Review

The merlin interacting proteins reveal multiple targets for NF2 therapy

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Abstract

The neurofibromatosis 2 (NF2) tumor suppressor protein merlin is commonly mutated in human benign brain tumors. The gene altered in NF2 was located on human chromosome 22q12 in 1993 and the encoded protein named merlin and schwannomin. Merlin has homology to ERM family proteins, ezrin, radixin, and moesin, within the protein 4.1 superfamily. In efforts to determine merlin function several groups have discovered 34 merlin interacting proteins, including ezrin, radixin, moesin, CD44, layilin, paxillin, actin, N-WASP, βII-spectrin, microtubules, TRBP, eIF3c, PIKE, NHERF, MAP, RalGDS, RhoGDI, EG1/magicin, HEI10, HRS, syntenin, caspr/paranodin, DCC, NGB, CRM1/exportin, SCHIP1, MYPT-1-PP1δ, RIβ, PKA, PAK (three types), calpain and *Drosophila* expanded. Many of the proteins that interact with the merlin N-terminal domain also bind ezrin, while other merlin interacting proteins do not bind other members of the ERM family. Merlin also interacts with itself. This review describes these proteins, their possible roles in NF2, and the resultant hypothesized merlin functions. Review of all of the merlin interacting proteins and functional consequences of losses of these interactions reveals multiple merlin actions in PI3-kinase, MAP kinase and small GTPase signaling pathways that might be targeted to inhibit the proliferation of NF2 tumors.
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1. Introduction

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder that predisposes individuals to the development of multiple tumors of the central and peripheral nervous system. Germline mutations of the *NF2* gene cause most inherited tumors of Schwann cell, meningeal or ependymal origin. Autosomal dominant NF2 is a relatively rare disorder with an incidence of 1 in 33,000 to 40,000 [1] and linkage analysis showed that NF2 is genetically homogeneous [2]. NF2 is characterized by bilateral vestibular schwannomas, and NF2 patients are also commonly burdened with spinal schwannomas, meningiomas, ependymomas, cutaneous neurofibromas, and cutaneous schwannomas [3–6]. Among sporadic tumors, virtually all schwannomas, 50% of meningiomas and ~5% of ependymomas and harbor *NF2* mutations [7–11].

The *NF2* gene was identified in 1993 in two labs and named merlin and schwannomin [12,13]. *NF2* was localized to chromosome 22q12 and consists of 17 exons and spans approximately 100 kb of genomic sequence. Exons of the *NF2* gene are alternatively spliced to form two common isoforms, referred to as isoform 1 and isoform 2 [14–16] (Fig. 1). In addition, a variety of other isoforms of the *NF2* gene have been described in tumors [17,18], and unique isoforms may exist in the mouse [15]. The *NF2* cDNA sequence predicts a protein of 595 amino acids (590 for isoform 2) with an estimated molecular weight of 65 kDa.

Merlin belongs to a superfamily of proteins that includes band 4.1 protein in erythrocytes, talin, the ezrin, radixin, moesin (ERM) family and also possesses homologies to the family of protein tyrosine phosphatases. This superfamily of proteins is involved in membrane organization with respect to the cytoskeleton. These proteins, with the exception of the protein tyrosine phosphatase family, have a common structural organization: a globular N-terminal domain followed by a long α-helical structure and a charged carboxy-terminal domain. Many of the proteins that interact with merlin also bind several proteins found in the protein 4.1 superfamily.

Since the identification of the *NF2* gene, significant advances have been made on determining merlin's function. Merlin interacts with multiple other proteins whose functions aid in the understanding of merlin function, but a specific pathway in which merlin actions suppress tumor growth in a manner associated with NF2 pathogenesis still remains undefined. This review will summarize information on each of the known merlin interacting proteins with cross-references that provide insight into merlin function. The review concludes with a comment on therapeutics that may be effective for treating NF2.

2. Merlin folding and function

The classic model for merlin interactions is ezrin and other ERM proteins, which are highly homologous to merlin. On average, the N-terminal domains among ERM proteins are 63% homologous to that of merlin, and the C-terminal domains are 28% homologous which is where merlin most diverges from the group. ERM proteins fold in a "head-to-tail" manner. The mechanism of ERM protein folding was first demonstrated for ezrin which is found in dormant monomers and active oligomers with itself and other ERM proteins in a manner regulated by ERM protein phosphorylation [19].

Merlin also binds the ERM proteins and interacts with itself [20–23]. Merlin's self-interactions were, as for ERM proteins, characterized in a head-to-tail manner, and changes in merlin folding are associated with altered tumor suppressor function of merlin. As first shown in ezrin and moesin, for the C-terminal domain to interact with the N-terminal domain the N-terminal
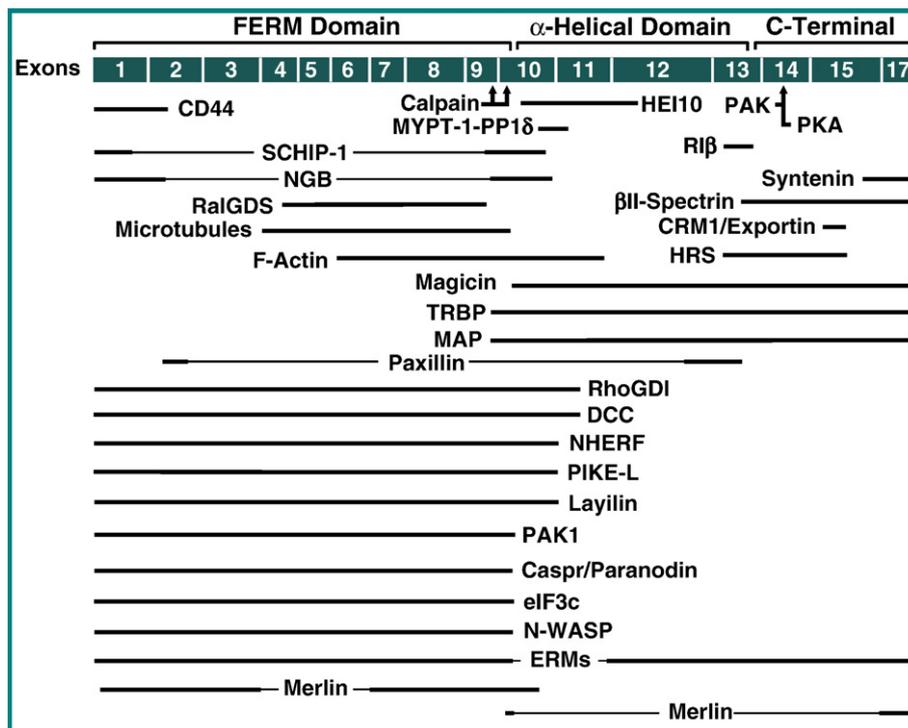
Fig. 1. Location of binding sites in merlin for all merlin binding proteins. Arrows indicate cleavage sites by calpain and the location of serine 518 phosphorylated by PAK and PKA. No proteins have been shown to interact with residues encoded by the alternatively spliced exon 16 of merlin isoform 2, which is not shown.

domain itself must first be folded (or "activated") [24,25]. By testing interactions between GST-merlin proteins and *in vitro* translated merlin proteins, Gutmann et al. showed a similar mechanism for merlin folding in which interaction between amino acids 8–121 and 200–320 within the N-terminal domain was required before interaction between amino acids 302–308 in the N-terminal domain and 580–595 in the C-terminal domain could occur. N–C-terminal domain folding does not occur in merlin isoform 2 and it is thought that this difference in

structure between the isoforms is why isoform 2 lacks a tumor suppressor function [26–28].

There are several studies that support this folding model for merlin and its requirement for merlin function. N-terminal domain folding is needed for merlin membrane localization and was disrupted by some *NF2* missense mutations [29]. Using affinity co-electrophoresis (ACE), Gonzalez-Agosti et al. [23] showed that N-terminal domain fragment of amino acids 1–332 interacted with a C-terminal domain fragment of amino acids
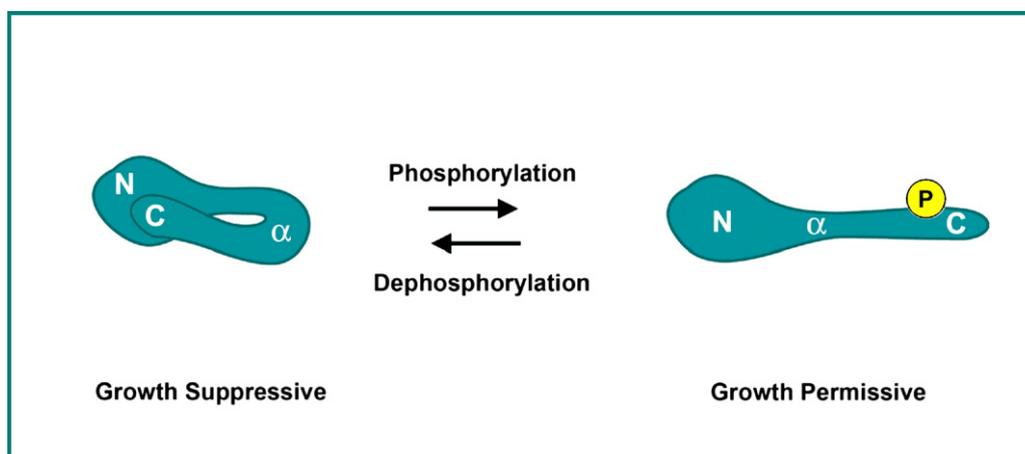
Fig. 2. Model for merlin folding. Merlin in its active form is a suppressor of growth and is folded and unphosphorylated at serine 518. Serine 518 phosphorylation in merlin prevents the C-terminal domain to bind the N-terminal domain, which itself requires intradomain folding before N–C domain interaction can take place. The folded N-terminal domain is represented by a globular shape. Merlin is phosphorylated at serine 518 by PKA and PKA and is dephosphorylated by MYPT-1-PP1δ. Merlin folding is also altered by alternative splicing and *NF2* mutation. Merlin folding can enhance or inhibit its interactions with other proteins. The N-terminal, α-helical and C-terminal domains are indicated.

340–595 of merlin isoform 1 but not amino acids 340–590 of merlin isoform 2. They also showed that N-terminal domain merlin binding sites are more available for NHERF/EBP50 interaction in merlin isoform 2 than in merlin isoform 1 by testing interactions between immobilized NHERF-GST fusion proteins with COS-7 cell lysates containing exogenous FLAG-merlin proteins [23]. Gonzalez-Agosti et al. [23] concluded that merlin isoform 1 can take on a conformation that partly masks the NHERF binding site in the merlin N-terminal domain. Yeast two-hybrid data support the model as well. Merlin isoform 1 N- and C-terminal domain fragments including residues 1–339 and 256–595 interacted while 1–167 and 252–595 did not [22]. By testing interaction between GST fusion proteins, Nguyen et al. [64] showed that the N-terminal domain of merlin could not bind the C-terminal domain deleted of amino acids 594–595 found only in merlin isoform 1.

Phosphorylation is another important regulator of merlin conformation and is required for proper merlin function. In ERM proteins inter- and intramolecular interactions and association with the actin cytoskeleton are regulated by tyrosine and threonine phosphorylation which unmasks ERM protein binding sites for actin and NHERF/EBP50 [19,30,31]. Merlin folding is regulated by phosphorylation at serine 518 by the p21-activated kinases (PAKs) and cAMP dependent-protein kinase A (PKA) [32–35]. Merlin phosphorylation prevents N–C domain folding in merlin, resulting in merlin inactivation and relocalization [32,34–37] (Fig. 2).

3. The Merlin interacting proteins

There are 34 proteins known to interact with merlin, including the three ERM proteins, calpain, four kinases (only one stringently verified as a direct interactor), one phosphatase, and 25 other proteins including one known only in *Drosophila* (Table 1). An illustration of where each binds in merlin is shown in Fig. 1. What follows is a description of each of the merlin interacting proteins in the same order as presented in Table 1. Because many of the interacting proteins have multiple functions the interacting proteins were organized into groups depending upon how they affect merlin function. Effort was also made to present the cell types in which each of the interactions were demonstrated, some of which were not relevant to NF2.

3.1. Activators and suppressors of merlin function

The proteins presented in this section represent a class of merlin interacting proteins that directly alter merlin structure resulting in either merlin activation or merlin inactivation. These include the phosphatases, kinases and proteases. The kinases and proteases were also briefly discussed in Section 2, above with regard to the effect of phosphorylation on merlin folding and function.

3.1.1. Merlin kinases and phosphatases (PAK, PKA, MYPT-1-PP1δ)

Phosphorylation of merlin is critical for the control of merlin folding and protein binding and has a significant effect on merlin

function and subcellular localization. Merlin phosphorylation occurs at serine 518 and can be accomplished by p21-activated kinases (PAKs) and cAMP dependent protein kinase A. Merlin dephosphorylation is accomplished by MYPT-1-PP1δ.

The p21-activated kinases constitute a family of conserved serine/threonine protein kinases that are activated by both Rac and Cdc42. There are six different PAK proteins, (PAK1-6) that are highly conserved [38]. Among these, PAK1 and PAK4 were shown unregulated in a wide variety of cancers [38]. Kissil et al. [33] demonstrated a direct interaction between PAK1 and merlin by GST pull-down assays. The PAK1 binding site in merlin was localized to within the merlin N-terminal domain residues 1–313, and the binding site in PAK was in the cdc42/Rac binding domain (PBD) [33]. Kissil et al. demonstrated that the interaction is dynamic, depending upon cell density. PAK1 inhibits the activity of merlin by phosphorylating it at serine 518 [33,34]. Merlin is also an inhibitor of PAK1 activity, preventing Rac to bind and activate PAK1 [33]. Therefore merlin loss by *NF2* mutation is associated with hyperactive PAK1 activity. Evidence for this was provided by reduced PAK1 activity in RT4 rat schwannoma cells upon overexpression of merlin [33], and the prior observation that merlin can negatively regulate Rac-mediated signaling and cell transformation [36]. Merlin also inhibits Ras activation which may also be a consequence of merlin inhibition of PAK [39]. Additionally, PAK1 specific inhibitors blocked merlin-deficient tumor cells but not merlin positive cells, demonstrating that PAK1 may be a highly effective therapeutic target for the treatment of NF2 [40]. Phosphorylation of merlin at serine 518 was also demonstrated by PAK2 and PAK6 which may not be highly expressed in Schwann cells and therefore are of uncertain relevance to NF2 [32,34]. Merlin is also phosphorylated by PKA [35]. Alfthan et al. demonstrated that PKA phosphorylation of merlin was PAK independent by showing merlin phosphorylation by PKA when PAK was specifically inhibited [35]. This was a useful demonstration because PKA also interacts with and phosphorylates PAK to inhibit its kinase activity.

Merlin dephosphorylation is accomplished by the myosin phosphatase MYPT-1-PP1δ. MYPT-1-PP1δ consists of a MYPT-1 regulatory subunit and PP1δ catalytic subunit encoded by different genes [41]. GST pull-down studies demonstrated that the MYPT-1 regulatory subunit interacts within merlin residues 312–341 and activates merlin by dephosphorylating serine 518 [42].

3.1.2. Calpain

Observations of merlin losses in tumors where no mutations could be identified to account for such losses were the bases for studies on proteolytic cleavage of merlin as a possible mechanism of NF2 tumorigenesis. Kimura et al. [43] demonstrated that *in vitro* proteolysis of a 35 kDa merlin fragment was abolished by addition of the specific calpain inhibitor carbobenzoxyl-leucinyl-leucinal (Z-LLAl). Additional proteolysis assays with *m*-calpain and various bacterially-purified merlin fragments fused to GST demonstrated calpain cleavage sites in merlin that specify for cleavage at the N-terminal sides of either of merlin lysines L295 or L299 [43,44]. Kimura et al.

Table 1
The merlin interacting proteins

Interacting protein	Function	Interacting region (amino acids)	Citation	Note
Merlin	Multifunctional tumor suppressor	8–121 binds 200–300 302–308 binds 580–595	Scoles et al. [53,161] Huang et al. [20] Grönholm et al. [22] Gutmann et al. [26]	2,3
Activators and suppressors of merlin function				
PAK	Merlin kinase	1–313 (PAK1)	Kissil et al. [32,33] Xiao et al. [34]	2,4,6,9
PKA	Merlin kinase	Not determined	Alfthan et al. [35]	6,8,9
MYPT-1-PP1δ	Merlin phosphatase	312–341	Jin et al. [42]	2,6
Calpain	Merlin cleavage	Not determined	Kimura et al. [43]	6,10,11
Structural proteins supporting merlin function				
βII-spectrin	Cytoskeletal structuring, focal adhesions	469–590	Scoles et al. [56]	1,2,3,4
Actin	Cytoskeletal structuring	178–367	Huang et at. [20] Xu and Gutmann [49] Brault et al. [29], James et al. [51]	2,4
Microtubles	Cytoskeletal structuring	122–302	Xu and Gutmann [49]	2,7
Growth activators suppressed by merlin				
Ezrin	Cytoskeleton-membrane interactions	1–313	Meng et al. [65], Nguyen et al. [210] Grönholm et al. [22] Gutmann et al. [21]	1,2,3
Radixin	Cytoskeleton-membrane interactions	Not determined	Gutmann et al. [21]	2
Moesin	Cytoskeleton-membrane interactions	1–332	Gonzalez-Agosti et al. [23]	2
CD44	Hyaluronan signaling, focal adhesions	1–50	Sainio et al. [68] Bai et al. [69]	1,2,4
Layilin	Focal adhesions, hyaluronan signaling	1–332	Bono et al. [72]	1,2
TRBP	Protein translation	288–595	Lee et al. [145]	1,2,3,4
elF3c	Protein translation	1–304	Scoles et al. [82]	1,2,3,4
PIKE	P13-kinase signaling	1–332	Rong et al. [102]	1,2,4
Syntenin	Axon growth, IL-5Rα signaling, endosomal localization	566–595	Jannatipour et al. [103]	1,2,3
NHERF	NHE and cMAP signaling, AJ formation	1–332	Murthy et al. [119]	2,3,4
RalGDS	Small GTPase signaling	1–288	Ryu et al. [128]	1,2,3,4
EG1/Magicin	Small GTPase signaling	340–590/595	Wiederhold et al. [130]	1,2,3,4
HEI10	Cell cycle regulation	306–339	Grönholm et al. [137]	1,2,3,4
N-WASP	Actin polymerization	1–332	Manchanda et al. [54]	1,2
Growth inhibitors that function with merlin				
MAP	Small GTPase signaling	288–595	Lee et al. [145]	1,2,3,4
RhoGDI	Small GTPase signaling	1–341	Maeda et al. [154]	1,2,4
Paxillin	Focal adhesions	50–70 and 425–450	Fernandez-Valle et al. [157]	1,2,4
HRS	Endoctytic trafficking	453–557	Scoles et al. [111]	1,2,3,4
DCC	Cell adhesion and neurite outgrowth	1–341	Martin et al. [168]	2
NGB	Tumor suppressor, GTPase	1–52 and 288–344	Lee et al. [170]	1,2,3,4
Drosophila expanded	Mediates control of fly development	522–635	McCartney et al. [180]	2,4
Role in merlin suppression of growth unclear				
CRM1/exportin	Nuclear export	539–551	Kressel and Schmucker [138]	6,12
RIβ	PKA function, myelination, learning, memory	463–480	Grönholm et al. [185]	1,2,3,4,8
Caspr/paranodin	Myelination, βI integrin signaling	1–314	Denisenko-Nehrbass et al. [197]	1,2
SCHIP-1	Unknown	1–27 and 280–323	Goutebroze et al. [198]	1,2,3,4,5

Supported by [1]co-immunoprecipitation, [2]*in vitro* binding assays, [3]yeast two-hybrid interaction, [4]subcellular co-localization.

[5]Interaction demonstrated only with partial or mutant merlins.

[6]Interaction assumed by functional analysis.

[7]There are several tubulin genes that express microtubule proteins.

[8]The PKA holoenzyme includes four subunits made from at least six different genes, including RIβ.

[9]PAK and PKA phosphorylate merlin at serine 518.

[10]Calpain cleaves merlin on the N-terminal sides of merlin lysines 295 and 299.

[11]There are at least 12 different calpain genes.

[12]The interacting region is assumed based on homology in this region with CRM1 nuclear export sequences.

[43] concluded with a model by which tumorigenesis can occur by merlin loss mediated by calpain dependent proteolysis. Subsequent studies have focused on meningiomas since about 50% of meningiomas lack *NF2* mutations. One study demonstrated that calpain activity was increased by oxidative stress in cultured meningioma cells, stimulated by ionomycin, H_2O_2, or $CaCl_2$, resulting in increased endogenous merlin proteolysis detected by immunoblotting [45]. However, in another study that included analysis of 50 meningiomas, 28 were observed to possess activated calpain but a correlation of calpain status with abundance of cleaved merlin could not be demonstrated [46]. A similar finding was observed for meningothelial meningiomas [47]. Therefore, calpain may be important for the regulation of merlin function but does not appear to be a causative factor in NF2.

3.2. Structural proteins supporting merlin function

Merlin is a member of the protein 4.1 superfamily and ERM family of proteins that are known for their structural roles in cytoskeletal organization with respect to the plasma membrane. This section describes merlin interactions with cytoskeletal structural proteins highlighting similarities in function for merlin in cytoskeletal-membrane organization to other protein 4.1 superfamily members.

3.2.1. Actin

Each of the ERM proteins has a well-characterized F-actin binding site in the C-terminal domain, including the last 34 amino acids in ezrin that is lacking in merlin [48]. Similar subcellular distribution between merlin and ERM proteins strongly suggested a similar mechanism in merlin for actin binding. However, while a homologous actin binding site in merlin has not been identified, efforts to identify such a site led to the finding of a unique actin binding site in the merlin N-terminal domain. Interaction between polymerized F-actin and merlin was demonstrated by GST pull-down studies showing that the actin binding site in merlin is within merlin residues 178–367 [49]. These authors showed that actin binding was similar for both merlin isoforms, and was not affected by the *NF2* missense mutations L64P, K413E, and L535P. Merlins with the missense mutations V219M and N220Y were also characterized as similar to wildtype merlin in their interaction with actin while the missense mutation L360P and various truncated proteins interacted actin more weakly [50]. Alterations in affinity by the N-terminal domain of merlin may be related to intradomain folding required for optimal actin binding [29]. Other investigators have been unable to demonstrate an interaction between actin and merlin isoform 1, suggesting the merlin interaction with actin is considerably weaker than other ERM proteins [20,51]. While ERM proteins interact with both F-actin and G-actin, merlin interaction with G-actin could not be demonstrated suggesting a distinct functional role for merlin [51].

Efforts to unravel the functional relevance of the merlin–actin interaction eventually led to considerable understanding of merlin actions in the Rho and Rac pathways [36,52].

Schwannoma cells were characterized as possessing disorganized actin including abnormal stress fibers and ruffling membranes. In support of this, work by the author demonstrated that knockdown of merlin expression in STS26T schwannoma cells by *NF2* antisense resulted in disorganized actin stress fibers [57]. Organization of stress fibers and ruffling membranes has been associated with the Rho and Rac pathways, respectively [53]. These schwannoma cell abnormalities could be returned to normal by overexpression of dominant negative Rho or dominant negative Rac [52]. Because *NF2* mutations are the most common abnormalities of schwannoma cells and merlin was demonstrated to directly interact with actin, it was intuitive to hypothesize that over-activation of Rho and Rac and disorganization of the actin cytoskeleton were due to merlin loss. Subsequent work by the same research team demonstrated that replacement of merlin reverses the actin cytoskeletal defects in human schwannoma cells [28]. These authors proved this merlin action exists for merlin isoform 1 but not for merlin with the L64P missense mutation or merlin isoform 2. Therefore, because merlin isoform 2 interacts with actin more strongly than merlin isoform 1 but does not reverse the actin cytoskeletal defects of primary schwannoma cells, the function of merlin on cytoskeletal maintenance may be largely mediated via the Rho and Rac pathways and the direct interactions between merlin and actin may serve other functional purposes. One such function by merlin isoform 2 may be to inhibit the disassembly of F-actin [51]. Additionally, merlin appears to regulate actin polymerization by direct interaction with N-WASP in a manner independent of actin binding [54].

3.2.2. βII-spectrin

Spectrin is a structural protein member of the membrane skeleton. β-spectrin heterodimerizes with α-spectrin in an anti-parallel manner and the α−β heterodimers then form head-to-tail tetramers that are linked at their ends by a complex of focal adhesion proteins including ankyrin, vinculin, paxillin, and adducin. β-spectrin also binds actin and provides a means for anchoring the cytoskeleton to the membrane skeleton. All spectrins are exceptionally large proteins that consist of small N- and C-terminal domains separated by multiple homologous spectrin repeats. There are a variety of genes encoding different forms of β-spectrin including βI to βV and a variety of splice variants, designated $\Sigma1–\Sigma4$ (see [55] for spectrin nomenclature).

The author identified βII-spectrin as a merlin interactor using the yeast two-hybrid method to screen an adult human brain cDNA library [56]. We verified the interaction between merlin and spectrin by showing that a merlin antibody immunoprecipitated βII-spectrin. We also showed that the interaction between merlin and spectrin is direct by testing the ability for a merlin fragment fused to GST to interact various immobilized fragments of βII-spectrin fused to maltose binding protein (MBP). The minimally interacting regions include merlin C-terminal residues 469–590, and spectrin residues 1774–1992 which includes spectrin repeats 15, 16, and part of 17, determined by *in vitro* binding assays [56]. We have also shown that merlin binds spectrin between spectrin

repeats 7–9 [57], subsequently verified by other investigators [58]. Merlin and spectrin co-localized in STS26T cells by confocal microscopy [56]. We also demonstrated that merlin is important for the organization of the actin cytoskeleton. Merlin was also shown to interact with a novel form of spectrin (β-SpII σ2) that localizes to the plasma membrane of Purkinje cell neurons supporting that merlin might have a role in the organization of neuronal actin [59]. Additionally, protein 4.1R also interacts with βII-spectrin, but at a site that is not conserved in merlin [60].

3.2.3. Microtubules

By investigating merlin interactions with the cytoskeleton it was discovered that merlin interacts with microtubules polymerized *in vitro* [49]. The interaction is mediated by a microtubule binding region in the N-terminal domain of merlin that is masked in the full-length protein, within merlin residues 122–302. Merlin isoform 1 failed to interact with polymerized microtubules while merlin isoform 2 and various merlin partial proteins (including the residues 1–302) interacted, suggesting the binding site is masked [49]. Subsequent work of the same research team demonstrated that L64P mutation in merlin isoform 1 unfolded the protein allowing interaction with microtubules [61]. Gutmann et al. [26] also tested a number of merlin partial proteins for interaction with microtubules including deletions lacking residues 1–121 narrowing the microtubule binding site to residues 122–302, resulting in the model for merlin folding described above.

The merlin interaction with microtubules was largely used as a tool to formulate the model for how folding in merlin affects interactions with other proteins and there has been little discussion on whether the interaction occurs *in vivo* and what the functional relevance might be. However, one study that showed *Drosophila* merlin is required for oocyte axis formation documented microtubule disorganization in *Drosophila* oocytes harboring mutation in the *Drosophila* merlin gene [62]. Additionally, a recent study showed that merlin binds microtubules in cultured glioma cells and that merlin promotes tubulin polymerization in cultured Schwann cells [63].

3.3. Growth activators suppressed by merlin

The merlin interactions described above provide a basis for understanding the control of merlin activation depending upon its status of phosphorylation and folding as well as interaction with the cytoskeleton. Described in this section are 14 merlin interacting proteins whose functions are primarily growth-stimulating. These constitute a unique class of proteins whose growth-promoting functions are inhibited by merlin interaction.

3.3.1. Ezrin/moesin/radixin (the ERM proteins)

A number of studies have demonstrated interactions between merlin and ezrin, radixin or moesin, the ERM proteins. These interactions are presented in a single section here because of similarity of intra- and intermolecular interactions among ezrin, radixin and moesin, including merlin binding characteristics.

Many features of merlin folding were described in Section 2, above, whereby merlin folds in a head-to-tail manner. Heterodimerization among the ERM proteins and merlin also involves N-terminal domain with C-terminal domain interactions. Merlin N-terminal domain affinity for the merlin C-terminal domain is weak compared to ezrin N-terminal domain affinity for the ezrin C-terminal domain [64]. The C-terminal domain of merlin binds the N-terminal domain of ezrin stronger than it binds the N-terminal domain of itself [64,65]. This dynamic interaction likely supports functions of both merlin and ezrin.

Phosphorylation of merlin promotes heterodimerization between merlin and ezrin, suggesting that ezrin preferentially binds inactive merlin in its unfolded conformation [35]. Supporting a functional connection between merlin and ezrin, an expression profiling study comparing mRNA levels between vestibular schwannomas and normal vestibular nerve showed ezrin expression was reduced in five of seven (∼70%) of the tumors studied [66].

The regions of interaction involved in merlin self-association are similar to those involved in binding ERM proteins. Binding sites in merlin for ezrin include the N-terminal domain residues 1–313 and the C-terminal residues of isoform 1 only, 359–595 [64]. Few studies have characterized merlin binding with radixin and moesin, demonstrating merlin binding for radixin in GST pull-down assays and narrowing the moesin binding site in merlin to within N-terminal FERM domain residues 1–332 [21,23].

3.3.2. CD44

Shortly after the discovery of the *NF2* gene Tsukita et al. showed that ezrin and CD44 were interacting partners, and thereafter it was hypothesized that merlin might also interact with CD44 based on the high degree of homology between the ezrin and merlin FERM domains [67]. Sainio et al. first demonstrated that CD44 interacted with merlin in a single GST pull-down experiment and observation of immunofluorescent co-localization of endogenous CD44 with exogenous merlin overexpressed in COS-1 cells [68]. The region in merlin where CD44 interacts was recently identified to be within merlin residues 1–50 [69]. Overexpression of merlin lacking these residues in Tr6BC1 human schwannoma cells resulted in elevated tumor growth in xenograft mice [69]. The mechanism for this action was merlin inhibition of CD44 binding of its ligand, hyaluronate [69].

Morrison et al. thoroughly characterized the merlin CD44 interaction, functionally. Merlin interacted with the C-terminal cytoplasmic tail of CD44 in a pull-down assay, a region in CD44 that is conserved among all CD44 isoforms [37]. Expression of the cytoplasmic tail of CD44 blocked the growth inhibition mediated by merlin. Cells overexpressing merlin that were treated with hyaluronate displayed more inhibition of growth compared to cells not overexpressing merlin. Changes in merlin phosphorylation status were also rapidly mediated by hyaluronate treatment. These authors concluded that merlin participates with CD44 in a molecular switch signaling cellular growth at low abundance when merlin is phosphorylated and

unbound to CD44 which is interacted with phosphorylated ezrin and moesin, or growth inhibition at high cell abundance when CD44 is bound to unphosphorylated merlin which then inhibits signaling mediated by Ras.

3.3.3. Layilin

Structural similarities between talin and ERM proteins and the observation that these proteins co-occur with the talin interacting protein layilin at ruffling membranes inspired testing of ERM proteins and merlin for interaction with layilin. Layilin is a transmembrane protein with a C-type lecitin domain that serves as a receptor for hyaluronan [70]. The function of layilin is not entirely certain and extremely little is published on this protein, but it appears that layilin is involved in the organization of focal adhesions controlling cell–cell interactions [71]. The merlin–layilin interaction was demonstrated by GST pull-down assays revealing the minimal regions of interaction to include merlin FERM domain residues 1–332 and layilin residues 330–374 [72]. This region in layilin includes the talin binding site, and excess talin was demonstrated to out-compete with merlin for layilin binding. Co-immunoprecipitation of endogenous layilin with exogenous merlin expressed in a tet-on cell line (RT4-D6P2T) also demonstrated a positive interaction between the two proteins [72]. While phosphatidylinositol 4-phosphate (PIP) enhanced the interaction between radixin to layilin, PIP had no effect on merlin affinity for layilin [72]. No interaction was demonstrated between layilin and ezrin. Bono et al. [72] concluded that since CD44 and layilin share hyaluronan as a ligand and both interact merlin that perhaps these two cell surface receptors also share like functional roles in cell growth and metastasis.

3.3.4. TRBP

The transactivation responsive RNA binding protein (TRBP) is a 366 amino acid protein encoded by the *tarbp2* gene located on chromosome 12p12.1-q13.1, with a pseudogene located on 8q22-qter [73]. Functional studies on TRBP demonstrated oncoprotein-like activity. TRBP interacts with ribosomes and its overexpression promotes cellular proliferation [74,75]. TRBP is a regulator of the interferon-induced ribosome-associated protein kinase PKR [76]. One known function of PKR is to inhibit protein translation upon binding double-stranded viral RNAs. Once bound to double-stranded RNAs, PKR becomes activated by autophosphorylation and then phosphorylates eIF2α to inhibit its action in protein translation [77]. TRBP overexpression is associated with reduced abundance of phosphorylated PKR and stimulation of protein translation [76]. eIF2α is overexpressed in some benign and malignant neoplasms of melanocytes and colonic epithelium [78,79].

TRBP was identified as a merlin interacting protein by Gal4-based yeast two-hybrid screening of a human adult brain cDNA library [80]. The region of merlin that interacts with TRBP lies within the C-terminal half of merlin residues 288–595. The portion of TRBP involved in interaction with merlin is within TRBP residues 237–366. The interaction was validated in a pull-down assay where GST-TRBP was incubated with extracts of NIH3T3 cells overexpressing full-length merlin or merlin fragments, and also by co-immunoprecipitation from NIH3T3 cells overexpressing both proteins [80]. Co-localization of both proteins expressed as merlin-RFP and TRBP-GFP fusions in NIH3T3 cells was observed at membrane and perinuclear structures by confocal microscopy [80]. TRBP was observed in the nucleolus [80] which is of interest considering both TRBP and PKR had previously been localized to the nucleolus [80,81] and we also had previously co-localized βII-spectrin and merlin in nucleoli [56].

Merlin inhibited proliferation mediated by TRBP overexpression. Lee et al. [145] studied the effects of merlin and TRBP on proliferation by generating stable NIH3T3 cell lines expressing combinations of merlin and TRBP. Growth rates were compared using a tetrazolium/formazan assay method. Vector transfected lines grew slowly compared to rapidly growing TRBP transfected lines, and lines transfected with both TRBP and merlin were intermediate [80]. The same cell lines were assessed in a soft agar clonogenic assay in which only the TRBP transfected lines formed significant numbers of colonies (∼30) after 15 days incubation while all other lines formed fewer than 5 colonies [80]. Lee et al. [145] also assessed the ability for these cell lines to form tumors in the nude mouse. With replicates of three, no mice injected with vector or NF2 transfected cells formed tumors, all mice injected with TRBP transfected cells formed tumors, and only one mouse formed a tumor when injected with cells transfected with both TRBP and NF2. These findings suggest that merlin can inhibit tumorigenesis mediated by TRBP and that merlin can regulate the protein translation pathways controlled by TRBP.

3.3.5. eIF3c

The author identified the eukaryotic initiation factor 3 subunit c (eIF3c) as a merlin binding partner by yeast two-hybrid screening of a lymphocyte cDNA library [82]. eIF3c is a 913 amino acid protein that is a member of the eIF3 complex that includes a total of eleven protein subunits. We validated the interaction between eIF3c and merlin in multiple systems. Verifications included three different yeast two-hybrid methods, including the pACT and pGAD10 Gal-4 based systems, and interaction between merlin and eIF3c in the Ras rescue yeast two-hybrid system. Merlin and eIF3c interacted *in vitro* and the interaction was also demonstrated *in vivo* in STS26T cells by co-immunoprecipitation of merlin with an anti-eIF3c antibody, and co-immunoprecipitation of eIF3c with two different anti-merlin antibodies. Further validation was obtained in cultured STS26T cells by showing a positive fluorescence resonance energy transfer (FRET) signal between endogenous merlin and exogenous myc-tagged eIF3c at cytoplasmic puncta, while FRET was negative in a paired experiment that utilized a myc-tagged control protein. The interacting regions included the FERM domain of merlin (merlin residues 1–304), and the C-terminal half of eIF3c including eIF3c residues 405–913. We also demonstrated that merlin expression countered eIF3c mediated proliferation of STS26T cells. Because approximately 50% of meningiomas lack merlin because of *NF2* gene mutation, we investigated correlated expression in meningiomas. We demonstrated that eIF3c and merlin expression were

inversely correlated in meningiomas suggesting that loss of the interaction may be a significant event in tumorigenesis [82]. There are few studies that similarly assessed the expression of a merlin interactor in tumors of the NF2 phenotype. One demonstrated a constant level of HRS expression regardless of merlin expression in meningiomas [21], and two others showed no correlations between merlin and calpain expression [46,47].

Interaction by merlin with eIF3c suggests a possible role for merlin in the regulation of eIF3c-mediated protein translation. Furthermore, this interaction suggests that drugs targeting mTOR may be effective for treating NF2 patient tumors. The eIF3 complex is required for the initiation of protein translation and a direct interaction between eIF3 and mTOR in the preinitiation complex has been demonstrated [83,84]. This interaction is the rate-limiting step in the transition from the G0/G1 to S phase of the cell cycle and is inhibited by rapamycin [83,85,86]. Note also that an analogous function was described for the homologue to merlin protein 4.1R. Protein 4.1R interacts with the eIF3 subunit eIF3g and had a significant effect on regulating protein translation through this interaction [87]. Additionally, a number of other eIF3 subunits are abnormally expressed in tumors. Overabundance of other eIF3 complex members was associated with pathogenesis of a variety of tumor types. eIF3h is amplified in breast and prostate cancers and eIF3h amplification was significantly associated with decreased survival in prostate cancer patients [88,89]. eIF3a is amplified in breast, cervical, esophageal, stomach, and lung cancers [90,91]. eIF3e is the Int-6 oncoprotein in the mouse, binds eIF3c, and causes malignant transformation in NIH/3T3 cells [92–95]. eIF3c overexpression is associated with testicular germ cell tumors (seminomas) [96,97]. The gene for eIF3c, *EIF3S8*, is located at CHR 16p11.2 within an unstable region capable of duplication, and intact duplication of the entire eIF3c gene has been demonstrated in multiple tissues [98]. An illustration of how merlin may regulate protein translation by way of its interactions with eIF3c and TRBP is shown in Fig. 3.

3.3.6. PIKE

Another means by which merlin could regulate mTOR signaling is by way of its interaction with the phosphatidylinositol 3-kinase (PI3-kinase) enhancer PIKE, a GTPase that interacts with and enhances PI3-kinase. PIKE has three splice variants including the brain-specific forms PIKE-L and PIKE-S, and a more widely distributed form PIKE-A [99]. PIKE-L and PIKE-A have a C-terminal domain with Arf-GAP function lacking in PIKE-S, and PIKE-A lacks an N-terminal domain found in PIKE-L and PIKE-S that prevents PIKE-A interaction with PI3-kinase and allows it to bind and activate AKT [99]. PIKE also regulates metabotropic glutamate receptors (mGluRs) by way of binding to the mGluR interacting protein Homer [100]. PIKE had previously been shown to interact with protein 4.1N [101]. In testing a hypothesis that merlin and PIKE might also interact based on merlin homology with protein 4.1N, Rong et al. demonstrated merlin interaction with PIKE at an N-terminal domain shared between both the L and S variants of PIKE, and was able to bind both PIKE-L and PIKE-S, but not PIKE-A. The minimal regions of interaction tested for both proteins, demonstrated by *in vitro* pull-down experiments and co-immunoprecipitation of exogenous proteins overexpressed in HEK293 or RT4-D6P2T cells, included PIKE residues 1–384 and merlin FERM domain residues 1–332. PIKE bound also to the full-length merlin but did not bind a C-terminal domain fragment of residues 342–595 or merlin containing the L64P missense mutation, and merlin failed to interact PIKE containing a P187L missense mutation. Supporting these observations, immunofluorescent microscopy demonstrated greater co-localization for PIKE and exogenous wildtype merlin than L64P merlin in RT4-D6P2T cells. The functional relevance of this interaction involves merlin regulation of PI3-kinase mediated
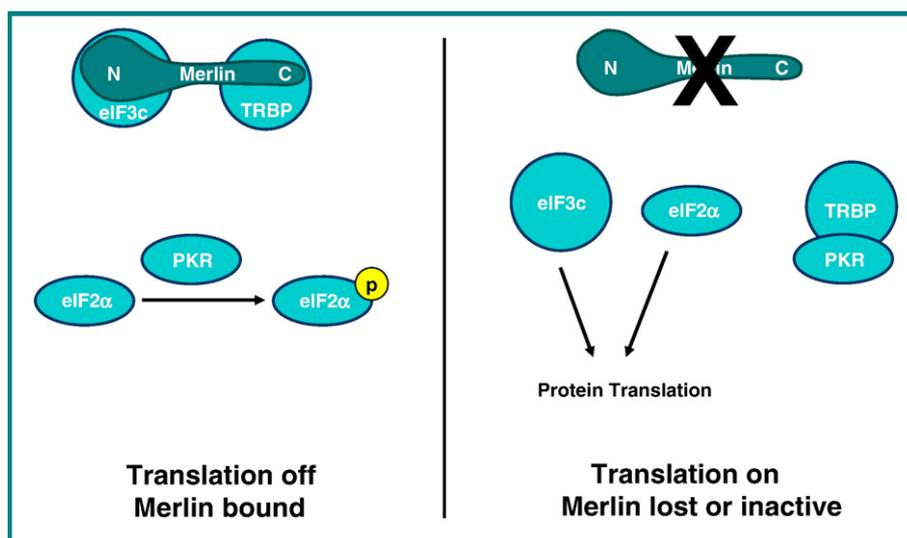
Fig. 3. Model for merlin regulation of protein translation. (A) Merlin interaction with eIF3c and TRBP involves the merlin N- and C-terminal domains respectively and merlin potentially can bind both simultaneously, blocking eIF3c action in protein translation and preventing TRBP inhibition of PKR leaving it free to phosphorylate eIF2α, further inhibiting protein translation. (B) Merlin loss by *NF2* mutation would leave eIF3c free to activate protein translation and TRBP available to bind and inhibit PKR. Merlin phosphorylation may also alter its interaction with eIF3c and TRBP.

by PIKE-L binding [102]. Increasing doses of merlin were associated with reductions in phosphatidylinositol phosphate (PIP) indicating reduced PI3-kinase activity. However, L64P merlin had no effect on phosphatidylinositol 3-phosphate (PI3P) abundance suggesting that merlin must bind PIKE-L to inhibit PI3-kinase. Overexpression of PIKE-L with the P187L missense mutation or knockdown of PIKE-L by RNA interference prevented merlin mediated reductions in cellular proliferation as determined by MTT assays. Rong et al. [102] concluded that merlin is an inhibitor of PI3-kinase signaling via its interaction with PIKE-L.

3.3.7. Syntenin

Jannatipour et al. [103] demonstrated syntenin as a merlin binding protein using the yeast two-hybrid method. Syntenin is a 30 kDa, 298 amino acid protein with two PDZ domains. The gene for syntenin, *SDCBP*, is located at CHR 8q12. While merlin isoform 1 interacted with syntenin, Jannatipour et al. [103] were unable to demonstrate syntenin binding by merlin isoform 2. The minimal binding region is within amino acids 566–595 in merlin isoform 1 and amino acids 120–273 in syntenin, encompassing both of its PDZ domains. The interaction required both of the PDZ domains of syntenin and was altered by point mutations to alanine within the last six amino acids of merlin [103]. Jannatipour et al. [103] verified syntenin as a merlin interacting protein by showing bacterially-purified GST fusions of merlin and syntenin interacted *in vitro*, and that an anti-syntenin antibody could immunoprecipitate merlin from human erythrocyte cell extracts. These authors also showed that GFP-merlin and GFP-syntenin localized at the membrane in HeLa cells, and that endogenous merlin was predominantly localized to granular structures in the cytoplasm of mouse fibroblast 3T3 cells after a full week of induced expression of syntenin antisense using a tet-inducible system [103].

Syntenin also interacts with syndecans, B-class ephrins, neurofascin, and the interleukin 5 (IL-5) receptor α-subunit (IL-5Rα) [104–107]. Syndecans are transmembrane proteoglycans that position heterogeneous heparan sulfate chains at the cell surface and possess a conserved cytoplasmic domain within which is the syntenin-binding region [107]. Syndecan-1 is a cell surface heparan sulphate proteoglycan whose expression is altered in various tumor types including breast cancers [108]. B-class ephrins are ligands of the B-class ephrin receptor tyrosine kinases [105]. Neurofascin is a widely-expressed member of the L1 immunoglobulin superfamily of cell adhesion molecules that is implicated in axonal growth in neurons, and cell attachment mediated primarily by interactions with axonin-1 and tenascin-R [109]. IL-5Rα is expressed in B-cells and granulocytes and associates with syntenin through interaction with its conserved cytoplasmic domain [104]. Activity of the transcription factor Sox4 is stimulated by syntenin association with IL-5Rα in the B-cell precursor line BaF3 [104]. Syntenin has been localized to vesicles in CHO cells [107], and to the Rab5 positive early endosome compartment in Madin–Darby canine kidney cells [110] where HRS and merlin also co-occur [111]. Syntenin binding

to phosphoinositide (PIP2) may be required for its localization to early endosomes [112].

Syntenin is a growth promoter that is overexpressed in melanomas and breast cancers [113,114]. More work is necessary to define how merlin might inhibit tumor progression possibly mediated by syntenin.

3.3.8. NHERF

The Na$^+$–H$^+$ exchanger regulatory factor (NHERF) was first described as a 44 kDa phosphorylated protein cofactor of the Na$^+$–H$^+$ exchanger (NHE3) that mediates regulation of Na$^+$–H$^+$ exchange transport in rabbit renal brush border membranes (BBM) [115–117]. NHERF interacts with and is required for cAMP-dependent protein kinase A regulation of the BBM Na$^+$–H$^+$ exchanger [118]. The ezrin binding phosphoprotein 50 (EBP50) was first described as a collection of polypeptides of 50 to 53 kDa in size eluted from an affinity column, consisting of the N-terminal domain of ezrin saturated with human placental proteins, that ran as a single 50 kDa band upon dephosphorylation [25]. Peptide microsequencing revealed that EBP50 was NHERF [25]. Reczek et al. [25] showed that NHERF/EBP50 also interacted with moesin, is strongly co-localized with ezrin in JEG-3 cells, and is widely expressed in a variety of human and murine tissues.

NHERF was identified as a merlin interacting protein by yeast two-hybrid screening of a human fetal frontal cortex library [119]. NHERF possesses two PDZ domains that are required for interacting with NHE3 [120]. The human NHERF is a 50 kDa protein of 358 amino acids in length. The minimal regions for interaction are amino acids 1–332 in merlin and amino acids 209–358 in NHERF, which is downstream of the PDZ domains in NHERF [23,119]. Murthy et al. [119] verified the interaction between merlin and NHERF by showing immobilized GST-N-terminal domain or full-length merlin isoform 1 could interact with exogenous NHERF overexpressed in COS-7 cells, and by co-localization of NHERF and merlin in COS-7 and HeLa cells in microvilli, lamellipodia, and filopodia.

NHERF is also an important link between merlin and adherens junctions. Merlin inhibition of MAP kinase signaling is mediated by the NHERF interacting protein erbin. Erbin also binds ErbB2 and controls cadheren abundances through interactions with catenins. Merlin association with adherens junction protein complexes was dependent upon erbin abundance and MEK demonstrating a MAP kinase dependency [121]. Merlin loss resulted in lack of adherens junctions and loss of contact dependent growth arrest [122].

3.3.9. RalGDS

Activated by Ras, the Ral guanine-nucleotide dissociation stimulator (RalGDS) is an activator of Ral A and Ral B GTPases, which play important roles in tumor cell anti-apoptosis and survival [123,124]. Knockout of Ral A in mice resulted in reductions in tumor incidence and proliferation compared to wildtype controls [125]. RalGDS is a 914 amino acid, 115 kDa ubiquitously expressed protein encoded by the *RALGDS* gene located within the *TSC1* critical region at Chr 9q34.1 [126,127].

RalGDS was identified as a merlin interacting protein by yeast two-hybrid screening [128]. Yeast two-hybrid interaction testing also identified the minimal regions of interaction. RalGDS interacted with merlin between merlin residues 1–288, but RalGDS failed to bind a 1–288 residue merlin fragment lacking residues 131–201. RalGDS also bound merlin fragments including residues 141–595, 1–288, and 288–595. Therefore, merlin residues 131–201 are required for the interaction, but the data suggested that residues 141–288+ are involved, and that merlin residues 141–288 represent the minimal binding region. Merlin bound the C-terminal half of RalGDS within residues 568–914. The interaction was validated as occurring directly between the two proteins by GST pull-down studies whereby ^{35}S-methionine labeled merlin generated by *in vitro* reverse transcription and translation (TnT) was interacted with purified GST-RalGDS. The interaction was validated to occur *in vivo* by immunoprecipitation of endogenous merlin with an anti-RalGDS antibody from NIH/3T3 cell extract. Co-immunoprecipitation in the opposite direction was successful from NIH/3T3 extracts containing exogenous merlin and exogenous RalGDS. Evidence for *in vivo* interaction was also demonstrated by co-localization of exogenous merlin and endogenous RalGDS in NIH/3T3 cells by confocal microscopy. The co-localization study included transfection of a merlin protein lacking residues 131–201, which did not co-localize with RalGDS as predicted by the interaction study.

Merlin inhibited the growth-promoting activities of RalGDS. Knowing that RalA more tightly binds Ral binding protein 1 (RalBP1) when RalA is GTP bound, and that RalGDS promotes the abundance of GTP bound RalA, Ryu et al. [128] investigated the effect of merlin abundance on the ability for anti-RalBP1 antibody to co-immunoprecipitate GTP bound RalA. They demonstrated that transfection of merlin in NIH/3T3 cells reduced the quantity of GTP bound RalA that could be immunoprecipitated with the anti-RalBP1 antibody, but not in NIH/3T3 cells transfected with the merlin protein lacking residues 131–201 that fails to bind RalGDS. The investigators also showed that the quantity of GTP that could be detected by thin layer chromatography (TLC) from RalA immunoprecipitated from COS7 cells was reduced by transfection of merlin. Ryu et al. [128] also demonstrated that merlin inhibited NIH/3T3 colony formation in soft agar mediated by co-transfection of RalGDS and Ral A. Reductions in RalGDS-mediated cell migration was also shown in NIH/3T3 cells by using a tissue culture scrape test whereby the scraped area was repopulated more slowly in cultures that were not overexpressing merlin, and by using the transwell method showing reduced migration to a lower serum containing chamber from an upper serum-free chamber by COS7 cell cultures overexpressing merlin.

3.3.10. EG1/magicin

The endothelial derived gene 1 (EG1) was first described as a gene that was upregulated in mitogen stimulated endothelial cells [129]. EG1 was later identified as a merlin interacting protein by yeast two-hybrid screening and given the name magicin, for merlin and Grb2 interacting cytoskeletal protein [130]. Magicin is a 24 kDa protein that interacts with the C-terminal half of merlin within merlin residues 340 to the end of either merlin isoforms, and binds merlin isoform 2 stronger than merlin isoform 1. Magicin did not interact with moesin. Immunoprecipitation experiments of endogenous magicin and merlin demonstrated that the two proteins could interact *in vivo* in T-47D cells. Immunoprecipitation experiments using exogenous merlin with either the S518D or S518A missense mutations demonstrated that these mutations in merlin do not abrogate magicin interaction. Magicin was characterized as a widely-expressed protein present in a variety of tissues and cell lines that co-localized with merlin and actin at neurites and below plasma membranes in CAD cells [130]. The CAD cell line is CNS derived with the ability to differentiate into neurons [131]. Wiederhold et al. [130] also demonstrated that magicin and merlin co-localized at the Triton X100 insoluble cytoskeleton of CAD cells using electron microscopy.

The functional relevance of the EG1/magicin–merlin interaction might relate to magicin action in Ras signaling downstream of receptor tyrosine kinases. Magicin was found to contain a consensus sequence for interacting the SH3 domain of Grb2, and was shown to directly interact Grb2 in pull-down experiments *in vitro* and in T-47D cells by immunoprecipitation [130]. Wiederhold et al. [130] also demonstrated that magicin and Grb2 occurred in a complex with merlin. Supporting magicin role in Grb2 signaling, Lu et al. showed EG1 formed a complex with map kinase activators including Src and demonstrated that EG1 overexpression activated ERK1/2, JNK, and p38 [132]. EG1 was also overexpressed in breast, colon, and prostate tumors compared to normal tissues [133]. Merlin can also regulate ERK signaling through Raf upstream of MLK3 in osteosarcoma cells [134][1].

3.3.11. HEI10

The gene for *Homo sapiens* enhancer of invasion 10 (*HEI10*) was originally identified as a growth regulatory gene from a HeLa cDNA library in a screening assay to identify cDNAs promoting agar invasion by yeast [136]. *HEI10*, mapped to chromosome 14q11.1, encodes a 277 amino acid, 32 kDa protein that directly interacts with cyclin B1 and E2 ubiquitin-conjugating enzyme UbcH7, and possesses a RING finger critical for invasion [136]. Demonstrating a role in cell cycling, HEI10 had increased nuclear localization in exponentially growing cells, and increased cytoplasmic localization in quiescent cells. Using an *in vitro* ubiquitin transfer assay, HEI10 was also demonstrated as an E3 ubiquitin ligase that requires UbcH7 for activity [136]. Toby et al. were unable to prove or disprove that HEI10 mediated cyclin B1 ubiquitination.

HEI10 was demonstrated as a direct interactor with merlin by yeast two-hybrid screening [137]. Using a combination of yeast

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[1] Note that the author is not aware of evidence demonstrating a direct interaction between Grb2 and merlin despite one report claiming the proteins interact that also stated that Wiederhold et al. [130] confirmed the direct interaction between the two proteins [135]. Likewise, there are various reports that merlin directly interacts with MLK3, but to date it has only been shown that MLK3 and merlin can be co-immunoprecipitated suggesting they can form a single complex [134].

two-hybrid tests of interaction and GST-based pull-down assays with full-length merlin or merlin fragments, the critical region for the interaction was identified within merlin residues 306–339, and HEI10 residues 116–200. Grönholm et al. [137] also verified the interaction by co-immunoprecipitation of exogenous merlin and HEI10 from 293HEK cells, and co-localization of endogenous merlin and HEI10 at ruffling membranes in newly plated U2OS osteosarcoma cells. Analysis of nuclear and cytoplasmic localization of merlin and HEI10 in synchronized U2OS cells demonstrated patterns for each suggestive of a partnership in growth regulation. Early on in the G1-phase both proteins were located in the nucleus and exited during the late G1-phase. HEI10 began its nuclear accumulation in the S-phase increasing in the G2 phase when merlin remained perinuclear. In merlin inducible tet-on RT4-D6P2T rat schwannoma cells, overexpression of merlin resulted in a reduction of nuclear HEI10 while cells expressing low merlin levels had nuclear HEI10. Note that the merlin interactor CRM1/exportin [138] is known for mediating nuclear transport during the G1 phase [139], and therefore the observation of G1-phase exit of nuclear merlin is consistent with a role involving CRM1/exportin in merlin nuclear translocation. Grönholm et al. [137] also showed data supporting that the active, unfolded merlin elicits a reduction of HEI10 expression. They demonstrated that HEI10 abundance was more reduced in cells overexpressing a constitutively active truncated merlin 1–547 that cannot fold than in cells overexpressing full-length merlin.

Sing et al. [140] further investigated the functional relationship between merlin and HEI10 in cell migration. Their study was somewhat surprising as it demonstrated that HEI10 is required for cellular proliferation, but unlike in yeast it is a negative regulator of invasion in mammalian cells. These authors demonstrated that HEI10 depletion inhibited the proliferation, migration, invasion and reattachment of U2OS cells, and elevated the expression of B-type cyclins. They concluded with the hypothesis that merlin loss results in abnormal HEI10 accumulation in the nucleus and impaired HEI10 ability to regulate cyclins involved in interphase migration [140].

3.3.12. N-WASP

The Wiskott–Aldrich syndrome is caused by mutation in the *WAS* gene, located at chromosome Xp11.23–11.22, leading to altered function of the gene product WASP [141,142]. N-WASP is a ubiquitously distributed 55 kDa member of the WASP family of proteins, that when activated by cdc42 binds the Arp2/3 complex to nucleate actin polymerization leading to branched actin structures [143]. In an effort to identify merlin interacting proteins, Manchanda et al. [54] conducted GST pull-down experiments from 293T cells identifying WASP as a protein that interacts with merlin and other ERM proteins. *In vitro* transcribed and translated N-WASP interacted with the GST-FERM domains of ERM proteins including amino acids 1–332 in merlin demonstrating direct interactions. The minimally interacting region in N-WASP that tested positive for merlin binding included N-WASP amino acids 1–158. This region comprises a domain designated WH1 known for its interaction with the WASP inhibitory protein WIP, and is a region where

most *WAS* mutations accounting for Wiskott–Aldrich syndrome are localized [144]. WASP and merlin also interacted *in vivo* as demonstrated by co-immunoprecipitation of endogenous proteins from 293T cells. Immunoprecipitations with anti-merlin antibodies demonstrated no co-immunoprecipitated ERM proteins suggesting that merlin and WASP forms a stronger interaction with WASP than do other ERM proteins [54]. These investigators also confirmed interaction *in vivo* by co-immunoprecipitation of FLAG tagged merlin FERM domain with myc tagged N-WASP WH1 domain.

Through their interaction with WASP, merlin and ERM proteins are negative regulators of actin polymerization. Manchanda et al. [54] demonstrated this by conducting component based *in vitro* polymerization assays in which they added dosewise amounts of purified merlin FERM domain to a mixture of purified Arp2/3, WASP, and pyrene-labeled actin, and quantified abundances of polymerized actin based on indirect fluorescence increases that take place upon pyrene–actin polymerization. They demonstrated highly significant dosewise reductions of polymerized actin in response to dosewise changes of merlin or moesin FERM domains, but not when they used the C-terminal halves of these proteins or when a truncated WASP protein was used. Finally, Manchanda et al. [54] conduced an assay to demonstrate that radixin could inhibit *Shigella flexneri* mediated actin polymerization. The *S. flexneri* pathogen harnesses the Arp2/3 complex stimulating actin polymerization as a means of translocation through host cells. Merlin therefore appears to be both a stabilizer of the actin cytoskeleton mediated by its direct interaction with actin or its effects on the Rho and Rac pathways as described above, and an inhibitor of actin polymerization mediated through interaction with N-WASP.

3.4. Growth inhibitors that function with merlin

Constituting another class of merlin interacting proteins are those whose functions are growth suppressive. There is evidence that some of these proteins are required for merlin function while others are not required but enhance merlin growth inhibition either additively or synergistically.

3.4.1. MAP

The merlin associated protein (MAP) is a 749 amino acid, 84.5 kDa protein thought to be a regulator of Rab GTPases. MAP was identified as a merlin interactor by yeast two-hybrid screening [145]. The regions of interaction between MAP and merlin were narrowed by secondary yeast two-hybrid interaction tests and *in vitro* GST pull-down assays demonstrating that the MAP binding site in merlin lies within the C-terminal domain residues 288–595. The interaction was also verified to occur *in vivo* in NIH/3T3 cells by co-immunoprecipitation of overexpressed merlin with endogenous MAP, overexpressed MAP with endogenous merlin, and by co-immunoprecipitation of endogenous MAP with endogenous merlin. The interaction was supported by co-localization of MAP and merlin tagged with green fluorescent protein (GFP) and red fluorescent protein (RFP), respectively [145].

MAP possesses TBC and RUN domains suggesting MAP has significant functions in regulation of Rab GTPases. TBC domains are common in the Rab GTPases and function to increase GTP hydrolysis [146]. The RUN domain was first characterized in an effector of Rab4, Rabip4, as mediating its membrane localization at endosomes [147], the RUN domain protein RUFY possesses a FYVE domain that functions in phosphatidylinositol phosphate binding important for membrane localization and vesicular trafficking [148], and the *C. elegans* UNC protein with a RUN domain functions in synaptic vesicle trafficking [149]. Since Rab proteins are important for endocytic trafficking, potentially merlin localization with HRS at early endosomes [111] might be mediated by interaction with MAP. While there are few studies showing RUN domain proteins or Rab GTPases signal to Ras/MAP kinases, Lee et al. [145] hypothesized that the interaction between MAP and merlin implicated the two in MAP kinase signaling. They tested merlin's ability to effect MAP mediated inhibition of the activator protein 1 (AP-1) complex promoter, activated by the Ras Map kinase pathway, using an AP-1-luciferase reporter assay. They demonstrated that increases in MAP in NIH/3T3 cells inhibited serum mediated AP-1 activation, and that the addition of merlin further inhibited AP-1 [145]. Co-expression of both merlin and MAP also inhibited NIH/3T3 colony formation better than either protein alone [145]. The study convincingly demonstrated that MAP and merlin cooperate to inhibit cellular proliferation at least additively.

3.4.2. RhoGDI

RhoGDP dissociation inhibitor (GDI) α is a ubiquitously expressed member of the RhoGDI family which includes the tissue specific proteins RhoGDIβ and RhoGDIγ. RhoGDIs control cytoskeletal remodeling by preventing GDP dissociation by Rho family members Rho, Rac and Cdc42 [150]. RhoGDIα is a 20 kDa protein encoded by the *ARHGDIA* gene on chromosome 17q25.3 [151,152].

A previous finding that FERM domains of ezrin, radixin and moesin interacted with RhoGDI [153] encouraged the investigation on whether or not merlin might interact with RhoGDI. Maeda et al. tested the interaction between merlin and RhoGDI using an *in vitro* binding method in which purified fragments of RhoGDI fused to GST were interacted with reverse transcribed and translated (TnT) ^{35}S-labeled merlin or merlin fragments [154]. The *in vitro* binding assays demonstrated that RhoGDI interacted with merlin at the merlin FERM domain within residues 1–341. The *in vitro* assays also suggested that RhoGDI interacted with full-length merlin isoform 2 but not with full-length merlin isoform 1 due to its folded conformation [154]. RhoGDI interacted with merlin isoform 1 with approximately the same affinity as for isoform 2 when a small portion of the N-terminal domain was deleted. This was revealed when the investigators learned that the proteins synthesized in their TnT reactions were a mixture of merlins including a species lacking an undetermined N-terminal portion including the epitope for the Santa Cruz A-19 antibody at amino acids 2–21. The interaction between merlin and RhoGDI was validated by co-immunoprecipitation of endogenous RhoGDI with endogenous merlin, and by partial co-localization of endogenous RhoGDI with exogenous merlin at membranes. Scatchard analysis determined a K_d for merlin and RhoGDI of 0.2 μM, similar to that for merlin and radixin of $K_d = 0.6$ μM [153,154]. By testing both GST-merlin and GST-radixin for interaction with ^{35}S-labeled RhoGDI, Maeda et al. [154] demonstrated that RhoGDI binding by radixin was reduced as merlin concentration increased suggesting that radixin and merlin compete for RhoGDI binding.

RhoGDI is an inhibitor of proliferation that has been observed in elevated concentrations in some tumors [155]. Hypothetically, merlin as a tumor suppressor is an enhancer of RhoGDI function.

3.4.3. Paxillin

Obermiski et al. demonstrated that merlin co-immunoprecipitated and co-localized with βI-integrin in Schwann cells [156]. Subsequent work by the investigators to determine the nature of the merlin–βI-integrin interaction led to the demonstration that merlin interacts directly with paxillin in a complex including focal adhesion kinase (FAK), and that paxillin binding mediated merlin localization with βI-integrin at the cell membrane [157]. Paxillin is a 557 amino acid protein whose gene, *PAX*, is located at CHR 12q24. Paxillin was validated as a merlin binding partner by co-immunoprecipitation and co-localization of endogenous proteins, and by *in vitro* binding studies. The *in vitro* binding studies demonstrated both merlin isoforms interacted with paxillin, and that there exist two paxillin binding sites in merlin, including merlin residues 50–70 and 425–450. Merlins with the naturally occurring missense mutations Trp60Cys and Phe62Ser that occur within the paxillin binding site in merlin, failed to interact with paxillin in *in vitro* binding assays.

The significance of merlin interaction with integrin with regard to NF2 pathogenesis may relate to signal pathways of integrin and ErbB2. Merlin in confluent cells is phosphorylated and inactive while subconfluent cells possess non-phosphorylated merlin that is active and localized to the plasma membrane [37]. Fernandez-Valle et al. [157] demonstrated that in subconfluent cells ErbB2, merlin and paxillin co-localized with βI-integrin at the plasma membrane while in confluent cells ErbB2 was localized with merlin and paxillin at the perinuclear cytoplasm. As pointed out by Fernandez-Valle et al., this observation is consistent with a merlin role of internalizing cell-surface receptors. In other studies, merlin overexpression inhibited trafficking of EGFR, while the merlin interacting protein HRS facilitated EGFR trafficking to the perinuclear region [158,159].

3.4.4. HRS

The hepatocyte growth factor receptor tyrosine kinase substrate (HRS) is an endosomal protein that is required for the trafficking of receptor tyrosine kinases (RTKs) from the early endosome to the lysosome where they are degraded. HRS is a 777 amino acid protein encoded by the *HGS* gene located at CHR 17q25. The author identified HRS as a merlin binding protein by yeast two-hybrid screening of an adult human brain

cDNA library [111]. The screen was conducted with the full-length merlin isoform 2 cDNA clone resulting in a full-length cDNA clone of HRS. The interaction was validated by co-immunoprecipitation of endogenous HRS and merlin from STS26T cell extracts, co-localization in STS26T cells, and by *in vitro* binding assays [111]. Yeast two-hybrid tests of interaction identified the interacting region in merlin to within the C-terminal half of the protein, including merlin residues 256–590 [111]. Further *in vitro* testing narrowed the HRS interacting region in merlin to merlin residues 453–557 [21] and the merlin interacting region in HRS to the HRS coiled-coil domain including residues 470–497 [160].

Merlin and HRS appear to function similarly in STS26T and RT4-D6P2T cells. HRS or merlin overexpression each caused decreased abundance of active Stat3 in RT4 or STS26T cells [161]. Inducible expression of merlin and HRS also inhibited EGF activation of Stat3 in RT4 cells [158]. Merlin and HRS similarly inhibit DNA synthesis in RT4 cells [21,161]. Additionally, HRS overexpression resulted in abnormal cell spreading and motility like that seen when merlin was overexpressed [21,26].

HRS is required for trafficking of receptors from the early endosome to the lysosome, and HRS loss results in loss of multivesicular body maturation. A *Drosophila* study demonstrated that multivesicular body formation was lost in HRS knockout embryos which had malformed and incompletely invaginated endosomes [162]. Consistent with this HRS function, EGFR was more abundant in HeLa cells in which HRS was knocked down by RNA interference [163]. Studies by the author demonstrated that HRS trafficked exogenous EGFR to the lysosome and inhibited EGFR phosphorylation mediated by EGF in RT4 cells [158]. Therefore it is interesting that merlin's ability to inhibit colony formation in MEF cells was impaired in MEF cells generated from an HRS knockout mouse suggesting that merlin requires HRS to inhibit cellular proliferation [160]. We therefore hypothesized that merlin participates with HRS in the trafficking and downregulation of cell surface receptors in Schwann cells. However, merlin may also serve to inhibit EGFR signaling by preventing its internalization, as was demonstrated by Curto et al. (2007) in liver epithelial cells [159].

HRS is also required for the trafficking of traffic Toll-like receptor 4 (TLR4). TLR4 mediates innate immunity when activated by bacterial lipopolysaccharide (LPS), HSP60 or low density lipoproteins (LDL) [164]. TLR4 activation results in activation of p38 kinase, NF-κB, JNK, STAT1, and PKR [165]. It's thought that PKR activation by TLR4 causes cells to enter apoptosis [166]. Therefore, another means by which merlin might contribute to protein translational control apart from PIKE, eIF3c and TRBP is HRS regulation of PKR.

3.4.5. DCC

The deleted in colorectal cancer (*DCC*) gene encodes one member of a complex of proteins that comprise the netrin-1 receptor functioning in neuronal guidance [167]. The protein product, DCC is a member of the immunoglobulin superfamily. Efforts to characterize the functions of DCC in tumor

suppression resulted in the elucidation of its regulatory actions on cell adhesion demonstrating that HT-29 human colon cells overexpressing DCC had increased cell–cell adhesion, but loss of cell–matrix interactions, as well as loss of focal adhesions and actin stress fibers [168]. Martin et al. noted a putative ERM binding domain in the juxtamembrane region of DCC and demonstrated that DCC interacted with both ezrin and merlin by both GST pull-down assays. GST fusions of the ezrin N-terminal domain amino acids 1–341 or the merlin N-terminal domain amino acids 1–341 interacted with GST fused to the DCC cytoplasmic tail C-terminal amino acids 1156–1447. Further interactions between DCC and ezrin were demonstrated *in vivo* by co-immunoprecipitation of exogenous proteins and co-localization in HT-29 cells. Martin et al. concluded that DCC regulates cell adhesion via interaction with ERM proteins. Neurite outgrowth mediated by netrin involves DCC-dependent hydrolysis of phosphatidylinositol bisphosphate (PIP2) in cortical neurons, and therefore there is the implication that merlin could participate in DCC functioning on a wider scale. This is because ezrin was shown to directly interact with PIP2 within the conserved N-terminal domain [169], and it has subsequently been speculated by many that merlin might interact with PIP2 as well.

3.4.6. NGB

Lee et al. [170] described the interaction between merlin and NGB (NF2-associated GTP binding protein) in a comprehensive study including the identification of the interaction and several relevant functional features. An N-terminal domain merlin fragment (amino acids 1–374) was used in a yeast two-hybrid screen of a brain cDNA library resulting in the identification of a cDNA fragment expressing 243 central residues of the NGB protein [170]. NGB is 633 amino acids in length with a GTP-binding domain and a coiled-coil residing downstream of the merlin binding region. A combination of pull-down and yeast two-hybrid experiments demonstrated two regions in merlin that interact with NGB, including merlin residues 1–52 and 288–344, and that the merlin binding region in NGB lies within the G-protein homology region including NGB residues 150–449. Note that the NGB binding sites in merlin are nearly identical to the merlin regions that interact with SCHIP-1 (Fig. 1). Supporting the interaction, both NGB and merlin co-localized to the perinuclear region of 82HTB rhabdomyosarcoma cells. Lee et al. also demonstrated that NGB with lysine 395 and arginine 394 replaced by a single alanine (NGB-K395/R394A) failed to bind merlin.

Functional analysis of the merlin–NGB interaction demonstrated that NGB is a growth inhibitor that supports merlin function that also may require merlin to inhibit growth. Analysis of NGB expression in twelve cancer cell lines demonstrated its highly variable expression. Expression of NGB inhibited the proliferation of JS1 rat schwannoma cells, demonstrated both by tritiated thymidine incorporation assay and growth of JS1 cells expressing NGB in nude mice. NGB expression also reduced cell migration attachment and aggregation. The authors then screened NGB sequences in 17 human gliomas and identified two naturally occurring NGB mutations, one of which

was in the NGB coding region resulting in the P561R missense. NGB proteins with either this mutation or the NGB-K395/R394A mutation were functionally impaired in their abilities to inhibit proliferation. The authors speculated that merlin is required for NGB-mediated inhibition of cell growth. Lee et al. demonstrated that NGB was capable of binding and hydrolyzing GTP, and showed that the presence of merlin did not alter this NGB GTPase activity suggesting that merlin acts downstream of NGB. Merlin is a ubiquitinated protein that when misfolded is degraded by the ubiquitin–proteasome pathway [171]. Lee et al. [170] showed that NGB expression inhibited merlin ubiquitination by applying a combination of methods including NGB RNA interference, merlin pulse-chase turnover assays and detection of ubiquitinated merlin complexes by immunoblotting. Finally, in follow-up to their previous study showing that merlin inhibited proliferation mainly through cyclin D1 inhibition [172], the authors investigated the effects of NGB on cyclin D1. Cyclin D1 expression was reduced in JS1 schwannoma cells by over-expression of NGB and increased by transfection of NGB small interfering RNA. The ability for NGB or merlin to inhibit cell proliferation was abrogated by overexpression of cyclin D1. The authors also presented evidence that the effect of NGB on cyclin D1 may be mediated by merlin. Since cyclin D1 is a translationally controlled protein [173], it is possible that NGB and merlin signaling converge at eIF3c mediated control of protein translation.

3.4.7. Expanded

Expanded is a 1429 residue *Drosophila* member of the protein 4.1 superfamily that has N-terminal FERM domain homology with merlin and functions as a growth suppressor [174]. Expanded received its name because mutations in the *Drosophila Expanded* gene resulted in wide wings [175]. McCartney et al. [180] demonstrated that *Drosophila* Merlin and Expanded directly interact and cooperate synergistically to regulate cellular proliferation and developmental changes in *Drosophila* eyes and wing blades. In flies, Merlin and Expanded work in parallel to mediate signaling between the protocadherin FAT to the Hippo tumor suppressor pathway important in imaginal epithelial cell development and cellular proliferation [176–178]. Merlin and Expanded are also important for the regulation of adhesion receptors and EGFR [179]. A direct interaction between Merlin and Expanded was demonstrated by a series of blot overlay experiments [180]. At 635 amino acids in length, the *Drosophila* Merlin protein is 40 amino acids longer than human merlin. Full-length *Drosophila* Merlin and a Merlin fragment consisting of residues 522–635 interacted with an N-terminal FERM domain Expanded fragment of residues 1–399 but not a longer Merlin fragment of 345–635 amino acids. The result suggested that *Drosophila* Merlin and Expanded interact in a head-to-tail manner similar to interactions between human Merlin and other human ERM proteins, and also suggested that the larger Merlin fragment is folded, masking the Expanded binding site. Additionally, Expanded interacted itself, with self-interaction sites demonstrated within residues 1–399 and 773–1429. However,

Expanded did not interact with *Drosophila* Moesin consistent with the previous observation that *Drosophila* Moesin and Merlin have unique functional properties [181]. Support for the direct interaction between Expanded and Merlin was provided by positive subcellular co-localization in imaginal disc cells [180].

The relevance of the *Drosophila* Merlin and Expanded interaction to NF2 remains unclear. Blast searching with Expanded of human protein sequences deposited in GenBank demonstrated the Expanded FERM domain possesses homologies to the human protein 4.1 family protein tyrosine phosphatases and merlin, but other domains in Expanded have poor homologies to human ERM or protein 4.1 family member proteins. Consequently, the Merlin–Expanded interaction might be limited in its relevance to NF2.

3.5. Proteins with uncertain roles in merlin action

Not necessarily of lesser importance are four merlin interacting proteins whose roles are not yet clear on merlin growth suppression. CRM1/exportin mediates merlin exit from the nucleus, a function that has not yet been demonstrated required in any way for merlin growth inhibition. Caspr/paranodin is included here because it is expressed in neurons but not Schwann cells, and RIβ is expressed primarily in the brain. These proteins may be significant to NF2 where merlin might mediate signaling between neurons and Schwann cells. The last is SCHIP1, which only interacts with truncated merlin proteins, which may not exist in tumors.

3.5.1. CRM1/exportin

There have been occasional observations of merlin inside the nucleus of various cells, which are particularly pronounced for some partial proteins of merlin [17,29,57]. Kressel and Schmucker [138] examined the features of merlin that might account for nuclear localization. They observed that residues encoded by exon 2 promote cytoplasmic retention, and they identified a CRM1/exportin nuclear export signal (NES) encoded by merlin exon 15. The study suggested that merlin interacts with CRM1/exportin, a 112 kDa protein encoded by the *XPO1* gene located at chromosome 2p16. Combined deletion of exon 2 and either inhibition of CRM1-mediated nuclear export by leptomycin B treatment or deletion of the NES sequence led to merlin nuclear localization in nearly all cells [138]. Alternative splicing between the two common merlin isoforms 1 and 2 did not alter the degree of nuclear localization. The NES sequence in merlin is located at merlin residues 539–551 and has the form LNELKTEIEALKL. This sequence is altered by the two naturally occurring merlin missense mutations L539H and L542H. This region is noted in Fig. 1 as the minimal region in merlin for CRM1 interaction, however it has not been formally demonstrated that merlin directly interacts with CRM1. Kressel and Schmucker [138] hypothesized that cytoplasmic retention mediated by exon 2 encoded amino acids might be the result of some form of cytoplasmic anchoring. To test whether exon 2 encoded amino acids that anchor merlin to actin might prevent nuclear

translocation, Muranen et al. disrupted the actin cytoskeleton with cytochalasin D but found no effect on nuclear accumulation for merlin. Muranen et al. also demonstrated that wildtype merlin trafficking to the nucleus was dependent upon the cell cycle in a manner whereby merlin was primarily located in a perinuclear space during the G2 and M phases, and in the nucleus in the early G1 phase in subconfluent cells [182]. This observation is consistent with CRM1/exportin mediating export of NES-containing proteins in a cell-cycle dependent manner with peak expression in the G1/M phases [139], and function of merlin as a cell cycle regulator through its interaction with HEI10 and export of nuclear merlin during the G1 phase [183]. While direct interaction between CRM1/exportin and merlin has not been demonstrated, these studies point to significant direct actions of CRM1/exportin in merlin function.

3.5.2. RIβ

Grönholm et al. [185] demonstrated that merlin interacts with the cAMP dependent protein kinase A (PKA) regulatory subunit RIβ in hippocampal neurons. RIβ is a protein of 330 amino acids in length and the gene for RIβ, *PRKAR1B*, is located at CHR 7pter-p22 [184], and is most abundantly expressed in the brain [185]. The investigators demonstrated that the two proteins interact by co-immunoprecipitation of endogenous merlin with RIβ fused to the green fluorescent protein (GFP) from Cos7 and HEK 293 cell extracts, and by co-localization of endogenous merlin and RIβ in cultured hippocampal neurons. By using *in vitro* pull-down studies and yeast two-hybrid tests of interaction, RIβ was shown to interact within merlin residues 252–546 [185]. By screening an array of 20-mer merlin peptides with ^{32}P-labeled RIβ, a region including merlin residues 463–480 was identified as critical for the interaction [185].

Merlin interaction with RIβ suggested a possible role for merlin in learning and memory based on what was already known about RIβ in neurons [185]. Changes in the balance of the relative abundances of the regulatory and catalytic subunits of the PKA holoenzyme alters synaptic plasticity in Aplysia [186]. In *Drosophila*, flies lacking RIβ showed defective olfactory learning [187]. Investigation of hippocampal neurons in RIβ knockout mice showed that RIβ was important for normal neuronal function, including long-term potentiation and depotentiation [188].

The association between merlin and cAMP signaling through PKA also suggests a role for merlin in myelination. Schwannoma cells not in contact with the neuron lose expression of a host of myelinating proteins [189], and one important event in Schwann cell myelination is PKA stimulation by cAMP upon neuronal contact [190]. PKA activation in cultured cells by treating cells with forskolin and the phosphodiesterase inhibitor IBMX to raise cAMP abundance, resulted in merlin phosphorylation at serine 518 by PKA [35]. Additionally, because NHERF is required for cAMP mediated activation of NHE3 [118] merlin may be involved in cAMP signaling at multiple levels including feedback inhibition of merlin signaling by interaction with RIβ.

3.5.3. Caspr/paranodin

Caspr, also known as paranodin, is a 190 kDa member of the neurexin superfamily of transmembrane proteins encoded on chromosome 17q21 [191,192]. Caspr/paranodin localizes to the nodes of Ranvier in the paranodal region of myelinating Schwann cells and regulates axon–glial interactions and adhesion by interacting with contactin [193–195].

The previous finding that protein 4.1B interacted caspr/paranodin [196] encouraged Denisenko-Nehrbass et al. to test the interaction between caspr/paranodin and merlin resulting in validation of the caspr/paranodin–merlin interaction [197]. Residues 1–314 including the merlin FERM domain interacted with the C-terminal cytoplasmic domain of caspr/paranodin including a conserved motif (termed GNP) and proline rich region, but merlin could not bind when the GNP motif was deleted [197]. The interaction was demonstrated by an *in vitro* binding method in which purified fragments of caspr/paranodin fused to GST were interacted with reverse transcribed and translated ^{35}S-labeled merlin or merlin fragments. The interaction was validated by immunoprecipitation of merlin with exogenous C-terminal fragment of caspr/paranodin from COS7 cells, and by co-immunoprecipitation of endogenous merlin with endogenous caspr/paranodin from rat brain lysates.

The functional significance of the merlin–caspr/paranodin interaction may relate to myelination and adhesion involving βI-integrin. Merlin had previously been shown to form complexes with βI-integrin [156]. Denisenko-Nehrbass et al. [197] demonstrated that endogenous merlin and exogenous caspr/paranodin immunoprecipitated with an antibody against βI-integrin from Cos7 cells, and that exogenous caspr/paranodin enhanced the βI-integrin–merlin association. Since caspr/paranodin is absent in Schwann cells [193], presumably the neuronal caspr/paranodin–merlin–βI-integrin complex regulates myelination in an axon–glial mechanism.

3.5.4. SCHIP1

SCHIP1 was identified as a merlin interacting protein by yeast two-hybrid screening using an N-terminal domain merlin fragment of amino acids 1–314 as the bait to screen a fetal brain cDNA library [198]. SCHIP1 is a novel protein with little similarity to other proteins except for its coiled-coil domain which shares high homology to that of FEZ proteins and *C. elegans* UNC-76 which are involved in axonal growth [199]. Merlin residues 1–27, and 280–323 interacted with SCHIP1 [198]. These two regions of interaction are also involved in NGB binding (Fig. 1). Immunoprecipitation studies have not demonstrated an interaction between exogenous full-length merlin and SCHIP1, however, truncated merlin proteins of both isoforms 1 and 2 strongly bind [198]. The regions in SCHIP1 where merlin interacts are not fully understood, but it is clear that the SCHIP1 coiled-coil domain, residues 419–479, strengthen binding [198]. SCHIP1 also occurs naturally as a 564 amino acid fusion protein with the calmodulin binding protein IQCJ, designated IQCJ-SCHIP1 [200]. The fusion protein results from a novel transcript that spans the two adjacent *IQCJ* and *SCHIP1* genes located at 3q25, and is widely expressed but most highly in a normal brain. It is not

known whether merlin can interact with IQCJ-SCHIP1, but the region where merlin apparently binds SCHIP1 appears to be present in IQCJ-SCHIP1.

The functional significance of the merlin–SCHIP1 interaction remains unclear. No "gain-of-function" has ever been associated with *NF2* mutations, and truncated merlin proteins are not detected in NF2 patient tumors [201]. Although an interaction has not been demonstrated between SCHIP1 and full-length merlin, it remains possible that this is due entirely to experimental conditions or weak binding and that there indeed exist interactions between these proteins with relevance to NF2.

4. Conclusions

Merlin is a multifunctional tumor suppressor that interacts with several other proteins involved in the signaling of a number of pathways. Despite the considerable abundance of work on identifying merlin function, it remains unclear if any of the known merlin regulated signaling pathways is key to the development of NF2 tumors. Perhaps there is no one particular aspect of merlin function that is significant to the development of tissue specific NF2 tumors, but rather it is the loss of a unique combination of signaling pathways characteristic of merlin loss that is pathogenic. Review of the merlin interacting proteins and the pathways in which they reside reveals functional relationships (Fig. 4). These relationships suggest potential pathways that might be targeted with existing drugs. Merlin interaction with the ErbB2–Paxillin complex, PIKE, and eIF3c, effect of

NGB and merlin on cyclin D1 expression, and functional consequences of merlin loss on proliferation mediated by these interactions suggest that signaling in the ErbB2→PI-3 kinase→mTOR→eIF3c→cyclin D1 pathway may contribute to NF2 pathogenesis. Drugs targeting this pathway therefore may be useful for inhibition of NF2 tumors with overactive mTOR signaling, including herceptin, LY294002, AKT inhibitors, UCN-01, Rad-001, CCI-779, and AP23573. Merlin interactions with MAP, magicin, RalGDS and RhoGDI suggest that merlin regulates Ras, Rho and Rac signaling and the map kinases downstream. Studies have shown the small GTP binding proteins as potential therapeutic targets and several new compounds against GTPases are available that merit investigation for treating NF2 [202–204]. Such drugs include p38 map kinase inhibitors KC706 or VX-702 that may be effective inhibitors of NF2 patient tumors with overactive MAP kinase signaling. Additionally, because PAK activates Ras, Rho, and Rac signaling pathways and is inhibited by merlin, specific PAK inhibitors may be useful for treating NF2, including FK228, PP1, AG879 and PAK inhibiting peptide PAK18, some of which were useful for treating NF1 deficient breast cancer in xenograft mice [205–208]. Finally, both MAP kinase and PI3-kinase/mTOR converge at the initiation of protein translation [209] and therefore targeting these pathways may be particularly effective for NF2. Efforts to assess the effectiveness of these drugs singly and in combination on NF2 tumors are needed to determine effective treatment strategies for NF2.
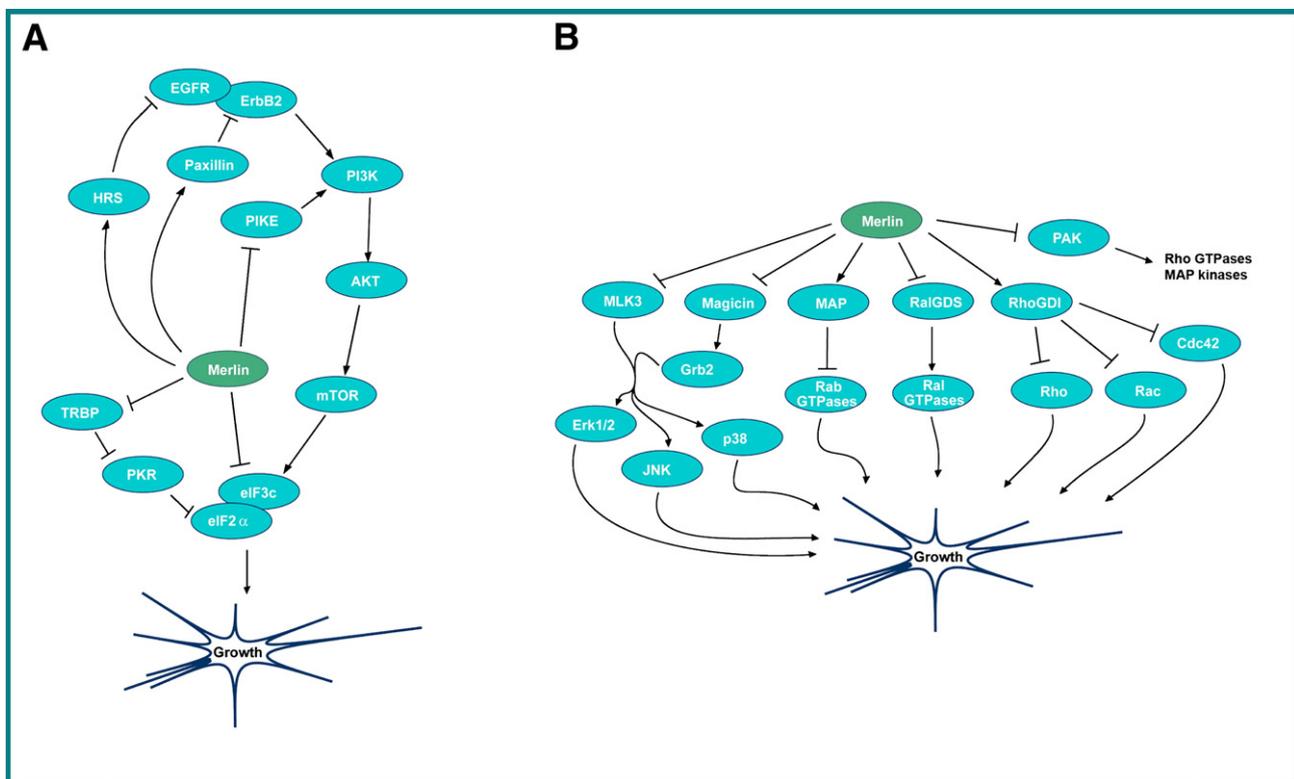

Fig. 4. Pathway maps for merlin action in PI3K signaling and small GTPase signaling. (A) Merlin inhibits signaling in the PI3K pathway at multiple points including direct inhibition of PIKE, eIF3c and TRBP, and inhibition of ErbB2 and EGFR by way of paxillin and HRS. Merlin loss leaves mTOR activation of protein translation unregulated. (B) Merlin interacts with multiple proteins that affect signaling of the small GTPases (Rab, Raf, Rho, Rac, Cdc42, Ral, Ras) resulting in growth inhibition.

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