

Merlin: a tumour suppressor with functions at the cell cortex and in the nucleus

Wei Li^{1*}, Jonathan Cooper^{1*}, Matthias A. Karajannis² & Filippo G. Giancotti¹⁺⁺

¹Cell Biology Program, Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, and ²NYU Cancer Institute and Department of Pediatrics, NYU Langone Medical Center, New York, USA

Inhibition of proliferation by cell-to-cell contact is essential for tissue organization, and its disruption contributes to tumorigenesis. The FERM domain protein Merlin, encoded by the *NF2* tumour suppressor gene, is an important mediator of contact inhibition. Merlin was thought to inhibit mitogenic signalling and activate the Hippo pathway by interacting with diverse target-effectors at or near the plasma membrane. However, recent studies highlight that Merlin pleiotropically affects signalling by migrating into the nucleus and inducing a growth-suppressive programme of gene expression through its direct inhibition of the CRL4^{DCAF1} E3 ubiquitin ligase. In addition, Merlin promotes the establishment of epithelial adhesion and polarity by recruiting Par3 and aPKC to E-cadherin-dependent junctions, and by ensuring the assembly of tight junctions. These recent advances suggest that Merlin acts at the cell cortex and in the nucleus in a similar, albeit antithetic, manner to the oncogene β -catenin.

Keywords: Merlin; type 2 neurofibromatosis; contact inhibition; tumour suppression; Hippo

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See the Glossary for abbreviations used in this article.

Introduction

Normal cells cease to proliferate when they come into contact with each other and assemble stable intercellular junctions. Contact inhibition of proliferation is essential for tissue organization and its loss is a characteristic of cancer [1]. Since its discovery as the tumour suppressor that is inactivated in neurofibromatosis type 2 (NF2) almost two decades ago [2,3], the FERM domain protein Merlin has emerged as a major effector of contact inhibition [4–7]. Furthermore, genetic studies in mice have shown that Merlin has a relatively

broad tumour suppressor function (Sidebar A). In addition to NF2, loss of Merlin function contributes to the development of sporadic meningiomas, ependymomas and schwannomas, a significant fraction of malignant pleural mesotheliomas, and a small subset of renal cell carcinomas, melanomas, glioblastomas and colorectal cancers in humans (Sidebar B; [8–14]). As for other oncogenic mutations, it remains unclear why loss of Merlin contributes to tumorigenesis in some but not other tissues (Sidebar C). Cell-type-specificity in the wiring of signalling pathways, previous accumulation of cooperating mutations and/or differences in the stromal microenvironment could all contribute to the tissue specificity of the phenotype.

Although *Nf2*-knockout embryos deteriorate at an early developmental stage due to defects in extraembryonic structures [15], tissue-specific ablation experiments have provided insight into some of the developmental roles of Merlin. Deletion of *Nf2* in the skin causes defects in epithelial adhesion and polarity that disrupt its barrier function [16]. Inactivation of *Nf2* in the liver causes a large expansion of progenitor cells, suggesting that Merlin inhibits stem-cell renewal or amplification [17,18]. Intriguingly, deletion of *Nf2* in the entire haematopoietic compartment leads to an expansion of progenitor cells that is largely secondary to the expansion of their perivascular niche [19], suggesting that Merlin can regulate stem-cell expansion by a non-cell-autonomous mechanism.

Merlin has significant sequence homology to members of the Ezrin/Radixin/Moesin (ERM) family of proteins, which in their open conformation link various cell-adhesion receptors to the cortical actin cytoskeleton [20]. On this basis, it has been argued that Merlin mediates both contact inhibition and tumour suppression by directly modulating mitogenic signal transduction at or near the plasma membrane [21,22]. Analysis of various cell types indicated that Merlin can potentially affect a variety of mitogenic pathways, such as Rac-PAK signalling [7,23–25], activation of mTORC1 independently of Akt [26,27], the EGFR-Ras-ERK pathway, the PI3K-Akt pathway and FAK-Src signalling [28–31]. In addition, genetic studies in *Drosophila* and mice showed that Merlin contributes to the activation of the Hippo tumour-suppressor pathway [18,32,33].

Against the backdrop of this rich biology, recent studies have revealed that Merlin can interact with α -catenin and Par3 at nascent adherens junctions [16], as well as with the scaffold and signalling protein Angiomotin at tight junctions [34]. In addition, it has become clear that Merlin translocates to the nucleus to modify

¹Cell Biology Program, Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 216, New York, New York 10065, USA

²NYU Cancer Institute and Department of Pediatrics, NYU Langone Medical Center, New York, New York 10016, USA

*These authors contributed equally to this work

Corresponding author. Tel: +1 212 639 7044; Fax: +1 212 794 6236;
E-mail: liw@mskcc.org

Corresponding author. Tel: +1 212 639 6998; Fax: +1 212 794 6236;
E-mail: f-giancotti@ski.mskcc.org

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gene expression through inhibition of the E3 ubiquitin ligase CRL4^{DCAF1} [35]. In this review, we discuss these recent results and the models of Merlin function they suggest, in an effort to provide a framework for future studies.

Merlin structure and modifications

ERM proteins consist of an amino-terminal FERM domain followed by a coiled-coil segment and a hydrophilic tail [20]. Although Merlin has the same domain organization and considerable sequence homology to canonical ERM proteins, it also contains unique sequence motifs [36,37]. In particular, the FERM domain of Merlin contains an evolutionarily conserved Blue box motif (residues 177–183), which is absent in canonical ERM proteins, whereas the carboxy-terminal domain of the protein lacks a canonical actin-binding motif that is present in all ERM proteins. In addition, Merlin has an extended 17 amino-acid-long N-terminal segment that is not found in other ERM proteins (Fig 1A).

Canonical ERM proteins are maintained in a dormant state by an intramolecular association between the FERM domain and the C-terminal tail [38–40]. In response to upstream activation of Rho, Rho kinase phosphorylates a threonine residue in the C-terminal domain of ERM proteins, disrupting the head-to-tail association that maintains the closed conformation (Fig 1B). Once ERM proteins adopt the open conformation, their FERM domain can associate with the cytoplasmic segment of cell-adhesion receptors—such as CD44 and ICAM—and their C-terminal domain can interact with actin filaments, regulating the organization of the cortical cytoskeleton [38,41,42].

Biochemical and mutational studies suggest that Merlin undergoes a similar conformational transition in response to PAK-mediated phosphorylation of Ser 518 (Fig 1C; [43]). However, recent evidence suggests that additional post-translational modifications might be required to fully disengage the α -helical segment of Merlin from the FERM domain [44]. The putatively open form of Merlin might associate with the cytoskeleton by forming head-to-tail heterodimers with canonical ERM proteins [45–47], as well as through additional potential mechanisms [48], whereas the closed form of Merlin seems to mediate growth inhibition *in vitro* and is thereby considered active [25,43,49–51]. Several lines of evidence support the latter point. Mutation of Ser 518 to alanine enhances the growth inhibitory activity of Merlin, whereas its change to aspartic acid eliminates this activity [52]. Overexpression of a Blue box mutant form of Merlin—presumed to be constitutively open [37,39,40]—induces overproliferation in the wing of *Drosophila*, and the murine analogue promotes cellular transformation *in vitro*, both presumably through a dominant-negative mechanism [4,53]. Finally, numerous missense mutations detected in patients affected by NF2 map to the F2 segment of the FERM domain and the most parsimonious truncation mutants lack only the C-terminal segment, which is predicted to interact with the F2 segment (Fig 2). Furthermore, structural considerations based on the analysis of the crystal structure of the closed form of Moesin—which is the only closed structure available—suggest that virtually all pathogenic missense mutations found in NF2, including the few affecting the α -helical portion of Merlin, disrupt the extended surface that mediates the interaction of the FERM domain with the C-terminal segment of the protein [39,40]. Interestingly, a recent study found that Merlin isoform 2—which lacks the five C-terminal residues found in the canonical Merlin isoform 1 necessary for interdomain

Glossary

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
Akt	protein kinase B
AMP	adenosine monophosphate
aPKC	atypical PKC
AXL	AXL receptor tyrosine kinase
Cdc42	cell division control protein 42 homologue
CRL4	cullin-ring E3 ligase 4
CRM1	chromosome region maintenance protein 1
DCAF1	DDB1- and CUL4-associated factor 1
DDB1	DNA damage-binding protein 1
EGFR	epidermal growth factor receptor
ErbB2/3	v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2/3
Erbin	ErbB2 interacting protein
ERK	extracellular-signal-regulated kinase
FAK	focal adhesion kinase
FERM	4.1 protein/Ezrin/Radixin/Moesin
HEK-293	human embryonic kidney 293
ICAM	intercellular adhesion molecule
IGF1R	insulin-like growth factor 1 receptor
Kibra	kidney and brain protein
Lats1/2	large tumour suppressor 1/2
MDCK	Madine–Darby kidney cancer
MEFs	mouse embryonic fibroblasts
Moesin	membrane-organizing extension spike protein
MST1/2	macrophage stimulating 1/2
mTORC1/2	mammalian target of rapamycin complex 1/2
MYPT1	myosin phosphatase targeting subunit 1
PAK	p21-activated kinase
Pals1	protein associated with Lin-7 1
Par3	partitioning defective 3 homologue
Patj	Pals1-associated tight junction protein
PDGFR	platelet-derived growth factor receptors
PI3K	phosphatidylinositol 3-kinase
PIKE-L	phosphoinositide 3-kinase enhancer isoform 1
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC α	protein kinase C alpha type
PP1 δ	serine/threonine-protein phosphatase PP1- δ catalytic subunit
PTEN	phosphatase and tensin homologue
Rac	Ras-related C3 botulinum toxin substrate
Ras	rat sarcoma
Rich1	RhoGAP interacting with CIP4 homologues 1
Roc1/Rbx1	RING box protein 1
S6K1	S6 kinase 1
TAZ	transcriptional coactivator with PDZ-binding motif
TEAD	TEA domain family member
TGF- β	transforming growth factor- β
TSC1/2	tuberous sclerosis complex 1/2
YAP	Yes-associated protein

binding *in vitro* [50]—suppresses *Nf2*^{−/−} Schwann cell proliferation to the same extent as Merlin isoform 1 [54]. As these results contradict previous findings [50,55], further investigation is required to understand how Merlin's conformation affects its activity and how Merlin isoform 2 functions (Sidebar C).

The levels of closed, active Merlin increase substantially in cells undergoing growth arrest due to contact inhibition, loss of matrix adhesion, deprivation of growth factors or exposure to hyaluronic acid [6,56], suggesting that Merlin integrates various anti-mitogenic signals (Fig 3A). Promitogenic signals—which are initiated by integrins and receptor tyrosine kinases and transduced by Cdc42

Sidebar A | Mouse models of NF2

Nf2-knockout mice succumb *in utero*, whereas heterozygous *Nf2* mutant mice develop multiple malignancies, especially if an allele of p53 is simultaneously inactivated. The tumours arising in *Nf2* mutant mice include hepatocellular carcinomas and osteosarcomas among others, but not schwannomas or meningiomas, suggesting that Merlin is a haploinsufficient tumour suppressor in several tissues [117]. Conditional biallelic inactivation of *Nf2* in Schwann cells leads to Schwann cell hyperplasia and schwannoma formation [118], mimicking human neurofibromatosis type 2, which results from loss of heterozygosity at the *Nf2* locus [8]. Furthermore, biallelic inactivation of *Nf2* in arachnoidal cells leads to the formation of meningiomas, which have significant histological similarity to the corresponding human tumours [119]. In a similar fashion, *Nf2*^{+/-} mice have increased sensitivity to the carcinogenic effect of inhaled asbestos [120], and conditional deletion of *Nf2* in mesothelial cells cooperates with loss of Ink4a/Arf and p53 to drive malignant mesothelioma [121]. Biallelic loss of Merlin in the liver was recently shown to result in hepatomegaly and formation of malignant tumours [17,18]. Although the issue has not been completely resolved, it seems that biallelic *Nf2* loss in the liver leads to the expansion of a progenitor population able to differentiate at least partly along the ductal lineage [99]. The complete penetrance observed in this model indicates that Merlin is a potent regulator of liver size and tumour suppression, and future studies using this model could provide great insight into the normal biological role of Merlin and its tumour suppressor activity.

and Rac—activate PAK, which directly phosphorylates Merlin at Ser 518. This phosphorylation disrupts the binding between the N-terminal FERM domain and the C-terminal tail, thereby inactivating Merlin [7,25]. Conversely, engagement of cadherins or loss of mitogenic signalling inactivates PAK, leading to an accumulation of the closed form of Merlin [56]. Although most studies have placed PAK upstream from Merlin, there is also evidence suggesting that Merlin can suppress PAK activation [24,57]. Recent studies suggest that this potential feed-forward mechanism is restricted to epithelial cells that express Erbin. Binding to Erbin allows Merlin to inactivate PAK2, disabling one branch of non-canonical TGF- β signalling [58]. Ser 518 can also be phosphorylated by PKA, suggesting that Merlin can also be inactivated by the cyclic AMP–PKA pathway, a signalling axis that regulates gene expression, cell growth and cell cycle progression in Schwann cells [59]. Increases in dephosphorylated Merlin might be a result not only of PAK inhibition but also of the activation of a Merlin phosphatase—such as MYPT1-PP1 δ —which dephosphorylates Ser 518, thus activating Merlin [26]. Overexpression of CPI-17, an MYPT1 cellular inhibitor, induces neoplastic transformation *in vitro*, underscoring the importance of Ser 518 dephosphorylation [29]. However, whether MYPT1-mediated dephosphorylation of Merlin is necessary and sufficient for activation of Merlin *in vivo* remains to be determined.

Phosphorylation at Ser 518 seems to be the primary post-translational modification that drives the activation–inactivation cycle. Nevertheless, additional phosphorylation events or other post-translational modifications might be required to disengage the extended contacts that the α -helical and C-terminal segments of Merlin form with the FERM domain in the closed conformation (Sidebar C; [39]). In agreement with this hypothesis, band-shift experiments suggest that Merlin is post-translationally modified at multiple sites in response to mitogenic stimuli [56]. Overexpression studies suggest that AKT can phosphorylate Merlin at Thr 230 and Ser 315, stabilizing the open conformation and

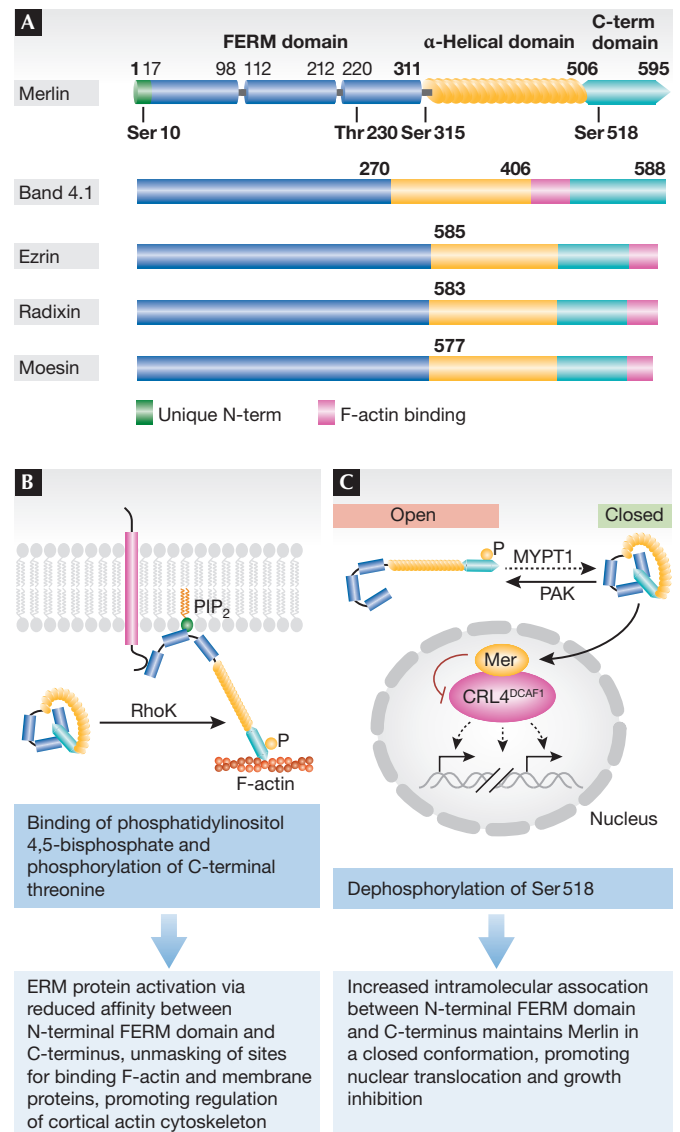


Fig 1 | Merlin and ERM protein domain organization and phosphoregulation.

(A) Merlin and other canonical ERM proteins have similar domain organizations consisting of an amino-terminal (N-term) FERM domain that is divided into three subdomains, an α -helical coiled-coil domain and a carboxy-terminal (C-term) hydrophilic tail. Canonical ERM proteins contain an actin-binding C-terminal ERM-associated domain (C-ERMAD; pink), whereas Merlin does not. It also has an extended N-terminal motif (green) unique among ERM proteins, illustrating its divergent structure with respect to other ERM proteins. Merlin phosphorylation sites are indicated. (B) Canonical ERM proteins are maintained in an inactive state by intramolecular interaction between the C-terminal tail and FERM domain. Phosphorylation of a C-terminal threonine by Rho kinase—which might be aided by ERM protein recruitment to membrane regions rich in phosphatidylinositol 4,5-bisphosphate—activates the protein by disrupting the head-to-tail interaction. (C) Conversely, Merlin's dephosphorylated and closed form is active and functions in tumour suppression and contact inhibition. Phosphorylation by PAK and PKA at Ser 518 renders the protein inactive in its putatively open form. ERM, Ezrin/Radixin/Moesin; FERM, 4.1 protein/Ezrin/Radixin/Moesin; MYPT1, myosin phosphatase targeting subunit 1; PAK, p21-activated kinase; PKA, protein kinase A; PIP₂, phosphatidylinositol 4,5-bisphosphate; RhoK, Ras homologue gene family, member K.

disrupting binding to PIKE-L—a GTPase that binds to PI3K and enhances its activity [60]. **These observations suggest that constitutive activation of PI3K–Akt signalling, as occurs in cells lacking PTEN or carrying activating mutations in PI3K, could lead to inactivation of Merlin and potentially eliminate a negative-feedback loop that restrains the activation of PI3K.** In addition, Akt-mediated phosphorylation has been proposed to promote Merlin ubiquitination and proteasome-mediated degradation [60]. However, the significance of these events remains unclear because cycloheximide chase experiments suggest that endogenous Merlin is not significantly degraded by the ubiquitin–proteasome system (UPS; [35]). Finally, PKA phosphorylation of Ser 10 was recently shown to alter the organization of the actin cytoskeleton without affecting Ser 518 phosphorylation [61].

Merlin localization

Due to its homology with classical ERM proteins and seemingly prevalent co-localization with cortical actin below the plasma membrane, Merlin has been proposed to regulate mitogenic signalling by organizing membrane domains [48]. The accumulation of Merlin at cell-to-cell junctions in confluent epithelial and endothelial cells [5,7], within lamellipodia in various types of cell [62–64], and on the surface of endocytic vesicles in *Drosophila* epithelial tissues [53,65] is consistent with this general hypothesis (Sidebar C).

Recent studies have shown that the distribution of Merlin can change dynamically in response to various signals and is more diverse than previously anticipated. For example, analysis of the behaviour of the Blue box and the S518A mutant forms of Merlin suggests that the open conformer of Merlin generated by integrin-mediated activation of PAK accumulates underneath matrix adhesions [7]. Skin-epithelium-specific deletion of Merlin in mouse shows that the protein transiently associates with primordial intercellular adhesions and regulates their maturation into adherens junctions, as well as the formation of tight junctions [16]. Although this study failed to detect any association of Merlin with mature adherens or tight junctions, another recent study indicated that Merlin is enriched at tight junctions in confluent MDCK cells and co-localizes with E-cadherin at the paranodes and Schmidt–Lanterman incisures of myelinating Schwann cells [34]. Finally, a significant fraction of Merlin is known to be associated with the soluble, cytosolic fraction in many cell types [64,66], and a smaller amount can move to a detergent-insoluble fraction that has been interpreted as lipid rafts in response to contact inhibition [67].

The above results support the hypothesis that Merlin functions at or near the plasma membrane. Nevertheless, the closed form of Merlin has recently been shown to suppress tumorigenesis by translocating to the nucleus, where it inhibits the E3 ubiquitin ligase CRL4^{DCAF1} [35]. These findings are fully consistent with the widely held view that only the closed form of Merlin is able to suppress tumorigenesis [43,50,52] and with reports suggesting that Merlin shuttles in and out of the nucleus [68,69]. However, most previous studies had failed to detect Merlin in the nucleus, possibly because the antibodies used are directed against an epitope of nuclear Merlin that is masked under standard fixation and permeabilization conditions. In fact, Merlin was observed in the nucleus in multiple cell types by using an antibody that recognizes its C-terminus and an enhanced permeabilization technique [35]. Similar problems initially hampered the detection of β -catenin in the nucleus [70,71]. In addition, consistent with the nucleo-cytoplasmic shuttling model,

Sidebar B | Clinical features of NF2

Neurofibromatosis type 2 (NF2) is an autosomal dominant genetic disorder with an incidence of approximately 1 in 40,000. It is caused by inactivation of the *NF2* gene located on chromosome 22q [2,3]. NF2 patients develop multiple central nervous system (CNS) and peripheral nervous system (PNS) tumours. The locations and types of CNS and PNS tumours seen in these patients are highly specific. Schwannomas arise from Schwann cells that form the myelin sheath surrounding the sensory and motor neurons. The hallmark of NF2 is the development of bilateral vestibular schwannomas (VS), which arise at the vestibular branch of the VIIIth cranial nerve. In addition to VS, most NF2 patients develop schwannomas in other locations, such as other cranial nerves and peripheral nerves, including nerve roots. Most NF2 patients experience progressive hearing loss in adolescence or young adulthood due to bilateral VS. Life-threatening neurological complications occur when these tumours reach a critical size. In addition, schwannomas involving other cranial nerves can impair swallowing, vision and facial function. Other CNS and PNS tumours seen in NF2 patients include meningiomas, which arise from arachnoid cap cells, and ependymomas, which arise from ependymal cells lining the ventricles and central spinal canal. There is a clear association between genotype and phenotype in NF2 patients, with nonsense/frameshift mutations being associated with earlier onset of symptoms, larger tumour burden and shorter life expectancy [122]. Sporadic VS, which occur in non-NF2 patients, consistently lack expression of detectable Merlin [123], and genetic inactivation of the *Nf2* gene also occurs in the majority of sporadic meningiomas [124–126], indicating similarities in tumour biology between sporadic and NF2-related VS and meningiomas. Sporadic VS have an incidence of roughly 3,000 per year in the USA, which seems to be rising [127], and sporadic meningiomas are the most common type of brain tumour, accounting for approximately 25% of primary intracranial tumours in the USA [128]. Despite major advances in neuroimaging and neurosurgical techniques over the past decades—including microsurgery and stereotaxis—the neurosurgical management of NF2 patients remains challenging. The identification of effective drugs to treat these patients would also be relevant for large patient populations without NF2, as there is no known effective treatment option for unresectable or progressive sporadic meningiomas.

Merlin was recently found to move along microtubules towards the nucleus in *Drosophila*, in a kinesin-1- and dynein-dependent manner [72]. Thus, Merlin could potentially have a similar mechanism of intracellular transport in mammals (Sidebar C). Although Merlin does not have a canonical nuclear localization sequence, a deletion mutant lacking four amino acids near the N-terminus does not localize to the nucleus, suggesting that these residues are essential for Merlin nuclear translocation (W.L. and F.G.G., unpublished observation). In addition, Merlin contains a motif in exon 15 that promotes export via the CRM1–exportin pathway [68], and truncation mutants lacking this sequence have a prominent nuclear localization [35,54]. In the closed form, intramolecular interactions could block the recognition of this export sequence, contributing to Merlin nuclear translocation.

Recent mutational analyses have attempted to distinguish between plasma membrane and nuclear models of Merlin function. Merlin has been reported to associate with the plasma membrane by binding to phosphoinositides, particularly PIP₂, through six charged residues within the FERM domain that are conserved in other ERM proteins. Although the interaction of Merlin with phosphoinositides is dispensable for Ser 518 phosphoregulation, it seems necessary for Merlin's localization to the plasma membrane and for some aspects of growth suppression [73]. **In addition, deletion of the 17 N-terminal residues that are not found in other ERM**

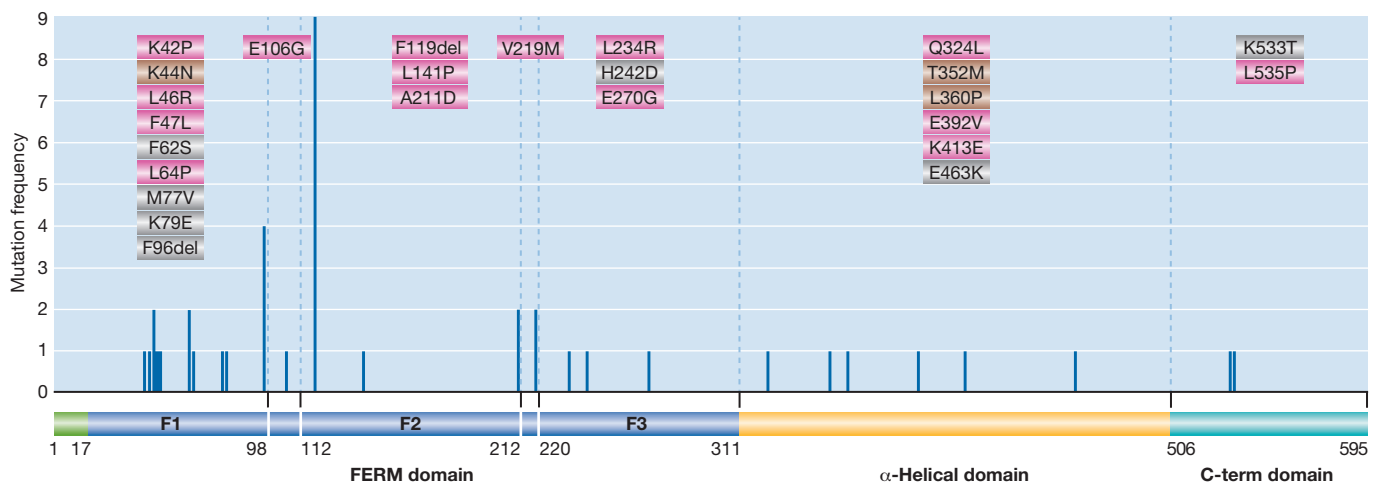


Fig 2 | Merlin missense mutations and single residue deletions in NF2. The position, frequency and type of mutation are plotted on a schematic diagram of Merlin. The pathogenicity of mutations supported by biological evidence is shown in pink, by genetic evidence in grey and by both biological and genetic evidence in brown. These mutations either correlate with disease in multiple members of a family, are found in two or more unrelated patients and/or there is biological evidence of their pathogenicity. Data were obtained from Ahronowitz *et al*, 2007 [18] and Li *et al*, 2010 [35]. FERM, 4.1 protein/Ezrin/Radixin/Moesin.

Sidebar C | In need of answers

- (i) What is the atomic structure of unphosphorylated, closed Merlin?
- (ii) What is the impact of tumour-derived missense mutations?
- (iii) Which conformational changes underlie the transition from the closed to the putatively open form of Merlin?
- (iv) Which post-translational events drive these conformational changes?
- (v) What are the mechanisms that govern the association of Merlin with the cortical cytoskeleton at various subcellular locations?
- (vi) Which mechanisms govern Merlin nuclear entry and exit?
- (vii) Does Merlin mediate contact inhibition and tumour suppression through distinct or overlapping mechanisms?
- (viii) How does Merlin contribute to each of these two functions?
- (ix) Why does Merlin deficiency drive tumour development in only a subset of tissue types and anatomical locations?
- (x) Which oncogenic mutations cooperate with inactivation of Merlin to drive tumorigenesis in sensitive tissues?

proteins has been shown to prevent the association of Merlin with the cortical cytoskeleton in confluent hepatocytes, preventing downregulation of EGFR [21]. However, an elegant analysis of a series of Merlin–Ezrin chimeric constructs revealed that the suppression of proliferation exerted by Merlin does not require this N-terminal sequence or association with the actin cytoskeleton in Schwann cells, which are a primary target of *NF2*-dependent tumorigenesis [54]. In fact, this study has confirmed that the sequences of Merlin that are involved in the intramolecular interaction—the F2 subdomain of the FERM domain and the C-terminal segment—are essential for growth inhibition in Schwann cells.

Inhibition of receptor tyrosine kinase activation

Merlin has been proposed to suppress proliferation by reducing the amount of transmembrane growth factor receptors at the plasma membrane [22,74,75]. Genetic analysis in *Drosophila* showed that Merlin, cooperating with the FERM domain protein Expanded, promotes endocytosis of several transmembrane receptors, including

some involved in mitogenic signalling [75]. Imaginal epithelial cells lacking functional Merlin and Expanded have elevated levels of Notch, EGFR, Patched, Smoothened and DE-cadherin at their surface. Pulse–chase labelling of Notch in living tissue indicated that Notch protein clearance from the membrane and degradation is deficient in these double mutant cells. This suggested that Merlin, cooperating with Expanded, controls the clearance of transmembrane receptors, regardless of their activation status. A similar phenomenon was observed in mouse Schwann cells, which were found to accumulate elevated levels of ErbB2, ErbB3, IGF1R and PDGFR at their surface when *Nf2* is deleted [74]. However, at least in Schwann cells, loss of Merlin was proposed to not decrease internalization but rather accelerate export of membrane receptors to the cell surface. Thus, although the results of both studies attribute to Merlin a role in regulating subcellular trafficking of multiple transmembrane receptors, the underlying mechanisms appear to be divergent. Genetic studies in *Drosophila* indicate that such trafficking regulation functions through the Hippo signalling pathway [76,77], as **receptor accumulation on the surface of imaginal epithelial cells** can similarly be seen when other components of the Hippo pathway are depleted. Importantly, such accumulation depends on Yorkie [77]. **Finally, studies on contact inhibition suggest** that Merlin controls EGFR signalling by regulating the availability of activatable EGFR at the cell surface [21,22]. Activated EGFR was found to partition in a higher density membrane fraction in MEFs, osteoblasts and liver-derived cells that have become fully confluent. Loss of Merlin attenuates this effect, leading to the hypothesis that Merlin sequesters EGFR at a specific membrane compartment where the activated receptor cannot access its downstream targets. Notably, membrane extracts, rather than total cell lysates, were used to examine the activation state of downstream signalling components—such as ERK and Akt—leaving open the question of whether effectors in the cytosolic compartment are also influenced. Thus, both genetic studies in *Drosophila* and cell biological studies in mammalian cells support the hypothesis that Merlin controls the number of activatable growth factor receptors

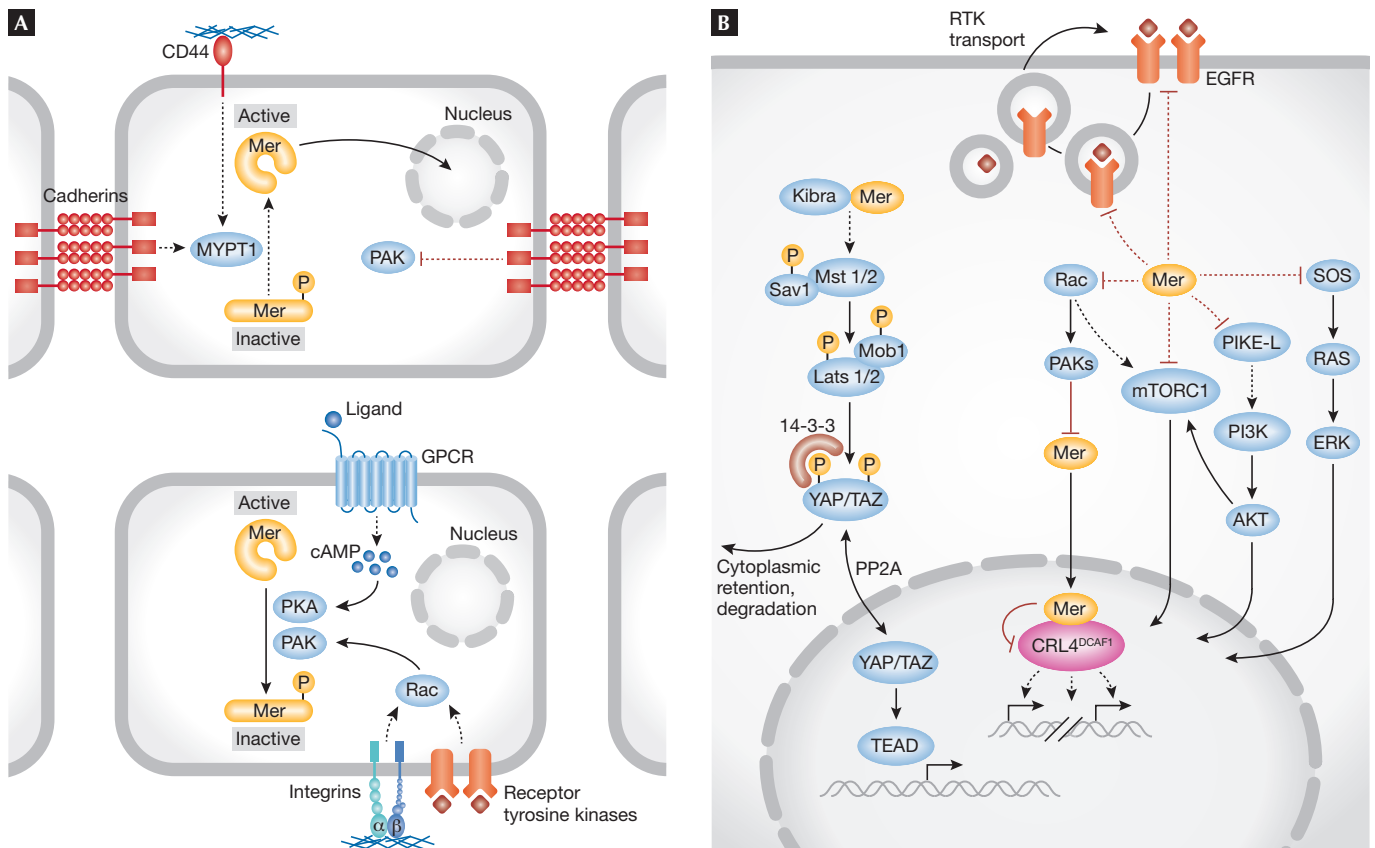


Fig 3 | Merlin activation and downstream signalling. The convergence of several upstream adhesion receptors regulates Merlin (Mer) activation and subsequently controls downstream mitogenic pathways. (A) The assembly of cell-to-cell adhesions and CD44 activation by hyaluronic-acid-rich matrix activates MYPT1, which dephosphorylates Merlin Ser 518 and maintains it in a closed and active conformation. Conversely, in sparse cells exposed to growth factors, integrins and receptor tyrosine kinases activate PAK, phosphorylating Ser 518. PKA—activated by increased cAMP—also phosphorylates Ser 518. (B) Merlin can affect a variety of mitogenic signalling pathways, including Rac–PAK signalling, mTORC1, EGFR–Ras–ERK and the PI3K–Akt pathway. In addition, Merlin contributes to the activation of the Hippo tumour-suppressor pathway. The active form of Merlin can enter the nucleus, bind to and inactivate the E3 ubiquitin ligase CRL4^{DCAF1}. Akt, protein kinase B; cAMP, cyclic AMP; CRL4, cullin-ring E3 ligase 4; DCAF1, DDB1- and CUL4-associated factor 1; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; mTORC1, mammalian target of Rapamycin complex 1; MYPT1, myosin phosphatase targeting subunit 1; PAK, p21-activated kinase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; Rac, Ras-related C3 botulinum toxin substrate; Ras, rat sarcoma.

at the cell surface (Fig 3B), but the biochemical mechanisms underlying this phenomenon are unclear and its significance in tumour suppression remains to be tested (Sidebar C).

Inhibition of Rac signalling

It has long been known that cultured schwannoma and meningioma cells have abundant lamellipodia and membrane ruffles, which are reminiscent of fibroblasts expressing activated forms of Rac [78–80]. Loss of Merlin was later shown to activate Rac signalling, which has been attributed to the release of negative regulation that Merlin exerts on PAK, consistent with the hypothesis that Merlin functions both upstream and downstream from PAK in a positive feedback loop (Fig 3B; [7,23–25,79]). Furthermore, biochemical and imaging studies indicated that the closed form of Merlin inhibits the recruitment of Rac to the plasma membrane in confluent cells [7]. Notably, inactivation of Merlin enables normal confluent cells to exit contact inhibition and re-enter the cell cycle, which could occur at least partly through the activation of Rac signalling [7]. In agreement with

this model, the expression of a membrane-targeted, but not constitutively active, form of Rac enables exit from contact inhibition, albeit not as efficiently as inactivation of Merlin [7]. Another mechanism for the regulation of Rac signalling by Merlin was recently reported [34]. Merlin can form a tight-junction-associated protein complex with Angiomotin, Patj and Pals1; Merlin binds tightly to Angiomotin and displaces Rich1, a small GTPase-activating protein (GAP) for Rac1, thereby inhibiting Rac1 and PAK. As anticipated from previous studies, inhibition of PAK attenuates Raf to ERK signalling [7]. These results suggest that loss of Merlin activates Rac signalling by multiple, potentially synergistic mechanisms.

Inhibition of mTORC1 signalling

Merlin is a negative regulator of the mTORC1 kinase complex, which regulates cell growth, proliferation, motility and survival by phosphorylating p70–S6K1 and 4E-BP1 [81]. Integrin-mediated adhesion was found to promote activation of mTORC1 signalling through PAK-mediated inactivation of Merlin, and Merlin-deficient

mesothelioma cell lines were shown to have activated mTORC1 signalling, whereas other cell lines do not [27]. **Similarly, constitutive** activation of mTORC1 signalling was found in meningioma and vestibular schwannoma cells from NF2 patients [26,27]. Surprisingly, loss of Merlin was shown to activate mTORC1 independently of AKT or ERK, which activate mTORC1 signalling in response to various mitogenic stimuli. Furthermore, re-expression of Merlin attenuates mTORC1 activity in Merlin-deficient cells, which requires the TSC1–TSC2 complex. The mechanism by which loss of Merlin induces hyperactivation of mTORC1 remains unknown. However, recent studies have shown that Rac can recruit mTORC1 to the plasma membrane [82], raising the possibility that Merlin antagonizes mTORC1 signalling by limiting Rac recruitment to the plasma membrane (Fig 3B; [7]). The significance of mTORC1 as an important downstream effector of Merlin is emphasized by the observation that the mTORC1 inhibitor rapamycin inhibits the proliferation of meningioma cells from NF2 patients and mesothelioma cells lacking Merlin [26,27].

Activation of the Hippo pathway

Increasing evidence implicates the Hippo signalling pathway as a major mediator of contact inhibition of growth. In agreement with this model, genetic analysis in *Drosophila* and mice showed that this pathway restrains cell proliferation and promotes apoptosis to limit organ size and suppress tumorigenesis [83–85]. The core kinase cascade of this pathway—Hippo (MST1/2)–Salvador (WW45)–Warts (Lats1/2)—has been well characterized in *Drosophila* and is conserved in mammals, whereas its upstream regulation, which is rather complex, seems to have diverged after the separation of arthropods and chordates. In *Drosophila*, the atypical cadherin Fat and the apical polarity protein Crumbs activate the core kinase cascade through the FERM domain protein Expanded [84,86]. Interestingly, genetic epistasis experiments showed that Merlin cooperates with Expanded to activate the Hippo pathway in the fly [32]. Mammalian cells lack a clear functional homologue of Fat [87,88]. **A recent study suggested that the FERM6/Willin protein**—which is a putative homologue of human Expanded—can activate the Hippo pathway in HEK-293 cells [89], **but this property of FERM6 cannot be recapitulated in the human breast cancer cell line MDA-MB-231, in which FERM6 expression inhibits proliferation** [90]. Therefore, whether FERM6/Willin is a functional homologue of Expanded is still uncertain. Similarly, engagement of E-cadherin has been reported to be sufficient to activate the Hippo pathway in human MCF10A mammary epithelial cells and MDA-MB-231 cells [91]. However, studies in the HaCaT human keratinocyte cell line did not support this role of E-cadherin in activation of the Hippo pathway [92], so the issue remains unresolved. Nevertheless, loss of Crumbs [93] or the tight junction component Angiomotin [94–96] inactivates the Hippo pathway in mammalian cells, suggesting that signals originating from both adherens and tight junctions can contribute to its activation.

Although several studies support the hypothesis that Merlin can activate the Hippo pathway, the molecular mechanisms remain largely undefined. Overexpression of Merlin promotes phosphorylation and nuclear extrusion of YAP, a downstream target of the Hippo kinase cascade [33,94], which functions as a co-activator of TEAD transcription factors (Fig 3B; [97,98]). By contrast, silencing of Merlin induces TEAD-dependent transcription [92]. **Two studies** have recently shown that liver-specific deletion of *Nf2* using

albumin-Cre results in massive liver overgrowth, followed by the development of multiple malignant tumours that seem to arise from hyperplastic lesions of a relatively differentiated ductal population [99], and finally overt hepatocellular carcinoma [17,18]. Interestingly, simultaneous deletion of *Yap* suppresses liver overgrowth and the ensuing tumorigenesis in *Nf2* mutant mice [18]. This effect is remarkable, as loss of a single *Yap* allele is sufficient to suppress liver overgrowth and tumorigenesis triggered by *Nf2* loss, suggesting that YAP is necessary for this Merlin-deficient phenotype. This robust genetic evidence, and the fact that *Nf2* deficiency in the liver reduces YAP phosphorylation, suggests that Merlin could regulate YAP signalling [18]. However, as YAP is necessary for the expansion of liver epithelial progenitors [18], its deficiency might suppress Merlin-dependent tumorigenesis by reducing the size of the stem cell or transit-amplifying compartment that sustains the enlargement of the liver, as well as its subsequent neoplastic transformation. Furthermore, treatment with the EGFR kinase inhibitor Erlotinib inhibits AKT activation and reduces hepatic tumour cell proliferation in mice with a liver-specific deletion of *Nf2*, suggesting that upregulation of EGFR signalling might also contribute to tumour development [17]. Although most of the genetic evidence suggests that YAP is necessary for liver overgrowth and tumorigenesis driven by loss of Merlin [18], further studies will be needed to compare the effect of conditional ablation of YAP or the EGFR on hepatomegaly and liver tumorigenesis. It will also be important to assess whether YAP is necessary for tumorigenesis in other mouse models of Merlin-deficient tumorigenesis.

Several mechanisms have been invoked to explain the effect that Merlin exerts on YAP-dependent transcription. Two additional upstream components of the Hippo pathway in *Drosophila* have been recently identified: Kibra [100–102] and Angiomotin [94–96]. Genetic analysis in *Drosophila* suggests that Kibra functions upstream from the Hippo kinase cascade. Although Merlin is required for some functions of Kibra, including control of organ size [100], evidence supports that both Kibra and Merlin cooperate with Expanded. Indeed, Merlin, Expanded and Kibra interact to form a protein complex [100–102] that subsequently binds to the Hippo–Salvador complex [102]. The biochemical mechanism underlying this interaction and how it can activate the kinase cascade are unknown, but overexpression studies suggest that Merlin can bind to Kibra and activate the canonical Hippo kinase cascade also in mammalian cells (Fig 3B; [18]).

Angiomotin has been shown to have a distinct role in Hippo signalling in mammalian cells [94–96,103]. It localizes to primordial tight junctions in response to the assembly of the apical polarity complex [104] and retains YAP and the related co-activator TAZ at the cell cortex, preventing them from acting in the nucleus [96,103]. Furthermore, it has been suggested that Angiomotin can also bind to Mst2 and Lats2 and function as a scaffold to enhance signal propagation through the canonical kinase cascade to YAP [94].

Nuclear inhibition of CRL4^{DCAF1}

Tandem affinity purification followed by mass spectrometry showed that wild-type Merlin—but not mutants obtained from tumours, such as L64P—interacts with the E3 ubiquitin ligase CRL4^{DCAF1} with high affinity [35]. Merlin binds directly to DCAF1, the substrate receptor subunit of CRL4^{DCAF1}, and inhibits CRL4^{DCAF1}-mediated ubiquitination of target proteins. The closed form of Merlin, which is able to mediate growth inhibition, translocates to the nucleus and

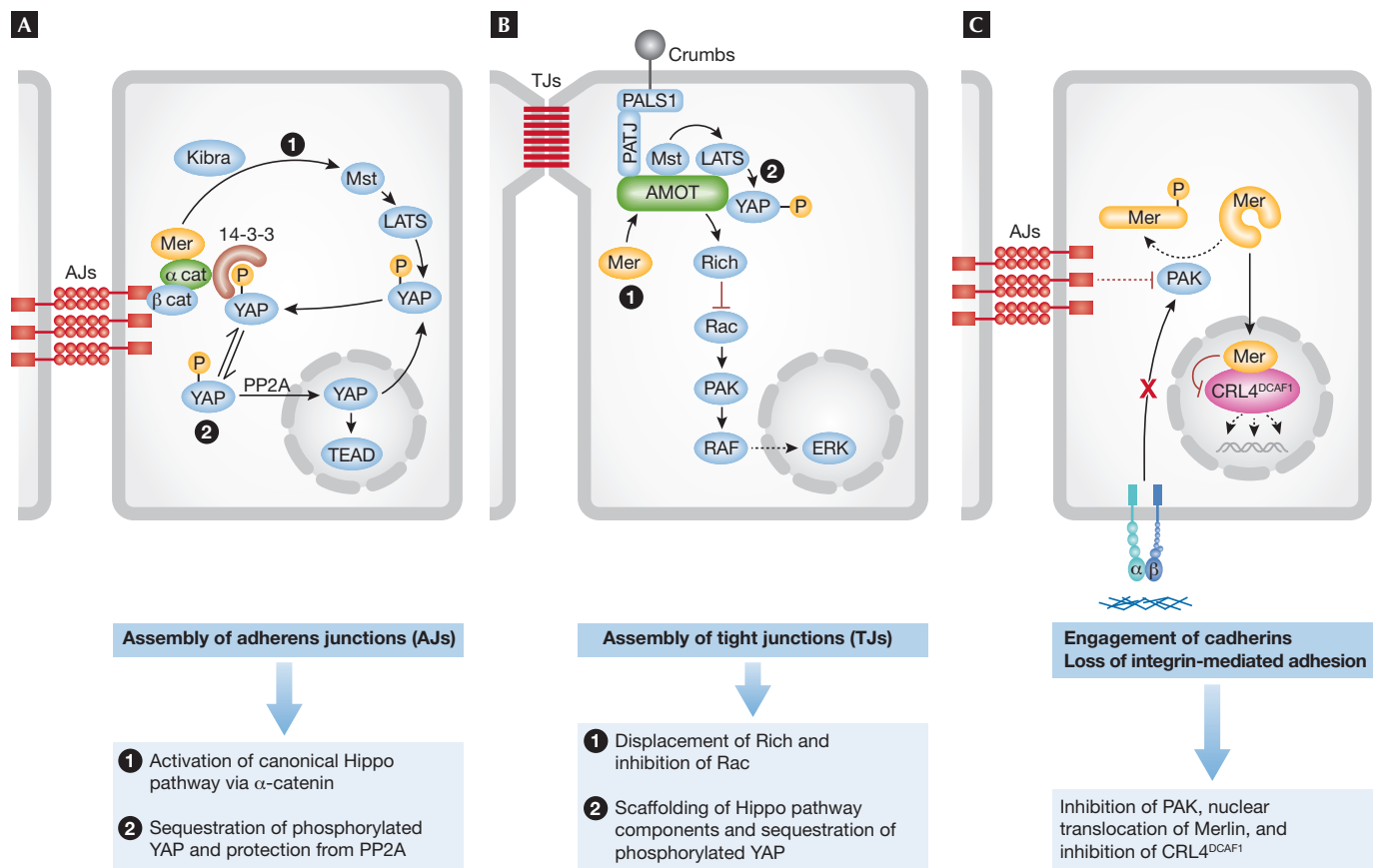


Fig 4 | Emerging mechanisms of Merlin-mediated epithelial adhesion, polarity and inhibition of proliferation. (A) Merlin (Mer) is recruited to nascent adherens junctions by α -catenin and contributes to the activation of Hippo signalling by cooperating with Kibra to activate the classical core kinase cascade, or by enabling α -catenin to sequester phosphorylated YAP in the cytoplasm through 14-3-3 proteins. (B) Upon assembly of tight junctions, Merlin binds to Angiomotin and displaces the Rac GAP Rich, thereby inhibiting Rac. In addition, Merlin might assist Angiomotin in coordinating the activation of the Hippo core kinase cascade. (C) Engagement of E-cadherin or loss of integrin-mediated adhesion leads to inactivation of PAK, promoting an accumulation of the de-phosphorylated (active) form of Merlin. Active Merlin enters the nucleus and inhibits the E3 ubiquitin ligase CRL4^{DCAF1}, thereby suppressing the expression of multiple pro-oncogenic genes. CRL4, cullin-ring E3 ligase 4; DCAF1, DDB1- and CUL4-associated factor 1; ERK, extracellular-signal-regulated kinase; LATS1/2, large tumour suppressor 1/2; Mst1/2, macrophage stimulating 1/2; PAK, p21-activated kinase; PALS1, protein associated with Lin-7 1; PATJ, Pals1-associated tight junction protein; Rac, Ras-related C3 botulinum toxin substrate; TEAD, TEA domain family member; YAP, Yes-associated protein.

binds to CRL4^{DCAF1}, whereas the putatively open form, which is inactive, does so to a much more limited extent. Genetic epistasis analysis in human Schwann cells, endothelial cells, mesothelial cells and mouse schwannoma cells indicates that Merlin inhibits growth and suppresses tumorigenesis by inhibiting CRL4^{DCAF1}. For example, depletion of DCAF1 blocked the hyperproliferation caused by loss of Merlin in human Schwann cells and endothelial cells. Conversely, enforced expression of a Merlin-insensitive mutant of DCAF1 counteracted the anti-mitogenic effect of Merlin in human mesothelioma cells. In addition, re-expression of Merlin and silencing of DCAF1 induced an overlapping tumour-suppressive gene expression programme in Merlin-deficient mouse schwannoma cells, suggesting that Merlin inactivation induces oncogenic gene expression by deregulating CRL4^{DCAF1} activity. Notably, a detailed biochemical and functional analysis of several tumour-derived mutants of Merlin revealed that pathogenic mutations fall into three main classes: some of the missense mutants that map to the FERM domain are defective in nuclear translocation; others fail to bind to DCAF1; and

the C-terminal truncation mutants accumulate in the nucleus and bind to DCAF1 but fail to suppress E3 ligase activity. Finally, depletion of DCAF1 suppresses the ability of Merlin-deficient schwannoma cells to hyperproliferate *in vitro*, to grow in soft agar or to form tumours after subcutaneous injection in nude mice. Together, these findings suggest that Merlin needs to enter the nucleus, bind to DCAF1 and suppress CRL4^{DCAF1} in order to inhibit tumorigenesis, although additional mechanisms are also possible.

CRL4^{DCAF1} belongs to a large subfamily of cullin-ring E3 ligases. These ligases consist of a catalytic subunit (Roc1/Rbx1), a scaffold (cullin 4), an adaptor protein (DDB1) and one of multiple WD40-domain-containing substrate receptors [105,106]. DCAF1 is the substrate receptor of CRL4^{DCAF1}. Members of the CRL4 E3 ligase family regulate chromatin remodelling, DNA replication and the response to DNA damage. Although the physiological substrates of CRL4^{DCAF1} have not yet been identified, gene expression analysis suggests that CRL4^{DCAF1} regulates a broad gene expression programme, consisting of more than 1,000 genes [35]. CRL4^{DCAF1} could exert this effect by

promoting the poly- or mono-ubiquitination of histones, chromatin-remodelling factors or transcription factors, as it has been established for other members of the CRL4 subfamily [107–110]. Indirect targets of CRL4^{DCAF1} include important growth regulators, such as receptor tyrosine kinases (RTKs), their downstream target-effectors, and various cell cycle regulators and anti-apoptotic proteins. For example, AXL, a RTK recently found to regulate mesothelioma proliferation and invasiveness [111], **seems to be inhibited at the transcriptional level** by Merlin expression as well as DCAF1 knockdown [35]. Irrespective of the specific mechanism by which CRL4^{DCAF1} regulates gene expression, the breadth of the oncogenic gene expression programme it induces and the identity of some of the genes regulated suggest that Merlin could suppress several mitogenic signalling pathways by inhibiting CRL4^{DCAF1}. Notably, Merlin expression or silencing of DCAF1 coordinately regulates a subset of Hippo pathway target genes, suggesting a connection between CRL4^{DCAF1} and YAP-dependent transcription [35].

Cortical and nuclear models

In addition to rescuing normal cells from contact inhibition, loss of Merlin accelerates their transit through the G1 phase of the cell cycle [26,27,112], suggesting that Merlin functions as a brake on cell cycle progression in both sparse and confluent cells. In addition, Merlin promotes the maturation of adherens junctions and the assembly of tight junctions in skin epithelium [5,16], **suggesting that loss of Merlin can contribute to disruption of cell adhesion and polarity during tumorigenesis**. These observations imply that Merlin restrains tumorigenesis by promoting epithelial adhesion and polarity and by restraining proliferation. As discussed above, Merlin exerts these effects through multiple, non-mutually exclusive mechanisms (Fig 4).

The first model of Merlin function, which emphasizes the role of contact inhibition in tumour suppression, places it downstream from the E-cadherin–catenin complex and upstream from Hippo signalling (Fig 4A). In one variant of this model, Merlin is recruited to nascent adherens junctions by binding to α -catenin [16] and activates the canonical Hippo kinase cascade through Kibra [18,91]. In the other variant, Merlin fosters maturation of adherens junctions [16], enabling α -catenin to bind to 14-3-3 and thereby to the phosphorylated inactive form of YAP [92]. Disassembly of the junctions or loss of α -catenin—which is a tumour suppressor on its own [113]—releases phosphorylated YAP from adherens junctions. Finally, dephosphorylation by PP2A allows activation and nuclear accumulation of YAP [92]. **These mechanisms probably mediate contact inhibition**, but their contribution to Merlin-mediated tumour suppression remains to be examined.

The second model proposes that Merlin enforces contact inhibition and suppresses tumorigenesis by binding to Angiomotin (Fig 4B). In one variant of this model, the binding of Merlin to Angiomotin displaces the Rac GAP Rich, suppressing Rac–PAK signalling [34]. In another variant, binding of Merlin is necessary for Angiomotin to function as a scaffold in the activation of the Hippo pathway. This latter possibility is consistent with the ability of Angiomotin to function as an upstream component of the Hippo pathway [94–96,103] but inconsistent with the fact that Angiomotin is required for tumour development in a xenograft model of NF2 [34]. Although Merlin binding to Angiomotin might contribute to contact inhibition, as suggested by studies implicating inhibition of Rac in this process [7], its role in tumour suppression remains to be

fully investigated. In fact, Merlin binds to Angiomotin through the α -helical and C-terminal segments, independently of whether it is in its open or closed conformation [34].

The third model is based on the fact that the closed form of Merlin translocates into the nucleus to bind to DCAF1—thereby suppressing CRL4^{DCAF1}-mediated E3 ligase activity—whereas the putatively open form does not (Fig 4C; [35]). The contribution of this pathway to contact inhibition remains to be tested; however, its involvement in tumour suppression is supported by strong genetic evidence, including the analysis of a large number of tumour-derived missense mutants of Merlin. Furthermore, this model is compatible with a role for YAP in NF2-dependent tumorigenesis. In fact, the observation that some of the genes regulated by YAP are concordantly regulated by CRL4^{DCAF1} suggests that CRL4^{DCAF1} could regulate YAP-dependent transcription [35]. As Merlin can promote phosphorylation of YAP independently of MST1/2 [91], CRL4^{DCAF1} could inhibit the Hippo pathway by acting on a target downstream from MST. In addition, CRL4^{DCAF1} probably ubiquitinates other targets. Future challenges include the identification of the physiological targets of CRL4^{DCAF1} and understanding how it regulates oncogenic gene expression.

Conclusions and perspectives

Recent studies strengthen the idea that Merlin can function in the nucleus as well as at the cell cortex. At the cell cortex, Merlin promotes the assembly of cell junctions by recruiting Par3–aPKC [16] as well as by locally inhibiting Rac signalling [7,34]. Once formed, both adherens and tight junctions can function as signalling hubs for the initiation of antimitogenic signals. Merlin can contribute to the initiation of some of these signals through interactions with Kibra, α -catenin or Angiomotin at the cell cortex, although additional signals are probably required to enforce contact inhibition. In particular, inactivation of PAK allows the accumulation of the closed form of Merlin, which migrates into the nucleus to inhibit CRL4^{DCAF1} and induce a growth-suppressive programme of gene expression [35]. Conversely, mitogenic stimulation induces activation of PAK and thereby accumulation of the putatively open form of Merlin, which remains in the cytoplasm, removing a block to cell cycle progression [7,25]. Taken together, these findings suggest that Merlin-mediated tumour suppression functions in an opposite manner to the Wnt– β -catenin signalling pathway [114,115]. Confirmation of this general model will require a careful assessment of the contribution of each of the signalling mechanisms outlined above to both contact inhibition and tumour suppression (Sidebar C). Considering the recent progress made in this field, it is reasonable to anticipate a quick answer to the most important outstanding questions. With a more definitive view of Merlin-mediated tumour suppression, it will be possible to design therapies that inhibit the oncogenic signalling pathways activated by the loss of Merlin [116].

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CONFLICT OF INTEREST

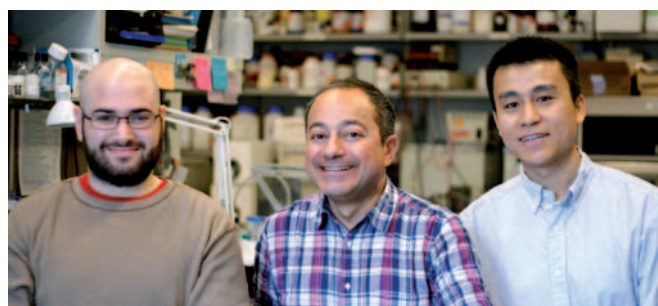
The authors declare that they have no conflict of interest.

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Jonathan Cooper, Filippo G. Giancotti & Wei Li



Matthias A. Karajannis