

The *Nf2* Tumor Suppressor, Merlin, Functions in Rac-Dependent Signaling

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Summary

Mutations in the neurofibromatosis type II (*NF2*) tumor suppressor predispose humans and mice to tumor development. The study of *Nf2*^{+/-} mice has demonstrated an additional effect of *Nf2* loss on tumor metastasis. The *NF2*-encoded protein, merlin, belongs to the ERM (ezrin, radixin, and moesin) family of cytoskeleton:membrane linkers. However, the molecular basis for the tumor- and metastasis-suppressing activity of merlin is unknown. We have now placed merlin in a signaling pathway downstream of the small GTPase Rac. Expression of activated Rac induces phosphorylation and decreased association of merlin with the cytoskeleton. Furthermore, merlin overexpression inhibits Rac-induced signaling in a phosphorylation-dependent manner. Finally, *Nf2*^{-/-} cells exhibit characteristics of cells expressing activated alleles of Rac. These studies provide insight into the normal cellular function of merlin and how *Nf2* mutation contributes to tumor initiation and progression.

Introduction

Neurofibromatosis type 2 (NF2) is a familial cancer syndrome that features the development of tumors of the

nervous system, particularly schwannomas and meningiomas (Gutmann et al., 1997). The disease is caused by inherited mutation of the *NF2* tumor suppressor gene; sporadically occurring tumors of these types frequently exhibit inactivation of both *NF2* alleles. In addition, *NF2* mutations are found in malignant mesotheliomas (Gusella et al., 1999). Mice heterozygous for an *Nf2* mutation are predisposed to developing osteosarcomas, fibrosarcomas, and hepatocellular carcinomas that have lost the wild-type *Nf2* allele. Strikingly, most tumors arising in *Nf2*^{+/-} mice metastasize, which sharply contrasts with the generally rare incidence of metastasis in the mouse. Indeed, experimental evidence supports a role for *Nf2* loss in metastatic progression (McClatchey et al., 1998).

The *NF2*-encoded protein, merlin, is highly related to the cytoskeleton:membrane linking proteins ezrin, radixin, and moesin (the ERM proteins). Although the function of the ERM proteins is not well understood, the study of merlin function has largely paralleled that of the ERMs (reviewed in Bretscher et al., 2000). There are important distinctions and similarities between merlin and the ERMs. Merlin and the ERM proteins share similar subcellular localization to regions of dynamic cytoskeletal remodeling such as membrane ruffles, actin-containing microspikes, and the cleavage furrow (Gusella et al., 1999). All four proteins interact with membrane-associated partners via an amino-(N)-terminal domain and with cytoskeletal components via a carboxyl-(C)-terminal domain (Bretscher et al., 2000). However, the ERMs interact directly with actin via a domain that is not conserved in merlin. In fact, the ERM proteins are more closely related to each other than to merlin. Merlin does share binding partners with the ERM proteins, including the hyaluronic acid receptor CD44, NHE-RF/EBP50, and RhoGDI (Sainio et al., 1997; Murthy et al., 1998; Maeda et al., 1999). However, the functional consequences of interaction of the ERMs or merlin with these proteins remains unclear.

The membrane:cytoskeletal linking activity of the ERM proteins appears to be regulated through intra- and perhaps intermolecular association of their N- and C-terminal domains (Bretscher et al., 2000). Phosphorylation and phospholipid binding weaken self-association of the ERM proteins, stimulating their ability to link membrane proteins and F-actin into dynamic actin structures such as microvilli, membrane ruffles, and uropods (Bretscher et al., 2000). Recent studies suggest that merlin is also regulated by self-association, and can form homo- and heterodimers with ERM family members (Sherman et al., 1997; Huang et al., 1998; Gronholm et al., 1999; Nguyen et al., 2000).

Recently, we and others have shown that the small GTPase RhoA is an important regulator of the ERM proteins. The Rho family of GTPases affects reorganization of the actin cytoskeleton and regulates such cellular activities as motility, invasiveness, and proliferation through the regulation of several signaling pathways (for review see Zohn et al., 1998; Van Aelst and D'Souza-Schorey, 1997). RhoA activity was found to be necessary and sufficient for the phosphorylation of a conserved

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residue (T558 in moesin) in the F-actin binding domain of the ERM proteins and the subsequent formation of ERM-containing apical microvilli (Shaw et al., 1998a; Matsui et al., 1998; Oshiro et al., 1998). Phosphorylation of T558 inhibits the association of the N- and C-terminal domains of moesin in vitro, unmasking its F-actin and membrane protein binding potential (Matsui et al., 1998; Simons et al., 1998; Huang et al., 1999). Both Rho-kinase and phosphatidylinositol 4-phosphate 5-kinase have been implicated in affecting this activation of the ERM proteins in vivo (Matsui et al., 1999). Importantly, while the sequence surrounding this site is well conserved in all three ERM proteins, it is quite distinct in merlin.

Merlin is also phosphorylated on serine/threonine residues in response to a variety of conditions in culture (Shaw et al., 1998b). The simplest interpretation of the available data is that phosphorylation inactivates merlin. High levels of a hypophosphorylated form of merlin are associated with conditions of growth arrest by serum deprivation, increased cell density, and loss of adhesion. Interestingly, these are all conditions under which Rho GTPase signaling is downregulated (Kjoller and Hall, 1999; Ren et al., 1999). Given the links between RhoA and ERM phosphorylation, merlin phosphorylation and growth control, and loss of merlin function leading to metastatic tumors in mice, we have investigated whether merlin is also regulated by members of the Rho family of GTPases. In this study, we find that merlin is targeted by and is a regulator of the Rac/Cdc42-pathway, providing a framework for understanding how merlin normally functions as a tumor and metastasis suppressor.

Results

Merlin Is Phosphorylated in a Rac/Cdc42-Dependent Manner

By SDS-PAGE, merlin migrates as a doublet, representing hyper- and hypophosphorylated forms of the protein (Shaw et al., 1998b). Phosphoamino acid analysis and two-dimensional phosphopeptide mapping experiments revealed that merlin is phosphorylated at multiple sites, predominantly on serine residues, but also on at least one threonine residue (Shaw et al., 1998b; see Supplemental Figure S1 at <http://www.developmental.com/cgi/content/full/1/1/63/DC1>). To address whether Rho GTPase activation affects merlin phosphorylation, NIH3T3 cells were transfected with wild-type merlin and either activated or dominant-negative alleles of the Rho family members RhoA, Rac1, and Cdc42Hs. Importantly, the pattern of phosphorylation of exogenous merlin under various conditions in culture is comparable to that of endogenous merlin (see Figure 1A, first two lanes; Shaw et al., 1998b). For example, in serum-starved cells (Figure 1A, second lane) the ratio of the faster migrating, hypophosphorylated form to the hyperphosphorylated form of exogenous merlin is high. Strikingly, while coexpression of activated Rho did not significantly affect merlin phosphorylation, expression of two different activated alleles of Rac (RacV12, RacL61) strongly increased it. An activated allele of Cdc42 (Cdc42V12) also induced merlin phosphorylation (Figure 1A), which may reflect the activity of a common effector pathway or the

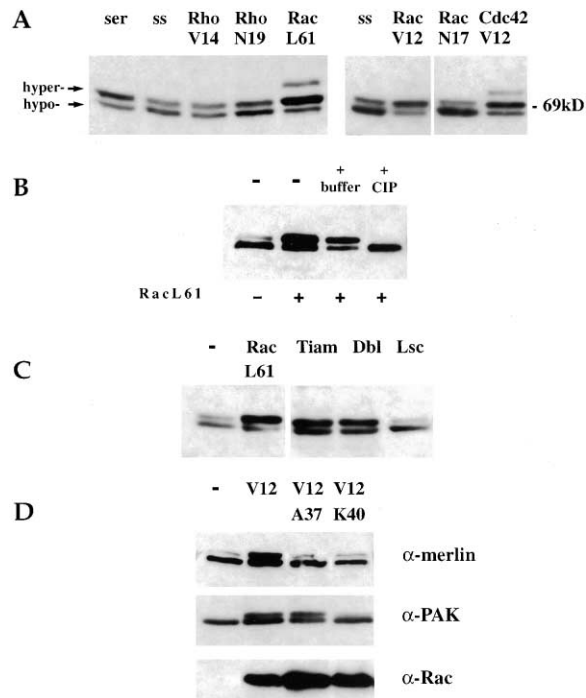


Figure 1. Activation of Rac or Cdc42, but Not Rho, Induces Merlin Hyperphosphorylation

(A) Wild-type merlin was transiently transfected with or without myc-tagged activated and dominant negative alleles of Rac, Rho, or Cdc42, as indicated, in NIH3T3 cells, then analyzed by immunoblotting with anti-merlin (sc331) antisera. All GTPases were expressed in serum-starved cells. First and second lanes show merlin phosphorylation in cycling (ser) and serum starved (ss) cells. Equivalent levels of activated and dominant negative GTPases were confirmed by anti-myc immunoblotting (data not shown).

(B) Wild-type HA-merlin was transfected with activated Rac, immunoprecipitated with anti-HA antisera, and treated with calf intestinal phosphatase (CIP), with CIP buffer alone or boiled in sample buffer as indicated. Merlin was detected by anti-HA immunoblotting.

(C) Wild-type merlin was transfected with Tiam1, Dbl, Lsc, or activated Rac as indicated and analyzed as above.

(D) Wild-type merlin or HA-tagged Pak1 were transfected with Rac effector mutants. Merlin was analyzed as above; Pak1 hyperphosphorylation resulting in a mobility shift detected by anti-HA immunoblotting indicates that RacV12A37 but not RacV12C40 activates Pak1.

sequential activation of Rac by Cdc42 (Nobes and Hall, 1995). A third merlin species with even slower mobility was also noted in the presence of activated Rac or Cdc42; this band may represent a super-phosphorylated form of merlin. Phosphatase treatment of immunoprecipitated merlin from Rac-transfected cells demonstrated that the Rac-induced mobility shift is due to phosphorylation (Figure 1B).

To examine whether activation of endogenous GTPases modulates merlin phosphorylation, merlin was transfected with members of the Dbl family of guanine nucleotide exchange factors (GEFs), each of which activates a subset of Rho GTPases in vivo. For example, Dbl activates Rac, Rho, and Cdc42 while Tiam-1 and Lsc are specific activators of Rac and Rho, respectively (Van Aelst and D'Souza-Schorey, 1997). As shown in Figure 1C, overexpression of either Tiam-1 or Dbl induced merlin phosphorylation, while expression of Lsc

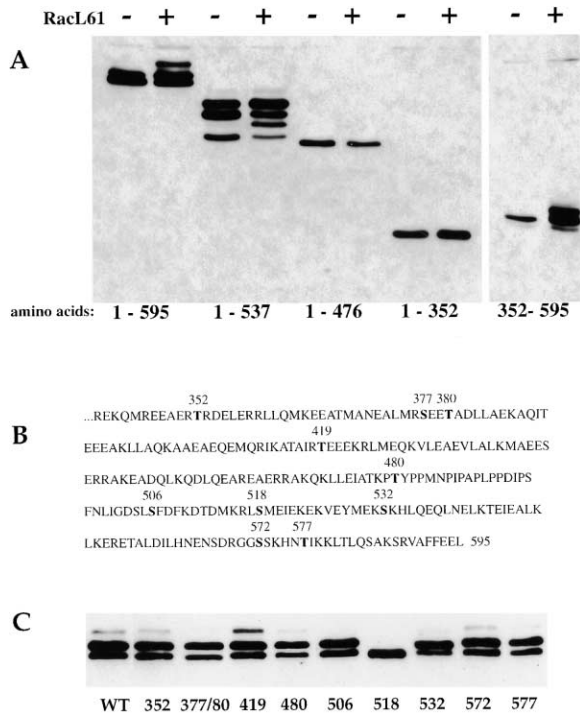


Figure 2. Identification of Rac-Dependent Phosphorylation Sites
(A) A series of HA-tagged merlin truncations were transfected with and without activated Rac and analyzed by immunoblotting. Residues contained within each truncation are indicated below the corresponding lanes.
(B) Amino acid sequence of the C-terminal half of merlin, with serine or threonine residues matching known kinase phosphorylation motifs or conserved in *Drosophila* numbered above the sequence.
(C) Potential phosphorylation sites were mutated individually (or in the case of 377 and 380, in combination), to alanine in the context of full-length HA-tagged merlin. These constructs were then transfected with activated Rac and analyzed by immunoblotting.

had no effect (Figure 1C). These results suggest that activation of endogenous Rac, and perhaps Cdc42, can mediate merlin phosphorylation.

Many serine/threonine kinases are known to be activated by Rac and/or Cdc42, and are thus candidates for mediating the effect of Rac activation on merlin phosphorylation (Aspenstrom, 1999). However, neither of two well-characterized Rac effector mutants (RacV12A37, RacV12K40) was capable of stimulating merlin phosphorylation (Figure 1D). As most known Rac effector pathways can be activated by one of these two effector mutants (Westwick et al., 1997; Lamarche et al., 1996; Joneson et al., 1996), this result suggests that multiple effector pathways are required for merlin hyperphosphorylation or that an uncharacterized pathway that is dependent upon both amino acids 37 and 40 of Rac is responsible for this effect.

Identification of Rac-Induced Phosphorylation Sites

To identify Rac-induced sites of phosphorylation in merlin, we transfected activated Rac with a series of epitope-tagged merlin truncation mutants and examined their phosphorylation by electrophoretic mobility shift (Figure 2A). As described previously, the N-terminal half

of merlin (residues 1–352) migrates as a single species (Shaw et al., 1998c); activated Rac had no additional effect on the mobility of this fragment. In contrast, the C-terminal half of merlin (residues 352–595) migrates as a doublet; introduction of activated Rac increased the amount of the upper species. Further deletion mapping narrowed the Rac-responsive region to residues 476–537 (Figure 2A). Orthophosphate labeling experiments revealed that although both the N- and C-terminal halves of merlin are phosphorylated in serum-starved cells, the introduction of activated Rac leads to increased incorporation of radioactive label in the C-terminal half only (data not shown). These data suggest that while there may be many phosphorylation sites in merlin, the Rac-responsive site(s) lie in the C-terminal half.

Within this region are ten serine/threonine residues, eight of which are surrounded by consensus kinase recognition motifs (Figure 2B). Each candidate residue was individually mutated to alanine in the context of the full-length molecule and transfected with and without activated Rac, to determine whether loss of any one site could affect Rac-dependent phosphorylation. As shown in Figure 2C, conversion of T577 to alanine did not effect basal or Rac-induced merlin phosphorylation; T577 is analogous to T558 of moesin, which is a target of RhoA-induced phosphorylation (Matsui et al., 1998, 1999). This is consistent with our observation that Rho activation had no effect on merlin phosphorylation. Instead, mutation of S518 had an appreciable effect on Rac-stimulated phosphorylation. In fact, the S518A mutant is not phosphorylated in response to serum and migrates as a single species. Orthophosphate labeling and phosphopeptide mapping experiments revealed that while the S518A mutant is still phosphorylated in cells, prominent phosphopeptides are absent (see Supplemental Figure S1). A synthetic peptide containing residue S518, but not one containing a S518A substitution, can be phosphorylated *in vitro* in a Rac-dependent manner, indicating that S518 is phosphorylated directly (J. Kissil and T.J., unpublished data).

Effect of S518 Phosphorylation on Merlin Function

We next investigated whether Rac-induced phosphorylation of merlin regulates its activity. Phosphorylation of the ERM proteins inhibits self-association and promotes their ability to link membrane and cytoskeletal partners. Thus, we investigated the effect of Rac-dependent phosphorylation on the ability of the N- and C-terminal halves of merlin to interact. First, the epitope-tagged, N-terminal half of merlin was transfected into NIH3T3 cells together with untagged versions of either full-length merlin or the C-terminal half. Immunoprecipitation of the N-terminal portion followed by immunoblotting for the C-terminal half revealed the association of the two (Figure 3A). Mutation of S518 to aspartic acid, which is predicted to mimic phosphoserine, inhibited the interaction of either the full-length or C-terminal forms of merlin with the N-terminal half. In contrast, mutation of S518 to alanine in the context of either the full-length or C-terminal half of merlin resulted in a stronger association with the N-terminal half. These data imply that the S518A mutant is not stably “closed” and therefore unavailable for interaction with the HA-N-terminal portion. This idea is supported by the recent demonstration

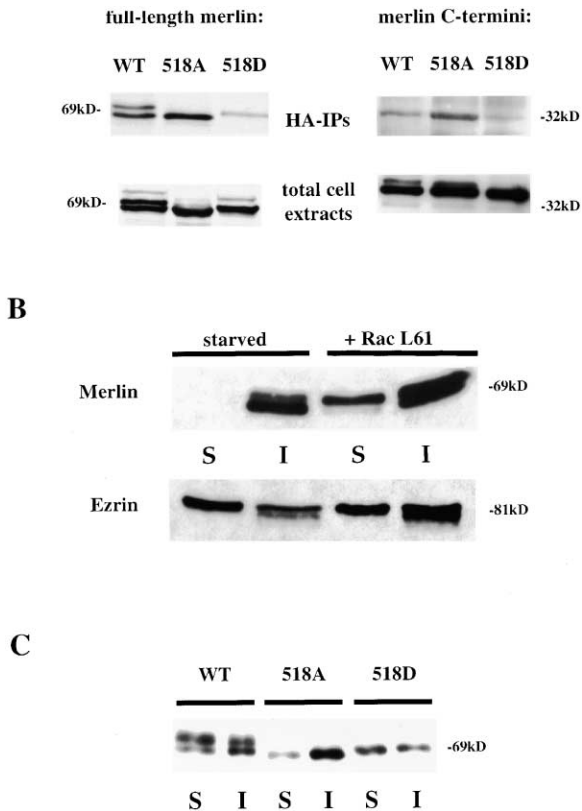


Figure 3. Rac Regulates the Intramolecular and Cytoskeletal Association of Merlin

(A) Untagged full-length or C-terminal merlin fragments were transfected with HA-tagged N-terminal merlin. Bound merlin was visualized by immunoprecipitation with anti-HA-antisera and immunoblotting with anti-merlin antisera directed against the final 20 amino acids of the protein (sc332). Five percent of the total cell extracts were immunoblotted to confirm equivalent expression of wild-type and mutant merlin isoforms. Control experiments indicate that merlin is not nonspecifically associating with the HA-antisera or protein A beads (data not shown).

(B) HA-tagged merlin or HA-tagged ezrin was transfected with or without activated Rac into NIH3T3 cells, which were then serum-starved. Triton X-100 soluble and insoluble fractions were then prepared and analyzed by anti-HA immunoblotting.

(C) Full-length wild-type, S518A- or S518D-expressing plasmids were transfected into NIH3T3 cells in 15% serum; Triton X-100 soluble and insoluble fractions were prepared and analyzed by immunoblotting with an anti-merlin antibody.

that association of the N- and C-terminal halves of merlin is a weaker and more dynamic interaction than that of the ERM proteins (Nguyen et al., 2000). We also examined these interactions directly by mixing ³⁵S-labeled in vitro-translated versions of full-length merlin with the HA-tagged N-terminal half of merlin and immunoprecipitating using anti-HA antisera; these experiments gave similar results (data not shown). These data suggest that phosphorylation of S518 disrupts merlin self-association, and perhaps oligomerization.

Given the distinct localization of merlin to areas of dynamic actin reorganization and its potential to interact with membrane and cytoskeleton proteins, localization is likely to be critical for its function. Thus, we examined the effect of Rac-dependent phosphorylation on the detergent-solubility of merlin. In serum-starved cells both

hyper- and hypophosphorylated merlin species were found in the detergent insoluble fraction (Figure 3B). When activated Rac was introduced, a significant amount of hyperphosphorylated merlin appeared in the soluble fraction. In contrast, activated Rac had no effect on ezrin solubility (Figure 3B). Consistent with this observation, examination of the detergent-solubility of expressed full-length S518A and S518D mutants revealed the relative insolubility of S518A and solubility of S518D compared to wild-type (Figure 3C). Together, these results suggest that Rac-induced phosphorylation of merlin weakens both its head-to-tail interaction and association with the cytoskeleton.

Overexpression of Merlin Blocks Rac-Mediated Transformation and Signaling

Overexpression of activated Rac can transform fibroblasts in vitro, and Rac activity is required for Ras-induced transformation (Khosravi-Far et al., 1995; Qiu et al., 1995). It has been previously reported that merlin overexpression can block Ras-induced transformation (Tikoo et al., 1994). We have confirmed this result and also examined the ability of merlin to block Rac-induced anchorage-independent growth in soft agar as a measure of transformation. We found that full-length merlin efficiently inhibited Rac-induced colony formation in this assay (Figure 4A).

We next examined the effect of merlin overexpression on signaling pathways that are activated downstream of Rac. A major downstream target of Rac is activation of the *c-jun*-N-terminal kinase (JNK) (Coso et al., 1995; Minden et al., 1995). We measured the effect of merlin overexpression on endogenous JNK activity by transfecting fibroblasts with full-length merlin along with a GFP-expressing plasmid. We analyzed basal JNK activity specifically in the transfected cells by fluorescence-activated cell sorting (FACS) and replating of the GFP-expressing cells. The levels of JNK activity were measured using an antibody that specifically recognizes phosphorylated (active) JNK. We found that merlin overexpression significantly reduced the level of phosphorylated JNK in cycling cells (Figure 4B, middle). Under these conditions, merlin overexpression had a negligible effect on the levels of phosphorylated extracellular regulated kinase (ERK) in these cells (Figure 4B, bottom).

A well-established consequence of JNK activation is increased activity of the AP-1 transcription factor, composed of heterodimeric combinations of jun and fos family members (Ip and Davis, 1998). After first ascertaining that merlin could also specifically block Rac-induced AP-1 activity, we asked whether this ability was dependent on merlin's phosphorylation state (Figure 4C). Our studies of merlin phosphorylation predict that hypophosphorylated merlin is the active, tumor suppressor form of the protein and that hyperphosphorylation inactivates it. To link merlin phosphorylation to its ability to block Rac signaling, we asked whether phosphorylation-defective (S518A) or phosphorylation-mimicking (S518D) forms of merlin could also block Rac-induced signaling. Strikingly, we found that while the S518A mutant inhibited Rac-induced activation of AP-1 reporter activity as well or better than the wild-type protein, S518D was clearly compromised in its ability to block Rac-induced signaling relative to wild-type (Figure

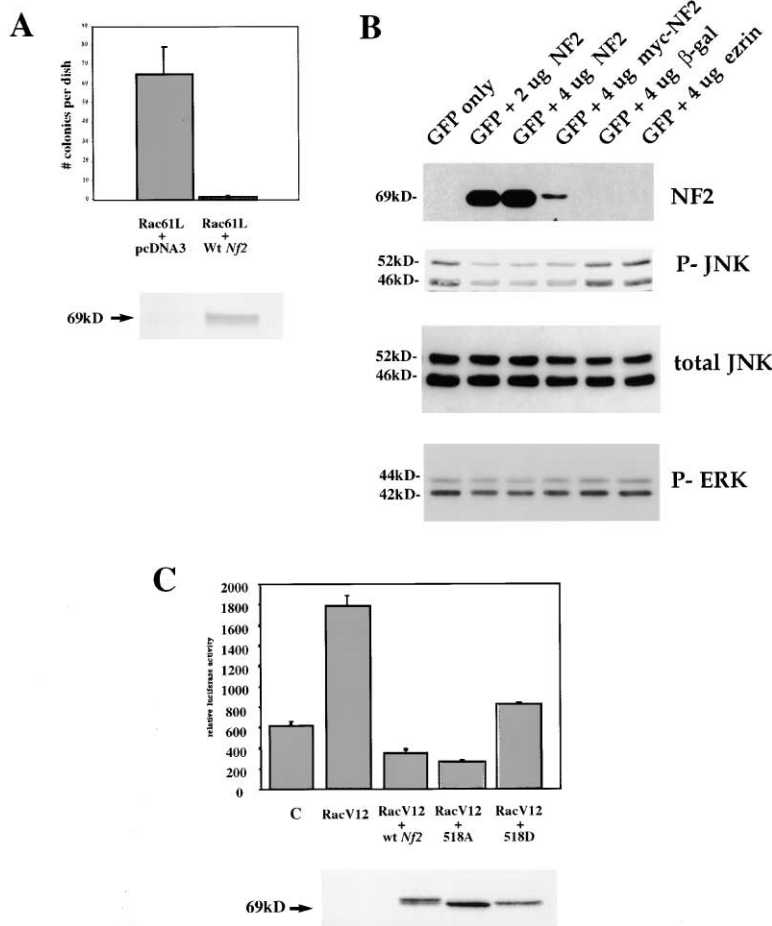


Figure 4. Merlin Inhibits Rac-Dependent Transformation and Signaling

(A) NIH3T3 cells stably expressing activated Rac with or without wild-type merlin were assayed for their ability to proliferate under anchorage-independent conditions. Cells were seeded in growth medium containing 0.3% agar, and colonies were visualized and quantitated after 14 days (top). Total cell lysates were immunoblotted using anti-merlin (sc331) or anti-Rac antisera to ensure expression of wild-type merlin (bottom) and Rac (data not shown). Experiment shown is representative of three independent experiments done in triplicate.

(B) Immortalized fibroblasts were transfected with a GFP-expression plasmid, with or without untagged or myc-tagged wild-type merlin, lacZ, or HA-tagged ezrin. GFP-positive cells were isolated by FACS and replated in 15% serum for 24 hr prior to harvesting. Total cell lysates were then immunoblotted with anti-merlin (sc331), anti-phospho-JNK, anti-phospho-ERK, or anti-JNK antibodies as indicated.

(C) Cells were transfected with the AP-1 reporter plasmid with or without an EF1α-GST-RacV12 expression plasmid and 50 ng of wild-type or mutant merlin expression plasmid, as indicated. Total cell lysates were immunoblotted with anti-merlin (sc331; below) or anti-GST (not shown) antibodies.

4C). Immunoblotting confirmed that equivalent levels of wild-type and mutant merlin protein were expressed in these cells (Figure 4C). These results are consistent with the possibility that Rac-induced phosphorylation inactivates merlin.

Examination of Rac-Dependent Signaling in *Nf2*-Deficient Fibroblasts

Given a link between Rac signaling and merlin phosphorylation and regulation, we wanted to examine the integrity of Rac signaling in the absence of *Nf2*. We previously generated a *Nf2* mutant strain of mice and found that *Nf2* homozygous mutant embryos fail at gastrulation, precluding the isolation of embryo-derived fibroblasts directly (McClatchey et al., 1997). Therefore, we generated primary *Nf2*^{-/-} fibroblasts from chimeric embryos composed of both wild-type and *Nf2*^{-/-} cells (see Experimental Procedures). In addition, several spontaneously immortalized fibroblast lines were generated for each genotype; these cells were used where indicated.

First, we compared the levels of phosphorylated (active) JNK in wild-type and *Nf2*^{-/-} fibroblasts by Western blot analysis. As shown in Figure 5A (left), we found that basal JNK activity was elevated approximately 4-fold in cycling *Nf2*^{-/-} fibroblasts and was further induced upon stimulation with serum or UV treatment. The same results were obtained by kinase assay (Figure 5A, right) and in *Nf2*^{-/-} ES cells (data not shown).

To determine whether AP-1 activity was also upregulated in these cells, we introduced an AP-1 luciferase reporter into wild-type and *Nf2*^{-/-} immortalized fibroblasts. As seen in Figure 5B, basal AP-1 activity was indeed increased in *Nf2*^{-/-} fibroblasts. Moreover, reintroduction of *Nf2* expression into *Nf2*^{-/-} fibroblasts resulted in a dose-dependent decrease in basal AP-1 activity to the levels of wild-type cells (Figure 5C). Taken together these results support the conclusion that loss of merlin function is responsible for deregulated AP-1 activity in *Nf2*^{-/-} cells.

In nearly every cell system examined, Rac plays a central role in the formation of membrane ruffles during cell spreading, cell motility, and growth factor stimulation (reviewed in Kjoller and Hall, 1999). To address the effects of merlin deficiency on this classic cytoskeletal response, we plated primary fibroblasts on coverslips and examined cell spreading by immunostaining for F-actin and the ERM proteins, which highlight the dynamic cortical cytoskeleton. In contrast to wild-type cells, at one hour post-plating, *Nf2*^{-/-} cells demonstrated prominent membrane ruffles rich in F-actin and ERM proteins (Figure 6A). At this time point, wild-type cells exhibited abundant apical microvilli, but few peripheral membrane ruffles. Notably, in addition to membrane ruffles, increased vesicles are also seen in the *Nf2*^{-/-} cells (see Figure 6A). Both of these phenotypes are observed in cells overexpressing activated Rac (Van Aelst and D'Souza-Schorey, 1997).

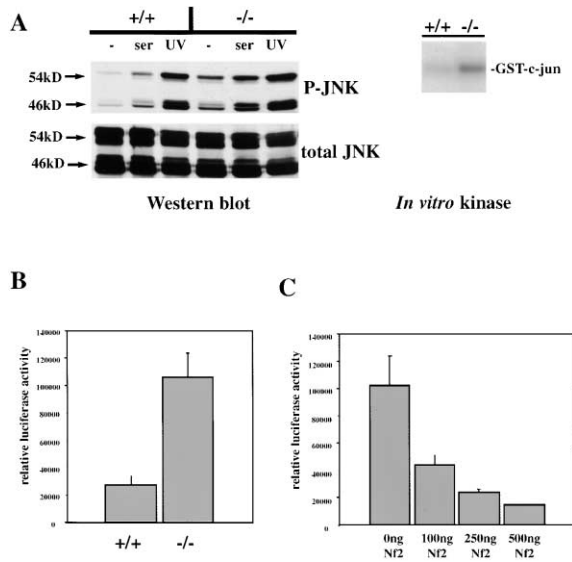


Figure 5. *Nf2* Deficient Fibroblasts Show Increased JNK and AP-1 Activity

(A) Cycling immortalized wild-type and *Nf2*^{-/-} fibroblasts were stimulated with serum or UV (80 J) for 30 min. Levels of activated JNK were evaluated by immunoblotting using an antibody that recognizes phosphorylated JNK (left). The corresponding levels of total JNK are shown below. In vitro kinase assay (right) yielded similarly elevated basal JNK activity in *Nf2*^{-/-} cells.

(B) An AP-1 luciferase reporter was transfected into wild-type and *Nf2*^{-/-} immortalized fibroblasts and basal luciferase activity was measured.

(C) The same AP-1 reporter was transfected into *Nf2*^{-/-} fibroblasts alone or with 50, 100, 250, or 500 ng of wild-type *Nf2* expression plasmid and resulting luciferase activity was measured. The data shown in (B) and (C) are representative of twelve independent experiments carried out using four different immortalized cell lines.

To examine whether *Nf2*-deficiency affected Rac-mediated cytoskeletal changes in another setting, we "scrape-wounded" confluent monolayers of wild-type and *Nf2*^{-/-} primary fibroblasts with a plastic pipette tip and then treated them with PDGF. As shown in Figure 6B, fifteen minutes after wounding and PDGF treatment, dramatic membrane ruffling was seen at the leading edge of the wounded *Nf2*^{-/-} monolayer. At this time point, wild-type cells at the leading edge of the "wound" displayed little membrane ruffling.

A positive role for Rac signaling in cell motility is well established. The link between merlin function and Rac signaling together with the metastatic nature of murine *Nf2*^{-/-} tumors suggested that *Nf2*-deficient cells may have enhanced motility. Thus, monolayers of wild-type and *Nf2*^{-/-} MEFs growing in serum were wounded and followed by phase contrast microscopy over the next 36 hr (Figure 6C). Strikingly, *Nf2*^{-/-} cells migrated to close the wound approximately two to three times as fast as wild-type cells (~15 vs ~36 hr). Similarly, *Nf2*^{-/-} immortalized fibroblasts migrate faster than wild-type fibroblasts in transwell chamber assays (data not shown). A recent analysis of the function of Rho GTPases in wound-healing of fibroblast monolayers revealed that while Rho activity was needed to maintain adhesion, Rac and Cdc42 activity were more critical in

promoting motility (Nobes and Hall, 1999). Therefore, the behavior of *Nf2*-deficient cells is most consistent with increased Rac- or Cdc42-dependent motility.

Discussion

Cloning of the *NF2* gene several years ago revealed that its encoded protein, merlin, is a member of a family of cytoskeleton:membrane linkers whose function is not well understood. Thus, merlin occupies an intriguing subcellular location for a tumor suppressor. The generation and study of *Nf2*-mutant mice confirmed its role as a tumor suppressor and suggested an additional role as a metastasis suppressor (McClatchey et al., 1998). Nevertheless, the molecular basis for the growth suppressing function of merlin remains elusive. We now provide several lines of evidence linking merlin function to signaling by the small GTPase Rac. Given the large body of evidence linking Rac activation to cell transformation, cell motility and invasion, these results suggest a mechanism whereby merlin exerts its tumor and metastasis suppressing activities.

Our results indicate that merlin is regulated by Rac. Although phosphopeptide mapping experiments indicate that merlin phosphorylation is complex (see Supplemental Figure S1), the existing data suggest that Rac-induced phosphorylation inactivates merlin. Phosphorylation of the Rac-responsive S518 residue inhibits merlin self-association, weakens its association with the cytoskeleton, and compromises its ability to inhibit Rac-mediated signaling. This suggests that the closed or oligomerized form of merlin is the active, growth suppressing form. This is consistent with the observations of Gutmann and colleagues, who found that the same amino acid residues necessary for self-association were also required for the growth- and motility- suppressing function of merlin in schwannoma cells (Sherman et al., 1997; Gutmann et al., 1999). Importantly, Rac is activated by growth factor stimulation and matrix adhesion, conditions which also stimulate endogenous merlin phosphorylation (reviewed in Kjoller and Hall, 1999; Shaw et al., 1998b). Our finding that merlin is phosphorylated in response to activation of Rac/ Cdc42 and not Rho would indicate that merlin and the ERMs are regulated in distinct ways. Together with the observation that merlin can heterodimerize with ERM proteins (Gronholm et al., 1999; Huang et al., 1998), this suggests that this family of proteins could serve to coordinate the activities of these GTPases.

The observation that merlin is phosphorylated in response to Rac activation might have suggested that merlin is an effector of Rac and thus required for some downstream functions of Rac. Instead, our results are consistent with a model wherein merlin normally acts to attenuate Rac signaling. First, overexpression of merlin inhibits JNK activity as well as Rac-induced AP-1 activity and transformation. Second, *Nf2*-deficient cells exhibit phenotypes that are consistent with hyperactivation of Rac signaling including increased activation of JNK, AP-1, membrane ruffling, and motility. Notably, a recent study also demonstrated that overexpression of merlin inhibited cell spreading and motility (Gutmann et al., 1999). Together these observations are consistent with a

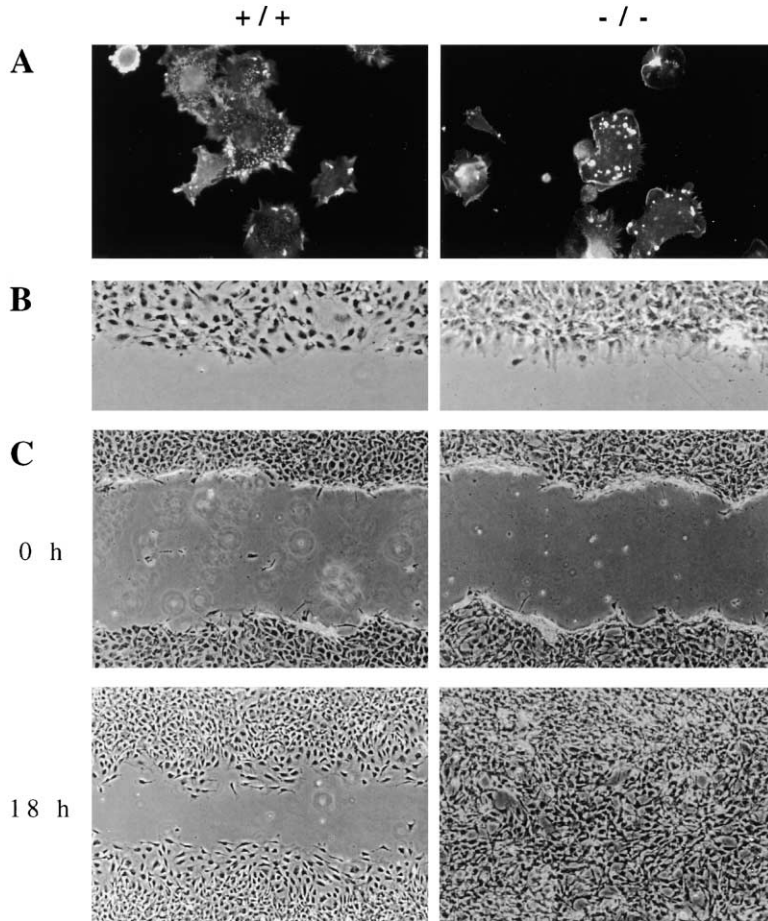


Figure 6. *Nf2*-Deficient Cells Exhibit Increased Ruffling in Response to PDGF and Substrate Reattachment and Exhibit Increased Rates of Migration during Wound Healing

(A) Serum-starved primary fibroblasts were plated on glass coverslips and fixed 1 hr post-plating. Cells were then processed for immunocytochemistry using a pan-ERM antibody to detect endogenous ERM protein localization. The ERM proteins are particularly concentrated in membrane ruffles in the *Nf2*^{-/-} fibroblasts.

(B) Monolayers of wild-type and *Nf2*^{-/-} primary fibroblasts were wounded and treated with 6 ng/ml PDGF. Increased protrusive ruffling is observed in the *Nf2*^{-/-} cells.

(C) Monolayers of wild-type and *Nf2*^{-/-} primary fibroblasts in full serum were wounded and examined by phase contrast microscopy for the next 36 hr. Photos shown were taken at the time points indicated. *Nf2*^{-/-} cells closed the wound 2-3× faster than their wild-type counterparts. Results shown are representative of three independent experiments.

model in which merlin functions as a sensor of Rac signaling. Phosphorylation of merlin in response to Rac activation or other stimuli inactivates merlin, thereby potentiating Rac signaling; dephosphorylation would restore its inhibitory function. This type of mechanism is analogous to the “feed-forward” mechanism of cell cycle progression established via inactivation of the cell cycle inhibitor p27 by cyclin-dependent kinase 2 (cdk2)-dependent phosphorylation and subsequent degradation (Vlach et al., 1997).

Several recent reports have demonstrated that the ERM proteins can similarly both regulate the activity of RhoA and be regulated by phosphorylation downstream of RhoA. As described above, RhoA-induced phosphorylation of the ERM C terminus is necessary and sufficient for the formation of apical microvilli (Matsui et al., 1999; Oshiro et al., 1998; Shaw et al., 1998a). Conversely, a role for the ERM proteins upstream of the Rho GTPases has emerged from several studies (Takahashi et al., 1998; Lamb et al., 2000). In fact, the ERM proteins can directly bind to RhoGDI and Dbl, negative and positive regulators, respectively, of Rho GTPases (Takahashi et al., 1997, 1998). Thus the ERM proteins may serve to localize regulators of the GTPases to particular sites of action at the membrane.

We propose that merlin functions as a tumor and metastasis suppressor through its ability to inhibit Rac-dependent signaling. Human *NF2* patients are predisposed to developing Schwann cell tumors while *Nf2*^{+/-}

mice develop a variety of metastatic cancers. Although little is known about the function of Rho GTPases in Schwann cells, human schwannoma cells exhibit actin cytoskeleton defects consistent with increased activity of these GTPases (Pelton et al., 1998). It is well established that Rac activity is necessary and sufficient for transformation of several other cell types (Zohn et al., 1998). In addition, a large body of literature indicates that Rac activity controls cell motility and invasion. For example, the Rac exchange factor Tiam-1 was identified in a screen designed to identify genes that promote cell invasion in vivo (Habets et al., 1994). In addition, several studies in normal and tumor cells have found that Rac is a critical determinant of cell motility and invasion (Keely et al., 1997; Shaw et al., 1997; Nobes and Hall, 1999). Finally, NIH3T3 cells expressing activated Rac display increased metastatic potential when injected into mice (del Peso et al., 1997).

The mechanism by which Rac causes transformation or affects cell motility is not known; however, multiple downstream targets are likely required for the full transforming properties of Rac (Joneson et al., 1996; Lamarque et al., 1996; Westwick et al., 1997). For example, AP-1, a key target of Rac signaling, plays an important role in cell proliferation and cellular invasion. Known AP-1 targets include members of the matrix metalloproteinase family, which have been repeatedly linked to the process of tumor metastasis, suggesting a mechanism by which *Nf2* loss leads to metastatic tumor formation

(Ip and Davis, 1998). Alternatively, *Nf2* loss may alter the function of its direct membrane binding partner CD44, whose function has been linked to metastatic progression of a wide variety of human tumors (Sherman et al., 1994). These two hypotheses are not mutually exclusive. Indeed, CD44 ligation can activate Rac under certain conditions, perhaps via direct interaction with the Rac-specific exchange factor Tiam-1 (Oliferenko et al., 2000; Bourguignon et al., 2000). Thus by directly binding CD44 and negatively regulating Rac signaling and AP-1 transcription, merlin may be uniquely poised to negatively regulate tumor metastasis.

Our data do not define the mechanism by which merlin controls Rac signaling. Extensive mutational analysis of Rac suggests that the downstream effector pathways controlling cytoskeletal and transcriptional responses are distinct. Given that overexpression of merlin effects both (Figure 5, Gutmann et al., 1999), and both are deregulated in *Nf2*-deficient cells (Figures 5 and 6), merlin may act to modulate Rac directly, or to modulate the activity of one of its enzymatic regulators. We have been unable to detect appreciably elevated levels of active Rac-GTP in *Nf2*^{-/-} cells by measuring the amount of (active) Rac-GTP bound to its effector Pak (data not shown; Bagrodia et al., 1998; del Pozo et al., 2000). This may be due to subtle changes in Rac activity coupled with the relative insensitivity of this assay or to the fact that merlin actually functions downstream of Rac as suggested by the data in Figure 4. In fact, like the ERM proteins, merlin can also bind to RhoGDI (Maeda et al., 1999). One possibility is that merlin controls the subcellular localization of Rac or RhoGDI. We have not detected alterations in the subcellular distribution of either Rac or RhoGDI in *Nf2*^{-/-} fibroblasts by crude cell fractionation; however, a higher resolution analysis is necessary before this hypothesis can be ruled out.

It is likely that merlin normally controls Rac signaling in a context-dependent manner, an idea that is supported by the relatively modest elevation of JNK activity in *Nf2*^{-/-} cells (Figure 5A). Interestingly, a recent study demonstrated that JNK activation by UV and serum is inhibited by cell confluence (Lallemand et al., 1998). Disruption of the actin cytoskeleton reversed this effect, suggesting cytoskeleton-based, confluence-sensitive regulation of JNK. Because merlin levels and phosphorylation are also regulated by cell confluence (Shaw et al., 1998b); merlin is therefore a candidate to mediate this effect. In fact, we have found that *Nf2*^{-/-} fibroblasts are not contact inhibited, growing to an extremely high saturation density and forming a multilayer rather than a monolayer (our unpublished data).

These studies suggest that the molecular basis for the tumor and metastasis suppressing function of merlin is at least partly due to its ability to negatively regulate Rac signaling. This, in turn, suggests many potential avenues of research and allows us to consider potential therapeutic strategies targeted specifically to Rac signaling. For example, Rac is posttranslationally modified by geranylation (Adamson et al., 1992). Thus, by analogy to the use of farnesyltransferase inhibitors to block post-translational modification of Ras, newly developed geranylgeranyltransferase inhibitors may show efficacy in slowing or preventing tumorigenesis and metastasis in *Nf2*^{+/-} mice, and ultimately in treating tumors in NF2

patients (Sebti and Hamilton, 2000). Finally, these studies also provide a broad link to the role of the membrane:cytoskeleton interface in tumor development and progression.

Experimental Procedures

Cell Culture

NIH3T3 cells (ATCC) were grown in DMEM plus 10% calf serum (Hyclone). To generate primary *Nf2*^{-/-} fibroblasts, we utilized *lacZ*⁺;*Nf2*^{-/-}, and *lacZ*⁺;*Nf2*^{+/+} embryonic stem (ES) cell lines generated de novo from blastocysts isolated from ROSA26;*Nf2*^{+/-} intercrosses (McClatchey et al., 1997). ES cells of each genotype were used to generate chimeric embryos by blastocyst injection. Embryonic fibroblasts harvested from E12.5 chimeric embryos were expanded and selected in G418 (400 μg/ml) to obtain pure populations of each genotype. Each *Nf2* mutant allele as well as the ROSA26 transgene contains a single copy of the *neo^r* gene. Multiple ES cell lines of each genotype were used for each experiment. After one passage in selection an aliquot of cells was taken for PCR analysis and lacZ staining to determine whether selection was complete (McClatchey et al., 1997). Primary cells were used between passages 4 and 8. Spontaneously immortalized cells were also generated using the 3T9 protocol (Todaro and Green, 1963).

For wounding assays, a pipette tip (p20) was used to scrape a monolayer wound; 6 ng/ml of PDGF was added immediately at the time of wounding. For UV treatment, growing cells were treated with 80J UV-C (Stratalinker; Stratagene) after rinsing with PBS. Following treatment, fresh media was placed back on the cells for the time indicated (30 min).

Antibodies

sc331 and sc332 antibodies (Santa Cruz) were used at 1:100 to detect merlin by immunoblotting. Anti-P-JNK, P-ERK, and ERK antisera were all purchased from New England Biolabs and used according to manufacturer's recommendations. Anti-JNK and anti-GST antisera were purchased from Santa Cruz. Anti-HA (12CA5) and anti-Rac antisera were purchased from Boehringer Mannheim and UBI, respectively. Anti-ERM antisera (CR-22) was provided by S. Tsukita.

Plasmids

Merlin truncations were generated by PCR amplification of the specified coding region using oligonucleotides containing flanking Sal I restriction sites. These PCR products were ligated into the Sal I site of the previously described HA-modified pCDNA3 or pCI-Neo (Shaw et al., 1998c). Site-directed mutagenesis of merlin and Rac was performed using the Quickchange kit (Stratagene) according to manufacturer's instruction. Each mutant was sequenced in its entirety. pEBG-RacV12 was supplied by J. Blenis (Chou and Blenis, 1996). All other GTPase and merlin constructs were previously described (Shaw et al., 1998a, c). CMV-Lsc and CMV-Dbl were supplied by J. Glaven and R. Cerione (Glaven et al., 1999). CMV-Tiam1 was supplied by C. Carpenter. CMV-HA-hPAK1 was supplied by J. Chant (Brown et al., 1996). AP-1 (7X), SRF, CMV-lacZ, and TK-renilla luciferase reporters were purchased from Stratagene.

Transcriptional Reporters

Nf2^{-/-} and wild-type immortalized cells were transiently transfected using Lipofectamine Plus (LTI) according to manufacturer's instructions. Cells were transfected with the indicated amount of reporter, a GFP expression plasmid to monitor transfection efficiency, a TK-renilla luciferase plasmid, and, where indicated, untagged wild-type, S518A or S518D merlin, and/or GST-RacV12 expression vectors. Dual luciferase quantitation was determined using kits from Promega.

Indirect Immunofluorescence

Cells plated on glass coverslips were rinsed in PBS and fixed in cold 3.7% paraformaldehyde for 15 min. Cells were then permeabilized in 0.2% Triton X-100 for 10 min and processed for indirect immunofluorescence as previously described (Shaw et al., 1998c).

Protein Analysis

Total cell lysates were made by washing cells in PBS, followed by addition of boiling SDS-lysis buffer (10 mM Tris 7.5, 1% SDS, 50 mM NaF, 1 mM NaVO₄) to the plate. Protein concentration was calculated using the BCA assay (Pierce) and Western analysis was performed as previously described (Shaw et al., 1998b).

Phosphorylation Analysis

NIH3T3 cells seeded at a density of $\sim 0.2 \times 10^6$ per 60 mm plate were transfected using Lipofectamine Plus (LTI) according to the manufacturer's instructions. Cells were placed in DMEM without serum 18 hr later. Six hours later, cells were washed in PBS and lysed in boiling SDS lysis buffer. Phosphatase treatment, orthophosphate labeling, and detergent extraction analysis were performed as previously described (Shaw et al., 1998a).

In Vitro Kinase Assays

Kinase assays were performed essentially as described (Hibi et al., 1993). Briefly, total JNK immunoprecipitated with an anti-JNK antibody and protein A agarose beads (Pierce) was pelleted and resuspended in 30 μ l kinase buffer with 5 μ g GST-c-jun (Santa Cruz), 20 μ l ATP, and 5 μ Ci [γ -³²P]ATP and incubated at 30°C for 20 min. Samples were resolved by SDS-PAGE, transferred to Immobilon-P (Millipore), and subject to autoradiography.

Merlin Self-Association Assays

NIH3T3 cells were transfected with 3 μ g of each construct per 60 mm plate and lysed in cold NP40 lysis buffer (50 mM Hepes [pH 7.5], 150 mM NaCl, 1% NP40, 50 mM NaF, 0.5 mM EDTA, 1 mM Pefabloc and Complete protease inhibitors [Boehringer Mannheim]). After pelleting insoluble material, lysates were precleared for 1 hr with Protein A-Sepharose (PAS) beads alone. After pelleting, supernatants were added to 50 μ l of premixed 12CA5/PAS beads and incubated at 4°C for 2 hr with rocking. Bound material was pelleted and washed 3 \times with NP40 lysis buffer prior to boiling in sample buffer.

Anchorage-Independent Growth Assays

NIH3T3 cells transfected as described were selected in 0.4 mg/ml G418 (pCDNA3 NF2) and 0.1 mg/ml hygromycin B (pCGN Rac1L61). Stably expressing cells were pooled and seeded at a density of 7.5×10^4 cells per 60 mm dish in DMEM containing 0.3% agar and 10% calf serum. Colony number was visualized after 14 days.

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