Helsinki Graduate School in Biotechnology and Molecular Biology

The Neurofibromatosis 2 tumor suppressor merlin in cytoskeleton organization and cell cycle regulation

Taru Muranen

Program of Molecular Neurology
Department of Pathology
Biomedicum Helsinki
Faculty of Medicine
University of Helsinki
Helsinki, Finland

Academic dissertation

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Thesis supervisors

Professor Olli Carpén, M.D., Ph.D.
Department of Pathology,
University of Turku and Turku University Hospital
Program of Molecular Neurology, Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Mikaela Grönholm, Ph.D.
Program of Molecular Neurology, Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Thesis reviewers

Marko Kallio, Ph.D
VTT Medical Biotechnology and Turku Centre for Biotechnology
University of Turku
Turku, Finland

Professor Kari Majamaa, M.D., Ph.D.
Department of Neurology
University of Turku
Turku, Finland

Thesis opponent

Helen Morrison
Institute of Age Research and Fritz-Lipmann-Institute
Jena, Germany
Forschungszentrum Karlsruhe, Institute of Toxicology and Genetics,
Karlsruhe, Germany

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On ne voit bien qu'avec le coeur.
L'essentiel est invisible pour les yeux.

Le Petit Prince, Antoine de Saint-Exupéry

CONTENTS

ABBREVIATIONS

aa	amino acid
A	Alanine
AKAP	Protein kinase A-anchoring protein
cAMP	Cyclic adenosine mono phosphate
C-ERMAD	C-terminal ERM association domain
Cdk	Cyclin-dependent kinase
CPI-17	17-kDa protein kinase C potentiated inhibitor
CRM1	Chromosome region maintenance 1
D	Aspartic acid
EBP50	ERM-binding phosphoprotein 50
EGFR	Endothelial growth factor receptor
ErbB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2/Her-2/neu
ERK	Extracellular-signal regulated kinase
ERM	Ezrin-radixin-moesin
EVH1	Enabled/VASP-1
FAK	Focal adhesion kinase
FERM-domain	Band four-point-one ezrin-radixin-moesin homology-domain
FRAP	Fluorescence recovery after photobleaching
G1 (phase)	Gap 1 (phase)
G2 (phase)	Gap 2 (phase)
G-actin	Globular actin
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HEI10	Human Enhancer of Invasion 10
Hrs	Hepatocyte growth factor receptor substrate
ICAM	Intracellular adhesion molecules
JNK	C-Jun N-terminal kinase
mAb	Monoclonal antibody

M (phase)	Mitosis (phase)
MAP	Microtubule-associated proteins
Mdm2	Mouse double minute 2
MEF	Mouse embryonic fibroblast
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MYPT-1-PP1δ	myosin phosphatase
NF2	Neurofibromatosis 2
NGB	NF2-associated GTP binding protein
NHE	Sodium-hydrogen exchanger
NHERF1	NHE regulatory factor 1
N-WASP	Neural Wiscott-Aldrich syndrome protein
pAb	Polyclonal antibody
PAK	P21-activated kinase
PDGFR	Platelet derived growth factor receptor
PDZ	PSD-95/Disc large/ZO-1
PI3K	Phosphoinositol-3 kinase
PIKE-L	PI3-kinase enhancer
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKCα	Protein kinase Cα
PKCθ	Protein kinase Cθ
PTB	Phospho-tyrosine binding
pVHL	Von Hippel Lindau protein
S	Serine
S (phase)	Synthesis (phase)
RalGDS	Ral guanine nucleotide dissociation stimulator
Rho-GDI	Rho-guanine dissociation inhibitor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following submitted manuscript and original publications, which are referred to in the text by their Roman numerals.

I **Taru Muranen***, Mikaela Grönholm*, G. Herma Renkema and Olli Carpén. Cell cycle-dependent nucleo-cytoplasmic shuttling of the neurofibromatosis 2 tumor suppressor merlin. (2005). *Oncogene* 24:1150-1158.

II Mikaela Grönholm*, **Taru Muranen***, Garabet G. Toby, Tamara Utermark, C. Oliver Hanemann, Erica A. Golemis and Olli Carpén. A functional association between merlin and HEI10, a cell cycle regulator. (2006). *Oncogene* 25:4389-4398.

III **Taru Muranen**, Mikaela Grönholm, Aurelie Lampin, Dominique Lallemand, Fang Zhao, Marco Giovannini and Olli Carpén. The tumor suppressor merlin interacts with microtubules and modulates Schwann cell microtubule cytoskeleton. (2007). *Hum Mol* Genet. 16:1742-1751.

IV Minja Laulajainen*, **Taru Muranen***, Olli Carpén and Mikaela Grönholm. Protein kinase A mediated phosphorylation of the NF2 tumor suppressor protein merlin at serine 10 affects actin cytoskeleton. Submitted.

* equal contribution, the publications I and II have also been used in the thesis work of Mikaela Grönholm.

ABSTRACT

Neurofibromatosis 2 (NF2) is a dominantly inherited disorder, which predisposes to multiple tumours of the nervous system, typically schwannomas and meningiomas. Biallelic inactivation of the *NF2* gene occurs both in sporadic and NF2-related schwannomas and in most meningiomas.

The *NF2* gene product merlin (or schwannomin) is structurally related to the ERM proteins, ezrin, radixin and moesin, which act as molecular linkers between the actin cytoskeleton and the plasma membrane. Merlin partly colocalizes with the ERM proteins in regions of dynamic cytoskeletal remodeling and forms heterodimers with ezrin. Like ezrin, merlin undergoes conformational regulation, which is at least partially mediated by phosphorylation. Merlin is a tumor suppressor that participates in cell cycle regulation. Merlin's phosphorylation status appears to be associated with its tumour suppressor activity, *i.e.* non-phosphorylated merlin functions as a tumour suppressor, whereas protein phosphorylation results in loss of functional activity. At least two kinases, p21-activated kinase (PAK) and cyclic AMP (cAMP) dependent protein kinase (PKA), have been shown to phosphorylate merlin.

This thesis study was initiated to investigate merlin's role as a tumor suppressor and growth inhibitor. These studies show, that like many other tumor suppressors, also merlin is targeted to the nucleus at some stages of the cell cycle. Merlin's nuclear localization is regulated by cell cycle phase, contact inhibition and adhesion. In addition, a potential nuclear binding partner for merlin was identified, Human Enhancer of Invasion 10 (HEI10), a cyclin B interacting protein, which functions as an ubiquitin ligase for cyclin B and controls its accumulation during cell cycle. HEI10 undergoes nucleo-cytoplasmic shuttling and colocalizes with merlin in the nucleus at the G1 phase of the cell cycle and at the plasma membrane during other phases of the cell cycle. Increased merlin expression in primary human schwannoma cell cultures, results in changes in the subcellular localization of HEI10. Merlin also regulates the amount of HEI10 by increasing its degradation.

Many tumor suppressors interact with microtubules and this thesis work shows that also merlin colocalizes with microtubules in mitotic structures. Merlin binds microtubules directly, and increases their polymerization *in vitro* and *in vivo*. In addition, primary mouse Schwann cells lacking merlin displays disturbed microtubule

cytoskeleton. This is of interest since cytoskeletal defects occur also in schwannomas that are isolated from patients.

Fourth part of this thesis work began from the notion that in addition to C-terminal phosphorylation, PKA phosphorylates also an unidentified site from the merlin N-terminus. Therefore the N-terminal phosphorylation was investigated further. Our studies show that serine 10 is a target for PKA and modulation of this residue regulates cytoskeletal organization, lamellipodia formation and cell migration. Dephosphorylation of this residue inhibits lamellipodia formation and decreases the amount of actin filaments.

In summary, this thesis work shows that merlin's role is much more versatile than previously thought. It has a yet unidentified role in the nucleus and it participates in the regulation of both microtubules and the actin cytoskeleton. These studies have led to a better understanding of this enigmatic tumor suppressor, which eventually will aid in the design of specific drugs for the NF2 disease.

REVIEW OF THE LITERATURE

1. The Cell-division cycle

Cells originate from other cells. Cell division is essential for unicellular organisms that use it to propagate and for multi-cellular organisms, which need cell division for production of the gametes (meiosis) and for growth and development (mitosis). It is the method by which a single fertilized egg develops into a mature organism, and by which tissue homeostasis is maintained. The somatic cell cycle of all animals consists of a series of events that leads to the duplication of all cellular material and to the birth of two genetically identical daughter cells. Errors in these finely tuned series of events can lead to genetic aberrations and cancer.

The cell cycle consists of four different phases (Fig. 1): Mitosis (M), Gap 1 (G1), Synthesis (S) and Gap 2 (G2). During these four phases the cell must replicate its genome, double its mass and duplicate the organelles in the cytoplasm. M phase is the physical separation of the chromosomes and cytoplasm to two daughter cells. During S phase the cell replicates its genetic material. G1 and G2 phases separate M and S phase and prepare the cell for the next phase in its cycle. During G2 the cell doubles its mass for the preceding cell cleavage at M phase. By this means daughter cells maintain their size during every round of symmetric cell division. Cells that are not dividing exit the cell cycle in G1, become quiescent and enter phase G0. The cell-division cycle is controlled by various checkpoints and the cycle is considered to be irreversible.

Three key classes of molecules regulate the progression of the cell-division cycle: cyclins, cyclin-dependent kinases (Cdks) and phosphatases (Hartwell, 2002, Hunt, 2002, Nurse, 2002, Boutros et al, 2007). Cyclins form the regulatory subunits and the Cdks the catalytic subunits of the heterodimer. Together a Cdk-cyclin heterodimer is an active kinase, which phosphorylates its downstream targets, ultimately leading to cell cycle progression. Phosphatases on the other hand function on the opposite manner, by removing phosphate groups from their targets. Precise timing is also important in the regulation of phosphatases during different cell cycle stages. They have the power to remove inhibitory phosphates from Cdks (Cdc25 and Calcineurin) (Boutros et al, 2007, Kahl & Means, 2004) thus promoting cell cycle progression or to remove inhibitory phosphates from cell cycle controllers (such as pRb, protein

phosphatase 1) (Tamrakar et al, 2000) thus inhibiting cell cycle progression. Different sets of Cdks, cyclins and phosphatases regulate different phases of the cell cycle. The amount of cyclins varies in a recurring fashion during cell cycle. For example degradation of an M-phase cyclin, cyclin B, is required for exit from mitosis. Yeast cells require only one Cdk, which binds all classes of cyclins and drives all phases of the cell cycle by changing cyclin partners. Vertebrate cells, however, contain 11 different Cdks, four key Cdk-cyclin complexes are considered to drive the cell cycle; Cdk2-cyclin E complex that governs cell cycle progression from G1 to S phase and Cdk1/2-cyclin A that ensures progression through S phase and entry to G2/M, Cdk1-cyclin B that is needed for mitosis, and Cdk4/5-cyclin D that controls entry into and progression of G1, and entry back into the cell cycle from G0 requires the Cdk4/6-cyclin D complex.

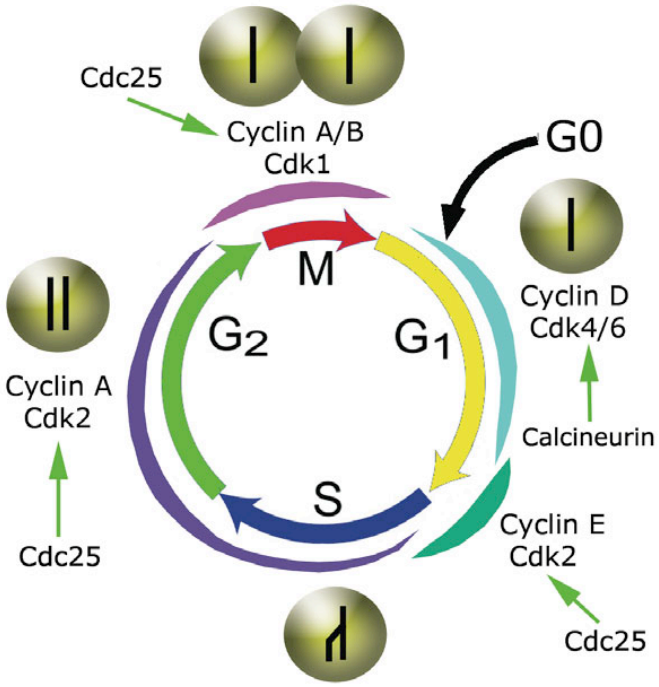

Figure 1. Four phases of the mammalian cell division cycle. The cell cycle consists of four distinct phases (G1-S-G2-M), which are normally irreversible. Progression through these phases is controlled by Cdks, their cyclin partners and phosphatases. The resting phase is called G0. Cell's genetic material is replicated in S phase, the cell doubles its mass in G2 phase and the division takes place in M phase. Cdc25 and Calcineurin remove inhibitory phosphate groups from Cdks (green arrows).

Cyclin partners do not simply activate Cdk's; they also direct the kinase to its specific targets. As a result, each Cdk-cyclin heterodimer phosphorylates a different set of substrates, leading to different outcomes, during different phases of the cell cycle

(Bloom & Cross, 2007, Hartwell, 2002, Hunt, 2002, Murray, 2004, Nurse, 2002, Wikman & Kettunen, 2006).

2. Genes and cancer

Cancer is uncontrollable growth of a cell population that originates from normal tissue and is able to invade and/or metastasize. The path that leads to a healthy cell becoming an invasive cancer cell has many steps that in most cases involve consecutive accumulation of genetic changes. Mutations that cause cancer usually appear in chromosomal areas, which encode for genes that regulate cell growth. These mutations are usually caused by chemical carcinogens, irradiation or by viruses that are able to insert their DNA into genome. Mutations may also occur spontaneously and it is possible to inherit a mutated gene, which makes a person more susceptible to cancer. In these cases cancer can be inherited. Cancer risk increases with age as our cells harbor more genetic alterations.

Cancer cells have many unique characteristics (Fig. 2). They are able to evade apoptosis, they have unlimited growth potential due to reactivation of telomerase and they do not require growth factors from outside. In addition, cancer cells have faster cell cycle rate and their ability to differentiate is altered. They show no contact inhibition of growth and they are able to invade neighboring tissues and to metastasize and to promote growth of blood vessels (http://www.nature.com/nrc/poster/subpathways/index.html) (Kastan, 2007, Sherr & McCormick, 2002, Stewart & Weinberg, 2006, Wikman & Kettunen, 2006).

2.1 Oncogenes

A proto-oncogene is a normal gene that becomes an oncogene as a result of a mutation or altered gene expression. Cell growth, cell division, survival and differentiation are all processes driven by proto-oncogenes. Typical proto-oncogenes are c-Myc and Ras; their mutations are detected in numerous human cancers. C-Myc functions normally as a transcription factor that promotes growth and also inhibits Cdk inhibitors. However, its functions are heavily dependent on mitogenic signaling. Ras on the other hand operates at the membrane where it can initiate multiple signaling cascades leading to cell proliferation. Gene duplications, chromosomal

translocations and mutations in proto-oncogenes can alter their functions, increasing their activity (Hemann & Narita, 2007).

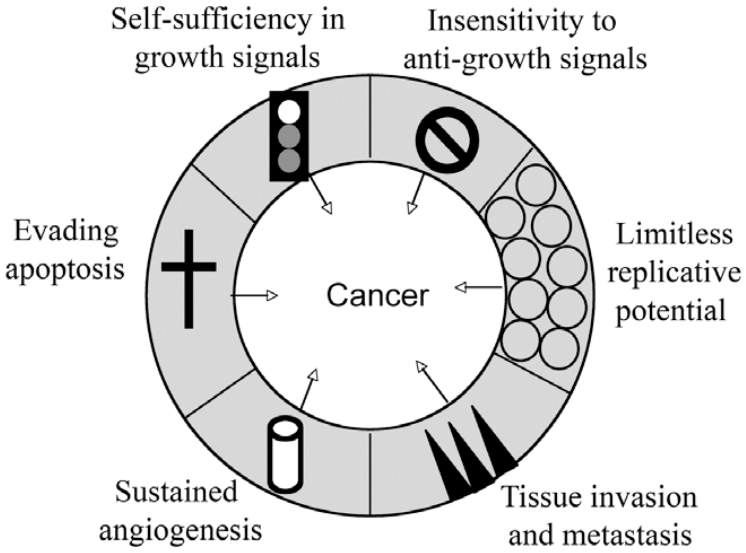

Figure 2. Alterations leading to cancer (modified from Hanahan and Weinberg, 2000) (Hanahan & Weinberg, 2000).

Recently, microRNAs (miRNAs), a class of non-protein-coding small RNAs, have been indicated in the regulation of cell proliferation and apoptosis. They influence gene expression by translational repression of their target genes, by mRNA cleavage and mRNA decay. These miRNAs are involved in several human cancers, where they can act as both oncogenes and tumor suppressors (Dalmay & Edwards, 2006, Zhang et al, 2007).

2.2 Tumor suppressor genes

The discovery of oncogenes proposed the presence of a distinct class of anti-oncogenes. Over the last 17 years such genes, tumor suppressor genes, have been identified (reviewed in Sherr, 2004). Tumor suppressor genes regulate a wide variety of cellular functions. They are involved in cell cycle checkpoint responses, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signaling, differentiation, migration, and tumor angiogenesis (Sherr, 2004). Tumor suppressor genes provide protection against cancer, and the presence of only a single allele of a tumor suppressor gene is in most cases sufficient for its protective function. Thus, tumor suppressor genes require a ‘two-hit’ inactivation of both alleles for

cancer to develop (Knudson, 1971). In cancers, tumor suppressor genes harbor loss-of-function mutations or are completely deleted. In hereditary cancer syndromes patients carry one inactivated tumor suppressor gene in their germ line and an additional somatic mutation, loss-of heterozygosity or an epigenetic mechanism is required for the complete loss of gene function. The same tumor suppressor genes are frequently mutated in sporadic cancers (Sherr, 2004) and therefore, studying these tumor suppressors is beneficial for understanding cancer in general.

2.2.1 Gatekeepers, caretakers and landscapers

Gatekeepers are tumor suppressor genes that directly regulate cell growth by inhibiting it or by promoting cell death. The most classical examples are p53 and pRb, the retinoblastoma gene product. pRb was the first tumor suppressor to be characterized (Knudson, 1971). It physically interacts with transcription factors and represses genes that regulate cell cycle progression, apoptosis and differentiation. It prevents cells from entering S phase, serving a cell cycle checkpoint function. Phosphorylation of the pRb-protein by mitogen-activated Cdk's cancels the pRb-mediated repression. This provides a link between Cdk's, extracellular signals and cell cycle checkpoint control; loss of pRb dissociates the cell cycle from pRb-mediated extracellular signals (reviewed in Sherr 2004). p53 has a cell cycle checkpoint function and it becomes activated by DNA damage. Activation of p53 causes it to arrest the cell cycle at G1. In response to various cellular stresses such as DNA damage and osmotic shock p53 activation leads to a transcriptional response that either inhibits cell proliferation or induces apoptosis (Kastan et al, 1991). Many cancers have both Rb and p53 inactivated, and for instance human papilloma virus can inactivate both tumor suppressors to promote growth (Scheffner et al, 1991, Slebos et al, 1994).

Gatekeepers form one point of restriction for tumor formation whereas caretakers that, for example, repair DNA or control oncogene expression, act as 'caretakers' of the genome. Inactivation of a caretaker gene does not promote tumor formation but rather increases mutation rate, thereby increasing the probability of tumorigenic mutations (Kinzler & Vogelstein, 1997). Therefore, persons carrying germ line mutations that affect caretaker tumor suppressor genes are often more prone to

tumors, as several mutations are required for the full development of cancer (Hanahan & Weinberg, 2000).

Landscapers are cancer susceptibility genes that work through less direct mechanisms than gatekeepers or caretakers. The discovery of the landscapers raised essential questions about the relation between tumor cells and other cells that together constitute a tumor mass. Landscaper defects are not yet well characterized. One example of a landscaper effect is found in the juvenile polyposis syndrome. The epithelial cells within and surrounding the polyp are initially devoid of neoplastic features, but are nevertheless at increased risk of becoming malignant as a result of an abnormal microenvironment. This effect can be thought of as a "landscaper" defect in the epithelial cells (Kinzler & Vogelstein, 1998).

3. The Cytoskeleton

The cytoskeleton is essential for all eukaryotic cells. It is formed by three different, but interconnected filament structures (Fig. 4): the actin cytoskeleton, microtubules and intermediate filaments. The mechanical properties of these structures provide shape and strength to the cell, justifying the use of the term cytoskeleton. Cytoskeleton also enables the cell to move. In addition, it is required for cell division and transport of material inside the cell. The term cytoskeleton is misleading, however, in the sense that it suggests a static structure. Cytoskeletal polymers are in fact highly dynamic, capable of polymerizing, depolymerizing, and moving within the cytoplasm on a time scale of seconds to minutes. Many pathogenic conditions, such as invasive cancer, immunity, and host cell infection by pathogens, are linked to defects in the cytoskeleton (Fuchs, 1996).

3.1 Actin cytoskeleton

The actin cytoskeleton forms the ‘muscles’ of a cell and helps to maintain cell shape. Actin filaments, also known as microfilaments or F-actin, are polar, helical and flexible fibers that are formed through polymerization of globular actin monomers (G-actin). In muscle tissue actin filaments participate in muscle contraction; in non-muscle cells actin filaments contribute to various cellular activities such as changes in

cell shape, spreading, motility, cytokinesis and polarity. Actin filaments are concentrated just beneath the plasma membrane where they form various structures that help cells to move. These protrusive structures of the plasma membrane are called lamellipodia, filopodia and pseudopodia (Fig. 3). All of these structures contain different, specialized actin networks, depending on the accessory proteins participating in network formation (Pantaloni et al, 2001, Pollard et al, 2000).

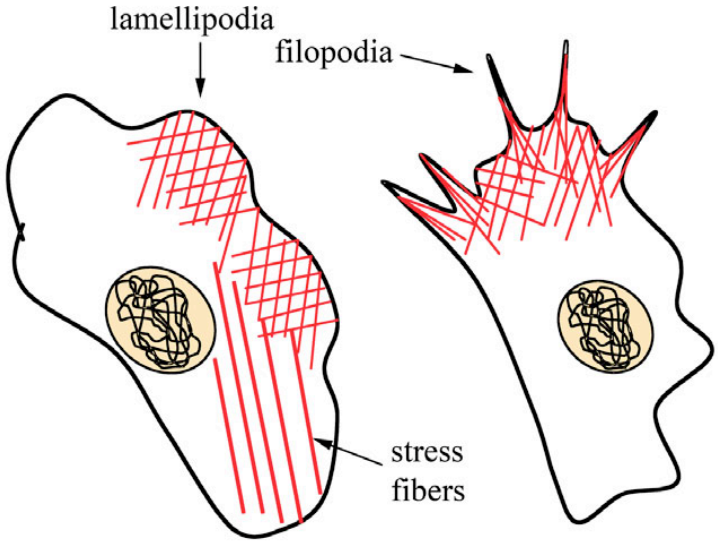

Figure 3. A schematic presentation of various actin structures. Lamellipodia are a characteristic feature at the front, the leading edge, of motile cells. They pull the cell forward during cell migration. Lamellipodia are formed by a two-dimensional actin meshwork. Within the lamellipodia are protrusions of actin called filopodia. They are formed from actin filaments cross-linked into bundles by actin-binding proteins. Filopodia form focal adhesions with the substratum, linking them to the cell surface. Stress fibers form when a cell makes stable connections with a substrate via integrins (transmembrane adhesion molecules), and they provide mechanical support to cells.

3.2 Microtubules

Microtubules are polymers of tubulin protein found in all dividing eukaryotic cells and in most differentiated cell types (Table 1). During cell division, a large dynamic array of microtubules, the mitotic spindle, functions to physically segregate the chromosomes and to orient the plane of cell cleavage. In non-dividing cells, microtubules organize the cytoplasm, position the nucleus and organelles, transport vesicles and serve as the principal structural element of axons, dendrites, flagella and cilia.

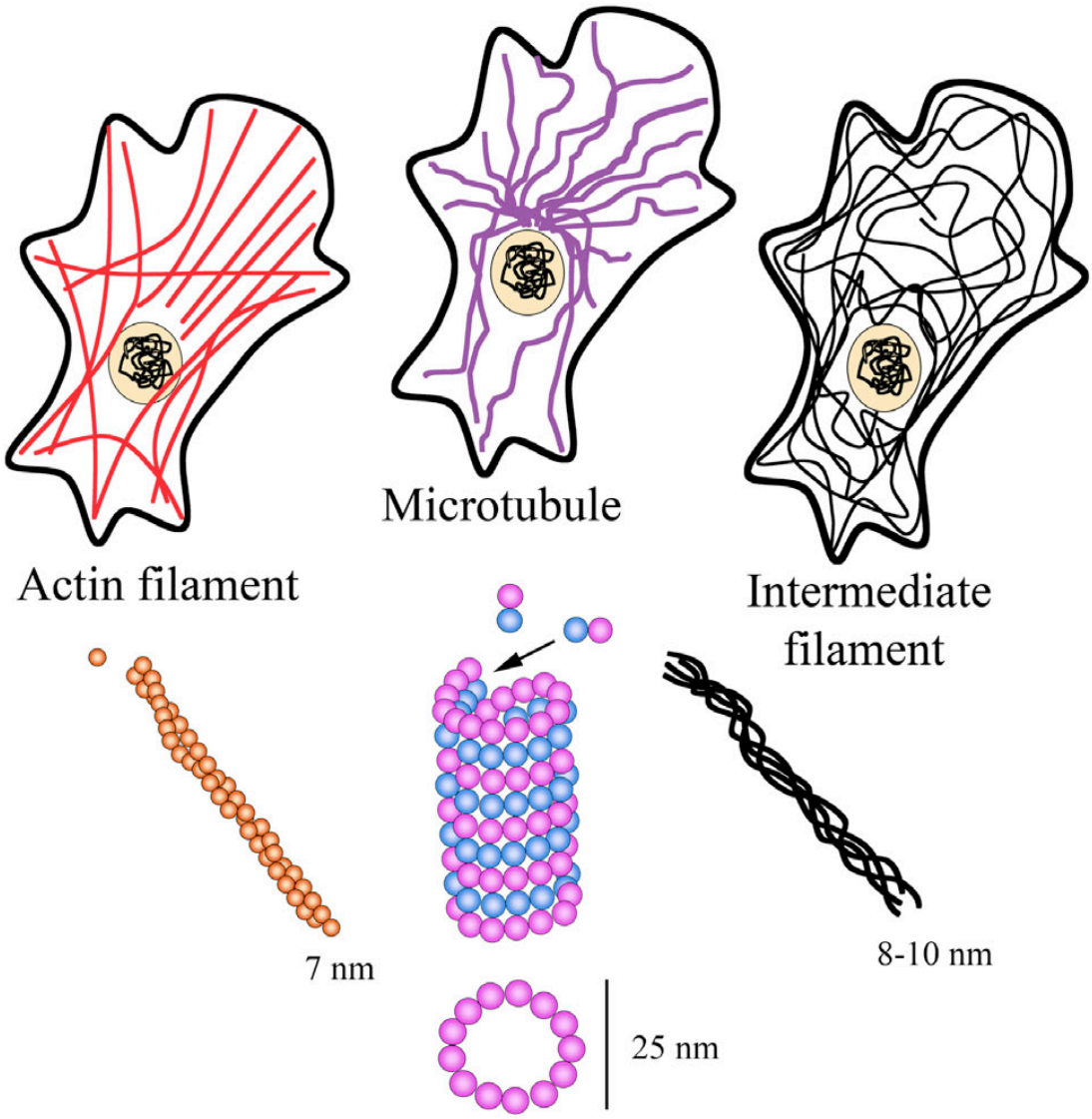

Figure 4. A schematic drawing of different cytoskeletal networks and proteins. Actin filaments are formed from actin proteins. They supply cell with structural support and enable it to move. Microtubules are hollow sylinders made from tubulin dimers (arrow). They participate e.g. in mitosis and in vesicle transport. Intermediate filaments provide structural support to cells and help to keep epithelial cells together. They are formed from filamentous proteins.

Microtubules are more rigid structures than actin filaments with an intrinsic resistance to bending and compression. They are hollow cylindrical filaments formed of globular α- and β-tubulin dimers. Microtubules are also polar structures, usually with the minus end of the microtubule attached to the centrosomes. They are the motorways of the cell. They allow intracellular trafficking as vesicles glide along the microtubules with the help of motor proteins (such as kinesin and dynein) to their targets. Microtubules are very dynamic in most structures, especially the mitotic spindle where the non-kinetochore microtubule turnover is only 20 seconds and in

kinetochore microtubules 300 seconds (Cimini et al, 2006). These dynamics are highly regulated by microtubule-associated proteins (MAPs), especially in neurons, where some structures are stabilized and some structures need rapid reformation. MAPs are proteins that bind microtubules and induce their polymerization/depolymerization (catastrophins). Another proteins are needed for microtubule nucleation (γ-tubulin) (reviewed in Desai and Mitchison 1997). To date, many tumor suppressors have been shown to bind or regulate microtubules (Chaudhuri et al, 1999, Dallol et al, 2004, Fisk et al, 2002, Gregory et al, 1993, Hergovich et al, 2003, Jiang & Yeung, 2006, Moshnikova et al, 2006, Sankaran et al, 2005, Vos et al, 2004).

Tubulin proteins	Microtubule associated proteins (MAPs)

α-tubulin (isotypes I-VI)	MAP1A	Tau
β-tubulin (isotypes I-VI)	MAP1B	Stathmin/OP18
γ-tubulin	MAP2a	BRCA1
Δ-tubulin	MAP2b	Survivin
ε-tubulin	MAP2c	pVHL
	MAP4	Eg5
	MAP7	MAP215/TOG

Table 1. Various tubulin and microtubule associated proteins. Δ- and ε-tubulin are also involved in cetrosome formation. MAP-proteins participate both in microtubule polymerization and depolymerization, and include some motor proteins as well.

Microtubules have become popular targets for cancer therapy. Microtubule-targeting agents, such as paclitaxel, colchicine and vinca alkaloids are used in cancer treatment. Their effectiveness is based on their ability to effect microtubule dynamics. This has an impact on cancer cells, which are constantly dividing, and require a functional mitotic spindle. These microtubule-disrupting agents hinder cancer cells from dividing. However, cancer cells have developed multiple ways of evading these microtubule-disrupting agents. They become resistant to these drugs for example by over-expressing *mdr1* gene that encodes for an efflux pump that keeps the drugs out of the cells. Alternative strategies for cancer cells to avoid death by these drugs are altered tubulin gene expression, altered interaction of the drugs with the microtubules or inadequate induction of apoptotic signaling. New and better microtubule-disrupting

agents are sought, and the conventional drugs (colcisin and paclitaxel) have also unwanted side effects such as neurotoxicity (due to high amount of microtubules in neurons) (Dumontet & Sikic, 1999). Another emerging field of research focuses on MAPs as possible cancer therapy targets (Bhat & Setaluri, 2007).

3.3 Intermediate filaments

Intermediate filaments are ropelike protein fibers that tolerate stretching and bending. They constitute the internal three-dimensional structure of the cell and the protective cage around the nucleus, the nuclear envelope. They also keep epithelial cell sheets together, help neuronal cells to extend axons and form tough appendages, such as hair and nails. Intermediate filaments are made up of filamentous proteins. So far, 67 genes encoding intermediate filament proteins have been identified, which makes this gene family one of the largest in the human genome (Table 2). Many members of the intermediate filament protein family are expressed abundantly and differentially in complex patterns during embryonic development and in the terminally differentiated cell types, making intermediate filaments cytoskeletal 'identity cards'. Intermediate filaments also provide each cell type with unique cytoskeletal architecture. One of the most well known families of intermediate filament proteins is the keratin-family, which form our skin, nails and hair.

The individual proteins of intermediate filaments are elongated molecules with an extended α-helical domain that forms a parallel coiled-coil with another molecule. This dimer then associates with another dimer to form a tetramer. The assembled intermediate filament lacks the polarity that is characteristic of microtubules and microfilaments (Chang & Goldman, 2004, Helfand et al, 2004).

Subtype I and II	Subtype III	Subtype IV	Subtype V	Subtype VI
Epithelial keratins	Desmin	α-Internexin	Lamins	Nestin
Trichocytic (hair) keratins	Glial fibrillary acidic protein	Neurofilaments (H/L/M)		
	Peripherin	Synemin α/β		
	Vimentin	Syncoilin		

Table 2. Intermediate filament subtypes (http://www.interfil.org/index.php)

All three cytoskeletal systems are interconnected via proteins that are able to bind the different cytoskeletal proteins. For instance, during cell division and cell migration, microtubules and actin cytoskeleton need to perform their tasks in an orderly manner. However, this crosstalk is not yet completely understood, and the mechanisms by which these cross-linking proteins function to connect these two systems are still somewhat unclear. Small GTPases, such as Rac, Rho and Cdc42 have been shown to influence both networks, but they do this by modulating signaling networks rather than binding actin or tubulin directly (Kodama et al, 2004).

4. FERM-domain proteins

Ezrin-**r**adixin-**m**oesin-proteins (ERM) form a family of closely related intracellular proteins that crosslink actin filaments to the plasma membrane, and participate in signal-transduction pathways. They regulate the structure and function of specific domains of the cell cortex. They are widely expressed, membrane-associated proteins, that are highly homologous and they also share a common feature, the band **f**our-point-one **e**zrin-**r**adixin-**m**oesin homology-domain (FERM-domain) that makes them members of the band 4.1 protein superfamily. The Neurofibromatosis 2 tumor suppressor merlin (also known as schwannomin) contains the FERM-domain and is highly homologous to the ERM-proteins from its FERM-domain (reviewed in Bretscher et al 2002).

4.1 The FERM-domain

The FERM-domain is a widespread protein module of approximately 300 amino acids that is involved in localizing proteins to the plasma membrane. The FERM-domain was originally identified in the band 4.1 protein that was isolated from human erythrocytes (Leto & Marchesi, 1984). The 30-kDa FERM domain of protein band 4.1 is a cysteine-rich, basic globular module (Chishti et al, 1998). Most FERM-domain proteins function as conformationally regulated membrane:cytoskeleton cross-linkers in actin-rich cell surface structures (reviewed in Bretscher et al 2002).

The structure of FERM-domain has been resolved by protein crystallography (Pearson et al, 2000). This globular domain consists of three sub-domains (F1, F2 and F3) that are arranged in a cloverleaf-like fashion. Even though there seems to be no

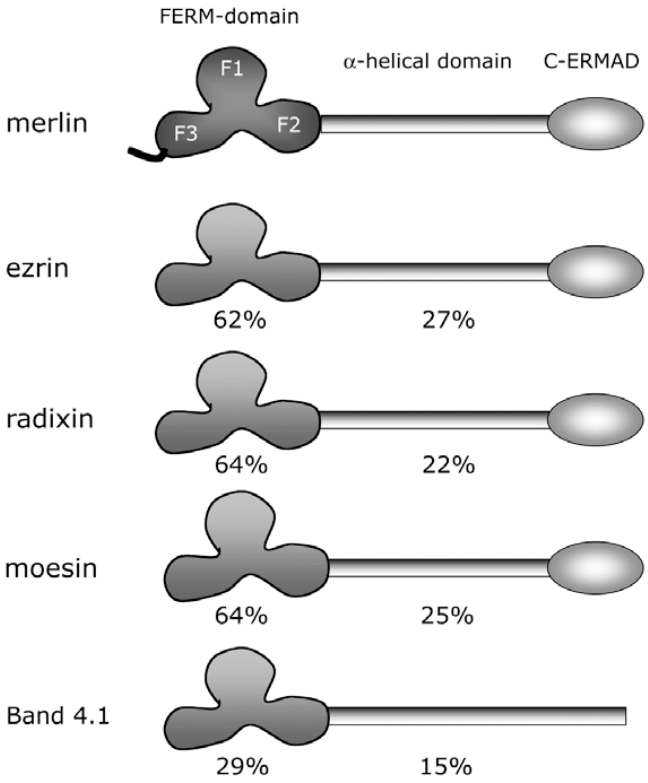

Figure 5. Domain structure of merlin in comparison to the other ERM-proteins. ERM-proteins consist of the amino-terminal FERM-domain (three sub-domains have been marked F1, F2 and F3), the central α-helical domain and the C-terminal domain (C-ERMAD, C-terminal ERM association domain. Percentages indicate the amino-acid sequence similarity.

evident sequence conservation, all three sub-domains are homologous to protein domains that have been described before: the F1-domain resembles ubiquitin, F2 resembles Acyl-CoA binding protein and F3 shows structural homology to the phospho-tyrosine binding (PTB), pleckstrin-homology and Enabled/VASP-1 (EVH1) domains (Pearson et al, 2000). The FERM-domain binds peptide and lipid ligands in signaling and cytoskeletal proteins. The FERM-domains of ERM-proteins and their homologues from different species show remarkable sequence conservation. ERM-proteins show 74-82% identity between their FERM-domains and also merlin displays 60% identity to ezrin's FERM-domain. This indicates that the structure has been exceptionally well conserved (Bretscher et al, 2002).

4.2 ERM-proteins and merlin

Ezrin, radixin and moesin, together with tumor suppressor merlin comprise one class of the band 4.1 superfamily (Fig. 5) (Rouleau et al, 1993, Sato et al, 1992, Trofatter et al, 1993). They link various membrane proteins to the actin cytoskeleton and

participate in a wide range of signaling pathways, such as the Ras-pathway. They also regulate the structure and function of specific domains of the cell cortex. Ezrin was the first family member that was identified as a component of actin rich membrane structures, such as microvilli and membrane ruffles (Bretscher, 1983, Pakkanen et al, 1987). Radixin was isolated from hepatic adherens junctions (Tsukita et al, 1989), and was shown to localize to microvilli (Amieva et al, 1994, Henry et al, 1995) and the cleavage furrow (Sato et al, 1991) in several cell types. Moesin is also enriched in actin rich membrane structures (Amieva & Furthmayr, 1995, Franck et al, 1993), although it was originally discovered for its ability to bind heparin (Lankes & Furthmayr, 1991).

ERM-proteins link F-actin to membrane proteins in a regulated fashion (Tsukita et al, 1994). They contain an F-actin binding site in their C-terminal part, however, this binding site is masked in the dormant molecule (Nakamura et al, 1999, Pestonjamasp et al, 1995, Turunen et al, 1994). Regulated attachment of membrane proteins to actin is important in many cellular functions, such as determination of cell shape, polarity, cell adhesion, motility and integration of signaling pathways with membrane transport.

Merlin was found in 1993 when the Neurofibromatosis 2 (NF2) tumor suppressor gene was identified (Rouleau et al, 1993, Trofatter et al, 1993). The subsequent protein product joined the family of ERM-proteins as it showed high similarity structurally and functionally to ERM-proteins, and hence, the protein was named merlin for **m**oesin-**e**zrin-**r**adixin-**li**ke protein (Trofatter et al, 1993), or schwannomin (Rouleau et al, 1993), describing its suppressor role in schwannoma formation. Like the other ERM-proteins, also merlin links the plasma membrane to the cytoskeleton and participates in conveying signals from the plasma membrane to the cell interior. However, merlin functions as a tumor suppressor. Despite the apparent sequence homology between merlin and ERM-proteins, merlin seems to perform a defined set of functions that no other protein is able to compensate, whereas the ERM-proteins seem to have overlapping functions (Takeuchi et al, 1994). Merlin knock-out in mice is embryonically lethal (McClatchey et al, 1997) whereas ezrin knock-out mice are viable although they have problems in epithelial organization in the developing intestine (Saotome et al, 2004). Radixin knock-out mice are normal at birth but develop mild liver injuries at the age of 8 weeks (Kikuchi et al, 2002), and moesin

knock-out mice appear to be normal, showing no change in ezrin or radixin expression (Doi et al, 1999).

Although the FERM-domains in all four proteins are quite similar (60% homology between human ERMs and merlin) (Pearson et al, 2000), there are regions in merlin that are conserved between mammalian and *Drosophila* merlin but that are distinct from the ERM-proteins. The FERM-domain structure shows that most divergent residues between merlin and the ERM-proteins are found clustered at the surface of the FERM-domain where these exposed residues might serve as sites for effector binding or regulatory interactions (reviewed in Bretscher et al., 2002) (Bretscher et al, 2002). Another conserved feature between mammalian and *Drosophila* merlin is the 'blue box' domain, a perfectly conserved seven-amino-acid stretch of basic residues (aa 177-183) (LaJeunesse et al, 1998) which has been shown to be essential for merlin's functions both in flies (LaJeunesse et al, 1998) and humans (Stokowski & Cox, 2000).

ERM-proteins and merlin are capable of forming homo- and heterotypic associations with the other ERM-proteins, thus forming dimers and oligomers (Berryman et al, 1995, Gary & Bretscher, 1993, Gronholm et al, 1999). The accessibility of merlin and ERM-proteins is regulated by intramolecular association between their N- and the C-terminal domains (Fig. 6). When the FERM-domain binds the C-terminal domain it forms a 'closed' protein molecule that masks many of the binding sites and inhibits interactions with some of the binding partners as well as the homo- and heterodimerization (Grönholm et al, 1999). Activation is needed to 'open' the molecule and for the separation of the two domains. This activation takes place in response to various cellular signals, such as phosphorylation and binding of lipids. Phosphorylation of ezrin, radixin and moesin on a conserved threonine (T558 in moesin, T567 in ezrin and T564 in radixin) and binding of the lipid, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2, or PIP_2), has been shown to open and 'activate' the molecule (Gautreau et al, 2000, Hirao et al, 1996, Nakamura et al, 1995, Nakamura et al, 1999). Several kinases phosphorylate the conserved threonine: Rho kinase (Matsui et al, 1998, Tran Quang et al, 2000), protein kinase Cα (PKCα) (Ng et al, 2001), protein kinase Cθ (PKCθ) (Pietromonaco et al, 1998,

Simons et al, 1998) and phosphatidylinositol-4-phosphate-5-kinase (Matsui et al, 1999).

ERM-proteins anchor F-actin to the plasma membrane by binding transmembrane receptors, such as hyaluronan receptor CD44 (Tsukita et al, 1994) and intracellular adhesion molecules (ICAM's) (Heiska et al, 1998). ERM-proteins bind CD44 only in the presence of PIP_2 (Hirao et al, 1996) and further studies have revealed that PIP_2 is required to unmask the F-actin binding site in ERM-proteins *in vitro* (Nakamura et al, 1999).

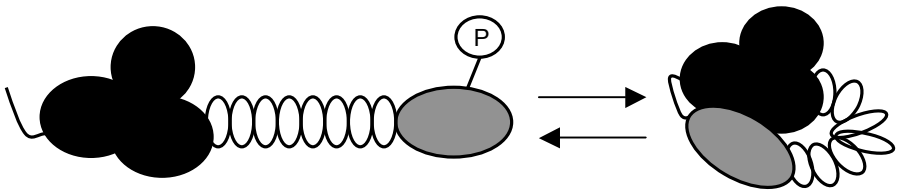

Figure 6. Model of conformational regulation of merlin and ERM-proteins. Merlin's FERM-domain (black) forms a clover-leaf like structure which is able to bind its C-terminus (grey) to form a closed conformation (right). Upon phosphorylation (P) molecule is activated and conformation becomes opened (left).

5. Neurofibromatosis 2 (NF2)

NF2 disease is an autosomal dominant, needing only one affected allele from either parent to inherit the disease manifested as multiple tumors of the central nervous system, in particular schwannomas and meningiomas. Biallelic inactivation of the *NF2* gene is also commonly found in sporadically occurring schwannomas and meningiomas (Lomas et al, 2005, Ruttledge et al, 1994, Stemmer-Rachamimov et al, 1997b). In addition, inactivation of the *NF2* gene has been reported to occur in asbestos exposed mesotheliomas of the lung (Bianchi et al, 1995, Sekido et al, 1995), sporadic thyroid carcinomas (Sheikh et al, 2004), hepatocellular carcinomas and in perineurial cell tumors (Lasota et al, 2001, Pineau et al, 2003, Sheikh et al, 2004).

The incidence of the NF2 disease is low, 1 in 35.000-70.000, with high penetrance (>95%) (Antinheimo et al, 2000, Evans et al, 1992). The most typical hallmark of the disease is the formation of bilateral schwannomas (Schwann cell tumors) around the vestibular branches of the eight cranial nerves. In addition, some patients develop multiple schwannomas associated with other cranial nerves, meningiomas (tumors

derived from meningeal cells), astrocytomas (derived from astrocytes) and intraspinal ependymomas (derived from ependymal cells) (reviewed in Baser et al. 2003). The tumors in NF2 are usually slowly growing and benign. They respond poorly to radiation and are difficult to operate due to their location and therefore, the morbidity among NF2 patients is high. Vestibular schwannoma growth rates among young patients are generally high and extremely variable even among members of the same family with the same age (Mautner et al, 2002). This indicates that other factors than simply the mutation in the *NF2* gene affect the outcome of the disease. Genotype-phenotype correlations have been examined in NF2. Patients with nonsense or frame-shift mutations usually have a more severe disease than patients with missense mutations, in-frame deletions or large deletions (Baser et al, 2002, Baser et al, 2005). It has been shown that all schwannomas lack functional NF2 protein, for meningiomas, the case is not as simple. All NF2 related meningiomas lack functional *NF2* gene, but in sporadic meningiomas only 50% lack functional *NF2* gene, suggesting that additional factors influence the formation of sporadic meningiomas. In addition, *NF2* status strongly depends on the meningiomas subtype, depending on the subtype, 20-80% of meningiomas lack functional *NF2* gene (Hanemann & Evans, 2006, Riemenschneider et al, 2006).

The *NF2* gene is located on chromosome 22q12 (Rouleau et al, 1993, Trofatter et al, 1993) and encodes the merlin protein. It is composed of 17 exons, with two alternatively spliced major isoforms, I and II (Hara et al, 1994). Isoform I lacks exon 16, whereas isoform II contains exon 16 but lacks exon 17, thus replacing 16 C-terminal residues with 11 new residues. In addition, more alternatively spliced isoforms have been reported (Hara et al, 1994, Schmucker et al, 1999), however, these isoforms are not widely expressed and at least some of these isoforms are degraded by the ubiquitin-proteasome pathway (Gautreau et al, 2002).

5.1 Merlin

During mouse embryonic development, high amount of merlin is detected in extra-embryonic tissues. Merlin is also expressed in nervous and skeletal systems and in the heart of mice and human. In adult tissues, merlin is widely expressed but expression levels are low. Merlin mRNA is found in heart, brain, spleen, lung, liver, skeletal muscle and kidney. Significant amounts of the protein are expressed in Schwann

cells, meningeal cells, lens and nerve cells whereas smaller amounts of the protein are expressed in lung, intestine, muscle, spleen, kidney and erythrocytes (Claudio et al, 1995, Gronholm et al, 2005, Hara et al, 1994, Huynh et al, 1996, Jindal et al, 2006, Rouleau et al, 1993).

Studies of merlin localization in cells have been complicated by the low amount of the endogenous protein. Therefore, many of the studies were done by expressing exogenous protein, which may not reflect the true localization of the endogenous protein. However, in cultured mammalian cells, both endogenous and over-expressed merlin are localized to actin-rich structures at the membrane and to cell:cell contacts, perhaps reflecting merlin's role in mediating contact dependent inhibition of growth (Gonzalez-Agosti et al, 1996, Lallemand et al, 2003, Sainio et al, 1997). Merlin is also shown to localize diffusely to the cytoplasm and to punctuate structures. These are thought to represent localization to intracellular vesicles or to lipid rafts (McCartney & Fehon, 1996, Stickney et al, 2004). In the central nervous system (CNS) merlin is expressed in coarse cytoplasmic granules in glia and neurons and in synaptic junctions in cultured polarized neurons (Grönholm et al, 2005, Stemmer-Rachamimov et al, 1997a).

5.2 Model organisms

Merlin orthologs have been found in a wide variety of species (*Drosophila melanogaster*, *Anopheles gambiae*, *Apis mellifera*, *Caernohabditis elegans*, *Xenopus laevis*, *Danio rerio*, *Oryzias latipes*, *Gallus gallus, Mus musculus, Homo sapiens*) (Golovnina et al, 2005), but not in yeast (*Saccharomyces cerevisiae*).

Mouse and human merlin show 98% sequence identity. Three different *Nf2* mutant mice have been engineered that have a homozygous germ-line mutations of the *Nf2* gene. These mice are not viable, due to embryonic death at early stage of development. Embryos fail to initiate gastrulation, and to form extra-embryonic structures (Giovannini et al, 2000, McClatchey et al, 1997). Merlin function is also required during *D. melanogaster* development and lack of merlin leads to overproliferation of cells of *D. melanogaster* (Fehon et al, 1997, LaJeunesse et al, 1998). Heterozygous *Nf2* mutant mice develop bone tumors that have lost the *Nf2* wild type allele (Giovannini et al, 2000, McClatchey et al, 1997), but do not spontaneously develop tumors that are classical for the NF2 disease in humans

(reviewed in McClatchey and Giovannini 2005) (McClatchey & Giovannini, 2005). However, inactivation of both *Nf2* alleles in only Schwann cells produces mice with features found in NF2 patients; these mice develop Schwann cell hyperplasia, Schwann cell tumors and cerebral calcifications, but not meningiomas (Giovannini et al, 2000). However, *Nf2* mice with targeted deletion of the *Nf2* gene in leptomeningeal cells display meningioma formation (Kalamarides et al, 2002).

5.3 Schwann cells

Schwann cells express ERM proteins and merlin, but only the absence of merlin results in NF2-related schwannomas. This implicates a special role for merlin in this cell type. Schwann cells are a subtype of neuroglia, which provide insulation to neurons by forming myelin sheaths around axons (Fig. 7). Schwann cells provide myelin for the axons of the peripheral nervous system, whereas oligodendrocytes myelinate axons of the central nervous system. Myelination increases the rate of conduction of the action potential dramatically, and thus reduces the necessity to increase the axon diameter. The myelin sheath is interrupted at regular intervals by the nodes of Ranvier, where sodium channels of the axon are concentrated. Schwannoma cells that are isolated from the NF2 patients show cytoskeletal changes in their actin and intermediate filament cytoskeletons when compared to normal Schwann cells (Bashour et al, 2002, Pelton et al, 1998, Rosenbaum et al, 1998, Utermark et al, 2005).

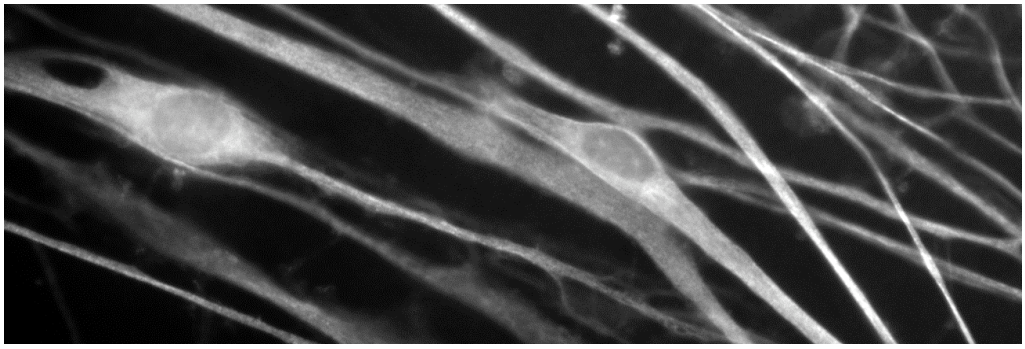

Figure 7. Mouse primary Schwann cells. Stained for α-tubulin, 1000x magnification.

6. Functions and regulation of merlin

Already early experiments with merlin showed its potential as a tumor suppressor. Over-expressed wild type merlin inhibits cell proliferation in NIH3T3 (Lutchman & Rouleau, 1995) and schwannoma cells (Sherman et al, 1997), and can reverse the Ras-induced malignant phenotype in NIH3T3 cells (Tikoo et al, 1994). Merlin also negatively regulates cyclin D1 levels, a major contributor of cell cycle progression in G1 (Xiao et al, 2005). In addition, merlin enhances the stability of p53, by degrading a p53-inhibitor mouse double minute 2 (Mdm2) (Kim et al, 2004). Heterozygous *Nf2* mice develop a wide range of tumors (Giovannini et al, 2000), and cultured cells lacking merlin fail to undergo contact dependent growth arrest (Lallemand et al, 2003). Due to merlin's localization to the areas of the plasma membrane:cytoskeleton interface, it has been considered a unique type of tumor suppressor. Therefore, merlin has been studied mainly in this cellular compartment.

6.1 Interactions with the cytoskeleton and the plasma membrane

Merlin binds F-actin directly with its FERM-domain, but lacks the C-terminal F-actin binding site found in other ERM-proteins (Brault et al, 2001, James et al, 2001, Xu & Gutmann, 1998). Merlin can also bind actin indirectly via its binding partners β-spectrin/fodrin (Scoles et al, 1998), paxillin (Fernandez-Valle et al, 2002), other ERM-proteins (Grönholm et al, 1999, Meng et al, 2000) and neural Wiskott-Aldrich syndrome protein (N-WASP) (Manchanda et al, 2005). Merlin's interaction with N-WASP has been shown to regulate actin dynamics *in vitro*, since this interaction inhibits actin polymerization (Manchanda et al, 2005). In addition, merlin stabilizes actin polymers in vitro by directly binding to actin filaments through a lateral association (James et al, 2001). These two functions appear to be independent from each other (Manchanda et al, 2005).

Merlin can anchor actin fibers to the plasma membrane by binding to transmembrane receptors, such as CD44 (Morrison et al, 2001, Sainio et al, 1997), layilin (Bono et al, 2005), β1-integrin (Obremski et al, 1998), ErbB2 (Fernandez-Valle et al, 2002) and paranodin (Denisenko-Nehrbass et al, 2003). Additionally it can bind membrane proteins indirectly by binding PDZ-domain containing protein NHE regulatory factor 1/ERM-binding phosphoprotein 50 (NHERF1/EBP50) (Murthy et al, 1998). NHERF1/EBP50 can link merlin to other membrane proteins, such as platelet-derived

growth factor receptors (PDGF) (Voltz et al, 2001). Through its interaction with transmembrane receptors merlin is thought to convey signals from the extracellular matrix inside the cell. For example its interaction with hyaluronic acid receptor CD44 is regulated by cell density and by the degree of merlin phosphorylation (Morrison et al, 2001). It is thought that merlin together with CD44 forms a molecular switch that conveys signals of growth arrest and proliferation (Morrison et al, 2001).

Recently, merlin has been linked to growth factor receptor recycling and endocytic trafficking (reviewed in McClatchey and Giovannini 2005). The first clue in this direction came from studies where merlin was shown to interact with hepatocyte growth factor receptor substrate (Hrs), which is an important controller of lysosomal trafficking of membrane receptors (Scoles et al, 2000), and regulates receptor tyrosine kinase trafficking to the degradation pathway (Lloyd et al, 2002). Merlin also interacts with other proteins with roles in growth factor receptor signaling (Table 3.). These include growth factor receptor-bound protein 2 (Grb2), magicin/MED28 (Wiederhold et al, 2004) and NHERF-1/EBP50 (Murthy et al, 1998). Merlin also inhibits platelet derived growth factor receptor (PDGFR) degradation (Fraenzer et al, 2003). It localizes to vesicular structures in both, mammalian cells and *Drosophila* (Maitra et al, 2006, Scoles et al, 2000). Recently merlin was shown to regulate endothelial growth factor receptor (EGFR) recycling and turnover in *Drosophila* (Maitra et al, 2006) and in mouse embryonic fibroblasts (Curto et al, 2007), and to have a role in ErbB2 receptor recycling in Schwann cells (Lallemand et al., personal communication).

Merlin's binding partners:

cytoskeletal:	*transmembrane:*	*receptor recycling:*	*signaling pathways:*	
tubulin	CD44	Hrs	RIβ	RalGDS
ERM	laylin	Grb2	PAK	magicin
F-actin	β1-integrin	NHERF1/EBP50	PIKE-L	TRBP
N-WASP	ErbB2	ErbB2	HEI10	syntenin
βII-spectrin	paranodin		Rho-GDI	NGB
paxillin			MYPT-1	MAP
			eIF3c	

Table 3. Merlin's known binding partners classified according to their function. (Transactivation-responsive RNA-binding protein (TRBP) (Lee et al, 2004b, Lee et al, 2006a), syntenin (Jannatipour et al, 2001), merlin-associating protein (MAP) (Lee et al, 2004a)), eukaryotic initiating factor subunit 3c (eIF3c) (Scoles et al, 2006).

6.2 Phosphorylation and conformational regulation

Merlin undergoes analogous conformational regulation between its N- and C-terminus as the ERM proteins (Grönholm et al, 1999, Gutmann et al, 1999, Gutmann et al, 2001, Huang et al, 1998, Meng et al, 2000, Neill & Crompton, 2001, Rong et al, 2004a), but the activation mechanism for merlin is still unclear. The threonine (T576) involved in conformational activation of ERM-proteins is conserved in merlin, but this residue has not been shown to play any role in its conformational activation. However, another C-terminal residue, not found in the other ERM-proteins, serine 518 (S518), is phosphorylated in merlin and appears, at least to some extent, to regulate merlin's conformation. Phosphorylation of S518 inactivates the protein's tumor suppressor mechanism and promotes growth (Morrison et al, 2001, Shaw et al, 2001). Two kinases have been shown to phosphorylate merlin at S518, p21 activated kinase (PAK) 1 and 2 (Kissil et al, 2002, Shaw et al, 2001), and cAMP activated PKA (Alfthan et al, 2004). PAKs 1-3 are downstream effectors of Rho GTPases Rac and Cdc42 (see below). PAKs mediate signals of cytoskeletal reorganization, cell motility and transcriptional activation (reviewed in Kumar et al., 2006). Merlin is not only phosphorylated by PAK, but is also able to modulate PAK, by binding and inhibiting PAK activity, thus forming a negative feedback loop. Binding is enhanced by cell confluency (Hirokawa et al, 2004, Kissil et al, 2003) perhaps indicating that it is the dephosphorylated merlin that binds PAK.

PAK and PKA act independently from each other since PKA phosphorylates merlin in cells where PAK activity is suppressed (Alfthan et al, 2004). cAMP regulates a wide variety of cellular processes and it has been shown that cAMP driven activation of PKA leads to proliferation in Schwann cells (Kim et al, 1997), and is required for myelin formation (Howe & McCarthy, 2000). Merlin also binds the PKA regulatory subunit RIβ and may work as a protein kinase A-anchoring protein (AKAP) in neurons (Grönholm et al, 2003). An additional kinase indicated in merlin phosphorylation is the Slik-kinase, found to affect merlin phosphorylation in *Drosophila*. However, it is still unclear whether this phosphorylation is direct (Hughes & Fehon, 2006).

A recent paper describes myosin phosphatase MYPT-1-PP1δ as the first phosphatase that dephosphorylates merlin at S518 (Jin et al, 2006). Inhibiting MYPT-1-PP1δ with a 17-kDa protein kinase C potentiated inhibitor (CPI-17), results in merlin

phosphorylation, Rac activation and transformation (Jin et al, 2006), indicating a crucial role for merlin dephosphorylation in growth suppression.

6.2.1 Rho GTPases

Rho GTPases regulate cell polarity and motility and they play a major role in actin cytoskeleton remodeling. They activate actin nucleators, which in turn induce different types of actin organization. The best studied members of this family are Rho, Rac and Cdc42, of which Rac and Cdc42 induce plasma membrane protrusions, such as lamellipodia and filopodia and Rho regulates vesicle trafficking and stress fiber formation (reviewed in Ridley 2006). Rho GTPases bind GTP and GDP, and they have intrinsic GTPase activity. In their GTP-bound state they are able to activate downstream target proteins. Their activity is regulated by guanine nucleotide exchange factors (GEF's), which result in the release of GDP, thus allowing GTP to bind (Ridley, 2006).

Ras is also a small GTPase protein and its activation takes place upstream of Rac activation (Scita et al, 1999). Rac1 activation leads to merlin phosphorylation, probably through PAK (Kissil et al, 2002, Shaw et al, 2001, Xiao et al, 2002). However, merlin in turn inhibits Ras and Rac activation by uncoupling them from growth factor signals (Morrison et al, 2007), not by direct binding but by counteracting ERM-dependent Ras and Rac activation (Morrison et al, 2007). In schwannomas, lack of merlin leads to increased Rac activity, and to the interruption of the neuron-Schwann cell alignment, a process which needs Rac regulation (Nakai et al, 2006). Merlin has also been shown to interact with a Rho-guanine dissociation inhibitor (Rho-GDI) (Maeda et al, 1999), which inhibits nucleotide exchange of the Rho family members, and with Ral guanine nucleotide dissociation stimulator (RalGDS) (Ryu et al, 2005). Thus, merlin could anchor these nucleotide exchange factors to plasma membrane thereby affecting Ras and Rac downstream signaling (McClatchey & Giovannini, 2005, Morrison et al, 2007). Recently, merlin was also shown to interact with a novel tumor suppressor that has intrinsic GTPase activity, NF2-associated GTP binding protein (NGB), which suppresses growth in a merlin-dependent fashion (Lee et al, 2007). NGB is a GTP-binding protein conserved from yeast to mammals, and it suppresses growth co-operatively with merlin by reducing

Cyclin D1 levels and increases merlin turnover by reducing its ubiquitinylation (Lee et al, 2007).

6.3 Merlin regulation of signaling pathways

Merlin is implicated in the regulation of several proteins that affect cell-signaling pathways (Fig. 8). Focal adhesion kinase (FAK) is up-regulated in many cancers and promotes cell migration and invasion. Down-regulation of merlin in mouse embryonic fibroblasts leads to increased spreading and invasion, whereas merlin expression leads to increased FAK phosphorylation. This, in turn, disrupts its interaction with the regulatory subunit of PI3K, p85 and Src (Poulikakos et al, 2006), an interaction required for cell migration (Shen & Guan, 2001).

Recently, merlin, together with tumor suppressor protein Expanded, a related FERM-domain protein, were found to negatively regulate the Hippo signaling pathway in *D. melanogaster* (Hamaratoglu et al, 2006). The Hippo signaling pathway consists of a kinase cascade that ultimately leads to the inhibition of a transcriptional co-activator Yorkie, which normally controls cell growth, survival and proliferation (reviewed in Edgar 2006). Merlin and Expanded activate Hippo/Salvador kinase in response to a yet unidentified signal from a membrane receptor. The activated kinase complex then phosphorylates its downstream target, the Warts/Mats kinase complex, which in turn phosphorylates Yorkie. In its phosphorylated state Yorkie is inactive and inhibits transcription (Edgar, 2006, Hamaratoglu et al, 2006). This signaling cascade forms a negative feedback loop, as Yorkie also regulates transcription of merlin and Expanded (Hamaratoglu et al, 2006). All components of this pathway are conserved in humans, but so far it is only the lack of merlin that causes tumors.

Merlin has also been shown to interact with a novel cytoskeletal protein, magicin (merlin and Grb2 interacting cytoskeletal protein) (Wiederhold et al, 2004), also known as MED28 (Sato et al, 2004). Merlin forms a complex with magicin and Grb2, that can regulate receptor tyrosine kinase and Ras signaling (Wiederhold et al, 2004).
Merlin also interacts with the PI3-kinase enhancer (PIKE-L), which is a brain specific GTPase that binds PI3-kinase and increases its lipid kinase activity. By binding PIKE-L merlin inhibits the PIKE-L:PI3-kinase interaction, thus inhibiting the PI3-kinase.

Interestingly, knocking down PIKE-L abolishes merlin's tumor suppressor activity in RT4 rat schwannoma cells (Rong et al, 2004b).

Lack of merlin leads to upregulation of both phosphorylated extracellular-signal regulated kinase (ERK) (Chadee et al, 2006, Jung et al, 2005, Lim et al, 2006, Rangwala et al, 2005), c-Jun N-terminal kinase (JNK) and PAK (Chadee et al, 2006, Kaempchen et al, 2003). Upon phosphorylation ERK translocates into the nucleus where it activates downstream targets, which ultimately leads to cell growth and proliferation. Activation of the Rac1-PAK-JNK-pathway also leads to transcriptional activation and cell proliferation.

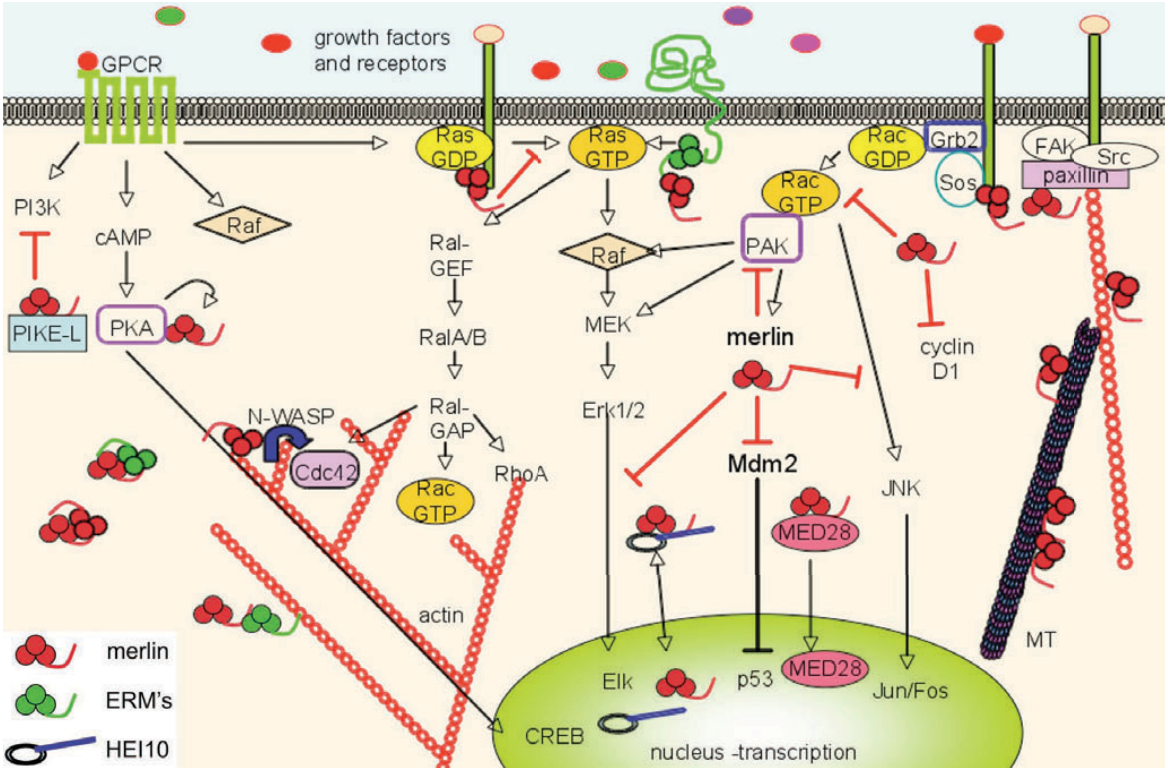

Figure 8. Merlin signaling pathways. Extracellular growth-factors bind to growth-factor receptors. This results in various downstream signaling cascades, that merlin is able to regulateor participate. These signaling cascades ultimately lead to the activation or repression of transcription. (GPCR (G-protein coupled receptor) conveys signals that lead to activation of adenylyl cyclase and increase in cAMP. It also activates phospholipase C, Src and PI3K, which then leads to Raf, Ras and Akt activation (not shown)). These molecules represent only a fraction of all the proteins that take part and regulate these processes.

Thus, merlin's role in cell proliferation includes participation in several signaling pathways that merlin is able to regulate, perhaps by affecting phosphorylation or interactions of signaling cascade partners at the cell membrane. Despite all the studies, the precise mechanism of action how merlin performs its specific functions in

a spatio-temporal manner and in different tissues remains unclear. Clarifying these mechanisms would be of great importance to cancer biology as this knowledge may translate into therapeutic opportunities in the treatment of NF2 patients.

AIMS OF THE STUDY

At the beginning of this study, merlin was considered as a unique type of tumor suppressor, which performs its function at the membrane. However, preliminary observations of merlin's localization during different stages of the cell cycle led us to challenge this view. Therefore I set out to study what additional functions merlin could have, and what purpose would these novel functions serve. The specific aims of this study were:

1) To study merlin's localization during different stages of the cell cycle and to determine the mechanisms and consequences of merlin's nucleo-cytoplasmic shuttling.
2) To identify interaction partners for merlin in the nucleus, and to study the functional relevance of these interactions.
3) To study merlin's association with the microtubule cytoskeleton, and to examine merlin's role in the regulation of the microtubule cytoskeleton in primary mouse Schwann cells and other relevant cell types.
4) To further study the role of phosphorylation in the regulation of merlin, and more specifically, to analyze the consequences of N-terminal phosphorylation of merlin by PKA.

MATERIALS AND METHODS

The materials and methods used in this study are listed below, and are described in detail in the original publications, which are referred to here by using their Roman numerals.

Antibodies and probes used in this study:

NAME	DESCRIPTION	REFERENCE	USED IN
A-19 (sc-331)	Merlin pAb	Santa Cruz Biotechnology	I, II, III, IV
C-18 (sc-332)	Merlin pAb	Santa Cruz Biotechnology	
1398 NF2	Merlin pAb	(den Bakker et al, 1995a)	I
schwannomin	Merlin pAb	(Lutchman & Rouleau, 1995)	I
HM2175	Merlin pS518 pAb	(Kissil et al, 2002)	I
1C4	Merlin mAb	(Gonzalez-Agosti et al, 1996)	II
KF10	Merlin mAb	(den Bakker et al, 1995b)	I, II
α-tubulin	α-tubulin mAb	Sigma-Aldrich	III, IV
β-tubulin	β-tubulin mAb	Sigma-Aldrich	III
N356	mAb α-tubulin	Amesham Pharmacia Biotech.	I, II
Acetyl-tubulin	Acetylated tubulin mAb	Sigma-Aldrich	III
α-HEI10	HEI10 pAb	(Toby et al, 2003)	II
α-porin	α-porin mAb	Invitrogen-Molecular Probes	I
ERK2	ERK2 pAb	Santa Cruz Biotechnology	
p75	p75 mAb	(Huber & Chao, 1995)	III
MAT-1 fl-309	Mat1 pAb	Santa Cruz Biotechnology	I
Hermes	CD44 mAb	(Jalkanen et al, 1987)	I
α-Myc	Myc mAb	Covance Research Products	I
α-HA	HA mAb	Roche	I
GST	GST pAb goat	GE Healthcare	

X63	mAb, negative control	ATCC	I
TO-PRO 3	Nucleic acid stain	Invitrogen-Molecular Probes	I, II
phalloidin	Oregon green and rhodamine conjugated	Invitrogen-Molecular Probes	I, IV

Cell lines used in this study:

CELL LINE	DESCRIPTION	SOURCE	USED IN
U2OS	Human osteosarcoma	ATCC	I, II
U251MG	Human glioma	Japan cell line collection	I, II, III
293HEK	Human kidney epithelial	ATCC	I, II
293A	Human kidney epithelial	Stratagene	III, IV
RT4	Rat schwannoma	(Morrison et al, 2001)	II
RT4-5-4	Rat schwannoma	(Morrison et al, 2001)	II
Schwann	Human primary	(Hanemann et al, 1998)	II
Schwannoma	Human primary	(Rosenbaum et al, 1998)	II
Schwann	Mouse primary	(Manent et al, 2003)	III, IV
COS-7	Monkey kidney	ATCC	IV
Nf2 -/- MEFs	Mouse embryonic fibroblasts	(Giovannini et al, 2000)	IV

Expression vectors used in this study:

PLASMID	DESCRIPTION AND INSERTION	SOURCE	USED IN
pEF-BOS T402E PAK2	Constitutively active PAK2 with myc-HA tag	(Alfthan et al, 2004)	I
pEBB-ME PAK2 1-248 H82/85L	Dominant negative PAK2 with myc tag	(Alfthan et al, 2004)	I
pcDNA3	Mammalian expression vector containing various merlin (aa): 1-595,	Invitrogen	I, II, IV

	1-590, 1-547, 1-537, 1-569, 1-587, 1-314, 492-595, 1-547/ins307 and HEI10 constructs		
pYESTrp2	Yeast two hybrid prey vector with merlin (aa) 1-547 construct with transposon mutations	Invitrogen	II
pGEX4T-1	Bacterial expression vector containing several merlin (aa): 1-314, 314-595, 492-595, 492-595 S518A, 1-100, 492-547, ezrin 1-585 and HEI10: 1-277, 46-277, constructs	Amersham Biosciences	II, III, IV
JG4-5	Yeast two hybrid prey vector containing various HEI10 constructs (aa): 1-277, 1-143, 1-200, 40-250, 116-277, 175-277	(Gyuris et al, 1993)	II, IV
EG202	Yeast two hybrid bait vector containing various merlin constructs (aa): 1-595, 1-595 S518A, 1-595 S518D, 1-595 T576A, 1-595 T576D, 1-590, 1-339, 1-546, 252-595, 252-595 A468P, 252-595 L316W, 252-595 L316T, 339-595, 306-478, 492-595 and ezrin (aa): 1-585, 1-309, 278-585	(Gyuris et al, 1993)	II, IV
pEGFP-Tub	Mammalian expression vector containing tubulin-GFP	Invitrogen	III
pBaculoGold	Baculovirus vestor containing merlin isoform 1 and (aa) 1-547	BD Biosciences	III
pShuttle	Shuttle vector for adenovirus vector pAdEasy containing merlin isoform 1, merlin isoform 1 S10A/S10D	Stratagene	III, IV
pAdEasy	Adenovirus vector with merlin isoform 1, isoform 1 S10A/S10D	Stratagene	III, IV
pENTR3C	Gateway entry vector with merlin	Invitrogen	IV

	isoform 1		
pDEST-27	Gateway target vector containing merlin isoform 1	Invitrogen	IV
GFP-α-actinin	Mammalian expression vector containing GFP-α-actinin	(Hotulainen & Lappalainen, 2006)	IV

Methods used in this study:

METHOD	USED IN STUDY
Cytochalasin D treatment of cells	I
Subcellular fractionation	I
Flow and laser scanning cytometry	I, II
Cell culture	I, II, III, IV
Cell transfections	I, II, III, IV
Production of recombinant DNA constructs	I, II, III, IV
Cell cycle synchronization	I, II, III
Immunofluorescence and laser scanning confocal microscopy	I, II, III, IV
Western blot analysis and immunoblotting	I, II, III, IV
Co-immunoprecipitation	I, II, IV
Insertion of random mutations by transposon insertion system	II
Primary culture of Schwann and schwannoma cells	II, III
Production of recombinant protein in *E.coli*	II, III, IV
Yeast two hybrid analysis	II, IV
Production of recombinant protein by *in vitro* translation	II, III
Site-specific mutagenesis of DNA constructs	II, IV
Protein affinity precipitation	II, IV
Tubulin purification from bovine brain	III
Tubulin pull-down assay	III
Baculovirus production	III
Production of recombinant protein in insect cells	III
Fluorescence recovery after photobleaching (FRAP)	III

Deconvolution and image analysis	III
In vitro and *in vivo* tubulin polymerization assay	III
Live cell imaging	III, IV
Adenovirus production	III, IV
In vitro phosphorylation assay	III, IV
Adenovirus infections	III, IV
Latrunculin B treatment of cells	IV
Production of recombinant protein in mammalian cells	IV
Wound healing assay	IV
Metabolic labeling of mammalian cells	IV

RESULTS AND DISCUSSION

1. Nucleo-cytoplasmic shuttling of merlin (I)

In our earlier studies we had seen a subset of cells display nuclear localization of endogenous merlin. The vast majority of tumor suppressors localize to the nucleus, at least in some phases of the cell cycle (Fabbro & Henderson, 2003). Merlin, however, was considered a cytoskeletal tumor suppressor. Previous studies had shown that some rare isoforms of merlin localize to the nucleus (Kressel & Schmucker, 2002, Schmucker et al, 1999), however, these studies were done using merlin mutants that were later shown to be degraded by the ubiquitin-proteasome pathway (Gautreau et al, 2002). We decided to study the localization of merlin further and used two different cell lines, U2OS osteosarcoma cells and U251 glioma cells, which contain detectable amounts of endogenous merlin (article I, Fig. 1 and 2). Since only 15% of the cells contained nuclear merlin we first studied whether we could increase the amount of merlin in the nucleus by inhibiting chromosome region maintenance 1 (CRM1)/exportin-dependent nuclear export with Leptomycin B. This treatment increased the amount of nuclear merlin significantly, indicating that merlin is dependent on CRM1/exportin nuclear export (I, Fig. 3). As only a small subset of cells displayed nuclear localization in normal growth conditions, we wanted to see whether this is dependent on cell cycle stage or confluency. We tested this by synchronizing the cell cycle or growing cell cultures to confluency and analyzing the cells for nuclear merlin. According to our results merlin enters the nucleus after mitosis, at early G1 and leaves the nucleus as the G1 phase proceeds (I, Fig 4 and 5). Confluent cells were completely devoid of merlin. However, merlin still shuttled to the nucleus in these cells since Leptomycin B treatment caused merlin to accumulate into the nucleus. This indicates that the export rate of merlin exceeds that of the import rate in confluent cells.

Mitotic cells have a round shape. After mitosis they gain their normal shape and adhere more firmly to the substratum, which reminds of the situation of cell adhesion. We therefore tested whether it is the adhesion that plays a role in the nuclear localization of merlin during cell cycle, not necessarily the cell cycle stage per se. Indeed we saw merlin localizing to the nucleus of adhering interphase cells, which would suggest that after mitosis, adherence induces merlin's nuclear localization (I,

Fig. 7). However, in contrast to ERK, this localization was not dependent on an intact actin cytoskeleton (I, Fig. 8) since merlin localized to the nucleus of adhering cells even if the actin cytoskeleton was disrupted by Cytochalasin D treatment. We also studied whether merlin phosphorylation plays a role in nuclear shuttling. In nuclear extracts, both phosphorylated forms of merlin could be found, and stimulation of PKA or PAK did not inhibit nuclear localization, suggesting that nuclear localization is not, at least directly, regulated by phosphorylation of serine 518 (I, Fig. 6).

Our finding that, like so many other tumor suppressor, also merlin localizes to the nucleus is interesting since merlin is considered to be an essential part of the cytoskeleton. Why would a cytoskeletal protein undergo nucleo-cytoplasmic shuttling? Of interest are results where merlin-binding partners such as paxillin, syntenin (Aplin & Juliano, 2001), PIKE-L (Rong et al, 2004b) and magicin/MED28 (Lee et al, 2006b, Sato et al, 2004) have been shown to undergo nucleo-cytoplasmic shuttling or which have been implicated in transcriptional or translational regulation. For instance paxillin and syntenin have been shown to play a role in transcription (Aplin & Juliano, 2001), but it is unclear whether merlin regulates these functions. One possibility for studying whether merlin regulates paxillins nuclear functions, would be to inhibit the merlin-paxillin interaction. Paxillin has been shown to bind polyA-binding protein 1 (PABP1) and to enhance PABP1 bound mRNA export from the nucleus (Woods et al, 2005). Paxillin targets mRNA to focal adhesions, where protein translation takes place. Localized, efficient protein translation also has an important role in cell migration (Woods et al, 2005). If merlin plays a role in paxillin dependent mRNA transport, then either inhibiting the merlin-paxillin interaction or creating a stable merlin-paxillin interaction at the membrane, could have role in paxillin dependent mRNA transport, focal adhesion formation and cell migration. Recently, another binding partner of merlin, actin, was shown to localize to the nucleus and control gene expression (Vartiainen et al, 2007).

The role of merlin in the nucleus and its nucleo-cytoplasmic shuttling is still unclear. However, increasing evidence suggests that the nucleus is not merely a convenient compartment where cytoskeletal proteins can be relocated when they are no longer needed in their more conventional locations (e.g. plasma membrane), but they also have other functions in the nucleus, perhaps independent from their cytoplasmic roles. In the case of merlin this could be tested by transfecting cells with a form of merlin, which contains a mutated cytoplasmic retention and nuclear export signals (Kressel &

Schmucker, 2002). This merlin would be trapped in the nucleus and its effects on cell cycle and migration could be studied. Another possibility would be to mutate merlin's, yet unidentified, nuclear import signal.

2. Interaction of merlin with a cell cycle-regulator HEI10 (II)

In a yeast-two hybrid screen in search for merlin-interacting proteins, we identified a novel binding partner for merlin, a cyclin B-binding protein and cell cycle regulator HEI10 that has been shown to localize to the nucleus (Toby et al, 2003). Our aim was to study whether HEI10 could be a nuclear binding partner for merlin and to study the functional consequences of the merlin-HEI10 interaction. HEI10 had been previously characterized as a protein capable of enhancing invasion in yeast. It is a cell cycle regulator both in yeast and mammals, controlling the accumulation of cyclin B. HEI10 is a 277 amino acid protein containing an N-terminal RING-finger motif, often found in E3 ubiquitin ligases, a coiled-coil domain and a C-terminal domain which is phosphorylated by cyclin B/Cdc2. HEI10 interacts with the UbcH7 E2 ubiquitin conjugating enzyme and with cyclin B and regulates its degradation (Toby et al, 2003). A recent publication shows that HEI10 also negatively regulates cell migration and metastasis, as HEI10 depleted cells migrate and invade more efficiently than wild type cells. In addition, depletion of HEI10 in these cells results in upregulation of p130Cas, paxillin, Cdk1 and cyclin B2, but not merlin (Singh et al, 2007).

We showed that merlin and HEI10 interact through their coiled-coil α-helical regions, which are domains frequently involved in protein interactions (II, Fig. 1-3). In addition, we analyzed merlin and HEI10 localization in U2OS osteosarcoma cells in which both proteins are expressed endogenously. The two proteins localize underneath the cell membrane during the entire cell cycle, and in a small subset of cell, they also colocalize in the nucleus (II, Fig. 4 and 5). HEI10 is expressed mostly in the nucleus (also during S-phase), whereas merlin and HEI10 colocalize in the nucleus only after mitosis at G1 and during cell attachment. We also studied whether merlin could influence HEI10 expression. In RT4-5-4 rat schwannoma cells and human Schwann (expressing merlin) and schwannoma (no merlin expression) cells that also express HEI10. Expression of merlin translocates HEI10 from the nucleus to the cell membrane (II, Fig. 6). In addition, we tested whether merlin expression influences HEI10 protein amount in 293HEK cells. When cells were transfected with

a mutant merlin lacking the C-terminus (merlin 1-547), the amount of HEI10 protein was reduced by 59% ($P<0.01$), and with the wild type protein, HEI10 amount was reduced by 23% ($P<0.05$). This degradation was seen after 72 hours of transfection (II, Fig. 7), which might indicate that merlin influences HEI10 degradation only when cells are beginning to reach contact inhibition of growth.

In this study we identified HEI10, a cell cycle and cell migration regulator, as a merlin binding partner. This is the first potential binding partner for merlin in the nucleus, and the two proteins also colocalize during mitosis at the centrosomes. HEI10 regulates cell cycle by degrading cyclin B, whereas merlin regulates HEI10 levels. This provides a link for merlin to cell cycle regulation, worth further investigation. It would be of interest to express a merlin construct that would stimulate constitutive HEI10 degradation, to see whether this would have any impact on cyclin B degradation or on cell cycle progression during mitosis. It is shown that depletion of HEI10 leads to enhanced motility and migration and up-regulation of several pro-motility genes (Singh et al, 2007). Does the depletion of the whole protein mimic the situation where its amount is regulated by degradation? Perhaps merlin is required for the regulation of HEI10, that it is then needed for efficient cyclin B degradation at the end of mitosis. This could be tested by studying MEF's lacking merlin; do these cells have altered cell cycle or cyclin B levels with or without HEI10 expression?

Whether these two proteins act together at the membrane or in the nucleus to govern cell cycle progression, is still unknown. Our unpublished data shows that merlin and HEI10 could together influence cell cycle progression, by blocking the cell cycle either in G1 or S phase. Constitutively open merlin and HEI10 block the cell cycle in S phase, whereas wild type merlin together with HEI10 block it in G1 even more strongly than merlin alone. Another interesting piece of data is the finding that HEI10 expression is different in tumors and in normal tissue. HEI10 is up-regulated in melanomas (Smith et al, 2004) and a translocation involving HEI10 has been reported in uterine leiomyoma (Mine et al, 2001). It would be of interest to study whether this up-regulation in HEI10 coincides with the lack of merlin in tumors.

3. Merlin regulates the microtubule cytoskeleton of primary Schwann cells (III)

Another common feature of tumor suppressors, in addition to their nuclear localization, is their ability to localize to mitotic structures and to interact with microtubules (Fisk et al, 2002, Hergovich et al, 2003). For example Von Hippel Lindau tumor suppressor protein (pVHL) interacts with microtubules and stabilizes them against depolymerization. In addition, patient mutations residing in the tubulin-binding area of pVHL influence its tubulin binding ability, suggesting that at least some of the patient phenotypes are caused by its inability to bind tubulin properly (Hergovich et al, 2003, Lolkema et al, 2004). We had previously seen merlin localize to mitotic structures (I, II) and this led us to study merlin in context with microtubules. Merlin has previously been shown to bind microtubules *in vitro* (Stokowski & Cox, 2000, Xu & Gutmann, 1998), however these studies have been controversial and no additional *in vivo* evidence has been presented. Therefore, we wanted to further investigate the merlin-tubulin interaction.

In this study we identified two tubulin-binding sites in merlin, one in the N-terminus (aa 1-100) and other in the C-terminus (aa 492-547) of merlin (III, Fig. 2). We saw the two proteins colocalize in the centrosomes and mitotic spindle during mitosis and in the mid-body during cytokinesis in U251 glioma cells (III, Fig. 1). Merlin is able to form a closed conformation by intramolecular association, which blocks many of its binding sites (Grönholm et al, 1999). We therefore studied whether intramolecular association or serine 518 phosphorylation, thought to open the molecule, could regulate merlin-tubulin binding. Merlin's intramolecular association, and also phosphorylation of the S518, inhibited merlin-tubulin binding, implicating a more complex mechanism for merlin activation than simply phosphorylation of S518 (III, Fig. 3). This would suggest that merlin exists in a conformation where it is open but unphosphorylated on S518.

Many microtubule-associated proteins regulate tubulin polymerization and, according to this study, also merlin modulates microtubule polymerization in an *in vitro* assay by enhancing the rate of polymerization (III, Fig. 4). It is of interest, that the full-length protein was required for this effect, whereas C-terminally deleted mutant merlin (aa 1-547) could not induce polymerization, even though it contains both

identified tubulin binding sites, which further shows the importance of the correct regulation of merlin.

To gain further *in vivo* insight in the merlin-tubulin interaction we used *Nf2* -/- primary mouse Schwann cells (Giovannini et al, 2000). In these cells no clear colocalization with adeno-infected wild type merlin and tubulin could be seen. Only partial colocalization was seen under the cell membrane where both proteins resided (III, Fig. 1). However, when these cells lost merlin, their microtubule cytoskeleton became disorganized, and the cell morphology changed dramatically (Fig. 9); the cells lost their spindle shape and obtained a more spread morphology (III, Fig. 5).

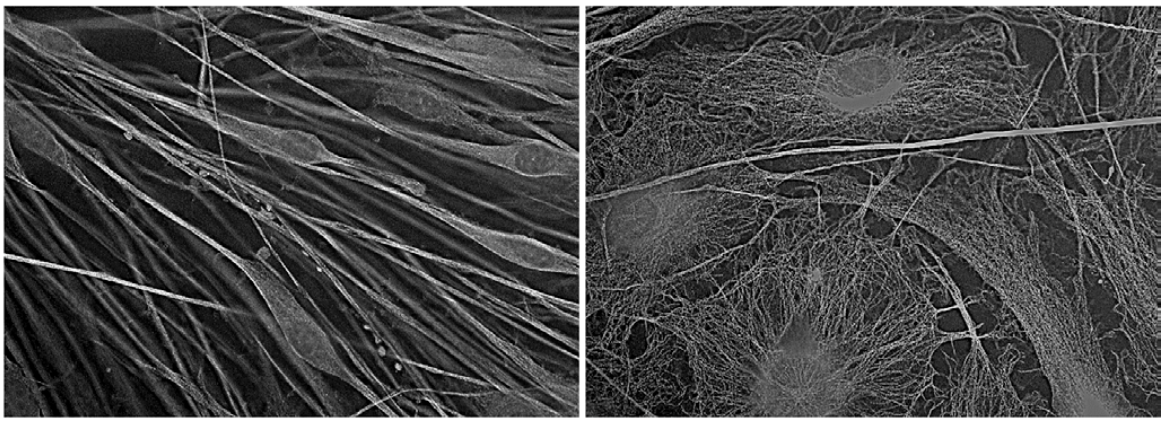

Figure 9. Mouse schwann cells expressing merlin (left) and lacking merlin (right). α-tubulin staining, 1000x magnification.

This change in the microtubule cytoskeleton is probably due to differences in microtubule dynamics, which has been shown previously to be responsible for fluid sheer stress dependent cell shape change (Malek & Izumo, 1996). The *Nf2* -/- Schwann cells contained more soluble tubulin in Triton-X 100 lysates. However, the overall tubulin amount in -/- and merlin expressing cells was similar when analyzed from 6M Urea lysates (III, Fig. 5). This might suggest that more tubulin is associated with microtubules in merlin expressing cells. We then tested whether merlin enhances microtubule polymerization also *in vivo*. Merlin expression increased the tubulin polymerization rates in Schwann cells treated with Nocodazole, a drug that depolymerizes microtubules, since they were able to re-polymerize their microtubules faster than the -/- cells. Using FRAP-analysis we could also see that merlin expressing Schwann cells had enhanced microtubule dynamics (III, Fig. 6).

According to our study, merlin-tubulin binding is under strict regulation. These proteins colocalize only briefly and it still remains unclear if this interaction affects merlin's tumor suppressor function. One possibility is in the regulation of endocytosis. Microtubules regulate endocytic pathways (Murray & Wolkoff, 2003, Petiot et al, 2003) and it has been shown that also merlin plays a role in endocytic processes by localizing to endocytic structures and regulating growth factor receptor availability (Fraenzer et al, 2003, Lloyd et al, 2002, Maitra et al, 2006, Scoles et al, 2000, Curto et al, 2007). Endocytic processes regulate growth-factor availability and recycling, and it has been shown that also microtubules play a role in these processes. Fast and slow microtubules sort their vesicles into different compartments, fast ones more often deliver their cargo to lysosomes for degradation than the slow ones. This rapid movement and maturation is completely dependent on microtubules; if microtubules are disrupted, the dynamic vesicles will non-selectively join all early endosomes and are less efficiently degraded (Lakadamyali et al, 2006). We hypothesize that merlin could function as a linker at the plasma membrane, helping receptor-containing endocytosed vesicles to attach to microtubules. In cells lacking merlin, this receptor recycling might be slower, and the growth factor receptors would be cleared from the membrane less efficiently. Merlin could have a dual role at the membrane of Schwann cells, partly by transferring early endocytic vesicles to microtubules and partly by increasing the microtubule polymerization rate, thus guiding them to lysosomes. *Nf2* -/- and add-back Schwann cells have differences in their growth factor receptor recycling rates (D. Lallemand, manuscript). If the merlin-tubulin interplay is important for clearing up the receptors one would hypothesize that Nocodazole treatment would increase the amount of receptors at the membrane of both Schwann cell lines. However, if merlin alone is responsible for this receptor recycling, the *Nf2* -/- cells would still have more receptors at their membrane.
As we did not see disorganized microtubules in any other cell types lacking merlin, this might be a way for merlin to specifically regulate Schwann cell proliferation.

4. Phosphorylation of merlin N-terminus by PKA (IV)

Our previous studies had identified a PKA phosphorylation site in the merlin FERM-domain, in addition to the previously described C-terminal site (Alfthan et al, 2004). It has also been shown that PKA activity increases the proliferation rate of Schwann

cell (Kim et al, 1997). For these reasons we wanted to study PKA mediated merlin phosphorylation further.

In our study we identified serine 10 (S10) as the N-terminal phosphorylation site. In addition to S10 and S518, no additional PKA phosphorylation sites were found (IV, Fig. 1). We studied whether S10 phosphorylation affects S518 phosphorylation, but could not see any effect when serine 10 was mutated either to alanine (A) or aspartic acid (D) (IV, Fig. 2), mimicking the unphosphorylated and phosphorylated residue, respectively. S518 phosphorylation has been shown to promote heterodimerization with ezrin (Alfthan et al, 2004). However, merlin phosphorylated at S10 bound ezrin similarly as unphosphorylated merlin (IV, Fig. 1).

The first 18 amino acids are unique to merlin. They are not included in the FERM domain, and are indicated in the merlin binding of actin (Brault et al, 2001). Therefore, we hypothesized that N-terminal phosphorylation could play a role in the modulation of the merlin-actin interaction. Our first clue came from transfection experiments where merlin S10 mutants induced more diverse morphologies than did the wild type merlin in COS-7 and *Nf2* -/- MEFs (IV, Fig. 3). Cells transfected with merlin wild type had an increased amount of membrane extensions compared to untransfected cells, whereas cells expressing merlin S10A displayed much elongated extensions not seen with wild type merlin or S10D. S10D transfection resulted in a different phenotype, producing short filopodia-like structures and rarely long extensions seen with S10A. When the same experiment was performed with merlin isoform II the phenotypes were even more pronounced (IV, Fig. 3), which is of interest as it is shown to bind actin more strongly than the wild-type merlin (James et al, 2001).

We next studied the F-actin structures in transfected *Nf2* -/- MEFs (IV, Fig. 4). Cells expressing wild type merlin had a dense, fine network of actin in lamellipodia areas. The actin meshwork structure was seen in wild type, S10D and S518A transfected cells; however, in S10A transfected cells the F-actin staining was reduced compared to wild type or S10D cells and the meshwork was missing. Since it has been shown that merlin can stabilize actin filaments (James et al, 2001) we then tested whether merlin mutants could protect F-actin from Latrunculin B treatment (an agent that inhibits actin polymerization). We noticed that cells expressing S10D were more resistant to the depolymerizing effect of Latrunculin B than cells with wild type or S10A merlin (IV, Fig. 5). We then studied whether S10 plays a role in cell motility.

When MEFs were transfected with various merlin constructs, cells expressing merlin S10A were unable to produce lamellipodia and migrated slower (IV, Fig. 6).

One of merlin's actin binding sites lies within the first 18 amino acids, however, when we tested merlin S10A ability to directly bind actin it was not affected (unpublished data). Therefore we hypothesize that it must be through a different mechanism than direct actin binding, that S10 phosphorylation of merlin affects cytoskeletal organization, lamellipodia formation and migration. This effect could be mediated through merlin's binding partners involved in the regulation of actin dynamics; the most promising candidates are β-spectrin, paxillin, N-WASP and ERM-proteins, or through the Rac-signaling pathway, but this requires further study. It will be of interest to study how S10A mutant affects the interplay between merlin and its binding partners.

CONCLUDING REMARKS

AND FUTURE PERSPECTIVES

This study began in 2002 with the observation on merlin's surprising subcellular localization in the nucleus and centrosomes. This direction of research seemed a bit hazardous at the time as most research efforts were targeted to identifying kinases phosphorylating merlin, mechanisms for its membrane:cytoskeleton interaction, and how these interactions modulated merlin's tumor suppressor activity. Since then, the areas of merlin research have vastly expanded, now covering various aspects of cellular functions that merlin is able to regulate, and identifying more and more interaction partners for merlin. It is now acknowledged that merlin has a versatile role in cells, perhaps depending on cell type or developmental stage of the organism.

The studies presented in this thesis have expanded merlin's role and found alternative functions that might explain why schwannomas are the typical hallmark of the NF2 disease. Our studies have shown that merlin actively shuttles in and out of the nucleus (I), and more and more evidence is published, showing nuclear localization of merlin binding partners (Aplin & Juliano, 2001, Rong et al, 2004b, Vartiainen et al, 2007). Unfortunately our research efforts have still not answered the question of what the functional consequence of merlin's nucleo-cytoplasmic shuttling might be. We hypothesize that merlin could regulate the localization of nuclear binding partners, perhaps retaining them in or transporting them to the cytoplasm. In the case of HEI10 this could be studied by identifying and mutating the nuclear import signal in merlin, to see whether this influences HEI10 localization and/or function. Alternatively, merlin could, as a cytoskeletal scaffolding protein, participate in re-building the nuclear envelope, after cell division, based on its nuclear localization at G1. To this point we have no evidence that merlin itself would bind RNA or DNA directly, but this needs further investigation. In our study of the HEI10-merlin interaction, we discovered that these two proteins can localize to the nucleus simultaneously, however, so far we have no data showing their interaction in the nucleus, since these experiments have been technically difficult to perform. We could only show that merlin can influence HEI10 protein levels and localization, retaining it at the membrane. There are not many studies published on HEI10, but recently Singh et al.

(2007) showed that depletion of HEI10 enhances cell migration by the upregulation of cyclin B/Cdk1. They hypothesize that merlin could relocate HEI10 in cells so that it would not be able to enhance migration (Singh et al, 2007). Our merlin-HEI10 study connects cyclin B to merlin. As cyclin B/Cdk1 regulates passage through M phase and merlin is not shown to influence the progression of mitosis it is unlikely that merlin would regulate this aspect of HEI10 function. However, our preliminary results show that merlin has some role in mitosis or alternatively in centrosome replication as 293HEK cells expressing constitutively open merlin display increased amount of multipolar spindles. Whether this is through a direct mechanism or through HEI10 is still unknown, as no role for HEI10 in causing spindle abnormalities has been shown. It is perhaps more likely that merlin affects other HEI10 mediated functions, such as migration or metastasis, linking the proteins to tumor formation.

The third study in this thesis expanded our early notions of merlin's localization to mitotic structures. This work showed for the first time, that merlin influences microtubules, their structure and dynamics. The finding is important, since microtubules are involved in various cellular processes, including cell growth and division. What makes this finding even more intriguing is that we could only see microtubule disorganization in primary Schwann cells lacking merlin, indicating that merlin has an important role in regulating Schwann cell microtubule cytoskeleton. This may partly explain the presence of such specific tumors, vestibular schwannomas, in NF2 patients. However, the link between merlin, microtubule organization and membrane traffic still needs to be clarified.

Finally, the fourth study in this thesis identified a novel phosphorylation site in merlin. This phosphorylation seems to regulate merlin's impact on the actin cytoskeleton. The finding is interesting as the first 18 amino acids containing the phosphorylated serine are not included in the FERM-domain. We believe that the extreme N-terminal residues could form a flexible tail that participates in regulating binding to interacting partners, depending on its phosphorylation status. Our experiments suggest that this tail does not modulate actin cytoskeleton directly as we could not see any difference in actin binding of the phosphorylated vs. unphosphorylated N-terminus. However, merlin has several binding partners that are able to modulate actin cytoskeleton, and it might be that it is through these binding partners that merlin regulates lamellipodia formation. Another interesting aspect is that merlin regulates PAK (Kissil et al, 2003), Rac and Ras activity (Morrison et al,

2007), which provides another link to cytoskeletal regulation. It will be interesting to study, through which pathway the S10 phosphorylation performs its role.

Despite the progress in NF2 research, we are still lacking specific treatment for the NF2 disease. This thesis work has widened the field and brought upon new avenues of research that may eventually offer new therapeutic targets in the fight against NF2 disease. In the beginning of a study it is hard to evaluate the relevance of its outcome. Yet, it is the basic research that lays the foundation for effective treatment and drug design.

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