Novel Functions of the Neurofibromatosis 2 Tumour Suppressor Protein Merlin

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To my grandparents and parents
who taught me to value curiosity, knowledge and understanding

”Jag söker den fråga på vilken människolivet är ett svar”

”I search for the question to which life is the answer"

(Willy Kyrklund, Mästaren Ma, 1952)

CONTENTS

ABBREVIATIONS

ABD	Actin binding domain
AKAP	A-kinase anchoring protein
APC	Adenomatosis polyposis coli
BRCA	Breast cancer gene
cAMP	Cyclic adenosine 5'-monophosphate
Cdk	Cyclic-dependent kinase
CNC	Carney complex
CNS	Central nervous system
CC	Coiled-coiled
CCD	Cytochalasin D
DAL 1	Differentially expressed in adenocarcinoma of the lung
E3KARP	NHE3 Kinase A Regulatory Protein
EBP50	Ezrin binding protein 50
ECM	Extracellular matrix
E.Coli	Escherichia coli
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ERM	Ezrin, Radixin, Moesin
ERMAD	ERM association domain
EVH1	Enabled/VASP-1 domain
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FBD	FERM binding domain
FERM	Four-point-one, Ezrin, Radixin, Moesin
G-actin	Globular actin
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanosine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GST	Glutathione S-transferase
GTPase	Guanosine triphosphatase
HA	Influenza virus hemagglutinin
HEI-10	Human enhancer of invasion
HGF	Hepatocyte growth factor
HRS	HGF regulated tyrosine kinase substrate
ICAM	Intracellular adhesion molecule
kDa	Kilodalton(s)
LMB	Leptomycin B
LOH	Loss of heterozygosity
LTD	Long term depression
LTP	Long term potentiation
mAb	Monoclonal antibody
MAP	Merlin-associated protein
MAP2	Microtubule associated protein 2

MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
MRP2	Multidrug resistance protein 2
MBS	Myosin binding subunit of myosin phosphatase
Mdm2	Mouse double minute 2
MRP2	Multidrug resistance protein 2
NF2	Neurofibromatosis 2
NHE	Sodium-hydrogen exchanger
NHE-RF	NHE regulatory factor
N-WASP	Neural Wiskott-Aldrich syndrome protein
ORF	Open reading frame
PAK	p21 activated kinase
PAP	Peripheral astrocytic processes
PDGF	Platelet derived growth factor
PDZ	PSD-95/Discs large/ZO-1
PH	Pleckstrin homology
PIP_2	Phosphatidylinositol-4,5-biphosphate
PI3 kinase	Phosphatidylinositol 3-kinase
PIKE	PI3-kinase enhancer
PKA	Protein kinase A
PSD	Postsynaptic density
PTB	Phosphotyrosine-binding domain
Rho-GDI	Rho GDP dissociation inhibitor
SAB	Spectrin-actin binding domain
SAP	Synaptic-associated protein
S.cerevisiae	Saccharomyces cerevisiae
SCHIP-1	Schwannomin-interacting protein 1
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRE	Serum response element
TAR	HIV-1 transactivating response
TNF	Tumour necrosis factor
TRBP	TAR RNA-binding protein
TSC	Tuberous sclerosis complex
VHL	Von Hippel-Lindau

ORIGINAL PUBLICATIONS

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

I **Mikaela Grönholm,** Markku Sainio, Fang Zhao, Leena Heiska, Antti Vaheri and Olli Carpén. Homotypic and heterotypic interaction of the Neurofibromatosis 2 tumor suppressor protein merlin and the ERM protein ezrin. *Journal of Cell Science* 112, 895-904 (1999).

II Taru Muranen*, **Mikaela Grönholm***, Herma G. Renkema and Olli Carpén. Cell cycle-dependent nucleocytoplasmic shuttling of the neurofibromatosis 2 tumour suppressor merlin. *Oncogene* 10;24(7):1150-8 (2005).

III **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. Manuscript submitted.

IV **Mikaela Grönholm,** Tambet Teesalu, Jaana Tyynelä, Katja Piltti, Tom Böhling, Kirmo Wartivaara, Antti Vaheri, Olli Carpén. Characterization of the neurofibromatosis 2 protein merlin and the ERM protein ezrin in the central nervous system. *Molecular and Cellular Neuroscience* 28, 683-693 (2005).

V **Mikaela Grönholm,** Lutz Vossebein, Cathrine R. Carlson, Juha Kuja-Panula, Tambet Teesalu, Kaija Alfthan, Antti Vaheri, Heikki Rauvala, Friedrich W. Herberg, Kjetil Taskén and Olli Carpén. Merlin links to the cAMP neuronal signaling pathway by anchoring the RIβ subunit of protein kinase A. *Journal of Biological Chemistry* 278 (42), 41167-72 (2003).

* equal contribution

ABSTRACT

The ERM (ezrin, radixin, moesin) proteins act as linkers between plasma membrane proteins and the actin-containing cytoskeleton. Ezrin and a related protein, merlin, are functionally and structurally quite homologous but have opposite effects on cell proliferation. Lack of functional merlin is involved in the tumourigenesis of nervous system tumours in the dominantly inherited neurofibromatosis 2 (NF2) disease, while increased ezrin expression is associated with enhanced cell growth and poor prognosis of malignant tumours. To find reasons for the tumour suppressor function of merlin we have analysed the molecular interactions of merlin and ezrin and pathways that differentially regulate the conformation and activity of both proteins.

ERM proteins are components of the cortical cytoskeleton and their function can be regulated through homotypic and heterotypic interactions. In this work we showed that merlin shares these properties with ERM-proteins. Merlin colocalised with ezrin underneath the plasma membrane in subconfluent cells and a concomitant redistribution of both proteins could be seen in confluent cells. The interaction domains for self-association and ezrin-binding corresponded to known ERM association domains. These data functionally linked merlin to ERM proteins. However, merlin appeared to be regulated differently than ezrin.

Most tumour suppressors are involved in the regulation of cell proliferation, which is often linked to their nuclear targeting. Because of its association with the cortical actin cytoskeleton, merlin has been considered an unusual tumour suppressor. We showed that the subcellular localisation of merlin is more versatile than previously thought. Merlin underwent nucleo-cytoplasmic shuttling that was dependent on the CRM1 nuclear export pathway and regulated by cell cycle phase, adhesion and confluency. Interestingly, we identified the recently discovered cell cycle regulator HEI10 as a novel binding partner for merlin but not ezrin. Merlin and HEI10 colocalised during certain phases of the cell cycle; at mitosis in the mitotic spindle and the contractile ring, and at early G1 in the nucleus. During most of the cell cycle, both proteins were also present at the plasma membrane. It is of particular interest that expression of merlin and HEI10 alone and together affected cell cycle progression. In addition, merlin could affect the localisation and integrity of the HEI10 protein. These

findings suggested that merlin possesses novel functions in the nucleus and plays a role in the control of cell division.

In addition to the tumour suppressor function of merlin, we are interested in its other functions in the brain. We performed an extensive study comparing the localisation and expression of merlin and ezrin in human, rat and mouse brain, which showed that they are widely but differentially expressed. Both merlin and ezrin were present in embryonic mouse neurospheres, but in differentiated cells, merlin was predominantly found in neurons while ezrin was expressed in astrocytes. Subcellular analysis revealed that ezrin was specifically localised to filopodia of adherent neuronal progenitor cells and to fine filopodial structures in astrocytes, while merlin was detected in neuronal synaptic junctions. As a potential binding partner for merlin in the synaptic junction, we identified one of the four regulatory subunits of PKA, RIβ, which is mainly expressed in neurons of the hippocampus. Merlin serves as an A-kinase anchoring protein (AKAP) by binding RIβ through a conserved α-helical AKAP motif, and may regulate the cAMP/PKA pathway important in neuronal signalling. The widespread expression of merlin in the central nervous system (CNS) and a neuronal merlin interacting protein suggest yet unidentified functions for merlin in the brain.

In summary, we have described novel and partly unexpected properties for the cytoskeletal protein merlin, which suggest a role for merlin in the nucleus, in cell cycle regulation and perhaps in synaptic plasticity.

REVIEW OF THE LITERATURE

1. THE CELL DIVISION CYCLE

Cell division lies at the foundation of the biology of all organisms. Single cell organisms employ cell division as a means to propagate, while multi-cellular organisms require cellular proliferation during development for organ and tissue building. In the adult organism cell proliferation is important in maintaining organ and tissue homeostasis by replacing cells that are lost or die.

Cell division occurs by an ordered series of metabolic and morphogenic changes that are collectively called the cell cycle. The duration of the cell cycle varies between different cell types. In most mammalian cells it lasts between 10 and 30 hours. Before division the cell must faithfully replicate its genome, double its mass and duplicate its cytoplasmic organelles. The cell cycle can be divided into four distinct phases (Fig. 1). During the G1 phase the cell monitors its size and environment for growth promoting or suppressing signals. During S phase (synthesis) DNA is replicated and a copy of each chromosome is formed. Thereafter, in G2, the cell ensures that the DNA has been properly replicated and gets ready for division. During M phase (mitosis) the replicated chromosomes separate (nuclear division) and cells divide by cytokinesis (cytoplasmic division), as a result of which a pair of genetically identical daughter cells are formed. When cells cease to proliferate, they exit the cycle and enter a non-dividing, quiescent state, G0. To ensure proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one (Hartwell, 1989; Norbury, 1992 Sherr, 1994).

Cell cycle progression is mediated in part by the coordinated activity of cyclin-dependent kinases (Cdks), a group of serine/threonine kinases. Following binding to their regulatory subunits, cyclins, Cdks form active heterodimeric complexes, which are sequentially activated to coordinate cell cycle progression (Fig. 1). The activation of the cyclin D-cdk4,6 complex, triggered by the presence of mitotic growth factors, controls progression of G1 or the re-entry of resting G0 cells into the G1 phase of the cell cycle. Thereafter, the G1/S transition is controlled by the activation of the cyclin E-Cdk2 complex. The DNA replication in the S phase and the transition to the G2 phase is regulated by the activation of the cyclin A-Cdk2 and cyclin A-Cdk1

complexes. Finally, the main regulator of the G2/M transition is the cyclin B-Cdk1 complex. The balance in maintaining control of the cell cycle is essential. Errors in this rigorous regulation can lead to defective embryogenesis, chromosomal aberrations and cancer (Nurse, 1990; Hunt, 1991; Norbury, 1992; Sherr, 1994; Grana, 1995; Morgan, l997; Sherr, 2000).

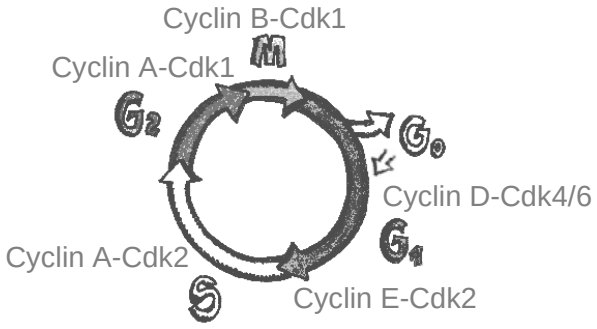

Figure 1. The mammalian cell cycle is divided into four phases; G1, S, G2 and M phase. G0 represents exit from the cell cycle. Cyclin-Cdk complexes that regulate the progression of the cell cycle are shown.

2. CANCER GENES

The process by which normal cells develop into malignant cancerous cells involves the successive acquisition of genetic alterations. Mutations that contribute to cancer development belong to two categories; gain-of-function mutations that activate oncogenes by selectively facilitating the growth of cancerous cells or their precursors and loss-of-function mutations that inactivate tumour suppressor genes thereby disabling the regular growth control of the cell (Vogelstein, 1988, 1993).

2.1 Oncogenes

Changes in proto-oncogenes that alter their normal cellular properties result in oncogenes that can cause cancer (Parada, 1982; Tabin, 1982; Varmus 1984). Oncogenic mutations can be manifested in a variety of ways such as protein overexpression, gene amplification, fusion proteins resulting from chromosomal translocations and gain-of-function mutations (Bishop, 1991). These mutations often affect genes involved in the regulation of the cell cycle, cell division control and differentiation.

2.2 Tumour suppressor genes

Loss-of-function mutations in tumour suppressor genes also enhance cancer susceptibility (Macleod, 2000). Most tumour suppressor gene abnormalities can be inherited as well as acquired. In the hereditary disease there is one inactivated copy of a tumour suppressor gene in the germ line and a somatic mutation causing the loss of the wild type allele, leading to the formation of a tumour. In sporadic tumours there must be two somatic mutations inactivating both copies of the tumour suppressor gene. Although inherited cancer syndromes are quite rare, the tumour suppressors behind these syndromes are frequently found to be somatically mutated in sporadic cancers. This points out the vital role these molecules play in growth control and differentiation. These syndromes are inherited in a dominant Mendelian fashion and are often associated with developmental defects and non-neoplastic phenotypes such as benign tumour formation (Knudson, 1971, 1993; Ponder, 2001).

Tumour suppressor proteins can be divided into gatekeepers and caretakers, based on their mechanism of action. Gatekeepers regulate the growth of tumours directly by inhibiting proliferation or promoting death; such as Rb, APC and NF1. Inactivation of these gatekeeper genes normally leads to a very specific tissue distribution of cancer. Caretakers, such as mismatch, nucleotide excision and double strand break repair genes, are involved in mediating DNA repair. Inactivation of caretaker genes promotes neoplasia indirectly and leads to genetic instability, which results in increased mutations of all genes (Kinzler, 1997). Some tumour suppressor gene defects, called landscapers, do not reside in the neoplastic cell population itself but changes the tumour microenvironment, which facilitates the induction, selection and expansion of neoplastic populations (Liotta, 2001). In the last years a few cytoskeletal proteins have been shown to function as tumour suppressors, such as Protein 4.1R, Protein 4.1B, DAL 1, and merlin, which all belong to the Band 4.1 superfamily of proteins (Ben-Ze'ev, 1997; Tran, 1999; Huang, 2001; Gutmann, 2001b; Sun, 2002).

3. THE ACTIN CYTOSKELETON

A complex, highly dynamic network of filamentous cytoskeletal proteins extends throughout the cytoplasm of eukaryotic cells. The cytoskeleton consists of three types of protein filaments; actin filaments, microtubules and intermediate filaments. Actin is

an essential, conserved protein of all eukaryotic cells that is necessary for a large number of cellular functions including cell motility and morphogenesis, vesicle trafficing, cytokinesis, establishment of cell polarity and intracellular signal transduction. In order for the actin cytoskeleton to play such diverse roles, actin filament assembly must be spatially and temporally controlled and its different functions regulated by a wide variety of actin binding proteins (reviewed in Ayscough, 1998).

3.1 The actin cytoskeleton in disease

Interactions between the actin cytoskeleton and cell membrane components allow the cell to coordinate cell movement and signalling, cell-cell and cell-extracellular matrix (ECM) interactions, to adopt a variety of cellular shapes and establish specialised membrane domains. The deregulation of these complexes and of proteins regulating the composition of the actin cytoskeleton are involved in a variety of diseases including infections, muscle disorders, cardiovascular and neurodegenerative diseases and cancer. An intact actin cytoskeleton is essential for the invasion, motility and dissemination of various bacteria, viruses and other parasites. The release of cytoskeletal elements into the extracellular space may contribute to allergies, coagulation defects and cystic fibrosis. The mechanical fragility of dystrophic muscle cells can be caused by defects in linking the actin network to the cell surface. Moreover, changes in the expression of actin, actin associated proteins and membrane-actin linkers participate in the abnormal growth properties of tumour cells, their ability to adhere to tissue and their increased ability to metastasize (reviewed in Janmey, 1995; Higley, 1997; Jordan, 1998; Towbin, 1998). As previously mentioned, cytoskeletal tumour suppressor proteins Band 4.1R, Band 4.1B, DAL-1 and merlin (Ben-Ze'ev, 1997; Tran, 1999; Gutmann, 2001b; Huang, 2001; Sun, 2002) have an inhibitory effect on cell proliferation, while studies have linked increased expression of ezrin, another Band 4.1 superfamily protein, with enhanced cell growth and metastasis (Geiger, 2000; Khanna, 2001; Nestl, 2001; Koon, 2004; Yu, 2004).

4. THE BAND 4.1 PROTEIN SUPERFAMILY

The Band 4.1 protein superfamily includes several membrane-associated signalling and cytoskeletal proteins; among them erythrocyte Band 4.1 protein, which connects

the integral membrane protein glycophorin to the subcortical actin-spectrin network (Conboy, 1986); talin, which links the fibronectin receptor to the actin cytoskeleton via vinculin and α-actinin (Bennett, 1989; Rees, 1990); protein-tyrosine phosphatases (Koyano, 1997; Neel, 1997); and the ERM protein family (Gould, 1989; Takeuchi, 1994a). A common domain in these proteins is the multifunctional protein- and lipid binding site called the FERM domain (Four-point-one, Ezrin, Radixin, Moesin) (Chishti, 1998) (Fig.2).

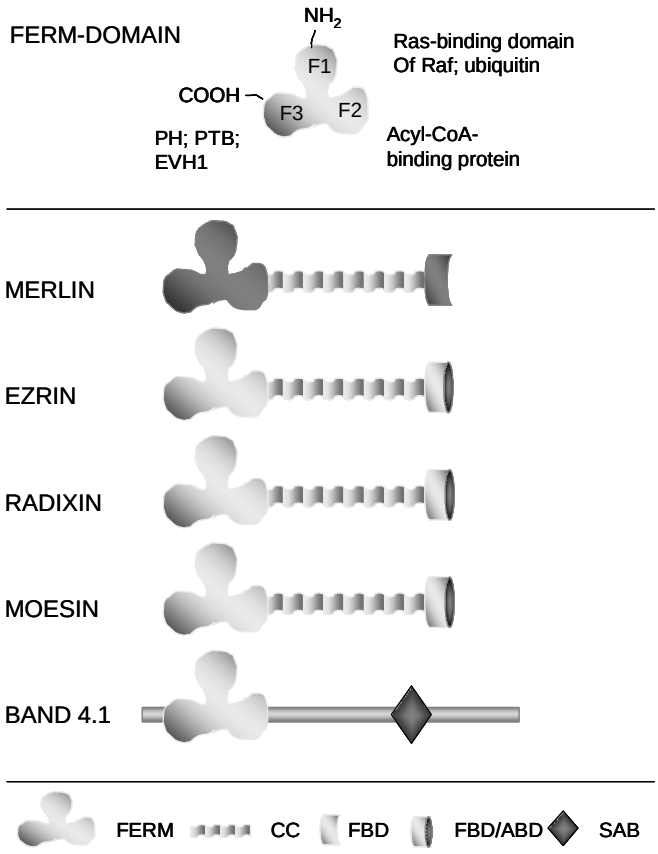

Figure 2. The clover leaf structure with the three subdomains of the FERM-domain; F1, F2 and F3, and the proteins/domains that share each subdomain fold. The ERM-proteins consist of an amino-terminal FERM-domain, an extended α-helical coiled-coiled domain and a charged carboxy-terminal domain with a FERM-binding domain (FBD) and in ERM proteins an actin binding domain (ABD). ERM-proteins belong to the Band 4.1 protein superfamily. The Band 4.1 protein has a FERM-domain and a 4.1 spectrin-actin binding domain (SAB).

4.1 The ERM protein family

The ERM proteins refer to three closely related proteins; ezrin (Gould, 1989; Turunen, 1989), radixin (Tsukita, 1989; Funayama, 1991) and moesin (Lankes, 1991), which share 75-80% amino acid sequence homology. The tumour suppressor merlin, which is encoded by the *NF2* gene (Rouleau, 1993; Trofatter, 1993), shares the overall

domain structure (Fig.2) and possesses some functional properties of ERM proteins. In this study we have worked with two of the ERM protein family members, ezrin and merlin.

4.1.1 The ERM proteins

Ezrin was the first characterised member of the ERM-family. It was cloned in 1989 and encodes for a 585 amino acid protein with a predicted molecular mass of 81 kDa (Gould, 1989; Turunen, 1989). The chromosomal localisation of the ezrin gene is 6q25-q26.

ERM proteins play a role in cell adhesion, signal transduction, membrane transport and in the regulation of cell growth (reviewed in Bretscher, 2002). They participate in the assembly of membrane- and cytoskeleton-associated protein complexes (Edwards, 1994; Henry, 1995; Martin, 1995; Helander, 1996) and are located in various actin containing cell surface structures, including microvilli, filopodia and membrane ruffles where they colocalise with actin (Bretscher, 1983; Pakkanen, 1988; Sato, 1992; Berryman, 1993; Franck, 1993; Winckler, 1994). These functions are partially redundant within the ERM family members. Experiments where the expression of all three ERM proteins was blocked by antisense oligonucleotides indicated that ERM proteins have at least partially overlapping functions (Takeuchi, 1994b). In *Drosophila* only a single ERM gene, D-moesin, has been found, with closest homology to human moesin (McCartney, 1996). Although ERM proteins are coexpressed in most cultured cells, they have a tissue specific expression pattern in the body. Ezrin is predominantly expressed in the intestine, stomach, lung and kidney; moesin in lung and spleen; and radixin in liver and intestine (Tsukita, 1989; Funayama, 1991). Ezrin is found in epithelial and mesothelial cells, and moesin in endothelial and hematopoietic cells while hepatocytes express only radixin (Berryman, 1993; Amieva, 1994; Nakamura, 1995; Schwartz-Albiez, 1995). Some unique functions are known for the different ERM proteins. Ezrin anchors podocalyxin in specialised podocyte foot processes on the apical surface of kidney epithelial cells (Takeda, 2001). In epithelial cells of the kidney proximal tubule, ezrin is linked to the ion transporter NHE-3 by EBP50/NHE-RF. Ezrin in turn links the complex to the actin cytoskeleton and recruits the kinase by binding the PKA regulatory subunit RIIα (Weinman, 1997; Weinman, 2000; Bagorda,

2002). Moesin is thought to restrict CD43 to the distal pole complex that forms opposite to the immunological synapse in T-cells (Cullinan, 2002). Radixin localises to the apical microvilli of bile canaculi cells in the liver and its loss in mice leads to abnormal microvillus architecture and loss of the anionic transporter MRP2 from the apical surface of the cells (Kikuchi, 2002). The ezrin knock-out mouse showed that ezrin is essential for epithelial organisation and villus morphogenesis in the developing intestine (Saotome, 2004). The moesin knock-out mouse, however, does not have any discernable phenotype (Doi, 1999).

4.1.1.1 Structure of the ERM proteins

The globular FERM domain in ERM proteins is composed of three subdomains; F1, F2 and F3, which are arranged as a clover leaf-like structure (Fig. 2). Although the subdomains have no sequence homology to other protein domains their structures are homologous to previously described folds; F1 to ubiquitin and the Ras-binding domain of Raf, F2 to Acyl-CoA binding protein and F3 to a domain variously described as the pleckstrin homology (PH), phosphotyrosine-binding domain (PTB) or Enabled/VASP-1 domain (EVH1), which binds peptide and lipid ligands in signalling and cytoskeletal proteins (Pearson, 2000). The highly conserved FERM domain is followed by an α-helical coiled-coil domain, which is less conserved between the ERM proteins. In addition, mammalian ezrin and radixin have a region rich in prolines at the end of the α-helical region, the function of which is unknown. The charged carboxy-terminal domain contains the C-ERMAD (C-ERM association domain) also known as the FBD (FERM binding domain) and an actin binding domain (Fig. 2). The C-ERMAD can extend across the FERM-domain surface forming a "head-to-tail" closed molecule, potentially masking recognition sites for other proteins. When this interaction is weakened, e.g. by phosphorylation, the molecule adopts a more extended structure which exposes interaction domains for other proteins (Pearson, 2000).

4.1.2 The NF2 protein, merlin

Neurofibromatosis 2 (NF2) is a tumour suppressor syndrome that predisposes to tumours of the nervous system. The *NF2*-gene codes for a protein called merlin (for moesin-ezrin-radixin-like protein) or schwannomin. It was identified in 1993 and

localised to chromosome 22q12. Merlin is composed of 18 exons, with two major alternatively spliced NF2 variants expressed *in vivo*. Isoform I, lacking exon 16, encodes for a 595 amino acid protein with a predicted molecular mass of 66 kDa (Rouleau, 1993; Trofatter, 1993). Isoform II contains exon 16, which inserts 11 unique carboxy-terminal amino acids followed by a termination codon and prevents translation of exon 17 (Bianchi, 1994; Haase, 1994; Hara, 1994; Gutmann, 1995).

Merlin, like ERM proteins, is a cytoskeleton-associated membrane-organising protein and thus a unique type of tumour suppressor. In cultured cells, merlin is localised underneath the plasma membrane in a pattern typical of ERM proteins and is mainly seen in membrane ruffles and filopodia (Gonzalez-Agosti, 1996; Sainio, 1997). Transfected and endogenous merlin partly colocalise with ezrin, although in cells with a poorly developed actin cytoskeleton, merlin replaces ezrin in filopodia and ruffling edges (Sainio, 1997).

Merlin is widely expressed but the expression level in most tissues appears low. Merlin mRNA is present in heart, brain, spleen, lung, liver, skeletal muscle and kidney (Hara, 1994; Rouleau, 1993). The merlin protein is expressed in lung, intestine, muscle, lens, spleen, kidney, spinal cord and brain (Claudio, 1995; den Bakker, 1995, 1999), and is highly abundant during mouse fetal development in extraembryonic tissues, heart and the nervous and skeletal systems (Huynh, 1996b). However, the cell type distrbution and subcellular localisation of merlin in these tissues is poorly understood.

Merlin is critical at early stages of development and plays a surprisingly broad role across many different cell types. Homozygous *Nf2* mutant murine embryos ($Nf2^{-/-}$) fail in development at day 7 of gestation caused by a collapsed extraembryonic region and the absence of organised extraembryonic ectoderm (McClatchey, 1997). Heterozygous *Nf2* mutant mice ($Nf2^{+/-}$) spontaneously develop various tumours including osteosarcomas and hepatocellular carcinomas that show loss of the *Nf2* wt allele. The unusually metastatic behaviour of these tumours indicates that *Nf2* loss also facilitates tumour metastasis (McClatchey, 1998). The $Nf2^{+/-}$ tumour phenotype can be dramatically accelerated in the presence of a targeted mutation in another tumour suppressor gene, p53 (McClatchey, 1998). Although $Nf2^{+/-}$ mice do not spontaneously develop schwannomas or meningiomas, a conditional deletion of *Nf2* in Schwann and arachnoidal cells leads to hyperplasia and development of

schwannomas and meningeal neoplasias, respectively, indicating that loss of the *Nf2* wt allele is rate limiting for the development of these tumours in mice (Giovannini, 2000; Kalamarides, 2002). In *Drosophila* a merlin ortholog has been found, called D-merlin (McCartney, 1996). D-merlin functions in axis speicification during oocytes, and D-merlin mutants show defects in nuclear migration and mRNA localisation in the oocyte (MacDougall, 2001).

4.1.3 Ezrin and merlin in neoplasia

Ezrin expression is altered in several tumours (Böhling, 1996; Mäkitie, 2001). Increased ezrin expression has been noticed in invasive cells (Hiscox, 1999; Ohtani, 1999; Wick, 2001) and has been linked to enhanced cell growth, tumour metastasis and poor prognosis of malignant tumours (Akisawa, 1999; Geiger, 2001; Khanna, 2001, 2004; Nestl, 2001; Koon, 2004; Tynninen, 2004; Yu, 2004).

Mutations in the *NF2*-gene have been found in both sporadic and NF2 associated schwannomas and meningiomas. Biallelic *NF2*-gene inactivation has also been demonstrated in mesotheliomas, melanomas, breast and colon carcinomas and lung and ovarian tumours (Arakawa, 1994; Lee, 1999; Pineau, 2003). Although NF2 is quite rare, sporadic schwannomas and meningiomas are among the most common nervous system tumours in humans. They are generally benign but may be intractable because of their location, recurrence and in NF2 patients, their multiplicity (Louis, 1995; Baser 2003).

4.1.3.1 Neurofibromatosis 2, NF2

NF2 is an autosomal dominantly inherited disease that affects around 1 in 40.000-87.000 individuals (Evans, 1992; Antinheimo, 2000) with high penetrance (>95%) and an onset at puberty (Evans, 1992). Inactivation of the *NF2*-gene predisposes to tumours of the nervous system. Early symptoms of NF2 are dysfunction of the acoustic and vestibular nerves leading to hearing loss, tinnitus and problems with balance. Other symptoms relate to the location of the tumour and include seizures, weakness, muscle wasting and root pain. The hallmark of NF2 is the development of bilateral vestibular schwannomas. The other main tumour features are schwannomas of other cranial, spinal and peripheral nerves, meningiomas, both intracranial and intraspinal, and some low grade CNS malignancies; ependymomas and gliomas. NF2

patients may also be affected by peripheral neuropathy, juvenile lens opacities and retinal hamartomas (Gutmann, 1997; Evans, 2000). The disease phenotype varies from mild (late onset and slowly growing vestibular schwannomas, but few other tumours) to an aggressive form (early onset and multiple rapidly growing tumours) (Baser, 2003).

The majority of inherited *NF2* gene mutations of schwannomas are small deletions and insertions that create splice-junction mutations, frame shifts and non-sense mutations. Inactivating mutations have been detected predominantly in exons 1-15 without mutational hot spots (Lutchman, 1996). There is a partial correlation between the type of merlin mutation and the severity of the disease. Missense mutations correlate with mild disease while splicing errors, frame shifts and protein truncations, which result in loss of function, cause a more severe disease (Ruttledge, 1996). At least some of the mutant deletion products of merlin observed in NF2 patients are efficiently degraded by the ubiquitin-proteasome pathway (Gautreau, 2002).

5. MOLECULAR INTERACTIONS AND FUNCTIONS OF MERLIN AND EZRIN

Regulated attachment of membrane proteins to the actin cytoskeleton is essential for many fundamental processes in the cell. By assembling and stabilising complexes of membrane- and cytoskeleton-associated proteins, ERM proteins and merlin participate in the formation and organisation of specific cell surface domains.

5.1 Association with the cytoskeleton

The idea that ezrin functions as a cytoskeleton-plasma membrane linker is supported by overexpression of ezrin domains in cultured cells. When amino- and carboxy-terminal halves of ezrin were transfected into cultured fibroblasts, they were targeted to plasma membranes and actin-filament bundles, respectively (Algrain, 1993). Actin binding was found to involve the last 34 amino acids in the carboxy-terminal domain (Turunen, 1994), while the RRRK motif at the end of the FERM-domain, is critical for G-actin binding and morphogenic activity (Pestonjamasp, 1995; Martin, 1997; Roy, 1997). In addition, ezrin binds the cytoskeletal protein palladin (Mykkänen, 2001).

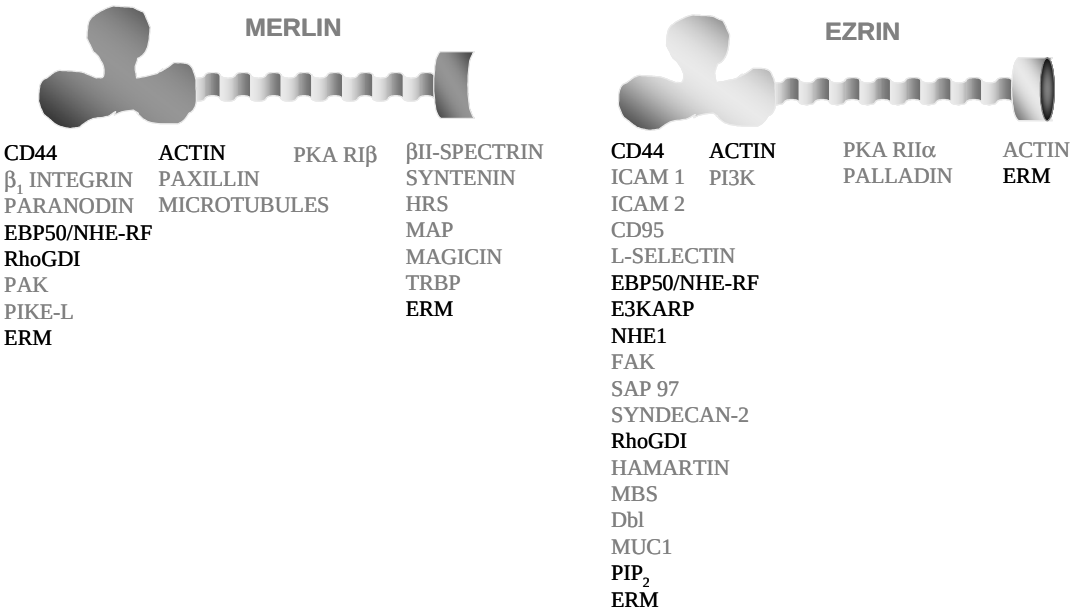

Figure 3. A list of molecules interacting with merlin and/or ezrin. Molecules in black are known to interact with both merlin and ezrin, while proteins in grey interact only with one of the two, or has not been tested for both proteins. The location of the molecule's name under merlin or ezrin indicates the approximate position of the interaction site.

Merlin, like ezrin, is localised to cortical cytoskeletal structures and is partly retained in the detergent-insoluble fraction of cell lysates (den Bakker, 1995; Gonzalez-Agosti, 1996; Sainio, 1997). Merlin does not, however, have an actin binding domain in the carboxy-terminus, but instead binds actin and microtubules with its amino-terminus (Xu, 1998; Stokowski, 2000; Brault, 2001; James, 2001; Muranen, in preparation). In addition, merlin binds cytoskeletal proteins βII spectrin (Scoles, 1998) and paxillin (Fernandez-Valle, 2002).

Changes in cell morphology are regulated in a complex manner by both the amino- and carboxy-terminal domain of ERM proteins (Henry, 1995; Martin, 1995, 1997). Transfection of cells with full length ERM proteins gives no response in cell morphology but transfection with truncated proteins result in drastic changes by induction of cellular extensions (Edwards, 1994; Henry, 1995; Martin, 1995, 1997). In contrast, overexpression of full-length merlin can induce morphogenic changes, such as cell surface protrusions and elongation of the cell body; however, the changes are more drastic with carboxy-terminally truncated proteins (Sainio, 1997; Laulajainen, in preparation).

5.2 Association with the plasma membrane

Ezrin and merlin bind plasma membrane components directly by binding cytoplasmic tails of transmembrane proteins (Fig. 3, 4). The FERM domain of ezrin binds adhesion molecules CD44 (Tsukita, 1994; Yonemura, 1998), ICAM-1 and ICAM-2 (Helander, 1996; Heiska, 1998; Yonemura, 1998). Both CD44 and ICAMs have a positively charged amino acid cluster in the juxtamembrane cytoplasmic domain which is responsible for binding to ezrin (Heiska, 1998; Yonemura, 1998). Furthermore, ezrin binds adhesion molecule L-Selectin (Ivetic, 2002), the tumour necrosis factor (TNF) family receptor CD95 (Parlato, 2000), a member of the mucine family MUC1 (Bennett, 2001) and the ion transporter NHE1 (Denker, 2000). Merlin binds transmembrane proteins CD44 (Sainio, 1997; Morrison, 2001), β1-integrin (Obremski, 1998) and paranodin (Denisenko-Nehrbass, 2003).

Ezrin and merlin can also associate with plasma membrane components indirectly by binding scaffolding proteins that often contain PDZ-domains (Fig. 3, 4). Ezrin binds EBP50/NHE-RF (Reczek, 1997), E3KARP (Yun, 1998), synaptic-associated protein SAP 97 (Bonilha, 2001) and the heparan sulphate proteoglycan syndecan-2 (Granes, 2000). Merlin binds EBP50/NHE-RF (Murthy, 1998) and syntenin (Jannatipour, 2001).

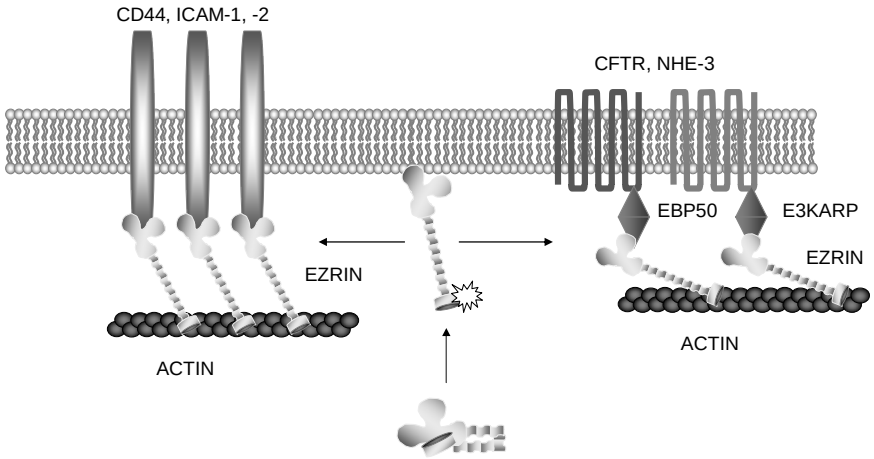

Figure 4. A model of ezrin plasma membrane-cytoskeleton interaction. Ezrin is present as a closed and inactive molecule in the cytoplasm and needs to be activated to make binding sites accessible. Activation involves phosphorylation and phospholipids. Ezrin can bind transmembrane receptors indirectly through the interaction with PDZ-containing proteins EBP50 and E3KARP. The carboxy-terminus of ezrin contains an actin-binding site.

5.3 Cell adhesion and cell-cell communication

ERM proteins and merlin colocalise with, and bind to, several adhesion molecules in cultured cells and play important roles in maintaining cell adhesion structures, in the regulation of cell-ECM interactions and in intercellular communication. When the expression of all three ERM proteins in cultured cells was blocked by antisense oligonucleotides, microvilli disappeared and cellular adhesion was disrupted (Takeuchi, 1994b). In addition, loss of D-moesin or expression of dominant-negative ezrin in mammalian cells disrupts cell-cell communication (Crepaldi, 1997; Speck, 2003). The ezrin-CD44 interaction is needed for cell motility (Legg, 2002) while the ezrin-ICAM-2 interaction is important for the recruitment of ICAM-2 to the uropod of natural killer cell targets (Helander, 1996). In addition to the ezrin-binding adhesion molecules mentioned before, ezrin binds the tuberous sclerosis complex 1 (TSC1) tumour suppressor protein hamartin, which regulates cell adhesion through ERM proteins and Rho (Lamb, 2000). Recently, ezrin was shown to control the formation of adherens junctions through its activation of Rac1, which perturbs the localisation of E-cadherin to the plasma membrane (Pujuguet, 2003). Ezrin also binds focal adhesion kinase (FAK) and promotes its phosphorylation, but this effect does not require cell adhesion (Poullet, 2001).

Antisense oligonucleotides to merlin result in the rounding up of cells and their detachment from the substratum (Huynh, 1996a). The merlin-CD44 complex in Schwann cells participates in contact inhibition of proliferation while the merlin-CD44-ERM-Met-complex promotes mitotic signalling (Orian-Rousseau, 2002). Furthermore, merlin's association with the paxillin-integrinβ1-ErbB2 complex is important for cell-ECM interactions (Fernandez-Valle, 2002). In *Nf2* wt cells, merlin colocalises and associates with adherens junctions, while *Nf2* deficiency leads to the loss of contact inhibition and the absence of adherens junctions. Although core adherens junction complexes assemble at the membrane of *Nf2* deficient cells, they remain diffusely localised (Lallemand, 2003). This indicates that merlin is involved in the assembly or stabilisation of the mature adherens junction, perhaps through interactions with the actin cytoskeleton. The loss of adherens junctions can lead to tumour development and metastasis *in vivo* (Nollet, 1999).

5.4 Cell proliferation

Merlin and ezrin appear to have opposite effects on cell proliferation. Transfection of ezrin into NIH3T3 fibroblasts promotes cell proliferation via loss of contact inhibition (Kaul, 1996), and fos-induced morphological transformation increases the level of ezrin (Jooss, 1995). Ezrin expression is altered in several types of tumours (Böhling, 1996; Mäkitie, 2001) and recent studies have linked increased ezrin expression with enhanced cell growth and poor prognosis of malignant tumours (Akisawa, 1999; Geiger, 2000; Khanna, 2001; Nestl, 2001; 2004; Koon, 2004; Tynninen, 2004; Yu, 2004). In addition, ezrin associates with the regulatory subunit p85 of phosphatidylinositol 3-kinase (PI3-kinase), participating in cell survival signalling through the PI3K/Akt pathway (Gautreau, 1999).

On the other hand, overexpression of merlin isoform I inhibits cell proliferation in rat schwannoma cells and NIH 3T3 cells, and suppresses a v-Ha-Ras-induced malignant phenotype (Tikoo, 1994; Lutchman, 1995; Sherman, 1997), while isoform II or truncated constructs of isoform I fail to influence schwannoma growth (Sherman, 1997). Transfection of merlin into human primary schwannoma cells reduces cell proliferation and promotes the accumulation of cells in G0/G1 (Schulze, 2002). Conversely, suppression of merlin in tumour cells induces proliferation and a targeted disruption of the *Nf2* gene results in increased cell proliferation and tumour formation (reviewed in McClatchey, 2001)

By assembling and stabilising complexes at the plasma membrane, merlin and ezrin co-ordinately control cell-ECM and growth factor receptor signalling and, hence, control both tumour growth and invasion. According to a model by Morrison et al. merlin controls contact-dependent inhibition of cell proliferation through an interaction with CD44. At low cell density a complex is formed with CD44, ezrin and phosphorylated or hyperphosphorylated merlin. Under these conditions, the tumour suppressor function of merlin is inactivated and the complex promotes mitogenic signalling, e.g. through the Ras pathway. At high cell density, CD44 interacts with the growth suppressive, hypophosphorylated form of merlin, ezrin is not present in the complex, and cell proliferation is blocked (Morrison, 2001). Other pathways may affect this regulation. CD44 is present in a complex together with growth factor receptors such as Met and the epidermal growth factor receptor (EGFR) family

member ErbB2 and seems to facilitate signalling via these receptors (Bourguignon, 1997; Orian-Rousseau, 2002).

Growth factor receptors can be regulated by their association with ERM protein- and merlin-organised membrane complexes, but it is not yet known whether ERM proteins and merlin directly control growth factor receptor signalling or transport. If it is the case, loss of merlin could lead to defective growth factor receptor turnover and persistent mitogenic signalling. One of the most potent stimuli for Schwann cell proliferation and motility is HGF (Krasnoselsky, 1994). Merlin interacts with the HGF regulated tyrosine kinase substrate HRS (Scoles, 2000; Gutmann, 2001a), which is present at the cytoplasmic surface of early endosomes and may regulate growth factor receptor internalisation (Hayakawa, 2000; Clague, 2001). HRS is a homologue of the yeast protein Vps27, that controls lysosomal sorting of several cell-surface molecules, including EGFR (Stahl, 2002). Regulated overexpression of HRS in rat schwannoma cells has the same consequences as merlin overexpression (Gutmann, 2001a), raising the possibility that HRS participates in merlin growth suppression.

Both ERM proteins and merlin can interact with EBP50, which in turn interacts with platelet derived growth factor receptor (PDGFR) (Maudsley, 2000). Merlin can promote PDGFR degradation and thus inhibit extracellular receptor-linked kinase (ERK) in a schwannoma cell line (Fraenzer, 2003). By inhibiting the activation of the Ras–ERK pathway and ERKs downstream substrate Elk, merlin suppresses SRE-dependent transcription, which is important for the progression of the cell cycle in G1 (Lim, 2003). Results from *Drosophila* demonstrate that loss of D-merlin function during larvae development results in a 2-3 fold increase of proliferation. and that merlin may function antagonistically with the EGFR pathway (LaJeunesse, 2001). In addition, D-merlin interacts with *expanded*, a tumour suppressor and member of the Protein 4.1 family, and together they regulate cell proliferation and differentiation (McCartney, 2000).

Merlin is a negative regulator of Rac (Shaw, 2001). The Rac/JNK pathway is upregulated in *Nf2* deficient fibroblasts and merlin negative primary human schwannoma cells (Shaw, 2001; Kaempchen, 2003). It has previously been shown that Rac activity is needed for malignant transformation by Ras and that activated forms of Rac have oncogenic properties (Symons, 1995). Increased Rac activity is also

associated with increased cellular motility and might therefore affect metastatic potential (Morrison, 2001; Crowe, 2004).

Several indirect links between merlin and cell growth regulation have been reported. Merlin may be a positive regulator of p53 by inhibiting the Mdm2-mediated degradation of p53 (Kim, 2004). Moreover, merlin represses NF-κB activation (Kim, 2002a), suppresses PI3-kinase activity by binding to the PI3-kinase enhancer PIKE-L (Rong, 2004b) and inhibits the tumourigenesis induced by the merlin binding protein TRBP (transactivation-responsive RNA binding protein) (Lee, 2004b). Merlin expression is upregulated in mammalian fibroblasts arrested in G1 in response to actin inhibition, which is dependent on the retinoblastoma protein (Rb) (Lohez, 2003). Furthermore, merlin inhibits abnormal cell proliferation that is activated via Ras by repressing Rb phosphorylation, blocking the increase of cyclin D1 protein level and inhibiting the activation of AP-1- and E2F-1-dependent transcription in NIH3T3 cells (Kim, 2002b). The merlin binding protein MAP decreases the AP-1-dependent promoter activity (Lee, 2004a), and magicin, a protein that interacts with both merlin and Grb2, links merlin to Ras signalling (Wiederhold, 2004).

6. REGULATION OF MERLIN AND EZRIN

Even if many functions are partially redundant, differences exist in the functional properties of ERM proteins and merlin; their phosphorylation patterns, regulation, and response to growth factors and proteases.

6.1 Phosphorylation

ERM proteins are rapidly phosphorylated on tyrosines 145 and 353 following EGF or HGF stimulation (Bretscher, 1989; Krieg, 1992; Fazioli, 1993; Franck, 1993; Jiang, 1995; Crepaldi, 1997), on tyrosine 145 after activation of Lck tyrosine kinase (Autero, 2003) and on tyrosines 145 and 477 after Src activation (Heiska, 2004; Srivastava, 2005). Substitution of tyrosine 145 and 353 with phenylalanine decreases the morphogenic and motility response of epithelial cells to HGF (Crepaldi, 1997). Phosphorylation of ezrin on tyrosine 353 is also important in protecting cells against apoptosis by activating the PI3K/Akt-pathway (Gautreau, 1999) and phosphorylation on tyrosine 477 by Src induces a phosphospecific association between ezrin and kelch-repeat protein family member, KBTBD2 (Heiska, 2004).

At least three kinases phosphorylate the conserved carboxy-terminal threonine in ERM-proteins (ezrin T567); Rho kinase (Matsui, 1998; Tran, 2000), PKCα (Ng, 2001), and PKCθ (Pietromonaco, 1998; Simons, 1998). Phosphorylation of ezrin on threonine 567 reduces the affinity of the C-ERMAD for the FERM-domain and weakens the "head-to-tail" interaction. Thereby other binding sites become accessible, such as those for CD44 and EBP-50 (Nakamura, 1995; Hirao, 1996; Matsui, 1998; Simons, 1998). The phosphorylation also results in a redistribution of ERM proteins to cell surface structures such as microvilli (Kotani, 1997; Lamb, 1997; Oshiro, 1998; Hayashi, 1999; Yonemura, 2002). Recently, serine 66 of ezrin was shown to be phosphorylated by PKA in response to histamine stimulation in parietal cells (Zhou, 2003). During apoptosis, dephosphorylation of ERM proteins results in their translocation from the plasma membrane and in loss of microvilli (Kondo, 1997). Furthermore, ezrin binds the myosin binding subunit of myosin phosphatase (MBS) (Fukata, 1998).

Merlin is phosphorylated on both serine and threonine residues but tyrosine phosphorylation has not been detected (Shaw, 1998, 2001). The phosphorylation status of merlin in cell culture models varies in response to growth conditions. At low cell density merlin is phosphorylated, whereas high cell density, serum starvation or loss of adhesion results in increased merlin expression and dephosphorylation (Shaw, 1998). At least three differently phosphorylated forms of merlin are present in cell lysates; hypophosphorylated, phosphorylated and hyperphosphorylated. Hypophosphorylated merlin is insoluble and enriched in cell culture under inhibitory growth conditions and is probably active as a growth suppressing molecule (Shaw, 1998).

p21 activating kinase (PAK) phosphorylates merlin on serine 518 (Kissil, 2002; Xiao, 2002), which weakens the self association, increases solubility and is believed to inactivate the growth suppressing activity of merlin (Shaw, 1998, 2001). It also impairs the ability of merlin to bind CD44 and HRS (Rong, 2004a). Interestingly, cAMP-dependent protein kinase, PKA, phosphorylates the same residue, serine 518, and an additional amino-terminal residue, which has not been mapped (Alfthan, 2004). PKA phosphorylation of serine 518 enhances the association between merlin and ezrin (Alfthan, 2004). Merlin might be regulated by signals from two or more different signal transduction pathways since both PKA and PAK, although activated

in response to different stimuli, phosphorylate the same site. Interestingly, a cross-talk between PKA and PAK has been demonstrated in some cell types, which regulates MAPK signalling and cytoskeletal integrity (Howe, 2000a).

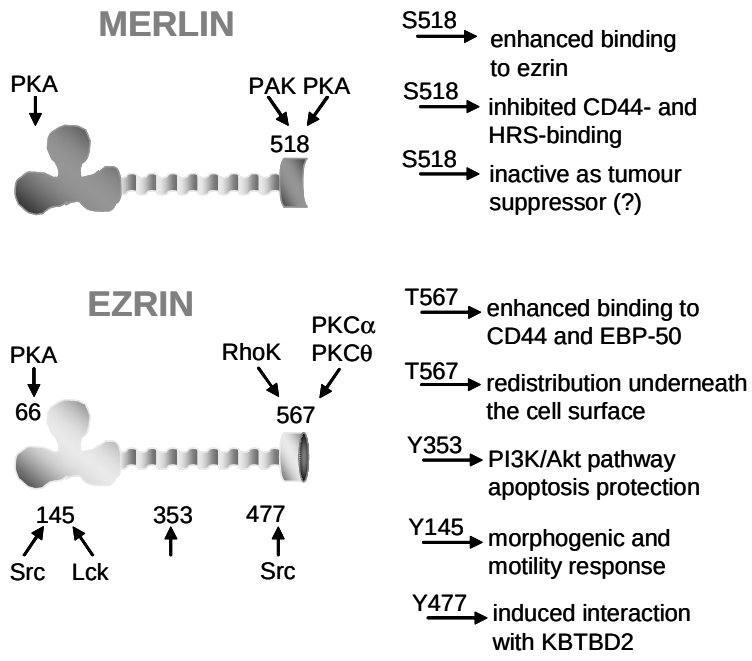

Figure 5. Phosphorylation of merlin and ezrin. Known merlin and ezrin kinases and their target residues. Known consequences of the phosphorylation are listed.

The cyclic AMP (cAMP)-PKA pathway elicits a wide array of metabolic and functional processes including cell growth and differentiation, actin cytoskeleton rearrangements, transcription and ion channel conductivity. Activation of the cAMP/PKA pathway promotes cell growth and cell cycle progression in Schwann cells (Kim, 1997) and is required for myelin formation (Howe, 2000b). In its inactive form PKA is a tetramer that consists of two regulatory and two catalytic subunits. There are three catalytic subunit isoforms (Cα, Cβ, Cγ) and four regulatory subunit isoforms (RIα, RIβ, RIIα, RIIβ), the expression of which varies among cell types and tissues. Cyclic AMP activates PKA by binding to its regulatory subunits, causing the tetrameric complex to disassociate, thus activating the kinase (Skalhegg, 2000). Although PKA has broad substrate specificity, it is able to selectively phosphorylate individual substrates in response to distinct stimuli. Selectivity is achieved by isoform multiplicity, by differential subunit expression patterns and through interactions with

A-kinase anchoring proteins (AKAPs). AKAPs bind PKA via the regulatory subunit and direct the kinase activity towards specific substrates at distinct intracellular locations. By binding additional signalling molecules, AKAPs may coordinate multiple signal transduction pathways (Colledge, 1999; Edwards, 2000). Ezrin serves as an AKAP in gastric parietal cells by binding RIIα, thus anchoring the kinase to critical regions in the canalicular target membranes (Dransfield, 1997; Zhou, 2003).

6.2 RHO GTPases

Both ERM proteins and merlin are regulated by members of the Rho family of GTPases. Rho GTPases are members of the Ras superfamily of monomeric GTP-binding proteins. The best characterised members are Rho, Rac and Cdc42, which control actin cytoskeleton remodelling, cell proliferation and survival, gene expression and cell morphology. Rho GTPases cycle between an inactive GDP-bound form and an active GTP-bound state, which can interact with effector molecules to initiate a downstream response. GTP hydrolysis returns the proteins to the GDP-bound state. There are numerous proteins that positively or negatively regulate the cycling between the GTP- and GDP-bound states. The guanosine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP while GTPase activating proteins (GAPs) increase the intrinsic rate of GTP hydrolysis of Rho GTPases, thus facilitating the inactivation process. Rho GTPases interact with membranes via post-translational lipid modifications, but can also be sequestered to the cytoplasm in their inactivated state by Rho GDP disassociation inhibitor (RhoGDI), which inhibits the exchange of GDP to GTP (reviewed in Etienne-Manneville, 2002).

As mentioned above, Rho induces the phosphorylation of the conserved carboxy-terminal threonine in ERM proteins by Rho kinase (Matsui, 1998; Tran, 2000). ERM proteins also positively regulate Rho activity (Mackay, 1997; Lamb, 2000). ERM-proteins bind Rho-GDI and release inactive Rho from Rho-GDI, which results in activation of the Rho pathway (Takahashi, 1997; Maeda, 1999). In addition, ERM proteins interact directly with Dbl, a positive regulator of Rho (Takahashi, 1998). Genetic analysis in *Drosophila*, however, showed that D-moesin deficiency leads to increased RhoA activity (Speck, 2003). The fact that ezrin functions both upstream and downstream of Rho implies that there could be a feed back loop for Rho-pathway autoregulation.

In contrast to ezrin, phosphorylation of merlin is induced by activated forms of Rac and Cdc42, but not by activated Rho. As mentioned above, PAK, a downstream target of both Rac and Cdc42, phosphorylates merlin (Kissil, 2002; Xiao, 2002). Merlin also interacts directly with the Cdc42/Rac binding domain of PAK1 and inhibits PAK1 activity (Kissil, 2003). Overexpression of merlin blocks Rac1 induced signalling and transformation, whereas *Nf2*-deficient fibroblasts have excessive Rac1 activity (Shaw, 2001). These data suggest that merlin is regulated by, and can serve as a negative regulator of the Rac/Cdc42 signalling pathway. Merlin can also interact with Rho-GDI (Maeda, 1999) but it is not known if it interacts with Dbl.

6.3 Homotypic and heterotypic binding

Homotypic and heterotypic associations of ERM proteins have been described as ways to regulate protein function. The binding domain in the amino-terminus of ezrin, N-ERMAD, has been mapped to amino acids 1-296, which corresponds to the domain called the FERM-domain. The carboxy-terminal binding domain, C-ERMAD, has been mapped to amino acids 479-585 (Gary, 1993, 1995; Magendantz, 1995). When the C-ERMAD binds to the FERM-domain, the carboxy-terminal residues adopt an extended structure that binds to and covers an extensive area of the FERM-domain surface (Pearson, 2000).

Intramolecular or intermolecular associations regulate ERM protein function (Gary, 1995; Henry, 1995; Magendantz, 1995; Martin, 1995). The folded state of the monomers and ERM protein oligomers represent the inactive form of the protein. Signals disrupting these interactions expose functional sites, allowing them to bind other proteins. The ezrin FERM-domain binding sites, at least for EBP50, Rho-GDI, CD44 and F-actin, are masked when associated with the C-ERMAD (Takahashi, 1997; Reczek, 1998; Hirao, 1996; Simons, 1998). Additionally, the morphogenic activity of ezrin appears to be masked by inter- and intramolecular interactions (Martin, 1995).

6.4 Other mechanisms of regulation

Phosphoinositides participate in the activation of ERM proteins (Yonemura, 2002). ERM proteins and merlin have a phosphatidylinositol-4,5-biphospahte (PIP_2) binding site in the FERM-domain (Niggli, 1995; Gonzalez-Agosti, 1999). PIP_2 can regulate

the binding of ezrin to ICAM-1, ICAM-2 (Heiska, 1998) and CD44 (Hirao, 1996). It binds between F2 and F3 in the FERM domain and may induce a conformational change that reduces the affinity of the C-ERMAD for the free FERM-domain (Hamada, 2000). Local production of PIP_2 is thought to recruit ERM-proteins to the plasma membrane. This places them in a location where they can be phosphorylated and, thereby, activated to bind transmembrane proteins, scaffolding molecules and actin and participate in signalling pathways (Fig. 4).

Furthermore, ERM and merlin protein levels and function are regulated by ubiquitin-proteasome or calpain mediated proteolysis (Kimura, 1998; Shcherbina, 1999; Gautreau, 2002; Kaneko, 2001).

AIMS OF THE STUDY

When this study was initiated, no cellular functions or interaction partners for merlin were known. Merlin was viewed as a structural protein associated with the plasma membrane-cytoskeleton interface and no association with cell cycle control had been reported. Therefore, merlin was regarded as a unique type of tumour suppressor, with growth inhibitory mechanisms linked to cell surface organisation and adhesion. The fact that merlin and its closest homologue, ezrin, have opposite effect on proliferation was of particular interest. Our aim was to elucidate the biological function of merlin by identifying binding partners specific for merlin or ezrin, and by analysing pathways that differentially regulate their functions. This could provide us with clues on how merlin exerts its growth regulatory activity.

Initial specific aims:

1) To determine whether merlin is a functional member of the ERM protein family.

2) To determine whether unique molecular interactions can explain the opposite effect of merlin and ezrin on cell proliferation.

As a result of these studies, we identified two molecules that specifically interact with merlin; the cell cycle regulator HEI10 and the neuron-specific PKA regulatory subunit, RIβ. We also noticed that the subcellular localisation of merlin was more versatile than previously thought.

Subsequent specific aims:

3) To analyse the mechanisms, regulation and functional consequences of the observed nucleo-cytoplasmic shuttling of merlin.

4) To study the role of the intercation between merlin and HEI10, and its potential involvement in the regulation of cell proliferation.

5) To characterise the expression pattern of merlin, ezrin and RIβ in the CNS.

6) To characterise the merlin-RIβ interaction and the role of merlin in neurons of the CNS.

METHODS

The materials and methods are described in detail in the original publications, which are referred to here using Roman numerals.

METHODS	USED IN STUDY
Production of recombinant DNA constructs	I, II, III, V
Cell culture and cell transfections	I, II, III, IV, V
Primary culture of rat embryo hippocampal cells	IV, V
Primary culture of mouse progenitor cells	IV
Primary culture of Schwann and schwannoma cells	III
Cell cycle synchronisation	II, III
Subcellular fractionation of nuclei	II
Flow and laser scanning cytometry	II, III
Immunofluorescence and laser scanning confocal microscopy	I, II, III, IV, V
Immunohistochemistry	IV, V
Coimmunoprecipitations	I, III, V
cAMP-pull downs and coimmunoprecipitation from rat brain	V
Fractionation of synaptosomes from rat brain	IV
Chemical cross-linking	I
Production of recombinant proteins	I, III, V
In vitro protein translation	III
Yeast two-hybrid analysis library screen mating assay determination of β-galactosidase activity	 III I, III, V I
Protein blot overlay	I
Protein affinity precipitation	I, III, V
Peptide array screening and sequence alignment	V
Western blot analysis and immunoblotting	I, II, III, IV, V

The main results of the studies are summarised here. The results are presented and discussed in detail in the accompanying original publications, which are referred to using Roman numerals.

1. HETEROTYPIC AND HOMOTYPIC INTERACTIONS OF MERLIN AND EZRIN (I)

1.1 Heterotypic interactions

To find out whether merlin shares known properties of ERM proteins, we studied the subcellular distribution of merlin and ezrin in human U251 glioma cells, which express both proteins endogenously. We showed that the subcellular distribution of both merlin and ezrin was affected by an increase in confluency. In subconfluent cells, double staining of merlin and ezrin revealed a highly overlapping distribution at cell surface projections resembling ruffling edges. In confluent cell cultures, only few cells with accumulation of merlin and ezrin at the cell periphery could be observed. Instead, most of the cells showed a diffuse or punctuate cytoplasmic pattern. The results demonstrated a coregulation of merlin and ezrin distribution under certain growth conditions (I, Fig. 1).

By coimmunoprecipitation experiments of endogenous and transfected proteins, we showed that merlin and ezrin not only colocalise, but form a complex in cells. After treatment of cells with a chemical cross-linker, novel bands, at sizes between 140-170 kDa appeared in merlin and ezrin immunoblots of cell lysates. Based on the sizes, the bands likely correspond to a merlin homodimer and a merlin-ezrin heterodimer (I, Fig. 2).

The merlin-ezrin heterodimerisation was also detected by several *in vitro* techniques, which showed that the interaction was direct. As previously reported for ERM proteins (Gary, 1995), also the merlin-ezrin heterotypic interaction occurred as "head-to-tail", the amino-terminus binding the carboxy-terminus. The interaction appeared to be under conformational regulation since only truncated, but not full length, proteins bound, indicating that the association sites in ezrin and merlin had to be unmasked for heterodimerisation (I, Figs 5-8). The result is thus analogous with

previous findings for two ezrin monomers, which need to be activated or truncated for homotypic binding (Gary, 1995).

1.2 Homotypic interactions

Our experiments of ezrin homotypic interactions were in accordance with previous studies. Homotypic binding between two ezrin molecules needed conformational regulation in analogy with the heterotypic binding between ezrin and moesin (Gary, 1995). Full-length ezrin molecules did not interact, but truncated ezrin proteins bound in a "head-to-tail" manner (I, Figs 5-8).

For homotypic binding of merlin, the functional regulation appears to be different. This was suggested by the fact that merlin, in contrast to ezrin, did not require exposure of the association domains for homotypic binding. Full length merlin was capable of homotypic interactions. Removal of the last 10 amino acids from one of the binding partners retained the interaction. However, no binding was seen after removal of the carboxy-terminal residues from both proteins. These results further supported "head-to-tail" binding of merlin (I, Figs 5-7).

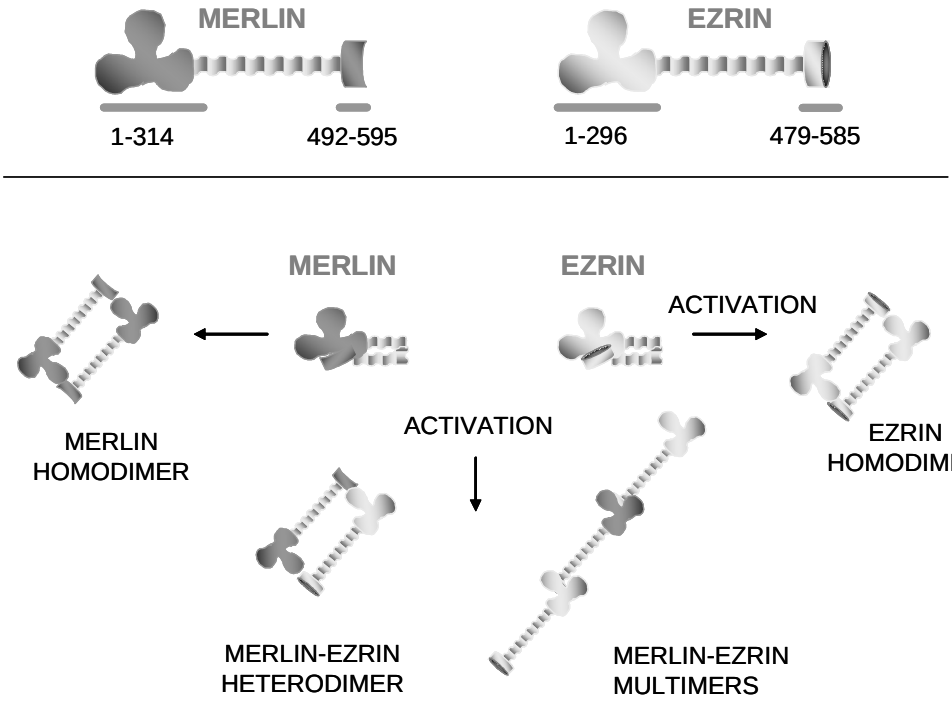

Figure 6. Model of homotypic and heterotypic interactions. Merlin and ezrin need to be activated to release an intermolecular association domain and to adopt a more extended conformation. This may result in homotypic and heterotypic binding. Homotypic interaction of merlin does not to require activation.

1.3 Merlin N-ERMAD and C-ERMAD

In ezrin, the amino-terminal association domain, N-ERMAD, contains residues 1-296 (Gary, 1995). Binding of merlin to carboxy-terminal merlin or ezrin proteins occurred via residues 1-339. Later we have mapped the merlin N-ERMAD to residues 1-314 (unpublished data). Thus, the amino-terminal association domain of merlin seems to be very similar to the N-ERMAD of ezrin (Fig. 6).

Based on the fact that the residues of the C-ERMAD of ezrin are poorly conserved in merlin, it had been suggested that the carboxy-terminus of merlin would not allow self-association (Gary, 1995). Our results indicate that this is not the case. In fact, the carboxy-terminus of merlin not only bound to the amino-terminus of merlin, but also to ezrin. In our study, the C-ERMAD of merlin could not be mapped as precisely as that of ezrin, which is contained in residues 479-585. However, we have since shown that a construct containing the carboxy-terminal residues 492-595 of merlin is sufficient for amino-terminal binding (unpublished data) and, hence, the C-ERMAD of merlin is functionally very similar to that of ezrin. A similar feature for the binding domain of merlin and ezrin is the absolute requirement of the last residues of the carboxy-terminus (Fig. 6).

The amino acid identity between merlin and ezrin is 61% in the amino-terminal domain, but the carboxy-terminal domain of merlin shares only 22% identity with ezrin (Turunen, 1998). The structure of all three ERM-proteins and merlin can, however, be roughly superimposed showing that the residues on the N-ERMAD and C-ERMAD binding interface are strongly conserved between merlin and the ERM proteins (Pearson, 2000). Although the overall structure of the merlin N- to C-ERMAD interface is similar to that of ERM proteins, there are some differences. Most divergent residues are found clustered on the surface of the N-ERMAD/FERM-domain possibly allowing for merlin specific interactions (Kang, 2002; Shimizu, 2002). Among the divergent residues is a seven amino acid stretch, the so-called blue box. The blue box is not found in ERM proteins but is perfectly conserved in D-merlin and essential for D-merlin function (LaJeunesse, 1998).

Other groups have later confirmed merlin homodimerisation and merlin-ezrin heterodimerisation (Gonzalez-Agosti, 1999; Gutmann, 1999; Meng, 2000). Heterotypic binding between ezrin and moesin (Gary, 1995) and merlin and moesin

(Gonzales-Agosti, 1999) have also been reported, reflecting a conservation of the association domain between ERM proteins and merlin. The protein 4.1B/DAL-1 also interacts with ERM proteins and merlin (Gutmann, 2001b), raising the possibility that additional members of the Protein 4.1 family are able to form intra- and intermolecular complexes.

In addition to the "head-to-tail" interaction, merlin forms an intramolecular interaction within the amino-terminus, which is needed for the N- to C-ERMAD interaction, the localisation of merlin underneath the plasma membrane and its association with actin. Known NF2 patient mutations in the amino-terminus of merlin impair these intramolecular interactions, suggesting that the interactions are important for the tumour suppressor function of merlin (Gutmann, 1999; Brault, 2001). Merlin isoform II has a different carboxy-terminus than isoform I and is not active as a tumour suppressor. In contrast to isoform I, it has been shown to bind full length ezrin, indicating that the intermolecular association in the two isoforms is regulated differently (Meng, 2000).

1.4 Regulation of the dormant and active states

The mechanism of a regulatory domain binding another part of the molecule to mask or inhibit its activity is not restricted to the ERM protein family. Conformational activation is found in the cytoskeletal protein vinculin, whose F-actin and talin binding sites are masked by a "head-to-tail" interaction that may be unmasked by PIP_2 (Bakolitsa, 1999). It has also been suggested that N-WASP homotypic interactions, necessary for activating the Arp2/3 complex, can be unmasked by activated Cdc42 (Rohatgi, 1999).

Our studies show that the regulation of the homotypic "head-to-tail" association of merlin is different from that of ezrin. As discussed in the introduction, the homotypic binding retains ERM proteins in a dormant state, in which binding sites for other molecules are masked. ERM proteins need to be activated and "opened" to allow them to interact with other molecules. The intramolecular association of ezrin is more stable than that of merlin (Nguyen, 2001). But merlin displays conformational activation, which is a prerequisite for heterodimerisation but not for homodimerisation. A merlin S518D mutation, which mimics the phosphorylated protein, blocks interactions between the C-ERMAD and N-ERMAD, while the S518A

mutation, mimicking the unphosphorylated protein, promotes this interaction (Shaw, 1998, 2001; Rong, 2004a). Phosphorylation of serine 518 by PAK2 was shown to impair the ability of merlin to bind to CD44 and HRS (Rong, 2004a) and we recently showed that PKA phosphorylation of merlin serine 518 directly enhances the association between merlin and ezrin (Alfthan, 2004). This suggests that phosphorylation of merlin directly modulates merlin intramolecular and intermolecular associations, which are important for the ability of merlin to function as a tumour suppressor. Differential phosphorylation of merlin may be a way to switch between different merlin containing complexes, which regulate downstream signalling.

Although our interaction studies were only semiquantitative, we repeatedly detected a stronger heterotypic binding between merlin and ezrin than the homotypic binding of ezrin or merlin. This was later confirmed by a study which showed that the merlin C-ERMAD has a stronger preference for binding the N-ERMAD of ezrin over its own and that merlin forms more stable interactions with ERM proteins than with itself (Nguyen, 2001). Therefore, the proportion of “active” ezrin in the cell could have implications on the activity of merlin and *vice versa.* Signals that effect the activation of merlin and ezrin can regulate the rank of order for their different binding partners. The merlin-ezrin coimmunoprecipitation experiments strongly suggest that at least a fraction of merlin and ezrin in cells are in a conformation that allows them to associate heterotypically.

In the tumour suppressor model by Morrison et al. (2001) and Ponta et al. (2003) (Fig. 7), distinct functions are given for the merlin and merlin-ERM complex. Contact inhibition of cell proliferation is mediated through interaction between CD44 and hypophosphorylated merlin while the CD44/merlin/ezrin-complex including phosphorylated merlin and ezrin, is growth promoting. The "open" ezrin molecule is called active, since binding domains for many interaction partners are exposed. In contrast, the hypophosphorylated, "closed" merlin molecule has been called active, since it appears to be active as a tumour suppressor. These terms may be oversimplified since both proteins are regulated in a complex manner, which involves several ways of activation and inactivation. At least three forms of phosphorylated merlin have been identified, and in *Drosophila*, the unfolded form of D-merlin is also active *in vivo*. Truncating mutations in D-merlin that remove the carboxy-terminus

and do not allow "head-to-tail" binding can still provide full genetic rescue of a null merlin mutant (LaJeunesse, 1998). This might be due to differential regulation or to merlin binding partners unique to *Drosophila*. Further analysis of the functional consequences of heterotypic binding between merlin and ezrin and the complex interplay between the different activating and inactivating modifications could provide novel information on how merlin and ezrin regulate proliferation.

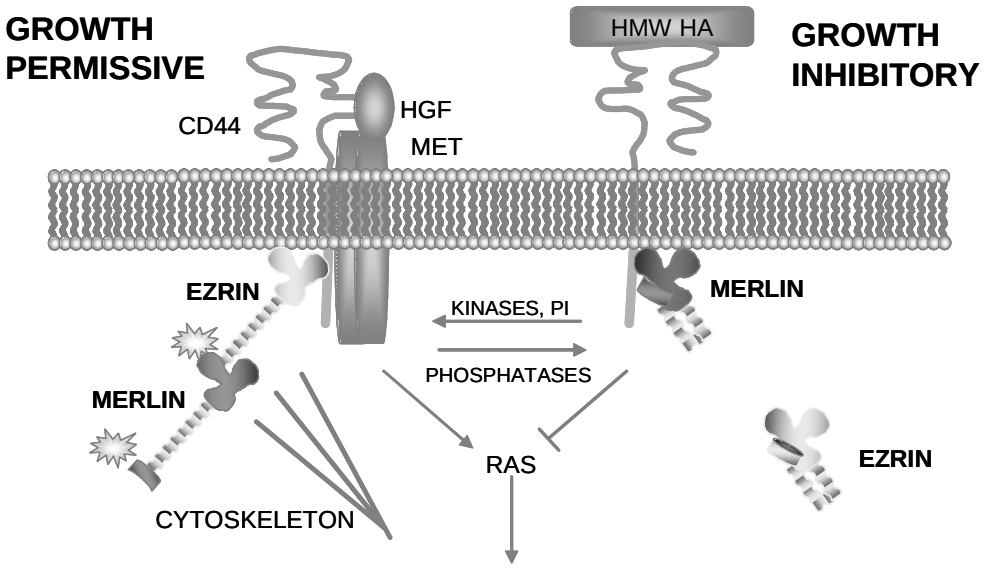

Figure 7. Model for the control of cell proliferation and growth arrest. Growth factors cause the formation of a growth promoting complex. Phosphorylation of ezrin allows it to bind the cytoplasmic tail of CD44, which can be in complex with e.g. Met. Phosphorylated merlin is bound to ezrin, and they link the cytoskeleton to these transmembrane receptors. At high cell density, high molecular weight hyaluronan (HMW HA) binds CD44. Ezrin is dephosphorylated, inactive and present in the cytoplasm as a closed monomer. The hypophosphorylated, closed merlin can replace ezrin as a binding partner for CD44 and is active as a tumour suppressor, thereby blocking Ras signalling and the link to the cytoskeleton. Adapted from Morrison et al., 2001 and Ponta et al., 2003.

2. NUCLEO-CYTOPLASMIC SHUTTLING OF MERLIN (II)

In general, functions of tumour suppressor proteins include the regulation of cellular responses to growth promoting signals, DNA damage and cell cycle checkpoints. These activities typically require nuclear localisation of the tumour suppressor protein, which may occur in a cell cycle specific manner (Fabbro, 2003).

To study if merlin shares these common properties of tumour suppressors, we used U251 glioma and U2OS osteosarcoma cell lines, which express endogenous merlin. We found that merlin was concentrated underneath the cell membrane in

subconfluent conditions in line with earlier studies. A small fraction of cells, however, demonstrated nuclear staining of varying intensity. Subcellular fractionation experiments with isolated nuclear and cytoplasmic fractions confirmed the presence of merlin in the nuclear as well as in the membrane fraction. Furthermore, the nuclear localisation of merlin was affected by cell density. A nuclear merlin signal was not detected in confluent cells, instead, merlin was localised to the cortical membrane and the cytoplasm. Laser scanning cytometric quantification of cells double-stained for merlin and DNA verified these findings (II, Figs 1-3).

2.1 Nucleo-cytoplasmic shuttling is dependent on the CRM1/exportin pathway

Nucleo-cytoplasmic shuttling of proteins is an active process regulated by various import and export pathways (Weis, 2003). The CRM1/exportin-mediated nuclear export can be blocked by Leptomycin B (LMB) (Kudo, 1998). Previous studies using transfected mutant merlin constructs have shown that they can be targeted to the nucleus and their export is regulated by the CRM1-pathway (Kressel, 2002). We showed that also the nuclear export of endogenous merlin was dependent on the CRM1 pathway. After LMB treatment, most of the subconfluent cells demonstrated strong nuclear merlin staining, and some nuclear accumulation was also seen in confluent cells (II, Fig. 3).

2.2 Nucleo-cytoplasmic shuttling is cell cycle-dependent

The fact that nuclear localisation was seen in actively proliferating cells but not in confluent or contact inhibited cells, suggested that nuclear targeting might occur in a cell cycle-specific manner. A similar pattern of expression has been shown for the APC and VHL tumour suppressors, which mainly localise to the cytoplasm of confluent cells, but shift to the nucleus in subconfluent cells and affect growth in a cell cycle-dependent manner (Lee, 1996; Zhang, 2001). In cells about to enter mitosis (late G2/M), merlin was localised in the cytoplasm and concentrated to the perinuclear region. During early mitosis, merlin was condensed around the breaking nuclear envelope, especially in nuclear envelope invaginations, *i.e.* regions, in which microtubules are concentrated in prophase (Burke, 2002). As mitosis progressed, merlin was present in mitotic spindles and in the midbody during cytokinesis. After mitosis, at early G1, merlin accumulated in the nucleus, but was also detected

underneath the plasma membrane. Progression of the cell cycle to late G1 phase resulted in export of merlin from the nucleus. At G1/S, merlin was concentrated to the membrane, and less prominently to the perinuclear region (II, Figs 4 and 5).

Previous studies have shown that merlin can bind tubulin *in vitro* (Xu, 1998; Stokowski, 2000) but have not demonstrated cellular colocalisation between merlin and tubulin. Our imaging results provide an explanation for the apparent controversy by showing that the association between merlin and tubulin may be transient and occur only during mitotic division. Preliminary results suggest that merlin associates with microtubules in a regulated manner and that the association may be important for the organisation of the mitotic spindle (Muranen, in preparation).

2.3 Nucleo-cytoplasmic shuttling is dependent on cell adhesion

Adhesion and anchorage to the substratum are known to regulate cell cycle progression at early G1 (Assoian, 1997). We studied merlin localisation during cell attachment and spreading, which also take place shortly after mitosis at G1. No nuclear merlin was seen in non-adherent cells, where merlin was concentrated at the submembranous regions. Strong nuclear merlin staining was detected at early phases of reattachment, both after trypsination and mitotic shake off. At later time points, when the cells were spread, merlin was again concentrated underneath the membrane and the nuclear localisation disappeared (II, Fig. 7).

Nuclear targeting of phosphorylated ERK is required for cell cycle progression at G1. Its activity depends on integrin-mediated cell adhesion and the presence of an intact actin cytoskeleton (Aplin, 2001). A downstream target of ERK in the nucleus is the Elk-1 transcription factor. Phosphorylation of Elk-1 increases its affinity to SRE and enhances transcription of growth-related proteins. This ultimately leads to the induction of cyclin D1 that regulates the passage through G1 (Marais, 1993; Whitmarsh, 1995; Vanhoutte, 2001). Interestingly, merlin inhibits phosphorylation of ERK as well as ERK-dependent nuclear Elk-1 phosphorylation (Lim, 2003). In addition, merlin prolongs the G1 phase of the cell cycle (Schulze, 2002). Therefore, we investigated if there is a functional interplay between ERK and merlin for their nuclear localisation. Both merlin and ERK were localised to the nucleus of adhering cells and at early G1. However, the nuclear localisation of merlin was not dependent on the nuclear localisation of ERK or an intact actin cytoskeleton (II, Fig. 8).

2.4 Regulation of the nucleo-cytoplasmic shuttling

We still do not know the molecular interactions of merlin in the nucleus or how the nucleo-cytoplasmic shuttling is regulated. Since merlin function is affected by phosphorylation, it is tempting to speculate that phosphorylation regulates its nuclear localisation. However, we found phosphorylated merlin both in the nuclear and cytoplasmic fractions, and did not find direct evidence that activation or inhibition of PAK and/or PKA would have an effect on its nuclear localisation (II, Fig. 6). But we cannot exclude an indirect link or transient effect between PAK- or PKA-dependent phosphorylation of merlin and its subcellular targeting. For two additional molecular partners of merlin; paxillin and syntenin, nucleo-cytoplasmic shuttling has been demonstrated (Jannatipour, 2001; Fernandez-Valle, 2002; Woods, 2002; Zimmermann, 2001). Both proteins also possess transcriptional potential (Aplin, 2003). However, it is not known whether they can bind merlin in the nucleus. Furthermore, a study demonstrated that human polyoma virus T-antigen, which is able to transform cells of neural origin, is in complex with merlin in the nucleus of malignant peripheral nerve sheath tumours (Shollar, 2004). Merlin pull down experiments from purified nuclei will hopefully clarify whether any of the known interaction partners or novel molecules, such as nucleic acids, bind merlin in the nucleus.

Several isoforms of band 4.1 proteins are targeted to the nucleus by nuclear localisation signals (NLS) (Correas, 1991; Lallena, 1997; De Carcer, 1995). An important NLS in band 4.1 proteins is a stretch of basic residues, KKKR, which is suggested to bind to a negatively charged motif of importin (Gascard, 1999). Recently localisation of ERM proteins to the nucleus was reported, and a functional NLS mapped to $_{435}$RRRK$_{438}$ in ezrin (Batchelor, 2004). Merlin contains an analogous stretch of basic residues, $_{309}$RRRK$_{312}$, which may be involved in its nuclear targeting. Merlin also has a cytoplasmic retention signal in exon 2, which can keep merlin in the cytoplasm (Kressel, 2002). Paxillin binds to the region of merlin encoded by exon 2 and facilitates the localisation of merlin to the cell membrane where it can interact with cell surface proteins, such as CD44 and ß1-integrin (Fernandez-Valle, 2002; Obremski, 1998). Thus, there may be several signal motifs with opposite effects whose interplay dictates the cell cycle-dependent distribution of merlin.

3. INTERACTION WITH HEI-10 CONNECTS MERLIN TO CELL CYCLE REGULATION (III)

To find explanations for the opposite effects merlin and ezrin play on cell proliferation, we looked for specific interaction partners for merlin, but not ezrin, in a yeast two-hybrid screen against a HeLa cDNA library. We found one cDNA that bound a merlin construct containing the α-helix and carboxy-terminus (amino acids 252-595) but not a similar ezrin construct (amino acids 278-585). The same gene was independently identified in a genetic screen aimed at identification of human cDNAs that promote invasion in yeast (Toby, 2003). Interestingly, it turned out to be a cell cycle regulator, mainly localised to the nucleus, and was given the name HEI10 (Human Enhancer of Invasion). The gene encodes for a 277 amino acid protein which consists of an amino-terminal RING-finger like motif characteristic of E3 ubiquitin ligase, a coiled-coil domain and a carboxy-terminal domain with a VSPSR motif, which is phosphorylated by cyclin B/cdk1 (Toby, 2003) (Fig. 8). The amino-terminal part of HEI10 interacts with the UbcH7 E2 ubiquitin conjugating enzyme and with cyclin B. HEI10 controls the accumulation of cyclin B, thereby regulating the passage through G2 in both yeast and vertebrates (Toby, 2003). Interestingly, recent reports have demonstrated that the *HEI10* gene is a component of a translocation fusion to the HMG1C gene in uterine leiomyoma (Mine, 2001) and altered HEI10 expression has been detected in melanomas (Smith, 2004). These results imply that deregulation of HEI10 may have consequences for tumour development.

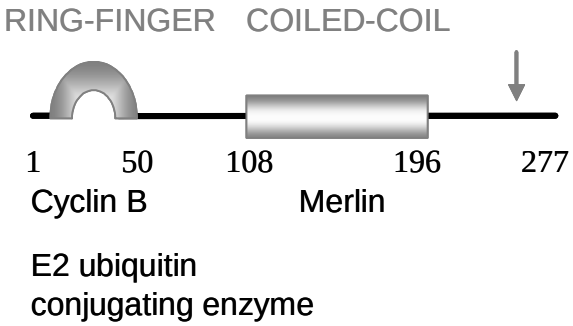

Figure 8. Structure of the HEI10 protein. HEI10 encodes a 277 amino acid protein with an amino-terminal RING finger domain, and a coiled-coil structure. Known protein binding partners are shown below. The arrow indicates the cyclin B/cdk1 phosphorylation site.

3.1 Merlin and HEI10 interact through coiled-coil domains

Based on binding studies, constructs containing the α-helical region of merlin interacted with the coiled-coil domain of HEI10. Further mapping using a transposon mutation library of merlin, identified residues 306-339 in merlin critical for the binding interphase, and indicated that the carboxy-terminus of the FERM domain may be involved in the regulation of binding. The interaction forms between two coiled-coil domains (III, Figs 1-3), which are known protein interaction domains, particularly for protein oligomerisation (Burkhard, 2001). Interestingly, although the sequence in ezrin is very similar to the binding site in merlin, ezrin does not bind to HEI10.

Only few interaction partners are known for the α-helical part of merlin. One of them is the regulatory subunit RIβ of PKA. A mutation which inhibits the RIβ-binding amphipathic helix, did not affect the HEI10 interaction. Based on previous studies, merlin interacting proteins that only bind to merlin isoform I after its conformation has been "opened", can bind to isoform II, which appears to have a more open conformation (Meng, 2000; unpublished data). HEI10, however, did not interact with merlin isoform II (III, Fig. 3).

Two known NF2 patient missense mutations, L316W and L316F, have been identified within residues 306-339. These mutations did not, however, affect binding of merlin to HEI10, which indicates that disuption of the interaction is not the likely cause for NF2 in these patients (III, Fig. 3). It has also recently been shown that PAK1 can be directly inhibited by merlin through two separate domains (Hirokawa, 2004). The HEI10 binding site lies within one of the domains comprising amino acids 288-359, indicating that this domain may be important in the regulation of PAK function.

3.2 Merlin and HEI10 interaction requires regulation of both proteins

Our results indicate that the association between merlin and HEI10 needs conformational activation of merlin, but so far, we do not know what regulates the merlin-HEI10 interaction. No binding was detected between HEI10 and merlin 1-595 containing mutations mimicking the phosphorylated and unphosphorylated serine 518 (S518D and S518A). Neither did mutation of threonine 576 (T576D and T576A), the residue whose phosphorylation in ERM proteins open the molecule, affect merlin binding to HEI10.

Also HEI10 appears to need activation for its interaction with merlin. The carboxy-terminus of HEI10 is phosphorylated by cyclin B/Cdk1 (Toby, 2003) and HEI10 appears to form homotypic interactions (unpublished data), which might represent a means to regulate its molecular interactions.

3.3 The colocalisation of merlin and HEI10 is dependent on cell adhesion, cell cycle stage and merlin expression levels

To find out whether merlin and HEI10 share subcellular localisation, we studied the proteins in varying culture conditions. During early reattachment of trypsinised cells, both merlin and HEI10 localised to the nucleus. As cells started to spread, merlin and HEI10 could be seen in punctuate structures at the plasma membrane. A fraction of HEI10 was still present in the nucleus (III, Fig. 4). When the cells were spread, merlin and HEI10 could be seen diffusely in the cytoplasm and underneath the membrane where HEI10 colocalised with focal adhesion proteins (unpublished data).

In analogy to merlin, the localisation of HEI10 varied in a cell cycle specific manner and HEI10 colocalised with merlin during parts, but not all, of the cell cycle. In early G1 merlin and HEI10 were present in the nucleus but both proteins rapidly disappeared from the nucleus as cells progressed in G1. HEI10 returned to the nucleus in S-phase, remaining in this compartment until mitosis. In contrast, merlin remained at the cell periphery until late G2, when the protein accumulated in the perinuclear region. During the entire cell cycle a fraction of both proteins were also found at the membrane. During mitosis both proteins associated with microtubules at the centrosome, mitotic spindle and contractile ring (III, Fig. 5). The partial colocalisation between merlin and HEI10 can indicate functional interplay between merlin and HEI10 in certain cell cycle phases.

Schwannomas are the primary manifestation of NF2. Therefore, it was interesting that Schwann cells express HEI10. We noticed a difference in HEI10 distriburion in primary human Schwann cells and schwannoma cells, which due to a genetic defect lack merlin, and in rat schwannoma cells expressing low or high levels of merlin. In cells deficient for merlin expression, HEI10 was frequently seen in the nucleus, whereas in cells expressing increased levels of merlin, HEI10 was cytoplasmic and localisesd to the plasma membrane. The results indicate that merlin is, either directly or indirectly, involved in targeting of HEI10. An indirect effect

might result from the fact that merlin expressing cells accumulate in G1 (Schulze, 2002), and during G1, HEI10 localised mainly to the cell membrane and cytosplasm. Since both proteins are exported from the nucleus at a similar time frame during G1, it is also possible that merlin contributes to the transport of HEI10 from the nucleus (III, Fig. 6). Cells treated with LMB to block the CRM1 export pathway caused an accumulation of merlin in the nucleus; however, no effect was seen for HEI10 (unpublished data), indicating that the CRM1 pathway is not the main export route for HEI10.

The nucleo-cytoplasmic shuttling may provide means for merlin and HEI10 to affect cell growth, although no nuclear function for either protein is known so far. Since merlin is concentrated around the nuclear envelope and in the nuclear groove during early stages of mitosis, it may play a role in nuclear envelope breakdown, as already demonstrated for HEI10 (Toby, 2003). Of interest is the potential interplay between merlin and merlin interacting proteins during mitosis and their role in the organisation of the centrosome, mitotic spindle and midbody. An earlier report stated that merlin could be immunoprecipitated in complex with the microtubule-associated protein kinesin-1 (Hakimi, 2002), which plays a role in microtubule motor-linked activities, regulation of cell adhesion and in cytokinesis (Krylyshkina, 2002; Glotzer, 2003). Band 4.1 protein isoforms are associated with mitotic spindles during early mitosis (Mattagajasingh, 1999; Krauss, 1997) and HEI10, PAK and PKA are localised to centrosomes, the mitotic spindle and/or the contractile ring (Li, 2002, Banerjee, 2002, Chiroli, 2003; Witczak, 1999). The centrosome also contains γ-tubulin (Stearns, 1991; Zheng, 1991) and cyclin B (Bailly, 1992). The centrosome is thought to function as a scaffold that brings together proteins needed for cell cycle regulation. There is, however, increasing evidence that the centrosome is home to several proteins that have nothing to do with centrosomal function, and may use association with the centrosome as a means of ensuring segregation at mitosis, or as a way to increase local protein concentration. A potential functional interplay of merlin and HEI10 during mitotic progression with their binding partners mentioned above, as well as their role in spindle formation and cytokinesis, need further study.

3.4 Merlin and HEI10 affect cell cycle progression

Since both merlin and HEI10 are linked to the regulation of cell proliferation, we studied their effect on the cell cycle. Transfection experiments demonstrated that merlin and HEI10, both independently and in combination, affected cell cycle progression. In cells expressing merlin, the amount of cells in G0/G1 phase was increased in agreement with previous results (Schulze, 2002). This effect was not seen in cells expressing constitutively open merlin. Similarly, expression of HEI10 prolonged the G0/G1 phase and with combined expression of merlin and HEI10 the amount of cells in G0/G1 was even higher than with either protein alone. In contrast, coexpression of HEI10 and constitutively open merlin resulted in an increase of cells in S phase. These findings suggest interplay between merlin and HEI10 in cell cycle control.

Although full length merlin did not interact with HEI10 in cells, constitutively open merlin could bind HEI10, perhaps through an interaction domain that normally needs activation to be exposed (III, Fig. 2). This interaction appeared to alter HEI10 function and its effect on cell cycle progression and interestingly, it also affected the integrity of HEI10 (III, Figs 7 and 8). In the cells coexpressing constitutively open merlin and HEI10, western blot analysis showed a significant decrease of the full length HEI10 protein and the presence of an abnormal HEI10 band, possibly a result of increased degradation of HEI10. Whether this abnormal HEI10 protein allows cells to pass the G1/S checkpoint, or whether constitutively open merlin can block HEI10 nuclear entry at S-phase, thereby affecting cell cycle progression, remains to be studied.

HEI10 is ubiquitinated and may function as an E3 ubiquitin ligase for cyclin B (Toby, 2003). Interestingly, Mdm2 functions as an E3 ubiquitin ligase for p53 (Honda, 1997) and merlin functions as a positive regulator of p53 by causing the degradation of Mdm2 (Kim, 2004). The decrease of the full length HEI10 protein by constitutively open merlin may be caused by merlin induced ubiquitination of HEI10. It could also be a result of the induction of other degradation pathways, protein cleavage or by regulated transcription. The effect of merlin on the integrity of the HEI10 protein and its consequences for HEI10 function is of great interest and needs further study.

In a recent report, adenovirus-mediated expression of merlin in a *NF2* deficient mesothelioma cell lines, caused the accumulation of cells in G1 concomitant

with a decreased expression of cyclin D1, inhibition of Cdk4 activity and dephosphorylation of Rb, which are important for G1/S transtition. This decrease was caused by merlin's inhibitory effect on PAK, a known upstream activator of cyclin D1 transcription (Xiao, 2005). In NIH 3T3 cells, however, expression of merlin caused a decrease in cyclin D1 transcription by inhibiting the ERK-Elk1 pathway (Lim, 2003) which was not affected in mesothelioma cells (Xiao, 2005). It is of interest to study whether the accumulation of cells in G1 by HEI10 expression is caused by inhibition of the same pathways as merlin expression.

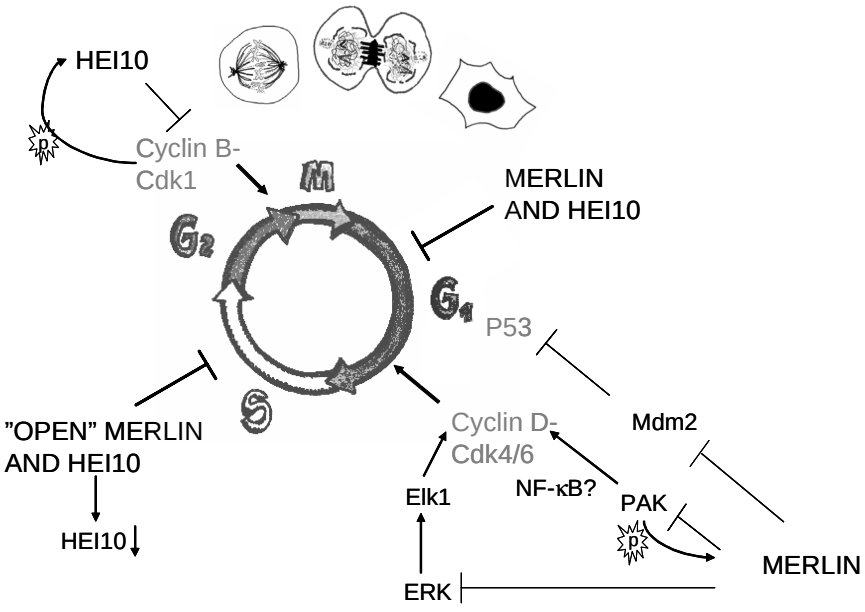

Figure 9. Possible pathways regulated by merlin and HEI10 during the cell cycle. At the G2/M phase HEI10 can affect cyclin B-levels and is phosphorylated by cyclin B-Cdk1. During mitosis merlin and HEI10 localise to the mitotic spindle, centrosome and midbody. After mitosis, in early G1, both proteins reside in the nucleus. Merlin and/or HEI10 cause the accumulation of cells in G0/G1, while expression of a constitutively open form of merlin together with HEI10 increases the amount of cells in S phase, and causes a decrease in HEI10 protein levels. The presence of merlin can affect cyclin D1 transcription either through the ERK-Elk1 pathway or by inhibiting PAK, which could inhibit NF-κB mediated transcription of cyclin D1 (Lim, 2003 and Xiao, 2005). Merlin can also positively regulate p53 by inhibiting the p53 inhibitor Mdm2 (Kim, 2004).

Previously, models for the tumour suppressor role of merlin at the membrane-cytoskeleton interphase have been proposed. We have shown a more versatile localisation of merlin, which is regulated by cell cycle phase, an interaction with the cell cycle regulator HEI10 and a functional interplay between merlin and HEI10 in the regulation of cell cycle progression (Fig. 9). This suggests that merlin performs

several functions in cells, which may all be linked to its function as a tumour suppressor.

4. MERLIN IN THE CNS (IV)

Several reports have analysed the tumour suppressor activity of merlin, but so far little is known of its additional functions. An indispensable role in early development and rather wide tissue expression pattern suggest yet unidentified functions for merlin (McClatchey, 1997). Of special interest is the CNS, in which high expression levels of merlin have been detected during and after organogenesis (Claudio, 1995; Stemmer-Rachamimov, 1997a). In our study, we have thoroughly compared the expression and localisation of merlin and ezrin in the CNS.

4.1 Analysis of merlin and ezrin in the developing and adult brain

We showed that merlin and ezrin are widely expressed in the CNS of mouse, rat and human. However, they have a markedly different expression pattern. In neurospheres, which can self-renew and generate both neuronal and glia cells, both proteins were present and partially colocalised. In attaching neurospheres, ezrin was predominantly seen in the outer cell layer in short filopodia, while merlin was present in the cell body. As cells started to differentiate, merlin was more prominent in neurons and ezrin almost exclusively found in astrocytes (IV, Figs 6 and 7). A potential role for merlin and ezrin in the differentiation process remains to be elucidated. From mouse E8 through E10, the CNS is composed of mostly undifferentiated neuroepithelia but by E11-E13 the brain and spinal cord are well developed. We could detect an abrupt neoexpression of merlin at E11 in lysates from whole fetus, which interestingly coincides with the onset of neurogenesis. Various amounts of ezrin were present from E5 onward (IV, Fig. 1).

Lysates of different human brain regions were immunoblotted for merlin and ezrin. Merlin was present in most regions with strongest staining in the brain stem, cerebellum, diencephalon, basal ganglia, corpus callosum, hypophysis and optic nerve. A double band of merlin, possibly representing differently phosphorylated forms (Kissil, 2002; Xiao, 2002; Alfthan, 2004), was seen in the brain stem and diencephalon. The strongest ezrin staining was detected in the cerebral cortex, basal

ganglia, hippocampus, hypophysis and optic nerve. Comparison of grey and white matter of the frontal lobe demonstrated stronger expression in the grey matter for both merlin and ezrin (IV, Fig. 2).

In analogy with the differentiated neuronal progenitor cells, merlin was detected predominantly in neurons, and ezrin in astrocytes in most human brain tissues studied. However, in the brain stem, astrocytes were negative for ezrin and instead strong ezrin immunoreactivity was seen in neurons. Although ezrin staining was weak in adult brains, a very strong ezrin staining was detected in the Purkinje cell layer and in part of the molecular layer of infant brain. However, Purkinje cells were ezrin negative. Merlin, on the other hand, was expressed in Purkinje cells and there was no difference in the intensity of merlin immunoreactivity between infant and adult brain (IV, Figs 3-5).

4.2 Subcellular distribution of merlin and ezrin in brain cells

Ezrin is an unlikely interaction partner for merlin in the adult CNS because of the lack of coexpression of merlin and ezrin after the onset of neuronal progenitor cell differentiation and in adult brain. Both proteins were specifically localised to distinct actin rich structures. When neurospheres were allowed to attach, ezrin positive microspikes could be seen extending from the border cells and migrating cells, which may suggest a role for ezrin in migration of differentiating glia cells (IV, Figs 6 and 7). Peripheral astrocytic processes (PAPs) of cultured hippocampal astrocytes were strongly labelled for ezrin (IV, Fig. 8) in agreement with Derouiche et al. (2001), who suggested a potential role for ezrin in the regulation of PAPs morphogenic properties and its connections to signalling pathways. An important role for glial cells in regulation of synapse structure and function has recently been described. The astrocytic processes that engulf the synaptic terminal modulate synaptic activity and effectively take up transmitters released by neurons (Haydon, 2001).

Merlin staining was strong in the cell soma of neurons and along dendritic extensions in a punctuate manner. Merlin and a marker of the postsynaptic density, PSD-95, colocalised in many punctuate structures, whereas presynaptic marker synapsin-containing structures were not stained with the merlin antibody. The presence of merlin in synaptic junctions was confirmed by synaptic fractionations of rat brain lysates (IV, Figs 8 and 9).

5. RIβ, A POTENTIAL INTERACTION PARTNER FOR MERLIN IN THE SYNAPTIC JUNCTIONS (IV, V)

A potential merlin interacting partner in the synaptic junctions is the PKA regulatory subunit RIβ, which is mainly expressed in neurons of the hippocampus (Clegg, 1988; Solberg, 1991). Multiple characterised protein interactions are involved in the targeting and regulation of the other PKA regulatory subunits, RIα, RIIα and RIIβ, but no such interactions had been described for RIβ. Ezrin has previously been shown to interact with RIα (Dransfield, 1997).

5.1 The merlin-RIβ interaction

We studied the expression of RIβ in the brain, which showed that both RIβ and merlin are present in neurons of human brain tissues (V, Figs 1 and 2). RIβ was localised to neuronal synaptic junctions as detected by immunofluorescence of rat hippocampal neurons and fractionation studies from rat brain (IV, Figs 8 and 9). To investigate a possible interaction between merlin and RIβ, we studied their association in rat brain homogenates. Both merlin and RIβ were present in the cytoskeletal fraction of rat brain lysate from which they could be coprecipitated. Furthermore, merlin was coprecipitated with a cAMP-agarose bead / RIβ-complex from rat brain, indicating that merlin and RIβ are present in the same complex in rat brain (V, Fig. 4).

The localisation and interaction between merlin and RIβ were further studied by transfection experiments. Merlin and RIβ could be coprecipitated from transfected cells and a partial colocalisation between merlin and RIβ-GFP could be seen in regions underneath the cell membrane. In these cells, ezrin formed a complex with merlin but not with RIβ (V, Figs 5 and 6).

5.2 Regulation of the merlin-RIβ interaction

Full length merlin did not interact with RIβ in the yeast two-hybrid experiment, indicating that it needs activation for the interaction. Full-length merlin with introduced mutations mimicking the phosphorylated and unphosphorylated serine 518 also did not bind RIβ, indicating that the interaction is not regulated by the phosphorylation status of serine 518 alone. According to coimmunoprecipitation

experiments RIβ is specifically associated with the hypophosphorylated but not the phosphorylated or hyperphosphorylated forms of merlin. In accordance with this, the amount of coprecipitated RIβ and merlin was increased in confluent cells, in which the amount of the hypophosphorylated form was highest (V, Figs 6 and 8).

5.3 Mapping of the AKAP interaction site

Binding studies demonstrated a direct interaction between merlin and RIβ, and suggest that the interaction domain resides within the α-helical part of merlin. Proteins that bind PKA regulatory subunits, AKAPs, function as multivalent scaffolds that assemble and integrate signals from multiple pathways. An AKAP should contain a sequence motif for binding regulatory subunits, an ampipathic helix with hydrophobic residues aligned along one face of the helix and charged residues along the other (Carr, 1991; Newlon, 1999, 2001). Comparison with known AKAPs identified a sequence between residues 463-480 of the α-helical domain of merlin, which shares most of the functionally relevant hydrophobic residues previously reported for RII-binding (Carr, 1991; Newlon, 1999, 2001). Comparison between merlin and D-AKAP1, a protein known to bind RI, identified six additional identical or conserved amino acids, which may be associated with the specificity of the interaction between these AKAPs and the RI regulatory subunits. Proline substitution within the merlin AKAP consensus site, A468P, fully abolished the interaction and another substitution, L472P, reduced binding to RIβ. Interestingly, our results demonstrate that ezrin does not interact with RIβ and merlin does not bind to RIIα (V, Figs 7-10). Thus, although merlin and ezrin share many common interaction partners, and a fairly similar AKAP consensus sequence, they demonstrate selectivity in their interactions with PKA subunits.

5.4 Potential functions for the merlin-PKA association

In neurons, extensive signal transduction machinery is localised to thousands of small compartments at presynaptic and postsynaptic sites. Postsynaptic densities (PSDs) are present at the tips of dendritic spines, which are contact sites for most excitatory synapses in the brain. They undergo morphological changes in response to activities associated with neuronal plasticity, learning and memory functions (Yuste, 2001). The

PSD is localised to the postsynaptic membrane in register with the active zones of the presynaptic terminal and provides a structural framework for localising functional molecules, regulating adhesion, controlling receptor clustering and regulating receptor function (Siekevitz, 1985). There is a link between synaptic activation and changes in spine morphology through the regulation of actin turnover, providing a possible mechanism of learning and memory in brain (Star, 2002). Drugs that inhibit actin dynamics suppress long term potentiation (LTP) and block shape changes in dendritic filopodia and spines (Kim, 1999; Krucker, 2000).

An important signalling route involved in learning and memory and in synaptic plasticity in the PSD, is the cAMP-PKA pathway (Brandon, 1997; Albright, 2000; Waltereit, 2003; Bauman, 2004). Hippocampal synaptic plasticity including LTP and long term depression (LTD) are defective in mice carrying a targeted disruption of the gene encoding for RIβ (Huang, 1995; Brandon, 1995). Since merlin and RIβ are expressed in the same cell types in the CNS, including the hippocampus, form a complex there, and colocalise in synaptic structures in cultured neurons, merlin may participate in neuronal PKA signalling and memory functions. In addition to PKA-RIβ, merlin binds to βII-spectrin/fodrin (Scoles, 1998), actin and tubulin (Xu, 1998; Brault, 2001; James, 2001), which are all integral components of the PSD (Kennedy, 1993). Merlin also binds to integrins (Obremski, 1998) and forms a complex with cadherins (Lallemand, 2003), which are both components of synaptic junctions. Cadherins have been suggested to play a role in synaptic junction formation and synaptic plasticity (Tanaka, 2000; Tang, 1998) and integrins in synaptic maturation (Chavis, 2001). Furthermore, the merlin binding partner paranodin (Denisenko-Nehrbass, 2003) is also enriched in synapses in the hippocampus (Murai, 2002). Whether or not merlin interacts with any of these proteins in the synapse needs further study. A potential role for merlin in the regulation of actin rearrangement in the dendritic spine and as a scaffolding protein in the synaptic junction should be further studied, which may suggest a novel function for merlin in connecting neuronal cytoskeleton to PKA signalling.

The link between merlin and PKA is also interesting for the understanding of tumour cell growth in schwannomas. In cultured Schwann cells, induction of the PKA signalling pathway promotes cell growth and cell cycle progression, which can be blocked by inhibitors of PKA activity (Kim, 1997). In addition to RIβ, merlin also

interacts with the regulatory subunit RIα (unpublished data). Disease causing mutations in the Carney complex (CNC) tumour suppressor syndrome results from mutations in the PKA regulatory subunit RIα, which leads to increased PKA activity (Kirschner, 2000). Interestingly, one of the manifestations of CNC is the formation of schwannomas. Thus, it is possible that the molecular pathways altered in CNC and NF2 overlap, and may be connected through merlin.

In Figure 10, a model is presented which is modified from that in Figure 7. It shows how merlin and ezrin containing complexes may affect cell proliferation in Schwann cells. The phosphorylation of merlin by PKA after cAMP stimulation, leads to merlin-ezrin association and induces downstream signalling, resulting in cell proliferation.

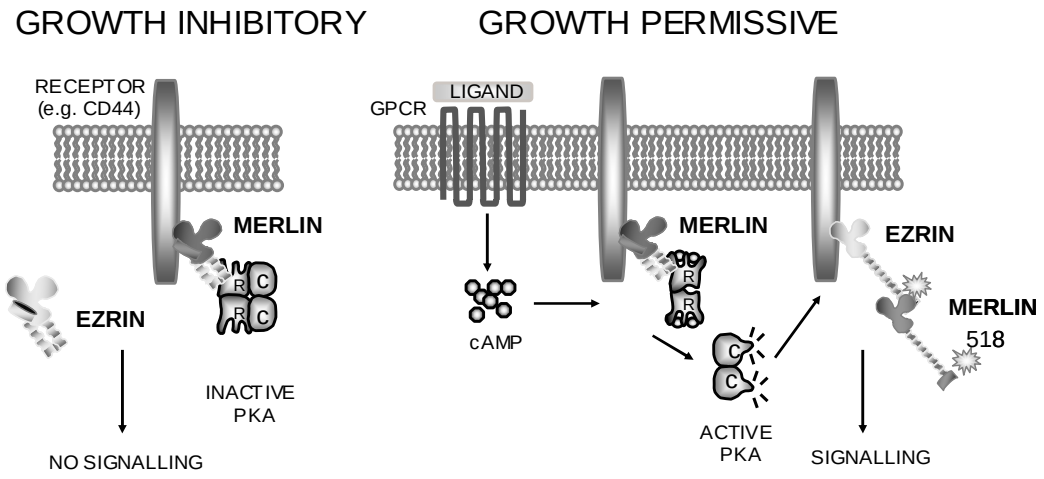

Figure 10. A model for the regulation of merlin activity by PKA phosphorylation. Hypophosphorylated merlin inhibits cell growth, possibly by blocking receptor mediated signalling (such as CD44) and Ras activation, whereas phosphorylated merlin permits growth. In the growth inhibitory complex, merlin may bind the regulatory subunit (R) of inactive PKA. PKA promotes growth of Schwann cells with yet unknown mechanisms. Binding of a ligand (e.g. hormones, neurotransmitters) to the G-protein-coupled receptor (GPCR) causes accumulation of cAMP. Following cAMP binding to the PKA regulatory subunits, active PKA catalytic subunits (C) are released. This may involve phosphorylation of merlin at serine 518, which increases heterodimerisation between merlin and ezrin. In this growth promoting complex phosphorylated ezrin is bound to the receptor resulting in downstream signalling and cell proliferation. An analogous chain of events may result from activation of PAK.

CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion this study identified four novel interaction partners for merlin; merlin, ezrin, HEI10 and RIβ. Our aim was to determine whether unique molecular interactions could explain the opposite effect of merlin and ezrin on cell proliferation. The merlin interaction with RIβ in neurons will most likely not explain the tumour suppressor function of merlin. It can, however, provide information about novel roles merlin plays in the CNS. We speculate that merlin participates in neuronal PKA signalling but further studies in animals models with a targeted neuronal disruption of the *Nf2*-gene are needed to further address this question. The identification of merlin as a regulator and substrate of the PKA complex is of interest also for the regulation of cell proliferation, since it links merlin to the cAMP/PKA signalling pathway known to promote cell growth and cell cycle progression in Schwann cells. Merlin can, as an AKAP, regulate PKA function but is also a target for PKA-induced phosphorylation. This promotes heterodimerisation between merlin and ezrin, an event suggested to convert merlin from the growth suppressive to the growth permissive state. Of interest is if PKA induced phosphorylation of merlin regulates the antiproliferative activity of merlin in Schwann cells.

Many binding partners to the ERM protein family members have been found since this study began. They bind signalling molecules, cytoskeletal proteins and transmembrane receptors. It is likely that merlin and ezrin function as scaffolding proteins which can associate with transmembrane receptors, the cytoskeleton, kinases, their substrates and perhaps phosphatases. Thereby they may spatially and temporally affect the formation of complexes that can regulate cell signalling, morphology, migration, adhesion, proliferation and differentiation. These complexes may look very different depending on cell types, subcellular localisation and stages of development and differentiation. Merlin and ezrin-containing complexes can consist of different components and merlin and ezrin can be present in the same complexes, which in different situations are regulated by distinct regulatory cues leading to different downstream effects. Whether merlin and ezrin function cooperatively and/or antagonistically is still not clear. With new functional models available we can attempt to elucidate whether the presence of ezrin can inhibit merlin function, thereby

promoting tumour growth and metastasis - and conversely, whether loss of merlin causes ezrin activation.

Merlin may be involved in the regulation of cellular proliferation at the plasma membrane by regulating signalling pathways and cell adhesion signals, as suggested. But the fact that merlin localises to the nucleus in a cell cycle-dependent manner opens up new possibilities for how merlin suppresses cell growth. We still do not know the functional consequences of the nuclear localisation of merlin. Further studies are needed to understand how the nucleo-cytoplasmic shuttling of merlin is regulated, to find the nuclear binding partners of merlin, and to explain its function in the nucleus. So far, described nuclear roles for cytoskeletal proteins include regulation of transcription, RNA splicing and transport and providing the cell with a cytoskeletal scaffold inside the nucleus.

Furthermore, interactions with molecules involved in cell cycle regulation, such as HEI10, are of special interest in trying to understand the molecular basis of the growth regulatory activity of merlin. The potential involvements of merlin in nuclear envelope breakdown and mitotic entry and in centrosome and spindle morphology, which all regulate the progression of the cell cycle, are of great interest. It appears that merlin is linked to cell cycle regulation in several ways; through different pathways and at different cell cycle stages.

What was once thought to be a passive contributor to cytoskeletal architecture is now known to play a significant role in many vital processes in cells. The extensive research of recent years has produced novel information of merlin's interaction partners, its involvement in various signalling pathways and its different ways of regulation. However, the mechanism by which merlin acts as a tumour suppressor is not yet understood in sufficient detail to reach the ultimate goal for our research, to help patients with NF2 by providing specific treatments against the disease.

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