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14. ABSTRACT
The NF2 tumor suppressor gene encodes an intracellular membrane-associated protein, called merlin, which belongs to the band 4.1 family of cytoskeleton-associated proteins that link cell surface glycoproteins to actin cytoskeleton. Merlin suppresses PI 3-kinase/Akt signaling through directly binding and inhibiting PIKE-L's stimulatory activity on PI 3-kinase. In Nature Cell Biology paper (2007), we have demonstrated that Akt directly binds to and phosphorylates merlin on residues Thr 230 and Ser 315, which abolishes merlin NTD/CTD interactions and binding to merlin's effector protein PIKE-L and other binding partners. Furthermore, Akt-mediated phosphorylation leads to merlin degradation by ubiquitination. In Cancer Research paper (2009), we show Akt phosphorylation and PI(3, 4, 5) P_3 binding mediate the tumor suppressive activity of merlin. The extreme N-terminus of merlin directly interacts with phosphatidylinositols, for which the unfolded conformation is required. Moreover, Akt phosphorylation enhances merlin binding affinity to phosphatidylinositols and inhibits its pro-apoptotic actions. Further, Akt phosphorylation and phosphatidylinositols increase merlin binding to CD44. EGF treatment and Akt phosphorylation provoke merlin to aggregate in the ruffled plasma membrane and promote cell migration. Thus, these results suggest that PI 3-kinase signaling regulates merlin's tumor suppressive activity via both Akt phosphorylation and phosphatidylinositol lipids binding to merlin. Recently, we show that phosphorylation of merlin also regulates its sumoylation. The biological significance of this finding is under investigation.

15. SUBJECT TERMS
Akt phosphorylation, Phosphoinositols, merlin, binding partners

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Introduction

The NF2 tumor suppressor protein merlin is structurally related to the Protein 4.1 family of molecules and, specifically, a subgroup including ezrin, radixin and moesin (ERM proteins). Like the ERM proteins, merlin contains an N-terminal domain (NTD, residues 1–302), which is highly conserved among all members of the Protein 4.1 family and is thought to mediate interactions with the cytoplasmic tail of cell surface glycoproteins such as glycophorin C and CD44 (1). The second half of the molecule contains a predicted α-helical domain (residues 303–478) and a unique C-terminus (CTD, residues 479–595). ERM proteins link the actin cytoskeleton to cell surface glycoproteins. Merlin can associate with polymerized actin *in vitro* by virtue of an amino (N-) terminal actin binding domain including residues 178-367. Merlin actin binding is not affected by natural NF2 patient mutations or alternatively spliced isoforms (2). Phosphatidylinositol lipids have been implicated in ERM protein activation. For example, $PI(4,5)P_2$ enhances the ability of full-length radixin, but not its N-terminal domain, to bind the cytoplasmic tail of CD44. However, the tail of CD44 does not bind $PI(4,5)P_2$, suggestive of an activating effect on full-length radixin (3). Analysis of recombinant moesin T588D, which mimics phosphorylated moesin, suggests an auxiliary role for $PI(4,5)P_2$ in unmasking the C-terminal F-actin binding site. Recent work using either unphosphorylated or T558 phosphorylated moesin purified from platelets revealed a dual requirement for T558 phosphorylation and the presence of a detergent and a phospholipid, such as $PI(4,5)P_2$, for F-actin binding (4). In support of a two-step activation process in which $PI(4,5)P_2$ renders moesin susceptible to C-terminal phosphorylation, transfection studies have suggested that enhanced phosphatidylinositol 4-phosphate 5-kinase activity results in enhanced phosphorylation of the C-terminal threonine (5).

Merlin is phosphorylated on both serine and threonine residues, and the phosphorylation status of merlin varies in response to growth conditions (6). Previously, we demonstrated that PAK2 phosphorylates S518 and abolishes merlin N-term/C-term binding. PAK2 phosphorylation impairs the ability of merlin to bind to two interacting proteins, CD44 and hepatocyte growth factor regulated tyrosine kinase substrate (HRS), both critical for merlin growth suppression (7). In addition to phosphorylation of S518, PKA has recently been showed to phosphorylate S10 and affect actin cytoskeletion, mediating cell migration (8). Most Recently, we show that Akt directly binds and phosphorylates merlin on residues T230 and S315, which abolishes merlin intramolecular NTD/CTD interaction and merlin binding to PIKE-L and other binding partners. Furthermore, Akt-mediated phosphorylation leads to merlin degradation by polyubiquitination (9). Phosphorylation and $PI(4,5)P_2$ binding coordinately mediate the activation of ERM proteins (5, 10). However, whether the dual regulatory machinery also controls merlin activity remains elusive. In this report, we show that the NTD of merlin directly associates with phosphatidylinositols, for which the “open” conformation is required. Moreover, Akt phosphorylation enhances the interaction between merlin and phosphatidylinositols. Akt phosphorylation blocks its pro-apoptotic activity. Interestingly, Akt phosphorylation of merlin substantially inhibits its pro-apoptotic activity and blocks its tumor suppressive activity on cell migration (11).

Most recently, we have determined that merlin can also be sumoylated, and this process is modulated by Akt and PAK phosphorylation. Since cell density regulates merlin phopshorylation status through regulating Akt and PAK phosphorylation, merlin sumoylation is also regulated by cell density. Mapping experiments support that K76 might be the major sumoylation site.

Body

In this report, we show that we have successfully accomplished most of the work proposed.

Task 1.To determine whether Akt phosphorylation on merlin regulates its conformation and subcellular localization (months1-12).

a. To determine Akt phosphorylation site on the NTD of merlin (months 1-3)

b. To determine the effect of Akt phosphorylation of merlin on its intramolecular and intermolecular interaction (months 4-8)

c. To determine whether Akt phosphorylation mediates merlin subcellular localization (months 9-12)

Task 2. To determine the effect of phosphorylation and phosphatidylinositol lipids on merlin's binding activity (months 13-36).

a. To investigate whether Akt phosphorylation regulates merlin's binding activity to critical effectors essential for merlin growth suppression (months 13-18)

b. To examine whether phosphatidylinositol lipids mediate merlin's binding activity (months 19-24)

c. To determine whether Akt phosphorylation and PI (3,4,5)P3 lipid cooperatively control merlin's binding activity (months 25-36)

Task 3. To determine the effect of Akt phosphorylation on merlin's tumor suppressive activity (months 37-48)

a. To determine the effect of merlin phosphorylation by Akt on cell growth (months 37-40)

b. To determine the effect of merlin phosphorylation by Akt on cell motility (months 41-43)

c. To determine the effect of merlin phosphorylation by Akt on cell survival (months 44-48)

However, we have observed merlin sumoylation effect. Although the physiological significance of this observation remains elusive, our exciting preliminary study suggests that other post-translational modification on merlin might also implicate in regulating this tumor suppressor as well. In this final report, we will provide these exciting new data and ask for non-cost extension of this current project for one more year to wrap up the new findings.

Results

The main tasks of this project have been successfully executed. Two papers that report our results have been published in Nature Cell Biology(9) and Cancer Research (11), respectively. In this report, we are presenting the follow-up novel observation on merlin sumoylation. Small ubiquitin-related modifier (SUMO) is a member of a family of ubiquitin-like proteins that regulates cellular function of a variety of proteins (12). Although having fairly low amino acid sequence identity with ubiquitin, the SUMO enzymes

exhibit similar tertiary structures. The mechanism for SUMO conjugation is analogous to that of the ubiquitin system, relying upon utilisation of E1, E2 and (potentially) E3 cascade enzymes (13). Unlike ubiquitinylation, which leads, *inter alia*, to a degradative pathway, SUMO modification of target proteins is involved in nuclear protein targeting, formation of sub-nuclear complexes, regulation of transcriptional activities, and control of protein stability. For example, SUMO modification of p53 represents an additional regulator of p53 tumour repressor protein stability and may contribute to activation of the p53 response (14).

Merlin can be sumoylated in vitro and in vivo

A short sequence containing the consensus Ψ-K-X-D/E (where lysine is the modified amino acid, Ψ is a large hydrophobic residue and X is any amino acid residue) is thought to be necessary for proteins to be sumoylated. Protein sequence analysis reveals that merlin contains numerous putative sumoylation sites. To explore whether merlin can be sumoylated in vitro, we conducted an in vitro sumoulation assay with purified His-merlin recombinant proteins. Merlin was robustly sumoylated by wild-type sumo1 but not mutated sumo1 (Figure 1A, right panels). As a postive control, p53 is also markedly sumoylated by wild-type but not mutated sumo1 (Figure 1A, left panels). To test whether merlin can be sumoylated in intact cells, we transfected GFP vector or GFP-merlin construct into His-sumo1 stable cells, and pulled down sumo1 with Ni2+ beads. Immunoblotting analysis demonstrated that GFP-merlin was selectively sumoylated (Figure 1B). Cotransfection of Myc-merlin and GFP-sumo1 also display similar results (Figure 1C). Hence, merlin can be sumoylated in vitro and in intact cells.

Akt phosphorylation mediates merlin sumoylation

Since Akt phosphorylates merlin provokes its polyubiquitination and degradation. It is possible that Akt phosphorylation might also regulates its sumoylation as well. To test this idea, we transfected GFP-tagged merlin wild-type, unphosphorylate mutant (T230AS315A) and phosphorylation mimetic merlin (T230DS315D) into His-sumo1 stable cells. Ni2+ beads pull-down assay supported that merlin (T230DS315D) was more robustly sumoylated as compared to wild-type control, whereas merlin (T230AS315A) was barely sumoylated (Figure 2A). To further investigate whether Akt phosphorylates PIKE-A regulates its sumoylation, we are now cotransfecting GFP-merlin and myc-sumo1 with wild-type Akt, constitutive active Akt-CA (T308DS473D) and kinase-dead Akt-KD (K179A) into HEK 293 cells, we will immunoprecipitate merlin and monitor its sumoylation status by immunoblotting. To test whether endogenous Akt plays any role in mediating merlin sumoylation, we will pretreat GFP-merlin and Myc-sumo1 cotransfected cells with PI 3-kinase inhibitor wortmannin, LY294002, DMSO, Akt inhibitor Akt1/2 for 30 min, followed by EGF stimulation for another 10 min. The transfected merlin will be immunoprecipitated and monitored by immunoblotting with anti-Sumo1 antibody. These experiments are ongoing.

Cell density regulates merlin sumoylation

Merlin mediates contact inhibition of growth through signals from the extracellular matrix. At high cell density, merlin becomes hypo-phosphorylated and inhibits cell growth in response to hyaluronate (HA), a mucopolysaccharide that surrounds cells. At low cell density, merlin is phosphorylated, growth permissive, and exists in a complex with ezrin, moesin, and CD44 (2001, 15, 968). To examine whether cell density also regulates merlin sumoylation, we cultured a few stable inducible merlin transfected schwannoma cell lines. The induced cells were grown at different densities. Immunoprecipitation with anti-merlin antibody showed that at 50% density but not 100% density, wild-type merlin ws markedly sumoylated in 5_4 cells. By contrast, control IgG failed to immunoprecipitate any detectable sumoylated merlin (Figure 3A, right panels). Accordingly, merlin was evidently phosphorylated by Akt on S315 at low density, in alignment with Akt activation status (Figure 3A, left panels). Interestingly, merlin L64P, an unfold mutant was unable to be sumoylated regardless of cell density. Consistently, merlin L64P S315 phosphorylation was not altered at 50% or 100% densities, fitting with Akt phosphorylation status (Figure 3B). On the other hand, merlin S315D, a PAK phosphorylation mimetic mutant, was strongly sumoylated at both 50% and 100% densities (Figure 3C). As expected, merlin was more robustly phosphorylated at S315 at 50% density than 100% density, so was Akt S473 phosphorylation (Figure 3C, left panels). Hence, hyperphosphorylated merlin is prone to sumoylation.

Merlin K76R mutation affects its subcellular residency

Protein sequence analysis suggests that merlin contains at least 4 putative sumoylation motifs (75LKMD78; 363MKEE366; 454LKQD457; 542LKTE545) (Figure 4A). To determine which residue is a potential sumoylated site, we mutate each of the Lysine residues into Arginine in GFP-tagged full-length merlin and monitor its subcellular residency. As expected, wild-type merlin predominantly localized on the plasma membrane, so were K364R, K455R and K543R. However, approximately 25% of the K76R mutant not only distributed on the plasma membrane but also aggregated inside the cells (Figure 4B and C). Therefore, K76 might be one of the major sumoylation sites on merlin. To confirm this possibility, we will cotransfect GFP-merlin K76R with myc-sumo1 into HEK293 cells, we will pretreat the cells with PI 3-kinase inhibitors or Akt inhibitors, followed by EGF stimulation. Transfected merlin will be immunoprecipitated with anti-GFP, and immunoblotted with anti-sumo1 antibody. We expect that merlin sumoylation signal will be substantially decreased, if K76 is the major sumoylation site. However, if merlin has multiple sumoylation sites, mutation of K76R alone might not significantly diminish merlin sumoylation signal. This study is actively pursuing in my laboratory.

Discussion

In this report, we show that merlin can be sumoylated in vitro and in vivo. This post-translational modification is regulated by Akt phosphorylation, as merlin unphosphoryaltion mutant by Akt displays impaired sumoylation activity, and phosphorylation mimetic mutant exhibits increased sumoylation effect. In alignment with this observation, cell density, which also regulates Akt phosphorylation status, mediates merlin sumoylation. PAK phosphorylation on S518 further escalates merlin sumoylation even at high density, indicating that Akt and PAK might synergistically regulate merlin sumoylation. The available data demonstrate that K76R

mutation can alter merlin plasma membrane residency. Conceivably, K76 is, if not the only sumoylation sites, the major sumoulation reside in merlin.

To address whether endogenous merlin is sumoylated by Akt under physiology conditions, we are overexpressing or knocking down Akt in a variety of human cancer cells to investigate merlin sumoylation alteration. Moreover, we are exploring whether sumoylation of merlin regulates its tumor suppressive activity, its binding affinities to various binding partners including PIKE-L, CD44, Erzrin etc. Hopefully, in next few months, we should be able to have clear answers to these questions.

Merlin subcellular localization and growth suppression has been shown to be modulated by merlin phosphorylation (15). Using an antibody directed against merlin phosphorylated on S518, Kissil and colleagues demonstrated that phosphorylated merlin is mislocalized to the cytoplasm, and is not expressed at the cell membrane. In addition, merlin mutant that mimics S518 phosphorylation (S518D) cannot suppress cell growth or motility in RT4 rat schwannoma cells, and results in dramatic changes in cell morphology and actin cytoskeleton organization (16). These results demonstrate that the S518D merlin mutation, which mimics merlin phosphorylation, impairs merlin growth and motility suppression activity. Recently, we provide further evidence showing that phosphorylation of merlin by Akt can also provoke merlin relocation and loss its growth inhibitory activity. The synergistic effect by PAK and Akt on merlin sumoylation might provide insight into how the signaling cross-talks affect merlin biological actions. Recently, we show that Akt feeds back and robustly phosphorylates merlin on both T230 and S315 residues and unfolds merlin, leading to its polyubiquitination and degradation. Although either Akt or PAK phosphorylating merlin results in its binding to phosphatidylinositol lipids, merlin S518 phosphorylation by PAK can not provoke merlin polyubiquitination and degradation (9). This finding suggests the unique aspect of the regulatory role of Akt in mediating merlin tumor suppressor. Nonetheless, it remains unclear whether PAK phosphorylation alone plays any role in triggering merlin sumoylation.

Key Research Accomplishments

- Two papers have been published (Nature Cell Biology and Cancer Research)
- Merlin can be sumoylated in vitro and in vivo.
- Akt phosphorylation of merlin regulates its sumoylation
- S518 phosphorylation increases merlin sumoylation
- Cell density regulates merlin sumoylation
- K76R mutation interferes merlin subcellular distribution

Reportable Outcomes

The above results and more in vivo functions of merlin sumoylation are being actively pursued in my laboratory. Hopefully, in a few months, we will have a manuscript ready for submission.

Conclusion

Akt directly binds to and phosphorylates merlin on residues Thr 230 and Ser 315, which abolishes merlin NTD/CTD interactions and binding to merlin's effector protein PIKE-L and other binding partners. Furthermore, Akt-mediated phosphorylation leads to merlin degradation by ubiquitination. These studies demonstrate that Akt-mediated merlin phosphorylation regulates the function of merlin in the absence of an inactivating mutation. Akt phosphorylation and PI(3,4,5)P3 binding mediate the tumor suppressive activity of merlin. The extreme N-terminus of merlin directly interacts with phosphatidylinositols, for which the unfolded conformation is required. Moreover, Akt phosphorylation enhances merlin binding affinity to phosphatidylinositols. Further, Akt phosphorylation increases merlin distribution on the cell-cell contact plasma membrane and association with CD44. Akt phosphorylation of merlin blocks its pro-apoptotic action. EGF treatment and Akt phosphorylation provoke merlin to aggregate in the ruffled plasma membrane and promote cell migration. Thus, these results suggest that PI 3-kinase signaling regulates merlin's tumor suppressive activity via both Akt phosphorylation and phosphoinositol lipids binding to merlin. Furthermore, Akt also provokes merlin sumoylation through phosphorylating merlin. Whether this modificatiomn regulates merlin's tumor suppressive activity, protein stability, binding affinities to other binding partners remain the major topics in the ongoing project.

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Figure legends

Figure 1. Merlin can be sumoylated in vitro and in intact cells

A. Merlin can be sumoylated in vitro. In vitro sumoylation assay with purified His-merlin recombinant proteins using sumoylation kit. P53 was employed as positive control.

B, Merlin can be sumoylated in transfected cells. GFP-merlin and GFP vector were transfected into His-sumo1 stable transfected F293 cells. Sumoylated proteins were pulled down by Ni2+ beads, and the precipitates were analyzed by immunoblotting with anti-GFP antibody.

Figure 2. Akt phosphorylation regulates merlin sumoylation

A, Akt phosphorylation enhances merlin sumoylation. GFP-merlin T230AS315A, T230DS315D and wild-type constructs were transfected into His-sumo1 stable cell line F293 cells. Sumo proteins were pulled down by Ni2+ beads, and analyzed by immunoblotting with anti-Sumo1 antibody.

Figure 3. Cell density regulates merlin sumoylation

A, wild-type merlin is high sumoylated at low density. Rat RT4 schwannoma cells that were stable transfected with wild-type merlin were culture at 50% and 100% density and the transfected merlin were induced by puramycin. The induced erlin was immunoprecipitated with anti-merlin antibody, and analyzed by anti-sumo1 antibody (right panels). Merlin, Akt phopshorylation status were analyzed (left panels).

B, Merlin L64P cannot be sumoylated regardless of cell density

C, Merlin S518D is strongly sumoylated at both high and low cell densities.

Figure 4. Merlin K76R mutation affects its plasma membrane residency.

A, Four putation merlin sumoylation motifs are listed.

B, Merlin K76R mutation aggregates merlin intracellularly. GFP-merlin wild-type and 4 point mutants were transfected into HEK293 cells. The distribution of GFP-merlin was analyzed under immunofluorescent microscope.

C, K76R mutation affect merlin plasma membrane residency. The transfected cells were quantitatively analyzed under fluorescent microscope. Approximately 500 cells were counted in 10 different fields. The data represent mean ± SEM.

Figure 1

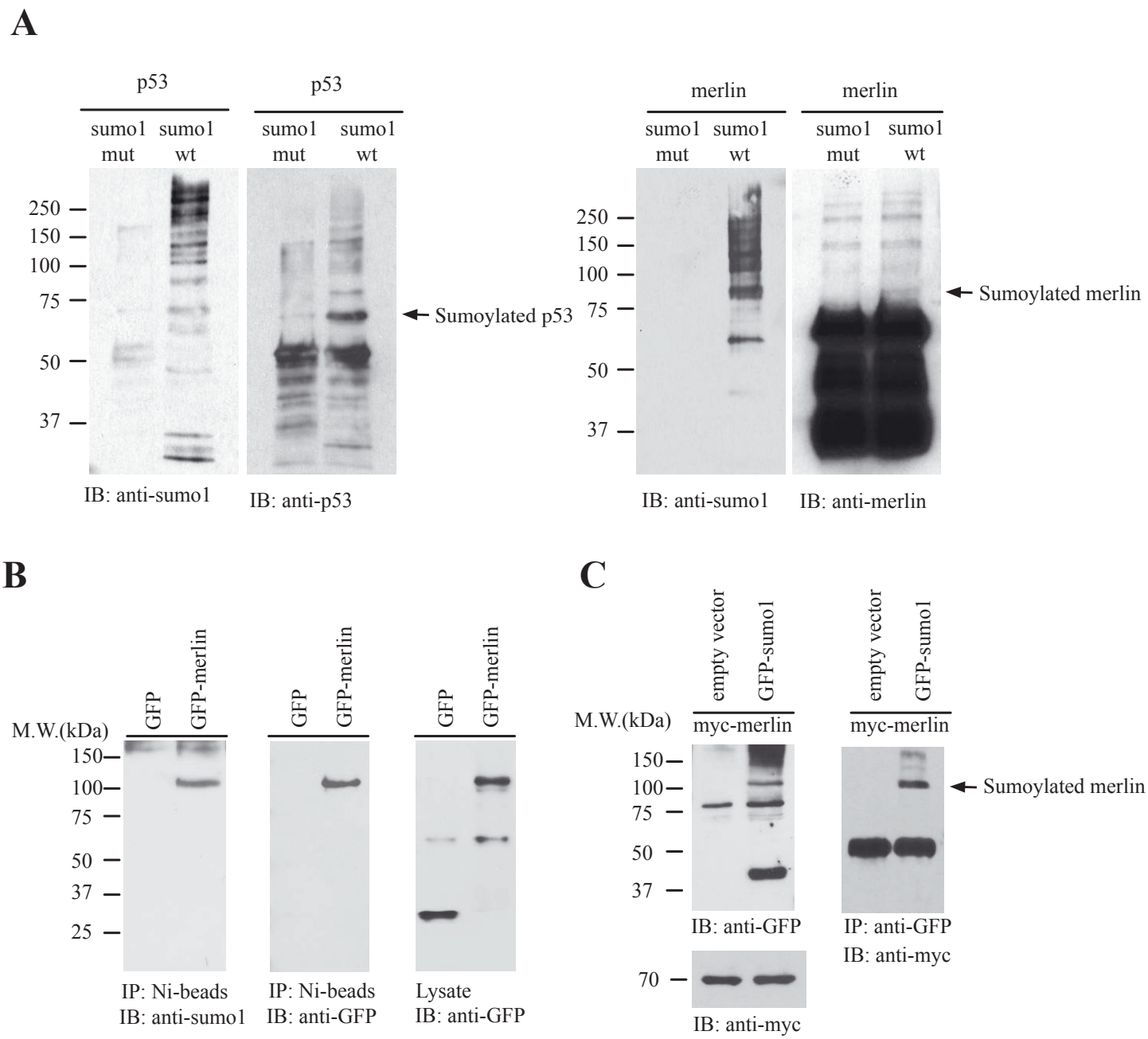
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Figure 2

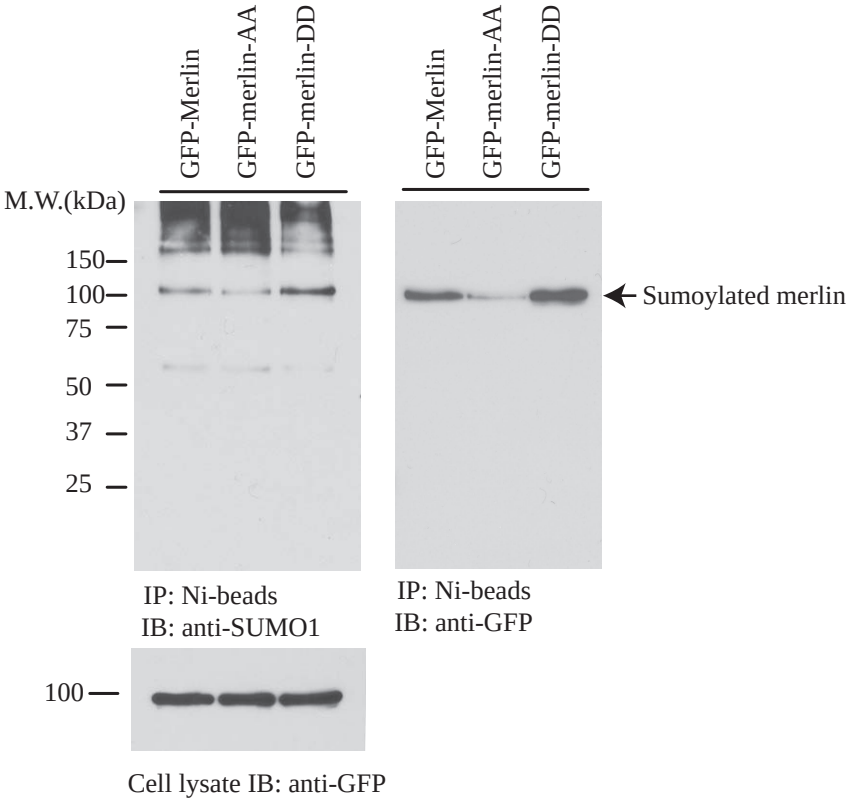
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Figure 3

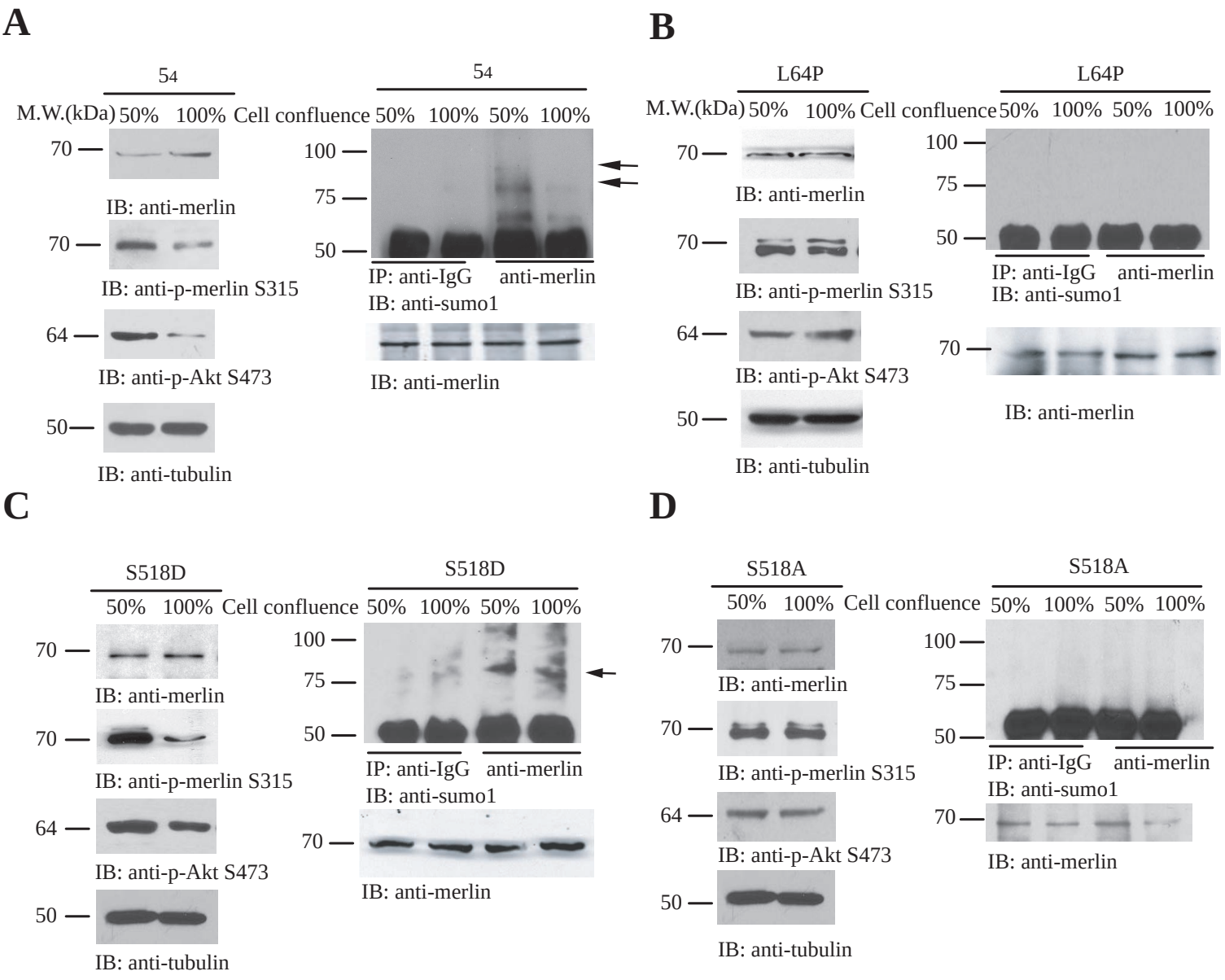
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Figure 4

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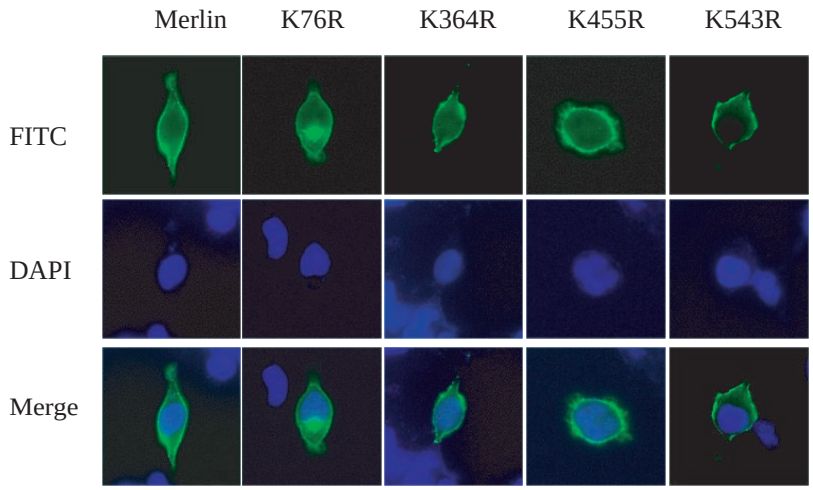
B



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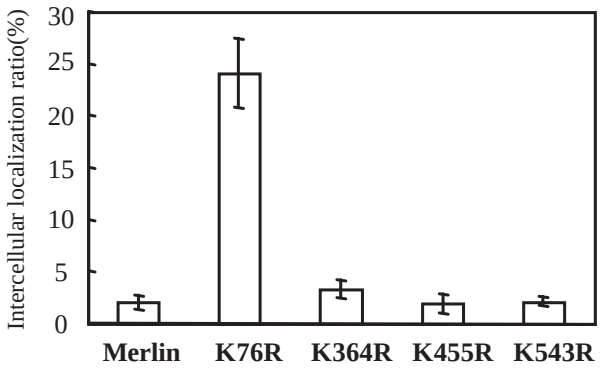

Akt Phosphorylation of Merlin Enhances Its Binding to Phosphatidylinositols and Inhibits the Tumor-Suppressive Activities of Merlin

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Abstract

The NF2 tumor suppressor gene encodes an intracellular membrane-associated protein, called merlin, which belongs to the band 4.1 family of cytoskeleton-associated proteins that link cell surface glycoproteins to the actin cytoskeleton. Merlin suppresses phosphatidylinositol 3-kinase (PI3K)/Akt signaling by directly binding and inhibiting the stimulatory activity of PIKE-L on PI3K. Akt feeds back and phosphorylates merlin and provokes its polyubiquitination and degradation. Here, we show that Akt phosphorylation and PI(3,4,5)P_3 binding mediate the tumor-suppressive activity of merlin. The extreme NH_2 terminus of merlin directly interacts with phosphatidylinositols, for which the unfolded conformation is required. Moreover, Akt phosphorylation enhances merlin binding affinity to phosphatidylinositols and inhibits its proapoptotic actions. Furthermore, Akt phosphorylation and phosphatidylinositols increase merlin binding to CD44. Epidermal growth factor treatment and Akt phosphorylation provoke merlin to aggregate in the ruffled plasma membrane and promote cell migration. Thus, these results suggest that PI3K signaling regulates the tumor-suppressive activity of merlin via both Akt phosphorylation and phosphatidylinositol lipids binding to merlin. [Cancer Res 2009;69(9):4043–51]

Introduction

The NF2 tumor suppressor protein merlin is structurally related to the protein 4.1 family of molecules and, specifically, a subgroup including ezrin, radixin, and moesin (ERM proteins). Like the ERM proteins, merlin contains an NH_2 terminal domain (NTD; residues 1–302), which is highly conserved among all members of the protein 4.1 family and is thought to mediate interactions with the cytoplasmic tail of cell surface glycoproteins, such as glycophorin C and CD44 (1). The second half of the molecule contains a predicted α-helical domain (residues 303–478) and a unique COOH terminal domain (CTD; residues 479–595). ERM proteins link the actin cytoskeleton to cell surface glycoproteins. Merlin can associate with polymerized actin *in vitro* by virtue of an amino (NH_2) terminal actin binding domain, including residues 178 to 367. Merlin actin binding is not affected by natural NF2 patient mutations or alternatively spliced isoforms (2). Phosphatidylinositol lipids have been implicated in ERM protein activation. For example, PI(4,5)P_2 enhances the ability of full-length radixin, but not its NTD, to bind the cytoplasmic tail of CD44. However, the tail of CD44 does not bind PI(4,5)P_2, suggestive of an activating effect on full-length radixin (3). Analysis of recombinant moesin T588D, which mimics phosphorylated moesin, suggests an auxiliary role for PI(4,5)P_2 in unmasking the COOH terminal filamentous actin (F-actin) binding site. Recent work using either unphosphorylated or T558-phosphorylated moesin purified from platelets revealed a dual requirement for T558 phosphorylation and the presence of a detergent and a phospholipid, such as PI(4,5)P_2, for F-actin binding (4). In support of a two-step activation process wherein PI(4,5)P_2 renders moesin susceptible to COOH terminal phosphorylation, transfection studies have suggested that enhanced phosphatidylinositol 4-phosphate 5-kinase activity results in enhanced phosphorylation of the COOH terminal threonine (5).

Merlin is phosphorylated on both serine and threonine residues, and the phosphorylation status of merlin varies in response to growth conditions (6). PAK2 phosphorylates S518 and abolishes merlin NH_2/COOH terminal binding. PAK2 also impairs the ability of merlin to bind to two interacting proteins, CD44 and HRS, both critical for merlin growth suppression (7). In addition to the phosphorylation of S518, PKA has recently been shown to phosphorylate S10 and affect the actin cytoskeleton, mediating cell migration (8). Most recently, we show that Akt phosphorylates merlin on residues T230 and S315, which abolishes merlin intramolecular NTD/CTD interaction and merlin binding to PIKE-L and other binding partners, leading to merlin degradation by polyubiquitination (9). Phosphorylation and PI(4,5)P_2 binding coordinately mediate the activation of ERM proteins (5, 10). However, whether the dual regulatory machinery also controls merlin activity remains elusive. In this report, we show that the NTD of merlin associates directly with phosphatidylinositols, for which the "open" conformation is required. Moreover, Akt phosphorylation enhances the interaction between merlin and phosphatidylinositols. Akt phosphorylation blocks its proapoptotic activity. Finally, we show that Akt phosphorylation of merlin substantially inhibits its proapoptotic activity and blocks its tumor-suppressive activity on cell migration.

Materials and Methods

Cells and reagents. Both 5_4 and 6_7, which are RT4-D6P2T schwannoma cells stably transfected with wild-type merlin (merlin T230DS315D and T230AS315A stably expressed cells), were maintained in DMEM including 10% fetal bovine serum, 100 units of penicillin-streptomycin, 500 μg/mL of G-418, and 1 μg/mL of puromycin. L64P, S518A, and S518D cells are patient-derived mutant (L64P), and S518 phosphorylation mutant stably transfected RT4-D6P2T cells. Merlin proteins were induced by 1 μg/mL of doxycycline treatment and then incubated for 24 h. HCT116 and HCT116 $PTEN^{-/-}$ cells

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

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were maintained in McCoy's 5A, including 10% fetal bovine serum and 100 units of penicillin-streptomycin. All cells were maintained at 37°C with 5% CO_2 atmosphere in a humidified incubator. Wortmannin, LY294002, and glutathione *S*-transferase (GST)–horseradish peroxidase were from Sigma. Akt1/2 inhibitor was from VWR, and PAK inhibitor IPA-3 was from Sigma. Phosphatidylinositol-conjugated beads and various phosphatidylinositols diC16 were from Echelon, Inc.

Cosedimentation of merlin recombinant proteins with lipid vesicles. The analysis of merlin-lipid interactions by cosedimentation of proteins with multilamellar liposomes was performed as described (11). Multilamellar liposomes were prepared from phosphatidylcholine and $PI(3,4,5)P_3$, $PI(4,5)P_2$, $PI(3,4)P_2$, and phosphatidylinositol in a buffer containing 20 mmol/L of HEPES (pH 7.4) and 0.2 mmol/L of EGTA. Proteins were subsequently incubated for 15 min at 25°C in the presence of 130 mmol/L of KCl and in the absence of liposomes, followed by further incubation in the absence or presence of liposomes for 15 min. The mixtures were subsequently centrifuged for 30 min at 100,000 × *g* at 4°C. The pellets were solubilized in 50 to 100 μL sample buffer. After SDS-PAGE, the amount of protein present in pellets and supernatants was quantified by scanning the bands of the Coomassie blue–stained gels. The amount of merlin sedimented in the absence of liposomes was subtracted from that sedimenting in the presence of lipid.

***In vitro* binding assay between GST-CD44 CTD and merlin in the presence of lipid micelles.** The glutathione beads binding to GST-CD44 CTD or GST alone were pretreated with radioimmunoprecipitation assay buffer [0.1% SDS, 0.5% deoxycholic acid, 1% NP40, 50 mmol/L Tris-HCl (pH 8.0), and 150 mmol/L NaCl] for 20 min at room temperature. They were washed with 20 vol of buffer containing 10 mmol/L of HEPES (pH 7.5), 1 mmol/L of $MgCl_2$, and 40 mmol/L of NaCl. After brief centrifugation, the beads were resuspended in 130 μL of buffer containing 50 μg/mL of phosphatidylserine and a variety of 5 μg/mL phosphatidylinositols. The mixture was sonicated, and the resulting vesicles were mixed with the glutathione beads and 2.6 μg of purified merlin. The reaction mixture was incubated at room temperature with constant rotation, followed by immunoblotting.

Wound-healing assays. RT4 schwannoma cells were stably transfected with inducible T230DS315D and T230AS315A mutant constructs. Three independent clones for each mutant were used for the migration assay. The cells were induced by doxycycline and split into 10-cm dishes and incubated until 90% to 100% confluent. After dishes were scratched by blue tip (0 h), cells were incubated for different time courses. The pictures were taken at 0, 6, 12, and 18 h, respectively. Phase contrast images were taken by Olympus IX71. Measurement of the wound-healing gap distance was performed using the computer program Image J. Results were expressed as mean ± SE. $P \leq 0.05$ was considered significant (Student's *t* test).

Results

The NH_2 terminus of merlin binds phosphatidylinositols. To examine whether phosphatidylinositol lipids are involved in binding to merlin, we performed *in vitro* binding assays. $PI(3,4,5)P_3$ selectively interacted with both NTD and full-length merlin, but not CTD or negative control GST. By contrast, $PI(4,5)P_2$ weakly associated with both NTD and CTD, but not full-length merlin (Fig. 1*A*). Truncation assay showed the extreme NH_2 terminal 1–82 and 1–132 fragments associated with $PI(3,4,5)P_3$; in contrast, all the other truncates lacking the NH_2 terminal 1–132 region failed to bind $PI(3,4,5)P_3$ (Fig. 1*B, top*).

As a measure for bilayer association, cosedimentation of proteins with liposomes containing 20% of a variety of phosphatidylinositol lipids and 80% phosphatidylcholine was analyzed. Control values of protein sedimenting in the absence of lipids were subtracted. Lipid interactions were measured under physiologic ionic strength conditions, as described in Materials and Methods. GST-merlin NTD strongly cosedimented with $PI(3,4,5)P_3$ liposomes (25 ± 5%). It revealed comparable affinity to both $PI(4,5)P_2$ and $PI(3,4)P_2$ with 18 ± 4% and 20 ± 3%, respectively. However, its affinity to PI(3)P was substantially decreased to 6 ± 2%. By contrast, GST-merlin CTD almost did not bind to 3′-phosphorylated phosphatidylinositol lipids, but it potently cosedimented with $PI(4,5)P_2$ (26 ± 6%). GST alone revealed negligible cosedimentation activity to all liposomes (Fig. 1*C*). Therefore, these findings support that merlin NTD possesses much stronger $PI(3,4,5)P_3$ binding affinity than its CTD, which, instead, reveals tighter affinity to $PI(4,5)P_2$.

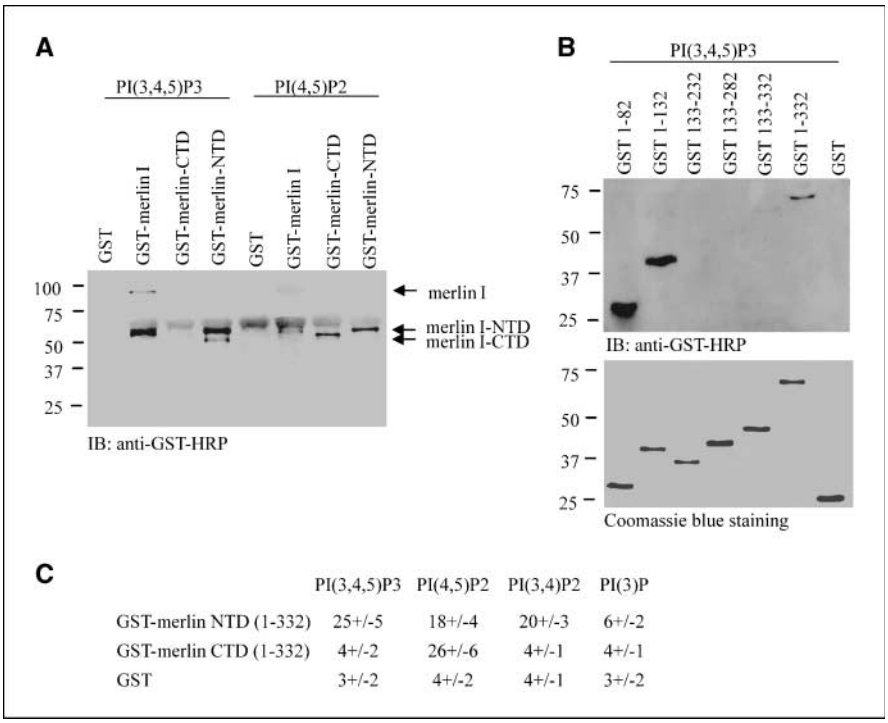

Figure 1. NH_2 terminus of merlin binds to phosphatidylinositol lipids. *A,* NTD of merlin directly associates phosphatidylinositol lipids. Purified GST and GST-merlin recombinant fusion proteins (5 μg each) were mixed with 30 μL of $PI(3,4,5)P_3$-conjugated or $PI(4,5)P_2$-conjugated beads for 3 h at 4°C, respectively. Lipid-protein complexes were separated by SDS-PAGE and analyzed by immunoblotting (*IB*) against GST. *B,* $PI(3,4,5)P_3$ binds to the extreme NH_2 terminus of merlin. *C,* quantitative analysis of cosedimentation of purified GST-merlin recombinant proteins with liposomes. The amount of protein present in the pellets and the supernatant was quantified by scanning the bands of the Coomassie blue–stained gels. The presented data stand for [protein in the pellets / total protein with liposomes (%)] − [proteins in the pellets / total proteins without liposomes (%)]. The amount of proteins in the pellets in the absence of liposomes was subtracted from that sedimenting in the presence of liposomes. Data are presented as mean ± SD of five independent experiments.

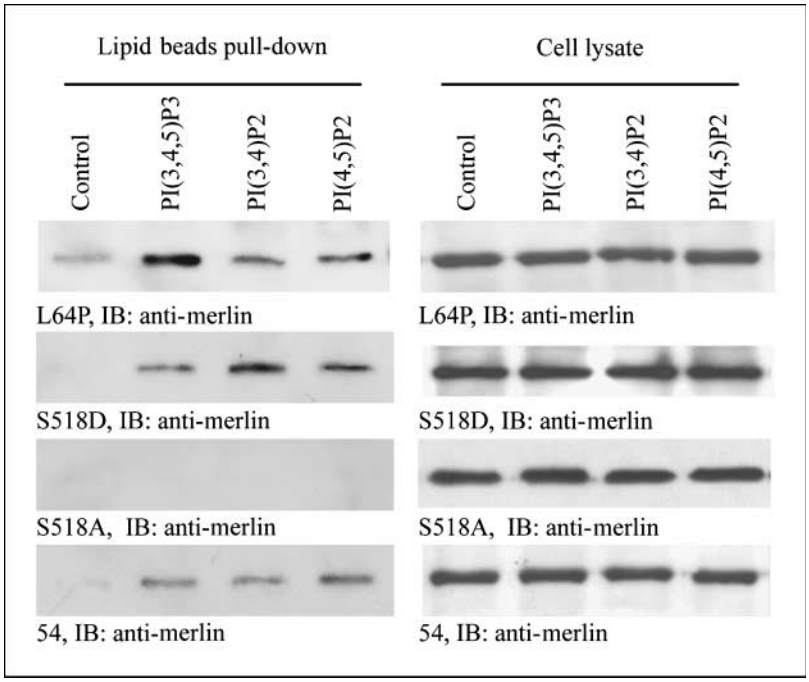

Figure 2. The unfolded conformation is required for the association between merlin and phosphatidylinositols. RT4 schwannoma cells transfected with various inducible merlin constructs, including wild-type (5_4), L64P, and S518 phosphorylation mutants. After merlin induction by doxycycline, the cell lysates (1 mg) were incubated with 30 μL of PI(4,5)P_2-conjugated, PI(3,4)P_2-conjugated, or PI(3,4,5)P_3-conjugated beads, and then proteins were separated by SDS-PAGE and analyzed by Western blotting (*left*). Equal amounts of cell lysates were used in the binding assay (*right*).

The unfolded conformation is required for the association between merlin and phosphatidylinositols. The NH_2 terminus of ezrin reveals more potent binding activity to PI(4,5)P_2 than full-length ezrin in the presence of physiologic ionic conditions, suggesting that folding conformation mediates its binding affinity to phosphatidylinositol lipids (11). To explore whether merlin folding conformation mediates its binding effect to phosphatidylinositol lipids, we used three forms of well-characterized merlin mutants: L64P, S518D, and S518A. The patient-derived L64P mutant displays an open inactive conformation, and so does S518D. However, S518A possesses a folded active conformation. We examined the binding activity in RT4-D6P2T schwannoma cell lines stably transfected with inducible wild-type and mutated merlins. The parental RT4-D6P2T schwannoma cell line expresses a negligible amount of merlin (7). Interestingly, no binding activity was observed with S518A. Surprisingly, S518D robustly associated with phosphatidylinositol lipids (Fig. 2, *left, second row*). Both wild-type and L64P merlin interacted with PI(4,5)P_2, PI(3,4)P_2, and PI(3,4,5)P_3. However, wild-type merlin in 5_4 cells displayed the strongest binding activity to PI(4,5)P_2. By contrast, L64P revealed the most prominent interaction with PI(3,4,5)P_3. These observations strongly support the notion that PI(3,4,5)P_3 or PI(3,4)P_2 robustly bind to open merlin whereas PI(4,5)P_2 preferentially interacts with native merlin.

Akt phosphorylation of merlin increases its affinity to phosphatidylinositols. To explore whether Akt phosphorylation of merlin mediates its binding activity to phosphatidylinositols, we conducted an *in vitro* binding assay. Under control conditions, the NTD strongly bound to PI(3,4,5)P_3, whereas full-length merlin barely associated with PI(3,4,5)P_3. In the presence of active Akt, both the NTD and full-length merlin displayed substantially elevated binding affinities to PI(3,4,5)P_3 (Fig. 3*A*). To examine the effect of Akt phosphorylation on merlin's binding behavior to phosphatidylinositols in intact cells, we infected 5_4 schwannoma cells with adenovirus expressing plasma membrane–localized active myristoylated Akt and short hairpin RNA of Akt1. Under control conditions (Fig. 3*B, bottom left*), wild-type merlin exhibited a binding effect to various phosphatidylinositols, with the strongest activity to PI(4,5)P_2, a phenomena similar to what was observed in Fig. 2*A*. However, when Akt1 was depleted, merlin lost its binding activity to both PI(3,4)P_2 and PI(3,4,5)P_3, but it still retained affinity to PI(4,5)P_2, indicating that Akt phosphorylation is not required for merlin to bind to PI(4,5)P_2 (Fig. 3*B, middle left*). In the presence of active Akt, merlin displayed obviously increased binding effect to both PI(3,4,5)P_3 and PI(3,4)P_2, whereas merlin remained comparable affinity to PI(4,5)P_2 as the control condition (Fig. 3*B*, *top left*), supporting that Akt phosphorylation provokes merlin binding to 3′-phosphorylated phosphatidylinositols. Both GSK3β and merlin S315 phosphorylation tightly correlated with Akt1 expression and activation profiles (Fig. 3*B, right*). Compared with wild-type NTD, purified recombinant GST-merlin 1–332 (T230DS315D) revealed much stronger binding activity to PI(3,4,5)P_3 (Fig. 3*C*). We have made similar observations with mammalian cells transfected with green fluorescent protein (GFP)–merlin. The phosphorylation mimetic merlin (T230DS315D) displayed markedly higher affinity to both PI(4,5)P_2 and PI(3,4,5)P_3 than wild-type merlin. By contrast, unphosphorylated merlin (T230AS315A) failed to bind either lipid (Fig. 3*D*), similar with S518A. These data further support that Akt phosphorylation is indispensable for merlin to interact with PI(3,4,5)P_3. Thus, Akt plays a critical role in dictating the binding of merlin to phosphatidylinositols.

PI(3,4,5)P_3 lipid enhances merlin binding affinity to CD44. To explore whether phosphatidylinositols regulate the interaction between merlin and CD44, we conducted an *in vitro* binding assay in the presence of phosphatidylserine or PIPx/phosphatidylserine micelles. Under the control condition (50 μg/mL phosphatidylserine), GST-CD44 CTD, but not GST, selectively interacted with merlin (Fig. 4*A, lanes 9* and *10*). Phosphatidylinositol did not significantly elevate the interaction between merlin and CD44 CTD, whereas PI(3,4)P_2 and PI(4,5)P_2 notably augmented the association (Fig. 4*A*). Remarkably, PI(3,4,5)P_3 elicited the strongest binding affinity between CD44 CTD and merlin (*lane 8*), indicating that

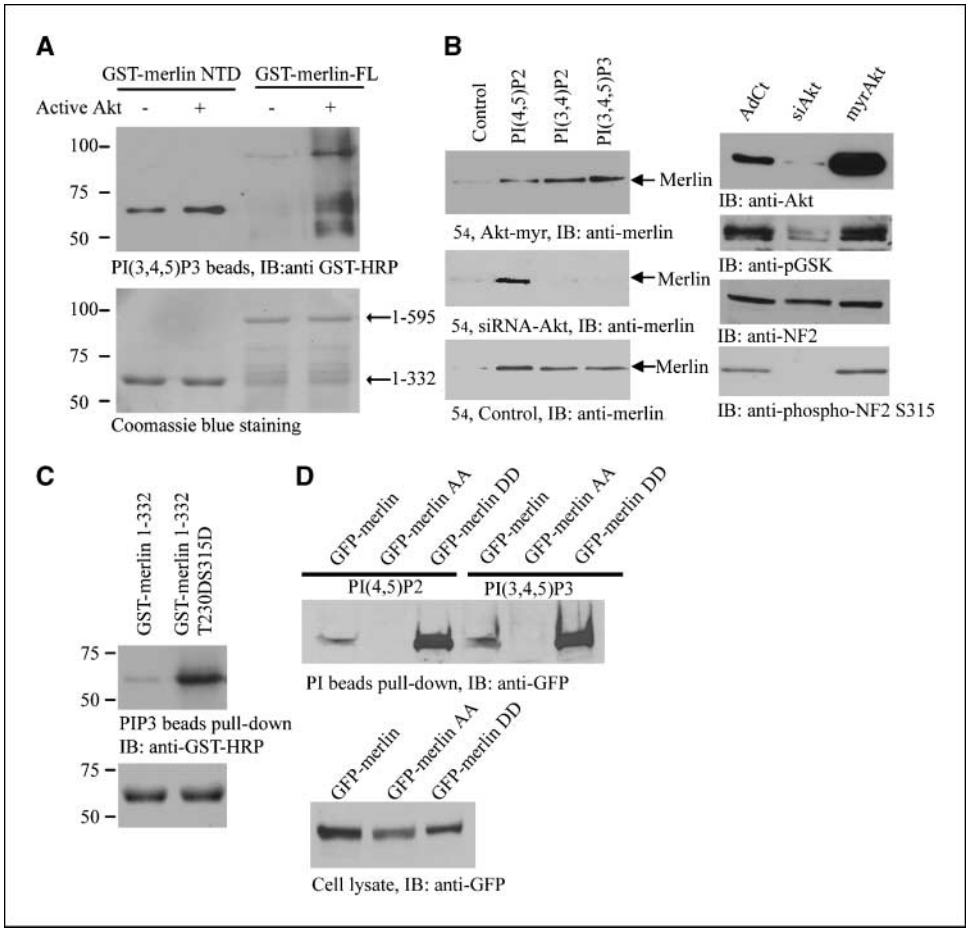

Figure 3. Phosphorylation of merlin by Akt enhances its lipid-binding activity. *A,* Akt enhances merlin-PIP3 interaction *in vitro*. *B,* Akt regulates merlin-PI(3,4,5)P_3 interaction. RT4 schwannoma cells were induced and infected adenovirus expressing control, siAkt, or myrAkt, and were incubated for 48 h. Cell lysates were mixed with phosphatidylinositol-conjugated beads and incubated for 3 h at 4°C. *C,* Akt phosphorylation of mimetic merlin mutant possesses stronger PI(3,4,5)P_3 binding activity. *D,* phosphorylation of mimetic mutant strongly interacts with PI(4,5)P_2 and PI(3,4,5)P_3.

phosphatidylinositol 3-kinase (PI3K) might up-regulate the merlin-CD44 association.

Inactivating mutations of PTEN tumor suppressor gene are found in a wide range of human cancers. PTEN is a lipid phosphatase that converts the second messenger PI(3,4,5)P_3 to PI(3,4)P_2 (12). To further test whether PI(3,4,5)P_3 mediates the interaction between CD44 and merlin in intact cells, we used the PTEN knockout HCT116 colon cell line, a previously described isogenic set of HCT116 cells in which PTEN genes had been deleted (13). We cultured the wild-type and PTEN-deleted cells at high density (100% confluence) and low density (50% confluence) and then immunoprecipitated merlin. The CD44-merlin association was substantially enhanced at the low-density culture compared with the high-density culture in wild-type HCT116 cells. Interestingly, significantly more CD44 was coprecipitated with merlin in highly confluent PTEN-null HCT116 cells than in the low-density cells (Fig. 4*B, left*). Akt phosphorylation was augmented in low-density HCT116 cells compared with high-density cells. As expected, Akt was highly phosphorylated in PTEN-null cells whether the cells were confluent or not. Accordingly, merlin S315 phosphorylation tightly correlated with Akt activation pattern (Fig. 4*B, right*). These data suggest that Akt phosphorylation and PI(3,4,5)P_3 lipid regulate the association between CD44 and merlin.

Because PTEN antagonizes PI3K signaling by hydrolyzing PI(3,4,5)P_3 back into PI(4,5)P_2, we wondered whether the elevated concentrations of PI(3,4,5)P_3 and hyperactive Akt in PTEN knockout cells would affect merlin's subcellular distribution. In wild-type HCT116 cells, endogenous merlin mainly localized in the cytoplasm and the cell-cell contact region. Epidermal growth factor (EGF) treatment relocalized merlin to the whole cytoplasm, and the concentration in the cell-cell contact was significantly decreased (Fig. 4*C*). This effect was substantially blocked by PI3K inhibitor wortmannin pretreatment (data not shown), indicating that Akt phosphorylation and PI(3,4,5)P_3 binding might contribute to the redistribution of merlin in the cytoplasm. However, in HCT116 PTEN-null cells, endogenous merlin was distributed in the whole cells, resembling the patterns observed in EGF-treated wild-type cells. It is worth noting that merlin was highly enriched in the microvilli and small protrusions (Fig. 4*D, top, white arrows*). By contrast, the actin filament was enriched in the cell boundaries. Strikingly, EGF provoked a tremendous number of small protrusions on the plasma membrane, where merlin and F-actin were aggregated and colocalized (Fig. 4*D, bottom, white arrows*). These results support that PI(3,4,5)P_3 binding and Akt phosphorylation synergistically facilitates merlin relocation and regulates its cellular effect on F-actin organization. Immunostaining with anti–phosphorylated S315 antibody revealed similar results (data not shown).

Akt phosphorylation of merlin inhibits its proapoptotic activity. Transduction of merlin into human schwannoma cells decreases cell proliferation by inducing apoptosis (14). In *Drosophila*, merlin and expanded, similar to other components of the hippo pathway, are required for proliferation arrest and apoptosis in developing imaginal discs (15). Thus, merlin is somehow implicated in triggering apoptosis. To explore whether

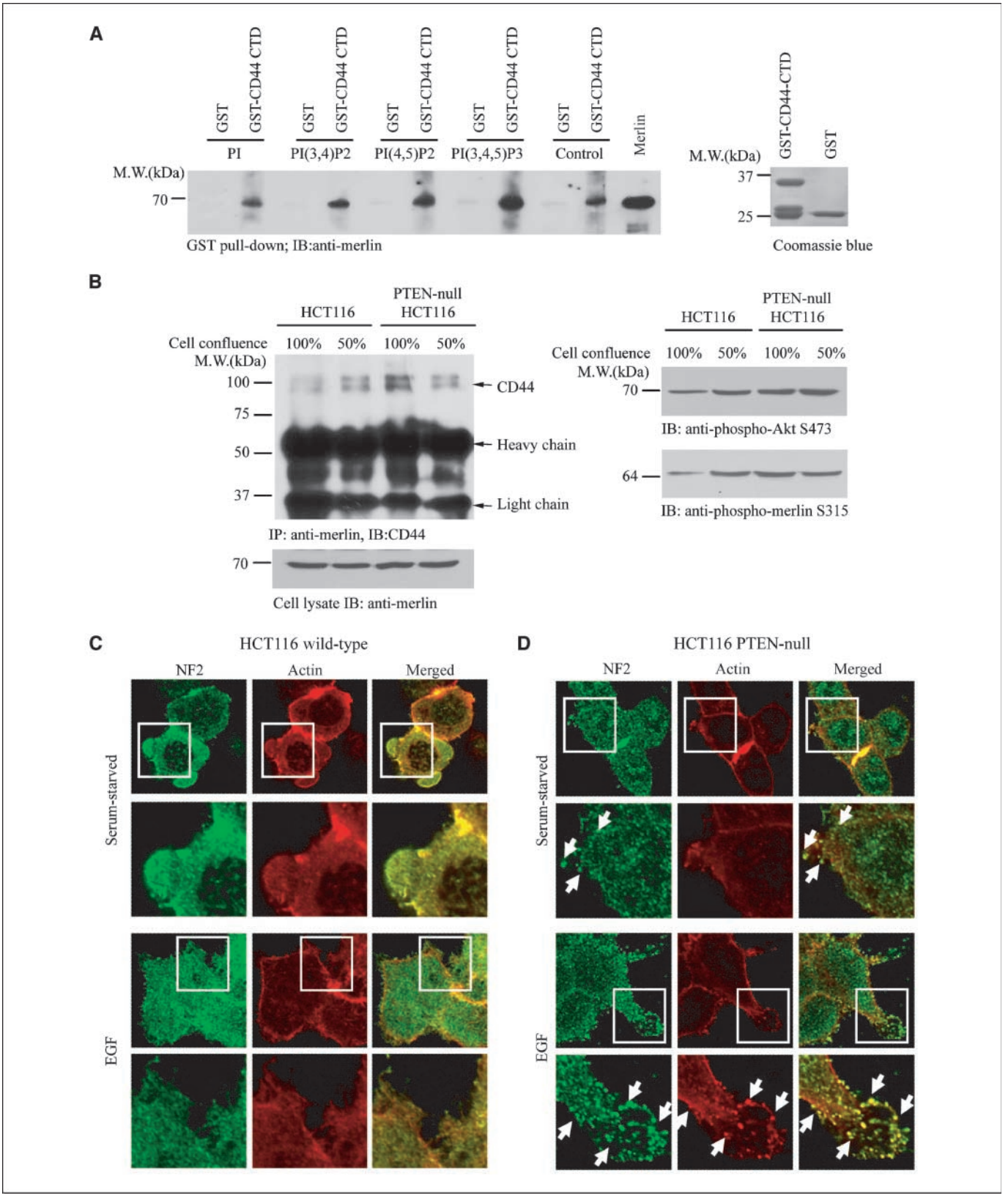

Figure 4. Akt phosphorylation and phosphatidylinositols enhance merlin binding to CD44. *A, in vitro* binding assay with GST-CD44 CTD. Glutathione beads–conjugated GST or GST-CD44 CTD were incubated with 2 μg of purified merlin in the presence of various vesicles containing phosphatidylserine, phosphatidylserine + PI, phosphatidylserine + PI(3,4)P_2, phosphatidylserine + PI(4,5)P_2, and phosphatidylserine + PI(3,4,5)P_3, respectively. The beads binding merlin were monitored by immunoblotting. *B,* Akt phosphorylation in PTEN-null cells up-regulates merlin-CD44 complex formation. *C* and *D,* EGF provokes merlin aggregation and colocalization with F-actin in PTEN-deficient HCT116 cells. In PTEN knockout cells, EGF triggered merlin to aggregate in the small protrusion, colocalizing with F-actin. *Arrows,* merlin accumulating in the dot-like structures (*D*).

the Akt phosphorylation of merlin mediates its proapoptotic effect, we cotransfected a variety of GFP-tagged merlin constructs into HEK293 cells with constitutively active Akt (Akt-CA) or kinase-dead Akt (Akt-KD) and monitored cell apoptosis. Compared with controls, overexpression of wild-type merlin provoked significant apoptosis, which was further elevated by unphosphorylated mutants (T230AS315A). By contrast, apoptosis was substantially decreased with the mutant (T230DS315D). Cotransfection of active Akt with wild-type merlin markedly diminished its proapoptotic actions. The apoptotic activity was recovered when merlin was cotransfected with Akt-KD (Fig. 5*A*). We also monitored cell death with trypan blue assay (Fig. 5*B*). Poly(ADP-ribose) polymerase (PARP) cleavage and caspase-3 activation were in alignment with the quantitative apoptotic results (Fig. 5*C*), underscoring that merlin phosphorylation by Akt is required to block its proapoptotic action.

The extreme NH_2 terminus of merlin is implicated in binding phosphatidylinositol lipids. It remains unknown whether this interaction is implicated in the proapoptotic action of merlin. Wild-type merlin and phosphorylated/mutated merlin, such as S518A and S518D, display different binding affinities to PI(3,4,5)P_3 and two different PIP_2. To address whether lipid binding by merlin plays any role in mediating its proapoptotic action, we monitored cell survival activity. When merlin is in open conformation, like S518D, the proapoptotic action of merlin was substantially diminished as in T230DS315D transfected cells; in contrast, when merlin is in close conformation, like S518A, it displayed more potent apoptotic action than wild-type merlin. Interestingly, truncation of the 1–133 region in merlin had no effect on merlin's proapoptotic action (Fig. 5*D*). PARP cleavage and caspase-3 activation correlated with apoptosis results (data not shown). Hence, PIP2 or PI(3,4,5)P_3 binding is not essential for the proapoptotic activity of merlin.

Merlin phosphorylation by Akt affects its subcellular distribution and cell migration. To explore whether Akt phosphorylation alone regulates merlin's subcellular residency, we transfected HEK293 cells with Myc-merlin constructs. T230DS315D mainly accumulated in the small protrusion structures on the

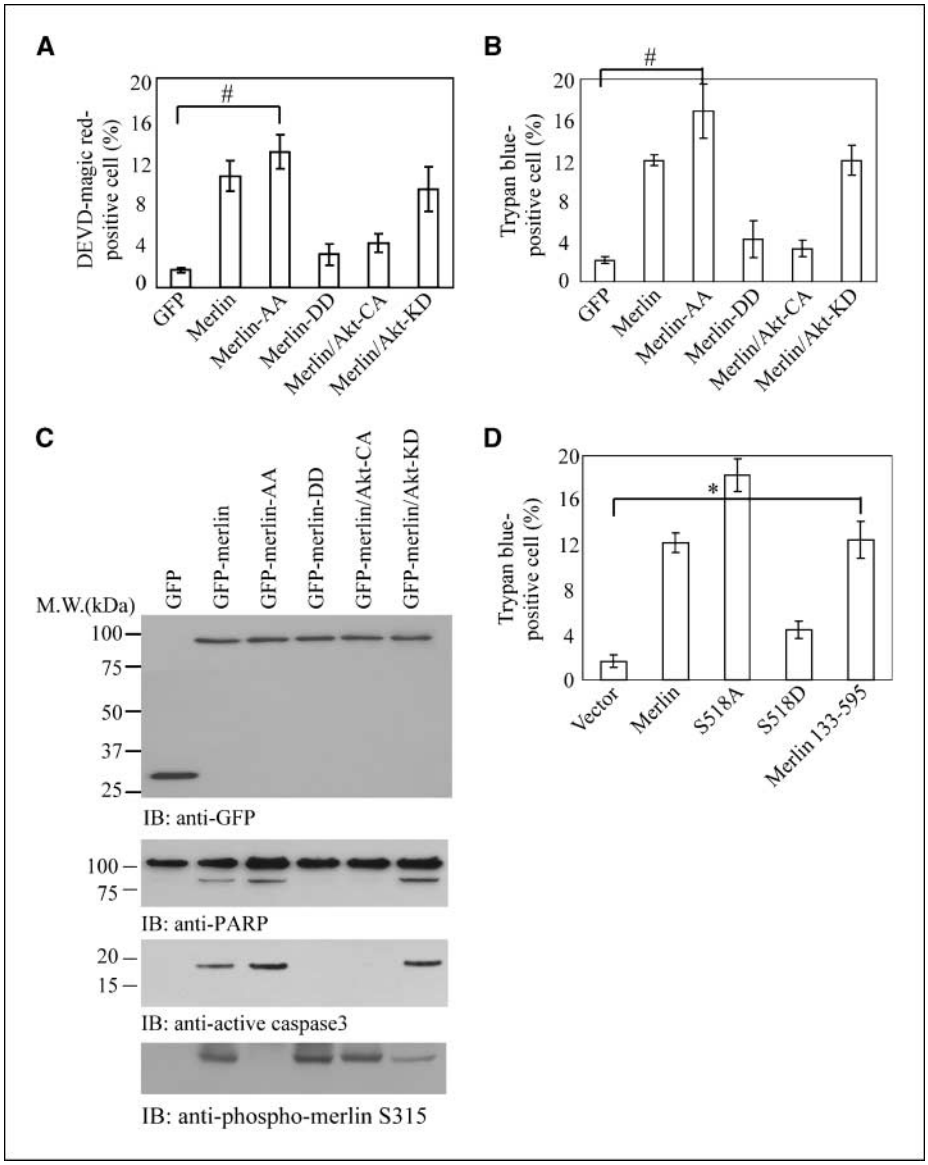

Figure 5. Akt phosphorylation of merlin blocks its proapoptotic activity. *A* and *B,* quantitative analysis of merlin-provoked apoptosis. The transfected cells were analyzed under fluorescent microscope for apoptosis. *Red,* apoptotic cells. *A,* in total, ~500 GFP-positive cells were calculated. *B,* trypan blue assay. *Columns,* means calculated from five determinations; *bars,* SE (#, $P < 0.05$). *C,* immunoblotting analysis. PARP was cleaved, and caspase-3 was activated in wild-type merlin and merlin (T230AS315A) cells. Merlin p-S315 was confirmed (*bottom*). Verification of transfected GFP-merlin constructs (*top*). *D,* phosphatidylinositol binding is not required for the proapoptotic activity of merlin. *Columns,* means; *bars,* SE (*, $P \leq 0.01$, Student's *t* test).

ruffled membrane, long cell extension, and cytoplasm in most of the transfected cells. Actin filaments strongly colocalized with merlin T230DS315D in the long cell extension (*white arrow*). In contrast, merlin mutants (T230AS315A) predominantly resided on the smooth plasma membrane without the aggregation in the protrusion (Fig. 6*A*). EGF also provoked Myc-merlin accumulation in the ruffled membrane, which was blocked by wortmannin (Fig. 6*B, top*), underscoring that Akt phosphorylation of merlin dictates its subcellular distribution. Immunostaining with anti–phosphorylated merlin S315 confirmed the observation (Fig. 6*B, middle*). To further test the notion that Akt phosphorylation regulates merlin aggregation in the ruffled plasma membrane, we cotransfected GFP-merlin into HEK293 cells with constitutive active Akt-CA and Akt-KD. GFP-merlin was strongly phosphorylated by Akt-CA, but not by Akt-KD, and accumulated on the disheveled plasma membrane (Fig. 6*C*). We made a similar observation in GFP-merlin and RFP-Akt cotransfected cells (Supplementary Fig. S1). Therefore, Akt phosphorylation plays an essential role in regulating merlin's subcellular distribution.

To explore whether Akt phosphorylation regulates the effect of merlin in cell motility, we conducted a wound-healing assay with RT4 schwannoma cells. The stable clones were transfected with inducible T230DS315D and T230AS315A mutants. Both cell lines transfected with wild-type merlin (5_4 and 6_7) loosely filled the wound. However, T230DS315D cells migrated faster and completely sealed the gap. Nevertheless, T230AS315A cells moved much slower than wild-type controls, and only about half of the gap was filled (Fig. 6*D, left*). These data support that Akt phosphorylation of merlin plays a critical role in mediating its effect on cell migration, fitting with our previous observation in Matrigel Boyden chamber (9).

Both Akt and PAK are implicated in phosphorylating merlin and regulate its binding to phosphatidylinositol lipids, but it remains unknown which signaling is essential for dictating the effect of merlin in cell migration. To quantitatively analyze this effect, we conducted a time course cell migration assay. In Supplementary Fig. S2, we show that merlin S518D cells migrated much faster than 5_4 cells. Blocking Akt by Akt1/2 or wortmannin inhibitor largely diminished both wild-type 5_4 and S518D cell migration; by contrast, inhibiting PAK3 markedly decreased wild-type, but not S518D, cell migration. This finding suggests that PI3K/Akt signaling is critical for schwannoma cell migration, for which merlin phosphorylation by Akt might be critical. On the other hand, S518 phosphorylation by PAK is also implicated in this event. Compared with merlin lacking V_1 schwannoma control cells, induction of wild-type merlin markedly decreased cell migration in 5_4 cells in a time-dependent manner. Notably, induction of S518A further inhibited cell migration. As expected, phosphorylation of S518 significantly abolished the inhibitory effect of merlin in cell migration, as the migratory speed was substantially increased in S518D cells (Fig. 6*D, right*).

Discussion

In this report, we show that merlin directly binds phosphatidylinositols, and this action is mediated by merlin folding conformation and Akt phosphorylation. Wild-type merlin reveals a stronger affinity to $PI(4,5)P_2$ than $PI(3,4,5)P_3$, whereas the patient-derived L64P, which possesses an unfolded conformation, exhibits a more potent affinity to $PI(3,4,5)P_3$ than $PI(4,5)P_2$. This observation was further supported by the finding with S518D, which also displays an open structure. By contrast, the folded S518A mutant fails to bind any of the phosphatidylinositols, underscoring that the open inactive conformation is required for merlin to bind $PI(3,4,5)P_3$. Moreover, we found that the interaction between merlin and $PI(3,4,5)P_3$ was evidently enhanced by Akt phosphorylation. Nevertheless, Akt phosphorylation is dispensable for merlin to associate with $PI(4,5)P_2$. Although binding to phosphatidylinositol lipids require merlin to maintain an inactive status, the functional relevance of this interaction remains obscure. Truncation of the 1–133 region in the NH_2 terminus, which is implicated in binding to $PI(3,4,5)P_3$, does not alter the proapoptotic action of merlin (Fig. 5*D*), suggesting that phosphatidylinositol binding to merlin is dispensable for this effect.

Previously, it has been reported that hypophosphorylated merlin, but not ezrin or moesin, binds the cytoplasmic tail of CD44 at high cell densities. At low cell densities, ezrin, moesin, and the phosphorylated form of merlin are associated with CD44, and this CD44-merlin association is likely to be indirect through ERM proteins (16). Consistently, we observed the increased interaction between CD44 and merlin in low-density HCT116 cells compared with high-density cells. Strikingly, the strongest CD44-merlin association occurred in high-density PTEN-null cells, which was slightly decreased when cell density was reduced (Fig. 5*B*). It remains unclear why, in PTEN-null cells, the association between CD44 and merlin was attenuated in low density compared with high density. Although the PI3K/Akt signaling cascade is markedly increased, when PTEN is depleted, other cell signalings that are dictated by cell/cell contact remain intact in PTEN-null cells. For instance, contact inhibition can be triggered by the addition of cell membrane preparations to dividing cells *in vitro* (17, 18). Adhesion molecules, including particular cadherins and integrins, induce cell cycle arrest upon contact with specific components of the extracellular matrix or with neighboring cells (19, 20). Presumably, these mechanisms operate in concert or in hierarchy to mediate cellular responses to contact with extracellular matrix and with other cells in addition to PI3K/Akt. This notion is indirectly supported by the cell migration observation that PI3K inhibitor wortmannin and Akt inhibitor Akt1/2 slightly but significantly decreased HCT116-PTEN cell migration; by contrast, PAK inhibitor exhibited a negligible effect (Supplementary Table S1). The weak inhibitory effect by Akt inhibitors might be due to the hyperactivity of Akt in PTEN-null cells.

To address the functional consequences of the enhanced binding affinity by Akt-phosphorylated merlin to CD44, we knocked down CD44 with its small interfering RNA in T230DS315D stably transfected cells, but we failed to observe any significant effect on cell migration compared with control small interfering RNA (data not shown). This finding indicates that the increased interaction between CD44 and Akt-phosphorylated merlin is not implicated in cell migration. This result is consistent with the previous finding that CD44 is not required for hyaluronan-induced vascular smooth muscle cell migration, which is dependent on PI3K-mediated Rac activation (21).

$PI(4,5)P_2$ strongly binds full-length wild-type merlin from schwannoma 5_4 cells (Fig. 3), although it fails to interact with the purified recombinant full-length merlin (Fig. 1*A*). This discrepancy might result from posttranslational modification of merlin in 5_4 cells. For example, phosphorylation of merlin might alter its folding conformation, unveiling the binding motif for $PI(4,5)P_2$ lipid. Unlike other ERM proteins, both the NTD and CTD of merlin bind to $PI(4,5)P_2$; however, $PI(3,4,5)P_3$ selectively associates with the NTD

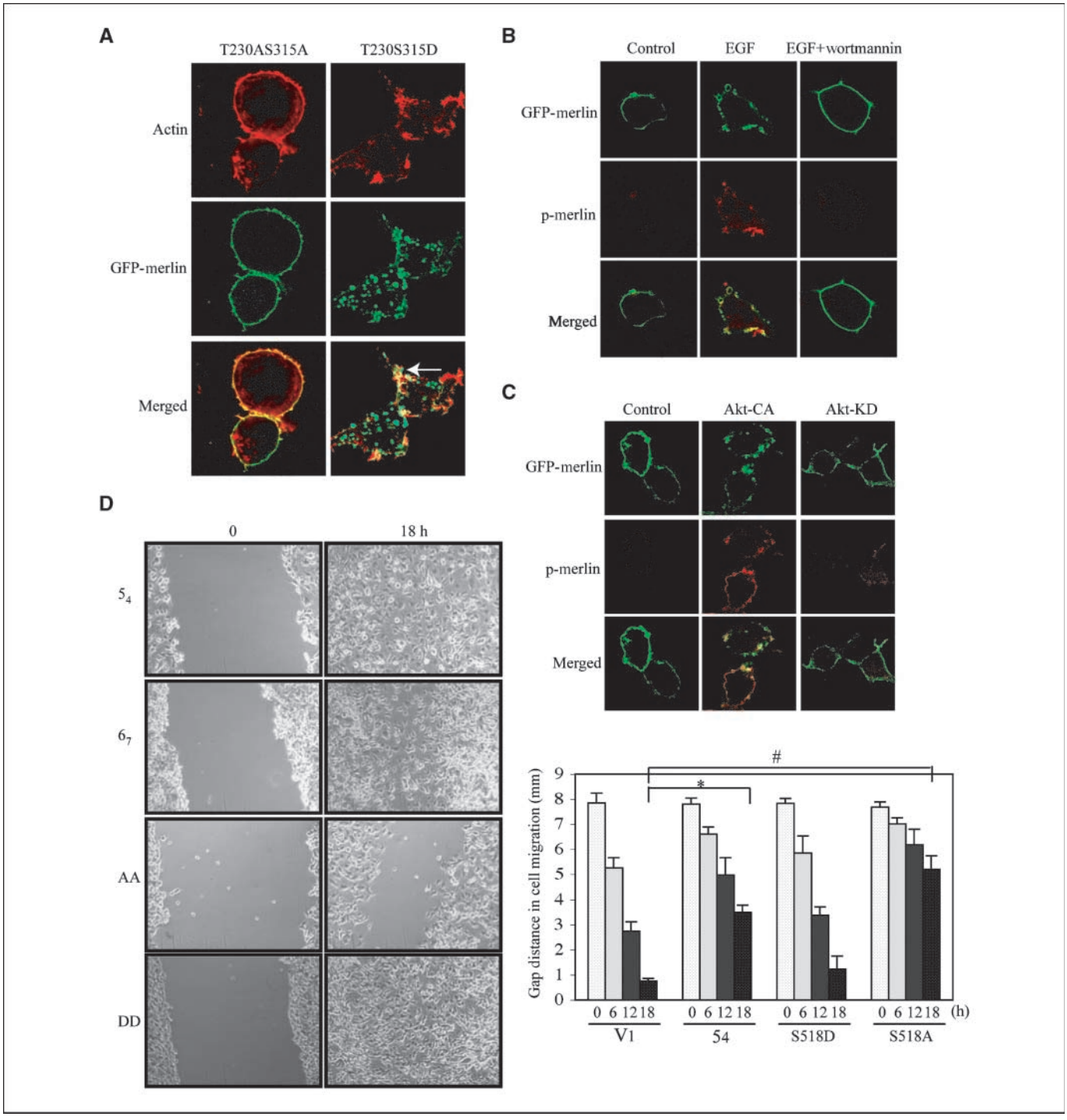

Figure 6. Merlin phosphorylation by Akt regulates its subcellular distribution and cell migration. *A,* Akt phosphorylation of mimetic mutant T230DS315D aggregated in the ruffled plasma membrane and relocated in the cytoplasm. HEK293 cells were transfected with T230AS315A and T230DS315D constructs, respectively. The transfected cells were stained with rhodamine-phalloidin. T230DS315D colocalized with F-actin in long cell extension (*arrow*). It was also distributed in the cytoplasm. *B,* EGF treatment provoked S315-phosphorylated merlin to accumulate on the disheveled plasma membrane. *C,* Akt-CA, but not Akt-KD, promoted merlin S315 phosphorylation and aggregation on the plasma membrane. *D,* wound-healing assay. RT-4 schwannoma cells stably transfected with inducible merlin wild-type (5_4 or 6_7), T230AS315A, or T230DS315D mutants were split in a 10-cm dish. Three independent clones for each mutant were used for the migration assay. One representative photo from three clones was presented (*left*). Quantitative analysis of the effect of merlin S518 phosphorylation in cell migration. Induction of wild-type merlin in 5_4 cells elicited a time-dependent inhibitory effect in cell migration (*right*). *Columns,* mean; *bars,* SE (*, $P \leq 0.01$; #, $P < 0.05$, Student's *t* test).

of merlin. Under subconfluent conditions, a portion of merlin might be phosphorylated by PAK on S518 in the CTD, leading to an unfolded conformation of merlin. $PI(4,5)P_2$ binds merlin and docks it to the plasma membrane. When growth factors are introduced, Akt is immediately activated and translocated to the plasma membrane, wherein its PH domain binds to newly generated $PI(3,4,5)P_3$ by PI3K, and the active Akt then attacks the NTD of merlin and phosphorylates it, resulting in a fully opened

conformation. PI(3,4,5)P_3 subsequently tightly binds to the NTD, which in turn, further enhances its phosphorylation by Akt and many other kinases, including PKA, PAK, etc., leading to the full inactivation of merlin.

Merlin directly associates with PIKE-L GTPase that binds PI3K and enhances its kinase activity (22, 23). Merlin exerts its growth-inhibitory effect, at least in part, by inhibiting PI3K/Akt signaling. This suppressive activity was achieved by blocking the stimulatory effect of PIKE-L on PI3K (23). Recently, we showed that Akt feeds back and robustly phosphorylates merlin on both T230 and S315 residues and unfolds merlin, leading to its polyubiquitination and degradation. Although either Akt or PAK phosphorylating merlin results in its binding to phosphatidylinositol lipids, merlin S518 phosphorylation by PAK cannot provoke merlin polyubiquitination and degradation (9). This finding suggests the unique aspect of the regulatory role of Akt in mediating merlin tumor suppressor. Nonetheless, it remains unclear whether phosphatidylinositol binding plays any role in triggering merlin degradation or plasma membrane residency. Here, we provide biochemical evidence revealing that PI(3,4,5)P_3, an essential second messenger generated by active PI3K, strongly binds Akt-phosphorylated merlin. Conceivably, this phosphatidylinositol lipid binding action might coordinately inactivate merlin with Akt. The dual regulation of merlin by phosphatidylinositol binding and Akt phosphorylation provides a novel mechanism explaining how the oncogenic PI3K signaling crosstalks with the tumor suppressor merlin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Akt phosphorylation regulates the tumour-suppressor merlin through ubiquitination and degradation

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The *neurofibromatosis-2* (*NF2*) tumour-suppressor gene encodes an intracellular membrane-associated protein, called merlin, whose growth-suppressive function is dependent on its ability to form interactions through its intramolecular amino-terminal domain (NTD) and carboxy-terminal domain (CTD)[1–3]. Merlin phosphorylation plays a critical part in dictating merlin NTD/CTD interactions as well as in controlling binding to its effector proteins[4–7]. Merlin is partially regulated by phosphorylation of Ser 518, such that hyperphosphorylated merlin is inactive and fails to form productive intramolecular and intermolecular interactions[8,9]. Here, we show that the protein kinase Akt directly binds to and phosphorylates merlin on residues Thr 230 and Ser 315, which abolishes merlin NTD/CTD interactions and binding to merlin's effector protein PIKE-L and other binding partners. Furthermore, Akt-mediated phosphorylation leads to merlin degradation by ubiquitination. These studies demonstrate that Akt-mediated merlin phosphorylation regulates the function of merlin in the absence of an inactivating mutation.

Analysis of the merlin protein sequence revealed that amino acids 225–231 (RNKKGTE) and 310–316 (RRKADSL) correspond to consensus Akt phosphorylation motifs previously identified in numerous Akt substrates. Using an *in vitro* kinase assay, we showed that both full-length merlin and its NTD only were robustly phosphorylated by active Akt. By contrast, other AGC kinase family members (the family that Akt belongs to), including $p70^{S6K}$ and $p90^{RSK}$, failed to phosphorylate merlin, indicating that Akt specifically phosphorylates merlin (Fig. 1a). Neither rapamycin (which inhibits the kinase mTOR) nor rapamycin in combination with PD98059 (a MAP-kinase kinase (MEK1) inhibitor) attenuated Akt S473 and merlin S315 phosphorylation, although they completely blocked $p70^{S6K}$ phosphorylation, consistent with our *in vitro* kinase results that showed that $p70^{S6K}$ failed to phosphorylate merlin (Supplemental Fig. S1).

To define the Akt-phosphorylated residues in merlin, we showed that the NTD (1–332) fragment was strongly phosphorylated, whereas the fragments 1–232 and 1–282 were only weakly phosphorylated. No phosphorylation was detected in other fragments (Fig. 1b, top panel), indicating that amino acids RNKKGTE (225–231) and RRKADSL (310–316) are the likely Akt phosphorylation sites. Phosphorylation of merlin containing either a T230A or S315A mutation was lower than with wild-type NTD, and was completely abolished in T230A/S315A double mutants, indicating that both the T230 and S315 residues are merlin Akt phosphorylation sites (Fig. 1c). Metabolic labelling with [^{32}P] H_3PO_4 reveals that phosphatidylinositol-3-OH kinase (PI(3)K inhibitors), but not a MEK1 inhibitor, significantly reduced merlin phosphorylation (Fig. 1d, top left panel). The T230A mutant had decreased merlin phosphorylation, and significantly lower phosphorylation took place in the S315A mutant. Moreover, epidermal growth factor (EGF)-induced phosphorylation was not detected in the T230A/S315A merlin mutant, demonstrating that both T230 and S315 are major phosphorylation sites. Furthermore, neither rapamycin nor rapamycin in combination with PD98059 decreased EGF-provoked merlin phosphorylation, indicating that other AGC family kinases are not implicated in phosphorylating merlin.

To determine whether merlin S315 was phosphorylated *in vivo*, we generated a merlin antibody that specifically recognized phosphor-S315. The immunoreactivity of this antibody was blocked by a phosphopeptide antigen, but not by non-phosphopeptide (Fig. 1d, right panels), confirming the specificity of the antibody. Immunoblotting with this antibody correlated with the metabolic labelling results (Fig. 1d, left, second panel). S315 was phosphorylated in wild-type merlin, and this phosphorylation was abrogated in the S315A mutant (Fig. 1e). Similarly, active Akt strongly stimulated merlin phosphorylation, whereas depletion of Akt abolished merlin phosphorylation (Fig. 1f). The reduction of merlin S315 phosphorylation in the T230A mutant under both serum-starvation and EGF-stimulation conditions suggests that T230 somehow affects S315 phosphorylation. Thus, T230/S315 phosphorylation is hierarchal (Fig. 1g). Taken together, our data support the idea that merlin is a physiological substrate of Akt in mammalian cells.

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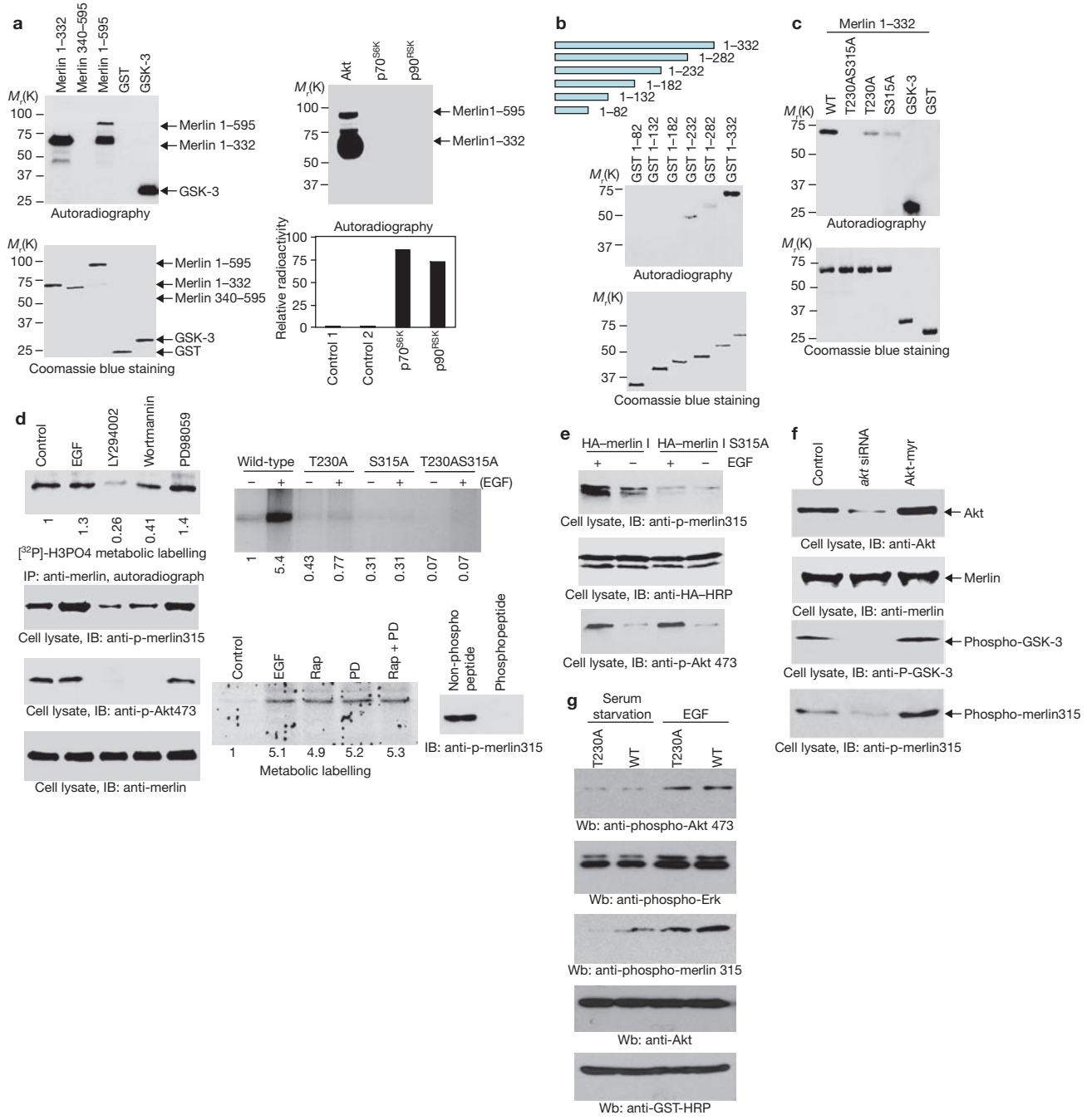

Figure 1 Merlin is a physiological substrate of Akt. (**a**) *In vitro* kinase assay with merlin recombinant proteins. The reaction mixture was resolved on 10% SDS–PAGE. Glycogen synthesis kinase (GSK) and GST alone were employed as positive and negative controls, respectively (upper left panel). Coomassie blue staining of purified GST proteins (lower left panel) is shown. $p70^{S6K}$ and $p90^{RSK}$ were unable to phosphorylate merlin. However, they robustly phosphorylated the peptide substrates (right panels). (**b**) *In vitro* Akt kinase assay with various N-terminal fragments of merlin. (**c**) T230 and S315 are Akt phosphorylation sites on merlin. T230A or S315A decreased the NTD phosphorylation, whereas phosphorylation was completely blocked in the double mutant T230A/S315A (upper panel). Coomassie blue staining of purified GST proteins (lower panel). (**d**) RT4-schwannoma cells (5_4), which expresses wild-type merlin, were cultured in regular medium and pretreated with 20 nM wortmannin, 10 μM LY294002, 10 μM PD98059 for 30 min, followed by metabolic labelling in $[^{32}P]$-H_3PO_4 for 4 h. Labelled cells were stimulated with 50 ng ml^{-1} EGF for 10 min. Merlin was immunoprecipitated and resolved on SDS–PAGE. PI(3)K inhibitors potently blocked merlin and Akt phosphorylation (left panels). The relative quantity of the signals is indicated below the gel. Mutation of T230 reduced EGF-initiated merlin metabolic labelling, whereas S315A mutation completely blocked EGF-provoked phosphorylation (right upper panel). Rapamycin by itself of in combination with PD98059 did not affect merlin phosphorylation (right lower panel) (see Supplementary Fig. S4 for a bigger image). Phosphopeptide antigen, but not the non-phosphopeptide, blocked merlin S315 phosphorylation (right lower panel). Merlin S315 and Akt phosphorylation and merlin expression were verified (left second to bottom panels). (**e**) EGF triggers merlin wild type but not S315A phosphorylation in intact cells. EGF enhanced S315 phosphorylation in wild-type merlin, which was abolished in S315A mutant transfected cells (top panel) (see Supplementary Fig. S4 for a bigger image). (**f**) Akt phosphorylates merlin in mammalian cells. Akt and merlin protein levels in cell lysates were confirmed (top and second panels). Positive control GSK-3 and merlin S315 phosphorylation were verified (third and bottom panels). (**g**) T230 affects S315 phosphorylation in merlin. WT, wild type.

GST pulldown assays revealed that both the NTD and the CTD fragements of merlin I (contains 1-595 residues) robustly associated with Akt, whereas the CTD of merlin II (contains 1-590 residues) had a low level of binding to Akt. Interestingly, neither full-length merlin I nor merlin II interacted with Akt (Fig. 2a, top panel). Because merlin I forms an intramolecular complex requiring NTD/CTD binding[1], we sought to determine whether EGF mediates the interaction between merlin and Akt. We employed the RT4-D6P2T rat schwannoma cells with doxycycline-controllable expression of either wild-type (WT; 5_4 and 6_7) or patient-derived (L64P) mutant merlin[6,10]. In the absence of EGF, endogenous Akt associated with L64P, which does not form a productive intramolecular complex, whereas negligible binding was observed with wild-type merlin. By contrast, EGF stimulated a robust interaction between wild-type merlin and Akt in both merlin-inducible (5_4 and 6_7) cell lines with an affinity similar to that of the L64P mutant, indicating that EGF facilitates merlin–Akt association, probably by activating Akt, which leads to merlin unfolding following Akt-mediated phosphorylation (Fig. 2b, top panel). Wild-type merlin phosphorylation status correlated with its association with Akt (Fig. 2b, bottom panel). Surprisingly, L64P was barely phosphorylated, although it abnormally interacted with Akt regardless of EGF stimulation. EGF-induced Akt–merlin complex formation was disrupted by PI(3)K inhibitors, but was not affected by an MEK inhibitor or PKC (protein kinase C) inhibitor (GF109203X), again suggesting that Akt activation is required for Akt–merlin association (Fig. 2c, top panel). In addition, merlin S315 phosphorylation status on the precipitated beads tightly correlated with its interaction with Akt (Fig. 2c, second panel). Interestingly, merlin S315 phosphorylation in Akt immunoprecipitation is stronger than that in cell lysates. Presumably, other kinases, which are enriched by the Akt antibody during immunoprecipitation, nonselectively provoked Akt-bound merlin phosphorylation. Constitutively active Akt (CA; T308D/S473D) strongly bound to merlin, whereas WT Akt weakly interacted with merlin. A kinase-dead (KD; K179A) Akt mutant failed to associate with merlin, indicating that Akt kinase activity is necessary for its association with merlin (Fig. 2d, top panel). Consistent with these observations, merlin was robustly phosphorylated by a CA and WT, but not kinase-dead, Akt mutants (Fig. 2d, bottom panel).

Recent studies have shown that the kinase mTOR and the mTOR–rictor complex (mTORC2) regulates Akt 473 phosphorylation, such that depletion of rictor reduces Akt phosphorylation in some cell lines[11,12]. Experiments using rictor knockout mice also support the idea that rictor is required for Akt 473 phosphorylation[13,14]. To further explore the effect of Akt activation on the association between endogenous Akt and merlin, we knocked down rictor and observed that Akt phosphorylation was completely abolished. Consequently, merlin S315 phosphorylation was totally blocked, and the association with Akt was also disrupted (Supplemental Fig. S2). Thus, Akt activation and subsequent merlin phosphorylation are essential for endogenous merlin binding to Akt.

Mapping experiments demonstrated that Akt fragments containing either the catalytic domain or the regulatory and at least half of the catalytic domain bound to merlin, whereas the pleckstrin homology (PH) and regulatory domains by themselves did not (Fig. 2e, left, second panel). These findings suggest that the catalytic domain of Akt is critical for binding to merlin. Presumably, merlin phosphorylation by endogenous Akt triggers its association with the exogenously transfected kinase-deficient Akt fragment #4 (half of the catalytic domain and the tail domain). To test this notion, we conducted a GST pulldown assay with Akt construct fragment #4. The binding of phosphorylation mimetic S315D merlin mutant to Akt fragment was much stronger than that of WT merlin, whereas the nonphosphorylatable S315A mutant failed to bind (Fig. 2e, right panels). Thus, these data support the idea that merlin phosphorylation is essential for the association.

Cell density regulates merlin phosphorylation and merlin growth-suppressor activity. To determine whether cell density also mediates merlin S315 phosphorylation, we analysed merlin S315 and Akt S473 phosphorylation in NIH3T3 cells at different cell densities using phosphospecific antibodies. We found that both Akt and merlin phosphorylation was significantly decreased as cell density increased (Fig. 2f). This inverse relationship between merlin S315 phosphorylation, Akt activity and cell density suggests that merlin phosphorylation by Akt takes place under physiological conditions. Moreover, the tight association between endogenous merlin and endogenous Akt gradually decreased as the cell density progressively increased (Fig. 2f, fifth panel). Hence, Akt forms a complex with merlin under physiological conditions, and Akt kinase activity and merlin phosphorylation by Akt are required for this action.

In the absence of Akt, the merlin CTD bound to the NTD, which was blocked in the presence of an active Akt molecule (Fig. 3a, top panel). The phosphorylation-mimetic GST–NTD-S315D mutant exhibited weaker affinity for merlin CTD than the non-phosphorylatable GST–NTD-S315A mutant did. Akt phosphorylation further decreased the binding to NTD-S315D, whereas it slightly affected the association with NTD-S315A. These observations suggest that Akt phosphorylation of the merlin NTD disrupts merlin NTD/CTD binding. Merlin normally migrates as a doublet containing both hyperphosphorylated and hypophosphorylated protein species. The merlin CTD I (340–595) bound to the hypophosphorylated merlin NTD more avidly than the CTD II (340-595). Merlin NTD/CTD binding was completely blocked by EGF stimulation, and this effect was abolished by wortmannin pretreatment (Fig. 3b, top panel). Therefore, this observation indicates that Akt-induced phosphorylation is essential for disrupting the intramolecular binding of merlin.

We next examined whether Akt phosphorylation also affected the association of merlin with several interacting proteins, including PIKE-L, CD44 and ezrin. In the absence of Akt phosphorylation, both full-length merlin and merlin NTD strongly interacted with PIKE-L, whereas the merlin CTD did not bind to PIKE-L, which is consistent with our previous findings[15]. By contrast, after Akt phosphorylation, full-length merlin weakly associated with PIKE-L, and the NTD failed to bind PIKE-L, indicating that Akt phosphorylation of residues within the NTD inhibits the association between merlin and PIKE-L (Fig. 3c). Compared with the untreated cells, EGF blocked CD44–merlin binding, whereas wortmannin increased CD44–merlin binding. PD98059 and GF109203X had no significant effects on CD44–merlin association (Fig. 3d, top panel). Akt-mediated merlin phosphorylation completely abolished its binding to ezrin as well (Fig. 3e, top panel). Collectively, these experiments demonstrate that Akt phosphorylation disrupts the ability of merlin to form both intramolecular and intermolecular associations that are important for its tumour-suppressor activity.

Merlin contains an F1 motif, similar to that in the protein FERM. The F1 motif in FERM has a ubiquitin-like structure[16] and facilitates MDM2 degradation and stimulates the ubiquitination and degradation of TRBP, a double-stranded RNA-binding protein[17,18]. To determine whether the

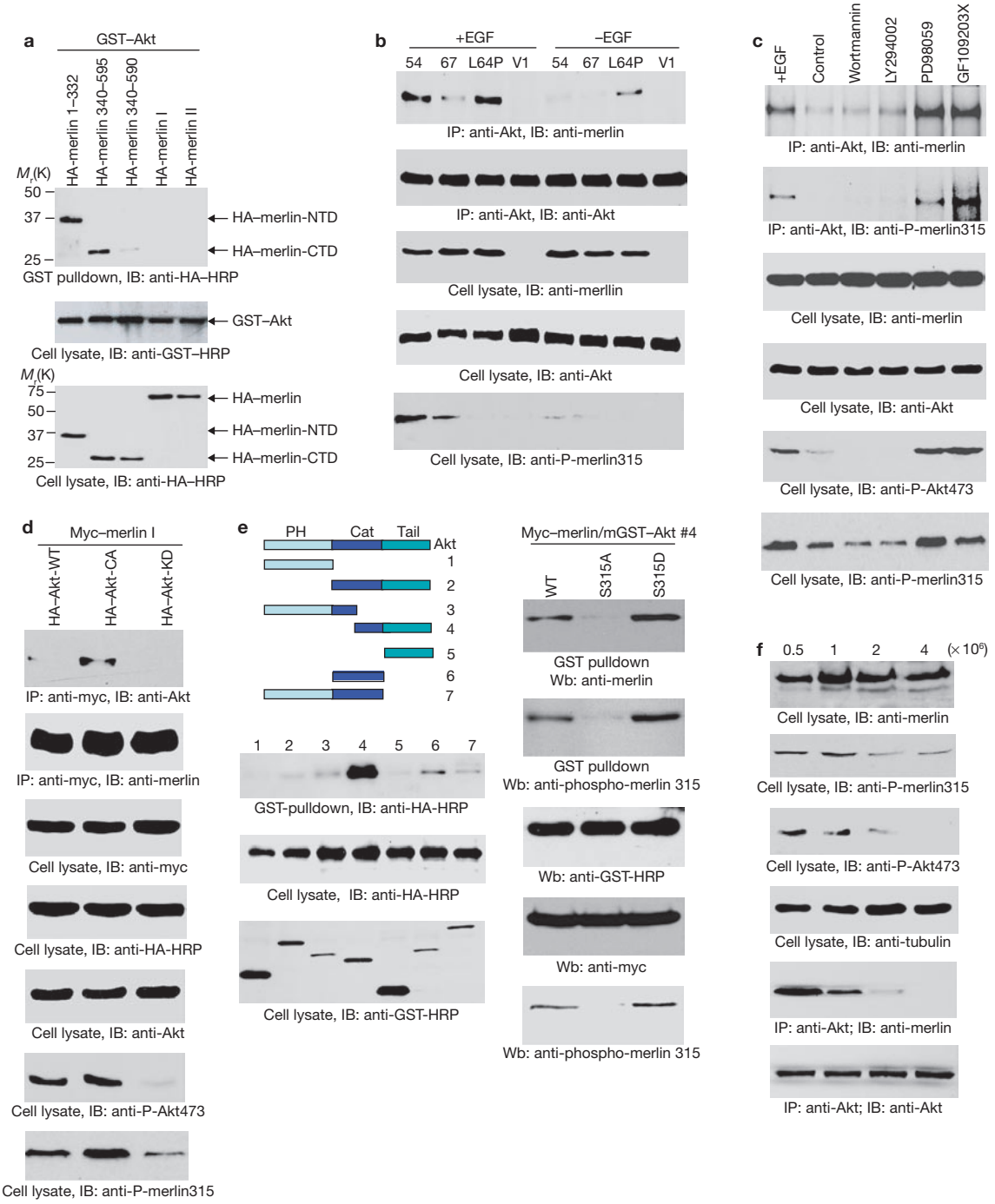

Figure 2 Merlin associates with Akt. (**a**) Both the NTD and CTD from merlin I, but not from merlin II, bind to Akt. The NTD and the merlin I CTD strongly bound to Akt, whereas the merlin II CTD weakly associated with Akt. By contrast, the full-length merlin I or II did not bind to Akt (top panel). Expression of the transfected constructs was verified (second and bottom panel). (**b**) EGF enhances the association between merlin and Akt. In the absence of EGF, only merlin (L64P) bound to Akt. In the presence of EGF, wild-type merlin (54 and 67) and L64P mutant robustly bound to Akt. No interaction was detected in control V1 cells (top panel) (see Supplementary Fig. S4 for a larger image). (**c**) PI(3)K inhibitors inhibit the interaction between merlin and Akt. PI(3)K inhibitors abolished the interaction between merlin and Akt, whereas it remained intact in the presence of PKC or MEK1 inhibitor (top panel). Coimmunoprecipitated merlin was also phosphorylated (second panel) (see Supplementary Fig. S4 for a larger image). (**d**) Akt kinase activity is required for its association with merlin. Akt WT and constitutively active (CA) Akt but not kinase-dead (KD) Akt robustly bound to merlin (top panel) (see Supplementary Fig. S4 for a larger image). (**e**) C-terminal regulatory and half catalytic (CAT) domain of Akt binds to merlin. Merlin phosphorylation status dictates its association with the Akt C-terminal regulatory and half catalytic domain. GST-Akt fragment 4 (#4) associated proteins were analysed with anti-merlin and anti-phospho-S315, respectively (right top and second panels) (Supplementary Fig. S4 for a larger image). PH, pleckstrin homology domain; Tail, regulatory domain. (**f**) Cell confluence dictates merlin and Akt phosphorylation and association. The phosphorylation of merlin S315 and Akt S473 decreased with increasing cell confluence (second and third panels). Equal amount of protein was loaded (top and fourth panels). The interaction between endogenous merlin and endogenous Akt in NIH3T3 cells was reversely coupled to cell density (fifth panel).

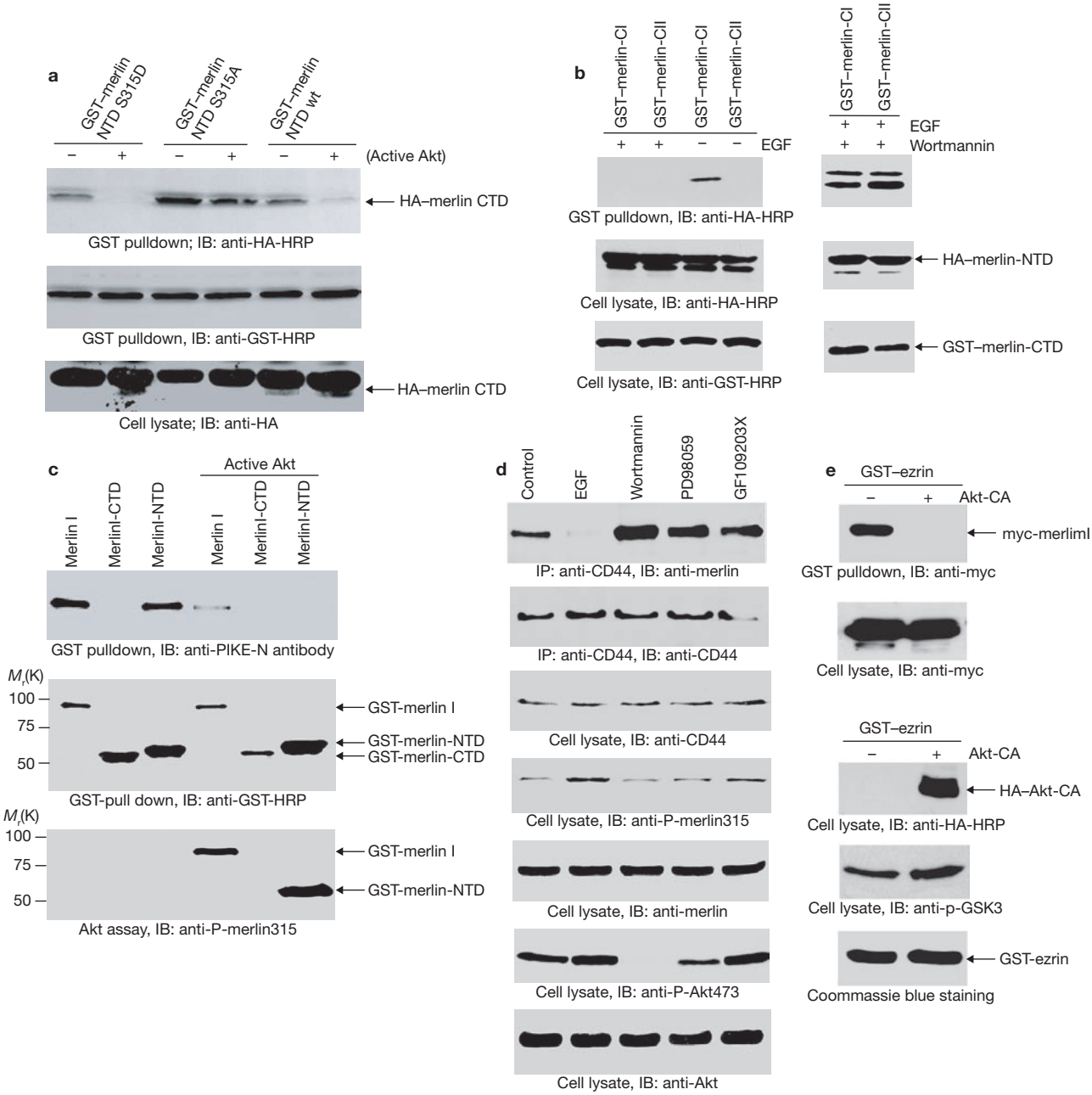

Figure 3 Akt phosphorylation disrupts the intramolecular and intermolecular binding of merlin. (**a**) Akt phosphorylation disrupts merlin NTD binding to CTD *in vitro*. S315D-NTD exhibited decreased binding affinity to merlin CTD compared with S315A, and Akt treatment further reduced the interaction (top panel). The expression of HA–merlin CTD and GST–merlin NTD was verified (second and third panels, respectively). (**b**) PI(3)K/Akt signalling mediates merlin NTD binding to its CTD. CTD I, but not CTD II (shown as GST-merlin-CI and -CII in the figure), bound to the NTD fragment, which was completely abrogated by EGF stimulation. In the presence of wortmannin, both CTD I and II robustly bound to the merlin NTD (left top panel). The expression of transfected proteins was confirmed (middle and bottom panels). (**c**) Akt phosphorylation decreases merlin binding to PIKE-L. Compared with controls, Akt phosphorylation of merlin reduced its interaction with PIKE-L (top panel) (see Supplementary Fig. S4 for a larger image). Coomassie blue staining of purified GST–merlin proteins is shown (middle panel). Full-length merlin and its NTD were potently phosphorylated by Akt (bottom panel). (**d**) PI(3)K/Akt signalling mediates merlin–CD44 interaction. EGF treatment abolished the interaction between CD44 and merlin, which was increased by wortmannin pretreatment (top panel) (see Supplementary Fig. S4 for a larger image). Merlin S315 and Akt S473 phosphorylation and corresponding protein levels were verified (second to bottom panels). (**e**) Akt phosphorylation decreases merlin binding to ezrin. Akt phosphorylation blocked the association between merlin and ezrin (upper left panel). The expression of the transfected proteins and Akt kinase activity were confirmed (lower left and right panels).

F1 motif in the merlin NTD is involved in ubiquitin-mediated degradation, we studied merlin degradation in high-grade human glioma cell lines that lack inactivating *NF2* mutations. We found that one cell line (U87MG) exhibited high levels of Akt activation and low levels of merlin (Fig. 4a, left panels). Treatment with a proteasome inhibitor (MG132) alone did not restore merlin expression in U87 cells. Interestingly, merlin S315 phosphorylation in U87MG was readily detectable and robustly enhanced upon MG132 treatment (Fig. 4a, third panels). A previous

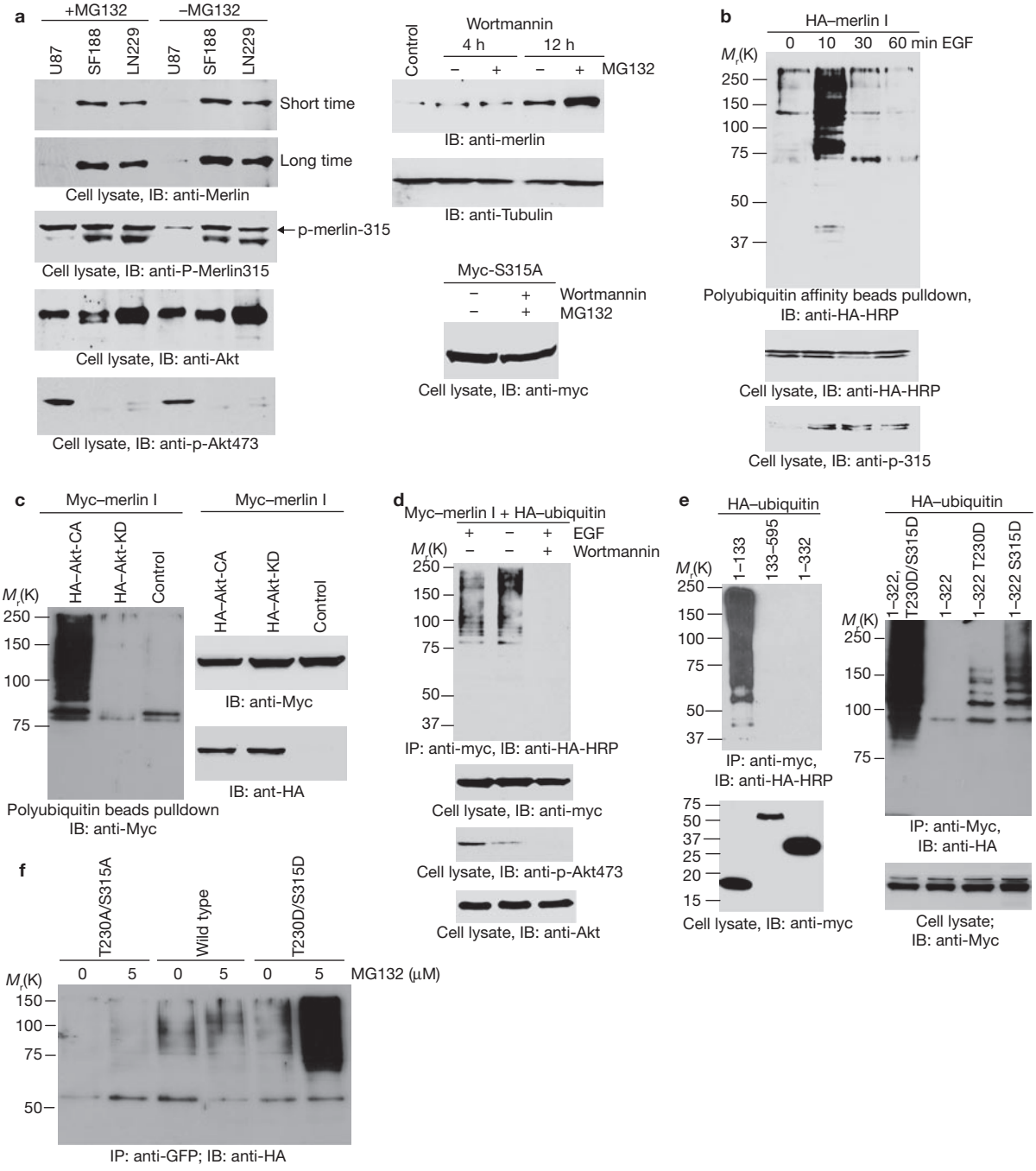

Figure 4 Akt mediates merlin degradation through ubiquitination. (**a**) Several high-grade glioma cell lines were treated with or without MG132 (20 μM) for 4 h. Merlin expression levels inversely correlated with the Akt phosphorylation status. However, MG132 did not significantly change merlin protein levels, but it readily upregulated merlin S315 phosphorylation in U87MG cells (left top, second and third panels). Compared with untreated controls, merlin expression was substantially increased after 12 h wortmannin treatment in U87MG cells (right top panel). Transfected S315A merlin expression level in HEK293 cells was not altered by wortmannin and MG132 treatment (right bottom panel). (**b**) EGF promotes merlin ubiquitination. After 10 min of EGF treatment, merlin was strongly ubiquitinated, which decreased at 30 min, and returned to baseline by 60 min (top panel). (**c**) Active but not kinase-dead (KD) Akt induces merlin ubiquitination. (**d**) Wortmannin pretreatment suppresses merlin ubiquitination. (**e**) The N terminus of Merlin is necessary for ubiquitination. The merlin fragment containing residues 1–133 was robustly ubiquitinated, whereas merlin fragments containing residues 133–595 and 1–332 fragments were not (upper left panel). The expression of the transfected merlin constructs was verified (lower left panel). T230D/S315D merlin NTD was robustly ubiquitinated (right panels). (**f**) MG132 upregulates ubiquitination of the phosphorylation-mimetic merlin mutant T230D/S315D. T230A/S315A merlin was not ubiquitinated. Compared with wild-type merlin, T230D/S315D was strongly ubiquitinated after MG132 treatment.

study demonstrates that wortmannin prevents Akt-induced phosphorylation and ubiquitination, increasing protein stability of p53[19]. As expected, wortmannin slightly enhanced merlin expression at 4 h and substantially elevated merlin level at 12 h. The additive effect was obvious with the combination of MG132 and wortmannin. By contrast, the treatment had no effect on the expression of transfected merlin S315A

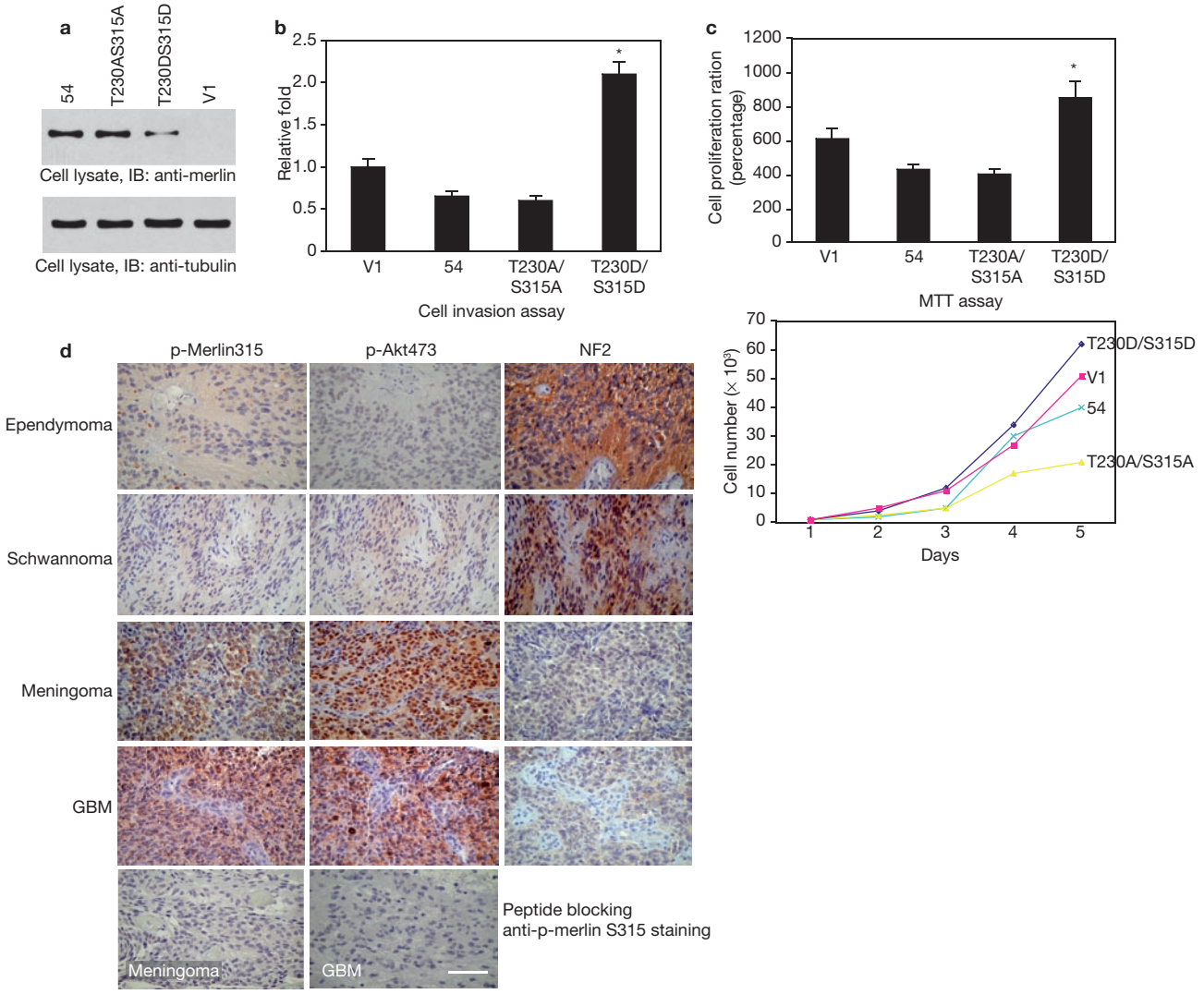

Figure 5 Akt phosphorylation of merlin increases its motility. (**a**) Expression of induced merlin wild-type and mutants in stable cells. (**b**) Merlin phosphorylation regulates its effect on cell invasion. Compared with control cells (V1), expression of the T230A/S315A merlin mutant reduced RT4 motility. The means ±s.d. for each condition are shown. The histogram summarizes the invaded cell numbers from three independent experiments (for V1, n=27,31,47; for S4, n=20,26,34; for T230A/S315A, n=14,21,29; for T230D/S315D, n=83,97,117). Asterisks denote statistical significance using student's t-test (P<0.05). (**c**) Merlin phosphorylation regulates its effect on cell proliferation. Compared with control cells (V1), expression of the T230A/S315A merlin mutant resulted in decreased RT4 cell growth as assayed by the MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay (left panel). The histogram summarizes the results from three independent experiments. Error bars indicate s.d. Direct cell counting revealed similar results (right panel). (**d**) Immunohistochemistry of human primary nervous-system tumours. Human ependymoma, schwannoma, meningioma and GBM tumours were stained with anti-phospho-S315, anti-phospho-Akt 473 and anti-merlin antibodies. Approximately 70% of the tumours showed either strong phospho-Akt immunoreactivity and low merlin expression or strong merlin immunoreactivity and negligible phospho-Akt/phospho-S315 staining. Phosphopeptide antigen blocked phospho-S315 staining on the patient samples (bottom panel). Scale bar represents 150 μm.

(Fig. 4a, right panels). Merlin was maximally ubiquitinated after 10 min in the presence of EGF (Fig. 4b). By contrast, merlin T230A/S315A was not ubiquitinated (data not shown). Transfection of CA Akt, but not KD Akt, induced evident merlin ubiquitination (Fig. 4c), indicating that Akt phosphorylation enhances merlin ubiquitination. Co-transfection of merlin with HA-tagged ubiquitin resulted in robust merlin ubiquitination regardless of EGF stimulation; ubiquitination was completely abolished by wortmannin pretreatment, suggesting that PI(3)K/Akt activity is required for merlin ubiquitination (Fig. 4d).

Using a series of merlin truncation mutants, we found that merlin NTD 1–133 residues are necessary for merlin ubiquitination; however, the NTD is not ubiquitinated (Fig. 4e, left panel). Nevertheless, we found that the NTD containing T230D or S315D was ubiquitinated, and the strongest ubiquitination took place on the T230D/S315D mutant (Fig. 4e, right panel), suggesting that merlin phosphorylation by Akt is necessary for its ubiquitination. Further, wild-type merlin was weakly ubiquitinated and the T230D/S315D mutant was strongly ubiquitinated, and ubiquitination of the mutant increased with MG132 treatment. By contrast, no polyubiquitination of the T230A/S315A mutant was detected regardless of MG132 treatment (Fig. 4f).

To determine the physiological relevance of phosphorylation of merlin on residues T230 and S315, we prepared stable and inducible RT4 rat schwannoma cell lines with nonphosphorylatable (T230A/S315A) and phosphorylation-mimetic (T230D/S315D) merlin mutants. Compared

with control cells (V1), induction of T230A/S315A merlin resulted in reduced cell proliferation and decreased motility. By contrast, the T230D/S315D mutant increased RT4 rat schwannoma cell growth and motility (Fig. 5b, c). We made similar observations with T230 and S315 single mutants. However, the effects of S315A and S315D mutants were stronger than those of the T230A and T230D mutants (Supplementary Fig. 3), suggesting that phosphorylation of merlin on both residues T230 and S315 abrogates merlin growth and motility suppression.

To determine whether merlin phosphorylation by Akt mediates merlin's degradation in primary tumours, we performed immunohistochemistry on a panel of human primary nervous-system tumours, including ependymoma (18 samples), schwannoma (18 samples), meningioma (15 samples) and glioblastoma multiformes (GBM; 28 samples). When Akt was activated (strong Akt-P immunoreactivity), total merlin expression was negligible. Conversely, when merlin immunoreactivity was strong, both merlin S315 and Akt S473 phosphorylation were modest. Phospho-S315 staining was completely blocked by the phospho-antigen, indicating that the staining was specific (Fig. 5d). Approximately 70% of the tumours exhibit one of these two staining patterns. Collectively, these data suggest that Akt phosphorylation-mediated merlin ubiquitination and degradation is also operative in primary tumours.

Akt binds to phosphorylated but not unphosphorylated merlin. It has been shown before that Akt can exist in a stable complex with several of its substrate proteins (for example, Mdm2, TSC2 and EDG-1) and can also interact with many proteins that do not serve as Akt substrates, but rather seem to play a modulatory part in Akt regulation[20]. Here, we provide compelling evidence supporting the idea that merlin is a physiological substrate of Akt, and that it also robustly associates with active Akt in a phosphorylation-dependent manner. This indicates that formation of this complex is tightly regulated by cell-proliferation signals. However, the possibility that merlin could be indirectly regulated by Akt can not be absolutely ruled out. Merlin might also act in a similar way to many other Akt binding partners to mediate Akt kinase activity. Our previous study shows that merlin suppresses PI(3)K/Akt signalling through inhibiting PIKE-L[15]. Presumably, it can directly downregulate Akt activity by physical interactions as well.

Merlin is key growth regulator in numerous cell types, and its activity is regulated by both cell density and phosphorylation events[5,6,8,9,15]. Whereas previous studies have shown that merlin binding to critical interacting proteins is abrogated by phosphorylation and that mutant forms of merlin can be degraded by a ubiquitin-mediated mechanism[21], the relationship between phosphorylation and ubiquitination of merlin levels has not been previously established. p21-activating kinase (PAK) or PKA mediates S518 phosphorylation[4,22]; however, we found no phosphorylation with the S518D mutant (data not shown), suggesting that Akt, but not PAK or PKA dependent, phosphorylation selectively elicits merlin ubiquitination. Spectrin binds to merlin[23–25], and spectrin has been reported to possess ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase (E3) activities[26]. Presumably, Akt-provoked ubiquitination of merlin is mediated through its binding partner, spectrin (also known as fodrin).

In addition to merlin loss in tumours, Akt-mediated merlin degradation provides another dynamic mechanism for controlling merlin expression under physiological conditions. Previous studies on another tumour-suppressor protein, neurofibromin, the protein product of the *neurofibromatosis-1* gene, revealed that rapid degradation of neurofibromin was observed after growth-factor stimulation[27]. Similar to our results on merlin, neurofibromin degradation was mediated by the ubiquitin–proteasome pathway. Our observations, coupled with those on neurofibromin, suggest a dynamic relationship between tumour-suppressor protein stability, growth-factor stimulation and phosphorylation. □

METHODS

Plasmids and reagents. GST-tagged merlin, merlin NTD (residues 1–332) and merlin CTD (residues 342–595) in pGex vector were kindly provided by Vijaya Ramesh (Molecular Neurogenetics Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA). Mouse monoclonal anti-HA–HRP, anti-Myc–HRP and anti-GST antibodies were from Sigma (St Louis, MO). Mouse monoclonal anti-Ser 473, and anti-Akt antibodies were from Cell Signaling. Rabbit polyclonal anti-merlin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A/G-conjugated agarose beads were from Calbiochem (San Diego, CA). Glutathione Sepharose 4B was supplied by Pharmacia Biotech (Piscataway, NJ).Purified active $p90^{RSK}$ and $p70^{S6K}$ assay kit were from Upstate Biotechnology (Waltham, MA). All the chemicals not included above were purchased from Sigma. The sense sequence for rictor lentivirus of shRNA is: 5′-CGGGCAGCCTTGAACTGTTTAACTTCCTgTCATTAAACAGTTCAAGGCTGCTTTTTG-3′.
The anti-sense sequence: 5′-AATTCAAAAAGCAGCCTTGAACTGTTTAATGACAGGAAGTTAAACAGTTCAAGGCTGC-3′.

Cell culture. The rat RT4-D6P2T schwannoma cells were stably transfected with empty vector, merlin (L64P) or wild-type merlin (5_4, 6_7 clones; gifts from Peter Herrlich, University of Karlsruhe, Karlsruhe, Germany). They were maintained in DMEM, supplemented with 10% fetal bovine serum (FBS), 2 mg ml^{-1} glutamine, 100 units penicillin–streptomycin, 500 μg ml^{-1} G-418, 1 μg ml^{-1} puromycin at 37 °C with 5% CO_2 atmosphere in a humidified incubator. Protein expression was induced by 1 μg ml^{-1} doxycycline for 24 h incubation.

$P70^{S6K}$ and $p90^{RSK}$ kinase assay. For the $p70^{S6K}$ kinase assay, we used the S6 kinase kit from Upstate Biotechnology (Temecula, CA), and employed S6 kinase/Rsk2 substrate peptide (KKRNRTLTK) as a substrate. The reaction mixture were incubated in 1× kinase buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, 15 mM $MgCl_2$) with 100 μM of ATP, 10 μCi (0.37 MBq) [γ-^{32}P] ATP. For the $p90^{RSK}$ kinase assay, we used the long S6 peptide according to the manufacturer's protocol using purified active His–$p90^{RSK1}$ (Upstate Biotechnology). As the negative control, the peptide and reaction mixture were incubated in the absence of active protein kinase. The reaction was performed at 30 °C for 30 minutes and analysed on p81 phosphocellulose paper. The phosphorylated proteins were quantified on a liquid scintillation counter.

Coimmunoprecipitation, *in vitro* binding assay and Akt kinase assay. The experimental procedures for coimmunoprecipitation, *in vitro* binding assay and Akt kinase assay have been previously described[10,28]. Akt was immunoprecipitated with agarose beads covalently linked to an anti-Akt antibody.

Ubiquitinated protein pulldown. Polyubiquitin affinity beads, in which the ubiquitin-associated domain of Rad23 was immobilized, and control beads were purchased from Calbiochem–Novabiochem (San Diego, CA). Whole-cell extracts from HEK293 cells were obtained using lysis buffer consisting of 50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100 and complete protease inhibitor (Roche Applied Science, Indianapolis, IN). 1 mg of each whole-cell extract was incubated with 40 μl of beads in lysis buffer for 4 h at 4 °C, with constant mixing. The beads were then washed three times with the same buffer, and the bound proteins were extracted in SDS buffer and analysed by western blotting using anti-NF2 or anti-HA–HRP antibodies.

Cell motility assay. Cell motility was determined in Transwell (Corning, Wilkes-Barre, Pennsylvania, chambers containing 8 μm membranes. Briefly, the bottom surface of the membrane was coated with Matrigel (Fisher Scientific, Houston, TX) and 10,000 cells, grown for 24 h either in the presence or absence of doxycycline, were seeded on the outside of the chamber and allowed to attach for 1 h. Cells were gently washed and then the Transwell chambers were inverted for 48 h

at 37 °C to allow for migration. Cells were then fixed in cold methanol for 30 min before staining with a LeukoStat staining kit (Fisher Scientific) and counted visually. The number of migrating cells was counted in quadruplicate and the mean and s.d. were determined for each condition. Each experiment was repeated three times with identical results.

Generation of anti-phosphomerlin S315 specific antibodies. Synthesized S315 phosphorylated peptide (CRRRKAD-(PO_3)S-LEVQQMKAQAREEKARK-COOH) was conjugated to keyhole limpet hemocyanin (KLH) and injected into rabbits. Antibodies were affinity purified from serum using unphosphorylated peptide crosslinked to Affi-Gel 10 or Affi-Gel 15, according to the manufacturer's recommendations (BioRad, Hercules, CA).

Immunohistochemistry staining of human primary tumours. A panel of human primary ependymoma, schwannoma, meningioma and GBM tumour sections (10 μm thickness) were deparaffinized by standard techniques. The tissues were stained with anti-phospho-Akt 473, anti-phospho-Ser 315 and anti-merlin antibodies, respectively. The staining procedures were performed using the protocols recommended by the manufacturers (Cell Signaling, Danvers, MA).

Statistical analysis. The results were expressed as means±s.d. calculated from a specified number of determinations. A student's *t*-test was used to compare individual data points with control values.

Note: Supplementary Information is available on the Nature Cell Biology website.

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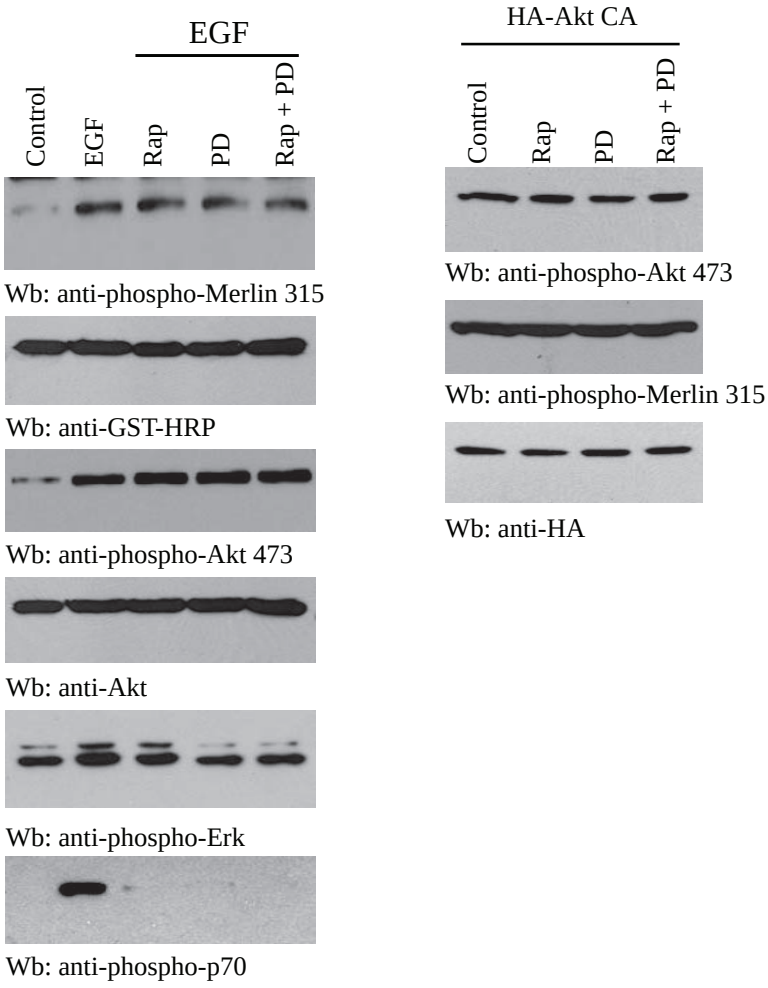


Figure S1. Rapamycin or rapamycin in combination with PD98059 does not inhibit merlin phosphorylation. HEK293 cells were transfected with or without HA-Akt-CA, and treated in the presence or absence of 100 nM rapamycin or rapamycin combined with PD98059 (10 μM) for 24 h, followed by 50 ng/ml EGF stimulation for 10 min. Cell lysates were analyzed by immunoblotting with anti-phospho-S315 (top left panel), phospho-Akt-473 (3rd left panel), phospho-P70^{S6K} (bottom left panel). Anti-p-Akt 473 and anti-p-S315 were not affected by rapamycin or rapamycin combined with PD98059 (right top and 2nd panels). Equal amount of HA-Akt-CA was transfected (bottom panel).

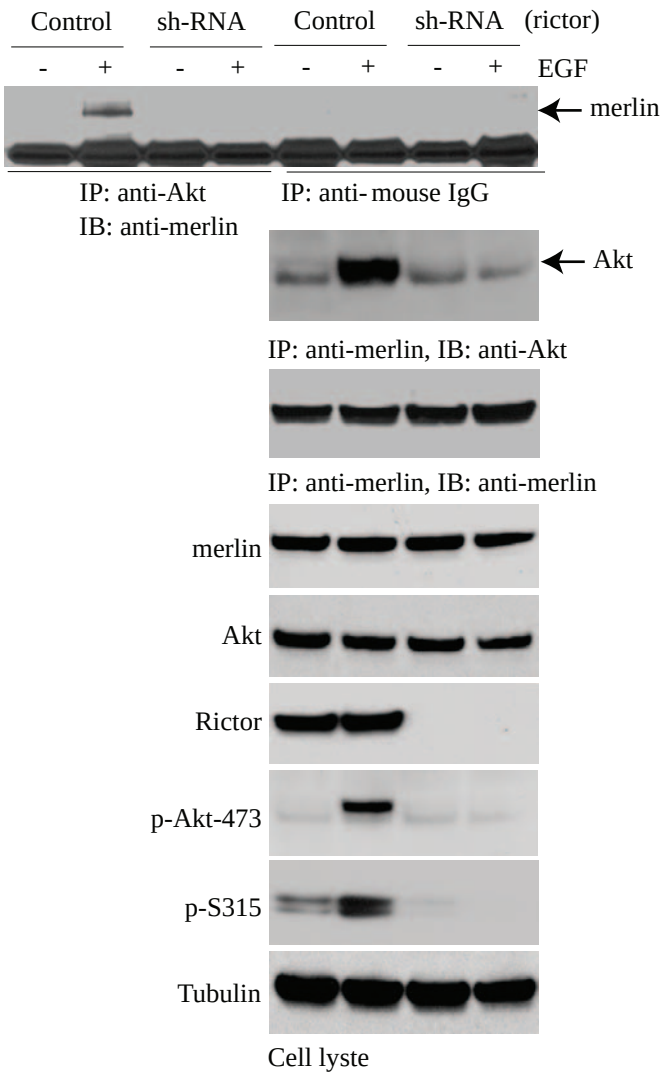


Figure S2. Depletion of rictor abolishes Akt and merlin phosphorylation and Akt-merlin binding. HEK293 cells were infected with control lentivirus or lentivirus containing rictor shRNAi. Infected cells were cultured in normal growth medium (10% FBS) for 48 h, followed by EGF stimulation for 10 min. Akt was immunoprecipitated and the binding proteins were analyzed by immunoblotting with anti-merlin antibody. Merlin association with Akt was abrogated after rictor knockdown (top and 2nd panels) (see SI-figure 4 for big image). Cell lysates were analyzed by immunoblotting with anti-rictor, anti-Akt, anti-phospho-Akt 473, anti-phospho-S315 and anti-tubulin antibodies, respectively (4th to bottom panel).

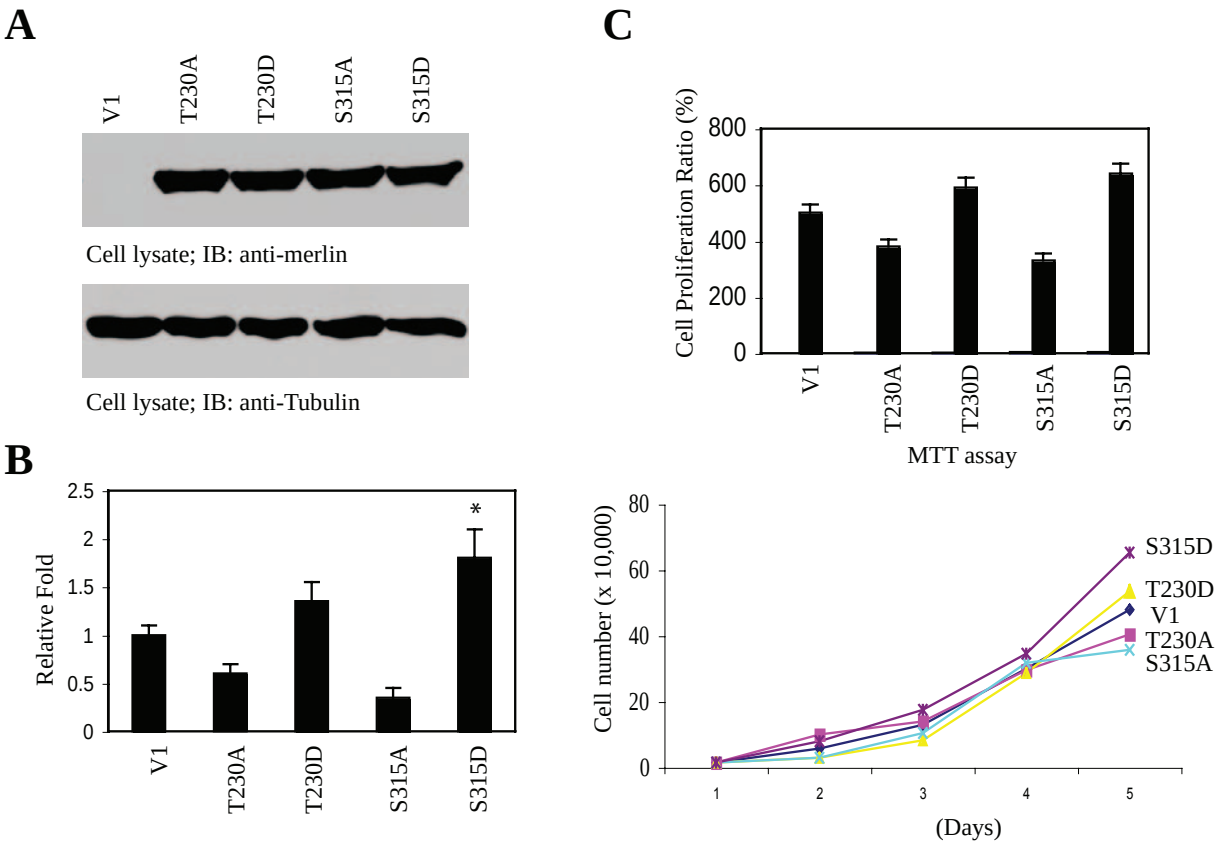


Figure S3. Merlin T230 or S315 phosphorylation regulates its tumor suppressive activity. **A,** Expression of induced merlin single point mutants in stable cells. **B,** Phosphorylation of merlin on T230 or S315 residue affects its effect on cell invasion. Compared to vector-containing cells (V1), expression of the T230A or S315A merlin mutant reduced RT4 motility. By contrast, T230D or S315D increased cell motility. The means and standard deviations (s.d.) for each condition are shown. Histogram summarizes invaded cell numbers from three independent experiments (n = 72-275). Asterisks denote statistical significance using Student's t-test ($P<0.05$). **C,** Phosphorylation of merlin on T230 or S315 residue affects its effect on cell proliferation. Compared to control cells (V1), expression of the T230A or S315A merlin mutant slightly decreased RT4 cell growth, whereas T230D and S315D increased cell proliferation as assayed by the MTT assay (upper panel). Histogram summarizes the results from three independent experiments. Error bars indicate standard derivation. Direct cell counting revealed similar results (lower panel).

Figure 1D right panels & 1E

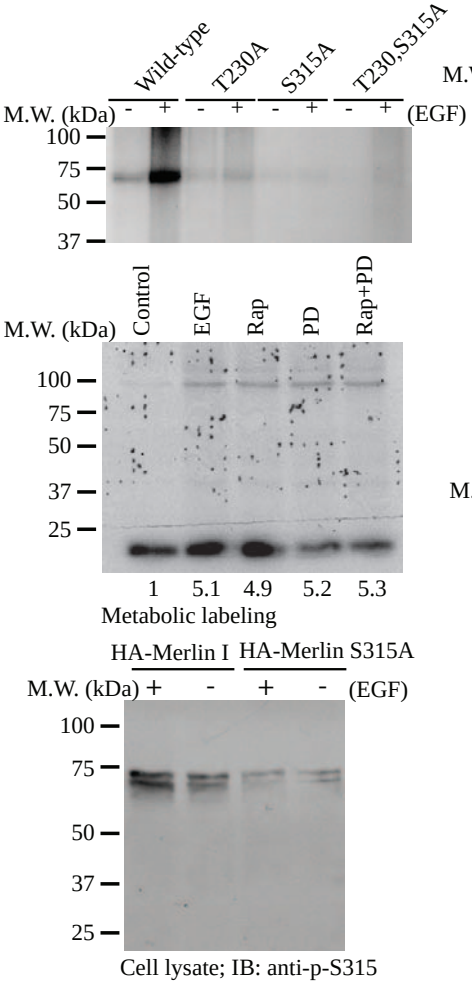


Figure 2B, C, D, E top panels

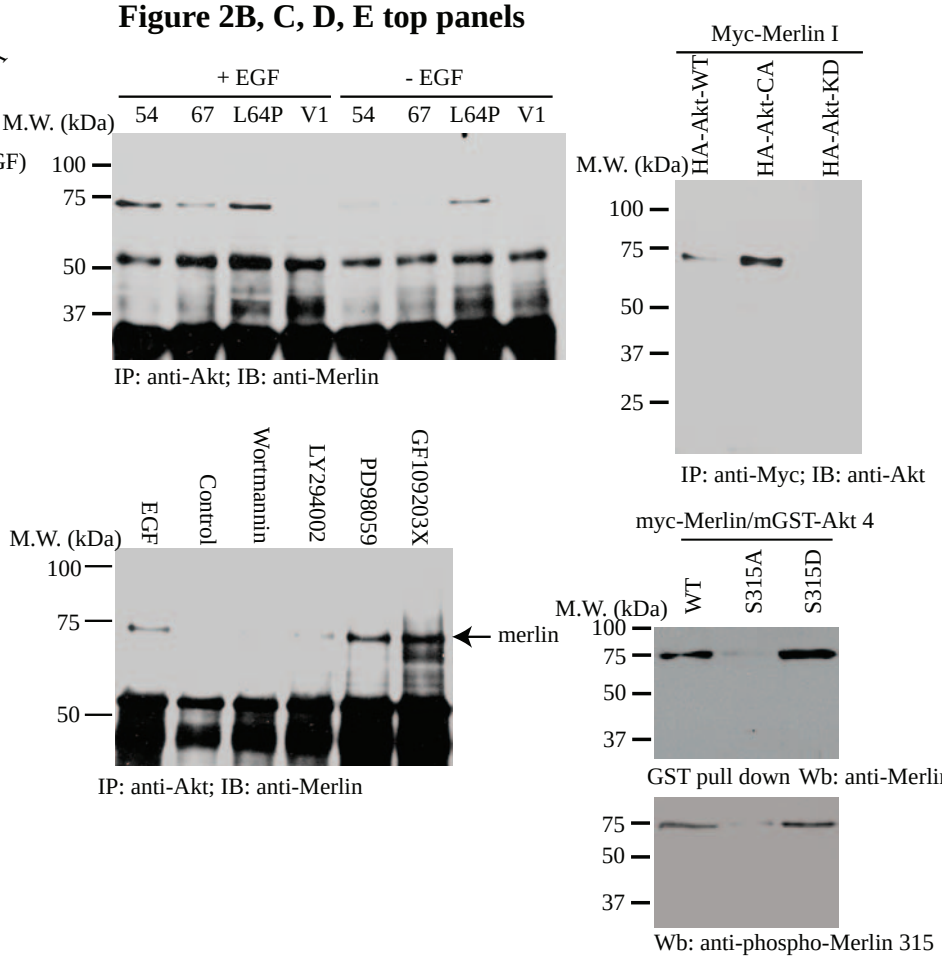
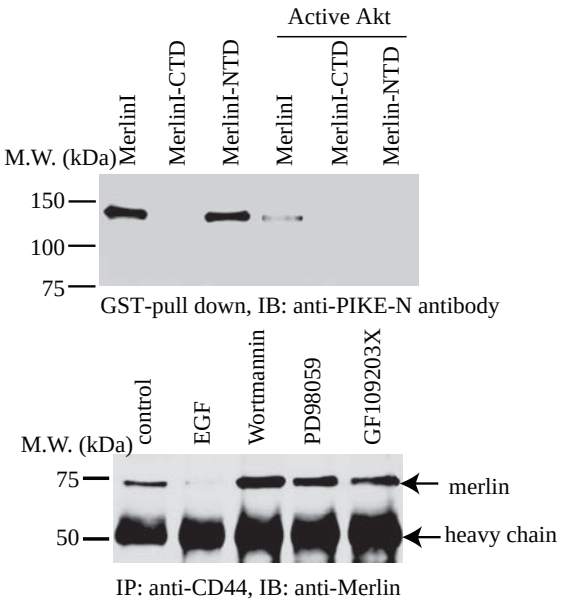


Figure 3C and D top panels



Supplemental Figure 2 (top and 2nd panels)

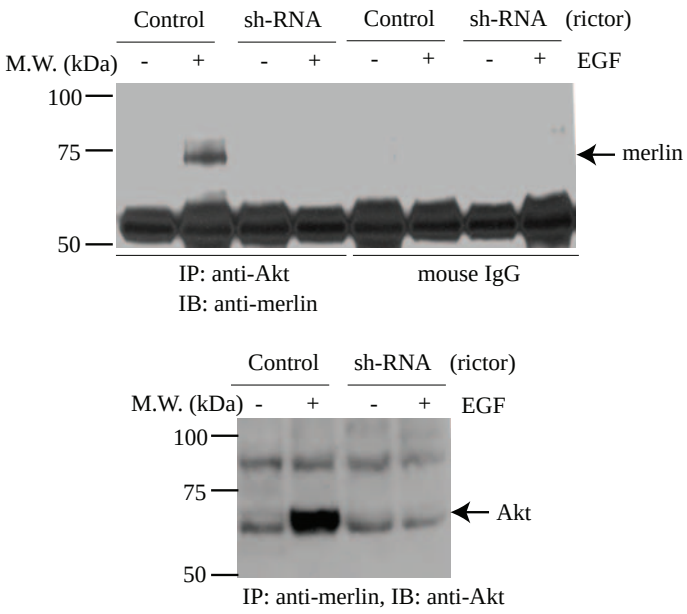


Figure S4. Full Scans