies. Bar, 200 μm.

Table 1. Formation of tumors in nude mice.

Cells Injected [a]	*Tumor Formation*
3T3-BABE	0/3
3T3-Mer^{WT}	0/3
3T3-Mer^{BBA}	5/5

[a] At least three independently-generated infected lines were injected for each cell type.

Discussion

We have shown that expression of a FERM domain mutant of Nf2 ($Nf2^{BBA}$) leads to complete transformation of NIH3T3 cells. Abnormalities in the 3T3-$Nf2^{BBA}$ cells include disruptions of the actin cytoskeleton, cell adhesion defects, loss of contact inhibition of growth, anchorage-independent growth and the ability to form tumors in nude mice. Our studies support the hypothesis that merlin may be important in regulating cell-cell and cell-matrix contacts and argue that merlin may regulate intracellular pathways important for both proliferation and actin cytoskeleton processes.

Our initial characterization of the phenotype of the 3T3-$Nf2^{BBA}$ cells revealed a severe deficiency in cell adhesion, both on tissue culture dishes and with respect to the ability of the cells to grow under anchorage-independent conditions. Similar cell adhesion defects have been observed by groups expressing merlin mutants and are indicative of disruptions of cell-matrix interactions (Koga *et al.*, 1998; Stokowski and Cox, 2000). It is well established that cell adhesion is important for normal cell growth of adherent cells; in fact, anchorage-independent growth is a criterion for cellular transformation (Hynes, 1992; Nur *et al.*, 1992). Microinjection of *Nf2* cDNAs into cultured fibroblasts demonstrates that yet other mutations in the FERM domain affect cell adhesion, causing cell detachment (Koga *et al.*, 1998). This data suggests that FERM domain mutations may affect cell membrane-cytoskeleton signaling, which consequently could disrupt cell-matrix interaction. Thus, merlin may act as a positive regulator of cell-matrix attachment and disruption of this activity may be an initial step in tumorigenesis.

Interestingly, merlin interacts with β1 integrin in Schwann cells and NIH3T3 cells (Obremski *et al.*, 1998; KCJ and TJ, unpublished data). Studying the consequence of such an interaction may be important to uncovering the role of merlin in cell adhesion. β1 integrins, like

merlin, have been shown to suppress anchorage-independent growth (Giancotti and Ruoslahti, 1990; Tikoo *et al.*, 1994), and, most interestingly, β1 integrin interactions with the extracellular matrix and cytoskeleton have been implicated as part of a signal transduction complex necessary for Schwann cell myelination (Fernandez-Valle *et al.*, 1994). Perhaps loss of merlin expression leads to aberrant signaling downstream of β1 integrins resulting in abnormal growth/proliferation of Schwann cells.

The second striking phenotype exhibited by the 3T3-$Nf2^{BBA}$ cells was a lack of contact inhibition of growth, which suggests that merlin may play an important role in the response to cell-cell contact. Recent work suggests that merlin helps to mediate contact inhibition of growth through interactions with the membrane protein CD44 (Morrison *et al.*, 2001). In rat schwannoma cells, which exhibit contact inhibition defects, merlin is expressed at very low levels. However, exogenous expression of wild-type merlin, but not a FERM domain point mutant, can partially rescue this phenotype by inhibition of cell division at high density, thereby exerting contact inhibition signals. These data support a model in which under growth inhibitory conditions (such as confluency) merlin is active, hypophosphorylated, and bound to CD44, where it may participate in downregulating signaling pathways that lead to cellular proliferation. In addition, cultured, patient-derived *Nf2*-deficient Schwann cells exhibit many phenotypes identical to those seen in 3T3-$Nf2^{BBA}$ cells, including lack of contact inhibition, anchorage-independent growth and increased cell proliferation (Rosenbaum *et al.*, 1998). Taken together with our results, these data suggest that mutation of *Nf2* leads to misregulation of pathways that direct proper responses to growth inhibitory signals, such as cell-cell contact.

Oncogenic *Ras* alleles and signaling molecules that act both upstream and downstream of Ras are known to transform NIH3T3 cells (reviewed by Vojtek and Der, 1998). Therefore,

efficient transformation of NIH3T3 cells by Nf2[BBA] may indicate that wild-type merlin normally acts to negatively regulate specific Ras signaling pathways. Notably, an early study implicates merlin in the reversal of a Ras-induced malignant phenotype in cultured fibroblasts (Tikoo *et al.*, 1994). Alternatively, the Nf2[BBA] allele may stimulate Ras signaling though some other mechanism. Based on our observations, and previous data, the most obvious candidates downstream of Ras for merlin regulation are the Rho GTPase proteins, several of which have been identified as critical regulators of oncogenic Ras transformation (reviewed by Zohn *et al.*, 1998). The Rho GTPases, including Rho, Rac, and Cdc42, have been implicated in the establishment of cell-cell contacts and cell-matrix interactions, as well as in the regulation of the actin cytoskeleton and transcriptional activation (reviewed by Schmitz *et al.*, 2000). Interestingly, *Nf2*-/- schwannoma cells exhibit abnormal membrane ruffling (a process inhibited by expression of dominant-negative Rac) and disorganized stress fibers (which can be blocked by expression of dominant-negative Rho) (Pelton *et al.*, 1998). In addition, primary mouse embryo fibroblasts lacking *Nf2* display increased levels of activated JNK, a known Rac effector (Shaw *et al.*, 2001). Taken together, data linking loss of merlin function to alterations in the cytoskeleton and cell-cell and cell-matrix interactions support a role for wild-type merlin in regulating Ras signaling, perhaps at the level of the Rho GTPases.

Studies discussed here and elsewhere have demonstrated that in-frame exon deletions in regions of the FERM domain have led to disruptions in normal cellular architecture suggesting that the sequence, and most likely structural, integrity of the FERM domain is critical to the proper functioning of merlin as a tumor suppressor. The crystal structure of moesin reveals that its FERM domain forms a tri-lobed structure that can interact with the tail domain using specific FERM domain residues (Pearson *et al.*, 2000). An overlay of the merlin sequence on this

structure suggests that the BBA mutation may disrupt merlin self-association, as this mutation may affect part of the second lobe of the FERM domain in a region that is hypothesized to be important for intramolecular association with the tail domain. Importantly, recent data strongly support the hypothesis that the closed/oligomerized, hypophosphorylated form of merlin is the active, growth-suppressive form (Shaw *et al.*, 2001). In addition, the same amino acid residues needed for self-association are also required for the growth- and motility- suppressing function of merlin (Gutmann *et al.*, 1999a). Recent structural data suggests that "blue box" mutations may not affect the overall structure of the merlin FERM domain but may alter physico-chemical properties that are important for the function of this region in merlin (Shimizu *et al.*, 2001). Thus $Nf2^{BBA}$ may act by inhibiting head-to-tail association of $Nf2^{BBA}$ itself and/or disrupting normal head-to-tail interactions of endogenous merlin molecules. Alternatively, the $Nf2^{BBA}$ protein might sequester normal merlin-binding partners, while lacking key residues in the N-terminus to couple those proteins to their proper regulatory proteins. While the nature of these sequestered merlin-binding partners is not known, this cell system may be useful in dissecting the pathways normally controlled by merlin.

Prior studies have lead to the suggestion that perhaps some mutations in merlin may act in a dominant manner. Loss of cell attachment still occurs when *Nf2* deletion mutants are co-expressed with wild-type merlin in fibroblasts (Koga *et al.*, 1998). In addition, transgenic expression of an *Nf2* mutant lacking exons 2 and 3 specifically in the Schwann cells of an *Nf2*+/+ mouse leads to the development of schwannomas (Giovannini *et al.*, 1999). Moreover, the tumorigenic activity of this *Nf2* mutant allele is dependent upon the number of wild-type *Nf2* alleles *in vivo*, giving support to the hypothesis that such a mutant allele might act in a dominant-negative manner. Finally, there are isolated examples of human schwannomas in which both a

full-length and a truncated form of merlin were found co-expressed (Harwalkar *et al.*, 1998). Here, we have demonstrated that exogenous expression of the $Nf2^{BBA}$ allele in the presence of endogenous merlin in NIH3T3 cells leads to aberrant phenotypes predicted from loss of merlin function. In support of this, the effects observed with overexpression of $Nf2^{BBA}$ mimic some of the phenotypes of *Nf2*-deficient fibroblasts, including proliferation to a high saturation density, presumably due to lack of contact inhibition (Shaw *et al.*, 2001).

Although we have not confirmed that $Nf2^{BBA}$ acts as a dominant-negative allele in the context of murine merlin, our data showing similar phenotypes to those observed in *Nf2*-deficient fibroblasts and *Nf2*-deficient Schwann cells strongly supports this hypothesis. If $Nf2^{BBA}$ is acting as a dominant-negative protein in fibroblasts, as has been observed with the equivalent mutant in *Drosophila*, our results confirm the notion that wild-type merlin function is required for proper mediation of cell-cell and cell-matrix interaction. Addition of $Nf2^{BBA}$ to NIH3T3 cells may cause disruption of the complexes required for the molecular switch that specifies growth arrest upon cell-cell contact, thereby leading to constant stimulatory signals for proliferation beyond confluency. Confirmation of the putative dominant-negative effects of the $NF2^{BBA}$ allele in NIH3T3 cells could be carried out by "knocking down" endogenous expression of merlin in these cells using stable suppression of gene expression by RNAi. Using such cells, and employing the transformation assays described here, the phenotype of loss of merlin expression in NIH3T3 cells could be ascertained and compared to that of the 3T3-$Nf2^{BBA}$ cells.

Finally, a recent study conducted by Stokowski and Cox investigating the phenotype of cultured epithelial cells following introduction of mutant *Nf2* alleles revealed that a human homologue of the *Drosophila* $Mer^{\Delta BB}$ (deletion of the "blue box" region) caused no adhesion defects (Stokowski and Cox, 2000). This human $Nf2^{\Delta BB}$ allele exhibited near wild-type behavior

in most assays, including solubility and self-association. In contrast, the $Nf2^{\Delta BB}$ protein had a slightly altered subcellular localization, and cells expressing this mutant had a mild change in cell shape. Given that both the ΔBB and BBA alleles behaved identically when expressed in *Drosophila*, one may have expected similar results in a mammalian context. Perhaps the inconsistencies are due to cell-type or expression-level differences between our experiments and those of Stokowski and Cox. It is also possible that deletion versus alanine substitution in this region may lead to different changes in the mammalian proteins (perhaps due to folding) that ultimately affect their functions in the cell.

In conclusion, we have provided further evidence supporting a role for merlin in tumor suppression. We have shown that expression of a FERM domain mutant of merlin is sufficient to transform established murine fibroblasts in culture. Expression of mutant merlin was associated with actin cytoskeletal abnormalities, lack of contact inhibition, and adhesion defects including anchorage-independent growth. These observations support a broader role for merlin in regulating cell-cell and cell-matrix signals, an area that deserves further investigation. Activation of such signaling pathways due to loss of merlin function may contribute to tumorigenicity in NF2 patients.

Materials and Methods

Plasmids

The $Nf2^{BBA}$ mutant was created by site-directed mutagenesis of wild-type isoform I *Nf2* cDNA ($Nf2^{WT}$) in pcDNA3. The $Nf2^{BBA}$ cDNA was sequenced to verify the presence of the introduced mutations. $Nf2^{WT}$ and $Nf2^{BBA}$ cDNAs were amplified with primers that added a 5' BamHI site and a 3' EcoRI site and then cloned into the pBABE-puro retroviral vector.

Retroviral transduction

NIH3T3 cells (ATCC) were infected with high-titer retrovirus stocks produced by transient transfection of ϕNX cells (gift from G. Nolan, UCSF). The efficiency of infection was always >90% (data not shown). The day before infection, NIH3T3 cells were plated at 10^6 cells per 10-cm dish. Infected NIH3T3s were selected for 3 days with 2 μg/ml puromycin (Sigma) and replated for the corresponding assays.

Tissue culture conditions

Dishes and wells were rinsed once with PBS, and then coated with 10 μg/ml fibronectin in PBS overnight at 4°C. Plates were rinsed twice with PBS following coating and blocked with 10% calf serum (10% CS; Sigma) in DME for 1 h at 37°C before plating cells. All infected cells were maintained in 10% CS, 5 mM glutamine, and penicillin/streptomycin at 37°C in 5% CO_2.

Immunoblotting

Whole cell extracts of exponentially growing cells were prepared in RIPA lysis buffer (50 mM TRIS-HCl, pH 7.5, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM

EGTA, 1mM sodium orthovanadate, and 1mM NaF) containing Complete protease inhibitor tablets (Roche). Protein concentrations were determined using the BCA protein assay reagent (Pierce). Immunoblot analysis for merlin was performed using the merlin sc331 antibody (Santa Cruz, 1:1000). Actin (sc-1616; Santa Cruz, 1:2000) was used as a loading control.

Immunofluorescence

Cells plated on glass coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.5% NP-40. Filamentous actin was observed on a Zeiss microscope following staining with Oregon Green 455-phalloidin (Molecular Probes, 1:500). Nuclei were visualized by DAPI staining (0.5 μg/ml).

Proliferation Curves

Proliferation rate, as described here, is a measurement of cell accumulation over time and, therefore, does not take into account cell death. Proliferation curves were generated by plating 5×10^4 cells into each well of fibronectin-coated 12-well plates then trypsinizing and counting cells every 24 h over an 8 day period. The cells were counted using a Coulter Counter (Z1 series, Beckman Coulter). Measurement of cell proliferation was performed in triplicate for each cell line. Cells were fed every 3 days throughout the assay. Statistical analysis was performed using the student's T-test.

Cell cycle analysis

10^6 cells were plated on 10-cm plates and harvested 1 or 6 days post-plating. Cells at day 1 represent exponentially growing cells. Those at day 6 had been confluent for ~3 days. Cells

were harvested by trypsinization, fixed in 70% ethanol (–20°C, overnight), and rehydrated/ washed with PBS. Samples were incubated at 37°C for 30 min with 0.2 mg/ml propidium iodide (Sigma) and 0.1 mg/ml Rnase A (Sigma). After 12 h at 4°C, samples were processed by a FACScan (Becton Dickinson) and analyzed using ModFit LT Software (Becton Dickinson).

Focus formation assays

Infected NIH3T3 cells were seeded with uninfected NIH3T3 cells in fibronectin-coated 6-cm dishes at a ratio 1:100. Media was changed every 3 days, and the cultures were followed for 3 weeks. Visualization of cells was done with Giemsa (Sigma).

Soft agar assays

25,000 cells were resuspended in 0.34% low melting point agarose (LMP; Gibco, BRL) in DME/HEPES supplemented with 15% CS, penicillin/streptomycin and 5 mM glutamine. Cells were plated onto 6-cm dishes coated with 0.5% LMP agarose in DME. Cultures were maintained at 37°C and were supplemented with 2 ml of 0.34% LMP agarose in DME containing 15% CS once a week. Colony formation was followed for 3 weeks.

Tumorigenicity assays

Infected NIH3T3 cells were trypsinized, washed with PBS, and resuspended at 10^7 cells per ml in PBS. 10^6 cells (100 μl) were injected subcutaneously into the flanks of 5 week-old nude mice (BALB/c nu/nu, Jackson Laboratories). Mice were monitored for 6 weeks following injection. At this time, the mice were sacrificed and the tumors resected for histological examination.

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Chapter 3

Signaling pathway analysis following merlin loss in 3T3-Nf2[BBA] cells and *Nf2*-conditional mouse embryo fibroblasts

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The author contributed 100% to the work described in this Chapter.

Abstract

Neurofibromatosis type 2 (NF2) is an inherited cancer disorder which arises following loss of function mutations in the *NF2* tumor suppressor gene. The *NF2* protein, merlin, shares significant homology with a family of membrane-cytoskeletal linkers, indicating that it may mediate its tumor suppressor function via such interactions. Despite extensive mutational analysis of merlin, little is understood concerning its role as a tumor suppressor. We have previously demonstrated that expression of a putative dominant-negative allele of *Nf2*, $Nf2^{BBA}$, results in complete transformation of NIH3T3 fibroblasts. Here, I extend my analysis of this allele to other cell types in an effort to understand better genetic cooperation in transformation downstream of merlin mutations, and I attempt to characterize further the defects in 3T3-$Nf2^{BBA}$ cells with regard to cell-matrix adhesion, cell motility, and cell shape/cytoskeletal changes. Second, I show that the phenotype of *Nf2*-deficient mouse embryo fibroblasts is similar to that of 3T3-$Nf2^{BBA}$ cells and use these two cell types to analyze the effect of merlin loss on the cell cycle via investigation of various signaling cascades. Finally, I demonstrate that upregulation of cyclin D1 is necessary for transformation of NIH3T3 cells by $Nf2^{BBA}$.

Introduction

The neurofibromatosis type 2 tumor suppressor gene is found mutated in familial and sporadic schwannomas and other benign tumors of the central nervous system (Gusella et al. 1999). The *NF2* encoded protein, merlin, shows great similarity to the ERM proteins (ezrin, radixin and moesin), members of the band 4.1 family of membrane-cytoskeletal linkers (Rouleau et al. 1993; Trofatter et al. 1993). The strongest region of homology between merlin and the ERMs lies in the N-terminal half of the proteins, called the FERM domain (4.1, ERM), where merlin is 61% identical to ezrin. The homology between merlin and the ERMs declines significantly in the C-terminal halves of the proteins, and merlin does not encode a conserved actin binding domain found near the C terminus of the ERMs.

Several informative animal models of NF2 have been created which support its assignment as a tumor suppressor gene. Interestingly, heterozygous *Nf2* mice develop highly metastatic sarcomas, very distinct from the benign CNS tumors in humans (McClatchey et al. 1998). These data implicate merlin in the suppression of cell motility and perhaps invasion and metastasis, at least in the mouse. Further mouse models have been developed that more accurately represent the human disease, including an *Nf2*-conditional mouse expressing cre from a P0 transgene which resulted in specific deletion of *Nf2* in Schwann cells and caused schwannomas (Giovannini et al. 2000). Genetic studies have implicated *Nf2* in inhibition of cell proliferation in *Drosophila*, as a loss-of-function mutant leads to an overproliferation phenotype in the fly wing (LaJeunesse et al. 1998).

Both merlin and the ERMs localize to regions rich in cortical actin, such as membrane ruffles and filopodia (Tsukita and Yonemura 1999). The FERM domain of merlin has been shown to associate with some of the same membrane proteins as the ERMs, such as CD44.

However, given the lack of conservation of the C-terminal actin-binding domain in merlin, the consequences of such interactions are not apparent.

Despite signficant sequence homology and similar localization patterns as the ERMs, the activity of merlin is not as well understood. Recent studies indicate that merlin, like the ERMs, exists in a closed, self-associated state, and that the protein is likely opened by phosphorylation of its C terminal domain. The phosphorylation of merlin occurs via the p21-activated kinase (Pak) downstream of Rac/Cdc42 activation (Kissil et al. 2002; Xiao et al. 2002). Yet, unlike the ERM proteins, it appears that the open/phosphorylated form of merlin is inactive. In addition, merlin expression is known to be regulated by factors that are growth inhibitory for cells, including cell-cell contact, loss of adhesion and serum starvation (Shaw et al. 1998b).

Merlin has been reported to interact with many proteins and to inhibit a number of signaling pathways (Bretscher et al. 2002; Sun et al. 2002), but the contribution of these individual activities to tumorigenicity downstream of loss of merlin function are not at all clear. Indeed, initial studies with merlin revealed that its overexpression resulted in suppression of Ras-induced transformation of NIH3T3 fibroblasts (Tikoo et al. 1994). However, it is not at all apparent what pathways downstream of Ras are inhibited by merlin. Many investigations of the function of merlin mutants have been carried out, and all have revealed an importance of the conserved N-terminal FERM domain for the function of the protein, as mutation in this domain leads to many abnormal phenotypes that are cell-context-dependent, including, disruption of the actin cytoskeleton, adhesion defects, cell-cell contact defects, and mislocalization (Bretscher et al. 2002). More recent data revealed that loss of merlin function in fibroblasts leads to an increase in membrane ruffling and cell motility (Shaw et al. 2001). In addition, *Nf2*-deficient fibroblasts seem to have hyperactive signaling downstream of Rac, as both JNK and AP-1

activities were upregulated in the absence of merlin (Shaw et al. 2001). Taken together, these data indicate that merlin may function to downregulate the Rac pathway. Our recent studies implicate merlin directly in the downregulation of Pak1, perhaps via a feed-forward loop, given the recent identification of Pak as a negative regulator of merlin function (Chapter 4 and J. Kissil et al., submitted).

Previously, we demonstrated that expression of $Nf2^{BBA}$, a putative dominant-negative allele of merlin, results in stress fiber abnormalities, cell adhesion defects, contact inhibition defects, adhesion-independent proliferation, and, ultimately, complete transformation of NIH3T3 cells in culture (Johnson et al. 2002). A major goal of my thesis project was to use these cells as a tool to understand the mechanism(s) by which $Nf2^{BBA}$ transforms fibroblasts in an effort to understand more fully the cell biological and biochemical consequences of loss of merlin function. My hope was that such studies would allow me to understand better the role of merlin as a tumor suppressor. First, I have characterized the expression of $NF2^{BBA}$ in other cell types in an effort to identify possible genetic interactors/pathways leading to transformation. Second, in an effort to more fully characterize the result of $Nf2^{BBA}$ expression, I examined other aspects of cells expressing this allele, including motility. During the course of these experiments, I was fortunate to obtain another genetic tool, *Nf2*-conditional MEFs, which I demonstrate harbor many of the same phenotypes as $Nf2^{BBA}$-expressing cells. Thus, I was able to employ both cell lines in the biochemical analyses of the result of loss of merlin function. Although the data obtained in this aspect of the project did not lead to an elucidation of the mechanism of transformation by the $Nf2^{BBA}$ allele, and while many questions remain unanswered regarding the mechanism of tumor suppression of merlin, these experiments have thoroughly addressed many of the relevant signaling pathways that might have been involved in this system.

Results and Discussion

Nf2^{BBA} transforms Rat1 fibroblasts

I began this study with an extension of my analysis on Nf2^{BBA}-expressing cells in order to more fully characterize their phenotype and identify further defects that would lead to a clearer picture of merlin function. First, I wanted to extend the analysis of Nf2^{BBA} to another cell type to determine whether transformation by this *Nf2* allele was transferable to other systems/cells or was specific to NIH3T3 cells.

In addition to transforming NIH3T3 cells, Nf2^{BBA} transforms Rat1 fibroblasts. Although Rat1-Nf2^{BBA} cells did not display adhesion defects or actin cytoskeletal defects as observed following staining with phalloidin (Figure 1a), the cells grew to a high density showing a disregard for contact inhibition signals. As demonstrated by the proliferation curve in Figure 1b, Rat1-Nf2^{BBA} cells continued to proliferate beyond the point at which contact inhibition signals slowed the growth of Rat1-babe and Rat1-Nf2^{WT} cells. In addition, Rat1-Nf2^{BBA} cells proliferated faster than control cells, as cell cycle analysis revealed a 2-3-fold increase in the percentage of cells in S-phase even during exponential growth (day 3 of proliferation curve) (Figure 1c). Consequently, these cells formed many small foci on the tissue culture dish (Figure 1d). Additionally, Rat1-Nf2^{BBA} cells formed small, slow-growing colonies in soft agar (Figure 1e). Finally, these cells formed tumors in nude mice, although not nearly as large or as quickly as those formed by 3T3-Nf2^{BBA} cells [(Johnson et al. 2002), Chapter 2, and data not shown]. In summary, it appears as though Nf2^{BBA} can transform Rat1 fibroblasts, although this allele has a weaker transforming capability in Rat1 cells than in NIH3T3 cells. Interestingly, preliminary studies indicate that Nf2^{BBA} cannot transform Swiss3T3 fibroblasts (data not shown).

It is likely that the differences in transformation activities of this allele as demonstrated between Swiss3T3 fibroblasts, Rat1 fibroblasts and NIH3T3 fibroblasts is due to known diversity in signaling pathways leading to transformation in these different cell lines (Pruitt and Der 2001). In general, sensitivity to transformation is extremely inconsistent among immortalized cell lines and likely varies due to differences in pathways downstream of Ras. In certain strains of NIH3T3s, Ras, Rho, and Rac can promote transformation, whereas in others, Ras, Rho and Rac are incapable of exerting an effect on transformation (Khosravi-Far et al. 1996). In addition, crosstalk between the RhoGTPases differs between Swiss 3T3s and NIH3T3s. Cdc42 stimulates Rac, which stimulates Rho, in Swiss 3T3 fibroblasts (Van Aelst and D'Souza-Schorey 1997). However, Cdc42 and Rac inhibit Rho activity in NIH3T3 cells (Leeuwen et al. 1997). Therefore, our data would indicate that perhaps inhibition of Rho is required for efficient cellular transformation by $Nf2^{BBA}$. Finally, whereas Pak1 activation of ERK is necessary for Ras transformation in Rat1 fibroblasts, expression of dominant-negative Pak in Ras transformed NIH3T3 cells has no effect (Tang et al. 1997). However, at this point, it is not clear how differences in Pak might affect the efficiency of transformation by $Nf2^{BBA}$ in these different cell types. Nevertheless, information regarding these pathways may assist in the understanding of the mechanism(s) by which $Nf2^{BBA}$ causes transformation.

BBA merlin expression in other cell types (genetic cooperation)

NIH3T3 cells were originally created through a 3T3 immortalization protocol (every 3 days Transfer $3x10^5$ cells to a new 6cm dish) of NIH mouse embryo fibroblasts. The resulting immortality of this cell line has been attributed to deficiency at the INK4 locus which harbors the cyclin-dependent kinase (cdk) inhibitors *p19ARF* and *p16INK4a*. Because $Nf2^{BBA}$ transforms

NIH3T3 cells, I wanted to test whether expression of this allele cooperated with mutation(s) in these and other genes to cause transformation of primary mouse fibroblasts. The goal of such experiments was to determine if the p16 and/or p19 pathways played a role in transformation by $Nf2^{BBA}$ and to identify what other pathways might cooperate with mutation of *Nf2* to cause cellular transformation in an effort to understand how NF2 tumors might arise.

Therefore, I employed wild-type mouse embryo fibroblasts (MEFs) and those with knock-out alleles of *INK4a* (*p16-/- and p19-/-*) and *p53*. Each MEF line was infected with vector control (babe), $Nf2^{WT}$, or $Nf2^{BBA}$ retroviruses. Merlin expression was tested by western blot following retroviral infection and selection (data not shown). Although the $Nf2^{BBA}$ MEF lines proliferated to a slightly higher saturation density than the wild-type and mock infected counterparts, none of the lines were transformed according to soft agar and nude mouse injection assays (Figure 2 and data not shown). First, these data indicate that expression of $Nf2^{BBA}$ likely cooperates with a genetic alteration other than *p16/p19*-deficiency to transform NIH3T3 cells. In addition, although we were unable to induce transformation of primary MEFs and thus gain insight into *Nf2* function using these methods, we were able to eliminate *p19, p16* and *p53* as cooperators with loss of *Nf2* function resulting in cellular transformation of primary MEFs. Finally, we have established that expression of a mutant form of merlin leads to contact inhibition defects in a variety of cellular contexts, thereby supporting a role for merlin in maintenance of inhibition of cellular growth upon cell-cell contact.

Investigation of cell shape, cell-cell and cell-matrix defects

Several groups, including ours, have previously reported that expression of merlin mutants leads to cytoskeletal defects. Therefore, I wanted to utilize NIH3T3 cells expressing

BBA as a way to investigate the role of merlin in regulating specific aspects of the cytoskeleton. I have previously shown that BBA expression in NIH3T3 cells led to abnormal cell shape and disorganized actin stress fibers, disruption of cell adhesion to the tissue culture dish, cell-cell contact inhibition defects, and proliferation defects at high cell density ultimately resulting in transformation. In order to characterize more deeply the cause of the cell shape phenotype of 3T3-$Nf2^{BBA}$ transformed cells, I performed several pilot experiments to compare these cells to the control cell lines. First, I reasoned that $Nf2^{BBA}$ could exert its effects on the cell and the cytoskeleton by means of abnormal subcellular localization. Since merlin is normally localized to both the cytosol (soluble) and membrane/cytoskeleton (insoluble) fractions, if $Nf2^{BBA}$ were mislocalized to just the soluble/cytosolic fraction, it may not be available for interactions with the cytoskeleton, thereby perhaps affecting cell shape and contacts. In addition, in *Drosophila*, an equivalent blue box mutant of merlin was shown to localize differently than wild-type merlin and when expressed together, the blue box mutant caused a relocalization of wild-type merlin (LaJeunesse et al. 1998). Figure 3a demonstrates that the solubility of BBA and wild-type proteins is similar in these cells. In addition, by immunofluorescence, BBA merlin localized to the same subcellular compartments as the wild-type protein, including diffusely in the cytoplasm and in membrane extensions (data not shown). Therefore, it is unlikely that BBA merlin causes a mislocalization of the wild-type protein in mouse fibroblasts, as was shown in *Drosophila* S2 cells. Given its localization pattern, it is formally possible that BBA merlin occupies interaction sites on critical proteins in the cell normally bound and regulated by wild-type merlin. However, given its mutation, perhaps BBA merlin cannot link these bound proteins to critical downstream partners like its wild-type counterpart.

The ERM proteins are critical regulators of the cytoskeleton exerting their effects on such processes as those seen disrupted in 3T3-$Nf2^{BBA}$ cells, stress fiber assembly and cell shape. Additionally, studies have shown that merlin can heterodimerize with the ERM proteins (Nguyen et al. 2001). Yet, the functional consequence of these interactions is unknown. Therefore, given the observed cytoskeletal defects, one could imagine that expression of $Nf2^{BBA}$ might affect the levels or localization of the ERM proteins. As shown in Figure 3b, the expression levels of the ERM protein ezrin by western blot analysis was similar in $Nf2^{BBA}$ cells and controls, indicating that merlin expression does not affect ERM levels in the cell. In addition, expression of BBA merlin did not affect the subcellular localization of the ERM proteins (data not shown). This data is consistent with experiments performed in rat schwannoma cells which demonstrated that there were no changes in ERM expression in the presence and absence of merlin expression (Gutmann et al. 1999). Nevertheless, these experiments do not assess whether BBA merlin can interact with the ERM proteins, and, if so, whether these interactions are productive allowing for normal ezrin function.

I have demonstrated that 3T3-$Nf2^{BBA}$ cells exhibit severe cell adhesion defects that were able to be rescued by coating the tissue culture dishes with fibronectin. Although the detailed consequences of the interaction have not been elucidated, merlin has been shown to be in a complex with paxillin and focal adhesion kinase (FAK) at the cytoplasmic face of the β1 integrin receptor in adherent Schwann cells (Taylor et al. 2003). It is possible that wild-type merlin interaction with this membrane protein assists in the maintenance of cell adhesion perhaps by linking mitogenic signals through β1 integrin to critical downstream pathways, and that loss of merlin expression causes loss of this regulation. Therefore, we tested the ability of BBA (and wild-type) merlin to interact with β1 integrin in NIH3T3 cells. Both BBA merlin and wild-type

merlin interact with $\beta 1$ integrin as shown by co-immunoprecipitation experiments (Figure 3c). Perhaps in 3T3-Nf2^{BBA} cells, BBA merlin occupies the endogenous merlin binding site on $\beta 1$ integrin and is unable to regulate the links between $\beta 1$ integrin and critical downstream pathways. Thus, further experiments are needed to address this hypothesis.

Finally, given the highly motile phenotype of *Nf2*-deficient MEFs, we believed that perhaps 3T3-Nf2^{BBA} cells had similar properties. In addition, we argued that cells expressing Nf2^{BBA} were likely to be more motile and invasive, given the aggressive growth of these cells in nude mouse tumorigenicity assays. In order to test the motile properties of these cells, we employed a sophisticated time-lapse videography system to track the movement of cells over 2 hours. Frames were taken every 5 min, and 15 cells were traced through 25 frames. The speed of these cells was assessed using DIAS software, and the resulting data are shown in the box and whiskers plot in Figure 3d. Although there was a slight increase in the motile rates of 3T3-Nf2^{BBA} cells and a slight decrease in the 3T3-Nf2^{WT} cells when compared to the vector control cells, these changes were not significant (p-value >0.05).

Interestingly, a study performed in rat embryo fibroblasts suggests the partial inhibition of Rho leads to increases in cell migration, whereas complete Rho inhibition causes cell detachment (Nobes and Hall 1999). In addition, activation of Rac/Cdc42 in NIH3T3 fibroblasts leads to downregulation of Rho by as yet unidentified mechanisms (Sander et al. 1999). Therefore, perhaps expression of Nf2^{BBA} leads to significant upregulation of pathways downstream of Rac/Cdc42 which results in complete inhibition of Rho and loss of cell attachment, a phenotype of 3T3-Nf2^{BBA} cells. In the case of complete inhibition of Rho, I would not expect these cells to have increased cell migration, which is what was observed in the present assay. However, increased cell migration was observed in *Nf2*-deficient fibroblasts, where,

perhaps loss of *Nf2* leads to only a partial inhibition of Rho. Further studies, including Rho activity assays in the presence and absence of merlin expression will need to be carried out in order to address these hypotheses.

Evaluation of the behavior of $Nf2^{BBA}$ as a dominant-negative allele

The phenotype of 3T3-$Nf2^{BBA}$ cells is consistent with the hypothesis that BBA merlin acts as a dominant-negative (antimorphic) allele in the context of the mouse. Nevertheless, to support this hypothesis further, I undertook a genetic proof-of-principle experiment in NIH3T3 cells. The genetic definition of an antimorph is that its expression in the background of wild-type protein expression should result in the same effect/phenotype as deleting gene expression altogether. With the advent of RNA interference (RNAi) technology, one can knock down the expression of their protein of interest by introducing short interfering (or short hairpin) RNAs (siRNAs). Cloning of the short hairpin RNA sequence in the context of a retroviral LTR allows for its integration in the genome, and, therefore, constant expression, resulting in stable knock down of expression. The goal of my experiments was to stably knock down the expression of merlin in NIH3T3 cells (3T3-siNf2) and compare their phenotype to that of 3T3-$Nf2^{BBA}$ cells. If $Nf2^{BBA}$ is truly a dominant-negative allele, then 3T3-siNf2 cells should have the phenotype of $Nf2^{BBA}$ expressing cells. Two different short hairpin RNA sequences were chosen corresponding to distinct 19 nucleotide sequences: (1) beginning 49 bp from the start ATG codon ($siNf2^{49}$), and (2) beginning 300 bp upstream of the stop TGA codon ($siNf2^{1388}$). Both siRNAs should be able to knock down expression of the two major isoforms of merlin (I and II), as 300 bp upstream from the stop codon is upstream of the alternatively spliced exons 15-17. $siNf2^{49}$ and $siNf2^{1388}$ were cloned into the pSIRIPP-puro retroviral vector under the H1 RNA promotor. NIH3T3 cells

were infected with recombinant retroviruses and then selected using puromycin for stable integration/expression of the puro selection cassette. Expression of merlin following siRNA infection was assayed by western blotting. As shown in Figure 4, the expression of merlin was significantly knocked down in the 3T3-siNf2 cells compared to mock infected. However, the incomplete merlin knock down may not be enough to recapitulate complete loss of merlin function. Indeed, these cells did not form colonies in soft agar or tumors in nude mice (data not shown). Therefore, I attempted to infect the NIH3T3 cells with both siRNAs in an effort to achieve a more complete knock down. A pSIRIPP-neo vector was employed and pairwise combinations of the recombinant retroviruses were used in infections. However, infection with two siRNAs had no greater effect on the level of knock down than expression of each alone (data not shown). Therefore, I was unable to address the dominant-negative nature of this allele using this method because of incomplete levels of merlin knockdown. Nevertheless, as demonstrated by studies using *Nf2* conditional MEFs, in many ways, 3T3-Nf2^{BBA} cells behave like fibroblasts that genetically lack merlin expression (see discussion below and Figure 5).

***Nf2*-conditional MEF characterization**

In order to understand what cell signals are perturbed when merlin expression is lost, I undertook a project to study the phenotype of and signaling pathways in *Nf2* conditional knock-out MEFs. To this end, MEFs were isolated from embryos of the following genotypes: Nf2$^{flox2/flox2}$ and Nf2$^{flox2/-}$. (Note: "-" denotes the *Nf2* null allele; "flox2" denotes the unrecombined *Nf2* conditional allele.) Nf2$^{flox2/-}$ and Nf2$^{flox2/flox2}$ MEFs were treated with adenoviral-cre recombinase (ad-cre) which resulted in the recombination of the conditional allele (Figure 5a) and loss of merlin expression from the recombined allele. As shown in Figure 5b,

merlin protein was undetectable 96 hrs post ad-cre infection. Control infection with an empty adenovirus (ad) or adeno-GFP resulted in no effect on merlin expression. While *Nf2*-deficient cells had no apparent proliferation defect during the exponential phase of growth, they did proliferate to a higher density, apparently overcoming contact inhibition signals (Figure 5c) and piled up on one another forming small foci (Figure 5d). In addition, these cells had adhesion defects at high cell densities; the entire cell layer would peel off the plate. Interestingly, as was the case with 3T3-$Nf2^{BBA}$ cells, coating the plates with fibronectin rescued the cell adhesion defect.

A recent study by Lallemand and colleagues, published after the work presented here was completed, suggests that the contact inhibition defects in these cells might be due to abnormalities in adherens junctions (Lallemand et al. 2003). Whereas wild-type MEFs and keratinocyes have normal adherens junction formation, *Nf2*-deficient cells cannot form stable cadherin-containing cell:cell junctions. Interestingly, merlin was shown to interact with components of adherens junctions in confluent cells, thereby indicating that loss of merlin expression in cells might lead to contact inhibition defects and eventually tumorigenesis via disruption of cell:cell contacts. It was hypothesized that merlin might function to locally stabilize actin filaments at the site of adherens junctions. These studies provide a plausible explanation for merlin's role in loss of contact inhibition of growth. Although the role of adherens junctions and signaling from this site to the nucleus is still under investigation, it is plausible that disruption of merlin at this site results in abnormal signaling to the nucleus which results in inappropriate cell proliferation upon cell-cell contact.

Signaling downstream of merlin loss of function

Having demonstrated that *Nf2*-deficient fibroblasts and 3T3-Nf2^{BBA} cells exhibit similar phenotypes, I wanted to use both cell types in order to look at signaling pathways that might be deregulated in the context of merlin loss. Notably, in cycling cells, where the proliferation rates between merlin expressing and non-expressing cells were similar, I observed no differences in the levels of phospho-signaling molecules as assessed by western blotting. Figure 6a shows that the cell cycle profiles of MEFs are the same with and without merlin expression under conditions of exponential cell growth, and that under these same conditions, levels of the cell cycle regulator cyclin D1 and various upstream signaling molecules are constant (Figure 6b). Although the cell cycle profile of 3T3-Nf2^{BBA} cells is altered (50% more cells in S-phase and 50% fewer in G2/M compared to controls) under conditions of exponential growth (Figure 6c), as with *Nf2*-deficient MEFs, there were no observable changes in cyclin D1 levels or levels of the signaling proteins assayed (Figure 6d). Because I did not see any changes in phenotype or signaling in cells under these conditions, my goal was to examine the effects of loss of merlin function under conditions were merlin is normally required and upregulated. Therefore the following experiments focused mainly on the condition of serum starvation.

My initial studies revealed that cyclin D1 protein levels were significantly elevated in serum-starved 3T3-Nf2^{BBA} cells (Figure 7a). Upregulation of cyclin D1 expression normally occurs in response to mitogenic signaling and results in phosphorylation and release of pRB from E2Fs allowing for progression through the G1/S transition (Sander et al. 1999). In normal cells, in the absence of serum, cyclin D1 levels should be low and the cell cycle is halted at the G1/S transition. Regulation of cyclin D1 expression occurs via many signaling pathways at both a transcriptional and a translational level (Pruitt and Der 2001). Therefore, given the high levels of

cyclin D1 protein under serum-starved conditions, I tested the levels of cyclin D1 transcripts in cells using real-time PCR. As demonstrated in Figure 7b, the levels of cyclin D1 transcripts in 3T3-$Nf2^{BBA}$ cells was 4-5 fold higher than that of serum-starved control cells. As expected, due to higher cyclin D1 levels, serum-starved 3T3-$Nf2^{BBA}$ cells failed to undergo a G1 arrest and continued to cycle, with almost 30% of cells in S phase (Figure 7c). Interestingly, by both western blotting and real-time PCR, there were no significant differences in cyclin D1 levels in *Nf2*-deficient MEFs compared to wild-type (Figure 7d). Consistent with this observation, there were no observable differences in the cell cycle profile between *Nf2*-deficient and wild-type MEFs following serum starvation (Figure 7e). This was in contrast to recent data suggesting that *Nf2*-deficient MEFs are not growth arrested following serum starvation (Lallemand et al. 2003). Interestingly, the authors note that these cells continue to proliferate *slowly* under serum-starved conditions. It is not clear what the differences may be between these studies and the data presented here, but it is evident from my studies that neither cyclin D1 levels nor S-phase percentages are discernably higher in serum-starved *Nf2*-deficient MEFs.

One of the most well characterized transformation systems has been oncogenic Ras transformation of NIH3T3 cells. As such, a significant amount of information is known concerning the pathways downstream of Ras that are required for complete transformation in this system (Pruitt and Der 2001). Interestingly, while dominant-negative Rho GTPases inhibit Ras transformation, they are only weakly transforming when expressed as activated molecules on their own (Roux et al. 1997). Expression of activated Rho and Rac causes contact inhibition defects, as the cells grow to a higher saturation density. Transformation by activated Rho alleles is dramatically increased when they are expressed with activated Raf. Interestingly, although previous data has suggested that disruption of merlin function leads to an upregulation of Rac

pathway activity, it is possible, given the full transforming capabilities of the $Nf2^{BBA}$ allele, that there are other pathways including the Raf pathway that must be disrupted in cells expressing this allele.

Significant effort has been placed into understanding the upregulation of cyclin D1 levels downstream of Ras activation, although there is still not a complete picture of these regulatory pathways (Downward 1997). Upregulation of cyclin D1 has been shown to be necessary for Ras transformation by antisense studies, but is not sufficient, as overexpression of cyclin D1 alone cannot support cellular transformation (Quelle et al. 1993; Filmus et al. 1994; Liu et al. 1995; Pruitt et al. 2000). Ras upregulation of cyclin D1 has been attributed mainly to Ras activation of the Raf/MEK/ERK pathway (Liu et al. 1995), although studies have revealed other important pathways in certain cell types. Ras leads to an upregulation of cyclin D1 levels by both transcriptional and post-transcriptional mechanisms. The cyclin D1 promotor contains several binding sites for transcription factors which are regulated downstream of Ras, including an Ets-2 binding site activated via the MEK/ERK pathway (Lavoie et al. 1996), a CRE promoter element activated via CREB (D'Amico et al. 2000), a TCF promoter element activated via the WNT/β-catenin pathway (Simcha et al. 1998; Tetsu and McCormick 1999), and an AP-1 site activated via c-jun (Wisdom et al. 1999). Interestingly, transcriptional activation from the AP-1 site was shown to be dispensible for fibroblast transformation by Ras (Albanese et al. 1995; Pruitt et al. 2000). In addition, NFκB regulation of the transcription of cyclin D1 is required for Ras transformation, perhaps downstream of RalGDS activation or via a Rac-Pak connection (Henry et al. 2000). Interestingly, a recent study suggests that wild-type merlin is effective in suppressing NFκB signaling pathways in NIH3T3 cells and that mediation of this suppression likely occurs through the FERM domain of merlin (Kim et al. 2002). Finally, post-

transcriptionally, cyclin D1 protein stability is decreased by phosphorylation. PI3K activation of AKT, downstream of Ras activation, leads to phosphorylation and inactivation of GSK3β, which otherwise phosphorylates cyclin D1, priming it for ubiquitin-mediated degradation (Diehl et al. 1998). Given that expression of $NF2^{BBA}$ leads to upregulation of cyclin D1 at the transcriptional level, and perhaps at a post-transcriptional level, I wanted to investigate what pathways are misregulated in these cells.

We chose to assay signaling pathways upstream of cyclin D1 regulation following serum starvation in order to assess how BBA mutation of merlin leads to upregulation of cyclin D1 levels. As expected, there were no differences in signaling molecules upstream of cyclin D1 in *Nf2*-deficient MEFs when compared to wild-type MEFs, as shown in Figure 8a. Conversely, I expected to observe an upregulation of some pathways upstream of cyclin D1 in serum-starved 3T3-$Nf2^{BBA}$ cells given their cycling profile and high levels of cyclin D1. However, as shown in Figure 8b, I was unable to detect any differences in the levels of phospho-signaling proteins between these cells and control cells that maintained low levels of cyclin D1 following starvation. These results (and data not shown) are summarized in Table 1. In addition to looking at pathways downstream of serum-starved conditions in these cells, we stimulated several of the assayed pathways using serum and other mitogens. Even under these conditions, there were no detectable differences in signaling pathways leading to cyclin D1 expression (data not shown).

Despite the fact that I was unable to identify which pathways were upregulated upstream of cyclin D1 in serum-starved 3T3-$Nf2^{BBA}$ cells, I was able to establish and eliminate those pathways that are not involved in the upregulation of cyclin D1 by BBA merlin under serum-starved conditions: ERK1/2, MEK, AKT, p38MAPK, and MKK4 (see Table 1). Perhaps my inability to observe any detectable changes in these pathways was due to minor changes that

would be below the sensitivity of western blotting. In addition, although the total levels of these phospho-proteins could be unchanged in these cells, perhaps their proper localization is affected following expression of BBA merlin. An example of this has recently been noted in β1 integrin mutant MEFs, where total levels of phospho-ERK are normal but the phospho-ERK is not localized to the nucleus where its activity is required, thereby leading to a downregulation of the activity of this signaling pathway despite normal protein levels (Hirsch et al. 2002). Future studies will be required to determine if pathways are affected in this way in these cells.

Upregulation of cyclin D1 expression, and, therefore, progression through the cell cycle, is both mitogen- and adhesion-dependent in normal cells (Zhu et al. 1996). I have shown that in 3T3-$Nf2^{BBA}$ cells, cyclin D1 levels are increased even in the absence of mitogens. Therefore, I wanted to test these cells and *Nf2*-deficient MEFs under another condition normally required for proliferation. Preliminary studies indicate that when placed under the condition of loss of adhesion, *Nf2*-deficient MEFs retained high levels of cyclin D1, whereas merlin-expressing MEFs downregulated cyclin D1 (Figure 9a). Consistent with this observation, suspended *Nf2* deficient MEFs ($Nf2^{flox2/flox2}$ + ad-cre) had almost twice as many cells in S phase as their merlin-expressing (ad) counterparts (Figure 9b). Interestingly, when 3T3s expressing $Nf2^{BBA}$ were placed under the same assay conditions for loss of adhesion, they showed only slight differences in cyclin D1 levels (Figure 9c), and an approximate 50% increase in S-phase percentage (Figure 9d). Therefore, although these two cell types have similar phenotypes, it is evident that there may be slightly different cellular/signaling disruptions leading to these phenotypes. Nevertheless, cell adhesion is normally required for proper proliferative signaling via integrins. Therefore, loss of merlin function likely leads to misregulated signaling downstream of integrins in the absence of cell adhesion, which results in improper entry into the cell cycle. Further

studies should be carried out to address the effects of loss of adhesion on signaling in *Nf2*-deficient MEFs in order to understand what pathways are disrupted leading to deregulation of cyclin D1.

Interestingly, following these studies, Lallemand and colleagues reported that cyclin D1, c-jun and both JNK and ERK pathways were upregulated in *Nf2*-deficient fibroblasts under confluent conditions (Lallemand et al. 2003). Under these conditions, they demonstrated that a much higher percentage of *Nf2*-deficient MEFs were in S phase than in the wild-type MEFs. In addition, this study demonstrated that *Nf2*-deficient MEFs did not differ from wild-type under suspension conditions (16 hrs.). However, my preliminary data suggests otherwise: following 12 hours of cell suspension, *Nf2*-deficient MEFs retain high levels of cyclin D1 and slightly higher percentage of cells in S-phase than the wild-type controls. Perhaps the differences in incubation times under suspension conditions led to the different observations in these cases. Nevertheless, the data obtained using confluent conditions is noteworthy given the presumed role for merlin in regulating contact inhibition. It would be interesting to follow up on these studies to more exactly identify the stage at which merlin disrupts the JNK and ERK pathways. Our recent identification of merlin regulation of Pak in rat schwannoma and *Nf2*-deficient fibroblasts might help to explain these data, as upregulation of Pak in cells can lead to increases in both JNK and ERK signaling (Chapter 4, and J. Kissil et al., submitted).

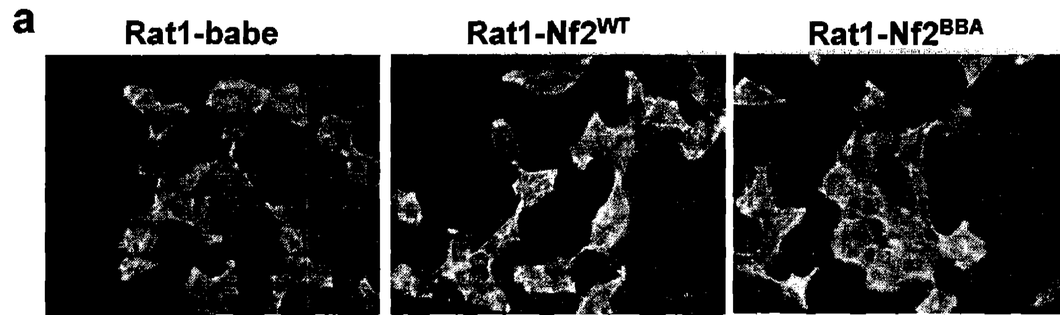
CyclinD1 expression is necessary for efficient Nf2^{BBA} transformation

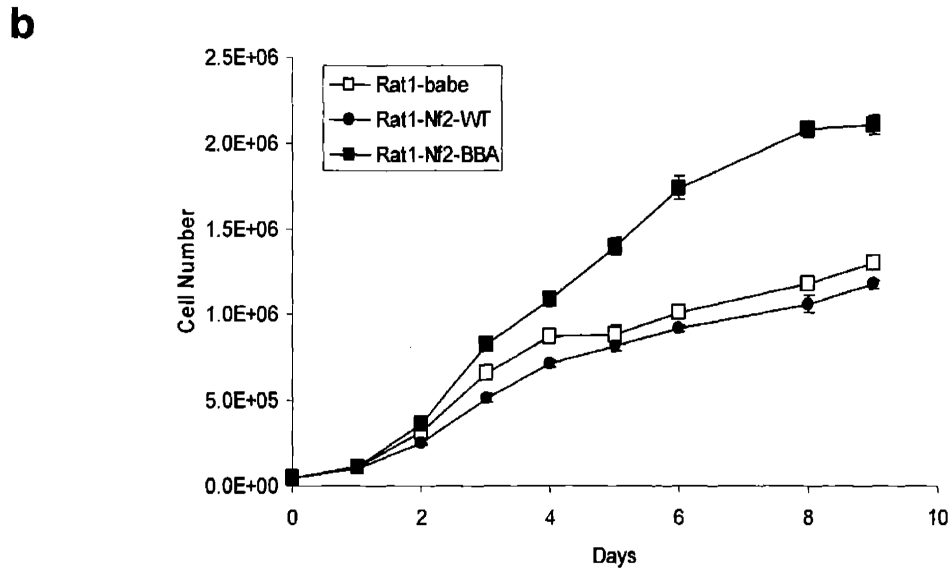
Finally, despite the lack of information regarding which pathways are upregulated downstream of merlin loss, I believed that the upregulation of cyclin D1 was critical to the tumorigenicity of the 3T3-Nf2^{BBA} cells. Previous studies have demonstrated that antisense

knock down of cyclin D1 levels in Ras-transformed cells leads to a decrease in their proliferative and transformative capacity (Liu et al. 1995). Therefore, in order to assess the contribution of upregulation of cyclin D1 to the transformation phenotype of 3T3-Nf2^{BBA} cells, we sought to knock down the high expression in these cells. To this end, we employed a recently developed lentilox vector system to deliver two different siRNAs directed against cyclin D1 (designated, si1-6 and si2-2) to 3T3-Nf2^{BBA} cells and control, 3T3-babe cells (Rubinson et al. 2003). In addition to containing the siRNA, this vector encodes constitutively expressed eGFP making identification and sorting of cells containing the siRNA feasible. 3T3-Nf2^{BBA} and 3T3-babe control cells were infected with the cyclin D1 lentilox viruses either alone or in combination to achieve the maximal amount of cyclin D1 knock down. Following infection, cells were sorted for GFP expression and the highest expressing cells were collected for experiments. As shown in Figure 10a, by western blot, cyclin D1 protein levels were knocked down significantly in cells that expressed si1-6, and only slightly in cells that expressed si2-2. Interestingly, knock down was more efficient in cells infected with an amphotropic (A) lentivirus than with an ecotropic (E) virus. In addition, real-time PCR analysis revealed that levels of cyclin D1 transcripts were reduced to as low as 3% of that observed in empty vector control cells (Figure 10a). It is noteworthy that with passaging, these cells regained some of the cyclin D1 expression, but the levels remained significantly lower than the control cells (data not shown). These cells were subjected to injection into the flanks of nude mice in order to assess whether downregulation of cyclin D1 in 3T3-Nf2^{BBA} cells resulted in a reduced capacity for transformation. As shown in Figure 10b, tumor growth was significantly reduced in cells expressing Nf2^{BBA} that also expressed the si1-6, si2-2 or both cyclin D1 siRNAs. Thus, even a small amount of cyclin D1 knck down had an effect on the tumorigenicity of the NF2^{BBA} cells. These data indicate that

upregulation of cyclin D1 expression is necessary for the transformation of these cells downstream of $Nf2^{BBA}$ expression. However, further studies are needed to characterize what signaling pathways lead to this upregulation. Perhaps there are as yet unidentified pathways that become perturbed following loss of merlin function, leading to an upregulation of cyclin D1 levels and cellular transformation.

In summary, although we have begun to eliminate some signaling pathways as important for cellular transformation by $Nf2^{BBA}$, it is clear that there are still many aspects to be explored and understood. Nevertheless, identification of the signaling pathways leading to cyclin D1 deregulation in these cells might assist in a more complete understanding of the mechanisms of transformation by loss of merlin, thereby leading to more effective treatments for tumors bearing *NF2* mutations.





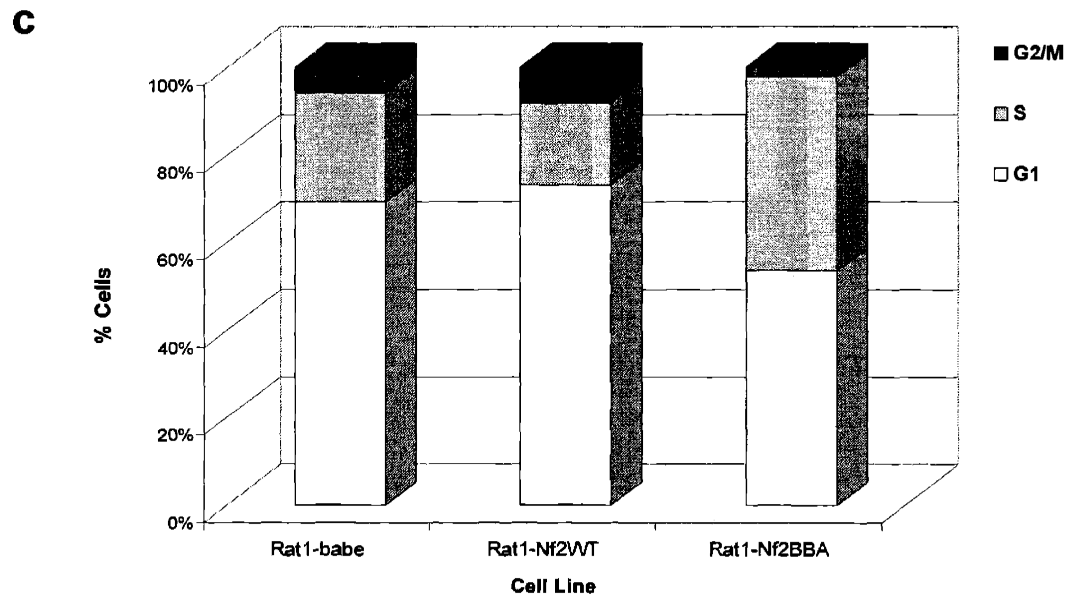
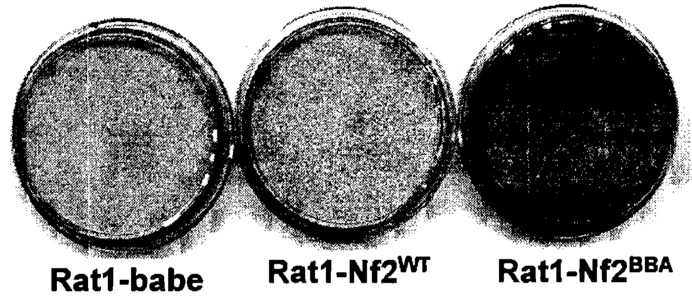


Figure 1. $Nf2^{BBA}$ transforms Rat1 fibroblasts. ***(a)*** Oregon-green phalloidin staining of the actin cytoskeleton. ***(b)*** Proliferation curve of *Nf2*-infected Rat1 cells counted every day for 8 days. ***(c)*** Graphic representation of cell cycle profiles of exponentially growing *Nf2*-infected Rat1 cells.

d



e

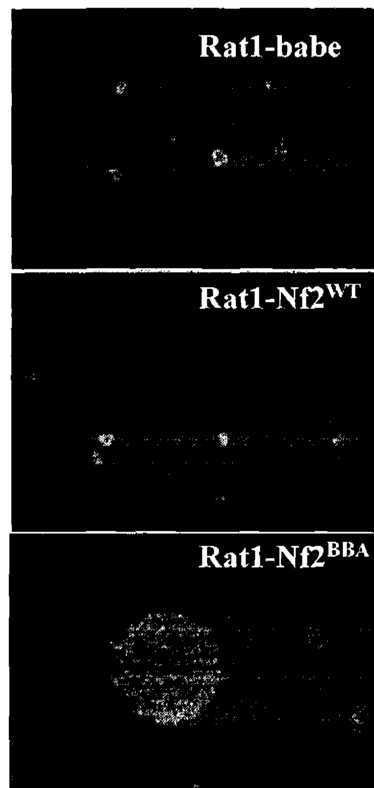
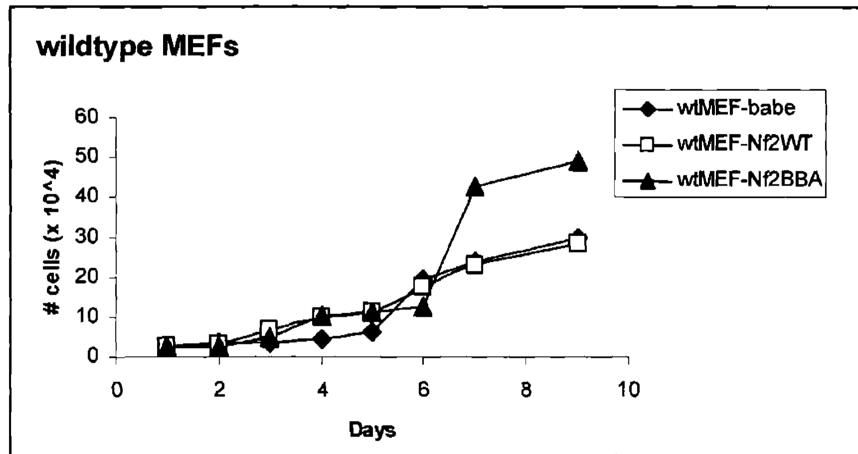
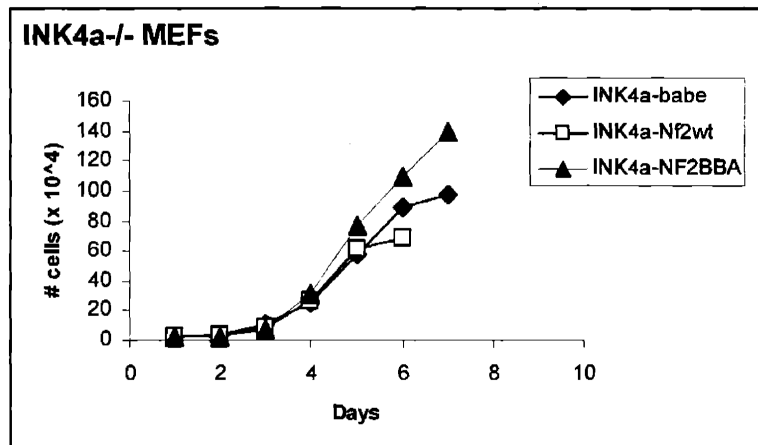


Figure 1 (cont). *(d)* Focus formation of cells on a monolayer visualized 4 weeks following plating by Giemsa staining. *(e)* Colony formation of *Nf2*-infected Rat 1 cells suspended in soft agar.





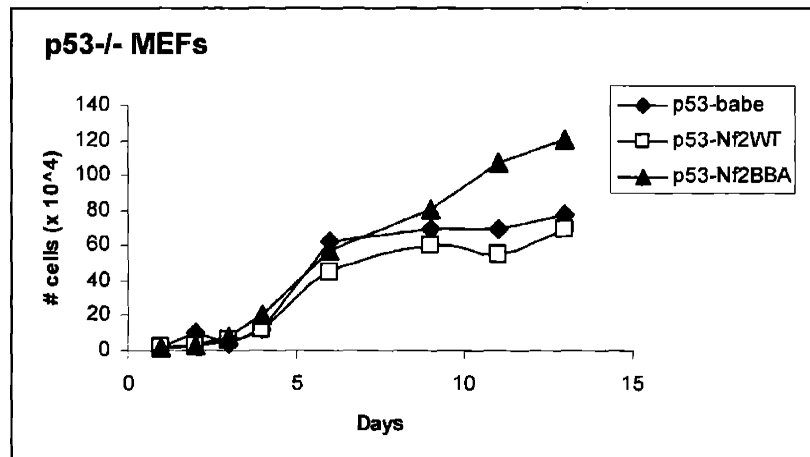


Figure 2. $Nf2^{BBA}$ causes proliferation to a slightly higher saturation density in several primary MEF lines. Proliferation curves of wild-type MEFs (*wtMEF*), INK4a-deficient (*INK4a-/-*), and p53-deficient (*p53-/-*) cells counted every day for 6 days.

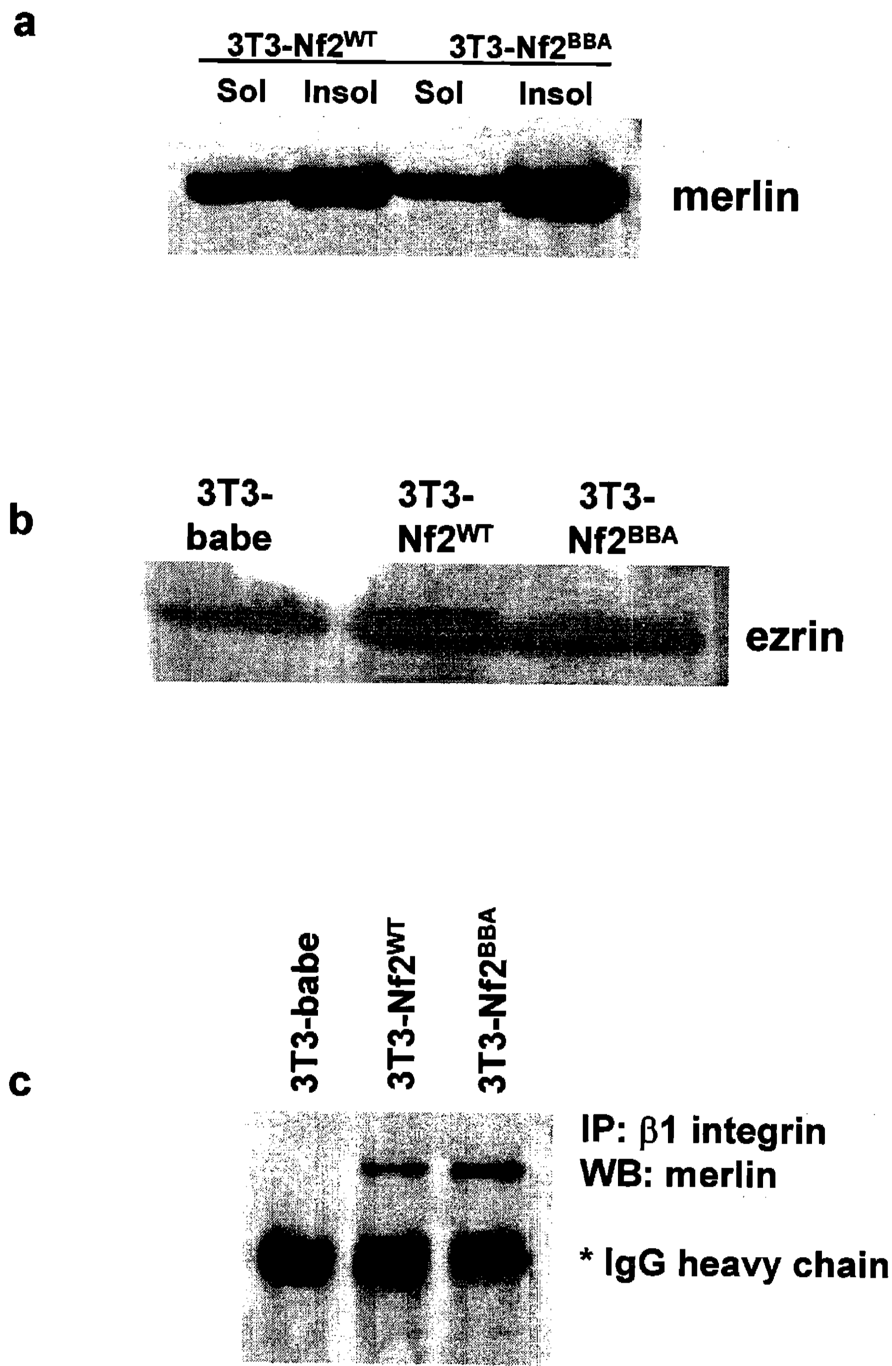


Figure 3. BBA mutation of *Nf2* does not cause altered solubility, ERM expression, β1 integrin interaction or motility of NIH3T3 cells. ***(a)*** Western analysis of merlin from 1% Triton X-100 soluble and insoluble fractions of 3T3-Nf2^{WT} and 3T3-Nf2^{BBA} cells. ***(b)*** Western analysis of ezrin expression in 3T3-Nf2^{BBA} and control cells. ***(c)*** Western blot analysis of merlin co-immunoprecipitated with β1 integrin from 3T3-Nf2^{WT} and 3T3-Nf2^{BBA} cells.

d

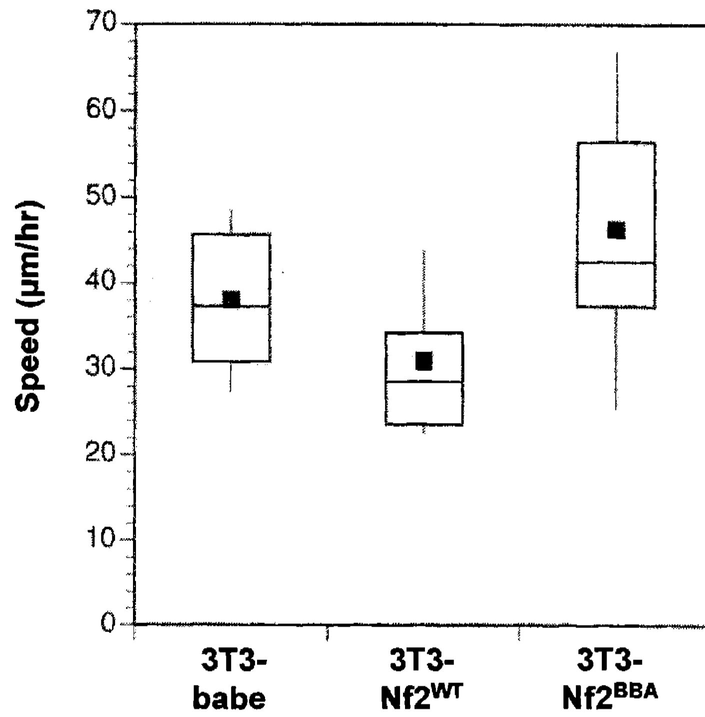


Figure 3 (cont). *(d)* Box and whiskers plot of motility of 3T3-Nf2^{BBA} and control cell speeds analyzed by time lapse microscopy (ANOVA p-value > 0.05).

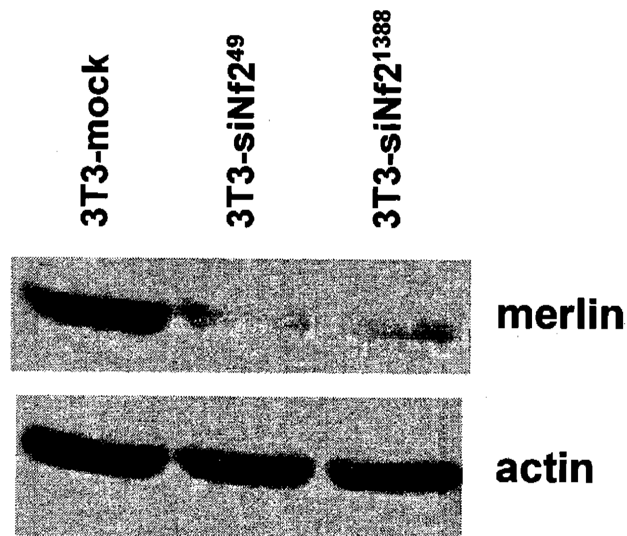


Figure 4. siRNA knockdown of merlin expression. Western blot analysis of merlin levels in wild-type NIH3T3 cells following mock infection or infection with pSIRIPP-siNf2^{49} or pSIRIPP-siNf2^{1388} retroviruses.

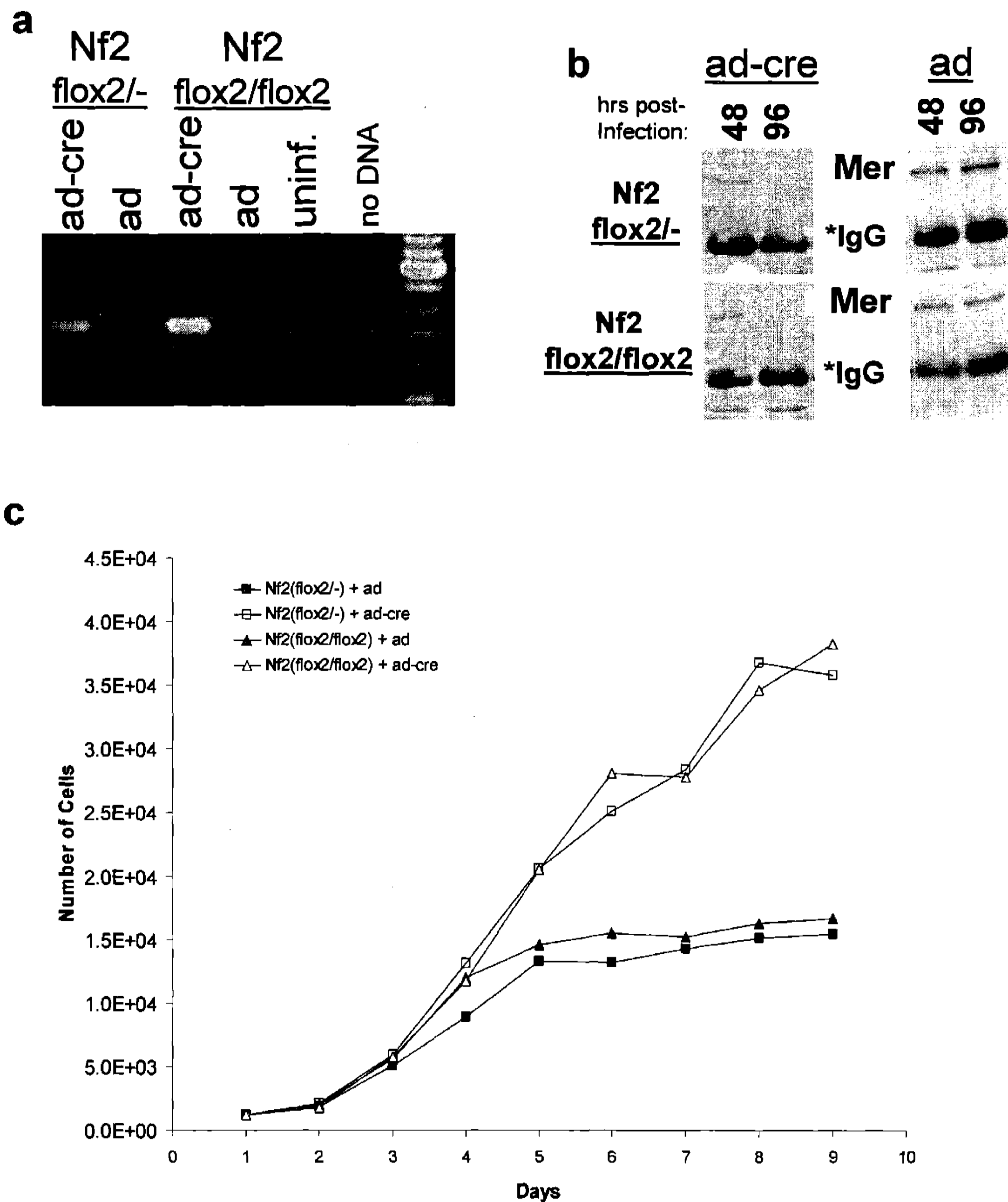


Figure 5. Phenotype of *Nf2*-deficient MEFs. ***(a)*** PCR analysis of recombination of the *Nf2* conditional allele 24 hrs following control, adenoviral GFP (ad), or adenoviral cre (ad-cre) infection of $Nf2^{flox2/flox2}$ or $Nf2^{flox2/-}$ MEFs. No infection (uninf) and no DNA samples are shown as controls. ***(b)*** Western analysis following immunoprecipitation of merlin from cells 96 hrs following control infection (ad) or ad-cre infection. ***(c)*** Proliferation curves of *Nf2*-conditional MEFs treated with ad or ad-cre counted everyday for 8 days.

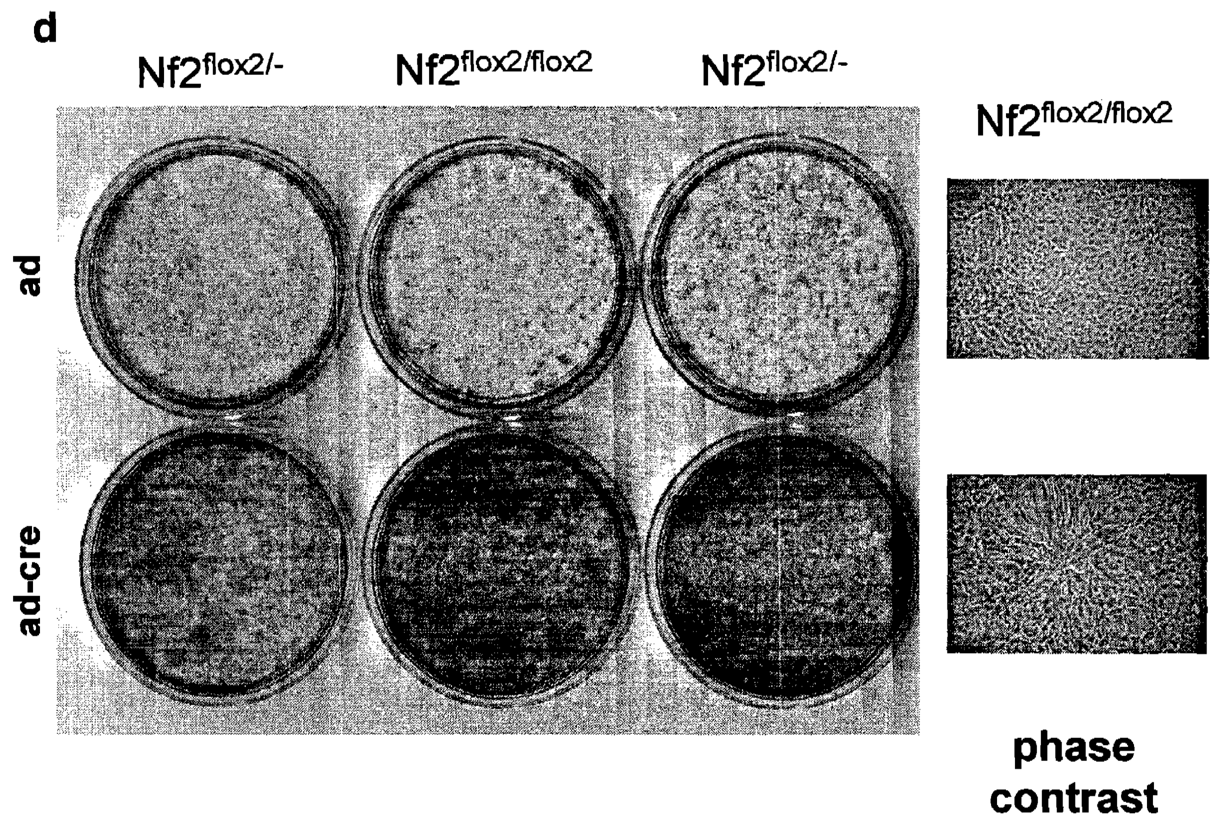
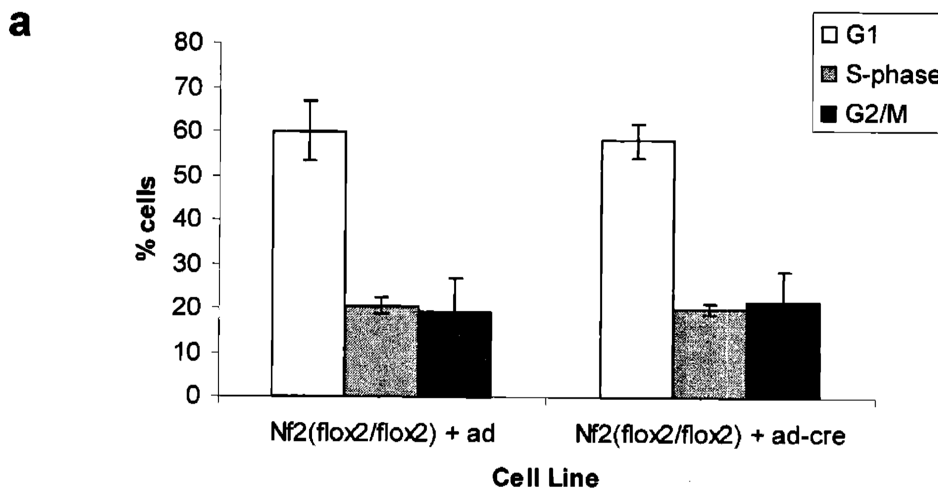


Figure 5 (cont). ***(d)*** Focus formation of *Nf2*-conditional MEFs visualized by Giemsa stain 4 weeks following plating. 4x phase contrast photomicrographs for the $Nf2^{flox2/flox2}$ cell line allow better visualization of the foci.

a



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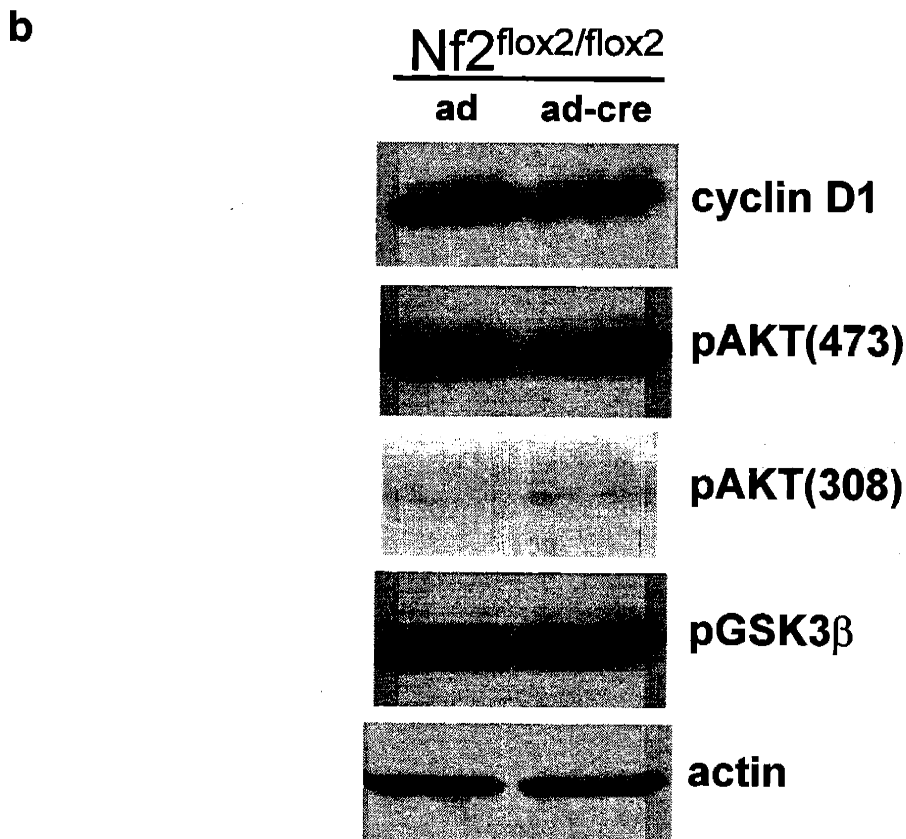
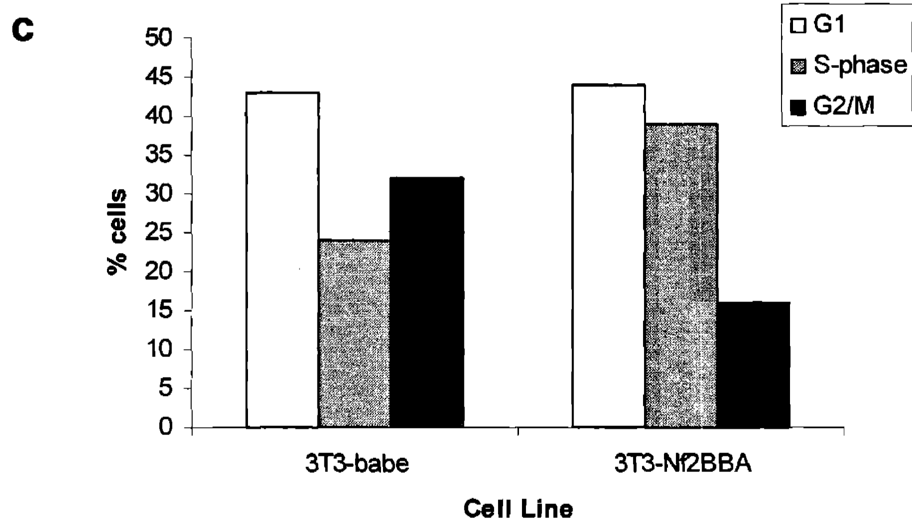


Figure 6. Cell Cycle and signaling in exponentially dividing *Nf2*-deficient MEFs and 3T3-Nf2^{BBA} cells. ***(a)*** Graphical representation of the percentage of cells in each phase of the cell cycle in exponentially growing *Nf2*-deficient MEFs compared to controls. ***(b)*** Western blot analysis of phospho-signaling proteins in exponentially growing *Nf2*-conditional cells compared to controls.



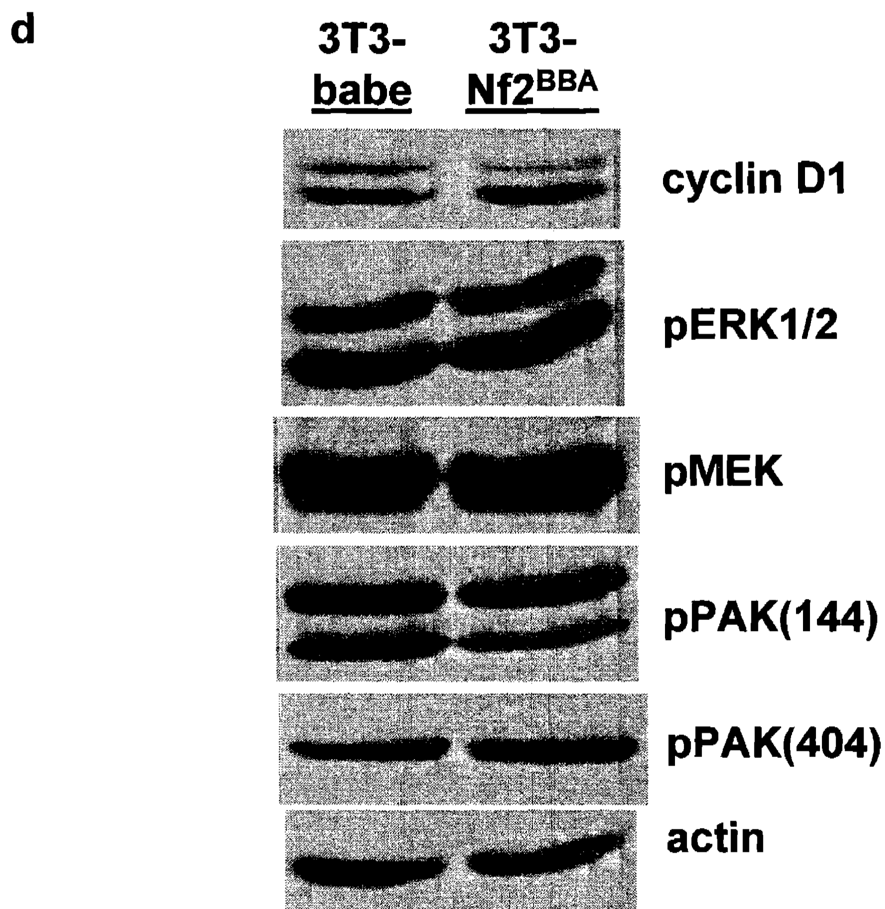


Figure 6 (cont). *(c)* Graphical representation of the percentage of cells in each phase of the cell cycle in exponentially growing 3T3-Nf2^{BBA} cells compared to controls. ***(d)*** Western blot analysis of phospho-signaling proteins in exponentially growing 3T3-Nf2^{BBA} cells compared to controls.

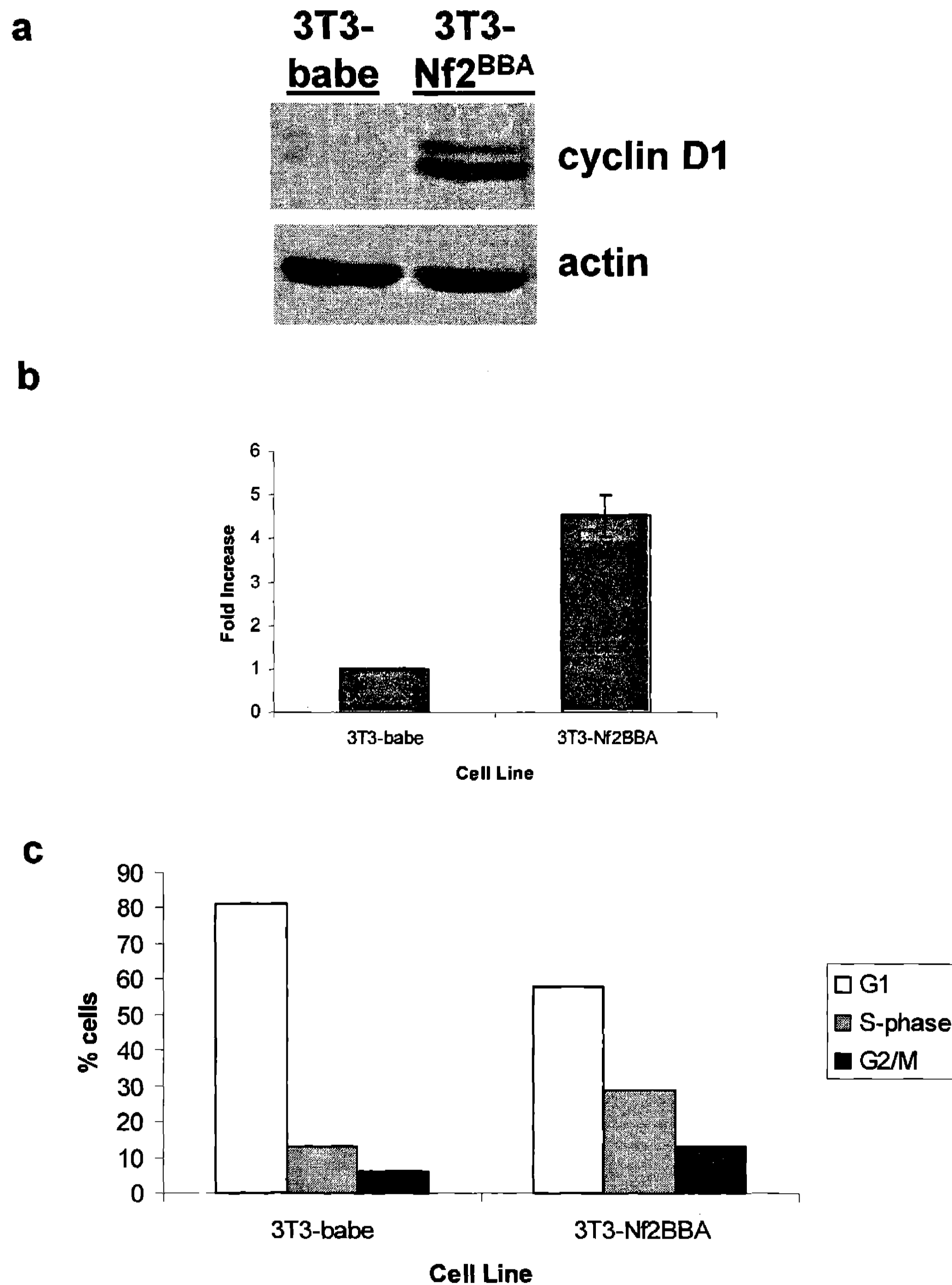


Figure 7. Cyclin D1 levels in serum-starved *Nf2*-deficient MEFs and 3T3-Nf2^{BBA} cells. ***(a)*** Western blot analysis of cyclin D1 levels in serum-starved 3T3-Nf2^{BBA} cells compared to controls. Actin is shown as a loading control. ***(b)*** Graphical representation of fold-increase of cyclin D1 mRNA transcripts in serum-starved 3T3-Nf2^{BBA} cells compared to 3T3-babe control cells as assessed by real time PCR. ***(c)*** Graphical representation of percentage of cells in each phase of the cell cycle in serum-starved 3T3-Nf2^{BBA} and control cells.

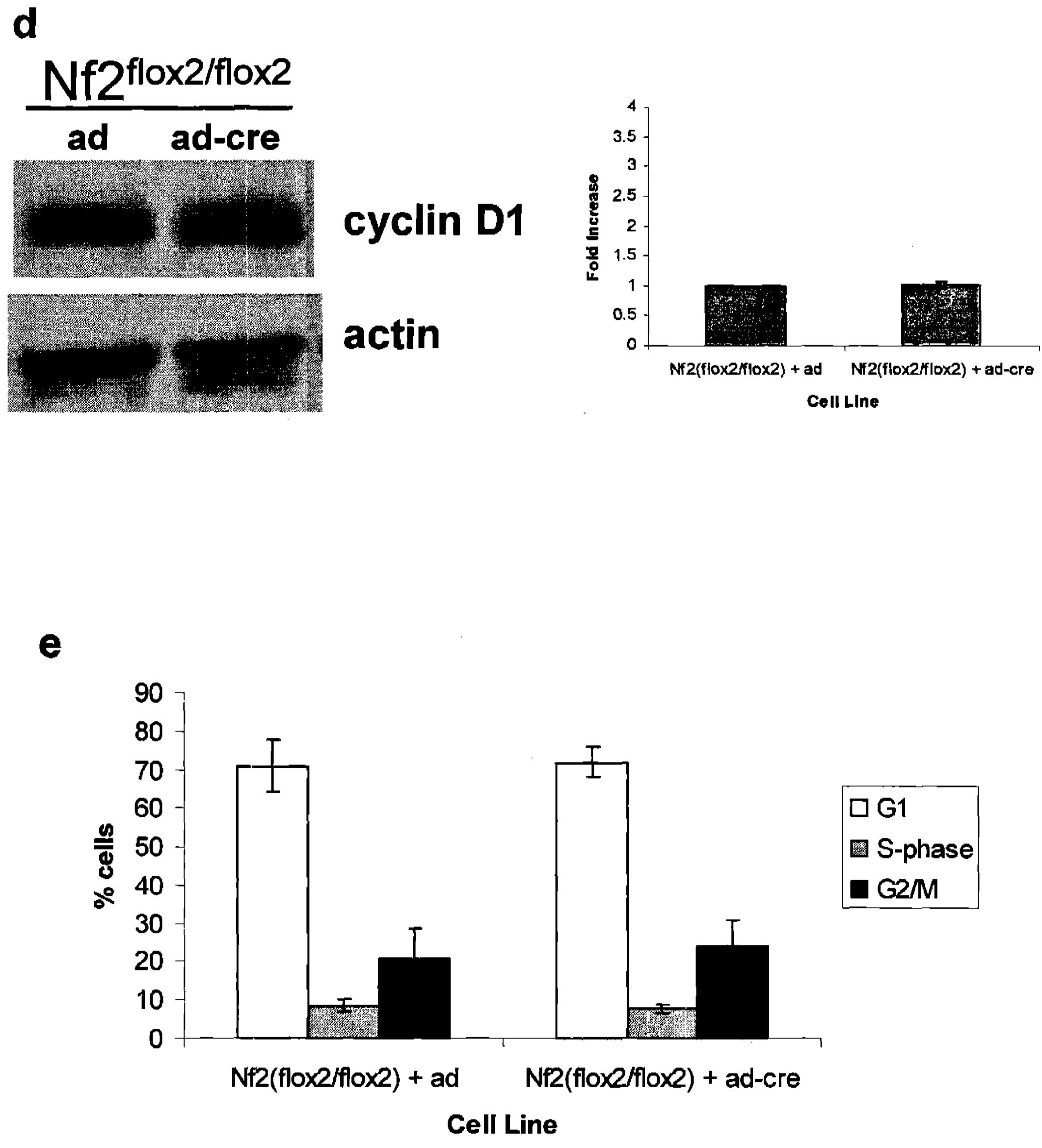


Figure 7 (cont). *(d)* Western blot analysis of cyclin D1 levels in serum-starved *Nf2*-deficient MEFs compared to controls and graphical representation of relative levels of cyclin D1 mRNA transcripts in serum-starved MEFs as assessed by real-time PCR. Actin is shown as a loading control for the western blot. *(e)* Graphical representation of percentage of cell in each phase of the cell cycle in serum-starved *Nf2*-deficient and control MEFs.

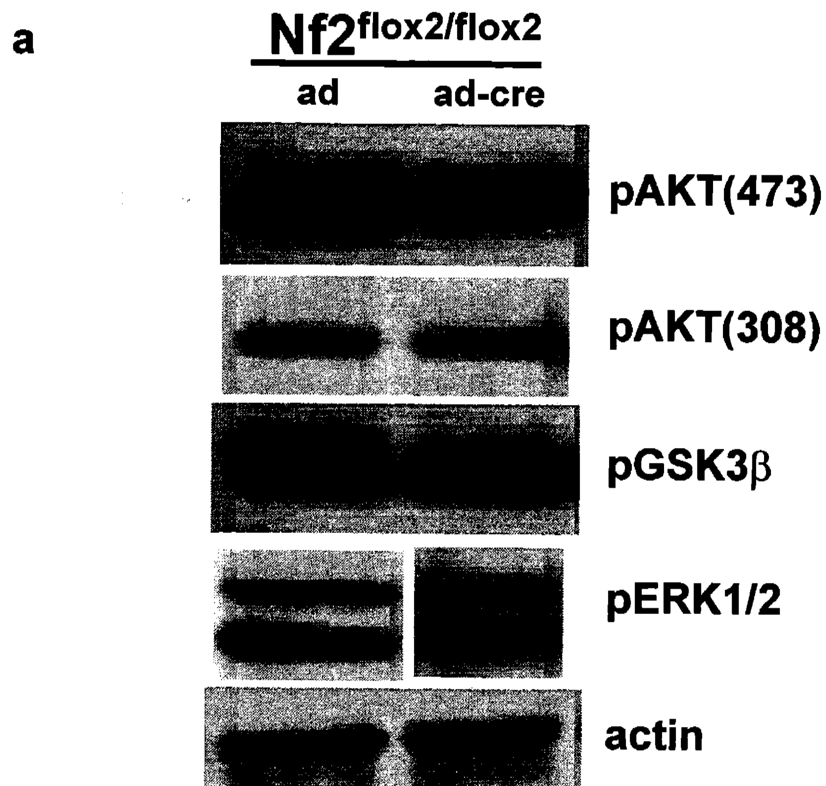


Figure 8. Signaling pathways in serum-starved *Nf2*-deficient MEFs and 3T3-Nf2^{BBA} cells. ***(a)*** Western blot analysis of phospho-signaling proteins in serum-starved *Nf2*-deficient and control MEFs. Actin is shown as a loading control.

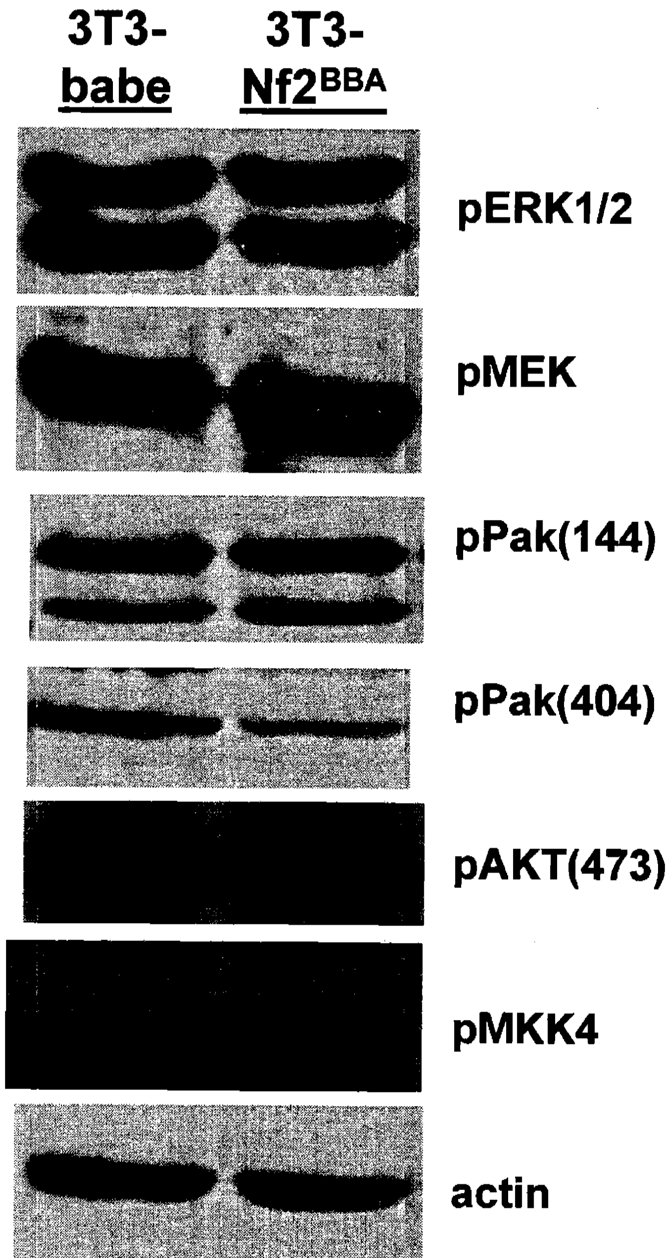
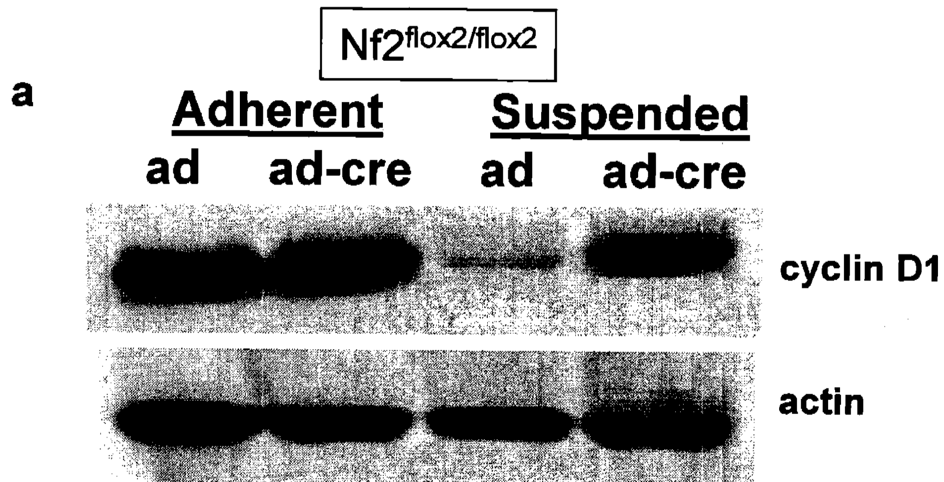


Figure 8 (cont). *(b)* Western blot analysis of phospho-signaling proteins in serum-starved 3T3-Nf2^{BBA} cells compared to controls. Actin is shown as a loading control.

Table 1. Summary of phospho-signaling analysis.

Antibody	Cell Line *serum starved*				Cell Line *exponential growth*			
	Nf2(flox2/ flox2)+ad	NF2(flox2/ flox2)+ad-cre	3T3-babe	3T3-NF2^{BBA}	Nf2(flox2/ flox2)+ad	NF2(flox2/ flox2)+ad-cre	3T3-babe	3T3-NF2^{BBA}
cyclinD1	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
p-AKT (308)	N.C.	N.C.	n.d.	n.d.	N.C.	N.C.	N.C.	N.C.
p-AKT (473)	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
p-GSK3b	N.C.	N.C.	n.d.	n.d.	N.C.	N.C.	n.d.	n.d.
p-MKK4	N.C.	N.C.	N.C.	N.C.	u.d.	u.d.	N.C.	N.C.
p-p38 MAPK	N.C.	N.C.	N.C.	N.C.	u.d.	u.d.	N.C.	N.C.
p-ERK1/2	N.C.	N.C.	N.C.	N.C.	n.d.	n.d.	N.C.	N.C.
p-MEK	n.d.	n.d.	N.C.	N.C.	n.d.	n.d.	N.C.	N.C.
p-Pak (144)	n.d.	n.d.	N.C.	N.C.	n.d.	n.d.	N.C.	N.C.
p-Pak (404)	n.d.	n.d.	N.C.	N.C.	n.d.	n.d.	N.C.	N.C.

N.C., No change
n.d., not determined
u.d., undetectable



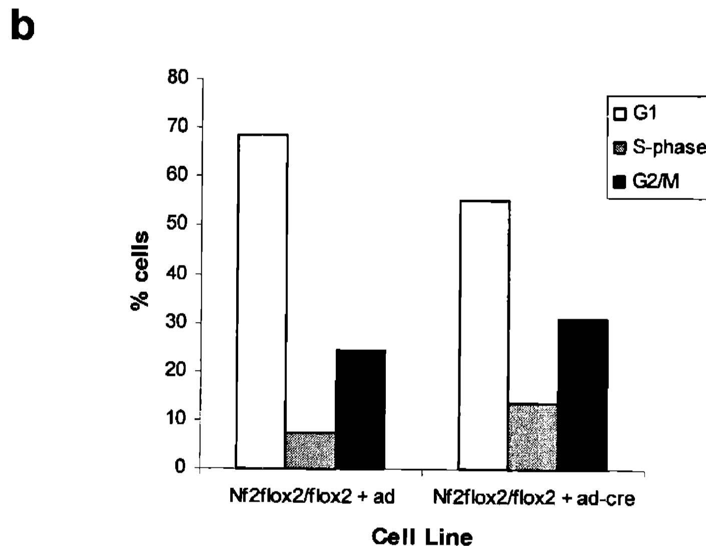
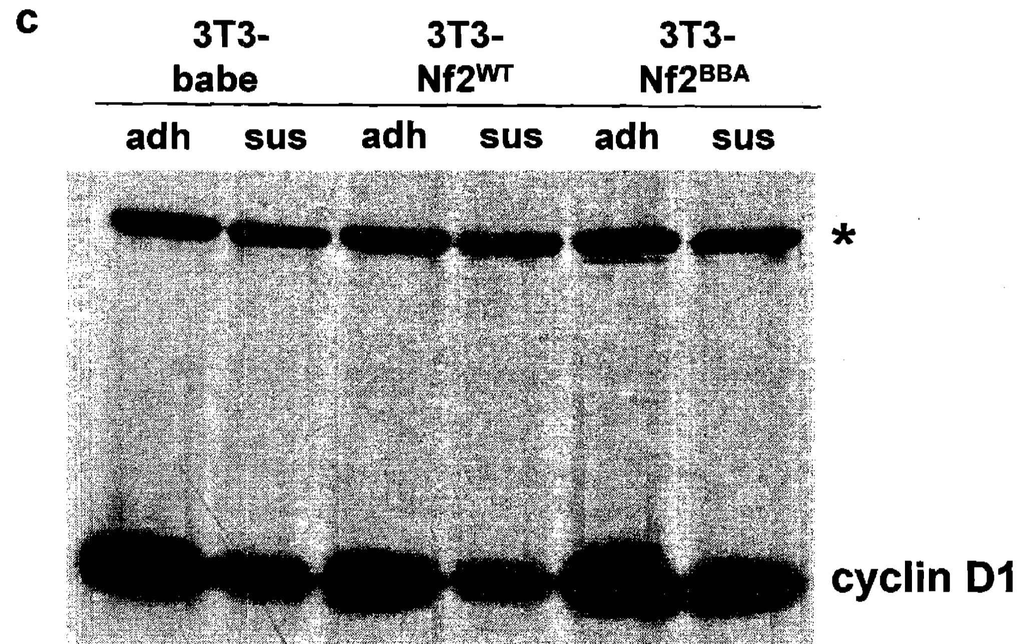


Figure 9. Cyclin D1 levels and cell cycle profiles following loss of adhesion in *Nf2*-deficient MEFs and 3T3-Nf2^{BBA} cells. ***(a)*** Western blot analysis of cyclin D1 levels in suspended (12 hrs) and adherent *Nf2*-deficient MEFs compared to controls. Actin is shown as a loading control. ***(b)*** Graphical representation of percentage of cells in each phase of the cell cycle in suspended *Nf2*-deficient and control MEFs.



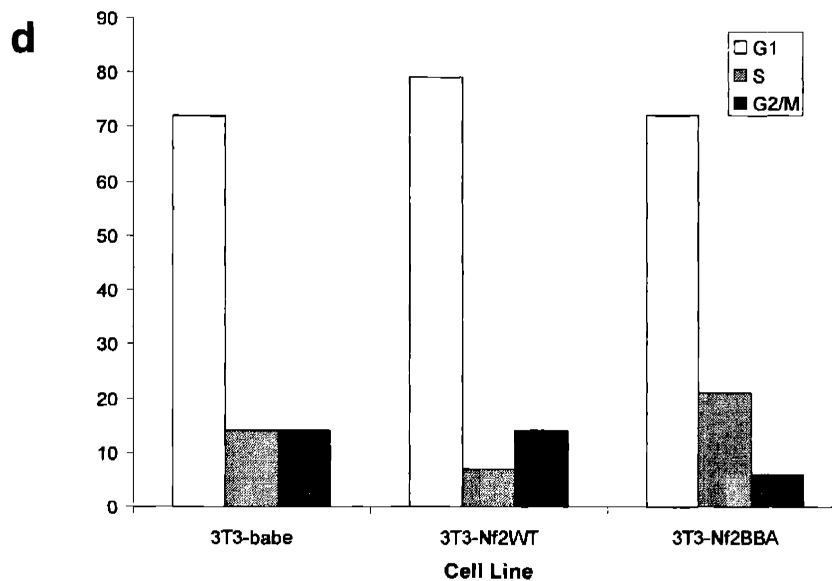
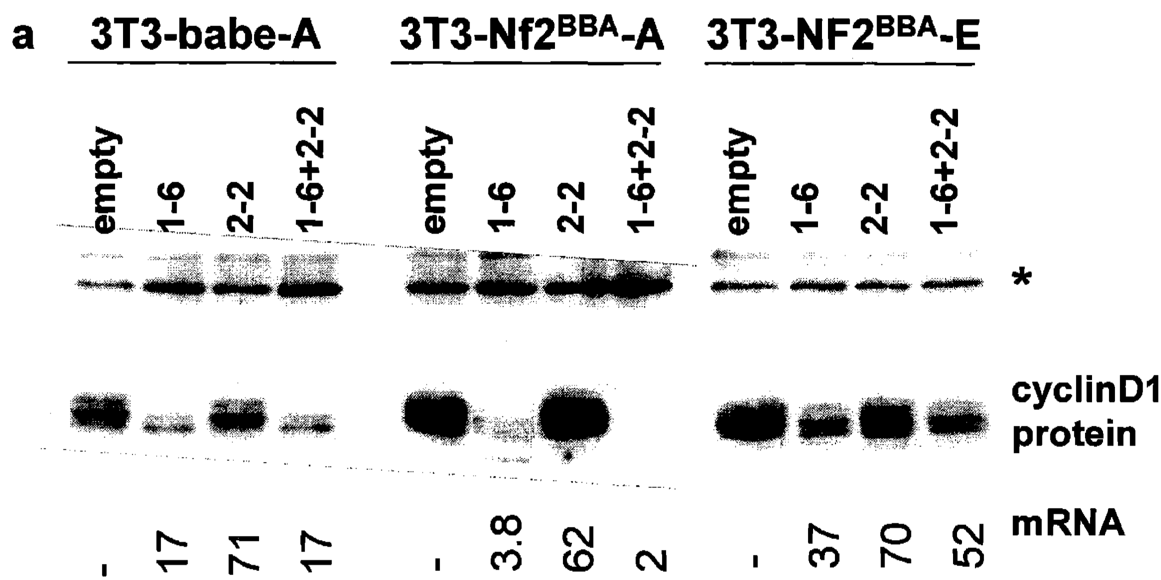


Figure 9 (cont). *(c)* Western blot analysis of cyclin D1 levels in suspended (12 hrs) and adherent 3T3-Nf2^{BBA} cells compared to controls. * crossreacting band indicating equal loading. *(d)* Graphical representation of percentage of cell in each phase of the cell cycle in suspended 3T3-Nf2^{BBA} and control cells.



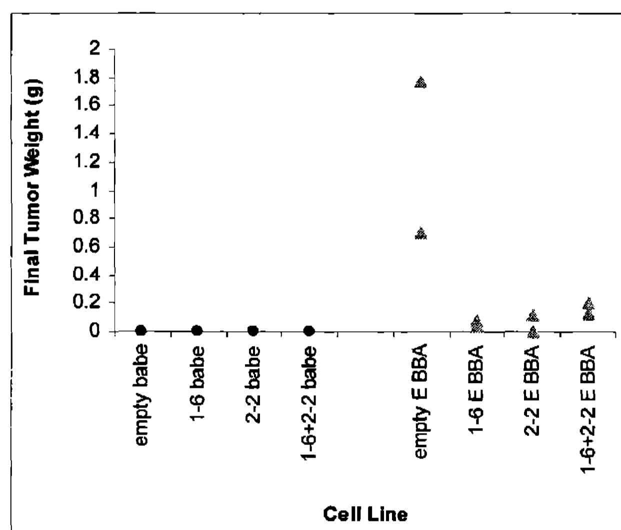


Figure 10. Knock down of cyclin D1 in 3T3-Nf2^{BBA} cells. *(a)* Western blot and real-time PCR analysis of cyclin D1 protein and transcript levels, respectively, in 3T3-Nf2^{BBA} and 3T3-babe cells following infection with Lentilox si-cyclin D1 vectors. Transcript levels are represented under the western blots as a percentage of empty-vector infected transcript levels measured in cells of the same genotype. "empty": infection with an empty lentilox siRNA vector; "1-6": infection with si1-6 vector; "2-2": infection with si2-2 vector; "1-6+2-2": infection with both si vectors. "A": amphotropic lentivirus; "E": ecotropic lentivirus. * crossreacting bands indicate relatively equal loading. *(b)* Graphical representation of growth of tumors in the flanks of nude mice measured once per week following injection with cyclin D1 siRNA (ecotropic) infected 3T3-Nf2^{BBA} and 3T3-babe cells.

Materials and Methods

Cell culture, transfections, and retroviral and lentiviral infections

NIH3T3 cells were cultured in DME plus 10% calf serum, glutamine and antibiotics. Rat1 fibroblasts, Swiss3T3 fibroblasts, MEFs, ΦNX, and 293T cells were cultured in DME plus 10% fetal calf serum, glutamine and antibiotics. Retroviral infections were carried out as described previously (Johnson et al. 2002). Ecotropic and amphitropic lentiviral infections were carried out on 3T3 cells using supernatants from 293T packaging cells 24 hrs following transfection with the appropriate vectors as described (Locker et al. 2000). MEFs were infected with adenoviral cre (ad-cre) or adenoviral GFP (ad) (Gene Transfer Vector Core Facility, University of Iowa College of Medicine) at ~ 100 PFU/cell. Medium was changed 24 hours post infection. Cells were assayed for recombination by PCR as described (Giovannini et al. 2000). Cell biological and transformation assays, including Oregon-green phalloidin staining, proliferation curves, cell cycle profiling using propidium iodide and FACS analysis, focus formation assays, soft agar assays and tumor formation in nude mice, were carried out as described (Johnson et al. 2002). Cells assayed under serum-starved conditions were cultured in 0.1% serum for 24 hrs prior to the assay.

Plasmids

pBABE-empty, pBABE-Nf2^{WT}, and pBABE-Nf2^{BBA} are as described (Johnson et al. 2002). pSIRIPP-siNf2^{49} and pSIRIPP-siNf2^{1388} were created by cloning annealed DNA oligos, corresponding to a stem loop structure created from selected *Nf2* 19-mer sequences, into the BglII and HindIII sites of pSIRIPP (kind gift of S. Losnick, Golub Laboratory). Sequences are as follows si49: 5'-GCAGCCCAAGACATTCACG-3', and si1388: 5'-

GCAGAGCGAAGAGCCAAGC-3'. pLL3.7-cycD1(1-6) and pLL3.7-cycD1(2-2) were created following annealing of DNA oligos corresponding to sequences forming a stem loop structure using cyclin D1 19-mer sequences (1-6: 5'- GATGAAGGAGACCATTCCC -3' and 2-2: 5'- CCTGGGCAGCCCCAACAAC -3') and cloning them into the XhoI and NotI sites of the pLL3.7 vector (kindly provided by Carla Kim, Jacks lab). Other lentilox system vectors used for packaging, including pVSVG, pECO-FMV, and pΔ8.2, were a kind gift of the Van Parijs laboratory (Center for Cancer Research, MIT).

Western blotting, immunoprecipitations, and antibodies

Cell lysates were prepared using either an SDS boiling lysis buffer (1% SDS, 100 mM TRIS, pH 6.8, 100 mM sodium orthovanadate, 1mM sodium fluoride), or a 1% Triton buffer (1% Triton X-100, 2mM EDTA, 150 mM NaCl, 50 mM TRIS, pH 7.5 and protease and protease inhibitors), and protein concentrations were assayed in lysates by BCA (Pierce) or Bradford (BioRad), respectively. 1% Triton X-100 soluble and insoluble fractions were obtained as described (Shaw et al. 1998a). Antibodies used in the described experiments were the following: cyclin D1 (sc-8396 or sc-718), merlin (sc-331 and sc-332), actin (sc-1616); β1 integrin rabbit polyclonal (kind gift of RO Hynes), ezrin (sc-6407). All signaling antibodies were rabbit polyclonal purchased from Cell Signaling: pERK1/2, total ERK1/2, pJNK, total JNK, pAKT(473), pAKT(308), total AKT, pGSK3β, pp38MAPK, pMKK4. Immunoprecipitations were carried out using 5 μl of β1 integrin antibody on 1 mg cell lysate (prepared using 1% NP40 buffer: 1% NP40, 10 mM TRIS, pH 6.8, 100 mM NaCl and protease and phosphatase inhibitors) precleared with protein A beads. Antibody-lysate mixtures were rotated for 4 hours at 4°C, then for 1 hour following addition of

protein A beads. Immune complexes were washed extensively and subjected to western blot analysis.

Time lapse cell motility assays

10^5 cells adapted to microscopy media (low bicarbonate) were plated on a ΔT dish (Bioptechs) coated with 10 μg/ml fibronectin (Becton Dickenson) and blocked with 1 mg/ml BSA (Sigma). After 1 hr, movies were started. 10X phase movies were 4.5 hrs long with frames taken every 5 min using IPLabs software on a NikonTE300 microscope. Movies were analyzed with DIAS Software (Solltech). All cells analyzed remained entirely within the field of view, did not more than transiently touch other cells, and did not divide. Cell paths were generated from centroid positions and speed was computed using the central difference method in DIAS. 15 cells over 25 frames were analyzed for each cell type.

Suspension assays

For adherent vs. suspended cell assays, 1×10^6 cells were plated either on regular tissue culture dishes (adh) or on polyHEME coated Petri dishes (sus). 12 hrs following plating cells were trypsinized off the adh plates, washed and pelleted before lysing for cyclin D1 western blotting or treatment for FACS analysis. Floating cells (sus) were collected by pelleting from the media on the polyHEME coated plates. Cell pellets were washed before lysing for cyclin D1 western analysis or treatment for FACS analysis.

Real-time PCR

Total RNA was isolated from the 2×10^6 cells on a 10-cm dish using an RNAse easy Mini kit

and RNase-free DNase set according to manufacturer's instructions (Qiagen). Reverse transcription was performed with the Superscript II Kit (Ambion). The cDNA products were diluted to a final concentration of 1 μg/ul in sterile water. PCR primer pairs were designed with the Primer Express 1.5 software that accompanies the ABI Model 7000 sequence detector (PE Applied Biosystems). Primers were designed to overlap an intron. Primer sets were tested to verify the amplification of a single band of the correct predicted size. SybrGreen probes were purchased from PE Applied Biosystems. To normalize the data with respect to total RNA, each sample was normalized to TATA binding protein (TBP) as an internal control. CyclinD1 primer sequences were as follows: (cycD1-F) 5'- ACAAGCAGACCATCCGCAAG -3';
(cycD1-R) 5'- AGGGTGGGTTGGAAATGAACT – 3'. TBP control primers were as follows:
(TBP-F) 5' –AATGCTTCATAAATCTCTGCTCTAACTTTA – 3';
(TBP-R) 5' – TGGAAAAGTTGTATTAACAGGTGCTAA – 3'.

Acknowledgements

We are grateful to Carla Kim for the creation of the pLentilox-cycD1 siRNA vectors and Han You and Shomit Sengupta for technical assistance with real-time PCR. This work was supported in part by grants from the Department of the Army. KCJ was a David Koch graduate fellow during many of these studies. TJ is an Investigator of the Howard Hughes Medical Institute.

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Chapter 4

The *Nf2* tumor suppressor protein, merlin, downregulates the Rac pathway via a direct interaction with the p21 activated kinase, Pak1.

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The author performed the majority of the work presented here, with the exception of the 2-dimensional gel electrophoresis assays (Figures 2 and 3), which were carried out by Joseph Kissil.

Abstract

The Neurofibromatosis type 2 tumor suppressor gene (*NF2*) is lost in inherited and sporadically-occurring central nervous system tumors. The *NF2* protein, merlin, shares high similarity with a family of membrane-cytoskeletal linkers suggesting a role for merlin in regulating cytoskeletal function. However, the mechanism of the tumor suppressor function of merlin is poorly understood. Cell-based studies have pointed toward merlin regulation by and of the Rac pathway, as merlin is phosphorylated by Pak, a Rac effector, and *Nf2-/-* fibroblasts have elevated JNK activity, respectively. Here, we report a novel function for merlin as an inhibitor of the p21 activated kinase, Pak1. The details of the interaction between merlin and Pak and its implications are discussed.

Introduction

Neurofibromatosis type 2 (NF2) is a dominantly-inherited cancer disorder that results in development of tumors of the central nervous system (Gusella et al. 1999). The *NF2* tumor suppressor gene was cloned in 1992, and its protein product, termed merlin, was found to have significant homology to the ERM proteins (ezrin, radixin and moesin), members of the band 4.1 family of membrane cytoskeletal linkers (Rouleau et al. 1993; Trofatter et al. 1993). A traditional mouse model of NF2 has been created which develops highly metastatic sarcomas (McClatchey et al. 1998). In addition, a conditional mouse model of NF2 which utilized P0-cre, leading to loss of merlin specifically in Schwann cells, closely mimicked the disease phenotype (Giovannini et al. 2000).

Based on the metastatic phenotype of *Nf2-/-* tumors and the phenotypes of expression of mutant alleles of *Nf2* (or loss of *Nf2*) in various cellular contexts, it is thought that merlin may play a role in maintenance of cell adhesion, contact inhibition, cell shape and control of cell migration (Bretscher et al. 2002; Gautreau et al. 2002). Moreover, merlin expression is upregulated by growth-inhibitory signals that would be consistent with these observations, including loss of cell adhesion, confluency and serum starvation (Shaw et al. 1998). Early studies suggested that merlin was capable of inhibiting Ras induced transformation (Tikoo et al. 1994). More recently, merlin has been placed in a pathway downstream of CD44 in the regulation of contact inhibition in rat schwannoma cells (Morrison et al. 2001), and loss of merlin has been shown to disrupt adherens junction organization in fibroblasts and keratinocytes (Lallemand et al. 2003).

Previous studies from our lab have demonstrated that merlin is phosphorylated on a serine residue in its C-terminal domain (S518) downstream of activation of Rac or Cdc42, small

GTP-binding proteins that integrate signals at the cell cortex that result in cytoskeletal rearrangements (Shaw et al. 2001). Experiments indicate that phosphorylation of merlin at this residue disrupts the self-association of the protein, possibly affecting its ability to function as a growth suppressor. Interestingly, in concert with its role in cytoskeletal function, recent cell-based studies have indicated a role for merlin in downregulation of the Rac/Cdc42 pathway (Pelton et al. 1998; Shaw et al. 2001). Loss of merlin in fibroblasts leads to increased signaling through JNK and the AP-1 transcription factor, as well as increased membrane ruffling and an increased rate of migration (Shaw et al. 2001). Merlin S518D, a mutant that mimics constitutive phosphorylation, and thus inactivation, was found to associate less with the cytoskeleton and had a reduced capability to inhibit AP-1 transcriptional activity. Its active counterpart, merlin S518A, has been shown to be more tightly associated with the cytoskeleton, strongly inhibits AP-1 activity, and restores contact inhibition and adherens junction organization to *Nf2-/-* fibroblasts (Lallemand et al. 2003). Recently, we and others reported that p21 activated kinase (Pak) mediates phosphorylation of merlin on serine 518 (Kissil et al. 2002; Xiao et al. 2002). In summary, it appears that merlin is both regulated by and itself regulates the Rac/Cdc42 pathway via a negative feedback loop. However, the details of the negative regulation and what role these functions play in the tumor suppressive activity of merlin remain unclear.

The p21 activated kinases (Pak 1, 2, and 3; group I Paks) are serine/threonine kinases which are molecular targets for the small GTP-binding proteins Rac and Cdc42 (Jaffer and Chernoff 2002). Extensive study on the group I Paks has revealed their important roles in actin cytoskeleton reorganization, cell motility, gene expression, and more recently, apoptosis (Kumar and Vadlamudi 2002). Less is understood about the recently identified group II Paks, Pak 4-6, which have been demonstrated to have both overlapping and distinct roles from the group I Paks.

Studies using dominant-negative alleles of Pak demonstrate that Pak1 is necessary for high efficiency transformation downstream of Ras, but that Pak activation alone is not sufficient for transformation (Tang et al. 1997; Tang et al. 1998; Tang et al. 1999). In certain cell types, Ras activates Pak and Pak potentiates signaling through ERK (Tang et al. 1999; Chaudhary et al. 2000; Eblen et al. 2002). Note, however, that the role of Pak1-3 in transformation remains under debate (Joneson et al. 1996; Lamarche et al. 1996; Westwick et al. 1997). Pak inhibition of Ras transformation seems to be very cell type specific with most observations being recorded in Rat1 fibroblasts (Tang et al. 1997). Surprisingly, there seems to be no link between Ras and Pak activation in NIH3T3 cells (Tang et al. 1998).

Pak activity is regulated by a variety of mechanisms. Structural studies indicate that, in the absence of G protein binding, group I Pak kinase activity is repressed by an intramolecular interaction between N-terminal regulatory and C-terminal catalytic domains. Rac/Cdc42 binding to the N-terminal CRIB (Cdc42/Rac interacting binding) domain of the Paks releases this repression and allows for autophosphorylation (Thr423) of the C-terminal catalytic domain and, thus, kinase activation in an adhesion- and growth factor- dependent manner (Zenke et al. 1999; del Pozo et al. 2000; Lei et al. 2000). In addition to activation by small G proteins, Pak has been shown to be regulated by lipids, such as sphingosine (Bokoch et al. 1998), and adaptor proteins, such as βPIX/p85COOL, paxillin, p95PKL, and Nck, that act by recruiting Pak from the cytoplasm to focal adhesions or the cell membrane (Manser et al. 1998; Turner et al. 1999; Brown et al. 2002). Stimulation through receptor tyrosine kinases as well as sensing of adhesion via integrins can affect Pak recruitment to the membrane, and, thus, its activation (Hashimoto et al. 2001).

Pak has many downstream effectors which mediate its regulation of different cellular processes. Pak exerts its function on cytoskeletal dynamics through a variety of known actin remodeling proteins. In addition, filamin (Vadlamudi et al. 2002) and paxillin (Hashimoto et al. 2001), both signaling scaffolds for cytoskeletal rearrangements, are regulated by Pak phosphorylation and binding. Finally, Pak promotes gene transcription and cell survival by directly phosphorylating kinases of the MAPK signaling cascade, including Raf (Chaudhary et al. 2000) and MEK (Frost et al. 1997), by phosphorylation and inactivation of the pro-apoptotic protein, Bad (Schurmann et al. 2000), and by upregulating the activities of JNK and the NFκB transcription factor (Bagrodia et al. 1995; Zhang et al. 1995; Frost et al. 2000).

Based on the recent identification of merlin as a negative regulator of Rac signaling, we investigated the possibility that merlin mediates its inhibitory function via control of Pak activity. Here, we demonstrate a novel function for merlin as an inhibitor of Pak. To date, only a few negative regulators of Pak have been identified, including a novel protein, hPIP, which inhibits GTPase-stimulated activity of Pak by binding to the CRIB domain (Xia et al. 2001). In this report, we investigate the domains that mediate the interaction between merlin and Pak. In addition, we examine the effect of merlin regulation of Pak on known downstream effectors. Finally, we propose several models for the regulation of Pak by merlin.

Results

Merlin inhibits Pak activity

In order to begin to assess at what level merlin functions to inhibit the Rac pathway, we wanted to examine the effect of merlin expression on levels of activated Rac in cells in culture. Two cell types were employed to determine the effects of merlin on Rac activity in this assay (and, also, later for determination of Pak activity): two *Nf2*-conditional MEF lines, treated with adenoviral cre recombinase (ad-cre) or empty adenovirus (ad), and rat schwannoma cells containing an inducible allele of merlin (RT4-67) (Morrison et al. 2001). In addition to carrying an $Nf2^{flox2}$ allele, the conditional MEFs harbored either an *Nf2* deletion allele rendering them heterozygous for *Nf2* ($Nf2^{flox2/-}$) or a wild-type *Nf2* allele, rendering them wild-type at the *Nf2* locus ($Nf2^{flox2/+}$). Following addition of ad-cre, which catalyzed the recombination of the floxed allele, the MEF lines became *Nf2*-deficient and *Nf2* heterozygous, respectively, thereby allowing for analysis of all possible genotypes in this study. Merlin loss in the conditional MEFs was assessed by two methods following ad-cre treatment: (1) PCR for the recombined allele (data not shown) and (2) immunoprecipitation of merlin followed by western blotting. As shown in Figure 1a, treatment with ad-cre, but not ad, in the $Nf2^{flox/-}$ MEFs resulted in loss of detectable merlin expression 96 hours post infection, while $Nf2^{flox/+}$ MEFs showed no alteration in merlin levels with either treatment (empty ad or ad-cre). Secondly, tetracycline-inducible rat schwannoma cells (RT4-67) were employed in order to assess whether merlin expression inhibits the Rac pathway at the level of Rac, itself. Previous studies have demonstrated that basal levels of merlin in RT4-67 cells are low, and that upon doxycycline induction, merlin expression is significantly increased (Morrison et al. 2001) (see also, Figure 1b).

Rac activity was assessed in RT4-67 cells through the use of a GST-fusion of the CRIB domain of Pak immobilized on agarose. GST-CRIB was used as bait to pull down all GTP-bound, active Rac from equivalent amounts of the different cell lysates. Rac-GTP levels were evaluated following extensive washing of the beads, addition of SDS sample buffer, and boiling to release the bound protein. Western blotting was carried out using an antibody against total Rac. Whole cell lysates blotted with Rac antibody were used as a loading control. Induction of expression of merlin 48 hours prior to lysate harvest in RT4-67 cells resulted in no change of Rac-GTP levels (Figure 1b). There were no differences in total levels of Rac in the cell. These data indicate that overexpression of merlin does not lead to a change in levels of activated Rac in the cell. Therefore, because merlin has been shown to regulate the Rac pathway, we sought to examine pathways downstream of Rac, itself.

Based on the observation that merlin regulates Rac signaling at some level downstream of Rac, we decided to test the whether mediation of this inhibition occurred through Pak, one of the best characterized Rac/Cdc42 effectors. The phosphorylation status of Pak serves as a direct indication of the activation of the kinase (Buchwald et al. 2001; Chong et al. 2001). In order to examine differences in the phosphorylation state of Pak1, we employed 2-dimensional gel eletrophoresis, a method used successfully by Garcia Arguinzonis and colleagues to separate the different forms of activated Pak (Garcia Arguinzonis et al. 2002). As a proof-of-principle experiment, we collected lysates from wild-type MEFs serum starved overnight (MEF-untreated) or from cells serum starved overnight followed by serum stimulation with PDGF for 5 minutes (MEF+PDGF). Following quantification, equal amounts of each lysate were separated by isoelectric focusing on an immobilized pH 4-7 gradient. Subsequently, the extracts were subjected to separation in the second dimension by SDS-PAGE, transferred to membranes, and

analyzed by western blotting using an anti-Pak1 antibody. As shown in Figure 2a, whereas only two forms of Pak1 were detected under conditions of serum starvation (MEF-untreated), likely corresponding to un- or hypo-phosphorylated Pak, several additional, hyperphosphorylated spots were detected upon stimulation with PDGF (MEF+PDGF), consistent with known Pak activation following serum stimulation. The additional spots, which are more acidic than those seen in the untreated sample, follow a signature pattern expected from a phosphoprotein that contains multiple phosphorylation sites. In order to establish that the additional spots were, in fact, due to hyperphosphorylation of the protein, extracts from PDGF-treated cells were lysed and treated with protein phosphatase 1 (PP1) (MEF+PDGF+PP1) prior to their separation by 2-D gel electrophoresis. As shown in Figure 2a, treatment with the phosphatase resulted in the disappearance of the additional, more acidic forms of Pak seen following PDGF treatment. When treated with PDGF, PP1 *and* phosphatase inhibitors, the additional spots reappeared (data not shown). Finally, as further confirmation that results from this assay bear relevance to Pak activity, lysates prepared from cells + PDGF treatment efficiently phosphorylated the substrate myelin basic protein (MBP) in an *in vitro* Pak kinase assay (Figure 2b).

Nf2 conditional fibroblasts were employed to test the activity of Pak in the presence and absence of merlin expression. 2-D analysis was performed on lysates harvested from serum starved MEFs plated 5 days after ad or ad-cre infection. Under these conditions, control MEFs (*Nf2*$^{flox/-}$ + ad, *Nf2*$^{flox/+}$ + ad, and *Nf2*$^{flox/+}$ + ad-cre) showed no activation of Pak, as demonstrated by detection of only hypo-phosphorylated forms of the protein (Figure 3a). Conversely, *Nf2*-deficient MEFs (*Nf2*$^{flox/-}$ + ad-cre) showed a striking activation of Pak, as demonstrated by the appearance of additional phosphorylated forms of the kinase. Thus, loss of merlin expression in MEFs was coincident with an upregulation of Pak activity under conditions which are normally

associated with Pak inactivity (serum starvation) (see Figure 2). These data are consistent with merlin functioning as an inhibitor of Pak.

Next we wanted to assess Pak activity in rat schwannoma cells in the presence of overexpressed merlin. Basal Pak activity was very high in RT4-67 cells following 24 hours serum starvation in the absence of merlin expression (RT4 - dox) (Figure 3b). However, upon induction of merlin expression (48 hrs prior to lysate harvest; RT4 + dox), we saw a significant reduction in the level of Pak activity, as demonstrated by fewer spots seen by 2-D gel analysis. Thus, reintroduction of merlin into RT4 schwannoma cells, which display a high level of basal Pak phosphorylation, results in the inhibition of Pak activity. These data, together with that generated from the MEFs, support the hypothesis that merlin is a negative regulator of Pak.

The FERM domain of merlin mediates interactions with Pak

In order to understand the mechanism by which merlin regulates Pak activity, we sought to test the interaction between merlin and Pak. Co-immunoprecipitation studies were used to test for a stable interaction between merlin and Pak in NIH3T3 and rat schwannoma (RT4-DP6) cells. Expression vectors for both Pak and merlin were transiently transfected into NIH3T3 cells 48 hours prior to lysate collection. In the case of RT4-DP6 cells, the interaction between endogenous merlin and Pak was assessed. Immunoprecipitations were performed in both directions using either a Pak1 antibody or a merlin antibody, followed by western blot detection using merlin and Pak antibodies, respectively. As shown in Figure 4a, merlin and Pak co-immunoprecipitate in both cell types. These data indicate that merlin and Pak interact in cultured cells, but do not indicate whether this interaction is direct or mediated through other proteins. Therefore, we employed an *in vitro* GST pulldown assay to determine possible direct interaction

between these two proteins. Full-length Pak was produced in bacteria as a GST fusion protein and purified on glutathione agarose beads. ^{35}S-labeled merlin was produced by *in vitro* transcription / translation and was incubated with GST-Pak agarose or with GST agarose, as a negative control. As shown in Figure 4b, merlin interacted with GST-Pak, but not with GST alone. Thus, the interaction between Pak and merlin is likely to be direct.

Having established a direct connection between merlin and Pak, we wanted to determine what domain(s) of merlin mediated this interaction. To examine whether the C-terminal tail or N-terminal FERM domain mediated the interaction with Pak, we created and tested such fragments in the pull-down assay. As shown in Figure 5a, the FERM domain interacted with Pak, whereas various C-terminal derived proteins did not. One of the tested C-terminal constructs, C1, interacted strongly with GST-Pak. However, it was later realized that the N-terminal region of this construct encoded the second half of the F3 domain, which was shown to be responsible for its binding to GST-Pak (as demonstrated by F3-1/2 and Cterm1 pulldown data; Figure 5a).

In order to narrow down the region of the merlin FERM required for Pak interaction, C-terminal deletion constructs which encoded subregions of the merlin FERM domain, including the F1and F2 lobes and the F3 lobe, were used. Both of these fragments bound to Pak, even more robustly than the F1-F3 FERM domain as a whole (Figure 5a). These data indicate that although the FERM domain mediates the interaction between merlin and Pak, there is likely some intramolecular tertiary structure within the FERM domain which masks part of the Pak-binding site. This is consistent with other reports in which intramolecular binding within the FERM domain exists and conceals merlin binding sites for other proteins, such as actin

(Gutmann et al. 1999). Therefore, it is likely that in a cell, merlin may require certain conditions and/or subcellular contexts for its interaction with and regulation of Pak.

Finally, we tested the ability of merlin phosphomutants to bind to GST-Pak. It is thought that substitution of serine 518 with alanine (S518A) mimics an unphosphorylated, active state of the protein, whereas substitution of this residue with aspartic acid (S518D) mimics a phosphorylated, inactive state (Shaw et al. 2001). Given our data implicating wild-type merlin in the binding and regulation of Pak activity, we wanted to determine how phosphorylation of merlin affects its ability to bind Pak. As shown in Figure 5a, the active, S518A form of merlin interacts with GST-Pak better than its S518D inactive counterpart. Therefore, it is likely that Pak phosphorylation of this site in merlin might regulate the ability of merlin to interact with and inhibit Pak function. The effects of these phosphomutants on Pak activity is currently under investigation.

Merlin binds to the Pak CRIB domain

In order to understand the mechanism by which merlin via its FERM domain regulates Pak activity, we performed experiments to determine what domain of Pak is required for this interaction. Pak activity is regulated by the binding of many adaptors which recruit Pak to the membrane and also by Rac and Cdc42 via their binding to the CRIB domain. Although few inhibitors of Pak have been identified to date, one, hPIP, mediates its negative regulation of Pak through its interaction with the CRIB domain and subsequent inhibition of Rac/Cdc42-stimulated Pak activity. Therefore, first, we wanted to determine with which domain of Pak merlin interacts. As the Pak kinase is highly toxic to bacteria when produced as a GST-fusion, we were limited in our ability to isolate domains of the kinase for pull-down assays. However, the

interaction between merlin and a fragment of Pak lacking its N-terminal 69 amino acids was tested (aa 70-545), as was the Pak CRIB domain (aa 70-150). Deletion of the N-terminal region of Pak had no effect on full-length or N-terminal merlin interactions with Pak, indicating that the Pak domain that mediates the interaction is likely outside this N-terminal domain (data not shown). Interestingly, this N-terminal region of Pak contains two proline-rich regions, one of which is required for binding to the adaptor/regulator Nck. Therefore, it is likely that merlin has no effect on Pak-Nck interactions, and thus does not mediate its inhibition of Pak activity via disruption of this interaction (see Figure 6 and text below).

Surprisingly, merlin interacted with the isolated Pak CRIB domain in the GST- pulldown assays (Figure 5b). The interaction between various merlin fragments and GST-CRIB mimicked the intensity and specificity of that seen in the full-length GST-Pak pull down assay. (These data are summarized in tabular form in Figure 5c.) Additionally, as demonstrated in binding assays to full-length Pak, preliminary evidence suggests that merlin (S518A) interacts more strongly with the CRIB domain of Pak than does its inactive (S518D) counterpart (data not shown). Although the binding of merlin to the CRIB domain is likely highly relevant to its function as an inhibitor, it is important to note that the GST-CRIB fragment used in the pulldown assays also contains the overlapping autoinhibitory domain (AID). The AID region is thought to interact with the kinase domain of Pak and negatively regulate its function until the inhibition is released via binding of Rac/Cdc42 to the overlapping CRIB domain. The present assay does not distinguish between merlin's binding to subregions within the GST-CRIB fragment. In addition, our data do not rule out the possibility that merlin may also bind to other, more C-terminal, domains of Pak in addition to binding to the CRIB domain. Interestingly, full-length ezrin, which is 61% identical to the merlin FERM domain, did not directly interact with Pak in this assay (data not shown),

indicating that the binding and regulation of Pak by merlin is likely specific and not a phenomenon of the entire ERM family.

Effect of merlin on the interactions between Pak and its upstream adaptors

We have begun assessing the direct effects of merlin's binding to Pak, by testing the effects of merlin on Pak's interactions with some of its binding partners. As mentioned above, Nck is an important adaptor that binds a proline rich domain in the N-terminal region of Pak (amino acids 1-70), recruiting it to the membrane and assisting in its activation. Although our *in vitro* interaction data suggested that merlin would not affect the interaction between Pak and Nck, we wanted to test this hypothesis directly. RT4-67 schwannoma cells were transfected with a plasmid encoding HA-tagged Nck, and Nck-Pak interactions were tested by co-immunoprecipitation in both directions. As shown in Figure 6, Pak and Nck do co-immunoprecipitate from these cells (- dox). However, as expected, there was no observable change in the interaction of these two proteins following dox-induction of merlin overexpression (+ dox), indicating that under these conditions, merlin expression has no effect on the interaction between Pak and its adaptor, Nck.

Disruption of merlin has no observable consequence on known Pak downstream effectors

Having established merlin as an inhibitor of Pak activity and defined the physical interaction domains between the two proteins, we sought to investigate the possible downstream signaling effects of merlin regulation of Pak. Pak activity is associated with upregulation of many signaling molecules that are coupled to cytoskeleton rearrangement processes. Although both overexpression and loss of merlin have been implicated in disruptions of the actin

cytoskeleton, including those involving cell-cell contact and cell adhesion, the molecular mechanisms underlying these phenotypes are unknown. Therefore, we reasoned that by examining the status of Pak downstream effectors in cells with and without merlin expression, we might uncover an explanation related to the effects of merlin on the cytoskeleton. In order to test this, we transfected RT4-67 cells, treated +/- dox to induce merlin expression, with exogenous Pak in order to upregulate the downstream effector pathways. We serum starved the cells 24 hours prior to harvesting the lysates and performed western blot analyses using phospho-antibodies for many of the downstream effector proteins. Downregulation of Pak activity should result in lower levels of phospho-cofillin (Ser3) and phospho-MEK (Ser298). As shown in Figure 7a, induction of merlin expression in RT4-67 schwannoma cells had no discernable effect on the levels of phosphorylation of either of these target proteins. In addition, we tested the effects of deregulation of Pak on the important focal adhesion scaffold protein, FAK. Phosphorylation of FAK has been correlated with activation of Pak in certain cellular contexts (Howe and Juliano 2000; Stoletov et al. 2001). Again, there were no observable changes in the level of phospho-FAK (P-Tyr407) in response to overexpression of merlin. The other FAK tyrosine phosphorylation sites examined in these cells, P-Tyr397 and P-Tyr576, were not detectable under the assay conditions. Where possible, antibodies against total protein corresponding to the individual phosphorylated proteins were used as loading controls. In all cases, total protein loading was assessed using an antibody against actin. In addition to looking at the downstream effectors in rat schwannoma cells, we assessed the levels of some of the phospho-signaling molecules downstream of Pak in *Nf2*-deficient MEFs. The same MEF lines used for Pak 2-D analysis were utilized in these studies. Lysates were collected from cells plated 5 days post-infection with ad or ad-cre. In the case of phospho-Raf, equivalent amounts of cell

lysates were subjected to immunoprecipitation using a total Raf antibody followed by western blotting with a phospho-Raf (Ser338) antibody. Activated Pak has been shown to phosphorylate Raf on Ser 338 leading to its activation, and, thus, we would expect, that in the absence of merlin, cells would have higher levels of phospho-Raf. However, as was seen in RT4-67 lysates, loss of merlin in *Nf2*-conditional MEFs following ad-cre treatment did not have any detectable effect on the levels of Pak downstream effectors (Figure 7b). Finally, some of the same pathways were examined in *Nf2*-deficient tumor cell lines that were either mock infected or infected with a retrovirus that allowed re-expression of merlin (data not shown). Again, no significant changes were observed in downstream effectors as a result of merlin expression in this cell type (Table 1). A couple of representative blots are shown for each cell type, and all of the data investigating downstream effectors are summarized in Table 1.

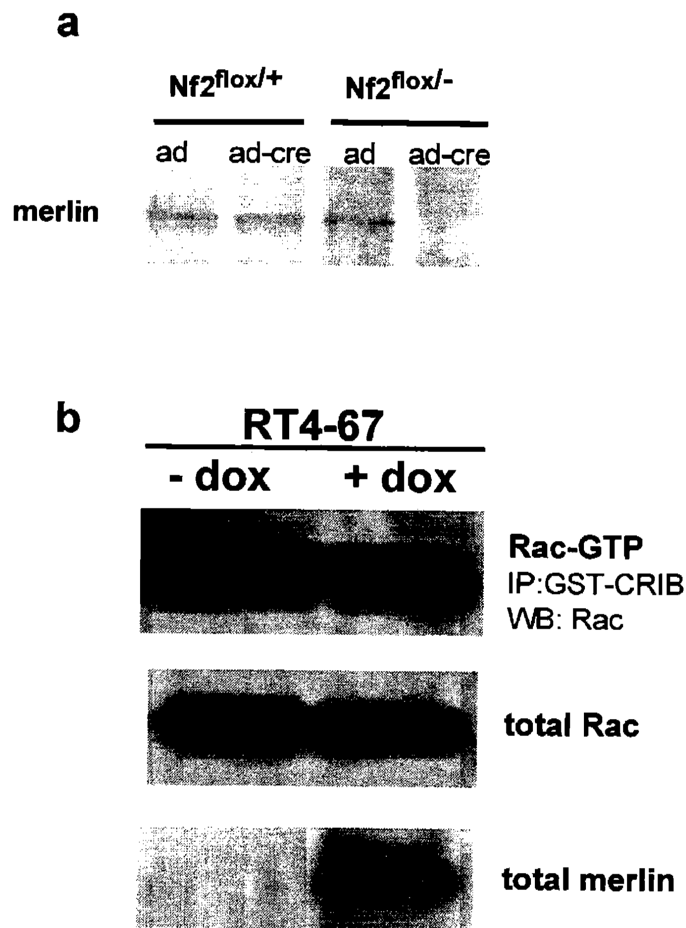


Figure 1. Analysis of the effect of merlin on activated Rac. ***(a)*** IP-Western blot analysis of loss of merlin expression in *Nf2*-conditional MEFs 5 days following ad-cre or control ad treatment. ***(b)*** Western blot analysis of activated Rac levels measured via CRIB pulldown of GTP-loaded Rac in the presence and absence of merlin expression in RT4-67 cells. Total Rac levels from whole cell lysates are shown as a control. Merlin expression levels induced in RT4-67 cells following doxycycline treatment (+ dox) are shown in comparison to untreated cells (-dox) as a control.

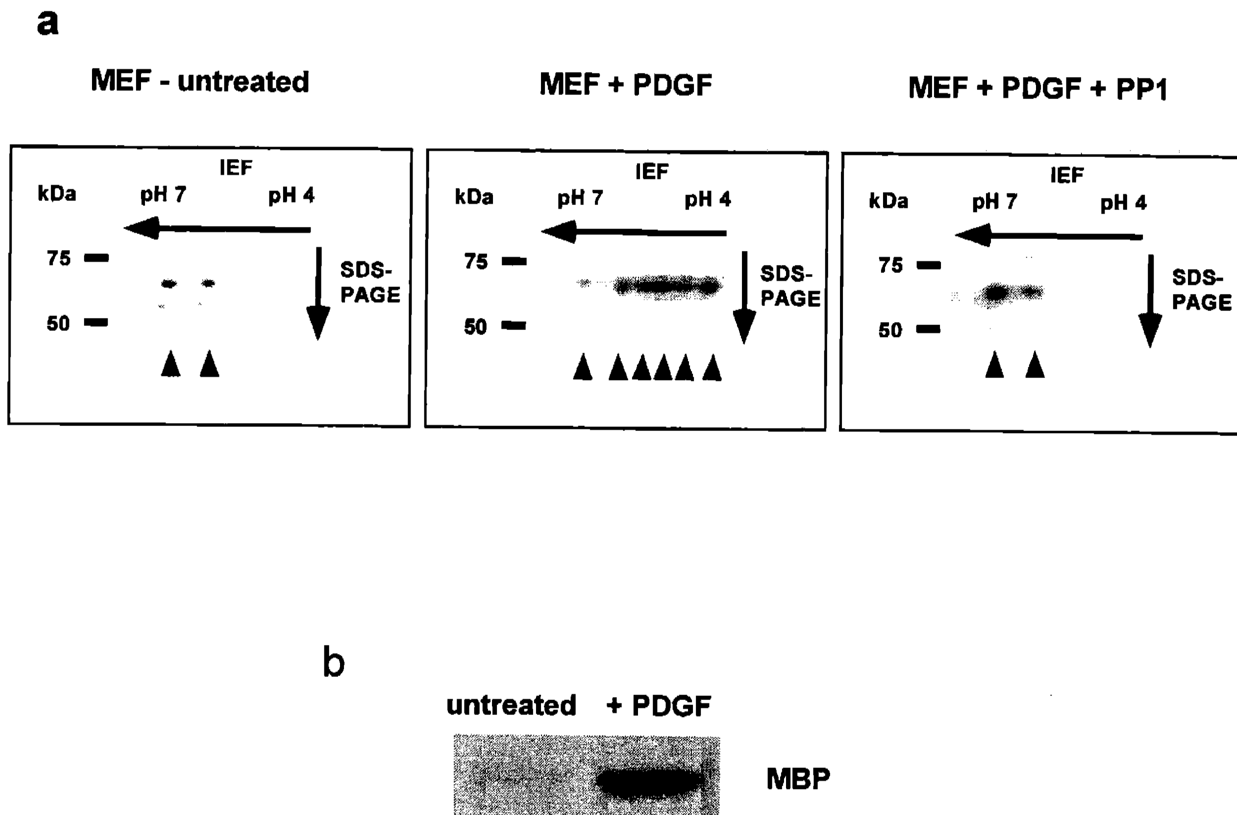


Figure 2. Analysis of Pak1 phosphorylation by 2-dimensional gel analysis. ***(a)*** Lysates were subjected to isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. Western blot analysis of Pak1 in wildtype MEFs serum starved (untreated), serum starved and treated with PDGF (+PDGF), or serum starved and treated with PDGF and PP1 (+PDGF+PP1). Various phospho-forms of Pak are indicated by arrows. ***(b)*** *In vitro* kinase assay of Pak1 immunoprecipitated from serum starved (untreated) or PDGF stimulated (+PDGF) NIH3T3 cells, employing MBP as a substrate.

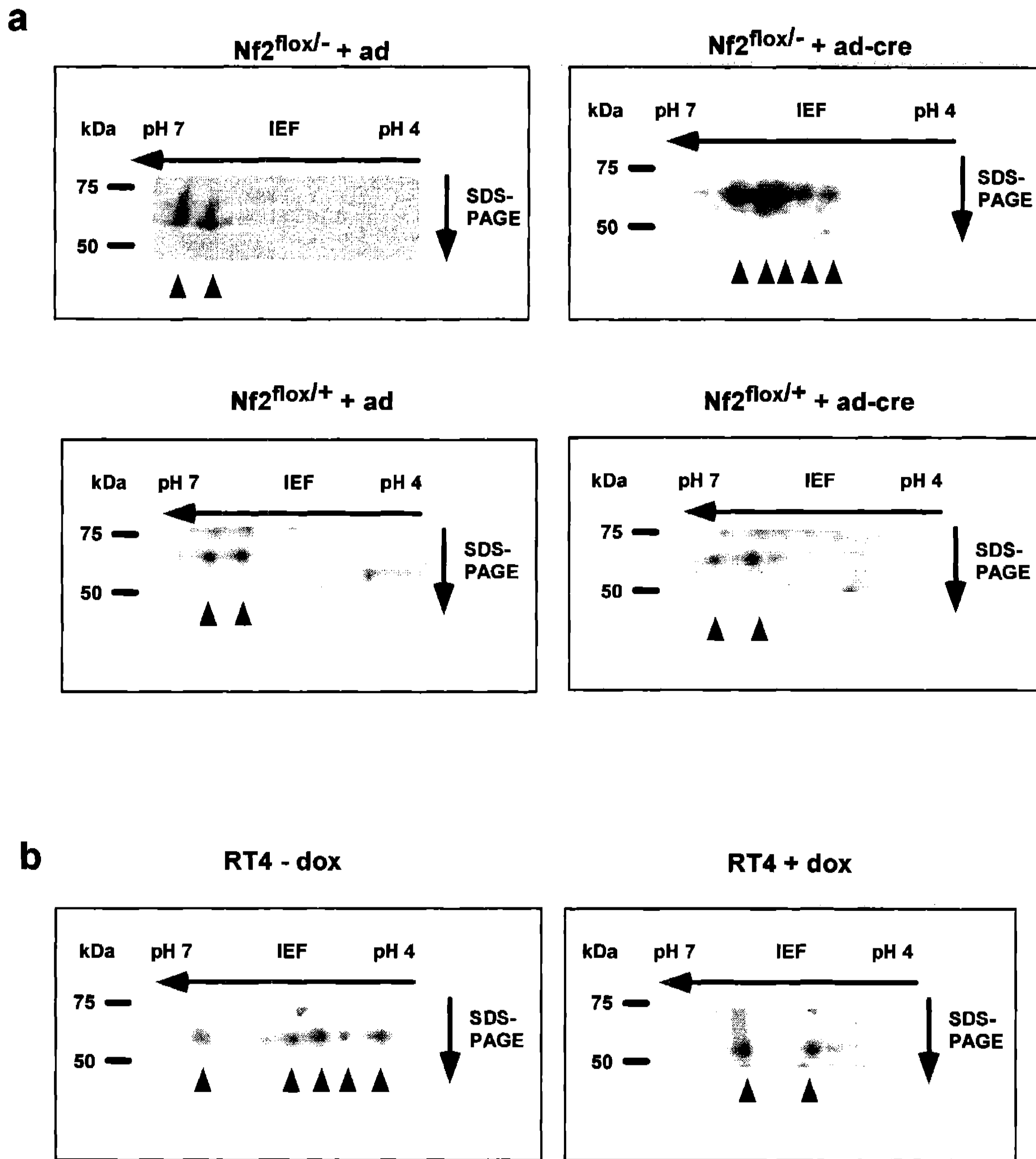


Figure 3. Analysis of Pak1 phosphorylation by 2-dimensional gel analysis in cells with and without merlin expression. ***(a)*** *Nf2*-conditional MEFs treated with ad-cre to cause loss of merlin expression (*Nf2*$^{flox2/-}$ + ad-cre) or empty ad and ad-cre as merlin expressing controls (*Nf2*$^{flox2/-}$ + ad, *Nf2*$^{flox2/+}$ + ad, and *Nf2*$^{flox2/+}$ + ad-cre) were serum starved and subjected to Pak 2-D analysis. ***(b)*** 2-D analysis of Pak from uninduced RT4-67 cells (RT4 - dox) or from those overexpressing merlin (RT4 + dox). Phospho-forms of Pak are indicated with arrowheads.

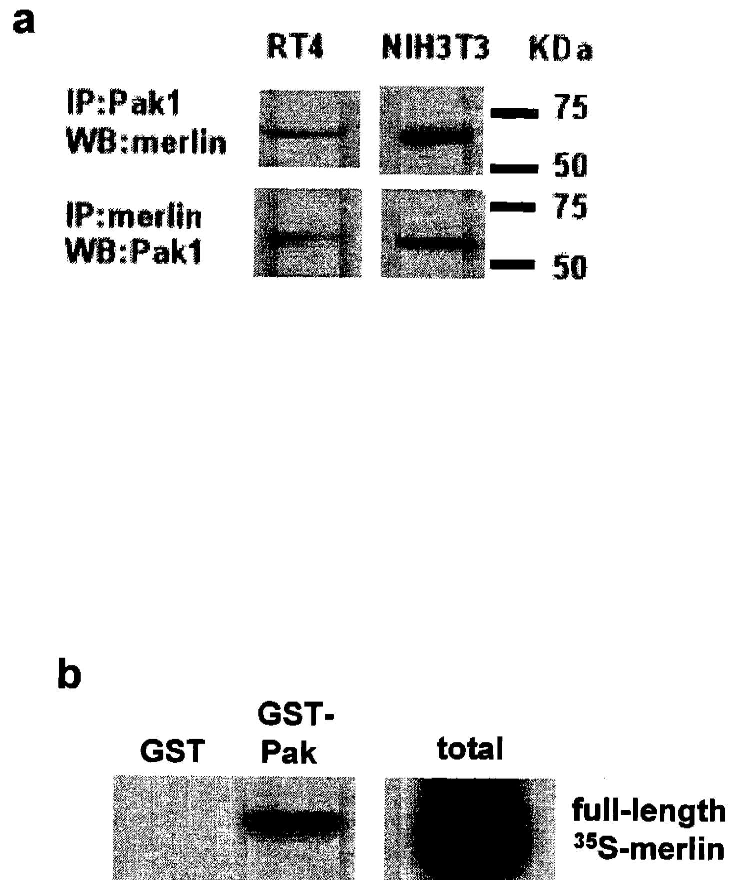


Figure 4. Interaction of Pak and merlin. ***(a)*** Western blot analysis of endogenous Pak and merlin co-immunoprecipitated from RT4 cells, and analysis of co-immunoprecipitates from NIH3T3 cells transfected with expression vectors for Pak and merlin. ***(b)*** *In vitro* direct interaction of ^{35}S-labeled full-length merlin (aa 1-595) with GST-Pak or GST (control).

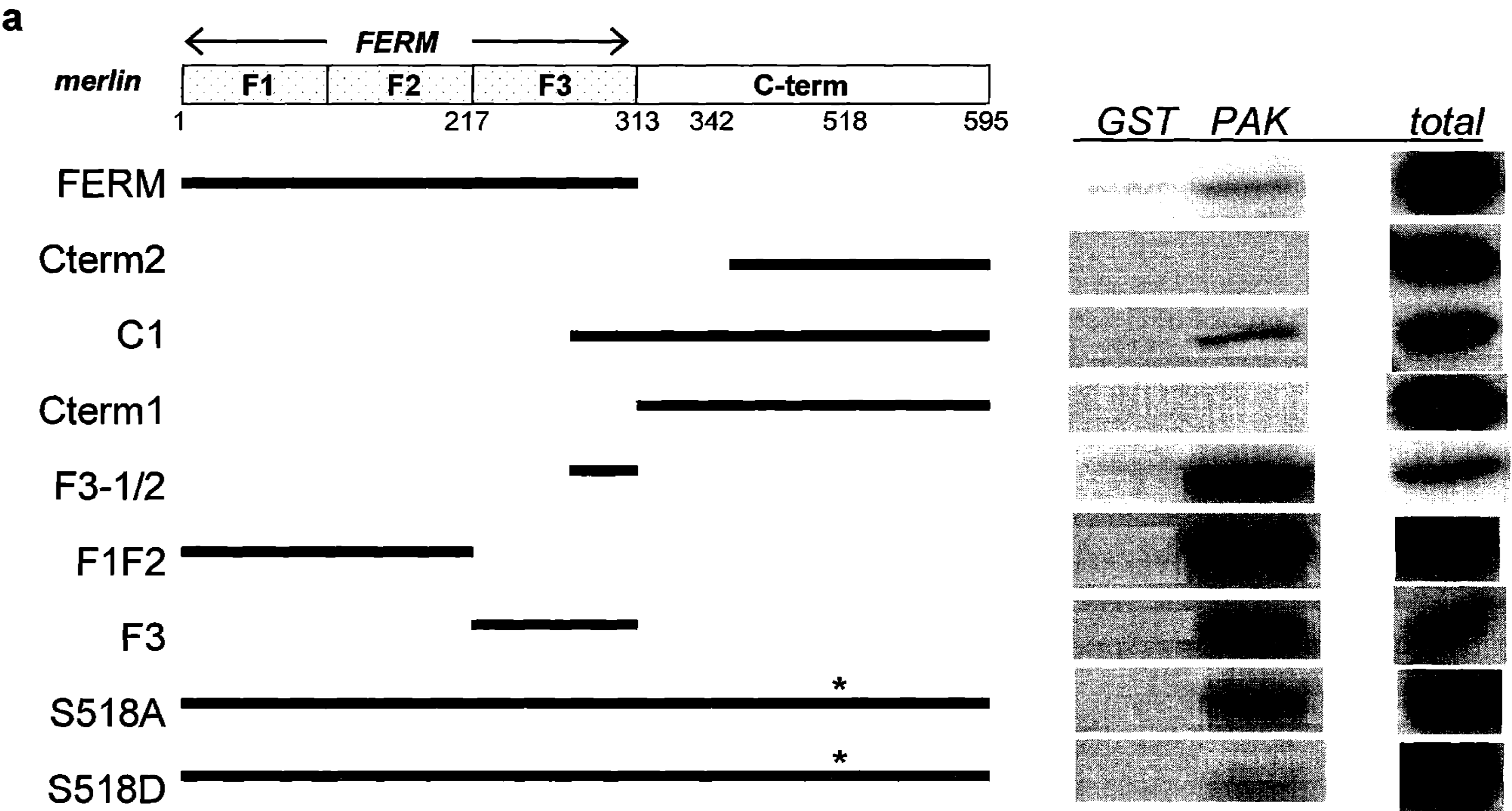


Figure 5. Isolation of domains mediating Pak-merlin interactions. ***(a)*** *In vitro* interactions between GST (negative control) or GST-Pak (Pak) and different merlin fragments (amino acids encoded by the fragments are in parentheses): FL1 (isoform 1: 1-595), FERM (1-313), F1F2 (1-217), F3 (218-313), F3-1/2 (252-313), C1 (252-595), Cterm1 (314-595), Cterm2 (354-595), and the phosphomutants, S518A and S518D. Total input of ^{35}S-merlin is shown on the right as a control. Note: Differences in intensity of bands in "total" lane is due to varying levels of ^{35}S labeling as a result of different numbers of methionines encoded in each merlin subfragment.

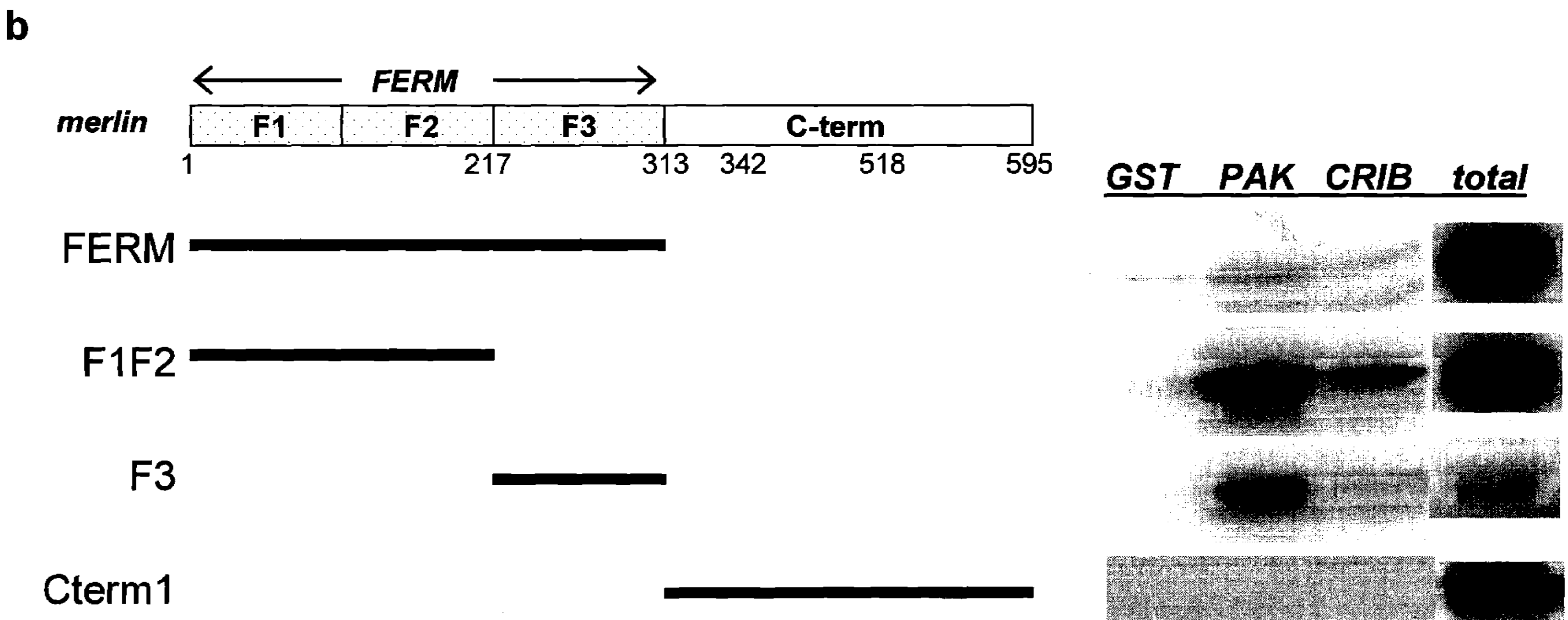


Figure 5 (cont). ***(b)*** *In vitro* interactions of some merlin fragments with GST-CRIB (70-150). Note: Differences in intensity of bands in "total" lane is due to varying levels of ^{35}S labeling as a result of different numbers of methionines encoded in each merlin subfragment.

c

Merlin	GST-Pak	GST-CRIB
FL1	+	+*
FERM	+	+/-
F1F2	++	++
F3	++	++
Cterm2	-	-*
C1	++	++*
Cterm1	-	-
F3-1/2	++	++*
S518A	++	++*
S518D	+	+*

Figure 5 (cont). *(c)* Summary of Pak – merlin interactions *in vitro*. n.d., not determined. *, data not shown.

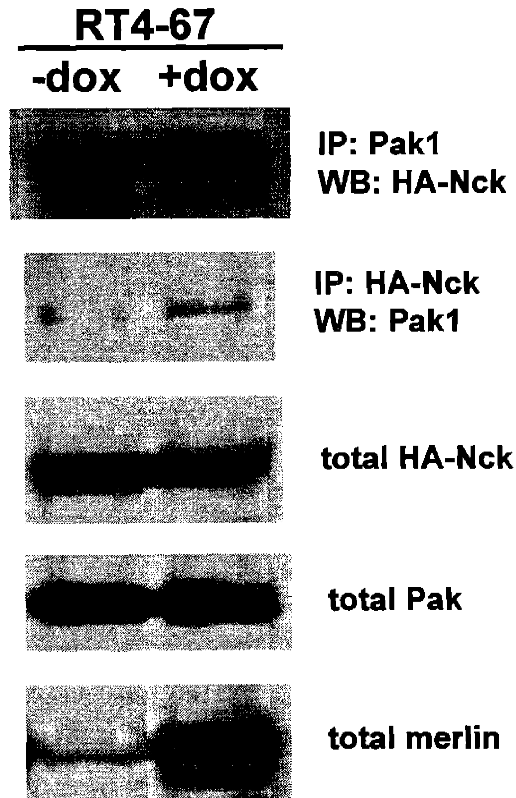
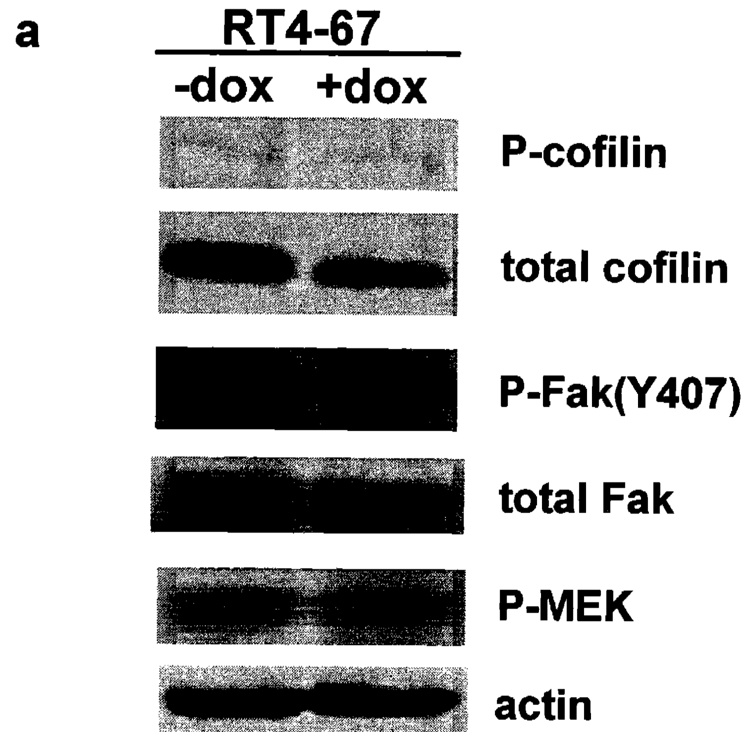


Figure 6. Interaction of Pak and its adaptor, Nck. Co-immunoprecipitation of exogenously expressed HA-tagged Nck with endogenous Pak from RT4-67 cells with (+ dox) and without (- dox) induction of merlin expression. Western blot analysis of co-IP in both directions, with blots showing total levels of both proteins and merlin in whole cell extracts as controls.



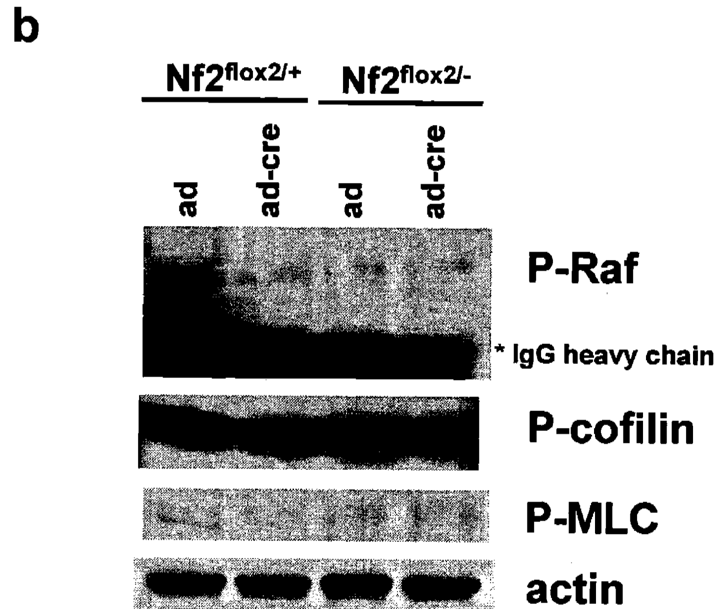


Figure 7. Effect of merlin expression on Pak downstream effectors. Western blot analysis of Pak effectors in whole cell extracts from uninduced (-) dox-induced (+) RT4-67 cells *(a)* or ad or ad-cre treated *Nf2*-conditional MEFs *(b)*. Actin is shown as a loading control.

Table 1. Summary of downstream effector analysis.

	Cell Lines		
Antibodies	**RT4-67[a]**	**Nf2 MEF[b]**	**Nf2 tumor cells[c]**
P-Raf (Ser338)	n.d.	N/C	N/C
P-MEK (Ser298)	N/C	N/C	n.d.
P-MLC (Ser18/19)	u.d.	N/C	u.d.
P-cofillin (Ser3)	N/C	N/C	N/C
P-FAK (Tyr397)	u.d.	n.d.	n.d.
P-FAK (Tyr407)	N/C	n.d.	n.d.
P-FAK (Tyr576)	u.d.	n.d.	n.d.
P-Tyr (total)	n.d.	N/C	n.d.

[a] Cells were treated +/- dox.

[b] flox/+ and flox/- MEF lines were used as in Figure 1.

[c] Nf2-/- tumor cell lines were infected with empty retrovirus or merlin expressing retrovirus for determination of the effects merlin reintroduction on these pathways.

n.d. not determined

u.d. undetectable

N/C no change

Discussion

Previous studies have demonstrated a link between merlin and the Rac pathway. First, merlin was shown to be phosphorylated downstream of activated Rac and Cdc42, and, more recently, this was demonstrated to be mediated by Pak (Shaw et al. 2001; Kissil et al. 2002; Xiao et al. 2002). In addition to its being regulated by Rac, merlin seems to negatively regulate the Rac pathway perhaps helping to explain its role as a tumor suppressor. Overexpression of merlin has been shown to inhibit Ras transformation in fibroblasts (Tikoo et al. 1994). Loss of merlin leads to increases in cell motility and membrane ruffling and increased levels of phospho-JNK and AP1 activity, all consistent with deregulated Rac activity (Shaw et al. 2001). Finally, overexpression of merlin has been shown to negatively regulate the NFκB transcription factor, downstream of Rac (Kim et al. 2002). Therefore, our demonstration that merlin inhibits Pak activity is consistent with merlin's link to downregulation of many aspects of the Rac pathway. In addition, the loss of function phenotype of merlin correlates well with that of Pak activation. Paks have been shown to be involved in the formation of focal complexes, induction of membrane ruffling and in stress fiber dissolution in various cell types. Some reports have concluded that activated Paks can lead to upregulation of p38MAPK and JNK activity downstream of Rac (Bagrodia et al. 1995). Finally, endogenous merlin is upregulated under conditions of cellular stress (such as loss of attachment, serum starvation, and confluency). Some of these same conditions have been shown to be inhibitory for Pak activation in cells in culture (Howe and Juliano 2000; Howe 2001). In fact, recent experiments suggest that the interaction between merlin and Pak is dynamically regulated under these growth inhibitory conditions (Kissil et al., submitted).

It was interesting to discover that the N-terminal domain of merlin mediated the interaction with Pak. In fact, overexpression of an N-terminal construct of merlin was shown to partially inhibit Ras transformation (Tikoo et al. 1994) in one report and was shown to inhibit NFκB activity in another report (Kim et al. 2002). Indeed, mutation in the conserved N-terminal FERM domain results in cancer phenotypes in humans, flies and mice. Therefore, this study furthers the hypothesis that the highly conserved FERM domain of merlin is critical for its function as a tumor suppressor. Interestingly, our study revealed that full-length ezrin, whose FERM domain is 61% identical to merlin, does not directly interact with Pak. Although we have not formally tested it yet, it is likely that the inhibition of Pak activity by merlin requires the C-terminal half of merlin, which may bind to other cellular proteins and localize the merlin-Pak complex to certain subcellular compartments. It has been clearly demonstrated that specific subcellular localization of Pak is critical for its activation. In one model, merlin might disrupt Pak activity by sequestering it away from a subcellular compartment normally required for its full activation.

To our surprise, merlin was found to interact with the functionally critical CRIB domain of Pak. Recently, another negative regulator of Pak was identified and characterized. hPIP, a human ortholog of Pak regulators in fission and budding yeast, interacts with Pak1 and inhibits the Cdc42/Rac-stimulated kinase activity through the N-terminal autoregulatory domain of Pak1, which includes the CRIB domain (Xia et al. 2001). Transfection of hPIP1 into mammalian cells inhibits Pak-mediated JNK and NFκB signaling pathways. The behavior of this Pak inhibitor, hPIP, is very reminiscent of previous observations following merlin overexpression, and, now, observations concerning its link to and regulation of Pak.

Activated Rac and Cdc42 bind to the CRIB domain of Pak and catalyze the activation of the protein via a conformational change. Here, using *in vitro* studies, we have demonstrated that merlin binds to the same domain of Pak. Perhaps merlin mediates its inhibition of Pak activity by exclusion of Rac/Cdc42 interaction with Pak and, thus, their activation of Pak. As a result, Pak may be suspended in an inactive conformation when bound by merlin. Then, already activated Pak, perhaps from another subcellular pool, may phosphorylate and inactivate merlin releasing its binding and inhibition of Pak, allowing for Rac/Cdc42 to bind and activate. This hypothesis is also consistent with the aforementioned model of merlin sequestration of Pak to a subregion of the cell away from a region normally required for its activation. In addition, our data demonstrating that an active, hypophosphorylated mimic of merlin (S518A) binds much better to GST-Pak (and GST-CRIB) than the inactive, phospho-mimic (S518D) is compatible with these models. It is likely that in a particular cellular context, interaction between Pak and the FERM domain of active, unphosphorylated merlin is critical for regulation of Pak activity perhaps via interactions of the C-terminal domain of merlin with other cellular proteins. Finally, the idea of merlin preventing Pak's interaction with its activator, Rac, is currently under investigation.

Alternatively, merlin binding to the CRIB domain of Pak could affect the interaction of Pak with other binding partners. A new report suggests that the interaction between Pak and filaminA (FLNa), a protein involved in F-actin crosslinking, receptor anchoring, and cellular signaling, is important for the subsequent activation of both proteins and is critical for Pak induction of membrane ruffling (Vadlamudi et al. 2002). Interestingly, FLNa interacts with the CRIB domain of Pak and its binding stimulates Pak kinase activity. In a model similar to that stated above for Rac, perhaps merlin binding to the CRIB domain of Pak excludes FLNa binding

and activation and the subsequent activities of the interacting proteins, such as stimulation of membrane ruffling. This hypothesis is consistent with the observation that loss of merlin results in increased membrane ruffling.

Merlin binding to the CRIB domain may bring about an even broader interpretation of merlin function. There are over 25 CRIB domain containing proteins in mammals, which are regulated in a manner similar to the Paks by the small GTP binding proteins, Rac and Cdc42 (Pirone et al. 2001). The fact that merlin can bind to the CRIB domain of Pak might indicate a larger role for merlin function in the regulation of many or all proteins containing a CRIB domain. However, it is important to note that there is a loose consensus sequence for the CRIB domain, which may argue against such a hypothesis. Nevertheless, the phenotypes of overexpression of several of these CRIB domain containing proteins is consistent with the phenotypes observed under conditions of deregulation of merlin. For example, Mig-6 (mitogen inducible gene 6) stimulates activation of JNK and NFκB (Makkinje et al. 2000; Tsunoda et al. 2002). Par-6 (partitioning defective) is critical for maintenance of cell polarity and negatively regulates epithelial cell-cell junction organization, such that overexpression of Par-6 inhibits assembly of tight junctions (Gao et al. 2002). It would be interesting to investigate whether merlin binds to the CRIB domains of these and other proteins, and, if so, how merlin binding affects the activities of these proteins.

As expected, merlin did not interfere with the binding of the adaptor Nck to Pak. However, several other proteins bind to and regulate Pak activity, including, βpix, p95PKL, and paxillin. Preliminary data suggests that merlin does not affect the interaction between βpix and Pak, as might be predicted given that the binding domain for βpix (aa 182-203) is outside of the CRIB domain (J. Kissil, pers. comm.). In addition, Pak can interact directly with paxillin or via

Nck/βPIX/p95PKL. Studies have demonstrated that Pak-paxillin interactions at focal adhesions are critical to the activation of Pak. One might hypothesize that merlin could exhibit its inhibitory action on Pak in part by sequestering it (and perhaps its binding partners) in the cytosol thereby preventing its localization to focal adhesions. However, preliminary data suggests that there is no difference in the solubility of Pak, p95PKL or paxillin in RT4-67 cells +/- merlin (see supplementary data; S1). Studies of the interactions between Pak and paxillin (and other adaptors) in the presence or absence of merlin expression under different cellular conditions are ongoing. In addition, Pak has been shown to be negatively regulated by several phosphatases, including POPX1/2 (Koh et al. 2002). Perhaps merlin serves to recruit these phosphatases or strengthen the interaction between the phosphatases and Pak leading to Pak downregulation.

Interestingly, recent data suggests that merlin binds to paxillin through two different domains, one between amino acids 50-70 (exon 2) and the other in the C-terminal region of the protein (Fernandez-Valle et al. 2002). However, the effect of the interactions between merlin and paxillin is not yet understood. Mutation of residues in the N-terminal paxillin binding domain of merlin corresponding to patient mutations (such as, W60C and F62S) leads to reduced binding of merlin to paxillin. Surprisingly, our *in vitro* studies suggest that although mutation of these amino acids almost completely abrogates merlin binding to Pak (FL-W60C, FL-F62S, and N-W60C), in one case the same merlin mutation that decreased the affinity of merlin for paxillin actually resulted in strong binding between ^{35}S-merlin and GST-Pak (N-F62S; see supplementary data; S2). Note that the well-known and studied patient mutation L64P was not tested in the paxillin binding studies. Interestingly, the L64P mutation did not abrogate merlin-Pak interaction in the context of either full-length or the N-terminal domain of merlin. The

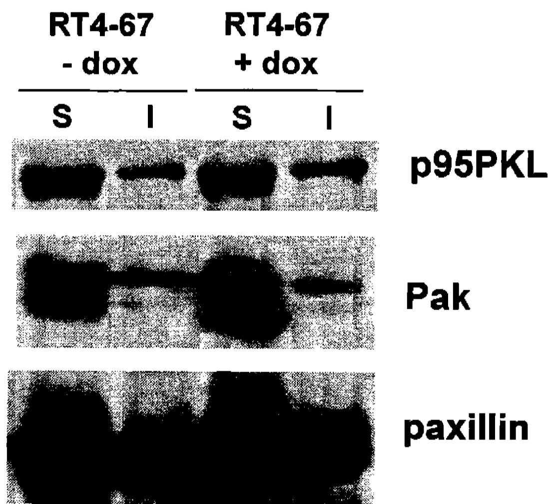
biochemical and cellular implications of these results are currently under investigation. In addition, further studies are needed in order to establish the potential importance of merlin binding to both Pak and paxillin.

Finally, we were surprised to see that expression of merlin had no observable consequence on the activity of the downstream effectors of Pak, given the effects of merlin on Pak activity. Conceivably, there are novel pathways, not yet identified, downstream of Pak activation with effectors whose activity may be inhibited by merlin expression. In addition, experiments to test the activation of known Pak effectors under various cellular conditions (e.g., high cell density) are underway. Perhaps in association with the hypothesis that Pak activity may be inhibited only in certain subcellular compartments, we should examine the activity of Pak effectors in individual subcellular regions. We are currently evaluating the feasibility of such experiments. If, for example, a small, localized pool of Pak is activated in the absence of merlin, it may only have a consequence on a local pool of Pak effectors. Therefore, an examination of the phosphorylation status of effectors in whole cell lysates (as we have done) may mask more local, yet important, effects.

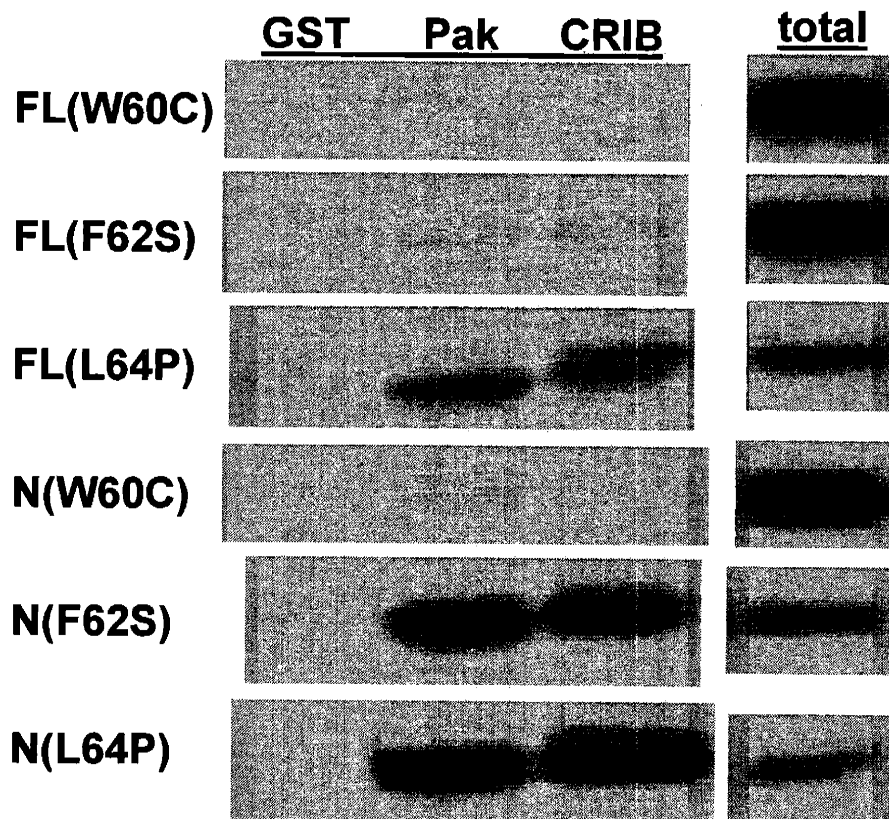
As a final point, it would be interesting to determine the effect of loss of merlin expression on the group II Paks, specifically Pak 4, whose activation has been directly implicated in tumorigenesis. Activation of Pak 4 was shown to be both necessary and sufficient for induction of transformation in Rat Intestinal Epithelial (RIE) cells as well as NIH3T3 fibroblasts (Callow et al. 2002). In addition, increased Pak4 expression was documented in most human tumor cell lines assayed from the National Cancer Institute human tumor panel (Callow et al. 2002). Interestingly, recent studies have implicated Pak4 as a primary mediator of cytoskeletal changes induced by Cdc42 rather than a key player in Cdc42 downstream signaling pathways. In

fact, unlike for the activation of group I Paks, activation of Pak4 does not affect the activities of MLCK, p38 MAPK or ERK (Qu et al. 2001). In addition, overexpression of activated Pak4 does not increase levels of phospho-JNK above that of overexpression of wild-type Pak4, indicating that specific activation of Pak4 does not play a role in JNK regulation (Qu et al. 2001). Given our data relating to the lack of observable consequence on Pak1 downstream effectors following merlin expression, it is intriguing to hypothesize that while merlin may exhibit mild inhibitory effects on the group I Paks, perhaps it exerts a greater inhibitory effect on Pak4 (or other group II Paks). Hence, in the context of merlin loss, a cell might upregulate Pak4 activity directly resulting in transformation via as yet unidentified effector pathways.

In summary, the experiments described here demonstrate the regulation of the Rac/Cdc42 effector, Pak, by the *Nf2* tumor suppressor protein, merlin. Merlin mediates its inhibition, at least in part, via direct interaction with the CRIB regulatory domain of Pak. The N terminal conserved FERM domain of merlin mediates the direct binding with Pak. All of the experiments described in this manuscript were performed using Pak1, which has been shown to be neccessary for cellular transformation downstream of Ras and Rac in certain cell types. Therefore, the binding of merlin to Pak and its subsequent regulatory consequences links an established tumor suppressor in the process of Pak1 regulation, possibly linking Pak1 deregulation to tumorigenesis. Understanding whether the deregulation of the Pak proteins in the absence of merlin is relevant to NF2 or sporadic tumors bearing *NF2* mutations, is a critical next step. This information and a more complete characterization of the impact of merlin on Pak signaling pathways could lead to the discovery of specific inhibitors that may be useful in the treatment of tumors bearing mutations in *NF2*.



S1. Solubility of Pak and select Pak binding proteins. Western blot analysis of 1% Triton X-100 soluble and insoluble fractions of RT4-67 cells with (+ dox) and without (- dox) induction of merlin expression. Data shown are for Pak and 2 adaptor proteins, paxillin and p95PKL.



S2. Interaction of Pak and merlin mutants. *In vitro* interactions between GST (negative control), GST-Pak (Pak) and GST-CRIB (CRIB) and different merlin mutants. Mutations correspond to several patient point mutations identified in exon 2 of *NF2* (identity of the amino acid substitutions is in parentheses). Total input of ^{35}S-merlin is shown on the right as a control. FL, full-length merlin. N, N-terminal merlin.

Materials and Methods

Cell Culture Conditions and transfections

RT4-67 and RT-DP6 rat schwannoma cells, *Nf2*-deficient tumor cell lines (F099 and F1503) and MEFs were grown in DME plus 10% fetal calf serum (FCS). NIH3T3 cells were grown in DME plus 10% calf serum (CS). In RT4-67 cells, merlin expression was induced by the addition of 1ug/ml doxycycline 48 hours prior to the experiment. Recombination of loxP sites in *Nf2* conditional MEFs was achieved by treating the 10^6 cells with ~10^8 PFU of adenoviral-cre (Gene Transfer Vector Core Facility, University of Iowa College of Medicine). The media was changed one day after infection. Recombination was assessed at the DNA level as described (Giovannini et al. 2000). For transfections, cells were plated at 10^6/10cm dish. Transfections were carried out using Fugene 6 (Roche) or Lipofectamine (Invitrogen) according to manufacturer's instructions. For experiments examining Pak1 activation, cells were plated at 10^6/10cm dish 48 hours prior to harvest and were serum starved in 0.1% FCS in DME for 24 hours prior to harvest. For some experiments, Pak1 activation was stimulated by adding 5 ng/ml PDGF-BB (Sigma) for 5 minutes. PP1 was used at 2.5 U/ml.

Plasmids and Antibodies

Expression plasmids used for transfection were pCMV-Pak1 (Sells et al. 1997), pcDNA3-HA-*Nf2* (Kissil et al. 2002) and pEFBOS-HA-Nck (Lu and Mayer 1999). The plasmids for *in vitro* transcription/translation were contructed by PCR from a full-length *Nf2* cDNA using primers containing a BamHI site on the 5' end and an EcoRI site on the 3' end and then cloned into the pcDNA3-HA vector, which contains an HA epitope tag between the BglII and BamHI sites of the multiple cloning region of pcDNA3. The following plasmids were

created with the amino acids corresponding to the polypeptide expressed in parentheses: pcDNA3-HA-FL1 (isoform1: 1-595), pcDNA3-HA-FERM (1-313), pcDNA3-HA-F1F2 (1-217), pcDNA3-HA-F3 (218-313), pcDNA3-HA-F3-1/2 (252-313), pcDNA3-HA-C1 (252-595), pcDNA3-HA-Cterm1 (314-595), and pcDNA3-HA-Cterm2 (354-595). pcDNA3-HA-Mer(S518A) and pcDNA3-HA-Mer(S518D) were created by site directed mutagenesis of the pcDNA3-HA-FL1 construct (QuikChange, Stratagene). Plasmids corresponding to NF2 patient mutations, pcDNA3-HA-FL(W60C), pcDNA3-HA-FL(F62S), pcDNA3-HA-FL(L64P), pcDNA3-HA-N(W60C), pcDNA3-HA-N(F62S), and pcDNA3-HA-N(L64P), were created by site directed mutagenesis of the corresponding wild-type merlin vectors. pGEX-Pak1 (L404) was used for production of full-length rat Pak for pulldown assays (Zhao et al. 1998). This plasmid was used as a template for PCR construction of the N-terminal deletion construct, pGEX-Pak (70-545), which encodes amino acids 70-545 of Pak fused to GST. All plasmids were sequenced following their construction.

Antibodies used for immunoprecipitations (2 μg/IP) and western blotting (used at 1:1000) are as follows: sc-331 and sc-332 for merlin, sc-881 for Pak1, sc-290 for Nck, sc-1616 for actin, sc-557 for FAK, sc-227 for Raf-1, sc-9449 for MLC, and sc-219 for MEK, (Santa Cruz), 9E10 for myc epitope (Sigma), 23A8 for Rac, 07-339 for phospho-MEK, and 05-534 for phospho-Raf (S338) (Upstate Biotech), 12CA5 for HA epitope (Boerhinger Mannheim), 3311 for phospho-cofilin (Ser3), 3312 for cofilin, and 3674 for phospho-MLC (Cell Signaling), 44-624 for phospho-FAK(Y397), 44-650 for phospho-FAK(Y407), and 44-652 for phospho-FAK(Y576) (Biosource).

Immunoprecipitations, kinase assays, cell fractionation, and Rac activation assays

For immunoprecipitations, cells were extracted into 0.5% NP40 buffer (0.5% NP40, 150 mM NaCl, 50 mM TRIS, pH 8.0, complete mini protease inhibitor tablets (Roche), phosphatase inhibitors: 1 mM $NaOVO_4$, 1 mM NaF and 2 mM pefabloc). Protein concentrations were quantitated using a Bradford assay (BioRad). Equal amounts of lysates were precleared for 1 hour on Protein A or G beads (Pierce), and then incubated with 2 μg of the appropriate antibody for 4 hours, 4°C, rotating. Protein A or G beads were added for an additional hour of rotation at 4°C. Immune complexes were washed with an excess of lysis buffer (3 times for 5 min and 2 times for 15 min) followed by separation by SDS-PAGE. Pak1 kinase assays were performed as described (Kissil et al. 2002) except that Pak1 was immunoprecipitated from the extracts and 0.5 μg/ml MBP (Upstate Biotech) was used as the substrate. In order to isolate 1% Triton X-100 soluble and insoluble fractions, cell fractionation was carried out as described (Shaw et al. 2001). Rac activation assays were performed using the Rac activation assay kit (Upstate Biotech) exactly according to the manufacturer's instructions.

2-dimensional analysis of Pak activity

Cells were harvested directly into sample buffer (9.8 M urea, 2% CHAPS, 5 ml IPG buffer 4-7, 15 mg/ml DTT). Extracts were incubated on ice for 10 min, centrifuged at full speed for 10 min to clear insoluble material, and protein concentrations were quantitated using a Bradford assay. 100 μg of each extract was cup loaded onto 7cm pH 4-7 IPG strips (Amersham Pharmacia) and resolved using a IPGphor unit (Pharmacia Biotech) at 50mA/strip for 100V/30', 200V/30', 400V/30', 1000V/60', 3500V/5h and 500V to a total of 20,000 volt hours. Strips were then washed in wash solution (50 mM TRIS-HCl, pH 8.8, 6 M urea, 30% glycerol, 2%

SDS) supplemented with 20 mg/ml DTT for 10 min at room temp, followed by a wash in wash solution supplemented with 25 mg/ml iodoacetamide for 10 min. The strips were then loaded onto a standard SDS-PAGE (10%), separated and transferred to ImmobilonP (Millipore). The blots were then used for western analysis for Pak. Equal loading of protein was determined by bloting with an actin antibody.

In vitro binding assays

^{35}S-labeled HA-tagged full-length merlin and fragments of merlin were produced *in vitro* using the TnT quick coupled T7 kit according to manufacturer's instructions (Promega). GST alone and GST-tagged full-length Pak1 were produced in bacteria as described (Thiel et al. 2002). Correct protein production and assessment of protein concentration was controlled using SDS-PAGE followed by Coomassie stain. GST-CRIB (aa 70-150) was purchased from Upstate Biotech. *In vitro* pulldown assays were performed by incubating 30 µl of each GST-tagged protein bound to glutathione beads (0.5 mg/ml) to equal amounts of ^{35}S-labeled merlin in binding buffer (50 mM TRIS-HCl, pH 7.5, 120 mM NaCl, 10 mM $MgCl_2$, 5% glycerol, 1% Triton X-100, complete mini protease inhibitor tablets, 2 mM $NaOVO_4$, and 2 mM pefabloc) at 4°C for 4 hours. The complexes were washed 5 times with an excess of binding buffer (3 times for 5 min, 2 times for 15 min). SDS sample buffer was added, the beads were boiled, and released proteins were separated by SDS-PAGE. Total ^{35}S-labeled merlin produced by *in vitro* transcription/translation was assessed by running 5% of each reaction alongside the pulldown assay. The gels were fixed (10% acetic acid, 15% isopropanol), treated with AMPLIFY (Amersham Pharmacia), dried down and exposed to film at -80°C.

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Chapter 5

Discussion and Conclusions

The molecular basis of Neurofibromatosis Type 2 has evaded scientists since the discovery of the genetic basis for the disease over a decade ago. While we know that mutation of one copy of the *NF2* tumor suppressor gene predisposes individuals to tumors of the central nervous system, we have not been able to answer the question "why?". What is it about the *NF2* gene and its protein product, merlin, that protects the cell from uncontrolled/cancerous growth? How would a putative membrane-cytoskeletal linker protein exhibit control over activities in the nucleus of the cell? Is the mechanism by which loss of merlin function results in tumor formation observed in the genesis of other types of cancer? The answers to these questions and more are critical to our understanding of this disease and our investigation of possible avenues for therapy. Studies presented here have begun to answer some of these pressing molecular questions and have uncovered a new angle for NF2 research with a whole new set of questions relating to NF2 and to tumorigenesis more broadly.

The importance of the FERM domain and the role of merlin in cell-cell and cell-matrix adhesion

The earliest hypotheses addressing the function of merlin were based on its homology to the ERM proteins. With such great sequence conservation in the N-terminal FERM domain, it was hard to ignore the possibility that merlin, too, acted as a membrane-cytoskeletal linker. But, whether this was in fact the case and whether this potential function had anything to do with merlin's role as a tumor suppressor remained to be determined. Interestingly, early studies determined that merlin did not share a conserved actin-binding domain located in the C-terminal half of the ERMs, thereby putting a question mark on whether this newest member of the family maintained a similar cellular behavior. Studies since have demonstrated the presence of a βII-spectrin binding domain near the C terminus of merlin and several potential actin-binding

domains in the N-terminal half of the protein. Nevertheless, despite the high degree of identity between merlin and the other ERM family members, it is important to recognize that these proteins have opposing functions in the cell. Whereas many reports have demonstrated that ezrin promotes cell motility and cell proliferation and suppresses cell death, merlin behaves as an inhibitor of cell proliferation. Where, then, does the information lie to specify one protein as growth promoting and the other as growth inhibitory?

Our studies of BBA merlin, along with other reports, suggest that the N-terminal half of merlin, which encodes the highly conserved FERM domain, is critical to its growth suppressive function. However, it is difficult to envision how such specificity arises from a domain that is so highly similar to an entire family of proteins (the ERMs) which are known to be growth promoting. The C-terminal domain of merlin differs quite dramatically from the ERM proteins, and therefore, likely plays a role in specifying the function of merlin. Indeed, merlin and the ERMs are regulated by phosphorylation downstream of different Rho GTPases. In addition, many studies have shown that merlin cannot suppress cell proliferation in the absence of its C-terminal half (see Appendix C). In fact, many of the more severe cases of NF2 result from nonsense mutations in the FERM domain that result in truncation of the C-terminal domain. Therefore, it is likely the coordinate regulation between the N- and C-terminal domains of the protein that lend to the specificity of merlin as a tumor suppressor. This coordination may result from interdomain interactions or interactions between merlin and other cellular proteins. Indeed, these mechanisms are still far from evident.

Our studies have begun to uncover some of the differences between merlin and the ERMs and their behavior(s) in the cell. Observations by *Drosophila* geneticists revealed that there is a stretch of seven amino acids in the FERM domain of merlin (the blue box) that is exactly

conserved in mice, humans and flies, but diverges in the ERM proteins of these species. Mutation of this domain in *Drosophila* merlin resulted in a dominant negative allele which caused an overproliferation phenotype, thereby indicating the critical nature of these residues to proper merlin function. Interestingly, recent structural studies revealed that the blue box residues make up one of three patches of residues on the surface of the merlin FERM domain that are distinct from the corresponding ERM sequence. Based on their location at the surface of the globular structure, these patches are likely to mediate interactions with binding partners of merlin, perhaps distinguishing merlin function from that of the ERMs. Interestingly, many missense mutations have been identified in NF2 patients that occur within the FERM domain of the protein further indicating the critical nature of the encoded amino acids.

I took advantage of the observations made in *Drosophila* and the structural studies and utilized a mammalian version of the blue box mutant allele to study the effects of merlin mutation on the growth and behavior of fibroblasts in culture. To our surprise, blue box mutant mouse merlin caused complete transformation of NIH3T3 cells in culture. This indicated the critical nature of these residues not only for the function of *Drosophila* merlin, but also for mammalian merlin, and, in addition, led us to many important observations of the effects of mutation of merlin on the behavior of cells in culture. Importantly, the goal of such studies was to understand more about the function of the protein in order to learn how to treat the disease more effectively.

The most important observation from the studies with BBA merlin (Chapter 2) was the critical nature of merlin in cell-matrix adhesion (at high cell densities) and maintenance of contact inhibition through cell-cell contact, phenotypes that were also observed in my studies with *Nf2*-deficient primary mouse fibroblasts (Chapter 3). Prior studies have localized merlin to

the periphery of the cell consistent with its assignment as a membrane-cytoskeletal linker. Indeed, some studies revealed the binding of merlin to integral membrane proteins, such as CD44 and β1 integrin. These findings were consistent with a role for merlin at sites of adhesion, but still did not reveal the mechanism(s) by which merlin exerted its effects at these sites.

Notably, there are several types of integrin-associated matrix adhesion sites, including focal complexes present at the edges of lamellipodia and induced by Rac (Nobes and Hall 1995; Clark et al. 1998), focal contacts which are focal complexes transformed by activation of Rho (Clark et al. 1998; Rottner et al. 1999), and focal adhesions which are the most stable of the three types. Interestingly, to date, more than 50 proteins have been shown to localize either transiently or stably with integrins in focal adhesions. The components of focal complexes and focal contacts likely include many of the same proteins, but the exact composition of all of these adhesion complexes remains to be elucidated and likely depends considerably upon cell type and context. Integrin-mediated adhesion complexes which include adaptor proteins, kinases, GTPases, GAPs, GEFs, actin-binding proteins, and actin, act as sensors to integrate both ECM-integrin binding signals and growth factor signals from the outside of the cell to intracellular signaling pathways. Thus, there is an extremely complex web of proteins leading to the induction of cell proliferation only when a cell is situated in the proper physical environment. Likewise, although perhaps not as complex, adhesion complexes at cell-cell junctions integrate signals in order to regulate cell proliferation in response to the physical environment. Through its interaction with several of these adhesion-associated proteins, merlin is placed at the center of this intricate adhesion-signaling network. We must, therefore, appreciate the probable complexity of merlin function in the cell. Thus, many studies are required to determine the role

of merlin at these cell adhesion sites and the resulting affect of loss of merlin on the structure and function of adhesion complexes.

Despite the fact that we are still far from understanding the exact role of merlin at sites of cell-matrix and cell-cell adhesion, my studies revealed that merlin acts a positive regulator of cell-matrix attachment and an inhibitor of cell proliferation under conditions of cell-cell contact. It is plausible that disruption of this activity of merlin may be an initial step in tumorigenesis. In addition, the residues in the FERM domain of merlin (specifically the blue box domain) are critical to its ability to carry out these functions. As was suggested for human ΔBB merlin and NHERF, perhaps mouse BBA merlin is less capable of binding particular membrane proteins because of a conformational change or the critical nature of these residues for mediating direct binding. My studies revealed that BBA merlin retained its ability to bind to β1 integrin, but further investigation is required to determine the effect of this mutation on merlin binding to other membrane partners. Perhaps such studies would reveal which binding partners are critical to the ability of merlin to mediate its tumor suppressor function.

Merlin has been shown to be capable of self-association, a conformation critical to its growth-suppressive function, and homodimerizing. Therefore, BBA merlin may not be able to interact in a head-to-tail fashion and/or may act by inhibiting normal head-to-tail interactions of endogenous merlin molecules. Alternatively, the C-terminal half of BBA merlin might sequester normal merlin-binding partners, while lacking key residues in the FERM domain to couple those proteins to their proper regulatory proteins. In this way, merlin may be thought of as an adaptor, perhaps even at cell adhesion sites linking up and regulating proteins through its N- and C-terminal domains. Recently, a similar function has been assigned to ezrin which binds transmembrane proteins through FERM domain interactions with NHERF or E3KARP and

regulates the membrane protein function through its C-terminal binding to PKA (Dransfield et al. 1997). Ultimately, it would be very informative to identify the important protein complexes with which merlin associates in order to begin to understand how mutation in merlin or loss of merlin expression leads to disrupted cell adhesion and aberrant responses to cell-cell contact. The NIH3T3 BBA merlin cell system, which I have designed and characterized, would be a valuable tool to carry out such studies.

Finally, a discussion of the dominant-negative function of the murine $Nf2^{BBA}$ allele is warranted. ΔBB behaved as a dominant-negative allele in all *Drosophila* assays. In this system, investigators were able to assess the nature of this mutation using several genetic proofs. However, given the complexity of this approach in mammalian cell culture, and the inability to completely knock down *Nf2* expression by siRNA in NIH3T3 cells (Chapter 3), we were unable to directly assess whether $Nf2^{BBA}$ behaves as a dominant-negative allele in a mammalian context. Therefore, our data do not exclude the possibility that $Nf2^{BBA}$ functions as a neomorph, causing phenotypes not normally associated with loss of merlin function. Although we performed several assays to assess biochemically the effect of BBA merlin expression on wild-type merlin, many further studies are required. Several studies could be carried out with our current set of reagents in order to determine the dominant-negative nature of the BBA allele. First, we could express $Nf2^{BBA}$ in the background of *Nf2*-deficiency in order to show that BBA merlin has no effect on the phenotype of these cells. Second, biochemical studies of the nature of the interactions between BBA merlin and wild-type merlin, such as those performed in *Drosophila*, could be carried out. Third, we have assayed the effect of expression of $Nf2^{BBA}$ on the phenotype of wild-type primary MEFs. Expression of $Nf2^{BBA}$ recapitulated the contact inhibition phenotype observed in *Nf2*-conditional knock-out MEFs. However, given the

prominence of the motility and membrane ruffling defects in *Nf2*-deficient MEFs, it would be valuable to characterize the same phenotypes in wild-type MEFs expressing $Nf2^{BBA}$. Interestingly, we observed that expression of $Nf2^{BBA}$ in NIH3T3 cells did not affect significantly the motile properties of the cells. These data argue against the assignment of $Nf2^{BBA}$ as an antimorph in the mammalian system. Nevertheless, it is apparent that further studies are required to confidently assign a function to the BBA allele.

Significance of Pak activation in absence of merlin

For a couple of years, it has been hypothesized that merlin regulates the Rac pathway. Data from several systems revealed that loss of merlin function resulted in abnormal cellular behaviors reminiscent of Rac activation, such as increased cell motility, increased membrane ruffling, and aberrant activation of JNK and the AP-1 transcription factor. The exact point of convergence between merlin and this pathway has remained elusive until now. We have identified a biochemical/molecular interaction between merlin and the Rac pathway via Pak1. Our studies revealed that merlin directly interacts with Pak1 and downregulates its activity, resulting in a hypophosphorylated form of the kinase. Conversely, absence of merlin results in high levels of active, hyperphosphorylated Pak1. To make things more complex, data from our lab and others has demonstrated that the Rac pathway, via Pak, controls merlin activity via phosphorylation. Therefore, not only does merlin converge on the Rac pathway upstream of Pak, but it is poised as an effector downstream of Pak. Interestingly, again, merlin is demonstrated to follow a model of ERM function, as the ERMs are both positively regulated by and positively regulate Rho.

Merlin and the group II Paks

Our experiments focused on Pak1, and, therefore, we did not investigate a possible role for merlin in the regulation of the recently identified group II Paks, Pak4, Pak5, and Pak6. Like the group I Paks, these Paks regulate cytoskeletal changes, for example, membrane ruffling and cell motility via Pak4 (Abo et al. 1998; Wells et al. 2002; Zhang et al. 2002) and neurite outgrowth via Pak 5 (Dan et al. 2002). However, structural differences between the group I and group II Paks, including the lack of an autoinhibitory domain in group II, might result in different mechanisms of regulation and different downstream effectors between the two classes. Given that most of the Paks are expressed either ubiquitously or specifically in the brain, it is possible that any/many of the Paks could participate in merlin function. Interestingly, data from another group revealed that in addition to active Pak2, active Pak1 and wild type Pak6 were able to induce merlin phosphorylation (Xiao et al. 2002). Therefore, as we have proposed a feedforward loop between merlin and the group I Paks, it is also plausible that merlin might be regulated by and might regulate the group II Paks.

Truncated versions of both Pak4 and Pak6 which contain only the kinase domain have greater kinase activity and, in one report, a greater effect on the cytoskeleton, than the full-length proteins, indicating a negative regulatory role for the N-terminal domain of the protein which harbors the CRIB motif (Abo et al. 1998; Yang et al. 2001; Wells et al. 2002). The authors of these studies propose that contrary to previous theories, the group II Paks may be regulated intramolecularly, but by different mechanisms than the group I Paks, since they do not contain an autoinhibitory domain. However, an alternative hypothesis is that the negative regulation in the N-terminal portion of the molecule is mediated through merlin binding to the CRIB. The

development of more reagents for these newly identified family members should facilitate such experimentation.

Despite relative little connection between the group I Paks and human cancer, the group II Paks, Pak 4 and 6, have been demonstrated to be frequently overexpressed in human tumor cell lines of different tissue origins (Callow et al. 2002). In addition, expression of activated Pak4 leads to cellular transformation in NIH3T3 cells, a behavior not observed from studies of the group I Paks. Expression of a kinase deficient Pak4 led to an inhibition of Ras and Dbl transformation. These data indicate that Pak4 activation is both necessary and sufficient for cellular transformation, and implicate a role for Pak4 more broadly in human cancer. Given that expression of $Nf2^{BBA}$ alone induces cellular transformation of NIH3T3 cells, it is tempting to speculate that, at least in this cell type, loss of proper merlin function leads to an upregulation of Pak4 activity, which results in transformation. More broadly this theory implies that perhaps merlin has the ability to regulate the function of all the Pak proteins, but does so in a cell type / context specific manner.

Implications of merlin binding to the CRIB domain

It is interesting to hypothesize that the tumor suppressor role of merlin may be broad-reaching and may affect many components of cytoskeletal dynamics, including cell-cell adhesion, cell-matrix adhesion, and cell polarity, all of which are required for the maintainence of normal cellular growth and tissue formation. There are many proteins downstream of the Rho GTPases that are responsible for carrying out these cytoskeletal functions. Given that merlin has been established as a negative regulator of the Rac pathway, and that it binds a functionally conserved domain in Rac/Cdc42 effector proteins, it is tempting to hypothesize that merlin may

mediate its affects on the cell not only through interactions with Pak, as we have identified in Chapter 4, but perhaps also through other Rac effectors. In mammals, over 25 CRIB-domain containing proteins have been identified, and they are classified into two groups, kinase and non-kinase (Pirone et al. 2001). These CRIB proteins carryout a wide range of mainly cytoskeletal functions following activation by Rac/Cdc42, including phosphorylation of targets required for actin cytoskeletal changes and regulation of tight junction assembly. Given that merlin binds to the CRIB domain of Pak, it is fascinating to hypothesize that merlin might interact with and regulate many, if not all, CRIB-domain containing proteins in its role as a tumor suppressor. The fact that phenotypes associated with overexpression of many of these CRIB domain-containing proteins are consistent with the phenotypes associated with merlin loss makes this hypothesis more plausible. One could begin to test this hypothesis through *in vitro* binding experiments and co-immunoprecipitations, as we have done with Pak1 (Chapter 4).

Therapeutic implications

Although we have demonstrated a direct interaction between the FERM domain of merlin and the CRIB domain of Pak, we have not elucidated the role that this interaction plays in the resulting regulation of Pak activity. It is also important to note that our data do not exclude the possibility that merlin binds other regions in Pak, outside of the CRIB domain. Because the Paks are activated by both GTPase-dependent and independent mechanisms, there exist many possible levels for merlin regulation. In fact, one study suggests that *both* membrane targeting and GTPase stimulation are required for Pak activation (Lu and Mayer 1999). In addition, integration of signals from cell-matrix adhesion and growth factor stimulation are important in the regulation of Pak activity. The diversity of mechanisms of Pak regulation and the multitude

of components co-localizing with Pak at the cytoplasmic surface of cell-matrix adhesion sites potentially indicates that merlin regulation of Pak is extremely complex. An understanding of these mechanisms would be beneficial to our complete understanding of the molecular function of merlin. However, the mere knowledge that loss of merlin function results in upregulation of Pak activity is important.

Because of the demonstrated role for Pak downstream of Ras transformation and our demonstration of Pak deregulation in the absence of merlin expression, it is interesting to hypothesize that deregulation of Pak may contribute to tumorigenesis in NF2 patients. However, whether these newly identified functions of merlin are relevant to its ability to suppress tumor formation remain to be established. Therefore, it would be extremely valuable to study the levels of Pak (group I and group II) expression and activity in human NF2 tumor tissue. Furthermore, it would be interesting to investigate whether sporadic schwannomas (or meningiomas or mesotheliomas) lacking NF2 mutation revealed an upregulation of Pak activity. Studies such as these would allow us to demonstrate an important physiological and cancer-related role for upregulation of Pak expression and/or activity. If NF2-related or sporadic schwannomas or meningiomas, for example, were to demonstrate high levels of activated Pak, we could investigate inhibition of this kinase as a modality for treating these tumor types. Interestingly, a very recent report suggests that, among other changes, membrane localization of phosphorylated/active Pak is increased in *NF2-/-* human schwannoma cells when compared to normal schwann cells (Kaempchen et al. 2003).

There are several candidate small molecules that have recently been tested for inhibition of Pak activity downstream of Ras transformation (He et al. 2000; He et al. 2001; Nheu et al. 2002). As a first step, it would be worthwhile to obtain and test the effect of these small

molecules on the behavior and growth properties of *Nf2*-deficient cells. The most striking phenotype of *Nf2*-deficient cells is the lack of inhibition of growth upon cell-cell contact. Not only could we test known inhibitors for reversal of the contact inhibition defects, but we could design a large-scale screen for small molecules that would reverse this phenotype. Molecules identified as efficient at reversing this cellular phenotype could then be used in several assays to determine their effects directly on Pak activity, JNK activity, and cyclin D1 levels. In addition, other cell systems might be useful in identifying Pak inhibitors, such as the rat schwannoma cell line (Chapter 4) and 3T3-$Nf2^{BBA}$ cells (Chapter 2).

It is likely that the recent solving of the Pak1 structure will facilitate the specific design of new Pak inhibitors, such as those that directly affect the kinase active site (Lei et al. 2000). However, specific targeting of kinase active sites with small molecule inhibitors has been problematic in the past; there are several examples of broad, non-specific inhibitory effects of such molecules in other systems. In the case of the Paks, membrane targeting has been demonstrated to be critical for its activity. Therefore, the identification of small molecules that might interfere with the binding sites for membrane targeting partners, leading to retention of Pak in the cytosol, would be extremely valuable. In addition, we have shown that merlin binds to the CRIB domain of Pak, another region that is functionally critical. Another inhibitor of Pak, hPIP, has also recently been demonstrated to bind to this domain (Xia et al. 2001). Therefore, it might be valuable to design small molecules that would bind to the Pak CRIB domain and exclude the binding of the Rac/Cdc42 GTPases. However, given the number of CRIB domain containing proteins, such a method would have to be approached with caution.

In summary, the finding that merlin regulates the Rac signaling pathway via a direct interaction with Pak opens several avenues with respect to NF2 research and therapeutic targets.

It will be interesting to see what role merlin plays in regulating cell-cell contact and adhesion within the complexity of the adhesion/signaling complexes. In addition, it will be important to establish what known, or unknown, binding partners are critical for merlin function. It is likely that the mechanisms of regulation of cell proliferation by merlin will be very cell type and context specific. Therefore, studying merlin function in the relevant cell types, e.g., schwann cells or mesothelial cells, will be critical for proper answering of the question: "Why does loss of *NF2* expression lead to tumorigenesis?"

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Appendix A

The role of merlin in the morphology and motility of polarized epithelial cells

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These experiments (carried out entirely by the author between July 1999 and September 2000) laid the groundwork for a project that was never completed. Inducible cell lines were prepared and initial studies performed to assess their value for the described experiments.

Question

The goal of this project was to understand the role of the tumor suppressor gene, *Nf2*, in tumorigenesis and metastasis. *Nf2* is a novel type of tumor suppressor gene in that it most likely mediates its function in the cytoplasm through an association with the cytoskeleton. Very little is understood about the interface between the proliferative state of the cell and the cytoskeleton, which must reorganize during the normal processes of cell division and differentiation, as well as during the transformation and invasion steps of tumorigenesis. Given the highly metastatic phenotype of the *Nf2*-/- tumors harbored by *Nf2*+/- mice, I became interested in examining the role of the *Nf2*-encoded protein, merlin, in cell morphology and motility. To achieve this, I began to design and employ a cell biological approach to address the following questions: (i) do alterations in merlin affect the general morphology, cytoskeletal organization, or migratory properties of polarized epithelial cells, and (ii) if so, does merlin mediate its function through antagonism of ezrin, a protein that promotes these activities?

Introduction and Rationale

The *Nf2* protein, merlin, is closely related to the band 4.1 family of membrane-cytoskeletal linkers. Within this family, merlin is most homologous to ezrin, radixin, and moesin (ERMs), which are implicated in cellular remodeling and formation of membrane ruffles and microvilli. Biochemically, merlin shares certain properties with the ERM family, including subcellular localization to cortical actin structures and binding to several integral membrane proteins, including CD44. However, despite some sequence and functional similarity, it is clear that merlin has distinct properties not attributed to the ERMs. Most notably, the ERMs and merlin seem to have opposite effects on cell proliferation.

The ERM proteins are positive regulators of cell growth and migration. Although the ERMs have not been implicated in human cancer, ezrin, the prototypical family member, has been associated with transformation and increased cell proliferation *in vitro* (Tran Quang et al. 2000). In addition, ezrin plays a critical role in controlling cell shape and promoting cell motility downstream of the hepatocyte growth factor (HGF) tyrosine kinase receptor in LLC PK1 polarized epithelial cells (Crepaldi et al. 1997). Regulation of ezrin protein function seems to occur through its ability to self-associate. An interaction between the N- and C-terminal domains of ezrin can occur intramolecularly in closed monomers and intermolecularly in head-to-tail oligomers. Gautreau and colleagues have demonstrated that phosphorylation of ezrin on a conserved C-terminal threonine (T567) regulates the transition from membrane-bound oligomers to active monomers, which link the membrane to the cytoskeleton through their C-terminal F-actin binding domain and N-terminal domains for binding membrane proteins (Gautreau et al. 2000). These active monomers induce, and are part of, actin-rich membrane projections, including lamellipodia, membrane ruffles, and microvilli. Normal cell morphology and motility

are severely disrupted upon overexpression of a T567A ezrin phosphomutant or a dominant negative allele of ezrin in LLC PK1 cells.

Despite sequence homology between merlin and ezrin and emerging data regarding ezrin regulation and function, the role of merlin as a putative membrane-cytoskeletal linker in normal cell biology is still poorly understood. Similarities between merlin and the ERMs suggest that the growth-regulatory capabilities of merlin may be due to effects on cytoskeletal organization and/or function. A few reports indicate that overexpression of merlin causes modest inhibitions of cell proliferation and motility in specific cell types (Tikoo et al. 1994; Sherman et al. 1997). In addition, the highly metastatic phenotype of tumors in *Nf2* +/- mice suggests a role for merlin in cell-cell or cell-matrix contacts, most likely associated with the processes of cell migration or invasion (McClatchey et al. 1998). Data generated in our lab has shed light on some factors that regulate merlin protein levels and phosphorylation. Merlin levels are increased in response to growth arrest induced by cell-cell contacts as well as serum deprivation (Shaw et al. 1998). Furthermore, these factors as well as loss of adhesion seem to modulate serine/threonine phosphorylation of merlin suggesting that phosphorylation may occur in response to cell spreading or changes in cell shape. Recent studies have suggested that, similar to ezrin, merlin can be regulated by head-to-tail self-association (Gronholm et al. 1999). Studies from our lab indicate that merlin is phosphorylated in a Rac-dependent manner at a serine in its C-terminal half and that this phosphorylation event is responsible for disrupting the head-to-tail association of the merlin molecule, much like that seen with ezrin (Shaw et al. 2001). The role of phosphorylation on merlin function is not clear, but it is currently under investigation. Interestingly, merlin is not only capable of forming homodimers, but also heterodimers with ezrin both *in vitro* and *in vivo*. Considering that merlin and ezrin have opposite effects on cell

proliferation and migration, and that merlin can heterodimerize with ezrin, an investigation of whether merlin mediates its tumor suppressor function through antagonism of ezrin function would be valuable.*

* Data have been generated that argue against this hypothesis. Quantitative immunoblotting revealed that the molar ratio of merlin/ERM in cultured epithelial or non-epithelial cells was approximately 0.14 or approximately 0.05, respectively (Maeda, M., T. Matsui, M. Imamura, and S. Tsukita. 1999. Expression level, subcellular distribution and rho-GDI binding affinity of merlin in comparison with Ezrin/Radixin/Moesin proteins. *Oncogene* **18**: 4788-97.). Therefore, it may be unlikely that merlin mediates its tumor suppressor activity via interactions with the ERMs when its presence is 6-20-fold lower than the ERMs in most cells.

Results/Discussion

Because it has been clearly demonstrated that ezrin plays a critical role in LLC PK1 epithelial cell morphology and motility, and that merlin can heterodimerize with ezrin, I was interested in investigating the role of merlin in LLC PK1 polarized epithelial cells. Not only would this serve as a good model system in which to examine the role of merlin in epithelial cell morphology and motility, but it would also allow for investigation of merlin as a potential modulator of ezrin function. Based on results from previous studies, I hypothesized that exogenous overexpression of wild-type merlin would cause inhibition of epithelial cell motility and disruption of normal epithelial cell morphology. Conversely, I hypothesized that overexpression of BBA merlin (a putative dominant negative allele; ref. Chapters 2 and 3), which should mimic loss of merlin function, would cause an increase in cell motility.

Establishment of an inducible system for regulated expression of merlin in LLC PK1 cells

Previous studies from our lab and others have indicated that it is difficult to stably overexpress *Nf2*, presumably because unregulated expression of merlin is toxic to the cell and leads to growth arrest or cell death. In addition, others have documented that overexpression of merlin inhibits cell proliferation. Because of these drawbacks to merlin overexpression, I have undertaken efforts to establish an inducible system for conditional expression of merlin in LLC PK1 cells. Previously, Gutmann and colleagues have demonstrated successful use of a Zn+-inducible expression system to study the effects of merlin overexpression in rat schwannoma cells (Gutmann et al. 1999). For my *Nf2* studies, I chose to use the ecdysone inducible system. Developed for mammalian cells in the laboratory of Ronald Evans, this system allows for dose dependent, regulated expression of the introduced construct (No et al. 1996). To study the

effects of over expression of merlin, a full-length wild-type *Nf2* cDNA (wild-type) was cloned under the ecdysone inducible promoter. In addition, to observe the effects of loss of merlin function in epithelial cells, the putative dominant negative blue box allele, $Nf2^{BBA}$, was cloned into the same vector.

Stable LLC PK1 cell lines expressing the ecdysone receptor were created (termed LLC-EcR). I developed a luciferase reporter assay by which to screen these cell lines for uninduced/background vs. induced expression of the introduced construct. A wide range of ecdysone receptor expressing cell lines with no/low background and varying ranges of inducibility have been set aside for experimentation (Figure 1a). The inducibility of the best of the LLC-EcR lines was compared to the commercially available 293-EcR and F099-EcR, an *Nf2*-deficient tumor cell line that stably expresses the ecdysone receptor (Figure 1b).

Merlin in LLC PK1 cells

While developing the inducible system for expression of merlin in LLC PK1 cells, I performed preliminary studies on the effects of merlin expression in stable LLC PK1 cell lines. These "stable" cell lines demonstrated the need for an inducible system, as the lines that "stably overexpressed" wildtype merlin had only 10-50% of cells with detectable levels of merlin by indirect immunofluorescence (Figure 2b and data not shown). In order to determine the phenotype of merlin overexpression or loss of function in LLC PK1 cells, I assessed BBA and wild-type merlin localization by indirect immunofluorescence, observing general cell morphology, including cytoskeletal organization, and determined the effects of merlin expression on cell motility.

A. Localization

Because LLC PK1 cells express endogenous levels of merlin, I planned to carry out localization experiments using untagged and epitope tagged forms of merlin. Previous studies have indicated that N-terminally epitope tagged merlin behaves identically to an untagged form (KCJ and TJ, unpublished results). Initially, LLC-$Nf2^{BBA}$ and LLC-$Nf2^{wt}$ cells were plated on coverslips and processed for indirect immunofluorescence with anti-HA antibodies, rhodamine-phalloidin to detect the actin cytoskeleton and DAPI to visualize the nucleus. Preliminary studies suggest that BBA merlin is more strongly localized to the microvilli than wildtype merlin which is found diffusely in the cytoplasm with some localization to the cell periphery (Figure 2b and c). In addition, it may be the case that there are more microvilli present in the LLC-$Nf2^{BBA}$ cells. It would be interesting to investigate potential differences in microvilli morphology and number between the cell types using electron microscopy. LLC-par control cells displayed only diffuse non-specific background staining with the anti-HA antibody (Figure 2a).

B. Cell Cycle Effects

Expression of BBA merlin in LLC PK1 cells lead to an increase in the percentage of cells that were in S phase, as assessed by BrdU incorporation of log-phase proliferating cells (Figure 3). Interestingly, when serum was removed from the media for 52 hours, most of the parental LLC PK1 cells arrested in the G1 phase, however, almost 20% of the LLC-$Nf2^{BBA}$ cells were found in S phase, indicating that these cells continue to cycle in the absence of serum.

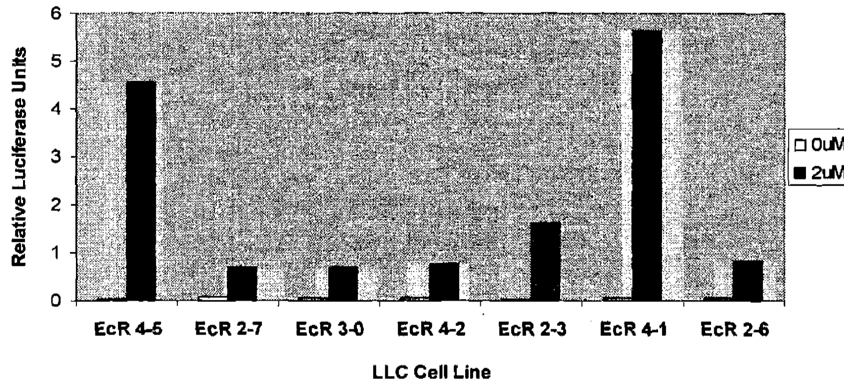
C. Migration and Invasion

The metastatic nature of *Nf2-/-* tumors and evidence from Gutmann and colleagues argue that wild-type merlin exhibits negative effects on cell motility. In order for tumor cells to undergo metastasis, they must break through the basement membrane, enter into the blood stream, and migrate to a distant site. In order to establish better the role of merlin in metastasis, I performed preliminary studies to investigate the migration and invasion properties of LLC-$Nf2^{wt}$ and LLC-$Nf2^{BBA}$ cell lines. HGF was first isolated as a factor that promoted scattering of epithelial cells *in vivo* and *in vitro* (Stoker et al. 1987). Since its original identification, many groups have demonstrated that HGF promotes migration of epithelial cells in many motility assays. Although the exact mechanism of HGF induction of cell motility is unclear, recent studies using LLC PK1 cells suggest that ezrin plays a crucial role in promotion of cell motility downstream of the HGF receptor. Therefore, I tested the migratory properties of the LLC PK1 cells in the presence and absence of HGF in wound healing assays. Not only would this assay (and others) allow for a direct examination of the effect of merlin on HGF-induced cell motility, but also would open a view to involvement of merlin in cell-cell and cell-matrix attachments which must undergo dynamic changes during the processes of invasion and migration during metastasis. My prediction was that overexpression of exogenous wild-type merlin would impair normal cell migration in response to HGF. Conversely, I predicted that BBA merlin would promote cell migration. Interestingly, BBA merlin promotes increased rates of cell motility (over LLC-par cells) in the absence of HGF/serum stimulation (Figure 4). Preliminary studies suggested that LLC-par, LLC-$Nf2^{wt}$ and LLC-$Nf2^{BBA}$ had similar motility/ wound healing rates when induced with HGF. Given that expression of BBA merlin leads to increased motility in the absence of serum, an investigation of how loss of merlin abrogates the need for growth factor

stimulation in epithelial cell migration should be undertaken. Finally, I observed merlin localization in the cells at the edge of the wound by indirect immunofluorescence and found that BBA merlin localized to the leading edge of the cell that faces the wound opening (Figure 5). However, in wild-type merlin expressing cells, which migrate slower than BBA expressing cells in the wound healing experiment, merlin was found more diffusely localized in the cytoplasm.

In summary, these data implicate loss of merlin function broadly in deregulation of epithelial cell shape and cell motility likely due to disruptions of the actin cytoskeleton network in the cell. Further experiments are required on many levels (some mentioned above) in order to establish a role for merlin in this cell type. In addition, it would be interesting to determine whether there is any crosstalk between merlin and ezrin in these cells and how such interactions might affect both of their functions. Because ezrin plays such a prominent role in the organization of the cytoskeleton of these epithelial cells, it would be advantageous to use the LLC PK1 cells with the inducible merlin expression system for such studies. Finally, I hoped that studies of this sort would lead to an appreciation for the normal role for merlin in the cell and in cytoskeletal regulation which may have resulted in a better understanding of why loss of merlin leads to tumorigenesis.

a



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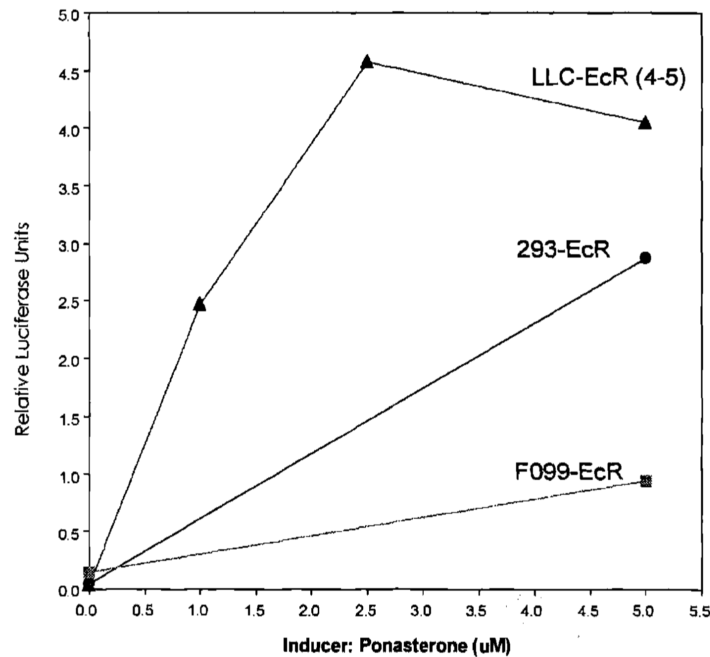

Figure 1. Inducibility of ecdysone-inducible cell lines. ***(a)*** Graphical representation of inducibility of various LLC-EcR clones as assessed by luciferase reporter assay. White bars represent background/uninduced levels. Black bars represent level of induction following 2 μM ponasterone A treatment for 24 hours. ***(b)*** Graphical comparison of inducibility following different levels of ponasterone A induction in the LLC-EcR (4-5) cell line compared to commercially available 293-EcR and ecdysone inducible *Nf2*-deficient tumor cell line, F099-EcR.

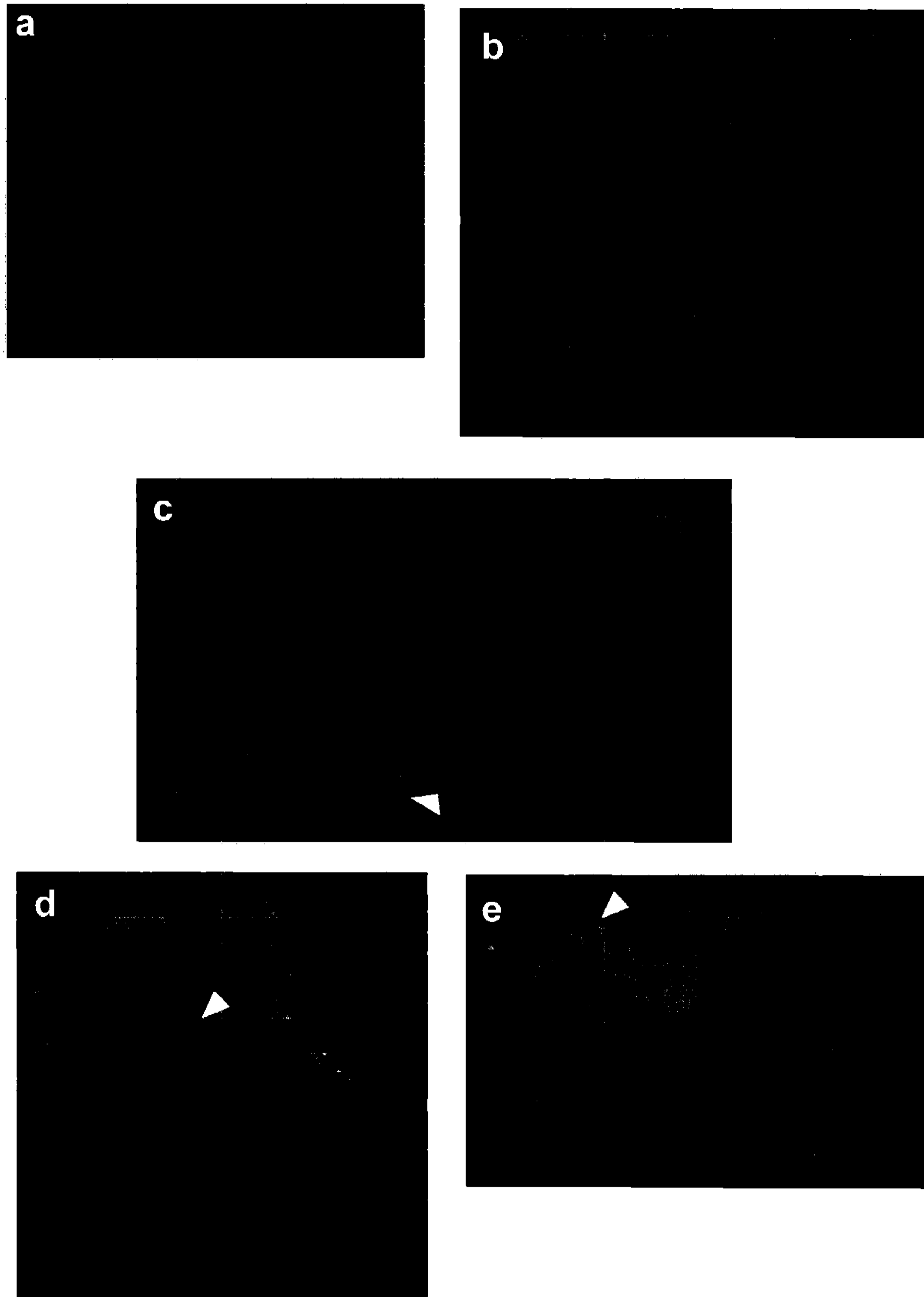

Figure 2. Merlin localization in LLC PK1 cells by indirect immunofluorescence. HA-tagged merlin (FITC- green) and nuclei (DAPI- blue) stained in LLC-Par cells ***(a)*** or LLC-Nf2^{wt} ***(b).*** LLC-Nf2^{BBA} cells stained for HA-merlin (green) only ***(c)*** or HA-merlin (green), nuclei (blue) and actin (red) ***(d&e)***. Arrowheads point to surface protrusions rich in merlin staining.

conditions / cell type	*parental*	*BBA merlin*
	% incorporation of BrdU / % in S-phase	
+ serum	13%	25.5%
serum starved (52 hours)	4.9%	18.45%

Figure 3. Cell cycle analysis of LLC PK1 cells. Chart represents percentage of cells in S phase under normally cycling conditions (+ serum) or serum starved conditions (serum starved 52 hrs) as assessed using BrdU incorporation. Parental = LLC-Par; BBA merlin = LLC-$Nf2^{BBA}$.

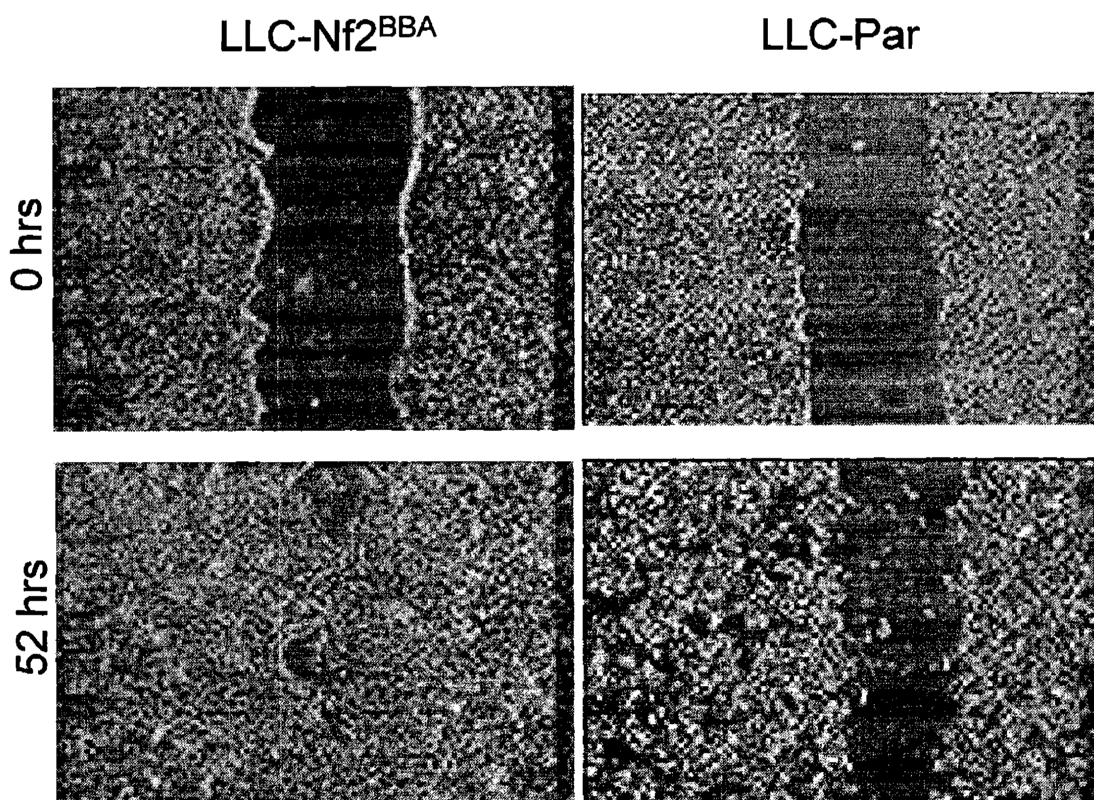

Figure 4. Wound healing capabilities of LLC PK1 cells. Representative photomicrographs 0 hours or 52 hours following scrape wounding of the monolayer of LLC-Par or LLC-$Nf2^{BBA}$ serum starved cells.

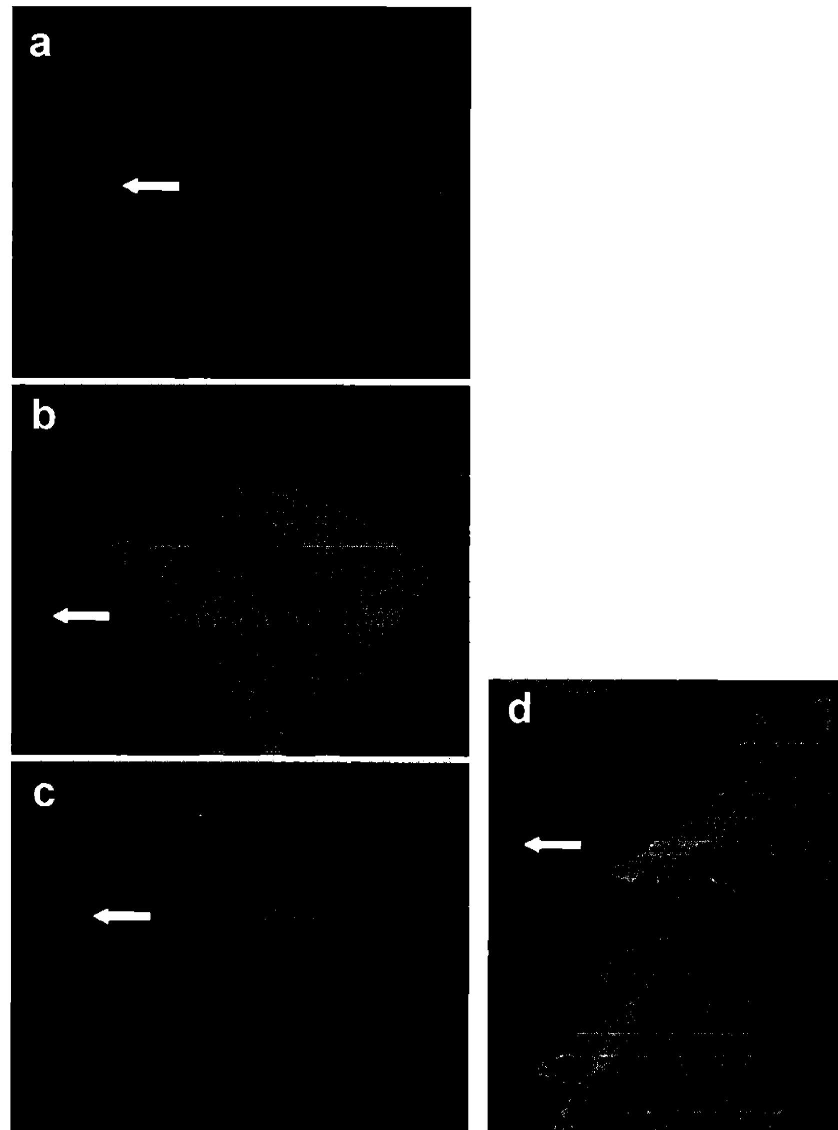

Figure 5. Merlin in cells at the edge of a wound. HA-merlin is visualized in green and nuclei in blue in LLC-Par cells *(a)*, LLC-Nf2^{wt} cells *(b)*, and LLC-Nf2^{BBA} cells *(c&d)*. Arrows point in the direction of cell movement/ into the wound.

Materials and Methods

Tissue culture conditions

All cells were maintained in 10% fetal calf serum, 5 mM glutamine, and penicillin/streptomycin at 37°C in 5% CO_2. HGF was used at 15 ng/ml.

Ecdysone Inducible System

LLC PK1 and F099 (*Nf2*-deficient fibrosarcoma) cells expressing the ecdysone receptor were created according to the manufacturer's instructions (Invitrogen). Briefly, cells were transfected with pVgRxR and selected for 1.5 weeks in 1 mg/ml zeocin. Stable clones were expanded and inducibility was assayed by transient transfection a pIND-Luciferase reporter construct (kind gift of Housman Laboratory) followed by induction with 0-5 μM amounts of ponasterone A. Dual-Luciferase reporter assays were carried out according to the manufacturers' instructions in order to assess luciferase expression (Promega).

Indirect Immunofluorescence

Cells plated on glass coverslips were fixed for 15 minutes in PBS with 4% paraformaldehyde, washed three times with PBS, permeabilized for 10 minutes in PBS containing 0.5% NP-40, and washed three times in PBS. HA-epitope tagged merlin was detected using the 12CA5 monoclonal antibody (1:1000; Becton Dickenson) followed by a FITC-conjugated goat anti-mouse antibody (1:200; Jackson Immunoresearch). Filamentous actin was visualized using rhodamine phalloidin (1:5000; Molecular Probes). Nuclei were visualized by DAPI staining (0.5 μg/ml). Cells were examined by conventional microscopy on a Zeiss microscope.

Brdu Incorporation

Cells plated on glass coverslips were either serum starved or left in serum for 52 hours. Cells were then incubated with 10 μM BrdU for 4 hours. Coverslips were processed as for indirect immunofluorescence above, except that the DNA was denatured following cell permeabilization (allowing for BrdU antibody detection) using hydrochloric acid. A mouse anti-BrdU primary antibody (1:50; Becton Dickenson) was followed by a FITC-anti-mouse secondary antibody. DAPI was used as a counter stain. Percentage of cells in S-phase was quantitated as a ratio of BrdU positive nuclei over total nuclei (DAPI).

Wound healing

Cells were plated at 5×10^5 per well of a 6 well dish and allowed to reach confluency (~ 48 hrs). A pipet tip (p20) was used to scrape a wound in the monolayer. Cells were either serum starved or HGF was added at the time of wounding. Cells were observed several times over the course of the experiment, and photomicrographs were taken at 52 hours. Wounded cell layers processed for indirect immunofluorescence were from cells plated and wounded on glass coverslips.

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Appendix B

Nf2$^{\Delta BB}$ transforms mouse fibroblasts

Kristen C. Johnson[1] and Tyler Jacks[1,2]

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All of the work in this appendix was performed by the author.

Results and Discussion

In *Drosophila*, deletion of the blue box region of merlin (ΔBB) resulted in the same phenotype as alanine substitution of these seven amino acids (BBA), the inability to rescue the lethality associated with merlin loss of function (LaJeunesse et al. 1998). However, notably, expression of a human ΔBB allele in epithelial cells resulted in near wild-type cellular appearance and behavior (Stokowski and Cox 2000). Nevertheless, we chose to make the equivalent allele in the context of murine merlin and test its behavior in transformation assays. Like 3T3-$Nf2^{BBA}$ cells, 3T3-$Nf2^{\Delta BB}$ cells are poorly adherent, form colonies in soft agar and tumors in nude mice (Figure 1 and data not shown) further implicating this region of the FERM domain as critical for the normal function of merlin.

Our data contrast with data from expression of human ΔBB in epithelial cells. While these studies demonstrated that dimerization between ΔBB and wild-type (wt) merlin was stronger than the wt-wt interaction, ΔBB showed a decreased affinity for one of its binding partners, NHERF, in *in vitro* binding experiments. These data indicate that mutation of the blue box region may result in disruption of the folding of the FERM domain making it unable to recognize its potential binding partners. In addition, it is likely that the blue box FERM domain disruptions lead to an opening of the protein allowing for greater interaction between the FERM of a wild-type molecule and the C-terminal half of ΔBB. Therefore, despite an increased affinity of ΔBB for wild-type merlin, it is likely that there is less self-association within the ΔBB molecule. However, further studies are required to support this hypothesis. Finally, as we have demonstrated with murine BBA merlin, there is no change in protein solubility of human ΔBB merlin. However, in stark contrast to our data with murine BBA merlin and, here, with murine

ΔBB merlin, cells expressing human ΔBB merlin had a rounded appearance with no/few cellular extensions and had no adhesion defects. Given our present study with mouse ΔBB and the high degree of homology between human and mouse merlin, it is likely that the differences observed in these two studies are due to cell-type specific responses to the expression of this allele.

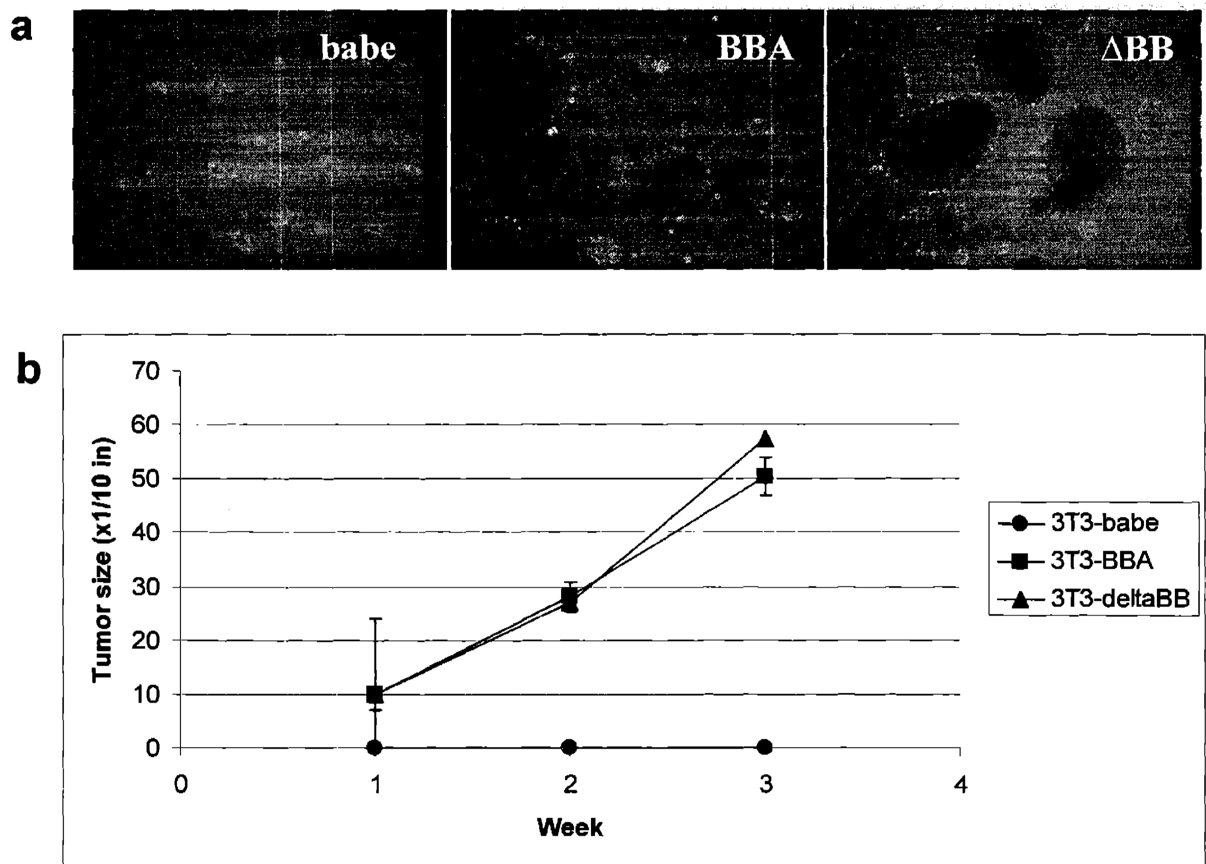

Figure 1. Soft agar colony formation and tumorigenicity assays in nude mice. ***(a)*** Representative photomicrographs of colony formation following a 3-week incubation of infected NIH3T3 cells in soft agar. ***(b)*** Graph representing the weekly diameter of tumors resulting from injection of infected NIH3T3 cells into the flanks of nude mice.

Materials and Methods

Retroviral transduction

NIH3T3 cells (ATCC) were infected with high-titer retrovirus stocks produced by transient transfection of ϕNX cells (gift from G. Nolan, UCSF). The efficiency of infection was always >90% (data not shown). The day before infection, NIH3T3 cells were plated at 10^6 cells per 10-cm dish. Infected NIH3T3s were selected for 3 days with 2 μg/ml puromycin (Sigma) and replated for the corresponding assays.

Tissue culture conditions

Dishes and wells were rinsed once with PBS, and then coated with 10 μg/ml fibronectin in PBS overnight at 4°C. Plates were rinsed twice with PBS following coating and blocked with 10% calf serum (10% CS; Sigma) in DME for 1 h at 37°C before plating cells. All infected cells were maintained in 10% CS, 5 mM glutamine, and penicillin/streptomycin at 37°C in 5% CO_2.

Immunoblotting

Whole cell extracts of exponentially growing cells were prepared in RIPA lysis buffer (50 mM TRIS-HCl, pH 7.5, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1mM sodium orthovanadate, and 1mM NaF) containing Complete protease inhibitor tablets (Roche). Protein concentrations were determined using the BCA protein assay reagent (Pierce). Immunoblot analysis for merlin was performed using the merlin sc331 antibody (Santa Cruz, 1:1000). Actin (sc-1616; Santa Cruz, 1:2000) was used as a loading control.

Soft agar assays

25,000 cells were resuspended in 0.34% low melting point agarose (LMP; Gibco, BRL) in DME/HEPES supplemented with 15% CS, penicillin/streptomycin and 5 mM glutamine. Cells were plated onto 6-cm dishes coated with 0.5% LMP agarose in DME. Cultures were maintained at 37°C and were supplemented with 2 ml of 0.34% LMP agarose in DME containing 15% CS once a week. Colony formation was followed for 3 weeks.

Tumorigenicity assays

Infected NIH3T3 cells were trypsinized, washed with PBS, and resuspended at 10^7 cells per ml in PBS. 10^6 cells (100 μl) were injected subcutaneously into the flanks of 5 week-old nude mice (BALB/c nu/nu, Jackson Laboratories). Mice were monitored for 5 weeks following injection.

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Appendix C

Effects of reintroduction of merlin into *Nf2*-deficient tumor cell lines

Kristen C. Johnson[1] and Tyler Jacks[1,2]

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All of the work presented in this appendix was performed by the author.

Rationale

In order to better understand the role of different domains and phosphorylation sites of merlin in a physiological setting, I undertook a project to reintroduce merlin into *Nf2*-deficient tumor cell lines. The goal, following these preliminary experiments, was to establish a system in which the biochemical function of merlin could be dissected with relationship to domains and phosphorylation. This system allowed for a simple readout for merlin function: reversal (or at least slowing) of the tumorigenic properties of the cells.

Results

Phosphorylation of merlin does not seem to affect its tumor suppressor function

The cell line employed for this study was derived from an osteosarcoma from an *Nf2*+/- mouse (termed, F1503). F1503 cells are *Nf2*-/- and form tumors in the flanks of nude mice and colonies in soft agar. F1503 cells were transduced with a recombinant pBABE retrovirus carrying either a wild-type *Nf2* cDNA ($Nf2^{wt}$), fragments of the gene [$Nf2^{Nterm}$ (aa 1-339) and $Nf2^{Cterm}$ (aa 252-595)] or merlin phosphomutants [$Nf2^{S518A}$ and $Nf2^{S518D}$]. An empty retrovirus ("babe") was used to control for possible effects of transduction on these cells. Puromycin resistant cells were selected and used for further experimentation. Figure 1 shows a western blot demonstrating the re-expression of merlin in the *Nf2*-deficient cells. F1503- $Nf2^{Cterm}$ cells were not used beyond this point, as the expressed merlin product is not of the correct size (~54kD); several products around 30-35kD specifically reacted with the merlin antibody (data not shown). This may have been due to misexpression from the retroviral plasmid or severe degradation of the Cterm protein following its production.

As expected, reintroduction of wild-type *Nf2* ($Nf2^{wt}$) slowed the proliferation of the tumor cells, whereas the N-terminal half alone ($Nf2^{Nterm}$) did not (Figure 2). These data are consistent with the effects observed following introduction of full-length or N-terminal merlin into rat schwanomma cells (Sherman et al. 1997). Surprisingly, reintroduction of either $Nf2^{S518A}$ or $Nf2^{S518D}$, serine 518 phosphomutants of merlin, slowed the proliferation rate of these cells. Previous studies have indicated that S518A is an "active" form of the protein, whereas S518D is "inactive" (Shaw et al. 2001). Therefore, in this present studied, we would have expected to see a slowing of tumor cell proliferation only in the presence of the $Nf2^{S518A}$ merlin expression. In addition, both of these merlin phosphomutants behaved similarly in a soft agar assay. As shown

in Figure 3, the number of colonies seen in the $Nf2^{S518A}$ and $NF2^{S518D}$ expressing cells is equivalent to that seen in the wild-type merlin expressing cells. In addition, there was no reduction in soft agar colony formation in $Nf2^{Nterm}$ expressing cells. These data indicate that both the N-terminal and C-terminal domains are likely required for the tumor suppressor function of merlin and that, in this assay and the proliferation assay, phosphorylation at residue 518 seems to have little effect on the growth suppressive function of this protein.

When injected into the flanks of nude mice, many of these cell lines behaved quite differently. There was significant variability in the tumor growth rates even within the same injected cell line (note large error bars in Figure 4a). What is first notable is that early in the experiment (weeks 1-2) there was a significant difference between the tumor diameter of all the experimental cells lines and the vector control line (p-value ≤ 0.023). However, as the experiment progressed (weeks 3-4), the cell lines that were slower growing at the start seemed to catch up to the faster growing, F1503, *Nf2*-deficient cell line. Measuring the diameter of the tumor mass weekly is a difficult method to assess the size of the tumor given the often abnormal shape. Therefore, at the end of the experiment, the tumor volume and weights of the resected tumors were measured. In the end, there was not a significant difference between the final tumor weight of any of the cell lines tested (Figure 4b). This data is obviously in opposition with the data generated in the tissue culture proliferation and soft agar assays. One hypothesis to explain the growth of tumors that overexpress inhibitory merlin molecules is the loss of expression of merlin in these cells or the outgrowth of a few cells in the population that began with no/low expression of the reintroduced *Nf2* cDNA. Either explanation is consistent with the later appearance of tumors from these injected lines. In order to test these hypotheses, whole cell extracts were prepared from resected tumor tissue (four weeks after injection) and were assayed

by western blotting for expression of merlin. As shown in Figure 5, there was variable expression of merlin in these tumors ranging from none to high levels. Therefore, more studies are required to establish the use of these cells as a model system for the dissection of merlin function.

Expression of BBA merlin versus wild-type merlin in Nf2-deficient tumor cell lines

A final experiment was carried out using the F1503 tumor cell line and reintroduction of wild-type merlin or BBA mutant merlin. The goal of this experiment was to obtain genetic evidence that BBA merlin acts as a dominant negative allele. The genetic definition of an antimorph is an allele that causes a loss of function phenotype when expressed in the presence of the wild-type allele (see Chapter 2 for experiments and discussion of this topic) and exhibits no phenotype in the presence complete loss of function. Therefore, the expression of BBA should not affect the transformed phenotype of *Nf2*-deficient F1503 cells. Interestingly, the data obtained from an independently generated F1503-Nf2^{wt} cell line was much more consistent than that seen for the above described experiments (Figure 6a). The diameter of the wild-type merlin overexpressing tumors was significantly different (p-value $\leq$ 0.003) than the vector control tumors across the entire 5 week dataset. In addition, the final tumor volume of the wild-type merlin overexpressing cells was 3-fold lower than the control cells (Figure 6b). Surprisingly, the BBA expressing cells showed an intermediate phenotype in the nude mouse tumorigenicity assay. Thus, it appeared that this *Nf2* allele may retain some of its growth suppressive function. However, in another assay of tumorigenicity, the BBA expressing cells formed many, large soft agar colonies equivalent to the levels observed with the vector control cells (Figure 6c). It is clear from experiments presented in Chapter 2 that the BBA allele acts as one would predict a

dominant negative-merlin allele to behave. However, the preliminary results presented here do not clearly demonstrate genetic proof. Therefore, it is important that these results be repeated using more independently-generated cell lines to follow up on these observations.

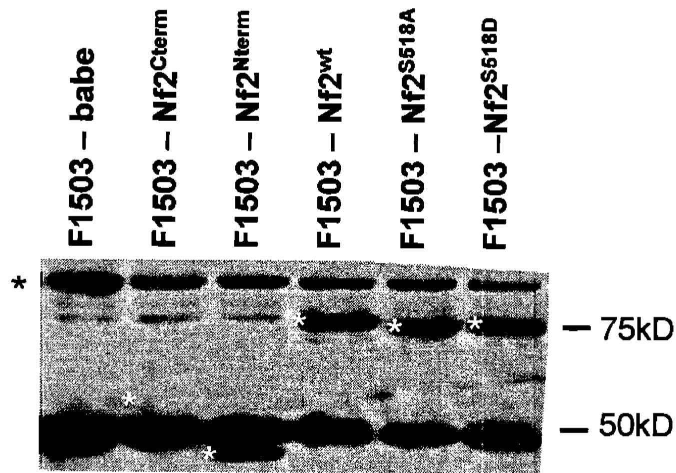

Figure 1. Overexpression of wild-type and mutant merlin in F1503 *Nf2*-deficient cells. Western blot of cell lysates prepared from retrovirally-infected F1503 cells following selection. Merlin (~70kD) is detected using the sc331 antibody. F1503-babe = F1503 cells infected with retroviruses carrying pBABE-puro empty vector; F1503-Nf2^{wt} = F1503 cells infected with retroviruses carrying wild-type *Nf2* cDNA; F1503-Nf2^{S518A} = F1503 cells infected with retroviruses carrying S518A mutant *Nf2* cDNA, F1503-Nf2^{S518D} = F1503 cells infected with retroviruses carrying S518D mutant *Nf2* cDNA, F1503-Nf2^{Nterm} = F1503 cells infected with retroviruses carrying the N-terminal half of the *Nf2* cDNA, and F1503-Nf2^{Cterm} = F1503 cells infected with retroviruses carrying the C-terminal half of the *Nf2* cDNA. * (white) indicates bands of expected size (or lack thereof). Note there is a non-specific, cross-reacting band that runs at almost the same molecular weight as merlin ~75kD (as indicated by the black * on the left side of the blot).

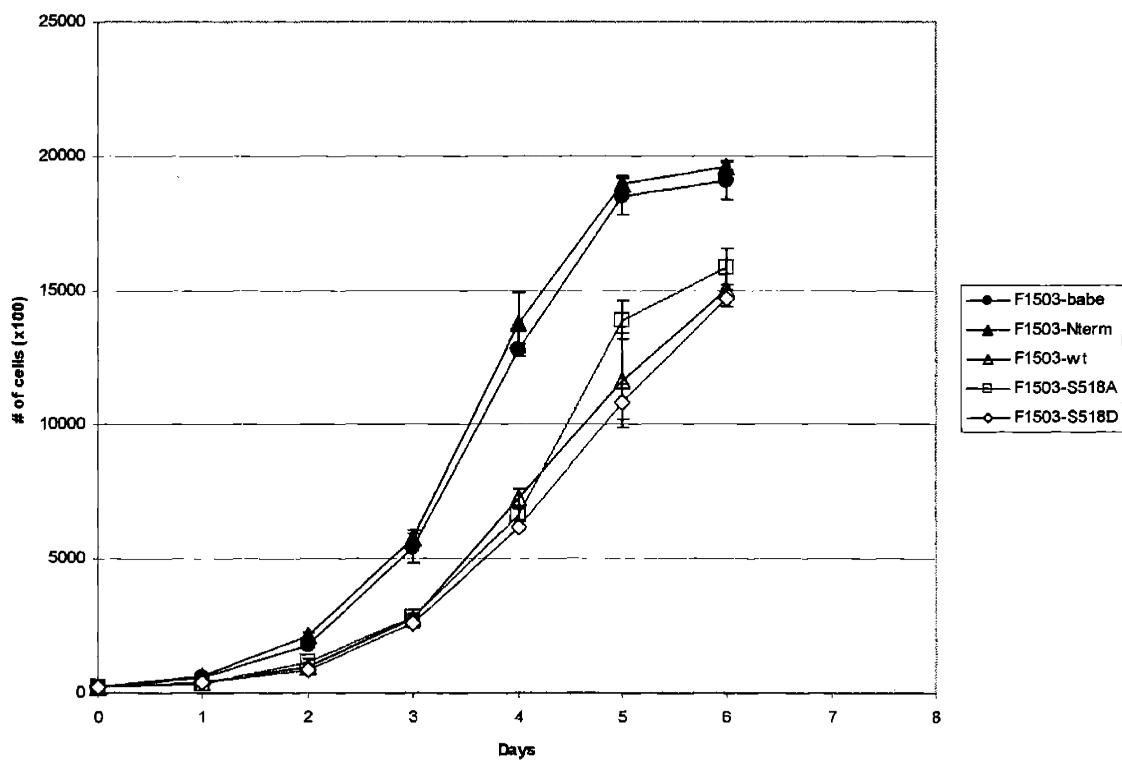

Figure 2. Wildtype and phosphomutant merlin suppress the proliferation of F1503 cells. Proliferation curves for infected F1503 cells. Data shown are from an assay performed in triplicate.

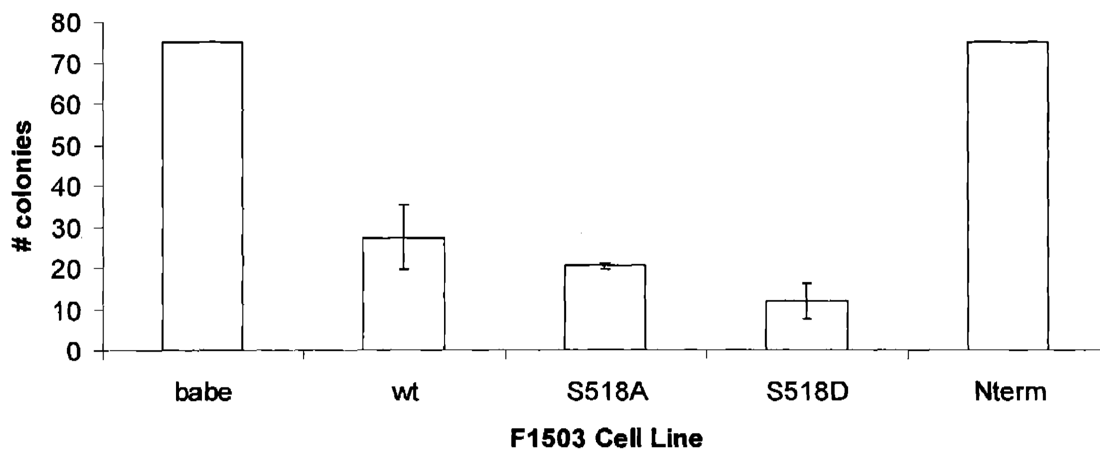

Figure 3. Soft agar colony formation. Graph representing quantitation of number of colonies for each infected cell line following 3 weeks incubation in soft agar .

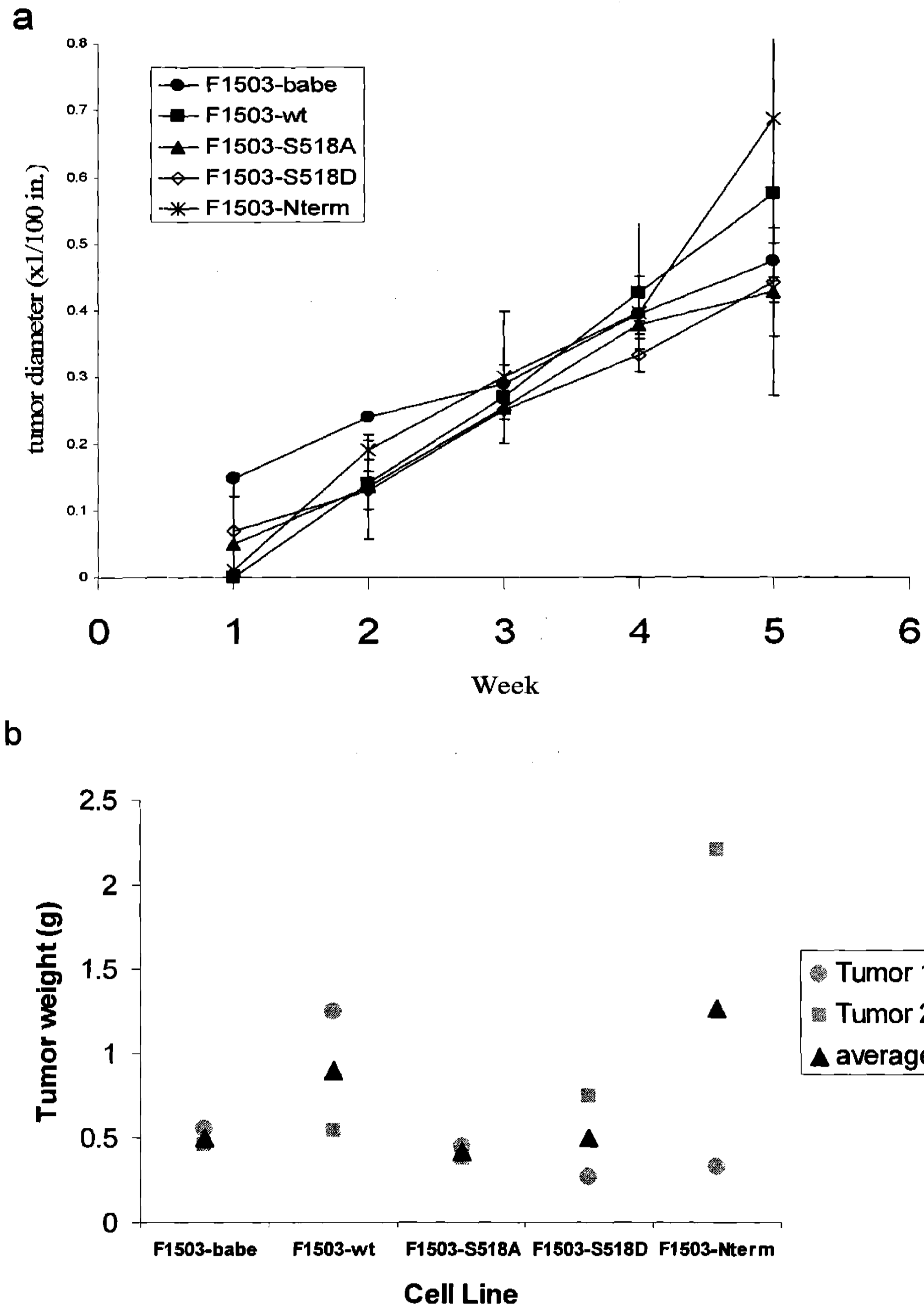

Figure 4. Tumor growth in nude mice. *(a)* Graph representing the weekly diameter of tumors resulting from injection of infected F1503 cells into the flanks of nude mice. *(b)* Graph representing the final weight of tumors resected from nude mice 5 weeks post-injection.

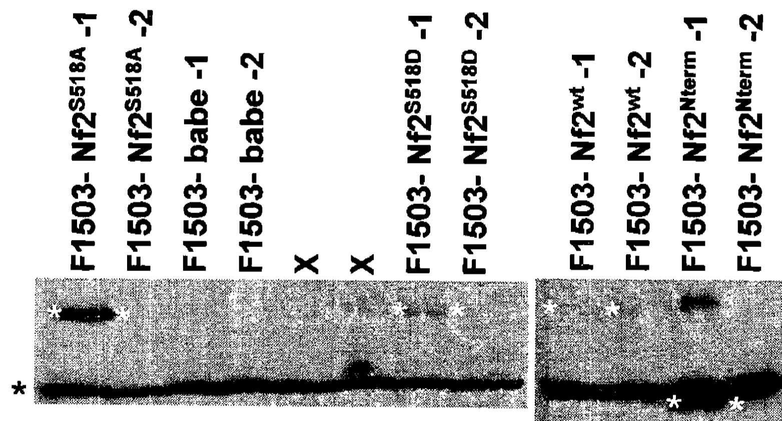

Figure 5 Merlin expression in nude mouse tumor tissue. Western blot analysis of merlin expression in homogenized tumor tissue from cell lines injected into nude mice 5 weeks post-injection.

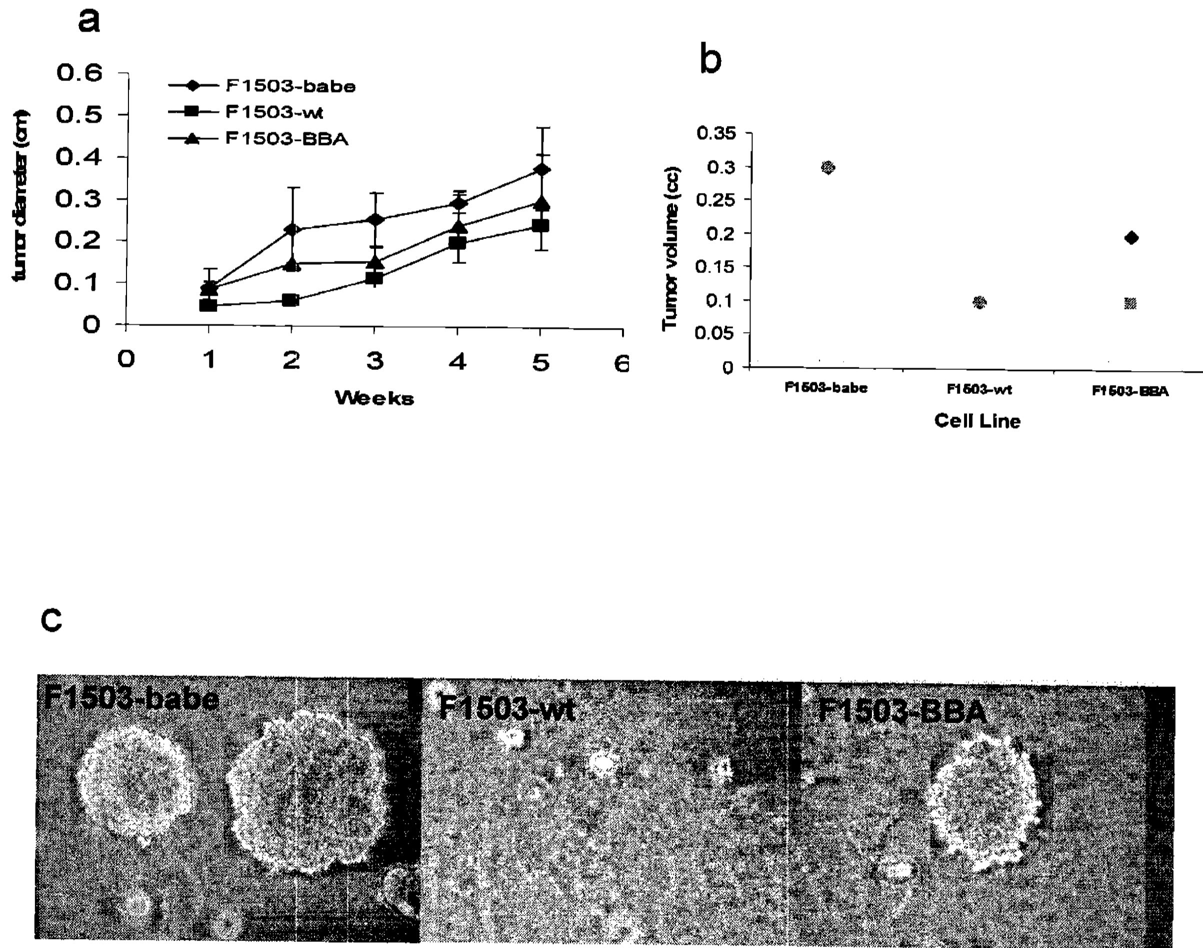

Figure 6. Tumor growth in nude mice and soft agar colony formation. ***(a)*** Graph representing the weekly diameter of tumors resulting from injection of infected F1503 cells into the flanks of nude mice. ***(b)*** Graph representing the final volume of tumors resected from nude mice 5 weeks post-injection. ***(c)*** Representative photomicrographs of colony formation following a 3-week incubation of infected F1503 cells in soft agar.

Summary

The results presented here, in part, demonstrate proof-of-principle that re-expression of wild-type merlin in *Nf2* deficient cells can reverse the transformed phenotype of these cells. The goal of the experiments was to determine the effects of re-expression of the N- and C-terminal halves of merlin as well as phosphomutants. Furthermore, the intention was to use these cell lines to dissect more about merlin function using assays described in Chapter 3 and 4. However, it is obvious, given the variability in this data that further experiments need to be carried out to confirm these preliminary results. Nevertheless, this may provide a useful system in which to dissect merlin function, particularly with respect to phosphorylation and the role of different domains of the protein in tumor suppression.

Materials and Methods

Plasmids

The $Nf2^{S518A}$ and $Nf2^{S518D}$ phosphomutants were created by site-directed mutagenesis of wild-type isoform I *Nf2* cDNA ($Nf2^{WT}$) in pcDNA3. $Nf2^{Nterm}$ (aa 1-339) and $Nf2^{Cterm}$ (aa252-595) cDNAs were amplified from wild-type isoform I *Nf2* cDNA ($Nf2^{WT}$) using primers that placed restriction enzymes at the 5' and 3' ends. All cDNAs were sequenced to verify the presence of the introduced mutations. All *Nf2* cDNAs in pcDNA3 were amplified with primers that added a 5' BamHI site and a 3' EcoRI site and then cloned into the pBABE-puro retroviral vector.

Retroviral transduction and tissue culture conditions

F1503 cells (derived from an osteosarcoma from an *Nf2*+/- mouse by Andi McClatchey) were infected with high-titer retrovirus stocks produced by transient transfection of ϕNX cells (gift from G. Nolan, UCSF). The efficiency of infection was always >90% (data not shown). The day before infection, F1503 cells were plated at 10^6 cells per 10-cm dish. Infected F1503s were selected for 3 days with 2 μg/ml puromycin (Sigma) and replated for the corresponding assays. All infected cells were maintained in 10% fetal calf serum, 5 mM glutamine, and penicillin/streptomycin at 37°C in 5% CO_2.

Immunoblotting

Whole cell extracts of exponentially growing cells or dounce homogenized tumor tissue were prepared in RIPA lysis buffer (50 mM TRIS-HCl, pH 7.5, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1mM sodium orthovanadate, and 1mM NaF) containing Complete protease inhibitor tablets (Roche). Protein concentrations were determined

using the BCA protein assay reagent (Pierce). Immunoblot analysis for merlin was performed using the merlin sc331 or sc332 antibody (Santa Cruz, 1:1000). Actin (sc-1616; Santa Cruz, 1:2000) was used as a loading control.

Proliferation Curves

Proliferation rate, as described here, is a measurement of cell accumulation over time and, therefore, does not take into account cell death. Proliferation curves were generated by plating 5 x 10^4 cells into each well of fibronectin-coated 12-well plates then trypsinizing and counting cells every 24 h over an 8 day period. The cells were counted using a Coulter Counter (Z1 series, Beckman Coulter). Measurement of cell proliferation was performed in triplicate for each cell line. Cells were fed every 3 days throughout the assay. Statistical analysis was performed using the student's T-test.

Soft agar assays

25,000 cells were resuspended in 0.34% low melting point agarose (LMP; Gibco, BRL) in DME/HEPES supplemented with 15% CS, penicillin/streptomycin and 5 mM glutamine. Cells were plated onto 6-cm dishes coated with 0.5% LMP agarose in DME. Cultures were maintained at 37°C and were supplemented with 2 ml of 0.34% LMP agarose in DME containing 15% CS once a week. Colony formation was followed for 3 weeks. At 3 weeks, quantification was achieved by counting the number of colonies visible to the eye under no magnification. (Counting was performed by two investigators and the numbers were averaged.)

Tumorigenicity assays

Infected F1503 cells were trypsinized, washed with PBS, and resuspended at 10^7 cells per ml in PBS. 10^6 cells (100 μl) were injected subcutaneously into the flanks of 5 week-old nude mice (BALB/c nu/nu, Jackson Laboratories). Mice were monitored for 5 weeks following injection. The diameter of the tumor was measured every week using calipers. At the 5 week timepoint, the mice were sacrificed and the tumors resected for final tumor weight/volume measurements.

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