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Merlin's tumor suppression linked to inhibition of the E3 ubiquitin ligase CRL4^{DCAF1}

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The mechanism by which the FERM domain protein Merlin, encoded by the tumor suppressor *NF2*, restrains cell proliferation is poorly understood. Prior studies have suggested that Merlin exerts its antimitogenic effect by interacting with multiple signaling proteins located at or near the plasma membrane. We have recently observed that Merlin translocates into the nucleus and binds to and inhibits the E3 ubiquitin ligase CRL4^{DCAF1}. Genetic evidence indicates that inactivation of Merlin induces oncogenic gene expression, hyperproliferation, and tumorigenicity by unleashing the activity of CRL4^{DCAF1}. In addition to providing a potential explanation for the diverse effects that loss of Merlin exerts in multiple cell types, these findings suggest that compounds inhibiting CRL4^{DCAF1} may display therapeutic efficacy in Neurofibromatosis type 2 and other cancers driven by Merlin inactivation.

Key words: Merlin, NF2, E3 ubiquitin ligase, CRL4, DCAF1, FERM domain protein

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Introduction

Increasing evidence indicates that the *NF2* gene, originally identified because of its inactivation in the familial cancer predisposition syndrome Neurofibromatosis type 2, has a broad tumor-suppressor function.[1,2] *NF2* encodes Merlin, a member of the Ezrin/Radixin/Moesin (ERM) family of proteins, which mediate linkage of cell adhesion receptors, such as CD44 and ICAM, to cortical actin. Because of sequence homology to ERM proteins and apparently prevalent localization to the cortical cytoskeleton, it has been suggested that functions at or near the plasma membrane to inhibit the transmission of promitogenic signals. In apparent agreement with this general model, it has been reported that Merlin interacts with multiple signaling proteins located at these cellular compartments and opposes activation of several pro-mitogenic signaling pathways.[1] Some reported interactions appear to be of low affinity, are not supported by convincing mutational analysis, and some of the effects observed are cell-type specific and perhaps irrelevant to the tumor suppressor function of Merlin. However, it seems clear that exogenous Merlin inhibits multiple signaling pathways in Schwannoma and meningioma cells carrying loss-of-function mutations at the *NF2* locus. The major pathways that appear to be modulated include membrane recruitment and activation of Rac and thereby PAK,[3-5] activation of mTORC1 independently of Akt,[6,7] the EGFR-Ras-ERK pathway, the PI3K-Akt pathway, and focal adhesion kinase (FAK)-Src signaling.[8-14] In addition, Merlin cooperates with Expanded and Kibra to activate the Hippo tumor-suppressor pathway in Drosophila[15-19] and a recent study has implicated YAP, the oncoprotein that lies downstream of the core Hippo pathway in mammalian cells, as a mediator of Merlin-dependent tumorigenesis in a mouse model of liver tumorigenesis.[20] In spite of these significant advances, the biochemical function of Merlin and hence the mechanism through which it suppresses tumorigenesis have remained, until recently, elusive.

Merlin Suppresses Tumorigenesis by Inhibiting CRL4^{DCAF1} in the Nucleus

We have used tandem affinity purification followed by mass spectrometry to identify

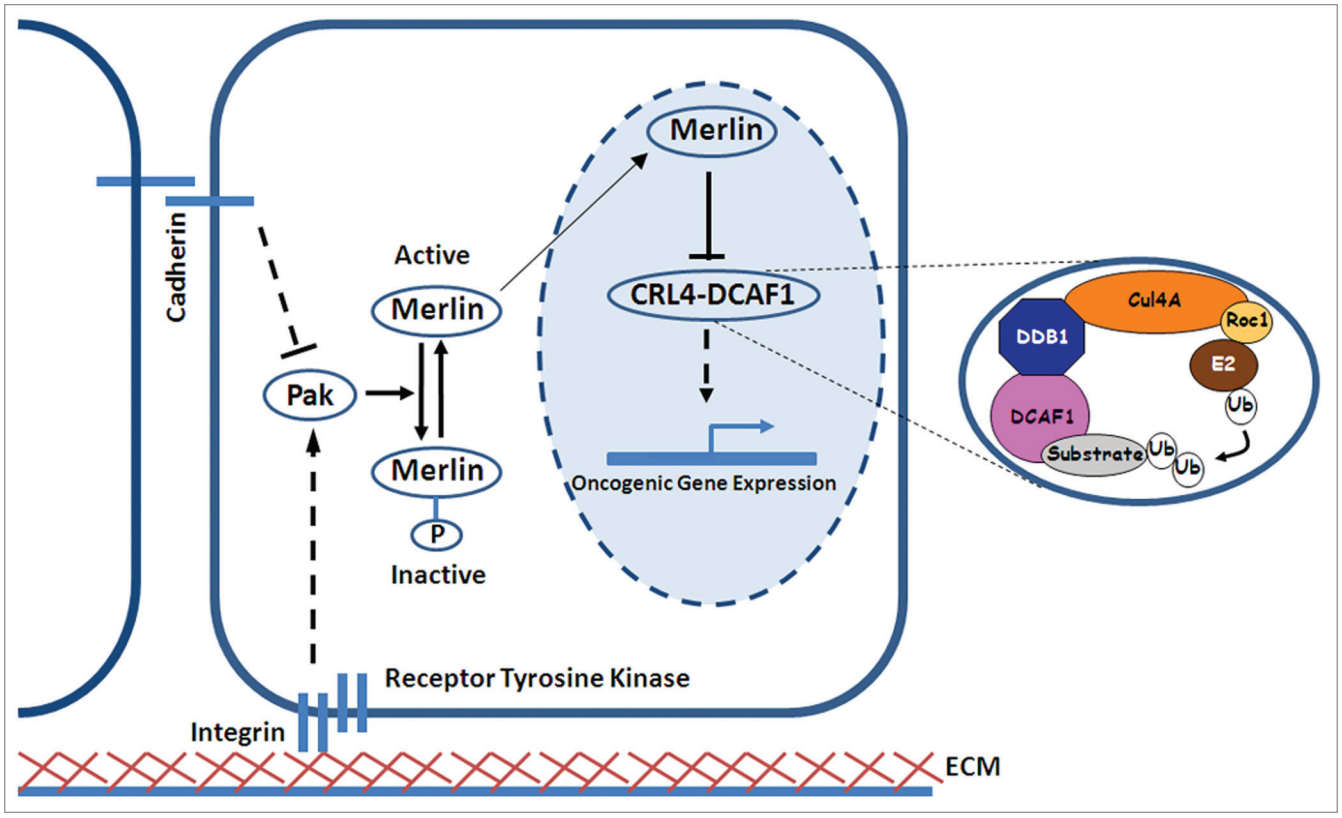

Figure 1. Merlin suppresses tumorigenesis by translocating into the nucleus and inhibiting the E3 ligase CRL4^{DCAF1}. Signals regulating cell growth from neighboring cells or extracellular matrix (ECM) regulate the phosphorylation status of Merlin through Pak. The active form of Merlin enters the nucleus and binds to the E3 ubiquitin ligase CRL4^{DCAF1}, thereby inhibiting its activity. Inset shows a model of the molecular architecture of the ligase. We posit that deregulated CRL4^{DCAF1} drives the oncogenicity of Merlin-deficient cells by upregulating the expression of multiple oncogenic genes.

proteins interacting with wild type but not a tumor-derived mutant form of Merlin. Our findings revealed that wild type but not mutant Merlin binds with high affinity to the E3 ubiquitin ligase CRL4^{DCAF1}.[21] Biochemical analyses indicated that Merlin binds directly to DCAF1, the substrate receptor subunit of CRL4^{DCAF1}, and inhibits CRL4^{DCAF1}-mediated ubiquitylation of target proteins. Further studies provided evidence that the unphoshorylated form of Merlin, which is presumably stabilized in the closed conformation and able to mediate growth inhibition, translocates into the nucleus and binds to CRL4^{DCAF1}, whereas the inactive phosphorylated form remains in the cytoplasm. To examine if Merlin mediates growth inhibition and suppresses tumorigenesis by inhibiting CRL4^{DCAF1}, we conducted genetic epistasis experiments in mammalian cells. Depletion of DCAF1 blocked the hyperproliferation caused by inactivation of Merlin in Schwann cells and mesothelial cells. Conversely, enforced expression of a Merlin-insensitive mutant of DCAF1 counteracted the antimitogenic effect of Merlin in mesothelioma cells. In addition, re-expression of Merlin and silencing of DCAF1 induced an overlapping tumor suppressive program of gene expression in Merlin-deficient Schwannoma cells, suggesting that inactivation of Merlin induces an oncogenic program of gene expression by deregulating CRL4^{DCAF1} activity. To further test if Merlin suppresses tumorigenesis through inhibition of CRL4^{DCAF1}, we conducted a detailed biochemical and functional analysis of several tumor-derived mutants of Merlin. We found that the pathogenic mutants under scrutiny fall into three major classes: some of the missense mutants mapping to the FERM domain exhibited defective translocation into the nucleus, others failed to bind to DCAF1, while the C-terminal truncation mutants accumulated in the nucleus and bound to DCAF1 but failed to suppress E3 ligase activity. Collectively, this study provided evidence that Merlin needs to enter the nucleus, bind to DCAF1 and suppress CRL4^{DCAF1} in order to suppress tumorigenesis. Finally, we examined if CRL4^{DCAF1} mediates the oncogenicity of Merlin-deficient cells. Depletion of DCAF1 suppressed the ability of Merlin-deficient Schwannoma cells to hyperproliferate in vitro, to grow in soft agar, and to form tumors upon subcutaneous injection in nude mice. Together, these results provided strong evidence that Merlin suppresses tumorigenesis by inhibiting CRL4^{DCAF1} in the nucleus.

Potential Connections to Other Signaling Pathways

CRL4^{DCAF1} belongs to a large subfamily of cullin-ring E3 ligases that consist of Roc1/Rbx1 (catalytic subunit), Cullin 4 (scaffold), DDB1 (adaptor) and one of multiple WD40 domain-containing substrate receptors.[22,23] The substrate receptor of CRL4^{DCAF1} is DCAF1 (see **Fig. 1**). CRL4 ligases have been implicated

in regulating chromatin remodeling, DNA replication and the DNA damage response. Although the physiological substrates of $CRL4^{DCAF1}$ have not yet been identified, our gene expression analysis suggests that $CRL4^{DCAF1}$ regulates a broad program of gene expression, consisting of more than 1,000 genes.[21] Therefore, it is conceivable that $CRL4^{DCAF1}$ exerts this effect by promoting the poly- or mono-ubiquitylation of histones, chromatin-remodeling factors or transcription factors, as it has been established for other members of the CRL4 subfamily.[23-25] Irrespective of the specific mechanism by which $CRL4^{DCAF1}$ regulates gene expression, the breadth of the oncogenic gene expression program it induces and the identity of some of the genes regulated suggest that Merlin may suppress multiple mitogenic signaling pathways by inhibiting $CRL4^{DCAF1}$.

Receptor tyrosine kinase signaling. Multiple mechanisms have been invoked to explain the inhibitory effect of Merlin on the EGFR-Ras-ERK signaling pathway. It has been proposed that Merlin suppresses EGFR signaling by binding to the receptor and sequestering it in an inactive conformation at cell-to-cell junctions.[13,26] Furthermore, whereas studies in the fly have indicated that Merlin accelerates the endocytosis of the EGFR and other signaling receptors,[27] studies in Schwannoma cells suggest that Merlin inhibits the export of the EGFR and other receptor tyrosine kinases to the plasma membrane.[28] Finally, it has been proposed that Merlin interferes with Ras activation downstream of receptor tyrosine kinases.[14] Our gene expression analysis has revealed that Merlin can regulate the transcription of several genes encoding components or regulators of signaling pathways jointly regulated by receptor tyrosine kinases and integrins.[21] Interestingly, expression of Merlin and silencing of DCAF1 cause a concordant up or downregulation of these genes, suggesting that Merlin regulates their expression by inhibiting $CRL4^{DCAF1}$. This observation provides a potential unitary explanation to the multiple effects that Merlin appears to exert on the signaling pathways activated by receptor tyrosine kinases and integrins.

The hippo pathway. The Hippo pathway is an evolutionarily conserved signaling cascade which controls organ size and suppresses tumorigenesis by inhibiting cell proliferation and promoting apoptosis.[29-31] The core pathway consists of two serine/threonine kinases and their adaptors—Hippo(MST1/2)-Salvador(WW45) and Warts(Lats1/2)-Mats(MOBKL1A/B)—and of a transcriptional co-activator Yorkie (YAPTAZ). Hippo phosphorylates and thereby activates Warts, which in turn phosphorylates Yorkie, promoting its exclusion from the nucleus. Yorkie promotes cell proliferation and survival by inducing the expression of various genes, including Cyclin E and DIAP. Although the function of the core Hippo pathway has been elucidated, the mechanism by which it is activated is not as well understood. In Drosophila, genetic analysis suggests that Merlin and another FERM domain protein Expanded function upstream of the Hippo kinase cascade to transduce extracellular signals.[16] Interestingly, Expanded can regulate the Hippo pathway by directly interacting with Yorkie, the downstream target of the Hippo kinase cascade.[32] How Merlin activates the Hippo pathway is not known. Recently, another component of the Hippo pathway, Kibra, was identified.[17-19] Genetic analysis suggests that Kibra functions upstream of the Hippo kinase cascade and is partially redundant with Expanded and Merlin. Furthermore, the Merlin-Expanded-Kibra complex interacts directly with the Hippo-Salvador complex in transfected cells, suggesting that Merlin-Expanded-Kibra might impinge directly on Hippo. In mammals, overexpression of Merlin activates the Hippo pathway and inactivates Yap, a homolog of Yorkie.[33] However, it remains to be determined whether Merlin suppresses cell proliferation and tumorigenesis, at least in part, through activation of the Hippo pathway and whether Merlin activates the pathway at or near the plasma membrane, as it appears to do in Drosophila. We found that re-expression of Merlin or inactivation of $CRL4^{DCAF1}$ in mouse Schwannoma cells induces the expression of a set of genes which are known to be regulated by Yap.[21,33] This finding supports the hypothesis that Yap is an effector of Merlin and that $CRL4^{DCAF1}$ is involved in its regulation. However, since our results suggest that Merlin needs to enter the nucleus and inhibit $CRL4^{DCAF1}$ to suppress cell proliferation and tumorigenesis, it will be interesting to investigate whether $CRL4^{DCAF1}$ regulates Hippo signaling in mammalian cells, the mechanism involved, and the nuclear function of Merlin is conserved in Drosophila.

Potential Drug Targets for NF2-Related Tumors

The *NF2* gene is inactivated in familial as well as in sporadic Schwannomas, meningiomas and ependymomas and in a large fraction of malignant mesotheliomas. Although Schwannomas, meningiomas, and ependymomas are slow-growing tumors and do not invade adjacent tissue, they arise in the brain and spinal cord and therefore can cause significant morbidity. In contrast, malignant mesotheliomas are aggressive tumors and do not respond well to classical chemotherapy.[34] Development of effective targeted therapies for these diseases requires a complete definition of the biochemical function of Merlin and the identification of the major signaling pathways that drive the expansion of Merlin-deficient tumors. We have found that inactivation of $CRL4^{DCAF1}$ through silencing of DCAF1 inhibits the proliferation of primary human Schwannoma cells but has no inhibitory effect on normal primary human Schwann cells.[21] In addition, depletion of DCAF1 suppresses the tumorigenic properties of *NF2*-deficient tumor cells but not of cells carrying other oncogenic mutations. These observations indicate that Merlin-deficient cells are dependent upon the signaling output of $CRL4^{DCAF1}$, suggesting that targeting this ligase or the signaling pathways activated by the ligase may constitute an effective therapeutic strategy. In addition, since depletion of $CRL4^{DCAF1}$ does not appear to inhibit the proliferation of cells expressing wild-type Merlin to a large extent, this strategy is predicted to afford a large therapeutic window. Modification of the cullin protein by NEDD8 is required for the cullin-RING ubiquitin ligase activity. Therefore, interfering with the neddylation of the ubiquitin ligase will affect

the ubiquitinylation-dependent cellular processes, such as cell cycle and cell survival. Notably, a potent and selective inhibitor of the NEDD8-activating enzyme, MLN4924, has recently been developed and shown to inhibit human colon tumor and lung tumor growth in mouse xenograft models.[35] It would be interesting to test whether this neddylation inhibitor could also be applied to inhibit the growth of schwannomas or other cancers caused by loss of Merlin function.

Perspective

Since the *NF2* gene was identified as a tumor suppressor inactivated in Neurofibromatosis type 2 and several sporadic cancers, intensive research has been carried out to elucidate the functions of its protein product, Merlin, and to understand how inactivation of this protein leads to tumorigenesis. Most anti-mitogenic functions of Merlin, especially related to contact inhibition of cell growth, were attributed to its roles in cortical cytoskeleton organization and signaling regulation. Whether these roles are critical in tumor suppression is unclear. We found that the closed/active form of Merlin translocates to the nucleus and binds to $CRL4^{DCAF1}$, thereby inhibiting its E3 ligase activity. This unexpected function is essential for tumor suppression by Merlin and might be responsible for regulating multiple mitogenic signaling pathways, some of which may also be affected by Merlin's functions at the cortical cytoskeleton. Identification of the substrates of this E3 ligase should help in understanding the relationship between Merlin's inhibitory effect on $CRL4^{DCAF1}$ and its role in regulating multiple mitogenic signaling pathways and ultimately may lead to generation of targeted therapies for tumors driven by loss of Merlin.

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