Functional Regulation of the Neurofibromatosis 2 Tumor Suppressor Merlin

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(née Laulajainen)

ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Medicine of the University of Helsinki, in Seth Wichmann Auditorium, Haartmaninkatu 2, Helsinki, on December 16th, 2011, at 12 noon.

Helsinki 2011
To the memory of a friend
# TABLE OF CONTENTS

**LIST OF ORIGINAL PUBLICATIONS**.............................................................. 6  
**ABBREVIATIONS**............................................................................................ 7  
**ABSTRACT**...................................................................................................... 9  
**REVIEW OF THE LITERATURE**.................................................................... 10  

## 1. Cancer........................................................................................................... 10  
   1.1. Tumorigenesis .......................................................................................... 10  
   1.2. Cancer genes .......................................................................................... 11  
      1.2.1. Oncogenes ....................................................................................... 12  
      1.2.2. Tumor suppressors ........................................................................ 12  
   1.3. Inherited Cancer Syndromes .................................................................... 13  

## 2. The Neurofibromatosis 2 disease .............................................................. 14  
   2.1. The **NF2** gene ..................................................................................... 16  
   2.2. **NF2** gene mutations .......................................................................... 18  
      2.2.1. Mutations in the NF2 disease ......................................................... 18  
      2.2.2. Mutations in NF2-related sporadic tumors ....................................... 19  
      2.2.3. Mutations in other sporadic tumors ............................................... 20  

## 3. The Cytoskeleton ......................................................................................... 21  
   3.1. Composition of the cytoskeleton ............................................................. 21  
   3.2. Regulation of cell shape and motility ...................................................... 22  
   3.3. Actin cytoskeleton in cancer ................................................................... 23  

## 4. ERM proteins ............................................................................................... 24  
   4.1. Ezrin ........................................................................................................ 27  

## 5. The NF2 protein Merlin.............................................................................. 28  
   5.1. Properties of merlin .............................................................................. 28  
   5.2. Functions of merlin ............................................................................... 29  

## 6. Regulation of Merlin and ERM activity................................................... 32  
   6.1. Conformational regulation ...................................................................... 32  
   6.2. Phosphorylation and phospholipid-binding ........................................... 34  
      6.2.1. PAK pathway ................................................................................... 36  
      6.2.2. PKA pathway ............................................................................... 37  
      6.2.3. Akt pathway .................................................................................. 38  
   6.3. Protein stability and the ubiquitin proteasome pathway ......................... 40  
      6.3.1. Degradation of merlin ................................................................... 42  

**AIMS OF THE STUDY**.................................................................................. 43  
**MATERIALS AND METHODS**..................................................................... 44  

## 1. Materials ..................................................................................................... 44  
   1.1. Cell lines (I, II, III) ............................................................................... 44  
   1.2. Antibodies (I, II, III) ............................................................................ 44
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I  Laulajainen M*, Muranen T*, Carpén O, Grönholm M.
Protein kinase A-mediated phosphorylation of the NF2 tumor suppressor protein merlin at serine 10 affects the actin cytoskeleton.
Oncogene. 2008 May 22;27(23):3233-3243.

II  Laulajainen M, Muranen T, Nyman TA, Carpén O, Grönholm M.
Multistep phosphorylation by oncogenic kinases enhances the degradation of the NF2 tumor suppressor merlin.

III  Laulajainen M, Melikova M, Muranen T, Carpén O, Grönholm M
Distinct overlapping sequences at the carboxy-terminus of merlin regulate its tumor suppressor and morphogenic activity.
Submitted.

* Equal contribution

Publication I was also included in the thesis of Taru Muranen (The Neurofibromatosis 2 tumor suppressor merlin in cytoskeleton organization and cell cycle regulation) in 2007.
ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ABS</td>
<td>actin binding site</td>
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<tr>
<td>AJ</td>
<td>adherens junction</td>
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<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
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<tr>
<td>AKT</td>
<td>AKT8 virus oncogene cellular homolog</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>C-</td>
<td>carboxy-</td>
</tr>
<tr>
<td>CAM</td>
<td>cell-cell adhesion molecule</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine 5’-monophosphate</td>
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<tr>
<td>CD43/44</td>
<td>cluster of differentiation antigen 43/44</td>
</tr>
<tr>
<td>Cdc42</td>
<td>cell division cycle 42</td>
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<tr>
<td>C-ERMAD</td>
<td>C-ERM association domain</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRL4</td>
<td>cullin-RING ligase 4</td>
</tr>
<tr>
<td>DCAF1</td>
<td>DDB1- and Cul4-associated factor 1</td>
</tr>
<tr>
<td>D-merlin</td>
<td>Drosophila melanogaster merlin</td>
</tr>
<tr>
<td>D-moesin</td>
<td>Drosophila melanogaster moesin</td>
</tr>
<tr>
<td>E3KARP</td>
<td>exchanger 3 kinase A regulatory protein</td>
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<tr>
<td>EBP50</td>
<td>ezrin-binding phosphoprotein 50</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>E. coli</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>ERM</td>
<td>ezrin-radixin-moesin</td>
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<tr>
<td>EVH1</td>
<td>Enabled/VASP homology 1</td>
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<tr>
<td>F-actin</td>
<td>filamentous actin</td>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>FBS/FCS</td>
<td>fetal bovine/calf serum</td>
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<tr>
<td>FERM</td>
<td>Four.1 protein, ezrin, radixin, moesin</td>
</tr>
<tr>
<td>FOXO</td>
<td>forhead box O</td>
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<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
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<tr>
<td>G-actin</td>
<td>globular actin</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GDI</td>
<td>guanine nucleotide dissociation inhibitor</td>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
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<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<tr>
<td>GSK</td>
<td>glycogen synthase kinase</td>
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<td>glutathione S-transferase</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>GTPase</td>
<td>guanosine triphosphatase</td>
</tr>
<tr>
<td>HEI10</td>
<td>human enhancer of invasion clone 10</td>
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<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
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<tr>
<td>HRS</td>
<td>hepatocyte growth factor-regulated tyrosine kinase substrate</td>
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<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton(s)</td>
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<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>Merlin</td>
<td>moesin-ezrin-radixin-like protein</td>
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<tr>
<td>MIM</td>
<td>Mendelian Inheritance in Man</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>mTORC1</td>
<td>mTOR complex 1</td>
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<tr>
<td>Myc</td>
<td>myelocytomatosis</td>
</tr>
<tr>
<td>MYPT-1-PP18</td>
<td>myosin phosphatase targeting subunit protein phosphatase 1</td>
</tr>
<tr>
<td>N-</td>
<td>amino-</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>N-ERMAD</td>
<td>N-ERM association domain</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export sequence</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis 1</td>
</tr>
<tr>
<td>NF2</td>
<td>Neurofibromatosis 2</td>
</tr>
<tr>
<td>NFs</td>
<td>Neurofibromatoses</td>
</tr>
<tr>
<td>NGB</td>
<td>NF2-associated GTP binding protein</td>
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<tr>
<td>NHE1</td>
<td>Na+/H+ exchanger</td>
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<tr>
<td>NHE-RF</td>
<td>Na+/H+ exchanger-regulatory factor</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>pAb</td>
<td>polyclonal antibody</td>
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<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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<tr>
<td>PBD</td>
<td>p21-binding domain</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDK1</td>
<td>3-phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PIKE-L</td>
<td>PI3K enhancer long isoform</td>
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<tr>
<td>PIP₂</td>
<td>phosphatidylinositol 4,5-biphosphate</td>
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<td>PIP₃</td>
<td>phosphatidylinositol 3,4,5-triphosphate</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PTB</td>
<td>phosphotyrosine binding</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue deleted on chromosome 10</td>
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<td>PTK</td>
<td>protein tyrosine kinase</td>
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<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
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<tr>
<td>Ras</td>
<td>rat sarcoma</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homologous member A</td>
</tr>
<tr>
<td>RhoGDI</td>
<td>Rho guanine-dissociation inhibitor</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SCHIP-1</td>
<td>schwannomin interacting protein-1</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis complex</td>
</tr>
<tr>
<td>UPP</td>
<td>ubiquitin-proteasome pathway</td>
</tr>
<tr>
<td>UVS</td>
<td>unilateral vestibular schwannoma</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VprBP</td>
<td>Vpr-binding protein</td>
</tr>
<tr>
<td>VS</td>
<td>vestibular schwannoma</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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Neurofibromatosis 2 (NF2) is an autosomal dominant disorder manifested by the formation of multiple benign tumors of the nervous system. Affected individuals typically develop bilateral vestibular schwannomas which lead to deafness and balance disorders. The syndrome is caused by inactivation of the NF2 tumor suppressor gene, and mutation or loss of the NF2 product, merlin, is sufficient for tumorigenesis in both hereditary and sporadic NF2-associated tumors.

Merlin belongs to the band 4.1 superfamily of cytoskeletal proteins, which also contain the related ezrin, radixin, and moesin (ERM) proteins. The ERM members provide a link between the cell cytoskeleton and membrane by connecting membrane-associated proteins to actin filaments. By stabilizing complexes in the cell cortex, the ERMs modulate morphology, growth, and migration of cells. Despite their structural homology, overlapping subcellular distribution, direct molecular association, and partial overlap of molecular interactions, merlin and ezrin exert opposite effects on cell proliferation. Merlin suppresses cell proliferation, whereas ezrin expression is linked to oncogenic activity. We hypothesized that the regions which differ between the proteins might explain merlin’s specificity as a tumor suppressor. We therefore analyzed the regions, which are most diverse between merlin and ezrin; the N-terminal tail and the C-terminus. To determine the properties of the C-terminal region, we studied the two most predominant merlin isoforms together with truncation variants similar to those found in patients. We also focused on the evolutionally conserved C-terminal residues, E545-E547, that harbor disease causing mutations in its corresponding DNA sequence.

In addition to inhibiting cell proliferation, merlin regulates cytoskeletal organization. The morphogenic properties of merlin may play a role in tumor suppression, since patient-derived tumor cells demonstrate cytoskeletal abnormalities. We analyzed the mechanisms of merlin-induced extension formation and determined that the C-terminal region of amino acids 538-568 is particularly important for the morphogenic activity. We also characterized the role of C-terminal merlin residues in the regulation of proliferation, phosphorylation, and intramolecular associations. In contrast to previous reports, we demonstrated that both merlin isoforms are able to suppress cell proliferation, whereas C-terminally mutated merlin constructs showed reduced growth inhibition.

Phosphorylation serves as a mechanism to regulate the tumor suppressive activity of merlin. The C-terminal serine 518 is phosphorylated in response to both p21-activated kinase (PAK) and protein kinase A (PKA), which inactivates the growth inhibitory function of merlin. However, at least three differentially phosphorylated forms of the protein exist. In this study we demonstrated that also the N-terminus of merlin is phosphorylated by AGC kinases, and that both PKA and Akt phosphorylate merlin at serine 10 (S10). We evaluated the impact of this N-terminal tail phosphorylation, and showed that the phosphorylation state of S10 is an important regulator of merlin’s ability to modulate cytoskeletal organization but also regulates the stability of the protein.

In summary, this study describes the functional effect of merlin specific regions. We demonstrate that both S10 in the N-terminal tail and residues E545-E547 in the C-terminus are essential for merlin activity and function.
REVIEW OF THE LITERATURE

1. Cancer

Cancer, the uncontrolled proliferation of cells, is caused by genetic changes. Multiple genetic alterations accumulate progressively during transformation when cells evolve from normal phenotypes via premalignant states towards malignant cancer. It has been estimated that between four and seven rate-limiting mutations are required for cancer cell transformation\(^1^2\). The premalignant mutated cells are converted into a neoplastic clone when the genetic alterations are transferred from the cancer cell to its progeny cells constituting a primary tumor\(^3\).

Cancers can be divided into familial and sporadic according to inherited or environmental causing factors. The sporadic cancers which occur spontaneously and result from acquired mutations, account for approximately 90% of all malignancies. Although many risk factors for cancer are recognized, no single factor increases the chance of malignancy as much as a genetic predisposition\(^4\). However, regardless of the underlying causing factor, tumor development in both sporadic and inherited cancers shares the same basic mechanism.

1.1. Tumorigenesis

More than 100 different forms of cancer exist, each with several subtypes. In spite of the large number of distinct tumors, they all evolve similarly. The transformation of normal cells into tumors; tumorigenesis, is a multistep process during which cells are genetically altered to confer a growth advantage. Malignant transformation involves six essential physiological changes: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, resistance to apoptosis, limitless replicative potential, induced angiogenesis, and activation of tissue invasion and metastasis\(^5\). Also genomic instability, inflammation, changed energy metabolism and the ability to evade the immune system are typical features of cancer progression\(^6\). These biological capabilities, acquired through changes in cancer cell genomes, are shared by most types of human tumors\(^5\).

The dependence on environmental stimulation is altered in malignant cells. Many antiproliferative signals maintain cellular quiescence within normal tissue by restricting the rate of cell division. Cancer cells are able to evade these signals by becoming insensitive to them, leading to their enhanced proliferation. Tumor cells also show reduced dependence on exogenous growth stimulation compared to normal cells, which are not able to proliferate in the absence of growth promoting signals. Transformed cells become sensitive to low levels of growth factors that would not normally trigger their proliferation by either overexpressing growth receptors or by favoring the receptors that transmit progrowth signals\(^5\).

Most cells carry a cell-autonomous program that restricts their multiplication, but this system is disrupted in cancer cells, which require limitless replicative potential during their
transformation into malignant tumors. The ability to multiply without limit, immortalization, is achieved by avoiding replicative senescence. This capability for unlimited proliferation is usually acquired by telomere maintenance\textsuperscript{5}, and 85-90\% of cancer cells succeed in sustaining their telomeres by upregulating the expression of telomerase, a telomere extending enzyme\textsuperscript{7}. Normal cells stop proliferating and enter into a quiescent state upon cell-cell contact, but cancer cells show loss of contact inhibition leading to tumor formation\textsuperscript{9}. However, neoplastic cell populations continue multiplying not only because of their higher rate of proliferation, but also due to decreased apoptosis that normally controls proliferation by eliminating excess cells. Tumor cells become resistant to cell death through inactivation of the apoptotic cascade, most commonly occurring by loss of the \textit{p53} tumor suppressor gene\textsuperscript{5}. The \textit{p53} protein functions as a DNA damage sensor that normally induces the apoptotic machinery, but the protein is inactivated in about 50\% of human cancers\textsuperscript{9}. As a consequence of both increased replicative potential and reduced apoptosis, transformed cells are able to expand to a size that constitutes a tumor.

Tumor growth also requires angiogenesis, a carefully regulated process by which existing quiescent vasculature is induced to grow new capillary blood vessels. A developing tumor needs to obtain the ability to stimulate angiogenesis, as the oxygen and nutrients supplied by the vasculature are necessary for cell survival. Thus, although angiogenesis does not initiate malignancy, it promotes tumor progression and metastasis. Different tumor types use distinct molecular strategies to activate angiogenesis, but many of them secrete angiogenic growth factors, such as vascular endothelial growth factor (VEGF)\textsuperscript{10}.

Benign tumors evolve to cancer when they obtain the ability to spread. The capability for invasion and metastasis enables malignant cells to break away from the tumor and migrate to distant organs. Cancer metastasis is a multistep process involving cell detachment from the primary tumor and invasion into the surrounding tissue, intravasation into the vascular or lymphatic system, spreading through the circulation, and extravasation and colonization in the target organ. Metastasis is mediated by different classes of genes affecting initiation, progression, and cancer cell virulence\textsuperscript{11}. It is enhanced by alterations in proteins that normally keep the cells of a tissue adherent or are involved in tethering cells to their surroundings. Several classes of proteins, such as cell-cell adhesion molecules (CAMs), integrins, and enzymes that cleave the extracellular matrix (ECM) are central to the acquisition of invasiveness and metastatic ability and the expression of these proteins is often altered in cancer cells\textsuperscript{5}.

1.2. Cancer genes

Almost 300 cancer associated genes have been identified, meaning that mutations in more than 1\% of our genes contribute to oncogenesis\textsuperscript{12}. Cancer genes are grouped into two broad classes; oncogenes and tumor suppressor genes. Tumorigenesis is induced by either activation of proto-oncogenes or by loss of tumor suppressor genes, but mutations in both these groups of genes have similar effects; increasing cell proliferation, differentiation, and survival.
1.2.1. Oncogenes

The genes with the potential to cause cancer by activating, gain-of-function mutations are called oncogenes. Individuals carrying activated oncogenes as a result of chromosomal translocations, gene amplifications, or mutations are highly tumor prone. Oncogenes are tumor-inducing constitutively active converted forms of normal proto-oncogenes. More than 100 proto-oncogenes have been identified, and many of them, such as Ras (rat sarcoma), Myc (myelocytomatosis oncogene cellular homolog), ERK (extracellular signal-regulated kinase), and EGFR (epidermal growth factor receptor), encode proteins that are involved in regulating cell proliferation and differentiation. The mutations generally involve only one of the oncogene alleles, which typically have a dominant effect on the cell, and most cancer-causing oncogene mutations are acquired, not inherited\textsuperscript{13,14}.

Oncogenes are involved in various signal transduction pathways, and alterations in these genes can lead to continuous signaling. Overactivating oncogene mutations are growth promoting by stimulating proliferation also in the absence of growth signals, thereby predisposing the cell to malignancy. Targeted cancer therapy takes advantage of the deregulated signaling in the tumor. Protein tyrosine kinases (PTKs) are the largest group of dominant oncogenes comprising about 0.3% of all genes, and somatic mutations in these genes cause a significant fraction of human cancers. Inhibition of oncogenic PTKs is often enough to slow tumor progression, and therefore specific PTK inhibitors have been developed. By analyzing signaling pathway activation in individual cancers, the growth inducing event of the tumor can be determined and used for drug selection, allowing for more personalized medicine\textsuperscript{15,16}.

1.2.2. Tumor suppressors

Genes with inactivating, loss-of-function mutations generating the cancer risk are tumor suppressor genes\textsuperscript{17}. These genes are protective genes whose protein products normally limit tumor growth. When a tumor suppressor gene is mutated leading to loss, inactivation, or reduction of its function, its growth inhibitory activity is decreased and the cell can progress towards malignancy\textsuperscript{18}. More than 30 tumor suppressors have been identified\textsuperscript{19}, and they regulate a wide range of cellular activities, including proliferation and cell cycle control, response to genetic damage, protein turnover, mitogenic signaling, cell differentiation, migration, apoptosis, and angiogenesis. Tumor suppressors can also prevent the formation of harmful mutations that could otherwise provide the cells with further genetic alterations\textsuperscript{20}.

According to Knudson’s two-hit model, two independent genetic events are required for tumor suppressor gene inactivation. Both alleles of these genes must be affected before an effect is manifested, as the presence of one functional allele is usually enough for the cancer-preventing function. Prototypic mutant tumor suppressor genes are therefore recessive. In hereditary cancers one of the mutations is passed through the germline and the second hit is spontaneously formed, whereas in sporadic tumors two somatic mutations inactivate both copies of the tumor suppressor gene, thus promoting tumor growth\textsuperscript{21}. Although most tumor suppressors follow this two-hit inactivation, also
exceptions exist. Some tumor suppressors are haploinsufficient, meaning that functional loss of only one allele is sufficient to confer a growth advantage that induces malignancy$^{22}$. Tumor suppressors can also be inactivated without genetic alterations by epigenetic silencing such as through methylation$^{23}$.

Tumor suppressors are divided into gatekeepers, caretakers, and landscapers depending on their function$^{24}$. Gatekeepers act directly to prevent tumor growth by inhibiting cell proliferation or by promoting apoptosis, and function for example as transcription factors. Both alleles of a gatekeeper gene must be mutated for tumor development and inactivation of these genes is the rate-limiting event in tumorigenesis. Individuals with a hereditary mutation in a gatekeeper gene have more than a 1000-fold greater risk of developing tumors than the general population. A tumor suppressor gene that functions as a caretaker operates indirectly to suppress growth by protecting the genome. Caretaker genes are involved in DNA-repair and genome integrity, and mutations in these genes lead to genomic instability, but not directly to cancer. However, caretaker mutations increase the rate by which mutations accumulate in other genes, thereby raising the probability that some gatekeeper function will be lost. Mutations in caretaker genes increase the risk of developing cancer by 5-50-fold$^{25}$. Landscaper tumor suppressors do not directly affect cellular growth, but they act by modulating the microenvironment of the tumor cells. These genes regulate ECM proteins, surface receptors, adhesion proteins, or secreted growth factors. Defects in landscapers generate an abnormal stromal environment that allows for the neoplastic transformation$^{24,26}$.

1.3. Inherited Cancer Syndromes

A cancer syndrome is a genetic predisposition to develop cancer. Although cancer is a common disease, only 5-10% of all malignancies arise as a result of inherited gene mutations$^{27}$. Germline mutations in cancer genes can be transferred from one generation to the next and result in a varying increased risk to develop cancer during lifetime, depending on the mutation and on environmental factors$^{4,28}$. Individuals with a definite cancer family history are predicted to have a two-fold risk of cancer compared to the general population, whereas those who carry a germline mutation in a cancer gene may have more than an 80% lifetime risk of forming a specific cancer$^{29}$. However, less than 0.3% of the population are carriers of a genetic mutation that has a strong effect on the cancer risk$^{30}$.

Over 200 hereditary cancer syndromes have been identified, but many of these conditions are rare$^{31}$. The majority of them are autosomal dominantly inherited and highly penetrant. Both tumor suppressors and oncogenes cause cancer susceptibility if inherited in a mutated state, but inactivating mutations in tumor suppressor genes are more common in inherited cancer predispositions than mutations in oncogenes$^{28}$, presumably because the loss-of-function mutation of a tumor suppressor is masked by the remaining normal allele during development, whereas gain-of-function mutations could be lethal$^{32}$.

In hereditary cancers, the first mutation in a cancer gene is inherited and recessive, whereas the second hit in the remaining allele of the same gene occurs somatically and initiates tumorigenesis$^{21}$. The second somatic event in individuals with a germ line
mutation often occurs via loss of heterozygosity (LOH) and is generally a larger molecular event such as a structural chromosome aberration. Inheritance of a single mutated allele is sufficient for tumor susceptibility, as only one additional mutation is required for complete loss of gene function, and inactivation of the normal remaining copy in the susceptible tissue is usually the rate-limiting step for tumor development. Thus, although the cancer predisposition has a dominant pattern of inheritance, the oncogenesis is recessive.

An inherited cancer syndrome can be suspected by typical clinical signs; a family history with multiple close relatives from several generations affected with the same type of cancer, an earlier age of onset than in sporadic cases of the same cancer type, and multiple primary tumors. Cancer syndromes not only increase the risk of malignancy, but some of the conditions are also associated with non-malignant features such as benign tumors and congenital anomalies which can help in identifying the syndrome. However, genetic heterogeneity is common among these conditions and because of phenotypic variability, mosaicism in which only a portion of cells contain the mutation, age-related or reduced penetrance, and gender-specific differences, many families with a cancer syndrome do not fulfill these criteria. Cancer is also relatively common in the general population, and it is therefore possible to have familial clustering of related cancers by chance without an inherited component. In families where the predisposing gene mutation has been identified, the cancer risk of healthy relatives can be assessed by genetic counseling and predictive genetic testing.

2. The Neurofibromatosis 2 disease

The neurofibromatoses (NFs) are a group of cancer syndromes characterized by the development of nerve sheath tumors. Two main forms of the NFs exist; NF1 and NF2, for which the diagnostic criteria were formulated at the National Institutes of Health (NIH) Consensus Development Conference in 1987. However, schwannomatosis is sometimes grouped to the NFs. The disorders belong to the phakomatoses, a group of tumor syndromes characterized by benign lesions where malignant transformation occurs only rarely. The most common Neurofibromatosis 1 (NF1), also called von Recklinghausen disease, is an autosomal dominant disorder caused by mutations in the NF1 tumor suppressor gene on chromosome 17q11.2 coding for neurofibromin. NF1 affects approximately 1 in 3500 individuals, and leads to the formation of cutaneous and plexiform neurofibromas, café-au-lait patches, iris Lisch nodules, skin fold freckling, optic nerve gliomas, and bony dysplasia. Schwannomatosis, a peripheral-nerve tumor syndrome estimated to have an incidence of 1 in 30,000, is induced in some patients as a result of a mutation in the INI1/SMARCB1 tumor suppressor gene on chromosome 22q11. The disorder is characterized by multiple schwannomas without the presence of vestibular tumors.
Neurofibromatosis 2 (NF2 [MIM 10100]) is an autosomal dominantly inherited tumor suppressor syndrome which was described already in 1822\(^43\) and its heredity first reported in 1920\(^44\). The disease is rare, affecting approximately 1 in 33,000 individuals\(^45\), although a study in Finland estimated an even lower occurrence of 1 in 87,000\(^46\). NF2 can be diagnosed when the criteria in Table 1 are fulfilled.

**Table 1. Diagnostic criteria for NF2.** The criteria include the main NIH criteria with additional Manchester criteria. Adapted from Evans 2009\(^47\).

<table>
<thead>
<tr>
<th>Main criteria</th>
<th>Additional criteria</th>
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<tr>
<td>Bilateral vestibular schwannomas <strong>or</strong></td>
<td>Unilateral VS <strong>and</strong> any two of: meningioma, glioma, neurofibroma, schwannoma, and posterior subcapsular opacities <strong>or</strong></td>
</tr>
<tr>
<td>Family history of NF2 <strong>and</strong></td>
<td></td>
</tr>
<tr>
<td>1) Unilateral VS <strong>or</strong></td>
<td></td>
</tr>
<tr>
<td>2) Any two of: meningioma, glioma, neurofibroma, schwannoma, posterior subcapsular lenticular opacities</td>
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NF2 predisposes to the development of multiple benign tumors of the nervous system, and the hallmark of the disease is bilateral schwannomas of the eighth cranial (vestibular) nerve (Fig.1). Schwannomas are encapsulated tumors of the Schwann cells. Normally Schwann cells grow around the neurons and produce the myelin sheath that surrounds axons in the peripheral nervous system. Over 90% of NF2 patients develop bilateral vestibular schwannomas (VS), but also schwannomas of other cranial and peripheral nerves are present in 25-50% of patients. The other main tumor features of NF2 are often multiple intracranial meningiomas (in 50% of patients, Fig.1), and some low grade central nervous system (CNS) malignancies such as ependymomas (in 20-50% of patients, Fig.1) and very rarely astrocytomas. Meningiomas are slowly growing mostly benign tumors arising from the brain and spinal cord meninges, whereas ependymomas form from the ependymal cells of the ventricular lining. At least two-thirds of individuals with NF2 develop spinal tumors; schwannomas, meningiomas, and ependymomas, which are often the most challenging to manage. Also ocular manifestations are common in NF2 patients and include reduced visual acuity and cataract (in 60-80% of patients). About 70% of patients have skin tumors, most of which are schwannomas, and café-au-lait patches are reported in 40% of patients. Many NF2 patients also form peripheral neuropathy\(^48,49\).
Review of the literature

NF2 generally presents in young adulthood with an average age of onset of symptoms between 18 and 24 years, but ranging from birth to 70 years. Diagnosis of the disease is delayed on average for 7 years with a mean age of diagnosis of 27 years. Most NF2-affected individuals form bilateral VS by the age of 30 years and develop symptoms such as hearing loss, tinnitus (ringing in the ears), and imbalance. The preference for tumors on the vestibular nerve remains unexplained. NF2-associated tumors are usually slowly growing and only rarely progress to malignancy, but their anatomical location and multiplicity can compress nearby nerves, causing pain and nerve dysfunction, or increase cranial pressure, leading to a variety of symptoms. The tumors gradually cause deafness, balance disorder, paralysis, and increasing neurological problems, and most patients will eventually need wheelchair assistance. NF2 patients face significant morbidity and reduced life expectancy; the average age of death is 36 years with a mean survival of 15 years from diagnosis. However, the actuarial survival is 60 years and improving with earlier diagnosis and better treatment in specialty centers. As the NF2-deficient tumors are not malignant, conventional chemotherapy is ineffective. Treatment of symptomatic NF2 tumors is therefore primarily surgical, although radiation therapy is also occasionally used[48,50].

2.1. The NF2 gene

The NF2 syndrome develops as a result of inactivation of the NF2 tumor suppressor gene, which is the only gene known to be associated with the disease. The NF2 gene was mapped to chromosome 22 in 1987[51,52] and identified on chromosome location 22q12.2 in 1993 (Fig.2)[53,54]. This gene fulfills the criteria for a classic tumor suppressor; transmission of the mutant gene through the germline of patients predisposes to tumor...
formation, which is initiated by a somatic mutation of the remaining wild-type (WT) copy of the gene\textsuperscript{51,55}.

The NF2 gene contains 17 exons and has two major splicing isoforms, isoform 1 and isoform 2 (Fig.2). Isoform 1 is expressed from exons 1-15 and 17, whereas isoform 2 has a 45 base pair (bp) insertion with a termination codon that prevents translation of exon 17, and is therefore coded by exons 1-16\textsuperscript{56}. The gene has an open reading frame (ORF) of 1788 bases distributed over 110 kilobases (kb) of DNA, and produces mRNAs of three different sizes; 7 kb, 4.4 kb, and 2.6 kb\textsuperscript{53,54}. Even though the phenotype of the NF2 disease is highly restricted, the NF2 gene is widely expressed in a variety of cell types from different tissues\textsuperscript{56,57}.

**Figure 2. The NF2 gene.** A. The NF2 gene is located on chromosome 22 (left), at the position 22q12.2 as shown in the ideogram (right). B. Schematic diagram of the NF2 gene exon organization and its two main isoforms. Each exon is indicated by the numbered boxes and above each box is the size of that exon in base pair. Numbers below each exon border indicates the position of the first codon of that exon. The alternatively spliced last exons 16 and 17 are shown in red and blue.

Orthologous genes to the human NF2 have been identified in many species. Among the metazoans, the gene is phylogenetically conserved in invertebrates, fish, mammals, and birds\textsuperscript{58}. The murine Nf2 gene maps to chromosome 11 in a region homologous to human chromosome 22, and shows 90% nucleotide sequence identity with the coding region of the human NF2 gene\textsuperscript{59-61}. No NF2 homolog has been found in the genomes of fungi, plants, or protozoa, implying that NF2 function is specific to multicellular metazoans\textsuperscript{58}.

In addition to its tumor suppressor function, the NF2 gene is essential for normal development and viability at least in fruit fly, roundworm, zebrafish, and mice\textsuperscript{62-65}. Nf2
knockout (Nf2-/-) mice die early during embryogenesis at day 7 of a failure to form extra-embryonic ectoderm and initiate gastrulation\textsuperscript{64}. Heterozygous Nf2 mice (Nf2+/-) are viable and develop a wide range of malignant highly metastatic tumors, predominantly osteosarcomas, fibrosarcomas, and hepatocellular carcinomas\textsuperscript{66}, but not the tumors typically observed in NF2 patients. However, targeted inactivation of Nf2 specifically in Schwann cells or meningeal cells in conditional Nf2 mutant mice, leads to the formation of schwannomas and meningiomas that resemble NF2 tumors\textsuperscript{67,68}.

### 2.2. \textit{NF2} gene mutations

Consistent with the Knudson’s two-hit theory of tumorigenesis, tumor development initiates when both alleles of the NF2 gene are inactivated. The NF2 gene has a high estimated mutation rate of $6.5 \times 10^{-6}$, and the occurrence of NF2 without family history is therefore common\textsuperscript{69}. Mutations of the NF2 gene are frequently detected not only in tumors of the NF2 disease, but also in their sporadic counterparts and in some malignant tumors unrelated to the syndrome.

#### 2.2.1. Mutations in the NF2 disease

In the NF2 syndrome, a germline defect in one of the NF2 alleles is inherited and followed by somatic inactivation of the remaining allele in individual Schwann or meningeal cells at some point later in life, which induces tumorigenesis. The disorder has a high penetrance of nearly 100% and individuals who inherit a pathogenic mutation will develop the disease in an average lifetime\textsuperscript{48}. As a result of the high spontaneous mutation rate of the NF2 gene, about half of NF2 cases represent \textit{de novo} germline mutations without a family history\textsuperscript{69}. Mutations in the NF2 gene are detected in 88% of familial and 59% of sporadic NF2 patients\textsuperscript{70}, indicating that loss of the NF2 protein is the main rate-limiting step in NF2 tumor formation\textsuperscript{71}. However, mathematical modeling of VS formation in NF2 patients has suggested that at least one more mutational event, in addition to the biallelic NF2 inactivation is required for human schwannoma development\textsuperscript{72}.

More than 200 different mutations in the NF2 gene have been described. The majority of the alterations are point mutations, of which C>T transitions causing nonsense mutations are the most common (Fig.3). Although truncating mutations are the most frequent germline events with nonsense and frameshift mutations occurring in approximately 50% of families and splice site mutations in 25%, also single and multiple exon deletions are detected. In contrast, missense mutations occur with an extremely low frequency in only 5% of NF2 families. Inactivating mutations in the NF2 gene are fairly evenly distributed throughout the first 15 exons, except for exon 9 which has a low frequency of mutations. However, exons 1-15 contain six CGA codons which are mutation hotspots for C>T transitions in CpG dinucleotides. Interestingly, no mutations have been detected in the alternatively spliced exons 16 and 17\textsuperscript{73,74}.

Partial correlation between the type of mutation and the NF2 disease phenotype has been observed. In general, patients with truncating nonsense or frameshift NF2 mutations
have a more severe disease, whereas missense mutations, large deletions, or somatic mosaicism are associated with mild phenotypes\textsuperscript{74-80}. Patients with constitutional truncating mutations typically have an earlier onset and increased prevalence of meningiomas, spinal tumors, and peripheral nerve tumors leading to a greater disease-related mortality than those with other NF2 mutations\textsuperscript{75,81-83}. The phenotype is more variable in patients with splice-site mutations, with a milder disease if occurring in the 3' half of the gene\textsuperscript{84}. The NF2 syndrome has a high rate of mosaicism with 25-33\% of patients without a family history of the syndrome, and especially milder cases, having a mosaic disease\textsuperscript{85-87}. Mosaicism is particularly likely when tumors are predominantly on one side of the body, and 60\% of NF2 patients with unilateral vestibular schwannoma (UVS) are somatic mosaics\textsuperscript{87}.

![Figure 3. Comparison of constitutional and somatic NF2 gene mutations by predicted effect. FS = frameshift, NS = nonsense, MS = missense, SS = splice site. Other mutations include both large deletions and insertions and in-frame deletions and insertions. Combined from Ahronowitz et al. 2007\textsuperscript{74} and Baser et al. 2006\textsuperscript{73}.]

\textbf{2.2.2. Mutations in NF2-related sporadic tumors}

In accordance with the two-hit model, acquired mutations in the NF2 gene contribute also to the development of sporadic tumors\textsuperscript{53-55}. Schwannomas and meningiomas are among the most common brain tumors in humans, accounting for about 30\% of adult CNS neoplasms. However, only 3-7\% of all schwannoma and 1\% of meningioma patients have NF2\textsuperscript{46,48}. Sporadic VS has an annual incidence of approximately 1.9 per 100 000 individuals\textsuperscript{88}, and UVS constitute 5-10\% of all intracranial tumors\textsuperscript{89}. Meningiomas account for 25\% of primary intracranial neoplasms\textsuperscript{90}, with an annual incidence of about 7.1 per 100 000 individuals\textsuperscript{31}, whereas the annual incidence of ependymoma is 2-4 per million in Europe\textsuperscript{92}. The sporadic tumors present with similar symptoms as their syndromic counterparts but later in life, with a median age of diagnosis of 48 years for schwannomas and 58 years for meningiomas\textsuperscript{46}. 
Somatic biallelic inactivation of the \textit{NF2} gene is detected in most sporadic schwannomas\textsuperscript{93}, in 50-70\% of sporadic meningiomas\textsuperscript{94}, and in 5-10\% of sporadic ependymomas\textsuperscript{95}, suggesting that \textit{NF2} inactivation is the main predisposing event in the formation of most schwannomas and about half of meningiomas\textsuperscript{74}. Partial or total deletion of chromosome 22 leading to LOH of the \textit{NF2} gene is the most consistent alteration reported in sporadic meningiomas\textsuperscript{96-98}, and it is associated with \textit{NF2} mutations in the tumors\textsuperscript{94,99} although the frequency of \textit{NF2} mutations varies among meningioma subtypes\textsuperscript{100}. LOH of the \textit{NF2} region is also detected in sporadic ependymomas\textsuperscript{101}. In addition, the two \textit{NF2} alleles are commonly altered in tumors from schwannomatosis patients, but the mutations differ between tumors in the same individual and are not found in their non-tumor tissues, implying that they represent secondary mutations\textsuperscript{102,103}.

Although the majority of somatic \textit{NF2} gene mutations are similar to germline mutations, there are some differences between their distribution and type (Fig.3). Of the somatic mutations in sporadic UVS over half are frameshift mutations, in contrast to constitutional mutations that are primarily nonsense and splice site. Somatic mutations also have the tendency to lie within the 5' end of the transcript in sporadic meningiomas and possibly also ependymomas, with a complete absence of mutations in exons 14 and 15 in meningiomas\textsuperscript{73,74}.

In addition to mutations, also epigenetic silencing of the \textit{NF2} gene has been described in tumors. Between 20-40\% of sporadic schwannomas are inactivated by \textit{NF2} promoter methylation\textsuperscript{104,105}, and aberrant hypermethylation is involved also in the development of sporadic meningiomas\textsuperscript{106} and some ependymomas\textsuperscript{107}.

### 2.2.3. Mutations in other sporadic tumors

Mutations in the \textit{NF2} gene are found in some sporadic malignant tumors unrelated to the \textit{NF2} syndrome, such as in carcinomas of breast\textsuperscript{56} and colon\textsuperscript{108,109}, and in melanomas\textsuperscript{56}. Somatic inactivation of \textit{NF2} has been identified in about 40\% of malignant mesotheliomas\textsuperscript{110-113}, and all tumors with \textit{NF2} mutations also exhibited LOH of the \textit{NF2} region\textsuperscript{112}. Coexistence of mutations and LOH at the \textit{NF2} locus has also been reported in perineural cell tumors\textsuperscript{114}, and the \textit{NF2} gene is homozygously lost in some hepatocellular carcinoma cell lines\textsuperscript{115}. In addition, allelic loss of \textit{NF2} has been described in thyroid carcinomas\textsuperscript{116} and in gastrointestinal stromal tumors\textsuperscript{117,118}. It is possible that \textit{NF2} mutations represent later stages in the tumor progression of these NF2-unrelated neoplasms\textsuperscript{56}.

Inactivation of the \textit{NF2} gene in tumors of different origin together with the studies in heterozygous \textit{Nf2} mice that develop sarcomas and liver tumors, suggest that the \textit{NF2} gene product might have a more general role in tumorigenesis\textsuperscript{56}. However, the highly metastatic tumors of \textit{Nf2+/-} mice are not observed in \textit{NF2} patients, human osteosarcomas do not have \textit{NF2} mutations\textsuperscript{119}, and the most prevalent human cancers have not been described to contain \textit{NF2} mutations\textsuperscript{65}. It is possible, however, that abolition of \textit{NF2} function occurs also through alternative mechanisms to mutation, such as through epigenetic silencing of the \textit{NF2} gene or post-translational inactivation of the \textit{NF2}-encoded protein\textsuperscript{103}.
3. The Cytoskeleton

3.1. Composition of the cytoskeleton

The cell cytoskeleton functions as dynamic scaffold providing structure and intracellular organization. This network of filamentous polymers and regulatory proteins is essential for many processes as it controls cell shape, adhesion, migration, and various signaling pathways. The cytoskeleton consists of three kinds of protein filaments; microfilaments, intermediate filaments, and microtubules (Fig.4).

![Cytoskeletal filaments](image)

**Figure 4. Cytoskeletal filaments.** The cytoskeleton is composed of three main types of filaments; actin filaments (AF), intermediate filaments (IF), and microtubules (MT).

Microfilaments, or actin filaments, are the thinnest cytoskeletal filaments with a diameter of 5-9 nm. In these filaments, globular actin (G-actin) monomers are assembled into filamentous polymers (F-actin). Actin filaments are present throughout the cell, but most highly concentrated in the cortex on the inner face of the plasma membrane, where they link transmembrane and cytoplasmic proteins together. The flexible polarized actin filaments provide mechanical strength and generate force, this way regulating both the shape and movement of cells\(^{120}\).

Intermediate filaments are fibers of an average of 10 nm in diameter, constructed of a heterogeneous class of intermediate filament proteins, such as keratin, vimentin, and lamin. These highly stable filaments create a fibrous network that increases the mechanical strength of the cytoskeleton. Intermediate filaments organize the internal structure by anchoring organelles but also serve as major components of the nuclear envelope. In addition, the filaments participate in cell connections, and are therefore involved in regulating adhesion\(^{121}\).

Microtubules are long, hollow tubes of about 25 nm in diameter built up by dimers of \(\alpha\)- and \(\beta\)-tubulin. These long and dynamic filaments typically extend from the centrosome to the cell periphery. Microtubules determine the positions of membrane-enclosed organelles, and these polymers are essential for directing cell transport and for cell
division\textsuperscript{122}. The cytoskeletal polymers formed by actin filaments, microtubules, and intermediate filaments interact with each other through many regulatory proteins that link the filaments to networks, which control the mechanics of the cell\textsuperscript{123}.

### 3.2. Regulation of cell shape and motility

Cell migration and morphological changes involve alterations in the cytoskeleton, the cell membrane, and cell-substrate adhesions. Especially actin and actin-associated proteins are involved in cell-shape determination, and a large number of actin-binding proteins regulate the assembly and organization of the actin cytoskeleton\textsuperscript{124}. By polymerizing actin filaments and microtubules against membranes, the cytoskeleton generates the forces that enable the cell to move and to change shape\textsuperscript{123}.

Actin filaments form many types of cell-surface structures. Branched networks of actin filaments make up sheet-like structures termed the lamellipodium which are often present at the front of motile cells (Fig.5). The thin 0.1-0.2 μm protrusive lamellipodia vary in breadth from 1-5 μm and are filled with a crosslinked meshwork of F-actin\textsuperscript{125}. Rodlike extensions called filopodia often protrude from the lamellipodia (Fig.5). These are protrusions of 0.1-0.5 μm in diameter composed of parallel F-actin bundles that extend 5-50 μm out of the cell\textsuperscript{126}. These dynamic structures are pioneers during protrusion formation, functioning as sensors for cells to probe their environments. They have an important role in cell migration guidance, wound healing, adhesion to the ECM, and neurite outgrowth. Short filopodia mostly embedded in the cell edge are called microspikes. Although filopodia are commonly found in association with lamellipodia, they can also form independently\textsuperscript{127,128}. In addition to lamellipodia and filopodia, non-muscle cells contain actin-rich stress fibers (Fig.5). Stress fibers are bundles composed of antiparallel arrays of actin filaments associated with myosin II. These acto-myosin structures provide the contractile force required for cell motility, adhesion, and morphogenesis\textsuperscript{129}.

The small GTPases of the Rho family are linked to the modulation of cell morphology as they are critical regulators of the actin cytoskeleton. The proteins are a part of a complex network of essential signal transduction pathways transmitting signals from extracellular stimuli to the downstream target effectors. Rho GTPases act as molecular switches by cycling between inactive GDP-bound and active GTP-bound states, and their activation is mediated by guanine nucleotide exchange factors (GEFs), while inactivation is promoted by GTPase activating proteins (GAPs). Twenty different mammalian Rho GTPases have been identified, but the best characterized members are RhoA (Ras homologous member A), Rac1 (Ras-related C3 botulinum toxin substrate 1), and Cdc42 (cell division cycle 42). Although there is cross-talk between the family members and the Rho GTPases can be activated in series, each protein induces specific morphological changes. Cdc42 triggers the formation of filopodia, Rac1 promotes lamellipodium development, whereas the formation of stress fibers and focal adhesions is regulated by RhoA\textsuperscript{130}.

Cell motility is a complex process, requiring constant restructuring of the actin cytoskeleton\textsuperscript{131}. The motility of a cell can be divided into three essential steps (Fig.5).
First, as a result of actin filament polymerization, protrusive structures such as lamellipodia and filopodia extend from the front of the cell in the direction of migration. Second, the cell attaches to the surface by forming new adhesions with the substratum under the leading edge. The formed protrusion is stabilized by attaching to the surrounding through receptors such as integrins whereas adhesion at the rear disassembles. Third, the rest of the cell body is translocated through actomyosin-based contractile forces. Stress fibers pull the rear of the migrating cell forward, leading to retraction of the trailing edge\textsuperscript{132}.

Figure 5. Cell migration. The cell migration process can be schematized into three separate phases. 1. Protrusion of the leading edge in the form of lamellipodia and filopodia. 2. Attachment of the leading edge through the formation of new focal adhesions and deadhesion of the rear. 3. Traction of the bulk of the cell through stress fiber contraction.

Localized activation of the Rho GTPases is essential for migration. The initial response of a cell to a migration-promoting agent is to polarize and extend protrusions in the orientation of migration to establish directionality. Cdc42 which is active in the front of migrating cells is a regulator of both cell polarity and filopodia formation, and activation of Rac at the leading edge promotes lamellipodia formation. Rho in contrast, acts in the rear of the cell and Rho-regulated contractility is required for tail retraction\textsuperscript{133}.

3.3. Actin cytoskeleton in cancer

As the actin cytoskeleton is critical for most cellular activities, malfunction of cytoskeletal proteins results in various human diseases, including cancer. The cytoskeleton plays an essential role in many of the functions that are important for cancer cells and their invasion, such as the cell cycle, morphogenesis, and migration. Cytoskeletal alterations are linked to changes in cell proliferation and ultimately tumor formation, since disorganization of the cytoskeleton is often associated with cellular transformation\textsuperscript{134}. Malignant cells display altered morphology with changes in their actin cytoskeleton, and often the expression of many actin-associated proteins in these cells is altered\textsuperscript{135}. Several of the Rho GTPases that regulate both morphogenesis and migration are overexpressed.
in various tumors, and contribute to most steps of cancer initiation and progression. Unregulated activity of Rho GTPases has been linked to cell transformation, invasion, and motility, and deregulation of these proteins correlates with poor clinical outcome. Also NF2-deficient schwannoma and meningioma cells display cytoskeletal abnormalities.

4. ERM proteins

The ERM protein family consists of three closely related molecules; ezrin, radixin, and moesin. These proteins belong to a larger superfamily of band 4.1-members, which regulate interactions between the cytoskeleton and plasma membrane. The founding member of this family, protein 4.1, is an important structural molecule particularly in erythrocytes, where it regulates the physical properties of membranes by stabilizing spectrin-actin interactions, this way controlling red blood cell shape. Band 4.1-protein family members are defined by the presence of a homologous amino (N)-terminal module of approximately 300 amino acids, a region called the FERM (Four.1 protein, Ezrin, Radixin, Moesin)-domain. This highly conserved globular structure is composed of three subdomains; F1, F2, and F3, that are arranged like a cloverleaf. The F1 subdomain is structurally similar to ubiquitin, F2 to the acyl-CoA-binding protein, and F3 to a module in phosphotyrosine binding (PTB)/pleckstrin homology (PH), and Enabled/VASP homology 1 (EVH1). The FERM-domain is involved in targeting the proteins to the plasma membrane and in mediating membrane-cytoskeleton interactions. The superfamily of FERM-domain proteins comprise more than 50 mammalian members, most of which are involved in signaling at the cell cortex.

Genes coding for ERM proteins have been identified in all multicellular metazoan organisms analyzed, but the number of family members varies from one to three depending on species, and all non-mammalian genomes sequenced so far contain only one ERM gene. The three ERMs in humans are encoded by genes residing on different chromosomes; the ezrin gene EZR is located on chromosome 6q25.3, the radixin gene RDX on chromosome 11q23, and the moesin gene MSN on chromosome Xq11.2. The ERM proteins have an overall homology of 75-80% on the amino acid level, and they share a similar structure consisting of three domains: the N-terminal FERM-domain (residues ~1-300 in ezrin), a highly charged α-helical segment (residues ~300-470 in ezrin), and a small charged carboxy (C)-terminal domain (residues ~470-585 in ezrin) (Fig.6). The α-helical domain of the proteins consists of three extended helices, whereas the ~100 residue C-terminus is composed of a β-sheet and four major and two minor α-helices that adopt an elongated structure. The exceptionally high level of identity among the ERMs implies that they arose by gene duplication within vertebrates. The high degree of structural conservation between the proteins also indicates that they have similar functions, and that their activities might be partially interchangeable. The single ERM proteins of non-vertebrates, such as Drosophila moesin (D-moesin), which is 56-58% homologous to human moesin and ezrin, may
therefore perform all the functions that the three ERM proteins possess in vertebrate tissues\textsuperscript{158}, where the ERMs have some functionally unique properties\textsuperscript{159}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Members of the ERM protein family. The overall homology of the full-length proteins compared with human ezrin is indicated in the last column, and the length of the molecules is in amino acids. The identity of different domains compared to the corresponding domains of human ezrin is shown beneath the relevant domains. Modified from Turunen et al., 1998\textsuperscript{160}.}
\end{figure}

Most cell types express one or several of the ERM family members\textsuperscript{161-163}, but their expression is developmentally regulated and organ and cell type specific. Ezrin is predominantly expressed in the intestine, lung, kidney, and stomach, moesin in lung and spleen, and radixin in liver\textsuperscript{144,161}. In these organs, ezrin is expressed in epithelial and mesenchymal cells, moesin in endothelial and hematopoietic cells, and radixin in hepatocytes\textsuperscript{161,164}. The ERM proteins interact with common molecules, localize similarly within cells, and have identical models of regulation. Studies in ERM knockout mice mostly support redundant functions for the three proteins\textsuperscript{157}, as defects are observed exclusively in the
Review of the literature

organs where only one of the ERM\textsuperscript{s} is expressed. A lack of ezrin, the only ERM protein expressed in the intestinal epithelium, results in abnormal villus morphogenesis and the ezrin-deficient mice die by 3 weeks of age as a consequence of defects in the gastrointestinal tract\textsuperscript{165}. Ezrin also plays a role in the formation of apical secretory canaliculi in gastric parietal cells, and therefore adult ezrin knockdown mice suffer from loss of gastric acid secretion from these cells in their stomachs\textsuperscript{166}. Mice lacking radixin develop normally but show mild liver defects\textsuperscript{167} and deafness\textsuperscript{168}, whereas moesin-deficient mice show no obvious abnormalities\textsuperscript{169}. The limited phenotypes of the ERM knockout mice could reflect compensation by other family members\textsuperscript{170}.

All three ERM proteins localize in the cell cortex to actin containing regions of membrane remodeling such as filopodia, microvilli, membrane ruffles, and cell adhesion sites\textsuperscript{161-163,171,172}. Ezrin is, for example, detected in intestinal microvilli\textsuperscript{173}, radixin at the cell adherens junctions (AJs)\textsuperscript{144} and in the cleavage furrow of dividing cells\textsuperscript{174}, and all ERM proteins are present in neuronal growth cones\textsuperscript{175,176}. In addition to their membrane localization, the proteins are found in soluble forms in the cytoplasm\textsuperscript{163,177}, and in the nucleus, where at least endogenous ezrin and moesin are relocated in a cell density-dependent manner\textsuperscript{178}.

\begin{center}
\textbf{Figure 7. The ERM family members function as linkers between the cell membrane and actin cytoskeleton.} The N-terminus (N) of the ERMs binds to transmembrane receptors whereas the C-terminal domain (C) is connected to actin filaments.
\end{center}

The ERMs provide a structural link between the cytoskeleton and membrane by connecting transmembrane proteins to cortical actin filaments, this way strengthening the cell cortex\textsuperscript{151}. This regulated attachment is essential for modulation of cell morphology, control of cell adhesion and motility, and integration of signal transduction pathways from membrane proteins\textsuperscript{157}. The FERM-domain localizes the ERMs to the plasma membrane, where they interact with membrane proteins via their N-terminal domains and with
cytoskeletal components by their C-terminal ends (Fig.7)\textsuperscript{179}. The ERM proteins have the ability to heterodimerize with each other\textsuperscript{180,181} and they share many binding partners. All three ERMs associate directly with the cytoplasmic tails of transmembrane proteins such as the hyaluronic acid receptor CD44\textsuperscript{182,183}, leukosialin CD43\textsuperscript{184}, intracellular adhesion molecules ICAM1, ICAM2, and ICAM3\textsuperscript{184-186}, and the Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE1)\textsuperscript{187}. The ERMs can also associate indirectly to ion channels and receptors via scaffolding proteins NHE-RF (NHE regulatory factor)/EBP50 (ezrin-binding phosphoprotein 50)\textsuperscript{188,189} and exchanger 3 kinase A regulatory protein (E3KARP)\textsuperscript{190}. The ERM proteins bind actin filaments through a conserved actin binding site (ABS) comprising the 34 most C-terminal residues containing the actin binding motif KYKTL\textsuperscript{191-193}, but besides the C-terminus, additional ABSs are present in the FERM-domains of the proteins\textsuperscript{194,195}. Suppression of ERM function leads to alterations in the cortical actin cytoskeleton\textsuperscript{196}, destroys cell surface structures, and disrupts cellular adhesion\textsuperscript{172}, indicating that the proteins have an important role in organizing the actin cytoskeleton. The ERM proteins also associate with many cytoplasmic signaling molecules and regulate signal transduction for example through the Rho GTPase pathway by interacting with both positive GEFs and negative Rho guanine-dissociation inhibitor (RhoGDI) regulators\textsuperscript{197,198}.

4.1. Ezrin

Ezrin (cytovillin, p81) was the first characterized ERM member and represents the prototype of the family. The ezrin gene \textit{EZR} (\textit{VIL2}) located on chromosome 6q25.3 consists of 13 exons distributed over approximately 24 kb of genomic DNA\textsuperscript{143,199}. The gene codes for a 585 amino acid protein with a molecular mass of 81 kilodalton (kDa)\textsuperscript{142,143}. The identity between the mammalian ezrin family members is 97%, making the family one of the most conserved classes of mammalian proteins\textsuperscript{160}. Ezrin was originally identified as a cytoskeletal component of intestinal microvilli\textsuperscript{173,200}, and a substrate for tyrosine kinase\textsuperscript{201}. Although ezrin is highly expressed in some tissues, such as the intestine, intermediate levels of the protein are found in most tissues except for liver and muscle\textsuperscript{142}. Ezrin is especially enriched on the apical surface of polarized epithelial cells\textsuperscript{161}.

As a plasma membrane-cytoskeleton crosslinker, ezrin serves as a binding partner for many different proteins, including adhesion molecules, ion transporters, scaffolding proteins and signaling molecules\textsuperscript{157}. Ezrin, like the other ERMs, contains the high affinity ABS in its C-terminus\textsuperscript{191}, but also N-terminal residues 13-30 and 281-310 are essential for both the G- and F-actin-binding activity of ezrin\textsuperscript{194,195}. By affecting actin organization, the protein regulates cell morphology and the formation of various surface structures. Expression of full-length ezrin in cultured cells does not induce morphological changes, but truncated proteins mimicking active molecules, provoke the appearance of microvilli and protrusions. Removal of the last 21 C-terminal amino acids is sufficient to generate this cell-extension phenotype\textsuperscript{195}, whereas overexpression of the N-terminal half of ezrin is able to suppress the formation of microvilli\textsuperscript{202}. The N-terminal domain apparently regulates the morphogenic activity of the C-terminal domain in full-length ezrin\textsuperscript{195,203}. 
Ezrin has oncogenic activities with a positive role in tumorigenesis. Although it does not represent a conventional oncogene as no EZR gene mutations have been detected\textsuperscript{204}, the protein enhances malignant transformation, survival, motility, and tumor invasion\textsuperscript{170}. Elevated ezrin levels are associated with increased cell proliferation\textsuperscript{205}, and both transformed rat fibroblasts\textsuperscript{206}\textsuperscript{207} and mouse sarcoma cells\textsuperscript{208} express enhanced amounts of ezrin. The protein is required for invasion of many different cancer cells\textsuperscript{209}\textsuperscript{211} and is an essential regulatory molecule for tumor metastasis\textsuperscript{212}. Ezrin is upregulated in many metastatic tumors\textsuperscript{213}\textsuperscript{216}, and promotes metastasis of at least osteosarcomas\textsuperscript{217}, rhabdomyosarcomas\textsuperscript{218}, mammary carcinoma\textsuperscript{219}, and melanoma cells\textsuperscript{220}. Ezrin is dynamically regulated during the metastatic cascade of osteosarcoma; it is active during the onset and early stages of metastatic progression and in later phases as established large metastases expand, but downregulated in between\textsuperscript{219}. As a component of cell surface structures, the protein has an important function in mediating both cell-matrix and cell-cell interactions, and may therefore have a role in the control of adhesion between cancer cells and the surrounding tissue, this way affecting metastasis and invasion\textsuperscript{212}\textsuperscript{221}. Ezrin expression, which is often altered in tumors, associates with tumor progression and is a prognostic indicator in several malignancies. Upregulation of ezrin correlates with poor clinical outcome at least in carcinomas of the breast\textsuperscript{222}, endometrium\textsuperscript{223}, head and neck\textsuperscript{224}, ovary\textsuperscript{210}, and colorectum\textsuperscript{225}\textsuperscript{226}, and in melanomas\textsuperscript{227}\textsuperscript{228} and different sarcomas\textsuperscript{217}\textsuperscript{229}.

5. The NF2 protein Merlin

5.1. Properties of merlin

The NF2 gene encodes the tumor suppressor protein merlin (or schwannomin). Merlin shares overall structural similarity with the ERM family of proteins after which it was named (moesin-ezrin-radixin like protein)\textsuperscript{53}\textsuperscript{54}. Similarly to the ERM proteins, merlin contains three structural domains (Fig.8); the N-terminal FERM-domain (residues ~1-314) consisting of three subdomains F1-F3, an α-helical region (residues ~315-491), and a charged C-terminal domain (amino acids ~492-595). The FERM-domain of human merlin is highly conserved and over 60% identical with the ERM-members, whereas the homology of the α-helical and C-terminal domains is only 20-30%. This makes the overall identity of merlin with the ERM proteins 45% (Fig.6)\textsuperscript{160}. Due to the marked sequence and structural similarity, merlin and ERMs are believed to have arisen early in metazoan evolution from a common ancestor\textsuperscript{58}. However, merlin contains 18 unique N-terminal residues outside the FERM-domain, which form an extended tail and are not conserved in the ERMs\textsuperscript{58}\textsuperscript{230}\textsuperscript{232}.

Merlin exists as two major isoforms as a result of alternative splicing of exon 16. Isoform 1, produced from exons 1-15 and 17, is a 595 residue protein with a molecular mass of 66 kDa\textsuperscript{54}. In isoform 2, exon 16 inserts 11 amino acids and a stop codon resulting in a 590 residue protein (Fig.2). Thus, the isoforms are identical over the first
579 residues but differ in their C-terminal sequences as isoform 1 has a hydrophobic helical C-terminus whereas isoform 2 contains a non-helical hydrophilic sequence\(^5^6\). Isoform 1 is the predominant type expressed in the eight cranial nerve\(^5^6\), although isoform 2 is slightly more prevalent in general\(^2^3^3\). Also a number of low transcript isoforms of merlin are found\(^2^3^3-2^3^5\), but their functional significance is unclear.

Figure 8. Domain structure of merlin isoform 1. Merlin consists of three distinct regions; the FERM-domain (exons 1-9), the \(\alpha\)-helical domain (exons 10-13), and the C-terminus (exons 14-17). The FERM-domain is made up from three subdomains; F1 (residues 19-100), F2 (residues 101-215), and F3 (residues 216-313). Merlin also contains an N-terminal tail region (residues 1-18).

Merlin is evolutionally conserved across species indicating that it has an important function\(^5^8\). The mouse protein is 98% identical to human merlin and similarly alternatively spliced\(^5^9-6^1\). A merlin ortholog exists also in Drosophila, called D-merlin that shares 55% amino acid identity with the human protein, but does not have alternatively spliced isoforms\(^1^5^8\).

Merlin is expressed in many tissues during rat embryogenesis, but has a more restricted tissue distribution in the adult organism\(^2^3^6\). The protein is widely expressed at low levels also in a variety of human tissues, but at the cellular level the presence of merlin has been demonstrated mainly in smooth muscle cells, neurons, and Schwann cells\(^2^3^7-2^3^9\). Although the protein is expressed in Schwann cells, meningothelial cells, and ependymal cells, the expression is not as pronounced as in neurons and other glia\(^2^3^8,2^4^0\), even though the NF2-associated tumors predominantly arise from these cell types. Despite the presence of both ERM proteins and merlin in Schwann cells, the absence of merlin function alone results in the development of schwannomas\(^9^3\).

5.2. Functions of merlin

Merlin is the first example of a human tumor suppressor, a gatekeeper, that serves as a cytoskeletal component between the membrane and cytoskeleton. Consistent with its function as a negative growth regulator, merlin plays an important role in inhibiting cell
proliferation at high cell densities. Overexpression of merlin suppresses the proliferation of mouse fibroblasts, rat, and human schwannoma cells, and human meninoma cells. Reintroduction of merlin in NF2-deficient schwannoma and mesothelioma cells, inhibits proliferation by inducing cell cycle arrest at G0/G1, but also increases apoptosis. Furthermore, merlin is able to reverse the malignant phenotype of Ras transformed NIH 3T3 cells and restore their contact inhibition of cell growth. Conversely, loss of D-merlin results in cellular overproliferation, and reduction of merlin expression enhances cell proliferation in Schwann-like and glial cells. Inactivation of merlin leads to exit from contact-dependent growth inhibition and promotes progression through the G1 cell cycle phase. Thus, merlin has distinct cell density dependent activities. It is inactive as a tumor suppressor in subconfluent cells, but becomes activated as the cells approach confluence, thereby affecting contact inhibition of growth. Although merlin and the ERM proteins share some common functions, there is no redundancy for merlin’s growth inhibitory mechanism.

Many tumor suppressors undergo nucleo-cytoplasmic shuttling that affects their function. Similarly, endogenous merlin is targeted to the nucleus in a cell cycle specific manner. The protein is accumulated perinuclearly at the G2/M phase, and localized to the nucleus at early G1 in cultured cells, whereas in schwannoma cells of VS, merlin resides in the cytoplasm during G0/G1 and translocates to the nucleus at S phase. No nuclear localization signal has been identified in merlin, but a nuclear export sequence (NES) is present in exon 15 between residues 535-551, while exon 2 contains a cytoplasmic retention factor. Studies have suggested that the FERM-domain is sufficient for nuclear entry, whereas the C-terminus can inhibit nuclear accumulation in the full-length molecule unless it associates with the N-terminus. The nucleo-cytoplasmic shuttling of merlin is tightly regulated and dependent not only on the cell cycle phase, but also on cell density and adhesion. Even though the nuclear function of merlin is not completely elucidated, it has been shown that merlin interacts with and inhibits the function of the CRL4-DCAF ubiquitin ligase upon entering the nucleus.

Merlin, like the ERM proteins, acts as a linker between plasma membrane molecules and cytoskeletal components. Many merlin-interacting proteins have been identified, and these include both membrane-associated and intracellular molecules. In analogy with ERM proteins, merlin associates via its FERM-domain with membrane proteins including CD44, β1-integrin, paranodin, and NHE-RF. Merlin also binds several molecules through its C-terminus, such as βII-spectrin, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), and syntenin. In addition, merlin directly interacts with cytoskeletal microtubules and F-actin. The high affinity ABS present in the C-terminus of the ERM proteins is not conserved in merlin, but instead merlin interacts with F-actin through two ABSs located in its N-terminus between residues 1-27 and 280-323. However, the actin binding of merlin is weaker than that of ERM proteins, but merlin is able to associate indirectly with the actin cytoskeleton through the ERM proteins, spectrin, or paxillin.

In addition to the growth inhibitory properties, merlin also has morphogenic effects. It localizes underneath the plasma membrane in cultured cells. The membrane targeting of merlin requires proper folding of the FERM-domain, and therefore mutants with intact C-terminal domains but with altered N-termini mislocalize around the
nucleus and diffusely in the cytoplasm\textsuperscript{279,280}. However, also full-length D-merlin has a punctate distribution in the cytoplasm and at the plasma membrane, and is concentrated in endocytotic compartments\textsuperscript{158}. The interaction between merlin and F-actin and their colocalization in areas of membrane remodeling\textsuperscript{263,278,281,282} suggests that merlin plays a role in maintaining cytoskeletal organization. Overexpression of merlin induces the formation of cell extensions, surface blebs, membrane ruffles, and filopodia\textsuperscript{259,263}. Interestingly, also merlin-deficient human schwannoma and meningioma cells display cytoskeletal defects and develop multiple cell extensions, show membrane ruffling and disorganized stress fibers, and exhibit increased cell spreading\textsuperscript{138-141}. Reintroduction of merlin in these NF2-deficient cells reverses the aberrant cytoskeletal phenotypes\textsuperscript{141,283,284}.

Merlin also regulates other actin cytoskeleton-mediated processes, and has an inhibitory effect on cell motility, spreading, and adhesion\textsuperscript{285,286}. Although the molecular mechanism by which merlin regulates motility is not known, several possibilities exist. Focal adhesions are dynamic protein complexes that link the ECM, integrins, and the actin cytoskeleton together to form cell-substrate adhesions. Merlin localizes to focal adhesions in fibroblasts\textsuperscript{287}, and interacts with the focal-adhesion component paxillin in a complex that contains both β-integrin and erbB2\textsuperscript{277}. The role of merlin in cell migration could be associated with the modulation of integrin signaling at focal contacts that coordinates cell motility\textsuperscript{288}. It has also been suggested that expression of merlin inhibits invasiveness by downregulating focal adhesion kinase (FAK), an essential protein in inducing cell motility and invasion\textsuperscript{285}. Overexpression of merlin does not only impair cell motility, but results in abnormal F-actin organization during cell spreading, and a transient decrease of cell adhesion\textsuperscript{286}. However, also reduction of merlin expression with antisense oligonucleotides or overexpression of merlin mutants reduces cell attachment\textsuperscript{250,259,280}, indicating that merlin’s function in mediating adhesion is complex and tightly regulated. Altered cell-matrix adhesions are typical for malignant cells and required for tumor invasiveness and metastasis. Heterozygous \textit{Nf2} mice develop highly invasive and metastatic tumors\textsuperscript{66}, further implying that merlin plays a role in regulating cell adhesion. However, no connection between merlin loss and metastasis in humans has been found, and NF2 patients do not have an increase in metastatic cancer forms\textsuperscript{289}.

The functional significance of the two merlin isoforms is not understood, but some divergences between the forms have been denoted. Merlin isoform 1 binds less efficiently than isoform 2 to several interaction partners, including NHE-RF\textsuperscript{275}, βII-spectrin\textsuperscript{290}, RhoGDI\textsuperscript{291}, HRS\textsuperscript{267,268}, and F-actin\textsuperscript{272}. In contrast to isoform 1, isoform 2 is unable to reverse the cytoskeletal defects of merlin-deficient schwannoma cells\textsuperscript{283} and does not impair motility or adhesion\textsuperscript{286}. Whereas merlin isoform 1 inhibits schwannoma growth, isoform 2 does not influence proliferation in rat schwannoma cells and is therefore thought to lack growth suppressive activity\textsuperscript{242}. However, merlin not only functions as a negative growth regulator in tumor formation, but also has an important role in embryogenesis and tissue differentiation. Interestingly, \textit{Nf2} knockout mice that express only isoform 2 develop normally\textsuperscript{157}.

Cadherin-containing AJs mediate cell-cell interactions and are important for transducing growth arrest signals during cell contact. Merlin localizes to cell-cell junctions\textsuperscript{240,253,291} and regulates both AJ and focal contact formation in several cell types\textsuperscript{141,253,292,293}. It colocalizes and associates with components of AJs, and controls the
assembly and stabilization of these structures, possibly by linking these complexes to the actin cytoskeleton. \textit{Nf2} deficiency leads to disruption of AJs and loss of contact-dependent inhibition of growth\textsuperscript{253}. Loss of AJs contributes to tumor development by enhancing invasiveness and metastasis\textsuperscript{294}, so the absence of stable AJs in \textit{Nf2}-deficient cells could therefore explain the metastatic potential of the tumors in heterozygous \textit{Nf2} mice\textsuperscript{170}.

Merlin stabilizes the membrane-cytoskeleton interface and organizes the distribution and signaling of membrane receptors\textsuperscript{151}. The protein has been suggested to control cell proliferation by regulating growth factor receptor abundance and thus signaling at the cell surface\textsuperscript{244,292,293,295}. Merlin regulates the presence of growth factor receptors by limiting their delivery to the plasma membrane\textsuperscript{252} or by sequestering them in membrane compartments from which they can neither signal nor be internalized\textsuperscript{293}, this way blocking downstream signaling upon cell-cell contact. Merlin is also believed to integrate receptor tyrosine kinase (RTK) and adhesion receptor CD44 signaling at the membrane\textsuperscript{296}. According to one model, merlin and CD44 function together as a molecular switch that regulates the growth-promoting and growth-inhibitory cell states. Upon cell contact, active merlin binds to CD44 and negatively regulates RTK signaling\textsuperscript{255}. Loss of merlin would lead to elevated growth factor receptor levels and persistent RTK signaling regardless of cell-cell contact, this way promoting proliferation\textsuperscript{151,296}.

Furthermore, merlin is involved in several intracellular signal transduction pathways through which it mediates the function as a negative growth regulator. It downregulates Ras-ERK\textsuperscript{297-299}, PI3K-Akt\textsuperscript{244,300}, mTORC1 (mammalian target of rapamycin complex 1)\textsuperscript{254,301}, Rac\textsuperscript{247,302,303}, and FAK-Src\textsuperscript{285,304} signaling. Merlin has been shown to regulate cell proliferation and survival also through the \textit{Drosophila} Hippo tumor suppressor pathway. It cooperates with the distantly related FERM superfamily member Expanded to activate Hippo signaling\textsuperscript{305-307} that plays important roles in restricting organ size and suppressing tumor development\textsuperscript{308}. Studies suggest that merlin participates also in Mst/Lats/Yap signaling which is the equivalent mammalian Hippo pathway\textsuperscript{308,309}.

### 6. Regulation of Merlin and ERM activity

Post-translational modifications regulate the biological function of merlin and the ERM proteins by several different mechanisms. The proteins can exist in active and inactive conformations, which modulate their ability to interact with other molecules. This conformational regulation is achieved by a combination of phospholipid-binding and phosphorylation. Ultimately, the activity of merlin is determined by its turnover rate.

#### 6.1. Conformational regulation

A characteristic feature within the ERM protein family is intra- and intermolecular association by head-to-tail joining (Fig.9). The proteins can exist in dormant, monomeric forms in which the N-ERM association domain (N-ERMAD), residues 1-296 in ezrin,
interacts with the C-ERM association domain (C-ERMAD), residues 479-585 in ezrin. This intramolecular folding regulates the functional activity of ERM proteins, as association between the ERMADs causes the proteins to adopt closed inactive conformations, where binding sites for other interacting molecules are masked. Dissociation of the ERMADs leads to a conformational change which unmasksmasked actin and membrane protein recognition sites, allowing ERMs to associate with these molecules. In addition to homodimerization, the ERMs also form heterotypic associations between family members, resulting in dimers and oligomers.

According to the crystal structure of the moesin N-ERMAD-C-ERMAD complex, the C-terminus adopts an extended structure that masks a large surface of the F2 and F3 lobes on the FERM-domain surface in the closed conformation. The C-terminal helices bind the N-terminus largely independently, implying that individual secondary structural elements make a binding contribution leading to the high affinity of the ERMADs to each other. Also the α-helical domain forms independent interactions with the N-terminal domain and together with the C-terminus they cover 25% of the accessible FERM-domain surface.

Although the complete structure of merlin has not been modeled, crystallographic analysis of the FERM-domain has been conducted and it is known that also merlin is conformationally regulated and exists in closed and open states. Similarly to the ERMs, merlin’s self-association in the closed conformation is mediated by the binding of its C-terminal domain to the N-terminus (Fig.9b), but the low level of sequence identity between the C-terminal domains makes it unclear whether the association is identical to that in ERM proteins. However, although the merlin C-terminus is unique, 81%
of the residues that are conserved between merlin and the ERMs lie on the interface between the FERM-domain and C-terminus, thereby predicting a similar conformation for merlin. Similarly to moesin, the merlin C-terminus folds to interact with the FERM subdomains F2 and F3 and together with central α-helical region interactions regulate the closed conformation. Two separate intramolecular interactions mediate the closed conformation in merlin; folding within the N-terminal domain between residues 8-121 and 200-320 and N- to C-terminal association requiring residues 302-308 and exon 17 (residues 580-595). Merlin isoform 2 with a variant C-terminus, as well as mutations that remove the C-terminal exon 17 of isoform 1, form only weak intramolecular complexes and are therefore thought to exist in constitutively open conformations. The different affinities of merlin isoforms to various interaction partners are believed to result from their distinct conformations, as conformational changes can mask binding sites of merlin associated proteins.

The protein conformation is relevant for the tumor suppressor function of merlin. Merlin is thought to modulate cell proliferation only if it is capable of forming intramolecular associations, and in contrast to the ERMs, merlin is believed to be active (growth suppressive) in its closed conformation. Thus, merlin isoform 2 and molecules with mutations in either the N- or C-terminal domain, which exhibit constitutively open conformations, are defective as growth regulators. However, self-association does not seem to be required for the activity of D-merlin, as both truncated molecules and the FERM-domain alone are fully functional in Drosophila. Also, recent fluorescence resonance energy transfer (FRET) studies have implicated that the conformational regulation of merlin is more complex than just a conversion between open and closed states.

Although merlin predominantly exists as a stable, closed monomer, it is also able to form oligomers by homodimerizing and by heterodimerizing with ERM proteins. The N- to C-ERMAD interaction of merlin is weaker and more dynamic than that of ERMs, and the merlin C-ERMAD forms more stable interactions with N-ERMAD of ezrin than with its own N-terminus. However, the heterodimerization of merlin and ezrin requires a conformational change in both proteins. The relevance of oligomer formation or heterotypic interactions between merlin and ERMs is not fully elucidated, but it has been speculated that heterodimerization could represent a mechanism to modulate the activity of the proteins.

6.2. Phosphorylation and phospholipid-binding

Phosphorylation, the substitution of a hydroxyl group for a phosphate group in serine, threonine, or tyrosine residues, serves as a mechanism for regulating the activity of many proteins. Proteins can be either activated or inactivated by phosphorylation, and it has been estimated that approximately one-third of all cellular proteins are phosphorylated at any given time. The phosphorylation event can affect the protein in several ways. It can lead to conformational changes in the substrate, or the attached phosphate group can be recognized by interaction partners, driving the assembly of protein complexes. The transfer of the terminal phosphate from ATP to the hydroxyl oxygen of the substrate
residue is catalyzed by protein kinases, whereas the phosphate groups are removed in a
dephosphorylation reaction by protein phosphatases. About 2% of human genes encode
protein kinases\textsuperscript{321}, and more than 500 known kinases and approximately 200 protein
phosphatases exist in the human genome\textsuperscript{322,323}. Protein kinases can be classified into two
main types; serine/threonine kinases that phosphorylate their targets on serine and
threonine residues and constitute about 80% of all kinases, and tyrosine kinases that
phosphorylate their substrates on tyrosines\textsuperscript{322}.

The molecular interactions and functional activities of merlin and ERM proteins are
regulated by a combination of phosphorylation and phospholipid-binding. Phosphatidylinositol
4,5-biphosphate (PIP\textsubscript{2}) binding to the groove between subdomains F1 and F3 in the N-terminus of ERM proteins\textsuperscript{324-326} is a major determinant of ERM
localization\textsuperscript{325}, as it activates the proteins and facilitates their association with membrane
targets\textsuperscript{182,186}. In addition, phospholipid-binding is required for the subsequent
phosphorylation and conformational change of ERMs\textsuperscript{327,328}. Also merlin binds
phosphoinositides via a conserved motif in its FERM-domain\textsuperscript{329,330}, and the phospholipid-
binding is required for membrane association, although it does not affect intramolecular
folding or phosphorylation of merlin. However, FERM-domain mediated phosphoinositide-
binding and membrane localization are critical for the growth inhibitory function of
merlin\textsuperscript{330}.

Phosphorylation regulates the function of ERM proteins, and all three ERM s are
phosphorylated on serine, threonine, as well as tyrosine residues. Numerous kinases,
such as Rho kinase (ROCK)\textsuperscript{331-333}, Akt\textsuperscript{334}, and different protein kinase C (PKC)
isoforms\textsuperscript{313,335-337} phosphorylate a conserved C-terminal threonine (ezrin T567, radixin
T564, moesin T558). Inactive closed ERM proteins in the cytoplasm are activated by
phosphorylation at the conserved threonine, which introduces a negative charge that
reduces the affinity of the C-ERMAD for the FERM-domain in all ERMs, leading to
molecules with open conformations. These molecules are translocated to the membrane-
cytoskeleton interface, where they acquire proper conformation to bind membrane
associated molecules and actin, thus becoming functional as membrane-cytoskeleton
crosslinkers\textsuperscript{152}. Besides the C-terminal threonine, ezrin is phosphorylated on several
other sites, for example on S66 by PKA\textsuperscript{338}, Y145 by Lck\textsuperscript{339}, EGFR\textsuperscript{340}, and Src\textsuperscript{341}, T235 by
Cdk\textsuperscript{342}, Y353 by Akt\textsuperscript{343} and EGFR\textsuperscript{340}, and Y477 by Src\textsuperscript{344}, making the regulation more
complex.

The phosphorylation status of merlin modulates cell growth arrest or proliferation, and
depends on growth conditions. Merlin is phosphorylated at low cell density, but serum
starvation, high cell density, or loss of adhesion leads to increased merlin expression and
dephosphorylation. The protein is phosphorylated on both serine and threonine residues,
but tyrosine phosphorylation has not been detected\textsuperscript{345}. At least three differentially
phosphorylated forms of merlin exist, as isoform 1 migrates as three distinct bands
around 70 kDa in electrophoretic mobility assays, which are believed to reflect
differentially phosphorylated forms of the molecule\textsuperscript{247,345,346}. The species with decreased
mobility is hyperphosphorylated, while the form with increased mobility is
hypophosphorylated. Merlin is phosphorylated on serine 518 (S518)\textsuperscript{247,346-348}, threonine
230 (T230), and serine 315 (S315)\textsuperscript{349}, but no other phosphorylation sites have been
identified. Phosphorylation or mutation of the C-terminal S518 causes a mobility shift in
merlin and has therefore been mostly studied. The C-terminal threonine phosphorylated in ERMs is conserved in merlin (T576), but phosphorylation at this residue has not been detected in mammals\textsuperscript{247,350}. However, in \textit{Drosophila}, the Ste20 kinase Slik has been suggested to phosphorylate merlin at the equivalent residue, T616\textsuperscript{351}. Only one merlin phosphatase has been identified, the myosin phosphatase MYPT-1-PP1-δ, which dephosphorylates merlin at S518\textsuperscript{297}.

\subsection*{6.2.1. PAK pathway}

The activation of merlin is at least partly regulated through the Rho GTPases, as phosphorylation of the merlin C-terminus is induced by active forms of Rac, and to a lesser extent Cdc42, but not Rho\textsuperscript{247,348}. The Rac-dependent phosphorylation at merlin S518 is mediated by p21 activating kinases (PAKs), including PAK1 and PAK2\textsuperscript{347,348}. The PAK members include six different isoforms; PAK1-6. These proteins are serine/threonine kinases that function as effectors for the Rho GTPases. They are recruited from the cytoplasm to the cell membrane, where their kinase activity is stimulated by binding of activated GTP-bound Cdc42 or Rac1 to the PAK p21-binding domain (PBD). GTPase binding to PAK disrupts an inhibitory interaction between the kinase and autoinhibitory domains, leading to PAK activation through phosphorylation and subsequent activation of downstream substrates. PAKs are key modulators of not only cytoskeletal dynamics and cell motility, but also involved in cancer-cell signaling networks and transformation\textsuperscript{352}. They promote cell proliferation and downregulate pro-apoptotic pathways leading to tumorigenesis and cancer cell metastasis. In accordance, alterations in PAK expression are detected in several human cancers, and both PAK1 and PAK4 are upregulated and activated in various tumors, whereas PAK2 has both pro- and anti-apoptotic functions\textsuperscript{353}.

Several cellular consequences of S518 phosphorylation are known. It directly modulates merlin conformation as well as participates in the control of cell proliferation, morphology and motility, all important for the ability of merlin to function as a tumor suppressor. Phosphorylation of the merlin C-terminus weakens self-association and is predicted to result in an unfolded, functionally inactive molecule\textsuperscript{247,350,354}, although FRET analysis have implicated that phosphorylation at S518 would lead to only subtle conformational changes\textsuperscript{260}. Besides disrupting intramolecular folding, S518 phosphorylation impairs merlin binding to the transmembrane receptor CD44\textsuperscript{255}. The S518 phosphorylated form of merlin is more soluble and relocates from the plasma membrane to the cytoplasm as a result of decreased cytoskeletal association\textsuperscript{247,347}. Expression of a S518D mutant, mimicking the phosphorylated form, leads to dramatic changes in both cell morphology and actin cytoskeleton organization and is unable to suppress both motility and cell proliferation\textsuperscript{350}. Thus, the hypophosphorylated, S518 unphosphorylated, presumably closed form of merlin is thought to represent the active growth inhibitory form\textsuperscript{247,348,350}. Dephosphorylation of S518 by MYPT-1-PP1δ phosphatase is believed to activate the tumor suppressor function of merlin, leading to growth arrest under confluent conditions\textsuperscript{297}. Interestingly, the S518 residue is not conserved among the ERM proteins, where the corresponding residue is an arginine, nor in D-merlin, where it is a threonine\textsuperscript{350}.
Both merlin and ERM proteins regulate signaling via Rho GTPases by associating with Rho regulators and effectors. Merlin is not only downstream of Rac, but it is also involved in modulating Rac activity as a negative regulator. Expression of merlin suppresses Rac-induced signaling by reducing downstream c-Jun N-terminal kinase (JNK) and PAK1 activity, leading to the downregulation of cyclin D1 and a block of transformation. S518 dephosphorylated merlin prevents Rac recruitment to ECM adhesions. In addition, merlin directly suppresses PAK1 activity through binding to PAK1 PBD and inhibits its recruitment to focal adhesions, which is required for PAK activation. Merlin and ERM proteins might also control Rho and Rac activity through their interaction with the negative regulator Rho-GDI. Although the mechanism is not completely defined, Rac, PAK, and merlin appear to operate in a negative feedback loop, with merlin both being regulated by the pathway and serving as an inhibitor of it. A model for how S518 phosphorylation regulates merlin’s tumor suppressive activity in rat schwannoma cells has been proposed. In growth permissive conditions, at low cell density, merlin is S518 phosphorylated, growth permissive, and exists in a complex with ezrin, moesin, and CD44. Under high cell density when cells are stimulated to undergo growth arrest, merlin is dephosphorylated, in its closed conformation, and directly interacts with CD44 to inhibit cell proliferation by abrogating Ras signaling.

Merlin-deficient schwannoma cells present upregulated Rac, Cdc42, and PAK1 activity and concomitant cytoskeletal alterations, which can be reversed by inhibiting Rac1 or RhoA. Many NF2-derived primary schwannomas also display highly elevated PAK1 activity. Activated PAK has been shown to redistribute to the leading edges of motile cells where it stimulates both motility and invasion. Inhibition of PAK activity in cells lacking merlin not only restores their normal motility, but also reverses their morphology, suppresses proliferation, and reduces their invasiveness and tumorigenicity, suggesting that PAK activity is essential for the tumorigenesis of NF2-deficient cells.

### 6.2.2. PKA pathway

Merlin is phosphorylated on S518 also by the cyclic AMP (cAMP) -dependent protein kinase A (PKA). PKA-mediated phosphorylation of the merlin C-terminus is detected when PAK activity is suppressed, indicating that these two kinases function independently to phosphorylate the same residue. In Schwann cells, merlin is phosphorylated on S518 through two separate pathways: by PAK as a result of ligand binding to β1-integrin, and by PKA following ligand binding to ErbB2/ErbB3 receptors. Although the functional consequence of S518 phosphorylation by two distinct kinases has not been defined, PKA-mediated phosphorylation at S518 promotes merlin-ezrin heterodimerization, whereas PAK2-induced S518 phosphorylation only has a weak effect on the interaction between merlin and ezrin.

PKA belongs to the AGC family of kinases that includes 60 members. These serine/threonine kinases are structurally related and have a high degree of sequence similarity in their catalytic domains. Full kinase activity of the AGC members depends on phosphorylation of the activation loops in their catalytic domains, but translocation to the...
plasma membrane is also essential for their optimal function\textsuperscript{361,362}. Most AGC kinases have distinct, but also overlapping substrate specificities and several of the family members can phosphorylate the same proteins\textsuperscript{360}.

PKA is a holoenzyme that in its inactive state exists as a heterotetrameric complex consisting of two catalytic and two regulatory subunits. There are four regulatory subunit isoforms; R\textsubscript{I\alpha}, R\textsubscript{I\beta}, R\textsubscript{II\alpha}, and R\textsubscript{II\beta}, and three catalytic isoforms; C\textsubscript{\alpha}, C\textsubscript{\beta}, and C\textsubscript{\gamma}, that can be assembled in different combinations. Binding of the cAMP second messenger to the regulatory subunits induces a conformational change that releases the active catalytic subunits from the complex. PKA also requires phosphorylation of its activation segment, either through autophosphorylation or phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1), to become fully functional. The released catalytic subunits are thereby activated to phosphorylate their over 370 target substrates both in the cytoplasm and nucleus\textsuperscript{360,363}.

The targets of PKA phosphorylation are specified by their subcellular location. Tethering of PKA to specific regions is mediated by interactions with A-kinase anchoring proteins (AKAPs). More than 50 different PKA recruiting AKAPs have been identified, which bind to the regulatory subunits of PKA heterotetramers thereby targeting the enzyme to a particular intracellular site. When PKA is compartmentalized and concentrated in proximity of its substrates, specific phosphorylation of selected proteins is enabled\textsuperscript{364}. The \alpha-helical domain of merlin binds to the PKA regulatory subunit R\textsubscript{I\beta}, and the hypophosphorylated form of merlin has been suggested to function as an AKAP in the CNS\textsuperscript{365}. Similarly, also ezrin is both phosphorylated by PKA\textsuperscript{338} and functions as an AKAP by binding the R\textsubscript{II\alpha} regulatory subunit in gastric parietal cells\textsuperscript{366,367}.

The cAMP-PKA signaling pathway regulates many cellular processes including proliferation, differentiation, apoptosis, actin cytoskeleton remodeling, and gene transcription by modulating the activity of a variety of proteins\textsuperscript{368,369}. The pathway is altered in several cancers, and overexpression of PKA1 or changes in the relative abundance of different PKA isoforms is a common trait of many tumors\textsuperscript{368,370}. Activation of PKA promotes cell growth and cell cycle progression also in Schwann cells\textsuperscript{371}, and both humans and mice with inactivating mutations in \textit{PRKAR1A}, encoding the R\textsubscript{I\alpha} regulatory subunit, develop schwannomas as a result of enhanced PKA activity\textsuperscript{372,373}.

6.2.3. Akt pathway

The serine/threonine kinase Akt (protein kinase B) has been shown to phosphorylate merlin on T230 and S315 residues\textsuperscript{349}. Akt is an AGC kinase family member fundamental to many cellular processes such as motility, growth, proliferation, and apoptosis. The Akt encoded kinase has an N-terminal PH-domain, a central catalytic domain, and a short C-terminal regulatory domain, and the kinase family contains three isoforms; Akt1, Akt2, and Akt3, with similar structure but some isoform-specific expression patterns. Akt1 is ubiquitously expressed at high levels, Akt2 is mostly expressed in insulin-sensitive tissues such as liver, skeletal muscle, and adipose tissue, whereas the highest expression of Akt3 is detected in the brain and testis\textsuperscript{374}.
The interaction of insulin and growth factors with their cell surface receptors leads to activation of phosphoinositide 3-kinase (PI3K) and induces the production of the phosphatidylinositol 3,4,5-triphosphate (PIP₃) second messenger at the inner surface of the plasma membrane. Phosphorylated PIP₃ recruits both Akt and PDK1 to the membrane by binding to their PH-domains. As for many AGC kinases, activation of Akt involves phosphorylation of two highly conserved regulatory motifs. The interaction of Akt with phosphoinositides induces a conformational change, which enables phosphorylation of threonine 308 (T308) in the activation segment by membrane-localized PDK1. T308 phosphorylation is necessary and sufficient for Akt activation, but additional phosphorylation of serine 473 (S473) in the hydrophobic motif is required for maximal Akt activity. Once activated, Akt phosphorylates its over 130 substrates at the plasma membrane, as well as in the cytosol and nucleus. The PI3K/Akt signaling pathway is growth-promoting in many human malignancies, and overexpression or hyper-activation of Akt plays an essential role in tumorigenesis. Akt-mediated phosphorylation modulates the function of many important regulatory proteins, resulting in inhibition of apoptosis and increased cell division, this way promoting cell survival. Both upstream components of the Akt pathway such as PI3K, and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) as well as downstream effectors like forkhead box O (FOXO), glycogen synthase kinase 3β (GSK3β), and tuberous sclerosis complex (TSC)1/TSC2-mammalian target of rapamycin (mTOR) are deregulated in malignant tumors. PTEN, that antagonizes PI3K activity, is a major negative regulator of Akt activation and the second most commonly mutated tumor suppressor in humans. The pathway is also one of the most frequently targeted signaling routes in sporadic tumors, with estimates that alterations in PI3K pathway components could account for up to 30% of all human cancers.

Activation of the Akt pathway has also been implicated in Schwann cell survival, differentiation, and proliferation, and several studies indicate an interplay between merlin and Akt. The Akt pathway is activated in both human schwannoma and malignant mesotheliomas from heterozygous Nf2+/- mice. Merlin controls the PI3K/Akt pathway by inhibiting Akt signaling. It suppresses PI3K activity by binding to PI3K enhancer long isoform (PIKE-L), preventing its association with PI3K. Not only merlin, but also ezrin regulates the pathway by activating PI3K leading to increased Akt phosphorylation and enhanced cell survival. Akt-mediated phosphorylation of T230 and S315 residues has been described to attenuate the intramolecular folding of merlin, to inhibit its association with PIKE-L, CD44, and ezrin, and to result in merlin degradation. Expression of a phosphomimetic T230D+S315D mutant increases cell motility and proliferation, whereas the T230A+S315A mutant has the opposite effect. Also apoptosis decreases markedly with the phosphomimetic mutant compared to WT, similarly to cotransfection of active Akt and WT merlin.

39
The intracellular signaling pathways that are involved in phosphorylating merlin are crosslinked (Fig.10), making phosphorylation-mediated regulation of merlin a complex process. The signaling routes not only interact, but the different kinases can enhance and in some situations also inhibit the activation of another signal transduction pathway. Rac is a downstream target of PI3K, but Rac can also stimulate the activation of PI3K. Rac also regulates Akt through PAK. The PI3K effector PDK1 activates not only AGC kinases Akt and PKA, but also PAK. In addition, PAK can be stimulated directly through PI3K or indirectly via Akt phosphorylation, as Akt phosphorylates and activates PAK. A cross-talk between PKA and PAK also exists where PKA negatively regulates PAK through phosphorylation.

6.3. Protein stability and the ubiquitin proteasome pathway

In addition to a protein’s expression level and post-translational modifications, protein activity is regulated by its turnover rate. Eukaryotic cells contain two major protein destruction pathways; the proteasomal pathway and the lysosomal apparatus. The lysosome with proteolytic enzymes plays a minor role in the turnover of cytosolic proteins as it primarily degrades membrane-associated or endocytosed extracellular proteins. Most intracellular proteins are degraded by the ubiquitin-proteasome pathway (UPP), which recognizes and eliminates misfolded and abnormal molecules by proteolysis, but also functions in protein turnover. By selectively degrading proteins whose concentrations quickly must vary in response to different cell states, it reduces protein lifetimes and is able to rapidly turn off the effect of its target substrate. The selective and controlled proteolysis of proteins by UPP has an essential regulatory role in a variety of cellular processes, including cell-cycle progression and proliferation, signal transduction, transcriptional regulation, differentiation, angiogenesis, apoptosis, and protein quality control.

In the UPP, proteins are targeted for degradation by attachment of ubiquitin molecules, highly conserved small proteins composed of 76 amino acids. In the process of ubiquitination, the C-terminus of the ubiquitin molecule is covalently linked to lysine residues in target substrates through the coordinated function of several enzymes. In the
first step of the cascade, ubiquitin is activated by an E1 ubiquitin-activating enzyme. Following activation, ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme, which associates with an E3 ubiquitin ligase capable of recognizing a specific target protein to be ubiquitinated. E2 facilitates the transfer of activated ubiquitin to the E3 ligase-protein complex, and the E3 ligase catalyzes the attachment of ubiquitin to the target protein. When the protein is tagged with a single ubiquitin; mono-ubiquitinated, it signals to attach additional ubiquitin molecules to form a polyubiquitin chain marking the protein for degradation. The polyubiquitin chain usually consists of at least four ubiquitin monomers and is formed through the same ubiquitination cascade with the facilitation of E4 chain elongation enzymes.

Polyubiquitination directs the protein to the proteasome complex for degradation. The proteasome is a large multicatalytic protease complex located in both the nucleus and cytoplasm of cells. The cylindrically shaped 26S proteasome is composed of a 20S catalytic core subunit, in which proteins are degraded, with one or two 19S regulatory subunits at either or both ends. The polyubiquitin chain on the target protein is recognized by ubiquitin receptors in the 19S complex, the chain is cleaved off the substrate, and the ubiquitin monomers are released by deubiquitination enzymes and recycled for future use. The target protein is then unfolded, inserted into the lumen of the 20S proteasome complex, and degraded by its proteolytic activity into short peptides of 3-20 residues, which can be further cleaved into amino acids and used in the synthesis of new proteins.

To prevent the random destruction of proteins, ubiquitination is tightly controlled. There are about 50 E2 enzymes, and more than 500 different E3 ligases. Distinct E3 proteins or E2-E3 complexes recognize specific degradation signals in their substrates allowing for selective tagging and thereby conferring high specificity for degradation. Some substrates contain constitutively active degradation signals, whereas other proteins require a post-translational modification of their sequence motif. A common way to activate a degradation signal on the target protein is by phosphorylation of specific sites, leading to the exposure of a normally hidden degradation motif. Different ubiquitin ligases are able to recognize distinct phosphorylation patterns in their target substrates. Hence, the specificity of UPP is increased by both post-translational substrate modifications and by regulation of the intracellular compartmentalization of the substrate and its E3 ligase.

Besides targeting proteins for destruction, different ubiquitin modifications function as regulatory cellular signals. Instead of a polyubiquitin chain, ubiquitin can be conjugated to target proteins as a monomer (monoubiquitination), as multiple monoubiquitins (multiubiquitination), or as short ubiquitin chains. The amount of attached ubiquitin molecules determines how the signal is interpreted and where the protein is targeted. Monoubiquitination regulates the activity and subcellular localization of diverse proteins, but ubiquitin conjugates also affect intermolecular interactions, as many proteins contain ubiquitin-binding domains that recognize and distinguish different types of ubiquitin modifications.
6.3.1. Degradation of merlin

Several reports have demonstrated a link between merlin and the UPP. Both full-length missense mutants and N-terminally deleted molecules of merlin show an increase in ubiquitination and are degraded by the UPP\textsuperscript{284,399}, indicating that the pathway is important in eliminating abnormal merlin products. Wild-type merlin is more stable, but also found ubiquitinated when overexpressed\textsuperscript{399}. As the mRNA expression levels of missense merlin mutants in NF2-associated tumors is unchanged in spite of their reduced protein expression, loss of function of the proteins has been suggested to result from a decrease in mutant protein half-life and increased degradation rather than from their abnormal function\textsuperscript{284}.

Akt-induced phosphorylation promotes polyubiquitination and proteasome-mediated degradation of merlin\textsuperscript{349}. In some cellular systems, merlin is targeted to the Roc1-Cul4-DDB1 E3 ligase complex through the WD40-containing adaptor protein VprBP/DCAF1 (DDB1- and Cul4-associated factor 1). Binding of merlin to the complex affects its stability by inducing polyubiquitination and consequent degradation through the proteasome\textsuperscript{400}. However, another study suggested that rather than serving as a substrate, the closed form of merlin translocates to the nucleus where it binds to E3 ubiquitin ligase CRL4\textsuperscript{DCAF1}, and inhibits its activity to mediate ubiquitination of other target proteins\textsuperscript{261}. NGB (NF2-associated GTP binding protein) has been shown to regulate the turnover of merlin by decreasing its ubiquitination, this way preventing degradation\textsuperscript{401}. Moreover spectrin\textsuperscript{266,402,403}, that possesses both E2 and E3 activities\textsuperscript{404}, and the cell cycle regulator human enhancer of invasion clone 10 (HEI10)\textsuperscript{405}, which can function as an E3 ubiquitin ligase\textsuperscript{406}, interact with merlin, so merlin ubiquitination could be mediated through several of its binding partners.

The F1 motif of merlin FERM-domain contains an ubiquitin-like structure that can stimulate ubiquitination and proteasomal degradation of other proteins\textsuperscript{150,230,232}. Accordingly, the FERM-domain of merlin has been shown to facilitate the degradation of the p53 negative regulator Mdm2 which is an E3 ubiquitin ligase\textsuperscript{407}, and to induce the ubiquitination and degradation of TRBP, a double-stranded RNA-binding protein with growth-promoting activity\textsuperscript{408}. Thus, merlin both serves as a substrate for the UPP pathway and acts to enhance the degradation of other proteins.

Also calpain-mediated proteolysis of merlin has been described. Calpains are a family of calcium-dependent cysteine proteinases that catalyze the proteolysis of many substrates, and their expression is often altered during tumorigenesis\textsuperscript{409}. Merlin is cleaved by calpain, and NF2-associated tumors display activation of the calpain system suggested to result in loss of merlin expression\textsuperscript{410}. 
AIMS OF THE STUDY

This study was undertaken to gain further insight into the function of the NF2 tumor suppressor protein merlin. Since merlin and the homologous ERM protein ezrin have opposite proliferative effects, we hypothesized that the regions which differ between the proteins might explain merlin's specificity as a tumor suppressor. The first 18 residues of merlin are unique and have no equivalents within the ERM proteins, and the C-terminus is the domain least conserved between the family members with a homology of only 22% between merlin and ezrin. Therefore, the aim of this study was to characterize the impact of these regions on merlin’s cellular and molecular properties. We were particularly interested in analyzing the impact on cytoskeletal organization, phosphorylation, and growth inhibition.

The specific aims set for this study were:

1. to characterize the N-terminal phosphorylation of merlin and to evaluate its functional consequence
2. to analyze the functional differences between merlin isoforms 1 and 2
3. to examine the morphogenic properties of merlin and to identify C-terminal regions required for this activity
4. to determine the effect of merlin’s C-terminal region on growth inhibition
5. to define the importance of the conserved C-terminal motif E545-E547, not present in the ERMs
MATERIALS AND METHODS

The materials and methods used are described in more detail in the original publications.

1. Materials

1.1. Cell lines (I, II, III)

293 human embryonic kidney (HEK) cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine/calf serum (FBS/FCS) (PromoCell) and antibiotics. Monkey kidney fibroblasts (COS-7 cells), mouse embryonic fibroblasts (MEFs), Nf2-/- MEFs, and ezrin-/- MEFs were all maintained in DMEM (Gibco-Invitrogen) with 10% FBS/FCS and antibiotics. Primary Nf2 knockout Schwann cells were isolated and cultured as previously described and used before passage 20. All cells were cultured at +37°C in a 5% CO2 humified incubator.

1.2. Antibodies (I, II, III)

Anti-merlin A-19 sc-331 polyclonal antibody (pAb), C-18 sc-332 pAb (both from Santa Cruz Biotechnology), and KF10 monoclonal antibody (mAb) were used to detect merlin. S518 phosphorylated merlin was detected with pS518 pAb (Biodesign). Rabbit pAb recognizing human merlin phosphorylated on S10 was produced by immunization of a 11-mer synthetic phosphopeptide IASRM(pS)FSSLK and subsequent purification of the antiserum using both negative and positive affinity purification methods. The specificity of the antibody was tested against in vitro Akt phosphorylated GST-merlin with and without S10A mutations. Ezrin was detected with 3C12 mAb. Alexa-568 conjugated phalloidin (Invitrogen-Molecular Probes) was used to stain F-actin, α-tubulin mAb (Sigma-Aldrich) to detect tubulin, and anti-GST pAb (GE Healthcare) to detect glutathione S-transferase (GST)-proteins. Pan-Akt and phospho-Ser473 Akt pAbs (both from Cell Signaling Technology) were used for Akt detection, phospho-GSK3α/β (Ser21/9) (Cell Signaling Technology) and GSK3α (Santa Cruz Biotechnology) antibodies for GSK3 detection, and anti-VprBP pAb (SMS-Gruppen) for DCAF1 detection. For detection of ubiquitin, Ub (P4G7) (Santa Cruz Biotechnology) was used. Ki-67 mAb sc-7846 (Santa Cruz Biotechnology) was used as a marker of cell proliferation, and the nuclei were stained with TO-PRO 3-iodide probe (Molecular Probes). Alexa-488-, Alexa-568- and Alexa-594-conjugated goat anti-mouse and goat anti-rabbit antibodies (Invitrogen-Molecular Probes) were used as secondary antibodies in immunofluorescence, and HRP-conjugated rabbit anti-mouse, swine anti-rabbit (both from DAKO A/S), and swine anti-goat (Santa Cruz Biotechnology) secondary antibodies in western blot analysis.
Materials and Methods

1.3. Constructs and samples (I, II, III)

Human merlin isoform 1 (WT, sequence coding for amino acids 1-595) and isoform 2 (sequence coding for amino acids 1-590) in pcDNA3 vector (Invitrogen) were used for transfection experiments of full-length proteins. C-terminally truncated constructs 1-587, 1-569, 1-547, 1-537, and 1-518 were created from merlin isoform 1 by digestion with restriction endonucleases and ligation into the pCDNA3 vector. For expression of recombinant GST-fusion proteins, the following constructs were used: merlin 1-100, merlin N-terminus (1-314), merlin α-helix (314-477), merlin 1-547, merlin C-terminus (492-595), and full-length ezrin, all in pGEX4T1 vector (Amersham Biosciences). Point mutations of merlin in all vectors were made by site-directed mutagenesis using the QuikChange Kit (Stratagene). The Δ2 merlin construct with a deletion of residues 50-70 in exon 2 has been described before. WT or S518A merlin in pDEST27 vector (Invitrogen) containing an N-terminal GST-tag was used for metabolic labeling, and merlin retro- and adenovirus constructs have been previously described. Akt WT in pUSEamp vector was from Upstate Biotechnology, and the cDNAs of the myristoylated Akt and myristoylated kinase dead K179A Akt were obtained from Dr. Rene H. Medema. DCAF1 in pRK5 vector was obtained from Dr. Filippo Giancotti and has been previously described. GFP in pCDNA3 vector was used as control in proliferation assays. Both the c-Ha-Ras C61L construct (Addgene plasmid 13485) used in soft agar experiments and GFP-α-actinin used in live cell imaging have been described before.

The frozen tumor material was obtained from the Neurofibromatosis Clinic of Massachusetts General Hospital, and contains a 1646 delT mutation in the NF2 gene and LOH compared to the matching blood sample.

2. Methods

2.1. Cell transfections and treatments (I, II, III)

Cells were transiently transfected using FuGENE6 reagent (Hoffmann-La Roche) or Lipofectamine 2000 (Invitrogen) for Schwann cells, and incubated for 48 h before analysis.

For Akt kinase activation and inhibition experiments, cells were cultured in serum-free medium for 24 h and treated before lysis with 0.2 μg/ml human recombinant epidermal growth factor (EGF) (Calbiochem) for 10 min, with 50 μM LY-294002 (Sigma-Aldrich) for 1 h, or with 0.1 μM Calyculin A (Calbiochem) for 10 min all at +37°C. For degradation assays, serum starved cells were treated for 24 h with 5 μM MG132 or 0.1 μM Bafilomycin A1 (both from Sigma-Aldrich). To activate or inhibit PKA activity, cells were treated with 50 mM IBMX and 25 mM forskolin or 10μM H89 (all from Sigma-Aldrich) for 24 h.
2.2. Immunofluorescence and immunoblotting (I, II, III)

For immunofluorescence, cells grown on glass coverslips were fixed in 3.5% paraformaldehyde (pH 7.5) in +4°C for 15 min followed by washes with phosphate buffered saline (PBS). The cells were permeabilized with Triton X-100, detected with appropriate antibodies using standard protocols, and imaged with a fluorescence microscope equipped with an Axiocam cooled CCD camera (Carl Zeiss).

For the Latrunculin B assay, Nf2-/- MEFs were fixed 48 h after transfection (zero timepoint) or treated with 2.5 mM Latrunculin B (Sigma-Aldrich) for 8 min. Cells were stained for merlin and phalloidin and analyzed with a fluorescence microscope.

For western blotting, cells were rinsed with PBS and lysed in ELB buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 0.5% Nonidet P-40 (NP-40), HALT phosphatase inhibitors (Pierce), and Complete protease inhibitors (Roche). Lysed cells were centrifuged at 13 000-20 000 g for 20-30 min at +4°C. The pellet (insoluble) and supernatant (soluble) were resolved in equal amounts of reducing Laemmli buffer. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad) when needed. Whole cell lysates were prepared by adding reducing Laemmli sample buffer on the culture plates. The tumor sample was dissolved in reducing Laemmli and sonicated. Samples were resolved in 8-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose filters, and analyzed by immunoblotting with appropriate antibodies using enhanced chemiluminescence (ECL) detection (Amersham-Biosciences).

2.3. Immunoprecipitations (II)

COS-7 cells were lysed 48 h after transfection in ELB buffer containing 0.5% NP-40, HALT phosphatase inhibitors and Complete protease inhibitors. Lysed cells were centrifuged at 13 000 g for 20-30 min at +4°C. The supernatants were diluted 1:1 to ELB-buffer without NP-40, and precleared with glutathione (G)-sepharose beads (Amersham Biosciences) for 1 h at +4°C. Beads were spun down, and cleared supernatants were incubated with appropriate antibodies overnight at +4°C under rotation. G-Sepharose beads were added and the samples were incubated for additional 4 h at +4°C under rotation. Immunoprecipitates were washed with ELB buffer, and bound proteins were eluted from the beads by boiling in non-reducing Laemmli buffer and analyzed by immunoblotting.

2.4. Metabolic labeling (I)

48 h after GST-merlin transfection 293 HEK cells were changed into serum- and phosphate free DMEM, and 1 mCi/ml of 32P labeled orthophosphate (Amersham Biosciences) was added for 2 h. Cells were treated with 50 µM IBMX and 25 µM forskolin or 40 µM H89 for 30 min or 2 h, respectively. Cells were lysed in ELB-buffer with 1% NP-40, phosphatase inhibitors (HALT, 1.5 µM Calyculin A (Calbiochem), Cocktail Set IV
Materials and Methods

(Calbiochem), 1.5 μM okadaic acid), and protease inhibitors, and centrifuged at 14 000 g for 30 min at +4°C. Samples were diluted to a final concentration of 0.5% NP-40, G-sepharose was added to the lysates, and the samples were incubated over night at +4°C. Beads were washed with ELB-buffer containing inhibitors, reducing Laemmli buffer was added, and samples were run in SDS-PAGE. Gels were silver stained following standard protocol, dried and exposed on a PhosphoImager plate, read by TyphoonImager 9400, and analyzed by ImageQuant TL2003 software (all by GE Healthcare).

2.5. In vitro phosphorylation assays (I, II)

GST-fusion proteins were expressed in E. coli DH5α and purified. G-sepharose beads to which ~4 µg of fusion protein was conjugated were washed in PKA buffer (20 mM Tris-HCl, 10 mM MgCl₂ pH 7.4), Akt buffer (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 15 mM DTT, 15 mM MgCl₂, 100 μM ATP), or PKC buffer (1/5 PKC lipid activator (Upstate Biotechnology), 20 mM HEPES, 10 mM MgCl₂, 0.1 mM EGTA, 500 μM ATP) containing Complete protease inhibitors. Phosphorylation reactions were carried out in a 30 µl buffer volume including 10 µCi of [γ-³²P] ATP (PerkinElmer Life Sciences) and 2 ng of purified human recombinant PKA (Sigma-Aldrich), 0.1 μg of human recombinant active Akt1 (ΔPH, S473D), Akt 2 (5PH, S474D), or Akt3 (117-end, S472D) (Upstate Biotechnology), or 5 ng of human recombinant PKCθ (Invitrogen) for 30 min at +30°C. Beads were washed in kinase buffer, reducing Laemmli buffer was added, and samples were resolved by SDS-PAGE. The gel was fixed and stained by Coo massie Blue followed by exposure on PhosphoImager plate and detection with Typhoon Imager 9400.

For Akt in vitro kinase assays of the full-length protein, COS-7 cells were transfected with merlin constructs and expressed proteins were immunoprecipitated with the C-terminal merlin KF10 mAb. Immunoprecipitated proteins were phosphorylated in vitro by Akt1 as described above.

2.6. Affinity pull-down (I, II, III)

Transfected COS-7 cells were lysed in cold binding buffer 1 (50mM HEPES pH 7.4, 150 mM NaCl, protease inhibitors) or binding buffer 2 (140 mM NaCl, 1 mM Tris pH 7.5) containing 0.5% Triton X-100 and Complete protease inhibitors, and the homogenates were cleared by centrifugation at +4°C for 20 min at 13 000 g. GST-merlin and ezrin fusion proteins were bacterially expressed and purified. G-sepharose beads carrying ~4 μg of proteins were washed in binding buffer and incubated with the lysates under rotation over night at +4°C. Samples were washed with binding buffer, reducing Laemmli buffer was added, and bound proteins were separated by SDS-PAGE. Proteins were detected with appropriate antibodies.

For the pull-down of phosphorylated merlin, G-sepharose beads carrying ~5 μg of merlin fusion protein were washed in PKA buffer and phosphorylated by human recombinant PKA as described above except that 200 μM of cold ATP (Sigma-Aldrich) was used in the reaction. After phosphorylation beads were washed in binding buffer 1.
Bacterially expressed full-length ezrin was cleaved from GST by thrombin (Sigma-Aldrich), and ~4 µg of ezrin was incubated with phosphorylated or unphosphorylated merlin constructs under rotation for 2 h at +4°C. Beads were washed with binding buffer, reducing Laemmli buffer was added, bound proteins were resolved in SDS-PAGE, and proteins were detected with ezrin and GST-antibodies.

2.7. In vitro wound-healing assay (I)

Nf2-/− MEFs and Nf2-/− Schwann cells (P18) were infected with retro- and adenovirus constructs, respectively, containing merlin or empty virus as previously described243,270. Plates were wounded 24-48 h after infection, and the wound-healing was monitored. After 72 h, cells were fixed and stained with merlin to control infection efficiency. Quantification of wound width was done by ImageJ (v10.2) image processing software.

2.8. Live cell imaging (I, III)

Nf2-/− MEFs, grown on LabTek glass chamber slides (Nunc), were cotransfected with GFP-α-actinin and merlin 24-48 h prior to imaging. Live cells expressing merlin were chosen by their GFP expression and imaged with an inverted Olympus IX81 microscope and CellR program at +37°C, 5% CO₂. Images were taken once every 30 seconds for 5 h. GFP-expression images were taken before and after the experiment, and the cells were stained for merlin to verify expression.

2.9. Soft agar colony assay and proliferation analysis (III)

For soft agar colony formation assay, Nf2-/− MEFs were transfected with c-Ha-Ras C61L CMV together with merlin constructs or empty pcDNA3 vector. 48 h after transfection 5 x 10⁴ cells were plated in triplicates in 0.3% agar containing DMEM and 10% FBS on a layer of precast 0.6% agar. After 2-3 weeks of growing in soft agar cells were stained by 0.005% Crystal Violet for 2-3 h. Plates were scanned and the number of the colonies on each plate was determined by count from three independent experiments.

For the proliferation assay, Nf2-/− MEFs transfected with merlin or GFP as a control were first serum starved followed by serum treatment for ~12 h. Cells were stained for merlin and the proliferation marker Ki-67. Ki-67 positivity from 400 cells of each construct from four independent experiments was quantified.

2.10. Quantifications and image analysis (I, II, III)

For quantification of actin, Nf2-/− MEFs transfected with merlin constructs were stained for merlin and with phalloidin. Cells were imaged with immunofluorescence microscopy using a fixed exposure of 900 ms for phalloidin. The intensity of the actin staining was
measured from whole cells by Image J software. Total fluorescence from twenty merlin transfected cells and untransfected cells from the same slides were analyzed separately, and the staining intensities were normalized to untransfected cells.

For quantification of cell-extensions, transfected Nf2-/− and ezrin-/− MEFs were stained for merlin and untransfected Nf2-/− MEFs for ezrin or phalloidin for ezrin-/− MEFs. Cells were imaged with fluorescence microscope and the extension length was measured from twenty untransfected cells and cells expressing the different constructs. The total length of all extensions from each cell was quantified and the mean length per cell calculated.

ImageQuant TL2003 software was used to analyze band intensities from immunoblots. Merlin intensities were normalized to tubulin to obtain the merlin/tubulin ratio, or the p-S518 amount to total merlin to obtain the p-S518/merlin ratio. For quantification of phosphorylation, the phospho-signal from the autoradiograph read by TyphoonImager 9400 was analyzed by ImageQuant TL2003 and normalized to the Coomassie-staining, and the background radioactivity (untransfected cells or empty GST plasmid) was subtracted from merlin samples.

Images of 293 cells transfected with WT merlin, taken with the Axiocam cooled CCD camera, were analyzed with the ImageJ software. Rectangular sections from the cell body towards extensions were chosen from the images, and pixel intensities were calculated for both the staining of transfected merlin and the endogenous ezrin.

All quantifications were done from at least three independent experiments and student’s t-test was used for calculation of P values.
RESULTS AND DISCUSSION

1. Merlin serine 10 is phosphorylated by both PKA and AKT (I, II)

Phosphorylation serves as a mechanism to regulate the tumor suppressive activity of merlin. Both the N- and C-terminal halves of the protein contain phosphorylated residues, and previous mass spectrometry analysis has shown that all four serines present in the N-terminal extended tail (S7, S10, S12, and S13) can be phosphorylated. Our earlier studies indicated that the N-terminus of merlin is phosphorylated in response to active PKA, and we therefore wanted to further characterize this phosphorylation. To determine the N-terminal phosphorylation site, the substrate residue was first mapped to the 100 most N-terminal amino acids of merlin by an in vitro PKA phosphorylation assay using truncated proteins, and then all potential kinase recognition motifs in this region were mutated to non-phosphorylatable alanines. Mutation of serine 10 (S10) abrogated PKA-mediated merlin phosphorylation, thus identifying the residue as a phosphorylation site in vitro (I, Fig.1c). In addition, mutation of both S10 and the previously identified C-terminal S518 in the same construct showed that these two amino acids represent the only PKA substrate residues in merlin (I, Fig.1d). PKA-mediated phosphorylation of merlin S10 was also confirmed by metabolic labeling, which demonstrated that activation of PKA leads to an increase in the phospho-signal despite the S518A mutation (I, Fig.1b). Thus, merlin S10 functions as a PKA phosphorylation site both in vitro and in vivo. Although S518 serves as a substrate residue for both PAK and PKA, S10 is probably not phosphorylated in response to PAK as activated Rac induces phosphorylation of only the merlin C-terminus.

A connection between merlin and Akt signaling has been described, and we therefore wanted to study also the Akt-mediated phosphorylation of merlin. Surprisingly, S10 was identified as not only a PKA site, but also as an Akt substrate residue by in vitro kinase assays of both merlin fragments and the full-length protein (II, Fig.1a and Fig.1c). The Akt dependent phosphorylation of merlin S10 was confirmed also in vivo by mass spectrometry analysis. Although earlier studies suggested that T230 and S315 would serve as Akt phosphorylation sites in merlin, we could not detect phosphorylation of these residues in vitro with any of the three Akt isoforms (II, Fig.1e). We also generated an antibody against phospho-S10 to specifically recognize the phosphorylated residue, but the affinity of the phospho-specific antibody was not sufficient to detect endogenous S10 phosphorylated merlin, even though the antibody showed specific reactivity against in vitro phosphorylated GST-merlin (II, Fig.1d).

The region around S10 (Fig.11) corresponds to the known PKA recognition motif R-X-S/T (where X denotes any amino acid). However, the motif is unique as an Akt phosphorylation site, since the preferred consensus sequence R-X-R-X-S/T contains arginines at positions -3 and -5, which are not present in merlin. However, authentic phosphorylation sites do not always conform to the consensus motifs, and many AGC-kinase substrate proteins are phosphorylated on non-optimal sequences, probably because they have other motifs that give the specificity for the interaction.
Figure 11. The sequence around serine 10. Consensus phosphorylation motifs for Akt and PKA are shown above the human merlin N-terminal tail sequence (amino acids 1-18). Serine 10 is underlined in the sequence. The corresponding region of merlin proteins from several species is shown below. Alignment adapted from Golovnina et al. 2005.

We mapped the Akt binding site of merlin to reside between N-terminal residues 100-314 (II, Fig.2b), and concluded that the interaction was not dependent on the phosphorylation state of S10 (II, Fig.2a and Fig.2c). Similarly, the binding motif of the PKA regulatory subunit R1β is located in the α-helical part of merlin, whereas the PKA phosphorylation site is present at the C-terminus. Docking motifs, the interaction domains between the kinase and its substrate, provide an additional mechanism for substrate recognition of serine/threonine kinases, and function to enhance substrate specificity by increasing the affinity of the kinase for its substrate. It is common that these docking motifs are distinct and often remote from the phosphorylation site. Thus, residues between 100-314 might comprise a docking motif that allows phosphorylation of the suboptimal S10 sequence by Akt.

The AGC kinases have similar substrate preferences and there are many examples of these kinases phosphorylating the same proteins. For example the N-terminal serine S21/9 residue in GSK3α/β is phosphorylated by at least five members of the AGC family. The ability of different kinases to phosphorylate the same substrates has probably evolved to allow different extracellular stimuli to regulate similar cellular responses. Likewise, S518 is phosphorylated by both PKA and PAK in response to distinct extracellular signals, neuregulin-1β for PKA and laminin-1 for PAK. As both PKA and Akt phosphorylate merlin on S10, we wanted to test whether the residue serves as a substrate motif for also other members of the AGC kinase family. Therefore, an in vitro kinase assay was conducted with PKCθ, another AGC member which has not been described as a merlin phosphorylating kinase, but phosphorylates ezrin. Our assays showed that PKC phosphorylated the merlin N-terminus (residues 1-314), but the S10A mutation had no effect on the reaction (II, Fig.1b), confirming that S10 represents a specific PKA and Akt phosphorylation site. This was further verified by Tang et al., who showed that AGC kinases p70S6K and p90RSK are unable to phosphorylate merlin. As the binding motifs for PKA and Akt are distinct despite the same N-terminal phosphorylation...
Results and Discussion

...the different docking motifs of the kinases might provide for the regulation of N-terminal merlin phosphorylation.

Taken together, we identify a novel phosphorylation site, S10, in merlin, and confirm that PKA, Akt, and PKC function as kinases phosphorylating the merlin N-terminus (Fig.12). The phosphatase dephosphorylating merlin at S10 remains to be discovered.

Figure 12. Merlin phosphorylation sites. Merlin is phosphorylated on S10 in response to both Akt and PKA, and on S518 by PKA and PAK. The previously identified Akt phosphorylation sites T230 and S315 could not be confirmed in this study. PKC phosphorylates the merlin N-terminus (1-314), but not S10.

2. Functional effects of S10 phosphorylation (I, II)

The identified PKA and Akt phosphorylation site, S10, resides in the N-terminal extended tail region that is excluded from the FERM-domain\(^{230-232}\), and which is not conserved in the ERMAs. The tail is, however, present in merlin proteins of various species\(^{58}\), and S10 is conserved in merlin orthologs of for example mouse, chicken, zebrafish, and Xenopus (Fig.11), suggesting that the residue has important functions. However, it should be noted that Drosophila merlin lacks the first 9 N-terminal residues, and does not contain S10. The N-terminal extension of merlin has been shown to be necessary, although not sufficient, for contact-dependent inhibition of proliferation\(^{415}\), so modifications on S10 could therefore have a large impact on merlin activity. Phosphorylation studies with merlin are challenging to conduct as endogenous merlin expression is very low in most cell lines and subtle changes in phosphorylation of a subfraction of molecules cannot be detected. We therefore took a mutational approach using transfected phosphomimetic S10D and non-phosphorylatable S10A merlin constructs to characterize the functional consequences of S10 phosphorylation.

When S10 mutants were transfected into fibroblasts, they induced apparent morphological changes. Expression of merlin S10A resulted in long cellular extensions whereas S10D expression induced a morphology with dense filopodia-like structures (I, Fig.2c and Fig.3). The 18 most N-terminal residues of merlin are involved in actin binding\(^{231}\) and necessary for association with the cortical cytoskeleton\(^{415}\). We therefore...
hypothesized that merlin S10 phosphorylation influences cell morphology through its effect on actin. Accordingly, the results showed that the phosphorylation status of S10 regulated actin cytoskeleton organization. The actin network at the cytoskeleton-membrane interphase and the amount of F-actin stress fibers were both affected by S10 mutations (I, Fig.4 and Fig.5). Merlin stabilizes actin filaments through lateral association and by slowing their depolymerization\textsuperscript{272}, and our assays indicate that the phosphorylation status of S10 has an impact on merlin’s ability to regulate F-actin organization. It has been suggested that phosphorylation of the N-terminal tail would also regulate the targeting of merlin to the plasma membrane\textsuperscript{415}, but both S10A and S10D constructs localized similarly to WT beneath the membrane (I, Fig.3). However, targeting may be regulated by phosphorylation of other N-terminal tail residues.

Since we observed changes in both the morphology and actin organization of cells expressing S10 mutants, we analyzed the effect of S10 on cell motility by wound-healing assays and live cell imaging. Merlin has an inhibitory effect on cell motility\textsuperscript{285,286}, and \textit{Nf2}-deficient MEFs migrate in wound-healing assays faster than their merlin expressing counterparts\textsuperscript{247}. This was observed also in our studies with both MEFs and Schwann cells, but interestingly, S10A merlin expressing cells migrated even slower than the WT merlin infected cells (I, Fig.6a). PKA is present at the leading edge during cell migration\textsuperscript{422}, similarly to merlin, so their interaction could lead to S10 phosphorylation in motile cells. Lamellipodia localizes the protein complexes that drive the actin polymerization required for cell motility\textsuperscript{125}. S10A expressing cells displayed altered lamellipodia formation as observed by live cell imaging (I, Fig.6b), possibly explaining the reduced ability of S10 mutants to migrate in wound-healing assays. Altogether, these results show that regulation of the merlin S10 phosphorylation state is critical for cell motility.

The detected effect of S10 mutants during cell migration probably reflects the role of merlin in cytoskeletal remodeling, since the S10A mutant, blocked in an unphosphorylated state, disturbs merlin’s normal regulative effect on the actin cytoskeleton, lamellipodia formation, and migration. We hypothesize that this is due to a different mechanism than direct actin association since the S10 mutants did not affect actin binding or depolymerization compared to the WT protein in our \textit{in vitro} assays. Merlin has several binding partners that are able to modulate the actin cytoskeleton\textsuperscript{262}, and the regulatory effect of merlin on actin organization could be mediated through these interacting proteins. It has been proposed that the N-terminal tail, containing S10, serves as a common protein-binding motif\textsuperscript{58}, and the region is required at least for the interaction with the schwannomin interacting protein-1 (SCHIP-1)\textsuperscript{423}. The extreme N-terminal residues could form a flexible tail that participates in the regulation of binding to interaction partners, depending on its phosphorylation status.

Our studies indicate that phosphorylation of S10 can also direct the protein for degradation, thereby down-regulating merlin stability and function. The effect was observed when active membrane-bound Akt was coexpressed with WT merlin, whereas degradation of the S10A mutant was significantly reduced (II, Fig.3a). The degradation is mediated by the proteasome, as the proteasomal inhibitor MG132 increased the amount of merlin in cells expressing active Akt (II, Fig.3c). Several studies have shown that inhibition of proteasomes does not increase the cellular level of merlin\textsuperscript{261,349,399,410}, and the protein exhibits a half-life of more than 24 hours in many cell types\textsuperscript{261}. It is possible that
efficient and fast proteasomal degradation of merlin requires a prior post-translational
modification, such as phosphorylation of S10 at a specific subcellular localization, to
reduce the protein half-life when its growth inhibitory activity is not needed in non-
confluent cells. Phosphorylation could this way provide an additional level of control to
merlin degradation, as the initial regulatory event would be carried out by the specific
kinase, while the E3 ubiquitin ligase would then recognize only the phosphorylated form
of the protein.

Previous studies demonstrated that merlin associates with the DCAF1/VprBP ubiquitin
ligase \(^{261,400}\), which targets the protein to the Roc1-Cul4/DDB1 E3 complex \(^{400}\). However,
merlin also functions upstream of DCAF1 as a negative regulator of the ligase in the
nucleus by inhibiting its ubiquitinating activity \(^{261}\). By using pull-down and
coimmunoprecipitation assays, we confirmed the interaction between merlin and DCAF1,
and mapped the binding region to residues 100-314 (II, Fig.5). Some conflicting results
about the effect of C-terminal S518 merlin phosphorylation on the DCAF1 interaction
have been presented, as the association was shown to be S518 phosphorylation
independent in one study \(^{400}\), whereas another report demonstrated that only nuclear,
closed, unphosphorylated S518A merlin associates efficiently with DCAF1 \(^{261}\). This could
reflect differences in merlin function in different subcellular compartments. Although we
did not detect any direct effect of S518 mutations on DCAF1 binding in our pull-down
analysis (II, Fig.5a), the results from the immunoprecipitates of soluble cellular fractions
indicate that merlin phosphorylated on both S518 and S10 would be most susceptible for
the interaction (II, Fig.5b). Li et al. \(^{261}\) proposed that a post-translational modification in the
nucleus could promote the interaction between merlin and DCAF1, and our results
suggest that S10 phosphorylation could represent such a mechanism.

Many tumor types harbor mutations of various components of the PI3K/Akt pathway
leading to deregulated PI3K signaling and increased Akt activity \(^{376,377}\). Activation of the
anti-apoptotic PI3K/Akt pathway could represent a way to inactivate merlin function in
tumors which lack NF2 mutations. Merlin is widely expressed despite the restricted
spectrum of NF2-associated tumors. However, mutations and loss of merlin expression
has been reported in also other tumor types than those associated with NF2 \(^{56,108-113}\) and
heterozygous \(Nf2^{+/-}\) mice develop a wide range of malignant tumors, suggesting that
merlin has a broader role in cancer development \(^{66}\). Expression of merlin is reduced in at
least malignant gliomas \(^{424}\), which also show activation of the PI3K signaling pathway \(^{425}\).
Akt pathway hyperactivation, a common feature of many malignant neoplasias, may
result in S10 dependent degradation of merlin, and thus increased proliferation.

3. Interplay between merlin phosphorylation sites (I, II)

S518 phosphorylation through PKA and PAK regulates the activity of merlin. To extend
our findings on the interaction between PKA signaling and merlin, the interplay between
the two phosphorylation sites was investigated. We determined whether the newly
identified phosphorylation site S10 affects S518 phosphorylation, and hence merlin’s
growth inhibitory effect, by performing electrophoresis mobility shift assays with S10
mutant molecules and by analyzing their C-terminal phosphorylation status by a phospho-

54
Results and Discussion

S518 specific antibody. The triplet electrophoretic migration pattern of merlin was present in S10A and S10D constructs (I, Fig.2a) indicating that both molecules are S518 phosphorylated. This was further confirmed with the phspo-S518 antibody, which detected both S10A and S10D merlin (I, Fig.2b). The phosphomimetic S518D mutant localizes predominantly to membrane protrusions and is present at the most peripheral regions of lamellipodia and microspikes in rat Schwann cells. A similar localization was detected when WT and S10 mutant expressing cells were stained for phspo-S518, where S518 phosphorylated merlin was present in shorter cellular protrusions, but absent from long extensions (I, Fig.2c). Only a subset of merlin molecules at specific membrane domains were S518 phosphorylated, possibly reflecting the presence of active kinases at these restricted areas of membrane remodeling. Since S10A and S10D mutations did not directly affect the localization or amount of the S518 phosphorylated protein, we conclude that the N-terminal phosphorylation of merlin is at least partly a separate event regulated independently of C-terminal phosphorylation. It is, however, possible that the S518 phosphorylation detected in our assays is mediated through PAK, and we can therefore not exclude the possibility that the S10 phosphorylation state would regulate PKA-mediated phosphorylation of S518.

By altering the conformation of merlin, phosphorylation has been implicated in modulating merlin interactions with its binding partners. Although PKA mediated phosphorylation of S518 enhances merlin-ezrin heterodimerization, phosphorylation of S10 did not increase binding to ezrin in our pull-down analysis (I, Fig.2d). These results suggest that N-terminal S10 phosphorylation does not modulate the conformation of merlin in a similar way as S518 phosphorylation. Thus, phosphorylation of the N- and C-terminal sites is likely to regulate distinct merlin functions. As S10 is located in the region outside of the FERM-domain it could be available for phosphorylation also in the closed conformation of merlin. To study if S10 phosphorylation is affected by the S518 state, immunoprecipitated full-length S518A and S518D mutants were phosphorylated in vitro by Akt. As the proteins were produced in mammalian cells, they should restore the structure reflecting their true conformation. In this assay, S518D merlin showed enhanced incorporation of phosphate compared to WT, whereas S518A merlin was less phosphorylated on S10 (II, Fig.4b). Thus, phosphorylation of S518 enhances S10 phosphorylation, probably by rendering merlin in a more open conformation.

Both PKA phosphorylation sites, S10 and S518, function as substrate residues for also other kinases, and S10 serves as a phosphorylation site for both PKA and Akt. Therefore, the regulation of phosphorylation needs to be both spatially and consecutively tightly controlled. Different extracellular stimuli at distinct intracellular localizations seem to trigger phosphorylation of S518. The phosphorylation status of merlin is probably also compartmentalized, as only membrane targeted constitutively active Akt was able to induce specific and efficient phosphorylation leading to merlin degradation. To better understand the biological significance of N-terminal phosphorylation, we addressed the link between PKA- and Akt-catalyzed S10 merlin phosphorylation. The experiments demonstrated that the Akt-induced merlin degradation is not PKA-dependent, as merlin was degraded also in the presence of PKA inhibitors. However, coactivation of Akt and PKA had an additive effect on the degradation (II, Fig.4a). It is therefore possible that also N-terminal PKA phosphorylation would lead to degradation of the protein if induced at
Results and Discussion

high levels. In accordance, PKA has been suggested to regulate the stability of merlin. Nf2 is post-transcriptionally lost in schwannomas from TEC3KO mice with increased PKA activity but lacking Akt activation\textsuperscript{372}, indicating that also enhanced PKA signaling leads to merlin downregulation.

\textbf{Figure 13. Model for post-translational merlin inactivation.}

Based on our findings we propose a model for post-translational merlin inactivation (Fig.13). When merlin inhibits proliferation, it exists in a closed conformation. Phosphorylation at S518 in response to either PAK or PKA leads to an open molecule where N-terminal S10 phosphorylation by Akt or PKA is enhanced. Merlin phosphorylated at both the N- and C-terminal residues binds DCAF1 or another ubiquitin ligase, leading to merlin degradation through the proteasomal cascade. This way merlin's tumor suppressive activity is lost.

Although Akt-mediated phosphorylation of T230 and S315 was not detected in our assays, the effect of T230 and S315 mutations on S518 phosphorylation was analyzed to understand the previously described effects of these mutants. Our assays showed that S518 phosphorylation was abolished in the double mutant T230A+S315A (II, Fig.4c). T230 and S315 reside at the end of the FERM-domain in the region required for N-terminal folding. The side chain of S315 in the N-terminal \(\alpha\)-helical segment makes a hydrogen bond with E317 that connects to subdomain F1, and the patient mutation S315F has been predicted to destabilize the \(\alpha\)-helical segment leading to disrupted N-terminal folding\textsuperscript{230}. As the N-terminal fold is essential for the closed conformation, the S315A mutation probably disrupts the intramolecular head-to-tail association of merlin, and T230 and S315 mutants are likely to have perturbed conformations where S518 can not be phosphorylated. Some of the effects reported for T230A+S315A mutants, such as their reduced cell proliferation, decreased motility, and increased apoptosis\textsuperscript{329,349} might therefore be a consequence of decreased C-terminal phosphorylation, rather than a direct impact of the phosphorylation of these sites on merlin function.
4. The importance of C-terminus for merlin properties (III)

Not much information is available on the functional domains of merlin. Especially the C-terminal part remains poorly characterized, although several patient mutations have been identified in this domain. As the C-terminus of merlin is the most divergent region from oncogenic ezrin, it is likely that proper merlin function and inhibition of proliferation requires an intact C-terminal region. The C-terminus of various merlin species shows extensive sequence variability, except for a C-terminal $\alpha$-helical structure, which is evolutionally conserved (Fig.14)\textsuperscript{58}. Especially the motif of residues E545-I546-E547 are highly homologous among different merlin species, but not conserved in the ERMs (III, Fig.4a), highlighting the importance of this region for merlin function. The isoleucine residue at position 546 in the merlin proteins is replaced by a leucine in the corresponding position in all ERM proteins. Interestingly, also a patient mutation substituting glutamic acid for lysine at codon 547 of this motif has been described\textsuperscript{427}, and it represents one of the most C-terminal NF2 missense mutations reported so far.

![Figure 14. Schematic model of merlin isoform 1 C-terminus.](image)

Figure 14. Schematic model of merlin isoform 1 C-terminus. The model is based on moesin structure and merlin predictions (combined from Pearson et al. 2000\textsuperscript{150} and Golovnina et al. 2005\textsuperscript{58}). Alpha-helices are indicated with helical structures, and numbers indicate amino acids. The C-ERMAD unique for isoform 1 is marked in purple, the conserved $\alpha$-helix is marked in orange, and the evolutionally conserved region of residues E545-E547 is marked in red.

By characterizing C-terminally deleted constructs we might gain insight to which regions are needed for different merlin functions. Therefore, we created truncation variants which produce proteins that end between residues 518-587 in exons 14, 15, and 17. To characterize the conserved domain of residues E545-E547, we also generated missense mutations that disrupt the motif and a mutant mimicking the patient mutation previously described. Analysis of patient missense mutations can provide valuable information about merlin function, as they should affect critical parts of the protein and are expected to designate important regions. Several of the truncations that we used in this study are similar to those detected in NF2 patients, such as Thr512fsX2\textsuperscript{70},...
Gln536X,70,75,428 and Glu547fsX270. These truncated merlin proteins often result in functionally inactive, unstable proteins.318,399,429,430 We could, however, detect expression of a merlin variant corresponding to residues 1-548 in a patient tumor sample (III, Fig.1d), demonstrating that C-terminally deleted proteins can be expressed in vivo. Few other studies have also shown that truncated proteins are detected in tumors430,431. Thus, the abnormal function of these proteins in some of the tumors apparently causes tumorigenesis, rather than the complete loss of merlin.

The C-terminus of merlin contains the well-characterized phosphorylation site S518, known to regulate merlin function. As the triplet electrophoretic migration pattern of merlin is believed to reflect differentially phosphorylated forms of the molecule,247,345,346 this assay was used to analyze the C-terminally truncated constructs. Full-length merlin isoform 1 migrates as a triplet in SDS-PAGE, but isoform 2 containing unique C-terminal residues and the shorter truncation constructs, migrated as single forms in our assays (III, Fig.6a). Interestingly, merlin 1-587 displayed all three migrating species similarly to isoform 1. To determine whether S518 phosphorylation is altered in some of the C-terminal mutants, the constructs were detected with the phospho-S518 antibody. The migration patterns did not correspond to the S518 phosphorylation status of the constructs, as isoform 2 and all the truncation mutants were S518 phosphorylated similarly to isoform 1 (III, Fig.6b). Thus, the extreme C-terminus is not directly required for S518 phosphorylation. It has previously been suggested that isoform 2 and C-terminally truncated constructs would have reduced PAK2-mediated phosphorylation of S518,354, but as PKA is able to phosphorylate at least isoform 2,346, the observed S518 phosphorylation in our assays could be PKA mediated. The migration pattern of merlin 1-587 indicates that the region of amino acids 580-587 is required for the formation of the slowly migrating band, as these residues are not present in isoform 2 or the shorter constructs that migrate as single bands (III, Fig.6c). As mutation of all potential phosphorylation sites in this region did not change the electrophoretic mobility of merlin (III, Fig.6d), it is unlikely that the migration pattern observed is a direct consequence of C-terminal phosphorylation. The different migration patterns of S518 phosphorylated and unphosphorylated merlin molecules may reflect changes in the protein resulting from phosphorylation rather than the S518 status. They could represent conformational differences that depend on intramolecular associations or other post-translational modifications, as intramolecular contacts affecting the structure can lead to differences in migration rates although SDS-PAGE electrophoretic migration assays are used on denaturated proteins.432 The shorter C-terminally truncated molecules, and isoform 2 lacking the regulational sites between residues 580-587, do not adopt the native three-dimensional conformation of full-length merlin isoform 1, and may therefore display altered electrophoretic mobilities.

Merlin undergoes conformational regulation and its ability to form intramolecular associations is believed to play an important functional role, as many of merlin’s interactions with other molecules depend on its conformation. Although the last 10 residues of merlin are required for the association with the N-ERMAD, they are not sufficient for the interaction, suggesting that the closed conformation involves interplay between several different regions.273 We determined the effect of C-terminal residues for merlin folding with a pull-down analysis where constructs with different C-termini were
allowed to interact with N-terminal fragments of merlin. The results showed that C-terminal truncations affect the intramolecular associations in varying degrees. Merlin isoform 2, 1-547, and E545K+E547K displayed increased binding in the assay (III, Fig.5c), suggesting that these constructs contain a C-terminal fold where the binding sites for the N-terminus are more accessible. Interestingly, although the E545K+E547K mutations do not locate within the region described as critical for N- to C-terminal folding\textsuperscript{317}, the mutations still impaired the intramolecular association of merlin allowing N-terminal binding. Thus, the last C-terminal helix in exon 17 is not the only determining factor for the intramolecular association. Isoleucine 546 is homologous to moesin L529, which is located at the end of the first C-terminal helix with other hydrophobic residues, contacting subdomain F\textsubscript{2}\textsuperscript{58,150}. Our results indicate that a similar interaction is present also in merlin, where the C-terminal helix participates in contacts with the N-terminal part of the protein, this way regulating conformation. According to these results, and supported by a recent FRET study\textsuperscript{260}, merlin forms complex intramolecular associations that require several C-terminal regions but are not S518 phosphorylation dependent.

5. Merlin-induced morphogenic changes (I, III)

Merlin functions as a linker between the membrane and cytoskeleton thus regulating cell morphology. The morphogenic properties of merlin may play a role in its tumor suppressor mechanism since both patient-derived schwannoma and meningioma cells display cytoskeletal abnormalities\textsuperscript{138-141}. However, it is unclear how these different merlin functions are linked, and why both merlin overexpression and deficiency results in cytoskeletal defects. We therefore further analyzed merlin’s morphogenic activity. Not only the N-terminal S10 (I, Fig.2c and Fig.3), but also the C-terminal S518 has been implicated in the regulation of merlin’s morphogenic properties\textsuperscript{350,426}. In addition, the C-terminus of ezrin has been reported to contain determinants for a cell-extension activity\textsuperscript{195}. To analyze the effect of merlin C-terminus on the morphogenic activity, and to define the essential region, several C-terminally truncated merlin constructs and the E547K and E545K+E547K missense mutants were expressed in various cell types. As reported before, expression of merlin resulted in changes in cell morphology in all cell types examined (III, Fig.2). Interestingly, the phenotypic alterations induced by merlin isoform 2 were more pronounced than those observed with full-length isoform 1. Thus, isoform 2 seems to have a more prominent role in modulating cytoskeletal processes. Also some of the truncation constructs induced a significant increase in cellular extensions, but the effect was dependent on the site of truncation. Besides isoform 2, especially merlin 1-587, 1-547, and E545K+E547K caused major changes in morphology by increasing both the total extension length and amount of extensions (III, Fig.2, Fig.4b, and Table 1). Merlin 1-547 induced the most drastic phenotype with long, thin cellular processes, similar to the previously described merlin construct 1-559\textsuperscript{259}. Merlin has been shown to promote the elongation of Schwann cell processes, and seems to stabilize the bipolar morphology of these cells\textsuperscript{433}. Expression of the 1-547 construct completely disrupted the normal Schwann cell morphology, and the protrusion phenotype with extremely long extensions resembled that of Schwann cells from NF2 patients\textsuperscript{139}. Based
on our results with C-terminally truncated molecules, we conclude that an internal sequence determinant for the cell-extension activity exists in the C-terminal region of merlin between residues 538-568. As expression of full-length merlin E545K+E547K was sufficient to induce the formation of long protrusions, the motif of E545-E547 is essential for merlin’s morphogenic activity.

The closed conformation of merlin isoform 1 seems to partially suppress the morphogenic activity of the C-terminal domain in the full-length molecule, similarly to ezrin. The more unfolded conformation of merlin isoform 2 and some of the mutants could make the proteins more prone to molecular interactions, this way increasing their cytoskeletal effect and leading to the observed changes in cell morphology. Since the morphogenic effect was not linearly dependent on the length of the construct, the C-terminus could also contain a silencer domain that prevents complete morphogenic activity of the full-length protein. The silencer domain could be deleted in some of the truncation constructs allowing a functional site inside the molecule to be unmasked, thus permitting merlin to interact with other molecules and influencing cell morphology.

The biological significance of merlin’s cell-extension activity has not been elucidated, but morphological changes are essential in many physiological situations. Extension formation is required when neurons grow their axons, dendrites, and dendritic spines in the CNS during neurogenesis. Merlin and ezrin are widely expressed and developmentally regulated in the CNS, suggesting that the proteins could have a regulatory role in neurogenesis\(^\text{237}\). Both the ERMs and merlin have also been linked to neuronal morphogenesis. ERMs are present in neuronal growth cones, and they have a function in the outgrowth of axons\(^\text{176}\). Merlin facilitates neurite outgrowth through its interaction with paxillin in neuroblastoma cells\(^\text{434}\), but inhibits neurite formation in differentiated neurons\(^\text{435}\) and in neuroblastoma if deficient in paxillin binding\(^\text{434}\). Thus, the merlin-induced cytoskeletal rearrangements and morphogenic changes might play a role in the formation of neuronal extensions.

To better understand the mechanism of merlin-induced extension formation, we analyzed the process by live cell imaging. Merlin isoform 2 was present in the imaged \(N\tilde{n}2^{-/-}\) MEFs in the lamellipodia at the leading edge but also as a gradient from the tails in the rear towards the center (III, Fig.3c and Fig.3d). Live cell imaging demonstrated that the merlin-induced cell extension phenotype is a dynamic process, where the protrusions are not actively formed through filopodia elongation, but rather their retraction during cell movement is altered (III, Fig.3b). The long protrusions are formed as a result of impaired release of adhesion points when the cell continues its movement to the opposite direction, consequently forming an actin and tubulin-containing tail (III, Fig.3a) behind the cell at the lagging edge. Adhesion disassembly is required for tail retraction\(^\text{133}\), and merlin localizes to cell-substrate contacts and associates with focal adhesion components such as FAK\(^\text{436}\), paxillin\(^\text{277}\), and \(\beta\)-integrin\(^\text{264}\). Merlin might control cell extension formation by connecting focal adhesion proteins to the actin cytoskeleton, and this way be essential for the regulation of morphological changes. Merlin, \(\beta1\)-integrin, ErbB2 receptors, paxillin, Cdc42, and PAK are all enriched at the distal tips of Schwann cell processes\(^\text{359}\), and the interaction between merlin and paxillin is involved in regulating actin-based morphological changes in these cells\(^\text{277}\). Thus, the morphological changes detected in our assays could be at least partly mediated by paxillin. Several other merlin interacting proteins are
Results and Discussion

associated with cell migration and could possibly explain the formation of the extended tail. Retraction of the rear requires Rho and ROCK, and inhibition of RhoA leads to the formation of an extended tail\textsuperscript{437-439}, similarly to overexpressed merlin. Thus, the tail retraction effect could be a consequence of merlin influencing the Rho signaling pathway, possibly indirectly through the other Rho GTPases. However, also active Rac is required for rear detachment of migrating cells, and expression of a dominant-negative Rac mutant leads to a phenotype where the tails are stuck to the substratum\textsuperscript{440}. Moreover, active PAK1 localizes to focal complexes at the tips of membrane protrusions\textsuperscript{441}, Dictyostelium cells deficient in PAK show impaired retraction\textsuperscript{442}, and merlin can antagonize the activity of PAK by inhibiting its recruitment to focal adhesions\textsuperscript{303}. Finally, the ERM proteins have also been implicated in tail retraction and polarization\textsuperscript{443}, making ezrin yet another possible interaction partner that could be involved in merlin-induced morphological changes. Thus, several molecular pathways could affect the formation of merlin-induced cellular processes, and it remains to be investigated which of these molecular interactions are essential for the activity.

6. The effect of ezrin on merlin-induced morphological changes (III)

Most cell types coexpress several ERM proteins together with merlin. The proteins interact through heterodimerization\textsuperscript{273,274}, and merlin often colocalizes with ezrin at the plasma membrane in cultured cells (Fig.15)\textsuperscript{263}. However, in cells with a poorly developed actin cytoskeleton, overexpressed merlin replaces ezrin in filopodia and ruffling edges\textsuperscript{263,291}. The subcellular colocalization of merlin and ezrin also depends on growth conditions in some cell types; in subconfluent U251 glioma cells the proteins colocalize, whereas in confluent cells, where merlin presumably suppresses proliferation, they do not distribute to the same subcellular locations\textsuperscript{273}. Although merlin and ezrin often showed equivalent subcellular distribution at the cell membrane in our stainings, there was no colocalization in the long merlin-induced protrusions observed in \textit{Nf2-/-} MEFs (III, Fig.3d and Fig.5a), suggesting that the proteins might not have cooperative functions in the formation of these structures.

Both N- and C-terminal truncations of ezrin increase the ratio of cell extensions, whereas the full-length molecule forms a self-complex that masks the availability of the domains needed for protrusions formation\textsuperscript{195}. Thus, merlin-induced regulation of cell morphology resembles the mechanism described for ezrin, where the cell extension activity of the full-length protein is reduced, but deletion of residues at either end of the protein releases the extension phenotype. However, in contrast to ezrin, also full-length merlin isoform 1 induces the formation of extensions, indicating that the effect on cell morphology of these homologous proteins is not identical. Since ezrin also causes morphological changes and the phenotypic effect of merlin may be mediated through ezrin, we investigated whether the merlin phenotype is ezrin dependent by expressing the truncation constructs in ezrin-deficient MEFs. The merlin truncations failed to induce similar protrusion formation in \textit{ezrin-/-} MEFs as in cells containing endogenous ezrin, and only the full-length merlin isoforms triggered a cell-extension phenotype (III, Fig.5b). The reduced effect of the truncated constructs in protrusion formation shows that the C-
terminally regulated extension formation is affected by the interplay with ezrin. However, it should be noted that these cells express also endogenous merlin, which could interfere with the C-terminally deleted molecules.

Figure 15. Merlin and ezrin colocalize at the cell membrane. 293 HEK cells transfected with merlin WT were stained for merlin (red) and endogenous ezrin (green). The plot profile displays a two-dimensional graph of the intensities of pixels along the rectangular section parallel to the protrusion. The x-axis in the graph represents the horizontal distance through the rectangular section from the cell body toward the end of the extension and the y-axis the vertically averaged pixel intensity. Merlin staining is shown in red and ezrin staining in green.

To study whether this reduced ability of merlin truncations to induce morphogenic changes would result from altered merlin-ezrin binding, a pull-down analysis with full-length ezrin was conducted. However, the interaction between merlin and ezrin was not affected by the truncations (III, Fig. 5c), suggesting that the reduced ability of the C-terminal variants to form protrusions in the absence of ezrin is not a consequence of a lack of this binding partner. Therefore, we conclude that ezrin is not required for the cell-extension activity of full-length merlin, but influences the formation and length of merlin-induced protrusions.

7. The effect of merlin C-terminus on growth inhibition (III)

Neither the N-terminus nor the C-terminus of human merlin is by itself sufficient for growth suppression, and both the F2 subdomain of the FERM-domain and the C-terminal region of 532-579 have been defined as essential for merlin-mediated inhibition of
Results and Discussion

Schwann cell proliferation. To further study the effect of merlin C-terminus on growth inhibition, two different assays were conducted. Anchorage-independent growth was analyzed by performing a soft agar analysis, and the proliferation status of the cells was determined by using the proliferation marker Ki-67. Interestingly, whereas the full-length isoforms suppressed growth in both these assays, the C-terminal mutants, both the truncation 1-547 and the missense mutant E545K+E547K, had only a minor effect on cell proliferation and anchorage-independent growth (III, Fig.7 and Table 1). The reduced growth inhibitory function of these constructs further defines the importance of the conserved residues around 545-547, and confirm that an intact C-terminus is required for the full growth inhibitory effect of human merlin. The results differ from experiments done with D-merlin, where the essential functions of the protein reside within the FERM-domain. In contrast to human merlin 1-547, D-merlin 1-600, with a deletion of the 35 most C-terminal residues, functions as a gain-of-function allele, acting as an activated protein in Drosophila. Thus, significant functional differences in human and fly merlin seem to exist, despite their high sequence conservation.

Table 2. Missense mutations in NF2 exon 15. Identified missense mutations in exon 15 (corresponding to residues 525-579) and their previously described cellular effects.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Protein</th>
<th>Phenotype</th>
<th>Reported effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>1598A&gt;C</td>
<td>K533T</td>
<td>severe NF2</td>
<td>reduced binding to βII-spectrin, HRS, p110, EBP50, and isoform 2, decreased self-interaction, increased solubility, impaired growth suppression, decreased half-life, impaired effect on motility, decreased adhesion, does not disorganize actin cytoskeleton, increased in microspikes</td>
</tr>
<tr>
<td>1604T&gt;C</td>
<td>L535P</td>
<td>mild NF2</td>
<td></td>
</tr>
<tr>
<td>1613A&gt;C</td>
<td>Q538P</td>
<td>variable NF2</td>
<td>reduced binding to βII-spectrin, HRS, p110, and isoform 2, decreased self-interaction, impaired growth suppression, does not disorganize actin cytoskeleton, impaired effect on motility</td>
</tr>
<tr>
<td>1616T&gt;A</td>
<td>L539H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1652T&gt;A</td>
<td>L542H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1639G&gt;A</td>
<td>E547K</td>
<td>neurofibroma-perineurioma</td>
<td></td>
</tr>
<tr>
<td>1736A&gt;T</td>
<td>K579M</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nd = not determined

Despite the fact that many of the identified patient mutations localize to the N-terminal part of merlin, also several interesting C-terminal mutations in exon 15 (corresponding to residues 525-579) have been described. Although missense mutations are rare and occur at a low frequency representing only 5% of all NF2 mutations (Fig.3), almost one third are located in exon 15. This implies that the region is essential for merlin function.
Results and Discussion

The identified missense mutations and their reported effects are shown in Table 2. These C-terminal mutations seem to cause variable clinical phenotypes, and most of them have been described to contain reduced growth inhibitory function. Most of the characterized disease-causing missense mutations in merlin are predicted to disrupt folding of the protein, indicating that the overall three-dimensional structure is essential for tumor suppressive function. For example mutations L535P and Q538P are predicted to break the conserved C-terminal helix, which probably disturbs merlin's function by altering the structural properties of the C-terminus. The importance of exon 15 and the region around 547 is further endorsed by the identification of the missense mutation E547K. Although the patient was not diagnosed with NF2, she had atypical hybrid neurofibroma-perineurioma. We speculate based on our binding assays that the functional defect caused by this mutation is a result of a structural change in the conformation of the protein rather than the direct consequence of a single residue substitution.

The construct that we characterized to have the most drastic effect on cell morphology, 1-547 is tumorigenic according to our proliferation assays. This is of interest since mutations resulting in truncations around merlin residue 547 are found in both NF2 patients and in sporadic schwannomas. These patient mutations further confirm that removing the very C-terminal residues of merlin impairs its function, leading to tumor formation. It is also possible that some of the truncations, also found in patients, could exert a dominant-negative effect by interfering with and possibly inactivating the function of WT merlin. This is supported by the fact that both full-length and truncated forms of merlin are coexpressed in some schwannomas, and the phenotypes for truncating mutations appear to be more severe than for whole gene deletions. The HEI-193 cell line, immortalized from schwannoma cells of an NF2 patient, expresses a protein consisting of exons 1-14 followed by an altered exon 17. This form lacking both exons 15 and 16 has been designated as merlin isoform 3 as it is detected also in normal cells at a low level. Isoform 3 was found to be expressed together with a mutant protein in tumors of patients with mild manifestations of NF2, and the isoform has been proposed to retain a partial tumor suppressive activity. This splice variant is also detected in many malignant mesotheliomas. In one study, deletion of NF2 exons 15-16 was described as the most frequent exon deletion found among NF2 patients, further confirming that these C-terminal exons are essential for merlin function. Thus, both the mutational analyses and our novel data indicate that alterations in exon 15 are enough to significantly reduce the growth inhibitory function of merlin.

8. Differences between merlin isoforms (III)

Not much information is available on the different merlin isoforms, and their functional significance is unclear. However, evolitional conservation together with similarly alternatively spliced mouse and human homologues suggest that the isoform 1 and 2 bear functional relevance. The mRNA of both isoforms is present in Schwann and meningeal cells, so they could equally be involved in NF2 tumorigenesis. Interestingly, knockout mice that express only merlin isoform 2 develop normally into healthy adults. Thus, isoform 2 is able to rescue the phenotype resulting from lack of
isoform 1, indicating that the isoforms have redundant functions during at least embryogenesis and growth.

Overexpression of merlin isoform 1 impairs cell motility, but such effects are not observed with isoform 2. Similarly, merlin isoform 1, but not isoform 2, reverses the cytoskeletal defects of Nf2-deficient schwannoma cells. Merlin isoform 2 induces the formation of long cell protrusions in our studies and associates more efficiently with F-actin than isoform 1. The actin binding properties of the two merlin isoforms are probably different because the N-terminal ABS is not available in the closed conformation of isoform 1. The different affinities for actin could explain the more pronounced morphogenic effects of isoform 2, as stronger interaction of isoform 2 with cytoskeletal components might cause increased focal adhesion stability, this way affecting detachment of protrusions. The stronger regulatory function in protrusion formation of isoform 2 may also partly explain why it is not able to reverse the morphology of merlin-deficient schwannoma.

Inactivating mutations in the NF2 gene occur in the first 15 coding exons, but have not been reported in the last exons 16 and 17 that distinguish the two isoforms from each other. The absence of tumor producing mutations in these exons has led to the belief that disruption of the proteins alternative C-termini would not be sufficient to eliminate merlin’s tumor suppressor function, although C-terminally truncated molecules have been described as incapable of influencing cell proliferation. The ability of merlin to function as a negative growth regulator has been suggested to be dependent on its intramolecular association. The distinct C-terminus of isoform 2 is predicted to result in a molecule with an open conformation, and also our studies demonstrated that isoform 2 exists in a structure that is more open than the conformation of isoform 1 (III, Fig.5c). However, according to our results, both full-length merlin isoforms 1 and 2 are equally able to suppress Ras-induced anchorage-independent cell growth and to decrease cell proliferation (III, Fig.7), regardless of their conformation. As isoform 2 retains growth inhibitory properties, full intramolecular association between the N- and C-terminal domains is not required to negatively regulate cell proliferation. This is in accordance with a recent study of Schwann cell proliferation. However, further studies are needed to explain the controversial results of isoform 2 on growth inhibition in some experimental systems.

To conclude, the merlin isoforms have different functions in regulating cytoskeletal changes, which seem to affect both cell morphology and migration. However, these merlin functions are probably distinct from the growth inhibitory activity, as isoform 2 with high cell extension activity is able to inhibit proliferation in our assays (III, Table 1).
CONCLUSIONS AND FUTURE PERSPECTIVES

While the hereditary cancer syndromes make up only a small fraction of the overall cancer burden, the study of these disorders is important as it not only provides insights into the regulatory molecular pathways of the syndromes, but also elucidates essential mechanisms in sporadic tumorigenesis. Merlin seems to be a key growth regulator also in nonhereditary nervous system malignancies, as NF2 gene inactivation is observed in a large fraction of sporadic schwannomas and meningiomas. Although NF2 is a rare disease, sporadic schwannomas and meningiomas are common, and population studies suggest that up to 1:300 of individuals will develop a tumor with an underlying sporadic NF2 mutation during their lifetime. Defining merlin’s normal physiological function in detail and understanding the molecular mechanisms behind NF2-related tumorigenesis is therefore needed to improve prevention and treatment of both the hereditary and sporadic tumors.

This work has resulted in several interesting observations on the biology of the NF2 tumor suppressor protein merlin. We hypothesized that the regions that are unique to merlin may, in part, be responsible for its specificity as a tumor suppressor. As the C-terminus of merlin is most divergent from ERM proteins, it is likely that proper merlin function requires an intact C-terminus. We therefore studied the role of the most C-terminal regions on merlin’s cellular and molecular properties, such as proliferation, cytoskeletal organization, phosphorylation, and intramolecular associations. The motif of amino acids 545-547 is conserved among the merlin proteins, and mutations affecting these residues have been speculated to affect protein function. According to our studies, the conserved C-terminal helix (amino acids 510-553), and in particularly the region between residues 538-568, functions as a regulatory domain of merlin-induced cell extension formation, which is a consequence of impaired detachment of adhesive structures. We also obtained evidence that the conformational regulation of merlin is highly more complex than previously thought. Truncation at 547 or substitution of the glutamic acid residues E545 and E547 disrupts the conserved helix and alters the conformation of the C-terminus sufficiently to eliminate productive intramolecular association. Thus, merlin forms more intricate conformations than just the “open” and “closed” states, and these structures require C-terminal residues other than exon 17.

Although the significance of isoform 2 still remains largely unknown, our study has given insights into its functions. It has been assumed that the closed state of merlin is the functionally active tumor suppressive molecule. Our results show that despite the different morphogenetic effects of the two isoforms, they possess equal growth inhibitory activities. This implies that the region distinguishing the two isoforms is not critical for merlin’s tumor suppressive activity, as both C-termini are capable of mediating this function. The open structure is probably not as such sufficient to disrupt merlin’s growth inhibitory function, but more complex conformational regulation is likely to be essential for this activity. It is of interest that no mutations have been identified in the last exons 16 and 17 that differ between the two isoforms. It is possible that such mutations would cause embryonic lethality, and would hence not be detected. Alternatively, mutations in the last exons are not harmful if they do not disturb the tumor suppressive activity of merlin, and do not
Conclusions

therefore cause tumorigenesis. To understand the lack of mutations in the last exons it would be of interest to sequence the NF2 gene from healthy individuals to analyze whether such mutations can be detected.

The results in this study show that overlapping but distinct C-terminal sequences of merlin mediate functions related to growth suppression, cellular morphology and control of molecular interactions (III, Table 1). The C-terminal α-helix, and especially the conserved residues of 545-547 were found particularly critical for all merlin functions analyzed. The importance of the region around 547 is further supported by the high conservation around the motif and identification of several pathogenic mutations within these residues that either truncate or cause missense mutations in the molecule. Although the region regulating protrusion formation overlaps with residues required for the tumor suppressive function, the morphogenic activity of merlin does not appear to be directly linked to growth inhibition. To further clarify whether the morphogenic properties are associated with contact inhibition, additional investigations of the molecular interactions essential for merlin’s morphogenic activity will be required.

The NF2-related intracranial tumors are clinically severe due to their location despite their benign and slow-growing features. Surgery and radiosurgery, the current treatments, target only single tumors and have associated adverse effects and major risks. As no other effective treatments are currently available, molecular therapies are urgently needed. Strategies to identify effective medical therapies for NF2 require the delineation of merlin’s molecular pathways, and analysis of the signaling events that mediate merlin activation could provide important molecular targets for future therapeutic drug design. Post-translational modification via phosphorylation is an important mechanism in the regulation of merlin function. The studies in this thesis identified a previously uncharacterized merlin phosphorylation site by confirming S10 as a substrate residue for both PKA and Akt. The identified phosphorylation site, S10, represents a novel Akt substrate recognition motif, R-X-S\(_{10}\), with an arginine at -2 position. Furthermore, PKC was shown to function as a merlin phosphorylating kinase, although the substrate residue was not mapped further than to the N-terminal region. Phosphorylation of S10 leads to modulation of the actin filament organization and cell morphology, indicating that normal regulation of the S10 phosphorylation state is critical for merlin’s cytoskeletal function. Our study also provides further evidence on the connection between merlin function and PI3K/Akt signaling, and implicates an important role for Akt in the regulation of merlin stability. Based on the work presented here, we propose that an unattributed consequence of Akt pathway overactivation in different malignancies is increased degradation of the tumor suppressor merlin. We postulate that temporal transient phosphorylation of S10 regulates the cytoskeletal functions of merlin, whereas constitutive phosphorylation in malignant cells leads to merlin degradation. However, the cross-talk and complexity between Akt, PKA, and PAK signaling makes evaluation of the biological impact of merlin phosphorylation challenging and demands further analysis.

Our findings are important since alternative mechanisms to mutation for merlin inactivation in NF2-associated tumors might exist. Some tumors lack detectable NF2 mutations, but lose merlin expression despite expression of NF2 transcripts. Approximately one-third of schwannomas have genetic evidence for inactivation of only
one of the NF2 alleles, and in about 10% of tumors genetic NF2 alterations have not been detected at all\textsuperscript{[93]}. Thus, many tumors may carry at least one intact copy of the NF2 gene, whose product must be inactivated for schwannoma development. These observations implicate the involvement of merlin degradation in tumor development. NF2 tumorigenesis might therefore involve alterations at many different levels, including post-translational modifications and protein turnover. Increased phosphorylation in tumors with hyperactivated Akt, such as in some VSs\textsuperscript{[383]}, could result in merlin degradation and subsequent inactivation of its growth inhibitory function. Phosphorylation by Akt could also function as a mechanism to inactivate merlin in normal benign cells during growth promoting conditions. It remains to be investigated in which situations the Akt mediated degradation of merlin occur, and if S10 phosphorylation plays a role in the normal functional regulation of merlin, or whether it is just a consequence of Akt overactivation in neoplasia.

Several compounds targeting signal transduction and kinases are used in treatment of human cancers, and inhibitors of the PI3K/Akt pathway are already in clinical trials\textsuperscript{[360]}. As merlin is phosphorylated by multiple kinases, many signaling events are potential targets and several possible levels for NF2 therapeutic intervention exist\textsuperscript{[457]}. Inhibitors of the PI3K-Akt pathway could potentially be used in restoring merlin function at least in tumors without NF2 mutations. OSU-03012, a PDK1 inhibitor, has recently been investigated as chemotherapeutic in preclinical studies for schwannomas, and this Akt pathway targeting compound suppressed primary human VS cell proliferation \textit{in vitro} and induced their apoptosis\textsuperscript{[459]}. It remains to be seen whether Akt inhibitors, among to their other effects, can restore the function of merlin in tumors where functional protein remains expressed. Also compounds effective in delaying proteasomal degradation could be tested, as modulation of this pathway should increase merlin’s half-life by reducing its degradation in tumors with hyperactivated Akt or PKA. Several proteasome inhibitors, which block proliferation and induce apoptosis, have undergone clinical trials as anti-tumor agents\textsuperscript{[460]}. Inactivation of some of these pathways could hopefully reduce tumor cell proliferation and have therapeutic potential for patients suffering from tumors associated with NF2.

The studies in this thesis have expanded our knowledge on the functional regulation of merlin. Our findings provide clues on the molecular mechanisms involved in the tumorigenetic events of NF2, by characterizing PKA and Akt mediated phosphorylation of merlin. These studies are highly relevant for understanding the normal function of merlin and will hopefully help in the ultimate goal of developing effective treatments for both NF2-associated and sporadic tumors.
SUMMARY

The main results of this study are:

(1) Merlin contains two PKA phosphorylation sites, serine 10 at the N-terminal domain and serine 518 at the C-terminus. Phosphorylation of serine 10 regulates merlin-induced cell morphology and affects actin filament organization and stability.

(2) The N-terminal serine 10 in merlin is also a substrate for the oncogenic kinase Akt. Merlin and Akt interact through an N-terminal Akt binding site, but the interaction is not regulated by S10 phosphorylation.

(3) Akt phosphorylated merlin is degraded through a proteasome-dependent pathway. Serine 10 phosphorylation could represent a non-genetically modified, post-translational mechanism of NF2 inactivation.

(4) Overlapping C-terminal regions of merlin mediate functions related to growth suppression, cytoskeletal organization, and control of molecular interactions.

(5) Merlin’s morphogenic properties are regulated by the C-terminus, particularly residues 538-568. C-terminally truncated proteins induce an increase in membrane-associated cellular projections, which are formed as a result of impaired tail retraction during cell movement.

(6) Both merlin full-length isoforms are able to inhibit cell proliferation and anchorage-independent growth, whereas C-terminally mutated merlin constructs show a decrease in growth suppression.
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References


77

References


References


References


References


References


References

References


