Merlin/Neurofibromatosis Type 2 Suppresses Growth by Inhibiting the Activation of Ras and Rac

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Abstract

The small G-protein Ras is a tightly controlled regulator of cell fate. Prolonged or persistent arrest in the activated GTP-loaded state by mutation of Ras as in lung cancer or in a Ras–GTPase-activating protein as in neurofibromatosis type 1 promotes tumorigenesis. We now show that the tumor-suppressor protein merlin (mutated in neurofibromatosis type 2) also controls Ras activity. Systematic analysis of growth factor signaling located the step of merlin interference to the activation of Ras and Rac. Merlin independently uncouples both Ras and Rac from growth factor signals. In the case of Ras, merlin acts downstream of the receptor tyrosine kinase-growth factor receptor binding protein 2 (Grb2)-SOS complex. However, merlin does not bind either SOS or Ras, but it counteracts the ERM (*e*zrin, *r*adixin, *m*oesin)–dependent activation of Ras, which correlates with the formation of a complex comprising ERM proteins, Grb2, SOS, Ras, and filamentous actin. Because efficient signaling from Ras requires Rac-p21-activated kinase–dependent phosphorylations of Raf and mitogen-activated protein/extracellular signal-regulated kinase kinase, merlin can also inhibit signal transfer from dominantly active Ras mutants. We propose that the interference of merlin with Ras- and Rac-dependent signal transfer represents part of the tumor-suppressive action of merlin. [Cancer Res 2007;67(2):520–7]

Introduction

The autosomal dominant tumor-prone hereditary disorder neurofibromatosis type 2 (NF2) results from mutation of one copy of the *nf2* gene. Upon loss of the second allele schwannomas, predominantly of the eighth cranial nerve, meningiomas and other brain tumors develop (1). Despite the benign nature of the tumors, the hereditary disease is severe in that close to 100% of all carriers die from multiple tumors. Disruption of the *nf2* gene in the mouse caused a pregastrulation block of embryogenesis (2). Mice heterozygous for the *nf2* deletion developed osteomas, aggressive osteosarcomas, and other tumors that had lost the second allele (3), indicating that *nf2* suppresses not only tumorigenesis in Schwann cells, but more generally, and, possibly, metastatic growth.

The *nf2* gene encodes the tumor-suppressor protein merlin. Merlin shares significant NH_2-terminal sequence homology with ERM proteins (*e*zrin, *r*adixin, *m*oesin; ref. 4) and, therefore, binds to identical or similar proteins of the plasma membrane (5, 6). The ERM proteins and merlin seem to act antagonistically on growth: Merlin is inhibitory whereas ERM proteins seem to enhance proliferation (7, 8). The activities of ERM proteins and merlin are regulated by phosphorylation in an opposite manner: Merlin is activated by dephosphorylation at Ser^{518} (8, 9), whereas ERM proteins are active upon phosphorylation of a critical threonine residue (ezrinT567, radixinT564, moesinT558; ref. 10). Merlin is activated (and ERM proteins are inactivated) upon cell-to-cell or cell-to-matrix contact; this activation step restores contact inhibition of growth to tumor cells (8).

Overexpression of merlin can counteract transformation of cells by oncogenic Ras (11, 12) or Ras-mediated signaling (13, 14), suggesting that merlin can act at the level of or downstream of Ras. Conversely, reduction of merlin abundance mimics transformation in that $nf2^{-/-}$ cells lack growth control at cell-to-cell contact, loose proper adherens junctions, and maintain elevated extracellular signal-regulated kinase (ERK) and c-Jun-NH_2-kinase (JNK) activity (9, 15). Although a number of properties of merlin have been revealed [e.g., merlin associates with the plasma membrane (8) and was coprecipitated with signaling components such as hepatic growth factor-regulated tyrosine kinase substrate (HRS; ref. 16) and p21-activated kinase 1 (PAK1; 17), with cytoskeletal components (18), and with components of the adherens junctions (15)], the mechanism of tumor suppression has remained unclear.

This study was initiated with the hypothesis that merlin would interfere with Ras-dependent proliferative signals. This hypothesis could be confirmed by showing that merlin blocks mitogen-activated protein/ERK kinase (MEK) and ERK activation induced by dominant active Ras. As expected, signal transduction from receptors that use the Ras pathway was also inhibited by merlin. However, the most important discovery is that merlin inhibits the activation of both Ras and Rac. Because Rac promotes Ras signaling, merlin can also inhibit the action of dominant-active Ras that cannot itself be blocked by merlin. Our data indicate that merlin exerts a specific effect relevant for tumor suppression—the inhibition of small G-protein activation.

Materials and Methods

Growth factors, antibodies, and reagents. Recombinant human platelet-derived growth factor (PDGF) BB (Biomol, Hamburg, Germany), Lubrol 17A17 (Uniqema, Pool, United Kingdom), recombinant human interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), epidermal growth factor (EGF), insulin-like growth factor (IGF), lysophosphatidic acid (LPA), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Igepal CA-630, Triton X-100,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and doxycycline (Sigma, Deisenhofen, Germany); hyaluronan (Healon, high molecular weight, Pharmacia & Upjohn, Erlangen, Germany); Raf-1 Ras binding domain glutathione agarose (Raf-1 RBD), glutathione *S*-transferase–growth factor receptor binding protein 2 (GST-Grb2; Upstate, Hamburg, Germany); CRIB-PAK glutathione agarose (Cytoskeleton tebu-bio, Offenbach, Germany); MEK inhibitor U0126 (Promega, Mannheim, Germany). Rabbit polyclonal antibodies against ERK1 (K23), p27 (C-19), SOS 1 (C-23), Raf-1 (C-12), PDGF receptor (PDGFR) β (958), c-Src (SRC2). Rabbit polyclonal antibodies against phosphorylated proteins merlin (Ser^{518}; Abcam, Cambridge, United Kingdom); ERK (Thr^{202}/Tyr^{204}); MEK1/2 ($Ser^{217/221}$); IκB ($Ser^{32/36}$); cyclic AMP (cAMP)–responsive element binding protein (CREB; Ser^{133}); signal transducers and activators of transcription 3 (STAT3; Tyr^{705}); Raf-1 (Ser^{338}); and pan-specific antibodies against PLCγ1, IκB, and CREB (New England Biolabs, Schwalbach, Germany). Mouse monoclonal antibodies hemagglutinin (HA) tag (12CA5; Boehringer Mannheim, Mannheim, Germany); phosphotyrosine (4G10), Grb-2 (3F2), Ras (RAS10), phosphatidylinositol 3-kinase (PI3K; Upstate); rabbit polyclonal antibodies against PI3K (Upstate); and Rac (Cytoskeleton).

Plasmids. Wild-type isoform 1 NF2 (pcDNA3; David Gutmann, Washington University School of Medicine, St. Louis, MO), RasL61 (19), RafBXB (ref. 20; Martin Schwartz, University of Virginia, Charlottesville, VA), MEK-1 DD (pcDNA3.1; ref. 21; Axel Knebel, University of Dundee, Dundee, United Kingdom), MEK-ERK fusion (Susanne Weg-Remers, Forschungszentrum Karlsruhe, Institute of Toxicology and Genetics, Karlsruhe, Germany), RacL61 and PakL107F (Alan Hall, University College, London, United Kingdom), and HA-tagged ERK-1 (Axel Ullrich, Max Planck Institute for Biochemistry, Martinsried, Germany).

Cell cultures. RT4-D6P2T schwannoma cell line and NIH3T3 mouse fibroblasts (European Collection of Animal Cell Cultures, Salisbury, United Kingdom). RT4tetNf2 cells (parental cell line RT4-D6P2T) carrying doxycycline-inducible merlin (wild-type and mutant 518A) were made as described by Morrison et al. (8). SC4-immortalized $nf2^{-/-}$ cell line and $nf2^{+/+}$ primary Schwann cells were from respective mice. Cells were grown in DMEM (Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Life Technologies) or 10% calf serum (Life Technologies) for NIH3T3. $Nf2^{+/+}$ primary Schwann cells were grown in DMEM+F12 (50:50) supplemented with N2 complement (Invitrogen), forskolin (Upstate), and heregulin β1 (Sigma). All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. The dose of doxycycline in all cell culture experiments was 1 μg/mL.

Stable and transient transfection of cells. Transfections were done using FuGENE-6 (Boehringer Mannheim) or LipofectAMINE (Invitrogen). To generate stable clones or pooled clones, cells were cotransfected with pCEP4 (for hygromycin resistance; Invitrogen) and selected in 100 μg/mL hygromycin (Roche, Mannheim, Germany).

Definition of growth condition in culture dishes. Low cell density (i.e., logarithmic or exponential growth) is defined as the density recorded at 24 h after seeding 500 cells/cm^2. High cell density (i.e., confluent growth condition) is defined as 24 h after seeding 5×10^3 cells/cm^2. High cell density for NIH3T3 and SC4 $nf2^{-/-}$ is defined as 24 h after seeding 1×10^5 cells/cm^2.

Affinity precipitation. For pulldowns with Raf-1 RBD-GST, PAK-CRIB-GST, and GST-Grb2, cells were lysed in 25 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1% Igepal CA-630, Lubrol 17A17 (Uniqema), 10% glycerol, 1 mmol/L EDTA 25 mmol/L NaF, 1 μmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Supernatants were incubated with 5 μg of recombinant fusion protein rotating at 4°C for 1 h. Immunoprecipitations are described in figure legends and in ref. 8.

Small interfering RNA. All small interfering RNA (siRNA) oligonucleotides were purchased from Ambion (Huntingdon, Germany). Merlin: mouse, sense, UACCGAGCUUCGACAUUAUUG; antisense, AUA AUGUCGAAGCUCGGUAUG. Ezrin: rat, sense, UCAACUAUUUCGAGAUCAAAA; antisense, UUGAUCUCGAAAUAGUUGAUC. Radixin: rat, sense, CUCGUCUGAGAAUCAAUAAGC; antisense, UUAUUGAUUCUCAGACGAGGU. Moesin: rat, sense, GGCUGAAACUCAAUAAGAAGG; antisense, UUCUUAUUGAGUUUCAGCCAA. Control 1: siRNA against firefly luciferase, sense, CGUACGCGGAAUACUUCGA; antisense, UCG AAGUAUUCCGCGUACG. Control 2: siRNA scrambled merlin, sense, AAUCCGGUUGCAUAGUUCAUG; antisense, UGAACUAUGCAACCGGAUUUG. Transfection of all siRNAs using LipofectAMINE (Invitrogen) were done as recommended by the manufacturer.

Results

Tumor-suppressive function of merlin through interference with the Ras-to-MEK transfer of signals. Immortalized $nf2^{-/-}$ Schwann cells are not contact inhibited but are nevertheless not tumorigenic. As we had shown previously that activated merlin reduced proliferation of cells (9), we examined whether reconstitution in $nf2^{-/-}$ Schwann cells with activated merlin at physiologic abundance would also inhibit proliferation. Reexpression of merlin at different levels indeed inhibited proliferation of $nf2^{-/-}$ Schwann cells in three independent clones [as measured by bromodeoxyuridine (BrdUrd) incorporation; strong BrdUrd staining represents cells in S phase (Fig. 1*A*)]. Figure 1*B* shows the levels of merlin in the three cell clones and a coarse comparison of clone 2 with merlin abundance in primary Schwann cells. The effect of merlin on BrdUrd incorporation resembled that achieved by a MEK inhibitor (Fig. 1*A*).

We then asked if loss of merlin from a cell line such as NIH3T3 cells will have an opposite result (i.e., enhanced BrdUrd incorporation). Down-regulation of merlin in NIH3T3 cells by two independent sequences of RNA interference (RNAi) strongly increased proliferation (Fig. 1*C*). These data show that approximately physiologic levels of merlin control proliferation. Loss of proliferation control may be the basis of tumor formation in NF2 patients.

Merlin has been shown to counteract the cellular transformation by oncogenic Ras (12). In the transformed ErbB2-driven rat schwannoma cell line RT4-D6P2T (RT4; ref. 22), which maintains elevated Ras activity, merlin overexpression caused a reduction in colony numbers (cells supplied with a doxycycline-inducible merlin expression construct: RT4tetNf2; ref. 8; Fig. 1*D*, control). To define the step of merlin interference with transformation, we introduced dominant-active components of the Ras pathway. Dominant-active Ras could not block the effect of merlin on colony growth (Fig. 1*D, RasLeu61*), nor could dominant-active Raf (Fig. 1*D, RafBXB*). Introduction of dominant-active MEK, however, prevented the reduction of agar colonies by merlin (Fig. 1*D*, *MEKDD* and *MEK-Erk*). Thus, merlin interferes with agar colony growth at a step above MEK. As a control experiment, the MEK activation inhibitor caused a similar reduction of agar colonies, indicating that the influence of merlin on the Ras-MEK-ERK pathway is important for its tumor-suppressor function (Fig. 1*D*, *MEK Inhibitor*). The block of the Ras-MEK-ERK pathway is likely related to a G_1 arrest caused by merlin as had been reported (23) and is reflected in the up-regulation of cell cycle inhibitors such as p21 (not shown) and p27 (Fig. 1*E*).

We have previously shown that the antiproliferative action of merlin is not a function simply of overexpression but requires merlin dephosphorylation (8, 9). Dephosphorylation is induced in soft agar (not shown) or by high cell density. To directly measure the action of merlin on the Ras signal transduction pathway, we studied oncogene-dependent activation of ERK in RT4tetNf2 (Fig. 1*F*) or NIH3T3 cells (Fig. 1*G*) transiently cotransfected with HA-tagged ERK and dominant-active oncogenes. Density-activated merlin prevented the phosphorylation of ERK by Ras and Raf, but not by MEK. This result suggests that merlin directly interferes with signal transduction, and, as in the agar colony experiment, merlin blocks at a level above MEK.

Specific interference of merlin with receptor tyrosine kinase–dependent signal transduction. Because Ras is involved in the signal transfer from numerous receptors, from receptor tyrosine kinases (RTK) to G-protein coupled receptors, we reasoned that merlin should interfere with several receptor-dependent signaling processes (as in the case of ErbB2 in RT4 cells). To examine this possibility, we determined activation of endogenous ERK by various RTKs in RT4tetNf2 under conditions of either low cell density, when merlin is inactive, or high cell density. The resulting activation of merlin by dephosphorylation under the latter condition is shown in Fig. 2*A* (*right*). Addition of FCS, which contains a number of receptor ligands, induced the phosphorylation of ERK that was reduced by active merlin (ref. 14; high cell density; Fig. 2*A*), but not by inactive merlin (low cell density; Fig. 2*A*). Moreover, the permanently inactive merlin point mutant L64P (8) did not inhibit signal transduction (not shown).

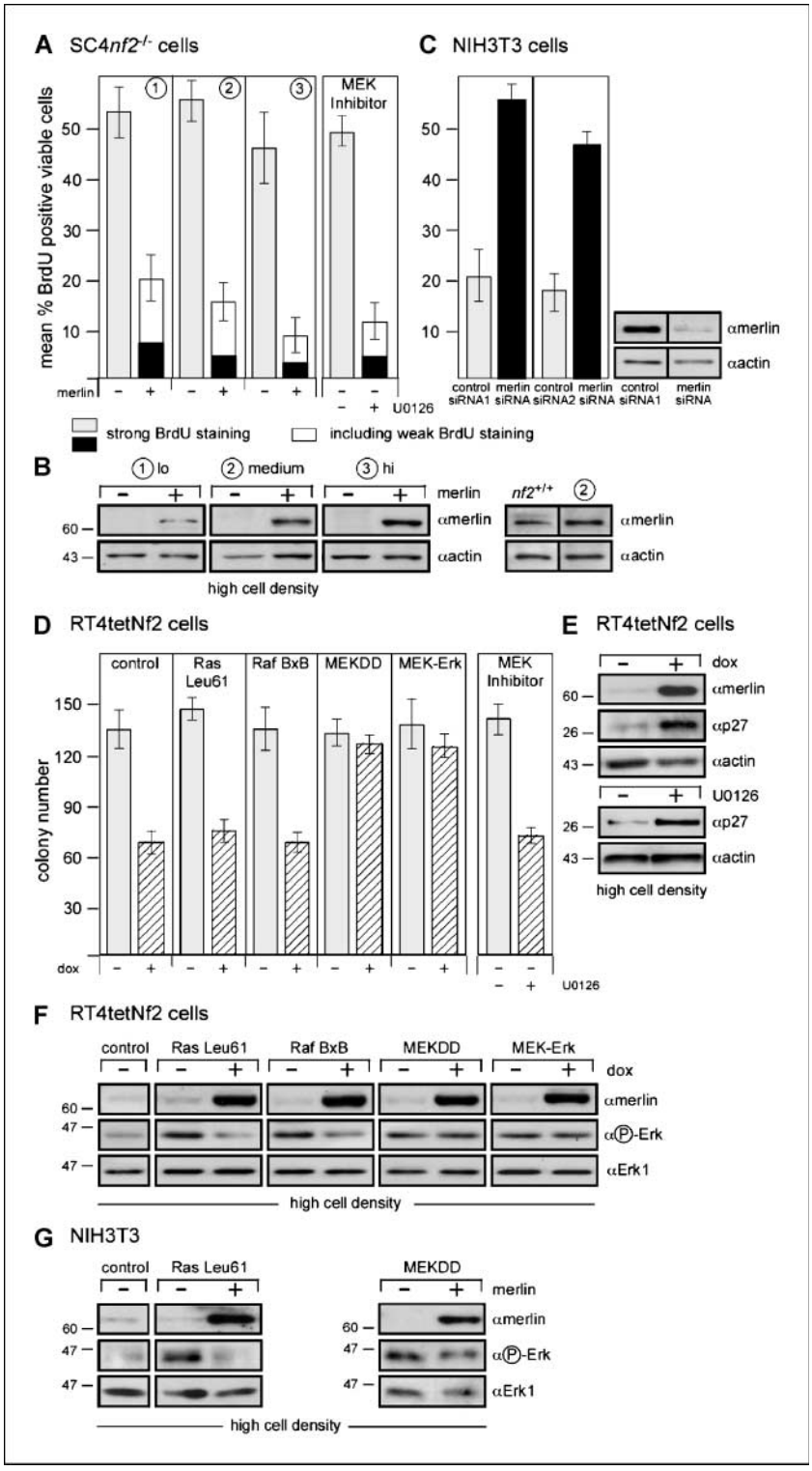

Figure 1. Down-regulation in NIH3T3 cells and reconstitution in $nf2^{-/-}$ cells of the tumor-suppressor function of merlin. *A* and *B,* physiologic levels of merlin inhibit proliferation. SC4-immortalized $nf2^{-/-}$ Schwann cells were stably transfected with a merlin expression construct. *B,* three independent clones were compared for merlin expression by immunoblotting (*1,* low; *2,* medium; *3,* high). The abundance of merlin in clone 2 resembled that of normal Schwann cells ($nf2^{+/+}$). *A,* three independent clones compared with the parental $nf2^{-/-}$ cells were plated at high cell density, labeled with BrdUrd (20 μmol/L, 30 min), and stained for incorporation using a biotinylated BrdUrd antibody (oncogene). For comparison, the effect of the MEK activation inhibitor UO126 (10 μmol/L) was included. *C,* down-regulation of merlin by RNAi in NIH3T3 cells caused an increase in proliferation. NIH3T3 cells were treated with siRNA against merlin for 36 h, replated at high cell density, labeled with BrdUrd (20 μmol/L, 2 h), and stained for incorporation using a biotinylated BrdUrd antibody (oncogene). Lysates were immunoblotted with antibodies as indicated. Two independent experiments, each with control siRNAs: unrelated siRNA complementary to luciferase and scrambled merlin siRNA. *D,* interference of merlin with oncogenic Ras, Raf but not MEK-dependent agar colony growth. RT4tetNf2 cells were stably transfected with cytomegalovirus promoter-driven constructs encoding oncogenic Ras, Raf or MEK, and were grown in soft agar [without (−) and with (+) doxycycline (*dox*); ref. 8]. For comparison, the effect of the MEK activation inhibitor UO126 (10 μmol/L) was included. *E,* increased levels of p27 upon inhibition of the Ras-MEK pathway by either merlin or a MEK activation inhibitor. RT4tetNf2 cells were plated at high density and treated with either doxycycline or 10 μmol/L MEK inhibitor (U0126) for 8 h. Lysates were immunoblotted (8) as indicated. *F* and *G,* interference of merlin with oncogenic Ras, Raf but not MEK-dependent ERK phosphorylation. *C,* RT4tetNf2 cells were transiently cotransfected in a 5:1 ratio with constructs encoding oncogenic Ras, Raf or MEK and a HA-tagged ERK1. Cells were densely plated, treated with doxycycline, and serum starved overnight before lysis. HA-tagged ERK was immunoprecipitated; proteins were resolved by 8% SDS-PAGE and immunoblotted as indicated. *D,* NIH3T3 cells were cotransfected in a 5:5:1 ratio with constructs encoding merlin, oncogenic Ras or MEK, and with HA-tagged ERK1. Cells were densely plated and serum starved overnight before lysis, and HA-tagged ERK was immunoprecipitated. Immunoblots were as indicated.

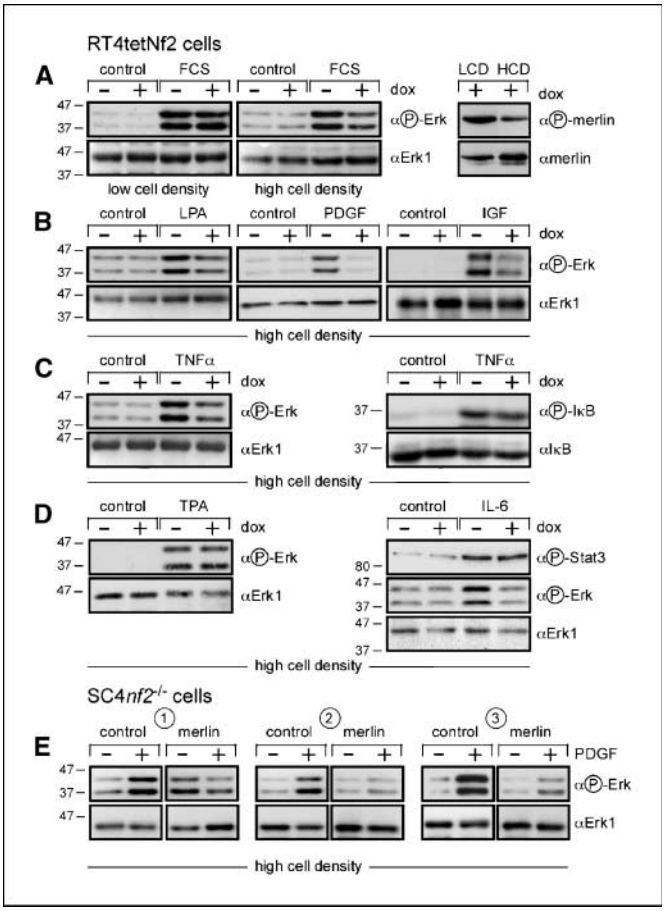

Figure 2. Specific interference of merlin with RTK-dependent signal transduction. *A,* active merlin inhibits serum-induced ERK phosphorylation. RT4tetNf2 cells were plated at low density (*LCD*) or high density (*HCD*), treated with doxycycline, and serum starved overnight before treatment with FCS (4% for 5 min). Immunoblots were as indicated. *B,* active merlin inhibits several receptor-dependent pathways. RT4tetNf2 cells were plated at high density, treated with doxycycline, and serum starved overnight before treatment with PDGF (10 ng/mL, 5 min), IGF (10 ng/mL, 5 min), or LPA (20 μmol/L, 3 min). Immunoblots as in Fig. 1*D*. *C,* specificity of merlin action. RT4tetNf2 cells were plated at high density, treated with doxycycline, and serum starved overnight before treatment with TNF-α (10 ng/mL, 5 min) or IL-6 (1 ng/mL, 5 min). Lysates were immunoblotted as indicated. *D,* merlin resistance by bypassing Ras with phorbol ester. RT4tetNf2 cells were plated at high density, treated with doxycycline, and serum starved overnight before treatment with TPA (100 ng/mL 5 min). Immunoblots as in Fig. 1*D*. *E,* physiologic levels of merlin inhibit PDGF-dependent ERK phosphorylation. SC4 *nf2*$^{-/-}$ cells and the merlin expression clones described in Fig. 1*C* were plated at high cell density, serum starved overnight before treatment with PDGF as in (*B*), and immunoblots were done as in Fig. 1*D*.

We activated ERK by individual growth factors contained in FCS and determined the effect of merlin. Activated merlin inhibited the phosphorylation of ERK induced by LPA (addressing a G-protein coupled receptor), by PDGF, by IGF (Fig. 2*B*), or by hepatocyte growth factor (HGF; not shown). A constitutively active ErbB2 drives the RT4 schwannoma cells, and no further stimulation by EGF could be detected. Therefore, EGF-induced ERK phosphorylation was tested in other cell types where merlin indeed inhibited EGF-dependent signaling (not shown). Moreover, in the *nf2*$^{-/-}$ Schwann cells, expression and activation of merlin inhibited the PDGF-induced phosphorylation of ERK (Fig. 2*E*). The data are compatible with the existence of a common mechanism of merlin interference with signaling pathways that use Ras.

Merlin is, however, not a general inhibitor of signaling. Although TNF-α–dependent ERK phosphorylation was blocked by merlin, the TNF-α–induced phosphorylation of IκB was not (Fig. 2*C*). Moreover, ERK activation in response to IL-6 was inhibited, but not the IL-6 dependent activation of STAT3 (Fig. 2*D, right*). The cAMP-induced phosphorylation of CREB and the transcription of a reporter gene in response to glucocorticoid hormone were also merlin resistant (not shown). Because merlin could not block if signaling was initiated by dominant-active MEK (Fig. 1*F* and *G*), we asked whether activation of ERK by TPA, which bypasses Ras, would be merlin resistant. This was indeed the case: ERK activation by TPA was not affected by merlin (Fig. 2*D, left*).

Dissection of the PDGF-dependent signaling pathway: merlin inhibits Ras and Rac activation. The experiments thus far suggest that merlin interferes with signal transduction below Ras. To pinpoint this step of merlin interference with signaling within a specific RTK-dependent pathway, we examined in detail the signaling steps in response to PDGF in RT4tetNf2 cells as well as in the merlin-transfected *nf2*$^{-/-}$ Schwann cells. Tyrosine phosphorylation sites in the activated PDGFR serve to assemble a number of adaptor proteins and enzymes (24). We immunoprecipitated PDGFR and determined its tyrosine phosphorylation and the coprecipitation of several binding proteins within 5 min of PDGF stimulation. The autophosphorylation of the receptor as well as the association of all proteins interacting with phosphotyrosines, including Grb2-SOS, was not affected by the expression and activation of merlin (Fig. 3*A, top*), indicating that merlin did not act at the receptor itself; it also did not interfere with the binding of components to the phosphotyrosines.

In lysates from the same experiments, we determined the activity levels of downstream components. The phosphorylations of CREB, ERK, and MEK in response to PDGF were inhibited by merlin (Fig. 3*A, bottom*). The PDGF-induced phosphorylations of JNK (not shown; see also ref. 9), and of Akt and protein kinase C (not shown), were also merlin sensitive. These results could be expected because activation of these signaling components depends, at least in part, on the Ras pathway and on Ras-dependent Rac activity (25). An indirect action through protein turnover or receptor internalization was ruled out because immunoblotting and fluorescence-activated cell sorting analysis (not shown) showed equal abundance of the respective components. In addition, inhibition of signal transduction was also achieved immediately after rapid merlin activation as occurs after treatment with hyaluronan (not shown; hyaluronan mimics high cell density; ref. 8).

In conjunction with the previous data, an interference mechanism below Ras would be plausible. The further analysis of the PDGF-induced signaling pathway revealed, however, an unexpected result: The GTP-loading of Ras as well as of Rac (17) was severely reduced by merlin (RT4tetNf2 in Fig. 3*A*; *nf2*$^{-/-}$ Schwann cells in Fig. 3*B*). A quantitation of critical blots is shown in Supplementary Fig. S1. Conversely, RNAi-mediated down-regulation of merlin in NIH3T3 cells enhanced basal and PDGF-induced activation of Ras at high cell density (Fig. 3*C*). These data clearly imply that merlin acts above Ras and Rac. In addition, these data imply that cells at a high density, compared with cells at a low density, carry lower amounts of Ras-GTP and are less responsive to growth factors. This was indeed the case: Activity levels and responses to PDGF or EGF were lower in high-density cultures compared with low-density cultures (Fig. 3*D*).

Merlin blocks the formation *in vitro* of a complex between ezrin, SOS, and Ras *in vitro*. We have previously shown that

merlin and ERM proteins are counterplayers in the control of cellular proliferation and that both require anchorage to the plasma membrane using identical binding sites (8). In the example of a coreceptor for the RTK, Met elimination of the common binding site interfered with HGF-dependent ERK activation (26). This led us to speculate that an ERM protein–containing complex was required for signaling and that merlin may disrupt this complex. Ras and Rac can be activated by the nucleotide exchange factor SOS. Based on our data obtained with merlin, the putative complex should contain the components needed for Ras activation (e.g., SOS and possibly Ras). In an attempt to form the putative ERM-SOS complex *in vitro*, we added GST-Grb2 to lysates and analyzed the pulled down components (Fig. 3*E*). The pulldowns revealed binding of SOS to Grb2 irrespective of whether the cells were treated with PDGF (Fig. 3*E*). This is in agreement with reported data showing constitutive association of Grb2 and SOS (27). Merlin did not disturb the association of Grb2 with SOS both *in vivo* (Fig. 3*A*) and *in vitro* (Fig. 3*E*). The apparent complex indeed contained ERM proteins, which were eliminated or reduced upon activation of merlin. Removal of ERM proteins from cells by RNAi prevented the formation of the pulldown complex *in vitro* (not shown) and prevented the activation of Ras (Fig. 3*F*). ERM knockdown normalized to actin in +PDGF samples reduced ERM expression by 77% and in −PDGF samples by 67%; the corresponding Ras-GTP levels were reduced by 90% (+PDGF) and 55% (−PDGF). Although the interaction of SOS with Ras is probably transient, we detected Ras in the pulldowns from lysates of cells treated with PDGF (Fig. 3*E*). Interestingly, similarly to that of the ERM proteins, the SOS Ras association was abolished by merlin. Although merlin expression is well detectable in the lysates, merlin did not associate with the Grb2-SOS–containing complex (Fig. 3*E*). We take these data as preliminary indications for a mechanism of Ras activation that involves a structured protein assembly, including ERM proteins, and that this assembly is disrupted by merlin.

Interference with Rac activation explains the inhibition of dominant-active Ras signaling by merlin. How can it be explained that merlin inhibits the activation of the small G-proteins Ras and Rac, but also blocks signaling and transformation by the dominant-active Ras? The GTP loading of the dominant-active Ras was not influenced by merlin (Fig. 4*C*). It is thought that efficient signal transduction from normal or mutated Ras (28) requires the phosphorylations of Raf at Ser^{338} and of MEK at Ser^{298}

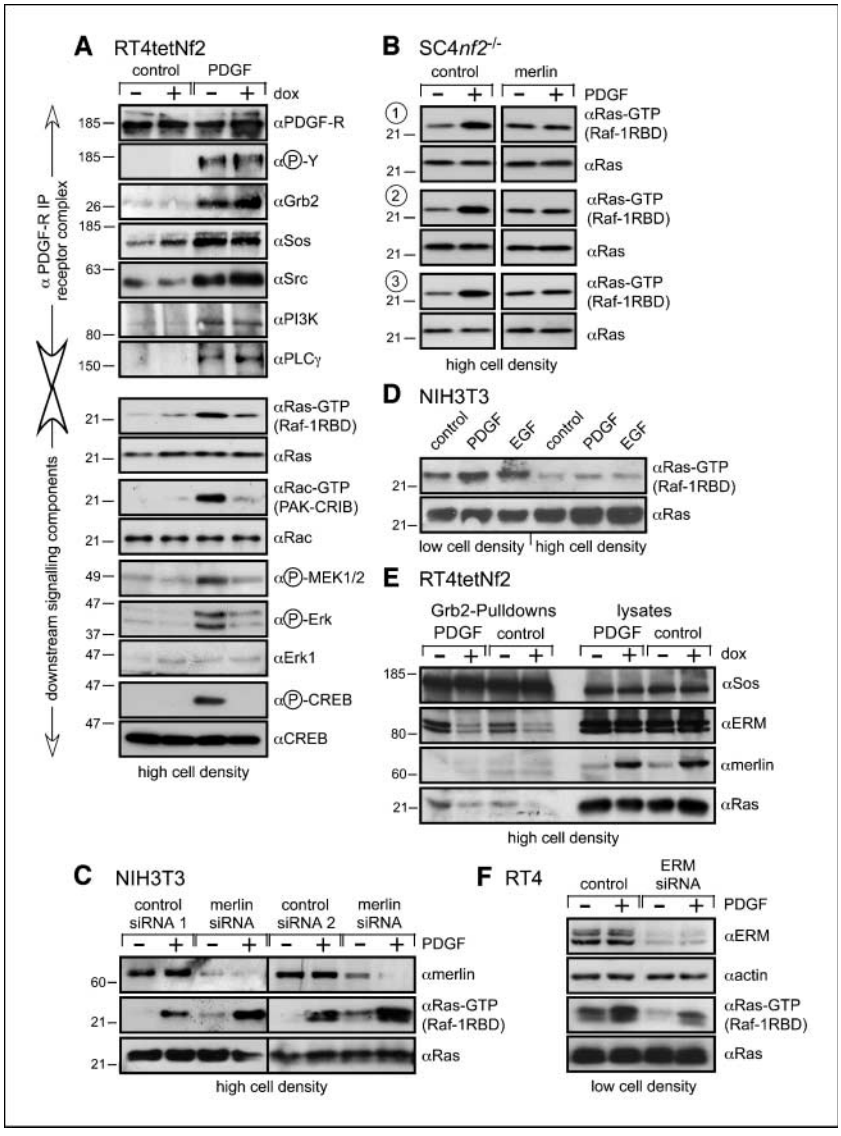

Figure 3. Dissection of the PDGF-dependent signaling pathway. *A,* autophosphorylation of the PDGFR as well as the binding of adaptor proteins and enzymes is not affected by active merlin. RT4tetNf2 cells were plated at a high density, treated with doxycycline, and serum starved overnight before treatment with PDGF for 3 min. From lysates, PDGFR was immunoprecipitated (8). Immunoblots were consecutively probed with antibodies against phosphorylated tyrosine, PDGFR, and the coimmunoprecipitated proteins Grb2, Sos, Src, PI3K, and PLCγ. Merlin inhibits PDGF-induced activation of the small G-proteins Ras and Rac. Lysates from the same experiments were immunoblotted for the activation of MEK, ERK, and CREB as indicated, or treated with either Raf-1RBD (for Ras-GTP) or CRIB-PAK (for Rac-GTP) to coprecipitate activated Ras and Rac, which was then detected by immunoblotting. *B,* physiologic levels of merlin inhibit PDGF-induced activation of Ras. The merlin expression clones in SC4 $nf2^{-/-}$ cells described in Fig. 2*B* were treated as in (*E*). Lysates were treated with Raf-1RBD to coprecipitate Ras-GTP, which was subsequently detected by immunoblotting. *C,* down-regulation of merlin by RNAi in NIH3T3 cells caused an increase in Ras activity. NIH3T3 cells were treated with siRNA against merlin or control siRNAs (as in Fig. 2*C*) for 36 h, replated at high cell density, and lysed. The lysates were treated as in (*B*). *D,* Ras-GTP in low- and high-density cells. NIH3T3 cells were grown to different densities (see Materials and Methods) and treated or not with PDGF and EGF. Ras-GTP was determined as in (*A* and *B*). *E,* merlin blocks the formation *in vitro* of a putative Ras activation complex. Components involved in the activation of Ras were assembled *in vitro* with GST-Grb2 added to the lysates. Pulldowns were as described in Materials and Methods. Immunoblots of the components are shown. *F,* down-regulation of ERM by RNAi in RT4 cells inhibited Ras activity. RT4 cells were treated with siRNA against ERM or control siRNAs (as in Fig. 2*C*) for 36 h, replated at low cell density, and lysed. The lysates were treated as in (*B*).

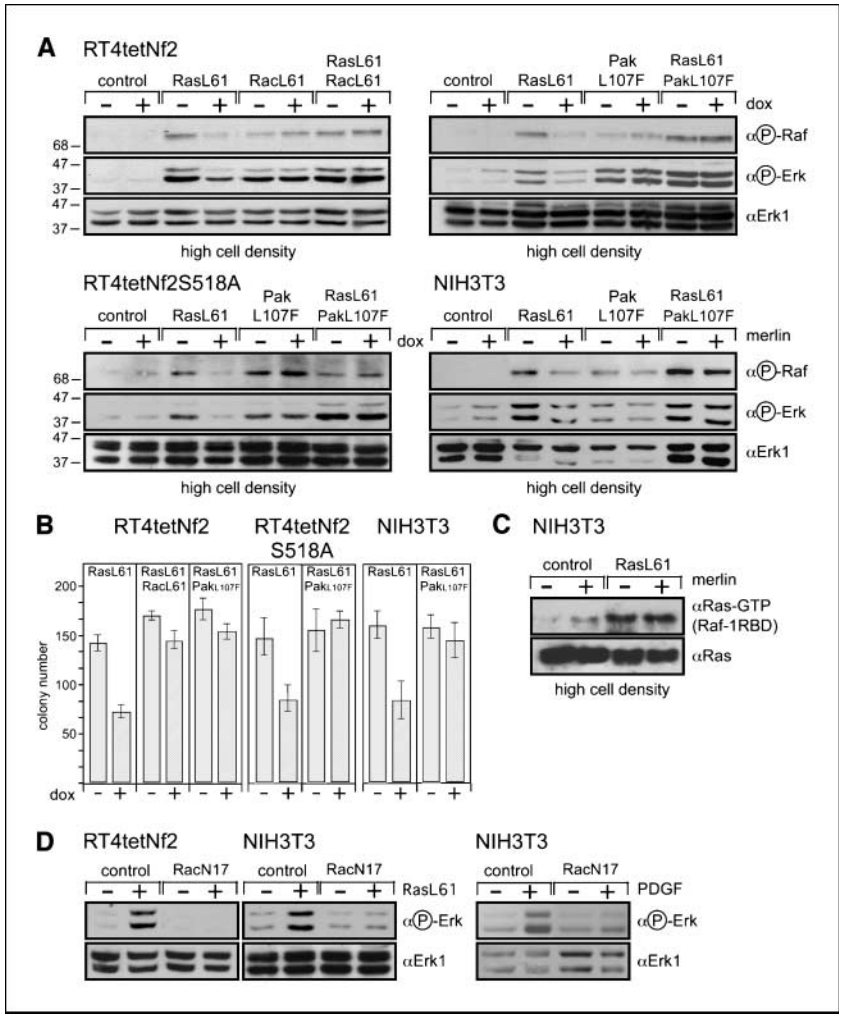

Figure 4. Interference with Ras and Rac activation is the only, or is the predominant, mechanism of merlin action. *A,* dominant-active Rac or dominant-active PAK prevents merlin inhibition of ERK phosphorylation triggered by dominant-active Ras. RT4 cells carrying inducible wild-type merlin (*RT4tetNf2*) or constitutively active mutant S518A (*RT4tetNf2S518A*), or NIH3T3 cells, were stably cotransfected with constructs encoding oncogenic Ras, Rac, and with a hygromycin resistance construct. Hygromycin-resistant cells were selected for 24 h. Cells were densely plated, treated with doxycycline, and serum starved overnight before lysis. Immunoblots are as indicated. *B,* dominant-active Rac or dominant-active PAK prevents merlin inhibition of dominant-active Ras–induced agar colony growth. Cells treated as in (*A*) were grown in soft agar (− and + doxycycline) for RT4tetNf2 and RT4tetNf2S518A (8). For NIH3T3 cells treated as in (*A*), 1.5×10^4 were grown in soft agar. *C,* GTP loading of dominant-active Ras is not inhibited by merlin. NIH3T3 cells were cotransfected with RasL61 and merlin (or vector control) and were plated at a high density. After 24 h, Ras-GTP was determined as in (*A* and *B*). *D,* the requirement for Rac in Ras signaling. Dominant-negative Rac blocks Ras or PDGF-dependent ERK activation. RacN17 (or vector control) were stably cotransfected with RasL61 (or vector control) as in (*A*) into RT4 or NIH3T3 cells. Twenty-four hours later, the phosphorylation of ERK was determined. *Right,* PDGF-induced ERK phosphorylation was determined in RacN17-transfected NIH3T3.

by PAK (29–31). PAK activity, in turn, depends on the activation of Rac, which is inhibited by merlin. The involvement of Rac-PAK regulation of the Raf-MEK-ERK pathway was confirmed in our cell systems: Indeed, a dominant-negative Rac (RacN17) inhibited the PDGF or dominant-active Ras-dependent ERK phosphorylation (Fig. 4*D*). Because merlin blocks the activation of Rac, we asked whether dominant-active Ras signaling and transformation could still be inhibited by merlin under conditions of persistent Rac or PAK activation. Although merlin inhibited dominant-active Ras–induced Raf and subsequent ERK phosphorylation (Fig. 4*A, left* and *right, lanes 3* and *4*), introduction of dominant-active Rac (RacL61; Fig. 4*A*, *left, lanes 7* and *8*) or dominant-active PAK (PakL107F; Fig. 4*A*, *right, lanes 7* and *8*) eliminated merlin action. Because PAK can inactivate merlin (phosphorylating at Ser^{518}; refs. 32, 33), we repeated the experiment by introducing dominant-active merlin S518A and obtained the same result (Fig. 4*A, bottom*; and not shown). Interestingly, dominant-active Rac or dominant-active PAK alone induced some Raf-Ser^{338} phosphorylation and ERK activation despite very little spontaneous Ras activity (Fig. 4*A*, *lanes 5* and *6*). Furthermore, dominant-active Rac or dominant-active PAK reduced the inhibitory effect of merlin on agar colony growth in NIH3T3 cells as well as in RT4tetNf2 cells carrying inducible wild-type merlin or mutant S518A (Fig. 4*B*). Taken together, inhibition of Ras and Rac activation seems to be the major growth-suppressive function of merlin.

Discussion

As the most important finding reported here, the tumor-suppressor protein merlin (*nf2*) interferes with the activation of Ras and Rac. This result was derived from a systematic study of the PDGF-induced signaling pathway. Neither the activation of the PDGFR itself nor the binding of adaptor proteins, such as Grb2-SOS, PLCγ, PI3K, or Src, was affected by merlin (shown in Fig. 3). The first detectable interference concerned the loading of Ras or Rac with GTP. It is well known that Rac can be activated after Ras activation (34). The reverse has also been reported for lymphoid cells (35, 36). This latter reverse signaling was not observed in our cell types (Supplementary Fig. S2). We conclude that merlin interferes with the activation of Rac and Ras independently for the following reasons: Interference by merlin with Rac activation was independent of the inhibition of Ras GTP loading as inhibition of Rac activation was not affected by either noninhibitable dominant-active Ras or dominant-negative Ras (not shown and Supplementary Fig. S3). Dominant-active Rac did not cause activation of Ras (Supplementary Fig. S2). We note, however, that dominant-active Rac caused some phosphorylations of Raf and ERK, both of which were resistant to merlin; this is quite in contrast to Raf and ERK activations by dominant-active Ras, which require the activation of Rac.

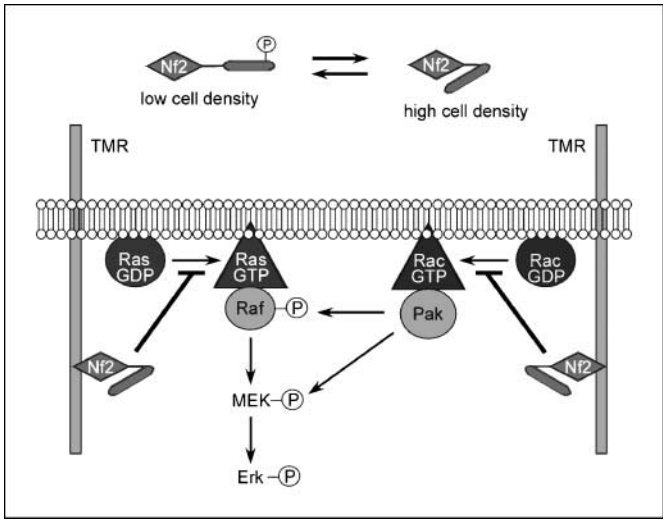

Figure 5. Proposed scheme of merlin action. Merlin (NF2) is activated by dephosphorylation. It needs to be bound to a plasma membrane protein (e.g., *TMR,* transmembrane receptor). Merlin blocks the activation of Ras and Rac. In the absence of Rac activation, Raf and MEK are not phosphorylated by PAK, which is an essential step in Ras-dependent signal transduction. Therefore, merlin also inhibits signaling from constitutively active Ras.

Coimmunoprecipitations did not reveal an association of merlin with either Ras or Rac (not shown). Merlin acts apparently after activation of an RTK that leads to guanine nucleotide-exchange factor (GEF) recruitment. Three possible reactions could be affected by merlin: (*a*) sequestering of GDP dissociation inhibitor (GDI). [This may be an attractive possibility because an interaction of merlin with RhoGDI has been reported (37)]; (*b*) activation of Ras-GAP and Rac-GAP activity; and (*c*) inhibition of the catalytic function or the association of GEF (e.g., SOS with Ras and Rac). Interestingly, an association of merlin with Ral-GDS, a GEF operating at the G-protein Ral, downstream of Ras, has been reported recently (38). Efficient tumor suppression by merlin may even be based on a combination of these molecular actions. Merlin function also permits the hypotheses on the action of the ERM proteins. The opposite effects of ERM proteins and merlin on proliferation control (8, 26) suggest that ERM proteins enhance Ras activation by one of the three steps proposed to be targeted by merlin. The RNAi data (Fig. 3*F*) strongly speak for a role of ERM proteins in Ras activation. The *in vitro* reconstitution experiments using GST-Grb2 suggest the existence of a Grb2-SOS-ERM-Ras complex [which also contains filamentous actin (F-actin); not shown], which is disrupted by merlin, thus favoring the hypothetical mechanism of an interference with GEF activity. Both ERM proteins and merlin require for their activity the association with membrane proteins, e.g., CD44 or integrins. Merlin competes for the same binding sites and thus releases the Grb2-SOS-ERM-Ras complex from the membrane anchorage (8). Although not yet proven, it is plausible that SOS requires the interaction with ERM proteins and F-actin to unfold and become catalytically active.

The inhibition of Ras and Rac activation (e.g., by interfering with their common GEF, SOS; ref. 34) also explains how merlin can interfere with transformation by a dominant-active Ras (Fig. 1; ref. 12) or dominant-active Rac (9). Dominant-active Rac or dominant-active PAK introduced together with dominant-active Ras abolished the inhibitory effect of merlin on signal transduction and on tumorigenic growth in soft agar (Fig. 4). We explain the result as follows: The efficient signal transduction and transformation by Ras requires PAK-dependent Raf and MEK phosphorylation (30, 39–41). Dominant-active PAK or persistent activation of PAK by dominant-active Rac eliminates the step addressed by merlin, the interference of Rac activation that blocks Ras signaling. Inhibition of signaling from dominant-active Raf-BXB (Fig. 1), which is not dependent on phosphorylation of Ser^{338} for activation, can probably be explained similarly: Raf-dependent activation of MEK at Ser^{218} and Ser^{222} (42) requires that PAK phosphorylates MEK on Ser^{298}. Dominant-active MEK is a 218D:222D double mutant and does not require PAK priming; thus, dominant-active MEK is merlin resistant. We conclude that the tumor-suppressive function of merlin is exerted by the combined inhibition of at least Ras and Rac activation (Fig. 5). The inhibition of Ras and Rac activation is consistent with data previously reported (e.g., the inhibition of PAK activity by merlin; ref. 17). PAK addresses several downstream signaling components, one of which is merlin itself (9, 32, 33). Like many other substrate-enzyme interactions that require high substrate specificity, e.g., JNK with Jun (43), PAK and merlin interact fairly stably and can be coprecipitated (17). Other substrates of the PAK family mediate the dramatic effect of Rac on the cytoskeleton (e.g., the formation of lamellipodia and the migration of cells). The block of Rac activation by merlin thus prevents the reorganization of the cytoskeleton, a feature of merlin that has been previously described (44).

Our results cannot explain the numerous interactions merlin is involved in. Most of these occur with the alternatively spliced merlin isoform 2. An interaction with some preference for isoform 2 has been reported recently: magicin, which forms a complex with merlin, Grb2, and F-actin (45). In our experiments, merlin isoform 1 was not associated with Grb2 (Fig. 3). Interacting proteins may be related to specific functions of this second splice variant, for example, HRS (16) that suggests a role in endocytosis or paxillin (18) that suggests a role in morphogenesis. That merlin can enhance endocytosis has been reported several years ago (46). In *Drosophila*, the absence of merlin increases the density on the cell surface of EGF receptor and Notch (47). A possible explanation for this finding could be that merlin-HRS promote the endocytic degradation pathway. In the absence of merlin, more activated receptors remain on or are recycled to the cell surface. In our RT4 cell system, we have not been able to see a merlin-induced reduction of PDGFR surface density (not shown). The Ras- and Rac-dependent pathways are targets of several tumor-suppressor proteins. Hamartin binds to ezrin and Rho (48), the neurofibromatosis type 1 protein is a Ras GAP (49), and the selective inhibitors of mitogenic signaling belonging to the family of mammalian sproutys prevent a Raf-activating step (50). Our results on merlin add to this list, underscoring the significance of proper Ras pathway control to prevent tumorigenic growth and metastases.

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