

# The *NF2* interactor, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), associates with merlin in the 'open' conformation and suppresses cell growth and motility

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**The neurofibromatosis 2 tumor suppressor protein, merlin or schwannomin, functions as a negative growth regulator; however, its mechanism of action is not known. In an effort to determine how merlin regulates cell growth, we analyzed a recently identified novel merlin interactor, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS). We demonstrate that regulated overexpression of HRS in rat schwannoma cells results in similar effects as overexpression of merlin, including growth inhibition, decreased motility and abnormalities in cell spreading. Previously, we showed that merlin forms an intramolecular association between the N- and C-termini and exists in 'open' and 'closed' conformations. Merlin interacts with HRS in the unfolded, or open, conformation. This HRS binding domain maps to merlin residues 453–557. Overexpression of C-terminal merlin has no effect on HRS function, arguing that merlin binding to HRS does not negatively regulate HRS growth suppressor activity. These results suggest the possibility that merlin and HRS may regulate cell growth in schwannoma cells through interacting pathways.**

## Introduction

Individuals affected with the inherited cancer predisposition syndrome, neurofibromatosis 2 (NF2), develop schwannomas and meningiomas with increased frequency (1). Because of this increased cancer risk, the *NF2* gene has been hypothesized to function as a tumor suppressor gene. Several observations support this idea. First, ependymomas, schwannomas and meningiomas from individuals with NF2 demonstrate homozygous inactivation of the *NF2* gene (2–5). In addition, nearly all sporadic schwannomas and 50–70% of sporadic meningiomas also demonstrate biallelic inactivation of the *NF2* gene, arguing that *NF2* functions as a critical growth regulator for these cells (6–8). Secondly, overexpression of

wild-type, but not mutant, *NF2* in schwannoma cell lines results in growth suppression (9–11). Thirdly, mice with a targeted mutation in the *Nf2* gene develop malignant tumors associated with inactivation of both copies of the *Nf2* gene (12).

The *NF2* gene was identified in 1993 by positional cloning, and was found to encode a protein termed schwannomin or merlin (13,14). Comparison of the predicted amino acid sequence of merlin demonstrated similarity with members of the Protein 4.1 family of protein and in particular three specific proteins, ezrin, radixin and moesin (ERM proteins) (15). Merlin is a 595 amino acid protein with three structural domains. The N-terminal domain (FERM domain), spanning residues 1–302, bears the greatest sequence conservation with the ERM proteins and is believed to mediate interactions with cell surface glycoproteins, like CD44 and intercellular adhesion molecules (ICAMs). The central domain (residues 303–478) contains a predicted  $\alpha$ -helix, which is also found in ERM proteins. The C-terminal domain of merlin (residues 479–595) is unique and lacks the conventional actin-binding motif found in ERM proteins.

ERM proteins have been shown to form both homo- and heterotypic interactions by virtue of head (N-terminal) to tail (C-terminal) associations (16–18). These inter- and intramolecular associations have been postulated to regulate ERM activity. Previously, we have shown that merlin forms two intramolecular associations (10,19). One such interaction involves residues in the N-terminal domain, while the second interaction requires binding of the C-terminus of merlin to N-terminal domain residues (20,21). We have shown that the ability of merlin to form these productive intramolecular associations is critical to its ability to function as a growth regulator (10,19). Failure to form these associations impairs the ability of merlin to inhibit cell proliferation and motility (9,10,22).

Studies from a number of laboratories have demonstrated that overexpression of wild-type merlin, but not merlin containing *NF2* missense mutations, can inhibit cell proliferation (9,10,23). In addition to cell growth regulation, merlin also regulates actin cytoskeleton-mediated functions, such as spreading, motility and attachment. Previous studies from our laboratory have demonstrated that regulated merlin over-

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expression dramatically reduces motility and disrupts the actin cytoskeleton during cell spreading (22). Antisense down-regulation of merlin also impairs cell attachment and increases cell proliferation (24). In addition, human schwannoma cells presumably lacking merlin expression have significant alterations in the actin cytoskeleton and in cell spreading (25), arguing that merlin may regulate intracellular pathways important for both growth and actin cytoskeleton processes.

In an effort to determine how merlin functions as a growth suppressor, several groups have employed yeast two-hybrid interaction cloning to identify novel merlin interactors (26–28). One such novel protein, hepatocyte growth factor (HGF)-regulated tyrosine kinase substrate (HRS) was recently identified (29). Since HGF is one of the most potent mitogens for Schwann cells and also promotes cell motility (30), we characterized HRS function with regard to the known properties of merlin. In this report, we demonstrate that HRS is a specific merlin interactor and that it associates with merlin via masked residues in the C-terminal domain of merlin. Moreover, we show that regulated HRS overexpression mimics the effect of merlin overexpression in rat schwannoma cells, suggesting that HRS is a good candidate for a merlin effector protein.

## RESULTS

### HRS uniquely binds to the C-terminus of merlin

Previously, we had demonstrated that HRS interacts with the C-terminus of merlin (29). In an effort to determine whether HRS is a specific merlin interacting protein, we performed *in vitro* glutathione *S*-transferase (GST) pull-down experiments to demonstrate that HRS does not interact with other ERM proteins. Whereas merlin binds to both full-length ezrin and radixin GST fusion proteins, we observed no binding of HRS to these two ERM proteins (Fig. 1A). Similar results were obtained with moesin (data not shown). To exclude the possibility that ERM intramolecular folding masked the ability of ezrin or radixin to bind HRS, we examined the binding of the C-termini of these proteins to associate with HRS *in vitro*. Using merlin–radixin hybrid proteins, we show that HRS only associates with the hybrid containing the C-terminus of merlin (radmer), but not the C-terminus of radixin (merad; Fig. 1B). Neither merad nor radmer can form intramolecular complexes, and they are unable to suppress schwannoma cell growth (9). The ability of merad to bind HRS suggests that the C-terminus of radixin lacks the residues required for HRS association, in contrast to radmer, which contains the C-terminus of merlin. Similar results were obtained with the C-terminus of ezrin (residues 284–585), which also fails to bind HRS (Fig. 1C). These results collectively suggest that HRS is a merlin-specific interacting protein and may be responsible for mediating merlin-specific functions.

To confirm that HRS binds to the C-terminus of merlin, we demonstrated that HRS associates with only the C-terminus (residues 299–595), but not the N-terminus (residues 1–302), of merlin (Fig. 1D). In addition, radmer contains merlin residues 341–595, thus narrowing the HRS binding domain to residues 341–579 of the full-length protein. This region is distinct from the binding domains reported for other merlin interacting proteins.

### Regulated overexpression of HRS impairs cell proliferation and anchorage-independent growth

Previous experiments in our laboratory utilized zinc-inducible merlin expressing RT4 cell lines (22). Doxycycline regulatable RT4 cell lines (rtTA RT4 rat schwannoma cell lines) have since been generated by Helen Morrison (Karlsruhe, Germany) (31,32). To determine whether regulated overexpression of HRS had similar effects as merlin overexpression, we generated several RT4 rtTA cell lines expressing full-length HRS under the control of a doxycycline regulatable promoter. Two clones (3 and 10) were chosen for further study. As shown in Figure 2A, increased HRS expression is observed within 4 h of doxycycline treatment and peaks at 24 h. We determined the level of HRS overexpression to be 5–7-fold for each of the cell lines by scanning densitometry on separate western blots using the HRS Ab-1080-2 polyclonal antibody (data not shown).

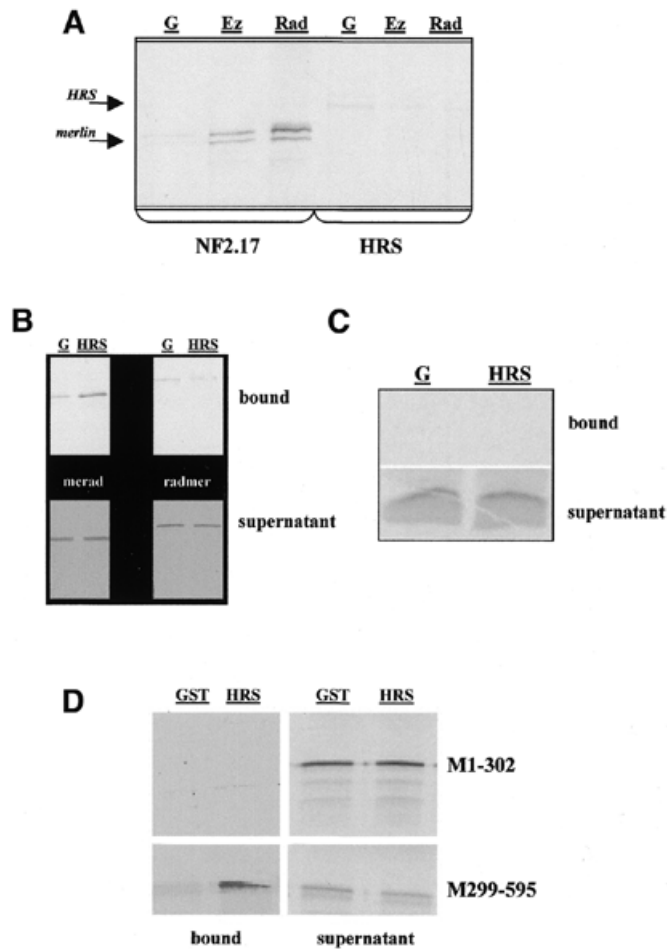
As we had reported previously for merlin, regulated overexpression of HRS resulted in significant decreases in cell proliferation as determined by thymidine incorporation (Fig. 2B). We routinely observed a 1.7–2.7-fold decrease in thymidine incorporation as a result of HRS overexpression. No changes in cell death were noted by Trypan blue exclusion, FACS analysis or the TUNEL method for assessing apoptosis (data not shown). In addition to decreased cell proliferation, we observed a 1.8–2.7-fold decrease in anchorage-independent colony formation in response to HRS overexpression (Fig. 2C). Lastly, HRS overexpression reduced RT4 cell growth in a colony formation assay (Fig. 2D). These results argue that regulated overexpression of HRS results in growth inhibition.

### Regulated overexpression of HRS results in abnormalities in cell spreading and motility

Previously, we had demonstrated that wild-type, but not mutant, merlin-regulated overexpression results in dramatic alterations in the actin cytoskeleton during cell attachment, as well as decreased cell motility (22). To determine whether HRS overexpression has similar effects on actin cytoskeleton-mediated processes, we assayed actin cytoskeleton organization upon cell attachment in RT4 cells induced to overexpress HRS. As shown in Figure 3A, doxycycline induction of HRS expression was associated with dramatic changes in the actin cytoskeleton as determined by phalloidin fluorescence cytochemistry. RT4 cells without HRS overexpression exhibit cortical actin rims at points of contact with the laminin substrate, whereas RT4 cells overexpressing HRS have a disorganized actin cytoskeleton and abnormal morphology. Similarly, HRS overexpression results in reduced cell motility assayed using a Boyden chamber as described in Materials and Methods (Fig. 3B). We routinely observed a 26–27% reduction in cell motility in this assay. The magnitude of the inhibition is similar to that observed for regulated merlin overexpression (data not shown).

### HRS binds to merlin in the open conformation

Previous work from our laboratory and others has demonstrated that merlin, like other ERM proteins, exists in ‘open’ and ‘closed’ conformations dictated by the ability of merlin to form an intramolecular association between the N- and



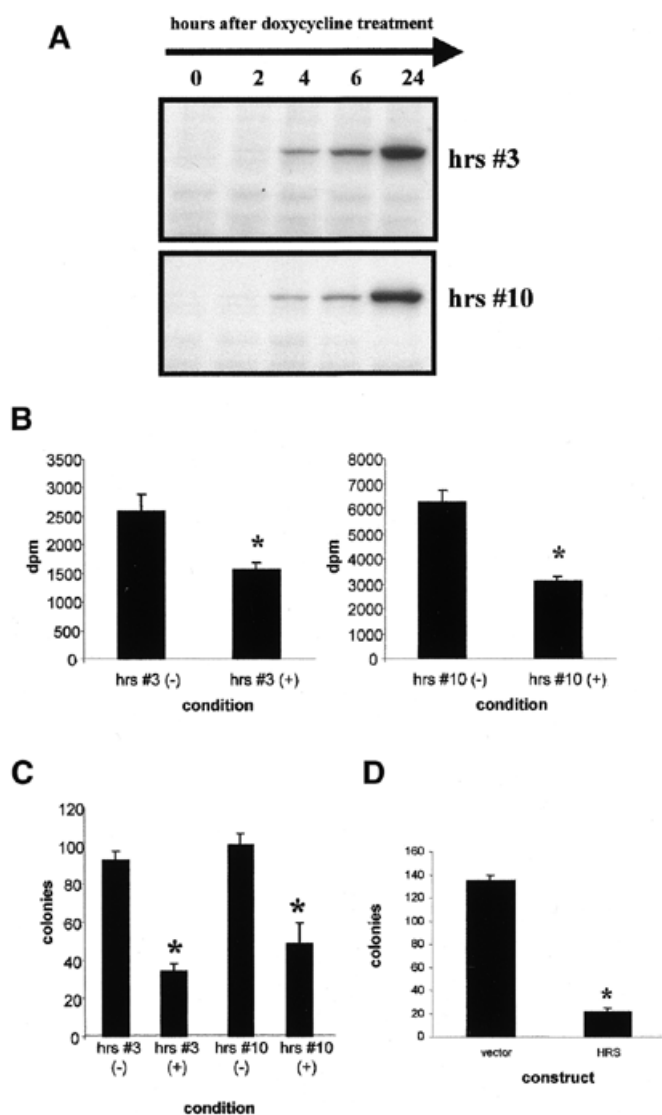
**Figure 1.** HRS interacts with the C-terminus of merlin *in vitro*. (A) HRS specifically interacts with merlin and not ezrin (Ez) or radixin (Rad). GST affinity chromatography pull-down experiments were performed with radiolabeled merlin (NF2.17) and HRS as described in Materials and Methods. No binding of merlin or HRS to the GST (G) beads alone was observed. Merlin bound to both ezrin and radixin, whereas no binding was observed with HRS. (B) HRS associates with the radmer, but not the merad, merlin–radixin hybrid. No binding was observed between either merlin–radixin hybrid or GST (G) alone. Whereas no binding was observed between HRS and merad (containing residues 1–340 of merlin), radmer (containing residues 341–595 of merlin) bound to HRS. The bound and supernatant fractions are shown. (C) HRS does not associate with the C-terminus (residues 284–595) of ezrin (ezrin C-term) *in vitro*. No binding was observed to GST (G) alone. The bound and supernatant fractions are shown. (D) HRS associates with the C-terminus of merlin. Radiolabeled N- (M1-302) and C-terminal (M299–595) fragments of merlin were interacted with GST alone (GST) or GST–HRS [full-length HRS (HRS)] as described in Materials and Methods. Whereas no binding was detected between N-terminal merlin and either GST or GST–HRS, the C-terminal fragment of merlin (residues 299–595; M299–595) binds specifically to GST–HRS and not GST alone.

C-termini of the protein (9,16,17,19–21). We further demonstrated that alterations in the extreme C-terminus of merlin impair the ability of the C-terminus to bind to N-terminal residues (e.g. merlin containing residues 1–568 and 1–557 or containing exon 16) (9). In addition, selected N-terminal domain mutations (e.g. L64P) also impair the ability of merlin to form a productive intramolecular association by disrupting the formation of an N-terminus self-association (19).

Initial experiments using the full-length merlin molecule failed to demonstrate significant binding to GST–HRS *in vitro* (Fig. 4A) and *in vivo* (Fig. 4B and C). This result is also reflected in a dramatic reduction in HRS binding using the yeast two-hybrid interaction system (29). Since merlin containing exon 16 (merlin isoform 2) was shown to strongly associate with HRS in the yeast two-hybrid system, we explored the possibility that HRS binding to merlin might be

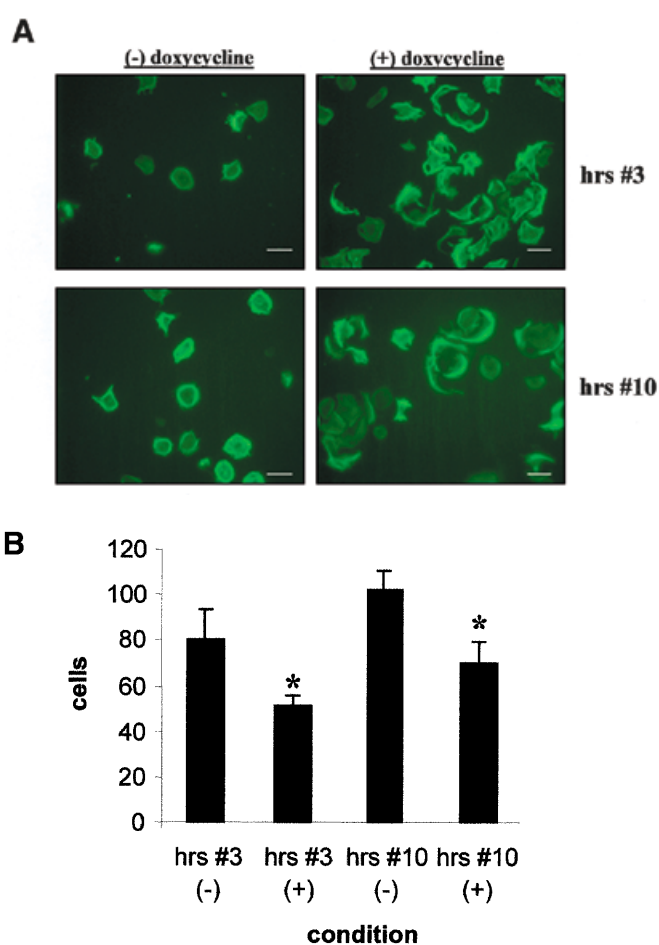
unmasked in the open merlin conformation. To demonstrate this, we used the L64P *NF2* missense mutant, which impairs the ability of merlin to form the N-terminal self-association required for merlin N-terminal:C-terminal folding (19). Whereas full-length wild-type merlin, which exists in the closed conformation, failed to bind significantly to HRS in this pull-down assay, the L64P merlin mutant demonstrated binding to HRS both *in vitro* and *in vivo* (Fig. 4A and C). These results suggest that merlin binding to HRS is partially masked by merlin self-association.

To further define the HRS binding domain within the C-terminus of merlin, we analyzed the ability of three additional laboratory-generated merlin deletion molecules. Merlin truncated at residue 568 (1–568) or 557 (1–557), or containing an internal deletion between residues 341 and 453 ( $\Delta$ 342–452) were used in the *in vivo* interaction assay (Fig. 4B



**Figure 2.** Regulated overexpression of HRS results in growth suppression. (A) RT4 rat schwannoma cells containing the rTA tetracycline transactivator were transfected with pUHD-10.3.HRS and two representative clones (3 and 10) were chosen for further study. Upon the addition of 1  $\mu\text{g/ml}$  doxycycline, an induction of HRS expression was seen over time. HRS was detected using the X-press antibody by western blot. (B) HRS induction results in a decrease in RT4 schwannoma cell proliferation in both HRS clones 3 and 10. Cell proliferation was measured using thymidine incorporation as described in Materials and Methods. There was 1.65–2.65-fold decrease in thymidine incorporation for cells expressing HRS compared with uninduced cells. (+) and (–) denote the addition or omission of doxycycline. The mean and standard deviation for each condition is shown. The asterisk denotes statistical significance using the Student's *t*-test ( $P < 0.05$ ). (C) Soft agar growth assays were performed in quadruplicate as described in Materials and Methods. A 1.8–2.7-fold decrease was seen in the number of colonies in cells expressing HRS compared with uninduced cells. (+) and (–) denote the addition or omission of doxycycline. The mean and standard deviation for each condition is shown. The asterisk denotes statistical significance using the Student's *t*-test ( $P < 0.05$ ). (D) Overexpression of HRS (pcDNA3.HRS) reduces RT4 cell colony formation after selection for 14 days in hygromycin compared to vector (pcDNA3) alone. The mean and standard deviation for each condition is shown. The asterisk denotes statistical significance using the Student's *t*-test ( $P < 0.05$ ).

and C). Whereas wild-type merlin failed to interact with HRS, significant interactions were observed between all three merlin mutants and HRS. These results in combination with the

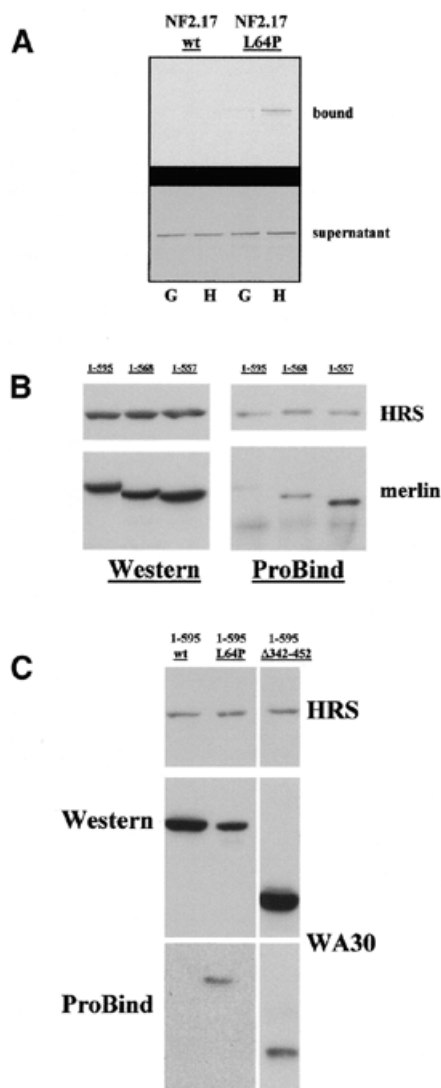


**Figure 3.** Regulated HRS overexpression impairs cell spreading and motility in RT4 schwannoma cells. (A) Induction of HRS was associated with abnormal spreading within 90 min of plating, as assessed using phalloidin-BODIPY immunofluorescence. Similar effects were observed for both HRS clone 3 and 10. No effect was observed with cell lines expressing the blank vector alone (data not shown). Photomicrographs were taken at 40 $\times$ . Scale bar denotes 20  $\mu\text{m}$ . (B) HRS RT4 cells were induced with doxycycline overnight and analyzed in a Boyden chamber motility assay as described in Materials and Methods. A reproducible 26–27% reduction in cell motility was observed after HRS overexpression in both RT4 clones. The mean and standard deviation for each condition is shown. (+) and (–) denote the addition or omission of doxycycline. Asterisks denote  $P < 0.05$  using the Student's *t*-test.

merlin–radixin hybrid data narrow the HRS binding domain to between residues 453 and 557 in the C-terminus of merlin.

#### Merlin C-terminal domain binding to HRS does not impair HRS growth suppression

The fact that merlin binds to HRS in the open conformation suggested that the interaction of the C-terminus of merlin with HRS might regulate HRS function. To determine whether merlin C-terminal binding to HRS reduced the ability of HRS to inhibit cell proliferation, we generated RT4-regulated HRS-overexpressing clones that additionally constitutively overexpress the C-terminus of merlin (residues 299–595), either as the wild-type form, which binds HRS, or as the L535P mutant, which demonstrates reduced HRS binding in the yeast two-hybrid interaction system (29). As shown in Figure 5A, one



**Figure 4.** HRS binds to the unfolded merlin molecule. (A) GST affinity interaction experiments were performed using GST alone (G) or GST-HRS (H) as described in Materials and Methods. Whereas no binding of wild-type full-length merlin (NF2.17) to HRS was observed, full-length merlin containing the L64P mutation binds to HRS *in vitro*. No significant binding was observed to GST alone. The bound and supernatant fractions are shown for each interaction. (B) ProBind pull-down experiments were performed by transiently transfecting HRS clone 10 with various merlin expression constructs as described in Materials and Methods. Full-length merlin (1–595) or merlin truncated at residue 568 (1–568) or 557 (1–557) were transfected and the total protein was analyzed by western blot (Western) to demonstrate both HRS and merlin fragment expression (left panel). Lysates were incubated with ProBind resin and bound proteins eluted for SDS-PAGE and western blot using X-press and WA30 merlin antibodies (right panel). HRS was detected in all three eluates; however, only merlin truncated at residues 568 or 557 was bound to HRS. Full-length wild-type merlin did not bind HRS as shown *in vitro* in Figure 5A. (C) ProBind pull-down experiments were performed by transiently transfecting HRS clone 10 with merlin expression constructs as described in Materials and Methods. Full-length merlin (1–595) or merlin containing the L64P mutation (1–595 L64P) or missing residues 342–452 (1–595  $\Delta$ 342–452) were transfected and the total protein was analyzed by western blot (Western) to demonstrate both HRS and merlin fragment expression (middle panel). Lysates were incubated with ProBind resin and bound proteins eluted for SDS-PAGE and western blot using X-press and WA30 merlin antibodies (upper and lower panels). HRS was detected in all three eluates; however, only merlin containing the L64P mutation or internally deleted of residues 342–452 bound to HRS. Full-length merlin containing the L64P mutation, but not wild-type merlin, bound HRS as shown *in vitro* in (A).

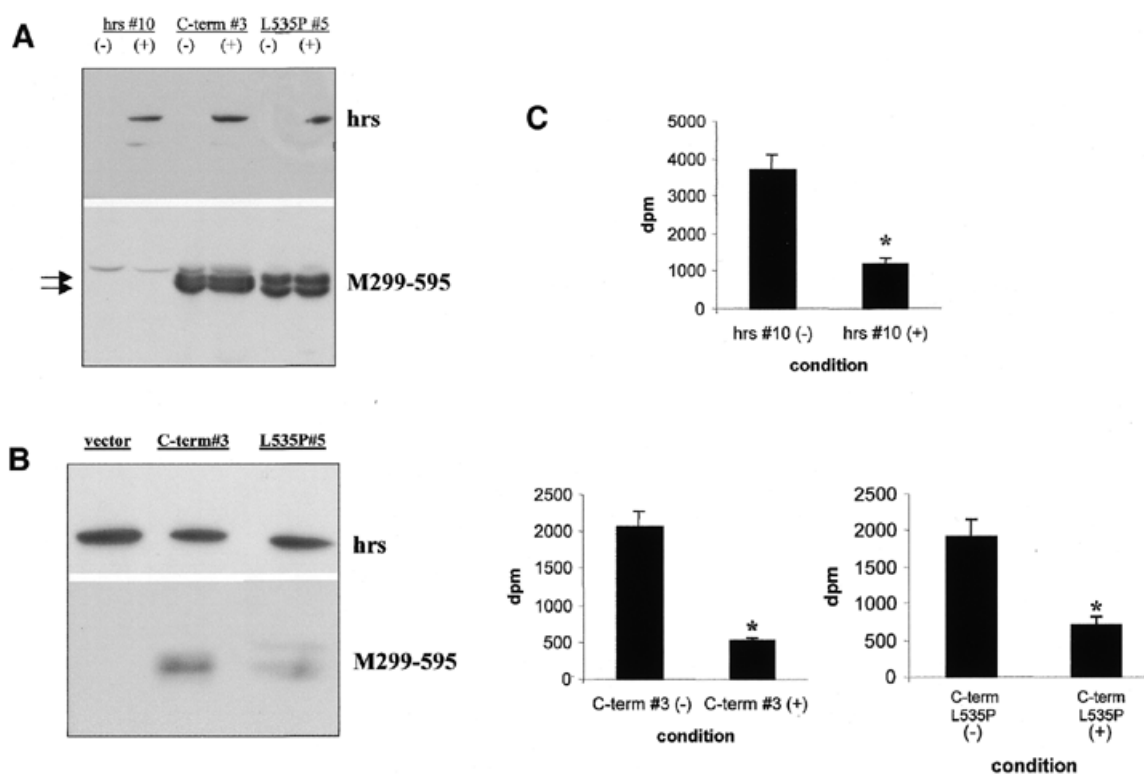
representative clone of each type demonstrate regulated HRS overexpression. The C-terminal no. 3 and L535P no. 5 clones additionally overexpress the wild-type and mutant C-terminal domains of merlin, respectively. The appearance of merlin C-terminal doublet bands (Fig. 5A) may represent phosphorylation of merlin (data not shown). To demonstrate that the C-terminus of merlin binds to HRS *in vivo*, pull-down experiments were performed using ProBind resin, which recovers the His-tagged HRS molecule, and eluates were analyzed for merlin C-terminus association. As shown in Figure 5B, the wild-type, but not the L535P mutant, merlin C-terminus binds HRS *in vivo*.

Thymidine incorporation experiments were next performed to analyze the effect of merlin C-terminus domain binding to HRS on HRS growth suppression. As shown in Figure 5C, no significant differences in growth suppression were observed between regulated HRS overexpressing clones containing wild-type or mutant merlin C-terminal domains compared with vector controls. These results argue that merlin C-terminal binding to HRS does not increase or impair the ability of HRS to regulate cell growth.

Although full-length merlin demonstrated reduced binding to HRS than the C-terminal construct alone, we explored the possibility that full-length merlin might interfere with HRS-mediated growth suppression. In these experiments, we used a doxycycline-regulatable merlin-expressing RT4 cell line (clone 6). Similar to our previous studies, regulatable overexpression of merlin resulted in increased merlin expression within 4–6 h of doxycycline treatment (Fig. 6A). Merlin induction is associated with a reduction in RT4 cell proliferation and anchorage-independent cell growth as determined by thymidine incorporation (Fig. 6B) and soft agar colony formation (Fig. 6C). No effect of doxycycline treatment on vector only containing cell lines was observed (C.A. Haipek and D.H. Gutmann, unpublished data). The effect of HRS and merlin expression on RT4 cell growth was determined using a colony formation assay. Overexpression of either HRS or merlin results in reduced RT4 colony formation; however, co-expression of both HRS and merlin was associated with further reduction in growth suppression (Fig. 6D). These results argue that the full-length merlin does not interfere with HRS growth suppression.

#### Loss of merlin expression in sporadic meningiomas is not associated with reduced or absent HRS expression

Since merlin and HRS exhibit similar functions in RT4 cells, we explored the possibility that loss of merlin expression in tumors was coordinately associated with loss of HRS expression. We chose to examine seven sporadic meningiomas, since 50–60% of these tumors have bi-allelic *NF2* inactivation and lack merlin expression. In contrast, all sporadic schwannomas lack merlin expression, and correlations between HRS and merlin expression would not be possible. As shown in Figure 7, loss of merlin expression (tumors 2, 3 and 7) is not associated with loss of HRS expression. Moreover, in tumors where merlin expression was retained, the level of HRS expression did not correlate with the level of merlin expression (tumors 1, 4 and 6). These observations suggest that the presence of HRS alone is not sufficient to compensate for loss of merlin.



**Figure 5.** Overexpression of C-terminal merlin fragments does not impair HRS growth suppression. (A) ProBind pull-down experiments were performed by stably transfecting HRS clone 10 with merlin C-terminal expression constructs (wild-type C-terminus; residues 299–595 and C-terminal merlin containing the L535P mutation) as described in Materials and Methods. Total protein was analyzed by western blot (Western) to demonstrate both HRS and merlin C-terminal fragment (M299–595) expression. The merlin C-terminal fragment appears as a doublet (arrows). HRS expression is regulated by the addition of doxycycline, denoted by (+) and (-). (B) Lysates were incubated with ProBind resin and bound proteins eluted for SDS-PAGE and western blot using X-press and C18 merlin antibodies. Vector refers to HRS 10 transfected with pcDNA3 alone. HRS was detected in all three eluates. Wild-type C-terminal merlin bound HRS, however, significantly reduced binding was observed with C-terminal merlin containing the L535P mutation. (C) Thymidine incorporation experiments were performed as described in Materials and Methods. HRS-mediated growth suppression was observed in all three clones. No significant differences in the magnitude of growth suppression were observed between these RT4 cell lines.

## DISCUSSION

### HRS is a specific merlin interactor

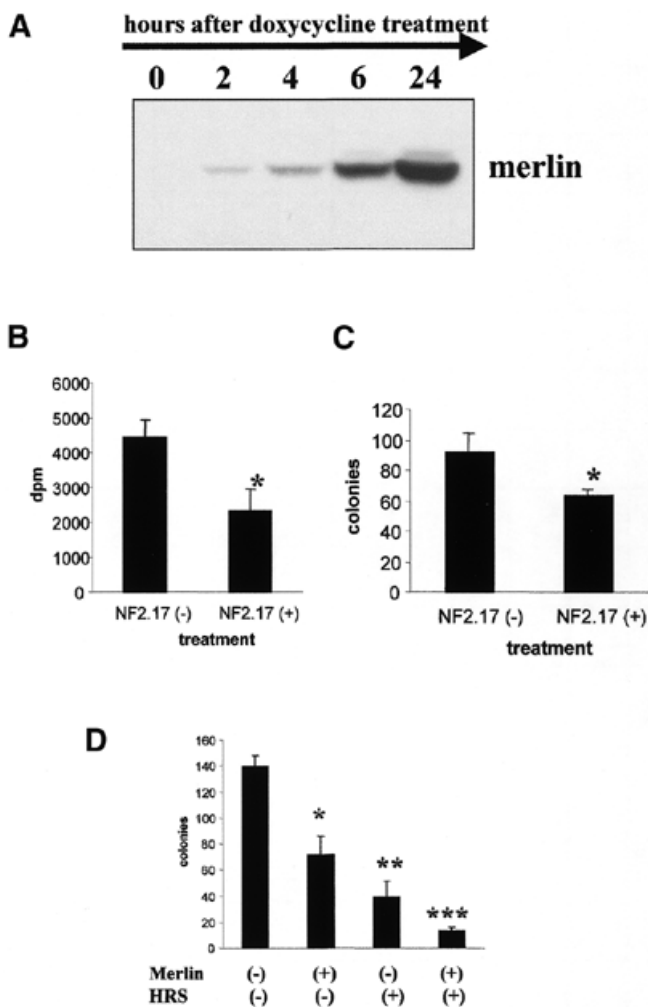
Several merlin-interacting proteins have been reported over the past few years, including actin (33),  $\beta$ II-spectrin (28), CD44 (34), NHE-RF (sodium hydrogen exchange regulator factor) (21,27,35) and SCHIP-1 (26). To evaluate the ability of these interacting proteins as effectors of merlin growth suppressor function, we sought to determine whether potential merlin interactors specifically interact with merlin and display some of the functional properties attributed to merlin. In this report, we demonstrate that the merlin interacting protein, HRS (29), specifically interacts with merlin and not other ERM proteins, and also exhibits all of the functional properties we have previously attributed to merlin. Based on these data, we propose that HRS is an attractive candidate for a merlin effector protein relevant to *NF2* growth regulation.

The identification of HRS as a unique binding partner for merlin suggests that HRS might be involved in mediating merlin growth suppression. In this report, we demonstrate that HRS binds specifically to merlin and not to ERM proteins. HRS is expressed in the same tissues as merlin and associates with merlin both *in vitro* and *in vivo* (29). We define the HRS

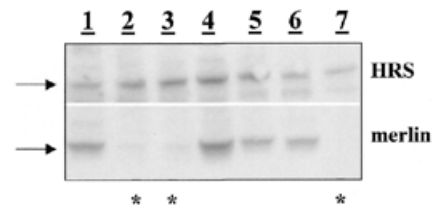
binding domain in merlin to between residues 453 and 557 using merlin-radixin hybrids and merlin truncation constructs. In support of this binding domain localization, HRS binding to merlin is dramatically impaired by a mutation at residue L535 that is contained within this domain (29). This HRS interaction region is distinct from the binding domains important for mediating merlin associations with NHE-RF (merlin residues 1–332) (21,35), SCHIP-1 (merlin residues 1–19 and 289–314) (26) and CD44 (merlin residues 1–300) (34), but overlaps partially with  $\beta$ II-spectrin binding domain (merlin residues 305–590) (28). Furthermore, this domain is located mostly within the predicted unique C-terminal domain of merlin (residues 479–595) which is not homologous to the C-terminus of other ERM proteins and is separate from the regions involved in merlin self-association, which involves residues 302–308 in the N-terminus and residues 579–595 in the C-terminus of merlin (19).

### Merlin binds HRS in the open conformation

Previous work from our laboratory and others has suggested that merlin exists in open and closed conformations which may be important for mediating merlin function and associations (10,19–21,26). We have shown that the ability of merlin to func-



**Figure 6.** Regulated overexpression of merlin results in growth suppression. (A) RT4 rat schwannoma cells containing the rtTA tetracycline transactivator were transfected with pUHD-10.3.NF2.17 and several clones were chosen for further study. One representative clone is shown (clone 6). Upon the addition of 1  $\mu$ g/ml of doxycycline, an induction of merlin expression was seen over time. Merlin was detected using the WA30 polyclonal antibody by western blot. (B) Merlin induction results in a decrease in RT4 schwannoma cell proliferation. Cell proliferation was measured using thymidine incorporation as described in Materials and Methods. There was a 2-fold decrease in thymidine incorporation for cells expressing merlin compared with uninduced cells. (+) and (-) denote the addition or omission of doxycycline. The mean and standard deviation for each condition is shown. The asterisk denotes statistical significance using the Student's *t*-test ( $P < 0.05$ ). (C) Soft agar growth assays were performed in quadruplicate as described in Materials and Methods. A 1.5-fold decrease in the number of colonies was seen in cells expressing merlin compared with uninduced cells. (+) and (-) denote the addition or omission of doxycycline. The mean and standard deviation for each condition is shown. The asterisk denotes statistical significance using the Student's *t*-test ( $P < 0.05$ ). (D) Overexpression of HRS and merlin results in additive reductions in cell growth. RT4 cells inducibly expressing merlin (RT4 NF2.17 6) were transfected with pcDNA3 (-) or pcDNA3.HRS (+) in the HRS row in the presence or absence of doxycycline to induce merlin expression denoted by the (+) or (-) in the merlin row, respectively. Either merlin (condition 2) or HRS (condition 3) alone resulted in RT4 growth suppression. Expression of both merlin and HRS (condition 4) resulted in a further decrease in cell growth. The mean and standard deviation for each condition is shown. The single, double and triple asterisks denote statistical significance using the Student's *t*-test ( $P < 0.05$ ) between each of the conditions.



**Figure 7.** HRS and merlin expression are not coordinately expressed in sporadic human meningioma tumors. Seven sporadic human meningioma tumors were homogenized and equal amounts of total protein were separated by SDS-PAGE prior to immunoblotting with the Ab-1080-2 HRS and WA30 merlin antibodies. Three tumors (2, 3 and 7) have absent merlin expression (denoted by the asterisks), but retain HRS expression. No correlation between the level of merlin and HRS expression was seen in the four tumors with retained merlin expression (1, 4, 5 and 6).

tion as a growth regulator or impair actin cytoskeleton-mediated events is dependent on the formation of two intramolecular associations involving N-terminal:N-terminal as well as N terminal:C-terminal interactions. Recent reports have demonstrated that the ability of merlin to interact with NHE-RF and SCHIP-1 is also regulated by merlin intramolecular associations (21,26). Merlin appears to bind both of these interactors more avidly in the open conformation, suggesting that merlin folding might mask the SCHIP-1 and NHE-RF binding sites. Previously, we had shown that merlin isoform 2 containing the 'unfolded' exon 16 sequences bound HRS more strongly than the 'folded' merlin isoform 1 containing exon 17 sequences in a yeast two-hybrid interaction assay (29). In this report, we demonstrate that mutations that disrupt merlin self-association and result in a protein in the open conformation are able to bind HRS *in vitro*. This differential HRS association argues that merlin intramolecular associations might also influence HRS binding to merlin.

The ability of merlin to associate with HRS in the open conformation suggests that merlin/HRS function may be dictated by changes in the folding of merlin *in vivo*. Two events have been implicated in the regulation of merlin folding. These include the interaction of merlin with interacting proteins and the phosphorylation of merlin. It is possible that merlin binding to CD44 or NHE-RF permits merlin unfolding and allows for HRS binding. In this fashion, a productive merlin-CD44 interaction might result in growth suppression through binding to HRS. Alternatively, merlin is phosphorylated on serine and threonine residues (36) and the unphosphorylated form appears to be the predominant protein species under conditions when merlin is active. Support for this hypothesis also derives from experiments in which the unphosphorylated merlin preferentially associates with CD44 under conditions of confluency (31,32). It is possible that phosphorylation of merlin dictates which binding partners merlin associates with, and in that fashion, regulates cell growth regulation. Additional studies aimed at determining the significance of merlin phosphorylation to merlin function and protein interactions are required.

#### Regulated HRS and merlin overexpression impair similar cellular processes

For HRS to represent a reasonable candidate for a merlin effector protein, we sought to demonstrate that HRS and

merlin have similar functional properties. Using regulatable RT4 cell lines, we showed that HRS overexpression resulted in reduced cell proliferation and anchorage-independent cell growth as well as impaired actin cytoskeleton-associated events. Previous work from our laboratory demonstrated that regulated overexpression of wild-type, but not mutant, merlin molecules resulted in decreased cell proliferation as assayed by direct cell counting, thymidine incorporation and anchorage-independent cell growth (9,10,19). This growth suppression was not the result of decreased cell viability as determined by FACS analysis and TUNEL labeling. In addition, regulated overexpression of wild-type, but not mutant, merlin molecules resulted in reduced cell motility and attachment as well as dramatic alterations in the actin cytoskeleton during cell spreading. These results collectively support the notion that HRS might function similarly to merlin; however, further studies will be required to definitively prove this hypothesis.

Since merlin binds to HRS in the open conformation by virtue of C-terminal residues, we explored the possibility that binding to merlin could regulate HRS function. In these experiments, we found no effect of C-terminal merlin overexpression on HRS function. Similarly, we also found no evidence for impairment of merlin growth suppression by N-terminal (residues 1–480) or C-terminal (residues 480–777) HRS fragments (C.A. Haipek and D.H. Gutmann, unpublished results). These observations suggest that it is unlikely that HRS functions upstream of merlin and raise the possibility that either (i) HRS is downstream of merlin in a common growth inhibitory pathway; (ii) merlin and HRS coordinately regulate different upstream signaling cascades that converge on a common pathway to regulate cell growth and actin cytoskeleton-mediated processes; or (iii) HRS binding is required for proper merlin function and localization. Likewise, merlin binding to HRS could activate HRS, provide the proper subcellular localization required for HRS function or displace a different HRS binding protein to allow for HRS function.

Merlin and HRS are both expressed in cells that give rise to the tumors seen in individuals with NF2. Since merlin and HRS seem to mediate similar functions within the cell, it is possible that merlin loss is associated with coordinated HRS loss to result in absent merlin/HRS growth suppression. We excluded this possibility by western blot in sporadic meningiomas and demonstrated no correlation between merlin loss and HRS expression. It is therefore unlikely that retained HRS expression can compensate for merlin loss.

HRS represents an attractive candidate for a merlin effector protein. HRS is associated with the HGF signaling pathway, which has been implicated in both mitogenic and motogenic regulation in Schwann cells. HGF is one of the most potent growth factors for Schwann cell proliferation and motility (30). Since merlin regulates both growth and motility in Schwann cells, it is tempting to associate merlin with the HGF signaling pathway. Recent studies have demonstrated a link between HGF signaling and CD44 function. The HGF receptor, c-met, can stimulate HA production in a PI3-kinase-dependent fashion (37) as well as result in increased CD44 expression (38). In addition, HGF binds to a variant of CD44 (CD44v3) to promote c-met activation and MAPK activation (39). These results suggest that merlin may function as a critical growth regulator for Schwann cells by integrating the CD44 and HGF signaling pathways. Further studies specifically aimed at

relating CD44 and HGF signaling as well as establishing a direct connection between merlin and HRS growth suppression are presently underway.

## MATERIALS AND METHODS

### Antibodies, cDNA constructs and cell lines

The rabbit polyclonal antibodies that specifically recognize merlin (WA30) and HRS (ab-1080-2 and X-press tag) have been described previously (6,29). The merlin and HRS cDNAs used in these experiments were of human origin. N-terminal (M1–302), C-terminal (M299–595), merlin fragments containing residues 1–568 and 1–557 or deleted between residues 341 and 453 ( $\Delta$ 342–452), merlin hybrids (merad and radmer), and missense merlin constructs have been described previously (9,19). The C-terminal ezrin construct was generated by cloning a *Bam*HI fragment of the full-length cDNA (residues 284–585) into pcDNA3.

HRS-expressing RT4 cell lines were established by transfecting RT4 cells containing the rTA reverse tetracycline transcriptional regulator (developed by H.Morrison) and puromycin resistance (pBABE.PURO) with pUHD10.3-HRS and pcDNA3 to confer G418 resistance (31,32). Several independent clones were selected in 500  $\mu$ g/ml G418 and 1  $\mu$ g/ml puromycin. Positive clones were screened for HRS expression using the X-press tag (Invitrogen) upon doxycycline addition, and two cell lines were maintained for further analysis (HRS clones 3 and 10). Additionally, wild-type merlin-expressing RT4 cell lines were generated and positive clones screened for regulatable merlin expression using WA30. Several clones were independently isolated and clone 6 is presented. A complete characterization of these wild-type and mutant merlin-regulatable RT4 cell lines will be presented elsewhere (D.H. Gutmann, A. Hirbe and C. Haipek, manuscript in preparation). RT4 cell lines transfected with pUHD-10.3 vector alone demonstrated no changes in cell proliferation, anchorage-independent growth, cell spreading and cell motility upon the addition of doxycycline.

Regulatable HRS RT4 schwannoma cell lines were also transfected with pcDNA3-C-term (residues 299–595) and C-term.L535P (residues 299–595 containing the L535P missense mutation) along with pCEP4 to confer hygromycin resistance. Clones were triply selected in G418, puromycin and hygromycin. Clones were selected that demonstrated regulatable HRS expression as well as constitutive C-terminal merlin expression using the Santa Cruz rabbit polyclonal antibody, C-18.

### Thymidine incorporation, colony formation assay, TUNEL staining and growth in soft agar

Thymidine incorporation was performed as described previously on subconfluent cultures of RT4 schwannoma cells containing the doxycycline-regulatable HRS (clones 3 and 10) (9,10). For HRS induction, 1  $\mu$ g/ml doxycycline was added to the medium for 24 h while 1  $\mu$ Ci/ml tritiated thymidine was added for the last 4 h. Each condition (with or without doxycycline) was performed in six duplicate wells and cells were harvested in 0.2 M NaOH. Thymidine incorporation was measured on a scintillation counter and the mean and standard deviation determined for each condition.



The colony formation assay was performed by transfecting RT4 cells with equimolar amounts of pcDNA3 (vector), pcDNA3.NF2 (full-length containing exon 17) and pcDNA3.HRS (full-length residues 1–777). Cells were then selected in 200 µg/ml hygromycin for 14 days. Quadruplicate dishes for each transfection were counted after staining in 0.5% Crystal violet.

TUNEL labeling was performed using the Boehringer Mannheim *in situ* cell death (POD) detection kit according to the manufacturer's instructions, and visualized using a Nikon fluorescence microscope.

Soft agar growth assays were performed in quadruplicate either in the presence or absence of doxycycline. Briefly, 1000 RT4 cells were plated in 24-well plates with medium containing 0.3% Noble agar for 14–21 days. The number of colonies was determined by direct counting on an inverted microscope and the mean and standard deviation determined for each condition. Each experiment was repeated at least three times with identical results.

### Cell spreading and motility

Glass coverslips were coated with 10 µg/ml of laminin (Sigma) in PBS overnight at 4°C. Coverslips were then aspirated. Inducible HRS RT4 cells, cultured in DMEM + 10% FBS were treated with 1 µg/ml doxycycline for 24 h, and then removed from dishes by trypsinization. Cells were washed twice in PBS, resuspended in DMEM + 10% FBS, with and without doxycycline, and plated onto the coverslips at ~100 000 cells/well. After 2 h, cells were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized in PBS containing 0.1% Triton X-100, and stained with BODIPY-conjugated phalloidin (Molecular Probes, 0.2 U in 50 µl) for 20 min. Coverslips were then washed in PBS, mounted in 1 drop of Fluoromount G (EM Sciences), and examined on a Zeiss Axiophot microscope (22).

Cell motility was determined in Transwell chambers containing 8 µ membranes. Briefly, the bottom surface of the membrane was coated with Matrigel (Collaborative Research) and 10 000 cells were seeded on the outside of the chamber and allowed to attach for 1 h. Cells were gently washed and then the Transwells were inverted for 48 h either in the presence or absence of doxycycline at 37°C to allow for migration. Cells were then fixed in cold methanol for 30 min prior to staining with a LeukoStat staining kit (Fisher Scientific) and counted visually. The number of migrating cells was counted in quadruplicate and the mean and standard deviation determined for each condition. Each experiment was repeated at least three times with identical results.

### HRS-merlin interaction *in vivo*

RT4 schwannoma cells with regulatable HRS expression and constitutive C-terminal merlin expression were lysed after a 24 h incubation in the presence or absence of doxycycline. Equal protein from these lysates was incubated with ProBind resin (Invitrogen) for 4 h followed by extensive washing in lysis buffer. Eluted proteins were separated by 10% SDS-PAGE and blotted with anti-merlin (C-18; Santa Cruz Biotechnology) and X-press tag antibodies to identify merlin C-terminal fragments and HRS, respectively. Total lysates were also separated by 10% SDS-PAGE for western blotting with the above antibodies.

In other experiments, merlin fragments cloned into pcDNA3 were transiently transfected into RT4 HRS clone 10 in the presence of doxycycline and lysed after 48 h. As above, equal protein from these lysates was incubated with ProBind resin (Invitrogen) for 4 h followed by extensive washing in lysis buffer. Eluted proteins were separated by 10% SDS-PAGE and blotted with anti-merlin (WA30) and X-press tag antibodies to identify merlin fragments and HRS, respectively. Total lysates were also separated by 10% SDS-PAGE for western blotting with the above antibodies. Each experiment was repeated at least three times with identical results.

### GST fusion protein affinity chromatography

GST-merlin fusion proteins were generated as described previously (9,10,19). Briefly, GST, GST-merlin (M8-320), GST-ezrin, GST-moesin and GST-radixin were transformed into DE3 (BL21) competent cells for fusion protein production. The GST-ERM proteins were kindly provided by Dr Heinz Furthmayr (Stanford University). The GST-HRS construct was generated by PCR using primers that amplify the full-length protein (residues 1–777; HRS-F: 5'-GGATCCCC-ATGGGGCGAGGCAGC-3' and HRS-R: 5'-GTCAGTCGA-ATGAAATGAGCTGGGCCTC-3') and subcloned into pGEX.3X for fusion protein expression. Each construct was sequenced in its entirety and cloned into pGEX.3X for fusion protein production. Bacteria were induced overnight in 0.4 mM IPTG at room temperature and GST-merlin fusion proteins collected on glutathione-agarose beads (Sigma) for the interaction experiments.

GST fusion proteins were prepared as above for merlin interaction experiments with *in vitro* transcribed and translated merlin and HRS proteins, as described previously (9,10,19). *In vitro* transcribed and translated merlin proteins were synthesized in the presence of [<sup>35</sup>S]methionine using the TnT protocol (Promega) according to the manufacturer's instructions and detected by autoradiography. In these experiments, radiolabeled proteins were incubated with equimolar amounts of GST fusion protein immobilized on glutathione-agarose beads for 4 h at 4°C. The unbound fraction was saved and the agarose beads were then washed four times in TEN buffer (10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) and eluted in 1× Laemmli buffer. An equal fraction of the supernatant and eluted bound fraction was separated by SDS-PAGE and analyzed by autoradiography. In all experiments, no significant binding was observed with immobilized GST alone (<2% total bound). Each experiment has been repeated at least twice with identical results.

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