

p21-activated Kinase Links Rac/Cdc42 Signaling to Merlin*

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The neurofibromatosis type 2 tumor suppressor gene, *NF2*, is mutated in the germ line of *NF2* patients and predisposes affected individuals to intracranial and spinal tumors. Moreover, somatic mutations of *NF2* can occur in the sporadic counterparts of these neurological tumor types as well as in certain neoplasms of non-neuroectodermal origin, such as malignant mesothelioma and melanoma. *NF2* encodes a 595-amino acid protein, merlin, which exhibits significant homology to the ezrin-radixin-moesin family of proteins. However, the mechanism by which merlin exerts its tumor suppressor activity is not well understood. In this investigation, we show that merlin is phosphorylated in response to expression of activated Rac and activated Cdc42 in mammalian cells. Furthermore, we demonstrate that merlin phosphorylation is mediated by p21-activated kinase (Pak), a common downstream target of both Rac and Cdc42. Both *in vivo* and *in vitro* kinase assays demonstrated that Pak can directly phosphorylate merlin at serine 518, a site that affects merlin activity and localization. These biochemical investigations provide insights into the regulation of merlin function and establish a framework for elucidating tumorigenic mechanisms involved in neoplasms associated with merlin inactivation.

The neurofibromatosis type 2 tumor suppressor gene, *NF2*, is mutated in the germ line of *NF2* patients and predisposes affected individuals to tumors of neuroectodermal origin (1, 2). *NF2* encodes a 595-amino acid protein (merlin), which exhibits significant homology to the highly conserved ezrin-radixin-moesin (ERM)¹ family of proteins (2, 3). However, the cellular function and regulation of merlin is not well understood.

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¹ The abbreviations used are: ERM, ezrin-radixin-moesin; Pak, p21-activated kinase; MLK, mixed lineage kinase; CIP, calf intestinal phosphatase; AID, autoinhibitory domain; HA, hemagglutinin; GST, glutathione *S*-transferase; JNK, c-Jun NH₂-terminal kinase.

Because of the similarity between merlin and ERM proteins, and the fact that ERM proteins are phosphorylated by Rho GTPase-mediated signaling (4, 5), we tested whether merlin is also regulated by members of the Rho family of GTPases. Our investigations have determined that merlin is phosphorylated in response to constitutively active Rac1 and, to a lesser extent, Cdc42, and this phosphorylation is mediated by the Rac/Cdc42 effector, p21-activated kinase (Pak).

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—A human NF2 expression plasmid was created by inserting a hemagglutinin (HA) epitope tag after the first methionine of full-length human NF2 cDNA (from D. H. Gutmann, Washington University, St. Louis, MO) and cloned into pcDNA3 vector (Invitrogen). NF2 S518A and NF2 S518D mutant constructs were made by polymerase chain reaction using a QuikChange site-directed mutagenesis kit (Stratagene). The oligonucleotide primers designed to introduce the mutations were as follows (codon changes are underlined): forward primer S518A, 5'-GACATGAAGCGGCTTGCATGGAGATAGAGAAAG-3'; reverse primer S518A, 5'-CTTTCTCTATCTCCATGGCAAGCCGCTTCATGTC-3'; forward primer S518D, 5'-GACATGAAGCGGCTTGCATGGAGATAGAGAAAG-3'; reverse primer S518D, 5'-CTTTCTCTATCTCCATGGCAAGCCGCTTCATGTC-3'. A HA-tagged NF2-C construct, encoding the carboxyl terminus (amino acids 299–595) of merlin was generated by PCR and cloned into pcDNA3 vector. Then the HA-NF2-C was used as a template to create truncated forms of NF2, containing S518A (HA-NF2-C Ala^{S18}) or S518D (HA-NF2-C Asp^{S18}), by site-directed mutagenesis as described above. Authenticity of various wild-type and mutant forms of NF2 was verified by nucleotide sequencing. Other plasmids included constitutively active Rho GTPases: pCMV6-Myc-RhoA Q63L, pCGN-HA-Rac1 Q61L (from C. Der, University of North Carolina, Chapel Hill, NC), and pRK5-Myc-Cdc42 Q61L (from A. Hall, Medical Research Council, London, UK); Rac1 effector mutants: pCGT-Rac1 V12L37 and pCGT-Rac1 V12H40 (both from D. Bar-Sagi, State University of New York, Stony Brook, NY); FLAG-tagged wild-type and kinase dead MLK3 (both from J. R. Woodgett, Ontario Cancer Institute, Toronto, Canada); HA-tagged wild-type ROK (from E. Manser, Glaxo-IMCB Group, Singapore); active forms of Pak1: pCMV5-Myc-Pak1 165 (from M. Cobb, University of Texas, Dallas, TX), pCMV6-Myc-Pak1 T423E, and pCMV-Myc-Pak1 L107F (6); dominant negative Pak1: pEBG-Pak1, auto-inhibitory domain (AID) (pEBG Pak1, 83–149); inactive dominant negative Pak1: pEBG-Pak1-AID L107F (pEBG-Pak1, 83–149 L107F); and pCMV6-Myc-Pak6 was constructed by inserted Pak6 cDNA into the *Bgl*III/*Eco*RI cloning sites of a pCMV6-Myc mammalian expression vector.

Cell Culture, Transfections, and Immunoblotting—NIH3T3 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum or 10% fetal bovine serum, respectively. Plasmid DNA was transfected into cells using GenePorter reagent (Gene Therapy System). After 24 h, cells were solubilized with lysis buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 1 mM Na₃VO₄, 1 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin). Samples were then centrifuged at 14,000 × *g* for 10 min at 4 °C, and the supernatants were collected. For phosphatase treatment, lysates from NIH3T3 cells cotransfected with HA-NF2 and Rac1 Leu⁶¹ were precipitated with HA.11 monoclonal antibody (Babco). Aliquots of the precipitated protein were treated with buffer alone, with 10 units of calf intestinal phosphatase (CIP, New England Biolabs) alone or with CIP plus 1 mM sodium vanadate and 10 mM NaF at 37 °C for 10 min. Reactions were terminated by adding SDS-PAGE sample buffer. Cell lysates or immunoprecipitates were electrophoresed on a 6% SDS-PAGE gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Inc.) for Western blotting. Antibodies included anti-Myc 9E10 and anti-FLAG M5 (both from Sigma), anti-GST (Santa Cruz), anti-Rac1 (Upstate Biotechnology), and anti-phospho-JNK (Cell Signaling). Antibody detection was by means of an ECL Western analysis system (Amersham Biosciences, Inc.).

In Vitro Phosphorylation of Merlin by Pak—Human Pak2 cDNA was subcloned into the pET28 bacterial expression vector (Invitrogen). BL21 (DE3) cells were transformed with this plasmid, and protein expression was induced by incubating a log phase culture in 0.5 mM

isopropyl- β -D-thiogalactopyranoside for 6 h at 30 °C. The recombinant protein was recovered by chromatographic purification on a Talon column (CLONTECH) using standard methods. The recombinant His₆-tagged Pak2 was stored in 10- μ l aliquots at -80 °C.

Ser⁵¹⁸ (wild-type), Ala⁵¹⁸, and Asp⁵¹⁸ forms of NF2-C (amino acid residues 299–595) were transiently expressed in HeLa cells and immunoprecipitated with anti-HA antibody. Each immunoprecipitate was washed extensively and then incubated in 25 μ l of protein kinase buffer (40 mM Hepes, pH 7.4, 10 mM NaCl, 1 mM MgCl₂, 1 mM MnCl₂) containing 20 μ M ATP, 2.5 μ Ci of [γ -³²P]ATP, and 60 ng of activated recombinant Pak2. The reaction was incubated at 30 °C for 30 min and terminated by adding SDS-PAGE sample buffer. The products were separated by SDS-PAGE, and the autoradiogram was made from the dried gel. Western blot analysis was performed using anti-HA antibody to verify equivalent amounts of merlin substrate among reactions.

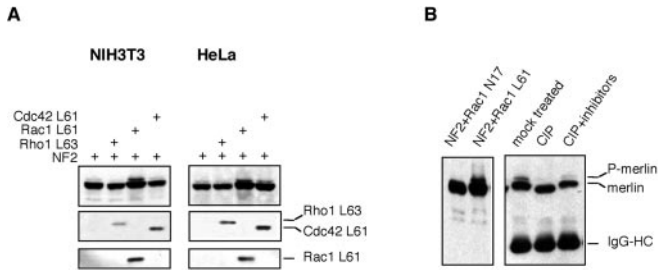


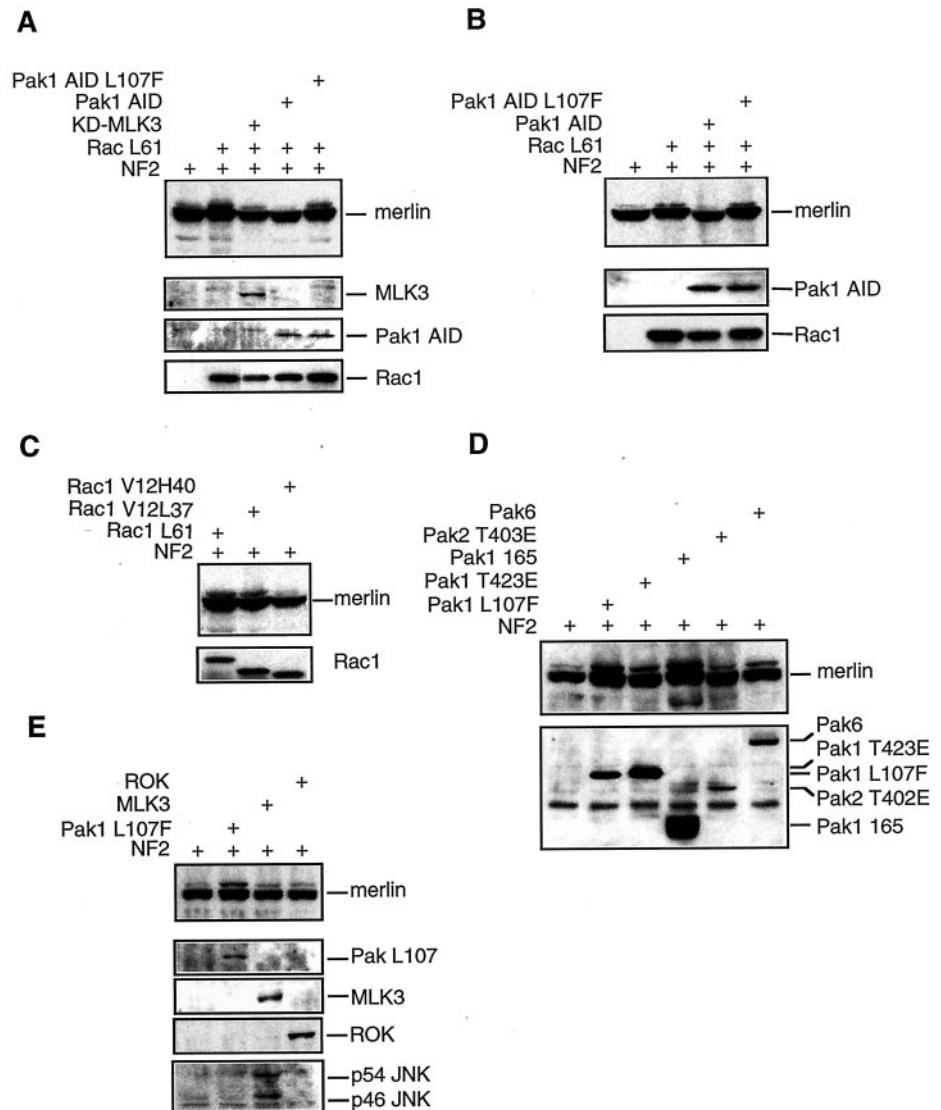
FIG. 1. Merlin phosphorylation by constitutively active Rac1 via Pak1. *A*, cells transfected with active Rac1 or, to a lesser extent, with active Cdc42 show faster and slower mobility bands, whereas cells transfected with RhoA exhibit only the faster mobility band. HA-tagged merlin was examined by immunoblotting with anti-HA antibody, and expression of Rho GTPases was verified with anti-Myc (for Myc-Rho Leu⁶³ and Myc-Cdc42 Leu⁶¹) or anti-HA (HA-Rac1 Leu⁶¹) antibodies. *B*, effect of phosphatase treatment on mobility of merlin. Lysates from NIH3T3 cells cotransfected with HA-NF2 and active Rac1 were precipitated with anti-HA antibody, and aliquots of the precipitated protein were treated with buffer alone, with CIP, or with CIP plus phosphatase inhibitors. CIP treatment eliminates the slower migrating form of merlin, whereas phosphatase inhibitors reversed the dephosphorylation effect of CIP.

RESULTS AND DISCUSSION

Previous studies have demonstrated that merlin is phosphorylated on serine and threonine residues and that merlin phosphorylation decreases with serum starvation, high cell density, or loss of adhesion (7). The ERM proteins play a role in cell surface dynamics and structure by linking the cytoskeleton to the plasma membrane (8, 9) and are regulated by Rho signaling (4, 5, 10). The Rho GTPases play crucial roles in regulating the organization of the actin cytoskeleton in mammalian cells, and Rho GTPases have been shown to regulate both cell-cell and cell-matrix adhesions and can influence the motile and invasive properties of tumor cells *in vitro* (11). Thus, we postulated

FIG. 2. Involvement of Pak in merlin phosphorylation.

A, cotransfected NIH3T3 cells showing abolishment or partial inhibition of the Rac-induced slower mobility form of merlin by dominant negative Pak1 (Pak1-AID) or kinase dead (KD) MLK3, respectively. *B*, cotransfected HeLa cells showing similar results as with NIH3T3 cells. *C*, cotransfected NIH3T3 cells demonstrating that Rac1 V12L37, but not Rac1 V12H40, stimulates merlin phosphorylation. *D*, cotransfected HeLa cells showing phosphorylation of merlin by active Pak1, active Pak2, or wild-type Pak6. Expression of the cotransfected plasmids was verified by immunoblotting using anti-Myc (for Myc-Pak1 T423E, Myc-Pak1 L107F, Myc-Pak1 165, Myc-Pak2 T403E, and Myc-Pak6), anti-HA (HA-Rac1 Leu⁶¹), anti-GST (GST-Pak1 AID and GST-Pak1 AID L107F), or anti-Rac (Rac1 Leu⁶¹, Rac1 V12L37, and Rac1 V12H40) antibodies. *E*, cotransfected HeLa cells showing phosphorylation of merlin by active Pak, but not by MLK3 or ROK. Expression of Pak, MLK3, and ROK was verified with anti-Myc, anti-FLAG, and anti-HA antibodies, respectively. Stimulation of endogenous JNK phosphorylation by MLK3 expression was revealed by anti-phospho-JNK antibody.



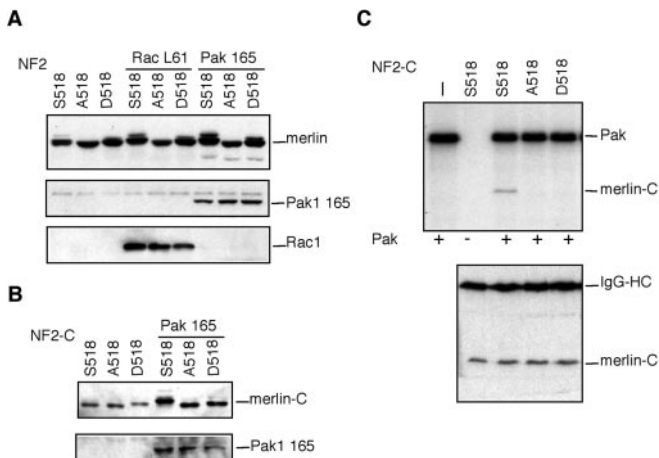


FIG. 3. Merlin Ser⁵¹⁸ is the phosphoacceptor site for Pak1. *A*, HA-tagged full-length NF2 Ser⁵¹⁸, NF2 Ala⁵¹⁸, or NF2 Asp⁵¹⁸ was transiently transfected into HeLa cells or cotransfected with either Rac1 Leu⁶¹ or Pak1 165 followed by immunoblotting with HA antibody. Although merlin Ser⁵¹⁸ shows faster and slower mobility bands in response to active forms of Rac1 or Pak1, NF2 Ala⁵¹⁸ migrates consistently as a single mobility form. Expression of Pak1 165 and Rac1 was verified by immunoblotting using anti-Myc or anti-HA antibodies. *B*, HA-tagged truncated forms of NF2 were transiently transfected into HeLa cells or cotransfected with Pak1 165 followed by immunoblotting with HA antibody. Truncated merlin Ser⁵¹⁸, but not the mutant forms Ala⁵¹⁸ or Asp⁵¹⁸, was phosphorylated by Pak1 165. Expression of Pak1 165 was verified by immunoblotting using anti-Myc antibody. *C*, *in vitro* phosphorylation of merlin by active Pak. HA-tagged truncated forms of NF2 were transiently expressed in HeLa cells and immunoprecipitated with anti-HA antibody, and the *in vitro* kinase assay was performed as described under "Materials and Methods." Note that wild-type merlin, but not mutant forms of merlin, are phosphorylated in the presence of Pak.

that Rho GTPase signaling could regulate the phosphorylation status of merlin. NIH3T3 cells and HeLa cells were transiently cotransfected with HA-tagged NF2 and individual constitutively active Rho GTPase constructs. Cells were lysed 24 h after transfection, and expression of merlin was examined by immunoblotting with anti-HA antibody. As shown in Fig. 1A, exogenous merlin was detected as two mobility forms. In cells transfected with active RhoA (RhoA Q63L), merlin migrated as a single mobility form. However, in cells transfected with active Rac1 (Rac1 Q61L), and to a lesser extent, in cells transfected with active Cdc42 (Cdc42 Q61L), merlin migrated as a doublet consisting of faster and slower mobility bands. To investigate the nature of the slower migrating form of merlin, lysates from NIH3T3 cells cotransfected with HA-NF2 and Rac1 Leu⁶¹ were precipitated with anti-HA antibody. CIP treatment eliminated the slower migrating form of merlin, whereas phosphatase inhibitors reversed the dephosphorylation effect of CIP (Fig. 1B). These data indicate that the slower migrating band represents the phosphorylated form of merlin, which is induced by either active Rac1 or Cdc42.

Earlier work identified several downstream target molecules of Rac. MLK3, a member of the mixed lineage kinase family, is able to associate with Rac and Cdc42 and, in turn, to activate JNK (12). Pak1, another effector of Rac, plays an important role in cell morphology and motility (6, 13–15). To examine which effector mediates merlin phosphorylation by Rac, NIH3T3 cells and HeLa cells were transiently cotransfected with HA-NF2, Rac1 Leu⁶¹, and a dominant negative form of MLK3 or Pak1 (Pak1-AID). Immunoblot analysis was performed as shown in Fig. 2A. The slower mobility (phosphorylated) form of merlin induced by active Rac1 was inhibited by a dominant negative form of Pak1 containing the autoinhibitory domain (Pak1-AID) (16, 17), but not by Pak1-AID L107F, which

is known to block the autoinhibitory effect of Pak1-AID (17). Similar results were obtained with HeLa cells (Fig. 2B). In addition, the slower mobility form of merlin was partially blocked by dominant negative (kinase dead) MLK3.

To confirm the involvement of Pak in merlin phosphorylation, we cotransfected HA-NF2 with the Rac effector mutant Rac1 V12L37, which is able to activate Pak1, or Rac1 V12H40, which cannot bind or activate Pak1 (18). Rac1 V12L37, but not Rac1 V12H40, stimulated merlin phosphorylation (Fig. 2C). Next, we cotransfected HeLa cells with HA-NF2 and individual active forms of Pak1 (Pak1 L107F, Pak1 T423E, Pak1 165) or Pak2 (Pak2 T403E), or wild-type Pak6, each of which was able to stimulate phosphorylation of merlin (Fig. 2D). Similar results were obtained with NIH3T3 cells (data not shown). These results show that both group I Paks and group II Paks can induce phosphorylation of merlin.

Because dominant negative MLK3 partially blocked merlin phosphorylation induced by Rac (Fig. 2A), we tested whether merlin can also be phosphorylated via MLK3 signaling. As shown in Fig. 2E, overexpression of MLK3 failed to stimulate merlin phosphorylation. Since MLK3 contains a partial CRIB (Cdc42-Rac interaction and binding) motif, the inhibitory effect of dominant negative MLK3 could be due to sequestering Rac from Pak. In addition, we determined that ROK, a downstream target of Rho, is also unable to stimulate merlin phosphorylation (Fig. 2E). Taken together, these findings indicate that merlin is phosphorylated by Rac specifically via Pak signaling.

Shaw *et al.* (19) has also recently observed that merlin functions in Rac/Cdc42-dependent signaling, although that work did not link Rac and merlin signaling with Pak. Their investigations also demonstrated that Rac-induced phosphorylation of merlin, at Ser⁵¹⁸, regulates its activity by weakening both its head-to-tail interaction and its association with the cytoskeleton. Since our data indicated that merlin is phosphorylated by Rac through Pak, we sought to determine whether Pak also phosphorylates merlin at Ser⁵¹⁸. To test this possibility, merlin Ser⁵¹⁸ was mutated to an alanine (NF2 S518A), which could no longer be phosphorylated at this site, or aspartic acid (NF2 S518D), which could mimic the effect of phosphorylation. Active Rac1 or Pak1 was transiently cotransfected with wild-type NF2, NF2 S518A, or NF2 S518D, followed by immunoblotting. As shown in Fig. 3A, mutation of this site had no effect on the level of expression of merlin. However, in cells transfected with either active Rac1 or Pak1, the NF2 S518A mutant was refractory to phosphorylation as indicated by a single mobility form, whereas NF2 S518D consistently migrated as a doublet whether cotransfected with active Rac1 or Pak1 or when transfected alone. Next, we transfected constructs encoding truncated forms of merlin into HeLa cells (Fig. 3B). Western blot analysis confirmed that truncated merlin Ser⁵¹⁸, but not the mutant forms Ala⁵¹⁸ or Asp⁵¹⁸, is phosphorylated by Pak1 165. Collectively, these data indicate that merlin Ser⁵¹⁸ is the phosphoacceptor site mediated not only by Rac, but also by Pak.

To determine whether merlin can be phosphorylated by Pak *in vitro*, we performed an immunocomplex kinase assay. Because full-length merlin comigrates with Pak, we used truncated forms of merlin (NF2-C Ser⁵¹⁸, NF2-C Ala⁵¹⁸, NF2-C Asp⁵¹⁸) as substrates. As demonstrated in Fig. 3C, truncated merlin Ser⁵¹⁸ was phosphorylated in the presence, but not in the absence, of active, recombinant Pak. Furthermore, truncated mutant forms of merlin (Ala⁵¹⁸ and Asp⁵¹⁸) were not phosphorylated by Pak. Collectively, these data indicate that Pak can directly phosphorylate merlin at serine 518.

Previous work indicates that overexpression of merlin in rat schwannoma cells inhibits their growth (20) and impairs cell motility, adhesion, and spreading (21). Merlin is hypophospho-

rylated in connection with serum deprivation, high cell density, or loss of adhesion (7). At low cell density, merlin is phosphorylated, growth-permissive, and exists in a complex with ezrin, moesin, and the hyaluronic acid receptor CD44 (22). These data indicate that the phosphorylation status of merlin specifies cell growth arrest or proliferation: *i.e.* hypophosphorylated merlin is growth-inhibitory and represents the functionally active tumor suppressor form of the protein, whereas hyperphosphorylation inactivates merlin and is growth-permissive.

Merlin loss has been associated with a high metastatic potential in an animal model reported by McClatchey *et al.* (23). The signal transduction studies presented here establish a framework for elucidating tumorigenic mechanisms involved in neoplasms associated with merlin inactivation. Interestingly, somatic mutations of merlin are common in human malignant mesothelioma (24, 25), a highly invasive and metastatic tumor type. Merlin loss of function by either phosphorylation or biallelic inactivation may contribute to tumor growth and invasiveness/metastasis. Thus, our characterization of merlin phosphorylation by Rac through Pak provides new insights into the regulation of its tumor suppressor function. Importantly, Pak has been shown to regulate motility in mammalian cells (6), and activated Paks can be transforming (26, 27). These data raise the intriguing possibility that merlin inactivation by Pak may play a role in tumor cell spreading and metastasis.

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